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Impact of the blue mussel *Mytilus edulis* on the microbial food web in the western Wadden Sea, The Netherlands

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ABSTRACT: To study the impact of juvenile blue mussels *Mytilus edulis* on the microbial food web in the Dutch Wadden Sea, natural sea water was first exposed to mussel filtration. Subsequently, filtered plankton communities were used in a dilution experiment to establish mussel-induced changes in bacterial, pico- and nanophytoplankton growth rates as well as heterotrophic nanoflagellates (HNAN) and ciliate-induced grazing mortality rates. During the experimental period, from July to September, mussel filtration had a size-selective impact on the plankton community; on average, nanophytoplankton, HNAN and ciliates biomasses were removed at equal rates, while bacterial and picophytoplankton biomasses were affected to a much lower extent. The reduction in HNAN predators by mussels significantly lowered the grazing mortality rates for picophytoplankton. For bacteria, grazing mortality did not change, while specific growth rates almost doubled (from 0.65 to 1.16 d^{-1}). There was an increase in HNAN biomass following the enhanced bacterial production. Single exposure to mussel filtration thus led to a stimulation of the bacterial-HNAN pathway. HNAN biomass, although seriously reduced by mussel filtration, recovered to pre-filtration levels within 24 h. Nanophytoplankton and ciliates did not recover completely within 24 h. The results from this study reveal potentially important effects of mussel filtration on the pelagic food web not disclosed when considering phytoplankton biomass alone.

KEY WORDS: Microbial food web · Dilution technique · *Mytilus edulis* · Filtration · Growth rate · Mortality rate · Carbon flux · Wadden Sea

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INTRODUCTION

The predators within the microbial plankton community play a central role in marine ecosystems (Azam et al. 1983). Bacteria and picophytoplankton (0.2 to 3 µm), particles too small to be efficiently used by most other organisms, are the main prey for heterotrophic nanoflagellates (HNAN), while ciliates prey on both HNAN and nanophytoplankton (3 to 20 µm). Consumption of HNAN and ciliates by larger zooplankton links the microbial food web to the classical food chain (Azam et al. 1983, Saiz & Calbet 2011). The specific growth rates of nano- and micro-sized predators (<200 µm) are high and in the same order as their prey. This allows for a tight control over picoplankton and, to a lesser extent, over nanophytoplankton biomasses (Riegman et al. 1993, Kuipers et al. 2003). The high growth and grazing rates result in high turn-over rates of organic carbon through the egestion, excretion and 'sloppy feeding' of small-sized grazers (Fuhrman 1992, Strom et al. 1997 and references therein). The dissolved organic matter

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(DOM) in turn stimulates bacterial production and, through the regeneration of nutrients, primary production. Predators within the microbial food web can thus be considered the most important remineralisers in the sea (Azam et al. 1983) (Fig. 1).

Although the existence of microbial food webs in both oligotrophic as well as eutrophic systems has long been recognised (Riegman et al. 1993), it was generally assumed that nano- and microzooplankton grazing dominated in oligotrophic systems, while grazing by larger zooplankton, i.e. the transfer of energy and matter through the 'classical food web', was the most important process in more eutrophic systems (Calbet & Landry 2004). Based on a meta-analysis of a wide diversity of systems around the globe, 59 to 75% of all primary production is consumed by nano- and microzooplankton (Calbet & Landry 2004), stressing the importance of the microbial food web in all marine pelagic ecosystems. Information on the importance of the microbial food web in shallow benthic (e.g. mussel-dominated) systems is lacking (Calbet & Landry 2004). Together with the introduction of bivalve cultures occurring often in pelagic systems, there is a need to gain insight into the impact of bivalves on the microbial food web.

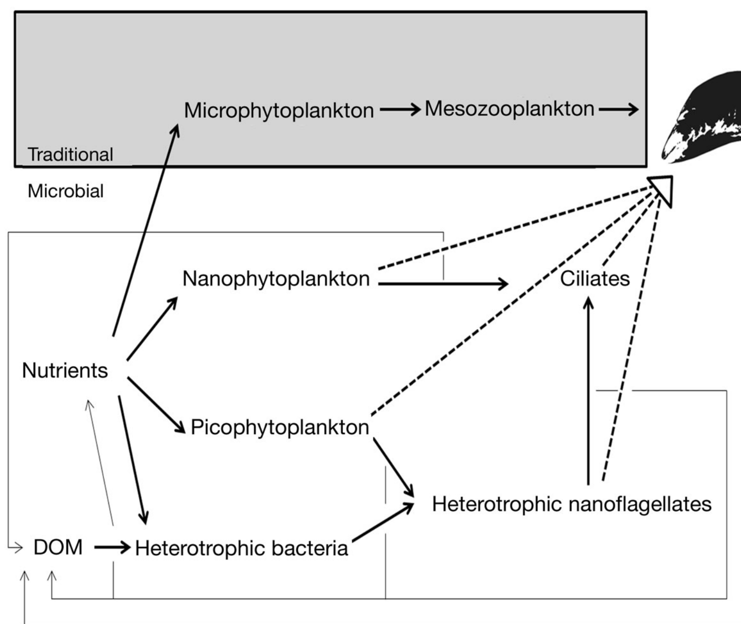

Fig. 1. A simplified marine food web indicating the classical food chain from microphytoplankton (>20 µm) to mesozooplankton (>200 µm) to higher trophic levels as well as the microbial food web. In the microbial food web, heterotrophic bacteria (<1 µm) and picophytoplankton (0.2–3 µm) are considered the main prey for heterotrophic nanoflagellates (HNAN) (2–20 µm), while nanophytoplankton and HNAN are considered main prey for ciliates (20–200 µm). Ciliates and HNAN, when consumed by mesozooplankton, provide the link between the 2 food webs. The dashed arrows represent the functional groups within the microbial food web potentially removed by juvenile blue mussels *Mytilus edulis*. Processes such as feeding produce dissolved organic matter (DOM) and release nutrients. These main remineralisation pathways are indicated by the thin lines

Several authors have suggested an impact of bivalve filter feeders on the microbial food web (Murrell & Hollibaugh 1998, Calbet & Landry 2004, Trottet et al. 2008, Greene et al. 2011, Froján et al. 2014). Bivalve filter feeders do not effectively remove small plankton but do feed on their predators, the HNAN and ciliates (Dupuy et al. 1999). This might result not only in a disruption of the link between small plankton and higher trophic levels (Dupuy et al. 1999, Wong et al. 2003, Greene et al. 2011) but also in complex indirect effects. The removal of nano- and microzooplankton predators by filter feeders might release prey from top-down control, resulting in biomass increases of bacteria and small phytoplankton. Bottom-up, bivalve excretion products result in an increase of dissolved and particulate organic matter as well as a greater availability of nutrients, stimulating both bacterial and phytoplankton production (Newell 2004, Van Broekhoven et al. 2014). At the same time, by removing suspended matter, mussel filtration improves the underwater light climate. Filtration by bivalves might dramatically change the size distribution of phytoplankton cells; nanophytoplankton are efficiently removed by mussels, while smaller cells are not. The pico-sized cells are better competitors for both light and nutrients, so improved growth conditions as a result of mussel filtration will likely favour the smallest cells (Riegman et al. 1993), potentially resulting in an increase in small cells at the expense of larger ones (Cranford et al. 2009). Finally, the removal of HNAN and ciliates, the main remineralisers, by mussels will likely alter the remineralisation process as well.

Surprisingly little research has been performed on this subject (Froján et al. 2014). Most studies focussed on the effect of bivalve cultures on phytoplankton biomass only, using total chlorophyll *a* as a proxy, or combined with the effect on larger mesozooplankton (Lehane & Davenport 2002, Wong et al. 2003, Nielsen & Maar 2007, Lonsdale et al. 2009). Recently, it was hypothesised that a focus on the classical food web led to an underestimation of the ecosystem impact of bivalve filter feeding (Greene et al. 2011). A recent study that included the effect of bivalves on the

microbial food web reported a decrease in ciliate biomass following the introduction of the clam *Corbula amurensis* (Greene et al. 2011). Froján et al. (2014) reported an *in situ* decrease in both nano- and microplankton while picoplankton remained unchanged inside a mussel *Mytilus galloprovincialis* raft culture. Authors of both studies concluded that bivalves altered the size structure of the microbial community. In both studies, the need to include the microbial food web when making an evaluation of bivalve modifications to the ecosystem was stressed.

In 2009, a small number of mussel seed collectors were introduced in the western Wadden Sea, The Netherlands. These collectors facilitate the settlement of large numbers of mussel *Mytilus edulis* larvae. After settlement, these larvae grow up to 25 mm in <6 mo, after which they are harvested (Jacobs et al. 2014). To assess the potential impact of an up-scaling of the number of collectors on the microbial food web, a paired set of experiments was designed. First, filtration experiments incubating juvenile mussels in natural sea water were performed. In these experiments, mussels were allowed to remove part of the available plankton biomass. After this mussel filtration experiment, the Landry and Hassett dilution technique (Landry & Hassett 1982) was applied to both mussel-filtered and unfiltered (control) water. This method allowed for an estimation of specific growth and grazing mortality rates of bacteria, picophytoplankton and nanophytoplankton as well as changes in these rates due to mussel filtration. Changes in HNAN and ciliate biomasses were also determined.

The aim of this study was to investigate the effect of mussel grazing on different species groups within the microbial food web. Changes in growth and mortality rates within the microbial food web caused by mussel filtration combined with calculations of the available carbon per functional group resulted in an estimation of the changes in carbon flow through the food web. Quantifying these pathways within the food web and the mussel-induced changes to these pathways provides a more realistic depiction of food web processes (Miehls et al. 2009). This analysis should allow for a better description of the direct and indirect effects of juvenile mussel filtration on the Wadden Sea food web.

MATERIALS AND METHODS

In 2011 and 2013, between July and September, 10 paired sets of experiments were executed. Juvenile mussels were incubated in water originating from the western Wadden Sea in a so-called 'filtration experiment'. Subsequently, both mussel-filtered as well as unfiltered (control) water was used in a 'Landry and Hassett dilution experiment' (Landry & Hassett 1982). In 2013, methodological improvements were introduced. The changes in 2013 compared to 2011 consisted of the use of 3 bottles per dilution level instead of 2 for the Landry and Hassett experiment and additional sampling for HNAN, ciliates and chlorophyll for both sets of experiments. Additionally, a larger experimental volume was used for both experiments. The main reason for using larger volumes in 2013 was to accommodate additional sampling for ciliates and chlorophyll. An unexpected, but positive effect of the use of larger volumes in the dilution experiments in 2013 might have been the reduction in variation between experimental units. Although it was assumed that the experimental volume would not impact the outcome of the results (Hammes et al. 2010), it cannot be excluded that in the present study the use of very small volumes in 2011 did have an effect on the outcome.

Mussel filtration experiments

Blue mussels *Mytilus edulis* were collected from a small collector placed in the Marsdiep (52° 58′ N, 4° 49′ E). This collector consisted of filamentous ropes facilitating mussel settlement (Xmas tree ropes, Donaghys). After settlement around June, mussels increase in size up to ~25 mm in October. The day before each experiment, ropes with juvenile mussels were collected, transported in sea water and stored at 4°C. On the day of the experiment, mussels were carefully removed from the rope, put in petticoat netting (0.5 × 0.5 cm mesh size) and acclimatised to the ambient sea water temperature. Mussels were placed in natural sea water, allowing them to resume feeding normally.

Experiments were conducted in two 3 l glass beakers (2011), filled with natural sea water. In 2013, 12 l polycarbonate carboys were used. Mussels were added ('mussel treatment') to 1 experimental unit, while the other unit served as a control. The number of mussels varied between 10 and 50 in 2011 and between 125 and 450 in 2013, with an average mussel shell length between 7.4 and 23.1 mm. During the experiment, the water was gently stirred. At the start of each experiment, water samples were taken for bacteria (2013), pico- and nanophytoplankton (2011 and 2013), heterotrophic nanoflagellates and ciliates (2013) and chlorophyll (2013). Samples were taken every 10 to 15 min, and phytoplankton cell counts were performed directly with a flow cytometer (see

'Pico- and nanophytoplankton') to monitor the decrease. Experiments lasted between 0.5 and 1.25 h in 2011 and between 1.5 and 3 h in 2013. Grazing pressure (G_p) is the fraction removed by mussels as compared to the fraction available in the control and was calculated per experiment as follows:

$$G_p = \frac{(N_C - N_M)}{N_C} \quad (1)$$

where N is the concentration of prey items (number l^{-1}) (bacteria, pico- or nanophytoplankton, HNAN or ciliates) or chlorophyll (µg l^{-1}) at the end of the mussel filtration experiment, C refers to the control treatment, and M refers to the mussel treatment. The number of mussels as well as their length differed between experiments. Although an effort was made to standardise the total biomass removed between experiments by adapting the duration of the filtration experiment, the variation between experiments is large (Table 1).

Dilution experiments

To study the effect of mussel filtration on the microbial food web, both mussel-filtered water ('mussel treatment') and natural sea water ('control') were serially diluted with filtered (Whatman GF/F filter) sea water. This dilution method (Landry & Hassett 1982) can be used to estimate specific growth rates (growth in the absence of grazers) (µ, d^{-1}) and grazing mortality rates (g, d^{-1}) of bacteria and phytoplankton (prey). In the present study, the specific growth and grazing mortality rates in bottles containing natural sea water were compared to the rates in bottles containing the plankton exposed to mussel filtration. The method is based on measuring the net rate of increase (k, d^{-1}) of prey (µ – g) along a gradient of dilutions. The net growth rate in each bottle was calculated as follows:

$$k \equiv \frac{1}{t}\ln\left(\frac{N_t}{N_0}\right) \quad (2)$$

where t is the duration of the experiment (in d), N_0 is the prey concentration at the start of the experiment, and N_t is the concentration at the end of the experiment. Assumptions for the dilution method are that prey growth is exponential and independent of the dilution level and that the ingestion rate of predators is linearly proportional to the concentration of prey (e.g. predators are not food saturated). If these assumptions are met, linear regression of the fraction of unfiltered sea water (f_u) against k should yield a slope and an intercept, with an error term ε, corresponding to the grazing mortality (g, d^{-1}) and the specific growth rate of prey (µ, d^{-1}), respectively (Landry & Hassett 1982):

$$k = \mu - g \cdot f_u + \varepsilon \quad (3)$$

For both the control treatments and the mussel treatment, a separate dilution series was prepared with a ratio of unfiltered:filtered water of 1:0 (100% unfiltered water), 3:1 (75%), 1:1 (50%) and 1:3 (25%). Since mussel grazing resulted in a reduction of larger plankton, further dilutions (below 25%) would result in concentrations of larger phytoplankton, HNAN and ciliates being too low to be determined reliably. The use of Whatman glass fibre filters (GF/F; nominal pore size of 0.8 µm) resulted in the passage of bacteria and occasional picophytoplankton cells through these filters. Cell counts were performed for all prepared dilutions. For both pico- and nanophytoplankton, the realised ratios of unfiltered to filtered water were close to the target ratios. For bacteria, due to the passage of cells through the filter, the lowest percentage of bactzeria in unfiltered water was 38% rather than the target 25%. For bacteria, pico- and nanophytoplankton, the realised dilution fractions were used in the calculations (Eq. 3). Another complication of the passage of bacteria cells through the GF/F filter is the potential increase in the specific growth rate (µ) for these

Table 1. Overview of the grazing pressure (G_p) exerted by mussels per experiment for each of the plankton groups. The grazing pressure is calculated as the fraction removed in the mussel treatment compared to the concentration in the control treatment (Eq. 1, 'Materials and methods: Mussel filtration experiments'). bact: bacteria; pico: picophytoplankton; nano: nanophytoplankton; hnan: heterotrophic nanoflagellates; cil: ciliates; chlT: total chlorophyll. An empty cell indicates that a parameter was not determined for that experiment

Expt	Date	$G_p$bact	$G_p$pico	$G_p$nano	$G_p$hnan	$G_p$cil	$G_p$chlT
1	28-Jul-11		0.72	0.90			
2	10-Aug-11		0.67	0.93			
3	9-Sep-11		0.77	0.85			
4	26-Sep-11		0.61	0.78			
5	2-Jul-13		0.25	0.16	0.26		
6	9-Jul-13		0.12	0.67	0.55	0.78	
7	24-Jul-13	−0.10	0.16	0.75	0.80	0.59	0.57
8	7-Aug-13	0.09	0.02	0.49	0.50	0.23	0.55
9	20-Aug-13	0.11	0.23	0.44	0.40	0.55	0.29
10	25-Sep-13	−0.12	0.29	0.62	0.83	0.67	0.42

cells in the most diluted concentrations, resulting in a non-linear relation between the net growth (k) and the fraction of undiluted sea water (Li & Dickie 1985). In the present study, no indication for non-linearity was found in the experiments with regard to bacterial growth.

In 2011, each dilution step was performed in duplicate (50 ml Greiner culture flasks), while in 2013, 3 replicates were used (500 ml polycarbonate bottles). Bottles and flasks were filled to the rim, to prevent air bubbles, by gently pouring the well-mixed water, after which they were attached to a slowly rotating plankton wheel for 24 h in a temperature-controlled room set at *in situ* temperature. Illumination was by daylight fluorescent tubes providing *in situ* light levels and applying a day-night regime. Changes in the plankton community were established after 24 h.

To calculate the grazing loss per day, the daily production as well as the net changes in biomass (production-consumption) for bacteria, pico- and nanophytoplankton, the following formulas were used (Landry et al. 2000, Calbet & Landry 2004) after first converting cell numbers into carbon (see 'Bacteria' and 'Pico- and nanophytoplankton' below):

$$P = \mu \cdot C_m \quad (4)$$

$$G = g \cdot C_m \quad (5)$$

$$C_m = C_0\left[e^{(\mu-g)t} - 1\right] / (\mu - g)t \quad (6)$$

where P is the production (µg C l^{-1} d^{-1}) for each functional group, µ is the specific growth rate (d^{-1}), and C_m is the integrated concentration during the incubation. G is the grazing loss (µg C l^{-1} d^{-1}) experienced by bacteria, pico- or nanophytoplankton, and g is the grazing mortality rate (d^{-1}). C_0 is the concentration for each functional group at the start of the incubation, and t is the duration of the incubation in days.

To calculate carbon-specific ingestion rates of predators, the following formula was used, in which I is the units of prey carbon ingested by 1 unit of predator carbon (d^{-1}):

$$I = G / C_{m,\ predator} \quad (7)$$

Sample analysis

At the start (t_0) and the end (t_{24}), 1 sample per bottle was analysed for bacteria, pico- and nanophytoplankton for all dilution levels. HNAN were enumerated for each of the 3 undiluted bottles only. Single subsamples for ciliates and duplicate subsamples for both total and fractionated chlorophyll were taken from the undiluted bottle at t_0 and a mixed sample of the 3 undiluted bottles at t_{24}. Not all parameters were measured in all experiments (Table 1).

Bacteria

Subsamples (1 ml) for enumerating bacteria were fixed with glutaraldehyde (0.5% final concentration), mixed and then stored at −80°C until analysis. Analysis was always within 1 mo. Analyses were performed using a flow cytometer (BD Accuri C6, excitation with 488 nm laser); samples were diluted with 10% Tris-EDTA buffer. SYBR® Green I (Invitrogen) stain was added (0.1% final concentration), and samples were incubated in the dark for 15 min. The 530 nm laser (FL1) was used to detect the stained cells. The average diameter of particles was calculated after calibration of forward scatter with size, using beads (3, 7 and 10 µm) (e.g. Li & Dickie 1985). Assuming a spherical shape, cell counts were converted to carbon biomass using a factor of 4.7×10^{-7} µg C µm^{-3} (Verity et al. 1992).

Pico- and nanophytoplankton

Phytoplankton cell counts were performed by means of flow cytometry. Water subsamples (1 ml) were processed unfixed, immediately after collection. Fluorescence at wavelengths > 670 nm (FL3) was ascribed to chlorophyll. Forward scatter was used as an indication of cell size, and a distinction between phytoplankton and debris was made based on the relative fluorescence to cell size. Phytoplankton cell counts were further divided in 2 size classes (<3 µm: pico and >3–20 µm: nano) using 3 µm beads (spherotech, BD Accuri). The definition of pico- and nanophytoplankton is based on the particle size effectively retained by mussels (Møhlenberg & Riisgård 1978). To convert pico- and nanophytoplankton cell counts into carbon biomass, spherical cell shapes were assumed. The conversion factors used were 4.7×10^{-7} and 2.2×10^{-7} µg C µm^{-3}, respectively (Verity et al. 1992). It must be noted that flow cytometry counts and subsequent conversion of counts into carbon biomass yielded much lower biomasses of picophytoplankton compared to biomasses based on fractionated chlorophyll. Using a fixed conversion factor of 20 g C g^{-1} chl (Riegman et al. 1993), picophytoplankton biomasses were 7- to 20-fold higher.

Heterotrophic nanoflagellates (HNAN)

HNAN were counted using flow cytometry applying a protocol slightly modified from that of Rose et al. (2004). Modifications included the use of a smaller volume (4 ml) and a higher final concentration of Lysotracker® Green (75 nM, Molecular Probes). A flow rate of 100 µl min^{-1} and core size of 40 µm was selected. Count time was 15 min; 2.5 µm beads (YG fluorescence, Polysciences) were used for volume and size reference. To convert cell counts to carbon biomass, the same procedure was applied as for nanophytoplankton.

Ciliates

For enumeration of ciliates, 1 subsample (0.5 to 1 l) was fixed in 2 to 4 ml acid Lugol and stored in brown glass bottles at 4°C until analysis. Samples were concentrated (10× to 20×) using the Utermöhl sedimentation technique (Verweij et al. 2010). For each sample, a minimum of 100 individuals was counted, or, at very low abundances, all individuals in a maximum of 10% of the concentrated sample were counted. Ciliate cells were counted and divided into 5 size classes (<20 µm, 20–40 µm, 40–60 µm, 60–80 µm and >80 µm) based on their length, using an inverted microscope. An oblate spheroid ($[4/3]\pi\ a^2b$) best represented the average shape of ciliates (Putt & Stoecker 1989). Using the middle of the size class/2 as *a* and the middle of the size class/4 as *b*, calculated cell volume was converted into carbon using a factor of 1.65×10^{-7} µg C $µm^{-3}$ (Verity et al. 1992).

Chlorophyll

For the determination of total and fractionated chlorophyll, duplicate subsamples (200 to 300 ml) were filtered over Whatman GF/F and 3.0 µm polycarbonate filters using low vacuum pressure (max. −0.4 bar). Filters were stored in the dark at −80°C for no more than 2 mo. Chlorophyll was extracted by homogenisation of filters in 90% acetone with the addition of glass pearls. Chlorophyll was determined fluorometrically (Holm-Hansen et al. 1965) using spinach chlorophyll *a* (Sigma) as a reference.

Data analysis

Linear regression analysis was performed for each experiment to estimate the specific growth rate (µ) and grazing mortality rate (*g*) per day for bacteria (when measured), pico- and nanophytoplankton (Table 2). On 3 occasions, non-linear responses were detected (Table 2), violating the assumption of linearity between predator ingestion rate and prey concentration. The occurrence of non-linear patterns is a common problem for the dilution method (Gallegos 1989, Evans & Paranjape 1992). Previously identified causes of non-linear regressions are the existence of a feeding threshold for grazers, occurring at high dilution levels (Quevedo & Anadón 2001), food saturation of grazers (Gallegos 1989, Evans & Paranjape 1992), a change in the microzooplankton community (Dolan et al. 2000), prey selection (Teixeira & Figueiras 2009) or nutrient limitation during the incubation (Landry & Hassett 1982). However, Teixeira & Figueiras (2009) reported that specific growth rates and grazing mortality are still robust when calculated over the linear part of the regression. This procedure was applied in the present study.

The estimated specific growth (µ, d^{-1}) and grazing mortality rates (*g*, d^{-1}) resulting from the regression analyses were used to calculate mean values. In the present study, the estimates from all experiments were considered, including those experiments in which grazing mortality rates were low (not significantly different from zero) or even negative (cf. Latasa 2014, Landry 2014). For the control experiments, it can be argued that including negative values in the mean value compensates for experiments in which rates were overestimated (Landry 2014), whereas leaving out low estimates results in an overestimation of the grazing mortality rates (Latasa 2014). For the mussel treatments, however, the occurrence of negative grazing mortality rates for both pico- and nanophytoplankton, indicated by a positive slope, occurred on a regular basis (Table 2). The most likely explanation for the occurrence of these positive slopes is the excretion of pseudofaeces by mussels. Pseudofaeces are relatively large particles, consisting of silt and algal cells, loosely bound in mucus. At the start of the dilution experiments, pseudofaeces were diluted accordingly, but algal cells bound in pseudofaeces were not enumerated by the flow cytometer due to the large size of the aggregation. During the 24 h incubations, these algal cells were released from the pseudofaeces and were then counted. This change resulted in a proportional increase in algal cells with the fraction of unfiltered water, and thus in a positive slope. Because the release of phytoplankton cells from pseudofaeces underestimates the grazing mortality, large positive regression coefficients (>0.20) were set to zero when calculating mean values.

Table 2. For each experiment, a linear regression analysis was performed on the fraction of unfiltered sea water against the change in bacteria, pico- or nanophytoplankton concentration in 24 h (Eq. 2). This analysis yielded a slope and an intercept (means ± SD) corresponding to the grazing mortality (g, d^{-1}) and the specific growth rate of prey (μ, d^{-1}) (Eq. 3), respectively. Analyses were performed for natural sea water (control experiments) and for mussel-filtered water. Non-linear responses were detected occasionally (in italic). In these cases, specific growth rates and grazing mortality rates were calculated using the linear part of the response (cf. Teixeira & Figueiras 2009, see 'Materials and methods: Dilution experiments'). These adapted values are given in the table below. (*)p < 0.05, *p < 0.01, **p < 0.001, ***p < 0.0001

Expt	Date	Control			Mussel		
	(d-mo-yr)	μ (d^{-1})	g (d^{-1})	R^2	μ (d^{-1})	g (d^{-1})	R^2
Bacteria							
7	24-Jul-13	0.67 ± 0.16**	1.08 ± 0.18***	0.78	1.06 ± 0.17***	0.81 ± 0.21**	0.66
8	7-Aug-13	0.09 ± 0.12	1.22 ± 0.16***	0.85	1.50 ± 0.12***	1.59 ± 0.17***	0.90
9	20-Aug-13	0.99 ± 0.23**	1.15 ± 0.27**	0.65	1.41 ± 0.12***	0.71 ± 0.15***	0.69
10	25-Sep-13	0.86 ± 0.33*	1.14 ± 0.39*	0.47	0.66 ± 0.18**	0.73 ± 0.21**	0.55
Picophytoplankton							
1	28-Jul-11	0.22 ± 0.09*	0.78 ± 0.13***	0.87	*0.02 ± 0.40*	*−0.29 ± 0.18*	*0.39*
2	10-Aug-11	0.12 ± 0.04*	0.46 ± 0.05***	0.93	0.11 ± 0.04*	−0.01 ± 0.05	0.01
3	9-Sep-11	0.13 ± 0.07	−0.06 ± 0.10	0.08	−0.27 ± 0.12(*)	−0.48 ± 0.17*	0.57
4	26-Sep-11	−0.09 ± 0.28	0.13 ± 0.40	0.02	0.25 ± 0.04***	−0.02 ± 0.06	0.02
5	2-Jul-13	0.64 ± 0.04***	0.07 ± 0.06	0.16	0.24 ± 0.07*	−0.15 ± 0.11	0.20
6	9-Jul-13	0.46 ± 0.05***	0.42 ± 0.07***	0.76	0.94 ± 0.04***	0.22 ± 0.06**	0.60
7	24-Jul-13	0.47 ± 0.04***	0.64 ± 0.06***	0.92	0.20 ± 0.08*	−0.21 ± 0.11	0.25
8	7-Aug-13	0.26 ± 0.13(*)	0.91 ± 0.19***	0.71	0.62 ± 0.06***	0.34 ± 0.09**	0.62
9	20-Aug-13	0.62 ± 0.11***	0.57 ± 0.15**	0.59	0.54 ± 0.04***	0.20 ± 0.05**	0.59
10	25-Sep-13	0.47 ± 0.06***	0.54 ± 0.09***	0.79	0.57 ± 0.03***	0.15 ± 0.05*	0.48
Nanophytoplankton							
1	28-Jul-11	0.13 ± 0.08	0.11 ± 0.11	0.13	*0.29 ± 0.22*	*−0.08 ± 0.28*	*0.02*
2	10-Aug-11	*0.24 ± 0.05***	*−0.01 ± 0.06*	*0.01*	0.05 ± 0.14	−0.30 ± 0.22	0.24
3	9-Sep-11	0.00 ± 0.15	−0.16 ± 0.26	0.09	−0.23 ± 0.28	−0.12 ± 0.39	0.09
4	26-Sep-11	−0.03 ± 0.18	0.06 ± 0.25	0.01	0.36 ± 0.10*	0.15 ± 0.16	0.12
5	2-Jul-13	0.82 ± 0.13***	0.27 ± 0.19	0.22	0.65 ± 0.07***	0.33 ± 0.12*	0.54
6	9-Jul-13	0.71 ± 0.12***	0.80 ± 0.20**	0.62	0.77 ± 0.16***	−0.29 ± 0.23	0.14
7	24-Jul-13	0.81 ± 0.07***	0.77 ± 0.12***	0.82	0.79 ± 0.19**	−0.33 ± 0.24	0.16
8	7-Aug-13	1.04 ± 0.09***	0.35 ± 0.12*	0.45	1.27 ± 0.06***	0.27 ± 0.08**	0.56
9	20-Aug-13	0.95 ± 0.10***	0.31 ± 0.12*	0.40	0.27 ± 0.18	0.42 ± 0.23(*)	0.26
10	25-Sep-13	0.69 ± 0.06***	0.30 ± 0.09**	0.52	0.42 ± 0.04***	0.16 ± 0.06*	0.44

To test for differences in growth and mortality rates in natural sea water and mussel-filtered water, paired *t*-tests were used. A significance level of α = 0.05 was applied. Statistical analyses were performed using R statistical software (R Development Core Team 2011).

RESULTS

Mussel filtration experiments

In 10 experiments, Wadden Sea water was exposed to mussel *Mytilus edulis* filtration, resulting in the removal of plankton biomass (Table 1). Both the duration as well as the number and size of mussels added differed between the experiments. This difference resulted in a different grazing pressure exerted on the plankton community for each experiment. The biomass removed was not equal for all plankton groups. On average, filtration by mussels led to a negligible removal of bacterial biomass and a relatively small but variable (average 20%) amount of picophytoplankton, while the removal of nanophytoplankton, HNAN and ciliates biomasses was substantial, with on average 50 to 60% of available biomasses removed. Total phytoplankton biomass, given as chlorophyll, was removed by half. In addition to filtering plankton, mussels also reduced the concentration of other suspended matter, like debris and silt, resulting in a greater underwater light availability (data not shown). Algae react rapidly to changes in the underwater light climate, and more light penetrating the water column might result in a reduction of the light-harvesting pigments such as chlorophyll (Perry et al. 1981). The occurrence of this so-called photo-adaptation was investigated in this study by comparing the net growth of the picophyto-

plankton fraction of chlorophyll with the picophytoplankton cell counts in mussel-filtered water after a 24 h incubation period. The lower net growth rate measured using chlorophyll was considered proof of photo-adaptation. Therefore, in this study, chlorophyll as a proxy for phytoplankton biomass is considered an unsuitable parameter to study the effect of mussel filtration, and results regarding chlorophyll will not be discussed further.

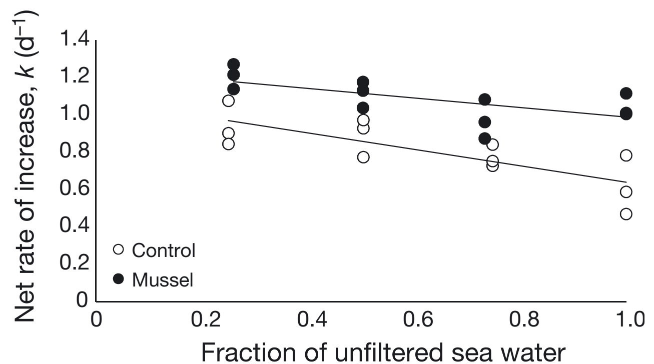

Fig. 2. An example of a dilution experiment to establish the specific growth and grazing mortality rate. The closed symbols indicate the change in cell concentration in 24 h in the mussel treatment; the open symbols indicate the change in the control (natural sea water). On the *y*-axis, the net rate of increase (in each bottle) is given as the natural logarithm of the change in cell concentration in 24 h (Eq. 2). The *x*-axis denotes the fraction of unfiltered sea water, 0 indicating 100% filtered sea water and 1.0 indicating 100% undiluted water. Regression of the net increase on the fraction of unfiltered water gives an estimate for the specific growth rate (μ, d^{-1}, the intercept) and the grazing mortality rate (g, d^{-1}, the regression coefficient)

Dilution experiments

After filtration by mussels, both mussel-filtered ('mussel treatment') water and unfiltered ('control') water were serially diluted and incubated for 24 h (Fig. 2, Table 2). The goal of this dilution experiment was to detect changes as a result of mussel filtration in both the specific growth rate (μ) and grazing mortality rate (g) for the different plankton functional groups within the microbial food web.

Microbial community in natural sea water

The results from the dilution experiments performed for the control treatments (natural sea water) provide specific growth (μ, d^{-1}) and grazing mortality rates (g, d^{-1}) of the plankton groups of the Dutch Wadden Sea for the study period (July through to September). The specific growth rate varied between 0.09 and 0.99 d^{-1} (average 0.65 ± 0.37) for bacteria, between −0.09 and 0.64 d^{-1} (average 0.33 ± 0.24) for picophytoplankton and between −0.04 and 1.04 d^{-1} (0.54 ± 0.41) for nanophytoplankton (Table 2). The average μ for bacteria and nanophytoplankton are both higher than the μ for pico-sized phytoplankton.

Grazing mortality rates varied between 1.08 and 1.22 d^{-1} for bacteria (average 1.15 ± 0.06), between −0.06 and 0.91 d^{-1} for picophytoplankton (average 0.45 ± 0.31) and between −0.16 and 0.80 d^{-1} for nanophytoplankton (average 0.28 ± 0.31). During the study period, there was an average net increase per day in predators of 0.65 ± 0.28 for HNAN and 0.31 ± 0.61 for ciliates in the control experiments (Fig. 3a). For bacteria and picoplankton, grazing mortality exceeded the production during the study period, while for nanophytoplankton, produc-

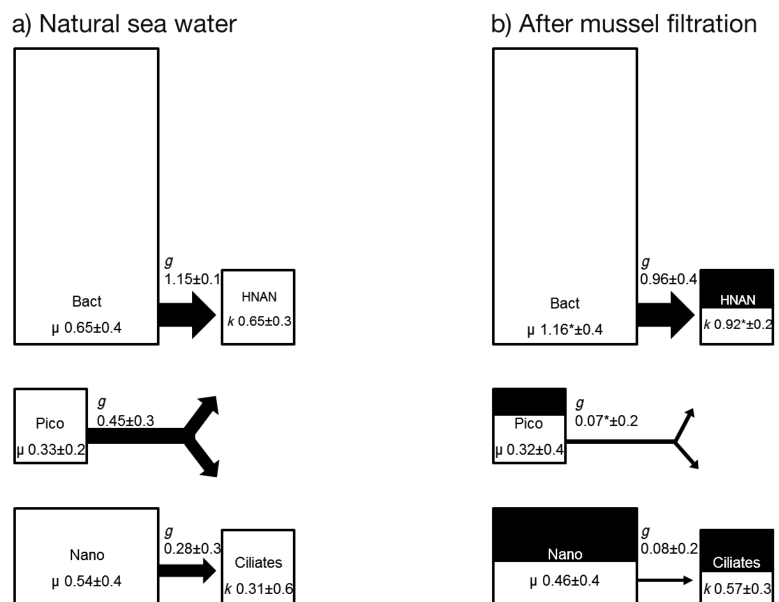

Fig. 3. Microbial food web structure in (a) natural sea water and (b) after mussel filtration. Box sizes are indicative of average biomass (μg C l^{-1}) in the Dutch Wadden Sea during the study period for each functional group. For bacteria, pico- and nanophytoplankton in each box, the average specific growth rate (μ) per day is given; the arrows indicate grazing mortality rates (g) per day. For heterotrophic nanoflagellates (HNAN) and ciliates, the changes in biomass were determined for the unfiltered fraction (f_u = 1) only, resulting in an estimate of the net change (k) per day (±SD). Average relative biomasses removed by mussels during the experiments are indicated by the black boxes. Significant changes in rates after mussel filtration are indicated with an asterisk (*)

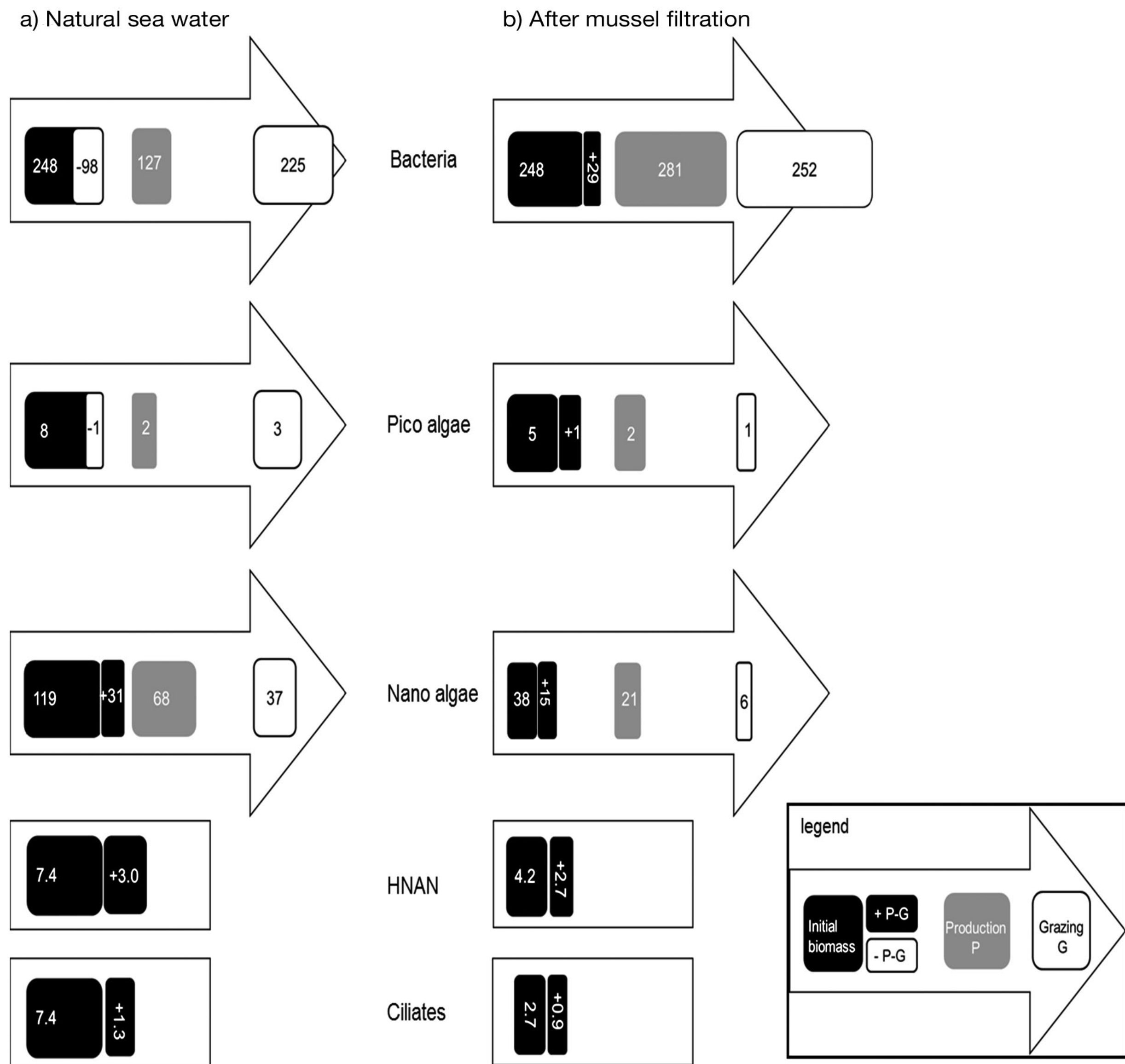

Fig. 4. Initial biomass (black box left) and net change in biomass after 24 h (+ and black box right in case of an increase or – and white box right in case of a decrease). For bacteria, pico- and nanophytoplankton, the net change is made explicit as the difference between production (grey box) and biomass removed by predators (white box in arrowhead). For heterotrophic nanoflagellates (HNAN) and ciliates, only biomass and net biomass change in 24 h is given. All boxes are in µg C l^{-1}; (a) natural sea water, (b) mussel filtration treatment

tion exceeded grazing losses (Fig. 4a). Nanophytoplankton daily specific growth and grazing mortality rates showed a seasonal pattern, with both rates decreasing from July to September.

Effect of mussel filtration on specific growth and grazing mortality rates

Mussel filtration affected the plankton groups considered in this study differently. For bacteria, the average specific growth rate (µ) almost doubled (0.65 to 1.16 d^{-1}) 24 h after mussel filtration (t = 3.84, df = 3, p = 0.031), while the average µ for both pico-and nanophytoplankton did not show significant differences between the control and mussel treatments (pico.: t = 0.08, df = 9, p = 0.94; nano.: t = 0.46, df = 9, p = 0.66) (Fig. 3).

Grazing mortality rates (g, d^{-1}) showed the opposite pattern to the specific growth rates: for bacteria and nanophytoplankton, after mussel exposure, g did not differ significantly from the rate measured in the

control treatments (t = −1.19, df = 3, p = 0.32; nano.: t = 1.86, df = 9, p = 0.096), while the remaining picophytoplankton after mussel exposure did experience a lower g (pico.: t = 4.24, df = 9, p = 0.0022) (Fig. 3). For the predators on bacteria and phytoplankton, HNAN and ciliates, only net growth rates ($\mu - g$, d^{-1}) were established. In the current study, mussel filtration resulted in increased net growth rates after 24 h for HNAN, present for ciliates, the differences in net growth rates between the control and mussel treatments were not significant (HNAN: t = 3.75, df = 4, p = 0.02; ciliates: t = 0.99, df = 4, p = 0.38) (Fig. 3).

Effect of mussel filtration on biomass

HNAN are considered the main predator of bacteria, and although mussel filtration in this study resulted in a substantial reduction of HNAN biomass (Table 1), this did not reduce the grazing mortality for bacteria. Instead, within 24 h after mussel filtration, the specific growth rates of bacteria increased, resulting in a substantial increase in bacterial production (Fig. 4). HNAN increased their net growth rate, and within 24 h, their biomass returned to pre-filtration concentrations (Fig. 4). During the 24 h incubations, HNAN concentrations were lower in the mussel treatment, but carbon-specific ingestion rates for HNAN on bacteria were higher; the fast-growing HNAN in the mussel treatment had a carbon-specific ingestion rate of 37 (µg C µg C^{-1} d^{-1}) compared to a rate of 22 in the control treatments. Picophytoplankton reacted differently to mussel filtration; the removal of a large part of their main predators, the HNAN, by mussels did result in a reduced g. This reduced grazing mortality enabled picophytoplankton to recover to pre-filtration concentrations within a day (Fig. 4). For nanophytoplankton, a similar pattern can be seen, but since mussels removed more nanophytoplankton biomass compared to picophytoplankton, the nanophytoplankton biomass did not recover to pre-filtration levels after 24 h. Ciliates, assumed to prey on nanophytoplankton and HNAN, also did not return to the biomass found before mussel filtration (Fig. 4).

DISCUSSION

Microbial community in natural sea water

Growth and grazing mortality rates vary in both time and space, making it difficult to compare rates between studies. However, the rates reported in the present study for the control experiments fall within the range of growth and mortality rates reported in previous studies (e.g. Gallegos et al. 1996, Calbet & Landry 2004). In natural sea water (undiluted fraction, f_u = 1), net growth rates ($\mu - g$, d^{-1}) for bacteria and picophytoplankton were positive on a few occasions, but for most experiments, grazing rates exceeded the specific growth rates, resulting in negative net growth (Table 2, Fig. 3a). In ambient water, bacterial as well as picophytoplankton growth is expected to be balanced by HNAN grazing, resulting in net growth rates oscillating around zero. Although net positive or negative growth rates are commonly reported (e.g. Quevedo & Anadón 2001, Pearce et al. 2011, Schmoker et al. 2013), it is difficult to determine whether the net growth rates in the current study are part of the expected oscillation or an artefact due to the experimental set-up (Del Giorgio et al. 1996, Dolan et al. 2000).

Impact of mussel filtration on the microbial community

Mussels (*Mytilus edulis*) removed a negligible amount of bacteria from the water column but impacted on bacteria indirectly; the increased specific growth rates for bacteria reported in this study are most likely the result of the mussel excretion products. Bacterial production can be stimulated by the excretion of particulate and dissolved organic matter (Azam et al. 1983). HNAN concentrations, substantially reduced as a result of mussel filtration, responded in the 24 h incubation period with increased ingestion and growth rates. Both ingestion and growth rates are known to increase with increasing food concentration before levelling off at saturating concentration, following a Monod response (Heinbokel 1978 and references herein). The simultaneous increase in bacterial specific growth rates and HNAN ingestion and growth rates after mussel filtration thus suggests a tight coupling between HNAN and bacteria as their main prey (Fig. 3). The lower HNAN concentrations during the 24 h incubation in the mussel treatments, combined with a higher ingestion rate, ultimately resulted in a larger part of bacterial carbon being removed (Fig. 4). Since only the net growth rates in the undiluted fraction were established ($\mu - g$, d^{-1}), it cannot be excluded that also a lower predation pressure experienced by the HNAN, due to the slow recovery of ciliate biomass (Figs. 3 & 4), contributed to the fast recovery of HNAN biomass. A reduced HNAN mortality due to the removal of larger zoo-

plankton (e.g. copepods) cannot be excluded but is considered less likely since juvenile mussels are inefficient predators on larger zooplankton (Horsted et al. 1988).

In addition to bacteria, picophytoplankton are also considered prey for HNAN, and although HNAN biomass was reduced substantially after mussel filtration, the rapid recovery was expected to enhance grazing mortality rates for picophytoplankton. Instead, grazing mortality rates decreased significantly over a period of 24 h (Fig. 3). In natural sea water, bacterial and picophytoplankton biomasses removed by predators seem more or less proportional to their availability (Fig. 4a). Mussel filtration reduced picophytoplankton concentration by ~20% in this study (Table 1) and increased bacterial production. This change caused a 'dilution' of picophytoplankton cells, resulting in a lower encounter rate of predators for picophytoplankton prey and hence lower predation rates. After mussel filtration, the remaining picophytoplankton biomass continued to grow with a comparable specific growth rate as before filtration, but since the grazing mortality rate was reduced, there was a consequential increase in picophytoplankton biomass (Figs. 3 & 4). After 24 h, picophytoplankton biomass recovered to pre-grazing concentrations. For nanophytoplankton, a similar pattern can be seen, but since mussels removed a larger part of nanophytoplankton biomass, nanophytoplankton biomass did not recover to the pre-filtration level within 1 d (Fig. 4). In the filtration experiments, both the duration of the experiments as well as the mussel biomass added differed between experiments, resulting in a different mussel grazing pressure for each experiment. This variation means that changes in both specific growth and grazing mortality rates reported attributed to mussel grazing should be regarded as qualitative rather than quantitative changes because mussel predation pressure was not standardised. At the same time, the differences in mussel grazing pressure in the present study allow for a first analysis between this mussel grazing pressure (G_p) and a recovery rate of the plankton community. It is hypothesised that there is a relation between the biomass of predators removed by mussels and the net growth rate of prey. In the present study, in those experiments in which a larger part of predator biomass was removed, the difference in grazing mortality rate of prey between the mussels and the control treatments was larger. HNAN biomass correlated with the change in picophytoplankton mortality rate (r = −0.52), and ciliates correlated with the change in nanophytoplankton mortality rate (r = −0.84). For bacteria, the number of experiments was too small to calculate a relation. Filtration pressure by mussels is thus an important parameter determining the ultimate effect on the microbial food web.

Conclusion

Results from this study show a size-selective removal of plankton by (juvenile) mussels resulting in relative changes in the different functional groups within the microbial food web. In the experiments, plankton were exposed to mussel seed filtration for a short period, after which the mussels were removed again. The measured effects, 24 h after this exposure, are the result of physical removal by filtration as well as chemical changes due to excretion products. The most important effect of the single exposure to mussel seeds was a stimulation of the bacterial-HNAN pathway, most likely due to excretion of DOM by mussels. Furthermore, picophytoplankton recovered faster than nanophytoplankton after mussel exposure due to reduced grazing losses by mussels.

Results from the present study revealed the direct as well as indirect effects of mussel exposure on the pathways within the microbial food web over a short period of time. Longer-term effects might include a shift from bacterial to picoalgal production due to complete remineralisation of mussel excretion products by bacteria and a stimulation of primary production due to increased growth conditions (more light and recycled nutrients). Whether HNAN biomass will continue to increase depends on the ability of ciliates to recover and control HNAN biomass. Recovery of ciliate biomass in turn might result in a further reduction of already low concentrations of nanophytoplankton. In the present study, the plankton community was exposed to mussel grazing for a single episode only. Continuous exposure to mussel grazing will likely change the outcome because mussels will effectively remove most HNAN and ciliate predators. High or continuous grazing pressure might, for example, result in a dominance of bacteria or picophytoplankton. Future experiments on the effect of bivalves on the microbial food web lasting longer than 24 h and with variable grazing exposures might be able to give more insight into the possible effects of filtration on an ecosystem level.

Several authors have stressed the need for research on the effect of bivalve filtration on the structure and composition of microbial food web (e.g. Murrell & Hollibaugh 1998, Calbet & Landry 2004, Trottet et al. 2008, Greene et al. 2011). To our knowl-

edge, the present work is the first study describing the short-term effect of mussel filtration on the different components of the microbial food web. The results from this study describe changes in growth and grazing mortality rates within the microbial food web induced by mussel filtration. With these changed rates, subsequent modifications in carbon flow though the food web were calculated. The results from this study allow for a better description of the direct and indirect effects of juvenile mussel filtration on the Wadden Sea food web.

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