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ORIGINAL PAPER

Settlement of *Macoma balthica* larvae in response to benthic diatom films

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Abstract The role of multi-species benthic diatom films (BDF) in the settlement of late pediveliger larvae of the bivalve *Macoma balthica* was investigated in still-water bioassays and multiple choice flume experiments. Axenic diatom cultures that were isolated from a tidal mudflat inhabited by *M. balthica* were selected to develop BDF sediment treatments characterized by a different community structure, biomass, and amount of extracellular polymeric substances (EPS). Control sediments had no added diatoms. Although all larvae settled and initiated burrowing within the first minute after their addition in still water, regardless of treatment, only 48–52% had completely penetrated the high diatom biomass treatments after 5 min, while on average 80 and 69% of the larvae had settled and burrowed into the control sediments and BDF with a low diatom biomass (<3.5 μg Chl *a* g^{-1} dry sediment), respectively. The percentage of larvae settling and burrowing into the sediment was negatively correlated with the concentration of Chl *a* and EPS of the BDF. This suggests higher physical resistance to bivalve penetration by the BDF with higher diatom biomass and more associated sugar and protein compounds. The larval settlement rate in annular flume experiments at flow velocities of 5 and 15 cm s^{-1} was distinctly lower compared to the still-water assays. Only 4.6–5.8% of the larvae were recovered from BDF and control sediments after 3 h. Nonetheless, a clear settlement preference was observed for BDF in the flume experiments; i.e., larvae settled significantly more in BDF compared to control sediments irrespective of flow speed. Comparison with the settlement of polystyrene mimics and freeze-killed larvae led to the conclusion that active selection, active secondary dispersal and, at low flow velocities (5 cm s^{-1}), passive adhesion to the sediment are important mechanisms determining the settlement of *M. balthica* larvae in estuarine biofilms.

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Introduction

An important challenge in estuarine benthic ecology is to understand the spatial and temporal variability in soft-sediment communities. Juvenile recruitment (i.e., the entry of juveniles into the adult population) is the foundation on which all subsequent interactions within the community

take place (Woodin et al. 1995). Most marine benthic invertebrates have a life cycle with a dispersive (i.e., pelagic) larval phase during which they are distributed and eventually settle into new habitats where they metamorphose into juveniles. Final recruitment success is determined by both pre-settlement processes (e.g., transport and planktonic mortality) and post-settlement processes (e.g., secondary dispersal and survival) (Pineda et al. 2009). Settlement of marine benthic invertebrate larvae or resuspended juveniles is mediated by many factors, e.g., flow characteristics (Pawlik et al. 1991; Pawlik and Butman 1993; Jonsson et al. 2004), organic content of the sediment (Grassle et al. 1992), sediment disturbance (Woodin et al. 1998; Marinelli and Woodin 2002; 2004), sediment grain size (Snelgrove et al. 1998), porewater and waterborne chemical cues (Pawlik 1992; Turner et al. 1994; Engstrom and Marinelli 2005), presence of conspecific juveniles or adults (Olivier et al. 1996; Hills et al. 1998; Snelgrove et al. 1999, 2001), metabolites of sympatric organisms (Woodin et al. 1993; Esser et al. 2008) and the presence of bacteria (Dobretsov and Qian 2006; Sebesvari et al. 2006). Furthermore, there is growing evidence that marine biofilms are instrumental in the settlement of many benthic organisms. Both facilitative and inhibitive effects of marine biofilms on larval settlement have been reported, which are generally attributed to waterborne bacterial extracellular polymeric substances (EPS) depending on origin, surface chemistry, microtopography, and metabolic activity of the biofilm (reviewed by Qian et al. 2007).

The composition of marine biofilms varies with time, forming complex aggregates of diatoms, bacteria, protozoa, and fungi (Decho 2000), all enmeshed in a matrix of EPS. The proportion of benthic diatoms in biofilms of estuarine tidal mudflats can be very high (Sabbe and Vyverman 1991; MacIntyre et al. 1996). Lam et al. (2003) showed that relative space occupation by diatoms can mediate larval settlement of the polychaete *Hydroides elegans*. Hence, in addition to the bacterial composition of a marine biofilm, the specific role of diatoms in the settlement of tidal flat invertebrate larvae is of interest. Moreover, recently settled herbivorous benthic invertebrates (post-larvae) often feed on diatoms. Consequently, recruitment success of these larvae may also depend on differences in diatom community composition because of their dietary requirements.

Marine biofilms have intensively been investigated with respect to their role in larval settlement of barnacles, ascidians, bryozoans, sea urchins, gastropods, and polychaetes (e.g., Keough and Raimondi 1995; Olivier et al. 2000; Harder et al. 2002; Lam et al. 2003, 2005; Dahms et al. 2004; Sebesvari et al. 2006; Chiu et al. 2007; Dworjanyn and Pirozzi 2008), but far less is known about the role of benthic diatom films (BDF) in bivalve settlement, especially in soft sediments. The Baltic tellin *Macoma balthica* is an infaunal surface deposit-feeding and facultative suspension-feeding bivalve (Rossi et al. 2004) with a pelagic larval stage (Caddy 1967). Recent genetic studies have revealed the occurrence of two subspecies: a mainly North Pacific subspecies *Macoma balthica balthica* with European populations inhabiting the White Sea and the Black Sea and the subspecies *Macoma balthica rubra*, which is distributed along the North Sea and the northeast Atlantic coasts (Väinola 2003; Nikula et al. 2007). In northwestern European tidal flats, *M. balthica rubra* is one of the most common bivalves, reaching densities of tens to hundreds of individuals m^{-2} (Beukema 1976; Ysebaert et al. 2003; Bocher et al. 2007; Van Colen et al. 2008, 2009). It is an important food source for wading birds, and benthic and epibenthic organisms (Hulscher 1982; Zwarts and Blomert 1992; Hiddink et al. 2002a, b, c). It influences the geochemistry of the sediment and thus the tidal flat energy cycling in general, due to its burrowing and feeding (e.g., Marinelli and Williams 2003). Hence, successful recruitment of *M. balthica rubra*, and bivalves in general, is important in tidal flat ecosystem function and is controlled by settlement processes (Bos 2005).

The present study examined larval settlement responses of the *M. balthica rubra* subspecies (further referred to as *Macoma balthica*) to axenic, multi-species, benthic diatom films (BDF), using still-water assays and flume experiments. Multiple choice flume experiments enable the determination of settlement preferences because bivalve larvae can select a preferred settlement site in a hydrodynamic environment (e.g., Grassle et al. 1992; Snelgrove et al. 1998; Engstrom and Marinelli 2005). In addition, still-water assays provide valuable information on the specific conditions that influence settlement within a given habitat (Marinelli and Woodin 2004). To assess whether the larval settlement of *M. balthica* is determined by BDF and whether larval settlement of *M. balthica* in response to BDF is an active or a passive, depositional process, the following null hypotheses were specifically tested:

- H_{01} Settlement and burrowing response does not differ between different ages of BDF in a still-water environment (Experiment 1).
- H_{02} Settlement choice is not influenced by BDF in a hydrodynamic environment (Experiment 2).
- H_{02a} Settlement choice is not influenced by flow velocity.
- H_{02b} Settlement choice does not differ from deposition of dead larvae and polystyrene mimics, thus settlement is a passive, depositional process.
- H_{03} In a hydrodynamic environment, the settlement response after primary settlement is not determined by BDF (Experiment 3).

Materials and methods

Collection and maintenance of *M. balthica*

Adult *M. balthica* were repeatedly collected from Paulinaschor (The Netherlands, 51°21′24″N, 3°42′51″W) at low tide in February–March 2008 and stored at 5°C in aerated basins (40 × 33 × 14 cm), prefilled with sieved sediment (1 mm) and 2-µm filtered seawater with a salinity of 27 (further referred to as FSW). Each basin contained ~150 individuals, which were fed three times week^{-1} with a mixture of concentrated non-viable algae (*Isochrysis galbana* and *Tetraselmis* sp., Reed Mariculture).

Larval production

Individual *M. balthica* were induced to spawn following the procedure of Honkoop et al. (1999) and Bos (2005). Adults were exposed to the selective serotonin re-uptake inhibitor (SSRI) fluoxetine, preceded by a Δ10°C temperature shock. SSRIs prevent the deterioration of neurotransmitters, so nerves are stimulated longer and more intensely than usual (Honkoop et al. 1999). On average, 35% of the adults could be induced to spawn. Fertilization was carried out by pipetting eggs of several females into a beaker and adding 1–3 ml of sperm suspension derived from at least five males. The resultant mixture was left undisturbed for 4 h at 15°C. Fertilized eggs (diameter ~100 µm) were then separated from all other matter by rinsing them over stacked sieves of 125 and 32 µm. Subsequently, they were transferred into 2-l glass bottles (further referred to as batches), containing 15°C UV-irradiated 0.2-µm filtered seawater with a salinity of 27 (further referred to as UV FSW) and dosed with 1.5×10^{-5} g l^{-1} penicillin G potassium salt and 2.5×10^{-5} g l^{-1} streptomycin sulfate. The bottles were placed on a rollertable (3 rpm) to avoid sinking of larvae.

Cultivation and maintenance

At day 4, all larvae (4972 ± 667 SE l^{-1}) had reached the D-stage, and from this moment on live *Isochrysis galbana* (10^5 cells ml^{-1}) was added to the UV FSW. The batches were refreshed every other day by rinsing the UV FSW and larvae over a 32-µm mesh sieve and transferring the larvae into new glass bottles containing UV FSW dosed with 1.5×10^{-5} g l^{-1} penicillin G potassium salt, 2.5×10^{-5} g l^{-1} streptomycin sulfate and live *I. galbana* (10^5 cells ml^{-1}). Subsamples were taken to measure larval mortality. During the cultivation, we observed a mortality of 36% on average by day 20, i.e., a mortality rate of about 0.02 day^{-1}, which is comparable to Bos et al. (2006). At 21–24 days after fertilization, the larvae developed a foot (i.e., pediveliger stage) and 25-day-old larvae (270 ± 4 SE µm), actively moving their foot and velum, were used in all experiments.

Settlement response in still water (Experiment 1)

Sediment processing

Sediment was collected from Paulinaschor at low tide. Collection was confined to the top 2 cm and sieved over a 1-mm mesh sieve in the laboratory to remove macrobenthic organisms and larger debris. Subsequently the sieved sediment was heated at 180°C for 4 h. This sediment had a median grain size of 89.6 ± 1.07 SE µm and the mud content was 30.8 ± 0.52 SE% (Malvern Mastersizer 2000 laser diffraction) and is further referred to as the control sediment. This sediment was preferred above muffled sediment as a control since the latter inhibited larval settlement, presumably due to dissolution of material from the muffled sediment into the water.

For the assays, 2.5 g of control sediment was transferred into each well of a sterile, 12-well microplate (3.8 cm² well surface area, TPP, Switzerland) resulting in a 7-mm sediment layer. To develop a benthic diatom film (BDF), the control sediments were inoculated with 3 ml of axenic diatom cultures and incubated at 18°C, on a 14 h light:10 h dark photoperiod (145 µmol photons m^{-2} s^{-1}). The diatoms used in this experiment were *Navicula phyllepta*, *N. gregaria*, *N. arenaria*, and *Cylindrotheca closterium.* These species were isolated from the tidal mudflat at Paulinaschor and were dominant components of the microphytobenthos at that site (Sabbe and Vyverman 1991; Forster et al. 2006). Cells for inoculations were harvested from monoclonal, exponentially growing axenic cultures at 19 ± 1°C and illuminated at a rate of 90 µmol photons m^{-2} s^{-1} on a 14 h light:10 h dark photoperiod. The experimental microcosms were inoculated with a fixed total biovolume of 1×10^8 µm³ (biovolume of *N. phyllepta, N. gregaria, N. arenaria* = 3×10^7 µm³; biovolume of *C. closterium* = 1×10^7 µm³). To obtain different BDF, sediments were incubated for 4, 11, and 21 days, and 1.2 ml of the F/2 medium (Guillard 1975) of all treatments was refreshed every day in a flow bench without disturbing the sediment. Control sediments were maintained under the same incubation conditions, but without addition of diatoms. This resulted in an averaged *N. phyllepta—N. gregaria—N. arenaria—C. closterium* relative biovolume of 26–31–32–14%, 17–26–34–22%, and 16–25–31–27%, for the 4-, 11-, and 21-day treatment, respectively. Experimental sediments were further characterized by their Chl *a* and EPS concentration. Chl *a* concentration was determined by HPLC analysis of the supernatant, extracted from the lyophilized sediment by adding 10 ml of a 90% acetone–10% milli-Q water solution. The EPS concentration was

measured spectrophotometrically using the phenol–sulfuric acid assay (Dubois et al. 1956) on the colloidal carbohydrate fraction of the supernatant extracted after lyophilization (De Brouwer and Stal 2001).

Experimental protocol

To observe the settlement and burrowing responses to different treatments (i.e., control, 4-, 11-, and 21-day BDF), larvae were labeled with fluorescent microparticles (Radglo, Radiant Color, N·V., Houthalen, Belgium) to obtain a contrast with the bioassay sediment. These microparticles are non-toxic and have a spherical diameter of 2–10 μm. Feeding larvae ingest these particles resulting in a gut filled with fluorescent pigment (Lindegarth and Jonsson 1991; Jonsson et al. 1991), which becomes visible by illumination of the larvae with UV-light (365 nm). Since the particles are insoluble in water, one droplet of detergent was added to facilitate suspension of these particles. Preliminary tests showed that mortality rate was not affected as a result of fluorescent labeling. To assure uptake by the larvae, fluorescent pigment particles were supplied to feeding larvae (10^5 particles ml^{-1}) 24 h prior to the experiments. Experiments were performed on two consecutive days using larvae originating from a different and independent batch on each day. Prior to each bioassay (1) larvae were picked out from the batch using a stereomicroscope and UV-light to check their viability and dyeing, and (2) 2 ml F/2 medium of each well was pipetted out and 2 ml of sterile UV FSW was added to the wells without disturbing the sediment. Subsequently, for each bioassay ($n = 6$ $batch^{-1}$), 15 larvae were gently added to a well with a glass pipette and timing started when the pipette was empty. All pipettes were checked for remaining larvae, i.e., larvae that were not added to the well. Over 5 min, the burrowing larvae were counted and their complete disappearance into the sediment was timed. After this time period, larvae that were still on the sediment surface were not counted as having settled and burrowed. All replicates for each treatment $batch^{-1}$ were sequentially performed within 1 h.

To quantify bacterial contamination of the BDF due to experimental handling procedures, bacteria were extracted from the biofilm, stained with Acridine Orange and bacterial cell densities were enumerated on 0–2 μm black polycarbonate filters under blue-green light excitation (480–495 nm). Recorded bacterial densities were marginal, on average 235 ± 136 SE cells mm^{-2} and did not differ significantly among treatments (*t* test; $P > 0.05$).

Statistical analysis (Experiment 1)

Burrowing time and percentage of larval burrowing (number of burrowing larvae/total number of larvae added) after 60, 120, 180, 240, and 300 s were used as response variables to identify settlement responses of larvae to the different BDF. Burrowing time data were root transformed and percentage of larval burrowing data were arcsine-transformed to gain normality (Shapiro-Wilks' tests) and homogeneity of variances (Cochran and Bartlett tests). The effect on burrowing time was investigated using two-factor analysis of variance with batch as random factor and treatment as fixed factor. Larval burrowing data were analyzed using a repeated measures design with batch as random factor and treatment and time as fixed factors. Tukey's multiple comparison tests were performed to investigate significant differences between treatments at different times. Since the sphericity assumption for repeated measurements was violated by our data, adjusted *F* tests using the Greenhouse-Geiser correction were calculated, resulting in more conservative *P* levels (Quinn and Keough 2002). Further, simple linear regression analysis was performed to investigate relationships between the percentage of larval settlement, averaged burrowing time and the BDF characteristics (Chl *a* and EPS).

Annular flume experiments (Experiments 2 and 3)

Annular flume characteristics

Following the Plymouth Marine Laboratory annular flume design (Widdows et al. 1998), a flume was constructed of polystyrene, with a circular channel 10 cm wide (inner diameter 44 cm, outer diameter 64 cm), 35 cm deep, and with a maximum volume of 60 l. The channel flow was driven by contact on the water surface with four PVC paddles (9 × 14 cm), which were attached to a rigid support system driven by a variable speed DC motor. On the bottom of the tank, PVC pots (inner diameter 5 cm) can be attached, flush with the flume bottom and O-rings sealed the pots to prevent water loss. The annular flume is a good compromise in terms of portability and surface area (0.17 m^2) and allowed simultaneous testing of treatments in a realistic, fully developed, benthic boundary layer where sediment treatments could easily be removed and recovered after each trial. The disadvantage of annular flumes, in general, is the effect of secondary circulation. However, secondary flows were kept to an acceptable minimum (~3% of tangential flow) with the 10-cm channel width of the flume in the current study (J. Widdows, personal communication). To characterize the fluid dynamic environment, velocity profiles were measured at 8 cm above the bottom with a SonTek Micro ADV (acoustic Doppler velocimeter), mounted through the bottom of the flume. A linear relation between free stream velocity and revolutions min^{-1} was found (free stream velocity = 1.7785 rpm − 0.5672 [$r^2 = 0.998$]).

Sediment processing

The same control sediment as for the still-water bioassays was used. To yield the BDFs, the PVC pots, prefilled with control sediment, were inoculated with a mixture of axenic diatom cultures (total biovolume = 4.68×10^8 µm³; relative biovolume = 30–30–30–10%, respectively, for *N. phyllepta, N. gregaria, N. arenaria* and *C. closterium*). Control and BDF sediments were incubated for 11 days at 18°C on a 14 h light:10 h dark photoperiod (145 µmol photons $m^{-2} s^{-1}$) and 10 ml of the F/2 medium was refreshed every day in both control and BDF sediments. Chl *a* and EPS concentrations of the upper 5 mm were determined according to the abovementioned methods (Experiment 1).

Settlement choice in a hydrodynamic environment (Experiment 2): protocol

The proportional distribution of live larvae, freeze-killed larvae (further referred to as dead larvae), and spherical polystyrene (PS) mimics (average diameter 264.5 ± 3.8 SD µm) between BDF and control sediments was tested in the first set of experiments to examine the processes affecting larval settlement (i.e., active habitat selection vs. passive deposition). Two BDF and two control sediments were screwed into the bottom of the flume (flume bottom surface occupied = 4.6%; distance between pots = 37.4 cm) and the flume was carefully filled with 50 l of FSW resulting in a water depth of 29.5 cm. For each experimental trial ($n = 4$), ~5,000 PS mimics and 500 live larvae were randomly added to the flume preceding initiation of the flow, which was maintained for 3 h at 5 or 15 cm s^{-1}. For each replicate trial per flow velocity, larvae were selected from one out of four independent batches. In addition, two trials at 5 cm s^{-1} and two trials at 15 cm s^{-1} were conducted with 500 dead larvae. Sinking velocities of the three types of 'settlers' in still FSW were 2.8 ± 0.5, 2.6 ± 0.2 SE mm s^{-1}, and 1.6 ± 0.2 mm s^{-1}, respectively, for live larvae, dead larvae, and PS mimics. Furthermore, no resuspension or bedload transport of the sediment was observed at 5 and 15 cm s^{-1} during pilot tests performed with neutral red-dyed sediment. Hence, secondary dispersal after primary settlement is expected to be due to active choice, rather than occurring passively by sediment resuspension or movement. After 3 h, the experimental sediments were closed with plates, the flume was drained, and the top 2 cm of the sediments was preserved in a 4% buffered formalin–tap water solution, stained with Rose Bengal and the settled larvae and mimics were enumerated under a stereomicroscope.

Settlement response after primary settlement (Experiment 3): protocol

A total of 30 live larvae were added to the control and BDF sediments and left to settle for 30 min. Subsequently, the supernatant was removed from each PVC pot and checked for unsettled larvae. For each experimental trial, two control and two BDF sediments with primary settled larvae originating from the same batch were screwed into the flume, flush with the flume bottom. Then, the flume was filled with 50 l of FSW and the flow was initiated at 5 cm s^{-1}. After 10 min, the flow was stopped and the experimental sediments were closed with plates. Subsequently, the flume was drained and the top 2 cm of the sediments was preserved in a 4% buffered formalin–tap water solution, stained with Rose Bengal, and the remaining *M. balthica* were enumerated under a stereomicroscope.

Statistical analyses (Experiments 2 and 3)

For Experiment 2, replicated G-tests for goodness of fit (Sokal and Rohlf 1995) were conducted to determine significant deviations from the 1/1 (i.e., even) distribution, the averaged distribution of the PS mimics, dead larvae, and the averaged distribution of live larvae, dead larvae, and PS mimics at 15 cm s^{-1}. The two BDF and the two control sediments per experimental trial were pooled and only the juvenile percentage inside sampling pots was retained for statistical analysis. All results were expressed as relative percentage recovered from BDF and control sediments, and the percentages were adjusted to give the composition, i.e., their cumulative abundance equaling 100%. As such, the weight of all replicates in a replicated statistical test is equal (Moens et al. 1999). Measurement of the pooled G statistic (G_p) enabled interpretation of the significance of the overall deviation from the tested distribution over all replicates. G_p was calculated at a critical probability of $\alpha' = \alpha/k$, with k equal to the number of multiple pairwise tests (i.e., Bonferroni approach). As such, *G*-tests for PS mimics and live larvae were performed at $\alpha = 0.008$ (i.e., 0.05/6). Experiment 3 was analyzed using a mixed model analysis of variance with batch and trial as random effects and treatment as fixed effect. The proportion of *M. balthica* remaining in the sediments was arcsine-square root transformed to meet assumptions of normality (Shapiro-Wilks' tests) and homogeneity of variances (Cochran and Bartlett tests).

Results

Benthic diatom film characteristics

Manipulation of the incubation time successfully resulted in different BDFs. Chlorophyll *a* and EPS concentration of

these BDFs (Table 1) were significantly different among treatments for each experiment (*t* test, $P < 0.05$). Slight erosion of the biofilm was observed during the first minute after initiation of the flow, which caused a reduction of the Chl *a* content of the BDF (−14, −12 and −29%; respectively, for 10 min at 5 cm s^{-1}, 3 h at 5 cm s^{-1}, and 3 h at 15 cm s^{-1}). However, differences between the control and BDF sediments remained large and significant (*t* test, $P < 0.05$).

Settlement response in still water (Experiment 1)

All larvae started to penetrate the sediments in all treatments and the control within the first minute after their addition to the wells. However, the percentage of larval settling and burrowing (i.e., number of completely penetrated larvae/number of larvae added, see "Materials and methods") significantly differed among treatments and times. Consequently, H_{01} was rejected, i.e., the settlement and burrowing response differed among different ages of BDF in a still-water environment. No significant differences between the two larval batches were found and the interaction between the factors time and treatment, nested in batch, was not significant (Table 2). In general, the settlement and burrowing response to control and 4-day BDF sediments was higher than in 11- and 21-day BDF. The percentage of larval settlement and burrowing increased with time for all treatments and, in Batch 1, significant differences remained between 11-day BDF and control sediments, even after 300 s (Tukey's test, $P < 0.05$) (Fig. 1). Consistently, the average burrowing time was significantly different between treatments with highest burrowing times in 11- and 21-day BDF for both batches (Table 3; Fig. 1). The percentage of larval settlement and burrowing was significantly negatively correlated with the Chl *a* concentration and the colloidal EPS fraction of the different BDFs ($r^2 = 0.68$ and $r^2 = 0.52$; respectively). No significant relationships were found between the average burrowing time per treatment and BDF characteristics.

Table 1 Chl *a* and EPS concentration (±SE) of the benthic diatom film (BDF) and control sediments in all experiments

	Chl *a* (μg g^{-1} dry sediment)	EPS (g glucose g^{-1} dry sediment)
Control sediment	$0.01 \pm 2.0 \times 10^{-4}$	$1.1 \times 10^{-4} \pm 7.9 \times 10^{-6}$
Experiment 1		
4-day BDF	3.13 ± 0.81	$1.6 \times 10^{-4} \pm 5.5 \times 10^{-5}$
11-day BDF	8.46 ± 0.59	$1.8 \times 10^{-4} \pm 5.9 \times 10^{-5}$
21-day BDF	15.35 ± 3.6	$2.3 \times 10^{-4} \pm 7.8 \times 10^{-5}$
Experiment 2 and 3		
11-day BDF	7.04 ± 1.17	$1.7 \times 10^{-4} \pm 2.9\ 10^{-7}$

Determination of BDF characteristics is based on the upper 7 mm (experiment 1) and the upper 5 mm sediment layer (experiment 2 and 3)

Settlement choice (Experiment 2)

Mean recovery rate of live and dead larvae was 98% in both flow velocities, indicating that loss of larvae due to stickiness to the walls and paddles was marginal. On average, 5.8 ± 1.5 SE% of the live larvae and 6.0 ± 1.5 SE% of the dead larvae were recovered in the control and BDF sediments at 5 cm s^{-1}. At 15 cm s^{-1}, the total percentages of settlement in control and BDF sediments were 4.6 ± 1.5 and 4.4 ± 1.4 SE%, respectively, for live larvae and dead larvae. Significantly more live larvae settled in BDF than in control sediments at 5 cm s^{-1} ($G_p = 36.6$, $P < 0.001$) and 15 cm s^{-1} ($G_p = 59.2$, $P < 0.001$), and the distribution of live larvae did not differ between the two flow velocities ($G_p = 2.9$, $P = 0.087$) (Fig. 2). Consequently, H_{02} is rejected, while H_{02a} cannot be rejected, i.e., settlement was influenced by BDF, but the settlement preference for BDF was independent of flow velocity.

The distribution of PS mimics did not differ significantly from an even distribution at both flow velocities ($G_p = 6.8$, $P = 0.009$; $G_p = 0.3$, $P = 0.56$, respectively, for 5and 15 cm s^{-1}). Consistently, the distribution of live larvae significantly differed from the passive deposition of PS mimics at both flow velocities ($G_p = 71.3$, $P < 0.001$; $G_p = 72.0$, $P < 0.001$, respectively, for 5 and 15 cm s^{-1}) (Fig. 2). Hence, H_{02b} is rejected, i.e., habitat selection for BDF is not a passive, depositional process. However, deposition of dead larvae was significantly higher in BDF at 5 cm s^{-1} (66%; $G_p = 20.8$, $P < 0.001$), whereas the distribution of dead

Table 2 Experiment 1. Mixed model ANOVA results for the effect of treatment, batch, and time on percentage of larval settlement and burrowing

	SS	*df*	MS	*F*	*P*	*P* (G–G)
Batch	0.13432	1	0.13432	0.2012	0.669487	
Treatment (Batch)	4.02861	6	0.67144	96.7442	**<0.001**	
Time	4.01163	4	1.00291	138.3899	**<0.001**	**<0.001**
Batch*time	0.02899	4	0.00725	1.0377	0.407856	0.99304
Treatment (Batch)*time	0.16657	24	0.00694	0.4967	0.977122	0.91774
Residual	2.51526	180	0.01397			

Values in bold are significant ($p < 0.05$)

Adjusted *P* levels are calculated for time effects based on Greenhouse-Geiser (GG) correction

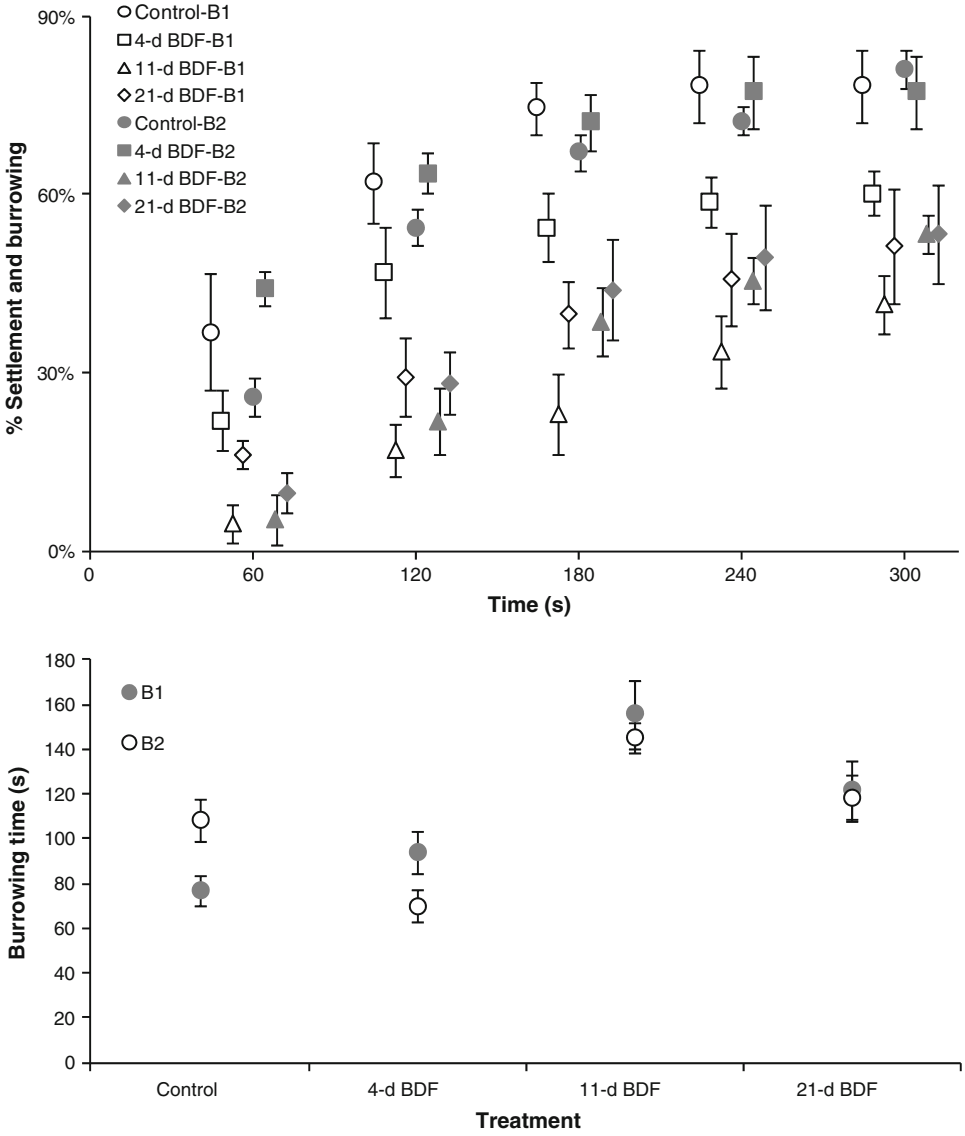

Fig. 1 Experiment 1. Settlement and burrowing of *Macoma balthica* larvae in still water in response to different benthic diatom film treatments (BDF, see Table 1), expressed as percentage of larval settlement and burrowing (±SE) in relation to time after addition of larvae (*upper panel*) and larval burrowing time (±SE) (*lower panel*). Data plotted are means of six replicates per batch (B1 = Batch 1, B2 = Batch 2)

Table 3 Experiment 1. Two-factor ANOVA results for effect of treatment and batch on burrowing time

	SS	*df*	MS	*F*	*P*
Batch	0.33	1	0.33	0.04	0.850811
Treatment	544.40	3	181.47	19.35	**<0.001**
Residual	3282.39	350	9.38		

Values in bold are significant ($p < 0.05$)

larvae did not differ significantly from an even distribution at 15 cm s^{-1} ($G_p = 1.0$, $P = 0.32$) (Fig. 2). Hence, based on comparison between distribution of dead and live larvae, H_{02b} could only be rejected at a flow velocity of 15 cm s^{-1}.

Settlement response after primary settlement (Experiment 3)

Analysis of the supernatant showed a larval addition efficiency of 100% in both control and BDF sediments.

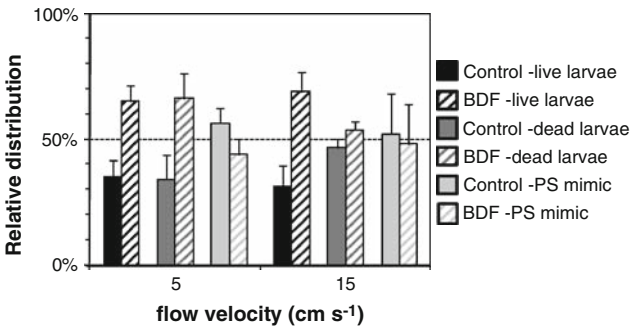

Fig. 2 Experiment 2. Settlement choice of *Macoma balthica* larvae in a multiple choice flume experiment, expressed as relative distribution (±SE) of recruited live larvae, dead larvae, and PS mimics in control sediments, and 11-day benthic diatom film (BDF) sediments after 3 h of flow at 5 and 15 cm s^{-1}

Retention rates of larvae were significantly higher in BDF (58%) as compared to controls (40%) (Fig. 3; Table 4). Despite the lower larval retention rate in BDF in Trial B of

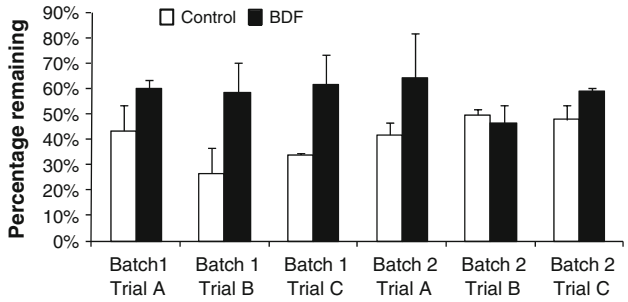

Fig. 3 Experiment 3. Secondary dispersal of *Macoma balthica* post-larvae, expressed as remaining percentage (±SE) of primary settled larvae in 11-day benthic diatom film (BDF) (*black bars*) and control sediments (*white bars*) after 10 min of flow at 5 cm s^{-1}

Table 4 Experiment 3. Mixed model ANOVA results for effect of treatment and trial on percentage of remaining post-larvae remaining in sediment treatments

	SS	*df*	MS	*F*	*P*
Treatment	0.034331	1	0.034331	10.799	**0.021805**
Batch	0.002726	1	0.002726	2.066	0.224013
Trial (batch)	0.005279	4	0.001320	0.415	0.792597
Residual	0.015895	5	0.003179		

Values in bold are significant ($p < 0.05$)

Batch 2, neither a batch nor a trial effect was found indicating that, overall, the magnitude of response did not significantly vary among replicates. Consequently, H_{03} was rejected, i.e., secondary dispersal after primary settlement was influenced by BDF.

Discussion and conclusions

In this study we investigated the role of multi-species benthic diatom films on the settlement of *M. balthica* larvae. Successful settlement is a crucial element in the recruitment of invertebrate larvae and thus in determining macrobenthic community structure. Settlement of invertebrate larvae is known to be mediated by marine biofilms, and facilitative and inhibitive effects have been demonstrated (Pawlik 1992; Wieczorek et al. 1995; Qian et al. 2007). Our results show that the settlement of *M. balthica* larvae was influenced by benthic diatoms and the outcomes of the different experiments suggest the underlying mechanisms.

In still water, the settlement and burrowing response was higher and the average burrowing time was faster in controls and younger BDF than in older BDF. All larvae settled and initiated burrowing within a minute after their inoculation, regardless of treatment; thus, no larval "rejection behavior" to any particular treatment, which would have indicated a diatom community-specific inhibitive settlement cue, was observed. Moreover, no significant differences were found between controls and 4-day BDF, either for percentage of settlement and burrowing, or for burrowing time. Complete settlement in 11-day BDF sediments in still water after 30 min, preceding addition to the flume in Experiment 3, and the negative relationship between the percentage of larval settlement and the Chl *a* and colloidal EPS concentration suggest that a physically mediated process is responsible for the differences in the settlement and burrowing response. We presume that a higher resistance during penetration into an older BDF with a higher diatom biomass and more associated sugar and protein compounds resulted in a slower penetration through the BDF and thus lower settlement and burrowing in the first few minutes. Hence, in the very short term, settling *M. balthica* larvae may be considered more susceptible to epi-and hyper-benthic predation (Hiddink et al. 2002a, 2002b, 2002c) in BDF with a higher diatom biomass and productivity. However, in the medium and longer term, a beneficial effect may be expected in such BDF, due to the better growing conditions as a result of a higher food supply and lower post-settlement resuspension (de Boer 1981; Tolhurst et al. 1999; Montserrat et al. 2008).

Settlement in a hydrodynamic environment is controlled by physical (e.g., larval fall velocity, near-bottom flow, and turbulence) and behavioral (e.g., cue detection and swimming capacities) processes (see Butman 1987 for review). The percentage of larval settlement into both BDF and control sediments was consistently lower in the flume experiments (4.6–5.8% in 3 h) compared to the still-water assays (100% within 1 min), which corroborates the observations of several authors that the highest settlement rates in shallow environments occur in very low flow conditions, e.g., around slack tides (Whitlatch et al. 2001; Tankersley et al. 2002), because (1) active selection processes may be limited and (2) fewer larvae are delivered to the bed at higher flow velocities (Butman 1987; Gross et al. 1992). Despite the overall low settlement in the flume experiments, a clear settlement preference of *M. balthica* larvae for BDF was still observed; i.e., significantly more larvae settled in BDF compared to control sediments. This distribution was not significantly different between flow velocities of 5 and 15 cm s^{-1}, but the underlying mechanism of habitat selection seemed to differ between the two flow velocities. At 15 cm s^{-1}, significantly higher proportions of larvae settled in BDF, whereas PS mimics and dead larvae both displayed an even distribution (i.e., no preference). However, like the live larvae, significantly more dead larvae were recorded from the BDF at 5 cm s^{-1}, suggesting passive deposition and adhesion of larvae to the BDF as a settlement mechanism at this flow velocity. The difference between inert, spherical PS mimics and non-spherical dead larvae suggests that flow-dependent adhesion to the biofilm was an

important settlement mechanism at the lower flow velocity. Adhesion to biofilms is a complex process that remains poorly understood, but biochemical (e.g., production of viscoelastic substances, wettability of the surface), behavioral, or physical (e.g., surface energy of the substratum) mechanisms may all be involved (Zardus et al. 2008). At higher flow velocities, substratum shear stress may be too high, inhibiting passive adhesion of dead larvae to the biofilm. The enhanced settlement of *M. balthica* larvae in BDF at 15 cm s^{-1} thus resulted from active selection. Furthermore, the results obtained from Experiment 3 highlight the importance of active post-settlement dispersal of *M. balthica* in final habitat selection. Hence, in addition to passive adhesion to the biofilm, active post-settlement behavior (i.e., rejection of the initial settlement site) is an important mechanism at low flow velocities. Whenever no suitable settlement site is encountered, *M. balthica* post-larvae can actively re-enter the water column after initial settlement by migrating to the sediment surface and secreting a byssus thread, which allows resuspension into the currents (i.e., byssus drifting, Beukema and Devlas 1989).

Higher recruitment success into BDF with a higher diatom biomass has been observed in the field for *M. balthica* (Van Colen et al. 2008) and for benthic invertebrates in general (e.g., Keough and Raimondi 1995). Wherever *M. balthica* occurs, primary settlement of larvae occurs predominantly on high, shallow, tidal flats, and offshore secondary dispersal occurs from late summer on toward the lower, less shallow, tidal flats (Reading 1979; Martini and Morrison 1987; Beukema and Devlas 1989; Van der Meer et al. 2003). Beukema and Devlas (1989) and Hiddink (2003) attribute this preference for primary settlement on high tidal flats to the lower predation pressure by epifaunal organisms and the lower disturbance by wave action at these sites. Furthermore, as a result of lower sediment resuspension, more stable, productive biofilms tend to develop on the more sheltered, upshore tidal flats (de Jong and de Jonge 1995). Taking our results into account, enhanced primary settlement of *M. balthica* larvae on the upper tidal flats may, in addition to the above-mentioned theories, also result from active habitat selection for biofilms, and the passive stickiness of the biofilm. Yet, the nature of the diatom-derived cues that influence active *M. balthica* larval settlement for BDF remain unknown. Such settlement cues have extensively been studied in relation to bacterial products in the biofilm (e.g., Bao et al. 2007), whereas the specific cues from diatoms have been investigated to a much lesser extent. Based on the manipulation of the different components of biofilms, Lam et al. (2003) reported that the settlement of the serpulid polychaete *H. elegans* was induced by the presence of capsular surface EPS, produced by specific diatoms. Such diatom-derived sugar compounds have also been identified as settlement and metamorphosis cues for barnacles, limpets, and bryozoans (Dahms et al. 2004; Patil and Anil 2005; Jouuchi et al. 2007). Further experiments, in which the chemical products derived from the different diatom communities (e.g., EPS) are manipulated, are needed to elucidate the specific diatom-derived cues for settlement of *M. balthica* larvae.

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