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In vivo exposure to northern diatoms arrests sea urchin embryonic development

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ABSTRACT

There are numerous reports indicating that marine diatoms may act harmful to early developmental stages of invertebrates. It is believed that the compounds responsible for these detrimental effects are oxylipins resulting from oxidized polyunsaturated fatty acids, and that they may function as grazing deterrents. Most studies reporting these effects have exposed test organisms to diatom extracts or purified toxins, but data from in vivo exposure to intact diatoms are scarce. We have conducted sea urchin egg incubation and plutei feeding experiments to test if intact diatom cells affected sea urchin embryo development and survival. This was done by exposing the common northern sea urchins *Strongylocentrotus droebachiensis* and *Echinus acutus* to northern strains of the diatoms *Chaetoceros socialis*, *Skeletonema marinoi*, *Chaetoceros furcellatus*, *Attheya longicornis*, *Thalassiosira gravida* and *Porosira glacialis*. The intact diatom cell suspensions were found to inhibit sea urchin egg hatching and embryogenesis. *S. marinoi* was the most potent one as it caused acute mortality in *S. droebachiensis* eggs after only four hours exposure to high (50 μg/L Chl*a*) diatom concentrations, as well as 24 h exposure to normal (20 μg/L Chl*a*) and high diatom concentrations. The second most potent species was *T. gravida* that caused acute mortality after 24 h exposure to both diatom concentrations. *A. longicornis* was the least harmful of the diatom species in terms of embryo development arrestment, and it was the species that was most actively ingested by *S. droebachiensis* plutei.

1. Introduction

For more than 100 million years the diatoms have evolved and adapted to a plethora of different environments. Estimates of the total range of species amounts from 100,000 to 200,000, and they are present in all marine environments (Armbrust, 2009). In northern temperate areas diatoms form the main food for planktonic and benthic marine invertebrates (Degerlund and Eilertsen, 2010). There are many indications that diatoms are chemically diverse (Barofsky et al., 2010; Huseby et al., 2013) and have high levels of bioactivity (Borowitzka, 1995; Prestegard et al., 2009; Mimouni et al., 2012). Northern temperate and Arctic diatoms are also special since they thrive at marginal environmental conditions (low temperatures and low light levels). This increases the likelihood of detecting so called extremophiles and thereby producers of bioactive secondary metabolites (Wilson and Brimble, 2009).

It used to be thought that diatoms are high-quality food for herbivores (Pohnert, 2005). Lately this has been disputed, and evidence has emerged that some diatoms produce secondary metabolites that may function as e.g. grazing deterrents. *Skeletonema costatum*, *Skeletonema marinoi*, *Thalassiosira rotula* and *Pseudonitzschia delicatissima* produces oxylipins, i.e. polyunsaturated aldehydes (Miralto et al., 1999; d'Ippolito et al., 2003; Adolph et al., 2004; Pohnert, 2005; Ianora et al., 1995, 1996, 2011). These compounds may affect the reproductive biology and ontogenesis of copepods (Miralto et al., 1999) and fertilization and early development of the ascidian *Ciona intestinalis* (Tosti et al., 2003). Apofucoxanthinoids are associated to *Thalassiosira pseudonana* (Shaw et al., 1995) and hydroxyl- and epoxy fatty acids and fatty acids hydroperoxides to *T. rotula*, *S. marinoi*, *P. delicatissima*, *Chaetoceros affinis*, and *Chaetoceros socialis* (Ianora et al., 2011).

Noxious effects of aldehydes against echinoderms have also

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been reported. Cellular extracts and 2-*trans*-4-*trans*-decadienal (DD) from *S. costatum* and *Nitzschia commutata* inhibited fertilization, embryogenesis and hatching success in a dose dependent manner of the sea urchins *Psammechinus miliaris* (Caldwell et al., 2002), *Sphaerechinus granularis* (Adolph et al., 2004) and *Paracentrotus lividus* (Romano et al., 2003). DD is known to disturb DNA replication and to prevent key mitotic events in *S. granularis* embryos (Hansen et al., 2004). Further Caldwell et al. (2005) detected sub-lethal effects of aldehydes on sea urchin larvae. Varrella et al. (2014) observed a dose dependent effect, but also recovery after exposure of the sea urchin *P. lividus* to the aldehydes heptadienal and octadienal. The common haptophycean *Phaeocystis pouchetii* produces toxic aldehydes that have deleterious effects on sea urchin (*S. granularis*) embryos (Hansen et al., 2003), cod (*Gadus morhua*) larvae (Eilertsen and Raa, 1995), and mammal blood cells (Stabell et al., 1999). Hence large focus has the late years been on the toxicity of phytoplankton species that are also assumed to be main constituents of marine invertebrate diets.

Almost all laboratory studies that have detected diatom toxicity have used in vitro aqueous immersion exposure (Tosti et al., 2003; Caldwell et al., 2005; Miralto et al., 1999; d'Ippolito et al., 2003; Adolph et al., 2004; Romano et al., 2003). Here animals have been exposed to solutions made by homogenizing and centrifuging diatoms or by dissolving pure toxin solutions. Some reports also conclude that in vivo exposure to intact diatom cells have deleterious effects on copepod egg production and hatching success (Lee et al., 1999) while other studies indicates that embryos of copepods and echinoderms incubated with intact diatoms or diatom exudates had normal hatching patterns (Campbell and Head, 2000; Tang and Dam, 2001; Irigoien et al., 2002). Caldwell et al. (2002) reported that embryos of the sea urchin *Psammechinus miliaris* incubated with intact diatom cells produced healthy larvae.

From this it is clear that there exists conflicting evidence whether certain naturally occurring diatoms are toxic or not. Then, also since most of the references cited above refer to organisms of South Atlantic or Mediterranean origin, the present work was aimed at detecting possible deleterious effects of intact northern diatom species on the embryonic development of the Northern sea urchin species *Strongylocentrotus droebachiensis*, (O.F. Muller) and *Echinus acutus* (Lamarck).

2. Materials and methods

2.1. Cultivation of diatoms

Monocultures of six centric diatom spring bloom species (Table 1) were cultured at two different temperatures (4.5 and 8.5 °C).

In terms of cell size these centric diatoms belongs to two classes, i.e. *Attheya longicornis*, *C. socialis*, *Chaetoceros furcellatus* and *S. marinoi* are small species (<300 μm^{-3}) while *Porosira glacialis* and *Thalassiosira gravida* are large ones (8000–35,000 μm^{-3}), and all are common constituents of the northern temperate diatom flora (Degerlund and Eilertsen, 2010).

The cultures originated from single cells or chains of the specimens, isolated from multi-species diatom cultures that had been started by germinating spores contained in bottom sediments or from water samples collected in the Barents Sea and along the coast of northern Norway (Table 1). All cultures were kept in vegetative state for ca. one year prior to the start of the experiments. The species *A. longicornis*, *C. socialis*, *P. glacialis* and *S. marinoi* were identified by applying morphological and molecular methods (18s rDNA, (SSU) and 28s rDNA (LSU)). *T. gravida* and *C. furcellatus* were identified from cleaned frustules and spores using light microscopy applying morphological characteristics (see Huseby et al., 2013).

The diatoms used in the experiments were batch cultivated in sterilized 1.5 L soft drink (PET) plastic bottles in temperature and light controlled rooms. Cultivation took place in nutrient sufficient medium (Sigma-Guillard's f/10) prepared from filtrated and autoclaved deep water from Malangen outside Tromsø, (69°29,73′N–18°23,60′E, 160 m). PAR scalar irradiance was 40 $\mu mol\ m^{-2}\ s^{-1}$ measured with a QSL-100 (Biospherical Instruments Inc.) sensor. Illumination was fluorescent tubes (Osram L 58W/954 Daylight) and photoperiod was L:D; 14:10. The positions of the cultivation bottles were altered randomly on a daily basis to ensure that they received the same amount of light. The diatoms were cultivated at approximately the same temperatures (4.5 and 8.5 °C) as the sea urchin experiments took place at, i.e. 8.0 ± 0.82 °C (mean ± SD) for *S. droebachiensis* and 4 ± 0.34 °C for *E. acutus*. The cultures were, by dilution, kept in an exponential state of growth at concentrations above 20 g Chl*a* L^{-1}. In vivo Chl*a* was monitored with a Turner TD-700 fluorometer. In vitro vs. in vivo Chl*a* L^{-1} calibration curves were obtained by measuring different concentrations of all diatom species using both methods. To measure in vitro Chl*a* L^{-1} we applied the method of Holm-Hansen et al. (1965) using ethanol as extractant while the in vivo measurements were performed by measuring the cultures directly at the cultivation temperatures.

2.2. Sea urchin experiments

Sea urchin embryo development experiments were performed in a laboratory (*S. droebachiensis*) and on board the research vessel Helmer Hanssen (*E. acutus*). Exposure to intact diatom cells was performed with both *S. droebachensis* and *E. acutus* (Table 2), while ingestion experiments were performed with *S. droebachenis* solely.

During the laboratory experiments (*S. droebachiensis*), seawater was retrieved from 20 m depth in Tromsøysund. Salinity was 33.9 ± 0.24‰ (mean ± SD). Prior to the experiments the seawater was filtered through a 0.22 μm membrane filter (Millipore) and stocked for one week to be aged before use. During the spring bloom along the coast of Norway and in the Barents Sea Chl*a* concentrations may reach 20–25 μg L^{-1} and during intense blooms even higher values may occur (Degerlund and Eilertsen, 2010). To mimic "normal" and "high" phytoplankton biomass concentrations, 20 μg Chl*a* L^{-1} (low dose, LD) and 50 μg Chl*a* L^{-1} (high dose, HD) were applied in the *S. droebachiensis* assays. In the *E. acutus* experiments onboard the research vessel only 20 μg Chl*a* L^{-1} (LD) was used. This was due to large mortalities during and after 32 and

Table 1
Applied diatom species and acronyms (in brackets), strain IDs and geographical origin (latitude °N). Cell volumes are from Huseby et al. (2013).

Species	Strain ID	Volume (μm^{-3})	Origin
Chaetoceros socialis (Cs) Lauder	AMB80	59	Barents Sea (74.5 °N)
Skeletonema marinoi (Sm) Sarno and Zingone	AMB39	126	North Norwegian coast, (69.5 °N)
Chaetoceros furcellatus; (Cf) Bailey	AMB61	201	Barents Sea (77.8 °N)
Attheya longicornis (Al) Crawford and Gardner	AMB20.1	296	North Norwegian coast (69.5 °N)
Thalassiosira gravida (Tg) Cleve	AMB85	8170	Ramfjord (69.4 °N)
Porosira glacialis (Pg) (Grunow) Jørgensen	AMB49.2D	35 366	Tromsøysund (69.4 °N)

Table 2
Diatom species and concentrations applied in the embryo development experiments. For diatom acronyms see Table 1. All experiments took place in Nunclon 4 well (4 × 2) mL chambers.

Development stage	Sea urchin applied	Diatom species applied	Concentration (mg Chl*a*/L)
Clevage and hatching	*S. droebachiensis*	Al, Cf, Cs, Tg, Pg, Sm	20 (LD)
Clevage and hatching	*S. droebachiensis*	Al, Cf, Cs, Pg	50 (HD)
Clevage and hatching	*E. acutus*	Al, Sm, Cs	20 (LD)
Ingestion experiments	*S. droebachiensis*	Al, Cf, Cs, Tg, Pg, Sm	20 (LD)

64 cell stages in 50 μg Chl*a* L^{-1} treatments (Table 2).

Prior to the experiments several pre-tests were performed, i. e. phytoplankton conentrations, duration of experiments and number of embryos in the wells.

To determine the stages of ingestion and digestion, a modification of the scale proposed by Strathmann Martinez-Fernandez et al. (2004) was used (Table 3). The gut contents were examined in a Primo Vert (Zeiss) inverted fluorescence and an Axio Vert A1 (Zeiss) compound microscope in Nunclon 4 × 2 mL chambers.

The experiments with *S. droebachiensis* were performed during March and April 2012. The spawning stock was from "Tromsø Kråkebolle" (sea urchin farm in vicinity of Tromsø). The animals were kept at a three months advanced light regime, ambient sea temperature (8 °C) and supplementary food added. The spawning procedure was performed at the farm. Individual animals were injected in the coelomic cavity with 3 ml of 0.5 M KCl through the peristomal membrane to stimulate emission of gametes. Gametes from three individual male and females were collected separately in 50 ml glass beakers and transported to the lab in an ice-container. Fertilization took place immediately after arrival by placing ovaries in 120 ml glass containers with filtered (0.22 μm) fresh sea water (FSW) kept at 8 °C and then adding a few drops of dense spermatozoa suspension. Ca. 40 min after fertilization, water was drained and replaced three times by fresh FSW to remove excess spermatozoa. The fertilized oocytes were then transferred to a 4 l glass jar containing (0.22 μm) FSW at a ~2 eggs mL^{-1} concentration. The larvae culture was aerated gently. Debris from the bottom of the jar was daily siphoned off to remove dead matters and ca. 2/3 of the vessel volume was replaced by aged natural FSW every 2nd day at the beginning and daily during the last part of the experiment.

During the experiments we only used oocytes with elevated fertilization membranes. The embryos were treated with diatoms in 4 well Nunclon (4 × 2 mL) plastic chambers with oxygen saturated unialgal suspensions (Table 2). Each well was stocked with ca. 70 fertilized oocytes (cleavage and hatching) or ca. 50 gastrula stage embryos with 4 replicates per treatment (for each species). FSW was used as control treatment. The incubation solution was renewed and aerated while dead larvae were collected and fixed in 4% formaldehyde daily.

The *E. acutus* experiments were performed onboard R/V Helmer Hanssen during a survey in May 2012. Adult specimens of *E. acutus* were collected with an Agassiz bottom trawl from a deep-water population (62° 58′ 644″ N, 008° 36′ 552″ E, 94–104 m, ~4 °C). Spawning was induced by warming up the animals to room temperature (20 °C) whereafter gametes from 2 females and 2 males were pooled. This suspension was transferred to a 50 mL polystyrene tube with 30 ml of FSW (0.22 μm, salinity 33.9‰). The tube was then placed in a refrigerated room (4.5 °C) to complete fertilization. When the fertilization membrane was elevated, the eggs were rinsed three times (decantation of upper water layer) to remove excess spermatozoa and debris. 21–23 eggs with well-developed fertilization membranes were distributed into each 2 mL well of the Nunclon chambers. FSW was used as control and treatments were set up with four replicates. The reason why different number of eggs per well were used in the *E. acutus* and *S. droebachiensis* experiments was that test experiments performed on beforehand revealed that the two echinoderm species suffered somewhat higher mortalities for densities 30–40% above the densities applied in the final experiments.

The ingestion experiments were conducted to quantify ingestion of diatom cells by the *S. droebachiensis* larvae. Samples of larvae from 4 and 6-arms development stages were sieved (85 μm nylon screen), washed and resuspended in FSW. Larvae to be used in experiments the next day were starved overnight under dim light at 8 °C and 30 active, healthy larvae were transferred to 100 mL glass beakers containing ca. 60 mL of each of the diatoms. All these experiments were performed with four replicates for each diatom (Table 2).

After 2 h treatment with diatoms, the larvae were washed in FSW and transferred to Nunclon chambers with FSW. The gut content was examined without delay in the inverted fluorescence microscope and thereafter in the ordinary inverted light microscope. Observations of the digestive gland content of the larvae were repeated 1, 2, 3, and 4 h after washing.

2.3. Statistics

To test for differences between treatments (species − concentrations) we applied One-Way ANOVA post hoc Fisher LSD tests.

3. Results

3.1. Cleavage and hatching

Fertilized eggs of *S. droebachiensis* started to divide 4 h after fertilization in both LD (*Al*, *Cf*, *Cs*, *Tg*, *Pg*, *Sm*) and HD (*Al*, *Cf*, *Cs*, *Pg*) treatments. The LD Sm treatment (Fig. 1) arrested division in 64% of the eggs followed by Pg (41%) while the lowest numbers of arrested eggs were in FSW and *Cf*. There were no dead cells observed in the

Table 3
Stages of *S. droebachiensis* larvae ingestion and digestion (modified from Strathmann Martinez-Fernandez et al., 2004).

Stage	Fluorescence	Characteristics
1	Red (intense)	Whole algal cells well defined in the stomach (abundant)
2	Red (weak)	Whole algal cells well defined in the stomach (single cells)
3	Tones of pink Orange or Yellow	Whole and lysed algal cells mixed in the stomach Or no whole cells present (lysed algae only)
4	No fluorescence	Empty stomach: larvae not fed or had finished digestion

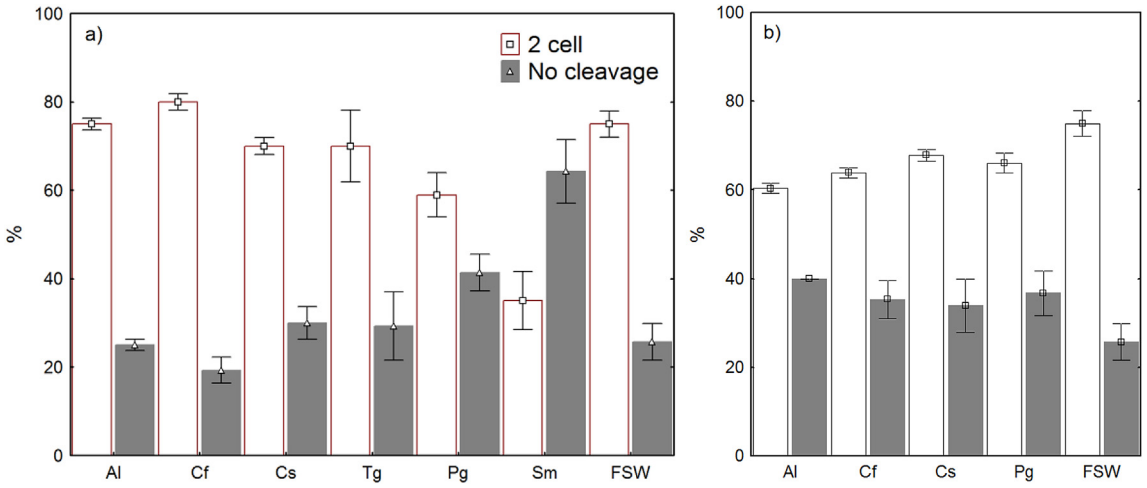

Fig. 1. *S. droebachiensis* development stages (mean% of four treatments) after 4 h treatment with 20 μg (a) and 50 μg Chl*a*/L (b) monocultures of six diatom species. For species acronyms see Table 1. FSW is control (fresh sea water). Treatments with 50 μg Chl*a*/L Sm and Tg are not included in the right figure (b) due to large acute mortalities. Vertical bars are SD for four treatments and n was 70 for each treatment.

LD treatments and in the HD treatments of Al, Cf, Cs, Pg and FSW. However, the Tg and Sm HD treatments experienced large acute mortalities (not shown) and the few eggs alive did not divide, so these were excluded from the analysis. In the HD treatments with Al and Pg 40 and 37% (respectively) were arrested, while in the control treatment (FSW) 25% of the eggs were arrested.

When *E. acutus* was treated with LD of three diatoms (*Al*, *Sm*, Cs) for four hours there was low mortality and high levels of first cell division was reached by the oocytes: In the control (FSW) all eggs had divided while in the diatom treatments 91–92% of the eggs divided (Fig. 2). When *E. acutus* was treated with LD cultures of diatoms for 24 h mortalities increased 47–51%, (Fig. 2). While 51.3% of the oocytes in FSW had reached morula no such development was observed in the diatom treatments. Of the surviving cells exposed to diatoms all were in 32–64 cell stages (48.7–50%).

After 24 h the *S. droebachiensis* oocytes had reached 32, 64 cell and morula stage (Fig. 3) and mortalities had started to occur. In the LD treatments the lowest mortalities were observed with Cf and Al (23.8 and 27.4%, respectively) while the highest was in the Pg treatment (45.2%). The larger part of the surviving oocytes had reached the morula stage and it was Cf and Al that had the highest rate (59.6 and 54%). In the LD Sm treatment almost all cells were dead and none had reached 16 or 32 cell stage (not shown).

In the HD *S. droebachiensis* treatments (Fig. 3) the lowest mortalities were in FSW (28.3%). No cells had reached morula stage and some oocytes still were in 32-cell stage, i.e. there was a significant arrestment of development relative to the LD treatments. Of the treatments *Al* had the highest mortality (43%). Further all 24-h exposures had slightly higher mortalities than the 4-h ones (Fig. 1 vs. Fig. 3).

The 4-arm plutei solely ingested Al (90%, Fig. 4). No dead individuals were observed in the Al treatment and plutei reached digestion stage 3 after 1–2 h. The other species in fact led to high mortalities, i.e. Sm 42.5%, Cf 65%, Pg 67.5%, Cs 70% and Tg that had 100% mortality (mean values, Fig. 4). In the 6-arm plutei experiments it was only the Sm cultures that induced mortalities. Again it was Al cells that were actively preyed upon, and occasionally *Cf*, *Cs*, *Pg*, and *Tg* were taken in. Direct video observations demonstrated that most cells (except Al) in fact were avoided or rejected from the oral hood. A video demonstrating rejection of Pg cells is uploaded on http://phaeocystis.com/Pg.wmv.

We did not observe the final digestion stage (4) for 4-arm plutei within a 4-h period after washing. The larvae showed some remaining fluorescence indicating digestion was probably still in progress. We observed significantly smaller amounts of intact *Al* cells in 6-arm larvae digestive glands throughout the experiment.

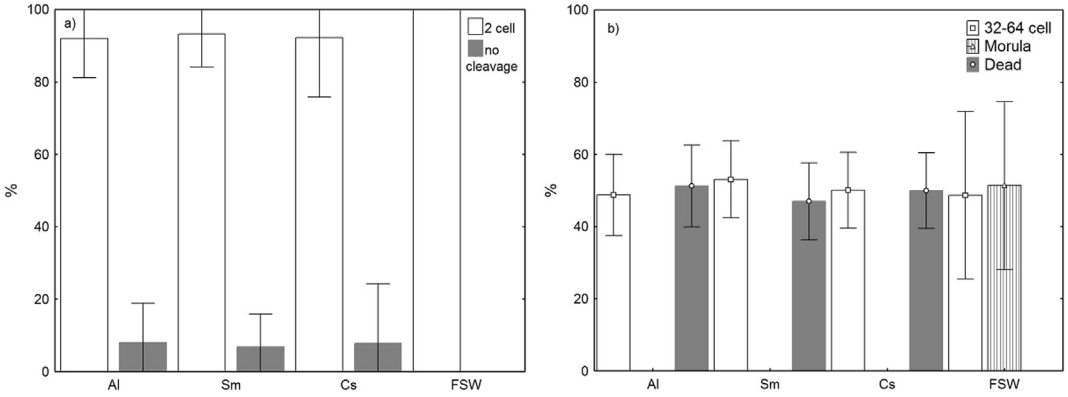

Fig. 2. *E. acutus* development stages (mean% of four treatments) after 4 (a) and 24 h (b) hours treatment with 20 μg Chl*a* L^{-1} monocultures of 3 diatom species. For species acronyms see Table 1. FSW is control (fresh sea water). Left figure shows first cleavage and right 32–64 cells and morula stage. Vertical bars are SD for four treatments and n was 21–23 for each treatment.

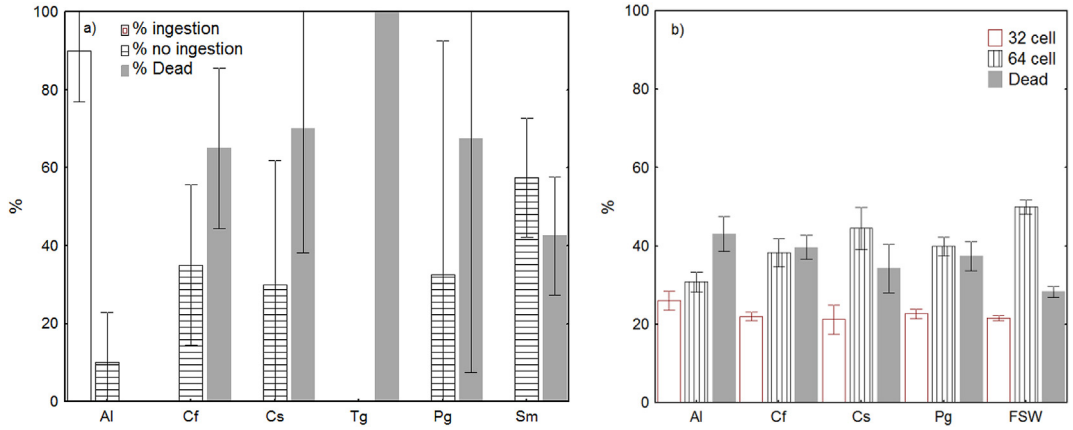

Fig. 3. *S. droebachiensis* developmental stages after 24 h treatment with monocultures of six species of diatoms. Left figure (a) 20 μg and right (b) 50 μg Chl*a* L^{-1} treatments. Vertical bars are SD for four treatments and n was 70 for each treatment. For species acronyms see Table 1.

Embryos at this stage digested cells faster reaching the final stage (4) within 2 h after washing.

3.2. Summary of statistical tests

In the *S. droebachensis* LD first cleavage experiments, Sm and Pg led to the highest number of eggs arrested at the 2-cell stage (Fig. 1). The one-Way ANOVA post hoc tests for differences showed that these were statistically significantly different from the FSW treatments ($p = 0.000$). The number of arrested eggs treated with Al, Cs and Tg were not significantly different from FSW ($p = 0.747$; 0.064; 0.118, respectively) while Cf in fact had significantly lower arrestment rates than FSW ($p = 0.008$). In the HD treatments (Sm and Tg excluded due to high mortalities) all surviving species (Al, Cf, Cs, Pg) had significantly higher cell arrestment rates than FSW ($p = 0.000$). For these four diatom species the HD treatments also showed significantly higher cell arrestments compared to the LD treatments; both Al and Cf had $p < 0.000$ and p for Cs was 0.032 and for Pg $p = 0.0129$ (Fig. 1).

In the *E. acutus* treatments (LD, Fig. 2) all diatom treatments had significantly higher cleavage arrestments than FSW (that had zero arrestment). The same pattern emerged after 24 h and high mortalities had occurred in the diatom treatments.

In the LD *S. droebaciensis* 24 h treatments (Fig. 3) Cs, Tg and Pg had significantly higher mortalities ($p = 0.009$; 0.006; 0.000) than FSW while Al mortalities were not distinguishable from FSW ($p = 0.063$) and Cf had lower mortalities ($p = 0.010$) than FSW. In the HD 24 h treatments all remaining diatom species had significantly higher mortalities than FSW ($p < 0.05$, Fig. 3).

4. Discussion

As was shown in our *S. droebachiensis* LD assays, intact cells of two out of the six diatom species applied, i.e. *Sm and Pg*, had statistically significant antiproliferative activity on fertilized eggs (first division) compared to the controls (FSW). Three of the diatoms that did not arrest cell division at this stage (*Al*, *Cs*, *Tg*) though caused non-viable hatched blastulas after 24 h and mortalities significantly higher than for FSW. *Sm*, that initially caused the highest degree of cell division arrestment, resulted in 100% dead cells after 24 h in LD and similar to Tg it also caused acute mortality at HD after 2 h. Since the 2 h HD treatments with *Al*, *Cf* and *Cs* had higher numbers of non-dividing eggs compared to the LD, and the same was the case for the 24 h experiments, we conclude that some toxic substance was present, and that it acted in an approximately dose dependent manner.

The fact that Sm and Tg had the most profound negative effects on *S. droebachiensis* eggs is maybe not surprising since both are amongst the most frequent reported and potent aldehyde producing diatoms (Ianora et al., 1996; Barofsky et al., 2010), and we therefore hold it likely that the toxin in question here is an oxylipin.

In our experiments the controls (FSW) generally had relatively

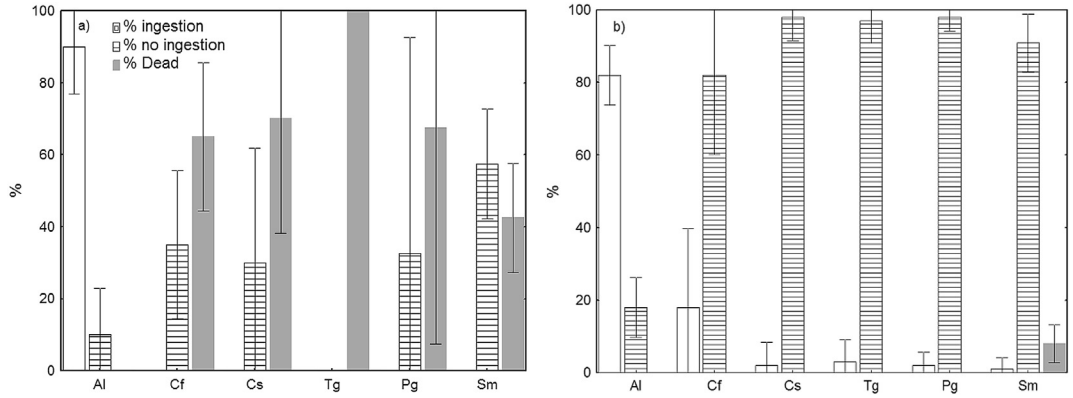

Fig. 4. Ingestion of monocultures of six diatoms (20 μg Chl*a* L^{-1}) by *S. droebachiensis* 4-arm (a) and 6-arm plutei (b). Percentages refer to amounts of larvae in each category with stomach content 1–3 according to Table 2. Vertical bars are SD for 4 treatments and n was 10 for 4 arms stage and 25 for 6 arms stage.

high numbers of non-dividing eggs and mortalities, and at some instances this was not discernible from the diatom treatments. One possible cause to this may be that the natural seawater we applied contained some toxic chemical compound or bacteria that passed 0.22 μm filter we used. It must also be considered that invertebrate larvae frequently can experience "natural" mortality rates above 0.1 day^{-1} (Strathmann, 1985). This may be the case both under natural conditions and in laboratory experiments, and the mortality may also be influenced by egg quality (George, 1996). We therefore conclude that the FSW mortalities were at the upper levels of published values, but though not uncommon and as such acceptable.

In the 4-arm plutei *S. droebachiensis* feeding experiments it was only Al that was ingested while the other species caused high mortalities. Tg in fact resulted in 100% mortality of the plutei. The 6-arm plutei also ingested large amounts of Al and some Cf while the other species (Cs, Tg, Pg, Sm) only were digested in minute amounts, and no mortality was observed. Since it was Sm, Tg and Pg that caused the most pronounced cell division arrestment in the egg incubation experiments, this possibly points towards selective gracing. Further we consider it unlikely that this was due to size preference since Sm, that induced large mortalities in eggs and was avoided by the plutei, is the second smallest species, while Al, that was the preferred food, is the fourth largest of the six diatoms applied. We therefore set up a separate experiment where we applied a microscope video recorder to monitor the feeding behavior of the plutei. In some of these recordings it appeared that certain species were taken into the gut and thereafter actively ejected (see video Pg on http://phaeocystis.com/Pg.wmv).

The fact that 6-arm plutei had negligible mortalities compared to the 4-arm ingestion experiments indicates that the insidious effects of the toxin had lesser effect on late developmental stages. Further we observed that *E. acutus* reacted similarly negatively to all the diatom cultures (Al, Sm, Cs), but only after 24 h exposure. This may have arisen from that different development stages or echinoderm species reacted different to the toxins. Also this may have resulted from that cell molecular composition and oxylipin levels in certain diatoms may vary with the growth phase of the cultures (Barofsky et al., 2010; Vidoudez and Pohnert, 2008; Huseby et al., 2013; Gerecht et al., 2013). Since the results from the present study do not allow for discrimination between such effects, we are not able to conclusively rank all the diatom species with regard to toxicity. We though mean our results clearly demonstrates that in vivo exposure to diatoms can affect early cell cleavage negatively in both *S. droebachiensis* and *E. acutus*, and also induce mortality at later developmental stages. Further there were indications that Sm was the most potent toxin producer followed by Tg and Sm. On the other side Al seemed to be the least toxic species.

The diatom Al was clearly the preferred food item by 4- and 6-arm plutei. The avoidance of the other species may be related both to cell size, types of spines and also possibly food quality (Rose and Baker, 1994; Lora-Vilchis and Maeda-Martinez, 1997). The larval fitness may vary and be influenced by food quality and quantity (Paulay et al., 1985; Hart and Strathmann, 1994; Bertram and Strathmann, 1998; Poorbagher et al., 2010), and we cannot rule out that such effects were present. Plutei are suspension feeders, actively responding to each food particle (Strathmann, 1971; Strathmann et al., 1972), and since there was no meaningful correlation between food size, shape and ingestion we assume that it was some allelopathic mechanism that caused this, as also indicated by our video observations of the feeding behavior.

Our results clearly conflicts with e.g. Caldwell et al. (2002) who reported that hatching and fertilization success of the echinoderms *Asterias rubens* and *Psammechinus miliaris* were unaffected by in vivo exposure to cultures of the diatoms *Nitzschia commutata* and *S. costatum*. All embryos incubated with intact cells this study developed beyond 128 cells stage and produced healthy normal larvae. Campbell and Head (2000) also showed that in vivo exposure to *Thalassiosira weissflogii* did not lead to increased mortalities in copepod eggs compared to FSW controls. As discussed earlier there are numerous reports indicating that in vitro exposure of diatom extracts or aldehydes are deleterious to copepods (Miralto et al., 1999; Pohnert, 2005; Ianora et al., 2011). Fertilization, embryogenesis and hatching in the sea urchins *Psammechinus miliaris*, *Sphaerechinus granularis* and *P. lividus* were found to be affected negatively by in vitro exposure to diatom aldehydes (Romano et al., 2003; Adolph et al., 2004). It is known that the quality and amounts of insidious compounds produced by diatoms might be affected by temperature (Huseby et al., 2013) diatom growth phase (Barofsky et al., 2010) and strain (Lakeman et al., 2009), and oxylipin production may be triggered by mechanical action (grazing, pressure, Pohnert, 2005). It is thus perhaps not surprising that discrepancies in the literature may occur, and we can not exclude that in our study exudates were present and influenced the results. The only sound conclusion to be made from this is therefore that certain diatom species may act deleterious towards echinoderm early life stages, and that the magnitude of this may vary with species and environmental conditions.

In Northern areas *S. droebachensis* spawns in concert with the phytoplankton spring bloom, while *E. acutus* spawns during autumn (Falk-Petersen, 1982). All the phytoplankton species applied in our experiments are present during the spring bloom. The magnitude of the diatom bloom may vary quite much between years, i.e. some years the haptophycean *P. pouchetii* may dominate the bloom (Degerlund and Eilertsen, 2010). Whether this influence the survival of pelagic invertebrates (e.g. copepods and echinoderm larvae) is an open question and will, even if targeted large-scale research is launched, in our opinion be difficult to answer conclusively.

Ethical statement

This will be arranged if manuscript is accepted, different authors on vacation and if each shall send form this can only be arranged later.

Conflict of interest

The authors declare The authors hereby declare that this manuscripthas not been published/submitted for publication elsewhere, and that no conflicts of interest exists.

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