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Directions in bivalve feeding

Mytilus sp. feeding at Houat Island, France.
Photo: Jean-Pierre Beurier

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THEME SECTIONS of Aquatic Biology (AB) present integrated multi-author syntheses initiated and coordinated by acknowledged experts. They highlight cutting-edge research areas or problems and/or bring together cogent bodies of literature on all aspects of the biology of organisms in freshwater and marine habitats.

AB Theme Section 3 focuses on bivalve feeding. On hard or soft substrates, in natural environments or farmed by man, various bivalve species may form vast assemblages comprising millions of individuals, thus conspicuously dominating many coastal habitats. Most bivalve species are filter-feeders and show complex particle processing, a major motor of pelago-benthic coupling. Merely by their number, and by their sophisticated feeding process, bivalves impact their environment in many ways.

The Theme Section presents 10 selected papers which were submitted to the journal over the course of 2008. Each paper deals with a certain aspect of bivalve particle processing, either indicating current directions or highlighting some of the 'hot' topics and topical approaches in the research on bivalve suspension feeding.

As for all current AB articles, we are pleased to make the online version of AB Theme Section 3 available with Open Access.

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Directions in bivalve feeding

Idea and coordination: **Peter G. Beninger**

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Introduction

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ABSTRACT: Manuscripts dealing with bivalve feeding and submitted to the Inter-Research journal Aquatic Biology over the course of 2008 were peer-reviewed in the standard manner, and the successful papers compiled for a Theme Issue entitled 'Directions in bivalve feeding'. The historical progression of research in this field is summarized, and the directions of current research highlighted in view of the compiled papers. Some suggestions for future work are presented.

KEY WORDS: Bivalve biology · Suspension feeding · Bivalve diet · Trophic ecology · Mechanisms · Measurements · Perspectives

Research in suspension feeding has been part of the landscape of bivalve biology since at least the early 20th century (Drew 1906, Dakin 1909, Kellogg 1915, Yonge 1923). There is, undeniably, a certain fascination with animals capable of providing for their metabolic needs by capturing microscopic particles, and which in conse-

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quence have largely given up locomotion somewhere along the evolutionary road. At the same time, investigation of this process presents such formidable obstacles (scale differentials, particle visualization, cryptic capture and processing due to occlusive structures such as valves, gill and palp troughs, or appressed surfaces), that many researchers simply turn to more accessible aspects of the bivalve world. That temptation is all the more acute today, when population biology, physiological ecology, aquaculture, and of course molecular biology, to name a few, offer attractive alternatives.

As a post-doc in the 1980s, I simply assumed, like everyone around me who had even thought about the question, that such a basic thing as feeding must be thoroughly understood by at least some researchers somewhere, in such an intensively-studied group as the bivalves. Wrong, of course. Bits and pieces of the most disparate and often contradictory sort were indeed available, but nothing anywhere close to an intelligible, coherent picture could be found. The pioneering work of the 19th and early 20th centuries was necessarily limited by the observational techniques of those times (although some of that work was remarkable despite these limitations, see especially Atkins 1936, 1937a,b,c, 1938a,b,c, 1943). Studies in the latter half of the 20th century tended to concentrate on aspects which did not require direct observation of the underlying mechanisms — a 'black box' approach — such as clearance and particle retention efficiencies (e.g. Møhlenberg & Riisgård 1978, Wright et al. 1982, Riisgård 1988a, Bacon et al. 1998, Hawkins et al. 1998). Outcome-based observational techniques such as automated particle counting, fluorimetry and flow cytometry were used to establish the basic characteristics of suspension feeding, and provided important clues to underlying mechanisms (e.g. Palmer & Williams 1980, Famme & Kofoed 1983, Cranford & Gordon 1992, Bayne et al. 1993, MacDonald & Ward 1994, Ward & MacDonald 1996, Barillé et al. 1997, Navarro & Widdows 1997, Newell et al. 2001, Filgueira et al. 2006, and see Ward & Shumway 2004 for a review of the voluminous literature on this subject). These studies yielded seminal information which would become at once a foundation and a prism through which we have continued to interpret the characteristics of bivalve feeding, and indeed the techniques are important tools in the panoply we use today. Adaptation of this approach to measurement in the field, either indirectly (via the biodeposition method, Cranford et al. 1998, Iglesias et al. 1998, Cranford & Hill 1999; or pumping from the field site to experimental chambers, MacDonald & Ward 1994) or directly (the InEx method, Yahel et al. 2003, 2005, 2006, 2009 this Theme Section) has allowed us to extend these observations much closer to the natural habitat. Simultaneously, application of the physical principles of fluid mechanics has yielded important insights into the hydrodynamic principles of particle movement underlying suspension feeding in bivalves (Jørgensen 1981, 1982, 1983, Jørgensen et al. 1984, Nielsen et al. 1993, Riisgård & Larsen 2001, 2005, 2007).

The 20th to 21st century interface saw rapid progress in the study of bivalve feeding, with the development or adaptation of new observational techniques, in conjunction with the 'old faithful' methods already in use. Techniques such as cilia and mucocyte mapping (Beninger et al. 1993, 1999, 2003, 2005, Beninger & Dufour 1996, Beninger & Veniot 1999, Beninger & Decottignies 2008), *in vivo* video-endoscopy (Ward et al. 1991, 1994, Chaparro et al. 1993, Tankersley & Dimock 1993, Ward et al. 1994, Beninger et al. 1997a, Beninger & St-Jean 1997), associated sampling in gill particle tracts (Beninger et al. 1992, 2004, 2008, Beninger & Decottignies 2005, Ward et al. 1993a, 1998, Cognie et al. 2003), confocal laser microscopy (Silverman et al. 1996a,c, Beninger et al. 1997b, Silverman et al. 1999), and enhanced micro-structural characterization (Beninger et al. 1994, 2005, Silverman et al. 1996a,b,c, Veniot et al. 2003, Beninger & Cannuel 2006, Cannuel & Beninger 2006, 2007) have either lent strong support to hypotheses arising from the outcome-based techniques, allowed direct testing of these hypotheses, or shown new possibilities in the field.

In addition to the 'hows' of bivalve feeding, recent techniques of diet determination, such as stable isotope and fatty acid signatures, have given us unprecedented insight into the actual diets of bivalves in the field. These are powerful tools for analyzing their place in food webs, potential trophic competition, and position with respect to carrying capacity (Riera & Richard 1996, 1997, Riera et al. 1999, Sauriau & Kang 2000, Page & Lastra 2003, Kasai et al. 2004, Rossi et al. 2004, Decottignies et al. 2007a,b, Dubois et al. 2007a,b, Silina & Zhukova 2007, Compton et al. 2008, Leal et al. 2008, Kang et al. 2009 this Theme Section).

We thus now have in hand many pieces of this puzzle, and while there are still large gaps in the picture, our understanding of feeding processes has moved forward substantially in recent years: an appropriate time to devote a Theme Section to the topic. The standard approach for Theme Sections is to contact the most eminent researchers in the field and request a contribution. While this does ensure good coverage of the theme, it does not necessarily reflect the diversity of researchers and approaches, the actual 'pulse' of the field. We at Aquatic Biology therefore decided to present a Theme Section which drew upon all bivalve feeding papers submitted to Inter-Research journals over the course of a year, to obtain an on-the-ground view of the different approaches and directions in this field today, by researchers of all

horizons (worldwide, senior and junior). The papers have undergone thorough quality control through rigorous peer review (3 to 5 reviewers per manuscript), and nearly half of the submitted manuscripts did not receive enough support to allow inclusion. Papers accepted at the beginning of our 'sampling interval' had to wait longer than those at the end of the interval to appear in print, and we consequently transferred to regular issues several for which the authors had very strict time imperatives. Our appreciation goes to the authors who have been able to wait for publication, our apologies to the authors who have been more pressed for time.

The articles accepted for this Theme Section suggest that current directions in bivalve feeding might be mapped out as:

(1) Dynamics of feeding responses, and implications for laboratory measurement of these responses (Robson et al. 2009 this Theme Section, and Pascoe et al. 2009 this Theme Section — in fact, the latter paper was the original impetus for the Theme Section). Both of these papers highlight the importance of methodology in the measurement of feeding responses.

(2) Processing of toxic, or potentially toxic, particles, and underlying mechanisms (Mafra et al. 2009a,b this Theme Section and Persson & Smith 2009 this Theme Section) show that oysters can consume not only dinoflagellates but also their resting cysts, with the obvious implications this has for toxin accumulation and for introduction of harmful algal bloom species through transplant vectors. Mafra et al. reveal why, in contrast to other bivalves, oysters do not accumulate large quantities of domoic acid. These papers tie into several recent studies showing them to be particularly efficient in qualitative selection, both in the laboratory and in the field; this may be related to their very highly-developed and particular gill structure.

(3) Pre-capture selection (Yahel et al. 2009). Qualitative selection in suspension-feeding bivalves has been assumed to be exclusively post-capture, but in the tropical species studied by Yahel et al. 2009, qualitative selection is shown to occur prior to retention. This forces us to re-examine what is meant by 'retention' (certainly different mechanisms for different gill types and sub-types) and how qualitative selection might intervene in some of these scenarios.

(4) The link between food type and digestive enzymes (Navarro et al. 2009 this Theme Section). This is a fascinating, yet little-studied aspect of bivalve feeding. It is obviously one that should be pursued in the future, since it constitutes a potentially important level of regulation of energy and metabolite acquisition.

(5) The effects of suspension-feeding bivalve populations on the plankton community (Maar et al. 2008, Peterson et al. 2008, Lonsdale et al. 2009 this Theme Section). In these papers, the impact of suspension-feeding bivalves on the plankton community are assessed, particularly with respect to culture operations. Such knowledge is obviously critical to both the understanding of benthic-pelagic coupling and the impact on plankton dynamics, and also to the effect of bivalve culture on these processes. Although Maar et al. (2008) and Peterson et al. (2008) have, due to author time constraints, been published in a previous issue of Aquatic Biology, they were originally processed for this Theme Section, and really do belong here.

(6) Particle clearance and retention in larvae (Tezuka et al. 2009 this Theme Section). There is a relative dearth of information concerning the characteristics and mechanisms of larval bivalve suspension feeding (Gerdes 1983, Gallager 1988, MacDonald 1988, Riisgård 1988b, Way 1989, Widdows et al. 1989, Baldwin & Newell 1991, MacIsaac et al. 1992, Baker & Mann 1994, Gallager et al. 1994, Baldwin 1995), compared to the numerous studies on adult bivalves. Information on retention efficiency and clearance in this cultured larval clam species is therefore welcome.

(7) Dietary fluctuations in field populations (Kang et al 2009 this Theme Section). Although sea squirts are a little exotic for this Theme Section, it is interesting to see how field diets vary between co-cultured oysters and sea squirts. The two have very different anatomical equipment for suspension feeding, and oysters here show a capacity to actively modify their diets, probably through the selection mechanisms for which they are becoming increasingly known.

It would be wrong to think that we know a great deal about bivalve feeding at this point in time. We certainly know much more than we did two decades ago, but, to paraphrase Darwin, it is always wise to correctly perceive our own ignorance. Examples of the exciting problems awaiting present and future researchers:

- Direct field study of clearance and retention in turbid or light-limited waters
- The exact mechanism(s) of qualitative particle selection (follow-up to Newell & Jordan 1983 and Ward & Targett 1989), and its corollary, the 'secret' of sorting and processing thousands of particles simultaneously
- Demonstration of the mechanism of particle capture in the vast majority of bivalves (e.g. heterorhabdic filibranchs, eulamellibranchs, pseudolamellibranchs)
- Fine chemical characterization (the equivalent of protein or DNA sequencing) of the different types of mucus which fulfill different processing functions
- An understanding of how precise mucus composition and spatial configuration affect processing
- Capture and ingestion probabilities for individual particles with specific physical and chemical characteristics, under different conditions of satiation.

There is enough in these future directions to keep us happily occupied for some time to come.

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Contribution to the Theme Section 'Directions in bivalve feeding'

Observations on the measurement and interpretation of clearance rate variations in suspension-feeding bivalve shellfish

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ABSTRACT: Mussels *Mytilus edulis* (35 ± 2 mm shell length) were fed on cultured *Isochrysis galbana* in a flow-through system, and measures were undertaken to quantify separate effects of inflow cell concentration, outflow cell concentration, percentage reduction (i.e. recycling), and flow rate and/or feeding history on clearance rate measures (CR; l h^{-1}). Findings identify the following features of physiological regulation: (1) a lag phase in the feeding response to changes in food concentration, of around 30 min; (2) a trigger level in algal cell (chlorophyll [chl] *a*) volume/concentration (4000 cells ml^{-1}, 0.5 µg l^{-1} chl *a*) below which filtering in most mussels ceases; (3) saturation reduction, or satiation, resulting in reduced CR and valve closure after feeding for over 2 h at 30 000 or more cells ml^{-1} (~6 µg l^{-1} chl *a*); and (4) at food levels between Features 2 & 3, CR increases to maximal rates (ca. 2 to 2.5 l h^{-1} $ind.^{-1}$ or 6 to 7.5 l^{-1} h^{-1} g^{-1} dry weight). Our findings also help resolve uncertainties associated with the measurement and interpretation of clearance rate variations. These uncertainties are associated with: differences that result from analyses of feeding responses to different components of the available seston; whether variations in seston availability are physiologically relevent; analyses of individual shellfish as compared with average responses computed for >1 ind.; and comparisons between short-term and integrated measures. We think it is important to consider whether measures of feeding are really required per individual in real time, and we stress that population averaging and temporal integration are required for the scaling up of results when simulating ecological interrelations between shellfish populations and their environments.

KEY WORDS: Filter feeding · Suspension feeding · Clearance rate · *Mytilus edulis* · Bivalve shellfish · Flow-through · Physiological regulation · Supply rate

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INTRODUCTION

The current understanding of filter feeding and its measurement in bivalve shellfish is beset by a number of controversies, and several reviews have summarised the current knowledge with a view to resolving these controversies (Jorgensen 1996, Bayne 1998, Riisgård & Larsen 2000, 2001, Riisgård 2001a,b). Riisgård (2001b) highlighted methodological shortcomings and possible misinterpretations of data, suggesting guidelines to help obtain reliable data for measures of the rate with which suspension-feeding bivalves remove particles from seawater (clearance rate, CR). Conclusions on the through-flow chamber method were collated in an appendix by Larsen (2001). These guidelines stimulated further work on the comparison of 3 different methods of measuring CR (Petersen et al. 2004), resulting in more comment and debate (Bayne 2004, Petersen 2004, Riisgård 2004). More recently Filgueira et al. (2006) again addressed the design and validation of the flow-through chamber method for CR measurements.

There is general recognition of the need to better understand the intriguing complexity of feeding processes in bivalve shellfish, which shows remarkable regulation and morphological adaptation of particle processing mechanisms within and between species, respectively (e.g. Beninger & St-Jean 1997, Beninger et al. 1997, 2008). Here, we address some key environ-

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mental factors and behavioural issues affecting variations in CR that are observed within species. Using mussels fed on cultured algae in a flow-through system, experiments were undertaken to quantify separate effects of inflow cell concentration, outflow cell concentration, percentage reduction (i.e. recycling) and flow rate and/or feeding history on CR measurements. Notable reductions or cessation of feeding were observed at both low (below trigger level) and high (through satiation) food concentrations. The interpretation of results is intended to help researchers ensure that the measures they use are 'fit for purpose', including whether averaging and integration may be required to simulate ecological interrelations between shellfish populations and their environments.

MATERIALS AND METHODS

Mussels *Mytilus edulis* L., at shell lengths of 35 ± 2 mm, were collected at Great Bull Point (50° 36.8' N, 3° 26.0' W), near the mouth of the Exe estuary, SW England (Hilbish et al. 2002). After removal of any epibiotic growth, individual animals were numbered and then maintained in a 7 l vessel linked to a 3000 l system of circulating seawater at 14 ± 1°C and 34 ± 1 psu for a period of 10 d before the experiments. During this time and between experiments, mussels were fed on a maintenance ration (approximately 2000 cells ml^{-1}) of the microalga *Isochrysis galbana*. In the feeding experiments, 14 ind. were each placed in a chamber within a flow-through raceway system as described by Hawkins et al. (1996) to measure CR (litres of water cleared of particles h^{-1}) over separate ranges of algal cell concentration, water flow rate and percentage reduction in food particles, as water passed from inflow to outflow. Mussels were orientated with their inhalant apertures towards the incoming flow. The volume of the chambers was approximately 200 ml, and experimental flow rates through each chamber ranged from 30 to 400 ml min^{-1}, which for comparative purposes equate to maximal current speeds of < 0.003 m s^{-1} through those chambers, measured with a Valeport 800-175 electro-magnetic current meter. As particle sedimentation within the mussel chambers was found to be negligible, 2 'control' chambers were maintained without mussels. At prescribed intervals, 20 ml aliquots from the outflows of the 2 control chambers, and from chambers containing an individual mussel, were analysed using a Coulter Multisizer II (100 µm diameter orifice) with Coulter Accucomp software. Both the total number and volume of particles between 3 and 6 µm equivalent spherical diameter, which spanned the size range of *I. galbana*, were measured and recorded in replicate 0.5 ml samples. We concede that using an algal monoculture as food may produce different responses from those using natural seston, but it was chosen here as a more consistent and comparable food source to study the experimental variables.

Clearance rate was determined by 2 equations that are in common use:

$$\mathrm{CR} = \mathrm{Fl} \times [(C_i - C_o)/C_i] \quad (1)$$

where Fl is the flow through the chamber, and C_i and C_o are the number of particles per millilitre within the inflow and outflow, respectively, and

$$\mathrm{CR} = \mathrm{Fl} \times [(C_i - C_o)/C_o] \quad (2)$$

where the outflow concentration is used as the denominator, and is normally used in steady-state conditions (Hildreth & Crisp 1976, Riisgård 2001b). Concentrations measured in the outflows from control chambers were used as the inflow concentration to chambers containing experimental mussels. To avoid errors associated with potential recycling, we have normally restricted calculations of CR to mussels effecting a reduction in particles between inflow and outflow of 5 to 30% (Hawkins et al. 1999). It should be noted that, for analytical purposes, greater reductions have in some cases deliberately been induced here.

Experiments were designed to resolve any separate effects of inflow cell concentration, outflow cell concentration, percentage reduction (recycling) and/or flow rate (current speed) on CR. To achieve this, 3 experimental phases were undertaken:

(1) Expt 1. To confirm the time course in response of CR to stepwise increases in food supply (cell concentration), CR was measured simultaneously in 14 mussels after 5, 30, 60 and 180 min following a change from the maintenance ration to between 4000 and 200 000 cells ml^{-1}. At each cell concentration, flow rates were adjusted to give between 5 and 30% reduction in algal cell concentration between inflow and outflow.

(2) Expt 2. To resolve the effects of flow rate, percentage reduction (recycling) and outflow cell concentration on CR, a series of experiments was undertaken using constant inflow concentrations (5, 10, 20, 30 and 50 000 cells ml^{-1}), and flow rates were varied to give mean percentage reductions in cell concentration between inflow and outflow of between 5 and 65%. On the basis of findings from our first experimental phase above, and as explained in the results, CR measures were standardised after 30 min under each condition.

(3) Expt 3. To assess the relative importance of inflow concentration versus outflow concentration, mussel chamber outflow concentrations were maintained as close as possible to 10 000 cells ml^{-1}, applying the same flow rates as in Experiment 2 above, and varying the algal dosing rate (inflow concentration) to give the

same percentage reductions in cell number as had been recorded at each flow rate during Experiment 2.

Because feeding history may affect the feeding responses of bivalve shellfish, care was taken to limit the time that each experimental mussel was exposed to high food rations, thus minimising any satiation that may have led to reduced CRs. On a day to day basis, experiments administering high rations were alternated with those at low rations. At each sampling point, individual mussels that were closed and not feeding, or which were producing pseudofaeces, were noted.

Chlorophyll *a* (chl *a*) was measured in filtered aliquots from the outflows of each control tray. Samples were collected on separate 47 mm Whatman GF/F filters that were frozen at –20°C before using standard procedures for acetone extraction and fluorometric analysis (Holm-Hansen et al. 1965).

Following feeding experiments, all soft tissues were excised from each mussel and dried at 60°C before weighing to a constant total soft tissue dry weight (mean ± 2 SE: 0.168 ± 0.019 g). Clearance rates presented here were not weight standardised. Instead, the stated mean total soft tissue dry weight affords the opportunity for others to do so at their discretion.

To resolve any effects of inflow or outflow cell concentrations, percentage reduction (recycling) and/or flow rates, the differences between mean CR measures were first tested for statistical significance with 1-way analysis of variance (ANOVA), applying a Bonferroni correction (Sokal & Rohlf 1995). Upon any suggested difference, specific pair-wise comparisons were undertaken using a Tukey's honestly significant difference test. These analyses were carried out using SYSTAT 11 (Systat Software Inc.).

RESULTS

Experiment 1

The effects on CR of stepwise increases in *Mytilus edulis* cell concentrations after 5, 30, 60 and 180 min following change from the maintenance ration to between 4000 and 200 000 cells ml^{-1} are summarised in Fig. 1. These CR values were measured under conditions when cell reductions ranged from 5 to 30%, and were computed using Eq. (1), with inflow concentration as the denominator. Findings illustrate that reduced average clearance rates were often associated with lower numbers of actively feeding animals. At low cell concentrations averaging <4000 cells ml^{-1} (less than ~0.5 µg chl *a* l^{-1}), CR remained at <1 l h^{-1} throughout the time course of up to 3 h, when low proportions of the mussels were actively feeding. At cell concentrations of 5000 to 200 000 cells ml^{-1} (~0.6 to 25 µg chl *a* l^{-1}), CR increased to values of between 1.93 and 2.38 l h^{-1} after 30 min, compared to <1 l h^{-1} below 4000 cells ml^{-1} ($p < 0.01$). 'Saturation reduction' occurred after 180 min at high concentrations as evidenced by a higher CR of >2 l h^{-1} at 30 000 cells ml^{-1}, compared with <1.5 l h^{-1} at >100 000 cells ml^{-1} ($p < 0.05$).

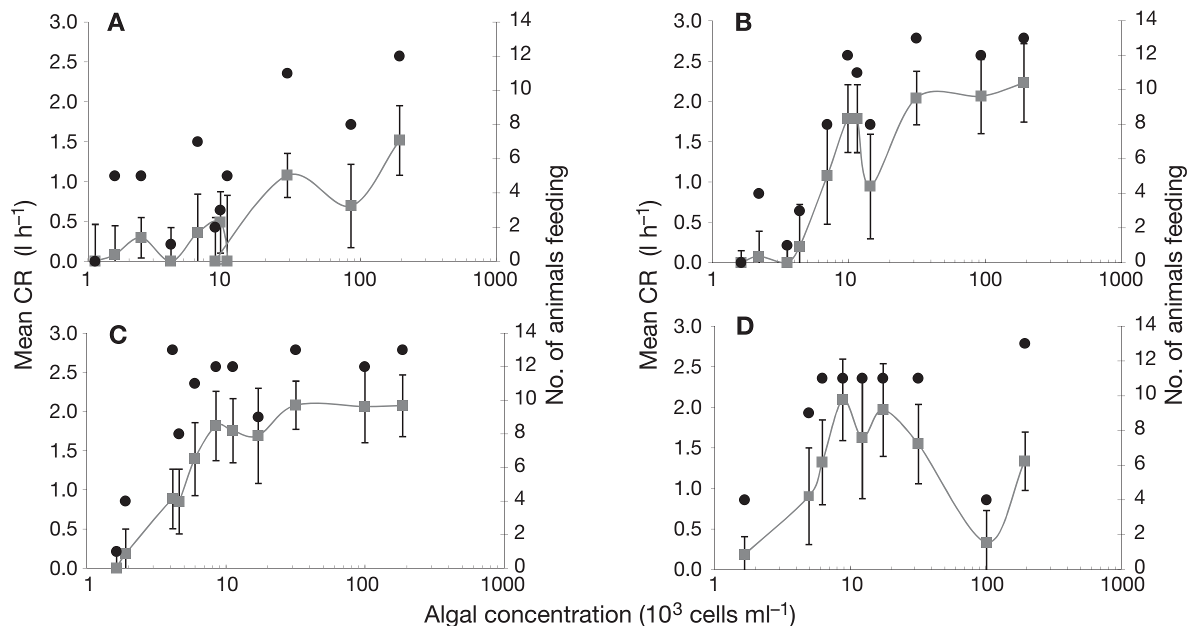

Fig. 1. *Mytilus edulis*. Relationships between clearance rate (CR; mean ± 2 SE) and algal cell concentration after different time intervals at each food ration. (A,B,C,D) Responses after 5, 30, 60 and 180 min at each ration, respectively. Data are for all animals, including those not feeding. (●) number of animals feeding

Experiment 2

On the basis of the above results, further CR measures were standardised to 30 min after any experimental change in feeding conditions. Fig. 2 illustrates all CR measures for which the percentage reduction in cell concentration between inflow and outflow was >5%, and shows that average CR increases with cell concentration from 5000 (mean = 1.58) to around 20 000 cells ml^{-1} (mean = 2.14) and then decreases to around 1.5 at higher concentrations of about 50 000 cells ml^{-1}. CR values at the lowest 3 concentrations were all significantly different from each other ($p < 0.01$), and that at 20 000 cells ml^{-1} was significantly different from all others ($p < 0.01$). Fig. 3 shows how, for given flow rates, CR calculated using Eq (1) increased with percentage reduction in cell concentration between inflow and outflow. Flow rate appeared to be the dominant parameter, as slopes of relations between CR and percentage reduction increased with increasing flow. Also, at low flow rates, percentage reductions were high, whilst CR remained low. The same data plotted according to Riisgård (2001b) show CR values approaching a plateau as flow rates rise above 150 ml min^{-1}, at which point virtually all values fall between the lines representing 5 and 30% reductions in particles (Fig. 4). At these higher flows of >150 ml min^{-1}, the maximal recorded average CR of around 38 ml min^{-1} (or 2.28 l h^{-1}) is similar to that predicted by Møhlenberg & Riisgård (1979) for *Mytilus edulis* of this size.

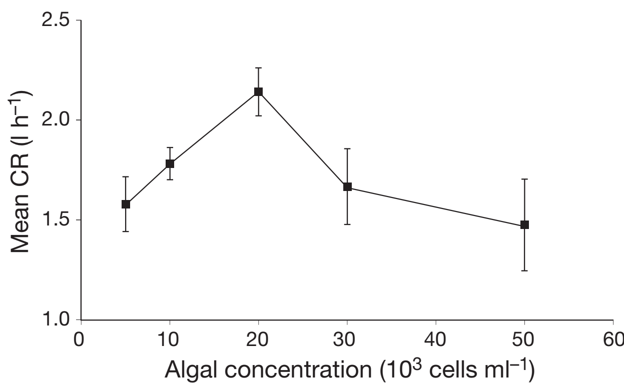

Fig. 2. *Mytilus edulis*. Relationships between clearance rate (CR) and algal cell concentration showing mean values (±2 SE) at each nominal concentration for all animals (with a >5% reduction in particles between inflow and outflow) and at all flow rates from Experiment 2

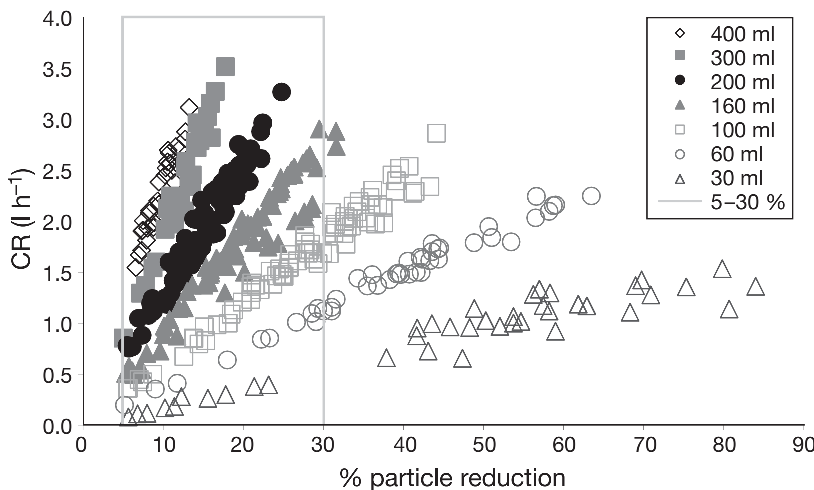

Fig. 3. *Mytilus edulis*. Clearance rate (CR) versus percentage particle reduction, plotted for all animals at each nominal flow rate (shown in the legend, ml min^{-1}). The box shows values lying between 5 and 30% reduction in particle numbers

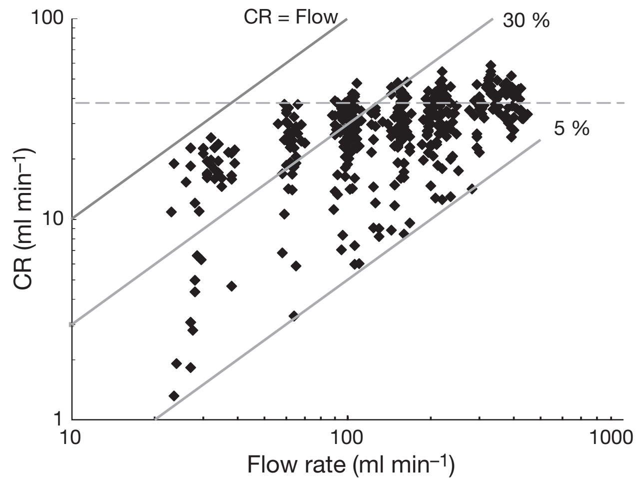

Fig. 4. *Mytilus edulis*. Relationships between clearance rate (CR) and flow rate following Riisgård (2001b), to validate the flow-through chamber design. Lines are plotted to show CR (i.e. flow rate), and the limits of 5 and 30% reduction in particles. Horizontal dashed line: theoretical CR (2.28 l h^{-1}) derived for the size of *M. edulis* used here, from the equation $CR = 7.45W^{0.66}$, where W is g dry soft tissue (Møhlenberg & Riisgård 1979)

Experiment 3

Fig. 5 illustrates relationships between CR and flow rate or percentage reduction according to inflow and outflow concentrations, including any differences resulting from the use of Eqs. (1) versus (2). Results are compared for feeding conditions with constant inflow (Fig. 5A & B) and outflow (Fig. 5D & C) concentrations, all of about 10 000 cells ml^{-1}. Fig. 5 Panels A & B show that at low flow rates, differences between cell concentrations in inflows and outflows (percentage reductions) were high due to likely recycling of

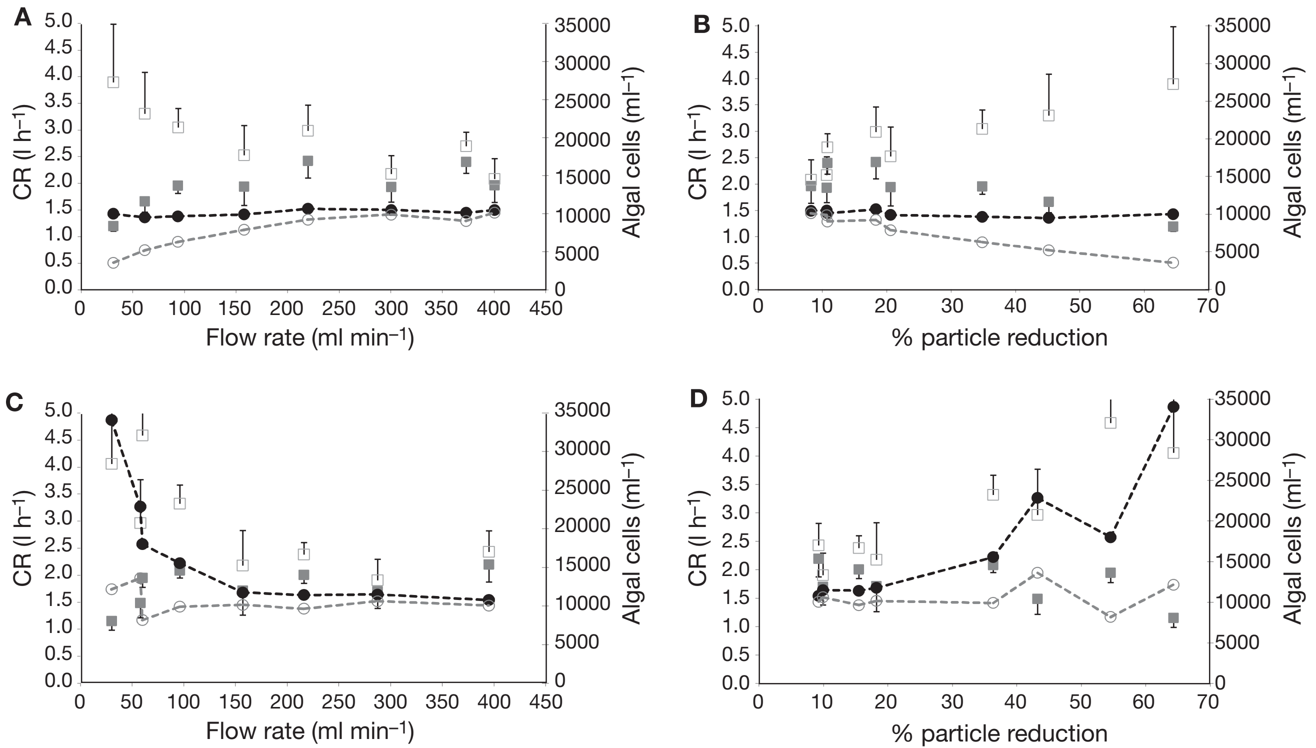

Fig. 5. *Mytilus edulis*. Relationships between clearance rate (CR; mean ± 2 SE) and both flow rate and percentage reduction in particles, for 2 separate conditions: (A,B) maintaining a constant inflow concentration of 10 000 cells ml^{-1} and (C,D) maintaining a constant outflow concentration of 10 000 cells ml^{-1}. CR values were derived from both Eq. (1) (■) and Eq. (2) (□), and cell concentrations are shown for both inflows (●) and outflows (○)

the water, which results in CR calculated using Eq. (2) being much greater than CR calculated using Eq. (1). This may highlight the lack of 'steady state' conditions in this case. Also, the latter may be depressed, as the concentration of algal cells falls towards the trigger level. At high flow rates, percentage reductions were very low, and, consequently, there is little difference between CR calculated using Eqs. (1) & (2). In this case, the animals are filtering at maximal rates, but they are only clearing a small percentage of the total supply, as most of the flow is bypassing them (see Larsen 2001). As the formulae would suggest, differences in CR values resulting from the 2 equations are clearly proportional to the percentage reduction. For example, given a reduction of 20 %, CR calculated using Eq. (2) will be 20 % higher than CR derived from Eq. (1).

Results from feeding conditions with constant outflows at about 10 000 cells ml^{-1} (Fig. 5D & C) are similar to those with constant inflow concentrations (Fig. 5A & B), since, although inflow concentrations at low flow rates are markedly different, the percentage reductions, and hence CR values calculated using either Eq. (1) or (2), are similar whether measured with constant outflows or inflows. The same data plotted against elapsed time show that CR calculated using Eq. (1) was inversely related to inflow concentration, and again illustrates a dependency related to flow rates <150 ml min^{-1} (Fig. 6). The effect of feeding history is also illustrated here, as CR values are lower and more variable after feeding at higher inflow concentrations and for a longer time.

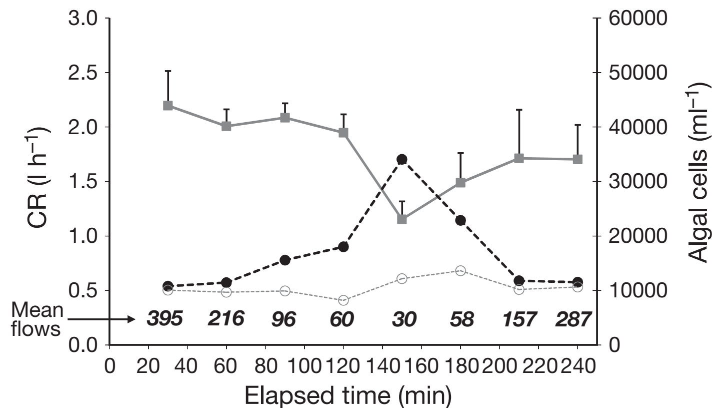

Fig. 6. *Mytilus edulis*. Interrelationships between mean clearance rates (CR; mean ± 2 SE) derived from Eq. (1) (■) and algal cell concentrations against time elapsed from food change (i.e. Expt 3). A constant outflow concentration of 10 000 cells ml^{-1} was maintained. Cell concentrations are shown for both inflows (●) and outflows (○), and flow rates for each measure are shown in ***bold italics***

DISCUSSION

Our findings are consistent with 'physiological regulation' of bivalve filter feeding, according to indicators of such regulation suggested by Riisgård (2001a,b): (1) a lag phase was evident in the feeding response to changes in food concentration, that lag being between 5 and 30 min in the present study, when mussels were acclimated to a low food ration of approximately 2000 cells ml^{-1} giving 0.25 µg l^{-1} chl *a*; (2) a trigger level was reached in algal cell (chl *a*) concentration, below which filtering in most mussels ceases, and which, in this study, appeared to be around 0.5 µg l^{-1} chl *a*; (3) saturation, or satiation, was reduced, resulting in reduced clearance rates and valve closure, after feeding for some time at high food rations, evidenced here after feeding for >2 h at ≥30 000 cells ml^{-1} (~6 µg l^{-1} chl *a*); and (4) at suitable flow rates and food levels between Points 2 & 3, CR increased to maximal rates, unless affected by other environmental conditions. There has been uncertainty over whether the transitions from no feeding to maximal CR, both at low and very high cell concentrations, entail abrupt stepwise events or a more gradual change resulting in a bell-shaped response of CR versus cell concentration. We suggest that different observations will result from measures based on instantaneous particle depletion by individuals, as compared with measures that average the responses of >1 ind., thus disguising significant inter-individual variability. Alternative methods may temporally integrate CR and other responses over longer time periods when measured as particle depletion within static seawater volumes (Widdows 1985, Petersen et al. 2004) or through the collection of faecal biodeposits (Cranford & Hargrave 1994, Hawkins et al. 1996, Cranford & Hill 1999, Navarro & Velasco 2003, Velasco & Navarro 2005). To illustrate this point, Fig. 7 compares differences between individual responses to food concentration in 3 mussels (Fig. 7A) with the averaged values for those same individuals (Fig. 7B).

It is clear that considerable variation in CR values can be produced by different methods of measurement. Problems have been recognised and recommendations advanced for the optimization and standardization of those methods, addressing chamber geometry, flow rate and the relative definitions of filtration rate and clearance rate (Riisgård 1977, Møhlenberg & Riisgård 1979, Widdows 1985, Riisgård 2001b). In the present study, one aim has been to re-assess our flow-through system as used by Hawkins et al. (1996, 1999, 2002) according to Riisgård's (2001b) suggested protocol. Figs. 3, 4 & 5 illustrate how—providing the normal limits of flow (>150 ml min^{-1}) and percentage reductions (5 to 30%) are met—measures made with our system are entirely consistent with the guidelines proposed. Indeed, maximal CR values of around 2.5 l h^{-1} described here for mussels of 0.168 g mean dry weight and 35 ± 2 mm shell length compare favourably with related measures of 2.29 l h^{-1} from CR = $7.45W^{0.66}$ (Møhlenberg & Riisgård 1979) and 2.42 from CR = $0.0012L^{2.14}$ (Kiørboe & Møhlenberg 1981), where W and L are dry soft tissue weight and shell length, respectively.

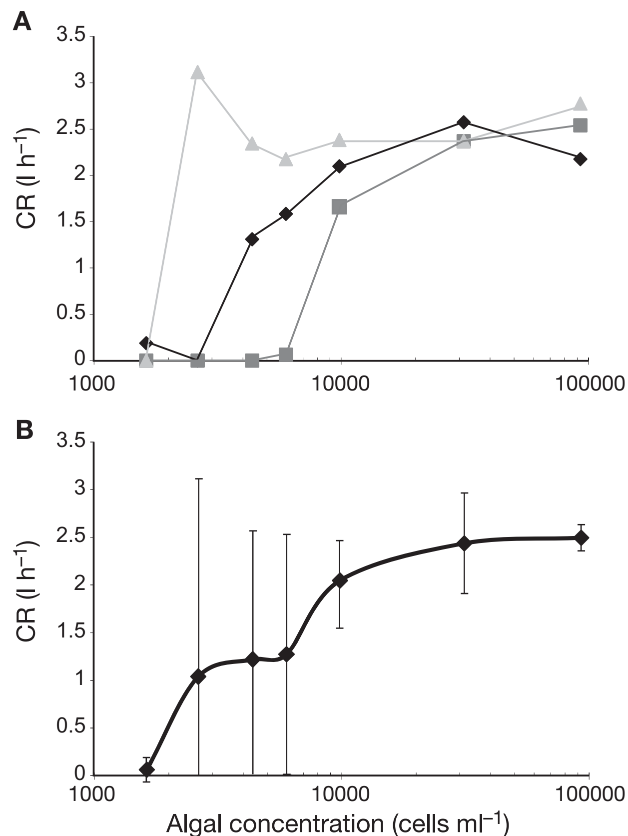

Fig. 7. *Mytilus edulis*. Relationships between clearance rate (CR,; mean ± 2 SE) and algal cell concentration, standardised to 30 min following changed feeding conditions, showing: (A) variation in 3 individual animals and (B) the mean values at each concentration for the same 3 animals

Since Riisgård's (2001b) review, further methodological investigations have included inter-calibration by Petersen et al. (2004), comparing the flow-through method, the biodeposition method and the indirect (or clearance) method, which further stimulated criticism and debate (Bayne 2004, Petersen 2004, Riisgård 2004). Although this inter-calibration (Petersen et al. 2004) still left some uncertainty over the correct methodology and measurements, their suggested maximal CR of 3.6 l h^{-1} is, in fact, close to estimates from early published equations, e.g. CR = $7.45W^{0.66}$ (Møhlenberg & Riisgård 1979). Widdows (1985) showed that the different methods (static, flow-through and steady-state) and their relevant equations should produce similar CR values. Also, as we have highlighted above, the dif-

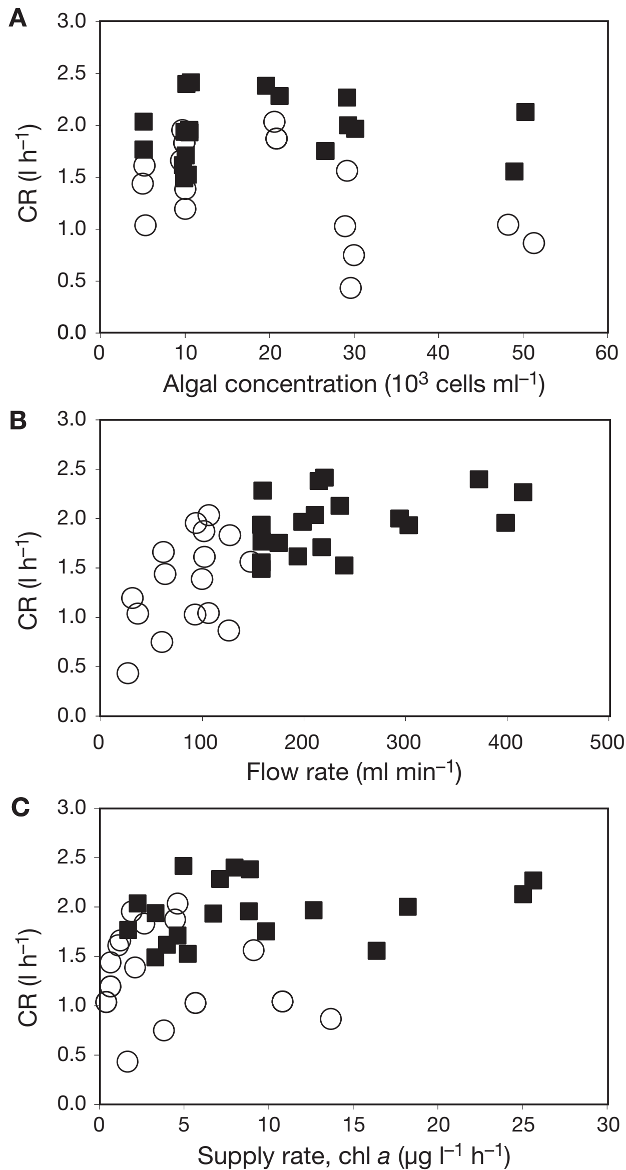

Fig. 8. *Mytilus edulis*. Mean clearance rates (CR) of up to 14 animals, at each flow rate and each inflow algal concentration, expressed as a function of: (A) inflow algal concentration, (B) flow rate and (C) supply rate (µg chl *a* l^{-1} h^{-1}). (□) flow rates of >150 ml min^{-1}; (●) flow rates of <150 ml min^{-1}

ference in CR values using Eqs. (1) & (2) for a flow-through system is proportional to the percentage reduction in particles. A clear validation of 2 different flow-through systems was made by Filgueira et al. (2006). They investigated the fluid dynamics in individual cylindrical experimental (flow-through) chambers (ICEC) and then, with *Mytilus galloprovincialis*, established that the system complied with the requirements for accurate CR measurements as suggested by Riisgård (2001b). Their proposed protocol for validation — identifying phases where CR is dependent on flow rate, where it is independent of flow, and the transitional phases — allows simple analysis of any flow-through chamber, even those used in previous studies. An average of 20% (range 13 to 25) reduction in particles is recommended for valid measurements, and our Eq. (1) is deemed correct for the flow-through chamber method. Accuracy and validity of the method relies to some extent on a suitable ratio of animal size to chamber size, and the suggested scaling of the chamber by reference to the allometric length–volume relationship of mussels is both simple and practical. Filgueira et al. (2006) also validate a mesocosm system by comparison with results from the ICEC, so, overall, their work represents a major step forward in the debate on measurement of clearance rates in bivalves.

Figs. 5 & 6 show the difference between CR values using the 2 equations, and confirm that Eq. (1) is the better representation of true CR. However, care needs to be taken over the limits of flow rate and percentage reduction, and also feeding history. Results presented here validate our methods for measurement of clearance rates, and confirm that guidelines for the limits of percentage particle reduction suggested by some previous authors were correct (Hawkins et al. 1999, 2002, Larsen 2001, Filgueira et al. 2006).

Our findings for *Mytilus edulis* complement those illustrating regulatory feeding behaviour in the New Zealand green-lipped mussel *Perna canaliculus* (Hawkins et al. 1999) and the Chinese scallop *Chlamys farreri* (Hawkins et al. 2002). CR has been observed to increase with the availability of natural suspended sediments in turbid environments, up to experimental maxima of as much as about 80 mg total particulate matter l^{-1} (e.g. Hawkins et al. 1996). However, particles suspended in turbid environments are dominated by silt, when chl *a* may only be present at up to about 3 µg l^{-1} (e.g. Iglesias et al. 1992, Newell & Shumway 1993, Hawkins et al. 1996). As reported here for *M. edulis*, it is notable that maximum CRs recorded in species from temperate latitudes typically average up to about 8 l h^{-1} g^{-1} dry soft tissue and are most commonly observed in mussels feeding on cultured unicellular algae at concentrations affording up to about 5 µg chl *a* l^{-1} (e.g. Møhlenberg & Riisgård 1979, Riisgård 1988, Clausen & Riisgård 1996, Jørgensen 1996, Hawkins et al. 1997). Alternatively, at higher concentrations of cultured unicellular algae, CR has frequently been observed to decrease in a negative exponential relation to increasing food availability (Winter 1978, Griffiths 1980, Iglesias et al. 1992, Arifin & Bendell-Young 1997, Hawkins et al. 1997, 1998, Yukihira et al. 1998, Denis et al. 1999). This is again consistent with observations for *M. edulis* here, when saturation reduction, or satiation, resulted in reduced CRs and valve closure after feeding for >2 h at 30 000 or more cells ml^{-1} (~6 µg chl *a* l^{-1}) (Fig. 1).

Three main points may be emphasised. Firstly, CR in suspension-feeding shellfish is highly flexible. Reductions from maximal rates occur, when costs of feeding (e.g. Widdows & Hawkins 1989) may outweigh potential returns, both at low particle availabilities and upon satiation at high food availabilities, when coincident rates of ingestion and absorption are nevertheless maintained, that is, until such elevated concentrations are reached to incur physical inhibition (e.g. clogging) of the feeding mechanism. Secondly, adjustments in CR are best understood in relation to the utilisable organic component of available seston, rather than the total suspended load; whether that utilisable component is measured as chlorophyll, as a marker for living organic matter, or as the remaining detrital organic matter, which is increasingly understood to play an important role in the nutrition of bivalve shellfish (Navarro et al. 2009, this Theme Section). Thirdly, responsive adjustments in CR are most evident at low availabilities of that utilisable component. Chl *a* may reach as much as 150 µg l^{-1} in dense blooms of algae within unmixed eutrophic waters. Yet, maximal CR in *Mytilus edulis* in the present study was observed at <~6 µg chl *a* l^{-1}. Similarly, maximal CR in *Chlamys farreri* occurred at about 5 µg chl *a* l^{-1}, this species experiencing between about 1 and 20 µg chl *a* l^{-1} during culture in China (Sun et al. 1996). More striking still, maximal CR in *Perna canaliculus* occurred at <2 µg chl *a* l^{-1} (Hawkins et al. 1999), normally experiencing between about 0.5 and 5 µg chl *a* l^{-1} during culture in New Zealand (Gibbs & Vant 1998). On this basis, variations in availability of suspended organic matter are of greatest physiological consequence in suspension-feeding bivalves whilst over the lower end of observed natural ranges, when responses are higher per unit change in food abundance and have the highest proportional impacts upon net energy balance (Hawkins et al. 1999).

A further advance in this field might be to formulate the relationship between the effects of both flow and food concentration on CR, by using the product of the two as units of food supply, provision, or availability, expressed as grams of chl *a* or carbon per litre per hour. Fig. 8 shows these relationships, both separately and combined as the supply rate, using data derived in the present study for chl *a* content in *Isochrysis galbana*. Over a wide range of supply rates, all the CR measures that we have judged to be valid in this study, i.e. those measured at flow rates of >150 ml min^{-1}, lie within a limited range of from 1.5 to 2.5 l^{-1} h^{-1}, averaging about 2 l^{-1} h^{-1} or 6 l h^{-1} g^{-1} when standardised according to dry soft tissue weight, and which are similar to the near-maximal or 'autonomous filtration' condition suggested by Riisgård (2001a) for moderate food levels.

In any continued quest for accuracy, it is important to consider what measures of feeding are intended for, and whether they are 'fit for purpose'. For example, we often measure feeding in individual bivalves and ignore the variation between individuals by taking mean values for given conditions. Extrapolating these data for use at the population or ecosystem level, for example, in modelling growth rates and impacts of bivalve feeding, is a significant step requiring some understanding of interactions between neighbouring individuals, the hydrodynamics of the system and, of course, temporal variations in food quantity and quality. In this context, there is a wealth of literature concerning the effects of flow or current velocity on bivalve feeding in nature (Wildish & Kristmanson 1997, Dolmer 2000, Newell et al. 2001, Widdows et al. 2002). It is worth emphasising that most or all of this research relates to relatively high current velocities (flows), including associated effects of external and internal pressure fields on the ciliary pump, neither of which are normally relevant to the effects of flow rate measured using a flow-through chamber system. These studies, whether using field measurements or attempting realistic simulations in a flume, usually involve flows well above 0.05 m s^{-1}, whereas maximal flow through our chambers was measured at <0.003 m s^{-1}.

Recently, Saurel et al. (2007) examined the feeding behaviour of mussel beds in the Menai Strait in relation to multiple environmental factors. Based on *in situ* video measurements of valve gape area (VA) over 2 tidal cycles, they concluded that mussel feeding was regulated solely by chl *a* concentration measured at 1 m above the mussel bed (0.6 to 2.5 µg l^{-1}), and not by flow velocity, changes in suspended particulate matter, or the presence of predators. However, at times of low flow during slack water, concentrations of chl *a* decreased to levels below the threshold for maximal CR and mussels consequently reduced their feeding activity. In fact, Saurel et al. (2007) estimated that maximal CR (VA of 80 to 100%) occurred for only ca. 45 min d^{-1}, suggesting that optimal feeding conditions are rarely experienced in the Menai Strait. Suggestions are made, with reference to the review by McKindsey et al. (2006), as to how these and other suitable *in situ* measurements may facilitate assessment and modelling of carrying capacity. Other work on *in situ* measurements has assessed the use of valve gape or exhalent siphon area as proxies for pumping rate in bivalves and propose these as useful methods for following the dynamic changes and physiological adaptations during natural bivalve feeding (Riisgård et al. 2003, 2006, Maire et al. 2007). Many of these field studies emphasise that CR varies greatly under natural conditions, such that dynamic modelling is required to assess ecological interrelations between bivalve sus-

pension feeders and their environment. Dynamic feeding responses have increasingly been established using the biodeposition method, which, through separate coincident collections of both true faeces and pseudofaeces and in contrast to measures of CR based solely upon particle depletion, not only integrates over time, but also enables calculations of ingestion rate and absorption efficiency (Hawkins et al. 1996, 2002, Cranford et al. 1998, Cranford & Hill 1999). Measured responses may then be used to simulate each physiological component of growth (Hawkins et al. 2002), for use in integrated ecological modelling (Ferreira et al. 2007, 2008).

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Contribution to the Theme Section 'Directions in bivalve feeding'

Valve gape and exhalant pumping in bivalves: optimization of measurement

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ABSTRACT: We examined the effect of sampling frequency of gape angle and exhalant pumping measurements on our ability to determine the behaviour of bivalves. We used the endangered freshwater bivalve *Margaritifera margaritifera*, the non-endangered mussels *Mytilus edulis* and *Mytilus trossulus*, the scallop *Pecten maximus* and the cockle *Cerastoderma edule*. Increasing sampling interval led to an underestimation of the rate of bivalve gape adduction and abduction events detected, an overestimation of the mean duration between gape adduction and abduction events, and a misunderstanding of the form of the gape adduction and abduction events and exhalant pumping profile. Our analyses suggest minimum appropriate sampling rates for archival tags to define gape behaviour of 2, 7 and 40 Hz in *M. margaritifera*, *C. edule* and *P. maximus*, respectively, and 18 Hz to describe the metachronal wave in exhalant pumping of *M. edulis*. We demonstrate that careful consideration has to be given to the selection of sampling intervals when using a non-continuous method of recording behaviour. Our results emphasize the importance of measuring fine-scale behaviour patterns in order to advance our understanding of bivalves. The potential loss of information associated with the choice of particular sampling intervals during measurements of single parameters, and the biases which can result from this choice, are effectively germane to all species.

KEYWORDS: *Mytilus* spp. · *Margaritifera margaritifera* · *Pecten maximus* · *Cerastoderma edule* · Sampling · Resolution · Recording · Frequency · Behaviour

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INTRODUCTION

Research on bivalve behaviour has produced insights on how organisms cope with highly fluctuating environments (e.g. Jørgensen et al. 1988). Some of the questions addressed have been aimed at providing an overall view of the behaviour of a particular bivalve species. Recording behaviour with high frequency measurements has allowed questions concerning fine-scale bivalve behavioural physiology to be addressed (e.g. Trueman 1966, Hoggarth & Trueman 1967, Wilson et al. 2005). This may involve assessment of valve gape, siphon movements (changes in aperture), filtration and pumping behaviour in relation to associated environmental parameters such as depth, light, temperature, particulate matter, food availability and predator interactions (e.g. Ropert-Coudert & Wilson 2004). Although archival tags have elucidated some remarkable animal behaviours (see e.g. Ropert-Coudert & Wilson 2004 for review), selection of the correct temporal resolution, defined by the sampling interval, is critical to defining the quantity and form of behavioural events (Boyd 1993, Ropert-Coudert & Wilson 2004). Controversy about many aspects of bivalve behaviour, such as feeding, partly results from difficulties in accurately recording high frequency measurements of bivalve filtration activity (Maire et al. 2007). Maire et al. (2007) also highlight the importance of recording short-term changes in valve gape and exhalant siphon area.

Direct observation of mussel gape and exhalant siphon area (e.g. Newell et al. 2001, Maire et al. 2007) has the advantage of being simple to perform; however, it does not lend itself to situations where turbidity is high or to burrowing bivalves. In addition, the effective resolution of visual-based systems to determine changing parameters and the frequency with which

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observations are conducted may profoundly affect the quality and interpretation of results (e.g. Wilson et al. 2005). The use of animal-attached remote-sensing technology, in particular Hall sensors, to measure bivalve gape (Wilson et al. 2005, Nagai et al. 2006, Robson et al. 2007) circumvents many of these problems because many measurements can be made per second and the animal may live in its normal substrate.

Maire et al. (2007) proposed that images acquired at a frequency of once every 15 s were sufficient to assess filtration activity precisely in *Mytilus galloprovinciallis*, although bivalve gape has also been recorded at 5 Hz (Wilson et al. 2005), 2 Hz (Robson et al. 2007), 1 Hz (Nagai et al. 2006) and once every 5 and 10 min (Riisgård et al. 2006). However, technology now exists for reliably measuring gape angle at a frequency of 32 Hz (Wilson et al. 2008).

Despite its endangered status, little is known about the behaviour of the endangered freshwater bivalve *Margaritifera margaritifera* or about how to measure its well-being in captivity (but see Trueman 1966). We suggest that archival tag technology (Cooke et al. 2004, Ropert-Coudert & Wilson 2004), such as that used by Wilson et al. (2005) on blue mussels *Mytilus edulis*, could change this by allowing identification of normal and stressed behaviour (Robson et al. 2007). In the present study we examine the effects of using different sampling frequencies on our ability to elucidate the behaviour of bivalves. The prime driver behind this study is the formalization of a methodology that is appropriate to examine — without bias — gape and pumping behaviours (general proxies for activity associated with respiration, feeding, excretion and their associated metabolic processes) of endangered freshwater pearl mussels, non-endangered mussels *Mytilus edulis* and *Mytilus trossulus*, scallops *Pecten maximus* and cockles *Cerastoderma edule*.

MATERIALS AND METHODS

Collection and maintenance of bivalves. All research detailed below was conducted in accordance with institutional, national and international guidelines relating to the use of bivalves in research.

Margaritifera margaritifera used in experiments were held at the Environment Agency Wales, Cynrig Hatchery, Brecon, Wales. *Pecten maximus* were collected from the Bay of Brest, France, and transferred to a flow-through aquarium system within 2 h. Intertidal *Mytilus edulis* and *Cerastoderma edule* were collected from Swansea Bay and Gower coast, Wales, UK, respectively, and *M. trossulus* from the coastline outside the Pacific Biological Station, Vancouver Island, Canada, at low tide and transferred to a flow-through aquarium system within 2 h.

Experimental design. To make relative valve gape measurements in mm between bivalves of different lengths, we used methods developed by Wilson et al. (2005) and modified by Robson et al. (2007) to quantify gape angle in mussels *Margaritifera margaritifera*, *Mytilus edulis*, *M. trossulus*, the scallop *Pecten maximus* and the cockle *Cerastoderma edule.* However, neither Wilson et al. (2005) or Robson et al. (2007) calibrated all possible gape angles with sensor output and extrapolated bivalve gape calibration curves beyond known limits. Some gape data >5° were thus probably overestimated. The valve gape calibration dilemma was avoided in the present study by killing the bivalves or using a muscle relaxant on them after experiments, and calibrating Hall sensor output in mV to gape (°) over all gape angles (but see Nagai et al. 2006 who used the Hall sensor to measure bivalve gape without the need for calibration). We recommend calibration to ensure best possible accuracy in valve gape measurements.

Briefly, quantifying bivalve gape involved using a Hall sensor (a transducer for magnetic field strength) attached to one shell valve reacting to a magnet attached to the other shell valve. Variance in gaping extent produced a corresponding variance in the magnetic field strength perceived by the Hall sensor (cf. Wilson et al. 2002). This was recorded by an archival tag. Since Hall sensor output is proportional to magnetic field strength and angle of impingement, the transducer output must be calibrated by comparing shell gape angle with sensor output over a wide variety of angles. A muscle relaxant (500 ppm buffered Tricaine methanesulfonate, MS-222) (Lellis et al. 2000) was used on the endangered freshwater pearl mussels *Margaritifera margaritifera* (note *M. margaritifera* were not killed) to allow calibration of all possible gape angles with sensor output. The adductor muscle(s) of *Mytilus edulis*, *M. trossulus*, *Pecten maximus* and *Cerastoderma edule* were simply severed with a knife and bivalves were immediately calibrated for gape over all possible gape angles (~5 min per bivalve). Subsequently, data of sensor output versus gape angle were curve-fitted (for details see Wilson et al. 2002, 2005, Wilson & Liebsch 2003, Robson et al. 2007). The curve-fit could then be used to determine any gape angle by converting the transducer output accordingly.

One type of archival logger used was a 13-channel JUV-Log equipped with 12 Hall sensors (Honeywell, SS59E) and 1 temperature transducer. Two other archival loggers used were 7-channel JUV-Logs equipped with 4 Hall sensors (Honeywell, SS59E) and also recorded light (lux), pressure (depth) and temperature (°C). Two further 13-channel loggers had Hall sensors linked to the logger (IMASEN, Driesen and Kern GmbH) and also recorded light, pressure and temperature. The 13- and 7-channel JUV archival loggers were powered by four 1.2 V 10 Ah NiMH D cells

and the IMASEN loggers by two 3.6 V ½ AA lithium batteries. Each had a 1 Gb flash random access memory and could be set to record at intervals up to a maximum frequency of 2, 12 and 30 Hz, respectively. The IMASEN and JUV-Log archival loggers had 16 and 22 bit resolution, respectively, both recording gape angle at better than 0.01°. The magnets used were 5 × 5 × 2 mm neodymium boron magnets.

Magnets and Hall sensors were glued to *Margaritifera margaritifera* and *Pecten maximus* using 5-minute epoxy adhesive (X003, Atlas Polymers) and Araldite® 90 Seconds (Huntsman Advanced Materials), respectively. The other bivalves kept in saltwater aquaria during experiments had their systems attached using aquarium sealant (Geocel®), and the bivalves kept in intertidal environments had systems attached using high strength epoxy adhesive (Power-Fast®+, Powers Fasteners). *M. margaritifera* had been in freshwater pumped from a local river for months before experiments began. *Mytilus edulis* and *Cerastoderma edule* were placed in an aerated flow-through aquarium system containing edible particulate matter-laden seawater from Swansea Bay for at least 1 mo before being used in aquarium experiments. *P. maximus* were placed in an aerated flow-through aquarium system containing edible particulate matter-laden seawater from the Bay of Brest for at least a 24 h before being used in aquarium experiments. Equipped *M. edulis* and *M. trossulus* used in intertidal experiments were returned to the intertidal within 24 h of initial collection.

Bivalve pumping. Lengths of PVC tubing (10 mm diameter, 1.5 mm wall thickness and lengths of 300 and 25 mm) were glued together at right angles using high strength epoxy adhesive (Fig. 1). A Hall sensor was attached (using aquarium sealant) to the outside of the 300 mm long PVC tube, 60 mm below the 25 mm length of tubing (Fig. 1). A vane 60.5 mm long, 18 mm wide and 0.05 mm thick, made of translucent green Silastic® (Dow Corning) or transparent polyethylene, had one end attached to the ~25 mm long PVC tubing using aquarium sealant (Fig. 1). A 0.1 g (in air) neodymium boron magnet was attached at the free end of the vane using aquarium sealant so that the magnet and Hall sensor were aligned (Fig. 1). Pumping sensors were kept in a fixed position in mussel tanks using PVC clamps. The study mussel was then placed in relation to the vane so that the water exhaled (from the top 10 mm of the inhalant siphon and whole of the exhalant siphon) caused the vane to move, bringing the magnet closer to the Hall sensor, thus causing a change in magnetic field intensity perceived by the transducer (in a manner similar to that used for determining gape angle, see above). It was imperative to keep the Hall sensors and magnets from the gape and pumping sensors sufficiently far apart so they did not interact.

In preliminary pumping experiments *Mytilus edulis* used their foot to move the translucent green Silastic® vane out of the path of their exhalant water current and stuck it to the outside of their shell. This did not occur over 12 mo of continuous pumping experiments using transparent polyethylene as the pumping sensor vane. Thus, transparent polyethylene was used as the pumping sensor vane in the present study. Sampling frequencies of 2 and 30 Hz were used to record *M. edulis* pumping. The new method for measuring pumping could not be used in strong currents because of the high sensitivity of the sensor. We did not attempt to calibrate the fine temporal and sensor resolution exhalant pumping data because of complications our system could not easily account for. Complications include: (1) *Mytilus edulis* exhalant pumping can occur from the top of the inhalant siphon in addition to the exhalant siphon — there is no defined barrier to exhalant pumping from the top of the inhalant siphon, and it may not be assumed that inhalant pumping occurs throughout the whole of the inhalant siphon area (and clearly not when

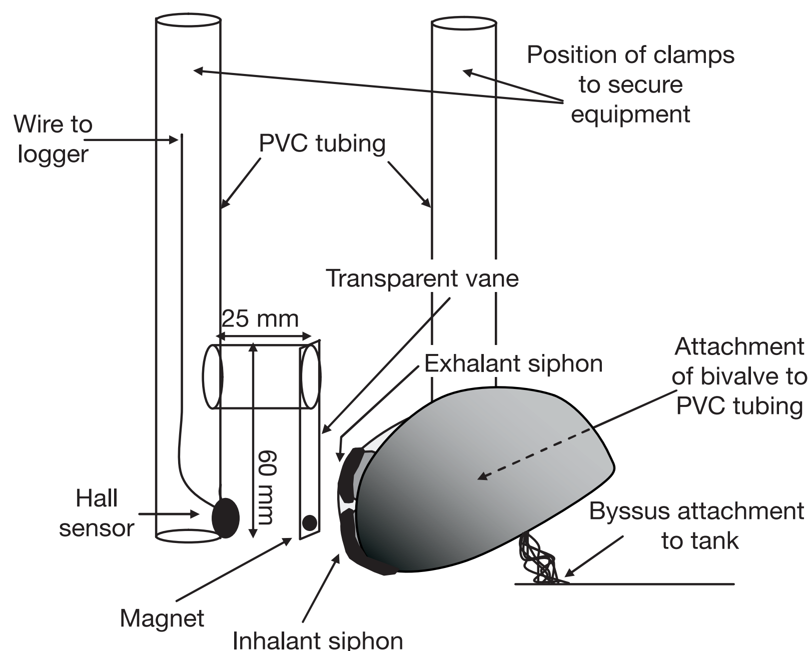

Fig. 1. *Mytilus* spp. Schematic diagram showing the bivalve pumping sensor for measurement of the flow of water out of the top of the inhalant siphon and whole of the exhalant siphon (aperture). See Wilson et al. (2005) for a schematic diagram showing the attachment of the Hall sensor and magnet system used for determining bivalve gape angle

exhalant pumping occurs from the top of the inhalant siphon) (2) Both changes in mussel siphon area and siphon orientation relative to the pumping sensor will change the force per unit area exerted on the pumping sensor. (3) *M. edulis* valve adduction events further complicate the measurement of exhalant pumping because maximum recorded exhalant pumping in this study was not produced by pumping (cilia beat) but by valve adduction (Appendix 1) (thus it is important to also measure valve gape in tandem with exhalant pumping at high temporal and sensor resolution so these two types of currents can be separated).

Experiments. Examples of bivalve gape behaviour at various sampling frequencies in the present study were selected as representative examples from a total of 6 *Margaritifera margaritifera*, 79 *Mytilus edulis* (gape and pumping in 48 *M. edulis*), 10 *Cerastoderma edule* and 7 *Pecten maximus* in laboratory aquaria as well as 52 *Mytilus* spp. in the intertidal zone (Atlantic and Pacific). Bivalves in their natural environments fed on natural seston and bivalves in aquarium experiments fed on seston pumped from their natural environment. Experiments with bivalves took place from December 2006 to April 2008.

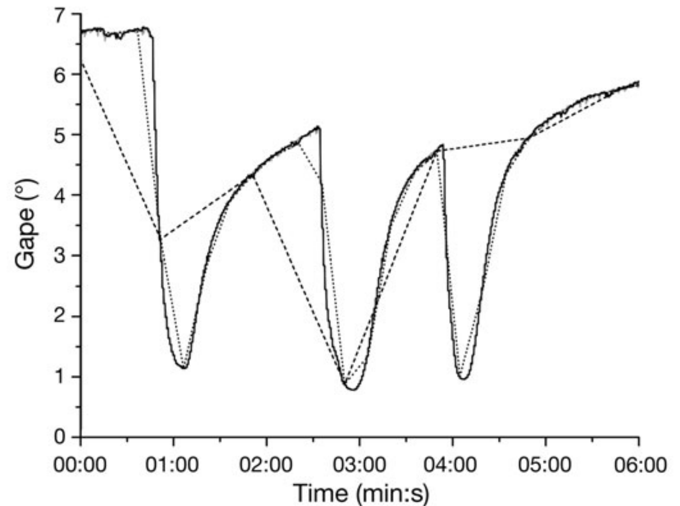

Fig. 2. *Mytilus edulis*. Example of the effect of sampling frequency on the gape data from a 70 mm long mussel in an aquarium at Swansea University, UK. Sampling frequencies: 30 Hz (—), 2 Hz (once every 0.5 s) (—), 0.067 Hz (once every 15 s) (···) and 0.017 Hz (once every 60 s) (---). The difference between valve gape recorded at 2 and 30 Hz is almost indistinguishable except between approximately 00:00 and 00:30 min:s

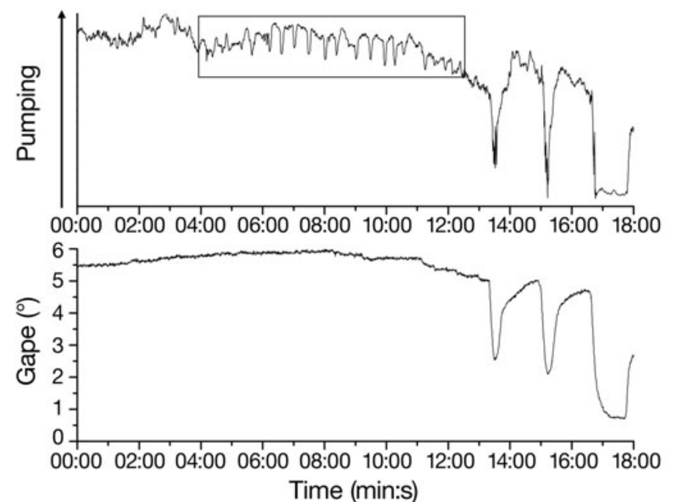

Fig. 3. *Mytilus edulis*. Detailed example of exhalant pumping and gape data recorded at 2 Hz from a 72 mm long mussel in a seawater aquarium at Swansea University, UK. Inset box in top panel highlights poorly defined variation in exhalant pumping

RESULTS

Bivalve gape

In preliminary investigations with live bivalves we made sure that our best-fit gape angle calibration curves for live animals were similar to those for sacrificed individuals. As an example, we used ANCOVA to compare 2 methods of gape calibration repeated in triplicate on one *Mytilus edulis*: (1) gape calibration on the live mussel and (2) gape calibration after the posterior adductor muscle was severed. Gape calibration method was the fixed factor and gape angle was the continuous variable. There was no significant effect of calibration method in the model ($F_{1,39}$ = 0.148, p = 0.702). We reiterate that calibration of maximum gape angle was not possible in live bivalves and highlight that the majority of any error in gape calibration curves was probably caused by human error (all best-fit calibration curves had $r^2 > 0.98$).

All major *Mytilus edulis* gape movements recorded at 2 Hz (0.5 s) followed the same general pattern as those recorded at 30 Hz (see Fig. 2). The rate of reduction in valve gape angle (adduction) was faster than the subsequent increase in gape angle (abduction), the latter having a roughly logarithmic form, in *M. edulis* (Figs. 2 & 3), *M. trossulus* (Fig. 4) and *Margaritifera margaritifera* (Fig. 5), with the rate decreasing near the endpoints of both adduction and abduction events. During recording of gape at 2 Hz in the smaller and faster-moving *Cerastoderma edule*, the rate of valve abduction did not always decrease near the endpoints of every abduction event (Fig. 6). Close inspection of *C. edule* gape data (Fig. 6) revealed that all valve adduction events occurred at a faster rate than the subsequent abduction event.

Reduction in gape sampling frequency was associated with a progressive change in the shape of the

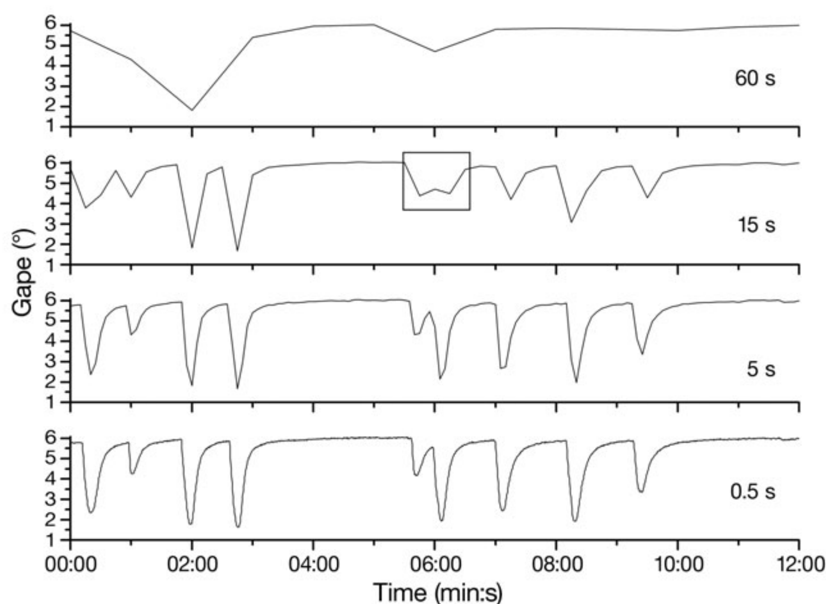

Fig. 4. *Mytilus trossulus*. Example of the effect of sampling frequency on gape data from a 55 mm long mussel in the Pacific intertidal zone, Vancouver Island, British Columbia, Canada. Sampling occurred once every 0.5, 5, 15 and 60 s. Box highlights the concatenation of adjacent gape adduction and abduction events in the data record that sampled once every 15 s

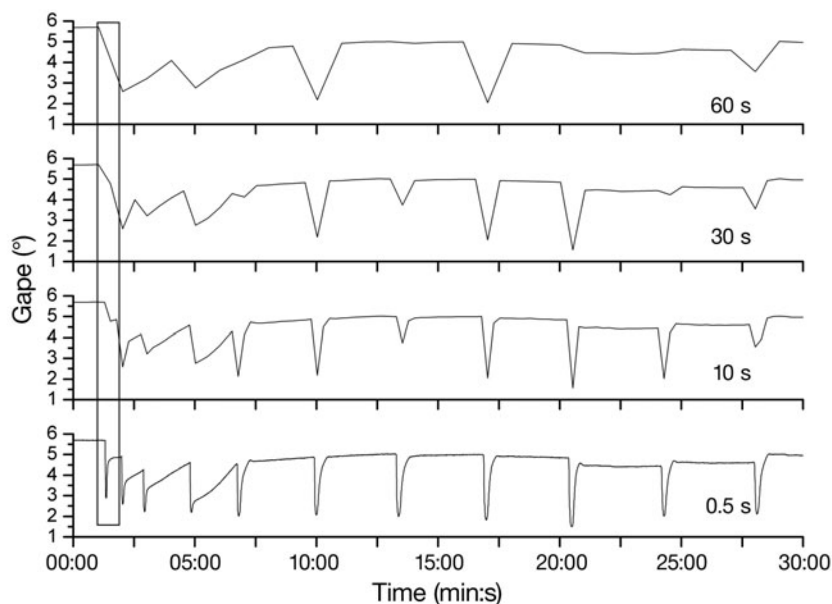

Fig. 5. *Margaritifera margaritifera*. Example of the effect of sampling frequency on burrowing gape data from a 100 mm long, freshwater pearl mussel in an aquarium. Sampling occurred once every 0.5, 10, 30 and 60 s. Box highlights the data loss of a valve adduction and subsequent abduction event with decreasing sampling frequency

gape angle versus time graph in both non-burrowing and burrowing bivalves in saltwater aquaria (Figs. 2 & 6, respectively) and in wild Pacific intertidal marine bivalves (Fig. 4). Reducing sampling frequency below 2 Hz (intervals of 0.5 s) made valve movements appear to be faster than they actually were (Figs. 2, 4–6). Accurate assessment of short-term changes in valve gape was only possible recording *Margaritifera margaritifera* gape at intervals of ≤0.5 s (Fig. 5). Increasing the sampling interval of gape data from 0.5 to 10 s resulted in the loss of some complete valve adduction and subsequent abduction events (e.g. Fig. 5). Visual observation of *M. margaritifera* burrowing behaviour backed up by recording gape at 0.5 s intervals (e.g. Fig. 5) highlighted the importance of valve movement for burrowing into sediment.

In one example, sampling at 1 to 5 s intervals, 45 valve adduction and subsequent abduction events over 1 h of *Margaritifera margaritifera* burrowing activity were plotted as a plateau with downward spikes. Increasing the sampling interval to ≥10 s concatenated some adjacent gape adduction and abduction events, with only 10 valve adduction and subsequent abduction events detected when sampling at 60 s intervals (Fig. 7). Over 1 h of burrowing activity, mean, median and minimum *M. margaritifera* gape angle increased as the sampling interval increased from 0.5 to 60 s (Table 1). Increasing the sampling interval from 0.5 to 60 s caused the interquartile range of *M. margaritifera* gape data to decrease by 0.59° and caused median gape to increase by 0.31° (Table 1). Maximum gape of *M. margaritifera* and *Pecten maxiumus* decreased by 0.1° and 4.72°, respectively, when the sampling interval was reduced from 0.5 to 60 s (Table 1). Over 1 h there was no change in mean gape but there was a reduction in maximum gape angle of *M. margaritifera* and *Mytilus edulis* when the sampling interval was increased from 0.5 to 5 s (Table 1). Also over 1 h there was no change in mean gape but there was a reduction in maximum gape angle of *Pecten maximus* when sampling frequency was decreased from 12 Hz (sampling interval of ~0.083 s) to once every 0.5 s (Table 1). However, over 1 h of *Cerastoderma edule* gape data, there was a change in mean gape and a decrease in maximum gape angle when the sampling interval increased from 0.5 to 5 s (Table 1).

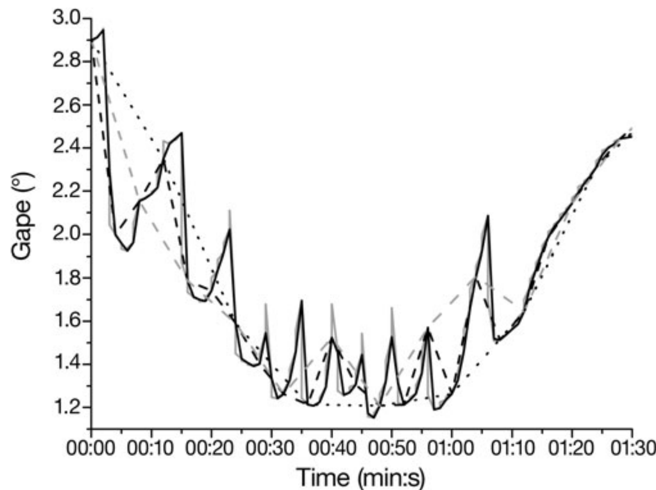

Fig. 6. *Cerastoderma edule*. Example of the effect of sampling frequency on burrowing gape data from a 30 mm long cockle in an aquarium at Swansea University, UK. Sampling occurred once every 0.5 s (—), 1 s (—), 4 s (– – –), 8 s (– – –) and 12 s (• • •)

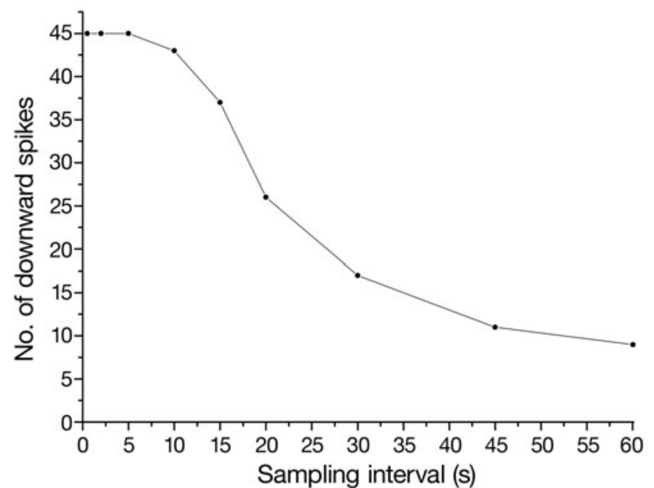

Fig. 7. *Margaritifera margaritifera*. Example of the effect of sampling interval on the number of detected downward spikes (i.e. valve adduction and subsequent abduction events) during 1 h of burrowing gape behaviour of a 105 mm long, freshwater pearl mussel in an aquarium

Table 1. Mean ± SD, median, maximum, minimum and interquartile range of gape data at different sampling intervals over 1 h from a burrowing, 100 mm long freshwater pearl mussel *Margaritifera margaritifera* in an aquarium, a 110 mm long scallop *Pecten maximus* in an aquarium, a 67 mm long *Mytilus edulis* immersed in the intertidal zone at Swansea Bay, UK, and a 28 mm long cockle *Cerastoderma edule* in an aquarium

Sampling interval (s)	Gape (°) Mean	Median	Max.	Min.	Interquartile range (°)
Margaritifera margaritifera					
0.5	3.76 ± 1.34	3.7	5.9	0.79	2.35
5	3.76 ± 1.34	3.7	5.87	0.8	2.32
10	3.76 ± 1.34	3.7	5.87	0.8	2.35
15	3.76 ± 1.34	3.72	5.87	0.87	2.33
30	3.80 ± 1.31	3.75	5.87	0.87	2.39
60	3.82 ± 1.31	4.01	5.8	0.87	1.76
Pecten maximus					
0.083	3.31 ± 1.58	3.44	10.81	0.97	2.76
0.5	3.31 ± 1.58	3.44	10.69	0.97	2.76
5	3.30 ± 1.57	3.53	10.03	0.98	2.76
10	3.31 ± 1.59	3.39	10.03	0.98	2.76
15	3.29 ± 1.54	3.54	8.52	0.98	2.75
30	3.27 ± 1.56	3.11	8.52	0.98	2.74
60	3.25 ± 1.53	3.02	5.97	0.98	2.83
Mytilus edulis					
0.5	3.28 ± 0.98	3.15	6.27	0.45	0.41
5	3.28 ± 0.98	3.15	6.24	0.48	0.4
10	3.28 ± 0.98	3.15	6.24	0.56	0.4
15	3.28 ± 0.98	3.16	6.19	0.72	0.41
30	3.28 ± 0.97	3.15	6.19	0.72	0.41
60	3.29 ± 0.94	3.16	6.09	1.37	0.35
Cerastoderma edule					
0.5	4.57 ± 0.52	4.76	6.36	1.18	0.53
5	4.56 ± 0.54	4.75	6.18	1.19	0.54
10	4.57 ± 0.54	4.75	6.18	1.36	0.54
15	4.55 ± 0.57	4.75	5.99	1.19	0.54
30	4.53 ± 0.60	4.74	5.49	1.36	0.54
60	4.51± 0.67	4.75	5.33	1.36	0.54

Pumping

A reduction in sampling frequency of bivalve pumping behaviour was associated with a loss in definition of short-term changes in exhalant pumping (Fig. 8). At fine scales (2 Hz), *Mytilus edulis* gape was well defined, while at the same frequency, pumping was apparently rarely constant and did not appear to be fully elucidated (e.g. Fig. 8). Mussel pumping recorded at 30 Hz revealed apparent and variable noise (a metachronal wave) in the pumping data of all animals (Fig. 9). We determined that the metachronal wave in the pumping data was biological in origin since it was not present when the pumping sensor was used on immersed dead mussels, or when gravity-fed water flowed out of an immersed, modelled mussel exhalant siphon (made from Silastic®, Dow Corning) towards the pumping sensor.

Measurements per event

Recording at 2 Hz, measurements (data points) per valve adduction and subsequent abduction event were counted for 50 events from 6 *Margaritifera margaritifera* (105 ± 1.4 mm length) and 10 *Cerastoderma edule* (28.6 ± 1.9 mm length). On average, fewer measurements were made per continuous valve adduction event compared to the subsequent abduction event in both *M. margaritifera* and *C. edule* (mean numbers of measurements per adduction and abduction event were 16.0 ± 5.7 and 44.3 ± 10.9, and 4.6 ± 1.5 and 9.1 ± 3.7 in *M. margaritifera* and *C. edule*, respectively), with a minimum of 10 and 3 measurements per adduction event in

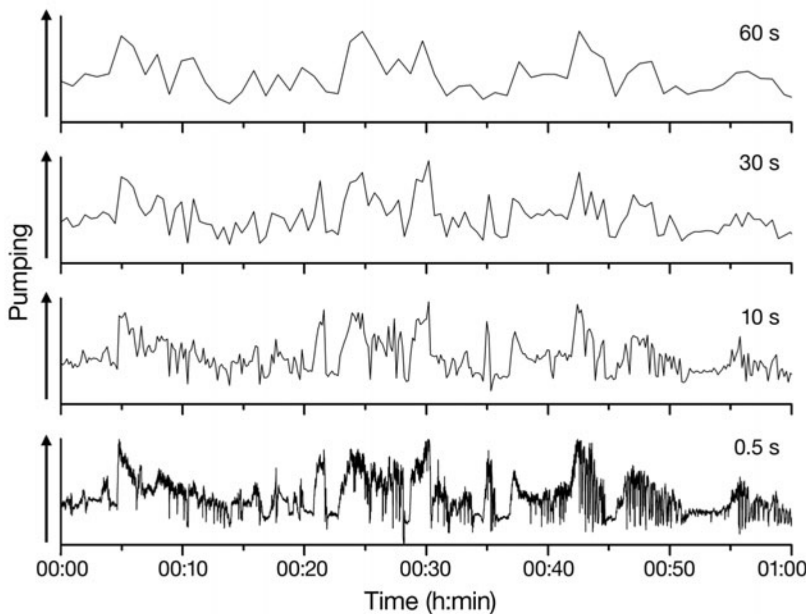

Fig. 8. *Mytilus edulis*. Example of the effect of sampling frequency on the exhalant pumping data from a 70 mm long mussel in an auqarium at Swansea University, UK. Sampling frequencies: 0.5, 10, 30 and 60 s

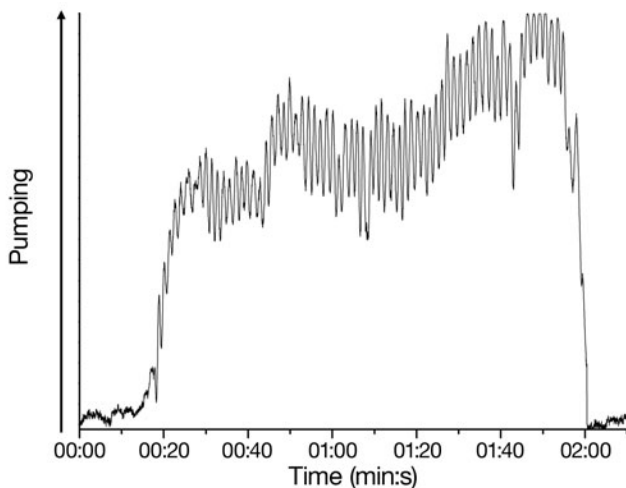

Fig. 9. *Mytilus edulis*. Example of a 75.5 mm long mussel eliminating faeces from the exhalant siphon in a seawater aquarium at Swansea University, UK. Pumping was recorded at 30 Hz with a metachronal wave evident in pumping data

M. margaritifera and *C. edule*, respectively. Complete *M. margaritifera* and *C. edule* valve adduction and subsequent abduction events had mean numbers of measurements per event of 54.5 ± 11.5 and 14.0 ± 4.7, respectively. Recording at 12 Hz, measurements per valve adduction and subsequent abduction event were counted for 50 events from 4 *Pecten maximus* (107.3 ± 1.7 mm length). Mean numbers of measurements per adduction and abduction event were 12.1 ± 6.8 and 789.2 ± 780.2, respectively, with a minimum of 3 measurements per adduction event. Complete *P. maximus* valve adduction and subsequent abduction events had a mean number of measurements per event of 1062.4 ± 766.1. Recording at 30 Hz, measurements per metachronal wave were counted for 50 metachronal waves from pumping data of 10 *Mytilus edulis* (69.8 ± 1.6 mm length). A mean of 30.5 ± 9.4 measurements was counted per metachronal wave, with a minimum of 17 measurements per wave.

DISCUSSION

Gape

The general patterns of *Margaritifera margaritifera* valve movements recorded at 2 Hz (e.g. Fig. 5) were the same as those for non-endangered *Mytlius* spp. (e.g. Figs. 2–4) and as previously described by Robson et al. (2007). Both the present study and the pioneering work by Trueman (1966) and Hoggarth & Trueman (1967) recorded *M. margaritifera* valve movements, although we have found no published material on the subject in the interim. We believe that bivalve valve adduction and subsequent abduction events constitute a normal part of bivalve behaviour of both endangered and non-endangered bivalves, occurring in the wild subtidal (e.g. Wilson et al. 2005) and intertidal (Fig. 4), simulated intertidal (Shick et al. 1986) and in laboratory aquariums (e.g. Figs. 2, 3, 5 & 6; Trueman 1966, Hoggarth & Trueman 1967, Robson et al. 2007).

Adult *Cerastoderma edule* are similar in size to the critically endangered little winged pearly mussel *Pegias fabula*, which rarely exceed 35 mm in length (Bogan 2002); therefore, gape data from *C. edule* (Fig. 6) may be a good proxy for small endangered bivalves. *C. edule* data (Fig. 6) also highlight that there can be greater variability in valve movements of smaller bivalves than in larger bivalves such as *Margaritifera margaritifera* (Fig. 5), indicating that recording gape of small endangered bivalves at higher frequency (i.e. >2 Hz, see 'Discussion — Sampling frequency and resolution of bivalve behaviour') may be appropriate (cf. Peters 1983).

Adult *Pecten maximus* are similar in size (15 cm maximum shell diameter) to another marine Pectinid, the IUCN Red Listed *Nodipecten magnificus*, which commonly approaches 20 cm in shell diameter (Waller 2007). *P. maximus* gape data highlight the rapid speed at which this scallop, and probably *N. magnificus*,

can adduct. The ratios of adductor muscle(s) volume/weight to shell volume/weight in *P. maximus* will undoubtedly be lower than in *Margaritifera margaritifera*—although due to their endangered status *M.margaritifera* could not be sacrificed to quantify the ratios—and may account for the rapid speed of valve adduction in *P. maximus* compared to *M. margaritifera* (see 'Discussion—Sampling frequency and resolution of bivalve behaviour').

Pumping

Although an accurate quantified measure of exhalant mussel pumping was not possible in the present study (see 'Materials and methods—Bivalve pumping') (cf. Ait Fdil et al. 2006), our results suggest that pumping should be measured over fine temporal scales because we found mussel pumping (and gape) to be often highly variable, even over periods as short as 1 min (cf. Robson et al. 2007). When measuring *Margaritifera margaritifera* exhalant pumping, especially in relation to gape angle, it may be beneficial to test whether an exhalant current exits from the top of the inhalant siphon as well as the exhalant siphon. *Mytilus edulis* has a mucociliary rejection pathway that functions via the inhalant siphon with pseudofaeces eliminated along the ventral side of the septum dividing the inhalant siphon from the exhalant siphon (Widdows et al. 1979, Beninger & St-Jean 1997, Beninger et al. 1999). Along with our own observations of *M. edulis* pseudofaeces strings being eliminated in an exhalant water current out of the top of the inhalant siphon (sometimes when the exhalant siphon was closed), we found it was appropriate to measure exhalant *M. edulis* pumping out of both the top of the inhalant siphon and the entire exhalant siphon.

Biological noise

Further research is necessary to determine the cause of the biological noise in the form of a metachronal wave of varying amplitude in *Mytilus edulis* exhalant pumping recorded at 30 Hz (e.g. Fig. 9). Wilson et al. (2005) reported biological noise in the gape data of bivalves (also present in our gape data) that was consistently higher in sand mussels *Astarte borealis* than *M. edulis* and suggested that some of it might be due to heart beat (cf. Curtis et al. 2000). While there is little known about the metachronal wave in mussel pumping, it may be an important parameter to measure in bivalves since the frequency of metachronal waves in pumping may vary according to biotic and abiotic factors (e.g. temperature).

Sampling frequency and resolution of bivalve behaviour

The present study reveals the degree to which intervals between sampling affect our ability to identify bivalve gape adduction and abduction events, the degree of variability in bivalve pumping and, ultimately, how this affects the descriptive statistics of gape and pumping behaviour. One effect of increasing the sampling interval was to concatenate adjacent gape adduction and abduction events in the data record (Figs. 2, 4–7), which resulted in an increased mean duration between gape adduction and abduction events and increased minimum gape angles (Table 1); this is an analogous process to the effect of increasing sampling interval on the diving behaviour of seals (Boyd 1993).

Another effect of increasing the sampling interval was the substantial change to the shape of bivalve gape adduction and abduction events (Figs. 2, 4–6) and pumping profiles (Fig. 8). Increasing the sampling interval from 0.5 to 60 s had relatively little effect on the mean gape of bivalves (Table 1). However, it was apparent that increasing the sampling interval from 0.5 to 5 s caused a reduction in maximum gape and thus a loss of definition in short-term changes in bivalve gape (Table 1).

It is essential to select the correct temporal resolution defined by sampling interval in order to detect and define fine-scale behaviour patterns. If the shape of an event is described via changing values in the measured parameter, then the recording frequency should be on the order of 10 measurements per event (Ropert-Coudert & Wilson 2004). Given this, our data analysis indicates that gape should be recorded at a minimum of 2, 7 and 40 Hz in *Margaritifera margaritifera*, *Cerastoderma edule* and *Pecten maximus*, respectively, and at 18 Hz to describe the metachronal wave in exhalant pumping of *Mytilus edulis*. Where the peak values in the measured event are important, such as peaks in bivalve pumping amplitudes (Fig. 9) and the exact start and fastest part of valve adduction events, 10 measurements per event may not adequately describe these extremes. We note that some *P. maximus* valve adductions could not be defined (10 measurements per event) with any of the loggers used in the present study or daily diary loggers (Wilson et al. 2008). From our experience measuring bivalve pumping, we speculate that an initial sampling frequency of 30 Hz would be required to determine the appropriate sampling frequency to measure fine-scale bivalve siphon movements (changes in aperture) of *Margaritifera margaritifera*.

An inherent problem in dealing with bivalve data measured at high sampling frequency (e.g. 2 to 30 Hz)

over days, weeks and months is data processing time. A computer with 8 GB RAM and a 3.4 GHz Pentium 4 processor takes ~40 min to convert 7 million gape data points (~64.8 h and ~40.5 d of data from an archival tag channel recording at 30 and 2 Hz, respectively) from only one bivalve in mV to degrees (°), using an exponential equation in the form $y = a + b \exp(-x/c)$ in Origin® version 7.5 (OriginLab). A way around this is to thin data so that curve-fits can be applied to much fewer data points. However, too few data points in the time series leads to poor resolution of behaviour which can lead to misinterpretation.

Temporal resolution

In the present study, with a 1 GB flash memory card and the system set to record at 30 Hz on 2 channels, recording bivalve gape and pumping simultaneously, the archival tag could record for ca. 70 d before the memory was full. Using 128 GB compact flash memory cards (Samsung) the recording times of the archival loggers could be multiplied by 128. A computer-programmed interface could stop the logger just before the memory card was full, the full memory card replaced and logger restarted within 10 min. Thus, it is possible to record high temporal resolution data almost continuously.

CONCLUSIONS

The potential loss of information associated with the choice of particular sampling intervals during measurements of single parameters, and the biases which can result from this choice, are effectively germane to all species (cf. Boyd 1993). The analyses presented here demonstrate that careful consideration has to be given to the selection of intervals between sampling when using a non-continuous method of recording behaviour. We believe that, where possible, all behavioural events should be recorded because they are likely to vary according to biotic or abiotic factors (e.g. Wilson et al. 2005, Robson et al. 2007). Given that the minimum appropriate sampling frequency has now been established for recording fine-scale *Margaritifera margaritifera* gape and, most probably, pumping behaviour, our ongoing research can test if the breakthrough in the ability to culture *M. margaritifera* (Preston et al. 2007) can be further improved by conditioning broodstock and providing juveniles with additional food. Advances in the understanding of bivalve feeding and reproductive strategies may be gleaned by recording behaviour with high temporal and sensor resolution over a range of ecological circumstances (according to factors such as depth, light, temperature, particulate matter, food availability and predator interactions) and may aid long-term survival of endangered bivalves including freshwater pearl mussels.

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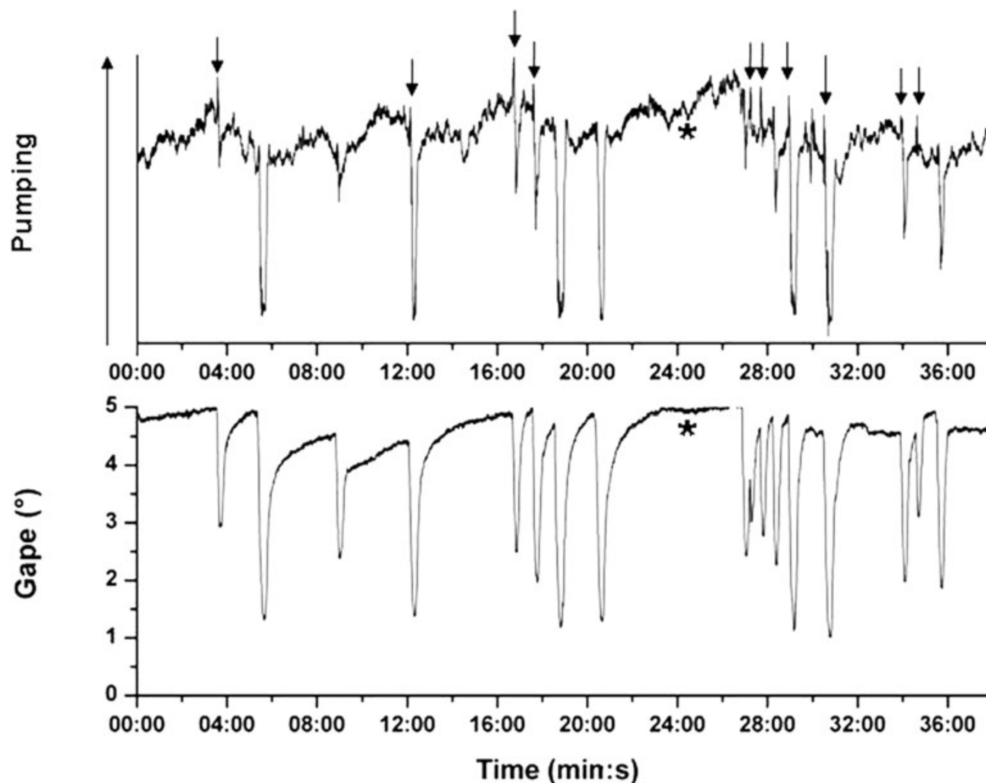
Appendix 1. *Mytilus edulis*. Example of gape angle and pumping rate of a 75 mm long mussel. Data recorded at 2 Hz. Arrows: spikes in pumping data caused by the force of rapid valve adductions; *elimination of a faeces string from the exhalant siphon over ~30 s



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Contribution to the Theme Section 'Directions in bivalve feeding'

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 µg 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 ml^{-1} (ca. 6 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 µg 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 ≥5 to 6 µm, 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 µg 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 µg g^{-1}. ND: not detected

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

In the present study, we used various *Pseudo-nitzschia multiseries* 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. multiseries* capture by feeding incapacitation due to initial toxin accumulation; (2) reduced CR when fed *P. multiseries* cells; and (3) active rejection of *P. multiseries* 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 multiseries* 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. multiseries* 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. multiseries* 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^{-2} s^{-1} 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. multiseries* 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^3) were determined with the particle counter. Cell length (L) and width (W) of all *Pseudo-nitzschia multiseries* 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. multiseries* 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^3) = $(0.6 \times L \times W^2) + (0.2 \times L \times W^2)$, where L and W are in micrometers.

Concentration of DA was measured in the particulate and dissolved fractions of *Pseudo-nitzschia multiseries* 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. multiseries* 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^{-1}, 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^{-1}) 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 resuspension 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 multiseries* (*Ps-m*) filtration, and hypotheses tested in each experiment. $CR_{Ps\text{-}m}$: 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 *Ps-m* cells	All experiments	H_0 (no closure): $CR_{Ps\text{-}m} \neq 0$ H_1 (shell closure): $CR_{Ps\text{-}m} = 0$
2. Reduced filtration when fed *Ps-m* cells	1	H_0 (no CR reduction): $CR_{T\text{-}Iso} = CR_{Ps\text{-}m}$ H_2 (reduced CR): $CR_{T\text{-}Iso} > CR_{Ps\text{-}m}$
2.1. Reduced filtration on toxic *Ps-m* cells	1	H_0 (no CR reduction): $CR_{non\text{-}toxic\ Ps\text{-}m} = CR_{toxic\ Ps\text{-}m}$ $H_{2.1}$ (reduced CR): $CR_{non\text{-}toxic\ Ps\text{-}m} > CR_{toxic\ Ps\text{-}m}$
2.2. Reduced filtration after prolonged exposure to *Ps-m* 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 *Ps-m* cells	1	H_0 (no CR reduction): $CR_{small\ Ps\text{-}m} = CR_{large\ Ps\text{-}m}$ $H_{2.3}$ (reduced CR): $CR_{small\ Ps\text{-}m} > CR_{large\ Ps\text{-}m}$
2.4. Reduced filtration on *Ps-m* cells at stationary phase	1	H_0 (no CR reduction): $CR_{exponential\ Ps\text{-}m}, = CR_{stationary\ Ps\text{-}m}$ $H_{2.4}$ (reduced CR): $CR_{exponential\ Ps\text{-}m} > CR_{stationary\ Ps\text{-}m}$
2.5. Reduced filtration on unialgal *Ps-m* diets	2	H_0 (no CR reduction): $CR_{mixed\ diet} = CR_{unialgal\ Ps\text{-}m}$ $H_{2.5}$ (reduced CR): $CR_{mixed\ diet} > CR_{unialgal\ Ps\text{-}m}$
2.6. Reduced filtration with increasing cell density of *Ps-m*	3	H_0 (no CR reduction): $CR_{100} = CR_{500} = CR_{1500} = \ldots = CR_{16,000}$ $H_{2.6}$ (reduced CR): $CR_{100} > CR_{500} > CR_{1500} > \ldots > CR_{16,000}$

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:

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

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

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

where C_i and C_f are initial and final particle concentrations (cells ml^{-1}) in the experimental chambers, respectively; Cc_i and Cc_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):

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

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 multiseries* clones in unialgal diets: the non-toxic CLNN-13 (mean cell length ± SE: 88 ± 3 µm) and the toxic CLNN-16 clone (92 ± 2 µm, 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. multiseries*. 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. multiseries* cells. The hypothesis of a reduced CR by contact with *P. multiseries* cells (H_2; Table 2) was tested by comparing the CR of oysters during the exposure to both toxic and non-toxic *P. multiseries* clones to the corresponding CR

on *I. galbana* (i.e. before and after exposure to *P. multiseries*, α = 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 (α = 0.05) than that on Clone CLNN-16. The effect of prolonged contact with *P. multiseries* 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 multiseries* 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 µm) or large (81 to 92 µm) *P. multiseries* clones in multiple trials, and growth phase ($H_{2.4}$) in a single trial using the clone CLN-20 (mean cell length ± SE: 25 ± 0.2 µm) harvested at exponential (11 d in batch culture) or stationary (41 d) phase (Student's *t*-test; α = 0.05).

Effects of diet composition on CR (unialgal vs. mixed suspensions). Two toxic *Pseudo-nitzschia multiseries* clones of contrasting cell length, CLN-20 (31 to 36 µm) and CLN-46 (82 µm), 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 ml^{-1} (0.9 and 3.6 mg DW l^{-1}) and Clone CLN-46 at 2000 cells ml^{-1} (2.9 mg l^{-1}). Mixed suspensions were prepared by replacing ca. half of the *P. multiseries* 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. multiseries* suspension and compared, using Student's *t*-test (α = 0.05), to the combined CR on both components of the mixed suspension. The hypothesis of a reduced CR on unialgal *P. multiseries* 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 (α = 0.05).

Effects of *Pseudo-nitzschia multiseries* cell density on CR. CR and FR of oysters fed a unialgal suspension of the toxic clone CLN-20 (26 to 36 µm) were calculated in multiple trials at various cell densities: 100, 500, 1500, 3000, 6500, 10 000 and 16 000 cells ml^{-1} (0.1 to 9.5 mg DW 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 ml^{-1} (4.1 to 12.0 mg DW l^{-1}). IR was then calculated from Eq. (3). Because the size of *Pseudo-nitzschia multiseries* 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. multiseries* avoidance by either shell closure, as inferred by a negligible CR at all cell densities (H_1), or reduced CR with increasing *P. multiseries* concentration ($H_{2.6}$), were tested by 1-way ANOVA (α = 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 multiseries* clones, CLN-20, CLN-46, CLN-50, CLNN-13 and CLNN-16, ranging in cell length from 24 to 100 µm, 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 = 8.5×10^{-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 multiseries* 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. multiseries* 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 multiseries* 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. multiseries* cells ($H_{2.3}$ and $H_{2.4}$).

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

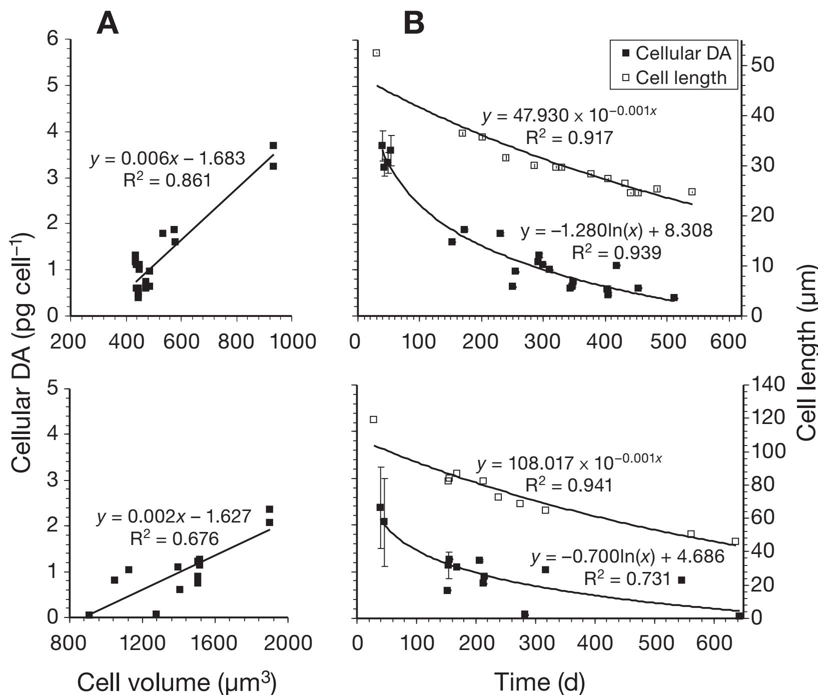

Fig. 1. *Pseudo-nitzschia multiseries*. Relationship between (A) cellular domoic acid (DA) concentration and mean cell volume, and (B) cellular toxicity and cell length (mean ± SE) over the time that 2 *P. multiseries* clones (CLN-20: upper panels and CLN-46: lower panels) were maintained in successive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

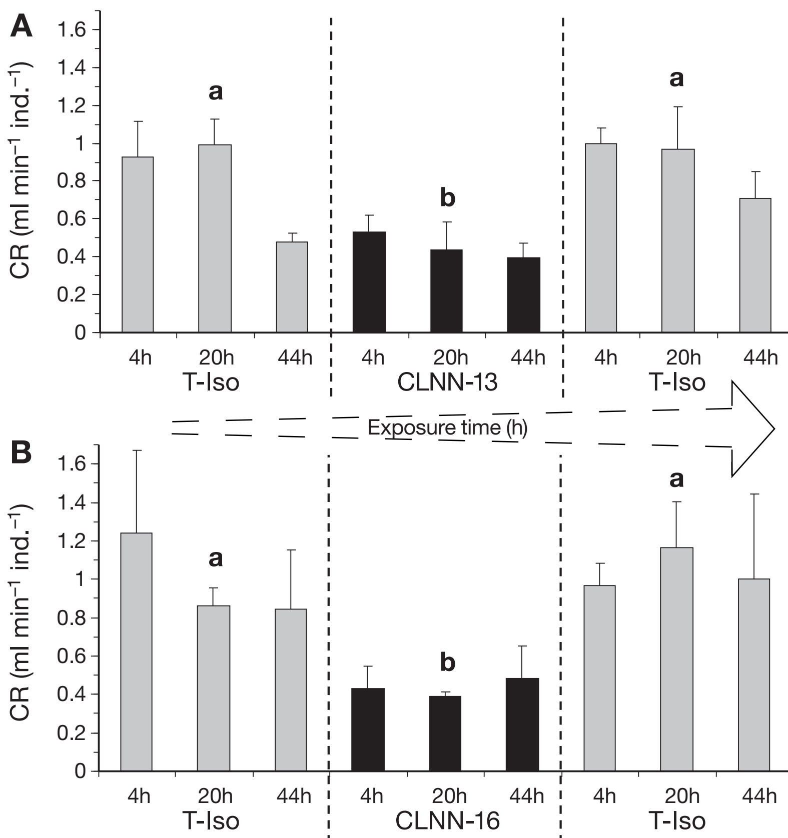

Fig. 2. *Crassostrea virginica* juveniles (mean shell height ± SE: 18.6 ± 0.6 mm). Clearance rate (CR) of oysters fed *Isochrysis galbana* (clone T-Iso) at 75 000 cells ml^{-1} (ca. 3.2 mg DW l^{-1}) for 44 h, then switched to an equivalent cellular volume of either (A) non-toxic CLNN-13 (88 µm cell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

or toxic *Pseudo-nitzschia multiseries* cells did not occur in the presence of a mixed phytoplankton assemblage, i.e. when ca. half of the toxic *P. multiseries* 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. multiseries* cell length (31 to 82 µm) and cell density (1000 to 6000 cells ml^{-1}, or 0.9 to 3.6 mg DW l^{-1}). 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. multiseries* 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 multiseries* in unialgal suspensions. Oysters exhibited a relatively constant CR (0.48 to 0.58 ml min^{-1} ind.$^{-1}$) with increasing *P. multiseries* concentration over a wide cell density range (100 to 16 000 cells ml^{-1}, or 0.1 to 9.5 mg DW l^{-1}; Fig. 4). Therefore, FR (CR × cell density) increased monotonically with increasing cell density. However, at densities >4200 cells ml^{-1} (ca. 2.5 mg DW l^{-1}), oysters started to reject an increasing number of *P. multi-*

Table 3. *Crassostrea virginica* juveniles (mean shell height ± SE: 18.6 ± 0.6 mm). Clearance rate (CR) of oysters fed *Pseudo-nitzschia multiseries* clones. The effect of *P. multiseries* cell length (µm) on CR was tested in multiple trials using all *P. multiseries* 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 µm). CR values (ml min^{-1} ind.$^{-1}$; mean ± SE) were weight-standardized to an average oyster of 0.02 g soft tissue dry weight

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

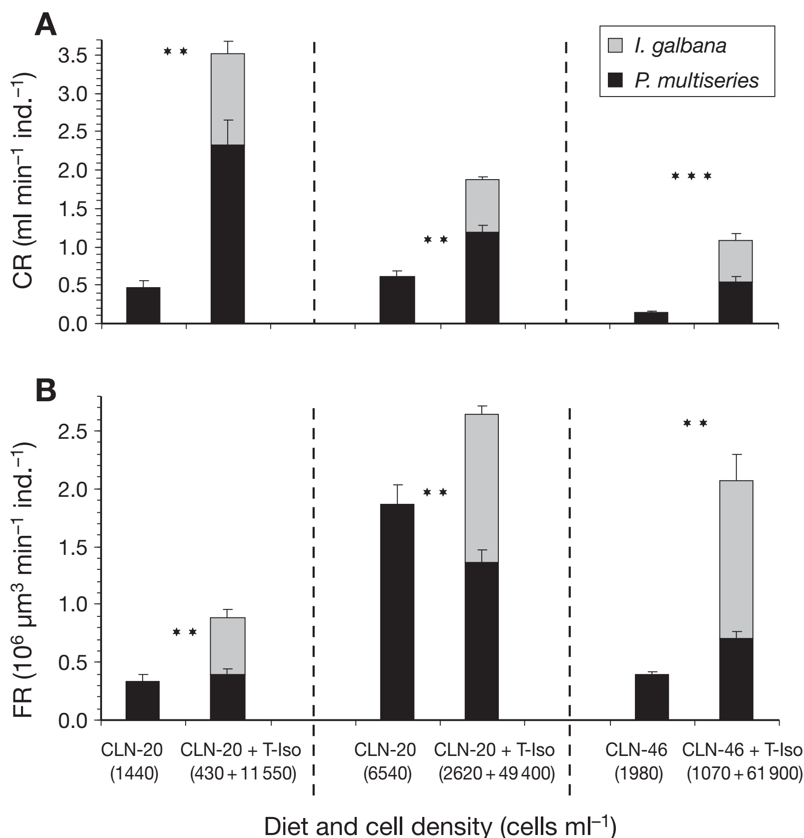

Fig. 3. *Crassostrea virginica* juveniles (mean shell height ± SE: 18.6 ± 0.6 mm). (A) Clearance rate (CR) and (B) filtration rate (FR) (mean ± SE; n = 4 to 5 chambers, 8 oysters per chamber) of oysters fed toxic *Pseudo-nitzschia multiseries* clones (CLN-20 and CLN-46) in either unialgal or mixed suspensions, with an equivalent cell volume of *Isochrysis galbana* (clone T-Iso). Total concentrations of the 3 mixed suspensions corresponded to those of unialgal diets: ~0.7, 4.2 and 3.7 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 g soft tissue DW. Cell length was 31 to 36 µm for CLN-20 and 82 µm for CLN-46

series cells in pseudofeces, and a maximum ingestive capacity was reached at 10 400 cells ml^{-1} (ca. 6 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. multiseries* 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 multiseries* 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 µm 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 × 5.3 µm (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. multiseries* cultures (e.g. Davidovich & Bates 1998), and is also reported in natural populations, where cells as short as 35 µm can be found (Bates et al. 1999). The wide range of cell size and toxicity found during *P. multiseries* blooms may therefore partly explain why the maximum DA level reported to date in field studies (7.2 pg cell^{-1}; Smith et al. 1990) is much lower than the 67 pg DA cell^{-1} achieved by large, young *P. multiseries* 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 pg cell^{-1}) and wide range in cell size (24 to 100 µm) 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. multiseries* strain, the clone CLNN-13.

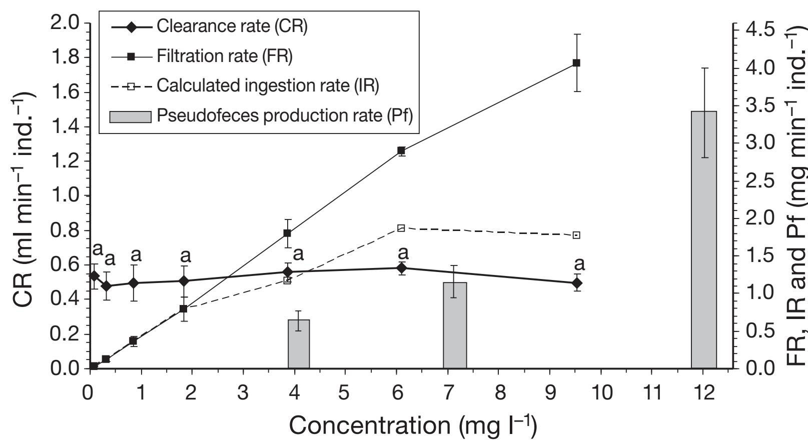

Fig. 4. *Crassostrea virginica* juveniles (mean shell height ± SE: 18.6 ± 0.6 mm). Clearance rate (CR), filtration rate (FR), calculated ingestion rate (IR) and pseudofeces production rate (Pf) of oysters with increasing concentration of *Pseudo-nitzschia multiseries* Clone CLN-20 (cell length = 26 to 36 µm). All rates (mean ± SE; n = 5 chambers, 8 oysters per chamber) were weight-standardized to an average oyster of 0.02 g soft tissue DW. IR = FR – Pf; same letters indicate no statistical difference (α = 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 multiseries*. 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 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 ± SD: 13 ± 1.1 cm), including shell closure and subsequent haemolymph acidosis and hypoxia after 4 h of exposure to toxic *P. multiseries* (1.6 pg DA cell^{-1}). However, Jones et al. (1995) used an extremely high *P. multiseries* cell density (330 000 cells ml^{-1}), contrasting with those found during natural blooms (100 to 15 000 cells ml^{-1}; Bates et al. 1998) and those used in our investigation (100 to 25 000 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 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 multiseries* 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. multiseries* 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. multiseries* 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 multiseries* either, but rather to other cell characteristics. This lower CR on *P. multiseries* 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. multiseries* clone CLN-50 (70 fg C µm^{-3} and 13 fg N µm^{-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. multiseries* 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 multiseries* 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 *Olisthodiscus luteus* and *Dunaliella tertiolecta*, this potential feeding deterrence caused by *P. multiseries* may be concentration dependent, since the CR of oysters feeding on *P. multiseries* unialgal suspensions was much lower than that on mixed suspensions with *I. galbana*, when only half of the *P. multiseries* cell density was offered. As expected, there were no differences in the CR of small and large *P. multiseries* cells (Table 3), given that all cellular dimensions (i.e. length, height and width) of the clones were ≥5 to 6 µm, 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 multiseries* cell densities, equivalent to seston concentrations of ca. 0.1 to 9.7 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 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 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. multiseries* 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. multiseries* and *Isochrysis galbana* at total concentrations of 3.7 and 4.2 mg DW l^{-1} were, respectively, 1.8 and 3.1 times lower than those exposed to the same suspension at 0.7 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 *Isocrhysis galbana* in our study were equivalent to 0.4 to 1.3 l h^{-1} g^{-1} soft tissues DW, which approximates the 1.4 ± 1.2 (SD) l h^{-1} g^{-1} measured by Palmer (1980), but is well below the 6.8 l h^{-1} 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 (~75 000 *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 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°C), which is much lower than those used in previous studies (e.g. 21°C; Palmer 1980; 27 to 29°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 wk at a

constant temperature) from <0.3 l h^{-1} g^{-1} at temperatures <9°C to between ca. 1.1 and 3.6 l h^{-1} g^{-1} at 20°C when filtering *I. galbana*. This was further confirmed with oysters exposed to a constant concentration of *P. multiseries* at temperatures ranging from 1 to 18°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 multiseries* cells in pseudofeces, ingestion rate (IR) reached an asymptote at ~6 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 ~2.5 mg l^{-1}, with pseudofeces production becoming increasingly important as a mechanism to reduce the ingestion of *P. multiseries* 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 ≥6 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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Contribution to the Theme Section 'Directions in bivalve feeding'

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

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ABSTRACT: Oysters accumulate relatively low levels of domoic acid (DA) compared to other bivalves. Mafra et al. (2009, in this Theme Section) identified feeding mechanisms of oysters that may lead to low DA accumulation during monospecific blooms of *Pseudo-nitzschia multiseries*. However, several different species of *Pseudo-nitzschia*, as well as other diatoms and flagellates, may co-occur during a bloom. Therefore, the present study investigates pre-ingestive feeding processes that operate when oysters *Crassostrea virginica* are exposed to mixed phytoplankton assemblages containing *P. multiseries* of varying cell length. Guided by video-endoscopy, material transported along the ventral and dorsal gill tracts was sampled and analyzed to determine the site for sorting of microalgae on the pallial organs. There was no preferential rejection of *P. multiseries* in pseudofeces when oysters were exposed to the alga in a mixed suspension with other diatom species (*Thalassiosira weissflogii* or *Chaetoceros muelleri*). In contrast, *P. multiseries* was preferentially rejected when mixed with the flagellates *Isochrysis galbana* or *Rhodomonas lens*, suggesting a qualitative mechanism for particle sorting. This occurred on the gills, followed by further selection on the palps. Oysters also preferentially rejected larger *P. multiseries* cells (82 to 90 µm) relative to smaller ones (24 to 28 µm) on the gills, while no further selection based on size occurred on the palps. This effect is attributed to the fact that *P. multiseries* cells with a length that exceeds the width of the principal filament aperture (ca. 68 µm) are more likely directed to the ventral tract and rejected in pseudofeces. These findings offer an additional explanation for the relatively low DA levels found in oysters during natural *Pseudo-nitzschia* spp. blooms.

KEY WORDS: Oyster · *Crassostrea virginica* · Particle selection · Pseudofeces · Selective ingestion · *Pseudo-nitzschia multiseries* · Domoic acid

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INTRODUCTION

Domoic acid (DA), a competitor for glutamic acid receptors in neuronal cells, has been linked to amnesic shellfish poisoning in humans (Wright et al. 1989) and marine animal intoxication outbreaks (e.g. Scholin et al. 2000) in several regions worldwide (reviewed in Hallegraeff 2003). This toxin, initially found in macroalgae (Daigo 1959), is also produced by diatoms, including several species of the genus *Pseudo-nitzschia* (reviewed by Trainer et al. 2008). Although suspension-feeding bivalve molluscs are the main vector for DA transfer to humans, toxin levels in oysters are often very low, even when DA concentrations exceeding the 20 µg g^{-1} regulatory level are present in other co-occurring bivalve species (reviewed by Mafra et al. 2009, this Theme Section).

Since DA uptake by bivalves depends on their capture and ingestion of toxic cells, regulation of the suspension-feeding process can noticeably increase or reduce the amount of toxin available for tissue incorporation. In previous laboratory experiments (Mafra et al. 2009), oysters exhibited a relatively low clearance rate (CR) when exposed to unialgal suspensions of

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either toxic or non-toxic *Pseudo-nitzschia multiseries* cells, but CR was restored when *P. multiseries* was combined with an alternative, nutritious food source. As other non-toxic phytoplankton species can be present in the water column during *Pseudo-nitzschia* spp. blooms, sometimes representing an equivalent or higher algal biomass (e.g. Fehling et al. 2006, Mafra et al. 2006, Spatharis et al. 2007), the low levels of DA commonly found in oysters could also be the result of an effective mechanism of particle selection. Pre-ingestive selection may occur on the gills and/or labial palps of bivalves (Ward et al. 1994, 1998a) and be affected by seston concentration (Barillé et al. 1993), particle morphology and size (Ward et al. 1998b), particle quality (i.e. organic vs. inorganic; Newell & Jordan 1983, Bayne et al. 1993, Cognie et al. 2003, Beninger et al. 2004) and other characteristics related to the organic content and/or coating of the cells, such as nutritional value (Ward et al. 1997), stickiness (Waite et al. 1995), surface charge (Hernroth et al. 2000) and secreted ectocrines (Ward & Targett 1989).

On the labial palps of bivalves, nutritious organic particles are typically transported to the mouth for ingestion, while undesirable components are rejected as pseudofeces, which provides a mechanism for enrichment of the food supply (Ward & Shumway 2004). In oysters, which are characterized by complex, heterorhabdic (i.e. differentiation of ordinary and principal filaments), pseudo-lamellibranch gills capable of bidirectional particle transport, the particle-sorting process starts earlier on the gills. As a result, oysters can selectively and efficiently ingest a given algal species from a mixed suspension while rejecting others as pseudofeces. Some studies have reported that diatom species are preferentially eliminated as pseudofeces from mixed suspensions with flagellates (e.g. Bougrier et al. 1997). However, diatoms may be an important food item for oysters during seasonal diatom blooms when other microalgae are scarce or a more refractory particle type is present (Decottignies et al. 2007).

The sorting capacity of the gills of oysters is also influenced by particle size and morphology. Using qualitative endoscopic observations, Cognie et al. (2003) showed that diatoms with all dimensional axes ≥70 µm cannot enter the principal filaments and thus cannot be directed efficiently to the dorsal ciliated tracts (dct) of the gills. Instead, such particles are obligatorily transported on the relatively exposed ventral ciliated grooves (vcg), from where a fraction may be lost in pseudofeces. In this case, particle selection can only be performed by the labial palps. Because *Pseudo-nitzschia* spp. cells are long, but narrow (ca. 5 µm wide), some cells can enter the principal filaments if they reach the gills in a dorsoventral orientation. Therefore, *Pseudo-nitzschia* spp. cells can be transported to both the ventral and dorsal tracts of the gills, as demonstrated by preliminary, qualitative endoscopic observations of the oyster *Crassostrea gigas* exposed to 80 µm long *Pseudo-nitzschia* sp. cells (J. E. Ward et al. unpubl. data). Additionally, the cell size of diatoms is highly variable, as it decreases over time due to successive vegetative divisions (Round et al. 1990), and *Pseudo-nitzschia* spp. of varying cell size, including *P. multiseries* cells as short as 35 µm, can be found during toxic blooms (Bates et al. 1999, Trainer et al. 2007). Thus, we hypothesize that, in oysters, the selective capacity of both the gills and labial palps limits the ingestion of *P. multiseries* cells, and that rejection is enhanced when oysters are exposed to long *P. multiseries* cells (up to 169 µm; Villac 1996).

In this study, a combination of feeding experiments and endoscope-directed *in vivo* sampling was used to investigate the magnitude and site of pre-ingestive selection on the pallial organs of the eastern oysters *Crassostrea virginica* that could lead to preferential rejection of toxic *Pseudo-nitzschia* spp. cells from mixed suspensions. Such a mechanism, combined with reduced CR of *C. virginica* on *Pseudo-nitzschia* spp. cells in a unialgal suspension (Mafra et al. 2009) could partially explain the low levels of DA accumulation in this and other oyster species. Feeding selectivity was examined via qualitative and quantitative analysis of pseudofeces produced from mixed suspensions of a *P. multiseries* clone with flagellates and other diatom species, and by determining the contribution of the gills and labial palps to this process. In addition, mixed suspensions containing *P. multiseries* clones of contrasting size were used to assess the effect of cell size on pre-ingestive selectivity. Post-ingestive mechanisms that could contribute to low accumulation of DA in oysters are the subject of ongoing research.

MATERIALS AND METHODS

Algal culture. Five toxic *Pseudo-nitzschia multiseries* clones, CLN-20, CLN-30, CLN-46, CLN-50 and CLNN-21, were kindly provided by S. Bates (Department of Fisheries and Oceans, Moncton, Canada) and grown in batch culture. All clones were cultivated in 1.5 l glass Fernbach flasks with autoclaved, 0.22 µm cartridge filtered seawater enriched with *f*/2 medium (Guillard 1975) at 16°C, 30 ppt salinity, a light intensity of 140 µmol quanta m^{-2} s^{-1}, and a 14 h light:10 h dark photoperiod. Five non-toxic algae from the Center for Culture of Marine Phytoplankton (CCMP, West Boothbay Harbor, ME) — the flagellates *Rhodomonas lens* (CCMP Strain 739), *Isochrysis galbana* (T-Iso clone, CCMP1324) and *Pavlova pinguis* (CCMP609), and the diatoms *Thalassiosira weissflogii* (Actin clone, CCMP

1336) and *Chaetoceros muelleri* (CCMP1316) — were also batch-cultured in *f*/2 medium, without added silicate for the flagellates. They were kept under the same culture conditions as *P. multiseries*, except for *I. galbana* and *P. pinguis*, which were cultured in either 20 l, aerated, plastic carboys or in a semi-continuous system (200 l photobioreactors) at 20°C. Cell density of *I. galbana*, *P. pinguis* and *C. muelleri* was determined with a Multisizer 3 particle counter (Beckman-Coulter). Cell density of *R. lens*, *T. weissflogii* and *P. multiseries* clones was measured with a microscope (Leica Model DMLB 100S). Dilutions for microscopy were made to obtain ca. 400 cells per Palmer-Maloney counting chamber.

Cell size (equivalent spherical diameter, ESD) and volume (μm^3) of *Rhodomonas lens*, *Isochrysis galbana*, *Pavlova pinguis* and *Chaetoceros muelleri* were obtained using the particle counter. For both *Thalassiosira weissflogii* and *Pseudo-nitzschia multiseries* clones, cell length and width (n = 30) were measured prior to each experiment using a microscope with a coupled Pulnix camera (Model TMC-7DSP) and image analysis software (Image Pro Plus Version 4.5, Media Cybernetics). Only cell length of *P. multiseries* clones is reported in Table 1, as width was similar among clones and over time (4.3 to 5.2 µm). Cellular volume of *T. weissflogii* was calculated by assuming a cylindrical shape, and that of *P. multiseries* using the formula described by Hillebrand et al. (1999) and modified by Lundholm et al. (2004) for *Pseudo-nitzschia* spp.: cell volume (μm^3) = $(0.6 \times L \times W^2) + (0.2 \times L \times W^2)$, where L and W are maximum length and width in micrometers, respectively.

DA concentration in *Pseudo-nitzschia multiseries* cultures was determined in triplicate by gently filtering 15 ml aliquots through Whatman GF/F glass microfiber filters (25 mm diameter, 0.7 µm minimum particle retention) followed by analysis of the fluorenylmethoxycarbonyl (FMOC) derivative in cellular and dissolved fractions by high performance liquid chromatography (HPLC) following the methods of Pocklington et al. (1990). All *P. multiseries* clones were harvested at the stationary phase of growth, when cultures exhibited the maximum intra-cellular toxicity throughout the growth cycle. Only cellular toxicities are reported in Table 1, as particulate DA is known to be the primary route for toxin accumulation (Novaczek et al. 1991). The clones were predominantly single-celled in the stationary phase, with <5% of the cells forming short chains of ≤4 cells, such that chain-formation was not a confounding variable in these experiments. Processing of *P. multiseries* chains by *Crassostrea virginica* is the subject of future investigation.

Selective feeding experiments. Experiments were conducted at the Marine Research Station, Institute for Marine Biosciences (MRS/IMB), National Research Council of Canada (NRC), Halifax, Nova Scotia. Juvenile eastern oysters *Crassostrea virginica* (first year cohort, mean shell height [SH] ± standard error [SE]: 21.4 ± 0.3 mm), were acquired from growers in Prince Edward Island, Canada, in October 2005 and kept in 1000 l insulated tanks containing active upwellers at ca. 1500 oysters per tank, 12°C and 30 ppt salinity. Shell height represented the maximum dimension from the umbo to the ventral margin of the shell. Oysters were fed *Pavlova pinguis* and *Isochrysis galbana*

Table 1. Characteristics of diets exposed to the eastern oyster *Crassostrea virginica* in endoscopic observations and feeding rate experiments. Diet composition and cell density, toxicity, cell size and total biomass concentration are presented. CLN and CLNN: *Pseudo-nitzschia multiseries* (*Ps-m*) clones; *C. muelleri*: *Chaetoceros muelleri*; *I. galbana*: *Isochrysis galbana*; *R. lens*: *Rhodomonas lens*; nt: non-toxic

Experiment	Diet	Composition (cell density in cells ml^{-1})	Toxicity (pg DA $cell^{-1}$) *Ps-m* /other spp.	Mean cell size (µm)	Total biomass concentration (mg l^{-1})
Feeding Mixed diets	M1	CLN-20 (9100) + *I. galbana* (87 000)	0.7 / nt	28[a] / 4.5[b]	7.4
	M2	CLN-46 (2000) + *I. galbana* (81 000)	0.8 / nt	82[a] / 4.5[b]	6.2
	M3	CLN-50 (1800) + *I. galbana* (70 000)	0.7 / nt	100[a] / 4.5[b]	6.7
	M4	CLN-50 (2400) + *R. lens* (9800)	5.9 / nt	68[a] / 7.6[b]	5.8
	M5	CLN-50 (2800) + *C. muelleri* (34 000)	5.9 / nt	68[a] / 5.4[b]	6.7
	M6	CLN-50 (2200) + *T. weissflogii* (3500)	0.7 / nt	100[a] / 23[a]	8.1
	M7	CLN-46 (4200) + CLN-20 (11 300)	1.2 / 0.6	82[a] / 28[a]	11.1
	M8	CLN-50 (1300) + CLN-20 (7300)	0.5 / 0.4	90[a] / 25[a]	5.7
Video-endoscopy	E1	CLNN-21 (2000) + CLN-30 (7000) + *I. galbana* (40 000)	0.2 / 0.03 / nt	99[a] / 35[a] / 4.5[b]	10.8
	E2	CLN-46 (3700) + *R. lens* (12 000)	0.05 / nt	46[a] / 7.6[b]	6.8
	E3	CLN-46 (1400) + *T. weissflogii* (1500) + *I. galbana* (40 000)	0.07 / nt / nt	69[a] / 23[a] / 4.5[b]	4.6

[a]Cell length; [b]ESD: equivalent spherical diameter

at a total cell density equivalent to 30 000 *I. galbana* cells ml^{-1} and acclimated to the experimental diet ca. 18 h before each trial. After being used in the selectivity experiments, oysters were stored frozen and then oven-dried at 80°C for 24 h to obtain the dry weight (DW) of soft tissue.

Clearance rate (CR, i.e. the volume of water cleared of particles per unit time, in ml min^{-1}) of oysters was measured in five 400 ml acrylic chambers (6 to 8 oysters per chamber), plus 1 chamber without oysters as a control for phytoplankton settlement. Mixing of the suspension was achieved 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 (Table 1) was gravity-fed to the chambers from a common 60 l header tank, and a peristaltic pump re-circulated the pooled outflow water from the chambers back to the header tank. Following a flow-through acclimation period, flow was interrupted and samples were taken from each chamber before and after a period that allowed oysters to deplete 15 to 30 % of the cells in suspension.

Pseudofeces production rate (Pf, i.e. number of cells rejected as pseudofeces per unit time, in cells min^{-1}) was measured in a similar experimental system, as described in Mafra et al. (2009). CR, filtration and ingestion rates (FR and IR, i.e. number of cells filtered and ingested per unit time; in cells min^{-1}) were calculated from the following equations:

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

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

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

where C_i and C_f are initial and final particle concentrations (cells ml^{-1}), Cc_i and Cc_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 means of C_i and C_f were used in the calculation of FR. All measured feeding rates (CR, FR, IR and Pf) were weight-standardized following the general allometric equation for suspension-feeding bivalves as reviewed by Bayne & Newell (1983):

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

where FdR_{std} is the weight-standardized feeding rate, W_{avg} is the soft tissue DW of an average oyster (0.02 g in our experiments), FdR_{exp} and W_{exp} are the experimental (i.e. measured) FR and soft tissue DW (in g), respectively.

To investigate selective ingestion/rejection of *Pseudo-nitzschia multiseries* cells, clones varying in cell size were tested in mixed suspensions with a second species, either a flagellate (Diets M1 to M4) or another diatom species (Diets M5 and M6; Table 1). All cellular dimensions (length, width, height) of the species used were >4.3 µm, which allows a minimum of ca. 80 to 87 % retention by the oyster gill (Riisgård 1988, Ward & Shumway 2004). Prior to every trial, cellular volume was calculated for both algal species and the suspension was prepared by adding equivalent cellular volumes of each alga to the 60 l header tank. Suspensions were monitored over time to ensure an approximate 50:50 volume ratio of both species throughout the trial, and total concentrations were sufficiently high (≥4.2 mg DW l^{-1}) to assure that pseudofeces were produced. Because *P. multiseries* cells are long but narrow (width = 4.3 to 5.2 µm), differential retention by the gills was investigated in mixed suspensions with a flagellate or other diatom of a different cell size. For each mixed suspension, CR measurements were taken from a different group of oysters over an interval that was adjusted to limit cell depletion to only 15 to 30 % (ranging from 14 to 19 min for Diets M3, M4 and M5, to 35 to 70 min for M1, M2 and M6). Reduced retention of *P. multiseries* cells by the gills (H_1; Table 2) is thus verified if the CR for *P. multiseries* is significantly lower than that for the second alga within a mixed suspension (paired t-test; $\alpha = 0.05$). Pseudofeces production was also measured for each component of the binary diets and used to calculate the IR from Eq. (3). Finally, FR (Eq. 2) was expressed in terms of total cell volume and then partitioned into IR and Pf. The proportion (p) of each algal clone rejected in pseudofeces was compared within each diet, and the hypothesis of selective rejection of *P. multiseries* cells was assessed by t-test ($\alpha = 0.05$), following an arcsine transformation.

In addition, cell-volume-based ratios of *Pseudo-nitzschia multiseries* to a second species were calculated for the suspension and pseudofeces. A greater ratio in pseudofeces than that offered in the suspension, as revealed by 1-tailed t-test ($\alpha = 0.05$), confirms preferential rejection of *P. multiseries* cells by oysters, as a result of selective filtration and/or rejection (H_2; Table 2).

Two additional suspensions (Diets M7 and M8; Table 1) were prepared by mixing 2 *Pseudo-nitzschia multiseries* clones with similar toxicity but different cell sizes. They were used to test the effect of *P. multiseries* size alone on selective feeding of oysters. Feeding trials were conducted as before, and ratios of large clone to small clone were calculated in the suspension and pseudofeces. Therefore, if the ratio in pseudofeces differs significantly from that in the offered suspension (2-tailed t-test; $\alpha = 0.05$), cell size can be considered as a factor affecting particle selectivity ($H_{2.1}$; Table 2).

Table 2. *Crassostrea virginica*. Possible pre-ingestive feeding mechanisms resulting in reduced filtration and/or ingestion of *Pseudo-nitzschia multiseries* cells, and hypotheses tested in each experiment. dct: dorsal ciliated tracts; EI: electivity index; $CR_{Ps\text{-}m}$: clearance rate of oysters on *Pseudo-nitzschia multiseries* (*Ps-m*) cells; $CR_{2nd\ sp.}$: clearance rate of oysters on a second species in mixed suspension with *Ps-m*; PF: pseudofeces; Susp: suspension; vcg: ventral ciliated grooves

Feeding process	Avoidance mechanism	Diets tested (experiment)	Underlying hypotheses
Filtration by the gills	1. Reduced retention of narrow *Ps-m* cells on the gills	M1 to M6 (feeding)	H_0 (no reduction): $CR_{Ps\text{-}m} \geq CR_{2nd\ sp.}$ H_1 (reduced retention): $CR_{Ps\text{-}m} < CR_{2nd\ sp.}$
Ingestion	2. Selective rejection of *Ps-m* cells in PF	M1 to M6 (feeding)	H_0 (no selection): ratio $[Ps\text{-}m/2\text{nd sp.}]_{PF}$ = ratio $[Ps\text{-}m/2\text{nd sp.}]_{Susp}$ H_2 (selective rejection): ratio $[Ps\text{-}m/2^{nd}\text{ sp.}]_{PF}$ > ratio $[Ps\text{-}m/2^{nd}\text{ sp.}]_{Susp}$
	2.1 Selective rejection of large cells in PF	M7 and M8 (feeding)	H_0 (no selection): ratio $[\text{large/small cell}]_{Pf}$ = ratio $[\text{large/small cell}]_{Susp}$ $H_{2.1}$ (size selection): ratio $[\text{large/small cell}]_{PF} \neq$ ratio $[\text{large/small cell}]_{Susp}$
	2.2 Particle sorting on the gills	E1 to E3 (endoscopy)	H_0 (no sorting): EI in the dct = EI in the vcg $H_{2.2}$ (sorting on the gills): EI in the dct ≠ EI in the vcg
	2.3 Particle sorting on the labial palps	E1 to E3 (endoscopy)	H_0 (no sorting): EI in pseudofeces = EI in the vcg $H_{2.3}$ (sorting on the palps): EI in pseudofeces ≠ EI in the vcg

Because all *Pseudo-nitzschia multiseries* clones were harvested in the stationary phase and mixed with another species harvested at the exponential phase, an additional experiment was performed to compare the relative rejection of exponential versus stationary *P. multiseries* cells. CLN-20 cells were harvested after 11 and 41 d of culture, representing mid-exponential and mid-stationary phases, respectively. Oysters were fed similar cell densities of unialgal CLN-20 diets at both growth phases. CR was measured (see Mafra et al. 2009), and pseudofeces were collected to quantify Pf. The proportion of cells rejected as pseudofeces was calculated in both treatments, and the hypothesis that *P. multiseries* cells in exponential and stationary phases are differentially rejected was tested by a *t*-test ($\alpha = 0.05$), following arcsine transformation of the proportions.

Video-endoscopic observations. The sites on the pallial feeding organs where selection of *Pseudo-nitzschia multiseries* cells could potentially take place were investigated *in vivo* by means of video-endoscopic techniques. Adult *Crassostrea virginica* (mean SH ± SE: 116 ± 2 mm) were acquired from AquaDelights Seafoods, NS, Canada, and kept in the laboratory under the same conditions as described for juveniles. Two weeks before the experiment, oysters were prepared by carefully trimming the antero-ventral, outer edge of the shell with an electric saw to allow insertion and prevent breakage of the optical insertion tube of the endoscope (Bricelj et al. 1998). Experiments followed methods described in Ward et al. (1991), using an endoscope mounted on a micromanipulator and attached to a colour video camera with an optical-zoom adapter.

Oysters were acclimated to the experimental temperature (18 to 20°C) for 2 wk and then transferred to 1 l containers. They were fed a diet of 40 000 *Isochrysis galbana* cells ml^{-1} for 30 min, a sufficient time to ascertain that they had opened their valves and were feeding normally. After this acclimation period, different mixed diets (Table 1) were offered in a flow-through system with a peristaltic pump at 160 ml min^{-1} for ca. 2 h. The first diet was composed of 2 *Pseudo-nitzschia multiseries* clones differing in cell size (Diet E1), the second of a *P. multiseries* clone mixed with the flagellate *Rhodomonas lens* (Diet E2), and the third diet was composed of a *P. multiseries* clone mixed with an equivalent cell volume of the diatom *Thalassiosira weissflogii* (Diet E3). In order to stimulate oyster feeding, 40 000 *I. galbana* cells ml^{-1} were added to Diets E1 and E3 only, as preliminary observations indicated that overall feeding activity was reduced in the presence of a suspension consisting exclusively of diatoms. Duplicate samples of the processed particles were collected from the dorsal ciliated tracts (dct) and ventral ciliated grooves (vcg) with a micropipette connected to a micro-peristaltic pump at a suction rate of 0.55 ml min^{-1} for ca. 5 min. The material in suspension was sampled every 5 to 10 min to obtain a time-integrated sample. Duplicate samples of pseudofeces, when available, were also recovered after rejection by the labial palps using a pipette. Electivity indices (EI), based on cell volume, were calculated for pseudofeces, dct and vcg samples using the formula of Jacobs (1974), modified by Ward et al. (1998b):

$$\mathrm{EI} = \frac{r - p}{(r + p) - (2rp)} \tag{5}$$

where *r* is the proportion of 'Alga B' in the post-capture samples (dct, vcg, pseudofeces), and *p* is the proportion of the same cells in suspension (food supply). A positive EI indicates enrichment of 'Alga B' in the

sample, whereas a negative EI indicates enrichment of 'Alga A', the first component of the binary mixed diet.

Isochrysis galbana, offered exclusively to stimulate feeding, was not taken into account for EI calculation. Particle selection on the gills ($H_{2.2}$; Table 2) is consequently confirmed if EI in the dct is statistically different from that in the vcg. Similarly, if EI of pseudofeces and vcg samples differ significantly, then further particle selection takes place on the labial palps ($H_{2.3}$; Table 2). EI was expressed as an average of 3 to 8 oysters, and the values were compared by 1-way repeated-measures analysis of variance (ANOVAR; $\alpha = 0.05$).

After the experiment, the gills of 5 oysters were removed and left in filtered seawater at 4°C for 2 h. Gills were then observed under a Wild Heerbrugg stereoscope (Model M5-48357) and 5 to 12 principal filament apertures (pfa) were measured from the 2 central demibranchs of each oyster. The pfa is defined as the space between adjacent plicae, which allows access to the principal filament (see Fig. 5). Measurements were taken at 32× magnification using a coupled camera and image analysis software as described previously for algal measurements. Since the aperture tends to be wider toward the ventral region, each pfa width was expressed as a mean of 7 to 12 measurements taken along the filament. In addition, comparative *in vivo* measurements were taken from the video images recorded during sampling. In this case, the ordinary filament width, a fairly constant dimension throughout the oyster gills, was measured from dissected oysters and used as a reference to calibrate the video images.

RESULTS

Oysters cleared *Pseudo-nitzschia multiseries* cells (i.e. removed from suspension by retaining on the gills) at comparable or higher rates than flagellates and other diatom species offered in mixed, binary suspensions (Fig. 1). The hypothesis that oysters could remove the narrow *P. multiseries* cells from suspension with lower efficiency than other species mixed in a binary diet (H_1; Table 2) was thus rejected. Surprisingly, when

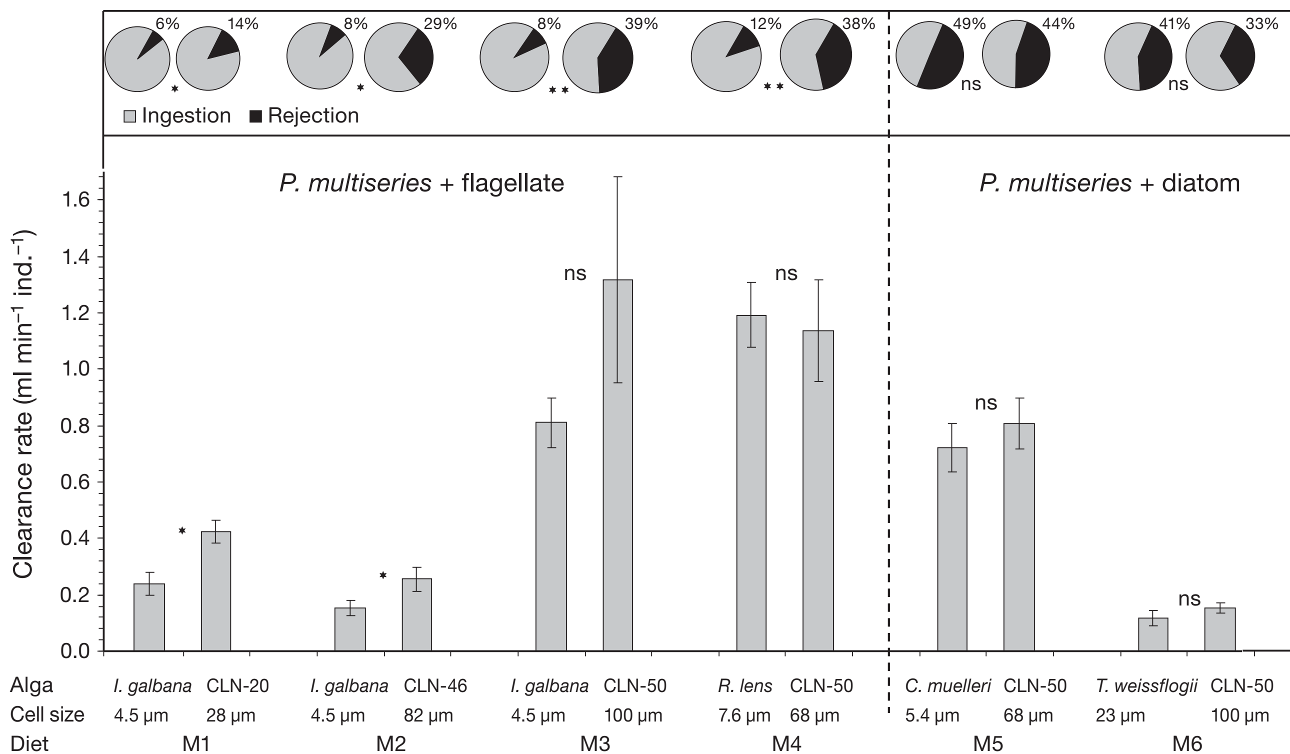

Fig. 1. *Crassostrea virginica* juveniles (mean shell height ± SE: 21.4 ± 0.3 mm). Clearance rate (mean ± SE, n = 5 chambers, 8 to 12 oysters per chamber) of juvenile oysters fed *Pseudo-nitzschia multiseries* clones (0.7 to 0.8 pg domoic acid [DA] cell^{-1}) in various mixed suspensions. Each binary diet was composed of equivalent cell volumes of 2 algae, with cell sizes (length for *P. multiseries* and *Thalassiosira weissflogii*, and equivalent spherical diameter for *Isochrysis galbana*, *Rhodomonas lens*, *Chaetoceros muelleri*) shown in the graph. Clearance rates for each pair of algae were statistically compared within every diet and results are shown above bars. Pie charts indicate the percentage of filtered cells that were rejected as pseudofeces (in black) in each diet, with statistical results shown between the pairs of charts. ns: non-significant difference; * $p < 0.05$, ** $p < 0.01$

3 different *P. multiseries* clones, ranging from 28 to 100 µm in cell length and 4.6 to 5.0 µm in cell width, were mixed with the small and nearly spherical flagellate *Isochrysis galbana* (ESD = 4.5 µm) in Diets M1 to M3, CR of the latter was significantly lower than that of the *P. multiseries* clone in 2 out of 3 diets tested (M1 and M2, $p = 0.02$ and 0.04, respectively), indicating a lower retention efficiency of *I. galbana* cells by oyster gills. In the third diet (M3), clearance of *I. galbana* was also lower than that of *P. multiseries*, but because of the high variability of the data the difference was not statistically significant ($p = 0.12$). Differential retention efficiency, as reflected in CR values, was not observed in Diets M4 to M6, in which *P. multiseries* clone CLN-50 was mixed with *Rhodomonas lens*, *Chaetoceros muelleri*, or *Thalassiosira weissflogii*, species with a cell size greater than that of *I. galbana* ($p = 0.78$, 0.22 and 0.58, respectively). Additionally, there was no relationship between CR and cell length of *P. multiseries*.

After retention on the gills, particles were either ingested or rejected in pseudofeces. In mixed suspensions, the percentage of particles that were rejected by the oysters varied as a function of particle size and algal species. When mixed with a flagellate (Diets M1 to M4), 14 to 39% of the filtered *Pseudo-nitzschia multiseries* cells were rejected as pseudofeces prior to ingestion (Fig. 1), with the greatest percent rejection for the larger clones, CLN-46 in Diet M2 and CLN-50 in Diets M3 and M4. In contrast, rejection of the flagellates *Isochrysis galbana* (4.5 µm ESD) and *Rhodomonas lens* (7.6 µm ESD) from the same suspensions was consistently low, ranging from only 6 to 12% of the filtered cells (Fig. 1). Percent rejection of *P. multiseries* cells was significantly higher than that of the flagellates in all diets ($p = 0.004$ to 0.037). However, when *P. multiseries* was offered in suspension with the smaller diatoms *Chaetoceros muelleri* (5.4 µm ESD; Diet M5) and *Thalassiosira weissflogii* (23 µm; Diet M6), both components of the binary diets were rejected in similar proportions, regardless of cell size and species ($p = 0.75$ and 0.10, respectively).

Due to both differential retention on the gills and rejection in the pseudofeces, the ratio of *Pseudo-nitzschia multiseries* to *Isochrysis galbana*, expressed in terms of cell volume (Fig. 2; Diets M1 to M3) increased substantially from ca. 1 in the mixed suspension to 3.6, 4.8 and 7.3 in pseudofeces ($p = 0.0002$,

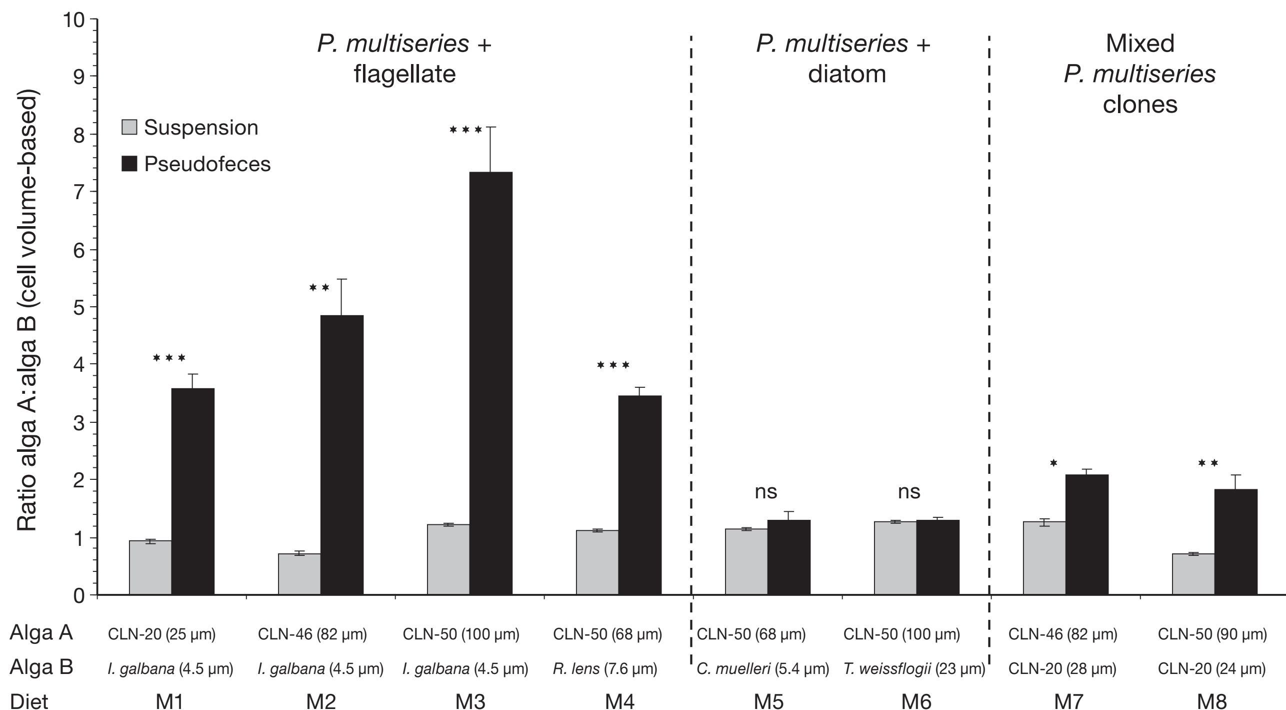

Fig. 2. *Crassostrea virginica* juveniles (mean shell height ± SE: 21.4 ± 0.3 mm). Ratio of 2 algae (Alga A:Alga B) in the mixed suspension and in pseudofeces produced by oysters (mean ± SE; n = 5 chambers, 6 to 9 oysters per chamber). Oysters were offered various *Pseudo-nitzschia multiseries* clones in a mixed diet with *Isochrysis galbana*, *Rhodomonas lens*, *Chaetoceros muelleri*, *Thalassiosira weissflogii*, or another *P. multiseries* clone of contrasting cell length. Each binary diet was composed of equivalent cell volumes of 2 algae (cell size as defined in Fig. 1 shown in parentheses). Statistical results of within-diet comparisons are shown above bars. ns: non-significant difference; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

0.001 and 0.0005, respectively). In addition, the ratio of *P. multiseries* to *Rhodomonas lens* increased significantly from 1.1 in suspension to 3.5 in pseudofeces (Diet M4; p = 0.009), which can only be explained by selective rejection, as the 2 algal species were removed from the mixed suspension at comparable rates. In contrast, no selectivity was observed when oysters were offered CLN-50 in mixed suspensions with the smaller diatom species *Chaetoceros muelleri* (Diet M5; p = 0.17) and *Thalassiosira weissflogii* (Diet M6; p = 0.29). Overall, the hypothesis of preferential rejection of *P. multiseries* cells in pseudofeces (H_2; Table 2) was only confirmed in diets where *P. multiseries* was mixed with a flagellate (Figs. 1 & 2).

When a large *Pseudo-nitzschia multiseries* clone was combined with the small clone CLN-20 (Diets M7 and M8; Fig. 2), oysters preferentially rejected the larger cells. The ratio of large to small *P. multiseries* cells increased to a lesser degree compared to the diets containing flagellates, from 1.2 in the suspension to 2.1 in pseudofeces for Diet M7 and from 0.7 to 1.8 for Diet M8 (Fig. 2). The differences, however, were statistically significant (p = 0.014 and 0.004, respectively), confirming the hypothesis that cell size affects the selective rejection of *P. multiseries* cells by the oysters ($H_{2.1}$; Table 2).

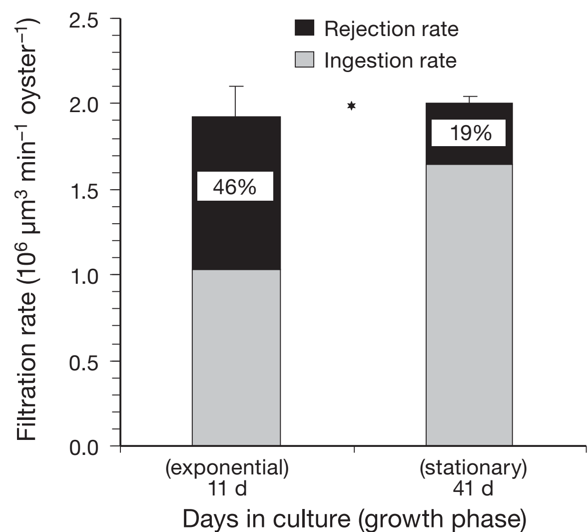

Fig. 3. *Crassostrea virginica* juveniles (mean shell height ± SE: 21.4 ± 0.3 mm). Filtration rate (composite bars, mean ± SE) and the relative allocation between ingestion rate and rejection, i.e. pseudofeces production rate (n = 5 chambers, 8 oysters per chamber) of oysters fed *Pseudo-nitzschia multiseries* clone CLN-20 at exponential and stationary phases (0.07 and 0.6 pg DA cell^{-1}, respectively). Star between bars indicates a significant difference in the proportion of cells rejected in pseudofeces ($p < 0.05$). Cell length was equivalent in both growth stages (25 µm)

Oysters removed comparable amounts of *Pseudo-nitzschia multiseries* cells in exponential and stationary phases from unialgal suspensions, as indicated by the similar FR (Fig. 3). However, because a greater number of cells in the exponential phase was rejected in pseudofeces, the proportion of cells that were actually ingested by the oysters was lower for cultures in exponential than for those in stationary phases (p = 0.027).

The video-endoscopy experiments allowed sampling of particles transported on the ventral and dorsal gill tracts, which further elucidated the site of particle selection. Adult oysters (mean pfa width ± SE: 68.2 ± 0.6 µm), exhibited negative EI values in the vcg when fed a mixed diet containing *Pseudo-nitzschia multiseries* clones of contrasting cell length, indicating considerable enrichment in larger cells (CLNN-21, 'Alga A' = 99 µm) relative to the suspension (Diet E1; Fig. 4). As CLNN-21 cells were preferentially directed to the ventral groove relative to the smaller cells of clone CLN-30 ('Alga B' = 35 µm), the dct were enriched in small CLN-30 cells, as reflected by positive EI values. The hypo-

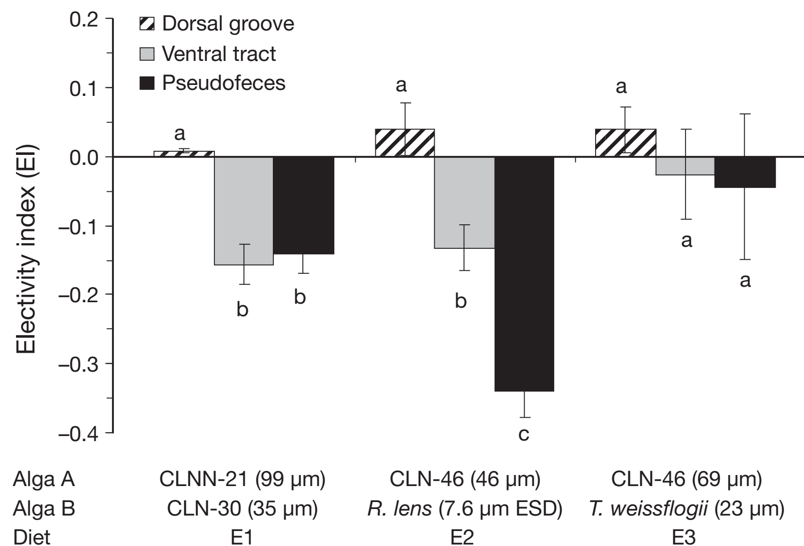

Fig. 4. *Crassostrea virginica* adults (mean shell height ± SE: 116 ± 2 mm). Electivity indices (EI, mean ± SE) in the dorsal ciliated tracts, ventral ciliated grooves and in pseudofeces of oysters fed *Pseudo-nitzschia multiseries* clones in 3 mixed suspensions: Diet E1: CLNN-21 + CLN-30; Diet E2: CLN-46 + *Rhodomonas lens*; and Diet E3: CLN-46 + *Thalassiosira weissflogii*. Diets 'E1' and 'E3' were enriched with *Isochrysis galbana* (40 000 cells ml^{-1}) to stimulate feeding. For each diet, a negative EI value indicates enrichment in 'Alga A', with a positive EI indicating enrichment in 'Alga B' at a given sampling site. Different letters indicate statistical difference in EI for each diet ($\alpha = 0.05$). Electivity indices represent the average of 8, 6 and 3 oysters in Diets 'E1', 'E2' and 'E3', respectively

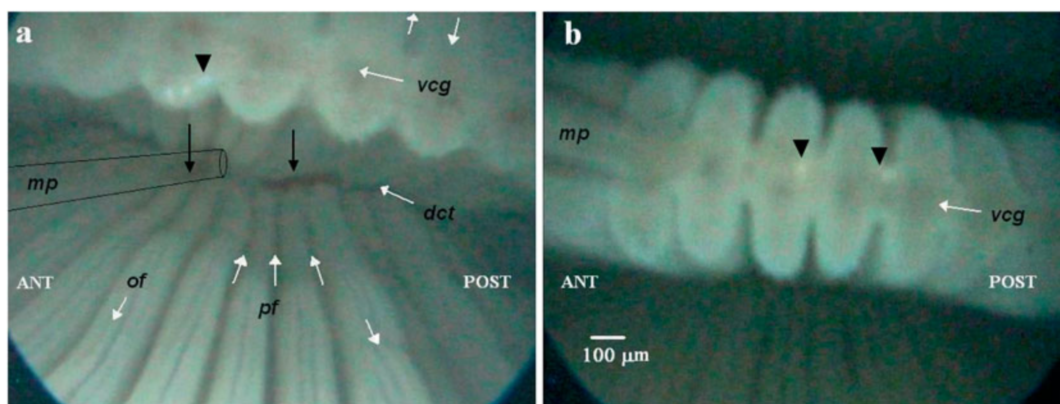

Fig. 5. *Crassostrea virginica*. Diet E2 (*Rhodomonas lens* + *Pseudo-nitzschia multiseries* clone CLN-46); video micrograph taken during endoscope-directed sampling of processed particles on the: (a) dorsal ciliated tract (dct) and (b) ventral ciliated groove (vcg). Note the dominance of reddish particles (*R. lens*; black arrows) carried on the dct contrasting to the more transparent, bright particles (*P. multiseries*; black arrowheads) transported on the vcg. White arrows indicate direction of particle transport on the dct, vcg, principal filaments (pf) and on the ordinary filaments (of) that compose the plicae. Samples were taken with a micropipette (mp, outlined in Panel a) connected to a micro-peristaltic pump (see 'Materials and methods'). ANT: anterior; POST: posterior

thesis of particle selection on the gills ($H_{2.2}$; Table 2) was strongly supported by the markedly different EI values between the dct and vcg (p = 0.001). In addition, the EI in pseudofeces did not differ from that in the vcg of the gills (p = 0.99), showing that no further sorting based on particle size took place on the labial palps.

Particle sorting also occurred on the gills of oysters fed the flagellate *Rhodomonas lens* in a mixed suspension with a relatively small (45 µm) *Pseudo-nitzschia multiseries* clone, CLN-46 (Diet E2; Figs. 4 & 5). Similarly to the first diet, the negative EI measured in the vcg differed significantly from the positive EI found in the dct (p = 0.018), indicating selective rejection of *P. multiseries* on the gills. This time, however, additional rejection of *P. multiseries* occurred on the labial palps, as indicated by the significantly greater negative EI of pseudofeces samples compared to the ventral groove samples (p = 0.021). This result shows that both the gills and labial palps can be active sites for particle selection in the oysters *Crassostrea virginica*. In contrast, no particle selectivity was found on the gills or on the labial palps of oysters fed *P. multiseries* clone CLN-46 (69 µm) in a mixed suspension with another diatom, *Thalassiosira weissflogii* (Diet E3; Fig. 4).

DISCUSSION

In a companion study (Mafra et al. 2009), we found that a combination of low CR and increasing pseudofeces production at higher cell densities led to reduced ingestion of *Pseudo-nitzschia multiseries* from unialgal suspensions by *Crassostrea virginica*. In the same study, oysters were found to exhibit a higher overall CR when *P. multiseries* clones were offered in a mixed suspension with the flagellate *Isochrysis galbana.* Since toxic *Pseudo-nitzschia* spp. blooms in the natural environment are often associated with high abundances of other algal species (Fehling et al. 2006, Mafra et al. 2006, Spatharis et al. 2007), including multiple *Pseudo-nitzschia* spp. (Rines et al. 2002, Kaczmarska et al. 2007), this finding suggests that DA intake by oysters could be facilitated in mixed suspensions with a more palatable food source. In the present study, oyster gills were shown to clear *P. multiseries* cells at similar or higher rates than flagellate species in a mixed suspension. However, after capture on the gills, *P. multiseries* cells were preferentially rejected by both juvenile and adult oysters in pseudofeces, leading to enrichment of the ingested food in flagellates and potentially reducing the DA intake by the shellfish. When *P. multiseries* clones were mixed in suspension with another diatom species, no selective feeding occurred and both algae were similarly rejected in pseudofeces.

In the present study, differential clearance of cells did not occur when juvenile oysters were fed the relatively long and narrow *Pseudo-nitzschia multiseries* in mixed suspensions with another relatively large but more spherical diatom species or the flagellate *Rhodomonas lens* (Diets M4 to M6; Fig. 1). However, when different *P. multiseries* clones, ranging in cell length from 28 to 100 µm, were mixed in a binary diet with the smaller flagellate *Isochrysis galbana* (4.5 ± 0.6 µm, mean ESD ± SD), the latter was removed from suspension at a consistently lower rate than *P. multiseries*, irrespective of clone size (Diets M1 to M3; Fig. 1). Since both algal particles theoretically approach the gills at the same velocity, the result suggests that *Crassostrea*

virginica gills may have retained *I. galbana* with lower efficiency than *P. multiseries* cells in the present study. For *C. virginica*, the reported size threshold at which particles are filtered with 100% efficiency varies somewhat among individual studies, but averaged data suggest that only particles ≥5 to 6 µm, slightly larger than *I. galbana* cells, can be fully retained on the gills (Ward & Shumway 2004). Moreover, exposure to high particle concentrations may reduce bivalve retention efficiency by changing the interfilamentary distance of the gills. For example, Palmer & Williams (1980) showed that retention efficiency of *C. virginica* for particles >4.35 µm dropped from 100% at 1.4 mg DW seston l^{-1} to 86% at concentrations as high as 6.5 mg l^{-1}. Because the total biomass concentrations of Diets M1 to M3 ranged from 6.2 to 7.4 mg l^{-1} in the present study, the lower clearance of *I. galbana* in mixed suspensions with *P. multiseries* is attributed to <100% retention efficiency of the former alga. Periodic variations in gill porosity were also observed by Haven & Morales-Alamo (1970) in *C. virginica* feeding at high seston concentrations (ca. 10 mg l^{-1}), and helped to explain how oysters can vary their CR while keeping the valves open (Palmer 1980). Variations of the oysters' retention efficiency would not affect filtration of *P. multiseries* cells at high cell densities because of their large size, and this is not expected to be a mechanism contributing to reduce DA intake from toxic cells at bloom densities. In fact, unialgal suspensions of 2 *Pseudo-nitzschia* species, *P. multiseries* and *P. pseudodelicatissima*, at common bloom concentrations (ca. 10^6 cells l^{-1}) were filtered by *C. virginica* at rates comparable to 2 other diatoms, *Thalassiosira weissflogii* and *Ditylum brightwellii* (Thessen et al. 2002).

Although the presence of a flagellate in mixed suspensions with *Pseudo-nitzschia multiseries* was shown to increase the overall CR in *Crassostrea virginica* (Mafra et al. 2009), some CR values reported in the present study were unexpectedly low, namely for Diets M1 and M2 (Fig. 1). High particle concentrations, such as those used in these diets, may lead to CR reduction in bivalves (Riisgård 2001). This was unlikely the case in the present study, however, as oysters exposed to other mixed suspensions at comparable concentrations (Diets M3 to M4; Table 2) showed much higher CR (Fig. 1). It is also improbable that the lower CR values observed in Diets M1 and M2 were due to artefacts in the measurement of clearance, since the method employed was the same for all diets and precautions to limit recirculation in the chambers by maintaining ≤30% depletion were taken. As Riisgård (2001) pointed out, even when suitable methods are applied, disparity in CR values between different experiments may reflect differences in bivalve condition and/or the influence of environmental factors.

We provide independent evidence from both feeding rate experiments with juvenile oysters and quantitative video-endoscopic observations of particle transport on the gills of adult oysters that the size of *Pseudo-nitzschia multiseries* cells is a critical factor influencing oyster pre-ingestive selection. The percentage of *P. multiseries* cells rejected in pseudofeces from mixed suspensions with *Isochrysis galbana* increased with an increase in cell length from 23 to 100 µm (Diets M1 to M4; Fig. 1). In addition, oysters preferentially rejected the larger *P. multiseries* clones CLN-46 (82 µm) and CLN-50 (90 µm) when offered in mixed suspensions with the smaller (24 to 28 µm) and similarly toxic clone CLN-20 (Diets M7 and M8; Fig. 2). Video-endoscopy-assisted sampling confirmed that the selective ability of oyster gills was affected when large cells were filtered. At least a portion of the larger *P. multiseries* cells, likely those not dorsoventrally oriented, was unable to enter the principal filaments, and was thus carried along the ventral grooves of *Crassostrea virginica* (Diet E1; Fig. 4). This confirms that a single algal species, *P. multiseries*, can be selected by *C. virginica* based exclusively on cell size, as previously demonstrated with polystyrene beads of contrasting diameters (40 and 275 µm) by Tamburry & Zimmer-Faust (1996). The gills were the only possible site for such selection, as we found no further selectivity on the labial palps (Diet E1; Fig. 4).

The present study also provides quantitative data that support the qualitative video-endoscopy observations made by Cognie et al. (2003) in the Pacific oyster *Crassostrea gigas*. Using the large, non-toxic pennate diatoms *Pleurosigma planctonicum* and *Rhizosolenia setigera*, and the centric diatom *Coscinodiscus perforatus*, these researchers found that cells with all axes greater than the pfa width (69 µm; Cognie et al. 2003) and some cells with 1 axis exceeding this width were unable to enter the principal filaments. Because the pfa width in our study (68.2 ± 0.6 µm, mean ± SE) was measured from relaxed, dissected gills, this measurement cannot be considered as a fixed threshold. During our video-endoscopic observations, this gap appeared to be variable and even disappeared as oysters stretched and contracted their gills. Our *in vivo* measurements of the pfa width from actively feeding *Crassostrea virginica*, however, were on average 66.7 ± 1.3 µm, mean ± SE, very similar to the values we obtained from dissected gills and those reported earlier for *C. gigas*.

Pseudo-nitzschia spp. cells are noticeably smaller and narrower (ca. 5 µm width) than the diatoms used by Cognie et al. (2003), in which the 2 pennate species were characterized by a length of 350 to 700 µm and a width of 35 to 40 µm. In the present study, we used *P. multiseries* clones of contrasting sizes to obtain a mixed suspension of cells either shorter or longer than the pfa

width, and confirmed that cells with 1 axis longer than this critical size are selectively rejected by oysters. Furthermore, our quantitative measures of feeding selection were obtained for a planktonic, toxic alga of public health relevance. Confirmation that oysters selectively reject high proportions of *P. multiseries* cells >70 µm in length is particularly important, given that at least 92% of cells in the natural environment exceed this size threshold (Bates et al. 1999), and that *Pseudo-nitzschia* spp. of highly varying cell size have been associated with shellfish closures during toxic blooms (Trainer et al. 2007). In addition, this finding has major ecological significance as *Pseudo-nitzschia* spp. are a ubiquitous component of the phytoplankton in coastal waters worldwide (Hasle 2002). Although cell toxicity may be directly related to the size of a given *P. multiseries* clone, we demonstrated that CR inhibition of oysters fed *Pseudo-nitzschia* spp. cells could not be attributed to DA toxicity (Mafra et al. 2009). Therefore, it is improbable that DA toxicity was the cause of the selective feeding of oysters exposed to *P. multiseries* in the present study. Furthermore, in previous endoscopic observations (L. L. Mafra et al. unpubl. data), larger *P. multiseries* cells (100 µm; 0.6 pg DA $cell^{-1}$) were preferentially rejected by adult *C. virginica*, even when mixed in suspension with a smaller but more toxic clone (24 µm; 1.1 pg DA $cell^{-1}$).

Results of our study suggest that factors other than cell size are involved in particle selection by the gills and labial palps of *Crassostrea virginica*. This conclusion is based on the fact that relatively small flagellates and diatoms (4.5 to 28 µm) were differentially rejected in pseudofeces when offered in a mixed suspension with a *Pseudo-nitzschia multiseries* clone, some of which had cell sizes smaller than the pfa width (<68 µm). Similar findings have been reported previously for *C. gigas* when delivered a mixed suspension of 2 qualitatively different particle types of comparable size, e.g. *Spartina* spp. detrital particles and *Rhodomonas lens* (Ward et al. 1997) and, more recently, when delivered a mixed suspension of the live diatoms *Actinoptychus senarius* and artificially cleaned, empty frustules (Beninger et al. 2008a). In the present study, we show conclusively that *C. virginica* was able to sort among algae of similar size from different taxa (i.e. diatoms vs. flagellates). While flagellated cells were mostly ingested (Fig. 1), up to 49% of the small diatoms (cell size <68 µm) were rejected in pseudofeces of oysters when offered in mixed suspensions with either *Isochrysis galbana* or a larger *P. multiseries* clone. Similarly, in a previous study, the diatom *Phaeodactylum tricornutum* was preferentially rejected in the pseudofeces of 5 other bivalve species when delivered in a mixed suspension with the dinoflagellate *Prorocentrum minimum* and the naked flagellate *Chroomonas salina* (Shumway et al. 1985). Prior studies have suggested that bivalves make use of chemical cues to discriminate among particles (Newell & Jordan 1983, Shumway et al. 1985, Ward & Targett 1989). Contact with the extracellular organic envelope, whose composition varies from diatoms to flagellates (Ward & Targett 1989) and even among diatom species (Volcani 1981), may be a major cue for selection by bivalve pallial organs. Alternatively, substances released from inside the cell or from its organic covering may be recognized by some bivalves, since *Crassostrea gigas* was able to selectively reject permeable microcapsules enclosing the diatom *Nitzschia closterium* while preferentially ingesting those containing the green alga *Tetraselmis suecica* (Espinosa et al. 2007).

Diatoms may contain metabolites that cause inhibitory effects on feeding and fitness parameters of suspension-feeding grazers (Shaw et al. 1995, Ianora et al. 2003). Whether such metabolites affect particle selection in bivalves, however, is not known. When Bougrier et al. (1997) offered 5 diets composed of a combination of 3 to 4 species from different taxa, *Crassostrea gigas* preferentially rejected in pseudofeces 3 relatively small diatom species (*Skeletonema costatum*, *Chaetoceros calcitrans* and *Nitzschia closterium*) compared with 3 similarly sized flagellate species (*Pavlova lutheri*, *Tetraselmis suecica* and *I. galbana*). In a study on the European oyster *Ostrea edulis*, Bricelj et al. (1998) reported that, compared to several dinoflagellates, the diatom *Thalassiosira weissflogii* was mainly transported along the ventral grooves of the gill where material is more likely to be rejected in pseudofeces. The present study shows that *Crassostrea virginica* were unable to sort between 2 diatom species, when *Pseudo-nitzschia multiseries* clones were mixed in a suspension with *T. weissflogii* or *Chaetoceros muelleri* (Diets M5 and M6; Fig. 2). This contrasts with the ability of *C. gigas* to preferentially ingest 2 out of 4 benthic diatom species from the Naviculaceae family, ranging in cell length from 22 to 60 µm (Cognie et al. 2001).

The present study provides the first evidence that selection of different microalgal species may occur concurrently in 2 distinct pallial organs of a heterorhabdic bivalve. In a mixed suspension with the flagellate *Rhodomonas lens*, selective rejection of *Pseudo-nitzschia multiseries* clone CLN-46 occurred simultaneously on the gills and labial palps of *Crassostrea virginica* (Diet E2; Figs. 4 & 5). This dual-site sorting capacity, suggested to be a general ability of heterorhabdic bivalves, has been confirmed in the scallop *Pecten maximus* (Beninger et al. 2004) and the oyster *Crassostrea gigas* (Beninger et al. 2008a), which were fed live and artificially cleaned, dead diatoms. However, oysters seem to have a more refined particle

selection mechanism than scallops. This idea was recently supported by the work of Beninger et al. (2008b), who found that, contrary to the scallop *P. maximus* (Beninger & Decottignies 2005), the oyster *C. gigas* was able to distinguish between live and naturally dead diatoms in a mixed suspension, the latter still covered by a peri-frustular envelope.

The chemical mechanisms involved in particle selection have not been elucidated, but the mapping of mucocytes on the labial palps and gill epithelium of different bivalve species is consistent with the use of mucus during the entire particle handling process (Beninger et al. 1993, 2005, Beninger & Dufour 1996). Mucocytes of the pallial organs secrete mucus of varying viscosity for different functions (Beninger & Dufour 1996, Beninger & St.-Jean 1997, Beninger et al. 2005). Except for the dorsal tracts and principal filaments, transport of particles occurs on relatively exposed sites of the oyster gills in a perpendicular or opposite direction to the prevailing inhalant current (Ward et al. 1994); thus, transport is only possible if particles strongly adhere to the ciliated epithelium by mucus (Beninger et al. 2005). Of all components of bivalve mucus, lectins (Fisher 1992) are the most probable to act as a particle agglutinant. Lectins are glycoproteins of non-immune origin that specifically and reversibly conjugate with sugars (Goldstein et al. 1980), including those covering algal cells (Waite et al. 1995, Cho 2003). A mannose-binding lectin has been shown to act as a feeding receptor for prey recognition by the heterotrophic dinoflagellate *Oxyrrhis marina* (Wootton et al. 2007), and recent studies suggest that lectins in mucus secreted by the feeding organs of *Crassostrea virginica* may be involved in particle selection (Espinosa et al. 2008). Affinity for different lectins varies among diatom species (Waite et al. 1995) and between diatoms and dinoflagellates, with the latter binding to a greater variety of lectin types (Cho 2003). Thus, if lectins associated with the feeding structures bind more effectively to certain groups of microalgae, this might explain why *C. virginica* showed no pre-ingestive selection among diatom species, but demonstrated selection between *Pseudo-nitzschia multiseries* and 2 flagellate species.

Oysters in the present study rejected greater amounts of exponentially growing *Pseudo-nitzschia multiseries* cells than those in the stationary phase. This finding could be explained by the fact that accumulation of cell-surface carbohydrates is lower in diatoms undergoing faster growth (Waite et al. 1995). Cell stickiness also tends to be lower in the exponential phase, which may influence adhesion between the cells and surfaces of the feeding structures. These factors may help to explain the preferential rejection of cleaned, empty frustules over intact diatom cells by *Pecten maximus* (Beninger et al. 2004) and *Crassostrea gigas* (Cognie et al. 2003), and rejection of naturally dead over live cells by *C. gigas* (Beninger et al. 2008b), assuming that the composition of the peri-frustular envelope changes after cell death. Therefore, it is unlikely that the use of *P. multiseries* cells in the stationary phase in our mixed diets caused their preferential rejection over flagellates by the oysters, since cells growing exponentially were rejected to an even greater degree in both unialgal (Fig. 3) and mixed diets (data not shown). Additionally, the similar feeding selectivity found in both juvenile (21.4 ± 0.3 mm, mean SH ± SE) and adult (116 ± 2 mm, mean SH ± SE) *Crassostrea virginica* confirms that the pallial organs were fully developed in the 1 to 1.5 yr old juveniles used in the present study. In fact, after 16 to 22 wk of rearing at 22 to 25°C, the complete mantle rejection system of *C. gigas* was functional in juveniles between 10 and 24 mm in shell height (Beninger & Cannuel 2006).

In conclusion, the relatively low DA levels found in *Crassostrea virginica* when exposed to toxic *Pseudo-nitzschia* spp. blooms can be explained at least partially by 2 processes: (1) pre-ingestive rejection of *P. multiseries* in mixed suspensions, which is more pronounced for larger cells (present study), and (2) reduced CR elicited in unialgal suspensions (Mafra et al. 2009). Indeed, 2 wk contamination experiments in the laboratory confirmed that *C. virginica* accumulates much less DA from *P. multiseries* than the mussel *Mytilus edulis* under the same experimental conditions, and that the difference was much greater when larger cells (>68 µm) were offered (L. L. Mafra et al. unpubl. data). In our study, *P. multiseries* occurred mostly as single cells (>95%); thus, the role of chain formation by *P. multiseries* on the selective ability of oysters requires further investigation. Nevertheless, we suggest that rejection of this toxic diatom by oysters will be more pronounced during natural blooms, when large cells are growing exponentially and favourable conditions trigger the formation of long, stepped chains. As a result, oysters in contact with multi-specific blooms, dominated by toxic *Pseudo-nitzschia* spp. and other non-toxic species, may filter a high biomass of phytoplankton without necessarily accumulating the levels of DA predicted from their clearance rates.

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Contribution to the Theme Section 'Directions in bivalve feeding'

Grazing on a natural assemblage of ciliate and dinoflagellate cysts by the eastern oyster *Crassostrea virginica*

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ABSTRACT: A natural dinoflagellate- and ciliate-cyst community from anoxic sediment collected from New Haven, Long Island Sound, was concentrated with a particle sorter and fed to oysters *Crassostrea virginica*. The total number of cysts and the species composition of cysts in beakers containing live oysters were measured before and after feeding by the oysters. The oysters significantly reduced the numbers of both dinoflagellate and ciliate cysts. Both empty cyst walls and filled (live) cysts were consumed. The oysters decreased the number of total cysts to less than half compared to control beakers containing empty oyster shells. Results from the experiment show that natural assemblages of cysts in sediment are degraded by the feeding activities of oysters. If toxic cysts were present in natural sediments resuspended from the bottom, digestion of resting cysts could lead to toxin accumulation in oysters. There was no major shift in species composition of cysts; cysts known as fossilizable were destroyed, as well as cysts not known to be very resistant or preservable.

KEY WORDS: Dinoflagellate · Cyst · *Crassostrea virginica* · Oyster · Grazing · Digesting

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INTRODUCTION

Many kinds of organisms emerge from sediment that is placed in culturing conditions after a resting period in cold and darkness. Among them, dinoflagellates and ciliates are very common. In Persson (2002), more than 46 different ciliate taxa and at least 47 different dinoflagellate taxa were germinated from sediment samples from the Swedish west coast. In Fenchel et al. (1997), 20 ciliate species were detected initially, but during 130 d of culturing, an additional 115 cryptic species were recorded.

Approximately 10 percent of the dinoflagellate species worldwide are known to produce resting cysts, but in temperate areas this increases to nearly one-third (Nehring 1997, Persson et al. 2000). Among the cyst-producers are many of the well-known, toxic species, for example paralytic shellfish poisoning (PSP)-producing *Alexandrium* spp. Resting cysts often are regarded as the over-wintering stage of coastal dinoflagellates; the cysts are formed at the end of the bloom and sink to the sea floor where they lay dormant before germinating when conditions again become suitable for vegetative growth (Anderson 1998, Dale 1983). Dinoflagellates that have a resting-cyst stage in their life history often spend most of the year as a resting cyst and only a brief period of a few weeks to months as vegetative cells (Dale 1983).

Other eukaryotic protists, such as ciliates, are known to produce cysts (Jones 1974), both division cysts (wherein cells divide by fission) and resting cysts. The resting cysts are thick-walled and can persist during adverse conditions; they are formed from vegetative cells as a strategy against environmental stresses, such as starvation (Gutiérrez et al. 2001).

Both ciliate and dinoflagellate cysts are known to be subjected to grazing by animals in nature (A. Persson pers. obs.). For example, resting cysts of dinoflagel-

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lates have been shown to be subjected to grazing (Persson & Rosenberg 2003, Tsujino & Uchida 2004, Persson et al. 2006, 2008, Persson & Smith 2009). It is clear that all resting cysts produced in the water column are not still present and alive for the next season following several loss factors, including grazing (Persson et al. 2000).

A major problem in studies of the effect of deposit feeders on dinoflagellate cysts is the quantification of the amount the animals need to eat in order to produce a certain amount of fecal deposits. A number of dinoflagellate-cyst grazing studies have been published (Kremp et al. 2003, Montresor et al. 2003, Persson & Rosenberg 2003, Tsujino & Uchida 2004), but none have included quantitative measurements of cyst numbers. There are difficulties with the interpretation of relative abundance data; even if most cysts were digested, the cysts could be more highly concentrated in the pellets if the sediment consumed is rich in easily digested organic material. If empty cysts are more fragile than live cysts, one could get the impression that more cysts remain alive after grazing than before without a quantitative measurement. If cysts are picked from pellets for germination experiments, only those cysts that appear alive are chosen, not empty cyst walls. If there are no major changes in the species composition of cysts, then one cannot detect if cysts have been affected by grazing.

To address this quantitative problem, we used a filter-feeding bivalve, the eastern oyster *Crassostrea virginica*, as a model grazer. Oysters were placed in clean containers to which known quantities of cysts were added, and exact cyst contents were counted in the food and in the waste (feces and pseudofeces). To our knowledge, this is the first quantitative study of the effects of filtering bivalves on live dinoflagellate and ciliate cysts concentrated from natural sediment. Other types of resting stages were not studied here. Planktonic diatoms, for example, produce spores, but those are of a different size (much smaller) and accumulate differently in a particle separator; therefore, we did not count them.

MATERIALS AND METHODS

Dinoflagellate and ciliate cysts. Anoxic sediment was collected at 10.2 m depth in Morris Cove, New Haven Harbor, Connecticut, USA (41° 15.658 N 72° 54.030 W), by divers. The uppermost 10 cm of the very fine-grained, black sediment was collected in buckets with lids.

For concentration of cysts, a sediment sorter designed for this purpose was used (Smith et al. 2009). A total of 16.5 l of sediment was sorted over 48 h in cold (6°C) and darkness; the most fine-grained material was collected (employing Stokes' Law) and immediately stored in a refrigerator (4°C). To concentrate the cysts further for the experiment, the flask containing the sorted fraction was shaken thoroughly, and 1 l of the slurry was removed. This slurry was sieved with cold-filtered seawater (0.1 µm) to concentrate the size fraction of the dinoflagellate cysts (20 to 100 µm) and then transferred to a 100 ml cylinder wherein the volume was adjusted to 100 ml. The sediment was kept as dark as possible during the process to prevent cyst germination.

Oysters. *Crassostrea virginica* from Long Island Sound were obtained 22 January 2003 from the Noank Aquaculture Cooperative in Groton, Connecticut. The oysters were kept in running, unfiltered seawater in the laboratory (Milford Laboratory, Milford, Connecticut) until use in the experiment (after 1 mo). The oysters were 30 ± 3 mm in shell height. Temperature was increased gradually from ambient by 2°C d^{-1} to 20°C. Before the experiment, oysters were brushed in filtered seawater and placed in aerated, filtered seawater for 4 d to empty stomach contents.

Experimental setup. Fifteen 1 l glass beakers were used in the experiment: 5 for feeding, 5 for controls, and 5 with unfed oysters. Two oysters were placed in each container on top of a stiff, plastic net (Fig. 1). The controls contained empty oyster shells to correct for any dinoflagellate cysts that may attach to the surface of experimental containers or otherwise become unre-

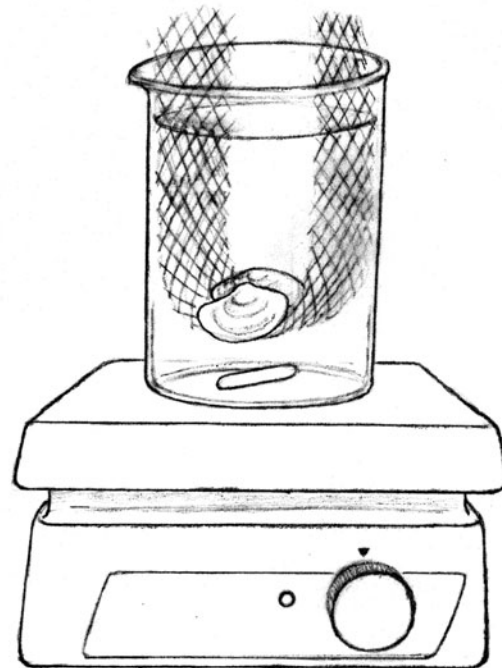

Fig. 1. Experimental setup used for the present and other studies (Persson et al. 2006). Two oysters are suspended on stiff plastic screen allowing free rotation of a stir bar in the bottom of the 1 l beaker

coverable. The same experimental setup was used to study grazing on culture-produced cysts of *Alexandrium fundyense* (Persson et al. 2006) and of *Scrippsiella lachrymosa* (Persson & Smith 2009).

Two ml of the sorted dinoflagellate cyst fraction were added to each experimental beaker (5 with live oysters and 5 controls with empty oyster shells) and the rest was saved for measurement of initial dinoflagellate cyst concentration and species composition. Five samples of 2 ml were used for preparation of permanent mounts, according to the description below, and observed microscopically to establish starting values.

The oysters were allowed to feed for 24 h at 18°C in darkness (covered with dark boxes). The sediment was kept suspended by gentle stirring with magnetic stir bars. At the end of the 24 h experiment, the temperature had increased to 28°C in the beakers because of friction from the stir bars, but the oysters were healthy and we concluded they had not been adversely affected by this. (In other experiments with the same setup, dark boxes were not used, and an insulating layer was used between stir plates and beakers, e.g. Persson et al. 2006.)

At the end of the experiment, each container was treated in the following way: the water was poured onto a 20 µm screen, the container and oysters were cleaned with a plant sprayer (Spray Doc #201P, Vermont American) and 0.1 µm filtered seawater onto the same screen until the container and the oysters were considered to be clean of particles. Then the material on the screen was collected quantitatively in a beaker, sonicated for 5 min (Sonicor SC-50) to remove material sticking to spines of cysts, and sieved again on the 20 µm screen, first with seawater, next with distilled water, and finally collected in a centrifuge tube. The tubes were placed in a 4°C refrigerator overnight.

To purge the oysters of any remaining cysts in the digestive system, they were returned to their respective containers with 1 l of filtered seawater and given a drop of yellow, plastic microbeads (fluorescent microspheres; Fluoresbrite® YG Carboxylate Microspheres 2.00 µm) overnight. The subsequent day, all oysters had defecated yellow fecal pellets. The same procedure as above was repeated to recover any cysts present in the fecal material. This material was sieved, sonicated, and settled as described above.

For preparation of permanent mounts for cyst counting and identification, the water above the sediment in each centrifuge tube was removed carefully with a Pasteur pipette. The sediment in each tube was mixed, and 10 µl of sediment was added to each of 5 slides (each tube corresponded to one beaker in the experiment) with a drop of glycerin. The slides were placed on a hot plate (50°C) and the contents were spread evenly. When the water had evaporated, a coverglass was placed on top of the glycerin drop and the slide was sealed with beeswax. The amount of sediment in each tube was determined by weighing distilled water in the clean tubes up to a mark made with a permanent pencil at the sediment surface. Slides of material from fecal pellets (that contained very little material) were prepared by dividing the material evenly on a known number of slides (5 or 6) to a desired density for counting. Otherwise fecal pellet samples were prepared the same way as sediment. Whole slides were counted (1 entire slide per container and per pellet sample).

RESULTS

The total number of cysts was significantly ($p = 0.0001$) reduced by oyster grazing (Fig. 2) to one-third of initial values and to less than half compared to the empty shell control (reduced by 57.5%). The control still contained 84% of the cysts from the start of the experiment, but the number of full (live) cysts had declined through germination; approximately 52% of the full cysts had germinated in the control (Fig. 2). When only full cysts are considered, a comparison between control and oyster treatment reveals that 43.8% of the full cysts were eliminated by oyster grazing activities. This, however, is probably an underestimation of the actual number of digested live cysts as many of the living cysts germinated in the control but were ingested long before they had time to germinate in the oyster treatment. Unfed live oyster treatments developed no cysts, full or otherwise, and no germinated cells.

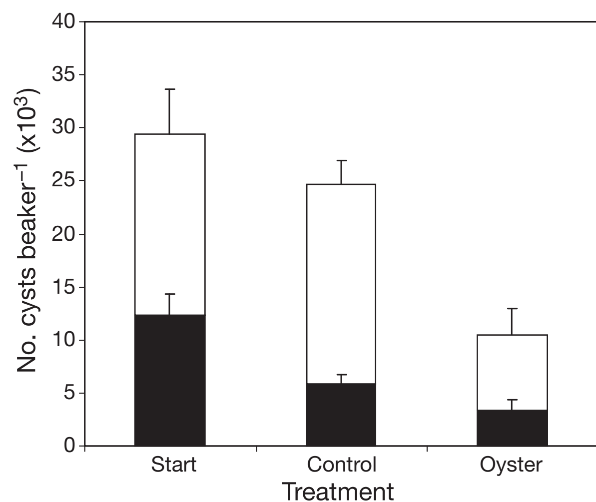

Fig. 2. Number of cysts per beaker (5 replicates/treatment). Filled (live) cysts are in black and empty cysts are in white. Error bars are SD of numbers of filled cysts (black) and total cysts (white). Start: total cysts in each treatment; control: empty shell treatments; oyster: filtering oysters

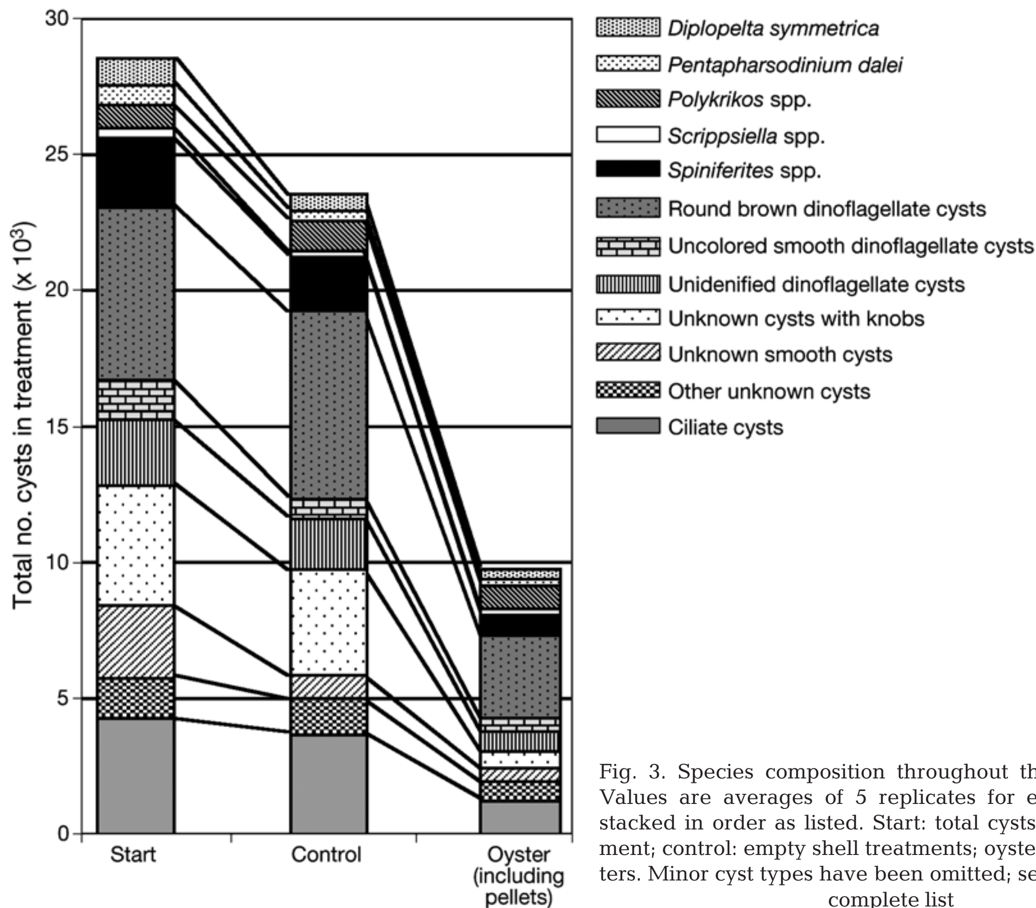

Fig. 3. Species composition throughout the experiment. Values are averages of 5 replicates for each treatment stacked in order as listed. Start: total cysts in each treatment; control: empty shell treatments; oyster: filtering oysters. Minor cyst types have been omitted; see Table 1 for a complete list

In Fig. 3 and Table 1 the cyst species composition is shown. There was a slight shift in species composition of cysts attributable to grazing: the proportion of cysts known to be fossilizable increased while cysts unknown as fossils decreased. Cysts of fossilizable species that are known to have hard and resistant walls (e.g. round, brown cysts of *Protoperidinium* spp. and *Diplopsalis*-like species and *Spiniferites* spp.), however, were reduced in numbers, as were cysts not known to have walls preservable over long time scales (e.g. smooth, uncolored cysts). Some cysts were easily germinated in the control: e.g. *Pentapharsodinium dalei*, *Plykrikos* spp., *Diplopelta symmetrica*, unidentified, uncolored dinoflagellate cysts, and ciliate cysts.

No significant changes in diversity of dinoflagellate cysts were found between experimental treatments ($p = 0.05$); approximately 30 different cyst species were found both before (mean ± SD, 32.8 ± 3.9) and after (28.6 ± 4.0) grazing. The control had 32.8 ± 3.3 different cyst species. No cyst species could be shown to disappear completely after grazing (although pellet samples contained fewer species [24.2 ± 5.4], but these samples were small).

DISCUSSION

The present study demonstrates clearly that benthic, protistan cysts are eaten and destroyed by the eastern oyster, and that empty cyst walls of fossilizable species, as well as unfossilizable cysts, are destroyed. This experiment has shown that *Crassostrea virginica*, when offered only this diet, reduced the number of cysts from the sediment to less than half of initial values in only 24 h of feeding. In nature, cysts are constantly grazed by filter feeders and deposit feeders, providing they are present in an oxic environment where animals can live. It is possible that the presence of sand grains causes a larger reduction in viable cysts through grinding, as in experiments with cultured cysts there was a smaller reduction in cyst numbers (Persson et al. 2006, Persson & Smith 2009). As much as 52 % of the filled cysts germinated during the experiment. As oysters from the same batch in other experiments cleared the same amount of sediment and cultured cysts within a few hours, it is unlikely that many cysts had time to germinate before being filtered by the oysters; however, cysts in the control remained in conditions favorable for germination during the entire 24 h experi-

Table 1. Relative abundance of cysts. Start: total cysts in each treatment; control: empty shell treatments; oyster: filtering oysters; pellet: cysts in oyster feces

Relative abundance (%)	Start	Control	Oyster	Pellet
Diplopelta symmetrica	3.4 ± 2.0	2.4 ± 0.5	3.7 ± 2.3	5.1 ± 3.9
Gymnodinium catenatum (cf.)	0.1 ± 0.1	0.1 ± 0.2	0.7 ± 0.7	0.3 ± 0.6
Lingulodinium polyedrum	0.4 ± 0.6	0.2 ± 0.3	0.3 ± 0.4	0.3 ± 0.4
Pentapharsodinium dalei	2.7 ± 0.7	1.4 ± 1.3	2.5 ± 2.5	2.6 ± 0.9
Polykrikos spp.	2.9 ± 0.9	4.5 ± 0.5	9.0 ± 3.1	6.0 ± 3.0
Protoceratium reticulatum	1.1 ± 1.2	1.6 ± 0.9	1.9 ± 1.3	1.4 ± 0.8
Protoperidinium americanum	0.2 ± 0.3	0.4 ± 0.3	0.9 ± 0.8	0.5 ± 0.7
Protoperidinium compressum	0.1 ± 0.1	0.2 ± 0.3	0.1 ± 0.2	0.3 ± 0.7
Protoperidinium conicoides	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0.1 ± 0.3
Protoperidinium monospinum	0.3 ± 0.2	0.2 ± 0.4	0.4 ± 0.4	0.1 ± 0.3
Protoperidinium nudum	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.8	0.0 ± 0.0
Protoperidinium oblongum	0.3 ± 0.4	0.4 ± 0.5	0.8 ± 1.2	1.0 ± 0.7
Scrippsiella spp.	1.0 ± 0.8	0.9 ± 0.7	1.6 ± 1.2	0.0 ± 0.0
Spiniferites spp.	8.7 ± 1.8	8.3 ± 3.4	7.9 ± 4.4	8.7 ± 3.0
Round, brown dinoflagellate cysts	22.2 ± 3.4	27.7 ± 3.6	28.9 ± 1.9	32.5 ± 3.4
Uncolored, smooth dinoflagellate cysts	4.8 ± 2.3	3.1 ± 2.4	4.9 ± 2.9	3.4 ± 4.0
Unidentified dinoflagellate cysts	12.5 ± 12.1	7.6 ± 4.6	6.9 ± 4.1	5.6 ± 3.3
Unknown cysts with knobs	13.3 ± 7.8	14.8±13.1	7.5 ± 4.2	9.0 ± 5.5
Unknown smooth cysts	10.7 ± 5.3	4.8 ± 3.0	6.2 ± 6.7	7.5 ± 6.0
Other unknown cysts	1.2 ± 0.5	6.4 ± 7.5	1.3 ± 1.0	3.7 ± 3.5
Ciliate cysts	14.1 ± 5.3	15.0 ± 8.2	14.1 ± 11.0	11.6 ± 2.7

ment. Cysts from anoxic sediments germinate very easily when transferred to culturing conditions, provided that the period of dormancy has passed and the cysts are in a state of quiescence (Anderson 1998, Persson 2002), awaiting suitable conditions for germination and continued growth. The presence of oxygen is thought to be the most important germination cue, but light is also known to trigger germination as well as a rise in temperature (Anderson 1998).

Some empty cysts that were found in initial samples could have been broken into smaller pieces from the stirring in the experiment and then sieved away during the rinsing process. This could account for some of the difference between initial and control numbers. Additionally, some cysts are not easily identified when they are empty (e.g. many of the round, uncolored cysts), which may also have caused some differences in cyst numbers found between initial and 24 h samples.

Anoxic sediments are known to have a different dinoflagellate-cyst composition than sediment from aerobic areas (Montresor et al. 1994, Zonneveld et al. 1997). Anoxic sediment was chosen for this feeding experiment based upon the assumption that the differences in species composition of dinoflagellate cysts between aerobic and anaerobic areas are caused, at least in part, by differences in the consumption and/or digestion of dinoflagellate cysts by animals (Persson et al. 2000, Persson & Rosenberg 2003). In anoxic sediment, cysts cannot be extensively modified by digestive processes of animals in the sediment, although some may have been subjected to zooplankton feeding while sinking. Cysts embedded in fecal pellets, however, would likely have been excluded from the fraction collected on the particle separator; thus it is reasonable to assume that the majority of cysts in the anoxic sediment used had not been previously consumed by other organisms.

There are 2 different views (research initiatives) of cysts that are of interest: the survey view and the bloom view. In a cyst survey, all cysts are of interest, not only live cysts. Some dinoflagellate cysts can only be identified to species level when empty (the opening, or archaeopyle, has a species-specific shape, but the cyst is simply round and brown when intact and alive). Surveys are very important for mapping the presence of species and it makes it possible to find dinoflagellate species that have a short bloom or are difficult to identify as free-living. For areas in which there is no current plankton survey program, dinoflagellate cyst surveys are crucial to find toxic species, such as *Alexandrium* spp. or *Gymnodinium catenatum*. With a bloom view it is important to consider such factors as cyst production, preservation, and germination. Mostly this is of interest for species that cause harmful or toxic blooms. Accordingly, the dynamics of *Alexandrium fundyense* blooms in the Gulf of Maine have been studied extensively (Anderson et al. 2005, McGillicuddy et al. 2005).

Information on ciliate resting cysts and literature on their identification is sparse, but growing. Recent information can be found in Gutiérrez et al. (2001), Kim et

al. (2002), Calvo et al. (2003) and Xu & Foissner (2005), and several tintinnid cysts were described by Reid & John (1978). Tintinnid cysts are often flask-shaped and closed with a plug in the opening from which the cell emerges during excystment (Reid & John 1978, Xu & Foissner 2005). Other ciliate cysts can be round with spines (e.g. *Stylonychia*) or round and uncoloured (e.g. *Euplotes*), and some ciliate cysts are often mistaken for dinoflagellate cysts (unidentified species or round, colorless cysts). Studies have been made on germination of cysts or emergence of cryptic cells from sediment samples (Fenchel et al. 1997, Finlay 1998, Persson 2002). In these studies, the germinated and/or emerging ciliates were identified, but the appearance and identity of different cyst types remained unknown. Both ciliate and dinoflagellate cysts can thus be smooth, colorless, light- to dark-brown, they can have spines of different shape and size, and they can have a cover of mucus to which debris attaches. These are most probably species-specific characters, not only for dinoflagellate but also for ciliate cysts (Xu & Foissner 2005). Most ciliate cysts remain unidentified. Approximately 200 dinoflagellate cysts have been identified (Dale 1983), but many remain unidentified.

A strong correlation has been shown between PSP toxicity levels in shoreline mollusks and abundance of toxic *Alexandrium* spp. cysts in sediments (Turgeon et al. 1990). The possibility that cysts sometimes may be the direct cause of toxicity in shellfish has been suggested by many authors (e.g. Yentsch & Mague 1979, White & Lewis 1982, Shumway et al. 1988, Anderson 1997, Bravo et al. 1998). Recently, Persson et al. (2006) showed that ingestion of toxic *Alexandrium fundyense* cysts caused accumulation of toxins in the eastern oyster *Crassostrea virginica*. The experiment in the present study showed that dinoflagellate cysts from natural sediments are ingested and digested by the same animal under the same experimental conditions (the same batch of oysters, experimental setup, room, etc.). There is no reason to believe that toxic cysts, if present in natural sediments, would escape or resist the action of suspension-feeding animals when sediment is resuspended. Thus, toxic dinoflagellate cysts can cause toxicity in shellfish at the decline of the bloom, when the cysts sink to the sediment, or even directly from the sediment. From these findings, it appears that a substantial reduction in numbers of toxic cysts may occur over time due to repeated grazing on cysts in oxygenated sediments, whereas anoxic sediment could act as a trap, preserving cysts of toxic (and other) species until they are resuspended by upwelling or a storm.

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Contribution to the Theme Section 'Directions in bivalve feeding'

In situ evidence for pre-capture qualitative selection in the tropical bivalve *Lithophaga simplex*

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ABSTRACT: Few feeding studies have been performed on tropical bivalves, and *in situ* feeding studies are lacking altogether. We investigated retention efficiencies for natural particles in the coral-boring tropical mytilid *Lithophaga simplex.* Using the *in situ* InEx technique (Yahel et al. 2005; Limnol Oceanogr Methods 3:46–58) SCUBA divers collected samples from the water inhaled and exhaled by undisturbed bivalves at the coral reef of Eilat (Gulf of Aqaba). Particle retention efficiencies were determined using flow cytometry analysis of the paired water samples. The photosynthetic bacterium *Synechococcus* (0.9 ± 0.1 µm) and larger eukaryotic algae (1 to 10 µm) were preferentially retained by the bivalve with removal efficiencies of up to 90% (1996 to 2000: averages of 69 ± 14% and 60 ± 17%, respectively, n = 74 individual bivalves). The minute photosynthetic bacterium *Prochlorococcus* (0.4 ± 0.1 µm) was also moderately retained (41 ± 19%). Only a small proportion of the non-photosynthetic bacteria (0.3 ± 0.1 µm) were retained (5 ± 18%, median of 9%), despite their numerical dominance in the plankton and considerable size overlap with *Prochlorococcus*. Size-independent preferential retention was also observed within particle types: (1) *L. simplex* more efficiently retained *Prochlorococcus* and picoeukaryotic algal cells with higher chlorophyll content and (2) the small fraction of non-photosynthetic bacteria retained did not differ in size, but had higher nucleic acid content (compared to the inhaled population) an indicator for viable and active bacteria. We conclude that particle retention is not strictly size-dependent in *L. simplex*, and probably involves other cell attributes such as cell surface properties and/or motility.

KEY WORDS: Suspension feeding · Nutrition · Selectivity · Coral reefs

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INTRODUCTION

Selective feeding is an important mechanism by which animals optimize their diet, e.g. by maximizing their energy gain or avoiding harmful food (Krebs & Davies 1997). The importance of selective feeding in structuring the pelagic microbial community is well documented (Pernthaler 2005), but much less is known about selection in benthic suspension feeders (see Yahel et al. 2006).

In the past 20 yr, bivalve suspension-feeding mechanisms have been vigorously investigated using a variety of relatively new approaches. Outcome-based techniques such as automated particle counting and flow cytometry have established the basic characteristics of suspension feeding, and provided important clues to underlying mechanisms. These have been investigated, in turn, using a variety of recently developed techniques to directly study various mechanism aspects (for reviews see Gosling 2003, Ward & Shumway 2004, Beninger 2009, this issue). While the mechanisms of particle transport, ingestion volume regulation, and pseudofaeces transport and rejection are all now reasonably well known (Gosling 2003),

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some aspects of particle processing by bivalves are still poorly understood. Among these, particle selection is, to date, a particularly intractable field, since it involves thousands of microscopic particles simultaneously passing over complex, and often occluding, processing structures, and no mechanism of recognition has yet been identified. It has long been known that bivalves are capable of particle selection (Ward & Shumway 2004). Direct observations have recently clarified the roles of the gills and the labial palps in particle selection in 3 of the 4 principal bivalve processing systems, and have established that the observed selection was effected post-capture (i.e. after the particles had been deviated from the through current onto the gill frontal surfaces: Beninger et al. 1997, 2004, 2007, Ward et al. 1998a, Cognie et al. 2003).

Our current knowledge of bivalve feeding is derived primarily from laboratory studies carried out with either laboratory-cultured algae or natural algal assemblages. It is yet unclear how valid the predictions and models based on laboratory experiments are to actual field situations; therefore, reliable methods are needed for *in situ* study of bivalve feeding (e.g. Cranford 1999, Cranford & Hill 1999, Kotta & Møhlenberg 2002, MacDonald & Nodwell 2003). The recent development of the InEx technique has enabled *in situ* sampling of inhalant and exhalent currents from undisturbed suspension feeders (Yahel et al. 2003, 2005, 2007).

Another gap in our understanding of bivalve feeding is related to the fact that the species investigated have overwhelmingly come from temperate, eutrophic, or mesotrophic habitats (Newell & Shumway 1993). These habitats are dominated by larger phytoplankton and characterized by relatively high loads of suspended sediments and large fluctuations of temperature and food availability. In contrast, few studies have investigated feeding characteristics and mechanisms in tropical suspension-feeding bivalves. Oligotrophic tropical waters are characterized by more stable conditions and are dominated by micron-size prokaryotic phytoplankton.

In the present study, we used the InEx sampling technique and subsequent flow cytometry analysis to quantify the *in situ* retention of naturally occurring phytoplankton and bacteria by the common tropical coral-boring mytild *Lithophaga simplex*.

MATERIALS AND METHODS

Study site. The study was carried out in the fore-reef (3 to 18 m depth) of the Eilat Coral Reef Nature Reserve, northern Gulf of Aqaba, Red Sea (Israel). For a description of the study site, see Genin et al. (2009) and references therein. The concentrations of chlorophyll (annual average: ~0.3 µg l^{-1}; Genin et al. 2009) and particulate organic carbon (~0.1 mg l^{-1}; Yahel et al. 2003) are low. The phytoplankton community in the Gulf of Aqaba is dominated by ultraphytoplankton (<8 µm; Yahel et al. 1998, Sommer et al. 2002). Small eukaryotic algae (0.6 to 4.0 µm) dominate during the winter mixing, whereas the cyanobacterium *Synechococcus* (0.9 to 1.2 µm; Sommer et al. 2002) dominates during spring blooms and is present at high concentrations (>10^4 ml^{-1}) throughout the year. *Prochlorococcus* (0.5 to 0.7 µm) is absent during the winter and dominates (numerically) the phytoplankton assemblage during the warm season, when the water is stratified and nutrient-depleted (Lindell & Post 1995). A thick (1 to 3 m) phytoplankton-depleted layer is usually found over the reef (Yahel et al. 1998), due to intense grazing by a rich guild of benthic phytoplanktivores, mainly soft corals, sponges, ascidians and boring bivalves (Yahel et al. 1998, Genin et al. 2009).

Lithophaga simplex. The Lithophaginae (Bivalvia: Mytilidae) are important borers of a wide variety of calcareous substrata, including dead and live coral skeletons. In the Red Sea, the mussel *L. simplex* Iredale, 1939 (Fig. 1) inhabits the massive coral *Astreopora myriophthalma* (Lamarck, 1816) (Fig. 1) and, less frequently *Goniastrea pectinata* (Mokady et al. 1992), but is specific to other corals in other localities (Morton 1983). The bivalve reaches a few centimetres in length and is completely enclosed by the coral skeleton (Fig. 1A), with only the distal end of the siphons (1 to 3 mm in diameter) extending into the surrounding seawater (Fig. 1B). As a mytilid, it probably possesses a homorhabdic filibranch gill structure (i.e. all filaments are of 1 simple tubular type, and the gill is not capable of post-capture qualitative selection), but we are not aware of any histological studies of the gill structure for this species. *A. myriophthalma* forms large colonies (up to several metres), often carrying dense populations of *L. simplex.* In the Gulf of Aqaba, several hundred *L. simplex* specimens may colonize a single coral head, with a mean density of 22 ± 11 mussels 100 cm^{-2} of coral surface (Mokady et al. 1998). The overall density of *L. simplex* at the study site was 2.5 ind. m^{-2}, about one-quarter of the overall *Lithophaga* spp. individuals we surveyed (Genin et al. 2009). The known benefits for the bivalves from this association include protection against predators and possibly the nutritional use of coral mucus (Shafir & Loya 1983). While boring bivalves are commonly regarded as parasites, *L. simplex* was shown to provide ammonium from its nitrogenous waste to the photosynthetic symbiotic algae of its coral host (Mokady et al. 1998).

Water sampling. An *in situ*, unintrusive technique, based on the simultaneous pairwise collection of the water inhaled and exhaled by the animal (InEx), was

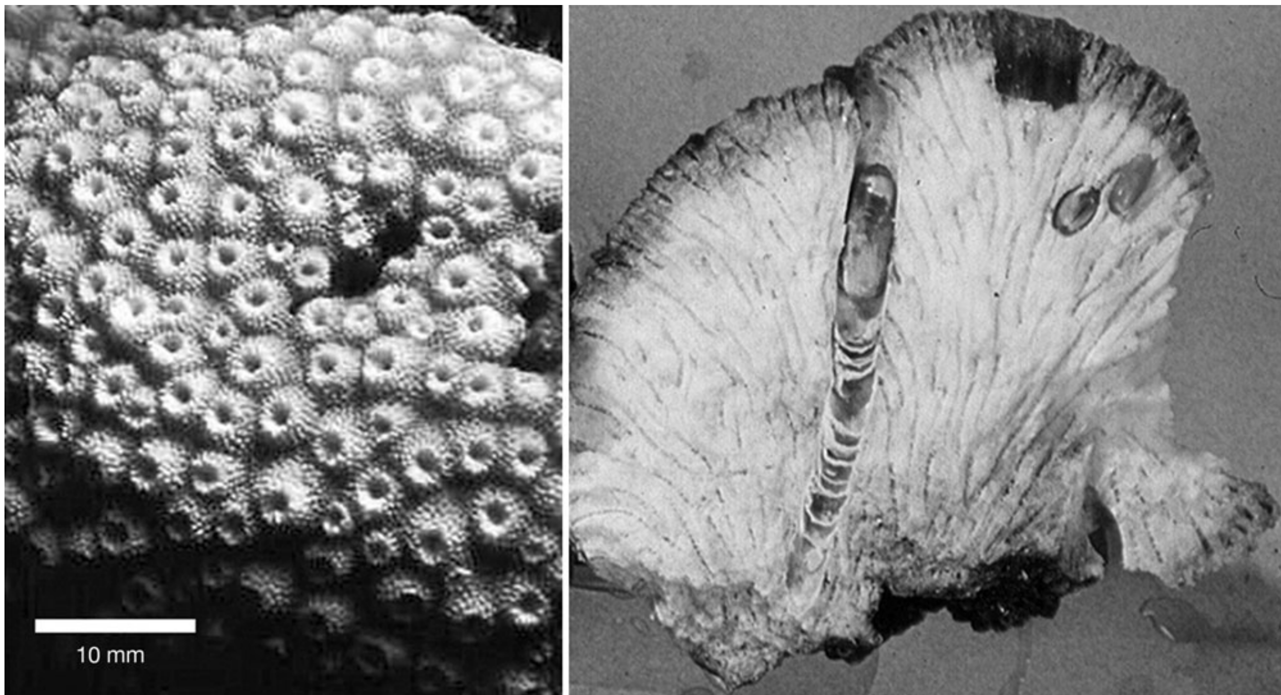

Fig. 1. (A) *Lithophaga simplex* inside the massive hermatypic coral *Astreopora myriophthalma*, as viewed from the outside (siphon diameter: ca. 2 to 3 mm). (B) The boring bivalve *L. simplex* inside its host *A. myriophthalma*. Photographs courtesy of I. Brikner and Y. Loya

used to directly measure the rate and efficiency of particle removal from the water filtered by the studied animals as detailed in Yahel et al. (2005). Briefly, a SCUBA diver sampled the inhaled water by slowly (~0.5 ml s^{-1}) withdrawing water into a modified plastic pipette, attached at its proximal end to a syringe, while holding its open (intake) end (3 to 5 mm) next to the bivalve inhalant siphon. A sample of exhaled water was taken simultaneously using an identical tube held within the exhalent jet, with the tube's intake end positioned <2 mm above the bivalve exhalant siphon, using the excurrent jet to flush and then fill the tube. We used 2, 3 and 7 ml samplers (LPt1, Pt5 and Pt10, respectively; in Yahel et al. 2005). Care was taken to avoid physical contact with the bivalve, and, when such contact occurred accidentally, the sample was aborted. Filling time (1 to 6 min, depending on the sampler volume) was determined individually for each pair so that it would last 150% of the time it took the exhalant jet to flush clear an identical tube pre-filled with fluorescein dye (measured a few minutes prior to each sampling). Sampling duration was >50 times longer than the few seconds it took the water to pass through the bivalve. Thus, each 'InEx' pair represented a several-minute integration of bivalve activity. The difference in particle concentration in the 2 samples provides a measure of the retention efficiencies of different particle sizes and types (Yahel et al. 2005).

The present study focused on the boring bivalve *Lithophaga simplex* living in *Astreopora myriophthalma* corals. Overall 74 pairs of InEx samples were collected from *L. simplex* in 16 dives during 10 sampling sessions spanning October 1996 to September 2000 and covering all 4 seasons.

Flow cytometry. A FACSort flow cytometer (Becton Dickinson, 15 mW, 488 nm, air-cooled Argon-ion laser) was used to measure the concentration and cell characteristics of non-photosynthetic microbes (hereafter referred to as non-photosynthetic bacteria [Bact]) and the 3 dominant autotrophic groups in the reef waters (*Prochlorococcus* [Pro], *Synechococcus* [Syn] and picoeukaryotes [Euk]). Taxonomic discrimination was made based on the orange fluorescence (Fl2) of phycoerythrin and the red fluorescence (Fl3) of chlorophyll (Marie et al. 1999), and on side scatter (SSC, a proxy of cell volume; Simon et al. 1994) and forward scatter (FSC, a proxy of cell size; Cunningham & Buonnacorsi 1992, Robertson et al. 1998). Each sample was analyzed twice. First, 150 to 300 µl of the sample water (>2.5×10^4 cells) was analyzed during 2 to 3 min, for determination of ultra-phytoplankton with the discriminator set to Fl3. A second run was used to analyze cells with no autofluorescence, i.e. non-photosynthetic

microbes. To visualize these cells, a 250 µl volume of the sample water was incubated with the nucleic acid stain SYBR Green I (20 min dark incubation at room temperature, $1:10^4$ of SYBR Green commercial stock) (Marie et al. 1999). About 50 µl of sample water ($>4 \times 10^4$ cells) was analyzed during a 2 min run with a low flow rate, and the discriminator was set to green fluorescence (Fl1). *Prochlorococcus* has very weak chlorophyll fluorescence near the surface, especially in summer. Thus, in some cases, when full separation from the noise was not possible, it was necessary to apply a Gaussian fit to a density distribution plot of the SSC or Fl3; this extrapolation allows better estimates of *Prochlorococcus* cell concentration. Only samples where the non-photosynthetic bacteria and *Prochlorococcus* populations could be fully resolved, from the noise and from each other, were used for subsequent analysis of cell population attributes.

Yellow-green beads (Polysciences; diameter: 1 µm) were used as an internal standard in each sample, and (unless stated otherwise in the text) all cellular attributes were normalized to the beads using the equation:

$$\overline{\text{Norm}}_{i,j} = \frac{\overline{\text{Population}}_{i,j}}{\overline{\text{Beads}}_{i,j}}$$

where $\text{Norm}_{i,j}$ is the normalized mean cell population i (e.g. *Synechococcus*) for the mean cell attribute j (e.g. SSC) (Marie et al. 1999). This normalization allows proper comparison of results obtained using different instrument settings.

The comparisons of cell properties were especially robust due to the paired sampling design applied in the present study (the same populations were compared in the same water prior to and after passage through the bivalve). The normalization to the calibration beads provided additional protection against instrumental drifts and shifts in sheath fluid properties.

Raw (list mode) data were recorded using 4 decades (10^0 to 10^4) log scale and 256 bins (channels) and analyzed using Cytowin (Version 4.1 developed by D. Vaulot, www.sb-roscoff.fr/Phyto/cyto.html#cytowin) or WinMDI (Version 2.8 developed by J. Trotter, http://facs.scripps.edu/software.html).

FSC is correlated to the size and the equivalent sphere diameter (ESD) of particles. Because these relationships are roughly linear for up to ~5 µm particles (Cunningham & Buonnacorsi 1992, Robertson et al. 1998, Cavender-Bares et al. 2001), FSC is widely used as a relative or absolute measure of size (Grob et al. 2007). For simplicity we report the ratio of FSC of particles to the FSC of 1 µm beads as cell size in micrometre units. Since we neither measured the cells microscopically, nor calibrated our flow cytometer measurements (Cavender-Bares et al. 2001, Shalapyonok et al. 2001, Grob et al. 2007), the sizes given here should be regarded as approximations, especially for larger particles (>8 µm) at the limits of the dynamic range used. Due to this limitation the flow cytometer counts were not transformed to cell biomass.

Log to linear transformation was done using the equation $x_{\text{lin}} = 10^{4x_{\text{log}}256^{-1}}$. No normalization to beads was applied to the density distribution examples presented below; thus, care was taken to average and compare only a few (>12) samples from adjacent water samples, which were run consecutively using exactly the same instrument settings.

Discrimination of the 4 particle types was not on the same taxonomic level. Moreover, the accuracy of discrimination was also variable; it was close to 100% for *Synechococcus* and the eukaryotic algae, but much lower for *Prochlorococcus* and non-photosynthetic bacteria that, on occasion, merged with the noise. To be conservative, we have omitted from the cell property analysis any samples in which the non-photosynthetic bacteria, *Prochlorococcus*, and noise could not be fully resolved. As a result, we have most likely overestimated the actual differences between the non-photosynthetic bacteria and *Prochlorococcus* populations. Population size has an important effect on the accuracy of statistical description of cell properties. Thus, larger confidence intervals were usually associated with smaller populations such as the eukaryotic algae (and in some seasons also *Prochlorococcus*), as well as with the reduced populations in the exhaled water.

Eventual differences in retention of live versus dead bacteria were investigated using the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining technique (Sieracki et al. 1999) for the InEx experiments undertaken in September 2000. An aliquot taken from each water sample was incubated in the dark, at room temperature, with 2 mmol l^{-1} CTC (Polysciences). Incubation was terminated after 15 min by the addition of 0.1% glutaraldehyde. The sample was then analyzed in a third run. In a positive control, established by enriching sample water with 1% LB medium, the CTC positive cell concentration doubled within 20 min.

Statistical analysis. The sampling design (InEx) was specifically developed as a 'pairwise comparison' (Yahel et al. 2005). Therefore, a 'within-subject' design (Rao 1997, i.e. paired *t*-test, repeated-measure ANOVA, and their nonparametric alternatives) was used throughout the analysis to test the null hypothesis of unselective retention. Data are reported as averages (±1 SD) unless stated otherwise. Statistical analyses were done using STATISTICA for Windows (Ver. 6.0, StatSoft).

Comparisons of cell size-frequency distributions were done within runs using samples stained with the nucleic acid stain SYBR Green I. *Prochlorococcus* was distinguished from the non-photosynthetic bacteria on the SSC versus red fluorescence cytogram based on their

red chlorophyll fluorescence. Bin averages were calculated for cell frequencies within each of the 256 logarithmically spaced bins (channels) and normalized as the percentage of the total number of cells in the respective population.

In classical grazing experiments, suspension-feeder activity affects food concentrations in the experimental vessel (Chesson 1983, Riisgård 2001a). Measuring direct filtration efficiency, as was done here, allows estimation of the Chesson selectivity index (α_i) as the maximum-likelihood estimator: $\hat{\alpha}_i = F_i\left(\sum_{i=1}^{m} F_i\right)^{-1}$ (Case 1 in Chesson 1983), where m is the number of particle types and F_i is the filtration efficiency for the ith particle type, calculated as: $F_i = (\mathrm{In}_i - \mathrm{Ex}_i)/\mathrm{In}_i$ (where In_i and Ex_i are the concentrations of the ith particle type in the water inhaled and exhaled by the studied animal, respectively). A separate α_i was calculated for each paired water sample. For better visualization α_i were rescaled to ε_i using the equation $\varepsilon_i = (m\alpha_i - 1)/[(m - 2)\alpha_i + 1]$, where m is the number of particle types available (Chesson 1983). Values of ε_i range from –1 when none of the ith type particles are taken, to 1 when the ith type particles are the only ones retained. However, since the statistical properties of the ε_i are not fully resolved, statistical inference was done solely with α_i values (Chesson 1983). To meet ANOVA requirements of homogeneity of variance and normality (verified by Cochran and Lilliefors tests, respectively), filtration efficiency was square-root and arcsine transformed, and Chesson α_i values were square-root transformed. Pairwise, post hoc comparisons of α_i values were made using the Tukey honestly significant differences (HSD) test for unequal n.

RESULTS

Ambient conditions

The water temperature at the study site ranged from 20 to 26°C, with rare occurrences of warmer (up to 28°C) days. The seasonal succession of phytoplankton was similar to that reported by Lindell & Post (1995), characterized by concentration shifts of several orders of magnitude for *Prochlorococcus* (null to 2.5×10^5 cells ml^{-1}) and eukaryotic algae (10^2 to 1.5×10^4 cells ml^{-1}). *Synechococcus* and the non-photosynthetic bacteria demonstrated much less variability, with changes between 10^4 to 3×10^4 cells ml^{-1} for *Synechococcus* (except for a few days during the spring blooms) and 0.5×10^6 to 1×10^6 cells ml^{-1} for non-photosynthetic bacteria. The ambient concentration of CTC positive (active) bacteria in September 2000 was $6 \times 10^4 \pm 3 \times 10^4$ ml^{-1} (n = 32).

Particle size and type

Over the entire study period, the mean size of non-photosynthetic bacteria populations in the ambient water averaged 0.3 µm (n = 140 flow cytometer runs) and those of the *Prochlorococcus* and *Synechococcus* were 0.4 and 0.9 µm, respectively. Eukaryotic algae were much larger with a mean FSC to 1 µm beads ratio of 7.4 ± 3.3. Within each water sample, the populations showed a wide size distribution, reflected by the large CVs of the population means (average: 262, 173 and 206% for the non-photosynthetic bacteria, *Prochlorococcus* and *Synechococcus*, respectively). As a result, the size distributions of the *Prochlorococcus* population within each water sample had typically >25% overlap with the non-photosynthetic bacteria on the one hand (Fig. 2) and with the *Synechococcus* population on the other (Fig. 3). Similarly, the size of *Synechococcus* cells overlapped with both the *Prochlorococcus* from below and the eukaryotic algae from above, albeit to a lesser degree.

Particle type retention

Particle retention by *Lithophaga simplex* varied according to particle type (repeated-measure ANOVA, $F_{3,117} = 254$, $p < 0.0001$; Fig. 3). *Synechococcus* cells

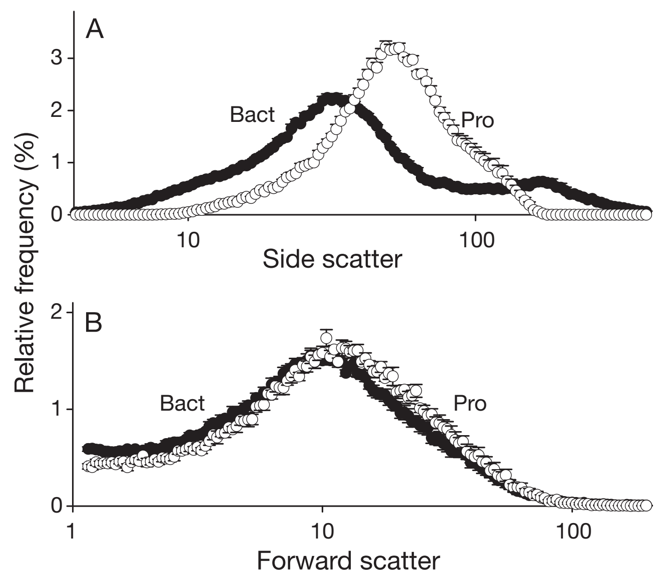

Fig. 2. Frequency distributions of the size and cellular properties of *Prochlorococcus* (Pro, ○) and non-photosynthetic bacteria (Bact, ●) in 10 representative samples collected in September 1998 from the ambient water next to the studied specimens. (A) Side scatter is related primarily to cell texture and volume of the cell. (B) Forward scatter is related to cell size. Horizontal axes are plotted using arbitrary, log-scale units. Error bars: 95% confidence intervals of the means. Note the large size overlap between the 2 bacterial populations

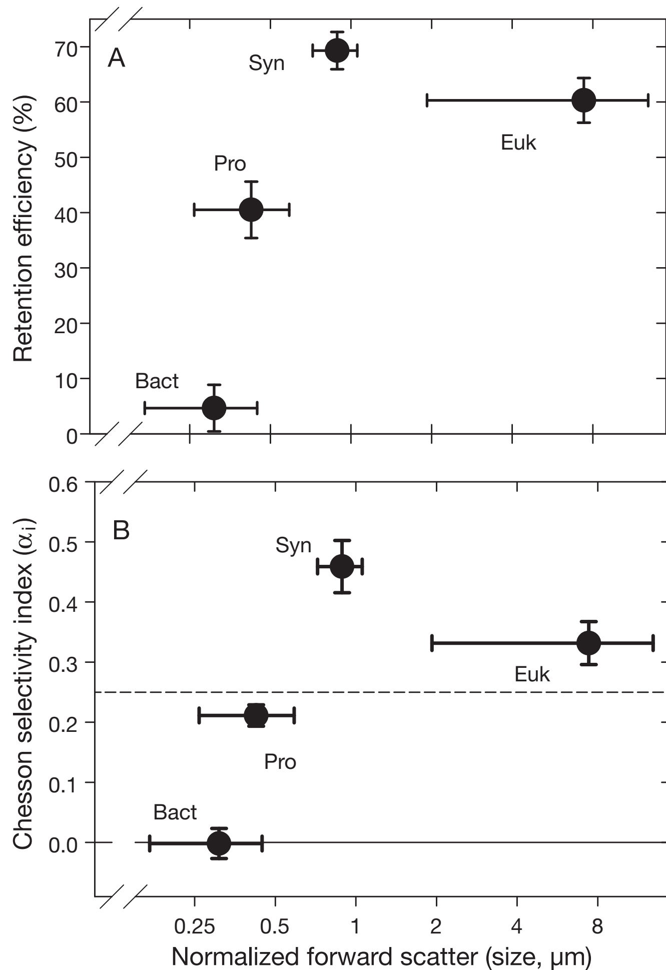

Fig. 3. *Lithophaga simplex*. (A) Average retention efficiency for each of the 4 particle types (Bact: non-photosynthetic bacteria; Euk: eukaryotic algae; Pro: *Prochlorococcus*; Syn: *Synechococcus*), plotted as a function of particle size. Vertical bars: 95% confidence intervals for mean retention efficiencies; horizontal bars: 90% prediction intervals for mean particle sizes. (B) Average selectivity index α_i (Chesson 1978) calculated for all InEx pairs when data for all 4 particle types were available. The expected value for non-selective feeding was 0.25 (dashed line). Vertical bars: 95% confidence intervals; horizontal bars: as in Panel A. Note the logarithmic scale of the *x*-axis

(0.9 ± 0.1 µm) were removed at efficiencies of up to 90% (average: 69 ± 14%; Fig. 3A). Larger eukaryotic algae (FSC: 7.4 ± 3.3) were removed with significantly reduced efficiency (60 ± 17%, paired *t*-test, $p < 0.001$), but comparison of selectivity indices indicated they were not significantly less preferred (Tukey HSD, $p = 0.066$). The minute photosynthetic bacterium *Prochlorococcus* (0.4 ± 0.1 µm) was also readily captured (41 ± 19%), but its removal efficiency was significantly lower (Tukey HSD, $p < 0.001$) than that of the other 2 photosynthetic groups. Surprisingly, only a small proportion of the non-photosynthetic bacteria (0.3 ± 0.1 µm) were removed (5 ± 19%) despite the large size overlap between those bacteria and *Prochlorococcus* (Fig. 2B). When present, >20% of the *Prochlorococcus* cells were removed in 88% of the InEx pairs (Fig. 4). In contrast, a removal of >20% of non-photosynthetic bacteria was observed in only 7 of 74 cases, with zero removal in 25% of the cases (Fig. 4).

Despite the marked seasonal changes in particle abundance, the removal of photosynthetic particles remained nearly constant, resulting in a quasi-linear functional response (Fig. 5A–C), and the particle retention ranking did not change in the different sampling seasons (Fig. 5E). Seasonal variations in retention efficiencies were only evident for the eukaryotic algae (Kruskal-Wallis ANOVA, $p < 0.05$), but without a clear pattern. Despite the low retention efficiency of the non-photosynthetic bacteria, their removal was also correlated with ambient (inhaled) concentrations ($r_p = 0.58$, $p < 0.001$), indicating that removal occurred mostly in higher than average bacterial concentrations ($>5 \times 10^5$ ml^{-1}; Fig. 5D).

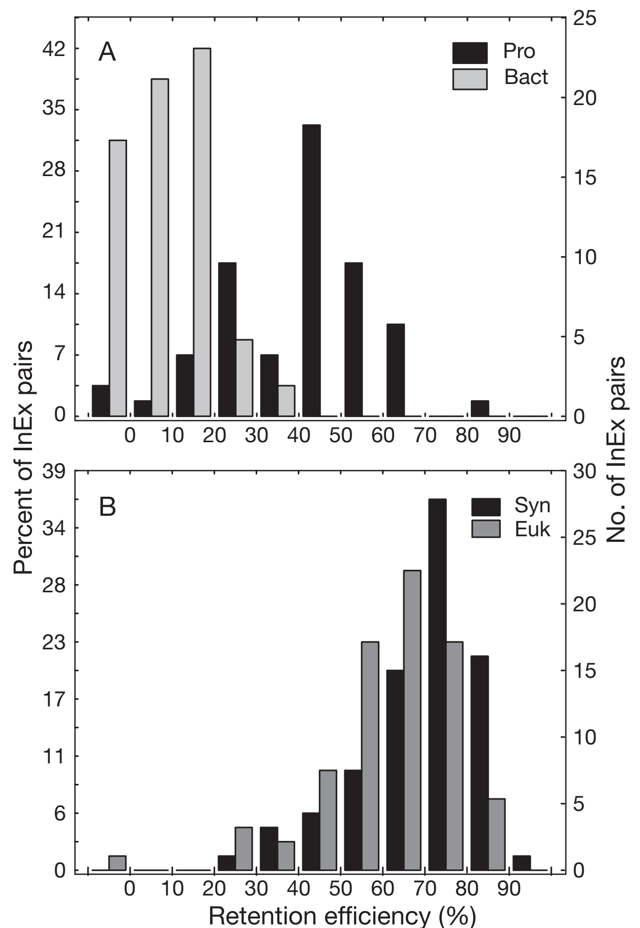

Fig. 4. Frequency of measured cell retention efficiencies for all 74 InEx pairs: (A) *Prochlorococcus* (Pro) and the non-photosynthetic bacteria (Bact) and (B) *Synechococcus* (Syn) and eukaryotic algae (Euk)

Selective retention within particle types

Selective retention was also observed within particle types. For example, within the *Prochlorococcus* and eukaryotic algae, cells with higher pigment content and higher SSC were always preferentially retained. Similarly, within the more homogeneous *Synechococcus* population such selectivity was observed at certain times of the year, notably the spring bloom of 1997. Here *Lithophaga simplex* exhibited a clear preference for *Synechococcus* cells with higher fluorescence (proxy of pigments and nitrogen content) and stronger SSC (proxy of cell texture and pigment content). Fig. 6 shows an example of a shift to the left in the optical properties distribution of the cells that were not re-

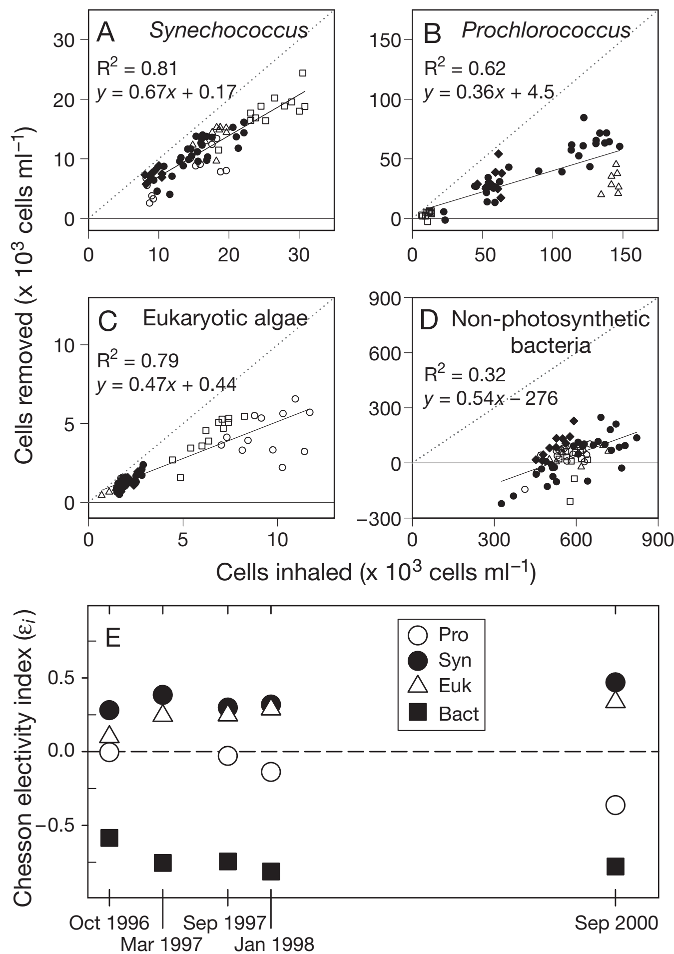

Fig. 5. Removal of each of the 4 ultra-planktonic particle types: (A) *Synechococcus* (Syn), (B) *Prochlorococcus* (Pro), (C) eukaryotic algae (Euk) and (D) non-photosynthetic bacteria (Bact), plotted against its ambient (inhaled) concentration. Dotted lines ($x = y$) represent 100% removal. Different symbols denote different sampling periods. ◆: October 1996; ○: March 1997; ●: September 1997; □: January 1998; △: September 2000. Linear regression statistics are for all seasons pooled. (E) Particle preference in the different sampling periods. Chesson α_i values were rescaled to ε_i so that they would be independent of the number of particle types available. ε_i values range from –1 to 1, where –1 indicates none of the ith type particles are retained, and an ε_i of 1 indicates cases when the ith type particles are the only ones selected. Zero is the expected value for ε if there is no selection. Error bars were omitted for clarity of presentation (Chesson 1983)

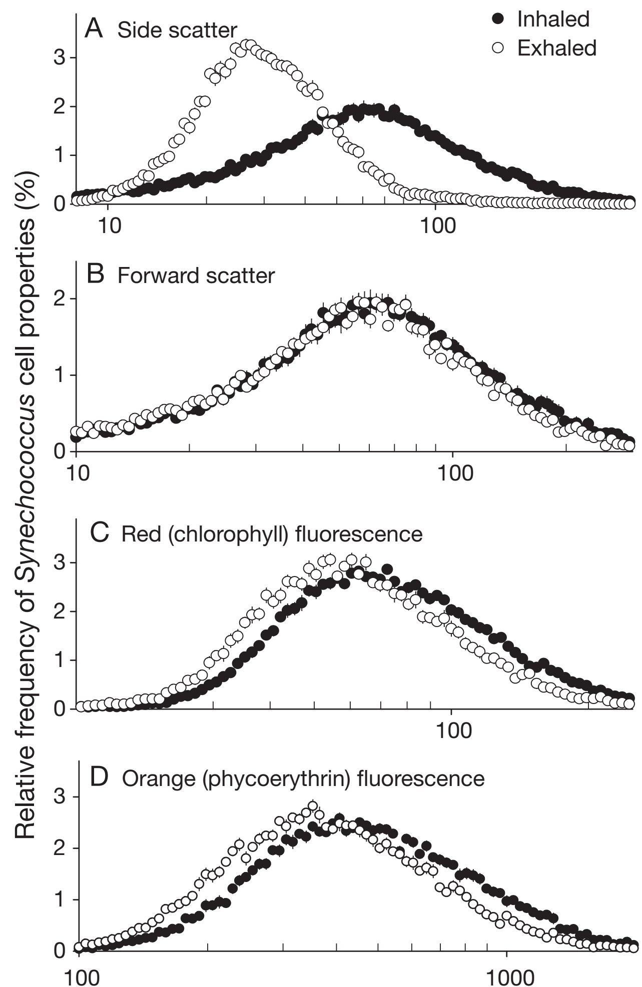

Fig. 6. An example of selective retention among a population of *Synechococcus* cells. The normalized frequency distributions of the optical cell properties (A to D) in the water inhaled (●) and exhaled (○) by 12 *Lithophaga simplex* were plotted. InEx samples were collected during 2 d in the 1997 eukaryote spring bloom (18 and 25 March 1997). As each sample was internally normalized, similar relative frequencies (y position) do not indicate similar concentrations (exhaled concentrations were considerably lower). List mode data were transformed from log to linear, and the skewness (Sk) and kurtosis (Ku) were calculated separately for each sample before normalization. The difference in the location of the distribution (population mean) was highly significant in all cases (paired t-test, $p < 0.001$). The shapes of the distributions were also significantly different for all but the phycoerythrin fluorescence distribution (Wilcoxon matched pairs test, $p < 0.05$ for the comparison of both the Sk and Ku within each pair). Orange fluorescence is related to *Synechococcus* phycoerythrin content (a light-harvesting and nitrogen-storage pigment). Horizontal axes are plotted using arbitrary, log-scaled units. Error bars: 95% confidence intervals of the means

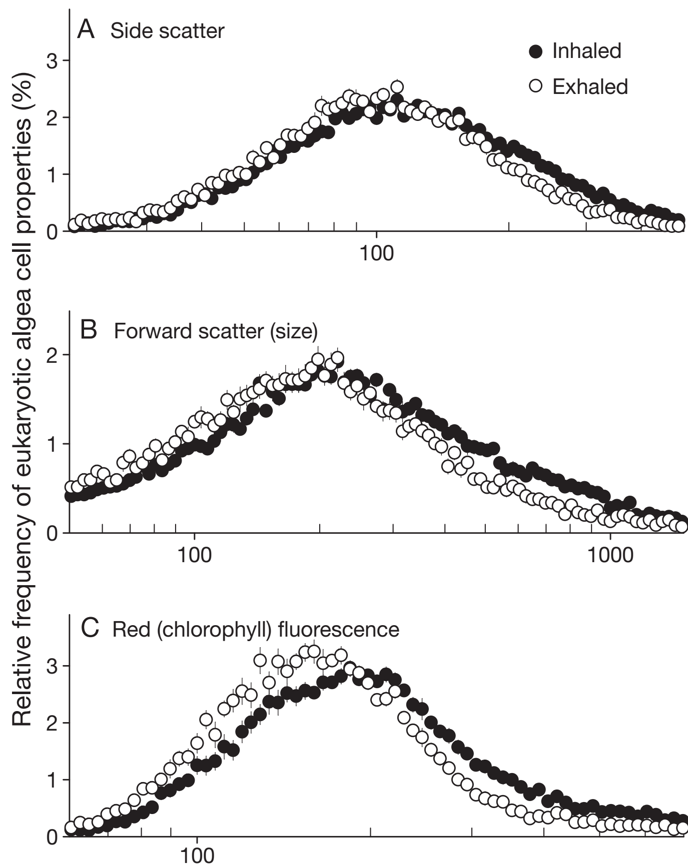

Fig. 7. As for Fig. 5A–C, but for eukaryotic algal (2 to 10 µm) cells. Since these cells normally do not contain phycoerythrin, the orange fluorescence signal was not plotted. The difference in the location of the distribution (population mean) was highly significant in all cases (paired *t*-test, $p < 0.001$). The shapes of the distributions were also significantly different for all but the side scatter distributions (Wilcoxon matched pairs test, $p < 0.05$)

tained by 12 *L. simplex* specimens. Selection for larger *Synechococcus* cells (shift to the left in the cells' FSC, a proxy of cell size) is much more muted in this example, but was evident in other seasons. Cell distributions were always peaked (Ku > 0), with a long right tail (Sk > 0) in both the inhaled and the exhaled waters, but the exhaled populations were significantly less skewed and less peaked (except for the red chlorophyll fluorescence). These results show that over and above the general preferential retention for *Synechococcus*, *L. simplex* preferentially retained higher quality *Synechococcus*. The same trend was also evident for the more diverse group of eukaryotic algae (Fig. 7).

The within-particle type preferences are summarized in Fig. 8, based on each of the cellular attributes recorded by the flow cytometer. Most notable is the preferential retention of cells with higher chlorophyll content within the populations of *Prochlorococcus* and eukaryotic algae (Wilcoxon matched pairs test, $p < 0.001$). *Lithophaga simplex* also preferentially retained larger *Synechococcus* and eukaryotic algae, whereas no such size preference was observed for the *Prochlorococcus* population. While *L. simplex* showed clear preference for *Synechococcus* cells with higher pigment content (e.g. Figs. 6 & 7) during some seasons (spring and summer 1997, autumn 2000), this was not the case during the autumn of 1996 and winter of 1998.

Interestingly, the small fraction (5%) of non-photosynthetic bacteria retained by *Lithophaga simplex* had significantly higher green fluorescence (nucleic acid content) in comparison to the nucleic acid content of the bacteria in the exhaled waters. A comparison between CTC positive and negative cells (Gasol et al. 1995) in 8 InEx pairs (September 2000) showed no significant difference (paired *t*-test, $p > 0.05$).

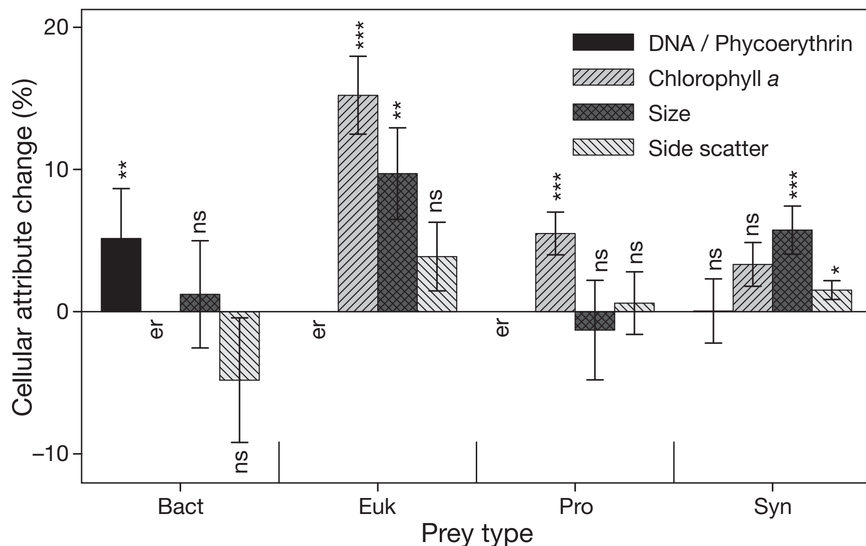

Fig. 8. Differences in cells' optical characteristics between inhaled and exhaled populations. Positive bars indicate positive selection for particles with higher cell attributes (fluorescence or scatter); negative bars indicate negative selection for the measured optical attribute of the cells. Each bar represents the average change calculated separately for each optical attribute in InEx pairs as: $100 \times (A_{In} - A_{Ex})A_{In}^{-1}$, where A is the respective attribute. The significance of the difference between inhaled and exhaled population attributes was tested using Wilcoxon matched pairs test (ns: no significant difference; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Attributes that were not relevant for the respective particle population are indicated as 'er'. Error bars: SE

DISCUSSION

The present study demonstrates *in situ* the selective, size-independent capture of planktonic microorganisms by the tropical boring mytilid *Lithophaga simplex*. Use of the InEx technique ensured that only the first step in the feeding process was targeted, namely, particle capture and retention from the highly diluted medium in which these tropical bivalves reside. Such pre-capture qualitative selection is biologically interesting from several standpoints. (1) It has not yet been documented for any bivalve (but see Yahel et al. 2006 regarding size-independent selectivity in sponges). (2) It obviously raises the question of possible mechanisms. Mucus trapping of particles deflected onto the gill filament frontal surface has been assumed to be the norm for homorhabdic-gill bivalves (Silverman et al. 1999, Beninger et al. 2003, Beninger & Decottignies 2008), but this is obviously incongruent with the results observed for *L. simplex.* (3) It is at variance with theoretical predictions of little or no selection under conditions of low particle concentration (Sierszen & Frost 1992). Concerning the latter point, it may be argued that suspension feeders in oligotrophic habitats are adapted to a different scale of relative particle concentrations.

The pioneering studies by Møhlenberg & Riisgård (1978), Palmer & Williams (1980), Jørgensen et al. (1984) and Riisgård (1988) established that retention efficiencies by a variety of bivalves representing the main processing systems were size-dependant, with an asymptotic increase in retention efficiency with increasing size of the food particles. Such size-dependant asymptotic curves may be typical for many particle types, but there is persistent evidence of partially size-independent retention for certain particle types (Kiørboe & Møhlenberg 1981; for review see also Ward & Shumway 2004). Reports of size-independent retention are particularly intriguing (Newell et al. 1989, Bougrier et al. 1997), since capture and retention are thought to be purely mechanical or hydro-mechanical processes (Jørgensen 1981, Beninger et al. 1992, 2003, Nielsen et al. 1993, Silverman et al. 1996b, 1999, Ward et al. 1998b, Beninger & Decottignies 2008, present paper).

To date, all studies reporting size-independent retention have used either cultured algae in laboratory experiments, or natural algal assemblages in laboratory experiments. Overwhelmingly, the majority of bivalve and algal species investigated have come from temperate, eutrophic, or mesotrophic habitats. Size-independent retention of phytoplankton has been reported for some bivalves feeding on >2 µm particles in temperate zones and conjectured to be due to differences in shape, flexibility, or motility (reviewed by Ward & Shumway 2004). Ward & MacDonald (1996) studied 2 sub-tropical bivalves in Bermuda (*Arca zebra*, Arciidae and *Pinctada imbricata*, Pteriidae). In the laboratory, both species showed typical size-dependant, pre-ingestive feeding responses, with an asymptotic increase in retention efficiency with increasing size of the food particles. *A. zebra*, but not *P. imbricata*, demonstrated particle selection, rejecting (in the pseudofeces) material with significantly higher carbon and lower nitrogen concentrations, thereby increasing the quality of material ingested by approximately 31%. Qualitative selection in the tropical pearl oyster *P. margaritifera* has also been inferred from gut content analysis (Loret et al. 2000); however, the techniques of both studies do not allow the distinction between pre- and post-capture selection.

The selective pattern observed for *Lithophaga simplex*, whereby pico-planktonic cells of similar size are retained in different efficiencies, argues for qualitative rather than mechanistic selection, presumably an adaption for enhancement of the quality of the retained particles. For example, the photosynthetic bacterium *Prochlorococcus* was efficiently removed (up to 88%), whereas non-photosynthetic bacteria that were essentially of the same size and shape were retained in null to very low efficiencies. The majority (90 to 95%) of the non-photosynthetic bacteria in the oligotrophic waters at our study site were CTC negative, indicating dead, inactive, starved, or growth-arrested cells (see Davidson et al. 2004 for a review and contrasting data). Such bacteria are less preferred by protozoan grazers (del-Giorgio et al. 1996, Jürgens & Matz 2002).

Retention efficiencies and Chesson selectivity indices were significantly different among all 4 prey taxa examined (non-photosynthetic bacteria, *Prochlorococcus*, *Synechococcus* and eukaryotic algae) despite the large overlap of the cell size distribution (typically >25%; Fig. 2, and compare Figs. 6B & 7B).

Within each of the photosynthetic cell populations, there was a general pattern of preferential retention of cells with higher chlorophyll content. Similarly, the small fraction of non-photosynthetic bacteria that was retained by the bivalve had a significantly higher nucleic acid content, compared to the rejected bacteria. These selectivity patterns prevailed regardless of large seasonal shifts in the planktonic community composition and abundance. The most notable exception was the reduced retention efficiency of *Prochlorococcus* in September 2000 (Fig. 5B,E).

It should be noted that, while only a small portion of the non-photosynthetic bacteria was retained by *Lithophaga simplex*, other homorhabdic bivalves such as *Geukensia demissa* and *Dreissena polymorpha* efficiently retained such small cells (Kreeger & Newell 1996, 2001, Silverman et al. 1996a). Nevertheless, assimilation of bacteria seems to be less efficient in comparison to phytoplankton (Kreeger & Newell 1996,

Nichols & Garling 2000). Microscopic observation using nucleic acid staining (DAPI) reveals that, while most of the picoplankton was freely suspended (unattached), some bacteria were attached to aggregates, mucus, or marine snow (G. Yahel unpubl. data). It is therefore plausible that the small proportion of non-photosynthetic bacteria removed by *L. simplex* may have been ingested with such aggregates (Kach & Ward 2008). Indeed, most of the bacteria removal occurred during spring blooms, when organic aggregates were prevalent in reef waters (G. Yahel pers. obs.).

Preference for cyanobacteria over diatoms (all >3 µm) has been reported for several freshwater unionids (Baker & Levinton 2000, 2003). However, in these cases, clear size differences existed between the different particle types, and selection was post-retention. The preferential retention of *Synechococcus* observed in the present study has also been observed in other coral reef suspension feeders examined using the InEx technique (Yahel et al. 2005, G. Yahel unpubl. data). The concentration of *Synechococcus* pigments was significantly reduced in comparison with other algal pigments in the water flowing over the reefs in Curacao, indicating its selective grazing (van Duyl et al. 2002). This contrasts with the situation observed for the pelagic habitat in the Gulf of Aqaba, where *Synechococcus* was shown to be the least-preferred cell type (Sommer et al. 2002). As the phytoplankton supply to the studied reef is mostly affected by pelagic processes (Genin et al. 2009), the seston available to reef benthic suspension feeders such as *L. simplex* may be impoverished of cells other than *Synechococcus*; thus, these benthic suspension feeders may have evolved to accept, or even select, these cyanobacteria.

Capture and retention of particles are generally considered to be purely mechanical or hydro-mechanical processes (Jørgensen 1981, Beninger et al. 1992, 2003, Nielsen et al. 1993, Silverman et al. 1996a,b, 1999, Ward et al. 1998b, Beninger & Decottignies 2008). This view is at variance with the observations of size-independent retention reported here. There is at present no theoretical or practical framework for understanding differential retention of particles of similar sizes, shapes, flexibilities and densities. The most prominent shift between the inhaled and exhaled populations (Fig. 6) is observed in the SSC, which is generally associated with internal complexity and external cell surface traits. It is thus tempting to suggest that selection is made according to external cell characteristics that are revealed by differences in SSC. However, preference for higher pigment content (see also Fig. 8) suggests that other mechanisms such as chemosensory detection may also be at work (Beninger et al. 2008a). Shimeta (1993) and Shimeta & Koehl (1997) suggested that the clearance of sub-micrometer particles should be greatly enhanced by cell motility. Indeed, swimming behaviour has been reported in several cultured *Synechococcus* strains (Brahamsha 1999), and these are the particles that were most efficiently retained in the present study. Motility has not been documented for *Prochlorococcus* cells, which were much less efficiently retained in the present study. Differences in cell motility may thus be a factor in size-independent particle retention.

Another feature that might help account for size-independent particle retention is some form of particle recognition prior to capture. Such recognition has been reported post-capture in 2 marine bivalve species for the perifrustular envelope of diatoms (Beninger et al. 2004, 2005, 2008a,b). This would be an interesting subject for future research on the mechanisms of pre-capture, size-independent particle retention.

If the preferential retention of *Synechococcus* is related to quality (e.g. Ward et al. 1997), it is of interest to determine what quality features may be discriminating. High-energy, low N and P coral mucus, rather than particles, was suggested as an important source of carbon for *Lithophaga simplex* (Shafir & Loya 1983). If this is so, then we suggest that the pre-capture qualitative selectivity observed in *L. simplex* may not maximize carbon or energy gain, but rather other nutrients, as has been observed in some freshwater bivalves (Nichols & Garling 2000) and oysters (Ward et al. 1997).

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Contribution to the Theme Section 'Directions in bivalve feeding'

Comparative utilization of phytoplankton and vascular plant detritus by the cockle *Cerastoderma edule*: digestive responses during diet acclimation

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ABSTRACT: Physiological components of food absorption (rates of particle clearance and ingestion and absorption efficiencies) were recorded in the cockles *Cerastoderma edule* fed variable concentration diets of phytoplankton (*Isochrysis galbana*) and detritus from salt-marsh vascular plants (*Spartina maritina*), after 2 and 12 d of acclimation to these diets. Corresponding digestive processes were also analysed, including determinations of gut passage time of food, gut fullness (GF) and digestive investments, the latter measured in terms of both metabolic faecal losses (MFL) and enzyme activities of the digestive glands. Digestive responses to increasing availability of phytoplankton included faster processing of food particles coupled to improved hydrolytic capacity of digestive glands that resulted in increased gut performance. Compared to phytoplankton, detritus utilization was constrained in the short-term by its refractory behaviour to digestion and the magnitude of metabolic faecal losses experienced by cockles fed these food materials. However, increased GF achieved during acclimation to detrital diets compensated for reduced digestibility, allowing for more food to be processed and thus cancelling out the effects of reduced passage time (and the consequent decline in absorption efficiency). Induction of the appropriate set of carbohydrases during the acclimation process resulted in increased amounts of reducing sugars released from natural substrates, with the additional benefit of diminishing the losses of endogenous materials accompanying each cycle of intracellular digestion.

KEY WORDS: Food absorption · Phytoplankton · Detrius · Digestive enzymes · Gut passage time · Bivalve molluscs · *Cerastoderma edule*

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INTRODUCTION

Seston accessible to marine filter feeders includes a diversity of organic particles varying in nutritional value, from living phytoplankton to detritus of different origins. Contribution of detritus to growth and production of benthic populations, particularly marine bivalves, has been a subject of special attention, since this material constitutes a significant fraction of suspended particulate organic matter (POM), particularly in estuarine systems and during the seasons not dominated by phytoplankton (Wienke & Cloern 1987). For instance, phytoplankton biomass estimated from chlorophyll concentration explains <40 % of POM, on average, even in the highly productive Galician rías (Cabanas et al. 1979, Navarro et al. 1996, Figueiras et al. 2002), while this proportion would appear considerably reduced in coastal areas affected by resuspension of bottom sediments: viz. 3 to 12 % in Marennes-Oleron Bay (Prou et al. 1994, Urrutia et al. 1996, Kang et al. 1999); 6 to 45 % in the Oosterschelde estuary (Smaal et al. 1986, Smaal & Haas 1997). In the area where the present study was conducted, this figure has been found to fluctuate, on a seasonal/tidal basis, between 3 and 28 % (authors' unpubl. data).

There has been considerable discussion in the literature concerning both the origin of detritus present in the different coastal systems and its nutritional value

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for suspension feeders (Kirby-Smith 1976, Seiderer et al. 1982, Stuart et al. 1982, Mann 1988, Fielding & Davis 1989, Langdon & Newell 1990, Alber & Valiella 1994,1995, Bustamante & Branch 1996, Levinton et al. 2002, Huang et al. 2003, Newell et al. 2005). Information on the subject has benefited extraordinarily from the generalized use of stable isotopes as 'signatures' to trace the origins of nutrients that contribute to tissue growth in marine species. Most of these studies have revealed that macrophyte-derived detritus might explain a significant amount of nutrients assimilated by bivalve populations growing in areas where these plants are relatively abundant (55 to 85% for kelp detritus: Bustamante & Branch 1996; 40 to 50% for salt-marsh vascular plants: Langdon & Newell 1990). Peterson et al. (1985, 1986) estimated 30 and 80% contribution of *Spartina alterniflora* to the nutrition of *Mytilus edulis* and *Geukensia demissa*, respectively, while Riera & Richard (1996, 1997) and Kang et al. (1999) reported spatial and seasonal variations in stable isotope composition of oyster *Crassostrea gigas* and cockle *Cerastoderma edule* tissues that reflected the incorporation of detritus of terrestrial origin into the water column.

Comparison of the above figures with direct physiological measurements of detritus utilization by marine bivalves reveals a complex situation whereby processing rates and assimilation efficiency vary according to both bivalve species and detritus origin. Tenore et al. (1982) suggested that vascular plants contain a high percentage of unavailable energy that can only be utilized after microbial decomposition, whereas detritus from seaweeds would be less refractory to digestion by bivalves and so could be more readily utilized. Accordingly, various authors have reported absorption efficiencies as high as 40 to 60% in the mussels *Aulacomya ater* and *Choromytilus meridionalis* (Stuart 1982, Stuart et al. 1982, Fielding & Davis 1989) and up to 87% in the scallops *Placopecten magellanicus* (Cranford & Grant 1990) fed kelp detritus (but see Charles 1993, Charles et al. 1996, concerning the deposit feeder *Abra ovata*). Meanwhile, measurements of carbon gross absorption efficiencies (GAEs) in other bivalves fed lignocellulosic detritus obtained from *Spartina alterniflora* ranged between 3% in the oyster *Crassostrea virginica* (Crosby et al. 1989) and 4 to 25% in the case of the mussel *Geukensia demissa* (Kreeger et al. 1990, Charles & Newell 1997, Kreeger & Newell 2001).

Regarding the ecological significance of salt-marsh plant detritus, the integration of physiological data with data about the environmental availability of cellulosic detritus in Canary Creek marsh (Langdon & Newell 1990) has revealed that the contribution to carbon and energy requirements of bivalves ranged from 0.7 to 2.1% in the oysters *Crassostrea virginica* and from 5.2 to 8.6% in the mussels *Geukensia demissa*. Although these figures might increase up to 20% in other systems, on account of the much higher detritus concentration in the seston (Crosby et al. 1989), they differ greatly from comparable estimations based on stable isotope enrichment. Consequently, a reconsideration of physiological data seems opportune, particularly data that concern assimilation efficiency values.

The aim of the present paper was to compare the utilization of phytoplankton and organic detritus from vascular plants by the infaunal bivalve *Cerastoderma edule*, a species living in food environments characterized by broad seasonal and spatial fluctuations in the relative importance of these 2 food sources (Navarro et al. 1997, Kang et al. 1999). Food assimilation was analyzed under a set of experimental conditions that resulted in a range of feeding rates and variable degrees of diet acclimation in an attempt to explore the effect of these factors on digestive performance for both food types. This approach is thought to provide physiological information useful in modeling growth and production of bivalve species inhabiting coastal systems submitted to a complex food regime.

MATERIALS AND METHODS

Collection and maintenance of animals. Cockles *Cerastoderma edule* measuring around 28 mm in shell height were collected in an intertidal mudflat in the Plentzia Estuary (Biscay, northern Spain) in February (Expt 1) and May 1999 (Expt 2). On each occasion, some 50 specimens were collected, immediately transported to the laboratory and placed in individual containers immersed in feeding tanks filled with seawater provided from a re-circulating system set at environmental temperature (12 and 16°C, for Expts 1 and 2, respectively) and 33‰ salinity. Cockles were divided into different groups each receiving a diet which differed in terms of either composition (phytoplankton or particulate organic detritus) or particle concentration; they were fed these diets for up to 12 d.

Characteristics of experimental diets. Diets were designed to have a common organic content, OC (~80%), but to be based on 2 different types of organic particles, either phytoplankton cells of the species *Isochrysis galbana* (Diet P) or particles of organic detritus (Diet D). The latter were obtained from freshly collected specimens of *Spartina maritima* that were freeze-dried and ground and the resulting powder was sieved to select the particle fraction <45 µm. OCs of the particulate suspensions of both phytoplankton and detritus were adjusted to the chosen values by adding ashed silt particles <45 µm obtained from natural sediments from the estuary.

Concentrated stocks of these diets were dosed into the feeding tanks at rates set to provide for stable particle concentrations that were checked by frequent monitoring of particulate volumes with a Coulter Multisizer. In Expt 1, Diets P and D were provided at a single particle concentration of 1.5 $mm^3\ l^{-1}$. In Expt 2, each composition was dosed at 2 concentrations of 1 (low concentration) and 2 $mm^3\ l^{-1}$ (high concentration). No pseudofaeces production was observed with any of these diets.

On 6 occasions, duplicated water samples taken from the feeding tanks were filtered onto ashed preweighted GF/C glass-fiber filters, which were subsequently processed to determine concentrations (mg l^{-1}) of total particulate matter (TPM), particulate inorganic matter (PIM) and POM (mg l^{-1}) in the feeding suspensions. Salts retained in the filters were rinsed out with a solution of ammonium formate seawater isotonic, and then filters were dried at 110°C, weighed, ashed at 450°C and weighed again. TPM and PIM were estimated, respectively, as the dry and ash weight increments of the filters, and POM was estimated as the weight loss of this filtered material on ashing. OC was finally estimated as POM/TPM.

Physiological measurements. Physiological determinations were carried out after 2 d (short term) and 12 d (long term) of acclimation to the above dietary conditions. Clearance rate (CR, l h^{-1}), ingestion and absorption rates (IR and AR, mg h^{-1}) and net absorption efficiency (NAE) were determined during Expt 1 according to the biodeposition method (Iglesias et al. 1998). To this effect, faeces produced by individual cockles were collected over defined time intervals, filtered onto GF/C filters and processed for inorganic and organic weight determinations of faecal material. The biodeposition method assumes conservative processing of inorganic particles within the gut of marine bivalves and, hence, the inorganic egestion rate (IER) would equal the inorganic ingestion rate (IIR, mg h^{-1}), whence CR could be estimated as IIR/PIM. Total IR and organic ingestion rate (OIR) were then calculated as the product of CR and TPM and CR and POM, respectively. The net absorption rate of organics (NAR, mg h^{-1}) was computed as the difference between OIR and the rates of organic egestion (OER), and NAE, as the ratio AR/OIR. Technically this measurement of absorption efficiency corresponds to the Conover method (Conover 1966) as it is based on the same principles (see Iglesias et al. 1998). Determinations of gut passage time of food (GPT, h) and GAE, performed during Expts 1 and 2, and IRs in the case of Expt 2, were based on the radioactive labeling of food particles using ^{14}C-formaldehyde (López & Crenshaw 1982, Charles et al. 1995). Pure cultures of *Isochrysis galbana* and concentrated suspensions of detrital particles obtained from *Spartina maritima* in seawater were incubated for 48 h with ^{14}C-formaldehyde (1.1 µm Ci mg^{-1} organics), then concentrated by centrifugation; the pellet was then re-suspended in 0.45 µm filtered seawater, in order to remove unreacted formaldehyde. This label was checked to remain stable for at least 48 h. OC of these diets was finally adjusted to the intended 80% by adding the appropriate amount of silt particles.

Radiolabeled diets were supplied to cockles in 1 h pulses at the particle concentrations specified above. Cockles were then transferred to unlabeled diets, and the faeces produced individually were collected at frequent intervals for up to 30 h. For radioactivity determination, samples of food and faeces were filtered on GF/C glass-fiber filters, processed in a Sample Oxidizer (Packard, Model 307) and then counted by liquid scintillation. A curve of cumulative isotope egestion (dpm) was fitted to each individual set of data, and GPTs were estimated as the time required for 95% ^{14}C egestion (Hawkins et al. 1990). ^{14}C ingestion (= ^{14}C added – ^{14}C remaining) was occasionally (Expt 2) used to compute IR using the specific radioactivity of diets. The efficiency of isotope absorption [(^{14}C ingested – ^{14}C egested) / ^{14}C ingested] was taken to represent the absorption efficiency of dietary organics (GAE), and the gross absorption rate (GAR, mg h^{-1}) was computed as the product OIR $\times$ GAE.

The difference between gross and net rates of absorption (GAR – NAR) has been termed metabolic faecal loss (MFL) (Hawkins & Bayne 1985, Bayne & Hawkins 1990), representing the endogenous materials contributed to digestion that are not reabsorbed.

Gut fullness (GF) was computed as the product of organic ingestion and GPT, to represent the amount of food (mg) held by the gut each moment. Digestive performance (DP) was defined as the ratio GAR/GF, or absorbed energy per unit weight of food held in the gut. By construction, it also represents the absorption efficiency (GAE) per unit GPT.

Digestive enzyme activities. Once physiological determinations were concluded, digestive glands of 5 to 7 ind. per condition were dissected, freeze-dried and stored at –40°C for further analysis. Individual digestive glands were homogenized in cold 0.01 M phosphate-citrate buffer (pH 6.9), centrifuged at 5100 × *g* for 30 min, and the clear supernatant was used for *in vitro* enzyme assays.

Two kinds of assays were performed with this extract: (1) standard carbohydrase and protease activity determinations based on commercial substrates (Expts 1 and 2) and (2) determinations of reducing sugar release from 'natural substrates' (Expt 2).

During standard determinations, crude extracts were tested against commercial starch, carboximethylcellu-

lose, laminarin and casein (for details see Ibarrola et al. 1996) in order to ascertain amylase, cellulase, laminarinase and total protease activities.

In enzyme activity determinations on 'natural substrates' crude extracts were assayed for total carbohydrase activity on lipid-free extracts of *Isochrysis galbana* cells and detrital particles of *Spartina maritima* that were obtained as follows: 60 l of phytoplankton culture were concentrated by continuous centrifugation up to a final volume of 200 ml; on the other hand, 2 g of the particulate *S. maritima* material used in the confection of detrital diets was suspended in 100 ml of filtered (0.45 µm) seawater. These concentrated stocks were lipid extracted with a mixture of chloroform/methanol/water (2:2:1), according to Bligh & Dyer (1959). The chloroform phase was discarded, and the procedure was repeated until total absence of coloration in the lipidic phase was observed. After centrifugation (20 min at 3300 × *g*), the supernatant was discarded and the pellet was rinsed 3 times with 0.2 M phosphate-citrate buffer (pH 6.9), and finally re-suspended in 100 ml of the buffer. Before the enzymatic assays, these re-suspensions were boiled for 30 min in order to obtain a homogeneous solution. Carbohydrase activity was measured following the Nelson-Somogyi method (Nelson 1944, Somogyi 1952).

Size standardization. At the end of experiments, the soft body of cockles was dissected out of the shell, dried at 80°C for 48 h and weighed. For cockles used in enzyme analysis, freeze-dried digestive glands and dried remaining tissues were weighed separately and total dry weight was obtained by addition. Physiological measurements, as well as the size of digestive glands, were standardized to an equivalent 200 mg dry-weight cockle according to the expression:

$$Y_s = (200/W_e)^b \, Y_e \quad (1)$$

where Y_s represent the standardized measurement, W_e is the actual dry weight, Y_e is the uncorrected measurement and b is the specific weight power that scales each measurement to body weight: b = 0.56 for rates of food processing by cockles (Newell & Bayne 1980), b = 0.41 for GPT (Hawkins et al. 1990) and b = 0.82 for dry weight of digestive gland (Ibarrola et al. 2008).

Digestive enzyme activities were expressed as specific activity (micrograms of released product per milligram of dry weight of digestive tissue) and total activity (micrograms of released product per standard-sized digestive gland).

Statistical procedures. Physiological measurements and digestive enzyme activities were compared for differences between treatments by means of multiple factor ANOVA, after homogeneity of variances was confirmed by means of a Bartlett test (Zar 1984). Nonlinear regression analyses were performed using SYSTAT 5.2 statistical package (SYSTAT 1992), and significant differences between coefficients were tested using a *t*-test (Zar 1984).

RESULTS

Physiological measurements

Expt 1

Characteristics of diets recorded during Expt 1 are reported in Table 1. Both total concentration (TPM and POM) and OC of suspended particles were kept constant irrespective of dietary composition (P vs. D). Differences in length of acclimation (short- vs. long-term), however, were significant for TPM and POM, but not for OC (Table 2).

Mean values of physiological variables measured in cockles *Cerastoderma edule* fed P and D diets for 2 and 12 d are given in Table 3. A 2-factor ANOVA applied to test the significance of mean differences for primary measurements (CR, NAE, GAE and GPT; Table 4) reveals the following. (1) Diet composition and time of acclimation per se had no significant effect on CR,

Table 1. Expt 1: characteristics of experimental diets recorded in short-term (2 d) and long-term (12 d) acclimation to diets of phytoplankton (P: *Isochrysis galbana* cells) and detritus (D: detrital particles from *Spartina maritima*). TPM: total particulate matter (mg l^{-1}); POM: particulate organic matter (mg l^{-1}); OC: organic content (fraction). Values are means of 6 to 13 determinations (±95% CI)

Diet	Acclimation	TPM	POM	OC
P	Short-term	1.54 ± 0.20	1.28 ± 0.15	0.83 ± 0.04
P	Long-term	1.78 ± 0.14	1.52 ± 0.09	0.86 ± 0.06
D	Short-term	1.57 ± 0.25	1.29 ± 0.21	0.81 ± 0.04
D	Long-term	2.18 ± 0.13	1.79 ± 0.14	0.82 ± 0.02

Table 2. Expt 1: Analysis of variance (ANOVA). Post hoc test (Fisher's protected least significant difference, PLSD) for mean significant differences in TPM, POM and OC. Abbreviations in Table 1. *$p < 0.05$

Effect	Mean difference	Critical difference	p
TPM			
Diet	−0.162	0.217*	0.1324
Acclimation time	−0.396	0.222	0.0009*
POM			
Diet	−0.104	0.177	0.2427
Acclimation time	−0.356	0.181	0.0003*
OC			
Diet	0.021	0.043	0.3365
Acclimation time	−0.017	0.044	0.4517

Table 3. *Cerastoderma edule*. Expt 1: physiological variables measured following short-term (2 d) and long-term (12 d) acclimation to diets of phytoplankton (P: *Isochrysis galbana* cells) and detritus (D: detrital particles from *Spartina maritima*). CR: clearance rate (l h^{-1}); OIR: organic ingestion rate (mg POM h^{-1}); GAE: gross absorption efficiency (fraction); NAE: net absorption efficiency (fraction); GPT: gut passage time (h); MFL: metabolic faecal loss (mg h^{-1}). Values are means of 5 determinations (10 to 15 for CR) (±95% CI)

Diet	Acclimation	CR	OIR	GAE	NAE	GPT	MFL/OIR
P	Short-term	0.50 ± 0.12	0.54 ± 0.13	0.88 ± 0.03	0.76 ± 0.06	12.68 ± 2.31	0.12 ± 0.05
P	Long-term	0.37 ± 0.07	0.54 ± 0.10	0.86 ± 0.02	0.79 ± 0.02	9.13 ± 2.30	0.07 ± 0.02
D	Short-term	0.30 ± 0.06	0.33 ± 0.06	0.77 ± 0.09	0.51 ± 0.10	15.25 ± 2.67	0.26 ± 0.11
D	Long-term	0.48 ± 0.05	0.85 ± 0.09	0.68 ± 0.05	0.56 ± 0.04	12.34 ± 3.31	0.12 ± 0.05

whereas the interaction of both did, indicating that CR tended to decrease (but not significantly) with acclimation in cockles fed P diets, while it increased significantly with acclimation to D diets. In other words, reduced CR values recorded in cockles fed *Spartina maritima* detritus are shown to increase following acclimation, to reach values characteristic of phytoplankton diets. (2) Composition of the diet had a very strong effect on GAE and NAE, detrital diets being absorbed with a reduced efficiency. (3) Both composition and acclimation had significant effects on GPT: phytoplankton diets are processed faster than detrital diets in the guts of cockles, and GPT tends to decrease with acclimation.

Table 4. *Cerastoderma edule*. Expt 1: summary of ANOVAs to test significance of both diet (POM in the case of OIR) and acclimation time on the physiological measurements reported in Table 3. *$p < 0.05$

Source of variation	df	SS	MS	*F*	p
CR					
Diet	1	0.026	0.026	1.071	0.3062
Acclimation time	1	0.006	0.006	0.241	0.6256
Interaction	1	0.283	0.283	11.672	0.0014*
Residual	45	1.091	0.024		
GAE					
Diet	1	0.101	0.101	29.170	0.0001*
Acclimation time	1	0.016	0.016	4.492	0.0501
Interaction	1	0.006	0.006	1.949	0.1928
Residual	16	0.055	0.003		
NAE					
Diet	1	0.286	0.286	60.496	0.0001*
Acclimation time	1	0.008	0.008	1.685	0.2127
Interaction	1	5×10^{-4}	5×10^{-4}	0.104	0.7516
Residual	16	0.076	0.005		
GPT					
Diet	1	37.148	37.148	4.852	0.0449*
Acclimation time	1	46.443	46.443	6.067	0.0273*
Interaction	1	0.451	0.451	0.059	0.8117
Residual	14	107.175	7.655		
OIR					
POM	1	0.005	0.005	0.202	0.6591
Acclimation time	1	0.128	0.128	5.339	0.0345*
Interaction	1	0.167	0.167	6.939	0.0180*
Residual	16	0.384	0.024		
MFL/OIR					
Diet	1	0.047	0.047	8.229	0.0111*
Acclimation time	1	0.045	0.045	8.027	0.0120*
Interaction	1	0.014	0.014	1.830	0.1949
Residual	16	0.091	0.006		

Since IR is directly affected by food concentration, possible effects of increased rations recorded along the acclimation period were analyzed by testing the effects of POM and acclimation time on OIR. Results of ANOVA (Table 4) led us to conclude that there were no effects of food concentration per se and significant effects of both acclimation and the interaction term. The latter results reflected opposite trends shown by CR during acclimation in the 2 diets (see above): a time-dependent reduction in phytoplankton-fed cockles appears compensated for by increased POM, while the strong increment of CR during acclimation to detrital diets enhances the effect of increased POM. In conclusion, feeding-response adjustments would be more important than differences in food ration concerning the overall increment of IR achieved during acclimation.

Computed values of MFLs per unit of organic ingestion (MFL/OIR), representing digestive costs per unit of food intake are also shown in Table 3. Being a relative magnitude, values taken by this index would not be affected by differences in rates of food processing associated with POM differences between diets, making comparisons particularly meaningful. These comparisons (Table 4) show that both food composition and acclimation exert significant effects. Thus, digestive costs per unit of ingestion are much higher for Diet D and tend to decrease with time of acclimation in both types of diets.

Expt 2

During this experiment cockles were fed 2 rations of either phytoplankton or detrital particles for 2 or 12 d before the labeled diets were used to determine physiological parameters. Characteristics of the diets are presented in Table 5. As intended, high rations had particle concentrations approximately twice those of low rations, except in the case of Diet D with long-term

Table 5. Expt 2: characteristics of experimental diets recorded in short-term (2 d) and long-term (12 d) acclimation to diets of phytoplankton (P: *Isochrysis galbana* cells) and detritus (D: detrital particles from *Spartina maritima*), dosed at 2 rations. TPM: total particulate matter (mg l^{-1}); POM: particulate organic matter (mg l^{-1}); OC: organic content (fraction). Values are means of 7 to 12 determinations (±95% CI)

Diet	Acclimation	Ration	TPM	POM	OC
P	Short-term	High	1.15 ± 0.23	0.88 ± 0.21	0.76 ± 0.05
		Low	0.62 ± 0.11	0.45 ± 0.06	0.75 ± 0.05
P	Long-term	High	1.33 ± 0.19	0.99 ± 0.16	0.77 ± 0.03
		Low	0.74 ± 0.09	0.54 ± 0.06	0.73 ± 0.05
D	Short-term	High	1.13 ± 0.16	0.88 ± 0.14	0.78 ± 0.03
		Low	0.62 ± 0.15	0.49 ± 0.13	0.77 ± 0.05
D	Long-term	High	1.70 ± 0.17	1.29 ± 0.14	0.76 ± 0.03
		Low	0.61 ± 0.10	0.44 ± 0.09	0.72 ± 0.03

exposure, where larger differences were obtained. Mean comparisons (Table 6) revealed that food concentration (both TPM and POM) increased significantly during acclimation, while OC decreased.

Values of physiological parameters (IR, GAE and GPT) estimated by using ^{14}C-labeled diets are given in Table 7. Variance analysis applied to these data (Table 8) reveals a complex picture arising from the interplay of the 3 different factors (e.g. dietary composition, ration and exposure time) acting upon the physiological variables.

(1) Food ration exerted significant effects on variables related to food processing (IR and GPT), either per se or in combination with other factors (type of diet and acclimation time). Parallel behaviour of both variables suggests a functional relationship based on the expectation that, in continuous feeders, gut residence time of food particles would decline with increased rates of feeding.

Table 6. Expt 2: ANOVA. Post hoc test (Fisher's PLSD) for mean significant differences in TPM, POM and OC. Abbreviations in Table 5. *p < 0.05

Effect	Mean difference	Critical difference	p
TPM			
Ration	0.678	0.107	<0.0001*
Diet	0.001	0.106	0.9908
Acclimation time	–0.164	0.106	0.0030*
POM			
Ration	0.537	0.088	<0.0001*
Diet	–0.008	0.088	0.8497
Acclimation time	–0.094	0.088	0.0369*
OC			
Ration	0.022	0.029	0.1472
Diet	–0.006	0.029	0.6893
Acclimation time	0.039	0.029	0.0090*

Table 7. *Cerastoderma edule*. Expt 2: physiological variables measured following short-term (2 d) and long-term (12 d) acclimation to diets of phytoplankton (P: *Isochrysis galbana* cells) and detritus (D: detrital particles from *Spartina maritima*), dosed at high and low food rations. IR: ingestion rate (mg TPM h^{-1}); GAE: gross absorption efficiency (fraction); GPT: gut passage time (h). Values are means of 5 to 7 determinations (±95% CI)

Diet	Acclimation	Ration	IR	GAE	GPT
P	Short-term	High	0.78 ± 0.14	0.83 ± 0.05	14.82 ± 2.12
		Low	0.45 ± 0.07	0.78 ± 0.03	16.66 ± 1.52
P	Long-term	High	0.87 ± 0.25	0.78 ± 0.05	13.27 ± 2.20
		Low	0.21 ± 0.05	0.79 ± 0.15	15.63 ± 4.76
D	Short-term	High	0.48 ± 0.19	0.64 ± 0.07	11.46 ± 2.20
		Low	0.60 ± 0.07	0.53 ± 0.04	13.32 ± 3.00
D	Long-term	High	0.50 ± 0.22	0.72 ± 0.15	19.41 ± 3.08
		Low	0.38 ± 0.12	0.59 ± 0.02	12.27 ± 2.52

To illustrate such dependence, individual values of GPT recorded in both experiments were plotted against IR and points were fitted with exponential functions of the form:

$$Y = Y_{max} \times e^{-B1X} \quad (2)$$

where Y_{max} represents the maximum GPT, X is IR, and $B1$ is the rate at which GPT declines with rising IR.

Table 8. *Cerastoderma edule*. Expt 2: summary of ANOVAs to test significance of diet, food ration and acclimation time on the physiological measurements reported in Table 7. *p < 0.05

Source of variation	df	SS	MS	*F*	p
IR					
Diet (A)	1	0.090	0.090	2.161	0.286
Ration (B)	1	0.801	0.801	19.142	<0.0001*
Acclimation time (C)	1	0.099	0.099	2.358	0.1312
Interaction AB	1	0.889	0.889	21.230	<0.0001*
Interaction AC	1	0.001	0.001	0.017	0.8961
Interaction BC	1	0.255	0.255	6.085	0.0173*
Interaction ABC	1	0.017	0.017	0.404	0.5281
Residual	48	2.010	0.042		
GPT					
Diet (A)	1	12.999	12.999	1.151	0.2896
Ration (B)	1	1.928	1.928	0.171	0.6816
Acclimation time (C)	1	32.052	32.052	2.838	0.0997
Interaction AB	1	52.758	52.758	4.672	0.0366*
Interaction AC	1	61.867	61.867	5.478	0.0242*
Interaction BC	1	60.244	60.244	5.334	0.0260*
Interaction ABC	1	52.903	52.903	4.684	0.0363*
Residual	41	463.029	11.293		
GAE					
Diet (A)	1	0.368	0.368	41.687	<0.0001*
Ration (B)	1	0.060	0.060	6.855	0.0122*
Acclimation time (C)	1	0.007	0.007	0.771	0.3850
Interaction AB	1	0.034	0.034	3.875	0.0556
Interaction AC	1	0.019	0.019	2.167	0.1474
Interaction BC	1	0.002	0.002	0.198	0.6588
Interaction ABC	1	0.003	0.003	0.378	0.5422
Residual	42	0.370	0.009		

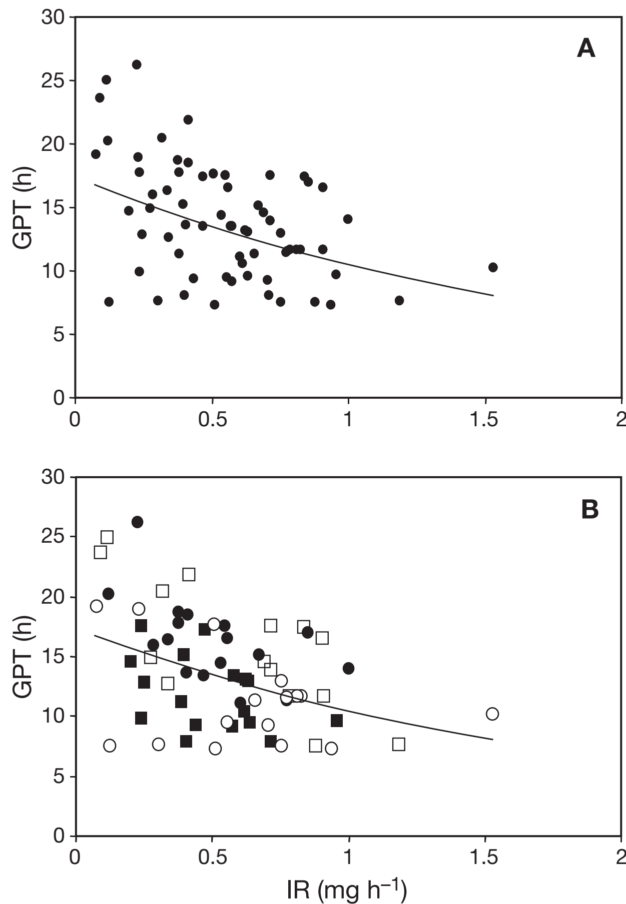

Fig. 1. *Cerastoderma edule*. Gut passage time (GPT, h) as a function of ingestion rate (IR, mg h^{-1}). (A) Pooled data for all combinations of diet and acclimation times in Expts 1 and 2. (B) Same data as in Panel A, with different symbols identifying the different experimental groups—circles: phytoplankton diets; squares: detritus diets; solid symbols: 2 d of acclimation; open symbols: 12 d of acclimation. Data were fitted with the exponential function (parameter value ± asymptotic standard error): GPT = 17.28 ± 1.46 × $e^{-0.49 \pm 0.16 \times IR}$, R^2 = 0.182, p < 0.001

Data for each combination of diet and acclimation time were independently fitted, and computed values were compared according to their confidence limits, to conclude lack of significant differences in either Y_{max} (t = 0.875) or b parameters (t = 0.562) ($t_{0.05}$ = 2.03). Consequently, a single relationship could be fitted to all data pooled (Fig. 1).

From Tables 5 & 6, it is clear that actual values of food concentration (TPM and POM) were consistently lower after short-term acclimation compared with long-term acclimation, which could account for significance interactive effects of ration × time on IR and GPT. When POM is introduced as a covariate (Table 9), comparison of means reveals no significant differences for any comparison criteria (diet or acclimation time).

Table 9. Expt 2: ANOVA. Summary of post hoc test (Fisher's PLSD) of IR, GPT and GAE, with POM as a covariate. Abbreviations in Table 7. *p < 0.05

Effect	Mean difference	Critical difference	p
IR			
Diet:			
P vs. D	0.080	0.110	0.1483
Acclimation time:			
2 vs. 12 d	0.084	0.110	0.1315
GPT			
Diet:			
P vs. D	0.930	1.943	0.3392
Acclimation time:			
2 vs. 12 d	–1.737	1.949	0.0793
GAE			
Diet:			
P vs. D	0.167	0.056	<0.0001*
Acclimation time:			
2 vs. 12 d	–0.034	0.056	0.2228

(2) Type of diet and food ration both exert significant effects on GAE (Table 8). Since GPT represents the available time for food digestion and absorption, ration effects on GAE very likely reflect the modification of rates of food processing within the gut. In modeling this relationship GAE was assumed to increase with rising GPT according to an asymptotic function that, according to Willows (1992), is written as:

$$Y = Y_{max}\,[1 - (1 + bX) \times e^{-B2X}] \quad (3)$$

where Y_{max} represents the maximum GAE, X is GPT and $B2$ is the rate at which this maximum is reached with rising GPT. Data for each combination of diet and acclimation time were independently fitted, and computed values were compared according to their confidence limits. The conclusion was that maximum GAE did not significantly differ between diet groups (t = 0.158) and the b parameter differed between diet groups (P vs. D, t = 2.535), but not within diet groups (i.e. 2 vs.12 d of acclimation, t = 0.147) ($t_{0.05}$ = 2.04). This confirms the results shown in Table 9 as to the significance of differences between GAE means for diet and lack of significance for acclimation time. Consequently, 2 different expressions were fitted for P and D diets (Fig. 2).

Digestive enzyme activities

Expt 1

Dry weight of the digestive gland and protein content of the digestive extract, together with data on enzyme activities recorded in this organ, are presented in Table 10. Three carbohydrase (amilase, cellulase

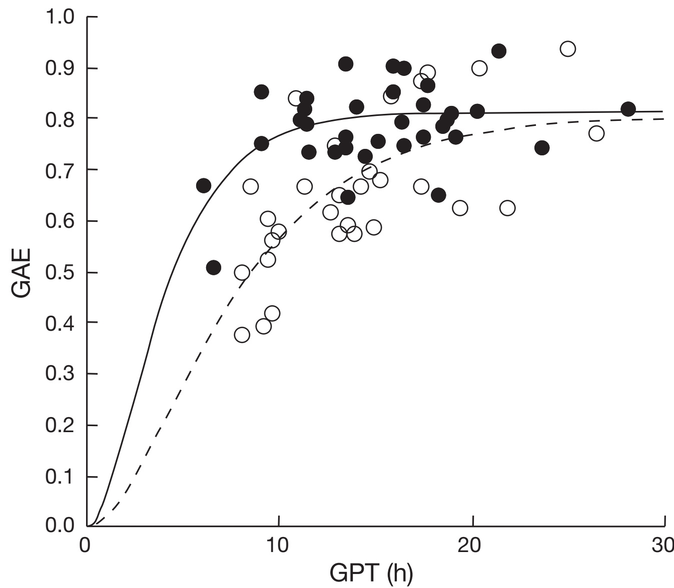

Fig. 2. *Cerastoderma edule*. Gross absorption efficiency (GAE, fraction) as a function of gut passage time (GPT, h). Solid symbols and continuous line: phytoplankton diets; open symbols and dashed line: detritus diets. Fitted parameters (± asymptotic standard error) were: phytoplankton diets: $Y_{max} = 0.8125 \pm 0.0182$, $B2 = 0.4166 \pm 0.0600$, $R^2 = 0.183$, $p < 0.001$; detritus diets: $Y_{max} = 0.8040 \pm 0.0507$, $B2 = 0.2450 \pm 0.0314$, $R^2 = 0.460$, $p < 0.001$

Table 11. Expt 1: summary of ANOVAs to test significance of both diet and acclimation time on digestive gland (DG) size and their protein content and total digestive enzyme activities. No significant differences were found in specific activities (omitted). *p < 0.05

Source of variation	df	SS	MS	F	p
Weight of DG					
Diet	1	0.274	0.274	0.030	0.8641
Acclimation time	1	17.504	17.504	1.941	0.1853
Interaction	1	0.2464	0.2464	0.027	0.8711
Residual	14	126.230	9.017		
Protein content					
Diet	1	8×10^{-4}	8×10^{-4}	1.429	0.2517
Acclimation time	1	1×10^{-4}	1×10^{-4}	0.120	0.7337
Interaction	1	1×10^{-7}	1×10^{-7}	2×10^{-4}	0.9884
Residual	14	7.4×10^{-3}	5×10^{-4}		
Total amylase					
Diet	1	19.278	19.278	3.735	0.0738
Acclimation time	1	0.331	0.331	0.064	0.8038
Interaction	1	8.019	8.019	1.553	0.2331
Residual	14	72.261	5.161		
Total cellulase					
Diet	1	0.800	0.800	0.861	0.3692
Acclimation time	1	3.518	3.518	3.784	0.0721
Interaction	1	3.177	3.177	3.417	0.0857
Residual	14	13.015	0.939		
Total laminarinase					
Diet	1	0.089	0.089	0.356	0.5601
Acclimation time	1	0.469	0.469	1.863	0.1938
Interaction	1	1.260	1.260	5.004	0.0421*
Residual	14	3.525	0.252		
Total protease					
Diet	1	0.189	0.189	1.240	0.2842
Acclimation time	1	0.117	0.117	0.771	0.3949
Interaction	1	0.139	0.139	0.914	0.3552
Residual	14	2.137	0.153		

and laminarinase) and total protease activities are presented as specific (milligrams of released maltose or tyrosine per milligram protein per hour) and total standard activities (milligrams of released maltose per hour per digestive gland). A 2-factor ANOVA (Table 11) showed that neither the type of diet nor acclimation had any significant effect on the size of the digestive gland, its protein content, or specific enzyme activities. Concerning total activities, the only significant effect refers to the interaction of diet and acclimation on laminarinase activity, accounting for a reduced laminarinase value in the long-term acclimation to diets of phytoplankton (Table 10). Importantly, whilst not statistically significant, cellulase showed consistent increments during acclimation to detrital diets, both in terms of specific and total activities.

Table 10. *Cerastoderma edule*. Expt 1: dry weight (W_{DG}, mg), protein content (PC, fraction) and enzyme activities of digestive glands (DG) of standard-sized cockles fed phytoplankton and detrital diets for 2 d (short-term acclimation) and 12 d (long-term acclimation). Ae, Ce, Le, Pe: specific amylase, cellulase, laminarinase (mg released maltose mg^{-1} protein h^{-1}) and protease (mg released tyrosine mg^{-1} protein h^{-1}) activities, respectively; A_{DG}, C_{DG}, L_{DG}, P_{DG}: total amylase, cellulase, laminarinase (mg released maltose digestive $gland^{-1}$ h^{-1}) and protease (mg released tyrosine digestive $gland^{-1}$ h^{-1}) activities, respectively. Values are means of 5 measurements (±95% CI)

Diet	Acclimation	W_{DG}	PC	Ae	Ce	Le	Pe	A_{DG}	C_{DG}	L_{DG}	P_{DG}
P	Short-term	19.036 ± 1.791	0.198 ±0.078	1.336 ±0.525	0.443 ±0.101	0.387 ±0.120	0.189 ±0.078	5.738 ±2.104	1.926 ±0.452	1.703 ±0.610	0.854 ±0.468
P	Long-term	20.785 ±1.917	0.242 ±0.053	0.857 ±0.345	0.389 ±0.175	0.179 ±0.073	0.242 ±0.053	4.123 ±1.724	1.970 ±1.164	0.846 ±0.388	1.194 ±0.398
D	Short-term	18.552 ±4.201	0.118 ±0.042	1.435 ±0.436	0.328 ±0.178	0.232 ±0.063	0.187 ±0.042	6.478 ±2.653	1.505 ±0.941	1.028 ±0.356	0.825 ±0.224
D	Long-term	20.772 ±2.702	0.163 ±0.035	1.478 ±0.151	0.651 ±0.157	0.259 ±0.120	0.163 ±0.035	7.548 ±1.1613	3.240 ±0.740	1.236 ±0.338	0.810 ±0.151

Expt 2

In this experiment, in addition to analyzing the effect of food concentration on enzyme activities, we tested the possibility that an assay system based on substrates present in natural food particles might be a more adequate procedure to reveal some digestive responses (of the type suggested by enhanced cellulase activities with prolonged exposure to detrital diets). To this effect, crude extracts of the digestive gland of specimens fed the different diets were simultaneously assayed for standard protease and cellulase activities and total carbohydrase activity using 'natural substrates' obtained as lipid-free extracts of *Isochrysis galbana* cells and detrital particles of *Spartina maritima*, as described in 'Materials and methods'. Results obtained in these assays (Table 12) can be summarized as follows. (1) Both food concentration and type of diet have a significant effect on the size of digestive glands (Table 13), these being consistently bigger in cockles fed phytoplankton at high ration. This helps to explain higher total protease activities with diets of phytoplankton and higher cellulase activity with the diet of phytoplankton at high concentration (significant interactive effect of diet and ration: AB in Table 13), since differences in specific activities are not significant. (2) Protein content of digestive glands significantly increases with detrital diets compared with phytoplankton diets. (3) The amount of maltose released in assays with 'natural substrates' is less than in assays with standard substrates (Tables 11 & 12), which could be attributed to a substrate concentration effect. This same effect could account for the fact that activities measured with detritus extracts are consistently higher than with phytoplankton extracts. (4) In both kinds of assays with 'natural substrates' a combined effect of diet and acclimation time is evident (Table 13), which is accounted for by the fact that digestive enzyme

Table 13. *Cerastoderma edule*. Expt 2: summary of ANOVAs to test significance of diet, particle concentration and acclimation time on digestive gland size and their protein content and total digestive enzyme activities reported in Table 12. Only significant interactions are included. *p < 0.05

Source of variation	df	SS	MS	F	p
Weight of DG					
Diet (A)	1	183.144	183.144	8.786	0.0047*
Ration (B)	1	169.798	169.798	8.146	0.0064*
Acclimation time (C)	1	77.744	77.744	3.729	0.0594
Residual	46	1000.517	20.844		
Protein content					
Diet (A)	1	0.0076	0.0076	9.009	0.0043*
Ration (B)	1	0.0004	0.0004	0.472	0.4955
Acclimation time (C)	1	0.0003	0.0003	0.296	0.5891
Residual	46	0.389	0.0008		
Total cellulase					
Diet (A)	1	1.629	1.629	3.828	0.0565
Ration (B)	1	0.095	0.095	0.223	0.6393
Acclimation time (C)	1	0.008	0.008	0.021	0.8857
Interaction AB	1	1.764	1.764	4.145	0.0475*
Residual	46	19.578	0.425		
Total protease					
Diet (A)	1	2.086	2.086	4.235	0.0455*
Ration (B)	1	0.248	0.248	0.503	0.4817
Acclimation time (C)	1	0.245	0.245	0.498	0.4839
Residual	46	21.279	0.493		
Total carbohydrase (phytoplankton extracts)					
Diet (A)	1	0.041	0.041	0.621	0.4348
Ration (B)	1	0.003	0.003	0.042	0.8388
Acclimation time (C)	1	0.546	0.546	8.181	0.0063*
Interaction AC	1	0.518	0.518	7.764	0.0070*
Residual	46	3.071	0.067		
Total carbohydrase (detritus extracts)					
Diet (A)	1	0.012	0.012	0.041	0.8405
Ration (B)	1	0.003	0.003	0.009	0.9215
Acclimation time (C)	1	1.403	1.403	0.223	0.6392
Interaction AC	1	0.184	0.184	4.820	0.0332*
Residual	46	32.569	0.528		

Table 12. *Cerastoderma edule*. Expt 2: dry weight (W_{DG}, mg), protein content (PC, fraction) and enzyme activities of digestive glands (DG) of standard-sized cockles fed phytoplankton and detrital diets for 2 d (short-term acclimation) and 12 d (long-term acclimation), dosed at high and low particle concentrations. C_{DG} and P_{DG}: total cellulase (mg released maltose digestive gland^{-1} h^{-1}) and protease (mg released tyrosine digestive gland^{-1} h^{-1}) activities, respectively; CHase: total carbohydrase activities (mg released maltose digestive gland^{-1} h^{-1}) recorded with lipid-free extracts of phytoplankton (*Isochrysis galbana* cells) and detrital particles of *Spartina maritima* acting as substrates (P and D, respectively). Values are means of 7 measurements (±95% CI)

Diet	Acclimation	Ration	W_{DG}	PC	C_{DG}	P_{DG}	CHaseP	CHaseD
P	Short-term	High	30.673 ± 5.738	0.224 ± 0.024	1.679 ± 0.631	2.004 ± 0.394	0.368 ± 0.216	0.790 ± 0.437
		Low	25.544 ± 2.180	0.244 ± 0.031	1.508 ± 0.273	1.814 ± 0.707	0.225 ± 0.183	0.871 ± 0.261
P	Long-term	High	26.224 ± 4.152	0.217 ± 0.017	1.611 ± 1.033	1.596 ± 0.525	0.321 ± 0.204	0.678 ± 0.287
		Low	22.554 ± 3.608	0.221 ± 0.023	0.888 ± 0.378	1.262 ± 0.441	0.282 ± 0.218	0.476 ± 0.312
D	Short-term	High	23.616 ± 2.278	0.255 ± 0.016	0.797 ± 0.422	1.076 ± 0.195	0.079 ± 0.096	0.475 ± 0.355
		Low	22.641 ± 1.613	0.239 ± 0.014	1.056 ± 0.479	1.242 ± 0.659	0.009 ± 0.018	0.599 ± 0.508
D	Long-term	High	24.213 ± 4.185	0.246 ± 0.015	1.071 ± 0.389	1.4171 ± 0.503	0.288 ± 0.163	0.961 ± 0.568
		Low	20.027 ± 1.309	0.261 ± 0.029	1.369 ± 0.356	1.264 ± 0.283	0.597 ± 0.335	0.899 ± 0.488

activity increases with acclimation in cockles fed detrital diets. As a result, released maltose that was initially lower in cockles fed *Spartina maritima* particles reached the amount released by phytoplankton-fed cockles after long-term acclimation to the detrital diet.

DISCUSSION

The present results have revealed that detritus derived from salt-marsh vascular plants can be assimilated with high efficiency (>50%), comparable to the efficiencies exhibited by bivalves feeding on natural seston (Bayne & Newell 1983). When values of ^{14}C are considered, representing the carbon GAE, these efficiencies reach 65%, whereas 82% is achieved for phytoplankton.

Discrepancies with the above-quoted results by Langdon, Newell and co-workers (Newell & Langdon 1986, Crosby et al. 1989, Kreeger et al. 1990, Langdon & Newell 1990), concerning the low AE values reported by these authors for mussels and oysters fed salt-marsh detritus (see 'Introduction'), may be interpreted in several ways. First, most of their results were obtained using lignocellulosic preparations (crude fiber extracts) obtained from vascular plant tissues. This rather refractory particulate material was chosen, since it appeared to more closely represent naturally occurring detritus after the labile components had been eliminated by fragmentation, eroding and colonization of organic particles by microorganisms (Newell & Langdon 1986). Available data seem insufficient to resolve the question as to whether composition differences between fresh and extracted detritus might have caused such large differences in absorption efficiency. Secondly, variable physiological conditions of bivalves induced by environmental factors might also play an important role. For example, Charles & Newell (1997) reported absorption efficiencies of ribbed mussels *Geukensia demissa* that were 72% higher than previous values in specimens of the same species collected from the same geographical location and tidal height (Kreeger et al. 1990). They attributed this difference to the induction of cellulase activity associated with the induction of absorption due to the presence of cellulose in the diet as well as to seasonal variability in cellulase activity. On the other hand, intertidal mussels absorbed lignocellulosic material with a higher efficiency than did subtidal mussels (Kreeger et al. 1990, Charles & Newell 1997), which was explained by the fact that, because of emersion, longer retention times of food within the gut occur in intertidal mussels, providing increased action time for digestive enzymes and favoring absorption efficiency (Bayne et al. 1988). Consequently, both diet acclimation and rates of food processing are acknowledged to influence the use of detrital energy by bivalves, most especially by affecting absorption efficiency.

As to the precise nature of plant detritus that is actually available to bivalves in the field, it should be pointed out that the objectives of the present work were primarily focused on exploring the suite of feeding–digestive adjustments that enable bivalves to exploit different vegetable sources rather than on the immediate ecological relevance of the results.

Digestive adjustments: gross absorption

Digestive adjustments are more clearly represented by the behaviour of GAR, as this measurement constitutes the overall component of energy gain from digestive processes. When GAR is plotted against food concentration (Fig. 3, Table 14) a clear difference between phytoplankton and detritus emerges. The capacity for obtaining energy from increasing amounts of suspended food appears to be greatly constrained in the case of *Spartina maritima* detritus in comparison with phytoplankton; however, the situation is clearly improved for detrital diets after 12 d of acclimation, when rates of absorption showed a rising tendency with increasing food concentration irrespective of food source (see ANOVA results in Table 14). Such beneficial effects of acclimation have been broadly documented in the cockles *Cerastoderma edule* (Ibarrola et al. 2000a,b) (for mussels *Mytilus edulis* see also Bayne 1993, Bayne et al. 1993). The digestive mechanisms underlying such acclimation responses can also be considered to differ between food types. GF, representing the total amount of food held by the gut at each moment, was independent of food concentration and remained invariable throughout the course of acclimation to phytoplankton diets, while it increased in cockles acclimated to moderate and high concentrations of detritus (Fig. 3, Table 14).

In continuous feeders, gut capacity and gut residence time of food together determine how much food can be processed per unit time (Sibly & Calow 1986, Willows 1992). Implicit to this statement is the existence of a functional relationship between IR and GPTs of food, which has been modeled as an exponential decaying function in bivalve mollusks (Bayne et al. 1984, 1989, Navarro & Iglesias 1993, Navarro et al. 1994). Present data for this relationship were fitted to a single function including pooled values for both food types after 2 and 12 d of acclimation (Fig. 1A), on account of the lack of differences between groups. A re-examination of data (Fig. 1B), however, suggests that, for a given rate of ingestion, GPTs tend to increase with acclimation time in detritus-fed cockles,

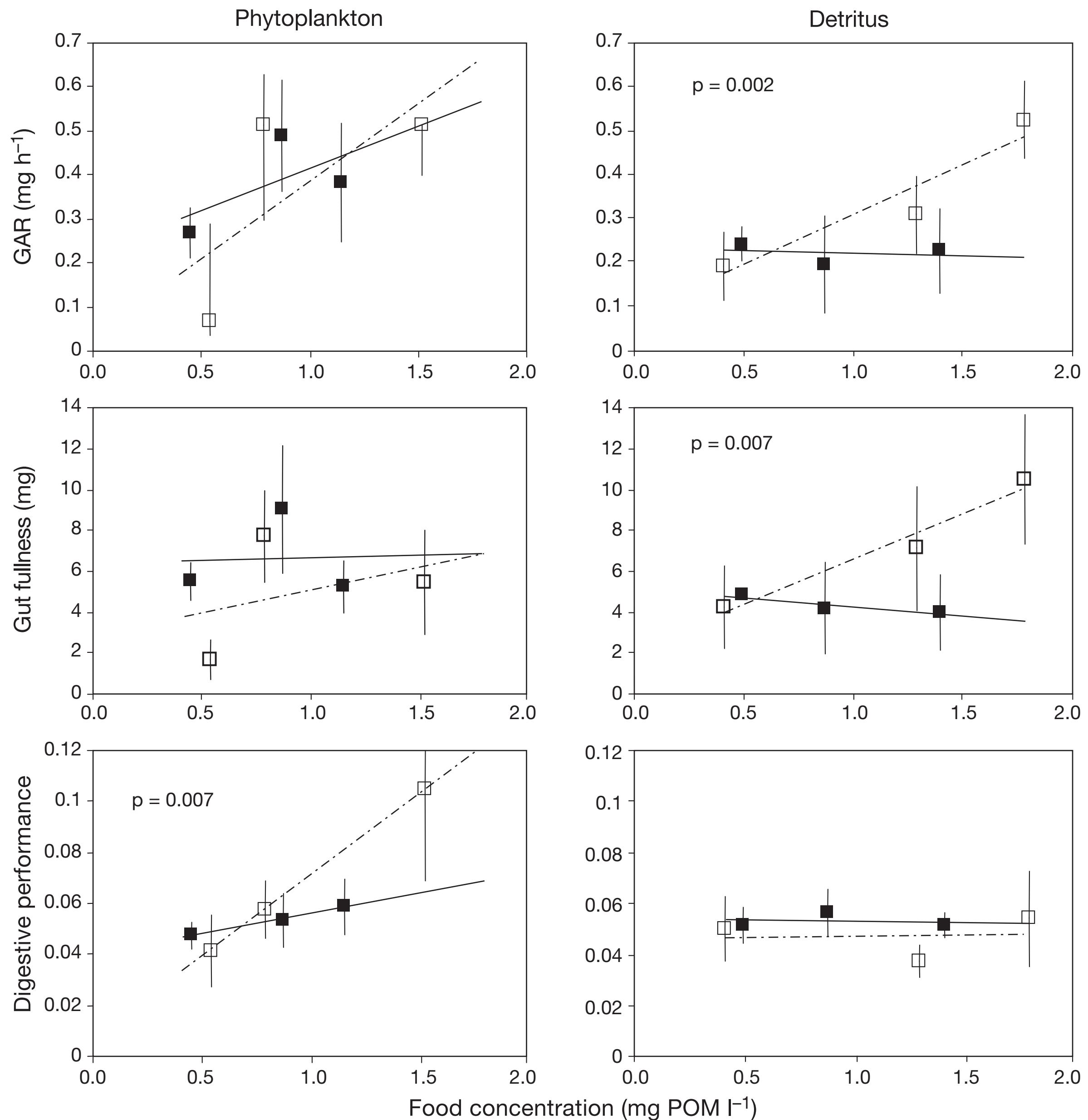

Fig. 3. *Cerastoderma edule*. Gross absorption rate (GAR), gut fullness (GF) and digestive performance (DP = GAE/GPT), as a function of food concentration in phytoplankton and detritus diets (see 'Discussion' for details). Points are mean values for the different experimental groups (±2 SE). Solid symbols: 2 d of acclimation; open symbols: 12 d of acclimation. To account for seasonal differences in feeding rates between Expts 1 (February) and 2 (May), thermal effects were assumed and data were standardized to a common temperature (16°C) applying a thermal coefficient (Q_{10} = 2.5) computed using data on seasonal variation of clearance rates of cockles (Newell & Bayne 1980, Ibarrola et al. 2008). Significant interactions between food concentration and acclimation time are indicated. ANOVA testing the effect of acclimation time with particulate organic matter (POM) as covariate given in Table 14

while they decrease in the case of phytoplankton diets, as revealed by significant interaction between diet and acclimation time (p = 0.02; see Tables 8 & 9). It is very likely that these opposite trends resulted in the cancellation of significant differences between food groups. In conclusion, enlarged gut capacity after complete acclimation to detrital diets (see ANOVA results in Table 14) would have enabled cockles to retain food longer in order to increase absorption efficiency.

The question now is why phytoplankton-fed cockles do not behave according to this 'strategy'. A possible explanation would be the different susceptibility of phytoplankton and detrital particles to digestive pro-

Table 14. ANOVA for testing the effect of acclimation time on GAR, GF and DP, with POM as covariate (see Fig. 3 for abbreviations). *p < 0.05

Factor	Phytoplankton						Detritus					
	GAR		GF		DP		GAR		GF		DP	
	F	p	*F*	p	*F*	p	*F*	p	*F*	p	*F*	p
Acclimation time	1.57	0.218	1.24	0.274	4.68	0.039*	5.09	0.031*	2.41	0.132	0.60	0.444
POM	10.57	0.002*	1.23	0.275	23.95	<0.0001*	8.47	0.007*	3.41	0.076	0.059	0.809
Interaction	0.75	0.392	0.18	0.675	8.46	0.007*	11.44	0.002*	8.58	0.007*	0.059	0.811

cesses, resulting in significantly different functions relating GAE and GPT for cockles fed microalgal and detrital diets (Fig. 2). Absorption efficiency has been proposed to increase asymptotically with increases in the residence time of food within the gut (Sibly 1981, Sibly & Calow 1986, Willows 1992), representing a basic feature of continuous-flow digestive systems that has been empirically proven in suspension-feeding bivalves (Bayne et al. 1987, 1988, Navarro & Iglesias 1993, Navarro et al. 1994). According to the present data (Fig. 2), cockles attained the same maximum GAE (81%) irrespective of food type, but the rate at which this value was approached with increasing GPT was almost halved in the case of *Spartina maritima* detritus (0.24) compared to *Isochrysis galbana* cells (0.42). Such reduced absorption efficiency per unit GPT could simply reflect the reduced digestibility of vascular plant detritus compared with phytoplankton. López & Levinton (1987) postulated similar digestive behaviour in deposit feeders with regards to the processing of different classes of food particles (benthic diatoms and seaweed and lignocellulosic detritus) present in marine sediments. Furthermore, the curves in Fig. 2 also served to illustrate that changes in GPT in the range of from 8 to 25 h (the empirical range observed in the present work) would produce a minimal effect in the GAE of phytoplankton (asymptotic zone), while dramatically affecting the efficiency with which detrital food is absorbed. In other words, cockles would greatly benefit from any increment in the gut residence time of detrital particles during the course of acclimation, while the improvement would be much less important in the case of phytoplankton diets. Indeed, different approaches based on optimality principles have predicted that extended gut residence times of food are associated with poor diets, accounting for the widespread finding that animals sustaining themselves on less digestible food items tend to develop larger guts (Sibly 1981, Sibly & Calow 1986).

Conversely, the virtual independence of GAE from GPT may help explain why acclimation to microalgal diets could, in spite of producing a faster passage of food, enhance digestive performance (see ANOVA results in Table 14), very likely enabling the exploitation of a further increase in food concentration.

Digestive enzyme activities

Compensatory adjustments considered up to this point, namely the increased digestive performance with reduced GPT in the highly concentrated phytoplankton diets, and the maintenance of digestive performance with increasing amounts of food processed by the gut in the course of acclimation to detritus diets, would imply an increment in the hydrolytic capacity of digestive structures. With respect to the digestive gland, such expectations are largely met by the present results on enzyme activity. For instance, cockles exposed to high rations of *Isochrysis galbana* showed increased protease and cellulase total activities that were partly based on the size increment of the digestive gland of these individuals. These results are coincident with those reported by Ibarrola et al. (1996, 2000b,c), who showed that the increment in various digestive enzyme activities (non-specific protease, cellulase and laminarinase) compensated for the decreased GPT of food produced by increasing the ration of *Tetraselmis suecica*, making absorption efficiency virtually independent of IRs (Ibarrola et al. 2000a,b). Also Bayne (1993) and Bayne et al. (1993) have reported that increments in carbohydrase activity correlated with the increment in ARs of both carbohydrates and total organics achieved during the acclimation of mussels *Mytilus edulis*, and Hawkins & Bayne (1992) reported changes in α-amilase and laminarinase activities in mussels held at high and low levels of ration. In the case of cockles, these changes have been found to involve the modification of several morphometric parameters of the digestive gland, as well as the volume fractions of digestive cell lysosomes and basophilic cells associated, respectively, with protease and carbohydrase activities (Ibarrola et al. 2000c).

On the other hand, maltose released from 'natural substrates' (lipid-free extracts of microalgal and detrital particles) in the assays performed with digestive gland extracts of cockles-fed detritus increased significantly in the course of acclimation. The induction of carbohydrases during the process of acclimation is strongly supported by the higher protein content of these glands. Fernández-Reiriz et al. (2004) reported that total carbohydrase activity in both the digestive

gland and crystalline style of *Argopecten purpuratus* significantly increased between 24 and 72 h of acclimation to detritus-rich diets, in contrast with the behaviour of scallops acclimated to diets in which phytoplankton was abundant. These changes mainly involved amilase and cellulase activities. According to results obtained in the present assays with standard substrates, carbohydrase induction would mainly be based on the increased cellulase activity that was shown to consistently occur in the course of acclimation to detrital diets (Tables 10 & 12).

Quantification of the activity of a few selected carbohydrases might not represent all digestive adjustments in bivalves. Since the composition of carbohydrates in available food is naturally variable, multiple types of glucanases are required for the breakdown of food particles. Consequently, other activities in addition to those of amylase, cellulase and laminarinase have been reported in the digestive system of bivalves (for review see Vonk & Western 1984). Although a significant increase in a given glucanase type is indicative of induction of this particular enzyme, no direct correspondence needs to exist between the increased rate of synthesis of a given enzyme and the rate of sugar release from food particles. In addition, total release of sugars depends on the sequential action of glucanases and glycosidases, so that slight increments in the activity of a given glucanase are likely to exponentially enhance total sugar release due to its synergic effects on glycosidases. This is especially true for those glucanases acting on structural carbohydrates, such as cellulase, because cell wall breakdown (Brock 1989) might improve substrate availability for other enzymes.

In this respect, the use of natural substrates represents a holistic approach, allowing the measurement of the overall activity of the whole pool of glucanases and glycosidases acting on a natural mixture of carbohydrates. The significant increment in the activity towards these natural substrates in the cockles acclimated to detrital diets (Table 12) might be a mere consequence of slight increases in cellulase activity that have been recorded, or, alternatively, it might come from changes in other (not determined) activities. In any case, reported results undoubtedly indicate the existence of digestive adjustments that allow cockles to improve detritus absorption.

Digestive adjustments: net absorption

Net rates of absorption are constrained by the magnitude of MFLs, that component of endogenous products contributing to digestion which is not reabsorbed (Hawkins & Bayne 1985, Bayne & Hawkins 1990). In animals, such as bivalves exhibiting predominantly intracellular digestion, the main component of endogenous matter defecated would derive from the fragmentation and subsequent release of digestive cell apices in the form of membranous 'fragmentation spherules' (Purchon 1977, Morton 1983), which are reputed to have a high lipid content (Owen 1972). This is consistent with the finding that assimilatory balance of lipids in cockles was reduced under feeding conditions characterized by high MFL (Ibarrola et al. 1996, 1998).

The magnitude of MFL increases with increasing rates of food processing; hence, for comparative purposes, the use of a relative index, such as MFL per unit of ingested organics or fractional costs of digestion, would be preferable. In the short-term exposure a clear difference between diets was evident, since these costs represented 12% in phytoplankton-fed cockles versus 26% in those fed detritus, but acclimation exerted an important reduction of the latter, to the point of a virtual cancellation of these differences (7 vs. 12%). It is important to note that this reduction of digestive cost induced by acclimation in detritus-fed cockles occurred concomitantly with the improvement of the digestive potential of digestive gland extracts measured in terms of the amount of reducing sugars released from 'natural' substrates. This coincidence suggests that increased efficacy in the intracellular processing of such refractory materials, mediated by the induction of the appropriate set of enzymes, would produce the additional benefit of reducing the losses of cell materials that accompanies each cycle of intracellular digestion. Whatever the precise mechanisms, both factors appear to function synergistically in order to greatly improve digestive capabilities during acclimation of cockles to detrital diets.

Concluding remarks

Concerning the practical question of the role played by different food types in sustaining growth of bivalves, it should be noted that the present study focused only on the carbon-energy balance, while algae and detritus might have different nutritional values due to differences in C:N composition. In particular, the relatively low nitrogen content of *Spartina maritima* might constitute a limiting factor, and the improved absorption of detrital organic matter shown in the present paper does not in itself enable cockles to exploit detritus effectively as a nutrient source. In this respect, results reported by Grant & Cranford (1991) indicated that, in the scope of growth measurements of the scallop *Placopecten magellanicus*, the actual growth rates recorded under nitrogen-limited diets of kelp detritus were greatly overestimated. Attempts to culture the scallop *Argo-*

pecten irradians (Kirby-Smith 1976) and the mussel *Mytilus edulis* (Williams 1981) on marsh-grass detritus alone were unsuccessful, although it is unclear from such studies whether poor growth was due to the nutritional limitations of detritus or due to its refractory behaviour to digestion. The present results account for some adjustments enabling cockles to compensate for the poor digestibility of *Spartina maritima* detritus. On the other hand, compensatory responses to reduced nitrogen availability have also been reported in mussels on a seasonal basis (Kreeger 1993).

Trophic relationships of suspension feeders in coastal environments revealed by recent studies based on stable isotope enrichment (Decottignies et al. 2007a,b, Dubois et al. 2007) depicted a complex structure: variability recorded in the relative contribution of different food sources (phytoplankton, microphytobenthos and plant detritus) to tissue growth included seasonal and site differences, as well as differences in selective behaviour between competitor species. Thus, for these relationships to be fully understood broad information on particulate food availability in the environment should be framed together with the knowledge of those aspects of feeding behaviour and nutrition concerning the differential utilization of living microalgae and plant detritus, including the assessment of species-specific variability.

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Contribution to the Theme Section 'Directions in bivalve feeding'

Influence of suspension-feeding bivalves on the pelagic food webs of shallow, coastal embayments

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ABSTRACT: Bivalve and microzooplankton community grazing on natural plankton assemblages were studied in 2 embayments of the Peconic Bay estuary (West Neck Bay, Long Island, New York) to determine the relative importance of each community on plankton mortality. Each embayment was characterized by a fringing salt marsh. The bivalve populations consisted of a re-stocked population of *Mercenaria mercenaria* (hard clam) and natural populations of *Geukensia demissa* (ribbed mussel) and *Mya arenaria* (soft-shell clam). Short-term (3 h) grazing experiments were conducted between May and October 2003 and 2004 using 59 l plastic buckets mounted on a floating platform at each study site. The total grazing pressure on phytoplankton by bivalves (i.e. percent bay volume cleared per day) was substantial, sometimes approaching or exceeding the daily flushing rate of the embayment, and was similar to microzooplankton grazing pressure. Experimental studies also showed that bivalves removed ciliates and copepod eggs of *Acartia tonsa* from the plankton. At times, bivalve-induced mortality was likely an important regulatory factor on the zooplankton. These findings demonstrate a complex trophic role for benthic suspension-feeders in shallow, coastal embayments. The effects of restoring shellfish populations will likely be beneficial to overall water quality by ameliorating some effects of eutrophication, but the resultant changes in pelagic food web structure are complex and will be difficult to predict.

KEY WORDS: Clam fisheries · Food webs · Long Island · *Geukensia demissa* · *Mercenaria mercenaria* · *Mya arenaria*

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INTRODUCTION

Benthic suspension-feeding bivalves filter plankton and other particulate matter in the water and, when present in high abundances, may provide a number of positive benefits to shallow bays, such as reduced turbidity and improved light availability for seagrass photosynthesis, prevention of unwanted algal blooms, sequestration of nutrients by burial of nitrogen and phosphorous in the form of biodeposits which may benefit cord grass growth, and enhanced denitrification (Jordon & Valiela 1982, Bertness 1984, Cerrato et al. 2004, Newell 2004, Newell & Koch 2004). In some cases, the impacts of bivalves on aquatic ecosystems have been well documented during the successful establishment of an exotic species (e.g. the zebra mussel *Dreissena polymorpha* in the Hudson River; Pace et al. 1998). It is more difficult, however, to establish the impacts of the loss of a once important suspension feeder because of additional environmental changes such as wetland loss or eutrophication. Indeed, there is debate about the efficacy of shellfish restoration for improving water quality in estuaries (Newell 1988, Gerritsen et al. 1994, Pomeroy et al. 2006, Heck & Valentine 2007).

An expectation of a decline in benthic suspension feeders, however, is that pelagic food web structure has shifted. It has been argued that the decline of ben-

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thic suspension-feeders such as oysters in the Chesapeake Bay has resulted in increased planktonic secondary production (Newell 1988, Jackson et al. 2001). Thus zooplankton grazers may be more abundant and play a larger role in structuring pelagic food webs when bivalve populations are reduced. Micro- and mesozooplankton display considerable feeding selectivity, consuming preferred species of algae and rejecting less desirable species (e.g. Stoecker et al. 1986). These less desirable species of phytoplankton may be less nutritious, toxic, or noxious, such as the brown tide alga *Aureococcus anophagefferens*.

Populations of large, suspension-feeding bivalves have declined in shallow embayments around Long Island, New York, for a variety of reasons. Stocks of commercially important species of bivalves, such as the hard clam *Mercenaria mercenaria* (Linnaeus, 1758), are a small fraction of those estimated to have been present 2 to 3 decades ago (Kassner 1993), particularly in Great South Bay on the south shore of Long Island. In addition, the total area of intertidal salt marshes has declined (e.g. New York State Office of Planning Services 1972, O'Connor & Terry 1972) and it is reasonable to presume that populations of the ribbed mussel *Geukensia demissa* (Dillwyn, 1817), which is endemic to salt marshes of this region, have declined proportionally.

Several observations suggest that maintaining an overall benthic suspension-feeding clearance rate of approximately 30 to 40% of bay volume per day is an appropriate restoration target for the shallow embayments on Long Island. Estimates by Kassner (1993) indicate that this level of clearance was present during the mid-1970s, prior to the precipitous decline in the hard clam population in Great South Bay. More recently, mesocosm experiments have demonstrated that this level of hard clam clearance prevented the build-up of phytoplankton biomass and net population growth of brown tide, even when the mesocosms were amended with high nutrient concentrations (Cerrato et al. 2004). Prevention of brown tides by clams could provide a positive feedback for the bivalves by maintaining the dominance of nutritious algae, as well as have beneficial effects on other shellfish and eelgrass by maintaining high overall water quality (Greenfield & Lonsdale 2002, Gobler et al. 2005, Lonsdale et al. 2007).

Autotrophic biomass and production in the water column is often dominated by small phytoplankton species in Long Island bays. In Great South Bay, for example, Lonsdale et al. (1996, 2006b) found that the <5 µm fraction consistently represented >80% of the total chlorophyll *a* (chl *a*) present. Dominance of small cells (1 to 4 µm plankton) including chlorophytes (e.g. *Nannochloris atomus*) and cyanobacteria (e.g. *Synechococcus* spp.) in coastal systems has been associated with eutrophication and poor growth in bivalves (Grizzle et al. 2001). In the absence of historically high abundances of benthic suspension-feeders in these bays, the primary consumers of these small cells are protists such as nanoflagellates and ciliates (mostly <40 µm in cell diameter; Boissonneault-Cellineri et al. 2001).

In the present study we measured and compared the relative grazing impacts on the phytoplankton community of the natural populations of *Geukensia demissa* and *Mya arenaria* (Linnaeus, 1758) (soft-shell clam), a restocked population of *Mercenaria mercenaria*, and microzooplankton in 2 shallow embayments characterized by a fringing salt marsh (~2 to 3 m wide). Predation rates of bivalves on microzooplankton (i.e. early life stages of copepods and ciliates) were also measured. Most of what is known about the ecology of salt marshes is derived from studies in meadow marshes (e.g. *Geukensia demissa*; Jordon & Valiela 1982, Kemp et al. 1990, Kreeger & Newell 2000), while there is comparatively little known from fringing salt marshes (Morgan et al. 2009). The goal of the present study was to characterize the impact of suspension-feeding bivalves on plankton community composition, dynamics, and pelagic ecological processes. Our results indicate that the ecological impact of some bivalves in the embayments may not be solely as regulators of primary production but also secondary production.

MATERIALS AND METHODS

Field site description and hydrography. Two small embayments located within West Neck Bay, Shelter Island (Fig. 1), served as our field sites (hard clam spawning sanctuary and control site) during 2003. The embayments are physically similar, exhibit no temperature stratification, and are characterized by a fringing band of *Spartina alternaflora* Loisel along the shoreline. The embayments are connected to the larger bay throughout the tidal cycle, and the inlets of both embayments are substantially constricted. The *S. alterniflora* band was approximately 2 to 3 m wide on all borders except the inlet. At each study site, *Geukensia demissa* was found along the edge and extended about 1 to 2 m into the *S. alterniflora* band. Unlike *G. demissa*, *Mercenaria mercenaria* initially occurred at low abundance at both sites. This was confirmed in qualitative surveys of the benthic community before hard clam restocking at the sanctuary site (data not shown). During the second year of the present study (2004), we conducted studies only at the sanctuary site, and included *Mya arenaria* as it had become a dominant member of the bivalve community. Temperature and salinity measurements were

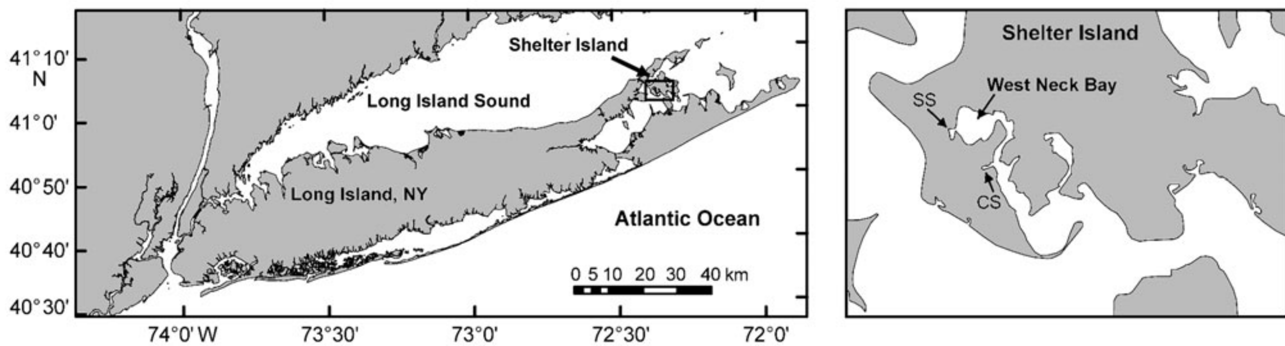

Fig. 1. Study sites in West Neck Bay, Shelter Island, New York, USA. CS: control site; SS: spawning sanctuary for *Mercenaria mercenaria*

taken throughout the experimental period using a YSI Model 30 salinity, conductivity, and temperature meter.

Bay volume, tidal flushing, and residence time of the control and sanctuary sites in West Neck Bay were determined (Holland 2004). Water depth was measured at random locations using a surveyor's leveling rod and GPS. Perimeter and area were estimated from measurements made with a measuring tape and GPS. Perimeter and area were verified in a GIS using a 1 m orthoimagery map. Because these bays are not closed systems (i.e. there is continual exchange of seawater with the main channel of West Neck Bay), these measurements were necessary to better assess the feeding impacts of the bivalve populations on the plankton community. The spawning sanctuary had a perimeter of 497 m, an average (±SE) depth of 1.03 ± 0.06 m (n = 50), an area of 14 136 m^2, and a volume of 14 600 m^3. The control basin was 241 m in perimeter, 0.88 ± 0.05 m in average depth, 3420 m^2 in area, and 3010 m^3 in volume. Mean tidal range was 0.54 m. Approximately 101 % of the bay volume of the hard clam sanctuary and 118 % of the control basin were flushed on a daily basis.

Shellfish censuses. The population size of *Mercenaria mercenaria* in the experimental site was estimated by counting the number of live and dead individuals within 0.25 m^2 areas sampled randomly along a series of bottom transects around the embayment using SCUBA. Plots were excavated to 15 cm. Population size in 2003 and 2004 was estimated by adjusting the population at planting (see below), which was well known, by the survival estimate from the SCUBA census. An estimate of the *Geukensia demissa* population in 2003 and 2004 was obtained by counting individuals within 0.25 m wide quadrats randomly located around the embayment and multiplying this abundance estimate by the perimeter of the bay. Quadrats were extended from shallow subtidal through the intertidal zone to cover the entire mussel distribution within the fringing cord grass. Size distribution of the ribbed mussel population was also determined by measuring individuals to the nearest mm. A SCUBA census of *Mya arenaria* was conducted in 2004 at random locations along a series of bottom transects. Abundance estimates were made by gently placing a 0.25 m^2 quadrat on the bottom and counting siphons. Plots were then excavated to verify the count.

Hard clam stocking. One of the 2 study sites served as a control and was left unmodified (Fig. 1). The spawning sanctuary (experimental site) was stocked with 'chowder size' *Mercenaria mercenaria* (approximately 80 to 90 mm in length) to bring the population to an average density of 15 m^{-2}, a density at which they could potentially account for a significant proportion of total grazing pressure on the phytoplankton community within the confined embayment. Assuming an average depth of 1 m and a clearance rate of 1 l h^{-1} $clam^{-1}$ (Hibbert 1977, Doering & Oviatt 1986), it was estimated that the clams could filter approximately 40 % of the embayment volume each day. This clearance rate by *M. mercenaria* has been shown to prevent the formation of blooms of *Aureococcus anophagefferens*, the brown tide alga, in experimental mesocosms (Cerrato et al. 2004). The hard clams were planted in fall 2002 by The Nature Conservancy (Long Island Chapter), and clam harvesting was closed in the spawning sanctuary at the same time.

Bivalve feeding. Bivalve feeding experiments were conducted bi-weekly to monthly from May to October 2003 and 2004. Measurements of decreases in total chl *a* in enclosures containing bivalves were used to estimate individual bivalve clearance rates (l $bivalve^{-1}$ h^{-1}) on the total phytoplankton community. Measurements of bivalve feeding on picoplankton (i.e. cyanobacteria [*Synechococcus*], photosynthetic eukaryotes, and bacteria) were made beginning in July 2003, and feeding on microplankton (>20 µm) during 2004.

Experiments were conducted in 59 l plastic buckets mounted on a floating platform at each study site. Buckets were filled with seawater collected from the sites and anchored within the embayments. Buckets were covered with 2 layers of neutral gray screening during 2003 to reduce light intensity, whereas polystyrene lids were fitted during 2004. Experimental buckets contained either *Mercenaria mercenaria* (2 to 4 per bucket; mean ± SD shell length = 89.0 ± 9.7 mm and 86.6 ± 0.9 mm for 2003 and 2004, respectively), *Geukensia demissa* (3 to 4 per bucket, shell height = 71.7 ± 2.6 and 74.1 ± 6.3 mm, 2003 and 2004, respectively), or *Mya arenaria* (4 to 5 per bucket, shell length = 61.5 ± 8.1 mm in 2004); 3 replicate buckets were used in each experimental treatment and control.

Experimental shellfish were collected from the study site on the day of the experiment, except for the first 2 experimental dates in 2004 when *Mya arenaria* were obtained from commercial sources on Shelter Island. Control buckets (n = 3) were also incubated during each experiment to examine phytoplankton net growth rate in the absence of benthic suspension-feeding. Phytoplankton growth in the control buckets was used to correct estimates of clearance rate within the experimental treatments (Coughlan 1969). After the first 3 experiments in 2003, nutrients (10 µM nitrate, 1 µM phosphate) were added to all buckets at the start of each experiment to minimize potential effects of bivalve excretion in experimental buckets on phytoplankton growth. Seawater samples for total chl *a*, picoplankton, and microplankton were removed from each bucket 1 h after experimental setup (t_0) to allow time for shellfish to acclimate, and again 4 h after setup (t).

Bivalve clearance rates (CR; l bivalve^{-1} h^{-1}) for total chl *a* and other planktonic taxa were measured as:

$$\mathrm{CR} = \frac{V}{n}\left[\frac{(\ln C_0 - \ln C_t)}{t_0} - a\right] \quad (1)$$

where V is the water volume, n is the number of clams or mussels, C_0 is the prey density at t_0, C_t is the prey density at t, and a is the cell growth rate (positive or negative) from the control containers (Coughlan 1969).

Seawater samples (n = 2) for total chl *a* analyses from each bucket at t_0 and t were collected in brown plastic bottles after gentle mixing of the buckets, stored in coolers with ice packs, and returned to the laboratory for analysis using standard procedures (Parsons et al. 1984) and a Turner Design fluorometer (Model 10-AU). Abundances of picoplanktonic cells (<4 µm) were determined by flow cytometry (Olson et al. 1993). Duplicated seawater samples (4.5 ml) were taken at both time points and preserved in gluteraldehyde (1% final concentration), stored in the cooler, and flash frozen in liquid nitrogen and stored at –80°C when returned to the laboratory. Bacterioplankton, *Synechococcus*, and photosynthetic picoeukaryotes were enumerated using a FACScalibur (BD®) flow cytometer based on fluorescence patterns and particle size determined by forward angle light scatter. Cell abundances of *Aureococcus anophagefferens*, the brown tide picoplankter, were measured using the monoclonal-antibody technique (Caron et al. 2003)

At each time point, seawater samples (180 ml; n = 1 per bucket) for microplankton (20 to 200 µm) were transferred to amber jars, preserved in acidic Lugol's iodine to achieve a final concentration of 10%, and stored in the dark (Stoecker et al. 1994). Samples were processed using standard settling techniques and inverted light microscopy (Utermöhl 1958). Microplankton were grouped into the following taxa: pennate and centric diatoms, flagellates, dinoflagellates, and loricate and non-loricate ciliates. Additionally, dinoflagellates were identified according to Steidinger & Tangen (1996) and Taylor et al. (2003) and counted using a Sedgewick-Rafter cell which was scanned with an inverted microscope (Olympus CK 2).

The initial densities of eggs and nauplii of the calanoid copepod *Acartia tonsa* Dana were determined at the beginning of each experimental date from 2 additional 59 l buckets that were filled with seawater at the same time as all other buckets. Twenty liters of seawater was removed from each of these 2 additional buckets (n = 2 for t_0) and filtered through a 64 µm mesh sieve; the contents on the sieve were preserved in buffered formalin (4% final concentration). The same seawater volume was also removed from each experimental and control bucket (n = 1) at the end of each experiment and processed similarly. Eggs of *A. tonsa*, total copepod nauplii, and other zooplankton (the latter data not shown) were counted in subsamples taken with a Stemple pipet and viewed with a dissecting microscope. A minimum of 200 organisms were counted for each microzooplankton and mesozooplankton sample (Omori & Ikeda 1984).

Clearance rates (l bivalve^{-1} h^{-1}) on total chl *a* were multiplied with population estimates to determine the total clearance capacity (% d^{-1}) of the bivalve populations. Errors in clearance capacity were obtained from the estimated variables by using approximate formulas for products and quotients of random variables (Mood et al. 1974). In the case of the ribbed mussel populations, individual clearance rates were adjusted for variation in body size because we found a wide range of shell lengths during each census (coefficient of variation > 30%). Clearance rate for individuals within each 5 mm size class present in the census was estimated from an allometric relationship suggested by Winter (1978):

$$CR_s = (W_s/W_m)^b CR_m \quad (2)$$

where CR_s is the estimated clearance rate for the size class, CR_m is the measured clearance rate per individual, W_s is the estimated dry tissue weight of a mussel in the size class, and W_m the measured dry tissue weight per individual. We used Winter's (1978) estimate of b = 0.76. Allometric relationships between dry tissue weight and shell length were used to estimate W_s for each size class. Ribbed mussels were assumed to feed for 6 h during a tidal cycle (Kreeger & Newell 2001). Size adjustment was not necessary for hard or soft-shell clams, as they did not exhibit large variations in shell length (coefficients of variation = 9 and 11%, respectively). We also assumed they fed throughout the tidal cycle.

Microzooplankton grazing. Microzooplankton grazing experiments (Landry et al. 1995) were conducted to estimate total phytoplankton growth and grazer-induced mortality coefficients. Seawater was gravity-filtered through 0.45 µm capsule filters for diluting whole seawater. Experimental dilutions of 100, 75, 50, 25, and 10% whole seawater were incubated *in situ* (~0.25 m water depth) in triplicate using 1.2 l polycarbonate bottles. Dilution bottles were amended with 10 µM nitrate and 1.0 µM phosphate (Caron et al. 2000). An additional set of whole seawater bottles was incubated without nutrients to assess the impact of nutrient amendments on phytoplankton growth rate and, if necessary, correct the estimate of the phytoplankton growth coefficient. One bottle containing filtered seawater with nutrient additions was also incubated. Whole and filtered seawater were sampled at the beginning of the experiment, and all bottles were sampled (n = 2 for each bottle) after 24 h incubation for total chl *a* determination as described above. Regression analyses relating the specific rate of change of total chl *a* (d^{-1}) with dilution treatment were conducted using Excel (Microsoft Office 2000®). Community grazing coefficients (g) and prey growth coefficients (k) can be calculated from the slope and y-intercept, respectively, of a plot of net growth rate of prey (y-axis) against the fraction of whole water (x-axis).

Statistical analysis. Unless otherwise stated, data were analyzed with a 2-way ANOVA with site or bivalve species as fixed factors and sampling date as a random factor. Prior to ANOVA, the F-max or Bartlett's test was used to test for homogeneity of variances. If standard transformations did not eliminate significant heteroscedasticity, a non-parametric, 2-way ANOVA was used where possible (equal sample sizes were required); the independent variables were experimental date and bivalve species. Unplanned multiple comparisons among means served as *a posteriori* tests for differences when significant date × site or date × bivalve species interactions were present.

RESULTS

Bivalve population census and chemical/physical factors

Results of annual bivalve population censuses indicated that approximately 67% of the planted hard clams survived for a period of 2 yr after planting (Table 1). A substantial fraction of the mortality during the first year probably occurred during the winter after planting because many of the clams failed to burrow. The total abundance of ribbed mussels was lower in the smaller control site compared to the sanctuary site in 2003. In the spawning sanctuary, the total ribbed mussel abundance was lower in the second year and there was a sizeable population of soft-shell clams that was not notable in 2003. There were no notable differences in water temperature between the control and spawning sanctuary sites, and salinity was only slightly lower at the latter location (Fig. 2).

Ambient plankton composition during grazing experiments

Total chl *a* (µg l^{-1}) concentrations were not significantly different between the spawning sanctuary and control site in 2003 (Table 2, Fig. 3). There was also no difference between years at the spawning sanctuary (nested ANOVA, df = 1,12, $F = 0.327$, $p = 0.859$) (Fig. 3). Photosynthetic picoeukaryotes were on average 2.7 times more abundant than prokaryotes (*Synechococcus*) in the spawning sanctuary, whereas the relative abundances of these assemblages were more equitable at the control site, with the picoeukaryotes approximately 1.2 times more abundant than eukaryotes. Overall, the microplankton community was dominated by dinoflagellates comprising on average 61% of the total community (Fig. 4). The next most abundant taxa were the non-loricate ciliates (27%) and tintinnids (7%). Pennate diatoms represented 4% of the total community and centric diatoms <1%.

Table 1. *Mercenaria mercenaria*, *Geukensia demissa*, and *Mya arenaria*. Mean (±SD) × 10^4 total abundances of bivalves within the 2 study sites in West Neck Bay. SS: spawning sanctuary; CS: control site; nm: not measured. *M. mercenaria* were planted in the SS in Fall 2002

Year	Site	*M. mercenaria*	*G. demissa*	*M. arenaria*
2002	SS	10.0	nm	nm
2003	SS	7.3 ± 5.9	65.1 ± 29.9	nm
2004	SS	6.6 ± 11.3	54.2 ± 17.1	86.9 ± 66.7
2003	CS	nm	11.4 ± 8.6	nm

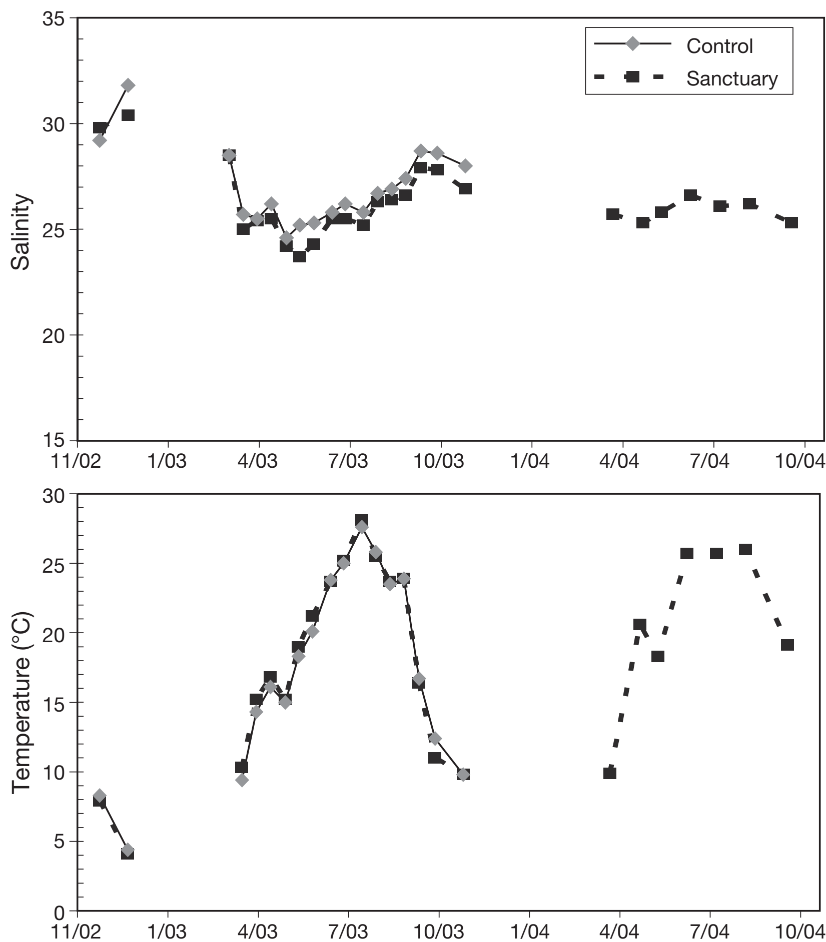

Fig. 2. Water temperature and salinity in West Neck Bay from November 2002 through October 2004

In 2004, dinoflagellates were comprised of 13 genera (data not shown). Among the potentially noxious or toxic dinoflagellates, *Prorocentrum minimum*, *Amphidinium carterae*, and *Akashiwo sanguinea* were most abundant. During June, the dinoflagellate community was comprised of ~60% non-toxic species and 40% noxious taxa. Later in the summer, the percent of non-toxic species decreased and heterotrophic forms (i.e. *Polykrikos schwartzii* and *Protoperidinium divergens*) became more abundant. The percent of potentially noxious species remained fairly constant. In late summer, dinoflagellate cysts were common.

Table 2. 2-way ANOVA of site differences in total chlorophyll *a* (µg l^{-1}) concentrations in 2003

	Effect	df	MS	*F*	p
Site	Fixed	1	0.1207	0.1328	0.728
Date	Random	6	8.8160	9.6948	0.007
Date × Site	Random	6	0.9094	4.2883	0.003
Error		28	0.2121		

Microzooplankton grazing on the total phytoplankton community

Significant grazing (g, d^{-1}) on total phytoplankton by zooplankton was detected on all experimental dates, and in general, the highest mortality rates were found during July and August (Table 3). In 2003, there was no significant difference in grazer-induced mortality rates between the spawning sanctuary (seasonal average ± SD, g = 0.77 ± 0.49 d^{-1}) and control site (0.69 ± 0.29 d^{-1}) (Wilcoxon signed-rank test, T = 10, p > 0.1).

The overall grazing impact by microzooplankton on total phytoplankton can be gauged by comparing the mortality coefficient (g, d^{-1}) to the specific growth rate (k, d^{-1}) of phytoplankton (i.e. g/k; Table 3). On average, these values were 0.90 ± 0.5 (SD) for the spawning sanctuary (2003 to 2004) and 0.81 ± 0.37 for the control site (2003). It has been suggested that dilution experiments may overestimate mortality rates if, at the highest dilution, microzooplankton are dying due to lack of food (Dolan & McKeon 2005). A re-evaluation of the results with the 10% whole seawater treatment removed from the regression analyses (data not shown) gave somewhat lower average grazing coefficients (g = 0.76 ± 0.65 and 0.67 ± 0.47 d^{-1}, respectively). The re-analysis resulted in a significant reduction in g for the experiments conducted in the spawning sanctuary (Wilcoxon signed-rank test, T = 5, p = 0.01; Sokal & Rohlf 1981), but not in the control site (T = 8, p > 0.01). Using the lower values of g, the average (2003 to 2004) grazing impact (g/k) of microzooplankton in the sanctuary was 0.72 ± 0.58. Clearly microzooplankton played a major role as phytoplankton grazers in both embayments.

In general, grazing on brown tide cells *per se* could not be measured because cell densities in the dilution treatments were below 5000 ml^{-1} (data not shown), the reliable detection limit (Caron et al. 2003). The one exception was on 18 June 2003 at the experimental site when the initial abundance of *Aureococcus anophagefferens* in whole seawater was 4.1 × 10^4 cells ml^{-1}. Although zooplankton grazing on the total phytoplankton community was measured (g = 0.3 d^{-1}; Table 3), grazing on brown tide cells was not detected (p > 0.05).

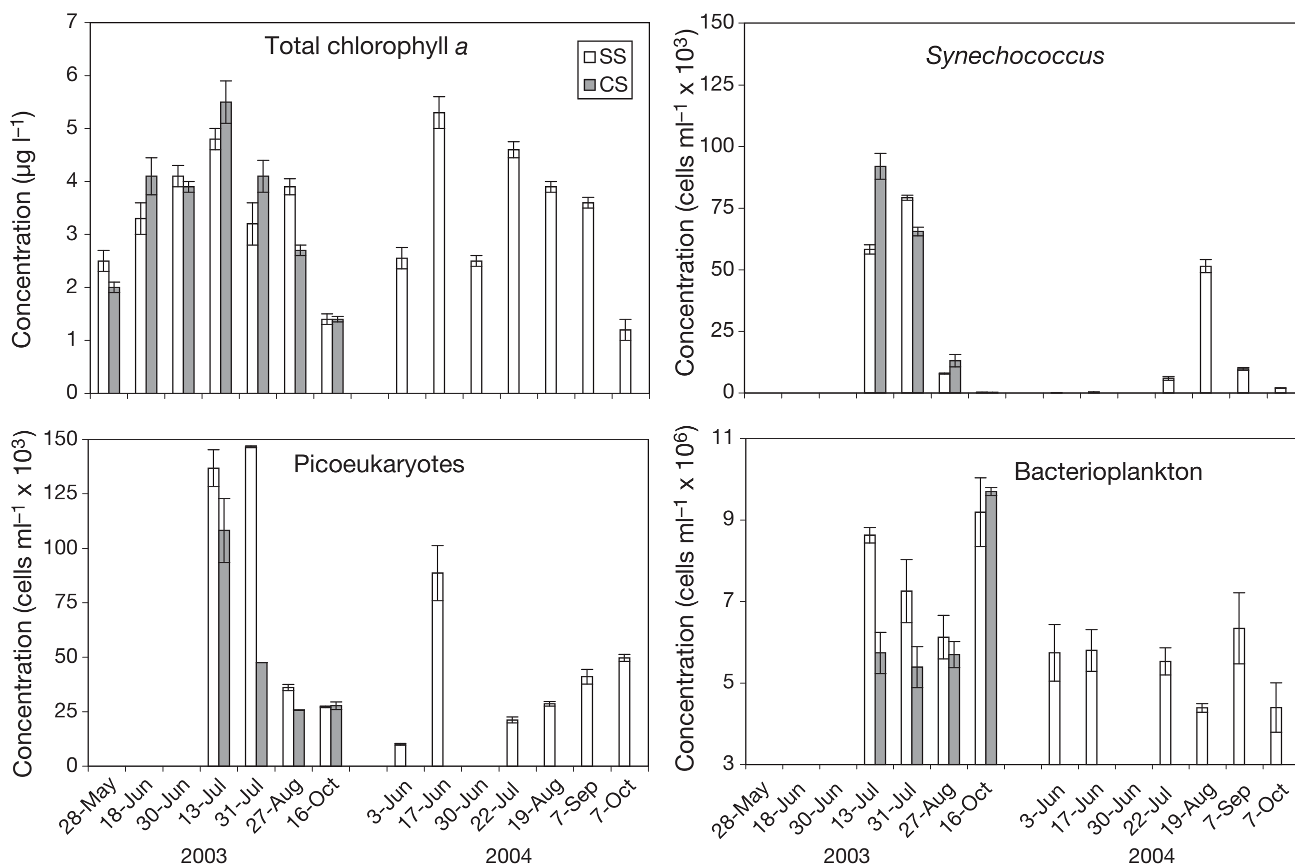

Fig. 3. Initial mean (±SE; n = 6) concentrations of total chlorophyll *a*, *Synechococcus*, photosynthetic picoeukaryotes, and bacterioplankton in seawater used in bivalve grazing experiments during 2003 and 2004 at the spawning sanctuary (SS) and during 2003 at the control site (CS). Flow cytometry samples for 30 June 2004 were not processed

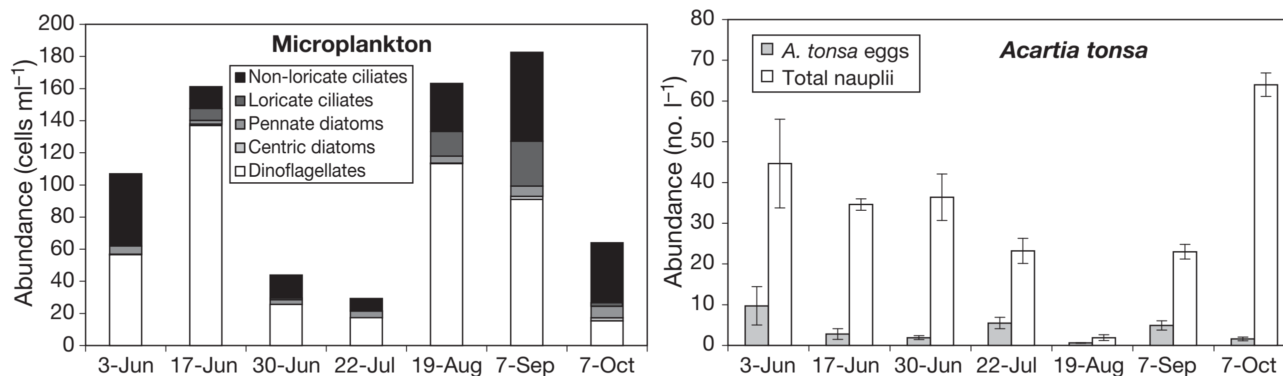

Fig. 4. Initial mean abundances of microplanktonic taxa and *Acartia tonsa* eggs in bivalve grazing experiments conducted in 2004

Bivalve feeding

Total phytoplankton community (total chl *a*)

Geukensia demissa and *Mercenaria mercenaria* in the sanctuary site during 2003 generally had similar clearance rates on the total phytoplankton community (Table 4, Fig. 5). There were no significant differences in clearance rates of ribbed mussels between the sanctuary and control sites. Averaged over the 2003 season, ribbed mussel clearance rates were 2.3 ± 1.5 l h^{-1}. Hard clam clearance rates averaged 1.4 ± 1.6 l h^{-1}. The one experiment where we did not detect any hard clam feeding was 18 June, when brown tide abundance (4.1×10^4 cells ml^{-1}) was above the laboratory-determined threshold for feeding inhibition (~3.5×10^4 cells ml^{-1};

Table 3. Microzooplankton grazing coefficient (g; ±SE; n = 15) and phytoplankton growth coefficient (k; ±SE; n = 15) determined from dilution experiments at 2 sites in 2003 and 1 site in 2004. SS: spawning sanctuary; CS: control site

Date	Site	g (d^{-1})	k (d^{-1})	r
2003				
18 Jun	SS	0.30 ± 0.11	0.87 ± 0.07	0.58
	CS	0.40 ± 0.04	0.69 ± 0.03	0.32
30 Jun	SS	0.62 ± 0.15	1.05 ± 0.09	0.45
	CS	0.82 ± 0.11	0.61 ± 0.07	−0.22
14 Jul	SS	0.96 ± 0.07	0.72 ± 0.04	−0.19
	CS	0.88 ± 0.08	0.84 ± 0.05	0.04
31 Jul	SS	1.60 ± 0.22	1.69 ± 0.13	0.20
	CS	0.94 ± 0.15	1.35 ± 0.09	0.31
28 Aug	SS	0.86 ± 0.08	1.05 ± 0.05	0.24
	CS	0.88 ± 0.09	0.95 ± 0.05	0.09
16 Oct	SS	0.26 ± 0.05	0.24 ± 0.03	0.13
	CS	0.22 ± 0.10	0.77 ± 0.06	0.58
2004				
4 Jun	SS	0.49 ± 0.05	1.17 ± 0.03	0.70
17 Jun	SS	0.53 ± 0.12	0.70 ± 0.07	0.32
30 Jun	SS	1.06 ± 0.07	1.30 ± 0.04	0.27
22 Jul	SS	2.07 ± 0.18	2.27 ± 0.11	0.38
19 Aug	SS	2.24 ± 0.16	0.98 ± 0.10	−1.13
7 Sep	SS	0.31 ± 0.09	0.89 ± 0.06	0.66
7 Oct	SS	0.94 ± 0.09	0.90 ± 0.06	0.03

Bricelj et al. 2001). However, a measurable clearance rate was obtained for *G. demissa* on that date.

During 2004, clearance rates of total chl *a* of ribbed mussels were always greater than rates of either hard or soft-shell clams, and on 3 dates (3 June, 30 June, and 22 July) the differences were significant (Table 4, Fig. 6). For example, during the 30 June experiment, the ribbed mussel clearance rate was 4.5 times greater than either clam species. In contrast, individual clearance rates of hard and soft-shell clams were similar ($p > 0.05$ for multiple comparisons among means) and generally <1.0 l bivalve^{-1} h^{-1}, with the exception of the 22 July experiment. On 22 July, the hard clam clearance rate was 2.5 l bivalve^{-1} h^{-1}. Average clearance rates (l bivalve^{-1} h^{-1} ± SD) of total chl *a* for the season were 0.8 ± 1.4 , 2.9 ± 2.3, and 0.6 ± 0.5 for hard clams, mussels, and soft-shell clams, respectively.

Clearance rates were combined with population estimates to determine the total clearance capacity of bivalve populations, and the results related to bay volume. In general, West Neck Bay resident mussels were estimated to have a greater clearance capacity of the total phytoplankton community than the planted clams in both years, but *Mya arenaria* was also an important grazer of total phytoplankton during 2004 (Fig. 7).

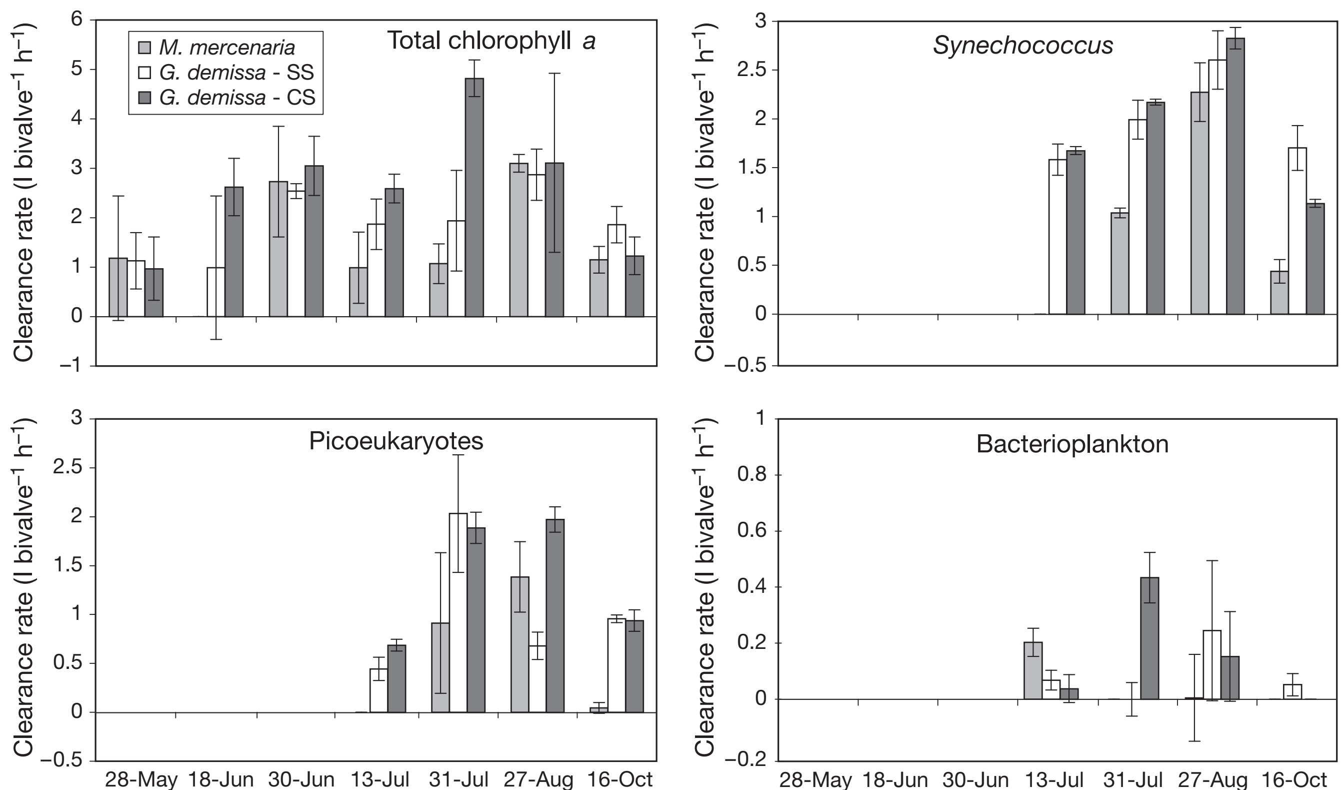

Fig. 5. *Geukensia demissa* and *Mercenaria mercenaria*. Mean (±SE; n = 3) bivalve clearance rates of total chlorophyll *a*, *Synechococcus*, photosynthetic picoeukaryotes, and bacterioplankton in 2003. Sampling of picoplankton began on 13 July. Negative clearance rates are shown as zero. CS: control site; SS: spawning sanctuary

Table 4. 2-way ANOVA of bivalve clearance rates (l bivalve^{-1} h^{-1}) on total chlorophyll *a* (chl *a*), picoplanktonic cells (*Synechococcus*, photosynthetic picoeukaryotes, bacterioplankton), and microplankton (dinoflagellates, ciliates, pennate diatoms, and *Acartia tonsa* eggs). Comparisons were made between *Geukensia demissa* and *Mercenaria mercenaria* in 2003 and among *G. demissa*, *M. mercenaria*, and *Mya arenaria* in 2004

			Effect	df	MS	*F*	p
Total chl *a*							
2003	Hard clam vs. ribbed mussel	Bivalve	Fixed	1	2.4211	3.9243	0.095
	Sanctuary site	Date	Random	6	5.0056	8.1133	0.011
		Date × Bivalve	Random	6	0.617	0.362	0.897
		Error		28	1.7043		
	Ribbed mussels	Site	Fixed	1	5.8666	2.7058	0.151
	Sanctuary vs. control	Date	Random	6	4.1933	1.934	0.221
		Date × Site	Random	6	2.1682	1.1348	0.368
		Error		28	1.9106		
2004	Hard, soft, ribbed	Bivalve	Fixed	2	34.079	8.1746	0.006
	Sanctuary site	Date	Random	6	8.4286	2.0189	0.141
		Date × Bivalve	Random	12	4.1821	3.7927	0.001
		Error		41	1.1026		
Synechococcus							
2003	Hard clam vs. ribbed mussel	Bivalve	Fixed	1	6.8873	13.1406	0.036
	Sanctuary site	Date	Random	3	3.3396	6.3718	0.081
		Date × Bivalve	Random	3	0.5241	4.1019	0.025
		Error		16	0.1278		
	Ribbed mussels	Site	Fixed	1	0.0018	0.00885	0.931
	Sanctuary vs. control	Date	Random	3	1.9766	9.5804	0.048
		Date × Site	Random	3	0.2063	2.4522	0.101
		Error		16	0.0841		
2004	Hard, soft, ribbed	Bivalve	Fixed	2	41.935	18.6229	0
	Sanctuary site	Date	Random	5	16.696	7.4147	0.004
		Date × Bivalve	Random	10	2.2518	0.4225	0.926
		Error		36	5.3296		
Picoeukaryotes							
2003	Hard clam vs. ribbed mussel	Bivalve	Fixed	1	1.6988	1.6246	0.292
	Sanctuary site	Date	Random	3	2.3279	2.2261	0.264
		Date × Bivalve	Random	3	1.0457	2.5954	0.088
		Error		16	0.4029		
	Ribbed mussels	Site	Fixed	1	0.7066	1.1048	0.37
	Sanctuary vs. control	Date	Random	3	2.1189	3.3132	0.176
		Date × Site	Random	3	0.6395	3.7329	0.033
		Error		16	0.1713		
2004	Hard, soft, ribbed	Bivalve	Fixed	2	17.085	3.357	0.077
	Sanctuary site	Date	Random	5	10.672	2.0968	0.149
		Date × Bivalve	Random	10	5.0895	3.1123	0.006
		Error		36	1.6353		
Bacterioplankton							
2003	Hard clam vs. ribbed mussel	Bivalve	Fixed	1	0.0854	1.9176	0.26
	Sanctuary site	Date	Random	3	0.1222	2.7446	0.215
		Date × Bivalve	Random	3	0.0445	0.6081	0.619
		Error		16	0.0732		
	Ribbed mussels	Site	Fixed	1	0.0297	0.2273	0.666
	Sanctuary vs. control	Date	Random	3	0.0566	0.4326	0.745
		Date × Site	Random	3	0.1307	3.2162	0.051
		Error		16	0.0406		
2004	Hard, soft, ribbed	Bivalve	Fixed	2	6.1924	1.7812	0.218
	Sanctuary site	Date	Random	5	0.5898	0.1697	0.968
		Date × Bivalve	Random	10	3.4765	1.81	0.094
		Error		36	1.9207		

Table 4 (continued)

			Effect	df	MS	*F*	p
Dinoflagellates							
2004	Hard, soft, ribbed	Bivalve	Fixed	2	73.117	6.5865	0.01
	Sanctuary site	Date	Random	6	13.396	1.2057	0.365
		Date × Bivalve	Random	12	11.117	1.0319	0.441
		Error		38	10.773		
Ciliates							
2004	Hard, soft, ribbed	Bivalve	Fixed	2	49.426	5.45	0.019
	Sanctuary site	Date	Random	6	19.149	2.1085	0.125
		Date × Bivalve	Random	12	9.0907	1.0553	0.422
		Error		38	8.614		
Pennate diatoms							
2004	Hard, soft, ribbed	Bivalve	Fixed	2	9.2202	0.7424	0.498
	Sanctuary site	Date	Random	5	22.0245	1.7809	0.2
		Date × Bivalve	Random	10	12.301	0.826	0.607
		Error		31	14.8914		
***Acartia tonsa* eggs**							
2004	Hard, soft, ribbed	Bivalve	Fixed	2	8.4096	0.496	0.619
	Sanctuary site	Date	Random	6	51.9378	3.0929	0.044
		Date × Bivalve	Random	12	16.7581	0.8212	0.628
		Error		34	20.4069		

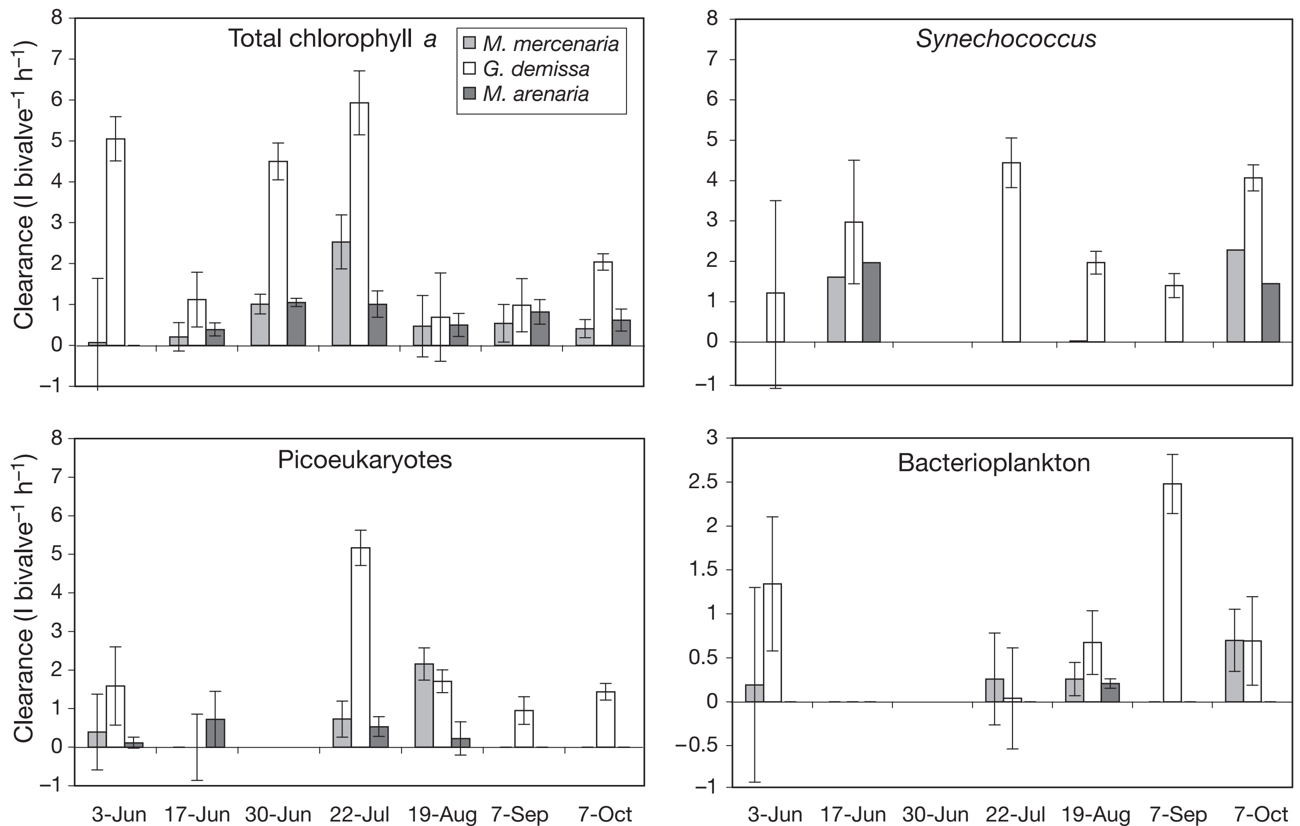

Fig. 6. *Geukensia demissa*, *Mercenaria mercenaria*, and *Mya arenaria*. Mean (±SE; n = 3) bivalve clearance rates of total chlorophyll *a*, *Synechococcus*, photosynthetic picoeukaryotes, and bacterioplankton in the spawning sanctuary during 2004. Flow cytometry samples for 30 June 2004 were not processed. Negative clearance rates are shown as zero

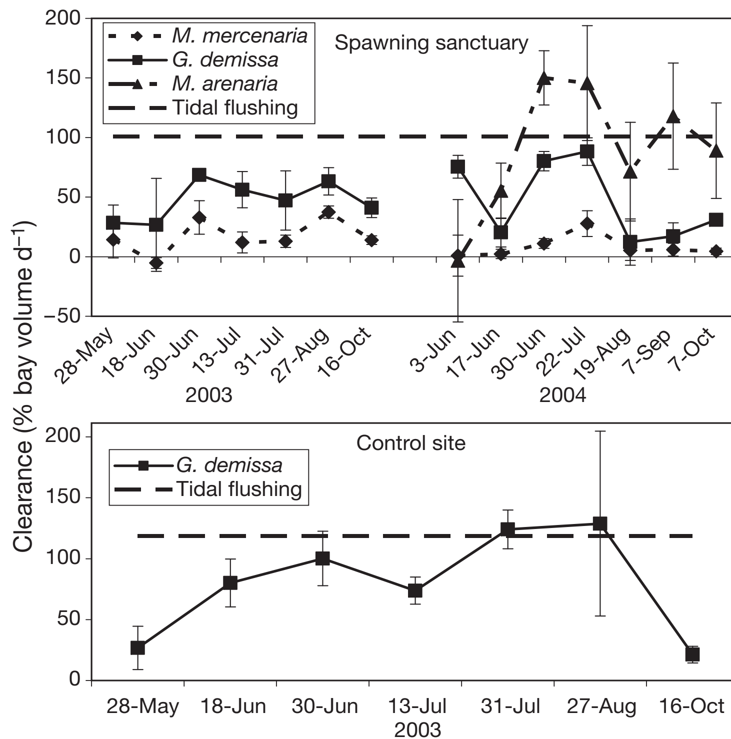

Fig. 7. *Geukensia demissa*, *Mercenaria mercenaria*, and *Mya arenaria*. Mean (±SE) clearance capacities of bivalves, determined from total chlorophyll *a* clearance rates (l bivalve^{-1} h^{-1}) and population abundances (n = 3 for clearance rate, n = 50 for bivalve abundance), relative to daily flushing of the control and spawning sites during 2003 and the latter in 2004

Table 5. *Geukensia demissa*, *Mercenaria mercenaria*, *Mya arenaria*. Mean (± SE) clearance capacities (% d^{-1}) of total bivalves on picoplanktonic cells (*Synechococcus*, photosynthetic picoeukaryotes, and bacterioplankton) determined from clearance rates (l bivalve^{-1} h^{-1}) and population abundances (n = 3 for clearance rate, n = 50 for abundance), relative to daily flushing of the experimental site in 2004

Date	*G. demissa*	*M. mercenaria*	*M. arenaria*
Synechococcus			
3 Jun	18 ± 48	0	0
17 Jun	54 ± 39	18 ± 8	282 ± 187
22 Jul	66 ± 13	0	0
19 Aug	35 ± 7	0	0
7 Sep	24 ± 7	0	0
7 Oct	62 ± 7	25 ± 8	208 ± 42
Photosynthetic picoeukaryotes			
3 Jun	24 ± 22	4 ± 15	17 ± 29
17 Jun	0	0	4 ± 147
22 Jul	77 ± 10	8 ± 8	77 ± 52
19 Aug	0	24 ± 10	33 ± 88
7 Sep	17 ± 9	0	0
7 Oct	22 ± 5	0	0
Bacterioplankton			
3 Jun	20 ± 16	2 ± 17	0
17 Jun	0	0	0
22 Jul	1 ± 12	3 ± 8	0
19 Aug	12 ± 9	3 ± 3	30 ± 11
7 Sep	43 ± 8	0	0
7 Oct	10 ± 11	8 ± 6	0

Picoplankton

A comparison of individual clearance rates of ribbed mussels between the sanctuary and control sites in 2003 showed no difference for any of the picoplanktonic taxa measured (Table 4, Figs. 5 & 6). With the exception of the 28 August 2003 experiment, ribbed mussels had significantly higher feeding rates on *Synechococcus* compared to clams during 2003 and 2004 ($p < 0.05$ for multiple comparisons among means), while there was no significant difference between *Mercenaria mercenaria* and *Mya arenaria* ($p > 0.05$). Averaged over 2003 and 2004, bivalve clearance rates (l bivalve^{-1} h^{-1}) on *Synechococcus* were 2.4 ± 2.2, 0.3 ± 2.2 and 0.3 ± 1.7 for ribbed mussels, hard clams, and soft-shell clams, respectively. There was no significant difference in clearance rates for *Geukensia demissa* (1.4 ± 1.8 l h^{-1}), *M. mercenaria* (0.1 ± 1.6 l h^{-1}), or *M. arenaria* (0.1 ± 0.9 l h^{-1}; $p > 0.05$) feeding on photosynthetic picoeukaryotes in either year. Although the average clearance rate on bacterioplankton was positive for *G. demissa* (0.5 ± 1.2 l h^{-1}) and on average not detected in hard (−0.1 ± 1.4 l h^{-1}) or soft-shelled clams (−0.2 ± 1.0 l h^{-1}), there was no significant difference among the species. In 2004, the clearance capacity of the *G. demissa* population on *Synechococcus* in the spawning sanctuary ranged between 18 and 66% d^{-1}. The population of *M. arenaria* was estimated to have the greatest clearance capacity, >200% d^{-1}, on the cyanobacteria on 2 dates — 17 June and 7 October — but on other dates it had no impact (Table 5).

Microplankton

Geukensia demissa consistently exhibited feeding on dinoflagellates for the 2004 season (average = 3.4 ± 3.5 [SD] l bivalve^{-1} h^{-1}) (Fig. 8). The average clearance rates of dinoflagellates by *Mercenaria mercenaria* (−0.2 ± 3.8 l h^{-1}) and *Mya arenaria* (0.8 ± 2.4 l h^{-1}) were significantly ($p < 0.05$) lower during the season than those by *G. demissa*, and the clam rates were not significantly different ($p < 0.05$) from one another (Table 4, and multiple comparison among means). Bivalve clearance rates of ciliates also were significantly related to bivalve species (Table 4, Fig. 8). Ribbed mussels exhibited higher seasonal clearance rates on ciliates (2.9 ± 3.5 l bivalve^{-1} h^{-1}) compared to hard (0.0 ± 3.5 l h^{-1}) and soft-shell clams (0.6 ± 2.0 l h^{-1}). The minimum total population clearance capacity of bi-

Table 6. *Geukensia demissa*, *Mercenaria mercenaria*, *Mya arenaria*. Mean (± SE) clearance capacities (% d^{-1}) of total bivalves on microplanktonic taxa (dinoflagellates, ciliates, and eggs of *Acartia tonsa*) determined from clearance rates (l bivalve^{-1} h^{-1}) and population abundances (n = 3, *n = 2, **n=1 for clearance rate and n = 50 for abundance), relative to daily flushing of the experimental site in 2004, nd = not determined.

Date	*G. demissa*	*M. mercenaria*	*M. arenaria*
Dinoflagellates			
3 Jun	110 ± 62	0	0
17 Jun	18 ± 07	13 ± 08	43 ± 88
30 Jun	96 ± 19	5 ± 38	307 ± 173
22 Jul	18 ± 21	0	0
19 Aug	55 ± 15	15 ± 13	258 ± 209
7 Sep	54 ± 45*	0	0
7 Oct	38 ± 10	8 ± 05	430 ± 253
Pennate diatoms			
3 Jun	0	0	17 ± 02**
17 Jun	nd	nd	nd
30 Jun	0	37 ± 16	258 ± 209
22 Jul	72 ± 74	0	316 ± 191
19 Aug	62 ± 41*	37 ± 12*	359 ± 182
7 Sep	109 ± 33	30 ± 09	445 ± 126*
7 Oct	47 ± 33	0	401 ± 249
Ciliates			
3 Jun	88 ± 20	0	0
17 Jun	40 ± 33	9 ± 14	72 ± 75
30 Jun	82 ± 41	23 ± 08	65 ± 115
22 Jul	10 ± 42	0	0
19 Aug	68 ± 07	12 ± 21	344 ± 125
7 Sep	0	0	43 ± 162*
7 Oct	40 ± 36	24 ± 16	258 ± 127
Copepod eggs			
3 Jun	53 ± 88	86 ± 42	1117 ± 263
17 Jun	0	12 ± 62*	275 ± 530*
30 Jun	114 ± 11*	0	0
22 Jul	101 ± 70	22 ± 13	309 ± 183
19 Aug	60 ± 41	10 ± 21	290 ± 200
7 Sep	2 ± 14	0	0
7 Oct	0	0	0

valves on dinoflagellates was 18% d^{-1} and on ciliates 10% d^{-1}, both occurring on 22 July (Table 6).

Due to a very low density of pennate diatoms on 16 June (some samples had no cells), we excluded this date for statistical analysis. No significant difference in clearance rate due to bivalve species was found (Table 4). Pennate diatoms were cleared from the plankton assemblage at an average rate of 2.1 ± 5.9 l bivalve^{-1} h^{-1}, 0.8 ± 2.8 l h^{-1}, and 2.3 ± 1.9 l h^{-1} for ribbed mussels, hard and soft-shell clams, respectively (Fig. 8). Although there was no difference in individual clearance rates among the bivalves, the *Mya arenaria* population exerted the highest clearance capacity (except on 17 June) on pennate diatoms due to their greater density in the embayment (Table 6).

The overall averages for bivalve clearance rate of eggs of *Acartia tonsa* were positive for all 3 species, although on 2 dates in late summer and 1 in spring egg predation by clams was not detected; on 1 date each in the spring and late summer mussels were not feeding on eggs either (Fig. 8). In cases where the experimental or control density of eggs was determined to be <1 l^{-1}, clearance rates were calculated assuming a density of 1 egg l^{-1} which resulted in more realistic (usually lower) estimates of bivalve clearance rates. Also, if the final density of eggs in a bucket was zero, the replicate was removed from the data set. *Geukensia demissa* cleared *A. tonsa* eggs at an average rate of 3.0 ± 4.2 l bivalve^{-1} h^{-1}, while *Mercenaria mercenaria* and *Mya arenaria* clearance rates were 1.4 ± 4.5 and 2.0 ± 4.3 l h^{-1}, respectively. There was no significant difference in clearance rates among the bivalve species (Table 4). On all dates the combined bivalve clearance capacity of eggs equaled or exceeded 10% d^{-1} (Table 6).

On most dates in 2004, bivalve predation on total copepod nauplii was not detected and the overall average clearance rates were negative (–0.55 ± 4.5 l bivalve^{-1} h^{-1}, –0.56 ± 3.2 l h^{-1}, and –0.11 ± 2.6 l h^{-1} for ribbed mussels, hard and soft-shell clams, respectively). Generally, the results did not change when only early-stage nauplii (NI to NIII) of *Acartia tonsa* were considered (average clearance rates were –1.6 ± 5.9 and –1.3 ± 4.8 l h^{-1} for mussels and hard clams, respectively), except for *Mya arenaria* which, on 4 out of the 7 dates, cleared these prey from the plankton assemblage (0.9 ± 5.3 l h^{-1}).

DISCUSSION

Although knowledge of the ecological roles of marine bivalves is largely drawn from estuaries with large salt marsh systems, we have shown that bivalve grazing on the autotrophic and heterotrophic components of the plankton is similarly important in relatively small, shallow embayments with only a fringing marsh. For example, in Great Sippewissett Marsh, Massachusetts, *Geukensia demissa* filtered a volume of water greater than the tidal volume of the marsh on each tidal cycle (Jordon & Valiela 1982), which is mostly higher than at our sites for the same species. However, the total grazing impact on chl *a* of all 3 bivalve species, *G. demissa*, *Mercenaria mercenaria*, and *Mya arenaria*, at times exceeded the total volume of the embayments flushed on a daily basis (Fig. 7). Our results also are in agreement with other studies that indicate a complex trophic role for bivalves in coastal environments (e.g. *G. demissa*; Kreeger & Newell 2000, Wetz et al. 2002). Complex food web effects are possible since bivalves are not only grazing on primary producers but simultaneously competing with and preying on planktonic secondary producers. For example, heterotrophic protists, a group we suspect will be maximally impacted by bivalve feeding, are an

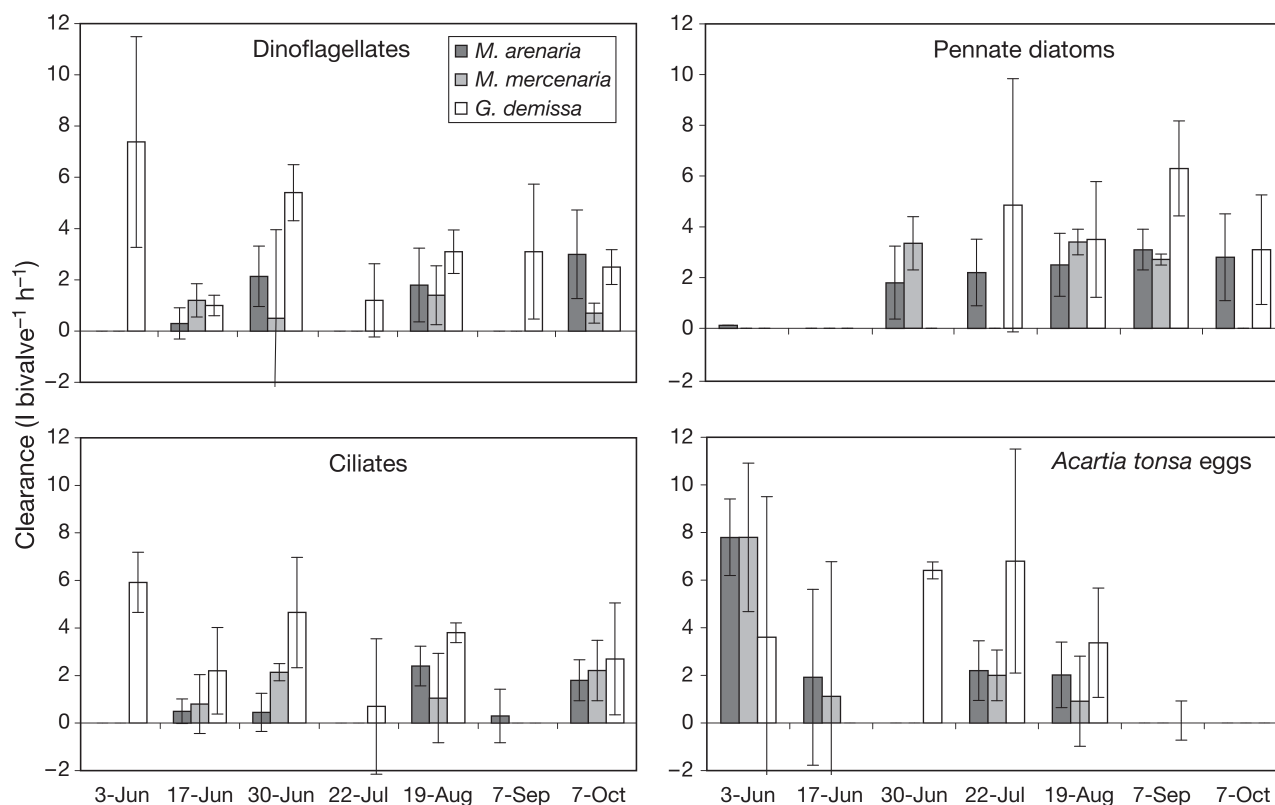

Fig. 8. *Geukensia demissa*, *Mercenaria mercenaria*, and *Mya arenaria.* Mean (±SE) bivalve clearance rates of dinoflagellates, ciliates, pennate diatoms, and *Acartia tonsa* eggs in the spawning sanctuary during 2004 (in general n = 3 for clearance rate, but see Table 6, n = 50 for bivalve abundance). Negative clearance rates are shown as zero

important food resource for other zooplankton such as copepods (Stoecker & Capuzzo 1990, Lonsdale et al. 1996). Moreover, *Acartia tonsa*, the dominant copepod in these bays in summer, was shown to be subjected to substantial egg predation by all 3 bivalve species.

Microplankton versus bivalve grazing impacts

Microzooplankton are significant grazers of phytoplankton in Long Island estuaries and elsewhere (Lonsdale et al. 1996, Boissonneault-Cellineri et al. 2001, Deonarine et al. 2006). Summarizing data from the literature on rates of growth of oceanic phytoplankton and microzooplankton grazing determined from dilution experiments, Calbet & Landry (2004) found that, on average, microzooplankton consumed 67% of phytoplankton growth on a daily basis. The present study has shown that the bivalve communities of the embayments of West Neck Bay had grazing impacts on the total phytoplankton (chl *a*) community comparable to or exceeding those of microzooplankton (Fig. 7, Table 3). The lowest clearance capacities (percent bay volume cleared per day) of the total bivalve community in the experimental site were ~35 to 40% d^{-1} in 2003 and ~75% d^{-1} in 2004 when soft-shell clams were abundant; the lower capacities occurred mostly during the spring and late summer. During the same times, the percent impact of microzooplankton (i.e. $g/k \times 100$) on phytoplankton growth was ~35 to 100% d^{-1}. In 2003 in the control site, ribbed mussels cleared a seasonal average of 47% d^{-1} of the embayment compared to 81% d^{-1} by microzooplankton. Our estimates of grazing impacts of bivalves on phytoplankton may be conservative. We selected the same nutrient treatments (nitrate and phosphate) for both the bivalve and microzooplankton grazing studies based on a standard protocol for nutrient-replete conditions in dilution experiments (e.g. Caron et al. 2000). If ammonia excretion by bivalves stimulated the growth of some phytoplankton in the experimental buckets, then clearance rates may have been underestimated. In addition, since our experiments were carried out in unmixed buckets under conditions where the water may have been re-filtered by the bivalves, our clearance rate estimates may be lower than we would have obtained under flow conditions.

Bivalve impacts on microplankton

The predation rates on microplankton indicate that bivalves could have had a substantial influence on the net population growth rate of some microplanktonic taxa, including heterotrophs, in the spawning sanctuary in 2004. The maximum daily growth rates of dinoflagellates can range between 1.0 and 3.5 d^{-1} (Smayda 1997). Additionally, on most dates except 22 July the total clearance of dinoflagellates from bivalve feeding was >50% d^{-1} and on 2 dates (30 June and 7 October) exceeded 350% d^{-1} (Table 5). Due to their high population density, *Mya arenaria* on some dates were largely responsible for the high rates of grazing mortality on dinoflagellates, while on other dates dinoflagellate grazing by this clam was not detected. This variable feeding behavior was also true for *Mercenaria mercenaria*. In contrast, *Geukensia demissa* consistently exhibited grazing on dinoflagellates, including dates when clams were not feeding. We cannot conclude decisively that the differences in feeding performance on dinoflagellates of the 3 bivalve species reflects differential sensitivity to potentially noxious species which were found at our study site. However, the difference in feeding behavior of hard clams and ribbed mussels (i.e. on 18 June in 2003) in the midst of a modest brown tide (4.1×10^4 cells ml^{-1}) suggests this is a possibility.

Population growth rates of planktonic ciliates can exceed 1.0 d^{-1} during summer months (e.g. 1.2 to 1.55 d^{-1}; Stoecker et al. 1983, Lonsdale et al. 1996) and, as found for dinoflagellates, bivalve-induced mortality in the embayments (Table 6) was sometimes higher than the potential growth rate of ciliates. This result suggests that the bivalves are a major source of mortality for protistan zooplankton and is in accordance with other findings in very different environments. For example, a decline in the abundance of tintinnids was associated with the invasion of the zebra mussel *Dreissena polymorpha* in the Hudson River, New York (Pace et al. 1998). Also, in a coastal lagoon in the northern Mediterranean, tintinnid abundance was reduced by a factor of 10 at a shellfish culture site compared to waters away from the site (Lam-Hoai et al. 1997). To our knowledge, this is the first report of feeding on planktonic ciliates by *Mya arenaria* and *Mercenaria mercenaria*.

Interactions with copepods

The present study also demonstrated that bivalve predation may directly regulate the population dynamics of copepods, at least of those species which broadcast their eggs as do species of *Acartia*. At temperatures between 18 and 25°C, the intrinsic rate of increase of *Acartia tonsa* ranges from 0.12 to 0.25 d^{-1} (T. Kiørboe pers. comm.) which also, at times, would have been exceeded by the clearance capacity of the bivalves (Table 6). The present study did not, however, detect bivalve predation on total copepod nauplii (NI to NVI) or earlier naupliar stages (NI to NIII) with the exception of *Mya arenaria* on several dates. These results are in contrast to studies indicating that other mussels, i.e. *Mytilus edulis* and *Perna canaliculus* (Davenport et al. 2000, Zeldis et al. 2004), and clams *Corbula* (*Potamocorbula*) *amurensis* (Kimmerer et al. 1994) prey on crustacean zooplankton, including copepod nauplii. In laboratory studies, Jonsson et al. (2009) found an inverse relationship between water turbulence and the ability of various developmental stages of *A. tonsa* to escape being filtered by *M. edulis*. Their results suggest that under turbulent conditions copepods have a reduced ability to detect the inhalant water current from mussel filtering. And, because water turbulence kept the eggs suspended in the water column, clearance rate on *A. tonsa* eggs by *M. edulis* also increased with turbulence and reached a maximum of ~3 l h^{-1} for a 35 mm mussel. This clearance rate is the same as the mean value we determined for larger *Geukensia demissa*. Thus the individual bivalve clearance rates and impacts on the *A. tonsa* population in West Neck Bay may be conservative estimates. We do not know, however, if an increase in turbulent water motion in the experimental buckets would have enhanced bivalve clearance of *A. tonsa* nauplii or eggs. Overall, it is appears that bivalves have the ability to substantially regulate the population dynamics of the dominant planktonic microzooplankton (Boissonneault-Cellineri et al. 2001) in these shallow embayments.

More evidence for complex benthic–pelagic interactions

The predation impact on *Acartia tonsa* eggs found in the present study helps to explain our previous observations in mesocosm experiments (using ambient seawater) in which hard clam abundance was manipulated, and where a negative relationship between hard clams and copepod abundance was found (Lonsdale et al. 2007). At the end of 3 separate experiments, biomasses of nauplii, copepodites, and adults of the dominant copepod, *A. tonsa*, were lower in tanks with clams compared to controls. Redundancy analysis indicated that total population clearance rate (l h^{-1}) by *Mercenaria mercenaria* was the single best predictor of composition of the planktonic community (carbon biomasses of diatoms, dinoflagellates, ciliates, and brown tide were also included in the analysis). Although food (i.e. diatoms and dinoflagellates) limitation could have contributed to low copepod abundance

in tanks with clam competitors, we hypothesized that these copepod reductions may have resulted from direct predatory impacts of clams on early life stages of *A. tonsa* and which is now supported by the present study.

In contrast, the predator–prey relationship between hard clams and ciliates reported herein does not match the results from the mesocosm experiments. In the latter, ciliate biomass was positively correlated with total clam clearance rate (Lonsdale et al. 2007). These contradictory results, however, can be explained: despite likely predation by clams, the conditions of no brown tide in the clam tanks likely resulted in much higher ciliate cellular growth and net population growth compared to control tanks without clams in which brown tide developed. In Great South Bay, negative population growth of ciliates was associated with a brown tide (Lonsdale et al. 1996).

Implications of bivalve diversity for shellfish restoration

Particle retention efficiencies for hard clams decrease below 4 µm in diameter (Riisgård 1988), while ribbed mussels are more capable of removing small particles. *Geukensia demissa* was more efficient at clearing particles in the 0.5 to 2.5 µm range than several other bivalve species (Wright et al. 1982), and equally capable of ingesting carbon from small bacteria (<1 µm estimated spherical diameter, ESD) and heterotrophic flagellates (3 to 5 µm ESD) (Kreeger & Newell 1996, 2001; see also Langdon & Newell 1990, Newell & Krambeck 1995). Clearance rates (l h^{-1} g^{-1}) on phytoplankton (i.e. *Isochrysis galbana*, 4.8 µm ESD) and benthic diatoms (15 µm ESD), however, were higher for ribbed mussels compared to bacteria and heterotrophic protists (see Fig. 1 in Kreeger & Newell 2001). Our field research has shown that in a natural plankton assemblage both clams and ribbed mussels removed *Synechococcus* (0.5 to 1 µm ESD) and photosynthetic picoeukaryotes (1 to 3 µm), albeit the mussels were more efficient on *Synechococcus*. Since the clearance rate estimates for each plankton group were obtained by sampling the same containers, the clam or mussel pumping rate was identical for each of the plankton groups. Differences in clearance rates among the plankton components within a date and treatment reflect, therefore, differences in retention efficiency (RE) and/or escape behavior of motile prey such as copepod nauplii. In West Neck Bay during 2003 and 2004, results for hard and soft-shell clams and ribbed mussels suggested that $RE_{\text{total chl } a} > RE_{Synechococcus} > RE_{\text{picoeukaryotes}} > RE_{\text{bacterioplankton}}$. Bacterioplankton were inefficiently filtered, having retention efficiencies that were almost always <10% of the values for total chl *a*. *G. demissa* consistently cleared dinoflagellates, ciliates, and copepod eggs from the water column and at rates comparable to those determined with total chl *a*. These results contrast with the field study by Kemp et al. (1990), who reported lower removal efficiencies for *G. demissa* of larger autotrophic cells (diatoms) and heterotrophic cells (ciliates and some dinoflagellates) compared to smaller autotrophic and heterotrophic cells (microflagellates). Other attributes besides cell size, including extracellular metabolites and polysaccharides and microalgal growth phase, also influence particle selection and rejection by *G. demissa* and other bivalves (reviewed in Pales Espinosa et al. 2008), and could explain the contrasting results.

The present study has shown the potential importance of *Geukensia demissa* in structuring plankton communities in shallow embayments such as West Neck Bay which may, in part, explain why there were no significant differences in total chl *a* or microzooplankton grazing rates between the control site (ribbed mussels only) and the spawning sanctuary (ribbed mussel and hard clam populations) in 2003. Any effect of hard clams on the autotrophic and heterotrophic plankton may have been swamped by ribbed mussels and other grazers, including microzooplankton. However, the experiments were conducted using incoming seawater from the main stem of West Neck Bay which likely explains the lack of difference. Nonetheless, the swamping scenario is supported by plankton sampling during ebb flow in the 2 embayments in 2003 (Lonsdale et al. 2006a). Sampling was conducted at 5 locations within each site monthly during the winter (2002 to 2003) when weather permitted, and bi-weekly from April through October. Contrary to our hypothesis that the planted hard clams would impact components of the plankton (e.g. *Aureococcus anophagefferens* cell abundance), there were no significant differences in abundances between the sites in total chl *a*, *Synechococcus*, photosynthetic eukaryotes, or *A. anophagefferens*; only bacterioplankton differed and it was higher in the spawning sanctuary.

Our results support the idea that multi-species restoration as opposed to single-species stock enhancement may be a better strategy for restoration of some shellfisheries including the hard clam fishery in Long Island bays. Specifically, the results indicate potential positive feedbacks to clams from the feeding activities of ribbed mussels. For example, in comparison to other microalgae, *Synechococcus* are inefficiently utilized by *Mercenaria mercenaria* (Bricelj et al. 1984). The cyanobacteria are sorted out and passed to the hind gut and not readily assimilated. Thus the higher filtration capacity of ribbed mussels on these cells and removal from suspension could lead to higher assimilation effi-

ciencies of hard clams. Our work also suggests the possibility that the feeding behavior of *Geukensia demissa* in nature is less sensitive to some noxious microalgae, including brown tide and perhaps some dinoflagellates. During a modest brown tide (4.1×10^4 cells ml^{-1}) in West Neck Bay, active feeding by ribbed mussels occurred but not by *M. mercenaria*. This observation is in keeping with laboratory studies showing brown tide had no effect on the ciliary activity of excised gill of *G. demissa*, whereas it caused a decrease in activity of *M. mercenaria* (Gainey and Shumway 1991). If we are correct, ribbed mussels could reduce the incidence of harmful algal blooms in shallow embayments and improve the quality of food for other suspension-feeders. Since ribbed mussels are restricted to intertidal marshes, however, their feeding effects on planktonic community structure would be limited in large bays (Pomeroy et al. 2006). Restoration of commercial shellfish in smaller embayments in which ribbed mussels have a major impact on the plankton community might prove to be a more robust strategy.

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Contribution to the Theme Section 'Directions in bivalve feeding'

Particle retention efficiency of asari clam *Ruditapes philippinarum* larvae

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ABSTRACT: Larval food abundance is one potential cause of recruitment variation in benthic invertebrates, including bivalves. Bivalve veliger larvae can clear particles <~10 µm. However, the particle retention efficiency (PRE) of bivalve larvae differs among species, so is necessary to determine larval food abundance in nature. We investigated the PRE of larvae of the asari clam *Ruditapes philippinarum*, which recently has greatly decreased in numbers in Japan. Artificially hatched larvae from the umbo to the full grown stage were exposed to natural food assemblages. Larval clearance rates were determined for particles in 32 size ranges from 0.8 to 18 µm. We observed significant clearance of particles in the ~1 to 8 µm size range, with the highest clearance rates being on 1.4 to 2.0 µm particles. Larval ingestion of the natural food assemblages ranged from 50×10^2 to 120×10^2 µm^3 ind.$^{-1}$ h^{-1}, which is below the hatchery food ration, suggesting larval food limitation in nature.

KEY WORDS: *Ruditapes philippinarum* · Clam larvae · Clearance rate · Particle retention efficiency

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INTRODUCTION

Events affecting larvae greatly influence the population dynamics of benthic marine invertebrates with planktonic larvae, including bivalves found in soft sediments (Thorson 1950, Ólafsson et al. 1994). Variation in larval food abundance is one potential cause of recruitment variation because it influences larval growth and survival (Liang 1995, Chícharo & Chícharo 2001, García-Esquivel et al. 2001, Bricelj & MacQuarrie 2007, Przeslawski et al. 2008). However, it is difficult to evaluate larval food abundance in nature because information on larval diet is limited (Bos et al. 2006).

The natural diet of bivalve veliger larvae is thought to be nanoplankton (Baldwin 1995, Raby et al. 1997, Sommer et al. 2000). In laboratory experiments with *Mytilus edulis* larvae, the highest clearance rate was seen for food particles in the size range of 2.5 to 3.5 µm (Riisgård 1980, Sprung 1984a). Particle retention efficiency (PRE) of bivalve larvae varies among species (Riisgård 1980, 1988, Sprung 1984a, Baldwin 1995, Riisgård et al. 2000), and thus it is necessary to determine the size range of the potential larval diet (i.e. the PRE) in order to assess larval food abundance in nature.

The asari clam (*Ruditapes philippinarum*; also known as the Manila clam and short-necked clam) is indigenous to coasts of East Asia and Japan. The fishery production in Japan decreased from a maximal production of 140 000 tonnes in 1985 to 35 000 tonnes in 2005 (Ministry of Agriculture, Forestry and Fisheries, Japan 2007). Although the reason for the decrease is unknown, decreasing recruitment may be one cause (Ishii et al. 2001, Toba et al. 2007). Measures of larval food abundance in nature can be used to determine if larval growth is limited by food availability.

The PRE of asari clam larvae has not been determined, although the larvae can be grown in hatcheries on standard algal diets for bivalve larvae (e.g. *Pavlova*

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lutheri or *Chaetoceros calcitrans*) (Miyama & Toba 1990, Yan et al. 2006). Information about the size of food particles in the natural larval diet may also improve larval rearing techniques in the hatchery. In this study, we investigated the clearance rate and PRE of asari clam larvae in natural seawater to better understand larval ingestion rates on natural food assemblages.

MATERIALS AND METHODS

PREs of asari clam larvae were examined in 4 separate experimental trials. Larval stage varied between trials; umbo stage to full-grown larvae were used. The clam larvae for the experiments were spawned and grown in our laboratory on standard algal diets (*Pavlova lutheri*, *Chaetoceros calcitrans*, or *Chaetoceros gracilis*). Natural seawater was collected for the experiments at a depth of ~2 to 5 m from the floating dock in front of our laboratory (water depth approximately 10 m) during mid- to high tide using a Niskin water sampler. This area is a natural habitat for the asari clam. Collected seawater was filtered through a 20 µm mesh screen and then poured into six 500 ml plastic bottles. Equal volumes of a uniform suspension of clam larvae were added to 3 of the bottles and the remaining 3 bottles were treated as controls. Larval densities were high enough for significant particle reduction during the experimental period (4 h), but were not adjusted to the same density in every trial (range 4.9 to 28 larvae ml^{-1}). An even distribution of particles and larvae was achieved in the experimental containers by continuous mixing, gentle bubbling in Trial 1 and with a plankton wheel (at 1 rpm) in the other 3 trials. Experiments were conducted at room temperature. Dates of each trial, larval size, larval density, temperature, salinity, and other experimental conditions are shown in Table 1.

Immediately after the addition of larvae and at the end of the 4 h experimental period, a sample of water (~50 ml) was taken from each bottle and filtered through a 20 µm mesh screen, then the particle concentration was determined using a Coulter Counter Multisizer 3 fitted with a 30 µm aperture tube (Beckman Coulter). The particle counter recorded the number and volume of particles in 0.25 to 0.3 ml of sample. Particle concentration was determined for 32 size ranges between 0.8 and 18 µm. The values separating the 32 size ranges were defined by $\ln(0.8) + n \times [\ln(18) - \ln(0.8)]/32$ for n = 0, 1, 2… 32. Particle counting was repeated 6× for each sample, and the particle concentrations were averaged. To determine larval counts and shell lengths, duplicate or triplicate 10 ml samples were taken from each experimental bottle at the end of the experiments and fixed with 3% neutral formalin or 1% glutaraldehyde. Larval numbers in the samples were counted using an inverted microscope. Larval shell length was determined from measurements of 50 larvae.

We calculated the rate of change in particle concentration (k) for each of the 32 size ranges from the equation of Frost (1972):

$$C_2 = C_1 e^{k(t_2 - t_1)} \quad (1)$$

where C_1 and C_2 are particle concentrations (µm^3 ml^{-1}) in control bottles at times t_1 and t_2, respectively. For each larval bottle, the grazing coefficient (g) was calculated from:

$$C'_2 = C'_1 e^{(\bar{k} - g)(t_2 - t_1)} \quad (2)$$

where C'_1 and C'_2 are particle concentrations in larval bottles at times t_1 and t_2, respectively, and $\bar{k}$ is the mean k of the 3 control bottles. For each larval bottle, the average particle concentration, $\bar{C}$, during time interval $t_2 - t_1$ was calculated as:

$$\bar{C} = \frac{C'_2 - C'_1}{(t_2 - t_1)(\bar{k} - g)} \quad (3)$$

For each larval bottle, the clearance rate (CR, µl ind.$^{-1}$ h^{-1}) was calculated as:

$$CR = g/d \quad (4)$$

where d is the larval density. We used the mean larval count from the 3 experimental bottles as the value for d because no statistically significant differences in larval counts were observed among the larval bottles in any experiment.

Table 1. Experimental conditions for each trial. Data are mean ± SD (n). Temperature and salinity were measured at the end of the experiments (n = 6)

Trial	Date (2007)	Temperature (°C)	Salinity (psu)	Shell length (µm)	Stage	Larval density (ind. ml^{-1})	Mixing method	Spawn date (2007)
1	19 Jun	24.6 ± 0.09	31.7 ± 0.05	205.4 ± 13.5	Full grown	14.4 ± 9.7 (6)	Gentle bubbling	5 Jun
2	13 Jul	24.5 ± 0.12	29.8 ± 0.15	141.1 ± 11.8	Umbo	28.0 ± 8.5 (9)	Plankton wheel	6 Jul
3	21 Aug	25.7 ± 0.00	31.8 ± 0.06	128.7 ± 6.0	Umbo	13.2 ± 2.0 (9)	Plankton wheel	15 Aug
4	30 Aug	25.8 ± 0.04	30.7 ± 0.00	191.9 ± 21.2	Full grown	4.9 ± 1.0 (9)	Plankton wheel	15 Aug

CRs over all particle size ranges were smoothed using the 'super smoother' function of the statistical software package R, which determines the best span for smoothing by using local cross-validation (Takezawa 2005, R Development Core Team 2008). The highest clearance rate (peak CR) and the particle size of the peak CR were determined from the highest value of the smoothed lines. For each larval bottle, the ingestion rate (IR, μm^3 ind.$^{-1}$ h^{-1}) was calculated as:

$$IR = \overline{C} \times CR \quad (5)$$

The cumulative IR was also calculated by summing IR beginning at the 0.8 µm size range. If the IR for a given size range was <0, that value was not included in the cumulative calculation.

RESULTS

Because we used natural seawater, the initial particle concentrations were different for each trial (Fig. 1). In Trial 1, there was a peak in particle volume at the 10 to 13 µm size, whereas in Trial 2, there were peaks at 4–6 and 10–13 µm. In contrast, there were no clear peaks in particle size distributions in Trials 3 and 4. Microscopic observation showed that the peaks in Trials 1 and 2 reflected the presence of several species of flagellates and diatoms, and were not due to a single dominant species.

There was a significant increase in particle concentration in the control bottles (Fig. 2; $k > 0$, $p = 0.05$) for small particles (<~1.5 µm) in all trials. Significant decreases ($k < 0$, $p < 0.05$) were seen in approximately 3 to 6 µm particles in Trials 1, 3, and 4. Values for k varied for the larger particles, especially in the >13 µm sizes in Trials 3 and 4, showing a large rate of decrease ($k < -0.1$).

We determined the CR for each size range in each trial (Fig. 3). Significant clearance was seen in the size ranges from 0.8–1 to 8–9 µm ($p < 0.05$). Overall, the CRs were highest for 1 to 3 µm particles. We also measured negative CRs for 4 to 6 µm particles in Trials 3 and 4.

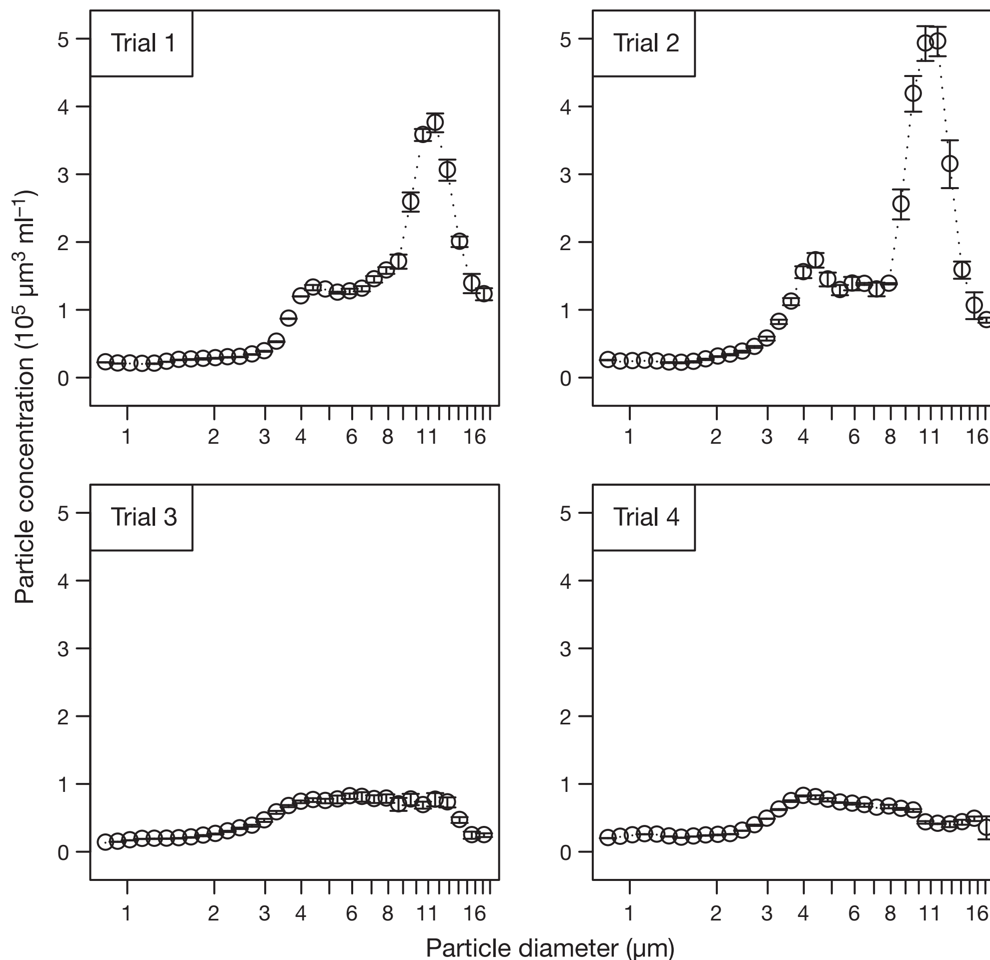

Fig. 1. Initial concentrations of particles in different size ranges for each trial in experimental bottles containing larvae (mean ± SD; n = 3 for all trials)

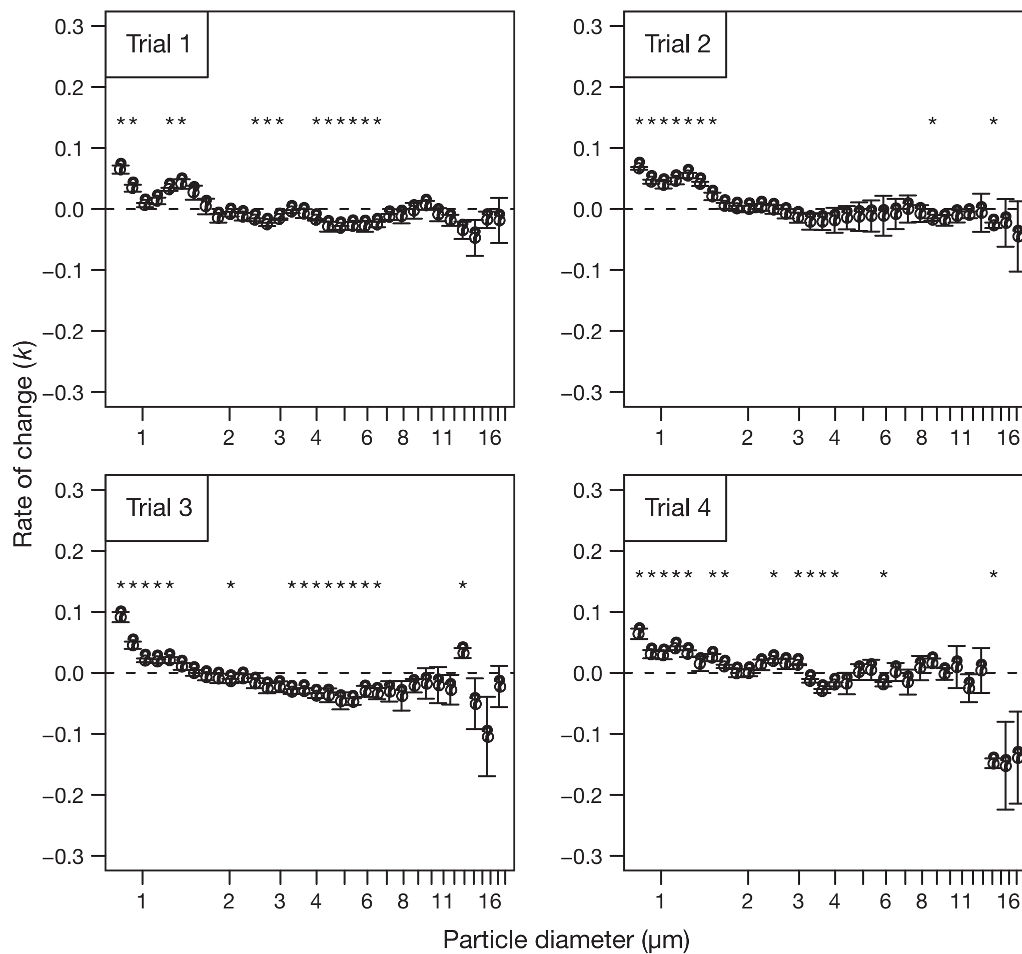

Fig. 2. Rate of change of particle concentrations in control bottles (k; see Eq. 1 in text) for each trial (mean ± SD of 3 bottles). *Rates significantly different from 0 ($p < 0.05$)

The peak CR in the 4 trials ranged from 5.8 to 23.4 µl ind.$^{-1}$ h^{-1}. Particle size of the peak CR ranged from 1.4 to 2.0 µm. Although particle size at peak CR seemed to increase with increasing larval shell length, the relationship was not statistically significant (Fig. 4; ANOVA, $p = 0.259$).

For each trial, the cumulative IR (µm^3 ind.$^{-1}$ h^{-1}) increased with increasing particle size and reached a maximum at a particle size of ~8 to 10 µm. The maximum cumulative IR in the 4 trials ranged from ~50 × 10^2 to 120 × 10^2 µm^3 ind.$^{-1}$ h^{-1} (Fig. 5).

DISCUSSION

In calculating CRs, we assumed that changes in the control bottles were applicable to the treatment bottles in the absence of larvae. It is important to examine the validity of this assumption. In the control bottles, we observed a significant increase in particles <1.5 µm in all trials (Fig. 2). These increases were probably caused by bacterial growth, as this rate of increase is within the range of growth rates of coastal bacteria (Yokokawa & Nagata 2005). If the presence of larvae had any stimulative or inhibitory effect on bacterial growth, then the applicability of changes in control bottles becomes questionable. Significant decreases were seen in 3 to 6 µm particles in the controls for Trials 1, 3, and 4. This might be from some grazing organisms that passed through the 20 µm screen or from adhesion to the inner bottle surface. High rates of decrease were also seen for larger particles (>~13 µm) in Trials 3 and 4. However, these decreases were not always statistically significant because of the large variance caused by the lower particle concentration in these size fractions in Trials 3 and 4 (Fig. 1). Because of this large variance, we suspect that these decreases were the same in the bottles containing larvae.

We observed similar patterns of PRE in the 4 trials. Significant CRs were seen in the 0.8–1 to 8–9 µm particle size fractions (Fig. 3). CRs were highest at the 1 to 3 µm particle sizes. CRs for 4 to 6 µm particles were

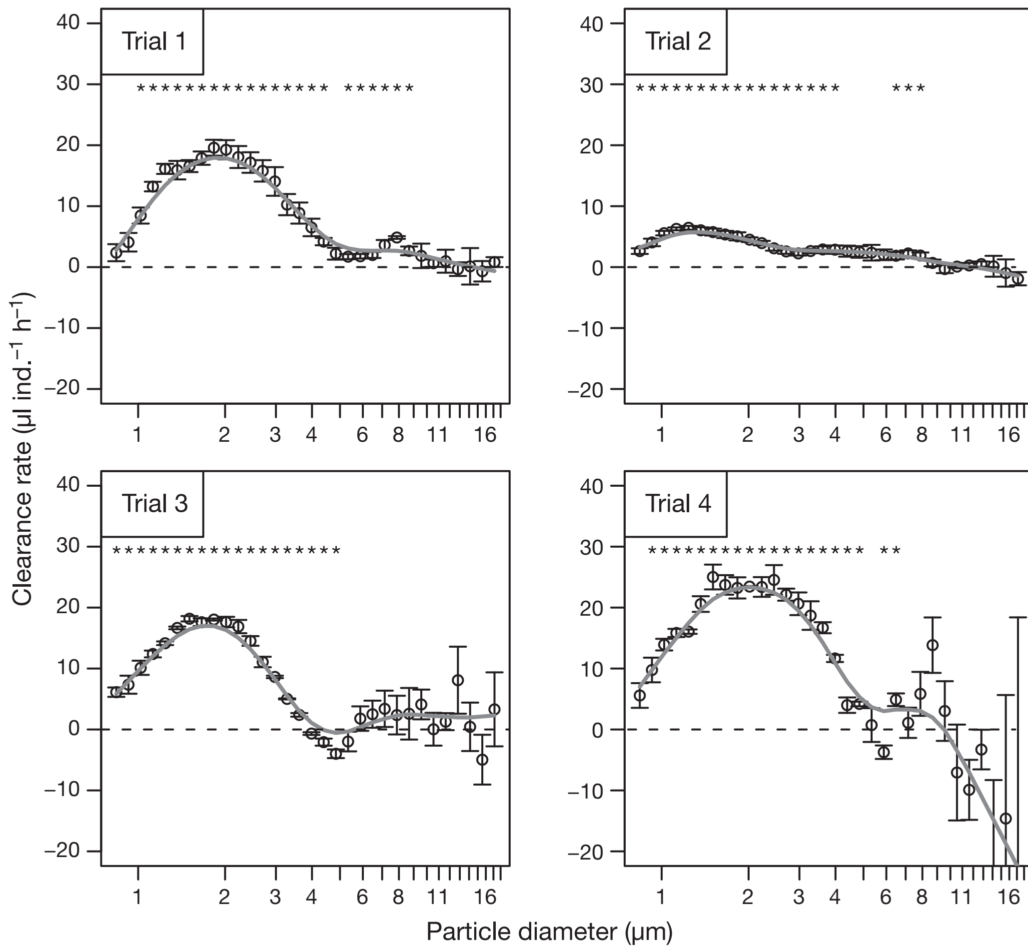

Fig. 3. *Ruditapes philippinarum*. Clearance rates (CR) by particle size for each trial. Error bars: SD of the 3 treatment bottles. *Rates significantly different from 0 ($p < 0.05$). (——) Clearance rate data after using a smoothing function (see text)

low in Trial 1, not significant in Trial 2, and negative in Trials 3 and 4. The negative values might reflect fecal excretion, although effects of fecal production were not part of this study. The different mixing systems did not seem to affect the results, although the systems were not compared under identical conditions.

The peak CRs ranged from 5.8 to 23.4 µl ind.$^{-1}$ h^{-1} (Fig. 3). Differences in larval size and density among the 4 trials would explain this. CRs of *Mytilus edulis* larvae range from 19 to 37 µl ind.$^{-1}$ h^{-1} for 156 to 195 µm larvae at 18°C (Sprung 1984a). CRs in our study (5.8 to 23.4 µl ind.$^{-1}$ h^{-1}) were lower despite the higher experimental temperature (24.5 to 25.8°C). This might have resulted from the high larval densities used in this study. Larval CRs are reported to decrease with increasing larval density above 5 larvae ml^{-1} (Hansen 1991). The lowest peak CR in this study (Trial 2; 5.8 µl ind.$^{-1}$ h^{-1}) coincided with the highest larval density (28 larvae ml^{-1}) and smaller larval size (141.1 µm).

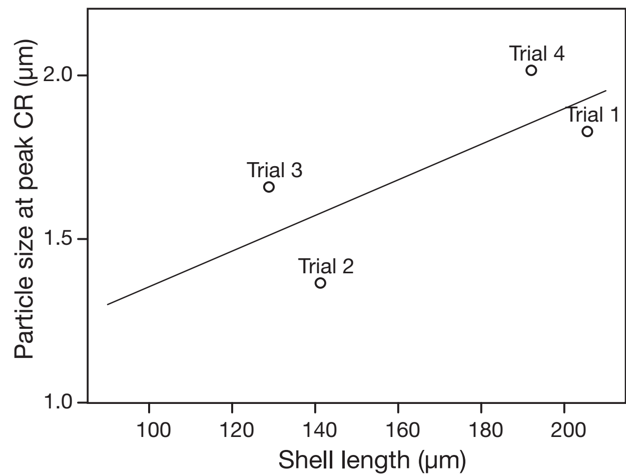

Fig. 4. *Ruditapes philippinarum*. Relationship between the particle size at peak clearance rate (CR, y) and shell length (SL) of larvae. A fit of the data to a linear model yielded $y = 0.81 + 0.00545 \times SL$, although the relationship was not significant (ANOVA, $p = 0.259$)

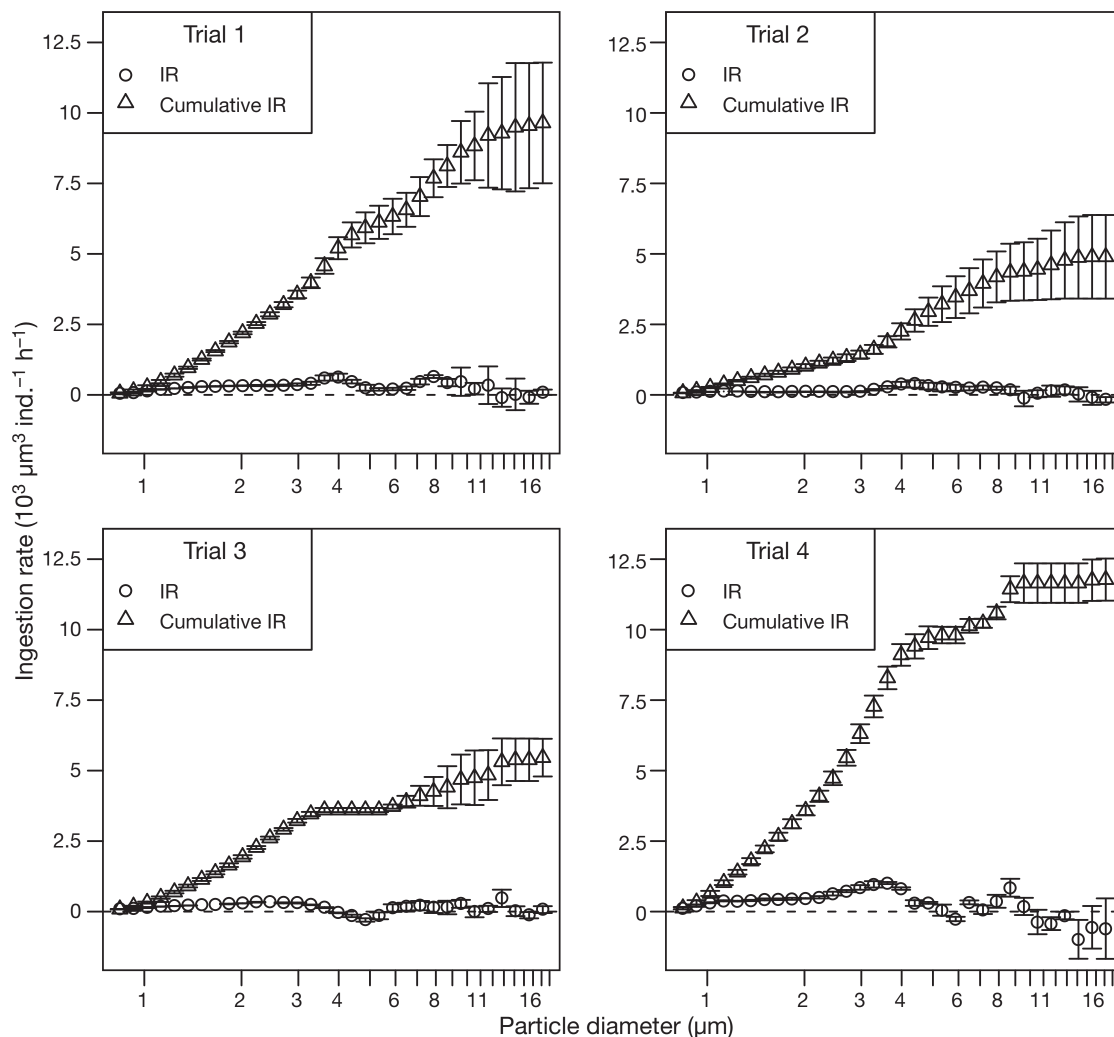

Fig. 5. *Ruditapes philippinarum*. Ingestion rate (IR) for each particle size range and cumulative IR. Error bars: SD of the 3 treatment bottles

Overall, larval densities in this study were much higher than in nature (Matsumura et al. 2001). The high larval density in Trial 2 might have reduced oxygen concentrations. At a larval respiration rate of 20 ng O_2 ind.$^{-1}$ h^{-1} (Sprung 1984b and references therein), 28 larvae ml^{-1} could consume 2.2 µg O_2 ml^{-1} during the 4 h experiment, corresponding to 30% of the oxygen in saturated seawater (7.5 mg l^{-1}). This reduction might have affected the larval CRs. It is also possible that the 4 h experimental period might have been too short to allow acclimation of larvae. Under our experimental conditions, the measured larval CRs might be lower than those in nature.

The particle size at the peak CR ranged from 1.4 to 2.0 µm (Figs. 3 & 4) and did not increase significantly with increasing larval shell length. Larvae of *Philine aperta* retained a broader size range of particles, particularly larger ones, as larval shell length increased (Hansen 1991). This difference between species might have resulted from the difference in size ranges of the larvae studied: 149 to 392 µm for *P. aperta* (Hansen 1991) and 128.7 to 205.4 µm for *Ruditapes philippinarum* (this study).

PREs of asari clam larvae were similar to those of *Mytilus edulis* larvae (Riisgård 1980). *M. edulis* larvae at 5 and 13 d cleared 2.5 to 3.5 µm particles at the highest rate, and particles <1 or >8–9 µm were not consumed. However, the particle sizes with highest CRs in this study were slightly smaller compared with those of other bivalve species: 2.5 to 3.5 µm in *M. edulis*, 4 µm in *Mercenaria mercenaria*, and 2 to 4 µm in *Crassostrea virginica* (Riisgård 1980, Sprung 1984a, Riisgård 1988, Baldwin 1995, Riisgård et al. 2000). The standard algal diets used in the hatchery, including species such as *Pavlova lutheri* or *Chaetoceros calcitrans* (4 to 5 µm), might not be of the optimum size for asari clam larvae.

The cumulative IR in this study ranged from ~50×10^2 to 100×10^2 µm^3 ind.$^{-1}$ h^{-1} (Fig. 5). These values correspond to 100–200 cells ind.$^{-1}$ h^{-1}, assuming the cell volume of an algal diet species (such as *Pavlova lutheri*) to be 50 µm^3 cell^{-1} (~4.6 µm in diameter). These values are well below the levels of food supplied in hatcheries (5000 to 15 000 cells ind.$^{-1}$ d^{-1}; ~200 to 600 cells ind.$^{-1}$ h^{-1}) for 120 to 200 µm larvae (Chiba Prefectural Fisheries Research Center 2004). Because the CRs we determined might be underestimates, the ingestion rates of *Ruditapes philippinarum* under the same food concentrations in nature might be higher than indicated in this study. However, our results suggest that the food supply in the natural seawater used in this study was not concentrated enough for maximal growth of the larvae. Further studies are required to determine variations in larval food abundance and larval ingestion in nature.

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Contribution to the Theme Section 'Directions in bivalve feeding'

Isotopic evidence of particle size-dependent food partitioning in cocultured sea squirt *Halocynthia roretzi* and Pacific oyster *Crassostrea gigas*

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ABSTRACT: To examine the trophic relationship between the sea squirt *Halocynthia roretzi* and the Pacific oyster *Crassostrea gigas* cocultured in suspension, their $\delta^{13}C$ and $\delta^{15}N$ values were monitored monthly on the southern coast of Korea for a period of 2 yr. Suspended particulate organic matter was fractionated as coarse (>20 µm, CPOM) and fine particles (<20 µm, FPOM), and the seasonal variations in the $\delta^{13}C$ and $\delta^{15}N$ were determined. CPOM $\delta^{13}C$ was slightly more variable than FPOM $\delta^{13}C$ over the sampling period, whereas $\delta^{15}N$ varied less for CPOM than for FPOM. Co- and monocultured sea squirts had a less variable $\delta^{13}C$, but a more variable $\delta^{15}N$ than cocultured oysters over the sampling period. The $\delta^{13}C$ and $\delta^{15}N$ of cocultured sea squirts were consistently lower than those of cocultured oysters. The more pronounced difference in the $\delta^{13}C$ between the cocultured suspension feeders was attributed to a striking isotopic change in oysters during their autumn to winter growing period. Differences in the $\delta^{13}C$ were significant between co-, monocultured, and wild sea squirts, but not between co- and monocultured oysters. These suspension feeders can use different POM size fractions within the same habitat. The marked ^{13}C-enrichment in oyster tissues, particularly during their fast growing period, may result from their strong selectivity of diatoms. In contrast, both the ^{13}C- and ^{15}N-depleted values in cocultured sea squirts indicate the importance of pico-/nano-size fractions as their dietary components. Size-related patterns in food resource exploitation between the cocultured suspension feeders may be due to different particle capture mechanisms.

KEY WORDS: *Halocynthia roretzi* · *Crassostrea gigas* · Coculture · Stable isotopes · Trophic relationship · Food partitioning

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INTRODUCTION

Aquaculture of suspension-feeding bivalves and other invertebrates is an area of tremendous economic potential in coastal waters worldwide. It is believed that populations cultured in suspension using a long, constantly submerged line system have the benefits of fast growth and high production over their wild counterparts (Rodhouse et al. 1984, Garen et al. 2004). Great attention has been paid to the ecological impacts of suspension-feeding communities on the quantity and quality of phytoplankton populations (Baker et al. 1998, Souchu et al. 2001). The grazing activity of cultivated animals may enhance nutrient recycling and increase light in the water column so that algal growth is stimulated and, in turn, food quality is improved by this positive feedback (Asmus & Asmus 1991, Prins et al. 1997). Alternatively, high stock densities of wild or cultivated suspension feeders may deplete phytoplankton biomass and suspended particulate matter in the water column (Cloern 1982, Jarry et al. 1990, Souchu et al. 2001). Such a negative feedback may result in lower growth rates in the cultivated stocks (Prins et al. 1998 and references therein).

The 2 most important aquaculture species in Korea are the edible sea squirt *Halocynthia roretzi* and the Pacific oyster *Crassostrea gigas*. The longline method for sea squirt production was expanded in 1980 and

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peaked at 42 800 tons in 1994, declining to 4500 tons in 2004 and 10 819 tons in 2006 (National Fisheries Research and Development Institute 2004). The decline in sea squirt production is attributed to a decrease in growth rate and an increase in mass mortality events. Causes of ubiquitous mass mortalities of sea squirts on Korean coasts are still unknown. Recently, fishermen developed a coculturing method, in which one line has sea squirts attached, and the next parallel line has oysters attached. With the expansion of this method, the mortality of *H. roretzi* was less pronounced. In contrast, during that time, mass mortalities happened much more frequently under an ascidian monoculture. In relation to the availability of food and the density of cultivated or wild populations, increasing the stocking density of cultured bivalves above the optimal level for a given site often leads to a significant reduction in growth and survivorship of the cultured stocks (Newell 1990, Parsons & Dadwell 1992, Raillard & Ménesguen 1994). Similarly, the declining growth rate and high mortality under ascidian monoculture conditions may be associated with the stocking density exceeding the trophic capacity of the system. In contrast, the reduced mortality under cocultures of ascidians and oysters may be indicative of a mitigation of food limitation. This may result from an insignificant competitive interaction for food resources between those 2 suspension-feeder stocks.

Several ascidian species co-occurring on the longline culture units of bivalves were considered to be fouling organisms or potential competitors with cultivated bivalves for food resources (Cayer et al. 1999, Carver et al. 2003). However, various laboratory feeding experiments have demonstrated that the co-occurring suspension-feeding ascidians and bivalves possess different mechanisms for particle capture (Stuart & Klump 1984, Lesser et al. 1992, Petersen 2007). Resource partitioning due to species-specific differences in qualitative selection capability in co-occurring mollusk communities was also found between an indiscriminate suspension-feeding gastropod *Crepidula fornicate* and a selectively feeding oyster *Crassostrea gigas* (Riera et al. 2002, Decottignies et al. 2007). In the same manner, as ascidians lack a particle sorting mechanism (Petersen 2007), partitioning food resources due to differences in particle selection capability between cocultured sea squirts and oysters may be expected.

The present study was carried out to elucidate the trophic interactions of sea squirts *Halocynthia roretzi* and Pacific oysters *Crassostrea gigas* cocultured in suspension using longlines. The $\delta^{13}C$ and $\delta^{15}N$ values of these 2 suspension feeders were monitored monthly and compared with those of suspended particulate organic matter (POM) as potential food sources. By comparing the $\delta^{13}C$ and $\delta^{15}N$ values of the suspension feeders with those of size-fractioned POM, we examined seasonal variations in the suspension feeders' food sources based on particle size ingested to provide evidence for food-resource partitioning.

MATERIALS AND METHODS

Field sites and sampling design. Our sampling occurred at 4 sites on the mid-south coast of Korea (Fig. 1). Cultivation of a variety of suspension feeders, including sea squirts (*Halocynthia roretzi* and *Styela clava*), oysters (*Crassostrea gigas*), and mussels (*Mytilus galloprovincialis*) is widely exploited in this area using ropes suspended from longlines. Over 90 % of Korea's oyster and sea squirt production comes from intensive longline cultures in this area (http://fs.fips.go.kr/index.jsp). Sea squirts and oysters were collected monthly at 4 sites from February 2005 to December 2006. Cocultured sea squirts and oysters came from Site C (Goseong), monocultured sea squirts from Site S (Youngun), monocultured oysters from Site O (Youngun), and a wild population of sea squirts came from Site D (Dae-

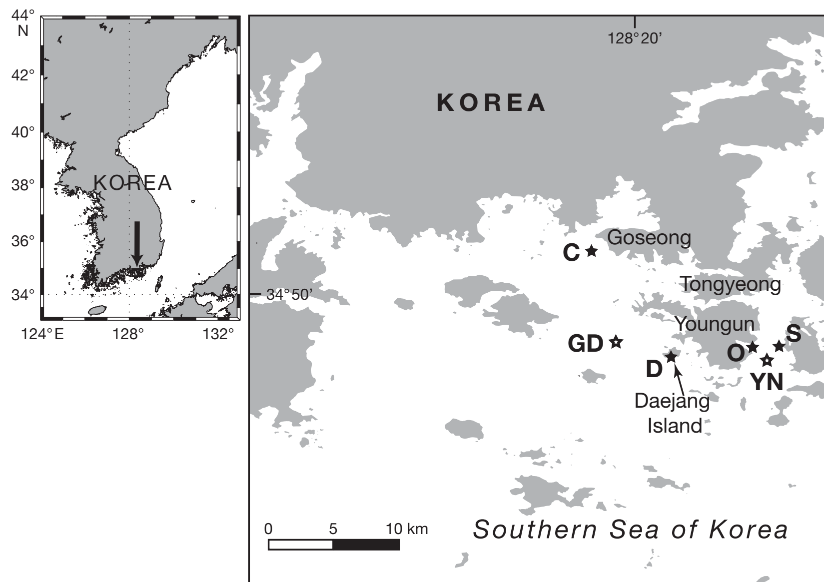

Fig. 1. Location of sampling sites on the mid-south coast of Korea. Site C: cocultured sea squirts and oysters; Site S: monocultured sea squirts; Site O: monocultured oysters; Site D: a natural population of sea squirts; Sites GD and YN: sites for suspended particulate organic matter sampling

jang). These sites were selected to take samples representative of different types of culture systems, e.g. mono- or coculture systems of oysters and sea squirts. Detailed information on the distribution, general ecology and habitat, seed collection, and cultivation processes of oysters and sea squirts in this area have been reported elsewhere (National Fisheries Research and Development Institute 2004).

The sampling sites in this study had a depth of <20 m. The tidal cycle of this area is semidiurnal, with maximal tidal amplitude of ~2.0 m on spring tides. Main currents are dominated by south- and northward reversing tidal current (10 to 20 cm s^{-1} near the sea surface) during the ebb and flood tides. The water temperatures showed a clear seasonal cycle typical of a temperate zone, with a summer maximum around 26.7°C in September and a winter minimum around 5.4°C in February. Salinities are lower during the summer monsoon period (around 28) and higher in the winter to spring dry season (around 34). These sites are free of large-scale river runoff. However, concentrated rainfall brings high nutrient concentrations during late spring to early summer, accompanied by high concentrations of chlorophyll *a* (chl *a*), indicating the summer phytoplankton bloom.

Sample collection and processing. The sea squirt and oyster larva collectors were moved to the nursery ground. Several months later, the collectors were taken to the farm and attached as vertical ropes hung from the longline (detailed procedures are in National Fisheries Research and Development Institute 2004). About 50 ind. of both species were randomly collected by hand from depths of 1 to 5 m at each site. Specimens were transported to the laboratory and cleaned to remove any attached epifauna. They were then acclimated overnight in filtered seawater at the *in situ* temperature to ensure gut evacuation and removal of fecal contents. After rinsing, the length, width, and height of the oyster shell and the sea squirt tunic were measured to the nearest 0.1 mm using vernier calipers. The animals were then dissected carefully, and their tissues were frozen and stored at –20°C. The shell valves and tunics were rinsed with distilled water and weighed 72 h after being placed in a drying oven at 50°C. The dry tissue weights of both species were determined after freeze-drying for a period of 72 h. A condition index was calculated from the dry tissue weight and tunic (for tunicates) or shell volume (for oysters) according to the formula: condition = (dry tissue weight/total dry weight or shell weight) × 100 (Petersen et al. 1997). Then, the dried tissues of 10 ind. from each site were pooled and mill ground into a homogeneous powder for isotope analysis.

Potential sources of organic matter available to cultivated invertebrates in the study area (i.e. particulate matter suspended in the water column [seston]) were sampled from duplicate samples in a volume of about 60 l of subsurface water (at a depth of about 1 m below the water surface) at 2 sites (Site GD for cocultured and wild sea squirt sites, and Site YN for monoculture sites, Fig. 1) using a van Dorn water sampler. Water samples were collected at these sites at midday high tide during spring tide periods to rule out waters filtered by intensively cultured organisms north of the sites during the ebb tides. The water samples were immediately passed through a 180 µm Nitex mesh to eliminate zooplankton and large particles, and collected in acid-washed plastic bottles. Water from these samples was then passed through a 20 µm sieve to obtain coarse suspended particulate organic matter (CPOM) comprised of mainly micro-sized phytoplankton. The remaining water was filtered once again through precombusted Whatman GF/F glass-fiber filters (nominal pore size = 0.7 µm) to obtain fine suspended particulate organic matter (FPOM) representing pico- and nano-plankton. Additional separation of seston was not attempted because of the difficulty in acquiring a sufficient sample for isotopic analysis. The sieved and filtered particulates for organic carbon isotope analysis were treated with 2 to 3 drops of 1 N HCl to remove any inorganic carbonate. Because acid washing can affect the nitrogen isotope ratios of organic material (Bunn et al. 1995), samples for nitrogen isotope analysis were not acidified. Invertebrate tissues were not acid washed before stable isotope analysis.

Stable isotope analysis. Stable carbon and nitrogen isotope assays were performed on small quantities of subsamples from homogenized materials. One milligram of powdered subsample was loaded into tin combustion cups and combusted at high temperature (1030°C) in an automated elemental analyzer (Euro EA 3000). The resulting CO_2 and N_2 gases were then analyzed using an interfaced continuous flow isotope ratio mass spectrometer (CF-IRMS, GV Isoprime). Two laboratory standards were analyzed with every tenth unknown sample. Stable isotope abundances were expressed in conventional delta (δ) notation, as deviation in parts per thousand (‰) relative to the Pee Dee Belemnite and atmospheric N_2 standards for carbon and nitrogen, respectively. The ratios were derived from the equation: $\delta X (‰) = [(R_{sample}/R_{standard}) - 1] \times 10^3$, where X is ^{13}C or ^{15}N, and R is the corresponding ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$. International Atomic Energy Agency (IAEA) CH-6 (sucrose, $\delta^{13}C = -10.45 \pm 0.07$‰) and IAEA-N1 (ammonium sulfate, $\delta^{15}N = 0.4 \pm 0.2$‰) with a known relationship to the international standard were used as reference materials. The SD of repeated measurements of internal peptone and urea standards was <0.2‰ for $\delta^{13}C$ and <0.3‰ for $\delta^{15}N$.

Table 1. 3-way ANOVA of effects of sampling site (GD and YN; see Fig. 1), size class of suspended particulate organic matter (POM, coarse POM, and fine POM), and sampling month on the $\delta^{13}C$ and $\delta^{15}N$ values of POM

Source of variation	df	$\delta^{13}C$			$\delta^{15}N$		
		MS	*F*	p	MS	*F*	p
Within + Residual	92	0.56			0.50		
Site	1	0.42	0.76	0.387	0.67	1.34	0.251
Size class	1	370.88	660.89	0.000	209.91	416.56	0.000
Month	22	8.96	15.96	0.000	3.88	7.70	0.000
Site × Size class	1	1.19	2.12	0.148	0.02	0.03	0.864
Site × Month	22	0.33	0.59	0.923	0.16	0.33	0.998
Size class × Month	22	2.57	4.58	0.000	1.61	3.19	0.000
Site × Size class × Month	22	0.35	0.62	0.903	0.23	0.45	0.982
Model	91	7.04	12.55	0.000	3.74	7.41	0.000
Total	183						

Statistical analysis. Data were tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Leven's test to meet the assumption of parametric statistics. Three-way analysis of variance (ANOVA) was used to test statistically significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values (dependent variables) of the POM due to the sampling site, POM type (size classes of CPOM and FPOM), and sampling month as factors. Because of significant interactions in the $\delta^{13}C$ and $\delta^{15}N$ values of the POM between the POM type and the sampling month, a Student's *t*-test was used to test isotopic differences between the CPOM and FPOM for pooled data from 2 sampling sites for each sampling occasion. One-way ANOVA and the Tukey test for multiple comparisons were used to test temporal differences in carbon and nitrogen isotopic values. Then, similarities in the seasonal variation of the $\delta^{13}C$ and $\delta^{15}N$ values between the culture types of the animals were compared using Spearman's rank correlation coefficient. A paired *t*-test was used to examine differences in the mean isotope values of animals between animal groups. All tests were performed using the SPSS 12.0 statistical software package.

RESULTS

$\delta^{13}C$ and $\delta^{15}N$ values of the suspended particulate organic matter

Results from the 3-way ANOVA tests showed that there was no significant difference in the $\delta^{13}C$ and $\delta^{15}N$ values of the POM between the GD and YN sites ($p = 0.387$ and 0.251 for $\delta^{13}C$ and $\delta^{15}N$, respectively; Table 1). In contrast, significant differences in both the $\delta^{13}C$ and the $\delta^{15}N$ values were found between the size classes of the CPOM and FPOM ($p < 0.001$). There was no significant interaction in the $\delta^{13}C$ and $\delta^{15}N$ values between the sampling site and the POM size class ($p = 0.148$ and 0.864, respectively), indicating consistent differences in the $\delta^{13}C$ and $\delta^{15}N$ values between the CPOM and FPOM at both sites. While there were significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values in the POM between months ($p < 0.001$), interactions between the sampling site and the sampling month were not significant ($p = 0.932$ and 0.998, respectively). On the other hand, strong statistical interactions in the $\delta^{13}C$ and $\delta^{15}N$ values in the POM between the POM type and the sampling month occurred ($p < 0.001$ for both the $\delta^{13}C$ and $\delta^{15}N$ values). These interactions revealed that the temporal variability in the $\delta^{13}C$ and $\delta^{15}N$ values was inconsistent between the CPOM and FPOM, i.e. the temporal variability in the values did not parallel the size classes. However, the insignificant interactions in the $\delta^{13}C$ and $\delta^{15}N$ values among the 3 factors (site × type × month) indicated that the temporal variability in the $\delta^{13}C$ and $\delta^{15}N$ values, depending on the types of POM, was common to both sites.

The $\delta^{13}C$ and $\delta^{15}N$ values of all size classes of the POM ranged from –23.7 to –15.4‰ and from 3.3 to 9.7‰, respectively, at Sites GD and YN (Fig. 2). The $\delta^{13}C$ values of the CPOM were slightly more variable, with a range of –21.9 to –15.4‰ (coefficient of variations [CVs] = 8.5 and 8.8 at Sites GD and YN, respectively), than those of the FPOM, with a range of –23.6 to –19.7‰ (CVs = 4.4 and 3.8, respectively) over the sampling period. In contrast, the $\delta^{15}N$ values of the CPOM were less variable, with a range of 6.2 to 9.7‰ (CVs = 9.2 and 10.5 at Sites GD and YN, respectively), than those of the FPOM, with a range of 3.3 to 8.6‰ (CVs = 20.2 and 19.2, respectively). As a result, while greatly elevated $\delta^{13}C$ values in the CPOM were found in the summer of 2005 and in summer to early fall of 2006, the $\delta^{15}N$ values in the FPOM peaked in the summer to early fall of 2005 (Tukey HSD test, $p < 0.05$). Although the unparalleled temporal variability in the $\delta^{13}C$ and $\delta^{15}N$ values between the CPOM and FPOM resulted in quite variable differences between the size classes, the FPOM was consistently depleted in ^{13}C and ^{15}N relative to the CPOM on each sampling occasion (Student's *t*-test, df = 6, $p < 0.05$ for $\delta^{13}C$ on all the 23 sampling occasions and for $\delta^{15}N$ on 22 occasions except for $p = 0.668$ in October 2005). The monthly differences between the 2 size fractions of the POM averaged 2.8 ± 1.1‰ (1 SD) and 2.1 ± 0.9‰ in the $\delta^{13}C$ and $\delta^{15}N$ values, respectively.

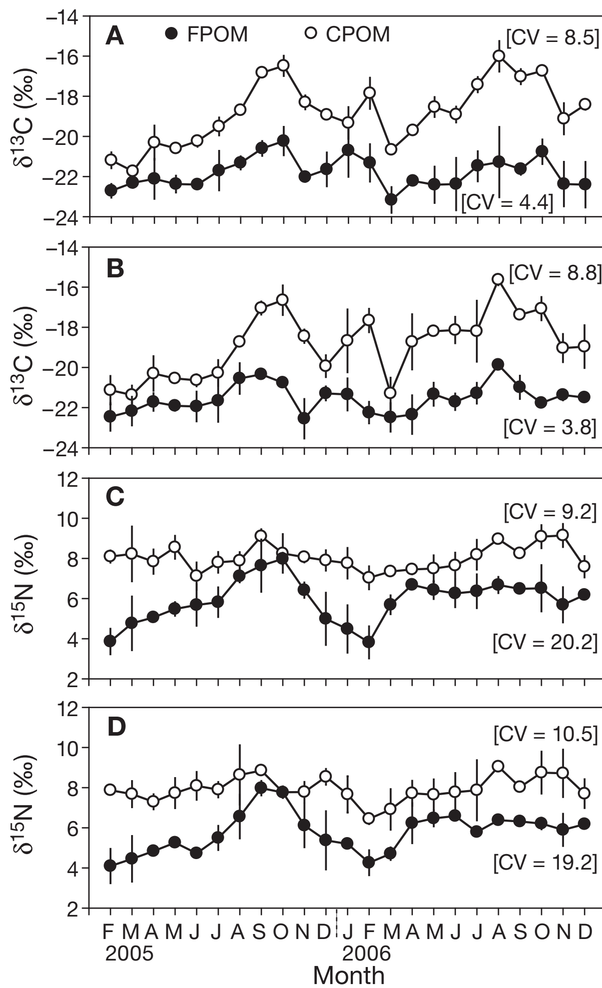

Fig. 2. Monthly means (±1 SD) of $\delta^{13}C$ and $\delta^{15}N$ values of suspended particulate organic matter (POM) at the GD (A,C) and YN (B,D) sites (see Fig. 1) from February 2005 to December 2006. FPOM: fine POM fraction <20 µm; CPOM: coarse POM fraction of 20 to 180 µm; CV: coefficient of variation

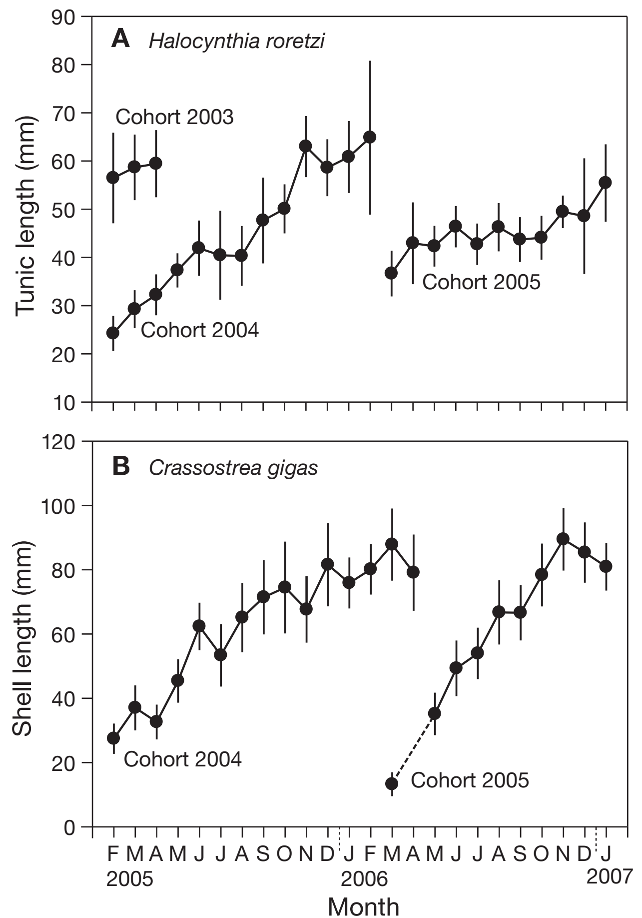

Fig. 3. *Halocynthia roretzi* and *Crassostrea gigas*. Seasonal growth patterns in: (A) sea squirt tunic and (B) oyster shell after the larva collectors were attached at Site C (see Fig. 1). Dashed line: no data

Seasonal growth patterns of suspension feeders

After the spat were taken to the culture system of the farm in late winter and early spring, seasonal patterns in shell and tunic growth were very similar for both suspension feeders at the coculture (Goseong) site (see Fig. 3). The shell and tunic growth of *Halocynthia roretzi* and *Crassostrea gigas* started from spat deployment in February to March, and continued until harvesting in winter. In contrast, their condition showed a clear seasonal pattern that was very similar for both species (Fig. 4). Relatively high values of the condition index at the beginning of the study (winter to spring) were followed by a progressive decline throughout the summer to autumn period to minimum levels in September to October. A subsequent rapid recovery in condition occurred in November in both years, after which the condition index reached maximum levels in late winter (February to March) during the main harvesting period.

δ^{13}C and δ^{15}N values of the suspension feeders

The $\delta^{13}C$ and $\delta^{15}N$ values of the sea squirts and oysters showed significant temporal variations (Fig. 5; 1-way ANOVA, $p < 0.001$ for all 5 cases of both elements). Similar to the isotopic values of the POM, the $\delta^{13}C$ values of the suspension feeders was less variable (CV range = 3.2 to 6.0) than the $\delta^{15}N$ values over the sampling period (CV range = 6.1 to 12.7). On the other hand, while the co- and monocultured sea squirts were less variable (CV = 3.5 and 3.4, respectively) than the cocultured oysters (CV = 6.0) in the $\delta^{13}C$ values, the former were more variable (CV = 8.7 and 12.7, respectively) than the latter group (CV = 7.3) in the $\delta^{15}N$ values over the sampling period. Along with the low variability in the $\delta^{13}C$ values

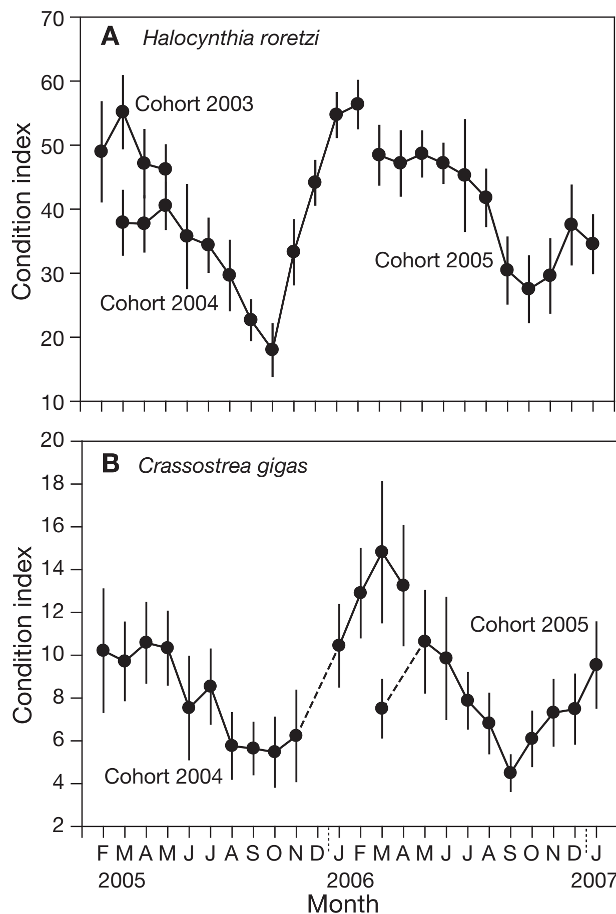

Fig. 4. *Halocynthia roretzi* and *Crassostrea gigas*. Seasonal variations in condition index [(dry tissue weight/dry tunic or shell weight) × 100] of: (A) sea squirt and (B) oyster after the larva collectors were attached at Site C (see Fig. 1). Dashed lines: no data

of the co- and monocultured sea squirts, their $\delta^{13}C$ values showed irregular seasonal fluctuation. In contrast, the $\delta^{13}C$ values of the co- and monocultured oysters displayed a clear seasonality, decreasing during winter to spring, and then increasing to a maximum in the late summer to autumn (Tukey HSD test, $p < 0.05$). The $\delta^{15}N$ values of the co- and monocultured sea squirts and oysters increased during the summer to autumn, and fell to a minimum in winter (Tukey HSD test, $p < 0.05$). From Spearman's rank correlation analyses (Table 2), similar seasonal trends in the variation in the $\delta^{13}C$ values between species, or between sites for the same species, were found in a few cases (4 out of 10 correlations), but in most cases of the $\delta^{15}N$ values (8 out of 10 correlations).

The $\delta^{13}C$ and $\delta^{15}N$ values of the cocultured sea squirts were consistently lower (range = –21.6 to –19.4‰ and 8.0 to 10.9‰, respectively) than those of the cocultured oysters (range = –20.2 to –16.5‰ and 10.3 to 13.6‰, respectively) over the sampling period (Fig. 5). The mean differences between the 2 groups were 2.1 ± 0.9‰ and 2.4 ± 0.8‰, respectively (paired *t*-test, p = < 0.001 for both groups; Table 3). While the $\delta^{13}C$ values of the cocultured sea squirts were slightly lower (mean difference = 1.3 ± 0.6‰) than those of the monocultured sea squirts (range = –20.5 to –18.1‰), the $\delta^{15}N$ values showed no significant difference between both groups (range of the latter group = 6.9 to 12.2‰, paired *t*-test, p = 0.492). Again, the cocultured sea squirts exhibited much lower $\delta^{13}C$ values than those grown in nature (range = –18.8 to –16.6‰) and monocultured oysters (range = –18.7 to –16.5‰), with mean differences of 2.2 ± 0.8‰ and 2.6 ± 0.7‰, respectively. The $\delta^{13}C$ values of the monocultured sea squirts tended to be between those of the other sea squirt and oyster groups, resulting in reduced mean differences between the monocultured sea squirts and wild sea squirts or oyster groups compared to those between cocultured sea squirts and these groups. No significant difference was found in the $\delta^{13}C$ values between wild sea squirts and co- or monocultured oysters (paired *t*-test, p = 0.558 and 0.383, respectively). While the mean differences in the $\delta^{13}C$ values between the sea squirt and oyster groups decreased from cocultured through monocultured sea squirts to insignificance in wild sea squirts. The large differences in the $\delta^{15}N$ values between the sea squirts and oysters were consistent (Table 3). The mean differences in the $\delta^{15}N$ values between the sea squirt groups were much lower than those between the sea squirts and the oysters.

Table 2. *Halocynthia roretzi*, *Crassostrea gigas*. Spearman's rank correlation analyses (ρ) on the $\delta^{13}C$ and $\delta^{15}N$ values between species, or between sampling sites for the same species

		Sea squirts Coculture	Sea squirts Monoculture	Sea squirts Natural	Oysters Coculture	Oysters Monoculture
		········	········	$\delta^{13}C$ (above diagonal)	········	········
Sea squirts	**Coculture**					
	ρ		0.702	–0.607	0.583	0.497
	n, p		23, <0.001	10, 0.854	21, 0.006	11, 0.120
	Monoculture					
	ρ	0.632		–0.006	0.603	0.391
	n, p	23, 0.001		10, 0.987	21, 0.004	11, 0.235
	Natural					
	ρ	0.758	0.806		0.333	–0.188
	n, p	10, 0.011	10, 0.005		9, 0.381	10, 0.603
Oysters	**Coculture**					
	ρ	0.536	0.263	0.533		0.758
	n, p	21, 0.012	21, 0.250	9, 0.139		10, 0.011
	Monoculture					
	ρ	0.664	0.864	0.685	0.830	
	n, p	11, 0.026	11, 0.001	10, 0.029	10, 0.003	
		········	········	$\delta^{15}N$ (below diagonal)	········	········

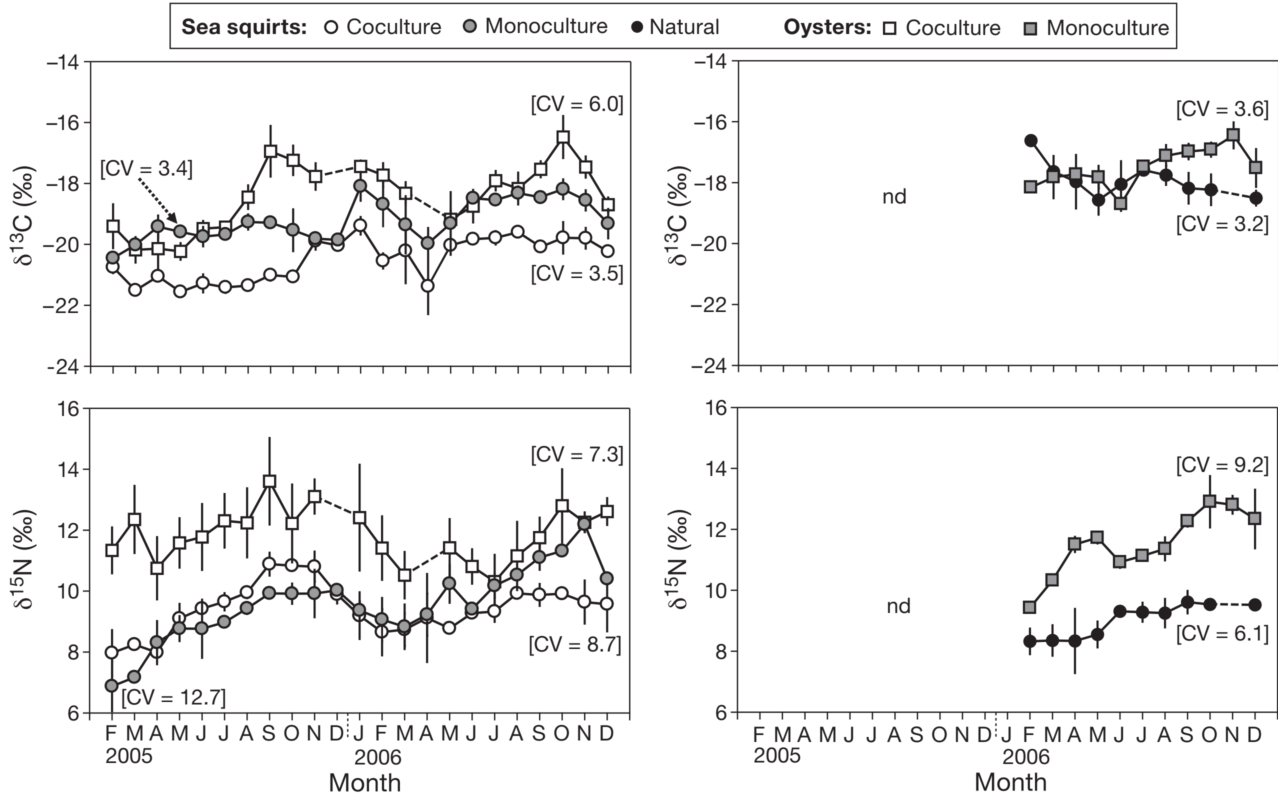

Fig. 5. *Halocynthia roretzi* and *Crassostrea gigas*. Monthly mean (±1 SD) $\delta^{13}C$ and $\delta^{15}N$ values. Cocultured oysters and sea squirts, monocultured sea squirts from February 2005 to December 2006; natural sea squirts and monocultured oysters from February to December 2006; CV: coefficient of variation; nd: not determined

Table 3. *Halocynthia roretzi* and *Crassostrea gigas*. Results of paired *t*-test on the $\delta^{13}C$ and $\delta^{15}N$ values between species, or between sampling sites for the same species

		Sea squirts			Oysters	
		Coculture	Monoculture	Natural	Coculture	Monoculture
		δ13C (upper right)				
Sea squirts	**Coculture**					
	Difference		1.25 ± 0.59	2.24 ± 0.83	2.06 ± 0.91	2.60 ± 0.67
	df, *t*		22, 10.224	9, 8.554	20, 10.402	10, 12.826
	p		<0.001	<0.001	<0.001	<0.001
	Monoculture					
	Difference	n.s.		1.03 ± 0.66	0.76 ± 0.92	1.39 ± 0.84
	df, *t*	22, 0.698		9, 4.934	20, 3.813	10, 5.469
	p	0.492		0.001	0.002	<0.001
	Natural					
	Difference	0.31 ± 0.26	1.03 ± 0.54		n.s.	n.s.
	df, *t*	9, 3.746	9, 6.044		8, 0.612	9, 1.022
	p	0.005	<0.001		0.558	0.383
Oysters	**Coculture**					
	Difference	2.43 ± 0.75	2.29 ± 1.35	2.34 ± 0.78		0.53 ± 0.64
	df, *t*	20, 14.655	20, 7.746	8, 8.952		9, 2.594
	p	<0.001	<0.001	<0.001		0.029
	Monoculture					
	Difference	2.17 ± 0.77	1.29 ± 0.5	2.38 ± 0.76	n.s.	
	df, *t*	10, 9.377	10, 7.525	9, 9.911	9, 0.073	
	p	<0.001	<0.001	<0.001	0.943	
		δ15N (lower left)				

DISCUSSION

Isotopic variations in size-fractionated marine particles

The isotopic variability of the suspended POM observed in the present study was mainly due to differences between size classes, i.e. the CPOM and FPOM rather than between sites. The $\delta^{13}C$ and $\delta^{15}N$ values of the FPOM (size fraction < 20 µm) were consistently lower by mean differences of 2.8 and 2.1‰, respectively, than those of the CPOM (size fraction > 20 µm). Such isotopic variations across the suspended POM size classes have been established in marine particles (Gearing et al. 1984, Minagawa & Wada 1986, Goering et al. 1990, Rau et al. 1990, Kopczyńska et al. 1995, Kukert & Riebesell 1998, Rolff 2000, Sato et al. 2006). Although researchers cannot completely explain the isotopic vari-

ability, these previous studies have shown that POM size-dependent isotopic variations are likely to be due to the different taxonomic composition in each size class. For example, the $\delta^{13}C$ values of the diatom-dominated CPOM is generally higher than that of the nanoplankton-dominated, e.g. nanoflagellates, FPOM (Gearing et al. 1984, Goering et al. 1990, Rolff 2000). The presence of pico-size bacteria or prymnesiophytes can be also attributed to the lowered $\delta^{13}C$ values of the FPOM (Bishop et al. 1977, Rau et al. 1990). Sato et al. (2006) speculated that the lower $\delta^{13}C$ values of the FPOM than those of the CPOM may be related to taxonomic (or cell-size-specific) differences in carbon isotopic discrimination during photosynthesis. Similar observations were obtained for differences in the $\delta^{15}N$ values, showing a lower value in the smaller size class of the POM compared to larger counterparts (Wada et al. 1987, Altabet 1988, Rau et al. 1990, Sato et al. 2006). In particular, the $\delta^{15}N$ values of isolated blue-green alga were 2 to 4‰ lower than those of a towed plankton mixture using a phytoplankton net (95 µm) in the East China Sea adjacent to the study area (Minagawa & Wada 1986). Rau et al. (1990) found that nanoflagellates, which are important consumers of bacteria, dominated the microbial biomass at a Mediterranean site, and their $\delta^{15}N$ value was lower than that of POM >20 µm. Thus N isotopic fractionation within the microbial food web may be different from that (3 to 5‰ fractionation per trophic level) of higher metazoan organisms. In addition, the possible existence of microzooplankton would partly explain the higher $\delta^{15}N$ values in the CPOM fraction.

Terrestrial/marine organic matter mixing can explain the observed isotopic variation (e.g. Rau et al. 1990, Sato et al. 2006). However, the atomic C/N ratios of the suspended POM in coastal waters adjacent to our sampling sites were typical of marine-origin materials (range = 3.7 to 10.2, mean = 7.7 ± 1.3 in the present study, versus 4.3 to 9.6 by Kang et al. 1999), which reject this hypothesis. Indeed, most of the $\delta^{13}C$ values of the suspended POM in the present study fall within the range of values found for marine particulates (e.g. Fry & Sherr 1984), and some high values (around −15‰) in the CPOM are commonly found during the spring to summer diatom blooms in coastal waters (Masan Bay) adjacent to the study area (Kim et al. 1994). The seasonal distribution of seston concentrations and the seston composition at the studied sites indicated that these sites can be characterized as an open bay or an oceanic system. The seston concentrations ranged from 1.75 to 8.80 mg l^{-1} and from 1.90 to 9.30 mg l^{-1} at Sites GD and YN, respectively (Fig. 6). These values were lower than those for estuarine or inner bay systems (cf. Navarro et al. 1993, Danovaro & Fabiano 1997, and also references therein). POM concentrations at the 2 sites were from 0.50 to 2.40 mg l^{-1} and 1.10 to 3.80 mg l^{-1}, respectively, representing high organic fraction between 20 and 60% of the total seston. No clear seasonality in either the seston or POM concentrations was found, indicating no significant seston load by riverine discharge. In addition to the low C/N ratios of the seston, low POC/chl *a* ratios (<200; authors' unpubl. data) found in the present study suggested the existence of a high amount of living material contained in the seston at the studied sites.

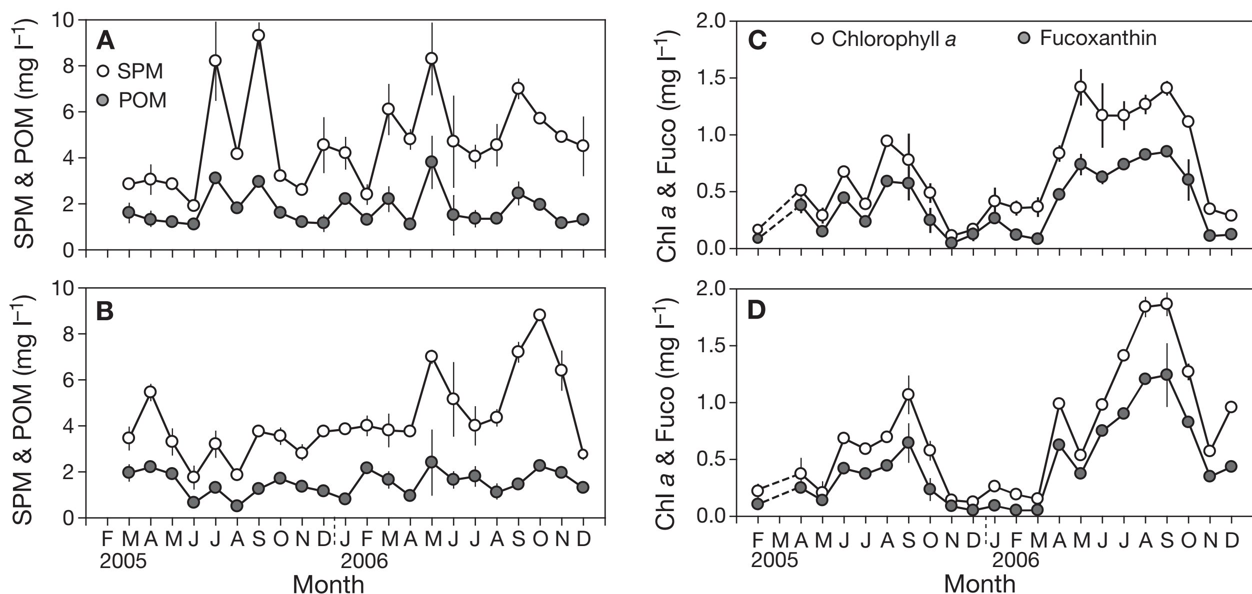

Fig. 6. Seasonal variations of suspended particulate matter (SPM), particulate organic matter (POM), chlorophyll *a* (Chl *a*) and fucoxanthin (Fuco, marker pigment for diatoms) in the water column at the GD (A,C) and YN (B,D) sites (see Fig. 1) from February 2005 to December 2006

A seasonally different variability in the $\delta^{13}C$ and $\delta^{15}N$ values between the size classes of the POM was also apparent in the present study (Table 1, Fig. 2). Such seasonal variations have been studied in relation to internal isotope variability of the autochthonous producers in various coastal waters (Rau et al. 1990, Sato et al. 2006). The $\delta^{13}C$ values of the CPOM were more variable than those of the FPOM over the sampling period, showing higher $\delta^{13}C$ values in the CPOM in the summer or summer to early fall, when the water temperature was high. The phytoplankton composition of the southern coastal waters of Korea is characterized by a high instability (Kim et al. 2004). High $\delta^{13}C$ values in the CPOM occurred when the chl *a* concentration was high. During that time, high fucoxanthin concentrations were detected, indicating a high contribution of diatoms to the phytoplankton biomass (Fig. 6). The $\delta^{13}C$ values of phytoplankton are known to be dependent on growth rate, dissolved inorganic carbon dynamics, and taxonomic group (Cifuentes et al. 1988, Fry 1996, Kukert & Riebesell 1998). Many diatoms are large, and their high $\delta^{13}C$ values could result from higher growth rates, larger cell sizes, and carbon content; thereby, they would have a higher carbon demand compared to other taxonomic groups (Laws et al. 1995, Kukert & Riebesell 1998). High temperatures in summer could decrease the pCO_2 value, and isotopic discrimination between HCO_3^- and CO_2 (aq) (Wong & Sackett 1978, Fontugne & Duplessy 1981). Furthermore, a high growth rate, i.e. increased CO_2 consumption, during the spring to summer phytoplankton bloom can lead to CO_2 limitation in the water column and a smaller isotope discrimination. Similar to results found in the Delaware estuary (Cifuentes et al. 1988), these effects resulted in very high $\delta^{13}C$ values of −9.2 to −15.4‰ during a plankton bloom in coastal waters (Masan Bay) adjacent to the study area (Kim et al. 1994).

In contrast to the $\delta^{13}C$ values, the $\delta^{15}N$ values of the CPOM were less variable than those of the FPOM, and the $\delta^{15}N$ values of the FPOM peaked in the summer to early fall of 2005. A higher seasonal variation of the $\delta^{15}N$ values in the FPOM than those in the CPOM has also been observed in other coastal waters (Rau et al. 1990, Sato et al. 2006). Nitrogen isotope discrimination of diatoms tends to decrease with growth rate, but not in other species (Montoya & McCarthy 1995). As supported by an inverse relationship between NO_3^- (or NH_4^+) ion concentrations and the $\delta^{15}N$ values of the POM, the $\delta^{15}N$ values of phytoplankton should be affected by differences in the availability of NO_3^- or NH_4^+ ions, supporting phytoplankton production (Wada & Hattori 1976, Benner et al. 1997, Sato et al. 2006). In fact, high sestonic $\delta^{15}N$ values during the course of algal growth can result from the assimilation of a nitrogen pool enriched in ^{15}N because of previous fractionation by phytoplankton (Cifuentes et al. 1988, Goering et al. 1990). However, although the concentrations of dissolved inorganic nitrogen (DIN) slightly decreased in the upper layer during the summer stratified season in the present study, the concentrations still remained at a high level of 1 to 4 µM (authors' unpubl. data). The atomic C/N ratio of the suspended POM ranged from 5.4 to 6.8 during the spring to summer phytoplankton bloom in the present study (authors' unpubl. data). This C/N range is typical of marine phytoplankton, implying that there was no limitation of nitrogen for phytoplankton growth. Indeed, during the summer season of heavy rainfall in the study area, the addition of excessive nitrogen via streams into the stratified coastal waters greatly proliferated the phytoplankton (mainly diatoms; see Kang et al. 1999). Along with a positive correlation between the $\delta^{15}N$ values of the POM and water temperature, a stronger temperature effect on the $\delta^{15}N$ values of the CPOM (compared to those of the FPOM) has also been reported (Goering et al. 1990, Sato et al. 2006). However, the temperature-dependent variation in the $\delta^{15}N$ values of the CPOM was unclear, and a greater seasonal variability was observed in the $\delta^{15}N$ values of the FPOM in the present study (Fig. 2). As a result, the large variability in taxonomy of the microbial community and the different physiological responses of the pico- and nano-size plankton from micro-size plankton were most likely responsible for the pronounced variations in the $\delta^{15}N$ values of the FPOM, as previously suggested (Rau et al. 1990, Sato et al. 2006).

Size-dependent food partitioning between cocultured sea squirts and oysters

A consistent discrepancy in the $\delta^{13}C$ and $\delta^{15}N$ values between cocultured sea squirts *Halocynthia roretzi* and oysters *Crassostrea gigas* indicates that these 2 suspension-feeding species feed in different proportions on the organic constituents of suspended particulate matter. This explanation seems reasonable because of the seasonal trends in their isotopic signatures. While the seasonality of the $\delta^{13}C$ values in cocultured oysters was similar to those of the CPOM, the cocultured sea squirts had a comparable seasonality of the $\delta^{15}N$ values to the FPOM. Accordingly, a seasonal trend in the $\delta^{13}C$ values of a species was not necessarily consistent with its $\delta^{15}N$ values.

A dual isotope plot for the trophic components, including suspension feeders and size-fractionated particles, enabled us to elucidate the dietary sources assimilated by the consumers (Fig. 7). In stable isotope analysis, dietary sources assimilated by a consumer

can be identified by assuming a trophic fractionation between the animal's tissue and its diet, which is generally accepted to be 0 to 1‰ for $\delta^{13}C$ and 3 to 4‰ for $\delta^{15}N$ (Fry & Sherr 1984, Minagawa & Wada 1984). Recently, laboratory feeding experiments showed that the ^{13}C and ^{15}N diet–tissue fractionations of *Crassostrea gigas*, were outside the abovementioned range (Dubois et al. 2007a, Yokoyama et al. 2008). However, Yokoyama et al. (2008) suggested that the ^{13}C fractionation for the whole soft body of the oysters is ≤1.0‰, and the actual $\delta^{15}N$ fractionation of the oysters in the field is smaller than that obtained from the rearing experiment on a monospecific microalgal diet. Accordingly, we assumed the generally accepted trophic enrichments of 1‰ for $\delta^{13}C$ and 3.5‰ for $\delta^{15}N$, respectively (e.g. Riera et al. 2002, Dubois et al. 2007b), and estimated the isotopic ranges of consumers assimilating a diet (indicated by the dashed line in Fig. 7). The results of the dual isotope plot confirmed the dietary switch between the cocultured suspension feeders.

The ranges of the $\delta^{13}C$ and $\delta^{15}N$ values of the cocultured oysters (–20.2 to –16.5‰ and 10.3 to 13.6‰, respectively) suggest that the CPOM was their primary dietary component. This conclusion may be supported by the seasonal variations in the isotopic composition of cocultured oysters. Seasonal peaks in the $\delta^{13}C$ values of cocultured oysters occurred in the summer or summer to early fall, when the CPOM was most ^{13}C-enriched by a seasonal diatom bloom. Consistent seasonal ^{13}C enrichment was also found for the monocultured oysters. Therefore, there was a slight difference in the $\delta^{13}C$ values between co- and monocultured oysters, but by only a mean of 0.5 ± 0.64‰, and no significant difference in the $\delta^{15}N$ values was found (Table 3). This result suggests that both co- and monocultured oysters rely largely on a CPOM diet source. In contrast, no pronounced ^{13}C enrichment appeared in the cocultured sea squirts during that time. A ciliary filter-feeding mechanism (termed cirri trapping) gives bivalves the ability to sort particles before ingestion; thus, Pacific oysters do not retain the smallest cells in the water column (Courties et al. 1994, Riisgård & Larsen 2000). Their preingestive selection to regulate the ingestion of particles by production of pseudofeces can also discriminate inorganic and organic particles, and different species of microalgae (Shumway et al. 1985). Such a qualitative selection capability can lead to preferential absorption and ingestion of microalgae by Pacific oysters (Shumway et al. 1985, Barillé et al. 1997, Cognie et al. 2001). As a result, a similar seasonality in the $\delta^{13}C$ and values between oysters and the CPOM, and the range of $\delta^{13}C$ and $\delta^{15}N$ values of oysters in this study, indicates the qualitative selection of microalgae (particularly diatoms) by oysters (cf. Decottignies et al. 2007, Marín Leal et al. 2008). On the other hand, oysters had more ^{13}C-depleted values relative to the remainder of the year, in early spring prior to the microalgal bloom, coinciding with that of the CPOM. While the seasonal difference in the $\delta^{13}C$ values between the 2 species during that time was reduced, the $\delta^{15}N$ values of oysters was still higher than that of sea squirts, reflecting the difference in $\delta^{15}N$ values between the size fractions of the POM. Accordingly, this result also suggests the oysters' ability to selectively ingest CPOM by size-dependent sorting.

The range of $\delta^{13}C$ and $\delta^{15}N$ values of the cocultured sea squirts (–21.6 to –19.4‰ and 8.0 to 10.9‰, respectively) were, on average, depleted 2.1 and 2.4‰, respectively, compared to the $\delta^{13}C$ and $\delta^{15}N$ values of oysters, implying an increased assimilation of the FPOM. Seasonal trends in the $\delta^{13}C$ and $\delta^{15}N$ values of cocultured sea squirts tended to parallel those of the FPOM. Although cocultured oysters had higher $\delta^{13}C$ values just after the summer or autumn diatom

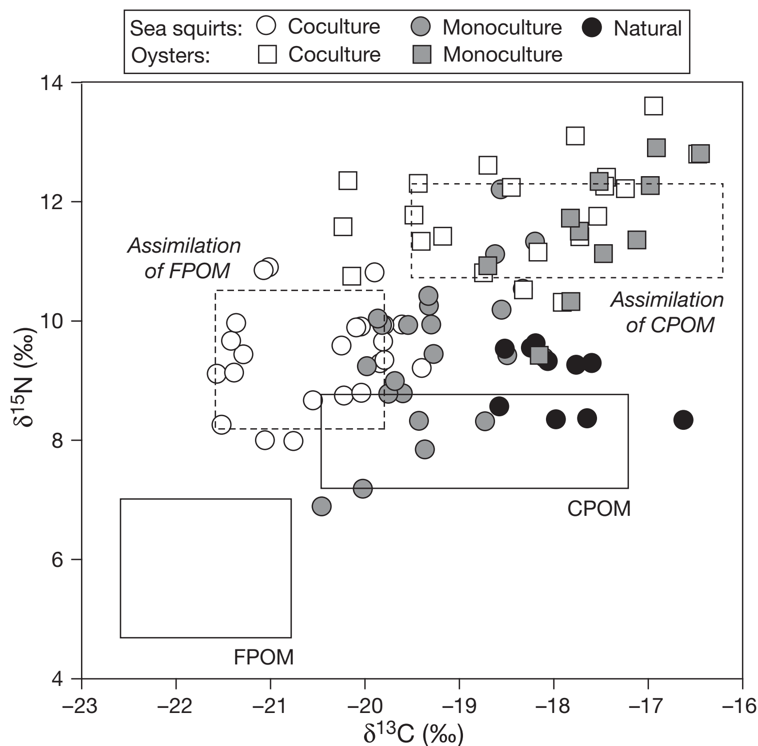

Fig. 7. Dual plot of $\delta^{13}C$ and $\delta^{15}N$ values for suspension feeders and size-fractionated suspended particulate organic matter (POM). Expected $\delta^{13}C$ and $\delta^{15}N$ ranges (as indicated by the dashed line) of consumers assimilating the fine and coarse POM (FPOM, CPOM) fractions were calculated by assuming trophic enrichments of 0.4 to 2.0‰ for $\delta^{13}C$ and 2.2 to 5.0‰ for $\delta^{15}N$, respectively

blooms, and no comparable ^{13}C enrichment was found in cocultured sea squirt tissue at the same time. Rather, the $\delta^{15}N$ values of cocultured sea squirts were greatly elevated, and coincident with the FPOM during that time. Effective use of the FPOM (pico- and nano-size plankton) by the cocultured sea squirts may be explained by their particle capture mechanism, characterized by net trapping (Riisgård & Larsen 2000). Ascidians deploy filter nets arranged in rectangular meshes of very fine filaments on the inner surface of the branchial basket (Flood & Fiala-Médioni 1981). The mucus net of ascidians can retain complete particles down to a size of 2 to 3 µm (see Riisgård & Larsen 2000, Bone et al. 2003). An experimental study on a similar ascidian species, *Halocynthia pyriformis*, demonstrated that with increasing sediment concentration the retention of small particles (2 to 5 µm) increased, while retention of larger particles (5 to 15 µm) decreased (Armsworthy et al. 2001).

On the other hand, while the $\delta^{13}C$ values of the sea squirts were different between co- and monocultured sites and wild habitats, there was no great difference in the $\delta^{13}C$ values between co- and monocultured oysters. This result suggests that unlike oysters, sea squirts lack particle-sorting mechanisms, and their indiscriminate feeding habits may lead to their dietary plasticity, depending on the availability of food resources (Coma et al. 2001, Petersen 2007). The higher $\delta^{13}C$ and $\delta^{15}N$ values of monocultured sea squirts during the summer to autumn of 2006, when the microalgal biomass peaked, compared to the rest of the study period, most likely reflect the increased use of diatom-derived microalgae. Further, the higher $\delta^{13}C$ values and lower $\delta^{15}N$ values of the wild sea squirts compared to co- and monocultured sea squirts indicates the assimilation of the specially ^{13}C-enriched and ^{15}N-depleted fractions in their diets. Considering that the habitats of the wild sea squirts are characterized by hard substrates colonized by macroalgae, the most probable candidates accountable for their isotope composition are macroalgal detritus, which have, on average, -17.2 ± 3.3‰ and 3.2 ± 1.3‰ for the $\delta^{13}C$ and $\delta^{15}N$ values, respectively (Kang et al. 2008).

The trophic relationships between cultivated oysters and co-occurring suspension feeders, slippersnails, or ascidians have also been exemplified in intertidal shellfish ecosystems on the French coast (Riera et al. 2002, Decottignies et al. 2007, Dubois et al. 2007b). These authors examined the dietary contributions of various potential food sources, including POM, benthic diatoms, macroalgae, C3 or C4 angiosperms, and terrestrial materials, to the animal tissues, using spatial or temporal variability in isotopic composition. They concluded that food resource partitioning due to a fundamental difference in feeding biology and the trophic plasticity of the co-occurring suspension feeders can limit interspecific competition for food. For example, Dubois et al. (2007b) found that, while oysters exhibited large spatial variations in isotopic signatures, ascidians had consistent isotopic signatures, showing the capability for greater trophic plasticity in oysters than in ascidians. These authors concluded that intraspecific variability in relative contributions of organic matter sources (i.e. terrestrial organic matter, microphytobenthos, *Ulva*, and marine particulate organic matter) according to the feeding biology and trophic plasticity of co-occurring suspension feeders can result in food resource partitioning in the intertidal ecosystem. Spatial variability in isotopic signatures of oysters and sea squirts found in the present study seems to be contradictory to the conclusion of Dubois et al. (2007b). However, considering the common trophic plasticity in consumers according to the availability of food items, such variability between systems seems to be reasonable. Our suspended culture system is in an open coastal area where the trophic base is largely dependent on water column production, and probably lacks benthic or terrestrial sources. In this system, cocultured suspension feeders, sea squirts, and Pacific oysters seem to partition their food resources in slightly different ways. Our isotopic data suggest that the 2 cocultured suspension feeders can exploit resources from the different size fractions of the entire POM pool. Laboratory feeding experiments for several suspension feeders from kelp beds have clearly shown a difference in concentrations of carbon and nitrogen ingested from different size fractions of particulate material between bivalves and ascidians (Stuart & Klump 1984).

Implications for the sustainability of aquaculture activity

The 2 suspension feeders initiate gametogenesis simultaneously with condition recovery in late autumn (Kang et al. 2000 for oysters, authors' unpubl. data for sea squirts). Although the spawning of sea squirts occurs in winter (mainly at the end of December), weight loss following spawning was undetectable using our monthly sampling scheme. In contrast, the spawning of oysters occurs in late spring to summer and accompanies a distinct loss in tissue weight. A progressive decline in tissue weight of the sea squirts also occurs throughout the summer to autumn period. Then, despite different reproductive strategies, their tissue growth appears to be completed from late autumn to spring, indicating temporal coupling in tissue growth. Adequate exploitation of food resources to meet high energy costs required during the critical period for growth and gametogenesis will determine

growth and production of these cultured stocks. Mass mortality of the monocultured sea squirts was encountered during the time of main growth and gametogenesis (winter to early spring). Ascidian mortality does not occur under cocultures of ascidians and oysters during the high energy-demand period. As previously shown for oysters in the neighboring culture system (Kang et al. 2000), a stocking density of sea squirts that exceeds the carrying capacity for the monoculturing ecosystem is likely to lead to food limitation, and thereby a decline in their growth rate and survival. If the reduced mortality under the coculture condition resulted from a mitigation of food limitation according to the trophic capacity of the system, then interspecific competition for food can be expected to be minimized. Our isotopic evidence confirms that a fundamental difference in the food particle capture mechanism between suspension-feeding sea squirts and oysters may reduce interspecific competition by food resource partitioning based on particle size. The coculture activity of ascidians and oysters retains high individual numbers and biomasses of suspension-feeding animals. However, the abovementioned evidence suggests that the exploitation of different food resources between the 2 suspension feeders may play an important role in reducing the nutritionally stressful effects of stocking density on growth and survival.

The present study demonstrates size-related patterns in food resource exploitation, presumably depending on the particle capture mechanism of cocultured suspension feeders. Further important ecological questions still remain: 'To what degree do suspension feeders actually retain each particle size fraction?', and 'To what degree does each size class support their C and N requirements?' Notwithstanding, this isotopic approach, based on size-fractionated suspended POM and associated biota, was found to be a simpler and more useful way to better understand trophic relationships and the nature of C and N cycling in these types of pelagic food webs.

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