



Full Length Article

Production of Aldehydes in Diatoms and Dinoflagellates and the Detrimental Effect on Copepod Grazers

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Abstract

Marine algae, including diatoms, are the primary food source of oceanic ecosystems, sustaining the marine food chain to top consumers and playing a key role in marine food webs. Their beneficial role is questioned due to the finding of harmful compounds in their secondary metabolites, such as polyunsaturated aldehydes, which impair reproductive and developmental processes of their grazers. To investigate the response of dominant copepod grazers in the Jiaozhou Bay to different diets and how aldehydes vary in batch algal cultures over time, we carried out experiments in which two diatoms *Skeletonema marinoi* and *Chaetoceros didymus* were fed to the copepod *Calanus sinicus*. Controls were run with two dinoflagellates *Prorocentrum micans* and *Scrippsiella trochoidea*. In our two set of experiments, we found a C-saturated aldehyde released by those four algal species, which is less reported. Our results showed that diatoms have greater potential for aldehydes production than dinoflagellates and the peak of aldehydes production occurred mostly in the first and second week, which clearly includes lag and exponential phase. However, the detrimental effect on copepods was species dependent. Some dinoflagellates are also potential aldehydes producers. Even though some dinoflagellates released fewer aldehydes than diatoms, the inhibitory effect on copepods was greater. © 2019 Friends Science Publishers

Keywords: Aldehydes; Diatoms; Dinoflagellates; Copepods

Introduction

Diatoms, one of the most common classes of autotrophic phytoplankton, contribute 40%-45% of the total primary production in oceanic environments, playing a key role in marine food webs (Yool and Tyrrell, 2003). Traditionally, diatoms are regarded as a good food source for primary consumers of herbivorous plankton, such as the small crustacean copepods. However, recently, the traditional view of marine food web energy flow from diatoms to fish by means of copepods is challenged with the discovery that some diatom species produce cytotoxic secondary metabolites, including polyunsaturated fatty acids (PUFAs) (Grima *et al.*, 1995; Li *et al.*, 2008; Pezzolesi *et al.*, 2017), polyunsaturated aldehydes (PUAs) (Ianora *et al.*, 2012; Pezzolesi *et al.*, 2017), and other compounds collectively termed as oxylipids. PUAs are mainly synthesized from enzymatic degradation of PUFAs upon cell wounding. It is confirmed that this wound-activated release takes place not only in the laboratory (Miralto *et al.*, 1999; Pohnert, 2000) but also in the field (Ianora *et al.*, 2004; Ribalet *et al.*, 2014). This process is a form of allelopathy, utilized by diatoms and other marine phytoplankton as a defense mechanism against

predation and inhibits competitors by producing certain chemicals (Strom, 2008; Ianora *et al.*, 2011). It has been reported that 36% of the 51 investigated diatom species released aldehydes showing a species-specific and strain-dependent production (Wichard *et al.*, 2005) and that the aldehydes are toxic to a wide variety of organisms, from bacteria to invertebrates, including phytoplankton (Pohnert, 2010). This defensive reaction can induce abortions, poor larval development and high offspring mortality in many invertebrates. Numerous studies (Miralto *et al.*, 1999; Wichard *et al.*, 2007; Ianora *et al.*, 2012; Lavrentyev *et al.*, 2015) indicate that aldehydes have an adverse effect on reproduction and development of copepods which Ban *et al.* (1997) described as "diatom-copepod paradox". However, the effect can not only be species-strain-specific, but also environmental-specific. Aldehydes composition and production can also depend on nutrient limitation and growth phase stage (Pohnert, 2002; Ribalet *et al.*, 2007). Additionally, the response of different copepod species to specific algal diets can vary (Ianora *et al.*, 2003; Md Amin *et al.*, 2011).

The aim of the study is to clarify the response of dominant copepod grazers in the Jiaozhou Bay (JZB) to

different diets and how aldehydes vary in batch algal cultures over time. We hypothesized that copepods would prefer dinoflagellates over diatoms and aldehydes production would increase with time. JZB is a typical semi-enclosed bay located in the west of the Yellow sea with a water area of 340 km² and an average depth of 7 m (Gao et al., 2014), where harmful algal blooms (HABs) occur often (Yuan et al., 2017). So far, the correlation of phytoplankton blooms with several environmental parameters has been investigated in detail (Mundy et al., 2014; Carstensen et al., 2015), while we should also focus on how phytoplankton blooms can alter oceans biochemically not only to the water body but also to their grazers.

Material and Methods

Copepod Sampling

Calanus sinicus are cosmopolitan, euryhaline and opportunistic copepod species. They were caught with a 500 µm net by vertical hauls on March 2009 from Jiaozhou Bay (36°06'N; 120°19'E), where they often account for the major part of the copepod assemblage. They were transported to the laboratory within one hour after sampling. Healthy mature females with intact appendages and active swimming behavior were sorted within 4 h and reared in 0.25 µm sieved ambient seawater.

Phytoplankton Cultures

Two diatoms *Skeletonema costatum* (SKE) and *Chaetoceros didymus* (CHA) and two dinoflagellates *Prorocentrum micans* (PRO) and *Scrippsiella trochoidea* (SCR) were selected as copepod food source. SKE and CHA are dominant diatom species in JZB, where they often form dense blooms. All the algae were collected from the ecological laboratory, Institute of Oceanology, Chinese Academy of Sciences, Qingdao. Monoclonal cultures were cultivated in f/2 medium at 20±0.5°C under a 12:12 light/dark cycle (Guillard, 1975).

Experimental Set-up

Two sets of experiments were carried out. The first was to detect how aldehydes vary in batch algal cultures within one month. The growth experiment were conducted in three independent replicates, inoculating four algal species in 5 L f/2 medium at 20±0.5°C under a 12:12 light/dark cycle. To investigate production of aldehydes during growth under standard conditions, several independent algal exudates were sampled every 7 days within one month, meanwhile, the growth curves (Fig. 1) were drawn according to the daily cell concentrations, which were monitored daily by Coulter Counter (Multisizer 3.5 µm aperture; measuring range: 2-30 µm). Every seven days, algal exudates were centrifuged and 3mL concentrated algae was stored in 20mL sealed headspace bottle at 20°C.

In a second set of experiments, we focused on the effects of different diets on the growth and recruitment of copepods. *Calanus sinicus*, after being captured, were acclimated in GF/F filtered seawater for 48 h. The egg production rate and hatching success of first day after sampling were recorded as the initial values to estimate the natural conditions. From the second day, females were acclimated 48 h under experimental conditions of various food types and concentrations. To study the effect of aldehydes on copepods, algal cultures were renewed with new f/2 medium to maintain cells in exponential phase (Fig. 1) and diluted to 0.5 µg C mL⁻¹ using GF/F filtered seawater daily for feeding.

The grazing experiment was conducted in five biologically independent replicates, placing six adult female copepods in four monoclonal (SKE, CHA, PRO, SCR) cultures (initial cell density 0.5 µg C mL⁻¹) respectively at exponential growth phase in 400 mL bottles. These bottles were plastic cylinders with a 220 µm sieve placed 5 cm above the bottom to separate eggs from copepods to prevent cannibalism of eggs. Each treatment with 300 ml algal suspension was incubated in a biochemistry cultivation cabinet under dim light at 6.5±0.5°C in accordance with the copepods sampling temperature.

Half a bottle of suspension was collected for egg counting and supplemented with equal amount of fresh algal culture. Over the period of 15 days, the number of surviving copepods was recorded and female survival (FS) as well as egg production rate (EPR) was calculated. After that, 30 eggs isolated from suspension were incubated in culture dishes with 20ml filtered seawater under dim light at 6.5±0.5°C. This process was monitored with a microscope every 24 h until 72 h to ensure all vital eggs had sufficient time to hatch. Hatching success was defined as the number of hatched nauplii which developed into naupliar stage NII. Finally, the cultivated suspension for feeding copepods was harvested by centrifugation. After removing the supernatant, 3 mL concentrated algae was stored in 20 mL sealed headspace bottle at 4°C.

Aldehydes Quantification

The quantification of aldehydes concentration was carried out by HSSE (Head space sorptive extraction)-GC-MS technique. Firstly, 2 mL O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride solution (PFBHA, Sigma-Aldrich, U.S.) was incubated at 75°C for 3.5 min for headspace absorption onto the PDMS/DVB fiber. The algal sample was ultrasonicated for 30 sec and then incubated at 75°C for 10 min. Afterwards, the PFBHA-absorbed PDMS/DVB fiber was put into the headspace bottle with treated algal sample and incubated at 75°C for 20 min. After that, PUA concentration was determined by GC-MS and molecular identification was done by comparison of retention times and mass spectra with those of available commercial standards: trans-2-hexenal (99%, Tokyo Chemical Industry,

Japan), trans, trans-2,4-heptadienal (94%, Tokyo Chemical Industry, Japan), trans-2-decenal (95%, Tokyo Chemical Industry, Japan), trans, trans-2,4-decadienal (95%, Tokyo Chemical Industry, Japan), heptaldehyde (1000 mg/L, Accustandard, U.S.) and trans-2-heptenal (1000 mg/L, Accustandard, U.S.).

Results

Aldehydes Production in different Growth Phases

We set the first seven-day as stage I and continue respectively. A time-dependent production of aldehydes was observed for all cultures (Fig. 2). The total aldehydes production of the four algae species all reached peak at stage I and stage II, which clearly includes lag and exponential phase (Fig. 1). Among the aldehydes, trans-2-hexenal ($0 \sim 263.6910^{-3} \mu\text{g/g DW}$) (DW represents dry weight of algae) and trans, trans-2,4-heptadienal ($0 \sim 469.19 \cdot 10^{-3} \mu\text{g/g DW}$) were always the most abundant, accounting for over 50%, during the whole period. Other aldehydes accounted for a lower proportion of total aldehydes, especially trans-2-decenal, which only appeared in trace amounts on SKE I and CHA III.

During the whole period, the average amount of aldehydes produced by diatoms was higher than the one produced by dinoflagellates (Fig. 3), which showed diatoms have greater potential for aldehydes production than dinoflagellates. Among them, CHA produced the maximum aldehydes and PRO the minimum. Additionally, there were six aldehydes released by diatoms, while only five were produced by dinoflagellates. Specifically, there was no trans-2-decenal in PRO and SCR and no trans-2-heptenal in PRO. The ability of diatoms to release aldehydes was significantly higher than the ability of dinoflagellates, especially in the late growth phase. Among all the aldehydes, we found a saturated aldehyde, heptaldehyde (Table 1), which is less reported.

Female Survival, Egg Production and Hatching Success

In the second set of experiments, female survival (FS), egg production rate (EPR) and HS (hatching success) of *Calanus sinicus* varied with different diets (Fig. 4-6). FS of females fed on PRO (Fig. 4) was higher (average 95.11%) during 15 days, while FS of other cultures declined sharply from the sixth day and stayed below 90% (on average, SKE, CHA and SCR were 79.35%, 81.08% and 84.52%, respectively). In general, viability of females fed on dinoflagellates was higher than of females fed on diatoms.

EPR and HS of females fed on PRO also remained at a high level (4.13 ± 1.64 (mean \pm s.d.) eggs female⁻¹day⁻¹ and $70.93 \pm 14.73\%$, respectively), even though they were lower compared to the initial value (7.13 ± 2.96 (mean \pm s.d.) eggs female⁻¹day⁻¹ and $81.11 \pm 2.94\%$, respectively). During the 15-day experiment, the average values of EPR for different diets ranged from 1.35 ± 1.29 eggs female⁻¹day⁻¹ to 4.42 ± 1.64 eggs female⁻¹day⁻¹. Despite the low rate of the CHA treatment at

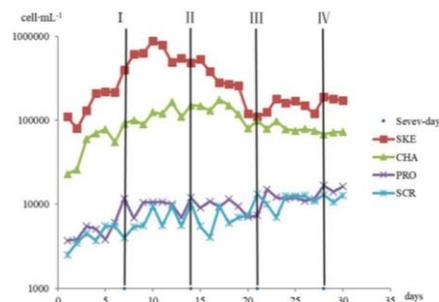


Fig. 1: Growth curves

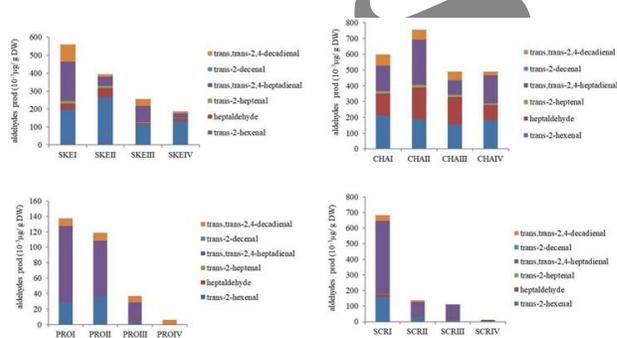


Fig. 2: Aldehydes production in every stage

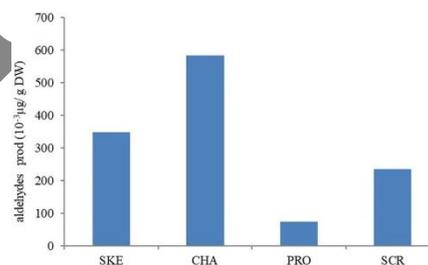


Fig. 3: Average aldehydes production during the whole period

the start, it climbed to 4.75 ± 1.33 eggs female⁻¹day⁻¹ at day thirteen and remained at 3.00 ± 1.57 eggs female⁻¹day⁻¹ after a small decrease. On the contrary, SKE and SCR treatments experienced a rapid increase at first, but then declined to below 2 eggs female⁻¹day⁻¹. HS of females fed on SKE, CHA and SCR were all below 60% ($56.43 \pm 22.34\%$, $55.91 \pm 18.81\%$ and $53.40 \pm 23.58\%$, respectively). There was no big difference among these three species. In summary, all diets resulted in decreasing female survivorship, viability and fecundity of eggs, while the PRO diet showed the lowest detrimental effect.

Aldehydes Production with Copepods Grazing

Aldehydes composition and production were distinctly different between diatom diets (SKE and CHA) and dinoflagellate diets (PRO and SCR) (Fig. 7). Firstly, the total amount of aldehydes released by diatoms was much higher

Table 1: Properties and structures of aldehydes

Name	Molecular Formula	Molecular Structure	CAS Number	Molecular Weight	Boiling Point(°C)
trans-2-hexenal	C ₆ H ₁₀ O		6728-26-3	98.15	81.6
heptaldehyde	C ₇ H ₁₄ O		111-71-7	114.19	153
trans-2-heptenal	C ₇ H ₁₂ O		18829-55-5	112.17	90
trans,trans-2,4-heptadienal	C ₇ H ₁₀ O		4313-03-5	110.15	84.7
trans-2-decenal	C ₁₀ H ₁₈ O		3913-81-3	154.23	78-80
trans,trans-2,4-decadienal	C ₁₀ H ₁₆ O		25152-84-5	152.23	115.2

than by dinoflagellates. Secondly, there were also six aldehydes released by diatoms, while only five were released by dinoflagellates, without trans-2-decenal in PRO and SCR. Four algal species, however, had in common that trans-2-hexenal and trans, trans-2,4-heptadienal were the most abundantly produced aldehydes, accounting for over 60%.

Discussion

Two diatom isolates (SKE and CHA) and two dinoflagellate isolates (PRO and SCR) were analyzed for aldehydes production over time or coexisting with copepods. The result of the first set of experiments showed that the intact diatoms and dinoflagellate cells could release aldehydes dependent on the growth stage. The peak of aldehydes production was mostly in stage I and II, which clearly includes lag and exponential phase. Vidoudez and Pohnert (2008) reported that intact cells of SKE released a pronounced burst of PUAs which only happened during a defined time span during stationary phase. Our results, however, showed that aldehydes release happened not only in stationary phase, but also in lag and exponential phase. What's more, the type and average amount of aldehydes released by diatoms was bigger than the one released by dinoflagellates, which demonstrated a great potential of aldehydes-formation of diatoms. Similarly, in the second set of experiments, diatoms also showed a better ability to produce aldehydes, when their cells were damaged by copepods grazing.

Traditionally, diatoms represent a quality food source for copepods, due to the well-balanced amino acid composition which can be stored in copepods eggs and utilized for nauplii development (Nahon *et al.*, 2010). Copepods are unable to synthesize essential amino acids, that are important in regulating cell membranes and precursors for tissue hormones, de novo. However, some diatoms lack or are deficient in some essential nutrients, such as C, N, P, polyunsaturated fatty acids (PUFAs), sterols and minerals (Brett and Müller-Navarra, 1997; Vehmaa *et al.*, 2011). Therefore, a mineral and biochemical inadequacy of a pure diatom diet cannot satisfy the nutrient demand of copepods. In the field, the growth peak of copepods and diatoms is always unsynchronized and copepods generally peak well after diatom blooms occur, suggesting that diatoms are not an ideal diet (Ianora *et al.*, 2004).

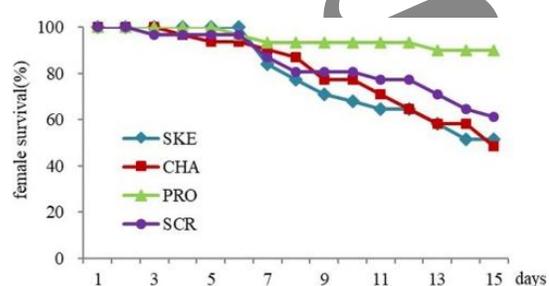


Fig. 4: Female survival (%) of *Calanus sinicus* fed on 4 diets (SKE: *Skeletonemacostatum*; CHA: *Chaetocerosdidymus*; PRO: *Prorocentrummicans*; SCR: *Scrippsiellatrochoidea*)

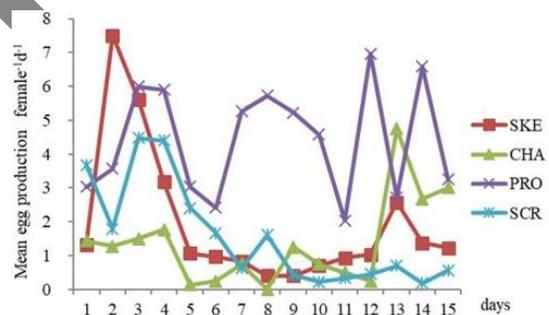


Fig. 5: Daily egg production of female *Calanus sinicus* fed on 4 diets (SKE: *Skeletonemacostatum*; CHA: *Chaetocerosdidymus*; PRO: *Prorocentrummicans*; SCR: *Scrippsiellatrochoidea*)

On the other hand, secondary metabolites released by diatoms are another important factor that is causing reproductive failure in copepods. Our results are consistent with studies (Ban *et al.*, 1997; Ianora *et al.*, 2004; Wichard *et al.*, 2007; Li *et al.*, 2008; Pohnert, 2010; Bruignano *et al.*, 2016) showing that aldehydes have a detrimental effect on the development and reproduction of copepods. Some studies (Niehoff, 2004; Poulet *et al.*, 2007) found that higher EPR and HS were produced on dinoflagellate diets rather than on diatom diets, while our results indicate that the adverse effect was species dependent. The aldehydes production of SKE (diatom) was about three times of the amount of SCR (dinoflagellate), while the EPR of SKE (diatom) was higher than the one of SCR (dinoflagellate) and their HS was almost the same. Thus, some dinoflagellates are also potential

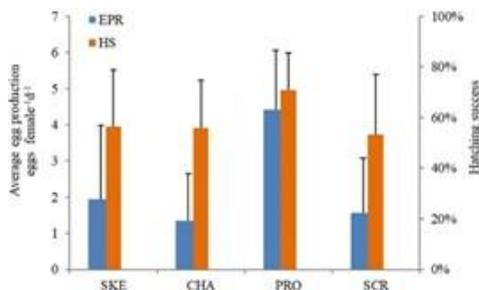


Fig. 6: Average egg production rate and hatching success of female *Calanus sinicus* fed on 4 diets (SKE: *Skeletonemacostatum*; CHA: *Chaetocerosdidymus*; PRO: *Proocentrummicans*; SCR: *Scrippsiellatrochoidea*)

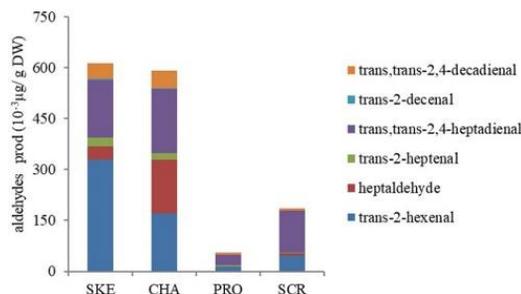


Fig. 7: aldehydes composition and production

aldehydes producer. We should not focus only on diatoms, but also on dinoflagellates in future studies. In addition, a mixed diet may serve to dilute toxins, a concept that is already explored in some studies (Ianora *et al.*, 2004; Li *et al.*, 2010).

Currently, most studies focus on unsaturated aldehydes, such as heptadienal, octadienal, octatrienal, decadienal and decatrienal (Pezzolesi *et al.*, 2017). In our study, however, we found a new released aldehyde, heptaldehyde, which is a C₇saturated aldehyde. At present, little is known about the effect of saturated aldehydes, which could be explored in the future work. Adolph *et al.* (2003) and Romano *et al.* (2011) reported that the proliferation inhibition activity of aldehydes depends on the degree of unsaturation and length of the chain. Unsaturated aldehydes showed greater activity than saturated aldehydes of the same chain length. Moreover, Pichierra *et al.* (2016) confirmed that the anti-cell-growth activity is stronger in longer-chain aldehydes than in shorter-chain ones. So far, the mechanism for aldehydes-mediated effect is not completely clear yet. Miralto *et al.* (1999) demonstrated that the decadienal can induce apoptosis in mammalian tumor cells. Subsequently, decadienal-induced apoptosis in the copepod *Calanus helgolandicus* and in sea urchin *Paracentrotus lividus* was demonstrated experimentally (Romano *et al.*, 2003). Lettieri *et al.* (2015) showed that decadienal can affect the expression level of genes involved in stress response and developmental processes. In our second set of experiments, diatoms released a lot more decadienal than dinoflagellates, thus this might be a reason

why diatoms have a greater inhibitory effect on copepods than dinoflagellates.

In conclusion, our results underline the importance that marine phytoplankton is not a passive participant in biotic interactions, and that they can actively alter their own susceptibility to attackers by releasing aldehydes, acting as a form of population control in the ocean.

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