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NOTE

Considerations on the toxigenic nature and prey sources of *Phalacroma rotundatum*

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ABSTRACT: The heterotrophic dinoflagellate *Phalacroma rotundatum* (Claparède & Lachman) Kofoid & Michener is considered a toxic species, but there is controversy about its toxigenic nature. In the present study, about one-third of the toxin analyses done with liquid chromatography-mass spectrometry (LC-MS) of *P. rotundatum* specimens picked from field populations in Galicia (NW Spain) between 2003 and 2005 revealed traces of lipophilic toxins — okadaic acid (OA) and/or dinophysistoxin-2 (DTX2) and/or pectenotoxin-2 (PTX2) (if any) — that mimicked the toxin profile of co-occurring toxigenic mixotrophic species of *Dinophysis* (*D. acuminata*, *D. acuta*, and *D. caudata*). Thus, during the period of study, *P. rotundatum* was never a relevant contributor to diarrhetic shellfish poisoning (DSP) toxins contaminating shellfish resources in Galicia. Observations of phycoerythrin-like autofluorescence in *P. rotundatum* and in its co-occurring potential ciliate prey — *Tiarina* cf. *fusus* — led to the suspicion that *P. rotundatum* had taken up toxins by feeding on this ciliate prey that had previously fed on *Dinophysis* spp. Nevertheless, toxins in *P. rotundatum* specimens with orange autofluorescence were under detection levels, and the source of these orange pigments may be a prey different from *Dinophysis* spp. (e.g. *Myrionecta* spp.). New results here add evidence to suggest that *P. rotundatum* does not produce toxins *de novo*, but acts as a vector from toxin-containing prey to shellfish, and that *M. rubra* may be one of its potential ciliate prey. Conclusive testing of these hypotheses is now under investigation with laboratory cultures of *Dinophysis* and *Phalacroma* spp. and *M. rubra*.

KEY WORDS: *Phalacroma rotundatum* · *Dinophysis* spp. · Diarrhetic shellfish poisoning · DSP · Pectenotoxins · PTX · Lipophilic shellfish toxins

INTRODUCTION

Phalacroma rotundatum (Claparède & Lachman) Kofoid & Michener (= *Dinophysis rotundata*) is a heterotrophic dinoflagellate widely distributed in cold to warm waters (Hallegraeff & Lucas 1988, Larsen & Moestrup 1992, Jensen & Daugbjerg 2009) and usually reported to occur at low cellular densities (1×10^2 to 2×10^2 cells l^{-1}). This species is included in the list of toxin-producing dinophysoids (Zingone & Larsen 2011) on the basis of one single high-performance liquid chromatography analysis with fluorescence detection (HPLC-FD) of cells, isolated from Japanese coastal waters, which revealed a high cellular content (101 pg $cell^{-1}$) of dinophysistoxin-1 (DTX1) (Lee et al. 1989). Different authors were not able to detect any lipophilic

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toxins either in dense net-hauls where *P. rotundatum* was not accompanied by other *Dinophysis* spp. (Cembella 1989, Caroppo et al. 1999) nor in single-cell isolates analyzed by liquid chromatography coupled to mass spectrometry (LC-MS) (Suzuki et al. 2009). In contrast, Miles et al. (2004) showed traces of lipophilic toxins in *P. rotundatum* cells picked from net-haul samples dominated by other *Dinophysis* spp. The possibility of false positives — due to interfering false peaks deriving from fluorescence reagents and biological matrices — has been suggested to explain discrepancies among different cellular toxin content observed in the same species using different analytical methods (Suzuki et al. 2009). An intriguing question concerning the mixotrophic species of *Dinophysis* has been whether the toxins are synthesized *de novo* or are derived from their prey. Recently, *de novo* production of okadaic acid (OA) derivatives and pectenotoxins in the mixotroph *D. acuminata* and the lack of toxins in its prey — the ciliate *Myrionecta rubra* — has been proved in laboratory cultures (Kamiyama & Suzuki 2009).

Phalacroma rotundatum was observed to feed on tintinnid ciliates (Elbrächter 1991) and to become replete of digestive vacuoles after feeding on laboratory cultures of the ciliate *Tiarina fusus* (Hansen 1991). Jacobson & Andersen (1994) observed vacuoles in field specimens of *P. rotundatum* under light (differential interference contrast [DIC]) microscopy. However, toxins were not analyzed by these previous authors.

In the present study, the toxigenic nature of *Phalacroma rotundatum* is questioned on the basis of LC-MS analyses of single-cell isolates of this species and co-occurring *Dinophysis* spp. collected in the Galician Rías Baixas (NW Spain) between 2003 and 2007. It is proposed that *P. rotundatum* does not produce toxins *de novo* but acts as a vector of diarrhetic shellfish poisoning (DSP) toxins and pectenotoxins accumulated in its ciliate prey that had previously fed on co-occurring toxin-producing *Dinophysis* spp.

MATERIALS AND METHODS

Plankton samples. Plankton samples were collected either by vertical net hauls (20 µm mesh; 0 to 20 m depth) or by size-fractioning (20 to 77 µm) through a set of superimposed meshes, between spring and autumn, 2003, 2005, and 2007, at one station from Ría de Pontevedra (Galicia, NW Spain) (Fig. 1) on board the RV 'J. M. Navaz'. Back in the laboratory, *in vivo* autofluorescence of *Dinophysis* spp. was observed under a Nikon Eclipse TE2000-S inverted microscope with epifluorescence, using blue-light excitation (excitation 450 to 490 nm, emission 520 nm long pass) and a short-pass filter set specific to select phycoerythrin fluorescence

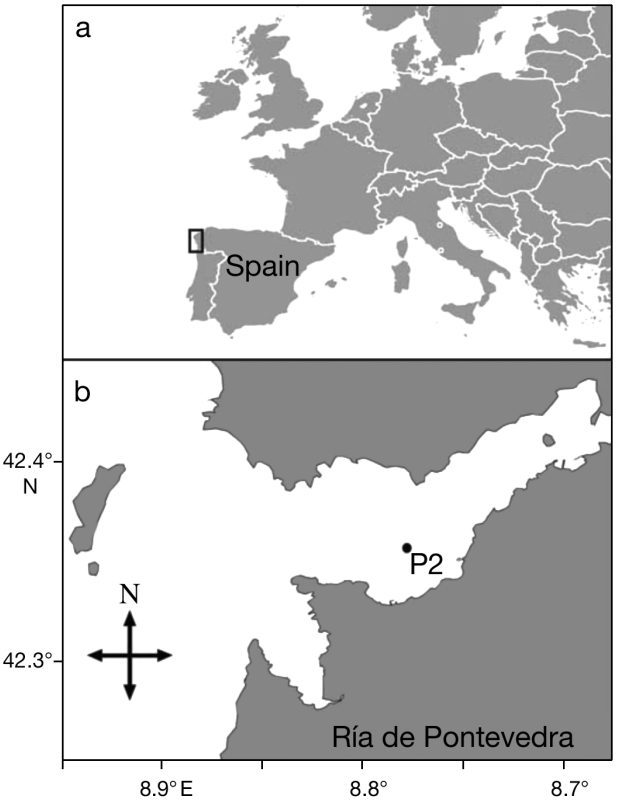

Fig. 1. Location of (a) Ría de Pontevedra (NW Spain) and (b) sampling station (P2)

(excitation 546 nm, emission 585 nm short pass). Nuclear staining was carried out with the DNA-specific fluorochrome SYBR Green I (Molecular Probes). Digitized micrographs (100× and 400× magnification) were taken with a Nikon D70 camera coupled to the microscope.

Cell isolation and extractions for toxin analysis. *Phalacroma rotundatum* cells were isolated one by one by micromanipulation with a microcapillary pipette, transferred 2 to 3 times through sterile seawater, and placed into 1.5 ml Eppendorf tubes with 500 µl methanol. Cells were sonicated for 1 min, another 500 µl of methanol was added, and the extract was frozen at –20°C. Prior to analysis, extracts were dried at 40°C (Speed Vac, Savant Instruments), re-suspended in 150 µl methanol, and filtered (0.45 µm filters, Gelman Nylon Acrodisc 13 mm) before injection into the LC-MS system.

Toxin analysis by LC-MS. Chromatographic separation was performed on a Thermo Finnigan Surveyor with a Waters XTerra C18 column 5 µm (2.1 × 150 mm) at 35°C. The mobile phase consisted of 2 mM ammonium acetate at pH 5.8 (solution A) and 100% methanol (solution B), with a flow rate of 0.2 ml min^{-1}. A linear gradient elution from 60 to 100% of solution B was run for 20 min and re-equilibration with 60% of solution B for 8 min was used. The sample injection volume was

variable (5 to 20 µl) depending on toxin concentration. The spectral measurements were performed using an ion-trap mass spectrometer (Thermo Finnigan LCQ-Advantage) equipped with an electrospray ionization interface (ESI) under the same operating conditions described in Pizarro et al. (2009). Calibration and quantification was performed using OA and pectenotoxin-2 (PTX2) certified reference standards (National Research Council of Canada) and quantified dinophysistoxin-2 (DTX2) (6 µg ml^{-1}) reference material provided by P. Hess (IFREMER, Nantes, France).

RESULTS AND DISCUSSION

Phalacroma rotundatum was present in very low cell densities (< 10^2 cells l^{-1}) and co-occurred with more abundant species of *Dinophysis*, and only on 8 occasions were there enough cells in the plankton concentrate to carry out single-cell isolation of *P. rotundatum*. Five out of 11 picked-cell samples presented detectable levels of lipophilic shellfish toxins (Table 1). Cells isolated from aliquots of the same plankton concentrate, or from plankton samples collected 1 d apart, showed different qualitative results (presence or absence and toxin profile). The selected ion chromatograms and mass spectra of the standards and reference material for OA/DTX2 and PTX2 in positive ion LC–MS analysis are shown in Fig. 2a,b. Within the samples from August 2003, only trace amounts of OA and PTX2 were found in 2 of the samples (the one from 26 August is shown in Fig. 2c,d) while only one showed a small amount of PTX2. This toxin (PTX2) was the only toxin detected in single- cell isolates of the co-occurring

Table 1. *Phalacroma rotundatum* and *Dinophysis* spp. Estimates of toxin content per cell (pg $cell^{-1}$) in picked cells of *P. rotundatum*. Relative abundance (%) of each *Dinophysis* species and of *P. rotundatum* in relation to the total number of toxic species (*Dinophysis* spp. + *P. rotundatum*). DTX2: dinophysistoxin-2; OA: okadaic acid; PTX2: pectenotoxin-2; nd: not detected

Sampling date	No. of cells	OA (pg $cell^{-1}$)	DTX2 (pg $cell^{-1}$)	PTX2 (pg $cell^{-1}$)	Total toxin (pg $cell^{-1}$)	Cell appearance	Relative abundance (%)
25/8/2003	38	nd	nd	0.8	0.8	Normal appearance of vegetative cells	*D. caudata* (66%) *D. acuminata* (16%) *P. rotundatum* (18%)
25/8/2003	40	0.3	nd	0.7	1.0	Normal appearance of vegetative cells	*D. caudata* (66%) *D. acuminata* (16%) *P. rotundatum* (18%)
26/8/2003	70	nd	nd	nd	nd	Small and pale non-vacuolated cells	*D. caudata* (66%) *D. acuminata* (16%) *P. rotundatum* (18%)
26/8/2003	50	nd	nd	nd	nd	Small and pale non-vacuolated cells	*D. caudata* (66%) *D. acuminata* (16%) *P. rotundatum* (18%)
26/8/2003	66	0.5	nd	0.1	0.6	Small and pale non-vacuolated cells	*D. caudata* (66%) *D. acuminata* (16%) *P. rotundatum* (18%)
26/7/2005	48	nd	nd	nd	nd	Normal appearance of vegetative cells	*D. acuminata* (82%) *P. rotundatum* (18%)
26/9/2005	51	0.7	0.9	nd	1.6	Normal appearance of vegetative cells	*D. acuminata* (60%) *P. rotundatum* (40%)
17/4/2007	142	nd	nd	nd	nd	100% vacuolated cells, some showing orange autofluorescence	*P. rotundatum* (100%)
29/10/2007	58	nd	nd	nd	nd	Vacuolated cells, some showing orange autofluorescence	*D. acuminata* *D. acuta*
29/10/2007	52	nd	nd	nd	nd	Vacuolated cells, some showing orange autofluorescence	*D. acuminata* *D. acuta*
3/12/2007	50	nd	nd	nd	nd	Normal appearance of vegetative cells	*P. rotundatum* (100%)

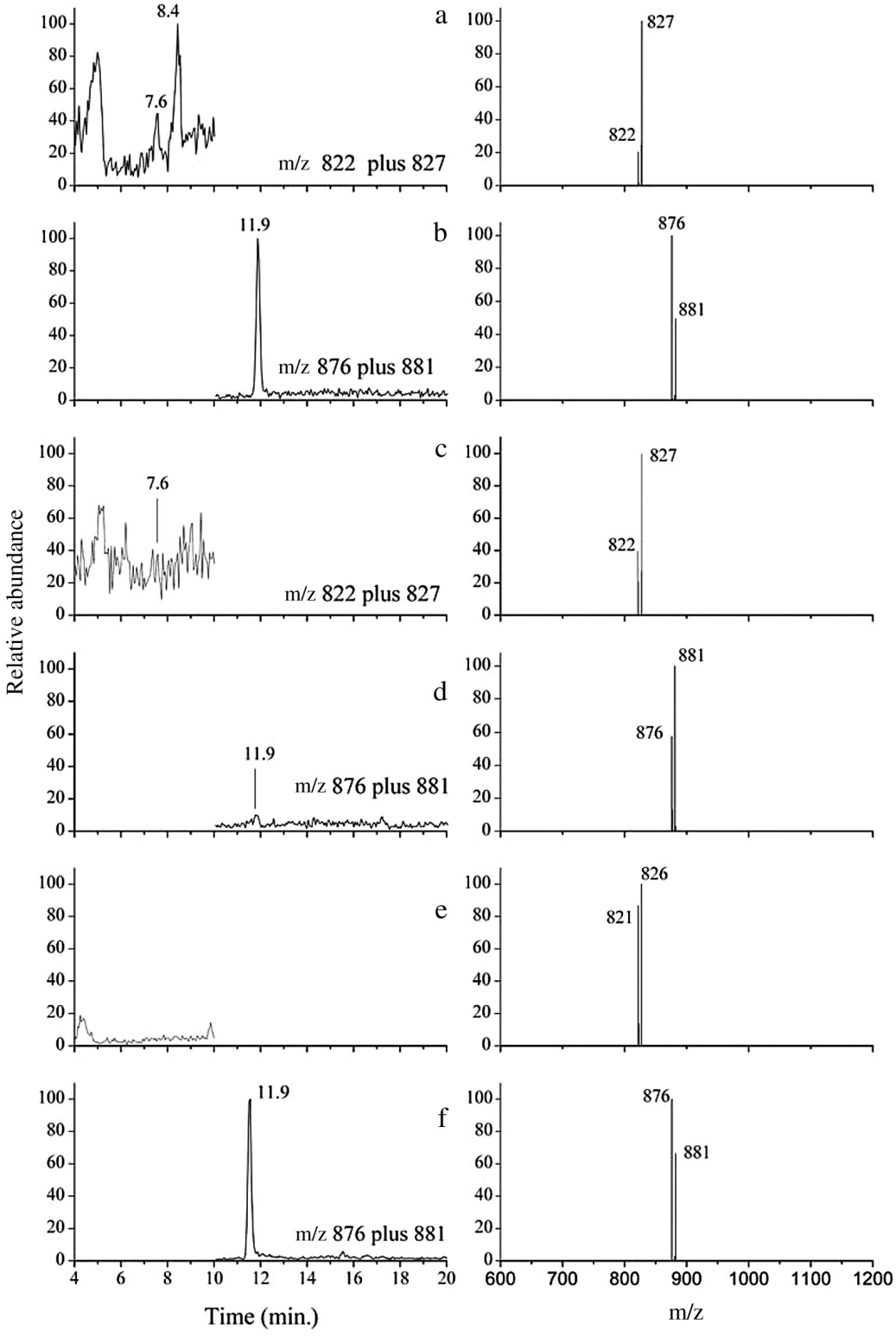

Fig. 2. *Phalacroma rotundatum* and *Dinophysis caudata*. Selected liquid chromatography-mass spectrometry (LC-MS) chromatograms (left) and mass spectra (right) obtained by selected ion monitoring (SIM) analysis, in positive ionization mode, for $[M+NH_4]^+$ and $[M+Na]^+$ ions of standards and of picked cells of *P. rotundatum* and *D. caudata* collected on 26 August 2003. (a) Okadaic acid (OA) (7.6 min) and dinophysistoxin-2 (DTX2) (8.4 min) standards with identical mass spectrum; (b) pectenotoxin-2 (PTX2) (11.9 min) standard; (c) OA (7.6 min) and (d) PTX2 (11.9 min) in picked cells of *P. rotundatum*; and (e) OA (not detected) and (f) PTX2 (11.9 min) in picked cells of *D. caudata.* m/z: mass to charge ratio

D. caudata (Fig. 2e,f), which was the most abundant *Dinophysis* species, followed by *D. acuminata* that August (Table 1). Toxins were not detected in cells analyzed in July 2005, while cells from September 2005 showed low levels of OA (0.7 pg cell^{-1}) and DTX2 (0.9 pg cell^{-1}). OA is the only toxin found in Galician strains of *D. acuminata* (Blanco et al. 1995) — which in the present study was the predominant species of *Dinophysis* in the plankton concentrate in September 2005 — and DTX2 is an important component in the toxin profile of *D. acuta* (Pizarro et al. 2009), a species that was present in the Galician Rías from April to November 2005 (Escalera et al. 2010). Neither OA nor DTX2 or PTX2 were found in 4 samples from April, October, and December 2007. Three of these samples had a high proportion of vacuolated cells of *P. rotundatum* (Table 1), and in some of them, vacuoles exhibited DNA rests when stained with SYBR Green I and observed under epifluorescence using a blue filter set (Fig. 3a,b), as well as an intense orange autofluorescence when a filter specific for phycoerythrin was used (Fig. 3c,d). Further, in the samples from April and December 2007, in which toxins were not detected, *P. rotundatum* was the only potential toxin producer present in the plankton concentrate.

Previous studies showing the association of *Phalacroma rotundatum* with lipophilic toxins found in plankton hauls and shellfish are ambiguous. Vershinin & Kamnev (2001) reported DSP toxins in mussels from the Black Sea when both *P. rotundatum* and *Dinophysis caudata* were present, so the species responsible for the outbreak could not be ascertained. Blanco et al. (1995), based on multiple regression analysis of toxin content (HPLC-FD analyses) in plankton net hauls versus *Dinophysis* spp. cell counts, estimated high cell-toxin levels (124 pg cell^{-1}) of OA in *P. rotundatum*. Miles et al. (2004) found toxins in picked cells of *P. rotundatum* that coincided with those present in co-occurring cells of toxigenic *Dinophysis* spp. Cembella (1989) found OA in net hauls — analyzed with an enzymatic assay in addition to HPLC — where *D. acuminata* plus *D. norvegica* were prominent, but no traces of diarrhetic toxins were found in hauls rich in *P. rotundatum* from eastern Canada. Masselin et al. (1992) found a decrease in toxin content in hauls when maximum concentrations of *P. rotundatum* appeared in addition to frequent populations of *D.* cf. *acuminata*, which led them to suggest that French strains of the former were not toxic. Caroppo et al. (1999), using a toxicity test (Microtox system), did not detect toxicity either in plankton concentrates rich in *P. rotundatum* or in picked-cell samples obtained when the species reached a seasonal maximum density (1460 cells l^{-1}).

In order to avoid analytical artefacts, all single-cell analyses in the present study were performed by LC-MS under identical chromatographic conditions. In all picked-cell samples of *Phalacroma rotundatum* in which lipophilic toxins were detected, the toxin profile mimicked that of co-occurring mixotrophic species of *Dinophysis*; samples of picked cells from plankton populations where *P. rotundatum* was the only potential lipophilic-toxin producer showed undetectable levels of toxins (Pizarro 2008). Cell-toxin content in *P. rotundatum* was extremely low whenever toxins were detected, and different toxin profiles were found for this species even in extracts of groups of

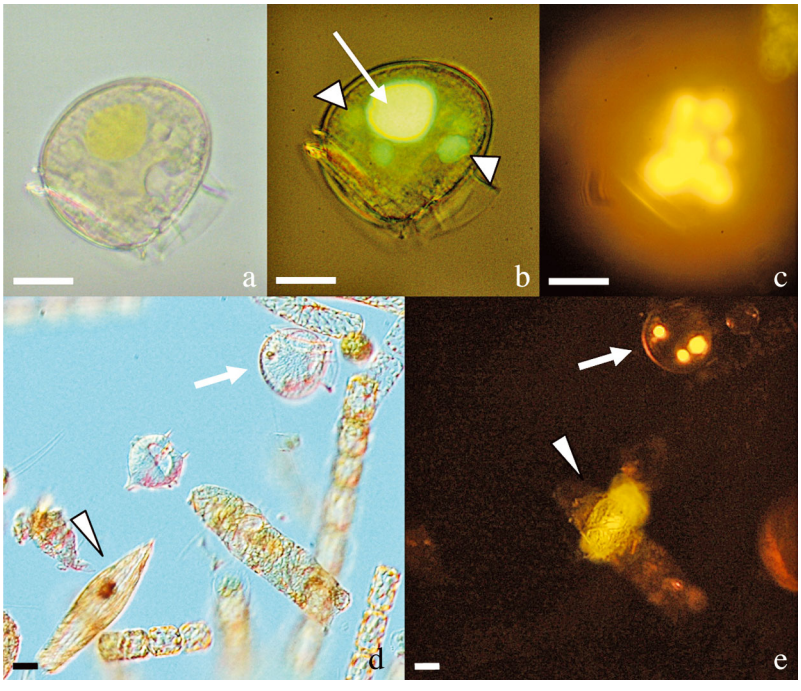

Fig. 3. *Phalacroma rotundatum* and *Tiarina* cf. *fusus*. Differential interference contrast (DIC) and epifluorescence micrographs of *P. rotundatum* specimens with digestive vacuoles. (a) DIC and (b) epifluorescence (excitation 450 to 490 nm, emission 520 nm long-pass filter set) micrographs of a *P. rotundatum* cell stained with SYBR Green I (arrow: nucleus, arrowheads: vacuoles); (c) epifluorescence (excitation 546 nm, emission 585 nm single-pass filter set, specific for phycoerithrin) micrograph of the same live *P. rotundatum* cell showing autofluorescent vacuoles; and (d) phase contrast and (e) epifluorescence (excitation 546 nm, emission 585 nm single-pass filter set) micrograph of a live *P. rotundatum* (arrow) and an actively swimming *T.* cf. *fusus* (arrowhead). In (d) and (e), both specimens exhibited the same phycoerythrin-like autofluorescence; micrograph (e) was taken 0.5 s later than (d). Scale bars = 20 µm

cells isolated from the same plankton concentrate (Table 1). From these results we can conclude that, at least between 2003 and 2007, *P. rotundatum* from the Galician Rías did not make a relevant contribution to overall plankton toxicity and did not pose a threat of shellfish contamination with DSP toxins.

Bright red-orange digestive vacuoles are rarely observed in mixotrophic species of *Dinophysis* (Jacobson & Andersen 1994), although Carvalho et al. (2008) found colorful vacuoles and cryptophyte-like bodies in the cytoplasm of *D. norvegica* cells from the Baltic Sea. *Dinophysis* species in culture do not feed directly on cryptophyte algae but can be grown successfully feeding the ciliate *Myrionecta rubra* that has previously been fed cryptophytes (Park et al. 2006). These cryptophyte plastids (hence the orange color) may remain active for >10 wk in *M. rubra* (Johnson et al. 2007). Similarly, *D. caudata* fed on *M. rubra* can also retain these plastids for a long period of time (Park et al. 2008), sometimes dispersed in the cytoplasm (Nagai et al. 2008) and not within vacuoles (García-Cuetos et al. 2010). A recent study verified the active uptake of plastids from *M. rubra* by *D. caudata*, which is clear evidence of kleptoplasty, but the possibility of some kind of semi-permanent plastids resident in *D. caudata* still cannot be excluded (Minnhagen et al. 2011).

Park & Kim (2010) found bright-orange fluorescent inclusions in the mixotrophic dinoflagellate *Fragilidium duplocampanaeforme* that had recently ingested *Dinophysis caudata* and *D. acuminata.* Thus, it seems that mixotrophic dinoflagellates may emit bright-orange autofluorescence from their ingested prey for a limited period of time. Observations in the present study constitute the first report of orange autofluorescence in digestive vacuoles of the heterotrophic dinoflagellate *Phalacroma rotundatum*. The same kind of orange autofluorescence was observed inside the lorica of the ciliate *Tiarina* cf. *fusus* (Fig. 3d,e), a potential prey of *P. rotundatum*, which was frequently observed co-occurring with *P. rotundatum*. These observations led us first to suggest the possibility that the orange autofluorescence in the digestive vacuoles of *P. rotundatum* were probably remains of mixotrophic species of *Dinophysis* taken up by *Tiarina* spp. ciliates that had previously fed on them, and that this transfer of *Dinophysis* spp. remains would explain the presence of toxins in *P. rotundatum* that mimicked those of co-occurring *Dinophysis* spp. Interestingly, picked cells of *P. rotundatum* with a high percentage of vacuolated cells that included observations of orange autofluorescence (samples from 2007 in Table 1) had no traces of toxins. It is unlikely — considering the myzocytotic preying behavior of *P. rotundatum* — that the orange autofluorescence in the vacuoles came from cryptophytes or cyanobacteria prey, i.e. the 2 groups of phycoerythrin-containing microalgae with no lipophilic toxins. Pelagic food webs are very complex and far from being understood completely (Jeong et al. 2002). The 2 possible interpretations of the orange-fluorescing vacuoles are that *P. rotundatum* either fed directly on phycoerythrin-containing *Myrionecta rubra*, or fed on tintinnids that had previously fed on *M. rubra* and still kept remains of its pigments in their digestive vacuoles.

To date, *Phalacroma rotundatum* and *Protoperidinium crassipes* are the only heterotrophic dinoflagellates reported to contain lipophilic shellfish toxins — DSP and azaspiracids respectively — but toxin production in laboratory cultures of phototrophic and mixotrophic microalgae has been linked to photosynthesis (Granéli & Flynn 2006, Kim et al. 2008). Recent findings showed that the small dinoflagellate *Azadinium spinosum* is the real producer of azaspiracids (Tillmann et al. 2009). Therefore, *Protoperidinium crassipes* (>100 µm) acts as a vector of azaspiracid shellfish poisoning (AZP) toxins accumulated from the ingestion of small (10 to 15 µm) AZP-toxin producers.

Results from the present study lead us to propose that: (1) *Phalacroma rotundatum* acts as a vector of toxins produced by mixotrophic co-occurring species of *Dinophysis* that previously had been eaten by *P. rotundatum*'s ciliate prey; and (2) orange autofluorescence in *P. rotundatum* vacuoles suggests that this species may include *Myrionecta rubra* as a prey organism. Conclusive testing of these hypotheses is now possible using laboratory cultures of *P. rotundatum* and *M. rubra*, and is the subject of ongoing investigations.

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