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Induction of larval settlement in the polychaete *Hydroides elegans* by extracellular polymers of benthic diatoms

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ABSTRACT: Larval settlement of the polychaete *Hydroides elegans* Haswell, 1883 is mediated by marine biofilms; complex agglomerates of bacteria, diatoms, fungi and protozoa enmeshed in a matrix of extracellular polymers (EPS). In our previous investigations, benthic diatoms were demonstrated to be potent mediators of larval settlement in *H. elegans*. The putative diatom-derived settlement cues were heat-stable components in close association with the diatom surface. For an in-depth investigation of the chemical nature of diatom-derived larval settlement cues, the EPS of the inductive (*Achnanthes* sp., *Nitzschia constricta*) and non-inductive (*Amphora tenerrima*, *Nitzschia frustulum*) diatoms were bioassayed for their effect on larval settlement. When EPS fractions larger than 100 kDa were immobilized in stable hydrogels to mimic their association to a solid substratum, they evoked an effect on larval settlement similar to that of the respective monospecies diatom films. The crude exopolymer samples mainly consisted of polysaccharides with a small proportion of proteinaceous sample components. After enzymatic removal of proteinaceous EPS components, the samples evoked an effect on larval settlement similar to that of the crude EPS samples, indicating the negligible role of large proteins or glycoproteins as settlement cues.

KEY WORDS: Diatoms · Larvae · Settlement · Biofilm · Exopolymers · *Hydroides elegans*

INTRODUCTION

Marine biofilms (MBF) are considered to be an important source of biochemical signals mediating surface exploration and substratum choice for larval settlement (i.e. attachment and metamorphosis) in many marine invertebrates (Pearce & Scheibling 1991, Slattery 1992, Tamburri et al. 1992, Keough & Raimondi 1996, Chan & Walker 1998). MBF mainly consist of attached bacteria, benthic diatoms, fungi and protozoa, all of which are enmeshed in a matrix of extracellular polymers (EPS) (Mihm et al. 1981, Wahl 1989, Holmström & Kjelleberg 1994).

Biofilms have been intensively studied with respect to their stimulatory and inhibitory roles on larval attachment and metamorphosis of polychaetes (Lau & Qian 2001) and barnacles (Wieczorek et al. 1995, Maki et al. 1998, Olivier et al. 2000). In this study, we extended previous investigations on larval settlement cues of the polychaete *Hydroides elegans* (Haswell). Biofilms effectively induce larval settlement of this polychaete (Hadfield et al. 1994, Beckmann et al. 1999), and the inductive effect is due to a small portion of bacteria and diatoms within the complex community of bacteria in biofilms (Lau & Qian 1997, Unabia & Hadfield 1999, Harder et al. 2002a, Lau et al. 2002). On a microscale, the initial contact of competent larvae with a marine biofilm can be regarded as a close encounter with a conglomerate of exopolymers from bacteria and diatoms (Lam et al. 2003, Lau et al. 2003). The sequential events of larval attachment and metamorphosis can be distinguished in chronological order

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as follows: larvae contact the film surface with increasing frequency, slow down and secrete sticky mucous threads, which eventually limit larval locomotion. Subsequently, larvae attach to the surface, secrete a primary tube and metamorphose into juveniles. The overall duration of attachment and metamorphosis is roughly 12 h (Carpizo-Ituarte & Hadfield 1998).

Differential larval settlement patterns on biofilm-covered surfaces of diverse origin and/or growth under different environmental and physiological conditions indicate a finely tuned and highly specific larval response towards biofilm-derived cues (Keough & Raimondi 1996, Qian et al. 2003). While bacteria-derived cues with an inductive effect on larval settlement have been separated in a number of cases (Zobell & Allen 1935, Hofmann et al. 1996, Maki 1999), the bioactive bacterial metabolites have rarely been purified and identified so far (Harder et al. 2002b). However, exopolymeric bacterial products have long been demonstrated to play an important mediatory role in larval settlement of marine invertebrates, e.g. the ascidian *Ciona intestinalis* (Szewzyk et al. 1991), the barnacle *Balanus amphitrite* (Maki et al. 1990, 1994, 1998, 2000), the polychaete *Hydroides elegans* (Lau et al. 2003) and the brittlestar *Amphipholis gracillima* (Hoskins et al. 2003).

In addition to bacteria, benthic diatoms are a dominant group of microorganisms in MBF, particularly in the photic zone. In terms of the surface coverage by individual biofilm components, benthic diatoms possibly occupy considerably larger surface areas than bacteria, due to their comparatively large cell sizes. We, therefore, hypothesized the mediatory role of diatoms on larval settlement to be potentially significant. Previously, we documented that various monospecies benthic diatoms were potent mediators of larval settlement in the polychaete *Hydroides elegans* (Haswell) (Harder et al. 2002a). In a follow-up study, the inductive effect of diatom films on larval settlement prevailed even after heat treatments of 121°C for 1 h, demonstrating that the putative diatom-derived settlement cues were heat-stable surface components in close association with the cell surface (Lam et al. 2003).

Based on this body of knowledge, we investigated the induction of the larval settlement of *Hydroides elegans* by marine benthic diatoms with particular emphasis on the role of extracellular polymers (EPS) as diatom-derived settlement cues. The primary objective of this study was to separate diatom EPS from 4 model diatom species (2 inductive and 2 non-inductive on larval settlement of *H. elegans*) and to investigate the effect of crude diatom EPS on larval settlement in laboratory-based still-water settlement assays. For this purpose, we adopted an embedding technique for organic compounds in stable hydrogels (Henrikson & Pawlik 1995).

MATERIALS AND METHODS

Larval culture procedure. From October 2002 to March 2003, PVC settling panels (100 cm^2) were submerged at 2 m depth at a fish farm in Yung Shue O, Hong Kong (22° 19′ N, 114° 16′ W). After this period, the panels were covered with a thick meshwork of tubes produced by the calcareous tubeworm *Hydroides elegans* around individual bodies of the solitary ascidian *Styela plicata*. In the laboratory, adult *H. elegans* were dislodged from the panels and induced to spawn by gently tapping the posterior end of the tube with a dissecting needle. The fertilized gametes of several males and females were filtered through 70 µm nylon mesh, rinsed with 0.45 µm-filtered seawater (FSW) and transferred into 1 l glass beakers containing FSW. Larval cultures were incubated at 24°C under a 15:9 h light:dark photo cycle and maintained according to Harder et al. (2002b). Larval competence was determined according to the larval morphology (Wisely 1958) and by a rapid bioassay in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Bryan et al. 1997). Batches of competent larvae were sieved through 110 µm nylon mesh. The retained larvae of a given batch were transferred into FSW and used for bioassays within 1 h. Replicates were performed with larvae from the same batch, whilst repeated experiments utilized single larval batches from different dates.

Diatom cultures and development of monospecies diatom films. In analogy to our previous study, 2 inductive (I) and 2 non-inductive (NI) diatoms were chosen as model strains. The stock cultures of *Achnanthes* sp. (I), *Nitzschia constricta* (I), *Amphora tenerrima* (NI) and *Nitzschia frustulum* (NI) were individually inoculated into 2 l Erlenmeyer flasks containing autoclaved *f*/2 nutrient medium in FSW (Guillard & Ryther 1962). Pure or monospecies diatom cultures were incubated for 2 to 4 wk at 24°C with overhead fluorescent illumination (0.3×10^{16} quanta s^{-1} cm^{-1}) under a 15:9 h light:dark photo cycle. After the development of visible diatom films in the culture flasks, the diatoms were dissociated with sterile brushes. Aliquots of 100 ml of the non-axenic diatom suspensions were filtered through 1 µm membranes (Osmonics), and in the case of *N. constricta* through 0.45 µm. The filter residues were washed with autoclaved FSW to reduce the number of non-attached bacteria. Subsequently, retained diatoms were resuspended in 250 ml FSW. The diatom-free bacterial filtrate was reduced to the original volume on a 0.22 µm filter membrane. The bacteria-containing diatom suspensions and the corresponding diatom-free bacterial samples were used to prepare diatom films and diatom-free bacterial films. Briefly, 5 ml aliquots of the suspensions were transferred into sterile Petri dishes with

replication (n = 8) and incubated for 24 h cell attachment and film development. Subsequently, filmed dishes were dip-rinsed in autoclaved FSW to remove loose cells. Prior to the larval settlement bioassay, 5 ml of autoclaved FSW were added to the dishes.

Development of marine biofilms. Sterile polystyrene Petri dishes (ID = 5 cm; FALCON #1006, Becton Dickinson) without lids were enmeshed in 125 µm nylon bags and anchored at 1.5 m depth at the sampling (see above) site for 5 d. The dishes were retrieved and immediately immersed in FSW to avoid desiccation. In the laboratory, biofilmed dishes were rinsed in autoclaved FSW and used as positive controls in larval settlement bioassays.

Isolation and purification of extracellular polymers (EPS). Prior to the EPS extraction, diatom suspensions were concentrated to 250 ml and fixed in 5% formalin in seawater. The isolation of diatom EPS was performed according to Bhosle et al. (1995, 1996). After rinsing the cells with 0.22 µm FSW, diatom EPS were dissociated from cells by light sheer force (150 rpm; Thermolyne shaker) in the presence of a chelating buffer (1 M NaCl, 0.05 M EDTA) at 24°C for 24 h. After the dissociation of EPS, the cells were spun down (1000 × *g*) for 5 min at 4°C. The supernatant was sterile-filtered through 0.22 µm nylon membranes (Millipore). Diatom EPS were concentrated in a stirred ultrafiltration cell (YM-10 kDa, Millipore), rinsed with distilled water and subsequently freeze-dried. The freeze-dried weight of crude diatom EPS and diatom cells (filter residue) was determined at the end of the experiment.

Size fractionation and gel-fractionation of crude diatom EPS. Crude diatom EPS obtained from the diatom species under investigation were separated into 3 size fractions (>100, 30 to 100, 10 to 30 kDa) by ultrafiltration (YM-100 kDa, YM-30 kDa, YM-10 kDa; Millipore). The filter residues from each fractionation step were freeze-dried, weighed and used for bioassays or chemical analyses.

Gel-immobilization. The immobilization of diatom EPS in hydrogels was adopted from Henrikson & Pawlik 1995. Hydrogels were prepared by addition of 0.4 g Phytagel™ (Sigma) into 20 ml of distilled water. The gel mixture was boiled and then cooled to 60°C prior to the addition of dry EPS aliquots of different molecular sizes to a final concentration of 50 mg diatom EPS per ml of distilled water. The mixture polymerized in a 50 ml Corning tube (∅ = 3 cm, CORNING, #2070). The resulting gel cylinders were sliced into 5 discs (2 mm thick) for bioassay purposes. Controls were prepared similarly by the addition of an aliquot of distilled water instead of diatom EPS into the gel.

Larval settlement bioassays. Larval settlement assays were performed with replication (n = 5). Twenty competent larvae were transferred in the smallest volume possible (ca. 200 µl) from a larval batch into Petri dishes containing experimental phytagel discs (Falcon #1006) and incubated for 5 h at 24°C under a 15:9 h light:dark photo cycle. Phytagel discs without EPS served as controls. Although the irreversible attachment of larvae could be clearly determined after 5 h under the dissecting microscope, an additional observation was made after 24 h to verify normal larval metamorphosis on the phytagel surface.

Bioassay-guided size-exclusion chromatography of diatom EPS. Diatom EPS obtained from each diatom species under investigation were concentrated 10 times by ultrafiltration (10 kDa) and fractionated by aqueous gel-filtration chromatography on Sephacryl S-400 HR (Pharmacia; 300 × 25 mm) with isochratic elution of water at 0.35 ml min^{-1} (Millipore) and dual wavelength detection at 201 and 254 nm. The different size fractions obtained were reconstituted at original concentrations for larval bioassays or were freeze-dried for subsequent chemical analyses.

Quantitative analyses of diatom EPS. The amount of total polysaccharides in diatom EPS was determined spectrophotometrically (595 nm) after phenol-sulphuric acid treatment according to Dubois et al. (1956), utilizing glucose as the standard. The protein contents of individual diatom EPS samples were determined according to Bradford (Berges et al. 1993) with bovine serum albumin as a standard. All polysaccharide and protein concentrations were expressed as weight per cell by dividing the values from standard curves by the total number of diatom cells in 2 l Erlenmeyer flasks.

Glycosyl composition analysis. The glycosyl composition of freeze-dried, biologically active fractions was analyzed at the Complex Carbohydrate Research Center, University of Georgia, USA according to Hoskins et al. (2003). The samples were analyzed by per-O-trimethylsilyl (TMS) derivatization of monosaccharides. Briefly, methyl glycosides were prepared by acidic methanolysis of the sample in 1 M methanolic HCl at 80°C for 18 to 22 h, followed by re-N-acetylation with methanolic pyridine and acetic anhydride in methanol. The released sugars were per-O-trimethylsilylated by treatment with Tri-Sil (Pierce Biotechnology) for 30 min at 80°C, and TMS-methyl glycosides were analyzed on a Hewlett Packard 5890 gas chromatograph interfaced to a 5970 mass selective detector in electron impact ionization mode. Separation was performed on a bonded-phase fused silica capillary column (Supelco DB5, 30 m × 0.25 mm). Prior to derivatization, 20 µg of inositol (Sigma) was added to both samples as an internal standard.

Bioassays with gel-immobilized monosaccharides. Three commercially available monosaccharides (i.e. glucose, galactose and mannose; Sigma) were immobi-

lized in hydrogels and bioassayed as above. The total amounts of monosaccharides embedded in phytagel were calculated by multiplying the mole percentages of the respective carbohydrate monomers obtained by glycosyl composition analysis of biologically active fractions with the total mass of analyzed diatom EPS (see Table 2). Controls were prepared by adding an aliquot of distilled water instead of monosaccharides into the gel.

Enzyme treatments of diatom EPS. The proteolytic enzymes trypsin and papain (Sigma) were used to digest the crude EPS obtained from *Achnanthes* sp. Crude diatom EPS were incubated at 37°C for 30 min at a working concentration of 0.01 trypsin and 0.2 mg ml^{-1} papain. Filtered seawater was treated accordingly and served as a control. The digestion products were separated from enzymes by membrane filtration through 30 kDa. The filter residue was further partitioned into fractions of >100 and <100 kDa and immobilized prior to the bioassay as described above.

Statistical analysis. Larval settlement bioassays were repeated 3 times. The software packages STATISTICA (StatSoft) and SPSS (SPSS) were used for statistical analyses. The percentage of larval settlement was arcsine-transformed before analysis. To improve the transformation results, a value of $(4n)^{-1}$ (n = number of larvae used in each replicate) was given to the replicates with zero larval settlement (Zar 1996). The normality of the data was checked with Shapiro-Wilks test (Shapiro & Wilk 1965). All data were treated non-parametrically due to non-homogeneity. This was achieved by transforming values to ranks and then applying 1-way ANOVA followed by Tukey's multiple comparison test (Conover & Iman 1981). The data presented in the figures are not transformed.

RESULTS

Bioassays with monospecies diatom films

The percentages of larval settlement were significantly higher on films of *Achnanthes* sp. and *Nitzschia constricta* than in the FSW-control (Tukey's test, $p < 0.05$; Fig. 1A). Larval settlement on films of *Amphora tenerrima* and *N. frustulum* was the same as in FSW (Tukey's test, $p = 1.00$; Fig. 1A). The abundance of diatoms in experimental films and 5 d old biofilms ranged from 2000 to 6000 cells mm^{-2}. This value was nearly consistent throughout the bioassays with different diatoms (Fig. 1B). The abundance of bacteria in diatom-free bacterial films ranged from 1340 ± 450 (SD) to 7600 ± 900 cells mm^{-2} (Fig. 1C). Although the bacterial abundance in non-axenic diatom films was the same as in diatom-free bacterial films (Tukey's test, $p = 0.84$; Fig. 1C), larval settlement on non-axenic diatom films was significantly higher than that on diatom-free bacterial films (Tukey's test, $p < 0.05$).

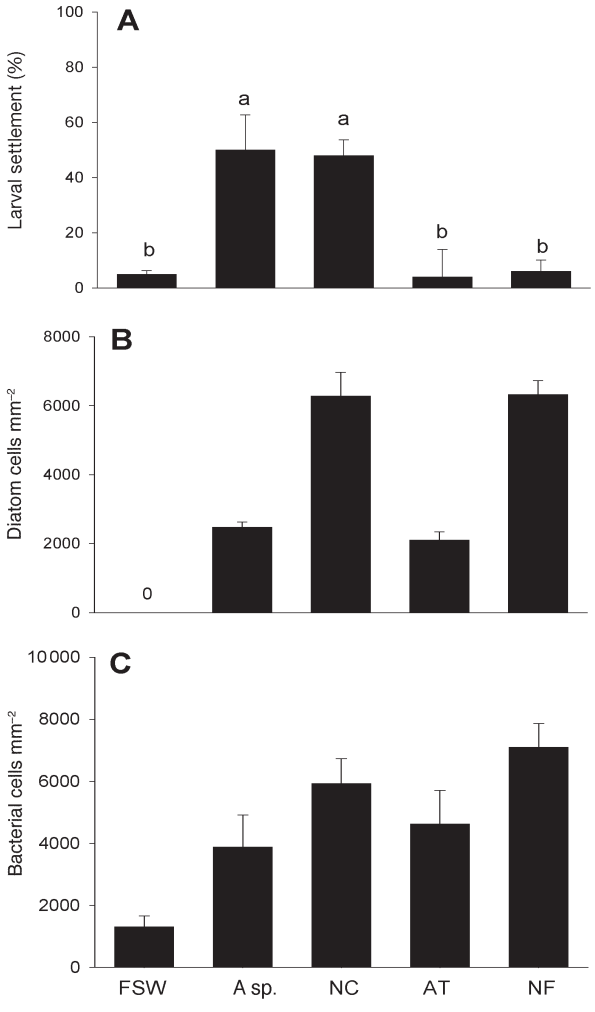

Fig. 1. *Hydroides elegans*. (A) Mean percentage of larval settlement after 24 h in response to diatom films of the inductive diatoms *Achnanthes* sp. (A sp.), *Nitzschia constricta* (NC) and the non-inductive diatoms *Amphora tenerrima* (AT), *N. frustulum* (NF) and a control of filtered seawater (FSW). Data that differ significantly (Tukey's test, $\alpha = 0.05$) are indicated by different letters. Data are means + SD of 5 replicates. (B) Mean density of diatoms. Data are means + SD of 3 replicates based on counts of 5 fields of view at 100× magnification. (C) Mean density of bacteria in diatom films. Data are means + SD of 3 replicates based on counts of 5 fields at 1250× magnification

Bioassays of gel-immobilized diatom EPS

In comparison to the clean dish and the pure phytagel control (Tukey's test, $p < 0.05$; Fig. 2), the

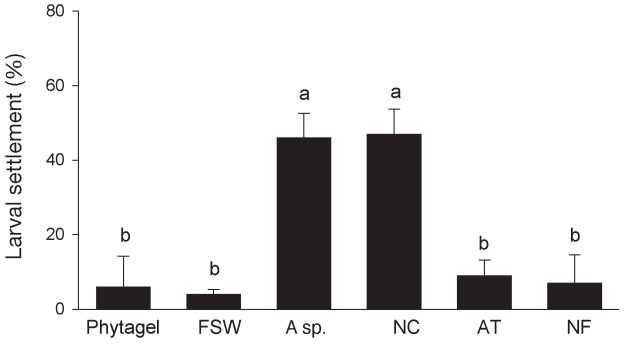

Fig. 2. *Hydroides elegans*. Mean percentage of larval settlement after 5 h in response to EPS of the inductive diatoms *Achnanthes* sp. (A sp.), *Nitzschia constricta* (NC) and the non-inductive diatoms *Amphora tenerrima* (AT), *N. frustulum* (NF) immobilized in phytagel. Phytagel without diatom EPS served as a negative control (Phytagel). Another control of filtered seawater only is indicated by FSW. Data with significant differences are indicated by different letters (Tukey's test, α = 0.05). Data plotted are means + SD of 5 replicates

gel-immobilized EPS of the inductive diatoms (*Achnanthes* sp. and *Nitzschia constricta*) evoked significantly higher percentages of larval settlement than the corresponding treatments prepared from the non-inductive diatoms *Amphora tenerrima* and *N. frustulum* (Tukey's test, p = 0.01; Fig. 2). In the inductive treatments, larvae that irreversibly attached on the gel after 5 h metamorphosed normally as indicated by the development of calcareous tubes after 24 h. In the case of different EPS size fractions originating from the inductive diatoms *Achnanthes* sp. and *N. constricta*, the fraction of >100 kDa evoked a statistically higher settlement response than the fractions of lower molecular weight (Tukey's test, p = 0.02; Fig. 3A). The percentages of larval settlement in all size fractions of *A. tenerrima*- and *N. frustulum*-treatments did not differ from that in the controls (clean dish and pure phytagel) (Tukey's test, p = 0.99; Fig. 3B).

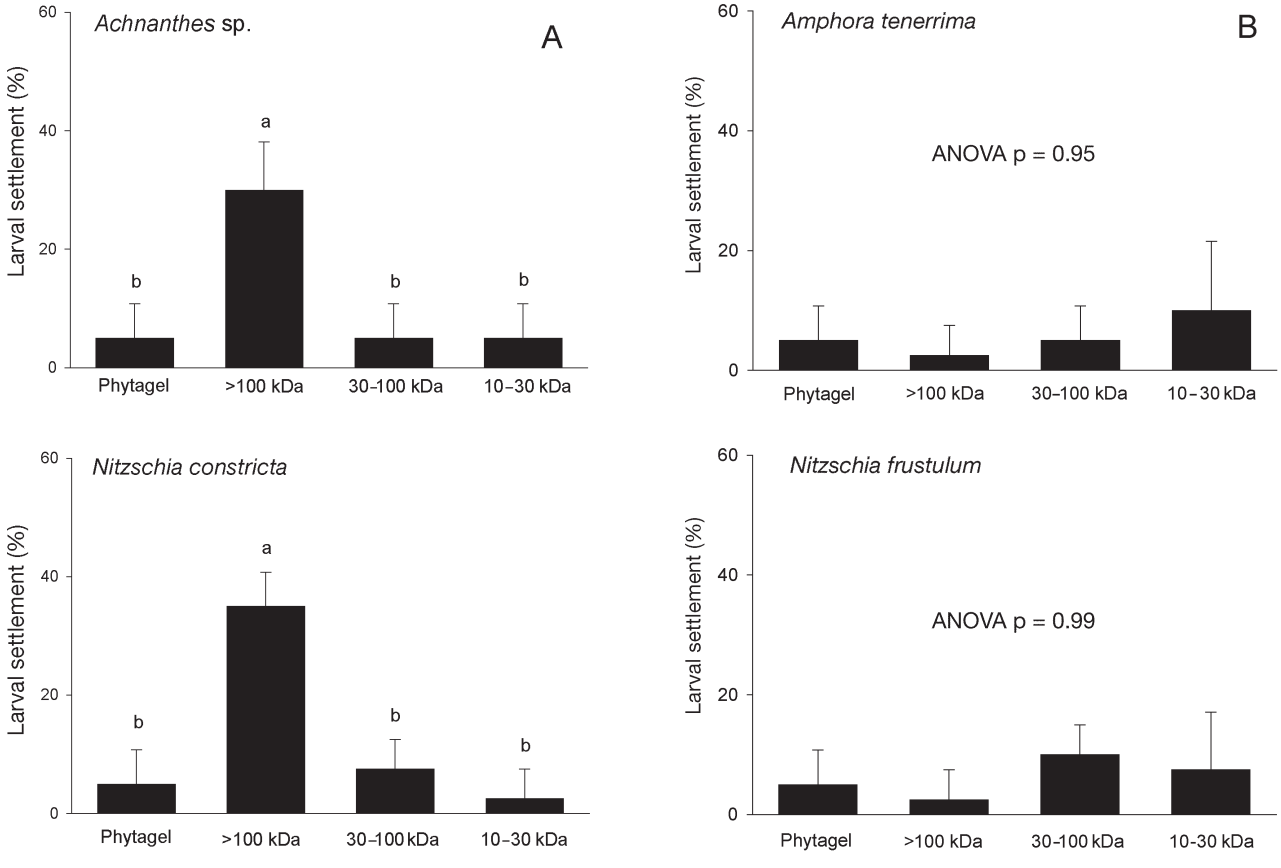

Fig. 3. *Hydroides elegans*. Mean percentage of larval settlement after 5 h in response to phytagel-immobilized EPS fractions of different molecular weight (in kiloDalton) obtained from (A) the inductive diatoms *Achnanthes* sp. and *Nitzschia constricta* and (B) the non-inductive diatoms *Amphora tenerrima* and *N. frustulum*; a phytagel control (Phytagel) is shown. Data with significant differences are indicated by different letters (Tukey's test, α = 0.05). Data plotted are means + SD of 5 replicates

Bioassays of purified gel-immobilized EPS fractions

The >100 kDa EPS fractions obtained from *Achnanthes* sp. (A sp.) and *Nitzschia constricta* (NC) were divided into 3 and 4 fractions in the retention windows bracketed by 40 and 200 min (Fig. 4). The percentage of larval settlement in response to Fraction 1 obtained from the separation of A sp. and NC was significantly higher than in the control (Tukey's test, $p = 0.023$). In both separations, the biologically active sample components eluted immediately after the column void volume.

Quantitative analyses of diatom EPS

The amount of carbohydrates in crude diatom EPS was higher than that of proteins. The protein and carbohydrate concentrations among the different diatom EPS crude ranged from 0.0204×10^{-4} to 0.0850×10^{-4} and 4.08×10^{-4} to 5.10×10^{-4} pg cell^{-1}, respectively (Table 1). The detection limits for proteins and polysaccharides of these methods were 0.0095 and 0.0874×10^{-4} pg cell^{-1}, respectively.

Glycosyl composition analysis

The bioactive diatom EPS fractions obtained by size exclusion chromatography (Fig. 4) consisted of different mole-percentages of total carbohydrates, i.e. 1.6% in *Achnanthes* sp. and 50.2% in *Nitzschia constricta*. Whilst the bioactive EPS fraction of *Achnanthes* sp. consisted only of glucose, galactose and mannose, the carbohydrate monomer profile in EPS fractions obtained from *N. constricta* was more complex and consisted of rhamnose, fucose, xylose, glucuronic acid, galactouronic acid and N-acetyl glucosamine (Table 2).

Bioassays of gel-immobilized monosaccharides

Although the concentrations of gel-immobilized monosaccharides were the same as those in biologically active SEC fractions of inductive diatom EPS, the percentage of larval settlement was comparatively low ranging from 5 ± 5 to 13 ± 4.5%. The percentages of

Table 1. *Hydroides elegans*. Total concentrations of polysaccharides (polysacch.) and proteins in crude diatom EPS samples obtained from the inductive diatoms *Achnanthes* sp. and *Nitzschia constricta*, and the non-inductive diatoms *Amphora tenerrima* and *N. frustulum*

Diatom	Protein (pg cell^{-1})	Polysacch. (pg cell^{-1})	Protein: Polysacch.
Achnanthes sp.	0.0204×10^{-4}	4.28×10^{-4}	1:209
Nitzschia constricta	0.0216×10^{-4}	4.88×10^{-4}	1:225
Amphora tenerrima	0.0270×10^{-4}	5.10×10^{-4}	1:188
Nitzschia frustulum	0.0805×10^{-4}	4.08×10^{-4}	1:50

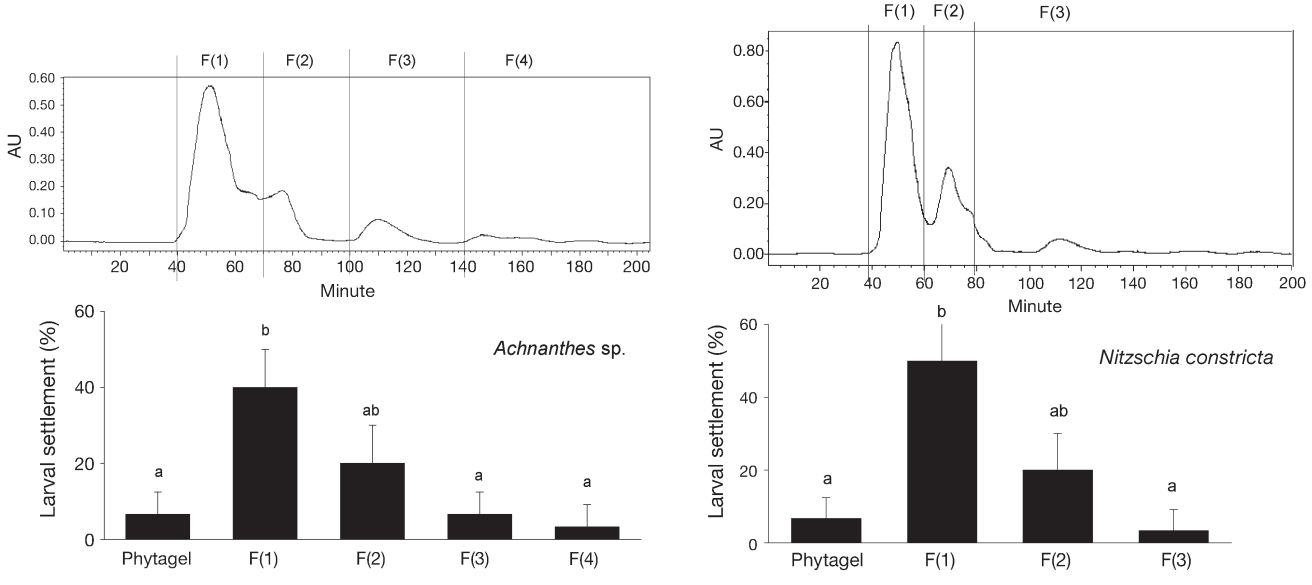

Fig. 4. *Hydroides elegans*. Bioassay-guided size fractionation of *Achnanthes* sp. and *Nitzschia constricta*, and the effect of individual, phytagel-immobilized size fractions (F) on larval settlement of the polychaete (after 5 h) in comparison to a control of pure phytagel (Phytagel). Vertical lines represent the fractionation width (min). Data with significant differences are indicated by different letters (Tukey's test, $\alpha = 0.05$). Data plotted are means + SD of 5 replicates. AU: absorption unit

Table 2. *Hydroides elegans*. Carbohydrate composition of diatom EPS from the inductive diatoms *Achnanthes* sp. and *Nitzschia constricta* as determined by glycosyl composition analysis

Monosaccharide(s)	Mole (%)	
	Achnanthes sp.	*Nitzschia constricta*
Rhamnose	–	7.0
Fucose	–	12.2
Xylose	–	12.9
Glucuronic acid	–	13.9
Mannose	9.8	10.3
Galactose	19.4	29.0
Glucose	70.8	11.2
Galactouronic acid	–	–
N-acetylglucosamine	–	3.5

larval settlement did not differ among galactose, glucose, mannose, a mixture of the 3 monosaccharides, and the phytagel control (Tukey's test, p = 1.14; Fig. 5).

Bioassays of gel-immobilized, protein-free diatom EPS

The qualitative protein analysis of the 30 kDa filter residue was negative indicating an effective elimination of proteinaceous EPS components of *Achnanthes* sp. The inductive effect of the protein-free, high-molecular weight diatom EPS (>100 kDa) on larval settlement was the same as in response to crude EPS

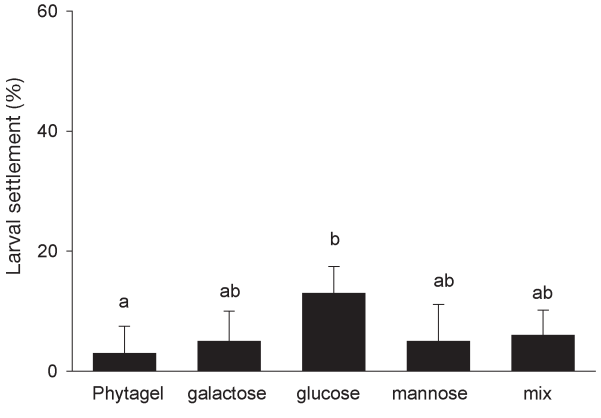

Fig. 5. *Hydroides elegans*. Mean percentage of larval settlement after 5 h in response to 3 monosaccharides (galactose, glucose, mannose) immobilized in 2% phytagel. Monosaccharide concentrations were calculated according to the abundance of biologically active fractions shown in Table 2. The mixture (mix) contained the 3 monosaccharides, and a phytagel control (Phytagel) was prepared from 2% phytagel without diatom EPS. Data with significant differences are indicated by different letters (Tukey's test, α = 0.05). Data plotted are means + SD of 5 replicates

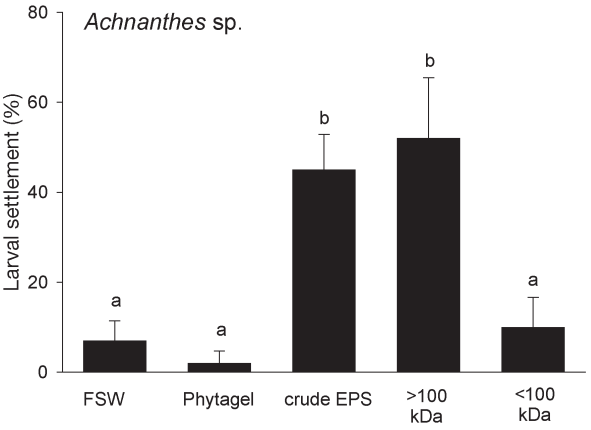

Fig. 6. *Hydroides elegans*. Mean percentage of larval settlement after 5 h in response to phytagel-immobilized, protein-free high (>100 kDa) and low (<100 kDa) molecular weight EPS fractions of *Achnanthes* sp., together with a gel-immobilized control of crude EPS of *Achnanthes* sp. The blank control of 2% phytagel is indicated as 'Phytagel', the filtered seawater control is indicated as FSW. Data with significant differences are indicated by different letters (Tukey's test, α = 0.05). Data plotted are means + SD of 5 replicates

(Fig. 6; Tukey's test, p = 0.85). The percentages of larval settlement in response to the protein-free, low-molecular weight diatom EPS (<100 kDa) was statistically the same as in the phytagel control (Fig. 6; Tukey's test, p > 0.5).

DISCUSSION

This study followed our previous investigations on the regulatory role of marine benthic diatoms on larval attachment and metamorphosis of the polychaete *Hydroides elegans* (Harder et al. 2002a). Out of 32 purified diatoms investigated, 12 were non-inductive, 14 were moderately inductive and 3 were highly inductive on larval settlement. In the case of the inductive diatoms, there was a clear density-dependent effect of diatom surface occupation on the induction of larval settlement (Lam et al. 2003). Larval settlement bioassays with choice option between filmed versus unfilmed surface revealed that the diatom-derived settlement cue was water-insoluble and associated with the diatom cell surface. The inductive activity of the diatoms *Achnanthes* sp. and *Nitzschia constricta* prevailed after heat treatments; thus, the diatom-derived settlement cues were hypothesized to be heat-stable surface components of diatoms, such as capsular EPS (Lam et al. 2003).

The objective of the present study was to investigate the effect of cell-dissociated EPS amongst a subset of previously investigated diatoms on larval settlement of *H. elegans*. The exopolymers were separated by pub-

lished procedures and subsequently immobilized in stable hydrogels in order to mimic their association to a solid substratum. The utilization of hydrogels as suitable matrices for exopolymers of high-molecular weight was based on the assumption that protruding macromolecules could be recognized by surface-exploring larvae. Since the larval settlement response on gel-immobilized diatom EPS was not only very similar compared to monospecies diatom films (Fig. 1), but also resulted in the onset of normal larval metamorphosis, we demonstrated the suitability of this methodology to investigate diatom cell-dissociated exopolymers. The crude exopolymer samples mainly consisted of polysaccharides with a small proportion of proteinaceous sample components (Table 1). Together with the observation of their high molecular weight (Fig. 2), these findings indicated that EPS may function as larval settlement cues in *H. elegans*. However, an unequivocal correlation of the presence of certain carbohydrate oligomers in diatom EPS with their bioactivity was confounded by a potentially high efficacy of small quantities of proteinaceous EPS components. This uncertainty was experimentally addressed by treatment of bioactive diatom EPS with proteolytic enzymes. Following this procedure, a bioactive filter residue of >30 kDa was obtained (Fig. 6). Under the assumption that proteins and/or glycoproteins were separated after enzymatic cleavage into subunits of less than 30 kDa, these bioassay results highlighted a potential role of extracellular polysaccharides as larval settlement cues.

The chemical compositions of diatom EPS has been the subject of previous studies (Allan et al. 1972, Bhosle et al. 1995, 1996, Hoskins et al. 2003). Diatom EPS have been described as complex mixtures of polysaccharides among other biopolymers, with glucose and mannose as the main constituents, and galactose, xylose, fucose and glucuronic acid as minor constituents. In this study, the crude diatom EPS were separated by bioassay-guided gel-filtration chromatography to purify and identify the carbohydrate composition in biologically active fractions. A clear difference in the major monomer compositions with respect to settlement induction activity of different diatom EPS was observed in these experiments. Glucose and galactose were the main EPS constituents obtained from the inductive diatom species *Achnanthes* sp. and *Nitzschia constricta* (Table 2), while galactose and rhamnose were the main constituents obtained from the non-inductive diatom species *Amphora tenerrima* and *N. frustulum*. However, due to the complex and heterogeneous nature of polysaccharides and the lack of monomer sequence information, we could not correlate the bioassay results with particular diatom EPS domains rich in glucose and/or galactose. Our experiments with gel-immobilized monosaccharides showed that *Hydroides elegans* larvae did not settle in response to any of the monosaccharides or their mixture under investigation (Fig. 5); thus, factors other than the qualitative and quantitative monosaccharide composition in diatom EPS may be causative for the observed inductive effect on larval settlement. Parameters in this regard may be the spatial orientation and/or the sequence of monomeric building blocks of polysaccharides.

Importantly, larval settlement on phytagel did not differ from that on other natural substrates; that is, larvae metamorphosed normally and developed calcareous tubes after 24 h. This observation was in contrast to Lau et al. (2003), who only observed the initial stages of larval attachment in response to gel-immobilized bacterial EPS. These results indicate a potentially different role of EPS in the induction of larval attachment and metamorphosis with respect to their origin, i.e. from benthic diatoms or bacteria. Whilst in the case of bacteria water soluble metabolites have been shown to act as inducers of larval settlement (Lau et al. 2003, Harder et al. 2002b), our experiments did not reveal any soluble diatom-dissociated settlement cues (Lam et al. 2003).

Generally, the maximum settlement response of larvae to diatoms and immobilized diatom EPS ranged between 30 to 50%, which was slightly lower compared to settlement rates of ca. 70% in the presence of MBF. This difference indicated that multiple cues, i.e. from both diatoms and bacteria, may be involved in the attachment and metamorphosis of *Hydroides elegans*.

Overall, our study not only highlights the role of diatom EPS as potent mediators of larval settlement, but also provides insight into the differences of the settlement response with respect to the composition and constitution of these macromolecular settlement cues. The ability of polychaete larvae to distinguish between these molecular features adds another level of complexity in the chemical–ecological understanding of the mediation of larval settlement by different components of marine biofilms.

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