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Feeding of *Fragilidium* cf. *duplocampanaeforme* and *F. subglobosum* on four *Dinophysis* species: prey specificity, local adaptation and fate of toxins

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ABSTRACT: We studied the feeding behaviour of 2 *Fragilidium* species — *F.* cf. *duplocampanaeforme* (strains VGO1120 and VGO692 from NW Iberia and the Mediterranean Sea, respectively), and *F. subglobosum* (IO97-01, W Iberia) — on *Dinophysis acuminata, D. acuta, D. caudata* and *D. tripos* from NW Iberia. Only the Atlantic strain of *F.* cf. *duplocampanaeforme* fed upon *D. acuminata* (2.52 ± 0.48 cells *Fragilidium*$^{-1}$ d^{-1}) and *D. caudata* (0.58 ± 0.32 cells *Fragilidium*$^{-1}$ d^{-1}); furthermore, this strain fed weakly on *D. tripos* (<0.10 cells *Fragilidium*$^{-1}$ d^{-1}) and not at all on *D. acuta*. Neither the Mediterranean *F. duplocampanaeforme* nor *F. subglobosum* were observed to ingest Atlantic strains of *Dinophysis* species. Deleterious effects caused by *Fragilidium*, namely cell immobilization or even death, were observed in some cases (the *D. acuminata* and *D. caudata* cultures). The Atlantic *F.* cf. *duplocampanaeforme* that had previously fed on *D. acuminata* and *D. caudata* showed low intracellular toxin levels at the end of the exponential growth phase (based on LC-MS). However, extracellular toxin levels were higher in the *Dinophysis* cultures exposed to the Atlantic *F.* cf. *duplocampanaeforme* than in those without *Fragilidium* (the contrary being true for the toxin amount per cell). Our results indicate that (1) *Fragilidium* cf. *duplocampanaeforme* feeds differentially on certain *Dinophysis* species (in particular *D. acuminata* and *D. caudata*), (2) inter-specific and intraspecific differences exist in *Fragilidium*-*Dinophysis* predator-prey interactions, (3) the amount of extracellular *Dinophysis* toxins is enhanced by the presence of *Fragilidium*, and (4) decreased final yields and higher frequencies of small cells of *Fragilidium* in mixotrophic cultures.

KEY WORDS: *Dinophysis* · *Fragilidium* · Prey specificity · Okadaic acid · DSP toxins transfer · Mixotrophy

INTRODUCTION

Dinoflagellates are marine protists which include photosynthetic and heterotrophic species but also many mixotrophic species that may take up nutrients through phototrophic and phagotrophic behaviour (Stoecker et al. 2006). Mixotrophy can be obligatory, as it is for those *Dinophysis* species that need to 'steal' plastids periodically from the ciliate *Mesodinium rubrum* (Park et al. 2006). However, in many cases, mixotrophic dinoflagellates contain constitutive plastids and maximize their growth rates when ingesting other algae (Stoecker 1999, Jakobsen et al. 2000, Jeong et al. 2005). Mixotrophic dinoflagellates can be found in most taxonomic orders, e.g. Gymnodiniales, Prorocentrales, Dinophysiales, Gonyaulacales, Blastodiniales, Peridiniales and Noctilucales (Stoecker 1999).

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Fragilidium is a facultative mixotrophic dinoflagellate genus, with 6 species described to date: *F. fissile, F. duplocampanaeforme, F. heterolobum, F. lacustre, F. mexicanum* and *F. subglobosum* (Lindemann 1924, Balech 1959, von Stoch 1969, Balech 1987, 1990, Nézan & Chomérat 2009). The mixotrophic nature of most *Fragilidium* species has been already reported (Skovgaard 1996, Jeong et al. 1999, Park & Kim 2010). According to these authors, *Fragilidium* feeds only on dinoflagellates, but different levels of prey selectivity have been observed for each species. Thus, *F. subglobosum* have been reported to feed only on *Ceratium* spp. and *F. duplocampanaeforme* on *Dinophysis* spp. (Park & Kim 2010, Hansen 2011), although a recent study (Amorim et al. 2013) described the predation of *F. subglobosum* and *F.* cf. *duplocampanaeforme* from Portuguese coastal waters on *Ceratium horridum*. In turn, *F. mexicanum* has been shown to feed on multiple dinoflagellate genera, such as *Akashiwo, Alexandrium, Ceratium, Lingulodinium, Prorocentrum, Protoperidinium* and *Scrippsiella* (Jeong et al. 1999). Furthermore, *Fragilidium* growth responds in different ways depending on its prey. For example, *C. tripos* has been reported to be a more suitable prey for *F. subglobosum* than *C. furca* and *C. fusus* (Hansen & Nielsen 1997).

Recently, Park & Kim (2010) described the prey specificity and feeding mechanism of *Fragilidium duplocampanaeforme* isolated from Masan Bay (South Korea). Based on a previous report of *F. duplocampanaeforme* containing *D. acuminata* and *D. caudata* from the French Atlantic coast, Nézan & Chomérat (2009) demonstrated that their isolate fed exclusively on *Dinophysis* when different dinoflagellates were offered as prey. These authors tested 4 species of *Dinophysis*: *D. acuminata, D. caudata, D. fortii* and *D. infundibulus*, from the same location as their strain of *F. duplocampanaeforme*. All *Dinophysis* species, except *D. fortii*, were ingested by *Fragilidium*, and deleterious effects on *Dinophysis* (motility decrease) were observed in all cases.

Shellfish harvesting closures due to contamination with diarrheic shellfish poisoning (DSP) toxins above regulatory levels are commonplace in the Galician Rías (NW Iberian Peninsula). Toxic outbreaks are associated with the occurrence of *Dinophysis* species, mainly *D. acuminata* but also *D. acuta* and *D. caudata*. In fact, *D. acuminata, D. sacculus, D. ovum, D. acuta, D. fortii* and *D. caudata* are common *Dinophysis* species in warm temperate seas (Reguera et al. 2012). Toxin profiles and cellular content are highly variable between *Dinophysis* species (and between strains of the same species), but all the aforementioned are known to produce okadaates (okadaic acid [OA] and its congeners the dinophysistoxins [DTXs]) and/or pectenotoxins (PTXs) (reviewed by Reguera et al. 2014). The term '*Dinophysis* toxins' (DsT) will be used throughout to indicate the sum of okadaates and PTXs produced by *Dinophysis* species.

Fragilidium species are common in Galician waters and can be easily recognized when forming ecdysal cysts. The presence of *F.* cf. *duplocampanaeforme* and *F. subglobosum* in the NW Iberian Peninsula was reported in a recent study (Amorim et al. 2013). However, to our knowledge, there are no studies on *Fragilidium* abundance, diversity and feeding behavior on *Dinophysis* spp. or on any other dinoflagellates in that area.

In the present study, we focused on the feeding behavior of 2 strains of *Fragilidium* cf. *duplocampanaeforme* (hereafter referred to as the Atlantic and Mediterranean *Fragilidium*) and 1 strain of *F. subglobosum* IO97-01 (W Iberia) on 4 *Dinophysis* species (*D. acuminata, D. acuta, D. caudata* and *D. tripos*). The potential impact of the Atlantic *Fragilidium* on *Dinophysis* is discussed on the basis of estimated grazing rates in the laboratory. DsT were also analysed in the Atlantic *Fragilidium* cells and culture filtrate to follow the fate of these compounds whenever *Dinophysis* spp. were exposed to and eventually ingested by the Atlantic *Fragilidium*.

MATERIALS AND METHODS

Cultures

Four *Dinophysis* species were isolated from the Galician Rías, NW Iberian Peninsula (Fig. 1), and established in mixotrophic cultures with their ciliate prey: *D. tripos* VGO1062 and *D. acuminata* VGO1063 (October 2009, Stn B1, Ría de Vigo, 42° 21.40′ N 8° 46.42′ W), *D. caudata* VGO1064 (April 2010, Stn P2, Ría de Pontevedra, 42° 8.22′ N, 8° 51.36′ W) and *D. acuta* VGO1065 (October 2010, Stn P2). Strains of the ciliate *Mesodinium rubrum* (AND-A0711) and the cryptophyte *Teleaulax amphioxeia* (AND-A0710), added periodically to the ciliate cultures as prey, were both isolated in 2007 in the course of weekly sampling of the Andalusian Monitoring Programme (Huelva, SW Spain). Cultures of *Fragilidium subglobosum* (IO97-01, Fig. 2A) were established by isolation of single cells from plankton-net haul samples in Portuguese coastal waters (Amorim et al. 2013). *Fragilidium* cf. *duplocampanaeforme* (Fig. 2B,C) were

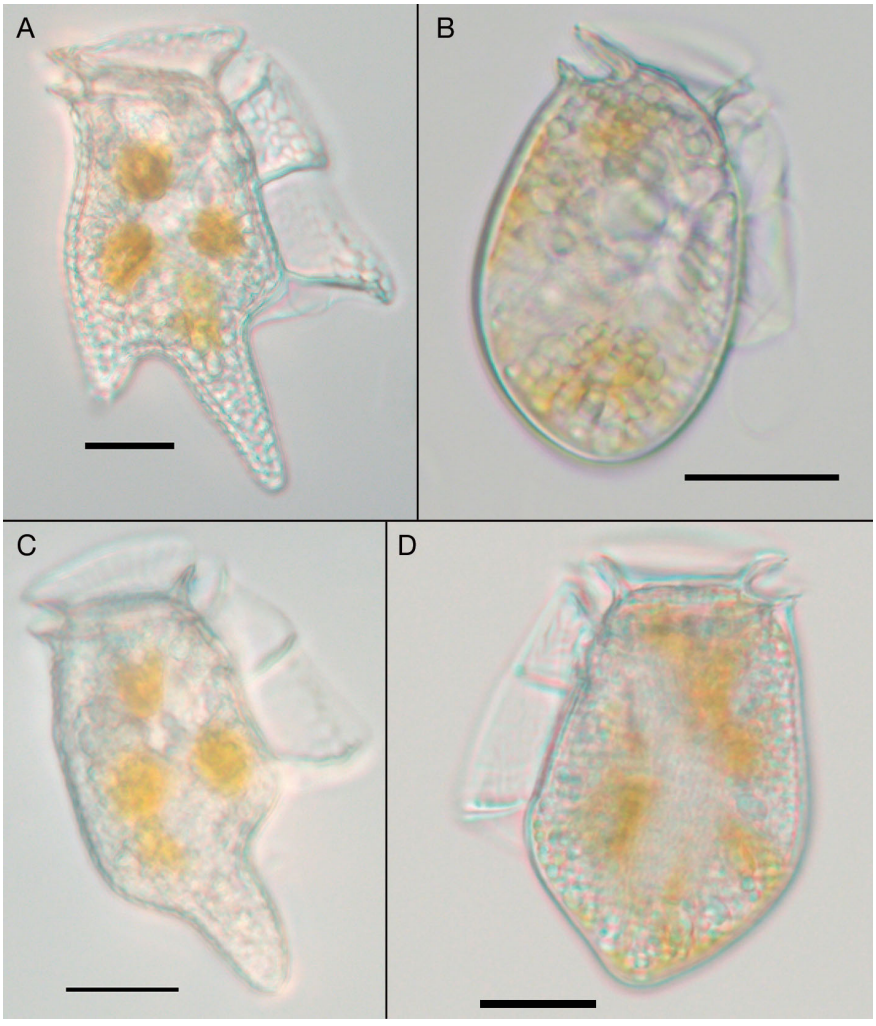

Fig. 1. Light micrographs (live cells) of the *Dinophysis* strains used in this study: (A) *D. tripos*, (B) *D. acuminata*, (C) *D. caudata*, (D) *D. acuta*. Scale bars = 20 µm

isolated during opportunistic samplings at Ría de Vigo (VGO 1120, Stn B1, July 2009) and Elefsis Bay, Saronikos Gulf, Greece (VGO 692, July 2003). Both isolates were genetically characterized by Amorim et al. (2013), and their morphologies matched that of *F. duplocampanaeforme* (Nézan & Chomérat 2009), but the lack of molecular data from the type locality led us to label these strains as *F.* cf. *duplocampanaeforme*. All cultures were grown in diluted (1/20) L1-Si medium at 19°C, 32 psu, under a 12 h light:12 h dark cycle with 150 µmol photons m^2 s^{-1} irradiance.

Feeding experiments

Three replicate cultures of the Atlantic *Fragilidium* and each of the 4 *Dinophysis* species were mixed in 50 ml glass flasks at an approximate initial concentration of 100 and 300 cells ml^{-1}, respectively. Cell counts were carried out using Sedgewick-Rafter chambers under a light microscope. The same approach was used for the Mediterranean *Fragilidium* and *F. subglobosum*, but mixed cultures were incubated in 24 well microplates (Thermo Scientific). Specific growth rates (r, t^{-1}) were calculated as follows:

$$r = \ln(Nt/N0)/\delta t \quad (1)$$

In the case of the Atlantic *Fragilidium*, 2 size classes typically described in *Dinophysis* spp. (normal vegetative and small forms) were distinguished and enumerated. The ingestion rates (I = *Dinophysis* cells eaten *Fragilidium*$^{-1}$ d^{-1}) were calculated following Eqs. (2–4) as described by (Frost 1972):

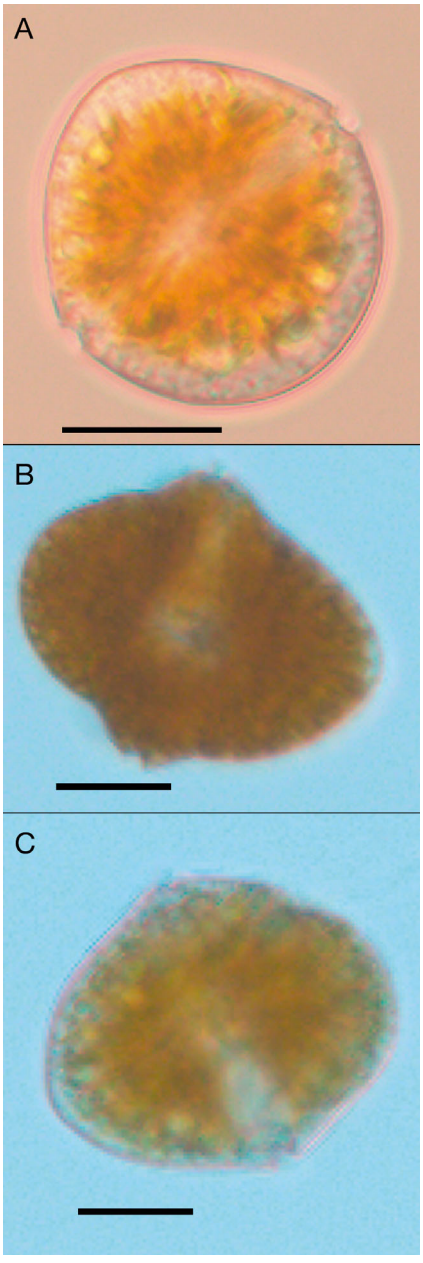

Fig. 2. Light micrographs (live vegetative cells) of the *Fragilidium* strains used in this study. (A) *F. subglobosum*, (B) Atlantic *Fragilidium*, (C) Mediterranean *Fragilidium*. Scale bars = 20 µm

$$C = C_1 \times [e^{(r-g)(t^2-t^1)} - 1]/(t_2 - t_1)(r - g) \quad (2)$$

where C_1 is the *Dinophysis* cell concentration at t_1, r is the specific growth rate for Dinophysis, g is the grazing coefficient, and C is the average cell concentration of *Dinophysis* during a time interval ($t_2 - t_1$);

$$F = Vg/N \quad (3)$$

where F is the volume swept clear (volume of ambient medium from which the cells are completely removed by *Fragilidium* to achieve the measured ingestion rate), V is the volume (ml) of the cultures, and N is the average number (cells) of *Fragilidium*; and

$$I = C \times F \quad (4)$$

Allelopathy experiments

Effect on *Dinophysis* species of exposure to Atlantic *Fragilidium* cf. *duplocampanaeforme* cells

In total, 0.2 ml of the Atlantic *Fragilidium* culture in exponential growth phase were added to *D. acuminata, D. acuta* and *D. caudata* cultures (2 ml volume at 300 cells ml^{-1}) in 24 well plates (Thermo Scientific). The predator:prey ratio was 1:3.6. Lugol-fixed samples were collected for cell counts, and observations were carried out under the light microscope at different exposure times (0.5, 1.5, 2.5, 3.5, 4.5, 6.5 and 23.5 or 24.5 h) to inspect any deleterious effect on *Dinophysis*.

Effect on *Dinophysis* species of exposure to supernatant of Atlantic *Fragilidium* cf. *duplocampanaeforme*

A 15 ml volume of the Atlantic *Fragilidium* culture in exponential growth (892 cells ml^{-1}) was centrifuged at 3000 × *g* for 10 min. The obtained supernatant from *Fragilidium* was added to 24 well culture plates containing 1 ml culture (~300 cells) of each *Dinophysis* species (*D. acuminata, D. acuta and D. caudata*) in triplicate treatments at different supernatant:*Dinophysis* ratios: 1:1 (T1), 0.5:1 (T2) and 0.1:1 (T3). Light microscopic observations were carried out at different time intervals (0.5, 1.5, 2.5, 3.5, 4.5 and 23.5 h) to follow any deleterious effect (e.g. cellular death or reduced mobility). Control treatments were done as above but adding 1 ml of culture medium to each *Dinophysis* species. Lugol-fixed samples were collected for cell counts at the end of the experiment.

Toxin analyses

Extraction

Samples from feeding experiment cultures (as previously detailed), control cultures of *Fragilidium* spp. (IO97-01, VGO692 and VGO1120) and monospecific cultures of *D. acuminata, D. caudata* and *D. acuta*

were collected at the end of the exponential growth phase. Control cultures of *Dinophysis* were grown after the feeding experiments (with the exception of *D. tripos* cultures that were lost).

Cells were filtered through Whatman GF/C glass fiber filters (1.4 µm, 25 mm ∅) (Whatman), and both filtered cells and culture filtrates were processed for toxin analyses as follows. Cellular toxins were extracted with MeOH, the culture material was sonicated and centrifuged at 5065 × *g* for 10 min at 10°C, the supernatant was removed, and the pellet was extracted again with MeOH following the same process. Considering that in some feeding experiments, all *Dinophysis* cell had been eaten, cellular toxin content was referred to as particulate toxin per volume (ng ml^{-1}) of filtered material.

Culture filtrates were loaded onto a Sep-Pak C18 light cartridge. Cartridges were conditioned with 3 ml of MeOH and equilibrated with 3 ml of Milli-Q water. Subsequently, samples were loaded (volumes ranged from 20 to 45 ml) and washed with 4 ml of MeOH: H_2O (2:8) to remove salts. Finally, toxins were eluted with 4 ml of 100% MeOH. Toxin amount was also expressed as ng ml^{-1} but in this case referred to as the dissolved (extracellular) toxin concentration.

Analyses by liquid chromatography

Paralytic shellfish poisoning (PSP) toxins in the Atlantic *Fragilidium* were analyzed according to Rourke et al. (2008).

Analyses by mass spectrometry

Particulate and extracellular lipophilic toxins — yessotoxins (YTXs), spirolides (SPXs) and azaspiracids (AZAs) — were analyzed by LC/MS in acidic conditions following the method by Gerssen et al. (2011). LC-MS analyses of DsT were performed on a Thermo Scientific Dionex High-Speed liquid-chromatography (LC) system coupled to an Exactive mass spectrometer (MS), equipped with an Orbitrap mass analyzer and a HESI-II probe for electrospray ionization. The instrument was mass-calibrated daily for positive and negative modes, and the capillary and tube lens voltages were optimized daily, using the automated script within the Exactive acquisition software in both cases. All analyses were performed using the 'balanced' automatic gain control setting with a 50 ms maximum injection time. Data were acquired using Thermo Scientific Xcalibur 2.1. Optimal ion source and interface conditions consisted of a spray voltage of 4 kV, sheath gas flow of 30 arbitrary units, auxiliary gas flow of 5 arbitrary units and a capillary temperature of 320°C.

Toxins were separated on an Acquity BEH C18 column (2.1 × 50 mm, 1.7 µm particle size) maintained at 40°C at a flow rate of 300 µl min^{-1}. The mobile phase consisted of (A) 2 mM ammonium acetate at pH 5.8 and (B) 100% MeOH. A linear gradient elution from 10% to 50% B was run for 3 min, 100% B was reached at Min 6.5 and held for 3 min before returning to the initial conditions of 10% B in 0.1 min, and this percentage was held until Min 12. Certified reference standard solutions of OA and dinophysistoxin-2 (DTX2) (*m/z* 805.4779 $[M+H]^+$, 822.5035 $[M+NH_4]^+$ and 827.4592 $[M+Na]^+$) and pectenotoxin-2 (PTX2) (876.5148 $[M+NH_4]^+$and 881.4696 $[M+Na]^+$) were purchased from the National Research Council (Canada). A standard working solution containing OA (0.617 ng $µl^{-1}$), DTX2 (0.266 ng $µl^{-1}$) and PTX2 (0.573 ng $µl^{-1}$) in MeOH was prepared.

RESULTS

Feeding experiments

The Atlantic *Fragilidium* fed heavily upon *D. acuminata* and *D. caudata* (Fig. 3A,B), only occasionally on *D. tripos* and not at all on *D. acuta* (Fig. 3C,D). The first captured cells of *D. acuminata* and *D. caudata* were observed from minutes to a few hours after the initiation of the experiments (see Video S1 in the supplementary information at www.int-res.com/articles/suppl/a072p241_supp/). The Atlantic *Fragilidium* did not appear to discriminate between *Dinophysis* spp. cell sizes (typical vegetative cells and small forms) enumerated in this particular experiment. Both size classes of *Dinophysis* showed the same trends, and only total abundances are detailed in Fig. 3. Maximum ingestion rates of *Dinophysis* spp. by Atlantic *Fragilidium* were observed for *D. acuminata* (Day 1, I = 2.52 ± 0.48 [SD] *Dinophysis* *Fragilidium*$^{-1}$ d^{-1}) with a prey:predator ratio of 3:1, and *D. caudata* (Day 3, I = 0.58 ± 0.32, prey:predator ratio 1:1). These ingestion rates were significantly different (*t*-test; n = 3; $p < 0.005$). Predation by Atlantic *Fragilidium* on *D. tripos* ($I < 0.10$) was confirmed by light microscopic observations, but predation on *D. acuta* was never observed.

Growth rates and cell densities of Atlantic *Fragilidium* were larger in the *D. acuminata* and *D. caudata* treatments (0.34 d^{-1} in both cases) relative to those of

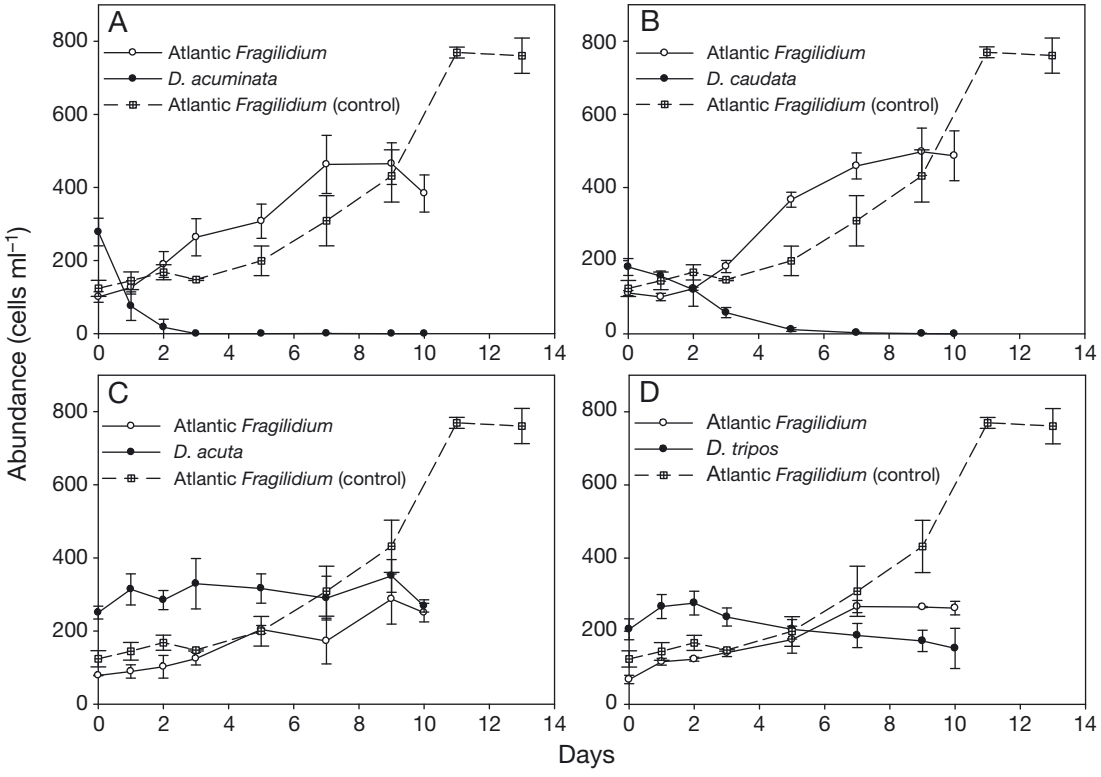

Fig. 3. Abundances (cells ml^{-1}) of Atlantic *Fragilidium* cultured with (A) *Dinophysis acuminata*, (B) *D. caudata*, (C) *D. acuta* and (D) *D. tripos*. Atlantic *Fragilidium* control treatment abundances for each *Dinophysis* species are also shown. Error bars are SD

D. acuta and *D. tripos* (0.22 d^{-1} and 0.19 d^{-1}, respectively). The Atlantic *Fragilidium* grew faster when feeding actively on *D. acuminata* and *D. caudata* during the first week of the experiment relative to its control treatment. After that, the cultures reached the stationary phase on Day 9. The Atlantic *Fragilidium* control showed a lower growth rate (0.17 d^{-1}) but a higher final yield because the stationary phase appeared later (Day 13).

At the end of the experiment, a higher frequency of small Atlantic *Fragilidium* cells were observed in the *D. acuminata* (59.5 ± 3.9%) and *D. caudata* (49.8 ± 10.6%) treatments, relative to the controls (34.1 ± 3.5%). The average frequency of small *Fragilidium* cells in each treatment was significantly higher than the control in the case of *D. acuminata* (*t*-test; n = 3; $p < 0.005$). In contrast, Atlantic *Fragilidium* co-cultured with *D. acuta* and *D. tripos* showed significantly lower proportions of small cells (19.4 ± 2.7% and 14.9 ± 0.8%, respectively) than in the controls (*t*-test; n = 3; $p < 0.005$). Cell measurements appeared distributed in 2 distinct size classes of small (diameter [D] = 26.7 ± 3.8 µm, length [L] = 29.5 ± 3.6 µm, n = 21) and normal vegetative (or planozygote) cells (D = 55.5 ± 5.6 µm, L = 59.6 ± 7.7 µm, n = 21).

As mentioned above, ingested *D. acuta* cells were never observed either in the Atlantic *Fragilidium* or in the Mediterranean *Fragilidium* and *F. subglobossum* cultures in this study (Fig. 4). Moreover, we could not find any evidence under the light microscope of grazing by either the Mediterranean *Fragilidium* or *F. subglobosum* on any *Dinophysis* spp. Their control cultures showed similar growth patterns to those of the Atlantic *Fragilidium* strain, with higher cell densities and growth rates than those mixed with *Dinophysis* in the second part of the experiment (Days 6 to 11). The only exception was the Mediterranean *Fragilidium* co-cultured with *D. acuminata*, which reached higher cell densities than the control (Fig. 4).

Cultures of the 3 *Dinophysis* species (*D. acuta, D. tripos* and *D. caudata*) showed cell densities slightly lower but not significantly different from the controls when exposed to the Mediterranean *Fragilidium* and to *F. subglobosum* (Fig. 4). Only *D. acuminata* showed lower densities than in the controls (*t*-test; n = 3; $p < 0.005$), despite the fact that predation was never observed. Deleterious effects were observed in this case under the light microscope. A high proportion of *D. acuminata* cells were immobile and probably dead by Day 11 when exposed to the Mediterranean

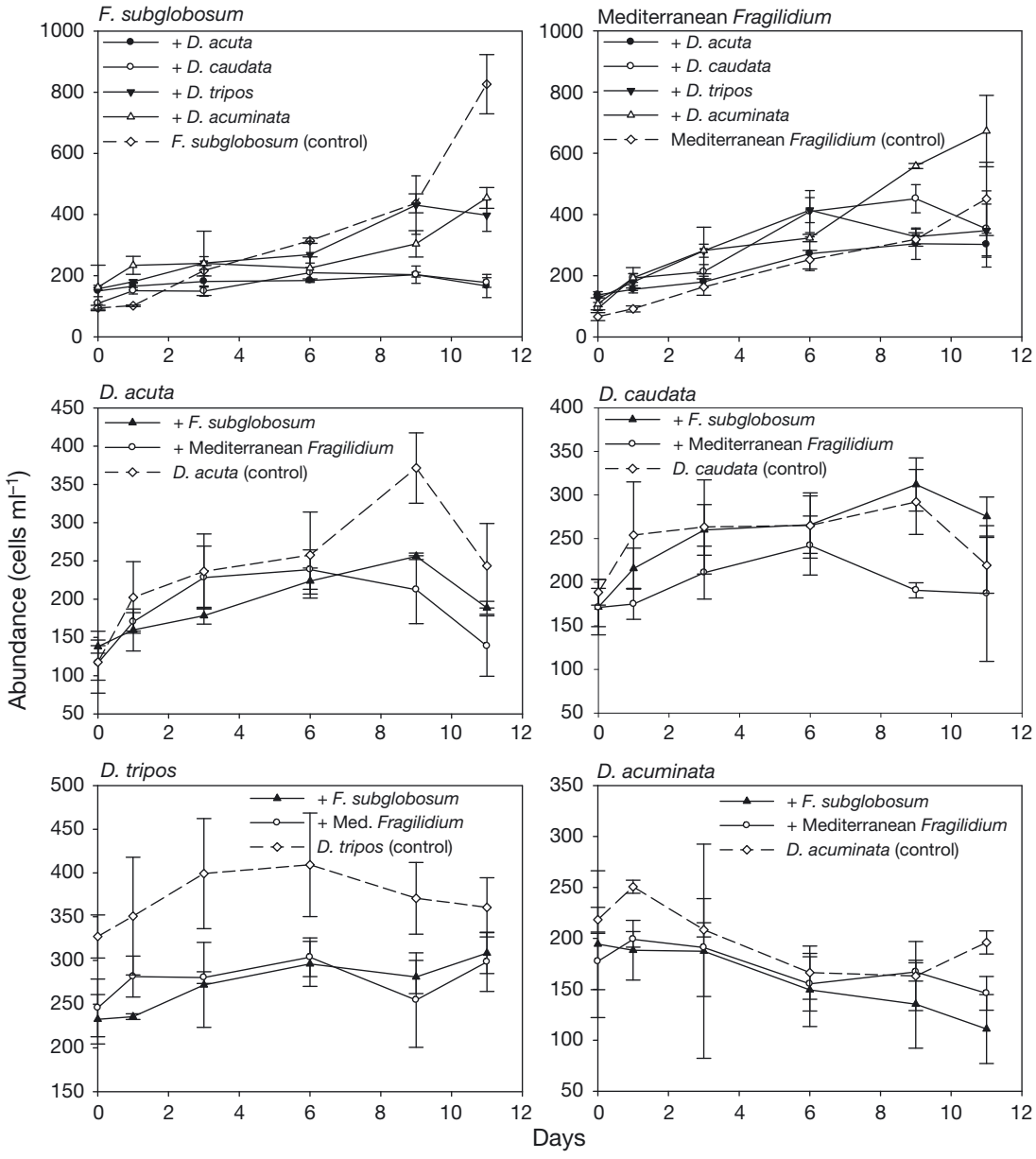

Fig. 4. Abundances (cells ml^{-1}) of *Dinophysis* spp. vs. *F. subglobosum* and Mediterranean *Fragilidium*. Upper panels: abundances of *F. subglobosum* and Mediterranean *Fragilidium* cultured with 4 *Dinophysis* spp. and their respective controls. Middle and lower panels: abundances of *D. acuta*, *D. caudata*, *D. tripos* and *D. acuminata* with both *Fragilidium* strains and control treatments. Error bars are SD

Fragilidium or to *F. subglobosum*, in contrast to cells from the control, which displayed normal swimming behavior.

Allelopathy experiments

Two short-term experiments were conducted to study the potential allelopathic effects of (1) full cultures of the Atlantic *Fragilidium* and (2) the (cell-free) supernatant of *Fragilidium* cultures on 3 *Dinophysis* species (*D. acuminata, D. acuta* and *D. caudata*). No allelopathic effects could be observed in either of the 2 treatments. The proportion of immobile cells of each *Dinophysis* species did not increase significantly when exposed to full cultures of *Fragilidium* (Fig. 5A–C, Panel 1). In addition, final yields of *Dinophysis* species exposed to full cultures of Atlantic *Fragilidium* were similar to the controls (Fig. 5, Panel 2). Likewise, the proportion of non-motile cells of *Dinophysis* species did not increase significantly when exposed to different volume ratios

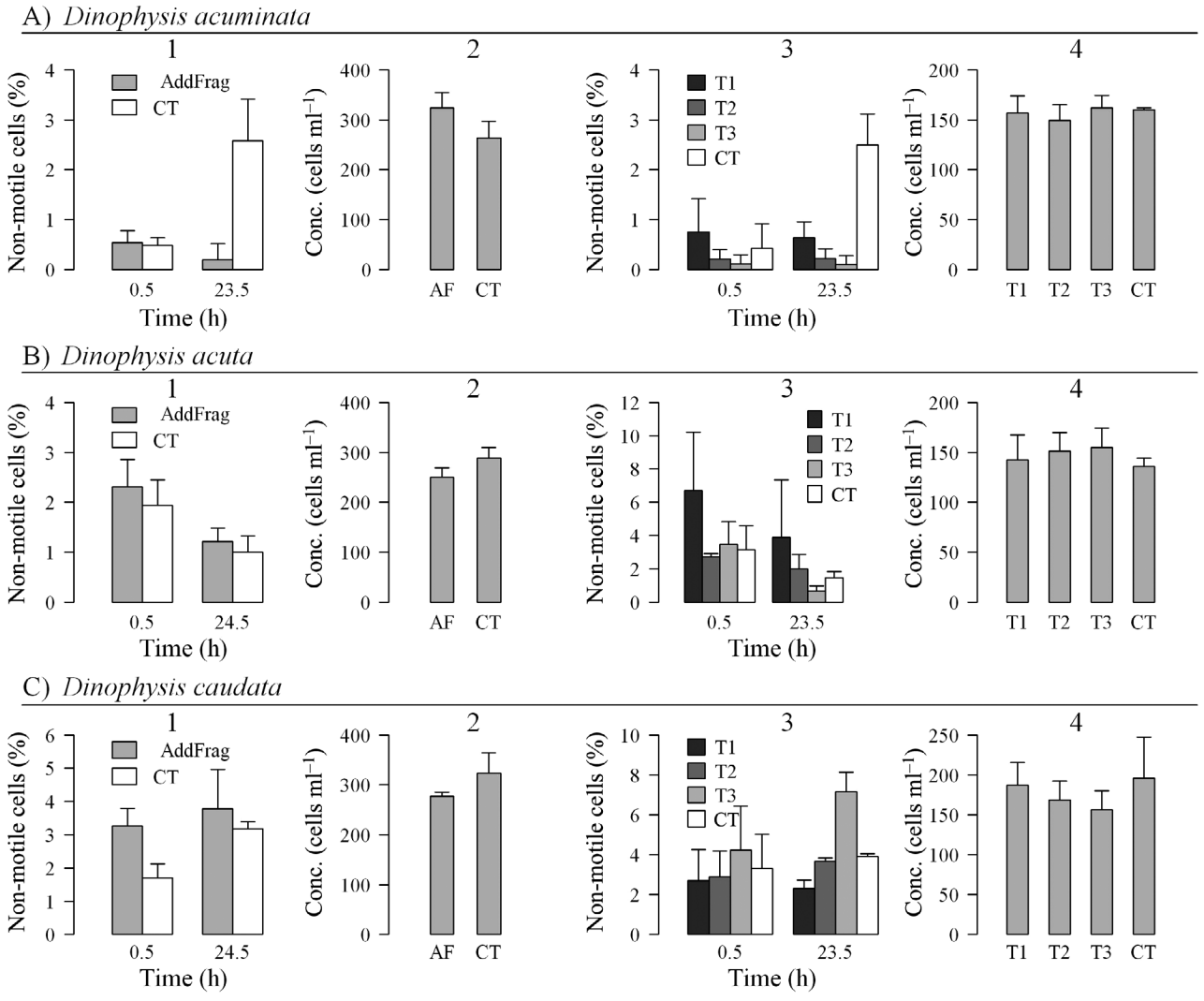

Fig. 5. Results from the allelopathic experiments for (A) *Dinophysis acuminata*, (B) *D. acuta* and (C) *D. caudata*. Panels 1 and 2: Experiment with full cultures of Atlantic *Fragilidium* — (1) % of non-motile cells of *Dinophysis* spp. at different times after adding full cultures of Atlantic *Fragilidium*, and (2) final concentration of *Dinophysis* spp. (AF: *Dinophysis* spp. with Atlantic *Fragilidium*; CT: control). Panels 3 and 4: Experiment with supernatant of *Fragilidium* — (3) % of non-motile cells of *Dinophysis* spp. (cells ml^{-1}) at different exposure times with different volume ratios of *Fragilidium* supernatant:*Dinophysis* (1:1 [T1], 0.5:1 [T2] and 0.1:1 [T3], CT: control), and (4) final concentration of *Dinophysis* spp. Error bars are SD

of the supernatant (T1, T2 and T3, see methods and legend in Fig. 5) of the Atlantic *Fragilidium* cultures (Fig. 5, Panel 3), and the final yield of the 3 *Dinophysis* species was similar in supernatant treatments relative to the controls (Fig. 5, Panel 4).

The only deleterious effect that could be seen at the end of both allelopathic experiments was a slower swimming speed in *D. caudata.*

Toxin analyses

PSP toxins, YTXs, SPXs and AZAs were not detected either in *Dinophysis* or in *Fragilidium* species. Moreover, DsT were not found in any of the control cultures of the 3 *Fragilidium* strains used here.

In this study, we focused our analyses in the detection and quantification of DsT in the Atlantic *Fragilidium* plus *Dinophysis* cells (particulate toxins) and in the culture filtrates. Monospecific cultures of *D. acuminata* and *D. acuta* showed the same toxin profiles (intracellular and extracellular; Fig. 6) as those in the feeding experiments: OA in *D. acuminata*, and OA, DTX2 and PTX2 in *D. acuta*. However, in the case of *D. caudata*, 3 different toxins (OA, DTX2 and PTX2) were found in the filtrate, whereas only PTX2 was found in the cells. Toxin analyses of *D. tripos* could not be performed, and PTX2 was the only com-

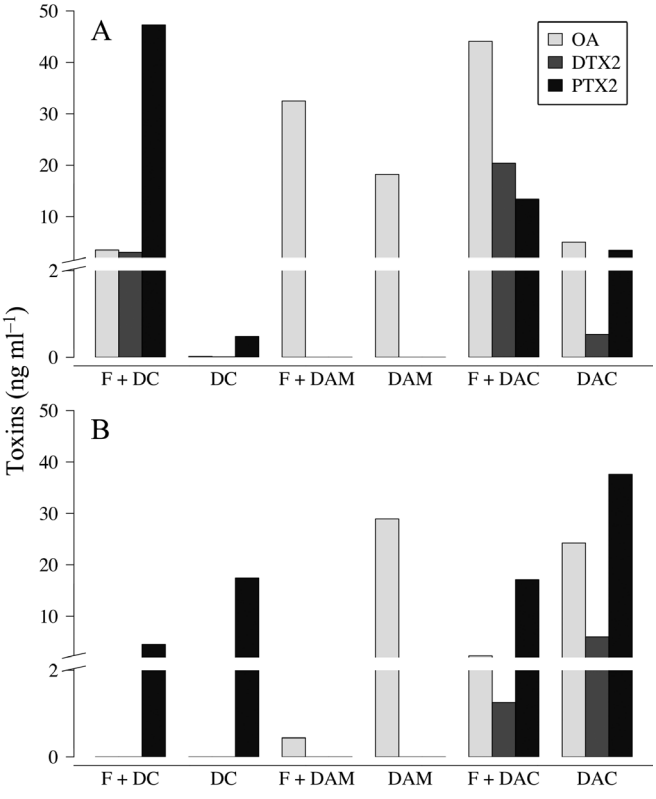

Fig. 6. Comparison of *Dinophysis* toxins (OA, DTX2, PTX2) between (A) culture filtrate and (B) particulate matter in the predation experiments (*Dinophysis* + Atlantic *Fragilidium*) and monospecific *Dinophysis* cultures. F+DC: *Fragilidium + Dinophysis caudata*, DC: *Dinophysis caudata*, F+DAM: *Fragilidium + Dinophysis acuminata*, DAM: *Dinophysis acuminata*, F+DAC: *Fragilidium + Dinophysis acuta*, DAC: *Dinophysis acuta*

pound detected in the mixed cultures with Atlantic *Fragilidium*. Quantitative results, expressed in ng ml^{-1}, revealed differences in the amount of OA, DTX2 and PTX2 between the monospecific *Dinophysis* cultures and those incubated with Atlantic *Fragilidium* (Fig. 6). The content of DsT in the particulate fraction was always higher in the monospecific *Dinophysis* cultures than in those from the feeding experiments with *Fragilidium*.

Conversely, estimations of extracellular toxins showed higher levels in the culture filtrates from the feeding experiments than in those from monospecific *Dinophysis* cultures (Fig. 6). For example, estimates of particulate PTX2 in *D. caudata* cultures were 17.5 ng ml^{-1} in the monospecific culture and 4.5 ng ml^{-1} in the culture with *Fragilidium*. This pattern was even more pronounced in the case of *D. acuminata* (28.9 vs. 0.4 ng OA ml^{-1}) and *D. acuta* (24.2 vs. 2.3 ng OA ml^{-1}; 6.0 vs. 1.3 ng DTX2 ml^{-1} and 37.6 vs. 17.1 ng PTX2 ml^{-1}).

DISCUSSION

Mixotrophy and growth in *Fragilidium*

Hansen (2011) described 2 types of growth response in mixotrophic dinoflagellates as a function of prey concentration. According to this author, *Fragilidium* belongs to 'Type 2' species (together with *Dinophysis acuminata, Karlodinium armiger* and *Paragymnodinium shiwaense*), which require high prey concentrations to attain maximum growth rates. Food uptake in 'Type 2' mixotrophs significantly increases the growth rate at low irradiances and only increases the growth rate in some cases when light is not a limiting factor. Some 'Type 2' species grow relatively fast at high irradiances without food (as is the case for *Fragilidium*), while other species grow slowly at high irradiances without food (*K. armiger*; Berge et al. 2008). In our case, we observed that food uptake (*D. acuminata* and *D. caudata*) in Atlantic *Fragilidium* accelerated its growth during the exponential phase, but stationary phase was reached sooner than in the *Fragilidium* controls, which attained higher final yields.

Differences in growth rates between purely autotrophic or mixotrophic cultures of *Fragilidium* have been reported before for *F. subglobosum* (Skovgaard 1996). Skovgaard (1996) showed that *F. subglobosum* growth was significantly enhanced in mixotrophic cultures at low irradiance (<100 µE m^{-2} s^{-1}), while small differences between mixotrophic and autotrophic cultures were found in high light conditions. Therefore, the light intensity used in our study (150 µE m^{-2} s^{-1}) might render minor differences in autotrophic vs. mixotrophic growth rates, but the influence of variable light conditions was not tested in our feeding experiments.

The higher growth rates achieved in mixotrophic cultures of Atlantic *Fragilidium* feeding on *D. acuminata* and *D. caudata* coincided with the formation of a larger proportion of small cells of *Fragilidium* that did not undergo further cell division, leading to the onset of the stationary phase earlier than in the controls. Feeding of these small cells on *Dinophysis* was never observed. These small *Fragilidium* cells have been claimed to be gametes, they display a slightly different plate arrangement (Amorim et al. 2013), and apparently they do not revert to the vegetative condition. The dimensions of small cells in the Atlantic *Fragilidium* fit within the range shown by Amorim et al. (2013) in their Portuguese isolate of *F.* cf. *duplocampanaeforme* and by Nézan & Chomérat (2009) in natural assemblages of *F. duplocampanaeforme* (26

to 28 µm). If these small cells were gametes, their formation could be argued to be the reason for the lower final yields in most feeding experiments relative to the controls. However, considering that higher final yields were achieved in every *Fragilidium* control vs. those observed in cultures with *Dinophysis*, we should look for alternative explanations, such as nutrient resource competition between the predator and its dinoflagellate prey or some allelopathic effects caused by *Dinophysis* spp. (or its toxins) on *Fragilidium*. It is likely that the different culture volumes used in the feeding experiments could have also influenced the final cell densities in 50 ml flasks vs. 24 microwell plates, but not the observed feeding behavior and selectivity on *Dinophysis*.

In summary, *Dinophysis* stimulated the short-term growth of *Fragilidium* but also the production of a higher proportion of small cells in laboratory cultures whenever the prey was available. Nevertheless, we should be cautious about extrapolation of these observations in culture conditions to the field. The onset of dense populations of *Fragilidium* would following *Dinophysis* proliferations has never been observed in the Galician Rías, despite the endemic occurrence of *Dinophysis* in the region. During a cruise in the northeastern Bay of Biscay, France, in July 2006, observations of abundant ecdysal cysts of *F. subglobosum* in the microphytoplankton fraction coincided with a decline of a preceding *D. acuminata* bloom (Velo-Suarez et al. 2010). Nevertheless, ingestion of *D. acuminata* (observations in fixed plankton samples) was only confirmed in specimens of *F. duplocampanaeforme,* a species present in a much lower proportion than was first described from this cruise data (Nézan & Chomérat 2009). These observations are in agreement with our experimental results.

Grazing impact of *Fragilidium* and prey (*Dinophysis*) specificity

Grazing impact studies of mixotrophic dinoflagellates are scarce (Hansen 2011). In the case of *Fragilidium subglobosum*, grazing rates on *Ceratium lineatum* ranged from 2.1 to 5.8 cells *Fragilidium*$^{-1}$ d^{-1} depending on light conditions (Skovgaard 1996). Our estimated maximum of 2.2 cells of *D. acuminata* per day ingested by the Atlantic *Fragilidium* is at the lower limit of this range, but the lowest ingestion rates in *F. subglobosum* were determined at irradiances higher than ~120 µE m^{-2} s^{-1}, with a peak at 63 to 107 µE m^{-2} s^{-1}. Grazing impact on *D. acuminata* in the laboratory cannot be directly extrapolated to the field. However, if we considered a theoretical example in which equally dense patches of predator and prey, no cell losses and maximum grazing rates on *D. acuminata* were observed, an initial population of 10^3 cells l^{-1} of *F. cf. duplocampanaeforme* could totally clear out a proliferation of *D. acuminata* of 10^4 cells l^{-1} in ~5 d. Thus, even if it has not been demonstrated yet, the ability of *Fragilidium* to remove significant densities of *D. acuminata* (and to a lesser extent *D. caudata*) in the field should be taken into account. Moreover, the predation of *F. duplocampanaeforme* on *D. acuminata* but not on *D. acuta* could play some role in the population dynamics of these species in late summer–autumn in the Galician coasts, when *D. acuminata* is progressively replaced by *D. acuta*. In this regard, during microscopic examination of samples from a widespread bloom of *Dinophysis* in the Galician Rías (October 2013), 1 *Fragilidium* specimen was observed that had recently ingested *D. acuminata*, but there was not any observation of *Fragilidium* recently fed on *D. acuta* despite the overwhelming dominance of the latter over *D. acuminata* (F. Rodríguez unpubl. data).

In an earlier study, Park & Kim (2010) showed the specific predation of *F. duplocampanaeforme* on several *Dinophysis* species. Some of these, like *D. acuminata* and *D. caudata*, were included in the present study, and our Atlantic *Fragilidium* also fed upon them. In addition, these authors observed feeding on *D. infundibula*, whereas *D. fortii* was not ingested, demonstrating the existence of prey selectivity within Korean *Fragilidium* and *Dinophysis* strains. In this sense, we observed that *D. acuta* was ignored by the Atlantic *Fragilidium* and *D. tripos* was rarely ingested. LSUrDNA analyses of several Portuguese *F.* cf. *duplocampanaeforme* isolates and the 2 *F.* cf. *duplocampanaeforme* strains included in our study (Amorim et al. 2013) clustered them together. Although we did not test the predation of our *Fragilidium* isolates on dinoflagellate genera other than *Dinophysis*, the ingestion of *Ceratium horridum* by a Portuguese isolate of *F. cf. duplocampanaeforme* indicates that this species may prey on other dinoflagellate genera in addition to *Dinophysis*. Park & Kim (2010) showed that their *Fragilidium duplocampanaeforme* did not ingest *Ceratium furca*, but given the selectivity observed toward certain *Dinophysis* species, it would not be surprising that it could ingest other *Ceratium* spp.

Selective feeding experiments using artificial particles showed that predatory dinoflagellates such as *Oxyrrhis marina* select their prey according to its

size, biochemical composition or cell surface charge (Roberts et al. 2011b). Size itself does not explain the selectivity toward certain *Dinophysis* species because, for example, *D. acuta* has a similar or smaller size than *D. caudata* or *D. tripos* and could be easily engulfed by *Fragilidium*. Rather, it seems that some kind of recognition mechanism could regulate this differential feeding on certain *Dinophysis* species. Lectins and carbohydrate-binding proteins have been shown to play a role as phagocytic receptors in protists (Roberts et al. 2006), and these could be part of the recognition mechanism used by *Fragilidium*. This hypothesis needs to be verified by further studies on the cell surface carbohydrate characterization of *Dinophysis* species (Raho et al. in press). Regarding the inability of *F. subglobosum* to ingest *Dinophysis*, there is no information available testing this hypothesis except the observations from the Bay of Biscay reported above.

Influence of local adaptations and toxins on *Dinophysis* predation by *Fragilidium*

In the case of the Mediterranean *F.* cf. *duplocampanaeforme*, it could first be hypothesized that this isolate does not consider *Dinophysis* among its potential prey or secondly, and more suggestively, that some type of local adaptation restrains its feeding on *Dinophysis* originating from the same region. This theory cannot be tested by other means than by feeding experiments including Mediterranean cultures or isolated cells of *Dinophysis*. If the second hypothesis were true, that would suggest some co-evolution of predator and prey within an 'arms race' that seems difficult to be found in planktonic organisms (Roberts et al. 2011a). Indeed, this has been suggested to be the case in some highly specific predator–prey relationships, such as those occurring between protist parasites and their hosts (Figueroa et al. 2010). In marine mixotrophic dinoflagellates exhibiting a certain level of prey specificity, such as *Fragilidium*, that hypothesis could not be totally discarded in favor of general chemical interactions expected in planktonic systems.

Differences in intracellular toxin content for each *Dinophysis* strain do not seem to explain different feeding efficiency and selectivity by *Fragilidium*. Nevertheless, the toxic nature of the prey has been suggested to affect in some way the grazing selectivity of *Fragilidium* cf. *mexicanum* (Jeong et al. 1999). Microalgal toxins are known to produce a variety of allelopathic effects to outcompete other organisms and act as a defense against protistan grazers or to cause cell immobilization before capturing other protists or even metazoan prey (Fistarol et al. 2003, Skovgaard & Hansen 2003, Granéli & Hansen 2006, Sheng et al. 2010, Berge et al. 2012, Blossom et al. 2012). In the case of *Fragilidium*, no PSP or lipophylic toxic compounds have been found in earlier studies or in the present study, but reduced mobility of some of its prey suggests other chemical cues involved in that process. In this sense, protein kinase and G-protein inhibitors have been shown to decrease the motile response and ingestion in a marine ciliate (*Uronema* sp.) and in the dinoflagellate *Oxyrrhis marina* (Hartz et al. 2008). In the present case, it would be the predator that would benefit from these chemical interactions to facilitate the capture of its prey.

Toxin profiles in *Dinophysis* controls coincided with those from the feeding experiments, and the discrepancies observed between the filtered cells and the culture filtrates in the case of *D. caudata* were likely due to detection of a very low biomass in the cells fraction because OA and DTX2 represented a very small percentage of the total toxin content of this species. In the case of *D. tripos*, although we could not include analyses from a monospecific culture, the detection of PTX2 in mixed cultures with *Fragilidium* matched the toxin profile observed for this strain in a previous study (Rodríguez et al. 2012). Overall, toxin analyses indicated that *Fragilidium* did not promote any metabolic transformations of the DsT, in contrast to those found in filter-feeding bivalves that accumulate lipophylic toxins (i.e. seco-acid derivatives of pectenotoxins in blue mussels; Vale & Sampayo 2002, Miles et al. 2006, Wilkins et al. 2006, Pizarro et al. 2013). However, quantitative results in the feeding experiments showed that *Fragilidium* presence was accompanied by a larger proportion of toxins in the extracellular fraction. A possible explanation for this result could be that *Dinophysis* cells release these toxins, although we could only speculate about the possible mechanisms (defense or deterrent mechanism, physical stress or 'sloppy feeding' during *Fragilidium* engulfment of *Dinophysis*, death processes, etc.). The defensive role of dinoflagellate toxins has been strengthened in the case of paralytic shellfish toxin-producers, such as species of the genus *Alexandrium* (Hansen 1989, Bagoien et al. 1996). However, in the case of OA-producing species, Sugg & VanDolah (1999) found that *Prorocentrum lima* had allelopathic effects (growth inhibition) on other dinoflagellates, but compounds other than OA were mainly responsible for these effects.

CONCLUSIONS

The Atlantic *Fragilidium* cf. *duplocampanaeforme* was the only strain able to feed on *Dinophysis* spp. from the Galician Rías, with different grazing efficiencies on *D. acuminata*, *D. caudata* and *D. tripos*. It did not ingest *D. acuta*. Our experiments using 2 strains of *F. duplocampanaeforme* and 1 of *F. subglobosum* showed that prey specificity of the mixotrophic dinoflagellate *Fragilidium* on *Dinophysis* is very different between different species from a similar area (*F. subglobosum* vs. Atlantic *F.* cf. *duplocampanaeforme*) and between different geographical isolates of the same species (Atlantic and Mediterranean *F.* cf. *duplocampanaeforme*). These inter- and intraspecific differences highlight the complexity of predator–prey interactions in mixotrophic dinoflagellates like *Fragilidium*, which display some specific traits that are not likely to be dependent on the prey size but rather depend on the chemical detection of its prey. Further studies are needed to evaluate the existence of local adaptations in the predator, suggested by the inability of the Mediterranean strain of *F.* cf. *duplocampanaeforme* to graze on Atlantic *Dinophysis* strains, and to find out whether isolates exist that are unable to feed on *Dinophysis* from the same area or whether there is some specific predator/prey relationship between *F.* cf. *duplocampanaeforme* and *Dinophysis*. No metabolic transformations of DsT could be attributed to the Atlantic *F.* cf. *duplocampanaeforme*. However, quantitative differences between particulate and extracellular toxin contents between the monospecific *Dinophysis* cultures and feeding experiments point to the possibility that *Fragilidium* promotes a higher release of toxins from *Dinophysis* cells, but the exact mechanism (i.e. cellular stress before or during predation, excretion from *Fragilidium*, etc.) is unknown.

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