

Zooplankton as a potential vector of diarrhetic shellfish poisoning toxins through the food web

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ABSTRACT: This study was carried out during an autumn bloom of *Dinophysis acuminata* in the Galician Rías Bajas (NW Spain). The main objective was to determine whether any of the predominant zooplankton species could ingest and transfer toxins through the pelagic food web. Grazing experiments showed that the copepods *Temora longicornis* and *Oithona nana* and the tintinnid *Favella serrata* fed on *Dinophysis* spp. whereas the copepods *Acartia clausi* and *Euterpina acutifrons* did not. However, only *F. serrata* seem to profit from the ingestion of the toxic dinoflagellates. Field populations of *F. serrata* increased whereas *T. longicornis* and *O. nana* densities declined after the toxic outbreak. Okadaic acid content found in the seston size fractions 100–200, 200–300 and 300–1000 µm showed a good correlation with *F. serrata*. These results confirm the hypothesis that tintinnids can transfer dinoflagellates toxins to higher trophic levels in the pelagic food web. The importance of faecal pellets with undigested dinoflagellates as an alternative pathway to transmit toxins to pelagic or benthic organisms is mentioned.

KEY WORDS: Zooplankton · Dinoflagellates · Toxins · *Dinophysis* spp. · DSP · Ingestion rates

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INTRODUCTION

Grazing zooplankton are potential vectors of phyto-toxins in the pelagic food web (Turner & Tester 1997, Turner et al. 1998). Field observations during blooms of dinoflagellates that produce paralytic shellfish poisoning (PSP) toxins (White 1981, Hayashi et al. 1982), and laboratory experiments with cultures of *Alexandrium* spp., have shown that zooplankton can ingest these toxins and accumulate them in their somatic tissues (Boyer et al. 1985, Turrieff et al. 1995, Teegarden & Cembella 1996). Zooplankton might thus transmit toxins to their predators. Mass mortalities of pelagic fishes such as herring and mackerel (White 1980, 1981, Beaulieu et al. 1996, Montoya et al. 1996, Castonguay et al. 1997) and marine mammals (Geraci et al. 1991,

Landsberg & Stidinger 1998) exposed to toxic dinoflagellate or diatom blooms, and accumulation of PSP toxins in anchovies (Montoya et al. 1998), have been reported. In all these cases, zooplankton presumably acted as a link between toxic phytoplankton and higher trophic levels.

However, there is little information about the interactions between zooplankton and phytoplankton that produce diarrhetic shellfish poisoning (DSP) toxins. This is due to the lack of established cultures of *Dinophysis*, the only phytoplanktonic genus so far known to produce DSP toxins. *Dinophysis* spp. usually occur at moderate concentrations (10^2 to 10^3 cells l^{-1}), and their effects are not as conspicuous as those caused by dinoflagellates which form dense blooms. There is only one report of fish mortality possibly associated with a dense bloom of *Dinophysis caudata* in the Seto Inland Sea of Japan (Okaichi 1967), but the cause of the mortality was not established with certainty.

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Dinophysis acuminata Ehrenberg and other *Dinophysis* spp. are regular components of the phytoplankton in the Galician Rías (NW Spain). They grow every year and render toxic bivalves which leads to closures of harvesting (Reguera et al. 1990, 1995). Maximum concentrations of *Dinophysis* in the area range from 0.5 to 10^5 cells l^{-1} ; here the term 'bloom' will be used for concentrations above 10^3 cells l^{-1} . In a field study, Turner & Anderson (1983) concluded that despite being the dominant species *D. acuminata* was not eaten by zooplankton. In contrast, Carlsson et al. (1995) reported that *D. acuminata* was grazed by 3 copepod species (*Acartia clausi*, *Isias clavipes* and *Centropages typicus*) in Biscayan waters. Significantly more individuals of *A. clausi* died than of the other copepod species, therefore these authors concluded that the okadaic acid content (OA) of *Dinophysis* may have deleterious effects on this copepod species. During a field survey carried out to study the interactions between a *D. acuminata* bloom and the microzooplankton community, Maneiro et al. (1998) found that 2 tintinnids, *Favella ehrenbergii* and *Tintinnopsis campanula*, co-occurred with the maximal abundance of *D. acuminata*. This coincidence suggested that there might be a trophic relationship between *Dinophysis* and the tintinnids.

In this study, the feeding response of the dominant zooplankton species was examined during an autumn bloom of *Dinophysis acuminata* in the Galician Rías. The main objective was to determine whether any of the dominant zooplankton species were capable of ingesting and accumulating DSP toxins.

METHODS

Field study. Samples were collected weekly during a bloom of *Dinophysis* spp. from 8 September to 5 October 1998 at 3 stations in Ría de Pontevedra (Fig. 1).

Five litre Niskin bottle casts were made at 2, 5, 10, 15 and 20 m. One litre aliquots from each depth were routinely concentrated with a 20 μ m mesh net and fixed in Lugol's iodine solution. But when *Dinophysis* was very common (following results of the local monitoring centre), 100 ml samples were fixed without previous concentration. Cell counts were made with an inverted microscope after sedimentation of 25 to 50 ml samples.

Vertical hauls were made with a Bongo net (opening diameter 23 cm, 20 μ m mesh size) fitted with a 'General Oceanic' flowmeter, from 20 m to the surface. These samples were divided into 2 aliquots; one was used to estimate toxin content, and the other for analysis of zooplankton composition. Each sample was sieved to obtain 4 different size fractions: 20–100, 100–200, 200–300 and 300–1000 μ m. Most *Dinophysis* cells were retained in the 20–100 μ m fraction. Samples for zooplankton composition and abundance estimate were preserved in formalin (4%) neutralised with borax. For the toxin analyses, each of the size fractions was resuspended in 1 l of filtered seawater, and four 75 ml subsamples were filtered through a pre-combusted baked glass microfibre filter (0.72 μ m mesh size) and immediately frozen for subsequent DSP toxin analyses.

Toxin analyses. Toxins were extracted by sonication (15 min, twice) of the filters with the seston fractions in 2 ml of 80% methanol. After centrifugation, the supernatant was decanted and the filter was sonicated again with 1 ml of 80% methanol. Supernatants were combined, and after addition of 1 ml of water to 2.5 ml of the methanol extract, toxins were extracted with 4 ml of chloroform. The chloroform extract was dried with Na_2SO_4 and an aliquot was evaporated for HPLC analyses. 200 μ l of 0.2% 9-ADAM methanolic solution was added to the dried seston extracts. The mixture was shaken for 2 min and kept for 1 h at 40°C protected against the light. The ADAM solution was prepared daily from small portions of the reagent kept at -30°C by adding some drops of acetone to facilitate the further solution in methanol, and was then filtered through 0.45 μ m. The clean-up step was identical to that described in Lee et al. (1987). HPLC analysis followed Lee's method (1987) with slight modifications. The fluorescent esters were separated using a Hewlett-Packard 1050 liquid chro-

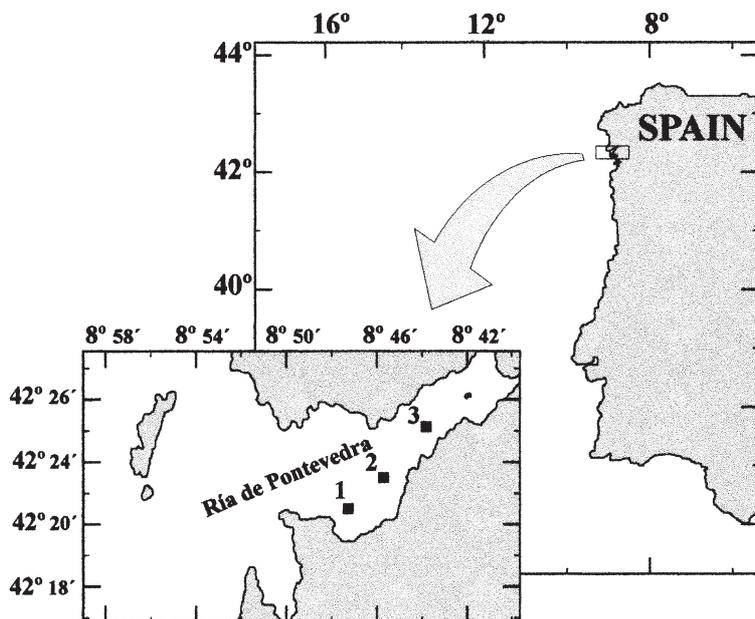


Fig. 1. Map of the Ría de Pontevedra showing location of sampling stations

matograph in a Merck Supersphere 100 column (Lichro-Cart 250-4) and detected in a Hewlett-Packard 1046A fluorescence detector. The mobile phase was acetonitrile:water 80:20, the column temperature was 35°C and the excitation and emission wavelengths were 365 nm and 412 nm, respectively. The flow rate was 1.1 ml min⁻¹. Okadaic acid from the National Research Council (Canada), DTX1 from Calbiochem and DTX2 (generous gift from Kevin James, Ireland) were used as toxin standards.

Grazing experiments. Phytoplankton and zooplankton samples were collected at Stn 3 on 8 September and at Stn 2 on 22 September. Vertical hauls were carried out from 20 m depth to the surface with a 20 µm mesh net. Samples were transported within 2 h of collection to the laboratory, and healthy individuals of the dominant zooplankton species were selected under a stereoscopic microscope for grazing experiments. After sieving through a 75 µm mesh net to remove grazers, the sample was concentrated on a 20 µm mesh and the concentrated material poured into a 1 l beaker and incubated at 18°C for 2 h. The upper part of the water (approximately 300 ml) was subsequently siphoned off into a clean 1 l container to select healthy swimming phytoplankton for the grazing experiment and to avoid sediment and unhealthy cells. These cells were then

resuspended in 0.45 µm Whatman GF/F-filtered seawater to provide *Dinophysis* concentrations ranging between 1 and 24 × 10³ cells l⁻¹ for the grazing experiments. Frost's (1972) equations were used to calculate initial (C_i) and control (C_x) concentrations, and copepod ingestion rates in experimental containers. Table 1 shows C_i and C_x obtained in both experiments. These concentrations were in the range of *Dinophysis* abundance observed in the field during the DSP outbreak (Fig. 2).

In the first experiment on 8 September, individuals of *Favella serrata* and adult females of *Temora longicornis* were isolated from the sample with a stereoscopic microscope, and groups of 10 *F. serrata* individuals and 1 *T. longicornis* were kept in plastic vessels with 20 ml of food suspension. Four replicates were used for each species in each experimental food concentration, and 4 initial trials and 4 controls with food suspension and no zooplankton were used to estimate initial and final phytoplankton concentration, respectively. In the second experiment on 22 September, 6 adult females of *Acartia clausi*, *Euterpina acutifrons* and *Oithona nana* were isolated and placed in individual plastic vessels with 10 ml of experimental food suspension. In both sets of experiments, vessels were kept at 18°C in darkness for 12 h. Zooplankton individuals were checked

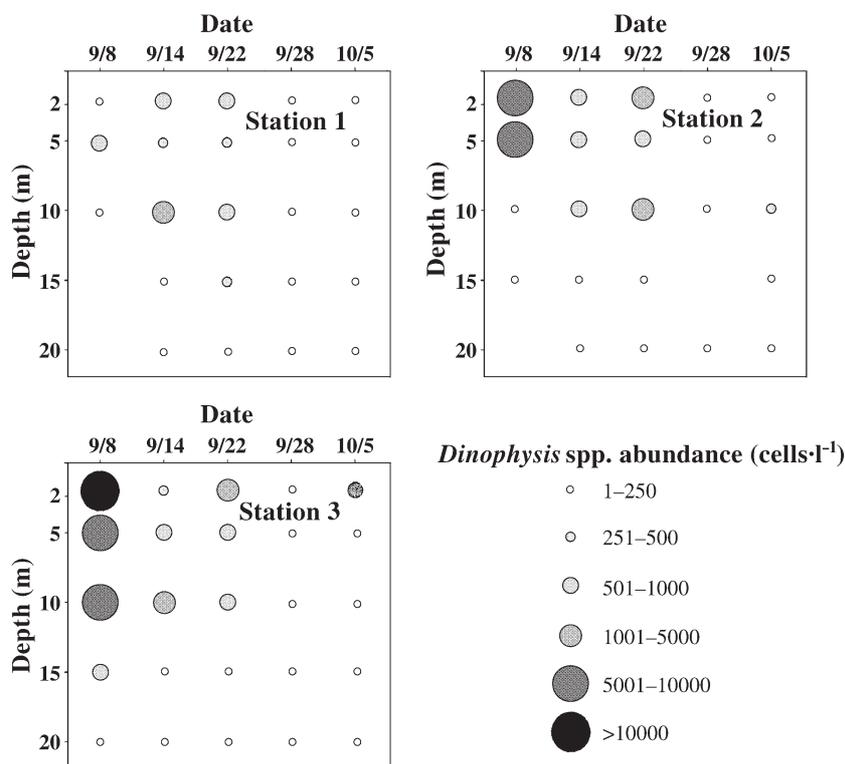


Fig. 2. Vertical distribution of the abundance of *Dinophysis* spp. (cells l⁻¹) in Ría de Pontevedra from 8 September to 5 October (see Fig. 1 for station positions)

Table 1. Initial concentrations (C_i) and average cell concentrations (C_x) of *Dinophysis* spp. in the grazing experiments (mean \pm SE, in cells l^{-1})

Experimental date	C_i	C_x
9 September	1600 \pm 73	1337 \pm 41
	3846 \pm 87	2486 \pm 223
	14091 \pm 891	13751 \pm 473
	24666 \pm 1778	27823 \pm 4405
22 September	8980 \pm 485	9907 \pm 148
	5627 \pm 282	7055 \pm 255
	2667 \pm 162	2760 \pm 203
	882 \pm 69	969 \pm 47

with a stereoscopic microscope at the end of the experiment. Samples were collected and preserved in Lugol's iodine for phytoplankton cell counting with an inverted microscope.

Manly's coefficient (α_i) (Manly 1974) was used to measure dietary preference of zooplankton species for *Dinophysis* spp.:

$$\alpha_i = \frac{\log \frac{e_i}{n_i}}{\sum_{j=1}^m \frac{e_j}{n_j}}$$

where n_i and e_i are the initial and final concentrations of *Dinophysis* spp. cells, n_j and e_j are the initial and final numbers of uneaten prey type j , and m is the number of phytoplankton species. Thus α_i is an estimate of the extent to which *Dinophysis* is selected.

RESULTS

Dinophysis acuminata was the predominant *Dinophysis* spp. during the period of the study with a pooled mean \pm SD considering all stations of 76.5 \pm 18.8 (range 41.9 to 94.7%). *D. rotundata* and *D. caudata* represented a low percentage of total *Dinophysis* population with a pooled mean \pm SD of 19.2 \pm 16.4 (range 4.8 to 55.5%) and 4.3 \pm 6.6 (range 0 to 20.4%), respectively. Fig. 2 shows *Dinophysis* spp. abundance in the water column during the sampling period. The highest values of dinoflagellate abundance appeared between 2 and 10 m at all sampling stations. The maximum cell density was found at Stn 3 on 8 September when 28 \times 10³ cells l^{-1} were observed at 2 m depth. Thereafter, dinoflagellate concentration decreased although the cell abundance remained higher than 10³ cells l^{-1} in the upper 10 m on 14 and 22 September. Concentrations decreased (>500 cells l^{-1}) on 28 September, and this levels remained until 5 October.

The accompanying phytoplankton populations changed over the sampling period. Fig. 3 shows percentage of

the 20–75 μ m size fraction phytoplankton species on 8 and 22 September. *Dinophysis* spp. represented up to 24% of the phytoplankton community on 8 September but less than 1% on 22 September.

Major differences were found in the grazing response of the different zooplankton species to *Dinophysis* spp. (Table 2). There were no significant differences in ingestion rate of *Euterpina acutifrons* and *Acartia clausi* at different concentrations of *Dinophysis*. A mean of 3.5 \pm 4.7 cells female⁻¹ d⁻¹ (mean \pm SE, n = 20) for *E. acutifrons* and 2.7 \pm 3.3 cells female⁻¹ d⁻¹ (mean \pm SE, n = 19) for *A. clausi* were found. However, in the case of *Favella serrata*, *Oithona nana* and *Temora longicornis* a significant increase in ingestion rate was found at increasing concentrations of *Dinophysis* spp. (Table 2, Fig. 4).

Dinoflagellate selection by zooplankton was found also to be species-specific (Fig. 5). *Oithona nana* did not present any trend in α_i for *Dinophysis* spp. However, *Temora longicornis* seemed to graze preferably on

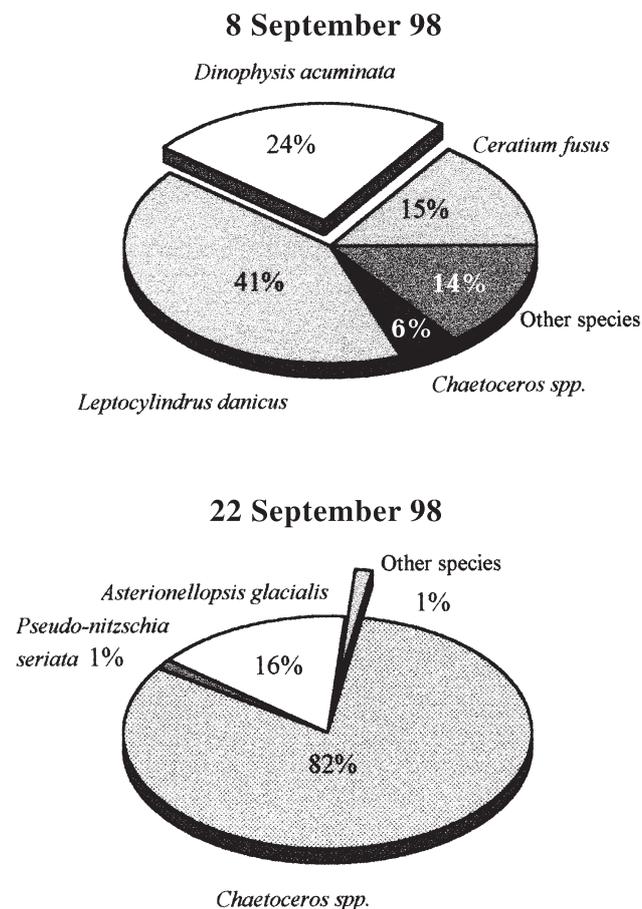


Fig. 3. Phytoplankton species composition of the size fraction 20–75 μ m used as food in the grazing experiments carried out from samples collected at Stn 3 on 8 September and Stn 2 on 22 September

Table 2. Summary of 1-way ANOVA comparing zooplankton ingestion rate on *Dinophysis* spp. as a function of dinoflagellate abundance

Source of variation	<i>Acartia clausi</i>				<i>Euterpina acutifrons</i>				<i>Favella serrata</i>				<i>Oithona nana</i>				<i>Temora longicornis</i>			
	df	MS	F	p	df	MS	F	p	df	MS	F	p	df	MS	F	p	df	MS	F	p
<i>Dinophysis</i> spp. abundance	3	92.1	0.4	0.754	3	36.6	0.1	0.974	3	11121.4	12.8	0.001	3	1273.2	5.9	0.007	3	19721.8	7.2	0.007
Error	15	229.3			16	511.7			11	869.2			16	216.6			10	2729.1		
Total	18				19				14				19				13			

these dinoflagellates as the phytoplankton concentration declined, whereas *Favella serrata* showed an increase in α_i as the phytoplankton abundance increased.

Fig. 6 shows mean abundance of field populations of *Favella serrata*, *Oithona nana* and *Temora longicornis* during the period that *Dinophysis* spp. abundance was higher (on 8, 14 and 22 September). *O. nana* and *T. longicornis* decreased after the bloom of *Dinophysis* whereas *F. serrata* concentrations were higher on 14 September and decreased on 22 September.

Fig. 7 shows the relationship between okadaic acid and *Dinophysis* abundance in the different size fractions and sampling stations obtained on 8 and 14 September. OA was too low to be detected on 22, 28 and 5 October. DTX1 and DTX2 were not detected in any of the size fractions obtained during the period studied. Assuming that the OA present in the 20–100 μm size fraction is only due to the *Dinophysis* spp. cells, it is clear that in the size fractions >100 μm there was more OA than expected from the abundance of *Dinophysis* spp. cells.

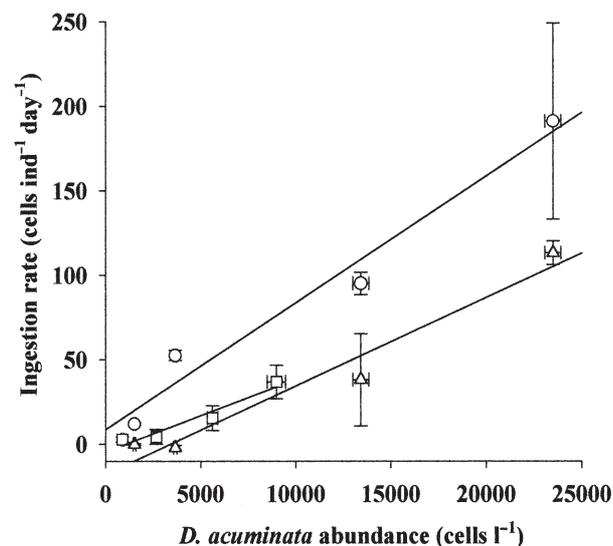


Fig. 4. Ingestion rates of *Oithona nana* (\square), *Temora longicornis* (\circ) and *Favella serrata* (Δ) on *Dinophysis* spp. Slopes are different from zero with a $p = 0.003$ for *O. nana*, $p = 0.025$ for *T. longicornis* and $p < 0.001$ for *F. serrata*

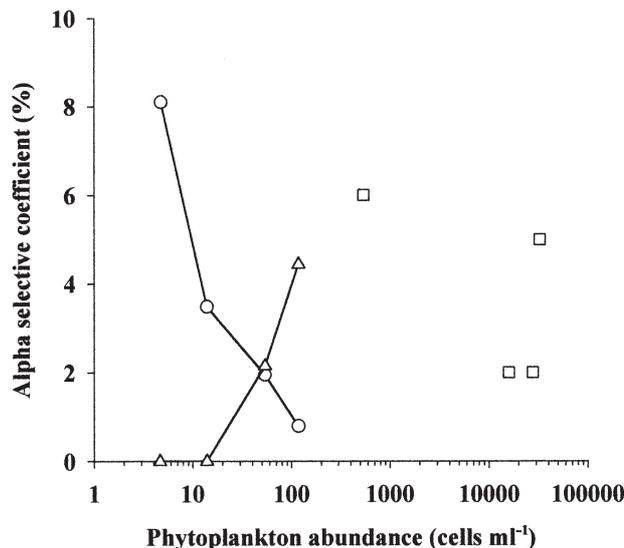


Fig. 5. Relationship between α selective coefficient of *Oithona nana* (\square), *Temora longicornis* (\circ) and *Favella serrata* (Δ) on *Dinophysis* spp. and phytoplankton abundance in the grazing experiments

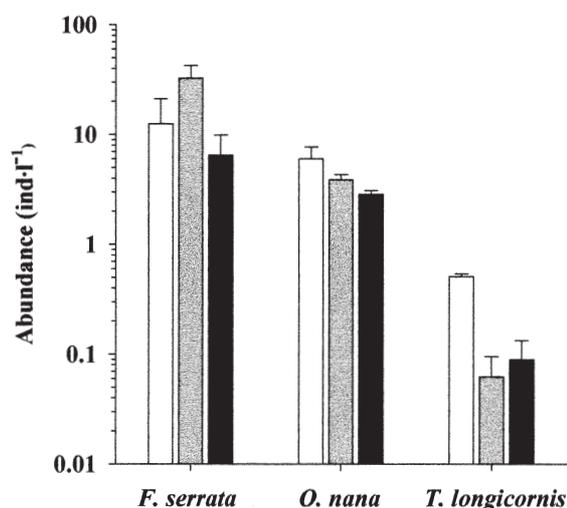


Fig. 6. Mean \pm SD abundance (ind. l^{-1}) of *Favella serrata*, *Oithona nana*, *Temora longicornis* on 8 September (white bars), 14 September (shaded bars) and 22 September (black bars) considering all sampling stations

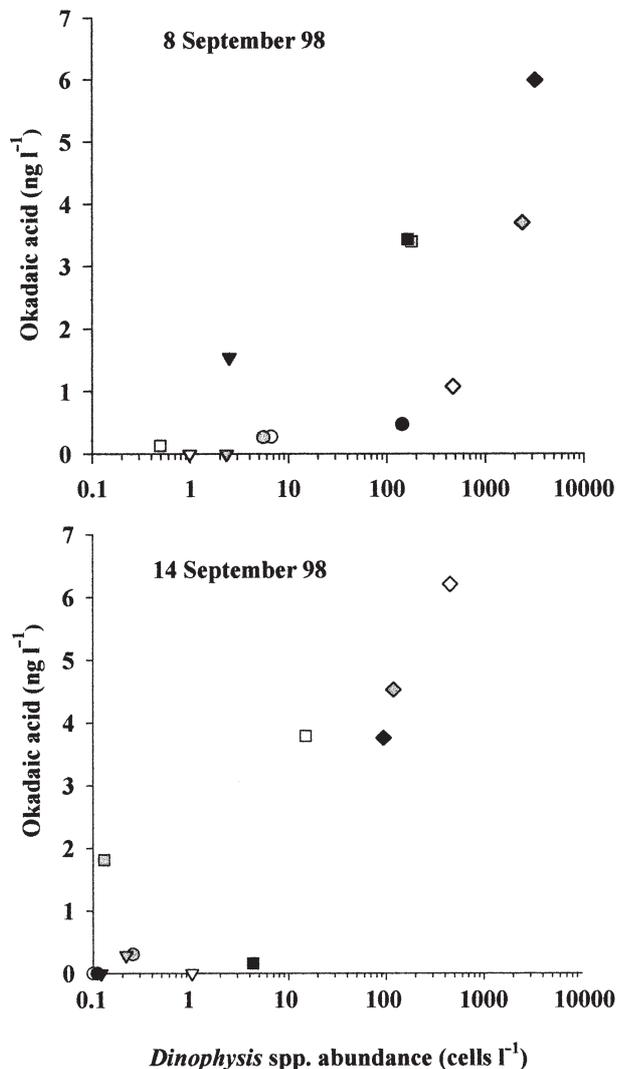


Fig. 7. Relationship between okadaic acid content (in ng l^{-1}) and *Dinophysis* spp. abundance (in cells l^{-1}) of the different seston size fractions 20–100 (\diamond), 100–200 (\square), 200–300 (∇) and 300–1000 (\circ) μm obtained at each station (white symbols are Stn 1, shaded symbols are Stn 2 and filled symbols are Stn 3) on 8 September and 14 September

Toxicity per cell (pg OA cell^{-1}) of *Dinophysis* spp. in the 20–100 μm size fraction was estimated considering the OA content and *Dinophysis* spp. abundance in that fraction. On 8 September a mean \pm SE of 1.8 ± 0.2 ($n = 6$) were found whereas this value increased on 14 September with a mean \pm SE of 30.1 ± 5.0 ($n = 6$). From these values we estimated the OA content which does not correspond to *Dinophysis* cells in the size fraction $>100 \mu\text{m}$, the 'zooplankton OA content' (ZOA). A step-wise multiple regression between the ZOA and the most abundant zooplankton species was performed for each sampling day. On both dates, *Favella serrata* abundance showed a significant positive correlation with

ZOA (Fig. 8). Non-significant results were found with the zooplankton species that did not ingest *Dinophysis* (*Acartia clausi* and *Euterpina acutifrons*) but also with species that ingested toxic dinoflagellates (*Oithona nana* and *Temora longicornis*).

DISCUSSION

Although the average OA concentration per cell of *Dinophysis* showed high variability, it was within the range of variation found in previous studies in the area (Blanco et al. 1995). This variability could result from the variation in the proportion of different *Dinophysis* spp. and to different physiological states of the cells as the bloom declined. Fernández et al. (2000) found large differences of toxicity per cell between different blooms or even between cells of the same species kept in the laboratory for a few days.

OA found in the seston size fraction $>100 \mu\text{m}$ indicates that OA is inside zooplankton that ingested *Dinophysis* spp. cells. Results from the grazing experiments suggest that *Acartia clausi* and *Euterpina acutifrons* did not ingest *Dinophysis* spp. cells. The response of the *E. acutifrons* is similar to the results of Bagøien et al. (1996), who showed that this copepod did not feed on toxic cultures of *Alexandrium minutum*, even when this dinoflagellate was the only food provided to starving adult females. Several studies describe the feeding response of *Acartia* spp. to toxic *Dinophysis* spp. Turner & Anderson (1983) observed that *Dinophysis acu-*

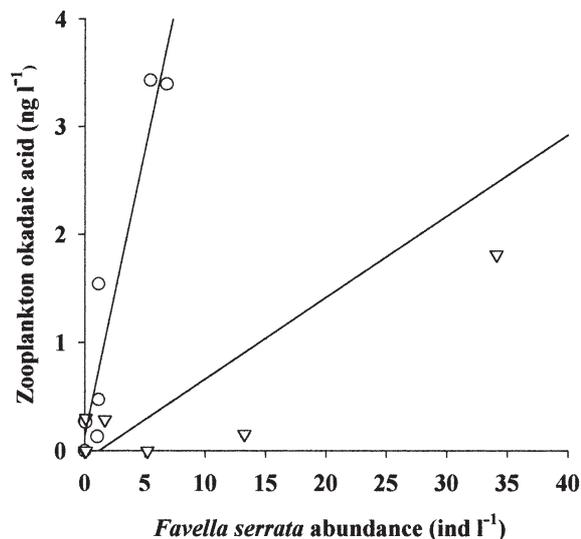


Fig. 8. Relationship between zooplankton okadaic acid content of different seston size fractions and *Favella serrata* abundance on 8 September (∇) and 14 September (\circ). Slopes are different from zero with a $p < 0.001$ on 8 September and $p = 0.001$ on 14 September

minata was not grazed upon by *A. clausi*, whereas Carlsson et al. (1995) found that *A. clausi* grazed significantly on *D. acuminata* when a 20–75 µm concentrated phytoplankton assemblage with a high content of this species was used as food. However, a higher agreement was found in the case of the interaction between *A. clausi* and *Alexandrium tamarense*. Several studies have shown that this copepod species grazed on this toxic dinoflagellate (White 1981, Turner & Anderson 1983, Ives 1985, 1987). Turner et al. (1997) suggested that the lack of co-evolutionary experience in feeding toxic cells could be the reason for the discrepancy in some experimental results. However, Dutz (1998) observed that *A. clausi* females from the German Bight were able to ingest toxic cells of *Alexandrium lusitanicum*, although the copepods had never been exposed to natural blooms of this Iberian strain.

Our results on the feeding response of *Acartia clausi* to *Dinophysis* spp. are in agreement with those obtained by Turner & Anderson (1983). However, Carlsson et al. (1995) observed that *Dinophysis acuminata* was one of the dominant phytoplankton species in the phytoplankton assemblage used as food, whereas in our grazing experiment dinoflagellates represented less than 1% by number of the phytoplankton concentrate. As *A. clausi* is a selective feeder (Donaghay & Small 1979) the accompanying phytoplankton might explain the different results found between both experiments.

In contrast with *Euterpina acutifrons* and *Acartia clausi*, *Temora longicornis* and *Oithona nana* fed on *Dinophysis* spp. However, neither species showed a positive correlation with the ZOA observed in their

size fraction. This suggests that, although they may eat *Dinophysis* spp., and could transmit cells in their guts to predators, accumulation and transmission of DSP through the food web by these zooplankton species is not important. At least in the case of *T. longicornis*, copepods may ingest the dinoflagellates but they do not digest them. This is supported by the observation of undigested *Dinophysis* cells in pellets from *T. longicornis* incubations (Fig. 9). An alternative explanation could be that population density of both copepod species was not high enough to have an important grazing effect on *Dinophysis* spp. In fact, population density of both copepod species decreased the following weeks after the bloom (Fig. 6).

Field studies have shown that some tintinnids, especially *Favella* spp., are often found associated with high *Dinophysis* spp. densities (Santhanam & Srinivasan 1996, Maneiro et al. 1998). Experiments show *Favella* spp. can graze on PSP toxins producing dinoflagellates (Stoecker et al. 1981, Hansen 1989). Results from this study seem to confirm that tintinnids can also ingest dinoflagellates that produce DSP. Moreover, the population density of *Favella serrata* increased considerably after the *Dinophysis* bloom, and a reduction was observed when the density of *Dinophysis* spp. declined (Fig. 6).

Our study also reveals that *Favella serrata* was able to retain OA. However, the slopes of the regression obtained between ZOA and *F. serrata* abundance were different on 8 and 14 September. The fact that the concentration of DSP toxins in tintinnids was not found to be a simple function of the abundance might be explained by the selection pattern showed by *F. serrata* on *Dinophysis* spp. (Fig. 5). Grazing experiments showed that at high concentrations of *Dinophysis* spp. (as on 8 September) *F. serrata* selectively preyed on dinoflagellates, whereas at low *Dinophysis* spp. densities (22 September) it did not graze preferably on dinoflagellates. This agrees with previous results in Ría de Pontevedra, where positive correlation of *Favella* spp. abundance and *Dinophysis acuminata* abundance were found only when a threshold of 1750 cells l⁻¹ was reached (Maneiro et al. 1998).

The high population density reached by *Favella serrata*, together with an active feeding selection on *Dinophysis* spp. at high concentrations could be the reason why, in this study, this microzooplankton species accumulate DSP.

The egestion of intact *Dinophysis* cells in faecal pellets of *Temora longicornis* could be interpreted as an alternative mechanism of toxin transfer that should be evaluated. These pellets with toxic cells within them can be eaten by coprophagous organisms in the pelagic food web. Alternatively, sedimented pellets can be eaten by a wide community of zooplanktonic

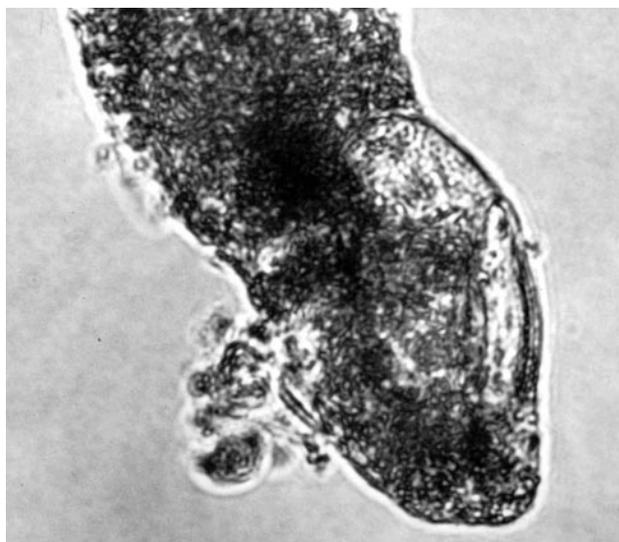


Fig. 9. Pellet of *Temora longicornis* with undigested *Dinophysis* spp. cell inside

organisms (ciliates, harpacticoid copepods, etc.) that are abundant on sediments rich in organic matter. Furthermore, the 'toxic pellets' can be eaten by detritivorous bivalves and other benthic species, that also become toxic during intense DSP episodes in the area. In this way, zooplanktonic organisms that do not assimilate the toxic dinoflagellate cells would make toxins from *Dinophysis* spp. available to bottom dwellers via sedimentation of faecal pellets.

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