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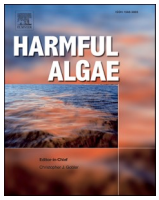

The influence of the toxin-producing dinoflagellate, *Alexandrium catenella*, on feeding, reproduction and toxin retention in *Calanus helgolandicus*

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ABSTRACT

Copepods of the genus *Calanus* dominate the biomass of pelagic ecosystems from the Mediterranean Sea up into the Arctic Ocean and form an important link between phytoplankton and higher trophic levels. Impacts from toxin-producing harmful algae (HA) have been recorded throughout this region over the last 50 years, with potentially negative effects on *Calanus* spp. populations and the ecosystem functions and services they provide. Here we examine how ingestion, egg-production and egg-viability in *Calanus helgolandicus* are affected by the relative abundance of the toxin-producing dinoflagellate *Alexandrium catenella* in their diet. Our four-day experiments demonstrate that the ingestion rate of *C. helgolandicus* declined significantly as the percentage of toxin-producing *A. catenella* within their diet increased, whereas egg production and egg viability were unaffected. Toxin profile concentrations for *A. catenella* are presented alongside body toxin-loads in *C. helgolandicus* after 4 days of feeding on these cells. The body toxin concentrations of *C. helgolandicus* were 3.6–356.6 pg STX diHCl eq. copepod^{-1}, approximately 0.02–3.3 % of the toxins ingested. Our work suggests that the effects of exposure to *A. catenella* may be negligible in the short-term but could manifest if bloom conditions persist for longer than our experimental duration.

1. Introduction

Copepods of the genus *Calanus* are one of the most common calanoid copepods in the North Atlantic, with populations ranging from the mid-Atlantic Shelf off the east coast of the United States, the Mediterranean Sea, and up into the Barents Sea north of Norway (Conover, 1988; Planque et al., 1997; Bonnet et al., 2005). *Calanus* is an important food source for many commercially important fish and also seabirds in the North Atlantic (Gaard and Reinert, 2002; Gislason and Astthorsson, 2002; Ringuette et al., 2002; Beaugrand et al., 2003; Steen et al., 2007; Wold et al., 2011). The warmer-water species, *Calanus helgolandicus*, is widely distributed throughout the Eastern North Atlantic, with particularly high abundances in the Western European Shelf region (Barnard et al., 2004; Bonnet et al., 2005; Choquet et al., 2017). The abundance and geographical distribution of *C. helgolandicus* in the Atlantic are positively related to temperature (Bonnet et al., 2005). Indeed, *C. helgolandicus* and other species associated with warmer water have shifted northwards by ten degrees of latitude over the last 7 decades with *C. helgolandicus* replacing the boreal species, *Calanus finmarchicus*, in regions of warming (Beaugrand et al., 2002; Choquet et al., 2017). Changing the geographical distribution and abundance may result in a possible mismatch between copepods and their phytoplankton prey, with wider consequences for ecosystem functioning (Edwards and Richardson, 2004). However, although there is a strong link between *C. helgolandicus* distribution and temperature, other factors relating to environmental change may also affect their ability to survive (e.g., Cook et al., 2007; Mayor et al., 2012).

Of the ~5000 species of extant marine phytoplankton, ~40 are able to produce potent toxins that can reach humans through fish and shellfish (Hallegraeff, 1993; Hallegraeff et al., 2021). Blooms of these harmful algae (HA) are a globally recurring issue, the distribution and frequency of which appear to have changed in recent years (Hallegraeff,

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1993; Edwards et al., 2006; Anderson et al., 2012). The apparent increase in the impacts from HA blooms can be attributed to increasing monitoring programs (Bresnan et al., 2021; Hallegraeff et al., 2021), but global climate change and anthropogenic pollution may also be contributing to the observed changes (Edwards et al., 2006; Hallegraeff et al., 2021; Nohe et al., 2020; Marampouti et al., 2021; Søgaard et al., 2021). Dinoflagellates are responsible for the majority of HA blooms (Sopanen et al., 2011) and are often associated with major environmental and economic issues (Hallegraeff, 1993; Anderson et al., 2012; Hallegraeff et al., 2021), causing disease and death in a variety of marine animals, including fish, seabirds, and mammals (Wang, 2008; Jensen et al., 2015; Kershaw et al., 2021), in addition to severe impacts on global aquaculture generated by large-scale climate anomalies (Pitcher et al., 2019; Díaz et al., 2023).

Toxic algae can affect copepod survival, feeding, and reproduction by decreasing ingestion, growth and egg production rates in their populations (Colin and Dam 2003; Barreiro et al., 2007; Jiang et al., 2009; Abdulhussain et al., 2020, 2021). The presence of algal toxins may cause copepods to reject prey cells (Teegarden, 1999; Xu and Kiørboe, 2018), or, once ingested, result in physical incapacitation that inhibits feeding (Sopanen et al., 2011). The paralytic shellfish toxin (PST)-producing dinoflagellate, *Alexandrium* spp., has been observed to adversely affect the feeding (Turriff et al., 1995; Campbell et al., 2004) and fitness (Roncalli et al., 2016) of *Calanus finmarchicus*. However, other studies have shown that *C. finmarchicus* consumed neurotoxic dinoflagellates and diatoms without apparent negative effects (Turner and Borkman 2005; Leandro et al., 2010) and *Calanus helgolandicus* is also reported to feed on the okadaic acid-producing dinoflagellate, *Dinophysis* spp., with no apparent impact on their ingestion (Wexels Riser et al., 2003). Copepods may accumulate PSTs when feeding on *Alexandrium* spp. (Teegarden and Cembella, 1996; Teegarden et al., 2003; Campbell et al., 2004) and transfer these to higher trophic levels, including marine mammals (Durbin et al., 2002; Doucette et al., 2006). However, most observations show that the tissues of copepods retain only a small fraction of the ingested toxins after feeding. *Calanus finmarchicus* fed on *Alexandrium* spp. in the field and laboratory accumulated toxins at a rate of 0.41–0.89 ng STX eq. copepod day^{-1} with a retention efficiency of 1–2 % of the total toxin ingested (Campbell et al., 2004). *Acartia hudsonica* fed *Alexandrium fundyense* at ~ 3000 cells mL^{-1} accumulated toxins up to 54 μg STX eq g^{-1} of wet weight within only 6 h of exposure, equating to ~ 10 % of the total toxin ingested (White, 1981). In addition, the toxin retention efficiencies of two copepod species, *Acartia tonsa* and *Eurytemora herdmani*, were typically < 5 % of ingested *Alexandrium* spp. toxins (Teegarden and Cembella, 1996). These low retention efficiencies suggest that toxins are either transformed and excreted as other compounds and/or are directly eliminated in dissolved form, perhaps by regurgitation (Guisande et al., 2002; Teegarden et al., 2003) or excretion. Even with low toxin retention efficiencies in copepods, species at higher trophic levels may still be at risk due to chronic exposure, as these toxins can accumulate in discernible levels, potentially impacting the overall health of marine ecosystems (Kershaw et al., 2021).

To date, the majority of studies examining how *Calanus* spp. respond to PST-producing HA have been conducted over ~ 24 h (Hassett, 2003; Teegarden et al., 2001; Turner, 2010; Turner and Borkman, 2005). This duration is sufficient to understand the immediate patterns of prey selectivity, but may not be sufficient to determine the effects of HA on reproduction as *Calanus* spp. may take > 24 h to convert ingested food into eggs (Hirche and Kwasniewski, 1997), and may also produce eggs from maternal biomass when feeding conditions are poor (e.g., Niehoff et al., 1999; Mayor et al., 2009). Here, we used 4-day incubation experiments to examine how feeding, egg production and egg viability in *C. helgolandicus* were affected by the relative abundances of the toxic dinoflagellate, *A. catenella* (1119/28), and the non-toxic species, *A. tamarense* (1119/33), in their diet. *A. catenella* [formerly *Alexandrium tamarense*, North American strain (Scholin et al., al.,1994) Group I (Lilly et al. (2007) reassigned taxonomically (John et al., 2014; Fraga et al., 2015), acknowledged in Prud'homme van Reine (2017) is widely distributed (Hallegraeff, 1993; Brown et al., 2010; Anderson et al., 2012) and reported as a nuisance species in Scotland, Iceland, the Faroe Islands and Norway. It can reach cell densities of between 1000 and 2000 cells L^{-1} and result in levels of PSTs in shellfish flesh that exceed the EU regulatory limit of 800 μg STX eq/KG for several weeks, resulting in closures of shellfish harvesting areas in Northern Europe (Bresnan et al., 2005; 2008; Brown et al., 2010). Our results are presented alongside a full toxin profile for *A. catenella*, as well as body toxin profiles for *C. helgolandicus* at the end of our experiments.

2. Materials and methods

2.1. Collection and culture conditions

Non-quantitative samples of adult female *Calanus helgolandicus* were collected from the Scottish Coastal Observatory site at Stonehaven in the Northwest North Sea (56° 57.8′N 02° 06.2′W) using a 1 m ring net with a 350 μm mesh, fitted with a non-filtering cod-end. A double oblique tow method was employed, to a depth of 40 m, and towing speed of 2 knots. Upon collection, copepods were diluted with fresh seawater and transported to the laboratory. Given the short transportation duration of 1–2 h, aeration was unnecessary as copepods can adequately respire in the fresh seawater provided during this period, ensuring they remained in optimal condition for subsequent experiments. Upon return to the laboratory, samples were immediately sorted in a controlled temperature laboratory set at 13 °C. The copepods were maintained in 10 L tanks with a 12 h photoperiod and aeration and fed Phyto Feast® Live (a mix of *Tetraselmis, Isochrysis, Pavlova, Nannochloropsis, Thalassiosira, Amphora* and *Synechoccus*) and Roti-Feast® (*Brachionus plicatilis* animals and eggs) produced by Reed Mariculture Inc., Campbell, California, USA. All experimental work was conducted in a controlled temperature room at 13 °C.

2.2. Collection and culture of organisms

The dinoflagellate, *Alexandrium catenella,* was isolated from Scapa, Orkney, UK, between 2007 and 2008, and cultured at Marine Scotland Science, Aberdeen, UK (Brown et al., 2010). The two species examined were toxic *A. catenella* (Strain W08/056/01; 1119/28; Culture Collection of Algae & Protozoa: CCAP) and non-toxic *A. tamarense* (Strain W07/069/01; 1119/33; CCAP). Due to ongoing nomenclature changes in the *Alexandrium* genus, strain designation has become crucial. For instance, *A. tamarense* Group I, which we used in our study, was reclassified several times, eventually as *A. catenella* by Fraga et al., 2015. *A. tamarense* Group III, the non-toxic algae in our work, retained its name across different studies. Given these shifting classifications, strain-specific toxin testing has become important.

All phytoplankton were grown in a temperature-controlled room at 15 °C with 12:12 (light:dark) photoperiod, using seawater from Stonehaven, UK, which was filtered (4.7 mm Whatman GFF filters, nominal pore size = 0.7 μm) and amended with L1 medium (3.5 mL of L1 medium for every 1 Litre following the protocol by Guillard and Ryther, 1962; Guillard and Hargraves, 1993). Prior to the experiment, the dinoflagellate cultures were monitored under a microscope to verify growth phase, and cultures were used in exponential phase typically determined within 1–2 weeks, depending on the initial culture condition.

2.3. Effects of toxic and non-toxic Alexandrium on the feeding and reproduction of C. helgolandicus

Food removal experiments (Båmstedt et al., 2000) were used to examine how ingestion rates for *Calanus helgolandicus* changed in response to changes in the relative abundances of *Alexandrium catenella* and *A. tamarense*. Adult female *C. helgolandicus* were carefully transferred via pipette into a 10 L bucket of 0.2 μm filtered seawater (FSW

hereafter) and incubated for 24 h to clear their guts. Experiments were conducted in 100 mL beakers, each containing a total of 400 µg C L^{-1} of algae (see analytical methods section) to ensure that feeding conditions were always saturating. The five experimental treatment levels contained 0 %, 25 % 50 %, 75 % and 100 % of *A. catenella*-derived carbon, with the remainder being provided via *A. tamarense*. These concentrations were achieved by first determining the cell concentrations of the *Alexandrium* stock cultures. Following this, the necessary volumes of *A. catenella* and *A. tamarense* cultures were added to the incubation beakers to achieve the required toxic and non-toxic algae ratios. The beakers were subsequently topped up with an appropriate volume of FSW. Nine beakers (3 × initial beakers = 0 h, 3 × control beakers = 24 h, and 3 × grazing beakers with copepods = 24 h) at each of the five treatment levels were initially set up (total n = 45; Fig. 1). For each treatment level, 50 mL samples were immediately taken from three randomly selected beakers. These samples were preserved with acidified Lugol's iodine to enumerate the initial cell concentrations at the start of each experiment. Three female *C. helgolandicus* were added to each of three grazing beakers at each treatment level and incubated alongside triplicate control beakers at each treatment level for four consecutive 24 h at 13 °C with a 12:12 h (L:D) photoperiod. At the end of each 24 h period, the copepods were removed from the grazing beakers using a 200 µm mesh screen and transferred into FSW. The number of motile copepods observed after mechanical stimulus was recorded. Only motile copepods were then transferred into new beakers containing fresh medium at the experimental conditions they had previously experienced. Any non-motile copepods found during this process were assumed to be dead and were not replaced in the new beakers or included in the toxin retention analysis. The eggs left in the beakers were collected daily with a 63 µm mesh screen, washed with FSW and counted using a binocular microscope (Wild M3) before determining their viability (see analytical methods Section 2.4). Ingestion was determined at the end of days 1 and 3 by preserving 50 mL water samples from each of the grazing and control beakers with acidified Lugol's iodine. At the end of day 4, the experimental copepods from each treatment level were grouped together for analysis to ensure sufficient detection levels of the toxins, transferred into single 1.5 mL Eppendorf tubes and frozen at −80 °C for toxin content analysis (see Analytical methods Section 2.4).

2.4. Analytical methods

The carbon contents and toxin concentration profiles were determined from the stock cultures of *Alexandrium catenella* and *A. tamarense* prior to their exposure to copepod grazing. The density of cells in each culture was counted from a 1 mL subsample using a Sedgewick Rafter cell and a ZEISS X200 inverted microscope. The average volume of a cell in each culture was calculated using the diameter of 30 cells, measured using a calibrated eyepiece graticule, and the equation for calculating the volume of a sphere. Carbon content of an average cell was calculated using the C:volume relationship for protist plankton excluding diatoms (Menden-deuer and Lessard, 2000; pgC $cell^{-1}$= 0.216 x $volume^{0.939}$). Cells from the experimental beakers preserved with acidified Lugol's iodine were counted from a 50 mL subsample settled for 48 h in a Utermöhl chamber. Copepod daily clearance (the volume of water completely cleared of food particles by a copepod per unit time) and ingestion rates (the amount of food ingested by an individual copepod per unit time) were calculated using established equations (Frost, 1972) and expressed as mL $copd^{-1}$ day^{-1} and µg C $copepod^{-1}$ day^{-1}, respectively.

Egg viability was examined using SYTOX® Green (Buttino et al., 2004); live cells are impermeant to this stain, making it a useful indicator of dead cells and hence eggs that will not hatch. In brief, the eggs were incubated for 50 min in chitinase solution (final concentration 1 mg mL^{-1} in FSW) at room temperature and subsequently stained using SYTOX® Green nucleic acid stain (final concentration 20 µM in DMSO) for 50 min in the dark at room temperature. The number of fluorescent eggs was counted using a Zeiss Axiovert 200 inverted fluorescence microscope and used to calculate the percentage of viable eggs likely to hatch.

The samples of *Calanus helgolandicus* and the *A. catenella* and *A. tamarense* cultures that were collected during the feeding experiments were analysed for PSTs using the PCOX method (Van de Riet et al., 2011). The *A. catenella* and *A. tamarense* culture samples (Table 1) were centrifuged at 3000 rpm for 20 min (multiple stages) to form pellets of ~250,000 cells in 2 mL Eppendorf tubes. The supernatants were removed using a pipette and the pellets were then stored at −20 °C until extraction. Glass beads (180 µm, 100 ± 20 mg) were acid-washed and

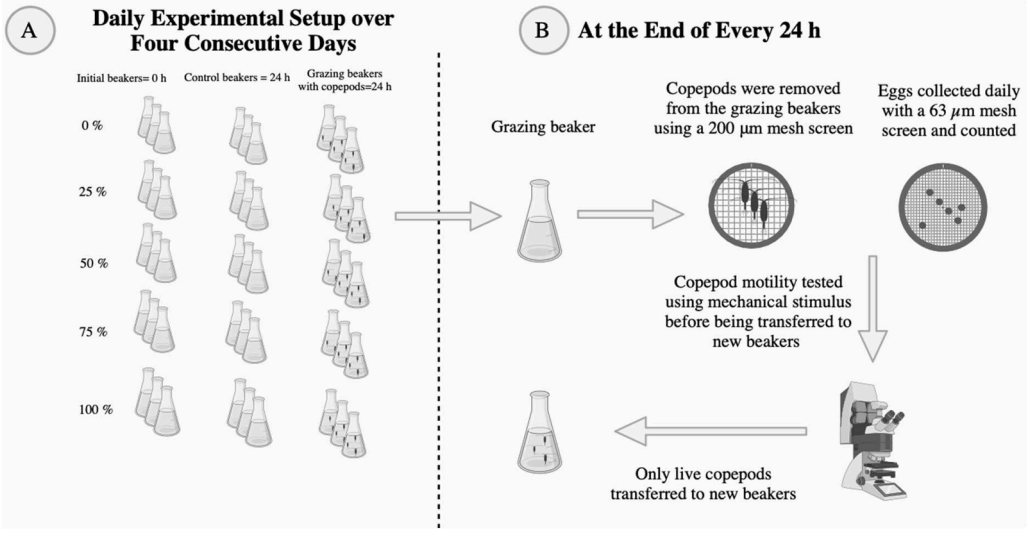

Fig. 1. Feeding and Egg Production Experiment over Four Consecutive Days.
Part A: Daily setup showing the percentage of toxicity (% Alexandrium catenella) in 45 beakers. The five experimental treatment levels contained a total of 400 µg C L^{-1} of algae in each beaker with 0 %, 25 % 50 %, 75 % and 100 % of toxic Alexandrium catenella, and the remainder being provided via non-toxic A. tamarense. In total, 45 copepods were placed in the grazing beakers, with 3 copepods per beaker, spread across 3 replicates per treatment. The initial (t0) and control (t24) beakers were preserved with acidified Lugol's iodine to enumerate the cell concentration at t0 and t24, respectively.
Part B: Procedure detailing the daily egg collection, motility checks for copepods every 24 h before the returned into fresh seawater and algae.

Table 1
Paralytic shellfish toxin content of the *Alexandrium catenella* and *Alexandrium tamarense* cultures used during the feeding and egg production experiment.

Sample type	Time of Analysis (d)	PST (fg STX diHCl eq. cell^{-1}) GTX1	GTX2	GTX3	GTX4	GTX5	dcGTX 2	dcGTX 3	STX	NEO	dcSTX	C1	C2	Overall Toxicity
A. catenella (W08/056/01: 1119/28)	0	0	36.9	615	0	28.4	0	15.8	1135	1236	0	5.7	846	3918.8
	1	0	46.6	555	0	19.2	0	26.2	1049	928	0	4.6	381	3009.6
	2	0	14	391	0	18.2	0	3.6	924	846	0	2.2	299	2498
	3	0	27.3	525	0	20.9	0	17.6	1068	1024	0	3.5	408	3094.3
A. tamarense (W07/069/01: 1119/33)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0

added to the Eppendorf tubes. The extraction solvent (0.5 M acetic acid, 100 μL) was then added with a calibrated pipette. The cells were extracted for two minutes at 25 Hz using a TissueLyser 2. Following microscopic confirmation that the cells had ruptured, they were centrifuged at 14,000 rpm for 5 min. The supernatants were transferred to 0.2 μm Ultrafree-MC centrifugal filters using a pipette fitted with a long tip and this was followed by centrifugation for five minutes at 10, 000 rpm. The *C. helgolandicus* samples (6 – 9 individuals per sample: Table 2) were extracted using the same techniques as the algae. Copepods were combined at the end of the experiment to ensure sufficient detection levels of the toxins. All extracted filtrates were transferred to pre-insert amber vials and were immediately analysed by HPLC following the PCOX method (Van de Riet et al., 2011). This method provides a very low limit of detection for the N-sulphocarbomoyl I toxins, whereas the limits of detection for the other PSTs are ~ 10 – 80 fold higher (Van de Riet et al., 2011: Table 1). We calculated the total toxicity values using the toxin equivalent factors (TEFs) as proposed by Oshima (1995). All the toxicity values are expressed as milligrams of Saxitoxin dihydrochloride equivalents (mg STXdiHCl eq) for consistency and ease of comparison (as presented in Tables 1 and 2).

2.5. Data analyses

The gross retention efficiency of toxins was calculated as:

$$\text{Retention efficiency } (\%) = \frac{\text{Body toxin concentration } (\textbf{pg STX diHCl eq.})}{\text{Total toxicity ingested } (\textbf{pg STX diHCl eq.})} * 100 \quad (1)$$

This assumes that the diet is proportionally consistent with the food available. The influence of the relative abundance of *Alexandrium catenella* and *A. tamarense* and sampling day on rates of 1) clearance, 2) ingestion, 3) egg production and 4) egg viability in *Calanus helgolandicus* were all examined using two-way analysis of variance (ANOVA) using backwards selection accounting for repeated measures in the latter two cases (where repeated observations = 4). The treatment levels '% *A. catenella*' and 'Day' were treated as continuous and categorical variables, respectively. All statistical analyses were carried out using the software Prism Graphpad (v.9.2).

3. Results

3.1. A. catenella toxin analyses and body toxin concentrations of C. helgolandicus

Toxin profile concentrations of *Alexandrium catenella, A. tamarense*, and *Calanus helgolandicus* are presented in Tables 1 and 2, respectively. Saxitoxin (STX), neosaxitoxin (NEO), gonyautoxin-3 (GTX3) and N-sulphocarbomoyl-2 (C2) were the four main toxins present in the *A. catenella* cells. No PSTs were detected in the *A. tamarense* culture used in this experiment (Table 1). The total toxicity retained in *C. helgolandicus* ranged between 3.6–356.6 pg STX diHCl eq. copepod^{-1}. Only NEO (sample day 4: 25 % *A. catenella*), C1 (samples day 4: 50 and 75 % *A. catenella)* and C2 (all 4 samples) toxins were detected in the copepods fed on mixtures of *A. catenella* and *A. tamarense*, demonstrating that the copepods had ingested the toxic *Alexandrium* cells.

3.2. Feeding of C. helgolandicus in the presence of toxic and non-toxic Alexandrium spp.

Clearance rates of *C. helgolandicus* ranged between 30 – 176 mL copepod^{-1} day^{-1}. The observed rates decreased significantly as the percentage of *Alexandrium catenella* in the diet increased and also differed between the experimental days (Fig. 2A,% *A. catenella: F* = 12.25, $p = 0.002$; Day: $F = 6.11$, $p = 0.02$, $R^2 = 0.53$). Total ingestion rates ranged between 8.6 – 17.9 μg C copepod^{-1} day^{-1} and declined significantly as a function of the relative abundance of *A. catenella* in the available prey field but was not affected by the experimental day on which the observations were made (Fig. 2B,% *A. catenella:* $F = 61.68$, $p < 0.001$; Day: $F = 1.82$, $p = 0.189$, $R^2 = 0.57$).

3.3. Egg-production and viability of Calanus helgolandicus in the presence of toxic and non-toxic Alexandrium spp.

The daily egg production rate of *Calanus helgolandicus* across all treatments ranged between 0 – 11.7 eggs copepod^{-1} day^{-1} (Fig. 3A). The observed rates were not significantly affected by the relative

Table 2
Toxin concentration profiles of experimental *Calanus helgolandicus* adult females.% *Alexandrium catenella* toxin retained calculated as shown in Eq. (1).

No. animals analysed (survived after experiment)	Exposure time (day)	% A. catenella in diet	PST (pg STX diHCl eq. copepod^{-1}) GTX 1	GTX 2	GTX 3	GTX 4	GTX 5	dcGTX 2	dcGTX 3	STX	NEO	dcSTX	C1	C2	Overall Toxicity	% Toxin Retained
9	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–
9	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–
9	**4**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–
6	**4**	25	0	0	0	0	0	0	0	0	102	0	0	2.4	104.4	1.7%
8	**4**	50	0	0	0	0	0	0	0	0	0	0	8.6	348	356.6	3.3%
9	**4**	75	0	0	0	0	0	0	0	0	0	0	1.2	51	52.2	2.9%
9	**4**	100	0	0	0	0	0	0	0	0	0	0	0	3.6	3.6	0.02%

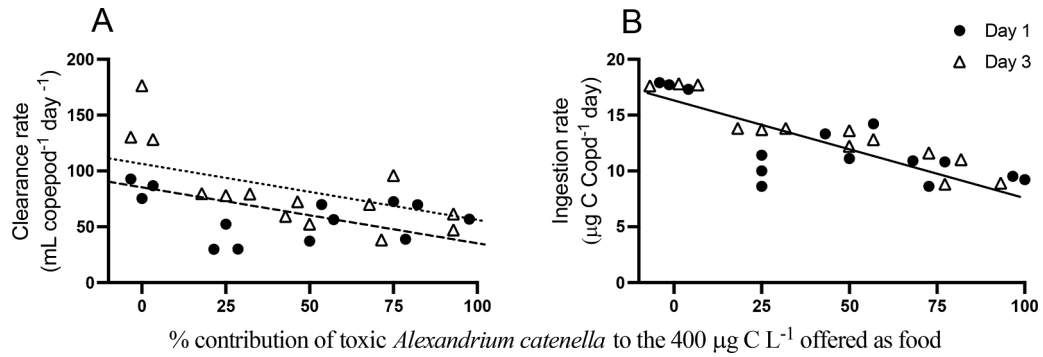

Fig. 2. Clearance (A) and ingestion (B) rates of *Calanus helgolandicus* fed 400 µg C L^{-1} with a variable contribution of toxic *Alexandrium catenella* and non-toxic *Alexandrium tamarense* after day 1 (circles) and day 3 (triangles). Straight lines indicate significant trends (p <0.05), with Fig. 1A showing significant differences between day 1 (dashed line) and day 3 (dotted line).

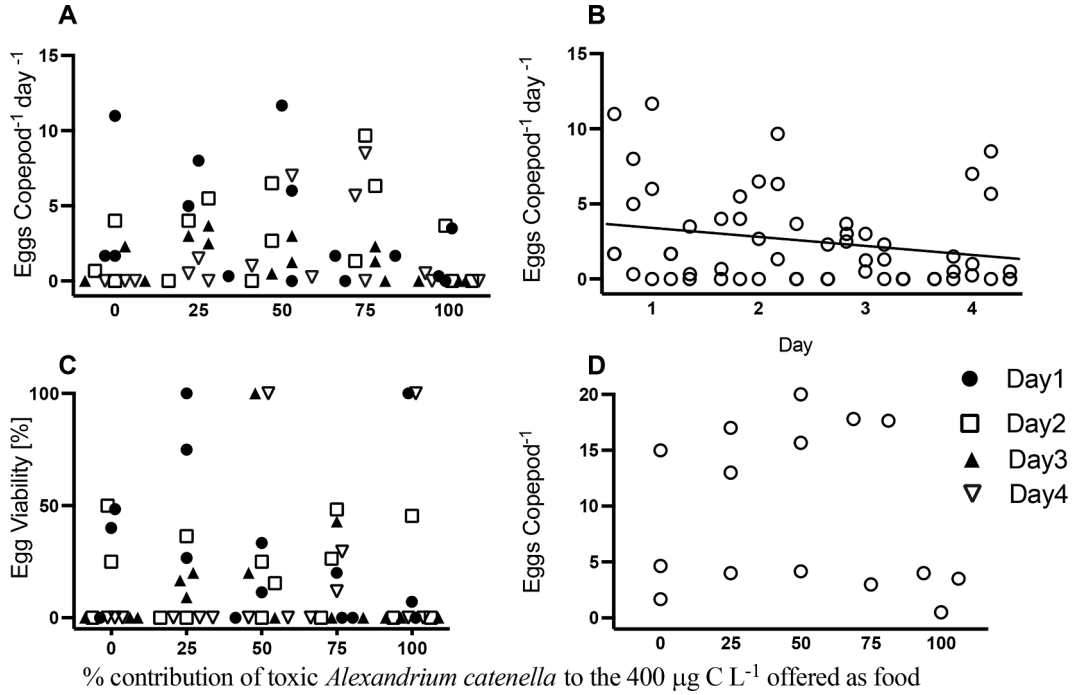

Fig. 3. Daily (A, C) and total over the whole duration (B, D) egg production and viability of *Calanus helgolandicus* fed 400 µg C L^{-1} with a variable contribution of toxic *Alexandrium catenella* and non-toxic *Alexandrium tamarense* over four consecutive 24 h incubation periods. See inset legends for symbols used. The straight line denotes a significant trend (p <0.05).

abundance of *Alexandrium catenella* in the available prey field (Fig. 3A,% *A. catenella:* $F = 0.465$, $p = 0.498$) but there was some evidence to indicate that they did decline as a function of time (Fig. 3B, Day: $F = 4.416$, $p = 0.040$, $R^2 = 0.28$). The viability of the eggs produced over the duration of the experiment ranged between 0 – 100%, and was not significantly influenced by either of the treatments examined (Fig. 3C,% *A. catenella:* $F = 0.049$, $p = 0.8266$; Day: $F = 0.956$, $p = 0.420$). The total numbers of eggs produced across the four days in the 100% *A. tamarense* and 100% *A. catenella* treatments ranged between 1.7 – 15 eggs copepod^{-1} and 0.5 – 4 eggs copepod^{-1}, respectively, and reached a maximum of 20 eggs copepod^{-1} in the 50% *A. catenella* treatment (Fig. 3D). Total egg production over the four-day experiment was not influenced by the relative abundance of *A. catenella* in the diet (Fig. 3D, % *A. catenella:* $F = 0.306$, $p = 0.590$).

4. Discussion

This study examined how the feeding, egg production and egg viability in *Calanus helgolandicus* were influenced by the relative abundance of toxic- and non-toxic dinoflagellates, *Alexandrium catenella* and *A. tamarense,* respectively. It also presents a full toxin profile for *A. catenella*, as well as body toxin concentrations of *C. helgolandicus* after feeding on *A. catenella* for four days.

4.1. Feeding of C. helgolandicus in the presence of toxic and non-toxic Alexandrium spp.

Calanus helgolandicus appeared capable of consuming a typical food ration, the amount required for their survival, growth, and reproduction, which is observed to range from 5 to 55 µg C copepod^{-1} day^{-1} (Harris et al., 2000; Meyer et al., 2002), over the duration of our experiment, even when offered a diet consisting of 100 % *Alexandrium catenella*. The clearance and ingestion rates observed in this study agree with those previously reported for copepods of the genus *Calanus* when feeding upon natural microplankton assemblages (Irigoien et al., 2000; Meyer et al., 2002; Wexels Riser et al., 2003; Mayor et al., 2006, 2009;

Castellani et al., 2008) and toxic dinoflagellates (Turriff et al., 1995; Campbell et al., 2004; Teegarden et al., 2008; Roncalli et al., 2016). Nevertheless, both clearance- and ingestion rates decreased as the percentage of *A. catenella* increased in the prey field (Fig. 2). *Calanus finmarchicus* is reported to be capable of distinguishing between toxic and non-toxic dinoflagellates (Campbell et al., 2004), and reduced food intake may have resulted because the animals were actively selecting against *A. catenella*. However, cells were clearly ingested in the 100 % *A. catenella* treatment and hence this explanation seems unlikely, particularly as the *A. catenella* and *A. tamarense* in our experiments were physically identical. We therefore suggest that the negative effect of increasing the relative abundance of *A. catenella* in the food on clearance and ingestion rates did not result from *C. helgolandicus* selecting against *A. catenella* cells on the basis of their physical attributes. It was not possible to discern between *A. catenella* and *A. tamarense* when counting the Lugol's samples from the grazing experiment, and thus we cannot investigate the potential role of physical size differences further. However, other studies indicate that *Calanus* spp. consume both toxic and non-toxic prey with little or no selectivity (Turner and Borkman 2005; Teegarden et al., 2008; Roncalli et al., 2016), and this is consistent with the understanding that the diet of *Calanus* is often proportionally equivalent to that of the food environment (Mayor et al., 2006, 2009; Castellani et al., 2008; Teegarden et al., 2008; Djeghri et al., 2018). We therefore suggest that the negative relationship between% *A. catenella* and food intake was attributable to a noxious effect of the ingested toxins. This effect could have been exacerbated by the experimental animals exuding copepodamides (also known as taurine-containing lipids; Mayor et al., 2015), which are reported to significantly increase the production of PSTs in dinoflagellates and their release into the water column (Wohlrab et al., 2010; Selander et al., 2015, 2019; Griffin et al., 2019). While it is indeed possible that secondary metabolites produced by *A. catenella* could contribute to the observed reduction in food intake, our study was specifically focused on the effects of PST, which is a well-documented toxin associated with this species. Nonetheless, a more comprehensive assessment incorporating both PSTs and secondary metabolites could provide a better understanding of their combined effects on feeding behavior. Future research should consider the analysis of both PSTs and secondary metabolites to more accurately discern their combined effects on feeding behavior.

Copepods are known to be able to feed on toxic algae with no ill-effects for several days (Roncalli et al., 2016), although ingestion rates have been shown to decrease over time (Guisande et al., 2002; Colin and Dam, 2003). We found that clearance rates were significantly higher during day 3, relative to those during day 1 of the experiment, although this result appears to be driven primarily by the 100 % *Alexandrium tamarense* treatment and hence seems unlikely to be related to any toxin-related effect. Total ingestion rates remained unchanged across the two time points examined and our results therefore suggest that the negative impact of *A. catenella* on feeding began at the onset of exposure, and did not increase over the duration of the experiment.

Alexandrium catenella negatively affected ingestion and could therefore impact upon the ability of *Calanus* spp. to successfully complete their lifecycle and contribute to vital ecosystem processes, including the transfer of biomass to higher trophic levels. However, realistically, in the natural environment there are multiple species of prey to feed upon. Given that HA cells typically form a relatively small proportion of the available prey biomass (e.g., Harris et al., 2000; Bresnan et al., 2008; Fehling et al., 2012), their impact on the feeding of *C. helgolandicus* and its congeners seems likely to be low (Turner and Borkman 2005; Leandro et al., 2010; this study). Overall, our results suggest that this might not be a significant threat to copepod fitness, given that *A. catenella* currently forms only low biomass HA blooms in the Northeast Atlantic. While our primary focus is the North Atlantic, there have been records of large *Alexandrium* blooms and resting cysts in the Chukchi and Beaufort Seas in the Pacific Ocean (Natsuike et al., 2013; Anderson, 2021). These occurrences in such remote and previously pristine locations emphasize the potential for toxic algal blooms to arise in regions once considered unlikely to experience them. This could potentially impact any copepod there, especially during summer where there is a high abundance of copepods in the Southern Chukchi Sea (Kim et al., 2020). Such blooms could impact the local copepod populations, disrupting the broader ecological balance.

4.2. Egg-production and viability of *C. helgolandicus* in the presence of toxic and non-toxic *Alexandrium spp.*

The egg production rates of *Calamus helgolandicus* feeding on *Alexandrium catenella* across the 4-day exposure experiments, 0 – 11.7 eggs copepod^{-1} day^{-1} (Fig. 3A), agree well with values previously reported for *Calanus* spp. feeding on natural microplankton (Pond et al., 1996; Jónasdóttir et al., 2005; Mayor et al., 2006, 2009; Castellani et al., 2008), and toxic *Alexandrium* sp. (Niehoff et al., 2000; Jansen et al., 2006; Madsen et al., 2008; Roncalli et al., 2016). The observed range of egg viability over the duration of the 4-day experiment, 0 – 100 % (Fig. 3C), did not differ significantly between the treatments, and agrees well with values previously reported for *C. helgolandicus* and *C. finmarchicus* (Jónasdóttir et al., 2005; Mayor et al., 2007).

Increasing the toxin concentration of *Alexandrium* sp. in the diet has previously been shown to negatively affect *Calanus* sp. egg production rates (Roncalli et al., 2016), but our results suggest that egg production of *C. helgolandicus* was not affected by the consumption of *A. catenella* (Fig. 3A and D). *Calanus* spp. are known to accumulate significant biomass reserves, and may adopt a spectrum of reproductive strategies, from capital breeding (from internal reserves), to income breeding (from ingested food) (Mayor et al., 2009; Sainmont et al., 2014). The available evidence suggests that *C. helgolandicus* is typically an income breeder, with egg production being positively associated with various descriptors of food availability (e.g., Pond et al., 1996). However, we did not determine if/how the biomass of *C. helgolandicus* changed throughout our experiments, and therefore cannot exclude the possibility that they were able to offset the negative effects of toxin ingestion and/or decreased food intake by producing eggs from stored reserves. Future experiments that use longer incubation periods and/or monitor the biomass of females over time are required before any negative effects of *A. catenella* on egg production in *C. helgolandicus* can be dismissed. In addition, *A. catenella* blooms are common on the coast of UK (specifically: Stonehaven, UK) throughout the range of experimentally collected *C. helgolandicus* (Bonnet et al., 2005; Bresnan et al., 2008; Brown et al., 2010, 2021), and it has been suggested that copepods that co-exist with HA may develop tolerance to their toxins (Turner and Borkman 2005; Teegarden et al., 2008). Thus, our observed result may be because *C. helgolandicus* has evolved tolerance to the toxins of *A. catenella*.

The marginally significant decline in egg production rate as a function of time observed across all treatments ($p = 0.04$: Fig. 3B) is not uncommon for *Calanus* spp. when incubated over several days. This could have occurred because the animals were not consuming enough food to sustain normal egg production rate (Mayor et al., 2007), or because the available food was lacking essential dietary components (Pond et al., 1996). Alternatively, if the eggs were produced from previously ingested food and/or stored reserves, the decrease in egg production through time may indicate that these reserves were becoming depleted. Regardless, these effects were apparent across all treatments, and were therefore not a response to the presence of *A. catenella*. Similarly, although egg viability was highly variable, it was not influenced by the relative abundance of *A. catenella* over the 4-day experimental period. It has been shown that both toxic and non-toxic species of *Alexandrium* produce extracellular allelochemicals, secondary metabolites that limit the growth of microalgae and heterotrophic protists (Tillmann and John, 2002; Tillmann et al., 2008), and these are also suggested to dramatically decrease egg production and hatching success in *Temora stylifera* and *C. finmarchicus* by interfering with fertilization or

egg viability (Ianora et al., 2004; Roncalli et al., 2016). Thus, the variable egg viability observed in the *A. catenella* and *A. tamarense* treatments is not surprising. However, there are other physiological reasons that might have resulted in egg viability being highly variable. For example, it could have resulted from the eggs being produced from nutritionally-deficient food ingested prior to experimentation (e.g., Pond et al., 1996; Jónasdóttir et al., 2002), or because the incubated females were at different phases of their spawning cycle. We made no attempt to assess if the females had previously mated, and hence some of the unhatched eggs could have also been attributable to individuals that had not previously mated. It is important to note that variability in egg viability in the real environment is often observed, e.g., Jónasdóttir et al. (2005) showed that the hatching success of *C. helgolandicus* in the North Sea varies greatly throughout the year at different sampling stations and egg viabilities of < 50 % in this species are not uncommon. Similarly, Miralto et al. (2003) showed that during diatom blooms in the northern Adriatic Sea, copepod egg production rates were high, but only 10 % of the eggs produced by C. *helgolandicus* were viable. *Calanus helgolandicus* egg viability is positively related to the total and relative contribution of specific taxa to the diet due to seasonal variability (Harris et al., 2000; Irigoien et al., 2000).

4.3. Body toxin concentrations of C. helgolandicus

The toxin profile analysis showed that the bodies of *Calanus helgolandicus* retained 0.02 – 3.3 % of the ingested toxins after feeding on *Alexandrium catenella* for four days, assuming that the diet is proportionally consistent with the prey field (Table 2). Toxin concentration was highest when the proportion of toxic cells in the diet was lowest. However, in all cases the body toxin concentration was very low (< 3.3 %) and we therefore refrain from over interpreting this result.

Retention efficiencies for *A. tonsa, A. hudsonica, Centropages hamatus, C. finmarchicus*, and *Eurytmora herdmani* fed *Alexandrium* spp. are also reported to vary between 0.2 – 10 % (White, 1981; Teegarden and Cembella, 1996; Teegarden et al., 2003; Campbell et al., 2004). These low retention efficiencies suggest that marine copepods are either able to metabolise toxins, or void them from their bodies via excretion, egestion or regurgitation (White 1981; Sykes and Huntley 1987; Guisande et al., 2002; Teegarden et al., 2003; Wexels Riser et al., 2003; Maneiro et al., 2002, 2005). Guisande et al. (2002) found low concentrations of PSTs in tissues and fecal pellets of *A. clausi* feeding on *Alexandrium minutum*, and suggested detoxification and excretion of dissolved toxins as the mechanisms of toxin loss. This interpretation would result in time-dependent increases in toxin degradation products within copepod tissues (e.g., metabolism of sulphocarbomoyl toxins to gonyautoxins and eventually to saxitoxins), as well as increased overall levels of toxins within their tissues. However, Teegarden et al. (2003) questioned this 'metabolic detoxification' pathway as studies suggesting detoxification (e.g., Teegarden and Cembella 1996; Guisande et al., 2002) did not observe these effects. An alternative explanation for the lower impact of toxins is that copepods feeding on HA lower the efficiency with which they absorb materials from their food.

Teegarden (1999) suggested that the absorption of both toxins and carbon may be lower for copepods feeding on toxic *Alexandrium* cells, relative to a non-toxic diet. For example, *A. tonsa, C. hamatus,* and *E. herdmani* fed non-toxic *A. tamarense* over the course of 24 h showed an increase in total body carbon (14–28 %), whereas copepods fed toxic *A. catenella* (as *A. fundyense*) either had no significant gains in body carbon (*C. hamatus*) or lost a significant amount of body weight (*A. tonsa,* and *E. herdmani*), despite the fact that the total carbon 'ingested' (32–63 % body weight day^{-1}) was not significantly different between the two diets offered (Teegarden, 1999). These results suggest that not absorbing carbon from toxin-producing *Alexandrium* sp. may help lower the impact of toxins. Alternatively, Sykes and Huntley (1987) suggested that toxic dinoflagellates may cause acute physiological reactions when ingested, inducing regurgitation. This idea was also supported by Teegarden et al. (2003), who suggest that regurgitation or sloppy feeding could explain the observed low efficiencies with which toxins are retained.

Regardless of the low toxin retention efficiencies in copepods, species at higher trophic levels may still be at risk due to chronic exposure (Kershaw et al., 2021). Forty different species of fish tested for toxin accumulation in Scottish waters found PST in 96.2 % of the samples. Since there were no HA recorded during the months when the fish were sampled, the concentrations reported in the study are unlikely to represent the acute doses ingested by fish during HA events (Kershaw et al., 2021). Therefore, even low toxin retention efficiencies may still enable toxins to be passed up the food chain and accumulate in higher trophic levels at discernible levels (Teegarden and Cembella, 1996; Teegarden et al., 2003; Campbell et al., 2004). Most previous experiments examined toxin accumulation in copepods over 24 h, and little is known about how increasing the exposure time will affect rates of detoxification, acclimatization or toxin accumulation in copepods. This considerable knowledge gap hinders our ability to understand how the transfer of toxins to higher trophic levels will change as the frequency and magnitude of toxin-producing HA blooms change in the future.

5. Conclusion

Blooms of the toxin-producing dinoflagellate, *Alexandrium catenella*, may have a direct or indirect impact on *Calanus helgolandicus*. Food ingestion by *C. helgolandicus* declined as the relative abundance of *A. catenella* in the available food increased. This may decrease the transfer of biomass from *C. helgolandicus* to higher trophic levels. Egg production and egg viability in *C. helgolandicus* were not affected by increasing the relative abundance of *A. catenella* in the available food. This may indicate that *C. helgolandicus* is able to tolerate the toxins produced, but could also suggest that eggs released within the 4-day experiments were produced either from food ingested prior to experimentation, or from maternal reserves. The body toxin concentrations within *C. helgolandicus* after 4 days of feeding on *A. catenella* were low, indicating that toxin retention efficiencies were also low. These results suggest that the immediate impact of *A. catenella* blooms on *C. helgolandicus* feeding behavior and short-term survival are likely to be minimal; however, even low toxin retention efficiency in copepods may still result in the transfer of toxins to higher trophic levels. Given the potential for toxin retention over longer periods, further studies are required to investigate the potential impacts on fecundity.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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