



# Mechanisms contributing to low domoic acid uptake by oysters feeding on *Pseudo-nitzschia* cells.

## I. Filtration and pseudofeces production

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**ABSTRACT:** Bivalve molluscs feeding on toxigenic *Pseudo-nitzschia* spp. are the main vector of domoic acid (DA) to humans. Although different oyster species rarely exceed the internationally adopted regulatory level for shellfish harvesting closures ( $20 \mu\text{g g}^{-1}$ ), these are often applied to all bivalve species in an affected area. This study examines the influence of diet composition and *Pseudo-nitzschia multiseries* cell size, density and toxicity on oyster feeding rates to determine potential pre-ingestive mechanisms that may lead to low DA accumulation in the oysters *Crassostrea virginica*. Clearance rate (CR) and pseudofeces production of juvenile oysters were quantified under varying laboratory conditions representative of natural *Pseudo-nitzschia* blooms. Oysters filtered increasing amounts of *P. multiseries* cells as cell density increased, but ingestion was limited by pseudofeces production at concentrations  $>10\,400$  cells  $\text{ml}^{-1}$  (ca.  $6 \text{ mg dry weight l}^{-1}$ ). Oysters significantly reduced their CR when fed both toxic and non-toxic *P. multiseries* clones in unialgal suspensions compared to *Isochrysis galbana*, and this rapid grazing inhibition was not related to growth stage, cell size, or exposure time. When offered mixed suspensions containing equivalent cellular volumes of the 2 species, however, relatively high CR was restored. Therefore, we suggest that DA intake by oysters from mono-specific *Pseudo-nitzschia* blooms would be limited by a persistently reduced CR and rejection of cells in pseudofeces. When an alternative, good source of food is present in a mixed phytoplankton assemblage with *P. multiseries*, no CR inhibition is expected and DA intake will be regulated by pre-ingestive particle selection on the feeding organs, as demonstrated in Mafra et al. (2009, in this Theme Section)

**KEY WORDS:** *Pseudo-nitzschia multiseries* · Domoic acid · Oyster · *Crassostrea virginica* · Feeding · Filtration · Pseudofeces

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### INTRODUCTION

Domoic acid (DA) was first recognized as the causative agent of an amnesic shellfish poisoning (ASP) outbreak in 1987, when 3 people died and 150 were hospitalized after ingesting contaminated mussels from Prince Edward Island (PEI), Canada (Wright et al. 1989). This neurotoxin binds to the synaptic receptors of glutamic acid in neuronal cells, and in humans it can lead to irreversible, short-term memory loss and, in extreme cases, death (Perl et al. 1990). In

the 1987 PEI outbreak, the diatom *Pseudo-nitzschia multiseries* was identified as the toxin-producing organism (Bates et al. 1989).

So far, DA has been found in at least 12 species of pennate diatoms, and various *Pseudo-nitzschia* species have been associated with toxic events (reviewed by Trainer et al. 2008). The associated risks for public health and marine fauna are highly variable, depending on temperature, bloom duration, cell toxicity, size and concentration, chain formation, composition of the phytoplankton assemblage and the

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presence of efficient grazers routing the toxin to higher trophic levels.

Bivalve molluscs, which can accumulate high DA levels by suspension-feeding on toxic diatoms, are the main vectors of the toxin to humans, yet they appear to suffer only minor (Jones et al. 1995) or no toxic effects. Shellfish containing  $>20 \mu\text{g DA g}^{-1}$  tissue wet weight are considered unsafe for human consumption (Wright et al. 1989). Since 1987, unsafe levels of DA have been detected in several regions worldwide, mainly in Canada, the Pacific coast of the USA, Scotland, Ireland, Denmark, Australia and New Zealand (Hallegraeff 2003, Trainer et al. 2008). DA has caused mortalities of marine fauna, including crabs, sea birds, fish and sea lions (e.g. Scholin et al. 2000), as well as severe economic losses due to harvesting closures of wild and cultivated shellfish stocks. Although closures are commonly applied to all harvested bivalve species in an affected area, DA contamination above this international regulatory limit is very unusual in oysters, even when high toxin levels are found in other bivalves at the same time and location (Table 1). This regulatory practice, which has affected cultured eastern oysters *Crassostrea virginica* in Atlantic North America, can also affect aquaculture of the most economically important oyster species worldwide, *Crassostrea gigas*, in areas where DA poses a threat. Mitigation of the economic losses to oyster growers could be achieved by implementing species-based management of DA

levels. Understanding and predicting the dynamics of DA accumulation in oysters is required to validate this management approach.

Since only negligible amounts of dissolved DA can be incorporated by bivalves (Novaczek et al. 1991), the bulk of accumulated toxin is derived from the ingestion of toxic diatoms, mostly *Pseudo-nitzschia* spp. Particles with dimensions  $\geq 5$  to  $6 \mu\text{m}$ , such as most of the highly toxic *Pseudo-nitzschia* spp., are expected to be completely retained by the gills of *Crassostrea virginica* (Riisgård 1988). Therefore, the ingestion rate (IR) of DA-producing cells depends on the oysters' clearance rate (CR, i.e. the volume of water cleared of particles per unit time), their capacity to reject those cells in pseudofeces, and the cell density.

Oysters and other bivalves living in coastal habitats experience high variability in both food quality and quantity and have developed various mechanisms to regulate both particle capture and ingestion, including changes in CR and in pseudofeces production (Pf) (Bayne & Newell 1983, Bayne 1993). The former controls the amount of food available for ingestion, the latter provides an important pre-ingestive mechanism to prevent overloading the digestive tract and to enhance the quality of ingested food by selectively eliminating less nutritious and noxious particles. We hypothesize that the relatively low levels of DA accumulated by oysters during toxic *Pseudo-nitzschia* blooms are at least partially explained by these 2 processes.

Table 1. Maximum domoic acid (DA) levels (in  $\mu\text{g per g}$  whole tissue wet weight) reported from various bivalve species during simultaneous sampling and maximum DA levels reported worldwide for key bivalve species. Regulatory DA limit =  $20 \mu\text{g g}^{-1}$ . ND: not detected

Species	Shellfish	DA ( $\mu\text{g g}^{-1}$ )	Location	Date	Source
<b>Simultaneous sampling</b>					
<i>Crassostrea virginica</i>	Oyster	ND	Richibouctou River,	Apr 2002	Canadian Food Inspection Agency (CFIA)
<i>Mytilus edulis</i>	Mussel	200.0	NB, Canada		
<i>Crassostrea gigas</i>	Oyster	ND	Penn Cove, WA, USA	Oct 2005	Trainer et al. (2007)
<i>M. edulis</i>	Mussel	46.0			
<i>Tapes philippinarum</i>	Clam	68.0			
<i>Ostrea edulis</i>	Oyster	0.3	Fokida, Greece	Apr 2003	Kaniou-Grigoriadou et al. (2005)
<i>Mytilus galloprovincialis</i>	Mussel	2.2			
<i>Venus verrucosa</i>	Clam	5.6			
<i>C. virginica</i>	Oyster	0.9	Neguac Bay, NB, Canada	Aug 2002	Canadian Food Inspection Agency (CFIA)
<i>M. edulis</i>	Mussel	20.0			
<i>O. edulis</i>	Oyster	5.6	Aveiro Lagoon, Portugal	Mar 2000	Vale & Sampayo (2001)
<i>M. edulis</i>	Mussel	6.4			
<i>Cerastoderma edule</i>	Cockle	16.5			
<i>Venerupis pullastra</i>	Clam	17.0			
<b>Maximum DA level reported</b>					
<i>C. gigas</i>	Oyster	30.0	Sequim Bay, WA, USA	Sep 2005	Trainer et al. (2007)
<i>Siliqua patula</i>	Razor clam	295.0	Kalaloch Beach, WA, USA	Oct 1998	Adams et al. (2000)
<i>M. edulis</i>	Mussel	790.0	Cardigan River, PEI, Canada	Nov 1987	Bates et al. (1989)
<i>Pecten maximus</i>	Scallop	1569.0	Tobermory Bay, Scotland	Dec 1999	Campbell et al. (2001)

In the present study, we used various *Pseudo-nitzschia multiseriis* clones to test the influence of cell size, toxicity, exposure time, cell density and diet composition (i.e. unialgal vs. mixed suspension) on feeding rates of the oyster *Crassostrea virginica* under laboratory conditions. The following physiological and behavioural pre-ingestive mechanisms that could explain the low levels of DA commonly found in oysters were investigated: (1) prevention of *P. multiseriis* capture by feeding incapacitation due to initial toxin accumulation; (2) reduced CR when fed *P. multiseriis* cells; and (3) active rejection of *P. multiseriis* cells from unialgal suspensions via pseudofeces production. Another important mechanism that could explain the relatively low capacity for DA accumulation in oysters, the selective rejection of *Pseudo-nitzschia* cells from mixed suspensions, is examined in Mafra et al. (2009, this Theme Section). Post-ingestive processes, such as absorption efficiency and detoxification, are the subject of ongoing research.

## MATERIALS AND METHODS

**Algal culture.** Four toxic *Pseudo-nitzschia multiseriis* clones, CLN-20, CLN-46, CLN-50 and CLNN-16, and the non-toxic clone CLNN-13, were obtained as offspring from the mating of other *P. multiseriis* clones from eastern Canada, using methods described in Davidovich & Bates (1998). They were kept in culture for a few months and then transferred to the laboratory at the Marine Research Station, Institute for Marine Biosciences (MRS/IMB), National Research Council of Canada (NRC), Halifax, Nova Scotia, where cell size and toxicity were monitored during the stationary phase over successive culture cycles. *P. multiseriis* clones were batch-cultured in 1.5 l glass Fernbach flasks with *f/2* medium (Guillard 1975) in autoclaved, 0.22 µm cartridge-filtered seawater at 16°C, 30 ppt salinity, 140 µmol quanta m<sup>-2</sup> s<sup>-1</sup> light intensity and a 14 h light:10 h dark photoperiod. The non-toxic flagellates *Isochrysis galbana* (T-Iso clone, CCMP1324), Strain 1324 and *Pavlova pinguis* (CCMP609), from the CCMP (Center for Culture of Marine Phytoplankton, West Boothbay Harbor, ME) used routinely as food for bivalves, were batch-cultured in *f/2* medium without silicate at 20°C in either 20 l aerated plastic carboys or in a semi-continuous system (200 l photobioreactors). Cell density of *P. multiseriis* clones was determined by counting of diluted samples (ca. 400 cells per Palmer-Maloney counting chamber) under a microscope (Leica Model DMLB 100S). Cell densities of *I. galbana* and *P. pinguis* were measured with a Multisizer 3 (Beckman-Coulter) particle counter.

Size of *Isochrysis galbana* and *Pavlova pinguis* cells (as equivalent spherical diameter, ESD) and cellular

volume (µm<sup>3</sup>) were determined with the particle counter. Cell length (*L*) and width (*W*) of all *Pseudo-nitzschia multiseriis* clones were measured prior to each experiment (n = 30) using the microscope coupled with a Pulnix camera Model TMC-7DSP and image analysis software (Image Pro Plus Version 4.5, Media Cybernetics). The cellular volume of *P. multiseriis* was calculated using the formula described in Hillebrand et al. (1999) and modified by Lundholm et al. (2004) for *Pseudo-nitzschia* spp.: cell volume (µm<sup>3</sup>) = (0.6 × *L* × *W*<sup>2</sup>) + (0.2 × *L* × *W*<sup>2</sup>), where *L* and *W* are in micrometers.

Concentration of DA was measured in the particulate and dissolved fractions of *Pseudo-nitzschia multiseriis* cultures by gently filtering triplicate 15 ml aliquots through Whatman GF/F glass microfiber filters (25 mm diameter, 0.7 µm minimum particle retention). Toxin was analyzed in both fractions by high performance liquid chromatography (HPLC) of the fluorenylmethoxycarbonyl (FMOC) derivative following methods of Pocklington et al. (1990), but only particulate DA is reported in the present study. *P. multiseriis* clones were harvested during the stationary phase after ca. 40 d, when cultures exhibited the maximum intra-cellular toxicity throughout the growth cycle. Chain formation was not a confounding variable in this study, as the clones were dominantly single-celled at that growth stage, with <3% of the cells forming short chains of ≤4 cells.

**Feeding rate experiments.** Juvenile eastern oysters *Crassostrea virginica* (first year cohort, mean shell height [SH] ± standard error [SE] = 18.6 ± 0.6 mm) were acquired from growers in PEI, Canada, in November 2005, and kept in 1000 l insulated tanks containing active upwellers at densities of 1500 oysters per tank, 12°C and 30 ppt salinity. SH represented the maximum axis in length measured from the umbo to the ventral margin of the shell. Oysters were fed *Pavlova pinguis* and *Isochrysis galbana* at a total cell density equivalent to 30 000 *I. galbana* cells ml<sup>-1</sup>, and acclimated to the experimental diet for ca. 18 h before each trial. All oysters used in feeding experiments were stored frozen at the conclusion of each experiment and then oven-dried at 80°C for 24 h to obtain the dry weight (DW) of soft tissues.

Trials to measure the CR (ml min<sup>-1</sup>) were conducted in five 400 ml acrylic chambers (8 oysters per chamber); 1 chamber without oysters was used to control for phytoplankton settlement. The suspension was mixed with a motor-driven magnetic stirrer held on the top of the chamber, which prevented disturbance and re-suspension of oyster biodeposits. The experimental diet was gravity-fed to the chambers from a common 60 l header tank, and a peristaltic pump recirculated the pooled outflow water from the chambers back to the header tank. Following this flow-through acclimation period, flow was interrupted and samples were

Table 2. *Crassostrea virginica*. Potential pre-ingestive feeding mechanisms resulting in reduction or avoidance of *Pseudo-nitzschia multiseriis* (*Ps-m*) filtration, and hypotheses tested in each experiment.  $CR_{Ps-m}$ : clearance rate (CR) of oysters fed *Ps-m*;  $CR_{4h}$ : CR of oysters after 4 h of exposure to *Ps-m*;  $CR_{100}$ : CR of oysters fed at 100 cells  $ml^{-1}$ ; T-Iso: *Isochrysis galbana*, clone T-Iso

Avoidance mechanism	Experiment	Underlying hypotheses
1. Shell closure when fed <i>Ps-m</i> cells	All experiments	$H_0$ (no closure): $CR_{Ps-m} \neq 0$ $H_1$ (shell closure): $CR_{Ps-m} = 0$
2. Reduced filtration when fed <i>Ps-m</i> cells	1	$H_0$ (no CR reduction): $CR_{T-Iso} = CR_{Ps-m}$ $H_2$ (reduced CR): $CR_{T-Iso} > CR_{Ps-m}$
2.1. Reduced filtration on toxic <i>Ps-m</i> cells	1	$H_0$ (no CR reduction): $CR_{non-toxic\ Ps-m} = CR_{toxic\ Ps-m}$ $H_{2.1}$ (reduced CR): $CR_{non-toxic\ Ps-m} > CR_{toxic\ Ps-m}$
2.2. Reduced filtration after prolonged exposure to <i>Ps-m</i> cells	1	$H_0$ (no CR reduction): $CR_{4h} = CR_{20h} = CR_{44h}$ $H_{2.2}$ (reduced CR): $CR_{4h} > CR_{20h} > CR_{44h}$
2.3. Reduced filtration on large <i>Ps-m</i> cells	1	$H_0$ (no CR reduction): $CR_{small\ Ps-m} = CR_{large\ Ps-m}$ $H_{2.3}$ (reduced CR): $CR_{small\ Ps-m} > CR_{large\ Ps-m}$
2.4. Reduced filtration on <i>Ps-m</i> cells at stationary phase	1	$H_0$ (no CR reduction): $CR_{exponential\ Ps-m} = CR_{stationary\ Ps-m}$ $H_{2.4}$ (reduced CR): $CR_{exponential\ Ps-m} > CR_{stationary\ Ps-m}$
2.5. Reduced filtration on unialgal <i>Ps-m</i> diets	2	$H_0$ (no CR reduction): $CR_{mixed\ diet} = CR_{unialgal\ Ps-m}$ $H_{2.5}$ (reduced CR): $CR_{mixed\ diet} > CR_{unialgal\ Ps-m}$
2.6. Reduced filtration with increasing cell density of <i>Ps-m</i>	3	$H_0$ (no CR reduction): $CR_{100} = CR_{500} = CR_{1500} = \dots = CR_{16,000}$ $H_{2.6}$ (reduced CR): $CR_{100} > CR_{500} > CR_{1500} > \dots > CR_{16,000}$

taken from each chamber before and after an interval generally ranging from 8 to 30 min, in order to allow typically 15 to 30% cell depletion. In only 2 trials, depletion time was longer (maximum = 60 min).

Pf (cells  $min^{-1}$ ) was measured in a similar experimental system, with acrylic chambers replaced by five 500 ml glass chambers containing 8 to 16 oysters each. Cell density was kept constant for 3 to 4 h, and all pseudofeces produced during that time were collected with a pipette and placed in a volumetric flask, which was then made up to 50 ml with filtered seawater. The diluted samples were vigorously shaken for 5 min and fixed in Lugol's solution for subsequent counting. CR, filtration rate (FR) and IR were calculated from the equations:

$$CR \text{ (ml min}^{-1}\text{)} = [(\log_e C_i - \log_e C_f) - (\log_e C_{c_i} - \log_e C_{c_f})] \times (V/t) \text{ (Coughlan 1969)} \quad (1)$$

$$FR \text{ (cells min}^{-1}\text{)} = CR \times \text{geomean}(C_i, C_f) \quad (2)$$

$$IR \text{ (cells min}^{-1}\text{)} = FR - Pf \quad (3)$$

where  $C_i$  and  $C_f$  are initial and final particle concentrations (cells  $ml^{-1}$ ) in the experimental chambers, respectively;  $C_{c_i}$  and  $C_{c_f}$  are the initial and final particle concentrations in the control chamber;  $V$  is the volume of the chamber (in ml, corrected for the volume occupied by the oysters); and  $t$  is the incubation time (in min). The geometric mean of  $C_i$  and  $C_f$  was used in the calculation of FR. All measured rates (CR, FR, IR and Pf) were weight-standardized following the general allometric equation for suspension-feeding bivalves, as reviewed by Bayne & Newell (1983):

$$FDR_{std} = (W_{avg} / W_{exp})^{0.616} \times FDR_{exp} \quad (4)$$

where  $FDR_{std}$  is the weight-standardized feeding rate;  $W_{avg}$  is the tissue DW of an average oyster (0.02 g in our experiments); and  $FDR_{exp}$  and  $W_{exp}$  are the experimental (i.e. measured) feeding rate and the tissue DW (g) of oysters used in each experiment, respectively. The various feeding experiments conducted and hypotheses tested are summarized in Table 2.

**Effects of cell toxicity on clearance rate.** Successive CR measurements were taken from oysters fed 2 different *Pseudo-nitzschia multiseriis* clones in unialgal diets: the non-toxic CLNN-13 (mean cell length  $\pm$  SE:  $88 \pm 3 \mu m$ ) and the toxic CLNN-16 clone ( $92 \pm 2 \mu m$ , toxicity = 1.5 pg DA cell $^{-1}$ ). Oysters were first fed the reference alga *Isochrysis galbana* at 75 000 cells  $ml^{-1}$  for 44 h and then, for the same amount of time, exposed to an equivalent cell volume of either CLNN-13 or CLNN-16, followed again by a 44 h recovery period on *I. galbana*. CR was measured after 4, 20 and 44 h of exposure to each diet. Cell depletion (15 to 30%) was measured during 14 to 30 min for *I. galbana* and 23 to 46 min for *P. multiseriis*. Data from all diets were combined and analyzed using 2-way repeated-measures analysis of variance (ANOVAR). Because of the high variability and heteroscedastic nature of the data, values were time-averaged in 3 groups: before, during and after exposure to *P. multiseriis* cells. The hypothesis of a reduced CR by contact with *P. multiseriis* cells ( $H_2$ ; Table 2) was tested by comparing the CR of oysters during the exposure to both toxic and non-toxic *P. multiseriis* clones to the corresponding CR

on *I. galbana* (i.e. before and after exposure to *P. multiseri*,  $\alpha = 0.05$ ). Toxicity was presumed to be the cause of feeding inhibition ( $H_{2.1}$ ) if the CR on Clone CLNN-13 was significantly higher ( $\alpha = 0.05$ ) than that on Clone CLNN-16. The effect of prolonged contact with *P. multiseri* cells on CR was also tested over the 44 h exposure to both clones ( $H_{2.2}$ ).

In addition, cell length and growth phase of *Pseudo-nitzschia multiseri* cells were investigated as possible alternative causes of feeding inhibition. The effect of cell length ( $H_{2.3}$ ) was tested by comparing the CR of oysters exposed to small (mean cell length = 31 to 36  $\mu\text{m}$ ) or large (81 to 92  $\mu\text{m}$ ) *P. multiseri* clones in multiple trials, and growth phase ( $H_{2.4}$ ) in a single trial using the clone CLN-20 (mean cell length  $\pm$  SE:  $25 \pm 0.2 \mu\text{m}$ ) harvested at exponential (11 d in batch culture) or stationary (41 d) phase (Student's *t*-test;  $\alpha = 0.05$ ).

**Effects of diet composition on CR (unialgal vs. mixed suspensions).** Two toxic *Pseudo-nitzschia multiseri* clones of contrasting cell length, CLN-20 (31 to 36  $\mu\text{m}$ ) and CLN-46 (82  $\mu\text{m}$ ), were offered to oysters as unialgal diets or as a mixed suspension with *Isochrysis galbana*. Clone CLN-20 was offered at ca. 1500 and 6000 cells  $\text{ml}^{-1}$  (0.9 and 3.6 mg DW  $\text{l}^{-1}$ ) and Clone CLN-46 at 2000 cells  $\text{ml}^{-1}$  (2.9 mg  $\text{l}^{-1}$ ). Mixed suspensions were prepared by replacing ca. half of the *P. multiseri* cells with an equivalent cell volume of *I. galbana*, so that the total algal cell volumes were comparable to those of the unialgal diets. For each cell density, CR was measured in oysters fed a unialgal *P. multiseri* suspension and compared, using Student's *t*-test ( $\alpha = 0.05$ ), to the combined CR on both components of the mixed suspension. The hypothesis of a reduced CR on unialgal *P. multiseri* suspensions relative to the combined CR on both components of the mixed suspensions ( $H_{2.5}$ ; Table 2) was thus tested. Cell depletion (15 to 30%) was measured over intervals ranging from 10 to 28 min, except for the unialgal CLN-46 suspension (60 min). Filtration rates were calculated with Eq. (2) and also compared for each pair of unialgal and mixed suspension using Student's *t*-test ( $\alpha = 0.05$ ).

**Effects of *Pseudo-nitzschia multiseri* cell density on CR.** CR and FR of oysters fed a unialgal suspension of the toxic clone CLN-20 (26 to 36  $\mu\text{m}$ ) were calculated in multiple trials at various cell densities: 100, 500, 1500, 3000, 6500, 10 000 and 16 000 cells  $\text{ml}^{-1}$  (0.1 to 9.5 mg DW  $\text{l}^{-1}$ ). For each cell density, cell depletion (15 to 30%) was measured over 25 to 30 min, following an 18 h acclimation period on the same diet. In addition, Pf (i.e. the number of rejected cells per unit time) was determined for oysters fed the same diet at 9000, 15 500 and 26 000 cells  $\text{ml}^{-1}$  (4.1 to 12.0 mg DW  $\text{l}^{-1}$ ). IR was then calculated from Eq. (3). Because the size

of *Pseudo-nitzschia multiseri* cells varied slightly among the different feeding experiments, feeding rates were calculated as the total seston concentration filtered, ingested, or rejected, in milligrams DW per unit time. The hypotheses of *P. multiseri* avoidance by either shell closure, as inferred by a negligible CR at all cell densities ( $H_1$ ), or reduced CR with increasing *P. multiseri* concentration ( $H_{2.6}$ ), were tested by 1-way ANOVA ( $\alpha = 0.05$ ) followed by Tukey's honestly significant difference (HSD) test, if needed. This and all other statistical comparisons were performed with the software SYSTAT 12.

## RESULTS

Five different *Pseudo-nitzschia multiseri* clones, CLN-20, CLN-46, CLN-50, CLNN-13 and CLNN-16, ranging in cell length from 24 to 100  $\mu\text{m}$ , were used in our feeding experiments. Cellular DA levels ranged from 0.1 to 1.9 pg DA cell $^{-1}$  among different clones and trials, except for CLNN-13, which did not produce detectable toxin levels (limit of detection =  $8.5 \times 10^{-5}$  pg cell $^{-1}$ ). All toxic clones experienced an exponential decrease in cellular toxicity when kept under batch culture conditions in the laboratory over a long time. This decrease was associated with a reduction in cell length, resulting in a positive linear relationship between cellular DA and cell volume, as illustrated for Clones CLN-20 and CLN-46 (Fig. 1).

Clearance rates of juvenile oysters were significantly reduced when exposed to unialgal suspensions of either toxic or non-toxic *Pseudo-nitzschia multiseri* clones, compared to *Isochrysis galbana* (Fig. 2;  $p = 0.027$ ), as stated in hypothesis  $H_2$  (Table 2). However, the CR of oysters fed the toxic clone was not statistically different ( $p = 0.48$ ) from that of oysters fed the non-toxic clone, leading to rejection of the alternative hypothesis of reduced CR by the presence of DA ( $H_{2.1}$ ). The hypothesis that oysters could potentially reduce CR over time as an avoidance mechanism during prolonged exposure to toxic *Pseudo-nitzschia* cells ( $H_{2.2}$ ) was also discarded, since CR remained nearly constant after 44 h of exposure to both toxic and non-toxic *P. multiseri* clones (Fig. 2). Finally, the interactive effect of clone and exposure time was not significant either ( $p = 0.67$ ).

There was no effect of *Pseudo-nitzschia multiseri* cell length or growth phase on the CR of juvenile oysters exposed to a unialgal suspension (Table 3), leading to the rejection of the alternative hypotheses that CR could be reduced in contact with larger or older *P. multiseri* cells ( $H_{2.3}$  and  $H_{2.4}$ ).

The feeding inhibition experienced by oysters when exposed to a unialgal suspension of either non-toxic

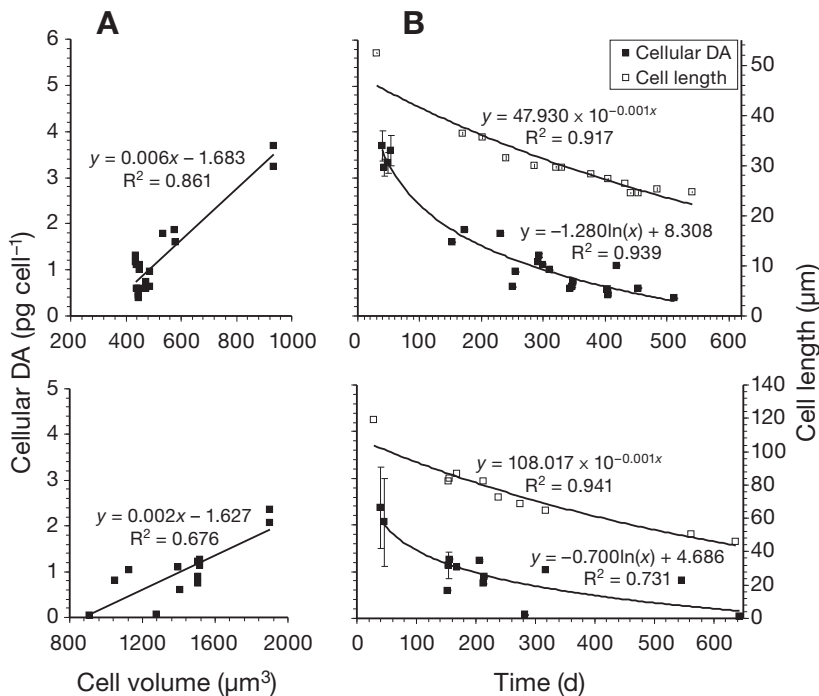
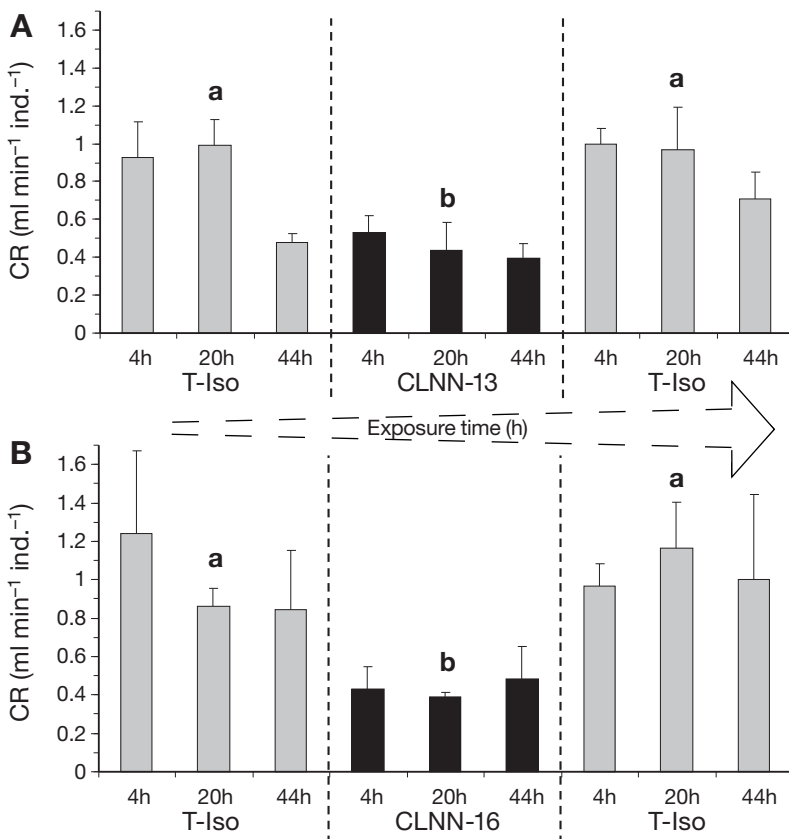


Fig. 1. *Pseudo-nitzschia multiseri*. Relationship between (A) cellular domoic acid (DA) concentration and mean cell volume, and (B) cellular toxicity and cell length (mean  $\pm$  SE) over the time that 2 *P. multiseri* clones (CLN-20: upper panels and CLN-46: lower panels) were maintained in successive batch cultures. Equations represent functions that best fit the data.  $R^2$ : coefficient of determination



or toxic *Pseudo-nitzschia multiseri* cells did not occur in the presence of a mixed phytoplankton assemblage, i.e. when ca. half of the toxic *P. multiseri* cells were replaced by an equivalent cell volume of *Isochrysis galbana*. Moreover, this effect was consistently observed in 3 independent feeding experiments, regardless of *P. multiseri* cell length (31 to 82 μm) and cell density (1000 to 6000 cells ml<sup>-1</sup>, or 0.9 to 3.6 mg DW l<sup>-1</sup>). The combined CR on both components of the mixed diets was 3- to 7-fold higher ( $p = 0.007$  to  $0.0001$ ) than on the corresponding unialgal diets (Fig. 3A). Therefore, oysters were able to filter comparable or higher cell volumes of toxic *P. multiseri* when only half of the cell density was offered (Fig. 3B), as long as an alternative nutritious source of food was available, in this case *I. galbana* cells.

In an additional series of feeding trials, we also rejected the hypothesis that high cell densities could be responsible for the low CR in oysters exposed to *Pseudo-nitzschia multiseri* in unialgal suspensions. Oysters exhibited a relatively constant CR (0.48 to 0.58 ml min<sup>-1</sup> ind.<sup>-1</sup>) with increasing *P. multiseri* concentration over a wide cell density range (100 to 16 000 cells ml<sup>-1</sup>, or 0.1 to 9.5 mg DW l<sup>-1</sup>; Fig. 4). Therefore, FR (CR  $\times$  cell density) increased monotonically with increasing cell density. However, at densities  $>4200$  cells ml<sup>-1</sup> (ca. 2.5 mg DW l<sup>-1</sup>), oysters started to reject an increasing number of *P. multi-*

Fig. 2. *Crassostrea virginica* juveniles (mean shell height  $\pm$  SE: 18.6  $\pm$  0.6 mm). Clearance rate (CR) of oysters fed *Isochrysis galbana* (clone T-Iso) at 75 000 cells ml<sup>-1</sup> (ca. 3.2 mg DW l<sup>-1</sup>) for 44 h, then switched to an equivalent cellular volume of either (A) non-toxic CLNN-13 (88 μm cell length) or (B) toxic CLNN-16 (92 μm cell length) *Pseudo-nitzschia multiseri* clones for another 44 h, then switched back to *I. galbana* for an extra 44 h period (mean  $\pm$  SE;  $n = 4$  to 5 chambers, 6 oysters per chamber). CR values (mean  $\pm$  SE) were weight-standardized to an average oyster of 0.02 g soft tissue dry weight. Same letters indicate no statistical difference ( $\alpha = 0.05$ )

Table 3. *Crassostrea virginica* juveniles (mean shell height  $\pm$  SE:  $18.6 \pm 0.6$  mm). Clearance rate (CR) of oysters fed *Pseudo-nitzschia multiseriis* clones. The effect of *P. multiseriis* cell length ( $\mu\text{m}$ ) on CR was tested in multiple trials using all *P. multiseriis* clones (CLN-20, CLN-46, CLN-50, CLNN-13 and CLNN-16), and the effect of growth phase (days in batch culture) was assessed in a single trial with Clone CLN-20 (cell length =  $25 \mu\text{m}$ ). CR values ( $\text{ml min}^{-1} \text{ind.}^{-1}$ ; mean  $\pm$  SE) were weight-standardized to an average oyster of  $0.02 \text{ g}$  soft tissue dry weight

Variable	Treatments	CR	p
Cell length	$34.7 \pm 0.9$ (n = 7)	$0.52 \pm 0.02$	0.11
	$87.3 \pm 1.6$ (n = 9)	$0.44 \pm 0.05$	
Growth phase	11 d (exponential)	$0.73 \pm 0.16$	0.82
	41 d (stationary)	$0.69 \pm 0.13$	

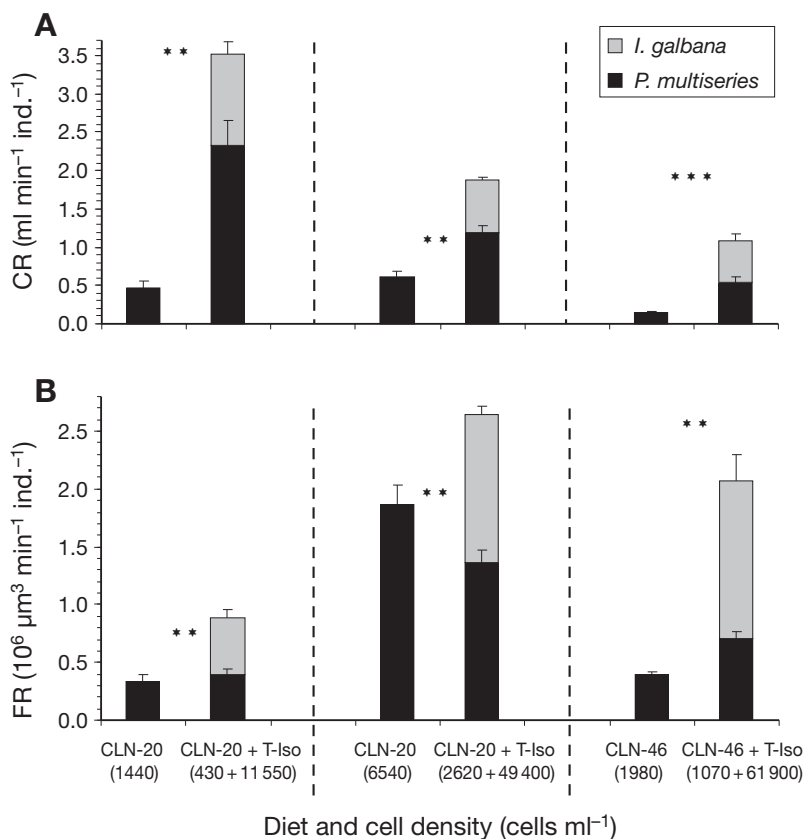


Fig. 3. *Crassostrea virginica* juveniles (mean shell height  $\pm$  SE:  $18.6 \pm 0.6$  mm). (A) Clearance rate (CR) and (B) filtration rate (FR) (mean  $\pm$  SE; n = 4 to 5 chambers, 8 oysters per chamber) of oysters fed toxic *Pseudo-nitzschia multiseriis* clones (CLN-20 and CLN-46) in either unialgal or mixed suspensions, with an equivalent cell volume of *Isochrysis galbana* (clone T-Iso). Total concentrations of the 3 mixed suspensions corresponded to those of unialgal diets:  $\sim 0.7$ ,  $4.2$  and  $3.7 \text{ mg DW l}^{-1}$ , respectively. Combined CR and FR for mixed diets (i.e. sum of the 2 components of binary diets) were compared to those of corresponding unialgal suspensions, with statistically significant differences shown between each pair of suspensions (\*\*p < 0.01; \*\*\*p < 0.001). Both rates were weight-standardized to an average oyster of  $0.02 \text{ g}$  soft tissue DW. Cell length was  $31$  to  $36 \mu\text{m}$  for CLN-20 and  $82 \mu\text{m}$  for CLN-46

*series* cells in pseudofeces, and a maximum ingestive capacity was reached at  $10\,400 \text{ cells ml}^{-1}$  (ca.  $6 \text{ mg l}^{-1}$ ; Fig. 4). The avoidance hypotheses of null ( $H_1$ ) or reduced CR with increasing cell density ( $H_{2,6}$ ; Table 2) were therefore both rejected (p = 0.96). A threshold *P. multiseriis* volume and seston concentration for the initiation of pseudofeces production was thus established for these juvenile oysters.

## DISCUSSION

Over the course of this study (ca. 20 mo), *Pseudo-nitzschia multiseriis* clones exhibited a progressive decline in particulate DA levels at stationary phase, and this was directly related to a continued reduction in cell volume (Fig. 1). A minimum viable cell length of  $24 \mu\text{m}$  was reached in our monoclonal cultures, which translates into a cell volume ca. 9.5 times lower than the maximum possible for this species, assuming a maximum size of  $169 \times 5.3 \mu\text{m}$  (Villac 1996). Such gradual size reduction, resulting from successive asexual cell divisions, is common to all diatoms and continues until sexual reproduction restores the maximum size typical of each species (see Round et al. 1990 for details). This is well known in *P. multiseriis* cultures (e.g. Davidovich & Bates 1998), and is also reported in natural populations, where cells as short as  $35 \mu\text{m}$  can be found (Bates et al. 1999). The wide range of cell size and toxicity found during *P. multiseriis* blooms may therefore partly explain why the maximum DA level reported to date in field studies ( $7.2 \text{ pg cell}^{-1}$ ; Smith et al. 1990) is much lower than the  $67 \text{ pg DA cell}^{-1}$  achieved by large, young *P. multiseriis* cells, produced by mating low-toxicity parent clones in the laboratory (Bates et al. 1999). Thus, the relatively moderate cellular DA levels ( $0.1$  to  $1.9 \text{ pg cell}^{-1}$ ) and wide range in cell size ( $24$  to  $100 \mu\text{m}$ ) used in our feeding experiments is a good representation of what oysters may experience in nature. In addition, to our knowledge, this study is the first to report the occurrence of a consistently non-toxic *P. multiseriis* strain, the clone CLNN-13.

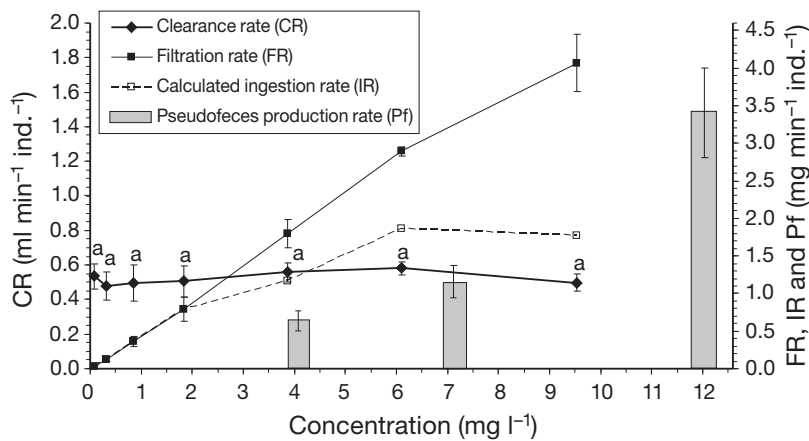


Fig. 4. *Crassostrea virginica* juveniles (mean shell height  $\pm$  SE:  $18.6 \pm 0.6$  mm). Clearance rate (CR), filtration rate (FR), calculated ingestion rate (IR) and pseudofeces production rate (Pf) of oysters with increasing concentration of *Pseudo-nitzschia multiseriata* Clone CLN-20 (cell length = 26 to 36  $\mu\text{m}$ ). All rates (mean  $\pm$  SE;  $n = 5$  chambers, 8 oysters per chamber) were weight-standardized to an average oyster of 0.02 g soft tissue DW.  $\text{IR} = \text{FR} - \text{Pf}$ ; same letters indicate no statistical difference ( $\alpha = 0.05$ )

Bivalves can regulate their CR and pseudofeces production, and therefore adjust the amount of ingested particles in response to different time-scale variations in food quality and quantity (Bayne 1993). In our investigation, CR of oysters was not reduced following prolonged exposure to a constant cell density of *Pseudo-nitzschia multiseriata*. In addition, shell closure was not observed, and the CR was maintained at similar levels after 4 to 44 h of exposure to both CLNN-13 (non-toxic) and CLNN-16 ( $1.5 \text{ pg DA cell}^{-1}$ ). These observations differ from those made by Jones et al. (1995), who reported a general stress response by adult *Crassostrea gigas* (mean SH  $\pm$  SD:  $13 \pm 1.1$  cm), including shell closure and subsequent haemolymph acidosis and hypoxia after 4 h of exposure to toxic *P. multiseriata* ( $1.6 \text{ pg DA cell}^{-1}$ ). However, Jones et al. (1995) used an extremely high *P. multiseriata* cell density ( $330\,000 \text{ cells ml}^{-1}$ ), contrasting with those found during natural blooms ( $100$  to  $15\,000 \text{ cells ml}^{-1}$ ; Bates et al. 1998) and those used in our investigation ( $100$  to  $25\,000 \text{ cells ml}^{-1}$ ). Contrary to findings for paralytic shellfish toxins (e.g. Bricelj et al. 1996), there are no reports to date of deleterious effects on growth or survival of juvenile and adult bivalves caused by ecologically relevant DA concentrations. Recently, however, relatively high dissolved DA concentrations ( $30$  to  $50 \text{ ng ml}^{-1}$ ) were reported to negatively affect development and survival of *Pecten maximus* larvae (Liu et al. 2007).

Although no acute harmful effect was found in the present study, oysters exhibited reduced CR when exposed to unialgal suspensions of *Pseudo-nitzschia multiseriata* clones, compared to *Isochrysis galbana*.

Such feeding inhibition was observed in oysters exposed to both small (L. L. Mafra et al. unpubl. data) and large, toxic *P. multiseriata* clones (Fig. 2B), but cannot be attributed to the presence of DA at the levels tested in the present study, because CR was similarly reduced in oysters fed a non-toxic *P. multiseriata* clone (Fig. 2A). Therefore, DA did not exhibit a feeding inhibitory effect on oysters as recently reported for karlotoxins (Brownlee et al. 2008). Other invertebrates have also been reported to feed at similar rates on both toxic and non-toxic *Pseudo-nitzschia* spp., namely copepods (e.g. Lincoln et al. 2001) and euphausiids (Bargu et al. 2003).

The reduced CR of *Crassostrea virginica* was not related to the growth stage of *Pseudo-nitzschia multiseriata* either, but rather to other cell characteristics.

This lower CR on *P. multiseriata* compared to that on *Isochrysis galbana* may be related to differences in food quality, as naked flagellates are generally characterized by a higher organic content than diatoms (reviewed by Menden-Deuer & Lessard 2000). For instance, both cellular organic carbon and nitrogen content of *P. multiseriata* clone CLN-50 ( $70 \text{ fg C } \mu\text{m}^{-3}$  and  $13 \text{ fg N } \mu\text{m}^{-3}$ ) were 5- and 6-fold lower, respectively, than those of the flagellate *Rhodomonas lens*. Several studies support this nutritional deficiency hypothesis as an explanation for the low egg production rates observed in copepods fed various diatom diets (reviewed by Ianora et al. 2003), including unialgal suspensions of both toxic and non-toxic *Pseudo-nitzschia* spp. (Lincoln et al. 2001). Nevertheless, other mechanisms have been suggested to explain why diatoms may sometimes be a sub-optimal food for invertebrates, such as their capacity to produce both toxic aldehydes from polyunsaturated fatty acid precursors (Miralto et al. 1999) and feeding-deterrent compounds such as apo-carotenoids derived from fucoxanthin (Shaw et al. 1997). Diatoms, however, are abundant in marine coastal waters and may constitute an important component of oyster diets, especially in the presence of more refractory material (Decottignies et al. 2007). Additionally, if no other food choice is presented, oysters increase their CR after 8 to 10 d of continuous exposure to *P. multiseriata* cells (L. L. Mafra et al. unpubl. data).

In bivalves, the feeding response to different algae is species-specific. The scallop *Argopecten irradians* cleared the diatom *Thalassiosira pseudonana* at a lower rate than the chlorophyte *Dunaliella tertiolecta*,



but the opposite was reported for *Crassostrea virginica* (Palmer 1980). In addition, the CR of the mussel *Mytilus galloprovincialis* was similar on 2 diatom and 3 flagellate species, including *Isochrysis galbana*, but lower when fed the toxic dinoflagellate *Alexandrium tamarense* (Matsuyama & Uchida 1997). Similarly, the oyster *Crassostrea ariakensis* reduced its CR when fed the ichthyotoxic raphidophyte *Heterosigma akashiwo* in mixed suspension with *I. galbana* (Alexander et al. 2008). Because oysters in the present study were presumably able to chemically discriminate between 2 different algal species and quickly reduce their CR when fed *Pseudo-nitzschia multiseriis* in unialgal suspensions as compared to *I. galbana*, the presence of possible deterrent compounds in this diatom needs further investigation. Moreover, as previously reported by Ward & Targett (1989) for *Olithodiscus luteus* and *Dunaliella tertiolecta*, this potential feeding deterrence caused by *P. multiseriis* may be concentration dependent, since the CR of oysters feeding on *P. multiseriis* unialgal suspensions was much lower than that on mixed suspensions with *I. galbana*, when only half of the *P. multiseriis* cell density was offered. As expected, there were no differences in the CR of small and large *P. multiseriis* cells (Table 3), given that all cellular dimensions (i.e. length, height and width) of the clones were  $\geq 5$  to  $6 \mu\text{m}$ , the size threshold for 100% particle retention efficiency by *C. virginica* gills (Riisgård 1988).

In addition to particle quality, it is well established that many bivalve species can also regulate their CR in response to particle concentration. In the present study, however, the CR of juvenile oysters remained constant over a wide range of *Pseudo-nitzschia multiseriis* cell densities, equivalent to seston concentrations of ca.  $0.1$  to  $9.7 \text{ mg DW l}^{-1}$  (Fig. 4). Such capacity to maintain a constant CR with increasing cell densities was previously reported in different sized *Crassostrea virginica* fed either a natural plankton assemblage (Tenore & Dunstan 1973) or unialgal suspensions (Palmer 1980). This oyster species (Newell & Langdon 1996) and other bivalves, such as the clam *Mulinia edulis* (Velasco & Navarro 2005), may maintain their CR near maximum levels at much higher cell densities (ca.  $25$  to  $30 \text{ mg l}^{-1}$ ), which allows them to actively feed at extremely high seston concentrations while rejecting undesirable or excess particles in pseudofeces. This contrasts with other bivalve species, which may rapidly reduce their CR as cell density increases within similar seston concentration ranges, such as the pearl oysters *Pinctada margaritifera* and *Pinctada maxima* (Yukihira et al. 1998), the clam *Mya arenaria* and the scallop *Placopecten magellanicus* (Bacon et al. 1998). The CR of mussels may also be

maintained nearly constant over a similar particle concentration range, but it is rapidly reduced at concentrations  $>10 \text{ mg l}^{-1}$ , as reported for *Mytilus chilensis* (Velasco & Navarro 2005) and *M. edulis* (Richoux & Thompson 2001). Thus, we found that oysters regulated the ingestion of *P. multiseriis* cells in unialgal suspensions via pseudofeces production rather than by adjusting the CR, which may be an evolutionary adaptation to the turbid environments they inhabit (Beninger & Cannuel 2006). It has been suggested that bivalve species whose primary regulation mechanism of particle ingestion is pseudofeces production would be more successful at exploiting turbid habitats than species that rely primarily on changes in CR (Bricelj & Malouf 1984). Even though the effects of cell density on CR were not tested in mixed suspensions by the present study, CR of oysters exposed to a mixture of *P. multiseriis* and *Isochrysis galbana* at total concentrations of  $3.7$  and  $4.2 \text{ mg DW l}^{-1}$  were, respectively,  $1.8$  and  $3.1$  times lower than those exposed to the same suspension at  $0.7 \text{ mg l}^{-1}$  (Fig. 3), suggesting feeding inhibition at high cell densities. However, this tendency cannot be generalized as CR may be highly variable, even when oysters are exposed to mixed suspensions of similar concentrations (see Fig. 1 in Mafra et al. 2009).

In general, the CR values reported in the present study were lower than those previously reported for *Crassostrea virginica*. For example, CR of oysters exposed to a unialgal suspension of *Isochrysis galbana* in our study were equivalent to  $0.4$  to  $1.3 \text{ l h}^{-1} \text{ g}^{-1}$  soft tissues DW, which approximates the  $1.4 \pm 1.2$  (SD)  $\text{l h}^{-1} \text{ g}^{-1}$  measured by Palmer (1980), but is well below the  $6.8 \text{ l h}^{-1} \text{ g}^{-1}$  reported by Riisgård (1988). Because CR remained unchanged with increasing cell density in our study, it is very unlikely that our low CR values were triggered by saturation of the alimentary tract due to the relatively high algal concentrations used ( $\sim 75\,000 \text{ I. galbana cells ml}^{-1}$ ), as critically discussed by Riisgård (2001). Furthermore, methodological problems, a potentially important source of error when measuring CR of bivalves (Riisgård 2001), were minimized in our study. Adequate and homogeneous water circulation was attained by using a motor-driven magnetic stirrer on the top of the measuring chambers and re-filtration was minimized by reducing the clearance time to between  $8$  and  $30 \text{ min}$  in most cases, typically allowing only  $15$  to  $30\%$  cell depletion. The lower CR values found in this study are more likely attributable to the experimental temperature ( $12^\circ\text{C}$ ), which is much lower than those used in previous studies (e.g.  $21^\circ\text{C}$ ; Palmer 1980;  $27$  to  $29^\circ\text{C}$ ; Riisgård 1988). From the data presented in Pernet et al. (2007), there appears to be an exponential increase in the CR of fully acclimated *C. virginica* (i.e. measurements taken after  $5$  to  $12 \text{ wk}$  at a

constant temperature) from  $<0.3 \text{ l h}^{-1} \text{ g}^{-1}$  at temperatures  $<9^\circ\text{C}$  to between ca. 1.1 and  $3.6 \text{ l h}^{-1} \text{ g}^{-1}$  at  $20^\circ\text{C}$  when filtering *I. galbana*. This was further confirmed with oysters exposed to a constant concentration of *P. multiseriis* at temperatures ranging from 1 to  $18^\circ\text{C}$  (L. L. Mafra et al. unpubl. data).

Maintenance of a constant CR in *Crassostrea virginica* resulted in a continuously increasing filtration rate as cell density increased over the entire range investigated in this study (Fig. 4). However, because oysters also rejected increasing amounts of *Pseudo-nitzschia multiseriis* cells in pseudofeces, ingestion rate (IR) reached an asymptote at  $\sim 6 \text{ mg l}^{-1}$ . Above this threshold, any further increase in FR was compensated by increased pseudofeces production. In all trials conducted during the present study, rejection of cells in pseudofeces was negligible below cell densities equivalent to  $\sim 2.5 \text{ mg l}^{-1}$ , with pseudofeces production becoming increasingly important as a mechanism to reduce the ingestion of *P. multiseriis* cells by *C. virginica* above this concentration. This mechanism is also important, in addition to changes in CR, for regulating the IR of bivalves such as *Placopecten magellanicus* (Bacon et al. 1998) and *Mytilus edulis* (Bayne 1993). In contrast, pseudofeces production and sorting capabilities appear to be less important for other bivalves such as *Mercenaria mercenaria* and *Mya arenaria* (Bricelj & Malouf 1984, Bacon et al. 1998). All oysters in the present study were acclimated to the experimental diet for at least 18 h preceding sampling, so that gut fullness cannot be considered a confounding factor affecting pseudofeces production.

Our laboratory experiments indicate that 2 different aspects of the feeding process of *Crassostrea virginica* may contribute to the low DA levels historically reported in this species during natural blooms. In monospecific, toxic *Pseudo-nitzschia* spp. blooms, DA intake would be limited by a combination of reduced CR and rejection of *Pseudo-nitzschia* spp. cells in pseudofeces. The relative importance of the first mechanism in reducing IR would be greater during low-density blooms, and the latter would be more effective in limiting the DA intake from high-density blooms, notably at concentrations  $\geq 6 \text{ mg l}^{-1}$ . Our results suggest that these mechanisms are expected to operate whenever oysters are exposed to a largely monospecific *Pseudo-nitzschia* spp. bloom, regardless of cell size distribution and cell toxicity. In the event of a multispecific bloom in which *Pseudo-nitzschia* spp. make a lesser contribution to the total food supply, no CR inhibition is expected (present study) and DA intake is therefore regulated by preferential rejection of *Pseudo-nitzschia* spp. cells in pseudofeces (Mafra et al. 2009).

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