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Growth conditions of benthic diatoms affect quality and quantity of extracellular polymeric larval settlement cues

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ABSTRACT: Extracellular polymers (EPS) produced by diatoms have been demonstrated as potent mediators of larval settlement in *Hydroides elegans*. Few studies have addressed the importance of environmental parameters (e.g. temperature and salinity) on quantity and quality of diatom EPS as larval settlement cues. The growth rates of the benthic pennate diatoms *Achnanthes* sp. and *Nitzschia constricta* reached a maximum and a minimum level at high and low temperature and salinity, respectively. A positive correlation was observed between the growth rate of diatoms and their production of EPS. In order to decouple the quantitative effect of diatom films from the potential qualitative effect of diatom EPS on larval settlement, the same amount of diatom EPS obtained from different growth conditions were immobilized in hydrogels and investigated in larval settlement assays. The diatom EPS obtained from high temperature treatments were more inductive to larval settlement than the low-temperature treatments, irrespective of the salinity setting. The qualitative differences of the diatom EPS samples under investigation were further analyzed by bioassay-guided gel-chromatography. The bioactive EPS fraction of *Achnanthes* sp. mainly consisted of large macromolecules, while the bioactive EPS fraction of *N. constricta* consisted of both large and small macromolecules. Due to their sensitivity to changes in environmental parameters, diatoms may serve as small scale proxies of substratum suitability for larval settlement of benthic marine invertebrates, analogous to the reported role of bacteria in marine biofilms.

KEY WORDS: Extracellular polymers (EPS) · Larvae settlement · Temperature · Salinity · *Hydroides elegans* · Diatoms · Biofilms

INTRODUCTION

The life cycle of a broad range of benthic marine invertebrates includes a pelagic larval stage. The dispersed larvae are destined to attach and subsequently metamorphose into sedentary juveniles once they reach competency (Crisp 1974, Chia 1978). Larval attachment and metamorphosis (hereafter referred to as settlement) is a specific process encompassing surface exploration and substratum choice guided by marine biofilms (Slattery 1992, Keough & Raimondi 1996, Wieczorek & Todd 1997, Chan & Walker 1998).

Marine biofilms (MBFs) consist of attached bacteria, benthic diatoms, fungi and protozoa, all of which are enmeshed in a matrix of extracellular polymers (Mihm et al. 1981, Charaklis & Cooksey 1983). MBFs have been intensively studied with respect to their stimulatory and inhibitory roles on larval settlement of polychaetes (Lau & Qian 2001), bryozoans (Wieczorek & Todd 1997) and barnacles (Wieczorek et al. 1995, Maki et al. 1998, Olivier et al. 2000). Differential larval settlement patterns on surfaces covered with biofilms of different origin and/or growth phase under different environmental and physiological conditions indicated a highly specific larval response towards biofilm-derived cues (Keough & Raimondi 1996, Qian et al. 2003). Although the role of marine biofilms on larval settlement has been largely attributed to bacterial components (Zobell & Allen 1935, Hofmann et al. 1996, Maki 1999), bacterial metabolites have rarely been purified so far

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(Harder et al. 2002a). Besides bacterial metabolites, extracellular polymeric bacterial products (EPS) play an important mediatory role in larval settlement of some marine invertebrates, such as the polychaete *Hydroides elegans* (Haswell) (Lau et al. 2003), the barnacle *Balanus amphitrite* (Maki et al. 1990, 1998, 2000), the ascidian *Ciona intestinalis* (Szewzyk et al. 1991) and the brittlestar *Amphipholis gracillima* (Hoskins et al. 2003).

In addition to bacteria, benthic diatoms are a dominant component of MBFs, especially in photic zones and have been described as larval settlement cues in barnacles (Hudon et al. 1983, Bourget 1988, Le-Tourneux & Bourget 1988). In terms of the relative surface coverage by biofilm components, benthic diatoms occupy considerably larger surface areas than bacteria due to their large cell size. Considering the relative surface areas occupied by different MBF components, we hypothesized the mediatory role of diatoms on larval settlement to be potentially significant, and we examined various monospecies (unialgal) diatom films as potential mediators of larval settlement in the polychaete *Hydroides elegans* (Haswell) (Harder et al. 2002b). We identified potent diatom-derived settlement cues as heat-stable EPS in close association with the cell surface (Lam et al. 2003). The mediatory effect of diatom films on larval settlement was not rendered by nutrient selectivity, since larvae of *H. elegans* are strictly planktotrophic (Wisely 1958). Importantly, the carbohydrate composition and the presence of certain monosaccharides in diatom EPS was correlated with the settlement induction effect (Lam et al. 2005).

Since growth and production of EPS by diatoms is largely affected by environmental parameters (Liu & Buskey 2000, Wolfstein & Stal 2002), the objective of this study was to investigate the effect of environmental parameters, such as temperature and salinity, on the quantity and quality of diatom EPS with respect to their mediatory role in larval settlement processes. Two diatoms that have previously been shown to induce larval settlement in *Hydroides elegans* were used as model strains in this study (Lam et al. 2003).

MATERIALS AND METHODS

Larval culture procedures. Larval culture techniques and assays to verify larval competency were performed according to Harder et al. (2002b). Briefly, adult *Hydroides elegans* were dislodged from the PVC settling panels and induced to spawn by gentle tapping on the posterior end of the tube with a dissecting needle. The gamete-containing seawater was filtered through 70 µm mesh to remove debris and then washed in 0.22 µm-filtered seawater (FSW) before being transferred into culture containers. The larval cultures were incubated at 24°C under a 15:9 h L:D photo cycle and maintained. Larval competence was determined according to larval morphology (Wisely 1958). Batches of competent larvae were sieved through a 110 µm nylon mesh. The larvae retained on the mesh were transferred into a container with FSW and used for bioassays within 1 h.

Diatom culture procedures. Following on from our previous studies (Lam et al. 2003, 2005), the inductive benthic pennate diatoms *Achnanthes* sp. and *Nitzschia constricta* were chosen as model species. The experiment was set up in an orthogonal 2-factorial design, with 2 levels of temperature (18 and 30°C) and salinity (18 and 30 ppt). The experimental temperatures and salinities selected here covered the range encountered in Hong Kong waters during different seasons. Stock cultures of *Achnanthes* sp. and *N. constricta* were inoculated into 40 ml culture tubes (Kimax) containing autoclaved f/2 nutrient medium and a mixture of streptomycin (3.5 mM) and penicillin (1.1 mM) in FSW (Guillard & Ryther 1962). The cultures were adjusted to the experimental salinities and incubated for 18 d with fluorescent illumination (0.3×10^{-16} quanta cm^{-1} s^{-1}) under a 15:9 h L:D photo period. Test tubes with autoclaved FSW served as blank controls. The growth of diatoms was determined by daily measurements of *in vivo* fluorescence (excitation wavelength: 400 to 660 nm; emission wavelength: 680 nm; Turner Designs).

Development of monospecies diatom films. After the growth of visible diatom films in 250 ml culture flasks, monospecies diatom suspensions were prepared by brushing the tube interior with a sterile paintbrush. Aliquots of 100 ml of non-axenic diatom suspensions were filtered through either 1.0 or 0.45 µm membrane filters (Osmonics), depending on the diatom cell size. The filter residues were washed with autoclaved FSW to reduce the number of non-attached bacteria. Subsequently, the retained diatoms were resuspended in 250 ml FSW. Briefly, 5 ml aliquots of the suspensions were transferred into sterile Petri dishes (diameter = 5 cm; Falcon #1006, Becton Dickinson) with replication (n = 8) followed by 24 h of incubation. The filmed dishes were dip-rinsed in autoclaved FSW to remove loosely attached cells. Prior to larval settlement bioassays, 5 ml of autoclaved FSW were added to the experimental dishes.

Isolation and immobilization of EPS. The diatom suspensions were concentrated and fixed in 5% formalin in seawater. The isolation of diatom EPS was performed according to Bhosle et al. (1995, 1996). Briefly, the cells were rinsed with 0.22 µm FSW, and EPS were dissociated from the cells by vigorous shaking of diatom suspensions in 1 M NaCl, 0.05 M EDTA at 24°C for 24 h. Subsequently, the cells were spun down ($1000 \times g$) for 5 min at 4°C, and the supernatant was filtered through

sterile 0.22 µm membrane filters (Millipore). Diatom EPS were concentrated in a stirred ultrafiltration cell (YM-10 kDa, Millipore), rinsed with distilled water, freeze-dried and weighed. After the EPS extraction, the freeze-dry weight of the diatoms was also determined. The individual EPS dry weights were divided by the sum of diatom and EPS weights to calculate the proportion of EPS in diatoms at each growth condition.

The immobilization of diatom EPS in stable hydrogels was performed according to Lam et al. (2005). Briefly, 50 mg of each EPS sample was dissolved in 20 ml of 0.02% Phytagel™ (Sigma). Solidified gel cylinders were cut into thin experimental discs of 2 cm in diameter.

Bioassay-guided gel-filtration chromatography of diatom EPS. EPS were concentrated 10 times by ultrafiltration (100 kDa) and fractionated by gel-filtration chromatography on Sephacryl S-400 HR (Pharmacia; 1.6 × 30 cm) with isochratic elution of water (Millipore) at 0.35 ml min^{-1} and 201 nm detection wavelength. The different size fractions obtained were reconstituted to the original concentration in Phytagel™ for larval bioassays.

Larval settlement assays. Larval settlement assays with immobilized diatom EPS were performed with replication (n = 5). Twenty competent larvae of *Hydroides elegans* were transferred in the smallest volume possible (ca. 200 µl) from larval batches of high density into Petri dishes (Falcon #1006) containing experimental Phytagel™ discs. Dishes were incubated for 5 h at 24°C under a 15 h:9 h L:D photo period. Phytagel™ discs without EPS served as controls. Assays were evaluated by recording the number of settled larvae under the dissecting microscope.

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–Wilk's W test (Shapiro & Wilk 1965). All data were treated non-parametrically by transforming values to ranks and 1-way ANOVA followed by Tukey's multiple comparison test (Conover & Iman 1981). The data presented in the figures were not transformed.

RESULTS

Diatom growth and EPS production

The 4 combinations of 2 temperatures (18 and 30°C) and 2 salinities (18 and 30 ppt) had a significant effect on diatom growth (Fig. 1). Overall fluorescence of the cultures of *Achnanthes* sp. (from 38 ± 9.0 to 79 ± 8.0 arbitrary units) and *Nitzschia constricta* (from 21 ± 2.2 to 124 ± 9.9 arbitrary units) reached a maximum and a minimum at high and low temperature and salinity, respectively. At a given salinity, the proportion of crude EPS in the diatoms *Achnanthes* sp. and *N. constricta* increased from 18 to 30°C; whilst the proportion of crude EPS was inconsistent when the salinity increased from 18 to 30 ppt at a given temperature (Table 1). Temperature played a more important role than salinity on the production of crude diatom EPS.

Bioassays with monospecies diatom films produced at 4 growth conditions

The percentage of larval settlement on films prepared from diatoms grown at 30°C was significantly

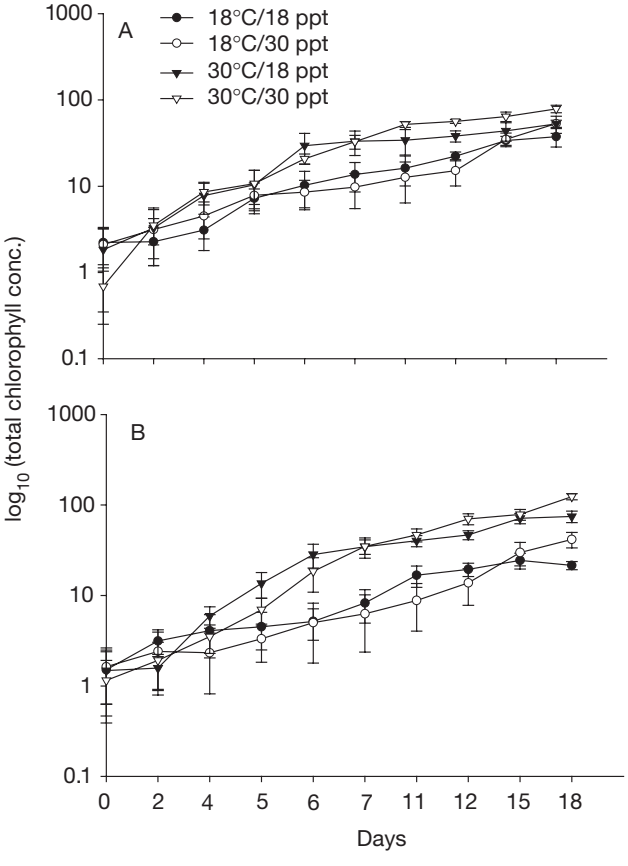

Fig. 1. Increase in fluorescence of the pinnate benthic diatoms (A) *Achnanthes* sp. and (B) *Nitzschia constricta* under 2 temperatures (18 and 30°C) and 2 salinities (18 and 30) measured by chlorophyll fluorescence. Data plotted are means ± SD of 3 replicates

Table 1. Proportion of crude extracellular polymer (EPS) produced by the diatoms *Achnanthes* sp. (A) and *Nitzschia constricta* (NC) grown at 4 combinations of temperature and salinity. Percent crude EPS were obtained by dividing EPS yields by the sum of EPS plus the diatom biomass after extraction

Diatom growth conditions	Σ(EPS yield + diatom biomass) (mg)	Crude EPS (%)
A (18°C; 18 ppt)	55.3	18.08
A (18°C; 30 ppt)	157.1	15.60
A (30°C; 18 ppt)	371.6	28.04
A (30°C; 30 ppt)	595.7	38.12
NC (18°C; 18 ppt)	140.6	26.17
NC (18°C; 30 ppt)	181.3	21.51
NC (30°C; 18 ppt)	558.1	37.74
NC (30°C; 30 ppt)	592.3	37.26

higher than that on films grown at 18°C, irrespective of the salinity setting (Tukey's test, $p = 0.023$ for *Achnanthes* sp. and $p = 0.002$ for *Nitzschia constricta*; Fig. 2). All diatom treatments evoked larval settlement significantly different from the FSW control (Tukey's test, $p < 0.05$). The settlement response for experimental diatom films was positively correlated with diatom density (*Achnanthes* sp.: $r^2 = 0.916$; $p = 0.043$; *N. constricta*: $r^2 = 0.857$; $p = 0.074$). When the same amount of EPS was used for the larval settlement bioassay, the immobilized diatom EPS obtained from the high-temperature treatments induced between 40 and 60% larval settlement, which was statistically higher than that for the low-temperature treatments, irrespective of the salinity setting (Tukey's test, $p = 0.045$ for *Achnanthes* sp., Fig. 3A; Tukey's test, $p = 0.021$ for *N. constricta*, Fig. 3B).

Bioassays of separated EPS fractions

The chromatographic peak profiles of the diatom EPS samples differed in both retention times and peak intensities. In the case of *Achnanthes* sp., the chromatographic properties of EPS samples obtained at 18°C/18 ppt and 18°C/30 ppt differed from the corresponding high-temperature treatments (Fig. 4). While the low-temperature treatments largely comprised small macromolecules (long retention time), the high-temperature treatments were characterized by the presence of mainly large macromolecules (short retention times; Fig. 4A–D). The settlement induction activity was correlated with the high-molecular-weight fractions obtained from the high-temperature treatments, especially at 30°C/30 ppt (Table 2).

In the case of *Nitzschia constricta*, the chromatograms of EPS samples obtained at all growth regimes also showed 2 bulk peaks. The small macromolecule fraction differed from *Achnanthes* sp. EPS due to shorter retention times. While *Achnanthes* sp. EPS did not reveal any low-molecular-weight fractions at high-temperature treatments, the 30°C/18 ppt treatment of *N. constricta* revealed the presence of small macromolecules in the corresponding EPS sample. This fraction also induced larval settlement in the bioassay (Table 2). This observation was in contrast to the most active crude EPS sample of *N. constricta* (30°C/30 ppt, Fig. 3B), which only revealed a biologically active fraction of high molecular weight (Fig. 4E–H).

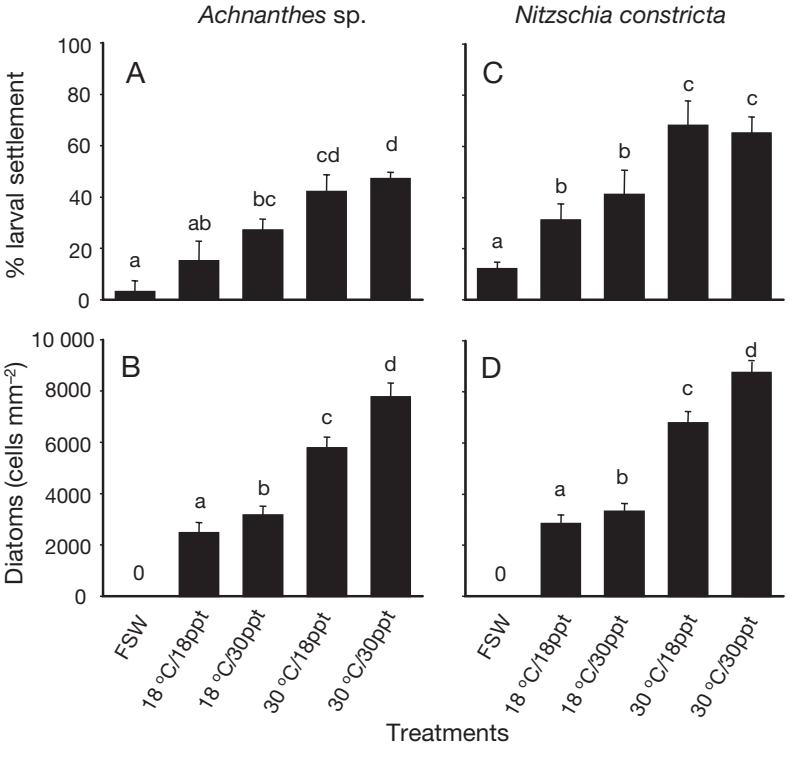

Fig. 2. (A,C) Mean percentage of larval settlement of *Hydroides elegans* after 24 h in response to diatom films of *Achnanthes* sp. (A) and *Nitzschia constricta* (C) for 4 combinations of 2 different temperatures and salinities. Filtered seawater (FSW) was used as a negative control. Data that differed significantly (Tukey's test, $p = 0.05$) are indicated by different letters. Data are means + SD of 5 replicates. (B,D) Mean diatom density of *Achnanthes* sp. (B) and *Nitzschia constricta* (D) for 4 films grown under the 4 combinations of temperature and salinity. Data are means + SD of 3 replicates

DISCUSSION

The EPS of diatoms have been intensively studied (Zobell & Allen 1935, Bourget 1988, Bhosle et al. 1995, 1996, Hoskins et al. 2003). A positive correlation was observed for diatom EPS production between temperature and salinity (Liu & Buskey 2000, Wolfstein & Stal 2002). Regarding the objectives of our study, previous investigations on the production of EPS at different levels of temperature and salinity (Lam et al. 2005) were of particular interest.

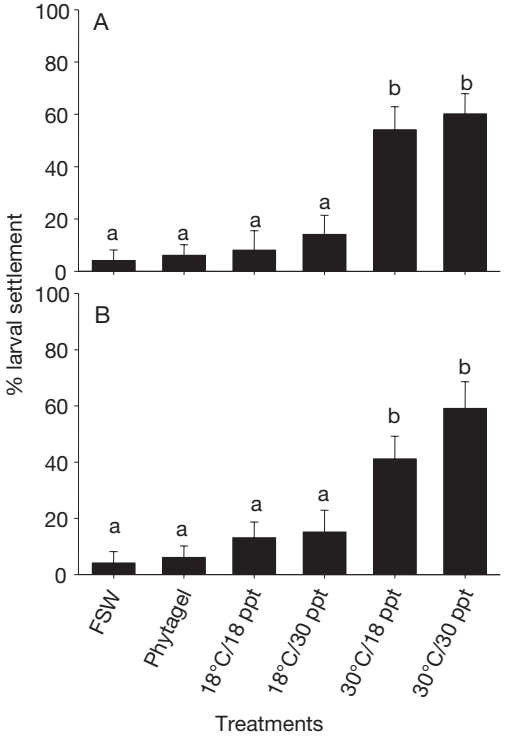

Fig. 3. Mean percentage of larval settlement of *Hydroides elegans* after 5 h in response to crude diatom EPS of (A) *Achnanthes* sp. and (B) *Nitzschia constricta* obtained from different growth conditions. FSW and Phytagel™ served as negative controls. Data that differed significantly (Tukey's test, p = 0.05) are indicated by different letters. Data are means + SD of 5 replicates

In accordance with the prior investigations of Geider (1987) and Wolfstein & Stal (2002), the different growth conditions utilized in this study significantly influenced the growth of *Achnanthes* sp. and *Nitzschia constricta* as evidenced by a direct positive relationship between high temperature and salinity treatments for fluorescence (Fig. 1). Larval settlement assays with monospecies (i.e. unialgal) diatom films grown at 4 combinations of temperature and salinity resulted in a significant correlation between the diatom film density and its inductive effect on larval settlement of *Hydroides elegans*. Considering the experimental difficulties to achieve total axenicity of unialgal diatom cultures, a mixture of antibiotics (streptomycin and penicillin) was added to each culture before diatom film development in order to lower the growth of bacterial contaminants. Furthermore, our previous study showed that bacterial contaminants separated by filtration had an insignificant effect on larval settlement of *H. elegans* in comparison to the corresponding diatom films (Harder et al. 2002b).

Previously, we observed that the magnitude of larval settlement correlated with the density of diatoms in biofilms (Lam et al. 2003). However, it remained unclear whether there was a direct effect of diatom growth conditions on EPS quality, and thus an influence of environmental growth conditions on larval settlement. Since our previous findings revealed that isolated diatom EPS might operate as larval settlement cues in *Hydroides elegans* (Lam et al. 2005), the same approach was applied in this study to investigate the settlement inductive effect of cell-dissociated diatom EPS obtained from cultures of different growth parameters. In order to decouple the quantitative effect of diatom film density on larval settlement from the potential qualitative effect of diatom EPS, the same amount of diatom EPS obtained from different growth regimes was

Table 2. *Hydroides elegans*. Retention times (see the chromatograms in Fig. 4) and percent larval settlement induction of *Hydroides elegans* for each EPS fraction obtained from the diatoms *Achnanthes* sp. (A) and *Nitzschia constricta* (NC). Settlement values denoted with an asterisk represent a significant difference compared to the control (FSW). na: not applicable

Diatom growth conditions	Retention times (min)			% larval settlement			
	Fraction 1	Fraction 2	Fraction 3	Fraction 1	Fraction 2	Fraction 3	Control
A (18°C; 18 ppt)	60–80	120–160	na	7.0 ± 5.7	10.0 ± 5.0*	na	1.0 ± 2.2
A (18°C; 30 ppt)	60–70	110–150	na	7.0 ± 2.7*	10.0 ± 8.4*	na	1.0 ± 2.2
A (30°C; 18 ppt)	30–40	50–80	na	16.0 ± 4.18*	21.0 ± 10.84*	na	1.0 ± 2.2
A (30°C; 30 ppt)	40–60	70–80	100–130	44.0 ± 9.8*	11.0 ± 6.5*	2.0 ± 2.7	1.0 ± 2.2
NC (18°C; 18 ppt)	40–70	100–140	na	9.0 ± 7.4	7.0 ± 4.5	na	6.8 ± 5.8
NC (18°C; 30 ppt)	40–90	100–140	na	5.0 ± 5.0	19.0 ± 14.3	na	6.8 ± 5.8
NC (30°C; 18 ppt)	40–70	100–140	na	18.0 ± 5.7*	28.0 ± 9.1*	na	6.8 ± 5.8
NC (30°C; 30 ppt)	40–80	100–120	na	42.0 ± 10.4*	8.0 ± 10.4	na	6.8 ± 5.8

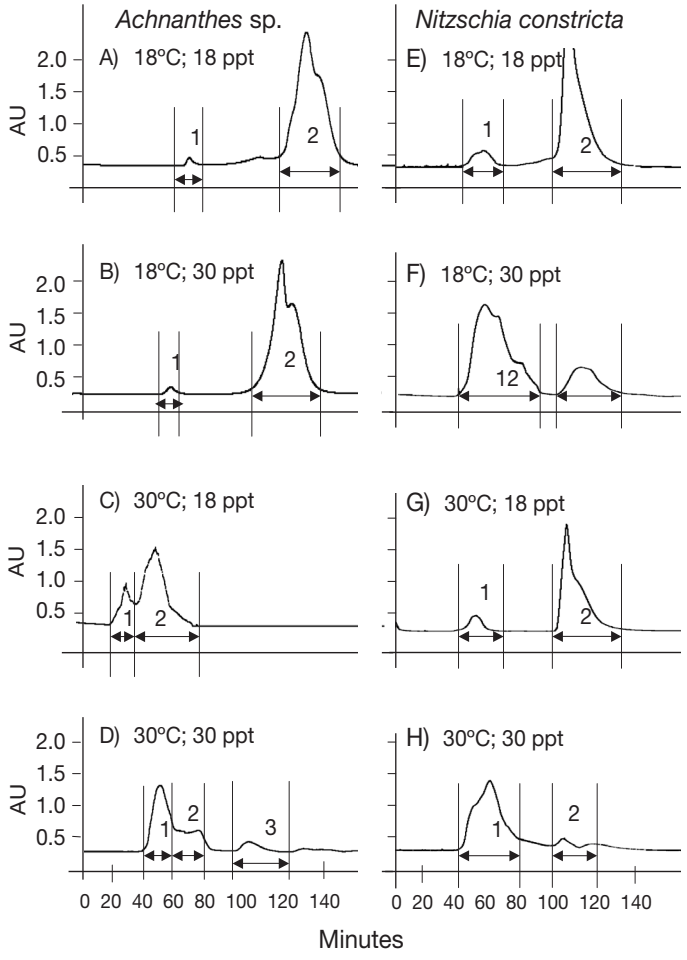

Fig. 4. Crude diatom EPS of (A–D) *Achnanthes* sp. and (E–H) *Nitzschia constricta* under different growth conditions were characterized by aqueous gel chromatography. Horizontal arrows denote the retention times of distinguishable (numbered) peak fractions

immobilized in stable hydrogels (outlined in detail in Lam et al. 2005) and subject to larval settlement assays. Given the significant settlement responses of larvae to the EPS obtained from high temperature and salinity treatments compared to the corresponding treatments at low temperature and salinity, these results clearly demonstrated a qualitative difference in the diatom EPS samples under investigation (Fig. 3). Our attempt to verify the qualitative role of diatom EPS on larval settlement did not allow us to determine the proportion of dissociated EPS after individual extractions.

Based on our previous knowledge that exopolymeric settlement cues of diatoms consist of macromolecular constituents larger than 100 kDa (Lam et al. 2005), the qualitative differences of the diatom EPS samples under investigation were further analyzed by bioassay-guided gel-filtration chromatography. The chromatographic profiles of EPS samples obtained in high- and low-temperature treatments were characterized by the presence of mainly large and small macromolecules, respectively, as indicated by short and long retention times (Fig. 4). In the case of *Achnanthes* sp., the settlement induction activity of the EPS sample obtained at high temperature and salinity was mainly comprised of a single high-molecular-weight fraction (Peak 1, Fig. 4D; Table 2). In case of *Nitzschia constricta*, the change in salinity from 18 to 30 ppt at 30°C resulted in 2 qualitatively different EPS settlement cues. While at low salinity the major inductive fraction was represented by a small macromolecular fraction (Peak 2, Fig. 4G; Table 2), a high molecular weight fraction with an inductive effect on larval settlement resulted in the high salinity treatment. However, this EPS constituent differed from the corresponding biologically active high molecular weight fraction obtained from *Achnanthes* sp. due to a different retention time.

In summary, these results demonstrate that growth conditions of the 2 diatoms under investigation not only influenced the quantity and quality of EPS, but also affected the biological activity of these EPS samples, as evidenced by significantly different response levels of larval settlement. From an ecological perspective, these findings correspond to the basic principles of larval settlement of sessile marine invertebrates that distribute via mobile, pelagic larval stages. Upon contact with a marine substratum, larvae actively explore the surface and assess its suitability for permanent, irreversible attachment and metamorphosis. Substratum suitability is largely determined by environmental parameters such as tidal height, flow regime and nutrient availability (Strathmann et al. 1981, Qian et al. 1999, 2000, 2003). For many marine invertebrates, larval settlement is effectively mediated by biofilms (Kirchman et al. 1982, Szewzyk et al. 1991, Lau & Qian 2001). Many larvae distinguish between biofilms of varying composition, physiological condition and growth phase (Neumann 1979, Szewzyk et al. 1991, Pearce & Scheibling 1991, Holmström et al. 1992, Anderson 1995, Wieczorek et al. 1995, Maki et al. 1998), indicating that biofilm components serve as important signposts for larvae seeking settlement substratum. Bacteria have been identified as small-scale proxies, since their composition and physiology in marine biofilms reflects the local environmental conditions (Anderson 1995, Wieczorek et al. 1995, Olivier et al. 2000). Our cumulative findings indicate that in addition to bacteria, benthic diatoms also play a significant role in determining substratum suitability for larval settlement of *Hydroides elegans* (Harder et al. 2002b, Lam et al. 2003, 2005). Our results demonstrate that there is wide range of chemical information encrypted in marine biofilms composed of benthic diatoms and bacteria.

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