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Impact of a *Dinophysis acuminata* Bloom on the Copepod *Acartia clausi*: First Indications

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Abstract: Faecal pellet production and content along with egg production of the dominant copepod species *Acartia clausi* were studied in the Thermaikos Gulf (NW Aegean Sea) during a pre-bloom and a bloom of the toxic dinoflagellate *Dinophysis acuminata*. Both faecal pellet production (6.8–8.6 ind^{-1} d^{-1}) and egg production (15.8–47.6 ind^{-1} d^{-1}) appeared unrelated to the *D. acuminata* bloom. Less than 11% of the copepod faecal pellets contained one or two *D. acuminata* cells, almost intact, whereas the other material in the pellets was broken into small pieces or amorphous shapes. The toxin outflux seemed to be insignificant when compared to the mean toxin concentration from the whole *D. acuminata* population. Finally, the potential grazing impact of *A. clausi* on *D. acuminata* during the study period was low.

Keywords: *Acartia clausi*; *Dinophysis acuminata*; egg production; faecal pellet; HAB

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1. Introduction

Harmful algal blooms (HABs) affect coastal marine ecosystems, on lower (plankton, bivalves) and higher trophic levels (fishes, birds and whales), as well as human health and cause large economical losses. In the Thermaikos Gulf (NW Aegean Sea), HABs result in substantial socio-economic impacts (economic losses of ~3 million euros every year), since the harvest of farmed mussels is banned for several weeks [1].

In the zooplankton–HAB blooms relationship there is a lack in our knowledge concerning the fate of toxic dinoflagellates ingested by zooplankton and outcomes appear situation-specific: redistribution in grazer tissues (e.g., [2,3]), eggs [4] or faecal pellets [5,6]. Faecal pellets could have a significant role in toxin transfer [6], as they are often the most important vector among all copepod products (reviews [7,8]).

Several studies indicate that phytoplankton toxicity is an adaptation of algae to escape grazing and toxic cells are selectively avoided by zooplankton when feeding on mixtures of different prey species (e.g., [9]). This avoidance is related to toxic phytoplankton affecting grazing, egg production and hatching rates (review by Turner and Tester [10]). Many studies on the harmful effects of phytoplankton on grazers have focused either on feeding activity (e.g., [5,11–14]) or on egg production and hatching rates (e.g., [15–18]). However, few studies have considered all these processes at the same time [4,14,19]), which is necessary if we are to fully evaluate the effects of harmful algae on grazers.

There is also little information on interactions between planktonic grazers and algae producing diarrhetic shellfish poisoning (DSP) toxins, due to unsuccessful attempts at

cultivation of the *Dinophysis* Ehrenberg genus [20]. The main DSP toxins (Okadaic acid (OA) and *Dinophysis* toxins) are produced usually by dinoflagellates that belong to the genera *Dinophysis* spp. [21] and epibenthic species of the dinoflagellate genus *Prorocentrum;* however, only the latter have been grown in cultures. Therefore, although *Prorocentrum* species have been used widely in studies on the transfer and fate of OA (e.g., [22–24]), field studies on the pelagic component of the food web (i.e., *Dinophysis* species) are important, in particular during bloom conditions.

Some copepod species graze on *Dinophysis* spp. [5,6,19,25,26], while some others do not (e.g., [5,19,26,27]). For *Dinophysis acuminata* Claparède et Lachmann, some grazing experiments have concluded that it is eaten by *Acartia clausi* Giesbreeht [25], whereas others have not [5]. Only a few studies have investigated what happens after the copepod ingestion of *Dinophysis* spp. cells, by examining if the faecal pellets contain cells [6,19,26]. These studies have dealt with the copepods *Temora longicornis* feeding on *Dinophysis* spp. [26] or *Temora longicornis*, *Calanus helgolandicus* and *Acartia* sp. feeding on *D. norvegica*. [6,19]. Finally, *D. norvegica* cells were not observed in the pellets produced by *Acartia* sp. [6,19].

The present study is the first attempt to understand the impact of a *D. acuminata* bloom on *A. clausi*. For this purpose, we examined, for the first time, the *A. clausi*'s faecal pellet production, egg production, toxin egestion and the occurrence of *D. acuminata* cells in pellets during the presence of *D. acuminata* in the sea water. Thermaikos Gulf, an area with little information concerning HABs, was chosen as the study site. In this area, *D. acuminata* blooms have been recorded during late winter–early spring [28,29].

2. Materials and Methods

Samples were collected from the inner Thermaikos Gulf (40°30′86″ N, 22°53′15″ E; NW Aegean Sea, E Mediterranean) during the first week of March 2003 and March 2004. Seawater samples (volume: 0.5–1.0 L) were collected from 2, 10 and 15 m depths. After GF/F filtration, chlorophyll (Chl-*a*) was determined by fluorometric measurements of acetone extracts [30] in a TURNER Designs TD-700 fluorometer. For phytoplankton composition analysis, seawater was collected from 2, 5, 10 and 15 m depth. Samples were fixed with alkaline Lugol solution and stored at 4 °C until analysis. Phytoplankton identification and counting were performed using the Utermohl method [31]. Conversion from *Dinophysis* cells to carbon was done using a value of 1194 pg C cell^{-1} [32]. Conversion of Chl-*a* to carbon was done with a conversion factor C/Chl-*a* equal to 50 [33,34].

For OA determination, the method used was based on Zhou et al. [35]. The sample was extracted with aqueous 80% methanol; distilled water was added and extracted again with dichloromethane. The extracts were cleaned-up with SPE (Solid Phase Extraction on silica). The clean eluate was derivatized with a mixture of 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (Fluka) and N-Ethyl-diisopropylamine solutions (0.1% in acetone). Analysis was performed with HPLC and fluorescence detection with isocratic conditions, and the mobile phase was acetonitrile/water 65:35. The OA concentration was calculated using a calibration curve based on injections of standard OA solutions.

Zooplankton for identification was collected with oblique tows from the bottom (~15 m) to the surface, using a 200 μm WP-2 net with a non-filtering cod end. The content of the cod end was fixed immediately and preserved in a 4% buffered-formaldehyde seawater solution. In the lab, all individuals were identified and counted in an aliquot (1/4 or 1/2) of the whole sample, which was obtained with a Folsom plankton sample splitter.

Copepods for the egg and faecal pellet production experiments were also collected by oblique tows within the 0–15 m layer, using a 200 μm WP-2 net equipped with a large non-filtering cod-end (10 L). On deck, the content of the cod end was diluted in a 25 L thermo-box containing seawater collected from the surface and 10 m depths, which was brought, within two hours, to the lab for estimation of the egg and pellet production.

There, *A. clausi* females were sorted under a dissecting microscope, and three to four undamaged individuals were transferred to each of ten 620 mL screwcap polycarbonate bottles filled with prescreened well-mixed seawater (150 μm) from the surface and 10 m

depth. In addition, six bottles without animals were used as controls. All bottles (5 + 3 for eggs and 5 + 3 for pellets) were left for 24 h in ambient temperature and dim light photoperiod. After that, eggs and faecal pellets were collected using a sieve (60 and 20 μm for eggs and pellets, respectively) and counted.

Eggs were kept for another 48 h in filtered (GF/F) seawater to estimate hatching success. *A. clausi* faecal pellets were kept for examination of their content. Faecal pellets were placed for 2 h in 2% glutaraldehyde in seawater, rinsed three times with seawater and placed for 30 min in a solution of 0.5% OsO_4 in seawater (all seawater used was filtered using GF/F). Faecal pellets were rinsed three times with filtered distilled water (0.45 μm) and were placed on glass plates covered with gelatine, after which they were dehydrated gradually from 30% to 100% ethanol. The samples were then dehydrated by CO_2 critical point drying system and coated with Au-Pd or Pt (20 nm). Pellets were observed in a scanning electron microscope (SEM) under 20 kv accelerating voltage and their content recorded.

3. Results and Discussion

D. acuminata densities were low during the first period (March 2003) of this study (<100 cells L^{-1}) whereas in the second period (March 2004) they reached 10,700 cells L^{-1}. The 2003 period represents a pre-bloom situation as the bloom occurred from April to May. The 2004 period, represents a bloom situation, which occurred between February and March. This is consistent with the *D. acuminata* blooms period in the Thermaikos Gulf mostly occurring between late December and early May (as reported from 2000 to 2004) with densities varying from ~2000 to 85,000 cells L^{-1} [29,36]. Although the percentage of *D. acuminata* cells in both years was always less than 1% of the total phytoplankton density, in 2004 it made up to 18% of the total phytoplankton carbon (Table 1).

Table 1. The range of Chl-*a*, phytoplankton density and *D. acuminata* parameters over the sampling period.

Period (March)	2003	2004
Chl-*a* (μg L^{-1})	4.1–5.3	0.2–2.7
Total phytoplankton density ($\times 10^5$ cells L^{-1})	19.0–46.0	9.9–28.8
Range of *D. ac.* density (cells L^{-1})	<100	600 to 10,700
D. ac.% in total phytoplankton (% cells)	<0.01	<0.5
D. ac.% in total phytoplankton (% carbon)	<0.06	1.7–18
OA in *D. acuminata* cells (pg $cell^{-1}$)	3.7–8.6	4.4–14.0

In 2003, mesozooplankton abundance included cladocerans (28–50%), appendicularians (30–42%) and copepods (6–12%); whereas in 2004 included lamellibranch larvae (28–64%), appendicularians (11–37%) and copepods (21–27%). *A. clausi* was the dominant copepod for both years, constituting approximately ~60% of the total copepods abundance (Figure 1). *A. clausi* is the typically dominant copepod in this area during winter and early spring [37,38].

The *A. clausi* egg production rate was much higher in 2004 than in 2003 ($F = 5.48$, $p < 0.05$, $n = 8$) indicating no or a not detectable effect by the higher density of *D. acuminata* recorded in 2004. Concerning hatching success, the lack of measurements in 2003 does not allow us to compare with the values obtained in 2004, which fall within the literature range for *A. clausi* feeding with non-toxic (e.g., [39–41] and toxic food (*Alexandrium minutum*: [4]). Clearly, the increase of the egg production rate in 2004 could be related to the food quality rather than the food quantity. This is supported by the fact that the faecal pellet production rate between the two periods was similar (Table 2), despite the decrease of phytoplankton density in 2004 (Table 1).

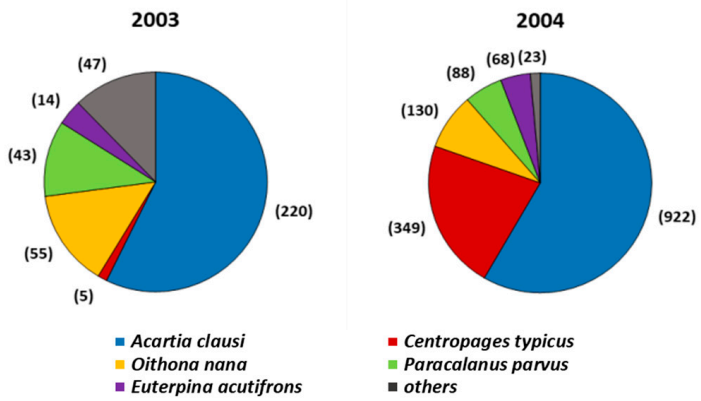

Figure 1. The mean water column copepod abundance (ind m^{-3}) during early March 2003 and March 2004 in Thermaikos Gulf.

Table 2. The mean water column values of *A. clausi* parameters over the sampling period (mean ± SE).

Period (March)	2003	2004
faecal pellet production (pellets ind^{-1} d^{-1})	6.8 ± 2.6 (n = 9)	8.6 ± 2.8 (n = 3)
egg production rate (eggs ind^{-1} d^{-1})	15.8 ± 5.4 (n = 7)	47.6 ± 9.0 (n = 4)
egg hatching success (% d^{-1})	Not measured	69.1 ± 3.7 (n = 5)
D. acuminata cells per faecal pellet	0.05 ± 0.2 (n = 30)	0.11 ± 0.4 (n = 53)

On the other hand, the relative densities of dominant phytoplankton groups between the two periods were comparable (Figure 2). The *A. clausi* pellets examined in both periods, contained essentially broken *Chaetoceros* sp. and *Pseudonitchia* sp. (covering approximately 80% of the surface examined, the rest being amorphous material), indicating a lack of any striking phytoplankton differences in the feeding conditions. Concerning the microzooplankton food component, it was essentially composed by tintinnids (90–150 μm) in 2003, whereas in 2004, small aloricate forms (mainly oligotrichida) dominated (70% being <50 μm) (Giannakourou, A., unpublished data).

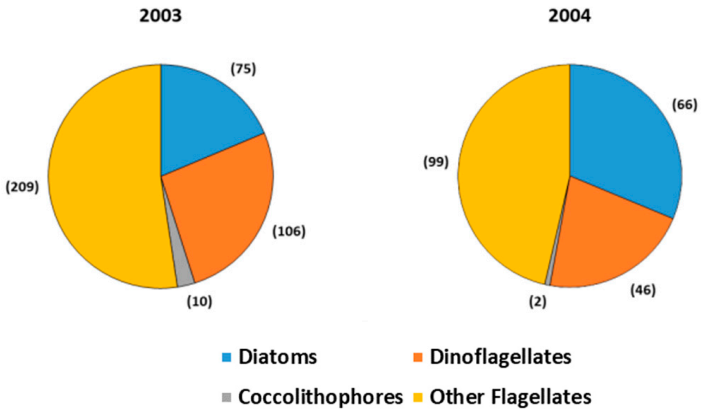

Figure 2. The mean phytoplankton composition (cells 10^4 L^{-1}) during early March 2003 and 2004 in Thermaikos Gulf.

In both periods, no tintinnid loricates were observed in *A. clausi* pellets. Our hypothesis was that the amorphous material of the pellets is originated essentially from oligotrichida forms, as *A. clausi* prefers oligotrichous ciliates, when feeding on a culture of mixtures of oligotrichous ciliates, dinoflagellates and diatoms [42]. Hence, the dominance of small oligotrichous ciliates in 2004 might sustain the much higher *A. clausi* egg production, masking, at the same time, a possible impact of the *D. acuminata* bloom.

However, beyond these speculations, a robust explanation for this increase cannot be safely formulated, as the *A. clausi* egg production rate is affected in opposite ways depending on the different food species and their mixture [40]. Finally, although the *D. acuminata* bloom reached up to 10,700 cells L^{-1} in 2004, it appeared that, without its dominance in the available food items, no impact on *A. clausi* egg and faecal pellet production could be identified.

No *D. acuminata* cells were found in the *A. clausi* pellets examined during the pre-bloom period (2003), which is clearly related to the low density of *D. acuminata* in the phytoplankton community in terms of both cells and carbon (Table 1). During 2004, 11% of the pellets, included one or two intact *D. acuminata* cells, which corresponds to an egestion rate of ~1.0 *D. acuminata* cell ind^{-1} d^{-1} (considering the pellet production rate in 2004; Table 2). This could suggest that these cells were occasionally ingested by *A. clausi* and could explain why grazing experiments at similar *D. acuminata* cell densities and percentages of the total phytoplankton density, as in the present study, showed a low ingestion rate, concluding that *D. acuminata* was not ingested by *A. clausi* [5].

The SEM micrographs (Figure 3) showed that all *D. acuminata* cells in the pellets were almost intact (only one slightly open), thus, incompletely digested. In contrast, the other material accompanying the intact *D. acuminata* cells (essentially *Chaetoceros* sp. and *Pseudonitchia* sp.) was broken into small pieces, and no *D. acuminata* or other dinoflagellate fragments were present, indicating a good digestion. Incomplete digestion of *Dinophysis* has been also found for *Calanus helgolandicus* feeding on *Dinophysis norvegica* [6,19] and *Temora longicornis* feeding on *Dinophysis* spp. [5,26].

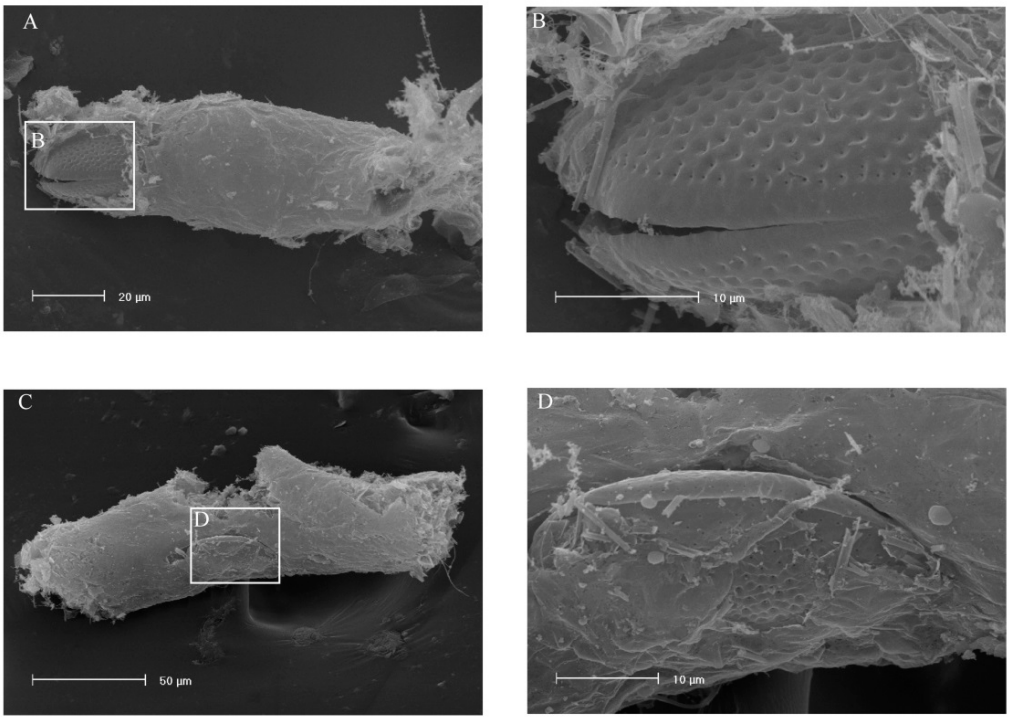

Figure 3. Scanning electron micrographs of *A. clausi* faecal pellets content collected in the Thermaikos Gulf. (**A,C**): faecal pellets covered partially with the peritrophic membrane showing a *Dinophysis acuminata* cell magnified in micrographs (**B,D**), respectively.

The toxin outflux by *A. clausi* was calculated, during the bloom in 2004, when *D. acuminata* cells density was high and the species represented up to nearly 20% of the total phytoplankton biomass. For that purpose, we used the given *A. clausi* egestion rate (1.0 *D. acuminata* cell ind^{-1} d^{-1}), the mean values of *D. acuminata* density, *A. clausi* abundance and measured *D. acuminata* cell toxicity (Table 1).

For the calculations, depth integrated values of *D. acuminata* density and *A. clausi* abundance were used (a 15 m water column considered as representative of the area). The resulting toxin outflux from the *A. clausi* population in the study area was close to 110 ng OA m^{-2} d^{-1} or lower depending on the retention of OA by *A. clausi*. This retention is probably low, as it is for the DSP toxins ingested by *A. clausi* [43] and as for *Centropages typicus* feeding on *D. acuta* [44]. The resulting toxin outflux is insignificant compared to the mean toxin concentration from the whole *D. acuminata* population in the Thermaikos Gulf (888×10^3 ng OA m^{-2}).

The potential grazing impact of the dominant copepod *A. clausi* was estimated from literature values of its ingestion rate, feeding upon *D. acuminata* at similar conditions as in the present study. In such conditions (i.e., *D. acuminata* representing <1% of the phytoplankton cell numbers), the ingestion rate is low (2.7 ± 3.3 cells ind^{-1} d^{-1}, [5]). Assuming that such a low ingestion rate is also the case in the Thermaikos Gulf, then the grazing impact of *A. clausi* on *D. acuminata* during the study period was low (close to 0.01% per day). This is probably also valid for other years.

In fact, maximum abundances of *D. acuminata* usually reported in Thermaikos Gulf, range from 50,000 to 85,000 cells L^{-1} [29,36,45], although patches with densities of *D. acuminata* as high as 1.0×10^6 cells L^{-1} were recorded in the port of Thessaloniki (Thermaikos Gulf) in April 2004 [29]. Within this range, even if *A. clausi* increases its ingestion rate up to ~200 cells ind^{-1} d^{-1} (which can happen above 30,000 cells L^{-1} of *D. acuminata*: [25]) and is present at the maximum density reported in the area (4500 ind m^{-3}: [46]), the grazing impact would still be low (close to 1% per day).

4. Conclusions

Summarizing the outcomes of this study, the faecal pellet and egg production of the dominant copepod *A. clausi* was not associated with the *D. acuminata* bloom in Thermaikos Gulf. Despite the blooming *D. acuminata* densities, reaching 10,700 cells L^{-1} during the experiment (or 85,000 cells L^{-1} during the phytoplankton growth period of that year), it is hypothesized that *A. clausi* feeds upon other prey, such as ciliates. The literature ingestion rate values at similar conditions also indicate a low grazing impact on *D. acuminata* cells. Considering that *D. acuminata* was not dominant in the available food items, this could be also related to the differential digestion of the toxic cells.

As also indicated by the *Dinophysis* cells condition in the faecal pellets, it is suggested that the ingestion of toxic cells does not always mean their digestion and impact on the grazer, such as in egg production. In addition, the toxin outflux through the *D. acuminata* cells found in *A. clausi* faecal pellets was negligible compared to the toxin content of *D. acuminata* cells in the water column. Finally, the output of this study can significantly contribute as a base for future research on the interactions between planktonic grazers and algae producing DSP toxins, considering all available food items as well as food selectivity.

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Conflicts of Interest: All authors declare that they have no conflict of interest to disclose in the context of this study.

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