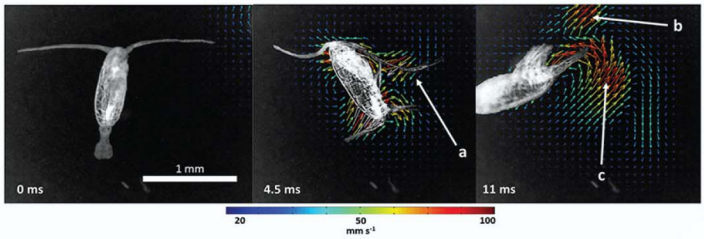
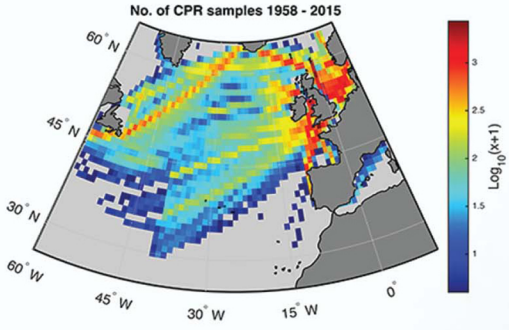
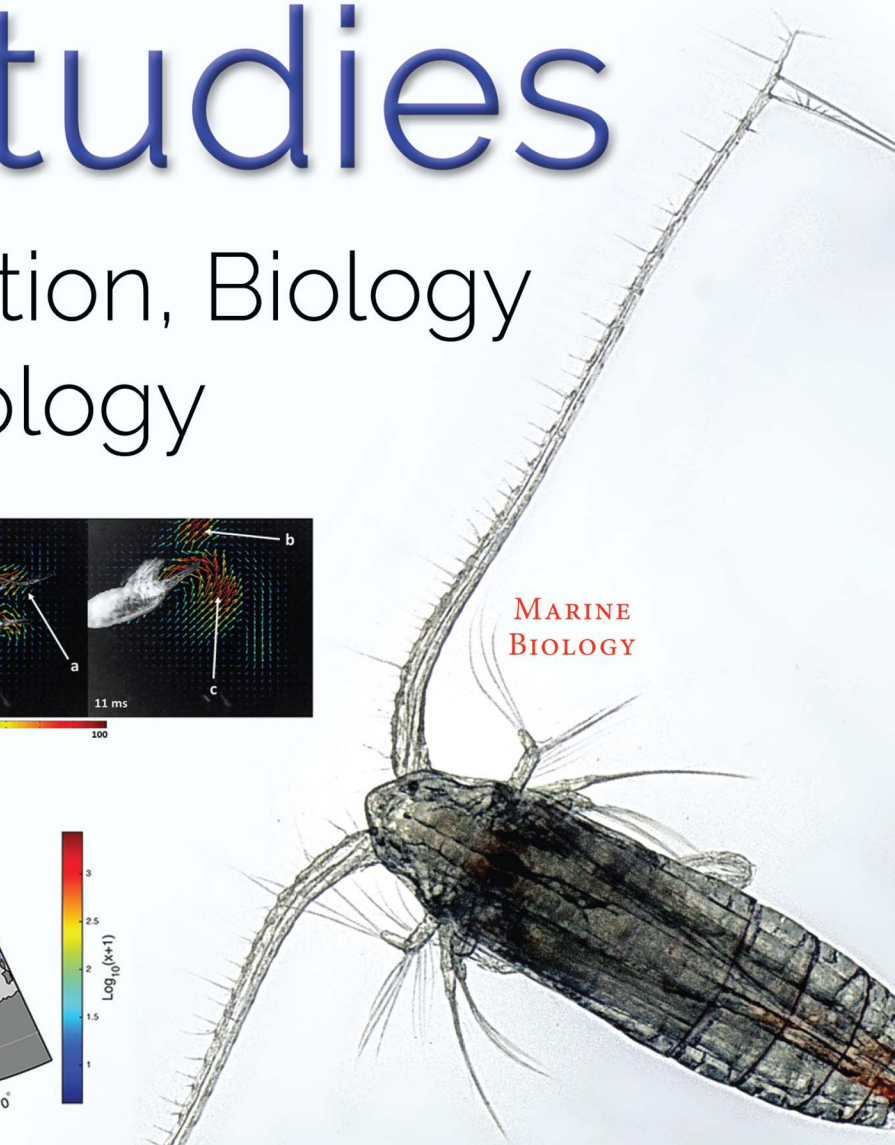
Trends in Copepod Studies

Distribution, Biology and Ecology







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MARCO UTTIERI
EDITOR

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to Maria Grazia, Maurizio, J. Rudi and Enrico,
my mentors,
with whom everything started

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CONTENTS

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PREFACE

When I first met up with copepods, during my undergraduate classes in “Biological Oceanography” and “Planktonology”, I became fascinated by their extreme diversity, plasticity and pivotal role in ecosystem dynamics. Today, several years later, I am still surprised at discovering the astonishing complexity of these tiny crustaceans. It is with this overwhelming feeling that I accepted the editorship of this volume, in the hope of conveying to our readers the same sense of wonder.

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Chapter 1

TRENDS IN COPEPOD STUDIES

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ABSTRACT

Being present in almost any aquatic system and owing to their ecological role, copepods have been the focus of a large number of studies from taxonomy to global patterns. However, despite the wealth of information available today, our knowledge of their distribution, biology and ecology is still incomplete. Apparently, the more we learn about them, the more we are spurred on to advancing our research on these tiny crustaceans to fully grasp their significance and role. Through the contributions collected, this volume aims at providing new insight in copepod studies and a new foundation for future studies.

Keywords: copepods, distribution, biology, ecology

1. INTRODUCTION

The "insects of the sea", the most abundant metazoans on Earth: over the years, copepods have been labelled in several different ways owing to their diversity of life

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forms and exceptional colonising ability (Huys and Boxshall, 1991; Hardy, 1970; Wiebe *et al.*, 1992). As reviewed by Schminke (2007), copepods are as successful as insects in terms of both absolute success and – equating swimming to flying – relative success thanks to several features including (but not limited to) phylogenetic age, speciosity and size.

Since Milne Edwards (1840), copepods have been established as a separate class. Huys and Boxshall (1991) identified ten orders, which were subsequently reduced to nine by Boxshall and Halsey (2004) upon the discovery of the Fratiidae family (Ho *et al.*, 1998). Among them, Calanoida are the most effective colonizers of the pelagic environment (Bradford-Grieve, 2002), with a predominance of marine species (Mauchline, 1998; Razouls *et al.*, 2005-2017) compared to freshwater ones (Boxshall and Defaye, 2008).

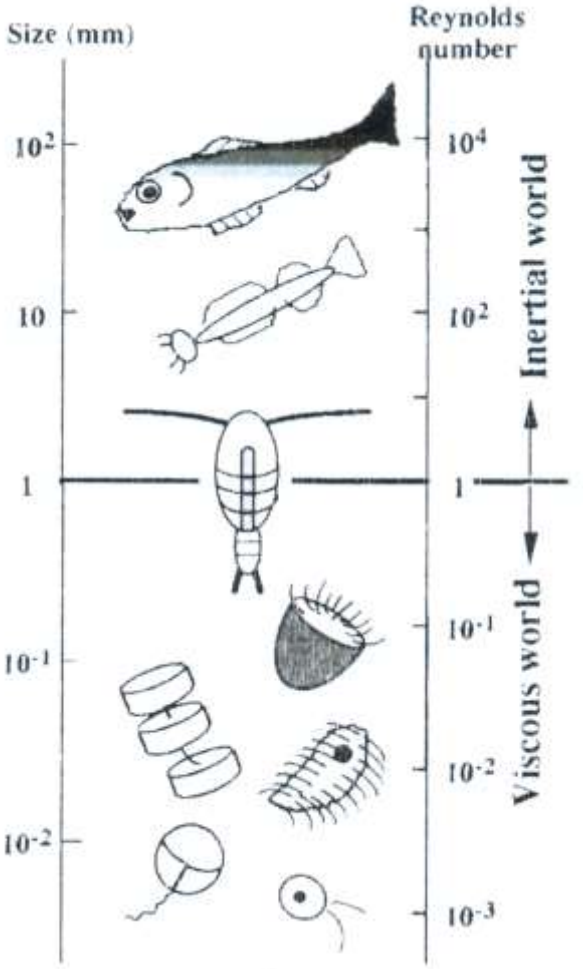

Figure 1. Ecological role of copepods, linking lower and higher trophic levels, respectively characterised by low (< 1) and high (> 1) Reynolds numbers (Reproduced from Naganuma, 1996, with permission).

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Free-living copepods have populated any available habitat, from the pelagic to the subterranean ones (Huys and Boxshall, 1991), through several independent colonisations (Boxshall and Jaume, 2000; Bradford-Grieve, 2002). Copepods are present in any aquatic environment, from deep-ocean trenches to mountain lakes over a vertical range of approximately 20 km (Huys and Boxshall, 1991), and occupy a wide temperature gamut, from polar waters to hydrothermal vents (Walter and Boxshall, 2017).

Copepods represent up to 90-97% of the total biomass of marine zooplankton (Bradford-Grieve *et al.*, 1999), also being abundant in freshwaters (Boxshall and Defaye, 2008). Copepods are primary actors in the functioning and shaping of aquatic ecosystems. They are the linchpin of food webs, preying upon phytoplankton (Marshall, 1973; Turner, 2004) while at the same time representing staple food for higher trophic level organisms, such as chaetognaths (Feigenbaum and Maris, 1984) and fish larvae (Poulet and Williams, 1991). Owing to this, copepods link the viscous and inertial realms, characterised by low and high Reynolds numbers respectively (Naganuma, 1996) (Figure 1). In addition, copepods support the vertical fluxes of carbon through the release of fecal pellets (Fowler and Knauer, 1986), as well as the availability of ammonia to sustain recycled production (Verity, 1985).

The importance of copepods, however, is not just restricted to this. For example, copepods can be used as beacons of climate change (Beaugrand *et al.*, 2002; Richardson, 2008) and as indicators of the effects of ocean acidification on marine biota (Vehmaa *et al.*, 2013). Nearly half of described species live in association with other organisms (Humes, 1994), host pathogens or have parasitic habits (Bron *et al.*, 2011). These crustaceans can also be extremely useful as model animals for ecotoxicological studies and environmental genomics (Raisuddin *et al.*, 2007; Kulkarni *et al.*, 2013), and a great number of species are well documented as invasive (Zenetos *et al.*, 2012; Sabia *et al.*, 2015). Copepods can also be used as control agents of disease vectors (Kalimuthu *et al.*, 2017). Moreover, despite their small size copepods display peculiar swimming behaviour which mediate their interactions with other organisms and with the surrounding environment (Sabia *et al.*, 2014).

Despite all this, our knowledge on the distribution, biology and ecology of copepods is still incomplete. For example, despite the numerous morphological and phylogenomic records available to date, the total number of copepod species is still indefinite. Over the last fifty years, this figure has risen from approximately 7,500 (Kaestner, 1970) to 14,747 (Walter and Boxshall, 2017) (Figure 2), also thanks to the adoption of molecular techniques particularly useful in the identification of cryptic species (Bucklin *et al.*, 2010; Blanco-Bercial *et al.*, 2014). However, considering that Humes (1994) guessed a grand total of 75,374, subsequently raised by Schminke (2007) to the staggering figure of 450,000 (based on the hypothetical Harpacticoida species counts by Seifried, 2004), much more needs yet to be done.

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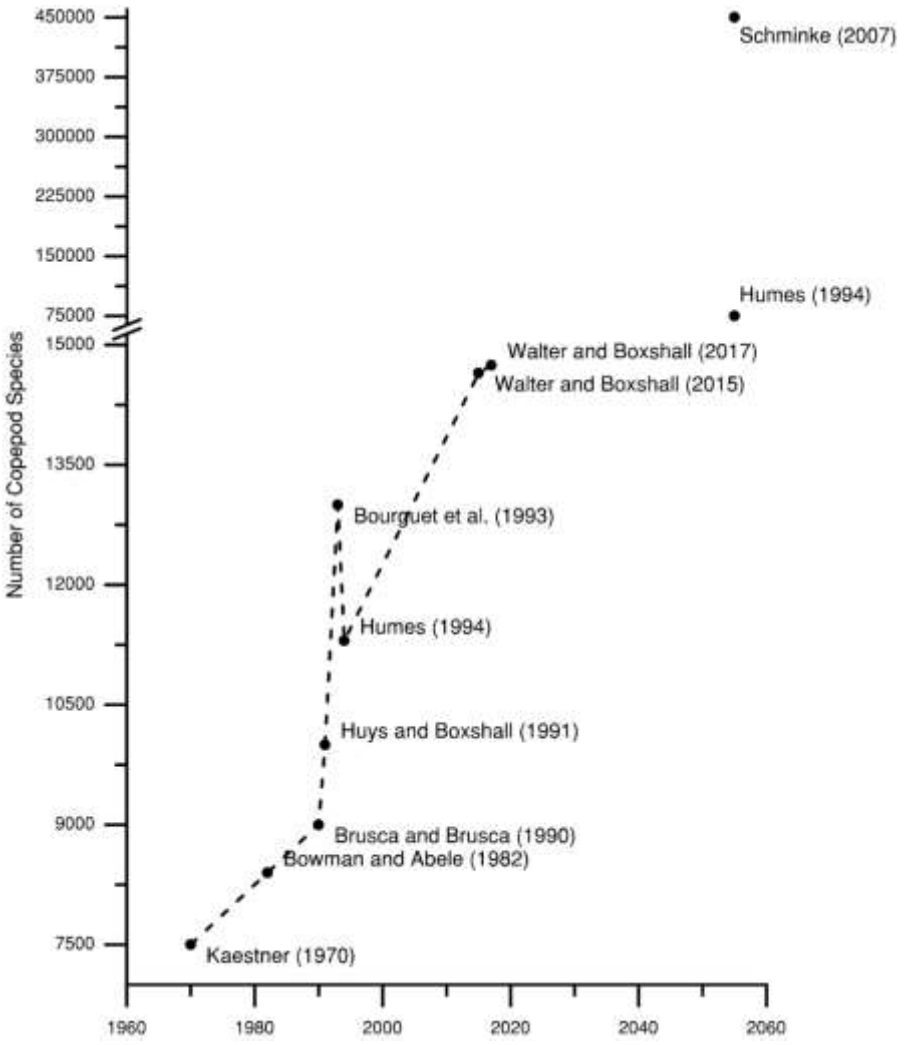

Figure 2. Evolution of the number of copepod species. The future projections by Humes (1994) and Schminke (2007) refer to estimates in the total number of species. It is to note the different line ticking before and after the break along the ordinate axis.

2. TRENDS IN COPEPOD STUDIES – SUMMARY OF CONTRIBUTIONS

When I was proposed to act as editor of a volume on copepods, I asked myself "Do we really need another book on them?" – a question that many readers may equally pose. After initial indecision, I realised that the answer was simply "Yes". As indicated in the previous section, current copepod studies are exploring multiple lines of research at a fast pace. The intended purpose of the present volume is to provide an up-to-date snapshot of some hot topics in the study of the distribution, biology and ecology of these ubiquitous crustaceans. The following chapters focus on a wide range of processes and scales, from

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global distribution to molecular investigations, witnessing the interest of the scientific community at different levels of investigation.

Wootton *et al.* (2018) discuss the functioning, policy issues, strengths and limitations of the survey carried out through the Continuous Plankton Recorded (CPR) since its inception in the 1930s. The authors review the copepod taxa collected by the CPR all over the world, including their biological traits and geographical areas of occurrence of each species. The authors then provide examples of applications of CPR data, and discuss about future perspectives and applications of this unique plankton sampler.

Fernández de Puelles *et al.* (2018) provide a synthesis of the global distribution of copepods from the tropical and subtropical sectors of the Atlantic, Indian and Pacific Oceans (35°N-40°S) over 15 biogeographical provinces. Samples were collected during the Malaspina Circumnavigation Expedition, from the epipelagic to the bathypelagic. The results highlight horizontal and vertical patterns, and point out great similarities in the assemblage composition among the three oceans, depth playing a pivotal role in their distribution.

The chapter by Zagami *et al.* (2018) is centred on the invasive cyclopoid *Oithona davisae*. The authors first review the biogeographic distribution, habitat characteristics, ecological traits and dispersal mechanisms of this species. Then, they report on the introduction and establishment of *O. davisae* in two coastal lakes in the Central Mediterranean Sea, where this cyclopoid has become a dominant species in the zooplankton community. Through a one-year study, the sex ratio and the seasonal variation in abundance for adults, copepodites and nauplii are studied and compared with the literature.

Another invasive copepod, the calanoid *Acartia tonsa*, is the focus of the contributions by Villate *et al.* (2018), Delpy and Pagano (2018) and Marques *et al.* (2018). Villate *et al.* (2018) report on the impact of *A. tonsa* on the Acartidaee assemblage from the estuaries of Bilbao and Urdaibai along the Basque coast (Spain) over a multiannual period (1998-2005). Their results show that both areas were colonised almost contemporaneously since 2003, with a preference for the innermost salinity habitat of both estuaries, but with distribution characteristics dependent upon the specific features of each estuary. The arrival of *A. tonsa* determined a niche shrinking in the native *Acartia clausi* in both sites, and a seaward shift in *Acartia bifilosa* in the estuary of Urdaibai, while the impact on less abundant Acartiidae species was less easily discernible.

Delpy and Pagano (2018) compare the relationship between *A. tonsa* and *A. clausi* in the Berre Lagoon (southeast France) before and after a rehabilitation period aimed at reducing salinity fluctuations. Following its introduction in the 1980s, *A. tonsa* dominated the zooplankton community restricting *A. clausi* to the neighbouring coastal area. Upon stabilisation of the salinity fluctuations following the rehabilitation of the lagoon, however, *A. tonsa* decreased its abundance, and the two species started to coexist in the

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Berre Lagoon with a clear seasonal succession. These signs, in the authors' opinion, might be considered an encouraging starting step in the recovery of the lagoon.

Marques *et al.* (2018) investigate the distribution of *A. tonsa* and *A. clausi* in the Mondego Estuary (Portugal) over a decade (2003-2013). *A. tonsa* was first observed in 1994, efficiently adapting to the newly invaded site. A spatial segregation between the two species was demonstrated, with *A. tonsa* restricted to the inner part of the estuary and *A. clausi* confined seaward. These results confirm *A. tonsa* as an opportunist species, taking advantage of periods of temperature increase and reduction in freshwater flow recorded in the area.

In their contribution, Svetlichny *et al.* (2018) provide a detailed review of the behavioural and physiological adaptations in key copepod species from the Black Sea. In particular, the authors focus on specific aspects such as salinity tolerance, effect of temperature on physiology and behaviour, and resistance to oxygen limitations. The examples discussed demonstrate the dependence of copepod distribution and vital rates on environmental parameters, which should be always taken into consideration to fully appreciate the ecological role of these tiny crustaceans.

Lenz and Hartline (2018) present a comprehensive description of the biology of myelin in calanoid copepods. Using examples from different species, they open with a description of the structure and function of myelin, linking its occurrence to the evolution of copepod families. Subsequently, the authors report on the role of myelin in terms of reaction times and localisation of stimuli, and finish by focusing on the niche separation between myelinate and amyelinate species.

The chapter by Gemmell and Buskey (2018) concentrates upon the escape strategies used by copepods as a response to the predation threat posed by different foes. The processes of predator detection and the mechanism of escape generation are discussed using examples from the available literature, together with a differentiation between the strategies used by visual and non-visual predators. The role of environmental features (water motion, temperature and viscosity) are also taken into account.

Langhoff *et al.* (2018) push the boundary of current knowledge on copepod mating, focusing on the detection by male *Temora longicornis* of the ratio of chemical compounds in the pheromone trails released by the female. Through the design of an olfactory apparatus and the implementation of a Simulated Annealing algorithm for the selection of synaptic weights, the authors demonstrate the capability of ratio detection in copepods, opening to further research on this topic.

The closing chapter by Amato and Carotenuto (2018) reviews the latest "omics" advances in copepod studies. The authors describe the methodologies presently in use, and provide a review of the most recent advances in molecular studies in planktonic copepods, focusing on gene expression approach, metabolomics and proteomics. The chapter closes with some future perspectives on this rapidly evolving field of research.

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The contributions collected in this volume point out the latest developments and case studies on a number of research issues, and will promote discussion and stimulate advances in each field of investigation. As editor, I am confident that readers will appreciate the contents of each chapter and will find in them inspiring suggestions for their research, or even just for their curiosity.

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I wish to thank all the authors for having accepted the invitation to contribute to this volume. It was a pleasure and an honour to interact with them all, sharing comments and building together the book as you can read it. I also wish to thank all the colleagues and friends with whom, over these years, I discussed about the different aspects of copepod life – listing them all would take many pages! Insightful comments by Maria Grazia Mazzocchi and Geoff A. Boxshall improved an earlier version of this chapter. I also owe a vote of thanks to Carra Feagaiga and all the staff at Nova Science Publishers, Inc. for supportive feedback during every stage of the publication process. Thanks are also due to Ian Stewart at Inter-Research Science Center for reprint permission of Figure 1.

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Chapter 2

USING THE CONTINUOUS PLANKTON RECORDER TO STUDY THE DISTRIBUTION AND ECOLOGY OF MARINE PELAGIC COPEPODS

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ABSTRACT

The Continuous Plankton Recorder (CPR) Survey, operated by the Sir Alister Hardy Foundation for Ocean Science (SAHFOS), is the longest running, most geographically extensive marine survey in the world. Since its inception in 1931, the Survey has monitored near surface planktonic communities, including pelagic copepods, providing essential baseline information on the state of the marine environment. Initial observations focused on the North Sea. However, today, in cooperation with sister CPR surveys that have started independent monitoring programmes, the scope extends around the globe, operating across basin scales, providing a truly unique and invaluable dataset for the international community.

CPR data have allowed the description of the geographical distribution of almost 700 planktonic taxa across the North Atlantic, North Pacific and Southern Ocean, monitoring their changes over time. Although both phytoplankton and zooplankton are regularly identified in the Survey, a substantial proportion of the routine analysis is dedicated to the group Copepoda (over 300 taxonomic entities) and forms the focus for this chapter. The CPRs multi-decadal time series allows seasonal cycles and natural variation to be disentangled from changes occurring over a much longer period, such as multiannual oscillations and long-term trends of key copepod species. CPR data have provided

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evidence for northward distributional shifts of indigenous copepod species, range expansions of non-native species and the presence of pathogenic bacteria on copepods' exoskeletons.

In this chapter we summarise the main findings on pelagic copepods based on CPR data collected all over the world, highlighting the key policy issues that they have contributed to over time. The strengths and limitations of CPR observations will also be discussed.

Keywords: *Calanus*, regime-shift, policy, phenology, biogeography, CPR, long-term monitoring

1. INTRODUCTION

In the 1920's, Sir Alister Hardy invented a plankton sampling device designed to demonstrate the patchiness (fluctuations in type and quantity) of plankton that he had observed whilst trying to study young herring in the North Sea. Engineered to collect a continuous line of observations, the sampler was first deployed on the Discovery Antarctic expedition (1925-1927) and was given the name Continuous Plankton Recorder (CPR). In subsequent years the sampler underwent a re-design, tailored towards being towed behind commercial vessels and, apart from some relatively minor modifications, is the CPR we see in operation around the globe today. Although regular deployment of the CPR in the North Sea began in the 1930's, consistent data using present-day methodology is available for the period after the Second World War (*i.e*., post 1948) and represents the longest running and most geographically expansive marine survey in the world.

Over the last 85 years the Survey has evolved into a unique marine monitoring programme, providing the community with its best long-term measure of the state of oceanic plankton (Richardson *et al*., 2006). Since 1991, the CPR Survey and resulting dataset have been managed by the Sir Alister Hardy Foundation for Ocean Science (SAHFOS, http://www.sahfos.ac.uk), in Plymouth, UK. Today, in cooperation with sister Surveys that have started independent monitoring programs (at present comprised of 12 regional Surveys) the CPR's scope extends around the globe, operating across basin scales, providing a truly unique and invaluable dataset for the international community. Known as the Global Alliance of Continuous Plankton Recorder Surveys (GACS) (http://www.globalcpr.org), this cooperative union of CPR surveys share their data and expertise with the marine community worldwide.

Truly global studies that use a consistent methodology are rare for planktonic organisms, including copepods, and even more so for open ocean regions. The CPR Survey and GACS community aim to address this but gaps in knowledge still exist, in particular for tropical waters. A recent study by Fernández de Puelles *et al*. (2018) tackles

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this issue and impressively describes the tropical to subtropical biogeography of copepods in all three major oceans, from the epipelagic to bathypelagic zones.

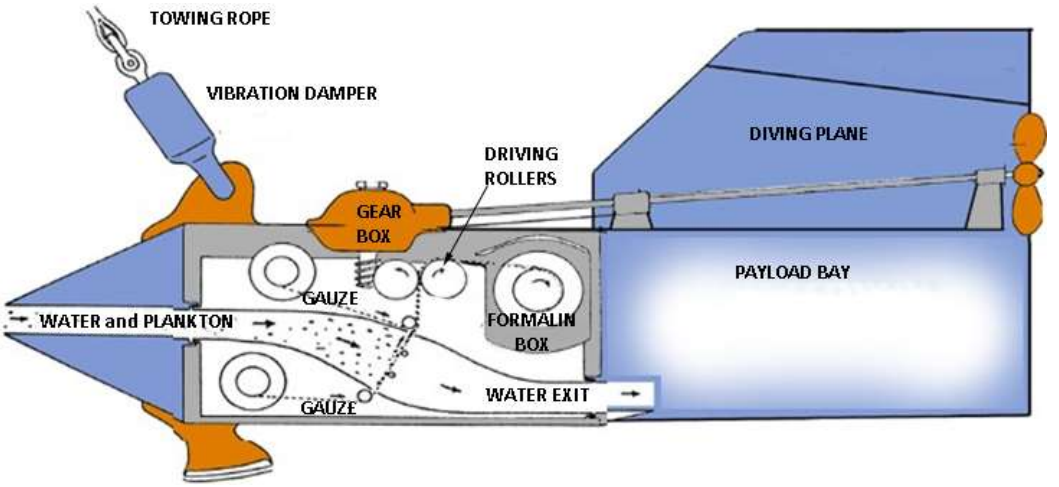

Figure 1. Schematic longitudinal section of a CPR internal mechanism and external body. Water enters through the front aperture and is filtered by a moving band of silk gauze; a second layer of gauze, moving in synchrony, covers the filtering mesh thereby trapping the captured plankton forming a plankton sandwich. As the tow progresses, driving rollers wind the sample into a storage tank containing buffered formaldehyde (fixing the plankton) allowing for the replenishment of fresh gauze and a new plankton sample to be collected.

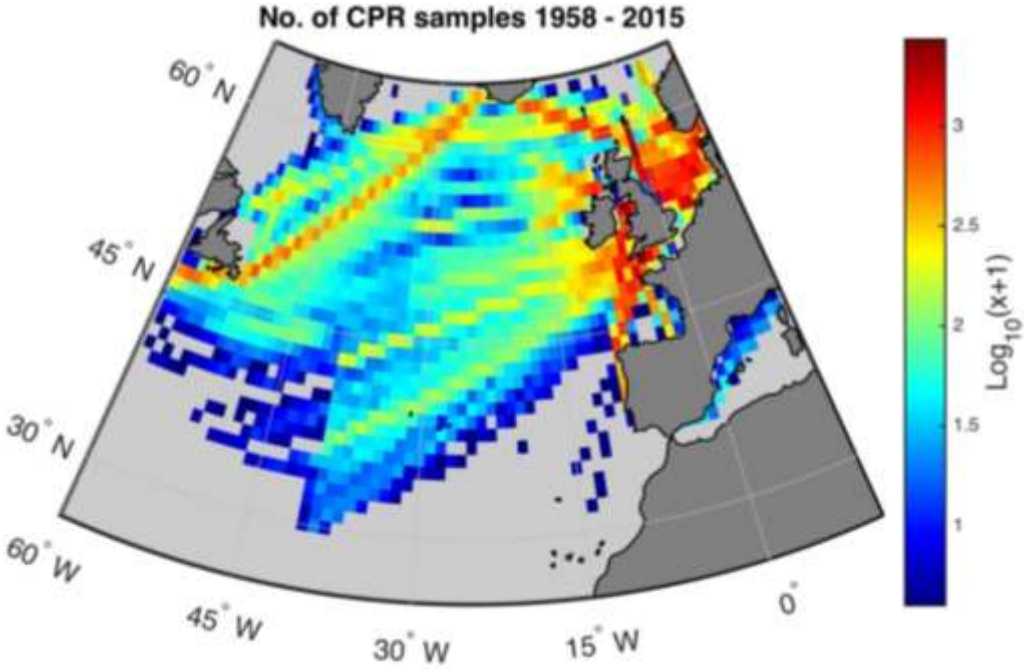

Figure 2. Map of the number of CPR samples collected between 1958 and 2015 in the North Atlantic, gridded onto a 1 by 1 degree grid. Each sample represents 10 nautical miles (18.5 km) of tow.

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A Continuous Plankton Recorder (CPR) is a robust semi-automated sampling device that monitors the near-surface plankton communities over hundreds of nautical miles. Towed at approximately 7-9 m depth (Hays and Warner, 1993) behind a Ship of Opportunity (SoOP) the CPR typically collects epipelagic organisms, including a wide range of copepod taxa. Using a simple and reliable mechanism the CPR collects planktonic organisms using a 270 µm silk mesh, which continuously advances as the CPR is towed through the water, typically at a rate of 10 cm per 10 nautical mile sample (Figure 1). Unlike many other sampling devices, the CPR is capable of towing continuously across 500 nautical miles with minimal supervision; the longest single transect towed to date being 3,000 nautical miles, from Canada to Japan. For a detailed description of sampling and analysis methods please refer to Richardson *et al*., (2006). At present the SAHFOS CPR Survey operates across 10,000 nautical miles of ocean each month on 23 routes passing between 20 different countries, with over 6.5 million miles sampled in total over the history of the Survey (see Figure 2). Having undergone minimal modification in design since 1931, the simple yet efficient design of the CPR means that non-scientists can easily deploy the device. Ships' crews require only brief initial training in CPR deployment and operation before becoming fully competent and part of the CPR Survey fleet. As a result, the Survey is able to operate across an extensive geographical range, on a monthly resolution, at significantly reduced cost compared with relying upon specialised research vessels. The collaboration and support of the shipping community is undoubtedly the key to the success and longevity of this unique time-series.

1.1. Why Use a CPR and CPR Methodology?

There are a variety of monitoring programmes and sampling methods used in the study of copepods, each with their own advantages and disadvantages depending upon the research question being asked. Although a detailed debate about the usefulness and limitations of the numerous types of sampling gear in operation is beyond the scope of this chapter, here we briefly highlight some of the reasons why the CPR and the CPR analysis methodology is a useful tool in copepod research (Owens *et al*., 2013).

As previously mentioned, the CPR is capable of sampling over large distances, up to 3,000 nautical miles at present, unlike traditional net sampling which typically sample a single point in space. Depending upon the CPR Survey, each sample represents either 5 or 10 nautical miles of tow, hence the CPR is the ideal tool for large or basin scale studies.

Typical routine analysis of CPR-collected copepods involves identification down to species or genus level for most taxa. Many programmes and analysis methodologies that are able to collect and process such high volume of samples similar to the CPR Survey

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(such as the digital identification systems ZooSCAN (Grosjean *et al*., 2004) and FlowCam (Le Bourg *et al*., 2015) are able to record 'bulk' indicators such as total zooplankton, total copepods and large *vs* small copepods, and can provide an estimate of bio-volume (and therefore coarse biomass and size spectra). Bulk indicators can provide a valuable quick general picture of processes that take place, but are often limited by their lack of taxonomic resolution. For example, research by Sydeman *et al*., (2010), using CPR data from the North Pacific, showed that seabird species were more closely related to zooplankton taxonomic groups rather than "bulk" measurements of zooplankton abundance or diversity. Many successful studies have been carried out using these automatic identification tools; however, the use of bulk indicators can often mean that subtle differences and relationships between members of the planktonic community are lost. Ultimately the decision on which level of taxonomic resolution is necessary, and hence choice of methodology, depends on what the data generated is to be used for.

The North Atlantic sibling species *Calanus finmarchicus* and *Calanus helgolandicus* are almost morphologically indistinguishable; however, their presence can indicate different water masses as they both have differing thermal niches (Helaouet and Beaugrand, 2007). For this reason they are currently used as copepod 'indicators' (Edwards *et al*., 2008) and are routinely identified to species level in the SAHFOS CPR Survey. Detailed information about plankton community composition at the species level can only be obtained through microscope-based or molecular analysis. Such detailed information, as provided by the CPR Survey, is essential in providing an accurate insight into changes in biodiversity, food web dynamics and wider issues such as climate change.

A CPR is a compact, robust and reliable piece of sampling equipment. With an average tow success rate of > 90%, a CPR works using simple mechanical engineering. Unlike other devices, such as multiple plankton samplers or the Longhurst-Hardy Plankton Sampler, a CPR is free from electrical firing mechanisms which can commonly cause sampling failure. Many other types of sampling gear need to be deployed and monitored by several skilled operators and are only really suitable for use on specifically designed research vessels. In contrast, a CPR can be deployed by non-scientific staff on non-specialised SoOPs (*e.g*., passenger ferries and container ships), requiring minimal supervision whilst sampling is underway. The body of a CPR also offers a payload area where other sampling equipment can be attached (*e.g*., temperature, chlorophyll and depth sensors). With oceanographic research vessels costs often prohibitive, it is hard to find a more cost efficient and reliable zooplankton sampler than a CPR.

However, as with any piece of equipment and monitoring programme, there are limitations and the CPR is no exception. Volume of water filtered per sample (3 m^3) is relatively small and sampling depth (typically 7 m) is restricted to the upper water

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column. Despite this apparent shallow depth, it has been speculated that the actual community sampled may be deeper than originally thought. As the CPR is towed behind a vessel at speed, it is probable that with a large ship draft, possessed by ships used in the Survey, enough turbulence will be caused to mix the water down to do approximately 25m, dependant on ship size, thus allowing the CPR to collect plankton from a wider depth range.

Common to many methods of sampling gear, a consistent CPR tow depth is achieved by deploying a set amount of tow-wire. Since the beginning of the CPR Survey, ship speed has undoubtedly increased over time and has raised questions as to its effect on sampling depth. Data gathered from depth sensors attached to CPRs have shown that tow depth is independent of ship speed (Batten *et al*., 2003).

Due to the nature of sample collection and the subsequent analysis process, some planktonic components are less well represented in the CPR dataset than others. For example, the CPR methodology could underestimate components of the plankton *e.g*., large plankton like euphausiids, delicate gelatinous plankton, and plankton smaller than the 270 μm mesh (a standard WP2 has a mesh size of 200 μm). In terms of copepods, the CPR Survey likely undersamples the smaller species of *Oithona* and *Oncaea* and some juveniles of larger species. The CPR method of sample collection and analysis is indeed somewhat different to other commonly used techniques, yet comparisons made with other types of sampling gear have demonstrated the CPR's ability to measure changes in seasonal cycles in accordance with other samplers (Richardson *et al*., 2004). In addition, when compared with another sampler (Norpac net) using the same 270 μm mesh, both abundance and species composition have been found to be comparable (Hunt and Hosie, 2003). However, direct comparisons of absolute abundances inevitably can vary within and between sampling devices, including the CPR. Regarding the CPR, this has been well documented and users of CPR data are advised of its limitations and help from experienced SAHFOS researchers is always offered in data interpretation (Owens *et al*., 2013).

Unlike many other sampling initiatives, including some long-term programmes, the strength of the Survey lies in its consistency in sampling technique and analysis methodology for over half a century. There have been very little changes in design and analysis methodology since 1958, and metadata on changes has been kept. Whilst the Survey might underestimate some taxa, it has operated in a consistent manner over the time period, meaning that long term trends in these taxa are still valid. In addition, as new scientific questions arise, plankton taxa can be speciated or grouped according to need, whilst keeping the core dataset intact.

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1.2. Copepod Taxa Recorded by the Survey

Of the 700 taxonomic entities (phytoplankton and zooplankton) recorded by the SAHFOS CPR Survey, 306 belong to the subclass Copepoda, many of which are identified to species level (Table 1). To the end of 2015, this dataset amounts to over a quarter of a million samples analysed for plankton taxa. This equates to over 175 million plankton abundance records; just under a third of these records are related to Copepoda. SAHFOS CPR data can be obtained for *bona fide* research (see https://www.sahfos.ac.uk/data/data-request-form/), and are subject to a data licence agreement. Presence only data for all planktonic taxa analysed by SAHFOS can be accessed via the Ocean Biogeographic Information System (OBIS) portal (http://www.iobis.org/) (Copepoda - http://www.iobis.org/explore/#/taxon/616102).

Table 1 details the copepod taxa recorded in the SAHFOS CPR Survey and their biological traits. For several taxa of specific interest, sex and copepodite stage are also recorded by the Survey, but for simplicity this information has been omitted from the table. For standard North Atlantic analysis it is general practice to include copepodites and adults in the identification level chosen for that taxon, but only where an accurate identification is possible. For example, a record of *Centropages hamatus* would include males, females and most likely all copepodite stages collected, as identification to species level even in juveniles is relatively simple. However, a record of *Calanus helgolandicus* would only include adult males, females and stage V copepodites, because it is generally thought of as impossible to separate *C. helgolandicus* from its sibling species at younger stages using traditional light microscopy. Each taxon in the CPR database will have with it associated metadata such as this, and should be consulted before any interpretations are made.

Biological traits are used to study functional diversity which provides a link between organisms and their ecosystems. With a wide range of trait descriptors used to characterise the role of a species in terms of ecological functioning, here we list the most common: morphological (body size, measured as total length); trophic position (feeding method, defined as herbivore, omnivore, predator or parasite); physical environment (habitat, defined as neritic, oceanic or cosmopolitan) and distribution (sea areas). It must be noted that many of the feeding method traits have been inferred, as the majority of species have not been specifically studied. In this case, traits have been inferred from similar taxa *i.e.*, species within a genus, or in some cases not explicitly detailed in the reference but alluded to (Johns and Wootton, 2013). Each taxon in the table is also matched with a corresponding Aphia ID, a unique numerical identifier given to each taxon as listed in the World Register of Marine Species (http://www.marinespecies.org/).

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Table 1. Copepod taxa identified in the SAHFOS CPR Survey and their biological traits. Ant, Antarctic; IO, Indian Ocean; Med, Mediterranean; NA, North Atlantic; SA South Atlantic; P Pacific

Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Acartia amboinensis	346049	1.3-1.5	herbivore	neritic	IO, P
Acartia danae	346026	0.7-1.3	herbivore	cosmopolitan	IO, Med, NA, NS, P
Acartia longiremis	346037	0.7-1.2	herbivore	neritic	Med, NA, SA,P, North Sea
Acartia negligens	346030	0.8-2.1	herbivore	cosmopolitan	IO, Med, NA,SA, P
Acartia spp.	104108	0.1-2.7	herbivore	cosmopolitan	Arctic, IO, Med, NA, North Sea, SA, P,
Acartia tonsa	345943	0.8-1.5	herbivore	neritic	IO, Med, NA, P, North Sea
Acartia tumida	346025	1.8-2.1	herbivore	neritic	Arctic, P
Acrocalanus spp.	104192	0.7-1.8	herbivore	cosmopolitan	IO, Med, NA,SA, P
Aetideopsis armatus	356886	2.8-4.5	predator	cosmopolitan	Arctic, IO, Med, NA, SA, P, North Sea
Aetideus acutus	104273	1.2-2.0	omnivore	cosmopolitan	IO, Med, NA,SA, P
Aetideus armatus	104275	1.3-2.3	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Aetideus giesbrechti	104276	1.1-2.2	omnivore	cosmopolitan	IO, Med, NA, SA
Aetideus spp.	104112	1.3-3.0	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea,
Alteutha spp.	115427	0.5-1.3	unknown	neritic	Ant, Arctic, IO, Med, NA, SA, North Sea, P
Amallothrix spp.	104221	1.3-5.4	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P
Anomalocera patersoni	104722	2.5-4.1	predator	neritic	IO, Med, NA, North Sea
Augaptilus spp.	104137	2.0-6.5	predator	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P
Bradyidius armatus	146735	1.5-2.7	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Bradyidius spp.	104115	0.9-4.9	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Calanoides acutus	342434	3.5-5.7	herbivore	cosmopolitan	Ant
Calanoides carinatus	104462	1.6-4.0	herbivore	oceanic	Med, IO, NA, P
Calanopia americana	104723	1.2-2.1	predator	neritic	IO, Med, NA, SA
Calanopia elliptica	104724	1.4-2.1	predator	neritic	IO, Med, NA, SA, P
Calanopia minor	220919	1.1-1.4	predator	neritic	IO, Med, SA, P
Calanopia spp.	104207	1.0-2.6	predator	cosmopolitan	IO, Med, NA, SA, P
Calanus finmarchicus	104464	2.4-5.0	herbivore	cosmopolitan, neritic, oceanic	Arctic, NA, North Sea
Calanus glacialis	104465	3.1-6.0	herbivore	oceanic	Arctic, NA
Calanus helgolandicus	104466	1.9-3.5	herbivore	cosmopolitan	NA, Med, North Sea

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Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Calanus hyperboreus	104467	5.3-10.0	herbivore	oceanic	Arctic, NA
Calanus marshallae	196770	2.9-4.5	herbivore	neritic	Arctic, P
Calanus pacificus	196771	2.2-5.3	herbivore	oceanic	P
Calanus propinquus	342435	4.8-6.0	omnivore	oceanic	Ant, SA
Calanus simillimus	342436	2.5-4.0	herbivore	oceanic	Ant, SA
Calanus spp.	104152	1.9-10.0	herbivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Calocalanus spp.	104193	0.5-1.5	omnivore	cosmopolitan	IO, Med, NA, SA, P, North Sea
Candacia armata	104474	1.7-3.2	predator	cosmopolitan	Med, NA, North Sea
Candacia bipinnata	104475	1.9-3.2	predator	cosmopolitan	Med, IO, NA, SA, P
Candacia columbiae	349800	3.1-5.0	predator	cosmopolitan	IO, P
Candacia curta	104476	1.5-2.9	predator	cosmopolitan	IO, Med, NA, SA, P
Candacia ethiopica	104478	2.0-3.0	predator	cosmopolitan	IO, Med, NA, SA, P
Candacia giesbrechti	104479	1.7-2.3	predator	neritic	Med
Candacia longimana	104481	2.4-3.9	predator	cosmopolitan	IO, Med, NA, SA, P
Candacia norvegica	104482	2.3-3.6	predator	cosmopolitan	IO, Med, NA, SA, P, North Sea
Candacia pachydactyla	104483	1.5-3.4	predator	cosmopolitan	IO, Med, NA, SA, P
Candacia spp.	104157	1.2-5.1	predator	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Candacia tenuimana	104485	2.4-3.4	predator	cosmopolitan	IO, Med, NA, SA, P
Candacia varicans	104486	1.9-2.7	predator	cosmopolitan	IO, Med, NA, SA, P
Canthocalanus pauper	220834	1.0-2.0	omnivore	cosmopolitan	IO, Med, P
Centropages abdominalis	196774	1.2-2.1	omnivore	neritic	Arctic, P
Centropages brachiatus	104490	1.3-3.0	omnivore	neritic	IO, Med, NA, SA, P, Ant
Centropages bradyi	104491	1.3-2.5	omnivore	cosmopolitan	IO, Med, NA, SA, P
Centropages calaninus	104492	1.7-2.2	omnivore	cosmopolitan	Med, IO, SA, P
Centropages chierchiae	104494	1.5-2.3	omnivore	cosmopolitan	IO, Med, NA, SA
Centropages furcatus	104495	1.4-1.9	omnivore	cosmopolitan	IO, Med, NA, SA, P
Centropages gracilis	220903	1.7-2.2	omnivore	cosmopolitan	IO, Med, NA, SA, P
Centropages hamatus	104496	0.9-1.4	omnivore	neritic	Med, NA, SA, P, North Sea
Centropages spp.	104159	0.2-3.0	omnivore	cosmopolitan	IO, Med, NA, SA, P, Ant, Arctic, North Sea
Centropages typicus	104499	0.8-2.0	omnivore	cosmopolitan	Med, NA, SA, North Sea
Centropages violaceus	104500	1.8-2.2	omnivore	cosmopolitan	IO, Med, NA, SA, P
Clausocalanus spp.	104161	0.5-2.0	omnivore	cosmopolitan	IO, Med, NA, SA, P, North Sea, Ant
Clytemnestrinae	587514	0.4-1.6	unknown	cosmopolitan	IO, Med, NA, SA, P, North Sea
Copilia spp.	128721	1.4-9.0	unknown	cosmopolitan	IO, Med, NA, SA, P
Corycaeus speciosus	128800	0.8-2.3	predator	cosmopolitan	IO, Med, NA, SA, P

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Table 1. (Continued)

Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Ctenocalanus spp.	104162	0.8-1.7	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P
Ctenocalanus vanus	104510	0.8-2.0	omnivore	cosmopolitan	IO, Med, NA, SA, P
Cyclopinoides longicornis	357973	0.5-1.1	unknown	neritic	P, NA, Med
Diaixis hibernica	104521	0.7-1.2	unknown	neritic	Arctic, Med, NA, SA, North Sea
Diaixis pygmaea	104522	0.7-1.0	unknown	neritic	Med, NA, SA
Drepanopus forcipatus	342452	1.1-1.9	omnivore	neritic	Ant, SA, P
Epilabidocera amphitrites	254330	2.3-4.0	predator	neritic	Arctic, P
Epilabidocera spp.	254329	2.3-4.0	predator	neritic	Arctic, P
Eucalanus bungii	254427	4.8-8.0	omnivore	neritic	Arctic, P
Eucalanus hyalinus	345781	3.2-8.3	omnivore	cosmopolitan	Arctic, IO, Med, NA, SA, P, North Sea
Eucalanidae	104085	2.8-8.5	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Euchaeta acuta	104550	3.2-4.8	predator	cosmopolitan	IO, Med, NA, SA, P, North Sea
Euchaeta marina	104552	2.3-3.9	predator	cosmopolitan	IO, Med, NA, SA, P, North Sea
Euchaeta media	104553	3.1-4.8	predator	cosmopolitan	IO, Med, NA, SA, P
Euchaeta paraconcinna	196780	2.3-3.2	predator	cosmopolitan	NA, SA
Euchaeta pubera	104554	2.9-4.4	predator	cosmopolitan	IO, Med, NA, SA, P
Euchaeta spinosa	104555	5.2-7.2	predator	cosmopolitan	IO, Med, NA, SA, P
Euchaetidae	104086	2.1-11.6	predator	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea
Euchirella amoena	104296	2.7-4.0	omnivore	cosmopolitan	IO, Med, NA, SA, P
Euchirella bella	104297	3.1-4.9	omnivore	cosmopolitan	IO, NA, SA, P
Euchirella curticauda	104299	2.5-5.6	omnivore	cosmopolitan	IO, NA, SA, P, North Sea
Euchirella maxima	104300	6.1-8.7	omnivore	neritic	IO, Med, NA, SA, P
Euchirella messinensis	104301	2.8-6.2	omnivore	cosmopolitan	IO, Med, NA, SA, North Sea, P
Euchirella pseudopulchra	196792	3.4-4.4	omnivore	neritic	P
Euchirella pulchra	104302	2.9-4.4	omnivore	cosmopolitan	IO, NA, SA, P, North Sea
Euchirella rostrata	104303	2.0-4.1	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Euchirella spp.	104120	2.0-8.7	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Eurytemora pacifica	232028	0.9-1.3	omnivore	neritic	Arctic, P
Eurytemora spp.	104240	0.9-2.3	omnivore	neritic	Arctic, NA, P, North Sea

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Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Euterpina acutifrons	116162	0.4-0.9	herbivore	neritic	IO, Med, NA, SA, North Sea, P
Farranula gracilis	128813	0.7-1.1	predator	cosmopolitan	Ant, IO, Med, NA, SA, P
Farranula spp.	128636	0.6-1.1	predator	cosmopolitan	Ant, IO, Med, NA, SA, P
Gaetanus minor	104312	1.7-2.4	omnivore	cosmopolitan	IO, Med, NA, SA, P
Gaetanus pungens	342623	2.0-3.8	omnivore	cosmopolitan	Ant, IO, NA, P
Gaetanus simplex	358653	2.7-3.6	omnivore	neritic	NA, P
Gaetanus spp.	104121	1.7-9.7	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Gaetanus tenuispinus	237965	1.8-4.0	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Giardella callianassae	128775	0.8-2.5	parasitic	neritic	North Sea
Giardella thompsoni	128776		parasitic	neritic	North Sea
Haloptilus acutifrons	104422	2.1-4.7	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Haloptilus longicornis	104431	1.2-2.6	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P, North Sea
Haloptilus spiniceps	104437	2.6-5.5	omnivore	cosmopolitan	IO, Med, NA, SA, P
Hemicyclops aberdonensis	413311	1.3-1.6	parasitic	neritic	North Sea
Heterorhabdus abyssalis	104576	2.0-3.7	predator	cosmopolitan	IO, Med, NA, SA, P, North Sea
Heterorhabdus austrinus	343728	2.4-4.0	predator	cosmopolitan	Ant, SA, P (south)
Heterorhabdus clausi	358813	2.0-2.7	predator	cosmopolitan	IO, Med, NA, SA, P
Heterorhabdus norvegicus	104579	2.6-4.6	predator	cosmopolitan	Arctic, NA, SA, North Sea
Heterorhabdus oikoumenikis	346540	2.1-3.5	predator	oceanic	IO, NA, P
Heterorhabdus papilliger	104580	1.6-2.7	predator	cosmopolitan	Ant, IO, Med, NA, SA, P
Heterorhabdus spinifer	104582	1.6-2.0	predator	cosmopolitan	IO, Med, NA, SA, P
Heterorhabdus spp.	104178	1.6-4.6	predator	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P
Heterorhabdus tanneri	196782	3.0-4.9	predator	cosmopolitan	P
Heterostylites longicornis	104586	2.3-4.3	predator	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Isias clavipes	104501	1.3-1.7	omnivore	neritic	Med, NA, SA, North Sea
Labidocera acuta	104726	2.3-3.6	predator	neritic	IO, Med, NA, SA, P
Labidocera acutifrons	104727	3.3-4.7	predator	cosmopolitan	Ant, IO, Med, NA, SA, P
Labidocera aestiva	104728	1.8-3.0	predator	neritic	NA, SA, P
Labidocera spp.	104208	1.1-4.7	predator	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea

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Table 1. (Continued)

Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Labidocera wollastoni	104736	2.0-2.6	predator	neritic	IO, Med, NA, SA, North Sea
Lophothrix spp.	104225	2.7-7.4	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P
Lubbockia spp.	128672	1.0-3.0	omnivore	oceanic	Ant, IO, Med, NA, SA, P
Lucicutia spp.	104183	1.0-10.3	omnivore	cosmopolitan	Arctic, Ant, IO, Med, NA, SA, P
Macrosetella gracilis	116382	0.9-1.8	herbivore	oceanic	IO, Med, NA, SA, P
Mecynocera clausi	104616	0.8-1.3	omnivore	cosmopolitan	IO, Med, NA, SA, P
Mesocalanus tenuicornis	104468	1.5-3.4	grazer, herbivore	cosmopolitan	IO, Med, NA, SA, P, North Sea
Metridia gerlachei	344689	2.2-4.3	omnivore	cosmopolitan	Ant, IO, SA, P (south)
Metridia longa	104632	1.6-4.5	omnivore	cosmopolitan	Ant, Arctic, NA, SA, North Sea, P
Metridia lucens	104633	1.5-4.0	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea
Metridia okhotensis	196783	2.8-4.5	omnivore	cosmopolitan	P
Metridia pacifica	196784	1.7-3.5	omnivore	cosmopolitan	Arctic, P
Metridia spp.	104190	1.3-10.5	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea
Microcalanus spp.	104164	0.6-1.1	omnivore	cosmopolitan	Ant, Arctic, IO, NA, SA, P, Med, North Sea
Microsetella spp.	115341	0.3-0.8	omnivore	cosmopolitan	Ant, Arctic, IO, NA, SA, P, Med, North Sea
Miracia efferata	116383	1.2-2.2	herbivore	cosmopolitan	IO, Med, NA, SA, P
Monstrilla longiremis	119805	2.0-4.5	parasitic	neritic	Arctic, IO, NA, P, Med
Nannocalanus minor	104469	1.2-2.5	grazer, herbivore	cosmopolitan	Ant, IO, NA, SA, P, Med
Neocalanus cristatus	104470	6.7-10.4	grazer, herbivore	oceanic	Arctic, P
Neocalanus flemingeri	353708	3.8-6.3	grazer, herbivore	oceanic	Arctic, P
Neocalanus gracilis	104471	1.6-4.4	grazer, herbivore	cosmopolitan	Med, NA, SA, IO, P, North Sea
Neocalanus plumchrus	196772	4.0-6.3	grazer, herbivore	oceanic	Arctic, P
Neocalanus robustior	104472	2.8-4.7	grazer, herbivore	oceanic	IO, Med, NA, SA, P

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Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Neocalanus spp.	104155	1.6-10.4	grazer, herbivore	cosmopolitan	Ant, Arctic, Med, NA, SA, IO, P
Neocalanus tonsus	344701	3.1-4.6	grazer, herbivore	oceanic	Ant, IO, SA, P
Oculosetella gracilis	116384	0.7-1.4	herbivore	cosmopolitan	IO, Med, NA, SA, P
Oithona spp.	106485	0.3-2.0	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA,SA, P, North Sea
Oncaea spp.	128690	0.2-1.7	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA,SA, P, North Sea
Paracalanus spp.	104196	0.5-1.5	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Paracandacia bispinosa	220915	1.4-2.2	predator	cosmopolitan	IO, Med, NA, SA, P
Paracandacia simplex	220914	1.6-2.3	predator	cosmopolitan	IO, Med, NA, SA, P
Paraeuchaeta antarctica	344974	5.1-10.4	predator	cosmopolitan	Ant, SA, P (south) IO
Paraeuchaeta elongata	196779	4.1-8.4	predator	neritic	P
Paraeuchaeta glacialis	104560	6.2-11.0	predator	cosmopolitan	Arctic, NA, North Sea
Paraeuchaeta gracilis	104561	5.1-7.0	predator	cosmopolitan	IO, NA, SA, P
Paraeuchaeta hebes	104563	2.6-3.7	predator	cosmopolitan	Med, NA, SA, North Sea
Paraeuchaeta norvegica	104566	5.5-11.0	predator	cosmopolitan	Arctic, Med, NA, North Sea
Paraeuchaeta tonsa	359879	5.1-6.7	predator	cosmopolitan	P
Paraheterorhabdus robustus	368129	2.9-5.3	predator	neritic	IO, NA, SA, P
Parapontella brevicornis	104686	1.3-1.6	unknown	neritic	Med, NA east, North Sea
Parathalestris croni	116598	1.7-2.3	unknown	cosmopolitan	Arctic, NA, North Sea
Phaenna spinifera	104698	1.5-3.0	omnivore	cosmopolitan	IO, NA, SA, P, Med
Pleuromamma abdominalis	104637	2.4-4.5	omnivore	cosmopolitan	IO, Med, NA, SA, P, North Sea
Pleuromamma borealis	104638	1.4-2.5	omnivore	cosmopolitan	IO, Med, NA, SA, P, North Sea
Pleuromamma gracilis	224184	1.5-2.6	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA,SA, P, North Sea
Pleuromamma indica	220900	1.7-2.7	omnivore	oceanic	IO, Med, SA, P
Pleuromamma piseki	104640	1.7-2.4	omnivore	cosmopolitan	IO, Med, NA, SA, P
Pleuromamma robusta	104642	2.1-4.7	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, North Sea, P
Pleuromamma spp.	104191	1.2-6.4	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, North Sea, P
Pleuromamma xiphias	104643	3.3-6.4	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P
Pontella mediterranea	104739	2.4-3.3	predator	neritic	Med, NA (east)
Pontellina plumata	104743	1.0-1.9	predator	cosmopolitan	IO, Med, NA, SA, P
Pontellina securifer	196786	3.2-4.6	predator	cosmopolitan	IO, Med, NA, SA, P

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Table 1. (Continued)

Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Pontellopsis perspicax	360249	1.8-4.3	predator	cosmopolitan	IO, NA, SA, P
Pontellopsis regalis	104745	1.6-4.5	predator	cosmopolitan	IO, Med, NA, SA, P
Pseudocalanus spp.	104165	0.5-2.4	herbivore	cosmopolitan	Arctic, Med, NA, SA, North Sea, P
Pseudochirella spp.	104126	3.2-9.7	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P
Pseudodiaptomus marinus	157680	0.8-1.8	herbivore	neritic	P, North Sea, Med, IO
Pseudolubbockia dilatata	128895	1.4-3.4	omnivore	cosmopolitan	NA, P
Rhincalanus cornutus	104542	2.4-4.2	omnivore	cosmopolitan	IO, Med, NA, SA, P
Rhincalanus gigas	220837	6.9-10.0	omnivore	oceanic	Ant, SA, IO, P (south)
Rhincalanus nasutus	104543	2.7-6.1	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, North Sea, P
Sapphirina spp.	128722	1.0-8.0	predator	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Scaphocalanus echinatus	104793	1.3-2.6	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P
Scaphocalanus spp.	104228	0.9-5.8	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P
Scolecithricella spp.	104229	0.7-6.0	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea
Scolecithrix bradyi	104820	1.0-1.6	omnivore	cosmopolitan	IO, Med, NA, SA, P
Scolecithrix danae	104821	1.7-2.5	omnivore	cosmopolitan	IO, Med, NA, SA, P
Scolecithrix spp.	104230	1.0-6.0	omnivore	cosmopolitan	IO, Med, NA, SA, P
Scottocalanus persecans	104832	3.9-5.8	omnivore	cosmopolitan	IO, Med, NA, SA, P
Scottocalanus securifrons	104833	3.8-5.3	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P
Siphonostomatoida	10052	< 1.0 - > 22.0	parasitic	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea
Spinocalanus spp.	104236	0.8-5.1	omnivore	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea
Subeucalanus crassus	104544	2.4-4.6	omnivore	cosmopolitan	IO, Med, NA, SA, P, North Sea
Subeucalanus longiceps	345366	3.9-5.1	omnivore	cosmopolitan	Ant, IO, SA, P (south)
Subeucalanus monachus	104545	1.8-2.8	omnivore	cosmopolitan	IO, Med, NA, SA, P
Subeucalanus mucronatus	104546	2.5-3.5	omnivore	cosmopolitan	IO, Med, NA, P
Subeucalanus pileatus	104547	1.7-2.5	omnivore	cosmopolitan	IO, Med, NA, SA, P
Temora discaudata	220898	1.6-1.8	omnivore	cosmopolitan	IO, Med, P
Temora longicornis	104878	1.0-1.5	omnivore	cosmopolitan	Arctic, Med, NA, SA, North Sea

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Taxon Name	Aphia ID	Adult total length (mm)	Feeding method	Habitat	Sea areas
Temora stylifera	104879	1.4-1.9	omnivore	cosmopolitan	IO, NA, SA, Med, P
Temora turbinata	104880	1.3-1.6	omnivore	neritic	NA, SA, Med, IO, P
Tortanus discaudatus	157684	1.4-3.1	predator	neritic	Arctic, NA west, P
Undeuchaeta intermedia	196795	3.6-4.5	omnivore	oceanic	IO, P
Undeuchaeta major	104342	3.0-6.5	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Undeuchaeta plumosa	104343	2.8-4.7	omnivore	cosmopolitan	IO, Med, NA, SA, P, North Sea
Undeuchaeta spp.	104128	2.8-6.7	omnivore	cosmopolitan	Ant, IO, Med, NA, SA, P, North Sea
Undinula vulgaris	367334	1.8-3.3	omnivore	cosmopolitan	IO, Med, NA, SA, P
Urocorycaeus spp.	128639	1.2-3.1	predator	cosmopolitan	Ant, IO, Med, NA, SA, P
Xanthocalanus spp.	104204	1.2-8.9	predator	cosmopolitan	Ant, Arctic, IO, Med, NA, SA, P, North Sea

2. WHAT CAN CPR DATA BE USED FOR?

2.1. Mapping Copepod Biogeography

With such a large spatial coverage, data from the CPR Survey is ideally placed to generate biogeographical maps of copepod presence and abundance. Distribution maps are able to be generated for any of the taxa listed in Table 1 and maps of the most common of these taxa in the North Atlantic can be found in Barnard *et al*. (2004).

In addition to sample position, each copepod taxon in the CPR database has metadata associated with it, such as: date taxa first counted, date of collection, abundance, time of collection (day/night). Together, these parameters mean a comprehensive picture of copepod distribution and abundance can be mapped over varying time periods, from short-scale seasonal cycles to long-term decadal or inter-decadal variability, at genus or species level.

Figure 3 shows the average abundances of large (> 2 mm) and small (< 2 mm) copepods, based on mean adult total length, across the North Atlantic from CPR samples. Although copepods are found throughout the entire region, it can be seen that there is a clear geographical difference in abundance between these two groups, with small copepods dominating in coastal regions. The small cyclopoid copepod *Oithona* is thought to be one of the most abundant and ubiquitous metazoans in the ocean (Gallienne and Robins, 2001), and it is perhaps not surprising that smaller copepods are found widely across the Atlantic. In contrast, the highest abundances of larger copepods seem to be

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restricted to subarctic waters, notably the Grand Banks and Labrador Sea regions which are known to be amongst the most productive waters in the world.

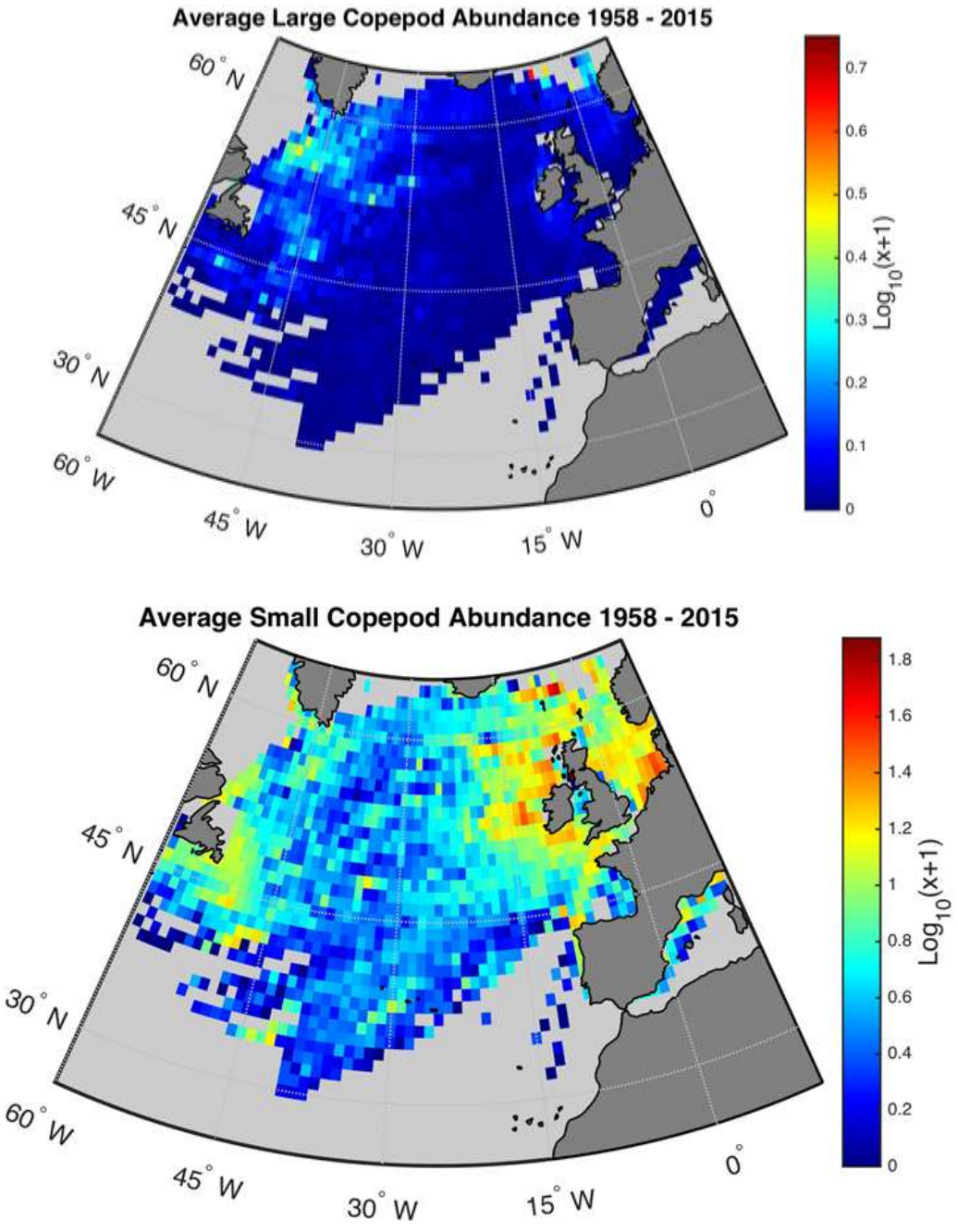

Figure 3. Map of the average monthly abundance of 'large' (> 2 mm) (a) and 'small' (< 2 mm) (b) copepods, counted in CPR samples between 1958 and 2015 for the North Atlantic, gridded onto a 1 by 1 degree grid.

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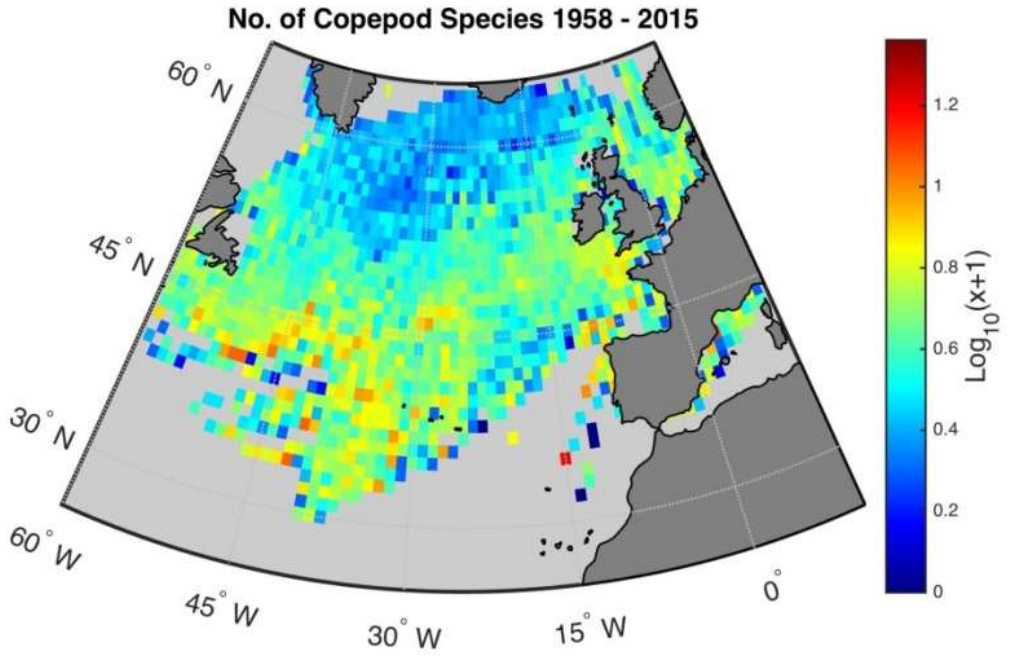

Figure 4. Map of copepod species richness recorded from CPR samples between 1958 and 2015 in the North Atlantic, gridded onto a 1 by 1 degree grid.

It is interesting to note that copepod abundance does not necessarily relate to species richness. Figure 4 shows the number of different copepod species recorded for a given area of ocean. Comparing this with Figure 3 demonstrates that the most speciose regions, such as the Sargasso Sea, coincide with lower copepod abundances. With temperature known to be a crucial environmental variable affecting marine species richness (Tittensor *et al.*, 2010), the map displays the classical theory of species number increasing with latitudinal gradient towards low latitudes, and a decrease in species (but an increase in body-size) towards the poles (Rombouts *et al.*, 2009). CPR data have shown that rising sea temperatures in the North Sea have been linked with an increase in overall species diversity (Beaugrand *et al.*, 2015). With sea temperatures predicted to rise, it will be interesting to see how this map will change over time.

2.2. Disentangling Short-Term and Rhythmic Variations from Long-Term Trends

Long-term (> 30 years) marine monitoring datasets are rare (Edwards *et al.*, 2010), but are essential in helping disentangle natural variation *e.g.*, seasonal cycles, from changes in the environment *e.g.*, regime shifts. Extensive time-series provide a baseline

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of data, a form of "yard-stick," against which new measurements can be compared and changes identified. Since its beginning, the CPR Survey has grown and developed with marine management drivers, linking copepod and other plankton abundances with both short-term events (*e.g*., upwelling, diel vertical migration) and long-term events (*e.g*., climate change, fish stock fluctuations) (Edwards *et al*., 2010). For instance, CPR research has shown that the Atlantic Multidecadal Oscillation accounts for the second most important large scale trend in North Atlantic plankton records, and is responsible for habitat switching (abrupt ecosystem/regime shifts) over multi-decadal scales (Edwards *et al*., 2013); without long-term monitoring programmes like the CPR Survey, our understanding of plankton community drivers would be, at best, limited.

2.3. Monitoring Northward Shifts and Range Expansions

Planktonic copepods are excellent indicators of environmental change (Beaugrand *et al*., 2008). They are quick to respond to changes in their environment *e.g*., temperature, have rapid generation times and are not generally a harvested resource and consequently they are affected less by direct anthropogenic pressures.

Much of the planktonic research carried out in the North Sea has focused on *Calanus finmarchicus*, a large, lipid rich calanoid copepod (Melle *et al*., 2014). This cold-water species forms the main food source for juvenile cod (Thorisson, 1989) and other fish species, and prefers a temperature range of 0-15°C, with peak abundances from 0-9°C (Melle *et al*., 2014). Its sister species, *Calanus helgolandicus*, similar in size, prefers temperatures between 9-20°C, with peak abundances from 13-17°C (Bonnet, 2005). *C. helgolandicus* abundance has two maxima during the year, the first one from May to June and the second one from September to October (Wilson *et al*., 2016). *C. finmarchicus* on the other hand has its annual maximum around May and is abundant throughout the year (Fromentin and Planque, 1996). Since the 1950s, CPR data have shown a large geographical shift in the distribution of these two species (Beaugrand *et al*., 2001), causing trophic mismatches (Edwards and Richardson, 2004). Notably, there has been a swing in the ratio from a *C. finmarchicus* dominated North Sea to a *C. helgolandicus* dominated North Sea (Figure 5). Coupled with a 70% reduction in total *Calanus* biomass, this has directly exacerbated the decline in the overexploited Atlantic Cod (*Gadus morhua*) stocks since the mid -1980s (Beaugrand *et al*., 2003), as larvae depend on this prey item for survival (Thorisson, 1989). This step-wise transition, termed a regime shift, is linked to the northward movement of the 9-10ºC isotherm in the region and has had effects not only on copepod species biogeography, but on phytoplankton, other types of zooplankton and terrestrial taxa too (Reid *et al*., 2016).

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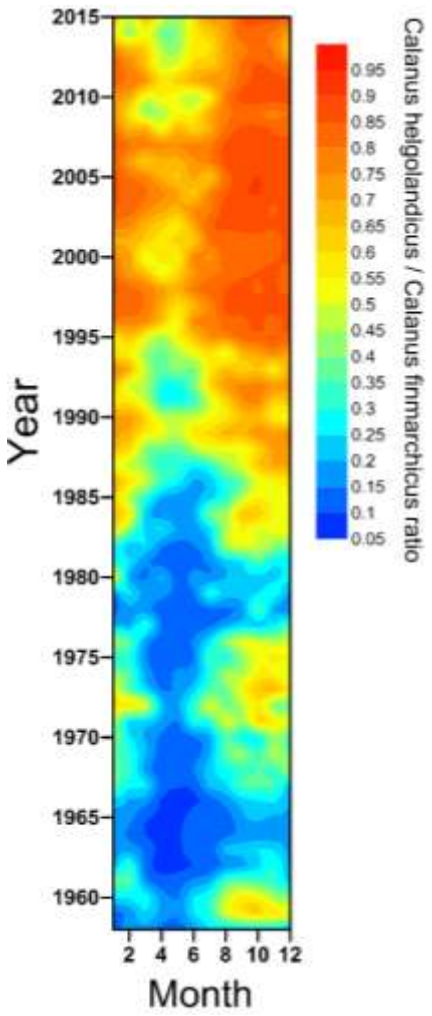

Figure 5. Ratio between the warm-water species (*Calanus helgolandicus*) and the cold-water species (*Calanus finmarchicus*) per month from 1958-2015 in the Central North Sea. Red shades indicate a dominance of the warm-water species and blue the dominance of the cold-water species.

Results from North Atlantic CPR data indicate that the northwards advancement of calanoid copepods is estimated to be 23 Km per year and is closely linked to increasing sea surface temperatures (SSTs) (Beaugrand *et al*., 2009). A similar pattern is also reflected in the North Pacific, with CPR data displaying a close correlation between warm-water copepod species assemblages (examples of taxa incorporated in this grouping include *Mesocalanus tenuicornis*, *Candacia bipinnata* and *Clausocalanus spp.*) and a positive Pacific Decadal Oscillation (PDO). A positive PDO, resulting in warmer SSTs in the northeast Pacific, corresponds with the northward movement of warm water copepod species (Batten and Walne, 2011).

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2.4. Observing Phenological Changes

Phenology is defined as the study of cyclic and seasonal natural phenomena, especially in relation to climate, plant and animal life. CPR data have shown that within the copepod assemblage, seasonal timings of peak abundance can have impacts throughout the marine ecosystem (Burthe *et al*., 2012), and propagate throughout multiple trophic levels, from primary producers to secondary consumers such as fish (Beaugrand *et al*., 2003) and seabirds (Fauchald *et al*., 2011; Reiertsen *et al*., 2014). Due to seasonal variations of the upper-ocean environment, copepods encounter changes that are both intense and prolonged compared to their life spans (Mackas *et al*., 2012). Changes in zooplankton phenology are often correlated with anomalies of one or more environmental variables, including water temperature (Edwards and Richardson, 2004). Most work on phenology in the zooplankton has focused on functional groups as opposed to individual species (Edwards and Richardson, 2004; Thackeray *et al*., 2016). Among copepods, the bulk of studies have concentrated on *Calanus*, due to their keystone position in many marine ecosystems (Melle *et al*., 2014 and references therein).

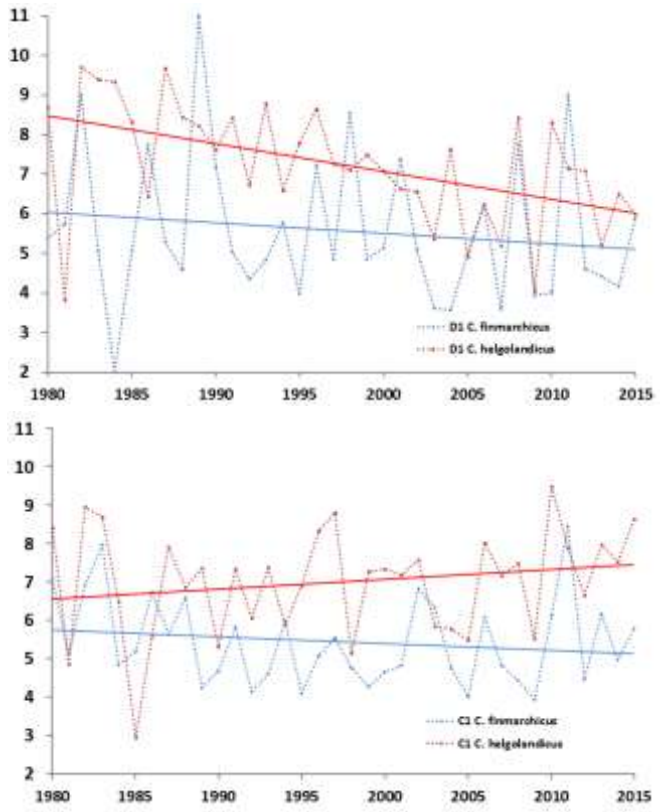

Figure 6. Time of seasonal peak changes in two species of *Calanus* (*Calanus finmarchicus* (blue) and *Calanus helgolandicus* (red)) in the North Sea (D1: Southern North Sea; C1: Central North Sea) from CPR data (Johns, 2017).

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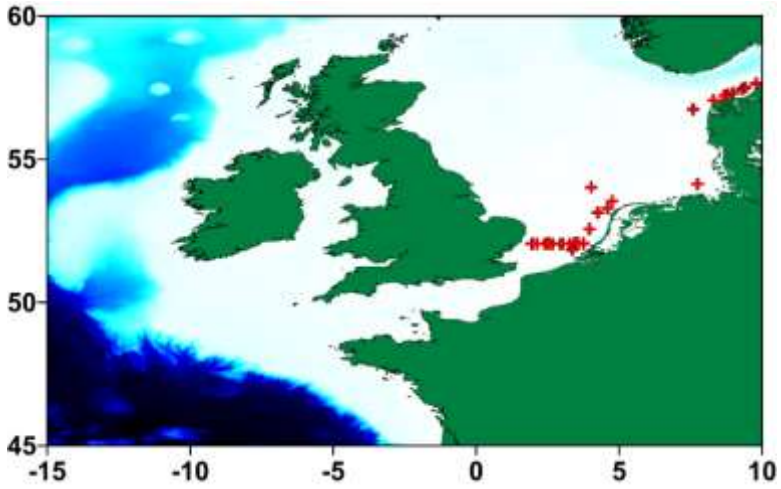

Figure 7. Occurrences of *Pseudodiaptomus marinus* in CPR samples from 2011-2016.

Figure 6 shows the month of seasonal peak for C. *finmarchicus* and C. *helgolandicus*, in two regions of the North Sea (CPR Standard Areas D1, Southern North Sea and C1, Central North Sea). The seasonal peak provides an insight into the phenology of the species in a given year, and the solid lines show long term trends of peak seasonality between 1980 and 2015. It can be seen that although *C. finmarchicus* is appearing slightly earlier in the year nowadays, in both areas *C. helgolandicus* has pronounced differences, moving earlier in the southern area and shifting to a later occurrence in the more northerly region. Both of these species are extremely important to higher trophic levels, such as fish and seabirds, and shifts in their peak periods of abundance can be propagated through the ecosystem – seasonal timing of prey species are often critical for the successful survival of higher trophic levels. This is known as a 'trophic-mismatch,' with seasonal timings becoming asynchronous. The examination of seasonal timings is crucial when looking at such keystone species as *Calanus*, as long-term trends in abundance only inform on part of the impact.

2.5. Detecting Non-Native Species

Research has shown the CPR to be a valuable tool in the discovery and monitoring of non-native species. With an extensive history of regular monthly sampling, the CPR Survey is able to provide a baseline for species diversity. In winter 2011 the Survey collected and identified specimens of the non-native copepod *Pseudodiaptomus marinus* in the southern North Sea (Jha *et al*., 2013). Native to north-western Pacific coastal waters, this species was first recorded in the Atlantic Ocean sector and North Sea in 2010 (Brylinski *et al*., 2012). However, the CPR records highlighted its northwards range extension since its arrival in European waters. Thought to have been introduced to the

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region via ballast water, *P. marinus* is both eurythermal and euryhaline. Not surprisingly, due to its adaptability, this non-native copepod has established itself throughout coastal regions in North America, the Mediterranean and now the North Sea (Sabia *et al*., 2015). Regularly found in North Sea CPR samples, since 2011, the Survey continues to monitor the spread and persistence of *P. marinus* around European waters (Figure 7).

2.6. CPR Copepod Data and Policy

Since the 1930s, the CPR Survey has co-evolved with marine management drivers, from monitoring North Sea plankton blooms for herring fisheries, to ecosystem-based management today (Edwards *et al*., 2010). The uniqueness of the CPR dataset means SAHFOS holds a distinctive position in the marine scientific policy community. The consistent methodology of the Survey allows exploration of long-term datasets that can be interrogated depending on policy questions. For instance, CPR research has contributed to the most recent Intergovernmental Panel on Climate Change (IPCC) reports (Pörtner *et al*., 2014) and the UN's World Ocean Assessment (Malone *et al*., 2015). Assessments such as these provide a mechanism to transfer scientific information on the state of the marine environment to decision makers, to facilitate evidence-based development of monitoring programmes and policy measures. For example, the European Union's Marine Strategy Framework Directive (MSFD) Directive 2008/56/EC (European Parliament and Council, 2008) seeks to achieve Good Environmental Status (GES) of European seas by 2020. By taking a holistic, ecosystem approach to marine management, this Directive relies on a suite of indicators being developed and monitored, at the member state and OSPAR (Oslo-Paris Convention) level, and monitored towards environmental targets which represent GES. Given the pivotal importance of copepods to the marine web, their inclusion as an indicator group for the monitoring of pelagic habitats is crucial. CPR data have played an integral role in the development of indicators at both national and international levels for multiple components of this Directive and, through the CPR Survey, will continue to contribute to the monitoring of these indicators in an effort to attain GES.

3. FUTURE OF THE CPR SURVEY

3.1. Sample Archive and Molecular Methods

SAHFOS has an extensive sample archive of approximately half a million samples, dating as far back as the late 1950s. Despite samples being preserved in formalin,

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recently developed molecular techniques have proven to be successful in the genetic analysis of copepod specimens (Kirby *et al*., 2007) as well as other planktonic taxa (Licandro *et al*., 2010). With the development of new techniques, and as interest in how molecular information can inform us about the ecology and physiology of copepods gains momentum (Amato and Carotenuto, 2018), further analyses of these archived samples are possible, thus progressing our understanding of the oceans.

3.2. Copepods and Human Health

Vibrio bacteria are numerous and abundant in the marine environment and are known to be associated with chitinous plankton, such as copepods. Many *Vibrio* species are pathogenic to humans (causing cholera, septicaemia and seafood-related gastroenteritis) as well as causing mass mortality to a range of marine life (Vezzulli *et al*., 2010). *Vibrio* genetic material has successfully been extracted from archived CPR samples, dating as far back as the 1960s, and has shown that during the last 50 years there has been an increase in the occurrence of *Vibrio* in the North Sea, linked with increased SSTs in the region (Vezzulli *et al*., 2012). Although there are still many questions regarding the relationship between copepod abundance and *Vibrio*-related outbreaks of disease, the predicted rise in climate change induced SSTs can only warrant further investigation into this subject.

3.3. Instrumentation

To gain a holistic picture of copepod biology and ecology it is important to accurately record many parameters associated within a copepods environment. Since 1994 a range of sensing technologies have been trialled on the CPR, and SAHFOS maintains an archive of these datasets alongside the contemporary presence and abundance data collected. These instruments include miniloggers for temperature and sensors for conductivity, temperature, chlorophyll and depth measurements. Going forward, new techniques are being developed to keep abreast of technological developments. For example, the autonomous Water and Microplankton Sampler (WaMS), which has been developed in collaboration with the Centre for Environment, Fisheries and Aquaculture Science (CEFAS), has already proven its capability to detect bacteria, naked flagellates and other microplankton that would be impossible to be detected with light microscopy alone (Stern *et al*., 2015). SAHFOS has recently invested in a new FlowCam® Macro for copepods and zooplankton, to be used to advance research capabilities. This newly developed instrument will complement microscopic analysis and SAHFOS' proven world-class taxonomic expertise.

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CONCLUSION

Since its inception in 1931 the CPR Survey has grown into the world's most geographically extensive and longest running marine biological survey. Despite sampling and recording a variety of phytoplankton and zooplankton taxa, copepods remain at the core of the Survey in terms of both taxonomic and research expertise. The use of consistent methodology coupled with long-term monitoring has allowed long-term trends to be identified from complex spatial and temporal variations, with data showing range shifts and changes in seasonal timing of copepod species in response to warming waters and climate change.

With an extensive sampling effort over large areas of ocean, CPR data are ideally suited to generate abundance and distribution maps for a wide range of species. Information on over 300 copepod taxonomic entities are routinely collected, yet the majority of research, published both by SAHFOS and the wider scientific community, seems to focus on the sister species *Calanus finmarchicus* and *Calanus helgolandicus*. Important to higher trophic organisms and indicators of different regimes, these two species certainly warrant intense study; however, with regional species diversity on the rise in response to a warming planet, the wealth of other copepod taxa stored in the CPR dataset perhaps also deserve attention and investigation.

As national and international leaders begin to realise the importance of the marine environment, policy and decision makers will be looking for suitable metrics to measure and monitor the health of our oceans. The CPR is the only sampler that covers the extent of the Northwestern European shelf, making it extremely useful for long-term and regional assessments of marine health. At a European level, The CPR Survey has been instrumental in the design of such indicators for the pelagic environment, and has been involved in a number of national and international assessments. With marine protection and policy continuing to progress alongside modelling and statistical techniques, it is likely that the use of such long-term vast spatial time-series will develop and they will become increasingly indispensable.

Criticised by some as antiquated, the CPR method of collection and sample analysis is robust and consistent; however, limitations to this method are acknowledged and advances are being made to expand and modernise the CPR's capabilities in the future. Molecular material has successfully been extracted from copepod specimens collected over 50 years ago and research carried out on a range of subjects from human health to population genetics. Hosting the largest known plankton archive in the world, and with the emergence of new techniques and technologies, the CPR Survey holds in its hands a unique and globally significant asset waiting to be explored.

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Chapter 3

GLOBAL DISTRIBUTION OF TROPICAL AND SUBTROPICAL COPEPODS

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ABSTRACT

Here we show the main distribution characteristics of marine copepods across the subtropical–tropical latitudes and to bathypelagic depths in the Atlantic, Indian and Pacific Oceans (35ºN–40ºS). The copepod samples were collected from December 2010 to June 2011 during the Malaspina Circumnavigation Expedition. Epipelagic (0–200 m), mesopelagic (200–1,000 m) and bathypelagic strata (1,000–3,000 m depth) were sampled using an opening and closing Hydro-Bios Multinet at the following depths: 0–200, 200–500, 500–1,000, 1,000–2,000 and 2,000–3,000 m. As expected, copepods were the most abundant contributors to the zooplankton community (84%), with more than 290 taxonomic categories identified. Other marine organisms observed were chaetognaths (5%), siphonophores (3%), ostracods (2%) and euphausiids (1%). The general distribution patterns observed included low abundances, irregular spatial distributions across the three oceans, and a large decrease of abundance as the depth of the water increased. The lowest abundance was found in the southern and western regions of the Pacific Ocean, while the highest abundances were found close to the upwelling systems of the northeastern Pacific Ocean, off the Cape Vert Islands and in the Benguela current.

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Large differences were not observed among oceans where depth played the most important role in the structure of the copepod communities.

Keywords: copepods, subtropical–tropical, vertical distribution, global distribution, Malaspina expedition

1. INTRODUCTION

Marine copepods play a key role in the transfer of matter and energy from the richer upper layers to the deep sea (Gartner, 1997). They are the main components of the zooplankton biomass, they are abundant throughout the whole water column and they are the most abundant group in the marine pelagic ecosystem (Siokou-Frangou *et al*., 2010). They contribute to the ecosystem by transporting organic matter to the deep ocean and egesting faecal pellets during their diel vertical migration (Longhurst, 1995; Hernández-León and Ikeda, 2005). Due to their abundance, biomass, diversity and fast response to environmental fluctuations, they are also good indicators of climate change (Beaugrand *et al*., 2002; Hays *et al.*, 2001). Moreover copepods are one of the most important links to higher trophic levels and a relevant component of the biological pump and the functioning of pelagic food webs (Richardson, 2008). The knowledge of their distribution and structure is useful for the management of marine ecosystems and the assessment of their status and health. Overall distribution and biology in the worldwide oceans are relatively well documented (Razouls *et al*., 2005-2017; Wootton *et al.*, 2018). However, a global-scale baseline assessment of copepod biodiversity and distribution, including long-term monitoring and retrospective analysis, is lacking. Gathering such information will provide a contemporary benchmark that will allow changes in the ocean to be followed over time.

The deep sea is the largest habitat on Earth and also the one that is least known. About 88% of the ocean environment is deeper than 1 km, and 76% of the environment is between a depth of 3 and 6 km (Hering, 2002). The exploration of these zones has been slow because of the inherent difficulties of sampling at great depths. These difficulties are gradually being overcome, which will likely result in an increase in the discovery and identification of new species. Below a depth of 1,500 m, the low abundance of most species requires large volumes of water to be filtered, and sampling takes place over many hours using large systems deployed from research vessels to collect a substantial number of individuals (Bucklin *et al.*, 2010). In addition, zooplankton communities in subtropical–tropical regions are poorly studied, particularly those of the southern hemisphere. They are widely unexplored in comparison to coastal areas, and most studies

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have been carried out in neritic waters of northern ocean areas (Richardson, 2008). The data on the abundance and distribution of zooplankton is sparse, and it corresponds to different ocean expeditions, mainly in the Atlantic (Gaard *et al*., 2008; Schnack-Schiel *et al*., 2010; Vereshchaka *et al*., 2017). An expedition never covers more than one ocean, and different expeditions use different methodologies, which makes it difficult to compare information about how organisms are distributed and relevant spatial scales. The opportunity to explore three different oceans and to use the same methodologies from surface to the deep ocean in all sampling sites would allow for the generation of a global description of the zooplankton community structure, which is of importance for future studies.

Accordingly, the main goal of this chapter was to give a global view of the copepod distribution down to bathypelagic depths in the subtropical–tropical latitudes in the Atlantic, Indian and the Pacific Oceans. A better understanding of global patterns will provide a baseline for observing future changes.

2. MATERIALS AND METHODS

During the Malaspina Circumnavigation Expedition carried out between December 2010 and July 2011 in the Atlantic, Indian and Pacific Oceans (35ºN–40ºS), samples of zooplankton were collected from 15 biogeographical provinces (Longhurst, 1995), including some poorly studied regions of the Indian and south-western Pacific Oceans (Figure 1). Samples were collected from the surface layer down to a depth of 3,000 m using an opening–closing Multinet that was equipped with a 300 µm mesh. Sample collection took place in five strata at depths of 0–200, 200–500, 500–1,000, 1,000–2,000 and 2,000–3,000 depth m, and samples were always collected during the day (10:00 to 13:00 local time). All samples were preserved on board in 5% buffered formaldehyde. Later, in the laboratory, a total of 38 stations and 198 samples were analysed under a Nikon SMZU stereomicroscope. The main zooplankton groups found were standardized to the number of individuals per m^3 (ind. m^{-3}). Copepods were identified at least to the level of genus and, when possible, to the level of species, using published literature from around the world (Bradford-Grieve, 1994; Bradford-Grieve *et al.*, 1999; Conway *et al.*, 2003; Razouls *et al.*, 2005–2017). For the analysis of copepods and main group linkages, the Primer-6 software package was used (Clarke and Warwick, 2005). A similarity matrix was calculated from the copepod abundance using Log (x + 1) and, after averaging main stations by oceans and strata for dendrograms, a cluster analysis was generated based on the Bray–Curtis similarity measure. Due to the particular distribution Benguela station was not included in Figure 5b.

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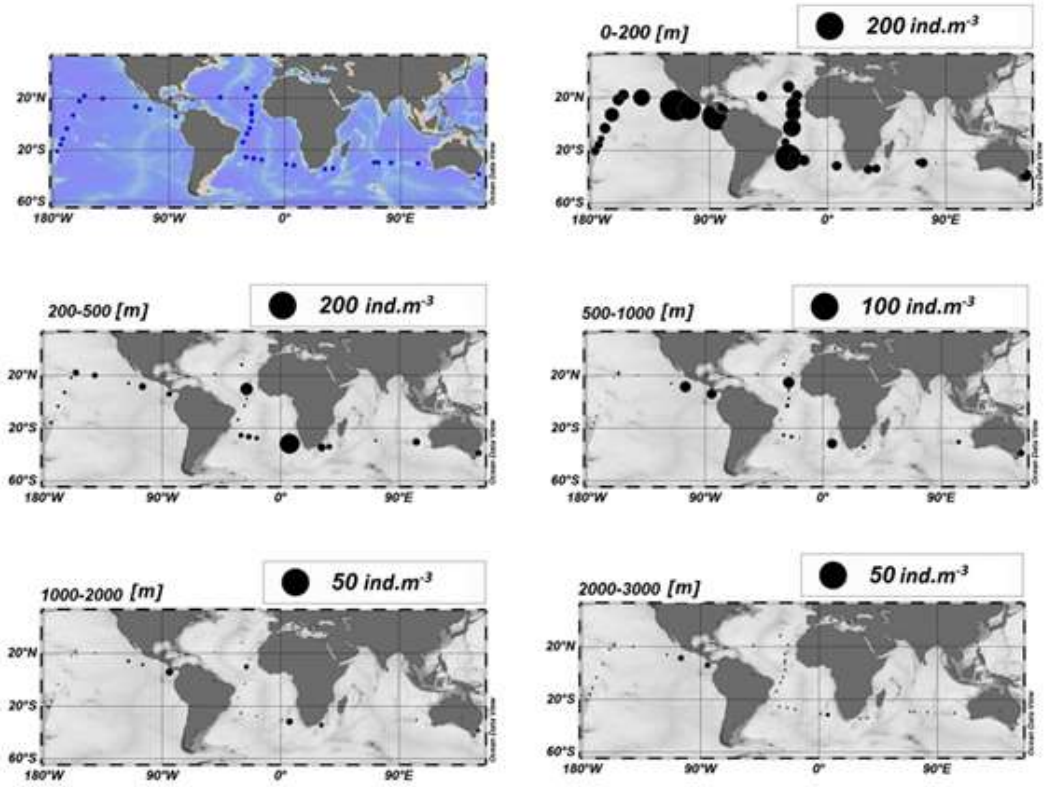

Figure 1. Map of stations and copepod abundance (ind. m^{-3}) for the five layers sampled at each station (circle) during the Malaspina expedition.

3. RESULTS

3.1. Abundance and Distribution

A low abundance of zooplankton was generally found across the entire study area (< 50 ind. m^{-3}), with the highest values corresponding to the upper 200 m of water in the eastern side of the Atlantic and Pacific Oceans (Figure 1). The spatial variability of the copepod abundance was observed among sampling stations, which covered oligotrophic areas with normal trophic conditions and rich areas and upwelling systems (for example, the northeastern Pacific [NEP], the Costa Rica Dome [CRD], off of the Cape Vert Islands [CV] and the Benguela current [BC]).

A drastic decrease of zooplankton abundance was observed as the depth of the water increased. In the epipelagic strata, only a few areas showed values greater than 200 ind. m^{-3}, for example, in the Brazilian waters, NEP and CRD. Large abundances in the mesopelagic strata off of CV and BC were also identified (> 80 ind. m^{-3}). In the bathypelagic strata, very low abundances were generally observed (< 2 ind. m^{-3}), with the

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exception of slightly higher abundances in NEP waters, CRD and BC (< 5 ind. m^{-3}). When samples from the stations of each ocean and strata were averaged (Figure 2), the epipelagic waters of the Pacific Ocean exhibited a higher percentage of copepods (79%) compared to the Atlantic (67%) or the Indian Oceans (43%). In the mesopelagic strata (200–1,000 m depth), the Atlantic and the Indian Ocean copepods were more abundant than in the Pacific. It was interesting to observe that the upper mesopelagic of the Indian Ocean (200–500 m depth) showed a higher percentage of copepods (31%) than the Atlantic (21%) or the Pacific Oceans (8%). Below a depth of 1,000 m, a very low abundance was found in all three oceans.

Seventeen different zooplankton groups were found, but only seven groups had an abundance higher than 1%. Copepods were the dominant group, and they were always found in an abundance higher than 82% (87% in the Indian Ocean). The main characteristic observed in each strata and ocean was the increase in the contribution of copepods at deep strata (Figure 3). However, in the upper mesopelagic (200–500 m depth), the percentage of copepods was the lowest (75%) because other groups such as chaetognaths, ostracods, siphonophores, pteropods and euphausiids were found in higher densities.

Accordingly, the vertical abundance of copepods in the three oceans defined the vertical pattern of total zooplankton, with a decrease from the epipelagic to bathypelagic strata (Figure 4A). The largest decrease was observed in the Pacific Ocean from the upper (42%) to the mesopelagic strata (19%), while in the Indian Ocean the contribution of copepods was higher in the bathypelagic (59%) than in the epipelagic strata (23%). In the Atlantic, abundance of copepods in the epipelagic and mesopelagic copepod strata was similar (35% and 33%, respectively), with a larger decrease down to the bathypelagic depths (19%).

3.2. Copepod Community Structure

In this study, 290 taxonomic categories of copepods were observed, and the highest number of species were found in the epipelagic strata, as expected (Figure 4B). In the mesopelagic layer, the number of species decreased sharply, particularly in the Atlantic Ocean; an increase in species was observed at a depth of 500–1,000 m, similar to the number found in the epipelagic layer. The sampling from the Indian Ocean exhibited a similar pattern to the Atlantic Ocean. In the Pacific Ocean, a higher number of copepod species was found in the upper bathypelagic layer at a depth of 1,000 to 2,000 m. The vertical profiles of the species number were not linear; an increase was observed at mid-depths and also at deeper water depths in the Pacific Ocean than in the Atlantic or in the Indian Oceans.

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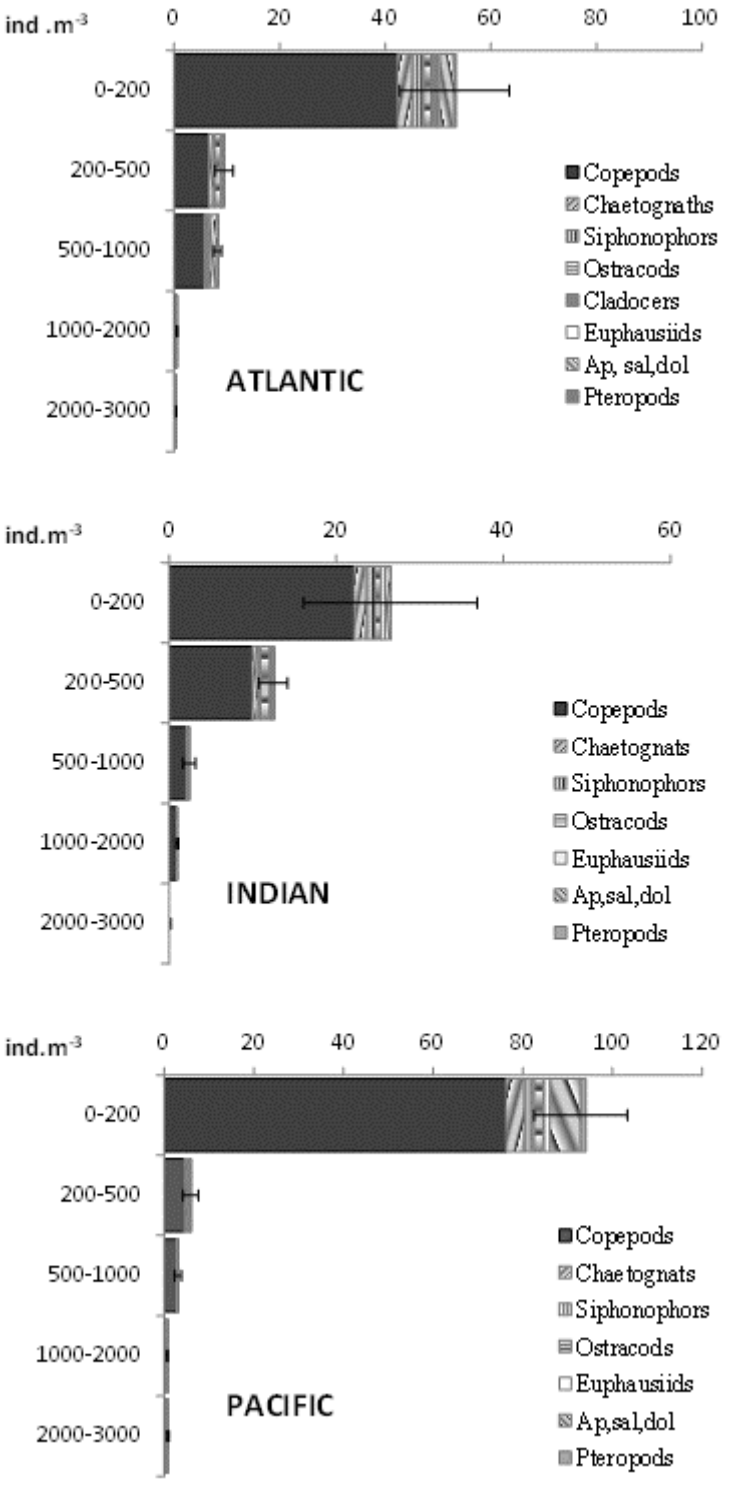

Figure 2. Average zooplankton abundance and their vertical distribution (as ind. m^{-3}) in the Atlantic, Pacific and Indian Oceans (Ap, sal, dol: appendicularians, salps and doliolids).

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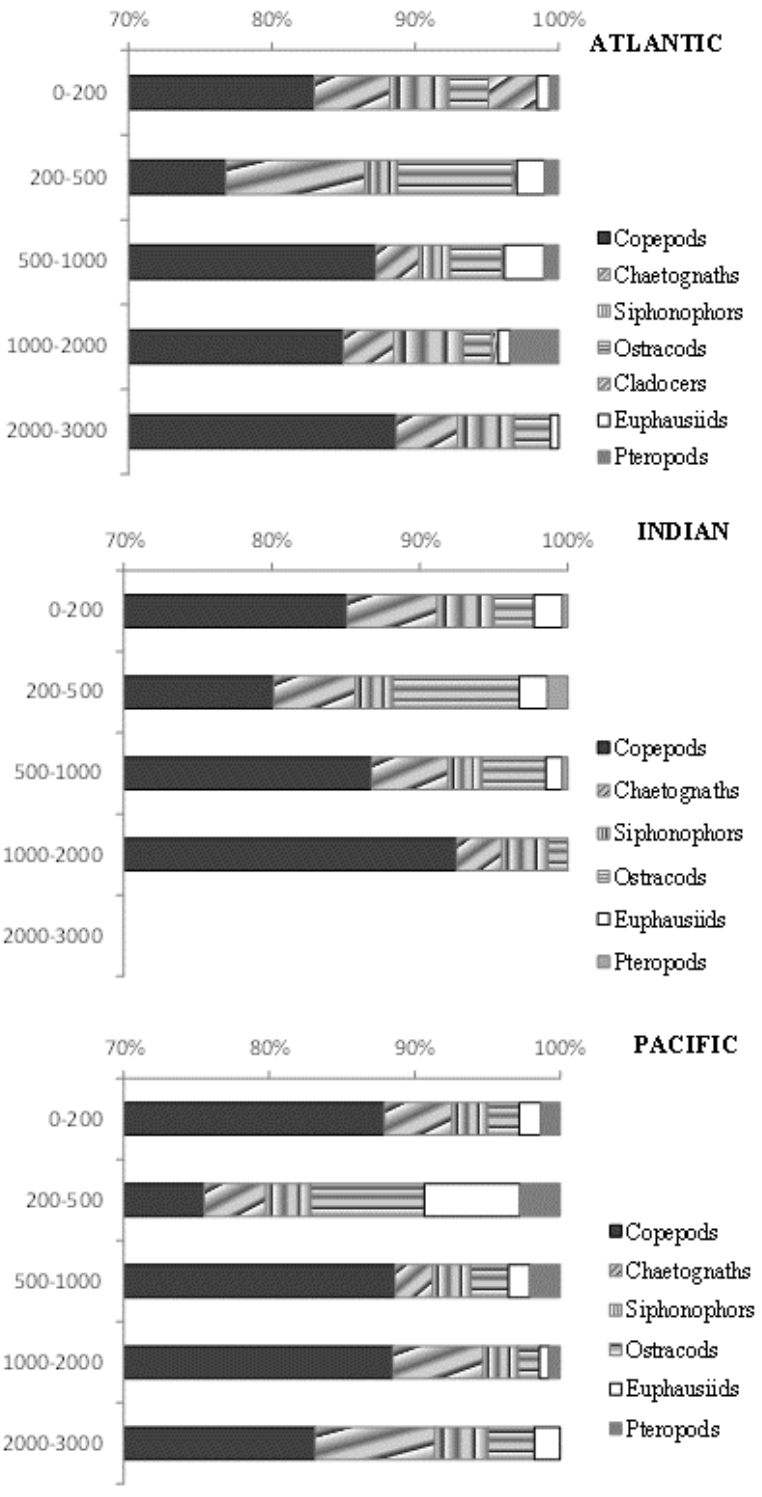

Figure 3. Vertical distribution (%) of the main zooplankton groups in the Atlantic, Pacific and Indian Oceans.

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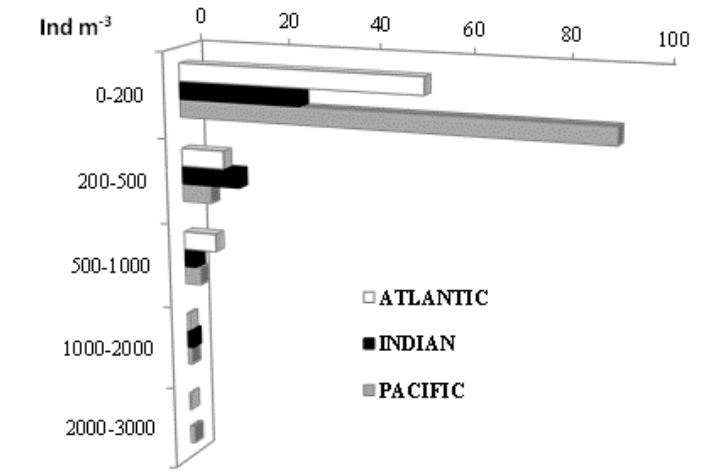

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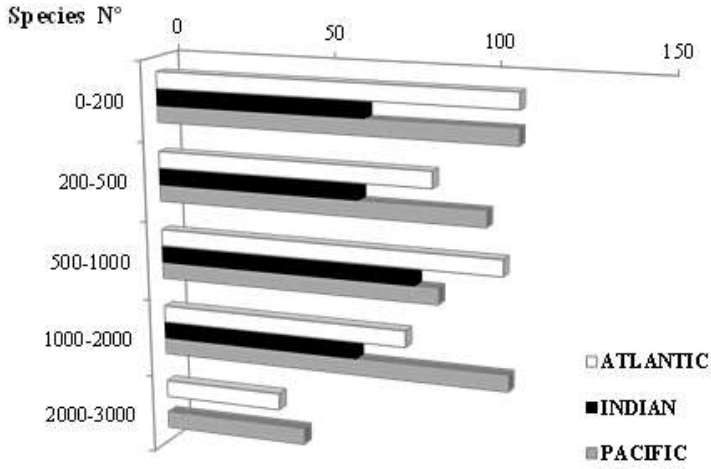

(B)

Figure 4. (A) Comparison of average abundances (ind. m^{-3}) and vertical copepod distributions for the Atlantic, Pacific and Indian Oceans. (B) Number of copepod species identified in the Atlantic, Pacific and Indian Oceans.

Although more than 230 species were identified, 55% were consistently rare (less than 1% of abundance). Similar numbers of species were found in the Atlantic and the Pacific Oceans (203 and 206, respectively), and a lower number of species were found in the Indian Ocean (150). Of the calanoids in the epipelagic strata, Clausocalanidae family (16%) had the highest abundance, followed by the Calanidae (14%), Paracalanidae, *Euchaeta* and *Acartia*; all of these accounted around 50% of the total copepods (Table I). Among the non-calanoids, *Oithona* and *Oncaea/Triconia* were dominant (18%). In all three oceans, small cosmopolitan copepods (that is, copepods with a body length ≤ 1 mm) were most common.

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Table I. Main groups of copepods found at the different strata: epipelagic (0-200 m), mesopelagic (200-1,000 m) and bathypelagic (1,000-3,000 m)

EPIPELAGIC	%	MESOPELAGIC	%	BATHYPELAGIC	%
Clausocalanidae	16	*Pleuromamma*	20	Paracalanidae	15
Calanidae	14	Calanidae	10	*Oncaea/Triconia*	13
Paracalanidae	12	Clausocalanidae	8	Calanidae	12
Oithona	9	*Oncaea*	7	*Metridia*	7
Oncaea/Triconia	9	*Lucicutia*	6	*Oithona*	7
Euchaeta	7	*Oithona*	6	*Lucicutia*	4
Corycaeidae	6	*Pareucalanus*	5	*Pleuromamma*	4
Acartia	5	*Scolecithricella*	4	*Monacilla/Spinocalanus*	4
		Heterorhabdus	4		

Although highly dispersed throughout the mesopelagic strata, *Pleuromamma* was the dominant copepod taxa, and among the non-calanoids *Oncaea* and *Oithona*. Some *Clausocalanus*, *Lucicutia*, *Pareucalanus* and *Heterorhabdus* were frequently found in the mesopelagic layer. In the bathypelagic layer, although *Oncaea/Triconia* among the non-calanoids were very abundant, some *Paracalanus*, *Metridia*, *Lucicutia* and *Monacilla* were also found. It was noteworthy that at a depth below 1,000 m, a large number of *Neocalanus* juveniles and *Calanoides carinatus* were observed in the Atlantic upwelling system.

A cluster analysis was performed in order to check for similarities between the oceans and the strata by using two way analysis of similarity (ANOSIM; Clarke and Warwick, 2005). Despite the differences in the total abundances between the oceans, the dendograms that resulted from the cluster analysis revealed that at a Bray–Curtis similarity level of 53%, there were more similarities between the Indian and Atlantic Oceans compared to the Pacific Ocean. No significant differences were observed between the three oceans (ANOSIM R: 0.105; significance level of 0.2%) (Figure 5A). However, a significant difference was found between the strata, particularly between the epipelagic layer and other zones (ANOSIM R: 0.508; significance level of 0.1%). The bathypelagic stratum (1,000–3,000 m depth) showed the highest similarity between the upper and the lower strata (40%). The cluster analysis among main stations, copepods and strata (Figure 5B) revealed the presence of three main groups: epipelagic (0–200m), mesopelagic (200–1,000 m) and bathypelagic layers (1,000–3,000 m).

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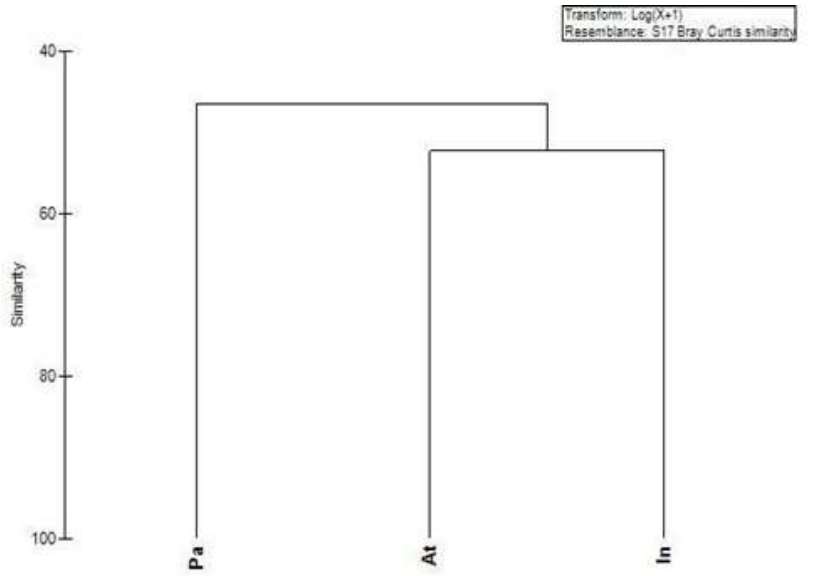

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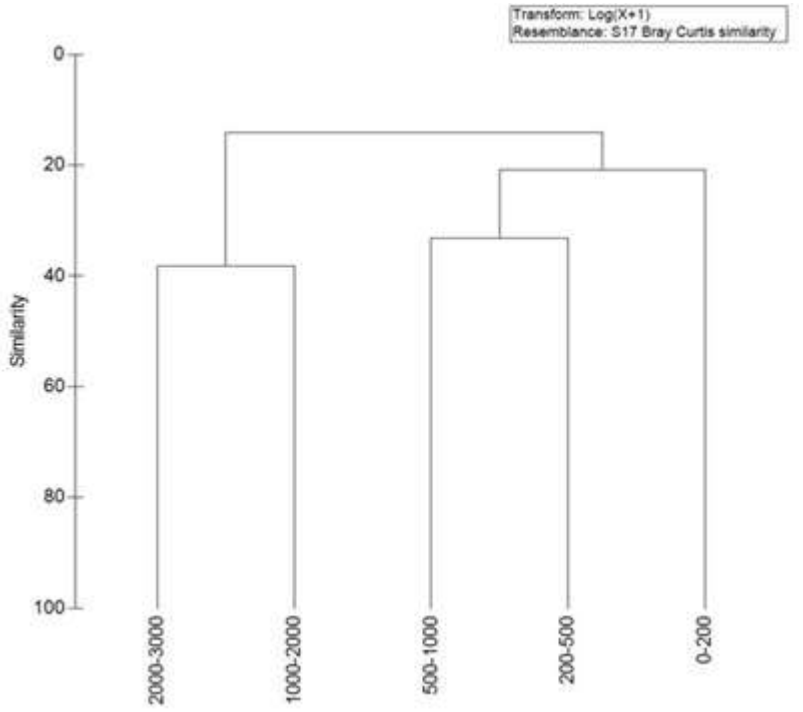

(B)

Figure 5. (A) Clusters of copepod abundances and stations in the Atlantic (At), Indian (In) and Pacific (Pa) (calculated using the Bray–Curtis similarity index after the data (x) had been transformed to Log (x + 1)). (B) Cluster showing copepod abundance at different sampled layers (calculated using the Bray–Curtis similarity index after the data had been transformed to Log (x + 1)).

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DISCUSSION

We analysed the abundance and composition of the zooplankton, and in particular the copepod community, in the water column (0–3,000 m depth) of the subtropical and tropical latitudes. The findings presented in this chapter demonstrate for the first time that three oceans have been sampled using the same methodology. Copepods were always dominant in the three oceans and at all depths (> 70% of total zooplankton abundance), as is usually observed in open ocean areas (Longhurst, 2007; Gaard *et al*., 2008). The dominant copepods observed at each depth were similar to what has been reported by other studies (Bradford-Grieve, 1994; Bradford-Grieve *et al*., 1999; Conway *et al*., 2003; Dias *et al*., 2010; Razouls *et al*., 2005–2017). The large decrease in abundance that was observed from the epipelagic to the bathypelagic zones was also found by different authors either in the Atlantic or in the Pacific Oceans (Fernández-Álamo and Farber-Lorda, 2006; Schnack-Schiel *et al*., 2010; Vereshchaka *et al*., 2017); this vertical discontinuity is a common pattern. Smaller copepods are usually more numerous at the surface layers, while larger copepods are less abundant at the surface layers and are mainly found in deep waters (Deevey and Brooks, 1977; Webber and Roff, 1995; Brugnano *et al*., 2012).

The high number of copepods observed here is a common pattern in many zooplankton studies, demonstrating the relevance of this group in transferring energy between different levels of the trophic web. They play a key role in the marine plankton food web by controlling primary production and microzooplankton, and by providing food for animals at higher trophic levels (Chank and Fang, 2004). Knowledge of the vertical distribution of copepods and their variations in space are relevant to understanding the dynamics in pelagic communities and the vertical flux of organic matter through the water column.

An overall finding of this study was the high number of copepod species found; however, the majority of them were found in an abundance of less than 1%, as other authors have reported for the Atlantic Ocean (Piontkovski *et al.*, 2003). The number of species and their abundances tended to decrease with increasing depth, which was another general trend that was observed for the distribution of copepods in the pelagic strata (Longhurst, 1995; Angel, 2003). Nevertheless, the decrease in the number of species was not linear, as a peak was normally found at mid-water and continued to decrease at deeper layers. This was another trait that has already been mentioned with regard to deep oceans and low latitudes (Bucklin *et al.*, 2010). In the present work, the results from the three oceans did not show significant differences between the compositions of copepods, as 50% of species were common for all three oceans; however, depth was the main factor affecting their distribution. In addition, deeper-dwelling copepods were less likely to be endemic (restricted to a particular region) and more likely to be geographically widespread (Bucklin *et al*., 2010; Yamaguchi *et al*.,

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2015). Moreover, feeding mode varied across all the strata, with filter-feeding herbivorous copepods observed more frequently in the upper layers and omnivores and carnivores more abundant in deeper layers (Benedetti *et al.*, 2015).

Another finding was the widespread distribution of small copepods, such as *Clausocalanus, Paracalanus, Oithona* and *Oncaea*, which is in contrast to other authors who reported limited distribution (Maycas *et al.*, 1999). As expected from their vertical migratory behaviour, large copepods, such as *Pleuromamma*, *Euchaeta*, *Pareucalanus, Metridia* and *Lucicutia,* were found in deeper layers (Bradford-Grieve *et al.*, 1999; Razouls *et al.*, 2005–2017). Other copepods, such as *Neocalanus* juveniles with an ontogenetic distribution, were mainly found in upwelling systems and were limited to deep waters and the oxygen-minimum zone (Cavalcanti and Larrazábal, 2004; Fernández-Álamo and Farber-Lorda, 2006; Verheye *et al.*, 2005; Gaard *et al*., 2008; Escribano *et al*., 2009; Teuber *et al.*, 2013).

In summary, we investigated the main characteristics of the copepod community in the subtropical and tropical latitudes (30ºN–40ºS) in the Atlantic, Indian and Pacific Oceans and down to bathypelagic depths. Our results showed irregular spatial abundance from the oligotrophic to the upwelling regions and a large decrease in abundance from the epipelagic to the bathypelagic zones. A large number of copepods were found mainly in the epipelagic layer, but they were also observed at mid-water depths. The overall distribution of copepods in the three oceans was similar; differences between the oceans were mainly due to small differences in genera and species rather than total abundances, and depth was the most relevant factor affecting the copepod distribution. In addition, the oceanic waters were characterized by cosmopolitan small-sized copepods, which dominated the community in the whole water column. In order to understand the role of copepods in the ocean ecosystem, we must expand our knowledge of organisms in deeper water, the patterns of distribution and the diversity of dominant species. This is especially relevant in the deep layers of the subtropical and tropical oceans, which are poorly understood due to the sampling challenges.

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Chapter 4

BIOGEOGRAPHICAL DISTRIBUTION AND ECOLOGY OF THE PLANKTONIC COPEPOD *OITHONA DAVISAE*: RAPID INVASION IN LAKES FARO AND GANZIRRI (CENTRAL MEDITERRANEAN SEA)

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ABSTRACT

The Asiatic copepod species *Oithona davisae* was initially described from the type locality of the Sacramento San Joaquin Estuary, California, USA (Ferrari and Orsi, 1984). Following recent reports of *O. davisae* in the Black Sea, we researched the biogeographical distribution and ecology of this species, based on original data and on a critical review of the literature. *O. davisae* was not recorded for the Mediterranean Sea before 2003, although it is now becoming established in Italian transitional environments. The literature indicates that *O. davisae* is a typical coastal and estuarine copepod species that was originally endemic in the temperate coastal waters of East Asia, and its spread to other regions was due to human-related vectors. *O. davisae* is less than 1 mm in length, and its abundance has often been underestimated because many sampling programmes have used net mesh sizes of 200 μm or larger, which cannot quantify small cyclopoid species. *O. davisae* has most often been recorded in transitional environments that are characterised by high trophic levels and stable waters, which allow its reproduction throughout the year. In Lakes Faro and Ganzirri, its highest abundance occurs in the late spring to early summer, coincident with the highest abundance of small-sized flagellates.

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The occurrence of dense populations of *O. davisae* in these transitional environments that are used for aquaculture activities confirms its invasive behaviour, with competitive exclusion of the indigenous species *Oithona nana*. After its introduction, *O. davisae* has become the most abundant species of the copepod assemblage in a short time. To date, there do not appear to be negative consequences at the community and ecosystem levels. Ship-ballast waters and bivalve transplants appear to be the main means of dispersion of *O. davisae*.

Keywords: copepods; biogeography; ecology; interspecific competition

1. INTRODUCTION

Global human activities have amplified the biogeographical ranges of many invasive species over the last decades. Invasive species are those that are introduced to, and successfully establish themselves in, a region that is outside of their natural range. These have often been considered major threats to ecosystem functions and the maintenance of biodiversity (Carlton, 1989 and 1996; Carlton and Geller, 1993; Ruiz *et al*., 1999; Geller, 1999). The recent increase in studies on such coastal invaders has provided new insights into the human-related vectors, with four main paths of invasion proposed: ship-ballast water, hull fouling, and new connections between water bodies through canals and aquaculture transfers (Ruiz *et al*., 1997; Elston, 1997; Galil, 2009; Zenetos *et al*., 2010). Thus, lagoons, estuaries and coastal marine systems are among the most highly invaded environments for non-indigenous species.

The genus *Oithona* is the most important cyclopoid copepod group in the world oceans in terms of abundance and productivity (Gallienne and Robins, 2001; Turner, 2004). This includes cosmopolitan species, like *Oithona similis* and *Oithona nana*, as well as species with narrower ranges of distribution, such as *Oithona vivida* and *Oithona robusta* (Dahms *et al*., 2015 and references therein). These small-sized oithonids have many ecological roles. They might be an important prey source for the larval stages of some key fishery species (Viñás and Ramirez, 1996), or conversely they might represent energy sinks in metazoan foodwebs (Atkinson and Snÿder, 1997). They directly affect the downward flux of calanoid copepod faecal-pellet material in pelagic zones (González and Smetacek, 1994), contribute to regeneration of nutrients that support primary production (Hiromi, 1995), and facilitate complex trophic interactions between the protozoan and metazoan foodwebs (Nakamura and Turner, 1997). Oithonids differ from better-studied suspension-feeding calanoid copepods in being primarily raptorial predators using hydromechanical signals to detect and capture motile prey (Svensen and Kiørboe, 2000).

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Oithona davisae was originally endemic to the temperate coastal waters of East Asia. Over the last forty years, it has been recorded in other regions, which now extend from the Pacific and Atlantic Oceans to northern and southern European waters (Nishida *et al*., 1977; Ferrari and Orsi, 1984; Hirakawa, 1988; Lee *et al*., 2001; Saiz *et al.*, 2003; Cordell *et al*., 2009; Lawrence and Cordell, 2010; Temnykh and Nishida, 2012; Cordell *et al*., 2015; Cornils and Wend-Heckmann, 2015; Uriarte *et al*., 2015; Doğan and İşinibilir, 2016). *O. davisae* shows high tolerance to different environments, which allows it to survive and reproduce in many coastal and transitional waters. This ecological plasticity may contribute to its success as an invasive species.

Passive dispersion of *O. davisae* by ship-ballast waters has been widely proposed (Ferrari and Orsi, 1984; Nishida, 1985; Hirakawa, 1988; Cordell *et al*., 2009; Lawrence and Cordell, 2010). Other dispersion vectors have not been considered to have a role in *O. davisae* dispersion, although aquaculture transfer as a possible means of introduction and spread of invasive non-indigenous copepod species is a well-known possibility (Carlton, 1992a, b; Naylor *et al*., 2001). Aquaculture activities involving the transport of live shellfish, and especially oysters, have been identified as the most important human vector for the introduction of invasive species into coastal marine and estuarine systems (Molnar *et al*., 2008).

The Mediterranean Sea is particularly vulnerable to ship-transported bio-invasions, which have increased greatly in frequency over the last two decades (Galil, 2009; Coll *et al*., 2010; Costello *et al*., 2010). Among the 955 alien species recorded in the Mediterranean Sea, 42 are planktonic copepods (Zenetos *et al*., 2010), three of which have recently been identified in Lakes Faro and Ganzirri, two coastal lakes located in the Central Mediterranean Sea (Sicily, Italy): *Pseudodiaptomus marinus* Sato, 1913; *Acartia tonsa* Dana, 1849; and *O. davisae* Ferrari and Orsi, 1984 (Brugnano, 2006; Zagami and Brugnano, 2013; Pansera *et al*., 2014; Sabia *et al*., 2014; Sabia *et al*., 2015). These above mentioned species have rapidly invaded many coastal and transitional European sites, showing different patterns of coexistence and/or competitive exclusion with indigenous species (Brugnano *et al*., 2011; Altukhov *et al*., 2014; Uriarte *et al*., 2015; Delpy and Pagano, 2018; Marques *et al*., 2018; Villate *et al*., 2018).

In this contribution we first provide a brief revision of the biogeography and ecology of *O. davisae*. We then present results from a year-long sampling programme in Lakes Faro and Ganzirri, showing the seasonal variations in abundance of this species. For the first time we report the introduction of *O. davisae* through aquaculture rather than ballast water, evidenced by the lack of water exchange among the two lakes and the sea. The dynamics observed in these lakes match those recorded in other sites worldwide, provide evidence for the invasiveness of this species and point to further hypothetical alternative vehicles for dispersion. This information broadens our knowledge of the invasive dynamics of this species, and provides new understanding about its biology and ecology.

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2. DISTRIBUTION AND ECOLOGY OF *OITHONA DAVISAE*

2.1. Biogeographic Distribution

Oithona davisae is a coastal and estuarine copepod species endemic in the temperate coastal waters of East Asia. *O. davisae* was originally described by Ferrari and Orsi (1984) for Californian estuarine waters, with the type locality of the Sacramento San Joaquin Estuary. It has also been recorded in Chile (Hirakawa, 1988) and in the north-western Mediterranean (Nishida, unpublished observations, cited by Saiz *et al.*, 2003). More recently, *O. davisae* was reported in the Bay of Varna by Mihneva and Stefanova (2013), in the Estuary of Bilbao (eastern Atlantic Ocean, Spain) (Uriarte *et al.*, 2015), Wadden Sea (Cornils and Wend-Heckmann, 2015), Puget Sound (Washington State, USA) (Cordell *et al.*, 2015), Marmara Sea and Golden Horn Estuary (Isinibilir *et al.*, 2016) and Venice Lagoon (northern Adriatic Sea) (Pansera, personal communication) (Figure 1). Data on the literature search on *O. davisae* biogeography are available from Razoul *et al.* (2005-2017) and Walter (2015).

Figure 1. Global distribution of *Oithona davisae* based on the literature data: ● Sacramento San Joaquin Estuary, California (Ferrari and Orsi, 1984); ◘ Chile (Hirakawa, 1988); ◓ Puget Sound, Washington State (Cordell *et al.*, 2015); ■ Eastern Atlantic Ocean, Spain (Uriarte *et al.*, 2015); ▼ North-western Mediterranean (Saiz *et al.*, 2003); ▲ Venice Lagoon, Adriatic Sea (Pansera, personal communication); ◊ Wadden Sea (Cornils and Wend-Heckmann, 2015); ◣ Bay of Varna (Mihneva and Stefanova, 2013); ◗ Black Sea (Temnykh and Nishida, 2012); ◓ Korea (Lee *et al.*, 2001; Orui-Sakaguchi *et al.*, 2011); ◂▸ Japan (Nishida, 1985; Ohtsuka *et al.*, 2008); ▸ Lakes Faro and Ganzirri, Central Mediterranean (present work).

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It is closely related to *Oithona nana*, *Oithona aruensis* and *Oithona brevicornis* (Nishida and Ferrari, 1983; Ferrari and Orsi, 1984). The morphological features that allow *O. davisae* to be distinguished from these other species are: sharply pointed rostrum; very long distal spine on the first inner lobe of the maxillule; and endopod of the maxillule carries only one seta. Owing to this morphological closeness, *O. davisae* has also been misidentified several times, including as *O. brevicornis* f. *minor* (Nishida *et al.*, 1977; Nishida and Ferrari, 1983), *O. aruensis* (Nishida, 1985; Ohtsuka *et al.*, 2008) for the coastal waters of Japan, and as *O. aruensis* in Korea (Lee *et al*., 2001; Orui-Sakaguchi *et al.*, 2011).

It also appears likely that the *O. brevicornis* specimens that have been reported as recently introduced into the Black Sea (Zagorodnyaya, 2002; Gubanova and Altukhov, 2007; Selifonova, 2009) are also *O. davisae*. This is supported by the specimens collected in an offshore area in the Black Sea off the Crimean Peninsula that were previously identified as *O. brevicornis*, and upon re-examination showed to be *O. davisae* (Temnykh and Nishida, 2012).

The geographic distribution of *O. davisae* shows that this species was originally endemic to the temperate coastal waters of East Asia, in contrast to *O. brevicornis*, which was widely distributed in the subtropical-tropical coastal waters of the Indo-Pacific (Giesbrecht, 1891; Nishida, 1985). The recent record in European waters confirms this non-indigenous species as typical of temperate environments. However, the records of the occurrence of *O. brevicornis* in the Mediterranean Sea (Carazzi and Grandori, 1912; Pesta, 1920; Vaisierre and Seguin 1980; Shuvalov, 1980) should be revised, because these studies were not based on description of the morphological characters that are now known to distinguish *O. brevicornis* from the other related species.

2.2. Habitat Characteristics

O. davisae has mainly been reported in eutrophic land/sea transitional zones, such as coastal lakes, estuaries, lagoons and coastal marine environments, including near-shore waters, inlets and bays. *O. davisae* can tolerate wide salinities from 12 to 35, and temperatures from -1.8 to 29°C (Ferrari and Orsi, 1984; Kimmerer, 2004; Gubanova and Altukohv, 2007; Temnykh and Nishida, 2012; Mihneva and Stefanova, 2013; Cornils and Wend-Heckman, 2015; Doğan and İşinibilir, 2016; Isinibilir *et al*., 2016), although it appears to thrive in relatively warm conditions (20 to 25°C). In the temperate eutrophic inlet of Fukuyama Harbour (Uye and Sano, 1998), in the San Francisco Estuary (Bollens *et al.*, 2011) and inner Estuary of Bilbao (Uriarte *et al*., 2015), *O. davisae* seasonal highest abundances occurred in warmer periods, between late spring and early summer. In Sevastopol Bay (Crimea), the peak abundances of *O. davisae* had high interannual variability, occurring at the end of October (Gubanova and Altukhov, 2007), mid-June

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and in autumn (Altukhov *et al*., 2014). In the western Black Sea, *O. davisae* abundances peaked during September-October (Mihneva and Stefanova, 2013).

Most of the transitional environments where *O. davisae* has been reported to be abundant, especially in the inner part of estuaries, inlets and semi-enclosed bights, have narrow mouths and shallow channels, limiting water exchange with the sea. This therefore results in little water circulation, eutrophic conditions, and wide salinity variations (Ferrari and Orsi, 1984; Uye and Sano, 1998; Bollens *et al*., 2011; Uriarte *et al*., 2015). These habitat parameters appear to be beneficial for the growth of *O. davisae*. In the Mediterranean Sea, *O. davisae* has only been recorded in some of the many sites that possess these characteristics. One explanation might be that *O. davisae* has often been misidentified as *O. brevicornis* (*e.g*., see Zagami and Brugnano, 2013; Pansera *et al*. 2014), while another might be that the optimal conditions for the success of *O. davisae* invasions are isolated eutrophic environments, resulting in disjunct distributional patterns, and reducing the chances of dissemination of *O. davisae* among coastal transitional environments. In addition, *O. davisae* may have been missed because of the mesh sizes used for copepod sampling. Owing to its small size (< 1 mm body length), *O. davisae* might not be efficiently collected through the WP-2 plankton net (*i.e*., 200 µm mesh size) (UNESCO, 1968; Sameoto *et al*., 2000), which remains the most commonly used net for marine zooplankton sampling. This would lead to underestimation of cyclopoid abundances (Gallienne and Robins, 2001; Pansera *et al*., 2014), but not to their total absence.

2.3. Ecology

With its high egg production rate (11.6 eggs female^{-1} d^{-1} in summer; Uye and Sano, 1995) and invasiveness, *O. davisae* may require only a short time to become the most abundant copepod species in zooplankton communities of many coastal transitional environments (Uye and Sano, 1998; Bollens *et al*., 2011; Altukhov *et al*., 2014; Cornils and Wend-Heckman, 2015; Uriarte *et al*., 2015). O. davisa*e* shows high numerical peaks in both its original and its newly invaded environments, ranging from 5.1×10^3 to 6.0×10^5 ind. m^{-3} (Uye and Sano, 1995). Its highest numbers were recorded for swarms of adults and copepodites attaining 1.34×10^6 ind. m^{-3} for the mud flats of Ariake Bay, Kyushu (Hirota and Tanaka, 1985). The reasons for such high *O. davisae* abundance might be the low species diversity and the high primary production that characterise these segregated environments, which can result in little inter-specific competition and sufficient or unlimited food supplies.

In several environments like Sevastopol Bay (Altukhov *et al*., 2014), the Marmara Sea and Golden Horn Estuary (Isinibilir *et al*., 2016), and Estuary of Bilbao (Uriarte *et al*., 2016), *O. davisae* has occupied the niche of the indigenous species *O. nana* and it

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has become the most abundant species of the copepod assemblage. The preferred food of *O. davisae* consists of motile prey, such as flagellates, ciliates and copepod nauplii (Uchima, 1988; Uchima and Hirano, 1986a, b; Saiz *et al*., 2003; Henriksen *et al*., 2007), with either sinking or jumping considered typical foraging movements (Cheng *et al.*, 2014). Also *O. nana* appears to ingest significant levels of non-motile prey, such as diatoms (Lampitt and Gamble, 1982). *O. davisae* might have a significant role in transferring energy from the nanoflagellates and microflagellates and ciliates to the higher trophic levels in eutrophic environments, such as transitional waters (Uye and Sano, 1998).

The dominance of the invasive species *O. davisae* over *O. nana* might be evidence of competitive exclusion of the indigenous species from the zooplankton community. Laboratory experiments and field studies have highlighted the competitive advantage of *O. davisae* over *O. nana*. *O. davisae* is a widely euryhaline species (Svetlichny and Hubareva, 2015; Svetlichny *et al*., 2018), while *O. nana* is a stenohaline species (Kovalev, 1966). In addition, the frequency and speed of routine jumps, and the maximum escape reaction speed in *O. davisae* are significantly higher than for *O. nana* (Isinibilir *et al*., 2016). Comparisons of the biological attributes between these two species show that, at the same temperature, *O. davisae,* has shorter development time than *O. nana,* 17 days (Uye and Sano, 1998) *vs.* 21-24 days (Haq, 1965), higher daily egg-production rate, 11.6 (Uye and Sano, 1995) *vs.* 7.4 eggs female^{-1} day^{-1} (Cepeda *et al*., 2015), and larger clutch size, 28.5 (Uye and Sano, 1995) *vs.* 12.4 eggs female^{-1} (Temperoni *et al*., 2010; Cepeda *et al*., 2015). All of these factors may indicate the higher adaptive potential of *O. davisae* respect to *O. nana*.

2.4. Dispersal

Previous reports on *O. davisae* have shown that it was originally endemic in temperate Japanese coastal waters. Until now, its occurrence in other regions of the world has been explained exclusively by ship-ballast water transport (Ferrari and Orsi, 1984; Nishida, 1985; Hirakawa, 1988; Cordell *et al*., 2009; Kaysan, 2010; Lawrence and Cordell, 2010). Other human-related vectors have not been considered to play a role in *O. davisae* dispersion. However, ballast transport alone does not completely explain the biogeographic distribution pattern of *O. davisae*. Around the world, *O. davisae* has been found mostly in highly anthropogenic environments, and particularly in segregated coastal features, such as bays, estuaries and lagoons, where it can attain high abundances. The optimal conditions for *O. davisae* growth occur in confined eutrophic environments that are characterised by little water circulation, leading to a varied distribution pattern that reduce the chances of the dispersion of this species. Therefore, *O. davisae* occurs in embayments both with and without ballast water discharge.

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3. *OITHONA DAVISAE* IN THE CENTRAL MEDITERRANEAN SEA (LAKES FARO AND GANZIRRI, SICILY, ITALY)

3.1. Study Area

Lakes Faro and Ganzirri are formed by two coastal basins in the central area of the Mediterranean Sea (Figure 2). Lake Faro (38°160' N, 15°380' E) is a small coastal basin (0.263 km^2) located in the north-eastern tip of Sicily (Italy), and is connected to the adjacent Lake Ganzirri and to the Strait of Messina by shallow channels. It has a funnel-shaped bottom profile, whereby it has a wide nearshore water area before it reaches its maximum depth of 29 m, in the central part. In this deepest part, Lake Faro is characterised by typical features of a meromictic temperate basin, with its upper oxygenated mixolimnion (up to 15 m in depth) and its lower anoxic and sulphidic monimolimnion (Genovese, 1963; Truper and Genovese, 1968). The nearshore waters of the lake are oxygenated down to the bottom.

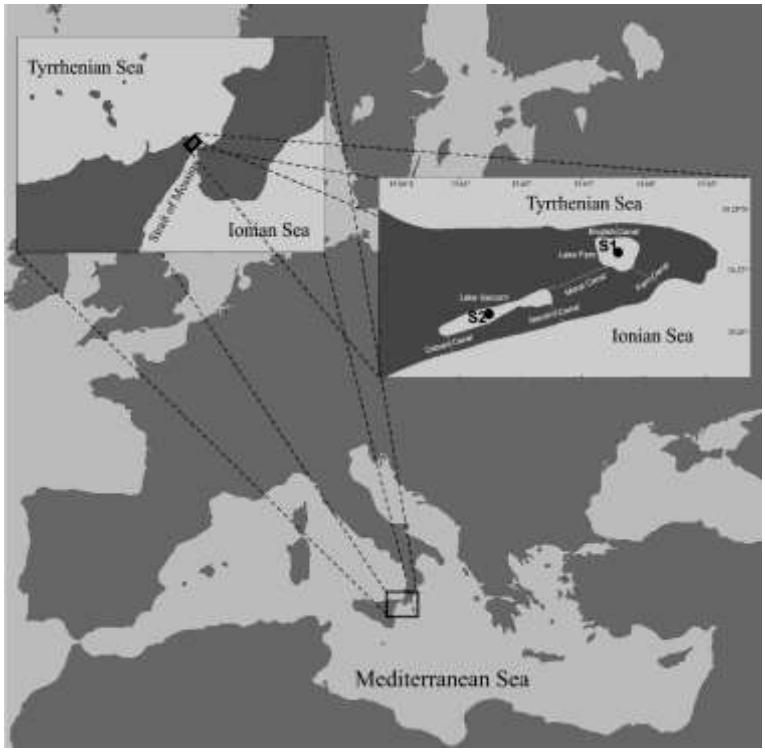

Figure 2. Map of the locations of Lakes Faro and Ganzirri.

Lake Faro shows large seasonal fluctuations in physico-chemical parameters, particularly for temperature (10-28°C) and salinity (28-37) (Crisafi, 1955; Pansera *et al.*, 2014). Its physical and chemical stratification between the surface water and the water

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column is significant, particularly in summer when anoxia extends up to the lower mixolimnion zone, resulting in large blooms of photosynthetic sulphur bacteria (Sorokin and Donato, 1975).

Lake Ganzirri (38°150' N, 15°360' E) has an elongated shape with shallow waters, reaching a maximum depth of 5 m thus maintaining oxygenated bottom waters. Through the year, Lake Ganzirri also has large fluctuations in its physico-chemical parameters, again for temperature and salinity in particular, which range from 10.4°C to 30.1°C, and from 21.2 to 28.7, respectively (Crisafi, 1955; Genovese, 1963; Ferrarin *et al*., 2013).

The very long and shallow channel that connects Lake Ganzirri to the sea results in a low marine water turnover rate. Thus, Lake Ganzirri is characterised by typical brackish waters that are supplied more by the surface rainwater than by marine water (Ferrarin *et al*., 2013). For both lakes, the water movements are influenced by the currents of the Strait of Messina. The marine water turnover rate is higher for Lake Faro than Lake Ganzirri, because of its shorter communication channel with the sea, thus Lake Faro has a more marine character than Lake Ganzirri (Genovese, 1963; Ferrarin *et al*., 2013).

In the segregated sectors of both lakes, very little water exchange occurs, leading to ecotoxicological alterations in the zooplankton communities (Minutoli *et al*., 2008). Lake Faro is an important centre for shellfish aquaculture, whereby living bivalves are imported from many Atlantic (Spain, northern France, The Netherlands) and Mediterranean (Venice Lagoon, Thau Lagoon) sites.

The mesozooplankton of Lakes Faro and Ganzirri include a relatively small number of taxonomic groups, of which copepods constitute by far the dominant species (Genovese, 1963; Crisafi *et al*., 1973; Zagami and Guglielmo, 1995). The permanent copepod communities in Lakes Faro and Ganzirri include only a few copepod species, including *Paracartia latisetosa* Krichagin, 1873, *Acartia margalefi* Alcaraz, 1976, and *Oithona nana* Giesbrecht, 1892. For Lake Faro only, there are also temporary coastal copepod species that enter and exit via tides from the Straits of Messina. A very complex current system driven by the flow of waters from the Ionian Sea to the Tyrrhenian Sea, and *vice versa* (Bossolasco and Dagnino, 1959; Mosetti, 1988), takes place in this area. This flow changes every 6 h, and thus has a large influence on the coastal and Lake Faro zooplankton communities (Zagami and Guglielmo, 1995; Zagami *et al*., 1996; Zagami and Brugnano, 2013).

3.2. Materials and Methods

Zooplankton sampling was carried out in Lakes Faro and Ganzirri at approximately monthly intervals from January to December 2014, at a station in the central, deepest area in each lake (Figure 1, S1, S2, respectively). The samples were collected using a net (mouth area, 0.125 m^2; mesh size, 80 μm; Apstein), with a digital flowmeter (Hydro-

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Bios, Kiel) mounted on the mouth of the net to calculate the filtered water volume. For Lake Faro, each net tow was vertical and taken within the oxygenated layer, from 15 m depth to the surface. For Lake Ganzirri, because of its lesser depth, each net tow was horizontal in the sub-subsurface layer of the water column (*i.e*., 0-1.5 m in depth). A multiparametric conductivity, temperature and depth probe (ISY 6600V2) was used to measure the temperature and salinity at each sampling station. In the laboratory, the zooplankton samples were fixed in 4% neutralised formalin in lake water, and *Oithona davisae* specimens were counted under a stereomicroscope (Wild MDG-17). The body appendages (*i.e*., mandible, maxillule) were removed under a stereomicroscope and mounted on microscope slides for taxonomic identification based on morphological comparisons according to Giesbrecht (1891) and Ferrari and Orsi (1984).

3.3. Results

3.3.1. Seasonal Variations of Environmental Factors

For Lake Faro, the surface water temperature ranged from 12.8°C in January to 29.0°C in August, and a similar range occurred in Lake Ganzirri, 12.5°C to 28.8°C in January and August, respectively (Figure 3). No significant temperature differences were recorded between these two lakes (Student's t-test; $p = 0.89$; n = 24).

For Lake Faro the salinity varied from 26.5 in March to 35.0 in October, while for Lake Ganzirri it varied from 21.3 in April to 28.9 in September. (Figure 4). In general, only salinity showed significant differences between Lakes Faro and Ganzirri (Student's t-test, $p < 0.01$; n = 24).

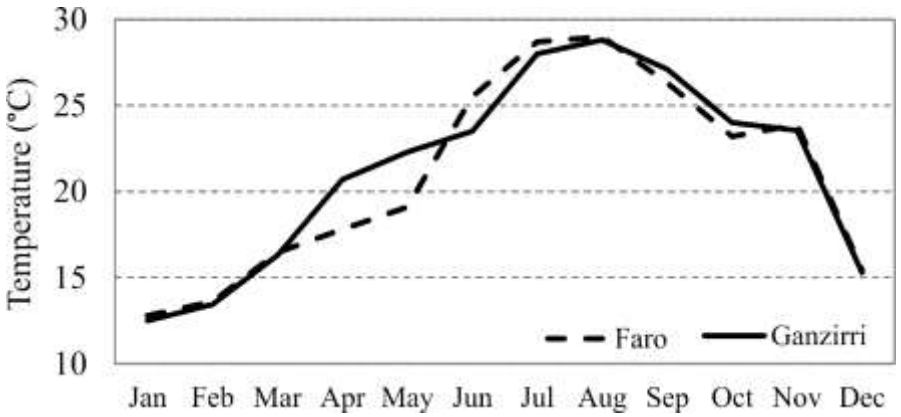

Figure 3. Seasonal variations of surface water temperatures for Lakes Faro and Ganzirri, from January to December 2014.

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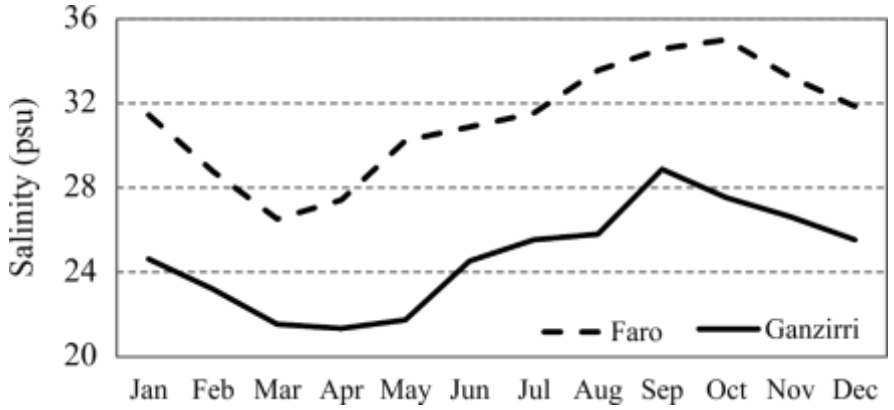

Figure 4. Seasonal variations of surface water salinity for Lakes Faro and Ganzirri, from January to December 2014.

3.3.2. Seasonal Variations in Abundance of Oithona davisae

O. davisae was found in Lakes Faro and Ganzirri during the entire sampling period. Females carrying ovisacs and nauplii were also seen throughout the year, and the two lakes had similar seasonal abundance dynamics. In terms of the mean density of *O. davisae* including adults plus all copepodite stages, abundances at Lake Faro were lowest in winter (January), at 3,312 ind. m^{-3}. From May to June, abundances increased exponentially, peaking in June at 116,512 ind. m^{-3}. Thereafter, from summer to winter, the abundances decreased gradually (Figure 5).

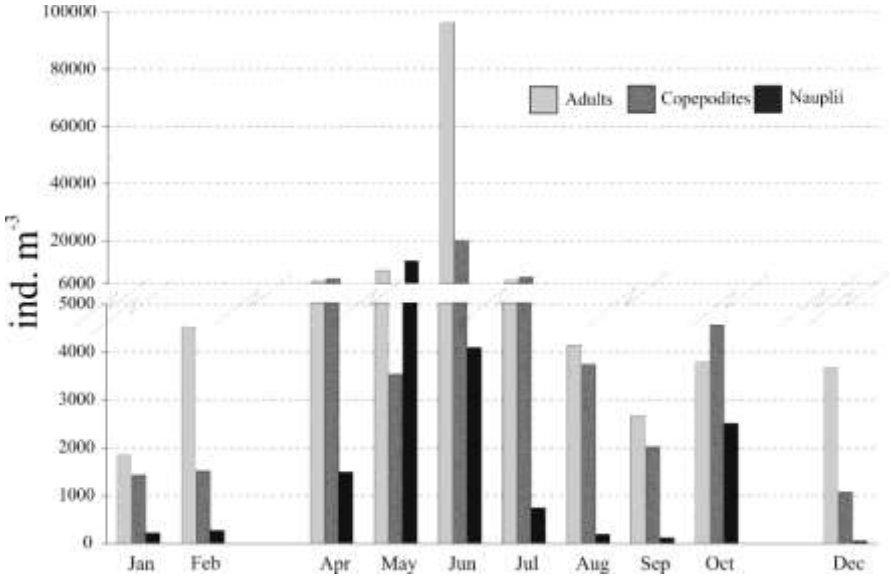

Figure 5. Seasonal variations in the abundance of *Oithona davisae* in Lake Faro.

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For Lake Ganzirri, the abundances also increased from winter (970 ind. m^{-3} in January) to spring, when they increased exponentially through May to June, peaking at 211,617 ind. m^{-3}. Abundances remained high all summer, and then decreased rapidly beginning in September, to the lowest for the year in December, at 92.6 ind. m^{-3} (Figure 6).

In both lakes, the naupliar abundances followed similar seasonal trends to the adults and copepodites. The nauplii abundance for Lake Faro peaked in May, at 13,000 ind. m^{-3}, and for Lake Ganzirri in June, at 242,692 ind. m^{-3}. Females always outnumbered males, with annual means of 85.4% females for Lake Faro and 87.2% females for Lake Ganzirri. Ovigerous females occurred throughout the year for both Lakes Faro and Ganzirri, although they showed greater relative abundance in spring/early summer (28.7%, 30.2%, respectively) than in winter (16.7% and 17.4%, respectively). Overall, the total annual mean *O. davisae* population for Lake Faro (19,141 ± 34,423 ind. m^{-3}) was lower than for Lake Ganzirri (55,420 ± 78,470 ind. m^{-3}), although this difference was not statistically significant (paired Student's t-test, $p = 0.19$; n = 20). The contribution of *O. davisae* to the total mean copepod abundance was also lower for Lake Faro compared to Lake Ganzirri (Figure 7). During the periods of peak abundance of *O. davisae*, its relative abundance in terms of the total copepod densities reached 98.3% and 99.6% for Lakes Faro and Ganzirri, respectively.

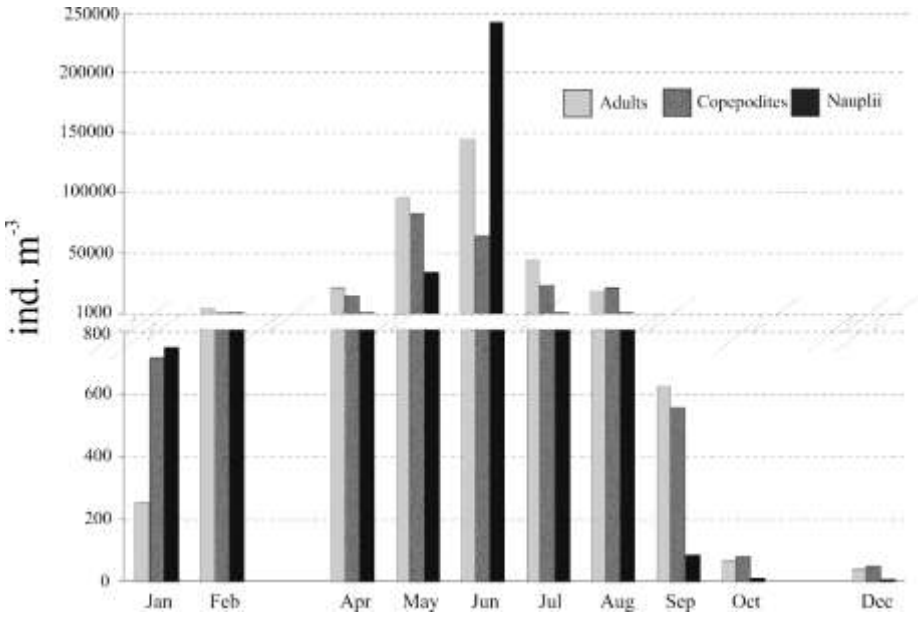

Figure 6. Seasonal variations in the abundance of *Oithona davisae* in Lake Ganzirri.

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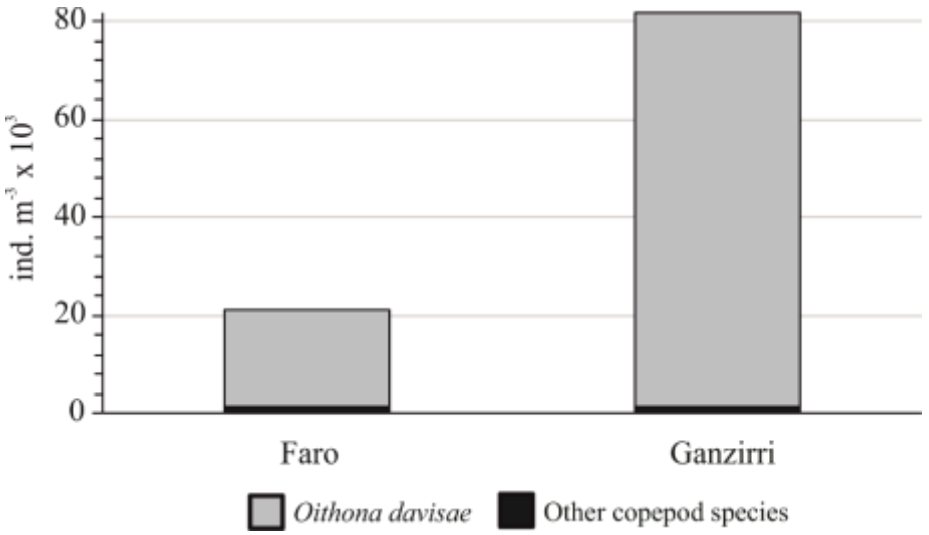

Figure 7. Mean annual contribution of *Oithona davisae* to the total copepod abundance in Lakes Faro and Ganzirri.

CONCLUSION

The passive dispersion of *Oithona davisae* due to synanthropic introduction in many regions of the world has rapidly widened its biogeographical distribution, with a circumglobal, though scattered distribution pattern. *O. davisae* occurs mostly in temperate waters of the boreal hemisphere. It is also found in both cold-temperate latitudes, as the northern Wadden Sea, and austral hemisphere, as southern Pacific Ocean (Chile).

The new records of *O. davisae* in Lakes Faro and Ganzirri confirm that this invasive species is typical of temperate environments. These lakes have physical characteristics similar to those of other locations where this species has been reported. The specimens identified as *Oithona nana* by Zagami and Guglielmo (1995) in these lakes were re-examined and confirmed to be only *O. nana*. In contrast, the specimens identified as *O. nana* and *Oithona brevicornis* by Brugnano (2006); Zagami and Brugnano (2013) and Pansera *et al.* (2014) in Lake Faro showed only a few specimens of *O. nana*, and high numbers of *O. davisae*. All of the *Oithona* specimens collected during the present study were identified as *O. davisae*. It thus appears that *O. davisae* has eliminated *O. nana* in Lakes Faro and Ganzirri.

Differences in abundances between Lakes Faro and Ganzirri can be ascribed to the different sampling methodologies employed: vertical and horizontal tows, respectively. The highest abundances were recorded in the sub-superficial layer (Pansera *et al.* 2014), so the vertical sampling throughout all the water column may have underestimated abundances.

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In Lakes Faro and Ganzirri, the sex ratio of *O. davisae* is female skewed, with values close to those reported in literature (Uye and Sano, 1995; Ceballos and Kiørboe, 2011). Females can reproduce throughout the year, and thus across temperatures ranging from 12.5 to 29.0°C. The high abundance peaks of the adults, copepodites and nauplii, the relatively high proportions females carrying ovisacs, and the occurrence of brooding females and nauplii during winter, all indicate that this invasive species has become a permanent component of Lakes Faro and Ganzirri. As reported elsewhere (Uye and Sano, 1995), the specific egg production rate of *O. davisae* increases linearly with increasing temperature, up to ~22°C, although at temperatures > 22°C this rate no longer increases, and can even decrease.

The density peaks of *O. davisae* in Lakes Faro and Ganzirri were recorded in late spring, when water temperatures were from 23°C to 25°C, in agreement with records from other temperate environments (Uye and Sano, 1998; Bollens *et al.*, 2011; Uriarte *et al*., 2016). These also co-occurred with the seasonal peaks of flagellates, both autotrophic and heterotrophic (Giuffrè and Pezzani, 2005), and ciliates (Saccà and Giuffrè, 2013), so *O. davisae* might have an important role as a predator in the regulation of an eventual dystrophic crisis, due to recurrent summer blooms of flagellates. Conversely, *O. davisae* summer decreases were correlated with temperatures over 25°C.

Before 1992, *O. davisae* was not recorded from Lakes Faro and Ganzirri (Crisafi *et al*., 1973; Zagami and Guglielmo, 1995). However, recent studies on the zooplankton community in Lake Faro in 2004-2005 showed gradual decreases eventually resulting in almost total disappearance of *O. nana*, with progressive replacement by *O. davisae*, although the latter was largely misidentified as *O. brevicornis* (Brugnano, 2006; Zagami and Brugnano, 2013). Therefore, *O. davisae* appears to have penetrated into Lake Faro between 1993 and 2004. However, it might have been present in Lake Faro earlier, but the absence of regular zooplankton monitoring in these lakes does not allow pinpointing the year of its introduction. After a few years of coexistence between *O. nana* and *O. davisae*, the non-indigenous *O. davisae* excluded the indigenous *O. nana*, which has not been reported since 2009 (Pansera *et al*., 2014; Zagami, personal observations). The replacement of *O. nana* by *O. davisae* agrees with reports from other locations (Altukhov *et al*., 2014; Isinibilir *et al*., 2016; Uriarte *et al*., 2015).

In Lakes Faro and Ganzirri, the low resilience of the *O. nana* native population to *O. davisae* invasion might have been caused by short anomalous high peaks in the water temperatures. These exceeded 32°C in both lakes, with Lake Faro also showing upwelling of the bottom anoxic waters during the summer period for some of the years over the last decade. These effects resulted in the death of the entire zooplankton community, and also bivalve cultures (Zagami, personal observations). The resulting temporarily empty niche in the zooplankton community might have favoured *O. davisae* establishment in Lakes Faro and Ganzirri, also enhanced by human activities.

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Coastal estuarine and marine environments are heavily subjected to non-indigenous species invasions. Ecological and evolutionary consequences of invasions can be observed at species, community and ecosystem levels. After its introduction, an invader must either find a niche that is not occupied or it must compete for an occupied niche (Di Castri, 1990). In Lakes Faro and Ganzirri, *O. davisae* has become the most abundant species of the copepod assemblage in a short time. To date, no negative consequences appear to have been reported for the ecosystem functioning of these lakes, apart from the disappearance of the indigenous species *O. nana*. Small proportions of the *O. nana* and *O. davisae* numbers enter the planktonic foodweb as prey for the pelagic fish species *Atherina boyeri*, as seen from gut-content observations (Zagami personal observations), whereas the major proportions of *O. nana* and *O. davisae* are not consumed by pelagic predators, but instead flow into the benthic systems to maintain the microbial loop (Nakamura and Turner, 1997).

Lakes Faro and Ganzirri are shallow-water environments, closed to transoceanic ship traffic. Due to their shallow channels, and the weak *O. davisae* adaptation to the strong currents of the Messina Strait (Mosetti, 1988), it is improbable that this species was introduced by ship ballast waters. Furthermore, *O. davisae* has not been reported in recent samplings of the adjacent Ionian and Tyrrhenian Seas (Zagami, personal observations), and its introduction from adjacent basins is thus highly improbable. In addition, Lake Faro is an important importation centre for living bivalve**s** coming from many Atlantic and Mediterranean sites, thus it is more probable that aquaculture activities are responsible for *O. davisae* introduction. Monitoring of imported molluscs and further samplings in the Messina harbour would provide further evidence in support of this hypothesis. Recently, the identification of new copepod species for Lake Faro (Baviera *et al.*, 2007; Zagami *et al*., 2008; Brugnano *et al*., 2010) and reports of not only plankton species typical of different biogeographic regions (Zagami *et al*., 2005; Cosentino *et al*., 2009; Cosentino and Giacobbe, 2011; Saccà and Giuffrè, 2013; Sabia *et al.* 2014 and 2015) can probably be ascribed to the importation of these molluscs for aquaculture. Indeed the Thau Lagoon, a shallow water system on France's Mediterranean coast, is one of the major hotspots of marine species invasions in the world (Verlaque, 2001), and also an important exportation centre of living bivalves to other aquaculture sites. Consequently, the high numbers of non-indigenous species introduced into Thau Lagoon has a high probability of being transported to other shellfish farming sites in Europe. Thus, it is likely that *O. davisae* was introduced into Lakes Faro and Ganzirri via aquaculture acitivities. The passive dispersion of *O. davisae* by ballast water has already been widely demonstrated (Ferrari and Orsi, 1984; Nishida, 1985; Hirakawa, 1988; Cordell *et al*., 2009; Kaysan, 2010; Lawrence and Cordell, 2010) and the present study represents the first record of the introduction of *O. davisae* through aquaculture.

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Chapter 5

IMPACT OF THE INVASIVE SPECIES *ACARTIA TONSA* ON THE DISTRIBUTION OF AUTOCHTHONOUS ACARTIIDAE SPECIES IN ESTUARIES OF THE BAY OF BISCAY

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ABSTRACT

The impact of the settlement of the invasive copepod species *Acartia tonsa* on the spatial and temporal (seasonal and interannual) distribution of native Acartiidae species in two estuaries of the Bay of Biscay of contrasting morphology, hydrology, and level of pollution, namely the estuaries of Bilbao (polluted) and Urdaibai (weakly polluted), was assessed. Until the arrival of the non-indigenous species *A. tonsa* in 2001-2002 and its occurrence in high density in the inner zone of both estuaries in 2003, *Acartia clausi* dominated the assemblage of Acartiidae species in the estuary of Bilbao, whereas in the estuary of Urdaibai *A. clausi* dominated in the high salinity zone and the brackish species *Acartia bifilosa* in the low salinity zone. The settlement of *A. tonsa* caused (directly or indirectly) the spatial distribution of *A. clausi* to shrink in both estuaries, the latter species being almost driven out from the 30 salinity zone in the estuary of Bilbao. Low oxygen levels in the low salinity zone of the estuary of Bilbao may have helped the settlement of *A. tonsa*, which is more tolerant to hypoxic conditions than *A. clausi*. In the estuary of

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Urdaibai, which has a wider range of salinity habitats to sustain brackish planktonic copepods, the *A. tonsa* population optimum was at a lower salinity than in the estuary of Bilbao, and there was large overlapping of the spatial and temporal distributions of *A. tonsa* and *A. bifilosa*. Competitive pressure of *A. bifilosa* likely restricted the seasonal distribution of *A. tonsa*, and contributed to its decline from 2003 to 2005, a period during which temperature decreased. Conversely, the settlement of *A. tonsa* caused the displacement of the annual maximum of *A. bifilosa* from summer to late spring, as well as its spatial displacement seaward. The impact of *A. tonsa* on other Acartiidae species that are scarce in the estuaries of Bilbao (*Acartia discaudata* and *Acartia margalefi*) and Urdaibai (*Paracartia grani*) could not be clearly assessed. *A. discaudata* and *A. margalefi* almost disappeared when *A. tonsa* settled, but the concomitant deterioration of environmental conditions for the former species argues against the hypothesis of a biotic interaction with *A. tonsa* as the main cause for their decline. In contrast, the summertime species *P. grani* was the only one that showed highest abundance at the same time as *A. tonsa*, likely as a common positive response to temperature increase.

Keywords: *Acartia tonsa*, invasive species, Acartiidae assemblages, estuaries, Bay of Biscay

1. INTRODUCTION

After habitat loss or degradation, biological invasions are the major challenge for the conservation of biodiversity and natural resources (MEA, 2005). Non-indigenous species are able to become invaders because they may displace or replace indigenous species, and affect ecosystem structure and functioning, causing losses of native genotypes, habitat changes, and alterations in community structure, food web properties and ecosystem processes. They can thus prevent the provision of ecosystem services, resulting in negative effects on human health and large economical losses (Grosholz, 2002; Perrings *et al.*, 2002; Wallentinus and Nyberg, 2007; Molnar *et al.*, 2008; Vilà *et al.*, 2010). In a globalized world like today's, the fast increase in trading, travelling and transportation over the last decades has accelerated marine biological invasions, notably in estuaries due to their vulnerability (Nehring, 2006), through maritime transport (*e.g.*, ballast water discharge, biofouling), channel construction for navigation, aquaculture and use of aquariums (Hulme 2009; Katsanevakis *et al.*, 2013).

The Convention on Biological Diversity (CBD, 2000) has recognized the need to compile and spread the information about non-indigenous species which threaten ecosystems, habitats, or native species, in order to use them in a context of prevention and mitigation actions. CBD (2000) has also issued a call to intensify research on the

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impact of non-indigenous species on biological diversity. In the case of the European Union, the Marine Strategy Framework Directive (EU, 2008) considers non-indigenous species as the main threat to biodiversity and ecosystems health in Europe.

Acartia tonsa is among the listed non-indigenous species that can become invasive in European marine systems and can have a high impact on ecosystems services or diversity, although both positive and negative impacts have been attributed to this species (Katsanevakis *et al.*, 2014). *A. tonsa* competes with native copepods, especially congenerics, and may become dominant in zooplankton communities. It may also modify food webs and trophic flows within invaded ecosystems (Katsanevakis *et al.*, 2014), as it can be a significant prey for pelagic fish (Detwyler and Houde, 1970) and it can control algal blooms (Leppäkoski *et al.*, 2002).

This species has become widespread across the world, but it is reported to be native to American and Indo-Pacific waters (Leppäkoski and Olenin, 2000). *A. tonsa* has been colonising new coastal areas and estuaries by self-propagation and/or anthropogenic introduction, due mainly to its ability to cross geographic barriers owing to its capacity to develop resistance stages (Belmonte and Potenza, 2001) and to tolerate wide ranges of environmental factors (Holste and Peck, 2006). This species has become a well-known non-indigenous species in European waters since the early 1900s (Brylinski, 1981).

In the Bay of Biscay, *A. tonsa* has been reported since 1983 in the Gironde estuary (David *et al.*, 2007), the largest estuary flowing into this bay. In the small estuaries of the Basque coast, however, it was detected for the first time only in 2001 in the estuary of Bilbao, where it has become the dominant zooplankton calanoid in the inner estuary since 2003 (Aravena *et al.*, 2009). *A. tonsa* was observed for the first time in the nearby estuary of Urdaibai later than in the estuary of Bilbao, in 2003 (Aravena, 2009). In addition, *A. tonsa* has shared from the beginning the occupation of the upper reaches of both estuaries with *Oithona davisae*, a typical coastal and estuarine species that has been found to be a rapid invader of transitional environments in the Mediterranean (Zagami *et al.*, 2018), and since 2010 with *Pseudodiaptomus marinus* in the estuary of Bilbao (Uriarte *et al.*, 2016). The last two non-indigenous species are both native to the Indo-Pacific region (see Mihneva and Stefanova, 2013; Sabia *et al.*, 2015).

In this chapter we aim to show the impact of the arrival of the non-indigenous *A. tonsa* in the estuaries of Bilbao and Urdaibai on the spatial and temporal distribution of the other Acartiidae species inhabiting these areas. Since these two estuaries differ largely in morphological and hydrological properties, as well as in the level of human pressure and system health, we have also dealt with the role of system inherent characteristics in constraining or enhancing the impact of the non-indigenous species on co-occurring Acartiidae species.

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2. MATERIAL AND METHODS

2.1. Study Area

The estuary of Bilbao (also known as Ibaizabal-Nerbioi estuary or Nervión estuary; 43º23'N, 03º07'W) and the estuary of Urdaibai (also known as Gernika estuary, Mundaka estuary or Oka estuary; 43º22'N, 02º43'W) are located in the Basque coast (inner Bay of Biscay) (Figure 1). Because of their proximity to each other, they share a temperate-oceanic climate. However, they differ largely in geomorphology, hydrodynamic characteristics, and level of anthropogenic impact, which result in differences in their water environments (Iriarte *et al.*, 2016).

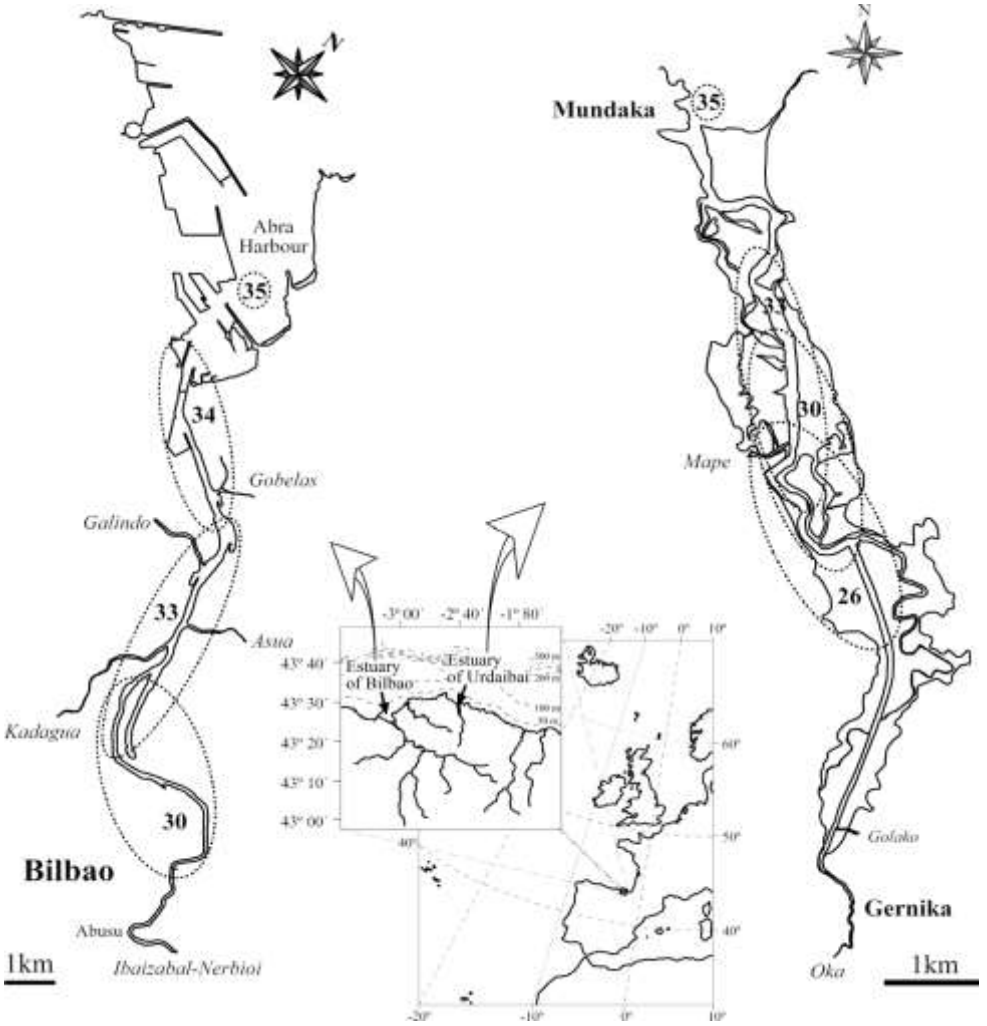

Figure 1. Map and location of the estuaries of Bilbao and Urdaibai, showing the 26, 30, 33, 34 and 35 salinity zones where samplings were carried out in each estuary.

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2.1.1. Estuary of Bilbao

The estuary of Bilbao has two clearly different areas: a funnel-like harbour (23 km long, 3.8 km wide (on average) and 10-25 m deep) called Abra, and a man-made channel (15 km long, 50-150 m wide and 2–9 m deep) that extends from this harbour area up to the Ibaizabal river. The estuary is partially mixed in the outer area and highly stratified in the inner area. High salinity waters (> 30) usually penetrate up to the inner reaches at the bottom, whilst freshwater flows seaward at surface and is progressively mixed with seawater. This results in a two-layer circulation with landward net flux in bottom layers and seaward net flux in surface layers, and much higher residence time in waters below the halocline (3-12.6 days) than in waters above the halocline (0.5-3 days) (Uriarte *et al.*, 2014). The original features of the estuary were dramatically modified due to (i) land reclamation for urban, industrial and port developments, and (ii) channelization and dredging works to make navigation easier. As a result of these, the estuary has lost most of its original intertidal areas (Cearreta *et al.*, 2004). By the 1970s, the channelled area was extremely polluted with high heavy metal and organic matter concentrations, and hypoxic/anoxic conditions in waters and sediments (Cearreta *et al.*, 2000), which gave rise to extensive benthic areas devoid of fauna (González-Oreja and Saiz-Salinas, 1998). Since 1979 the estuary is in a rehabilitation process, as a consequence of the industrial decline in the area surrounding the estuary, the treatment of wastewaters from the Metropolitan Area of Bilbao and new environmental protection policies, resulting in a significant decrease in heavy metal, ammonia and organic matter loadings, and an increase in oxygenation and biodiversity (García-Barcina *et al.*, 2006; Borja *et al.*, 2010; Pascual *et al.*, 2012; Villate *et al.*, 2013).

The first plankton studies in this system, carried out around 1980, were restricted to the Abra harbour, where both phytoplankton biomass and neritic zooplankton abundance where shown to strongly decrease toward the inner-eastern zone due to the effect of the polluted estuarine plume coming from the channel mouth (Villate, 1991a and 1994). In these studies, *Acartia clausi* was the only species of Acartiidae found. Later studies initiated in 1996 also included the channelled zone and reported the occurrence in low numbers of two more Acartiidae species *i.e.*, *Acartia margalefi* and *Acartia discaudata*, which were observed landward from the area occupied by *A. clausi* (Uriarte, 2001).

2.1.2. Estuary of Urdaibai

The estuary of Urdaibai, with a maximum and minimum width of 1.2 km and < 20 m in the outer area and the inner channel respectively, is shorter (12.5 km), shallower (mean depth of 3 m) and physically less modified (*e.g.*, less channelized, with less land reclamation) than the estuary of Bilbao. Reed beds and salt marshes at its upper and middle reaches border the central channel, and relatively extensive intertidal flats of mainly muddy (in the inner part) and sandy (in the outer part) sediments occupy the outer half of the estuary. The watershed area is small and river inputs are usually low when

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compared to the tidal prism. As a consequence, most of the estuary is seawater-dominated at high tide, while a stronger axial gradient of salinity is only found in the upper reaches, where it receives freshwater inputs from its main tributary, the Oka river (Villate *et al.*, 2008). In the outer zone, tidal flushing is so high that waters of salinities > 34 are flushed out of the estuary at each tidal cycle. The outer half of the estuary remains well mixed most of the time, while the inner half is partially stratified. In the upper reaches, the estuary receives relatively large amounts of nutrients and organic matter from an old primary waste water treatment plant (Franco *et al.*, 2004). Nevertheless, this estuary is among the healthiest coastal systems of the Basque coast, and constitutes the most valuable natural resource of the Biosphere Reserve of Urdaibai.

The first plankton studies in this system, in the early 1980s, showed the presence of three Acartiidae species segregated along the salinity gradient: the neritic *A. clausi*, dominant in the highest salinity waters; the brackish *Acartia bifilosa*, dominant in < 33 salinity waters; and *Paracartia grani* (*Acartia grani* in previous publications), which was less abundant than the former two species, and occurred in an intermediate spatial position (Villate, 1991b and 1995). The abundance of the latter species has been shown to be much lower since 1996 when studies on zooplankton were resumed (Uriarte and Villate, 2005).

2.2. Data Source

Zooplankton and environmental data shown in this chapter come from an ongoing monitoring program carried out in the estuaries of Bilbao and Urdaibai since 1997, and correspond to the time-series of the period 1998–2005, for which zooplankton identification and counting has been completed for all salinity sites in both estuaries at present. Data were obtained from monthly samplings (usually during the last week of the month) conducted at high tide during neap tides at the 35, 34, 33 and 30 salinity zones of the estuary of Bilbao, and at the 35, 33, 30 and 26 salinity zones of the estuary of Urdaibai (Figure 1), using a Lagrangian type of sampling strategy. This consisted in obtaining the biological and environmental information in water masses of a given salinity instead of at spatially fixed sampling sites (see Kimmerer *et al.*, 1998; Modéran *et al.*, 2010). The salinity zones were selected according to previous studies (*e.g.*, Villate, 1991b; Uriarte, 2001), which allowed to define the main salinity habitats within each estuary on the basis of the distribution of main zooplankton species. Water masses of around 30 salinity constitute the innermost salinity habitat occupied by mesozooplankton copepods in the estuary of Bilbao, while water masses of around 26 salinity represent the optimal inner limit for brackish mesozooplankton copepods in the estuary of Urdaibai (Villate *et al.*, 1993).

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Zooplankton samples were obtained from below the halocline by 2–3 min. horizontal tows using a 200 µm mesh size net (mouth diameter 0.25 m) equipped with a Digital Flowmeter, and preserved in 4% buffered formalin. Zooplankton counting and identification to the lowest possible taxonomic level was made under a stereomicroscope Olympus IX70. In Acartiidae species, female, male and copepodite stages were distinguished, but for the purposes of the chapter only the total abundance of each species was taken into account. Vertical profiles of salinity, water temperature and dissolved oxygen saturation (DOS) were measured *in situ* using a WTW LF 197 thermosalinometer and a YSI 55 oxymeter, but only data from the depth of zooplankton sampling are presented in this chapter. Water samples were collected also at the depth of zooplankton sampling using a Niskin bottle for measuring chlorophyll *a* (Chl *a*), which was determined spectrophotometrically in triplicate samples according to the monochromatic method with acidification (Jeffrey and Mantoura, 1997). Secchi disk depths (SDDs) were also recorded to assess water turbidity at each sampling site.

Salinity stratification was calculated from salinity profiles obtained at each sampling site in each sampling day as the maximum difference in salinity at 0.5 m depth intervals in the water column (Δ salinity). This calculation has been put forward as an index to reflect the sharpening of the salinity gradient associated to the narrowing of the halocline layer, and was found to explain dissolved oxygen saturation dynamics below the halocline in the estuary of Bilbao better than a stratification index calculated from the difference between bottom and surface salinities (Villate *et al.*, 2013).

River flow data for the Ibaizabal-Nerbioi river (main tributary of the estuary of Bilbao) and for the Oka river (main tributary of the estuary of Urdaibai) were obtained from the Provincial Council of Bizkaia. In the case of the Oka river, missing values were filled with values obtained using a regression model ($y = 0.3892x$, $R^2 = 0.9333$) performed with data from the nearest station (Oleta (LE02) hydro-meteorological station) with a complete series. Daily values of river flow were averaged for each month.

2.3. Data Treatment

The impact of *A. tonsa* and the effect of environmental variables on Acartiidae species were assessed. To that purpose, the spatial (in relation to salinity) and temporal (seasonal and interannual) variations of abundance of all Acartiidae species, together with the niche breadth and overlap based on distribution of the dominant species (*A. clausi*, *A. bifilosa* and *A. tonsa*) were compared for three periods in the 1998-2005 data time series. The first two periods correspond to years prior to the large increase of *A. tonsa* abundance, but with different environmental conditions *i.e.*, the 1998-2000 and the 2001-2002 periods. The third period extends from 2003 to 2005 and was characterized by the dominance of *A. tonsa* in the low salinity waters of both estuaries at least during one

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month of the year. Prior to data analysis, Acartiidae species abundances were log-transformed (log (x + 1)).

Principal components analysis (PCA) was used to assess the influence of environmental factors on spatial and seasonal changes in Acartiidae species abundance. The eigenvectors allowed identification of the variables contributing most to the principal components of environmental variability. The three first principal components were used to describe the main environmental gradients in each estuary. The plot of Acartiidae species within the space configured by such environmental gradients was used to describe the segregation of Acartiidae populations along environmental gradients and determine the ecological niche of these species in each estuary.

Data normality was tested by means of the Kolmogorov-Smirnov test. Data comparisons were carried out by Kruskal-Wallis and Mann-Whitney U tests. Relationships between variables were assessed by Spearman rank order correlations and regressions (linear and quadratic).

Spatial (salinity sites) and seasonal (monthly) uniformity of distribution of individuals of the dominant Acartiidae species for each time period was estimated by Levins' standardized niche breadth (B_A), suggested by Hurlbert (1978):

$$B_A = \frac{B-1}{n-1} \qquad \text{being} \quad B = \frac{1}{\sum p_j^2} \tag{1}$$

where:

B_A = Levins' standardized niche breadth
n = number of possible resource states
B = Levins' measure of niche breadth
p_j = proportion of individuals found in or using resource state j

Additionally, to measure spatial (salinity sites) and seasonal (monthly) niche overlap between *A. tonsa* and *A. clausi* in the estuary of Bilbao, and *A. tonsa*, *A. clausi* and *A. bifilosa* in the estuary of Urdaibai for each time period, the simplified Morisita index proposed by Horn (1966) was calculated:

$$C_H = \frac{2\sum_i^n p_j p_k}{\sum_i^n p_j^2 + \sum_i^n p_k^2} \tag{2}$$

where:

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C_H = simplified Morisita index of overlap between species j and species k
p_{ij} = proportion of individuals *i* of species *j* found in resource state
p_{ik} = proportion of individuals *i* of species *k* found in resource state
n = total number of resource states

3. RESULTS

3.1. Environmental Conditions

Table 1 summarizes the abiotic and biotic data considered to describe and compare the environmental conditions in the estuaries of Bilbao and Urdaibai during the 1998-2005 study period. None of the variables showed normal distribution (Kolmogorov-Smirnov; $p < 0.001$).

The variations of river flow and salinity stratification index were similar in both estuaries (Figure 2), showing the typical seasonal patterns of river flow and stratification, with highest values in late autumn-winter and lowest values in summer. However, a clear anomaly in the autumn-winter period of 2001-2002 occurred, when river flow and salinity stratification were unusually low. In addition, summer river discharge and stratification were higher (Kruskal-Wallis test; $p = 0.005$) in 2002 than in the other years of the series. Between-estuary differences were evident in terms of fresh water discharges into the estuary and degree of stratification, both being much higher in the estuary of Bilbao than in Urdaibai (Table 1) due to the characteristics of the tributaries (much extensive watershed in the estuary of Bilbao) and morphological features of the estuarine basin (deeper and channelized in the estuary of Bilbao).

Table 1. Mean (± standard deviation, SD), minimum and maximum values of environmental variables obtained for the estuaries of Bilbao and Urdaibai during 1998-2005. *P*-values of Mann-Whitney U tests for between-estuary differences are also shown

	Estuary of Bilbao			Estuary of Urdaibai			
	Mean ± SD	Min.	Max.	Mean ± SD	Min.	Max.	*p*
River flow ($m^3 s^{-1}$)	21.48 ± 19.83	2.57	74.56	0.64 ± 0.55	0.06	2.08	< 0.001
Stratification (Δ salinity)	7.67 ± 5.92	0.10	31.90	2.28 ± 3.55	0.00	20.50	< 0.001
Salinity	32.92 ± 1.84	28.10	35.50	30.99 ± 3.40	23.30	35.60	< 0.001
Temperature (ºC)	16.08 ± 3.32	10.20	24.20	16.34 ± 4.36	8.00	26.30	0.947
DOS (%)	72.01 ± 31.33	0.30	152.90	86.95 ± 16.93	29.80	137.60	< 0.001
SDD (m)	1.79 ± 1.45	0.20	11.00	3.10 ± 2.56	0.20	8.00	< 0.001
Chl *a* (μg l^{-1})	3.02 ± 4.53	0.00	31.33	2.53 ± 3.59	0.13	25.78	0.583

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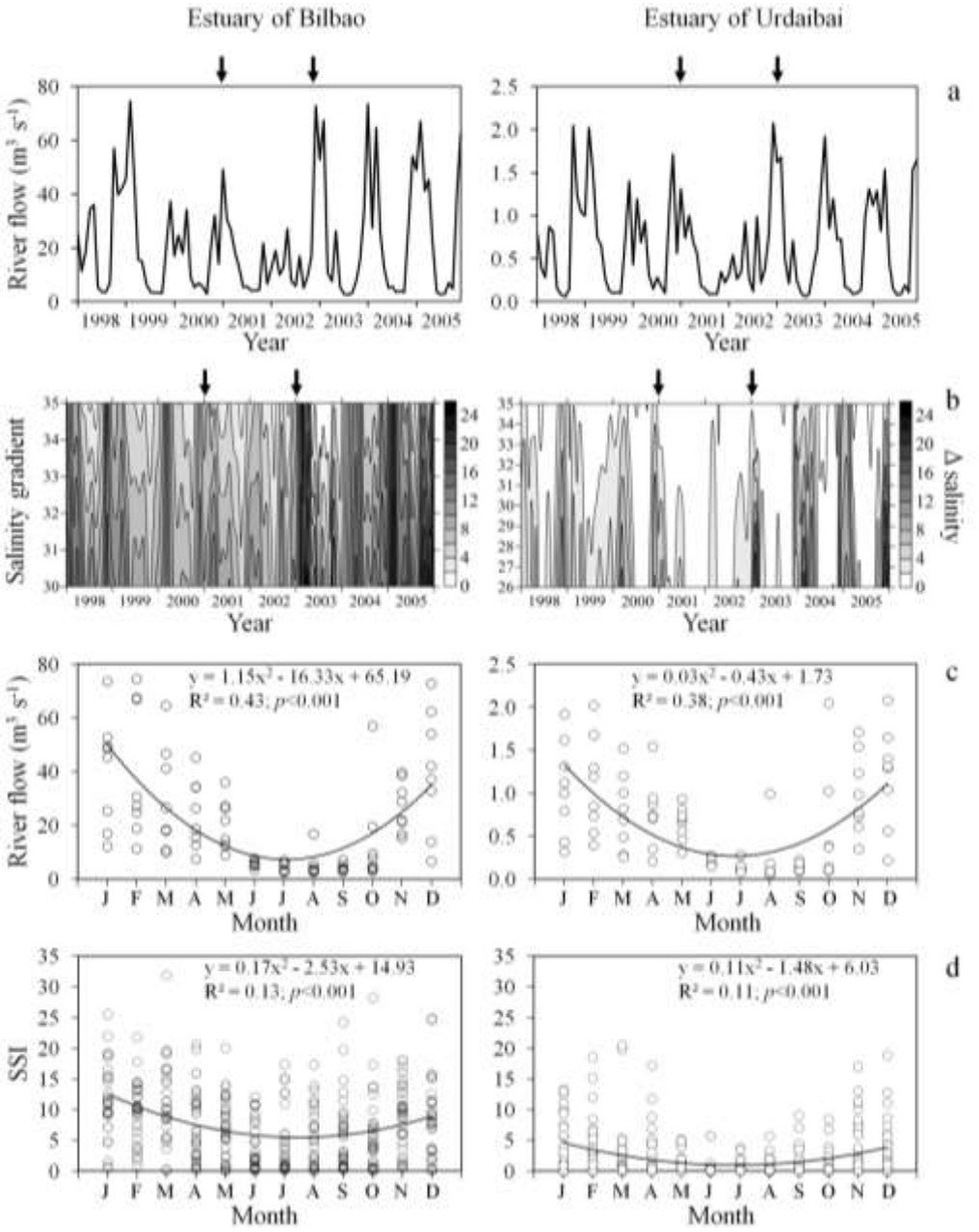

Figure 2. (a) Temporal variations of the monthly mean river flow ($m^3 s^{-1}$) of the main tributaries of the estuary of Bilbao (Ibaizabal-Nerbioi river) and Urdaibai (Oka river), and (b) spatiotemporal variations of the salinity stratification (Δ salinity) along the salinity gradient of the estuaries of Bilbao and Urdaibai, for the 1998-2005 period. Arrows indicate the division between the three periods distinguished in the series. (c) Seasonal pattern of the river flow ($m^3 s^{-1}$) of the main tributaries of the estuary of Bilbao (Ibaizabal-Nerbioi river) and Urdaibai (Oka river) and (d) seasonal pattern of the salinity stratification index (SSI) in the estuaries of Bilbao and Urdaibai. Results of the quadratic regressions are showed.

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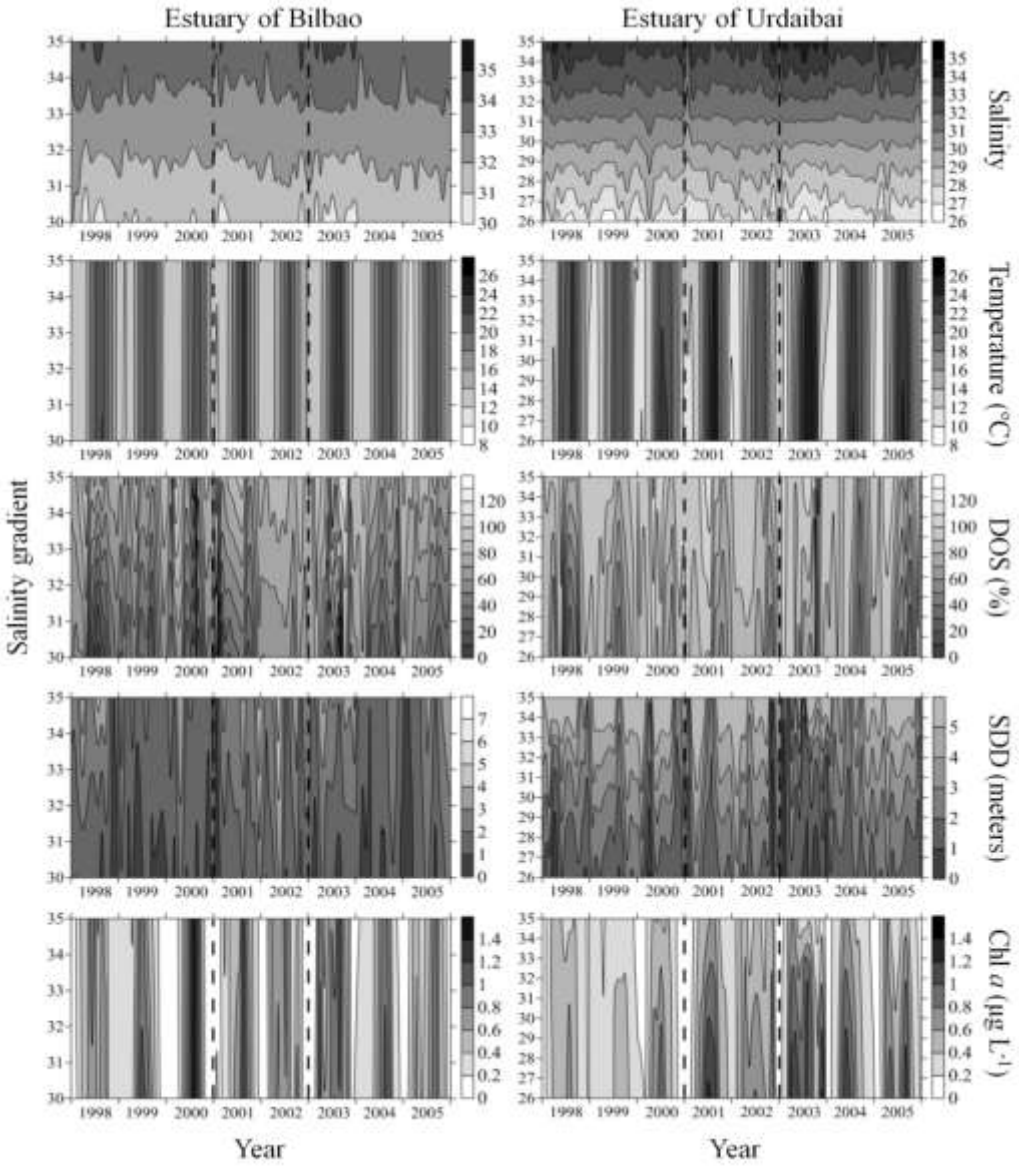

Figure 3. Spatiotemporal variations of salinity, temperature (ºC), dissolved oxygen saturation (DOS; %), Secchi disk depth (SDD; meters) and concentration of chlorophyll *a* (µg L^{-1}) along the salinity gradient of the estuaries of Bilbao and Urdaibai for the 1998-2005 period. Vertical dashed lines indicate the limits between the three periods distinguished in the series.

Figure 3 shows the spatiotemporal variation of abiotic (salinity, temperature, DOS and SDD) factors and chlorophyll *a* (a variable that indicates food availability for zooplankton) in the two estuaries during the period 1998-2005. Salinity values were almost constant at each sampling site over the study period as a result of the Lagrangian-type of sampling method. Salinity ranged from 35.5 at the 35 salinity site to 28.1 at the 30 salinity site in the estuary of Bilbao, and from 35.6 at the 35 salinity site to 23.3 at the 26 salinity site in the estuary of Urdaibai. Temperature showed the typical seasonal pattern

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in temperate areas with summer maxima and winter minima (Figure 3 and Table 1), but with a wider range of variation in low salinity waters (14.1ºC and 18.3ºC in the estuary of Bilbao and Urdaibai, respectively) than in high salinity ones (11.2ºC and 14.3ºC in the estuary of Bilbao and Urdaibai, respectively). Between-year differences were similar in both estuaries. In the period 1998-2000 temperature showed smaller differences between years (range of variation – maximum temperatures: 0.7°C and 1.6°C; minimum temperatures: 1.2°C and 1.6°C, in the estuaries of Bilbao and Urdaibai, respectively) than in the second period (range of variation – maximum temperatures: 2.1°C and 4.2°C; minimum temperatures: 1.7°C and 3.6°C, in the estuaries of Bilbao and Urdaibai, respectively), the latter being characterized by an increase of summer temperatures in 2001 followed by a decrease in 2002. The highest summer temperatures of the series were found in the last period, with maximum values of 24.2°C and 26.3°C in the estuaries of Bilbao and Urdaibai, respectively, in 2003. DOS showed higher values and lower differences between salinities in the estuary of Urdaibai (with a range from 29.8% to 137.6%) than in the estuary of Bilbao (with a range from 0.3% to 152.9%). DOS values were more homogenous in the estuary of Urdaibai than in the estuary of Bilbao. Hypoxic conditions (< 30% of DOS) were frequent during the warm season in the 30 salinity site of the estuary of Bilbao in the first (1998-2000) and last (2003-2005) periods. In the middle period, at the lowest salinity site of this estuary the highest values of DOS were observed in 2002. Turbidity increased, in general, with decreasing salinity in both estuaries (Spearman rank correlation: $R = -0.603$, $p < 0.001$ in the estuary of Bilbao and $R = -0.698$, $p < 0.001$ in the estuary of Urdaibai), but was clearly higher in the estuary of Bilbao (Table 1). Chlorophyll *a* showed a clear decreasing trend with increasing salinity in the estuary of Urdaibai (Spearman rank correlation: $R = -0.309$, $p < 0.001$), whereas in the estuary of Bilbao its distribution was rather uniform along the salinity gradient (Spearman rank correlation: $R = -0.094$, $p = 0.065$) and showed seasonally well defined cycles with maxima in summer. In addition, chlorophyll *a* fluctuated from year to year without a clear trend in the estuary of Bilbao ($R^2 = 5 \times 10^{-5}$, $p = 0.881$), but showed a slight but significant increasing trend over the study period in the estuary of Urdaibai ($R^2 = 0.029$, $p = 0.001$).

3.2. Spatial and Temporal Distribution of Acartiidae Species

The densities of Acartiidae species (*Acartia clausi*, *Acartia discaudata*, *Acartia margalefi*, *Paracartia grani*, *Acartia bifilosa* and *Acartia tonsa*) in the estuaries of Bilbao and Urdaibai in each salinity zone and in the three periods distinguished in the time series are summarized in Table 2 and Table 3, respectively.

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Table 2. Mean ± standard deviation of the density (ind. m^{-3}) of the Acartiidae species at the 35, 34, 33 and 30 salinity sites in the estuary of Bilbao and the 35, 33, 30 and 26 salinity sites in the estuary of Urdaibai

			Estuary of Bilbao	
Species	35	34	33	30
A. clausi	793.7 ± 1267.5	777.6 ± 1563.4	213.6 ± 539.4	50.4 ± 120.1
A. discaudata	3.1 ± 9.2	9.2 ± 32.3	3.7 ± 12.9	0.6 ± 2.3
A. margalefi	0.5 ± 1.9	5.8 ± 20.1	4.4 ± 13.1	3.1 ± 12.1
P. grani	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
A. bifilosa	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
A. tonsa	64.9 ± 617.7	29.5 ± 158.5	318.8 ± 1649.3	618.2 ± 2379.4
			Estuary of Urdaibai	
Species	35	33	30	26
A. clausi	684.4 ± 1571.2	372.9 ± 2488.7	47.2 ± 103.6	106.5 ± 891.1
A. discaudata	0.3 ± 2.3	0.1 ± 1.0	0.1 ± 1.1	0.03 ± 0.3
A. margalefi	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. grani	0.03 ± 0.3	4.5 ± 28.6	6.3 ± 46.9	0.2 ± 2.1
A. bifilosa	4.2 ± 20.4	258.5 ± 743.6	1541.1 ± 6392.2	1380.0 ± 3175.9
A. tonsa	0.0 ± 0.0	37.3 ± 159.5	131.4 ± 778.1	1251.5 ± 10289.8

Table 3. Mean ± standard deviation of the density (ind. m^{-3}) of the Acartiidae species of the estuaries of Bilbao and Urdaibai for each of the three periods distinguished in the time series

		Estuary of Bilbao	
Species	1998-2000	2001-2002	2003-2005
A. clausi	442.6 ± 1159.9	569.6 ± 1290.1	392.5 ± 834.0
A. discaudata	3.8 ± 22.1	6.9 ± 18.9	2.5 ± 12.0
A. margalefi	2.9 ± 15.2	8.3 ± 18.4	0.7 ± 3.4
P. grani	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
A. bifilosa	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
A. tonsa	0.0 ± 0.0	0.9 ± 4.4	700.1 ± 2407.7
		Estuary of Urdaibai	
Species	1998-2000	2001-2002	2003-2005
A. clausi	343.7 ± 2159.7	322.9 ± 1325.5	248.4 ± 789.6
A. discaudata	0.1 ± 0.9	0.2 ± 2.1	0.1 ± 1.1
A. margalefi	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P. grani	0.03 ± 0.3	0.03 ± 0.02	7.4 ± 44.7
A. bifilosa	1335.3 ± 5651.1	137.8 ± 544.8	695.4 ± 1644.4
A. tonsa	0.0 ± 0.0	0.0 ± 0.0	946.8 ± 8421.4

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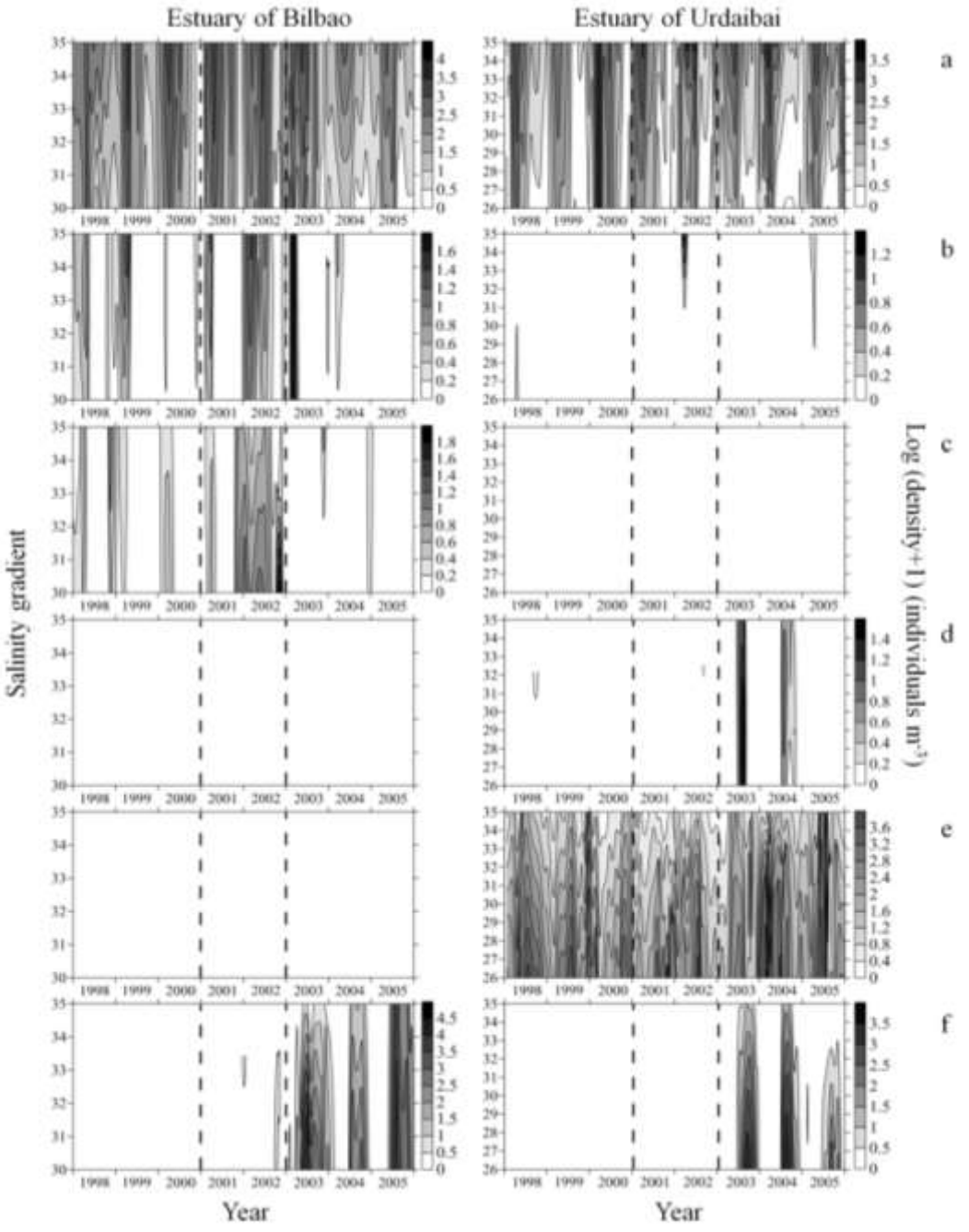

Figure 4. Spatiotemporal distribution of the density (log (x + 1), individuals m^{-3}) of (a) *Acartia clausi*, (b) *Acartia discaudata*, (c) *Acartia margalefi*, (d) *Paracartia grani*, (e) *Acartia bifilosa* and (f) *Acartia tonsa* along the salinity gradient of the estuaries of Bilbao and Urdaibai from 1998 to 2005. Vertical dashed lines indicate the division between the three periods distinguished in the time series.

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The dominant neritic species *A. clausi* showed a decrease of abundance with decreasing salinity in both estuaries (Spearman rank correlation: R = 0.425, $p < 0.001$ in the estuary of Bilbao and R = 0.410, $p < 0.001$ in the estuary of Urdaibai) (Figures 4 and 5). In addition, the seasonal cycle of this species showed the annual maximum earlier (in March) and was more constrained to the spring period in the estuary of Urdaibai than in the estuary of Bilbao, where it peaked between April (during the first and the third period) and June (in the second period) (Figure 6). Throughout the study period, *A. clausi* fluctuated from year to year without a clear temporal trend until 2003 in both estuaries, but showed a clear decrease during the last period (2003-2005) in waters of salinities below 35 (Kruskal-Wallis test; $p < 0.001$, for both estuaries), this decrease being more evident at the lowest salinity zone of the estuary of Bilbao (Figure 4).

A. discaudata and *A. margalefi* appeared in low abundances in the estuary of Bilbao during the first period (1998-2000). They reached highest densities, mainly *A. margalefi*, during the second period, in 2002, and were rarely observed since 2003 (Figure 4). In most of the years, *A. discaudata* and *A. margalefi* showed the highest density in late winter-early spring (February-March), although the seasonal distribution of both species was extended throughout the year during the second period, in 2002, when secondary peaks in early summer or autumn were also observed (Figure 6). Spatially, both species peaked at lower salinities than *A. clausi*, but the distribution of *A. discaudata* in the estuary skewed toward high salinity, whereas that of *A. margalefi* skewed toward low salinity (Figures 4 and 5). *A. discaudata* was also found in the estuary of Urdaibai, but only in one occasion during each period of the series (Figure 4).

P. grani was only recorded in the estuary of Urdaibai: in the first two periods it was found occasionally, but in the last period it was found in relatively high abundance in 2003 and in much lower abundance in 2004 (Figure 4). Seasonally, the occurrence of *P. grani* was restricted to the warmest period, mainly to July and August (Figure 6), showing a spatial preference for intermediate salinity (33-30) sites (Figure 5).

The brackish species *A. bifilosa* was only observed in the estuary of Urdaibai, where it dominated the assemblage of Acartiidae species in low salinity waters (Figures 4 and 5). *A. bifilosa* showed a rather variable seasonal pattern during the period of study (Figure 6) and marked interannual variations, with lowest abundances and atypical seasonal distributions in the second period (Figure 4). The seasonal increase and annual maximum were found to occur earlier in the last period, from 2003 to 2005.

A. tonsa was recorded for the first time in autumn 2001 in the estuary of Bilbao, and from 2003 onward it became very abundant in this system (Figure 4). In the estuary of Urdaibai it also occurred in high density in 2003, but in contrast with observations in the estuary of Bilbao it showed a marked decrease in 2005. The population of this species decreased with increasing salinity in both systems (Spearman rank correlation: R = -0.368, $p < 0.001$ in the estuary of Bilbao and R = -0.294, $p < 0.001$ in the estuary of Urdaibai), where the highest densities were observed at the lowest salinity sites of 30 and

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26 in the estuaries of Bilbao and Urdaibai, respectively (Figure 5). Seasonally, *A. tonsa* showed maxima in the warm period, but with a wider seasonal distribution in the estuary of Bilbao (Figure 6).

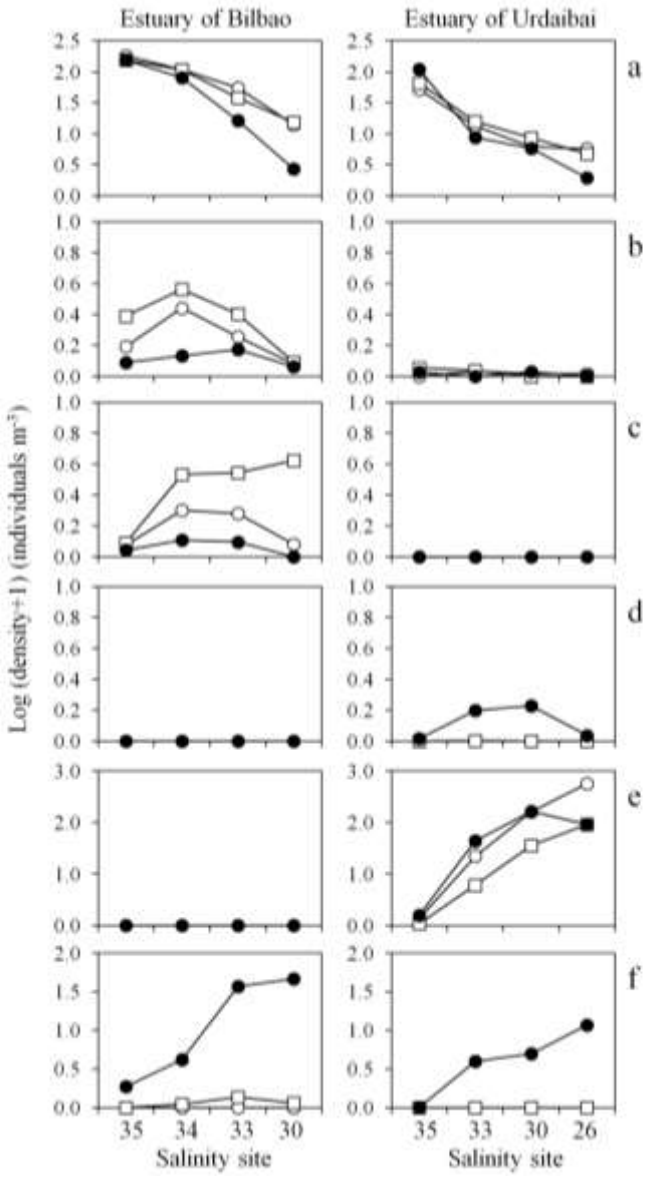

Figure 5. Mean density (log (x + 1), individuals m^{-3}) values of (a) *Acartia clausi*, (b) *Acartia discaudata*, (c) *Acartia margalefi*, (d) *Paracartia grani*, (e) *Acartia bifilosa* and (f) *Acartia tonsa* at the 35, 34, 33 and 30 salinity sites in the estuary of Bilbao and the 35, 33, 30 and 26 salinity sites in the estuary of Urdaibai for the three different periods of the series: 1998-2000 (open circles), 2001-2002 (open squares) and 2003-2005 (full circles).

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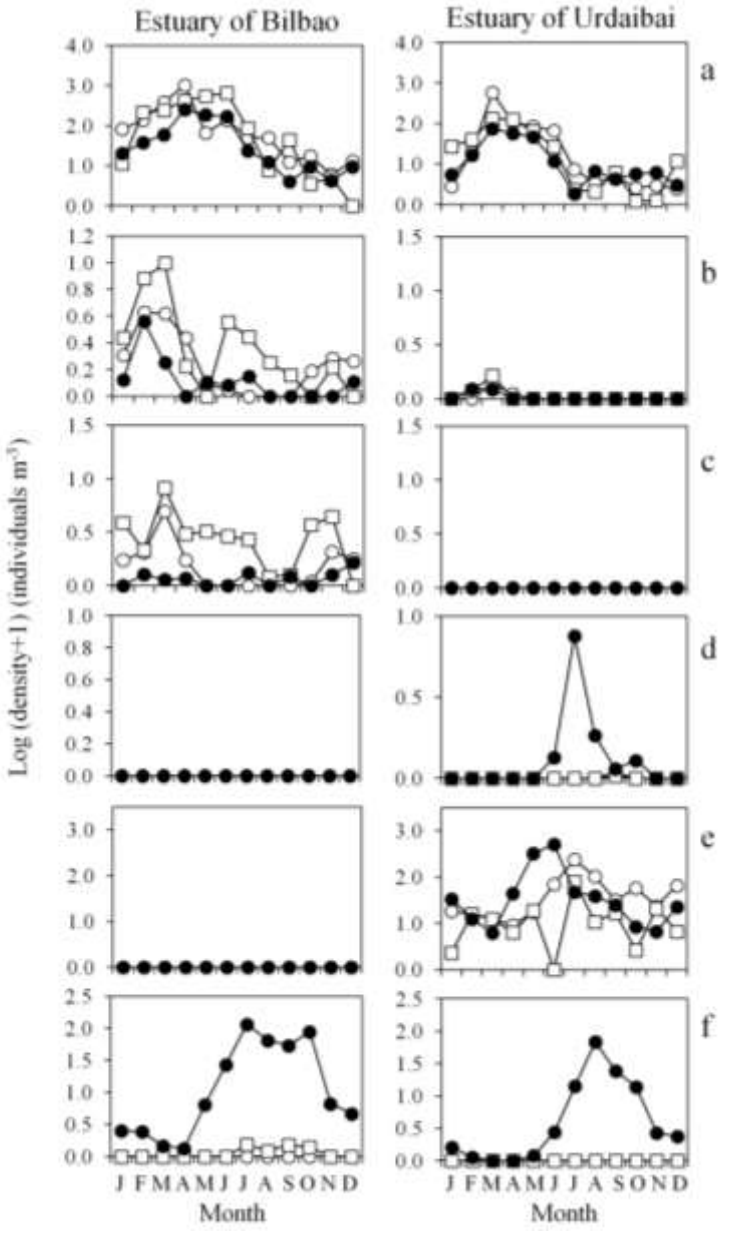

Figure 6. Monthly mean density (log (x + 1), individuals m^{-3}) values of (a) *Acartia clausi*, (b) *Acartia discaudata*, (c) *Acartia margalefi*, (d) *Paracartia grani*, (e) *Acartia bifilosa* and (f) *Acartia tonsa* in the estuaries of Bilbao and Urdaibai for the three different periods of the series: 1998-2000 (open circles), 2001-2002 (open squares) and 2003-2005 (full circles).

3.3. Species Segregation in Environmental Gradients

Three main modes of environmental variation were extracted by the PCA of environmental variables in each estuary (Table 4). The first one was mainly explained by

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salinity, DOS and SDD in opposition to stratification in the estuary of Bilbao (37.07% of variance), and by salinity and SDD in opposition to stratification in the estuary of Urdaibai (30.85% of variance), reflecting the spatial gradient of salinity in both estuaries. The second one was mainly explained by river flow in opposition to temperature in the estuary of Bilbao (27.51% of variance) and by temperature and chlorophyll *a* in the estuary of Urdaibai (22.35% of variance), while the third one was mainly explained by chlorophyll *a* in the estuary of Bilbao (14.94% of variance) and by river flow in opposition to DOS in the estuary of Urdaibai (17.38% of variance). Both the second and the third factors reflected temporal environmental variations.

The plot of species abundance in the space configured by the three main factors of environmental variability in the two estuaries showed the spatial and temporal segregation pattern of Acartiidae species along environmental gradients (Figure 7). In the estuary of Bilbao, the segregation of species along the spatial gradient determined by salinity, DOS, stratification and turbidity showed the succession *A. clausi – A. discaudata – A. margalefi – A. tonsa* from the outer to the inner estuary; however, *A. clausi* and *A. tonsa* showed a wider distribution along this gradient than *A. discaudata* and *A. margalefi*. Along the seasonal gradient related to river flow and temperature, *A. clausi*, *A. discaudata* and *A. margalefi* were all similarly positioned under conditions of higher river flow and lower temperature than *A. tonsa*, which was more restricted to low river flow and high temperature conditions. By contrast, *A. clausi* showed a wider distribution along this environmental gradient than the other three species. Similarly, *A. clausi* had a wider distribution than the other congeneric species along the trophic gradient accounted for by chlorophyll *a* concentration.

Table 4. Loadings of environmental variables for the three main rotated components extracted by PCA in the estuaries of Bilbao and Urdaibai. Percentage of variance explained by each component is shown in parentheses

	Estuary of Bilbao			**Estuary of Urdaibai**		
	Component			Component		
	1 (37.07%)	2 (27.51%)	3 (14.94%)	1 (30.85%)	2 (22.35%)	3 (17.38%)
River flow (m^3 s^{-1})	-0.049	**0.896**	-0.080	0.022	-0.225	**0.828**
Stratification (Δ salinity)	**-0.737**	0.431	-0.058	**-0.676**	-0.291	0.165
Salinity	**0.895**	0.041	-0.042	**0.874**	-0.143	-0.035
Temperature (°C)	0.004	**-0.830**	0.363	0.140	**0.919**	0.081
DOS (%)	**0.895**	0.238	0.151	0.369	-0.348	**-0.668**
SDD (m)	**0.669**	-0.263	-0.355	**0.811**	-0.180	-0.059
Chl *a* (µg l^{-1})	0.005	-0.349	**0.868**	-0.353	**0.640**	-0.214

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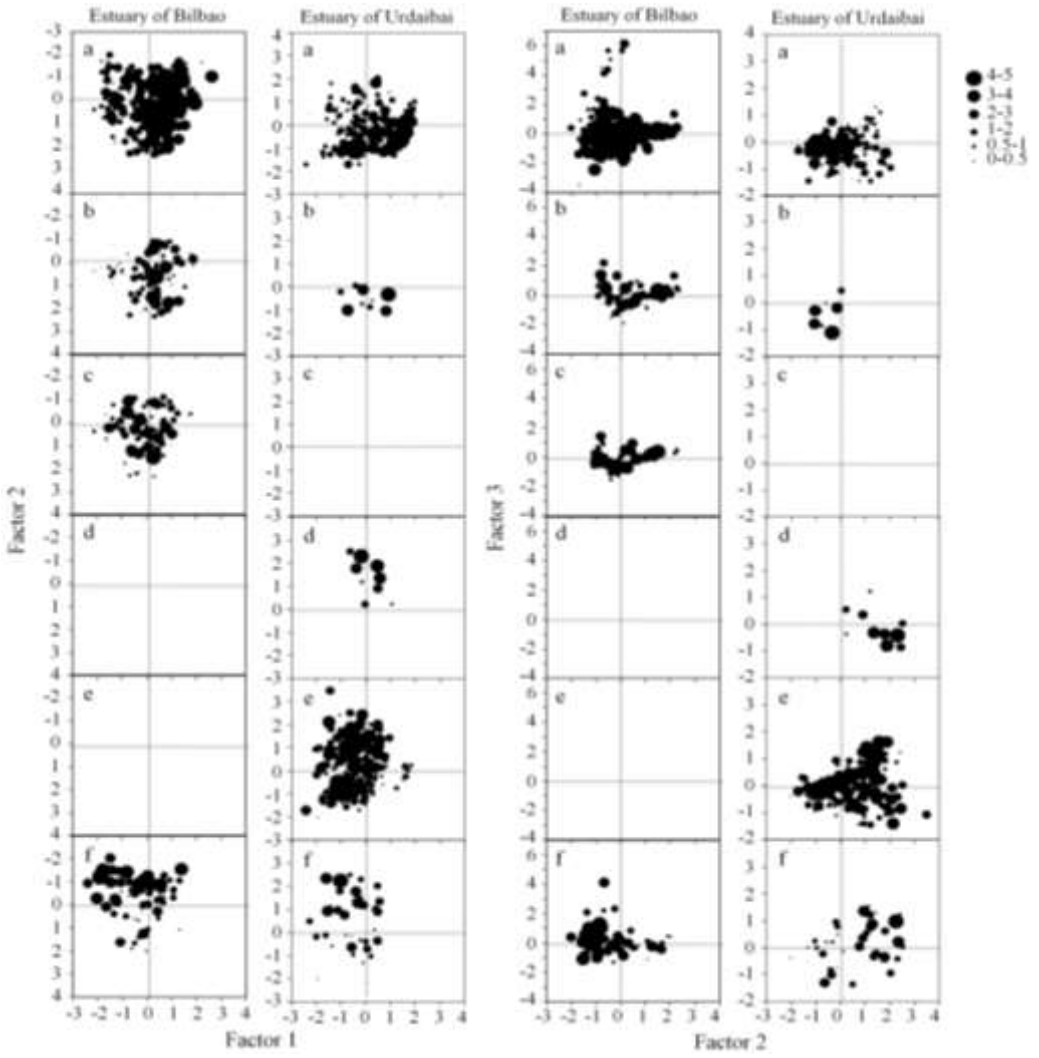

Figure 7. Plot of density values (log (x + 1), individuals m^{-3}) of (a) *Acartia clausi*, (b) *Acartia discaudata*, (c) *Acartia margalefi*, (d) *Paracartia grani*, (e) *Acartia bifilosa* and (f) *Acartia tonsa* in the space configured by the first and second factors (left figures) and the second and third factors (right figures) of environmental variability in the estuaries of Bilbao and Urdaibai during the 1998-2005 period.

In the estuary of Urdaibai, the segregation of species along the spatial gradient determined by salinity, turbidity and stratification showed the succession *A. clausi – A. discaudata – P. grani – A. bifilosa – A. tonsa* from the outer to the inner estuary. The spatial segregation between the outermost species *A. clausi* and the innermost species *A. tonsa* was more marked than in the estuary of Bilbao. Along the environmental gradient driven mainly by temperature and secondarily by chlorophyll *a* concentration, *A. bifilosa* showed the widest distribution, whereas more restricted distributions and a clear segregation along temperature gradients were found for *P. grani* and *A. tonsa*, associated to highest temperatures, and *A. discaudata*, associated to low temperatures.

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3.4. Niche Breadth and Overlap

The niche breadths estimated in relation to salinity sites (spatial) and months (seasonal) of the three dominant species *A. clausi*, *A. bifilosa* and *A. tonsa* in the estuaries of Bilbao and Urdaibai for each of the three periods distinguished in the time series are shown in Table 5.

Table 5. Levin's niche breadth (B_A) of *Acartia clausi*, *Acartia bifilosa* and *Acartia tonsa* in relation to salinity (spatial) and months (seasonal) in the estuaries of Bilbao and Urdaibai for each of the three periods distinguished in the time series

	Period	Niche breadth (B_A)					
		Spatial			Seasonal		
		A. clausi	*A. bifilosa*	*A. tonsa*	*A. clausi*	*A. bifilosa*	*A. tonsa*
Estuary of Bilbao	1998-2000	0.997	---	---	0.985	---	---
	2001-2002	0.960	---	---	0.951	---	---
	2003-2005	0.706	---	0.652	0.887	---	0.687
Estuary of Urdaibai	1998-2000	0.501	0.702	---	0.771	0.878	---
	2001-2002	0.622	0.607	---	0.763	0.876	---
	2003-2005	0.273	0.679	0.539	0.870	0.916	0.451

Table 6. Simplified Morisita Index for spatial niche overlap (C_H), in relation to salinity (spatial) and months (seasonal) between *Acartia clausi* ($_c$), *Acartia bifilosa* ($_b$) and *Acartia tonsa* ($_t$) in the estuaries of Bilbao and Urdaibai for each of the three periods distinguished in the time series

	Period	Niche overlap (C_H)					
		Spatial			Temporal		
		C_{Hcb}	C_{Hct}	C_{Hbt}	C_{Hcb}	C_{Hct}	C_{Hbt}
Estuary of Bilbao	1998-2000	---	---	---	---	---	---
	2001-2002	---	---	---	---	---	---
	2003-2005	---	0.53	---	---	0.64	---
Estuary of Urdaibai	1998-2000	0.41	---	---	0.68	---	---
	2001-2002	0.42	---	---	0.71	---	---
	2003-2005	0.22	0.16	0.95	0.87	0.53	0.52

The spatial niche of *A. clausi* was reduced from the first two periods to the third in both estuaries. The spatial niche breadth of *A. bifilosa* in the estuary of Urdaibai did not differ largely between periods, but it was lowest in the second period, this coinciding with the highest spatial niche breadth of *A. clausi*. In the third period, when *A. tonsa* was established in both estuaries, the spatial niche breadth of *A. tonsa* was smaller than that of *A. clausi* in the estuary of Bilbao and than of *A. bifilosa* in the estuary of Urdaibai.

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The seasonal niche of *A. clausi* diminished from the first two periods to the third in the estuary of Bilbao, the niche breadth in the third period being higher than that of *A. tonsa*. In the estuary of Urdaibai, however, the seasonal niche of both *A. clausi* and *A. bifilosa* expanded in the third period, when the niche breadth of *A. tonsa* was much lower than those of the two former species.

Results of niche overlap between the three dominant species *A. clausi*, *A. bifilosa* and *A. tonsa* in the estuaries of Bilbao and Urdaibai for each of the three periods distinguished in the time series, in relation to salinity (spatial) and months (seasonal) are shown in Table 6. The spatial niche overlap between *A. clausi* and *A. tonsa* was much higher in the estuary of Bilbao than in Urdaibai, and that between *A. bifilosa* and *A. tonsa* in the latter estuary was the highest found between the dominant *Acartia* species in any of the two estuaries. Seasonally, the highest niche overlap was obtained between *A. clausi* and *A. bifilosa* in the estuary of Urdaibai, and the overlap between *A. tonsa* and *A. clausi* was higher in the estuary of Bilbao than in Urdaibai. In addition, in the 2003-2005 period the spatial overlap between *A. clausi* and *A. bifilosa* decreased while the seasonal overlap increased in the estuary of Urdaibai.

CONCLUSION

Before the arrival of the non-indigenous species *Acartia tonsa* in the estuaries of Bilbao and Urdaibai, the Acartiidae assemblage was constituted by the perennial neritic species *Acartia clausi* and the occasional species *Acartia discaudata* and *Acartia margalefi* in the estuary of Bilbao, and by the perennial neritic species *A. clausi*, the perennial brackish species *Acartia bifilosa* and the occasional species *A. discaudata* and *Paracartia grani* in the estuary of Urdaibai. This indicates that each estuary, in spite of the geographic proximity, was inhabited by different assemblages of Acartiidae species. Such differences cannot be attributed only to differences in the health status, but also to the extent of salinity habitats able to support perennial brackish species populations. The role of system features in the composition of the Acartiidae assemblage is corroborated by the variety of species combinations reported in different types of estuarine systems of the Bay of Biscay and nearby European Atlantic coasts. Without taking into account the invasive *A. tonsa*, for example, *A. clausi*, *A. bifilosa* and *A. discaudata* have been reported in the Ems estuary (Baretta and Malschaert, 1988), *A. clausi*, *A. bifilosa*, *A. discaudata* and *A. margalefi* in Southampton Water (Muxagata, 2005), *A. clausi*, *A. bifilosa*, *A. discaudata* and *A. grani* in the Marennes-Oléron Bay (Sautour and Castel, 1993), and *A. clausi*, *A. discaudata*, *A. margalefi* and *A. grani* in the Ría de Vigo (Alcaraz, 1983).

A. tonsa reached high abundances in the estuaries of Bilbao and Urdaibai at the same time, in 2003. This may have been favoured by a common environmental event in both

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systems, such as the marked increase of temperature in 2003 after the dry period of 2001-2002. In fact, already by this period *A. tonsa* was occasionally found in the estuary of Bilbao. The most plausible hypothesis is that *A. tonsa* arrived in this estuary (maybe even repeatedly) via ballast water from ships that brought it to the outer port area of the Abra harbour. In 2002 the penetration of this species to the upper reaches was presumably favoured by the decrease of river flow, which enabled a higher penetration of marine waters upstream, a mechanism reported for this species in the Gironde estuary too (David *et al.*, 2007). There is no commercial shipping transport in the estuary of Urdaibai but small boat circulation between this estuary and that of Bilbao is frequent, being likely responsible for carrying plankton organisms between them. This has been claimed to be an important way of secondary spread for crustacean zooplankton (Kelly *et al.*, 2013). Although the exact mechanism by which *A. tonsa* arrived in the estuaries of Bilbao and Urdaibai cannot be known for certain, genetic analysis of individuals from both estuaries suggested a secondary invasion from a European source to Basque estuaries (Albaina *et al.*, 2016).

A. tonsa reached the highest population growth in the innermost salinity habitats of both estuaries in spite of the differences in salinity range, which was wider in the estuary of Urdaibai. *A. tonsa* is found to be an extremely euryhaline species well adapted to instantaneous variations of salinity (Svetlichny *et al.*, 2018), with high reproductive success over wide ranges of temperature and salinity (Holste and Peck, 2006) and optimal adaptation to salinities between 15 and 22 (Cervetto *et al.*, 1999). It is abundant from oligohaline (0.5-5 salinity) to polyhaline (18-30 salinity) waters, with usual maxima in the mesohaline (5-18 salinity) region of large estuaries like Chesapeake Bay and the Gironde (Kimmel and Roman, 2004; David *et al.*, 2007). Thus, *A. tonsa* population settled preferentially in the available innermost salinity habitat of the estuaries of Bilbao and Urdaibai which were in the limit between euhaline and polyhaline waters (around 30 salinity) in the former, but in polyhaline (18-30 salinity) waters in the latter. This shows the ability of this species to adapt to different salinity habitats in transitional waters of estuarine systems, depending on their availability.

The colonization of *A. tonsa* seems to be facilitated by environmental conditions close to its temperature and salinity optima (Chaalali *et al.*, 2013). However, although salinity conditions in the estuary of Urdaibai were nearer to the optimal, the settlement of *A. tonsa* was more successful in the estuary of Bilbao than in Urdaibai, where this species showed a more restricted seasonal presence and decreased from 2003 to 2005. This may be the result of the combined effect of biotic and abiotic factors, such as competition, temperature and ecosystem health. The lack of perennial brackish *Acartia* species in the inner estuary of Bilbao, in contrast to the development of a large population of *A. bifilosa* in the inner estuary of Urdaibai, suggests a stronger competitive pressure for *A. tonsa* in the estuary of Urdaibai, which could be enhanced when environmental conditions were less favourable for this species than for *A. bifilosa*. *A. tonsa* is well known as a warm-

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water species (Katajisto, 2006) which reaches its maximum abundances and dominance in summer-early autumn in a wide variety of estuaries and coastal systems (Soetaert and Rijswijk, 1993; Mouny and Dauvin, 2002; David *et al.*, 2005; Purcell and Decker, 2005; Marques *et al.*, 2007; Feike and Heerkloss, 2008; Mackas *et al.*, 2012; Rowshan *et al.*, 2014), and usually disappears from the water column in winter or winter-spring for long periods in which it stays on the bottom as resting eggs (David *et al.*, 2005; Katajisto, 2006; Milligan *et al.*, 2011). The absence or lower abundance of this species during the cold season may be caused by low water temperature, high river discharge and high water export from the estuary (Mortazavi *et al.*, 2000). River flow is also reported to govern *A. tonsa* dynamics in the inner Mondego estuary by advective transport and turbulence (Marques *et al.*, 2018). *A. bifilosa*, however, has been found to have a wide thermal niche, and it is usually a perennial species which may peak from spring to summer depending on the year (our own results; Hernroth and Ackefors, 1979; Viitasalo, 1992; David *et al.*, 2005). *A. tonsa* decreased as temperature decreased from 2003 to 2005 in the estuary of Urdaibai but not in the estuary of Bilbao, this suggesting that the temperature decrease which can be unfavourable in itself for this species could have an additional negative effect by enhancing the competitive pressure by *A. bifilosa*. In the estuary of Bilbao, on the contrary, *A. tonsa* increased from 2003 to 2005, likely due to the lack of brackish competitors and to the deterioration of oxygen conditions in the estuary of Bilbao, which were found to confer a competitive advantage to *A. tonsa* over the neritic species *A. clausi* in this system (Aravena *et al.*, 2009). At very low dissolved oxygen concentrations *i.e.*, 0.5 mL L^{-1}, *A. tonsa* shows high mortality, but it can tolerate dissolved oxygen concentrations as low as 1.0 mL L^{-1} and adapt well to hypoxia (Decker *et al.*, 2003; Kimmel *et al.*, 2009). The differences observed in the colonising success of *A. tonsa* in the inner estuarine habitats of the estuaries of Bilbao and Urdaibai corroborate the finding that both natural and anthropogenic forcings can explain the successful settlement of this species in estuaries, as reported in the Gironde estuary (David *et al.*, 2007). Our results also exemplify the plasticity of this species, a property which helps to explain why *A. tonsa* is a key species in many estuaries around the world (Derisio *et al.*, 2014). Once this species is established, its replacement by other *Acartia* species might require strong environmental changes such as reported for Berre lagoon, where rehabilitation processes resulting in an increase of salinity have led to the replacement of *A. tonsa* by *A. clausi* (Delpy and Pagano, 2018). The ongoing rehabilitation processes in the estuary of Bilbao may also lead to changes in the relative abundances of these species, but possibly linked to modifications in trophic and/or water quality parameters rather than to unlikely changes of salinity due to anthropogenic activities in the system.

Another difference in the distribution of *A. tonsa* attributable to the peculiarities inherent to the system was that its abundance decreased seaward with increasing salinity in both estuaries, but dropped sharply in 35 salinity waters of the estuary of Urdaibai, as the abundance of *A. bifilosa* did. This may be related to the much stronger effect of tides

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on the dynamics of water masses in the estuary of Urdaibai, where water masses of 35 salinity are removed from the estuary at low tide during each semidiurnal tidal cycle, thus enhancing dispersal of brackish species populations. The marked differences in environmental and plankton dynamics of the 35 salinity site of the estuary of Urdaibai compared to lower salinity sites have also been emphasized for dissolved oxygen and phytoplankton biomass (Villate *et al.*, 2008; Iriarte *et al.*, 2014).

The settlement of *A. tonsa* in the estuaries of Bilbao and Urdaibai caused an impact on the spatial and seasonal distribution of Acartiidae species that coexisted in these estuaries before its arrival, in different ways depending on the estuary characteristics and the brackish or neritic origin of species. The spatial distribution of *A. clausi* was displaced seaward and its salinity niche breadth shrank in both estuaries after the settlement of *A. tonsa*, but the direct effect was more evident in the estuary of Bilbao, where both the spatial and the seasonal niche overlaps between *A. clausi* and *A. tonsa* were higher than in the estuary of Urdaibai. This was the result of the smaller spatial salinity range available for the distribution of these two species in the estuary of Bilbao and of the wider seasonal distribution of both species as compared to the estuary of Urdaibai. Therefore, a strong spatial segregation can be expected when salinity habitat range increases and both species coexist in time, as it has been reported also for the Mondego estuary and the Ria de Aveiro (Azeiteiro *et al.*, 2005; Leandro *et al.*, 2014). In the estuary of Urdaibai, however, the seasonal occurrence of *A. tonsa* was more restricted than in the estuary of Bilbao, and *A. clausi* showed a longer period of coexistence with *A. bifilosa*. This latter species showed higher spatial and seasonal niche breadth, and higher spatial and seasonal overlap with *A. clausi* than *A. tonsa*. Consequently, a greater effect of *A. bifilosa* – than of *A. tonsa* – in the spatial and seasonal distribution of *A. clausi* can be expected in the estuary of Urdaibai. Since both the spatial and seasonal distributions of *A. bifilosa* changed when *A. tonsa* settled in the estuary of Urdaibai, the likely impact of *A. tonsa* on *A. clausi* could be better understood as an indirect effect via the impact of *A. tonsa* on *A. bifilosa*. Our results indicate that *A. bifilosa* population maximum was displaced seaward along the salinity gradient, from 26 to 30 salinity, and the seasonal maximum came earlier, being displaced from summer to late spring. This was due to the fact that *A. tonsa* occupied preferentially the lowest salinity habitat and reached the annual maximum in summer when it colonized the estuary of Urdaibai. The spatial segregation pattern of *A. tonsa* and *A. bifilosa* observed in this estuary agrees with that observed in other estuaries where both species coexist after the colonization by the former. For instance, Soetaert and Rijswijk (1993) reported that *A. bifilosa* occupied preferentially the central part and *A. tonsa* the upstream part of the Westerschelde estuary, and that *A. bifilosa* could not penetrate more upstream in summer because of the occurrence of *A. tonsa* in this area. Seasonally, *A. bifilosa* also showed a phenological change upon *A. tonsa* establishment in the Gironde estuary, with the production period occurring one month earlier than before, which resulted in a clear seasonal segregation

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between the two species (David *et al.*, 2007; Selleslagh *et al.*, 2012). In an interannual context, after the occurrence in large numbers of *A. tonsa* in 2003, this species showed a decline in the estuary of Urdaibai, while the congeneric brackish species *A. bifilosa* recovered from lower abundances during the period prior to the occurrence of *A. tonsa*. This is in contrast with observations in the Gironde estuary, where the colonization process of *A. tonsa* was more progressive and consisted in a succession of long phases from its initial occurrence in low numbers in 1983 to the point of becoming more abundant than *A. bifilosa* from 1999 onward (Chaalali *et al.*, 2013). In any case, the period of coexistence of *A. tonsa* and *A. bifilosa* analyzed in the estuary of Urdaibai is still short to be able to explain the pattern of joint population dynamic of these species in this system, and an extension of the study to include more years is needed. Changes in environmental conditions may also play a key role in the variations of brackish species in the estuary of Urdaibai as inferred from the strong decline of *A. bifilosa* prior to the occurrence of *A. tonsa*, in the period characterized by the anomalous river flow regime during 2001-2002 and low summer temperatures in 2002.

The impact of *A. tonsa* on the much less abundant and occasional Acartiidae species, such as *A. discaudata* in both estuaries, *A. margalefi* in the estuary of Bilbao and *P. grani* in the estuary of Urdaibai, could not be easily assessed on the basis of our data. These species show an intermediate spatial position in the salinity gradient between the neritic species *A. clausi* and the brackish species *A. bifilosa* and *A. tonsa*, but they also appear spatially (*A. discaudata* and *A. margalefi* in the estuary of Bilbao) or seasonally (*A. discaudata* and *P. grani* in the estuary of Urdaibai) segregated among them. In the estuary of Bilbao, both *A. discaudata* and *A. margalefi* increased in the period prior to the settlement of *A. tonsa*, coinciding with an improvement of water environmental conditions in the inner estuary, and they almost disappeared after the settlement of *A. tonsa*. The arrival of *A. tonsa* has been suggested as the cause for the disappearance of the formerly abundant *A. margalefi* in some Mediterranean systems (Sei *et al.*, 1996; Sei and Ferrari, 2008) and the Black Sea (Gubanova *et al.*, 2014). However, it is difficult to attribute the strong decline of *A. discaudata* and *A. margalefi* to the negative impact of *A. tonsa* in the estuary of Bilbao when it coincided with a general deterioration of environmental conditions, namely a decrease in dissolved oxygen and an increase in river flow and temperature. Such conditions were similar to those found in the period 1998-2000, in which *A. tonsa* was not present but *A. discaudata* and *A. margalefi* were also scarce. The sensitivity of *A. margalefi* to environmental changes is corroborated by results from other studies. In the lagoon of Venice *A. margalefi* density also declined with the introduction of *A. tonsa*, but the reported simultaneous disappearance of this species from other Italian estuarine and lagoon systems, as discussed in Ribera d'Alcalà *et al.* (2004), suggested that larger scale environmental changes might be responsible (Bandelj *et al.*, 2008).

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The case of *P. grani* in the estuary of Urdaibai was clearly different, as this species showed a very weak presence until 2003 when it showed the highest abundance at the same time as *A. tonsa* did. This only allows concluding that the occurrence of *P. grani* was linked to high temperatures, and that it responded to this factor in the same way as *A. tonsa* did. The presence of *P. grani* in the plankton also appears restricted to the summer period in Mediterranean lagoon systems (Boyer *et al.*, 2013). At an interannual scale, the occurrence of *P. grani* in the estuary of Urdaibai was erratic and with long periods of absence. Its highest abundance and seasonal presence were recorded in previous studies during the years 1981-1982 and the summer of 1990 (Villate, 1982; Uriarte *et al.*, 2000), but it was almost absent from 1997 until 2003. Similarly, this species was reported in the 1960s but not in more recent studies (2001-2002) in Southampton Water (Muxagata, 2005). This indicates that this species might be undergoing a long term decline in these systems. However, the present scarcity of *P. grani* in the estuary of Urdaibai precluded the analysis of competitive relationships with other Acartiidae species, as is the case of the invading *A. tonsa*.

In summary, the colonization of the inner salinity habitats of the estuaries of Bilbao and Urdaibai by the non-indigenous copepod *A. tonsa* since 2003 was found to be directly or indirectly responsible for spatial and temporal changes in the distribution of the autochthonous dominant neritic species *A. clausi* in both systems, and the autochthonous dominant brackish species *A. bifilosa* in the estuary of Urdaibai. No clear impact of *A. tonsa* on the less abundant and occasional species *A. discaudata*, *A. margalefi* and *P. grani* was observed, presumably because the development of these species was primarily limited by environmental constraints. Differences in the spatial, seasonal and interannual distribution of *A. tonsa* between the two estuaries showed that population dynamics were affected by biotic and abiotic features specific to each system, and that *A. tonsa* benefited from the worse environmental conditions and the lack of a dominant brackish species in the inner estuary of Bilbao. The synoptic scheme shown in Figure 8 summarizes the role of specific competition and hydroclimatic factors in the changes observed for Acartiidae species throughout the 1998-2005 period in the estuaries of Bilbao and Urdaibai. Results are preliminary because they cover a short period of time after the occurrence of *A. tonsa* in the estuaries of Bilbao and Urdaibai. An extension of the study encompassing more years would be advisable to obtain a more robust picture of changes in the Acartiidae congenerics, and determine the invasive character of *A. tonsa* in these systems. For this last purpose, future studies should be expanded to address also the impact of this species at the ecosystem level, considering ecosystem services too. Comparative studies covering a wide variety of systems colonized by *A. tonsa* would also be desirable in order to determine how system typology and level of anthropogenic disturbance affect the invasive character of this species. In addition, long term studies based on ongoing time series to analyse the response of *A. tonsa* to current modes of

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climate variation should be promoted. They could provide useful information to predict future impacts of this species under different climate change scenarios.

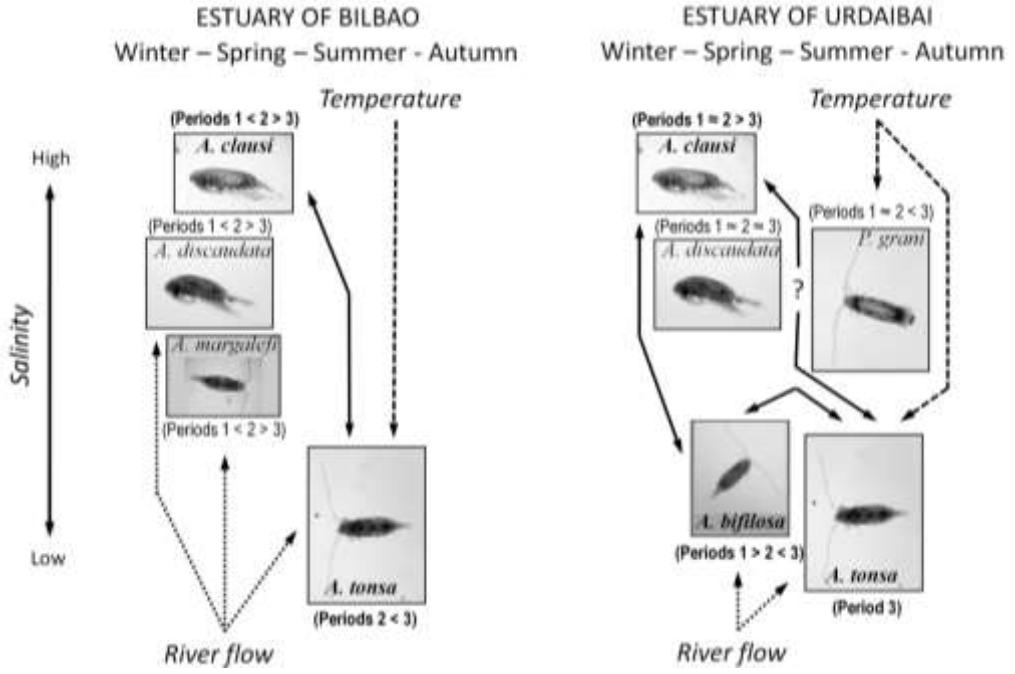

Figure 8. Synoptic diagram of the spatial and temporal distribution of Acartiidae species in the estuaries of Bilbao and Urdaibai, with indication of the observed effects of species competition and hydroclimatic factors in the variations in abundance of *Acartia clausi*, *Acartia discaudata*, *Acartia margalefi*, *Paracartia grani*, *Acartia bifilosa* and *Acartia tonsa* during the 1998-2005 period. Continuous lines: competitive interactions resulting in reduction or displacement of spatial and/or seasonal distributions, or changes in abundance between periods (1: 1998-2000, 2: 2001-2002 and 3: 2003-2005). Dashed lines: negative effect of river flow and positive effect of temperature.

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Chapter 6

CAN CHANGES IN THE DISTRIBUTION OF TWO CONGENERIC COPEPODS (*ACARTIA CLAUSI VS. ACARTIA TONSA*) CONSTITUTE A SIGN OF RECOVERY FOR THE ANTHROPIZED BERRE LAGOON (FRANCE, MEDITERRANEAN SEA)?

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ABSTRACT

The impact of rehabilitation processes on *Acartia* distributions in the anthropized Berre Lagoon was investigated comparing a study performed in 2010-12 to ones achieved before the rehabilitation period. In 1966, the opening of a hydroelectric powerplant led to the establishment of a strong unidirectional salinity gradient. The invasive copepod *Acartia tonsa*, introduced in the 1980's, dominated a low-diversity zooplankton community with another common brackish species, the rotifer *Brachionus plicatilis*. At that time, *Acartia clausi* was restricted to the adjacent coastal area. Initiated since the mid 1990's, the rehabilitation processes have managed to reduce salinity fluctuations and maintain it above 15. The time and space partitioning of both *Acartia* species was modified, since *A. tonsa* and *A. clausi* coexisted over the whole lagoon. A seasonal succession pattern was then outlined throughout the year, with *A. clausi* dominant from winter to spring and *A. tonsa* from summer to autumn. Likewise, a spatial segregation was observed in the entire lagoon, as *A. clausi* remained in more marine areas. *A. tonsa*

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was less abundant, highlighting that a balance seemed established between these two congeneric species. However, environmental variables did not display any clear direct relationship with these distributions, suggesting more complex mechanisms, such as trophic interactions. Nevertheless, these changes in the distribution of *Acartia* species following the rehabilitation processes constitute a sign of a hoped recovery.

Keywords: anthropized lagoon, salinity gradient, rehabilitation processes, *Acartia tonsa*, *Acartia clausi*

1. INTRODUCTION

Due to their short generation time, copepods, as other zooplankton organisms, quickly respond to environmental changes, a response that could be considered useful to appreciate the health status of aquatic ecosystems and assess their variability at distinct space and time scales (Cairns *et al.*, 1993; Beaugrand, 2005; Fernández de Puelles *et al.*, 2018; Wootton *et al.*, 2018). Community structure modifications (*e.g.*, species composition, size spectra) following perturbations, as well as changes in the spatial distribution of 'steno' species depending on different types of gradients (haline, trophic, pollution, *etc.*), are often considered tools to acknowledge the variability of marine, coastal and brackish water environments (Etilé *et al.*, 2009; Delpy *et al.*, 2012; Serranito *et al.*, 2016; Svetlichny *et al.*, 2018). For instance, at large scale, alterations in copepod communities have been associated to hydroclimatic changes in the North Atlantic Ocean (Beaugrand and Reid, 2003). At the regional scale, the impact of pollution, water quality, eutrophication and brief climatic events have been evidenced on copepod communities (Vincent *et al.*, 2002; Etilé *et al.*, 2009; Delpy *et al.*, 2012; Serranito *et al.*, 2016). Their monitoring may thus represent a powerful tool for a sustainable management of coastal lagoons, which are major sites of socioeconomic interests (*e.g.*, artisanal fisheries, aquaculture, tourism, various aquatic leisure activities). They represent ~13% of worldwide coastlines and are highly impacted by various anthropogenic pressures (*e.g.*, urbanization, chemical industries, agriculture, marine commercial traffic) which combine with natural forcing such as temperature and salinity variations matching seawater and/or freshwater inputs. Coastal lagoons are then very selective to copepods, and particularly to species which may be considered reliable bio-indicators (Capuzzo, 1980; Bianchi *et al.*, 2003; Marcus, 2004; Beaugrand, 2005; Etilé *et al.*, 2009; Delpy *et al.*, 2012; Falcão *et al.*, 2012; Serranito *et al.*, 2016).

Among the noteworthy copepod families, Acartiidae represent typical coastal and brackish water copepods that have been used to characterize water quality and hydrological changes in coastal ecosystems (*e.g.*, Alcaraz, 1983; Bianchi *et al.*, 2003; David *et al.*, 2007; Aravena *et al.*, 2009; Etilé *et al.*, 2009; Marques *et al.*, 2018; Villate *et al.*, 2018). In this chapter, we focus on the only two *Acartia* species (*Acartia tonsa*

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Dana, 1849 and *Acartia clausi* Giesbrecht, 1889) observed in a French Mediterranean coastal lagoon (Berre Lagoon), which has been particularly impacted by major hydrological changes. Berre Lagoon has been subject to strong anthropogenic pressures for several decades (Warner, 2012). The development of chemical and petrochemical industries (1920-70), as well as a massive urbanization of surrounding cities (1973-90), led to large inputs of organic and inorganic pollutants. This was in addition to the contribution of three small tributaries (*i.e.*, Touloubre, Durançole and Arc) which drained a watershed of ~1,300 km², bringing annually 275 tons of $N\text{-}NO_3$ and 36 tons of $P\text{-}PO_4$ into the lagoon (Gouze *et al.*, 2008; Warner, 2012). In 2005-06, floods were responsible for supplying up to 33% of nitrate, 53% of phosphate and 99% of suspended matter (Gouze *et al.*, 2008). Moreover, strong winds (*i.e.*, Mistral and East wind) contributed to the release of stored phosphorus within the sediment. In 1966, a derivation canal of the Durance River into the northern part of the lagoon was built to supply a hydroelectric powerplant. Releases of freshwater (3.3×10^9 m^3 y^{-1}, 3.7 times Berre volume) and silt (520,000 t y^{-1}) were realized with no or very few controls causing intense eutrophication (Minas, 1976a; Gaudy *et al.*, 1995; Gouze *et al.*, 2008) and significant anoxia events (Minas, 1976b; Nerini *et al.*, 2000). In 1994, the rehabilitation processes were initiated with a reduction in freshwater (1.2×10^9 m^3 y^{-1}) and silt (100,000 t y^{-1}) inputs through Barnier Plan. However, these restrictions were not enough as large fluctuations of salinity and eutrophication were still observed. In 2006, a litigation of the European Union imposed stricter limitations with a further reduction of silt inputs (< 60,000 t y^{-1}) and a mandatory smoothing of both freshwater and silt releases over a weekly basis. The objective was the maintenance of salinity above 15.

As other coastal environments, Berre Lagoon was also impacted by the introduction of several alien species probably *via* ballast waters of commercial ships (Gaudy and Vinas, 1985; Delpy *et al.*, 2012 and 2016). Among them, two species (the copepod *A. tonsa* and the ctenophore *Mnemiopsis leidyi*) were known to be particularly invasive, modifying food webs drastically (Gaudy *et al.*, 1995; Katsanevakis *et al.*, 2014; Albaina *et al.*, 2016; Delpy *et al.*, 2016). Thus, *A. tonsa* can alter trophic fluxes to higher trophic levels competing with native copepods, and exert a potential control on primary producers (Chaalali *et al.*, 2013; Katsanevakis *et al.*, 2014; Marques *et al.*, 2018; Villate *et al.*, 2018). In Berre Lagoon, *A. tonsa* was observed for the first time in the 1980's (Gaudy and Vinas, 1985) and then co-dominated the zooplankton community with another euryhaline and eurythermal species, the rotifer *Brachionus plicatilis* (Cervetto, 1995; Gaudy *et al.*, 1995). Conversely, *A. clausi* was an autochtonous species observed in Berre Lagoon before 1966 when it had the characteristics of a marine environment (Blanc *et al.*, 1967). Afterwards, the introduction of *A. tonsa* and the powerplant-induced freshwater discharges drove it out from the lagoon, limiting it to the adjacent coastal area where it represented one of the most abundant species (Cervetto, 1995; Cervetto *et al.*, 1995). While *A. tonsa* and *A. clausi* exhibited an opposite spatial distribution in the

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lagoon and its adjacent coastal waters before any rehabilitation effort (Cervetto, 1995; Gaudy *et al.*, 1995 and 2000), they now coexist in both areas (Delpy *et al.*, 2012; unpublished data).

Several environmental parameters may be responsible for the maintenance of *A. tonsa* within invaded areas. Temperature, salinity and diet (*i.e.*, type and amount of available food) can differently influence the metabolism of congeneric species, particularly in terms of reproductive success and feeding activity (Jeffries, 1962; Gaudy *et al.*, 2000; Calliari *et al.*, 2006; Boyer *et al.*, 2013; Marques *et al.*, 2018; Svetlichny *et al.*, 2018; Villate *et al.*, 2018). For instance, *A. clausi* and *A. tonsa* are known to accept a wide range of salinity (1-65 and 1-72, respectively), but with different optimal values (24-30 and 15-22) (Cervetto *et al.*, 1995 and 1999). Therefore, the presence of many *Acartia* species in the same area generally matched a seasonal succession (Jeffries, 1962; Lee and McAlice, 1979; Wooldridge and Melville-Smith, 1979) and/or a spatial segregation especially in brackish environments presenting a unidirectional salinity gradient (Greenwood, 1981; Alcaraz, 1983; Azeiteiro *et al.*, 2005; Aravena *et al.*, 2009; Falcão *et al.*, 2012; Leandro *et al.*, 2014). These segregations, whether temporal and/or spatial, highlighted that *A. tonsa* presents competitive advantages compared to other species, regarding the production of resistance stages (Belmonte and Potenza, 2001; Svetlichny *et al.*, 2018) and its large tolerance to low oxygen concentrations (Decker *et al.*, 2003; Kimmel *et al.*, 2009).

In this context, the present chapter, based on a comparison of our current results and studies achieved before the rehabilitation period (Gaudy *et al.*, 1995; Cervetto, 1995), aims at: (1) analyzing the relationships between *A. clausi* and *A. tonsa* abundances and their responses to environmental conditions, and (2) evaluating the effects of the rehabilitation processes on the respective distribution of these species. This comparison shows in particular that, linked to strong environmental changes after the rehabilitation, the two *Acartia* species are now cohabiting within the lagoon, representing a sign of recovery, but display seasonal succession and spatial segregation.

2. METHODS

2.1. Study Site and Sampling Strategy

Located northwest off Marseille (southeast France), Berre Lagoon is a large (155 km²) and shallow (7 m average depth) brackish water basin composed of two parts: the main lagoon and Vaïne Lagoon in the east (Figure 1). Freshwater inflows are concentrated in the northern part with three small rivers (*i.e.*, Touloubre, Durançole and Arc) and the man-made Durance River derivation canal supplying a hydroelectric

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powerplant at Saint-Chamas. In the southwestern part, Caronte Canal is its only connection to the Mediterranean Sea.

Between 1966 and 1994, releases of freshwater by the hydroelectric powerplant into Berre Lagoon were realized with no or very few controls leading to large fluctuations of salinity (4.0 to 28.0). Since 1994, rehabilitation processes (*i.e.*, gradual reduction and smoothing of inputs) were performed to maintain the salinity above 15. To evaluate the responses of *Acartia clausi* and *Acartia tonsa* distributions to these different environmental conditions, we compared a recent study we performed in 2010-12 (referred to "after rehabilitation") to previous ones by Gaudy *et al.* (1995) and Cervetto (1995) conducted in 1985 and 1992-93, respectively just before the first rehabilitation efforts (referred to "before rehabilitation").

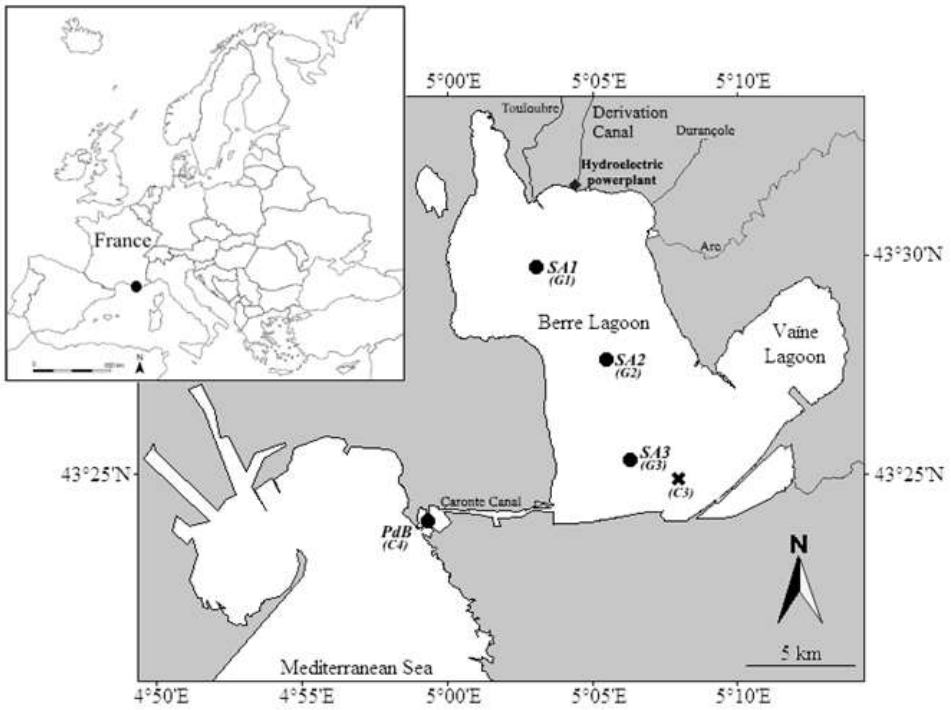

Figure 1. Map of Berre Lagoon and its adjacent coastal area. Station location: SA1, SA2, SA3 and PdB sampled in 2010-12; G1, G2 and G3 in 1985 (Gaudy *et al.*, 1995); C3 and C4 in 1992-93 (Cervetto, 1995).

In our study, monthly sampling was realized from January 2010 to January 2012 (39 samplings) at three mooring stations in Berre Lagoon (SA1, SA2 and SA3) and at Port de Bouc (PdB) (Figure 1). Located in the northern part, SA1 (43°29.735'N / 5°2.953'E, 6.9 m deep) is impacted by freshwater inflows. In the middle, SA2 (43°27.598'N / 5°5.404'E, 7.8 m deep) represents an intermediate situation. In the southern part, SA3 (43°25.300'N / 5°6.219'E, 9.5 m deep) is influenced by bottom inflows of marine water through the Caronte Canal. Situated at the mouth of the Caronte Canal, PdB (43°23.944'N /

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4°59.299'E, 6.5 m deep) presents almost typical marine characteristics of the Mediterranean Sea with a minimal influence of brackish water surface inflows from the Berre Lagoon.

In Gaudy *et al.* (1995), data were collected in February, June, October and November 1985 at stations close to SA1, SA2 and SA3 (G1, G2 and G3, Figure 1). In Cervetto (1995), sampling was performed between March 1992 and March 1993 (22 samplings) at a station slightly southeast of SA3 and another one close to PdB (C3 and C4, Figure 1). Thus, Gaudy *et al.* (1995)'s dataset allowed a seasonal comparison of the spatial distribution of *Acartia* assemblages over the whole lagoon, while Cervetto (1995) was used to evaluate the impact of the rehabilitation processes on their higher-frequency temporal distribution in the south of the lagoon and its adjacent coastal area.

2.2. Environmental Parameters

In our study, each mooring station in Berre Lagoon was equipped with multiparametric probes CTD SBE37 (Seabird) located at ~1 m from the surface and the bottom. Daily averages of temperature and salinity were computed from a high frequency sampling (96 times per day) dataset. At PdB, vertical profiles of temperature and salinity were realized with the multiparametric probe CTD SBE19 + (Seabird) at each sampling date.

To estimate chlorophyll *a* (Chl *a*) concentrations, water was collected at sub-surface (0.5 m) and at ~1 m from the bottom using an 8 L Niskin bottle. Triplicate samples of 20-100 mL were filtered directly onboard onto GF/F filters (0.7 µm), then frozen with liquid nitrogen and stored at -20°C until further analysis. Chlorophyll *a* content was extracted using acetone (90%) in the dark, at 4°C, for 24 h. Measurements were performed by fluorimetry (Turner Design – TD700 fluorimeter) according to Lorenzen (1967). Chlorophyll *a* concentrations were obtained by averaging triplicate values.

Temperature and salinity were measured by Gaudy *et al.* (1995) in the surface and deeper layers using a salinograph YTECH, whereas Cervetto (1995) realized vertical profiles with a multiparametric probe (ME Hydrodata). In both studies, chlorophyll *a* concentrations were evaluated at the same depths using a fluorimetric method.

2.3. Metazooplankton Community

In our study, to sample metazooplankton (defined as planktonic metazoans), a haul was performed at each station with a modified WP2 plankton net (1.2 m long, 50 cm diameter of opening area and 80 µm mesh size). The net was towed vertically, from bottom to surface, at a speed of ~1 m s^{-1}. The cod-end content was immediately fixed

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with a formaldehyde - buffered seawater solution (4% final). Sub-samples (1-20%) were realized with micropipettes (1 and 5 mL) and analyzed using a dissecting microscope (Leica MZ6). An average of 948 individuals were counted per sample and the enumeration error was estimated at ± 6.5% (Postel *et al.*, 2000). The metazooplankton community of Berre Lagoon presented only two *Acartia* species: *A. clausi* and *A. tonsa*. Adults of these two congeneric copepods were identified and counted according to Rose (1933) and Razouls *et al.* (2005-2017). *Acartia* copepodite stages were counted, but they could not be discriminated at the species level. Abundances of remaining metazooplankton were also estimated and expressed as ind. m^{-3}.

In Gaudy *et al.* (1995) and Cervetto (1995), metazooplankton was collected in the surface and deeper layers with a Clarke Bumpus type net (80 µm) and a WP2 plankton net (60 µm), respectively. Data were averaged over the water column to allow relevant comparisons.

2.4. Data Analysis

Only the dataset obtained from Cervetto (1995) allowed a higher-frequency comparison matching our dataset and was used for statistical analyses.

Non-parametric Mann-Whitney U tests (MWU thereafter; Mann and Whitney, 1947) were used to evaluate differences between studied periods, stations and depths. Environmental parameters (*i.e.*, temperature, salinity and chlorophyll *a* concentrations) and biological data (*i.e.*, abundance and relative abundance) were not normally distributed (Shapiro-Wilk test) and considered independently of one another.

The relationships between environmental variables (*i.e.*, temperature, salinity and chlorophyll *a* concentrations) and zooplankton (*A. clausi* and *A. tonsa*, other - *i.e.*, non-*Acartia* - copepods, meroplanktonic larvae and other zooplankton organisms) were assessed using principal component analyses (PCA) performed on log-transformed data of 1992-93 and 2010-12 for the two shared stations (C3/SA3 and C4/PdB) using ADE4 software (Thioulouse *et al.*, 1997). Partial correlation analyses were also carried out on log-transformed data to explore the relationships between the two *Acartia* species and the same environmental and zooplankton variables. In these multivariate analyses, abundances of *Acartia* species were estimated by cumulating abundance of adults and copepodites. As *Acartia* copepodite stages could not be discriminated at the species level, their abundance in each species was estimated at the *pro rata* of adult abundance.

Furthermore, two dimensional surface plots were drawn to show the effects of salinity and chlorophyll *a* concentrations on *A. clausi* and *A. tonsa* abundances. They were performed on pooled datasets of 1992-93 and 2010-12 with least square adjustment using Statistica software 7.1.

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3. RESULTS

3.1. Environmental Parameters

Before and after the rehabilitation processes, temperature displayed comparable range values (4.7 to 26.2°C in Berre Lagoon, 6.5 to 23.8°C at Port de Bouc) and similar seasonal pattern with higher values in summer and lower ones in winter (Figures 2 and 3). These observations corresponded to the typical seasonal cycle in temperate areas.

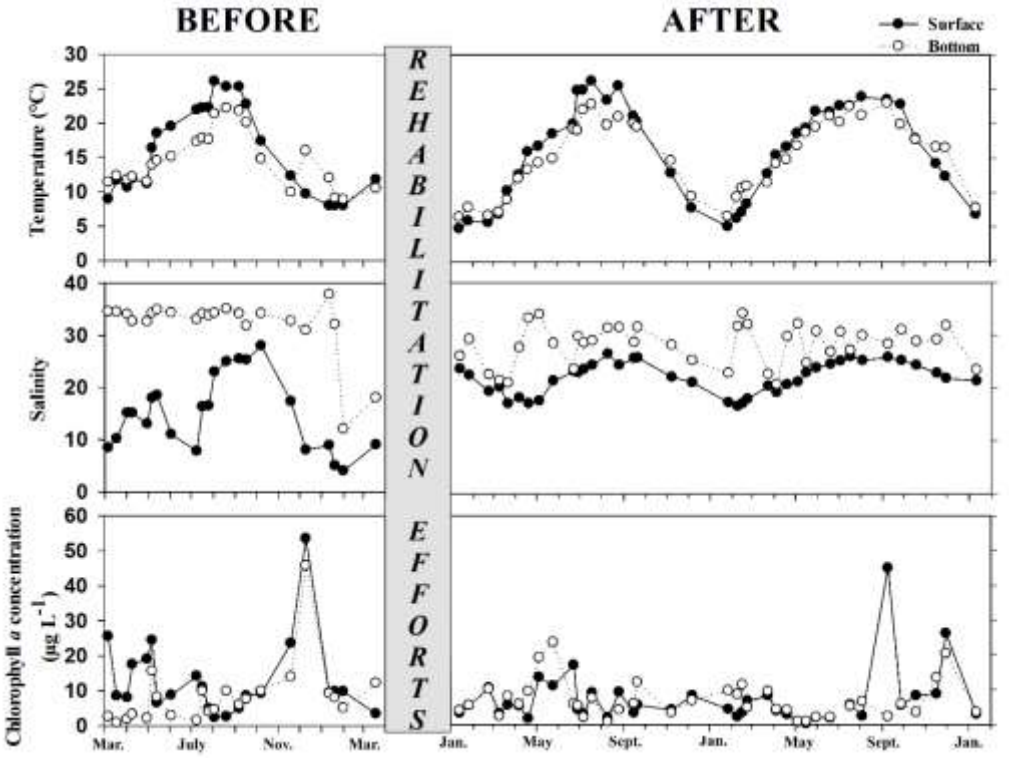

Figure 2. Temporal variations of temperature (°C), salinity and chlorophyll *a* concentrations (µg Chl *a* L^{-1}) in the south of Berre Lagoon (C3/SA3) before (1992-93 – Cervetto, 1995) and after (2010-12 – this study) the rehabilitation efforts.

For both periods, salinity displayed a clear seasonal pattern that matched the temperature pattern (Figures 2 and 3). Lower values were observed in winter corresponding to higher freshwater inputs related to increased activity of the hydroelectric powerplant to fulfill heating requirements (Warner, 2012). However, in the lagoon (C3/SA3 stations), this temporal variability was significantly lower after the rehabilitation efforts, particularly regarding surface values (4.0 to 28.0 in 1992-93 *vs.* 16.6 to 26.5 in 2010-12; MWU, $p < 0.001$) (Figure 2). A lower vertical stratification was also observed after the rehabilitation compared to before (difference bottom-surface: 6.2

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to 29.0 in 1992-93 *vs.* 0.4 to 17.1 in 2010-12; MWU, $p < 0.001$). At Port de Bouc (C4/PdB stations), surface salinity significantly increased after the rehabilitation (13.0 to 32.2 in 1992-93 *vs.* 21.0 to 35.4 in 2010-12; MWU, $p < 0.001$) (Figure 3). This salinity change matched the gradual reduction in freshwater released by the powerplant since 1994 and the mandatory smoothing over a weekly basis realized since 2006 (Delpy *et al.*, 2012; Warner, 2012). They led also to lower brackish water outputs through the Caronte Canal into the adjacent coastal area.

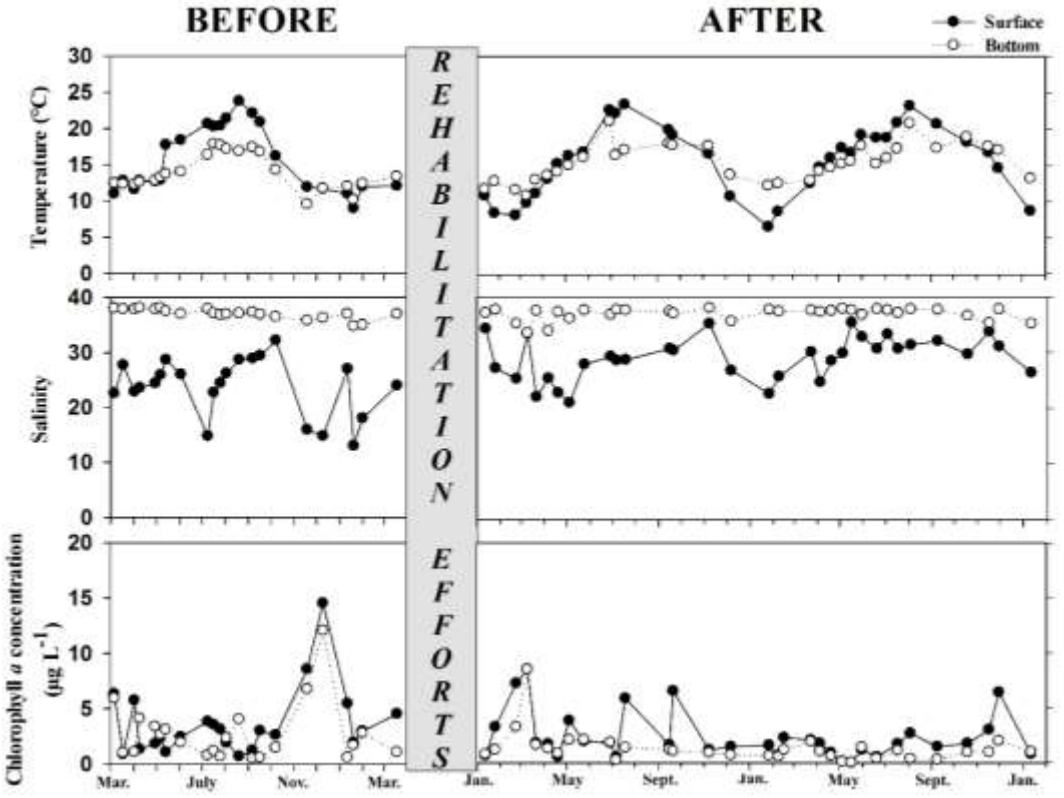

Figure 3. Temporal variations of temperature (°C), salinity and chlorophyll *a* concentrations (µg Chl *a* L^{-1}) at Port de Bouc (C4/PdB) before (1992-93 – Cervetto, 1995) and after (2010-12 – this study) the rehabilitation efforts.

Chlorophyll *a* concentrations presented no clear seasonal pattern during both periods. In Berre Lagoon, significantly lower values were observed after the rehabilitation (7.0 ± 6.8 µg Chl *a* L^{-1} in 2010-12) compared to before (10.7 ± 10.5 µg Chl *a* L^{-1} in 1992-93) (MWU, $p < 0.05$) (Figure 2). However, very high concentrations were occasionally observed in 1992-93 (53.5 µg Chl *a* L^{-1} in December 1992), and even in 2010-12 (45.0 µg Chl *a* L^{-1} in September 2011). At Port de Bouc, chlorophyll *a* concentrations presented a significant decrease after the rehabilitation processes with mean values of 1.8 ± 1.9 µg Chl *a* L^{-1} in 2010-12 *vs.* 3.2 ± 2.9 µg Chl *a* L^{-1} in 1992-93 (MWU,

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$p < 0.001$) (Figure 3). The maximum value observed in 1992-93 was 14.5 µg Chl *a* L^{-1}, whereas it was only 8.5 µg Chl *a* L^{-1} in 2010-12. Accompanying the drastic changes in salinity conditions, a modification of the trophic status was then observed with lower chlorophyll *a* concentrations in 2010-12. It is worth noticing that the persistence of high peaks of chlorophyll *a* concentrations is typical of coastal and shallow ecosystems. In this type of environments, development of phytoplanktonic blooms depended also on short-term episodic weather events like heavy rainfall and strong winds (*i.e.*, Mistral and East wind in the studied area), and not only on traditional seasonal variations in plankton succession (Cloern, 1996; Pinazo *et al.*, 2004; Cook *et al.*, 2010; Liess *et al.*, 2016).

3.2. Spatiotemporal Variations of Zooplankton Community and *Acartia* Populations

At all stations, total zooplankton abundance followed a clear seasonal pattern before and after the rehabilitation efforts. Minimal values were observed in winter, followed by a progressive increase in spring to maximal values reached in early summer (from 0.8 to 200.6 x 10^3 ind. m^{-3}) (Figure 4), matching seasonal variations of temperature previously described (Figures 2 and 3). Before the rehabilitation, peaks of total zooplankton abundance were characterized by an increase of *Acartia* abundance (copepodites, *A. clausi* and *A. tonsa* adults) (Figure 4). After the rehabilitation, *Acartia* assemblages were particularly abundant in winter/early-spring, as well as in summer but to a lesser extent.

In the lagoon (C3/SA3 stations), seasonal pattern and range of abundance were not significantly different between both periods (40.0 ± 43.1 x 10^3 ind. m^{-3} in 1992-93 *vs.* 42.0 ± 34.6 x 10^3 ind. m^{-3} in 2010-12; MWU, $p > 0.05$). Zooplankton communities presented comparable structures with comparable relative abundances for copepods (43.6 ± 26.0% in 1992-93 *vs.* 40.5 ± 28.8% in 2010-12) and other zooplankton organisms (56.4 ± 26.0% in 1992-93 *vs.* 59.5 ± 28.8% in 2010-12) (MWU, $p > 0.05$). At Port de Bouc (C4/PdB stations), the same seasonal pattern was observed whereas total zooplankton abundance nearly doubled after the rehabilitation processes (32.1 ± 24.8 x 10^3 ind. m^{-3} in 1992-93 *vs.* 56.0 ± 41.7 x 10^3 ind. m^{-3} in 2010-12; MWU, $p < 0.01$). Maximal value was 99.7 x 10^3 ind. m^{-3} in 1992-93 and reached 200.6 x 10^3 ind. m^{-3} in 2010-12. Compared to the lagoon, copepods contribution to total abundance was significantly higher (54.8 ± 17.6% in 1992-93 *vs.* 68.3 ± 17.1% in 2010-12) and occurred at the expense of other zooplankton organisms (45.2 ± 17.6% in 1992-93 *vs.* 31.7 ± 17.1% in 2010-12) (MWU, $p < 0.05$).

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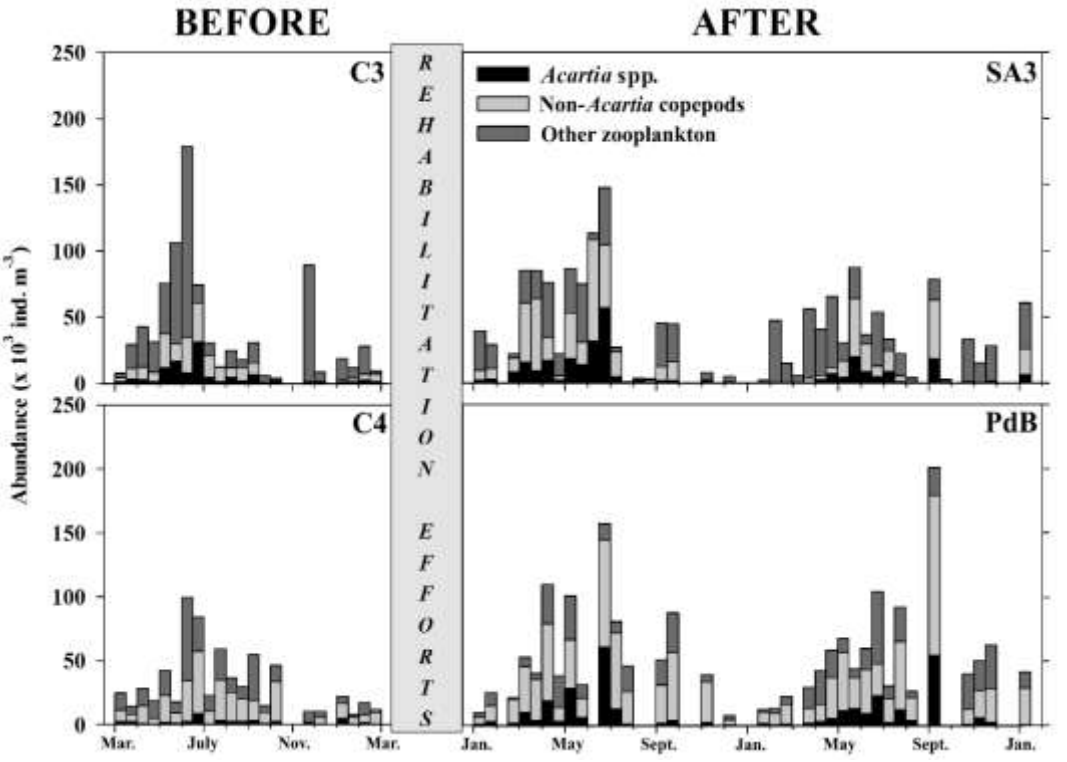

Figure 4. Temporal variations of abundances (x10^3 ind. m^{-3}) of *Acartia* spp. (black), non-*Acartia* copepods (grey) and other zooplankton organisms (dark grey) before (1992-93 – Cervetto, 1995) and after (2010-12 – this study) the rehabilitation efforts, in the south of Berre Lagoon (C3/SA3) and at Port de Bouc (C4/PdB).

The relative contributions of *A. tonsa* and *A. clausi* to total Acartiidae revealed a clear change before and after the rehabilitation processes (Figure 5). In 1992-93, *A. tonsa* was almost the only *Acartia* species in the lagoon (C3) representing up to 100% almost year round. Conversely, at Port de Bouc (C4), *A. clausi* and *A. tonsa* successively dominated the *Acartia* population. In 2010-12, a similar temporal pattern was observed at both stations (SA3 and PdB). At the spatial scale, *A. clausi* presented a higher relative abundance at Port de Bouc (63.1 ± 41.6%), while *A. tonsa* displayed a correlative higher one in the lagoon (65.1 ± 39.4%) (MWU, $p < 0.05$). Nevertheless, at Port de Bouc, it is worth noticing that in 2010-12 the main predominance periods of each species (winter to spring for *A. clausi vs.* summer to autumn for *A. tonsa*) were longer than in 1992-93 exhibiting alternation of five dominance peaks for each species.

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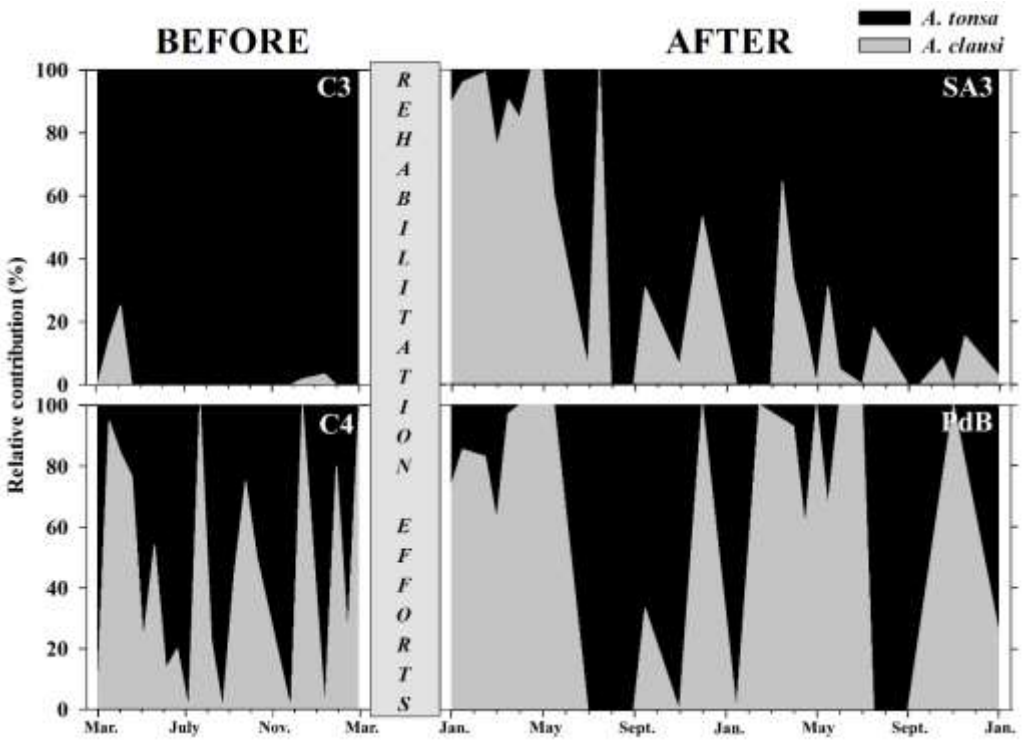

Figure 5. Temporal variations of relative abundance (% of total Acartiidae) of *A. tonsa* (dark) and *A. clausi* (grey) adults before (1992-93 – Cervetto, 1995) and after (2010-12 – this study) the rehabilitation efforts, in the south of Berre Lagoon (C3/SA3) and at Port de Bouc (C4/PdB).

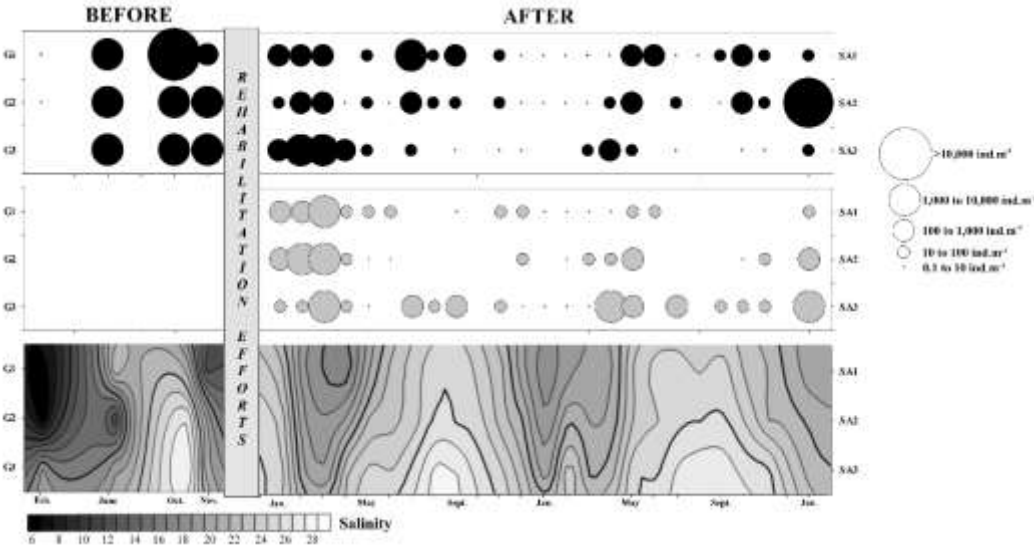

Figure 6. Bubble plots representing abundances of *A. tonsa* (black) and *A. clausi* (grey) adults before (1985 – Gaudy *et al.*, 1995) and after (2010-12 – this study) the rehabilitation efforts from North (G1/SA1) to South (G3/SA3) of Berre Lagoon. Size of bubbles is proportional to the range of abundance (ind. m^{-3}). The bottom graph represents the temporal variations of the salinity (average of surface and bottom values) gradient within the lagoon.

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The spatial distribution of *Acartia* populations in Berre Lagoon at both periods was compared to salinity variations in Figure 6. Before the rehabilitation, the salinity presented a clear seasonal variation with lowest values (~6) observed in winter in the northern part of the lagoon and highest ones (~18) in summer in the southern part (Figure 6 bottom). *A. clausi* was already absent from Berre Lagoon (G1-G2-G3), whereas adults of *A. tonsa* dominated the zooplankton community with a mean abundance of 3,458 ± 4,352 ind. m^{-3} from June to November. In February, low abundance (2.1 ± 3.2 ind. m^{-3}) corresponded to the development of a large population of the rotifer *Brachionus plicatilis* (Gaudy *et al.*, 1995). After the rehabilitation, the salinity presented the same seasonal variations, but to a lesser extent, with a range of values from 17 to 28 (Figure 6 bottom). The mean abundance of *A. tonsa* was twice higher than the one of *A. clausi* (469 ± 1,632 ind. m^{-3} and 221 ± 536 ind. m^{-3}, respectively). Despite the inter-annual variability, our study emphasizes that highest abundances of *A. clausi* occurred in winter/early-spring, and those of *A. tonsa* in summer following the same trend as in 1985 (Figures 4 and 6). Even if the two *Acartia* species were present over the whole lagoon, a spatial segregation was generally observed with a predominance of *A. tonsa* in the northern and intermediate parts (SA1-SA2) and a strong presence of *A. clausi* in the southern area (SA3). A reverse trend was only observed from January to April 2010.

3.3. Effects of Environmental Factors on *Acartia* Distribution

The PCA analyses on the lagoon data before and after the rehabilitation processes explained 57.5% and 54.6% of the variance, respectively (Figure 7 top). Before the rehabilitation, *A. clausi* was poorly correlated with the first axis (33.5%) contrarily to *A. tonsa.* These two congeneric species, together with other copepods and meroplankton, were opposed to high chlorophyll *a* concentrations. On the second axis (24.0%), both species, together with the other zooplankton groups and chlorophyll, were opposed to high temperature and salinity. After the rehabilitation, the first axis (30.2%) opposed the two *Acartia* species to high temperature and salinity, whereas the second axis (24.3%) opposed *A. tonsa*, temperature and salinity to chlorophyll.

The PCA on Port de Bouc data before and after the rehabilitation explained 60.5% and 58.2%, respectively (Figure 7 bottom). Before the rehabilitation, the first axis (42.3%) opposed high abundances of meroplankton and copepods (including both *Acartia* species) to high chlorophyll, whereas the second axis (18.2%) opposed *A. clausi* to *A. tonsa*. After the rehabilitation, on the first axis (34%), non-*Acartia* copepods and other holoplankton were associated to high temperature and opposed to *A. clausi* and chlorophyll. The second axis (24.4%) clearly opposed the two *Acartia* species with *A. tonsa* associated to high salinity. Thus, the role of temperature, salinity and chlorophyll on the two *Acartia* species did not appear very clearly on the basis of the PCA analyses.

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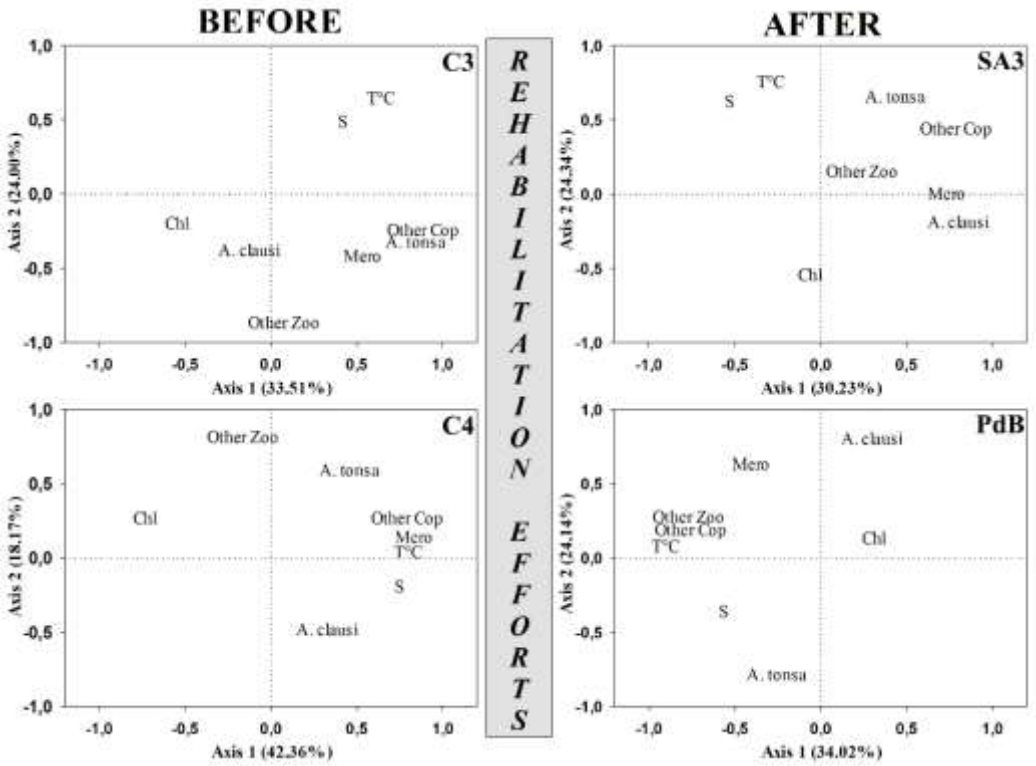

Figure 7. Principal component analyses (PCA) realized on datasets obtained before (1992-93 – Cervetto, 1995) and after (2010-12 – this study) the rehabilitation efforts in Berre Lagoon (C3/SA3) and at Port de Bouc (C4/PdB). T°C: temperature, S: salinity, Chl: chlorophyll *a* concentrations, A. tonsa: *A. tonsa* adults and copepodites, A. clausi: *A. clausi* adults and copepodites, Other Cop: non-*Acartia* copepods, Mero: meroplanktonic larvae and Other Zoo: other zooplankton organisms.

The partial correlation analysis confirmed this tendency as no correlation was found with temperature, salinity or chlorophyll for both species in the lagoon at the two periods (Table 1). However, in Port de Bouc, after the rehabilitation, both species were negatively correlated with temperature, whereas *A. tonsa* was also positively correlated to salinity. Furthermore, the analysis showed high positive correlation between *Acartia* and non-*Acartia* copepods in the lagoon, for the different situations considered (before and after the rehabilitation and pooled data of the two periods) in the case of *A. tonsa* and only after the rehabilitation processes in the case of *A. clausi*. The two *Acartia* species were negatively correlated in both sites (Berre and Port de Bouc) after the rehabilitation or when considering pooled data of the two periods. Before the rehabilitation, negative correlation between the two species was only found when considering pooled data of the two sites, which illustrated the reverse spatial trend at this period with *A. clausi* only present in PdB and *A. tonsa* quite exclusively present in Berre.

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Table 1. Partial correlation coefficients between the two *Acartia* species, environmental and zooplankton variables. n = number of data. Significance values ns = non-significant, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$

	Acartia tonsa						*Acartia clausi*					
	Berre		Port de Bouc		Total		Berre		Port de Bouc		Total	
Before												
n	*22*		*22*		*44*		*22*		*22*		*44*	
Temperature	0.000	ns	-0.249	ns	0.007	ns	-0.422	ns	-0.439	ns	**-0.371**	*
Salinity	-0.011	ns	-0.052	ns	-0.108	ns	0.379	ns	-0.301	ns	0.235	ns
Chlorophyll	0.050	ns	-0.368	ns	-0.062	ns	-0.377	ns	-0.366	ns	**-0.449**	**
Other copepods	**0.897**	***	0.262	ns	**0.358**	*	0.111	ns	0.463	ns	0.252	ns
Meroplankton	-0.080	ns	0.264	ns	0.133	ns	-0.217	ns	0.368	ns	0.083	ns
Other holoplankton	0.240	ns	0.074	ns	0.095	ns	0.309	ns	-0.273	ns	0.063	ns
Other Acartia	-0.138	ns	-0.488	ns	**-0.452**	**	-0.138	ns	-0.488	ns	**-0.452**	**
After												
n	*39*		*32*		*71*		*39*		*32*		*71*	
Temperature	0.045	ns	**-0.523**	*	-0.043	ns	-0.084	ns	**-0.515**	*	-0.238	ns
Salinity	-0.125	ns	**0.521**	*	**-0.272**	*	-0.251	ns	0.347	ns	-0.165	ns
Chlorophyll	-0.355	ns	0.296	ns	-0.036	ns	0.036	ns	0.254	ns	0.069	ns
Other copepods	**0.566**	**	0.397	ns	**0.506**	***	**0.657**	***	0.316	ns	**0.598**	***
Meroplankton	0.164	ns	-0.047	ns	0.077	ns	0.222	ns	0.215	ns	0.195	ns
Other holoplankton	-0.034	ns	0.270	ns	-0.094	ns	-0.067	ns	0.190	ns	-0.112	ns
Other Acartia	**-0.425**	*	-0.732	***	**-0.574**	***	**-0.425**	**	**-0.732**	***	**-0.574**	***
Before + After												
n	*61*		*54*		*115*		*61*		*54*		*115*	
Temperature	-0.047	ns	-0.225	ns	-0.050	ns	**-0.297**	*	**-0.341**	*	**-0.307**	**
Salinity	-0.067	ns	0.137	ns	-0.167	ns	0.056	ns	-0.038	ns	0.028	ns
Chlorophyll	**-0.284**	*	0.042	ns	-0.022	ns	-0.178	ns	-0.043	ns	-0.079	ns
Other copepods	**0.644**	***	0.257	ns	**0.444**	***	**0.548**	***	**0.315**	*	**0.478**	***
Meroplankton	0.042	ns	0.130	ns	0.154	ns	0.239	ns	**0.362**	*	**0.277**	**
Other holoplankton	0.069	ns	-0.013	ns	-0.082	ns	-0.101	ns	-0.151	ns	-0.165	ns
Other Acartia	**-0.501**	***	**-0.588**	***	**-0.549**	***	**-0.501**	***	**-0.588**	***	**-0.549**	***

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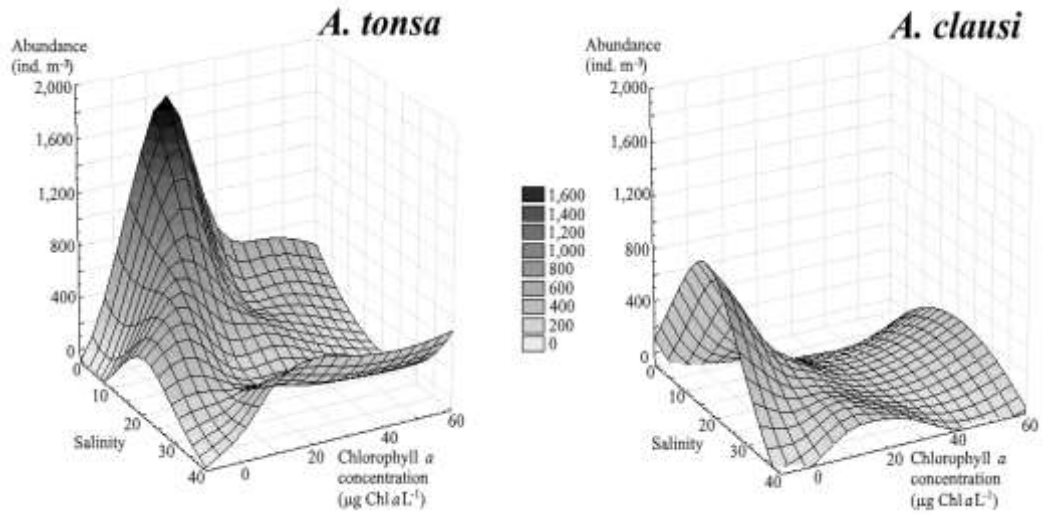

Figure 8. Surface plots of *A. tonsa* and *A. clausi* abundances (ind. m^{-3}) depending on salinity and chlorophyll *a* concentrations (µg Chl *a* L^{-1}). Datasets of 1992-93 (C3 and C4 – Cervetto, 1995) and 2010-12 (SA3 and PdB – this study) were pooled in these analyses.

In summary, if the negative relationship between the two *Acartia* species illustrated their space and/or time partitioning, the absence of clear direct relationship with environmental variables suggests complex mechanisms as interaction or competition to explain these distributions.

To better explore this issue and better understand the effects of salinity and trophic changes after the rehabilitation, we represented, on the same surface plots, the *Acartia* abundances relative to salinity and chlorophyll *a* concentrations by combining the data obtained in 2010-12 and in 1992-93 (Figure 8). The two species showed different patterns. *A. tonsa* presented clear optimal ranges with higher abundances at low salinity (< 10) and medium chlorophyll *a* concentrations (5 to 25 µg Chl *a* L^{-1}). The abundance of *A. clausi* was also dependent on these two environmental parameters with a peak outlined at medium salinity (5 to 25) and low chlorophyll *a* concentrations (< 10 µg Chl *a* L^{-1}). The start of another slight peak can be observed at medium salinity (5 to 25) and very high chlorophyll *a* concentrations (> 50 µg Chl *a* L^{-1}).

CONCLUSION

In the anthropized Berre Lagoon, the rehabilitation processes did not seem to have any significant effect on seasonal pattern of zooplankton abundance. Thus, the rough community structure remained unchanged with similar relative contributions of copepods and other zooplankton organisms, despite the lower chlorophyll *a* concentrations observed after the rehabilitation processes. Conversely, at Port de Bouc, total

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zooplankton abundance increased significantly in 2010-12 reaching values as high as in the lagoon. Copepods gained prominence in the zooplankton community reaching relative contributions (*ca.* 70%) that are currently observed in coastal marine conditions (Siokou-Frangou *et al.*, 2010). The effects of the rehabilitation processes were then perceived as far as the adjacent coastal area, confirming a lower influence of surface brackish water originating from Berre Lagoon. Environmental restorations have already shown positive impacts on plankton communities, and particularly on copepod assemblages. For instance, in a coastal marine system of Tunisia, the removal and containment of phosphogypsum has allowed a diversification of several plankton compartments (Kobbi-Rebai *et al.*, 2012; Rekik *et al.*, 2015). Likewise, the taxonomic composition of zooplankton has drastically changed in Berre Lagoon after the rehabilitation, with a more diverse community including several typical coastal marine species as evidenced by Delpy *et al.* (2012) for the copepods *Centropages typicus*, *Paracalanus parvus* and *Acartia clausi*. The lower variability in salinity likely allowed the development of these marine species brought into the lagoon through the Caronte Canal. Even if both *Acartia tonsa* and *A. clausi* were present in the entire studied area, they tended to succeed one another over time and space.

A clear succession of predominance periods was observed in Berre, as well as at Port de Bouc, with *A. clausi* dominating from winter to spring and *A. tonsa* from summer to autumn. Thus, the reappearance of *A. clausi* in Berre Lagoon and its predominance in winter/spring coincided with the disappearance of the rotifer *Brachionus plicatilis* which had been reported missing after the rehabilitation processes (Gaudy *et al.*, 1995; Delpy *et al.*, 2012). These observations corresponded to the well-defined influence of temperature on the population dynamics of both species (Jeffries, 1962). *A. clausi* is preferentially present in winter/spring as its growth rate increased with increasing temperature and food level (Klein Breteler and Schogt, 1994). In summer, egg production of *A. clausi* was negatively impacted by high temperature (Boyer *et al.*, 2013). This matched the development of the warm-water species *A. tonsa,* mainly depending on production and hatching time of its resting eggs (Katajisto, 2006; Mackas *et al.*, 2012; Svetlichny *et al.*, 2018).

At both periods, a spatial segregation of both *Acartia* species occurred along the salinity gradient from the northern part of Berre Lagoon to Port de Bouc. Before the rehabilitation processes, *A. clausi* was restricted to the marine coastal area, while *A. tonsa* occupied the entire brackish lagoon. This opposite distribution was highlighted in many estuarine systems with *A. clausi* observed downstream and *A. tonsa* upstream (Alcaraz, 1983; Azeiteiro *et al.*, 2005; Aravena *et al.*, 2009; Leandro *et al.*, 2014; Marques *et al.*, 2018; Villate *et al.*, 2018). After the rehabilitation, increasing salinity allowed *A. clausi* penetration into Berre Lagoon, as highlighted in the Mondego estuary by Marques *et al.* (2018). *A. tonsa* dominated *Acartia* assemblages in most of the lagoon, except in the southern part where *A. clausi* was more abundant, matching optimal salinity range of

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both species (Cervetto *et al.*, 1995 and 1999; Svetlichny *et al.*, 2018). In the adjacent coastal area, *A. clausi* was still predominant, but followed closely by *A. tonsa*. Falcão *et al.* (2012) showed that restoration measures can lead to a relative homogenization of zooplankton community downstream of the Mondego estuary.

A focus on the two environmental factors modified by the rehabilitation processes (*i.e.*, salinity and chlorophyll *a* concentration) highlighted the first signs of ecological niche for *A. tonsa* and *A. clausi*. After the rehabilitation processes, *A. clausi* co-dominated *Acartia* assemblages with the non-indigenous species *A. tonsa*. The drastic change in salinity conditions may partly explain this evolution, although the salinity effect was not shown in the multivariate analyses in Berre Lagoon. Higher salinity (mean value of 23.6) was less favorable for *A. tonsa*, whose optimal adaptation for metabolism (respiration and excretion) was found at 15-22 (Cervetto *et al.*, 1999; Gaudy *et al.*, 2000; Calliari *et al.*, 2006). The decrease in *A. tonsa* abundance in 2010-12 also corresponded with the installation of *A. clausi* population throughout Berre Lagoon. Its penetration into the lagoon was also linked to salinity increase as *A. clausi* was shown to exhibit reduced ingestion and highly elevated cost of growth at low salinity (< 20) (Calliari *et al.*, 2006). Its optimal salinity range was found to be 24-30, matching the new salinity conditions observed in the lagoon (Cervetto, 1995; Gaudy *et al.*, 2000). Short-term variations of salinity may also explain the spatial segregation observed in Berre after the rehabilitation processes. Following this hypothesis, due to its higher tolerance to sudden salinity variations (Cervetto *et al.*, 1999; Hubareva *et al.*, 2008) and its ability for osmoregulation (Svetlichny *et al.*, 2018), *A. tonsa* would be better adapted to the northern part, which suffered influences of freshwater releases in surface. *A. clausi* would prefer the southern part with marine conditions closer to that observed in the adjacent coastal area.

Concerning chlorophyll, Boyer *et al.* (2013) showed that this factor played a far lower importance than salinity and temperature on egg production of *Acartia* species in another Mediterranean coastal lagoon (Thau Lagoon). However, the following development stages of *A. tonsa* selected their prey according to their physiological need: P-rich prey for the rapid growth of nauplii and N-rich prey for the slower growth of copepodites (Meunier *et al.*, 2015). For *Acartia* adults, the feeding behavior is even more complex since they are omnivorous (Tiselius, 1989; Kleppel, 1993). Even if the peaks of *A. tonsa* and *A. clausi* abundances appeared in Berre Lagoon at medium and low chlorophyll *a* concentrations respectively, the quality of prey could be more important than its quantity. While the feeding regime of *A. tonsa* was more oriented toward proteinic food than *A. clausi*, no relationship between ingestion and food concentration was observed for both species (Gaudy *et al.*, 2000). Thus, *A. tonsa* could be present in less eutrophic environments as it exhibits a fast response to food and a strong efficiency to remain inside ephemeral and thin patches (Tiselius, 1992). Likewise, *A. clausi* also occurred in hyper eutrophic environments in African sites (Arfi *et al.*, 1989). Nevertheless, in 2011, the succession of predominance periods was less evident with a

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higher relative contribution of *A. tonsa* from April. This predominance coincided with chlorophyll *a* concentrations twice higher in surface waters (MWU, $p < 0.05$), values close to those observed before the rehabilitation processes (Cervetto, 1995; Gaudy *et al.*, 1995). Increased abundances of *A. tonsa* have often been associated to increase in chlorophyll and turbidity in other coastal systems (Biancala *et al.*, 2014; Derisio *et al.*, 2014).

The role of temperature, salinity and chlorophyll appeared not to be so obvious, as described in other coastal ecosystems (Jeffries, 1962; Alcaraz, 1983; Gaudy *et al.*, 2000; Calliari *et al.*, 2006; Boyer *et al.*, 2013; Marques *et al.*, 2018; Svetlichny *et al.*, 2018; Villate *et al.*, 2018). Kimmel *et al.* (2012) showed that environmental and food web changes can be involved together to explain the decline in *A. tonsa* population in the central Chesapeake Bay. Then, a combination of site-specific factors has to be considered to explain modifications in *Acartia* assemblages (Lakkis, 1994; Calliari *et al.*, 2006). Trophic interactions, like competition and predation, can move out species from their optimal temporal and/or spatial distribution. Villate *et al.* (2018) emphasized a restricted seasonal distribution of *A. tonsa* due to a competitive pressure with *Acartia bifilosa* in the estuary of Urdaibai.

In conclusion, the rehabilitation processes have managed to reduce salinity fluctuations and maintain it above 15. These modifications led to a space and time partitioning of *A. tonsa* and *A. clausi* along the salinity gradient as described in other brackish water systems. Changes in the distribution of *Acartia* species following the rehabilitation processes constitute a sign of a hoped recovery. Thus, even if Acartiidae constitute an important link to higher trophic levels in brackish environments (Alcaraz, 1983; Azeitero *et al.*, 2005; Werbrouch *et al.*, 2016; Villate *et al.*, 2018), the contribution of both *Acartia* species to the food web may be different. Werbrouch *et al.* (2016) highlighted that the replacement of *A. clausi* by *A. tonsa* is detrimental for higher trophic levels as *A. tonsa* presents a lower content of fatty acids (*i.e.*, DHA and EPA) in membrane lipids for the same body size. In Berre Lagoon, the cephalothorax length of *A. tonsa* was significantly lower than the one of *A. clausi* with mean values of 0.98 ± 0.12 cm ($n = 272$ ind. for the 2010-12 survey) and 1.08 ± 0.06 cm ($n = 419$ ind. for the 2010-12 survey), respectively (MWU, $p < 0.001$; data not shown). Before the rehabilitation processes, the energy transfer was then likely less effective since *A. tonsa* was the only Acartiidae observed in the lagoon. The arrival of *A. clausi* in Berre Lagoon, resulting from salinity increase, could eventually drive to a rich and diverse pelagic ecosystem which is actually very damaged by the anthropogenic pressures of these last decades. For the moment, a positive influence on higher trophic levels is still expected as food web is dominated by gelatinous zooplankton considered as a trophic dead end, particularly the indigenous scyphozoan *Aurelia* sp. (Marques *et al.*, 2015) and two alien species (*i.e.*, the ctenophore *Mnemiopsis leidyi* (Delpy *et al.*, 2016) and the hydrozoan *Gonionemus vertens* (F. Delpy, June 2013, pers. obs.)). Nonetheless, the presence of some

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planktivorous fish whether inhabiting the lagoon (*e.g.*, goby and atherine) or coming from the Mediterranean Sea through Caronte Canal (*e.g.*, sardine and anchovy) can offer hopes for a next improvement (GIPREB, pers. com.). These results constitute a very first step in the long road to Berre recovery.

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Chapter 7

THE IMPACT OF CONSPICUOUS ENVIRONMENTAL CHANGES ON THE SPATIAL AND TEMPORAL DYNAMICS OF *ACARTIA TONSA* AND *ACARTIA CLAUSI*: A DECADAL STUDY IN A TEMPERATE ESTUARY (MONDEGO, PORTUGAL)

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ABSTRACT

This chapter aimed to identify the role of natural environmental factors in the distribution of the congeneric species *Acartia tonsa* and *Acartia clausi* in the Mondego estuary, central Iberian Peninsula, over the period 2003-2012. *A. tonsa* was the dominant species during the study period, representing 84% of the total abundance of *Acartia*. The distribution patterns of *Acartia* species revealed a spatial segregation along the estuary, with *A. tonsa* confined to upstream areas, while *A. clausi* was restricted downstream.

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Both species showed seasonal variation, peaking during warmer months. Since 2007, *A. tonsa* exibithed yearly averages consistently above the long-term mean abundance. The results identified a clear effect of temperature warming on the ecosystem, favoring and accelerating the settlement of the non-indigenous species *A. tonsa* at an unexpectedly rapid rate.

STATICO analysis revealed that 2007, 2010 and 2011 were years with strong temporal patterns, which best fitted the compromise analyses between *Acartia* abundance and environmental parameters. *A. tonsa* showed an overall positive relationship with temperature, salinity, and total suspended solids (TSS), while *A. clausi* presented a positive association with chlorophyll *a*. Local variability of freshwater flow was the dominant signal in the present time-series, and explained the observed dynamics in *Acartia* populations structure at shorter temporal scales. However, this effect could be masked by larger time-scale phenomena occurring simultaneously, such as a rise in water temperature, leading to a higher abundance of opportunistic tolerant-species, as *A. tonsa*.

These results suggest that copepods living in highly dynamic ecosystems are prone to shifts in community equilibria that show complex, non-linear responses to climatic oscillations.

Keywords: *Acartia tonsa*, *Acartia clausi*, Mondego estuary, environmental variability

1. INTRODUCTION

Zooplankton constitutes the ocean's essential secondary producers, representing a key link between primary producers and higher trophic levels (Turner, 2004). Their spatial and temporal variability are known to be linked with hydrological patterns, seasonal fluctuations, trophic status and pollution (*e.g.*, Kimmerer, 1993; Lawrence *et al.*, 2004; Uriarte *et al.*, 2005; Aravena *et al.*, 2009). Often, zooplankton communities are reported as more sensitive indicators of change than environmental variables themselves (Taylor *et al.*, 2002, Hays *et al.*, 2005), acting as integrators of hydroclimatic forcing and providing an accurate diagnosis of the ecosystem state (Beaugrand, 2005). Zooplankton is highly sensitive to short-term environmental variability, with studies reporting changes in zooplanktonic communities according to seasonal, tidal, and diurnal variations (*e.g.*, Kimmerer, 1993; Roman *et al.*, 2001; Lawrence *et al.*, 2004; Marques *et al.*, 2009; Menéndez *et al.*, 2012; Leandro *et al.*, 2014). Furthermore, zooplankton communities have been useful to track large-scale climate-driven environmental changes (Hays *et al.*, 2005; Richardson, 2008; Aravena *et al.*, 2009; Edwards *et al.*, 2013), and recent evidences of long-term studies indicate variations in the seasonal window and timing around the world (*e.g.*, Hinder *et al.*, 2012; Mackas *et al.*, 2012), as well as the reduction and redistribution of several species (*e.g.*, Hinder *et al.*, 2012; Edwards *et al.*, 2013; Gubanova *et al.*, 2014).

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Global climate change has led to a sustained increase in ocean temperatures in the last decades. In the North Atlantic, for instance, sea surface temperature (SST) has increased by about 0.5°C during the past 50 years (IPCC, 2013). Copepods play a fundamental role in the energy transfer to higher trophic levels, in the transport of organic matter to depth (*via* fecal pellets), in the dynamics of their main prey (phyto- and microzooplankton), and in the mineralisation of nitrogen and carbon *via* grazing (Turner, 2004). Thus, it is mandatory to know how these communities are affected by this increased climatic variability. Further, the phenotypic plasticity and tolerance to hydrological variability will also determine the zooplankton community structure in highly unstable ecosystems, such as estuaries (Dam, 2013).

Copepods clearly dominate the numerical abundance of zooplankton, particularly calanoid copepods (Mauchline, 1998). These small microcrustaceans graze upon phytoplankton and microzooplankton, including nauplii of their own class (Turner, 2004). Among calanoids, members of the genus *Acartia* exhibit a clear supremacy in the plankton communities across several temperate and subtropical estuaries (Brylinski, 1981; David *et al.*, 2007; Marques *et al.*, 2007) and other semi-enclosed marine areas (Kimmerer, 1993; Roman *et al.*, 2001; Leandro *et al.*, 2014). Their spatial and temporal variability are known to be linked with hydrological patterns, seasonal fluctuations, trophic status and pollution (*e.g.*, Kimmerer, 1993; Lawrence *et al.*, 2004; Uriarte *et al.*, 2005; Aravena *et al.*, 2009). Recently, non-native species of *Acartia* are efficiently colonizing many coastal areas and estuaries, increasing the pressure on autochthonous copepod communities (*e.g.*, Comaschi *et al.*, 2000; Seuront, 2005; David *et al.*, 2007; Delpy *et al.*, 2018; Villate *et al.*, 2018). The increase in ocean temperature has been pointed out as an important factor for the successful establishment of these non-native species (Aravena *et al.*, 2009; Chaalali *et al.*, 2013; David *et al.*, 2007). Non indigenous *Acartia* species have beeen responsible for changing the seasonal distribution pattern of zooplankton leading to a phenological shift in the native copepod *Acartia bifilosa* production period at the Gironde estuary (David *et al.*, 2007), the decreased abundance of the autochtonous *Acartia clausi* in the estuary of Bilbao (Aravena *et al.*, 2009; Villate *et al.*, 2018), or the complete replacement of formerly abundant *Acartia margalefi* in the South Adriatic Sea (Brugnano *et al.*, 2011). Changes in the ecosystem also favoured the non-native *Acartia* species to the detriment of the indigenous copepods in the early 1970s in the Black Sea (Gubanova *et al.*, 2014): in this case, increased pollution and eutrophication have been identified as the main factors determining the replacement of native species, rather than the increase in temperature (Gubanova *et al.*, 2014). Also, changes in salinity proved to be unfavorable for the autochthonous species of *Acartia* congeners in the Gironde estuary (David *et al.*, 2007) and in the estuary of Bilbao (Aravena *et al.*, 2009). Non-native species are extremely efficient at colonizing brackish waters, which are more vulnerable to the establishment of alien species owing to the greatest natural ‘indigenous species minimum’ (Paavola *et al.*, 2005).

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Relevant interannual variability observed in the weather conditions over the Iberian Peninsula attests to the decreasing trend in annual precipitation in Portugal (Rodrigo and Trigo 2007; Santos *et al.*, 2010). In the particular case of the Mondego estuary, located in the Portuguese Atlantic coast, lower annual precipitations have led to a reduction in river flow, influencing also the extent of the estuarine salinity gradient and of the spatial distribution of planktonic species (Marques *et al.*, 2008, 2014; Primo *et al.*, 2011, 2015). This highlights the strong influence of water exchanges with the Atlantic Ocean on the composition and dynamics of local pelagic communities (Bento *et al.*, 2016; D'Ambrosio *et al.*, 2016). As in other temperate estuaries, *Acartia tonsa* represents the main species of planktonic communities of this estuary, only outreached by its congener *A. clausi* (Marques *et al.*, 2006; Primo *et al.*, 2009). The congeners present different seasonal and spatial distributions patterns in the estuary and are generally linked with estuarine and marine water masses, respectively.

The main aim of this contribution was to identify the role of natural environmental factors in the populations of the coexisting species *A. tonsa* and *A. clausi* in the Mondego estuary over a 10-year period (2003-2012). To answer this objective, we analysed and described the influence of environmental factors (hydrology and biology) on *Acartia* species dynamics and assessed the effects of different environmental regimes on congeneric spatial segregation. Our approach reveals that variability of freshwater flow explained the observed dynamics in *Acartia* populations structure at shorter temporal scales. However, this effect could be masked by an increase in water temperature, leading to a higher abundance of opportunistic tolerant-species as *A. tonsa*.

2. METHODS

2.1. Study Area

The Mondego estuary (40º 08' N, 8º 50' W) is located in a warm temperate region on the west coast of Portugal (Figure 1). This system experiences a Mediterranean temperate climate and is classified as a mesotidal estuary. About 7 km from the sea, the river branches into 2 arms (northern and southern), which converge again near the mouth. The two branches exhibit different hydrographic characteristics: the northern branch presents a low residence time (< 1 day, Kenov *et al.*, 2012), is deeper (4–8 m during high tide), and is the location of the Figueira da Foz harbour, constituting the main navigation channel and being subjected to regular dredging activities. The southern branch is shallower (2–4 m deep, during high tide) with longer residence time (2–8 days) (Kenov *et al.*, 2012).

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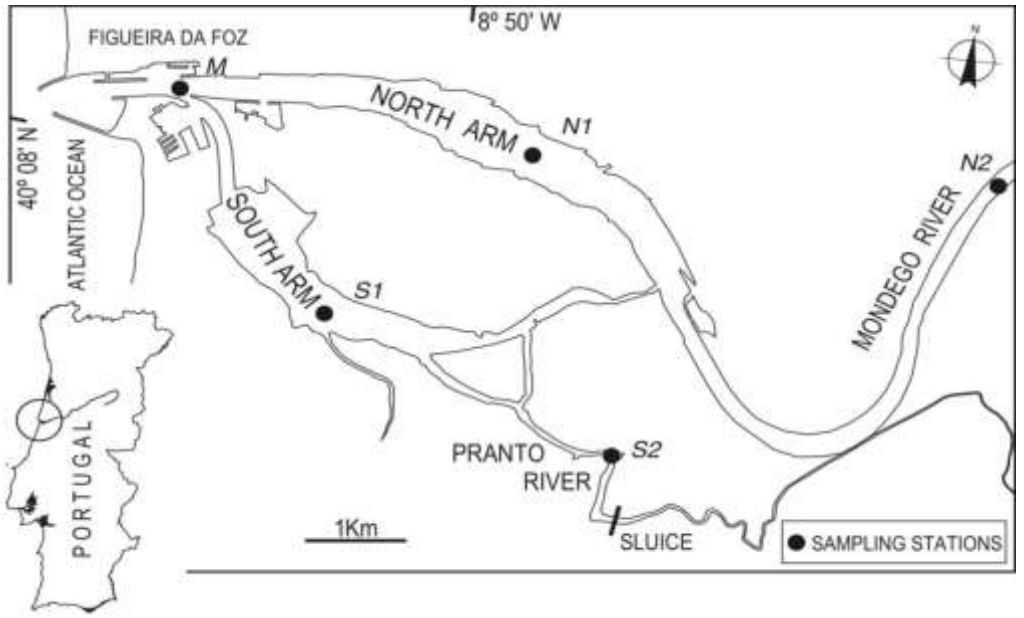

Figure 1. Map of the Mondego estuary showing the location of the sampling sites. M (mouth site), S1 and S2 (southern arm sites), N1 and N 2 (northern arm sites).

2.2. Environmental and Biological Data

Data on the abundance of *Acartia clausi* and *Acartia tonsa* cover a 10 year period (2003 to 2012) and were obtained from monthly samples at 5 stations distributed throughout both branches (M – mouth, N1 and N2 – northern branch, S1 and S2 – southern branch; M, S1 and N1– downstream areas, S2 and N2 – upstream areas) (Figure 1). This constitutes a unique time series dataset for an estuarine system of the West Iberian coast (which started in 2003), considering the consistency in the sampling methods and number of samples. Zooplankton samples were collected by subsurface horizontal hauls, using a 335 μm mesh bongo net (mouth diameter: 0.5 m). Subsequently, all collected organisms were fixed and stored with buffered formaldehyde (4%). The zooplankton analysis was carried out under a Leica M80 stereomicroscopice and subsamples (a minimum of 500 individuals were counted) were obtained for numerical abundance using a Folsom-splitter. Records of *Acartia* were only based on adults, since the mesh size used under-estimates the early life stages. Abundance data were standardized as the number of ind. m^{-3}.

In situ surface salinity and water temperature were recorded with appropriate sensors (WTW Cond 330i) simultaneously to zooplankton sampling. Water samples were also collected for determination of total suspended solids (TSS, mg l^{-1}; APHA, 1992) and chlorophyll *a* concentration (Chl *a*, mg m^{-3}; Parsons *et al.*, 1984) as a measure of total phytoplankton biomass.

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2.3. Statistical Analysis

Interannual variability of biological and environmental variables was represented by standardised anomalies (z-scores), computed as deviations from the mean of the time-series divided by the respective standard deviation. Additionally, the relationship between environmental variables was determined using the Spearman's non parametric correlation ρ, using SigmaPlot v12.5 (Systat Software). When applicable, results were presented as mean ± standard deviation (STD).

In order to investigate the influence of the environmental conditions on *Acartia* populations, species abundance and environmental parameters (temperature, salinity, Chl *a* and TSS) for each year and sampling site were combined to generate two series of tables: one for the environmental parameters and another for *Acartia* species densities. Each pair of tables corresponded to the same sampling year (in rows). Therefore, both tables (biological and environmental) were composed of 10 matrices (2003-2012). Prior to calculations, *Acartia* abundance was log(x + 1) transformed, in order to minimize the dominant effect of exceptional values, and environmental data were normalized.

In order to detect change points in *Acartia* abundance and environmental parameters, the cumulative sum (CUSUM) of the deviations from the mean of the reference period 2003 to 2012 were computed. The interpretation was based on the sign and steepness of the slopes, which reflect the deviation of a period from the time-series mean value (Ibañez *et al.*, 1993).

The STATICO method (Simier *et al.*, 1999; Thioulouse *et al.*, 2004) was carried out to analyze the two series of tables (Figure 2). In this chapter, the common structure between environmental and *Acartia* abundance tables and the stability of this structure over the sampling period were assessed. The samples, which must be the same for both paired tables but may vary between the pairs, correspond to a monthly sampling. The STATICO method proceeds in three steps: (1) the interstructure, which is achieved by performing a PCA for analyzing each table by a one table method (normed for the the environmental variables and centred for the species data); (2) the compromise analysis gives an ordination of the environmental parameters and an ordination of the *Acartia* species representing the average *Acartia*-environment relationship across the years; the compromise analysis links each pair of table Co-inertia analysis (Dolédec and Chessel, 1994) providing an average image of the co-structure (species-variables cross-table); and (3) the trajectories analysis involves the projection of the individual tables onto the axes of the compromise. This step allows visualisation of the similarities and differences amongst the years' structures *i.e.*, environmental and *Acartia* cycles. A Partial Triadic Analysis (Thioulouse and Chessel, 1987) was used to analyze the series of species and environmental variables cross-tables. STATICO allows building compromise maps composed of compromise axes on which observation (*i.e.*, monthly samples), species abundance and environmental factors from each original table are projected. Hence, it is

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possible to observe the correlation between species distribution and environmental factors. Calculations and graphs shown in this work were done using ADE-4 software (Thioulouse *et al.*, 1997; available at http://pbil.univ-lyon1.fr/ADE-4).

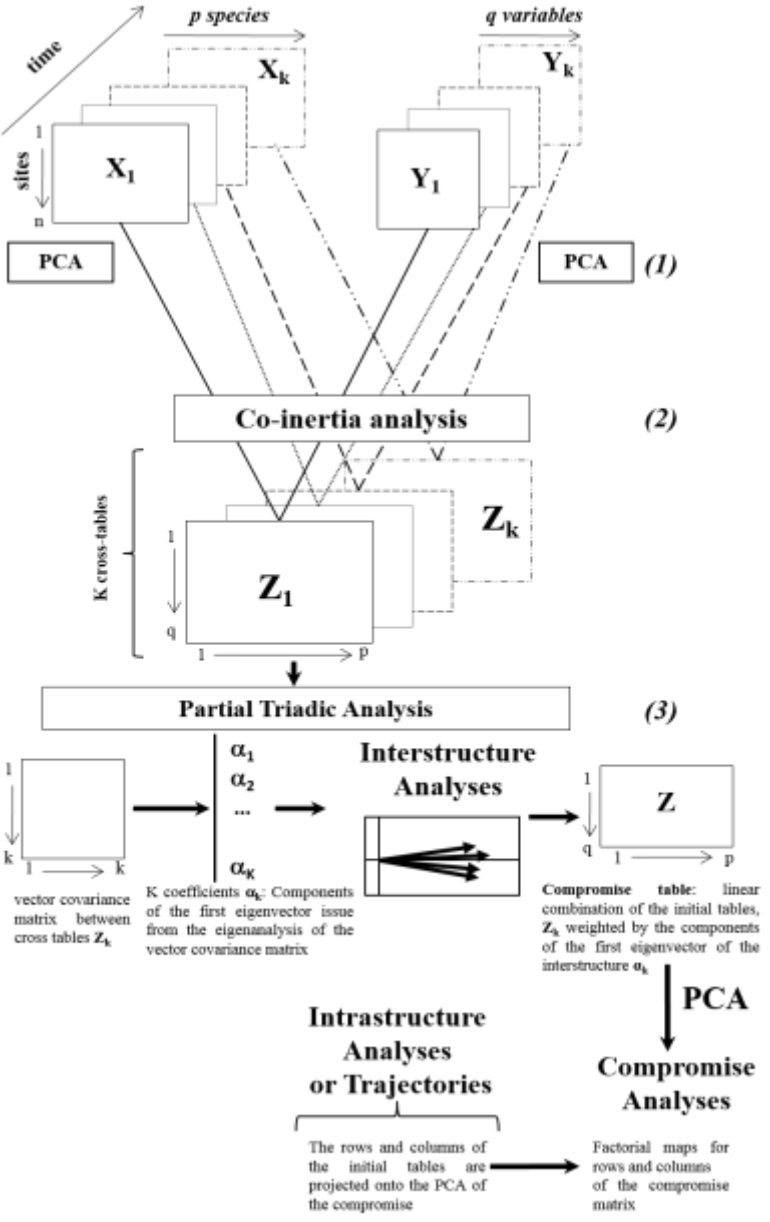

Figure 2. STATICO scheme. The data structure is a sequence of K paired ecological tables. X_k and Y_k are respectively the species (dimension n_k x p) and environmental (dimension n_k x q) tables in the pair. Z_k is the cross-table at k occurences; p is the number of species, q is the number of environmental variables, n_k is the number of rows at k different dates. (1) Basic analyses (PCA for species abundance and environmental tables) are performed on each table; (2) Co-inertia analyses allow linkage of the pairs of PCA-PCA, producing a sequence of K cross-tables; (3) PTA is finally used to analyze this sequence.

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All data regarding precipitation and freshwater runoff were acquired from the Portuguese Water Institute (INAG, www.snirh.inag.pt) stations Soure 13/01G and Açude Ponte de Coimbra 12G/01A (nearby the city of Coimbra, since no meteorological station was present in the study area). In accordance with the reports of the Portuguese Institute for Sea and Atmosphere (IPMA, https://www.ipma.pt/pt/), three major drought events were considered during the study period: 2004-2005, 2007-08 and 2012 (García-Herrera *et al.*, 2007; Trigo *et al.*, 2013).

3. RESULTS

3.1. Variabilty of Environmental Parameters and *Acartia* Abundance

The years sampled included a very wide range of environmental conditions, encompassing extreme climate events (Figure 3). Compared to the mean precipitation regime for central Portugal during 1981-2010 (winter: 92.4 mm; spring: 58.3 mm; summer: 8.8 mm; autumn: 87.1 mm, http://www.ipma.pt/pt/oclima/normais.clima), it was possible to distinguish three periods where drought regimes prevailed, namely 2004-2005, 2007-2008 and 2012 (García-Herrera *et al.*, 2007; Trigo *et al.*, 2013). According to IPMA, 2009 was considered a regular to dry year, while 2010 and 2011 regular years. The drought episode of 2004-2005 was considered the most severe in terms of meteorological data, extent of the area affected, and impacts on different socio-economic and environmental sectors (https://www.ipma.pt/pt/).

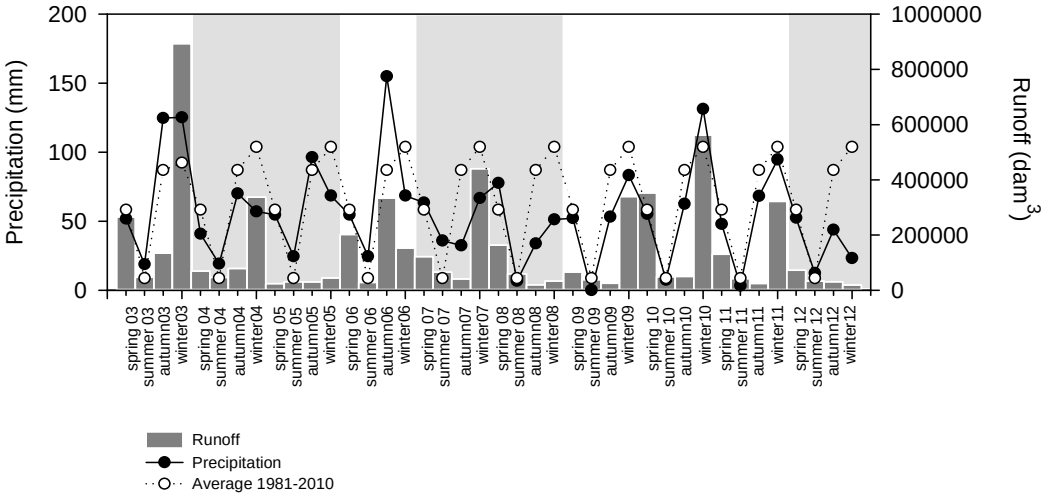

Figure 3. Seasonal precipitation (mm) and runoff (dam^3) for the 10-year study period (2003-2012) in the Mondego estuary. Shaded areas represent drought periods. Black circles – precipitation (mm), white circles – average precipitation for 1981-2010, and grey bars – runoff (dam^3).

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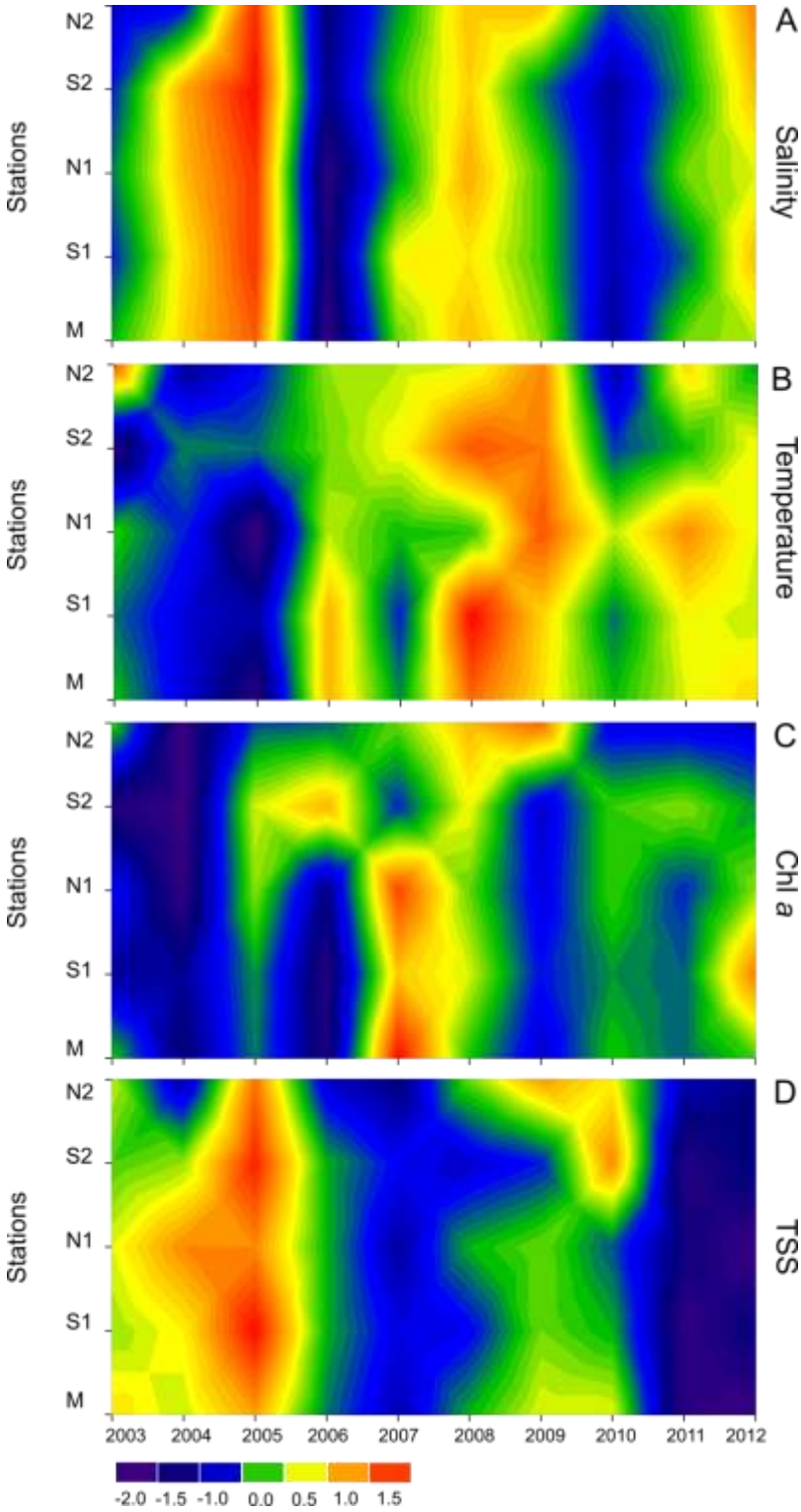

Figure 4. Standardized anomalies of mean annual (A) salinity (surface), (B) water temperature, (C) chlorophyll *a* (Chl *a*) and (D) total suspended solids (TSS).

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Table 1. Spearman correlations coefficient between the environmental parameters anomalies: temperature (°C, Temp), salinity (Sal), chlorophyll a (mg m^{-3}, Chl *a*), total suspended solids (mg m^{-3}, TSS), precipitation (mm, Precip) and runoff (dam^3). Significant *p*-values are in bold; **p* > 0.05; **0.05 > *p* > 0.01; ***0.01 > *p* > 0.001

	Sal	Chl *a*	TSS	Precip	Runoff
Temp	-0.164	0.136	**-0.413*****	-0.233	-0.104
Sal		0.229	0.204	**-0.612*****	**-0.812*****
Chl *a*			-0.166	-0.164	-0.245
TSS				**0.368****	0.073
Precip					**0.673*****

The variations of freshwater discharge into the Mondego estuary were clearly influenced by precipitation (Figure 3), showing a highly significant positive relationship between these two parameters (Spearman rank, $p < 0.001$, R = 0.67, Table 1). Maximum salinity values were typically observed during the lowest runoff periods described above, especially in 2004-2005, due to a drought started in 2004 and extended to an extreme event in 2005 (Figure 4A). In comparison, low salinity values were observed in 2003, 2006 and 2010, concomitant with the years of highest river runoff registered during the study period. The salinity anomalies values exhibited a highly significant negative correlation with runoff ($p < 0.001$, R = -0.81, Table 1) and precipitation ($p < 0.001$, R = -0.61, Table 1). Dealing with water temperature, the ten years presented the typical annual pattern for temperate ecosystems, with lower values in winter (9.0-15.2ºC) and higher in summer (15.4-26.7ºC) (data not shown). Positive anomalies were recorded in 2006 (particularly downstream), with highest positive deviation in 2008-09 and 2011-12 (Figure 4B).

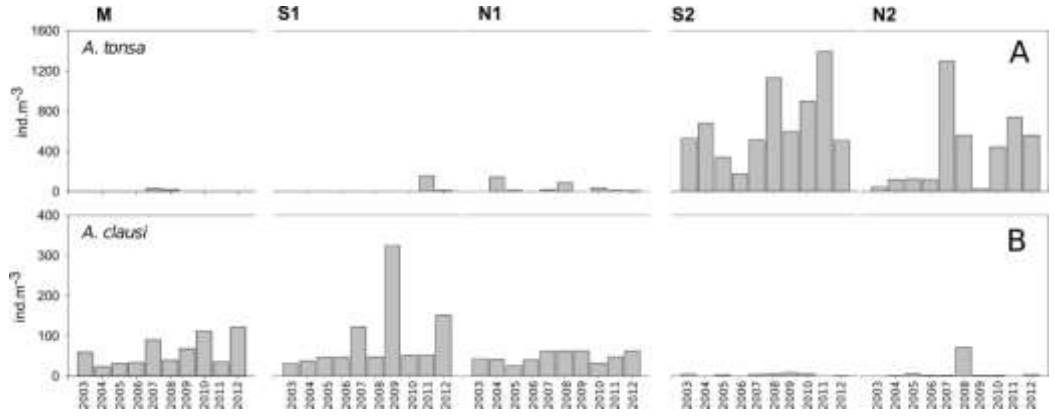

Figure 5. Variability of (A) *Acartia tonsa* and (B) *Acartia clausi* abundance (ind. m^{-3}) over the 2003-2012 period.

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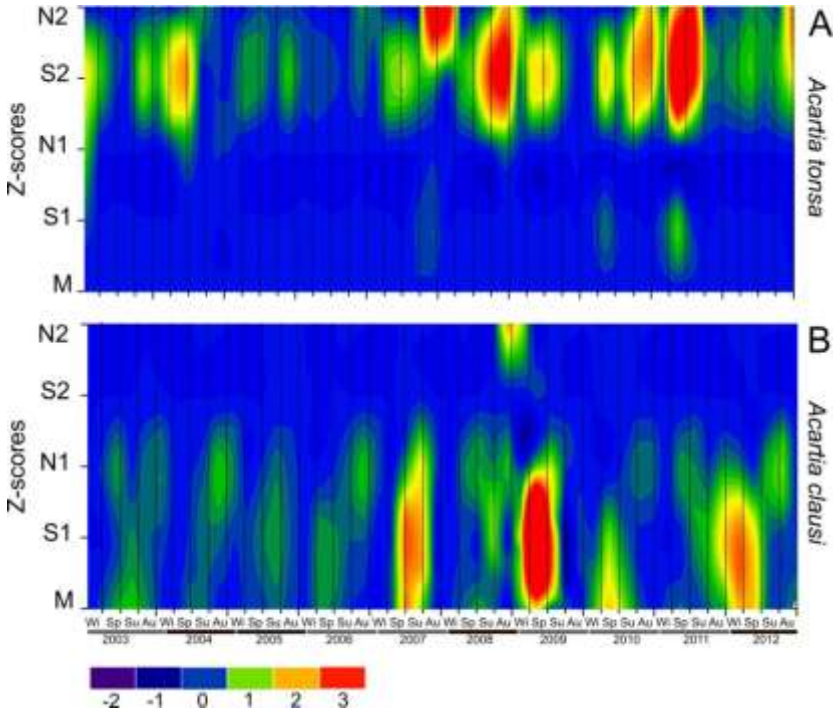

Figure 6. Z-scores of (A) *Acartia clausi* and (B) *Acartia tonsa*, abundance in the Mondego estuary over the 2003-2012 period (winter-Wi, spring- Sp, summer – Su, autumn-Au).

Chlorophyll *a* values varied between 0.9 and 29.9 mg m^{-3} (mean = 6.7 ± 5.4 mg m^{-3}) during the 2003–2012 period. This parameter was generally above the long-term average in 2007 (downstream), 2008 (upstream), and 2012 (downstream) (Figure 4C). Higher positive average of TSS were recorded between 2003 and 2005, and in 2009-2010, more pronounced at upstream, while the lowest were observed in 2007 and 2011-12. The correlation between TSS anomalies and water temperature anomalies was highly significant ($p < 0.001$, R = -0.41, Table 1).

Over the 2003-2012 period, *Acartia tonsa* was the dominant species, making up 84% of the total abundance of *Acartia* (Figure 5). The distribution pattern of *Acartia* species revealed a spatial segregation along the estuary. On one hand, *A. tonsa* was confined to the upstream areas, with an average annual density of 540.5 ± 401.0 ind. m^{-3} (Figure 5A). The anomalies in *A. tonsa* abundance revealed a cyclical variation, with peaks during the warming seasons (Figure 6A). After 2007, *A. tonsa* abundance increased considerably, with yearly averages consistently above the long-term mean. In autumn 2007-2008, 2010 and 2012, the species exhibithed higher amplitude of its seasonal maxima compared to other years (max. 4.089, 2.618, 2.075, 1.605 ind. m^{-3}, respectively) and extended its period of presence until winter in 2010 and 2012. On the other hand, its congener *Acartia clausi* was more restricted to the lower estuary (mean = 67.2 ± 58.0 ind. m^{-3}). A clear general pattern was observed over time, with a bimodal increase of *A. clausi* abundance in spring and summer. The highest positive anomalies were detected in summer 2007

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(377 ind. m^{-3}), spring 2009 (1,235 ind. m^{-3}), spring and winter 2012 (291 and 287 ind. m^{-3}, respectively) (Figure 6B). Noticeable changes also occurred in the phenology of *A. clausi*, with an earlier timing of seasonal peak after 2007, with the occurrence of major abundance peaks in late winter/early spring.

3.2. *Acartia* Variability in Relation to Environmental Parameters

3.2.1. CUSUM Analysis of Time-Series

Regarding environmental variability, water temperature showed two main periods, delimited by a breakpoint in 2008 (Figure 7A). The first period (2003-08) was characterized by lower seasonal values. Afterwards, an upward change in temperature was observed. Salinity showed higher variability: two main periods of positive slope, 2005-early 2006 and late 2007 until 2010, were highlighted by the cumulative sums (Figure 7B). Chl *a* presented a clear upward trend after 2007 (Figure 7C), while TSS showed positive slope in 2006-07 and during the period 2009-2012 (Figure 7D). As for *Acartia*, the abundance of both species exhibited a synchronized increase after 2006 and 2007, for *A. tonsa* and *A. clausi*, respectively (Figures 7E, F).

Overall, despite the variability in the CUSUM analysis, a significant positive correlation was observed between water temperature and Chl *a* ($R = 0.43$, $p < 0.001$), which may have also played a role in influencing the changes in *Acartia* abundance, since a positive correlation was also detected between them and Chl *a* (*A. clausi*, $R = 0.57$, $p = 0.001$ and *A. tonsa*, $R = 0.70$, $p = 0.001$). TSS showed negative significant correlations with *A. clausi* ($R = -0.59$, $p = 0.001$) and *A. tonsa* ($R = -0.53$, $p = 0.001$).

3.2.2. Interstructure Analysis of the Years

The first axis represented 94.5% of the total inertia (3.4% for the second axis, Figure 8A). Therefore, no structure inversion was evidenced from one sample to the other, and distribution patterns of *Acartia* were (at least partially) common to all sampling years. Weights (reflecting the contribution of each sub-matrix in the construction of the compromise) and cos^2 (indicating of how much the compromise expresses the information contained in each table) values (Table 2) showed that each year shared characteristics with the compromise, despite this has some individual features. From the analysis of the correlation and weights (Table 1, Figure 8), the common temporal pattern appeared to be stronger in 2007, 2011, 2010 and 2006 (higher weight and longer arrows), exhibiting a strong association with PC1; this in turn indicated similar structures and meant that the compromise would be more influenced by these years. The years of 2004, 2005, 2008, 2009, 2012 presented lower weight (short arrows) indicating that their corresponding tables were less structured and consequently their contribution to the compromise was lower.

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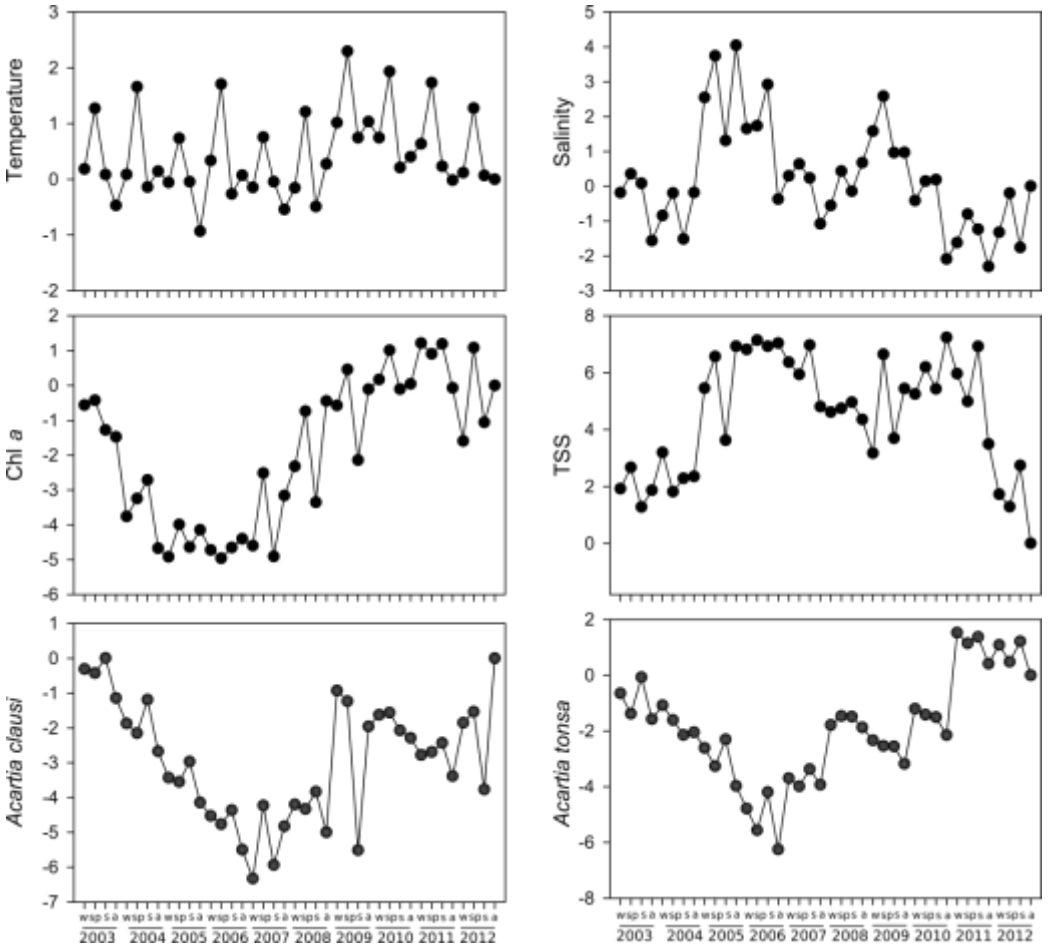

Figure 7. Cumulative sums of normal standard deviates of (A) water temperature, (B) salinity, (C) clorophyll *a* (Chl *a*), (D) total suspended solids (TSS), (E) *Acartia clausi* abundance (F) *Acartia tonsa* abundance; 2003 to 2012 (winter -w, spring - sp, summer-s and autumn-a).

Table 2. Correlation matrix and typological value indices: Weights = weights of tables in the compromise; cos^2 = square cosine between table and approximated compromise

Year											Weights	cos^2
2003	1000										0.277	0.629
2004	230	1000									0.116	0.081
2005	712	293	1000								0.273	0.621
2006	601	65	571	1000							0.387	0.572
2007	623	61	611	496	1000						0.456	0.645
2008	434	70	378	383	412	1000					0.206	0.250
2009	440	200	447	524	354	175	1000				0.245	0.400
2010	573	306	571	509	509	410	592	1000			0.403	0.622
2011	589	269	638	503	666	393	420	501	1000		0.407	0.640
2012	476	247	537	462	555	241	422	569	525	1000	0.218	0.477

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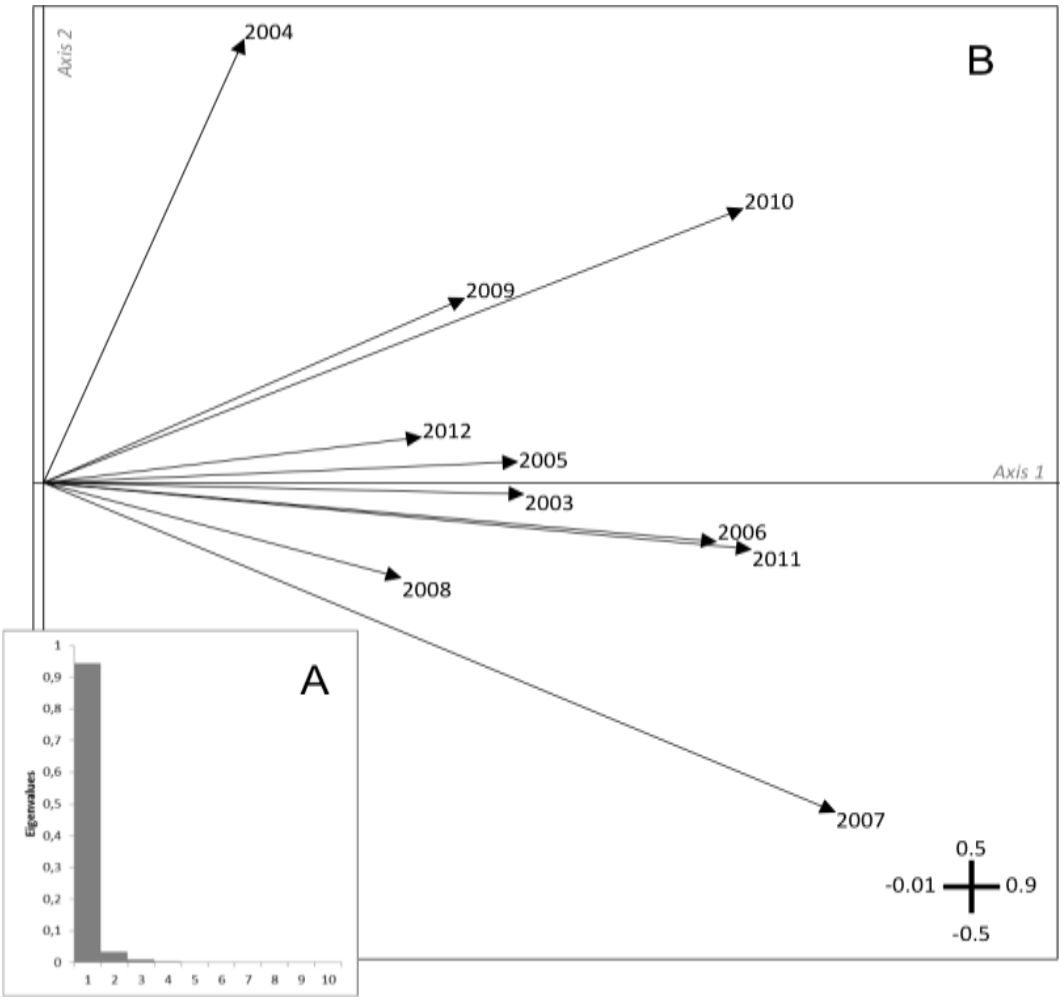

Figure 8. Results from the interstructure analysis. (A) Histogram of eigenvalues. (B) Factorial map for years. The contribution of sampled years to the compromise map is indicated by the strength of association with each principal component (PC1, x-axis; PC2, y-axis) extracted from the analysis. The y-axis is the second principal component (PC2). Scales for axes is given in the bottom right corner.

3.2.3. Compromise Analysis

The factor map of the first two axes are shown for the *Acartia* populations (Figure 9A) and environmental variables (Figure 9B). Axis 1 accounted for 53.9% of the explained variance and differentiated the samples with higher and lower abundance, whilst Axis 2 (10.63% of the explained variance) indicated a clear separation in the distribution patterns of *A. clausi* and *A. tonsa* within the estuary (Figure 9A). As can be seen from the environmental compromise analysis (Figure 9B), water temperature and salinity were displayed on the positive section of Axis 1, confirming that high abundance were typically observed in years of higher salinity and temperature. Overall, chlorophyll *a* was positively linked to Axis 2, whereas TSS was negatively associated to this axis.

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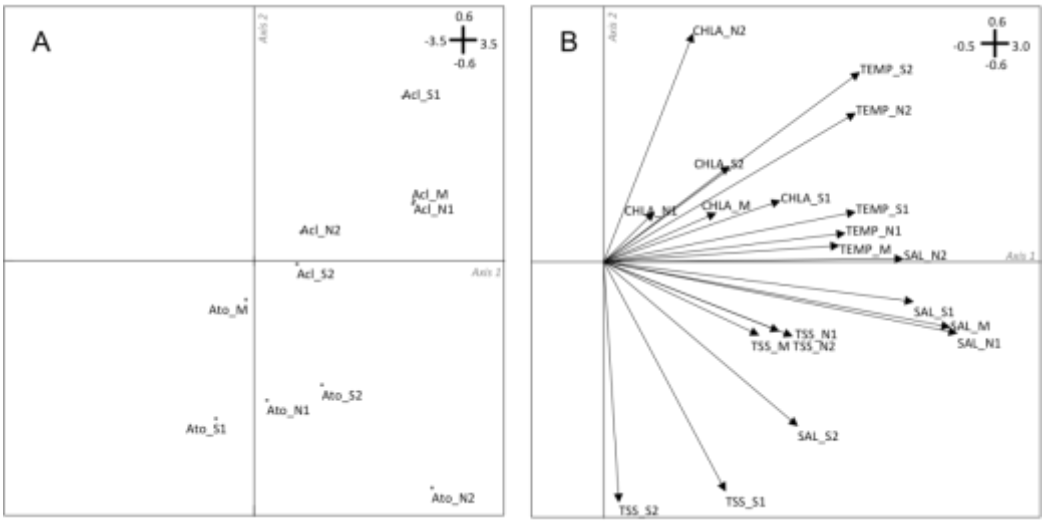

Figure 9. Compromise factor maps of the STATICO analysis. This plot represents the typical (A) *Acartia* population structure and (B) environmental structure in the Mondego estuary. Ato – *Acartia tonsa*, Acl– *A. clausi*, CHLA – chlorophyll *a*, TEMP – water temperature, SAL – Salinity, TSS – total suspended solids. The scales for axes are given in the right upper corner.

In the downstream areas of the estuary (sites M, S1, N1), the higher abundance of *Acartia* were associated to *A. clausi*, whereas in the upstream areas (sites S2 and N2) they were associated to *A. tonsa*. These results confirmed the positive relationship between *Acartia* abundance with temperature and salinity. Axis 2 also permitted to evidence the positive association between Chl *a* and *A. clausi*, while *A. tonsa* was more related with TSS.

3.2.4 Trajectories Analysis

The projection of the environmental variables and *Acartia* abundance on the compromise axes is showed in the factorial map of trajectories (Figure 10). High interannual variability in the *Acartia* populations and environmental conditions was evidenced, and differences between years were predominantly driven by variablity of salinity and temperature. The characteristic structure of the species-environmental dynamics, revealed by the compromise analysis, was well expressed in 2007, 2010 and 2011, which corresponded to the highest abundance peaks of *A. tonsa*. A different scenario emerged in 2003, 2005 and 2009, when a spatial segregation in *Acartia* species abundance was not so evident.

The co-structure analysis (divided according to sampling years) clearly showed the dynamics of the *Acartia* species-environment relationships and highlighted seasonal differences (Figure 11). Whatever the date, the species points (circles) were more stable than the environmental points. This expresses the steady establishment of the *Acartia* assemblages, despite the high environmental variability (salinity and water temperature, in particular). The end of the arrows (environmental variables) had comparatively

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different values (generally presented separated) and simultaneously close *Acartia* abundances (the circles were in general in the same area). Indeed, considering the species, the sites were regularly projected on the right-hand side of the first axis, characterized by the highest *Acartia* abundances (see Figure 9A).

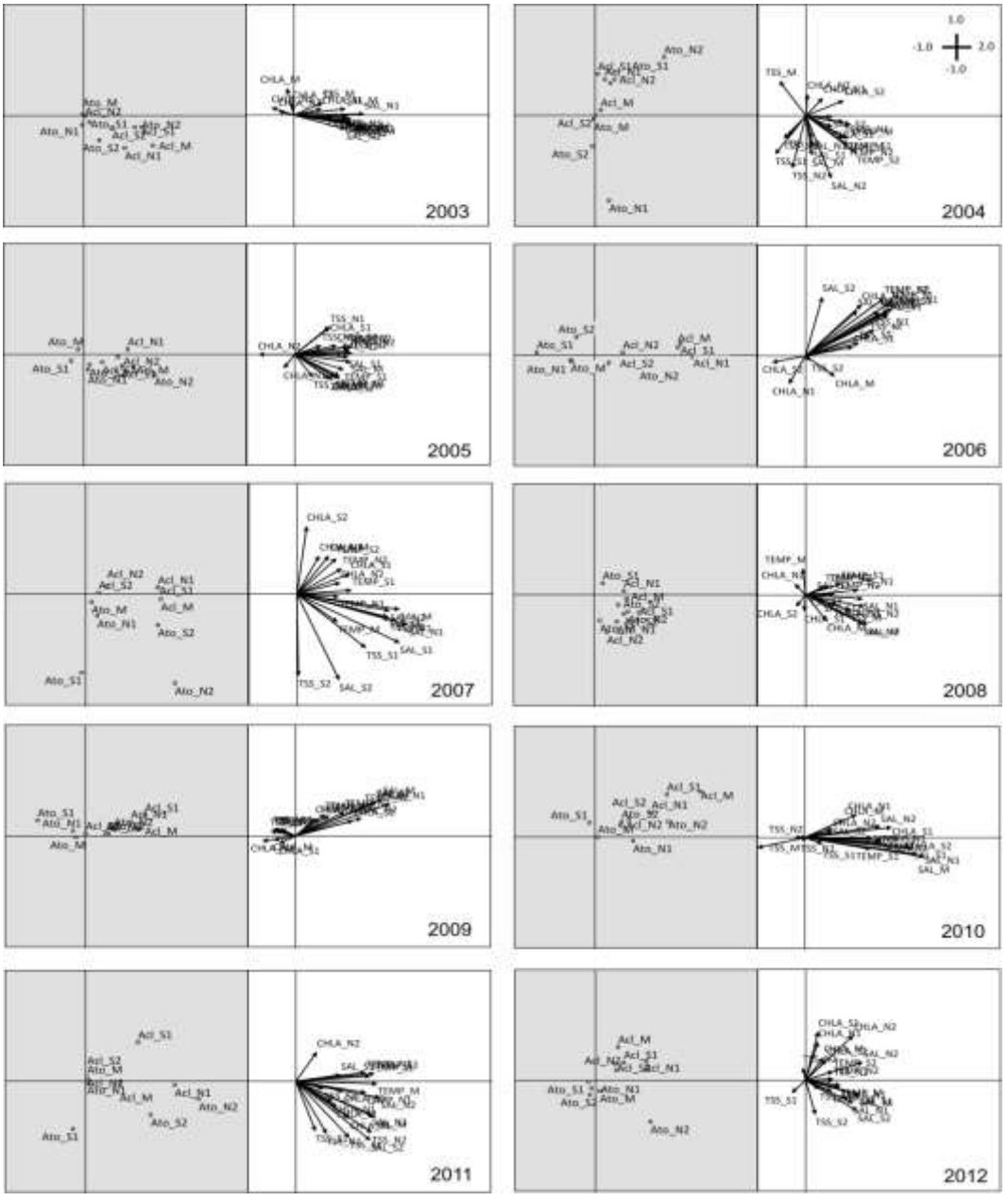

Figure 10. Factorial map of trajectories for species (grey) and environmental parameters (white). Depicted are annual projections of *Acartia* abundance and environmental variables along the two principal components of STATICO analysis. Scale for axes are given at the top right corner.

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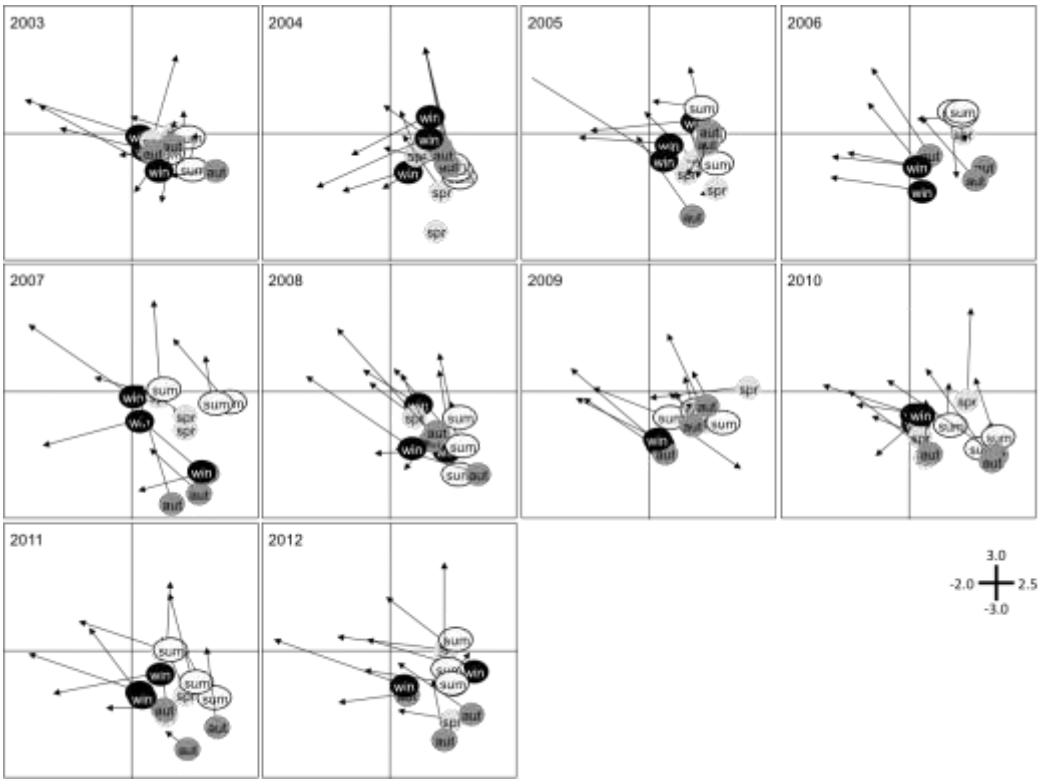

Figure 11. Trajectories factor plots of the STATICO analysis: projection of the samples in respect to seasons on the first factorial plan of the compromise analysis. Each sample is represented by two points: one is the projection of the row of the *Acartia* table (circle: origin of arrows), and the other is the projection of the row of the environmental table (end of arrows). The length of the connecting line reveals the disagreement or the consensus between the two profiles (*Acartia*–environment) *i.e.*, the length of the line is proportional to the divergence between the datasets. When the datasets strongly agree, the arrows will be short. Likewise, a long arrow demonstrates a locally weak relationship between the environment and *Acartia* features for that case. The scales for axes are given in the right lower corner.

In general, and notwithstanding the strong dispersion of the environmental points and a poor fit between the *Acartia* species and environment parameters (long arrows), summer, autumn and winter were regularly grouped together, expressing a better consensus in the species-environment relationship. In fact, this was most clear (in general) for 2007, 2010 and 2011. Overall, in winter the environmental points (given by the end of arrows) were located on the left-hand side of the first axis, corresponding to "lower environmental values". On the other hand, environmental points (end of arrows) corresponding to summer (followed by autumn) were located on the right-hand side of the first axis, which means "higher environmental values". In sum, species and environmental parameters denote the absence of a clear coupling between environmental and copepod trajectories.

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CONCLUSION

Estuaries are higly dynamic and variable environments, which promote not only a low level of species diversity, but also the co-existence of congeneric species. Our results revealed considerable complexity in the processes structuring the dynamics of the two congeneric *Acartia* species, *Acartia clausi* and *Acartia tonsa*, which represent nearly 60% of the total zooplankton abundance of the Mondego estuary (Marques *et al.*, 2006), a shallow coastal ecosystem under the influence of river discharge. In these ecosystems, zooplankton abundance is characterized by a high degree of spatial and temporal variability (Kimmel, 2011). Our results showed the existence of a spatial segregation between congeneric species: *A. clausi* was restricted to the downstream areas where salinity is relatively higher, while *A. tonsa* occurred at upstream sections of the estuary. Environmental pressures characterized in the present chapter (*e.g.*, salinity, temperature and chlorophyll *a*), together with biotic and physiological aspects already observed by other authors (*e.g.*, Gaudy *et al.*, 2000; Leandro *et al.*, 2014), explain the spatial distribution pattern for these two species at the Mondego estuary.

A. tonsa was first observed in the estuary in 1994 by Azeiteiro *et al.* (1999), and since then persistent proliferation and swarming demonstrate the adaptive behavior of this species. In this sense, Gaudy *et al. (*2000) described the influence of temperature and salinity on the metabolism of *A. clausi* and *A. tonsa* and concluded that, for several temperatures tested, at the salinity of 35 psu, respiration rates were lower in *A. clausi* than in *A. tonsa*, with the contrary being observed at the lowest salinity. High respiration rates reflect the existence of some physiological constrains (*e.g.*, osmotic pressure) that are overtaken through costly energetic processes. If more energy is available for growth and reproduction, that will be reflected on the population structure of a given copepod species – higher abundance and high reproductive rate. A dense population of *A. tonsa* occurring at a specific location as a result of the combination of optimal environmental conditions (temperature, salinity and food) will have a negative effect on other pelagic copepod population like *A. clausi* given its predatory impact on other copepod species.

Results from the present work corroborate findings suggesting that *A. tonsa* is an opportunistic-tolerant species, which can take advantage of thermal increase. An increment in water temperature has been suggested as a key factor to explain the introduction of *A. tonsa* in some estuarine ecosystems (Kimmel and Roman, 2004; David *et al.*, 2007). According to Leandro *et al. (*2006 a, b), *A. tonsa* has a stronger response to temperature than the majority of marine and estuarine calanoid copepods, including *A. clausi*, exhibiting higher growth rates and lower developmental times. By developing faster, *A. tonsa* populations will be able to dominate the estuarine zooplankton community. Coastal waters have warmed during the last century, leading to profound consequences for the dynamic regime of coastal ecosystems (Scavia *et al.*, 2002; Goberville *et al.*, 2010). In fact, the abundance of this species showed an increase after

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2007-2008 in the inner upstream areas of the estuary, concomitantly with an increase in water temperature and chlorophyll *a* concentration. In the western Iberian coast, previous studies showed that hydrographic modifications covary with the secular trend of both sea surface temperatures and the North Atlantic Oscillation (NAO) (Pérez *et al.*, 2010). Indeed, the interannual changes of the NAO showed enhanced variance (twofold higher) after 2008 that encompassed a dramatic drop in the NAO during 2010–2011, followed by a marked reversal change of the signal (Marques *et al.*, 2017). In turn, the physical environment in the Mondego estuary showed prominent monthly variations of hydrological conditions that exhibited a larger variance around 2007–2010 as well. This suggests a close link between the environmental conditions at the Mondego estuary and the NAO through the influence of the later on regional atmospheric variables in the Northeast Atlantic coast, such as temperature, atmospheric pressure, wind and precipitation, and whose variability permeated into the environmental conditions in the estuary, as suggested by the temporal patterns of autotrophic and heterotrophic communities (Marques *et al.*, 2017). Overall, the observed structural change in the *Acartia* populations lies in the direction of pervasive structural changes in Northeast Atlantic coastal ecosystems. Indeed, increased abundances of copepod cosmopolitan species in the western English Channel have been shown to be linked to hydroclimate changes acting in the North Atlantic Ocean (Reygondeau *et al.*, 2015).

The STATICO approach used in this work proved to be an efficient tool to analyse sequences of paired ecological tables. This technique has previously been used by Simier *et al.* (2006) to study the spatial and seasonal variability of fish assemblages in Gambia estuary. Recently, Mazzocchi *et al.* (2012) applied this statistical method in planktonic communities in order to describe the variability of copepod assemblages in relation to local environmental dynamics. In both works, it was possible to visualize the variations in the species distribution and abundance patterns as a function of different environmental scenarios. As already demonstrated by Mendes *et al.* (2011), the STATICO advantage over other classical methods, such as canonical correspondence analysis (CCA) and redundancy analysis (RDA) commonly used in community ecology, is that it provides a complete and consistent analysis framework and the results presented are thus interesting and important for understanding estuarine dynamics. The findings reported in the STATICO compromise analysis suggested that water temperature and salinity were key factors determining the inter-annual occurrence and abundance of *Acartia* species in the Mondego estuary. In estuaries with a wide range of brackish habitats, salinity is thought to determine the spatial segregation of *Acartia* species (*e.g.*, David *et al.*, 2007; Aravena *et al.*, 2009; Leandro *et al.*, 2014). During regular years (in sense of precipitation) the Mondego Estuary presented a dynamic horizontal salinity gradient, with values increasing gradually from the inner to the lower estuary. These hydrographical conditions induced a spatial segregation in the distribution of *A. tonsa* and *A. clausi*.

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A. clausi is a neritic species that occurs in the Mondego estuary as a result of water mass transport, namely advection during the flood, and its population dynamics is not directly dependent on the hydrological estuarine characteristics. The relatively stability of nertitic waters is reflected on the constant occurrence of *A. clausi* at the most downstream station of Mondego estuary between 2003-2012. Despite the evidence that *A. clausi* can tolerate a broad range of salinities (*e.g.*, Cervetto *et al.*, 1999; Gaudy *et al.*, 2000), our results indicate that a decrease in salinity had an unfavourable effect on *A. clausi* populations, which was revealed by their distribution restricted to the outer part of the estuary. This agrees with observations in other estuarine systems of the European Atlantic coast, where *A. clausi* populations reach higher densities at higher salinity areas (*e.g.*, Uriarte *et al.*, 2005; Albaina and Irigoien, 2007).

In Portugal, a high inter-annual variability and irregular distribution of precipitation was observed between 2003 and 2012, which was responsible for altered hydrological regimes in the Mondego estuary. As a result, drought conditions gave rise to prolonged periods of reduced freshwater inflow. This is in accordance with regional climate models that estimate sligh precipitation decreases for southern Europe by the end of the 21st century (Miranda *et al.*, 2006; IPCC 2007), including in the Iberian Peninsula (Lehner *et al.*, 2006). The reduced river flow, caused by the general evolution of climate conditions, was an important factor that induced the observed trends in *A. tonsa* and *A. clausi* dynamics in the Mondego estuary. First, it is well known that the diffusive and advective properties of freshwater discharge play a critical role in the population distribution patterns and richness, as well as in their temporal variability (*e.g.*, Licandro and Ibanez, 2000; Sundby, 2000; Roman *et al.*, 2001; Lindley and Daykin, 2005). During dry years, the lower advective transport also allowed stabilization of the environmental estuarine conditions, and the recolonization of important species such as *A. tonsa* (Marques *et al.*, 2007).

However, the success of an invader population over time cannot be linked solely to abiotic parameters such as temperature or salinity. Other important factors in the Mondego estuary were total suspended solids and clorophyll *a*. These can be helpful to explain the lower abundance of *A. tonsa* at the downstream stations. The low concentrations of appropriate food in seawater (Paffenhöfer and Stearns, 1988) and the qualitative nature of food (less proteinic material in marine seston) (Gaudy *et al.*, 2000) could explain the difficulties of *A. tonsa* to develop in coastal marine waters, because of its inability to filter sufficient food at lower concentrations. Therefore, it is reasonable to consider that processes such as interspecific competition and distinct feeding strategies might also have played a significant role in the observed distribution patterns of the two copepod species.

In sum, local variability of freshwater flow was the dominant signal in the present time-series, and explained the observed dynamics in *Acartia* populations structure at shorter temporal scales. However, this effect could be masked by larger time-scale

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phenomena occurring simultaneously, such as a rise in temperature, leading to a higher abundance of opportunistic tolerant-species as *A. tonsa*. Indeed, more than one mechanism was probably operating simultaneously, thus establishing the complex nonlinear relationships between climate variability and zooplankton dynamics (Hays *et al.*, 2005), an issue that needs to be assessed based on longer observation periods.

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Chapter 8

TEMPERATURE, SALINITY AND OXYGEN CONCENTRATION IN LIFE CYCLE TRAITS OF THE BLACK SEA COPEPODS

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ABSTRACT

In the Black Sea copepod species from marine and coastal areas (including Ponto-Caspian and boreal relicts, old Mediterranean immigrants and recent Atlantic and Indo-Pacific invaders), the salinity tolerance ranges and osmotic responses to salinity changes were estimated. Also, the effect of temperature and dissolved oxygen concentration on respiration rate, feeding, growth and locomotion activity was studied in these copepods. In comparison with native marine species, conspecific alien copepods possessed wider salinity tolerance ranges and were able to osmoregulate. Moreover, the Ponto-Caspian relict *Calanipeda aquaedulcis*, capable to survive as in fresh as in hypersaline water, was found to be an exceptional osmoconformer over the salinity range of 0.2 to 40 psu. A population strategy of overwintering (unknown for cyclopoid copepods) was first reported for the warm-water Indo-Pacific cyclopoid *Oithona davisae*. The unique specific adaptation to local temperature and oxygen regimes was shown in *Calanus helgolandicus* (dominating species in the open zone of the Black Sea). Due to diel vertical migrations

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from surface warm oxygenated layers to deep cold hypoxic zones, the late developmental stages of this species can decrease their energy requirements and increase the duration and efficiency of lipid accumulation in the form of wax esters. Therefore, the values of definitive body size, lipid amount and productivity in the Black Sea *C. helgolandicus* are as high as those in *Calanus* species from the more productive North Atlantic seas. This review aims to synthesize the understanding of the processes of behavioral and physiological adaptation of the copepod species to the highly diversificated Black Sea environment.

Keywords: Copepoda, effect of temperature, salinity, oxygen concentration, Black Sea

1. INTRODUCTION

The Black Sea is nowadays the world's largest brackish and anoxic semi-isolated basin. Mean salinity of the sea upper layers amounts to about 18 psu (nearly half than that of the surface layers of the World Ocean). As a rule, water salinity decreases near the river creeks down to 15 psu and increases up to 22 psu at the depths bordering upon the hydrogen sulfide zone. In the coastal areas, the temperature varies from 0°C in winter to 28 – 29°C at maximum summer insolation. However, even in summer the upper layer temperature of the Black Sea may change sharply by 15 – 20°C due to the winds (Zaitsev, 2006). In the deep-sea regions, the cold intermediate layer (CIL) with the temperature of 6 – 8°C is located permanently under the upper quasi-homogeneous zone at the depth of 30 – 100 m. Below the CIL, temperature and salinity increase slightly whilst oxygen concentration decreases dramatically down to zero at a depth of 100–200 m, where a permanent halocline separates oxygenated waters from the sulfide-rich deep waters (Sorokin, 1983).

Copepods constituting the major Black Sea mesozooplankton fraction play the key role in transferring the primary production to higher trophic levels. Modern taxonomic composition of the Black Sea copepods (Gubanova *et al.*, 2014) reflects the general geological processes and modern trends of ecosystem formation (Mordukhay-Boltovskoy, 1960; Zaitsev and Alexandrov, 1998; Zaitsev and Oztiirk, 2001). According to the origins of the species, copepods inhabiting open and coastal Black Sea regions can be divided into six groups:

1) Mediterranean-Ponto-Caspian relict *Calanipeda aquaedulcis* (Grindley, 1969 and 1984). The most ancient inhabitant and primitive form is found in the waters with low salinity.
2) Palaearctic halophylic species like *Arctodiaptomus salinus* (Boxshall and Defaye, 2008) living in salty and brackish inland lakes, common in the mouths of rivers, discharge areas and estuaries of the Black Sea (Mordukhay-Boltovskoy, 1972) and also in the hypersaline lakes of Crimea (Belmonte *et al.*, 2012).

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3) Boreal-Atlantic immigrants: *Calanus helgolandicus* (*euxinus*)*, *Pseudocalanus elongatus* and *Oithona similis*, because in the Black Sea these three species form the united deep-sea complex of cold-water copepods (Nikitin, 1929). Probably, in the period of melting of glaciers, the cold waters brought by rivers from the northern seas filled the ancient Black Sea (Polischuk, 1984; Zaitsev, 2006). This fact is in agreement with the results of Unal *et al.* (2006) reporting the absence of substantial genetic differentiation between the Black Sea *C. helgolandicus* and *P. elongatus* and the same species from the English Channel. According to Papadopoulos *et al.* (2005), the divergences between the Mediterranean and the Black Sea populations of *C. helgolandicus* are much older than the estimated dates of colonization of the Black Sea. Perhaps, the last connection between the North and Black Seas could have been about 2800 - 2700 BP in the Late Holocene (Polischuk, 1984). Nowadays, *C. helgolandicus, P. elongatus* and *O. similis* constituted more than 90% of the Black Sea mesozooplankton inhabiting open zones of the sea.
4) Mediterranean immigrants like *Paracalanus parvus*, *Centropages ponticus*, *Acartia clausi*, *Pontella mediterranea*, *Anomalocera patersoni* that have invaded and ultimately adapted to local environmental conditions after the connection of the Mediterranean and Black Seas. These species form up to 90% of the total mesozooplankton in the Black Sea coastal area. Most of them prefer warm upper layers of the sea. This group also includes *Acartia margalefi*, *Paracartia latisetosa* and *Oithona nana*, recently they have disappeared from the Black Sea (Gubanova *et al.*, 2014).
5) Freshwater species: mainly calanoid copepods of the family Diaptomidae (Samchyshyna, 2011) and Cyclopoida introduced by river discharges, which usually occur in the sea water during the maximum river run-off.
6) Alien species *Acartia tonsa* and *Oithona davisae* introduced by ship ballast waters from the countries where these species are distributed. *A. tonsa* was transferred to the Black Sea via Baltic-Black sea connection (Belmonte *et al.*, 2011) probably starting from the North American Atlantic coastal regions which are considered to be a native area of this species (McAlice, 1981). *O. davisae* is a representative of the Indo-West Pacific Oithonidae (Ferrari and Orsi, 1984) which are distributed widely all over the world after the synanthropic introduction, probably from Japanese coastal waters.

Since the Mediterranean Sea (which connects the Black Sea with the Atlantic Ocean) is a highly saline and oxygenated water body with moderate temperature regime, one could suggest that the cold, brackish and hypoxic Black Sea was inhabited by the eurybiotic species. This hypothesis may be tested by studying the adaptive potential of

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the Black Sea copepods and mechanisms of their physiological adaptation to salinity, temperature and oxygen changes.

Table 1. Capture location and sampling and experimental conditions of studied species

Species	Capture location	Habitual conditions		Experimental conditions		
		Temp. (°C)	Salinity (psu)	Temp. (°C)	Salinity (psu)	Dissolved Oxygen (mg O_2 L^{-1})
*Calanipeda aquaedulcis**	Coastal salt lakes of the Black Sea	20 - 22*	18*	20 - 22	0 - 60	0.15 – 9.8
*Arctodiaptomus salinus**		20 - 22*	18*	20 - 22	0 - 70	0.22 – 9.8
Calanus helgolandicus	Ionian Sea	15	39	20	18 - 39	7
	Marmara Sea	15 - 20	22 - 38	20	18 - 40	7 - 9
	Black Sea	8	18	2 - 24	10 - 40	0.15 - 10
Pseudocalanus elongatus	Black Sea	8	18	6 - 22	18	7 - 10
Acartia clausi	Marmara Sea	18 - 22	20 - 22	20 - 22	22 - 39	7 -9
Acartia clausi	Black Sea	18 - 25	17 - 18	20 - 22	2 - 45	6.5 - 10
Acartia tonsa		18 - 25	17 - 18	20 - 22	0 - 70	5 - 9
Oithona similis		8 - 10	18	8 - 10	7 - 33	8 - 10
Oithona davisae		18 - 25	17 - 18	6 - 28	0 - 60	5 - 9
Oithona nana	Marmara Sea	20 - 25	18 - 20	20 - 22	10 - 32	7 - 9

*Cultured for 4 years under laboratory conditions

Nikitin (1926) was the first to determine the temperature preferences of mass copepod species of the Black Sea. Nikitin and Malm (1932) studied their tolerance to oxygen, hydrogen ions and carbon dioxide concentrations. Kovalev (1966) investigated the effect of salinity on survival of the mass Black Sea copepods. The influence of temperature on growth and development of copepods was investigated by Sazhina (1987). Svetlichny (1989) analyzed the locomotor activity of *C. helgolandicus* under different temperatures.

This review aims to synthesize the understanding of the processes of behavioral and physiological adaptation of the key copepod species to the highly diversified Black Sea environment. In particular, the impact of changing salinity, temperature and oxygen concentration on survival, respiration rate, moving behavior, egg formation and lipid accumulation will be discussed for native and introduced species. This work is based on

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laboratory and field studies carried out by the Department of Animal Physiology and Biochemistry, Institute of Biology of the Southern Seas (IBSS) from 1984 to 2014.The list of the studied species and experimental conditions used are given in Table 1.

2. SALINITY TOLERANCE OF THE BLACK SEA SPECIES AND THEIR CONGENERIC POPULATIONS FROM THE MARMARA AND IONIAN SEAS

Salinity tolerance was studied in females of all the species listed in Table 1 (except *Pseudocalanus elongatus*). The experiments were carried out on board of research vessels "Professor Vodyanitsky," "Bilim" and "Knorr" during the cruises and in the laboratories of the Institute of Biology of the Southern Seas (Sevastopol), Istanbul University (Turkey) and Marine Biological Station, University of Salento (Italy).

To study the effect of salinity on the survival of copepods, we placed 20-30 (for species with the body length greater or equal to 1 mm) or 40-50 (for species with the body length of about 0.5 mm) active females of every species (in 3-5 treatments) into transparent 100 mL beakers filled with filtered seawater and exposed (at natural habitat temperature) to a gradual decrease or increase in salinity over the periods ranging from 2 to 10 h at a rate of 2–3 psu h^{-1} (Hubareva *et al.*, 2008; Svetlichny *et al.*, 2012a and b; Hubareva and Svetlichny, 2016). The range of salinity changes was determined according to the results of preliminary experiments (Kovalev, 1966; Svetlichny *et al.*, 2006a). The salinity tolerance range of copepods was estimated basing on the values of the median lethal salinity (LS_{50}). To access the ability of copepods to shift the salinity tolerance range, in some experiments the females which had survived at the LS_{50} were exposed to a slow uniform increase in salinity due to natural evaporation of seawater. Salinity tolerance of *Calanus helgolandicus* was estimated taking into account behavioral and physiological response of separate individuals to salinity change.

2.1. Salinity Tolerance in *Calanipeda aquaedulcis* and *Arctodiaptomus salinus*

Calanipeda aquaedulcis and *Arctodiaptomus salinus* are representatives of two taxonomically and ecologically close families: Pseudodiaptomidae and Diaptomidae. According to Grindley (1984), "Pseudodiaptomidae have arisen from the sea and represent an intermediate stage in adaptation to the freshwater environment" while "Diaptomidae appear equally well adapted to the fresh-water environment." Although *C. aquaedulcis* and *A. salinus* are not strictly marine copepods, these two species have been considered for comparison with the marine ones.

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According to our results, *C. aquaedulcis* and *A. salinus* are able to develop successfully as in fresh water, as in the Black Sea brackish water. These species have been kept for 4 years in the laboratory at a room temperature and salinity of 18 psu, and two months prior the experiment half of the culture was transferred to fresh water. For fresh water copepod generations, the salinity tolerance ranges were within 0.2 to 17 psu in *A. salinus* and 0.2 to 35 psu in *C. aquaedulcis*. For individuals of both species acclimated to 18 psu (Black Sea water) the borders of salinity ranges were shifted to 35-40 psu (*A. salinus*) and 50 psu (*C. aquaedulcis*) (Figure 1). In our experiments 50% of population of *C. aquaedulcis* could survive gradual salinity changes during 8 - 10 h up to 30 psu, whereas that of *A. salinus* tolerated only salinity alterations limited to a range of about 20 psu.

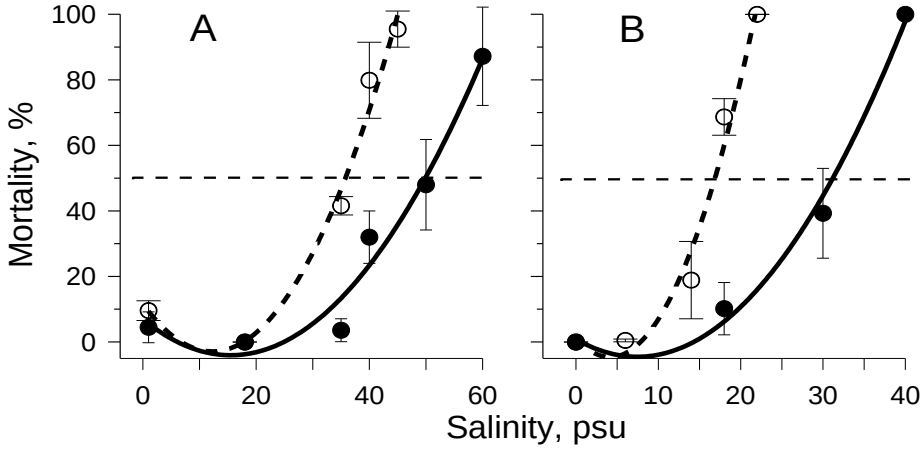

Figure 1. Salinity tolerance of *Calanipeda aquaedulcis* (A) and *Arctodiaptomus salinus* (B) reared at 0.2 psu (○) and 18 psu (●) in 5–10 d after gradual salinity acclimation. The salinity tolerance range in females and males of both species was between 0.1 psu; the horizontal long-dashed line denotes 50% mortality (from Svetlichny *et al.*, 2012a with changes).

2.2. Salinity Tolerance of Native *Acartia clausi* and Alien *Acartia tonsa*

Copepods of the genus *Acartia* inhabit many coastal and offshore environments where they are usually among the most abundant zooplankton species. *Acartia tonsa* possesses a wide range of tolerance to salinity whilst *Acartia clausi* is considered to be a more stenohaline species (Stalder and Marcus, 1997; Calliary *et al.*, 2006 and 2008). *A. tonsa* adapted to high food concentration (Paffenhöfer and Stearns, 1988) usually is more abundant in the near-shore environments and estuaries. Similar trend in the distribution of these species is observed in the Black Sea. After the introduction of *A. tonsa* in the Black Sea presumably in 1976, both species can coexist (Gubanova, 2000); however, *A. clausi* dominates in the open zone of coastal areas, while *A. tonsa* occurs in semi-closed bays (Delpy and Pagano, 2018; Marques *et al.*, 2018; Villate *et al.*, 2018).

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Salinity tolerance ranges for females of *A. clausi* and *A. tonsa* collected at 18 psu in Sevastopol Bay (Black Sea) were estimated as 10-35 and 3–30 psu, respectively (Figure 2). However, after 1 week of acclimation to 30 psu the salinity tolerance ranges were extended up to 50 psu in *A. tonsa*. Therefore, in comparison with *A. clausi*, *A. tonsa* introduced in the Black Sea possessed the salinity tolerance range shifted to lower salinity (up to 3 psu) and, at the same time, this species was able to acclimate to high-saline water (50 psu).

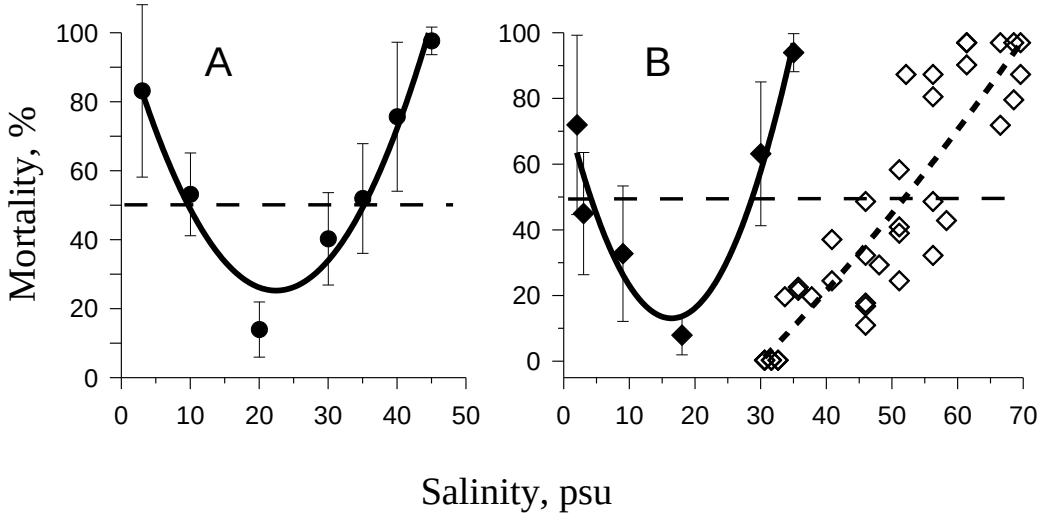

Figure 2. Mortality of *Acartia clausi* (A, ●) and *Acartia tonsa* (B, ♦) acclimated to 18–20 psu in 3 days after gradual salinity change. ◊ - Effect of salinity on mortality of *A. tonsa* acclimated during one week to 30 psu. (Figure 2B from Svetlichny and Hubareva, 2014a with changes).

Consequently indigenous *A. clausi*, living at the quasi-permanent salinity of 18 psu in natural environment but demonstrating the ability to survive in the high-saline water, showed the "genetic memory" about the conditions of its oceanic origin. Salinity tolerance range of *A. tonsa* inhabiting the Black Sea was close to that of the same species from Øresund kept for several generations at *ca.* 33 psu in the laboratory (Calliari *et al.*, 2006), also being capable to sustain the decrease in the salinity down to 2 psu. Thus, *A. tonsa* may be considered as an extremely euryhaline species widely distributed in the eutrophic regions of the World Ocean.

2.3. Salinity Tolerance of the Copepods from the Genus *Oithona*

The effect of salinity on mortality of Oithonidae was studied (Figure 3) at 20 ºC for warm-water *Oithona davisae* and *Oithona nana* (Svetlichny and Hubareva, 2014a) and at 8-10 ºC for cold-water *Oithona similis* (Hubareva and Svetlichny, 2016). Salinity tolerance range (5–45 psu) of the Black Sea invader *O. davisae* (Ferrari and Orsi, 1984)

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in 3 days after the gradual salinity acclimation at a rate of 2-3 psu h^{-1} was significantly wider than that of indigenous copepods *O. similis* (10–30 psu) and especially *O. nana* (15–28 psu). Narrow salinity tolerance range of *O. nana* was known from the studies of Kovalev (1966) and, probably, may be one of the reasons of the elimination of this species from the Black Sea as the most vulnerable component of the zooplankton community which underwent dramatic changes during last decades (Gubanova *et al.*, 2014).

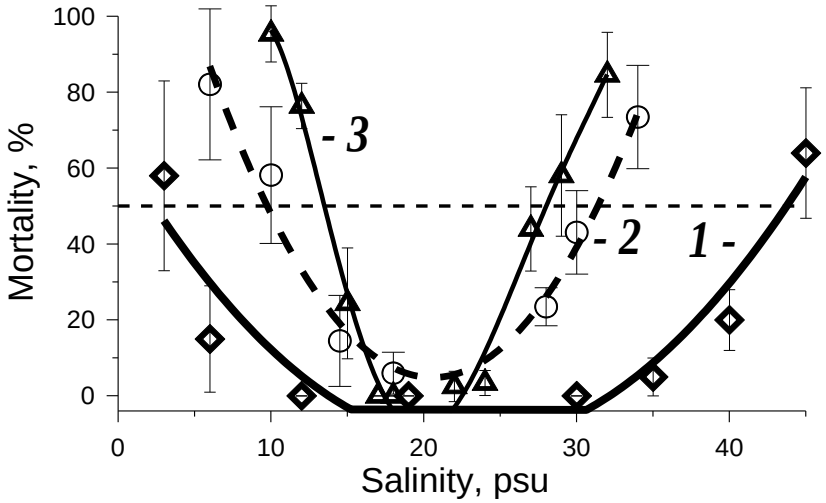

Figure 3. Salinity tolerance of *Oithona davisae* (1), *Oithona similis* (2) and *Oithona nana* (3) (from Isinibilir *et al.*, 2016).

On the contrary, high salinity tolerance of *O. davisae* seemed to facilitate the expansion of this species from Eastern Asia waters to the European and American seas.

An extraordinary sustainability of *O. davisae* was confirmed by our experiments (unpublished data) when 63.8 ± 20.5% of females were alive during 3 days after the transfer from 18 to 35 psu and 86.9 ± 9.7% of individuals survived after the transfer from 35 to 18 psu during the same period. After sharp increase in salinity the bodies of copepods shrunk and flattened. Such individuals descended to the bottom of the aquaria and kept the immobility for several hours. After that, their bodies reshaped and the copepods began to swim in the water again.

2.4. Salinity Tolerance of *Calanus helgolandicus*

Calanus helgolandicus is widely distributed in the North Atlantic seas. *Calanus euxinus* in the Black Sea is considered to be a phenotypic variation of *C. helgolandicus* (Papadopoulos *et al.*, 2005; Unal *et al.*, 2006; Yebra *et al.*, 2011). To evaluate the adaptive potential of this species, we studied the effect of gradual salinity changes on

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survival, locomotion and feeding (as mostly sensitive indicator of animal health) of *C. helgolandicus* living at the quasi-homogeneous salinity of about 38 and 18 psu in the Ionian and Black Seas, respectively, and in the Marmara Sea with a two-layer salinity structure (about 20 and 38 psu in the upper and lower layers, respectively).

In 0.5 L aquaria, the individuals of this species usually aggregate near the bottom whilst after salinity changes these copepods ascend to the surface and swim there. In *C. helgolandicus* collected in the Black Sea at 18 psu, a gradual decrease and increase in salinity at a rate of 2 psu h^{-1} within the range of 10-40 psu resulted in an increase in locomotion activity up to maximum values at 14 and 31 psu, and dramatic decrease at the following salinity change (Figure 4A). Although after salinity increase up to 40 psu the copepods remained alive, the muscles of these individuals became opaque indicating irreversible tissue changes (Svetlichny *et al.*, 2010b). More than 50% of studied copepods died in 3 – 5 days after gradual salinity increase higher than 30 psu (Figure 4B).

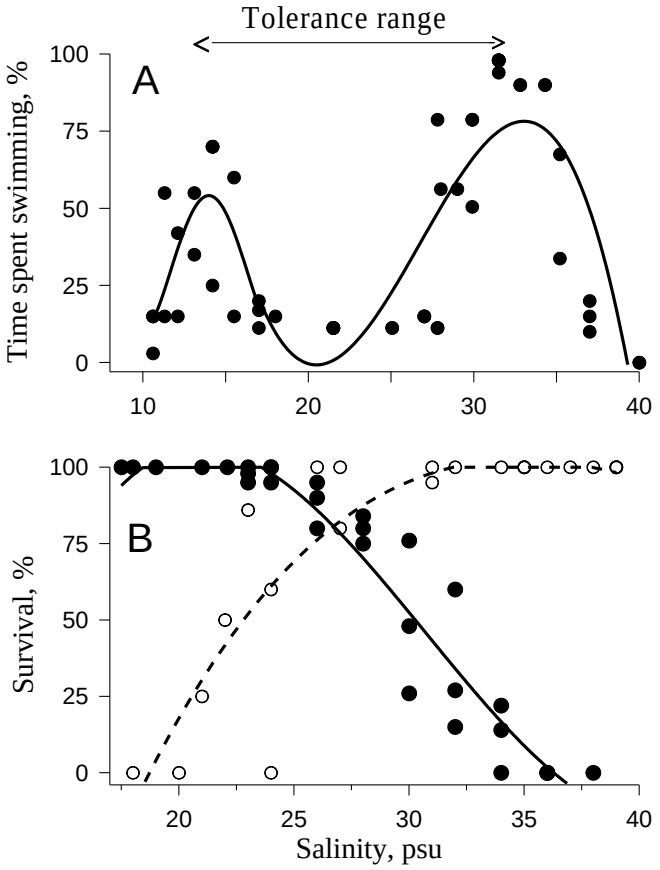

Figure 4. Time spent routing swimming (A) in the Black Sea *Calanus helgolandicus* as a response to gradual salinity changes and survival (B) of *C. helgolandicus* from the Black (black circle) and Ionian (open circle) Seas after 72 and 24 h of salinity changes, respectively (data on *C. helgolandicus* from the Ionian Sea from Isinibilir *et al.*, 2011).

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In the feeding experiments (see details in Svetlichny *et al.*, 2010b) a gradual salinity increase from 17 to 25 psu during 1.5 h did not affect dinoflagellate *Prorocentrum minimum* consumption rate in females from the Black Sea. The anterior part of the gut was constantly full of algae in all individuals. During the following salinity increase, every change in the salinity (by 2–3 psu) brought to rapid gut evacuation and renewed feeding during the period of acclimation to new salinity. The duration of salinity acclimation period changed from 15-40 min at 17–25 psu to 80–120 min at 28-30 psu. When the salinity exceeded 31 psu, copepods completely stopped consuming algae. However, after the acclimation to 27 psu during about 3 days *C. helgolandicus* kept the ability to feed.

In *C. helgolandicus* inhabiting the Ionian Sea, the gradual salinity transition from 39 to 26 psu did not affect the survival of preadults and adults; however, after further reduction of the salinity down to 18 psu all animals died within 24 h (Figure 4B) (Isinibilir *et al.*, 2011). According to the LS_{50} criteria, the critical salinity for survival of population in the Ionian Sea was equal to 22 psu. Therefore, without the pre-adaptation the salinity tolerance range in *C. helgolandicus* living at constantly high salinity amounted to 22-40 psu whilst that for this species from the brackish Black Sea was about 15-30 psu (Svetlichny *et al.*, 2010b).

In the Marmara Sea with two types of water masses (brackish Black Sea and high-saline Mediterranean water), *C. helgolandicus* inhabit the whole water column performing diel vertical migrations (Svetlichny *et al.*, 2010b). From this perspective, the Marmara Sea can be considered as the zone of natural wide amplitude acclimation. In *C. helgolandicus* females sampled in deep high-saline layers of the Marmara Sea near the Prince Islands, moving activity did not change significantly after gradual (during 4 h) salinity increase from 22 to 40 psu and only at 40-50 psu we observed a pronounced depression of locomotion (Svetlichny *et al.*, 2010b). We found no regular trends in changing of locomotor activity of females kept for several days at 38.5 psu and then exposed to a short-term (2.5 h) gradual salinity decrease down to 22 psu. During gradual increase and further decrease in salinity within the range of 22–38.5 psu the copepods did not stop consuming food. The copepods were alive even after the acute transfer from 22 to 39 psu (Svetlichny *et al.*, 2010b).

2.5. Types of Osmotic Response in the Black Sea Copepods

According to Mauchline (1998), most marine copepod species are osmoconformers with the internal osmolality following the molality of the surrounding water. In this case, the mass body density of these animals should be proportional to the surrounding water density. It is advantageous for floating in the water because the buoyancy and sinking speed are constant, and energy losses to keep the body in the water are independent of the

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salinity. In the benthic copepod *Tigriopus brevicornis* mass density (determined in the formalin preserved individuals with the density test medium) linearly increased from 1.036 to 1.085 g cm^{-3} (McAllen *et al.*, 1998) with a salinity increase from 5 to 100 psu (Figure 5A). The authors considered this species to be a euryhaline osmoconformer. In the Black Sea, marine indigenous *A. clausi*, coastal *C. aquaedulcis* and *A. salinus* inhabiting the Crimean coastal hypersaline lakes the body mass density (measured in accordance with sinking speed of anesthetized individuals) varied in the range of 18–40, 0.2–43 and 0.2-40, respectively. This was directly in accordance with the theoretical expectation suggesting that copepod body volume is constant and that water content is iso-osmotic to the surrounding water (Figure 5A). In males, non-ovigerous and ovigerous females of *Eurytemora affinis* from Seine estuary (Seuront, 2006), sinking speeds did not change significantly within the salinity range from 0 to 35 psu, indicating that this estuarine species is also as strong osmoconformer, as the above mentioned Black Sea species. Therefore, an osmoconformic response to salinity changes can be attributed not only to marine copepods, but also to the eurybiotic species, such as *C. aquaedulcis* and *A. salinus*.

Nevertheless, some copepods may possess homeostatic mechanisms which permit physiological compensatory osmoregulation. The ability to regulate the inorganic ion content of hemolymph was found in the benthic copepod *Tisbe reticulata* (Battaglia and Bryan, 1964) and pelagic copepods *Calanoides acutus* and *Rhincalanus gigas* (Sartoris *et al.*, 2010). Osmoregulatory responses to salinity alterations in organic osmolyte content were reported for the marine copepod *E. affinis* (Roddie *et al.*, 1984) and estuarine copepods *T. californicus* (Goolish and Burton, 1989) and *Temora longicornis* (Tang *et al.*, 2000). The ability to keep a body fluid homeostasis allows species-osmoregulators to overcome abrupt salinity changes at rain, evaporation, river flow and tides. Jeffries (1962) suggested that estuarine *A. tonsa* had developed an efficient osmoregulatory mechanism. Nevertheless, Lance (1965) found no clear evidence that *A. tonsa* could effectively control its water balance although in laboratory experiments the body fluid of this species was hyper-osmotic to the external medium salinities ranging from 90% to 15% sea water for 12 h.

Our results (Svetlichny and Hubareva, 2014a) demonstrated the clear evidence of osmoregulation in *A. tonsa* from the Black Sea within the salinity tolerance range of 2–30 psu (Figure 5B). Females of *A. tonsa* collected at 18 psu and acclimated to 3 psu for 2 weeks, kept quasi-constant body density of 1.064 g cm^{-3}, however, responded hyper-osmotically at the salinity increase from 30 to 50 psu. It is worth noting that hyper-osmotic increase in body mass density of *A. tonsa* after the salinity increase from 30 to 50 psu in our experiments is not in accordance with the data on hypo-osmotic changes in free amino acid pool (Farmer and Reeve, 1978) and hemolymph Na (Farmer, 1980) in *A. tonsa* after the increase in external salinity from 34 to 39 psu. Probably, the results of these authors may reflect not only the osmoregulation processes but also an inadequate

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metabolic response of the organisms under the extreme salinities because such experimental artifacts as starvation may affect *A. tonsa* body composition.

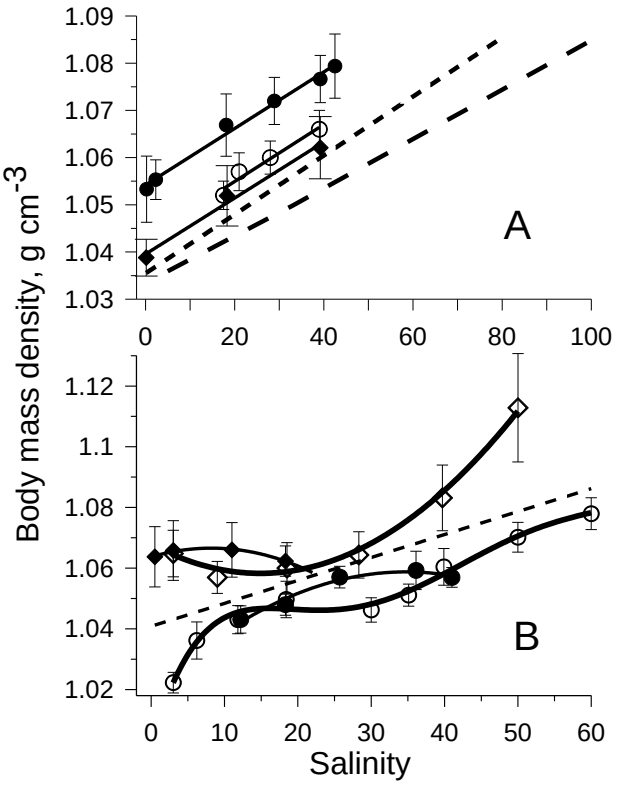

Figure 5. A: Effect of salinity on body mass density of females of *Arctodiaptomus salinus* (shaded diamonds) and *Calanipeda aquaedulcis* (shaded circles) reared at 18 psu for 4 years (from Svetlichny *et al.*, 2012a with additions and changes) and *Acartia clausi* (open circles) collected at 17.5 psu (unpublished data). Short-dashed line: theoretical change in body mass density in the case of ideal osmoconformity of copepods with a water content of 76% of the body volume. The long-dashed line shows the relationship between body mass density and salinity in *Tigriopus brevicornis* (McAllen *et al.*, 1998). B: Effect of salinity on body mass density of *Acartia tonsa* acclimated to 18 (◊, open diamonds) and 3 psu (♦, shaded diamonds), and *Oithona davisae* acclimated to 18 (○, open circles) and 41 psu (●, shaded circles). Short-dashed line shows the isometric changes of body mass density (from Svetlichny and Hubareva, 2014a with changes).

In *O. davisae* within the range 3–60 psu, three types of reaction to salinity changes were found: a) hypo-osmotic response: abrupt decrease in body mass density due to pronounced swelling and watering of the body after salinity changes from 6 to 3 psu; b) homeostatic regulation: maintenance of constant body mass density at salinity increase from 18.4 to 35.1 psu in females collected in the sea at 18 psu, and at salinity decrease from 41 to 25.7 psu in females acclimated for one week to 41 psu. This phenomenon may be due to low permeability of *O. davisae* integuments, providing an osmotic isolation from the external medium as a mechanism of physiological regulation of ion exchange in stressful habitats (Lee *et al.*, 2012); c) iso-osmotic response: body mass density changes

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equiproportionally to surrounding water salinity decrease from 18 to 6 psu and increase from 40 to 60 psu in the copepods collected in the sea at 18 psu.

Wide salinity range and osmoregulation ability in *A. tonsa* and *O. davisae* seem to be the features that facilitate the formation of self-reproducing populations of only these two species in the Black Sea (Gubanova *et al.*, 2014), despite the fact that a great number of copepods was brought with ship ballast waters from adjacent and distant seas (Selifonova, 2011).

2.6. Copepod Egg Salinity Tolerance

Despite the fact that females of some marine copepods (for example, *A. tonsa*) possess the ability to osmoregulate, the volume of their eggs immediately follows the salinity changes as a perfect osmometer (Calliari *et al.*, 2006; Hansen *et al.*, 2012), and the egg density alters as well (Miller and Marcus, 1994). At the decrease in salinity from 31 to 15 psu, mass density of eggs in *A. tonsa* from the Gulf of Mexico reduced from 1.087 to 1.066 g cm^{-3} (Miller and Marcus, 1994). In the Black Sea *A. salinus* acclimated to fresh water, a salinity increase from 0.2 to 18 psu resulted in an increase in the mass density of both resting and subitaneous eggs (Figure 6).

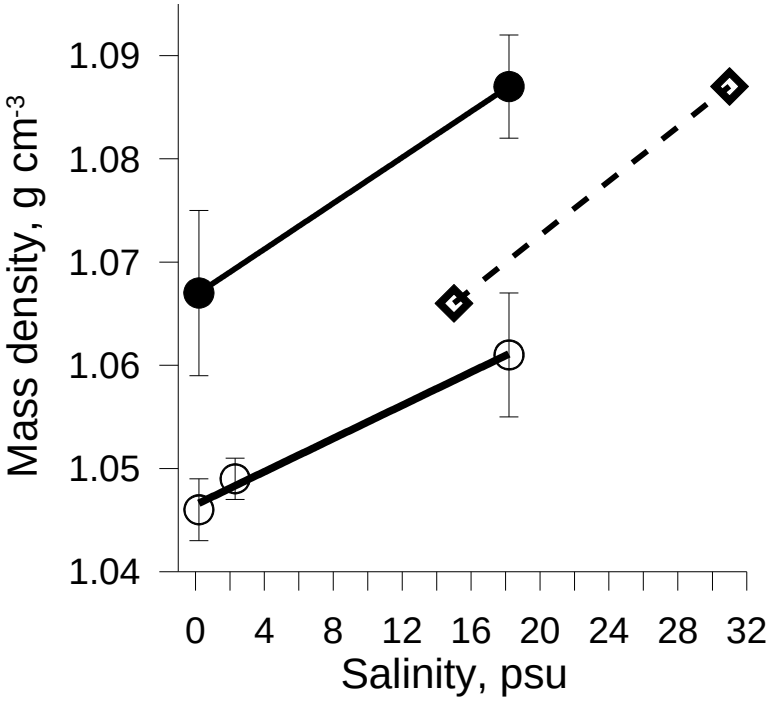

Figure 6. Effect of salinity on mass density of subitaneous (open circles) and resting eggs of *Arctodiaptomus salinus* (shaded circles) (data from Table 2 of Svetlichny *et al.*, 2012a) and subitaneous eggs of *Acartia tonsa* (open diamonds) (from Miller and Marcus, 1994).

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As a whole, in marine copepods hatching success of subitaneous eggs is in accordance with the parental salinity tolerance. Egg hatching success of widely euryhaline *A. tonsa* remained high as at extremely low salinity of 2 psu, as at high salinity of 39 psu (Calliari *et al.*, 2006; Svetlichny *et al.*, 2010a).

For copepods with narrower salinity tolerance range, egg hatching success is due to a parental shift in salinity acclimation. In *A. clausi* from the Black Sea almost all eggs died after the gradual salinity increase from 18 to 39 psu (Figure 7A); however, up to 50% of eggs of this species living in the Marmara Sea at changing salinity (22-39 psu) kept the ability to develop into nauplii at 39 psu (Svetlichny *et al.*, 2010a). On the contrary, egg hatching success in *A. clausi* maintained for several generations at a salinity of *ca.* 33 psu decreased dramatically at salinity lower than 20 psu (Calliari *et al.*, 2006).

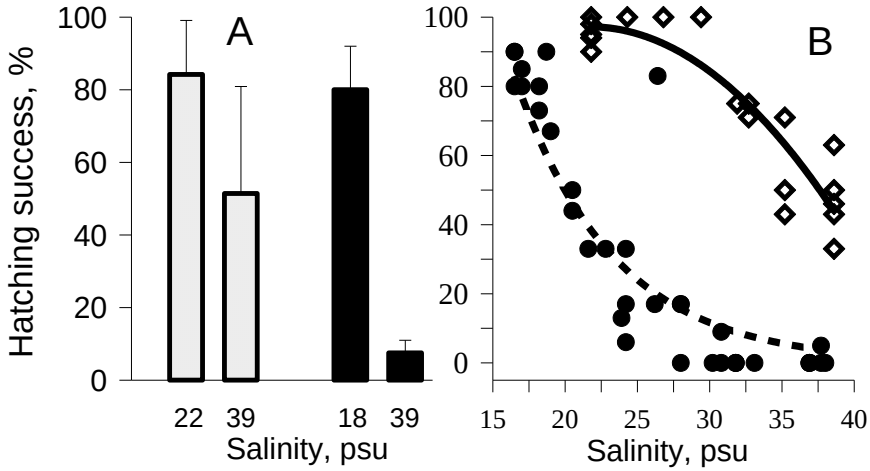

Figure 7. Hatching success of egg in *Acartia clausi* females from the Marmara (□) and Black (■) Seas (A) and *Calanus helgolandicus* females from the Marmara (◊) and Black (●) Seas (B) at different salinity (from Svetlichny *et al.*, 2010a with changes).

Eggs laid by the Black Sea *C. helgolandicus* females at 17–18.7 psu died at salinities > 30 psu, in contrast to 47 ± 11% of eggs laid by the Marmara Sea females which produced nauplii successfully after the gradual salinity change up to 39 psu (Figure 7B).

Hansen *et al.* (2012) proposed that the embryo of *A. tonsa* can be protected from the salinity stress by its plasma membrane and that water exchange driven by osmosis was restricted to the perivitelline space of the egg. However, under extreme hypo-saline (0 psu) or hyper-saline (76 psu) conditions the eggs swelled or compressed, respectively, due to passive osmosis and killed the embryo.

We observed similar effect of salinity on the eggs of *C. helgolandicus* from the Marmara Sea living at changing salinity. The eggs laid at 22 psu became wrinkled during gradual salinity up to 38.5 psu (Svetlichny *et al.*, 2010a) due to the loss of water (Figure 8). However, at long-term (1-2 days) keeping at this salinity the shape of survived eggs recovered and nauplii hatched successfully from these eggs.

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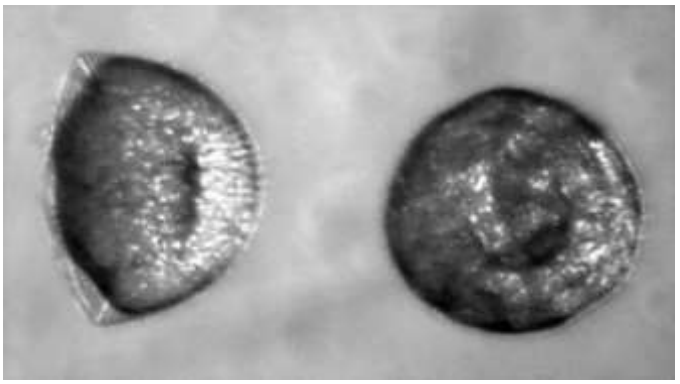

Figure 8. Eggs of *Calanus helgolandicus* from the Marmara Sea after salinity increase from 22 to 38 psu.

However, our results showed that copepod eggs are more sensitive to extremal salinities than the adults. Anufriieva (2014) found adults of *A. salinus* in hyper-saline lakes of Crimea at the salinity of about 300 psu. In our experiments, some *A. salinus* specimens survived for more than 14 d at the salinities of about 70 psu, and up to 15% of *C. aquaedulcis* females were alive for 10 d at 60 psu. However, we did not observe hatching of nauplii from the ovisacs of *A. salinus* and *C. aquaedulcis* females at salinities higher than 50 psu. Probably, this phenomenon was due to osmotic effects during hatching**.** According to Marshall and Orr (1972) and Davis (1959), nauplii burst out from eggs after cracking the outer membrane due to a sharp increase of pressure inside the inner case. The pressure increase seems to be caused by active absorption of water through the inner membrane. This suggestion may be confirmed by the increase in respiration rate of eggs at the hatching time (Nielsen *et al.*, 2007). According to Rokneddine and Chentoufi (2004), the reproductive potential of *A. salinus* from the Zima salt marsh in Morocco decreased 5-fold with an increase in salinity to the upper boundary of its tolerance range. In other estuarine copepods, the critical salinity for survival of their juvenile stages was also found to be lower than in adults of *E. affinis* (Ishikawa *et al.*, 1999; Lee *et al.*, 2007) and *P. annandalei* (Chen and Suzuki, 2006).

2.7. Effect of Salinity on Copepod Respiration Rate

Variations in respiration rate of copepods at the salinity changes are considered to be connected with additional energy losses for osmotic regulation (Gyllenberg and Lundqvist, 1978; McAllen and Taylor, 2001). Theoretically, the minimum osmotic work for ion transport constitutes only from 1 to 5% of the total metabolic energy requirements in brackish and freshwater animals (Potts, 1954). However, Goolish and Burton (1989) showed that the daily energy required for adjusting metabolism to hyperosmotic stress in *T. californicus* acclimated to constant salinity amounted to 11.6% of the total energy

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respired. In copepods metabolic rates of active individuals can exceed the basal metabolism by 6-fold (Buskey, 1998; Svetlichny and Hubareva, 2005); therefore, the energy of muscular activity associated with typical copepods avoidance response to unfavorable conditions may mask the losses for osmoregulation. Probably, therefore, the results of numerous studies concerning the effect of salinity on respiration rates of copepods are very contradictory and are highly dependent on the functional mobility of the species.

For example, an increase in respiration rate of *A. clausi* and *A. tonsa* (Calliari *et al.*, 2006) and *Calanus finmarchicus* (Anraku, 1964; Marshall and Orr, 1972) followed to the increase in water salinity, while in *T. brevicornis* (McAllen and Taylor, 2001) an increase in salinity caused a decrease in oxygen consumption rate and activity as well. There was no evidence of salinity-associated respiratory distress in respiration experiments with *E. affinis* in the range of 0-40 psu (Roddie *et al.*, 1984). The respiration rate of *Pseudodiaptomus hessei* females also showed no differences within the salinity range of 3-31 psu (Isla and Perissinotto, 2004). According to Newrkla (1978), respiration rate and moving activity of *Arctodiaptomus spinosus* did not vary within the salinity range of 3–66 psu. In *C. helgolandicus* collected from the deep layers (38.5 psu, 15ºC) of the Marmara Sea and kept under laboratory conditions (20°C) at a salinity of 38.5 psu during 6 d and 22 psu over 13 d, weight-specific respiration rates did not differ significantly amounting to 1.62 ± 0.05 and 1.76 ± 0.2 μg O_2 mg^{-1} h^{-1}, respectively (Svetlichny *et al.*, 2010b). In the Black Sea for osmoconformers *A. salinus*, *C. aquaedulcis*, *A. clausi* and for osmoregulators *A. tonsa* and *O. davisae* there was also no significant difference in weight-specific respiration rates within the salinity tolerance ranges (Hubareva *et al.*, 2008; Svetlichny and Hubareva 2014a; Svetlichny *et al.*, 2012b).

3. Effect of Temperature

3.1. Effect of Temperature on Respiration Rate

Respiration rates of ectothermic animals are known to be affected by temperature with the Van't Hoff coefficient (Q_{10}) varying within the range of 2.0-2.5 (Prosser, 1973). According to Winberg, (1983), a mean Q_{10} value of 2.25 can be applied to the majority of hydrobionts within the range of tolerable temperatures. After the analyses of literature and own data on temperature dependence of respiration rate in planktonic copepods, Lee *et al.* (2001) concluded that most of them fitted well to Van't Hoff rule, however, the exceptions were found which may be interpreted as a dissimilarity of eurythermic and stenothermic responses in copepods (Conover, 1956).

Copepods inhabiting permanently the coastal zone of the Black Sea undergo mainly seasonal gradual changes in temperature (up to 30°C), whilst species from the open sea

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regions experience diel temperature variations during the warm season because nearly all these animals are able to migrate from warm (17 – 25°C) surface layers to cold (6-8°C) mixed strata. At the winter-spring homothermia, the temperature dependence of respiration rate for both copepod groups may be similar. In our study for cold-water *Calanus helgolandicus* and *Pseudocalanus elongatus* and overwintering (warm-water) *Oithona davisae*, respiration rate followed the Van't Hoff exponential rule (Figure 9) with the Q_{10} of 2.07 (Svetlichny *et al.*, 2009), 2.08 (unpublished data) and 2.06 (Svetlichny *et al.*, 2016), respectively. In the Marmara Sea *C. helgolandicus* the Q_{10} varied near 2 for all development stages from nauplii to adults (Svetlichny *et al.*, 2010b). The Q_{10} varying from 1.93 to 2.25 was found in *Acartia clausi* for the range of 10 - 20ºC (Gaudy *et al.*, 2000). Similar Q_{10} values of about 2 were obtained for *Pseudocalanus newmani* (Lee *et al.*, 2001), *Calanus pacificus* (Vidal, 1980b) and other copepod groups (Ikeda *et al.*, 2001). However, in the experiments of some authors the temperature dependence of respiration rate in copepods was not found (*A. clausi*, see Anraku, 1964), or the Q_{10} was extraordinary high (about 6 in *Pseudocalanus* sp., see Isla *et al.*, 2008). Since the energy cost of swimming constitutes the main part of metabolism, these data discrepancies may be due to the specific behavioral response to temperature. Nevertheless, Hirche (1987) reported that in Arctic copepods respiration rates increased with temperature following the Arrhenius equation being independent of the activity. One should take into account that the trends in the temperature dependence of respiration rate of copepods may be affected by the combinations of methodical errors due to several factors including duration of experiment, capture and starvation stress (see review of Ikeda *et al.*, 2000).

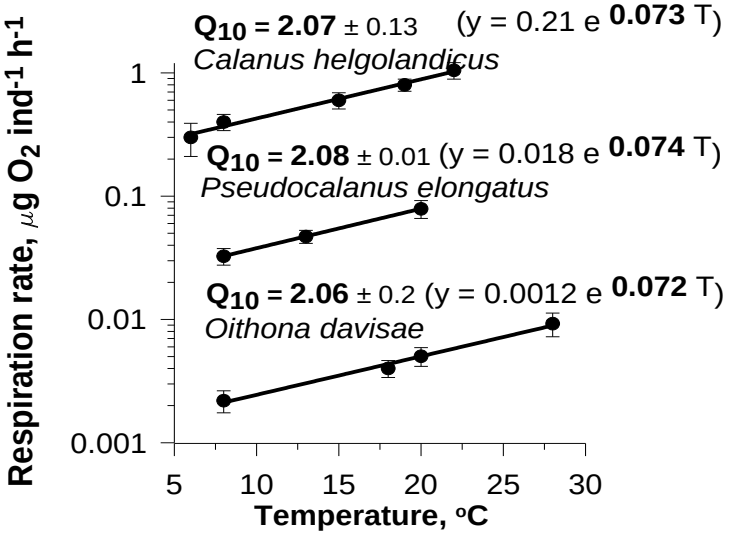

Figure 9. Effect of temperature on respiration rate of *Calanus helgolandicus*, *Pseudocalanus elongatus* and *Oithona davisae* (regression lines are constructed based on the tabular data from Svetlichny *et al.*, 2009 and 2016).

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3.2. Effect of Temperature on Moving Activity

In calanoid copepods active swimming is realized by two principally different modes: uniform locomotion using the mouthparts limbs and unsteady jerk locomotion using mainly the thoracic limbs.

The effect of temperature on the parameters of swimming in copepods may be due to the temperature dependence of muscle contraction and neurogenic rhythm controlling muscle action (see a review of Lenz *et al.*, 2005). For example, the contractile rates of vertebrate skeletal muscle were temperature-dependent with the Van`t Hoff temperature coefficients Q_{10} of 1.95 – 2.42 (Bennett, 1985), and a pyloric rhythm in the intact crab increased significantly with the Q_{10} = 2–2.5 (Tang *et al.*, 2012).

Nevertheless, the Q_{10} values of locomotor parameters are predominantly lower than those mentioned above.

The frequency of locomotor patterns shows a strong effect of temperature on locomotor activity and behavior both in free swimming (Hirche, 1987) and tethered copepods (Lenz *et al.*, 2005; Gill and Crisp, 1985). In *Temora longicornis* frequency of beats by mouthpart limbs within the temperature range of 5-20ºC increased from 11.5 - 15.4 to 23.8 – 30.2 Hz (Gill and Crisp, 1985) (our calculated Q_{10} = 1.66 ± 0.2). In the experiments of Larsen *et al.* (2008) the swimming velocity of *Acartia tonsa* in the temperature range of ~ 6 - 20ºC changed from 0.17 to 0.45 cm s^{-1} (Q_{10} = 1.7, calculated according to their equation in Figure 1). In behavior experiments of Lenz *et al.* (2005) with *Calanus finmarchicus* tethered to a force transducer, force transients produced by swimming legs and kick frequency during escape reaction increased with temperature with a Q_{10} value ranging from 1.23 to 1.86 and from 1.28 to 1.86, respectively.

In the Black Sea *C. helgolandicus*, force production and beat frequency of mouthpart limbs at routing swimming increased with the Q_{10} of 1.34 and 1.57 under strictly exponential law while the changes of power occurred in accordance with a power equation (Figure 10A, B). The period of the circadian rhythm of locomotor activity in copepod attached to a semiconductor force sensor in darkness was inversely related to the temperature described by the exponential equation (Figure 10C). It should be noted that the daily rhythm of 24 h was observed at a temperature of about 6^0C, which is close to the temperature of the cold mixed layer of the Black Sea where in total darkness *Calanus* spend the daytime during the warm season.

Parameters of quick and short-term escape reaction were also temperature-dependent with the Van`t Hoff temperature coefficients Q_{10} of 1.65 for the maximum jump frequency and 3.75 for mechanical power (Figure 11). Stronger temperature dependence of mechanical power both for routine and escape locomotion (Figure 10B and Figure 11) in comparison with the Q_{10} of respiration rate (about 2) seems to be due to the auxotonic

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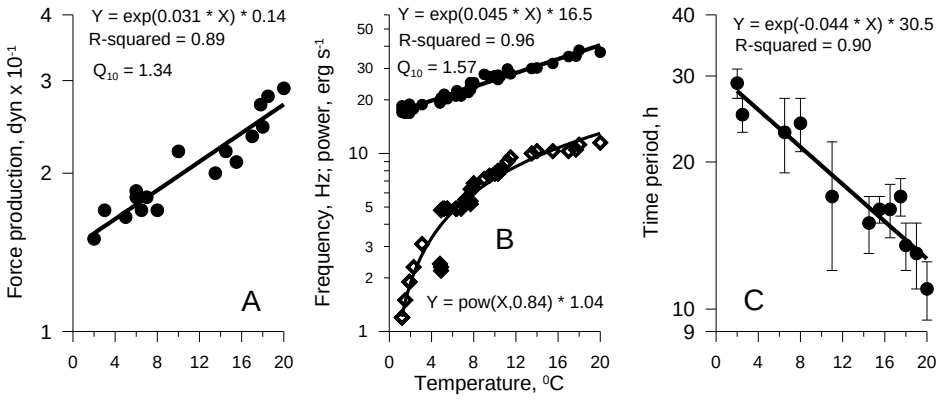

Figure 10. Effect of temperature on force production (A), beat frequency (●) and mechanic power (◊) of mouth limbs at routing swimming (B) and the period of circadian rhythm of activity (C) in *Calanus helgolandicus* (from Svetlichny, 1989).

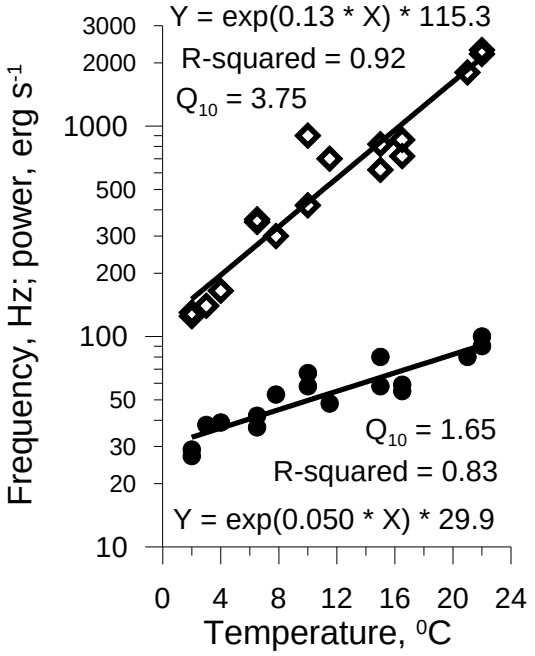

Figure 11. Effect of temperature on kick frequency (●) and maximum power (◊) of stroke phase of kicks during escape swimming of *Calanus helgolandicus* (from Svetlichny, 1989).

type of muscle contraction during swimming when force, power and energy efficiency of muscles increase following the increase of their contraction. Therefore, one can suggest that maximum swimming speed should correlate with the temperature optimum for copepods.

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3.3. Temperature Impact on the Life Cycle and Respiration Rate of the Black Sea Native and Alien Species

Due to lack of low-temperature tolerance in subitaneous eggs (capable to descend to the cold layers during development period) and nauplii (Svetlichny *et al.*, 2010a), *A. tonsa* population in the Black Sea inhabits shallow zones (mainly the bays) in summer and autumn, while in winter it endures cold period in the stage of resting eggs.

O. davisae is a perennial species. No records on production of diapausing eggs by cyclopoids are known (Alekseev and Starobogatov, 1996), and no evidence of any diapause stage exists for copepods from Oithonidae family (Marcus, 1996). Therefore, population of *O. davisae* can not be temporarily intermittent but depends on year-round recruitment of mated females, and its abundance significantly depends on seasonal temperature alterations (Uye and Sano, 1995; Altukhov *et al.*, 2014; Svetlichny *et al.*, 2016). Our field observation and laboratory experiments (Svetlichny *et al.*, 2016) indicate that *O. davisae* population in the Black Sea can survive winter low temperature of about 8°C in the stage of pre-fertilized females similarly to *Cyclops strenuus* (Nsess and Nilssen, 1991). In our experiments, overwintering females transferred from the Black Sea to the laboratory began to lay viable eggs progressively to temperature higher than 10°C. The share of ovigerous females in *O. davisae* population increased in proportion to temperature reaching 100% at 24°C (Figure 12A).

In another experiment (Svetlichny *et al.*, 2016) with long-term exposures for *O. davisae* females (without males) fed *ad libitum* by heterotrophic dinoflagellates *Oxyrrhis* sp. during 34, 56 and 71 days at 8ºC, copepods began to lay eggs on the day 2 - 3 after the increase of temperature from 8 to 20ºC, and the viable nauplii hatched on day 5. The maximum number of ovigerous females was observed after 56 days of exposition at 8ºC (Figure 12B). The number of eggs in the ovisacs varied from 1 to 9 (mean value of 4.1 ± 1.5) and was independent of the exposition at 8ºC.

Consequently, the maintenance of *O. davisae* population in the Black Sea during the cold period depends on the ability of females to keep the sperm in a spermatheca until the spring increase in temperature. However, in spite of high share of overwintering females which are capable to lay eggs, their clutch size was small, both in freshly collected from the sea and experimental *O. davisae*. The most probable explication to this phenomenon was the age of females affecting greatly the productivity of *O. davisae* (Ceballos and Kjorboe, 2011). On the contrary, in females developed in Sevastopol Bay in summer-autumn natural populations, the clutch size reached up to 19.4 ± 5.5 eggs female^{-1}.

Similar temperature shift was found in *C. helgolandicus* from the seas with different temperature regimes (Figure 14). In the Black Sea at the permanent temperature of

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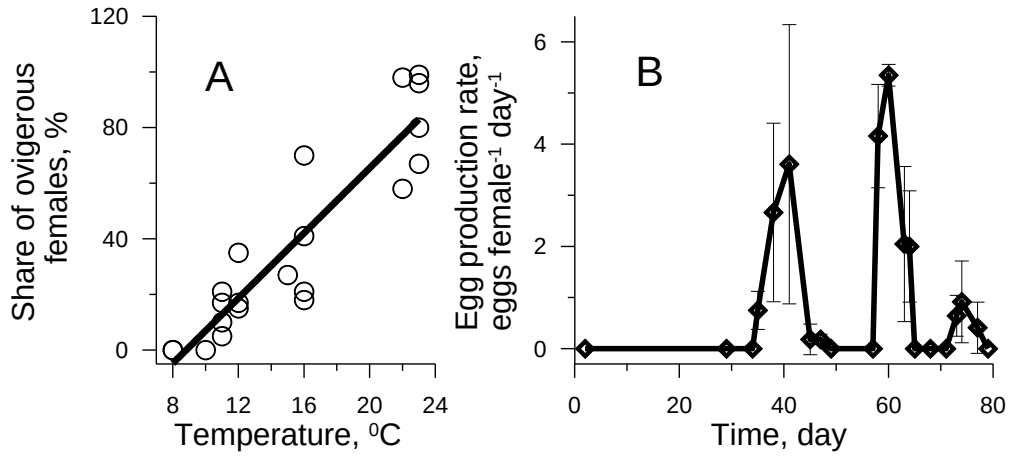

Figure 12. Effect of temperature on the reproductive activity of freshly collected overwintering females (A) (from Hubareva and Svetlichny, 2013) and egg production rate (B) of *Oithona davisae* females at 20ºC after the incubation during 34, 56 and 71 days at 8°C. All females used in the experiments were collected at the beginning of March.

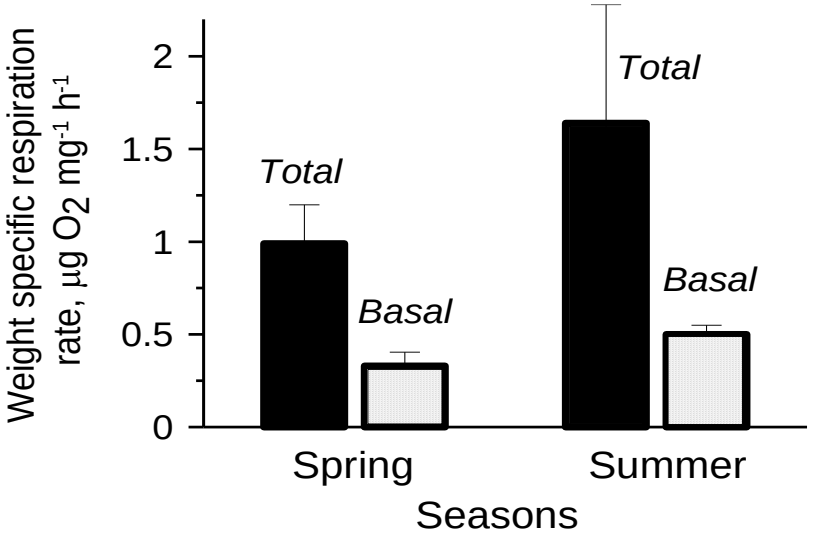

Figure 13. Weight-specific respiration rate of active and anesthetized *Oithona davisae* females collected during spring and summer. Values are presented as mean ± SD (constructed based on the data from Table 1 in Svetlichny *et al.*, 2016).

daytime habitat (6 – 8°C), weight-specific respiration rate of *C. helgolandicus* females (1.2 ± 0.36 µg O_2 mg^{-1} h^{-1}) was 1.6-fold and 1.34-fold lower than those in the Marmara and Ionian Seas, respectively, where this species developed at 13-15°C. This value was also 1.5-fold lower than weight-specific respiration rate of the Black Sea females cultivated from eggs at 18°C.

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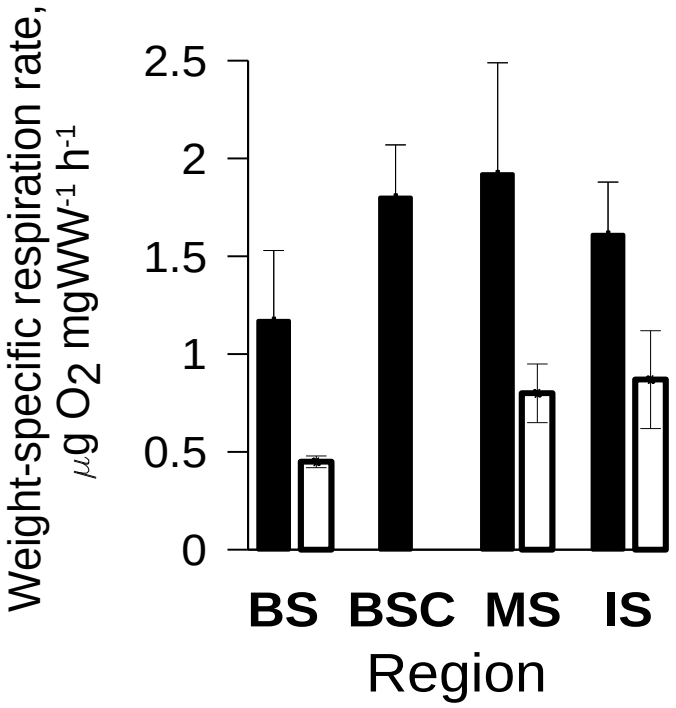

Figure 14. *Calanus helgolandicus*. Weight-specific respiration rate at 20°C of active (■) and anesthetized (□) females from the Black (BS), Marmara (MS), Ionian (IS) Seas and the Black Sea females reared in the laboratory at 18°C (BSC) (calculated from the data in Table 2 in Svetlichny *et al.*, 2010b).

Probably, this is due to the same patterns of changes in the basal metabolism when females inhabiting the seas with different temperature regimes have close metabolic energy losses for activity varying between 0.74 and 1.12 μg O_2 mg^{-1} h^{-1}.

However, it is necessary to take into consideration a higher salinity of the Marmara and Ionian Seas, which can accelerate the metabolism of copepods as well (Svetlichny *et al.*, 2010b).

4. Tolerance of the Black Sea Copepods to Oxygen Deficiency Stress

In the entire Black Sea below a depth of about 150 m, the oxygenated layers are replaced by hydrogen sulfide anoxic zone (probably, the largest one on the planet). The North-Western shelf of this basin and some shallow costal areas are affected by seasonal hypoxia caused by the enrichment of seawater by nutrients from rivers.

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Nikitin and Malm (1932) first estimated the lower habitat border for copepods in the Black Sea as 0.1 – 0.3 mL O_2 L^{-1} where *Calanus helgolandicus* aggregate during daytime. These authors reported that under conditions of experimental hypoxia the individuals of this species died at the close oxygen concentrations of 0.2-0.3 mL O_2 L^{-1}. Further studies (Flint, 1989; Vinogradov *et al.*, 1992; Arashkevich, 1996) showed that in summer-autumn season the daytime aggregation of *C. helgolandicus* had a two-layer structure. The lower layer formed by diapausing CV (CV_d) was located where the oxygen concentration was near 0.2 mg O_2 L^{-1}. The upper layer consisting of migrating CV (CV_m) and adults was located at the oxygen concentration of about 0.8 mg O_2 L^{-1}.

Vinogradov *et al.* (1992) reported that the respiration rate of CV_d decreased about 3-fold from 0.13 to 0.043 μL O_2 mg^{-1} h^{-1} at the oxygen concentrations changing from 9.2 to 0.2 mg L^{-1}. They also found that CV_d were able to survive for some days under oxygen deficiency (up to 0.08 mg O_2 L^{-1}), however, the oxygen concentration of 0.06 mg L^{-1} was pointed as the lethal one.

4.1. Effect of Oxygen Concentration on Energy Metabolism of the Migrating and Diapausing *Calanus helgolandicus*

More detailed studies of the effect of dissolved oxygen on respiration rate and ammonia excretion in active and anesthetized (both diapausing and migrating copepods) *C. helgolandicus* were conducted in shipboard experiments (Svetlichny *et al.*, 1998, 2000 and 2002) with the individuals collected in offshore regions of the Black Sea.

In CV and adult females of *C. helgolandicus* performing daily vertical migrations, specific values of the total and basal metabolic rates, as well as the ammonium excretion rates as an indicator of protein catabolism, did not differ significantly. Therefore, the data obtained on migrating CV_m and females were joined and represented as one environmental group (CM).

It was shown that when the oxygen concentration decreased from 9 to 0.8 mg L^{-1}, in the CM collected at night in the sub-surface layers the weight-specific metabolic rate at 8°C reduced 2.7-fold from 7.0 to 2.6 J mg^{-1} h^{-1} (Figure 15A). At the same temperature, the energy of the protein catabolism was independent of the oxygen concentration varying randomly within the range from 3.1 to 4.6 J mg^{-1} h^{-1}. Constituting about 60% of total energy metabolism under the normoxia, protein catabolism exceeded the respired energy by 50% at the oxygen concentration of 0.8 mg L^{-1}. These data point to the great contribution of the anaerobic protein catabolism to the total energy metabolism in CM during daytime aggregation in the hypoxic layers.

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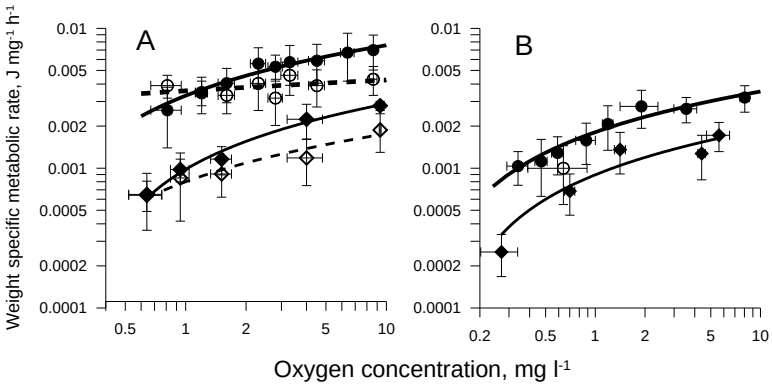

Figure 15. Energy equivalents (J mg^{-1} h^{-1}) of weight-specific respiration and ammonia excretion rates in CM (A) and diapausing CV_d (B) of *Calanus helgolandicus* at different oxygen concentrations and constant temperature of 8°C. A: respiratory energy of active (●) and anesthetized (♦) CM and ammonia excretion rate of active (○) and anesthetized (◊) CM; B: energy of total metabolism (●) and catabolized protein (○) of active diapausing CV_d and energy of basal metabolism in anesthetized CV_d (♦) (From Svetlichny *et al.*, 2002 with changes).

In the anesthetized individuals from the CM group, both respired energy and the protein catabolism significantly decreased proportionally to the oxygen concentration. Due to this reason, in the individuals from the CM group, when the oxygen concentration reduced, the ratio between total metabolic energy of active and anesthetized CM increased from 2.5 under normoxia to 4.5 at 0.8-0.9 mg O_2 L^{-1} indicating a high cost of activity in CM under hypoxic conditions.

In CV_d collected at night in the hypoxic layers, the total energy metabolism also depended upon the oxygen concentration (Figure 15B). Higher metabolic rate of untreated CV_d in comparison with anesthetized individuals indicated that during diapause *C. helgolandicus* were able to show locomotor activity necessary for controlling the habitat depth near the hydroxide sulfide zone border. At the oxygen concentrations of 0.6 – 0.8 mg O_2 L^{-1} (minimum values for CM), the total energy metabolism in untreated CV_d was on average 2.3-fold lower than that in active CM, whilst the metabolic rates in anesthetized CM and CV_d were close. However, the ability of CV_d to live at the lower oxygen concentration (about 0.3 mg O_2 L^{-1}) allows them to reduce 4-fold their metabolism in comparison with the possible minimum metabolism of active and anesthetized CM. As a result, the metabolism of CV_d aggregating in the active state in the hypoxic layers was 7-fold lower in comparison with the metabolic rate of active CM living at normoxia, whilst there was 28-fold difference between the metabolic rates of active CM and torpid CV_d under normoxic and hypoxic conditions, respectively. This difference was significantly higher than that between the specific metabolic rates in active CV and overwintering torpid CV_d of *C. finmarchicus* and *C. helgolandicus* (about 7 times) from the Norwegian fjords (Hirche, 1983), and *Calanoides carinatus* (factor of 11) from the Antarctic region of the Southern Ocean (Drits *et al.*, 1994) where the hypoxic zones are absent.

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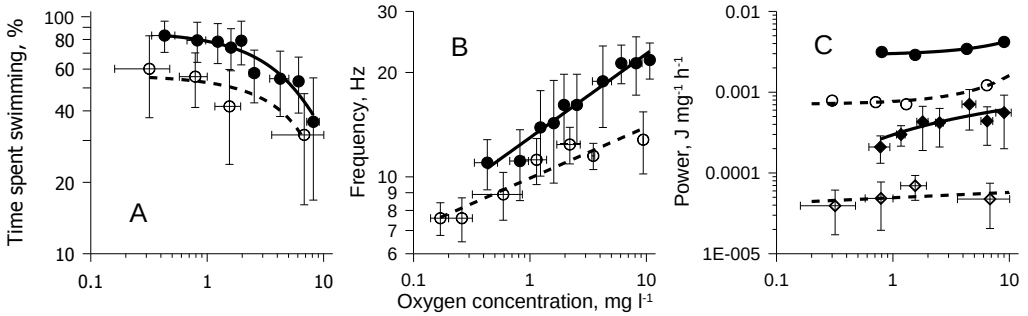

Figure 16. C*alanus helgolandicus.* Effect of oxygen concentration on locomotor parameters at 8ºC. A: time spent swimming (% of total time) and B: beat frequency (Hz) of mouthparts in CM (●) and CV_d (○), and C: active metabolism (J mg^{-1} h^{-1}) of CM (●) and CV_d (○) and mechanical power (J mg^{-1} h^{-1}) of CM (♦) and CV_d (◊) (From Svetlichny *et al.*, 2002 with changes).

The effect of the oxygen concentration on the parameters of locomotion activity was examined in the individuals attached to a semiconductor force sensor. In the experiments with CM, the oxygen concentration was decreased by bubbling the water with gaseous nitrogen during 2-3 hours imitating oxygen concentration changes during diel vertical migrations (Svetlichny *et al.*, 2000; Mutlu, 2003). In our study the individuals of CV_d collected at the depth by the plankton net experienced gradual oxygen concentration decrease in the similar mode. At every value of oxygen concentration, copepods were kept about 10 min when force production by mouth and swimming limbs, their beat frequency and duration of main behavioral acts were recorded.

Under experimental conditions, the decrease in oxygen concentration brought to an increase in the regularity of routine swimming of CM using mouth appendages (Figure 16A), until at about 1 mg O_2 L^{-1} the activity of copepods became nearly persistent. Such type of behavior in CM seems to be due to the necessity to control the habitat depth in the hypoxic layers located above the anoxic zone. These experimental results were confirmed by the numerous field acoustic studies of vertical migrations of *C. helgolandicus* in the Black Sea conducted by Mutlu (2003 and 2007). However, an increase in the regularity of swimming was followed by the decrease in beat frequency of mouthparts (Figure 16B) reflecting the speed of limb strokes. As a result of such adverse hypoxic effect, active metabolism of CM estimated from the difference in the energetic equivalents of active and anesthetized individuals insignificantly depended on the oxygen concentration, whilst the mechanical power of locomotion calculated from the force production by mouth and thoracic limbs and their circular speed (Svetlichny *et al.*, 1998) weakly decreased. Consequently, the total efficiency of the transformation of mechanical energy to biological one also reduced following the decrease in the oxygen concentration from 13 to 7% (Figure 16C).

The parameters of activity in CV_d showed similar trends of oxygen concentration dependence while their absolute values were significantly lower, especially the locomotion efficiency constituting only 3-4%. One should take into consideration that the

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absence of complete torpidity in CV_d may be due to the increase in their activity resulting from capture and maintenance in the oxygenated seawater.

4.2. Effect of Oxygen Concentration on Respiration Rate of *Calanipeda aquaedulcis* and *Arctodiaptomus salinus*

In contrast to *C. helgolandicus*, in *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* females a significant response of respiration rate to oxygen concentration changes over the range of 1 - 8 mg O_2 L^{-1} was not found. However, when oxygen concentration decreased from 0.7 mg O_2 L^{-1} to sublethal values (about 0.2 mg L^{-1} in *C. aquaedulcis* and 0.4 mg L^{-1} in *A. salinus*), which experimental copepods can tolerate only for 1 - 2 h, respiration rates fell dramatically (about 5 times) in both species (Figure 17).

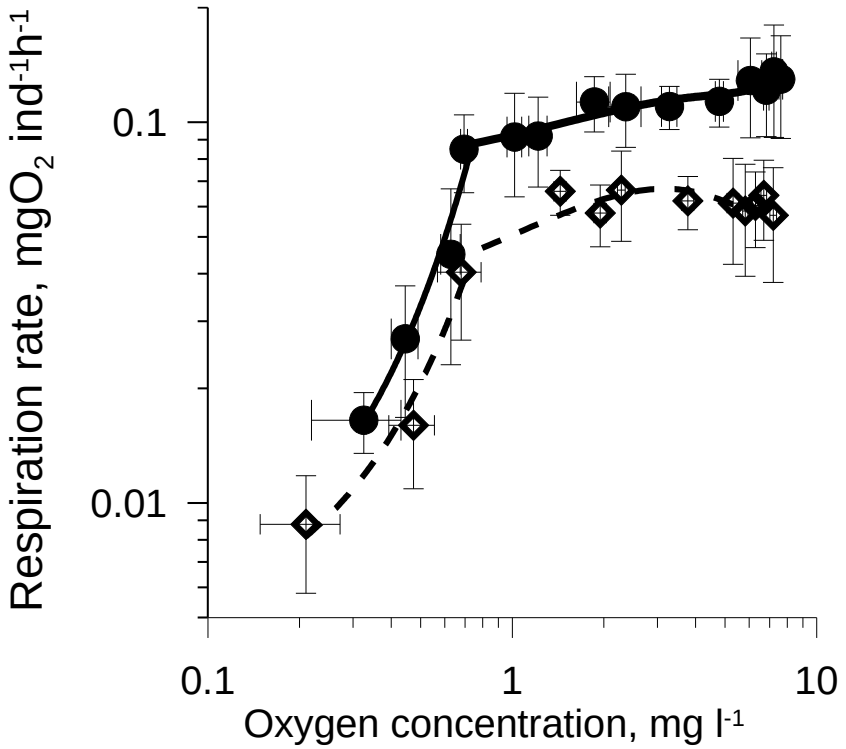

Figure 17. Effect of the oxygen concentration on respiration rate of *Arctodiaptomus salinus* (●) and *Calanipeda aquaedulcis* (◊) (Recalculated from Svetlichny *et al.*, 2012b).

Consequently, according to our results, the Black Sea deep-water *C. helgolandicus* and coastal *C. aquaedulcis* and *A. salinus* perform two types of reaction to oxygen concentration changes (see Prosser, 1973): oxyconformic response, when animals

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steadily reduce their energy metabolism with decreasing ambient oxygen concentration; and oxyregulation response, when animals keep nearly constant respiration rate up to critical value of oxygen concentration, after which oxygen consumption declines. In *C. aquaedulcis* and *A. salinus* a critical oxygen concentration of 0.7 – 0.8 mg O_2 L^{-1} (or 0.5 – 0.6 mL O_2 L^{-1}) was found, lower than 1-2 mL O_2 L^{-1} which is the shift value for hypoxic and normoxic sea conditions (Middelburg and Levin, 2009). Nevertheless, these species may be considered as the oxyphilic copepods similar to *Acartia tonsa*, which experienced sub-lethal effects of hypoxia below the oxygen partial pressure of 3.1 mg L^{-1} (2.3 mL L^{-1}) (Elliott *et al.*, 2013) and died at the oxygen concentration of 0.7 mL L^{-1} (Marcus *et al.*, 2004).

4.3. Energy Benefits of the Development of *Calanus helgolandicus* in the Black Sea Environment

C. helgolandicus is widely distributed in the North Atlantic seas. Fleminger and Hulsemann (1987) were the first who put attention to the fact that the definitive body size of females from the Black Sea exceeds greatly that of females of *C. helgolandicus* from the West, Mid-North, East–North Atlantic and Mediterranean Sea. Only in the Celtic Sea (Williams and Robins, 1982) and North Sea (Hirche, 1983), the body length of *C. helgolandicus* approximates to that of the Black Sea individuals. CVs and adults from the Black Sea population are able to accumulate large amount of lipids, mainly wax esters (Yuneva *et al.*, 1997 and 1999) reaching up to 30% of body volume (Svetlichny *et al.*, 1998) which is close to lipid content of *C. helgolandicus* from the North-East Atlantic regions (Miller *et al.*, 2000). However, in the North-East Atlantic the development of *C. helgolandicus* population is timed with algae bloom at very high chlorophyll *a* (Chl *a*) concentration up to 7.6 μg L^{-1} (Ceballos *et al.*, 2004), while the Black Sea population develops at low algae concentrations (0.29–0.68 μg Chl *a* L^{-1}) (Yuneva *et al.*, 1997). We suggest that diel descending of *C. helgolandicus* from upper layers to cold hypoxic zone of the Black Sea facilitates the utilization of the energy of consumed food for growth and lipid accumulation. The ability to decrease energy expenditure is of great importance especially during summer season, when chlorophyll *a* concentration reduces to 0.22 μg L^{-1} (Yunev *et al.*, 2005).

To test this hypothesis, we conducted comparative studies of ontogenetic changes of size, lipid content, molting patterns and respiration rate of *C. helgolandicus* collected in the Black Sea (BS), Marmara Sea (MS) and Ionian (IS) Sea. The phases of molting cycle were determined in CV basing on changes in the mandibular gnathobase during tooth formation (Miller *et al.*, 1991; Arashkevich *et al.*, 2004; Svetlichny *et al.*, 2006b).

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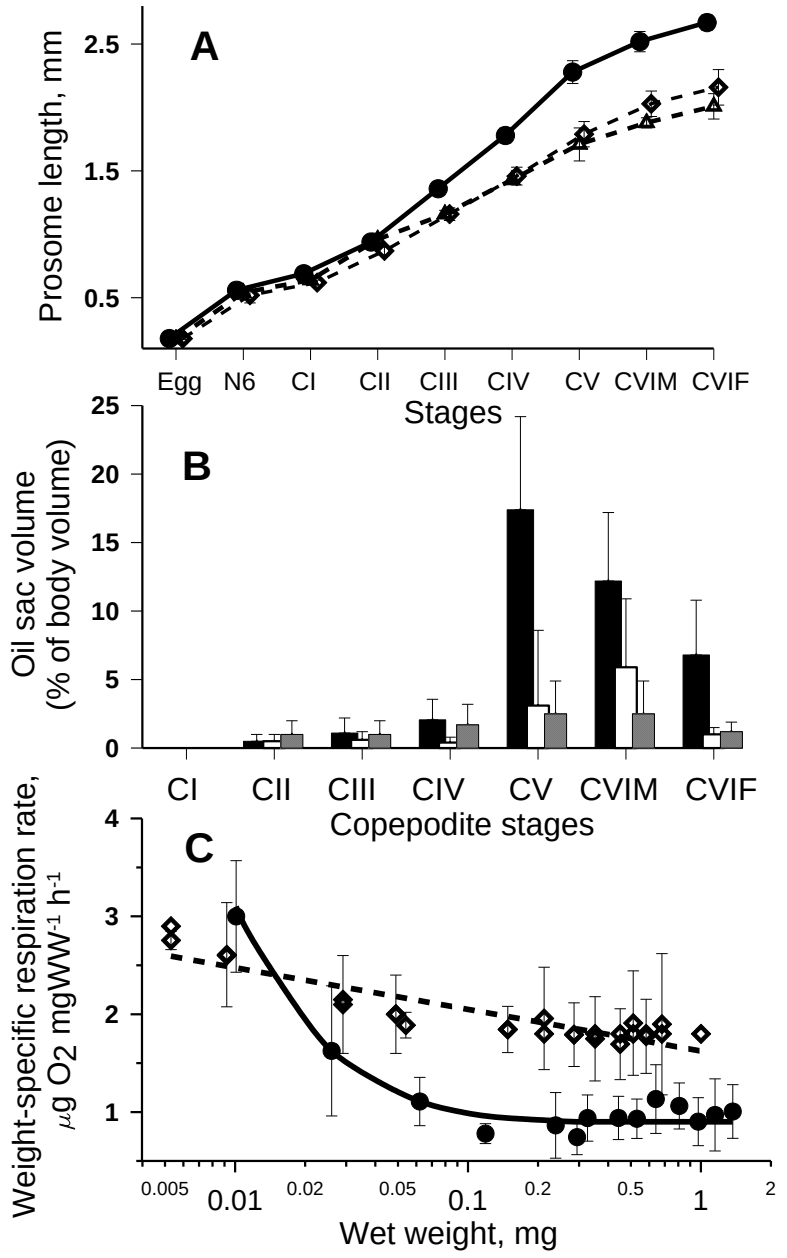

Figure 18. Ontogenetic changes of size (diameter of eggs, total length of nauplii and prosome length of copepodites) (A) in *Calanus helgolandicus* populations from the BS (●), MS (◊) and IS (▲), oil sac volume (B) in populations from the BS (■), MS (□) and IS (▒) and weight-specific respiration rate (C) of the BS and MS *C. helgolandicus* from nauplii to adults during winter-spring period (Recalculated and combined data from Isinibilir *et al.*, 2009; Svetlichny *et al.*, 2010b; Svetlichny and Hubareva, 2013).

In all studied areas, the modal intervals of the egg diameter varied in the same range of 172–180 μm, which also was close to the egg diameter of this species in the English Channel (Marshall *et al.*, 1953, Guisande and Harris, 1995, Poulet *et al.*, 1995).

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In *C. helgolandicus* from the BS, MS and IS the body sizes of nauplii and early copepodites were close as well. However, starting from CIII, the divergence of prosome length began to increase, and resulted in 20 - 25% excess of prosome length in CV and adults from the BS in comparison with the MS and IS (Figure 18A). In *C. helgolandicus* from the MS and IS, lipid accumulation in the oil sac occurred almost uniformly and reached maximum values in females (similar to the populations of this species in the North Sea, see Rey-Rassat *et al.*, 2002). By contrast, in the Black Sea *C. helgolandicus* CVs showed the maximum rate of lipid storage, which was spent then by females and males in the reproductive process (Figure 18B).

In our opinion, the following factors may contribute to a more efficient accumulation of lipids in the Black Sea *C. helgolandicus*: 1) an adaptation to periodic hypoxia during diel vertical migrations from subsurface to cold, hypoxic layers shifts the total energy metabolism of CVs to a lower level, as compared to non-migratory individuals (Figure 18C), due to lower level of the basal energy metabolism (Figure 14); 2) a decrease in temperature and oxygen concentration during vertical migration reduces the metabolic rates of CVs to the level of the basal metabolism due to the inhibition of their motor activity; both these factors lead to an increase in efficiency of use of food consumed for the formation of lipid reserves; 3) under hypoxic conditions during the daytime, the catabolism of lipids is depressed whilst the synthesis of fatty alcohols from non-lipid components in the oil sac may be facilitated (Sargent and McIntosh, 1974); 4) the development rate at the reduced metabolic level is decreased and CVs molted from CIVs have a longer period to accumulate lipids.

This phenomenon may be confirmed by the fact that, in contrast to CVs from MS and IS, in the BS CVs group during all seasons predominantly consisted of the postmolts (Figure 19A). In April 2003, during the cruise of the R/V "Knor," the postmolts with soft integument, low lipid content and small gonads and also the postmolts, intermolts and premolts with high lipid content and increased gonads were found simultaneously at night in the upper layers of the Main Rim Current of the BS (Figure 19B), whilst in the hypoxic layers diapausing postmolts without gonads and with high lipid content aggregated. Consequently, the population of *C. helgolandicus* concurrently included lipid-poor migrating CVs postmolts (just after molting), migrating CVs postmolts with medium lipid content and lipid-rich diapausing CVs postmolts (Svetlichny *et al.*, 2009). One can suggest that the main pool of reserved lipids is accumulated in the Black Sea *C. helgolandicus* in the phase of the postmolts of CVs. In any case, in the Black Sea the postmolts of CVs possessed 8-fold higher lipid content than CIVs (Figure 18B) collected during numerous cruises in 1996-2010 (Svetlichny *et al.*, 1998, 2006b, 2009; Svetlichny and Hubareva, 2014b).

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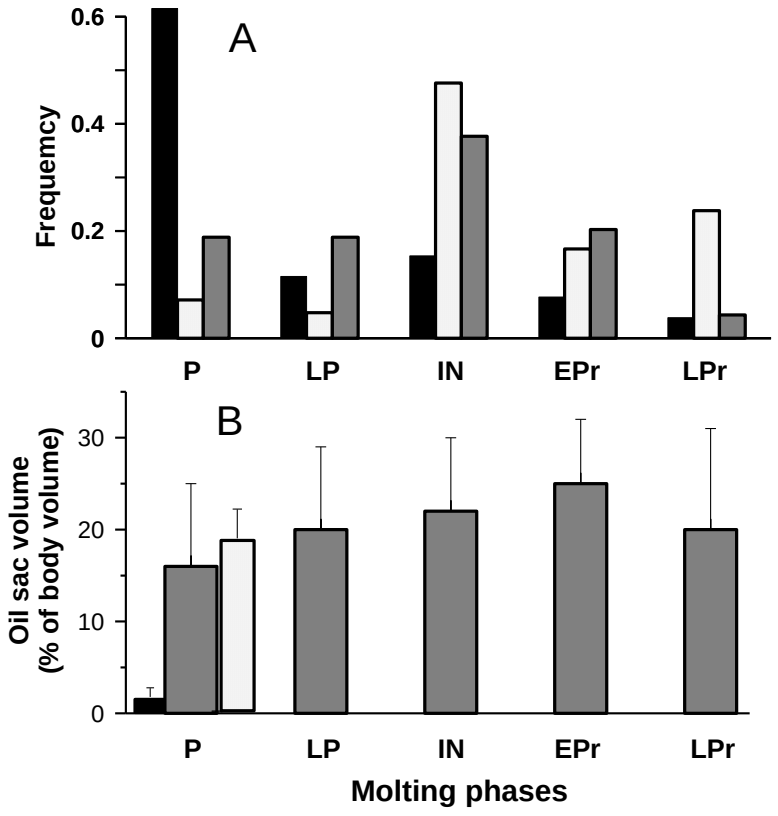

Figure 19. Frequency distribution of molting cycle phases (A) in CVs of *Calanus helgolandicus* from the BS (■), MS (□) and IS (▩) and mean oil sac volume of the Black Sea *C. helgolandicus* CVs molting groups (B) just after molting from CIVs (■), diapausing postmolts (□) and migrating (▩) postmolts (P), late postmolts (LP), intermolts (IN), early premolts (Epr) and late premolts (LPr) (Recalculated from Isinibilir *et al.*, 2009, and Svetlichny and Hubareva, 2014b).

To estimate the duration of the development stages and lipid accumulation in the Black Sea *C. helgolandicus*, a reconstruction of their age dynamics was made basing on the field studies (April 2003) of molt increments of the body weight, lipids content and specific growth rate as a function of stage specific metabolic rates and growth efficiency at 8°C (Svetlichny *et al.*, 2009). According to our estimates, the period of intensive lipid reserve formation from late premolts of CIVs to late postmolts of CVs took approximately 20 d while total duration of CVs is about 30 d. This is significantly higher than median development time (9.6 to 12.1 d) of CV in *Calanus* elsewhere at ~8°C (Vidal, 1980a; Thompson, 1982; Corkett *et al.*, 1986; Campbell *et al.*, 2001). Therefore, total development time from eggs to adults lasted about 66 d, which is in accordance with the development time only in subarctic *C. finmarchicus* (Miller and Tande, 1993; Jensen *et al.*, 2006).

Due to such characteristics of growth and development, the maximum daily production of 132 mg C m^{-2} day^{-1} (Svetlichny and Hubareva, 2011) for *C. helgolandicus* in the Black Sea was slightly lower than those for this species in the phytoplankton-rich

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North Sea (140 mg C m^{-2} day^{-1}) (Hay, 1995) and for *Calanus algulhensis* in the South Atlantic (164.4 mg C m^{-2} day^{-1}) (Hutchings *et al.*, 1995), and significantly lower in comparison with that for the North Sea *C. finmarchicus* (910 mg C m^{-2} day^{-1}) (Williams and Lindley, 1980).

CONCLUSION

In summary, when comparing the adaptive potentials of the Black Sea copepods, one should note the extraordinary euryhaline abilities in the Pontian relict *Calanipeda aquaedulcis* and Palaearctic *Acartodiaptomus salinus* when 50% of specimens of these species underwent salinity changes in the range of 0.2 – 50 and 0.2 – 35 psu, respectively. Both species collected in salt lakes of Crimea were able to be cultivated in the brackish and fresh water. Such osmotic relations of organisms with their environment characterize euryhaline amphiosmotic osmoregulators that originated from freshwater environments (Khlebovich and Aladin, 2010) although, according to our results, both studied species are osmoconformers as most marine copepods. It is important to emphasize that these abilities are genetically determined in both species as they were maintained at constant salinity of 18 psu for about 4 yr before the experiments. According to our results, up to 15% of *C. aquaedulcis* specimens survived at 60 psu during 10 d, and some individuals of *A. salinus* were alive for more than 14 d at salinities up to 70 psu (Svetlichny *et al.*, 2012a). This fact may be considered as extraordinary because even the intertidal harpacticoid copepod *Tigriopus japonicus*, active in a salinity range of 0 to 60 psu, became dormant at salinities above 80 psu (Finney, 1979). Similar to harpacticoids, *C. aquaedulcis* and *A. salinus* are not sensible to Artenminimum barrier (5 to 8 psu) of Remane (1934), 'critical salinity' postulated by Khlebovich (1969), or 'the horohalinicum' (Kinne, 1971) dividing marine and freshwater species.

At the same laboratory conditions *C. aquaedulcis* (whose specific name means 'fresh water') withstood higher increase in salinity than *A. salinus* (whose specific name means 'salty'). In the Aral Sea *A. salinus* was a dominating species at low salinity (< 13 psu), however after the salinity increase up to 20 psu the invader *C. aquaedulcis* completely substituted for *A. salinus*. The effect of substitution can be related to the trophic factor, nevertheless when *C. aquaedulcis* disappeared from the Aral Sea after the increase in salinity up to 50 psu, *A. salinus* did not return to the sea from the adjacent lakes (Aladin *et al.*, 2004).

Widely euryhaline *C. aquaedulcis* is also an eurythermic species capable to develop within the temperature range from 3 to 30°C (Bledzki and Rybak, 2016), whilst the lower temperature border for *A. salinus* was near 13°C (Anufrieva and Shadrin, 2014), which is significantly higher than the winter temperature of the Black Sea. *A. salinus* survive cold season in the stage of diapausing eggs.

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According to the response to oxygen concentration changes, both relict species are oxyphylic copepods.

A wide adaptive plasticity was found also in the alien species *Acartia tonsa* and *Oithona davisae* which was limited by critical salinity from 2 - 3 to 40 - 50 psu. According to Deaton and Greenberg (1986), the most pronounced changes in ionic composition of diluted seawater are attributed to the salinities lower than this value, therefore the salinity of 2 – 3 psu to a greater extent may be considered to be a physico-chemical barrier for brackish animals than "critical salinity" reported by Khlebovich (1969). Both of these marine species were able to osmoregulate in the salinity tolerance ranges.

Thermophylic *A. tonsa* and *O. davisae* used two different strategies of surviving at the low temperature during the assimilation in the Black Sea. Due to the absence of low-temperature tolerance, *A. tonsa* in the Black Sea survive winter in the stage of resting eggs, while population of *O. davisae* can overcome late winter to mid-spring period in the state of mating females.

Salinity tolerance range of the Mediterranean immigrants, the eurythermic *Acartia clausi* and the cold-water stenothermic *Oithona similis*, obtained in our experiments were typical for marine species. *A. clausi* collected in the Black Sea at 18 psu withstood gradual salinity changes from 10 to 35 psu, whilst the individuals collected in the Marmara Sea at 22 psu were not sensitive to salinity about 40 psu. Within that salinity range *A. clausi* performed the iso-osmotic response. For *O. similis* a salinity tolerance range turned out to be narrower (10 – 30 psu), however this fact may be due to the difficulties of keeping this species under laboratory conditions. According to Nikitin and Malm (1932), both species are able to survive at the oxygen concentration decrease to 0.17 mL L^{-1}, while only *O. similis* can descend to the hypoxic layers of the Black Sea.

The most abundant inhabitant of the open zone of the Black Sea, *Calanus helgolandicus* is well adapted to low temperature, oxygen concentration and salinity, although this species can tolerate a salinity about 40 psu after gradual salinity acclimation (Svetlichny *et al.*, 2010b). All these abiotic factors which decrease the metabolism, especially hypoxia, determine the successful development of *C. helgolandicus* in the Black Sea. The North Atlantic *C. helgolandicus* was considered to be transferred into the Black Sea after flooding of the Black Sea with Mediterranean waters and to form there a phenotypic isolated population which was recognized as a distinct species by Fleminger and Hulsemann (1987) with the name of *Calanus euxinus* (Hulsemann, 1991). However, the genetic divergences between the North Atlantic, Mediterranean and Black Sea populations are much lower than congeneric interspecific divergences in calanoid copepods (Papadopoulos *et al.*, 2005; Unal *et al.*, 2006). In addition, within the limits of revealed genetic variations in local populations in Swedish and Norwegian Fjords, Oceanic Inflow, North-East Atlantic, Adriatic Sea, Mljet Island, Aegean Sea and Black Sea the lowest level of genetic difference was obtained between the Fjords and Black Sea

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populations (Yebra *et al.*, 2011). Fjords, as a land-enclosed water bodies, have a long residence time, little water exchange and often exhibit temperature and oxygen depletion in deeper waters. Therefore, if the hypothesis of Polischuk (1984) about the penetration of boreal species into the Black Sea directly from the northern seas is right, one can suggest that this species has been already pre-adapted for living in its highly stratified environment. In the Black Sea, *C. helgolandicus* is the most abundant copepod of the Main Rim Current system which is slightly sensitive to the global ecological trends. Therefore, the population of *C. helgolandicus* seems not to be affected by warming events so profoundly as the coastal copepod populations.

During several decades, coastal copepod species as *Paracartia latisetosa*, *Acartia margalefi* and *Oithona nana* were eliminated from the zooplankton community of the Black Sea. The ecological niches of *A. latisetosa* and *A. margalefi* were occupied by the invader *A. tonsa* (Gubanova, 2000), while since 2001 a new species *O. davisae* (Gubanova *et al.*, 2014) identified first as *Oithona brevicornis* (Zagorodnyaya, 2002) appeared instead of *O. nana* in the Black Sea.

Among our studied species, *O. nana* has extremely narrow salinity range (15 – 28 psu) indicating a narrow ecological specialization of this species, nevertheless this copepod is widely distributed in the high-saline and even hyper-saline Adriatic Sea (Razouls *et al.*, 2005 - 2017). At the beginning of the 1990s *O. nana* was eliminated from the Black Sea after the invasion of *Mnemiopsis leidyi*. According to Kovalev (2007), an inhabitant of the surface layers, broadcasting *O. nana* was subjected to the most severe elimination because females of this species were eaten by *M. leidyi* together with the brood. This copepod species survives until now in the brackish upper layers of the adjacent Marmara Sea (Isinibilir *et al.*, 2011) and Golden Horn Estuary (Dorak and Temel, 2015), probably due to lower abundance of *M. leidy*i in these regions. However, *O. nana* did not return to the Black Sea after the trophic balance restore in the late 1990s as a result of the appearance of another predator, the ctenophore *Beroe ovata* feeding on *M. leidyi*. The Bosphorus Strait as an ecological barrier may be one reason for this situation (Öztürk and Öztürk, 1996; Svetlichny *et al.*, 2006a; Oğuz and Öztürk, 2011). In order to return to the Black Sea, *O. nana* needs to use the Bosphorus bottom counter current with the salinity of about 38 psu which is harmful to this species. Another factor of its failure in the Black Sea may be the fact that its ecological niche from the early 2000s was occupied by the more competitive *O. davisae*.

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Chapter 9

THE BIOLOGY OF MYELIN IN CALANOID COPEPODS

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ABSTRACT

Myelin is an evolutionary innovation by the nervous system that greatly speeds nerve impulse conduction, thus reducing communication delays within an organism and enhancing its information processing capabilities. The discovery of myelin in approximately half of calanoid copepod taxa came as a surprise. The evolution of myelin is usually associated with large organism size and/or complex nervous systems, not small organisms with simplified nervous systems. Myelinate and amyelinate species occur in similar numbers, with both groups being highly abundant and key members of marine planktonic communities. Myelinates and amyelinates have similar size distributions, similar antennule to prosome length ratios, overlapping maximum escape speeds scaled to copepod length, and similar sensory and motor system organization. Nevertheless, the biogeographic distributions and functional ecological groups of myelinate and amyelinate taxa differ markedly, suggesting niche separation based on nervous system architecture. Behavioral differences between the amyelinate and myelinate forms are apparent: not only are myelinate copepodites quicker than amyelinates in responding to a sudden hydromechanical stimulus but they are also better at localizing and escaping away from its source. The enhanced performances conferred by nervous system myelination in calanoids in combination with biogeographic observations supports the conclusion that myelination provides extra protection in habitats characterized by high risk from visual predators. In contrast, amyelinate calanoids may depend on strategies that reduce encounter rates with predators, such as diel vertical migration, dormant eggs and reduced activity levels.

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1. INTRODUCTION

A fundamental paradigm shift in ecological theory came with the realization that major interactions such as the impact of predators on prey, competition for limiting resources, habitat use, and the nature of food webs could not be understood without incorporating behavior (Schmitz *et al*., 2008; Valdovinos *et al*., 2010; Sih *et al*., 2012). In turn, animal behavior is determined by the nervous system: information is acquired through sensory systems, which is transmitted for central processing prior to generating a behavioral output. The nervous system is critical to an organism's behavioral repertoire, and sets constraints on its niche. Thus, any major change to the nervous system in a group of organisms not only affects their behavior directly, but it has significant impacts on ecological patterns. The evolution of myelin in half of calanoid copepods is a prime example of such a change (Davis *et al*., 1999). Myelin is a radical innovation of the nervous system that increases the speed of nerve impulse conduction by an order of magnitude (Ritchie, 1984). This potentially endows a myelinate species with a multitude of advantages over similar amyelinate species, including greater success in escape from predators, greater success in predatory attacks, improved coordination in muscle contraction, greater compactness of nervous systems, more rapid processing of complex information, maintenance of timely communication over greater body distances (*e.g.*, whales and dinosaurs) and enhanced precision in event timing (Hartline and Colman, 2007; Hartline, 2008). Thus, the observed split of the calanoids into either amyelinate or myelinate taxa is of major significance not only to the animal's behavioral performance but also to its ecology.

How has the evolution of myelin affected calanoid copepods and planktonic communities in general? While it is unlikely that this question can be answered directly, behavioral studies in combination with analyses of plankton community structure and trophic cascades, placed against an understanding of the properties of myelinated nerves (Hartline and Colman, 2007; Rosenbluth, 1999), can provide insights into niche separation between myelinate and amyelinate taxa. Here, we review the biology of myelin in calanoids with two major goals: 1) to describe the current knowledge of the structure and function of myelin including its development; and 2) to discuss the significance of myelination to behavior and its potential role in the ecology of zooplankton communities. We propose that myelin plays an important role in the effectiveness of a copepod's escape response, which is key to niche separation between myelinate and amyelinate taxa.

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2. Structure and Function of Copepod Myelin

2.1. Copepod Myelin is an Axonal Sheath Composed of Multiple Concentric Layers of Membrane

Detailed anatomical studies of *Calanus finmarchicus* (myelinate) and *Epilabidocera amphritites* (amyelinate) show that the two species are similar in the overall organization of their central and peripheral nervous systems (Lowe, 1935; Park, 1966). The number and type of sensory setae on the antennules are similar in myelinate and amyelinate copepods (Huys and Boxshall, 1991; Ohtsuka and Huys, 2001). Myelin stands out as the major difference between the nervous systems of myelinate and amyelinate calanoids.

The difference between a myelinated and an unmyelinated copepod axon is dramatic, as illustrated by the electron micrographs in Figure 1 (A, B). The unmyelinated axon consists of a single simple cell membrane surrounding microtubule-containing cytoplasm (termed "axoplasm"; Figure 1A). The myelinated axon is ensheathed in many concentric layers of membrane compacted together along one face (Figure 1B). The layers in the copepod are unusual in being without any gaps or seams, forming in mature myelin, concentric tubes surrounding the length of the axon and interrupted only in places by myelin-free patches termed "nodes" (Davis *et al*., 1999; Weatherby *et al*., 2000).

2.2. Copepod Myelin is Produced by Nerve Cells, not Glia

Myelination of the nervous system is gradual and occurs throughout development. In the paracalanid *Bestiolina similis*, the first nauplius stage (NI) has been found to lack myelin completely (Wilson and Hartline, 2011a). Wilson and Hartline (2011a) were able to follow reidentifiable axons from one stage to the next and demonstrate how "naked" axons transition into myelinated axons through several intermediary stages as the copepod develops first through the six naupliar and then six copepodite stages. The first sign of myelin occurs in the second nauplius (NII) with the appearance of "partial" myelin in a single axon of the proto-ventral nerve cord. Two more axons commence myelination in the NIII stage. These three reidentifiable pairs of myelinated naupliar axons are likely involved in relaying sensory information from posterior mechanoreceptors to anterior centers involved in escape behavior. "Partial" myelin originates as a single internal cisterna adhering to a portion of the inner surface of the axon membrane (Figure 1C) (Wilson and Hartline, 2011a and b). As development progresses, additional cisternae are added internally against the layer previously laid down to form a stack. As the myelin develops further, the stack expands around the inside of the axon (Figure 1D) to eventually envelop it completely in a seamless

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multilamellar sheath of myelin (Figure 1B) (Wilson and Hartline, 2011a and b). The number of myelin layers surrounding individual axons is greater in larger fibers, as is the case with vertebrate myelin. The formation of myelin internally to the axon is a radical departure from all other known cases of myelin formation, in which it is derived from the surrounding glial cells (Raine, 1984). "Copepods break all the rules" (T.M. Weatherby, personal communication).

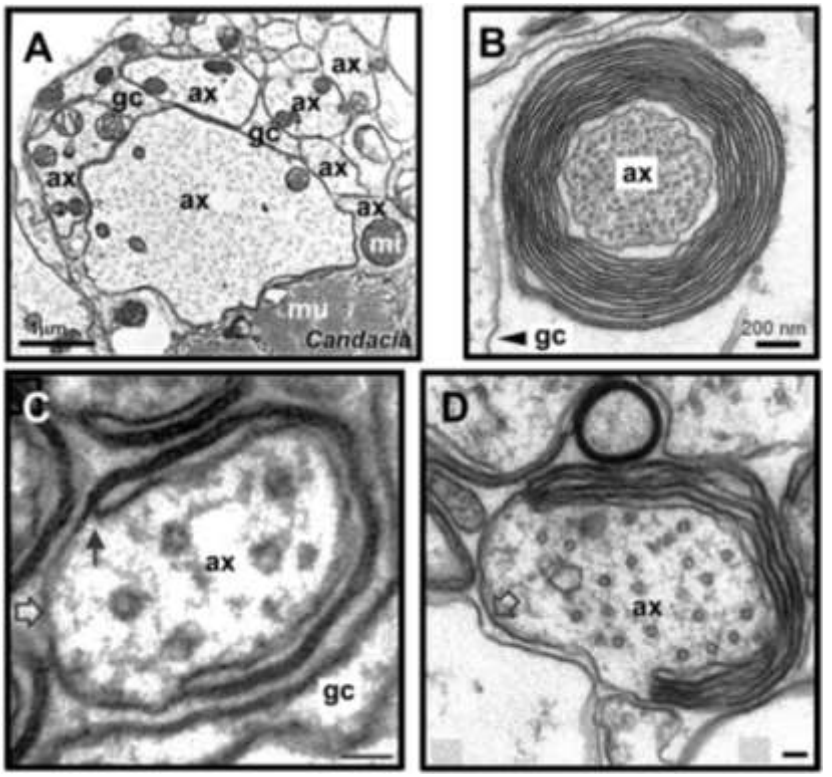

Figure 1. Ultrastructure of calanoid copepod myelin. A. Cross section through the antennular nerve of an amyelinate, *Candacia aethiopica*. Axons (ax) have at most a simple single-layer glial sheath (gc); other abbreviations: muscle (mu); mitochondria (mi). Note the large size of one mechanosensory axon presumed to mediate rapid responses to hydrodynamic disturbances. B. Cross section through a myelinated axon from the central nervous system of *Bestiolina similis*. Note the multiple continuous concentric layers of membrane tightly apposed along one face to neighboring membrane. Glial cells (gc) are not involved in forming the myelin. C. Cross section through an early stage in the development of a myelinated axon. A single internal cisterna (solid arrow) is apposed to the inner surface of the axon. The investment is incomplete, leaving a portion of the axonal membrane exposed on both intracellular and extracellular faces (open arrow). D. Cross section through an axon showing a somewhat later stage of myelin development: four cisternae are stacked together against the inner surface of the axon. Scale bars: A: 1 µm; B: 200 nm; C, D: 50 nm. A: from www.pbrc.hawaii.edu/~petra/copepod.html, with permission. Modified from image by A. Davis, B-D: Modified from Wilson and Hartline (2011b), with permission.

Large changes in form, including a reorganization of the nervous system, occur between the nauplius and the copepodite stages (Mauchline, 1998). The transition is

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characterized by the loss of the three pairs of large myelinated naupliar axons (Wilson and Hartline, 2011a). Myelination of the central nervous system and the antennular nerves is still at an early stage in the first copepodite, with just a few axons so ensheathed. The first axons to become myelinated in the early copepodite are the giant axons in the ventral nerve cord, which presumably are involved in escape responses (Lowe, 1935). Increases in both the number of myelinated axons and the number of layers are observed throughout the copepodite stages. Myelination is not complete until the final molt into the adult (Wilson and Hartline, 2011a). The overall pattern of myelin formation includes an anterior to posterior progression, large axons myelinating first, as happens also in vertebrates, and myelin developing earlier in the central nervous system than in the peripheral nervous system (*i.e.*, antennules) (Wilson and Hartline, 2011a).

These studies show that myelination, and by inference the conduction speed enhancement it provides, increases continuously throughout development. In adult calanoids, nearly all axons are myelinated, even very small, presumed chemosensory axons of the first antenna (Weatherby *et al.*, 2000). The advantages accruing to the organism from the faster conduction speed are expected to increase in parallel.

2.3. Myelin Functions by Electrically Insulating Axons

Myelin speeds nerve impulses by providing an insulating sheath around an axon that prevents leakage of electrical current needed to regenerate and propagate the impulses (Ritchie, 1984). Direct measurements of conduction speed have not been made in copepods owing to their small size. Nevertheless, the same principles are expected to govern its properties as for all other cases of myelin (Hartline, 2008). Consistent with properties of other myelinated nerve, extracellularly-recorded impulses in myelinate copepods are shorter in duration and smaller in amplitude than those in amyelinates (Lenz *et al.*, 2000).

The better the insulation the faster the conduction. A nerve impulse is initiated when current through an axonal patch raises the trans-membrane voltage above a threshold level. Thereupon, voltage-gated sodium channels open sufficiently to cause regenerative entry of sodium ions that sustain the impulse. In unmyelinated fibers, the electrical current thereby produced expends itself charging near-by regions ahead of the travelling impulse to bring them to threshold. When those near-by regions are electrically insulated with myelin, the current passes unimpeded farther down the axon to the next uninsulated channel-bearing membrane (typically a node), where rapid charging to threshold results in a faster conduction speed. The seamless concentric leak-free construction of the copepod myelin along with the small-amplitude impulses observed suggests that the insulating properties may be better than those of other myelin sheaths.

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3. COPEPOD MYELIN IS CONFINED TO MORE RECENTLY EVOLVED SUPERFAMILIES

Calanoid diversity is characterized by a nearly 50:50 split between amyelinate and myelinate taxa (Lenz, 2012; Lenz *et al*., 2000). The Calanoida have been organized into 10 super-families (Park, 1986). While this organization into super-families is generally supported by more detailed morphological analysis and molecular-based phylogenetic tree, it is likely to be an oversimplification of the evolution of the taxon (Blanco-Bercial *et al*., 2011; Bradford-Grieve *et al*., 2010). Nevertheless, the super-families provide a well-supported framework for the absence/presence of myelin (Davis *et al*., 1999; Lenz *et al*., 2000). Myelin has only been found in calanoids, where it is absent in taxa belonging to basal superfamilies, but is present in the more recently evolved ones (Figure 2). Amyelinates surveyed included representatives from the families Metridinidae in the Augaptiloidea, and Acartiidae, Candaciidae, Centropagidae, Pontellidae, Temoridae and Tortanidae (added since) in the Centropagoidea. Myelinate species were members of the families Calanidae, Megacalanidae (added since) and Paracalanidae in the Megacalanoidea; Eucalanidae in the Eucalanoidea, and Aetideidae, Clausocalanidae, and Euchaetidae in the Clausocalanoidea. Thus, myelinate taxa are largely absent from freshwater systems, which have been colonized primarily by centropagoidean (amyelinate) taxa (Boxshall and Jaume, 2000). So far no members of the superfamilies Bathypontioidea or Spinocalanoidea have been surveyed, but based on their phylogenetic placement, both are likely to be myelinate.

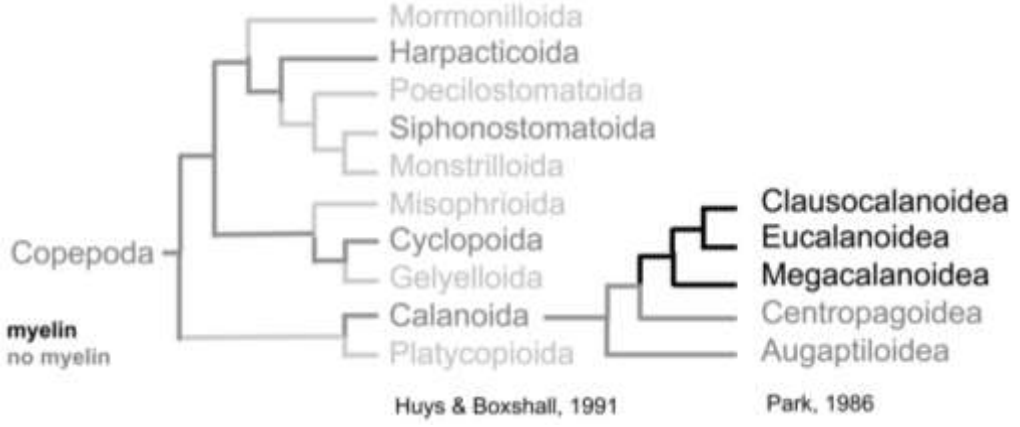

Figure 2. Phylogenetic tree of the Copepoda showing the hypothesized relationships between amyelinate (medium grey) and myelinate (black) taxa. Myelin appears only in the more recently-evolved superfamilies of the Calanoida. Light grey indicates unknown. Phylogeny of the order Copepoda from Huys and Boxshall (1991); that of the Calanoida from Park (1986).

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The identification of myelin (or lack thereof) requires examination of nerve tracts using transmission electron microscopy (Figure 1B-D), since myelin is poorly resolved at lower magnification. Copepods that have been checked for myelin using TEM have either shown no evidence of myelin, or they have a fully myelinated nervous system with nearly all, if not all axons enveloped by myelin (Figure 2). For example, a detailed study of the antennule of *Euchaeta rimana*, a member of the Clausocalanoidea, has shown that motor, mechanosensory and chemosensory axons are all enveloped by myelin sheaths ranging from just a few layers to *ca*. 50 layers (Weatherby *et al*., 2000). Ultrastructural studies of harpacticoids, cyclopoids and siphonostomatoids have not found myelinated axons in these copepod orders (Fahrenbach, 1962 and 1964; Gresty *et al*., 1993). Thus, myelin appears to have evolved a single time in the Copepoda - in the Calanoida, most of which are free-living and planktonic. Some evidence suggests evolutionary trends within the different myelinate groups, for example a greater tendency for more complete membrane condensation in the Clausocalanoidea (*E. rimana*). No examples of copepods representing intermediate stages in myelin evolution have been found, nor any examples of myelin loss. However, the number of species that have been investigated is small, and additional studies are needed to either confirm or refute these generalizations.

4. Body Size Does not Correlate with Myelination

Since one major benefit of myelin is a ten-fold increase in nerve impulse conduction speed, it has been suggested that the emergence of myelin in the vertebrates was an important contributor to the evolution of larger size (Castelfranco and Hartline, 2016; Salzer and Zalc, 2016; Zalc, 2016). By analogy, one might predict that myelin would lead to the evolution of larger copepods. An analysis of total length of myelinate and amyelinate taxa does not support this prediction (Figure 3A, B). The mean lengths of myelinate and amyelinate species in a broad selection of calanoids from the North Pacific figured in a plankton atlas (Yamaji, 1976) were 2.4 mm (n = 36) and 2.3 mm (n = 57), respectively (Student's t-test; $p = 0.74$). A similar analysis based on data from copepods in the Mediterranean Sea (Benedetti *et al*., 2016) confirmed no difference in size between the two groups (myelinate taxa: average length = 2.2 mm, n = 68; amyelinate taxa: average length = 2.2 mm, n = 54; Student's t-test, $p = 0.86$). However, calanoid copepods were significantly larger than non-calanoid copepods (cyclopoids, poecilostomatoids and mormonilloids: average length = 1.4 mm, n = 69; Student's t-test, $p = 1 \times 10^{-4}$; data from Benedetti *et al*., 2016).

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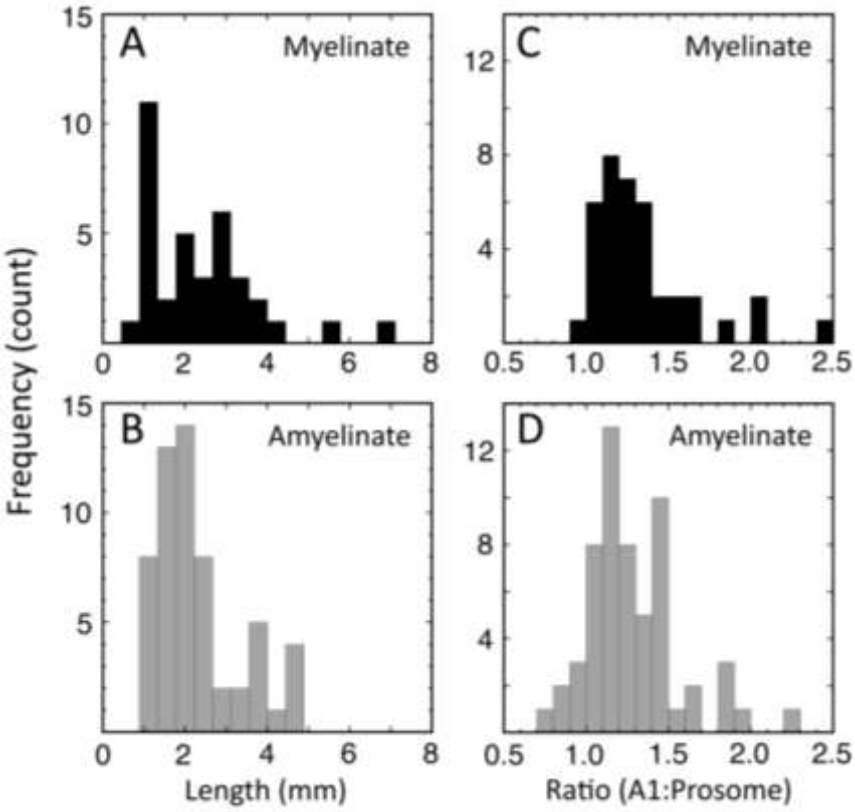

Figure 3. Histogram distributions of calanoid lengths (A, B) and antennular (A1) to prosome length ratios (C, D) of adult females in myelinates (black) and amyelinates (grey). Data were obtained from Yamaji (1976). Prosome and antennular lengths were measured from illustrations using ImageJ software. Calanoid lengths were obtained from descriptions, when a range of lengths were given a median value was used for the histogram. Calanoids were categorized into myelinate/amyelinate according to Lenz *et al.* (2000). Total number of observations – A: 36; B: 57; C: 38; D: 58.

While the calanoid body is quite compact, the antennules are long, typically as long or longer than the prosome length. Longer sensor-bearing antennules provide a copepod with greater sensitivity to water-borne disturbances that signal potential predatory threats Kiørboe and Visser, 1999), but at the expense of a longer delay in the arrival of that information in the central nervous system (CNS) where it must be acted upon to produce a behavioral response. Faster conduction of this sensory information would alleviate the disadvantage of long antennules. Thus, it might be predicted that antennular length is greater in myelinate than in amyelinate species. The data do not support this prediction (Figure 3C, D) – the median ratio of antennule length to prosome lengths is 1.3 (n = 38) and 1.2 (n = 58) in myelinate and amyelinate species, respectively (Wilcoxon-Mann-Whitney Rank Sum Test; $p = 0.50$). Thus, the general predictions of myelin leading to the evolution of larger bodies and longer communication distances between remote regions in calanoids are not supported.

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5. Myelinate Copepods have Shorter Reaction Times than Amyelinates

While expanding body size does not appear to be a factor in copepod myelination, reaction times are a different matter. Two studies compared response latencies in amyelinate and myelinate species, with somewhat different results. Lenz *et al*. (2000) used brief controlled displacements of a small plastic sphere to produce a calibrated near-field dipole flow stimulus triggering escapes in copepods tethered to a force transducer, so that the time between the onset of the stimulus and that of the force transient of the escape was measured with sub-millisecond accuracy. In two myelinate (*Neocalanus robustior* and *Undinula vulgaris*) and two amyelinate (*Labidocera madurae* and *Pleuromamma xiphias*) species tested (> 2 mm in prosome length), minimum response latencies of myelinate taxa were significantly shorter than those of the amyelinate ones (Figure 4A). A study using an acoustic stimulus and high-speed video to record escapes in free-swimming copepods (myelinate: *Paracalanus parvus*, *Mesocalanus tenuicornis*, *Subeucalanus pileatus*; amyelinate: *Acartia spinata*, *Centropages typicus*, *Pontella marplatensis*, *Pontella* sp., *Pontellopsis brevis*, *Temora turbinata*) failed to find a similar pattern (Waggett and Buskey, 2008). Most of the measured average response latencies ranged between 2 and 4 ms irrespective of the state of myelination or copepod size (prosome length: 0.6 – 2.3 mm). However, the experimental set-up may not have been optimized for this type of analysis given the ambiguous nature of the acoustic stimulus, the temporal resolution (1 ms) and synchronization between the stimulus and the camera (± 0.5 ms). The predicted difference in response latency would be less than 1 ms in the small species tested and requires sub-millisecond resolution to determine reliably. In addition, the two outliers, one myelinate (*S. pileatus*) and one amyelinate (*T. turbinata*) species, responded with longer delays, suggesting that these escapes were mediated by a slower escape circuit, possibly an alternative to the giant fiber circuit (Park, 1966; Lowe, 1935). While not much studied in copepods, arthropods including crustaceans possess multiple neuronal escape circuits having different response latencies (Herberholz and Marquart, 2012). Additional measurements of minimum response latencies to precisely controlled stimuli with high temporal resolution are needed to resolve differences in reaction times among these small organisms.

6. Myelinates Localize Sudden Hydrodynamic Disturbances Better than Amyelinates

Another, more recent study examined the question of whether the presence of myelin might affect escape responses other than through response latencies (Buskey *et al*., 2017; Figure 4). This study, motivated by the presence of exceptionally small myelinate

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calanoid copepods (< 1 mm total length) compared the directionality of the escape response between myelinate and amyelinate species (Figure 4B, C). Using a precisely controlled abrupt movement of a small plastic sphere to produce a rapidly rising deformation in the surrounding water, Buskey *et al*. (2017) discovered that myelinate copepodites and adults are better at localizing the stimulus and redirecting their escape away from it than amyelinates (Figure 4B, C). This difference was pronounced in copepodites that were initially oriented towards the stimulus (Figure 4C), while those oriented away directed their escape away regardless of state of myelination (Figure 4B).

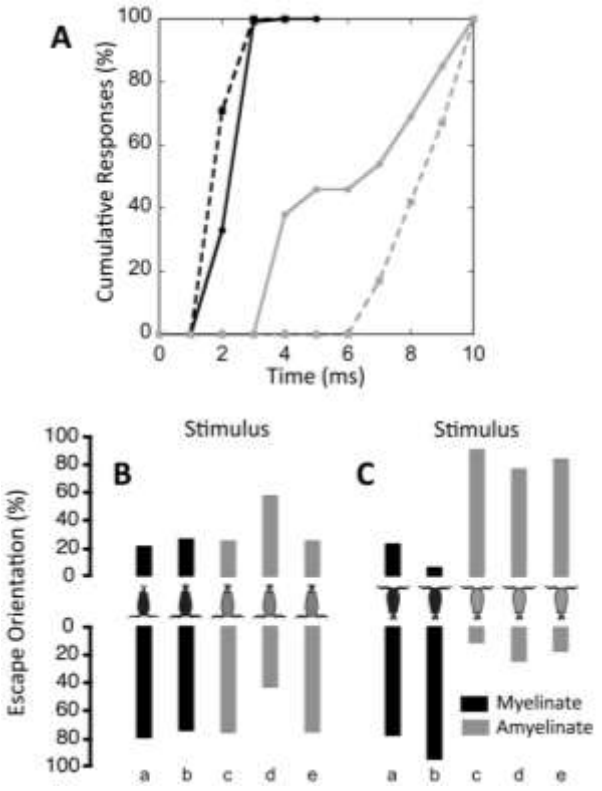

Figure 4. Calanoid copepod responses to a mechanosensory stimulus. A. Latencies to an abrupt stimulus. Cumulative distribution of the percent of responses within 10 ms after the stimulus was triggered. Black: myelinate species (*Undinula vulgaris* [circles, solid line; n = 77], *Neocalanus robustior* [squares, dashed line; n = 7]; grey: amyelinate species (*Labidocera madurae* [circles, solid line; n = 13], *Pleuromamma xiphias* [squares dashed line; n = 12]). Response latencies measured for individuals tethered to a force transducer. Data redrawn from Lenz *et al*. (2000; Figure 5). Number of responses with delays greater than 10 ms of the stimulus trigger: *U. vulgaris* (n = 0), *N. robustior* (n = 0), *L. madurae* (n = 3), *P. xiphias* (n = 14). B and C. Direction of escape swims of myelinate and amyelinate free-swimming copepods oriented away from (B) or towards (C) the stimulus. Direction of escape response of copepodites (CI-CIII, and CVI) triggered by an abrupt stimulus located at the top of the observation chamber. Species tested: myelinate species - a: *Bestiolina similis*, b: *Parvocalanus crassirostris* (black bars); amyelinate species - c: *Acartia tonsa*; d: *Eurytemora affinis*; e: *Centropages hamatus* (grey bars). Data presented as percentages; number of observations for panel B - a: 14; b: 19; c: 12; d: 37; e: 4, number of observations for panel C – a: 73; b: 67; c: 54; d: 117; e: 35. Figure redrawn from Buskey *et al*. (2017), with permission.

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While the difference in the ability to locate the stimulus was highly significant in copepodite stages (CI-CVI), it was not observed in the nauplii (Buskey *et al*., 2017). The absence of the long antennules and the early stage of myelination of the naupliar forms likely explain this difference.

7. DO MILLISECONDS MATTER?

How might the speed advantages of myelin play out in a predatory attack on a copepod? A number of behavioral studies have quantified various aspects of the copepod escape response including outcomes of interactions between copepod prey and fish predators (Bradley *et al*., 2013; Burdick *et al*., 2007; Buskey *et al*., 2002 and 2012; Clarke *et al*., 2005; Coughlin and Strickler, 1990; Coughlin *et al*., 1992; Gemmell and Buskey, 2011 and 2018; Gemmell *et al*., 2012; Jackson and Lenz, 2016; Waggett and Buskey, 2007). While these studies do not answer the question directly, they provide data that can be applied to hypothetical situations to estimate how differences in response time might affect the outcome of a predator-prey interaction. A copepod's peak escape speed matches that of a fish 10 to 30 times its body length (Lenz *et al*., 2004). Thus, a copepod 1 mm in body length can "outrun" a fish 1 to 3 cm in length. Pelagic fish larvae are in this size category and they are major predators of planktonic copepods (Sampey *et al*., 2007). If it takes 1 ms for a 1-mm copepod accelerating at a rate of 200 m s^{-2} (Buskey *et al*., 2002) to exceed the peak ram speed of ~200 mm s^{-1} of a 1-cm fish larva ($v_{(mm/s)} = 45 \times BL_{mm}^{0.68}$) (Lenz *et al*., 2004), the copepod can travel 0.1 mm in this time ($s = 0.5\ at^2$; s = distance; a = acceleration; t = time). However, there is an expected response-time delay of some 2 ms between detection and the initiation of the escape behavior by amyelinate copepods of this size (*Acartia*: Buskey *et al.*, 2002). The fish would thus have at least a 3-ms head start on the copepod in which time it can cover a distance of 0.9 mm if it is capable of the same acceleration. Thus, the copepod can out-distance the fish if the strike distance is > 1 mm, which is comparable to reported strike distances (China and Holzman, 2014; Coughlin, 1994). Nerve fibers in ~1 mm calanoid escape circuits are ~3 μm in diameter (Figure 1A; Wilson and Hartline, 2011a) and are expected to conduct at around 1.7 m s^{-1} if unmyelinated and around 8 m s^{-1} if myelinated (Bullock and Horridge, 1965). A myelinate calanoid of this size can thus shave ~1.2 ms off its conduction time along a 2.25 mm pathway (antennule + ventral nerve cord; see also Lenz *et al.*, 2000). So the fish would have to reduce its strike distance to 0.2 mm for a successful capture (absent suction) – less easily achieved without alerting the copepod to the fish's approach.

Another important component of the escape is its direction. On a purely random distribution of escapes relative to the direction to the predator, half of escapes would be toward it and half away. While some of the escapes toward the predator might be sufficiently off the line of attack to elude capture (absent strong suction), those directed

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away are on average more likely to be successful. Thus, an effective escape often involves a reorientation by the prey (Figure 4B, C), but this adds an additional delay to the escape swim. *Acartia tonsa*, for example, takes 1 ms or more to turn 100° (Buskey *et al*., 2002). For a strike from a distance of 0.9 mm, a 1.2 ms faster reaction would allow a myelinate copepod to reorient by up to 120º without exceeding the time needed to achieve a successful escape speed. While the discussion here is speculative, it provides specific hypotheses that might be tested experimentally using high-speed videography to quantitatively compare predatory lunges with escape behavior of copepod prey.

8. ECOLOGY OF MYELIN

8.1. Myelinates Dominate over Amyelinates in Marine Environments with High Visibility

Anti-predator strategies can be divided into adaptations that either 1) lower encounter rates with predators, or 2) allow the prey to escape from a predator once an encounter has occurred (Langerhans, 2007). While calanoids possess numerous adaptations that decrease encounter rates with predators such as transparent and small bodies, diel vertical migration, "sit-and-wait" strategies, and dormancy (Bollens and Frost, 1991; Pasternak *et al*., 2006; Thuesen *et al*., 1998; Strickler *et al*., 2005), the behavioral studies described above support the conclusion that myelin is an adaptation that enhances escape performance, and that it might be a factor in niche partitioning. Since myelinate and amyelinate taxa are widespread and co-occur in all marine environments, this raises the question how this difference in escape performance affects ecological success, measured either by diversity or abundance. A biogeographic study of the distribution of myelinate and amyelinate taxa in oceanic and estuarine environments addressed this question, and concluded that myelinate taxa are more abundant and diverse in environments where a better escape response may effectively decrease risk from visual predators (Lenz, 2012).

Epipelagic oceanic environments are characterized by high water transparency, low standing stocks of phytoplankton and an abundance of vertebrate and invertebrate predators. Resident calanoid copepods in the upper 100 m are typically dominated by myelinate taxa (Lenz, 2012; Fernández de Puelles *et al*., 2018). While amyelinates are important members of these communities, most of them are predominately diel vertical migrators, which enter the upper water layers primarily at night, thus avoiding day-time encounters with visual predators (Lenz, 2012). Thus, an analysis of the vertical

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distribution of amyelinate and myelinate taxa in the North Pacific gyre shows a pattern of narrow vertical distributions for the dominant myelinate species, and broad distributions extending into mesopelagic depths for the amyelinates (Figure 5; data from Ambler and Miller, 1987).

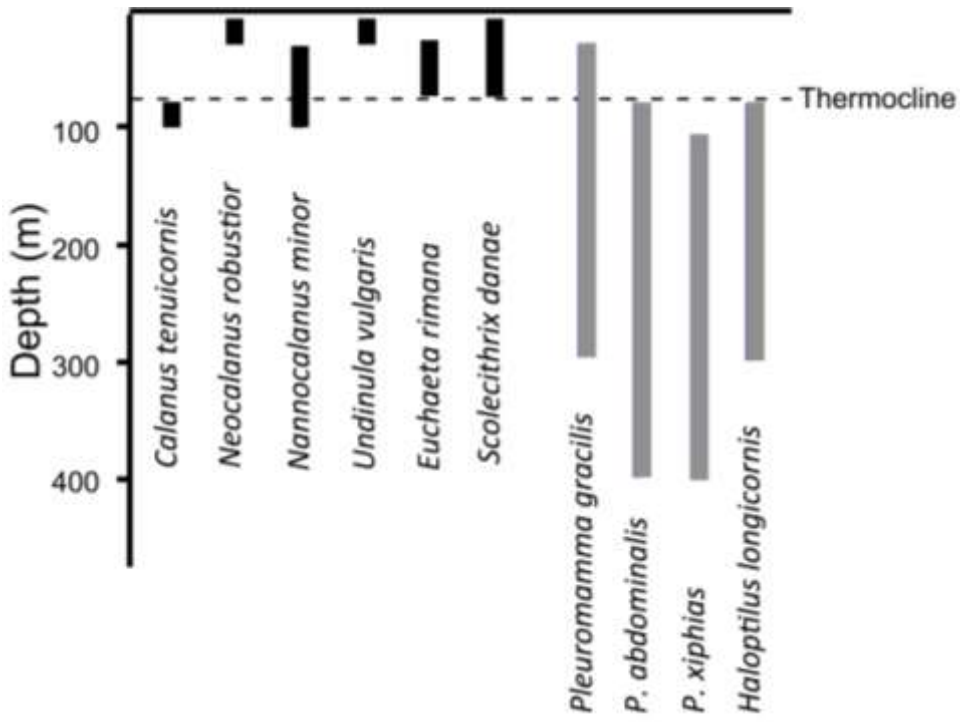

Figure 5. Vertical distribution of 10 abundant calanoids of the North Pacific gyre. Bars represent the range over which the indicated species are found. Myelinates (left set of short bars - black) show little diel vertical migration, hence their bars are short. Amyelinates (long, grey bars on the right) are found deeper and many undergo extensive diel vertical migration, so they are found at different depths at different times of the day, greatly expanding their vertical range. Data from Ambler and Miller (1987).

Near-shore coral-reef-associated habitats, which are characterized by high transparency and low seasonality, have a predominance of myelinate taxa (Lenz, 2012). These habitats harbor diverse communities of planktivorous fishes, and hence present high risk from visual predators. This is in contrast to most estuarine environments, which are typically dominated by amyelinate calanoid copepods. Many of the latter environments are characterized by high turbidity, which reduces visual predation. Temperate estuaries also experience strong seasonality, so amyelinate calanoids in these environments produce overwintering eggs, a strategy that enhances survival during unfavorable environmental conditions including periods of high predation risk (Marcus, 1984 and 1996; Castro-Longoria and Williams, 1999).

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8.2. Myelin is Correlated with Niche Separation between Co-Occurring Myelinate and Amyelinate Species

Niche separation between myelinate and amyelinate species within a zooplankton community is supported by a re-analysis of a study characterizing copepods by their ecological role in the Mediterranean Sea (Benedetti *et al*., 2016). The study found evidence for six distinct ecological niche groupings (= "functional groups") after clustering 191 copepod species by similarity using data on vertical distribution/migration, swimming behavior, feeding habits/trophic position and morphology. The analysis included 122 calanoids (68 myelinate and 54 amyelinate taxa), 61 cyclopoids/poecilostomatoids, six harpacticoids and two mormonilloids. While five of the six functional groups had a significant number of calanoid species, myelinate and amyelinate taxa did not contribute to these groups proportionally ($p \leq 0.001$; Chi-square test; Figure 6). Nearly half (49%) of myelinate taxa belonged to a single functional group (group 4), which consists of species that are mostly herbivorous, oceanic and contribute significantly to carbon flux. Of the remaining myelinate taxa 34% were assigned to functional group 6, which includes a large number of detritivores and small cruising herbivores. Amyelinate taxa were over-represented in three functional groups (70%, groups 1, 2 and 3), which include the large and small carnivores (groups 1 and 2, respectively) and mostly neritic species (group 3).

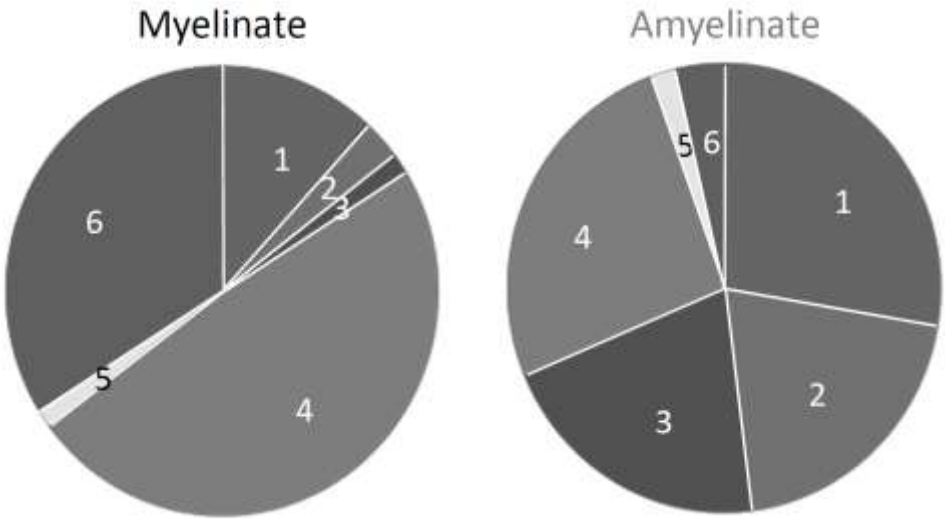

Figure 6. Distribution of myelinate vs amyelinate copepod species of the Mediterranean Sea among 6 functional groups defined by Benedetti *et al*. (2016). Myelinates dominate in groups 4 and 6; amyelinates in groups 1-3. Total number of species: myelinate, 68; amyelinate 54.

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Metabolic studies of deep-living copepods provide additional evidence for niche separation based on the presence/absence of myelin. Calanoid taxa that occur in mesopelagic (200 – 1000 m depth) and bathypelagic habitats (1000 - 4000 m depth) include suspension feeders, detritivores and carnivores from both amyelinate and myelinate taxa. While in general deep-living calanoids have lower metabolic rates than epipelagic ones (Ikeda, 2008), adjusted metabolic rates (AMR), protein, energy content and condition factor index (CFI = dry weight/prosome length^3) were all significantly higher in myelinate calanoids compared with amyelinates (Ikeda *et al*., 2006a, 2006b and 2007). These indicators suggest that myelinates in these deep environments are more active than amyelinates.

Deep-living calanoids differ in their ATP metabolism and this difference correlates with myelin. Eukaryotes have two pathways for producing ATP: an aerobic one for which the activity level of citrate synthase is an indicator, and an anaerobic one, characterized by activity of lactate dehydrogenase (LDH). The anaerobic pathway is important when oxygen supply is insufficient to sustain high muscle activity. Using these two indicators, Thuesen *et al*. (1998) examined the relative importance of these pathways in calanoids. In general, they found that LDH activity levels are high in mesopelagic and bathypelagic calanoids compared with surface dwelling ones, while citrate synthase activity is low. Thus, they concluded that the deep-living calanoids have a higher dependence on anaerobic metabolism, which is presumably important for episodic energy demands such as burst swimming. A re-analysis of Thuesen *et al*.'s (1998) data suggests that myelinates and amyelinates differ in LDH activity levels (Figure 7). With the exception of the giant calanoids, LDH activity was lower in myelinate calanoids than in amyelinates. Thus, deep-living myelinate calanoids, which are metabolically more active than amyelinate ones (Ikeda *et al*., 2006a), have a much less pronounced difference in the relative importance of aerobic and anaerobic energy production. By eliminating the need to restore sodium concentrations along the inactive insulated stretches of axons after an impulse, myelin is thought to greatly reduce demand on metabolic energy sources supporting neural activity. These phenotypic differences between myelinate and amyelinate calanoids may thus be indicators of differences in life history strategies related to optimizing survival. Reduction of activity level is one strategy to decrease encounter with and/or detection by potential predators (Langerhans, 2007). While this may be an important strategy for all mesopelagic and bathypelagic calanoids, it may be even more important for amyelinate taxa.

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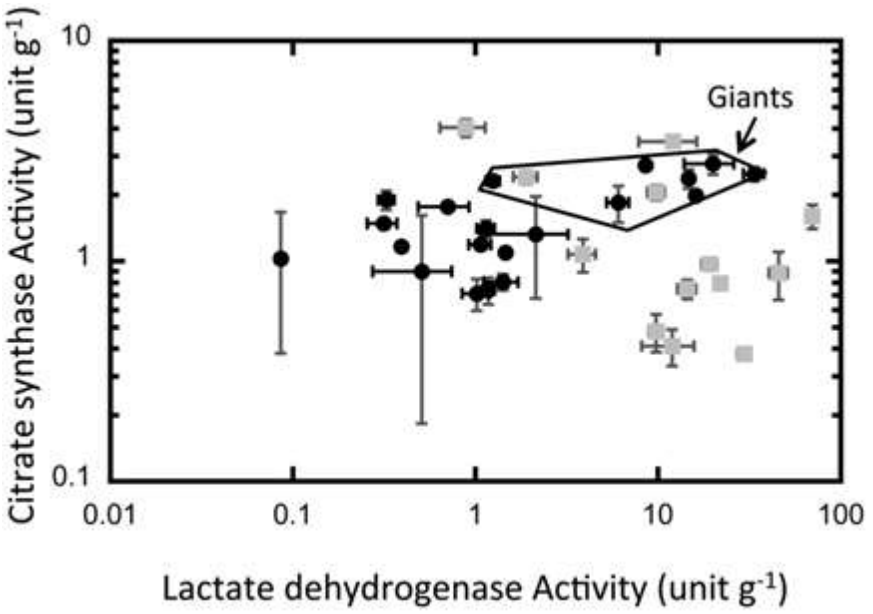

Figure 7. Relationship between citrate synthase activity and lactate dehydrogenase activity in copepods collected from depths between 200 and 2000 m. Black circles: myelinate taxa (*Onchocalanus magnus*; *Landrumius gigas*, *Gaetanus* spp., *Megacalanus* spp., *Bathycalanus* spp., Euchaetidae); grey squares: amyelinate taxa (*Metridia princeps*, *Pleuromamma abdominalis*, *Gaussia princeps*, *Arietellus plumifer*; Heterorhabdidae, Augaptilidae, Lucicutiidae). Giants: *Megacalanus* spp., *Bathycalanus* spp., *Gaussia princeps* (males, females). Data redrawn from Thuesen *et al*. (1998; Figure 8). Calanoids were categorized into myelinate/amyelinate according to Lenz *et al*. (2000). Error bars: standard errors.

9. Invasion of the Pelagic Environment and Evolution of Myelin

While copepods inhabit a variety of environments and niches that include interstitial, benthic, troglodytic and even terrestrial ecosystems, their importance as key members of pelagic communities in marine and freshwater habitats stands out. It has been proposed that planktonic taxa have evolved from benthic forms (Huys and Boxshall, 1991). The invasion of the pelagic may have occurred during a period of high turbidity during the Devonian (~400 million year ago [Mya], "Age of Fish"), which made the benthos a less favorable environment due to low light, decreasing food resources and low oxygen availability (Marcotte, 1999). High-speed miniaturization assured the success of many taxa including copepods, since it allowed them to selectively feed on small food particles (phytoplankton) mixed with inert ones (Marcotte, 1999). However, high turbidity also meant limited visibility, providing an opportunity for benthic forms to invade the water column during a period of relatively low risk from visual predators. The pelagic Arietelloidea (= Augaptiloidea) and Diaptomoidea (= Centropagoidea) may have evolved during this early period (Bradford-Grieve, 2002). High turbidity was followed by a period

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of high transparency during the Permian (~275 Mya). Based on the fossil record, diversity in pelagic species decreased dramatically, while there was an increase in benthic crustaceans *i.e.,* decapods (Marcotte, 1999). Increased predation risk from visual predators due to high transparency might have been a driving selective force. Thus, Bradford-Grieve (2002) hypothesized that the evolution of more-recent super-families such as the Calanoidea (Megacalanoidea) and Clausocalanoidea along with myelin occurred during this time.

CONCLUSION

The unexpected discovery of myelin in half of all calanoid copepods has raised questions regarding the advantages of this innovation in small organisms with simple nervous systems. Ted Bullock (1996), a comparative neuroscientist and founding father of neuroethology, concluded that there was an inherent paradox in adaptations that increase conduction speeds in small organisms, since conduction delays are essentially negligible. The existence of taxa with and without myelin in the calanoids provides a unique opportunity to address the paradox and quantify the benefits of this innovation in small organisms. We have reviewed studies comparing escape reactions and predator-prey ecology between myelinate and amyelinate copepods, arguing that several lines of evidence support the hypothesis that the presence of myelin confers greater resistance to predation, especially from visual predators. The fact that there are few confounding differences between myelinates and amyelinates strengthens this conclusion: they share similar body forms, size distributions, antennule to prosome length ratios, maximum scaled escape speeds, and organization of sensory and motor systems.

Lower susceptibility to visual predators has led in turn to differences in biogeographic distributions and ecological functional groups of myelinate and amyelinate taxa, suggesting niche separation based on nervous system architecture. Early-stage development of myelin is observed in calanoid nauplii, and its presence may confer some benefit to these young stages. However, the extent of myelination of axons in the central and peripheral nervous systems is much greater in the copepodite stages and progressively increases to the adult stage. It is in the copepodites that behavioral differences between the amyelinate and myelinate forms become apparent. “Negligible” conduction delays notwithstanding, response latencies are shorter in myelinate copepodites than amyelinates, and myelinates are better at localizing and escaping away from a stimulus source.

Planktonic communities are characterized by high predation pressure and both phytoplankton and zooplankton exhibit a variety of adaptations that lower predation risk. Myelin is an adaptation that is likely to increase the chances of survival once an encounter has occurred. Thus, differences in escape performance are expected to lower

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predation risk in environments with high encounter rates, especially with visual predators that stalk unwary prey and then attempt capture with a sudden attack. The evolution of myelin is a key innovation in the Calanoida that undoubtedly promoted radiation within several superfamilies.

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Chapter 10

EVASION FROM PREDATION: UNDERSTANDING COPEPOD ESCAPE BEHAVIOR IN RELATION TO PREDATOR CAPTURE STRATEGIES

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ABSTRACT

Copepods are a key link in marine food webs and are consumed by a wide range of predators. As a result, copepods have evolved numerous adaptations for avoiding predation. The escape response of calanoid copepods is arguably one of the most important adaptations as it is well-developed in most species and developmental stages and functions against a variety of predatory modes. Copepods have evolved sensitive mechanoreceptors in the antennae to detect the presence of predators, and respond with powerful swimming strokes which can produce speeds in excess of 500 body lengths per second. Copepods also respond with one of the shortest response latencies of all aquatic organisms, and can react to a hydrodynamic disturbance in as little as 2 milliseconds. Yet many predators are capable of capturing copepods with high success. However, success in capturing copepods varies with predatory mode and developmental stages of the copepod. The great abundance of copepods within the marine environment and number of species that rely on them as food indicate the importance of understanding these interactions. Here we discuss the interactions between predators and their copepod prey, the ability of copepods to evade predators, and several of the mechanisms predators employ to capture copepods.

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1. INTRODUCTION

Copepods are an evolutionarily successful group, as they are among the most numerous multicellular animals on earth (Humes, 1994). On a global scale copepods are estimated to process 7.5-10.5 gigatons of carbon per year (Calbet and Saiz, 2005). Therefore, this group of animals plays a key role in marine food webs which makes their behavioral adaptations to predation important to understand. Copepods in the order Calanoida are considered to be the most ecologically significant group, in part, because they often exceed other copepod groups within the neritic and pelagic zones in terms of abundance or biomass (Dagg and Turner, 1982). Calanoid copepods are important grazers on microplankton in marine food webs (Banse, 1995) and are, in turn, preyed upon by a wide range of predators with diverse feeding adaptations. In addition to their ecological roles, copepods are known to be a superior food source when compared to traditional feeds used in aquaculture such as enriched *Artemia* sp. (Shields *et al.*, 1999). Thus, understandings of copepod-predator interactions have both direct ecological and economic implications.

In nature, planktonic copepods are subject to predation from a wide array of different taxonomic groups. As such, they require a variety of adaptations that aid in minimizing detection, capture and ingestion from a host of different predation strategies. In this chapter many of these different predation modes will be explored with respect to a copepod's ability to detect, respond and ultimately survive encounters. Non-visual predators are the most taxonomically diverse and consist of cnidarian medusae, ctenophores, chaetognaths and even other copepods. There are also a host of benthic invertebrate predators that feed on copepods which include: bivalve molluscs (*e.g.*, mussels), crustaceans (*e.g.*, barnacles), cnidarians (*e.g.*, corals). Visual predators consist primarily of fish but also include cephalopods, large mammals (*e.g.*, baleen whales) and occasionally some bird species. Since fishes themselves are a highly diverse group, there is a variety of predation adaptations which result in high variation in predation success on copepods.

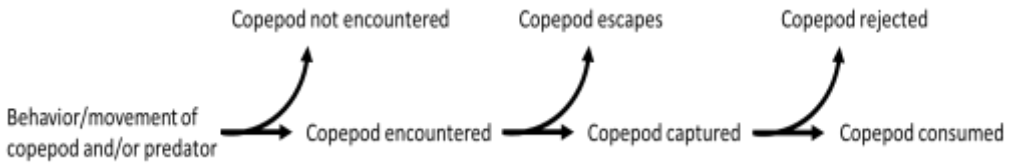

Figure 1. Potential outcomes of copepod interactions with a predator. The primary focus of the chapter will be on the factors that influence results of post-encounter situations that determine whether a copepod escapes or is captured.

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In order to survive in an ocean teeming with predators, copepods possess an array of adaptations to prevent detection, capture and ingestion (Figure 1). At each of these steps predators and prey are involved in an evolutionary arms race. Predator adaptions are aimed at increasing encounter, capture and ingestion rates while copepods possess adaptions that aid in minimizing these. The primary focus of this chapter will be on the factors that determine the outcome of post encounter interactions with predators. However it is worth identifying some of the other important factors that allow copepods to avoid succumbing to predators both pre-encounter and post capture.

The result of any predator-prey encounter will depend upon many factors. One central factor that emerges from the behavioral ecology of copepods is that there is no place to hide from a diverse assemblage of predators in open water. In order to limit encounters with predators, nearly transparent tissues help copepods lower their conspicuousness to visual predators. However this has implications for feeding because ingesting pigmented food during daylight hours can partially mitigate this effect (Giguere and Northcote, 1987). A common behavioral mechanism to minimize encounters with predators is diel vertical migration. Copepods migrate downwards to depths with reduced light intensity during daylight hours to avoid the suite of visual predators that rely on light for prey detection (Bollens and Frost, 1989). In cases where non-visual invertebrate predators dominate and follow a similar migration pattern as copepods, a reverse diel vertical migration can occur where copepods move into surface waters during the day to minimize encounters with the dominate predator (Frost and Bollens, 1992). Another behavioral adaptation copepods can employ to minimize encounters is modifying their swimming patterns. Swimming kinematics that generate low hydrodynamic disturbance are likely to minimize encounters with rheotactic predators relying on the detection of flows created by moving prey (Titelman and Kiørboe, 2003; Tiselius *et al.*, 1997; Ohman, 1988).

If a copepod fails to avoid detection by a predator, it can still survive the encounter if it can promote rejection by the predator. Some copepods possess spines, which are known to cause rejection by small fish feeding on planktonic crustaceans (Barnhisel, 1991). Certain species of copepods can physiologically produce bioluminescence (Latz *et al.*, 1987; Herring, 1988) in response to a physical disturbance simulating the approach of a predator (Hartline *et al.*, 1999), and flashes of bioluminescence can trigger a startle response (Buskey and Swift, 1985) or potentially distract potential predators (Herring, 1988). However, the escape response which rapidly propels the animal away from a potential predator is arguably the most important mechanism in avoiding predation once an encounter has occurred.

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2. DETECTION OF PREDATORS

In order to respond to a potential threat, there must be reliable mechanisms in place to detect the predator. Calanoid copepods have three sensory modalities by which they can potentially sense an approaching predator prior to executing an escape: photosensory, chemosensory and mechanosensory receptors. The visual system of copepods often consists of three pigment cups that combine to form a naupliar eye (Ong, 1970). This eye is not capable of forming images, only responding to rapid changes in light intensity such as flashes of light or shadows (Buskey *et al.*, 1986). Copepods can detect and respond to rapid changes in light with an escape response which may be an adaptive response to the presence of a diurnal predator overhead (casting a shadow) or to a bioluminescent predator such as a ctenophore (Buskey *et al.*, 1986). It therefore makes sense for both types of photic stimulation to elicit an escape response in copepods. Although copepods can detect sudden changes in light intensity, they appear unable to distinguish between sources. This is supported by observations that bioluminescent dinoflagellates, which are a food source of copepods, can elicit escape responses from copepods by producing their own bioluminescence (Buskey and Swift, 1983; Buskey *et al.*, 1986). Because these flashes of light from the dinoflagellate appear indistinguishable from those of a potential predator, the dinoflagellates may use this as a defense to disrupt the normal feeding behavior of the copepod.

Chemical stimuli can be detected via chemosensory cells located on the first antenna (Boxshall and Huys, 1998) but do not appear important in generating an escape response (Fields and Yen, 1997) or triggering vertical migration in the water column (Bollens *et al.*, 1994). Instead the chemosensory cells found in copepods likely function in prey and mate detection (Weissburg *et al.*, 1998; Yen *et al.*, 1998; Langhoff *et al.*, 2018). The first antennae is also lined with setae (or sensilla), which are small structures that are innervated by sensory cells (Figure 2; Strickler and Bal, 1973; Yen and Nicoll, 1990). Mechanical disturbances are detected from the deformations of fluid movement (Yen *et al.*, 1992). Depolarization causes the transmission of an action potential to a motor neuron which stimulates muscles and generates the escape response. The mechanosensory systems of pelagic adult copepods are well developed. The first antennae (antennules; A1) mechanoreceptors of the adults are highly sensitive (Hartline *et al.*, 1996) and have many microtubules (500 to 3000) which fill the distal dendrites of the mechanosensory neurons (Weatherby *et al.*, 1994). Each pair of dendrites is surrounded by a well-developed scolopale and by two sheath cells, one of which is firmly attached to the cuticle via microfilaments (Weatherby and Lenz, 2000). These characteristics make the system particularly rigid and thus contribute to its high mechanosensitivity (Hartline *et al.*, 1996).

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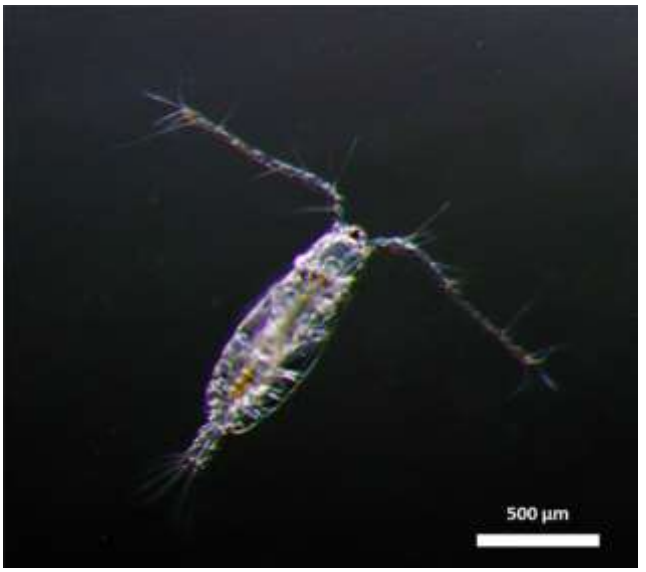

Figure 2. Photograph of the copepod *Acartia tonsa*. This species exhibits very short response latencies and high swimming velocities in response to hydrodynamic disturbances created by predators, which are detected using the large setae on the prominent antennule.

In order for a copepod to survive an attack from a predator, it must be able to detect the approach of a predator and perform an appropriate escape response. However, the strength of detection and escape vary depending on the developmental stage (Buskey, 1994). This is likely due to the fact that the setae on the distal tip of the antennae are primarily responsible for the detection of predators (Lenz and Yen, 1993), but the distal portion of the antennae does not fully resemble the adult until developmental stage N6 (Boxshall and Huys, 1998). This suggests that predator detection ability increases throughout each molt during the nauplii stages. During the transition from nauplii (N6) to copepodite (C1), the predator detection capabilities also increase (Buskey, 1994). This trend continues through each copepodite stage as the number of segments and setae proximal to the tip increase with subsequent molts (Boxshall and Huys, 1998), which provides a plausible mechanism for the continued improvement in sensitivity with each molt. Although the distal tip resembles that of the adult at the N6 stage, sensitivity may also still improve at the distal tip due to continued development of the sensory neurons involved in detecting hydrodynamic disturbances, but little is known about the internal structures of antennae during development.

3. Generation of an Escape Jump

Rapid escape swimming may be the most important anti-predator adaptation copepods possess once encountered (Figure 3). During the peak of a typical escape,

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copepods exceed speeds of 100 body lengths per second (bl s^{-1}) and some species can achieve instantaneous speeds in excess of 500 bl s^{-1} (Trager *et al.*, 1994; Buskey *et al.*, 2002; Lenz *et al.*, 2004). Due to their small size, copepods perform rapid escape swimming under low/transitional Reynolds numbers (Re) from less than 100 (van Duren and Videler, 2003) to roughly 500 (calculated from Buskey *et al.*, 2002). In order to achieve such speeds, copepods must produce a large amount of force. Escaping copepods are capable of producing more than 100 dynes per jump (Lenz and Hartline, 1999) which is more energy per gram of body weight than almost any other animal. While achieving high relative swimming speeds through powerful swimming muscles is a clear advantage for surviving encounters with predators, the ability to react quickly to a perceived threat could be equally important. Copepods have one of the shortest reaction latencies known for aquatic organisms and can respond in as little as 2 ms to a hydrodynamic disturbance (Lenz and Hartline, 1999; Buskey *et al.*, 2002; Waggett and Buskey, 2007a).

Generally, there are six naupliar stages (N1-6) and five copepodite stages (C1-5) before a copepod molts into an adult (Lawson and Grice, 1970), and the escape response is present in all stages of copepod development (Buskey, 1994; Titelman, 2001; Green *et al.*, 2003). The escape response is produced by a different set of appendages in the youngest (naupliar) stages compared to the adult and copepodite stages (Gauld, 1959). In addition, many of the mechanoreceptors for hydrodynamic sensing of an approaching predator are missing in the youngest stages (Weatherby and Lenz, 2000). Interestingly, escape speed in terms of body lengths per second is similar for both nauplii and copepodites (Buskey *et al.*, 2002; Bradley *et al.*, 2012).

In the adult and copepodite stages, copepods are primarily propelled forward by anterior-to-posterior metachronal strokes of the thoracic pereiopods (Strickler, 1975). During the initial stages of the escape when the animal achieves maximal acceleration, the use of the telson as a thrust generating appendage is also very important (Figure 4). The sweeping motion of the telson creates a large jet directed posteriorly to the animal such that both pereiopods and telson motion contribute to forward thrust. Using these appendages, copepods accelerate within milliseconds to speeds up to 800 bl s^{-1} (Lenz *et al.*, 2005). However, the mechanism used for generating thrust for escapes is considerably different in the naupliar and copepodite stages. Nauplii lack pereiopods so in order to generate a rapid escape they beat the 1st antennae, 2nd antennae and mandibular palps sequentially (Gauld, 1959). In comparison, the 1st and 2nd antennae contribute very little to the propulsive forces that are generated during an escape for adults and copepodites as the antennae become folded against the body making the copepod more streamlined (Lenz *et al.*, 2004). The emergence of pereiopods in copepodites results in an escape that is stronger and therefore allows the animals to propel themselves a greater distance from a potential predator (Landry, 1978). With each subsequent molt from C1 to C5, a new pair of pereiopods emerges and older ones become larger and presumably more powerful.

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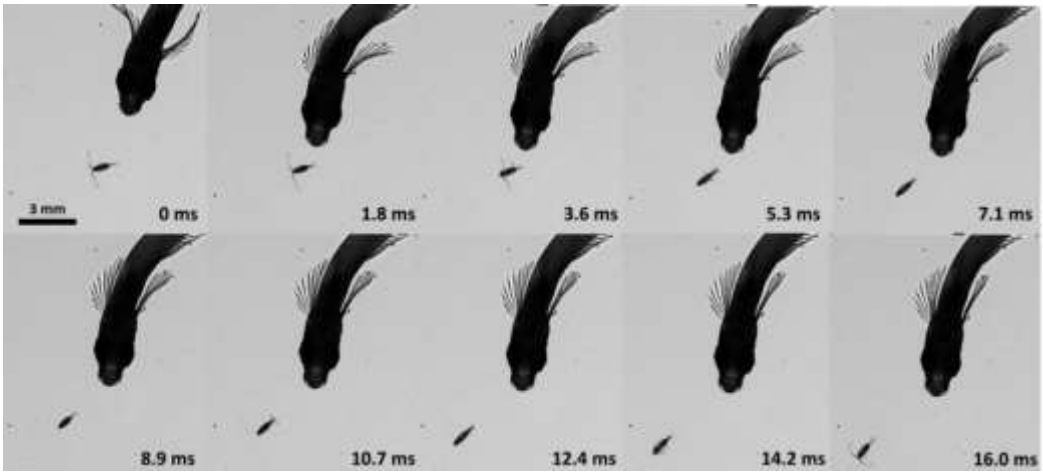

Figure 3. The copepod *Acartia tonsa* responding to the approach of a juvenile pinfish (*Lagodon rhomboides*) with rapid escape swimming.

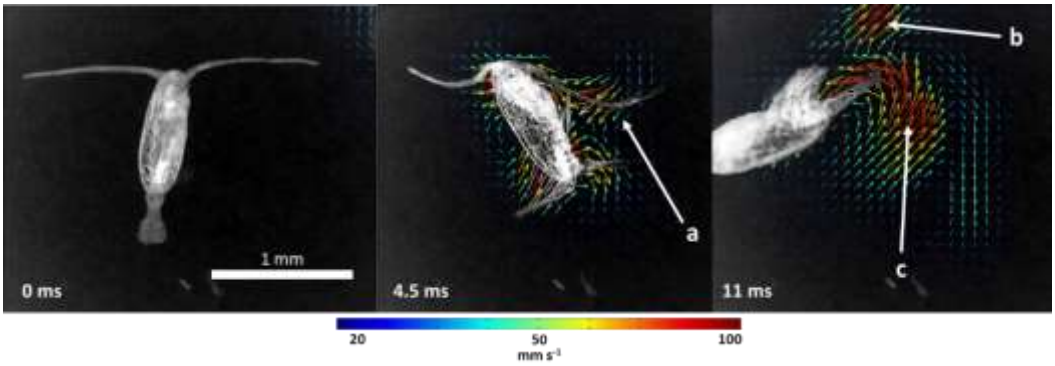

Figure 4. *Acartia tonsa* responding to a hydrodynamic disturbance. The left image is time zero, before initiation of the escape jump. In the center image, 4.5 ms later, the antennae is used to generate thrust and provide the initial reorientation away from the disturbance (a) and the telson is pulled back perpendicular to the prosome. In the right image at 11 ms, completion of the sequential movement of the pereiopods results in a jet of fluid ejected behind the copepod (b). However, the sweeping movement of the telson appears to generate most of thrust during the initial acceleration as evidenced by the much larger jet it produces (c).

Adult copepods are also able to change orientation of escape in response to an approaching predator (Buskey *et al.*, 2002). Escapes responses for adult *Acartia tonsa* usually begin with a rapid, random reorientation from the source of the stimulation which causes the escape, and the animals do this by turning at a rate of approximately 30° ms^{-1} (Buskey *et al.*, 2002). Adults reorient either by the asymmetrical sweep of the A1, or through a backward summersault produced by a combination of pereiopod and urosome movement. Directional capabilities are therefore likely to depend on differences in stimulus strength at the two A1 tips, as well as timing in the sensory neurons (Buskey *et al.*, 2017). The ability to produce a powerful escape with directionality is important, but another key to surviving an attack from a predator is being able to respond rapidly. Some

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species within the order Calanoida also possess myelination around neurons which, as in vertebrates, allows faster transmission of the action potential and thus faster responses to stimuli (Davis *et al.*, 1999). The reaction times of myelinated species can be 2 to 5 times faster than some non-myelinated species (Lenz *et al.*, 2000). This may provide an advantage by which myelinated species can respond more quickly to a hydrodynamic signal from a predator. However, non-myelinated species can also exhibit short response latencies, and some small non-myelinated copepods can exhibit minimum response latencies comparable to those of myelinated species (Waggett and Buskey, 2008). It was also observed that myelination did not result in increased survivorship when exposed to a visually hunting fish predator (Waggett and Buskey, 2007a). Because of this, it has been suggested that perhaps myelin functions more as an energy saving mechanism due to more efficient transfer of action potential which provides a likely advantage in low food oceanic habitats where myelinated species are most prevalent (Waggett and Buskey, 2008), or for more accurate detection of the direction of approach of a predator (Buskey *et al.*, 2017). This topic is discussed in more detail in the chapter by Lenz and Hartline (2018).

The dominance of planktonic copepods within the mesozooplankton results in part from their highly developed detection and anti-capture adaptations. As with many species, predation risk is not uniform with age or developmental stage, and in copepods predation is greatest on the younger developing stages compared to the adults (Sell *et al.*, 2001). One reason is likely that younger stages are less capable of detecting a potential predator (Buskey, 1994), and even when an approaching predator is detected in time to generate an escape response, the escape of a young copepod is often much less effective than an adult's (Sell *et al.*, 2001; Titelman, 2001). However, with respect to relative escape speeds (*i.e.*, bl s^{-1}) copepods outperform fish by an order of magnitude, suggesting that an escaping copepod can keep ahead of a pursuing fish up to 30 times longer than the copepod itself (estimated from Lenz *et al.*, 2004). The changes in the escape system from nauplius (stages N1 to N6) to copepodite (C1 to C5) to adult in response to hydrodynamic and bioluminescent stimuli are substantial, but poorly understood. Most focus has been on research involving external changes to appendages, but behavioral studies are required to determine the significance in relation to interactions with predators which will have adaptive and evolutionary significance on both predator and prey.

4. NON-VISUAL PREDATORS

Predators of copepods can be classified into two main groups; visual and non-visual predators. The non-visual class includes a wide variety of organisms from entangling predators, such as medusae and ctenophores, to suspension feeders, such as bivalve mollusks and even other copepods. Chaetognaths (arrow worms) are raptorial predators

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on copepods and locate prey using the hydrodynamic disturbances created by copepod swimming (Feigenbaum and Reeve, 1977). Corals (Sebens *et al.*, 1996), barnacles (Trager *et al.*, 1994) and even larger copepods (Landry, 1980) are known to prey on smaller copepods. For avoiding non-visual predators, mechanisms such as transparency are not effective; instead, reducing hydrodynamic signals to limit detections and exhibiting high sensitivity to hydrodynamic disturbance created by predators is vital.

Some cnidarian medusae create fluid motion during swimming to entrain prey and bring them in contact with tentacles (Costello and Colin, 1994) which contain immobilizing nematocysts. This flow over the bell creates a hydrodynamic regime different from surrounding water, and this shear stress potentially provides a signal for detection by copepods. The strength of the flow field is a function of medusa bell diameter. Medusae with bell diameters less than 7 cm produce weaker flow fields, and therefore highly evasive prey such as adult copepods will be negatively selected for, while prey which exhibit low escape velocities will be captured frequently (Costello and Colin, 1994). Thus, adult copepods should only be captured at high rates by larger medusae which can create high flow velocities which could exceed escape velocities of copepods. Suchman and Sullivan (2000) found that when *Acartia hudsonica* encountered the scyphomedusae *Aurelia aurita* and *Cyanea* sp., less than 1% of encounters resulted in ingestion suggesting that copepods could successfully detect and escape from these predators. This result is interesting as several studies have concluded that gelatinous predators such as medusae can exert top-down control on copepod populations (Lindahl and Hernroth, 1983; Matsakis and Conover, 1991; Behrends and Schneider, 1995; Omori *et al.*, 1995). Perhaps turbulence in nature may increase encounter rates or mask the hydrodynamic signals available for copepods to detect a potential predator. Also, the impact on copepod populations may not be occurring strongly at the adult stage but instead on developing ones, which are known to have lower sensitivity to hydrodynamic disturbances (Buskey, 1994). Indeed developing copepods were found to be captured at a higher rate than adults (Suchman and Sullivan, 2000), which may explain the results suggesting an ability of medusae to exert top-down control of copepod populations under certain conditions.

Ctenophores have been found to be more effective predators on copepods than most cnidarian medusae, with clearance rates 1.2 times greater by volume and 3 times greater by carbon biomass (Purcell and Decker, 2005). The feeding mode of lobate ctenophores such as *Mnemiopsis* sp. is different than that of medusae, and appears specialized to capture evasive prey such as copepods. Studies by Waggett and Costello (1999) revealed two major routes by which prey are encountered and captured. The first is through feeding currents generated by the auricular cilia. This is the predominant mechanism producing encounters with smaller prey such as copepod nauplii (Waggett and Costello, 1999). The second mechanism is by entrapment on the broad oral lobes which is most effective with the larger, rapidly swimming prey such as adult *Acartia tonsa*. Entrainment

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through the tentillae selects for prey whose swimming speeds are less than the flow field velocities generated by the auricular cilia (Waggett and Costello, 1999). Nauplii rarely attempt to escape while being carried by the auricular flow towards the auricles and tentillae. Therefore the nauplii are thought to often fail to detect the predator's presence. This is supported by experiments by Fields and Yen (1997) which find that *A. tonsa* nauplii are much less sensitive to shear in flows than older stages and escape much less frequently in a suction flow. In contrast, adult *A. tonsa* are more active and stronger swimmers (Buskey, 1994) and, if entrained, should respond as shear rates increase near the tentillae and auricles. However, the use of cilia by lobate ctenophores creates a mechanism capable of processing large volumes of water but creating virtually no hydrodynamic signal (Colin *et al.*, 2010). This mechanism is likely responsible for ctenophores being able to consume large quantities of copepods, as they can successfully avoid triggering escape response.

Bivalve mollusks such as clams and mussels are generally considered herbivorous suspension feeders consuming mainly phytoplankton, and are known to occur in high densities in the benthos (Meadows *et al.*, 1998). Because many bivalves can process large volumes of water (Davenport and Woolmington, 1982), they are known to substantially affect the overlying planktonic community in shallow water and can be important in determining not only phytoplankton dynamics (Cloern, 1982 and 1991; Møhlenberg, 1995; Dolmer, 2000), but also zooplankton communities (Green *et al.*, 2003; Davenport *et al.*, 2000; Maar *et al.*, 2008). Adult copepods and copepodites are occasionally captured by bivalves but nauplii are captured more frequently (Zeldis *et al.*, 2004). However, even nauplii often responded to the fluid deformation created by the feeding current of the bivalve siphon (Green *et al.*, 2003). N1 nauplii of *Temora longicornis* are captured more often than those of *A. tonsa*, which reflect their sensitivity to shear, and both species are captured more frequently when they are located furthest away from exhalent siphon where the fluid deformation rate is lowest. In this region nauplii are assumed to be unable to detect the hydrodynamic disturbance in time to escape (Green *et al.*, 2003).

Chaetognaths are small planktonic predators which often remain motionless while suspended in the water column. Among the mesozooplankton, they are often second only to copepods in abundance and biomass (Froneman and Pakhomov, 1998; Fernández de Puelles *et al.*, 2018). Chaetognaths are one of the main sources of predation on the copepod community and have a substantial influence on the population structure of lower trophic levels (Pearre, 1980; Feigenbaum and Maris, 1984). Because chaetognaths are small, travel with ambient flow and do not actively swim while hunting, copepods are unlikely to detect the presence of a chaetognath unless an attack occurs. Copepods produce hydrodynamic disturbances when they swim or feed and these stimuli are detected by chaetognaths through sensory hairs (Feigenbaum and Reeve, 1977). Chaetognaths lie motionless in the water column until a copepod comes to within range

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of 1-3 mm (Horridge and Boulton, 1967). The rapid strike of a chaetognath provides only a brief time period in which to react before grasping spines are used to secure prey. Therefore, in order to escape from these non-visual predators, a short response latency is likely to be the most important adaptation and perhaps species exhibiting myelinated nerves may have a slight advantage for surviving these encounters.

Some copepod species exhibit carnivory and will readily feed on other copepods (Greene, 1988). This is a rather interesting scenario from the perspective of the copepod's escape since the sensory and motility structures used to avoid predators are the same ones being used to detect and pursue them. While the structures (antennae and pleopods) may be present on both predator and prey, it has been argued that prey perception depends on the absolute magnitude of the fluid velocity generated by the moving prey, while predator perception depends on the magnitude of one or several of the components of the fluid velocity gradients (deformation rate, vorticity, acceleration) generated by the predator (Kiørboe and Visser, 1999).

To successfully avoid detection by carnivorous copepods, the prey species must minimize the hydrodynamic disturbance generated (Kiørboe and Visser, 1999; Kiørboe *et al.*, 2014). One strategy for doing this is to decouple feeding and swimming (Tiselius *et al.*, 1997). Species in which feeding and swimming are separate processes are able to generate hydrodynamic disturbances with a much faster dissipation rate and are able to display 'quiet' propulsion (Kiørboe *et al.*, 2014). If detected, prey copepods must have a very short response latency and/or be capable of generating very high swimming speeds. Since speed is often proportional to body size, it is not surprising that predatory copepods capture higher proportions of small-bodied copepods (Yen, 1983) and nauplii (Lonsdale *et al.*, 1979). Cannibalism is also prevalent among copepods and occurs primarily on naupliar stages, even when sufficient phytoplankton is present (Hada and Uye, 1991). For omnivorous species, cannibalism makes up less than 10% of the minimum daily food requirement but can still result substantial mortality of naupliar stages (Hada and Uye, 1991).

5. VISUAL PREDATORS

Predators that hunt visually upon copepods are most commonly fish (Confer *et al.*, 1978) although other visually hunting species also consume copepods. Some species of birds are known to consume copepods (Hunt and Harrison, 1990). Birds have been documented feeding on large copepods which accumulate at oceanographic fronts (Hunt and Harrison, 1990), but no information exists on the success rates of birds feeding on copepods. Investigations into the interactions between these visual predators and copepods are needed to determine potential ecological effects and trophic interactions. The fact that copepods are known to react with escape jumps to shadows (Buskey and

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Hartline, 2003) suggest that perhaps this may be effective in limiting predation from birds feeding from above, but further investigation is necessary.

Larval squid also consume copepods which can make up a significant portion of their diet, and predation success rate increases as larval squid age and learn about capture of evasive prey (Chen *et al.*, 1996). Copepods are also used in aquaculture to raise *Loligo* sp. (Yang *et al.*, 1983). Some predatory zooplankton species such as mysids may use vision to assist with prey capture but are probably unable to capture evasive copepods (Viitasalo *et al.*, 1998). Whales (baleen) and some large sharks (basking, whale), although visual animals well known to consume large quantities of zooplankton including copepods (Lowry *et al.*, 2004), are not considered visual zooplanktivors due to the fact that they filter their food in massive volumes and do not visually track and locate individual copepods.

Teleost fishes are the most well-known visual predators on copepods (Confer *et al.*, 1978). They possess many sophisticated sensory systems including vision, chemoreception, hearing, and a lateral line. Because most fish are visual hunters, the color, size, and motion of prey should significantly affect the prey's chance of survival. Transparency, which is ineffective against non-visual hunters, acts to reduce contrast and reduce visual conspicuousness to visually hunting predators (Aksnes and Giske, 1993; Buskey, 1994). However, susceptibility can increase in species which bear highly visible clutches of eggs, as females have the highest encounter rates with fish and the egg-clutch is a major determinant of their susceptibility, while males are least successful in escaping once encountered (Svensson, 1992). It was hypothesized that this difference in escape reaction may have evolved because of sex-specific requirements in mate encounter and mate location, with male copepods using hydrodynamic disturbances to locate mates possibly leading to higher signal thresholds for initiation of escape behavior (Svensson, 1992). Another important aspect to consider when transparency is used to avoid visual predators is that feeding can increase visual conspicuousness by concentrating pigmented food in the gut (Giguere and Northcote, 1987). Copepods have been shown to exhibit a nocturnal feeding pattern (Stearns, 1986) which may be an adaptive strategy to limit conspicuousness during daylight hours. Brightly colored parasitic copepods visible through the cuticle of their amphipod secondary host can also alter visual conspicuousness and lead to more encounters with visual predators, including their primary fish host (Bakker *et al.*, 1997).

Diel vertical migration, where copepods move into deeper waters during daylight hours and back into surface water during darkness, is a mechanism to avoid visually hunting fish predators (Zaret and Suffern, 1976; Pearre, 1979; Bollens and Frost, 1989). Some copepods do not vertically migrate and remain in the brightly lit surface waters during the day. Pontellid copepods are one family containing several species that reside in the neustonic environment. These animals are often highly pigmented to reduce the effects of damaging UV radiation (Byron, 1982; Hansson *et al.*, 2007), and are larger in

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comparison with many other copepod species. This large size, combined with pigmentation, makes these copepods more visually conspicuous, and thus should be preferred by visual fish predators (Brooks and Dodson, 1965; Morgan and Christy, 1996). This would appear to put these copepods at a significant disadvantage, but at least two species, *Anomalocera ornata* and *Labidocera aestiva*, have been shown to make aerial escapes, whereby the animals break the surface tension of the water and travel many body lengths through the air, reentering the water beyond the perceptive field of the fish predator (Gemmell *et al.*, 2012) (Figure 5). The energetic cost of breaking the surface tension is high (copepods lose up to 88% of their kinetic energy), but the vastly lower density of air allows them to travel many times further from a predator than they could underwater.

Fish create a fluid disturbance when feeding, and copepods can receive a signal to alert them to the presence of a predator. To counter this, many fish exhibit adaptive morphology and behavior of their own. Many planktivorous fish feed by suction (Coughlin and Strickler, 1990; Coughlin, 1994; Holzman and Wainwright, 2009). In order to capture copepods in this manner, a fish must get sufficiently close to its prey to allow the suction flow to overwhelm the prey and draw it into the mouth. Both swimming towards the prey and suction flow create a hydrodynamic disturbance, which can elicit an escape response by the copepod. To overcome the bow wave created by swimming towards a copepod, the fish rapidly opens its mouth creating suction, thereby reversing fluid deformation (Holzman and Wainwright, 2009). Therefore, during a strike many fish can be detected only by its suction-induced disturbance, rather than the disturbance from the bow wave. These fish are able to produce a more subtle disturbance than expected based on their flow speeds and mouth size alone. Jaw protrusion and the rapid opening of the mouth during the strike both help to minimize the signal available to the prey (Holzman and Wainwright, 2009). It is likely that the jaw protrusion observed in many planktivorous fish is an adaptation to minimize the initiation of copepod escape responses.

Fish can also limit their hydrodynamic conspicuousness prior to the strike, during the approach phase. It is crucial for fish to get close to evasive copepods prior to initiating a feeding strike, given that suction-based feeding produces flows that are exceptionally short lived, lasting only 10–50 ms, and are restricted to an area very close to the mouth (Ferry-Graham *et al.*, 2003; Van Wassenbergh and Aerts, 2009; Day *et al.*, 2005). To avoid detection by copepods prior to a feeding strike, fish can mask their hydrodynamic signatures by utilizing either morphology or behavior. Seahorses are an example of fish that can overcome a copepod's sensory defense through morphology as the narrow, elongated snout, separated from the blunt head is associated with minimal fluid deformation, which allows these fish to approach evasive copepods to within 1 mm

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(Gemmell *et al.*, 2013b). Other fish appear to behaviorally minimize the fluid deformation profile in front of the mouth by creating minor suction to offset the water being pushed forward (Gemmell *et al.*, 2014). Both of these strategies reduce the fluid deformation in the strike zone of the fish to levels just below detection limits of copepods, and illustrates the evolutionary "arms race" occurring and this important trophic link.

In the absence of modifications to minimize fluid disturbances, high strike speeds can also be successfully employed by some planktivorous fish. Small reef dwelling fish (blennies, *Acanthemblemaria* sp.) that live within small holes on coral reefs, wait for potential meals to swim or drift by in the current. Once a copepod is located visually the blenny attacks its prey by lunging forward, mouth agape to ingest its prey. The speed of attack is ≈230 mm s^{-1} (Waggett and Buskey, 2007a). Although rapid, this strike velocity is lower than the maximum escape velocity of *Acartia tonsa* (≈500 mm s^{-1}). This results in most copepods being able to sense and escape successfully from blennies under still water conditions (Clarke *et al.*, 2005). It is only under moderate turbulence that the reduced ability to hydrodynamically sense a predator results in higher capture success by the fish (Clarke *et al.*, 2009).

Copepods exhibit continuous to intermittent modes of swimming. Species that maintain a relatively smooth swimming pattern and nearly constant frontward motion are termed 'continuous cruisers.' Species that only swim intermittently are known as either 'hop-and-sink' swimmers, where brief forward jumps are followed by a short period of sinking when appendage motion ceases, or 'cruise-and-sink' swimmers, which exhibit longer periods of forward swimming followed by short periods of sinking (Bainbridge, 1952). The pauses during intermittent swimming are believed to increase the perceptual abilities of the copepod by reducing any self-generated hydrodynamic noise (Bundy and Paffenhöfer, 1996; Yen, 2000; Kramer and McLaughlin, 2001). However, an intermittent swimming pattern can act to increase conspicuousness to a visual predator (Peterson and Ausubel, 1984; Buskey *et al.*, 1993). Even within a species, male and females can have different swimming patterns and this can translate into higher capture rates of males (Saito and Kiørboe, 2001). Alteration of swimming patterns behavior can also be used by fish such as herring to increase their encounters with prey. At low copepod densities, fish will increase speeds by approximately 100% compared to speeds at high copepod densities (Munk and Kiørboe, 1985). It is also noteworthy that fish, like copepods, appear to have increased perceptive abilities when not actively swimming. In cod larvae 94% of copepod prey are perceived during glide events where the fish are not actively swimming (Hunt von Herbing and Gallager, 2000). This has important implications for determining realistic encounter rates.

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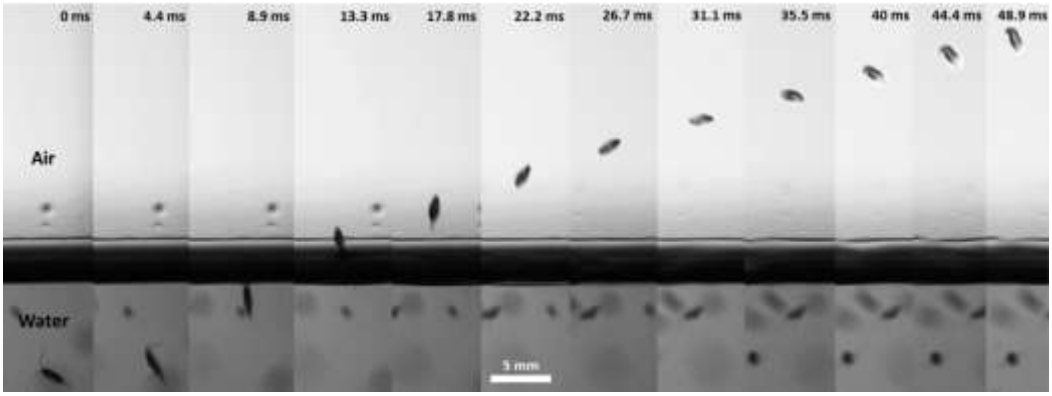

Figure 5. Aerial escape behavior of the pontellid copepod *Labidocera aestiva*. This is one of only a few species known to break the surface tension of the water during an escape and travel many times their own body length through air before re-entering the water.

Due to their smaller size, nauplii should be less visually conspicuous and therefore be less vulnerable than larger stages to visual predators such as fish (Eiane *et al.*, 2002). This is in contrast to most findings on encounters with non-visual predators, where nauplii are often captured at significantly higher rates than later developmental stages (Costello and Colin, 1994; Waggett and Costello, 1999; Suchman and Sullivan, 2000). As copepods molt and become larger, this should make them more conspicuous to a visual predator which may translate into increased frequency and distance of attacks from a predator. However later stages have greater sensory abilities and greater escape responses (Buskey, 1994) which may act to offset the increase in visual conspicuousness.

6. Effect of Water Motion

The escape response involves rapid acceleration which is energetically costly, using over 400 times the normal energetic expenditure (Strickler, 1975; Alcaraz and Strickler, 1988). Therefore copepods must maintain a balance between being able to successfully avoid predators and conserving energy. In order to conserve energy, copepods of all developmental stages display escape behaviors only when a stimulus is detected above a certain threshold. This prevents a copepod from performing energetically costly escape responses when they are not necessary. However, when copepods are constantly stimulated above the threshold for escape, for instance in a turbulent environment, they have the ability to habituate to these stimuli which may reduce their ability to detect an approaching predator and result in a greater likelihood of capture (Costello *et al.*, 1990; Hwang *et al.*, 1994).

Most behavioral studies with marine copepods have been done under still water conditions, but turbulence has been shown to play an important role in determining how

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predator-prey interactions involving copepods operate in more realistic conditions (Clarke *et al.*, 2005 and 2009; Robinson *et al.*, 2007; Finelli *et al.*, 2009). Turbulence was originally thought of as a mechanism that simply increased encounter rates between predators and copepods (Rothschild and Osborn, 1988), but turbulence can impact capture and escape success of both predator and prey (Clarke *et al.*, 2009). The exact impact turbulence has on capture success is often difficult to predict because it has two opposing effects. Turbulence can create erratic movement patterns of prey particles making a fish (predator) more likely to miss during an attack or abort a pursuit completely (MacKenzie and Kiørboe, 2000).

Turbulent water motion can also mask the signals that copepods use to avoid capture making their reaction distances to stimuli shorter (Robinson *et al.*, 2007), which will increase the predation risk for evading a visual predator (Clarke *et al.*, 2009). The escape response and capture rates of the copepod *Acartia tonsa* were examined in laboratory flumes that created both unidirectional and oscillatory flow conditions. The reactive distance to a siphon remained the same as still water in low-flow conditions, but was reduced by 25% at elevated flow speeds, indicating a decline in the copepods' ability to detect velocity gradients formed by the siphon (Robinson *et al.*, 2007). Turbulence appears to make it more difficult for a copepod to respond to a potential threat and may also affect the strength of the response. Escape speed of copepods can be slower under turbulent conditions (Lee *et al.*, 2010) but may not impact the total escape distance (Waggett and Buskey, 2007b). Copepod developmental stage and predator species are also important to consider in the context of water motion. Some planktivorous fish exhibit similar capture success rates under both calm and flow conditions, whereas other fish species display strong differences both within and across copepod developmental stages. For example the dwarf seahorse, *Hippocampus zosterae*, captures both copepodites and nauplii with very high success (> 90%) under calm conditions compared to the blenny, *Acanthemblemaria paula*, which captures nauplii at a significantly higher rate than copepodites (Gemmell and Buskey, 2011). Under flow conditions however the blenny is able to maintain similar capture rates on both nauplii and copepodites but the seahorse success rate plummets to nearly zero.

7. Effect of Temperature and Viscosity

In addition to turbulence, physical characteristics of water such as viscosity and temperature can affect escape performance in copepods. As water becomes cooler, it also becomes denser and more viscous. For example, a decrease of temperature from 20°C to 10°C increases viscosity from 0.0109 Pa s to 0.0139 Pa s **(**Bolton and Havenhand, 2005**).** Therefore, the change in temperature alters not just the metabolic rate of organisms but also the physical characteristics of the ambient fluid, which in turn affects the ability of

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very small organisms to feed, move or escape within the water column. These effects of temperature are very important at hydrodynamic scales where viscous forces dominate motion (Re < 1) because low Re has a marked influence on the drag that operates against the feeding and swimming structures of small aquatic ectotherms such as copepods (Koehl and Strickler, 1981; Lagergren and Stenson, 2000).

This inverse relationship of water temperature and viscosity has particular importance for copepods living in temperate and sub-tropical coastal environments, where they are subject to large fluctuations in water temperature throughout the course of a year (from 5-30°C). Here, the escape response will be subject to water temperature variations from local weather (*e.g.,* cold fronts), seasonal variations, and large-scale global patterns (*e.g.,* climate change). Because viscosity fluctuates inversely with temperature, the impact of high viscous drag on small organisms' routine locomotion has substantial effects on swimming speeds (Bolton and Havenhand, 1997; Podolsky and Emlet, 1993; Fuiman and Batty, 1997; Muller *et al.*, 2000; Larsen *et al.*, 2008). However, the effect on escape behavior is unknown because non-escape (routine) locomotion occurs at Re = 0.1 (for copepod nauplii) based on body length and cruising speed, and escaping nauplii exhibit unsteady motion and their flow environment can rapidly transit to Re = 6 (Gemmell *et al.*, 2013a).

Although altering viscosity will undoubtedly influence locomotion, the effect on predator and prey will not be equal. This is because larger bodies operate at a higher Re and exhibit less viscous drag than smaller ones. Because predators (*e.g.,* fish larvae) are often much larger (an order of magnitude or more) than the nauplii themselves, the predator will be less affected by viscosity (Herbing, 2002) and should capture nauplii more effectively when viscosity increases. Small organisms should escape less effectively at colder temperatures (Fuiman, 1986). However, field studies on copepod nauplii predation have shown no evidence that nauplii are captured more successfully at colder temperatures (Paul, 1983; MichaudI *et al.*, 1996) suggesting an ability of nauplii to compensate for viscosity disadvantages at reduced temperature. Gemmell *et al.* (2013a) showed that the early nauplii of *Acartia tonsa* can indeed compensate to a certain degree and maximize the effectiveness of escape swimming at both ends of their natural thermal range. This is accomplished by a shift in timing of appendage motion which creates an increase in power stroke duration relative to recovery stroke duration at low temperatures. The shift in power stroke duration relative to recovery stroke duration is found to be regulated by the temperature dependence of swimming appendage muscle groups (Lenz *et al.*, 2005; Gemmell *et al.*, 2013a), not a dynamic response to viscosity change. While some copepod nauplii have natural adaptive mechanisms to compensate for viscosity variations with temperature, it would not appear to function in situations in which viscosity varies independent of temperature, such as in some phytoplankton blooms (Seuront *et al.*, 2006).

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CONCLUSION

A copepod's success in escaping from predators depends on its ability to detect the predator's approach and to respond quickly and effectively, and the result of any predator-prey encounter will depend upon many factors. There is no place for copepods to hide from a diverse assemblage of predators in the pelagic ocean, and they have evolved escape performances matched by few other organisms, vertebrate or invertebrate.

Copepods provide model organisms to investigate the kinematics of escape in the aquatic environment, correlate it with physiological and morphological changes through development, and compare these results to measurements of predator susceptibility and changing environmental conditions. Previous studies have examined the changes in copepod vulnerability to predators through developmental stages, although changes in escape kinematics with development were not considered. By studying the developing stages of calanoid copepods, their ability to avoid predation at various stages of development and how environment affects these interactions, we can begin to understand which stages of copepods are most susceptible to different types of predators. Through the use of emerging techniques such as micro-Particle Image Velocimetry (μPIV) (Gemmell *et al.*, 2014) and 3D methods (Gemmell *et al.*, 2013a and 2014; Malkiel *et al.*, 2003) we are beginning to understand the role that the physical fluid environment plays in mediating these interactions. Eventually this may help to understand the factors that control plankton community structures, localized abundances or deficiencies of both predator and prey in the marine food webs, and predict the robustness of species to seasonal and long-term changes in climate.

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Chapter 11

CHEMOSENSATION AND A POTENTIAL NEURONAL MECHANISM OF RATIO DETECTION IN A COPEPOD

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ABSTRACT

Male copepods of the species *Temora longicornis* are able to follow a pheromone trail laid out by a female. Moreover, the male is able to change the direction of its movement if it initially follows the trail in the wrong direction. Previously, we proposed that the female pheromone may be blend of multiple compounds with different chemical properties and that the male senses their ratio rather than an absolute concentration. This allows for a better method to decide in which direction to move. Here we implement a simple, yet efficient design for the olfactory apparatus using the Leaky Integrate-and-Fire neuronal model. We implement a Simulated Annealing algorithm for the selection of optimal synaptic weights and show that the circuit enables ratio detection over a wide array of input signals. Our results encourage further research on similarities of brain

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organization in copepods and airborne arthropods in which ratio detection plays an important role.

Keywords: chemosensation, ratio detection, *Temora longicornis*, neuronal model

ABBREVIATIONS

LIF: Leaky Integrate-and-Fire
LNs: Local Neurons
ORNs: Olfactory Receptor Neurons
SA: Simulated Annealing

1. INTRODUCTION

At a population density of about one individual per liter of water in the world ocean, copepods are the most abundant class and the greatest reservoir of organic carbon among all animals. They constitute a key link between autotrophic phytoplankton and higher trophic levels in freshwater and marine food webs. Thus their ecology has tremendous implications on other life forms, all the way to land and air based animals. Current challenges to their well-being include anthropogenic acidification and chemical pollution of their natural habitats. It is imperative to understand their ecology, in particular their mating behavior, to predict whether and how these challenges may be met by the world's copepods.

Copepods are sexual animals and their mating behavior has been a fascinating research topic for two centuries. Katona (1973) proposed that copepods use chemical signaling mechanisms to find each other in the dark and three-dimensional water column. This view was substantiated in a series of works at the turn of the 21st century (Bagøien and Kiørboe, 2005; Doall *et al.*, 1998; Seuront, 2013; Tsuda and Miller, 1998; Weissburg *et al.*, 1998; Yen *et al.*, 2004). Males often display swimming adaptations aimed at increasing the encounter success with conspecific females (Nihongi *et al.*, 2004; Uttieri *et al.*, 2007; Yen and Lasley, 2011). A key observation by Doall *et al.* (1998) was that when a male *Temora longicornis* finds the trail laid by a female, but begins the pursuit in the wrong direction, he is able to turn around and to continue until he has reached the female. This happens within a remarkably short time, after only 1-2 s of pursuit in the wrong direction (Figure 1). The observations of Doall *et al.* (1998) do not indicate that the males know the direction of the female at the moment of finding the trail.

In earlier work (Hinow *et al.*, 2017) we presented a simple mathematical description of how the directional information can be encoded in the pheromone trail left behind by

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the female. Even in quiet and undisturbed water, any compound emitted by the female is subject to diffusion and chemical decay reactions. In the fixed frame of the moving female, the concentrations of the compound(s) form an idealized one-dimensional trail of concentrations that decrease with the distance from the female. A searching agent (the male) can be equipped in one of two ways. On the one hand, it can attempt to detect a change in the absolute concentration of a single compound. If the concentration is increasing in the direction of the motion, it should continue in that direction; if the concentration is decreasing in the direction of the motion, it should turn around. On the other hand, the female can emit two compounds at roughly the same rate that differ however in their decay or diffusion rates. At a distance from the female, the male will encounter these compounds in a ratio that differs from 1. The direction of the motion should be maintained if the ratio approaches 1 and it should be reversed otherwise. Using mathematical modeling and simulation we found evidence that an agent able to detect a ratio outperforms an agent that is dependent on the gradient of a single compound (Hinow *et al.*, 2017). Indeed, if the initial direction of motion is opposite to the direction of the moving female, it is possible to enter the trail further away from the female and still make the turn. Moreover, the threshold in the signal change required for turning can be chosen bigger.

Extracting directional information from chemical cues is an important task for a large number of animal species though the precise mechanisms often remain only partially understood. A well-known example are dogs (*Canis familiaris*) with special breeds for tracking purposes (Hepper and Wells, 2005; Gadbois and Reeve, 2014; Wells and Hepper, 2003). Ratio detection of chemicals has been investigated in a number of species (Clifford and Riffell, 2013; Wyatt, 2010), most notably in moths and other lepidopterans (Belmabrouk *et al.*, 2011; deBruyne and Baker, 2008; Zavada *et al.*, 2011). In various moth species where the chemical components of the pheromone blends have been identified, sympatric sister species use identical components of the mixture, albeit in different ratios. Zavada *et al.* (2011) proposed a simple competition-based neuronal model that is capable to compare the strengths of two signals and to lead a search towards a region where the signals are in the desired ratio. Their model is composed of sets of Olfactory Receptor Neurons (ORNs) that excite Local Neurons (LNs) of different types. The local neurons, in turn, are connected by inhibitory synapses. The presence of multiple compounds in the moth pheromone blend indicates that species-specific information is also conveyed. This can help to maintain species integrity, provided that the ratios of the components remain stable in the plume. Hinow *et al.* (2017) postulated that the ratio of components needs to change over time so that the trail can point toward the animal that laid it.

The goal of this chapter is to revisit the observations of Doall *et al.* (1998) in light of newer insights gained from chemically modulated mating behavior in airborne insects. We begin by reviewing in part the Schlieren optical technique pioneered by Töpler

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(1866) and its application to the capture of small translucent animals in water in Section 2. In Section 3 we consider a simplified version of the neuronal model proposed in Zavada *et al.* (2011). Instead of modeling the neurons by sophisticated models such as the one by Hodgkin and Huxley (1952), we use the Leaky Integrate-and-Fire (LIF) model (Gerstner *et al.*, 2014). This model can be traced back to Louis Lapicque (1907) from a time long before mechanisms generating neuronal action potentials were known (Abbott, 1999; Brunel and van Rossum, 2007). The LIF model is widely used today. Its main advantage, the computational simplicity, allows a focus on questions of design of circuitry. In Section 4 we implement the Simulated Annealing (SA) (Press *et al.*, 2007) algorithm to optimize the synaptic weights of the network. We find that a simple network model consisting of LIF neurons with conductance-based synaptic weights can implement a ratio detection mechanism. The optimal synaptic weights for the model indicate a strong mutual inhibition of the specialist and generalist local neurons. The relative simplicity of the network topology lends credibility to the thesis that ratio detection evolved early on in the ancestry of today's marine copepods.

2. OBSERVATIONS OF *TEMORA LONGICORNIS*

Most if not all photographs taken by the public at large are of "amplitude objects". In such an object, spatial differences in color values make it visible on the image. To image a clear wine glass submerged in clear water is one of the biggest challenges in photography. Special lighting at special angles may reflect from the surfaces to show parts of the glass. What has not been used in this case is the fact that the glass has a different refractive index than water. In such an environment, the glass should be looked at as a "phase object", and the optical techniques of making phase objects visible would render a distinct image of the glass. Phase objects with their refractive index slow down the light, creating a difference between light passing through the object and light from the background. Schlieren and shadowgraph techniques (Settles, 2001) render images based on differences in refractive indices. To employ these optical techniques became easier with the advent of single-line lasers and optics that allow collimated light beams carrying the information of an object for a long distance. For example, in our case, copepods swimming in a 1.5 L volume of water (10 × 10 × 15 cm) were imaged by a camera 2.16 m away with a resolution of 0.1 mm (Doall *et al.*, 1998; Strickler, 1977 and 1998; Strickler and Hwang, 1999).

Using collimated laser beams emitting laser light at one wavelength adds another component that can be used to observe particles of different sizes in 3D and suspended in volumes of water. Schlieren optical pathways were employed using white spectrum point sources, as well as laser in the visible and near-infrared emission range (Strickler, 1977; Strickler and Balázsi, 2007; Strickler and Hwang, 1999). Doall *et al.* (1998) used the

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advantage of the long distance between the object and the image to split the original collimated laser beam in two beams crossing each other perpendicularly in the vessel of the animals. The subsequent combination of the two beams in one gave us a beam that carried the information of the front and side view of each phase object swimming around in the vessel. The result was then a dark-field picture of the vessel with white to light grey images of the objects. A single video camera was used to register the events. The task to evaluate the videos in the late 20th century was to click on the object frame-by-frame, giving us the 3D coordinates at 60 Hz.

Figure 1 shows the time before a mating event as observed by Doall *et al.* (1998). The female starts at position *a'* and the male a little later at position *a*. The male meets the track of the female at position *b*, takes a turn and follows the track of the female. However, the male follows the track in the wrong direction. After 1.2 s it turns and back-tracks to catch up with the female at position *c*. In 27 of 67 pursuit observations (40%) the male started in the wrong direction, which does not suggest a good method to determine the direction at the moment of finding the trail. In 22 out of 27 observations when the initial swimming direction was incorrect the male turned around (81%). Thus, in a total of 92% of cases, the male eventually followed the female in the correct direction, a remarkable achievement for any member of the animal kingdom. Back tracking with similar time components was also observed in presence of trails simulating tracks from swimming zooplankters (Yen *et al.*, 2004).

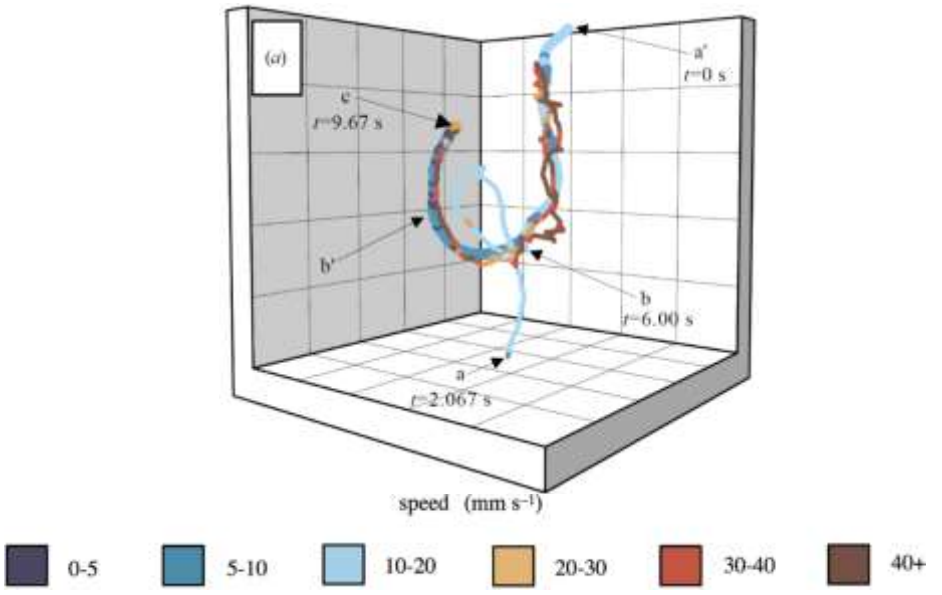

Figure 1. Trails of a female and a male from Doall *et al.* (1998). The fat and thin trails are that of the female and the male, respectively, while colors indicate swimming speeds. The male encounters the female's trail at position *b* and proceeds for the next ≈1.2 s in the wrong direction before turning around and finally capturing the female at position *c*. The grid unit is 1 cm. Reprinted from (Doall *et al.*, 1998) with permission from the Royal Society.

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3. THE SPIKING NEURON MODEL FOR RATIO DETECTION

Simple phenomenological spiking neuron models are very useful for investigation of neural coding, memory and other functions, as they are easier to analyze than detailed electrophysiological neuron models; see Gerstner *et al.* (2014) for a thorough introduction to both classes. The simplest form of the LIF model for a single neuron is given by the following differential equation for the membrane potential v

$$\tau \frac{dv}{dt} = -v(t) + RI(t), \tag{1}$$

where τ is the membrane time constant, R is the resistance of the membrane and I is an external current, if present. Spikes are generated whenever the membrane potential reaches a threshold ϑ. Then the membrane potential is reset to the resting potential v_r,

$$\text{if } v(t-) = \vartheta, \text{ then } v(t+) = v_r,$$

which also defines t as the spiking time of the LIF neuron. A schematic output of a LIF neuron with a single excitatory input is shown in Figure 2. For simplicity, a spike of the presynaptic neuron causes an immediate increase in the membrane potential.

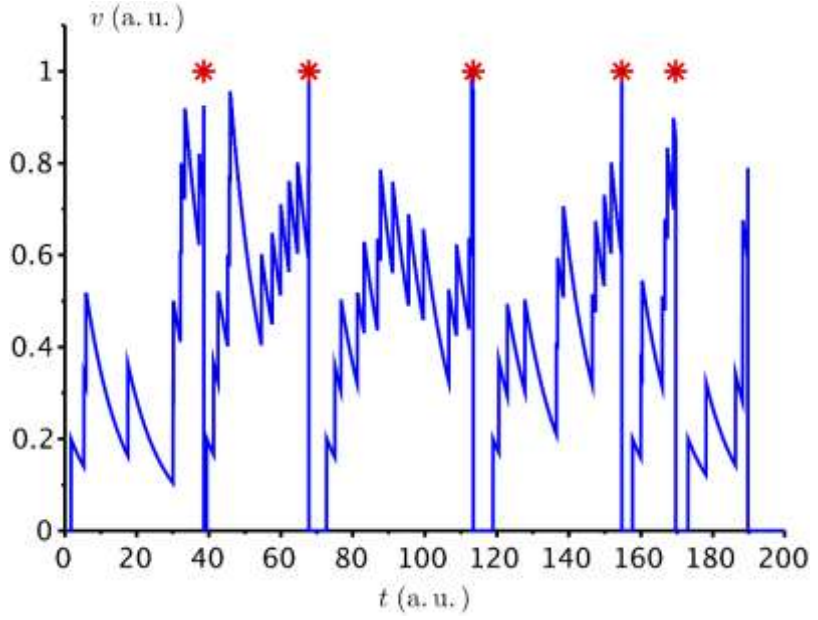

Figure 2. The membrane potential v of a LIF neuron governed by Equation (1) with $I \equiv 0$ that receives a single excitatory input ("a.u." = auxiliary unit). At each incoming spike, v is increased by 0.2. The spiking times of the neuron are indicated by red dots.

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To model the synaptic connections between neurons we use the conductance-based model as described by Vogels and Abbott (2005). The sub-threshold membrane potential V and the excitatory, respectively inhibitory conductances g_{ex} and g_{inh} are governed by

$$\tau \frac{dV}{dt} = (V_r - V) + g_{ex}(E_{ex} - V) + g_{inh}(E_{inh} - V), \tag{2}$$

$$\tau_{ex} \frac{dg_{ex}}{dt} = -g_{ex}, \tag{3}$$

$$\tau_{inh} \frac{dg_{inh}}{dt} = -g_{inh}, \tag{4}$$

as long as no spiking takes place. We choose the resting potential V_r=-60 mV, the relaxation time constant for the membrane τ = 20 ms and the synaptic time constants τ_{ex} = 5 ms and τ_{inh} = 10 ms, respectively (Vogels and Abbott, 2005). The key difference in the influence of the excitatory and inhibitory conductances is that the two reversal potentials are chosen to be E_{ex} = 0 mV and E_{inh} = -80 mV, respectively. Once the membrane potential reaches the threshold of ϑ = -50 mV, the time is recorded as a spiking time and V is reset to V_r. Upon spiking of a presynaptic neuron at time t, all postsynaptic neurons have their excitatory respectively inhibitory conductances changed by a certain amount, depending on the nature of the synapse. If the synapse is excitatory, the excitatory conductance of the postsynaptic neuron is increased,

$$g_{ex}(t+) = g_{ex}(t-) + w_{ex}, \tag{5}$$

if the synapse is inhibitory, the inhibitory conductance of the postsynaptic neuron is increased,

$$g_{inh}(t+) = g_{inh}(t-) + w_{inh}. \tag{6}$$

The amounts of increase in the respective conductances of the postsynaptic neuron are called the weights of the synapse. Their selection will be discussed in greater detail in Section 4. Note that these weights are non-negative in both cases and that only one of them characterizes a synapse.

A network topology for a ratio detection mechanism was proposed by Zavada *et al.* (2011). ORNs of types *a* and *b* are excited upon binding of their respective ligand. These are modeled as Poisson sources with firing rates r_a and r_b, respectively. Precisely, the probability that there are k spikes in a time interval Δt is

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$$P\{k\ spikes\ during\ \Delta t\} = \frac{e^{-r\Delta t}(r\Delta t)^{k}}{k!}.$$

Each ORN's firing rate grows linearly with respect to the logarithm of the ligand concentration u. This has been shown to hold for several orders of magnitude in the moth *Antheraea polyphemus* (Kaissling, 1996; Zack, 1979). For lack of better resources, we use the relationship

$$r = 48\lambda + 400$$

where λ is the logarithm of the concentration of the compound, see Figure 3 in Kaissling (1996), ranging from -8 to -2, and r is the firing rate in Hz. This is the hypothetical response curve for both components of the mixture.

The ORNs are connected to two types of LNs by excitatory synapses. Each ORN of type a, respectively b, is connected to a specialist LN of the same type and these receive excitatory input only from the corresponding ORNs. Simultaneously, the ORNs also are connected to a generalist LN, that receives excitatory input from both types of ORNs. In both cases, there is a convergence ratio of N ORNs feeding a single LN. For simplicity, we choose this convergence ratio to be the same for all excitatory connections. The LNs of all three types are connected by inhibitory synapses. We pick the smallest number possible, namely just one LN_a, one LN_b and one LN_{gen}. The synaptic connections are characterized by five weights where we make the following symmetry assumptions for the target ratio 1:1

1. the connections $ORN_x \to LN_x$ have the same weights for $x = a$ and $x = b$,
2. the connections $ORN_x \to LN_{gen}$ have the same weights for $x = a$ and $x = b$,
3. the mutual inhibition between LN_a and LN_b is symmetric,
4. $LN_{a/b}$ act the same way on LN_{gen}, and
5. LN_{gen} acts the same way on $LN_{a/b}$.

The network is depicted schematically in Figure 3. The output of the network is the firing rate of LN_{gen}. Note that by Dale's principle this output is necessarily inhibitory, as LN_{gen} already inhibits LN_a and LN_b. This signal is processed by further local intermediate neurons and projection neurons that we do not include in our model.

The output of the generalist neuron LN_{gen} *i.e.*, its firing rate r_{gen} is the output of the mechanism, as a function of the firing rates r_a and r_b. If the mixture components a and b are present at the ratio 1:1, both LN_a and LN_b inhibit each other. Thus the ORNs excite directly LN_{gen}. If, however, component a is present at a much higher concentration than component b, then LN_a will silence both LN_b and LN_{gen}, similarly if b is present at a much higher concentration than component a.

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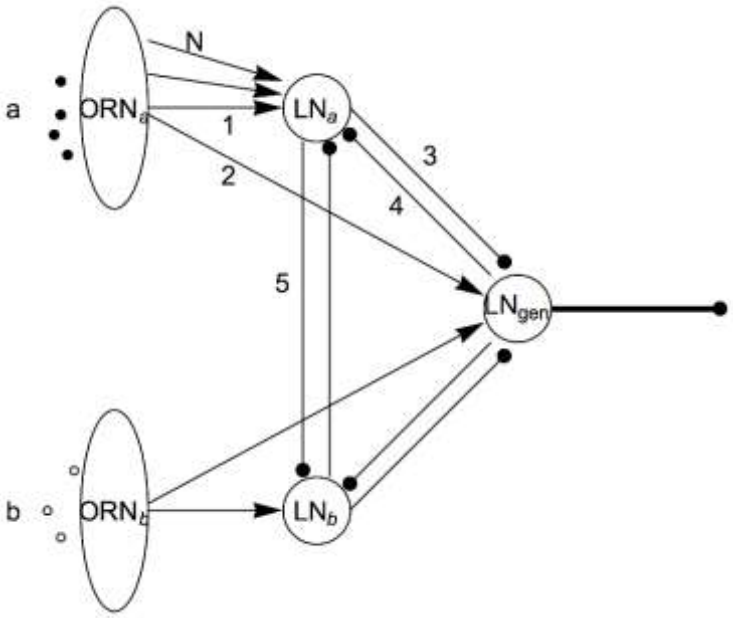

Figure 3. The topology of the ratio detection mechanism adapted from Zavada *et al.* (2011). Ligands of type *a* and *b* bind to the respective ORNs. The convergence from ORNs to LNs is indicated only once for clarity. Pointed and blunt arrows indicate excitatory respectively inhibitory relationships; labels indicate the independent weights.

4. SYNAPTIC WEIGHT SELECTION

The spiking neuron model in Equations (2)-(6) is almost complete except for the choice of the synaptic weights. Recall that the weights are non-negative numbers associated with each synapse. Thus the problem of choosing weights can be viewed as an optimization problem, where we optimize the network's behavior with respect to the desired output as a function of the five numerical weights. For each such weight vector we simulate the network behavior for selected ratios of r_a:r_b. Specifically, we use rates

$$r_a^i = r_0 \cdot 1.3^i \,,\; r_b^j = r_0 \cdot 1.3^j \,, \tag{7}$$

for i, j = 0,..., 9 and r_0 = 10 Hz. This corresponds to logarithmic concentrations ranging from 10^{-8} to 10^{-6}. While the ORN firing rates span a much larger range, we chose this range to demonstrate ratio detection at very low concentrations. The firing rate of LN_{gen} is recorded for each such simulation in a 10 × 10 response matrix **R**. The numerical cost for each weight vector is defined to be the negative of the Frobenius inner product of the response matrix **R** with a convolution kernel **T** as in Figure 4,

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$$C_T(w) = -\sum_{i,j=0}^{9} R_{i,j} T_{i,j} \ . \tag{8}$$

Note that for example negative off-diagonal entries in **T** strongly penalize against positive entries in the corresponding positions in **R**.

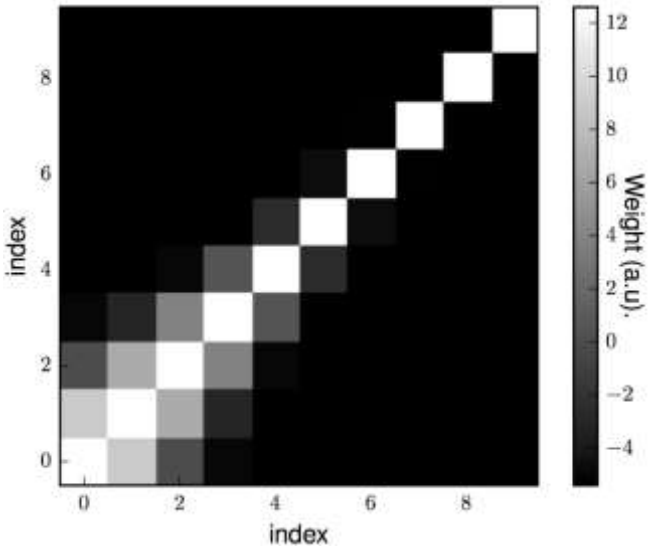

Figure 4. The convolution kernel **T** to determine the "cost" of the response matrix **R** used in Equation (8). The indices *i* and *j* are those from Equation (7).

We have implemented a SA algorithm (Press *et al.*, 2007, Section 10.12) to optimize the weights using the cost function in Equation (8). The PYTHON code is available from the github repository (Langhoff, 2017). The SA algorithm is built on an analogy from thermodynamics, namely the freezing and crystallization of liquids. Provided that the liquid cools sufficiently slowly, the constituents are able to align and to form ordered structures many orders of magnitude larger than the typical particle size. This amounts to a global minimization of the energy, as opposed to a rapid cooling that results only in a "quenched" or "amorphous" state corresponding to a local minimum. In practice, if a location x has been found, a random perturbation Δx is added. If $\Delta f = f(x + \Delta x) - f(x) < 0$, then $x + \Delta x$ is chosen as the next point of the iteration. If $\Delta f = f(x + \Delta x) - f(x) > 0$, then $x + \Delta x$ is chosen with a certain probability that is proportional to $\exp\left(-\frac{\Delta f}{T}\right)$, where T is the quantity analogous to temperature. Thus any improvement in the cost function is taken, while a worsening is sometimes accepted, but less and less likely as the temperature decreases. The main choices to be specified are the generator for the random perturbations Δx and the method for decreasing T, called the "annealing schedule". Here

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we select Δx from a uniform distribution and enforce the constraint that all synaptic weights are non-negative. The temperature is multiplied by 0.85 every fifth iteration.

5. RESULTS

We choose the convergence ratio of $N = 100$ ORNs feeding onto a single LN, for all possible ORN → LN connections. The optimal synapse weights are listed in Table 1.

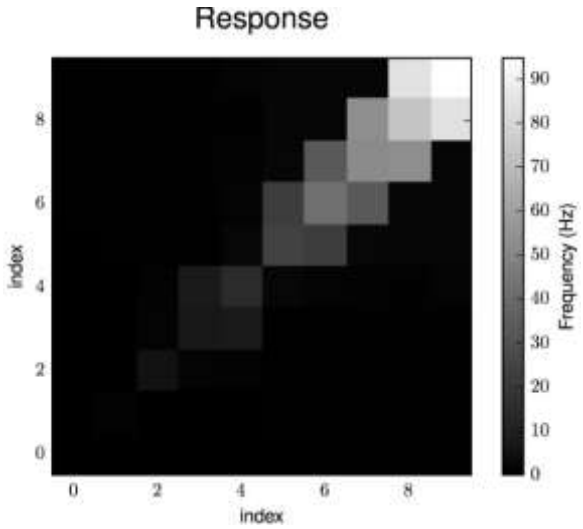

Figure 5. Optimal output of the network m defined as the LN_{gen} firing frequency. The *(i, j)*-entry corresponds to ORN firing frequencies of $10 \cdot 1.3^i$ Hz and $10 \cdot 1.3^j$ Hz respectively.

Table 1. Optimal synaptic weights determined by the SA algorithm

Connection	Weight (μS)
$ORN_{a/b} \rightarrow LN_{a/b}$	0.14025 (w_{ex})
$ORN_{a/b} \rightarrow LN_{gen}$	0.06980 (w_{ex})
$LN_{a/b} \rightarrow LN_{b/a}$	0.77945 (w_{inh})
$LN_{a/b} \rightarrow LN_{gen}$	0.64391 (w_{inh})
$LN_{gen} \rightarrow LN_{a/b}$	0.59438 (w_{inh})

We note that the excitatory connections from the ORNs to the specialist LNs are stronger than to the generalist LN. Moreover, the mutual inhibition relations between the local neurons are roughly of similar strength. The model is somewhat limited in its discriminatory power at the lower end of the concentration ranges which may be caused by its strong simplification. In future work one may increase the number of LNs in the mutually inhibitory groups in the triangle in Figure 3 and include the subsequent intermediate LNs and projection neurons.

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CONCLUSION

It is difficult for us humans to imagine how challenging pelagic copepod life must be. They live at low Reynolds number, at low population densities, and in the dark and three dimensional ocean. With the adult males of some species even lacking functional mouthparts, finding mates can absolutely not be left to chance. It is therefore natural for the males to respond to specific chemical signals (Yen and Lasley, 2011). To the best of our knowledge it is still an open question whether the copepod sex pheromones are specifically produced by the female or whether they are incidental byproducts of naturally occurring metabolism as, say, would be CO_2. Another important open question is how the well defined trails that were observed in water at rest are deformed and perhaps torn in the actual oceanic habitat of *Temora longicornis*.

We have shown that even a minimalistic, simplified neuronal model is capable of ratio detection. This represents a significant simplification from the previous model (Zavada *et al.*, 2011), where a full Hodgkins-Huxley model in addition to a rate-based Hodgkins-Huxley model of the neuron were used. Phenomenological models like the LIF model considered here do not explicitly model the individual ion channels in a neuron and treat spikes as formal events (Abbott, 1999). By omitting some of the biological details, we can gain insight into the behavior we seek to understand. The simplification is of course more relevant for much more complex networks, containing for example 10^5 neurons. In our case we see the relative weight of the neural pathways used for ratio detection. In reality, the ratio detection network will consist of more than just three LNs. At present little is known about the neuroanatomy of copepod brains and peripheral nervous systems, but there is evidence from the species *Tigriopus californicus* that it is endowed with a complex brain (Andrew *et al.*, 2011). In the future it will be valuable to investigate and to model the "spatial structure" that arises from the presence of ORNs on both antennas of the copepod sending signals to a pair of LN structures. We anticipate that further impulses for research will come from comparison with airborne arthropods due to the high level of conservation of neural circuitry in the pancrustaceans (the clade comprising crustaceans and hexapods).

So far only few semiochemicals used by copepods have been identified. One example is isophorone which is used by the parasitic sea louse *Lepeophtheirus salmonis* to locate its host, the Atlantic salmon (*Salmo salar*) (Ingvarsdóttir *et al.*, 2002); see Figure 6. It has a molar mass of 138.21 g/mol and is used by airborne arthropods as semiochemical as well (El-Sayed, 2017). Very recently Selander *et al.* (2015) identified so-called "copepodamides" that signal to phytoplankton the presence of their zooplankton predators and thereby induce the production of toxins as a defense. Identification of such chemicals in seawater can be done by coupled liquid chromatography and mass spectrometry (LC-MS), and an electroantennographic detector (EAD) to confirm the response of the animal's antenna at precisely the moment that the chemical is detected. If

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there are candidates for the pheromone components, behavioral assays such as the Y-tube assay (Ingvarsdóttir *et al.*, 2002) can be used. Selander *et al.* (2016) present a list of 87 exudates from male and female *T. longicornis*. Their list contains nine compounds that are produced mainly or even exclusively by the females. These compounds did not initiate the pursuit reaction in the male, but this can be because there were other volatile compounds that were not retained. Future research is needed to investigate the decay and diffusion rates of the compounds and to locate potential differences. The number of compounds, their relatively large molecular masses (300-700 g/mol) and their likely complicated chemical structures indicate that a host of information should be available from their combined presence for the trained "observer".

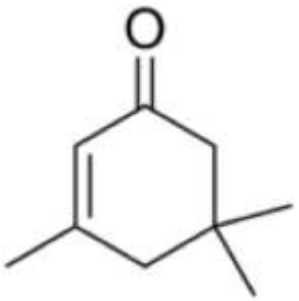

Figure 6. Isophorone is a kairomone used by the parasitic sea louse *Lepeophtheirus salmonis* to locate its host (Ingvarsdóttir *et al.*, 2002). Structural formula from Wikipedia (2017).

All marine fauna are currently challenged by increasing chemical pollution and decreasing oceanic pH values, both of human origin. Changes in the background chemical landscape have harmful effects on olfactory-mediated behaviors in fish and crustaceans (Olsén, 2011). Due to their critical linking position in the marine food web, it is of high value to identify the chemical components of copepod sex pheromones and how their mating behavior is affected in the presence of pollutants.

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Chapter 12

PLANKTONIC CALANOIDS EMBARK INTO THE "OMICS ERA"

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ABSTRACT

Since the very first DNA fragment was sequenced in early 70s and the 1980s Nobel Prize was awarded to Sanger and Gilbert "for their contributions concerning the determination of base sequences in nucleic acids," both biology and medicine have taken great steps forward. In particular, marine biologists have taken advantage of technical development and are increasingly applying molecular techniques to address biological and ecological questions. Recently, the growing interest in marine and freshwater organisms expressed by private companies involved in pharmaceuticals, nutriceutics, and bioproduction have boosted interest and applications of genomics, transcriptomics, proteomics and metabolomics. In parallel, molecular techniques like genetic transformation, genome modification, and gene silencing have been developed for non-model species. This improvement of molecular biology-related approach has enhanced academic research in biology, ecology and physiology of a plethora of metazoan non-model species. Copepods are the most species-rich class among the marine arthropods, are globally distributed and inhabit every environment from coastal to oceanic waters, where they represent the dominant group of zooplankton, and can also live as symbionts or parasites. This diverse array of life strategies and environments make copepods highly interesting as target species for genomic and transcriptomic studies. In the present

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contribution, we provide an overview of the state of the art about 'omics' studies in calanoid copepods, focusing on marine free-living species. Finally, we will suggest some future perspectives for possible applications of functional genomics studies in copepods.

Keywords: calanoid copepods, transcriptomics, metabolomics, proteomics

1. INTRODUCTION

The phylum Arthropoda is one of the most successful among animals on our planet, with more than one million species (Telford *et al.*, 2008) inhabiting every environment. Among Arthropoda, copepods belong to the class Maxillopoda (Sub-phylum Crustacea, sub-class Copepoda), which is thought to have arisen during the Paleozoic, between the Cambrian and the middle Devonian (Regier *et al.*, 2005).

Copepods represent the most abundant group of multicellular animals, and more than 14,000 species have been described to date (Humes, 1994). Their adaptive radiation is astonishing: they colonised literally every subaqueous, subaerial and subterranean environment (Huys and Boxshall, 1991; Boxshall and Halsey, 2004). The same authors suggested that all orders of copepods are derived from ancestral marine epibenthic forms, and that the invasion of the pelagic environment was accompanied by strong morphological specializations. Besides their free-living life-style, copepods have evolved symbiosis and parasitism as well (Ho, 2001). It has been hypothesised that during evolution, copepods have left the free-living habitus on at least 11 independent occasions (*e.g.*, Bayly and Boxshall, 2009). Because of their species richness and incredibly high biomass, copepods have been dubbed the 'insects of the sea' (Huys and Boxshall, 1991). In a review paper, Schminke (2007) has even emphasised this assumption, in saying that insects should be called 'the terrestrial copepods', due to the evidence that Crustacea and Insecta may form a monophyletic group called Tetraconata or Pancrustacea (Schminke, 2007). The relationship between these two sister groups has been recently validated by molecular and morphological phylogeny (Legg *et al.*, 2013). However, unlike insects, the studies on genetics and genomics of copepods is in its infancy. Here we will focus on free-living marine calanoid copepods because the ecological role they play in marine ecosystems as primary consumers is crucial for the whole food-web. Bron *et al.* (2011) have already presented the state of the art in copepod genomics in a very elegant and omni-comprehensive review. In particular, genomic resources have been used to address issues related to species identification (*e.g.*, Bucklin *et al.*, 2009; Sabia *et al.*, 2017; Di Capua *et al.*, 2017), biogeography (*e.g.*, Rubao *et al.*, 2012), cryptic species detection (*e.g.*, Schoville *et al.*, 2012; Cornils and Held, 2014), symbiosis and parasitism (*e.g.*, Fallang *et al.*, 2005; Fast *et al.*, 2007), and acute and sub-lethal responses to environmental stressors (*e.g.*, Raisuddin *et al.*, 2007; Hansen *et al.*, 2011). In this chapter,

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we aim to update the state of the art, paying particular attention to the techniques developed since 2011. We will emphasise recent gene expression studies on free-living keystone copepod species, and then will give a more thorough description of 'omics' studies (transcriptomics, proteomics and metabolomics), addressing specific questions in copepod biology and ecology. Finally, we will give some future perspectives for functional genomics studies in copepods.

2. TECHNIQUES

In the present section, the molecular biology techniques that will be discussed in the following sections are described.

2.1. RT-qPCR

The acronym RT-qPCR stands for Real-Time quantitative Polymerase Chain Reaction. This technique is an improvement of the conventional end-point PCR and takes advantage of fluorophores. Such fluorophores are used in two different ways: 1) they can directly bind in a stochiometric way to double stranded DNA so they emit photons when incorporated into the amplicon or 2) they are linked to a probe binding to the region wich contains the DNA fragment to be amplified. The probe is linked to both the fluorophore and to a quencher which hampers fluorescence if it is in the vicinity of the fluorophore. Polymerase binds to the region to be amplified and unwinds it. Because the probe is bound the one portion of this region, polymerase removes the probe, the quencher and the fluorophore are separated, and fluorescence is released. In both cases, photons are released and detected at each PCR cycle.

The main goal of RT-qPCR is to quantify the amount of template DNA present in the sample. In the present chapter, studies based on RT-qPCR have been used to determine gene expression levels under two experimental conditions. This is achievable by amplifying complementary DNA (cDNA) samples obtained by reverse transcribing RNA from specimens under a given experimental condition. RNA is not amplifiable by PCR so it is necessary to synthesise cDNA first. To do so, a viral enzyme, the RNA-dependent DNA polymerase, colloquially called reverse transcriptase is used. This enzyme synthesizes DNA from RNA templates. When RT-qPCR is used to determine gene expression levels, the relative quantity of the template cDNA from a gene of interest (GoI) is measured in comparison to the relative quantity of a series of reference genes (RG). This procedure is needed to normalise cDNA quantity used for the reaction mix. RG's are usually house-keeping genes whose expression does not change over a broad range of experimental conditions. In order to define the suitable RG's among a set of

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candidates, different algorithms are used. To be valid, RT-qPCR-based works should rely on at least 3 RG's. For a wide review of RT-qPCR we suggest Valasek and Repa (2005) and Bustin *et al.* (2009).

2.2. Microarray

Microarrays, or the DNA chip technique, is based on the principle of nucleotide hybridisation. Oligomer probes (short DNA fragments) are spotted on a glass microscope slide. Oligomers can be fragments of genes or transcripts. The slide is then put in contact with nucleic acid extracts (DNA or cDNA) from two samples: one control and the other from the experimental condition. If cDNAs are hybridised to the microarray, the outcome is the gene expression level in the experimental vs the control condition. In order to obtain such a comparison, RNA samples are reverse transcribed and labelled with fluorescent dyes of a different colour. Individual fragments in template cDNAs, complementary to the probes spotted onto the chip, will anneal. The chip scanner reads the relative intensity of each dye and gives relative quantification of a given template fragment in one sample compared to the other. In the case of microarrays internal or external controls are needed to normalise the expression levels (Lippa *et al.*, 2010). In order to rule out any bias due to differential efficiency in the reverse transcription and labelling, a dye swap (*e.g.*, Hori *et al.*, 2015) is carried out. This procedure is performed by hybridising twice the chip with the same sample pair, but with the samples labelled alternately with the two different fluorescent dyes. This constitutes a technical replicate, with cDNA that is reverse transcribed separately from the same RNA sample to label with different dyes the first cDNA strand. In addition, it is also a methodological control, possibly reducing the occurrence of false positives due to the bias of fluorescent dyes (Mary-Huard *et al.*, 2008). Microarrays can be used to measure the expression of thousands of genes simultaneously, but lack the ability to detect transcripts whose sequence is unknown. This makes such technology unsuitable to work on non-model organisms, where little or no genomic and transcriptomic information is available. Microarrays are currently applied mostly in molecular diagnostics because protocols have been standardised and validated for health and clinical investigations (*e.g.*, Yoo *et al.*, 2009; Govindarajan *et al.*, 2012).

2.3. EST and SSH

Expressed Sequence Tag libraries (EST) are sets of sequenced cDNAs from specific conditions or tissues and are used primarily for gene discovery or tissue-specific gene expression analyses (Adams *et al.*, 1991). cDNA synthesised under specific conditions or

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from tissues are inserted into vectors like plasmids, cosmids or fosmids and then transformed into competent bacterial cells. This procedure enables sequencing of unknown mRNA transcripts or random genomic DNA fragments. ESTs have been very useful in the field of molecular ecology to understand the population structure, and the genetic basis of phenotypic variation and adaptation of species. Through the discovery of new molecular markers, ESTs may provide a means for investigating gene family and genome evolution (Bouck and Vision, 2007).

If the problem to address is to identify genes differentially expressed between an experimental condition and that of the control (or in one tissue compared to another), one of the ways to enhance the yield of differentially expressed genes is to produce subtraction libraries by means of suppressive subtraction hybridization (SSH) (Diatchenko *et al.*, 1996). This technique enriches the library from the sample of those transcripts that are differentially expressed compared to the control. The basis of such an enrichment is the elimination of those transcripts that are present in both samples. For an extensive and exhaustive description of the method we suggest Rebrikov *et al.* (2004).

2.4. RNA-Seq and *De Novo* Assembly of Transcriptomes

In the studies reviewed in the present chapter, RNA sequencing (RNA-seq) for transcriptome production have been mainly performed using Illumina or 454 sequencing platforms. These sequencing techniques differ from the Sanger sequencing in many aspects, most importantly in that Sanger sequencing relies on capillary electrophoresis of a population of ddNTP-terminated dye labelled products that are separated by molecular weight and the dye detected. Each colour corresponds to one of the four nucleotides. Sanger sequencing is based on DNA synthesis. Illumina and 454 sequencing are based on solid-phase bridge PCR amplification, and emulsion PCR amplification, respectively. The 454 method produces longer reads compared to Illumina sequencing and with its newly released version of the platform (454 GS FLX+) can equal Sanger sequencing in read length. Although softwares exist to assemble short reads with a very high degree of effectiveness, longer reads can help with scaffold assembly in *de novo* assemblies. Sequencing technology advances very rapidly and a complete overview of all the techniques presently available is beyond the aims of the present section. Here we will briefly describe only the two major sequencing techniques currently used in marine copepod transcriptomics analyses. The most up-to-date sequencing method is the nanopore sequencing technology (Feng *et al.*, 2015). Single molecules electrophoretically pass through a pore that separates two reservoirs, called the *cis* and the *trans* reservoirs. When the analyte passes through the pore, the voltage through it is blocked and by statistically analysing the amplitude and the time of such blockage, the nature of the molecule can be identified. This techniology needs still improvements (Feng *et al.*, 2015)

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but will reduce costs and manipulation of samples (*e.g.*, Quick *et al.*, 2017). For a detailed description of all the Next Generation Sequencing (NGS) technologies we suggest the following papers: Branton *et al.*, 2008; Shendure and Ji, 2008; Quail *et al.*, 2012; Rhoads and Au, 2015; Wang *et al.*, 2015; Feng *et al.*, 2015; Yuan *et al.*, 2016.

Briefly, RNA-seq is based on the great technological advancement of NGS that allow obtaining billions of base pairs (Gb) and millions of short reads between 30-400 bp long per run without any cloning steps. As discussed in the above-cited papers, together with the augmentation of sequence power, the costs have dramatically decreased, enabling obtaining transcriptomes to be more widely available. An RNA-seq experiment has to be thoroughly planned beforehand with the proper questions to address. The amount of data generated by a single RNA-seq experiment is enormous and the bioinformatics analyses required are quite demanding. A transcriptome is usually produced for two main reasons; 1) to identify new genes specifically expressed in a given condition or tissue; 2) to pinpoint differentially expressed genes in different conditions or tissues or even different developmental stages. In addition to these scenarios, the only limitation to the technique is the researcher imagination. Consider the case 2. Total RNA is extracted from specimens under the experimental and control conditions. The control can be arbitrarily chosen. cDNA is synthesised as described above and libraries are constructed. The library construction depends on the sequencing technology of choice (*e.g.*, Pease and Sooknanan, 2012; Baran-Gale *et al.*, 2015). In order to be statistically robust *i.e.*, to obtain sound results with the lowest false positive rate possible, replicates are required. Usually triplicates are accepted (Conesa *et al.*, 2016) although it has been recently demonstrated that at least six biological replicates are needed in order to be able to statistically identify differentially expressed genes (Schurch *et al.*, 2016). The output of sequencing is a variable number of variably long sequences (depending on the technique) called reads. Reads are quality filtered *in silico* in order to obtain mainly sequences from messenger RNAs *i.e.*, expressed genes, and other RNA types like micro RNAs (miRNA), long non-coding RNA (lncRNA), etc. The assembly of short reads into full length genes is a complex bioinformatic process. Assembly can either rely on a reference genome, if available, which is usually the case for model organisms; or can be done *de novo*. In the former case, a series of algorithms (pipelines) align the reads to the reference genome sequence; in the latter, reads have to be assembled without a guide (*e.g.*, Haas *et al.*, 2013). Once the reads have been assembled into contiguous overlapping regions, referred to as contigs, which can eventually match with full length transcripts, annotation is performed. For a review on read assembly see Miller *et al.* (2010). Annotation is a bioinformatic procedure which assigns to contigs gene models (Yang and Kim, 2015; Conesa *et al.*, 2016) or other features like *e.g.*, retrotransposons or transposable elements (Criscione *et al.*, 2014; Jin *et al.*, 2015). With appropriate normalization procedures, the number of reads can be quantified and the expression level of each transcript defined. Differential expression analyses can be now performed using adequate software (Conesa

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et al., 2016). RNA-seq is devoid of problems derived from technical issues inherent to microarray probe performance such as cross-hybridization, non-specific hybridization and limited detection range of individual probes. Additionally, RNA-seq technology does not require species- or transcript-specific probes (Zhao *et al.*, 2014).

A recent study by Wang *et al.* (2009) compared RNA-seq, microarray and cDNA sequencing techniques in transcriptome profiling and found that RNA-seq was superior in detecting low abundance transcripts, differentiating biologically critical isoforms, and allowing the identification of genetic variants. RNA-seq also demonstrated a broader dynamic range than microarrays, which allowed for the detection of more differentially expressed genes with higher fold-change (see Table 1 in Wang *et al.*, 2009).

3. THE TARGET SPECIES

A few gene expression and transcriptomic studies have been published to date on free-living marine copepods. The most investigated genera are the calanoid *Calanus* and the harpacticoid *Tigriopus*. Calanus has been used as model organism for studies on mechanisms inducing diapause, on the life cycle in general (Tarrant *et al.*, 2008; Lenz *et al.*, 2012), and responses to microenvironmental discontinuity (Unal *et al.*, 2013). *Tigriopus* has been used for investigating temperature tolerance of different populations, ecotoxicology and for phylogenomic purposes (Raisuddin *et al.*, 2007; Schoville *et al.*, 2012). However, as it is not a planktonic species, *Tigriopus* is not dealt with in the chapter.

Only a few transcriptome studies have been performed on *Calanus* despite the extensive knowledge on its biology and ecology. The focus of these studies has been to better understand the molecular basis of their physiological responses to intrinsic and extrinsic factors. *Calanus finmarchicus* and *Calanus helgolandicus* are two of the most widely studied *Calanus* species in terms of biology (Niehoff, 2007) and distribution (Mackas *et al.*, 2012; Maud *et al.*, 2015). *C. finmarchicus* is a very abundant copepod species possessing a subarctic distribution with a latitudinal range from 40°N to 80°N (Bryant *et al.*, 1998) and inhabiting shallow to very deep waters. Its ecological importance is linked to its abundance and subsequently to the impressive amount of energy that flows through this primary consumer in most ecosystems from the primary phytoplanktonic production to economically relevant fish larvae (Prokopchuk and Sentyabov, 2006). Similarly, *C. helgolandicus* is one of the dominant zooplankton species in European waters, living in open and coastal waters of the temperate Eastern Atlantic Ocean and the Mediterranean Sea (Northern Adriatic Sea) (Bonnet *et al.*, 2005). Recent surveys showed that both species distributions are gradually shifting northward due to the increase in water temperature, with *C. finmarchicus* moving into Arctic waters and *C. helgolandicus* expanding its area towards the North Sea (Beaugrand *et al.*, 2002).

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As indicators of different water masses, both species are routinely identified by the Continuous Plankton Recorder (CPR) Survey, operated by the Sir Alister Hardy Foundation for Ocean Science (SAHFOS) in Plymouth (UK) (Wootton *et al.*, 2018).

Within the *Calanus* genus, it is notable that *Calanus sinicus* is a widespread cold-temperate species that inhabits the Northwest Pacific Ocean from the Yellow Sea to the Sea of Japan and the South China Sea and Taiwan Straits (Huselmann, 1994; Hwang *et al.*, 2006). *C. sinicus* has been one of the target species of the China-GLOBEC program (Sun, 2005), because it is a strong link between the anchovy and sardine fisheries and the copepod secondary production in this area (Uye, 2000; Yang *et al.*, 2014). Because of its ecological relevance, this species has recently received much attention using RT-qPCR and RNA-Seq approaches (see section 4).

4. GENE EXPRESSION APPROACH

4.1. RT-qPCR-Based Works

Gene relative expression and expression profiling can be achieved by different means *i.e.*, RT-qPCR, DNA microarrays (section 4.2), and RNA-seq (section 4.3). The former is limited to genes already identified and for which a sequence and possibly a function are already known. This RT-qPCR approach has been extensively applied in the ecotoxicology field because stress-related genes responding to environmental stressors have been identified in copepods (*e.g.*, Hansen *et al.*, 2008 and 2013). Recently, a RT-qPCR approach was also used to explore the deleterious effects of harmful algal diets on copepod gene expression. In particular, the expression levels of genes involved in generic stress responses, defence systems and apoptosis regulation in *Calanus helgolandicus* feeding on the diatom *Skeletonema marinoi* were investigated (Lauritano *et al.*, 2011a and b). *S. marinoi* is able to produce cytotoxic metabolites called ‘oxylipins’ from the oxidative metabolism of fatty acids (Fontana *et al.*, 2007; Gerecht *et al.*, 2011; Di Dato *et al.*, 2017). In *C. helgolandicus*, the diatom induced the regulation of α and β tubulins, possibly revealing cellular rearrangement, and of several other genes related to stress response (heat shock protein 40), detoxification (several aldehyde dehydrogenases ALDHs) and apoptosis (cellular *apoptosis* susceptibility, CAS and inhibitor of apoptosis, IAP). These studies provided the first molecular evidence that the primary defence system that would be activated to protect copepods against toxic algae could be inhibited (Lauritano *et al.*, 2011a and b). Interestingly, this approach enabled testing for subtle differences among the responses of *C. helgolandicus* populations isolated from North Sea, Atlantic Ocean and Mediterranean Sea. Despite the only three bp difference in a 518 bp-long mitochondrial COI fragment among the three *C. helgolandicus* populations, the

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Mediterranean population showed stronger gene down-regulation compared to the others (Lauritano *et al.*, 2012).

Similar results were obtained with the congeneric species *Calanus sinicus* fed on *S. marinoi* for two and five days (Lauritano *et al.*, 2015). At day five, *C. sinicus* showed up-regulation of several ALDHs, catalase (CAT), and glutathione-S-transferase (GSH-S), known to enzymatically reduce free radicals and reactive species (RS).

These studies provided new insights for understanding copepod population- and species-specific responses to toxic algae and co-evolution between toxic algae and detoxification mechanisms in copepods.

The results presented above, were partially corroborated by an in situ study carried out on wild *C. helgolandicus* specimens collected at the end of the spring diatom bloom in the Northern Adriatic Sea during an oceanographic cruise (Lauritano *et al.*, 2016). Surprisingly, detoxification genes did not revel consistent regulation. This complicates our understanding of this complex phenomenon in copepods and calls for more detailed investigations. The response to a possibly noxious diet could be different if the copepods are fed with phytoplankton other than diatoms. In fact, when *C. helgolandicus* was fed on a non-toxin producing strain of the dinoflagellate *Karenia brevis* for three, five and eight days, the results were completely different (Lauritano *et al.*, 2013). β tubulin, ALDHs, CAS and HSP40 were significantly down-regulated, but only after eight days of exposure to this food. This could be due to a different effect of unknown compounds produced by the dinoflagellate. Chemical analyses of the same strain were performed but they did not reveal any harmful compound (Turner *et al.*, 2012). Such a discrepancy suggests how intricate the copepod-prey ecotoxicological landscape can be and highlights the need to more detailed functional studies.

4.2. Microarrays and EST Libraries

Microarrays or DNA chips, suppressive subtractive hybridization (SSH), expressed sequence tag (EST) libraries and RNA-seq (section 4.3) are useful techniques that have been applied to disentangle copepod transcriptional responses to different environmental stressors and different diets or to identify genes involved in reproduction and molting.

The first *C. finmarchicus* microarray was produced by Lenz *et al.* (2012). Before the target genes to spot on to the chip were defined, < 11,000 ESTs were sequenced, assembled and annotated together with the other ESTs publicly available at that time for the species. Around one thousand target genes were selected according to their function. 50mers (synthetic oligonucleotides composed of 50 bp) were spotted on the chip. The microarray was first tested with *C. finmarchicus* under food stress compared to adequately fed animals. A second test was performed comparing lipid-rich and lipid-poor animals. In both cases, several genes resulted differentially expressed. This pioneering

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work led to the conclusion that copepods respond to food limitation similarly to other organisms and they tend to save energy.

The '*Calanus* physiological microarray' (Lenz *et al.*, 2012) was then used in an in situ investigation by Unal *et al.* (2013) who, in 2008, compared gene expression in deep and surface *C. finmarchicus* individuals. The custom 995-gene microarray was hybridised with cDNA produced from 9-12 copepod RNA extractions. Chip hybridisations involved comparison between surface and deep females, and surface females with deep juvenile stages (copepodite V, CV). No CV were detected at the surface in that sampling occasion. The differential expression patterns displayed by surface and deep individuals presented a scenario where in deep waters individuals recently emerged from diapause were completing their developmental process ready to migrate to the surface. The individuals at the surface were metabolically more dynamic with grazing and predator escaping activities (Unal *et al.*, 2013). These observations are in accordance with the bimodal vertical distribution of the species. In spring copepodites emerge from diapause and migrate to the surface to feed and reproduce. This is corroborated by the up-regulation of genes involved in development and phototransduction, as well as of some genes involved in late embryogenesis. In deep females, the set of differentially expressed genes suggested that diapause emergence, early embryogenesis and tissue remodeling take place. Despite the absence of dye swap, the data in Unal *et al.* (2013) were statistically robust because all the possible normalization and quality controls were performed. Moreover, the work represented a novel approach.

In 2014, the first transcriptional profiling study of a copepod exposed to a harmful algal diet (the oxylipin-producing diatom *S. marinoi*) was performed by Carotenuto *et al.* (2014) on *C. helgolandicus* using a SSH technique. *Rhodomonas baltica* was used as food for control copepods. Although this method did not provide as many sequences as the RNA-seq analysis (less than 1k), it generated two reciprocal EST libraries composed of longer sequences (on average 370 bp) compared to RNA-seq. This enabled a more robust annotation of the database, with about 62% of ESTs showing a BLAST match compared to 40% reported in Lenz *et al.* (2014), and about 81% of ESTs functionally annotated into Gene Ontology (GO, http://www.geneontology.org) categories (475 out of 583) compared to 13% (5k out of 38k) (Lenz *et al.*, 2014). The annotation statistics improved when the ESTs were eventually assembled into longer contigs (on average 420 bp, and 70% with a Blast-hits). A differential expression analysis, performed on the two reciprocal ESTs libraries using the Fisher's Exact Test with Multiple Testing Correction of False Discovery Rate (FDR < 0.01), showed that several GO terms were significantly enriched in both libraries. More specifically, in *Rhodomonas*-fed *C. helgolandicus*, macromolecule biosynthetic process, ribosome biogenesis and cell cycle processes were enriched. This suggests that animals are in an active state and that they are healthy. In *Skeletonema*-fed copepods the enriched GO-terms were biological regulation, nucleotide binding, signal transduction and protein folding. This transcriptional scenario can reveal a

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stressing state the organisms have to cope with. The results were validated and confirmed by RT-qPCR, and led to the conclusion that in *C. helgolandicus*, the diatom diet was inducing a generalised Cellular Stress Response mechanism, characterised by over-expression of molecular chaperones aimed at re-establishing the cellular homeostasis of the copepod.

4.3. RNA-Seq

In 2013 the complete transcriptome of *C. siniucs* was sequenced using the 454 GS FLX method (Ning *et al.*, 2013). Copepodites and adult males and females were collected from the field and cDNA was obtained from total RNAs extracted from pools of CIV-CV and adult males and females. Almost 1.5×10^6 350 bp reads were obtained in total from both libraries sequenced, 7% of which did not pass quality and size control. The transcriptome was *de novo* assembled and ca. 15k genes annotated. RNA-seq data were analysed for differential expression and ca. 2k genes resulted up- and 2k down-regulated in copepodite vs.adult library. Among these genes, some were of great interest because linked to diapause regulation. In this study, RT-qPCR validation of 7 transcripts was performed on the same cDNAs used for sequencing and all of them were significantly differentially expressed validating the RNA-seq results. This validation adds robustness to the results. In the study by Ning *et al.* (2013) $> 2 \times 10^5$ single nucleotide polymorphisms (SNPs) and $> 3 \times 10^4$ insertions/deletions (indels) were found. The sequencing was performed on a mixture of individuals so the intrapopulation level variability could be detected. SNPs can be of great utility for phylogenomic studies as in bacteria and coral systems (*e.g.*, Bryant *et al.*, 2016; Rosser *et al.*, 2017) or population genetics (Coates *et al.*, 2011).

Later on, the transcriptome of *C. sinicus* was also sequenced by means of Illumina technology (Yang *et al.*, 2014). The results obtained from this second study corroborated and enriched that of Ning *et al.* (2013), giving a deeper coverage of the transcriptome with nearly 6×10^6 100 bp reads sequenced. In Yang *et al.* (2014), GO term assignment was similar to that reported by their colleagues for 'molecular function' (30% vs 32%) and for 'biological process', being in both cases the most represented term. Given that Yang *et al.* (2014) obtained seven times more enzyme code (EC) assignments *i.e.*, KEGG gene pathways annotations (KEGG: Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) with 14K vs 2.9K transcripts compared to Ning *et al.* (2013), the most represented pathways were the same: carbohydrate, amino acid and energy metabolisms. In both studies, particular importance was paid to lipid metabolisms for its involvement in diapause. Noteworthy, most transcripts identified as diapause-related, such as a long chain fatty acid elongase (ELOV), several HSPs and ferretin, were differentially expressed between copepodites and adults (Ning *et al.*, 2013). Yang *et al.*

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(2014) identified stress- and immune-related genes as well. This is of great interest for future studies focusing on the effect of different pollutants on marine ecosystems in general.

In 2014 and 2016 *C. finmarchicus* was the target species for three Illumina RNA sequencing projects. The first produced from different developmental stages (Lenz *et al.*, 2014), the second dealing mainly with molting (Tarrant *et al.*, 2014 and 2016) and the third showing the copepod response to a toxic dinoflagellate (Roncalli *et al.*, 2016). These studies were aimed at obtaining new resources on key copepod species. Lenz *et al.* (2014) produced the first complete transcriptome for *C. finmarchicus* and immediately after Tarrant *et al.* (2014) produced a second complete transcriptome on the same species. The former *de novo* assembly was carried out using reads from six cDNA libraries built on RNA extracts from different developmental stages of the target copepod, namely embryos, early and late nauplii (NI-NII and NV-NVI, respectively), early copepodites (CI-CII), pre-adults (CV) and adult females (CVI). Ribosomal sequences were filtered from the raw data ($> 4 \times 10^8$ 100 bp-reads) and library-specific adaptors removed before transcriptome assembly. The number of unique contigs was 96k, of which ca. 38k presented a significant blast with protein-coding genes (about 40%). This number is in the same order of magnitude as the *Daphnia pulex* genome (ca. 31k reported genes; Colbourne *et al.*, 2011) but is twice as many putative genes as *Drosophila melanogaster*'s (ca. 18k genes; Hoskins *et al.*, 2015) and 2.5 times as many as Eriocheir sinensis crab's (14k genes; Song *et al.*, 2016). The second transcriptome by Tarrant *et al.* (2014) was carried out from pooled RNA of copepodites V individuals from two time points (three and 10 days growth). To this pool, *C. finmarchicus* RNA from wild CV's was added.

Without a complete genome sequenced, it is very difficult to extrapolate the number of genes composing a genome from RNA-seq data. On the other hand, this exercise has been done for a congeneric species, C. sinicus, and for Tigriopus californicus. In both instances, around 15k genes have been found to be expressed in these copepods' transcriptomes (Barreto *et al.*, 2011; Ning *et al.*, 2013). In comparison, the human genome has been estimated to be composed of around 19k protein coding genes (Ezkurdia *et al.*, 2014). The genome size of *C. finmarchicus* was indirectly estimated to be 6 Gb (http://www.genomesize.com), which is almost double the size of the human haploid genome.

In Lenz *et al.* (2014), the *C. finmarchicus* RNA-seq data were used to infer transcript differential expression among developmental stages. Usually, a differential expression analysis enables identification of genes that are specifically or preferentially expressed in one treatment compared to the condition set as control (*e.g.*, treated vs. non-treated). In their study, Lenz *et al.* (2014) carried out a differential expression analysis among the different developmental stages of a number of transcripts involved in lipid biosynthesis. The *C. finmarchicus* transcriptome provides a great amount of information for gene

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discovery. In this study at least one third of the transcripts are not expressed in any particular developmental stage. A few transcripts, though, showed pronounced stage-specificity: an acyl transferase expressed in adults and embryos; an ELOV and a Δ9 desaturase in copepodites. All these transcripts have EST support and are consistent with previous findings showing that copepodites stock fatty acids (FA) to be used in wax and the adults and embryos synthesise triacylglycerols (TAGs) suggesting a different lipid metabolism in the different developmental stages. This finding is consistent with Tarrant *et al.* (2014) as well. Voltage-gated sodium channels (Na_V) were also identified using D. melanogaster protein sequence. This protein is the target of saxitoxin poisoning and mutated Na_V proteins can be at the basis of resistant strains. Actually, different transcripts had Na_V identity. Unfortunately, in the transcriptome produced by the same group on *C. finmarchicus* fed with the toxic dinoflagellate *Alexandrium funndiense*, no Na_V genes were differentially expressed (Roncalli *et al.*, 2016). Actually, the entire detoxification machinery did not show any sign of regulation. In this second study, the authors identified cellular stress and homeostatic responses at day two and five, respectively, but no striking detoxification response as expected from comparisons with other organisms exposed to toxic compounds. Overall, this work enabled investigation of the subtle physiological response of the copepods to sub-optimal food conditions, namely the ingestion of a toxic alga, which did not reduce the copepod survival but could potentially lead to lower reproductive potential and population fitness.

Together with a previously produced database (~ 12k ESTs; Lenz *et al.*, 2012), gene model predictions and sequences can be used to design primers for specific genes involved in biological processes or environmental responses. Once an annotated transcriptome or genome becomes publicly available for a species (especially a non-model species), the research on it gets a kick onwards and thus ecologically, biologically and biotechnologically relevant questions are addressed. Moreover, the *C. finmarchicus* transcriptome will be used as reference for new RNA-seq studies, like it has been done in Roncalli *et al.* (2016).

In 2015, the transcriptome response to thermal stress of the temperate *C. finmarchicus* vs. the arctic *Calanus glacialis* was compared using an Ion Torrent RNA-seq experiment (Smolina *et al.*, 2015). Results, validated by RT-qPCR, suggest that *C. finmarchicus* responds to thermal stress activating HSPs, chaperons and proteins involved in ROS detoxification. The Arctic *C. glacialis*, on the contrary, did not show differential expression of transcripts due to the treatment. The conclusion was that the *C. glacialis* lacks the machinery to respond to thermal stress, like other organisms adapted to low temperatures (*e.g.*, Clark *et al.*, 2008). This finding does not indicate that *C. glacialis* is not stressed by temperature. On the contrary, at high temperatures the animals appeared to be dormant. Results suggest that this species is less resilient to temperature variations and hence is more vulnerable to higher temperatures. Global warming would thus endanger *C. glacialis*.

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5. METABOLOMICS AND PROTEOMICS

The analyses of either endogenous or exogenous molecules like peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, that are usually below 1.5 KDa is called metabolomics. Typically, these analyses are performed by Nuclear Magnetic Resonance (NMR) or Mass Spctrometry (MS) techniques, coupled with statistical tools such as principal component analysis (PCA) and partial least squares (PLS). Metabolomics is a broad and multidisciplinary field and the description of the different techniques is beyond the aims of the present chapter. A main distinction has to be made between the non-targeted unbiased analysis of large sets of low molecular weight organic metabolites, and the targeted-metabolomic approach. The former complements genomics, transcriptomics and proteomics, and represents the closest link to phenotype; the latter is aimed at measuring defined groups of chemically characterised metabolites. For a detailed description of protocols, we suggest the book by Metz *et al.* (2011) or the review paper by Zhang *et al.* (2012).

Proteomics is a term that was first introduced in the early 90s mirroring the word 'genomics', which identified the investigation of the totality of genes present in a given genome. Actually, the word proteomics identifies a plethora of different analyses like expression proteomics, which highlights differentially expressed proteins in two conditions or samples, protein-protein interactions, proteome fingerprinting, *etc*. Techniques are mainly based on gel separation like Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE), two dimentional PAGE (2D-PAGE), or high performance liquid chromatography (HPLC) or MS.

Here we will give a short overview of recent works in targeted- and non-targeted metabolomics and proteomics on planktonic calanoids. Recently, the effect of ocean acidification (OA) and temperature variations were investigated in two planktonic calanoids, Paracalanus sp. (Garzke *et al.*, 2016) and a nearly 1:1 mixture of *Calanus helgolandicus* and *Calanus finmarchicus* (Mayor *et al.*, 2015). Targeted analysis of FA composition in adult copepods was investigated by means of Gas Chromatography, together with analysis of body size. Results suggest that in the next two centuries, warming and OA would produce a shift in copepod body size and FA composition. This would impair copepod suitability as valuable food for higher trophic levels. Changes in FA compositions in copepods would be driven by variation in the different classes of FA in phytoplankton. Remarkably, docosaexahenoic acid (DHA, 22:6 ω3) would dramatically diminish with increasing temperature and decreasing pH. This, together with the variation of other FAs, might impair the development of copepods as well (Garzke *et al.*, 2016). The reduced productivity and abundance of copepod food due to warming (Behrenfeld *et al.*, 2006; Boyce *et al.*, 2010) would lead to food stress in the future for copepods. This scenario, integrated into the context of global warming, was investigated by means of non-targeted metabolomics based on chromatography/MS techniques

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(Mayor *et al.*, 2015). Copepodites (CmV) of *Calanus* species were exposed to two temperatures and two pCO_2 conditions and starved for five days. The four treatment groups did not show statistically significant differences after five days of starvation, however results were different when comparing the pre-experimental to the post-experimental animals (t_0 vs t_5). Almost all polar compounds, such as amino acids, were down-regulated at t_5, while saturated and monounsaturated FA were all up-regulated. Only DHA and eicosapentaenoic acid (EPA, 20:5 ω3) were down-regulated (Mayor *et al.*, 2015). Food-deprived animals also showed significantly higher levels of a new class of taurine-containing lipids, which the authors speculated might act as an active transporter of substrates to a catabolic site. Similar compounds were previously identified in *C. finmarchicus* and *Centropages typicus* (termed as 'copepodamides'), where they played a role as signal molecules modulating toxin production in the dinoflagellate *Alexandrium minutum* (Selander *et al.*, 2015). In conclusion, though copepods seem to be more strongly affected by starvation than by warming and OA, both influence the copepod survival by indirectly changing the phytoplankton lipid composition and productivity (Mayor *et al.*, 2015).

Non-targeted metabolomics studies in copepods are in their infancy. The pioneering environmental toxicometabolomic works by Hansen and co-authors on *C. finmarchicus* (Hansen *et al.*, 2010 and 2017) have clearly showed a relationship between copepod metabolic responses and sub-lethal xenobiotic exposure. As with transcriptomics (section 4.3), metabolomics studies as well deserve further investigation.

The first proteomics study on a planktonic calanoid, *Eurytemora affinis*, including protein sequencing, was published by Boulangé-Lecomte *et al.* (2016). The effect of two pollutants were investigated, namely phenylureic hericide commercially known as diuron (DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea) and a mixture of two alkylphenols, the 4-nonylphenol (4-NP) and the nonylphenol-ethoxy-acetic-acid (NP1EC), widely used in synthetic industry. The two pollutants produced strikingly different results, with DCMU inducing the highest protein variation. DCMU exposure induced a defense mechanism against cell damage which triggered an elevated energy request, as showed by up-regulation of ATP synthase and arginine kinase genes to cope with high ATP demand. Signal transduction and immune response machineries were regulated as well. For the latter, the 14-3-3 zeta protein contributes to oocyte mis-development, as also shown in other systems. Oxidative stress response was induced as well with GSH and superoxide dismutase proteins being up-regulated. In *E. affinis*, as in *C. helgolandicus* fed on a potentially toxic diatom (Lauritano *et al.*, 2011b), HSPs and protein-folding related proteins were up-regulated. Alkalylphenols produced an opposite result with most of the proteins down-regulated. This study, although suffering from the lack of a reference genome, shed light on copepod proteomic response to environmental concentrations of pollutants, but also posed the basis for further development of new biomarkers for water quality assessment.

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6. GENOME UP-DATES

In the last few years, two genome projects for calanoids were initiated, the 'Whole genome assembly of common copepod (*Eurytemora affinis*)' as part of the Baylor College of Medicine-Human Genome Sequencing Center (BCM-HGSC) i5k Pilot Project (Accession: PRJNA203087; ID: 203087) and 'Restriction site-Associated DNA sequencing (RAD) tag study of populations of *Centropages typicus*, a marine copepod' (Accession: PRJNA265130; ID: 265130), to detect population genetic structure and connectivity among North Atlantic populations of this species (Blanco-Bercial and Bucklin 2016).

The calanoid copepod *E. affinis* is a euryhaline epibenthic species that can live both on the sediment and in the water column and is able to adapte to wide salinity gradients. For this reason, this species can disperse quite easily from estuarine areas to inland waters. Since *E. affinis* can be a carrier of important pathogens, this ability has attracted researchers' interest to this species. In 2012 a genome sequence project of this species was started (https://www.hgsc.bcm.edu/arthropods/eurytemora-affinis-genome-project); the ~ 7K assembled contigs are available on the National Center for Biotechnology Information (NCBI) website under the ID 17731 (https://www.ncbi.nlm.nih.gov/genome/17731) where BLAST searches can be performed. A publicly accessible genome browser with automated annotation can be found at https://apollo.nal.usda.gov/euraff/jbrowse. The size of the genome was estimated to 0.6-0.7 pg DNA/cell (~587-685 Mb, assembly reaches ~ 500Mb) with a CG content of 35.8%.

This newly available resource has enabled a very elegant work on chemosensory-related gene families in arthropods, with particular focus on copepods (Eyun *et al.*, 2017). Most interesting was the discovery of gustatory receptors (GRs) in Pancrustacea and that in *E. affinis* (that contains 10 GR genes) they are poorly expressed like in insects. Also, it was demonstrated that Ionotropic Receptors (IRs) were differentially expressed in sexes. This finding could give an indication of their function. Moreover, it was found a gene family duplication of antenna IRs in males that would be a way to over-express these genes during mating.

CONCLUSION

The abovementioned studies on large-scale gene expression profiling, genomics, metabolomics and proteomics performed on planktonic calanoids have significantly improved our understanding of the mechanistic processes underlying fundamental biological and ecological issues in copepods (reproduction, molting, diapause, xenobiotic detoxification, ocean acidificiation and global warming). In the future, it is important that

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these analytical techniques would be integrated to better predict the species' response to changing environmental conditions, evaluate ecosystem health and environmental risk, and improve food security and nutrition. One of the main constraints limiting the full exploitation of high-throughput copepod transcriptomic studies is the lack of routine protocols for genetic transformation of copepods. These techniques will allow a better understanding of the functions of the newly sequenced genes. Such reverse-genetic tools have been already developed for the model crustacean *Daphina magna*, where exogenous RNA was injected into ovulated eggs to achieve specific gene silencing by RNA interference (RNAi) method (Kato *et al.*, 2011). Posttranscriptional gene silencing by RNAi method have been developed in *Lepeophtheirus salmonis* for identification of molecular targets of new drugs or vaccines for management of aquaculture fish parasites (Eichner *et al.*, 2014), and in *Tigriopus californicus* for testing the role of genes putatively involved in thermal adaptation (Barreto *et al.*, 2014.). In the future, we envision this approach could be also applied to other key calanoid species (*i.e.*, *Calanus*), to better elucidate the role of specific gene functions in copepod physiology and ecology, and also to generate transgenic individuals for biotechnological applications.

In conclusion, functional genomics studies in marine free-living copepods have grown in number since 2011, but the way to go is still very long. Only two genomes are currently available for planktonic calanoids and this slows down functional approaches as well as forward and reverse genetics for those species that are not yet sequenced. Once genomic resources become available for a wider number of copepods and different molecular tools will be routinely applied, the biological, ecological, ecotoxicological and eventually biotechnological research will take major steps further. Some species can then become models for marine invertebrates.

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I wish to express my personal gratitude to all the Reviewers who accepted to examine the chapters included in the present volume. They all did an excellent job, and their comments were invaluable in improving the overall quality of the book.

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INDEX

Complimentary Contributor Copy

B

C

Complimentary Contributor Copy

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Dr. Marco Uttieri received his Laurea degree (*magna cum laude*) in Environmental Sciences in 2001 from the University of Naples Parthenope (Italy). In 2006 he was granted a PhD in Dynamics of Marine Ecosystems by the Open University (UK). Dr. Uttieri is presently a researcher at the Stazione Zoologica Anton Dohrn of Naples (Italy); from 2012 to 2015 he was appointed as a researcher at the Department of Science and Technology of the University of Naples Parthenope. While there, he was also the professor of Fundamentals of Biology and Biodynamics of the Water Column, as well as member of the board of doctoral courses. Dr. Uttieri worked as a Principal Investigator on the MOKA (Modelling and Observation of zooplanKton Organisms) project, financed by the Italian Ministry of Education, University and Research (MIUR), and was responsible of two research units in the Flagship Project RITMARE - The Italian Research for the Sea. Dr. Uttieri is the author of 28 articles and 7 book chapters indexed in Scopus.

“Copepods are for aquatic systems what insects are for terrestrial – successful, widespread, and a key link in the food web. Yet knowledge, as well as textbooks, on copepod ecology is scarce. Therefore *Trends in Copepod Studies – Distribution, Biology and Ecology* is very timely and covers a wide array of subjects, including long-term population trends, predator-prey dynamics, neurology, chemical ecology, invasiveness, as well as the inclusion of copepod genetics into the new molecular, or –omics, era. The broad coverage, as well as the many insightful in-depth excursions into copepod ecology, together make this book warmly recommended reading for researchers and advanced students, as well as for devoted laymen.”

Professor Lars-Anders Hansson
Department of Biology/Aquatic Ecology
Lund University, Sweden

“New insights on a range of current topics in copepod biology and ecology are provided in the twelve chapters. This work will attract wide interest and stimulate future directions for research”.

Professor Roger Harris
Plymouth Marine Laboratory

"Scientists involved in copepod studies will find in this book a broad avenue to enhance their knowledge on this important and ever-growing research topic. The book's scope, the range of data explored, and the large reputation of chapter's contributors are an invitation to read and appreciate this essential contribution to the aquatic sciences".

Professor Rubens M. Lopes
Oceanographic Institute
University of São Paulo, Brazil

“The contents of this new book *Trends in Copepod Studies – Distribution, Biology and Ecology* span a variety of scientific approaches developed to study the biology and ecology of copepods. The volume offers a very interesting updated expertise dealing with the diversity of topics studied by copepodologists and confirm the key role of copepods in ecosystem studies”.

Professor Sami Souissi
Laboratoire d’Océanologie et de Géosciences (LOG)
Station Marine de Wimereux, Université de Lille, France

Front images: *Calanus helgolandicus* (L. Svetlichny); *Acartia tonsa* (Gemmell and Buskey, Chapter 10); CPR samples collected between 1985 and 2015 (Wootton *et al.*, Chapter 2).

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