CALIFORNIA SEA GRANT

Biennial Report of Completed Projects 1990–92

A Publication of the California Sea Grant College

CALIFORNIA
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The California Sea Grant College is a statewide, multiuniversity program of marine research, education, and extension activities, administered by the University of California. Sea Grant-sponsored research contributes to the growing body of knowledge about our coastal and ocean resources and, consequently, to the solution of many marine-related problems facing our society. Through its Marine Extension Program, Sea Grant transfers information and technology developed in research efforts to a wide community of interested parties and actual users of marine information and technology, not only in California but throughout the nation. Sea Grant also supports a broad range of educational programs so that our coastal and ocean resources can be understood and used judiciously by this and future generations.

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Biennial Report of Completed Projects

1990–92

California Sea Grant College
University of California
La Jolla, CA 92093
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Introduction

This biennial report presents the results of research activities undertaken by the California Sea Grant College during fiscal years 1990–92.

It is meant to be a technical record of our accomplishments for use by individuals in academia, government, and industry.

This publication contains only reports of completed projects (as opposed to descriptions of work in progress). It thus forms an important historical record of program achievement and an important document in terms of program accountability.

For readers unfamiliar with our program, the California Sea Grant College is the largest of 29 Sea Grant programs underway in more than half the nation's states. Its purpose is clearly stated in the 1966 National Sea Grant College and Program Act responsible for its creation: "to increase the understanding, assessment, development, utilization, and conservation of the nation's ocean and coastal resources by providing assistance to promote a strong educational base, responsive research and training activities, and broad and prompt dissemination of knowledge and techniques."

California's Sea Grant College is administered by the University of California and is headquartered at Scripps Institution of Oceanography, part of the University of California, San Diego.

James J. Sullivan
Director

Coastal Resources

Wastewater Wetlands: Pulsed Discharges to Protect Coastal Water Bodies

San Diego State University
R/CZ-87
1989–91

Rick Gersberg, Joy Zedler, and René Langis

In drought-stricken California, where most of the water is imported from outside the watershed, the landscape-level hydrologic pattern has shifted from winter-pulsed streamflows to year-round flows of municipal discharge. These streamflows reduce the salinity of the estuarine waters and sediments in unknown patterns. Carried along with these inflows are unknown quantities of nutrients and toxic materials. Once these materials reach the estuary, they may be concentrated near the inflows, bioaccumulated, or diluted and carried to the ocean.

From a broadly based literature on the topic of wastewater inflows and urban runoff entering estuaries (e.g., Groman et al., 1985; Hopkinson and Day, 1979; Otte, 1979; Smith and Bridges, 1977; Teal et al., 1982), we conclude that all estuarine organisms respond to these changes. Most of our locally occurring marine animals are negatively affected (Zedler et al., 1984). Nordby (1988, 1989) and Nordby and Zedler (1991) have documented the loss of fish and invertebrate populations in response to increased streamflows. Although some marsh plants may benefit temporarily, prolonged freshwater discharges allow invasion by aggressive brackish marsh species (cattails and bulrushes) which replace halophytes and jeopardize critical habitat (Zedler and Onuf, 1984; Zedler and Beare, 1986). Thus, it is essential that year-round inflows of wastewater be controlled.

The principal goal of this Sea Grant project was to understand how constructed wastewater wetlands, providing both nutrient and toxic metal removal, may be operated with a pulsed-discharge regime so that the impact of freshwater (sewage) flows on the downstream estuary would be minimized.

Constructed Wastewater Wetlands

Constructed wetlands are being used to treat municipal wastewaters throughout the United States (e.g., there are 96 registered wastewater wetland sites in the Midwest and 224 registered sites in the Southeast; Richardson and Nichols, 1985). Wetlands have been constructed to treat municipal wastewater in several European countries (Kiefer, 1968; Seidel, 1976, de Jong,1976). Constructed wetlands have been shown to meet National Pollutant Discharge Elimination System requirements (Watson et al., 1987).

Water quality improvement involves sedimentation, microbial action, precipitation, and adsorption, although the mechanisms are much better understood for nutrient removal than for metal immobilization (Godfrey et al., 1985). In a series of controlled experiments near San Diego, Gersberg and coworkers (1984a) found that artificial cattail (*Typha*) and bulrush (*Scirpus*) wetlands were capable of removing a wide variety of wastewater contaminants, including organic matter (measured as biological oxygen demand, BOD), suspended solids (Gersberg et al., 1984b), nitrate (Gersberg et al. 1984c, 1986), and bacteria and viruses (Gersberg et al.,1986, 1987). The nitrogen-removal function is particularly important for southern California coastal areas, where this nutrient stimulates algal blooms (Fong et al., 1987), which later decay, cause anoxia, and lead to fish kills in poorly flushed lagoons. Nitrogen, rather than phosphorus, limitation is indicated for coastal wetlands receiving urban wastewater (Howarth, 1988).

Nutrient removal and metal retention are not independent processes. Giblin (1985) pointed out several complexities of metal retention in wastewater wetlands and called for research to understand the ecosystem-level interactions between nutrient addition and sediment chemistry. Furthermore, these soil processes are intimately affected by hydroperiods, i.e., inundation and drainage regimes (Bayley, 1985, Giblin, 1985), and by hydraulic loadings (Richardson and Nichols, 1985). Thus, management of wastewater wetlands for nutrient and metal removal can be accomplished by manipulating hydroperiods, but one regime may favor denitrification, while another maximizes retention of one or more metals. Our study focused on obtaining a basic understanding of how pulsed discharges (and their coinciding inundation/drainage regimes) affect nutrient and metal removal by wetland soils.

Experimental Mesocosms

Wetland mesocosms (each 2.0 m^2) were located outdoors at the Pacific Estuarine Research Laboratory at the Tijuana River National Estuarine Research Reserve. They were constructed by filling reinforced plastic troughs (1000 liters) with 20 cm of coarse sand, and planting these with *Scirpus californicus* (bulrush). Rhizome cuttings from nearby planted stands of bulrush were transplanted (with the shoot cut to about 10 cm height) approximately 5–10 cm below the sand surface.

The experimental regime consisted of replicate mesocosms (5 reps. x 4 treatments = 20 mesocosms) assigned to four different hydroperiods with varying inundation and pulsed discharge regimes tested in a randomized block design.

The hydroperiods were:

H0 No pulse: continuous inundation and continuous overflow;

H0.5 1 pulse/2 days: 44 hr inundation with discharge for 4 hr every other day;

H11 pulse/day: 20 hr inundation with discharge for 4 hr once each day and

H2 2 pulses/day: 8 hr inundation with discharge for 4 hr twice each day.

Nutrient Removal

The constructed wetlands planted with *Scirpus californicus* were very effective at removing inorganic nitrogen with mean removal efficiencies (for all treatments) of 91.5% at the low nitrogen loading rate (hydraulic retention time = 2 days), and 77% at the high nitrogen loading rate (hydraulic retention time = 1.6 days) (Figure 1). There appeared to be no significant effect of hydroperiod regime on inorganic nitrogen removal at the low nitrogen loading rate. However, at the high loading rate, the inorganic nitrogen removal efficiency in the H2 (flooded twice daily) treatment was consistently higher than for all the other treatment regimes, and significantly higher ($P < 0.05$) on three of the eight dates tested (Figure 2). Because redox values were also highest in the H2 treatment, it would seem that increased oxygen penetration into the wetland soils in the pulsed (flooded twice daily) system increased sequential nitrification-denitrification, thereby enhancing overall inorganic nitrogen removal.

The constructed wetlands were less efficient at removing phosphorus compared to nitrogen, with a mean phosphorus removal efficiency of 58% at the lower phosphorus loading rate and 68% at the high phosphorus loading rate (Figure 3). The higher efficiency at the higher loading rate is surprising and may result from the fact that the higher loading rate was tested later in the course of wetland establishment when the plant biomass was at its highest levels. It is important to note that at both loading rates, the H2 (drained twice daily) treatment showed significantly higher removal efficiencies than all other treatments, pointing to the fact that the pulsed discharge regime confers superior treatment capability in this regard

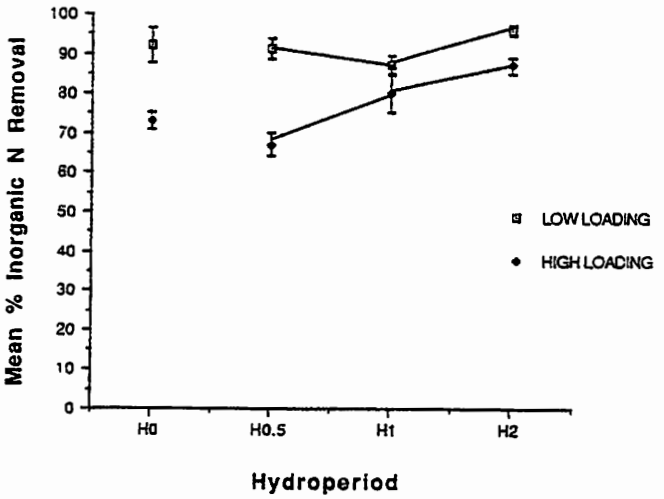

Figure 1. Removal of inorganic nitrogen under different hydroperiod regimes at low and high loading rates (error bars = ± 1 S.E.).

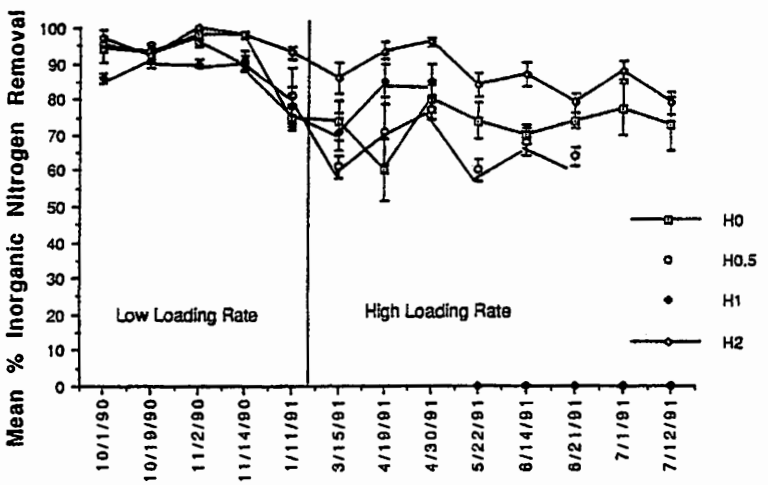

Figure 2. Removal of inorganic nitrogen under different hydroperiod regimes at low and high loading rates.

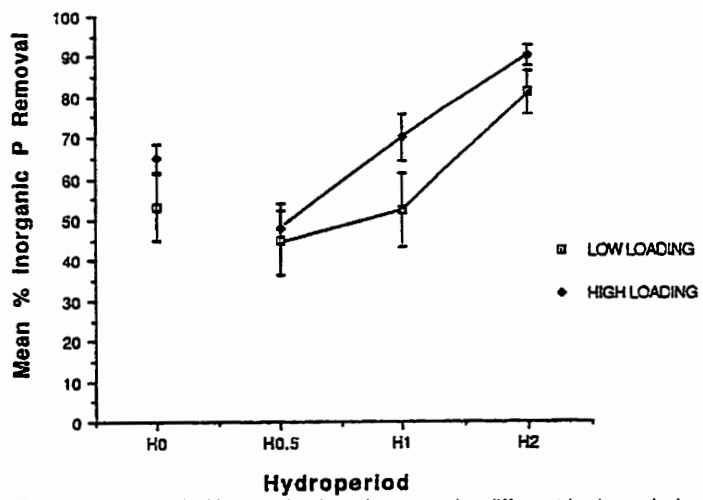

Figure 3. Removal of inorganic phosphorus under different hydroperiod regimes at low and high loading rates (error bars = ± 1 S.E.).

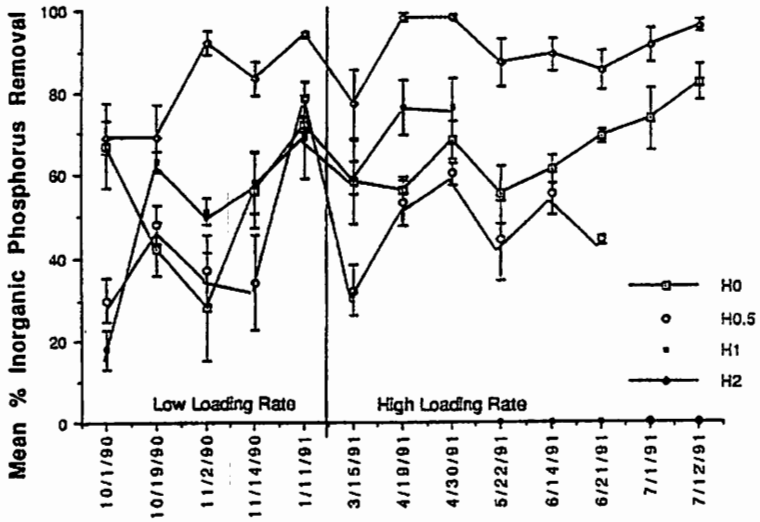

Figure 4. Removal of inorganic phosphorus under different hydroperiod regimes at low and high loading rates.

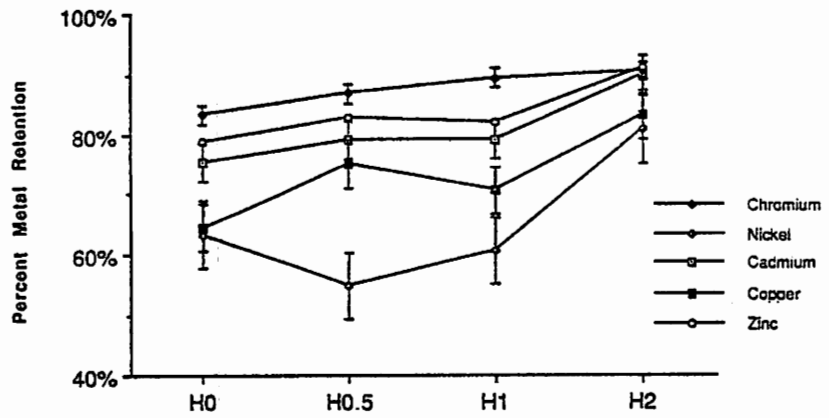

Figure 5. Mean metal retention of each hydroperiod at the low loading rate (error bars = ± 1 S.E.).

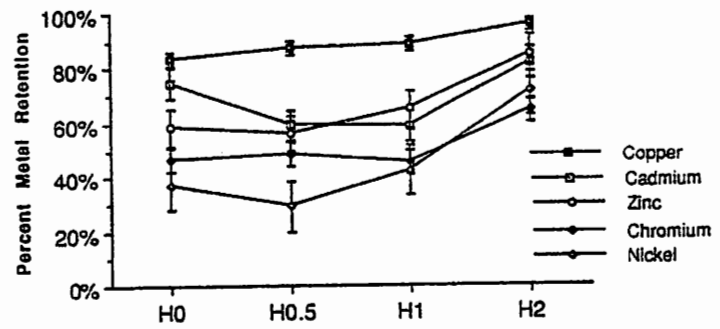

Figure 6. Mean metal retention of each hydroperiod at the high load rate (error bars = ± 1 S.E.).

(Figure 4). The frequent drying out of the wetland soils in the H2 regime increases penetration of oxygen and raises the redox level, thereby stimulating the formation of hydrated ferric oxides, which tend to absorb phosphate.

Metal Removal

The constructed wetland mesocosms effectively removed each metal added at the low loading rate, with mean removal efficiencies of 80–90% for cadmium, chromium, and zinc, and 65–70% for nickel and copper (Figure 5). Removal efficiencies decreased to 40–70% for all metals tested except copper (90%) at the high loading rate (Figure 6). At both loading rates, nickel showed the lowest removal efficiency.

When the various hydroperiod regimes were compared at the low loading rate, metal removal was generally equally efficient in all four treatments (Figure 5). At the high metal loading rate, however, highest metal removal value was observed in the H2 treatment with twice-daily drainage (Figure 6). Although the continuously flooded (H0) treatment had the highest concentration of sulfide in the pore waters, metal removal was not always as high as in the H2 treatment. These results indicate that the generally higher redox values prevailing in the H2 treatment lead to the formation of metal-absorbing iron oxyhydroxides, thus enhancing metal removal in the H2 treatment as compared to the more often flooded treatments.

The metal levels in the plant biomass did not exceed 0.05 mg cadmium g^{-1} dry weight (dw), 17.0 mg copper g^{-1} dw, or 70 mg zinc g^{-1} dw, even though the mesocosms had received inputs of 1.5 g cadmium, 1.8 g copper, and 11 g zinc by the time the plant metal levels were measured. Figure 7 shows the distribution of these three metals in the wetland mesocosms. Metal concentrations in the plant biomass and in the wetland effluent were measured directly, while the amount in the soil fraction was estimated by difference. The low metal levels in the aboveground plant biomass suggest that bioaccumulation of metals in the food chain

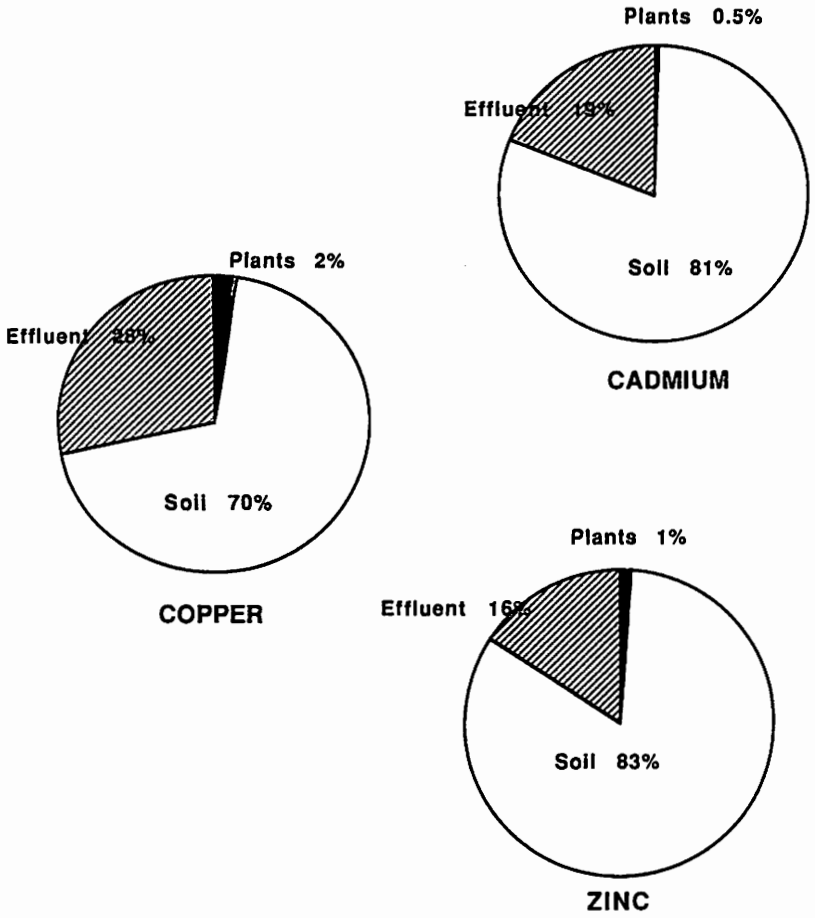

Figure 7. Proportion of cadmium, copper, and zinc in mesocosm compartments.

may not be a problem in these wetland systems.

Plant Biomass

The vegetative growth of bulrush (from rhizome cuttings) was very high in the wetland mesocosms. Plants were well established by July 1990, with a mean stem height of 94 cm and a mean stem density of 68 shoots per m^2 on July 18, 1990. By August 1991, the standing biomass level was extremely high, with means of 11,576 g m^2 for aboveground biomass and 5,352 for belowground biomass. Indeed the total plant productivity of 15,559 g m^2 yr^{-1} is among the highest estimates ever reported. This may be due to the high nutrient input, the warm climate of San Diego, and the fact that the bulrush leaves draw sunlight from an area about three times that of the mesocosm containers. Because of these very high levels of standing crop, the uptake of nutrients by plants was higher than in the typical wastewater wetlands. In our mesocosm, we estimate that 50% of nitrogen and 50% of phosphorus were removed from the influent wastewaters by plant uptake. The remaining fraction of nitrogen and phosphorus was removed by sequential nitrification-denitrification and adsorption on ferric oxyhydroxides, respectively.

Conclusions and Recommendations

This study points out the efficiency of a pulsed-discharge regime for a wetland treatment system upstream of the Tijuana Estuary. Wastewater may be impounded and released on a tidal cycle, with an improved nitrogen-, phosphorus-, and toxic-metal removal efficiency compared to a continuously flowing system. These constructed wetlands provide for pollutant removal, wildlife habitat, and hydroperiod control in a single system.

Because wastewater discharges (even of tertiary quality) to coastal streams are projected to increase in volume in the southern California region, it is imperative that nutrient and toxic removal, as well as salinity dilution, be managed to protect downstream estuaries.

Cooperating Organizations

California Coastal Commission: Provided permission for the facility to be developed in the coastal zone.

California Department of Parks and Recreation: Provided manpower and heavy equipment to prepare the mesocosm site; also donated miscellaneous supplies for construction of mesocosm facility.

City of Imperial Beach: Assisted with water hook-up and sewer connection.

NOAA's Sanctuaries and Reserves Division: Provided complementary research funds in 1990–91. A second trainee, Theresa Sinicrope, was funded to work on metal removal.

U.S. Fish and Wildlife Service: Provided use of land for mesocosm facility at the Tijuana Slough National Wildlife Refuge, Imperial Beach, California. The Refuge land is part of NOAA's National Estuarine Research Reserve.

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Experimental Studies on the Response of Cohesive Sediments to Wave Motion

University of California, Berkeley
R/CZ-89
1989–91

James R. Hunt and Mostafa A. Foda

Recent experimental studies have shown that water waves can sometimes fluidize soft soil beds. The fluidization process is observed as a rather sudden rise in the *mean* pore pressure inside the soil bed. In some locations, the magnitude of this rise sometimes approaches the value of the submerged weight per unit area of the soil column above. This corresponds to nearly reaching a stage of total fluidization, where a block of soil particles becomes effectively in complete suspension, with its weight now supported almost entirely by the intervening fluid, and thus accounting for the net rise in fluid pressure. Partial fluidization response corresponds to partial suspension of the soil particles and hence a smaller rise in the mean fluid pressure inside the bed. Limited laboratory wave flume measurements by Clukey et al. (1983) show that states of total or partial fluidization can be achieved in a soil trench below shallow water waves. This is a similar wave flume study, conducted at the Richmond Field Station, of the University of California, Berkeley to examine the fluidization process more closely and in greater detail.

Experimental Setup

A soil basin was built inside the 8 ft (W) × 5 ft (H) × 180 ft (L) experimental wave flume, which is equipped with a mechanical wave maker at one end and an absorbing sloping beach at the other end. The dimensions of the wooden soil basin are 3 ft in width, by 2 ft in depth, by 10 ft in length. Two movable internal walls, to divide the width and/or the length of the soil basin, were used to examine the response for different basin dimensions. The basin was placed on the floor of the 5 ft deep wave flume, and a false floor was placed flush around the rim of the basin, with a total elevated floor area (including the basin area) of 40 ft × 8 ft. Two wooden, gently sloping ramps were placed at the ends of the elevated floor to provide a smooth transition for the water waves as they propagated above. The clearance above the soil basin allows for an overlying 1.75 ft deep water layer (3.75 ft water depth from the flume real floor). The special and time-consuming task of filling the soil basin with soil took place before the flume was filled with water. The selected soil was a commercially available fine soil, which is of the silty type (mineral composition: silica, alumina, iron oxide, etc.) with a mean grain size D_{50} = 0.05 mm. A thick slurry is first made by thoroughly mixing amounts of soil and water in a container and then carefully and slowly placing the slurry into the basin. After a settling period, the separated overlying water layer is drained out and another slurry layer is placed. This process is repeated until the whole basin is filled with the saturated soil. Several days are then allowed for soil consolidation before the flume is filled with water up to a depth of 1.75 ft above the soil basin. Two columns of pressure transducers were placed inside the soil basin during soil placement to constitute the deployed two-dimensional array pore-pressure measurement scheme. One column of four pressure transducers (Diphram-type, Model AB of Data Instruments) was placed in the middle of the basin, and one with two or three pressure transducers was placed near one of the basin's walls (commonly near the beach side or transverse side walls of the basin). Data from the pressure transducers and wave gauges, placed above the soil basin, were processed with the aid of an IBM-AT personal computer.

Experimental Results

Figures 1, 2, and 3 show sample records of pore pressure measurements at selected points inside the soil basin, and under a monochromatic water wave of height H = 9.5 cm and period T = 1.8 sec. Figure 1 shows the measured water pressure at the mudline, or the interface between the water and soil layers. This represents the direct pressure loading from the propagating water wave on the underlying soil (checks well with the linear shallow water wave theory). This should be contrasted with Figures 2 and 3, which show the pore pressure response at depth 8 in. and 14 in., respectively, due to this almost steady harmonic mudline loading. Observe first that there is a gradual rise in the *mean* value of pore pressure, above its hydrostatic value, as soon as the wave starts to propagate above the soil trench.

Ultimately, the mean pore pressure reaches a new steady-state value at both depths. The net increase in the mean pore pressure is also observed to be approximately the same for depth 8 in (Figure 2) and depth 14 in (Figure 3). This net rise in the mean pore pressure is believed to be the result of a partial suspension of some of the bed sediments (above 8 in deep in this particular case). These suspended sediments are now entirely supported by the pore water, thus accounting for the rise in the mean pressure. Pore pressure measurements above the 8 in depth show smaller net rise in the mean pore pressure, indicating that the resuspension process is taking place in a surface layer at a depth of 8 in for this particular experimental run. Below this layer of resuspension, there is no further increase in the net rise in the mean pore pressure, i.e., no further sediment

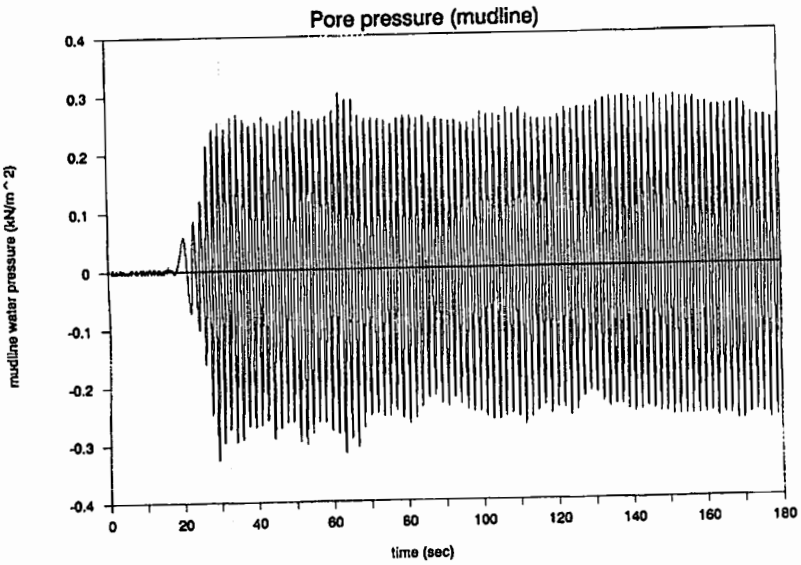

Figure 1. Mudline water pressure, water wave height $H = 9.5$ cm, and wave period $T = 1.8$ sec.

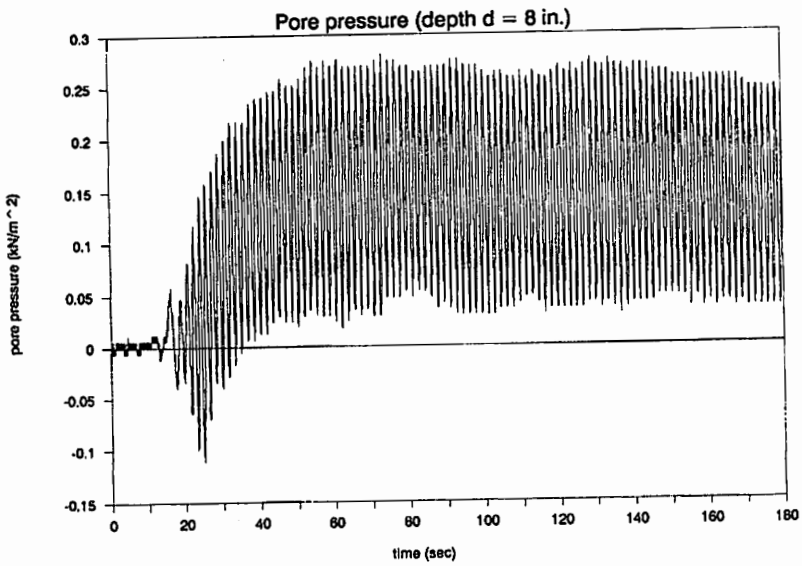

Figure 2. Pore pressure response at depth $d = 8$ in below mudline, $H = 9.5$ cm, $T = 1.8$ sec.

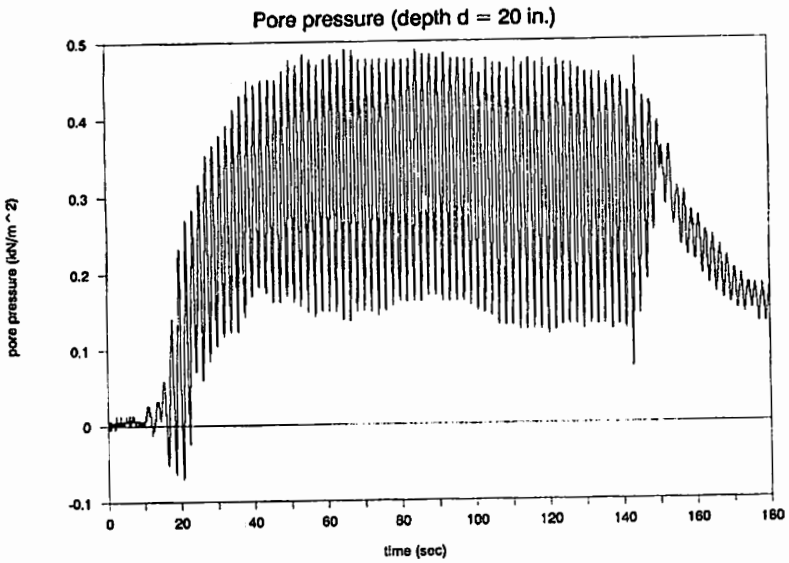

Figure 3. Pore pressure response at depth $d = 14$ in below mudline, $H = 9.5$ cm, $T = 1.8$ sec.

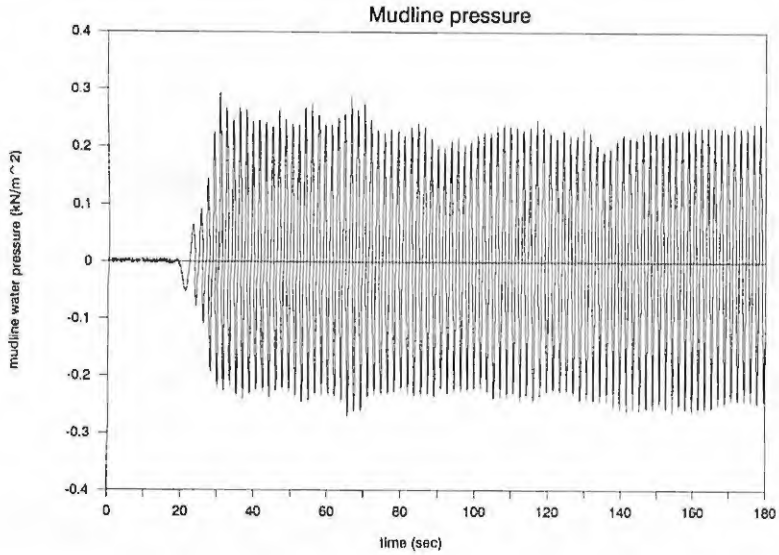

Figure 4. Mudline water pressure under water wave height H = 12.1 cm, and wave period T = 1.38 sec.

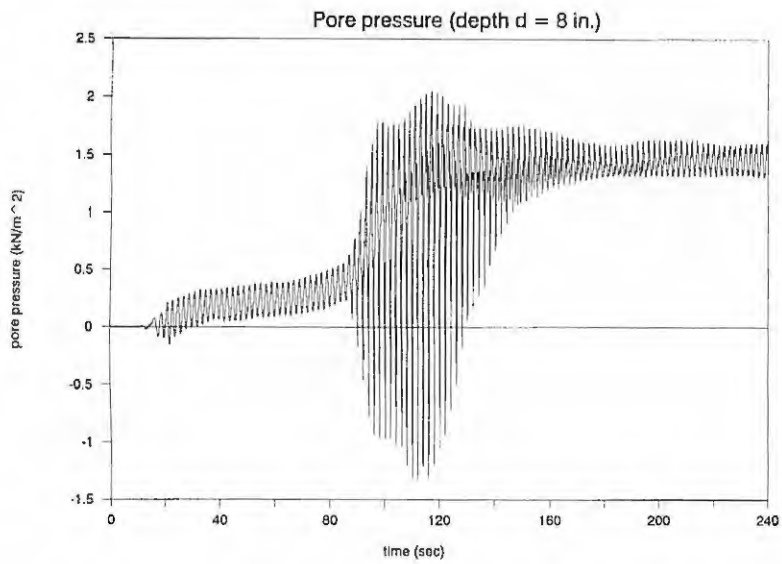

Figure 5. Resonant pore pressure response at depth d = 8 in below mudline, H = 12.1 cm, T = 1.38 sec.

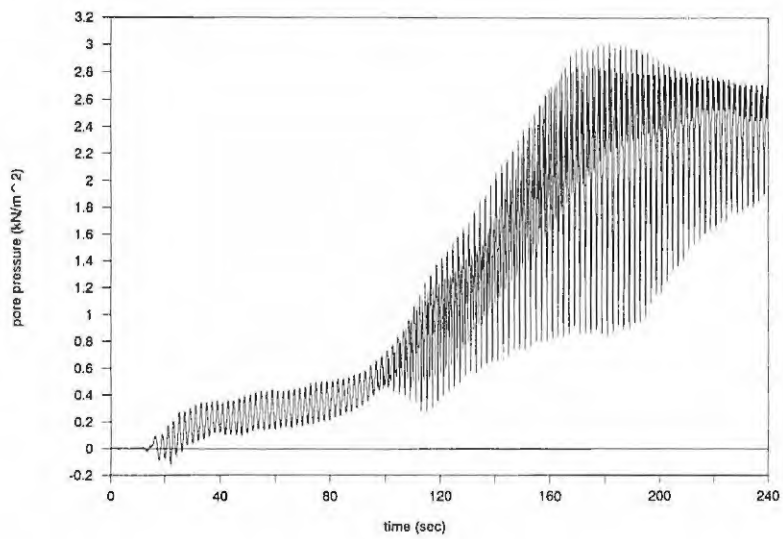

Figure 6. Resonant pore pressure response at depth d = 14 in below mudline, H = 12.1 cm, T = 1.38 sec.

suspension below this depth.

Figures 4, 5, and 6 show another set of pressure records under a somewhat larger wave of height $H = 12$ cm and period $T = 1.4$ sec. A very different pore pressure response is observed in this case, compared to the previous one. Here, there are typically three distinct phases of the pore pressure response in the soil bed. Initially, we start with a response similar to that shown in Figures 2 and 3, where there is a gradual rise in the mean pore pressure, on top of an oscillatory response. The gradual rise is associated with a partial suspension of the soil particles near the mudline. The oscillatory part of the response in the initial phase is found to check relatively well with predictions from the linear theory of poroelasticity due to Biot (1941). This theory assumes that the skeleton of the soil behaves elastically, following Hooke's law, and that the intervening viscous pore fluid obeys Darcy's law.

Turning to Figures 5 and 6, however, we note that both the amplitude of fluctuation and the net rise in the mean pressure during this initial phase are rather small in comparison with those in the following phases of the response. For example, in Figure 5 around time $t \sim 70$ sec, a second phase of the response is initiated, characterized by a rather sudden increase in pore-pressure fluctuations. This is brought about by the appearance of a new oscillation which grows in time to dominate the signal in less than ten cycles. Observe that the new oscillation, which has the same frequency as the forcing water wave, starts to appear as a very small departure from the linear poroelastic response of the first phase. This departure is magnified while maintaining a harmonic structure every following wave cycle, until the linear response is drowned, and becomes hard to detect, in this new resonated oscillation. Observe also from Figure 5 that much of the total rise in the mean pore pressure has occurred after the onset of this resonant oscillation. This may suggest that this new oscillation plays a significant role in the observed liquefaction process.

This short "resonance" phase is followed by a third or final phase, where the amplitude or oscillation as well as the net rise in the mean pore pressure will asymptotically reach steady-state values. These new steady-state values are larger than those predicted by the linear poroelasticity theory and observed in the initial phase as shown in Figures 5 and 6. This simply means that part of the solid skeleton of the soil mass, which was entirely self-supported at the beginning, has been converted to the fluid phase, or *liquefied*, and is now in suspension in the more energetic pore water. The weight of this liquefied part of the solid skeleton per unit area is equal, on the average, to this observed net rise in the mean pore pressure during this final steady-state phase. In all of our experiments, however, such observed mean pressure rise never reached the value of the submerged weight of all of the solid particles, per unit area, above any particular transducer. This implies that the observed *partial* liquefaction process has dismantled only a part of the soil's solid skeleton but has not totally demolished it under the conditions of our experiment.

Conclusion

The experimental results show that the response of a silty soil bed to water wave loading is typically composed of three distinct phases. Initially, there is a poroelastic response governed by the linear Biot's equation, plus a slow buildup of the pore pressure inside the bed. This slow buildup is believed to be associated with some inelastic particle-particle rearrangement process, such as the so-called soil shake-down effect. This is followed by a sudden resonant transition, where new harmonic oscillations start to appear and quickly dominate the response. These resonant events appear to be, at least initially, localized events. Complex transition will follow as these resonant oscillations reach maturity and asymptotically approach a new equilibrium state, signifying a third or the final phase of the observed response.

One major characteristic of this final steady-state phase is that there is a significant rise in the *mean* pore pressure, above its initial hydrostatic value. This means that in this new equilibrium phase, there has been a conversion of part of the solid skeleton to become, dynamically, a part of the pore fluid. The weight of this converted, or liquefied part of the skeleton is now fully supported by the intervening pore water, i.e., in suspension in the pore water and hence accounting for the net rise in the mean pore pressure.

Cooperating Organizations
Japanese Ministry of Education
Maizuru College of Technology, Maizuru, Kyoto, Japan.

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Southern California Waves: Model Verification and Utilization

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R/CZ-90
1989–92

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Surface gravity (i.e., wind and swell) waves are an important factor in coastal planning and resource utilization. It is not practical to measure waves at the large number of locations where information about wave conditions is needed within the Southern California Bight. The overall goal of this 3-year project was to develop and field test a wave model that uses a limited data set of observations at a few locations to estimate wave energy at uninstrumented sites. Additionally, the possibility of extending the effective spatial coverage of an existing data collection program within the Bight was explored. The project, jointly funded by Sea Grant and the California Department of Boating and Waterways, thus involved both model development and field data collection.

Two existing models, a spectral refraction and a spectral refraction-diffraction model, were adapted for the Bight. Spectral wave models are only now coming into routine use in the coastal engineering community, and two papers discussing these two models were published as part of this Sea Grant project, (O'Reilly and Guza, 1991; 1992). These models are unique in that the size of the area being modeled (the entire Southern California Bight) is much larger than typically considered.

In order to verify the accuracy of the models, two 3-month field deployments of shallow water (30 m) wave gauges were made. The first deployment, from mid-August to early November 1991, was designed to study Southern Hemisphere waves using 10 gauges. The second deployment, from early November to mid-February 1992, used 11 gauges and a directional deep-water buoy and was tailored to monitor Northern Hemisphere waves. Gauge sites for both deployments were selected by using an optimization technique known as "simulated annealing," which resulted in measurements that contained the minimum amount of redundant information, based on the predictions of the spectral refraction-diffraction wave model. Both deployments were extremely successful, with a combined data return of more than 95%. The resulting measurements are summarized in a data report (O'Reilly et al., 1992).

Initial analysis of the winter data suggests that potentially useful predictions of wave energy can be made in many areas within the Bight by using measurements from a deep ocean (outside the islands) directional buoy. The predictions are based on deep ocean wave spectra estimates derived from the buoy data using a spectral estimation technique known as the maximum entropy method (MEM). These deep ocean spectra are then used as input to the spectral refraction wave model to make wave predictions within the Bight. These preliminary findings have led to specific recommendations for the expansion of existing wave monitoring networks, as well as for future field verification experiments (O'Reilly and McGehee, 1992).

Preliminary comparisons of the measurements and predictions also show that more sophisticated prediction schemes are necessary in some of the more geographically complicated regions in the Bight (e.g., near coastal submarine canyons, the east end of the Santa Barbara Channel). Additional analysis of the field data is continuing with support from the California Department of Boating and Waterways. Specifically, mathematical techniques broadly defined as "inverse methods" are being used to combine both the buoy data and the shallow water gauge data to make higher resolution deep ocean directional spectra estimates. It is hypothesized that these inverse estimates are more accurate than those obtained using the buoy alone and that they will lead to more accurate wave predictions throughout the Bight when used as input conditions for the spectral wave model.

Cooperating Organizations

California Department of Boating and Waterways
U.S. Army Corps of Engineers

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Scales of Variability of Sewage-Influenced Water Column Properties in the Southern California Bight

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R/CZ-92
1989–91

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The nearshore waters of the Southern California Bight are intensively utilized by human populations, and the rate of utilization for all purposes has increased in the past 15 or 20 years.

One of the most obvious and controversial of these uses is sewage disposal. In 1985 about 4,500 million liters of sewage effluent were discharged daily in the Bight, mostly through four major submarine outfalls. This represents an input greater than the natural input from rivers, runoff, and storms.

There has been concern that these artificial rivers may affect water quality, and thus may have long-term chronic effects on the ecology of the area, not only at the local point-source scale but also on the entire Southern California Bight system. Numerous, very costly solutions to the problem have been proposed.

Because of the high natural variability in oceanic physical and biological properties, it is essential that we be able to distinguish natural from human-induced variations, prior to assessing a human effect on the variables studied. The study of long-time series can help in making this distinction.

Modern statistical methods of time-series analysis permit the data to be examined for temporal and spatial trends, dominant frequencies, and spatial dimensions of variability at different frequencies. Previous time-series analyses have shown that biological and physical properties in the Bight undergo synchronous, low frequency changes. Such changes are indicative of Bight-wide forcing factors, rather than of local sewage inputs.

Fortunately, there exist three excellent, concurrent time series of water-column monitoring data. These are the data collected by monitoring agencies for 15 or more years with weekly or monthly frequencies at stations near and far from (controls)—the sewage outfalls of Santa Monica Bay, Palos Verdes, and Point Loma. These time-series encompass a period during which there was an enormous increase in the population around the Southern California Bight, as well as natural oceanographic changes, including the powerful El Niño of 1983–84. These data (on temperature, light transmissivity, dissolved oxygen, and Secchi disk depth) are excellent proxies for describing the state of the system, and can be used to shed light on how water-quality measurements vary through time, and on how they relate to the anthropogenic discharge. Because of the spatial separation of the three outfalls, the relative importance of natural, large spatial scale (Bight-wide), environmental variability versus that of local point-sources can be studied.

Results

Most of the following discussion refers to the two water clarity properties (beam transmissivity and Secchi transparency). Temperature and dissolved oxygen were not analyzed to the same level of detail because preliminary analyses showed that these results were less original (temperature) or less conclusive (oxygen) than were the water clarity properties.

Beam transmissivity and Secchi transparency are affected by the concentration of particles in the water and are therefore influenced by both the living plankton, particularly phytoplankton, and other suspended matter. The sewage effluent affects water clarity by adding particles (suspended solid discharge) and by adding dissolved matter to the water (discharge flow).

Four major questions were addressed by this research: (1) What are the main patterns of spatial and temporal variability of the water clarity near the sewage outfalls in the Southern California Bight? (2) What are the main temporal patterns of sewage discharge in the Bight? (3) Can we establish a relationship between these and sewage discharge? (4) Can we distinguish between human-induced and natural variability in water column properties relating to water clarity?

With regard to the first question, time-series analysis showed that the main patterns of temporal variability in water clarity were similar across the Bight. Water clarity was highly variable over time, but displayed similar means and ranges at all three sites. Neither Point Loma nor Palos Verdes revealed long-term trends, but at Santa Monica the water became clearer over time. In other words, there is no indication of overall degradation in the water clarity at the three areas. Variance spectra at both Santa Monica and Point Loma were dominated by seasonal peaks, while those at Palos Verdes approached randomness.

Spatial analysis revealed that there are no long-shore spatial gradients "upstream" or "downstream" from any of the outfalls in the long-term means of water clarity. Instead, both water clarity properties were dominated by an inshore–offshore gradient, with murkier waters nearshore. As measured in Secchi depth, the nearshore reduction in transparency was accompanied by a specific reduction in the annual band of variability and the onset of random variability. This gradient seemed to be related to distance from the coast rather than to bottom topography.

Water clarity seems, therefore, to be dominated by different processes nearshore (less than 2 km) than farther from shore: the murky water

near the coast may be the result of land phenomena, such as runoff or bottom sediment resuspension, while the seasonally changing clarity offshore may depend on biological processes, such as phytoplankton blooms.

The main temporal patterns of sewage discharge are as follows. At all three locations, the overall flow discharge increased over the 15 years (showing upward trends), reflecting population increase. On the other hand, the curves of suspended solid discharge at the three sites were quite dissimilar, no doubt reflecting different histories of management and local regulation. Because of treatment improvements, suspended solid discharge decreased off Palos Verdes and Point Loma. However, this decrease was not reflected in an increase in water clarity at these locations. In Santa Monica, the pattern was different; a sharp augmentation in suspended solid discharge in the early 1980s was followed later in the decade by an equally sharp decline. This is the area where transparency increased over the 15 years. While most of the variability in water clarity resulted from seasonal changes (except for Palos Verdes), most of the variability in flow and suspended solids was due to interannual fluctuation, showing very different cyclicity between anthropogenic discharge and water properties.

In general, the temporal patterns of sewage discharge were different from those of water clarity. Most important, the difference in the sewage discharge pattern at the three outfalls confirms that across-Bight changes in water quality would not be caused by the human discharge, but rather by large-scale phenomena that can be approximated to natural variability.

Finally, we attempted to correlate water clarity and discharge variables across the Bight and within each area in order to see whether variability in water clarity is driven by Bight-wide oceanographic phenomena or by local sewage discharge. The rationale is that large spatial and temporal-scale changes in water clarity can be interpreted as most likely the result of natural causes *if* there is a pattern of correlation across the Bight and with the control stations, and *if*, at the same time, these changes are not correlated locally to sewage discharge. Such Bight-wide low-frequency variations on these scales are known to occur naturally in the sea surface temperature and in the plankton abundances of the Southern California Bight. Conversely, if water clarity is unrelated across the Bight, but locally related to the discharge, it can be interpreted as the result of anthropogenic modulation.

None of the time-series analyses showed evident association between water clarity and sewage discharge. There was no correlation at any lag between water clarity and sewage discharge; there was no serious difference between the control and the test subgroups in any of the analyses; systematic trends of water clarity and discharge were not related; water clarity was dominated by a seasonal signal while the main signal in the discharge properties was interannual; and there were Bight-wide correlations in water clarity, both in the raw and in the deseasonalized data. Furthermore, spatial analyses showed no relation between water clarity and distance from the outfall.

The across-Bight comparisons did not, however, provide a clear-cut picture. In fact, almost all test water clarity subsets were less correlated between sites than were controls (particularly once deseasonalized and detrended), which suggests that there may indeed be an effect, although minor, from the sewage discharge. Until more is known of the longer term and larger spatial-scale relationships of the natural variability of optical properties, plankton biomass, and temperature at multiple sites far removed from the possible influence of local sewage discharges, these latter observations will remain unresolved.

Cooperating Organizations

City of Los Angeles, Department of Public Works, Bureau of Sanitation
Los Angeles County Sanitation Districts
Point Loma Treatment Plant

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Assessment of Sublethal Toxic Effects in Marine Organisms by NMR Spectroscopy

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R/CZ-97
1990–93

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The toxicity of a chemical is a function of both its biochemical action and the presence and effectiveness of inherent detoxication mechanisms; environmental factors may contribute in synergistic or antagonistic fashion. The description of pollutant effects on marine organisms has previously involved toxicity testing, where behavioral or morphological responses are measured *in vivo* (Parrish, 1985; McKim, 1985). While such techniques are able to determine harmful seawater concentrations, they are not sensitive enough to detect sublethal stress, and they provide little information on mechanisms of toxic action. Conversely, description of the biochemical actions of toxicants in marine organisms has always involved invasive *in vitro* techniques, where neither whole organism responses, nor the interaction of natural stress factors, can effectively be measured (Mehrle and Mayer, 1985).

Techniques combining high sensitivity and use of intact, unstressed organisms to describe sublethal effects are needed to assess the long-term effects of pollutants, detect sublethal effects of presently unrecognized chemical hazards, and understand how marine animals respond to environmental stresses. The field of *in vivo* nuclear magnetic resonance (NMR) spectroscopy presents a major opportunity for investigation of sublethal effects in living marine organisms; biochemical processes may be measured *as they occur*, advancing our understanding of toxic action mechanisms and the interactive effects of environmental stress factors.

Many pollutants act by inhibiting or uncoupling mitochondrial oxidative phosphorylation (Corbett, 1984). Therefore, to develop *in vivo* techniques and demonstrate the ability of NMR to assess sublethal toxicity, we have described the effects of a representative agent, the wood preservative pentachlorophenol (PCP), on biochemical processes in a marine gastropod, the red abalone (*Haliotis rufescens*), and in a bivalve, the Pacific oyster (*Crassostrea gigas*). Both are representative of the wide variety of molluscs inhabiting intertidal regions, and their large size facilitates use of surface-probe NMR techniques, where sufficient organ size allows localized signal acquisition without interference from adjacent tissues.

NMR allows the measurement of biochemical responses *in vivo* by monitoring endogenous "energy" compounds. Using ^{31}P, *in vivo* changes simultaneously measurable include those in tissue levels of endogenous phosphagens such as phosphoarginine or phosphocreatine (PA and PC, respectively: high-energy storage molecules); nucleoside phosphates (NPs), such as ATP; phosphoesters; inorganic monophosphate (P_i); and intracellular pH (pH_i; Gadian, 1982). Such measurements allow description of the effects of either natural stress factors or pollutants on mitochondrial electron transport and oxidative phosphorylation.

Presently, acceptable environmental levels are determined using "no-observed-effect levels" (NOELs; Parrish, 1985). However, many marine populations continue to decline, indicating that energy metabolism may still be impaired. While adult die-offs are rare, growth rates and fecundities may be affected, reducing the quality of marketable species and their population sizes. Thus, we used standard toxicity tests to determine NOELs for use in later *in vivo* ^{31}P NMR experiments. Use of phosphagens to measure sublethal effects is similar in concept, but more sensitive than previously used "scope-for-growth" measurements. Emergence (part of the daily tidal cycle) and different seawater temperatures were used to determine the interactions of natural stress factors. In addition, we described the disposition (bioconcentration, biotransformation, and depuration) of PCP to obtain a better understanding of the inherent detoxication mechanisms mitigating the effects measured by *in vivo* NMR. Finally, we have developed specific criteria for use of NMR in assessing sublethal toxicity in marine organisms.

Project Objectives

The objectives of this investigation were as follows: (1) To modify the existing flow-through exposure system for use with NMR and radiotracers; (2) To develop high-pressure liquid chromatographic (HPLC) methods for analysis of PCP and its metabolites; (3) To measure the toxicity (6-h LC50s and NOELs) of PCP in abalones and oysters; (4)To describe the sublethal effects of both 6-h NOELs of PCP, as well as recovery, in abalones and oysters by *in vivo* ^{31}P NMR, correlating them to later HPLC measurements; (5) To describe the sublethal effects of the 6-h NOELs of PCP in abalones and oysters following acute re-exposure by *in vivo* ^{31}P NMR; (6) To determine the disposition of [^{14}C]PCP in both abalones and oysters, comparing them to both the NMR results and representative fishes; (7) To determine the interaction of air exposure (emergence) on the sublethal effects of PCP in abalones by *in vivo* ^{31}P NMR; (8) To determine the interaction of different seawater temperatures on the sublethal effects of PCP in abalones by *in vivo* ^{31}P NMR; (9) To develop criteria for use of NMR in assessing sublethal toxicity in marine organisms.

Research Results

Effects of PCP in Molluscs as Measured by ^{31}P NMR

The sublethal biochemical effects of PCP were investigated in live, intact red abalones, using a modified flow-through exposure system (Figure 1), by *in vivo* ^{31}P NMR. Based upon the results of range-finding tests (6-h LC_{50} = 1.6 mg L^{-1}; 6-h NOEL = 0.8 mg L^{-1}), abalones (n = 3) were exposed to 1.2 mg L^{-1} of PCP for 5 h, followed by a 13-h recovery period. Effects in foot muscle included both a decrease in the concentration of PA ([PA]) and an increase in [P_i], respectively (Figure 2A); both pH_i (Figure 2B) and [ATP] (Figure 2C) also declined.

Parallel *in vivo* experiments, incorporating static exposure and gas chromatography-mass spectroscopy, revealed that concentrations of the metabolic intermediates glycerol 3-phosphate, lactate, citrate, succinate, malate, and alanine also increased during PCP exposure, while those of glyceraldehyde 3-phosphate and glutamine remained stable (Figures 3A–D). Also, these effects were not evident until 2 h into exposure, possibly the time required for PCP to attain an effective concentration in foot muscle.

During recovery, while P_i declined to pre-exposure levels, [PA] completely recovered in only one individual. Also, realkalinization of pH_i was similar to recovery of [P_i], and ATP returned to near-initial levels, as did glycerol 3-phosphate, lactate, succinate, malate, and alanine; glyceraldehyde 3-phosphate, citrate, and glutamine levels declined. Recovery responses corresponded to the time for PCP clearance from foot muscle. Overall, the effects of PCP were similar to those observed in other aquatic organisms from hypoxia (Barrow et al., 1980; Dubyak and Scarpa, 1983; Ellington, 1983; Butler et al., 1985; van den Thillart et al., 1989), fatigue (Kamp and Juretschke, 1987; Thebault et al., 1987), or hypersalinity (Higashi et al., 1989). Also, since during PCP exposure both

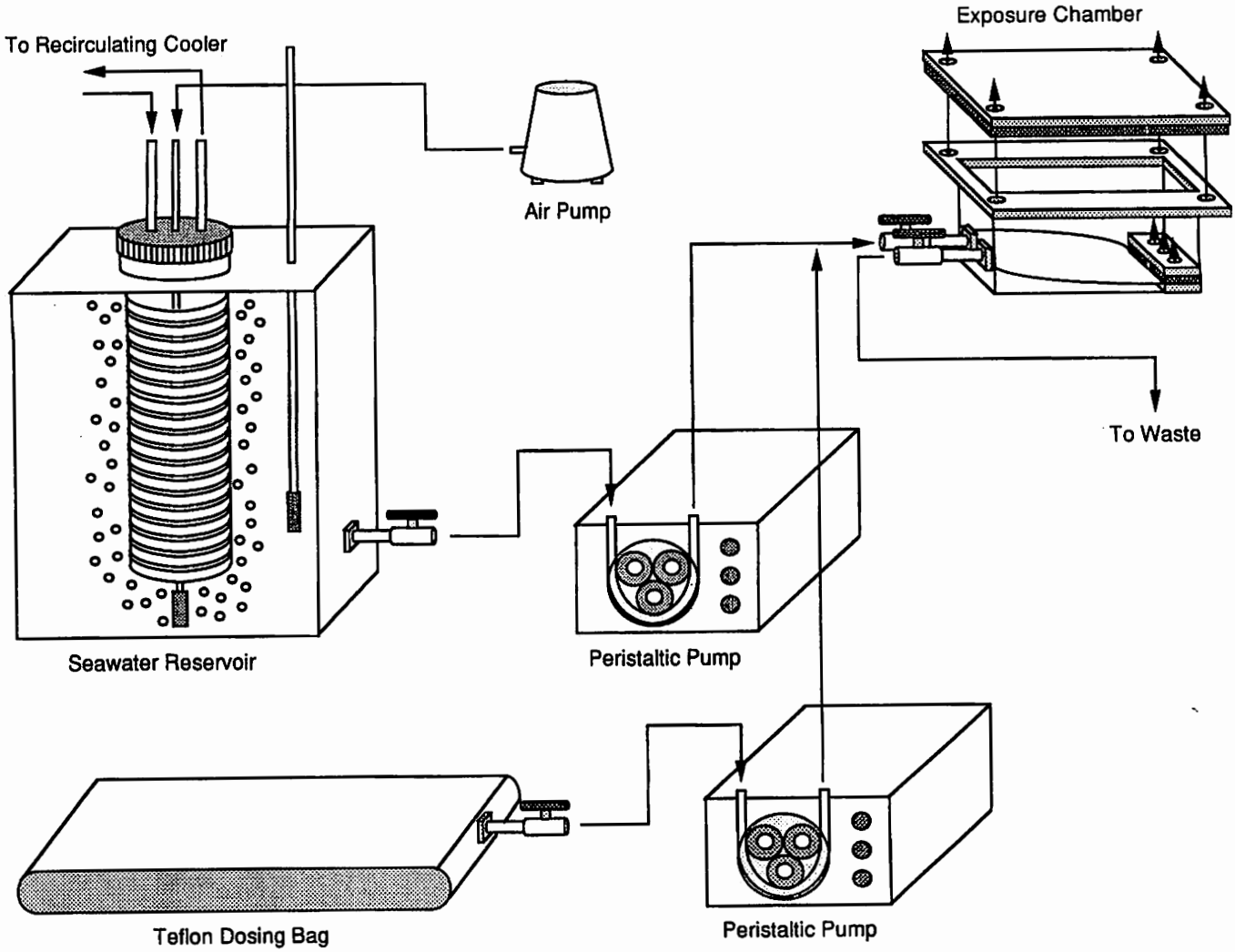

Figure 1. The flow-through exposure system for use with aquatic invertebrates and NMR.

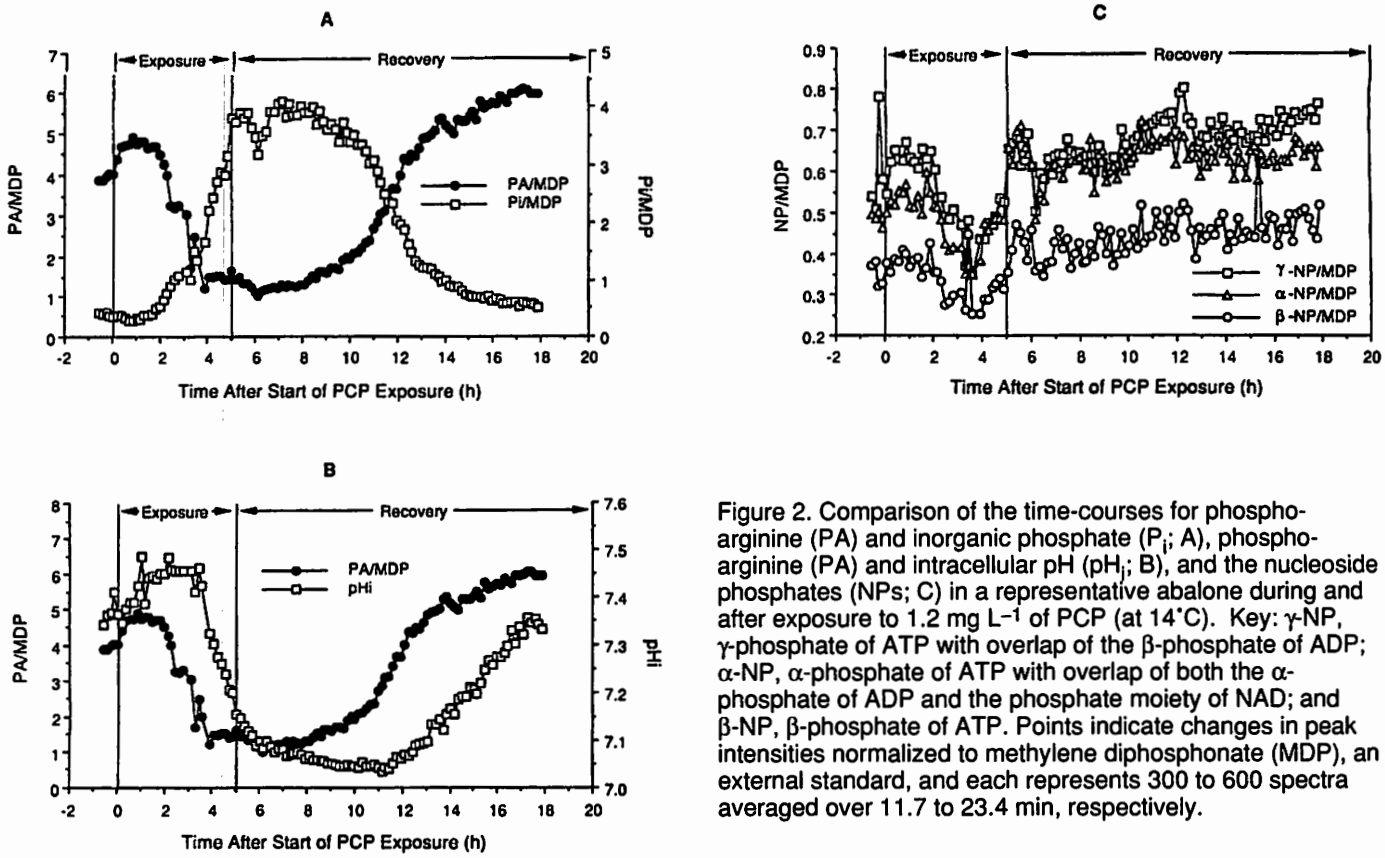

Figure 2. Comparison of the time-courses for phosphoarginine (PA) and inorganic phosphate (P_i; A), phosphoarginine (PA) and intracellular pH (pH_i; B), and the nucleoside phosphates (NPs; C) in a representative abalone during and after exposure to 1.2 mg L^{-1} of PCP (at 14°C). Key: γ-NP, γ-phosphate of ATP with overlap of the β-phosphate of ADP; α-NP, α-phosphate of ATP with overlap of both the α-phosphate of ADP and the phosphate moiety of NAD; and β-NP, β-phosphate of ATP. Points indicate changes in peak intensities normalized to methylene diphosphonate (MDP), an external standard, and each represents 300 to 600 spectra averaged over 11.7 to 23.4 min, respectively.

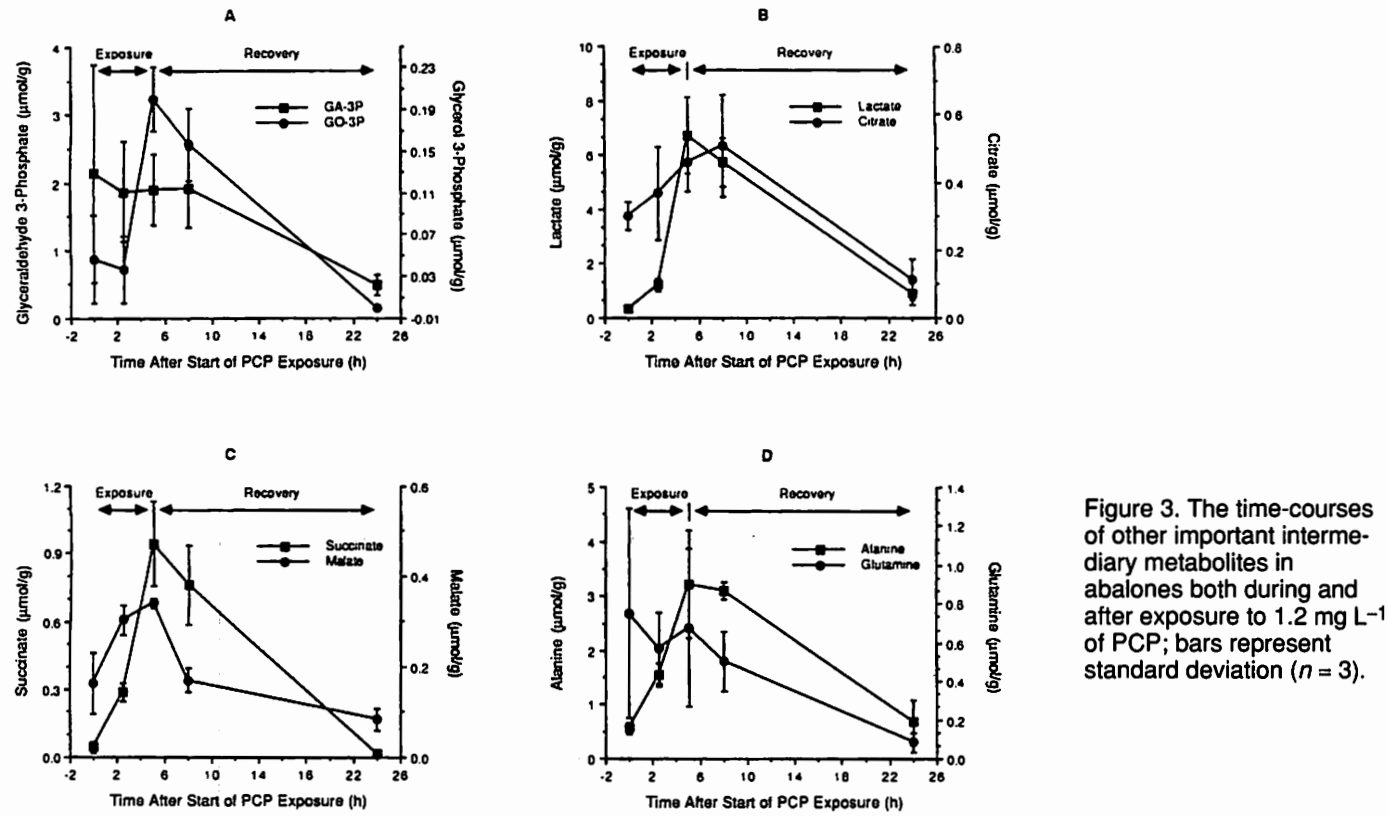

Figure 3. The time-courses of other important intermediary metabolites in abalones both during and after exposure to 1.2 mg L^{-1} of PCP; bars represent standard deviation ($n = 3$).

lactate accumulated and [PA] declined prior to complete depletion of [ATP], sublethal concentrations may also inhibit electron transport and arginine kinase, respectively, as well as uncouple mitochondrial oxidative phosphorylation in intact molluscs (Barrow et al., 1980; Dubyak and Scarpa, 1983; Zubay, 1988).

Because they lacked sufficient muscle mass, bivalves, including Pacific oysters and bay mussels (*Mytilus edulis*), did not provide a consistent strong signal by NMR, and thus they could not be reliably used for determination of sublethal effects. However, using appropriate organisms (with sufficient muscle mass), the biochemical effects of pollutants may now be measured as they occur, greatly improving our ability to assess the environmental effects of pollutants.

Effects of PCP and Hypoxia in Molluscs as Measured by ^{31}P NMR

The effects of hypoxia and PCP were investigated in red abalones using flow-through exposure and *in vivo* ^{31}P NMR. Following acclimation to clean seawater, abalones ($n = 3$) were exposed in the modified exposure system to 1.2 mg L^{-1} of PCP until the spectral resonance for P_i was half-height to that of PA; the endpoint assured that all individuals were metabolically equivalent at the start of air exposure. They were exposed to clean seawater for 2 h to allow the effects from PCP to stabilize, then air for 45 min, and finally clean seawater for 15 h to check recovery.

The effects of PCP in foot muscle again included a decrease in [PA] and an increase in [P_i] (Figure 4A), and decreases in both pH_i (Figure 4B) and [ATP] (Figure 4C). On air exposure, [PA] and pH_i rapidly declined further, [P_i] increased, and [ATP] did not change; the magnitudes were similar to those from emergence alone, which were similar to those previously observed from hypoxia in other organisms (Barrow et al., 1980; Dubyak and Scarpa, 1983; Ellington, 1983; Butler et al., 1985; van den Thillart et al., 1989). After resubmergence,

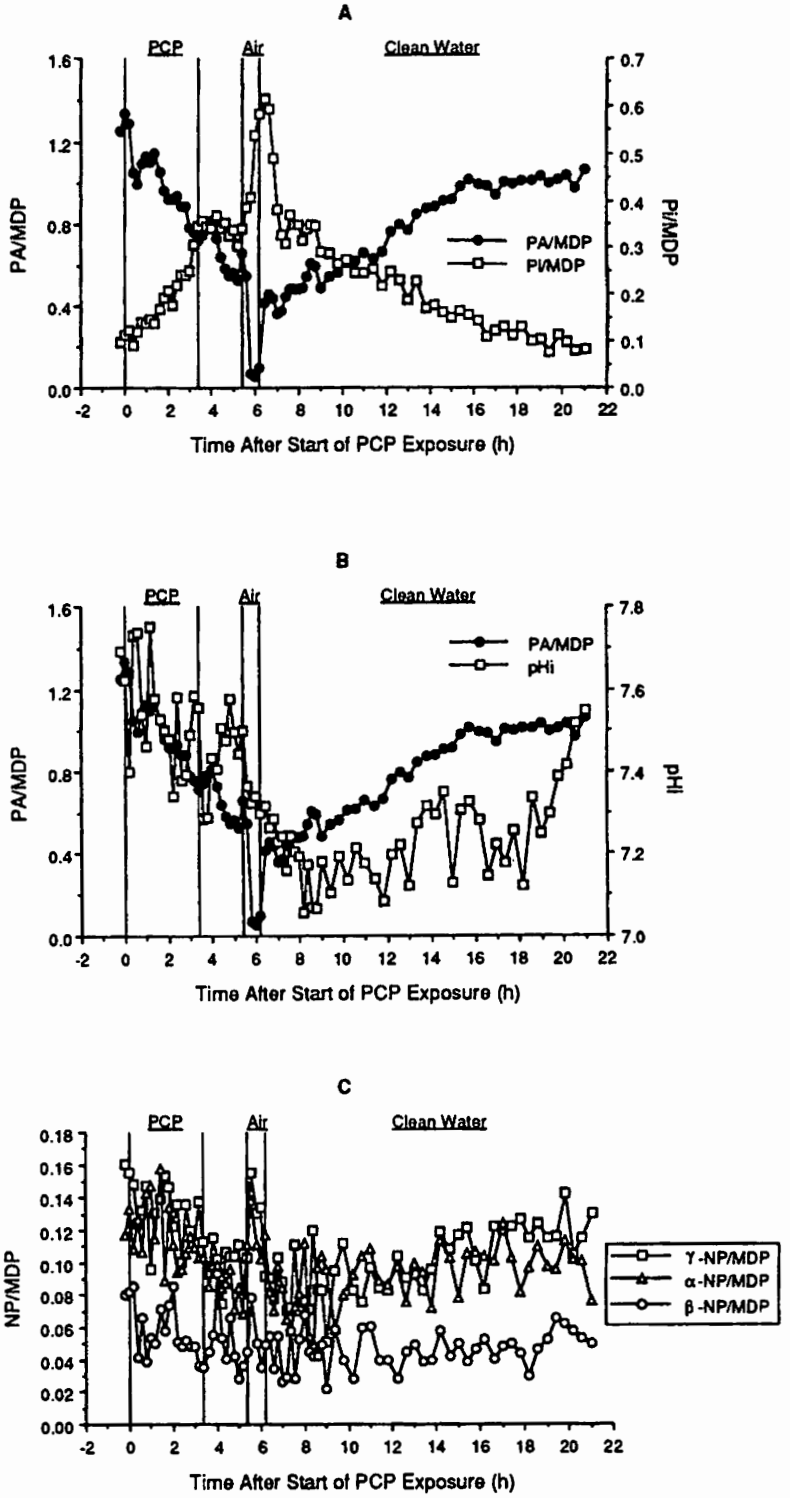

Figure 4. Comparison of the time-courses for phosphoarginine (PA) and inorganic phosphate (P_i; A), phosphoarginine (PA) and intracellular pH (pH_i; B), and the nucleoside phosphates (NPs; C) in a representative abalone during and after exposure to both 1.2 mg L^{-1} of PCP (at 14°C) and air. Please refer to Figure 2 for details.

both [PA] and [P_i] recovered rapidly from the effects of hypoxia, but slowly from those of PCP. While pH_i initially declined further, both [ATP] and pH_i recovered to near-initial levels after 15 h. In general, the metabolic effects of hypoxia alone (Figures 5A–C) did not change upon pretreatment with PCP; the biochemical interaction of the two factors was additive.

Other abalones (n = 3) were exposed to PCP at an environmentally relevant concentration of 120 µg L^{-1} for 6 h, air for 1 h, then the same PCP concentration for 2.5 h to check recovery. PCP produced no discernible effects, and those of hypoxia were similar to those without PCP (Figures 6A–C). Since PCP produced no additional effects with or without hypoxia, it does not significantly impact abalones at currently measured environmental levels.

Effects of PCP and Temperature in Molluscs as Measured by ^{31}P NMR

The influence of temperature on the effects of PCP was described in red abalones using *in vivo* ^{31}P NMR. Specifically, two effects were measured: the exposure times required for onset of PCP effects; and both the intensity of, and recovery from, such effects. Following acclimation to clean seawater, abalones (n = 3) were exposed to 1.2 mg L^{-1} of PCP at either 9° or 19°C until the spectral peak area for P_i was one-half that of PA; temperatures represented the seasonal range in California. The endpoint served to compare the times required to reach equivalent metabolic condition, and insured that all individuals were metabolically equivalent at the onset of exposure to clean seawater; abalones were maintained in clean seawater for up to 16 h to monitor recovery.

In foot muscle, PCP again caused a decrease in [PA] and [ATP], an increase in [P_i], and acidification of pH_i; effects were produced more rapidly at 19°C (Figures 7A–C), as the endpoint was attained in less than half the time (2.4 h) as was that at 9°C (5.4 h; $p < 0.05$; Figures 8A–C). Both average response to, and average recovery from, the biocide were greater in abalones exposed at 19°C. Upon exposure to clean

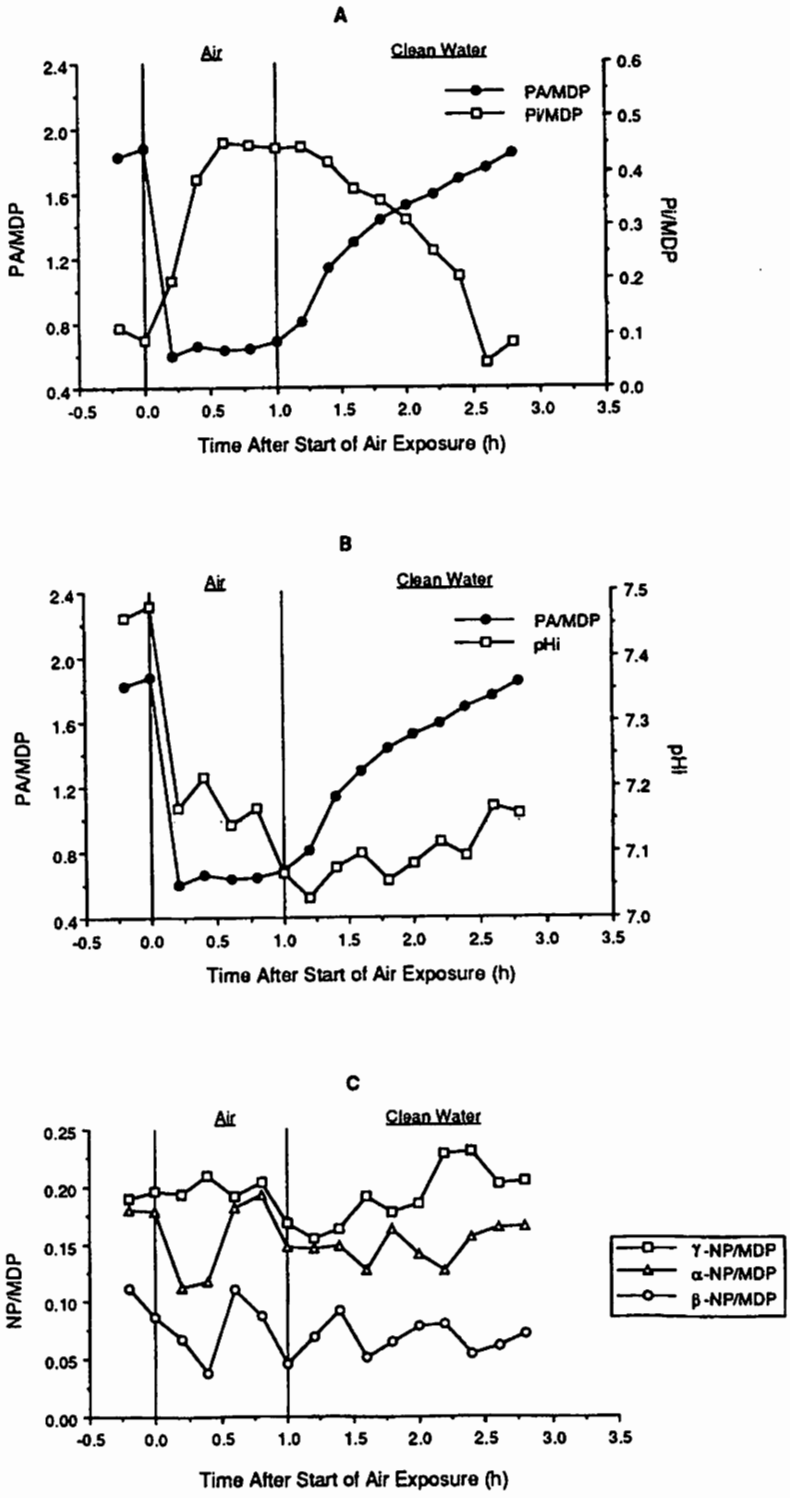

Figure 5. Comparison of the time-courses for phosphoarginine (PA) and inorganic phosphate (P_i; A), phosphoarginine (PA) and intracellular pH (pH_i; B), and the nucleoside phosphates (NPs; C) in a representative abalone during and after exposure to air. Please refer to Figure 2 for details.

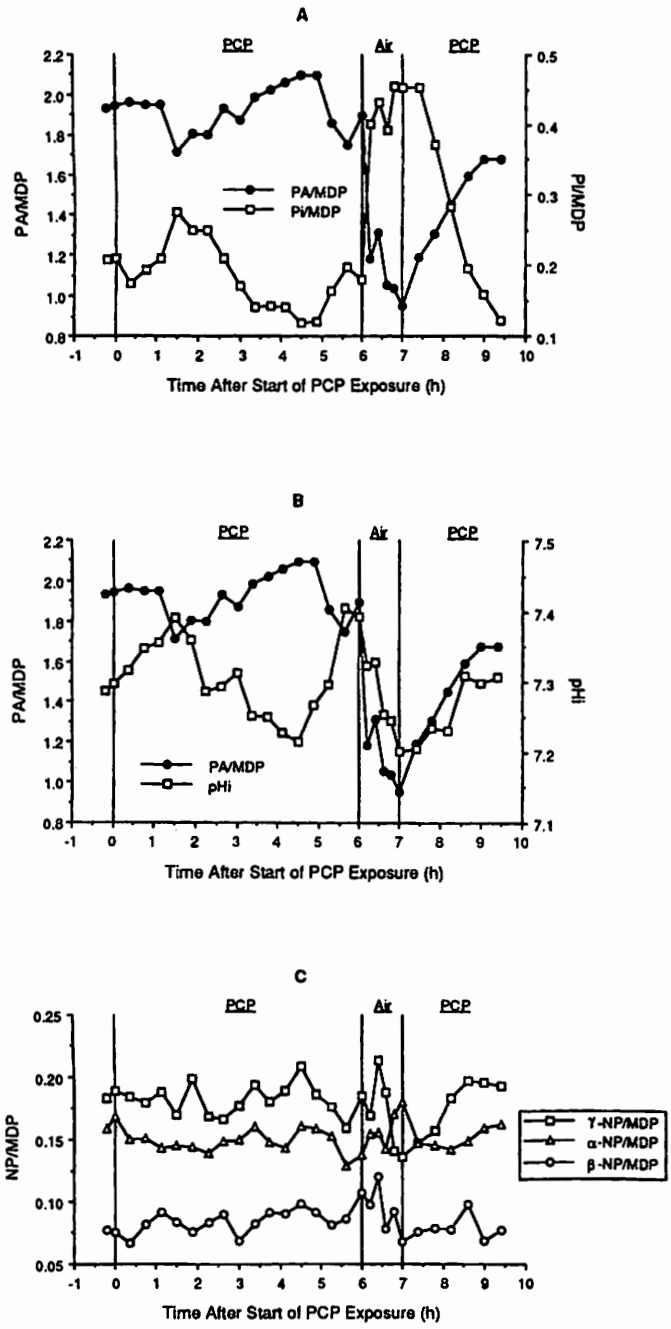

Figure 6. Comparison of the time-courses for phosphoarginine (PA) and inorganic phosphate (P_i; A), phosphoarginine (PA) and intracellular pH (pH_i; B), and the nucleoside phosphates (NPs; C) in a representative abalone during and after exposure to both 120 mg L^{-1} of PCP (at 14°C) and air. Please refer to Figure 2 for details.

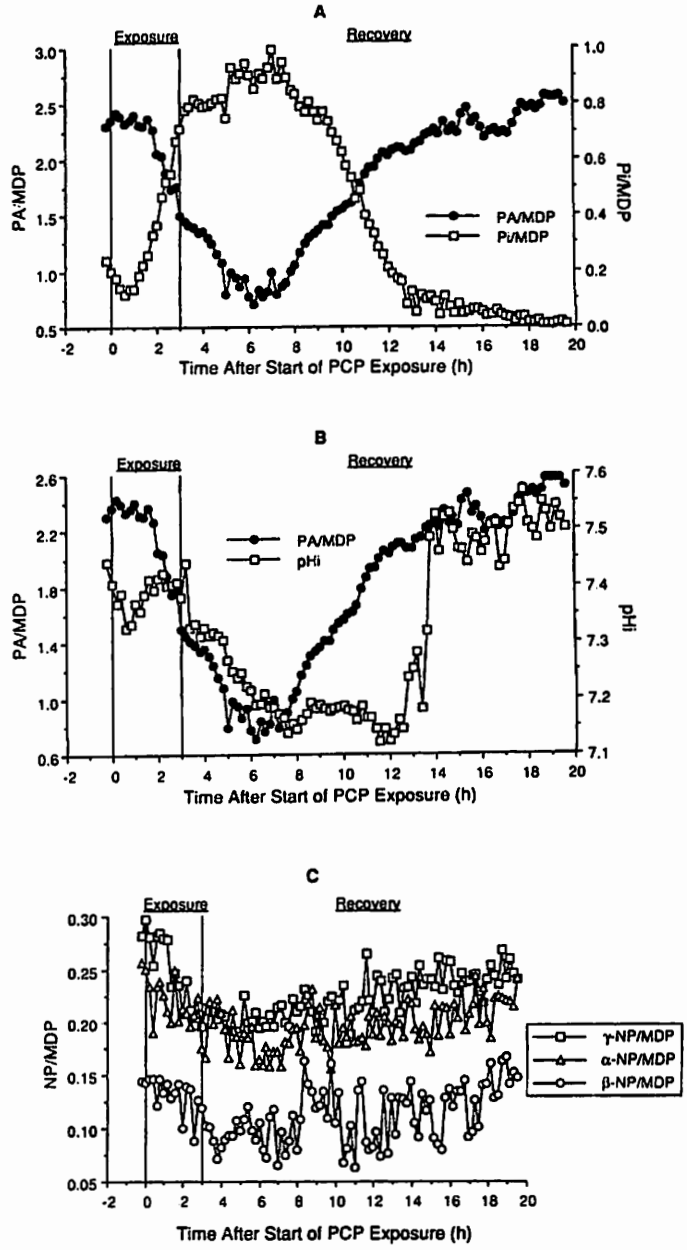

Figure 7. Comparison of the time-courses for phosphoarginine (PA) and inorganic phosphate (P_i; A), phosphoarginine (PA) and intracellular pH (pH_i; B), and the nucleoside phosphates (NPs; C) in a representative abalone during and after exposure to both 1.2 mg L^{-1} of PCP (at 19°C) and air. Please refer to Figure 2 for details.

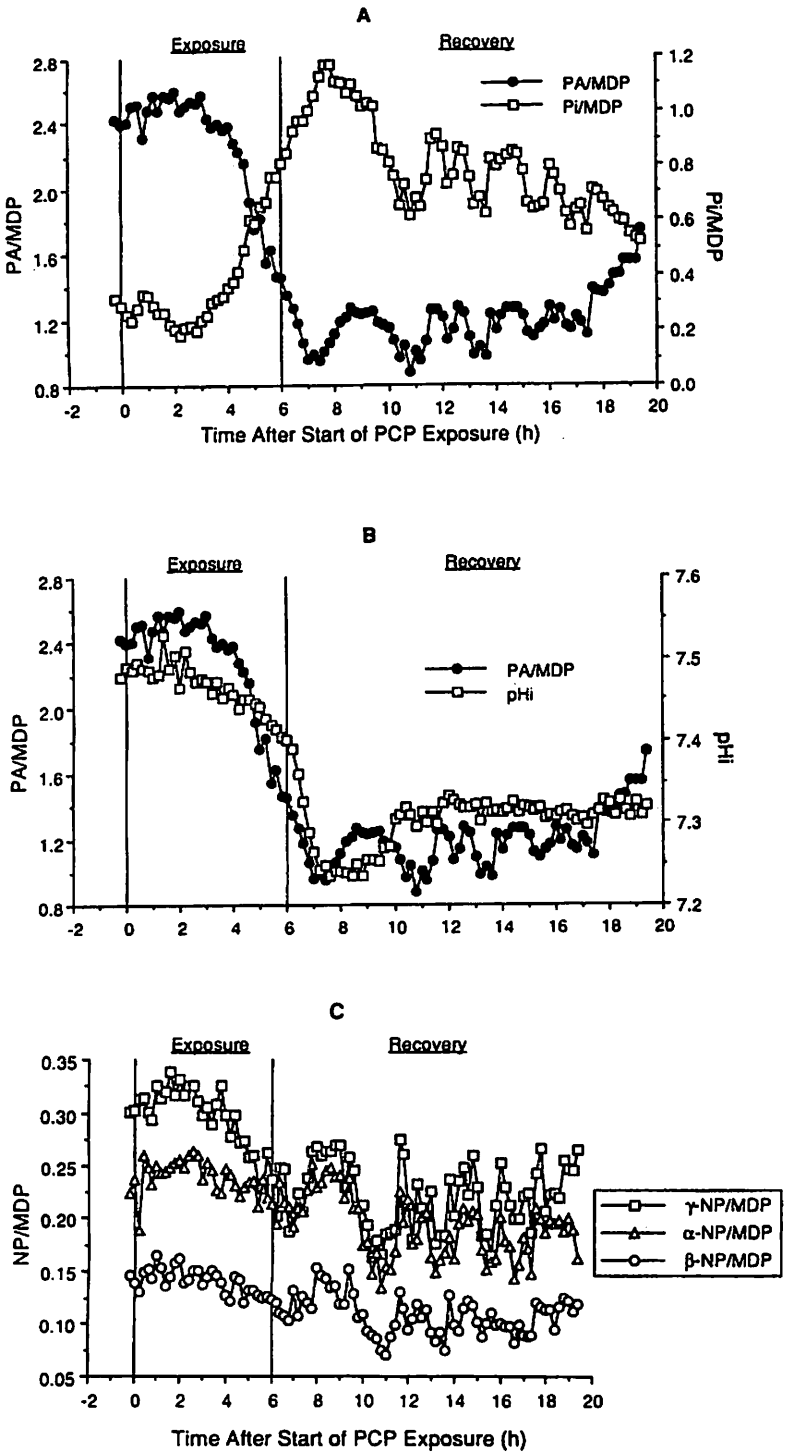

Figure 8. Comparison of the time-courses for phosphoarginine (PA) and inorganic phosphate (P_i; A), phosphoarginine (PA) and intracellular pH (pH_i; B), and the nucleoside phosphates (NPs; C) in a representative abalone during and after exposure to both 1.2 mg L^{-1} of PCP (at 9°C) and air. Please refer to Figure 2 for details.

seawater, all effects reversed within 10 to 16 h, and control abalones maintained at the two temperatures alone exhibited no adverse effects.

Finally, abalones ($n = 3$) exposed to 120 µg L^{-1} of PCP for 18 h at both 9° and 19°C exhibited no adverse effects, indicating that PCP does not impact them at current environmental levels. Thus, high temperature potentiates the time both for onset of and recovery from the sublethal effects of PCP in the red abalone.

Disposition and Biotransformation of PCP in Molluscs

In order to elucidate possible detoxication mechanisms influencing the sublethal effects of PCP measured by *in vivo* ^{31}P NMR, disposition and biotransformation of PCP were compared in three marine molluscs: two gastropods, the red and green abalone (*H. fulgens*), and a bivalve, the Pacific oyster. In the modified flow-through exposure system (Figure 9), and based upon the 6-h NOELs measured using traditional toxicity range-finding tests, abalones and oysters ($n = 6$ each) were exposed for 5 h to the following PCP concentrations: red abalones, 1.2 mg L^{-1}; green abalones, 500 µg L^{-1}; and Pacific oysters, 800 µg L^{-1}. In order to determine bioconcentration and tissue distribution after short-term exposure, three indviduals of each species were removed for analysis, while remaining animals were exposed to clean seawater for an additional 13 h to allow depuration of retained residues.

Residues were quantified by tissue digestion and liquid scintillation counting (LSC), and excreted residues were collected on XAD-4 resin and identified by HPLC, fraction collection, and LSC. The 5-h total concentration factor (TCF), similar to a bioconcentration factor but including both PCP and any metabolites; (Tjeerdema and Crosby; 1987,1988) ranged from 16.0 to 21.5 and 44.5 to 63.2 in red and green abalones, respectively, and from 25.1 to 44.5 in oysters.

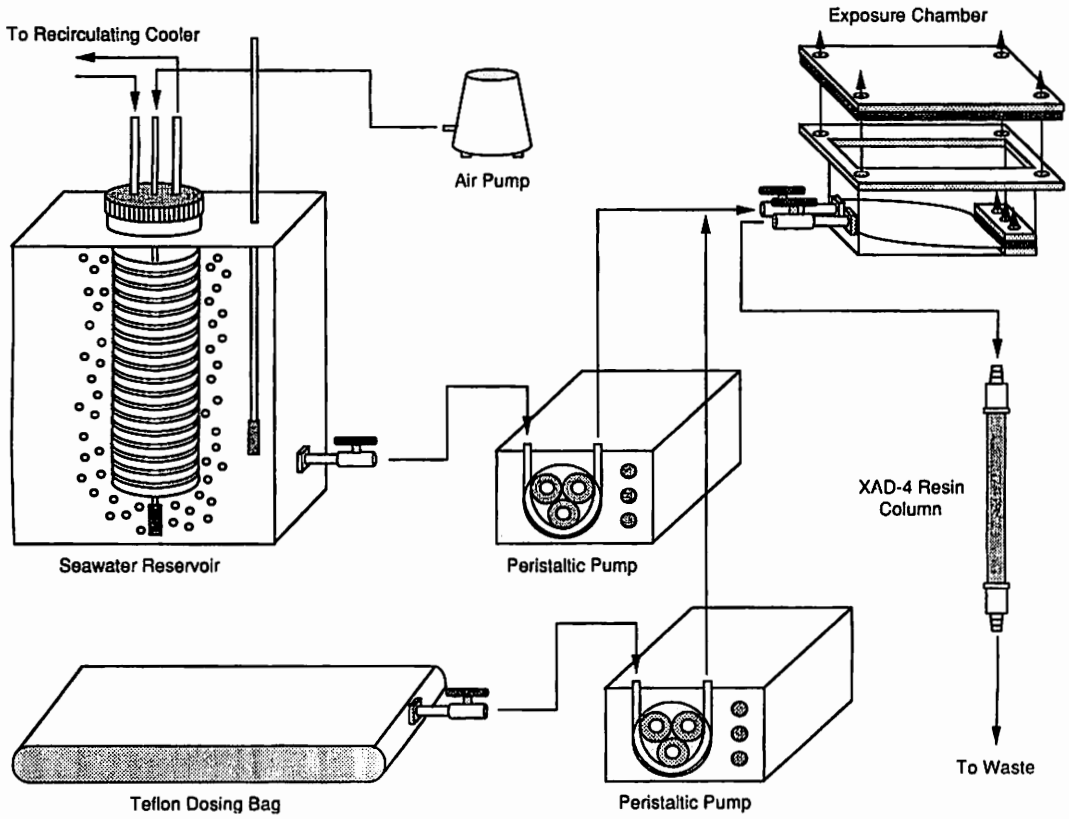

Figure 9. The flow-through exposure system for use with aquatic invertebrates and radiotracers.

Highest tissue concentrations were 133.4 nmol g^{-1} and 141.6 nmol g^{-1} in the gills of red and green abalones (Tables 1 and 2), respectively, and 109.3 nmol g^{-1} in oyster viscera (Table 3); tissues retaining the greatest PCP mass were the foot muscles of red (47.4%) and green abalones (39.0%), and oyster viscera (41.5%).

During 13-h recovery, depuration of retained residues was generally greatest from oysters (red abalones, 72.2%; green abalones, 23.3%; and oysters, 95.2%); concentrations in all individual tissues also declined. Finally, PCP biotransformation was limited in all three species. Both red and green abalones mainly excreted PCP unchanged (89.3% and 72.5%, respectively; Tables 4 and 5); red abalones also formed pentachloro-β-D-glucoside (7.9%), pentachloroanisole (1.3%), pentachlorophenyl-sulfate (0.9%), and tetrachloro-*p*-hydroquinone (0.6%; Figure 10), while green abalones produced pentachlorophenyl-β-D-glucoside (14.7%), tetrachloro-*p*-hydroquinone (7.3%), and pentachlorophenyl-sulfate (5.5%; Figure 11). In contrast, Pacific oysters depurated PCP (88.8%) and one metabolite, pentachlorophenylsulfate (11.2%; Table 5 and Figure 11). PCP is commonly detoxified by sulfation, glucuronidation, and/or methylation, all of which have been observed in other aquatic invertebrates (Kobayashi, 1969,1970a,b; Kobayashi, 1978; Kobayashi and Nakamura, 1979).

The sublethal effects of PCP in red abalones, as measured by *in vivo* ^{31}P NMR, parallel the kinetics of the biocide. During acute exposure, abalones rely more on biotransformation than depuration to detoxify residues since they cannot limit their exposure to PCP nor depurate retained residues as effectively as oysters. Pacific oysters limit the accumulation of PCP, possibly by closing their shells, and efficiently depurate retained residues to limit toxic effects.

Disposition and Biotransformation of PCP in Fishes

In order to compare the disposition of PCP with more metabolically active fishes, it was also determined for both the topsmelt (*Atherinops affinis*) and striped bass (*Morone saxatilis*). In a static chamber, topsmelt ($n = 9$) were exposed to a nontoxic concentration of [U-^{14}C]PCP (50 μg L^{-1}) for 24 h to determine bioconcentration of the biocide. Following exposure, they were placed in a recirculating flow-through metabolism system (Figure 12; Garnas and Crosby, 1979) for 24 h to allow depuration of retained residues, which were collected on XAD-4 resin; they were identified and quantified by HPLC, fraction collection, and LSC.

Using a modified flow-through exposure system (Figure13; Tjeerdema and Crosby, 1987), striped bass ($n = 6$) were also exposed to a nontoxic concentration

Table 1. Disposition of Residues in Red Abalones after 5-h Exposure to 1.2 mg L^{-1} of Pentachlorophenol and 13-h Depuration

	^{14}C Disposition					
	Exposure (5 h)			Depuration (13 h)		
Tissue	Total mass (μmol)[a]	%	Concentration (nmol g^{-1})[a,b]	Total mass (μmol)[a]	%	Concentration (nmol g^{-1})[a,b]
XAD-4 resin	—	—	—	2.55 (0.02)	72.24	70.05 (2.83)
Foot muscle	1.37 (0.13)	47.74	109.13 (14.63)	0.27 (0.01)	7.65	17.59 (2.26)
Viscera	0.39 (0.04)	13.59	136.18 (7.69)	0.14 (0.01)	3.97	86.68 (0.29)
Shell muscle	0.36 (0.11)	12.54	30.55 (5.23)	0.10 (0.01)	2.83	14.61 (1.70)
Epipodium	0.29 (0.03)	10.10	143.52 (14.52)	0.06 (0.01)	1.70	30.71 (0.85)
Head	0.14 (0.01)	4.88	101.54 (11.92)	0.04 (0.01)	1.13	27.19 (4.19)
Gonad	0.13 (0.01)	4.53	17.46 (0.16)	0.27 (0.05)	7.65	37.00 (11.87)
Gill	0.10 (0.03)	3.48	133.42 (37.04)	0.08 (0.01)	2.27	93.47 (18.03)
Mantle	0.09 (0.01)	3.14	81.12 (4.27)	0.02 (0.01)	0.56	22.83 (4.99)
Totals	2.87 (0.23)	100.00	71.86 (2.70)	3.53 (0.04)	100.00	96.85 (3.41)

[a]Mean (SD), $n = 3$
[b]Wet weight

Table 2. Residue Disposition in Green Abalones after Both 5-h Exposure to 500 μg L^{-1} of Pentachlorophenol and 13-h Depuration

	^{14}C Disposition[a]					
	Uptake (5 h)			Depuration (13 h)		
Tissue	Total mass (μmol)	%	Concentration[b] (nmol g^{-1})	Total mass (μmol)	%	Concentration[b] (nmol g^{-1})
XAD-4	—	—	—	0.48 (0.57)	23.3	22.2 (29.0)
Foot muscle	1.15 (0.73)	39.0	130.3 (32.9)	0.51 (0.28)	24.8	63.8 (32.3)
Viscera	0.64 (0.25)	21.7	121.8 (26.1)	0.44 (0.15)	21.4	65.5 (14.2)
Shell muscle	0.26 (0.02)	8.8	64.4 (11.4)	0.14 (0.09)	6.8	37.7 (21.9)
Gill	0.25 (0.12)	8.5	141.6 (21.6)	0.11 (0.04)	5.3	67.7 (6.3)
Gonad	0.21 (0.11)	7.1	135.9 (83.5)	0.13 (0.01)	6.3	62.3 (22.4)
Epipodium	0.19 (0.04)	6.4	138.0 (24.5)	0.10 (0.05)	4.9	73.8 (25.0)
Liver	0.13 (0.06)	4.4	123.3 (63.0)	0.08 (0.03)	3.8	83.2 (23.4)
Head	0.12 (0.04)	4.1	131.3 (34.1)	0.07 (0.03)	3.4	69.9 (21.3)
Totals	2.95 (0.73)	100.0	118.5 (5.9)	2.06 (0.67)	100.0	83.4 (10.5)

[a]Mean (SD), $n = 3$
[b]Wet weight

Table 3. Disposition of Residues in Pacific Oysters after Both 5-h Exposure to 800 μg L^{-1} of Pentachlorophenol and 13-h Depuration

	^{14}C Disposition[a]					
	Uptake (5 h)			Depuration (13 h)		
Tissue	Total mass (nmol)	%	Concentration[b] (nmol g^{-1})	Total mass (nmol)	%	Concentration[b] (nmol g^{-1})
XAD-4	—	—	—	920.0 (204.0)	95.2	127.9 (19.1)
Viscera	226.6 (87.2)	41.5	109.3 (9.5)	25.7 (35.3)	2.7	11.5 (13.9)
Mantle	145.3 (49.9)	26.6	70.4 (15.5)	10.8 (11.5)	1.1	3.8 (2.9)
Adductor	97.4 (30.5)	17.8	76.1 (21.4)	4.1 (4.6)	0.4	2.9 (2.4)
Gill	76.4 (54.7)	14.1	48.1 (13.6)	6.1 (4.5)	0.6	2.8 (1.3)
Totals	545.7 (172.3)	100.0	75.2 (5.3)	966.7 (55.9)	100.0	133.2 (16.0)

[a]Mean (SD), $n = 3$
[b]Wet weight

Table 4. Metabolite Profile of XAD-4 Resin Extracts after 13-h Depuration by Red Abalones

Component	Resin Recovery (%)[a]	^{14}C Distribution Concentration (nmol g^{-1})[b,c]	%
Pentachlorophenol	91.0	62.56 (2.68)	89.31
Tetrachloro-*p*-hydroquinone	91.2	0.44 (0.07)	0.63
Pentachloroanisole	92.1	0.89 (0.12)	1.27
Pentachloro-β-D-glucoside	—	5.56 (0.16)	7.94
Pentachlorophenylsulfate	—	0.60 (0.12)	0.85
Totals		70.05 (2.83)	100.00

[a]Average, $n = 2$; applicable to all biotransformation experiments
[b]Mean (SD), $n = 3$
[c]Wet weight

Table 5. Metabolite Profiles of XAD-4 Resin Extracts after 13-h Depuration by Pacific Oysters and Green Abalones

Metabolite	^{14}C Distribution[a] Oysters nmol g^{-1}	Oysters %	Abalones nmol g^{-1}	Abalones %
Pentachlorophenol	113.2 (12.8)	88.8	14.5 (17.8)	72.5
Pentachlorophenylsulfate	14.7 (7.1)	11.2	2.8 (4.7)	5.5
Pentachlorophenyl-β-D-glucoside	0.0 (0.0)	0.0	3.1 (3.8)	14.7
Tetrachloro-*p*-hydroquinone	0.0 (0.0)	0.0	1.9 (2.6)	7.3
Totals	127.9 (19.1)	100.0	22.2 (29.0)	100.0

[a]Mean (SD), $n = 3$ (wet weight)

Table 6. Metabolite Profile of XAD-4 Resin Extracts After 24-h Depuration by Striped Bass

Component	^{14}C Distribution[a] Concentration (nmol/g)[b]	%
Pentachlorophenol	7.8 (0.6)	71.5
Pentachlorophenylsulfate	2.2 (2.8)	20.3
Pentachlorophenyl-β-D-glucuronide	0.8 (0.5)	7.0
Tetrachloro-*p*-hydroquinone	0.1 (0.1)	1.2
Totals	10.9 (3.9)	100.0

[a]Mean (SD), $n = 3$
[b]Wet weight

of [U-^{14}C]PCP (60 µg L^{-1}) for 24 h; while three fish were removed for analysis, the remaining fish were exposed to clean seawater for an additional 24 h to allow depuration of retained residues, which were quantified by tissue digestion and LSC. Excreted residues were collected on XAD-4 resin and identified and quantified as described above.

The 24-h TCF for topsmelt averaged 278.0 ± 182.0, while for striped bass it ranged from 134.0 to 189.5. During a 24-h exposure to clean seawater, topsmelt depurated 32.9% of retained residues, and while PCP was primarily excreted unchanged (64.9%), pentachlorophenylsulfate (18.9%) and pentachlorophenyl-β-D-glucuronide (16.2%) were also formed (Figure 14). In contrast, striped bass depurated 36.4% of retained residues during a 24-h recovery period. Again, PCP was excreted mainly unchanged (71.5%; Table 6); however, pentachlorophenylsulfate (20.3%), pentachlorophenyl-β-D-glucuronide (7.0%), and tetrachloro-*p*-hydroquinone (1.2%) were also produced (Figure 15). PCP is commonly detoxified by sulfation, glucuronidation, and/or methylation, which have been observed in other fishes (Akitake and Kobayashi, 1975; Kobayashi et al., 1977; Kobayashi, 1978; Kobayashi and Nakamura, 1979; Huckins and Petty, 1983; Stehly and Hayton, 1989a,b).

While depuration of the biocide differed widely for the two fish species, it was comparable to that from both red and green abalones and lower than that from Pacific oysters. However, the more metabolically active fishes detoxified significantly greater proportions of the absorbed PCP, indicating that they rely more on biotransformation, and less on simple depuration, for the detoxication of accumulated PCP than molluscs.

Conclusions

This report describes the biochemical effects of a representative pollutant, PCP, both alone and in combination with important natural

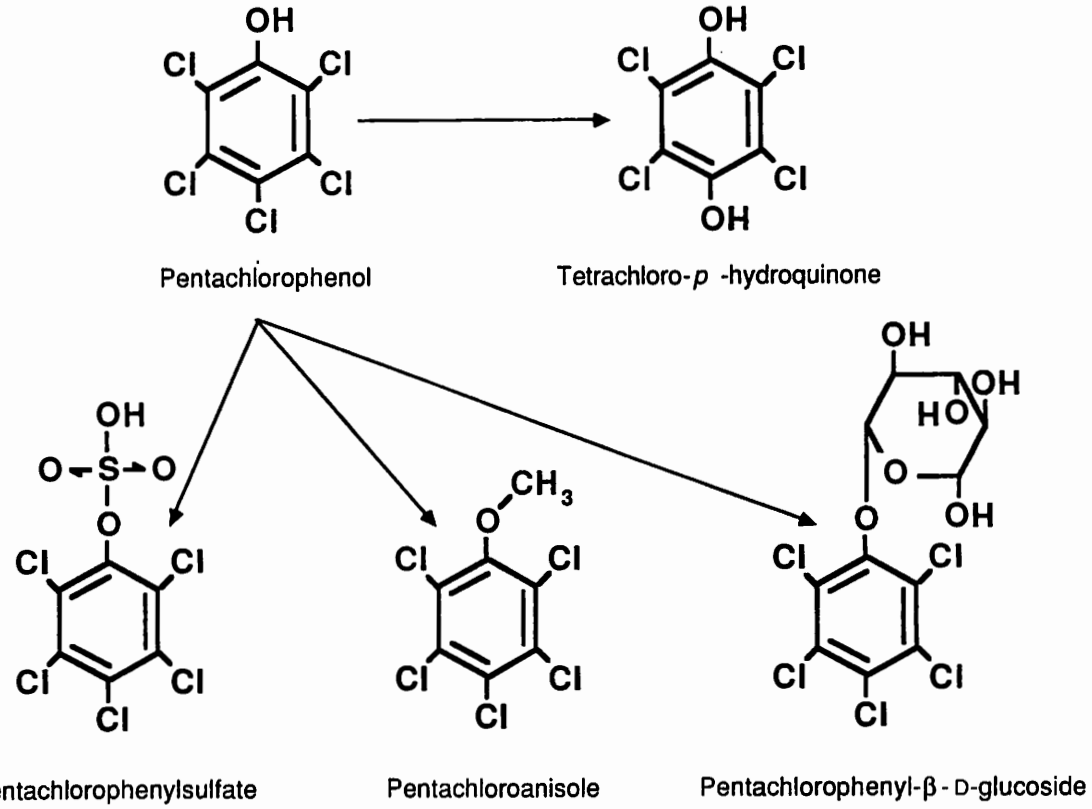

Figure 10. The metabolic pathways of pentachlorophenol in red abalones.

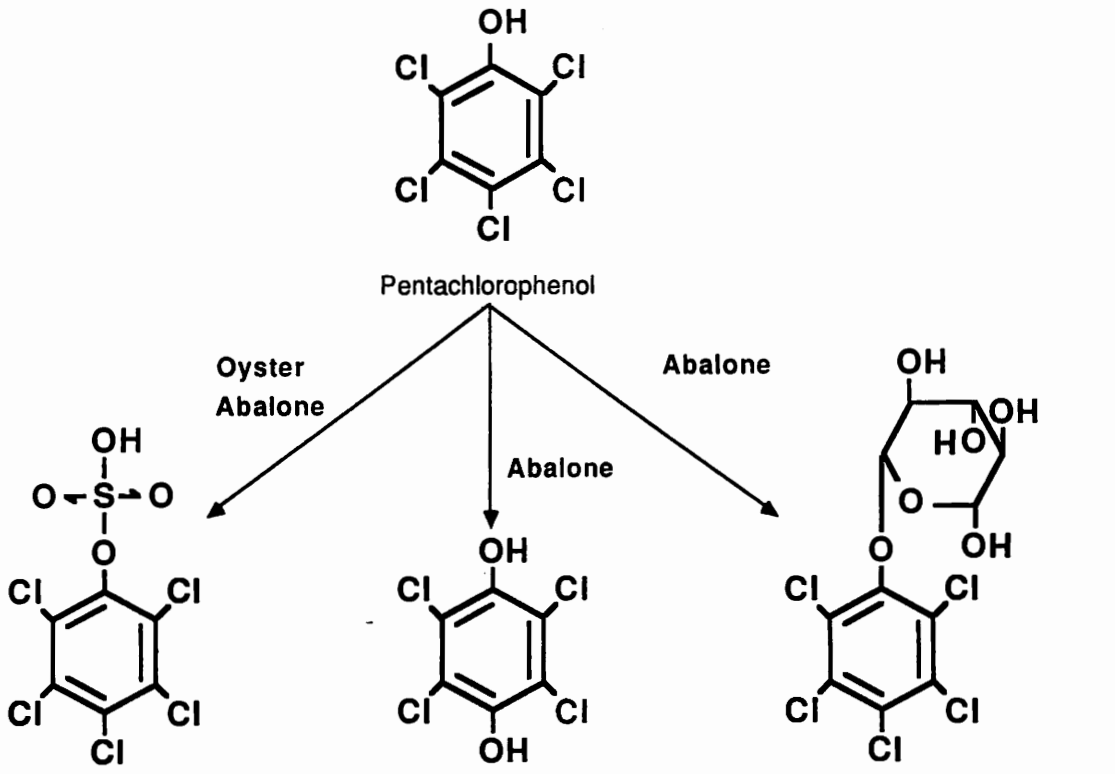

Figure 11. The metabolic pathways of pentachlorophenol in Pacific oysters and green abalones.

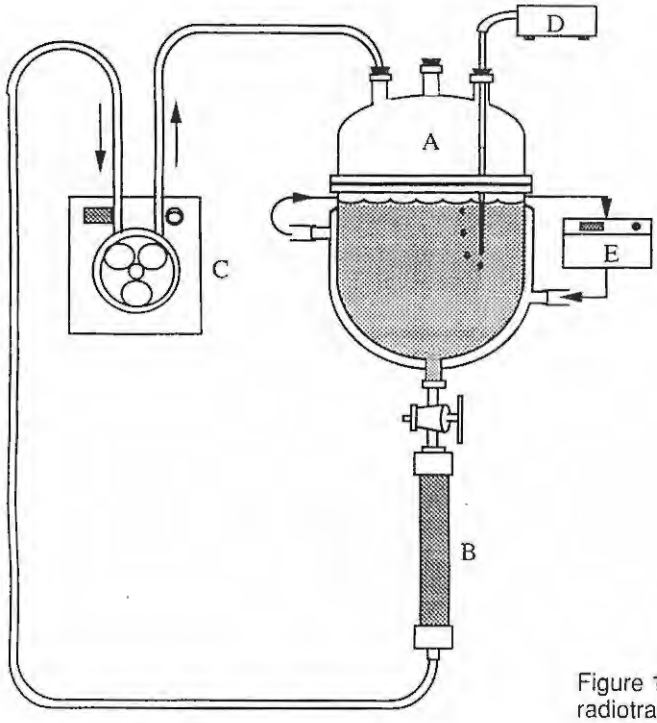

Figure 12. The flow-through metabolism system for use with topsmelt and radiotracers.

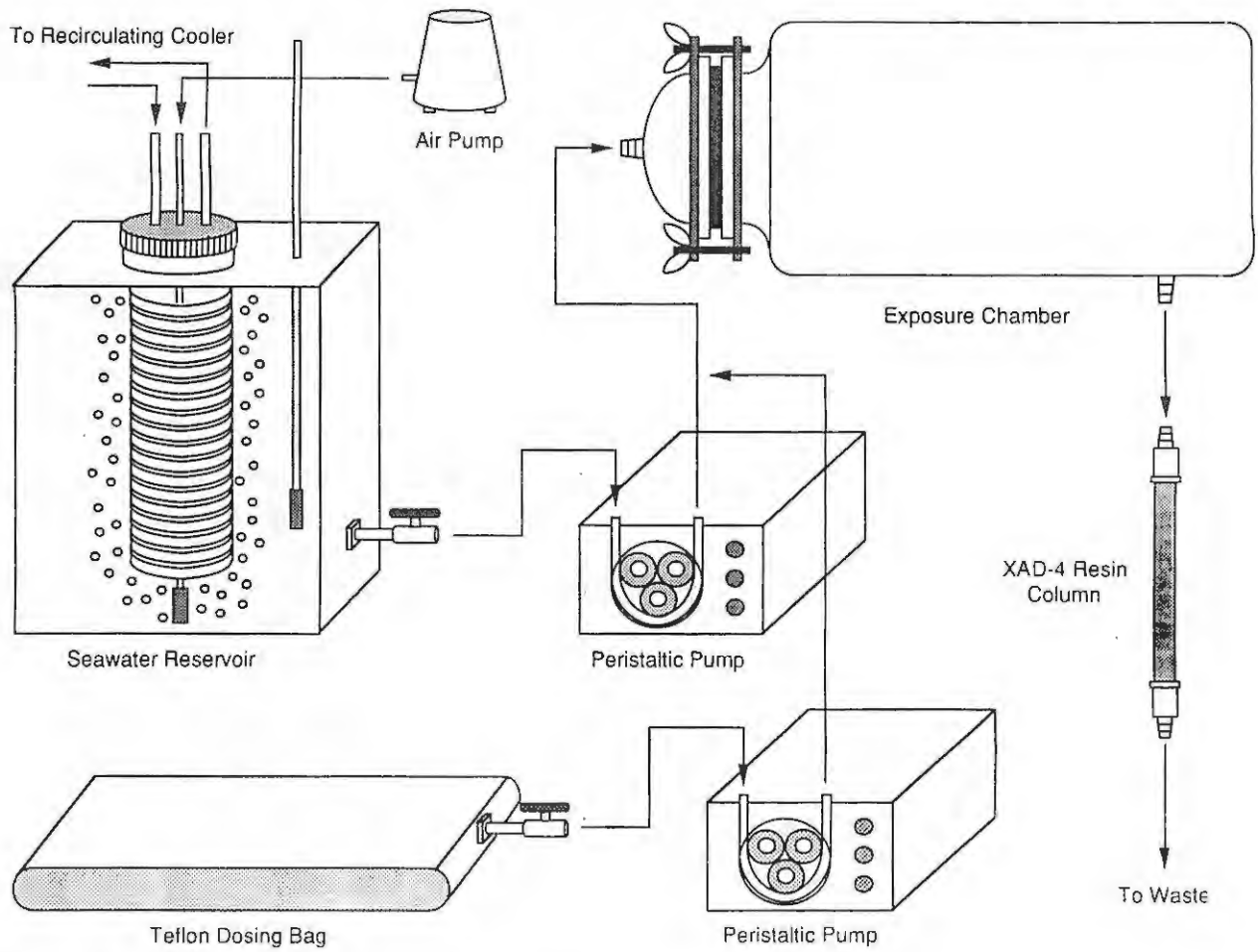

Figure 13. The flow-through exposure system for use with striped bass and radiotracers.

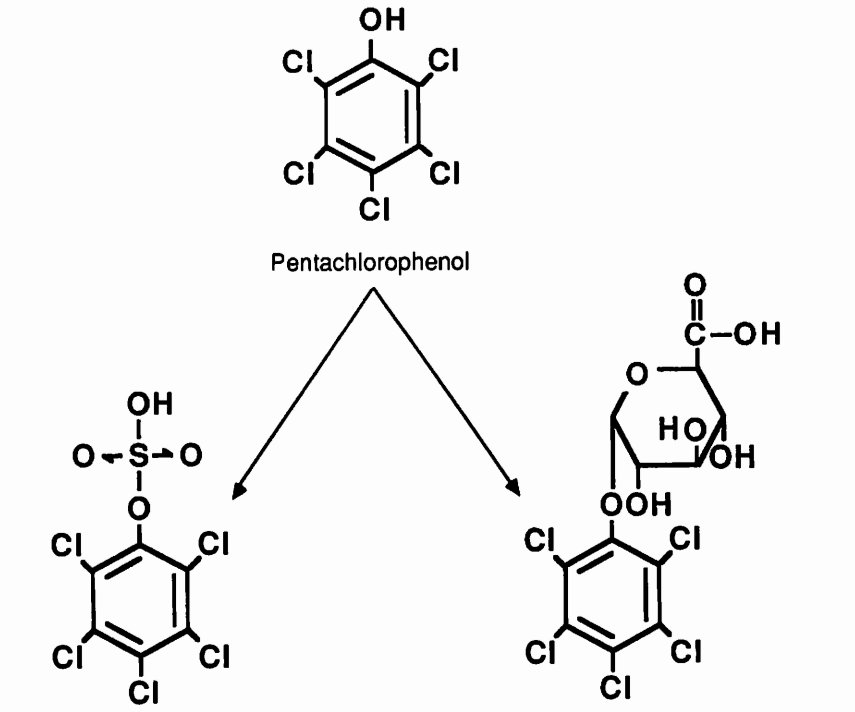

Figure 14. The metabolic pathways of pentachlorophenol in topsmelt.

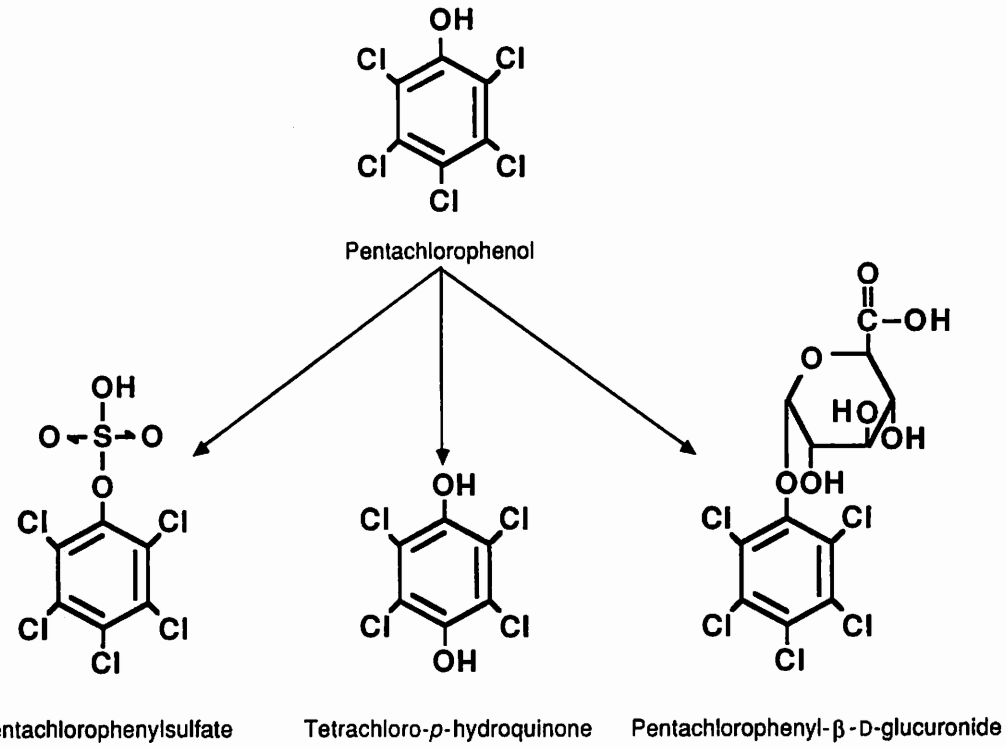

Figure 15. The metabolic pathways of pentachlorophenol in striped bass.

stress factors, in intact marine organisms as measured by *in vivo* ^{31}P NMR. *In vivo* NMR represents a sensitive, new approach for investigation of sublethal toxic effects, and the interactions of natural stress factors, in marine organisms. Important biochemical actions can now be measured in intact organisms as they occur (in real time), allowing repetitive multicompound analyses using a single organism. In combination with parallel toxico-kinetic studies, use of *in vivo* NMR can provide a more complete picture of the actions of toxic chemicals. As demonstrated by this investigation, the combination of intact organisms, appropriate exposure systems, and *in vivo* NMR can assist in determining both the molecular interactions of pollutants and natural stress factors (of interest to theoretical toxicologists) and the true sublethal water concentrations impacting marine populations (of interest to environmental regulators).

Guidelines for Use of *in Vivo* NMR

While specific criteria for use of *in vivo* NMR will change as the technique is further developed, the following guidelines will assist those using the new approach for establishment of sublethal water concentrations.

1. Range-finding tests. Classical aquatic toxicity tests (incorporating flow-through or static exposure) should be conducted to provide range-finding results for the pollutant of interest. Either lethality or a specific morphological defect may be used as the toxic endpoint, with the goal the establishment of an NOEL to guide NMR experiments. Pollutant water concentrations should be selected on a logarithmic scale, and should never exceed water solubility.

2. Nuclide selection. *In vivo* NMR should involve use of ^{31}P for determination of metabolic effects, but may incorporate other nuclei when appropriate; the biochemical endpoint of interest should guide the selection of the proper nucleus for use.

3. NMR exposure conditions. Flow-through exposure systems, similar to that developed for our investigation, should always be used for NMR experimentation unless air exposure is desired. Chemically inert materials should be used to construct the system, and water temperature, salinity, and pH should be closely controlled; adequate aeration should be provided.

4. Organism choice. Organism choice will vary with need, but one should consider compatibility with the magnet and exposure chamber. Organisms that naturally remain still for long time periods will provide the best results; anesthesia should not be used unless absolutely necessary to alleviate stress, as it may alter natural responses.

5. Chemical exposure. Organisms should be exposed within the magnet to sequentially decreasing water concentrations of a pollutant for periods of up to 12 h, allowing up to another 12 h to test recovery (as was done in our investigation). Once the smallest water concentration eliciting a sublethal effect is determined, it should be retested with at least another two individuals ($n = 3$). Because of the expense of operating an NMR instrument, minimal sample sizes are appropriate.

6. NMR conditions. Single-pulse, surface-probe NMR is most appropriate for larger organisms, such as abalones. It provides for optimal data retrieval from single tissues with minimal interference. Specific NMR parameters, such as pulse width, spectral width, pulse delay, etc. will vary with application and nucleus.

7. Environmental factors. Whenever appropriate, environmental factors should be incorporated into NMR testing, both alone and in combination with the pollutant under evaluation. Only by doing so will the true *environmental* effects of a pollutant be determined.

Using the above criteria, and our investigation for guidance, the application of *in vivo* NMR to important pollution concerns will provide more specific information as to exactly how toxicants act biochemically, and what water concentrations truly represent a threat to the survival of marine populations.

Cooperating Organizations

California Department of Fish and Game, Monterey, California
Moss Landing Marine Laboratories, Moss Landing, California
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Phenolic Compounds in Seawater: Rates and Mechanisms of Biodegradation

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Introduction

Phenolic compounds in the marine environment have numerous sources. Many are produced *in situ* by marine algae (Sieburth and Jensen, 1968, 1969; Carlson and Mayer, 1983), marine invertebrates (King, 1986, 1988), and by as yet unidentified sources (Charriere et al., 1991). In addition, phenols are released in tremendous quantities as a result of human activities. A recent report (U.S. Environmental Protection Agency, 1992) suggests that water and air releases are more than 125 Gg·year^{-1} in California alone. These releases reflect not only direct transfers, but also transfers to publicly-owned treatment works which, in turn, channel the materials into streams and ocean outfalls.

In addition to *in situ* production and human sources, considerable anthropogenic input of phenolics comes from another natural source, humic materials—that is, materials derived from the partial decomposition of plant or animal matter. Chemically, humic materials vary considerably between freshwater and marine systems (Malcolm, 1990). One difference is the high phenolic content in freshwater humic materials and low-to-no phenolic content in marine humic compounds. Equally important, data on changes in carbon-13/12 ratios (Williams and Gordon, 1970; Nissenbaum and Kaplan, 1972) suggest that humic substances are almost completely formed *in situ*.

Because the input of human-influenced phenolic compounds into the coastal environment is considerable and because naturally derived humic materials lose phenolic moieties during travel from riverine environments into the ocean, we chose simple phenolic compounds as models to assess microbial biodegradation in coastal marine environments. We have determined these rates of biodegradation of measurable concentrations of simple phenolics in several environments. The results suggest that seemingly recalcitrant humic organic materials carried in rivers to the sea are degraded rapidly by estuarine and marine bacteria.

Objectives and Experimental Description

Because this project was the first to assess the potential for biodegradation in marine waters off California, we envisioned a sequence of experimental efforts in order to obtain realistic rate estimates.

The first objective was to determine whether marine bacteria off the coast of California were able to degrade simple phenolic compounds. Populations of marine bacteria in water collected off Scripps pier were incubated in batch culture with several different phenolic substrates. We analyzed the disappearance of the phenolic substrate and the changes in total bacterial numbers over the duration of incubation. Growth and uptake (calculated on the basis of the disappearance of substrate) were then related to a rate of biodegradation.

A second objective was to develop sensitive techniques for measuring phenolic materials both qualitatively and quantitatively in several coastal environments. None of the available methods for the identification and quantification of phenolic materials in marine waters provided the sensitivity and specificity needed for our studies. Consequently, we developed a technique that uses aqueous acetylation, solid-phase extraction, and gas chromatographic and mass spectral analyses to identify phenolic compounds and measure their levels in samples of seawater. This was followed by the fabrication of an apparatus designed to extract phenolics from large volumes of seawater (5–40 l). Data were obtained that reflect phenolic composition of seawater samples down to 1 ng·l^{-1}.

A third objective was to develop methods for measuring utilization rates of phenolic materials by using substrates labeled with tritium. These substrates are available with about 1000 times higher specific activity (ratio of radioactivity to mass) than previously used substrates labeled with carbon-14 (Pfaender and Bartholomew, 1982; Bartholomew and Pfaender, 1983), therefore allowing increased sensitivity in analyses of "dilute" marine waters. Tritiated substrates have previously been used to assess the biological utilization kinetics of sugars (Azam and Holm-Hansen, 1973) and amino acids (Carlucci et al., 1986; Craven and Carlucci, 1989) in seawater. With the method developed, as little as 1·ng·l^{-1} of tracer ^{3}H-labeled *p*-cresol can be added to a seawater sample and utilization followed over time.

The fourth objective was to use both newly developed techniques to determine a reliable biodegradation rate for phenolic materials in coastal environments. Seawater was collected from various sites off the coast of northern and southern California. Subsamples were analyzed for representative phenolic compounds and incubated with ^{3}H-labeled *p*-cresol. Fractions representing uptake (^{3}H incorporated into cellular material), respiration (^{3}H converted to ^3H_2O), and residual ^{3}H-labeled *p*-cresol were assayed for radioactivity. The amounts of radioactivity were then related to the amount of *p*-cresol; specific activities were recalculated to account for ambient or natural levels of *p*-cresol level in the

sample. These values, in turn, were used to calculate utilization rates (uptake plus respiration) and turnover times.

Results and Discussion

Growth of Natural Marine Populations on Phenolic Substrates. When natural populations of marine bacteria are incubated with several phenolic materials in batch culture, cell growth is notably enhanced. Table 1 summarizes growth and utilization rates. Populations acclimated to *p*-cresol, *o*-cresol, 2,4-dichlorophenol, and 2-bromophenol grew relatively rapidly. A general trend of increasing growth with increasing concentration of the phenolic compound up to 1000 $\mu g \cdot l^{-1}$ was observed. An exception was 2-bromophenol incubations. This phenolic seemed to be inhibitory at higher concentrations.

Phenolic Compounds in the Coastal Environment. In an effort to understand the qualitative aspects of phenolic chemistry in the coastal environment of California, waters were sampled and assayed for representative phenolic compounds qualitatively and quantitatively. Large volumes of water (20–40 l) were taken in San Diego Bay, off White's Point Outfall (Los Angeles County), from the effluent of the Joint Water Pollution Control Point (JWPCP) and Hyperion treatment facilities (Los Angeles), and off the coast of Eureka (northern California), where effluent pipes of pulp mills are located. Table 2 shows the types and amounts of phenolic materials found at the various sites.

Utilization of *p*-Cresol. A tritium-labeled tracer phenolic compound, [2,6-^{3}H]-*p*-cresol, was purified and used in uptake experiments. The method developed to measure uptake and respiration consisted of short-term incubations of water samples with a tracer amount (1 $ng \cdot l^{-1}$) of added [2,6-^{3}H]-*p*-cresol. Because ^3H_2O (the respiration product of [2,6-^{3}H]-*p*-cresol) is subject to secondary utilization, incubation times were no more than 6 hours. After incubation, cells were filtered out and assayed for radioactivity (uptake). In order to measure the ^3H_2O produced by cellular

Table 1. Growth and Utilization Rates of Natural Marine Bacterial Populations Incubated in Batch Cultures with Phenolic Compounds

Substrate	Concentration ($\mu g \cdot l^{-1}$)	Growth Rate (cells $\times 10^5 \cdot l^{-1} \cdot hr^{-1}$)	Utilization Rate ($\mu g \cdot l^{-1} \cdot hr^{-1}$)	Half-life, in hours (days)
o-cresol	100	300	9.2	50 (2)
	500	520	14	240 (10)
	1000	750	16	335 (14)
p-cresol	100	280	2.8	295 (12)
	500	570	14	215 (9)
	1000	770	19	325 (13)
2,4-dichlorophenol	100	27	ND	ND
	500	65	ND	ND
	1000	79	ND	ND
2-bromophenol	100	19	ND	ND
	500	17	ND	ND
	1000	5.8	ND	ND

ND = Not determined.

Table 2. Sampling Sites and Depths and Concentrations of Identified Phenolic Compounds*

Sampling Location Coordinates Date	Depth	Phenolic Compounds Identified	Concentration ($ng \cdot l^{-1}$)
Spanish Landing, San Diego Bay	Surface	*o*-cresol	3 ± 0.5
		catechol	130 ± 21
32°43'40"× 117°12'42"		3-methylcatechol	33 ± 5
April 4 1992		4-methylcatechol	22 ± 3
Sweetwater Channel, San Diego Bay	11 m	phenol	228 ± 36
		o-cresol	5 ± 1
32°38'43"× 117°07'25"		catechol	188 ± 30
December 9 1992			
Simpson Outfall	Surface	phenol	32 ± 5
40°48'03"× 124°12'50"		*o*-cresol	22 ± 3
June 24 1992		*p*-cresol	15 ± 2
		2-methoxyphenol	*390–6600*
		2,3,5,6-tetramethylphenol	*12–200*
		2-methoxy-4-methylphenol	*17–280*
		4-ethyl-2-methoxyphenol	*110–1900*
		2-methoxy-4-isopopenylphenol	*13–220*
		4-hydroxy-3-methoxybenzaldehyde	*82–1400*
		4,5-dichloro-2-methoxyphenol	*19–320*
		1,1-dimethyl-ethyl-catechol	*66–1100*
Louisiana-Pacific Outfall	Surface	phenol	12 ± 2
40°49'05"× 124°12'10"		*o*-cresol	9 ± 1
June 24 1992		catechol	100 ± 16
		2-methoxyphenol	*34–580*
White's Point Outfall	34 m	phenol	328 ± 52
33°41'40"× 118°19'30"		*o*-cresol	8 ± 1
March 10 1993		*p*-cresol	60 ± 10
JWPCP effluent	NA	phenol	7000 ± 1120
NA		*o*-cresol	2000 ± 320
March 9 1993		*p*-cresol	2400 ± 380
Hyperion effluent	NA	*o*-cresol	170 ± 27
NA		*p*-cresol	516 ± 83
February 3 1993			

*Phenolic materials in italics are putative identifications based on mass spectral data.
JWPCP = Joint Water Pollution Control Point, NA = not applicable.

respiration during incubation, the remaining [2,6-^{3}H]-*p*-cresol was extracted from the filtrate. Radioassay of the aqueous fraction yielded the amount of ^3H_2O produced. The extracted [2,6-^{3}H]-*p*-cresol was also assayed to determine disappearance rate and percent respiration for the added label (Table 3).

Phenolic biodegradation was measured in coastal California samples by using [2,6-^{3}H]-*p*-cresol as a tracer. The dilution of the labeled material ([2,6-^{3}H]-*p*-cresol) by the ambient or natural level of the substrate was calculated, and turnover times were determined for phenolic compounds in natural environments. This work represents the first time ambient concentrations of phenolic compounds and the consequent dilution of labeled substrate have been taken into account when determining biodegradation rates. When the dilution of labeled substrate was included, the measured utilization rates increased by as much as 300 times. In general, rates were extremely fast for all sample sites (Table 4). Turn-over times were short and indicate dynamic utilization of "recalcitrant" organic materials in the coastal environment.

Probably the most intriguing finding of this project is the evidence supporting rapid biodegradation of more complex, humic-type materials in coastal environments. Where riverine input was high (San Francisco and Humboldt bays), a flux of phenolic materials occurred within the time of incubation (6 hours). It was concluded that biodegradation of riverine humic materials produced simple phenolic compounds that were used along with those phenolic materials identified chemically. As identified phenolic materials were similar in all sample sites, riverborne humic materials were assumed to be the additional source of simple phenolic moieties.

Table 3. Utilization, Percentage of Respiration, and Half-life of [2,6-^{3}H]-*p*-Cresol for Microbial Populations Incubated in Seawater (July 1993)

p-Cresol Concentration ($ng \cdot l^{-1}$)	Utilization ($pg \cdot l^{-1} \cdot hr^{-1}$)	Respiration (%)	Half-life, in hours (days)
1.05	1.67	97.9	315 (13)
4.15	1.83	61.9	1134 (46)
8.37	20.2	92.7	207 (9)
48.5	87.6	89.8	277 (12)
80.7	133	89.6	304 (13)
476	640	86.5	372 (16)

Table 4. Concentration of *p*-Cresol, Microbial Utilization Rate, and Turnover Time for Various Coastal California Sites

Sample Location	*p*-Cresol Concentration ($ng \cdot l^{-1}$)	Utilization Rate ($ng \cdot l^{-1} \cdot hr^{-1}$)	Turnover Time of *p*-Cresol (hr)
Sweetwater Channel	45.0 ± 1.3	1.56 ± 0.23	28.9 ± 5.8
Spanish Landing	37.6 ± 1.1	1.02 ± 0.15	37.0 ± 7.4
White's Point Outfall	303 ± 9.1	20.8 ± 3.1	14.6 ± 3.0
San Francisco Bay	61.1 ± 1.8	35.5 ± 5.3	1.72 ± .34
Humboldt Bay	9.26 ± 0.28	1.48 ± 0.22	6.26 ± 1.2

Summary

Marine and estuarine bacteria play a major role in the biodegradation of phenolic materials in coastal environments. In addition to rapidly utilizing anthropogenic phenols resulting from pollution, microbes are able to break down more complex, biogeochemically significant humic materials containing phenolic moieties. Marine bacteria thus play a significant role in the overall transformation of organic carbon in marine and estuarine systems.

Cooperating Organizations

Captain and crew of the *Celtic*
City of Los Angeles
County Sanitation Districts of Los Angeles County
Crew of R/V *La Mer*
Crew of R/V *Ocean Sentinel*
Regional Water Quality Control Boards of San Diego, Alameda, and Humboldt Counties

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T.J. Boyd. 1993. Ecological and biogeochemical aspects of microbial degradation of phenolic materials in the California coastal marine environment. Ph.D. dissertation, University of California, San Diego.

Genetic Detection of Red Tide Dinoflagellates

University of California, Santa Barbara
R/CZ-102
1990–93

Barbara Prézelin and Edward Triplett

Dinoflagellates uniquely contain peridinin, a photosynthetic carotenoid functionally and structurally analogous to the major light-harvesting carotenoids (e.g., fucoxanthin, hex-fucoxanthin) of other algae that contain chlorophyll *c*. Cellular concentrations of peridinin commonly exceed those of the other photosynthetic pigments (chlorophylls *a* and *c*) in dinoflagellates and give these organisms their characteristic brick-red coloration. (Peridinin is what makes red tides red.) Most, if not all, peridinin is organized into discrete complexes containing peridinin, chlorophyll *a*, and protein (PCP). These complexes serve as light-harvesting complexes (LHCs) and are organized in supramolecular aggregates on thylakoid membranes in close association with photosystem II (Prézelin, 1987).

Each species of dinoflagellate has a "family" of water-soluble brick-red PCP complexes with identical spectral signatures (400–700 nm) and similar molecular weights, and members of the family can be distinguished on the basis of their isoelectric point. Each PCP complex has just one chromophore (light-harvesting center). This chromophore is buried in a hydrophobic crevice of the protein; externally exposed hydrophilic amino acids confer the water-soluble characteristic of the total complex. Each chromophore has four molecules of peridinin, which are arranged as two dimers around a chlorophyll *a* monomer. The fluorescence lifetime of the peridinin increases and presumably accounts for the 100% efficient transfer of radiant energy that occurs from peridinin to cholorphyll *a* within 10 psec after peridinin absorbs light. Work on clonal isolates of dinoflagellates showed that all isoelectric variants of a PCP family were present in a single cell and not generated by protein degradation during isolation procedures. The isoelectric diversity within and between different species of dinoflagellates was thought to be due from minor differences in the amino acid composition of different PCP apoproteins.

In some dinoflagellate species, only a single polypeptide (molecular weight, 29–35 kD) is bound to a single PCP chromophore, whereas in other species, two polypeptides (molecular weight 14–15.5 kD each) are bound to the chromophore (Govind et al., 1990). Recently, an antibody to PCP from *Heterocapsa pygmaea* (Roman et al., 1988) was used to detect the presence of both monomeric (35 kD) and dimeric forms (15 kD) of PCP apoproteins in extracts isolated from several additional species of free-living and symbiotic dinoflagellates (Govind et al., 1990). These and other findings support our hypothesis that the genes for monomeric and dimeric PCP apoproteins of different dinoflagellates are closely related.

Jovine et al. (1992) detected 56–64 isoelectric (pI) variants of PCPs in *H. pygmaea,* which can be clustered into seven subgroupings on the basis of pI values. The high diversity of PCPs was not due to either protein phosphorylation or glycosylation. Rather, it appeared to be due to different rates of formation of apoprotein heterodimers derived from a multigene family of PCP apoproteins (Triplett et al., 1993; Jovine et al., 1992).

The distribution of chlorophyll *a* in dinoflagellates is analogous to that of chlorophyll *b* in green plants and phycobilin in red algae and cyanobacteria (Prézelin and Boczar, 1986). In dinoflagellates, a core of chlorophyll *a* composed of photosystems I and II is coupled together by a matrix of LHCs. The core receives most of its photosynthetically absorbed light energy (AQ) via a structural association with LHCs. The combination of photosystems I and II and the LHCs are termed a photosynthetic unit. The LHCs are divided structurally into two major components, one dominated by LHCs containing chlorophyll *c*, and the other representative of the PCP complexes.

A total of 28 cDNA clones encoding the PCP apoprotein of an LHC have been isolated from *H. pygmaea*, and five have been sequenced. Sequenced cDNA clones represent the final posttranslationally modified protein. Residues representing the first 24 amino acids (as determined by direct protein analysis) are included in some of the cDNAs. Open reading frames encode for proteins ranging from 16.7 to 17.2 kD. Hybrid release translation and immunoprecipitation of the product yielded a protein doublet of about 21 kD, indicating the primary protein contains a leader sequence not represented in clones thus far sequenced.

The longest open reading frame for the nucleotide sequence of cPCP1 encodes for 103 amino acids and has 315 base pairs of 3′ non-translated sequence followed by the poly A tail. A computer-generated Chou-Fasman diagram shows the polypeptide is very hydrophilic, with the 47% acidic and basic residues nonrandomly distributed along the length of the molecule. Several clusters of nonpolar residues are found in highly conserved regions between amino acid residues 1–15, 25–30, and 52–60. These deduced PCP apoprotein characteristics are completely compatible with the earlier findings. We hypothesize that the clustered nonpolar residues seen in all five cPCPs sequenced to date represent an integral part of the

PCP binding sites making up the hydrophobic crevice in which the chromophore is known to reside (Prézelin, 1987).

Feng-Doolittle alignment indicates that cPCPs fall into three major families. Sequence comparisons showed less than 11% sequence homology (i.e., random) between *H. pygmaea* PCP and LHCs from a variety of other photoautotrophs, including angiosperms, green algae, and cyanobacteria.

Evidence is now sufficient to conclude that *H. pygmaea* PCP apoproteins are encoded by a nuclear multigene family. (1) Polyadenylated *H. pygmaea* RNA is enriched for PCP mRNA, and polyadenylation is not a feature of chloroplast mRNA. (2) Nonadenylated *H. pygmaea* RNA does not contain PCP mRNA. (3) Studies with isolated *H. pygmaea* genomic, chloroplast, and mitochondrial DNA indicate that only nuclear DNA hybridizes to our cPCP probes. (4) cPCP1 hybridizes to four RNA size classes, in which the visual and radioactive intensities of the smaller bands depend on growth irradiance. (5) PCP is known to occur in several isoelectric forms (Prézelin, 1987; Jovine et al., submitted), there are at least five PCP cDNAs with distinct nucleotide and deduced amino acid sequences, and there are at least four size classes of PCP mRNAs.

Roman et al. (1988) found that *in vitro* translation reactions supplied with total RNA from cultures of *H. pygmaea* grown in high-light or low-light conditions showed about a twofold increase in translatable PCP mRNAs in low-light cells. In addition, PCP apoproteins appeared to be encoded as larger preproteins.The parallel increases in PCP apoprotein and translatable PCP mRNAs were our first indication that light regulation of PCP complexes likely occurs at the level of PCP mRNA abundances. More recently, we found that four mRNA size classes encode for PCP complexes; one appears to be constitutive, whereas three appear to be photoregulated by growth irradiance (Triplett et al., 1993).

When cells were batch cultured under blue, green, or white light, the amount of PCP apoprotein in all high-light cells remained remarkably constant (Jovine et al., 1992). In contrast, cells grown under low-light conditions had large increases in the amount of PCP apoprotein. These increases accounted for 15–30% of total cell protein and were up to 15-fold higher than that required to bind all available peridinin molecules into PCP complexes.

Assembled PCP complexes are present in many isoforms under all growth conditions. The relative abundance of specific PCP isoforms may change under different growth conditions.The relative abundance of the seven subgroups of the pI variants of PCP varied as a function of spectral growth irradiance. Lowering the irradiance, but not the spectral composition, of the light field shifted the PCP conformer pattern. As the amount of red light in the light field decreased, predominance by neutral isoforms shifted to predominance by acidic isoforms. The two most basic clusters of PCP isoforms (pI 7.94–6.8) predominate under blue high-light conditions.

In order to investigate the possible regulation of these isoforms, it is essential to determine the number of PCP isoelectric variants possible and the number of apoprotein genes that could be regulated under different physiological conditions. The presence of multiple genes accounts for five of eight putative expressed genes (Triplett et al., submitted). These genes provide the primary protein sequences from which all the isoforms are generated. We found no evidence of posttranslational modification such as glycosylation or phosphorylation of the PCP gene products. This suggests that all the isoforms are due to primary sequence–dependent pI variations. The large number of isoforms appears to be a result of random formation of dimers. The maximum possible isoforms derived from dimerization of eight gene products is 64, which correlates well with the 56–62 PCP isoforms detected in *H. pygmaea* (Jovine et al., submitted). The dimerization of PCP apoproteins would result in the formation of heterodimers of PCP to form isoelectric intermediates with different pIs. In the case of the PCP complexes of dinoflagellates, the extent of transcriptional, translational, or posttranslational controls contributing to the differences in apoproteins of PCP isomers is unknown.

Photoregulation of peridinin, chlorophyll *a*, and PCP apoprotein abundance appears to be differentially cued to different regions of the visible spectrum. On the basis of previous work (Nelson and Prézelin, 1990) we were able to make precise calculations of the wavelength-dependent values of absorbed light energy $AQ(\lambda)$ for populations photoadapted to different spectral irradiances. Using spectrally weighted $AQ(\lambda)$ values for 50-nm regions of the visible spectrum and comparing the changes in abundances of PCP components as a function of changing irradiances of $AQ(\lambda)$ for a discrete bandwidth, we were able to determine the spectral dependencies in peridinin, chlorophyll *a*, and PCP apoprotein abundances independently and to conclude that their photoregulation was loosely coupled at best. Such differential spectral photoregulation of PCP gene expression would not be evident if the spectral growth irradiances only were considered. Rather the differences in spectral photoregulation became evident when the amount of absorbed quanta [$AQ(\lambda)$], not the amount of available quanta (Qpar),was considered instead.

Concentrations of cellular chlorophyll *a* varied as a inverse function of the amount of red light absorbed and showed no consistent relationship with other spectral bandwidths where light absorption was attributable to a mixture of dinoflagellate pigments (data not shown). Concentrations of cellular peridinin varied as a inverse function of the amount of blue-green light absorbed, which is the region of the visible spectrum where peridinin dominates cell absorption, and showed no consistent relationship with other spectral bandwidths where peridinin absorption contributes little or nothing to the absorption capabilities of whole cells. At first glance, the obvious lack of red light in blue and green

light fields suggested that PCP apoprotein concentrations were down regulated by red light and that photoreceptors analogous to phytochromes might be involved. Although this is an interesting possibility, close examination indicated that PCP apoprotein also varied as an inverse function of AQ at all spectral bandwidths between 400 and 700 nm. Therefore, we cautiously suggest that PCP apoprotein photoregulation is spectrally independent and dependent on total AQ absorption by *H. pygmaea* cells and may involve one or more photoreceptors.

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Geochemical Control of Toxicant Release Rates from San Diego Bay Sediments

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Assessments of sediment contamination have often depended on measurements of bulk concentrations of toxic chemicals in the sediment. Such assessments provide little information regarding the possible mobility and bioavailability of the contaminants. For this reason this project was designed as a 1-year pilot study to demonstrate the usefulness of benthic flux measurements as indicators of the environmental risk posed by contaminated sediments and to determine how sensitive trace metal release rates are to natural factors at the sediment-water interface that might affect the solubility and migration of toxicants.

Our approach had two parts. First, we measured *in situ* benthic fluxes of the metals—copper, cadmium, nickel, zinc, lead, manganese, and iron—together with selected nutrients and metabolites with a benthic chamber device. This technique is new to environmental assessment work. Second, we monitored chemical and physical conditions at the sediment-water interface by measuring millimeter-scale pore water profiles of O_2, pH, H_2S, and electrical resistivity in the uppermost sediment column with an *in situ* microprofiling instrument.

The benthic chamber instrument, developed at the Naval Ocean Systems Center (NOSC) in San Diego, consists of an open-bottomed chamber mounted in a tripod-shaped framework with associated sampling gear, sensors, control system, power supply, and deployment/retrieval equipment (Chadwick et al., 1992). When deployed, the 40 cm by 40 cm by 25 cm polycarbonate chamber isolates a volume of water above the sediment. Samples are drawn off from this volume over time (in this case over 72 hours) and then these samples are analyzed for changes in toxicant (or nutrient) concentration. Fluxes are calculated based on the rate of change in concentration times the chamber volume, divided by the chamber area. Continuous measurements of temperature, salinity, pH and dissolved oxygen are also made during each deployment by sensors mounted through the chamber lid. To prevent the chamber water from becoming anoxic, readings from the oxygen sensor are used to trigger the introduction of O_2 from a supply bottle into the chamber through porous capillary tubing. Blank tests run with the chamber have shown negligible uptake or release of trace elements by the chamber materials.

The *in situ* microprofiler operates an array of vertically mounted microelectrodes that are attached to the bottom end-cap of a pressure case containing the system electronics and data logger (Reimers, 1987). An electric motor attached to a threaded rod drives the electrodes stepwise across the sediment-water interface and several centimeters into the sediment. During this study, the profiler was deployed on a small tripod framework similar to the one used for the benthic chamber. The time to complete a profile was limited to 4–5 hours.

Our sampling in San Diego Bay was focused at one site off a marina at Shelter Island where the water depth is 4–6m, depending on the tide. Our original plan to work at six sites was too ambitious for a 1-year pilot study. Sediment loadings of trace metals at the Shelter Island site are as high as many other nearshore sites where trace metals have been shown to be introduced anthropogenically (Table 1). Four deployments of the benthic chamber and five of the microprofiler were made between February 3 and 26 1992, and between June 18 and 29 1992, in hopes of comparing winter and summer conditions. In general, variations in temperature and chemical properties in the bay's water were small between the two periods, and microelectrode profiles through the sediment-water interface were quite similar. However, we are unable to compare benthic fluxes because the benthic chamber sampling system failed during the winter work.

In this report we will emphasize our results from the June 1992 deployments. These results are preliminary and still require complete error analysis. Figures 1 and 2 illustrate time series of chemical and physical conditions in the chamber during the two 3-day deployments. In Figure 1a and 2a time series of salinity, temperature, dissolved oxygen, and pH as indicated by the internal sensors are plotted. Although during each experiment a parcel of water is isolated in the chamber, these records indicate that this water is heated and cooled as the external water changes temperature in response to both the tidal circulation in the bay and the sun's radiation cycle. The sea bed also naturally experiences changes of at least 40 μM O_2 in its overlying water during each day (as determined by conductivity-temperature-depth-O_2 monitoring). In the chamber, decreases in oxygen concentrations caused by benthic oxygen consumption were initially larger than 40 μM, but our system, which bled oxygen into the chamber, did succeed in maintaining near ambient O_2 conditions for most of the duration of the June 19–22 deployment (Figure 1a) and probably the June 25–28 deployment as well (Figure 2a). (The end of the data file for the latter deployment was not properly stored by the chamber instrument's data logging system).

One external water sample was collected at the beginning of each

Table 1. Metal Concentrations (μg g⁻¹) in San Diego Bay Surface Sediments Compared to Other Nearshore Sites

Site (No. of samples)	Cu	Cd	Cr	Pb	Ni	Zn
Shelter Island Site (n=2)	104; 137	<0.05; 0.07	39; 50	29; 37	16; 22	97; 121
Sinclair Inlet (10 sites)[1]	5–360	.08–5.0	46–98	11–380	29–101	26–2800
Long Island Sound (2 sites)[2]	4.4; 35	0.41; 1.2	n.d.	15; 265	n.d.	55; 57

Data sources: [1]Chadwick et al. (1992); [2]Lyons and Fitzgerald (1980)

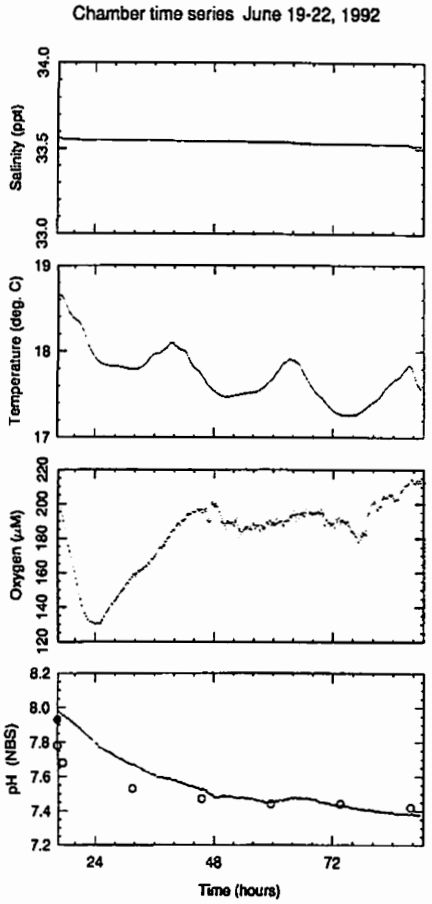

Figure 1a. Time-series data from the benthic chamber beginning at 16:30 hours on June 19, 1992 and continuing through June 22. Continuous records were recorded by sensors within the chamber. Open circles indicate pH values measured in water samples retrieved by the instrument's sampling system. These samples were not analyzed until after the deployment was completed. The filled circle indicates the pH of an outside bottom water sample collected before the deployment and analyzed promptly.

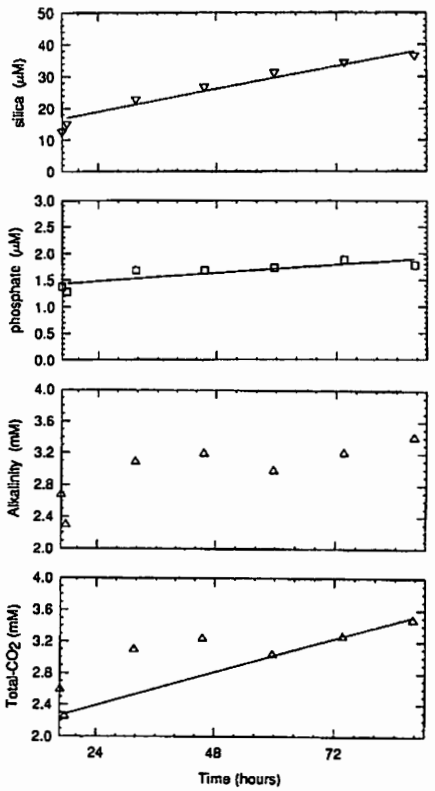

Figure 1b. Time series of silica, phosphate, alkalinity and total-CO_2 (calculated) concentrations in the benthic flux chamber during the June 19–22 deployment. The slopes of the linear regression lines shown were used to calculate fluxes reported in Table 2.

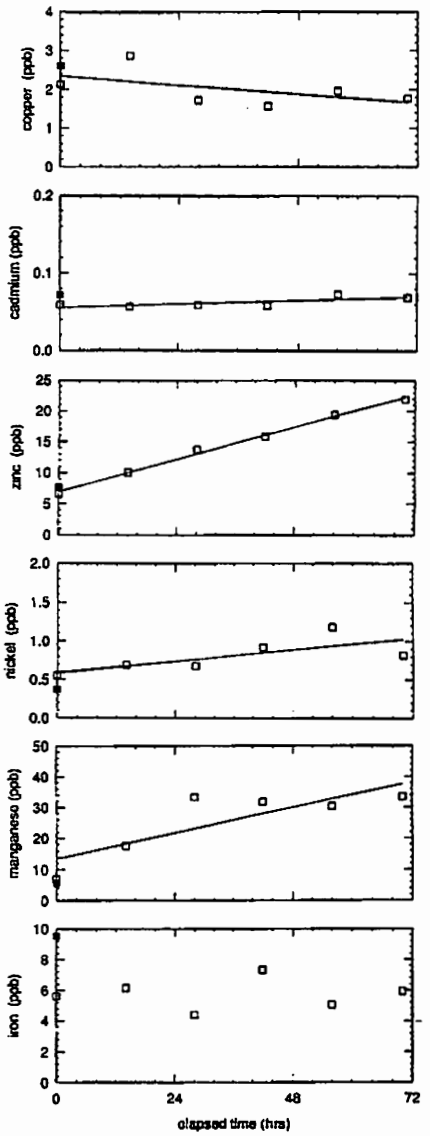

Figure 1c. Evolution of metal concentrations in the benthic flux chamber during the June 19–22 deployment. Filled squares indicate concentrations in water collected from outside the chamber. The slopes of the linear regression lines shown were used to calculate the fluxes in Table 3.

Table 2. Benthic Solute Fluxes (mmol $m^{-2}day^{-1}$) in San Diego Bay Compared to Other Coastal Environments

Environment	Total-CO_2	Silica	Phosphate
San Diego Bay	107; 83	1.8; 2.2	0.04; --
Gullmarsfjorden, Sweden[1]		0.17 to 6.26	
Skan Bay, Alaska[2]	42 ± 7		
Narragensett Bay, Rhode Island[3]	20	12 ± 2	0.7 ±. 2
Cape Lookout Bight[4]	97.5 ± 5.2		−0.5 to 2.9

Positive fluxes are out of the sediments. Data sources:[1]Rutgers van der Loeff et al. (1984);[2]Alperin et al. (1992);[3]McCaffrey et al. (1980); [4] Martens (1984) and Klump and Martens (1981).

flux experiment, and six time-series water samples were collected from inside the benthic chamber. The time-series samples filled into acid-washed, 500 cm^3 teflon (TFE) sampling bottles aboard the benthic flux instrument by hydrostatic pressure. After recovery, all seven samples were filtered through precleaned 0.45 μM cellulose nitrate membrane filters, 20 ml splits were removed for nutrient analyses, and the remainder was acidified to pH 2 with high purity nitric acid.

The 20 cm^3 splits were subdivided and analyzed for pH, total alkalinity, phosphate, and silica using methods recommended by Gieskes and Peretsman (1986). In Figures 1a and 2a the pH values of these samples are compared to the real-time sensor readings in the chamber. In Figures 1b and 2b, the phosphate, silica, and alkalinity time-series are plotted together with total-CO_2, which is calculated from alkalinity and pH. Linear regressions through the silica, phosphate, and total-CO_2 data indicate flux rates that are in line with other estimates for coastal sediments (Table 2). These data also suggest that while some solutes may have a constant flux rate and exchange direction throughout the course of an experiment, others may be variable.

Constituent metals of interest were extracted from the acidified water samples at NOSC by the ammonium pyrrolidine dithiocarbamate/methyl isobutyl ketone (APDC/MIBK) method (Kinrade and van Loon, 1974; Franson, 1981), and the extracted samples were analyzed by graphite furnace atomic absorption using the method of standard additions to develop a standard curve. Initial metal concentrations were of the same order of magnitude as levels in inshore surface waters of Monterey Bay, California (Knauer and Martin, 1973), except for manganese, which had higher concentrations here. Concentration changes inside the chamber over time indicated that San Diego Bay sediments may steadily release manganese, zinc, and nickel, but they are a sink for copper. A consistent flux direction for the elements cadmium, lead, and iron was not apparent in the data (Figure 1c and 2c; lead not shown because Pb concentrations were at or below our limit of detection, 0.2 ppb). We believe this indicates little mobility in the cases of cadmium and lead, and contamination problems for iron.

Working in western Sweden, Westerlund et al. (1986) hypothesized that for trace metals to be released from coastal sediments they first must be freed by the oxidation of carrier organic matter phases and then solubilized within a surface oxic layer of the sediments. Solubilization may depend on the equilibrium behavior of both free and complexed ions and adsorption-desorption reactions with solids (Lu and Chen, 1977). Redox reactions involving inorganic solids are another means to release trace metals. Conversely, if sediments are highly anoxic, the precipitation of metal sulfides and other insoluble authigenic solids effectively may prevent trace metal mobilization (Westerlund et al., 1986).

The high TCO_2 fluxes from the sediments at our study site indicate a high rate of organic carbon oxidation that could be either by reactions of oxic or anaerobic metabolism (e.g., MnO_2 and Fe_2O_3 reduction). Figure 3 shows that the first 5–8 mm of sediment is usually oxic but that oxygen is readily consumed in the surface layer. Daytime oxygen profiles exhibit a surface O^3 and pH maximum associated with photosynthesis. These peaks disappear at night. Hydrogen sulfide was not detectable by microelectrode in the uppermost 6 cm of these sediments, but the pH minimum at roughly 0.5 cm suggests the oxidation of reduced metabolites through reactions such as:

$$4Fe^{2+} + O_2 + 6H_2O \rightarrow 4FeOOH + 8H^+$$

and

$$4Mn^{2+} + 2O_2 + 4H_2O \rightarrow 4MnO_2 + 8H^+.$$

Formation factor profiles were variable, indicating nonuniform physical properties across the site as well.

Thus, our microelectrode studies suggest that conditions in the first centimeter of sediment in San Diego Bay are conducive to a host of competing metal mobilization and uptake reactions. We infer that the release of manganese and nickel may be coupled by the association of these metals in oxyhydroxide solids and that during our chamber experiments there was a net reduction of these phases. The release of zinc is suggested to be driven by the decomposition of organic matter, since both TCO_2 and zinc show high positive fluxes. The uptake of copper is probably by the thin, oxidizing, biologically active surface layer (Klinkhammer et al., 1982). A constant anthropogenic source of copper to San Diego Bay is the leaching of antifouling paints on boat hulls.

In Table 3 the metal fluxes we have estimated from our data for sediments in San Diego Bay are

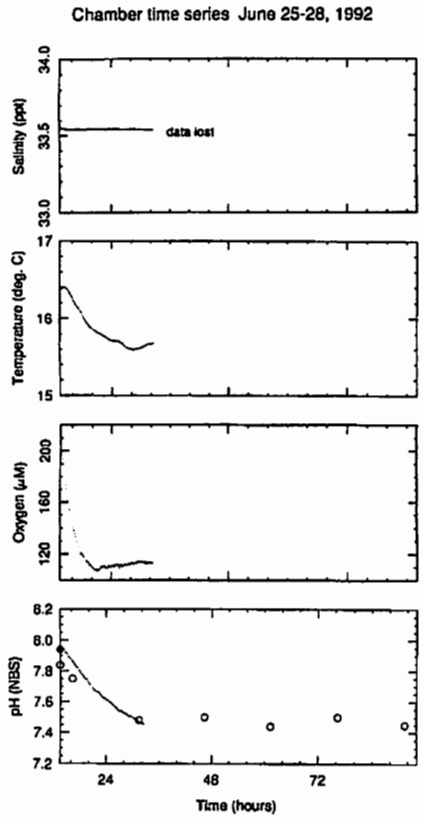

Figure 2a. Time-series data from the benthic chamber beginning at 13:30 hours on June 25, 1992 and continuing through June 28. See Figure 1b for explanation of symbols.

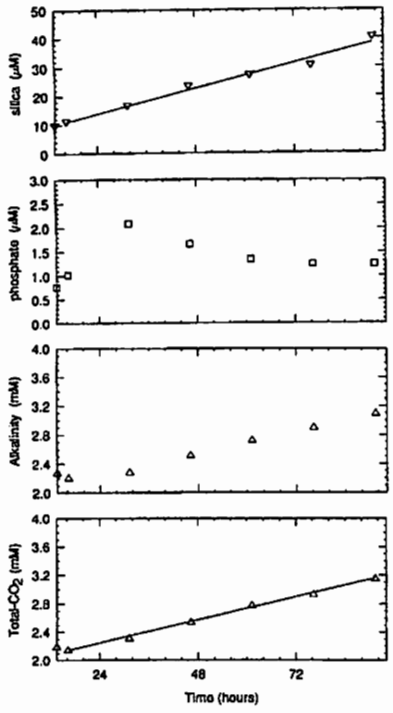

Figure 2b. Times series of silica, phosphate, alkalinity, and total-CO_2 (calculated) concentrations in the benthic flux chamber during the June 25–28 deployment. The slopes of the linear regression lines shown were used to calculate fluxes reported in Table 2.

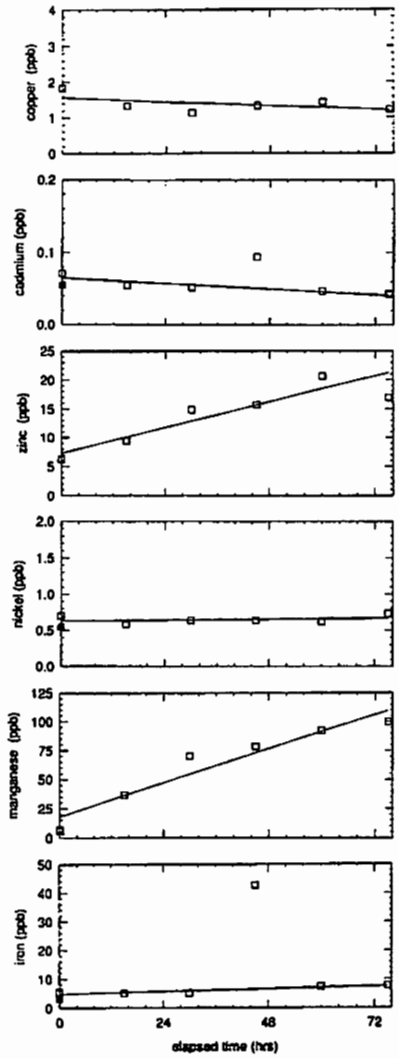

Figure 2c. Evolution of metal concentrations in the benthic flux chamber during the June 25–28 deployment. The slopes of the linear regression lines shown were used to calculate fluxes reported in Table 3.

listed with the sediment concentrations. The lack of a consistent ratio between these measurements reinforces the argument that the environmental risk of polluted sediments cannot be based on the sediment's concentration levels of pollutants. In this pilot study we have demonstrated that we can successfully assess trace metal fluxes from sediments in their natural state and that we can characterize many aspects of that natural state. Although, more work needs to be done to describe the natural variability of flux rates in relationship to environmental conditions, we believe these findings represent a significant beginning to predicting the fate of anthropogenic metals in the marine environment.

Table 3. Preliminary Estimates of Metal Fluxes (μg $m^2 day^{-1}$) from San Diego Bay Sediments Compared to Concentrations in the Surface Sediment (μg g^1)

Metal	Observed Fluxes	Sediment Content
Cu	–58; -30	104; 137
Cd	–2; 1	<0.05; 0.07
Ni	3, 38	16; 22
Zn	1100; 1300	97; 121
Pb	Indeterminate	29; 37
Mn	2100; 7500	35–160
Fe	Indeterminate; 230	26,000–37,000

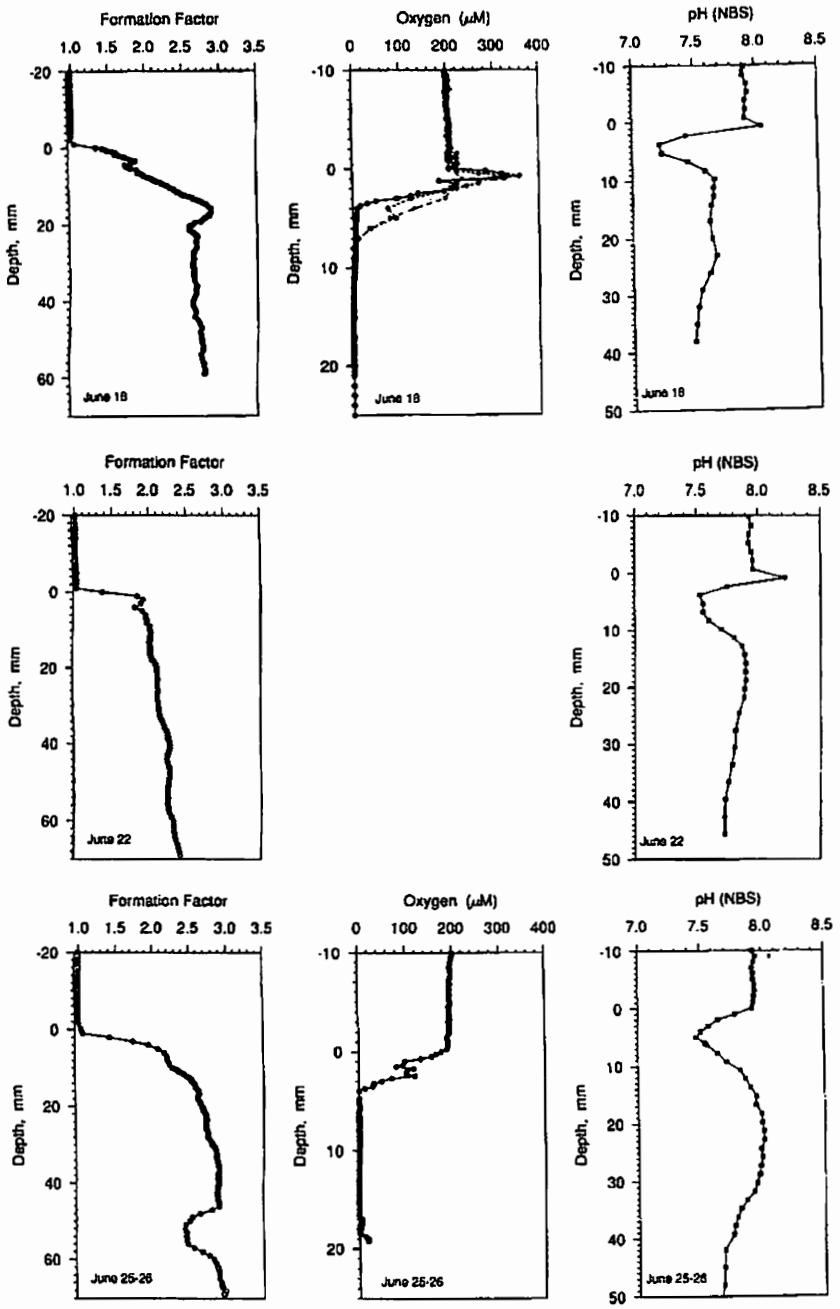

Figure 3. Microelectrode profiles of formation factor, O_2, and pH across the sediment-water interface of the Shelter Island study site. Formation factor equals the resistivity in the sediment/ resistivity of overlying water. Hydrogen sulfide electrodes detected no H_2S within these surface sediments during the same profiling experiments. The June 18 and 22 deployments were made during the middle of the day. The June 25–26 deployment was made during the middle of the night.

Cooperating Organizations

U.S. Naval, Command, Control and Ocean Surveillance Center, Marine Environmental Branch, San Diego, California

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Aquaculture

Capacitation and Cryopreservation of Shrimp Sperm

Bodega Marine Laboratory
R/A-70
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Penaeoidean shrimp culture, an industry representing one of the largest aquaculture cash crops in the world, has historically relied on wild stock. Domestication has been hampered by a lack of knowledge about the basic biology of shrimp gametes, but we have made considerable progress toward providing fundamental information concerning activation of and interaction between gametes of a model penaeoidean shrimp, *Sicyonia ingentis* (reviewed by Clark et al., 1984; Clark and Griffin, 1988; Pillai and Clark, 1990). Further, we have developed methods for the cryopreservation of sperm that will permit their long term storage (Anchordoguy et al., 1988). Originally we proposed to use what we have learned about these gametes to develop the technology required to ensure sperm availability for *in vitro* fertilization at any time of the year.

We have during the last four years accomplished this goal. Specifically, our laboratory has: (1) determined the temporal requirements for *in situ* capacitation of *S. ingentis* sperm; (2) morphologically described the site of capacitation, the seminal receptacles; (3) determined physiological alterations within sperm during capacitation; (4) isolated and partially characterized biochemical components of seminal receptacle fluid; (5) documented pH differences in uncapacitated versus capacitated sperm and seminal plasm versus seminal receptacle fluid; and (6) used the above to define a technique for *in vitro* capacitation of *S. ingentis* sperm taken from males. In addition, we (1) successfully fertilized *S. ingentis* eggs *in vitro* using freshly obtained and cryopreserved sperm capacitated *in situ* by females, as well as *in vitro* capacitated sperm; and (2) cryopreserved sperm of *Penaeus setiferus*.

To determine the time required for sperm to become capacitated after transfer to a female, we initiated laboratory mating studies between males and "virgin" females (females which have molted and no longer possess sperm in their seminal receptacles). The results of these studies demonstrated that *S. ingentis* sperm are capable of undergoing the first phase of the acrosome reaction (acrosomal exocytosis) within 48 hr of transfer to a female but require 5 to 7 days of storage in the female before they become competent to complete the acrosome reaction by forming an acrosomal filament (Clark et al., 1989; Wikramanayake et al., 1989).

Penaeoideans can reproductively be divided into two groups, those with open thelyca and those with closed thelyca. The thelycum is an exoskeletal specialization, located on the ventral cephalothorax of a female, onto which (in those species possessing open thelyca) or into which (in those with closed thelyca) spermatophores or sperm are deposited by the male during mating. *S. ingentis* females have closed thelyca and males do not produce a spermatophore; rather they produce a sperm mass that consists of a viscous seminal plasm containing sperm and nonmembrane-bound granules of vas deferens origin. This sperm mass is then encased in an acellular sheath (Almeida and Clark, in preparation). During mating male *S. ingentis* deposit these sausage-like sperm masses into the seminal receptacles (which are sac-like specializations of the thelyca) of females via laterally placed openings in the thelyca.

Thelyca and their seminal receptacles from both mated and "virgin" females were examined using light microscopy, scanning electron microscopy, and transmission electron microscopy (Suverkropp, 1990). Seminal receptacles are invaginations of the exoskeleton of the posterior region of the thelycum. The receptacles are structurally similar to arthrodial membranes; they are trilaminar and elastic (noncalcified). Each receptacle can be subdivided into three chambers, and each chamber is surrounded by secretory epithelia of at least two differing cell types. One of the secretory cell types (that resemble mast cells) produces nonmembrane-bound granules that contained acidic, sulfated glycosaminoglycans. Therefore, two different types of nonmembrane-bound granules (one of vas origin and one of female origin) were described in association with sperm or sperm-bearing structures. We found it interesting that within the seminal receptacles of previously mated females, sperm were embedded in a fluid (seminal plasm), but neither the nonmembrane-bound granules of vas deferens origin nor the heparin-like granules of female origin were present. By examining receptacles from mated and nonmated females at different stages of the molt cycle, we determined that secretory cells lining the seminal receptacle degranulate over a period of 24–36 hr postecdysis, with the contents filling the seminal receptacles; dissolution of granules and release to seminal receptacles occurs independent of mating or the presence of a male.

Early on in this project we fractionated and analyzed seminal plasm from both the vas deferens of males and seminal receptacles of mated females. These analyses provided evidence for the presence of a number of small molecular weight (MW) peptides and free amino acids but few polypeptides in seminal plasm from seminal receptacles. In contrast, seminal plasm from the vas deferens

contained numerous polypeptides but few small MW peptides or free amino acids. With the evidence that material was deposited into seminal receptacles shortly after ecdysis and before mating, we undertook an analysis of this fluid. Such analyses revealed the presence of serine protease-like activity with a broad range of specificity in seminal receptacle fluid of nonmated females. Since protease activity SDS-PAGE yields six bands of activity, we are uncertain at this time whether we are dealing with a single protease of broad specificity or a number of proteases.

In addition to the foregoing we found that (1) the intracellular pH of sperm rises from 7.2 to approximately 8.5 during the capacitory process; and (2) there is also a dramatic elevation of internal Ca^{++} in sperm cells during this period (Lindsay and Clark, 1990; 1992a,b).

Utilizing the above-described data we refined our techniques (described in a previous Sea Grant report) for *in vitro* capacitation of *S. ingentis* sperm. Uncapacitated sperm (removed from males) after being incubated in seminal receptacle fluid at pH 8.5 for 50 hr were capable of undergoing acrosomal exocytosis (80%) and acrosomal filament formation (60%). Capacitation is inhibited in the presence of protease inhibitors, soybean trypsin inhibitor, and aprotinin, indicating that the activity of the protease is necessary during capacitation. One trypsin inhibitor, para-aminobenzamidine, however, had the opposite effect; capacitation was enhanced in its presence. This molecule mimics the arginine binding site on trypsin, pointing to a role for the amino acid arginine in capacitation. Addition of arginine to *in vitro* capacitation media did enhance capacitation, suggesting that arginine or an arginine containing peptide may be the capacitation factor.

From these experiments we have been able to efficiently *in vitro* capacitate *S. ingentis* sperm and have generated a testable model of the mechanisms and factors involved.

The experience gained from successfully cryopreserving *S. ingentis* sperm has been utilized to cryopreserve the sperm of *P. setiferus*. Experiments with *P. setiferus* sperm indicated that these cells were more susceptible to freezing damage than were *S. ingentis* sperm. In order to cryopreserve *P. setiferus* sperm it was necessary to reduce the freezing rate and utilize different cryoprotectants. Cryopreserved *P. setiferus* sperm were stored for 10 months, and although we did not use these sperm for *in vitro* fertilizations (because of collecting problems in Texas we have been unable to collect ripe females for the past three years), we did activate such sperm with stored egg water.

In most animal systems fertilization (whether internal or external) is achieved by a motile sperm (released from a male) swimming to an egg, traversing one or more egg-associated extracellular coats, undergoing an acrosome reaction, traversing or penetrating any remaining egg coats, and binding to and fusing with the egg. In these systems, *in vitro* fertilization is achieved by introducing sperm to eggs (procured from females) that have been placed in a medium that mimics physiological *in vivo* fertilization conditions. Techniques for *in vitro* fertilization in such systems are now well defined but are not transferable to *S. ingentis* since the sperm are nonmotile and thus incapable of swimming to an egg or traversing thick extracellular egg coats such as a jelly layer (see Clark et al., 1984).

During *in vivo* fertilization in *S. ingentis*, the spawning female physically mixes eggs (released from paired ovipores) and stored sperm (from seminal receptacles) via the movement of her pleopods (Clark et al., 1984; Pillai et al., 1988). Sperm brought into contact with an egg bind to the outermost extracellular coat, a thin vitelline envelope that is closely associated with the egg plasma membrane (at spawning eggs do not possess a jelly coat). Sperm undergo the first phase of the acrosome reaction (exocytosis of the acrosomal vesicle) upon binding to the vitelline envelope, locally digest the vitelline envelope, and make contact with the plasma membrane of the egg (see Clark and Griffin, 1988, for review). There is a critical time constraint upon these events, because within seconds of exposure to seawater (regardless of whether or not sperm are present) *S. ingentis* eggs begin to form a jelly layer (Pillai and Clark, 1987; Clark et al., 1990). Jelly precursor is released from extracellular crypts in the egg and forms a corona around the egg; the corona is formed between the egg and the vitelline envelope; thus, as formation ensues the vitelline envelope is lifted off the surface of the egg. Because the sperm are nonmotile, any sperm that are brought into contact with the vitelline envelope after initiation of jelly corona formation are prevented access to the egg surface and thus cannot participate in fertilization.

To achieve *in vitro* fertilization in *S. ingentis* we first isolated and pooled sperm from female seminal receptacles as described by Griffin et al. (1987). Pooled sperm (10 cells/ml) were held in beakers containing chilled seawater, while gravid females were light cycled to induce ovulation (see Pillai et al., 1988). Unfertilized eggs for *in vitro* fertilization experiments were obtained by two techniques: (1) an ovulated female was sacrificed and eggs were removed directly from the ovary; or (2) ovulated females were induced to spawn (Pillai et al., 1988) but were prevented from releasing sperm stored in their seminal receptacles. This procedure involves immobilizing the fourth pair of periopods during spawning (Shoffner-McGee, 1989; Clark et al., 1992; Clark and Griffin, 1992). When utilizing this technique, both positive controls (where the female was allowed to release sperm along with eggs) and negative controls (where the female was prevented from releasing stored sperm and eggs were spawned into seawater that did not contain isolated sperm) were run on each individual spawn. Eggs were deemed fertilized if they underwent an equal first cleavage; unfertilized *S. ingentis* eggs undergo an unequal, abortive first cleavage (Pillai and Clark, 1987).

In experiments utilizing spawned ova (10 trials) control (natural)

fertilization averaged 68%. Negative controls, where females were immobilized using the Shoffner-McGee/Clark technique, yielded only 18% fertilization; however, when immobilized females spawned into sperm suspensions (*in vitro* fertilizations), fertilization was 66%. Additionally, we have successfully used cryopreserved *S. ingentis* sperm to fertilize eggs *in vitro*. Although this work is continuing, we have achieved 68% fertilization in five *in vitro* trials with cryopreserved sperm (compared to 77% utilizing freshly isolated sperm). Utilizing ovarian eggs (obtained by dissection of the ovaries of ovulated females), we have obtained greater than 48% *in vitro* fertilization. It should be noted that although our criteria for fertilization is an equal first cleavage, we have cultured eggs, fertilized *in vitro*, through hatching.

The ability to both cryopreserve sperm and obtain *in vitro* fertilization has been an important accomplishment. The inability, however, to obtain large numbers of competent sperm has been a limiting factor. As a result of the work accomplished in this project, we now have the technology to *in vitro* capacitate *S. ingentis* sperm, providing the numbers of sperm required for further fundamental studies of gamete physiology and eventual domestication.

Cooperating Organizations

Baylor College of Medicine
California Department of Fish and Game
Institute of Fishery Sciences, National Taiwan University
Institute of Oceanology, Quindao, Peoples Republic of China
Louisiana State University, Baton Rouge and New Orleans, Louisiana
McCormick and Associates
Medical University of South Carolina
Texas A & M University, Port Aransas, Texas
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Endocrine Stimulation and Regulation of Sturgeon Female Maturation

University of California, Davis
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Gary P. Moberg

Collaborative efforts between aquaculturists and University of California researchers have established a new aquaculture industry in California, producing highly prized white sturgeon (*Acipenser transmontanus*). The continued development of this industry depends upon the successful breeding of the domestic broodstocks established by the California farmers. Since 1987, aquaculturists have been able to utilize the semen of cultured males for artificial reproduction. However, sturgeon hatchery breeding still depends largely on the egg supply obtained from wild caught females.

Recently a small number of cultured females, 7–10 years of age, reached sexual maturity, and the first domestic spawning occurred in the spring of 1990. This past year, additional cultured females were spawned, demonstrating that cultured female sturgeon have the potential for being used in commercial operations. Our monitoring of ovarian maturation in a large number of commercial broodstock, however, has revealed several major problems in sturgeon broodstock management that prevent the total reliance on cultured females: the late age of puberty, varied recruitment into vitellogenesis among the fish of the same age, and the deleterious impact of abnormal environmental conditions on final ovarian maturation.

Economically efficient sturgeon breeding will depend on the early onset of puberty, synchronous vitellogenesis of fish of the same age group or size, and normal final ovarian maturation. In spite of the late maturity and long reproductive cycle in wild acipenserid stocks, our collaborative work with industry suggests that an approximately 30% maturation and spawning rate in 6 to 7-year-old cultured white sturgeon should eventually be achievable and that this level of maturation would be adequate to initiate commercial fingerling production.

Cultured white sturgeon grow rapidly, with the males reaching sexual maturity at 3–5 years of age. The females reach 30 kg of body weight at 7 years of age, but only a few reach reproductive maturity at that time, even though wild gravid females of this species with a body weight of only 13–16 kg have been caught and successfully spawned in commercial hatcheries.

Three years ago we began to study the endocrine control of sturgeon reproduction with three immediate goals: (1) to investigate the role of the endocrine system in reproductive development, (2) to evaluate in female sturgeon hormone treatments that have proved successful in inducing gonadotropin (GTH) secretion or reproductive maturation in other species, and (3) to monitor plasma hormone concentrations and ovarian development in captive broodstock held at commercial farms. Since publications are in preparation or the work is still in progress, we will describe results for each of these goals.

Endocrine Control of Reproduction

In order to use hormones to manipulate reproduction in sturgeon, it is first necessary to understand the role that the hypothalamic-pituitary-gonadal endocrine system plays in regulating reproduction in sturgeon. This system has been virtually unstudied in this species. The major block to such studies has been the failure to isolate and identify the gonadotropins in the sturgeon and then to establish assays for these hormones. We have now accomplished these tasks and have initiated a series of studies to define the role of the hypothalamic-pituitary system in regulating reproduction.

From purified sturgeon pituitary fractions provided by Dr. H. Papkoff (UC San Francisco), we have identified two pituitary fractions, GTH-14 and GTH-20, that appear to be functional analogs of GTH I and GTH II in salmonids (Kawauchi et al., 1989; Swanson et al., 1989). We have raised relative specific polyclonal antibodies to these two hormones and have used them to establish highly specific radioimmunoassays (RIA) for measuring the two gonadotropins (Table 1). We have now conducted the following studies to define the biological function of these two gonadotropins.

Table 1.
RIA for Measuring Gonadotrophins

	Cross Reactivity (%)	
Peptide	GtH-14 Assay	GtH-20 Assay
GtH-14	-	2.0
GtH-20	9.3	
Hake GtH	< 0.0095	< 0.0095
Salmon GtH	< 0.0095	< 0.0095
Tilapia GtH	< 0.0095	< 0.0095
Carp GtH	< 0.0095	0.7
Gillichthes GtH	< 0.0095	< 0.0095
Ovine GtH	< 0.0095	< 0.0095
Sturgeon growth hormone-Prolactin	0.5	0.6

1. Induction of Egg Maturation. Eggs collected by catheterization from wild spawning sturgeon were incubated in Leibovitz culture media with progesterone, GTH-14, GTH-20, or without treatment (control). The germinal vesicle breakdown (GVBD) response was 0% for controls, 100% for progesterone, 39% for GTH-14, and 77% for GTH-20. These data suggest that both GTHs can induce GVBD, but GTH-20 appears to have the greatest effect.

2. Pituitary Concentrations of GTHs. Both GTHs were found at low concentrations in immature males and females. In the mature male there is an increase in the concentration of both GTHs, with the concentration of GTH-14 being markedly higher than the concentration of GTH-20 (Figure 1). These are opportunistic studies that continue when material is available.

3. Plasma GTHs in Wild Spawned Females. Wild females being spawned at one fish farm were bled before an injection of carp pituitary extract and at spawning. While the plasma concentrations of both GTHs increased at spawning, it was GTH-20 that showed the most striking increase (Figure 2). Because carp pituitary extracts do not significantly cross react with our two antibodies (see Table 1), we believe that the carp pituitary extracts induced ovarian steroid synthesis and these steroids in turn induced GTH secretion. This experiment will be repeated when our UC Davis fish mature and we can use GnRHa to induce spawning. Nevertheless, these data support our conclusion that GTH-20 is the gonadotropin responsible for final maturation in the sturgeon.

4. GTHs in Maturing Cultured Females. We had been following changes in the plasma concentrations of the gonadotropins in cultured females for 2 years. During vitellogenesis, plasma GTH-14 is elevated but drops prior to spawning. At spawning there was a significant increase in GTH-20 with only a slight increase in GTH-14, suggesting that GTH-14 controls early ovarian development but GTH-20 is responsible for final maturation.

5. GnRHa Treatment of Males. Because access to females is limited, our initial studies to evaluate pituitary responsiveness to GnRH have been conducted with mature males. Since des-Gly^{10}-[D-Ala^{6}]-LH-RH ethylamide (GnRHa) is similar in structure to sturgeon GnRH (Sherwood and Lovejoy, 1989) and will induce spawning in sturgeon (Doroshov and Lutes, 1984; Fuji et al., 1989; Gontcharov et al., 1989), we have used this GnRH analog to

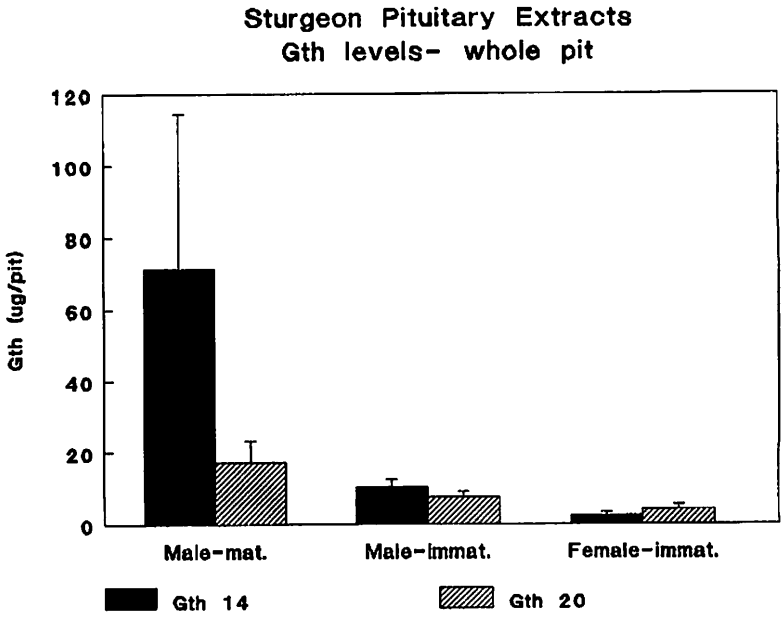

Figure 1.

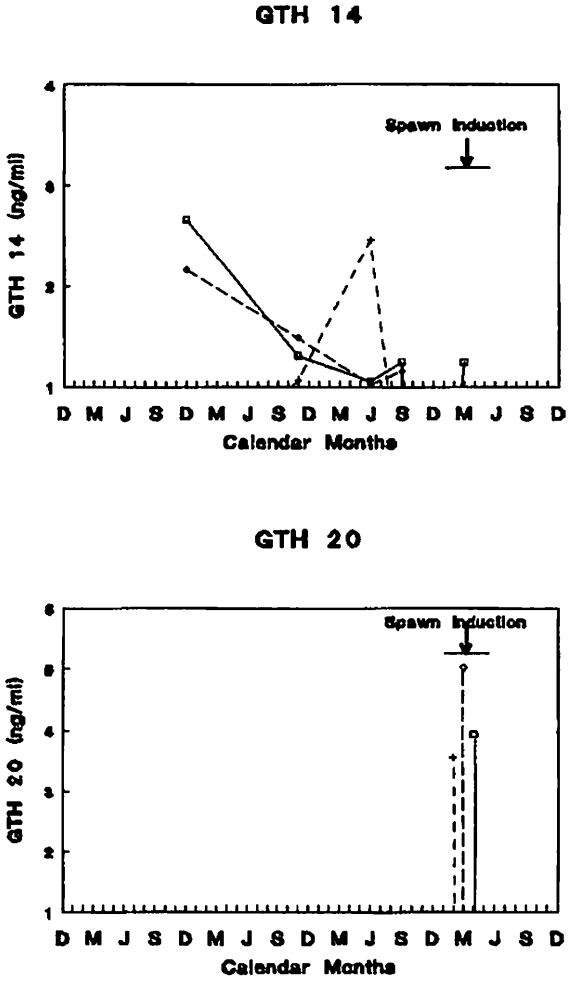

Figure 2.

study GTH secretion. The intramuscular injection of GnRHa (10 or 20 μg/kg) induced the secretion of GTH-14 with only a marginal effect on GTH-20. While we found the response was extremely variable, there appears to be a positive correlation between elevated plasma concentrations of testosterone prior to the injection of GnRHa and the ability of fish to respond to GnRHa treatment.

During the past year we have been studying the influence of season on the amount of GTHs secreted in response to GnRH stimulation. In August there was only a limited response to GnRHa stimulation. However, in December and April, which correlate with the initiation of spawning, both GTH-14 and GTH-20 showed a marked response to GnRHa treatment. These data suggest that season has a marked influence on the pituitary responsiveness of male sturgeon to GnRHa.

Because our goal is to induce ovarian development, we have evaluated the effect of long-term exposure to GnRHa. Pellets of GnRHa (using cholesterol, Dextran, and cocoa butter as the carrier) were implanted into mature males, and the GTH response to four doses of GnRHa (0, 50, 100, and 200 μg/kg) was evaluated. As with the single injections, the response was variable and appears to be dependent on the presence of elevated plasma concentrations of testosterone prior to treatment for the GnRHa to be effective. With the pellet implants there appears to be a second confounding factor with respect to the GTH response. In those animals that responded to the implanted GnRHa pellets, there was a negative correlation between the plasma concentration of GnRHa and GTH, suggesting that prolonged continuous exposure to a high dose of GnRHa may cause down regulation of the GnRH receptors as occurs in goldfish (Peter, 1980). Because of this finding we have initiated a series of *in vitro* studies to define the receptor dynamics of the sturgeon pituitary gonadotropes.

6. In Vitro Studies. Using a perifused pituitary system that we have established previously (Matteri et al., 1986; Swartz and Moberg, 1986), we have demonstrated that the perifused male pituitary will secrete both GTHs in response to GnRHa stimulation. We have now validated the parameters of the system.

A dose-dependency of GnRHa concentration on GTH-14 secretion was established. The level of GTH-14 secretion was raised 18.29% ± 12.2% above the control level upon treatment for 1 hr with 10^{-10} *M* GnRHa, while secretion increased by 48.59% ± 6.6% with 10^{-8} *M* GnRHa treatment and by 58.06% ± 7.4% with 10^{-6} *M* GnRHa treatment.

A similar dose relationship for GTH-20 secretion was established. Stimulation with 10^{-6} *M* GnRHa raised control secretion by 176% ± 132%, with 10^{-8} *M* GnRHa by 64.7% ± 26.0% and with 10^{-10} *M* GnRHa by 77.6% ± 63.1%. Although the amount of GTH-20 secreted was variable at each concentration of GnRHa studied, it appears that the analog had a more potent effect on the stimulation of GTH-20 than on GTH-14.

Further development and improvement of the perifusion system will allow us to pursue the study of down regulation of GnRH receptors and the role of steroid feedback on GTH secretion.

7. Induction of GTH Synthesis by Exogenous Testosterone. One possible approach to obtain the precocious induction of gonadal development may be by using the positive feedback action of testosterone to induce pituitary gonadotropin production. Administered in silastic implants, testosterone has been shown to stimulate the accumulation of pituitary gonadotropin in sexually immature rainbow trout when given over 1, 2, or 3 month periods (Crim and Evans, 1983). Furthermore, when coupled with the gonadotropin-releasing activity of GnRHa, testosterone can cause circulating gonadotropin concentrations to increase, which in turn stimulate the gonads. Such treatment has been effective in inducing precocious gonadal development in immature milkfish (Lee, 1988).

We have evaluated the effects of exogenous testosterone on pituitary gonadotropin content in undifferentiated 1-year old white sturgeon. The experimental groups were as follows: control receiving no implantation, 600 mg testosterone pellet for 10 days, 600 mg testosterone pellet for 21 days, and 600 mg testosterone pellet for 21 days followed by a 50 mg injection of estrogen on day 10.

The average GTH-14 pituitary content for control fish was measured at 663 ± 187 ng/mg pit. Testosterone treatment for 10 days raised this control level by 28% to an average of 848 ± 230 ng/mg pit, while treatment for 21 days increased it by 91% to an average of 1265 ± 126 ng/mg pit. The combined testosterone/estrogen treatment had an even greater effect, as it raised the control level by 156% to 1697 ± 567 ng/mg pit.

The effects on GTH-20 were much more pronounced when compared to GTH-14. The control value of 115 ± 23 ng/mg pit was increased 129% (263 ± 126.15 ng/mg pit) with testosterone treatment for 10 days and by 270% (425 ± 136 ng/mg pit) with testosterone treatment for 21 days. The combined testosterone/estrogen treatment was less effective than the testosterone alone for 21 days, raising the control level by 264% to 419 ±12.56 ng/mg pit.

From these data it appears that testosterone treatment in undifferentiated white sturgeon can induce a positive feedback on the pituitary production of both GTH-14 and GTH-20. Based on these results, we conclude that estrogen may play a permissive role with testosterone on the synthesis of GTH-14.

Summary. The results from these studies indicate that the sturgeon, like salmonids, have two GTHs. We believe that fraction GTH-14 (like GTH I in salmonids) is responsible for ovarian development while the GTH-20 fraction (like GTH II in salmonids) is responsible for final maturation. Testosterone appears to enhance the ability of the pituitary gonadotrope to respond to GnRHa stimulation. We are currently determining whether this enhancing effect is the result of increased GTH

synthesis and/or increased responsiveness of the gonadotrope to GnRHa stimulation in order that we can design future exogenous hormone treatments to control reproduction of sturgeon. We also have an indication that continuous exposure to GnRH may result in the down regulation of the pituitary GTH receptors.

Field Evaluation of Hormone Treatments

We have evaluated three possible approaches to inducing ovarian development in immature sturgeon by using techniques that had been successfully applied in other species.

1. Osmotic Pump. Since our experience with implanted GnRHa pellets suggested that continuous exposure to GnRHa may result in the down regulation of the GnRH receptor, we tested Alzet osmotic pumps that delivered daily pulses of 10 μg/kg for 60 days. Controls were implanted with pumps that released only the carrier. We found this treatment was ineffective in elevating the plasma concentrations of GTHs 14 and 20 estrogen or vitellogenin, and there was no indication of ovarian development. However, we did note that all fish implanted with pumps (treatment or control) showed substantial weight loss and a decrease in the condition factor. At biopsy, the ovarian tissue was hydrated and inflamed, apparently because the implanted pumps proved to be stressful to fish, possibly masking any positive response to the pulsatile treatment of GnRHa.

2. Clomiphene Treatment. Previously we had demonstrated that estradiol, with or without exogenous GnRHa, would induce vitellogenesis in immature females but not ovarian development or vitellogenin uptake (Moberg et al., 1991). Since these results may have stemmed from the negative feedback of estrogen (Lessman and Habibi, 1987) as well as the down regulation of the GnRH receptors, we readdressed this question using a modified approach. Estradiol was administered to immature females to induce vitellogenin synthesis and was followed 30 days later with the antiestrogen clomiphene (clom), since this drug had been demonstrated to induce GTH secretion in carp (Breton et al., 1975). In addition, one group of estradiol + clom fish were periodically injected with GnRHa. While estradiol was effective in elevating the plasma concentrations of vitellogenin, none of the treatments was effective in inducing GTH secretion or in inducing vitellogenin uptake by the follicles. As in the Alzet pump studies, the treated fish lost body weight, suggesting that the repeated, brief handling was stressful to the fish.

3. Methyl-Testosterone Treatment. In the studies with males that were discussed previously, it appeared that the ability of GnRHa to stimulate GTH secretion was positively correlated with elevated plasma concentrations of testosterone. Other investigators have reported that testosterone has a positive influence on the pituitary responsiveness to GnRHa and/or pituitary concentration of GTHs in trout (Goos, 1987; Goos et al., 1986).

Likewise, methyl-testosterone (MT) has been found effective in inducing reproductive maturation in milkfish (Lee, 1988) and increasing the pituitary concentrations of GTH in juvenile trout (Crim and Evans, 1983). Therefore, we implanted immature females with silastic capsules containing 17α-methyl-testosterone. Implanted and nonimplanted controls and the MT treated fish were challenged 29 and 56 days later with GnRHa. There was no indication that MT had any effect on the plasma concentration of estrogen or vitellogenin or on the ability of the females to secrete GTHs in response to the GnRHa challenge.

Summary. While each of the approaches evaluated had enjoyed some success in other species, they were ineffective in inducing GTH secretion or ovarian maturation in the cultured sturgeon females. Refinement of these approaches may eventually prove effective; however, such efforts should not be attempted until we have a better understanding of the endocrine regulation of sturgeon.

Monitoring Program

We monitor annually or biannually the plasma concentrations of hormones and the ovarian development in over 500 females maintained at several fish farms. These studies permit us to compare reproductive immature and maturing females under a variety of management conditions. These females provide the biological samples (blood, eggs, and ovarian tissue) that were used in the studies already discussed.

As we have previously reported, the data we have accumulated indicate that female maturation is characterized by the following stages. In the fall the granulosa and chorion differentiate; there are no visible yolk platelets. The next spring there is a deposition of yolk platelets and a dramatic increase in the yolk synthesis and oocyte growth throughout the summer. By the next fall some fish in captivity have black eggs (i.e., acquire melanin pigment granules), which is indicative of spawning in the spring. The others which did not develop black eggs cease vitellogenesis during the winter and will continue the process during the following summer. The fish with black eggs will enter the phase of final maturation the next spring, when eggs polarize and appear ready for spawning. Unfortunately, follicles in many of these fish do not respond with egg maturation to hormone stimulation in either the *in vitro* (progesterone) or the *in vivo* (carp pituitary extract). From our observations it appears that season and water temperature are two modulating factors determining reproductive maturity and spawning success. We are now addressing these issues. As discussed previously, we have already observed in males that season influences the pituitary response to exogenous GnRH. We are initiating *in vitro* studies to see if temperature also alters the pituitary response to GnRH.

Cooperating Organizations
Sturgeon Broodstock Development Committee

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Population Genetics of Commercial Pacific Oyster Stocks

University of California, Davis
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Until recently, the chief tools for gaining information on the genetics of populations were gel-electrophoretic separations of either proteins, followed by histochemical staining to reveal genetically encoded variation in the mobility of specific enzymes, or DNA fragments generated by the digestion of DNA with restriction endonucleases, enzymes that recognize and cleave specific nucleotide sequences. Both of these methods detect only a fraction of variation in DNA sequences, and neither allows study of very small amounts of tissue or larval forms.

A primary technical objective of this project, in its first year, was to adapt new technology for enzymatic amplification of DNA, the polymerase chain reaction (PCR) (Saiki et al., 1988), to the direct study of DNA sequence variation in Pacific oyster (*Crassostrea gigas*) populations. The advantages of this new technology are (1) sensitivity and specificity, allowing a particular target DNA sequence to be amplified a billionfold from a single larva, spat, or adult tissue sample; (2) the ability to utilize crude cellular extracts as well as highly purified DNA, obviating tedious DNA isolations in conducting population surveys; and (3) amplification of target DNA sequences to concentrations allowing direct sequencing by standard methods and subsequently the use of nonradioactively labeled probes to identify genotypes by simple hybridization methods.

The need to sample larval oysters was indicated by our earlier study of the genetics of hatchery-propagated populations of the Pacific oyster (Hedgecock and Sly, 1990). Utilizing enzyme electrophoresis of tissue extracts from juvenile oysters, we found that substantial genetic drift had occurred in two hatchery stocks isolated for three generations from the progenitor native population in Dabob Bay, Washington. From the amount of genetic drift, we estimated effective population numbers of only 40.6 and 8.9, in marked contrast to the hundreds of brood stock used to propagate commercial standing stocks of several million individuals. One hypothesis to explain this discrepancy between breeding and effective population numbers is the great variation in the numbers of offspring that females contribute to the brood stock of the next generation. Such variance in reproductive success is made possible by the enormous fecundity but highly variable fertility of oysters (Lannan, 1980). A test of this hypothesis requires methods for obtaining information on the genetics of spawning females and their larval offspring in mass cultures. PCR-based analysis of maternally inherited mitochondrial DNA (mtDNA) is the best approach to obtaining such information.

Adapting PCR methods to oysters required work in three areas: DNA isolation, PCR optimization, and sequencing of PCR products. Simple digestion of tissues, in as little as 5 µl buffered solution of proteinase K with nonionic detergent at moderate temperatures (37–56°C) for 1–3 hours, provides sufficient template for PCR. We isolated and amplified mtDNA from single larvae (200 µm shell height) and month-old spat, which were fresh or fixed in formalin, and from biopsied adult mantle tissues. The best PCR results were obtained with the formalin-preserved larvae, which facilitates collecting larval samples from commercial hatcheries or from the wild.

The first step in adapting PCR to the amplification of oyster mtDNA was the selection of pairs of oligonucleotides capable of priming the reaction by recognizing and binding, on opposite strands, to sequences flanking target sequences. We began with four sets of "conserved" primers previously designed to amplify portions of the mtDNA genes for 16S rRNA, 12S rRNA, cytochrome b, and the vertebrate noncoding control region (Kessing et al., 1989). Only the 16S rRNA and cytochrome b primers gave amplification, yielding 570 base pair (bp) and 420 bp products, respectively. Following standard symmetric PCR, which generates double-stranded DNA, we successfully performed asymmetric reactions for both the 16S rRNA and cytochrome b targets in order to generate single-stranded DNA for subsequent sequencing.

Samples selected for study were Pacific oyster adults (*Crassostrea gigas*) from the native population in Dabob Bay, Washington; Kumamoto oysters from commercial stocks cultivated by Coast Oyster Company in Humboldt Bay, California, by Taylor United in Puget Sound, Washington, and by Hog Island Oyster Company, in Tomales Bay, California; and native flat oyster (*Ostrea lurida*) from Humboldt Bay. Native flat oysters were used as an outgroup to aid in the construction of sequence phylogenies.

Working with DNA extracts from these different species of oysters and using the conserved primers, we found that each target sequence, each strand of each target in asymmetric PCR, and each species required, to some extent, the empirical development of specific PCR protocols and parameters. Experiments with reaction ingredients—relative concentrations of template and primer, Mg^{2+} concentration relative to template and primer concentrations, and dNTP concentrations—were carried out, as well as manipulations of thermocycling parameters, particularly annealing temperatures and

ramping times from annealing to extension temperatures. Once sequence information was obtained for the 16S rRNA gene, however, we were able to design oyster-specific primers, internal to the original conserved primers, which now give repeatably uniform PCR amplification of a 320 bp target sequence under stringent annealing conditions.

After successful amplification of mtDNA, our next objective was to identify polymorphic sequences that might serve for progeny identification. Single-stranded PCR products from asymmetric reactions were washed in Centricon filters to remove unincorporated nucleotides and primers. One-fourth to one-half of retentate volumes were dried or ethanol precipitated and used directly in Sanger dideoxy-nucleotide, chain-terminating, sequencing reactions (Sequenase kit, US Biochemicals) with 35S-dATP labeling. Sequencing reaction products were separated in 6% denaturing polyacrylamide wedge gels in an IBI apparatus and autoradiographed. Sequencing of both strands of the 16S rRNA PCR product was completed for six Pacific, eight Kumamoto, and two native flat oysters; sequences of one strand only were obtained for four other Pacific oysters and an additional Kumamoto oyster. Although a cytochrome b target was successfully amplified from two Pacific oysters, readable sequence from these PCR products was not obtained.

Initial comparison of a Pacific oyster 16S rRNA sequence with the homologous human sequence, using the University of Wisconsin Genetics Computer Group software, revealed a 63% sequence identity, thus confirming that our PCR product and sequence were the large subunit ribosomal RNA-coding gene of the mitochondrial genome. Among individuals within the three oyster species, we did not detect any DNA sequence variation, but differences among species were evident. As expected from their more distant relationship, the 16S rRNA sequence of the native flat oyster differed considerably from the two *Crassostrea* sequences by 62 and 60 nucleotide substitutions, one deletion, and five insertions. Six of the nine Kumamoto 16S rRNA sequences differed from the Pacific oyster sequence at seven nucleotide positions (2% sequence divergence); six of the nucleotide differences were transitions between purines or pyrimidines, one was a purine-to-pyrimidine transversion. These sequence differences suggest that the Pacific and Kumamoto oysters diverged from one another at least 1 million years ago (Wilson et al., 1985). The remaining three Kumamoto samples, however, had sequences identical to the Pacific oyster sequence, suggesting contamination of the commercial Kumamoto stocks.

We also surveyed protein variation in commercial Kumamoto stocks using starch gel electrophoresis and specific enzyme staining as described by Hedgecock and Sly (1990). Eleven protein-coding loci were screened in a total of 77 individuals from samples of 1987 year-class production stock, 1991 year-class hatchery seed, and several small lots of unknown age and provenance. Pacific oysters ($N = 78$) from the 1990 spatfall in Dabob Bay, Washington, were used as controls for scoring of allozyme electrophoretic mobilities. The Kumamoto oysters were found to be substantially different from the Pacific oysters, having an overall average genetic distance of 0.4 electrophoretically detectable amino acid substitutions per protein, including fixed, diagnostic differences for isocitrate dehydrogenase, malate dehydrogenase, and mannose phosphate isomerase.

These electrophoretic results confirm the earlier allozyme study of Buroker et al. (1979), and together with the mtDNA divergence and evidence for a one-way gametic incompatiblity (sperm of the Kumamoto oyster are unable to fertilize eggs of the Pacific oyster [Imai and Sakai, 1961; S. Allen and D. Hedgecock, unpublished observation]), support the suggestion of Ahmed (1975) that the Kumamoto oyster be recognized as a distinct species, *C. sikamea.*

The three supposed Kumamoto oysters that were found to have Pacific oyster mtDNA sequences were also found to be homozygous for Pacific oyster allozyme markers, indicating that these were Pacific oyster contaminants of Kumamoto stocks. Moreover, substantial changes in allelic frequencies between the 1987 and 1991 year classes of a commercial Kumamoto stock yielded an estimate of only four for the effective population number of this commercial brood stock (following methods of Hedgecock and Sly, 1990). The genetic resources of Kumamoto oyster stocks on the west coast may be in jeopardy because (1) commercial brood stocks have small effective population numbers and will become inbred at an appreciable rate unless managed more prudently, and (2) the Pacific and Kumamoto species have been confused, resulting in contamination of brood stocks. The potential for replenishing genetic resources from Japanese native stocks has, moreover, been greatly diminished by massive importations of the Pacific oyster into the Kumamoto Prefecture (Ozaki and Fujio, 1985; K. Cooper, personal communication).

Owing to its characteristic deep-cupped shape and small size, Kumamoto oysters command a good price in the restaurant half-shell trade. Kumamoto hatchery seed destined for the half-shell trade is often reared by more labor- intensive and costly methods than those used for Pacific oysters. In the past year, however, Kumamoto producers in California, Oregon, and Washington have reported that Kumamoto hatchery seed has been growing up with the slze and shape of Pacific oysters. This results not only in a loss of the price differential between the Kumamoto and Pacific products but a loss of cultchless production costs. Contamination of commercial Kumamoto brood stocks is thus having an adverse economic impact on this segment of the west coast industry.

Shell shape and size, the only characters currently used by commercial oyster breeders to distinguish brood stock of the two

species, are evidently not completely reliable, a conclusion reached also for the American oysters (Galtsoff, 1964; Hedgecock and Okazaki, 1984). In the extreme, the shell morphologies of the Pacific and Kumamoto oysters are distinctive, but intermediate shell shapes occur. Clearly, biochemical markers diagnostic for the two species are useful in screening brood stocks for species purity.

Differences between the 16S rRNA mtDNA types of the two species may be rapidly diagnosed by restriction enzyme digestion of the PCR product (Banks, 1991; Banks et al., 1993). The enzyme Dra I, which recognizes and cleaves the sequence TTTAAA, cuts the 16S product amplified from Pacific oyster samples into two pieces that are easily resolved on agarose gels stained with ethidium bromide (Figure 1). The Kumamoto PCR product, having a TTAAAA sequence at this site, is not cut by Dra I. Thus, the maternal parents of commercial hatchery spawns may now be diagnosed in just a few hours by PCR amplification and restriction enzyme digestion of the product. Paternal lineages can also now be identified by examining diagnostic allozymes in young spat; development of PCR methods for nuclear DNA differences are now needed to accomplish paternal identification at the larval stage.

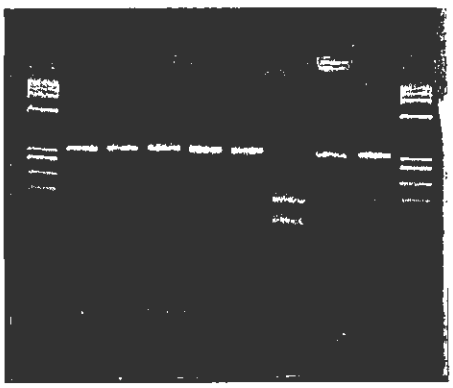

Figure 1. Ethidium-bromide stained agarose gel (3% NuSieve, 1% agarose) containing DNA size markers (ϕX174 phage DNA digested with Hae III) and a 319 base pair (bp) portion of the 16S rRNA-coding mitochondrial DNA, amplified by the polymerase chain reaction (PCR), from Pacific and Kumamoto oysters. After amplification, oyster DNA was digested with the restriction endonuclease Dra I, which cleaves the PCR product from the Pacific oyster into 178 bp and 141 bp fragments; the PCR product from the Kumamoto oyster lacks the Dra I recognition site and remains uncleaved. Lanes 1 and 10: ϕX174; lanes 2-6, 8, 9: PCR product from Kumamoto oysters; lane 7: PCR product from a Pacific oyster.

Cooperating Organizations

Coast Oyster Company, Quilcene, Washington

Hog Island Oyster Company, Marshall, California

Taylor United Company, Shelton, Washington

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Controlled Culture of a New Marine Model, the Sea Anemone *Nematostella Vectensis*

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1990–1993

Cadet Hand

The sea anemone *Nematostella vectensis* is an unusually widely distributed species. It is known from England, from Nova Scotia to Georgia on the Atlantic Coast, from Florida to Louisiana in the Gulf of Mexico, and from California to Washington along the Pacific Coast (Hand and Uhlinger, 1992). It is primarily an estuarine anemone and most commonly occurs in isolated pools in marshes of estuaries and bays. It is, however, tolerant of variations in salinity (8.96–51.54°/oo) and tolerates water temperatures from –1 to 28° C (Williams, 1983). It is a simple sea anemone and is a member of the family Edwardsiidae. Like other members of that family, it has an aboral, expandable digging structure, the physa, that allows it to burrow into soft substrates. It does not attach to solid substrates, and it does not have a true base.

Throughout most of its range, *N. vectensis* is abundant and can be readily collected. For instance, Williams (1983) estimated that a single pool in England contained more than 5 million, and Bleakney and Meyer (1979) found 1,816 individuals in a 15 × 15 cm sample in Nova Scotia. When found in the field, most individuals are usually less than 2 cm long, and the diameter of the column is only a few millimeters. The 12–16 tentacles are transparent and difficult to see in the field. The animals are best collected by gathering the top 2 cm of soft sediment from marsh pools. Subsequently, this material is spread out in a layer about 1 cm thick in large pans and covered with about 1 cm of water. The salinity of the water should be approximately the same as the salinity of the water from the pool from which the anemones were taken. After the mixture stands 1–2 hrs or longer the *Nematostella* will emerge from the sediment and can be removed with either a pipette or forceps. The specimens can then be maintained in the laboratory in small dishes free of sediment.

We have obtained living specimens of *N. vectensis* from England, Nova Scotia, Maryland, Georgia, California, Oregon, and Washington. We have maintained all of them in culture and used them in our research. More recently we have also obtained living material from New Hampshire. We now have those sea anemones in culture and are rearing them to sexual maturity.

Culture Methods

We have maintained our sea anemones in crystallizing dishes with plastic Petri dish parts as covers to reduce evaporation. The cultures have been kept at room temperatures, which have varied from 16° C–26° C and at a salinity of about 12°/oo. To prepare the culture water, we filter raw seawater through Whatman no. 1 filter paper. Normally, we dilute the filtered seawater with two parts of distilled water. That water is aerated in large flasks before being used, although the cultures are kept in standing water and no additional aeration is provided. The culture water is changed every 1–2 weeks.

The sea anemones from Maryland came from the Rhode River, a subestuary of Chesapeake Bay, and the offspring of those anemones have been used most often in our research. The salinity where these anemones were collected was about 12°/oo, and that fact and subsequent studies using various salinities caused us to select the one-third seawater as our standard culture medium.

The cultures are fed recently hatched brine shrimp nauplii (*Artemia* sp.). In some instances, we have fed the anemones every second day; at other times, we have fed them only three times a week, with no feedings on Saturday or Sunday. Large cultures of anemones have been maintained in about 100 ml of water; isolated anemones were cultured in 25 ml. We have found that many other foods as well as nauplii are accepted by *Nematostella*. In nature, this sea anemone eats small insects, crustaceans, polychaete worms, and small gastropods. In the laboratory, they will accept the yolk of hard-boiled eggs and small pieces of crustaceans and mollusks. After trying several sorts of foods to supplement the regular diet of nauplii, we settled on using small pieces of the ovaries of the California mussel *Mytilus californianus*. That tissue can be cut into 1 to 2-mm pieces and is readily accepted by the anemones. In qualitative studies, we determined that a regular diet of nauplii, supplemented once every 7 or 8 days with pieces of mussel ovary, led to maximal growth and reproductive output by the anemones.

Early Results

The *Nematostella* from Maryland were not sexually mature when first received, although feeding these anemones *Artemia* nauplii, supplemented with occasional pieces of mussel or shrimp meat, led to their sexual maturity and successful reproduction. We learned that males and females tend to spawn at the same time and that fertile eggs go through cleavage and become free planula larvae 36–48 hrs after fertilization. By about 7 days after fertilization, the planulae metamorphose into four-tentacled juvenile sea anemones about 500 µm-long. Growth, when the anemones are fed nauplii every second day, is relatively rapid; in about a month, the juveniles may grow to be 1–2 cm long and may have 12 tentacles. At 2 months of age, the young anemones are approaching sexual maturity

and have become 2–5 cm long and usually have 16 tentacles. Some individuals have reached full sexual maturity and spawned in as few as 69 days after fertilization, but more commonly, first spawnings occur at around 3–4 months.

Another early observation was that well-fed developing anemones began to divide asexually about the time of sexual maturity, that is, at 10 weeks of age or later. More recently, with heavy feeding, we have observed asexual reproduction in anemones only 5 weeks old. Asexual division in this sea anemone is by transverse fission, giving rise to fragments that vary in length from 1 mm to several centimeters. The oral fragments develop a new physal area in a few days, and the aboral fragments develop a new tentacular crown and begin feeding a few days after fission.

An important early observation was that the anemones in different culture dishes all tended to spawn on the same day when like culture regimens of feeding and care were used. By that time, we had several generations of *Nematostella* in culture, and some cultures consisted of several hundred anemones. Even though spawnings tended to be simultaneous, variation occurred, and we decided to take advantage of asexual division and obtain clones of *Nematostella*. Our presumption was that by using genetically identical clonemates, we should be able to obtain large numbers of individuals, all of which would spawn simultaneously if cultured identically.

The Production of Clones

We isolated two groups of sibling anemones, one a group of 20 and another a group of 10, each from different parents. These were anemones that had not yet become sexually mature. Each anemone was kept in a separate dish, containing 25 ml of 12% seawater. All were fed three to five drops of concentrated *Artemia* nauplii every second day and small pieces of mussel ovary every eighth day, and the water was changed every 1–2 weeks.

A few weeks after they had been isolated, all the anemones had reached sexual maturity and had begun to spawn. As we had discovered earlier, on numerous occasions over the next several months, many of the isolated anemones spawned on the same day and within a few hours of each other. These anemones also underwent asexual divisions as we had anticipated, and we were obtaining clones as we had hoped. The group of 10 anemones was kept in individual isolation for 264 days. These animals consisted of 4 females and 6 males. At the end of the 264 days the 4 females had become clones of 5, 1, 2, and 4 (1 represents no divisions in the period of isolation), and the 6 males had become clones of 2, 2, 1, 1, 5, and 4. The group of 20 isolated anemones consisted of 12 females and 8 males. All were kept isolated for 303 days. The 12 females of this group became clones of 3,18, 2, 2, 7, 0, 27, 38, 1, 1, 8, and 2 (0 represents a female that died during the 303 days without any divisions). In the same period, the 8 males became clones of 4, 1, 14, 9, 53, 96, 1, and 2.

Although we did not use them for our experiments, we also determined that what amounts to artificial asexual divisions can be achieved by cutting anemones into two or more pieces. This can be done by using sharp scissors or razor blades or by tightly constricting an anemone's body with fine nylon monofilament. In any event, the pieces produced will regenerate to normal anemones, just as fragments from natural transverse fission do, and clones can be readily created by this process.

Simultaneous Spawning

Having accomplished our goal of creating genetically identical clonemates, we were then prepared to see if our culture techniques could yield predictable, synchronous spawnings. We put each of 12 female clonemates and 12 male clonemates in individual dishes in 25 ml of 12% seawater as before. Every second day, each was fed a few drops of concentrated nauplii. On every eighth day, each was also fed two pieces of mussel ovary, and the water in the dish was changed. All 24 anemones were maintained on this rigid schedule of feeding and care for almost 8 months. During that period, the females spawned 322 times, and the males spawned 264 times. Of these totals, 75% of the female spawns and 64% of the male spawns occurred on the day after the anemones were fed mussel and the water was changed.

Using another group of 16 female and 16 male clonemates, we reduced the feeding schedule so that the anemones were fed nauplii every Monday, Wednesday, and Friday, and were fed mussel ovary and had the water changed on Mondays. With this regimen, most spawning occurred on Tuesdays, the day after the anemones were fed mussel and the water was changed. Thus, these anemones were spawning regularly and synchronously on a 7-day cycle.

A third group of clonemates was put on a schedule in which the frequency of receiving mussel as food varied. All were fed nauplii every second day, and the water was changed every eighth day. Some of these clonemates received no mussel as food, and some received mussel every 4, 8, 12, or 16 days. Each of the five feeding groups consisted of four anemones. The anemones receiving mussel every eighth day spawned quite predictably on the day after receiving mussel. The group receiving no mussel spawned erratically and less than the groups receiving mussel. Spawning in the group receiving mussel every 4 days was also erratic, but many spawned the day after eating mussel if that also was the day after the water in their dish was changed. Spawning was less regular in the groups receiving mussel every 16 days and every 12 days than in the group receiving mussel every 8 days, but again the spawning tended to occur after the anemones ate mussel and the water was changed. We concluded that somehow both the mussel food and the change in water were stimulating the anemones to spawn and that too heavy feeding led to irregular and less spawning than that in the group that was fed mussel and had the

water changed every 8 days.

To attempt to discover whether spawning was more likely controlled by the mussel feeding or by the water change, we set up 10 groups with 10 clonemates per group. This time each dish contained 10 clonemates in 100 ml of 12% seawater. All were fed nauplii every Monday, Wednesday, and Friday, and all received mussel ovary on Mondays. However, the water was changed in six groups on Mondays, in two on Tuesdays, and in two on Wednesdays. With these schedules, most spawnings occurred on the day after the change in water. We have concluded that if these sea anemones are fully mature and contain gametes nearly ready for release, the change in water appears to be the stimulus that sets off the events and the process that lead to actual release of gametes. We do not know what specific aspect of the change in water is involved in stimulating egg release. Further studies may show that it is changes in oxygen tension or the reduction in ammonia content that occurs with the change in water.

Asexual Reproduction

As noted earlier, we observed large variations in the frequency of asexual divisions among sibling anemones. Taking advantage of the asexual division during our studies of spawning in clonemates, we learned that the number of divisions by clonemates shows little variation among those on comparable feeding and care regimens. The 12 female clonemates that we kept isolated for 323 days divided 88 times, an average of 7.33 ± 1.15 divisions per female. The 12 male clonemates kept for the same period on the same diet and care regimen divided only 32 times, an average of 2.67 ± 0.89 divisions per male. On the basis of these and other observations, we have concluded that clonemates derived from rapidly dividing animals tend to continue to divide rapidly, whereas those from more slowly dividing animals continue to divide more slowly. This suggests that fission rate in this anemone is controlled by the heredity of the anemone, as does the variation in the number of clonemates obtained from isolated sibling anemones.

We also obtained data on the relationship of food intake and asexual reproduction. As described earlier, 20 female clonemates were separated into five groups with four anemones per group and fed a varied diet consisting of nauplii every second day and given no mussel or given mussel every 4, 8, 12, or 16 days. In 280 days, those receiving no mussel divided a total of 14 times. In the other groups, the numbers of divisions were 42, 25, 23, and 18, respectively, for anemones given mussel every 4, 8, 12, or 16 days. Thus, the group receiving the most mussel food divided three times more frequently than the group receiving no mussel. However, the sizes of the fragments produced by transverse fission were not significantly different among the groups, and we found no significant difference in the time (days) required for the aboral fragments to develop new tentacular crowns and begin feeding.

Are the Widespread Populations of *Nematostella* a Single Species?

Hand (1957) and Williams (1975) concluded that *Nematostella pellucida* Crowell (1946) was a junior synonym of *N. vectensis* and that the populations of *N. vectensis* in England and along the Atlantic and Pacific coasts of North America were all representatives of a single species. We had *N. vectensis* from many places in culture, and we learned that when the anemones were maintained on similar culture regimens, all the populations tended to spawn at the same time. This gave us the opportunity to make crosses between the populations obtained from various geographic locations. Unfortunately, we did not have representatives of both sexes from all locations. We had only males from Nova Scotia; only females from England, California, and Washington; and both males and females from Maryland, Georgia, and Oregon. Thus, we had females from six locations and males from four locations. Simultaneous spawnings allowed us to cross the six female types by the four male types. This yielded 24 healthy first generations from the 24 crosses, and those first generations matured and produced healthy second-generation anemones. These results add convincing evidence that the widely distributed *N. vectensis* is indeed a single species.

Summary

We have described the full life history of *N. vectensis*. We have developed defined, repeatable, controlled culture methods for the anemone and have determined the feeding and culture conditions that yield predictable synchronous spawning of the animals. We have learned much about both asexual and sexual reproduction in the species, and we have developed clones both by natural fission and by artificial surgical fission. We have shown that the use of clonemates in experiments yields uniform results, as might be expected. Our techniques make it possible to provide every stage of the life history for study and experiments, and, as *N. vectensis* remains reproductively active throughout the year, gametes, developmental stages, and all other stages can be made continuously available. We have provided a unique marine metazoan for use in bioassay studies; this anemone is now available for use in educational settings as well as research laboratories.

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Improvement of Striped Bass Rearing in Hatcheries

Joseph J. Cech, Jr.

Results

Exercise conditioning at 1.5 to 2.5 body length/sec for 60 days significantly improved the growth and swimming performance of both cultured and salvaged (from water-export pump screens) young-of-the-year (YOY) striped bass. Final weights in exercise-conditioned fish were significantly greater than controls in both cultured and salvaged groups (Figure 1). Moreover, salvaged fish had final weights significantly greater than cultured fish in both exercised and control groups.

Exercised fish also had a significantly higher critical swimming velocity than controls for both the cultured and salvaged fish (Figure 2).

Cross-sectional areas of red muscle in the control and exercised cultured fish were not significantly different from each other at any section (Figure 3). The same results were observed between controls and exercised salvaged fish. However, cross-sectional areas in the control and exercised salvaged fish were significantly greater than the control cultured fish at 65 and 80% standard length (SL).

Cross-sectional areas of white muscle in exercised cultured fish were significantly greater than the control at 50 and 65% SL (Figure 4). Cross-sectional areas in the control and exercised salvaged fish were not significantly different from each other at any section. However, they were both significantly greater than those of the control cultured fish in all sections.

Exercise conditioning improved stress responses for both cultured and salvaged fish in terms of earlier return to prestress levels of plasma cortisol and plasma osmolality and faster clearance rate of plasma lactate (Figures 5 and 6). Hematocrit levels were not different between

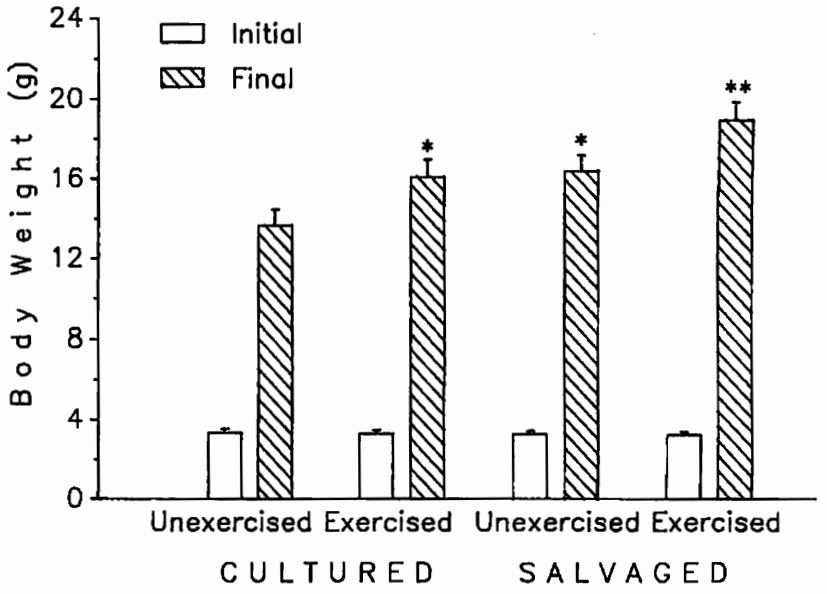

Figure 1. Initial and final body weights for unexercised and exercised cultured and salvaged fish. (n = 90; * significantly greater than unexercised cultured fish; ** significantly greater than *; error bar = S.E.M.).

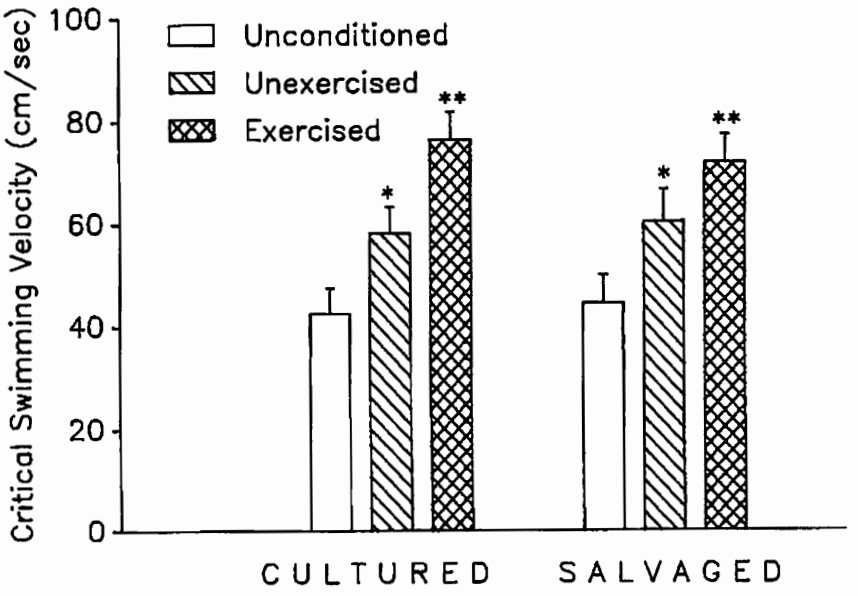

Figure 2. Critical swimming velocity for unconditioned, unexercised and exercised, cultured and salvaged fish. (n = 18; * significantly greater than unconditioned fish; ** significantly greater than *; error bar = S.E.M.).

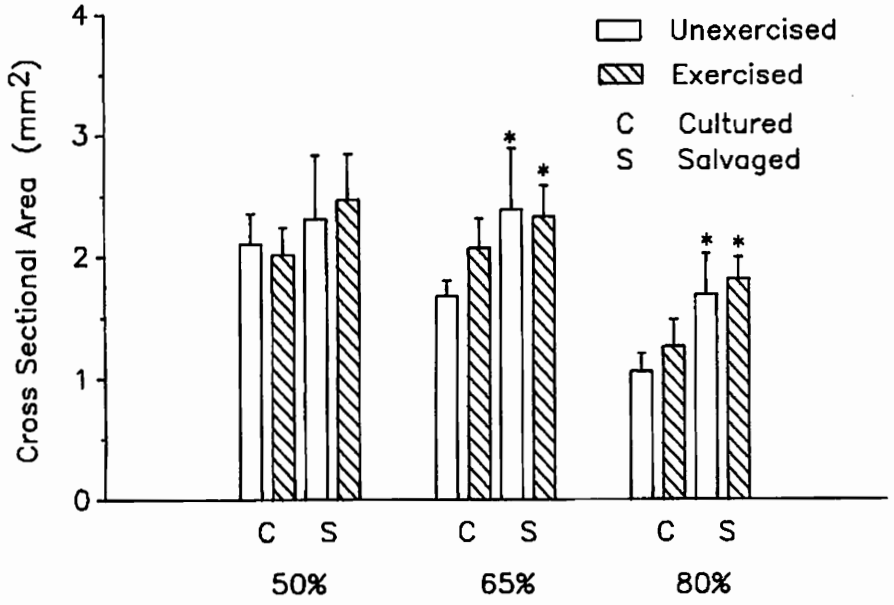

Figure 3. Cross-sectional area of red muscle at 50, 65, and 80% of standard length (SL) for unexercised and exercised, cultured and salvaged fish. (n = 18; * significantly greater than unexercised cultured fish; error bar = S.E.M.).

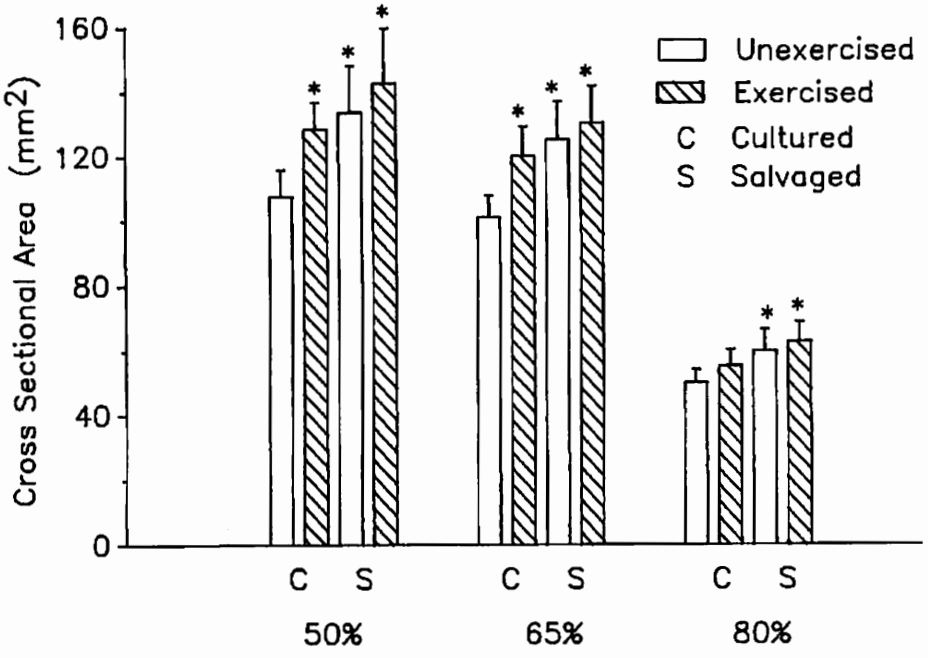

Figure 4. Cross-sectional area of white muscle at 50, 65, and 80% of standard length (SL) for unexercised and exercised, cultured and salvaged fish. (n = 18; * significantly greater than unexercised cultured fish; error bar = S.E.M.).

exercised and unexercised fish.

Unconditioned (not held in the laboratory for more than a week) cultured and salvaged fish did not differ in their swimming performance. The performances of both groups in swimming were significantly less than the unexercised and exercised cultured and salvaged fish held in the laboratory (Figure 2).

Stress responses to net confinement did not differ between unconditioned cultured and salvaged YOY striped bass in terms of plasma cortisol, osmolality, lactate, and hematocrit levels (Figure 7).

Discussion

Exercise conditioning enhances growth in YOY striped bass. Similar observations were also shown in Arctic charr fry (Christiansen et al., 1989; Christian-sen and Jobling, 1990) and in salmonids (reviewed by Davison, 1989) such as rainbow trout (Nahhas et al., 1982a; Houlihan and Laurent, 1987; Farrell et al., 1990), brook trout (Leon, 1986) and Atlantic salmon (Totland et al., 1987). This increase in growth has been shown to result from an increase in food intake and better food conversion efficiency (Davison and Goldspink, 1977; Greer Walker and Emerson, 1978; White and Li, 1985; Leon, 1986). Davison and Goldspink (1977) speculated that increased growth rate following training in trout is caused by lower stress hormones such as catecholamines and cortisol. Woodward and Smith (1985) showed that trained rainbow trout, indeed, have reduced levels of adrenaline, noradrenaline and cortisol. Our results, however, did not show any reduction in the resting level of cortisol in exercised fish.

Muscle is the active tissue involved in exercise training. In our study, we observed that exercise did not affect the red muscle but increased white muscle tissue in cultured fish. This increase in white muscle is isometric to the increase in its body weight—an integral part of growth. We also found no difference in the ratio of red to white muscle. Nahhas et al. (1982b) demonstrated that low speed

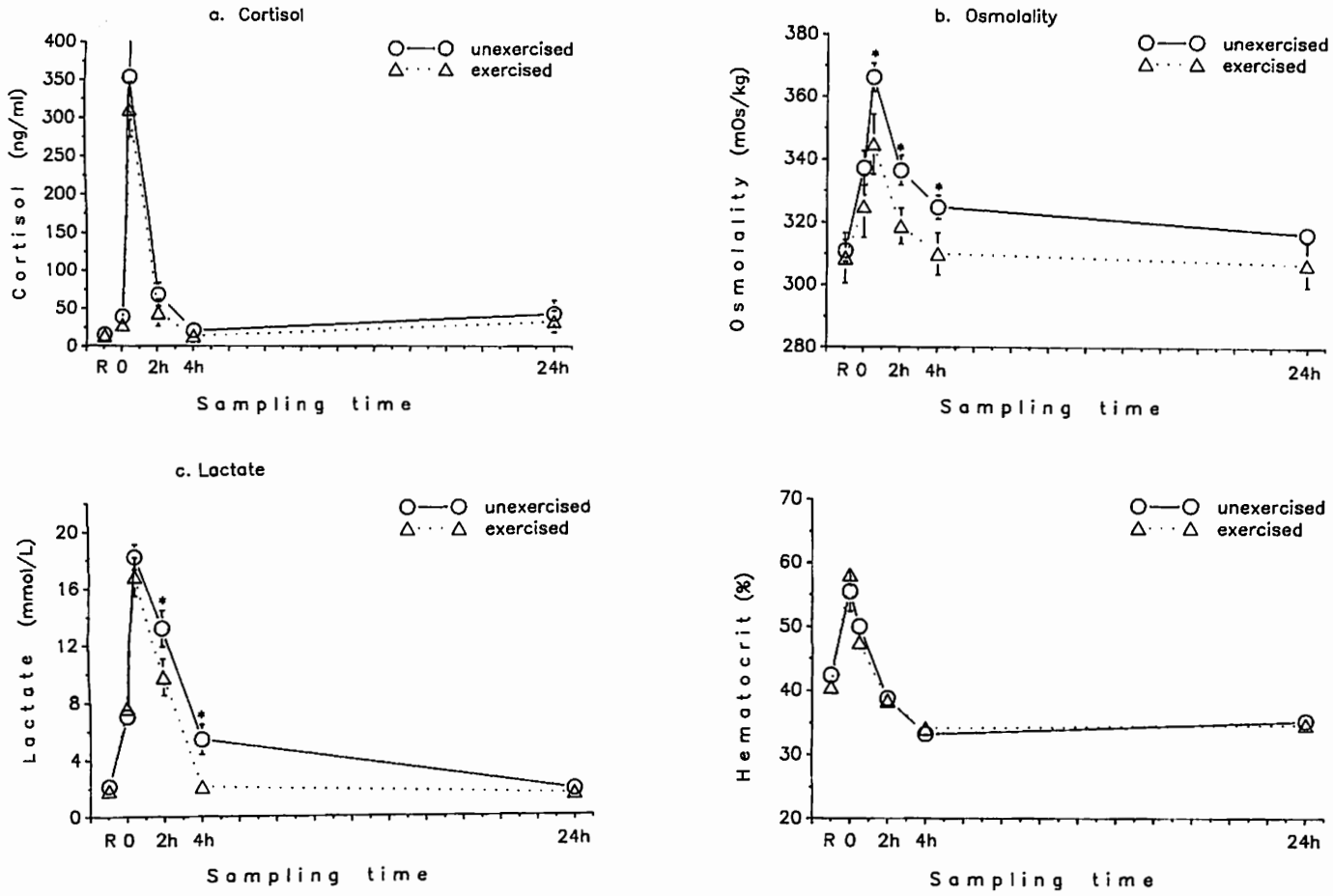

Figure 5. Plasma cortisol (a), osmolality (b), lactate (c), and hematocrit (d), of unexercised and exercised cultured fish at resting (R), immediately after net confinement (0), 2, 4, and 24 hours after net confinement. (n = 12; * significantly greater than without * at a specified sampling time; error bar = S.E.M.).

training in small rainbow trout did not alter the ratio of red to white muscle. However, at high speeds, the proportion of white muscle increased. This contradicted findings of Broughton et al. (1980), where high speed was used to elicit increase in *red* muscle in roach. Many previous studies have also reported increased white muscle size after exercise training (Davison and Goldspink, 1977, 1978; Greer Walker and Emerson, 1978; Johnston and Moon, 1980).

Many studies on exercise training in fish have focused on changes in the swimming performance. Our results showed that exercise conditioning improves the swimming performance of YOY striped bass. Similar results were shown in largemouth bass (Farlinger and Beamish, 1978), roach (Broughton et al,. 1980) coho salmon (Besner and Smith, 1983), brook trout (Leon, 1986), and rainbow trout (Nahhas et al., 1982b; Houlihan and Laurent, 1987; Pearson et al., 1990). The improved performance is related to (1) a lower 0_2 consumption rate at any given swimming speed (Nahhas et al., 1982b; Woodward and Smith, 1985) most probably due to increased aerobic capacity of red and white muscles (Davie et al., 1986); (2) decrease in levels of stress hormones (Woodward and Smith, 1985); (3) low blood lactate levels (Wendt and Saunders, 1973); and (4) improved efficiency as a result of conditioning to an exercise regime (MacLeod, 1967).

Recovery from net confinement in exercised YOY striped bass showed faster return to prestress levels of plasma cortisol and osmolality and a faster clearance rate of plasma lactate. Faster clearance of lactate in exercised fish after stress was also observed in young Atlantic salmon (Wendt and Saunders, 1973), rainbow trout (Hammond and Hickman, 1966; Woodward and Smith, 1985; Pearson et al., 1990) and chub (Lackner et al., 1988).

Unconditioned cultured and salvaged fish proved to have inferior swimming performance compared with unexercised cultured and salvaged fish held in the laboratory. The cultured fish taken from Central Valley Hatchery were raised in a raceway with water flow of <1.0 cm/sec. Salvaged fish taken from the Byron Grow-out Facility were held there for at least 2 months in a crowded condition with water flow of <1.0 cm/sec. The crowded condition in which they were held or the transport stress might account for the inferior performance of the unconditioned fish.

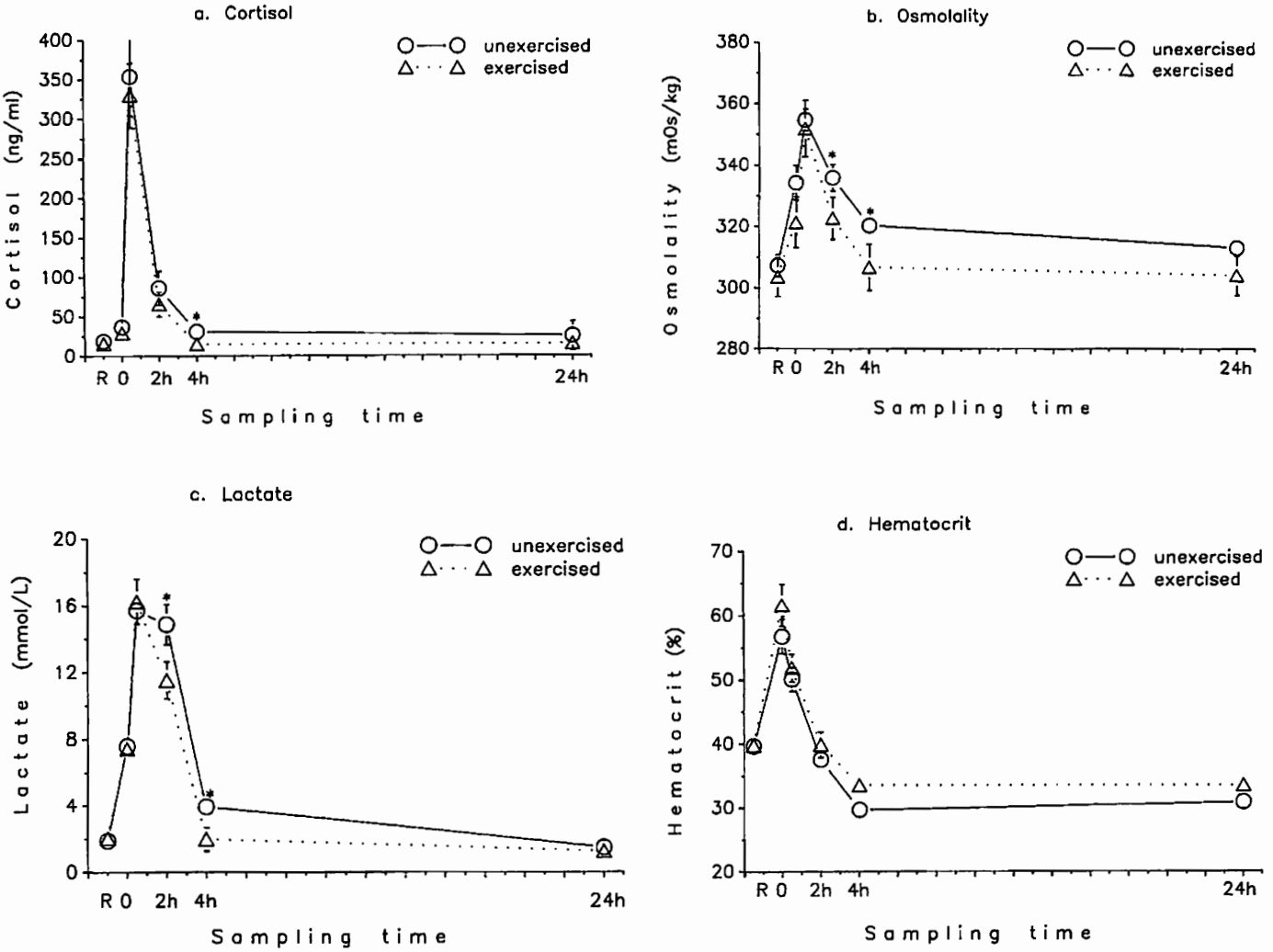

Figure 6. Plasma cortisol (a), osmolality (b), lactate (c), and hematocrit (d), of unexercised and exercised salvaged fish at resting (R), immediately after net confinement (0), 2, 4, and 24 hours after net confinement. (n =1 2; * significantly greater than without * at a specified sampling time; error bar = S.E.M.).

Application

Exercise conditioning in YOY striped bass improves growth, swimming performance, and stress responses. These are factors that directly impact survival of fish stocked into the natural environment. Higher growth rates shorten the vulnerability periods as potential prey (Werner et al., 1983) and increase prey-capture efficiency as a predator so that survival of fish is increased when stocked at a greater size (Junge and Phinney, 1963; Townsend and Calow, 1981). Better swimming performance improves feeding efficiency on prey and enhances both the escape response from predators and the capacity to maintain station against current (Beamish, 1978; Webb, 1984). Appropriate stress responses regulate physiological systems and increase survival after transport and stocking into the natural environment (Carmichael et al., 1984; Nikinmaa et al., 1984). If exercise-conditioned striped bass are used for mitigation, they could increase the percentage of return and recapture, as in Atlantic salmon (Wendt and Saunders, 1973) and brown trout (Creswell and Williams, 1983), thereby contributing in greater proportion to the next generation, potentially increasing the population of striped bass in the natural environment. Application of our results to Atlantic coast states might improve YOY survival in Chesapeake Bay, which has also shown reduced striped bass juvenile abundance and adult commercial landings (Boreman and Austin, 1985). Current declines in striped bass populations on both Atlantic and Pacific coasts provide a national scope and urgency for the application of our work.

We strongly recommend the incorporation of exercise conditioning

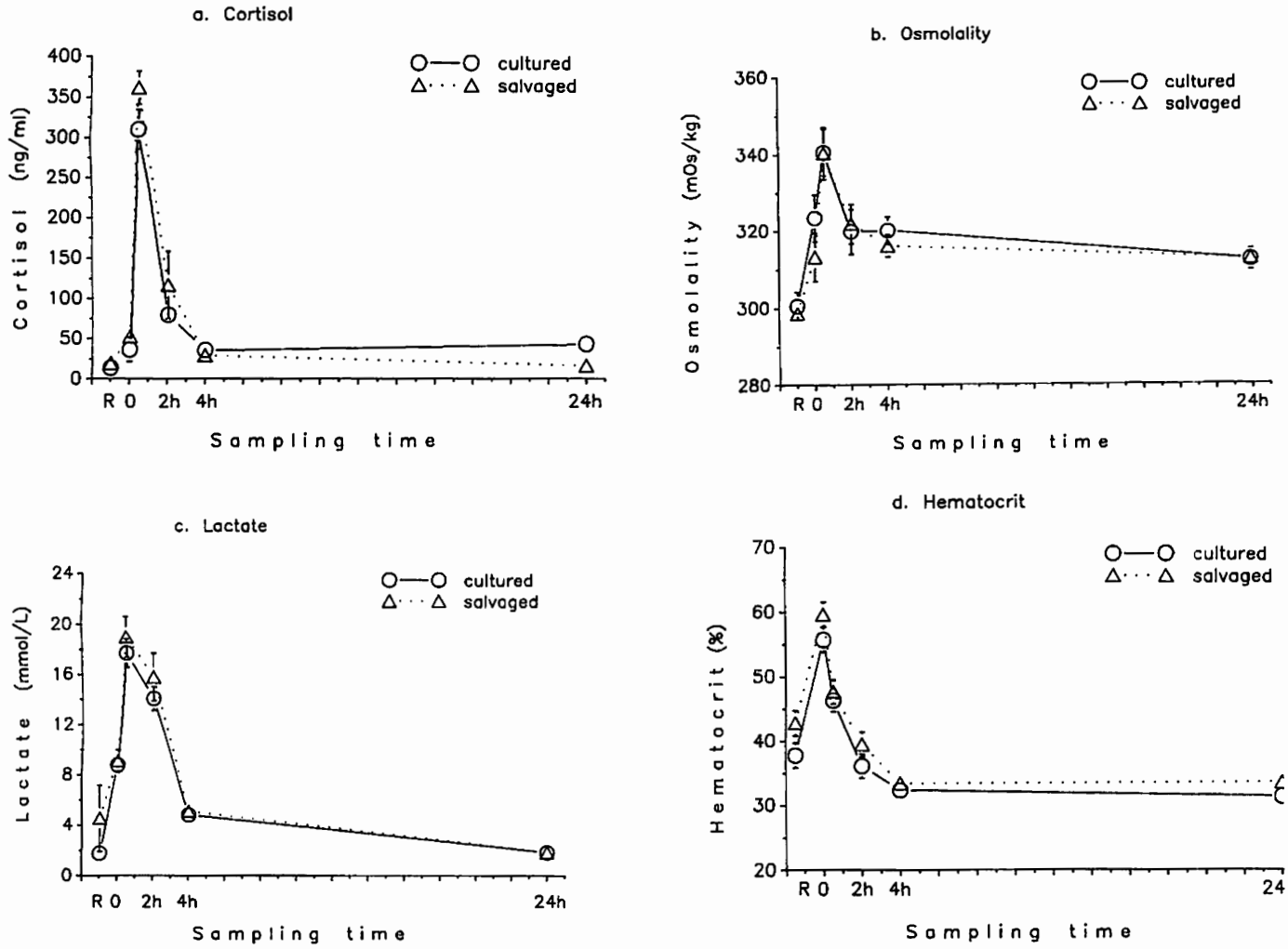

Figure 7. Plasma cortisol (a), osmolality (b), lactate (c), and hematocrit (d), of unconditioned cultured and salvaged fish at resting (R), immediately after net confinement (0), 2, 4, and 24 hours after net confinement. (n = 12; * significantly greater than without * at a specified sampling time; error bar = S.E.M.).

for striped bass and other fish species used for mitigation purposes. To exercise-condition the fish, culturists would need to fit some of their tanks with devices to create a current equivalent to 1.5 to 2.5 body length/sec. The simplest method is to release water into the tank at a suitable angle and velocity to induce a water current and to maintain this current with an air lift system. This is more effectively achieved with a round tank. For a raceway, it is possible to induce a current with a slowly moving propeller or a pump. Each of these systems has its own benefits and costs, and one may be more appropriate than another, depending on existing tank size and shape.

Cooperating Organizations

California Department of Fish and Game

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Environmental Effects on Ovarian Development in White Sturgeon Broodstock

University of California, Davis
R/A-83
1990–92

Serge Doroshov

The California sturgeon industry currently produces 0.5—1 million pounds of fish for the food market. However, it still depends on wild broodstock in fingerling supply for commercial rearing. Experience with captive breeding in California and Europe indicates that rearing temperature and artificial diets may affect broodstock reproductive performance. With the exception of recent work with cultured Siberian sturgeon (Pelissero and Le Menn, 1991; Le Menn and Pelissero, 1991), information on ovarian development and regulation of the reproductive cycle in sturgeon is highly inadequate. The objectives of our study were: (1) to determine the effects of low winter temperature and commercial diets with different lipid content on ovarian development in white sturgeon; (2) to examine endogenous regulation of the ovarian cycle. We collaborated with a commercial sturgeon grower (Sea Farms, Galt, California) and the laboratories of Dr. G.P. Moberg (endocrinology) and Dr. S.S. Hung (nutrition).

Two broodstocks, raised at a university facility and at a commercial sturgeon farm, were exposed to different temperature and dietary treatments over a 2-year rearing period. Ovarian development, plasma profiles of reproductive hormones and metabolites, and growth were monitored in each individually marked female by repeated samplings at 6-month intervals. University broodstock (87 females, 4–9 years old) were treated with both temperature and diets in a 2×2 crossed design. Farm broodstock (130 females, 2–6 years old) received only temperature treatment. Fish were raised in large tanks outdoors, under natural photoperiod. Different age cohorts were equally assorted to treatment tanks. Treatments were (1) constant year round temperature (17.6° C at the university and 19.5° C at the farm); (2) "vernalization" from December through February (11.5° C at the university and 9° C at the farm). Two commercial diets were used with the university stock: (1) trout broodstock diet, LL (10% lipid, estimated energy 3.52 cal/g, from Murray Elevators, Utah); and (2) salmon broodstock diet, HL (19% lipid, 3.87 cal/g, from Moore-Clark, Canada). The HL diet was used by Sea Farms.

Ovarian biopsies were processed by the paraffin histology method and scored for the development of ovarian follicle (Anonymous, 1991). Egg diameter and polarization index (distance between the germinal vesicle and animal pole divided by the egg) were measured by image analysis. Plasma estradiol, androgens, and two sturgeon gonadotropins were measured by radioimmunoassays (Moberg et al., 1991). Total plasma calcium (correlated with vitellogenin) was measured by flame atomic absorption, and plasma glucose and triglycerides by colorimetric assay kits (Sigma). Data were analyzed by the GLM procedure (S.A.S. Institute) and contingency tables and stepwise logistic regression (BMDP Statistical Software).

Sturgeon exhibit some similarity with amphibian oogenesis described in *Xenopus laevis* (Dumont, 1972). We classified the ovarian development of white sturgeon as follows: stage I—resting or quiescent follicle, diameter (d) 0.1–0.2 mm; stage II—endogenous growth phase, (d) 0.2–0.4 mm; stage III—previtellogenic follicle (synthesis of PAS-positive chorion and proliferation of granulosa cells), (d) 0.4–0.6 mm; stage IV—exogenous vitellogenesis, (d) 0.6–3.0 mm; and stage V—postvitellogenic oocyte undergoing polarization, (d) 3.0–3.5 mm. Stages III to V are shown in Figure 1. Neither temperature nor dietary treatments significantly affected proportions of the ovarian stages in the university stock ($p > 0.05$). Approximately 85% of the fish were recruited into stages II to V and 10% completed vitellogenesis (Figure 2). Age and sampling month (June or November) were significant predictors for the advancement from stage II to stage III, but body weight was a better predictor for change from stage III to stage IV (onset of vitellogenesis). Temperature significantly affected ($p < 0.05$) distributions of the ovarian stages in the Sea Farms stock, because increased proportions of young females advanced from stage I to stage II after vernalization (Figure 2). However, approximately equal numbers of fish in both treatments (65%) were in various stages of the ovarian cycle, and the greater proportion of vitellogenic females (stages IV and V) in constant temperature indicates acceleration of vitellogenesis. As with university stock, about 10% completed vitellogenesis (stage V). Age was a significant predictor for stage III, whereas body weight was for stage IV.

Factorial analysis revealed a significant ($p < 0.05$) dietary effect on growth rates and more rapid growth in the HL dietary treatment (Figure 3). Plasma triglycerides were at higher ($p < 0.05$) levels in fish fed the HL diets, but plasma glucose was not affected by diet. Temperature did not affect overall growth rates in the university stock, because of a compensatory growth in vernalization treatment during the stock's exposure to warm water (Figure 3). However, temperature produced a significant effect on growth of the Sea Farms stock (lower in vernalization treatment, Figure 3).

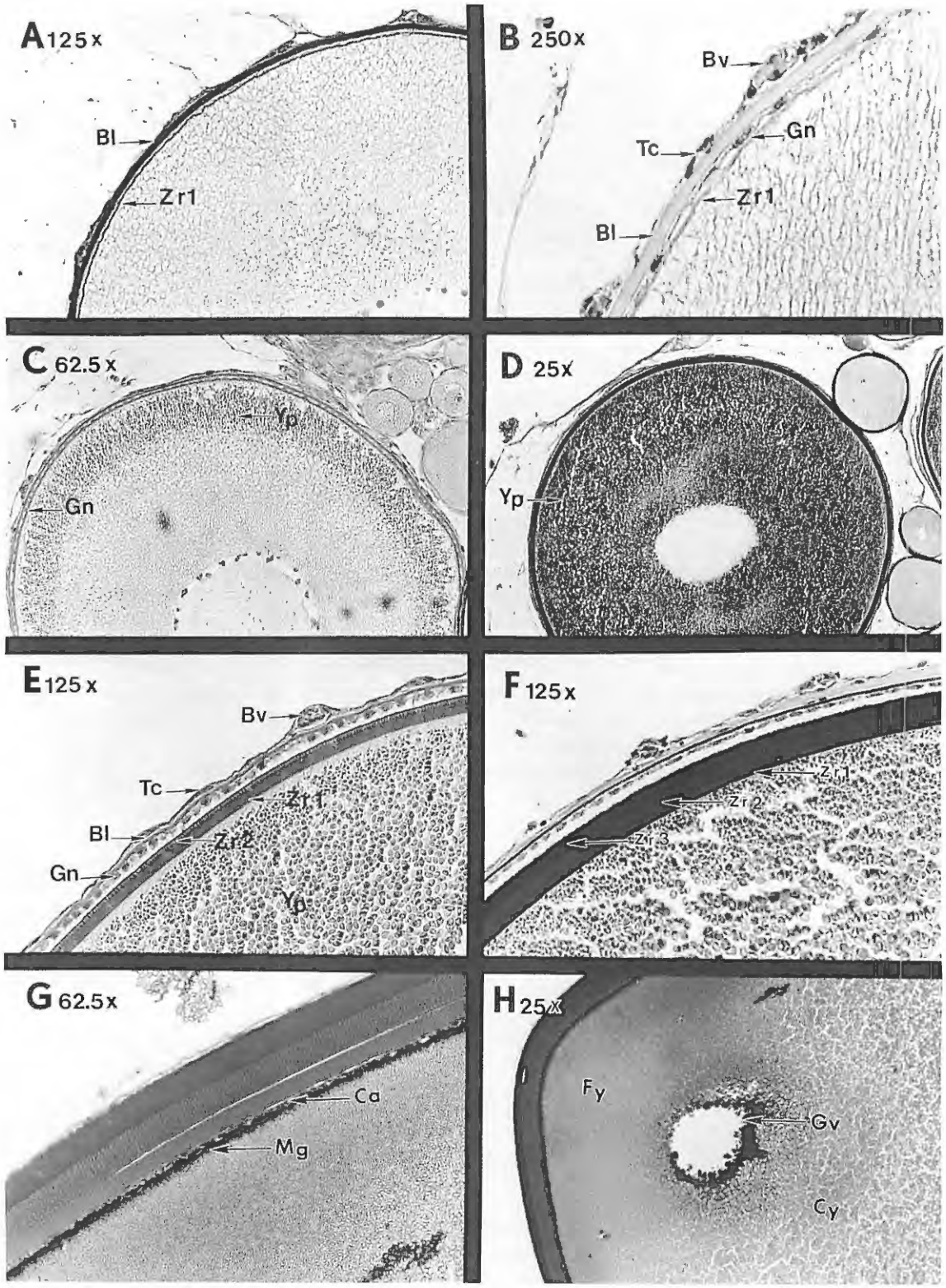


Figure 1. Development of the ovarian follicle in domestically raised white sturgeon females. Microphotographs of histological sections stained by PAS stain. The original magnification is shown in the upper left corner. A & B: differentiation of chorion (zona radiata, stage III). C–F: various stages of exogenous vitellogenesis (stage IV). G & H: polarization of the oocyte (stage V). Bl = basal lamina, Bv = blood vessels, Ca = cortical alveoli, Cy = coarse yolk, Fy = fine yolk, Gn = granulosa cells, Gv = germinal vesicle, Zrl = zona radiata 1, Zr2 = zona radiata 2, Zr3 = zona radiata 3.

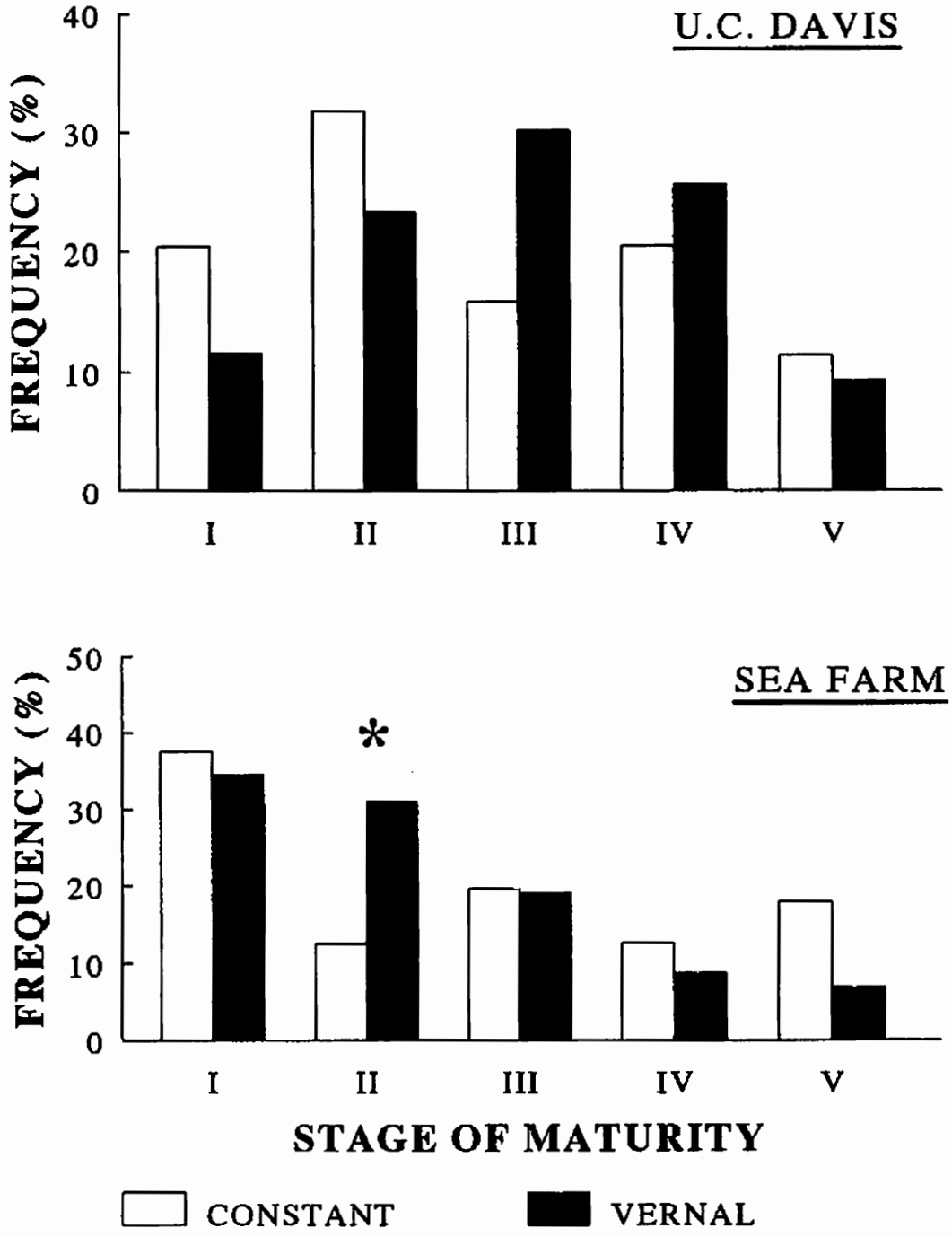
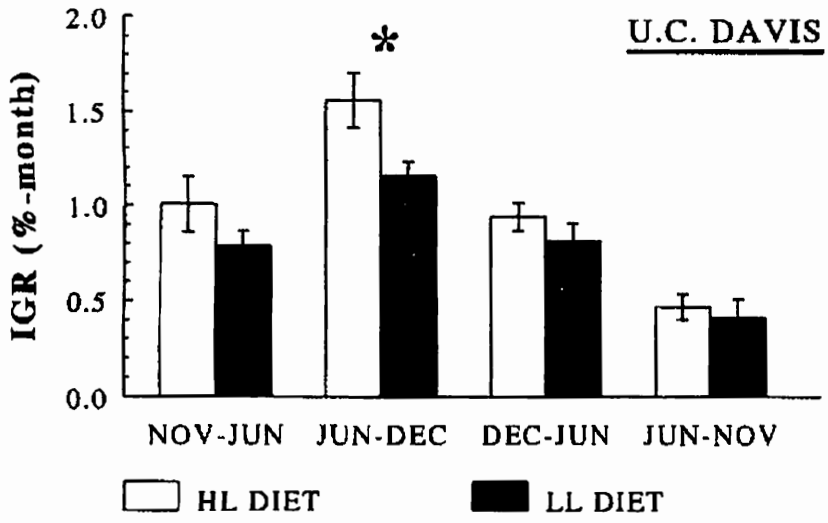
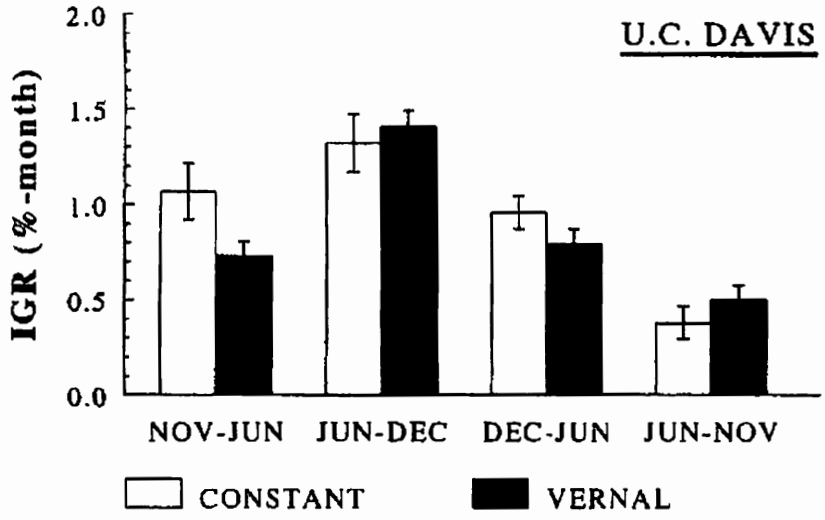


Figure 2. Histograms of the final ovarian maturation distribution of female sturgeon at different stages of ovarian development kept under constant temperature or exposed annually to a cold water period (VERNAL). Top, U.C. Davis stock: VERNAL, N = 43; CONSTANT, N = 44. Bottom, Sea Farm stock: VERNAL, N = 58; CONSTANT, N = 56.





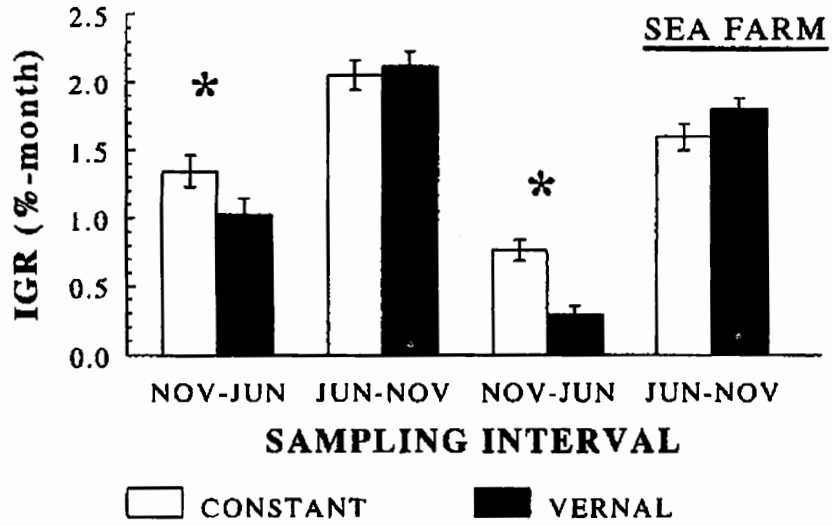


Figure 3. Mean (± SE) instantaneous growth rate (IGR) in body weight of sturgeon females during four sampling intervals. Top, U.C. Davis stock fed a high lipid diet (HL) or a low lipid diet (LL). Middle, U.C. Davis stock kept at constant temperature (CONSTANT) or exposed annually to a cold water period (VERNAL). Bottom, Sea Farm stock kept at constant temperature (CONSTANT) or exposed annually to a cold water period (VERNAL).

The duration of exogenous vitellogenesis (stage IV to V) was estimated by examining ovarian biopsies in 23 females that had completed vitellogenesis. Eighty-five percent of these fish exhibited a 12- to 18-month period of vitellogenesis, with some individuals ranging from 6 to 24 months. Average duration of exogenous vitellogenesis in constant temperature treatment was 15 + 1 month (n = 15) and in vernalization treatment 18 ± 1.2 month (n = 8).

The oocyte growth and plasma concentrations of calcium, sex steroids, and gonadotropins were monitored in nine females at the university (Figure 4). The egg growth and correlated increase in plasma calcium were observed between June and November and continued throughout the winter and summer until November of the following year. Egg growth rate decreased, and plasma calcium concentration returned to a previtellogenic level during the winter months before spawning. Plasma androgens continued to increase during vitellogenesis, but the estrogen reached peak concentration during the first half of vitellogenic growth. Both gonadotropin hormones (GTH-1 and GTH-2, Moberg et al., 1991) were close to the assay detection limit (0.86 ng/ml) throughout the entire vitellogenesis, but their concentrations dramatically increased at spawning.

Seven females at the university were separated and held at a water temperature of 14–16° C for spawning. Oocyte development (germinal vesicle migration) and final ovarian maturation were monitored by changes in polarization index (PI) and *in vitro* egg maturation assays. By March–April, 1992, the PI had reached the value 0.125 ± 0.003 (x ± SE) and the eggs in some females exhibited *in vitro* meiosis. Four females (57%) responded with 100% ovulation after hormonal injections (GnRHa or carp pituitary). They produced an average 100,000 eggs and 50% fertilization success. There was no apparent effect of previous dietary or temperature treatment on spawning success, since all four spawned fish originated from all treatments.

The fourteen females of the farm stock were held at a temperature of 18–20° C before spawning, since the irrigation water was not available to fish farmers after 1991. Germinal vesicle migration in all females was arrested (PI = 0.235 + 0.013), and the ovaries became atretic in March–April. Attempts to induce ovulation in several females failed. We conclude that the lack of seasonal fluctuation of the environmental temperature produces little effect on ovarian development of sturgeon during vitellogenesis. However, during the prespawning period, sturgeon females acquire high sensitivity to the elevated temperature. Optimization of temperature regime during the final ovarian maturation is required in order to achieve successful sturgeon spawning.

Cooperating Organizations

Sea Farms, California

Sturgeon Broodstock Research & Development Program, UC Davis

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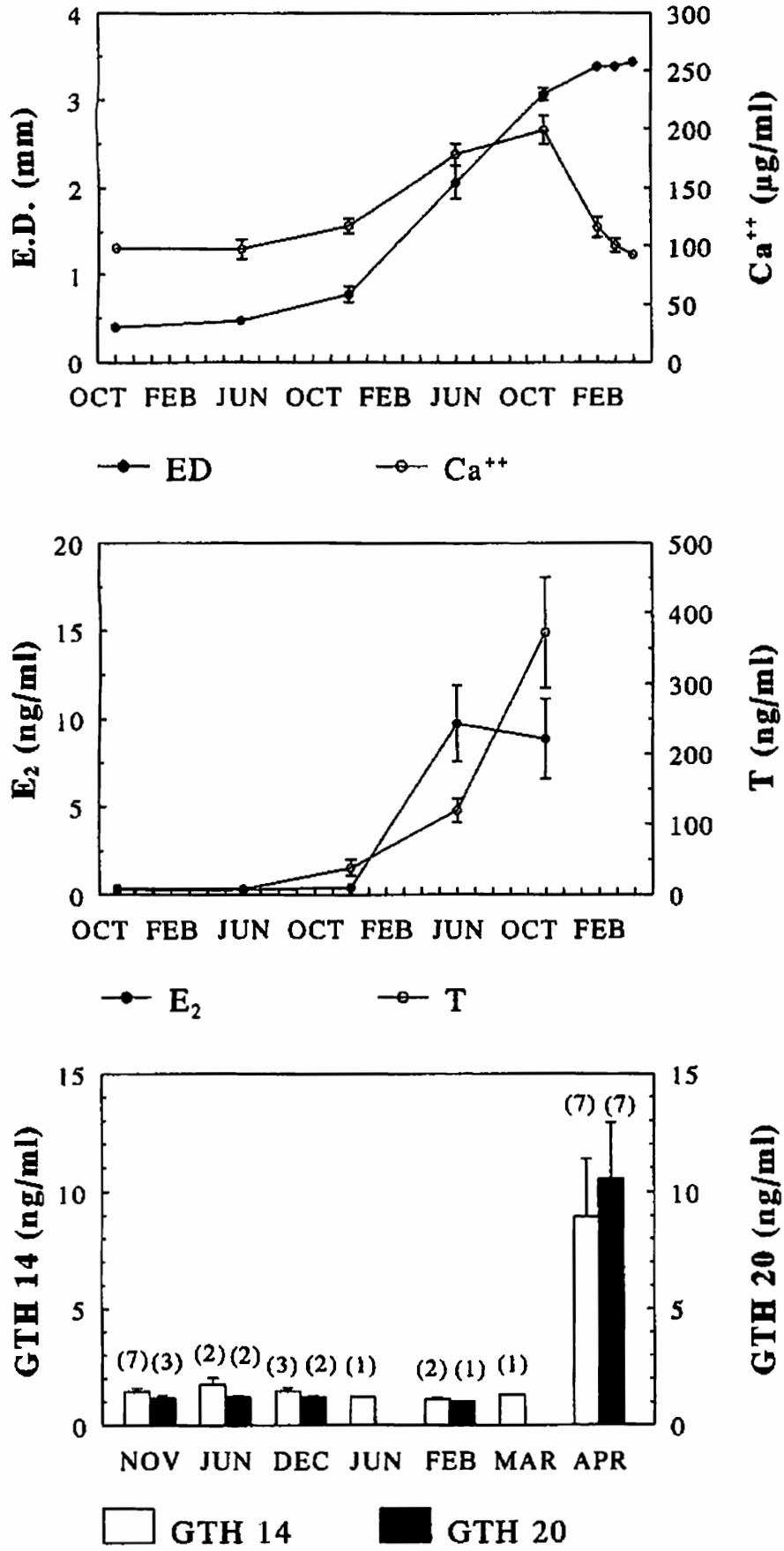

Figure 4. Top, Egg diameter (mean ± SE) and total plasma calcium concentration (mean ± SE) during the reproductive cycle of female white sturgeon ($n = 9$). Middle, Mean (± SE) of plasma estrogen and plasma testosterone throughout the reproductive cycle of female white sturgeon ($n = 9$). Bottom, Plasma gonadotropin concentration (mean ± SE) throughout the reproductive cycle of female white sturgeon (the sample size is in parenthesis in the graph).

Fisheries

Endocrinology and Physiology of the Development and Survival of Salmon and Striped Bass

University of California, Berkeley
R/F-117
1990–91

Howard A. Bern and Richard S. Nishioka (with Elisabeth S. Gray, Steffen S. Madsen, Kevin M. Kelley, Peter I. Tsai, Alberto Corrales, Jeanette Enderson, Juan Guerrero, Wynne Myint, Kimmakone Siharath, Robert Tsai, and Jenny Yang)

Our investigations in 1990–91 involved laboratory and hatchery-based studies concerning smoltification-related phenomena in salmonids and the basic physiology of the striped bass. We have focused on hormonal control of growth, hormonal regulation of ion transport, and the function and regulation of growth hormone receptors in these fishes. Useful information is channeled directly to hatchery managers of the California Department of Fish and Game, to advisers of the UC Cooperative Extension, and to our collaborators.

Growth Studies

The role of growth hormone (GH) in stimulating hepatic insulin-like growth factor I (IGF-I) secretion and in augmenting the response to IGF-I of ceratobranchial cartilage is of particular interest. However, there is as yet no clear evidence for circulating GH in striped bass. Various ways of looking for striped bass pituitary GH, including HPLC of pituitary incubates, PAGE, and Western blot analysis, show little if any GH-like material. However, immunocytochemistry of the pituitary clearly shows some GH-positive cells. GH turnover studies may illuminate the basis for the discrepancy.

Recent studies have shown that *in vivo* injections of unfed striped bass with ovine GH can induce a significant three-fold increase in basal growth of ceratobranchial cartilage over the control. Furthermore, this three-fold increase persists after addition of IGF-I *in vitro,* where a dose-related response to IGF-I can be demonstrated.

Other growth-related studies involve injecting intact striped bass with increasing doses of GH to determine if ceratobranchial growth would then respond to IGF-I *in vitro* in a dose-dependent manner. In addition, the optimal number of injections necessary to induce maximal growth response is being determined.

IGF-I circulates bound to a complex of specific IGF-binding proteins (IGF-BPs). These IGF-BPs appear to assist in promoting and/or inhibiting the growth effects of IGF-I. In addition, IGF-BPs are likely to play a role in targeting IGFs to their responsive tissues. IGF-BPs are being investigated in teleosts in four ways: (1) tissue source(s) of IGF-BPs will be identified by examining conditioned medium from tissue incubations by Western-ligand blot analysis to look for the three previously identified IGF-BPs (40-50 kDa, 31 kDa, and 29 kDa proteins) in liver, muscle, brain, kidney, bone, cartilage, heart, gut, and gill; (2) changes in circulating levels of IGF-BPs will be characterized during modifications of nutritional status; (3) changes in IGF-I bioactivity in the presence of IGF-BPs will be measured by determining the effect of adding 5–10% of the fish's plasma to the medium during radiosulfate incorporation; and (4) GH regulation of IGF-BP(s) will be determined by injecting GH (0.25, 1, 4, and 10 ng/g/day) for a period of 2 weeks while fish are kept on a limited diet.

We are comparing smoltification and growth of normally growing and rapidly growing populations of coho at Warm Springs Hatchery. Samples of blood, gills, and cartilage have been taken; data have yet to be analyzed.

Hormones and Ion Transport

The interaction of GH and prolactin (PRL) in seawater (SW) adaptation was examined in the steelhead trout. Because GH has been shown to have SW-adaptive effects in salmonids and PRL is essential for freshwater (FW) adaptation in a variety of teleosts, the hypothesis to be evaluated was whether PRL antagonizes the effect of GH during SW adaptation. To test this hypothesis, steelhead trout from Warm Springs Hatchery (California Fish and Game) were injected with various doses and combinations of doses of the two hormones. In one dose-response experiment, all PRL doses clearly antagonized the effect of simultaneously injected GH and resulted in poorer SW adaptation than in the controls, thus confirming the hypothesis. There is evidence that the mechanism involves antagonistic actions on gill Na^+,K^+-ATPase activity. There may be seasonal variation in the sensitivity of target organs. The results imply that elevated endogenous PRL levels and/or failure to reduce PRL levels may be critical and incompatible with SW adaptation.

As both GH and IGF-I have shown growth-independent SW-adaptive effects in salmonids, the possibility of a GH-IGF-I relationship in the development of gill Na^+,K^+-ATPase was investigated in cultures of coho salmon gill filaments. Preliminary data show that GH pretreatment of coho salmon results in increased gill sensitivity to rbIGF-I *in vitro.* Gill Na^+,K^+-ATPase from untreated fish is not responsive to IGF-I, whereas the enzyme shows a dose-related response to IGF-I (100–1000 ng/ml) in GH-treated fish. An important pathway for GH action during SW adaptation may include hepatic (and possibly branchial) IGF-I production, in line with the current Green dual-effector model for cartilage and adipose tissue growth in mammals.

Preliminary results show that striped bass obtained from the Central Valley Hatchery of California Fish and Game are fully euryhaline—i.e., they adapt quickly to varying salinities with only small

alterations of their internal osmotic environment. The mechanisms behind this "unusual" ability—compared with other known euryhaline teleosts—are being investigated. Compared to other species, FW striped bass have large numbers of chloride cells in their gills and opercular membranes. Also, activities of enzymes involved in transepithelial salt transport (Na^+,K^+-ATPase) and oxidative metabolism (succinate dehydrogenase) are high in FW and do not change after SW adaptation. These features are normally not found in FW fish but only develop after SW transfer or during smoltification of anadromous salmonids. The striped bass appears prepared at any time for either FW or SW life.

We have also investigated endocrine involvement during SW adaptation of striped bass. In preliminary experiments, we found that either cortisol or GH injected into intact fish stimulates salt-excreting mechanisms (Na^+,K^+-ATPase activity) in the gill and opercular membrane. However, the responses seem to be less than those described for salmonids. This may not be surprising, as the system is already in an activated state (see above). Cortisol has been shown to exert this effect on striped bass gill tissue *in vitro* as well, as has already been shown in salmonids.

Receptors for Growth Hormone

Endocrine regulation of salmon growth during smoltification was studied by surveying animals over time. Coho salmon raised in FW at the Bodega Marine Laboratory were sampled monthly from December 1989 to June 1990. Hepatic salmon GH receptor levels were analyzed by radioreceptor assay, using purified salmon GH (courtesy of Professor H. Kawauchi) as a tracer. IGF-I activity was estimated simultaneously by measuring $^{35}SO_4$ uptake by the cartilaginous zone of ceratobranchial bones from 4–6 gill arches per fish.

Both GH binding and $^{35}SO_4$ incorporation were low in December and January, and then increased in tandem in late February. In early April both measures decreased, again rising in late April. Both the timing and the bimodal patterns of GH binding and $^{35}SO_4$ incorporation resemble the pattern reported for plasma insulin in salmon. There is evidence from mammalian literature that insulin regulates IGF-I and GH receptors and may be regulatory in another teleost, *Gillichthys mirabilis* (K.M. Kelley, 1991). However, plasma insulin levels (assayed by Dr. E. Plisetskaya, University of Washington) from the surveyed salmon did not coincide with $^{35}SO_4$ incorporation and [^{125}I]salmon GH (sGH) binding; hence, it is unknown at this point if insulin regulates these factors in salmon.

In stunted coho salmon, [^{125}I]sGH-binding was decreased by 60% compared to that in normal smolts (Gray et al., 1990). However, *in vitro* $MgC1_2$ treatment of membranes to remove endogenous GH elevated specific binding, indicating receptor occupation. Free binding refers to binding by untreated membranes; total binding is that seen with $MgC1_2$-washed membranes. Only a limited number of stunts have been examined to date; total binding tends to be lower in stunts than in smolts. When stunts were transferred from SW to FW and held for 3 weeks, free binding was low if they were unfed (2.7 ± 1.0% of added [^{125}I]sGH) but increased to 6.6 ± 2.1% (P<O.OI) if they were fed.

In a pilot study, normal salmon were fasted for 5 days, and [^{125}I]sGH binding was compared to that of fed controls. Free binding by fasted salmon was below the level of detection; binding by fed fish was 5.1 ± 1.2% (n = 5). Total binding was not different between fasted and fed salmon (5.9 ± 1.3% vs. 5.8 ± 1.2%). In a second study, in which salmon were fasted for 3 weeks and sampled weekly, plasma glucose levels decreased relative to levels in fed smolts. Total [^{125}I]sGH-binding in fasted salmon tended to decrease after 1 week, and by 3 weeks binding was 50% lower than in fed fish (P<0.05).

Free [^{125}I]sGH-binding was not different between hypophysectomized (Hx) and sham-Hx coho salmon 2 or 3 weeks after surgery. Two weeks after Hx, total [^{125}I]sGH binding appeared to be lower in Hx than in sham-Hx salmon but was not different in salmon examined at 3 weeks. Hx salmon do not feed and hence sham-Hx salmon were not fed. The effect of withholding food from sham-Hx salmon may account for the lack of difference between them and Hx salmon at 3 weeks. rbIGF-I stimulated growth in slowly growing 2-year-old fish and in ration-limited 1-year-old fish but did not change free [^{125}I]sGH-binding. Immersion of presmolt salmon into water containing thyroxine (T_4) increased plasma T_4 from 2–4 ng/ml to 18–23 ng/ml but was without effect on free [^{125}I]sGH-binding throughout the 3-week study. $^{35}SO_4$ incorporation by T_4-treated fish was also not affected. Cortisol treatment of either presmolt or postsmolt fish did not appear to affect binding. Salmon GH receptors appear more sensitive to alterations in nutrition than in endocrine status.

Poor nutrition may also contribute to receptor down-regulation and low GH binding in stunted coho salmon. The rate of amino acid influx across stunt intestine is one-half of that in smolts (Collie, 1985); therefore, stunts may experience inanition-like conditions. Whereas stunt symptoms are reversible with transfer to FW, normal recovery is inhibited by withholding food, suggesting that stunting may in part reflect malnutrition of these fish.

Coho salmon liver microsomes were cross-linked to [^{125}I]sGH with *bis*(sulfo-succinimidyl)suberate and separated by SDS-polyacrylamide gel electrophoresis. Three cross-linked bands: I, II, and III, had apparent molecular weights (M_r) of 109 kDa, 87.5 kDa, and 79.5 kDa, respectively; the band materials specifically bound sGH and did not bind salmon prolactin. Bands II and III are not disulfide-linked subunits of band I, and all three appeared to be N-glycosylated but not by mannose. In immunoblots, salmon liver microsomes did not cross-react with an antibody to human GH-binding protein (the extracellular domain of the GH receptor). Cross-linked GH receptors from human, mouse, and rabbit are N-linked glycoproteins of

a M_r range of 60–130 kDa (Hubbard, 1987). Hence, the salmon GH receptor(s) appears similar to mammalian GH receptor with respect to glycosylation but probably has low sequence homology.

Membrane fractions from striped bass liver were assayed for teleost GH-binding sites. Hepatic GH receptors were present in striped bass and were similar to other vertebrate GH receptors in that specific GH binding increased with increasing amount of protein (100–800 g), was pH dependent (optimal at pH 7.0–7.5), and was enhanced by the addition of 10 mM $MgCl_2$ to the assay buffer. Striped bass GH receptors apparently do not differentiate between tilapia and salmon GH. Scatchard analysis indicates a single class of binding sites for salmon GH, with an affinity constant of $5 ¥ 10^8$ *M* and approximately 200 fmol receptor/mg protein. This affinity constant is relatively low; at this point it is unclear if this is due to unfavorable assay conditions or to the use of heterologous GH in probing for striped bass GH receptors.

Cooperating Organizations

California Department of Fish and Game:
Iron Gate Hatchery
Warm Springs Hatchery
Central Valley Hatchery

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Design and Development of a Sea Urchin Processing System

University of California, Davis
R/F-118
1989–92

R. Paul Singh

Sea-urchin processing involves a number of manually conducted operations that have been virtually unchanged on the West Coast of the United States since the sea urchin's initial commercialization in the early 1970s. Worker variability, random cracking of the test (urchin shell), and bulk handling lead to high product loss and impaired product quality. The overall goal of this project was to design and develop a mechanized processing system.

Figure 1 presents a diagram of the product flow in a typical sea-urchin processing plant. Sea urchins are received in wood bins approximately 4 ft square and 2 ft deep, weighing approximately 500 to 600 lb. A forklift handles the bins and overturns them on a receiving table. A rake is used to bring individual urchins to the edge of the table, where they are cracked into halves and placed in small bins or boxes. At present all material handling is done manually in a variety of plastic containers. Likewise, breaking the urchin test in half is a manual task employing a duck-bill-like plier that opens when the handles are squeezed. Cracking is accomplished by inserting (jamming) the duck bill into a circular soft tissue (peristomal membrane and mouth) area in the bottom of the test and expanding the plier (squeezing the handle), causing the test to separate along the vertical axis. The boxes of cracked urchins are distributed to a group of "spooners," who use long-handled spoons (soda spoons with an increased bowl angle) to scrape out the gonads. Dinner-plate-sized mesh trays are used to collect the gonads on one tray per urchin. Across a table from the spooners, an equal group of workers separate and clean the viscera out of the tray. Of relevance to mechanization are the breaking and spooning steps.

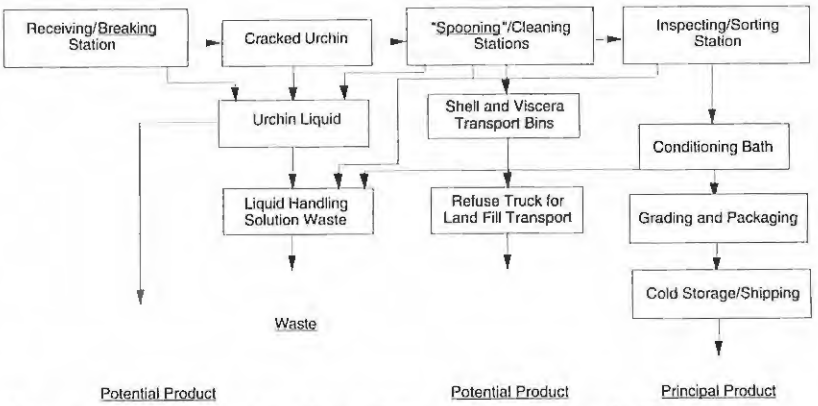

Figure 1. Diagram of flow through an existing urchin processing facility.

During the first year of this study, we obtained the physical properties needed for the design of such a system and conducted a substantial literature review. Numerous descriptions in the literature were found that generally reported variations of the existing labor-intensive process. A Japanese cracking machine was cited in one publication, but further search has not found the original source for details. An Australian patent for a powered cracking device was obtained; contacts were also made with researchers in Maine and a diver who is attempting to develop hardware for purple urchin in Santa Barbara. Limited information was found suggesting that mechanization efforts were undertaken by the University of Tromsoe in Norway, and the researchers were contacted for details. Because no prior system designs were found, bench scale trials were conducted to develop concepts that would assist in the design of a pilot-scale processing system during the second year.

Figure 2 shows a pull testing device used to simulate the function of the plier currently used. This device was successful in generating

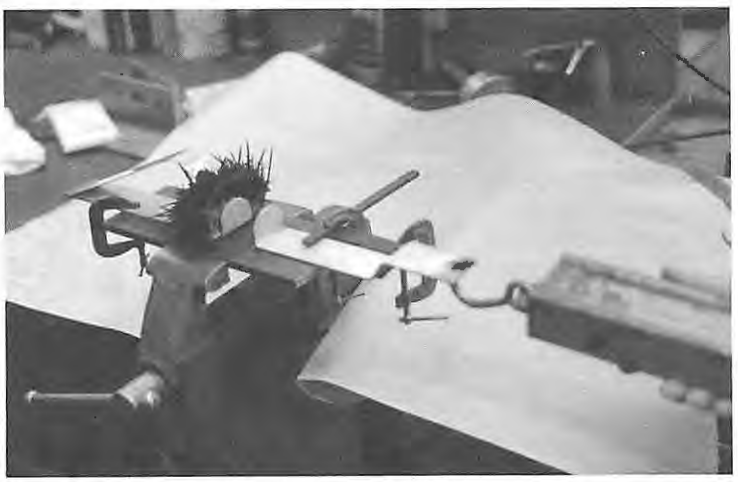

Figure 2. A system for determining breaking force.

half tests. Table 1 displays the results from some of the observations. As a result of plant visits and data shown in Table 1, a device was designed to accomplish the breaking function. Figure 3 displays the intended operational steps for the breaker. Several preliminary tests for automated gonad removal (mechanical spooning) were conducted using various water jets, both as water only and as an aid to mechanical scrapers. Figure 4 shows a combination device that scrapes around the curvature of the test and at the same time exhibits a water spray from orifices on its leading edge. Different sizes of rubber spatulas were also tested, with the thinner edge portion of a 3 in to 4 in wide by 2 in long spatula easily conforming to the test contour. The obstacles were test spikes around the test opening, and occasionally the hard mouth parts would interfere with the desired single continuous sweep of the open test. Encouraging results were observed, but it was decided to first verify the breaking performance and the ability to securely and reliably position the open urchin for the removal of the gonads.

A sea-urchin breaking and opening device was fabricated and tested. The device was found to be successful in breaking the tests into halves similar to manual breaking. This device was also useful in holding the tests securely enough for removing the gonads. A limitation was the presence of the tabs inserted into the test for the breaking step. This problem could be corrected with a movable tab that retracts as the test halves are separated and tilted for presentation to the spooning step.

Because in manual operation, random breaking of the tests results in some gonad damage, attempts were made to find external features of the sea urchin that might permit selecting a better method. It was noticed that, on top, the large spines that are grouped over the five lobes where the gonads are located can be easily detected. This might be done with an image processing system to assist in alignment decisions. It was also noticed that

Table 1. Preliminary Data on Dimensions and Breaking Force to Open Urchin Tests

	Exterior Dimensions				
	Major Diameter		Height from Flat Bottom		
	Shell OD	Spines OD	Shell OD	Spines OD	"Mouth Diameter"
1.00	5.00	7.50	3.00	4.00	1.50
2.00	5.00	7.50	2.50	3.75	1.50
3.00	5.25	8.00	2.50	4.00	1.25
4.00	5.25	8.00	3.00	4.50	1.25
5.00	4.75	7.00	2.50	3.75	1.38
6.00	5.50	7.50	3.00	5.00	1.50
7.00	5.50	8.50	2.50	4.75	1.50
8.00	5.00	7.50	2.50	3.75	1.50
9.00	5.00	7.00	2.50	3.75	1.38
10.00	4.50	6.75	2.25	3.50	1.25
11.00	4.25	6.25	2.00	3.25	1.25

	Exterior Dimensions	
	Major Diameter	Height from Flat Bottom
	Shell ID	Shell ID
1	4.75	2.25
2	4.50	2.00
3	4.13	2.00
4	4.50	2.38
5	3.75	1.88
6	4.38	2.50
7	4.75	2.31
8	5.00	2.38

Breaking Forces
45 #
52 #
47 #
55 #
54 #

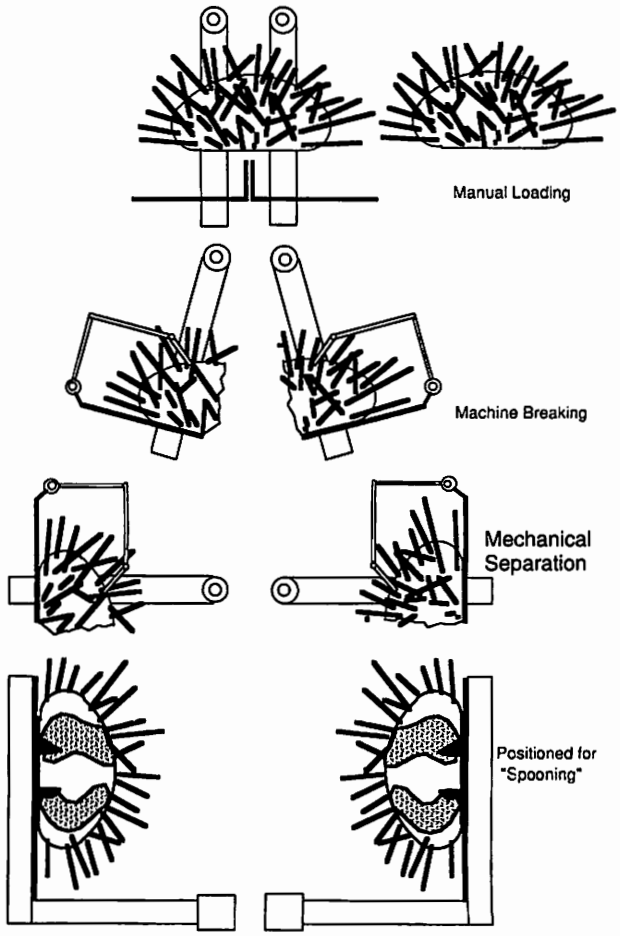

Figure 3. An urchin-test breaking system.

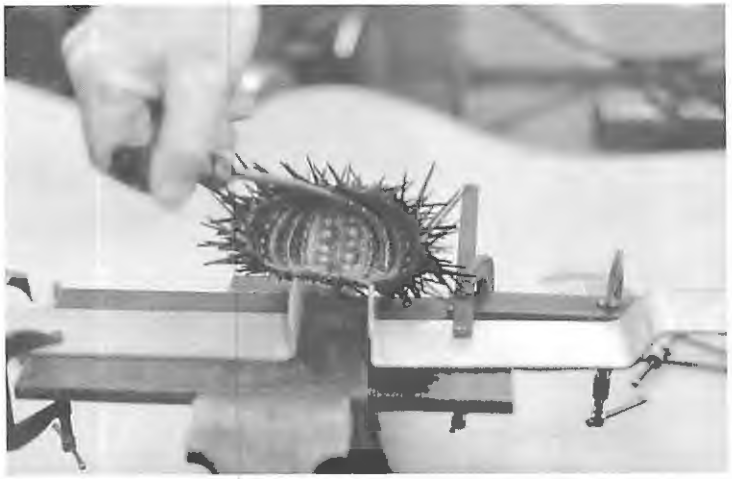

Figure 4. Combination water/jet mechanical scraper for gonad removal.

the opening in the test bottom is pentagon shaped. Attempts were then directed at simple cleaning and washing to make the pentagon shape clearer. Later, this led to scrubbing the bottoms on a piece of expanded metal to remove the spines and lightly wire brushing while simultaneously flushing with water. This procedure produced an obvious distinction between where the gonad lobes are located and where breaking should take place. The solid clusters of spine bases identify the gonad lobes, while the parallel rows offer references for separating the test. Cutting and prying schemes were used to force separation between the lobes, of the dark stripes, or two parallel lines between the dark stripes, producing only undamaged gonads. According to sea-urchin processors, the estimated loss from breaking gonads is about 15%, which is similar to the 20% mentioned in conversations with scientists at the Maine Department of Marine Research.

The next steps were to assemble a spine removal device, explore methods for controlled cracking, and explore vibration and shaking for gonad removal.

A spine removal device, as shown in Figure 5, was built and tested. Results indicate that only the expanded metal portion is required and that this technique is adequate for exposing lobe patterns on the bottom. We tried shaking the open tests using a scotch yoke shaker (Figure 6) with several combinations of stroke and frequency. Generally, this process produced better results with longer strokes and lower frequency. Although this method for gonad removal needs more testing, damage escalates rapidly when the gonads fail to separate on the initial or second cycle.

Single impact removal of the gonads appeared viable after observing the low-frequency shaking response. Figure 7 shows a modified creep measurement stand and test holding device, both on the shaker and the impact tester. Generally, the larger urchin gonads were readily removed with about an 8-in drop. Smaller urchins required about a 12-in drop. On a couple of urchins, an additional "bump" was required to completely remove the gonads, suggesting the need for additional testing using anvils that produced greater *g* forces during deceleration of the test. The test was held, open side down, at the end of a lever arm that impacted an anvil (hard rubber block) when it was raised and allowed to fall by gravity. For these initial trials the tests were first scrubbed (spines removed from the bottom) to expose the lobe pattern, then partially cut to force breaking between the lobes. When separating the test, it became apparent that breaking the bond of the peristomal membrane would be necessary. This was done by pushing it inward on the mouth with a thumb or finger to rupture and dislodge the attachment. Of more than 50 gonads removed, only three small broken pieces were found, or roughly 6% by number and far less than 1% by weight.

In-plant trials were conducted to collect data from a large number of samples with an improved test holder and anvils (stiff springs) that produce up to 160 *g* deceleration. Four repetitions of three breaking and removal methods with five urchins in each group were used. After standard cleaning by plant personnel, the gonads were separated into "whole" and "broken parts" categories, and weighed. This sorting does not entirely correlate with economic value but provides a comparison with existing procedures. Results of the following three breaking and removal methods are presented in Table 2.

1. Standard Breaking. Breaking by normal random orientation and removal by spoon, all by regular

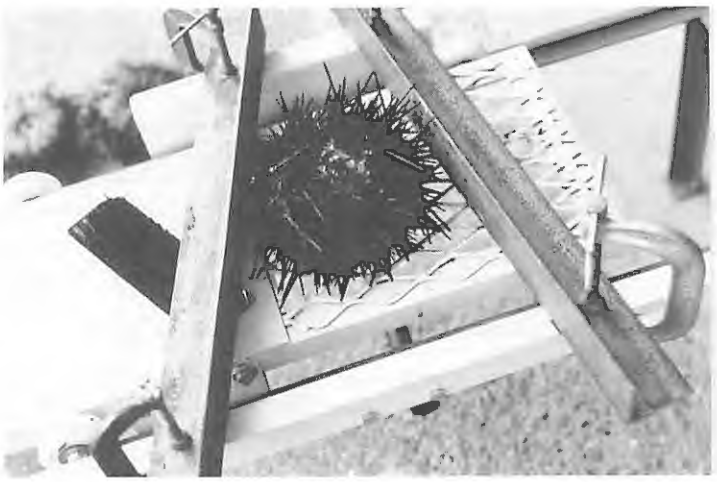

Figure 5. Spine removal with expanded metal top reciprocating table.

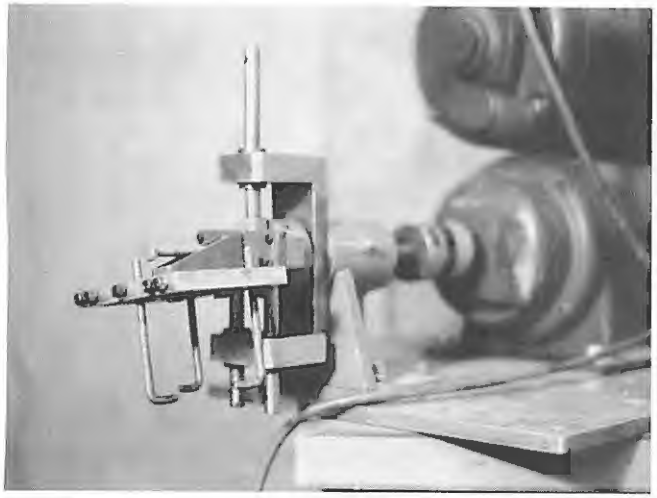

Figure 6. Test holder and scotch yoke variable stroke and frequency shaker.

Figure 7. Impact test setup with test holder, support arm, and rubber impact surface.

Table 2. Product Yield in Different Urchin-Test Breaking Systems

	Standard Breaking			Lobe Cut Breaking			Lobe Cut Impact Removal		
Urchin No.	Whole	Parts		Whole	Parts		Whole	Parts	Upgrade
1-A	50.8	12.5		78.1			74.3		three
2-A	65.2	2.8		88.0	2.3		70.6		difficult
3-A	19.2	11.6		18.0	11.7		52.6		
4-A	89.8			61.5	3.5		62.6	13.1	13.1
5-A	21.7	4.6		59.1	2.8	57.6	40.6	25.0	8.0
		11.32%			6.25%			12.67%	
1-B	88.8	1.5		63.8	1.6		62.4		
2-B	40.2	2.4		63.4			59.6		
3-B	57.8			65.1	2.2		56.3	1.6	1.6
4-B	67.4			88.3	2.3		67.0	7.6	7.6
5-B	72.2	3.5		82.5	1.9	59.8	39.0	26.0	5.2
		2.22%			2.16%			11.02%	
1-C	69.6	2.6		66.9		73.6	48.4	31.6	6.4
2-C	63.6			60.4			106.6		
3-C	65.6	11.1		68.0			53.9	10.9	10.9
4-C	70.9	22.7		59.1	1.6		66.2	12.1	12.1
5-C	65.0	15.1		54.8			58.4	8.5	8.5
		13.34%			0.52%			15.91%	
1-D	30.2	6.5		51.6	1.3		57.9	3.7	3.7
2-D	40.2	11.4		83.4			41.3	4.1	4.1
3-D	20.7	6.0		73.2			39.0	6.4	6.4
4-D	34.4	2.0		67.4	15.5		68.0	2.9	2.9
5-D	54.8			76.3			39.0		
		12.56%			4.77%			6.97%	
	1088.1	116.3		1328.9	46.7		1163.7	153.5	
						1226.7			90.5
Total		1204.4			1375.6			1317.2	1317.2
Parts, % of total		9.66%			3.39%			11.65%	6.87%

Weights in grams

Standard breaking, existing breaking, spooning, and cleaning.

Lobe Cut, remove bottom spines on scrubber table and saw cut on reference lines between lubes, then finish with standard spooning and cleaning.

Impact removal, lobe cut and break as above, then secure in spring-loaded gripper and drop/impact holder assembly against an anvil (stiff spring or rubber pad). Previous tests indicate approximately 160 *g* with spring and 60 *g* with rubber pad. Used spring on all but first few.

One urchin released the gonads on breaking and inverting. Two or three others had to be impacted repeatedly and remaining broken pieces removed by hand.

plant personnel. Gather gonads in sieve pans after cleaning step.

2. Lobe Cut Breaking. Remove bottom spines with scrubber table to make lobes/divisions visible for cutting with a hacksaw. Saw cut used to control break and not cut entirely through the test. Urchins with cracked tests were then delivered to plant spooners for removal of the gonads, which were retrieved in sieve pans after cleaning.

3. Lobe Cut with Impact Removal. Lobe cut as above, then placed into impact gripper with open side down. Impact arm then raised and dropped onto an anvil. In previous tests with an accelerometer the impact was measured at about 160 *g*.

The results presented in Table 2 indicate a substantial benefit from breaking between the lobes and that impact removal is probably worthy of additional consideration. Note the reduction in percent parts when the three difficult urchins were brought more in line with the standard breaking yield. This may be possible if a single impact is used, followed by inspection and manual cleanup.

After in-plant testing of an impact-type gonad removal device, comparison of the results obtained by this equipment with conventionally harvested gonads indicated substantial promise for mechanizing this step. As reported, cutting the test between the lobes was an important step in keeping overall damage comparable to the conventional method. Actually, this approach could reduce losses from traditional cracking and spooning.

In designing a prototype system that would combine the bench-top scale individual steps, it was decided to assemble two separate modules, one for removal of the gonads and the second for preparing the cut halves of the test. As planned, the preparation unit would have a station for scrubbing the bottoms of the test for lobe identification and a cutting station for cutting between the lobes. These operations would be primarily hand operations, with the separated halves then transferred to (slid across a common work surface) the removal unit. A removal unit as described above was fabricated.

The gonad removal unit was designed to be manually loaded at rates between one and two test halves per second into its 15 gripper arms. Actually, only three of the proposed arms were built. The test halves were to be loaded open side down into air-operated grippers that automatically opened when passing the loading station. The grippers were mounted on hinged arms that were attached to a central hub. Rotation of the hub propelled the arms on their support wheels along a cam surface. This cam surface rose approximately 8 in upon departure from the loading area, then terminated such that the arms would fall, striking an anvil and thus dislodging the roe skins into a sieve pan indexed to travel with the gripper. For testing, a sheet-metal table and manual indexing were used in place of a conveying mechanism. After the single impact, the grippers automatically rotate 90° to expose the open side of the test for inspection and occasional manual completion of removal. A delrin landing pad is used for impacting. After inspection, the grippers rotate back to their level position, open to discharge the empty test, and are ready for reloading.

In one test run, five urchins were scrubbed on the bottoms, cut between the lobes, and processed by the machine. Three half tests out of ten required minor hand cleanup, and there was less than 10% breakage of the roe skanes. This was consistent with the previous tests of individual elements of the system operated at the processing plant. Limited testing of the loading rate suggests the design goal can be achieved.

Evaluation and Enhancement of a Developing Claw and Whole Body Fishery for the Sheep Crab, *Loxorhynchus grandis*

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R/F-128
1989–91

This study began with a relative growth analysis of *Loxorhynchus grandis* to detect morphometric criteria for maturation as has been done for other spider crab species. Carapace length (CL) was chosen as the reference dimension. For males we analyzed the relative length of the claw (propodus). At the molt of puberty (CL ≥110 mm), the length of the claw increases dramatically. At this molt a diastema appears in the relatively evenly serrated gape of the juvenile claw. Animals with larger claws always exhibited a diastema in the gape. Such males are morphometric adults. At a smaller size (CL ≥ 70 mm) an inflection point was detected and termed the molt of prepuberty. No crab was ever observed to molt or even show signs of preparation for ecdysis after the molt of puberty. Crabs molt at least once, however, following the molt of prepuberty. For female crabs the allometry of the abdomen was studied. No molt of prepuberty was detected, but there was a dramatic increase in abdomen width at the molt of puberty (Culver, 1991).

Crabs parasitized by the rhizocephalan barnacle *Heterosaccus californicus* generally exhibited abdomen widths similar to those of unparasitized females. Some parasitized animals exhibited abdomen widths that were intermediate between juvenile and adult females. We suggest that these are parasitized and feminized males.

The detection of the molt of puberty enables us to determine maturation status of crabs at a glance, noting the diastema in the gape of the male claw or the wide abdomen of the adult female. When the commercial catch was examined it was evident that the fishery harvested morphometrically adult animals almost exclusively.

Molts of puberty were detected throughout the year but were most frequent in the fall. We invoke a seasonal size threshold in the molt of puberty to account for the very wide size range exhibited by adult males (CL ranging from 100 to 200 mm) (Culver and Kuris, 1992).

The existence of an obligate terminal molt in male spider crabs is highly controversial and greatly impacts management strategies for the snow (tanner) crab, an important commercial species of spider crab in Alaska, Far Eastern Russia, and Atlantic Canada. Our observations support a terminal molt for male *L. grandis* and are consistent with observations on male snow crabs. No morphometrically adult males have been observed to molt or enter premolt. Cuticular wear patterns document that adult males remain in the same instar for very long periods (> 1 year), whereas juvenile males of similar or even greater size molt relatively frequently. Wear patterns (and encrustation by epibionts) of adult male crabs resemble that of adult female crabs for which all agree that the molt of puberty is also the final ecdysis. Most importantly, we have recognized that limbs are not regenerated in morphometrically adult crabs of either sex. If a limb is autotomized, a calcified plate forms over the breakage plane in a few weeks. In contrast, a tough flexible membrane soon covers the breakage plane of an autotomized limb in a juvenile crab.

In the course of our studies of limb regeneration, we observed that an externally visible limb bud does not form as the regenerating animal passes through premolt stages. The small regenerate appendage forms beneath the tough flat membrane that seals the breakage plane. This appears to be a novel mechanism for limb regeneration in crustaceans. Studies of other crustaceans report the development of an external limb bud. Changes in the size and color of these buds have been used to predict the date of impending molts. Associated with the cryptic morphogenesis of the differentiating regenerate limbs of *L. grandis,* we noted that the newly regenerated limbs that appeared at the next ecdysis were quite small compared to newly regenerated limbs of other species of crabs.

Molt increments were large, ranging from 25 to 35% increase in CL. The percentage increase in size at molt decreased somewhat with premolt size for both males and females. For female crabs the molt of puberty was associated with a smaller molt increment than would have been predicted based on size alone (Culver, 1991). This pattern resembles molt increment observations in other species of spider crabs.

Information on the duration of the interval between molts was difficult to obtain and was less reliable than that for molt increments. Molt interval increases strongly with size for female crabs. Data for male crabs were quite sparse, but the general pattern seemed similar to that of female crabs. The principal difficulty in these studies was obtaining sufficient juvenile males for molting observations.

Combining the molt increment and interval data with unpublished observations on larval duration (Culver and Dugan), we developed a growth model for female crabs. We estimate that the typical female crab takes 2 to 3 years to reach the size threshold for a molt of puberty. We completely lack data on very small juvenile crabs. No crabs smaller than 10 mm CL were collected. From studies of larval development of other species of

spider crabs, we predict that the first true crab instar will have a CL of ~1.5 mm.

We were able to develop a sequence of cuticular wear stages for adult male *L. grandis* based on the loss of epicuticular pubescence, color changes, and wear of the tanned tips of the dactyls and other spines. Analysis of the monthly frequency of advanced wear stages indicated that it takes at least 9 months after the molt of puberty to attain these advanced stages. Combining these observations with the frequency of limb loss and the presence and size of epibionts (particularly barnacles) suggested that very worn animals have been adults for at least 2 years.

Following the studies of Laufer on the Atlantic spider crab *Libinia emarginata* (Laufer et al., 1990; Homola et al., 1991), we began a study of the relative weight of testes, sperm duct, and the large accessory gland associated with the distal portion of the sperm duct. Testes were well developed and sperm was present in the sperm ducts of morphometrically juvenile males. There was a modest increase in size of these structures after the molt of puberty. As in *Libinia emarginata*, the surprising finding is that these organs greatly hypertrophied several months later when relatively advanced cuticular wear stages had been reached. These observations call into question the simple notion that the molt of puberty establishes the onset of functional sexual maturity. Do the morphometrically juvenile crabs with sperm in the sperm ducts have the behavioral repertoire needed to mate? Do only the old, worn, morphometrically adult males with large reproductive systems successfully mate? Do they behaviorally dominate the other males?

The curious male accessory glands merit further anatomical and physiological study. Studies of other spider crabs (*Chionoecetes, Inachus*) do not report these large glands but rather indicate similar glandular lobules arrayed along the sperm duct. Our preliminary observations on *Taliepus nuttali* and *Pugettia producta* reveal accessory glands very similar to those of *Libinia emarginata* and *Loxorhynchus grandis*. These variations suggest that these glands may have taxonomic value at the generic or subfamilial level and may also indicate very different mating behavior patterns.

With H. Laufer and A. Sagi (University of Connecticut) we began a preliminary analysis of blood samples for methyl farnesoate (MF). This substance was not significantly associated with the continued development of the male gonads beyond the molt of puberty that we observed in the relative gonadal weight study. In females, MF titers increased after the morphometric molt of puberty. The pattern for female crabs is similar to that observed by Laufer and Sagi in their studies of *Libinia emarginata*.

Our preliminary observations, using ROVE, on the dense aggregates of *Loxorhynchus californicus* observed in the spring at the entrance to the harbor at Redondo Beach suggest that these pods consist almost exclusively of morphometrically adult females. Pods include hundreds to thousands of crabs. At the periphery of the pods, large adult males tend to occupy perches on rocks and ledges. These features suggest that this is an important element in the breeding biology of these crabs and that further study of this phenomenon would be rewarding.

Some ovigerous crabs were collected in all months for which samples were available. In both 1989 and 1990 the fewest females with eggs were captured in the early fall. Fecundity is positively associated with crab size. Minor losses of eggs to the newly discovered nemertean egg predator were quantified. The nemertean is probably an undescribed species of *Carcinonemertes*. Crabs parasitized by *Heterosaccus californicus* never carried eggs. This parasitic castrator also appears to induce maturity of the host at abnormally small sizes. Parasitized crabs with externae did not even overlap with the size range of uninfected adult female crabs. Parasitized crabs, and the rhizocephalan parasites themselves, had low MF titers. These are the first observations on the role of MF in parasitic crustaceans.

Cooperating Organizations

California Department of Fish and Game
Marine Fisheries Impact Program
Santa Barbara Shellfish Company
Sea Food Specialties

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The Biology and Fishery Potential of Hagfish

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R/F-129
1989–91

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Introduction

Little published information is available on the state of fisheries for hagfish in any country. Strahan and Honma (1960) and Honma (1960) described a small fishery for consumption of *Paramyxine atamii* in the Sado Strait, Sea of Japan. The hagfishery of Japan was described in a review article by Gorbman et al. (1990). A $70 million market has developed for leather products imported from Japan and Korea to the United States alone. Unfortunately, demand now exceeds supply because of apparent resource depletion. With the decline of their population in only a few years, other sources of hagfish have been investigated, including the west coast of the United States.

Studies on the ecology and basic biology of hagfish are few and generally incomplete (Brodal and Fange, 1963; Hardisty, 1979). Studies have shown hagfish to prefer mud bottom, in which they often burrow (Foss, 1962, 1963, 1968; Fernholm, 1974; Strahan, 1963; McInerney and Evans, 1970). Hagfish often keep their heads above the substrate while in their burrows to aid in gill ventilation and reception of food cues (Foss, 1968).

Hagfish have long been known as pests by fishermen and are known to be scavengers (Fernholm, 1974). Moller-Buchner et al. (1984) and Shelton (1978) investigated the feeding of *Myxine glutinosa* in the North Sea. The diversity and abundance of their prey suggested that this hagfish was a major benthic predator and opportunistic scavenger.

Some information is available on the reproduction of hagfish, but exact details are lacking regarding fertilization, fecundity, seasonality, gestation period, and mode of development. The shallow-water *Eptatretus burgeri* from Japan is easily obtained and has been well studied (Patzner, 1977, 1978, 1980). *E. burgeri* migrates seasonally to deeper water to spawn, as discerned by changes in the number and size of eggs, the state of spermiogenesis of the testis, and the changes in gonadosomatic index with season and depth (Tsuneki et al., 1983; Patzner, 1977, 1978). Similarly, Patzner (1980) and Patzner and Adam (1981) used hepatosomatic indices for *E. burgeri* and *M. glutinosa* because of the liver's seasonal role in egg development.

Several small-scale tag/recapture studies have been attempted, focusing only on tagging feasibility. Foss (1963) attached sprat tags through the dorsal musculature of *M. glutinosa*. Tag returns from hagfish at large as long as 29 months were obtained and did not affect growth or egg development. Walvig (1967) found that hagfish could successfully be marked with subcutaneous India-ink injections or tagged with small plastic chip insertions. Hagfish yielded a high return rate of 33.9% of the marked individuals and demonstrated a tendency to home. Tagged hagfish were used for growth studies in captivity only.

The Pacific hagfish, *Eptatretus stoutii,* and black hagfish, *E. deani*, are common members of the fish family Myxinidae off the northwest Pacific coast (Eschmeyer et al., 1983; Wisner and McMillan,1990). These hagfishes have been the subject of neurophysiological, sensory studies, but little is known about their general ecology. They occur from Southeast Alaska to Baja California, Mexico, and are found on or near the bottom (Eschmeyer et al.,1983). The Pacific hagfish lives shallower, from 18 to 944 m, while the black hagfish is deeper, from 155 to 1158 m.

Information about the size composition, depth distribution, feeding habits, reproduction, and habitat utilization patterns of these two species is often anecdotal, and few details are available. Both are thought to occupy muddy habitats, feed primarily as scavengers, and deposit sausageshaped egg cases on the bottom, with no free-living, larval stage (Eschmeyer et al., 1983). Information on spermiogenesis (Jesperson, 1975) and sex differentiation (Gorbman, 1990) in Pacific hagfish is available. However, more ecological details about reproductive strategies, cycling, and development are lacking for both species. Recent evidence from Oregon (Robert Demory, personal communication, 1989) indicates that the deeper-dwelling *E. deani* has a low fecundity, with an average of 12 eggs being produced by large females.

Reports of substrate preference, depth, seasonal migrations, and fishing techniques for Pacific hagfish vary widely in the available scientific literature. Several researchers report catching hagfish over soft, muddy substrate (Adam and Strahan, 1963; Honma, 1960; Jensen, 1966; McInerney and Evans, 1970; Isaacs and Schwartzlose, 1975), yet others (Dean, as reported by Conel, 1931; Worthington, 1905) reported catching hagfish over hard substrates in Monterey Bay.

Catch depths of Pacific hagfish have ranged from 10 to 50 fathoms in Monterey Bay (Conel, 1931; Worthington, 1905). McInerny and Evans (1970) found it at a minimum depth of 20 fathoms in the waters of British Columbia, Canada. Optimal fishing depths of Pacific hagfish are unknown and may indeed vary with season (Dean, as reported by Conel, 1931, Honma, 1960; Adam and Strahan, 1963; Tsuneki et al., 1981).

Despite their broad distribution (Miller and Lea, 1972; Hart, 1973; Eschmeyer et al., 1983), Pacific and black hagfish have remained an unutilized resource until quite recently, when exploratory fishing started throughout California and in Oregon and Washington. Production fishing for hagfish started recently off San Francisco, where several vessels landed considerable but variable amounts. More recently, fisheries have started, with varying degrees of success, from Santa Barbara to the Canadian border. These emerging fisheries presented an excellent opportunity to study the ecology of hagfish populations off the California coast.

Development of the California (and west coast) hagfish fishery has been hampered by a number of product quality and fishery technique problems. Information on the well-established Korean and Japanese hagfish fisheries has proved difficult to obtain, despite numerous contacts. Also, this fishery targets on a different, and much larger, species (*Eptatretus burgeri*).

Skins from hagfish caught in a variety of California locations tend to have perforations or "pinholes" along the anterior-posterior dorsal axis that are not related to slime glands. Traditional skinning practices place this axis along the center of the skin, which is used whole for various leather products, making removal of the perforated sections impractical. This inconsistent quality defect has led to the rejection of several container loads of frozen hagfish in Korea. Speculation on the source of these perforations includes environmental, biological, and handling factors.

Another unique aspect of the California fishery, and allegedly the Japanese fishery, is the use of anesthetics on the catch on deck to prevent hagfish from biting each other while in storage on the vessel. Live fish are delivered for quick freezing at –40°F. Unfortunately, anesthetics are expensive, and questions arise regarding food safety because of possible consumption of the flesh as a by-product.

Quickly killing the hagfish may be a possible alternative to anesthetics, but it also creates unique challenges with this hardy animal. Worthington (1905) reported that hagfish were extremely active several hours after decapitation, and healthy hagfish responded violently but survived exposure to warm water. Jensen (1966) reported that hagfish had poor tolerance to salinity changes. Their highly developed sense of smell (Sutterlin, 1975) suggests that they may be vulnerable to mild chemical treatment. Pacific hagfish are reported to have a very low rate of metabolism (Munz and Morris, 1965), suggesting suffocation techniques would be unsuccessful. Treatments combining temperature, salinity, and mild caustic chemicals may provide methods to quickly kill hagfish to minimize biting and perhaps perforations.

There is little literature on fishing techniques for hagfish. A number of traps and baits have been used to catch this abundant fish (Worthington, 1905; Adam and Strahan, 1963; Honma, 1960; Tsuneki et al., 1983). Given the well developed sense of smell in hagfish, which are often scavengers, bait selection could be critical to fishing success (Fernholm,1974; Strahan, 1963). Where to fish and how to catch commercial quantities of high-quality Pacific and black hagfish are currently unknown.

Thus, the overall objective of this study was to better understand the life history and comparative ecology of both species of hagfishes, relative to their developing fisheries. The specific goals were to: (1) perform an ecological survey in Monterey Bay to analyze population characteristics of Pacific and black hagfish; (2) evaluate the habitat utilization patterns and ecological associations of hagfish; (3) determine optimal fishing gear and onboard handling techniques; and (4) undertake laboratory studies examining tag-retention, growth, and behavior. The results of this study will help the fishery in California and throughout the West Coast develop intelligently to its full potential and provide the basic tools for management.

Results

We surveyed Monterey Bay for hagfishes using baited traps during 12 of the 15 months of the study. Nearly 6,500 Pacific (*E. stoutii*) and 3,500 black (*E. deani*) hagfish were captured and dissected for ecological analysis.

Because traps were used as samples and they occurred along a common groundline, we tested catch rates to determine whether they could be treated as independent sampling units. The length and weight of hagfish from independent traps and interdependent traps were compared over a two-day period. An analysis of variance showed no difference between days within groups, so they were pooled. No significant differences were found between pooled values for dependent and independent traps when compared using a *t*-test for either length or weight. Thus, we assume that traps, whether or not they were in a string, were independent samples of hagfishes.

Pacific hagfish were encountered in shallower waters (80–750 m) while black hagfish inhabited deeper waters (500–1000 m, the maximum coinciding with our deepest sample), with little overlap between these two species. Catches from 500 to 750 m infrequently included both species but usually involved only a few individuals. No temporal trends were found in the depth distribution of either species.

Both species exhibited normally distributed length and weight frequency distribution patterns and overlapped considerably. Pacific hagfish ranged between 100 and 500 mm, while black hagfish were 200 to 540 mm. One factor which may relate to the slightly larger size of the black hagfish could be mature egg size in females, which was ~24 mm for Pacific and ~34 mm for black hagfish.

During our surveys in Monterey Bay, we noticed sizes were more normally distributed and contained larger individuals in infrequently fished areas than in those frequently fished. Average catch per unit of effort from 100 m during comparable months was 0.125 hagfish per trap-hour in frequently fished areas versus 0.52 for infrequently fished areas. Because of these impacts from fishing in our initial study area,

we were forced to change our sampling design to include samples from other areas.

Females dominated the catches of both species, but sex ratio also appeared to be related to individual fish size. Pacific hagfish had 1.75 females to 1 male, while black hagfish had 2.56 to 1. Hermaphroditism was not commonly encountered, with only one distinct Pacific hagfish and 10 black hagfish hermaphrodites found. In both species, females outnumbered males up to a certain size (240 mm for Pacific and 400 mm for black hagfish), over which the sex ratio approached parity. Because there is no reason to believe that there was a sexual difference in capture rates, especially for small size classes, protogynous hermaphroditism may be occurring.

Pacific hagfish had a smaller size at which 50% of its females matured (370.7 ± 30.9 mm) than black hagfish (406.7 ± 31.3 mm). In contrast, male Pacific hagfish reached this stage at a larger size (365.4 ± 37.2 mm) than black hagfish (349.1 ± 33.7 mm).

Average fecundity of mature Pacific hagfish females was significantly higher (14.5 ± 5.4 eggs per female) than for black hagfish (12.0 ± 3.9 eggs). Although not statistically significant, both species had a weak, but positive, relationship between body size and fecundity. Once females reached a large enough size to reproduce, they generally produced offspring near the overall average. Variability in female investment probably depends upon food availability, which is itself highly variable and perhaps limiting.

No seasonal patterns in spawning were found for either sex or species. The frequency of neither male nor female maturity stages varied with season, although males were more variable. Females of both species had no seasonal patterns in either the hepatosomatic or gonadosomatic indices, a result which could perhaps be explained by the lack of distinct seasonal cues in their deep-sea habitat, and therefore little seasonal selection for juvenile survival.

Analysis of gut contents indicated that the two species had somewhat different diets. Pacific hagfish stomachs contained cephalopods (25.9% frequency of occurrence), sergestids (14.8%), fish parts (14.8%), polychaetes (14.8%), shrimp (11.1%), amphipods (11.1%), eggs (7.4%), and euphausiids (3.7%). Black hagfish ate sergestids (22.8%), polychaetes (22.8%), fish parts (15.8%), crustaceans (14.0%), copepods (3.5%), euphausiids (3.5%), birds (1.7%), mammals (1.7%), and crabs (1.7%).

As a part of this study we attempted to discover the particular habitat preferences of the Pacific hagfish within the Monterey submarine canyon, much like Fricke and Hissmann (1990) did for coelacanths. Such knowledge is important for complete understanding of the general ecology and for management of the emerging California hagfish fishery.

The Monterey Bay Aquarium Research Institute (MBARI) made archives of videotapes taken to depths of 450 m from their Remote Operated Vehicle (ROV) *Ventana* available to us for analysis of habitat utilization, and in addition we participated on several cruises to directly observe hagfishes in their natural setting.

MBARI's ROV is equipped with a Sony Betacam video system that transmits signals through a fiber optic cable up to the R/V *Point Lobos*, where it is recorded on 20 minute Beta videotapes. We examined videotapes from dives in Monterey Bay. The videos were used as a data base for describing the abundance and distribution of the Pacific hagfish at four sites within the bay. The coordinates of each dive were plotted to estimate the area covered in the surveys. A total of 24 dives from April 1989 to June 1990 was included in the analysis. These were originally taken by different scientists with a variety of objectives and had complete depth, longitude, and latitude records.

To randomize the sequences on the tapes, we analyzed individual frames at each 15 second interval. A frame, or photo quadrat, was accepted if it was well lit, clearly focused, not a close-up, and did not overlap with a previously accepted frame. We classified the topography according to geologic formation, general terrain, slope, substrate type, substrate features, and the presence of other fishes, invertebrates, and drift algae. Low numbers in many of the descriptor categories led us to examine the data using graphical rather than statistical comparisons and only substrate type, depth, and number of Pacific hagfish were used in the data analysis.

An analysis of randomly sorted frames was performed to determine the adequacy of describing the substrate types and habitats typical of each site. The cumulative number of habitat types leveled off at numbers of frames well below the actual number of frames examined at all four sites (Figure 1a: curves), indicating that the number of samples taken from each site adequately described the substrate types (Figure 1b: histograms).

Our null hypothesis was that the Pacific hagfish would occur equally in all substrate types. Our expected number of hagfish per substrate type was derived by dividing the number of hagfish seen in frames with a particular substrate by the total number of frames at that site, and multiplying by the total number of hagfish seen at that site. This gave the number of hagfish we would expect to see within each substrate type if they were distributed evenly throughout the site (in the same proportions as the substrate types).

At one site (Soquel), the number of hagfish observed exceeded the number expected in sedimentary areas (s), but were seen less than expected in areas of massive rocks (m) (Figure 2). At two other sites (Carmel and C4-C5), there was a close match between expected and observed numbers, and the hagfish appeared to occur equally on all substrate types. At the fourth site (Pt. Joe), there were higher than expected numbers of hagfish on sand/gravel (s/g), sand/cobble (s/c), sand (s), and boulder/sand (b/s) substrates, but fewer than expected in areas of massive rock formations.

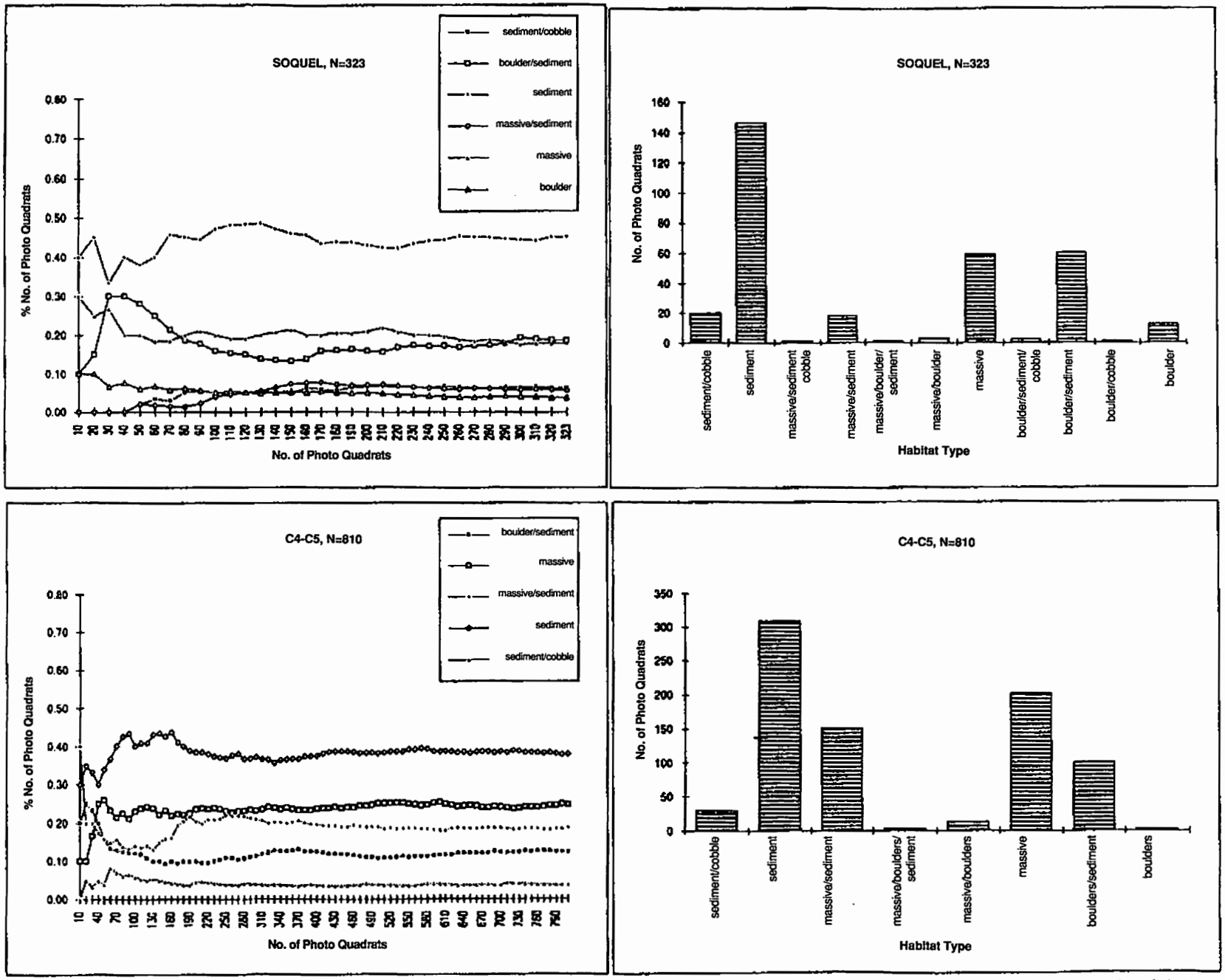

Figure 1a. Cumulative habitat curves (on left) expressed as the percent of photo quadrats from the ROV *Ventana* with a particular habitat type (see box) (*Y* axis) versus the number of photo quadrats (*X* axis) in each of four sites (Soquel, C4-C5, Pt. Joe, and Carmel). Histograms (on right) reflect the overall number of photo quadrats at each site which had one of the 11 habitat types.

Figure 1b. Histograms reflect the overall number of photo quadrats at each site which had one of the 11 habitat types.

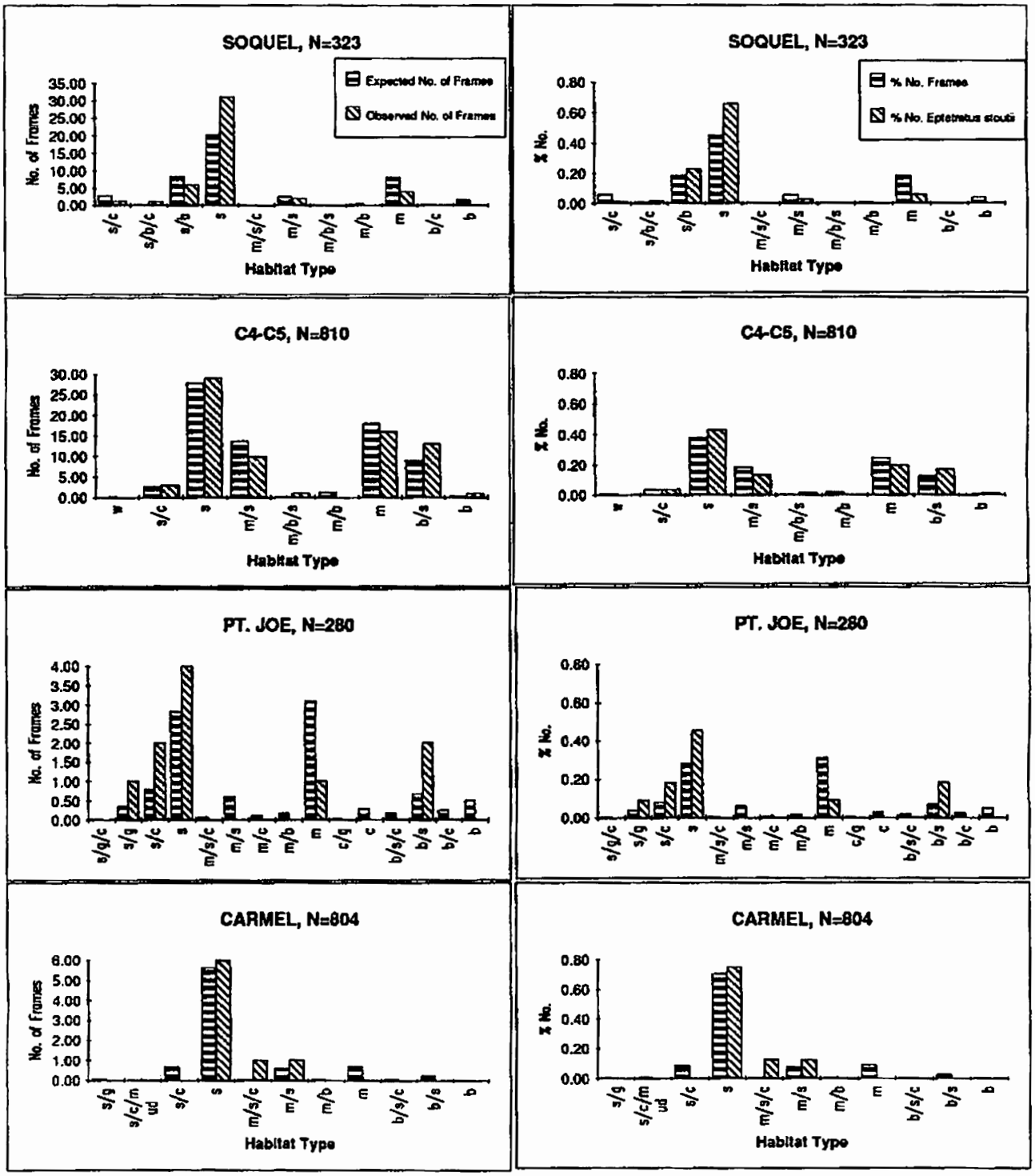

Figure 2. Histograms of the expected number (left side) and percent (right side) of photoquadrats (frames) to include Pacific hagfish for each of the habitat types (see Figure 1) at the four sites.

The general impression gained from previous studies (i.e. Adam and Strahan,1963; Foss, 1963; McInerney and Evans, 1970) and from trapping was that Pacific hagfish occur only on soft bottoms such as sand or mud. Randomly selected frames from MBARI's ROV dives showed a much wider habitat usage. All four sites examined showed that the majority of animals were seen in sand or mud areas, but a high percentage (30%) were seen in mixed substrate sections, and a surprising number (21 out of 142 total) were seen in areas consisting only of massive substrate.

The wider range of habitat types used by this species of hagfish may indicate either that they travel over a large area, or that they do not always require soft sediment to burrow into. Given the rapid response times of hagfish in general to baited traps, it seems plausible that they travel quite frequently in search of suitable food sources.

To check depth distribution and perhaps observe habitat utilization of the deeper-dwelling black hagfish, we participated in several submersible dives using WHOI's DSV *Alvin* off the *Atlantis II* and Russian submersibles *MIR I* and *MIR II*, off the Russian Research Vessel *Keldysh*, to habitats as deep as 3950 m. Unfortunately, except for occasional sightings of black hagfish attracted to baited cannisters in shallow (1,000–1,500 m dives), none were seen at these depths, thus confirming previous depth records (Eschmeyer et al., 1983).

Trap design experiments partially funded by Grant NA90AA-H-SK142 using ROV observations suggested that traps without side holes caught more hagfish than traps with side holes, but these results were not statistically significant. However, traps with two funnels caught significantly more hagfish per trap and significantly larger hagfish than traps with single funnels. Traps with double funnels and without side holes were the best design tested, because they caught more and larger fish.

Experimental traps were also set at different times of day and durations and using different baits. Neither the mean number per trap nor mean length of hagfish varied significantly with time of day, duration, or bait type. The tremendous variation in catches with soaks and among soak intervals made it impossible to reach definite conclusions, especially about nocturnal activity patterns. We concluded that the best bait is probably the one that is least expensive and most available in a given port.

Despite trace fishing levels and relatively poor prices coastwide in 1991, the eel skin market remains product-limited. The reason for poor price ($0.25 to $0.30 per pound) appears to be the inferior quality of the west coast product, particularly from California. Our results strongly suggest that if the product is kept cold and densities are kept to around 200 hagfish per 55 gallon barrel, skin quality can be improved and anesthetics eliminated. Dorsal holes in the skin greater than 0.5 mm (tears) and bites limit the value of the skins. Tiny pinholes (mostly <0.25 mm) were few and generally not limiting. Tears seem to be the result of temperature abuse and are in some cases caused by bites.

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Cooperating Organizations

California Department of Fish and Game Marine Resources Laboratory at Granite Canyon
California Polytechnic State University, San Luis Obispo
Clemson University
Monterey Bay Aquarium Research Institute
National Marine Fisheries Service, Tiburon Laboratory
Shoals Marine Laboratory
University of California, Santa Barbara
University of Washington

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Immunological Identification of Larval Fish Prey

University of California, San Diego
Scripps Institution of Oceanography
R/F-132
1989–91

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Introduction

The overall goal of this research was to develop a dietary immunoassay that would permit soft-bodied microzooplankton prey to be detected in the gut contents of fish larvae. The need for a new approach to the study of larval fish diets arises from artifacts associated with conventional methods. Soft-bodied protists cannot be detected in the gut contents of predators. Classical incubation methods damage the protists, generating biased predation rates. An improved understanding of the significance of "microbial loop" production to the feeding success and survivorship of teleost larvae requires new methods that recognize prey remains and do not require long-term incubations.

We successfully developed an immunochemical probe that accomplishes these objectives. We produced polyclonal antisera against planktonic ciliates of the genus *Strombidium*. The antibodies recognize intact planktonic ciliates as well as partially assimilated ciliate remains from the gut contents of first-feeding larvae of the northern anchovy, *Engraulis mordax*. We quantified the immunochemical reaction using enzyme-linked dot blots and reflection densitometry. An unexpected finding, tangential to the original goals, was the discovery and description of a new species of planktonic ciliate, *Strombidinopsis cheshiri* (Snyder and Ohman, 1991).

The research proceeded in three phases, each of which will be described briefly below. First, we perfected ciliate culturing methods and determined ciliate specific growth rates. Second, antibodies were produced against ciliates, then purified, characterized, and tested for cross-reactions. Third, the antibodies were used in predation experiments. The experiments compared immunochemical measures of ingestion with those determined by conventional prey disappearance experiments.

Ciliate Growth Rates

Several ciliate species were isolated from southern California coastal waters and initiated in culture. The primary focus of the research was *Strombidium* sp., a representative of the most abundant ciliate genus in the ocean (Sorokin, 1981). Comparative experiments were done with the scuticociliate *Uronema* sp. (Both ciliates appear to be new species; work in progress may result in additional new species descriptions.) A highly reproducible method was developed for culturing planktonic ciliates on a diet of washed planktonic bacteria or a mixed diet of bacteria together with heterotrophic microflagellates.

Both the specific growth rate and the yield of *Strombidium* sp. vary with the density of bacterial prey (Figure 1). Both characteristics also vary with the composition of the diet; the presence of microflagellates as prey, in addition to planktonic bacteria, markedly increases the rate of growth as well as the final cell density. Temperature influences the specific growth rate but does not influence the final yield (Figure 1).

Comparative experiments performed with *Uronema* sp. revealed that the scuticociliate attained a 9-fold higher specific growth rate and a 30-fold higher cell yield than *Strombidium* sp. raised on the same diet (Figure 2). This contrast existed whether the ciliates were fed cultured bacteria or enriched natural bacteria. Determinations of gross growth efficiency, K_1, were consistent with the conclusion that *Uronema* is an efficient bacterivore; *Strombidium*, however, utilizes a pure bacterial diet rather inefficiently (Table 1).

These results were used to optimize the production of milligram quantities of ciliate antigens for subsequent antibody production.

Antibody Production and Purification

Polyclonal antibodies were produced against *Strombidium* sp. in New Zealand white rabbits. The immunization protocol used was a refinement of the method of Vaitukaitus (1981). The immunoglobulin G (IgG) fraction was isolated from the crude antiserum by ammonium sulfate precipitation and DEAE ion exchange chromatography. This serum was then tested for titre and specificity. The antigens were characterized using SDS-PAGE gels, and the immunoreactive fraction identified using Western blots.

The antigen-antibody reaction was visualized using dot blots (Monroe, 1985; Theilacker et al., 1986; Ohman et al., 1991). The secondary antibody used is alkaline phosphatase-conjugated goat anti-rabbit IgG. We were able to quantify the intensity of the dot blot reaction by measurement with a reflection densitometer. By interfacing this instrument to a microcomputer we developed an efficient means to acquire, store, and process the dot blot results.

The results of dot blot assays showed that the anti-*Strombidium* IgG recognizes *Strombidium* antigens in different forms: from intact ciliates, from ciliates in the gut contents of anchovy larvae, and from ciliate remains egested in the fecal pellets of planktonic copepods. The antiserum recognizes *Strombidium* sp. from southern California as well as a different species of *Strombidium* from a Georgian salt marsh. It does not recognize 25 other taxa of planktonic bacteria, phytoplankton, and metazoans; hence, it appears to be

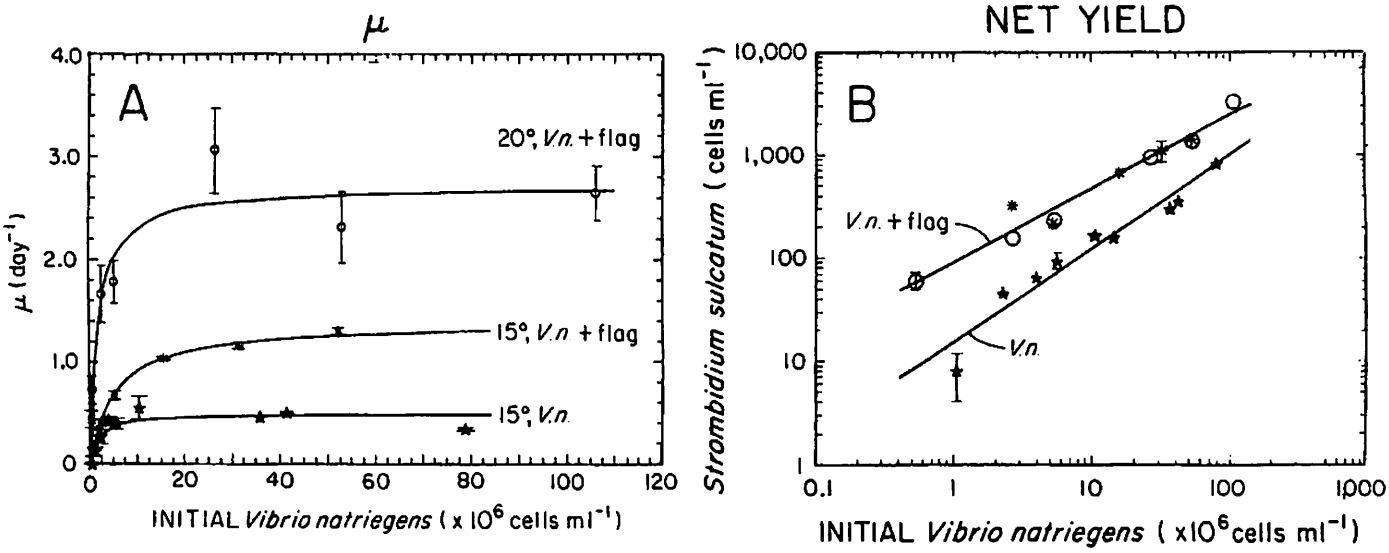

Figure 1. Specific growth rate (left) and net yield (right) of *Strombidium* sp., as a function of initial concentration of the prey bacterium *Vibrio natriegens*. The three treatments were 15°C, *V. natriegens* only (filled stars); 15°C, *V. natriegens* + microflagellates (asterisks); and 20°C, *V. natriegens* + microflagellates (circles).

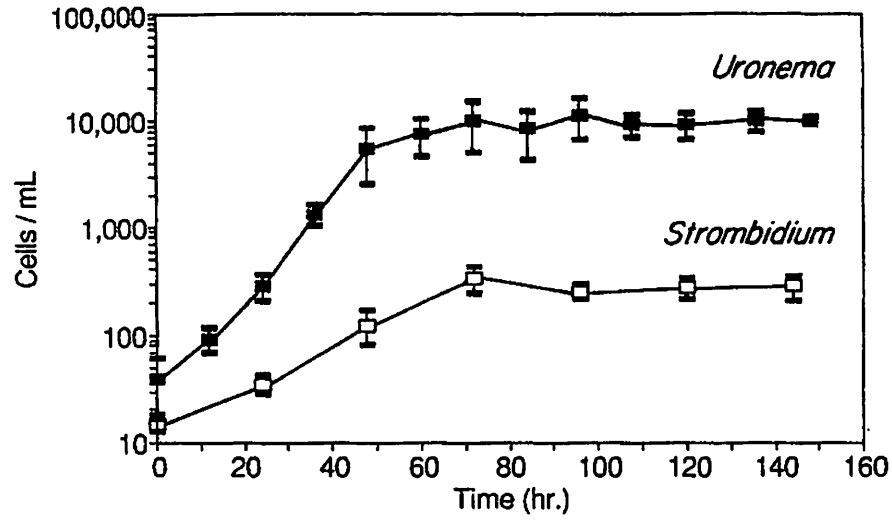

Figure 2. Comparative growth of *Strombidium* sp. and *Uronema* sp. when offered picoplankton from an enriched filtrate of 0.6 μm filtered natural seawater. (x) ± s.e.).

Table 1. Gross Growth Efficiency (K_1; ciliate production/bacteria consumed) of *Strombidium* sp. (n = 9) and *Uronema* sp. O (n = 3) Raised on *Vibrio natriegens*. (x ± 95%)

	Gross Growth Efficiency	
Constituent	***Strombidium* sp.**	***Uronema* sp.**
Protein	19.1 ± 7.6%	70.4 ±10.69%
Carbon	19.6 ± 7.8%	48.6 ± 7.3%
Nitrogen	11.0 ± 4.4%	54.5 ± 8.2%

a genus-specific antiserum.

One unexpected cross-reaction did occur. There was a strong reaction with anchovy larvae, which initially complicated the use of this serum for predation studies using the northern anchovy. Both immune and preimmune serum cross-reacted. However, we successfully eliminated the cross-reaction by immunoadsorption against a Sepharose 6MB affinity column. The immunoadsorbed anti-*Strombidium* IgG was used in predation studies employing larval anchovy and *Strombidium* sp. Figure 3 illustrates a calibration curve, showing that the intensity of the dot blot reaction varies with the amount of antigen present. The antiserum used for this calibration and for dietary immunoassays had been immunoadsorbed in the manner described.

Immunochemical Predation Studies

Predation experiments were carried out to test the feasibility of using the immunochemical probe as a quantitative estimator of ingestion of planktonic ciliates by fish larvae. For this purpose, first-feeding larvae of the northern anchovy were incubated with *Strombidium* sp. over prey concentrations ranging from 0.8 to 45 cells ml^{-1}. At the end of the incubation larvae were quickly frozen in liquid nitrogen. Larval gut contents were subsequently dissected out,

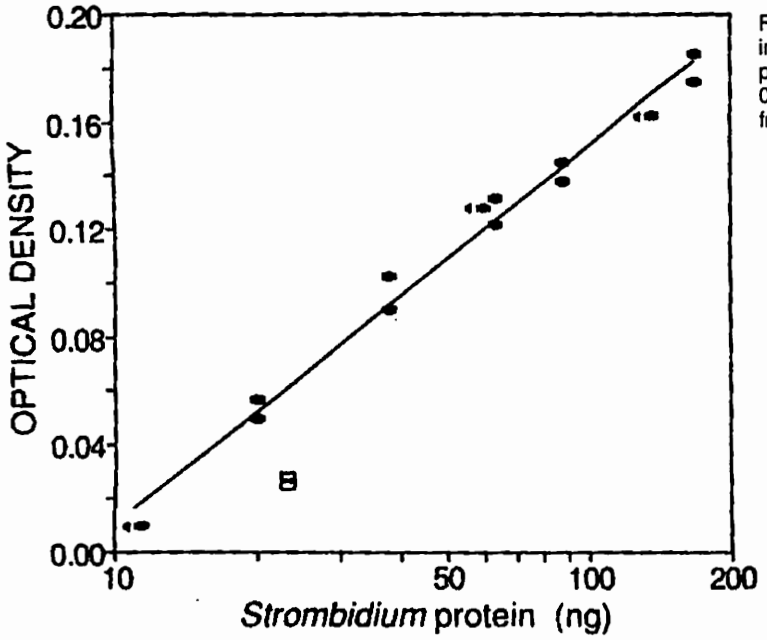

Figure 3. Calibration relation between the optical density of immunochemical dot blots and the quantity of *Strobidium* sp. protein (OD = 0.131 + 0.141[log protein]; $r^2 = 0.987$, $P < 0.001$). The outliers indicated by open squares were excluded from the regression. Overlapping data points are offset.

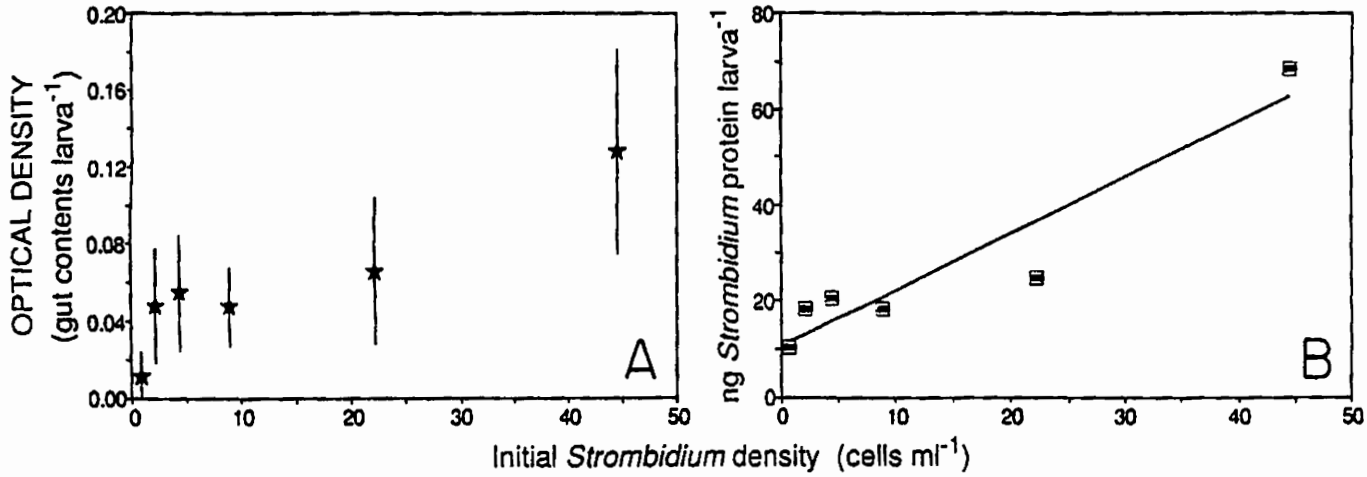

Figure 4. (A) Relation between the gut contents of first feeding anchovy larvae ($x \pm 95\%$) as determined by immunochemical dot blots and the concentration of ciliate prey. (B) As in part A, with gut contents expressed as ciliate protein larva^{-1} from the relation in Figure 3.

blotted onto nitrocellulose, and carried through the dot blot procedure. Figure 4 illustrates the results from this experiment. The intensity of the immunochemical reaction varies in proportion to the density of prey (Figure 4A). When converted to ciliate protein equivalents from the calibration relation in Figure 3, a significant positive relation exists between the amount of *Strombidium* protein detectable per larval gut and prey density (Figure 4B). Significant ingestion of ciliates occurred even at the lowest prey density offered.

The time course of uptake of ciliate antigens was also evaluated in separate experiments. Naive anchovy larvae took 2 hr to efficiently capture ciliates and fill their guts. The quantity of immunoreactive gut contents decreased after 2 hr, then reattained the previous level after 6 hr. This pattern reflects either discontinuous ingestion or egestion, or perhaps alterations in immunoreactivity of gut contents over time.

Conventional prey disappearance experiments were also carried out, to confirm the ingestion rates of ciliates by anchovy larvae and to calibrate the immunochemical method. Figure 5 illustrates the functional response of anchovy larvae, measured over a broader range of prey concentrations than in the immunochemical experiments. There was a significant positive relation between immunochemical estimates of gut contents (from Figure 3) and conventional prey disappearance experiments (from Figure 5; $r^2 = 0.834$, $P < 0.05$). The maximum ingestion rates are equivalent to 7–8 successful strikes

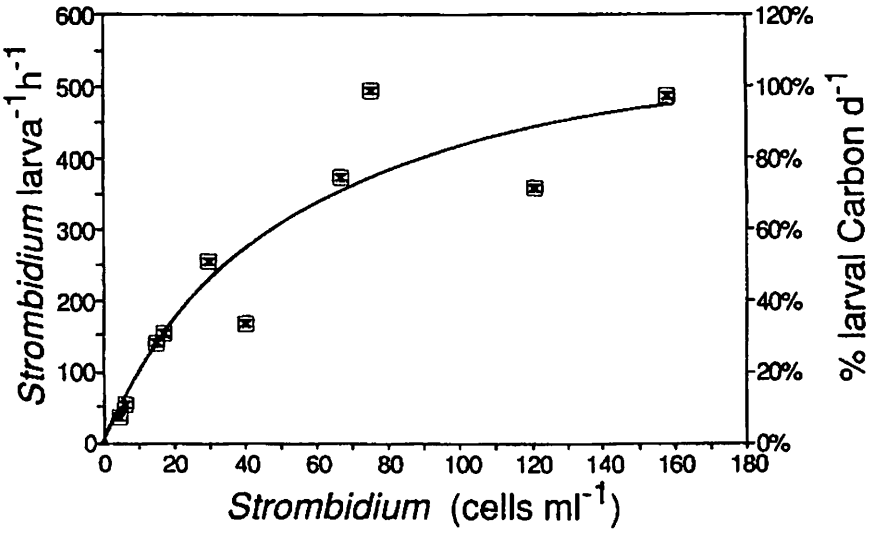

Figure 5. Ingestion rate (I) of first-feeding anchovy larvae as a function of concentration of the ciliate *Strombidium* sp. (C), determined by prey disappearance experiments. The ingestion rate is described by

$$I = \frac{639.5 * C}{54.6 + C}$$

min^{-1}. This compares with Hunter's (1977) determination of 5–7 strikes min^{-1} for first feeding anchovy larvae feeding on the dinoflagellate *Gymnodinium sanguineum* (= *splendens*). These results imply that ciliates are captured at rates comparable to those when larvae are fed an unarmored dinoflagellate that is thought to be an optimal prey item.

We have established the ability of fish larvae to capture and ingest planktonic ciliates and to do so at prey concentrations as low as 0.8 ciliates ml^{-1} using an immunochemical assay. The intensity of the immunochemical reaction is directly proportional to prey density and to the predator's ingestion rate. Soft-bodied ciliates and other natural microzooplankton prey, heretofore largely overlooked in larval fish feeding studies, may be important contributors to the survivorship of marine teleost larvae.

Cooperating Organizations

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NOAA National Marine Fisheries Service, Southwest Fisheries Science Center, La Jolla, California
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Lectures and Conferences

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Oxidative Metabolism of Polyunsaturated Fatty Acids in Fish: Mechanism and Physiological Function of Lipoxygenase

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Polyunsaturated fatty acids (PUFA) of the n-3 family are uniquely abundant in the tissues of marine fishes. The long-term optimal use of this marine resource will require an understanding of both the functioning of these components in fish and the chemistry that leads to their deterioration. In mammals it has been recognized that one of the functions of highly unsaturated membrane fatty acids is to serve as precursors to oxygenated signal molecules. Unfortunately, this property of high unsaturation (the basis of purported therapeutic effects in human diets) also causes these fatty acids to be extremely susceptible to the reactions of autoxidation. The consequences of lipid autoxidation to fish as human food include loss of therapeutic components, off-flavors, bleaching of vitamins and pigments, and protein cross-linking. Controlling lipid oxidation depends on understanding and controlling the initiating catalysts. This project characterized a family of enzymes in fish that catalyze the initial oxygenation reaction of PUFA and the role of these same enzymatic initiators in the production of flavors in postharvest tissue.

The oxidation of polyunsaturated fatty acids (PUFA) is well recognized as a source of volatile products that are perceived as off-flavors (Frankel, 1984). The potency of these volatile off-flavors derived from lipid oxidation is underscored by the significant quality losses of PUFA-containing foods attributed in large part to these processes (Allen and Hamilton, 1989; Hall and Anderson, 1983; Richardson and Korycka-Dahl, 1983) and the substantial measures taken to minimize them (Schuler, 1990). Somewhat paradoxically, then, is the developing appreciation that in many foods some of these same volatiles are critical components of the overall impact of "fresh" flavor. That is, certain molecules that are part of the collection of volatile breakdown products of autoxidation when detected separately are not off-flavors per se but in fact contribute to the flavors associated with freshness itself. This is best considered from the perspective of what fundamentally distinguishes freshness (Badings, 1990). Freshness is not simply a lack of off-flavor. There are distinct compounds that lead to this perception. Similarly, this is not a simple response to the sensory detection and transduction of olfactory signals but rather a cognitive decision based on the overall spectrum of sensory inputs. The mechanisms underlying the development of this cognitive decision relate to the ephemeral nature of certain volatiles and especially their production.

Thus, starting from the same precursors, the products of a nonspecific or random breakdown process are generally perceived collectively as deteriorative (off) flavors while controlled or specifically catalyzed reactions yield a different mixture that is ultimately perceived as flavor. In the case of "fresh" this is arguably related to the inherently biological origin of these compounds, that relates to the temporary or transitory nature of certain volatiles and notably their biosynthesis (Badings, 1990). The loss of freshness is a result of both an increase in deteriorative compounds and a loss of the volatiles themselves or the biosynthetic capacity that gives rise to them. Therefore, in searching for the candidates for "fresh" flavors, one must be drawn to those mechanisms that are particularly transient. In this regard the lipoxygenases are now recognized as important.

Autoxidation

The specificity and kinetics of lipoxygenases are conspicuous when contrasted with the known processes of autoxidation. For example, in autoxidation of, linoleic acid 18:2, the methylene carbon interrupting two nonconjugated double bonds is relatively easily oxidized by a variety of single electron oxidants, yielding an allyl radical that is very rapidly quenched by ground state triplet oxygen (Ingold, 1969). The resulting peroxy radical is itself an excellent single electron oxidant for methylene interrupted 1,4-diene carbon systems (Ingold, 1969). This is the fundamental driving factor for the propagation of autoxidation. The acyl hydroperoxides formed by this reduction are substrates for subsequent scission reactions leading to hydrocarbons, carbonyls, and alcohols, many of which are volatile, some with very low-flavor thresholds (Berger et al., 1985; Buttery, 1981).

One feature of importance of this well-described chemistry is the relative nonspecificity of oxygen addition sites. In linolelic acid both the 9 and 13 positions are attacked with similar frequency, and also while a chiral center is formed a racemic mixture of products results (Frankel, 1984).

Because many foods contain a mixture of polyunsaturated fatty acids, the possible number of products formed by purely autocatalytic events is thus large indeed. Fish containing abundant fatty acids with 4, 5, and 6 double bonds per molecule represent a food system in which the polyunsaturated fatty acids, the primary substrates for lipid oxidation, are the most *un*saturated typically encountered in foods. The volatile compounds generated in fish oils have been reported, and several mechanisms for their biogenesis have been proposed.

Importantly, the volatiles produced from pure fish oils and those from fresh fish tissues are dramatically different, as are the odor impressions (Josephson and Lindsay, 1986). This would imply that while the substrates are common, the mechanisms of volatile generation must be different. Our thesis in this work has been that enzymes as biological and hence ephemeral catalysts profoundly constrain the oxygen attack and subsequent breakdown reactions. This would mean that of all *possible* volatiles that would be generated by autoxidation, enzymes both increase specific subsets and decrease others, and when perceived by the olfactory center a cognitive decision deems the overall impression to be "fresh."

Enzymatic Oxidation via Lipoxygenases

Lipoxygenases can participate in the generation and proliferation of lipid products in several ways. Primarily is the catalytic reaction for which they are designated, the addition of molecular oxygen to a cis, cis 1,4-pentadiene containing unsaturated fatty acid releasing a fatty acyl hydroperoxide. These hydroperoxides can then be broken down by enzyme or nonenzyme-catalyzed scission reactions to yield specific chain length volatile compounds (Hiatt et al., 1968). The lipoxygenases can act at more than one methylene carbon on the substrate molecule to yield double oxygenation sites enzymatically (Yamamoto, 1991). The fatty acid hydroperoxides can also be broken down via homolytic cleavage pathways and catalyzing further oxidations of the parent molecule intramolecularly. This project studied the endogenous lipoxygenases in fresh fish tissue to determine the mechanisms by which these enzymes influence the specificity and time course of peroxidation reactions. Our first questions related to what precise reactions of polyunsaturated fatty acids the lipoxygenases catalyzed in fish tissue. We have thus described (1) the specificity of primary abstraction and oxygen addition sites by the enzymes present leading to monohydroperoxide derivatives of substrate PUFA, (2) the catalysis of secondary sites on the same molecule leading to dihydroxy derivatives, and (3) the accumulation of specific volatile flavors resulting from the decomposition of lipoxygenase-generated products.

Specificity of Fish Lipoxygenases

Lipoxygenases catalyze the initial peroxidation event in the oxygenation of PUFA. Employing a nonheme iron in the high spin FE III state as the single electron oxidant, the enzyme catalyzes the stereospecific hydrogen removal, oxygen addition, and peroxy radical reduction reactions, forming a stereospecific conjugated diene hydroperoxy fatty acid product. The specificity of formation of these hydroperoxides is predicted to be an important determinant of the final flavor profile. A graphic indication of such specificity is shown by the HPLC separation and UV detection of the products of oxidation of arachidonic acid by fish gill homogenates (Figure 1).

Separation on the same chromatographic system of the autoxidation products yields multiple peaks representing the mixture of the various oxygen addition positions described previously. The single dominant product from the fish gill tissue was the 12 hydroxy derivative of arachidonic acid.

Separation of the stereoisomers of these hydroxy products of arachidonic acid using chiral analyses by normal phase chiral HPLC and circular dichroism spectroscopy (data not shown) and NMR spectroscopy (Figure 2) further identify this to be the 12 (S) 5Z 8Z 10E 14Z HETE formed by the arachidonate 12-lipoxygenase. Further clarification of the substrate dependency and product specificity toward the other abundant PUFA in fish 20:5 n3 22:4 n6 22:5 n3 and 22:6 n3 indicated that the enzyme exhibits specificity toward the methyl rather than the carboxyl terminus and is more precisely described as an n-9 lipoxygenase.

Although these experiments illustrate the potential enzyme activity, *in situ* lipoxygenases oxygenate solely unesterified fatty acids released from membranes by the action of phospholipases. Hence experiments were conducted to determine if the lipoxygenases were a major contributor to the peroxidation of the actual fatty acids available for oxygenation in the tissue. Perfused, homogenized trout gill tissue was used to investigate the release and metabolism of *endogenous* fatty acids. The n-3 PUFA were most abundant esterified to phospholipids in membranes. After incubation, however, the free fatty acids were separated and analyzed, and arachidonic, eicosapentaenoic, and docosahexaenoic acids represented, quantitatively, the potential substrates for lipoxygenase catalysis. The monohydroxy derivatives formed endogenously from these liberated fatty acids were purified over SPE cartridges as methyl esters and derivatized to trimethyl silyl ethers, separated by GC and structures confirmed by GC/MS. Greater than 95% of metabolites were the respective monohydroxy derivatives expected from the activity of 12-lipoxygenase on these fatty acids (Figure 3).

The obvious question was, then, does this lipoxygenase influence volatile production from the fresh tissue in which the enzyme is active? Rational decomposition mechanisms have been proposed for this position-specific n-9 monohydroperoxide and conclude that the inordinate abundance of three 9 carbon products 3,6 nonadienal, 2,6 nonadienal, and 3,6 nonadien-1-ol isolated from fresh fish volatiles are due to this lipoxygenase's activity. Similarly, 8 carbon decomposition and rearrangement products could be expected from an abundance of lipoxygenase-generated hydroperoxides. This was tested in the fish gill model using two separate approaches: adding a selective inhibitor of the 12-lipoxygenase, esculetin at 1 μM, and adding exogenous polyunsaturated substrates to the tissue preparation. Results shown in Table 1 are consistent with formation of 1 octen-3-ol and 2 octen-1-ol from n-6 PUFA

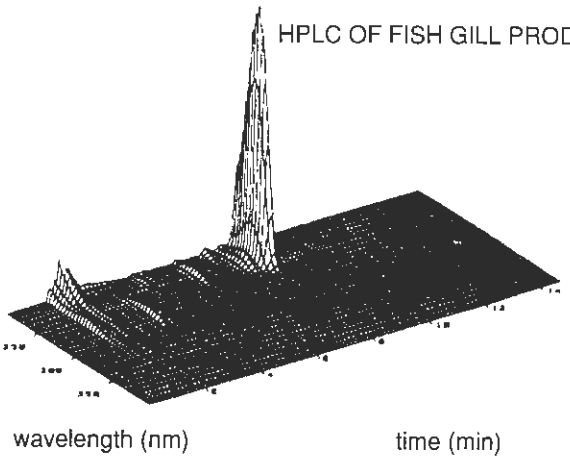


Figure I. HPLC separation and UV-VIS spectral detection of the products formed from exogenous arachidonic acid by trout gill homogenates. Fresh gill tissue was homogenized in 5 volumes (0.05 M phosphate buffer pH 7.5) and then incubated with 20 mM arachidonic acid for 20 min. Products were extracted with 2 volumes ethyl acetate, concentrated, injected onto a reverse phase C-18 HPLC column and eluted isocratically with a 70% methanol in buffered water solvent system. Spectra recorded at 1 sec intervals were converted into the two-dimensional chromatogram of elution time and UV-VIS absorption.

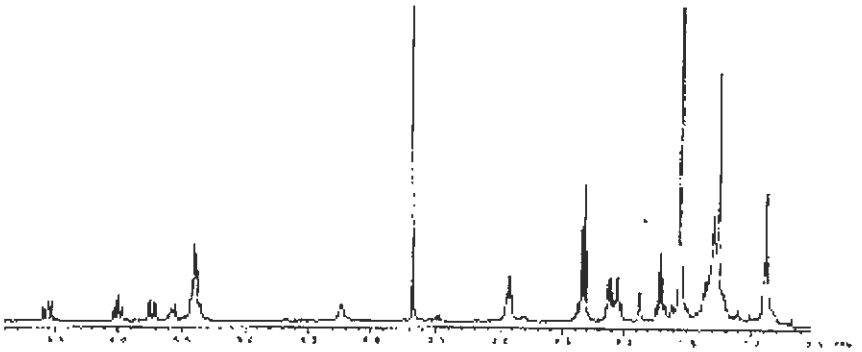


Figure 2. Proton NMR spectrum of the primary metabolite of arachidonic acid formed by trout gill. Products formed as described in Figure 1 were methylated using diazomethane and the methyl HETE fraction purified over silica solid phase extraction columns using hexane ether 75:25 as eluting solvent. Samples were dried under nitrogen, redissolved in CCl3D, and proton spectra recorded using a Nicholet NT-360 NMR spectrometer. Proton resonances were identified from standards and proton decoupling experiments. Proton chemical shifts for 100 mg 12 (S) HETE methyl ester were generated by trout gill lipoxygenase from arachidonic acid in CDCl3. Assignments were made from comparison with literature values and confirmed (or as necessary revised) by COSY and selective decoupling experiments.

Carbon number	^{1}Hδ (ppm)
1	
2	2.33
3	1.70
4	2.12
5	5.41
6	5.41
7	2.93
8	5.41
9	5.97
10	6.55
11	5.56
12	4.22
12′	1.88
13	2.37
14	5.41
15	5.58
16	2.09
17	1.30
18	1.30
19	1.30
20	0.88

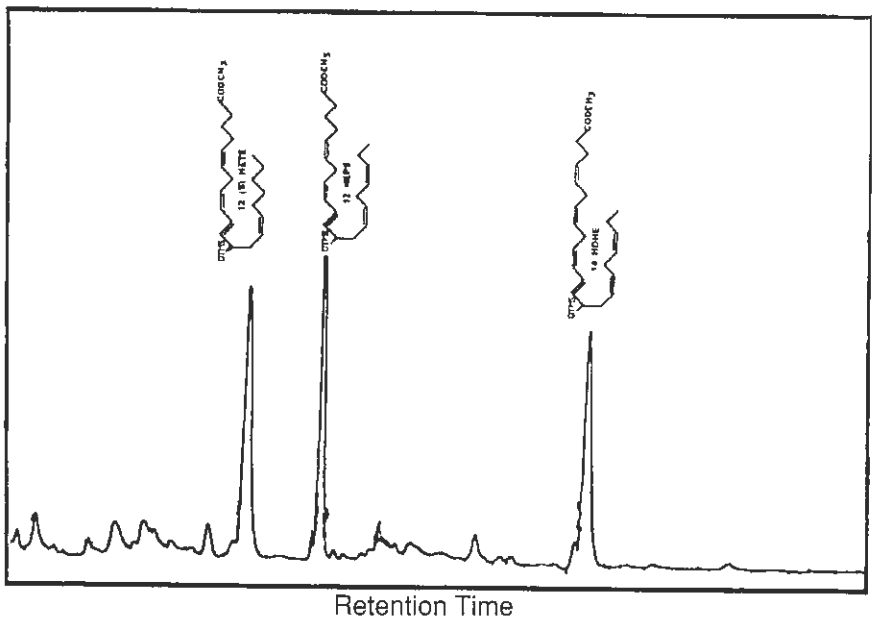


Figure 3. Gas chromatographic separation and flame ionization detection of the major hydroxylated polyunsaturated fatty acids formed from endogenous fatty acids in trout gill. Fatty acid structures shown were confirmed by GC/MS.

such as arachidonic acid and 1,5 octadien-3-ol and 2,5-octadien-1-ol from n-3 PUFA 20:5 and 22:6 all by the n-9 lipoxygenase.

These are not the only volatile flavor compounds formed in these and other fish, however. Similar analyses of volatiles released from fresh teleost fishes have found 5 carbon volatiles in some abundance. These 5 carbon volatile aldehydes and alcohols are the same as those identified in a variety of plants. In plant tissues these 5 carbon products have been shown to be the direct result of an n-6 lipoxygenase activity such as the type 1 soybean lipoxygenase. The observation of large quantities of these analogous compounds from fresh fish suggested that there might be a similar n-6 lipoxygenase present in fish as well. Our initial attempts to identify this activity were unsuccessful. Previous experiments searching for this enzyme in fresh tissue homogenates have not revealed significant quantities of this activity; recent experiments attempting to purify the 12-lipoxygenase have identified both the presence of the enzyme and at least a partial explanation for its obscurity.

During purification of the n-9 lipoxygenase, an increasing quantity of an additional conjugated diene product with hydroxyl function not at the n-9 but rather at the n-6 position suggested an n-6 lipoxygenase metabolic activity. These could have arisen via an alteration in the absolute specificity of the single n-9 lipoxygenase enzyme or the appearance of a previously undetected n-6 lipoxygenase enzyme. Tissue samples containing the 12-lipoxygenase enzyme activity applied to hydroxlapatite columns eluted the two lipoxygenase peaks separately (Figure 4). The first peak, eluting at low ionic strength was the enzyme similar in position and chirality to its catalytic reaction to the 15-lipoxygenase characterized in leukocytes, reticulocytes, and soybeans. Physical descriptions of the enzyme found an apparent molecular weight of 70 ± 5 kD, consistent with that found for mammalian lipoxygenases but significantly smaller than the soybean enzyme, that has a molecular weight of 100,000.

Table 1.
Concentrations of Discriminating Volatiles in Trout Gill Homogenates

Compound (µg/100 g fr. wt.)	Control	Esculetin	+ 5 µM 20:4 + 5 µM 22:6	
1-Octen-3-ol	100	58	130	133
1,5-Octadien-3-ol	152	93	155	207
2-Octen-1-ol	45	35	58	58
2,5-Octadien-1-ol	200	113	157	225
1-Penten-3-ol	n.d.	7	10	23

n.d., not detected.

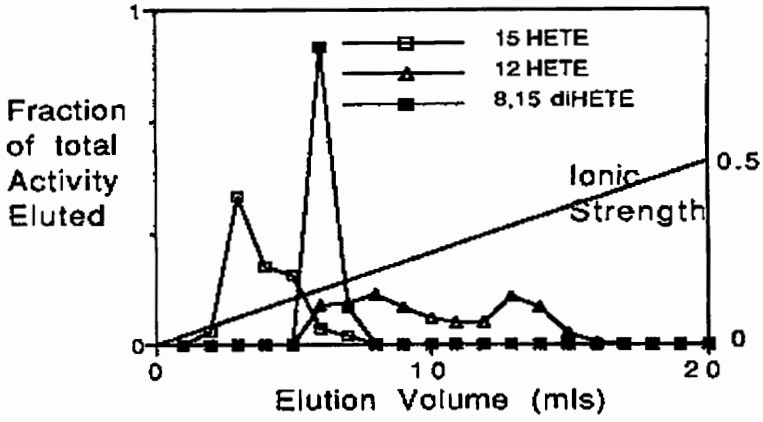


Figure 4. Elution of trout gill lipoxygenase activities from a hydroxylapatite column. The redissolved 45% ammonium sulphate precipitate was placed on a hydroxyapatite column and eluted with a linear gradient of sodium chloride. 1-ml fractions were collected and assayed for ability to convert arachidonic acid to chromophore containing mono and dihydroxylated products.

The substrate specificity of this new n-6 specific enzyme was determined by incubating a variety of fatty acids with the partially purified enzyme preparation. Fatty acids with double bonds at both the n-6 and n-9 positions were substrates for activity. Similarly, fatty acids with chain lengths from 18 to 22 carbons with the appropriate orientation of double bonds (n-6 n-9) were effective (though not equally reactive) substrates. The respective substrates tested and products formed as confirmed by HPLC and GC/MS are summarized in Table 2.

Table 2.
Substrate Specificity and Products Generated by the n-6 Lipoxygenase of Trout

Substrate Fatty Acid	Product Formed
18:2 n6	13(S) Hydroxyoctadecadienoic acid
18:3 n3	13 Hydroxyoctatrienoic acid
20:3 n6	15 Hydroxyeicosatrienoic acid
20:3 n3	15 Hydroxyeicosatrienoic acid
20:4 n6	15(S) Hydroxyeicosatetraenoic acid
20:5 n3	15 Hydroxyeicosapentaenoic acid
22:6 n3	17 Hydroxydocosahexaenoic acid

Thus, the fish gill tissue contains two separate lipoxygenases, each active toward polyunsaturated fatty acids but exhibiting different hydroperoxide addition sites. If we extrapolate from the enzymes as catalysts to their activity as producers of volatile precursors, the different lipoxygenases present would be predicted to result in distinct volatiles from the same fatty acids. Evidence in favor of this hypothesis came from parallel experiments on the inhibition of the two enzymes and the volatiles produced by trout gills. The phenolic inhibitor esculetin previously has been shown to strongly inhibit the 12-lipoxygenase. By assaying its inhibition of the trout gill system, it was found that the 15-lipoxygenase was not as sensitive to esculetin, and in fact the net product from this enzyme actually increased (Figure 5).

The volatiles from trout gill homogenates were also examined using similar protocols in which esculetin was added to the homogenates before incubation and subsequent trapping of the volatiles generated. When the volatiles released from these incubations were analyzed, whereas the 8 carbon volatiles (the putative 12-lipoxygenase-derived compounds) were reduced by esculetin as predicted from the reactions initiated by this enzyme, the 5 carbon product 1 penten-3-ol (a breakdown product of the 15-lipoxygenase reaction) increased (Table 1).

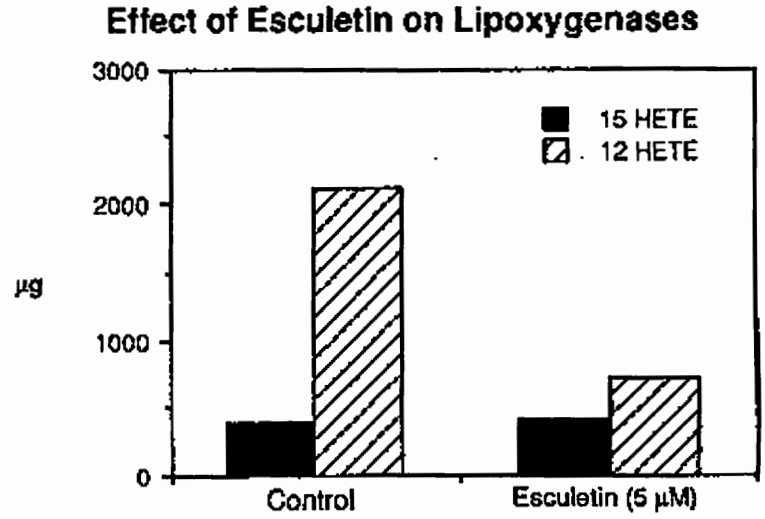
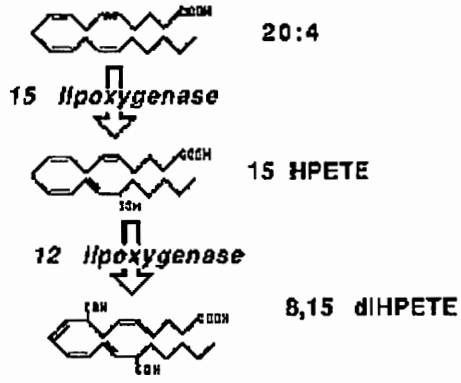


Figure 5. Effect of esculetin on the release of particular hydroxy fatty acids (HETEs) formed from exogenous arachidonic acid by trout gill homogenates. Esculetin or its negative control was incubated for 10 min prior to addition of arachidonic acid. After 20 min incubation, the products formed were analyzed by HPLC.

Sequential Reactions of Fish Lipoxygenases

Fatty acids from fish are conspicuous by the number of double bonds in a single fatty acid molecule. Increasing the number of double bonds in a fatty acid increases the number of carbons susceptible to hydrogen abstraction by both autoxidative and enzymatic reactions. This also increases the possibility of multiple oxygenations on a single molecule. Again, the possibility arises that such reactions could be favored by the presence of specific (enzymatic) catalysts. We have found oxidation products in fish tissues that arise by sequential attack by the two separate enzymes. The critical moiety on a PUFA substrate necessary for enzyme catalysis is the presence of the appropriate 1,4-pentadiene double bond system. In the case of 15-lipoxygenase, starting from arachidonic acid 20:4 n-6, it is the n-6 n-9 double bond system that is necessary, and for the 12-lipoxygenase it is the n-9 n-12 arrangement. Importantly, the 15 HETE product of the 15-lipoxygenase still contains the n-9 n-12 double bonds necessary for 12-lipoxygenase attack as described schematically below.



The most convincing evidence of the role of the respective enzymes is that these are both highly selective stereospecific reactions consistent with their enzymatic nature. Both the 15- and 12-lipoxygenases show very strong chiral selectivity, each producing the specific (L_S) monoHETE isomer. Therefore, the initial product of the 15-lipoxygenase is the 15(L_S). The second abstraction at carbon 10 and oxygen addition at carbon 8 (–2) retains the specificity of the 12(L_S) lipoxygenase for the L hydrogen to yield the 8(L_R) product. To resolve the chirality of the second oxygenation, the diHETE from trout was analyzed on RP HPLC relative to standard diHETE's, and results indicated an 8(L_R),15(L_S) structure. The chiral specificity of the 12-lipoxygenase for abstraction and oxygen addition was retained, but rather than oxygen adding +2 from the c-10 H abstraction carbon, it was reversed to –2 in forming the second hydroperoxide product on carbon 8. The stereospecificity of the arachidonic acid-derived compound was thus determined to be the 8(L_R),15(L_S) diHETE. The formation of this dihydroxy class of compounds has been verified for the three main PUFA substrates in fish: arachidonic, eicosapentaenoic, and docosahexaenoic acids (data not shown).

Properties of the Lipoxygenases Relevant to Their Role in Flavor Production

With the understanding that lipoxygenase enzymes in animal tissues generate different volatile patterns depending on the sub-

strates, activity of the enzymes, and condition of the tissues, we have investigated various properties of the catalytic proteins. One aspect particularly relevant to the lipoxygenases and their role in the production of "fresh" flavors is their stability. These enzymes as a class are remarkable for their *lack* of stability as catalysts. The lipoxygenases and the dioxygenases in general exhibit a coincidental self inactivation frequently termed suicidal. The cause is the product, in this case a hydroperoxide, which is the substrate for a secondary peroxidase reaction, which is (presumably artifactually) a self-destructive catalytic activity of the enzyme. The sensitivity of the lipoxygenase in fish gill to hydroperoxides is illustrated by the response to t-butyl hydroperoxide (Figure 6). This addition of this hydroperoxide irreversibly inactivates the enzyme. This inactivation by t-butyl hydroperoxides has been shown to be similar in both fish and mammalian lipoxygenases (40).

This sensitivity of the enzymes to product hydroperoxides and the instability of the enzymes in general leads to certain predictions for the role of these enzymes in the temporal aspects of flavor generation and especially to the production of "fresh" flavors. The unique pattern of flavors that results from the enzymatic activity in raw, healthy tissues will rapidly diminish as the enzymes deteriorate and autoxidative reactions predominate.

Similarly, variation among different species of fish would lead to distinct differences in flavor depending on the species alone. We have catalogued the activity of these enzymes across several species and evolutionary periods. Examination of the variation in products of the 12- and 15-lipoxygenases in fish gills across species and time illustrated several points relevant to flavor. Typical of these data are those shown by the summary figures following activity of the two enzymes in disrupted tissue stored at 0°C (Figure 7).

As typified by trout and carp, the two lipoxygenases are *not* equally distributed among fish species.

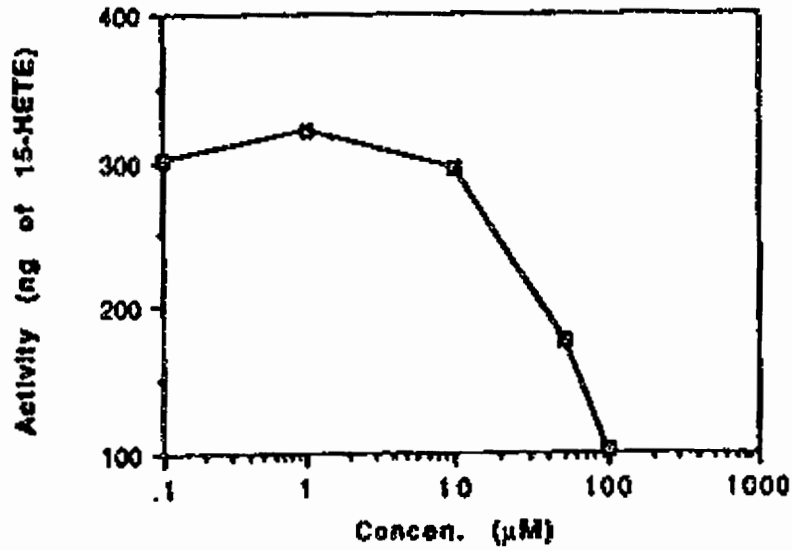


Figure 6. Effect of t-butyl hydroperoxide on the 15-lipoxygenase of sturgeon. Fresh homogenates of sturgeon gill were incubated for 10 min with the indicated concentration of hydroperoxide before addition of arachidonic acid. Activity was then determined by HPLC.

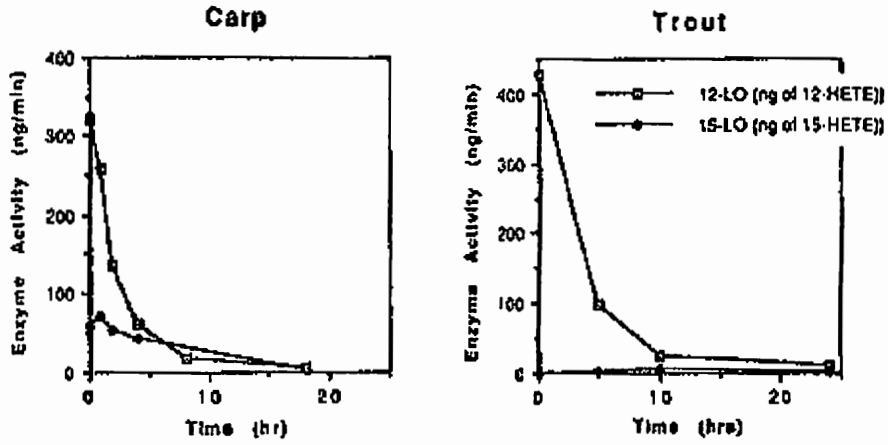


Figure 7. Lipoxygenase activity in carp and trout as a function of storage time at 0°C. After indicated times of storage the enzyme activity toward exogenous arachidonic acid was determined by HPLC as described.

Trout initially exhibited very high 12- and virtually undetectable 15-lipoxygenase activities. In carp, although the 12-lipoxygenase was most active, the 15-lipoxygenase was also relatively abundant. In sturgeon, a relatively ancient species, the 15-lipoxygenase was actually the predominant enzyme. Also evident from these data is the striking instability of these enzymes. The half time of the 12-lipoxygenase at 0°C. was less than 3 hours. The half time of the 15-lipoxygenase was greater than 10 hours. Thus, although the 12-lipoxygenase activity was predominant initially, in both carp and trout, after 24 hours of storage the 15-lipoxygenase was the major enzyme activity remaining.

These observations are potentially significant to the time course of flavor development in fresh fish tissues. Owing to the differences in incidence of the enzymes, fresh volatiles are predicted to differ between species. Even more important, the changing activities of the two enzymes with time will result in changing volatile patterns as the tissue ages.

As our knowledge advances, especially on the basis of instability of the enzymes and the subsequent lytic reactions, various technologies could make use of these enzyme systems in food applications. Their

activity is a sensitive index of the age and quality of the tissue. For example, a single rapid freeze-thaw cycle inactivates the enzymes. The lipoxygenases themselves or processing wastes in which they are present could be exploited as sources of marketable flavors for seafood products. As our understanding of the biogeneration of "fresh" flavors improves, even more opportunities will arise for maintaining this vital and elusive aspect of food quality.

Cooperating Organizations

Aquaculture and Fisheries Program, University of California, Davis
Facility for Advanced Instrumentation, University of California, Davis
National Marine Fisheries Service

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Abalone Wasting Disease: Role of Coccidian Parasites and Environmental Factors

University of California, Santa Barbara
R/F-135
1990–92

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To achieve a rapid, quantitative diagnostic procedure for black abalone (*Haliotis cracherodii*) withering syndrome (WS), we developed a fresh tissue smear technique to rapidly quantify life cycle stages of the coccidian parasite in both right and left kidneys of black abalone. We recognized oocysts, macrogamonts, and meronts in these smears. The parasite was present in all black abalones examined from the Channel Islands. Because this included all animals from San Nicolas Island, where there was, at the time of sampling, no evidence of either withering syndrome (WS) or of mass mortality, this did not support the hypothesis that the coccidian parasite was a cause of WS.

Before the death of diseased abalones, the foot becomes discolored and withers. Because we recognized that damage to the foot was the terminal pathologic indicator for a wide range of fatal ailments, much as loss of lung function is for humans, we initiated a study to detect earlier pathologic indicators to assist the recognition of WS at an earlier point in the disease process. To clarify this important issue we began a careful analysis of gross pathology, looking at the relative size of several organs (right kidney, gonad, digestive gland, foot, rest of viscera), as well as semiquantitative indicators of foot size, foot color, and the color and condition of the mantle margin. Using these variables, we were unable to detect a significant association between intensity of the coccidian and pathology. Thus, we conclude that this parasite is not the sole cause of WS and the associated mortality. If it is involved at all, a cofactor is necessary to permit the full elaboration of this disease.

To further clarify the pathogenic process, we began a principal components analysis (PCA) of relative organ weight. The PCA used randomly collected abalone and was designed to reveal earlier pathologic symptoms since study of WS was limited by use of terminal pathologic sequelae (the withered foot). We also recorded the color of the epidermis and the condition of the epipodium (mantle margin), as these also exhibit changes associated with WS. Our analysis included animals from San Nicolas Island (where no mortality had yet occurred), San Clemente Island, and San Miguel Island (where WS was then evident and populations were crashing), and Santa Cruz Island (where mortality had been catastrophic but is no longer occurring and few animals exhibit WS symptoms).

With the PCA we hoped to separate other biological processes associated with the "condition" of abalones from WS symptomology. Physiologic processes such as gonadogenesis required samples from healthy and diseased populations at times of both peak and minimal gonadogenesis. The relative growth and PCA studies showed that relative foot size varied between islands and the foot-to-shell ratio was significantly higher at San Nicolas Island. We confirmed that semiquantitative estimates of foot size in the field were reliable indicators of foot size and that the color and condition of the epipodium aided the reliable diagnosis of WS in the field.

The PCA did not succeed in further defining WS because there was actually little variation in foot weight compared with other variables such as gonad weight. In the PCA the first component, PC I, was interpreted as "size" because all loadings were of similar magnitude and sign. It accounted for 72% of the variance. The second component, PC II, was interpreted as "gonadogenesis" because gonad weight was contrasted with shell length (and also kidney weight). Twelve percent of the variance was included in PC II. Examination of the loadings on PC III revealed that this component represented "condition," as the loading of digestive gland weight contrasted with those of the kidney and gonads. Considering the results of the PCA, a discriminant functions analysis (DFA) was conducted by using foot weight for classification (dropping foot size as a variable). The DFA indicated that additional WS pathology was evident and included reduced gonad and digestive gland weight and hypertrophied right kidney weight. Animals from the islands with ongoing disease, San Miguel and San Clemente, had the smallest gonads.

The PCA was also used to investigate if coccidian intensity was associated with any pathology; whether or not this pathology was related to WS. To detect this association, coccidian intensity was added to the PCA as an additional variable with the data on organ sizes. Using coccidian intensity as such a probe enabled us to predict that PC I would still reflect size, as all variables but intensity would be of similar signs and magnitudes, and that PC II would be highly correlated with intensity. If so, the other strongly correlated variables would reflect pathology. The outcome of this analysis showed that PC I still represented size and that PC II represented coccidian intensity as predicted. Interpretation of the loadings on PC II showed that when intensity was high, the gonads were small. Further, PC II also represented intensity and yielded further information on pathology. For abalone with high intensities but whose gonads were ripe, the right

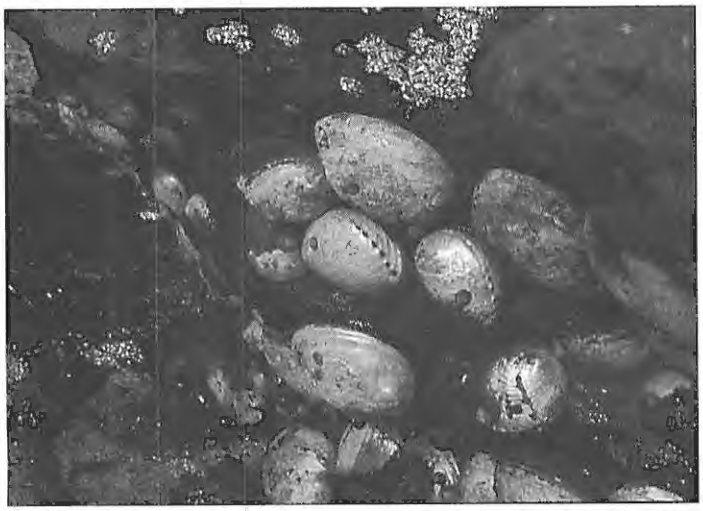

Figure 1. Intertidal black abalone abundance before mass mortality caused by withering syndrome (San Nicolas Island, November 1991)

Figure 2. Empty space and empty black abalone shells after mass mortality caused by withering syndrome (San Clemente Island, December 1991)

Figure 3. This photograph shows a healthy black abalone on the right (near the knife). Its foot is large (compared to the shell opening), color is dark and the animal is active, immediately trying to right itself. It had to be pried from the rock with an abalone iron. The abalone on the left exhibits withering syndrome and was easily detached by hand. Its foot is relatively small, its color is pale, and it is motionless. The third abalone on the bottom is in an earlier stage of withering syndrome. It shows some activity but its foot size is reduced and its color is pale.

kidney weight was low.

Our samples also yielded some additional information on the biology of the coccidian. Its intensity varied significantly among islands and was seasonal, being highest in winter samples. The abundance of coccidian life cycle stages in the left kidney generally reflected the abundance of stages in the right kidney.

To summarize the analysis of relative organ sizes, we concluded that, other than foot size, WS is hard to characterize; but negative effects on reproduction and condition were evident. Again, although the coccidian may be pathogenic, it is not likely to be the cause of WS.

Our field samples have provided some interesting information on other abalone parasites. An ectoparasitic pycnogonid, *Achelia chelata,* was consistently present on the surface of the foot and mantle of black abalone from Santa Cruz and Santa Rosa Islands, but not from San Clemente, San Nicolas, or San Miguel. The parasites make a distinctive lesion as they engage in suctorial feeding. The most commonly occupied sites are the tentacles and the epipodium. This is the first report of pycnogonids parasitic on abalones and only the second report of parasitism by sea spiders on a molluscan host.

Samples from San Clemente Island also revealed a low prevalence of larval gnathostome nematodes (*Echinocephalus pseudouncinatus)* (see Millemann, 1951). As ingestion of these living worms may cause *larval migrans* in humans, we recommend that black abalone from San Clemente Island not be used for ceviche, sushi, or other raw or undercooked preparations.

A translocation study moving healthy black abalone from San Nicolas Island to San Miguel Island and Anacapa Island (postmortality) was initiated in cooperation with Friedman, California Department of Fish and Game (CDF&G), Davis, Channel Islands National Park (CINP), Haaker (CDF&G), and VanBlaricom, U.S. Fish & Wildlife Service, (USF&WS) in the winter of 1992. The study is being conducted as part of a Master's thesis by Joan Ruediger at UC Santa Cruz. This experiment is designed to confirm whether the disease can be elicited in healthy abalone not previously exposed to the conditions present where mortality is now occurring. Healthy abalone on San Nicolas were individually tagged and translocated to Santa Rosa Island (where there is ongoing disease), San Clemente Island (ongoing disease) and Anacapa (epidemic). Other tagged abalone were translocated to other locations on San Nicolas Island to control for handling. To date the transplanted abalone have experienced a high mortality rate. They are dying at the same rate as the controls, however, suggesting that they have not acquired WS at the "diseased" locations. Dispersal, tag loss, and predation appear to account for most of the losses.

This study was further complicated by the onset of WS at San Nicolas Island in the summer of 1992. The WS symptoms affect up to 12% of the abalone at some locations on San Nicolas Island. Observations in October 1992 indicate that mortality has now increased at these locations.

Because the investigations of individual abalone from a parasitological and pathologic perspective have yielded distressingly little insight into the cause of WS, we (with Dr. Kevin Lafferty, UC Santa Barbara), considered that an epidemiological approach might permit us to test some of the hypotheses concerning the cause of the rapid decline in black abalone abundance (Lafferty and Kuris, 1992). To aid this process we attempted a Geographic Information Systems (GIS) analysis and acquired additional extramural support for this effort from the National Marine Sanctuaries Program. To enable this approach, the several agencies and individuals who have sampled black abalone on a long-term basis generously contributed their data (biologists at CNIP, USF&WS, CDF&G, Oregon State University, Pacific Gas & Electric Company, and the logs of several commercial fishermen). These data sets were prepared so that the spread of WS and associated mortality could be visualized and then associated with the several environmental cofactors that have been hypothesized as causative elements. The use of true GIS-type technology proved to be unsuitable and cumbersome as changes through time were not easily analyzed. Using more traditional procedures, however, we were able to test several hypotheses and provide bounds for some of the probable attributes of the still unknown causative agent (Lafferty and Kuris, 1993).

The decline in the black abalone population has been attributed to overfishing or WS. Suggested causes of WS included El Niño (or other causes of seawater temperature change; Tissot,1990), pollution, and infectious disease. Human activities may have spread WS from island to island.

If the fishery were responsible we would predict that fishing intensity and abalone density would be negatively associated. Further, the mortality rate should be higher for abalone larger than the legal size limit compared with the mortality rate for sublegal-sized abalone. We rejected overfishing as a cause of the die-off because there was a significant positive association between date of the die-off and the landings from each site. Additionally, at most sites, sublegal-sized abalone died at higher rates than did the larger animals. The cause of the mass mortalities seems to be WS, as abalone exhibiting this syndrome were always common at all sites where the die-off ensued.

If El Niño conditions were responsible, we expected die-offs to occur during warm years. Because El Niño reduces kelp abundance (the main food of black abalone), we might also expect a positive association between abalone and kelp density. Delayed effects might cause a time lag in the pattern of such associations. Die-offs did not occur more frequently during warmer than average years. However, high water temperatures in a given year were associated with higher mortality rates, and die-offs were more rapid at warmer locations. There was no

significant association between abalone and kelp abundance. Thus, we conclude that El Niño and associated environmental conditions such as food shortages were not related to WS or the rapid die-offs (mass mortalities).

The spread of WS from an initial focus would be indicative of either an infectious disease or a point source of a pollutant. These causes may be distinguished, as dilution would reduce the mortality rate at distant locations if pollution from a point source were the cause.

In 1985 WS was first observed by commercial fishermen on the south coast of Santa Cruz Island. We detected a significant association ($r = .698$, $p < .01$) between the date of the die-offs and the distance of sites from the south coast of Santa Cruz Island but mortality rates did not decline with distance. Thus, we reject the hypothesis that a pollutant from a point source caused WS. This correlation was strengthened when the direction of the prevailing current was included in the analysis. The disease spread more rapidly to down-current locations.

Our data are most consistent with an infectious agent as the cause of WS. Strong support for the infectious disease hypothesis comes from the geographic pattern of the spread of the disease and the increased mortality rate at elevated temperatures. However, the rate of mortality was not associated with abalone density at the various sites. Coupled with the inability to transmit the disease via contact to uninfected abalone in the laboratory (Haaker et al., 1992), this suggests that WS is not directly transmitted between abalone. Thus, an intermediate host, a vector, or a dormant stage may be required. The detection of such an infectious agent still eludes us.

The lack of a positive association between dates of die-offs and distances from major ports, as well as the lack of a negative correlation between dates of die-offs and fishing intensity, suggests that human activities are not directly responsible for the spread of WS.

In terms of practical applications, our studies provide no evidence that cultured abalone of other species are affected by WS and the presence of the coccidian in cultured abalone is not a cause for concern. The fishery does not appear to be implicated in either the cause or the spread of WS. Black abalone from San Clemente Island contain a significant human pathogen, *Echinocephalus pseudouncinatus,* and should not be consumed raw or in undercooked preparations such as ceviche or sushi. Our geographic analyses of causes of WS provides a model for the investigation of causes of other marine mass mortalities.

Cooperating Organizations

Ab Lab
California Department of Fish and Game
Channels Islands National Park
Commercial Fishermen
National Marine Sanctuaries Program
Nature Conservancy
Pacific Gas & Electric
U.S. Fish and Wildlife Service
U.S. Navy

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Lafferty, K. Geographic patterns in the decline of the black abalone. Annual Meeting of the Ecological Society of America, Honolulu, Hawaii, August 1992.

Northern California Red Sea Urchin Fishery

University of California, Davis
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The northern California fishery for the red sea urchin, *Strongylocentrotus franciscanus*, developed from a negligible level in 1984 to 13.8 × 10^6 Mt in 1988. The initial size limit of 75 mm was increased to 87 mm in 1990, and has been closed during the month of July to limit effort in the past few years. Since 1988, catch and catch-per-unit-effort (CPUE) in the fishery have declined to about half of their former values, and densities observed in fishery independent surveys have declined even more (Kalvass, 1992). The similarity between this fishery and patterns of decline in other urchin fisheries in Japan and the Caribbean has been a cause for concern (Tegner, 1989).

Adequate recruitment of red urchins may depend critically on the presence of adults through two different "Allee" mechanisms. First, a minimum density of adults is required for successful fertilization of gametes, which are spawned into the water column (Levitan et al., 1992). Second, adults provide a refuge from predation for young recruits beneath their spine canopy (Tegner and Dayton, 1977).

The formulation of rational management has also been hampered by the fact that growth and mortality rates were virtually unknown. One source of data for these rates, size distributions, varies in a way that was not understood. Size distributions for this species vary with location, typically having a single mode at larger sizes but occasionally having an additional mode at lower sizes. The presence of this mode has been associated with the spine canopy refuge mentioned above (Tegner and Levin, 1983). Because of both alongshore and cross-shelf variability in food resources (mainly kelp) and flow fields, growth and reproduction vary spatially. Mortality rates also vary with location due to differences in predators, including spiny lobsters, sheephead, sea stars, and sea otters.

Our research on this fishery has been aimed at understanding the population dynamics of this species and using that understanding to better manage the fishery. Our original approach, begun two years ago, was to measure the response of small experimental plots to different harvest policies. This approach of direct observation did not work well because urchins migrated into the experimental plots almost immediately after harvest. Because we were unsuccessful in finding a way to exclude them, and the Bodega Marine Laboratory got rid of the research vessel that we had been told we could use for the experimental harvest, we adopted a different approach. The new approach was to develop a method for estimating growth and mortality rates from size distributions and to use those estimates to determine the response of the population to various harvest policies. We are also taking benthic samples in harvested and unharvested areas to determine the effect of harvest on settlement and postsettlement survival. To obtain a more comprehensive understanding of the complete process of recruitment, we are sampling gonad indices throughout the year, obtaining efficiency of recovery data from the industry, and sampling settlement of juveniles on brush collectors.

Patterns of Size Structure

Formulation of management policy requires good estimates of growth and mortality rates, as well as knowledge of the mechanisms underlying broadcast spawning and protection from predation via the spine canopy refuge. For this species, the major source of data on which estimates of growth and mortality rates could be based is the size distributions of both harvested and unharvested populations. The occasional occurrence of bimodality in size distributions, coupled with observations of juveniles beneath the spine canopies of adults, led to the proposal that these phenomena were related (Tegner and Levin, 1983). To allow us to formulate a method for estimating growth and mortality rates, as well as to possibly learn something about the spine canopy refuge, we analyzed the way in which mortality and growth patterns shaped size distributions (see Barry and Tegner, 1990).

We used the size-structured version of the von Foerster equation to describe how the shape of the size distribution changes with time (Botsford et al., 1994). This expression shows that whether the slope of the size distribution is positive or negative will depend on the difference between the mortality rate and the rate of change of growth rate with respect to size (see Van Sickle, 1977; DeAngelis and Mattice, 1979). Size distributions decline with size when the mortality rate is greater (mortality dominated) and increase with size when the rate of change of growth rate with size is smaller (growth dominated).

We then relaxed the assumption of constant recruitment, allowing recruitment to be annual pulses, and added random variability among individuals (see Sainsbury, 1980). Basically, pulsed recruitment simply transformed the size distribution into pulses that were more closely spaced at larger sizes. Random variability in the growth parameter k of a von Bertalanffy growth expression smeared the distribution at lower sizes, obscuring the pulses at lower sizes, while random variability in maximum size L_∞ smeared the distribution at higher sizes and widened the upper mode in growth-

dominated populations.

From this analysis we concluded that the common mode at higher sizes in red urchin size distributions was due to random variability in L_∞ in a growth-dominated population. Because the addition of a mode at lower sizes requires a size range of decreasing slope (i.e., the upper half of the mode), we concluded that the presence of this mode could not be completely due to the addition of spine canopy protection. It had to involve higher mortality rates at intermediate sizes. The lower half of the mode could be due to lower mortality from the spine canopy, but it is near the size range at which selectivity of the sampling process affects the size distribution (i.e., divers probably have difficulty finding urchins smaller than 10 to 15 mm).

A New Method for Estimating Growth and Mortality Patterns

We used this description to develop a maximum likelihood method for estimating growth and mortality rates from size distributions and growth increment data (Smith et al., 1992). Because measurement of size is usually less expensive than determining age, size distributions can be an important source of information in fisheries. Several methods have been developed for estimating growth and mortality parameters from size distribution data, but virtually all of them involve some means of extracting individual cohort pulses from a mixture of them, then estimating mortality and recruitment rates from the resulting age structure. These would not work for the urchin because the size distributions do not contain cohort modes, except an occasional recruitment pulse at small sizes.

We formulated a method for estimating growth and mortality rates from size distributions that do not exhibit cohort modes (Smith et al., 1993). Estimation of L_∞ was possible using this method, but estimation of k, variability in L_∞, and mortality rates required that we incorporate the use of size increment data in this method. The size structured von Foerster equation given above was used to generate the size distribution. For the criterion function we used the sum of Schnute and Fournier's (1980) separation statistic for the size distribution data and the likelihood function corresponding to a Gaussian distribution for the increment data. We tested the sensitivity of bias, standard error, and estimated standard error to sample sizes, variability, and model characteristics with Monte Carlo simulations. The method was relatively robust with respect to reasonable sample sizes, levels of error, and model type. It typically estimated mortality rates, L_∞, variability in L_∞, and parameter k with biases on the order of 5 percent or less and standard errors on the order of 10 percent or less (e.g., Figure 1).

We used the method to estimate von Bertalanffy growth parameters with variable L_∞, and mortality rates (Table 1) (Smith et al., 1992). Because we do not yet have reliable growth increment data, we used growth increments from a laboratory study in which individuals were fed macroalgae *ad libidum* (unpublished data). We first applied this method to a size distribution from an unharvested site to estimate growth parameters and natural mortality rate (Figure 2a, Table 1). We then fixed natural mortality and growth parameters to estimate fishing mortality rate from a size distribution collected from harvested sites (Figure 2b, Table 1).

Control of Effort by Rotating Spatial Harvests

Although pulses of juvenile settlement are occasionaly still observed in northern California, measured densities of adult spawners are below the spawning threshold implied by the measurements described above. A fine scale comparison between harvested and unharvested areas in 1991 showed total urchin densities of 0.3 m^{-2} and 7.0 m^{-2} , respectively (Kalvass, 1992). Broad scale surveys in harvested areas showed a decline in

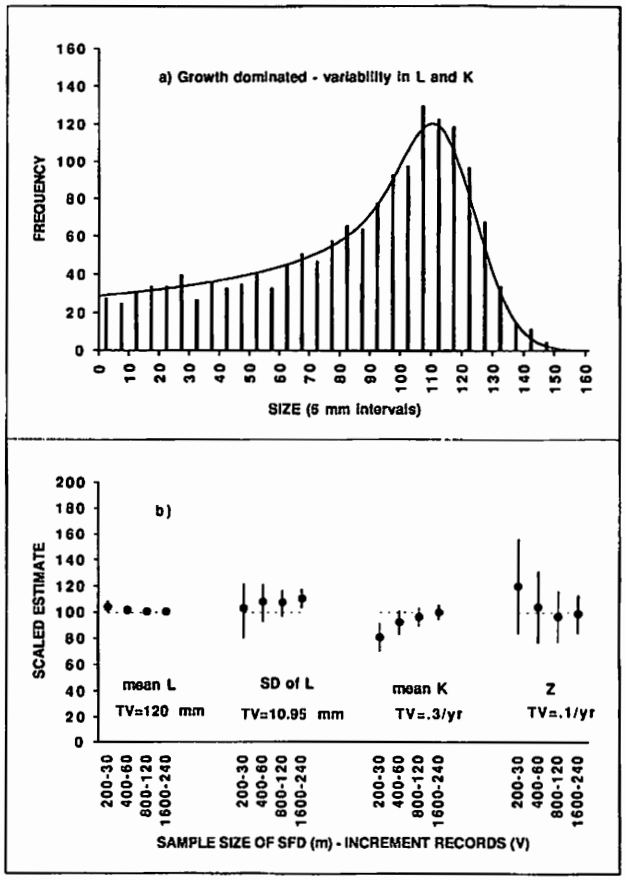

Figure 1. An example of the Monte Carlo evaluation of the estimation method in Smith et al., 1992. (a) The size frequency from which estimates were made. (b) Estimates and standard errors for various sample sizes for the size frequencies and the growth increments.

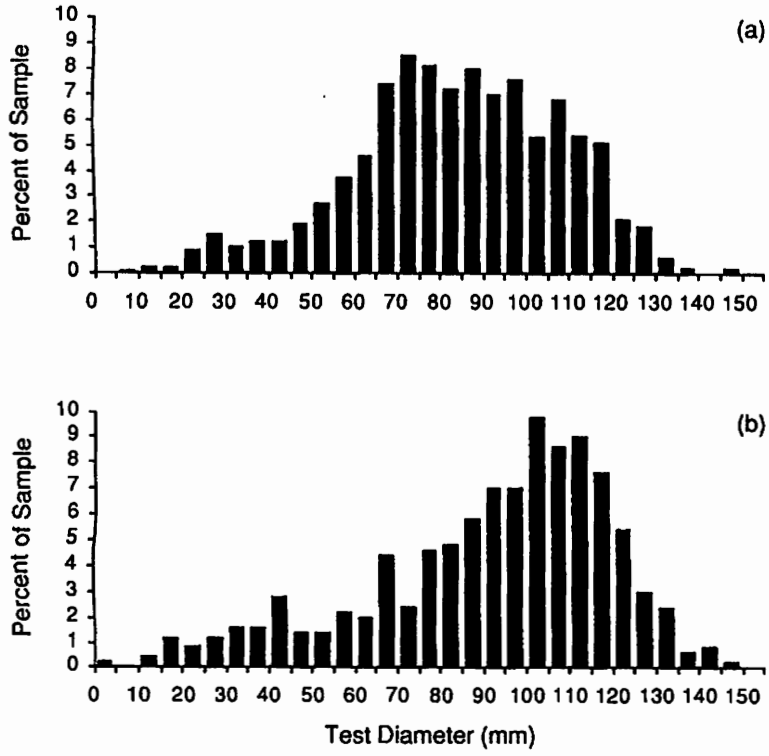

Figure 2. Size distributions of red sea urchins from harvested sites (a) at an unharvested site, the Point Cabrillo Marine Reserve; (b) (from Kalvass et al., 1991). The size measurement is test diameter.

Table 1. Estimates of Fishery Parameters from Size Frequency Data in Figure 2 and Growth Increment Data from Our Laboratory Growth Experiment*

Mean L_∞	112mm
Standard Deviation of L_∞	15 mm
von Bertalanffy Parameter, k	0.48 yr^{-1}
Natural Mortality Rate	0.14 yr^{-1}
Fishing Mortality Rate	0.25 yr^{-1}

*Using the methods in Smith et al., 1992

the density of individuals greater than 30 mm (the minimum size of spawning) from 1.2 m^{-2} in 1988 to 0.6 m^{-2} in 1991 (Kalvass, 1992).

Although fishing effort in general can be controlled in several different ways (e.g., limited entry, individual transferrable quotas), for sessile or nonmigratory species spatial management is also a possibility. A system of rotating spatial harvests can effectively limit effort at any one location, by allowing it to be harvested only occasionally. Such a scheme can be viewed as pulse fishing, but it also maintains constant fishing opportunity by having a constant number of areas available for harvest at any one time. The state of Washington uses such a system in its urchin fishery. Each area is typically opened for 6 months of harvest once every 3 years.

Values of yield-, reproduction-, and refuge-per recruit were computed from a simulation model that operated on a monthly time step. In the model, specified recruitment was distributed over several months in the spring at a mean size of 5 mm test diameter. Growth in terms of test diameter was computed from a von Bertalanffy function with parameter $k = 0.48\ yr^{-1}$, a small amount of variability in k, and variable dispersion which essentially mimicked variability in L_∞. Although reproduction, yield, and price vary seasonally, we have not yet represented this in the model, primarily because our initial focus was on the effects of interannual variability in harvesting. Refuge provision was computed from the size distribution at the time of settlement in each year (assumed to be August) as a specified fraction of the area covered by the spines. To compute total reproduction, gonad weight was computed from a typical value of prehatch gonado-somatic index (0.35) and the size distribution in March each year.

Because we had direct information on the dependence of spawning on density of reproducing adults, we also computed spawning density. Total numbers greater than 30 mm test diameter, the minimum size of spawning, were multiplied by a calibration factor based on actual observed spawning densities. The calibration factor was the ratio of the computed total number of spawners at the current lower size limit and fishing mortality rate to the current observed spawning density, 0.6 m^{-2} (Kalvass, 1992). To illustrate the use of rotating spatial closures, we chose 0.7 m^{-2} as the minimum density at which successful spawning occurs. Egg production was set to zero below this value.

The resulting plot of yield-per-recruit versus lower size limit and fishing mortality rate had the standard form, with a maximum at infinite fishing mortality rate and the size of maximum unfished biomass (Figure 3a). Egg production-per-recruit and refuge per-recruit were both similar to the egg-per-recruit plots typically obtained (Figures 3b and 3c), except that egg production was set to zero for spawner densities less than 0.7 m^{-2}. Above this value egg production and refuge increased as either fishing mortality rate or lower size limit decreased. Note that in going from the current operating point (point A in Figure 3a) to no harvest, spawning density varies by only a factor of 2, while the data comparing harvested areas to unharvested areas varies by a factor of 10. This may be due to aggregative distributions and fishermen harvesting denser aggregations first. One of the implications of this is that the values of spawning density in Figure 3d probably correspond to higher actual densities as density increases.

Because we are at a low or at least marginal spawning density, it would be desirable to change policy

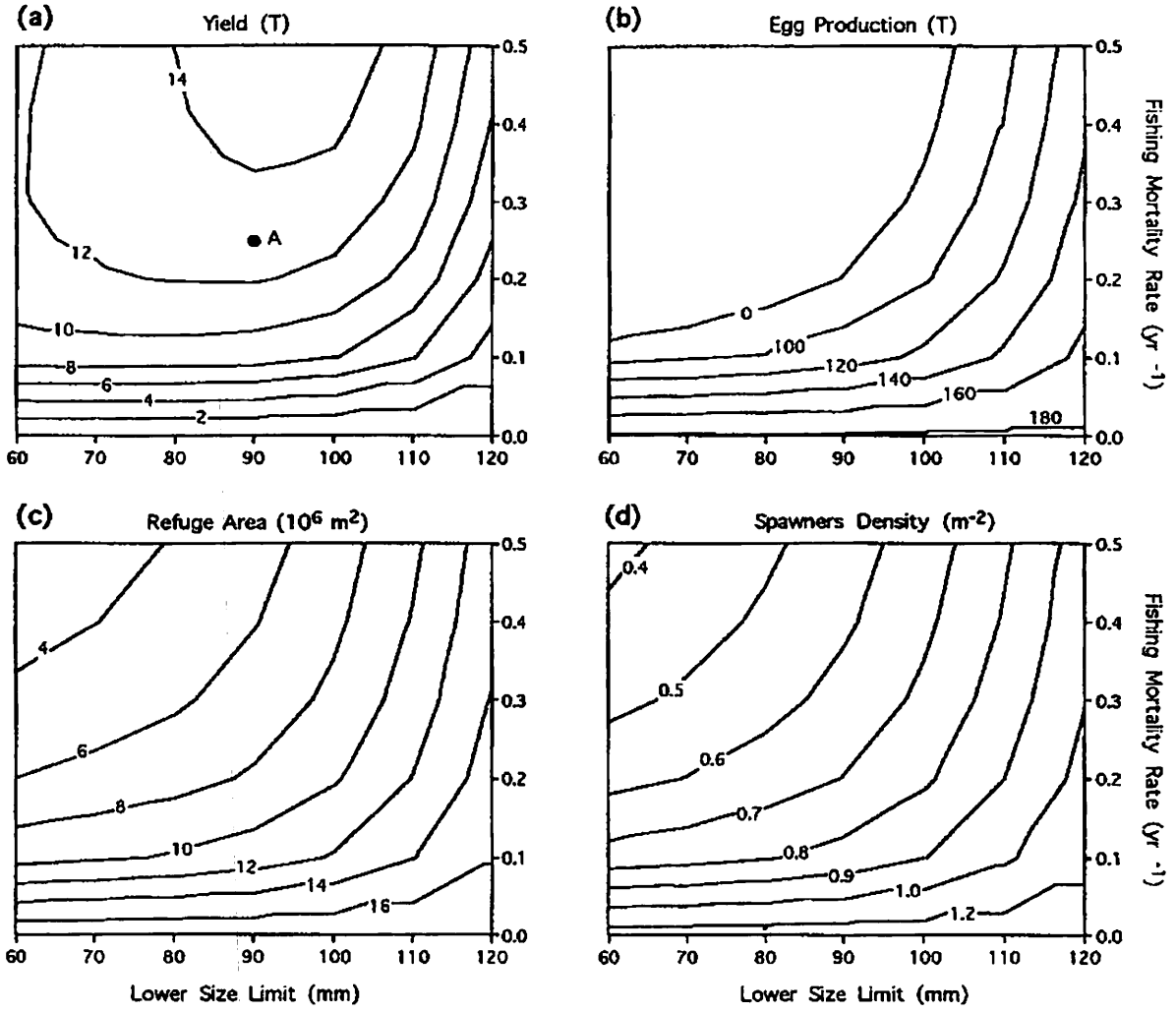

Figure 3. Yield (a), egg production (b), total refuge area (c), and spawner density (d), versus lower size limit and fishing mortality rate from a model of the northern California red sea urchin fishery. Recruitment was adjusted to give the current yield at the current lower size limit and fishing mortality rate (point A). Spawner density is calibrated to be the observed density at the same point. Egg production is set to zero when spawner density less than 0.7 m^{-2}.

from point A so that spawning density was increased. A change in policy that would increase spawning density would involve either further increase in the size limit or a decrease in effort. Both would decrease yield substantially. The latter would require a control on effort through transferable quotas or a similar device. As a possible means of avoiding the additional infrastructure associated with such a change, we investigated the potential advantages of periodic harvest.

To determine the effect of controlling effort through rotating spatial harvests, we computed average annual yield-per-recruit and average annual egg production, assuming that harvest occurred every 2, 3, 4, and 5 years (Botsford et al., 1993). Egg production was set to zero in years in which the spawner density was less than the threshold of 0.7 m^{-2}. How pulse fishing works depends on the response of fishermen, that is, whether they drop out of the fishery or fish less, for example, and ultimately on the consequent effect of this behavior on harvest mortality. The most parsimonious means of depicting harvest mortality with no prior knowledge of harvester behavior is to assume that the stock will be harvested down to the point at which it is no longer profitable to harvest. We made this assumption, and furthermore, we assumed that the population level at which harvest is no longer profitable is just below the current level.

The results of periodic harvesting with this description of harvester behavior are shown in Figure 4, with a rotation period of 1.0 corresponding to constant harvest. Assuming the current lower size limit of 90 mm, as we increase the period of rotation, yield declines moderately, but average egg production increases substantially. Note that for the current size limit (90 mm), as the period of rotation increases from 1.0 (i.e., fishing every year, as is now done), egg production goes from 0.0 to 60 metric tons. This happens because the population in each location is allowed to increase above the minimum spawning density of 0.7 m^{-2} (Figure 5). Rotating spatial harvests have a different effect on population structure than size limits or seasonal closures, resulting in

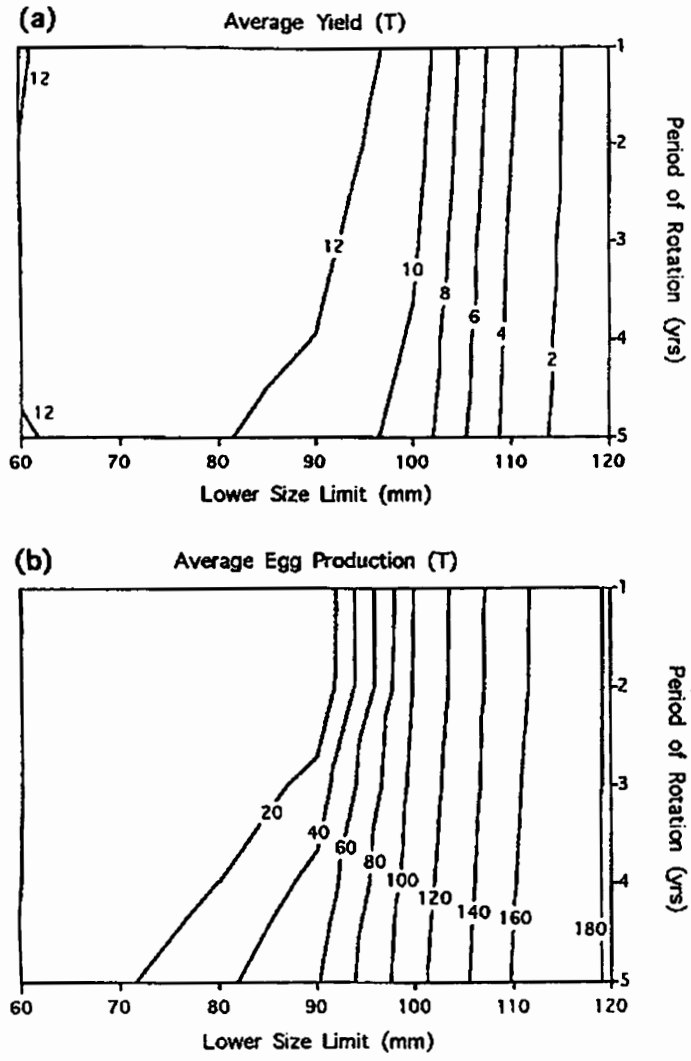

Figure 4. Average (a) yield, and (b) egg production, from the same model as in Figure 3 when the population is harvested in a rotating spatial closure scheme with various periods of rotation. Period 1 corresponds to constant harvest. The harvest continues each harvest year until the population is reduced to the biomass escapement that would correspond to constant effort of 0.26 yr^{-1} and lower size limit is 90 mm.

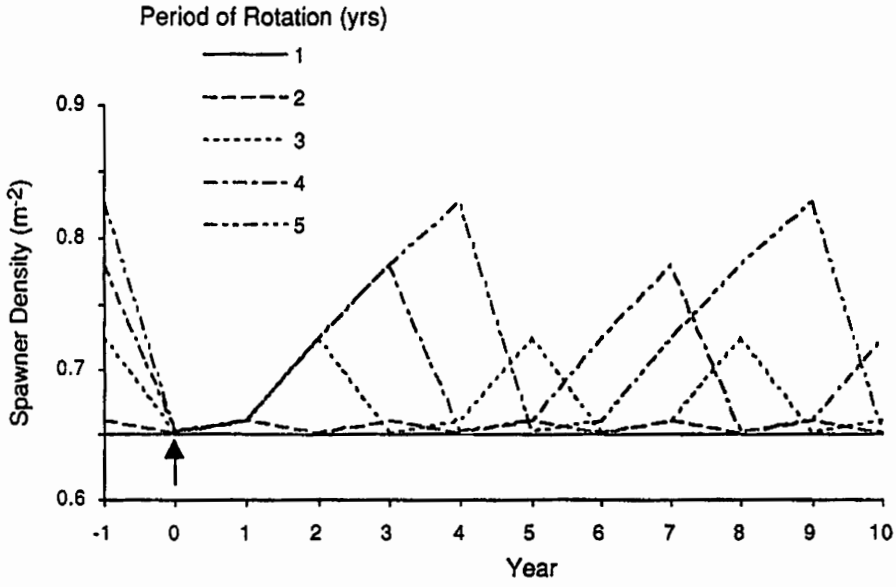

Figure 5. Spawning density under a management scheme of rotating spatial closures. Note that the population is above the threshold below which fertilization does not occur in the model (0.7 yr^{-1}) for progressively longer periods of time as the period increases.

alternating periods of high and very low density rather than a more constant density. The effects on overall recruitment depend on the relationship between adult density, and reproductive success. In particular, if reproductive failure occurs below some intermediate density, closure areas that are occasionally at high densities may contribute more to recruitment than areas managed conventionally with the same yield.

Recruitment

Our study of recruitment involved measurements at three points in the early life history. First, we collected monthly samples of gonad indices along a depth transect near Bodega Marine Laboratory. The results showed a sharp drop near the time of spawning (Figure 6), a result we have verified over broader space using data from a processor.

Second, we monitored recruitment of juveniles to Subsurface Collectors for Recruitment of Urchins to the Benthos (SCRUB) brush collectors suspended 1 m off the bottom at four different locations (about 100 km). We found little settlement in 1991 but substantial settlement in 1992 (Figure 6). We are currently analyzing these data in conjunction with physical data to determine how physical conditions influence urchin settlement.

Third, we are obtaining photo transects and air lift samples at several locations along the coast, in both harvested and unharvested areas. Preliminary indications are that postsettlement survival is less in areas with no harvest.

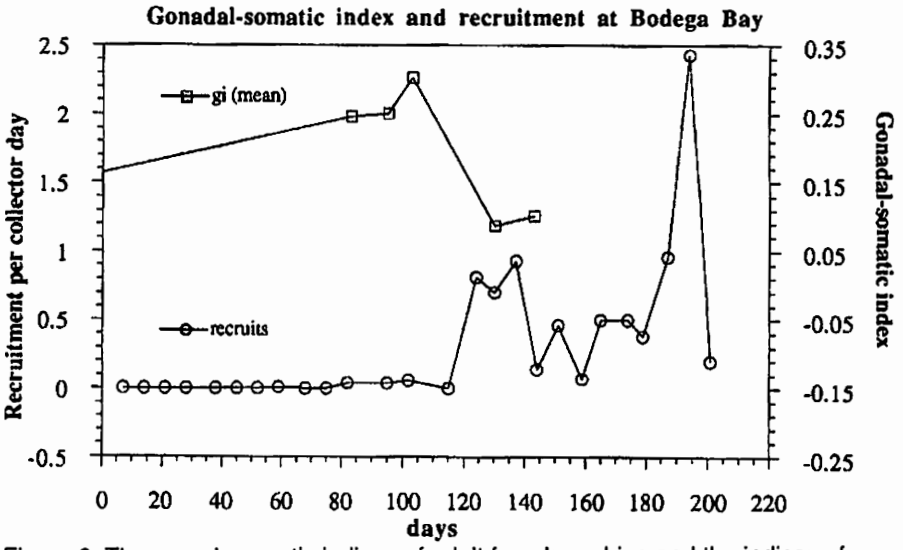

Figure 6. The gonadosomatic indices of adult female urchins and the indices of juvenile recruitment from SCRUB brush collectors at Bodega Bay.

Cooperating Organizations

California Department of Fish and Game
Catalina Offshore Products

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Variation in Coastal Pelagic Fish Populations Over the Past Millennium as a Response to Global Climatic Change and Biological Interaction: A Perspective from the Paleosedimentary Record

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R/F-143
1991–93

Introduction

Population sizes of small schooling fishes in the California Current have undergone significant changes during the last century. Investigation of the causes and even of the actual time scales of this variability is difficult, if not impossible, using traditional approaches. The principal goal of this project was to determine the fundamental time scales, and to examine possible causes of the variability in population sizes using "nontraditional" sources. These are the indirect record of abundances based on fish-scale accumulation found in sediments from a southern California coastal basin. Formation of the annually layered (varved) sediments in the anaerobic Santa Barbara Basin (Fig. 1) provides a memory of ongoing processes.

This project was designed around the retrieval and analysis of a high-quality, 2-m-long, Kasten core to reconstruct fish-scale deposition rates over the past 1000 years, with adequate resolution to define changes over periods as short as 10–15 years. Our overall objective was: (a) to determine the fundamental time scales of variability in coastal pelagic fish population sizes, and (b) to investigate the underlying regulatory mechanisms imposed by large-scale/global climatic change and internal population controls through interaction among and within the major species.

The existing data set of sardine and anchovy scale-deposition rates (SDRs) which was available at the beginning of this Sea Grant project has been plotted in Figure 2. These series extend from AD 270 to 1970 and are resolved into 10-year sample blocks. We began this

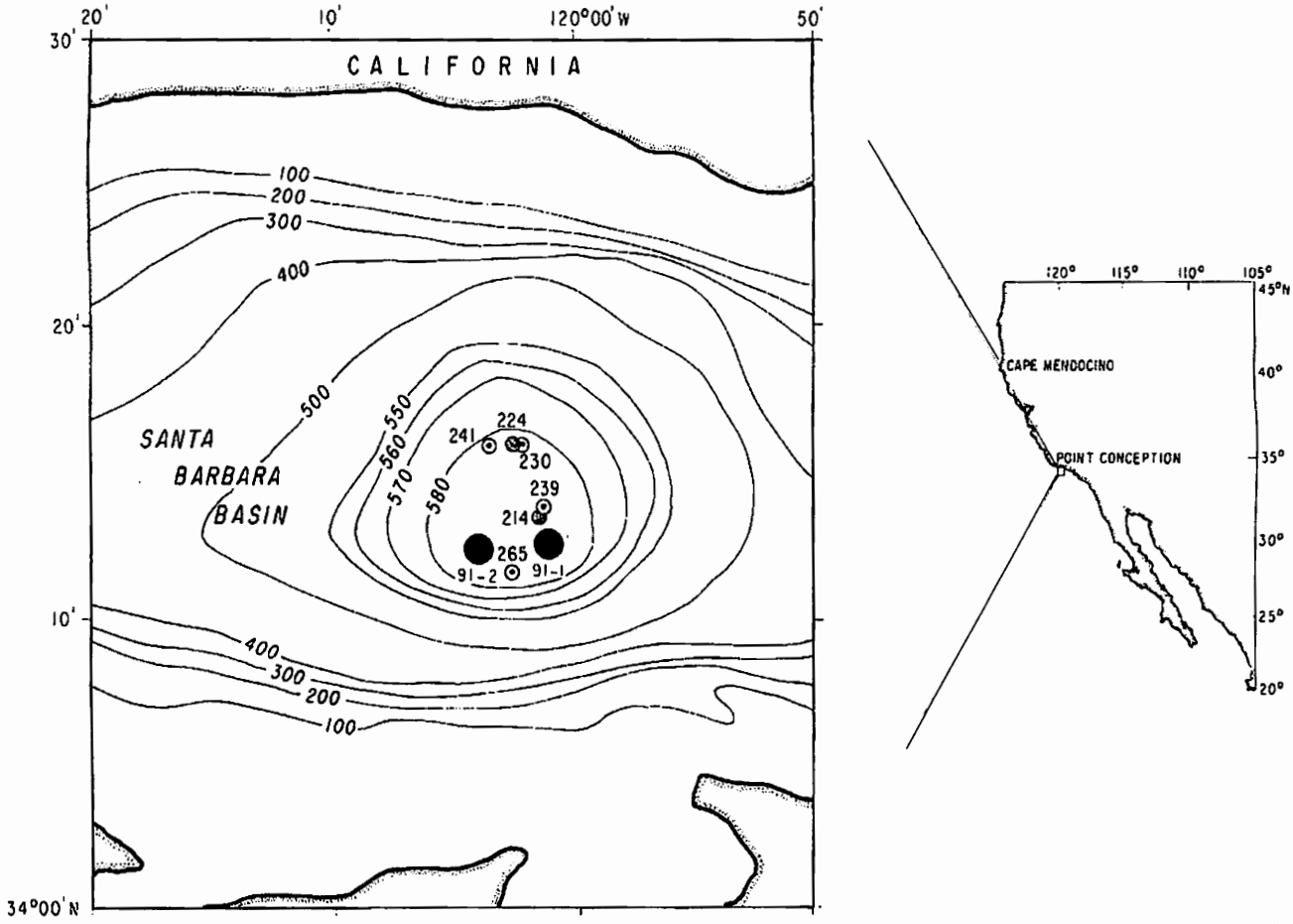

Figure 1. Location and bathymetry of the Santa Barbara Basin with positions of core sites mentioned in text. The larger darkened circles indicate the two sites (91-1 and 91-2) cored from the R/V *New Horizon* during October 1991 to collect material for this project: one Kasten core and one box core were recovered from each site. The existing 1700-year time series published in Baumgartner et al. (1992) was constructed from a composite of two piston cores shown by the smaller darkened circles (214 and 224; for data below 1810) and four box cores taken from the sites shown by circles with dots (230, 239, 240, 265; data above 1810).

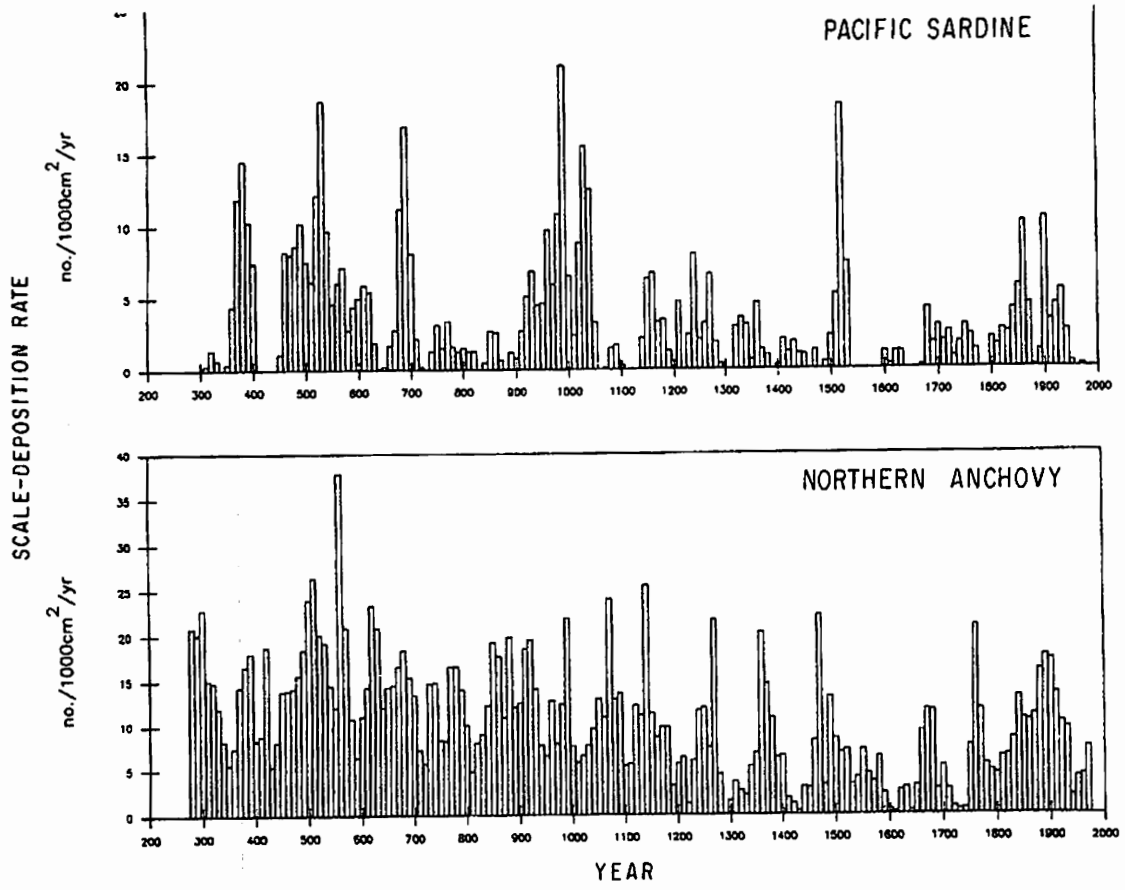

Figure 2. Time series of the Pacific sardine and northern anchovy scale-deposition rates (SDRs) developed from piston cores 214 and 224 and box cores 241, 230, 239, and 265 (from Baumgartner et al., 1992).

project with the development of an improved calibration of the SDR values plotted in Figure 2. This calibration is based upon regression of the scale-deposition rates on available biomass estimates of specific geographic stocks (Fig. 3). We then calculated the spectra on the detrended data (two right plots, Fig. 4). By separating the higher frequencies (periods <150 years length) from the total variability (two left plots, Figs. 4, 5), we were able to more clearly distinguish periods of approximately 60 and 70 to 80 years in the spectra for both species (two right plots, Fig. 5). These results were published in Baumgartner et al. (1992) and provide a summary of the temporal scales of variability in the existing SDR data for sardines and anchovies.

Recovery of New Core Material

During October 1991, we collected two Kasten cores and two box cores from the Santa Barbara Basin aboard the R/V *New Horizon*. One Kasten and one box core each were taken from sites labeled 91-1 and 91-2 in Figure 1. (It is necessary to take the shorter box cores in conjunction with Kasten cores in order to ensure that one obtains an undisturbed surface with the upper 100 years intact). Figure 6 compares the total area of depositional surface available (plotted against calendar years) to reconstruct the existing time series of SDRs published in Figure 2, and the amount of surface area sampled by the two Kasten and Box cores.

The length of the Kasten cores (2.58-m and 2.64-m long) exceeded our expectations, reaching sediment deposited approximately 1600 years ago. As a result, we altered our original objective and reconstructed a 1000-year series beginning at the bottom of the core and working upwards towards the present. Work on the upper 600 years has continued with our current Sea Grant project, which builds on this work with reconstruction of a more complete and accurate record of both fish scale deposition and critical environmental parameters over the past 1600 years.

Development of the New Scale-Deposition Time Series

Development of the new SDR time series was begun with Kasten core 9110-1301 (91-1 on Fig. 1). The sediment column of this core was sliced into uniform thick and thin slabs to provide replicates of high-resolution X-radiographs (two 1-cm-thick slabs), and for separation and counting of fish scales (four 2.5-cm-thick slabs). The first step in developing the chronostratigraphy was to identify and count the individual varves (the annual layers composed of a pair of seasonal laminae) on the radiographs, beginning at the bottom of the core. Our resulting varve chronostratigraphy is still preliminary because

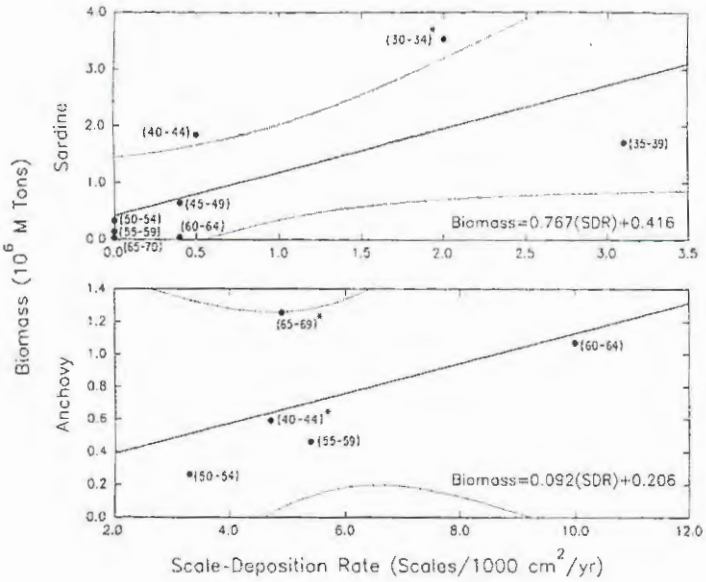

Figure 3. Calibration of SDRs in Figure 2 by regression of population biomass estimates of 2-year and older Pacific sardine and northern anchovy (5-year averages). Curved lines show the 95% confidence interval around the regression line (from Baumgartner et al., 1992).

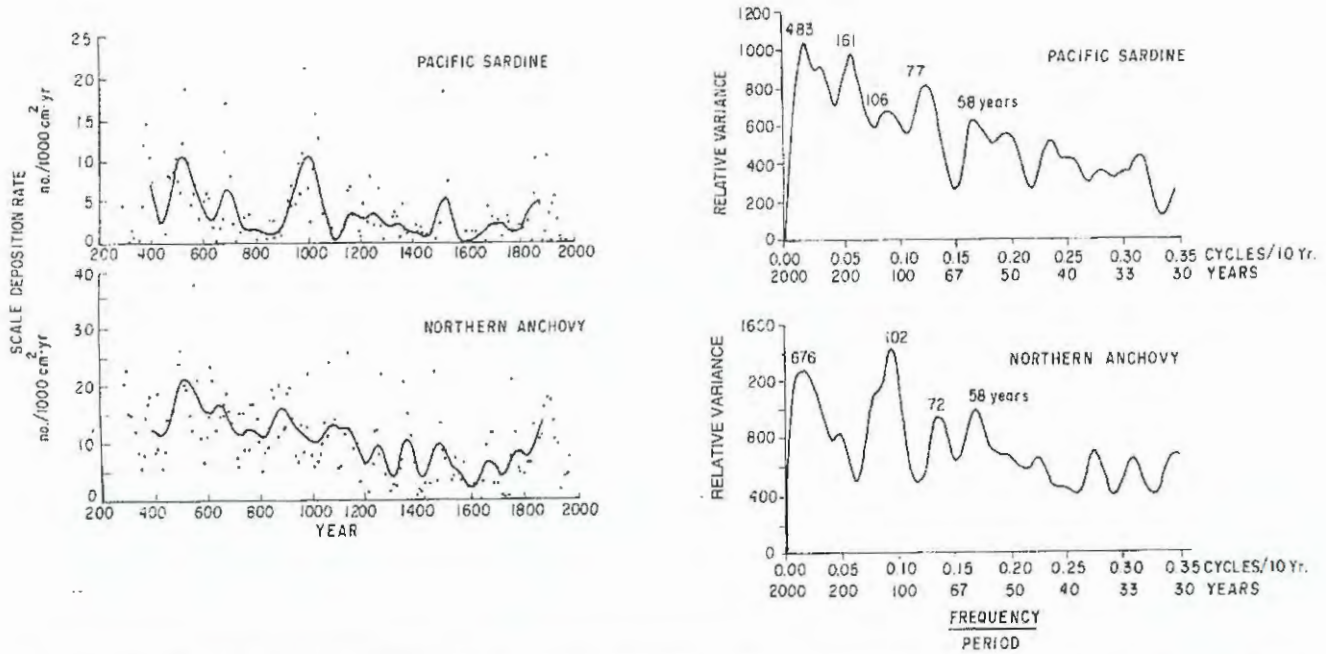

Figure 4. Two left plots are time series of the low-frequency components (continuous curves) of sardine and anchovy SDRs calculated by low-pass filtering data of Figure 2 (data points). The two right plots are smoothed power spectra of the sardine and anchovy SDRs after removal of linear trend from each series of Figure 2. Published in Baumgartner et al., 1992.

of the uncertainty resulting from the presence of poorly laminated and homogeneous sections through which the varve counts had to be extrapolated. This preliminary effort, however, is a major accomplishment in itself and an important contribution to our continuing refinement of sediment dating.

We sampled the four thick slabs up through the approximate calendar year of AD 1400 for fish scale analysis. Sample boundaries of 5-year intervals were identified from the radiographs of the thin slabs. The fish scales were concentrated for identification and counting by disaggregating and washing the samples through a 500-µm sieve. The processed sample was stored in buffered ethanol. Two hundred and six samples (of 5-year intervals) were prepared and sieved from three slabs (designated 1, 4, and 6) within the Kasten core, giving a total of 718 samples prepared. These samples make up three replicate sequences of observations over a period of 1030 years, running from approximately AD 400 to 1430.

The fish scales were examined and counted in a plastic counting tray using a Wild M5A stereo microscope. Figures 7, 8, and 9 are histogram plots of the time series of the sardine anchovy and hake scales. Besides these three species, the only others with sufficient abundance for a separate count,

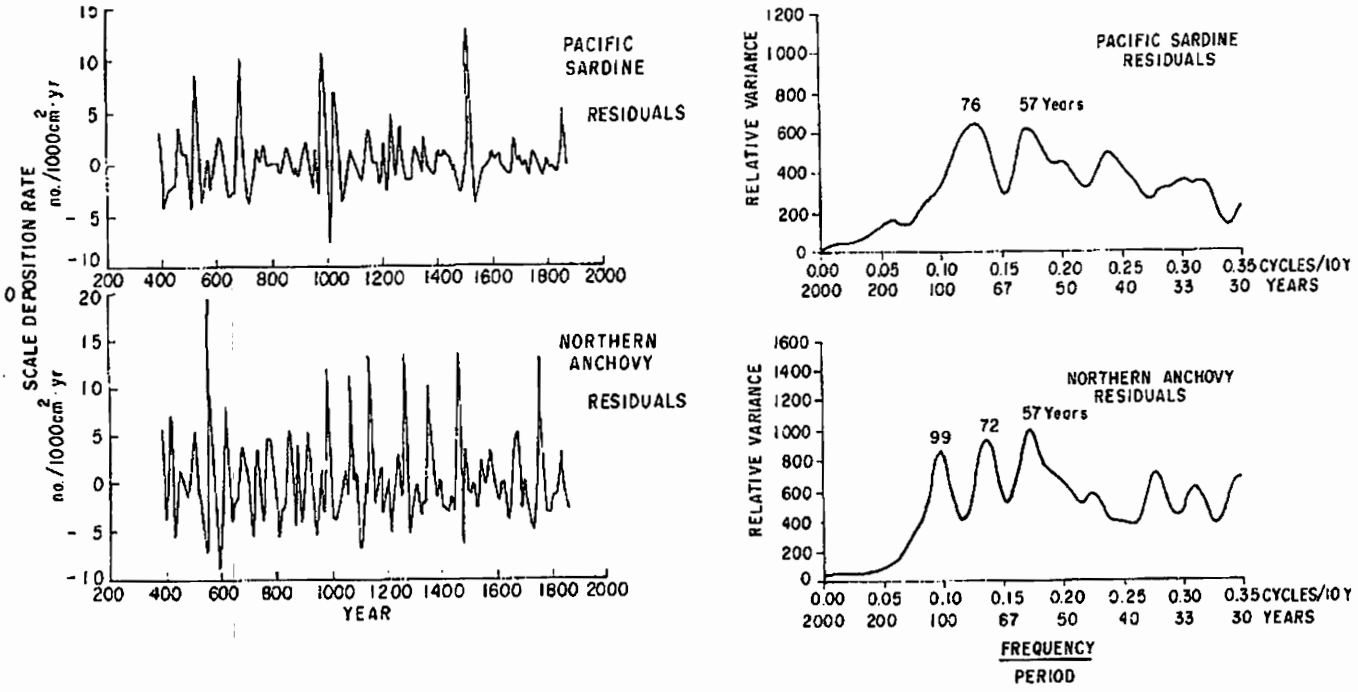

Figure 5. Two left plots are high-frequency variability in sardine and anchovy SDRs time series. These are the deviations of original data from filtered data in two left plots of Figure 4. Two right plots are power spectra of residuals (deviations) SDR series from the two left plots shown here (from Baumgartner et al., 1992).

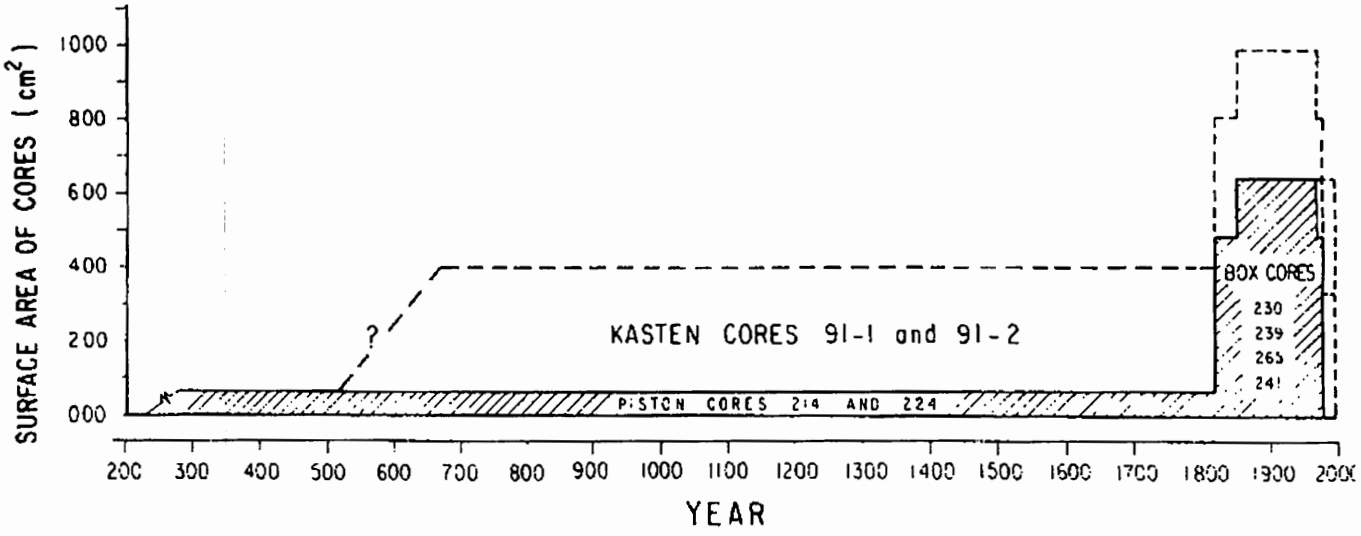

Figure 6. Diagram of the cumulative area of depositional surface sampled by the cores marked on Figure 1, plotted as a function of estimated calendar time sampled. The hatched area corresponds to cores used to construct the sardine and anchovy SDR time series of Figure 2. The open area represents the amount of material made available for analysis by the October 1992 recovery of Kasten cores KC-SB 9110-1301 and -1302, plus two accompanying box cores from the Santa Barbara Basin.

and whose scales were readily identifiable, is the Pacific saury, shown in Figure 14. All other scales were lumped into a group called "undifferentiated." Note that the scales of Pacific mackerel and the myctophids are poorly represented in these samples because their small size allows many or most to pass through the mesh of the 500-μm sieve.

We present the series of each of individual slabs (1, 4, and 6) in the upper three plots of these figures. The lower plot is the time series of averaged values of the 5-year sam-ples. The units of scale deposition rates, on the vertical axes, are simply the number of scales encountered in the 5-year sample blocks with an area of

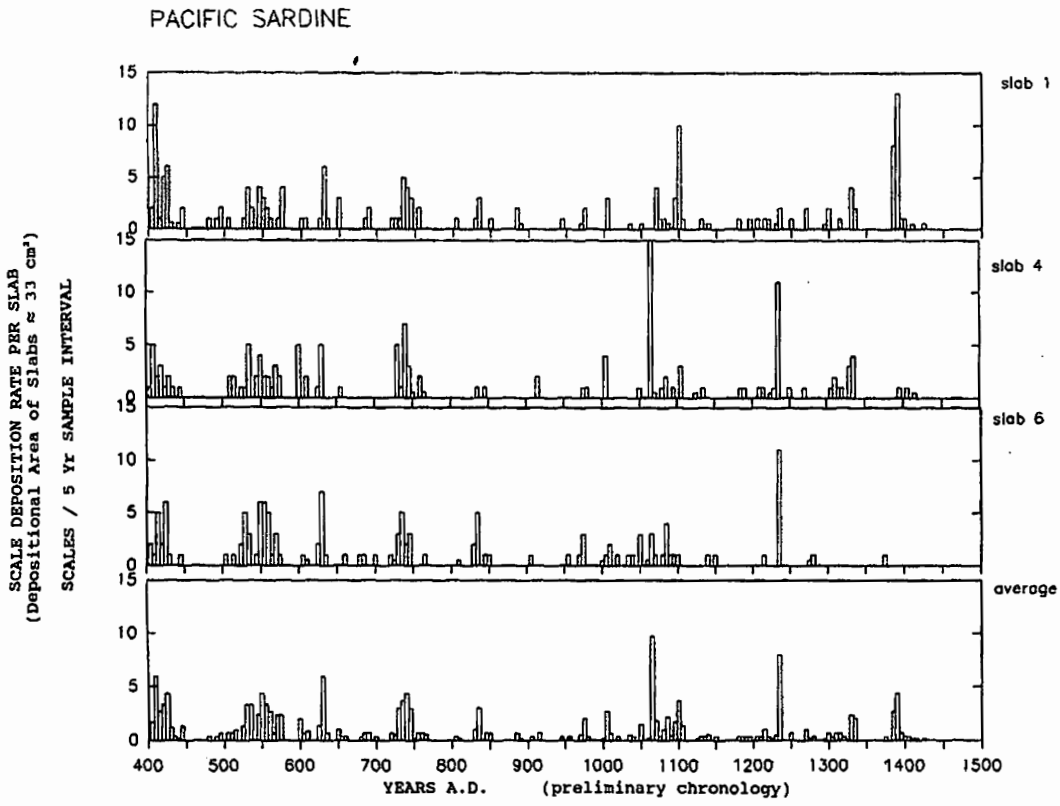

Figure 7. Time series of Pacific sardine SDRs for Kasten core KC-SB 9110-1301 developed during this project by separation and counting of the fish scales. Replicate time series were reconstructed from three slabs of the Kasten core (slabs 1, 4, 6). Each sample of the series represents a 5-year time interval based on varve counts from the chronostratigraphy developed from the thin-slab X-radiographs. Thus the units of SDR shown on the vertical axis is the number of scales deposited in 5 years within an area of approximately 33 cm^2. The total depositional area sampled by these three slabs is approximately 100 cm^2. The composite time series of averaged values over these three slabs is shown in the lower plot.

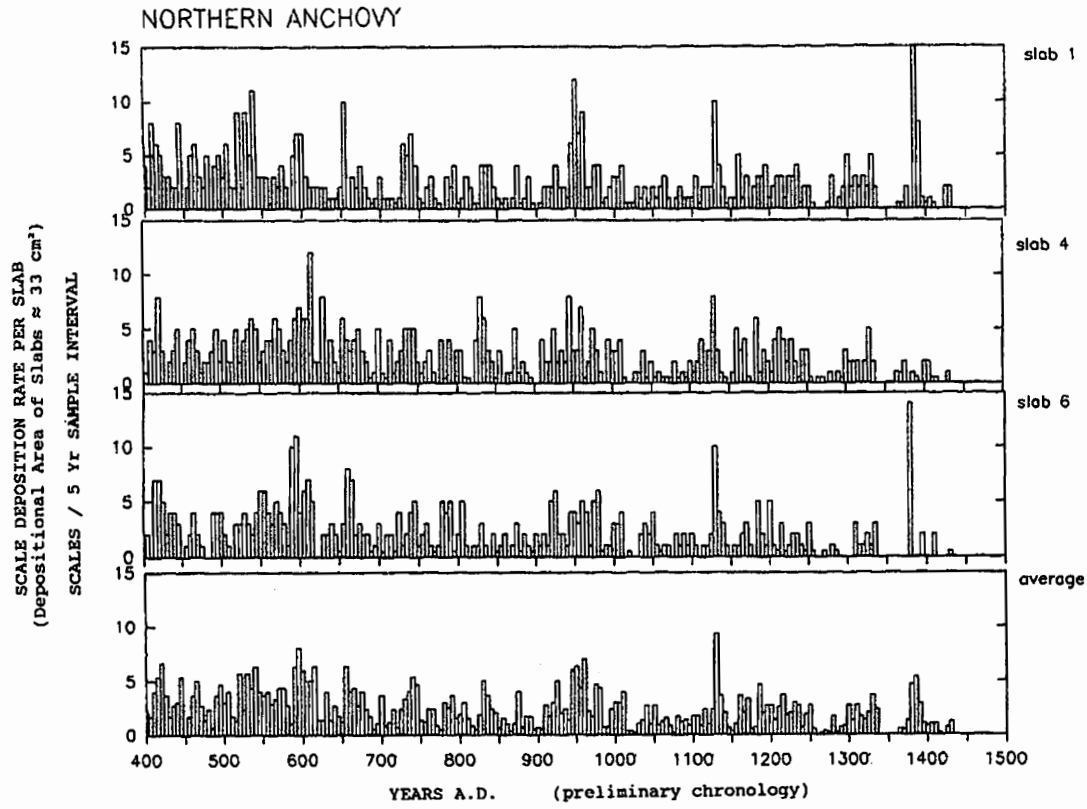

Figure 8. Replicate and composite time series of northern anchovy SDRs for Kasten core KC-SB 9110-1301 developed during this project by separation and counting of the fish scales. See caption of Figure 7.

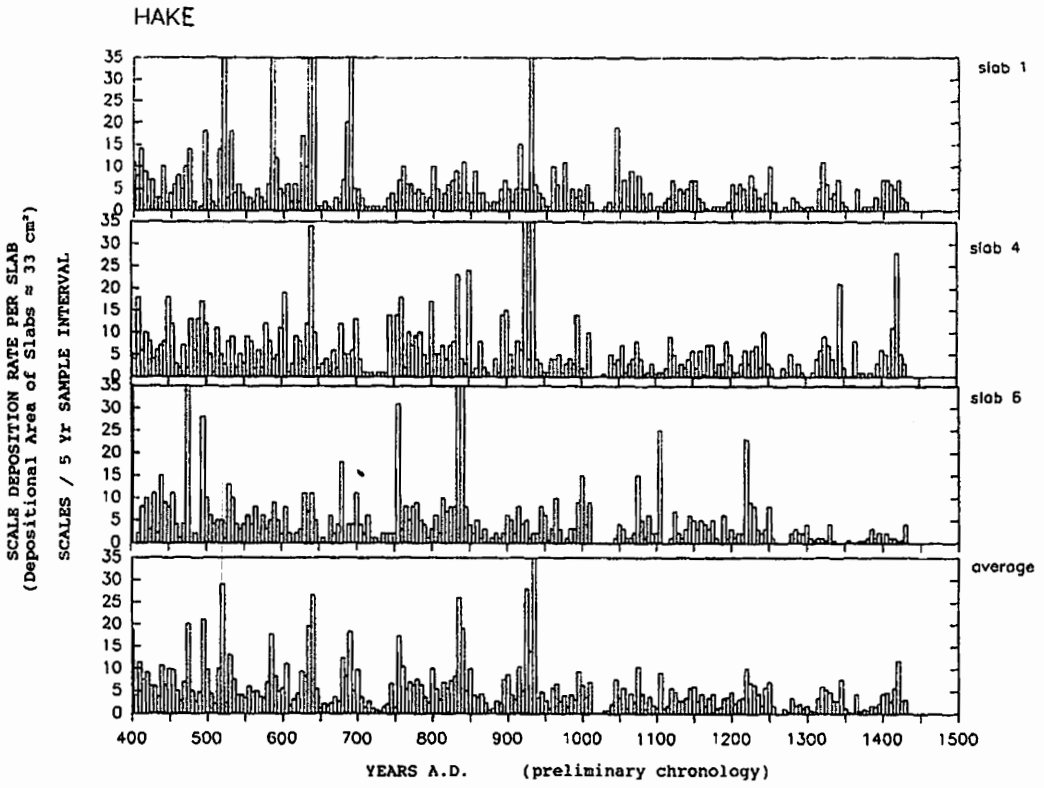

Figure 9. Replicate and composite time series of Pacific hake SDRs for Kasten core KC-SB 9110-1301 developed during this project. See caption of Figure 7.

depositional surface of approximately 33 cm^2. It should be noted that these are not the same units used in the series of Figure 2, but they can easily be converted.

AD 400 is the initial year of the chronology of the SDR time series in the Figures 7, 8, and 9. This date is our best estimate for the beginning of the varve count near the bottom of core KC 1301. This chronology of SDR values does not agree perfectly with the chronology used in Figure 2 (Baumgartner et al., 1992) for the existing SDR time series. This interplay among several versions of the chronology is a continuing process that is leading to greater refinement and accuracy.

Analysis of New Scale-Deposition Series from KC SB 9110-1301

As part of this project, we began to explore the nature of the signal-to-noise relationship in the existing sardine and anchovy data of piston cores 214 and 224, which were recovered from sites approximately 6 km apart. This was completed and published as part of Baumgartner et al. (1992; their Tables 2 and 3). We have now completed similar analyses for the series from the individual slabs of Figures 7–9 which are separated only by centimeters (Table 1). Comparison of these two sets of analyses indicates that the signal-to-noise characteristics obtained from the pair of time series reconstructed from sites several kilometers apart are comparable to those obtained with series taken very near to one another (i.e., the cm-scale distances within a single Kasten core). This preliminary comparison suggests that the principal spatial scale of variability in scale deposition is centimeters (perhaps meters), but not kilometers. Thus, we are on the way to answering the very important question concerning spatial variability of the scale-deposition process in the Santa Barbara Basin.

Figure 10 is a preliminary step in the examination of variance spectra of sardines and anchovy from Kasten core SB 9110-1301. These spectra were obtained from the time series of averaged values (lower plots, Figs. 7, 8, and 9). The units of variance on the vertical axes for anchovy and sardine are unequal so that the sardine spectrum fits under that of the anchovy. The 5-year sample interval now provides us with higher resolution of the temporal variability and gives us greater confidence in shorter period peaks. The spectra of total variance for the sardine and anchovy series in Figure 10 are generally similar to their counterparts for the original series of Figure 2. The hake spectrum (Fig. 11) is also similar to that of sardine and anchovy spectra of the original 1700-year time series (Fig. 2) from the piston plus box cores because of the concentration of variance around 75 and 55 years.

A major objective of this project has been a comparison and integration of our Kasten time series with the existing scale-deposition series. Figure 12 (upper plot) shows our progress comparing the new sardine data of Figure 7 (replotted as 10-year sample intervals from AD 400–1430) with the longer sardine and anchovy data series of Figure 2 plotted below. The alignment of the new and old series in

Table 1. Separate Analyses of Variance Using Input from Three Replicate Time Series for the Sardine, Anchovy, and Hake*

PACIFIC SARDINE

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
SAMPLES	1238.111	213	5.812728	2.480064	1.09E-15	1.211515
SLABS	2.049065	2	1.024533	0.437128	0.646178	3.016893
NOISE	998.4509	426	2.343782			
TOTAL	2238.611	641				

NORTHERN ANCHOVY

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
SAMPLES	2083.095	213	9.779791	3.378012	7.2E-27	1.211515
SLABS	11.67368	2	5.836838	2.016087	0.134444	3.016893
NOISE	1233.326	426	2.895132			
TOTAL	3328.095	641				

PACIFIC HAKE

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
SAMPLES	21775.78	213	102.2337	1.402693	0.001805	1.211515
SLABS	199.9836	2	99.99182	1.371934	0.254734	3.016893
NOISE	31048.52	426	72.88384			
TOTAL	53024.28	641				

*These analyses allowed us to examine the downcore signal-to-noise ratios, and the among-slab effects (i.e., difference/similarity), in a similar fashion as in Baumgartner et al. (1992; see their Tables 3 and 4) using the two piston cores 214 and 224. The comparison is made with the observed *F* ratios in the fifth column and the critical *F* ratios in the final column (the associated probability is in the sixth column). For example, the sardines have a downcore signal-to-noise ratio (shown in the "samples" row) of 2.48, which is over twice the critical value, indicating that the signal in which we are interested is significantly greater than the noise within the replicated series. Conversely, the ratio of the among-slab effect to the unexplained noise in the sardine data is only 0.44 (much less than the critical *F* value of 3.01), indicating no significant difference detected among the slabs.

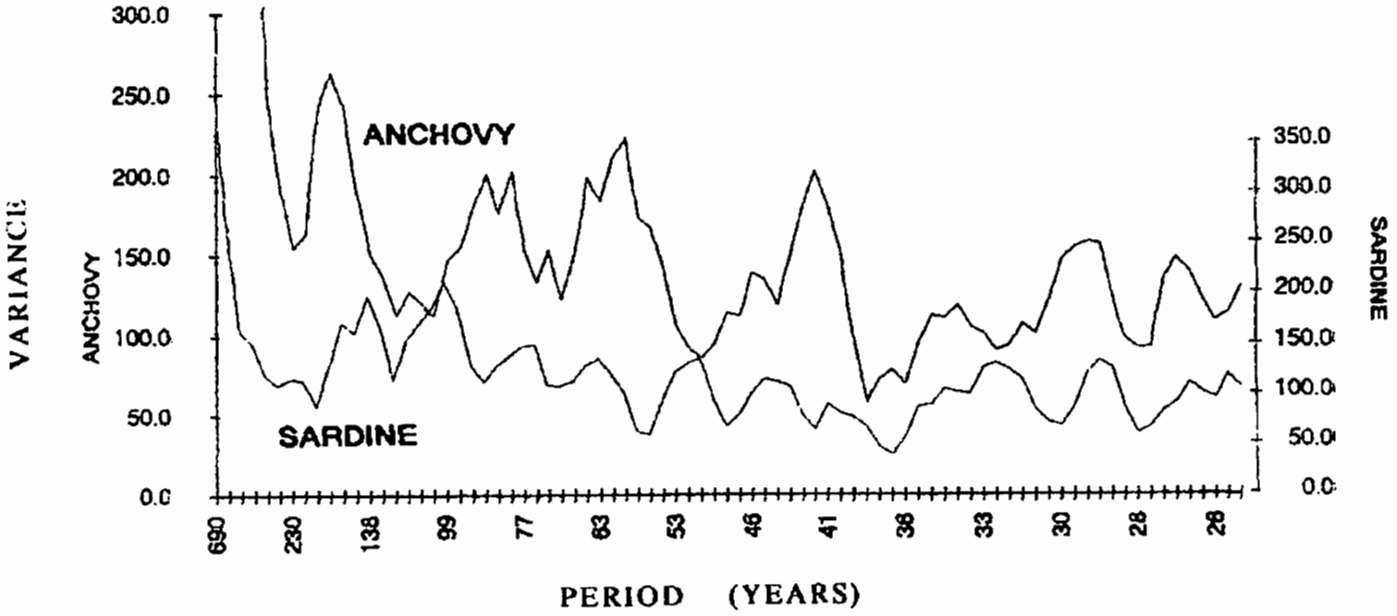

Figure 10. Variance spectra of the composite time series (lower plots in Figures 7 and 8) of the Pacific sardine and northern anchovy. The spectra have been smoothed with a five-point running mean. Note that these spectra were calculated without first removing the long-term trends.

Figure 12 (with a 70-year offset) provides the best match of the temporal pattern of the two series based on the cross-correlation of the two series (Fig. 13). This comparison provides a strong verification of the general temporal pattern of the existing scale-deposition series of cores 214 and 224 (Fig. 2).

Examination of Hypotheses

A final objective of the project was to begin the examination of three hypotheses: (1) Very low-frequency environmental change (periods >150 years, reflecting long-term global climate change) produce a generally similar response in both sardines and anchovies; (2) Interaction between major species, governed by density-dependent processes and uncoupled from extrinsic (environmental) forcing, produces variation in population sizes at a period of approximately 60 years (see Fig. 5); (3) Independent and sufficiently distinct responses of the different species to the same set of changing environmental conditions over high-frequency time scale (periods <150 years) govern changes in population sizes around the 60 year period.

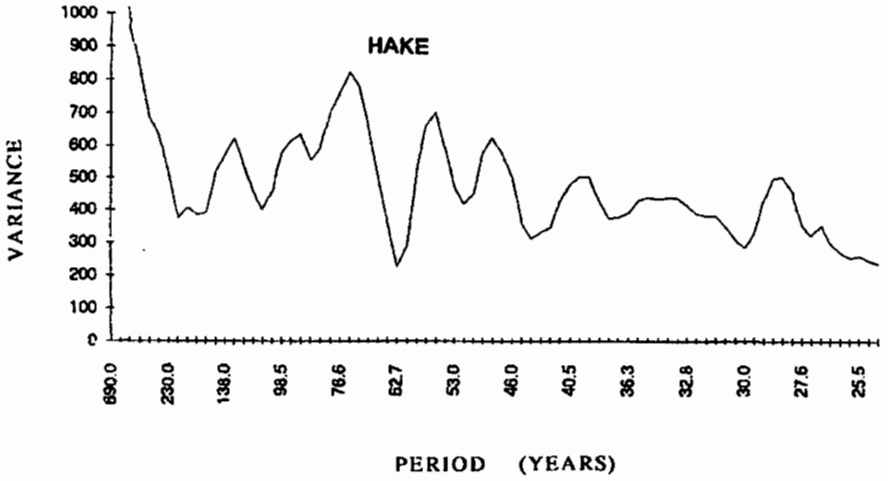

Figure 11. Variance spectrum of the composite time series (lower plot in Figure 9) of the Pacific hake. The spectrum has been smoothed with a five-point running mean. Note that the spectrum was obtained without first removing the long-term trends.

Hypothesis 1: Response to low-frequency global climate change. This hypothesis was formulated from the observation of important similarities in the pattern of temporal variability of the fish scale records with various proxy time series reflecting the behavior of large-scale climate over the past 1700 years. Based on these comparisons we postulated that much of the low-frequency variability in both the sardine and anchovy series reflects a response to environmental controls originating with global climate change. Examination of this idea has resulted in a model showing an obvious relationship between the low-frequency changes in sardine and anchovy populations.

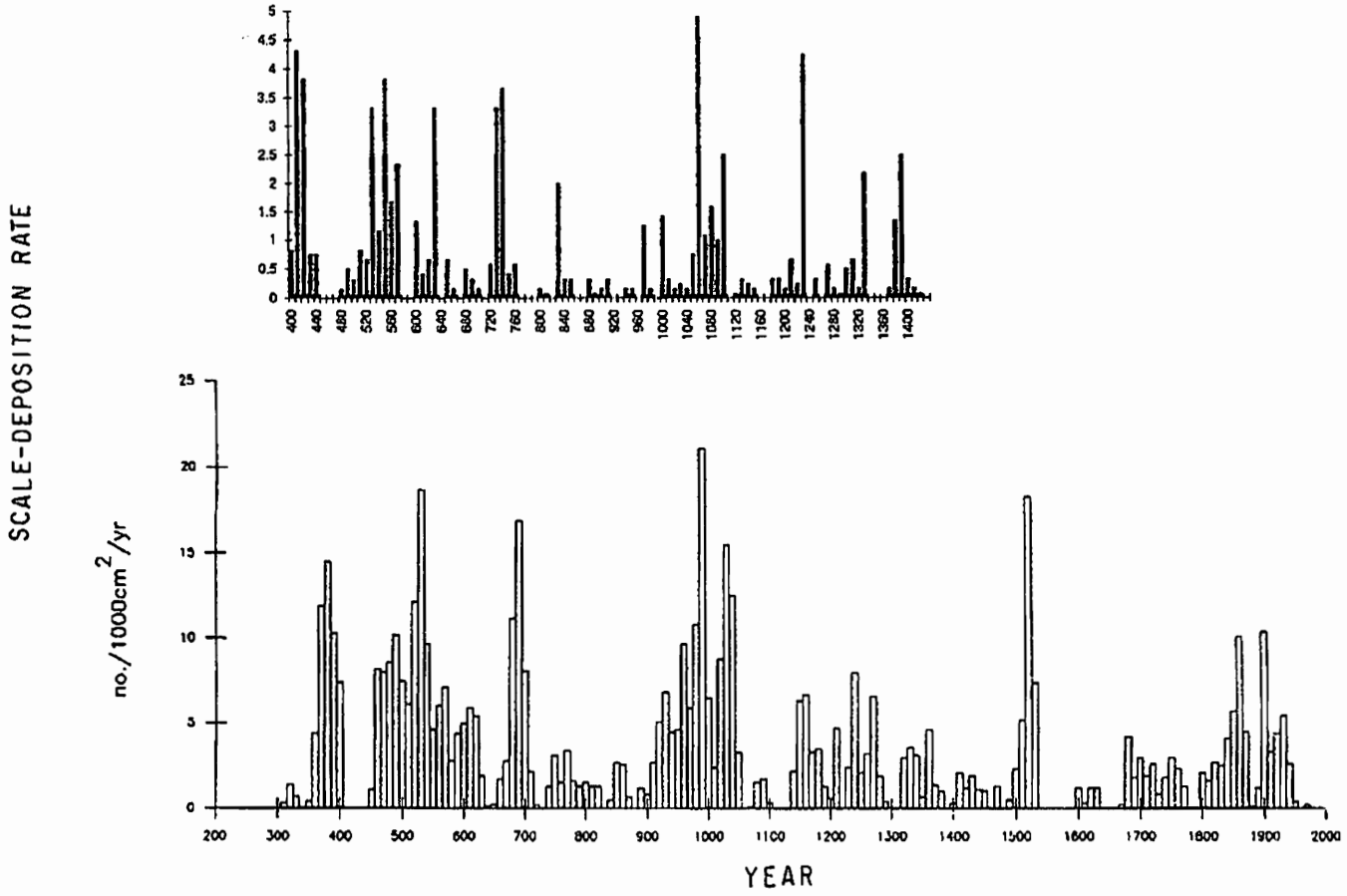

Figure 12. Comparison of the existing Pacific sardine time series developed from the piston and box core sites shown in Figure 1 (lower plot) and the composite sardine time series of Figure 7 developed during this Sea Grant project from Kasten core KC-SB 9110-1301 (upper curve). For this comparison the sam-ple intervals of the Sea Grant time series were converted from five-year to ten-year averages. The Sea Grant time series extends from AD 400–1430 according to our prelimin-ary varve chronostratigraphy. Note that the chronologies of these two series do not match; the comparison indicates a 70-year offset between the series if we arrange them to create a match with the double peak of sardine scale abundance on either side of the year AD 1000 of the original series. Thus the year AD 1000 of the original time series of Figure 2 is equivalent to AD 1070 in the new Sea Grant time series.

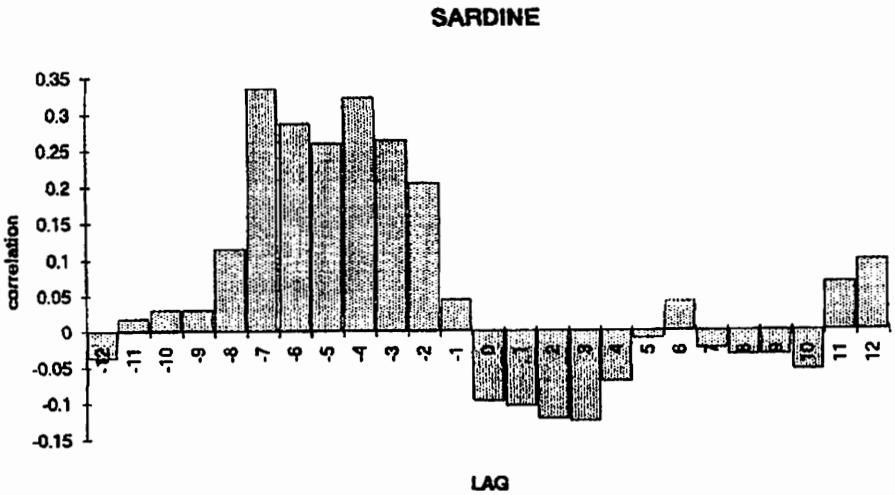

Figure 13. Cross-correlation function of the two time series in Figure 12. Note that there are positive matches at minus lag 4 and lag 7 (40- and 70-year offsets). This indicates two possible ways to align the two chronologies. We have chosen the 70-year offset to make the alignment shown in Figure 12.

Figure 14 embodies our reformulated and refined hypothesis of the relationship between the variability in the fish populations and global climate (using the more complete existing series of Fig. 2). This model is based on a sum of fish scale-deposition rates for sardines and anchovies and assumes that anchovies are more deciduous than sardines, with SDRs twice those of the sardines. At the core of this model is the notion that sardines and anchovies together respond to an overall carrying capacity of the environment which limits the total biomass of these two ecologically similar species on a time scale greater than 150 years. We also suspect now that the original chronology used in Figure 2 (Baumgartner et al., 1992) yields ages which are in error by approximately 70 years (see Figures 12 and 13); the postulated biomass model in Figure 14 is realigned with the proxy paleoclimate series to show our current best estimate of the comparison taking into account this chronological discrepancy. This combined biomass model yields a much better fit to the paleoclimate curve than do either of the individual sardine and anchovy series.

Hypotheses 2 and 3. High-frequency changes in population sizes. These hypotheses represent two extreme possibilities between which there may exist a gradation of explanations. The effects of fishing as a source of variability in the records can be disregarded if we consider only the prefishery period prior to this century. Figure 15 allows us to more closely compare the behavior of the decadal-to-century scale (our high-frequency) variability of sardine and anchovy, and to use this comparison to examine these hypotheses. The lower plot in this figure is the superposition of the anchovy and sardine series of residual scale-deposition rates taken from Figure 5. Plotting the ratio of sardine-to-anchovy residuals (upper plot, Fig. 15) shows a continuous succession of periods through which anchovy versus sardine dominance has alternated.

These observations lead us to choose the third hypothesis to represent the dominant control in the alternation of anchovy and sardine populations. Our study also suggests a refinement of the third hypothesis. That is, by stressing the importance of the differences in the niche characteristics of the two species we emphasize the "differential success" (Eldredge, 1985, p.82) of one of the two species as a response to a given set of environmental conditions. We therefore postulate that one or the other species tends to be favored as a function of the shifting of environment conditions between states which may persist over decadal time scales. This general hypothesis is a concrete improvement upon our original ideas and a valuable step in understanding the decadal-scale variability in sardine and anchovy populations.

Cooperating Organizations

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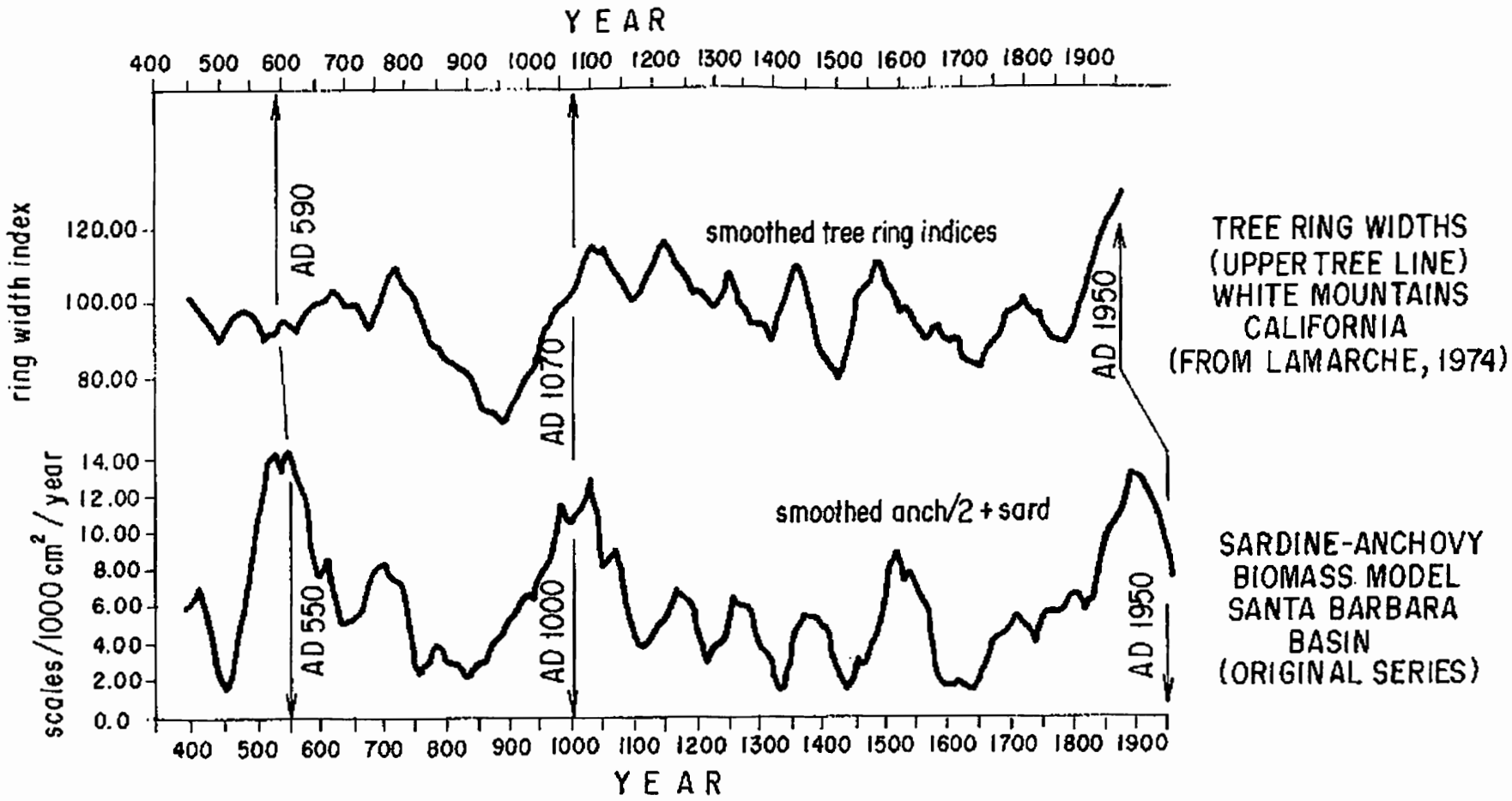

Figure 14. Comparison of a combined biomass model of sardine and anchovy to a proxy index of climate from the past 1600 years. The proxy paleoclimatic series (upper curve) is LaMarche's (1974) series of tree-ring widths from bristlecone pines at the upper tree line, White Mountains, California. The biomass model was constructed from the original series of observed Pacific sardine and northern anchovy SDRs (Figure 2); it is formed from the sum of the amplitudes of sardine SDRs and one-half the amplitudes of anchovy rates. Both the tree rings and SDRs are 10-year averages, smoothed with 13-term running mean. The alignment of the chronologies is based on the 70-year offset of the original SDR series with the new Sea Grant series presented in Figure 12. The 70-year offset is indicated by the middle arrow connecting AD 1000 of the original series with AD 1070 of the tree ring series. The best-fit alignments at either end of the series is shown by the other two arrows. Thus, there is a 40-year offset near the beginning of the series (Figure 13) and a 0-year offset at the end of the series at AD 1950.

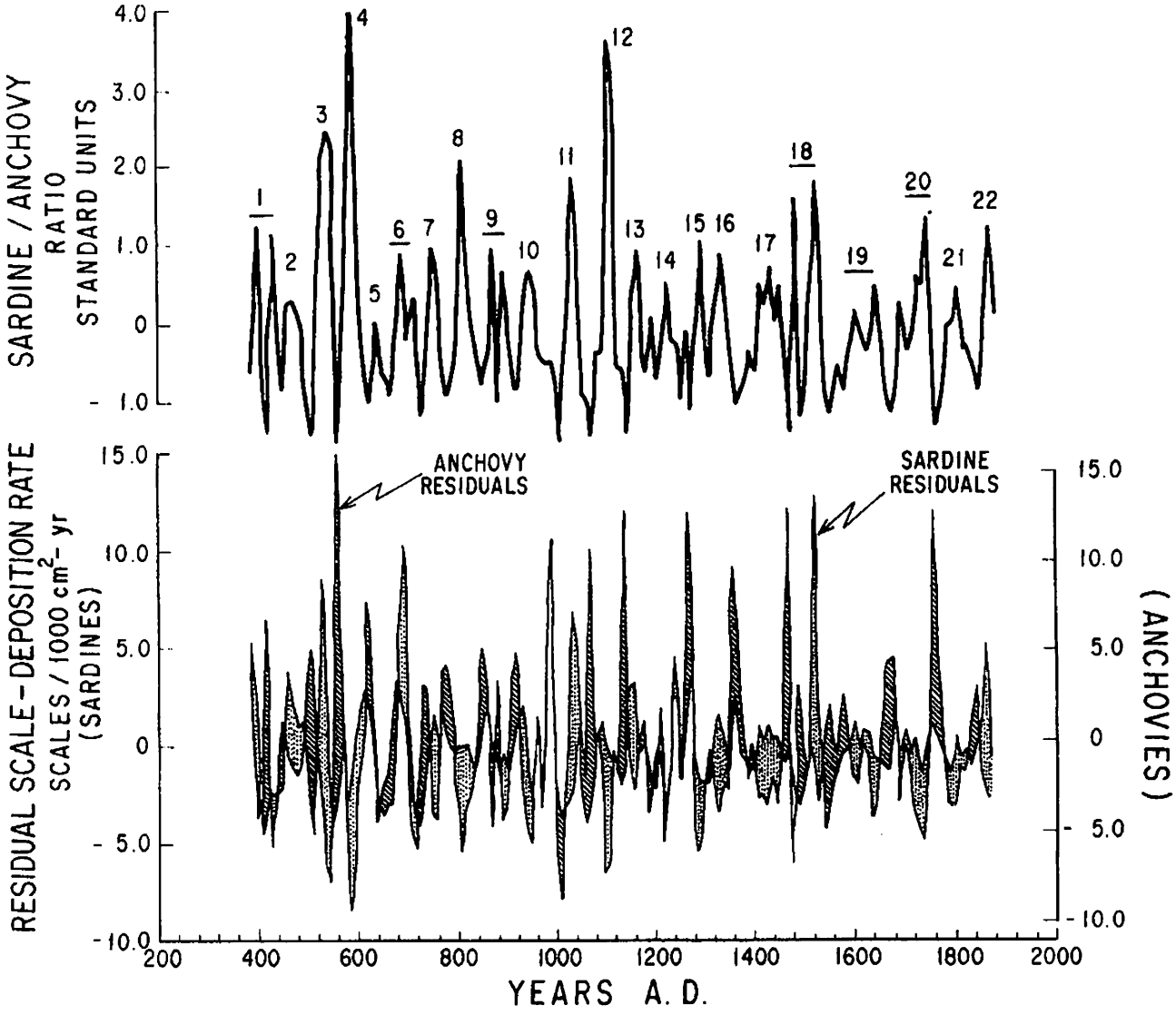

Figure 15. Comparison of the high-frequency components of the variability (periods <150 years in length) in sardine and anchovy SDRs of the original series of Figure 2. These high-frequency residuals were calculated by subtracting the deviations (i.e., residuals) from a smoothed curve for which periods <150 years had been removed by low-pass filtering (see Figures 4 and 5). The lower plot shows the superposition of the two time series of the high-frequency residuals of sardines and anchovies. This combined plot exhibits a general pattern of alternation between sardine and anchovy importance or dominance by dots (sardine SDRs > anchovy SDRs) and diagonal hatching (anchovy SDRs > sardine SDRs). The upper plot is a time series formed by the ratios of sardine residuals to anchovy residuals. The positive peaks are formed by periods in which sardine SDRs are greater than anchovy SDRs. The minima in the upper curve are formed by the maximum differences between anchovy and sardine SDRs over the intervals with anchovy SDRs > sardine SDRs. Note that we have identified 22 cycles of alternation between sardine and anchovy SDRs. Each cycle >40 years is labeled with its number in the sequence. Bars under some of the numbers indicate that the cycle includes more than a single peak.

Remote Sensing in Operational Fisheries: A Tool for the Utilization and Management of a Renewable Resource

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A/S-3
1990–91

At present, the U.S. fishing fleet uses antiquated remote-sensing technology and has almost no geographic information systems (GIS) capability. These deficiencies contribute to an inefficient and ineffective utilization of U.S. quotas on a species-by-species basis (e.g., tuna, swordfish, salmon). Under these circumstances, foreign fishing interests (e.g., Canadian, Japanese, Korean, Taiwanese and Russian), currently being equipped with RS/GIS technologies, would be able to utilize unused portions of the U.S. quotas, and present stock assessment tools probably would prove inadequate to insure the fecundity of the U.S. resources. Examples of this RS/GIS technology are the SORPU system currently under initial sea trials by the Commonwealth of Independent States, and the Salmon Early Assessment Grid (SEAGRID) under design and development by Canada.

This paper provides a brief overview of project A/S-3 which attempted to summarize the possible directions these efforts might take in the 1990s. Representative results from some geographically widely distributed fisheries were discussed in a technical report published by California Sea Grant in 1992: "Remote Sensing and Geographic Information Systems: Implications for Global Marine Fisheries."

The report outlines historical and anticipated uses of spacecraft data in support of operational fisheries in the United States, Canada, the Commonwealth of Independent States, Japan, France, Scandinavia, the People's Republic of China, and other nations. It describes global databases and Geographic Information Systems, notably the Global Resource Information Database (GRID) program and the Global Environmental Data Network.

It provides a basis for assembling a U.S. team of industry, resource management, and academic people to focus on implementing a U.S. strategy for the development of a remote sensing/geographic information system for use by the operational fisheries and oceanography community in the U.S. Exclusive Economic Zone. The combination of remote sensing capabilities with a geographic information system and its associated databases (e.g., hydrographic, bathymetric) can help eliminate the historical deficiencies in satellite support of operational fisheries—namely, no support for mid- and deep-water species and absence of information when clouds are present. Moreover, the strategy of an RS/GIS system, updated in near-real time, provides the possibility for a new and dynamic approach to fisheries resource management by federal and state assessment personnel.

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New Marine Products

Development of Selective Halogenation by Naturally Occurring Enzymes in Marine Organisms: A Biotechnological Approach

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R/MP-44
1989–92

Alison Butler

In the past three years (1989–92), the focuses of our Sea Grant project were (1) the discovery and characterization of haloperoxidase enzymes in marine organisms and, (2) the initial investigations of the enzyme-catalyzed biosynthetic pathways of halogenated marine natural products (particularly halogenated terpenes, indoles, and bi-indoles).

Haloperoxidases

Vanadium bromoperoxidase isolated from *Ascophyllum nodosum* is an exceptionally stable and versatile enzyme, which for these reasons is attractive to the biotechnical and pharmaceutical industries. Most of the reactivity studies of bromoperoxidase have been performed on the *A. nodosum* enzyme (Butler, 1992); the predominant halogenated compound produced by *A. nodosum* is bromoform. Because other marine algae and organisms produce halogenated compounds, it is important to investigate the characteristics and reactivity of the other marine haloperoxidases. We have isolated and characterized the vanadium bromoperoxidase from several other marine sources, including brown algae (e.g., *Macrocystis pyrifera, Fucus distichus*; Soedjak and Butler, 1990a, 1991) and a red alga *(Laurencia intricata)*. A small amount of bromoperoxidase was isolated from the green algae *Halimeda* (sp.) and *Penicillus* sp. Bromoperoxidase activity has been detected in *Laurencia pacifica* and *Bosiella orbigniana*. In addition, vanadium bromoperoxidase has been detected in the tunicate *Leptoclinides lissus*, which is the first discovery of this enzyme in invertebrates.

As isolated, the *Fucus* and *Macrocystis* enzymes contained less than a stoichiometric equivalent of vanadium per subunit; however, with the addition of vanadate, one equivalent vanadium/subunit was achieved. The striking result was a much higher enzyme activity than that of the *A. nodosum* enzyme (Soedjak and Butler, 1990a). We also found that vanadium bromoperoxidase was inactivated slowly by incubation in phosphate buffer (Butler et al., 1991). Inactivation arises by removal of vanadate from the enzyme active site. The activity can be fully restored by the addition of vanadate to the inactivated protein, after phosphate has been removed (Butler et al., 1991). In addition we have found that V-BrPO gives a strong immunologic response; thus we have now isolated and purified polyclonal antibodies to the V-BrPOs.

One of the most significant results of the haloperoxidase reactions was the discovery that V-BrPO catalyzes the oxidation of chloride by hydrogen peroxide (Soedjak and Butler, 1990b). Thus, the origin of the chlorinated marine natural products can now be addressed.

Indoles

Vanadium bromoperoxidase from the brown alga *A. nodosum* catalyzed the oxidative coupling of indole to the bi-indole, indigo, as well as the bromination and oxidation of indole (Figure 1). The oxidative coupling of indole to indigo is relevant to the biosynthesis of the halogenated and nonhalogenated bi-indole natural products indigo and the ancient dye Tyrian purple, both of which are specialty chemicals and antifungal agents (Figure 2). Oxidations of indole and its derivatives could arise from initial bromination followed by bromide displacement, resulting in an oxidation reaction. In this case the primary function of V-BrPO would be considered a bromoperoxidase. On the other hand, V-BrPO could also effect direct oxidation by singlet oxygen, in which case the primary function might be considered an oxidase. Vanadium bromoperoxidase is known to produce singlet oxygen without inactivating itself (Everett et al., 1990)

V-BrPO catalyzes the oxidative coupling of indole to indigo, in the presence of hydrogen peroxide and bromide, in a reaction that requires the presence of bromide but does not produce brominated indigo. V-BrPO also catalyzes the bromination of 1-methylindole, 2-methylindole, and 1,2-dimethylindole forming the 3-bromo-derivatives, and the oxidation of 3-methylindole forming 3-methyl-2-oxindole (Figure 2). Only one equivalent of hydrogen peroxide is required for complete reaction of indole and the indole derivatives. This stoichiometry indicates that the oxidized-bromine species is the reactive oxidizing or brominating species as opposed to oxidation or oxidative coupling by singlet oxygen. A singlet oxygen mediated oxidation would require consumption of two equivalents of hydrogen peroxide per equivalent of indole oxidized. The oxidative coupling of indole to indigo is unique to V-BrPO.The FeHeme haloperoxidases, chloroperoxidase, and lactoperoxidase, produce 2-oxindole exclusively (and not indigo), which must reflect the oxidative reactivity

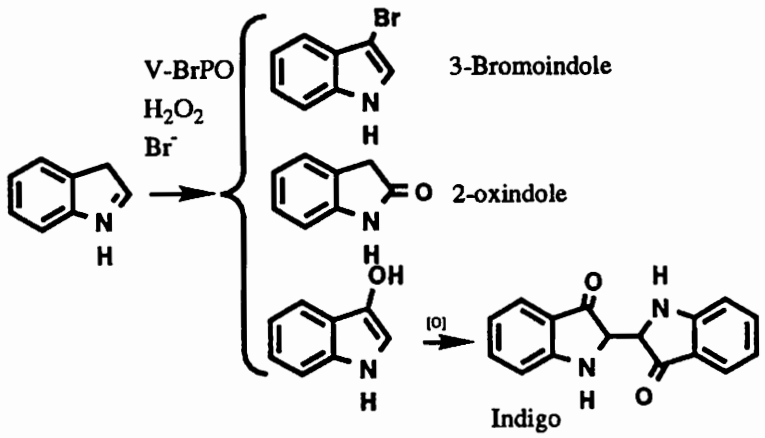

Figure 1

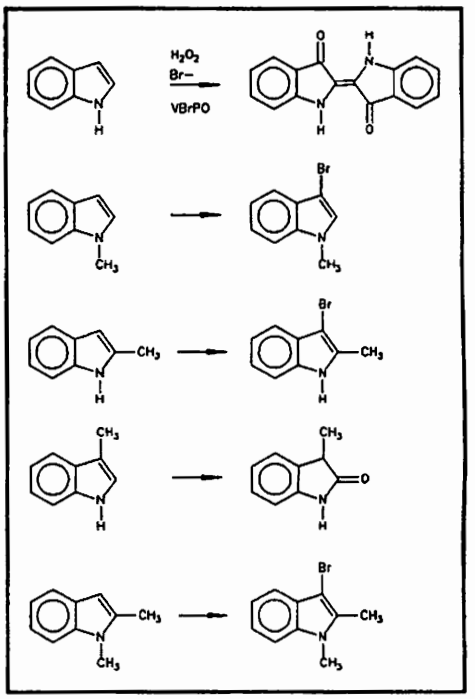

Figure 2

of the Compound I [FeO^{2+}-Heme$^+$] state. The origin of the oxygen atom in indigo is water and not hydrogen peroxide as established through ^{18}O-labeling studies of H_2O_2. This is consistent with bromination of indole followed by bromide displacement.

Terpenes: Geraniol, Farnesol

We also investigated the vanadium bromoperoxidase-catalyzed products of the mono- and di-terpenes geraniol and farnesol. Geraniol and farnesol are likely precursors of cyclic, halogenated mono-and di-terpene natural products. The products of the V-BrPO catalyzed reactions of geraniol and farnesol are the terminal bromohydrin and epoxide species (Figure 3). Enantioselectivity was not observed when the terpene esters of 2-R-phenylpropionic acid were used as the substrate, suggesting that under these conditions the reactive brominating intermediate was a "released" (i.e., not enzyme-bound) bromine species, such as HOBr, Br_2, or Br_3^-.

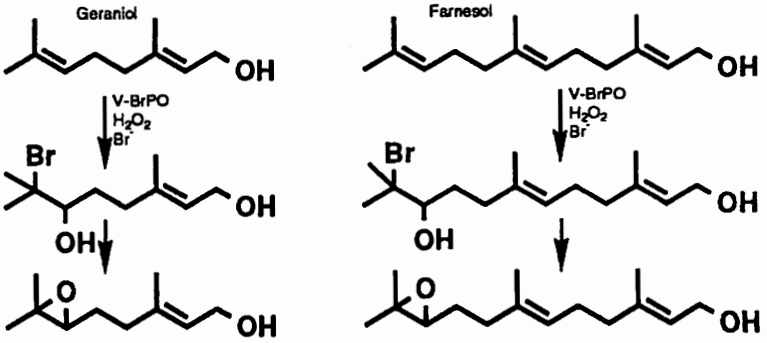

Figure 3

New Marine Enzymes and Marine Natural Products

In the course of our program in the isolation and characterization of haloperoxidase from marine algae, we discovered, working in conjunction with Professor R.S. Jacobs at UC Santa Barbara, a new marine natural product related to other anti-inflammatory natural products, as well as a new enzyme activity (Burgess et al., 1991). Aqueous extracts of the coralline red alga *Bossiella orbigniana* were shown to catalyze the enzymatic oxidation of arachidonic acid to a previously unreported ω-6 eicosapentaenoic acid product. This unique fatty acid contains a conjugated tetraene with absorption maxima at 293, 306, and 321 nm, and was identified by spectral methods of structural determination as 5(Z),8(Z),10(E),12(E),14(Z)-eicosapentaenoic acid. The compound was given the trivial name bosseopentaenoic acid. Bosseopentaenoic acid, along with several other conjugated tetraenes, was also present in the algae endogenously, as revealed by a comparison of the ultraviolet spectra and the HPLC chromatographic pattern of the purified product and the organic extract of *Bossiella*. Heated or boiled samples of the aqueous extract did not convert arachidonic acid to bosseo-pentaenoic acid, suggesting that a heat-sensitive enzyme is responsible for the conversion. The activity is indicative of a lipoxygenase or a cytochrome P-450 type enzyme.

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Allergan, Inc., Irvine, California

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Marine Natural Products in Pharmacology: Discovery and Development of New Chemotherapeutics from Marine Animals

University of California, Santa Cruz
R/MP-45
1989–92

Phillip Crews

Marine-derived bioactive substances with preclinical or clinical potential are beginning to emerge for many important disease areas. A brief overview of the highlights of past work on marine bioactive substances provides an interesting perspective. The first examples of a bioactivity-directed study of marine organisms appeared in the 1950s and included Nigrelli's (1959) work on sponges and Burkholder's (1958) studies on corals. The contemporary literature shows that marine antimicrobial agents are still being extensively isolated (Jiménez and Crews, 1991). Poisonous marine animals and plants represent another obvious source of bioactive substances; recent announcements of the labyrinthine structures of palytoxin (Moore, 1985), and of ciguatoxin (Murata et al., 1990) culminate work begun in the early 1960s. Enduring research efforts revealed the important anticancer agent lead didemnin B, first discovered in the 1960s by Weinheimer (under a National Cancer Institute contract program at the University of Oklahoma) during P388 antitumor screening, and characterized by Rinehart (University of Illinois) (Rinehart et al., 1988) during antiviral screening. It is now in phase II clinical trial for colorectal cancer chemotherapy (Abbruzzese et al., 1988). Similarly, important active metabolites are bryostatin-1 (Smith et al., 1985; May et al., 1987) and dolastatin-10 (Pettit et al., 1987) which are in, and about to enter, respectively, advanced stages of clinical development.

In 1984 our group at the University of California, Santa Cruz established a collaboration with Dr. Tom Matthews and his co-workers at the Institute of Antiviral and Antimicrobial Chemotherapy of Syntex Research. Our aim was to use antiparasitic primary screens to identify the most promising, among hundreds, of sponge extracts obtained from Indo-Pacific invertebrates. A number of antiparasitic active compounds were subsequently purified, many possessed novel chemotypes, and some of their properties have been described in both the patent and primary chemical literature. We recently completed a comprehensive review titled "The Search for Antiparasitic Agents from Marine Animals." This will appear in Dr. David Attaway's (National Sea Grant Office) volume on Marine Biotechnology, and it unites published literature with our extensive unpublished results.

Our collaboration with Syntex Research has since developed into a screening program that emphasizes antiviral assays (an anti-HIV assay and two anti-HSV assays). Since 1989, we have screened 360 organisms (890 separate extracts) in the anti-HIV assay. Pure compounds from our repository and from bioassay-guided isolations yielded a total of 55 compounds tested as potential anti-HIV agents. During this time, we also screened 257 organisms in the HSV assay; 45 pure compounds were tested. A second anti-HSV assay was recently incorporated into our screening program, and we have already screened 150 organism extracts and seven pure compounds.

Our program has begun to use the National Cancer Institute's *in vitro* anti-HIV screen. We have tested six pure compounds, but unfortunately we have no significant leads to date. Our future plans are to take full advantage of this important screening resource and screen the majority of the compounds in our repository, as well as newly isolated compounds.

The discovery and investigation of bioactive agents requires a combination of organic structure elucidation and pharmacological experimental methods. The most mature examples of these past achievements are represented by compounds that represent leads for antiparasite and antiviral development, covered by U.S. patents as follows: The bengazoles, the structures and anthelmintic properties of which were described in patent #4785017 (1988); the bengamides, having antiviral and anthelmintic data described in patent #4831135 (1989); anthelmintic dysinins of marine origin described in patent #4943589 (1990); and plakinidines with anthelmintic and antiRT properties as described in patent #4959370 (1990).

Other significant achievements of past work supported by Sea Grant involve bioactive alkaloids and oxygen-containing compounds. The alkaloids are divisible into more than a dozen structural classes. Joining the ketide-amino acids mentioned above (bengamides, bengazoles) are jasplakinolide (jaspamide), and mycothiazole. Jasplakinolide shows promise as a selective cytotoxin, and it seems appropriate to subject it to the antiprotozoal screen. Research continued on this compound under Sea Grant project R/MP-45, and our recent published results on jasplakinolide included: (a) the first comprehensive conformational dynamics study of its macrocyclic ring (Inman and Crews, 1989a), and (b) an analysis of molecular recognition properties resulting in the preparation of the Li^+ complex (Inman and Crews, 1989b). Jasplakinolide has also shown promising antitumor activity and is undergoing *in vivo* testing in anticancer assays.

The bengamides are also ketide-amino acids of continuing interest. Nine bengamides have been isolated to date by our group, and these consist of bengamides A–F

and iso-bengamide F (Quiñoá et al., 1986; Adamczeski et al., 1989), plus two new derivatives, G and H. Work under Sea Grant Project R/MP-45 led to the isolation and characterization of the latter two bengamides, along with reisolation of several of the others. During this period we also reported the complete absolute stereochemistry of all the bengamides based on a combination of NMR and synthetic chemistry efforts (Adamczeski et al., 1990). This announcement stimulated two scalemic total syntheses (Chida et al., 1991; Broka and Ehrler, 1991), and one partial synthesis (Gurjar and Srinivas, 1991) of the bengamides. Significantly, bengamide G (unpublished structure) has recently shown potency against HIV (IC_{50} = 0.3 μg/mL), and bengamide F showed moderate *in vivo* activity without toxicity in a mixed helminth assay in mice. More material must be isolated for follow-up assays. Bengamide A exhibited potent activity in the National Cancer Institute's 60 cell *in vitro* tumor assay and is now under evaluation for further studies.

Still other anti-infectious disease-active compounds have emerged from our research. Two compounds, plakinidines A and B (Inman et al., 1990), were isolated from the crude extract fractions of an Indo-Pacific sponge, which showed potent antigiardia activity. Unfortunately, the giardia assay was unavailable to us after these compounds had been purified and characterized. Alternatively, both have excellent *in vitro* anthelmintic activity, and only the former was mildly active against the RT enzyme. The result of our search for antiparasitic marine natural products has netted some the 17 moderately, and 21 strongly *in vitro* active anthelmintic compounds from sponges, a nudibranch, and a zoanthid, as represented by the various lead structures. Many of these lead structures are alkaloids as represented by: terpenoid alkaloids—axisonitrile-3 (Kambhampati et al., unpublished results); ketide-amino acids—mycothiazole (Crews et al., 1988); penazetidine A (Alvi and Crews, unpublished results; Kobayashi et al., 1991); novel amino acids—psammaplin D Jimémez and Crews, 1991; Quiñoá and Crews, 1987; Rodríquez et al., 1988; Arabshahi and Schmitz, 1987); epismenospongiarine (Rodríquez et al., 1992); reticulatine A (Jiménez et al, 1991a; Jiménez et al., 1991b; Roll et al., 1988). Many of the compounds were unknown before our research, while a few were discovered during prior work by others; examples are the dysinins (Horton et al., 1990; Dunlop et al., 1982) and kalihinols (Chang et al., 1987; Omar et al., 1988). Some were discovered during simultaneous work by others; examples are the psammaplins (Chang et al., 1987; Omar et al., 1988) and plakinidines (Rodríquez et al., 1992; West et al., 1991).

Developments that represent achievements to build on for the future are as follows. A vigorous field collection program, supported from both Sea Grant and non-Sea Grant funds, was set in motion. Since 1989 our collection repository has grown significantly as 679 new marine organisms were obtained and 290 were identified taxonomically. Hundreds of extracts were submitted for prescreen trials against the viral targets and, not unexpectedly, approximately 8% of the extracts have sufficient potency to be considered active. We have begun a process to evaluate organisms rich in photosynthetic microbial symbionts and have discovered a number of sponges for further examination that appear to be rich in the chlorophylls of cyanobacteria.

Cooperating Organization

Syntex Corp., Palo Alto, California

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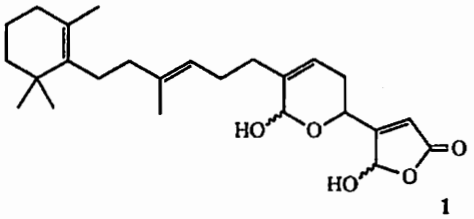
Novel amino acid derivatives and alkaloids from marine sponges. Structures, stereochemistry, and biological activities. Invited talk, 1989 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii, December 22, 1989.

Marine Pharmaceutical Discovery Program: Chemistry Component A

University of California, San Diego
Scripps Institution of Oceanography
R/MP-46
1989–92

D. John Faulkner

During the reporting period, 39 new marine natural products were submitted for pharmacological evaluation by Professor Robert S. Jacobs of UC Santa Barbara. More than half of these samples inhibit phorbol-induced inflammation in the mouse ear assay, show *in vitro* inactivation of phospholipase A_2, or *inhibit* the division of fertilized sea urchin eggs. These compounds have been retained for further study. Details of the biological screening data are reported by Professor Jacobs in the corresponding report for project R/MP-47. We have also provided samples of marine natural products such as manoalide, luffariellolide, and scalaradial, that were used for the thesis research of Sea Grant trainees at UC Santa Barbara. In addition, we have supplied marine natural products to Dr. Vivek Malhotra of the Biology Department at UC San Diego to be used in studies of basic cellular mechanisms.



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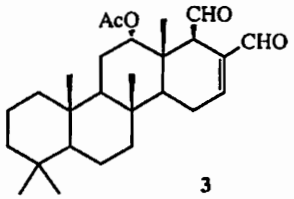

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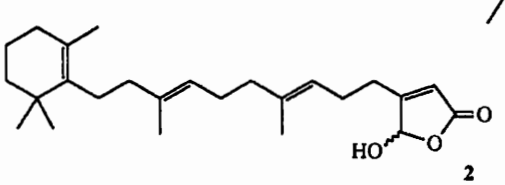

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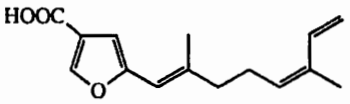

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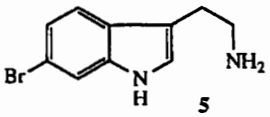


5

We have made a breakthrough in our research on the mechanism of action of manoalide (1), luffariellolide (2), and scalaradial (3), which are marine natural products that act as anti-inflammatory agents by inactivation of phospholipase A_2. We have now demonstrated that for all three compounds the first step in the inactivation mechanism is the formation of a Schiff base (or γ-aminobutenolide) with a lysine residue that is located at or near the active site on the enzyme. We can also partially reverse the previously irreversible binding of manoalide to the enzyme, and we can almost completely reverse the binding of luffariellolide to PLA_2.

Among the compounds that were submitted to UC Santa Barbara, the furanoic acid (4) is of particular interest because it shows excellent anti-inflammatory activity. The furanoic acid (4) was isolated from a soft coral from the Seychelles, but it was first described from *Sinularia gonatodes* from the Great Barrier Reef. It is unusual to find promising pharmacological activity associated with such a simple molecular structure. This was an obvious candidate for synthesis, and we have now synthesized several simpler analogs, some of which show comparable anti-inflammatory activity.

Two slightly different specimens of the encrusting gray tunicate *Didemnum candidum* were collected in the southern Gulf of California and were examined separately. 6-Bromotryptamine (5) was isolated from a specimen that was encrusting on mangrove roots and 2,2-bis-(6'-bromo-3'indoyl)ethylamine (6) and 2,5-bis-(6'-bromo-3'indoyl) piperazine (7) were obtained from specimens collected from submerged rocks. After initial evaluation in-house, 2,5-bis-(6'-bromo-3'indoyl)piperazine (7) was submitted to the National Cancer Institute (NCI) screening program. As a result of the preliminary screening, NCI has requested additional compound for *in vivo* studies.

The chemical studies associated with this project have resulted in the isolation and identification of more than 50 new marine natural products, representing a wide variety of structural types. The highlights are summarized below.

In collaboration with scientists from SmithKline Beecham, we identified didemnaketals A (8) and B (9), which are terpenoids from a species of *Didemnum* that inhibit HIV-1 protease.

A series of complex diterpenes of the spongian class were obtained from Dendroceratid sponges, and their value as defensive chemicals was compared with their pharmaco-

logical profiles. New diterpenes were also isolated from the gorgonian *Junceella gemmacea* and the brown alga *Dictyota divaricata*.

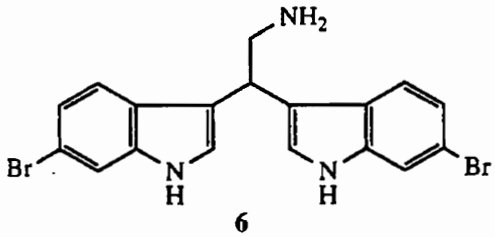
Four novel alkaloids were isolated from the sponge *Chelonaplysilla* sp.; one of these, chelonin C (10) shows strong anti-inflammatory activity. A new alkaloid, pictamine (11) was obtained from the tunicate *Clavelina picta*, and two new octacyclic alkaloids, eudistones A (12) and B (13) from a tunicate of the genus *Eudistoma*.

The first naturally occurring cyclic perlactone (14) was found in the Caribbean sponge *Plakortis angulospiculata*.

A Seychelles sponge of the genus *Smenospongia* contained four very unusual macrocyclic merosesquiterpenes, smenochromenes A–D (15–18).

Two new sesterterpenoids, luffolactone (19) and (4*E*,6*E*)-dehydromanoalide (20), were described as minor constituents of *Luffariella variabilis*.

The sponges *Damiria* sp., *Calyx podatypa*, and *Psammaplysilla purpurea* produced pyrroloquinoline alkaloids, N-methyl pyridinium salts, and brominated tyrosine derivatives, respectively.



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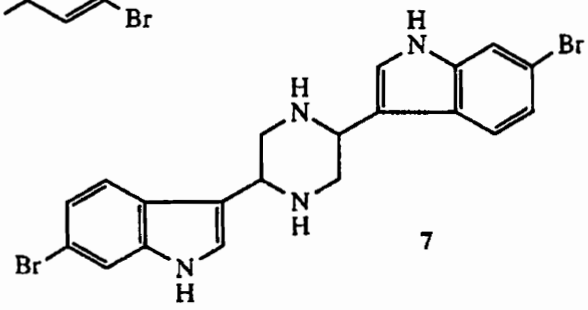

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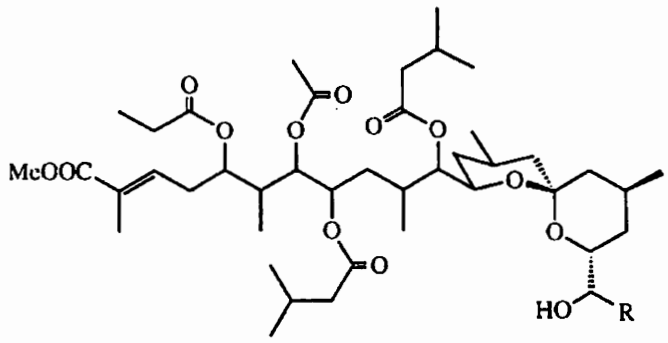

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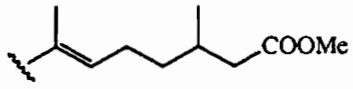

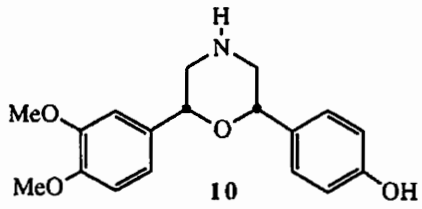

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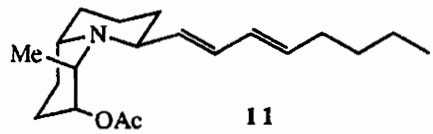

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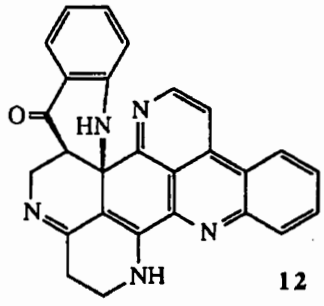

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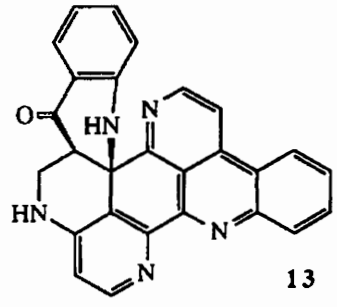

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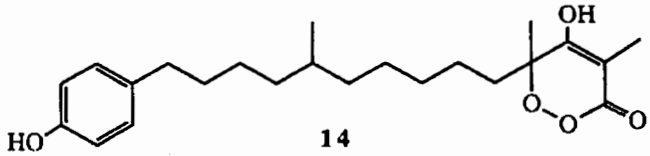

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Cooperating Organizations

OsteoArthritis Sciences, Inc.
SmithKline Beecham Research & Development

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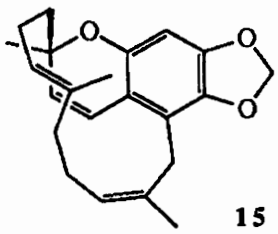

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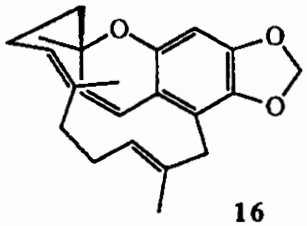

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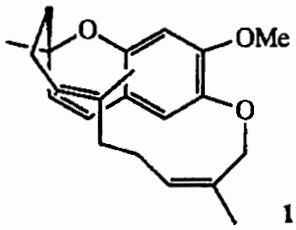

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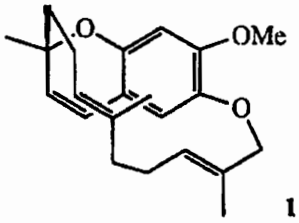

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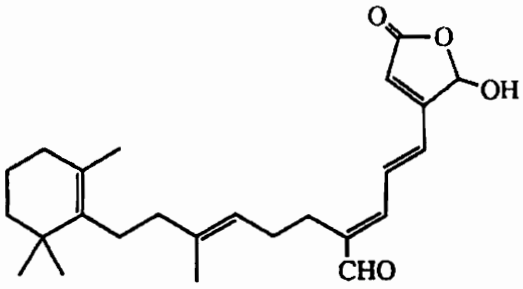

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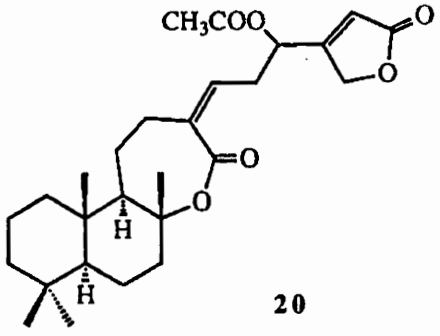

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Marine Pharmaceutical Discovery Program: Chemistry Component B

University of California, San Diego
Scripps Institution of Oceanography
R/MP-48
1989–92

William Fenical

The goals of the Marine Pharmaceutical Discovery Program were to explore marine plants and animals for new leads in the treatment of inflammatory diseases and related disorders in lipid metabolism and to further define existing discoveries, with a major emphasis on the elucidation of novel mechanisms of pharmacological action. Additional goals were to continue to develop the commercial application of the pseudopterosins and to initiate a research collaboration with Ligand Pharmaceuticals in La Jolla. Ligand is a new biotechnology firm dedicated to the drug discovery process by the application of cloned intracellular receptors (IRs) to the screening process. During the project, two research vessel expeditions to the Bahama Islands and to Belize were carried out as part of our new lead exploration program. Working with the research group of Professor Robert S. Jacobs, UC Santa Barbara, we investigated numerous collections and focused on several new active extracts. A major discovery during this grant period was the mechanistically exciting new anti-inflammatory agent, fuscoside B. A diterpene glycoside, this compound was isolated from the Caribbean gorgonian *Eunicea fusca*. Another anti-inflammatory agent of a novel type, salinamide A, has been discovered in a new microbial fermentation program. An exciting development is the final commercialization of the pseudopterosins, as anti-inflammatory agents in skin lotions. Details of these studies follow.

New Drug Discovery Research—Field Research Programs

As part of our collaborative field program to discover new drug leads, we have continued to collaborate with the Jacobs's group in the operation of the University of Miami's research vessel *Columbus Iselin*. During the summer of 1990, we performed onboard collaborative research consisting of collections, integrated chemistry, and biological assays, as part of a 21-day expedition to the Bahama Islands. During June–July 1991, we investigated numerous areas along the barrier reef in Belize. These studies have been enormously productive in identifying new sources for compounds with unique properties. The program is based upon NSF support, and the Sea Grant research performed is highly complementary to our NSF goals. In 1991, we established onboard bioassays that for the first time detected the presence of phospholipase A_2 in marine plants and invertebrate organisms. This discovery validated our hypothesis that marine invertebrates possess the same inflammation-producing biochemical pathways as humans. By following the same biochemistry in marine sources, we feel that our discovery program is rationally based.

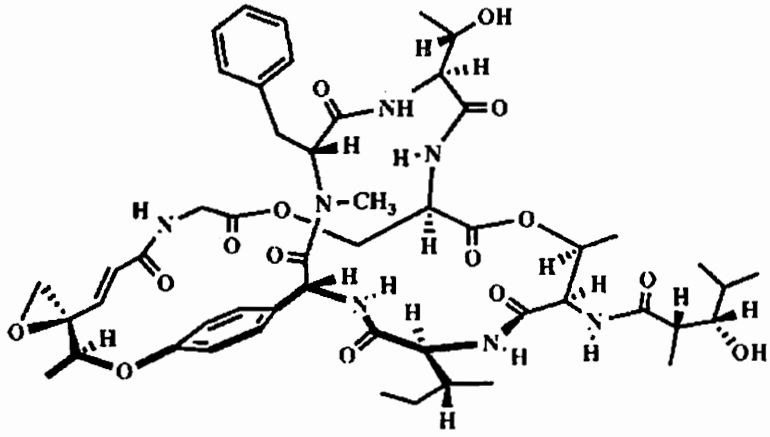
Another component of our field discovery program has been a project initiated by Sea Grant trainee Sarah Richards-Gross to investigate the ascidians from La Jolla and surrounding waters. Richards-Gross has developed a rigorous collecting program, which has already identified at least four new projects this year. Her research will lead to the discovery of new molecules with novel bioactivities.

New Microbioloay Program

In another project, we have made a sizable commitment to develop the microbiological, particularly bacterial, resources in marine environments. With the recognition that fermentation products from soil bacteria have provided the foundation of the modern pharmaceutical industry, we have devoted significant efforts to understanding the distributions, variability, and culture requirements of marine gm– and gm+ bacteria. Sea Grant trainee Jackie Trischman, for example, has developed methods to isolate and culture bacteria from the sediments of local estuaries. These bacteria produce antibiotics as part of a complex adaptation involving competitive interactions for available nutrients. By using this approach, Trischman has isolated several marine bacterial strains that are chemically unique. In one case, she isolated a unique anti-inflammatory agent, salinamide A (Figure 1), which is produced by a marine bacterium under saline fermentation conditions. This new program has also been interfaced with Ligand Pharmaceuticals, in La Jolla, who are dedicated to drug discovery through intracellular receptor biotechnology.

New Anti-inflammatory Agents

From a Caribbean gorgonian, *Eunicea fusca*, we have discovered two new glycosides, fuscosides A and B (Figures 2 and 3), each of which possesses arabinose sugars. Compound 2 is an arabinose pyranoside of a new class of diterpenoid molecules, while compound 3 is the side-chain arabinoside of fuscol. Both compounds, in subsequent testing, have been found to possess potent *in vitro* anti-inflammatory properties. These latter molecules resemble the pseudopterosins in that they are pentose sugar glycosides. There can be little mechanistic overlap between these compounds, however, as the aglycones are of completely different classes and possess no structural or functional similarities. These compounds are also physiologically mechanistically distinct from the pseudopterosins. In preliminary testing, the compounds



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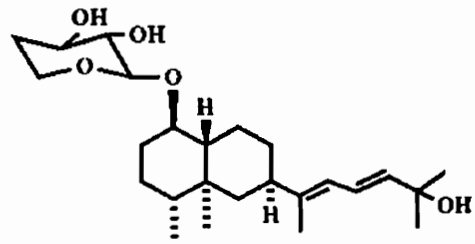

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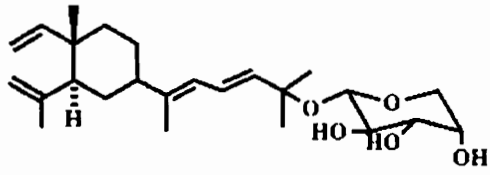

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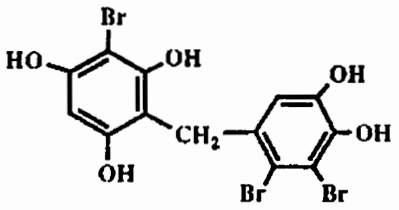
appear to inhibit leukotriene biosynthesis. This is a novel mechanism of action, and UC Santa Barbara Sea Grant trainee Peer Jacobsen has finished his Ph.D. dissertation on this topic. We now know that fuscoside B is an exciting molecule that could lead to a new generation of nonsteroidal anti-inflammatory agents. Research is in progress to initiate industrial involvement in this new class of drugs.

Another discovery from this program's *Columbus Iselin* expeditions are vidalols A and B (Figures 4 and 5). These unique phenyl ethers are potent anti-inflammatory agents that have received considerable investigation by the Jacobs's research group.

Developmental Research with the Pseudopterosins

During the past 3 years, we have continued to interact with the Jacobs's group and with several industries to foster the commercial development of the pseudopterosin class of anti-inflammatory agents. Our major goals were to determine the pharmacological mechanism of action of this group of compounds and to encourage their development in industry. Because the commercial development of these compounds depends upon knowledge of how they act to control inflammation, we have made this effort a major component of our interaction. We have worked closely with Sea Grant trainee Ed Luedke to perform various chemical and pharmacological experiments to solve this problem. At this point, we believe the pseudopterosins (exemplified by PsE, Figure 6) act by the direct inhibition of the arachidonic acid liberating enzyme phospholipase A_2. This conclusion is based on a series of experiments using inflammatory cells, PMN leukocytes, and macrophages, as well as isolated arachidonic acid pathway enzymes. It should be stated that compounds that inhibit PLA_2 represent a novel new approach to the development of anti-inflammatory drugs. The only other authentic PLA_2 inhibitor known is the sponge metabolite manoalide, which has been advanced in commercial development to the clinical trial stage. Our major successes in commercializing the pseudopterosins lies in their effective utilization in skin creams. A major U.S. skin care company has been awarded an option by the University of California to license this discovery and to market the pseudopterosins as part of a new line of outdoor skin care products. Complete clinical trials have been performed, and all toxicological data have been obtained. The pseudopterosins have been proved clinically to be superior to any anti-inflammatory agents used thus far. My research group has been working with this company's



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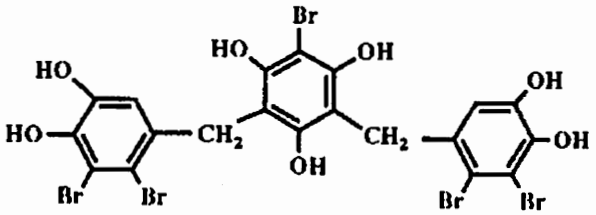

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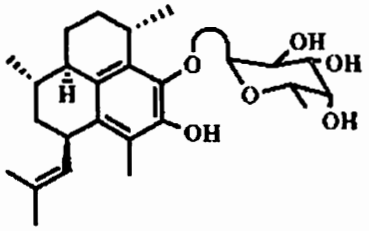

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research group to establish a reliable supply of these compounds for commercialization. A commercial collection program has been approved by the government of the Bahamas, and we have begun to oversee collection and processing of the air-dried animal. The intention is to use an ethanol extract of *P. elisabethae* as a "natural ingredient." We anticipate that this product will reach the market within 12 months.

Discovery of Selective Binding Agents

As stated, we have dedicated considerable efforts toward the introduction of our program to a new pharmaceutical company in La Jolla, Ligand Pharmaceuticals. The basis of Ligand's approach to drug discovery is the application of intracellular receptors (IRs) in drug screening. These receptors are the natural binding sites for drugs and natural hormones. By cloning these receptors and establishing novel bioassay methods, Ligand can screen for analogs of existing drugs and hormones that are as effective but much safer. An example is the vitamin D receptor. In principal, 1,25-dihydroxyvitamin D would be an effective drug to regulate calcium metabolism. Unfortunately, this drug has serious side-effects, which completely restrict its use in this regard. Our interactive program seeks to identify new molecules that bind to this and other receptors but that lack the serious toxicities often observed. Our main approach has been to screen many marine bacterial fermentation broths. In doing so, we have isolated and identified at least three new classes of selective binding agents. These observations are the first to prove that "mimics" of the natural agents can be isolated and possibly developed into new drugs. We have isolated a highly selective antagonist of the progesterone receptor, and it has been patented by Ligand and the University of California. The molecule discovered, from the green alga *Cymopolia barbata*, is mechanistically novel. The compound has been obtained in larger quantities, and it is now being subjected to subsequent *in vivo* evaluation.

Old and New Industrial Collaborations

As already mentioned, we have had significant interactions with industry over the past 2 years. These interactions have been rewarding in that new discoveries have moved rapidly toward the production of products. Our research to commercialize the pseudopterosins as drugs continues to attract attention. However, we have been disappointed with the rate at which these compounds have been developed. Studies in the Jacobs's lab have shown that these agents are effective anti-inflammatory

agents, particularly against conditions of rapid onset inflammation such as that observed in asthma. Unfortunately, there are still communication problems in interfacing Sea Grant research with the pharmaceutical industry. We are stimulated, however, by a new industrial interaction with a small biotechnology firm, OsteoArthritis Sciences Inc., located in Massachusetts. This organization wishes to negotiate an exclusive arrangement with our Sea Grant collaborative program, aimed at the development of anti-inflammatory agents. This new interaction represents a solid commitment of time and resources, which should stimulate the development of our discoveries.

Cooperating Organizations
Merck Sharpe and Dohme
Schering Plough Corporation
Sterling Drug Company
Ligand Pharmaceuticals
Wyeth-Ayerst Pharmaceuticals
Estée Lauder Company
OsteoArthritis Sciences Inc.

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Natural products chemistry of marine bacteria. Mini-Symposium, Department of Chemistry, University of British Columbia, July 1989.

PSP, paralytic shellfish poisoning. Plenary Lecture, Marine Medicine 89 Conference, Office of Continuing Education, University of California, San Diego, July 1989.

Natural products chemistry of symbiotic and free-living marine bacteria. Plenary Lecture, 6th International Symposium on Marine Natural Products Chemistry, Dakar, Senegal, July 1989.

The ocean, a biomedical frontier. Plenary National Congress of the Mexican Pharmaceutical Association, Mazatlan, Mexico, October 29, 1989.

University Lecturer—University of Naples, School of Pharmacy, a series of 3 lectures on various aspects of marine natural products chemistry and pharmacology, December 1–15, 1989.

The potential role of marine resources in new skin care products. Estée Lauder Company, March 26, 1990.

Studies in marine chemical ecology and biotechnology. Distinguished Ocean Scholar Series, Cornell University, March 28, 1990.

Natural products chemistry of symbiotic and free-living marine bacteria. Invited Lecture, American Society of Microbiology Annual Meeting, Anaheim, California, May 14, 1990.

New frontiers in marine natural products chemistry. Invited Lecture, School of Pharmacy, Purdue University, August 28, 1990.

New frontiers in marine natural products chemistry. Invited Lecture, Department of Chemistry, University of Iowa, September 18, 1990.

Marine toxins. Invited Lecture, School of Public Health, San Diego State University, September 24, 1990.

Marine microbial chemical ecology and its interface with biotechnology. Invited Lecture, Hopkins Marine Station of Stanford University, Pacific Grove, California, January 10, 1991.

Medicines from the sea. Invited Special Lecture, Division of Research Grants Administration, National Institutes of Health, Bethesda, Maryland, June 26, 1991.

Paralytic shellfish poisoning: The toxic dinoflagellates story. Invited Lecture, Marine Medicine 1991 Conference, Office of Continuing Education, University of California, San Diego, July 11, 1991.

New bioactive heterocyclic metabolites from marine microorganisms. Plenary Lecture, 13th International Congress of Heterocyclic Chemistry, Corvallis, Oregon, August 14, 1991.

New antitumor antibiotics from marine microorganisms. Plenary Lecture, 3rd Pacific-Asia Symposium on Biologically Active Natural Products, Noumea, New Caledonia, August 28, 1991.

Marine bacteria and symbiosis. Invited Symposium Lecture, International Marine Biotechnology Conference, 1991, Baltimore, Maryland, October 13–16, 1991.

Marine bacteria: A new biomedical resource. Sterling Drug Company, Malvern, Pennsylvania, October 17, 1991.

New metabolites from Indian Ocean marine invertebrates. Invited Lecture, Asian Symposium on Medicinal Plants and Spices, Manila, February 2, 1992.

Marine bacteria, a new biomedical resource. Invited Lecture, Annual Meeting of the American Association for the Advancement of Science, Chicago, Illinois, February 6, 1992.

Marine bacteria: Symbiosis and the production of bioactive metabolites. Invited Lecture, Gordon Research Conference on Marine Natural Products, Ventura, California, February 17, 1992.

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Marine Pharmaceutical Discovery Program: Pharmacology

University of California, Santa Barbara
Marine Science Institute
R/MP-47
1989–92

Robert S. Jacobs

Marine invertebrates and marine plants have provided a new source of pharmacological probes for the study of the mechanisms mediating inflammatory disease processes. New structural classes of anti-inflammatory compounds have been identified by our research group from pharmacological evaluation of pure marine natural products isolated from marine invertebrates. The identification of the biochemical targets for these anti-inflammatory natural products provides insight into the mechanisms of inflammation and provides lead compounds to use for the rational design of better therapeutic anti-inflammatory agents.

Our research has taken two paths for the study of marine natural products and metabolites. One is the use of marine organisms as pharmacological models of cellular and molecular processes, while the second approach targets the site and mechanism of action of marine natural products. These two directions have aided us in forming a more cohesive and complete picture with regard to the eicosanoid biosynthesis and cellular activity of marine organisms. The data indicate that both primitive marine invertebrates and algae can metabolize arachidonic acid to yield a variety of eicosanoid metabolites. These metabolites can in some cases cross-react with mammalian prostaglandin and leukotriene antibodies. Furthermore, marine natural products may function to selectively inhibit pathways involved in eicosanoid metabolism. In this report, we will summarize our most recent investigations of marine natural products having selective anti-inflammatory mechanisms.

Over this grant period we have completed our studies on several marine natural products. These include scalardial, fuscoside, pseudopterosin, and pseudopleroside (see Publications). In addition, several new discoveries have been made including chelonin C, furanoic acid, bromotopsentin, discoderanide, and two new peptides from marine bacteria.

These compounds all are anti-inflammatory. Our most recent progress is described below.

Manoalide and Scalaradial Studies of Phospholipase A_2

Scalaradial (SLD), a marine natural product isolated from the sponge (*Cacospongia sp.*), possesses anti-inflammatory properties *in vivo* and *in vitro* (Jacobs et al., 1993). In this study we characterize its effects against bee venom phospholipase A_2 (PLA_2; EC 3.1.1.4). SLD is a potent inactivator of bee venom PLA_2 with an IC_{50} value of 0.07 μM. Inactivation of bee venom PLA_2 occurred in a time-dependent, irreversible manner. The rate of inactivation followed first-order reaction kinetics and was dependent on the concentration of SLD. Kinetic analysis suggested a two-step mechanism of inactivation: an initial apparent noncovalent binding ($K_i = 4.5 \times 10^{-5}$ M), followed by covalent modification. The rate of inactivation was reduced markedly in the presence of excess phosphatidylcholine, suggesting that modification of the enzyme occurs at or near the substrate binding site. Inhibition of bee venom phospholipase A_2 (PLA_2) by manoalide and luffariellolide involves the initial formation of a Schiff base (imine) between a lysine residue on PLA_2 and an aldehyde group on each of the drugs. Model reactions using a primary amine in place of the lysine residue were studied by ^1H NMR spectroscopy. Amines reacted at the γ-hydroxybutenolide ring of 2 to produce γ-(alkylamino)butenolides, which are cyclized forms of the corresponding Schiff bases. Manoalide methyl analogue, which is a simple analogue of the reactive portion, reacted similarly. The γ-(*n*-butylamino)butenolide reacted with hydroxylamine to form the oxime, with concomitant release of *n*-butylamine. When the luffariellolide-PLA_2 and manoalide-PLA_2 adducts were treated with hydroxylamine, the PLA_2 activity was substantially recovered, but the activity was not recovered if the luffariellolide-PLA_2 adduct was reduced with sodium borohydride before hydroxylamine treatment. PLA_2 activity could be significantly recovered by treatment of the initial scalaradial adduct with hydroxylamine, but the final adduct, which is proposed to be a pyrrole, could not be cleaved.

Pseudopterolide Studies of Cytokinesis

Low concentrations (1–10 μM) of the marine natural product pseudopterolide inhibited cytokinesis and induced formation of multinucleate cells in fertilized *Strongylocentrotus purpuratus* embryos. As determined by immunofluorescence microscopy by using fluorescent stains for actin filaments, microtubules, and chromosomes, pseudopterolide inhibited cytokinesis selectively by disrupting the contractile ring, whereas spindle microtubule organization and mitotic chromosome segregation to opposite spindle poles were unimpaired. At somewhat higher concentrations (16–20 μM), pseudopterolide induced formation of microtubule spiral asters, that are believed to be caused by rotation of the cytoplasm relative to the cell cortex. The effects of pseudopterolide on cytokinesis were cell-cycle dependent. The actions of pseudopterolide in fertilized sea urchin embryos were strikingly similar to the actions of another marine natural product,

stypoldione, a structurally unrelated orthoquinone that reacts covalently with the sulfhydryl groups of glutathione, β-mercaptoethanol, cysteine, and a number of proteins (O'Brien et al., 1989). In the present study, pseudopterolide was also found to react with sulfhydryl groups of glutathione, β-mercaptoethanol, and cysteine. The results indicate that the cellular target for pseudopterolide, like the target for stypoldione, may be an especially sensitive sulfhydryl-containing protein involved in the formation or function of the contractile ring.

Fuscoside, Inflammation and 5-lipoxygenase

The biological and biochemical pharmacology of fuscoside, a novel anti-inflammatory marine natural product isolated from the Caribbean gorgonian *Eunicea fusca*, has recently been characterized by using murine (part I) and human (part II) models of inflammation. Topically applied fuscoside (FSD) effectively inhibits phorbol myristate acetate (PMA)-induced edema in mouse ears at levels comparable with indomethacin over a 3.3-hr exposure period, and is significantly more efficacious than indomethacin over 24 hr in the PMA model. Histological preparations and quantification of the neutrophil-specific marker, myeloperoxidase, demonstrate that FSD inhibits neutrophil infiltration into PMA-induced regions of edema and inflammation. In systemic studies, where FSD is injected intraperitoneally before the topical application of PMA, negligible effects on ear inflammation are observed. FSD does not inhibit bee venom or human synovial fluid phospholipase A_2 up to concentrations of 500 μM. In calcium ionophore-activated cultures of mouse peritoneal macrophages, FSD selectively and irreversibly inhibits leukotriene C_4 biosynthesis (IC_{50} = 8 μM), yet has negligible effects on prostaglandin E_2 production. FSD is also without effect on the conversion of arachidonic acid to prostaglandin E_2 by ram seminal vesicle cyclooxygenase. Chromatographic and spectroscopic studies suggest that FSD is not metabolized, and that drug uptake/binding by macrophages is time dependent, saturable, and independent of active transport mechanisms. These studies represent the first report of an anti-inflammatory marine natural product that selectively inhibits leukotriene biosynthesis. Fuscoside (FSD) is a potent and long-lasting anti-inflammatory drug that selectively inhibits leukotriene production in murine models of inflammation. In the present study, the effects of FSD on the lipoxygenase pathways in human polymorphonuclear leukocytes are explored in order to better understand the mechanism of action of this novel drug. In adherent and suspended polymorphonuclear leukocytes, FSD irreversibly inhibits leukotriene B_4 (LTB_4) synthesis (IC_{50} = 10 μM) and the release of ^{14}C-labeled LTB_4 from neutrophils prelabeled with [^{14}C]arachidonic acid. Unlike the reversible 5-lipoxygenase inhibitor L-651,896, FSD has no observable effect on LTB_4 biosynthesis in whole blood but does express activity as blood is successively diluted. In 10,000 × *g* supernatants of human platelets and polymorphonuclear leukocytes, FSD does not inhibit platelet 12-lipoxygenase, but is extremely effective in inhibiting the metabolism of arachidonic acid and 5-hydroperoxyeicosatetraenoic acid to LTB_4 via neutrophil 5-lipoxygenase. FSD had no effect on the conversion of leukotriene A_4 to LTB_4 in this system. Interestingly, concurrent with FSD inhibition of leukotriene synthesis is a concentration-dependent increase in 5-hydroxyeicosatetraenoic acid, suggesting that FSD may selectively inhibit the leukotriene A_4 synthase activity associated with human 5-lipoxygenase. FSD is therefore representative of a new class of nonantioxidant 5-lipoxygenase inhibitors that may be effective local therapeutic agents in the management of such diseases as psoriasis, arthritis, and inflammatory bowel and lung diseases.

Pseudopterosin

The pseudopterosins have been officially licensed by the University of California to Osteoarthritis Sciences, Inc. (OSI) for the treatment of inflammatory conditions. Five model compounds were selected by Dr. William H. Fenical, based on their chemistry and availability, which will be selected by myself and OSI for development of a clinical trial. We have assisted OSI in determining their levels of interest in this series for therapeutic purposes. Their investment in drug safety studies will be substantial.

New Discoveries

A. Cell division and cytokinesis. We have 13 new marine natural products that inhibit cell division.

B. Inflammation. There are currently 64 active leads based on preliminary test results for this past year.

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Ocean Technology

Extreme Loadings of Marine Structures: Wave-Induced Structural Loading

University of California, Berkeley
R/OE-12
1989–91

J. Randolph Paulling

A method developed under a previous Sea Grant project for the study of ship capsizing has been adapted to the prediction of the structural loads acting on a ship moving through high waves. The basis is a timestepping integration of the exact large amplitude equations of rigid body motion. External wave- and motion-dependent fluid forces, including the freesurface "memory effect" terms, are estimated by a hybrid method in which an exact computation is made of part of the force and the remainder is estimated by linear methods.

A "first generation" computer program has been written and tested, which computes the large amplitude motions and corresponding structural loading in a ship moving in regular head seas. In this procedure, the Froude-Krylov or dynamic pressure forces are computed for the exact wetted portion of the ship up to the instantaneous local water surface, and the hydrodynamic diffraction and radiation forces are approximated by linear, small amplitude wave theory. This is completely analogous to the procedure that was successfully used earlier to numerically simulate a ship capsizing in high waves.

The procedure has been tested for a simple barge form and for the SL7 container ship. An exact analytic solution can be obtained for the barge, and the these results were duplicated quite closely by the present procedure. In the case of the real SL7 container ship, conventional strip theory results for the midship shear and bending moment were used as a basis of comparison. Strip theory is purely linear and results in wave loadings that are proportional to wave amplitude. The present nonlinear procedure is found to predict a maximum sagging load that increases more rapidly than, and a hogging load that increases less rapidly than, the linear value. This is in accordance with scanty experimental evidence and is consistent with what is expected as a result of the geometry of the forward and after parts of this ship.

Publications

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Extreme Loadings of Marine Structures: Slow Drift Forces on Offshore Structures

University of California, Berkeley
R/OE-13
1989–92

William C. Webster and Peter Mull

This study was focused on the determination of the force system which is exerted on an offshore structure at frequencies well below those contained in the incoming wave system. This force system arises strictly from the nonlinear interaction between random waves and the platform. Current methods for its prediction are not appropriate for high sea states where the waves are nearly breaking. Slow drift forces which occur in these high sea states are responsible for very large excursions of moored ships and floating offshore platforms, excursions which can, and do, cause catastrophic loss of moorings.

The approach adopted in the study was based on the Green-Naghdi (GN) theory of fluid sheets. This is a new theory which recent research has shown to be a good model for ocean waves, especially the steep ocean waves of interest in this problem. Previous applications of Green-Naghdi theory have been limited to two dimensional problems and for wave systems without surface-piercing bodies because the implementation of the algebraic-intensive theory for these problems is easier. However, the slow drift force system for real, three-dimensional bodies is different in character from its two-dimensional counterpart. The basic problem was therefore to extend the GN theory to three dimensions and to allow for the flow about a real body. For the purposes of this study, the slow drift forces developed on a vertical cylinder exposed to long-crested, random waves in a flume were selected as the problem. This problem was chosen because there exists good experimental data to compare results.

The research conducted in this study investigated several different approaches to the problem. Initially it was hoped that the finite-element method would lead to a simpler formulation of the algebraic equations. However, the treatment of the boundary conditions on the cylinder were problematical.

The problem was reformulated as a finite difference scheme. To eliminate the need for a large number of grid lines and to eliminate the difficulties with the boundary conditions, an orthogonal curvilinear grid system was introduced, which included both the cylinder and the flume walls as boundary grid lines.

The most important single development was the development of the three-dimensional counterpart of the Thomas algorithm for advancing the computation one time step. This advance allows the stepping algorithm to be formulated as a sequential solution of sets of simultaneous equations of small dimensions rather than requiring the solution of one extremely large matrix. This approach permits the treatment to the slow drift problem on a high-end workstation rather than on a supercomputer.

Other developments included efficient techniques for treating the three-dimensional boundary conditions for the walls of the flume and the down-stream open boundary.

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Improved Fatigue Life for Moorings

University of California, San Diego
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R/OE-14
1990–92

Richard J. Seymour

Moorings are ubiquitous in our useful occupation of the ocean. They are used to secure instruments and other unattended apparatus for scientific, civil, and military uses and to fix the location for various lengths of time of manned platforms for mineral and hydrocarbon extraction and for marine transportation and ocean construction applications. Subject to wave and current loads, vibration from eddy shedding, and a hostile chemical environment, moorings fatigue and eventually fail if not replaced in time.

This research is directed toward significantly improving the reliability and the service life of ocean mooring systems by developing the use of nonconventional materials (particularly advanced composites) and constructions. The work will extend previous and present research by the investigators in highly related areas. Subscale mooring elements (lines, terminations, and flexures) will be tested for fatigue resistance in a seawater environment in the laboratory.

The overall project objective is to make substantial improvements in the service life and reliability of ocean moorings by using materials and constructions that are significantly less sensitive to fatigue and stress corrosion than conventional components.

This research follows from an initial National Science Foundation grant that led to the development of a promising material construction and its associated manufacturing processes. This construction consists of composite "wires" of continuous graphite fiber reinforcement in a matrix of Du Pont J-2 thermoplastic resin. Seven of the individual wires are then warm-formed (at a temperature high enough to allow deformation of the matrix without melting) into a twisted cable. This cable, in which the individual wires are free to slide over their neighbors, has a useful bending radius no greater than that of one wire, although it has potentially seven times the strength.

For this project there were four major goals. The primary goal was to develop efficient termination techniques (i.e., end fittings) for the existing seven-wire twisted graphite strand. The second goal, pursued in parallel with the first, was to develop a fatigue machine for testing the resulting strand/termination system. The third was to develop fatigue-resistant swivels to allow for 2 degrees of freedom motion of the tethers. The fourth was to begin the fatigue testing.

The first goal addresses a problem common to most composite structures loaded primarily in tension. That is, it is characteristically difficult to utilize the strength of the structure effectively because of limitations on the strength of the transition from the composite to some metal fitting. This fitting is often necessary in order to transition to another construction or to allow for replacement. It is usually difficult to terminate a rope without degradation of the basic strength. With graphite, because of its great strength and stiffness, the problems are enhanced. It is impossible to bend the rope around a small radius, and it is difficult, given the extreme stiffness of the material, to balance the load between all of the strands within the rope. As opposed to very elastic materials like Kevlar, graphite presents serious load sharing problems because it does not stretch very far before it breaks. Several types of industrial cable termination methods have been attempted and have been found to be inefficient when used on the graphite strand. A completely new method of termination, which melts the strand matrix in a plug-and-socket end-fitting, has been developed over the past year and has performed well. A custom-designed oven for thermoforming cable terminations was built to support these experiments. Following the recommendations of Dr. Francis Liu at Naval Civil Engineering Laboratory, we have improved the load sharing between wires by pretensioning each wire as the termination is made. The fitting along with added J2 material is then heated in an oven and the tapered plug pushed down until the wires are deformed into a solid ring filling the annulus between the plug and the socket.

We have used other novel termination methods, including resistive heating, but have found that they do not improve the thermoforming parameters achieved in the present method.

Fatigue failure of graphite tendons under heavy loading was much like that of the tensile test samples. Fractures in each case were sudden and catastrophic. Broken pieces of the failed tethers flew away at the instant of failure. Most of these pieces were still clearly composite, composed of both J-2 thermoplastic and carbon fiber. In each case the failure appears to be of a mechanical nature.

A different type of failure emerged with tethers at the lower load end of the fatigue loading scale. These samples, taking longer to fail, spent a significant time in the seawater bath and showed signs of significant composite breakdown. Individual carbon fibers could be seen separated from the composite matrix. Below the waterline, the J-2 thermoplastic disappeared, leaving only raw carbon fibers to support the load, while the composite structure above the waterline appeared to be intact. This mechanism of failure, which was first identified in this

project (Sloan 1991; Sloan et al; 1991, Sloan and Talbot; 1992). Sloan found that galvanic cells set up between the graphite and surrounding metal caused chemical reactions that degraded epoxy resins. It was assumed not to have been a problem in these tests because the resin did not contain the radicals that were attacked in Sloan's prior investigations, and because the major metal masses were isolated from the graphite by plastic insulators in the swivel attachments. Apparently, the small mass of metal in the termination is sufficient to cause the destructive reaction, and it will occur in the J-2 thermoplastic as well as in the epoxy thermoset resin. The obvious first answer is to incorporate electrical isolation within the termination itself.

The results of the fatigue tests showed that the strength of the graphite tendons in seawater does degrade faster (with fewer cycles) under larger varying fatigue loads as predicted by classical mechanics considerations, but that a second mechanism, which is time dependent and not cycle dependent, also affects those elements that remain in the seawater for a long time. This mechanism was not recognized before this research and is likely to be the most important element of this work.

Cooperating Organizations

The DuPont Corporation, Wilmington, Delaware
Texas A&M University, College Station, Texas
University of Texas, Austin, Texas

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Advanced Development of a 3-D Ultrasonic Imaging System

University of California, San Diego
Scripps Institution of Oceanography
R/OE-15
1990–91

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This report will chronicle progress that we have made in our California Sea Grant funded project "Advanced Development of a 3-D Ultrasonic Imaging System." The proposed work, as elaborated in our original proposal, contained several goals. First, we proposed to extend studies in our test tank facility to attempt to track animals in three-dimensions. Also, we proposed to develop a methodology for synthesizing complex sonar arrays using a relatively new polymer: PVDF.

During the first year of this project we performed several experiments that have allowed us to track single objects in three-dimensions in our test tank. This has been done using existing experimental apparatus. To perform the experiment, several goldfish were purchased in a local aquarium store and released in the test tank. Because these fish have a small air bladder to regulate their vertical position, they were considered to be good test targets. Also, their swimming speeds are usually slow enough so that the system frame rate (max = 10 frames/sec) would allow measurement of their three-dimensional trajectories without ambiguities in position.

A system consisting of a transmitting array of 2×4 transmitters and a single omnidirectional hydrophone for sensing was used. A special set of frequency hop codes that were previously designed (Jaffe et al., 1990), were used with some modifications. One aspect of our research has been to optimize the code design in order to generate a "best" set of signals in the water. Quantitative criteria for this best set consists of measuring both the coherent and incoherent cross correlations between the waveforms.

The system was calibrated by measuring the transmitted waveforms via positioning and recording from a small hydrophone in the far field of the array. Measurements were made on a 3×7 matrix that consisted of all positions both in the center of and in between the set of eight elements. The quality of the transmitted sound was judged by graphing a time varying spectral amplitude plot of the data. This was then compared to the theoretically predicted values. Excellent agreement was obtained, which indicated that both our transmit and receive electronics were functioning correctly.

In order to reduce the incoherent correlation between the codes, we experimented with various schemes. Application of a Hamming window (Richardson, 1989) was applied to each of the frequency "chips." This resulted in a large reduction in incoherent cross correlation in the experimental system. Unfortunately, one aspect of this window is that the individual code chips must be twice as long. This consumes more of the time-bandwidth product. A new technique of using intercalated code chips was invented in order to circumvent this difficulty. This resulted in equivalent autocorrelation properties; however, the cross correlation between the codes was more ambiguous.

In order to track individual animals in 3-D, the set of 21 waveforms that were recorded from the array were stored as a reference set. Subsequently, when an animal was in the field of view of the array, the set of eight transmitters were fired in fast sequence (~1/sec) and the set of reflected waveforms were digitized and recorded. Cross correlation of the reflected sound with the set of waveforms produced a very coarse (3×7) image, which depicted the degree of correlation of the reflected wave with each of the stored waveforms as a function of time. This produced our very first three-dimensional pictures. A reflective object was evidenced in three-dimensions at the approximate position of the fish. Moreover, this object could be easily tracked in three-dimensions.

We have also been pursuing our goal using PVDF for the design of complex array patterns. Toward this goal, our Sea Grant trainee, Duncan McGehee, has developed a new method for synthesizing PVDF arrays, placing them in a test facility, and measuring their beam patterns. First, the theoretical studies will be described, then the experimental methods will be elaborated.

In order to determine the best PVDF array pattern which matches a given desired beam pattern, we have developed two methods which allow the systematic design of a transmitting array given the desired pattern. In one case, a small set of transmitting elements is prescribed which "best approximates" the given desired pattern in a least squares sense. The algorithm that has been formulated allows the determination of both the size and the amplitudes of a finite number of transmitting elements. It has been proven (McGehee and Jaffe,1991) that the solution is a global minimum. That is, there are no other solutions that are a better fit in the least squares sense.

Another technique that we have been working on concerns the use of a random array which is systematically chosen—given, again, the design parameters for transmitting array. The empirical criteria that we have been using have permitted the fabrication of PVDF patterns consisting of a number of many small elements which, together, approach the desired array pattern. This has been validated in both theoretical studies and also by building a PVDF array whose output pattern agrees quite well with the theory.

In order to fabricate the PVDF transducer elements, it was decided that a method should be developed which allowed rapid testing our design results. By experimentation, a method was developed using a sheet of copper that could be etched free of the PVDF backing and then sandwiched between two layers of PVDF. This method was tested and seemed to work quite well. Now, the photoetching step can be separated from the rest of the procedure, and different parameters in creating the conductive photoetched layer can be tested without regard to the PVDF. This has facilitated a more rapid design cycle and also allowed us more flexibility in our experimental process. A paper recently authored on these subjects has been accepted for publication (McGehee and Jaffe, 1993).

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Results of Damaged Offshore Structural Steel Members Using Grouting

University of California, San Diego
R/OE-16
1991–92

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Damage to offshore drilling and production platforms is a topic of increasing concern in the United States and around the world because of the extraordinarily high stakes involved in terms of lost lives, environmental devastation, and wasted resources.

There are presently over 3,500 major platforms in U.S. waters, 25 of which lie off the coast of California (California Sea Grant College, 1992). A majority of these structures were designed for a 20-year life period, and are still in operation today after 30 and even 40 years. The cost to replace them is prohibitive, and economic realities thus create strong incentives to develop ways to rehabilitate aging platforms and even to increase their capacity.

Decisions regarding the safety of a damaged platform and the need for repair are derived from assessment of the residual strength of the damaged members. Operators or certifying officials use the results to determine what type and magnitude of repair is required, if any.

Grout has been used extensively in offshore applications because of its great strength, ease of placement, and low cost. Traditional repair techniques available to operators have included the use of bolted sections, welded sections, and clamps. Grouted repair offers a viable alternative to these repair methods, for it involves neither underwater welding nor the highly precise fabrication of repair components. There are two types of grouted repairs used for repair of damaged members. These include internal grouting and grouted steel clamps. Both are relatively simple repair techniques that do not require extensive amounts of work offshore.

Decisions regarding safety of a damaged platform and the need for repair depend on the critical assessment of the residual strength of damaged members under combined axial and bending loads. Previous analytical and experimental investigations into this problem have shown that even minor damage levels in the form of denting can cause substantial degradation in member strength.

Objectives

There is a lack of information on the strength and performance of damaged bracing members that have been repaired with grout. Practicing engineers have been designing grouted repairs based on extrapolated data from applications other than the repair of damaged members. Experimental and analytical work would verify the amount of increased load-carrying capacity produced by the grouted repair techniques. Therefore, a research study was conducted to determine the residual strength of damaged bracing members, and to assess the increased strength, if any, derived from grouted repair techniques.

The objectives of the research program were the following: (1) To experimentally investigate the residual strength of dent-damaged tubular steel bracing under direct axial loading and combined loading conditions through end eccentricity; (2) To assess present strength equations and analytical methods, as well as formulate new strength equations, for predicting the residual strength of dent-damaged tubular steel bracing under direct axial loading and combined loading conditions; (3) To experimentally investigate the use of internal grouting and external grouted steel clamps, respectively, in repairing and improving the residual strength of dent-damaged tubular steel bracing under combined loading; (4) To investigate experimentally the ultimate strength of nondamaged tubular steel bracing under combined loading in order to establish a baseline for assessing the strength of dent-damaged bracing; and (5) To perform preliminary analytical investigations using nonlinear finite element techniques to assess the applicability of the method in predicting damaged member strength.

Experimental Program

The size of the test specimens was chosen to closely represent a large-scale model of a typical diagonal bracing member found in fixed platforms in U.S. waters. Values for the slenderness ratio (kL/r) and the column slenderness parameter (λ) were selected from typical design parameters and set at 60 and 0.75, respectively. Representative outer diameter D to wall thickness t ratios (D/t) of 34, 45, and 64 were tested. The specimens represent two-thirds to full-scale models of typical diagonal bracing, depending on platform geographic location and water depth.

Structural analysis of platforms indicated that the ratio of member flexural force to axial force is equal to 0.20 D. Therefore, for the combined loading tests, the axial load was applied through an end eccentricity of 0.20 D from the centerline of the specimen. The specimen was loaded in single curvature to induce bending at the dented section, and was considered a worst-case scenario, where the dent was in compression.

To achieve the desired objectives, five series of tests were conducted in this study.

Series 1. Testing of dent-damaged tubular bracing under direct axial load, one dented specimen from each D/t group, in order to assess unrepaired residual strength.

Series 2. Testing of dent-damaged tubular bracing under combined loading, one dented specimen for each D/t group, in order to

assess unrepaired residual strength under such conditions.

Series 3. Testing of internally grout repaired specimens. A specimen from each D/t group was damaged, then repaired by internally grouting the full length of the member. Each specimen was tested under combined loading.

Series 4. Testing of a grouted steel clamp specimen with $D/t = 64$ under combined loading. A specimen with $D/t = 64$ was selected because of the member's susceptibility to fail under local buckling rather than column buckling. It was presumed that if this group could be strengthened by this means of repair, then the two lower D/t groups could be repaired by this means as well.

Series 5. Testing of a control set of nondamaged specimens, one from each D/t group under combined loading to provide baseline data for comparison with unrepaired and repaired specimen behavior.

A total of 13 tests were conducted. The experimental test matrix is presented in Table 1.

Table 1. Experimental Test Matrix

Spec. No.	$\frac{D}{t}$	End Eccentricity e	Dent Depth d_d	Description of Specimen
A1	34.5	0	0.10 D	Damaged, Nonrepaired
B1	46	0	0.10 D	Damaged, Nonrepaired
C1	64	0	0.10 D	Damaged, Nonrepaired
A2	34.5	0.20 D	0.10 D	Damaged, Nonrepaired
B2	46	0.20 D	0.10 D	Damaged, Nonrepaired
C2	64	0.20 D	0.10 D	Damaged, Nonrepaired
A3	34.5	0.20 D	0.10 D	Damaged, Internal Grout Repair
B3	46	0.20 D	0.10 D	Damaged, Internal Grout Repair
C3	64	0.20 D	0.10 D	Damaged, Internal Grout Repair
C4	64	0.20 D	0.10 D	Damaged, Grout Clamp Repair
A5	34.5	0.20 D	0	Nondamaged
B5	46	0.20 D	0	Nondamaged
C5	64	0.20 D	0	Nondamaged

Internal Grout Repair

The expected benefits when applying internal grout repair to dent-damaged bracing members arise from the arrest of a growth in the dent depth that has been shown to occur during loading of dented members in past experimental research (Taby, 1986). Ultimate load capacity is thus increased by eliminating this mode of failure. The ultimate capacity of internally grouted members can be estimated assuming composite action between the steel member and the grout.

Grouted Clamp Repair

A conservative design philosophy was taken in designing the clamp to be installed and tested. The steel clamp was designed to replace the lost capacity of the dented member, by completely replacing the axial strength of a nondamaged section of the dented tube.

A full design of a working grouted clamp was completed for the required clamp length and specimen geometry of this test program. The clamp was modeled after standard clamp technology presently used in offshore applications. The design consists of two pieces mated together about the dented section through bolted flange plates.

Test Setup

The 500 kip self-reacting test frame shown schematically in Figure 1 was designed and fabricated for the purpose of testing the specimens under compressive load. Care was taken in the development of the design criteria for the test frame to appropriately model assumed experimental parameters including pinned-end conditions, various combinations of applied axial load and bending, unrestrained rotation of specimen ends about any axis, and sufficient test frame capacity. Components of the test frame include: a reinforced concrete reaction block, a sliding load beam located on frictionless wheel casters and directed by a reinforced concrete guide block, two frictionless precision machined ball-and-socket bearing connections, and two high strength tension rods.

Experimental Behavior

Nondamaged Specimen Behavior (Series 5). Three specimens were tested without damage and subjected to combined axial and bending loads to provide baseline comparisons for damaged member tests. This series provided a means to evaluate present design criteria, including API's (American Petroleum Institute, 1989) allowable stress design (ASD) method, and the load and resistance factored design (LRFD) method.

Yielding initiated along the compression face near peak axial load. The nondamaged specimens all developed a local buckle in the tube wall at or near the midspan on the compression face after reaching their peak load. Slight curvature occurred in the specimens until after the peak load was reached. The local buckle transformed into major cross-section distortion because of the high D/t ratio. This failure mode is safeguarded against in API design by designating substitution of local buckling stresses into column buckling equations for members with D/t ratios greater than 60.

Damaged, Nonrepaired Specimen Behavior (Series 1 and 2). A total of six tests were conducted to assess the effect of combined axial and bending loads and direct axial load on damaged specimen behavior. One specimen of each D/t group was tested under direct axial load, and one under combined axial and bending load.

The specimens subjected to direct axial load attained higher ultimate capacities than did those subjected to combined axial and bending loads through an end eccentricity. The dent initially resisted applied loads through development of compressive strain. All six specimens failed shortly after yielding occurred in the saddle of the dent, where the axial load resistance deteriorated with continued axial shortening. Upon failure, the dent consistently was shown to rapidly grow inwards as shown in Figure 2.

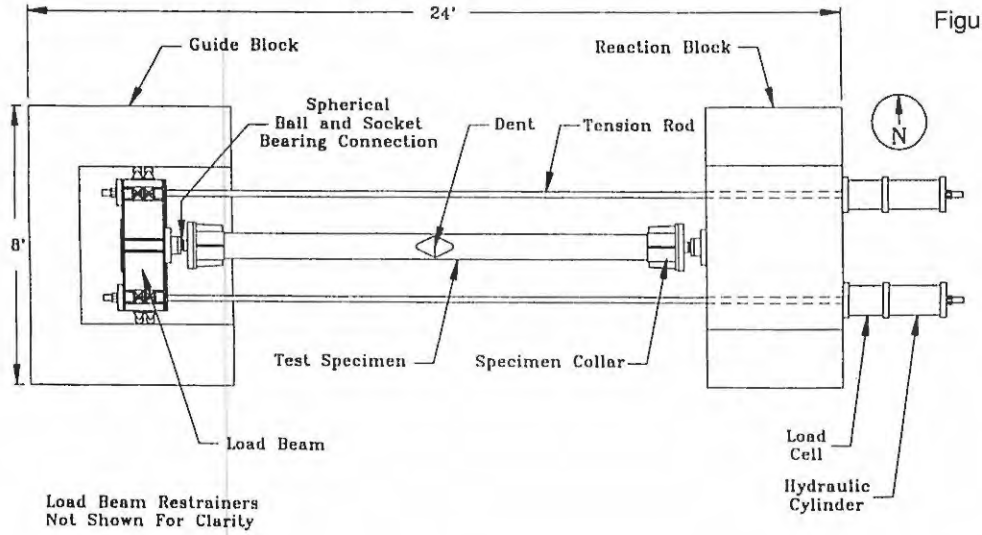

Figure 1. Plan view of test frame

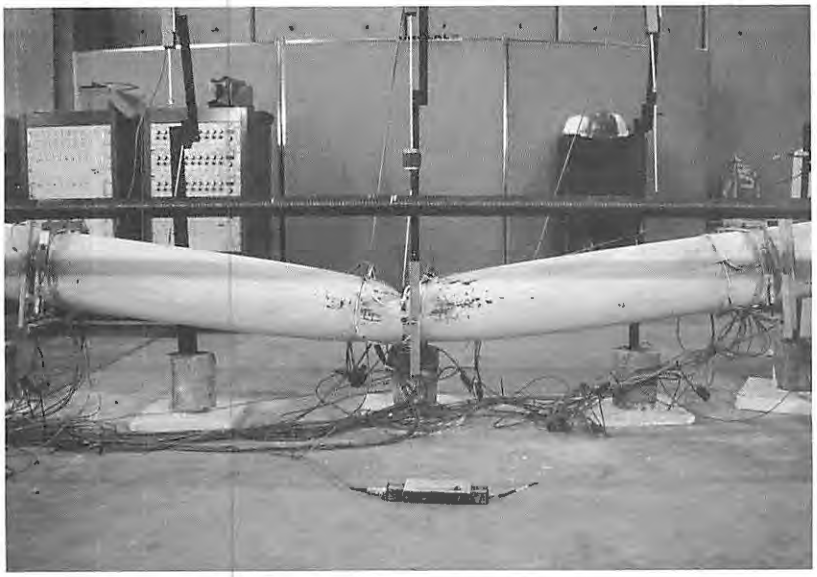

Figure 2. Two views of a test specimen following the formation of a plastic hinge at the dented section.

Damaged, Internal Grout Repaired Specimen Behavior (Series 3). Three damaged specimens were tested after having been repaired by internal grouting. The grout extended the full length of the specimen. One specimen from each D/t group was repaired and tested under combined loading to assess the performance of this technique.

Internal grout repair significantly restored the load carrying capacity of the tested specimens through arrest of the dent growth. The grout supported the dented section, thus eliminating this mechanism of failure. The specimens developed their peak axial load as the steel wall yielded in the dent saddle and along the compression face of the tube. An outward local buckle formed at each end of the dent after the peak load was developed in the specimen.

Damaged, Grouted Clamp Repaired Specimen Behavior (Series 4). One damaged specimen was repaired by placement of a grouted steel sleeve around the damaged section of the member. The repair technique was assessed by testing the specimen under combined axial and bending loads through end eccentricity.

The grouted sleeve was effective in repairing the damaged specimen of D/t ratio 64 by restoring the load carrying capacity. The sleeve averted the mechanism of failure observed in nonrepaired specimens by resisting the dent growth through containment of the tube cross section. Ovaling of the cross section was observed to follow dent growth in the earlier nonrepaired specimens. By resisting the ovaling, the dent was not allowed to grow and the mode of failure was transferred to outside the clamp.

Summary of Tests

Five series of tests, consisting of 13 steel tubular braces of various diameter to thickness (D/t) ratios, were tested to examine the effect of a dent damage of 0.10 D depth on their residual strength and to assess the effectiveness of internal grout repair and grouted steel clamp repair techniques. The braces were subjected to either direct axial loading or combined axial and bending loads through an end eccentricity of 0.20 D/t. Comparisons between the test series allowed direct evaluation of the effect of dent damage on specimens subjected to concentric and combined loading, the repaired residual strength of internal grout repaired specimens, and the repaired strength of a grouted steel clamp repaired specimen. Various existing analytical techniques and newly developed formulations used for predicting the residual strength of dent-damaged tubular braces were also assessed through comparison with experimental results.

Conclusions

Based on the study presented here, the following results are given: (1) A dent depth of 0.10 D leads to significant loss of strength; (2) Effects of an end eccentricity of 0.20 D on the residual strength is pronounced; (3) Internal grout repair of a 0.10 D dent-damaged brace is successful in reinstating the original nondamaged member strength by arresting the dent; (4) Grouted steel clamp repair of a 0.10 D dent damaged brace is successful in reinstating the original nondamaged member strength by restraining the cross section from ovaling, thereby preventing the dent from growing inwards; (5) Modification of strength equations for additional eccentricity of the applied axial load provides a lower bound on residual strength; (6) A computer program developed as part of this project agrees reasonably well with test results, providing a lower bound prediction for ultimate load capacity, and shows correct trends in relationships between residual strength and dent depth; (7) Preliminary nonlinear finite element analyses show that the method is effective in modeling the experimental tests, but care must be taken in the description of the material properties and refinement of the mesh; (8) The residual strength of the nonrepaired, damaged specimens is closely predicted by DENTA (an industry standard computer program); (9) The unity check method for dented members was found to agree closely with the experimental results, where the stability check controlled; and (10) Formulations for predicting the strength of internally grout repaired members was found to provide a reasonably close lower bound for the strength of the internally grout repaired specimens.

Cooperating Organizations

California State Lands Commission, Long Beach and Sacramento
Chevron Oil Company, La Habra, California
Hexcal, Dublin, California
National Science Foundation
Unocal Oil Company, Brea, California and Bangkok, Thailand

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Management of Human Error in Operations of Marine Systems

University of California, Berkeley
R/OE-17
1990–93

Robert G. Bea and William H. Moore

Generally, more than 80% of high-consequence marine disasters are caused by human and organizational errors (Bea, 1989). Recent examples include the grounding of the oil tanker the *Exxon Valdez*, (258,000 barrels of crude oil spilled) and the explosion and fire on the offshore oil platform *Piper Alpha* in the North Sea (167 workers killed). At the start of this research in 1990, engineers, operators, and regulators of marine systems had no structured, quantitative approaches to help them design systems that would be tolerant of these errors or include considerations of such errors in the design, construction, and operation of marine systems. The human element has generally been ignored.

The objective of our research was to develop engineering procedures that can be used to measure, assess, and manage human and organizational errors in the operation of tankers and offshore platforms. The most important goals were to understand how checks and balances can be put in place to reduce the prevalence of these errors and to learn how to take advantage of "early warning signs" to stop these errors before they become catastrophic (Construction Industry Research and Information Association, 1977; Dougherty and Fragola, 1986; Offshore Certification Bureau, 1988). The results are intended to be used by engineers, operators, managers, and regulators concerned with preventing and mitigating the effects of human and organizational errors, and thus increase the safety of marine systems.

At the present time, the California State Lands Commission and Chevron Shipping, in conjunction with the Department of Naval Architecture and Offshore Engineering at the University of California at Berkeley, are engaged in a Sea Grant-sponsored project designed both to verify and enhance the modeling procedures established in this research. In addition, Amoco Production Company has used the modeling procedure described in this paper to study the impacts of human and organizational-related factors for transporting mobile offshore drilling structures around the world. Work has also been initiated by the U.S. Ship Structure Committee to investigate how these methods might be applied to the design and construction of commercial ships.

Methods

The approach used can be divided into five primary tasks. Figure 1 shows the interrelation of these tasks.

The first task was to collect and analyze well-documented case histories of accidents on tankers and offshore platforms in which human and organizational errors were the root cause. Accident investigation reports prepared by the U.S. Coast Guard, Minerals Management Service, U.S. Geological Survey, and the National Transportation Safety Board, and material provided by United States ship and platform operators were the main sources of information. Accident investigation reports compiled by the Canadian Royal Commission (capsizing of the *Ocean Ranger*), the U.K. Department of Energy (*Piper Alpha* fires and explosions), and the Norwegian Petroleum Directorate (sinking of the *Alexander Keilland*) were obtained to provide additional case histories. Casualty data bases supplied by the U.S. Coast Guard, Veritec, and the Institut du Français Petrole provided the most current data base information relating human errors to marine casualties.

The second task was to develop a system for classifying and characterizing human and organizational errors. Classifications used by the marine industry were reviewed, and a practical system was developed for the modeling framework to follow.

The third task was to develop models that could be used to characterize how human and organizational errors interacted to cause the accidents. Influence diagrams (Bea, 1989; Paté-Cornell and Bea, 1989) provided the basic analytical framework. Simplified and generalized "templates" for tanker and offshore operations were developed. These templates preserved the central causative mechanisms rather than the unique aspects of a particular disaster.

The fourth task was to formulate analytical methods based on quantitative risk analysis. Procedures used for quantitative risk analysis included measuring techniques (probability encoding) and a probabilistic–heuristic approach to calculating a "safety index." The safety index (or, conversely, the risk index) provides a relative measure of human errors influenced by events, decisions, actions, environmental inhibitors, system and task complexities, stress, and routine. These quantitative data and the templates were then used to measure the influence of human errors on the likelihood that an accident would occur in various scenarios.

The fifth task was to establish methods and criteria for determining how well various alternatives reduced the prevalence and effects of human and organizational errors. Alternatives are generated through regulatory, operator, and heuristic judgments. The costs and benefits of various alternatives were determined, as well as acceptable risks. Two well-documented case studies

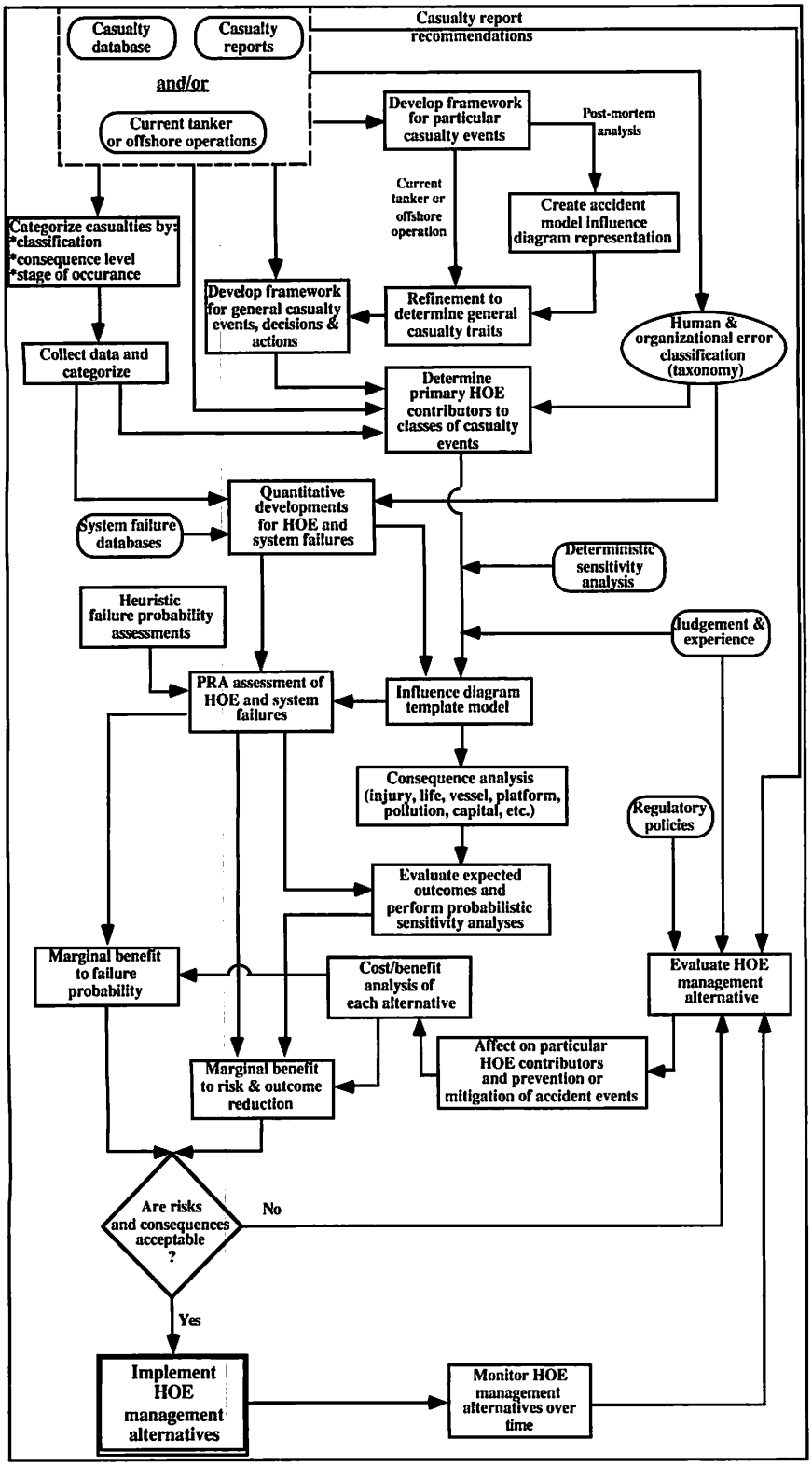

Figure 1. Flow chart of human and organizational errors (HOEs).

were used to illustrate the processes associated with developing effective strategies for managing human and organizational errors in operations of tankers and offshore platforms.

Results

During the first year of the three-year project, we completed the first task.

An analysis of the written accident reports and computerized data indicated that both were incorrect and insufficient in capturing the critical factors that lead to accidents. Much of the information focused on technical and system-related factors. Little was included on the complex human and organizational interactions involved. This was the result of poor understanding and knowledge of the complex interactions between humans, organizations, and systems. Because the existing data could not be used for our type of analysis, other methods of quantification were developed.

The classification system we developed (the second task) is based on previously proposed systems developed by the U.S. Coast Guard. To these we added several key measures of human and organizational behavior and performance, including violations, commitments to safety, and allocations of safety resources. Thirteen errors were detected and categorized into human, organizational, regulatory, and system-related factors (Figure 2). Each error was affected by two critical top-level management factors: *commitment to safety* and *resources*. These two factors permeate all levels of an operation or organization and were taken into account.

Figures 3–6 are the models developed to show the interactions of human and organizational errors in accidents involving oil tankers and offshore platforms. The models cannot be used to predict a particular accident sequence. Instead, they describe a general set of accident factors. For models of previous accidents, the templates do not preserve the unique aspects of the disaster but do include the general factors related to that particular class of accident (e.g. groundings and

collisions of oil tankers and high-pressure gas fires on offshore platforms, as shown in Figures 4 and 5). Models of existing operations were developed from knowledge and experience to illustrate potential casualty scenarios. An advantage of using influence diagram templates to model the interactions of contributing factors in marine-related casualties is that no quantitative analysis is required.

To reliably quantify human and organizational factors in marine-related casualties (fourth task), we developed a measuring technique called the human error safety index method. This method is used to measure organizational, task, and system complexities and environmental factors that affect an operator's ability to make decisions and take actions to prevent or mitigate accidents. Judgments and experiences of those familiar with the operations being modeled and objective data are used to formulate a safety index.

We also developed a method for collecting the objective data, the human and organizational error data quantification system. This method provides a way to update the information on the prevalence of human errors. The strength of this system is that it is self-correcting and can be updated and refined. The safety index method is used to determine the impact of organizational, system, and task complexities; stress; routineness; and environmental conditions on human errors and how these factors affect an accident, that is risk of failure of a system or operation. The safety index method and the data quantification system are used to update information on how often an error occurs, and the results are used to update the failure event index. That index can then be matched against the probability that a particular type of accident will occur. The functional relationship between the risk index and the probability of the accident is then determined. This enables us to forecast the likelihood that an accident will occur under various human operator conditions and thus to determine if these conditions are associated with an acceptable level of risk.

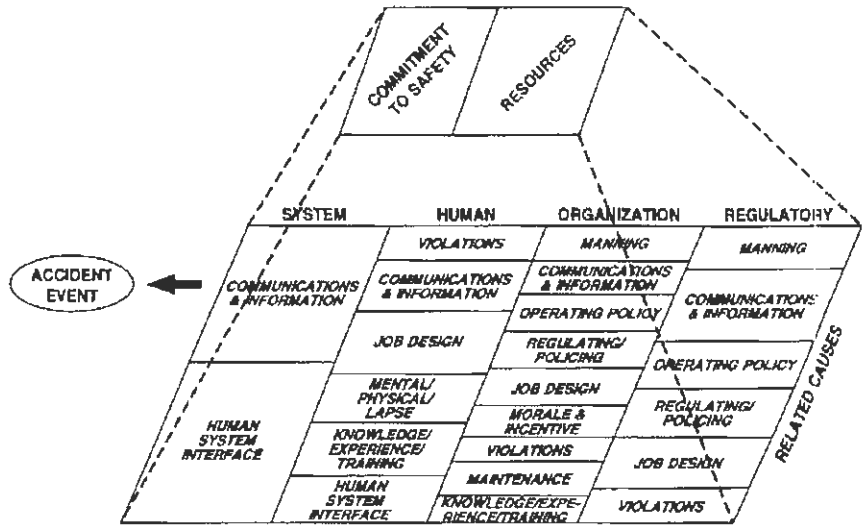

Figure 2. Classification of human and organizational errors significant in marine accidents.

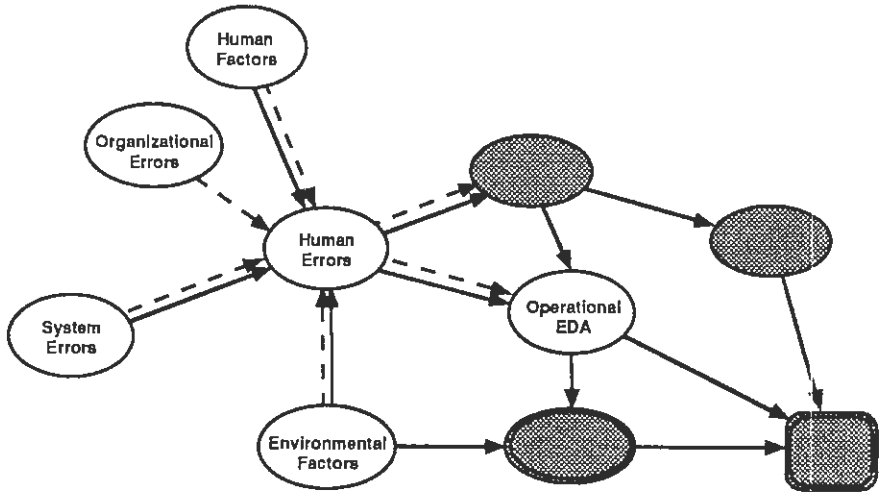

Figure 3. Influence diagram model of human errors.

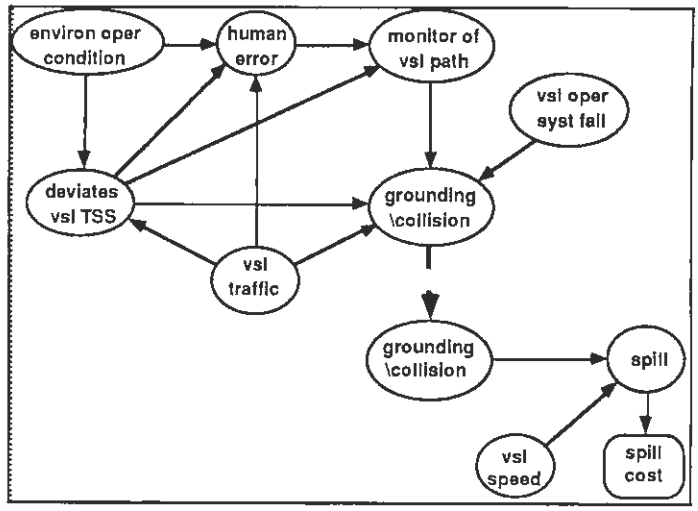

Figure 4. Influence diagram model of contributing factors for the grounding or collision of an oil tanker.

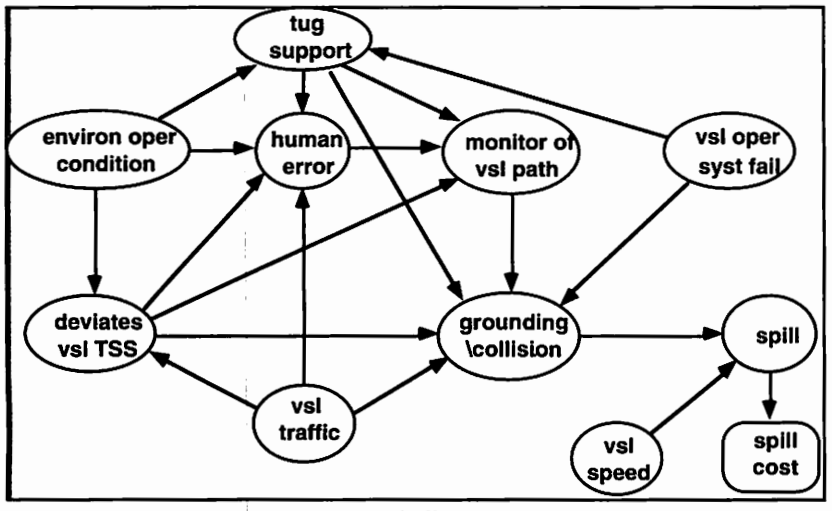

Figure 5. Influence diagram model of effect of tug support on grounding or collison of an oil tanker. See Figure 4 for explanation of abbreviations and acronyms.

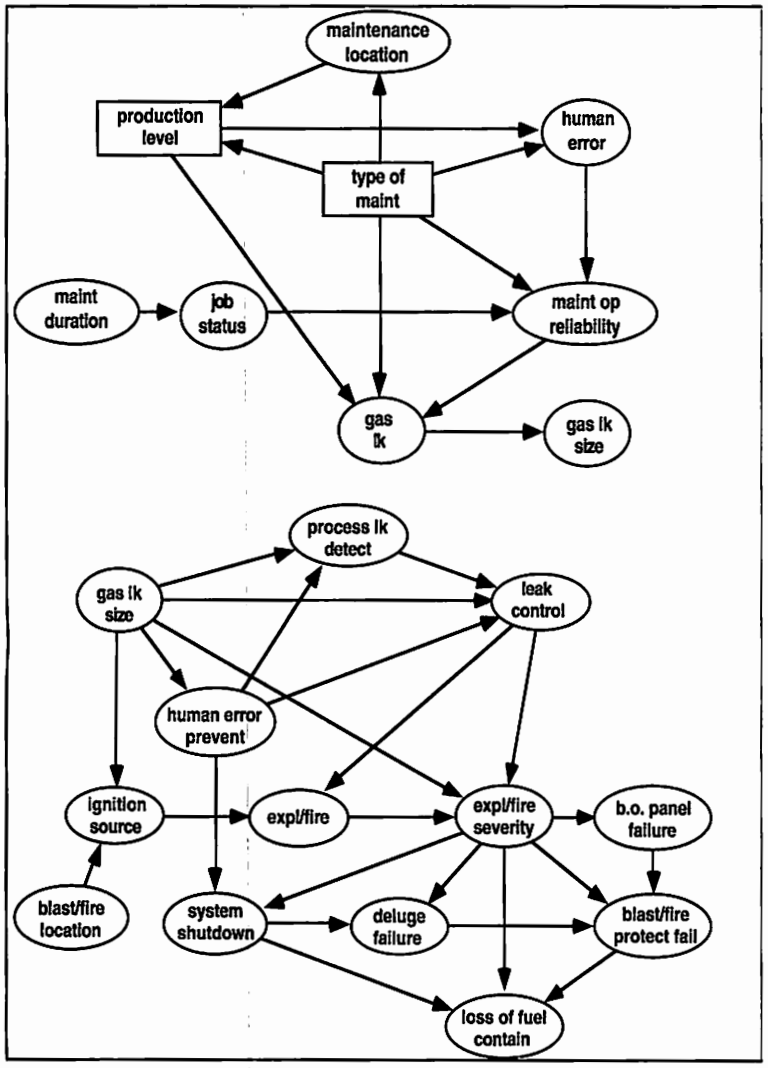

Figure 6. Influence diagram of eventual effects of offshore gas maintenance on a leak leading to loss of fuel containment.

In the first phase of the fifth task, statistics on the number of times an error occurred, influence diagrams, and cost-benefit analysis were selected as methods for evaluating alternatives that could be used to decrease human and organizational errors. The concept of "acceptable risk" was presented from two points of view: historical (what was acceptable to society at the time of interest) and *standards of practice* (the state of technology and the ability to prevent these accidents).

The next phase of this task was to illustrate the use of these methods. We analyzed two well-documented case studies: the grounding of *Exxon Valdez* and the *Piper Alpha* disaster. The models developed for these two accidents were used as a framework to construct general models for two classes of marine casualties: (1) tanker groundings or collisions and (2) leaks from offshore platforms due to simultaneous production and maintenance. For evaluation of alternatives, two illustrations were provided: (1) tug escorts for tankers (see Figure 5) and, (2) detection and control of gas leaks for production platforms.

Additional models were constructed for currently existing tanker and offshore operations: loading and discharge of tankers (Figure 7) and crane operations for offshore platforms (Figure 8). Examples of alternatives that might decrease human and organizational errors were provided for each of these models. For tanker loading and discharge, the addition of a deck master for offshore spread mooring facilities was examined. For crane operations, the relative impact of camera monitoring systems in decreasing human errors and the consequences of these errors was considered.

Discussion

Human errors are influenced by a wide range of individual characteristics and states, including fatigue, negligence, ignorance, greed, folly, wishful thinking, mischief, laziness, excessive use of drugs, bad judgment, carelessness, physical limitations, boredom, and inadequate training. External (to the system) and

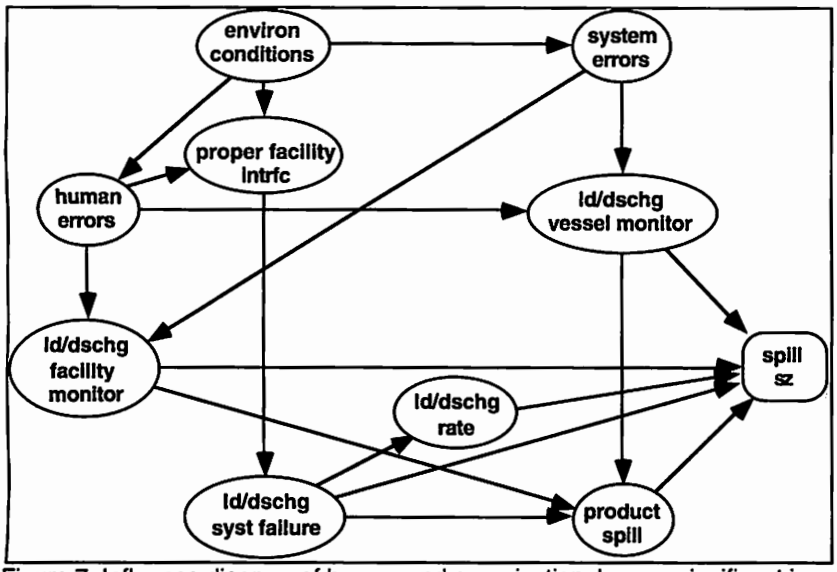

Figure 7. Influence diagram of human and organizational errors significant in an accident involving loading and discharge of an oil tanker.

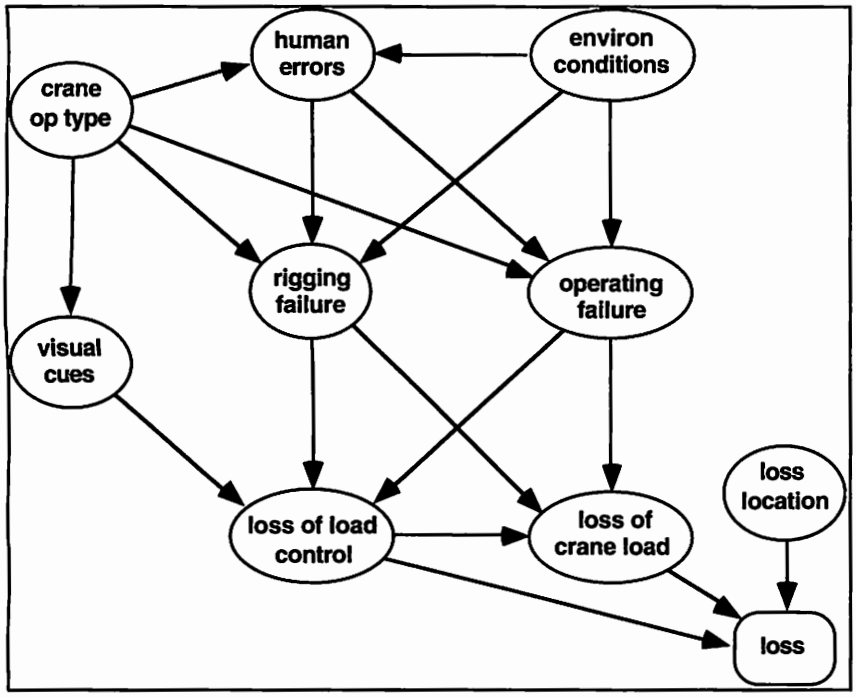

Figure 8. Influence diagram of human and organizational errors significant in an accident involving an offshore crane accident.

internal (in the system) environmental factors such as adverse weather, time of day, smoke, and temperature are additional factors. Selection of personnel (determination of an employee's ability to handle the job), training (particularly crisis management), licensing, discipline, verification and checking, and job design, all provide avenues for improving the performance of frontline operators.

Although human factors and the characteristics of a system are important, organizational aspects are frequently overriding. For instance, a focus on production at the expense of quality, ineffective and stifled communications, lack of the commitment and resources needed to achieve quality, excessive emphasis on time and profit, conflicting corporate objectives, and incentives that counter quality and integrity are often present in "low reliability" organizations. Generally, these aspects are the most difficult to address. Experience indicates that high-reliability organizations tend to improve, whereas low-reliability organizations do not improve or improve slowly.

The most important part of evaluating human and organizational errors is *qualitative*. A realistic and detailed understanding of the human, organizational, and system aspects involved, and their potential interactions must underlie the entire process. Quantitative aspects provide an important framework for evaluating the potential effectiveness of proposed "fixes" and for examining the detailed interactions of human, organizational, and system components.

No accepted and established data base provides accurate quantitative information on the contribution of human and organizational errors in marine accidents. In the case of specific accidents, existing data bases frequently give misleading indications of causes and consequences. Complex interactions frequently are undetermined or are lost in the reporting process. Study of past accidents can provide important insights into the complex interactions between humans, organizations, and systems and a basis for developing generic "templates" for evaluating similar systems. Study of near misses can show how potentially catastrophic sequences of actions and events can be interrupted and brought under control. No data base or archiving system for information on near misses is generally available.

As a result of this Sea Grant research, we have developed an adequate and understandable quantitative system for evaluating the contributions of human and organizational errors in marine accidents. Probability-based influence diagrams show the complex interactions and influences, and efficiently produce quantitative indexes that can indicate the effectiveness of various alternatives designed to decrease

the number of human and organizational errors. Because of the lack of accurate and definitive objective data for the quantitative models, structured safety index models have been developed so that subjective judgments can be included in the evaluation of probabilities. The human and organizational error data quantification system provides a structured framework that can be used to examine the complex interactions between events, environmental factors, systems, task complexity, and human and organizational errors. As yet, no method has been established for documenting casualties and near misses. Even the information that is currently being documented excludes critical factors such as minor and major violations that lead to accidents.

The system we developed for classifying human and organizational errors is reasonable and workable. It should provide a basis for developing a system for reporting accidents that involve marine operations. Investigators need to be well trained in the evaluation of human and organizational factors in marine accidents. An industry-wide computer data base needs to be developed to improve the efficiency of accident reporting and the analysis of results. Information on both accidents and near misses should be included in this data base.

Producing numbers should not be the primary objective in analyzing human and organizational errors. The primary objective should be to provide a disciplined and structured framework. This can then be used to produce insights and information for improving methods used to decrease the number of errors. Even in the absence of reliable data, quantitative assessments can be made. Judgments and experiences of operators, managers, regulators, and other decision makers are important to the decision process. Techniques for quantifying human and organizational errors, such as the safety index method, can provide valuable guidelines. If consistent methods are used in quantitative measuring, reliable comparisons between various alternatives for decreasing these errors can be assessed.

If we are to substantially improve the safety of marine systems, we must learn how to better manage the human elements of these systems. This work provides a disciplined and structured procedure to help identify and evaluate alternatives in the design of systems that are more tolerant of human error, and that better manage the human elements.

Cooperating Organizations

American Bureau of Shipping
Amoco Production Company
Amoco Transport Company
California State Lands Commission
Chevron Shipping Company
Chevron Research & Technology Company
Minerals Management Service—U.S. Department of the Interior
U.S. Coast Guard—Department of Transportation
Unocal Corporation

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Salinity-Powered Distillation of Freshwater from Seawater Using Plastic Sheet Heat Exchangers

San Diego State University
R/OE-18
1990–92

Preston Lowrey

Salinity concentration differences represent a large energy resource that has received relatively little research to date. What research has occurred (Isshiki, 1977; Nadev and Ophir, 1982; Olsson et al., 1979; Olsson, 1982; Seymour and Lowrey, 1992) has aimed at developing power. This research, instead, demonstrates that the concentration difference between seawater and saturated brine derived from seawater can drive the distillation of fresh water from a second stream of seawater (Ibrahim, 1991; Ibrahim and Lowrey, 1992). This could find wide application in arid seashore environments. Unlike conventional distillation, this approach would not need a fuel source so it could help reduce CO_2 production.

San Diego State University has engaged a patent consultant to contact industries and market this invention.

Objectives

Objectives for the first year were (a) to develop a detailed computer model of the governing heat and mass transfer, and (b) to design and test a batch-mode, proof-of-concept, laboratory model of salinity-powered distillation.

Objectives for the second year were (a) to refine the computer model developed in year 1, and (b) to design a scaled-up version of the device that would operate with flow-through (falling film) features.

Though not specified as objectives, we were also interested in evaluating candidate plastics, rather than metal, for the heat exchangers and projecting costs to the extent possible.

All of the specified project objectives have been met. To get this far we have wrestled with many problems, and I am indebted to the dedication of three master's students and three student volunteers.

Both objectives (a) and (b) for the first year were completed.

The computer model assumed a vertical falling film configuration. The films of seawater and brine fell side by side down the same sheet, separated by a perforated partition. This virtually eliminated both convective and radiation heat losses from one film to another. Heat losses had been a problem in the original configuration considered, which had the two falling films facing each other and separated by a small gap.

At the end of the first year of work our efforts increasingly assumed $MgCl_2$ would be the required brine solution since it was three times as effective as NaCl. In addition, an $MgCl_2$-rich solution called bittern (density 1.25) is widely available around the world as a waste product from the solar production of NaCl from seawater. If bittern is further concentrated by evaporation to density 1.35, most other salts precipitate out, leaving $MgCl_2$ of a crude purity (Hull et al., 1989). A simple, inexpensive, device to reconcentrate bittern in arid environments has been developed (Assaf, 1984).

In the original research proposal it was argued that the dominant heat transfer resistances are conduction through the liquid films coating both sides of the heat exchangers, so a good conductor like metal may not be required. Also, metal may be restricted by the corrosive saline environment. Our more complete computer model predicted that heat exchanger sheets made of 1 mil thick plastic would produce 90% of the fresh water possible with a metal heat exchanger. At this time, we had not, however, considered the ability of different materials to stay wetted by a falling film. This impacts performance and is discussed later.

Parametric studies with the computer code showed that the seawater and probably the brine flow rates should be kept as low as possible (see Figure 1, Ibrahim and Lowrey, 1992; Brauner et. al., 1986, 1987). For seawater this gives nearly the greatest fresh water production, with the minimum internal pumping power. For brine, increasing the flow rate fivefold increases fresh water production only 32%, which is probably not worth the pumping cost.

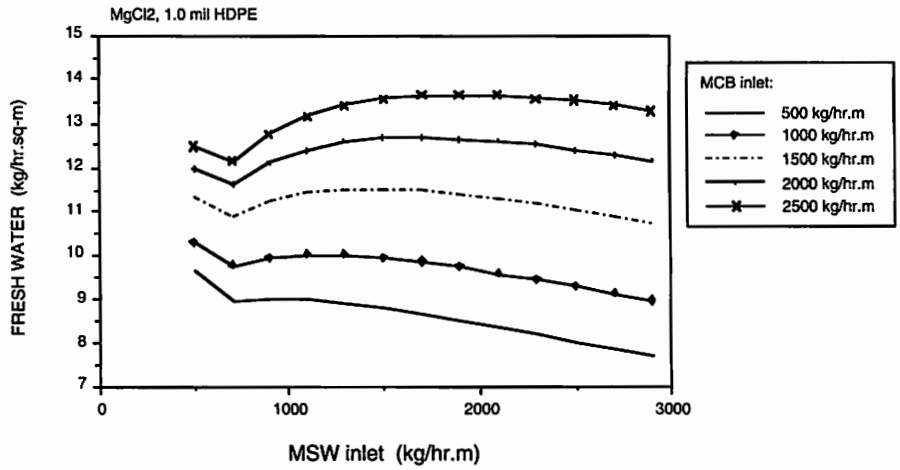

Figure 1. Variation of fresh water production with varying inlet flow-rates of seawaters (SW1 and SW2) and brine (CB).

Experimental Work

A first generation laboratory model was successfully constructed and debugged and produced fresh water in multiple runs using both aluminum and plastic heat exchangers (see Table 1). Getting the device to work involved multiple iterations. This work used four chambers fashioned from two aluminum pipes and interconnected by tygon tubing. Plexiglas view ports sealed with o-rings were set in the ends of the pipe. To reduce penetrations of the vacuum, the solutions were all stirred with magnetic stirrers. Important lessons absorbed were that everything had to be thoroughly insulated from the room, that small amounts of residual air could cripple performance, and that thermal transient as the operational temperature distribution developed could not be overlooked.

In theory the temperature increase could approach 12°C but did not because of heat losses to the room and pressure drops in the tubing. To project this, the uncorrected column was multiplied by (12°C/the actual temperature elevation) to give the corrected column.

Table 1. Year 1 Experimental Fresh Water Production Rates per Square Meter of Effective Heat Exchanger

	Heat Exchanger		Temperature	Uncorrected	Corrected
Run	1	2	Elevation	kg/hr.m^2	kg/hr.m^2
A	Aluminum	Aluminum	6.1°C	1.06	2.09
B	Aluminum	Aluminum	6.1°C	1.04	2.05
C	Aluminum	Aluminum	5.6°C	0.94	2.01
D	HDPE	Aluminum	7.8°C	1.18	1.82
E	HDPE	HDPE	5.6°C	0.78	1.67

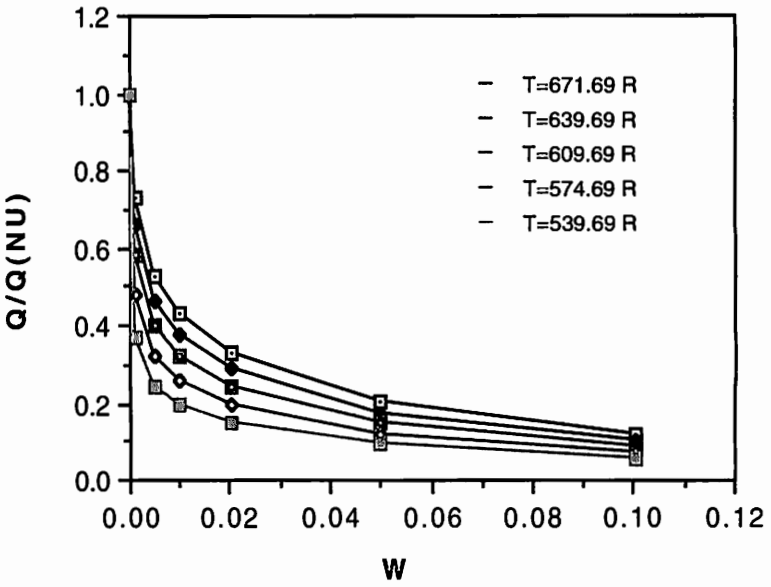

Figure 2. The air effect (W = % air) at different concentrations and saturation temperatures.

Year 2

The computer model was refined as follows. The thermodynamic data were expanded so that the model would work at higher temperatures. Production is faster at higher temperatures. It is necessary, however, to use the exiting spent streams of brine and seawater to preheat the incoming supply streams, and this involves heat losses. It was decided to moderately elevate the operational temperature to about 30–35°C.

A model for the brine reconcentration showers was included (Assaf,1984). These require pumping power. Consequently, the machine's internal pumping power and the reconcentration pumping could be optimized simultaneously.

In the first year of modeling we had assumed the absence of noncondensible gases, chiefly air, which comes out of solution or leaks into vacuum chambers. Figure 2, showing the large effect of noncondensibles (air), is adapted from Minkowycz and Sparrow (1966). Notice that a very small amount of air, 0.01% of the already thin steam (about 10^{-5} atm of air), can reduce the performance to 30% of that with no noncondensibles. In our case, 0.01% noncondensibles would reduce our predicted optimum fresh water production rate from 9.5 kg/(hr–m^2) to 2.85 kg/(hr–m^2). Clearly, Figure 1 from year 1 is qualitatively accurate but quantitatively optimistic. We have not determined what percentage of noncondensibles can reasonably be maintained, but this is a problem also faced by conventional distillation and the new technology of Ocean Thermal Energy Conversion (OTEC).

Design and Testing

A modular design made of heat exchanger sheets layered between plastic frames all clamped in a stack was developed. The whole stack was placed inside an evacuated pressure vessel. Our intent was to debug and test a single module of this stack this year, but we designed a pressure vessel and circulation systems suitable for the full stack of 13 modules. Based on our calculations, the pressure vessel was conservatively designed and contracted out. A spreadsheet computer model of the pumping flow resistance was developed and piping sized to minimize head loss. After careful consideration, centrifugal pumps were selected over positive displacement pumps. The centrifugal pumps take less power but must

be positioned 7 feet below the pressure vessel in order not to cavitate.

A mature version of this machine would be located next to the ocean and an outdoor brine source. At this experimental stage, however, small reservoirs of the seawaters and concentrated brine were included in the circulation lines to give us sufficient capacity for testing the system. A diagram of the reservoirs and piping design used for parts selection is shown in Figure 3.

Other research indicated the extreme importance of maintaining the thinnest possible continuous films of water on the heat exchanger surfaces (Brauner et al., 1985; Hartley and Murgatroyd, 1964; Petke and Ray, 1969). This simultaneously increases heat transfer and reduces internal pumping. If the flow rate is too low, however, the film will break down into discontinuous rivulets and become ineffective for heat and mass transfer. This critical flow rate is called the minimum wetting rate (MWR) and is a function of both the liquid and the material it falls across. Many plastics have high MWRs. Using both theory and experiment, we examined many candidate plastics and then focused on 4.5-mil-thick Mylar vellum. This gave MWRs in the range of metals, and was more effective than other plastics we had previously favored. In addition, Mylar has excellent strength, low cost and does not react with salt solutions. Mylar is manufactured by DuPont in sheets as thin as 0.5 mils. DuPont provided a range of samples with different surface treatments. Assuming identical, realistic flow rates, we calculated that 0.5-mil-Mylar should produce 92% (and 4.5-mil-Mylar, 51%) of the fresh water of an aluminum heat exchanger. Experiments revealed, however, that spraying water on 0.5-mil-sheets stretched in a frame caused vibrations of the sheet, which disrupted the film flows. It was therefore decided to demonstrate the use of plastic heat exchangers with the 4.5-mil-Mylar with the intent of extending our design to thinner Mylar and solving these additional problems in future research.

Two MWRs are typically specified, one on a dry surface and a lower value on a previously wet surface. There is limited recent literature suggesting a third mode of wetting, intermittent application of the flow (Maron et al., 1992). Experiments with this showed that a fully wetted surface could be maintained at even lower flow rates. Consequently, small sprinkler turbines were modified to slowly rotate and intermittently apply the water to the vertical surfaces. MWRs of 250 kg/(hr-m of heat exchanger width) were reliably achieved in this fashion. The MWRs are much lower when concentrated brines are used than they are when fresh or seawater is used, so intermittent application was not needed for the brine (Brauner et al., 1985).

Considerable debugging of the machine was required. The single module was positioned between two thick sheets of Plexiglas for viewing. As with the year 1 design, calculations showed that most of this Plexiglas and the other surfaces of the module needed insulation. It was also necessary to insulate all of the external plumbing and reservoirs. The drainage system needed improvement. A high water flow rate could flood the bottom of a chamber, overflow through the vapor passages in the central upright of a frame, and pollute the adjacent chamber. The second generation design used much flatter chambers and many more piping passages than the first generation. This created pockets for residual air and complicated the essential evacuation procedure. To solve this, the evacuation ports were relocated near the bottom of the chambers where the air tends to accumulate. In addition we adopted an established distillation industry practice. Small amounts of helium (1 in. of Hg) were introduced to the evacuated chambers. Then the vacuum pump removed this helium and most of the residual air. Also, vacuum leaks were systematically eliminated. With an aluminum sheet heat exchanger, there was a parasitic fin effect moving heat from the warm brine back to the first seawater. To solve this, the aluminum was cut vertically and separated by a 0.25-in. gap. The Mylar sheets were flexible and could bow because of the small pressure differences on them. Then the Mylar could touch and stall the turbine sprayers. This was solved with some bracing.

As improvements were made, the overall performance continually improved. Water production was repeatedly demonstrated. Our best results were our last successful runs. Using aluminum for the heat exchanger, we achieved a rate of fresh water production of 2.5 kg/(hr–m^2). Figure 3 again showed that 0.01% residual noncondensibles would lower the predicted fresh water production rate to 2.8 kg/(hr–m^2). We therefore got 89% of the fresh water rate predicted by the computer model and assuming a noncondensible level equivalent to 0.01% air. Using the Mylar the best rate was 0.6 kg/(hr–m^2). This successfully demonstrated the use of plastics for the heat exchangers; it should be recalled that this was using 4.5-mil Mylar. We predict 92% of the production rate on metal if we can learn to use the 0.5 mil-Mylar that DuPont manufactures.

The computer model assumes no flow resistance as the vapor travels from the seawater 1 to the brine and therefore predicts that the brine would heat the maximum 12°C above the seawater 1. In practice, in both year 1 and 2 designs a 6°C increase was typical (8–9°C on Mylar). Therefore, heat is being removed from the brine faster than vapor can arrive and fully heat the brine. Clearly we are experiencing some pressure drop, slowing the vapor transfer, and this needs to be remedied.

Many of the difficulties we overcame would be greatly diminished in testing a full stack of 13 modules. The plumbing and piping are all sized for 13 modules, so for a full stack the heat losses per module would be divided by 13. We evacuate the full volume of the pressure vessel whether it has one module or 13. Heat losses through the Plexiglas end faces are the same whether the stack has one module or 13. Because of the

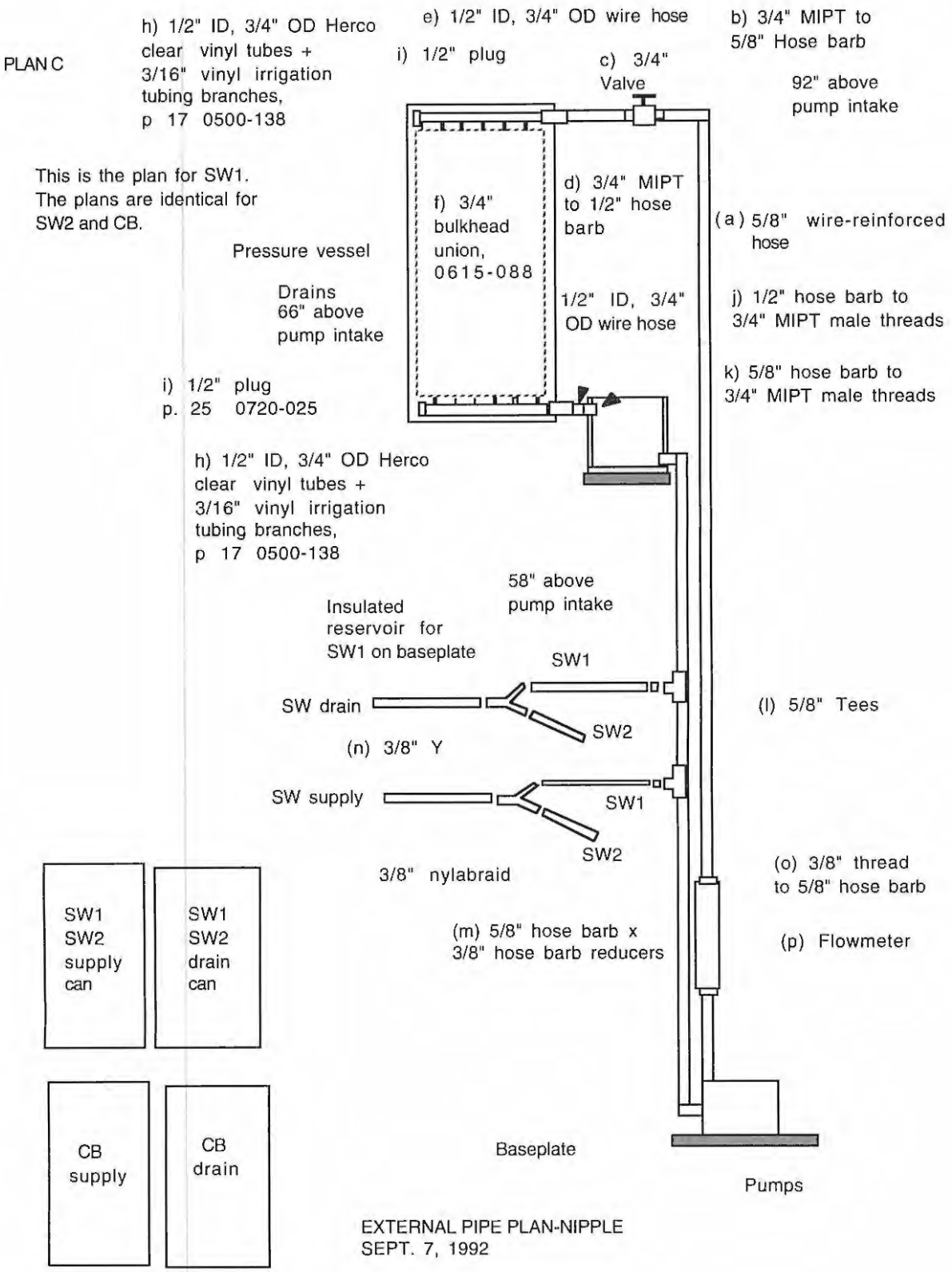

Figure 3. Piping diagram.

insulation required, we could not monitor the falling films precisely and tended to faster flow rates to be conservative.

I believe it is still premature to be conclusive about costs for this technology, but I am optimistic. Foremost, there is no cost for heat from fossil fuel. Also, we now see ways to dramatically reduce most component costs. The stack of modules must be clamped together. This pressure could be provided by deflection of the pressure vessel walls, and then these walls could be made thinner and cheaper. A mature unit would use PVC pipe at one-tenth the cost of the flexible hose used in experiments. The frames are currently made by hand out of PVC stock. They could eventually be made by injection molding, complete with every attachment fitting. In the next stage of development we would eliminate the seawater and brine reservoirs (see Figure 3). This would greatly simplify initial evacuation of the system and eliminate five valves. More precise control of flow rates would reduce the pumping further.

Summary

We have successfully demonstrated this new method of desalination without a fuel source, powered by the concentration difference between seawater and a saturated brine that can be derived from seawater by evaporation. All of the stated objectives have been met. We have created a detailed computer model and two generations of laboratory devices. We have demonstrated the possible use of plastics for the heat exchangers and are encouraged about the economics of this approach. The second generation design used a modular approach that can be expanded readily by an order of magnitude in capacity.

Acknowledgment

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Marine Affairs

International Management Regimes, the EEZ, and the U.S. Pacific Fisheries

University of California, Berkeley
R/MA-33
1990–92

Harry N. Scheiber

This project has investigated the impact of changing U.S. policies and international relations with respect to the management of fisheries in the Pacific since the Magnuson Act and the subsequent institution of Exclusive Economic Zones (EEZ) throughout the Pacific Rim. The research has embraced study of changes in commercial and political strategies of the U.S. flag fleets, new initiatives by rival fishing powers in the Pacific region and their impact on U.S. fishing industries and public policy, and qualitative analysis of issues associated with equity and efficiency in the allocation of U.S. offshore fishery resources in the EEZ. The study has investigated the roles of industry interest groups, political leadership, and the relationship of overarching U.S. foreign-policy objectives to marine policy issues. It has also been concerned with the issue of how influences have variously been exerted by specialists in fisheries management science in regard to changes in both domestic policy and the U.S. role in international fisheries regimes.

Research was conducted during 1990–91 in archival sources to provide data essential to the historical and policy analysis that is the main focus. A survey was made on-site of available National Archives diplomatic records. And data were obtained from Department of State and other archival records on U.S. policy respecting tuna, salmon, and other fisheries, documenting U.S. participation in international management and research agencies. American Tunaboat Association archival records newly acquired by the Scripps Institution of Oceanography Library Archives were also used intensively in 1990 and 1992 on-site, while the trainee began preparation of a data base that will result in publication of a guide to the papers of William C. Herrington, recently donated to the University of California, Berkeley.

Archival records in the Gerald Ford Presidential Library (Ann Arbor, Michigan) and the Jimmy Carter Presidential Library (Atlanta, Georgia) were obtained on-site and analyzed in 1992; these materials include White House files and related legislative files on territorial waters, fisheries policy, and Law of the Sea. Data on interest groups, congressional policy process, and Law of the Sea negotiations were also collected and processed from a major collection that includes the John Norton Moore Papers, in the Law of the Sea Collection at the University of Virginia Law Library.

The constitutional dimension of EEZ and extended jurisdiction questions is explored in an article (Scheiber and Carr, 1992) that deals with conflicting claims of the executive versus legislative branches from the 1930s to the Reagan administration. This study also presents data on the organized economic and other domestic interests, especially of the commercial fisheries and the Department of Defense, that played a formative role in the politics of the issue. The article also demonstrates how the international legal context—especially Cold War pressures and the progress of United Nations Law of the Sea negotiations and agreements—affected the changing posture of the executive branch with respect to extended offshore U.S. jurisdiction.

In progress now are a monographic analysis of the history of State of Hawaii and Pacific Islands Development Commission projects for expanded tuna fisheries research and development and functional linkage of this effort to U.S. Pacific fisheries management regimes and EEZ policy and a briefer monograph, invited for presentation at a scholarly meeting in 1993, on the U.S. and the International Whaling Commission (IWC) in relation to global EEZ politics and policies.

A paper (Scheiber and Schuele, 1993) presenting the findings of the project, plus data on the histories of the International Pacific Fisheries Commission, the IWC, the Inter-American Tropical Tuna Commission, and other international agencies in relation to U.S. EEZ policies. This paper was presented to the January 1993 Ocean Governance Study Group conference in Berkeley. It embraces a proposal for an interpretive model of how basic changes in the competiive order of marine fisheries have affected the content and implementation of U.S. fisheries policy in the EEZ and in relation to international Pacific fishery regimes since 1975.

The 1991 and 1992 workshop and conference meetings of the Ocean Governance Study Group afforded an opportunity to obtain data from agency officials and legislators, and formal interviews are continuing. A small phase of the project involved interviews of fisheries managers and researchers associated with the international commissions, as well as members of regional management councils and staff of the U.S. Marine Fisheries Service.

A continuing research enterprise that connects this project with prior research sponsored by Sea Grant and continuing studies is concerned with the relationship of fisheries science to management strategies and the policy process. This theme has been explored for the period centering on the Magnuson Act and early development of management council policies; research results have been presented in conferences and papers (see Publications).

As provided for in the initial project proposal, the project leader

also completed editing and supervised distribution of working papers from an international conference on ocean resources and industries (Scheiber, 1990). And the project's personnel have been involved in the ongoing preparation of papers analyzing current issues and options in ocean and coastal policy (see Cicin-Sain, 1992).

Currently in final drafting phase is a monograph providing an exhaustive account, based on archival and other sources, of the fisheries policy process and the Magnuson Act. This study will be especially concerned with the central policy issues surrounding objectives of decentralization (in the regional management councils) and establishment of alternatives (most notably, since 1980, the privatization initiatives associated with International Transferable Quotas) for which the Magnuson Act provided a policy and administrative framework.

Cooperating organizations

Carter Presidential Library
Ford Presidential Library
Hawaii State Archives
Scripps Institution of Oceanography Archives
Sho Sat Fund of Boalt Hall, UC Berkeley
University of Hawaii Library
University of Virginia Law School Library
University of Washington Libraries and Archives
U.S. National Archives, Washington

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Rapid Response

Population Studies on Yellowtail Rockfish: Management for the United States and Canada

Humboldt State University
R/NP-1-20A
1990–91

Timothy J. Mulligan

Introduction

Yellowtail rockfish, *Sebastes flavidus,* are a commercially and recreationally important species ranging from Kodiak Island, Alaska, to San Diego, California. Despite its economic importance, little is known about the population structure of this species, whose northern stocks have been declining since the mid 1970s (Tagart, 1990). This information is important because sound management of a fishery requires that subpopulations or stocks be identified and managed separately (Allendorf et al., 1987). The single allozyme study conducted on Pacific coast yellowtail rockfish offered no evidence of subgroupings (Wishard et al., 1980). However, discrete, nonmigratory schools have been observed in southeast Alaska (Carlson and Haight, 1972), and tagging evidence suggests that a yellowtail rockfish stock located off northern British Columbia may be distinct from populations to the south (Canadian Department of Fisheries and Oceans, 1989).

Mitochondrial DNA (mtDNA) analysis has been conducted on numerous fish species, but only a few studies have focused exclusively on marine fishes (e.g., skipjack tuna, *Katsuwonus pelamis*, Graves et al., 1984; orange roughy, *Hoplostethus atlanticus*, Ovenden et al., 1989; walleye pollock, *Theragra chalcogramma,* Mulligan et al., 1992). Conventional mtDNA analysis requires isolation of the mtDNA molecule, followed by digestion with a variety of type II restriction endonucleases. The resulting fragment patterns are then examined for polymorphisms within and among the populations. Although this method, restriction fragment length polymorphism (RFLP) analysis, is still widely used, the advent of polymerase chain reaction (PCR) (Saiki et al., 1988) has simplified the process of direct nucleotide sequencing to a level where it is increasingly being employed in the study of fish populations (Bartlett and Davidson, 1991; Carr and Marshall, 1991).

Our objective in this study was to employ a combination of molecular techniques to examine the genetic structure of three Pacific coast yellowtail rockfish populations. PCR was used to amplify a portion of the mtDNA molecule, which was then subjected to an RFLP analysis.

Methods

Approximately 30 yellowtail rockfish were collected from each of three Pacific coast localities:
(1) central west coast of Vancouver Island, British Columbia,
(2) Westport, Washington; and
(3) Cordell Bank, California. All fish were caught between January and March 1991. The mtDNA was extracted from the livers or ovaries according to Chapman and Brown (1989) with minor modifications. This method is most effective when applied to fresh samples, preferably ovarian tissue where mitochondria are highly concentrated. Because we were unable to acquire an adequate number of fresh ovaries and because the yields from frozen specimens were poor, we used the polymerase chain reaction to amplify a portion of the mtDNA molecule. Also, rather than amplify and sequence a small fragment (200–300 base pairs) from a few individuals, we amplified a large segment (1600 bp) from numerous individuals and conducted an RFLP analysis.

Amplification by PCR followed Kocher et al. (1989). The oligonucleotide primers were selected from highly conserved regions of the 12S and 16S ribosomal RNA genes of the human mtDNA molecule (Anderson et al., 1981; Kocher et al., 1989). Amplifications were performed using the GeneAmp PCR Reagent Kit (Perkin-Elmer Cetus, Emeryville, California).

Samples were digested with restriction endonucleases Hae III, Rsa I, Nde II, Hha I, Alu I, Taq I, Hinf I, Msp I, Mva I, and Eco RI according to manufacturer's instructions (Bethesda Research Laboratories, Grand Island, New York). The mixtures were loaded onto 1.5% regular agarose or 4% Nusieve (FMC Bioproducts, Rockland, Maine) agarose gels. After electrophoresis, the mtDNA fragment patterns were visualized by ethidium bromide staining and photographed. Genetic diversity was estimated by the diversity indices h and π of Nei (1987).

Results and Discussion

Successful amplification was achieved for 74 *Sebastes flavidus* samples. All amplifications yielded a single product of ca. 1,600 base pairs. Negative controls, consisting of all reaction components except template mtDNA, were run periodically with the sample reactions to rule out the possibility of contamination by foreign DNA. These produced no high molecular weight products, confirming the purity of the amplifications.

Restriction digests of the PCR amplified region, from the 74 *Sebastes flavidus* samples examined, revealed a total of 33 restriction sites (Table 1). Except for a single variant in the Westport Hha I digests, no variation was detected within or among the yellowtail rockfish populations. With only one variant observed across all individuals and all enzymes, genetic diversity was estimated to be extremely low ($h = 0.04$; $\pi = 0.000082$).

Table 1. Fragment Patterns Produced by Restriction Enzyme Digestions of Amplified *Sebastes flavidus* mtDNA

Enzyme	*n*	Fragment Sizes (bp)	Total Length[a]
Rsa I	74	480,260,260,250,250,75,50	1625
Msp I	68	520,520,285,145,130	1600
Eco RI	54	1480,120	1600
Hha I	73	1160,300,130	1590
	1	940,300,220,130	1590
Mva I	62	1500,100	1600
Hae III	67	794,282,245,178,105	1604
Nde II	64	600,400,320,170,100	1590
Taq I	74	1300,290	1590
Hinf I	68	1020,520	1540
Alu I	63	417,324,214,174,120,100, 90,60,48	1547

Total Length of the PCR Product, Determined for Each Enzyme, is Shown.
n = number of successful digestions.
[a]mean length = 1588.7 + 24.6, *n* = 11.

Genetic differentiation of subpopulations, within a species, is determined primarily by gene flow and genetic drift (Chakraborty and Leimar, 1987). The homogenizing effect of gene flow has been proposed to account for lack of population substructure in several marine fishes examined over broad geographic areas (e.g., *Chanos chanos*, Winans, 1980; *Katsuwonus pelamis*, Graves et al., 1984; *Mugil cephalus*, Campton and Mahmoudi, 1991). Results from these studies and those reported here support the consensus that marine fish species with high dispersal capabilities tend to be genetically homogeneous (Waples, 1987).

Yellowtail rockfish extrude their young live, primarily in February and March (Wyllie-Echeverria, 1987). Pelagic *Sebastes* larvae (4–10 mm SL) are predominantly distributed offshore (to 250 nautical miles) where they are a major component of ichthyoplankton assemblages (Moser and Boehlert, 1991). Older larvae and pelagic juveniles (15–50 mm SL) are found mostly in the upper 30–100 m of the water column. The pelagic juvenile stage may last from several months to a year, and during this time currents may direct them shoreward, usually during the spring and summer (Moser and Boehlert, 1991). For yellowtail rockfish, the transition to the bottom-dwelling juvenile stage occurs during the summer at about 50 mm in size (Tagart, 1990). At this time they recruit to hard substrates or macrophytes at depths shallower than those inhabited by adults (Love et al., 1991). This benthic stage may last up to several years, with the young tending to occupy the initial recruitment sites until migrating to deeper reef areas, which serve as adult habitat (Love et al., 1991). The shift to adult areas by juvenile yellowtail rockfish in Puget Sound, Washington, appears not to occur until the onset of maturity, at about age seven, when the fish migrate to coastal areas up to 144 km away from the nursery site (Matthews and Barker, 1983). Thus, extensive larval and early juvenile dispersal by ocean currents and later juvenile migrations could account for the lack of mtDNA haplotype diversity observed among the three populations of yellowtail rockfish examined in this study.

The overall low genetic variability within the yellowtail rockfish populations may be related to ecological and environmental parameters. Smith and Fujio (1982) compared allozyme heterozygosity values from 106 marine teleosts making up 10 orders and found the lowest levels of genetic variation in the Gadiformes and Scorpaeniformes. They hypothesized that heterozygosity levels are related to habitat type, with habitat generalists exhibiting lower variation than specialists. Generalists such as *Sebastes* would possess a few "wide-range alleles" and be characterized by low heterozygosities, whereas specialists such as Pleuronectiformes would require many "narrow-range alleles" and would exhibit higher levels of genetic variation.

It is possible that significant mtDNA variation does in fact exist within and among yellowtail populations which our methods failed to detect. Only 10% of the mtDNA molecule was examined, and only nucleotide sequences recognized by the 10 endonucleases were compared. Nevertheless, this technique allowed us to indirectly examine 133 base pairs, >0.8% of the mtDNA molecule, which is comparable to several RFLP studies (e.g., Graves et al., 1984; Johansen et al., 1990). In addition, this method offers several advantages. Only minute amounts of tissue are required, and it need not be in excellent condition. Frozen, preserved, and dried specimens can be used. A total genomic DNA extraction, simpler than mtDNA extraction, is sufficient for PCR, and restriction digests are, overall, less laborious and time consuming than sequencing and just as revealing if one uses an ample number of endonucleases on one or more amplified fragments. However, independent mtDNA sequence analyses or conventional RFLP analyses may provide more information on the genetic structure of Pacific Coast yellowtail rockfish.

Cooperating Organizations
National Marine Fisheries Service, Tiburon, California
Washington Department of Fisheries, Seattle
Canadian Department of Fisheries and Oceans, Ottawa

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Determination of Culture Conditions, Especially Light, and Their Effect on Survival of *Pacific* Hagfish in Captivity

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Marine Science Institute
R/NP-1-20B
1990–91

Miriam Polne-Fuller

A small Sea Grant Rapid Response grant, which started in January 1991, allowed us to perform initial evaluations of three aspects of hagfish fibers:

1. Initial evaluations of culture conditions for maintaining Pacific hagfish in tank cultures (feeding frequency, light levels, and quality).
2. Preliminary recordings of thread and mucus production and recovery in hagfish of different sizes over time in tank maintenance.
3. The potential toxicity of the two major components of the slime (threads and mucus) to small marine animals, to mammalian cell lines in culture, and as food or implants to mice.

Table 1. Effect of Light Levels and Water Motion on Feeding and Survival of Hagfish in Laboratory Tanks

Light: $\mu E/m^{-2}sec^{-1}$	Total *N*	Skin Damaged	%	% Dead	Feeding
1,200	15	15	100	100	Not at all
500	15	15	100	37	Hardly
200	15	3	20	7	Little
3	44	1	2	2	Well/weekly
Dark	15	0	0	0	Well/weekly

Light exposed animals were exposed for 6 weeks; survivors were allowed to recover in the dark.

General Maintenance

Although several researchers attempted unsuccessfully to maintain hagfish under laboratory conditions for long periods, we have been successful in doing so for the past three years. Our oldest population of nine hagfish was brought into the tanks three years ago. Eight of these fish are still alive, feeding actively and releasing slime. The one that died was a young fish, 3 g and 5 in long, which did not eat from the beginning.

Low light levels (less than 3 $\mu E/m^2/sec$, Table 1) and mild water motion were important requirements for fish health. Even at these low light levels, the animals chose to gather under abalone shells or other shaded spaces on the bottom of the tank. A second group of 35 animals was brought into the tanks 15 months ago. These have been heavily used for evaluations of slime-harvesting and for tests of light exposure. Five of these animals were lost to skin infections, all of which occurred under high light exposure, and three possibly to overharvesting. Additional data are being collected for confirmation.

The third group (78 fish) was collected two months ago. The fish are doing well and currently are used for light quality experiments and further accumulation of slime harvesting data. All fish have been maintained in running seawater tanks at local ocean temperatures (12–17°C). They are offered food at weekly intervals; however, most feed only every other week. High water motion from a localized hose (technical measurements for quantification of flow are planned in the future) did not seem to disturb the fish; however, whenever possible, they seem to prefer protected spaces where water motion, light, or both are minimal.

Light Quality and Quantity

Healthy fish avoided light exposure whenever possible. Animals that were kept in low light searched for shelter immediately upon exposure to higher light levels. If shelters were not found after 4–5 min, the fish settled and coiled up, possibly to minimize the exposure to damaging light. Daily sun exposure resulted in loss of interest in feeding, in skin damage, and mortality within 4–8 weeks (Table 2). The controls, (fish in dark tanks) fed actively, had healthy skins, and survived well. Neutral density-filtered light, as low as 200 $\mu E/min/sec$, was still damaging, resulting in slow or no feeding, but skin damage was not observed within eight weeks. In two cases fish that were kept in dark tanks developed skin infections and eventually died. It is not known whether the cause of the infection in the dark chambers was identical to that occurring in the light-exposed fish. Skin conditions did not spread to other fish in the tank, and the damaged-skin fish tended to keep away from the clustered healthy individuals, which accumulated under the abalone shells. No physical attacks among the fish were observed.

We are currently studying effects of light quality (blue, red, green) and quantity (200 $\mu E/^{-2}/sec^{-1}$ and lower).

Slime Production

Recovery of Slime Production. The 15 hand-harvested fish (1991 tests) recovered full mucus production within two months after harvest. Weekly harvest produced small quantities of threads and mucus and

Table 2. Feeding and Death under High Light Levels

	% Hags Feed of Anchovy >50%	% Hags Feed of Anchovy <50%	% Hags Coiled (no.)	% Skin Damage (no.)	% Dead (no.)
Week 1	0	40 Nibble	100	0	0
Week 2	0	0	100	0	0
Week 3	0	0	55 (11)	10 (2)	0
Week 4	0	0	30 (6)	35 (7)	0
Week 5	0	0	0	90 (18)	0
Week 6	0	0	0	90 (18)	10 (2)
Week 7	0	0	0	95 (19)	40 (8)
Week 8	0	0	0	100 (20)	70 (14)

By the third week of exposure, the fish coiled less, were generally less active, and stopped feeding.Twenty fish were tested under 1,200 $\mu E/^{-2}/sec^{-1}$ in four repeated experiments.

Table 3. Exposure of Marine Organisms to Total Hagfish Slime (mucus and threads combined)

Organism	(Numbers)	Exposure Time	Number Dead
Gillichthys mirabilis (long-jawed mud sucker)	(15)	2 hr	0
Sebastes diplopra (juvenile split mouth rockfish)	(25)	2 hr	2
Paralabrax clathratus (juvenile kelp bass)	(20)	2 hr	0
Emerita analog (sand crabs)	(20)	2 hr	0
Artemia salina (brine shrimp)	(>50)	2 hr	0
Bacteria from seawater and from hagfish skin		72 hr	7 strains are alive

seemed to increase feeding. More data are needed to evaluate the validity of these preliminary conclusions.

Quantities of Slime per Fish. Both large and small fish produced slime. Maximum recorded production was 0.05 g of dry thread/g live fish in one harvest. Some fish did not produce any slime. The quantities of dry fiber per gram of live fish were highly variable and poorly correlated with fish size or weight. Some small fish produced repeatedly larger quantities than some of the largest fish. Data from the silk industry indicated that genetic lines of high producing silkworms were selected over the years for commercial production. We need to collect additional data from larger numbers of hagfish to test the hypothesis that one can select for specific lines of prolific producers.

Toxicity of Threads and Mucus

Toxicity upon External Contact. Neither the threads nor the mucus, wet or dried, were toxic to the tested experimental animals. These two slime components were nontoxic to shrimp, fish, crabs, amoebae, and some marine bacteria, which were immersed in slime for 2–72 hr (Table 3). The lack of toxicity was evident even in highly viscous solutions presenting unnaturally high concentration of the mucus (1 g dry mucus dissolved in 50 ml of seawater. Normal concentrations of total released slime are about 0.01 g/500 ml). Tests of toxicity on additional small organisms and specific marine bacteria are currently being pursued (an ongoing MA project).

Toxicity Through Digestion. Toxicity was not observed in any of the feeding experiments in which ground threads were fed to the above organisms, as well as to 20 C-Bulb white infant mice (200–400 mg/day per mouse = 4–8% of the daily diet). The mice have been gaining normal weight during the five months of the experiment. The marine animals and the mice seem to like the threads, and eat it immediately upon presentation. Quantitative long-term measurements regarding the effects on growth or digestibility are being collected.

Subcutaneous Insertion of Threads. Sections of fiber "paper" (sheets made of fibers) were implanted in two mice in subcutaneous incisions. No toxic effects have been observed during the four months of the experiment. No inflammatory or tumerogenic reactions developed in any of the animals. The incisions healed well in the experimental animals as well as in the controls. One mouse was sacrificed one month after the implant was placed. Preliminary pathological sections indicated that the wound area was healing well and that macrophages accumulated in the vicinity (mouse macrophages were also shown to eat the fiber in our previous laboratory cell culture experiments). The second mouse is being maintained to study the potential total absorption of the threads over time. As of this writing (4 months into the implantation), no negative immune response or tumoregenic reaction has developed. A second set of mice should be tested in surgical stitching for longer periods to assure lack of negative effects over longer time.

The Development of Techniques for the Mass Culture of the Red Sea Urchin *Strongylocentrotus franciscanus*

University of California
Bodega Marine Laboratory
R/NP-1-20C
1991–92

Wallis H. Clark, Jr. and Douglas E. Conklin

The red sea urchin, *Strongylocentrotus franciscanus,* is the subject of one of the most important, if not *the* most important, commercial fisheries in the state of California. The impact on this fishery has been severe, and natural populations are rapidly becoming depleted. In response to this depletion, management measures are being implemented, and fisherman groups, working with California Sea Grant marine advisors, are seeking means to assure the long-term well-being of red urchin populations. One possibility that has aroused a great deal of interest is the potential of reseeding depleted fisheries grounds with young urchins, either just prior to settlement or as recently metamorphosed juveniles.

Previous information according to the literature stated that the maximum number of pluteus larvae that could be cultured to metamorphosis was one larva per 10 ml of water (Leahy, 1986). The larvae were fed the algae *Rhodomonas lens* at a density of approximately 6,000 cells/ml or at a concentration that could be cleared by the plutei within 24 hours (Leahy, 1986). Under such conditions metamorphosis took place in approximately 40 days. Percentage metamorphosis was moderate and variable.

Our laboratory has made great progress in working toward our objectives. We have: (1) cultured larvae to settlement at a maximum density of 5 larvae/ml; (2) determined an optimal algal feed, *Rhodomonas lens,* and algal density, 60,000 cells/ml; and (3) determined that the minimum number of days to the onset of metamorphosis, under optimal conditions, is 25.

Gravid animals were collected in the waters near the Bodega Marine Laboratory by local divers and maintained in flow-through seawater tanks. They were induced to spawn by injection of 0.5 *M* KCl through their peristomal membrane (Tyler, 1949). The eggs and sperm were mixed together in U.V. filtered seawater and maintained at two different water temperatures, 15° and 18°C. Several trials were attempted at 22°C, but in each culture, bacterial infection was heavy and the culture was lost.

At 18°C, the first cell division occurred 2 hr postfertilization. Hatching occurred within 18 hr, and gastrulation began by 26 hr. Development reached the prism stage (as evidenced by the presence of $CaCO_3$ spicules) by 48 hr postfertilization. By day three, larvae had reached the four-armed pluteus stage (pluteus is the terminal echinoid larval stage). At this stage feeding began, with the planktonic larvae feeding mainly on unicellular algae. By day ten, the pluteus larvae had grown to nearly 1 mm in width and had developed a total of eight arms. After approximately 14 days, rudimentary urchins were visible developing within the larvae. Rudiment development continued further until larvae initiated metamorphosis on approximately day 25. When an urchin was competent to metamorphose, it attached to the substrate, possibly because of a chemical cue produced by certain bacteria (Cameron, 1974), and slowly broke out of the larval structure like a bird from an egg. Unlike a bird, the urchin utilizes the majority of its "shell," either incorporating it into the urchin test (Hinegardner, 1969), or possibly as a nutritional source since the newly metamorphosed urchin has no mouth and is unable to eat for several days. The length of the larval period varied, depending on culture temperature, larval density, and nutritional factors.

The larvae of *S. franciscanus* are very sensitive to overcrowding. The deleterious effects of overcrowding could be minimized by completely exchanging their water with fresh 5 μm, U.V. filtered seawater on a daily basis. Trials of 500, 100, 50, 10, 5, and 1 larvae/ml were treated in this fashion, with only the three lower densities surviving (the trials at 10 larvae/ml survived, but naturally thinned themselves out to 5/ml). At higher densities, many larvae eventually retracted their arms, clumped together, and died.

Four algal varieties and several diets consisting of varying mixtures of each were tested, the diet of 100% *Rhodomonas lens* was determined to be the best. *Isochrysis galbana* also produced competent animals, but the time until metamorphosis was greater than with *Rhodomonas* (40 days compared to 25), and the percentage metamorphosis was decreased drastically. *Chaetoceros weisflogii* and *Thalassiosira gracilis* both proved insufficient for metamorphosis and possibly toxic to the larvae. An algal density of 60,000 cells/ml was determined to be optimal. This was the maximum density of algae that 5 larvae/ml could clear in one 24 hour period.

Under the optimal conditions (5 larvae/ml maintained at 18°C, fed 60,000 *R. lens*/ml, and water exchanged daily), metamorphosis began 25 days postfertilization. Larvae continue to metamorphose for at least one week if kept in the same container and under the same conditions. As many as 6,000 larvae settled per container. This represented approximately 65% metamorphosis. Animals reared and maintained at densities higher than 10/ml did not metamorphose.

By modifying culture conditions, we were able to maintain larvae at densities as high as 50/ml for periods of up to 50 days. Conditions

were as follows: (1) the water in which they were raised was exchanged *three* times per day; and (2) the larvae were fed after each cleaning (i.e., *three* times a day). Larval development rates at 50/ml, however, were more variable and overall much slower than rates observed at 5 larvae/ml. Within the same vessel (containing 50 larvae/ml), several larval stages were present at the same time. Additionally, metamorphosis did not take place at this high density. Although metamorphosis did not occur, many larvae developed a rudiment and appeared competent to metamorphose. In fact, if the densities of these 50-day-old larvae were decreased, metamorphosis occurred shortly thereafter, demonstrating that they were, in fact, competent.

Three probable, not mutually exclusive, explanations for the observed limits to larval density are suggested: (1) fouling; (2) starvation; and (3) social interactions (density effects of behaviors involved in growth and/or metamorphosis).

Larvae produce waste which is not only potentially toxic to them but also makes the culture more susceptible to bacterial infection. In addition, higher larval densities often result in greater larval mortalities, which further decreases water quality. Thus, maintenance of water quality (control of fouling) becomes more difficult as larval density is increased.

The observation that larvae exhibit slower and more variable growth rates at higher densities suggests a nutritional component. At a density of 50 larvae/ml, the total number of animals was ten times that of our optimal density, yet these animals received only three times the amount of algae. Algal clearance was rapid; thus, the larvae were subject to periods of starvation, especially overnight when feeding was suspended.

The fact that high density larvae would settle and metamorphose after a reduction of density suggests a crowding or social interaction effect. That is, larvae need to be maintained at or below a certain maximum density in order to proceed to metamorphosis.

Several other algal species have been suggested to us as possible improvements on *R. lens* but were not made available to us until after adult animals had passed through their spawning season. These alternative food sources should be tested to determine if our optimal culturing conditions can be improved. Additionally, our results thus far suggest that larval densities of greater than 5/ml may be plausible with more complicated culture modifications. Further tests will be needed to determine if they are practical.

Cooperating Organizations
California Institute of Technology
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Preliminary Studies of Radiometric Dating on Deep-Water Rockfishes (*Sebastes rufus* and *Sebastolobus* spp.) off California

Moss Landing Marine Laboratories
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Gregor M. Cailliet and Kenneth Coale

Deep-water fishes, such as the bank rockfishes (*Sebastes rufus*) and thornyhead rockfishes (*Sebastolobus altivelis* and *S. alascanus*), increasingly are being commercially harvested off the west coast of the United States (Jacobsen, 1991). However, their age composition, growth, age at maturity, and longevity have not been estimated. Estimates of age from growth zones in otoliths from these species suggest that they are quite long-lived and therefore could be vulnerable to heavy fishing pressure. Ages for these species have not been validated.

The basic objective of this study was to demonstrate the feasibility of using the radiometric age-determination approach (Bennett et al., 1982; Fenton et al., 1990; Campana et al., 1990) on bank rockfish (*Sebastes rufus*) and thornyhead rockfishes (*Sebastolobus altivelis* and *S. alascanus*) to validate life history information from otolith growth increment analysis.

To accomplish this objective, during the three months of this project, we have: (a) established the capability to use trace metal clean preparation and handling techniques (see Appendix 1) required for radiochemical analyses at low activity; (b) calibrated both the alpha spectrometer and radon emanation system for use with anticipated activity levels; (c) developed the techniques and local capability to plate polonium-210 (daughter/proxy for lead-210) and determine polonium-210 activity using isotope dilution alpha spectrometry; (d) measured the background activity of the two systems that will be used, as well as backgrounds on all chemicals and preparation technique blanks; (e) prepared otolith samples from bank rockfish for radiometric analyses; and (f) initiated work on the two thornyhead species.

Studies that have successfully conducted radiometric analyses of fish otolith material (Bennett et al., 1982; Fenton et al.,1990; Kastelle, 1991; Smith et al., 1991) have emphasized that the very low activity of the isotopes in the otolith require the use of clean techniques in sample preparation. We therefore have developed a preparation and cleaning protocol based on trace metal techniques (Fabry and Delaney, 1989; Linn, 1988) and established otolith preparation procedures (Bennett et al.,1982; Campana et al., 1990).

This protocol consists of coring each otolith and combining cores of similar ages. These cores are then cleaned repetitively with acid and milli-Q purified, deionized water, using only acid-cleaned equipment and keeping the sample dust-free, to remove the surface layer of the core which was exposed to handling and avoid contamination. They are then dissolved in acid and analyzed for radon-226 or polonium-210 as indicators of radium-226 and lead-210 activity. For details, refer to Appendix 1.

Each sample used for radon extraction must equilibrate for 30 days in a sealed container to allow the radon to build in before extraction. The extracted radon is then counted for approximately one week to calculate the radium-226 activity which is normalized per gram of calcium carbonate.

Samples prepared for polonium analysis are spiked with a yield tracer (polonium-208): the polonium-210 and tracer are autodeposited on a silver planchet (Flynn, 1968) and activity is determined over 3 weeks using an alpha spectrometer. Samples are typically alpha-counted for about 3 weeks.

Activities of the isotopes in these samples are very low (e.g., 83–550 μBq/g, where 1 μBq = 10^6 disintegrations per second) for lead and 830 μBq/g for radium (Bennett et al., 1982; Smith et al., 1991). Therefore, precise calibration of both systems was required. We have accurately defined polonium-208 (yield tracer) and polonium-210 channel intervals for each of the eight quadrants that make up the alpha spectrometer system and measured background activity for each quadrant. This was done by counting standards of known isotopes, determining energy-specific channels in each quadrant and extrapolating to the energies of the two isotopes of interest, and counting planchets containing only polonium-210 and polonium-208, respectively. Background counts (with blank silver planchets in the detectors) were determined for polonium-208 and polonium-210.

The use of clean reagents and procedures is very important in estimating these low activities. We have tested our clean preparation technique, fine cleaning reagents, and dissolution acid for lead contamination by plating them in the same manner that samples are plated, and determining activity using the alpha spectrometer. We found that the polonium-210 activity in these samples was not above background levels for the alpha spectrometer system.

The efficiency of the radon emanation system was determined by extracting radon from a series of known activity standards and counting decays using a lucas cell and photomultiplier tube. The calculated efficiency for our system is 72 ± 0.03%. No otolith samples have been counted. Background count for the system is less than 0.01 cpm.

The background counts for both our radon emanation and alpha spectrometer systems are comparable to those reported from other studies using similar techniques (Campana et al., 1990; Kastelle, 1991). Efficiency of the radon extraction system is slightly lower than previously reported (72 vs. 85%) but is much less variable than that of other studies (± 0.03 vs. 5%).

Bank rockfish otoliths, collected during 1987 and 1989 for Sea Grant Project R/F-113, were used for this project. Three hundred otolith sections were re-aged for consistency, and the other half of each otolith pair was placed into five age groups (1987 age groups 9–16 and 30+, and 1989 age groups 10–15, 20–29, and 35+). A total of 107 otoliths was cored, cleaned, and prepared for polonium-210 (lead-210) analysis, including 20–24 otoliths in each of the five age groups. The five age group samples currently are being analyzed on the alpha spectrometer.

Thornyhead otoliths were collected during a National Marine Fisheries Service, Southwest Fisheries Science Center, La Jolla, groundfish survey cruise conducted September 10–19, 1991 off Santa Cruz, California. A total of 628 otolith pairs was collected, 237 longspine (*S. altivelis*) and 391 shortspine (*S. alascanus*). Approximately 500 pounds of longspine thornyhead were brought back for processing as needed. In addition, thornyheads were collected during a September 1991 cruise near the Farallon Islands. We are in the preliminary stages of developing age and growth curves for these two species before assembling samples for radiometric analyses. Rockfish otoliths of 90 longspine and 35 shortspine rockfishes have been sectioned and aged.

We expected to finish the analysis of the bank rockfish otoliths by the conclusion of this project. Dr. Ross Williams, U.C. Santa Cruz, was instrumental in teaching us the radon emanation method. We have learned to operate the radon emanation system and conduct this portion of the analysis ourselves. Furthermore, we are in the process of salvaging Moss Landing Marine Laboratories' alpha spectrometer, which was damaged during the Loma Prieta earthquake. We hope to restore its preearthquake condition and produce counts of polonium-210 at our facility.

Appendix 1. Otolith Preparation and Cleaning

1. Otolith Storage
   a. All right otoliths will be stored dry for radiometric analysis.
   b. All left otoliths will be stored in 50–70% ethanol for sectioning, microscopic viewing, and aging.
2. Sectioning
   a. Embed in LR White resin using ice cube trays (or EM molds) with cavities close to the size of the otoliths.
   b. Mount each embedded otolith on a cardboard tag using epoxy.
   c. Section using a Buehler Isomet low-speed saw and low density diamond blade. Blade is lubricated with deionized water and Micro detergent. Four cuts are made around the otolith core (core dimensions are determined for each species individually from young whole otoliths).
3. Grinding
   a. Mount each block on a glass microscope slide with the proximal surface facing up, using histoclad (or LR White).
   b. Grind to the core edge using a Buehler Ecomet III grinding wheel and 600 grit silicon carbide grinding paper. For relatively large cores this can be done by hand vice mounting the blocks on a slide.
   c. Heat slide to flip block and repeat for the distal surface if necessary.
4. Rough Cleaning
   a. Soak slide and section in toluene for 10 minutes to free the block from histoclad (or other mounting medium).
   b. Combine blocks of similar age categories to obtain a sample weight of greater than 5.0 g (10 or more g if possible) for radon extraction or at least 1.0 g for samples on which only polonium analysis will be done. Record precleaning weight.
   c. Rinse in deionized water/Micro mixture three times.
   d. Rinse in deionized water three times.
   e. Rinse in Milli-Q water three times.
   f. Transfer sample to acid-cleaned container. (All subsequent steps will be completed using "clean" techniques, i.e., acid-washed containers and handling equipment and dust-free [covered at all times]).
5. Fine Cleaning
   a. Rinse in Milli-Q water three times.
   b. Soak 10 minutes in Milli-Q water with ultrasound.
   c. Soak 1 minute in 0.15 *N* nitric acid with ultrasound.
   d. Soak 10 minutes in 30% hydrogen peroxide buffered to pH 10 with continuous ultrasound.
   e. Rinse in Milli-Q water three times.
   f. Soak 1 minute in 0.15 *N* nitric acid with ultrasound.
   g. Soak 5 minutes in Milli-Q water with ultrasound and for 5 minutes after ultrasound.
   h. Rinse three times for 30 seconds with 0.001 *N* nitric acid with ultrasound.
   i. Rinse two times with Milli-Q water.
   j. Dry 12 hours in oven/desiccator and reweigh to determine material loss and weight from which radium-226 activity will be calculated.
6. Dissolution
   a. Add small amounts of 8 *N* nitric acid until the sample is almost completely dissolved.
   b. Add small drops until dissolution is complete.
7. Radon Analysis
   a. Transfer sample to acid washed gas-wash bottle with tubing and clips required for radon emanation in place.
   b. Heat to boiling point to remove any dissolved carbon dioxide.
   c. Seal gas-wash bottle using a small amount of silicon grease and vent lower clip to allow off-gassing during cooling.
   d. Bubble helium gas through the sample to remove any residual radon, secure tightly, and allow radon to build into the sample for 30 days before extraction.
8. Polonium Plating
   a. Dilute the solution to 0.5 *N* nitric acid with Milli-Q or nitric as required.
   b. Add polonium-208 spike.
   c. Plate in accordance with established procedures.

Cooperating Organizations

National Marine Fisheries Service, Southwest Fisheries Center, La Jolla, California
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Augmenting Organic Matter and Nitrogen to Accelerate Ecosystem Development in Constructed Wetlands

San Diego State University
R/NP-1-20H
1991–91

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Introduction

Legislative efforts to preserve the nation's dwindling coastal wetlands frequently require developers to construct man-made marshes to replace damaged or destroyed marshes. Unfortunately, there is little evidence to date that constructed wetlands can achieve the functional equivalence of natural marshes. On the contrary, a recent review of the status of wetland restoration and creation by the Environmental Protection Agency (Kusler and Kentula, 1989), with the help of 30 authors from around the nation, identified several gaps in our understanding of how constructed marshes function relative to natural marshes. Among the shortcomings listed was a tendency of constructed marshes to have lower sediment organic matter than natural marshes. Three examples illustrate this point.

Lindau et al. (1981) found that the natural wetlands on the coast of Texas had low sediment organic carbon (<1.3%) and nutrient content (total N <6 mg/g), but levels in the constructed marsh were even lower (0.5% and 1 mg/g, respectively). Craft et al. (1986, 1988) worked in salt marshes along the North Carolina coast, both natural and constructed marshes. The natural marshes had high levels of organic carbon (up to 8.6%) and total nitrogen (up to 1.68 mg/g). The constructed marshes had lower organic carbon matter (<1.8%) and lower nutrient levels (total N<l mg/g). The authors estimated that it would take 15–30 years for macro-organic matter nutrient pools in constructed marshes to approximate conditions in natural marshes. Langis et al. (1991) compared a constructed marsh with an adjacent natural marsh located on the San Diego Bay in southern California. The 4-year-old constructed marsh had significantly lower levels of total nitrogen (0.87 mg/g vs. 2.01 mg/g) and percent organic carbon (1.11% vs. 2.38%) than the natural marsh.

Organic matter plays an important role in several ecosystem processes. It affects nitrogen cycling and retention, water holding capacity, plant growth rates, and the detrital food chain. However, organic matter accumulation is a slow process. Adding organic matter to newly constructed marshes may therefore greatly accelerate their development. The present study tested the effect of organic matter and inorganic nitrogen additions on *Spartina foliosa* growth and on sediment nitrogen and organic carbon levels in a newly constructed intertidal marsh in southern California. We predicted that augmenting sediment organic matter would accelerate ecosystem development.

Methods

The study took place in a newly excavated intertidal marsh at San Diego Bay. Experimental plots were amended with organic matter and inorganic nitrogen on February 26, 1991. The site was opened to tidal flushing on March 5, 1991 and planted with *Spartina foliosa* on March 21, 1990. The experiment followed a randomized complete block design with four blocks and seven treatments. Each treatment plot was 1 m wide and 5 m long. Within each block, plots were randomly assigned to one of seven treatments: no modification (control), rototilling only, rototilling with inorganic nitrogen fertilizer, rototilling with straw, rototilling with alfalfa, rototilling with straw and inorganic nitrogen fertilizer (s+n), and rototilling with alfalfa and inorganic nitrogen fertilizer (a+n).

Results and Discussion

With the exception of the straw amendment, plant biomass was proportional to the amount of nitrogen initially present in the amendments (Figure 1). In October 1990, when biomass was greatest, there were significant treatment and block effects. Alfalfa + inorganic nitrogen had the highest biomass (174.5 g/m^2 ± 30.5 s.e.) and rototill-only the lowest (27.8 g/m^2 ± 15.0 s.e.) The rototill-only treatment was significantly lower than all other treatments, suggesting an inhibitory effect on plant growth. Differences between the control and the a+n amendment, and between the straw and both alfalfa amendments were also significant. Biomass declined over the winter, but by April 1991, biomass had regained levels found in October 1990. The rapid growth rates in the spring of 1991 suggest that peak biomass will surpass that of 1990.

The data (Figure 1) show that nitrogen-rich organic matter can significantly increase plant growth. Organic matter additions failed to accelerate the development of sediment nitrogen and carbon pools. Sediment organic carbon, total nitrogen, dissolved organic carbon, and porewater nitrogen were not significantly higher on the amended plots. Extractable NH^{4+} was significantly higher for alfalfa and alfalfa plus inorganic nitrogen in September 1990, but no differences were detected in February 1991. Sediment carbon levels in February 1991 were low (0.690 mg/g dry weight ± 0.21 s.e.) compared to the natural (2.11 mg/g dry weight ± 0.015) and 4-year-old constructed (1.08 mg/g dry weight ± 0.07) marshes described by Langis et al. (1991). Total nitrogen was also much lower in February 1991 on the experimental plots than on the natural or constructed marshes (0.55 mg/g ± 0.008 vs. 1.74 ± 0.13 and 0.94 ± 0.04, respectively).

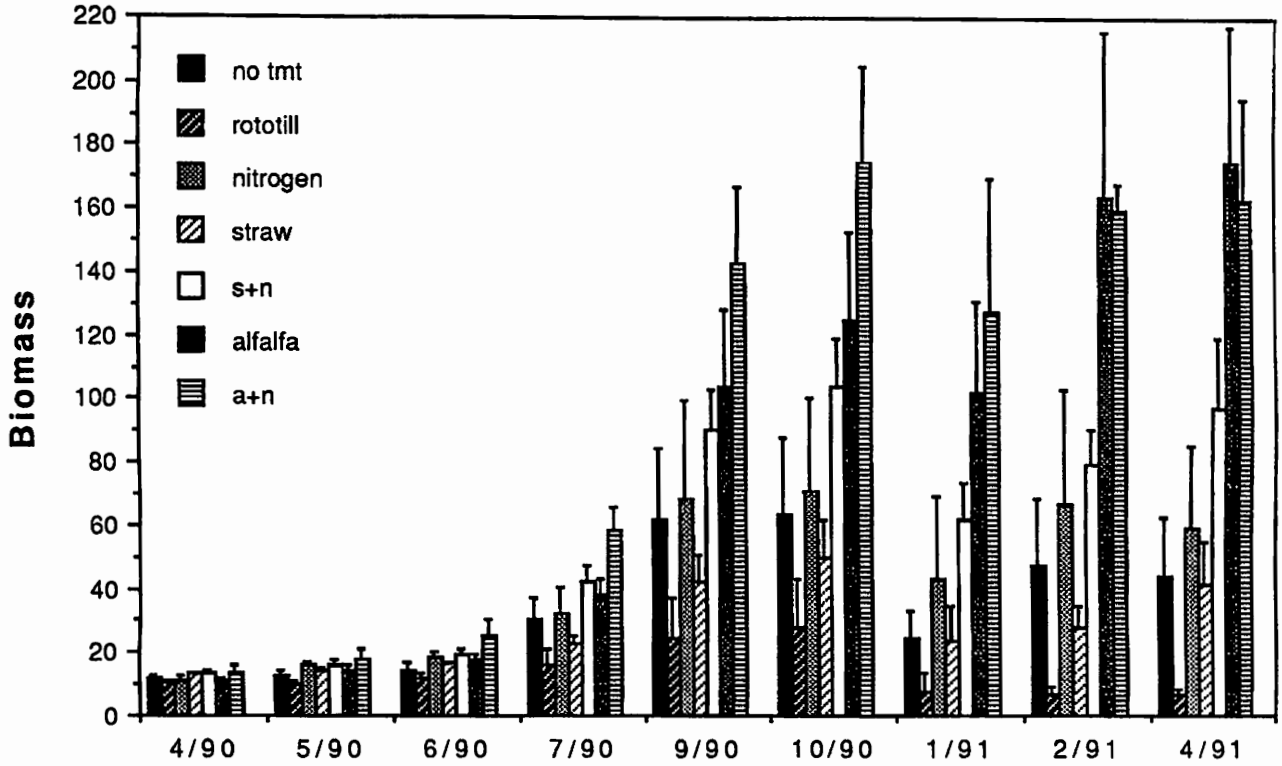

Figure 1. Biomass (g/m2) changes during the first year. Error bars are ± 1 s.e.; column means are from 4 plots.

The development of nutrient and organic matter pools was probably limited by rapid decomposition rates and by the sandy texture of the soil. Both organic matter types lost more than half of their dry weight during a 4 month litterbag study (Gibson, 1991). Alfalfa lost approximately 90% of its nitrogen, and straw lost approximately 60% during that same time period. Soil texture analysis showed that soils had a much lower clay content than soils found in the natural marsh (> 30% clay vs. approximately 50%). Soils with low clay content tend to be well drained and poor substrates for nutrient retention (Barbour et al., 1987).

The addition of nitrogen-rich organic matter stimulates cordgrass growth. Based on our initial results, the California Department of Transportation (Caltrans) required contractors to add alfalfa to the substrate of Marisma de Nacion, a 17-acre salt marsh that was planted in spring 1991. Plant growth will be followed periodically. The development of sediment nitrogen and organic carbon pools proved more elusive. Greater quantities of organic matter or repeated applications may be needed to overcome rapid decomposition rates and sandy soils on the constructed marsh. The study needs to be continued to assess the importance of sediment characteristics, such as texture and density to the development of mature marsh soil, and to determine the short-term effects of organic matter additions on decomposition rates and sediment chemistry (redox potential and sulfide production).

Cooperating Organizations

California Department of Transportation
U.S. Fish and Wildlife Service

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Interannual Variation in Growth and Fecundity of Dungeness Crabs

Humboldt State University
R/NP-1-21A
1991–93

D.G. Hankin

Background

The Dungeness crab, *Cancer magister*, supports important commercial fisheries along the Pacific Coast, from central California through southeast Alaska. Although commercial fisheries in California, Oregon, and Washington currently prohibit landing of female Dungeness crabs, British Columbia allows the taking of adult females that exceed the minimum legal carapace width for males, and Washington has considered adoption of a similar policy. Capture of female Dungeness crabs in recreational fisheries is allowed in California where female Dungeness crabs exceeding 5.75 in (≈ 146 mm) carapace width may be retained, and in Oregon where limited recreational harvest of females is allowed in bays.

The commercial catch of male Dungeness crabs has exhibited large fluctuations in all coastal areas (Botsford 1986; Jamieson, 1986). These fluctuations have been most regular ("cyclic") in the northern California fishery (McKelvey et al. 1980; Methot 1986). Extensive efforts have been directed at modeling these fluctuations so as to improve management of the resource. Modeling efforts during the late 1970s and early 1980s focused principally on possible internal population mechanisms which might influence or cause cyclic fluctuations, including at least predation by a parasitic worm, cannibalism by adults on juveniles, and density-dependent egg/larval survival (Botsford and Wickham, 1978; McKelvey et al., 1980; Botsford et al., 1983; Botsford, 1984). No real consensus has been achieved regarding which (if any) of these mechanisms may be the most plausible alternative endogenous control on abundance (see Botsford, 1981; McKelvey and Hankin, 1981; Hankin, 1985; Botsford, 1986).

There now seems little doubt that fluctuations in abundance of Dungeness crabs depend on both internal demographic (and possibly density-dependent) factors, and on external ocean environmental factors (Wild 1980, Johnson et al. 1986, Jamieson et al. 1989, McConnaughey et al. 1992). Internal demographic factors must primarily determine the total number of eggs that are extruded by females each year. The ocean environment must then have substantial influence on survival of these brooding crab eggs, on survival of pelagic larvae and, through ocean transport processes, on settlement success of metamorphosed larvae. Viewed from this perspective, internal population mechanisms determine the total number of eggs that are carried by a population of female Dungeness crabs in a given year and thereby determine an absolute upper limit to recruitment. The ocean environment together with possible internal population factors, such as density-dependent larval growth and survival or cannibalism by adults or juveniles on newly settled crabs, determine the survival rate of eggs, larvae, and early instars and establish eventual year-class strength.

The overall objective of this project was to improve our knowledge of those internal demographic factors that determine the total number of eggs that are extruded by a Dungeness crab population in any given year. In particular, we wished to collect new data concerning growth and reproduction of female Dungeness crabs, including size-specific molt increments, molting probabilities, and fecundities. Through comparison of results obtained in this study (1991–1992) with results from previous research (1981–1983), we hoped to develop an improved understanding of interannual variation in growth and reproduction of female Dungeness crabs in northern California.

Methods

We relied extensively on the cooperation and skills of our contracted commercial fisherman, Jim Gullett of Trinidad, for assistance in collection of our data concerning growth and reproduction of female Dungeness crabs in northern California. Mr. Gullett provided us with: (a) ovigerous female crabs taken from commercial traps during January–February 1992; (b) premating embrace females (for generation of molt increments); and (c) extensive measurements of male and female sizes in premating embrace pairs. We also fished 18 experimental small mesh (non-size-selective) traps (see Diamond 1983, Diamond and Hankin 1985) from Mr. Gullett's vessel at two locations and in two months during mid-summer 1992, after the 1992 molting season for females. At this time we measured female carapace widths, and we recorded a visual assessment of probable molting history (molted, probably molted, probably did not molt, did not molt) for each collected female. Our earlier research indicated that we could calculate size-specific molting probabilities for female Dungeness crabs from such "shell condition" data (Hankin et al. 1989).

Fecundities were estimated via subsampling of dried egg masses following methods detailed in Hankin et al. (1989). We used linear regression methods to describe relations between size-specific molt increments, fecundities, and (premolt) carapace widths, and to compare regression lines between years (Draper and Smith 1981). We used the methods of Mohr and Hankin (1989) to calculate estimates of size-specific molting probabilities

from shell condition data. A Sea Grant trainee was responsible for most field data collections, for generation of laboratory molt increments, and for fecundity estimates. The research carried out in this project will form the basis for the trainee's Master's degree in Fisheries Science at Humboldt State University (HSU). The Principal Investigator (Hankin) participated in all field sampling relating to generation of shell condition data so as to ensure continuity between data collected in earlier research (1981–1983), and in this study.

Results and Discussion

Overall, we were very successful in generation of desired data. We met or exceeded most of our proposed data collection objectives for the project. In particular:

1. We obtained fecundity estimates for more than 100 female Dungeness crabs based on late-stage egg masses (proposed = 40).
2. We generated laboratory molt increments from 181 female Dungeness crabs removed from premating embraces and returned to the HSU Marine Laboratory for molting (proposed = 120).
3. We used our experimental nonselective crab traps to obtain size frequency measurements and corresponding shell condition assessments from more than 1,800 crabs collected from two northern California locations during late June, 1992, and from more than 2,300 crabs collected from two locations during mid-July, 1992 (proposed = 1,000 each period).

In addition to substantial success in achieving our original proposed objectives, we were also successful in collecting additional data related to the topics of our research. Collection of these data had not been contemplated in our project.

With the assistance of our contracted commercial crab fisherman, we obtained 450 paired measurements of male and female Dungeness crabs collected from premating embraces. Hard-shell male Dungeness crabs clasp females that are near molting in these premating embraces. These females will molt in 1–3 days at which time they will be fertilized by the males while females are soft-shelled. These records of male and female sizes should prove very valuable in addressing the important management question of whether the intensive fishery for males leaves sufficient numbers of large males to ensure successful fertilization of females. Although it is an old issue, this subject has been the focus of recent discussion in the fisheries literature (Smith and Jamieson 1991).

Because the Sea Grant trainee on this project has not yet completed his Master's thesis based on project results, we can present only a brief and preliminary analysis of data generated by project R/NP-1-21A. Important results related to our project objectives are the following:

1. The trend of size-specific molt increments with premolt carapace widths of female Dungeness crabs during 1992 was not statistically different from similar established regression lines based on previous research. Thus, for females of a given carapace width, average size-specific molt increments have shown little variation across years.
2. As in our previous research (Hankin et al. 1989), we again detected significantly lower size-specific fecundities among crabs that did not molt in the most recent season(s) as compared with those that did. Our previous research showed that adult female Dungeness crabs may retain viable sperm for at least 2.5 years. They may extrude successive masses of viable eggs, fertilized by stored sperm. Reduced fecundity of such crabs may, however, reflect reduced numbers, motility, or viability of sperm stored for long periods of time.
3. In contrast to our finding regarding the apparent stability of size-specific molt increments, shell condition data collected during June and July of 1992 suggest that variation in size-specific annual molting probabilities may be extreme. In our previous Sea Grant research we found that size-specific molting probabilities declined precipitously with increasing carapace width from near 100% at a width of 135 mm to approximately zero at widths exceeding 155 mm (Hankin et al. 1989). Collections made during June and July of 1992, at two geographically separated areas, indicated that almost all female Dungeness crab, irrespective of premolt carapace width, molted during the 1992 molting season. Numerical calculations of size-specific molting probabilities based on Mohr and Hankin (1989) supported a conclusion of striking differences between 1992 data and earlier estimates of molting probabilities.

The extent of differences between years is, however, perhaps most clearly illustrated by a simple tabular summary of shell condition data collected during late May 1983 compared to mid-June 1992 (Table 1). For crabs exceeding 144.9 mm carapace width in 1983, a total of 126 had not molted whereas 76 had molted. For crabs exceeding 144.9 mm carapace width in 1992, a total of 30 crabs had not molted whereas 714 had molted.

Inspection of Table 1 makes it obvious that no sophisticated statistical analysis is required to support a conclusion that the trend of molting probabilities with carapace width was very different during 1992 as compared to 1983! To our knowledge, such extreme interannual variation in size-specific molting probabilities has rarely been observed and quantified in adult decapod crustaceans. Such variation, of course, must have profound impact on interannual variation in size at age. Variation in size at age must in turn have important repercussions for population egg production, etc. We hope to follow up on this remarkable and unexpected degree of interannual variation in future research.

Table 1. Summaries of Shell Condition Data for Female Dungeness Crabs

Width[3]	1983 DATA[1]		1992 DATA[2]	
	Molted	Non-Molted	Molted	Non-Molted
85	0	0	12	1
90	0	0	10	2
95	0	1	17	0
100	0	1	12	0
105	0	1	7	0
110	0	1	7	0
115	4	3	10	1
120	7	1	12	1
125	19	2	50	4
130	26	1	118	8
135	39	6	220	10
140	30	18	363	16
145	19	41	339	12
150	19	38	231	7
155	25	30	106	3
160	12	14	37	8
165	1	3	1	0
Totals	201	161	1,552	75

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Preliminary Studies of Hagfish Fibers for Commercial and Medical Applications

University of California, Santa Barbara
R/NP-1-21B
1991–92

Miriam Polne-Fuller

The major objective of this project was to collect data regarding the nutritional and medical properties of fibers from *Aptatretus stoutii*, formerly known as hagfish or slime eel. Because this species produces a silk-like protein thread, we are now calling it *silkfish*.

Specific objectives were: (1) to determine the long-term safety of using these fibers (or threads) as suture material in living organisms; and (2) to determine the long-term toxic effects of using these fibers as a food supplement.

To address the first objective, a year-long experiment was carried out on 4 × 2 mice, which were surgically subcutaneously inserted with silkfish protein fibers made into threads or 1 cm^2 "paper" sheets. The experiment had very promising results:

- All of the incisions healed completely;
- The silkfish threads were not toxic to any of the experimental animals throughout the year-long experiment;
- The threads were totally absorbed by the animal tissue, leaving no residue. This reinforces the results of our previous work with macrophages. The immune system of living mice also consumed these protein threads. The implication of this work may be important in eliminating the need for a second surgical procedure to remove stitches;
- No tumors, allergies, or other morphological or anatomical side effects were detected in any of the animals.

For our long-term study of the toxic and nutritional effects of silkfish threads, a five-month experiment was done on ten young mice, fed daily with 1 g of silkfish threads added to their regular diet. Results observed were:

- After five months no toxicity was detected. All animals developed normally, gained weight as expected, and did not have any detectable morphological or anatomical side effects;
- The experimental animals did not gain weight faster than did the controls.

We are continuing our work, with an eye on both the implications for aquaculture and the commercial potential of the threads.

Geographic Patterns of Settlement in Sea Urchins

San Diego State University
R/NP-1-21D
1991–92

J.D. Dixon, S.C. Schroeter, and T.A. Ebert

Knowledge of the patterns of settlement and recruitment of red and purple urchins is of great practical and theoretical value. Coupled with studies of the population dynamics of urchins after recruitment, such studies enable one to determine whether settlement is limiting to the population. Based on the density of emergent individuals, it seems unlikely that this is the case in southern California. However, it is possible that urchin populations in northern California may be settlement limited.

Data from our ongoing studies of settlement, begun in February 1990, will help resolve the problem. This work documented two pulses of settlement in southern California during the period from February 1990 through June 1991, and almost no settlement in northern California (Ebert et al., 1991). These results suggested that settlement is less frequent in northern than in southern California. However, observations of settlement during two settlement seasons is a shaky basis for management decisions. Support to continue the study for several more years, and determine the generality of these patterns was provided by volunteer work by us and colleagues throughout California, and funding by the Director's Sea Urchin Advisory Committee of the California Department of Fish and Game, and by Rapid Response funding from Sea Grant for technicians and supplies.

Red *(Strongylocentrotus franciscanus)* and purple (*S. purpuratus)* sea urchins have larvae that feed in the plankton for a minimum of one month and very likely several months before they settle (Strathman, 1978; Leahey, 1986; Cameron and Schroeter, 1980; Rowley, 1989; Carrol, unpublished data). Recruitment of small red and purple urchins (i.e., individuals between 1 and 3 cm in test diameter) is generally pulsed (Ebert, 1983; Ebert and Russell, 1988; Ebert et al., 1993; Harrold et al., 1991; Kenner, 1992). In addition, there appears to be a latitudinal cline in recruitment. Recruitment pulses in northern California, Oregon, Washington, and British Columbia, Canada generally do not occur every year (Bernard and Miller, 1973; Ebert, 1983; Ebert and Russell, 1988; Kenner, 1992; Harrold et al., 1991). This contrasts with observations from southern California and Mexico, where recruitment pulses appear to occur at annual or higher frequencies (Ebert, 1983; Tegner and Dayton, 1981). For purple sea urchins, there is an additional component of local variability. Recruitment tends to be lower near headlands than in areas between headlands. Ebert and Russell (1988) speculate that these differences in recruitment result from lower rates of larval settlement caused by greater offshore transport at headlands. If this hypothesis is correct, then one would expect to see patterns of settlement that correspond to patterns of recruitment. The present study provides one of the necessary pieces of data for deciding whether there is a linkage between settlement and recruitment to the benthos.

In order to accurately estimate settlement, one must reduce postsettlement mortality as much as possible. This requires frequent sampling. If settlement is spatially and temporally variable, one must also sample for at least a year, and very likely for several years. These constraints probably account for the fact that most of the data we have on settlement of marine organisms is for sessile intertidal organisms, primarily barnacles (see Connell, 1985 for references). These studies are almost all done at one or a few closely spaced sites (Connell, 1985). A notable exception, is the monumental work on sea star settlement undertaken by Loosanoff (1964).

Prior to our study, three field studies had attempted to separate settlement from postsettlement mortality for sea urchins. Harrold et al. (1991) monitored the settlement of *Strongylocentrotus* spp. monthly for 14 months in a kelp forest in Monterey, California. Rowley (1989) used a pulsed sampling scheme in a kelp forest near Santa Barbara, California that was able to more effectively reduce the possible effects of postsettlement mortality. Both studies found that settlement and recruitment were pulsed. Harrold et al. (1991) detected settlement in all but one of the months of their study, but clear pulses between November and December 1988 and May and June 1989. From 1984 to 1986, Rowley (1989) found a single pulse of recruitment of very small red and purple sea urchins beginning in the second week of May 1986. By comparing size ranges in the field with those obtained in laboratory settlement experiments, he inferred that this recruitment pulse was caused by a single settlement pulse that occurred between 5 and 17 days previously. Finally, Ebert (unpublished data) monitored the settlement of *Strongylocentrotus* spp. weekly on three kinds of scrub brush collectors placed in the shallow subtidal about a kilometer north of Ocean Beach from June through August 1988. Settlement was observed on the first sampling date and continued through early July. This work was preceded by studies to determine which of seven kinds of artificial substrates resulted in highest urchin settlement rates. The substrates were Astroturf, oyster shells, pea gravel, pieces of

upright coralline algae, and scrub brushes with natural, nylon, and plastic bristles. Settlement rates were highest on brushes with nylon bristles. These substrates have been used in all of our subsequent studies.

Newly settled sea urchins are preyed upon by small crabs (Rowley, 1989), and there is some evidence of significant mortality caused by crabs settling into artificial collectors along with sea urchins (Harrold et al., 1991). It is likely that there are other small urchin predators that might have similar effects (e.g., polychaetes and flatworms). We selected a one-week sampling interval, the shortest that was logistically feasible, to reduce as much as possible this source of post-settlement mortality.

Findings

During the period from December 1, 1991 through November 30, 1992, settlement of red and purple sea urchins was monitored weekly on artificial collectors at locations in northern and southern California. In southern California sites ranged from Gaviota near Point Conception to Point Loma in southern San Diego County. In northern California, there were sites at Fort Bragg and at Westport in Mendocino County.

Settlement in 1992 showed several general patterns for both species: (1) Settlement was pulsed or seasonal at most sites. (2) There was significant spatial variation both within and between geographical regions. Sites along the coast could be placed in three or four groups based on variations in settlement. One group comprised the three southernmost sites (Point Loma, Ocean Beach Pier, and Scripps Pier). Here pulses of settlement occurred from January through July. The June/July pulse was largest at all three sites. The site in Orange County and the sites in Santa Barbara County (Dana Point, Stearn's Wharf, Ellwood Pier, and Gaviota Pier) made up the second group. Here, settlement occurred in pulses between December and July. The final group consisted of the two northern California sites. Here, settlement pulses occurred between June and August, with the June pulse being the largest. (3) Purple urchins settle in greater numbers and on more sampling dates than red sea urchins in both northern and southern California. (4) Finally, settlement occurred over a greater proportion of the year in southern compared to northern California. However, at maximum, settlement was similar in both geographic regions. Differences in settlement between northern and southern California in 1992 contrasted sharply with patterns observed during the two previous years. In 1990 and 1991, settlement was somewhat higher in southern California, and much lower (virtually nil) in northern California. The resulting year-to-year variability was much greater in northern than southern California, and suggests that recruitment to the benthic population may be limited by the availability of settlers in northern California. In addition to exploring the possible linkage between settlement and recruitment, the patterns of spatial and temporal variability that we have documented can be combined with oceanographic data to form testable hypotheses about the possible mechanisms underlying these patterns.

Finally, we have shared our methods to measure urchin settlement with investigators at UC Davis. They are now using them to assess patterns of settlement at sites near Bodega Bay.

Cooperating Organizations

Director's Sea Urchin Advisory Committee, California Department of Fish and Game
EcoSystems Management Associates
Kelco Corporation
Orange County Marine Science Institute
San Diego Fisherman's Association
University of California, Santa Barbara

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The Cost of Livebearing in Yellowtail Rockfish, *Sebastes flavidus*

University of California, Davis
R/NP-1-21G
1992–93

Joseph J. Cech, Jr.

Introduction

The rockfish (*Sebastes* spp.) complex is the most important component of the Pacific coast groundfish fishery. The yellowtail rockfish (*S. flavidus*) has primary commercial importance in Oregon and Washington trawl fisheries and is the most landed rockfish in the northern California recreational fishery. Efficient management requires knowledge of the complex and unique reproductive life history of this species. Recent laboratory and field research has established the fish's viviparous mode of reproduction, high reproductive potential, spatial and temporal variability, and physiological plasticity with regard to egg resorption and interruption of spawning (Eldridge et al., 1991). In ongoing National Marine Fisheries Service studies designed to determine the rates of embryonic and larva development during gestation, captive fish displayed decreased activity, voluntary fasting, and hyperventilation during the latter stages of gestation. Previous measurements of oxygen consumption in pregnant Japanese *Sebastes schlegeli* have shown that the metabolic demands of live-bearing are significantly high (Boehlert et al., 1991). This has implications for maternal energy budgets and the ability of gestating fish to withstand stress such as high temperatures and low food availability as occurred during recent El Niño conditions.

The basis for any physiological, bioenergetic perspective on the demands of reproduction and its effects on other aspects of life history is the knowledge of routine metabolism. This study was designed to accurately determine the change in routine metabolism of female yellowtail rockfish throughout gestation. With this information, it was also possible to estimate the cost of viviparity by comparison of gestating females to nonreproducing adult females held in the laboratory for up to 2 years, males, and spent females. This is the first study to determine the relative cost of livebearing for any Eastern Pacific rockfish.

Materials and Methods

Fish capture and holding

Yellowtail rockfish were captured by hook and line from Cordell Bank, California; their air bladders were deflated, and they were transported to the University of California, Bodega Marine Laboratory, by using handling and transport procedures similar to those of Eldridge et al. (1991). Fish were segregated by sex and held indoors in small groups in circular 2200 L tanks with flowing aerated seawater (mean temperature = 11.93°C and salinity = 33.0 ppt), ambient photoperiod, and fed cut fish and squid to satiation every 3 days. Fish classified as sexually mature, nonreproducing females were captured the previous reproductive seasons (1989–90, 1990–91) and held in isolation in the laboratory, under the conditions just described. Food was withheld from fish for 2 days before the start of respiration measurements. Each fish had a numbered, stainless disc tag fitted loosely through the membranes of the dorsal fin to aid in identification. Fish were lightly anesthetized with 20–50 mg/L MS-222 (tricaine methanesulfonate), and the stage of embryos determined at 3- to 4-day intervals by using a staging scheme described by Yamada and Kusakari (1991). Estimates of the time course of development for embryos and larvae from fertilization to parturition were based on findings from 3 years of studies involving catheterization of individual pregnant yellowtail rockfish and the microscopic examination and staging of embryos and larvae throughout gestation.

After a minimum of 1–2 weeks of acclimation, fish were classified by reproductive stage for respirometry experiments. Female yellowtail rockfish classified as gestating were divided into early embryo, late embryo, and larval stages. Nongestating fish were either vitellogenic, spent, male, or nonreproducing adult females.

Oxygen consumption measurements

Immediately after the embryo/larval staging, reproducing females of the correct reproductive stage (or males or nonreproducing females) were lightly anesthetized and placed into 13-L flow-through respirometers in one of two 250-L insulated fiberglass tanks, which were interconnected with submersible pumps and siphons. Respirometry tubes were covered, except at the ends, with black plastic, and the tanks were partially covered with plywood so that the fish only received indirect light from each end of the respirometer. Each fish was used only once for oxygen consumption measurements. A thermostatted electronic relay controlled the inflow of cold seawater from a 40-L cooler into one of two 250-L mixing tanks as needed to balance a continuous 60–80-L/hr ambient inflow provided to prevent water quality degradation during the experiment. Water from one mixing tank was pumped through a filter, which removed particles ≥ 25 μm, and up to an insulated reservoir incorporating an overflow standpipe, which maintained a constant pressure head and flow of water to the respirometers. Mean temperature for all experiments was 10 ± 0.4°C.

Oxygen consumption was determined by flow-through respirometry by using a system of solenoids controlled by a commercial

sprinkler timer (Toro EL-12+), which sequentially shunted part of the outflowing water from a single respirometer or an inflow line, for 20–24 min every 2 hr, past a Nester O_2 electrode (electrode model 617034, meter model 8500). The O_2 meter output was plotted on a Soltec 310 chart recorder. Flow rates were determined gravimetrically by collection of water from the outflow of a respirometer when the shunting solenoid was closed (i.e., not sampling). Oxygen content of the outflowing water was never allowed to drop below 70% of air saturation.

An initial continuous 48-hr run was conducted to determine an appropriate acclimation period and the necessary length of O_2 consumption measurements. Fish were usually placed in the respirometers by 1100 hr. Subsequent to this first experiment, tests were conducted over 24-hr periods. While measurements of O_2 consumption were taken continuously, only data from 2200–1200 hr the following day were used in calculations. Several fish showed large temporary "spikes," or increases in oxygen consumption, when overhead fluorescent lights shot on or off at dawn and dusk, respectively; these values were discarded. Resting routine metabolic rate (Cech, 1990) was determined from the mean of the 10–12 values (not including the discarded "spike" values) recorded over the 12-hr measurement period (2000 to 0800 hr) for each fish. During one 12-hr measurement period for two of the males the chart recorder jammed, and mean oxygen consumption was determined from only eight values. Mean live body mass for all fish was 1.320 kg and ranged from 0.768 to 2.452 kg. Mass-independent metabolism (Heusner, 1984) or MO_2 in $mgO_2 \bullet kg^{-0.67} \bullet h^{-1}$ was calculated from:

$MO_2 = (O_2 \text{ in} - O_2 \text{ out}) \bullet (V_w \bullet 60) \bullet (M^{-0.67})$ where O_2 in = inflowing water $[O_2]$ in milligrams per liter; O_2 out = outflowing water $[O_2]$ in milligrams per liter; V_w = water flow in liters per minute; and M_b = live body mass of fish in kilograms.

Data analysis

Specific classification of test fish was done by the number of days postfertilization: 0 d (vitellogenic stage), 5–8 d (early embryo), 11–21 d (late embryo), 24–28 d (larval), and >29 d (spent). Data were analyzed using an analysis of variance with Bonferroni posthoc tests (Neter et al., 1985) performed on SYSTAT 4.0 (Wilkinson, 1988).

Results

A total of nine respirometry experiments were conducted between January 13, and March 26, 1992, using 34 individual yellowtail rockfish representing seven different reproductive stages.

Protocol revision

Two behavioral and physiological responses were observed in the initial 48-hr experiment that determined the protocol for subsequent tests (Figure 1). First, fish required approximately 12 hr of acclimation before accurate measurements of resting-routine metabolism could be taken. Second, most fish responded to light changes with dramatic increases in respiration rates. Consequently, we decided that the most accurate measures were to be obtained after 12 hr of acclimation and that the "spike" rate values within the measured period would be discounted from the calculation of resting-routine metabolism.

Metabolism results

The mean mass-independent, resting-routine metabolism of female yellowtail rockfish in the most advanced stage of gestation (i.e., larval stage) was significantly higher than those of males and adult females in stages of prefertilization (i.e., vitellogenic), early embryonic, and spent (t-tests, $p < .01$). *A posteriori* tests at $p = .05$ further indicated that late embryo and larval stage females were statistically similar and separate from all other stages. The overall pattern seen in Figure 2 shows a gradual distinct increase in metabolic demands as gestation progresses, followed by a rapid decrease after parturition. Males that served as models of metabolically unburdened fish had the lowest values of all fish measured.

Unexpected responses were observed in nonreproducing females. The mean oxygen consumption rate of four replicate females was 98.034 mg O_2/kg/hr for the weight-specific rate and 104.800 for the mass-independent metabolic rate. These rates were 36.7% and 24.4% greater than those of the highest metabolizing pregnant females containing larvae.

Discussion

Our study provided valuable information on the protocol for measuring metabolism in gestating marine rockfishes. Foremost in importance was the need for prolonged acclimation and test periods; 12 hr each were judged suitable for yellowtail rockfish. Handling, anesthesia, and adaptation to the confinement of the respirometer most likely contributed to the erratic pattern of oxygen consumption observed (Figure 1). Changes in light conditions, specifically when lights were turned off and on, also caused abnormally elevated consumption rates. For accurate estimates of resting-routine metabolism, we concluded that only the nonspike measurements averaged over the 12-hr postacclimation period were valid. In contrast, Boehlert et al. (1991), who conducted the first and most comparable studies of respirometry in the viviparous *Sebastes schlegeli*, acclimated their fish for 3–5 min followed by test measurements of 5–10 min of oxygen consumption. Their findings are representative of more active metabolism, although their fish were captive stock, presumably more accustomed to handling than our wild fish.

Metabolism significantly increased as gestation progressed. The observed pattern showed that supporting even unfertilized eggs has a cost (i.e., vitellogenic and gestating stages were higher than both spent females and males, Figure 2). As embryonic and larval development proceeded, metabolism significantly increased, especially during late embryo and larval stages,

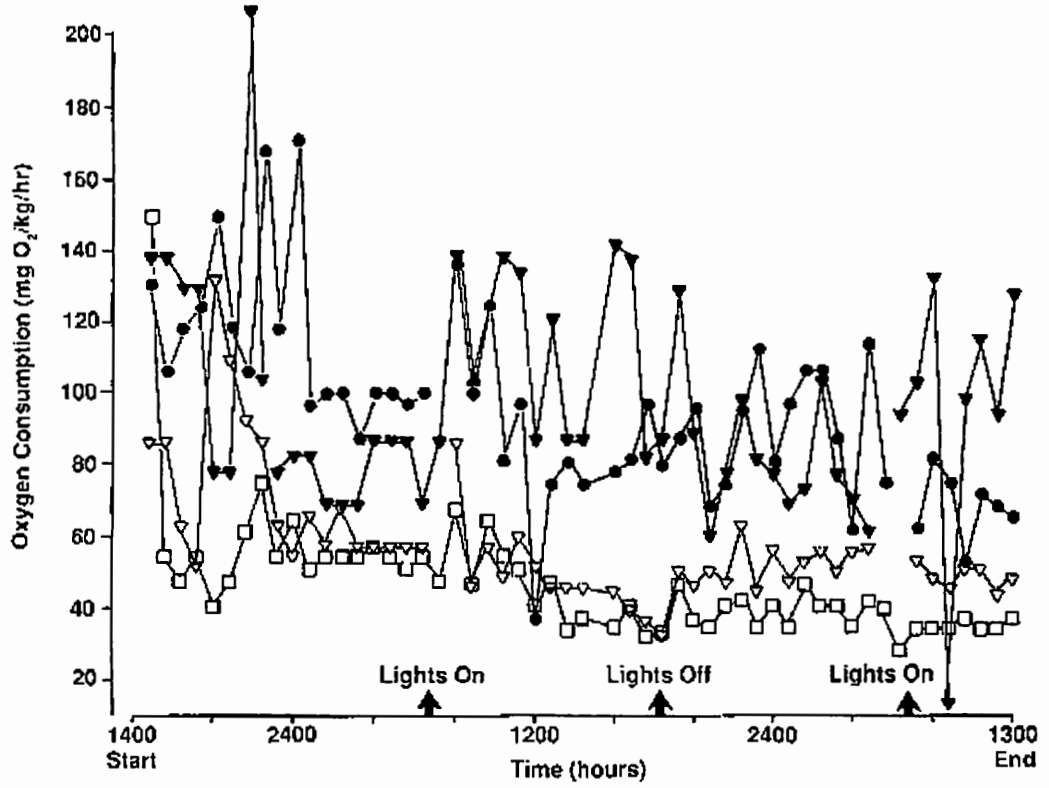

Figure 1. Oxygen consumption rates of four yellowtail rockfish over 48 continuous hours. Solid symbols represent nonreproducing adult females; clear symbols represent vitellogenic females.

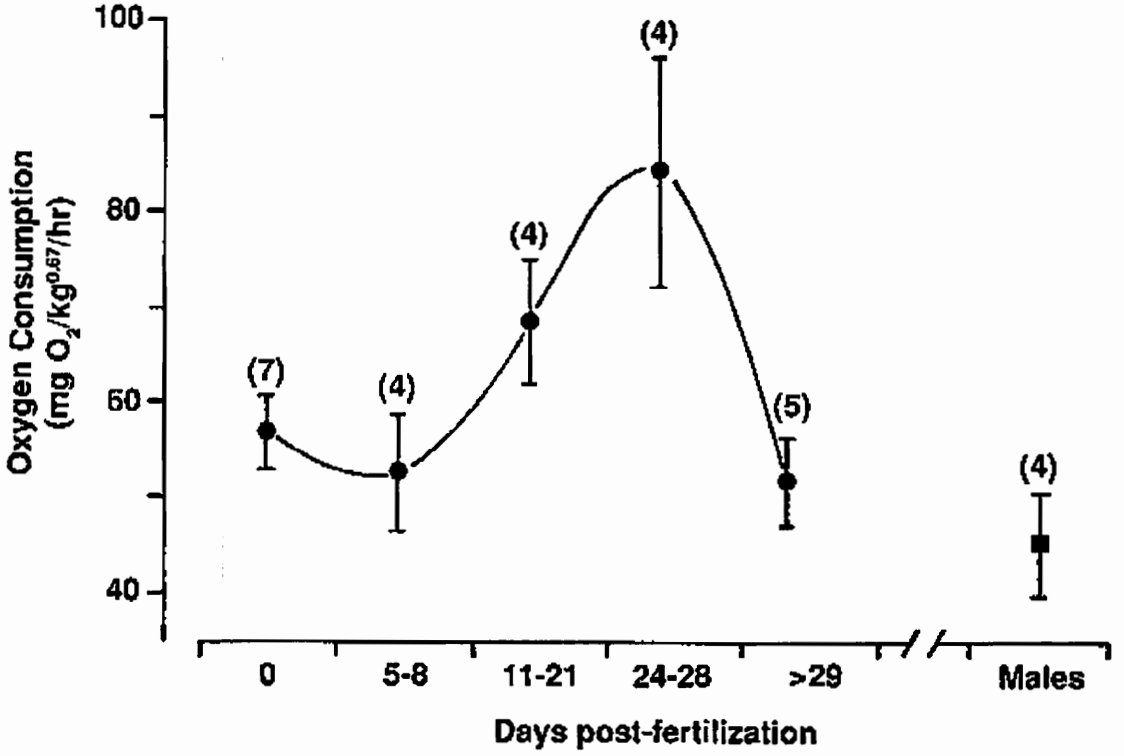

Figure 2. Mass-independent oxygen consumption rates of yellowtail rockfish in different reproductive stages. 0 days postfertilization = vitellogenic fish; 5-8 d = early embryo fish; 1121 d = late embryo fish; 24–28 d = larval fish. Numbers in parentheses represent replicate experiments of fish in each stage. Points are means of replicate measurements with standard error bars.

approximately 11–28 days after fertilization. Our study contributes a definitive perspective to the work of Boehlert et al. (1991), which pooled all gestating stages together and found significant differences between gestating and nongestating females and males. This study's findings indicate that it is the most advanced embryos and larvae that are responsible for the significant differences in metabolic rates. A comparison of the magnitude of the differences shows that gestating *S. schlegeli* respired at 68% higher levels than nongestating fish; *S. flavidus* females with larvae respired at 63% higher levels than spent fish and 86% higher levels than males.

An enigmatic unexpected result was the high metabolism of nonreproducing adult females that had been held captive for long periods. These fish respired at rates higher than all other categories, suggesting that performances of such specimens in the laboratory are not suitable for predictive extrapolation to the natural environment.

The 1991–92 reproductive season in the coastal waters off northern California experienced the warm water temperatures of an El Niño (Kerr, 1992). Field studies of rockfish mesenteric fat deposits, which serve as energy sources, have shown that warm water conditions are stressful (Lenarz and Echeverria, 1986). Proximate effects of high water temperatures were observed in our laboratory fish toward the end of the study as fish resorbed or aborted their embryos or larvae. We countered the problem by holding newly captured fish in seawater chilled to the experimental temperature of 10°C. The combined stress of high temperatures and the demonstrated high energetic demands of supporting advanced stage embryos or larvae could present a potential problem by reducing population fecundity or increasing adult natural mortality.

Cooperating Organizations

NOAA, National Marine Fisheries Service, Southwest Fisheries Science Center, Tiburon Laboratory, California

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Management of Agricultural Runoff in Shellfish Growing Waters

Humboldt State University
R/NP-1-21H
1991–92

Charles Chamberlin and Robert Gearheart

Introduction

Point source pollution, with definable sources such as sewage treatment plants, has historically been the primary focus of water quality control regulations. With increased environmental awareness there has been a shift in recent years to address nonpoint sources of pollution (NPSP) as well. NPSP is associated with storm water and runoff, generally from sources related to human land use and to natural processes in the watershed. Agriculture is a major source of nonpoint source pollution in the United States, contributing significant levels of total and fecal coliform into receiving waters. The possibility of treating NPSP with constructed wetlands has generated much interest, and many researchers have proposed and identified various bacterial removal processes in wetlands. There is, however, a lack of quantitative information from laboratory studies regarding bacterial removal and inactivation mechanisms at work in constructed wetlands. This project addresses the bacteriological contamination of shellfish harvesting areas in Arcata Bay, California by dairy runoff and evaluates the feasibility of treating this runoff with wetlands.

Farm runoff, secondary to activities such as dairy operations, cropland, rangeland, and feedlots, is the principal contributor of pollutants for 64% of the rivers and 57% of the United States lakes (Novotny and Chesters, 1981). Storm and runoff events can result in significantly increased coliform counts due to bacteria entering the stream from overland flow, subsurface flow, or the disturbance of organisms in bottom sediments (Bohn and Buckhouse, 1985). NPSP accounts for greater than 98% of the fecal and total coliform loading into receiving waters (Novotny and Chesters, 1981). With respect to shellfish harvesting in Arcata Bay, coliform contamination from agricultural sources is the primary nonpoint source pollutant (Anatec, 1982). The 1991 Management Plan for Commercial Shellfishing in Humboldt Bay, California established closure standards with criteria that include closing the shellfish harvesting beds after one-half inch of rain within a 24-hour period. During normal hydrological years, up to 100 days of winter harvesting have been lost because of high fecal coliform concentrations in Arcata Bay.

The possibility of treating NPSP with constructed wetlands has generated much interest. The success of small, constructed wetlands in treating municipal waste suggests that such systems may be appropriate for treating dairy waste (Barker et al., 1984; Moore et al., 1983; Schwer and Clausen, 1989; Wengrzynek, 1990). Wetlands have the potential to provide a relatively high level of secondary and tertiary treatment at a lower initial cost than conventional waste treatment systems, with lower operation and maintenance costs. Wetlands are relatively insensitive to hydraulic or pulse loading and can produce significant pathogen removal (Finney and Willis, 1990). Several pilot wetland studies have reported greater than 90% reduction in coliform bacteria (Spangler et al., 1976; Reed et al., 1988). The primary indicators measured in these pilot project studies have been total and fecal coliform groups.

There is a lack of *quantitative* information from laboratory studies regarding bacterial removal and inactivation mechanisms at work in constructed wetlands. A variety of bacterial decay mechanisms have been identified. *Physical* processes that produce bacterial decay include sedimentation, adsorption, coagulation, flocculation, and photo-oxidation. *Biochemical* processes include predation, algal inhibition, and microbial growth. *Physiochemical* factors include osmotic effects, pH, chemical toxicity, and redox potential (Tetra Tech and Chamberlin, 1985).

Wetland vegetation may play a significant role in bacterial removal. The surface of plants may act as a habitat for microorganisms that prey on allochthonous bacteria. Wolverton (1987) asserts that the reactions between environmental pollutants and wetland ecosystems are complex, and aquatic plants serve as more than just large surface areas for microorganisms. Gearheart et al. (1986) demonstrated that bacterial removal is dependent on aquatic vegetation. This study postulated that the biofilm associated with emergent vegetation may inactivate coliforms and that vegetation may help to stabilize the settled suspended solids to which bacteria have adsorbed. Vegetation reduces water velocity, which facilitates sedimentation. Cattail and bulrush debris and surface slime from marsh plants may also provide coagulation aids for bacterial aggregation, which further increases sedimentation rates. It has been proposed that some plants may contribute photosensitizers, which increase bacterial susceptibility to inactivation by photo-oxidation (Tetra Tech and Chamberlin, 1985).

Other bacterial removal processes such as photo-oxidation and physiochemical factors have also been supported by researchers. According to Klock (1971) coliform inactivation may be associated with endogenous metabolism and wastewater conditions unfavorable to some living organisms. Sarikaya and Saatchi (1987) found that bacterial die-off rate constants were inversely proportional to pond

depths due to ultraviolet inactivation. However, the presence of densely growing duckweed *Lemna minuata* in wetlands which limits light penetration, may indicate that photo-oxidation is not a significant mechanism for bacterial inactivation. Polprast and Hoang (1983) concluded that removal was more strongly influenced by water quality and filtration than by adsorption in a study of anaerobic filters of different specific surface areas with comparable removal rates under various hydraulic loadings (Finney and Willis, 1990). Efforts to determine which of these factors plays a dominant role in bacterial removal in wetlands can lead to improved design criteria and management models of constructed wetlands used for water treatment.

This project addresses the bacteriological contamination of shellfish harvesting areas in Arcata Bay from dairy runoff and evaluates the feasibility of treating this runoff with wetlands. The bacterial loadings into the Bay were estimated in order to identify the need for treatment. A series of laboratory tests as well as a bench scale wetland or mesocosm were used to identify the mechanisms of bacterial removal in wetlands. The results of this project are intended for use in the development of design criteria for constructed wetlands to treat dairy runoff.

Field Sampling

Liscom Slough, a tributary to the Arcata Bay, was chosen as the sampling site. The watershed drains into the shellfish beds of the Bay via Liscom and Mad River Sloughs. Dairy operations are the major land use, supporting approximately 200 cattle. The methods of bacterial transport from the watershed to the slough include overland flow, soil interflow, and to a lesser degree groundwater flow. In order to determine the extent of bacterial contamination in the slough, bacterial loadings were characterized from samples taken during ebb tides for baseline and during storm events. Samples were collected and analyzed by the membrane filter technique for total and fecal coliform and enterococci. Enterococci were of primary interest because data on this subgroup are limited and of particular concern for shellfish contamination. Historical fecal and total coliform data from the Department of Health Services had been collected near this site and were used to analyze bacterial trends.

Literature regarding the quality of pasture runoff from feedlots and dairies indicates that it is difficult to determine the extent of bacterial contamination based upon the number of animals and the land area (Doran et al.,1981; VanDonsel et al.,1967). Manure accumulates during the nonrainy season, and bacterial survival in manure has been reported to be up to 1 year (Doran et al.,1981). Rain intensity and duration affect to what degree manure is broken down and consequently how much will migrate to receiving waters.

Figures 1 and 2 show increased coliform counts during storm events for the months of October through December. These plots indicate that coliform concentrations increased after the first couple of days of the storm, perhaps indicating that the maximum coliform transportation/ mobilization occurs once the soil has reached saturation.

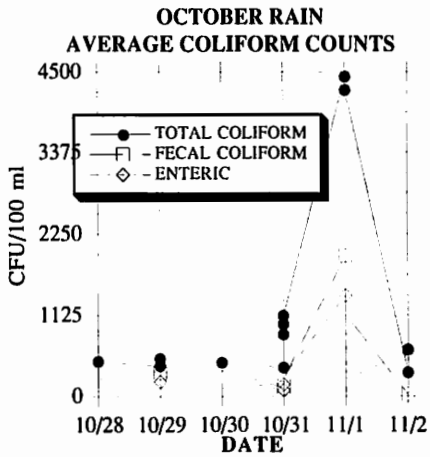

Figure 1. October storm sampling.

An increasing trend for all three bacterial parameters was noted. When samples were taken more than once during an ebb tide, the lower tide cycle had consistently

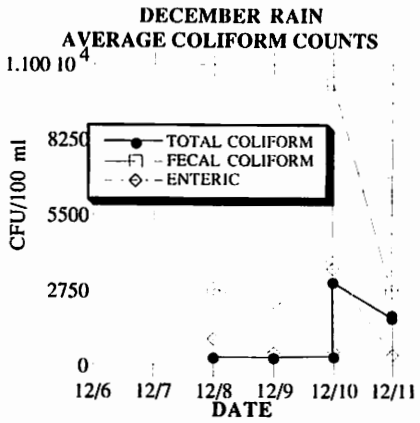

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Figure 2. December storm sampling.

higher bacterial counts, probably as a result of disturbance of the sediment layer where bacteria are adsorbed.

Bacterial loading from Liscom Slough into the Mad River Slough and the Arcata Bay are presented in Table 1. At the time of sampling, velocity and stage were measured. Loadings presented as CFU/hrs/ acre are based on an approximate total drainage area of 28.16 acres (Fitzgerald, 1987). Loadings will be higher during January-February since historical data from the Department of Health Services indicate that fecal and total coliform concentrations reach a maximum during those months. The shellfish harvesting limit is given as 70 MPN/ 100 ml for total coliform and 14 MPN/100 ml for fecal coliform (Anatec, 1982).

Log plots of coliform concentrations versus flow revealed no clear trends or valid correlations between these two parameters in the heavily tidally influenced slough. This is consistent with previous studies, which have been unsuccessful in drawing valid correlations between coliform concentrations and surface runoff flows (Olivieri, et al., 1977; Anatec, 1982).

The relationships between total and fecal coliform and enterococci was evaluated to identify possible relationships between the various bacterial parameters. While the correlation between the total coliform and the fecal coliform or enterococci parameters is weak (r^2 of .264 and .176), there is a strong

association between the fecal and enterococcal indicators (r^2 of .847). The mean fecal/enteric ratio from storm samples was 4.09. A strong association would be expected since enterococci are a subgroup of the fecal coliform group. This suggests the possibility that enteric concentrations can be estimated from fecal counts for similar conditions; however, the implications of a functional dependence are limited. A stronger association between total and fecal groups was expected, yet we considered that the total coliform counts were the least reliable because of overgrowth of abnormal colonies and greater subjectivity in distinguishing positive colony-forming units than the other tests. As Bonde (1977) discussed, organisms of mixed origin (human, animal natural source, industrial products) cannot be correlated in a strict sense with the amount of fecal matter or with strictly fecal organisms.

Table 1. Bacterial Loading—Liscom Slough

		Fecal	Enteric
Q (litres/hr) mean	3085.74		
Standard deviation	1365.58		
CFU/100 ml mean		1780	608
CFU/100 ml median		1156	403
CFU/100 ml mode		1950	1400
Range—smallest		61	85
largest		9000	2200
CFU/Hrs/Acre mean (x 10^6)		210.0	72.5
CFU/Hrs/Acre median (x 10^6)		33.5	40.7
CFU/Hrs/Acre maximum (x 10^6)		1076.3	263.1

Table 2. Water Quality Parameters

Wash Water Parameters		Slough Water Parameters	
Hardness	110 mg CaC03/l		
Alkalinity	138 mg CaC03/l	Alkalinity	120 mg/l CaC03/l
Dissolved oxygen	6.0 mg/l	Dissolved oxygen	7.1 mg/l
pH	6.6-8.3	pH	7.3-7.8
Salinity	2,300 umhos	Salinity	>36,000 umhos
Total solids	278.57 mg/l	Total solids	3720 mg/l
Total dissolved	203.57 mg/l	Total dissolved	3692 mg/l
Volatile dissolved	79.76 mg/l	Volatile dissolved	622 mg/l
Fixed dissolved	123.81 mg/l	Fixed dissolved	3070 mg/l
Total suspended	75.00 mg/l	Total suspended	28.4 mg/l
Volatile suspended	31.67 mg/l	Volatile suspended	4.40 mg/l
Fixed suspended	43.33 mg/l	Fixed suspended	16.0 mg/l
Turbidity	500 NTU	Turbidity	23 NTU
BOD	240 mg/l		
COD	470 mg/l		

Laboratory Test Results

Water that is used to wash down a 60-head dairy barn after milking currently flows onto pasture land located in the study watershed. This wash water was sampled before the addition of detergents or disinfectants and analyzed for various water quality parameters (Table 2). This water was diluted with distilled water for the laboratory tests, which included both standing jar tests and flocculation tests. These laboratory tests focused primarily upon the physical bacterial removal mechanisms of adsorption, sedimentation, flocculation, coagulation and photo-oxidation, and predation.

Jar Tests

Laboratory tests included jar and flocculation tests. In the jar tests, three series of seven 1-liter beakers, including a control, of dairy wash water at 0.1 dilutions with distilled water. The initial pH of the diluted wash water was between 6.6 and 7.4. Coagulation and flocculation processes are affected by the hydrogen ion concentration, and generally the presence of organic acids lowers the pH in natural wetland systems. These jar tests addressed only freshwater conditions and results would not be applicable to the saline slough conditions. In an attempt to isolate wetland mechanisms, each jar contained a separate single wetland constituent: (I) Control, (II) Cattail *Typha latifolia*, (III) Bulrush *Scirpus californicus*, (IV) Sand, (V) Gravel, (VI) Pasture soil with high clay content, and (VII) Darkness. The wetland plants were included to evaluate the effects of vegetation on bacterial removal. Each soil type varied in specific surface area and was used to evaluate differences in adsorption. The dark jar was included to evaluate bacterial decay in the absence of photo-oxidation effects. Bacterial levels in each beaker were measured and recorded daily for 11 days to determine how bacterial removal rates differed with each treatment. Linear regressions on the natural log of the coliform concentrations resulted in decay rates in each of the three separate series of jar tests as presented in Figures 3 and 4.

Analysis of variance of these results indicates that the variation in the decay rates between each series within a jar type is indistinguishable from the variation beween

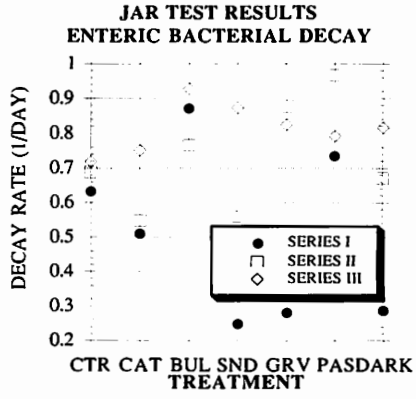

Figure 3. Enterococci decay rates.

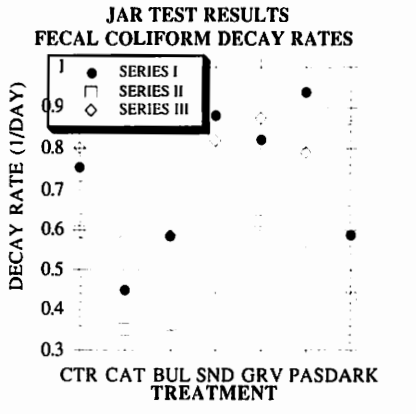

Figure 4. Fecal coliform decay rates.

the different jars. Conclusions therefore cannot be drawn regarding differences in decay rates between jars or treatments. These results exemplify the difficulty in setting up isolated laboratory tests to simulate a dynamic ecological system as complex as a wetland.

Flocculation Tests

In addition to the standing jar tests, flocculation tests were run on the dairy wash water samples in order to evaluate the effects of water movement. Three replicate tests were run over a 3-hour period. A series of five beakers including a control were spiked with wash water, flocculated at 25 revolutions per minute for 1 hour, and allowed to settle for 5 minutes; then samples were taken and tested for total, fecal, and enteric bacteria. Each beaker contained a wetland parameter that may relate to bacterial adhesion and sedimentation: plant surfaces (bulrush), sand, gravel, clay, and a control.

Sedimentation of bacteria can be the result of aggregation of bacterial particles to each other, which form larger conglomerates that sink, or the adsorption of bacteria to soil particles that settle. Soil is composed of finely divided minerals; plant, animal and microbial residues in various stages of decay, and microbiota. The processes of bacterial aggregation, adsorption, and sedimentation are a function of water velocity and shear stress on the particles. Sedimentation is hindered by turbulent flow conditions. Some level of turbulence aids in aggregation, but at higher levels the bacterial clumps may break apart.

Flocculation tests demonstrated both increases and decreases between tests, which probably reflects aggregation and disaggregation processes rather than die-off. The removal rates obtained from regressing the overall 3-hour results are presented per bacterial group in Figure 5 and reveal that the total coliform group generally experienced greater die-off or aggregation than either the fecal or enterococcal groups.

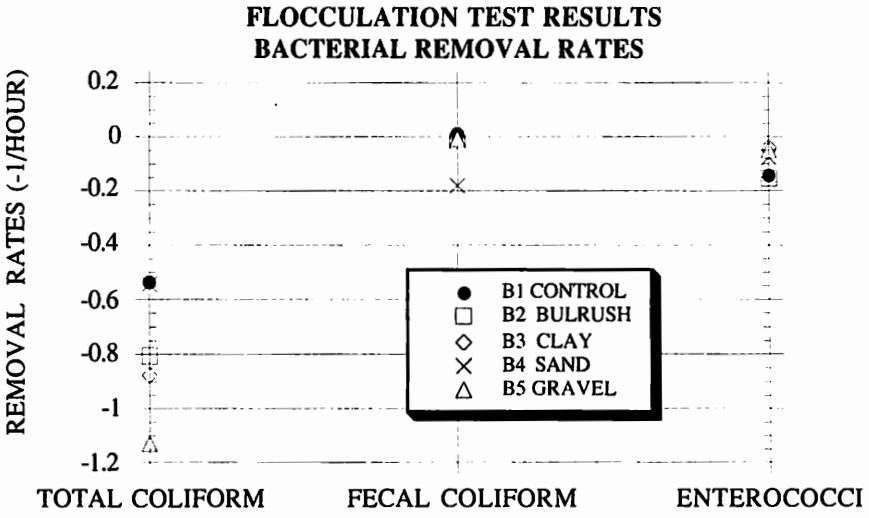

Figure 5. Flocculation test results.

Mesocosm

Testing is currently underway on the bench-scale wetland model, which has been designed, built, and planted with bulrush. This mesocosm consists of a series of three 4 × 2 × 2 foot metal tubs, connected by pipes and weirs, which are set up to isolate bacterial removal mechanisms. Tub 1 contains only gravel and is the sedimentation basin. Tub 2 contains 150 7/8 in diameter pvc pipes to simulate the physical influence of plants, absent of the biological processes. Tub 3 has been planted with bulrush and more realistically simulates wetland vegetative conditions. No conclusions have been drawn from preliminary results but will be presented when testing has been completed.

Summary of Results

The following conclusions have been reached from this study:

- Significant amounts of fecal coliform levels enter Arcata Bay shellfish beds via the surrounding pasture lands during storm runoff, and therefore a need for treatment exists.
- Coliform concentrations increase after the first couple of days of a storm, perhaps an indication that the maximum coliform transportation/mobilization occurs once the soil has reached saturation.
- Bacterial loading was noted to be as high as 9000 fecal coliform per 100 ml and 2200 enterococci per 100 ml.
- A strong association between fecal coliform and enterococci was noted (r^2 of .847), with a mean ratio of 4.09 fecal coliform to enterococci. The implications of a functional relationship, however, are limited to very similar conditions.
- The data showed no clear trends between bacterial concentration and flow in the heavily tidally influenced slough.
- The differences in decay rates noted *within* jar types between series was indistinguishable from the differences *between* the different jar types.
- Laboratory test results indicate the difficulty of isolating wetland mechanisms for study.
- Because photo-oxidation processes can be more easily isolated from the other proceses in dark versus light jars, the lack of statistically significant differences in decay rates may indicate that photo-oxidation is not a dominant bacterial decay factor in wetlands. This is supported by the fact that duckweed *Lemna minuata* and other vegetation often blocks light penetration.
- The flocculation tests showed that the total coliform group generally experienced greater decay or aggregation than the fecal or enteric groups.
- Continued testing of the mesocosm, simulating more realistic conditions than the jar tests, is expected to provide useful results.

- Further research, such as a pilot project built on a dairy farm, could provide valuable information on the use of wetlands as a treatment approach.

Discussion

There are obvious difficulties associated with wetlands built to treat dairy runoff as opposed to wetlands for municipal treatment. Raw livestock wastewater sampled from animal waste lagoons showed considerably higher concentrations of biological oxygen demand, ammonia, and total phosphorus than municipal wastewater (Surrency, 1992). The rain catchment area is much more diffuse in agricultural storm runoff, and therefore it is necessary to take advantage of natural water drainage courses for wetland placement. Because of the diffuse nature of nonpoint pollution sources, it appears that a wetland should be constructed near the primary source. Unfortunately, the ideal land area to capture primary sources near barns or in pastures is often the most valuable land to the dairy farmer. Hydraulic loading would vary widely from season to season, but it would be necessary to maintain water levels to sustain the wetland plants. Infiltration through saturated soil directly into the groundwater could still be a significant source of bacterial contamination (Burge and Parr, 1980).

A series of strategically placed wetland buffer areas on less valuable land near receiving waters could be utilized to catch the majority of contaminated runoff. It may be possible to plant existing drainage channels with wetland plants and place weirs in these areas (Brown, 1988; Dickey and Vanderholm, 1981; Phillips and Phillips, 1988). A comprehensive farm pollution management plan would facilitate the success of a constructed wetland (U.S. Soil Conservation Service, 1991). One viable option is to combine treatment of both point sources such as barn washwater with pasture runoff, thus providing a more consistent water supply to the wetland.

It is recognized that not all dairy runoff can be contained. However, decreasing the bacterial loadings with wetland treatment will decrease shellfish contamination. Given that dairy farmers are facing unknown costs to deal with nonpoint regulations and the need to protect shellfish harvesting beds from contamination, it is therefore important to consider wetland treatment alternatives.

Cooperating Organizations

California Department of Health Services—Environmental Health Services Section
Humboldt State University, Arcata, California
Oregon State University, Corvallis, Oregon—Department of Agricultural Engineering
U.S. Environmental Protection Agency—Environmental Research Center

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Management Issues and Policy Options in Ocean Governance

University of California, Berkeley
R/NP-1-211
January–June 1992

Harry N. Scheiber

The objectives of this project were all in support of a collaborative effort of Berkeley faculty members with students of marine affairs in a dozen other coastal states' institutions to analyze the outstanding issues in management of U.S. offshore waters. Organized as the Ocean Governance Study Group, this consortium of scholars established a detailed agenda of research at a workshop meeting at the University of Hawaii in January 1992, attendance at which by Berkeley students and faculty was supported by this grant. The publication *Ocean Governance: A New Vision* (Ocean Governance Study Group, 1992) contains papers presented at the workshop, including a presentation by Professor Scheiber (1992); and that publication is submitted herewith as a part of this report and as a principal evidence of progress of the studies supported.

The grant also supported the following additional major objectives during 1992.

First, research and writing of policy briefing papers was designed to serve the needs of state and federal agencies, and of Congress and the state legislatures, as well as user groups and scholars. Several of these papers were presented, after formal termination of this grant, at a meeting of 35 congressional committee staff concerned with marine policy. Among the presentations was one by Professor Scheiber on the subject of fisheries management and intergovernmental relations.

Second, faculty and staff time was supported in the Ocean Law and Policy Program at Boalt Hall School of Law, for organization of a major OGSG research conference on ocean governance at Berkeley in January 1993. With the assistance of the Sea Grant Trainee, Professors Caron and Scheiber both also prepared individual papers for this conference (see Caron, 1993).

Third, the efforts of Professors Caron and Scheiber, with staffs, were supported in their capacities as steering committee members of the OGSG for 1992 to: advance research by members of this group on specific issues in ocean and coastal management; cooperate with the Western Governors Association, and the Western Legislative Conference (Council of State Legislatures) in development of new concepts of cooperative state-federal offshore resources management; and promote dialogue with elective officials, agency officers, scientists and technical experts, representatives of industry, and public interest groups.

Throughout the period of the grant, and also subsequently, Professor Scheiber has been simultaneously involved with the National Research Council project on science/policy interaction in ocean studies, dealing with intersecting themes with those being explored by OGSG. At least one publication (Scheiber, 1993; see also Scheiber 1992b) will issue from that activity. Professor and former Chancellor I.M. Heyman of Boalt Hall has also been involved the National Research Council project.

Dissemination of the products of workshops and other research and coordinative activities has been in the form of: wide distribution of the publication, *Ocean Governance: A New Vision*; preparation and distribution of preliminary drafts of papers for the January 1993 Berkeley conference; and oral presentation at the congressional meeting.

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Refinement of Techniques for the Mass Culture of the Red Sea Urchin *Strongylocentrotus franciscanus*

University of California
Bodega Marine Laboratory
R/NP-1-21L
January–May 1992

Wallis H. Clark, Jr. and Douglas E. Conklin

The red sea urchin, *Strongylocentrotus franciscanus,* is the subject of one of the most important commercial fisheries in the state of California. The impact of commercial harvesting has been severe and natural populations are rapidly becoming depleted. In response to this depletion, management measures are being implemented, and those in the commercial urchin industry, working with California Sea Grant marine advisors, are seeking means to assure the long-term well-being of red urchin populations. One possibility that has aroused a great deal of interest is the potential of reseeding depleted fishery grounds with young urchins, either just prior to settlement, or as recently metamorphosed juveniles.

Until our recent studies, reports in the scientific literature held that the maximum number of pluteus larvae that could be cultured to metamorphosis was one per 10 ml of seawater (Leahy, 1986). The larvae were fed the algae *Rhodomonas lens* at a density of approximately 6,000 cells/ml or at a concentration that could be cleared by the plutei within 24 hr (Leahy, 1986). Under such conditions metamorphosis took place in approximately 40 days, and the percentage completing metamorphosis was moderate and variable.

In a 1991 California Sea Grant funded project ("The Development of Techniques for the Mass Culture of the Red Sea Urchin, *Strongylocentrotus franciscanus*"), our laboratory made great progress in increasing the density of *S. franciscanus* larvae in culture. We showed that a larval culture density of 5 larvae/ml could be obtained when fed on an optimal algal feed *(Rhodomonas lens)* and at an algal density of 60,000 cells/ml, as long as the water was changed daily. At 18°C, the temperature we found to be optimal, metamorphosis began as early as 24 days following fertilization, at rates of 56%. We were also able to maintain larval densities as high as 50/ml, but metamorphosis did not occur. The purpose of the present study was to determine conditions for increasing larval density while still achieving appreciable levels of metamorphosis, and we were able to show that intensive culture of *S. franciscanus is* indeed possible.

Gravid animals were collected in the waters near the Bodega Marine Laboratory by local divers and maintained in flow-through seawater tanks. They were induced to spawn by injecting 0.5 M KCl through the peristomal membrane (Tyler, 1949). The eggs and sperm were mixed together in UV-filtered (5 μm) seawater in containers holding 1.5 liters of seawater, equipped with stir paddles run by a small motor.

Under our culture conditions, the first cell division occurred 2 hr postfertilization. Hatching occurred within 18 hr, and gastrulation began by 26 hr. Development reached the prism stage (as evidenced by the presence of $CaCO_3$ spicules) by 48 hr postfertilization. By day 3, larvae had reached the four-armed pluteus stage (pluteus is the terminal echinoid larval stage). At this stage feeding began, with the planktonic larvae feeding mainly on unicellular algae. At this point, from day 4 and until the larvae are ready to settle, the larvae are cleaned regularly by rinsing them onto an 80 μm screen; the 1.5 liter container and stir paddle are rinsed; and the larvae are rinsed back into the refilled container. After cleaning, the larvae are fed with algae (60,000 cell/ml).

By day 10, the pluteus larvae had grown to nearly one millimeter in width and had developed a total of eight arms. After approximately 14 days, rudimentary urchins were visible developing within the larvae. Rudiment development continued further until larvae initiated metamorphosis on approximately day 25. When an urchin was competent to metamorphose, it attached to the substrate, possibly as a result of a chemical cue produced by certain bacteria (Cameron,1974), and slowly broke out of the larval structure. Once settlement began, the containers were not rinsed for one or two days, and the larvae settled and attached on the sides of the containers. The settled larvae and the containers were then submerged in an outdoor tank where the urchins could graze on the container and tank. Various types of macroscopic algae were provided to the young urchins as they grew, including kelp, Bull Kelp, and fleshy red alga. Some of the cultured urchins were 50 mm in test diameter at 14 months, but there was a high variability in test diameter at this age.

During culture, the larvae of *S. franciscanus* are very sensitive to overcrowding; at densities higher than 10/ml the larvae eventually clumped together and died. We had previously determined that the effects of overcrowding could be minimized by completely exchanging their water with fresh seawater on a daily basis. However, even under these modified conditions, larval development rates were more variable and overall much slower than rates observed at 5/ml, and metamorphosis did not take place.

Three probable, and not mutually exclusive, explanations for the observed limits to larval density are suggested: (1) fouling; (2) starvation; and (3) social interactions (density effects of behaviors involved in growth and/or metamorphosis). We addressed the possibility of fouling in the present study by changing the seawater more frequently, and found that changing the water three times daily and

constantly overnight was required for animals to metamorphose. Concurrently, feeding levels had to be increased to three times a day. Under these conditions, up to 25 larvae/ml could be maintained, but metamorphosis was reduced to 18.7%, and settlement was delayed to 30 days postfertilization.

During our study we also determined the efficiency of high surface area substrates on metamorphosis. A series of mylar corrugated sheets were placed vertically in the culture containers just below the circulation paddles. However, the increased surface area had no effect on rates of metamorphosis in the higher density cultures.

Overall, our results suggest that larval development and metamorphosis are only indirectly density dependent, reflecting a lack of antagonistic interactions between larvae in contrast to such invertebrates as shrimp. Metamorphosis at high densities, however, does seem to be directly dependent on food availability and/or frequent cleaning. Intensive culture of the red sea urchin systems does appear possible but will undoubtedly require the engineering of systems which provide continual feeding and water exchange.

Cooperating Organizations
California Institute of Technology
Louisiana State University
San Diego State University

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Gametophytic Development in the Giant Kelp, *Macrocystis pyrifera*

University of California
Bodega Marine Laboratory
R/NP-1-21M
1992–93

Muralidharan C. Pillai and Gary N. Cherr

The giant kelp, *Macrocystis pyrifera*, is the principal brown alga utilized commercially in the United States, and is one of the most important species in near-shore ecosystems. Mariculture of kelp is on the edge of commercial development in the United States, and techniques for its mass culture have been developed (Charters and Neushul, 1979; North, 1981). These techniques often involve growing the dioecious haploid gametophytes vegetatively and inducing sexual reproduction and fertilization in the laboratory (Sanbonsuga and Neushul, 1980; Waaland, 1983); the young diploid sporophytes are then outplanted for further growth. Therefore, a successful culture practice largely depends on the ability of gametophytes to produce a large quantity of viable gametes, and in the long term, the ability to manipulate these stages at the cellular and genetic levels. The success of this will largely depend on our understanding of its cell cycle events, as well as its genomic behavior, during reproduction and development (Lewis et al., 1986).

The objectives of our project were: (1) to elucidate the cell cycle events associated with early development in *M. pyrifera* gametophytes; (2) to determine the cellular DNA levels (and thus the ploidy) in cells of gametophytes (and gametes) over a fine developmental time course, and; (3) to initiate experiments on manipulation of DNA levels using inhibitors and modulators of both DNA synthesis and cell division. We have made great progress in working towards these objectives.

For our study, mature sporophylls were collected from the bases of *M. pyrifera* sporophytes at Santa Barbara and Bodega Bay, California. Induction of zoospore release was performed according to Anderson and Hunt (1988). Briefly, reproductive sporophylls were air dried for 1 hr at room temperature. They were then extensively washed in running seawater, blotted to dryness, and placed in "culture medium" (CM; 0.45 micron filtered seawater containing 0.01% penicillin G, pH 7.9) in the dark at 15°C. Sporophylls were monitored periodically for zoospore release, which usually occurred in 45–60 min. Sporophylls were removed once a high density of motile zoospores were present in CM as determined by darkfield microscopy (100 × magnification). Zoospores were cultured (7.5×10^4 cells/ml) in 5 ml Lab-Tek chamber slides (Nunc, Napperville, Illinois) at 15°C under continuous white fluorescent light adjusted to give 100 $\mu E/m^2/s$ (Deysher and Dean, 1984). Gamete culture was conducted according to Reed et al. (1991) using an enriched Provosoli's medium (Provosoli, 1968).

For light microscope studies of early developmental events, samples of cultures at different stages of development were fixed with seawater containing 1% paraformaldehyde and observed with an Olympus BH-2 light microscope equipped with Hoffman modulation optics. To determine the time course of germination and germ tube elongation, samples of zoospore cultures at different stages of development were fixed as described above. Successful germination was determined when the germ tube was equal to or longer than the diameter of the zoospore. Division and movement of nucleus (nuclear translocation) during gametophytic development were determined by probing the cells with the vital DNA stain Hoechst 33342.

Microspectrofluorometric analyses of DNA levels in cells at different stages of gametophytic development were conducted. These stages included recently released zoospores, germinated zoospores (prior to nuclear division), early gametophytes (after nuclear division and translocation), late gametophytes (differentiated into male and female), and gametes (sperm). For this, zoospores were cultured, at 7.5×10^4 cells/ml, on glass microscope slides (placed in 500 ml Corning culture bowls) in CM as described above. Cultures were monitored for successful development. Samples of monolayer cultures, at different stages of development, were fixed in 3:1 (95% ethanol: glacial acetic acid) for 1 hr at room temperature, washed with and stored in 75% ethanol. DNA analysis in single cells were carried out according to Goff and Coleman (1984). Briefly, cells were probed with the water soluble fluorochrome 4', 6-diamidino-2-phenylindole (DAPI) and examined using a Leitz photomicroscope equipped with an epi-illumination system. Measurements of fluorescence were made with a Leitz microspectrophotometer as described in Goff and Coleman (1984). This approach helped us elucidate the sequence of nuclear events leading to dramatic changes in DNA levels in cells during gametophytic development.

The important findings of this study are as follows. Zoospores of *M. pyrifera* are approximately 3–4 µm in diameter and are biflagellated. After their release from the sporophyll, the zoospores adhere to a substratum, lose their flagella, and initiate germination, which involves germ-tube protrusion from the zoospore cell as a "nipple-like" projection. Once the germination is initiated, the germ tube continues to grow until it reaches a maximum length of approximately 15 µm, within 18–20 hr. The vital

DNA stain Hoechst 33342 was used to investigate the nuclear behavior (events) within the cell during different stages of germination and germ-tube elongation. The zoospore possesses a single nucleus. Following completion of germ-tube elongation, the zoospore nucleus initiates division. During division, the nucleus is observed to undergo a constriction resulting in an apparent fission-like division. Following division, the two daughter nuclei are readily observed, and subsequently, the distal daughter nuclei immediately begins translocation along the germ tube. Once initiated, completion of nuclear division and translocation occur within 1 hr. Following this nuclear translocation, the first gametophytic cross wall is formed, resulting in two-celled gametophyte. Subsequent to cross wall formation, the second daughter nucleus remaining in the original zoospore body, undergoes repositioning, assuming a position in the germ tube near the newly formed cross wall.

Germ-tube elongation and nuclear translocation were found to be temporally distinct events. In all cases, nuclear translocation did not initiate until germ-tube elongation was completed and maximum germ-tube length was attained. There is a window of 3–5 hr between completion of germ-tube elongation and initiation of nuclear events, allowing one to manipulate each of these developmental events specifically. In addition, we found that the germination and nuclear events (nuclear division and translocation) are mechanically distinct developmental events. While the events associated with germination and germ-tube elongation are dependent on actin microfilament dynamics, the nuclear events are microtubule mediated. Cytochalasin D inhibited germ-tube elongation; however, it did not affect nuclear division or nuclear translocation (when applied after completion of germ-tube elongation). Colchicine and the plant specific antimicrotubule agent amiprophos methyl blocked nuclear division and translocation without any apparent effects on germ-tube elongation. Prior to nuclear division, bundles of microtubules were associated with the nucleus as visualized using a monoclonal antibody to b-tubulin. During nuclear division a bipolar spindle was observed. As translocation of the first daughter nucleus proceeded, the distal pole of the spindle appeared to be at the leading edge of the nucleus. Microtubule bundles, apparently associated with each pole, were observed to extend along the entire length of the germ tube as nuclear translocation was completed.

We utilized several probes to understand the cell cycle events, specifically the chromosome cycle, during early development in this species. We investigated the fine time course of DNA replication and levels of DNA levels in cells during gametophytic development. The amount of DNA in cultured sperm cells served as a reference point for comparisons with DNA levels in cells of gametophytes. Sperm from cultured gametophytes had the lowest levels of DNA. Zoospores (within 15 min of release) had four times the DNA levels as sperm, suggesting that DNA replication occurs shortly after meiosis. The populations of cells at 4, 8, and 12 hr post-release had increasing amounts of DNA. Maximal DNA level was observed at 16 hr post-release. These data suggest that additional DNA replication occurs during gametophytic growth. Following nuclear division and translocation (24 hr), the DNA levels in each nucleus was reduced to that of the zoospore nucleus (Figure 1).

We initiated experiments on manipulation of DNA levels using inhibitors and modulators of DNA synthesis and cell division. Colchicine and vinblastine, widely used antimitotic agents, inhibited cell division in the gametophytes; and the DNA levels in these cells remained at high levels (similar to that of a replicated but undivided nucleus). Actinomycin D, a potent inhibitor of DNA replication, was capable of blocking the DNA replication in gametophytes. In this case, the cellular DNA level remained at a level similar to that of recently released zoospores (Figure 2). These data suggest that drugs that block cell division can be used as tools to maintain higher DNA levels (and thus polyploidy) in the cells during gametophytic development.

In summary, the present investigation has described the key cellular events associated with early gametophytic development in *M. pyrifera*, an economically and ecologically important kelp species. Our study clearly indicates that germination (and germ-tube elongation) and nuclear events are both temporally and mechanistically distinct developmental events. In addition, the cellular DNA appears to undergo several rounds of replications during early development; thus, the genomic behavior is amenable to manipulation.

Cooperating Organizations
University of California, Santa Cruz

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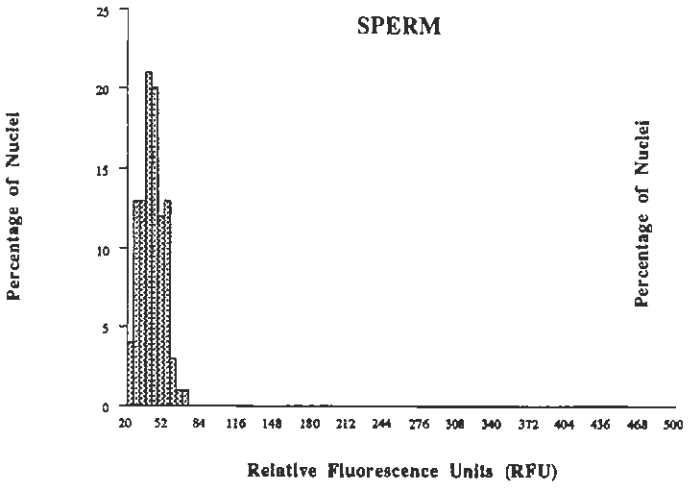

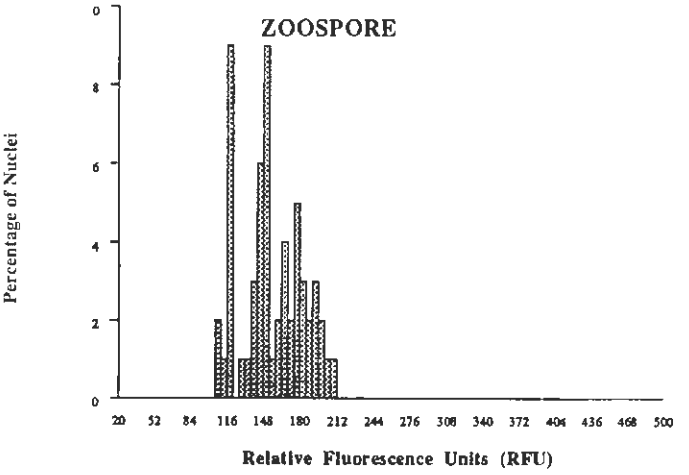

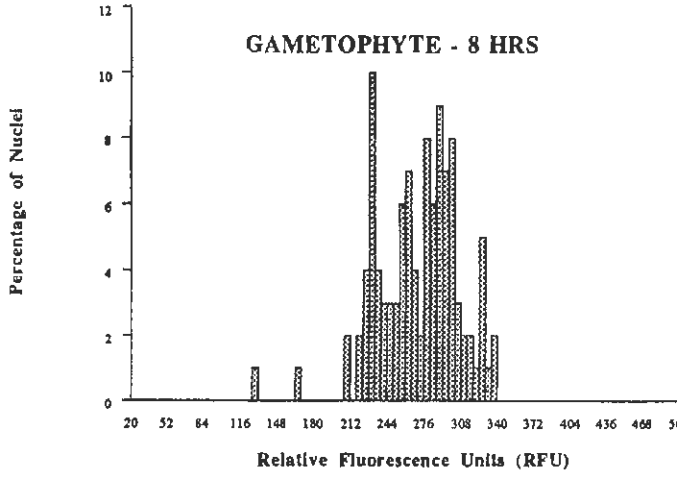

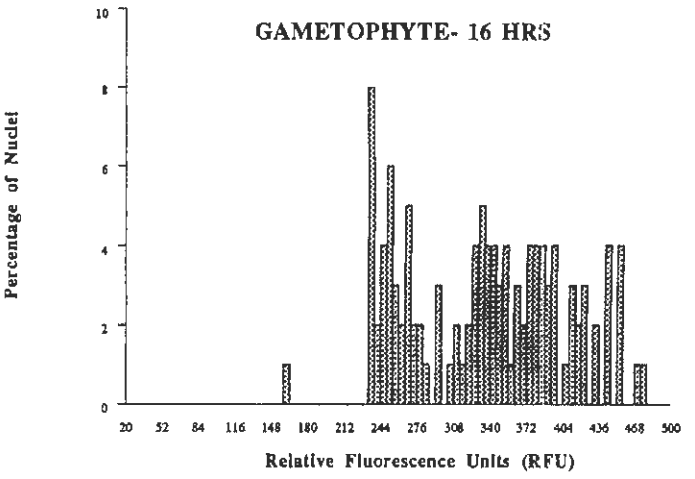

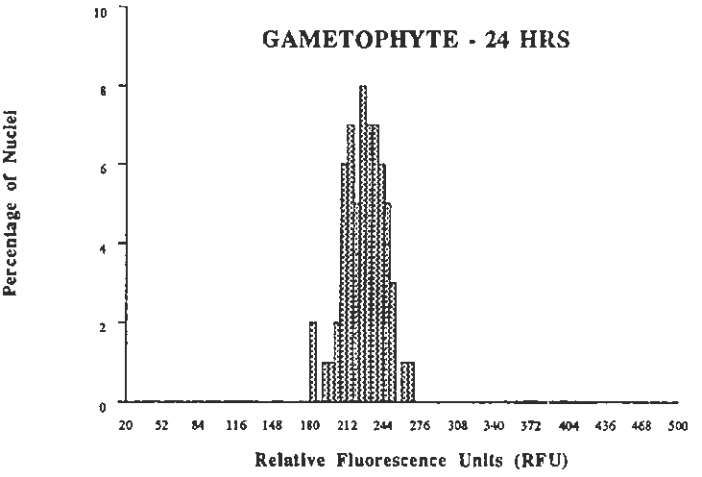

Figure 1. Histograms of the DNA levels measured as relative fluorescence units. Stages include sperm, zoospore, gametophytes 8 hr post-release, 16 hr post-release, and 24 hr post-release. Note that sperm have the lowest quantity of DNA and that the 16 hr gametophytes have the most. However, the population of cells at 16 hr appears to be a mixture of cells at different stages of DNA replication, hence the broad range of RFUs. The DNA values at 24 hr post-release represent the levels of only one of the divided nuclei.

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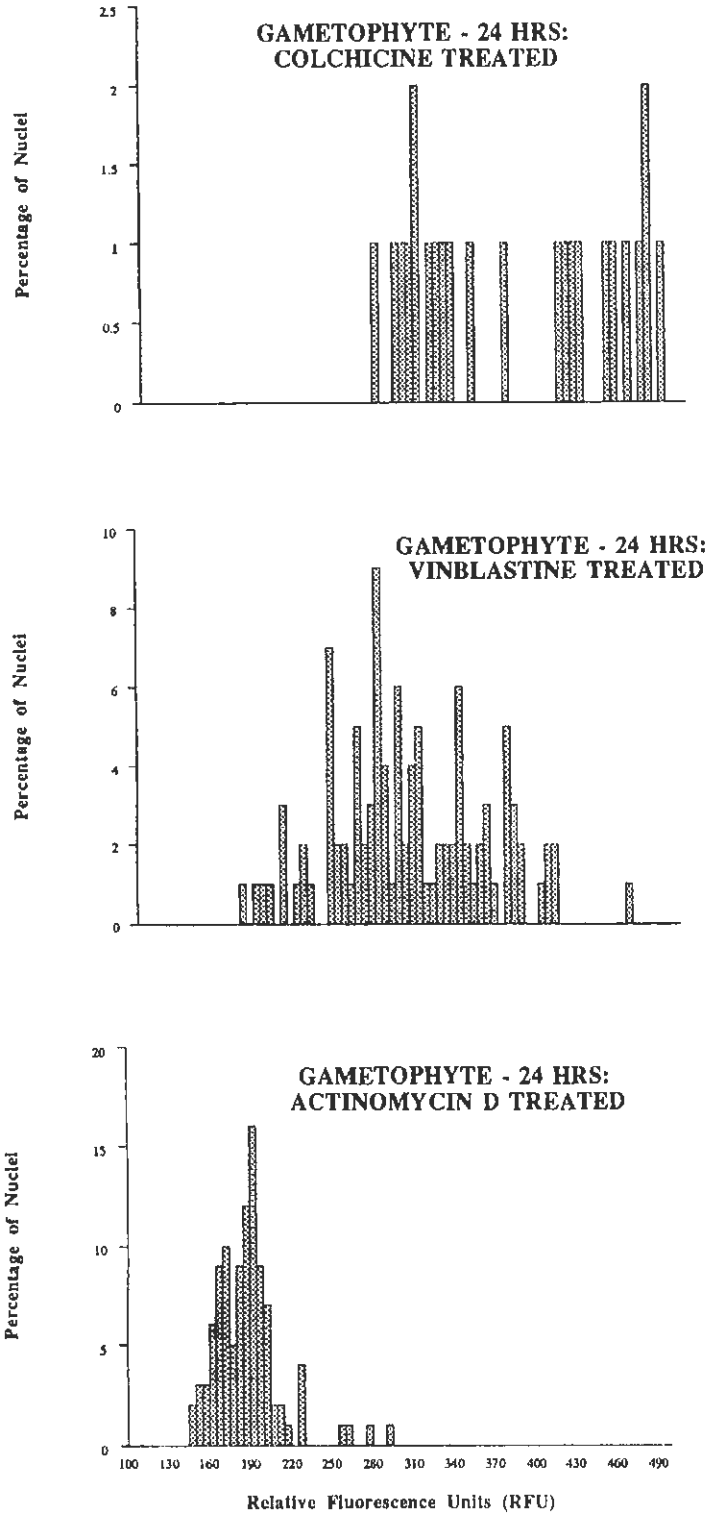

Figure 2. Histograms of the DNA levels of gametophytes at 24 hr post-release, which were treated with either colchicine (120 µg/ml), vineblastine (10 µg/ml), or actinomycin D (5 µg/ml) prior to DNA replication (4 hr post-release) in zoospores. Actinomycin D, a inhibitor of DNA replication, blocked synthesis of DNA. Vineblastine and colchicine, antimitotic drugs, inhibited nuclear division, but not replication as indicated by the increased RFU values.

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Information Necessary to Reevaluate Female Harvest Policies in Dungeness Crab Fisheries

Humboldt State University
R/NP-1-22-1PD
1991–93

D.G. Hankin

Background

The Dungeness crab fishery off the Pacific coast of North America, as for many other decapod crustacean fisheries, is managed on the basis of "3-S" (Sex, Size, Season) policies. Because males and females can be readily distinguished, commercial fishery harvest is usually restricted to the male sex. Prohibition of female harvest presumably prevents reduction of the reproductive potential (egg production) of a crustacean population. Minimum size limits for males are designed to allow males to breed with females on at least one occasion prior to fishery vulnerability, and to prevent capture of smaller and less valuable animals. Closed seasons are often adopted to prevent fishing on males after the molting season when males are in softshelled condition. Such closures prevent capture and sale of softshelled males that are in poor market condition, and minimize handling mortality on discarded softshelled males when fishermen sort for hardshelled males.

Management by "3-S" appears generally effective in the sense of preventing population collapse due to overfishing, but there are usually no fishery quotas for males (i.e., total fishery capture of males is unrestricted) and fishery exploitation rates for males can become quite high. Indeed, there is evidence that fishery exploitation rates for males often approach 90% in northern California's Dungeness crab fisheries (Gotshall 1978; Hankin 1985; Methot and Botsford 1982). The population dynamic consequences of such high fishery exploitation rates on males have been the subject of controversies among modelers (Botsford and Wickham 1978; McKelvey et al. 1980; Botsford 1981; McKelvey and Hankin 1981), but researchers have generally assumed that the intensive fisheries for males do not limit the reproductive potential of females in any fashion.

Previous California Sea Grant-supported studies of growth and reproduction of female Dungeness crabs (Diamond 1983, Diamond and Hankin 1985, Hankin 1985, Hankin et al. 1989, Mohr and Hankin 1989), have revealed a number of interesting features of the life history of female Dungeness crabs that seem pertinent to the possible impact of intensive male fisheries on female reproductive potential. First, studies by Hankin and his students have shown that annual molting probabilities of females are usually very low (near zero) for females in excess of about 150 mm carapace width. Although these large females generally do not molt annually, Hankin et al. (1989) found that female Dungeness crabs can extrude successive viable egg masses without molting and mating, thus reflecting an ability to store sperm for long periods of time (at least 2.5 years). Fecundity of such ovigerous "skipmolt" females is, however, reduced when compared to that of females of similar size that have recently molted and mated. Hankin et al. (1989) also provided evidence that annual mortality rates for large females (in excess of 155 mm) are very low (about 10%). Overall, Hankin's Sea Grant research suggested that large female Dungeness crabs (>150 mm carapace width) probably do not make a large contribution to total egg production in the northern California population in most years.

In British Columbia, commercial take of female Dungeness crabs is allowed so long as females exceed the legal size limit for males (154 mm carapace width). In California, Oregon, and Washington, commercial capture of females is prohibited. Prior to 1991, California was the only state to allow legal recreational capture of females and these had to exceed the legal size limit (159 mm) of males. Because relatively few females reach 159 mm carapace width in northern California (Diamond 1983), such recreational take must have been small. Based in part on the studies by Diamond (1983), Hankin et al. (1989), and Mohr and Hankin (1989), the recreational size limit for take of female Dungeness crabs in California was reduced to 146 mm in 1991. And, since 1992, Oregon has allowed recreational take of female Dungeness crabs in bays so long as they exceed 146 mm.

Although the above studies and regulations seem consistent with a contention that large female crabs (>150 mm carapace width) do not make a contribution to total population egg production, the supposition that intensive fisheries for males does not limit the reproductive potential of females has been recently questioned by Smith and Jamieson (1991). Smith and Jamieson noted that, at mating, male *Cancer* crabs are usually larger than their female mates. They speculated that female Dungeness crabs in excess of 140 mm carapace width might not have an adequate supply of larger males to ensure mating and fertilization of eggs because commercial fisheries remove most males in excess of current legal size limits. As evidence in support of their contentions they presented size frequency data for female Dungeness crabs collected in commercial crab traps in British Columbia, and they presented a scattergram of female and male carapace widths for crabs found paired in premating embraces (see Butler 1960). Smith and Jamieson's concerns are not new, however. Indeed, current size

limits have been generally designed to allow males to mate at least once prior to vulnerability (see above).

The principal objective of this study was to gather additional information concerning growth and reproduction of female Dungeness crabs in northern California so as to more accurately describe the possible contribution to total population egg production that might originate from large females (>150 mm). A secondary objective was to examine the concerns expressed by Smith and Jamieson regarding mating opportunities for large females in light of new data collected from the northern California population of Dungeness crabs.

Methods

We relied extensively on the cooperation and skills of a contracted commercial fisherman for assistance in collection of our data concerning growth and reproduction of female Dungeness crabs in northern California. Our contracted fisherman provided us with paired males and females found in individual mating embraces in commercial traps. Premating embrace females generally molted within 1–4 days of capture. Differences between premolt and postmolt size of such females were used to establish size-specific molt increments. Paired males and females were used in behavioral experiments carried out at Humboldt State University's (HSU) Marine Laboratory in Trinidad, California (see below). We also fished 18 experimental small mesh (non-size-selective) traps (see Diamond 1983; Diamond and Hankin 1985) from our contracted vessel at two locations and in two months during mid-summer 1993, after the 1993 molting season for females. At this time we measured female carapace widths, and recorded a visual assessment of probable molting history (molted, probably molted, probably did not molt, did not molt) for each collected female. Our earlier research indicated that we could calculate size-specific molting probabilities for female Dungeness crabs from such size frequency and "shell condition" data (Hankin et al. 1989).

Our behavioral experiments were designed to address the possibility that sizes of males and females found in premating embraces reflect not just required size differences between males and females, but also possible behavioral interactions among males. We carried these experiments out by placing the pairs of males and females found in mating embraces in individual cells approximately 4 square feet in area and about 8 inches in depth. A coarse sand substrate was provided (#3 grade of sandblasting sand), and recirculating seawater was supplied on a flow-through basis. To most original pairs of crabs, an additional male crab was added. This additional male was either larger or smaller than the original male, depending on the size of male originally in the embrace. We also kept some individual sublegal male Dungeness crabs with female Dungeness crabs to verify that mating embraces were achieved by these smaller males (in the absence of larger males). Observations of premating or mating embrace activities were taken on a 24-hour basis from collection until no more activities of this type were observed for at least three days.

We used linear regression methods to describe relations between size-specific molt increments and (premolt) carapace widths, and we will use regression methods to compare trend lines between years (Draper and Smith 1981).

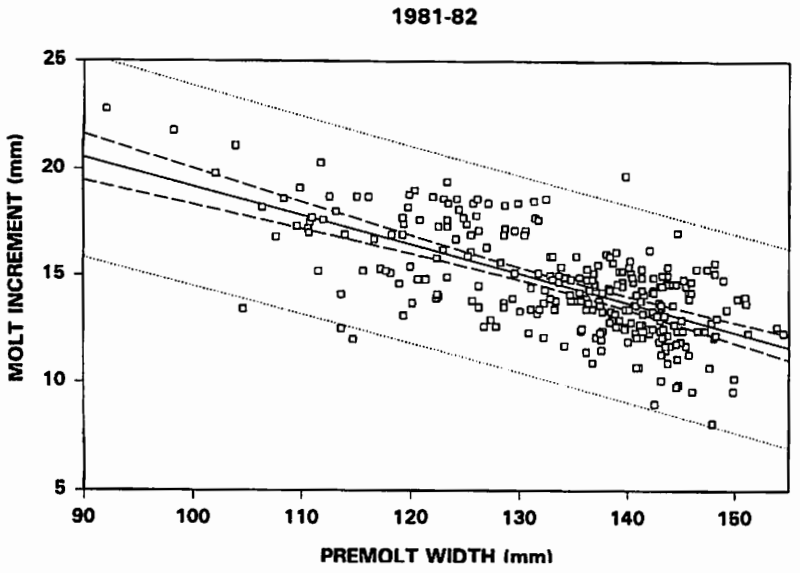
We used the methods of Mohr and Hankin (1989) to calculate estimates of size-specific molting probabilities from shell condition data. A Sea Grant trainee was responsible for most field data collections, for generation of laboratory molt increments, and for recording behavioral observations. The research carried out in this project will form the basis for the trainee's Master's degree in Fisheries Science at HSU. The Principal Investigator (Hankin) participated in all field sampling relating to generation of shell condition data so as to ensure continuity between data collected in earlier research (1981–1983) and in this study.

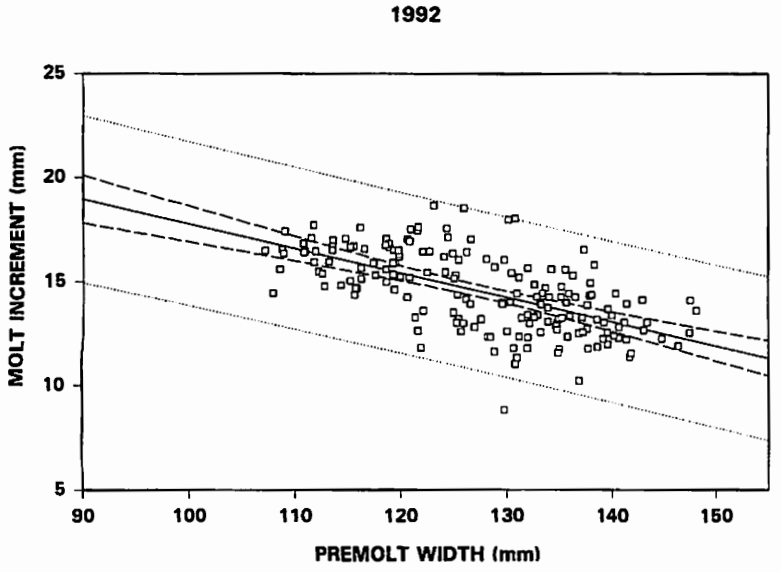
Results and Discussion

Because we are still in the process of analyzing data collected in our research, we can provide only a brief and preliminary analysis and discussion of our results at this time. Results from this study will probably form the basis for at least two journal articles. The first paper will describe interannual variation in growth of female Dungeness crabs. In a second paper, Hankin will be collaborating with Terry Butler, a distinguished crustacean biologist recently retired from Fisheries and Oceans, Canada, on a paper concerning the issues raised by Smith and Jamieson regarding effects of intensive fishing on mating opportunities of females.

Growth of crustaceans such as Dungeness crabs depends on two events: (a) molting, and (b) carapace width increase (molt increments). Obviously, the second event (width increase) is conditioned on occurrence of the first event (molting). Molt increment data collected in this study, when combined with those data collected in our previous studies of female Dungeness crabs, have allowed us to state with some confidence that interannual variation in size-specific molt increments is relatively small in northern California. That is, for crabs of a particular size, the mean and variance of molt increments appears fairly similar across years (Figure 1). Thus, interannual variation in molt increments does not appear to be responsible for substantial between year variation in size (and thereby reproductive potential) at age of female Dungeness crabs.

Size-specific molting probability estimates generated from data collected in this study were combined with data collected in our previous studies. Together, these estimates suggest that interannual variation in molting probabilities may make a substantial contribution to between year variation in growth and reproduction of female crabs. As Figure 2 illustrates, for the 1982, 1983, and 1993 molting seasons, estimated size-specific molting probabilities declined steeply from near 1 at carapace widths less than 135 mm to near zero at carapace





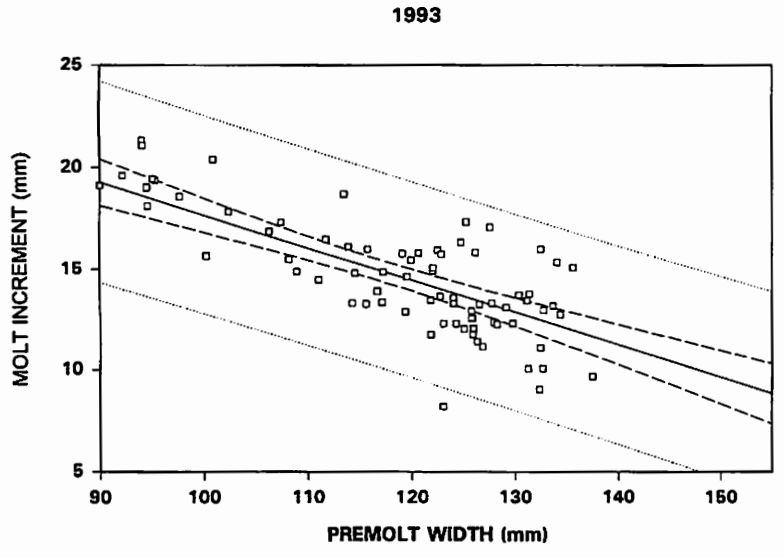


Figure 1. Molt increments plotted against premolt carapace widths for female Dungeness crabs collected in mating embraces from commercial traps in 1981–82 (n = 77), 1992 (n = 81), and 1993 (n = 82) in northern California near Trinidad Bay. Solid lines indicates least-squares linear regression lines; dashed lines indicate 95% confidence intervals about mean values of molt increments for fixed premolt width; dotted lines indicate 95% confidence intervals for predicted values of molt increments given fixed premolt width.

widths exceeding 155 mm. In contrast, for the 1992 molting season, size-specific molting probabilities seemed independent of female size—nearly all females molted in that year, regardless of female size. Further and more complete analyses of our data may make the contrast of molting probabilities between 1992 and other years less extreme than is suggested by Figure 2, but strong differences will definitely remain.

Figure 2 suggests that, at least in some years, large females may sometimes have a substantial probability of molting to a size that might exceed that of legal size males. Although such years may be relatively rare, this finding may have significance with respect to the concerns expressed by Smith and Jamieson regarding size requirements for males and females in matings of Dungeness crabs. Interannual variation in the fraction of large female crabs that molt and mate seems apparent also from plots of male and female sizes from paired premating embrace animals collected in 1992 and 1993. In 1992 (Figure 3 top) there were substantial numbers of females in excess of 140 mm carapace width that were collected in premating embraces, whereas in 1993 (Figure 3 bottom) there were essentially no females collected from mating embraces for which premolt carapace width exceeded 140 mm. Smith and Jamieson (1991) speculated that the frequent observations of "skipmolt" females in excess of 140 mm reflected the relative absence of

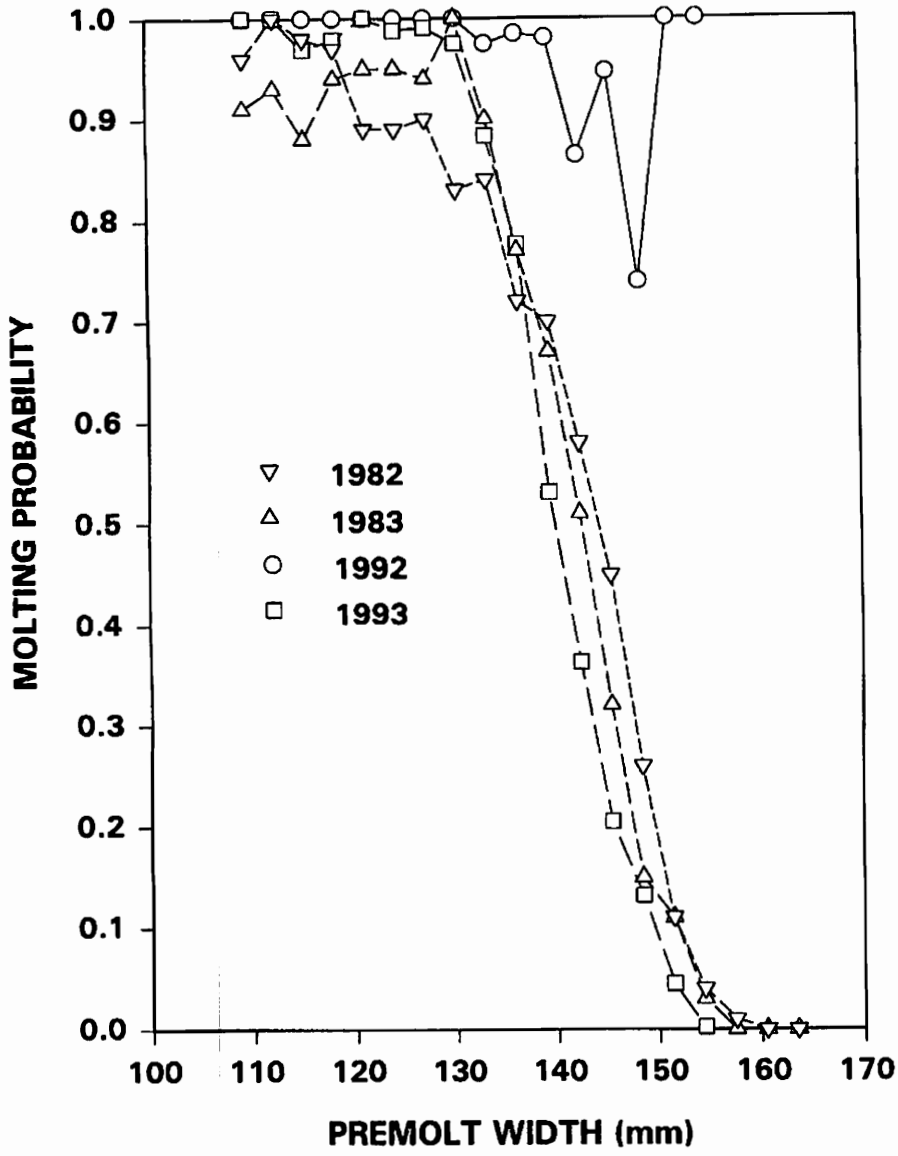


Figure 2. Estimated size-specific annual molting probabilities for female Dungeness crabs collected in experimental (non-size-selective) traps in northern California near Trinidad Bay. Estimates are based on application of methods of Mohr and Hankin (1989) to shell condition data collected from 1982 (n = 2,105), 1983 (n = 362), 1992 (n = 430), and 1993 (1,061) molting seasons. Estimates for 1992 and 1993 are preliminary and are not based all collected data.

large males that would be required to mate with large females. Our data suggest that presence or absence of skipmolt females is more likely indicative of interannual variation in molting probabilities that is independent of the abundance of males. Our Figure 3 does suggest that males generally have substantially larger sizes than their female mates, but one must also remember that our premating embrace females were collected from February through May. In northern California, the commercial fishery for male Dungeness crabs generally opens in December. The abundance of legal males had already been dramatically reduced by fisheries by the dates when we began collecting premating embrace measurements. Because molting of females takes place several months after the intensive removal of males, females have no way to "anticipate" the number of males that will remain unharvested by the time that they will actually molt.

Finally, preliminary analyses of our laboratory experiments regarding mating behaviors of male and female Dungeness crabs have suggested that larger males may be more successful in securing mates than smaller males. This relative success may not depend on physical necessity (i.e., a male must exceed width "y" before he can successfully mate with a female of size "x"), but may instead reflect male-male behaviors. When "small" and "large" males were both present with a female near molting, the large male was more frequently observed in an embrace with females. When only the small (generally sublegal) male was present, this male was observed in an embrace with a female. Thus, behavioral interactions among males may skew observations of male sizes in mating embraces toward larger males, even though such large males may not be required for successful copulation with females. Indeed, the almost nonexistent trends in increasing male carapace width with increasing female premolt carapace width among premating embrace pairs (Figure 3) is consistent with this same possibility. If there were a strong minimum size requirement that depended on female size, one would anticipate a strong trend of male size with female size among premating embrace pairs. Figure 3 provides little support for such a requirement.

To summarize, in northern California it appears that presence of large females that are destined to molt is highly variable across years. In most years, few females in excess of 140 mm may participate in molting and mating, although most of these females may produce viable egg masses utilizing stored sperm from earlier matings at a smaller premolt size. In some years, molting probabilities for larger females may be relatively high and these larger females may require large male mates. However, our collected data suggest that females of all sizes appear to find large male mates even after the fishery has removed the largest fraction of a year's catch. Thus, the mean size of a male paired with a female of

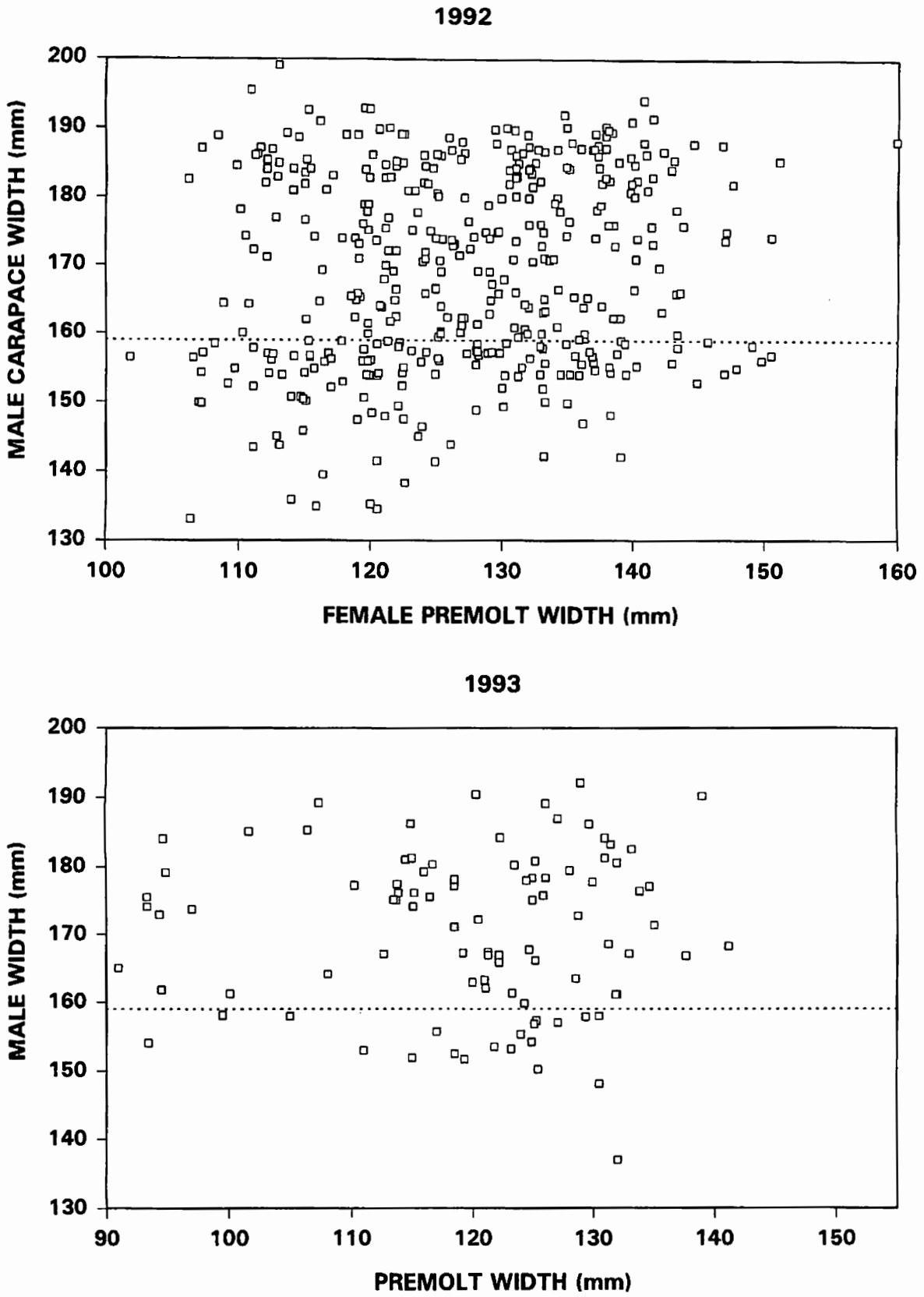


Figure 3. Plots of male carapace width against female premolt carapace width for paired male and female Dungeness crabs collected in commercial traps and removed from mating embraces during 1992 (n = 450) and 1993 (n = 103). Dotted line indicates minimum carapace width for legal retention of males in commercial fisheries in northern California.

100 mm premolt width is not so different from that of a female of 150 mm premolt width. The current minimum carapace width of 159 mm for legal commercial capture of males in California appears to be allowing sufficient males to remain to mate with females, even though fishery exploitation rates are very high.

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Education

Education

California Sea Grant's commitment to education is evident in the projects it supports for student at all levels.

The Trainee Program

Research projects supported by California Sea Grant generally include at least one graduate student trainee. The students work alongside university scientists and engineers in stimulating research environments, while working on or completing graduate degrees. These young scientists and engineers will be responsible for maintaining the high quality of U.S. marine research in the future. In 1990–91, 74 Sea Grant trainees conducted marine research with project leaders at California universities and colleges, and in 1991–92 there were 61 Sea Grant trainees.

Isaacs Scholarship

The tenth John D. Isaacs Memorial Sea Grant Scholarship was awarded in 1991 to Rose Marie Gregory, then a graduate of Burroughs High School in Ridgecrest, who focused her research on brine shrimp and pollution. Gregory is now in her junior year at UC Berkeley, where she is majoring in integrative biology. The 1992 scholarship went to Kenia Whitehead of Samuel Gompers High School in San Diego. Whitehead's study investigated the effects of ultraviolet light on oceanic phytoplankton. She is presently a sophomore at UC San Diego. The $10,000 award, allocated over a four-year period, recognizes the research excellence of California high school seniors and encourages students to continue their marine education at California colleges and universities. It is selected from other marine-related projects at the annual California State Science Fair.

California Sea Grant State Fellowship Program

This program provides a unique educational opportunity for graduate students who are interested in both marine resources and the policy decisions that affect those resources. The program matches highly motivated and qualified graduate student with "hosts" in the California state government or in state agencies for a nine-month paid fellowship. In 1990–91, there were three fellows: Rob R. Pollard of UC Berkeley, worked with the Senate Committee on Natural Resources and Wildlife; Linda Rao of Humboldt State University, joined the California legislature's Joint Committee on Fisheries and Aquaculture; and Susan Reidy of San Francisco State University, worked with the staff of Assemblyman Dan Hauser on issues related to natural resource management. In 1992, there were two fellows: Mark Evans from UC Davis, worked with the Pacific Fisheries Legislative Task Force; Melody Paige Tate, also from UC Davis, worked with the Senate Committee on Natural Resources and Wildlife.

Graduate Research Fellowship Program

In 1990 an experimental program was initiated to support independent thesis research in the marine sciences. The purposes of the program are (1) to provide support for meritorious independent graduate student research and (2) to recognize in an appropriate way the independent contributions of students. Since its inception, a total of 17 students have received the award. Six began their fellowships in1990: Charles F. Lester (UC Berkeley), Blaise J. Eitner (UC Los Angeles), Peggy Fong (San Diego State University), Kevin Lafferty and Erik V. Thuesen (UC Santa Barbara), and Melissa A. Gibbs (Moss Landing Marine Laboratories). Continuing fellowships went to Lafferty and Thuesen in 1991. New fellowships awarded in 1991 were: Stacey Baczkowski (San Diego State University), Sherry L. Fitzsimmons (UC Davis), Heidi M.Nepf (Stanford University), and James Kanihan and Steven A. Osborn (Moss Landing Marine Laboratories). Fitzsimmons received a continuing fellowship in 1992, with the new awards going to: Michael A. Banks (UC Davis), Hae Jin Jeong (UC San Diego), and Theresa Stevens (UC Santa Barbara).

Continuing Projects

Sea Grant Extension Program

The work of the California Sea Grant Extension Program (SGEP) is organized at present into four major program areas: Marine Fisheries, Seafood Technology, Coastal Resources, and Aquaculture.

Marine Fisheries

SGEP staff has divided the marine fisheries program into five subprogram areas based on needs assessment and available resources: (1) Fisheries Efficiency and Safety; (2) Fisheries Utilization and Management; (3) Fisheries Enhancement; (4) Fisheries Education; and (5) Fishing Gear Technology.

Fisheries efficiency and safety. Improving fishermen's safety practices and energy efficiency are the major emphases of this subprogram.

Energy efficiency. Fuel costs are high in most California commercial and commercial passenger fishing vessels. Increased fuel efficiency would lower harvesting costs and increase profitability. SGEP initiated an energy efficiency project in 1985, and its program, linked with the California Energy Extension Service's (CEES) low-interest loan program ($668,000), resulted in an average fuel savings of 17 percent for participants. Early in 1992, Governor Wilson signed legislation (SB1032) authorizing a new $1 million five-year low-interest revolving loan fund. SGEP currently has a two-year ($74,234) contract to continue fuel conservation research, education, and evaluation through June 1993.

During 1991-92, SGEP hired Luis Alberto Woll, a doctoral student in naval architecture at UC Berkeley, as the project research assistant. He completed 28 in-depth computer analyses of propellers and the pretest for a vessel energy retrofit with assistance from San Diego Marine Advisor, Leigh Johnson. SGEP staff met with the project advisory committee and CEES to develop eligibility criteria for the new loan program. CEES worked with banks to set up loan administration, and implementation is expected in late 1992 or early 1993.

Humboldt research associate Fred Jurick participated in a hearing by the California Air Resources Board on control of emissions from fishing vessels.

Fishing safety project. The Vietnamese language safety manual was revised by Connie Ryan to include new federal safety regulations. Leigh Johnson helped put together a safety workshop for 16 fishermen which covered hypothermia, emergency action planning, safety equipment, and new Coast Guard procedures. Special efforts were made to target Hispanic fishermen. She also participated in negotiations between the Navy and the California Urchin Producers Association about Navy weapons testing on San Clemente Island. Marine advisors publicized the new U.S. Coast Guard safety requirements. Bruce Wyatt worked with fisheries groups addressing the personal safety and rights of women working at sea as deckhands. Fred Jurick organized a "Weather Users Workshop" to address the lack of accurate weather data. John Richards began an innovative voluntary weather reporting system in the Santa Barbara Channel using cellular phones (to insure confidentiality).

Fisheries utilization and management. This subprogram was established to provide technical and research information on newly developed and established fisheries.

Innovative fisheries management techniques. Chris Dewees presented a paper on individual transferable quotas (ITQ) at the World Fisheries Congress in Greece. He also had a major ITQ paper published in *Society and Natural Resources* and authored a leaflet on ITQ considerations for the sea urchin fishery. Dewees also served as an advisor to the Pacific Marine Fisheries Council on ITQ plans for the halibut and sablefish fishery.

Jim Waldvogel was instrumental in organizing the Klamath Management Zone Fisheries Coalition to address community concerns resulting from severe restrictions on the salmon fishery. He served as technical advisor to the group which participates actively in the fishery management process. The group includes port districts, boards of supervisors, chambers of commerce, fisheries groups, local businesses, and city councils in the area. Waldvogel also published the results of his 1991 survey on the effects of salmon fishery restrictions on local communities.

Bruce Wyatt worked with his advisory committee and others to address rockfish problems. One was the need for improved catch data from the explosively growing hook-and-line fishery. Wyatt also helped fishermen resolve concerns about bycatch of rockfish by the whiting fleet operating at Cordell Bank.

Increasing Utilization of Bycatch and Joint-catch Fish Species: John Richards has been participating in a cooperative research project with the commercial fishing industry, U.C. Santa Barbara Marine Science Institute, and the Local Marine Fisheries Impact Program to increase utilization of bycatch and joint catch species on the south-central coast. At the completion of the project, he participated in analysis of the data and development of the final report by Michael Wagner entitled, *Final Report on Catching, Handling, Processing and Marketing Live Fish Using Trawl Gear.*

Richards and Carrie Culver advertised the study in the *SGEP Oil and Gas Project Newsletter for Fishermen and Offshore Operators.*

Increasing recreational use of underutilized fish and underutilized fishing opportunities. Fred Jurick assisted United Anglers with its proposal to build an artificial reef outside Humboldt Bay. He worked primarily on permit and funding issues. Chris Dewees distributed the results of a National Marine Fisheries Service-funded onboard refrigeration project.

Nearshore dive fisheries. An international conference on sea urchins, kelp, and abalone, sponsored by Sea Grant and the sea urchin industry, attracted 150 participants from six states and five Pacific Rim nations. Three publications and 11 Sea Grant preproposals were direct results of the conference. The publications included a conference summary with research, enhancement, and management recommendations; a compilation of foreign papers; and a research agenda.

Leigh Johnson completed field work on experimental feeding in the wild of urchins to improve their gonad quality and to reduce damage to kelp beds. Preliminary findings were presented at the International Urchin, Abalone, and Kelp Conference, and two San Diego divers are using the information to develop a supplemental business. Johnson presented a paper on conflict resolution between urchin and kelp harvesters to the national conference of the Coastal Society. Bruce Wyatt continued to examine the use of sea urchin processing waste for agricultural applications and presented his findings at the Sea Urchin, Kelp, Abalone Conference.

Chris Dewees organized the Sea Urchin, Abalone, Kelp Conference and edited the conference summary. He obtained $24,000 from the sea urchin industry to enable Ph.D. candidate Laura Rogers-Bennett to begin work on identifying important sea urchin nursery habitat and to set up experimental shellfish-rearing facilities at Bodega Marine Laboratory. Dewees continued to serve on the California Department of Fish and Game Sea Urchin Advisory Committee and made presentations on the potential benefits and problems of ITQs at two industry-funded workshops.

John Richards collected and analyzed sea urchin recruitment data from three coastal sites and one site on Anacapa Island in cooperation with John Dixon and Steve Schroeter at San Diego State.

A summary of the SGEP workshop, *Black Abalone Mortality: Establishing a Research Agenda, Summary of a Sea Grant Workshop*, was published and distributed to the research community, commercial abalone industry, recreational organizations, agencies, and interested public. Carrie Culver and John Richards continued to assist researchers with information and to act as liaisons between the industry and researchers.

Leigh Johnson worked with recreational and commercial spiny lobster fishermen, the California Department of Fish and Game, and the California Seafood Council to design a cooperative research program to resolve questions about effects of changes in lobster seasons.

Fisheries enhancement. Need exists to enhance fisheries through habitat improvement projects and improved fishery management techniques, to train public enhancement groups in new rearing and habitat techniques, and to improve communication among public enhancement groups.

Salmonid resource enhancement. Jim Waldvogel edited and distributed 300 copies of the *Proceedings of the Tenth California Salmon, Steelhead and Trout Enhancement Conference.* Fred Jurick continued to supply technical assistance to Humboldt County salmon enhancement groups. Bruce Wyatt served as secretary for the Winter-run Chinook Salmon Captive Rearing Program and helped secure funding to start up the captive breeding project at Bodega Marine Laboratory.

Salmonid management studies. Jim Waldvogel completed the twelfth year of the long-term escapement study for chinook salmon on Mill Creek (Smith River). Chinook salmon counts were again low, a result of six consecutive years of drought and poor ocean conditions. The eighth year of adult steelhead scale sampling on the Winchuck River reflected a continuing appearance of hatchery strays in this "wild fish" system.

Marine fisheries education. The overall objective of this fisheries subprogram is to supply research-based information to California citizens to increase their understanding of fisheries and fisheries issues.

Marine educator training. John Richards gave lectures and field trips to four Los Marineros classes and assisted the Channel Islands National Marine Sanctuary coordinators in setting up a speakers bureau of commercial fishermen to work with the project teachers during harbor field trips. Bruce Wyatt trained teachers and high school students to carry out the "steelhead in the classroom" project. This project was also done at the Juvenile Hall and at a San Rafael school where 20 Hispanic students were trained. Fred Jurick ran a fisheries education booth at Eureka's "Blessing of the Fleet." He also arranged to have fisheries and aquaculture included in Humboldt County's Annual Farm Tour. Chris Dewees and Leon Davies provided fisheries training to 25 4-H Junior Leaders at the State 4-H All Star Conference.

***California's Living Marine Resources* book**. *California's Living Marine Resources and Their Utilization*, first published by the California Department of Fish and Game (CDF&G), was printed and distributed. Several SGEP staff wrote and reviewed chapters for the book.

Fishing gear technology. This subprogram area involves developing techniques for supplemental fisheries, improving new gear technology, reducing bycatch, and reducing gear conflicts.

Sheep crab fishery studies with ROVs. Carrie Culver completed a study of ROV utilization in sheep crab fishery research and wrote a section of the local marine fisheries program final report, *Research on Remote Operated Vehicle (ROV)*

Utilization in Commercial Fisheries Operations and Studies by Steve Farris. Culver's section was entitled "The feasibility of using a ROV to conduct fisheries research on the West Coast spider crab, *Loxorhynchus grandis*."

Seafood Technology

During 1991-92, Robert Price and Pamela Tom conducted a workshop on "Seafood Quality Determination and Species Identification." Tom chaired the planning committee for the four-day international Pacific Fisheries Technologists annual meeting, and served on a national seafood symposium planning committee. Price chaired the national planning committee for a "Total Quality Management" short course, and gave 11 invited presentations on seafood quality at workshops, short courses, legislative hearings, and professional meetings.

Price and Tom completed preliminary drafts of 28 species identification and purchasing specification sheets. Price gave five seafood quality radio, television, and magazine interviews, and prepared two radio tapes. Tom obtained technical information for these media interviews.

John Richards presented information on "Harvesting and Processing Fish and Shellfish on the West Coast" during the Seafood Quality Determination and Species Identification workshop. Price and Richards completed a preliminary study on processing methods for sheep crab. Price, Tom, and all the marine advisors responded to information requests on seafood quality problems and concerns.

Price conducted two, and Tom one, "Better Process Control Schools" for food canners. Price and Tom gave 28 invited food safety presentations at workshops, short courses, and training meetings.

Price completed two chapters for a National Fisheries Institute Hazard Analysis and Critical Control Point (HACCP) Training Manual, and coauthored a "white paper" on food microbiology. Price and Tom authored or coauthored publications on "Domoic Acid," "Contaminants in Fish," "Residue Concerns in Seafoods," and "Natural Marine Toxins." Tom coordinated the translation of two publications into Spanish, and assisted a Spanish Broadcast and Media Services Coordinator in developing Spanish news releases on seafood publications.

Price gave 13 seafood safety radio, television, and magazine interviews, and prepared five seafood safety radio tapes. Price and Tom developed three slide sets (86 slides total) to accompany chapters in the National Fisheries Institute's HACCP Training Manual. Price, Tom, and all Marine Advisors responded to information requests on seafood safety problems and concerns.

Seafood processing waste. Waste is a major California seafood industry concern. Jim Waldvogel provided technical information to a coalition developing a seafood-processing waste-composting operation. Bruce Wyatt co-organized a composting conference in Madison, Wisconsin, and presented a paper on "Agricultural Uses of Sea Urchin Shells and Viscera." Wyatt provided information to three prospective composters, resulting in two new composting operations and the use of sea urchin processing waste by an organic farmer as a soil amendment. Wyatt analyzed urchin compost to determine its value as a soil conditioner, and in cooperation with the University of California Bodega Marine Laboratory, determined the contribution of viscera, water, shell, and stomach contents to processing plant waste streams. Wyatt presented a seminar on "Agricultural Uses of Seafood By-Products" to the University of California, Berkeley Soils Department, and cooperatively designed a study to determine the effect of adding calcium (oyster shell) during and after compost making. Price, Waldvogel, Wyatt, Johnson, and other marine advisors responded to requests for seafood information on this topic.

Coastal Resources

The objectives of this program are to (1) promote wise use, management, and conservation of coastal resources; (2) reduce conflicts among user groups; and (3) improve communication and cooperation among resource managers, key leaders, and resource users.

Marine ecosystem management. The objectives of this subprogram are to improve the ability of adults and children to make wise decisions regarding coastal resources.

Reducing agricultural impacts on coastal water quality. This project seeks to improve the ability of agricultural, environmental, and government interests to reduce agricultural impacts on estuarine and coastal water quality.

Leigh Johnson cooperated with Cooperative Extension Environmental Issues Advisor Valerie Mellano to conduct a national pilot program with funding from the U.S. Department of Agriculture. They summarized the regulatory framework for agriculture and coastal nonpoint source pollution (NPS) and the knowledge, concerns, and anticipated actions of stakeholders regarding the issue. Stakeholders received the preliminary research reports and then identified alternatives for addressing this issue. Johnson and Mellano met twice with the Steering Committee and received U.S. Environmental Protection Agency funding to develop a research and education program regarding NPS impacts on coastal waters, and agricultural best-management practices for reducing NPS. Johnson and Mellano presented a preliminary case study to the Cooperative Extension Western Region Workshop. Johnson also discussed the project in a paper presented to the National Public Policy Education Conference. Following a presentation on the project, Farm Bureaus in Riverside, Orange, and San Bernardino Counties established a tri-county steering committee to work with regulatory agencies on NPS management plans. Cooperators include University of California Cooperative Extension, San Diego State University, Farm Bureau, San Diego County, California Coastal Commission, U.S. Department of Agricul-

ture, U.S. Environmental Protection Agency, California Regional Water Quality Control Board, Tijuana Estuary National Estuarine Research Reserve, Environmental Health Coalition, Audubon Society, and several local estuary organizations.

San Diego Bay environmental management. Johnson has assisted the San Diego Interagency Water Quality Panel for several years. Legislation authorizing the Panel expires at the end of 1992. Objectives of this project are to evaluate Panel accomplishments, to improve Panel members' ability to examine and select alternatives for future cooperation, and to improve their ability to develop sound environmental research and management programs for San Diego Bay. Cooperators include the San Diego Interagency Water Quality Panel, the California Regional Water Quality Control Board, California Coastal Commission, California State Senator Lucy Killea, San Diego Unified Port District, U.S. Navy, U.S. Fish and Wildlife Service, San Diego County, Scripps Institution of Oceanography, San Diego Dockmasters, Environmental Health Coalition, San Diego Gas and Electric Company, and others.

A related 1989-91 project resulted in a Panel committee survey of water and sediment quality monitoring programs, submission of a National Estuary Program (NEP) nomination by the Environmental Health Coalition, and development of a five-year environmental management plan by the Port of San Diego. During 1991-92, Johnson reviewed the monitoring survey, provided data and review for the NEP nomination, and provided review and input to the Panel's 1991 annual report.

Johnson facilitated a Panel workshop and summarized and distributed its findings on alternative objectives and their consequences for a reauthorized Panel. State Senator Killea used workshop results to draft legislation reauthorizing the Panel for five years. The Senator recognized the value of SGEP participation by placing Sea Grant on the new Panel's Executive Committee.

John Richards served as coeditor of the *Morro Bay: State of the Bay Conference Proceedings* and coauthored two papers: "Commercial Fishing in Morro Bay" and "Oyster Culture in Morro Bay." The proceedings were advertised in the Oil/Fisheries Newsletter and distributed to California Marine Advisors. This project was completed in 1992.

Tomales Bay State of the Bay Conference. Bruce Wyatt served on the steering committee for the third biennial State of Tomales Bay Conference. He wrote the introduction to the conference proceedings, edited ten papers, and published the proceedings. Outcomes included initiation of a water quality subcommittee to the Tomales Bay Advisory Committee.

Small estuary management in Marin County. During residential development, the Peacock Gap Estuary was deepened and closed off to form a lake. Absence of tidal flushing resulted in water quality problems that killed aquatic life. Management decision-making was difficult given the diversity of responsible agencies.

Bruce Wyatt developed a simple, inexpensive water quality and flow monitoring program; educated the local homeowners association, operators of a nearby golf course, and the City of San Rafael on estuarine ecology; and recommended a management plan to avoid die-off. Program participants agreed to monthly flushing of the lagoon, which the city implemented in 1991.

Multiple use of coastal resources. The objectives of this subprogram are to promote wise resource use and to reduce conflicts.

Mitigation of oil/gas development impact. *The Oil and Gas Project Newsletter for Fishermen and Offshore Operators* continued to be published monthly by John Richards, Carrie Culver, and Maynard Silva. Newsletter readership increased to 600 in 1991-92. This project was a part of the offshore oil and fisheries communication and conflict resolution program which began in 1983 and was completed in October 1992.

Mariner's oil spill response team. During 1991-92, Fred Jurick participated in planning activities with the Humboldt County Mariner's Oil Spill Training and Response Team. The group includes certified volunteers who would be contacted in the event of an oil spill.

Richmond marina management study. Connie Ryan and Cooperative Extension Agricultural Economics Specialist Desmond Jolley participated in a management study of the Richmond Marina, organized and directed by Oregon Sea Grant Marine Economics Specialist Fred Smith. The team studied marina marketing, management, administration, pricing policies, and community relations. It also assessed how the marina functioned as an enterprise fund within the city's structure and how it had performed relative to the city's redevelopment goals and to other San Francisco Bay area marinas. The confidential study report was submitted to the city's Acting Director of Human Services.

Northern California coastal economic development. Fred Jurick continued to participate in the Humboldt County group, Citizens for Port Development. Goals of the organization are to enhance port development and waterfront utilization. Jurick also continued to assist the Redwood Economic Development Forum in long-range planning.

Coordinating San Diego regional pollution monitoring. Six ocean sewage treatment plant outfalls are under the jurisdiction of the California Regional Water Quality Control Board, San Diego Region. The Regional Board, U.S. Environmental Protection Agency, and Tetra Tech, Inc. requested SGEP assistance in obtaining public input for developing a regional outfall monitoring program. Leigh Johnson recommended academic, commercial and sportfishing, and environmental representatives for a mail survey, and advised and cooperated in conducting group interviews on public concerns. Results assisted project leaders in planning the monitoring program.

Coastal resources education. Education and information transfer are primary responsibilities of

SGEP, and this subprogram is a continuing effort.

Stream habitat restoration education. Jim Waldvogel and Cooperative Extension Natural Resources Advisor Greg Giusti received a Renewable Resources Extension Act grant to compile existing stream habitat and restoration educational materials.

Newsletters and public exhibits. Jurick, Wyatt, Richards, Johnson, and Dewees continued to provide ocean and coastal resources information to 7,400 marine educators, industry members, scientists, decision makers, and members of the public through their newsletters.

Marine Advisor Johnson and Sea Grant Information Specialist Gretchen Frederick presented a public information exhibit at the San Diego Sportfishing Council's Day at the Docks event.

Aquaculture

Marine advisors have directed extension activities at problems of concern to shellfish growers, with an emphasis on improving water quality and shellfish sanitation.

Aquaculture public service. Objectives of this project are to provide access to aquaculture information to citizens and to increase public awareness of aquaculture issues. Cooperators are the U.S. Soil Conservation Service, the California Department of Fish and Game, U.C. Cooperative Extension, National Shellfisheries Association, and Western Regional Aquaculture Association.

Fred Jurick participated on an advisory planning committee for a Eureka High School aquaculture program which initiated a pilot training program for pond culture.

Bruce Wyatt continued to serve as a resource person and university liaison to the Tomales Bay Shellfish Growers Association, assisting with the planning and coordination of the Tomales Bay State of the Bay Conference.

John Richards coauthored a paper with George Trevelyan on the statewide mussel aquaculture industry for *California's Living Marine Resources and Their Utilization* as well as a paper on the Morro Bay oyster industry for the *Morro Bay, State of the Bay Conference Proceedings.*

Water quality and shellfish sanitation. The objective of this project is to maintain and, where possible, enhance water quality in shellfish growing areas. Cooperators are the State and Regional Water Quality Control Boards (RWQCBs), California Department of Health Services, coastal county health departments, the U.S. Environmental Protection Agency, the California Department of Fish and Game, and the U.S. Soil Conservation Service.

Bruce Wyatt assisted in conducting three meetings with the State Public Health Services, the Tomales Bay Shellfish Growers Association, and Ann Huyer of California State University, Hayward, to develop a plan for a sanitary survey of Tomales Bay.

John Richards continued to serve as Technical Advisory Committee member to the Central Coast Regional Water Quality Control Board. The Board's research project on nonpoint source pollution in the Santa Barbara Channel and watershed was completed in the fall of 1992. Richards assisted in developing a plan for implementing study recommendations. Program Representative Culver will replace John Richards as member of the Technical Advisory Committee in the winter of 1992.

Species technology development. A project to determine the natural settling periods for economically important marine species (mussels, scallops, and urchins) was initiated in 1990. Pilot hatchery projects were initiated at San Diego State University and the Bodega Marine Laboratory in 1991. A proposal to expand this work to develop commercial scale hatchery culture of urchins in California was submitted by Richards and Culver in collaboration with Ben Beede and Dick Craig of the Cultured Abalone, a Santa Barbara-based shellfish company.

An 18-month data set of the weekly settlement patterns of postlarval mussels, scallops, and sea urchins at three sites in the Santa Barbara Channel has been completed by Richards, two UC Santa Barbara research assistants, and a high school minority student research trainee employed through San Diego State University with funding from the sea urchin industry and Sea Grant.

Communications

California, which is now home to 31 million people, 80 percent of whom live in coastal areas, stretches for more than 1,000 miles along the Pacific.

Not surprisingly, the state has developed a strong academic tradition in marine science. From modest turn-of-the century beginnings in San Diego under a University of California professor from Berkeley, marine research in the University of California has developed into the world's largest and most diverse academic program in ocean science and technology.

Today, on all eight general campuses of the University, marine studies are integrated into many departments. And on five of these campuses—Berkeley, Davis, Santa Barbara, Santa Cruz, and San Diego (i.e., the Scripps Institution of Oceanography)—there are also units devoted solely to marine studies.

In addition, there are strong marine science curricula at a number of private universities, including Stanford University, and at several of the California State University (CSU) campuses, including Humboldt State University, San Diego State University, and Moss Landing Marine Laboratories (sponsored by a consortium of CSU campuses).

In California, Sea Grant began in 1968 with an award to Scripps Institution of Oceanography. By the following year, the National Sea Grant Program was supporting separate projects as well at San Diego State University and UC Santa Barbara. Ultimately, in order to achieve greater coordination and reduce administrative expenses, programs at the various University of California and California State University systems consolidated into the University of California Sea Grant Program. In 1973, this program was designated a Sea Grant College "for sustained excellence in research, education, and public service dedicated to wise use of America's marine resources."

Today, the California Sea Grant College is the largest in the national network, with a reputation for supporting strong, cutting-edge research in marine science and technology. In the period from FY 1990–1992, the program supported 83 major research projects, plus rapid response projects as well, in the general areas of Coastal Ocean Research, Aquaculture, Fisheries, New Marine Products, Ocean Engineering and Instrumentation, and Marine Affairs. The projects are selected on the basis of competitive proposals.

In addition to research, the California Sea Grant College has an active extension component and a range of educational programs, chief among which is graduate training through its trainee program.

Communications Objectives

The Communications Office is integral to California Sea Grant's mission of sustainable development of our nation's coastal and marine resources.

Like its counterparts around the nation, the basic functions of California Sea Grant's Communications Office are to make the program widely known and understood, to disseminate information about the program's accomplishments, to increase understanding of marine-related issues, and to help the program communicate with its diverse audiences.

These objectives can be formally stated as follows:

- To inform a wide spectrum of audiences about the mission and activities of the state, regional, and national Sea Grant programs;
- To inform public, industry, scientific, legislative, and other audiences about the scientific findings arising from Sea Grant-sponsored research;
- To educate a wide spectrum of audiences about state, national, and international marine-resource issues
- To support the communications needs of program management.

The objectives are particularly challenging in a state like California, which is geographically large and has many centers of marine activity. Clearly, they can only be accomplished if the communications staff works in concert both with Sea Grant management and with the Sea Grant Extension Program, which in California comprises two marine specialists and seven advisors located up and down the thousand-mile coast.

Operations

Mechanisms for Information Gathering. Perhaps the single most valuable formal mechanism that the Communications Program has for gathering information is the Annual Progress Report, requested of each project leader by Joann Furse every October 1.

The Annual Progress Report has three major components (1) a questionnaire (to be completed in lay language); (2) a technical narrative report (to be written in language appropriate for peers); and (3) a trainee report.

The questionnaire asks project leaders about results-to-date in light of project objectives; practical applications of their work; press contacts; publications to date; cooperating organizations; international contacts, and so on. This document is used in a variety of ways. Here are just three:

• The questionnaire alerts the Communications Program to accomplishments that should be followed up and reported to the media or to federal or state agencies, for example.

• It helps us track what media coverage a project has received, either as a result of our efforts, or those of the project leader and his/her public affairs office;

• It provides lists of published articles, books, and conference presentations by project leaders against which to check what we have actually received. This ensures that our publications inventory and our archives are kept as complete and up-to-date as possible.

Another important component of the Annual Progress Report is the technical narrative. We require narratives (in language appropriate for peers) both from project leaders whose work is continuing and from those whose projects are complete. Reports on continuing projects are used by management to monitor progress toward objectives; they also provide in-depth background on research to the Communications Office. Reports of completed projects ("Completion Reports") are published in our *Biennial Report of Completed Projects*. This publication not only describes overall project accomplishments, but also provides a forum in which project leaders can discuss difficulties encountered, project modifications, public benefits, and so on. The technical Biennial Report contains lists of cooperating organizations and also a complete list of publications resulting from the project.

Further, because we encourage project leaders to update their reports when we later send them edited proofs, the Biennial Report provides a mechanism for following up on project accomplishments.

The Communications Coordinator participates in regular management and planning meetings with the director and assistant director. She attends meetings of the California Sea Grant Committee where research needs are discussed and projects evaluated. She attends California Sea Grant's Subject Area Meetings, where project leaders from various campuses meet to review work and to identify future research needs in specific subject areas. She attends major symposia and workshops sponsored by the program in order to determine whether, and in what format, the results of the conference should be published. She attends meetings of the Sea Grant Extension Program in order to provide advice and to stay informed about Extension activities. Lastly, to stay abreast of major scientific and policy advances, the Communications Coordinator attends selected national meetings. In the last biennium these included Coastal Zone '91 in Long Beach, California, the National Conference of the Marine Technology Society in Washington, DC, and the National Research Council Symposium on Interactions Between Coastal Science and Policy in Irvine, California.

Given the vast distances in California and the program's present financial constraints, the Communications Coordinator visits project leaders on their respective campuses only on an occasional basis, though more regular visits would be highly desirable. Most contact with project leaders, with Extension personnel, and so on, is conducted via phone, mail, and fax.

Management of Information. In order to encourage project leaders to report their publications to us (and to appropriately acknowledge Sea Grant support), the Communications Office pays for 100 journal article reprints, 50 of which we give to the author and 50 of which we keep for mandatory and other distributions.

In any given year, the research projects funded by California Sea Grant result in the publication of as many as 50 to 80 specialized articles in refereed journals, plus other publications such as theses and dissertations, technical reports, and conference papers. The fact that the program has the highest publication record in the network has ramifications throughout the area of information management and dissemination and necessitates our having a full-time Information Specialist.

The Information Specialist has responsibility for maintaining records on publications, for filing and distributing publications, and for filling requests. Specifically the Information Specialist:

(a) Maintains a cumulative, computerized inventory of Sea Grant publications dating back to 1968. There are at present 2,347 entries in this system; a breakdown appears in Table 1:

Table 1. Cumulative Publication Inventory

Publication Type	Type Count
SG Series	129
Reprint of Journal Articles	1228
Dissertation/Thesis	182
Conference Paper	241
Technical Report	99
Abstract	161
SG Extension Pub	172
Book	22
Proceedings	26
Working Paper	46
Miscellaneous CSG Publications	41
Total	2347

(b) Maintains a physical archive of these publications, which also includes those produced by Extension specialists and advisors;

(c) Maintains a sophisticated computerized database of nearly 6,000 names and addresses organized into 114 specialized groups. This database allows California Sea Grant to achieve highly targeted mailings—for example, to state legislators or to members of the Pacific Fishery Management Council or to high school teachers in Santa Barbara County. Much of the Information Specialist's efforts go into list maintenance: Merging, purging, and updating the various categories in our computer files represent ongoing activities;

(d) Maintains a "library" of journals, newsletters, and brochures that the program receives from other sources, including other Sea Grant programs in the network;

(e) Tallies the number of publications received and distributed each month, and enters this information into the computer. A spreadsheet produced from these data details monthly distribution and requests counts by type of publication. At the end of each quarter, a report is

generated consisting of the spreadsheet with monthly and year-to-date totals, an initial distribution analysis for publications received during the quarter, year-to-date totals of initial distributions, and a page of distribution notes commenting on noteworthy requests and distributions during the period. A listing showing how many times each publication was requested and/or distributed each month during the quarter is also included. A percentage breakdown on the affiliations of persons requesting information appears in Table 2, and a breakdown of publications by subject area appears in Table 2.

The Information Specialist also has a critical role in information dissemination.

(a) She handles distributions of reprints, announcements, press releases, and California Sea Grant series publications, and fulfills all mandatory distribution requirements, including those for Extension materials;

(b) She answers all phone, mail, and "walk-in" requests for general and specific information and fills all orders:

(c) Twice yearly she compiles and distributes Publications Lists containing newly received publications resulting from our programs. Each mailing goes to approximately 2,000 persons.

In FY 1991–92, the Information Specialist distributed reprints of 73 journal articles and 9 papers from published conference proceedings. In addition, she distributed publications in the California Sea Grant series and miscellaneous publications in a number of categories, for a total of 129 different items, or 10,918 pieces. Publication announcements, press releases, and awards announcements brought the number of pieces distributed to 44,470.

The Information Specialist not only handles initial distribution of publications, but also maintains a library of reprints and books from which to fill both specific and general requests for information. In 1991–92 there were 1,349 individual requests for information or publications, bringing the total number of pieces distributed to 50,092.

Table 2. Percentage Requests by Affiliation of Requestor

University Professors and Libraries	30%
Federal and State Government	24%
Industries and Trade Associations	21%
Public (including secondary schools, museums, aquaria, etc.)	25%

Table 3. Publications by Subject Area—1989 to Present

Subject Area	Count
Aquaculture	64
Coastal Ocean Research	47
Fisheries	99
Marine Affairs	14
New Marine Products	86
Ocean Engineering	44
Total	354

Information Dissemination

Once information on marine issues or program accomplishments has been gathered, the Communications Coordinator, acting in consultation with the director and with members of a Publications Committee, decides on appropriate methods for disseminating it. There are a number of communications vehicles that are used routinely. These include: a Program Directory, a Summary Report, a Biennial Report of Completed Projects, conference summaries and proceedings, technical and working papers, specialized publications, a bimonthly newsletter (Sea Grant In Brief), press releases, and announcements. These will be described more fully in the Progress Report and Proposed Activities sections of this proposal, which follow.

Cover design is usually done by freelancers or by the Design Department at the University of California San Diego, under the direction of the Communications Coordinator. Page makeup is done internally using Macintosh software, and printing is either done by the University or by outside vendors on the basis of solicited bids in accordance with University policy.

Publications

California Sea Grant's publications fall into three general categories: those about Sea Grant or Sea Grant research results, conference summaries, and specialized publications of different kinds.

In 1990–92, we published several core publications. These include the *Program Directories* for 1991 and 1992, the *Biennial Report of Completed Projects, 1988–90*, and a *Summary Report* covering 1991 and 1992.

The annual *Program Directory*, a 24- to 32-page publication printed in 4" x 9" format, presents short descriptions of each current research project, with emphasis on the project's potential applicability. The Directory also lists education projects, extension staff, and campuses on which research is being conducted. This publication presents a brief overview of the program, and it serves to meet our objectives of informing a wide spectrum of audiences about the mission and activities of California Sea Grant. This publication is disseminated widely. It is used to answer requests for general information about the program, distributed at meetings and conventions, and sent to potential investigators with the Call for Proposals. About 8,000 copies are distributed annually.

The *Biennial Report of Completed Projects* is a technical publication that serves as a historical record of program accomplishments and an important document in terms of program accountability. It also helps us to achieve wide dissemination of scientific and technical results, another of our major objectives. This publication is widely distributed to the marine scientific community, to appropriate libraries, and to resource managers. We published 1500 copies of the 256-page *Biennial Report of Completed Projects, 1988–90*, this past year and are presently working on the Report for 90–92.

During the biennium covered by this proposal, we also wrote and published a 36-page summary report of those research projects that relate to environmental quality. Titled *California Sea Grant and the Coastal Ocean Environment*, the publication

focused on recent projects that aim to better understand the effects of humans on the coast and coastal ocean environment, try to separate out human effects from natural variations, and diminish or mitigate those effects. This publication, which is written at a lay level in magazine style and illustrated with black-and-white photographs was sent to all 6,000 persons on our mailing list. It thus served to highlight program accomplishments to a wide variety of audiences including teachers, industry people, legislators, management agency personnel, and scientists.

A number of features from this publication were picked up verbatim by newsletters and other publications, including: *Currents* (Marine Technology Society), *Waterline, The Aquaculture News, Fish Farm News,* and *A'lul'quoy*, a joint project of the Channel Islands National Marine Sanctuary and the Santa Barbara Museum of Natural History.

We also published a 198-page handbook of benthic marine plants from intertidal and subtidal sites between the U.S.-Mexican Border and Orange County, California. Directed to students as well as field biologists, *Marine Algae and Seagrasses of San Diego County* provides an important introduction to a marine resource that has not heretofore been extensively surveyed and provides a means of recognizing and naming approximately 360 taxa. The publication was written by Dr. Joan Stewart of Scripps Institution of Oceanography.

Marine Pharmacology: Prospects for the 1990s represented a summary of a California Sea Grant workshop held at the University of California, Santa Barbara. Edited by Professor Robert S. Jacobs and Marianne de Carvalho, the 82-page publication reviewed California Sea Grant's pioneering involvement in the development of marine pharmaceuticals since 1977 and outlined five areas where advancement is likely in the 1990s: new chemistry and drug synthesis; drug receptors, screening, and discovery; natural product chemistry; new biochemical and molecular models; and drug receptors and cellular processes. Representatives of a number of major pharmaceutical firms participated in the workshop along with academics from around the country. Pharmaceutical firms represented included Ligand Pharmaceuticals, Inc., Merck, Sharp & Dohme Research Laboratories; Allergan Pharmaceuticals, Inc., Syntex Corporation, Wyeth-Ayerst Research, California Bio-Marine Products Company, and Amgen, Inc. The conference received coverage in the *Santa Barbara News-Press* and *Newsweek*.

We had earlier planned to publish a review of California Sea Grant's seminal work in marine pharmaceutical research, with an emphasis on potential therapeutic agents in the treatment of inflammation. Marine natural products have proved to be a valuable source of inhibitors of an enzyme, PLA_2, which mediates the inflammation response in conditions as diverse as arthritis and bee stings. Several of the anti-inflammatory agents discovered in this research program are considered to have the potential for commercial development, and one has proceeded as far as clinical trials. However, the author received an offer from the editor of *Journal of Natural Products* to publish a similar review, and we agreed that this would be a more effective forum for reaching our targeted audience and should take precedence over our proposed publication. The article, "Phospholipase A_2 Inhibitors from Marine Organisms" by Barbara Potts and D. John Faulkner of Scripps Institution of Oceanography and Robert S. Jacobs of the University of California, Santa Barbara, appeared in the December, 1992 issue of that journal.

The Taxonomy of Economic Seaweeds, edited by Dr. Isabella A. Abbott of the University of Hawaii, is an award-winning series of highly illustrated taxonomic guides to a potentially rich marine resource. The publications result from biennial working meetings held around the Pacific Rim at which some of the world's leading marine algal taxonomists work together to identify, describe, and classify subtropical and tropical red and brown algae groups, and particularly those that yield useful biopolymers, including agar, carrageenan, and alginate. In 1992, we published Volume III of the series. The 256-page publication received excellent reviews in *NAGA* and *The ICLARM Quarterly*. Reviewer Paul Silva, writing in the *Plant Science Bulletin*, commented that this third volume "sustains the high standards of scholarship and book production of the first two volumes." We are now in the process of editing Volume IV in this series, with a projected publication date in late 1993 or early 1994.

Another publication produced in 1992 was titled *Remote Sensing and Geographic Information Systems: Implications for Global Marine Fisheries*. The 28-page publication, written by Dr. James J. Simpson, Director of the Satellite Oceanography Center at Scripps Institution of Oceanography compared historical and anticipated uses of spacecraft data in support of fisheries in a number of countries, including Japan, the People's Republic of China, the Commonwealth of Independent States, the United States, and several European countries. The publication was reviewed favorably in *Sea Technology* and by the Marine Technology Society.

Two very different publications resulted from a major international conference on kelp bed resources sponsored by California Sea Grant and the California Department of Fish & Game in March 1992. The conference, organized by Sea Grant Extension Specialist Dr. Christopher Dewees and Sea Grant Subject Area Coordinator Wallis Clark of the University of California, Davis, attracted 150 participants from the United States, Canada, Mexico, Japan, Australia, and New Zealand. Three days of papers and discussions identified many information gaps and complex management issues. One product of this conference was a 54-page workshop summary, *Sea Urchins, Abalone, and Kelp: Their Biology, Enhancement, and Management*, which includes abstracts of presentations plus the research recommendations of small focus groups that broke out to discuss particular areas of concern, such as stock assessment or resource management. Because our overseas guests had so much

valuable information to share with U.S. scientists, industry people, and managers, we also made available an unedited collection of papers from foreign participants at the conference. *The Management and Enhancement of Sea Urchins and Other Kelp Bed Resources: A Pacific Rim Perspective* includes papers on the urchin fisheries of British Columbia, Chile, and Japan as well as U.S. West Coast states, and thus serves an important function as part of our Innternational Technology and Information Transfer program.

In addition, we published a second proposed national research initiative, *Marine Resource Development: Enhancement of Fish Growth and Development.* This 12-page document represented the summation of a project headed by Dr. Howard A. Bern of the University of California and Dr. E. Gordon Grau of the University of Hawaii, who co-chaired a Sea Grant working committee comprising members from seven states, Japan, and Canada. The document presents the strong case for a national research initiative to facilitate the development and transfer of growth enhancement technology to the seafood industry and lays out specific areas of needed study in the areas of endocrine regulation of growth and development and genetic methods to enhance growth and development.

We are presently working with Professor Robert Bea of the Department of Naval Architecture and Offshore Engineering at the University of California, Berkeley, on a third proposed initiative—this one to be focused on the reassessment and rehabilitation of aging marine structures. This effort, which is being co-chaired by Dr. Richard Seymour of Texas A&M University, involves a working committee representing prestigious engineering institutions in seven states, plus the National Office of Sea Grant. This document will address the aging and technological obsolescence of much of the U.S. infrastructure of marine structures, including breakwaters, piers, pipelines, outfalls, offshore platforms, barges, ships, and harbor structures. It will identify much-needed research on the reassessment and rehabilitation of marine structures and will be designed to provide the basis through which the United States can better meet the engineering challenges associated with its aging marine structures.

Lastly, we published three research agendas that were developed as a result of meetings sponsored by California Sea Grant with the intent of identifying critical research needs in California in fairly focused and timely areas. These were *Black Abalone Mortality* (Carrie Culver and John Richards, editors), *Kelp Bed Resources of the California Coast* (Dr. Susan L. Williams, editor), and *Restoring Sustainable Coastal Ecosystems on the Pacific Coast* (Dr. Susan L. Williams and Dr. Joy B. Zedler). These three documents, inexpensively produced, were part of California Sea Grant's management planning process.

We are presently completing production of a handbook for food retailers on an important new concept in food-quality assurance: The Hazard Analysis and Critical Control Point system (or HACCP). The HACCP system, which is not widely used at the present time, allows the seafood industry to predict risks to food safety and to prevent them before they happen. It does, however, require that technical expertise in food safety be properly developed. The HACCP system is recommended by the U.S. Food and Drug Administration (FDA), Department of Commerce (DOC), and Department of Agriculture for food processing and handling establishments. HACCP is also required as part of the new DOC inspection program and will be included in future FDA inspection programs.

The 44-page manual that we will be publishing, *Ensuring Food Safety—The HACCP Way,* is both an introduction to HACCP and a resource guide for delicatessen managers. The publication was written by Dr. Robert J. Price, California Sea Grant's Seafood Technology Specialist in collaboration with his assistant, Pamela Tom, and Dr. Kenneth Stevenson, Senior Director of Microbiology/Sanitation, National Food Processors Association. It will be published under a special grant from the Extension Service, U.S. Department of Agriculture, which will allow us to disseminate 5,000 copies, free or at very nominal fee, throughout the nation. Among the advisory board members who have reviewed the publication are representatives from Vons, Safeway, the Food Marketing Institute, and the California Department of Health Services. We are also working actively with these organizations to ensure that the publication will have distribution nationwide.

Also in production is the third, fully revised edition of one of our most frequently requested publications. *The Directory of Academic Marine Programs in California* is addressed to high school and college students and counselors who are interested in information about marine science programs at California's private and public colleges and universities. The publication includes information on degrees and courses offered, members of faculty, and research facilities.

We are also completing production of a five-year summary report, which will highlight California Sea Grant's recent accomplishments in research, education, and extension activities. Our aim will be to produce an interesting, illustrated description of the program that is appropriate for very diverse audience.

Appendices

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