On the presence of *Phalacroma rotundatum* in the southern Adriatic Sea (Italy)

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ABSTRACT: The genus *Dinophysis sensu lato* is often involved in episodes of Diarrhetic Shellfish Poisoning (DSP) contaminations and human intoxications. To date, several species of this genus have shown toxin production in the laboratory; however, it is likely that many others could have this capability. *Phalacroma rotundatum* (Claparède & Lachmann) Kofoid & Michener, allocated by some authors to the genus *Dinophysis*, is a non-photosynthetic species, which has the potential to produce okadaic acid and its derivatives DTX-1 and DTX-2. This species was observed for the first time along the southern Adriatic coast of Puglia (Italy) during a 2 yr monitoring program on potentially toxic phytoplankton. *P. rotundatum* dynamics was studied in relation to hydrobiological conditions; its seasonal changes appeared rather independent from chemico-physical factors, but were significantly associated with those of phytoflagellates. A toxicity test (Microtox system) on *P. rotundatum* cells proved negative.

KEY WORDS: *Phalacroma rotundatum* · Phytoflagellates · Hydrobiological factors · DSP · Adriatic Sea

INTRODUCTION

The repeated occurrences of Diarrhetic Shellfish Poisoning (DSP) mussel contaminations in the last few years (Boni 1992, Honsell et al. 1995) in the northern and middle Adriatic coastal waters have led Italian authorities to promote monitoring programs on potentially toxic phytoplankton along the entire Adriatic coastal belt, to collect information about the possible occurrence and abundance of harmful microalgae. Species of the dinoflagellate genus *Dinophysis* are considered the main DSP threat in the areas of the northern and middle Adriatic coasts where algal monitoring activities are carried out (Ammazzalorso et al. 1991, Fattorusso et al. 1992, Boni et al. 1993, Della Loggia et al. 1993, Tubaro et al. 1995).

Phalacroma rotundatum (Claparède & Lachmann) Kofoid & Michener is a dinophysoid non-photosynthetic species, whose genus name is often considered synonymous with *Dinophysis* (Abé 1967, Balech 1967 in Hallegraeff & Lucas 1988), but recently Hallegraeff & Lucas (1988) have supported the original generic distinction between *Dinophysis* and *Phalacroma* genera, based on physiological and ecological arguments. The morphological features separating the 2 genera: development and direction of the cingular lists in combination with the height and shape of epitheca, have been coupled with the presence or absence of chloroplasts and relative distribution (*Dinophysis*, mostly neritic and photosynthetic, and *Phalacroma*, mostly oceanic and heterotrophic), but there are several exceptions: e.g. *D. schuettii* and *D. hastata*, non-photosynthetic and *P. rapa*, autotrophic species (Hallegraeff & Lucas 1988, Steidinger & Tangen 1997). In addition, the highly variable chloroplast properties and the phagotrophic potential found in several photosynthetic *Dinophysis* species (Jacobson & Andersen 1994) still prevent a clearcut separation between the two and partially support the conclusion of Schnepf & Elbrächter (1988, in Jacobson & Andersen 1994) that the entire dinophysoid lineage is fundamentally heterotrophic and apochlorotic.

Phalacroma rotundatum exhibits food vacuoles in its cytoplasm (Hallegraeff & Lucas 1988, Jacobson & An-

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dersen 1994, Giacobbe 1995) which have been shown by electron microscopy to contain plastids, with internal pyrenoids, and rare prasinophyte scales (Jacobson & Andersen 1994). This heterotrophic species has been observed to feed upon prostomatid (*Tiarina fusus*, Hansen 1991), tintinnid and oligotrich ciliates (Jacobson & Andersen 1994). Hansen (1991) directly described its feeding behaviour via a peduncle (myzocytosis, Schnepf & Deichgraber 1983) extending from the flagellar pore, which fuses to the membrane of the prey and sucks on its cytoplasm.

Production of toxins of the okadaic acid group was detected in cells of *Phalacroma rotundatum* from Japanese waters by Lee et al. (1989) and in the Ria Pontevedra (NW Spain) populations by Blanco et al. (1995), whilst Cembella (1989) and Masselin et al. (1992) considered the populations from eastern coasts of North America and from French coasts, respectively, to be non-toxic. Probably these controversial results are due to regional and temporal factors affecting the toxigenicity of cells of the same species together with the lack of cultures (Blanco et al. 1995, Giacobbe 1995). In Italy, *P. rotundatum* was detected along the northern Adriatic coasts (Boni et al. 1993, Della Loggia et al. 1993, Sidari et al. 1995, Tubaro et al. 1995), along the Ionian coasts of Calabria (Giacobbe et al. 1993, Giacobbe 1995), and in the Strait of Messina (southern Tyrrhenian Sea, Giacobbe 1995).

The present paper reports on a survey on potentially toxic phytoplankton carried out from April 1995 to March 1997 in the southern Adriatic Sea. *Phalacroma rotundatum*, besides several other potentially toxic algal species (Caroppo et al. 1996), was detected during the sampling period. The dynamics of this species was studied in relation to hydrobiological conditions to contribute to knowledge of the distribution of harmful phytoplankton in the Mediterranean Sea.

In order to investigate the possibility of toxin production by the southern Adriatic populations of *Phalacroma rotundatum*, raw and sorted samples were tested on bioassay for the presence of okadaic acid.

STUDY AREA

Whereas the plankton of the northern Adriatic Sea has been much studied, only limited information is available for the southern part, specially with regard to the Italian coasts (Fanuko 1983/84, Marano & Rizzi 1985, Vilicic et al. 1995).

The southern basin of the Adriatic Sea, the deepest of the 3 Adriatic basins, is hydrologically important as it represents the transition zone of different waters: those coming from the northern Adriatic Sea (Adriatic

Surface Water, ASW) and those of the Ionian Sea (Levantine Intermediate Water, LIW) (Buljan & Zore-Armanda 1976). Ionian water originates in the Levantine Sea; in wintertime the low temperature (15°C) and high salinity (39.1‰) produce water with a high density that sinks and spreads at intermediate depths (200 to 600 m) and invades the southern Adriatic basin, between 40 and 150 m in depth (Magazzù pers. comm.). The gradient of density formed between the Adriatic (ASW) and the Ionian water (LIW) produces a circulation which moves southwards along the western coast in summer, and northwards along the eastern coast in winter. In spring and autumn the inflow and outflow intensities are similar (Zore-Armanda 1968).

The analyzed waters are essentially oligotrophic with reference to their inorganic nitrogen (NH_4^+ + NO_2^- + NO_3^-)-N and phosphorus (PO_4^{3-}-P) content, with averaged values of 0.9 and 0.1 µg l^{-1} respectively (Cardellicchio pers. comm.). The Ionian flow seems to be the major nutrient supply to the surface waters of the southern basin (Buljan et al. 1975, Cescon & Scarazzato 1979). The annual trend of the temperature in these waters is sinusoidal with wide variations between the winter minimum (8 to 10°C) and the summer maximum (28 to 29°C) (Marano pers. comm.). The dissolved oxygen shows an inverse sinusoidal trend to the thermal one, varying between 6.27 and 8.54 mg l^{-1}. The surface values often show marked oversaturation, while the rest of the water column does not fall below 84 % (Bregant et al. 1992, 1994). Chlorophyll a (chl a) values are consistently below 0.5 µg l^{-1} and rates of primary production lower than 1 µg C l^{-1} h^{-1} (Faganeli et al. 1989).

The coastal tract under investigation is tall and characterized by the presence of limestone sediments from the Mesozoic (Brambati 1992) and by the lack of remarkable waterways.

MATERIALS AND METHODS

Monthly samples were collected at 4 stations located 0.5 km (Stn 1), 3 km (Stn 2), 5 km (Stn 3) and 10 km (Stn 4) from the coast, along 4 transects: (a) Brindisi; (b) S. Cataldo; (c) Otranto and (d) Santa Maria di Leuca (Fig. 1). Samples were taken at 4 discrete depths (0, 5, 10, 20 or 50 m, total depth allowing) at each station, using a 5 l Niskin bottle.

Transparency was measured by Secchi disk. Temperature, salinity, dissolved oxygen and pH were recorded by an Idronaut Ocean Seven 501 multiprobe and compared to *in situ* (electronic thermometers) and laboratory (Guildline salt-metre, Autosal 8400 B, Winkler and pH-metre) measurements. Nutrient analyses

(NH_4^+-N, NO_2^--N, NO_3^--N, PO_4^{3-}-P, SiO_4^{2-}-Si) were estimated according to Strickland & Parsons (1972); chl *a* was determined by the Parsons et al. method (1984).

Phytoplankton analyses were performed on 50 to 100 ml subsamples, preserved with Lugol's iodine solution, allowed to settle for 24 to 48 h and examined for phytoplankton and *Phalacroma* cell counts (Utermöhl 1958), using an inverted light microscope at 200× and 400× magnification. For species identification, the principal taxonomic texts were those indicated in Zingone et al. (1990). Algae which could not be identified either to the species or the genus level were counted as: small (<10 μm) and large (>10 μm) phytoflagellates, small (<15 μm) and large (>15 μm) naked or thecate dinoflagellates. A distinction between photosynthetic and non-photosynthetic species was made using the information available in the literature (Chrétiennot-Dinet 1990, Larsen & Sournia 1991). *Phalacroma* identification was based on: Dodge (1982), Rampi & Bernhard (1980), Sournia (1986), Steidinger & Tangen (1997), Schiller (1931–33), Taylor et al. (1995). Correlation coefficients between hydrobiological data and *Phalacroma* cell concentration were computed and significance was tested by the Student's *t*-test.

The toxicity of the species was tested as follows: a 10 mg aliquot of centrifuged fresh algae, obtained by concentrating the bottle sample showing the highest *Phalacroma rotundatum* density (1460 cells l^{-1}), was extracted with 1 ml MeOH 90 %. In addition, 650 cells of *P. rotundatum* were picked up from a net sample, harvested in April 1998 by repeated vertical net tows (20 μm mesh), and extracted as above. The solution was stirred, sonicated (5 min at 30 to 40°C), then centrifuged and the supernatant collected. The extraction was repeated on the cell pellet; the resulting supernatants were pooled and tested using the Microtox system (Microbics, Inc.).

The bacterial bioassay Microtox system is based on 4 progressive dilutions of the extracted sample which are put in contact with equal concentrations of the phosphorescent marine bacterium *Vibrio fisheri*. The presence of toxins in the sample gives a diminution in the lightening of the microrganisms, and the values read by luminometer of the system, statistically processed by a software computer program in a trend with confidence limits, give the extinction coefficient value at 50 % of the bacterial lightening. The system was tested in the laboratory with various algal toxins, among which was okadaic acid (Gucci et al. 1989, Bruno et al.1990, Bruno et al. 1994).
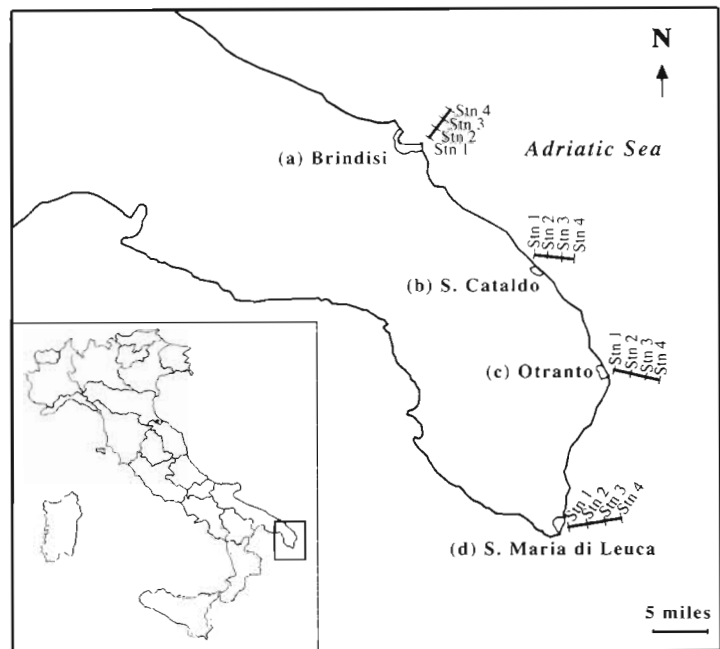

Fig. 1. Map of the study area with sampling stations and transects

RESULTS

Phalacroma rotundatum densities

Phalacroma rotundatum (Claparède & Lachmann) Kofoid & Michener was recorded for the first time in these southern Adriatic waters. It occurred over the whole sampling area, mainly in the autumn-winter months, at moderate densities. Light microscopic observations of specimens revealed cells round-oval in shape, 45 to 53 μm in width and 45 to 55 μm in length, with laterally visible, domed, epitheca and, occasionally, with large globules in the cytoplasm that could be interpreted as food vacuoles (Fig. 2a,b).

Phalacroma rotundatum seasonal appearence was characterized by slight fluctuations in the entire area with abundances ranging from 160 cells l^{-1} (S. Cataldo and Otranto, inner stations, surface layer, July 1995) to 1460 cells l^{-1} (S. M. Leuca, Stn 4, surface layer, February 1997). Annual distribution of *P. rotundatum* densities was similar in the 2 yr of survey.

Maximal densities were observed within the 0 to 10 m depth layer, but vertical cell segregation was not marked (Fig. 3). Distance from the coastline appeared to slightly affect the distribution of cells, which occurred more frequently at the outer stations, especially during the first year of the survey (Fig. 4). The highest frequencies were observed along the southern transects of Otranto and S. M. Leuca.

In spring *Phalacroma rotundatum* occurred sporadically along the transects of S. Cataldo, Otranto and S.
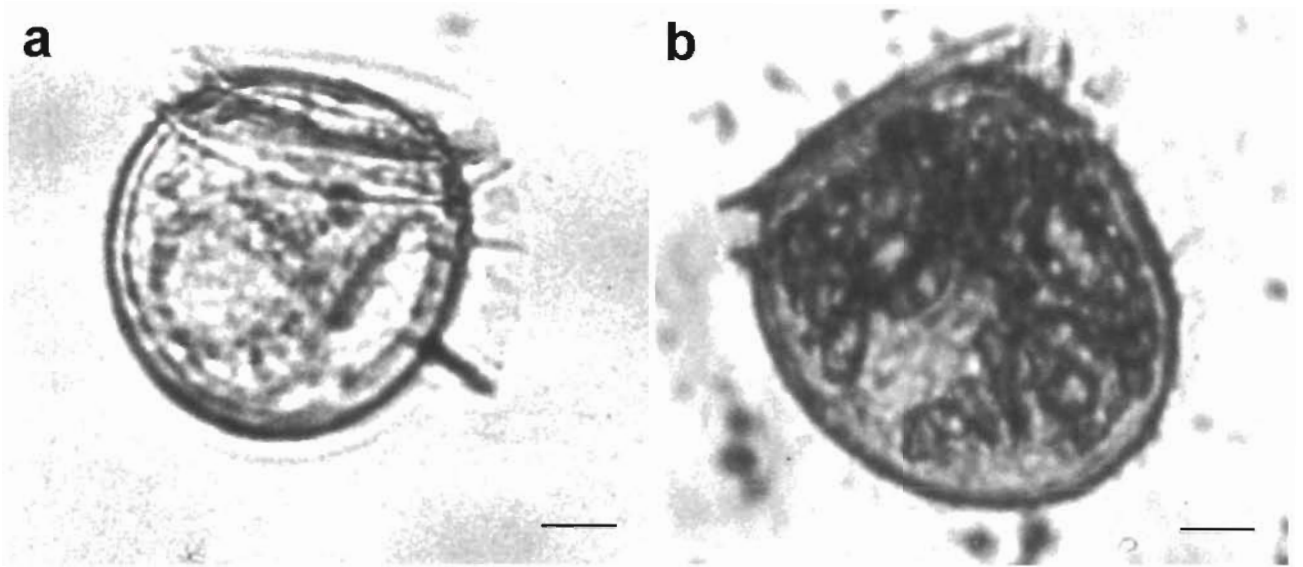
Fig. 2. *Phalacroma rotundatum.* (a) Cell from the sampling area. (b) Cell containing food vacuole-like globules. Scale bars = 10 μm
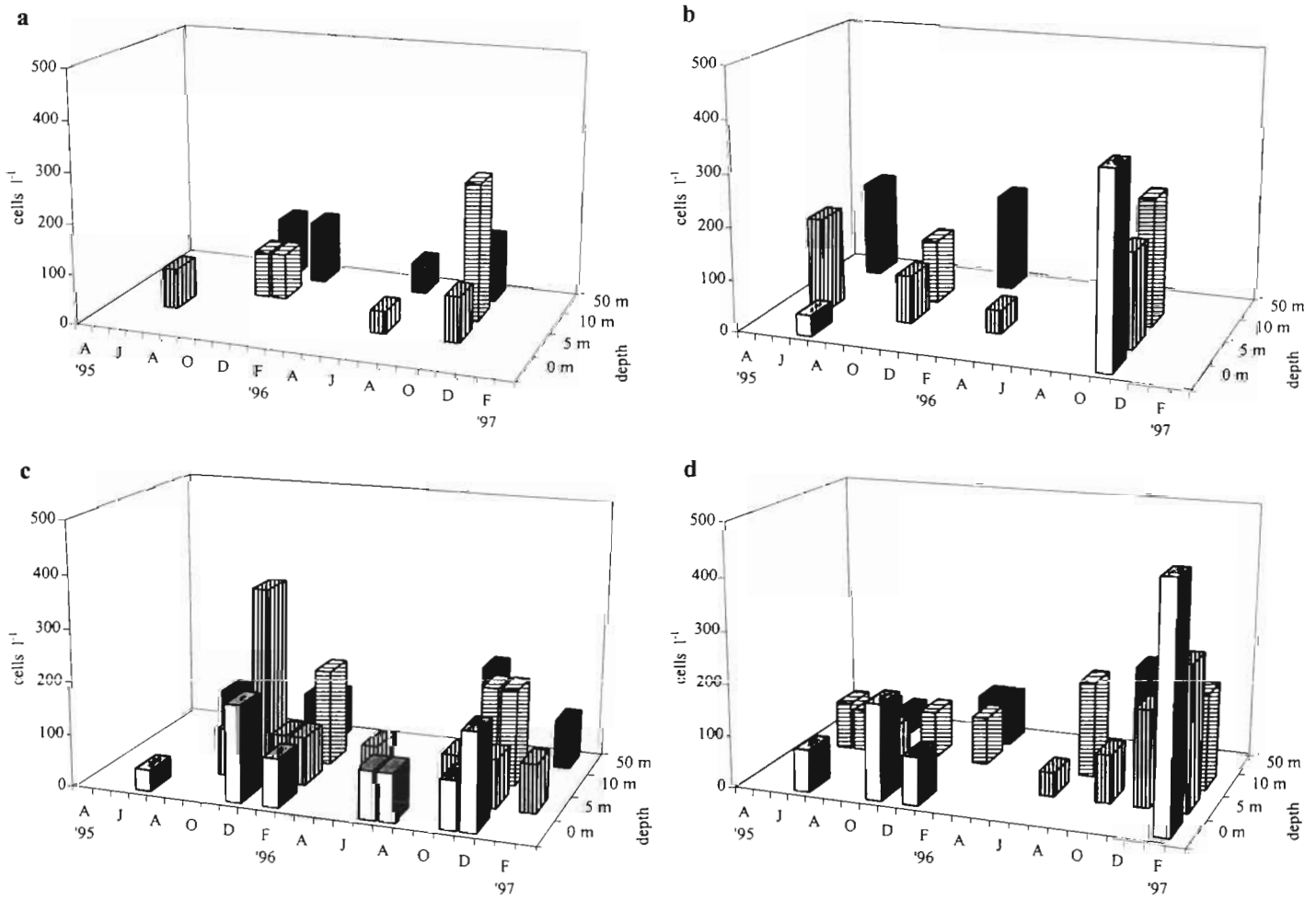

Fig. 3. *Phalacroma rotundatum.* Monthly variations of average concentrations at each depth of the examined stations and transects. (a) Brindisi, (b) S. Cataldo, (c) Otranto, (d) S. M. Leuca

M. Leuca except in May, when it was not detected at any stations. At S. Cataldo, this species was present mainly at the outer stations, with densities ranging from 180 cells l^{-1} (Stn 3, 5 m, April 1996) to 730 cells l^{-1} (Stn 4, 5 m, June 1995), whereas along Otranto and S. M. Leuca transects it was recorded once only, with the abundance of 365 cells l^{-1}, at Stns 4 (5 m, June 1996) and 2 (10 m, June 1995), respectively.

During the summer period, there was a general increase in cell numbers, more evident at Otranto and S. M. Leuca, although abundances were maintained at moderate levels: from 160 to 365 cells l^{-1}. At Brindisi, *Phalacroma rotundatum* was observed at Stns 1, 2 and 4, always in July, at depths of 5 m (up to 320 cells l^{-1}, Stn 1, 1995) and 50 m (180 cells l^{-1}, Stn 4, 1996). The only report of *P. rotundatum* along S. Cataldo was in July 1995 at Stn 1 (0 m, 160 cells l^{-1}). In the southern areas a wider distribution of this dinoflagellate was

observed. At Otranto *P. rotundatum* showed the highest concentrations within the surface layer (up to 365 cells l^{-1}, July and August 1996) and was also recorded once at 50 m (320 cells l^{-1}, Stn 3, July 1995). At S. M. Leuca it occurred at all depths, with maximal densities at 10 m. In September *P. rotundatum* was not recorded at any sampling sites.

In autumn, this species was observed with higher frequencies at all transects, stations and depths, especially in November. Along Brindisi and S. Cataldo transects *Phalacroma rotundatum* was detected mainly in November, when the maximum values (730 cells l^{-1}, Stn 1, 10 m, 1996) were reached. At Otranto and S. M. Leuca this species occurred in similar densities and more frequently.

In winter, frequencies declined at the northern transects where *Phalacroma rotundatum* occurred once only, in January and February 1995 respectively, both
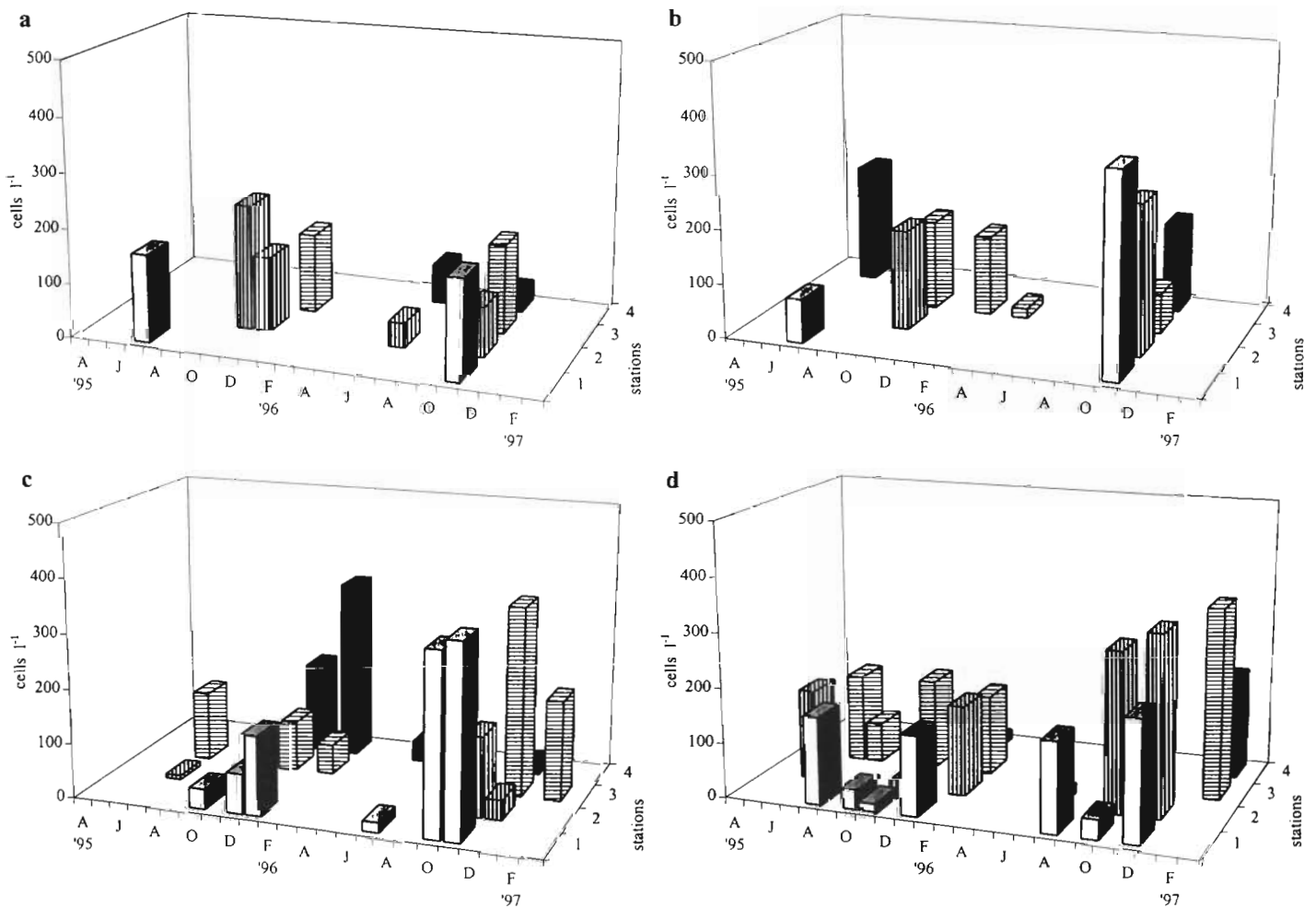

Fig. 4. *Phalacroma rotundatum*. Monthly variations of average concentrations at each station of the examined transects. (a) Brindisi, (b) S. Cataldo, (c) Otranto, (d) S. M. Leuca. The values are reported as 0 to 50 m integrated values
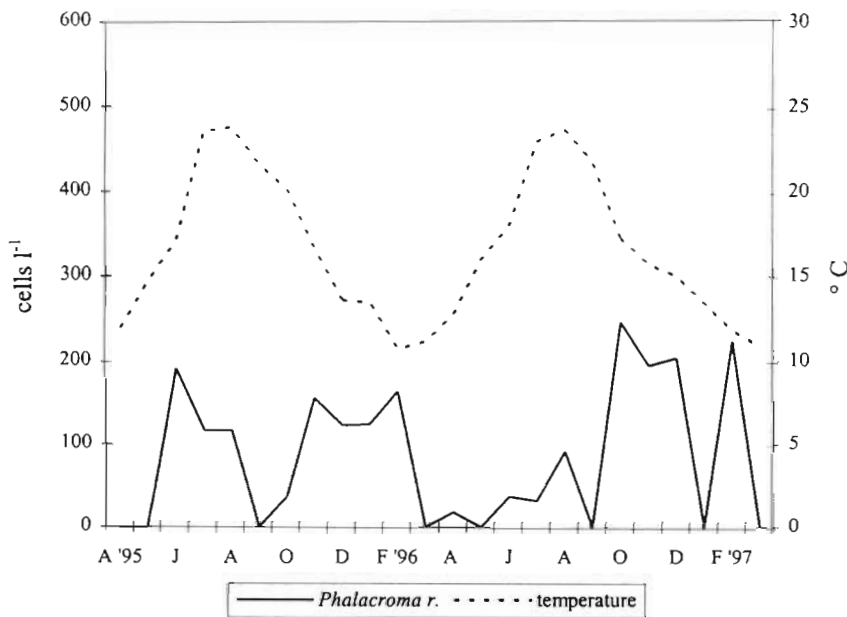
Fig. 5. *Phalacroma rotundatum*. Temporal trends of densities and water temperature averaged for all stations and transects
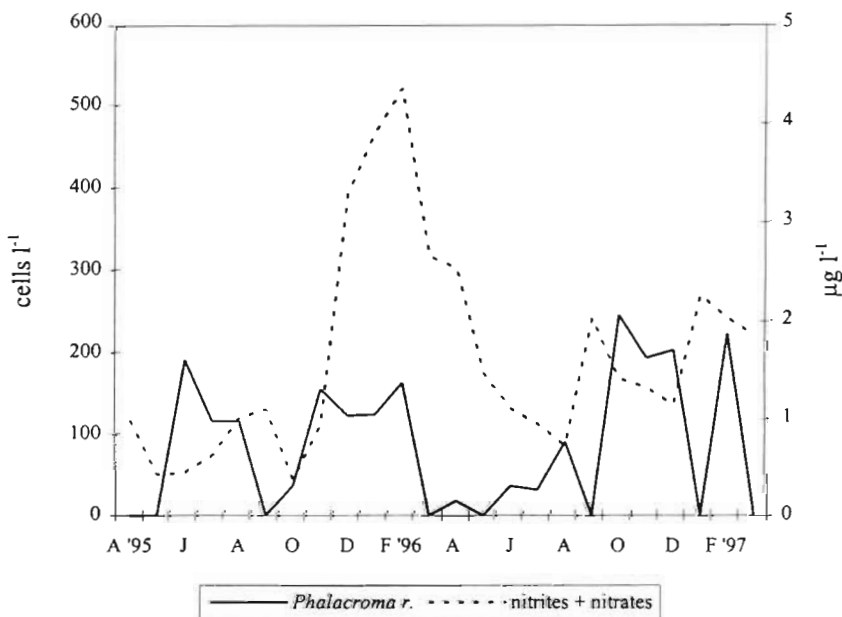

Fig. 6. *Phalacroma rotundatum*. Temporal trends of densities and N (nitrite + nitrate) concentration averaged for all stations and transects

at S. M. Leuca (Stn 4, 0 m, 1997). Then, in March, this species disappeared throughout the water column.

The Microtox assay gave negative results.

Abiotic and biotic factors

The average temperature had a seasonal cycle characterized by a minimum of 10.2°C in February and a maximum of 26.9°C in July. Water warming began in May, with temperature over 16°C, and peaked in July-August, when a sharp thermocline was established. Water stratification came to an end in November, with thermal homogeneity throughout the column, then in winter (January and February), when the cold ASW prevailed for its move southwards, a thermal inversion, particularly detectable along Brindisi and S. Cataldo transects, was observed again. At the southern transects this thermal inversion occurred with higher temperature values (13.8°C) probably due to the ingression of the LIW (Fiocca et al. 1998). Salinity reached minimal values (36.9 to 37.7‰) in winter. Dissolved oxygen ranged from 98.7% (January 1996) to 129.7% (December 1996).

N and P distribution and concentration were widely influenced by the global circulation in the basin and the higher values were reached in winter: NO_2^--N and NO_3^--N reached their maximum levels, of 1.35 (February) and 4.27 µg l^{-1} (January) respectively, at Otranto in 1996. NH_4^+-N was present at very low concentrations, ranging between 0.01 and 0.84 µg l^{-1} (S. Cataldo, August 1995), without significant fluctuations throughout the year. Also $PO_4^{3-}-P$ levels were low, at times undetectable, and quite constant, with average values below 0.1 µg l^{-1}. In the winter months the highest $SiO_4^{2-}-Si$ concentrations were detected too, which ranged from 0.31 µg l^{-1} (Otranto, October 1996) to 7.23 µg l^{-1} (S. M. Leuca, January 1996).

Phalacroma rotundatum appeared rather independent from thermal conditions as supported by correlation analysis (Table 1) and the only correlation be-

with the abundance of 365 cells l^{-1} and at the same station and depth (Stn 3, 50 m), whereas at Otranto and S. M. Leuca this species was present at all the sampling stations and depths with significant densities. The highest annual densities, both during the first and second year of the study, were observed in February: 730 cells l^{-1} at Otranto (Stn 4, 10 m, 1996) and 1460 cells l^{-1}

Table 1. *Phalacroma rotundatum*. Correlation coefficients and Student's *t*-test for dependent samples between densities and hydrobiological data
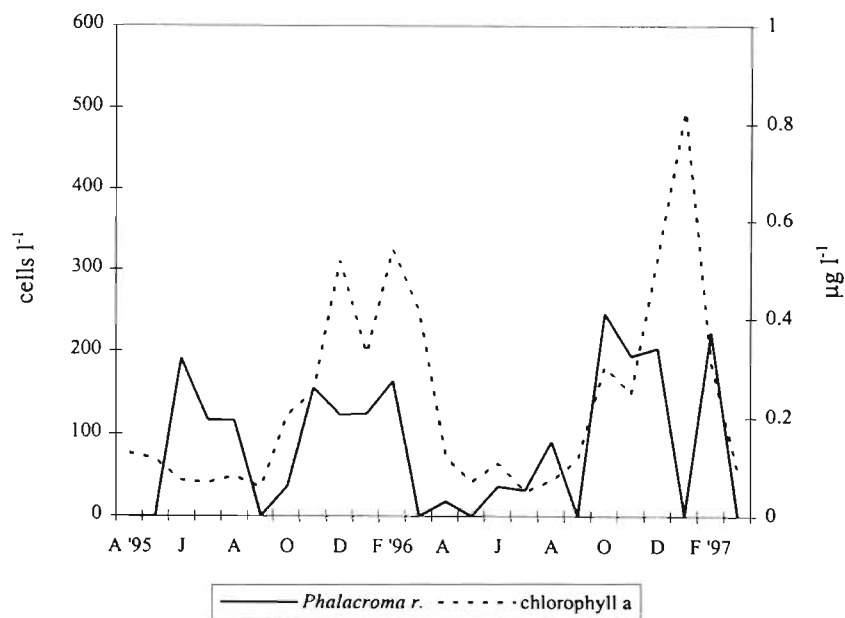
	(a) Brindisi				(b) S. Cataldo				(c) Otranto				(d) S. M. Leuca			
	r	p	*t*	p	r	p	*t*	p	r	p	*t*	p	r	p	*t*	p
Temperature	−0.26	ns	−5.35	<0.01	0.09	ns	−3.15	<0.05	−0.58	ns	−3.79	<0.01	−0.57	ns	−5.13	<0.01
Nitrites + nitrates	−0.60	ns	−6.16	<0.01	0.01	ns	−4.31	<0.01	0.38	ns	−4.42	<0.01	0.13	ns	−5.95	$<10^{-4}$
Chlorophyll *a*	0.20	ns	−6.25	<0.01	0.68	ns	−4.35	<0.01	0.76	<0.05	−4.45	<0.01	0.65	<0.05	−6.00	$<10^{-4}$
Diatoms	0.33	ns	3.17	<0.05	−0.10	ns	1.32	ns	0.30	ns	2.38	<0.05	−0.17	ns	3.78	<0.01
Dinoflagellates	−0.05	ns	4.25	<0.01	−0.41	ns	1.83	ns	0.23	ns	2.01	ns	0.12	ns	3.81	<0.01
Coccolithophorids	−0.35	ns	2.79	<0.05	−0.74	ns	2.25	ns	0.13	ns	3.06	<0.05	0.48	ns	7.15	$<10^{-4}$
Small phytoflagellates	0.96	<0.05	4.73	<0.01	0.80	<0.05	5.40	<0.01	0.69	<0.05	14.64	$<10^{-4}$	0.54	ns	7.32	$<10^{-4}$
Large phytoflagellates	0.58	ns	3.38	<0.05	0.41	ns	3.30	<0.05	−0.41	ns	4.47	<0.01	−0.19	ns	3.70	<0.01
Total phytoflagellates	0.90	<0.05	4.93	<0.01	0.56	ns	5.87	<0.01	0.10	ns	16.39	$<10^{-4}$	0.69	<0.05	2.38	<0.05
Phytoplankton	0.52	ns	4.38	<0.01	−0.34	ns	1.86	ns	0.29	ns	3.36	<0.01	−0.01	ns	7.84	$<10^{-4}$

tween nutrient (NO_2^--N + NO_3^--N) dynamics and *Phalacroma* was found along the Otranto transect during the second year of sampling (r = −0.756, p < 0.05; *t* = −2.976, p < 0.05). PO_4^{3-}-P was not considered in the analysis for its low and constant levels during the investigation, which did not permit any hypothesis as to its role in the dynamics of this species. In Figs. 5 & 6 the trends of *P. rotundatum*, temperature and NO_2^--N + NO_3^--N are represented, averaged for all transects.

Phytoplankton biomass (chl *a*) was correlated to phytoplankton densities (r = 0.59, p < 0.05; *t* = −4.63, p < 0.01) and was quite low, with mean values ranging from a minimum of 0.05 µg l⁻¹ (S. M. Leuca, July 1995) to a maximum of 1.25 µg l⁻¹ (S. Cataldo, January 1997); chl *a* concentration showed a seasonal trend with the highest values in winter (Fig. 7), more limited for S. M. Leuca transect (max value, 0.65 µg l⁻¹, January 1997). *Phalacroma* was significantly correlated to phytoplankton biomass along the southern transects (Otranto and S. M. Leuca) (Table 1).

Average phytoplankton densities showed generally low values, within 1 × 10⁵ cells l⁻¹, with the exception of winter levels in both years of investigation when, during the yearly blooming, average values of 3.3 × 10⁵ cells l⁻¹ (February 1995) and 10.4 × 10⁵ cells l⁻¹ (January 1997) were recorded.

As to quality, diatoms represented the larger component in the population (44.3%) together with phytoflagellates (39.6%); dinoflagellates and coccolithophorids, on the other hand, accounted for 10.3 and 5.8% respectively. During the year diatoms were dominant with high cell densities in the autumn-winter period,

Fig. 7. *Phalacroma rotundatum*. Temporal trends of densities and chlorophyll *a* concentration averaged for all stations and transects

phytoflagellates had a larger distribution over time and their average values varied between 4 and 55 × 10³ cells l⁻¹. They were mainly represented by small (68.5%) and large (17.8%) forms of uncertain taxonomic classification and cryptophyceans (8.3%), whereas the 'others' (prasino-, chryso-, euglenophyceans and silicoflagellates) accounted for only 5.4%. Phytoflagellates reached the minimum percentage at Brindisi (36.1%) against S. Cataldo (38.8%), Otranto (40.9%) and S. M. Leuca (43.6%) transects. In particular, at Brindisi, they were observed mainly in spring (41.4%) and autumn months (44.8%) while in the other transects they were detected mainly in summer (S. M. Leuca, 56.8%; Otranto, 53.7%; S. Cataldo, 49.5%).
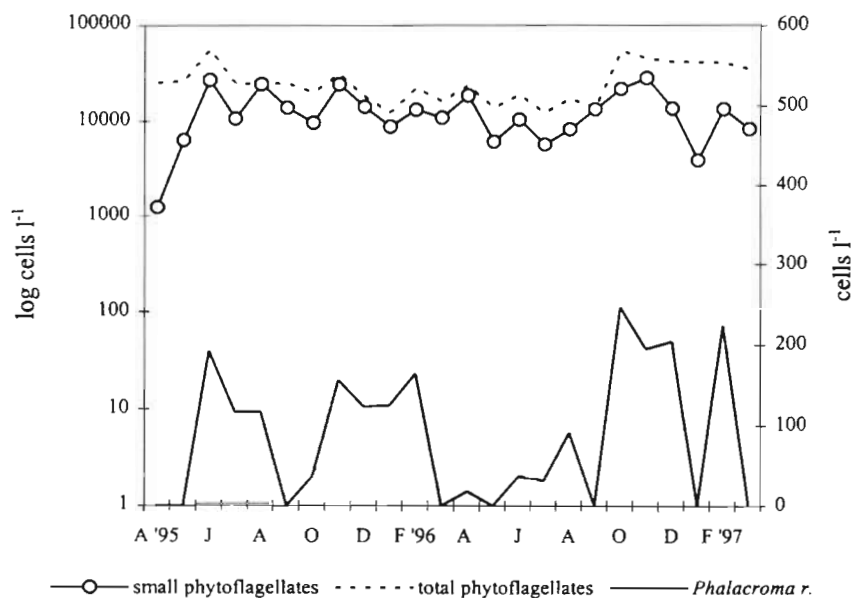
Fig. 8. Temporal trends of total phytoflagellates and small phytoflagellates (log scale) and *Phalacroma rotundatum* densities averaged for all stations and transects

Phalacroma rotundatum seasonal variations were significantly correlated with those of phytoflagellates, specially with the small form ones. This feature was evidenced analyzing spatially pooled distributional data (Fig. 8) and, more in detail, considering the correlation coefficients between the spatio-temporal patterns of *P. rotundatum* and those of phytoflagellate populations at the individual sampling transects (Table 1).

DISCUSSION

The seasonal occurrence of *Phalacroma rotundatum* in the southern Adriatic basin was similar in the 2 different years of the survey, showing a rather wide temporal distribution in both cases, although in low numeric densities. Thus this species seemed to show a certain adaptability to environmental variations throughout the year, as observed by Sidari et al. (1995) in the Gulf of Trieste (NW Adriatic Sea). On that occasion, *P. rotundatum* maximum abundances and frequencies were detected in spring, whereas in the southern Adriatic this dinoflagellate has occurred in the autumn-winter months. Both these findings could suggest a preference of *P. rotundatum* for conditions of high turbulence and active water mixing. This latter aspect could also explain the more frequent observations of *P. rotundatum* in the winter period along the more southerly transects, where a greater incidence of LIW would contribute to the development of denser

populations of this species, different from northern transects where the effects of ASW seem to prevail.

Particularly interesting was the association of *Phalacroma rotundatum* with the phytoflagellate assemblage, mainly with the nanoplanktonic component. The almost overlapping distributional patterns of this species and those of phytoflagellates seemed to go beyond a simple simultaneous fluctuation in the phytoplankton community. Although no experimental evidence yet exists to support a trophic link between this heterotrophic dinophysoid and phytoflagellates, a similar coincidence in the temporal cycles of *Dinophysis* spp. and *Tiarina fusus* was evidenced by Hansen (1991) when describing *Dinophysis rotundata* predation upon that prostomatid ciliate. It is known that phagocytosis of ciliates and other protists by marine dinoflagellates is a widespread phenomenon, both among heterotrophic (aplastidic) and mixotrophic (photosynthetic) taxa (Jacobson & Anderson 1996) which can, through a more or less selective mechanism (Jacobson & Anderson 1986), complicate the food webs in plankton communities. Further studies are needed to elucidate growth requirements of *P. rotundatum* and the role played by the phytoflagellates on its population dynamics.

The negative results of the Microtox bioassay, both on raw and sorted samples of the southern Adriatic *Phalacroma rotundatum* populations agree with the findings of Cembella (1989) and Masselin et al. (1992), but are contrary to those of Lee et al. (1989) and Blanco et al. (1995), indicating that further assays are needed to clarify the toxin production of this species and the possible relationships with its heterotrophic metabolism and spatio/temporal variations.

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