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Effect of dissolved domoic acid on the grazing rate of krill *Euphausia pacifica*

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ABSTRACT: Some amino acids released from phytoplankton into the water are known to be feeding stimulants to zooplankton. However, domoic acid (DA), a neuroexcitatory amino acid released by some species of the diatom genus *Pseudo-nitzschia*, has the potential to be a feeding deterrent due to its neurotoxicity. Euphausiids (krill) are important members of the zooplankton grazer community and key prey items for many high level carnivores in the world's oceans. Although it is known that krill do consume toxic *Pseudo-nitzschia* spp. during blooms, there is currently no information concerning the effect of dissolved DA on the rate of feeding or the grazing behavior of krill. Therefore, we conducted experiments in which *Euphausia pacifica* were fed non-toxic *P. pungens* along with different added levels of either dissolved DA or glutamic acid (GA), an amino acid with a similar structure to DA. Krill grazing rates did not change significantly with added GA, but decreased significantly in the presence of DA. The rapid response of krill to dissolved DA suggests that either direct toxic action on the filtering apparatus or a chemoreceptor-like mechanism may suppress grazing in the krill. We suggest that these dissolved DA concentrations are relevant, given the DA concentrations found in *Pseudo-nitzschia* spp. populations in Monterey Bay and the feeding biology of krill. In either case, the suppression of feeding by dissolved DA suggests that high dissolved DA concentrations, should they occur in the field, may be a mechanism whereby blooms are perpetuated due to reduced losses to grazers such as krill.

KEY WORDS: Dissolved amino acid · Domoic acid · Glutamic acid · *Pseudo-nitzschia* · Krill · *Euphausia pacifica* · Feeding response

INTRODUCTION

Toxins in phytoplankton are examples of secondary compounds produced from primary metabolic activities. Algal toxins are chemically diverse and their functions are still a topic of considerable speculation. While some secondary compounds can be released by phytoplankton into the water to sequester nutrients and vitamins (Fogg 1966, 1983), others can be retained in cells and used as feeding deterrents to marine grazers such as copepods (reviewed in Paul 1987, Hay & Fenical 1988, Turner & Tester 1997). However, the function of many commonly produced algal toxins is currently unknown. Recently, species of the diatom genus *Pseudo-nitzschia* have been found to produce the neurotoxin domoic acid (DA), a potent water-soluble, neuroexcitatory amino acid that can cause detrimental effects to animals (Bates et al. 1989a,b, Wright et al. 1989, Lefebvre et al. 1999, 2001, Scholin et al. 2000). This highly toxic secondary amino acid functionally mimics glutamate, a primary neurotransmitter of the central nervous system. The importance of DA for *Pseudo-nitzschia* spp., however, is still unclear, although a few studies have suggested potential roles for DA such as enhancement of iron chelation and/or copper detoxification (Ladizinsky & Smith 2000, Rue & Bruland 2001).

The diatoms *Pseudo-nitzschia* spp. are common worldwide, especially in coastal areas such as California, USA, Chile and Australia (Buck et al. 1992, Hasle

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1994, 1995, Hasle et al. 1996). In Monterey Bay, California, primary consumers such as planktivorous fishes can pass on the toxin from dense blooms of *P. australis* to their marine bird and mammal predators (Fritz et al. 1992, Work et al. 1993, Walz et al. 1994, Lefebvre et al. 1999, Scholin et al. 2000). Euphausiids (krill) are also important prey for many types of marine nekton. As herbivores they can play a critical role as direct vectors of phytoplankton toxins to higher trophic levels in pelagic food webs. The principal predators of Pacific euphausiids are planktivorous fishes (Chess et al. 1988, Starr et al. 1998, Yamamura et al. 1998), squid (Morejohn et al. 1978), baleen whales (Ponomareva 1966, Schoenherr 1991, Fiedler et al. 1998), and some seabirds such as Cassin's auklets (Ainley et al. 1996). Previous studies have shown that krill feed on toxic *Pseudo-nitzschia* spp. and that the quantities of cells consumed per unit weight and the feeding pattern over time are dependent on the species of euphausiid and the cellular DA content of the food (Bargu & Silver 2003, Bargu et al. 2002, 2003). These studies demonstrated that *Euphausia pacifica*, the most common euphausiid species in the California Current system, consumed both toxic and non-toxic *Pseudo-nitzschia* spp. at approximately the same rate in the laboratory. However, the temporal feeding pattern of krill on the toxic diatom species differed from that of the temporal feeding pattern of krill on the non-toxic diatom species, and may have contributed to the maintenance of toxin body burdens below a certain threshold, where no immediate ill effects were apparent (Bargu et al. 2003). Toxin concentrations similar to those found in krill in the laboratory study have also been found in field-collected specimens (Bargu et al. 2002), suggesting that krill may have a DA threshold above which ingestion of toxic cells is inhibited.

The goal of the present study was to determine the effect of dissolved DA on a key zooplankter, the euphausiid *Euphausia pacifica* (krill). We tested its feeding responses to non-toxic *Pseudo-nitzschia pungens* when varying levels of dissolved DA were added. We also compared the feeding responses of krill in the presence of dissolved DA versus dissolved GA, a naturally occurring amino acid of similar structure, in order to examine the specificity of the response.

MATERIALS AND METHODS

Field collection of krill. *Euphausia pacifica* were collected opportunistically at night from surface waters at various locations overlying the submarine canyon in Monterey Bay, California, USA, using 0.7 m bongo nets fitted with 333 µm mesh (Ocean Instruments). After krill were transported to the laboratory in 20 l plastic buckets filled with unfiltered seawater, samples were sorted by species and size, and healthy-looking, active, adult individuals (6 to 8 in each bucket) were placed in 4 l buckets containing 0.2 µm-filtered seawater. The krill were acclimated to laboratory conditions for 5 d prior to experiments at 11°C in the dark. Molts and fecal pellets were removed daily and live specimens were fed every other day with low concentrations of a mixed culture of *Thalassiosira* sp. and non-toxic *Pseudo-nitzschia pungens*.

Phytoplankton cultures and toxin analysis. A Monterey Bay clone of non-toxic *Pseudo-nitzschia pungens* (provided by P. Miller) served as a food species in the experiments. Cultures were grown in f/2 medium (Guillard & Ryther 1962) in 2 l flasks, at 16°C on a 12:12 h light:dark cycle. Cellular DA concentrations of *P. pungens* were measured via high-performance liquid chromatography of the fluorenylmethoxycarbonyl (HPLC-FMOC) method described by Pocklington et al. (1990), which involves pre-column derivatization of DA in cell extracts with 9-fluorenylmethylchloroformate (FMOC) reagent to form the fluorescent FMOC-derivative of DA. We filtered 3 aliquots of 100 ml each from batch cultures at their early stationary (9 d old) phase onto 3 µm pore-size, 25 mm polycarbonate filters and immediately froze them at –20°C until analysis.

Expt 1: 24 h grazing experiment with additions of varying levels of dissolved DA. In order to determine whether krill grazing rates change in the presence of various concentrations of dissolved DA, a 24 h feeding experiment was conducted. *Euphausia pacifica* were fed non-toxic *Pseudo-nitzschia pungens* in the presence of added dissolved DA. Krill were starved for 12 h prior to the experiment. Experiments were conducted in a temperature-controlled room (11°C) in the dark beginning at 19:00 h, to mimic ambient conditions during feeding. Exposure concentrations of 400 and 1000 µg DA l^{-1} were prepared by dissolving DA in grazing containers filled with 1.5 l of 0.2 µm-filtered seawater (n = 3 for each treatment), a concentration readily detected by our analytical technique; as controls 3 additional grazing containers were filled with 1.5 l of 0.2 µm-filtered seawater and no added DA. The diatom *P. pungens* and 4 individual *E. pacifica* were added to each container. Another set of control containers (n = 3) for each treatment consisted of algae without krill, allowing measurement of potential changes in diatom cell number not related to grazing. Krill were allowed to feed on *P. pungens* (a local species that does not produce DA) at an initial concentration of 2.6×10^6 cells l^{-1} for 24 h. At each 6 h time interval, three 50 ml water aliquots were withdrawn from each container and filtered onto 25 mm GF/F filters (Whatman). The filters were then extracted for 24 h in 90% aqueous acetone, and subsequently analyzed for

chlorophyll *a* (chl *a*) using a Turner fluorometer (Parsons et al. 1984). Krill were removed individually at the end of the experiment, rinsed 3 times with distilled water, and dried for 24 h at 60°C in an oven. Weights were determined on a Mettler analytical balance. The feeding results were then standardized using the weights of individual krill specimens, and rates recorded per mg krill dry weight. All krill specimens used in the experiment were either adult males or non-gravid females.

Ingestion rates were calculated from changes in chl *a* concentrations in the feeding containers relative to changes in chl *a* concentrations in control bottles using Frost's (1972) equations. Chl *a* values were then converted to cell numbers using laboratory determined values of chl *a* cell^{-1} measured in cultures obtained immediately prior to the feeding experiment.

Expt 2: 9 h grazing experiment with additions of dissolved GA and DA. The protocol for this experiment was the same as that described in the last subsection, with the following changes: of 9 grazing containers filled with 1.5 l of 0.2 µm-filtered seawater, 3 had dissolved GA added to produce a final concentration of 600 µg GA l^{-1}, 3 had dissolved DA added to produce a final concentration of 500 µg DA l^{-1}, and 3 control grazing containers had no added amino acids. The diatom *Pseudo-nitzschia pungens* and 2 individual *Euphausia pacifica* were added to each of the 9 containers; 3 additional containers were filled with 1.5 l of 0.2 µm-filtered seawater to determine potential phytoplankton growth alone in the absence of grazers. In grazing containers, krill were allowed to feed on *P. pungens* (initial concentration of 2.6×10^6 cells l^{-1}) for 9 h. At each 3 h time interval, three 50 ml water aliquots were withdrawn from each container and filtered onto 25 mm GF/F filters for chl *a* analyses. Krill were removed individually at the end of 9 h and processed to measure dry weight as described above.

Dissolved DA detection via HPLC-UV. Dissolved DA levels were measured from 1 ml samples taken from each exposure container at each time point during the grazing experiments. DA analyses were performed using an isocratic elution profile on a Hewlett-Packard 1090 HPLC equipped with a diode array detector (DAD) set at 242 nm with a bandwidth of 10 nm. The reference signal was set at 450 nm with a bandwidth of 10 nm. A reverse-phase Vydac C_{18} column (Catalog No. 201TP52, 2.1 mm × 25 mm, Separations Group, Hesperia) equipped with a Vydac guard column (particle size 5 µm) was used. Trifluoroacetic acid (TFA), analytical grade sodium chloride (NaCl), and Optima grade methanol (MeOH) and acetonitrile (MeCN) were obtained from Fisher Scientific. The mobile phase (90/10/0.1, water/MeCN/TFA) was degassed with helium for 10 min prior to analysis. A calibration curve was generated using DACS-1C DA standards (National Research Council of Canada, Institute for Marine Biosciences, 1411 Oxford Street, Halifax, Nova Scotia, Canada) of 0.15, 0.3, 0.5, 1.0, 2.0, 4.0, 8.0, and 16 µg ml^{-1} (r = 0.99). Nanopure water was used for standard preparation and standards were kept refrigerated and in the dark when not in use. The lowest detectable standard was 0.15 µg DA ml^{-1}. The instrument detection limit, which was equivalent to the concentration that corresponded to 3 times the standard deviation of the signal from the lowest detectable standard (n = 3), was 0.2 µg DA ml^{-1}. Injections were 10 or 25 µl, with a flow rate of 0.3 ml min^{-1}.

RESULTS

Expt 1

DA was not detected in any *Pseudo-nitzschia pungens* cultures used in this study. In addition, dissolved DA levels remained constant for both treatments throughout the entire experiment (400 ± 3 and 1000 ± 5 µg l^{-1}) and thus there was no evidence of toxin uptake by krill during the experiments. The mean dry weight of *Euphausia pacifica* used in this experiment was 5.9 ± 1.2 mg (n = 36).

The presence of dissolved DA significantly affected the food consumption rate of *Euphausia pacifica*. Over the 24 h period, cell consumption rates were highest when DA levels were 0 ($12 \pm 2.5 \times 10^4$ cells mg^{-1} dry wt d^{-1}), were intermediate for DA levels of 400 µg DA l^{-1} ($6.8 \pm 1.6 \times 10^4$ cells mg^{-1} dry wt d^{-1}), and were lowest for DA levels of 1000 mg DA l^{-1} ($3.3 \pm 0.9 \times 10^4$ cells mg^{-1} dry wt d^{-1}) (1-way ANOVA, F = 300, df = 2, $p < 0.001$). Assuming that krill feed most heavily after they ascend to surface waters, we compared the cell removal rates among the 3 treatments for the first 6 h of their feeding. Ingestion rates were significantly lower in the containers that contained dissolved DA than those in controls for the initial 6 h (1-way ANOVA, $F = 41.36$, df = 2, $p < 0.001$) (Fig. 1).

The time course of the feeding response also differed in the feeding containers as a function of the added DA levels. When DA was absent, *Euphausia pacifica* consumed non-toxic *Pseudo-nitzschia pungens* over the entire 24 h period, with a high initial 6 h rate (Fig. 1A). After the first 6 h, the rate of ingestion declined and stayed relatively constant thereafter. At a dissolved DA level of 400 µg l^{-1}, krill consumption rates were relatively constant for the first 12 h but began an obvious decline in the last 12 h (Fig. 1B). In the highest DA treatment (1000 µg DA l^{-1}), ingestion rates did not differ significantly over time (Fig. 1C).

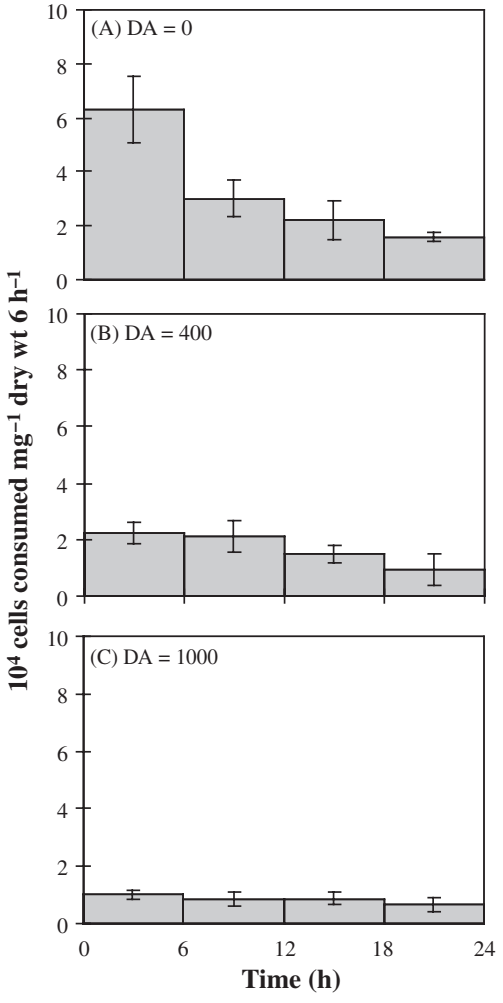

Fig. 1. *Euphausia pacifica*. Ingestion rates (means ± SD) of krill fed the non-toxic diatom *Pseudo-nitzschia pungens* during 24 h grazing experiment with additions of dissolved DA. (A) Control; (B) 400 µg DA l^{-1} exposure; (C) 1000 µg DA l^{-1} exposure

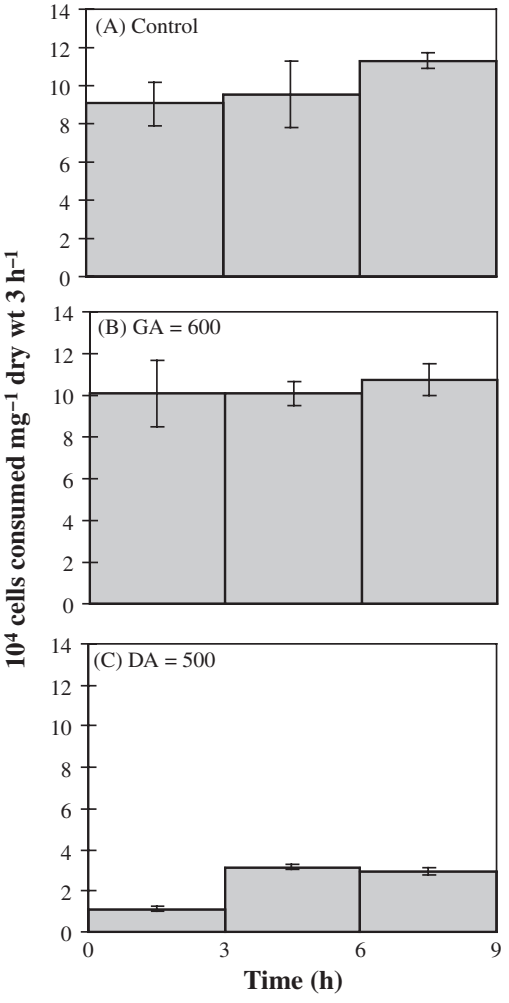

Fig. 2. *Euphausia pacifica*. Ingestion rates (means ± SD) of krill fed the non-toxic diatom *Pseudo-nitzschia pungens* during 9 h grazing experiment with additions of DA or dissolved GA. (A) Control; (B) 600 µg GA l^{-1} exposure; (C) 500 µg DA l^{-1} exposure

Expt 2

The second experiment was conducted to examine the specificity of the krill feeding response to dissolved DA. For this purpose, dissolved GA was chosen because of its chemical similarity to DA. Dissolved DA levels remained constant for the treatments throughout the entire experiment (500 ± 3 µg l^{-1}). The mean dry weight of *Euphausia pacifica* used in this experiment was similar to that in the first experiment at 5.8 ± 1.7 mg (n = 17). The krill feeding response differed depending on which of the 2 amino acids were dissolved in the water. Cell consumption rates over the 9 h period were 29 ± 1.6 × 10^4 cells mg^{-1} dry wt d^{-1} for control krill with no added DA or GA, 31 ± 1.6 × 10^4 cells mg^{-1} dry wt d^{-1} for krill with 600 µg GA l^{-1}, and 7 ± 1.6 × 10^4 cells mg^{-1} dry wt d^{-1} for krill with 500 µg DA l^{-1}. There was no significant difference in the cell consumption rates between krill in the control and GA treatments (Tukey test, p = 3, q = 1.39, p = 0.614). However, the 9 h cell removal rates in both the control vs. DA and GA vs. DA were significantly different (Tukey test, p = 3, q = 35, p < 0.001, Fig. 2).

The temporal pattern of the feeding responses varied between treatments. *Euphausia pacifica* ingested non-toxic *Pseudo-nitzschia pungens* at the high rate of 10 × 10^4 cells mg^{-1} krill dry wt 3 h^{-1} over a 9 h period with or without added GA (Fig. 2A,B). The small increase in grazing observed at 6 to 9 h with GA present (Fig. 2B) was not significant (Tukey test, p = 3, q = 0.352, p = 0.967). When dissolved DA was present in the grazing containers, *E. pacifica* consumed *P. pun-*

gens throughout the entire 9 h period. However, as compared to the rates with added GA, the ingestion rate was 10-fold lower initially; it then increased, and stayed relatively constant thereafter (Fig. 2C).

DISCUSSION

The goal of this study was to examine the impacts of dissolved DA exposure on krill feeding rates. Recent reviews of krill feeding behavior describe the characteristic food concentrating response of krill, which involves capturing cells through movements of thoracic appendages in a process called compression feeding (Hamner et al. 1983, Hamner & Hamner 2000). When phytoplankton are abundant, a food bolus (phytoplankton aggregate) can be produced, which is carried for a time and consumed or 'nibbled' as it is brought to the mandibules for mastication. Alternatively the bolus may be ejected when it becomes too large or the krill is satiated, or it may be stolen or fought over in a krill swarm (Hamner 1988, Hamner & Hamner 2000). To our knowledge, there are no measurements of the cell concentrations in the food bolus (i.e. volume of the bolus and the number of enclosed cells) but, since this aggregate has been readily observed both by divers in the water and by laboratory video (Hamner 1988, Hamner & Hamner 2000), the cell densities are presumably considerably higher than those in the surrounding water. During cell-concentrating processes, the raking motions of feeding appendages that scrape and maneuver the cells into the food concentrate (Hamner 1988) may break some cells (this is especially likely for the long, needle-shaped genus *Pseudo-nitzschia*), thus releasing dissolved constituents into the bolus. An analogy may be the dramatic increase of solute concentrations in small, concentrated aggregates compared with those in surrounding waters (Alldredge & Cohen 1987).

Currently, very few measurements of dissolved DA concentrations during toxic algal blooms are available. In June 1998, Trainer et al. (2000) detected low levels of dissolved DA ranging from 0.1 to 6.3 µg DA l^{-1} in surface waters off the central California coast. Doucette et al. (2002) found maximum levels of 40 µg DA equiv. l^{-1} at 5 m depth in Monterey Bay during an August to September 2000 bloom of toxic *Pseudo-nitzschia australis*. The lowest concentration of dissolved DA used in the present study (400 µg DA l^{-1}) is 10-fold greater than maximum concentrations reported in seawater to date from bulk water analyses such as those described above. Recent evidence, however, suggests that *Pseudo-nitzschia* spp. can aggregate in thin layers at very high concentrations (Rines et al. 2002) in some highly stratified waters, and such concentrations may result in correspondingly high dissolved DA levels in such strata.

Although the concentration of dissolved DA used in the present experiments is considerably higher than dissolved DA concentrations measured from water samples in Monterey Bay or elsewhere, we suggest that such concentrations may indeed be encountered when krill feed during toxic *Pseudo-nitzschia* spp. blooms. In Monterey Bay, *P. australis* blooms (i.e. periods when cell densities are $>10^5$ cells l^{-1}) were found in 15% of the 169 water samples obtained over the last 4 yr during routine surveys at deep-water sites where krill commonly occur (M. W. Silver unpubl. data). At these bloom sites, particulate DA averaged 9.1 ng ml^{-1} (M. W. Silver unpubl. data), which is approximately 1/44th the level of the lowest dissolved DA concentration used in the present feeding experiments. If compression feeding by *Euphausia pacifica* resulted in concentrating cells ≥44× and the DA in those cells was spilled into the food bolus, then the DA concentrations used in this study could be equaled or exceeded. Furthermore, about 2% of the samples taken during routine field monitoring had particulate DA levels ≥20 ng ml^{-1} (i.e. 1/20th the concentration of the lowest dissolved toxin level used in the present experiments: M. W. Silver unpubl. data). There are currently no data on the concentrations of cells in the food aggregate/bolus, but its visible size suggests that a concentration factor of 20 to 44× for krill feeding in the field is not unrealistic. The concentration of dissolved DA resulting from cell breakage would depend on the number of cells in the bolus, the water volume entrained, the rate of DA release from cells and its subsequent diffusion rate out of the bolus, and other factors. These are all unknown quantities at the present time. Hopefully our data will generate interest in characterizing the dissolved DA concentrations generated during compression feeding by krill in toxic bloom areas like Monterey Bay.

The intensity of a zooplankter's response to dissolved amino acids has been found to increase linearly with the stimulus concentration (Ache 1982). Feeding rates of krill exposed to 400, 500 and 1000 µg DA l^{-1} in this study appeared to be decreasing with increasing DA levels also. However, discriminating such relatively small differences would require more sample replication than that used in this study. All concentrations of dissolved DA used in this study fall within the concentration range in which behavioral responses have been reported previously for various crustaceans (Poulet & Ouellet 1982, Gill & Poulet 1988), suggesting that the responses seen in this study are not unexpected. Gill & Poulet (1988) noted that low concentrations of some amino acids are stimulatory to copepods, although increases in concentration of the same compound can be non-stimulatory or even toxic.

Results from previous laboratory studies on copepods have revealed various effects of exposure to dissolved DA, depending upon the types of assays performed and the taxa involved. Lincoln et al. (2001) found no effect of 1 µg dissolved DA l^{-1} levels on egg production of 2 calanoid copepods. Shaw et al. (1997) documented deaths of an harpacticoid copepod with an LC_{50} of 0.900 µg dissolved DA l^{-1}, whereas Windust (1992) found the LC_{50} values for 2 calanoid species to be 12 000 to 42 000 µg dissolved DA l^{-1}. We found feeding deterrence at 400 µg dissolved DA l^{-1} in euphausiids. Shaw et al. (1997) noted that decreases in feeding rates of copepods resulted from death of the zooplankters, whereas in our experiments with lower levels of dissolved DA, no enhanced mortality of DA-exposed krill compared with non-exposed krill was observed. Thus, it is apparent that effects are concentration dependent and also that different effects such as feeding inhibition, reduction in fecundity and mortality have different concentration thresholds. However, we realize that direct neurotoxic action of DA on the feeding apparatus may also occur and could also explain our results.

The evolutionary value of domoic acid production by *Pseudo-nitzschia* spp. is unclear. Some researchers have suggested that it may benefit diatoms by binding trace metals, such as iron and/or copper (Ladizinsky & Smith 2000, Rue & Bruland 2001). The results of this study on *P. pungens* suggest that dissolved DA may reduce grazing pressure by reducing krill feeding rates. *Pseudo-nitzschia* spp. populations that have reached stationary phase may especially benefit from releasing DA if this release reduces cell losses to grazing at the time when cells are not dividing. Further research is needed on the responses of other consumers of *Pseudo-nitzschia* spp. to particulate and dissolved DA and on the specificity of their response. Such data will lead to a better understanding of the role of zooplankton in promoting toxic blooms and vectoring toxins to higher trophic levels in pelagic environments.

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