Zur Homepage der Dissertation

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Do microbial or biochemical settlement cues on the sediment surface mediate larval settlement of *Polydora cornuta* and *Streblospio benedicti* (Polychaeta, Spionidae)?

Sind mikrobielle oder biochemische Signale an der Sedimentoberfläche für die larvale Ansiedlung von *Polydora cornuta* und *Streblospio benedicti* (Polychaeta, Spionidae) verantwortlich?

DISSERTATION

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„UND SIE DREHT SICH DOCH!“

Galileo Galilei

ERKLÄRUNG

VERÖFFENTLICHUNGEN

Teilergebnisse dieser Arbeit sind als Beiträge in Fachzeitschriften erschienen (Kapitel 2 und 3), in einer Diplomarbeit von Justus Lodemann behandelt (Teile von Kapitel 2 und 3), als Poster auf einer Konferenz vorgestellt (Teile von Kapitel 4-6; Untersuchung von Diatomeen als Siedlungssignal), als Manuskript eingereicht und auf einer Konferenz vorgetragen oder in einer Diplomarbeit von Mandy Winterberg verwendet (Teile von Kapitel 4-6; Untersuchung von Halometaboliten als Siedlungssignal). Mein Beitrag an der Erstellung der Arbeiten wird im Folgenden erläutert:

SEBESVARI, Z., ESSER, F., HARDER, T. 2006. Sediment-associated cues for larval settlement of the inbenthic spionid polychaetes *Polydora cornuta* and *Streblospio benedicti*. J. Exp. Mar. Biol. Ecol. 337, 109-120.

Etablierung der Aufzucht von *S. benedicti* und *P. cornuta* im Labor und Entwicklung des single-choice Testverfahrens durch F.E. und Z.S., dieses stellt die Basis der ersten larval-biologischen Siedlungstests und aller folgenden Untersuchungen dar. Kultivierung der Polychaeten im Labor durch F.E. und Z.S., experimentelle Arbeiten und Korrektur des Manuskripts F.E., Z.S., Überarbeitung durch Z.S. und T.H.

SEBESVARI, Z., ESSER, F., NEUMANN R., SCHULTE A., BRINKHOFF T., HARDER T. 2007. Monospecies bacterial films on sediment induce larval settlement of the infaunal spionid polychaete *Polydora cornuta* and *Streblospio benedicti*. J. Exp. Mar. Biol. Ecol. (Submitted 18.12.06).

Durchführung von Siedlungstests mit Larven von *P. cornuta* und *S. benedicti* auf Sediment konditioniert mit Bakterienisolaten durch Z.S. und F.E., Entwicklung des multiple-choice Testverfahrens durch F.E. und Z.S., ein weiteres fundamentales Verfahren, welches für larvale Ansiedlungstests angewendet wurde. Experimentelle Arbeiten und Konzeption des Manuskripts durch Z.S, F.E. Überarbeitung durch T.H.

Esser, F., Sebesvari, Z., Winterberg, M., Harder, T. 2007. Direct and indirect effect of halogenated metabolites from infaunal polychaetes on larval settlement of the spionid polychaete *Streblospio benedicti*. Mar. Ecol. Prog. Ser. (Submitted 24.05.07)

Durchführung von multiple-choice und single-choice Tests durch F.E. und M.W.; Aufzucht der Polychaeten im Labor durch F.E., M.W., A.S., Z.S., Durchführung der molekularbiologischen Untersuchungen durch F.E.; Gaschromatographische Messungen durch F.E. und M.W.; Erstellung des Manuskriptes durch F.E. und T.H.

WINTERBERG, M. 2007. Quantifizierung von 2,6-Dibromphenol und 1-Chlornonan in Zellgewebe von Polychaeten und Sediment per GC-MS. Diplomarbeit, Universität Oldenburg

Betreuung der Arbeit, Durchführung von multiple-choice Tests, gaschromatographische Messung von Wurm- und Sedimentproben und Bereitstellung der Polychaetenkultur durch F.E., Z.S. und T.H.

LODEMANN, J. 2005. Untersuchung zum Einfluss ausgewählter biologischer, chemischer und physikalischer Parameter auf das larvale Siedlungsverhalten von *Streblospio benedicti* und *Polydora cornuta*. Diplomarbeit, Universität Oldenburg.

Betreuung der Arbeit und Bereitstellung der Polychaetenkultur durch F.E., Z.S. und T.H.

KONFERENZBEITRÄGE

ESSER F., SEBESVARI Z., HARDER T.: Direct and indirect influence of halogenated metabolites from infaunal polychaetes on larval settlement of the spionid polychaete *Streblospio benedicti*. VII International Larval Biology Symposium, 25.08.-01.09. 2006, Coos Bay, Oregon. (Vortrag)

SEBESVARI Z., ESSER F., HARDER T.: Bacterial cues for larval settlement of the spionid polychaete *Polydora cornuta*. VII International Larval Biology Symposium, 25.08.-01.09. 2006, Coos Bay, Oregon. (Vortrag)

ESSER F., SEBESVARI Z., HARDER T.: Influence of biogeochemical sediment parameters on infaunal colonization of spionids in tidal flats (Wadden Sea, Germany). American Society of Limnology and Oceanography (ASLO), 20.-24.06. 2005, Santiago de Compostella, Spain. (Poster)

SEBESVARI Z., ESSER F., HARDER T.: Sediment related settlement cues for larvae of the polychaete *Polydora cornuta*. VI International Larval Biology Conference, 21.-25. 06. 2004, Hong Kong. (Vortrag)

ESSER F.: Influence of biogeochemical sediment parameters on infaunal colonization. Microsensor course - analysis in the Environmental Sciences, 23.04.-01.05. 2004 Ronbjerg, Denmark. (Vortrag)

ZUSAMMENFASSUNG

In tidenbeeinflussten Sedimenten des deutschen Wattenmeers wurde eine fleckenhafte Verteilung von spionieden Polychaeten (*Polydora cornuta* und *Streblospio benedicti*) mit indirekter larvaler Entwicklungsweise festgestellt. Diese Verteilungsmuster sind nicht durch Sedimentparameter wie z.B. Korngröße erklärbar. Es stellt sich die Frage, ob aktive Habitatwahl und Ansiedlung von Larven durch im Sediment assoziierte mikrobielle Signale, wie Biofilme, hervorgerufen werden können. In der vorliegenden Arbeit wurde zum ersten Mal die larvale Ansiedlung von Weichsubstratsiedlern unter Berücksichtigung von unterschiedlichen Biofilmkomponenten wie Diatomeen, Bakterien oder deren Epipolysaccharide oder halogenierte Metabolite untersucht. Diese Hypothese wurde in laborbasierten Tests mit und ohne Auswahlmöglichkeit mit Larven von *P. cornuta* und *S. benedicti* untersucht. Die Larven beider Polychaetenarten stammten aus Laborkulturen, die Larven in ausreichendem Maße zur Verfügung stellten. Larven beider Arten waren in der Lage zwischen Sedimenten unterschiedlicher Qualität zu unterscheiden. In Testreihen ohne Auswahlmöglichkeit zeigten Larven von *P. cornuta* und *S. benedicti* signifikant hohe Siedlungsraten auf natürlichem Sediment (zwischen 75% und 95%) im Vergleich zu 600°C veraschtem Sediment (zwischen 5% und 50%). Der prozentuale Anteil der larvalen Ansiedlung von *P. cornuta* auf durch Autoklavieren sterilisiertes Sediment war signifikant niedriger (zwischen 25% und 55%) als im natürlichen Sediment und unterscheidet sich von dem von *S. benedicti* (zwischen 70% und 90%). Vier unterschiedliche Sedimentbehandlungen (natürliches, steriles, veraschtes und mit Säure behandeltes Sediment) wurden durchgeführt und in Testreihen mit Auswahlmöglichkeit für beide Larvenarten gegeneinander getestet. Signifikant hohe Siedlungsraten beider Arten wurden in natürlichem Sediment im Vergleich zu allen anderen Sedimenten vorgefunden. In diesem Fall unterscheiden *S. benedicti* Larven aktiv zwischen natürlichem und sterilem Sediment, anders als in Ansiedlungstests ohne Sedimentauswahl.

Die Frage, ob Diatomeen als potentielles Siedlungssignal für Larven dienen könnten, wurde in larvalen Ansiedlungstests ohne Auswahlmöglichkeit auf drei unterschiedlichen Wegen untersucht. Erstens wurde natürliches Sediment mit einem Algizid (Cycloheximid) und Antibiotika (Penicillin, Streptomycin) behandelt. Dabei soll selektiv entweder der Bakterien-Anteil oder der Diatomeen-Anteil im

Sediment eliminiert werden. Die Wirkung des Algizides, bei einer Konzentration von 10 mg g^{-1} in natürlichem Sediment, zeigte 10^4 tote Diatomeenzellen pro mm^2 Sediment. Dies ist eine Größenordnung niedriger als die von lebendigen Diatomeenzellen in natürlichem Sediment oder in Sediment mit Antibiotikabehandlung. Geringe Bakteriendichten (< 10^5 Zellen g^{-1} Sediment) wuden in mit Antibiotika behandeltem natürlichen Sediment nachgewiesen. Sie waren zwei bis drei Größenordnungen niedriger als in natürlichem Sediment ohne Antibiotika- oder mit Algizidbehandlung. Zweitens wurde natürliches Sediment mit Licht bestrahlt oder im Dunkeln aufbewahrt und in larvalen Ansiedlungstests ohne Auswahlmöglichkeit eingesetzt. Für beide Larvenarten wurde die larvale Ansiedlung nicht durch das Vorhandensein oder die Abwesenheit der Diatomeen auf der Sedimentoberfläche beeinflusst. Drittens wurde veraschtes und steriles Sediment mit einer Suspension lebendiger Diatomeen beimpft, die von natürlichem Sediment stammte. Die Wiederbeimpfung war jedoch nur in geringem Maße erfolgreich, so dass nur eine geringe Anzahl an Diatomeen auf veraschtem und eine etwas höhere Anzahl auf sterilem Sediment wiederzufinden waren. Weder veraschtes Sediment, das mit der Diatomeensuspension beeimpft wurde, noch veraschtes Sediment mit Diatomeensuspension und einer zusätzlichen Antibiotikabehandlung zeigten eine prozentual höhere larvale Ansiedlung im Vergleich zu veraschtem Sediment.

Die Hypothese, dass die larvale Ansiedlung durch Epipolysaccharide (EPS) von Bakterien oder Diatomeen beeinflusst wird, wurde in drei unterschiedlichen Experimenten untersucht. Erstens wurde EPS von natürlichem Sediment extrahiert und auf veraschtes Sediment aufgebracht. Das larvale Siedlungsverhalten von *P. cornuta* auf veraschtem Sediment, welches mit EPS Suspension beeimpft wurde, war gleich mit dem von nur veraschtem Sediment und signifikant höher als auf sterilem oder natürlichem Sediment. Zweitens wurde in einem weiteren Experiment natürliches Sediment entweder mit einer Mischung aus unterschiedlichen Enzymen oder EDTA oder mit Ultraschall behandelt und in larvalen Ansiedlungstests ohne Auswahlmöglichkeit eingesetzt. Die larvale Ansiedlung wurde mit *S. benedicti* Larven getestet und war gleich auf natürlichem Sediment, welches mit Enzymen, EDTA oder Ultraschall behandelt wurde, jedoch signifikant niedriger als auf sterilem oder natürlichem Sediment. Drittens wurde steriles Sediment mit Lektinen (Concanavalin A und Peanut Lektin) behandelt, um die Lektin-Polysaccharid Bindung zwischen Lektinen der

Larve und Polysacchariden auf der Sedimentoberfläche zu blockieren. In Ansiedlungstests ohne Auswahlmöglichkeit war die larvale Ansiedlung von *S. benedicti* auf sterilem Sediment, welches mit Lektinen behandelt wurde, gleich zu der auf sterilem oder natürlichem Sediment. Viertens wurden Monosaccharide zu sterilem oder veraschtem Sediment hinzugegeben, um als Kohlehydrat Bindungsstelle für die Larven zu agieren. Der prozentuale Anteil der larvalen Ansiedlung von *S. benedicti* auf sterilem Sediment, welches mit jedem von vier Monosacchariden konditioniert wurde, war gleich zu der auf sterilem oder natürlichem Sediment. Veraschtes Sediment, welches mit Monosacchariden behandelt wurde, zeigte jedoch eine höhere signifikante Ansiedlung, im Verglich zu nur veraschtem Sediment.

In dieser Arbeit wurde die Rolle von Halometaboliten als potentielle Siedlungssignale erstmals in Ansiedlungstests ohne und mit Auswahl für *S. benedicti* Larven untersucht. Halometabolite haben antimikrobielle Wirkung. Der direkte oder indirekte Effekt von Halometaboliten auf die larvale Ansiedlung wurde in drei unterschiedlichen Wegen untersucht. Halometabolite wurden in Gewebe von adulten *S. benedicti* und *Capitella* sp. I unter Anwendung von gaschromatographisch-massenspektrometrischer (GC-MS) Analyse quantifiziert. In Gewebe von adulten *S. benedicti* konnte 70.57 ± 0.08 ng mm^{-3} Chlornonan und in *Capitella* sp. I 20.71 ± 1.65 ng mm^{-3} Dibromphenol nachgewiesen werden. Um den direkten Effekt von halogenierten Verbindungen auf die larvale Ansiedlung zu testen, wurde steriles Sediment, ohne lebendige Mikroorganismen, mit Chlornonan oder Dibromphenol zu unterschiedlichen Konzentrationen (1x und 10x) versetzt. Die larvale Ansiedlung auf mit Halometaboliten versetzten sterilen Sedimenten war in Ansiedlungstests ohne Auswahl gleich zu der auf sterilem oder natürlichem Sediment. In Ansiedlungstests mit Auswahl war die larvale Ansiedlung auf sterilem Sediment mit der zehnfachen Konzentration Chlornonan geringer im Vergleich zu sterilem oder natürlichem Sediment. Die larvale Ansiedlung auf sterilem Sediment versetzt mit Dibromphenol mit beiden unterschiedlichen Konzentrationen war gleich im Vergleich zu natürlichem oder sterilen Sediment. In Ansiedlungstests mit Auswahl war die larvale Ansiedlung auf sterilem Sediment versetzt mit Chlornonan und Dibromphenol gleich mit der auf sterilem Sediment, jedoch geringer im Vergleich zu natürlichem Sediment.

Zweitens wurde untersucht, ob Halometabolite, die von adulten Würmern in das Sediment abgegeben wurden, die mikrobielle Gemeinschaft auf dem Sediment

beeinflussen und indirekt eine Auswirkung auf die larvale Ansiedlung haben. Der indirekte Einfluss von Halometaboliten wurde in Ansiedlungstests ohne und mit Auswahl untersucht. In Ansiedlungstests ohne Auswahl wurden adulte Tiere von *S. benedicti* und *Capitella* sp. I in natürlichem Sediment eingesetzt und in natürichem Seewasser für 6 - 29 Tage konditioniert. Die larvale Ansiedlung von *S. benedicti* in natürlichem Sediment, konditioniert mit adulten *S. benedicti* oder *Capitella* sp. I, war gleich zu der in natürlichem Sediment ohne adulte Spezies. Eine Ausnahme waren Tag 8 und 29 in natürlichem Sediment konditioniert mit adulten *Capitella* sp. I. Dort war die prozentuale larvale Ansiedlug von *S. benedicti* geringer als die in natürlichem oder sterilem Sediment. Die larvale Ansiedlung von *S. benedicti* war in Ansiedlungstests mit Auswahl in natürlichem Sediment, konditioniert mit adulten *S. benedicti* oder *Capitella* sp. I, inkubiert in Seewasser für 7 Tage gleich zu der in natürlichem und sterilem Sediment.
Drittens wurde in Ansiedlungstests mit Auswahl natürliches Sediment mit 1-Chlornonan und 2,6-Dibromphenol in einer zehnfach höheren Konzentration versetzt, als in Gewebe von adulten Tieren gemessen wurde und für sieben Tage in natürlichem Seewasser inkubiert. Die larvale Ansiedlung von *S. benedicti* war in Sedimenten mit jedem von beiden Halometaboliten im Vergleich zu natürlichem Sediment gleich aber signifikant unterschiedlich zu der in sterilem Sediment. Genfragmente der 16S rRNA wurden anhand der Denaturierenden Gradientengelelektrophorese (DGGE) und anschließender Clusteranalyse in Sedimenten an Tag 0 und Tag 7 vor und nach Behandlung mit adulten Tieren oder künstlichen Halometaboliten untersucht. Die Analyse von 16S rRNA Genfragmenten von sedimentassoziierten bakteriellen Gemeinschaften in Sedimenten an Tag 0 und nach Inkubation von 7 Tagen entweder mit adulten Tieren von beiden Wurmarten oder Sedimenten mit künstlichen Halometaboliten sind signifikant unterschiedlich im Vergleich zu der in natürlichem Sediment an Tag 0 mit oder ohne Behandlung von Halometaboliten. Das Resultat dieser Experimente wird hinsichtlich dem potentiellen Zusammenspiel oder der Resonanz zwischen dichten infaunistischen Polychaeten-Kolonien und larvaler Ansiedlung im Hinblick auf potentielle bioaktive Metaboliten diskutiert, die von adulten Würmern produziert werden.

ABSTRACT

Distinctive, patchy abundance patterns of spionid polychaetes i.e. *Polydora cornuta* and *Streblospio benedicti* with indirect larval development were found in sediments of tidal flats in the German Wadden Sea. These patterns were not explicable through grain size or other sediment parameters. This led to the hypotheses that active site selection and initial settlement of larvae were triggered by sediment associated microbial cues of i.e. biofilms. This thesis addressed the first time questions about different biofilm components e.g. diatoms, bacteria or their epipolysaccharides or halogenated metabolites as potential settlement cues for soft sediment settlers. In single-choice and multiple-choice assays this hypothesis was tested with larvae of *P. cornuta* and *S. benedicti* obtained from laboratory stock cultures which provide a sufficient amount of larvae. Larvae of both species were able to accept or reject sediments in different sediment qualities. In single-choice assays *P. cornuta* and *S. benedicti* settled on natural sediment in high numbers (75 to 95 %). Significantly low settlement was found on ashed sediment (5 to 50 %). The percentage of larval settlement on sterile sediment of *P cornuta* was lower (25 to 55 %) and different to *S. benedicti* (70 to 90 %). In multiple-choice assays four different sediments were treated i.e. natural, sterile, ashed and acid-washed sediment and larval settlement for both species on natural sediment was significantly higher than on all other sediments. Unlike in single-choice assays, *S. benedicti* here distinguish actively between natural and sterile sediment.

To test the hypothesis that benthic diatoms may act as potential settlement cue larval settlement was investigated in single-choice assays in three different ways. Firstly, natural sediments were treated with algicides (cycloheximide) and antibiotics (streptomycin and penicillin) to selectively eliminate either the bacteria or diatom component of a natural biofilm. The efficiency of algicides in inserted concentrations (10 mg g^{-1}) in natural sediment was 10^4 non-viable diatom cells per mm^2 sediment, i.e. one order of magnitude lower than viable diatom cells in natural sediments or sediments treated with antibiotics. Low bacteria densities (< 10^5 cells g^{-1} sediment) were found in natural sediments treated with antibiotics i.e. two to three orders of magnitude lower than in natural sediment or sediment treated with algicides. Secondly, natural sediment was kept under illumination or constant darkness and larval single-choice assays were

performed. Larval settlement was not influenced in presence or absence of diatoms for both species. Thirdly, ashed and sterile sediments were inoculated with viable diatom suspension obtained from natural sediment. The recolonization was barely successful and only a low number of diatoms was attached on the surface of ashed and to a greater extend on sterile sediment. Neither did ashed sediment inoculate with diatom suspension nor did ashed sediment and diatom suspension plus the additional treatment of antibiotics exhibit a higher percentage of larval settlement than pure ashed sediment.

To experimentally address the hypothesis that bacteria or diatom EPS may influence larval settlement three different experimental designs were performed. Firstly, EPS was extracted from natural sediment and inoculated on ashed sediment. Larval settlement of *P. cornuta* on ashed sediment inoculated with EPS was the same than pure ashed sediment and significant higher on sterile or natural sediment. Secondly, natural sediment was treated either with a mixture of enzymes or EDTA or ultrasonication and single-choice assays were performed with *S. benedicti*.

Larval settlement was the same on natural sediment treated with a mixture of enzymes, EDTA or ultrasonication but significant lower than on sterile or natural sediment. Thirdly, sterile sediment was treated with lectins (Concanavalin A and peanut lectin) to block the lectin-polysaccharide bond that compete with an assumed larval lectin for receptor sites on the sediment surface. In single-choice assays the percentage of larval settlement of *S. benedicti* on sterile sediment inoculated with lectins was the same to sterile or natural sediments. Fourthly, monosaccharides were added on sterile or ashed sediment to act as carbohydrate binding sides for *S. benedicti* larvae. The percentage of larval settlement on sterile sediment conditioned with each of four monosaccharides or a mixture was the same to only sterile or natural sediment. But ashed sediment treated with monosaccharides revealed significant higher percentage of larval settlement of *S. benedicti* in comparison to only ashed sediment.

This thesis addressed for the first time the role of halometabolites as potential settlement cues for larvae of *S. benedicti* investigated in single-choice and multiple-choice assays. Halometabolites were thought to serve as antimicrobial agents. The direct and the indirect effect of halometabolites on larval settlement were investigated in three different ways. Halometabolites in tissue of adult *S. benedicti* and *Capitella* sp. I were quantified by coupled gaschromatography-

mass spectrometry. Tissue of adult *S. benedicti* contained 70.57 ± 0.08 ng mm^{-3} chlorononane and *Capitella* sp. I 20.71 ± 1.65 ng mm^{-3} dibromophenole. To test the direct effect of halogenated proxies on larval settlement sterile sediments, i.e. without viable microorganisms, were contaminated with individual compounds of 1-chlorononane or 2,6-dibromophenole in different concentrations (1x, 10x). In single-choice assays larval settlement was similar on spiked sediments in comparison to sterile or natural sediment. Multiple choice assays showed lower larval settlement in sterile sediment contaminated with the 10x concentration 1-chlorononane in comparison to sterile sediment. Larval settlement on sterile sediment contaminated with 2,6-dibromphenol at 1x and 10x concentration was the same in comparison to only sterile sediment but significant lower than on natural sediment.

Secondly, the hypothesis that halometabolites released by adults may influence the microbial community on the sediment surface and indirectly larval settlement was experimentally addressed. The indirect influence of halometabolites was tested by larval single-choice and multiple-choice settlement assays on natural sediment inhabited with adults of *S. benedicti* and *Capitella* sp. I. incubated in natural seawater. In single-choice assays larval settlement of *S. benedicti* larvae was the same on sediment treated with each of adult worms and conditioned for 6-29 days in natural seawater in comparison to natural sediment. Except sediment conditioned with adults of *Capitella* sp. I revealed lower larval settlement at day 8 and 29 in comparison to natural or sterile sediment. In multiple-choice assays the percentage of *S. benedicti* larval settlement on natural sediment conditioned with adults of *S. benedicti* or *Capitella* sp. I was the same in comparison to natural or sterile sediment.

Thirdly in multiple-choice assays natural sediment was spiked with synthetic 1-chlorononane and 2,6-dibromophenole at 10x concentration than found in tissue of adult worms and incubated for 7 days in natural seawater. Larval settlement of *S. benedicti* was the same in natural sediment treated with each of both halometabolites and natural sediment but significantly different to sterile sediment. Utilizing the molecular fingerprinting technique of denaturant-gradient-gel-electrophoresis (DGGE) of 16S rRNA gene fragments the shift in bacterial community patterns in sediments at day zero and day seven before and after exposure to adults and synthetic halometabolites, respectively, was analyzed. The analysis of PCR amplified 16S rRNA gene fragments of sediment associated

bacterial communities in sediment samples at day zero and after seven days incubation either with adult worm species or spiked with both halometabolites were different to natural sediments at day zero with or without halometabolites. The outcome of these experiments will be discussed with respect to the potential interaction and feedback between dense infaunal polychaetes colonies and larval recruitment via potentially bioactive metabolites produced by adults.

CONTENTS

1 GENERAL INTRODUCTION

1.1 FOCUS AND SCOPE OF THIS DISSERTATION

Distinctive patterns of abundance of spionid polychaetes with larval development were found in sediment cores along transects of tidal flats in the Wadden Sea (Germany) (Stamm 2000). These patterns were neither correlated with sediment characteristics such as silt content nor organic carbon and nitrogen content (Stamm 2000). These observations led to the hypothesis that the observed distribution patterns may have resulted from active site selection and initial settlement of larvae triggered by factors others than silt, organic carbon and nitrogen content. This dissertation ascertains if these distribution patters are due to microbial and/or bio-chemical cues associated with the sediment surface which in turn may actively mediate larval settlement of spionid polychaetes. The study organisms were *Polydora cornuta* (Bosc, 1802) and *Streblospio benedicti* (Webster, 1879) (Annelida, Polychaeta), both of them have as pronounced planktotrophic larval developmental phase. At the beginning of these works no information about active substrate selection in these two species was present.

Preliminary data suggested that biofilms on the sediment surface influenced larval settlement (Sebesvari et al. 2006, 2007). In the course of this thesis different biofilm components were investigated regarding their potential role as larval settlement cues, i.e. bacteria, diatoms, epipolysaccharides (EPS), lectins or monosaccharides. Additionally the influence of halomgenated proxies produced by adult worm species or spiked on sediments was researched in larval settlement assays. To investigate larval settlement of polychaetes under controlled and reproducible laboratory conditions I have developed a laboratory based larval culture and bioassay of *P. cornuta* and *S. benedicti* together with another PhD student, Z. Sebesvari. We managed to culture both spionid species and raise larvae in sufficient quantities for bioassay purposes year-round. Different larval stages of *P. cornuta* and *S. benedicti* were investigated for their ability to actively accept or reject sediments in still water single-choice (chapter 2, Sebesvari et al. 2006) and multiple-choice bioassays (chapter 3, Sebesvari et al. 2007). Sediment qualities under investigation were natural-untreated or sterilized, ashed or acid-washed sediments obtained from tidal flats. If larvae, as hypothesized, actively selected and differentiated between sediment qualities,

different preferences would be displayed in the settlement assays. Early results indicated that larvae of *P. cornuta* preferred natural sediment over sterile and ashed sediment, whereas *S. benedicti* larvae preferred natural and sterile sediment but rejected ashed sediment. The hypothesis that bacteria in sediments acted as prominent settlement cues was tested by isolation of bacteria from natural sediment and inoculated on ashed or sterile sediments with suspensions of detached but viable bacteria at different cell densities. Larval settlement of *P. cornuta* and *S. benedicti* was significantly higher in two out of 15 isolates i.e. sterile sediment treatments recolonized with bacteria strain 54 (*Flavobacteria*) and strain DF11 (α-*Proteobacteria*, *Roseobacter-clade*) induced similar rates of settlement as the control of natural sediment (chapter 3). These results were crucial to design manipulative experiments testing bacterial isolates in particular and individual components of biofilms in general, (chapter 4-6).

Biofilms are composed of a variety of microorganisms and their products. Next to bacteria, another highly abundant group of microorganisms are benthic diatoms. Previous studies about the role of diatoms as settlement cues mainly focused on invertebrate species with grazing juveniles such as abalones (Kawamura and Kikuchi 1992; Slattery 1992; Bryan and Qian 1998), sea urchins (Tani and Ito 1979) and sea cucumbers (Ito and Kitamura 1997). Few studies have focused on larval settlement induction by benthic diatoms with regard to larval settlement behavior of sedentary organisms such as the barnacle *Balanus balanoides* (Le Tourneux and Bourget 1988) and polychaete *Hydroides elegans* (Harder et al. 2002; Lam et al. 2003; 2005). Based on these studies I hypothesize that benthic diatoms influence larval settlement of *P. cornuta*. I addressed this hypothesis with three experimental designs by manipulating the diatom density and viability in biofilms. 1) Due to the migratory behavior of benthic diatoms to light I assumed that high and low diatom densities were found in illuminated and darkened sediments, respectively. These assumptions provided the basis of the experimental design to test larval settlement on natural sediments under illumination and darkness. 2) Diatoms and bacteria in natural biofilms were selectively eliminated with algicides and antibiotics to test the effect of the remaining group of microorganisms in larval settlement assays. 3) Furthermore, I addressed the question whether ashed sediment could be regain attractiveness for larvae by inoculation with viable diatom-suspensions. None of these

experimental designs have been investigated in further studies on larval settlement for soft sediment settlers.

As a fourth aspect the surface boundary layer of the sediment was investigated. Photosynthetic biofilms were densely populated microbial communities and characterized by a steep chemical gradient e.g. of oxygen and a very strong attenuation of light within a photic zone from <0.5 mm to a few millimeters in thickness (Kühl et al. 1996). The diffusive boundary layer above relatively smooth surfaces is about 0.1 – 0.2 mm thick at such current rates of 5 – 10 cm sec^{-1}, whereas it is abut 1 mm thick in stagnant water (Revsbech and Jorgensen 1986). The boundary layers are especially important to the exchange of nutrients between microbial communities and their aquatic environment. The hypotheses if the boundary layer thickness trigger larval settlement was tested on natural, sterile and ashed sediment by the use of microsensors. All experiments were detailed in chapter 4-6.

Besides the viable biofilm components of diatoms and bacteria, extracellular polymeric substances (EPS) encasing microbial cells are a major component of biofilms (Wingender et al. 1999; Flemming and Wingender 2001). To test the hypothesis if EPS triggered larval settlement I extracted EPS from natural sediment and incubated ashed sediment with obtained EPS solution to conduct larval settlement assays. The signal triggering larval settlement for *P. cornuta* could likewise derive from a conglomerate of exopolymers from bacteria and diatoms as demonstrated for hard substrate settlers (Kirchman et al. 1982a; Fitt et al. 1990; Harder et al. 2002). This hypothesis was addressed using three different experimental setups. 1) EPS was extracted from natural sediment and reapplied to ashed sediment and subject to larval settlement assays. 2) Natural sediment was treated with a combination of enzymes or/and EDTA (ethylene-diamine-tetraacetic acid, as chelating agent) to degrade or dissolve microbial EPS and subject to larval settlement assays. Due to the heterogeneity of extracellular polysaccharides in the biofilm, a mixture of enzyme activities was used for a sufficient destabilization and consequently, of the entire bacterial biofilm (Böckelmann et al. 2003). 3) Based on previous studies suggesting carbohydrate moieties in microbial EPS as well as carbohydrate binding proteins (lectins) as larval settlement cues (Kirchman et al. 1982b; Kirchman and Mitchell 1983; Mitchell and Maki 1988) I tested the hypothesis if lectins which are located on the outer surface of the larvae may mediate settlement of *S. benedicti* larvae. Lectins

are highly specific for their sugar moieties on the sediment surface and have binding affinities to carbohydrates e.g. glucose. I addressed this hypothesis with three experimental designs by conditioning sediment with either lectins or sugar monosaccharides to investigate larval settlement (chapter 4-6).

A fifth aspect of studies concerned the effect of halogenated metabolites produced by adult polychaetes on conspecifics larval settlement. The occurrence of natural halogenated organic compounds among temperate marine infauna is widespread (Woodin et al. 1987, Fielman et al. 2001). A variety of infaunal polychaetes and hemichordates contain high concentrations of halogenated noxious secondary metabolites which are released into the sediment in proximity of worm burrows (Ashworth and Cormier 1967; Higa and Scheuer 1975a, 1975b; King 1986; Woodin et al. 1987; Steward et al. 1992; Cowart et al. 2000, Fielman et al. 2001). In biological systems the specific incorporation of halogens into organic molecules is regulated by a haloperoxidase enzyme (Fenical 1979). In adults of many soft-bodied infaunal taxa such as hemichordates and polychaetes 2-3% of the total protein content is comprised of haloperoxidase enzymes and used in the biosynthesis of halogenated compounds (Chen et al. 1991).

In addition to the direct role of biogenic halometabolites as negative settlement cues for infaunal polychaetes (Woodin et al. 1993, 1997), in this study I tested the hypothesis that halometabolites may indirectly influence larval settlement of an infaunal polychaete *Streblospio benedicti*. The hypothesis was inspired by previous finding that competent larvae of *S. benedicti*, whilst significantly rejecting ashed sediment in comparison to natural sediment, were stimulated to settle in ashed sediment that was subsequently reinfected with viable microorganisms obtained from natural sediment (Sebesvari et al. 2006). Thus, I raised the question whether polychaete-derived halometabolites may alter the microbial abundance and richness in surface sediments and in turn indirectly affect settlement of polychaete larvae.

I experimentally addressed this hypothesis with two infaunal polychaetes, *Streblospio benedicti* and *Capitella* sp. I. The adult organisms qualitatively differ in their halometabolite contents; *S. benedicti* contains at least 11 chlorinated and brominated alkylhalides (Fielman et al. 1999) while *Capitella* sp. I contains 3 brominated aromatic compounds (Cowart et al. 2000). For both species the predominant halometabolites in worm tissues were determined and quantified by coupled gas chromatography-mass spectrometry. Commercially available

halogenated compounds identical or similar to the dominant halometabolites in worm tissue served as chemical experimental proxies in laboratory settlement assays with larvae of *S. benedicti*. In accordance with Cowart et al. (2000), *S. benedicti* mainly contained an unidentified isomer of chlorononane which was approximated with 1-chlorononane, *Capitella* sp.I mainly contained 2,6-dibromophenol which was commercially available as such.

To test the direct effect of halogenated proxies on larval settlement sterilized sediment samples, i.e. without viable microorganisms, were mixed with individual compounds at different concentrations and subjected to larval settlement assays. The indirect effect was tested accordingly but with natural sediment, i.e. sediment containing viable microorganisms, after different time intervals. The assays were accompanied with positive and negative controls of natural and sterile sediments, respectively. The concentration of added proxies was simultaneously measured when assays were evaluated for settlement rates of larvae. In a more ecologically realistic set up of larval settlement assays, halogenated proxies were replaced with adult specimen of *S. benedicti* or *Capitella* sp. I to contaminate natural sediments with halometabolites and possibly modify the microbial community composition.

To test and quantify the effect of halogenated proxies on the composition of bacteria associated with the sediment I applied the molecular fingerprinting tool of denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA bacterial gene sequences allowing to visualize and statistically compare the community profile in treated sediments with untreated controls. The analyses of the 16S rRNA molecule or its corresponding gene (16S rDNA) is by far the most widely used approach in the last decade (Amann et al. 1995). DGGE has received the most attention for studying complex microbial populations like in surface sediments, and has been successfully applied to several natural habitats (Murray et al. 1996; Teske et al. 1996; Vallaeys et al. 1997; Jensen et al. 1998).

The aim of this approach was to test whether the qualitative modification of the bacterial community associated with sediment due to exposure to halogenated metabolites resulted in a less attractive sediment for larval settlement. The verification of this hypothesis would indicate a new ecological role of halometabolites released by infaunal polychaetes on subsequent colonization of sediment patches already inhabited by adult polychaetes.

1.2 INFAUNAL POLYCHAETES

Polychaetes are the most species-rich and morphologically diverse class of the Phylum Annelida with over 5000 identified species. They occupy every part of the marine ecosystem but are especially abundant in the littoral zone e.g. in muddy sediments of the German Wadden Sea (Stamm 2000). Spionid polychaetes inhabit these near-shore regions mostly living at the sediment-surface interface and numbering several thousand per square meter in many cases (Zajac 1991). Spionid and other interstitial polychaetes are responsible for recycling much of the organic matter of the littoral zone. As such they are ecologically of interest as a major component of the meiofaunal community (Levin 1984). Spionids use a pair of ciliated tentacular palps extending from the head to capture particles at or near the sediment-water boundary and are referred to interface feeders, capturing particles from the sediment surface, in suspension, resuspension and in bedload transport (Dauer et al. 1981). Littoral polychaetes have also been used as pollution-indicator species (Pearson and Rosenberg 1978; Rice and Simon 1980; Jha et al. 1996; Reish and Gerlinger 1997). Spionids are one of the largest families in the group of polychaetes and only few investigations so far deal with larval settlement. Among spionid polychaetes only *Streblospio benedicti* was used in larval settlement assays so far to study the impact of pollutants (endosulfan and PAHs) (Chandler and Scott 1991; Chandler et al. 1997). Spionid larvae are generally brooded in the maternal tube until they develop a number of segments and are released into the plankton (Blake and Arnovsky 1999). Many of them disperse via planktonic larvae (Rouse and Pleijel 2001) until they find a suitable habitat.

The following chapters summarize relevant aspects of larval behavior, dispersal, recruitment, competence, metamorphosis and settlement.

1.3 DISTRIBUTION OF LARVAE IN THE SEA

Many benthic marine invertebrates such as oysters, barnacles and polychaetes have complex life cycles that consist of one or more planktonic larval stages and a benthic adult stage. Benthic invertebrates whose life history includes planktonic larvae, i.e. a life stage characterized by drifting and feeding in the open water, occupy a wider geographic range than those without such a stage. Species with a long planktonic larval development occupy a larger geographic area than species

spending only a short period of their life in the plankton (Crisp 1977; Jablonski and Lutz 1983). Passive dispersal is directly responsible for the geographic distribution of marine invertebrates. On a large scale, more than ten kilometers, larval dispersion is driven by hydrodynamic processes such as currents, tides and stagnations. Oceanic circulation has long been known to influence larval distribution (Thorson 1961; Scheltema 1971; Levin 1983; Banse 1986). In the old literature, it has been suggested that larvae ready to settle sink passively through the water column like “seeds in the wind” (Yonge 1937). More recently, the importance of more localized, small-scale processes has gained support from the coupling of information on realistic boundary-layer flow and the swimming behavior of planktonic larvae (Butman 1986a, 1987). Local current patterns, flow velocity and particularly near bottom flow dynamics play important roles in larval retention and larval settlement (Hannan 1984). Passive deposition determines where larvae initially encounter the sea bed. After encountering the sediment surface, active or passive redistribution of larvae takes place (Eckman 1979; Palmer 1988; Butman 1989). The benefits of larval dispersal are genetic exchange between different populations, avoidance of competition for resources with adults and increased ability to withstand local extinction (Levin 1984; Pawlik 1992; Pechenik 1999).

1.4 COMPETENCE, SETTLEMENT, METAMORPHOSIS

The selection of a suitable habitat by settling larvae often determines the long-term survival of juveniles and adults. Consequently, the larvae of a wide range of marine invertebrates do not settle and metamorphose unless they encounter specific conditions that are likely to ensure their growth and survival (Crisp 1974; Crisp et al. 1985; Snelgrove et al. 1999). For most species studied, the settlement of larvae is not random but correlates with biological, chemical and physical factors in the environment (Crisp 1974; Chia and Rice 1978; Pawlik 1992; Hadfield and Paul 2001). The term “*recruitment*” is used as an umbrella term including the release of larvae into the water column, their transport, planktonic mortality, settlement and post-settlement survival (Jenkins et al. 1999). “*Settlement*” is the process by which a planktonic larva moves toward the substratum, explores, attaches to the substratum, and begins its benthic life (Qian 1999). Larvae competent to metamorphose are typically able to remain pelagic in the water column until they encounter proper environmental cues

indicating suitable habitats (Hadfield 1998, 2000). Exceptions are small lecithotrophic larvae that may simply exhaust their energy reserves during extended swimming periods, e.g. ascidian tadpoles, bryozoan coronate larvae and barnacle cyprids. "*Competence*" typically arises in most marine invertebrates at a time, when the development of juvenile structures is all or mostly complete in planktotrophic larvae. The development of competence largely depends on the abundance of particulate food, and thus the age of the larvae at the onset of competence may vary with season and nutrient setting (Hadfield et al. 2001). Whether developed prior to or after hatching of the pelagic larvae, competence permits the marine-invertebrates larvae to continue to live a functional planktonic life while retaining the capacity to settle and metamorphose in response to an environmental cue that may be highly specific (Hadfield et al. 2001). "*Metamorphosis*" is the process by which a planktonic larva goes through morphological and physiological changes to complete the transition from planktonic larva to benthic juvenile (Qian 1999). Metamorphosis typically involves loss of larval characters and emergence or functionalization of juvenile characters. For most marine invertebrates, metamorphosis begins when a pelagic larva irrevocably settles to the sea floor and initiates degeneration of larva-specific characters. It ends when all essential juvenile structures have emerged and the juvenile functions (feeding, moving or is permanently attached) in the final juvenile adult habitat (Hadfield et al. 2001). Metamorphosis can commence even before larval settlement or occur concurrently with larval settlement or right after larval attachment to the substratum (Fenaux and Pedrotti 1988).

1.5 CURRENT KNOWLEDGE OF SETTLEMENT CUES FOR INFAUNA IN SOFT SEDIMENT

For soft sediment habitats, very little effort has been put into identifying cues for larval settlement (Woodin 1986). Several parameters, such as organic content of the sediment (Butman and Grassle 1992; Grassle et al. 1992), the presence of elevated sulfide concentrations (Cuomo 1985), sediment disturbance (Woodin et al. 1998; Marinelli and Woodin 2004), sediment grain size distribution (Pinedo et al. 2000), presence of conspecifics juveniles or adults in the habitat (Highsmith 1982; Olivier et al. 1996; Hardege et al. 1998) and haloaromatic metabolites of sympatric organisms (Woodin et al. 1993; Hardege et al. 1998) have been

discussed. In a similar manner for hard substrate settlers, the role of microorganisms as settlement cues has been suggested for larvae of benthic invertebrates settling on sand e.g. *Ophelia bicornis* and species of *Protodrilus* (Wilson 1955; Gray 1966, 1967). But only few continuative works identifying the role of sediment-associated microorganisms on infaunal recruitment patterns have been carried out since (Hadl et al. 1970; Herrmann 1975, 1995). However, recent experiments of my colleagues with *Polydora cornuta* showed high levels of larval settlement on natural and low levels on sterile and ashed sediment. Quite the opposite is true for *Streblospio benedicti* larvae accepting natural and sterile sediment in a similar percentage but rejecting ashed sediment (Sebesvari et al. 2006). These findings support the thesis that microorganisms influence larval settlement and furthermore, in the case of *S. benedicti*, that other substances e.g. EPS or secondary metabolites mediate larval settlement. *S. benedicti* larvae settled in high numbers on sediment with deadened microorganisms. The exact nature of the cue inducing settlement in this species has yet to be identified.

1.6 LARVAL SETTLEMENT CUES

Naturally, settling larvae exhibit a specific searching behavior upon contact with a suitable surface such as microbial film or macroalgae. Larvae may recognize favourable or unfavourable locations for settlement and metamorphosis through gregarious or associative cues (Crisp 1984). Habitat recognition by larvae is mediated by either positive or negative settlement cues in the form of single or mixed cues (Woodin 1991; Snelgrove and Butman 1994). Some larvae metamorphose in response to very specific cues, while others are generalists and respond to cues of various origins (Qian 1999). For organisms settling on hard substratum laboratory experiments have confirmed that exploring larvae respond to physical factors (Mullineaux and Butman 1991; Maida et al. 1994; Thiyagarajan et al. 2003) and chemical cues of biotic and abiotic origin (Pawlik 1992; Qian 1999; Steinerg et al. 2002). The focus in this study was to investigate biofilms or their components as a potential settlement cue for *P. cornuta* and *S. benedicti* larvae.

BIOFILMS

Microbial biofilms have been found to be important in the settlement processes of representatives of most marine invertebrate groups, e.g. corals (Negri et al. 2001), sponges (Woollacott and Hadfield 1996), cnidarians (Müller et al. 1976; Neumann 1979; Hofmann et al. 1996), annelids (Wilson 1955; Gray 1967; Hadfield et al. 1994), polychaetes (Kirchman et al. 1982a, 1982b; Hamer et al. 2001), bryozoan (Mihm et al. 1981; Brancato and Woollacott 1982; Maki et al. 1989), ascidian (Szewzyk et al. 1991; Wieczorek and Todd 1997), oysters (Fitt et al. 1990), mussels (Satuito et al. 1995), barnacles (Hadfield and Scheuer 1985; Avelinmary et al. 1993; Pechenik et al. 1993; Neal and Yule 1994; Wieczorek et al. 1995; O´Connor and Richardson 1996; Anil and Khandeparker 1998; Khandeparker et al. 2002a, 2003), sea urchins (Cameron and Hinegard 1974), starfishs (Johnson and Sutton 1994), and also algae (Mitchell and Kirchman 1984). Biofilms are a complex network of microorganisms (nonphotosynthetic and photosynthetic bacteria, diatoms, protozoa and fungi) all which are enmeshed in a matrix of extracellular polymeric substances (Mihm et al. 1981; Characklis and Cooksey 1983; Costerton et al. 1995). Each biofilm component may be a settlement cue for larvae. Surfaces are rapidly filmed by microorganisms and adsorbed organic matter. In short succession, the biofilmed surface is colonized by invertebrate larvae and macroalgae. The knowledge about cues transmitted by biofilms to settling larvae of hard substrate settlers has been useful in understanding spatial variations in larval settlement and developing control strategies for biofouling. Less effort has been put into investigating the impact of biofilms for inbenthic organisms in soft-sediment.

BIOFILM-COMPONENTS

1. BACTERIA

Zobell and Allen (1935) first suggested that the presence of bacterial films favored the subsequent attachment of marine macroorganisms. As part of microorganisms, bacteria were among the initial colonizers of new substrate placed in the marine environment (Zobell and Allen 1935; Mitchell and Kirchman 1984) and presented a metabolically active surface to any other micro- or macroorganisms that may approach. The types of bacteria in these films exhibit a patchiness both in their distribution and metabolic activity (Paerl 1985). Due to

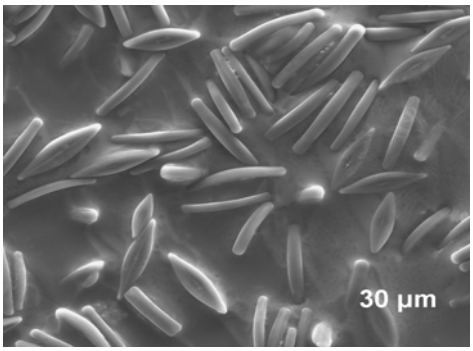
the responsiveness to changes in environmental conditions, bacteria are important informative signposts for surface-exploring larvae. The induction of larval settlement by bacteria is reportedly due to both water-born metabolites (Cameron and Hinegard 1974; Fitt et al 1990; Rodriguez and Epifanio 2000) and cell surface-associated signals (Kirchman et al 1982a; Maki and Mitchell 1985; Maki et al. 1990; Szewzyk et al. 1991). Bacterial films can either induce or inhibit larval settlement of polychaetes (Kirchman et al. 1982a; Hadfield et al. 1994; Bryan et al. 1997; Lau and Qian 1997; Bryan and Qian 1998). Inhibitory and inductive effects of bacterial films may be due to diffusible compounds released by bacteria or bacterial exoplolymers that change the surface matrix (Maki and Mitchell 1985), charges or free surface energy of the substratum or a combination of both (Qian 1999). Certain bacteria have been demonstrated to positively influence the recruitment of a number of marine invertebrate larvae to surfaces by the production of specific molecules (Bonar et al. 1986). Bonar et al. (1986) specified that in several species of gastropod mollusks, experimentally applied neuroactive peptides, e.g. GABA, serotonin, and choline compounds, have been shown to induce metamorphosis. The inhibitory effect by biofilms has been mainly attributed to their bacterial components (Maki et al. 1988; Keough and Raimondi 1995; Wieczored and Tood 1997; Khandeparker et al. 2002; Abdullahi et al. 2006).

2. DIATOMS (BENTHIC MICROALGAE)

Diatoms occur in many soft sediment aquatic habitats (estuaries, shallow subtidal seas, coral reef flats, lakes and rivers) and contribute up to 50% of the total autotrophic production in some ecosystems (Cahoon 1999; Underwood and Kromkamp 1999). They are early colonizers (Jackson and Jones 1988) of the sediment environment. Benthic diatoms occupy considerably larger surface areas than bacteria due to their comparatively large cell sizes (Meadows and Anderson 1968; Round 1971; Kingston 1999a, 1999b; Mitbavkar and Anil 2004). In soft sediments in the German Wadden Sea diatoms dominate these habitats with respect in terms of species diversity and cell density and often exceed cell numbers of more than 10^6 cm^{-2} sediment (Sauer et al. 2002; Meyer et al. 2005). Sediment-inhabiting diatoms migrate vertically in order to stay within the photic zone as the tides, wind, waves, water currents and micro- and meiofauna cause continuous deposition of material onto the surface of the sediment resulting in

burial, loss and detachment of cells. Thus they exhibit a rhythmic, vertical migratory behavior which results in an upward migration onto the sediment surface, generally with the onset of light, followed by a downward movement, generally with incoming tides or when light intensity decreases (Fig. 1).

A: Complete darkness



B: white light

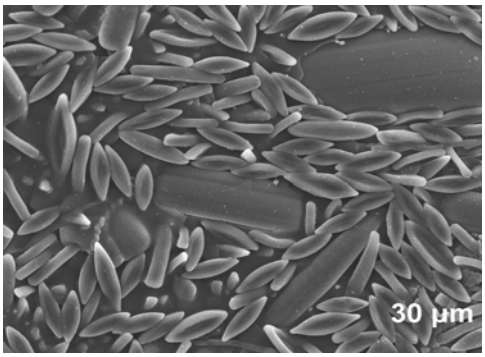


Fig. 1: Electron micrographs of sediment surfaces (magnification; 500X). A: sediment treatment after 3 h incubation in complete darkness and B: in white light of 10 µmol photons^{-2}s^{-1}, received from Dr. Erhard Riel, University of Oldenburg and (Sauer et al. 2002)

In diatoms, this motility is generated by the production of extracellular polymeric substances primarily polysaccharides (Hoagland et al. 1993; Lind et al. 1997); of which carbohydrates are a major component (Smith and Underwood 2000). This migratory behavior was used to investigate larval settlement on sediments in illumination or in darkness.

Algal biofilms provide a major source of energy for higher trophic levels and play a major role in nutrient recycling and mediate the cycling and consumption of O_2 (Dong et al. 2000). The surfaces of biofilms often have uneven topography, and their metabolism is strongly influenced by the diffusive boundary layer (Liehr et al. 1989, 1990). The use of the oxygen microelectrode technique aroused from the idea that the boundary layer thickness can mediate larval settlement. The diffusive boundary layers were created by viscous forces, which cause a thin film of water to “stick” to the surface. The bulk water flows above the sediment. The diffusive boundary layer covers the sea bottom as a thin (most often between 0.3 and 4 mm thick) blanket and surface exploring larvae encounter this layer and may decide if it enters the sediment or not. The water in these films does not participate in the general circulation. Because of the diffusion barrier, this may limit the exchange of dissolved molecules between the surface and the bulk of the liquid. High metabolic rates (due to high densities of microorganisms) on the

sediment surface combined with molecular diffusion acts as the major transport mechanism and result in steep chemical gradients in to sediment in depth of < 50-100 µm (Jorgensen and Revsbech 1985; Revsbech and Jorgensen 1986). The typical sequence found is an upper oxygenic photosynthetic layer with concurrent oxygen respiration and a lower anoxic layer with denitrification and sulfate reduction as the predominant respiratory processes and with anoxygenic photosynthesis, provided sufficient light is penetrating from above (Jorgensen et al. 1983; Revsbech et al. 1989; Kühl 1993; Revsbech et al. 1998).

3. EXTRACELLULAR POLYMERIC SUBSTANCES (EPS)

The abbreviation “EPS” is used as a more general and comprehensive term for different classes of macromolecules such as polysaccharides, proteins, nucleic acids, (phospho)lipids, and other polymeric compounds. The EPS fill and form the space between the cells and is important in the biostabilisation of sediments, especially in estuarine environments (Yallop et al. 1994; Cooksey and Wigglesworth-Cooksey 2001). EPS is responsible for the architecture and morphology of the matrix in which the cells live (Flemming and Wingender 2001). Most works dealing with EPS as meditative settlement cues were processed on hard-substrate settlers (Jensen and Morse 1990; Maki et al. 1990; Szewzyk et al. 1991; Zimmer-Faust and Tamburi 1994; Harder et al. 2002; Lau et al. 2003). There were no further attempts to investigate EPS as a tool for larval settlement of soft bottom settlers. Most bacteria are able to produce extracellular polymers, either as capsules attached to the cell wall or as mucoid secretions in the extracellular environment. These polysaccharides are essentially very long, thin molecular chains with molecular mass of the order of 0.5-2.0*106 Da, but they can associate in a number of different ways (Sutherland 2001). Polysaccharides have been visualized as fine strands attached to the bacterial cell surface and forming a complex network surrounding the cell, electrostatic and hydrogen bonds are the dominant forces (Mayer et al. 1999). They consist mainly of homo- and heteropolysaccharides of glucose, fucose, mannose, galactose, fructose, pyruvate and mannuronic-acid- or glucuronic-acid-based complexes (Brisou 1995) and can be enzymatically degraded (Aldridge et al. 1994; Brisou 1995; Sutherland 1995). The question of the meditative effect of EPS on larval settlement of *P. cornuta* and *S. benedicti* was subject to different larval settlement experiments.

4. LECTINS, MONOSACCHARIDES

Lectins, a class of naturally occurring proteins or glycoproteins exist in almost all living organisms and can recognize and bind carbohydrates specifically and non-covalently. The settlement of a polychaete, *Janua brasiliensis* was also mediated by lectins on the larval surface that were suggested to “recognize” and bind to extracellular polysaccharides or glycoproteins produced by bacterial films containing glucose (Kirchman et al. 1982a). Larvae settled on surfaces coated with bacterial films but did not do so in the presence of concanavalin A (conA), a lectin which binds to glucose and mannose. It was suggested that the lectin had already saturated all the polysaccharide chains. Kirchman et al. (1982a) specified that processes mediated by lectines were inhibited by monosaccharides. Monosaccharides i.e. glucose was the only monosaccharide that blocked settlement and metamorphosis. In the case of *Crassostera*, an oyster, neither the lectins nor monoclonal antibodies blocked the ability of *Alteromonas colwelliana* films to trigger larval settlement (Weiner et al. 1989). However, recently *Lens culinaris* agglutinin sugar chains bound to the settlement-inducing protein complex have been implicated in the settlement of *Balanus amphitrite* (Matsumura et al. 1998). Lectins occur in a broad spectrum of organisms and play a key role in cell adhesion and processes which involve specific recognition between cells during development. The question of whether or not lectins influence larval settlement on soft sediment is not known and is addressed in this thesis.

HALOGENATED SECONDARY METABOLITES (HALOMETABOLITES)

A variety of infaunal polychaetes and hemichordates contain and release high concentrations of noxious e.g. halogenated secondary metabolites (halometabolites) into the sediment (Ashworth and Cormier 1967; Higa and Scheuer 1975a, 1975b; King 1986; Woodin et al. 1987; Steward et al. 1992). They might act as negative settlement cues (Woodin et al. 1993, 1997) but although a number of positive larval responses to biogenic chemical moieties have been described (Highsmith 1982; Suer and Phillips 1983). Within several temperate benthic assemblages 40 taxa were investigated and 43% of the taxa contained halometabolites. This indicates the widespread occurrence of natural halogenated organic compounds among temperate marine infauna (Fielmann et al. 1999). More research was taken on the American coast e.g. on terebellid

polychaete *Thelepus crispus* of the coast of Washington State (USA) but only a few polychaetes were investigated at the German North Sea e.g. the polychaete *Lanice conchileca* (Buhr 1976; Weber and Ernst 1978), both species are high abundant and have a strong odor and their congeners are known to produce several brominated aromatic metabolites.

In previous studies the effect of halometabolites on microorganisms (Zsolnai 1960, Higa and Scheuer 1975b, Sheikh and Djerassi 1975, Stockdale and Selwyn 1971a, 1971b, King 1988, Reineke 2003), fish (Casillas and Myers 1989, Malins et al., 1987) and fish eggs (Reineke 2003) was generally toxic. Halogenated molecules were found in the sclerotized body parts such as seta, operculum, periostricum, skeleton and cuticle of tunicates, mollusks, polychaetes, arthropods, corals and sponges (Hunt 1984) and interfere with respiration and membrane integrity (Escher et al. 1996). The potent deterrent effect of halometabolites on epibenthic predation by fishes and crabs at environmentally realistic sediment concentrations in the nano- to micromolar range indicated a potential ecological role of these compounds in sediments populated by infaunal organisms (King 1986, 1988, Reineke 2003, Cowart et al. 2000). However, thus far the role of halometabolites as biogenic antimicrobials has not been unequivocally identified. For example, whilst bromophenols were assumed to selectively target microbial activity in worm burrows (King 1986, 1988, Giray and King 1997a, 1997b), other studies did not detect any significant antimicrobial effect of this compound class (Jensen 1992, Steward et al. 1992, 1996, Steward and Lovell 1997, Lovell et al. 1999). Besides their antimicrobial efficiency biogenic haloaromatic metabolites have been demonstrated to reduce recruitment of heterospecific, non- producing infauna and deter predators via allelopathy (Woodin et al. 1993, 1997, Yoon et al. 1994, Fielman and Targett 1995, Steward and Lovell 1997). The general hypothesis in all these studies was based on the assumption that the production of halometabolites serves as a defense strategy against microorganisms, predators and interspecific competition.

The antimicrobial properties of halogenated metabolites arises the idea that they influence larval settlement directly, tested on sterile sediments contaminated with halogenated proxies. Or that halogenated proxies spiked on natural sediment firstly influence the bacterial community richness of surface sediments and secondary influence larval settlement of *S. benedicti*. If so, than

halometabolites originated from adult worms (e.g. *S. benedicti* and *Capitella* sp.I) will indirect influence their recruits and mediate larval settlement. Further only halometabolites were put into natural sediment to investigate the larval settlement behavior of conditioned sediment samples.

1.7 PREVIOUS WORK TO INVESTIGATE LARVAL SETTLEMENT CUES FOR *P. CORNUTA* AND *S. BENEDICTI*

The development of repeatable single-choice and multiple-choice assays of me and my colleagues was the precondition to investigate larval settlement for the soft settlers *P. cornuta* and *S. benedicti*. The construction and performance of these assays is described in the following chapters 2 and 3. The single-choice assays with three sediment treatments (natural, sterile, ashed) display clear differences in larval settlement of both species. Natural sediment induced positive larval settlement for *P. cornuta* and *S. benedicti*. Sterilizing and ashing of natural sediment significantly decreases the settlement rate of *P. cornuta* (Sebesvari et al. 2006). The inductive effect on larval settlement was retained for *S. benedicti* on sterile sediment in contrast to *P. cornuta*, whereas ashing of sediment significantly decreased the settlement rate (Fig. 2).

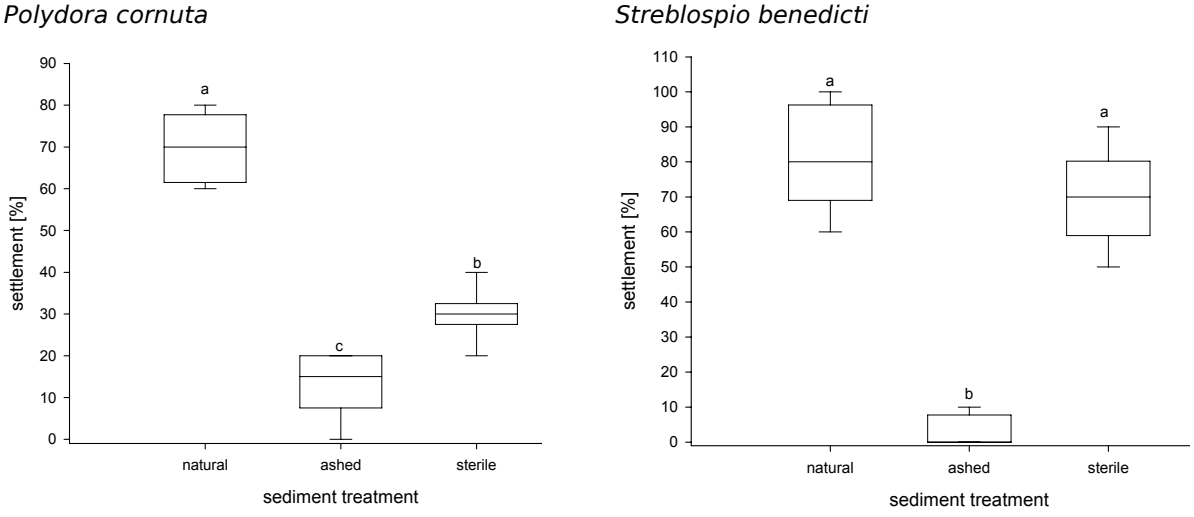


Fig. 2: Larval settlement [%] of *Polydora cornuta* (0.97 ± 0.14 mm body length, 15 setigers) and *Streblospio benedicti* (0.78±0.11 mm body length, 14 setigers) per treatment in a single-choice assay after 1 h. Larval response to natural, ashed, sterile sediment. Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 6 replicates shown in a box-and-whisker diagram.

In the multiple-choice assay a fourth treatment (acid-washed sediment) was added to natural, sterile and ashed sediment treatments (Fig. 3). Both species settled in low rates in acid-washed sediment in comparison to the other sediment treatments. The larval settlement of *P. cornuta* and *S. benedicti* in acid-washed sediment was comparable to the settlement rates in sterile sediment. The harsh treatment of acid washing changed various physical and chemical sediment properties and killed the microorganisms in the sediment. The multiple-choice assay was more sensitive than the single-choice assay sign up in the clear assortment of *S. benedicti* to natural rather than sterile sediment. With *P. cornuta* larvae the results of the multiple-choice assay corresponded to the ones obtained with the single-choice assay (Sebesvari et al. 2006).

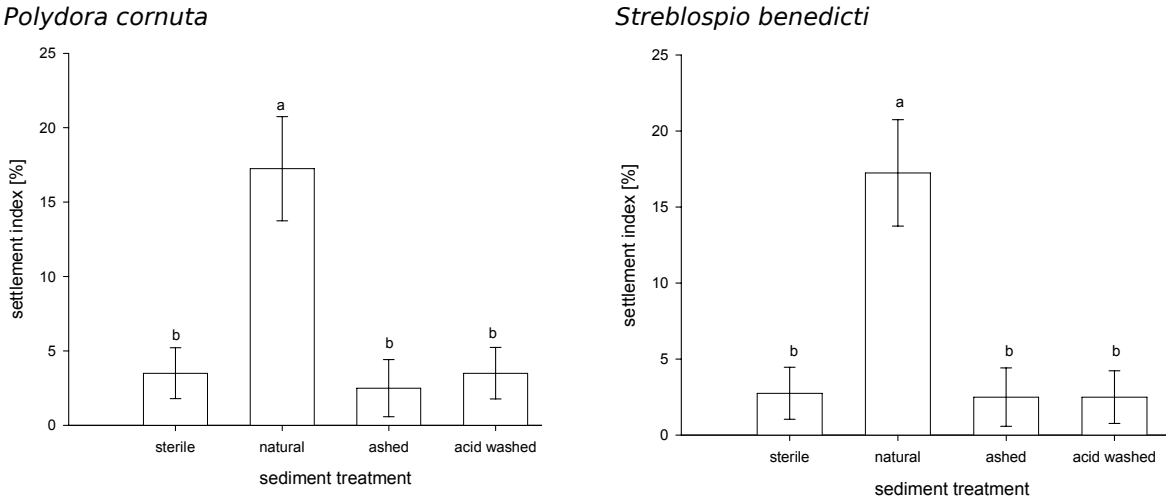


Fig. 3: Larval settlement index [%] of *Polydora cornuta* and *Streblospio benedicti* in a multiple-choice assay after 20 h in response to sterile, natural, ashed and acid-washed sediment in two different assays. Statistical differences are indicated by different letters above the bars (α = 0.05, Tukey's test). Total number of settled larvae was 410 for *P. cornuta* and 383 for *S. benedicti* respectively.

Low settlement rates in ashed sediment treatments in both assay types demonstrated that complete removal of organic material influenced the attractiveness of such sediments dramatically, although other influences such as modified grain size distribution due to fracturing of sand grains might have caused differences in larval settlement response. The effect of material of the ashed sediment dissolving in the overlying water body and consequently influencing larval settlement negatively could be excluded (Sebesvari et al. 2006). The low settlement rates must have been brought about by other changes in sediment quality.

The settlement behavior of *S. benedicti* larvae was not significantly different in single-choice assays in contrast to multiple-choice assays. The larval settlement in sterile in comparison to natural sediment was influenced by e.g. sediment properties susceptible to autoclaving, such as modified sediment colour, texture, structure and/or the elimination of microbial viability. The fact that *S. benedicti* larvae accepted more unattractive sediments in the absence of attractive sediments correspond with the opportunistic character of this species (Levin 1986). The influence of light on larval settlement and of the non-toxic vital stain Neutral Red were not considerable and did not affect larval settlement of either species (Lodemann 2005, Sebesvari et al. 2006).

In summary, both polychaete species were able to accept or reject sediment and choose between different sediment qualities. At this stage, it appeared likely that the loss of “sediment-attractiveness” as a consequence of autoclaving or acid-washing might have been caused at least partially by the removal of sediment-associated microbial viability.

Based on this knowledge (chapter 2 and 3) I investigated larval settlement of *P. cornuta* and *S. benedicti* in order to find out whether microorganisms (bacteria, diatoms), their epipolysaccharides, lectins or their secondary halometabolites influence larval settlement. A series of experiments and methods were performed and described in detail in chapter 4-6.

1.8 OBJECTIVES:

The main question in this dissertation arose why variability in abundance patterns of spionid polychaetes in tidal flats of the Wadden Sea (Germany) exist. Chemical parameters such as organic carbon, nitrogen or silt content were not responsible for these patterns (Stamm 2000). Larval single-choice and multiple-choice settlement experiments with *Polydora cornuta* and *Streblospio benedicti* showed active choice behavior (Sebesvari et al. 2006, 2007), and chose different sediment types. Larvae of *P. cornuta* displayed high settlement on natural and low on sterile and ashed sediment (chapter 2 and 3). Larvae of *S. benedicti* displayed high settlement on natural and sterile and low on ashed sediment. Furthermore both species settled in two cases on sediment inoculated with one bacteria strain similar to the settlement rate on natural sediment. This evidence leads to the assumption that biofilms may influence larval settlement. In particular, I focused on the role of different biofilm components such as benthic diatoms, epipolysaccharides, lectins and monosaccharides as mediators on the larval settlement. Moreover I focused on the meditative settlement effect of direct and indirect influence of halometabolites released from adult worm species into the sediment.

- Do benthic diatoms affect larval settlement of *P. cornuta* and *S. benedicti*?
- Does the thickness of the surface boundary layer of natural, sterile or ashed sediment mediate larval settlement?

The main emphasis of that approach was to focus on the role of the high abundant group of sediment associated benthic diatoms as potential triggers of larval settlement in *P. cornuta* and *S. benedicti*. At the one side natural sediment was treated with algicides or antibiotics to deactivate either the diatom or the bacterial part of surface associated microorganisms. The viable diatoms were tested as meditative settlement cues. At the other side the migratory behavior of diatoms to light was used to gain high diatom densities in sediment in illumination and low densities in sediment kept in darkness. This sediment was used to conduct larval settlement assays with both spionid species. Further on, diatoms were isolated from natural sediment and reestablished on sterile sediment to test the influence of diatoms on larval settlement.

- Do bacteria and diatom EPS extracted from natural sediment and inoculated onto ashed (unattractive) sediment influence larval settlement?

The signal triggering larval settlement may derive from epipolysaccharides as another main component of biofilms produced of bacteria or diatoms. EPS was extracted from natural sediment and transferred onto ashed sediment to test the influence of EPS on larval settlement.

- Do lectins and monosaccharides inoculated on sediment influence larval settlement?

The “lectin receptor” on the outer surface of the larvae presented high binding affinities to carbohydrates found on the sediment surface. The larvae may recognize special binding sides which trigger larval settlement. To test these hypothesis lectins were used to saturate all polysaccharide chains on the sediment surface and larval settlement was investigated. Further sugar monosaccharides were given onto sterile and ashed sediment to test their effect as a meditative settlement cue.

- Do secondary halometabolites at concentrations mimicking those found in adult specimen influence larval settlement of *S. benedicti* larvae directly?

The main emphasis of this approach was to focus on the role of halometabolites as trigger for larval settlement. To test if halometabolites affect larval settlement directly sterile sediment was spiked with commercially available halogenated compounds identical or similar to the dominant halometabolites in worm tissue and larval settlement was investigated.

- Do artificial halometabolites similar to the dominant halometabolites in worm tissue influence larval settlement of *S. benedicti* larvae directly or indirectly?
- Do halometabolites released from adult worms inhabiting natural sediment influence larval settlement of *S. benedicti* larvae indirectly?
- Do GC-MS measurements of halometabolites give indices about the concentration of halometabolites in tissue of adult worm species and in treated sediments?
- Do halometabolites influence the bacterial community on the sediment surface?

Halometabolites acted as antimicrobial agents and may affect the bacterial community. Gas chromatography-mass spectrometry (GC-MS) measurements shall give evidence of the compounds of halometabolites in tissue of adult *S. benedicti* and *Capitella* sp.I and in spiked or untreated sediments. This assumption was used to conduct a time series experiment in use of natural sediment either spiked with halogenated proxies at different concentrations or with adult worms which release these metabolites into the sediment conditioned for seven days in natural seawater. The larval settlement behavior was tested at day zero and day seven and compared to molecular biology data obtained from the same sediments. After extraction of bacterial DNA in treated and untreated sediments, the 16S rDNA fragments were amplified using PCR and denaturing gradient gel electrophoresis (DGGE) to visualize and statistically compare the effect of halogenated proxies on the community composition of bacteria assoziated with the treated and untreated conrol sediments. The aim of this study was to investigate the composition of sediment-associated bacterial community at day zero and after seven days to test if there was a shift in the bacterial community before and after the experimental time. For soft sediment settlers this idea was not detailed in previous investigations.

2 SEDIMENT-ASSOCIATED CUES FOR LARVAL SETTLEMENT OF THE INBENTHIC SPIONID POLYCHAETES *POLYDORA CORNUTA* AND *STREBLOSPIO BENEDICTI*

SUMMARY

Patchy distribution patterns of infaunal polychaetes in the German Wadden Sea were found. In the present study the hypothesis was tested if active site selection of larvae were influenced by sediment associated microbial cues. Two spionid polychaetes *Polydora cornuta* and *Streblospio benedicti* were reared in laboratory brood cultures yielded a sufficient number of larvae with planktotrophic development for bioassays. A single-choice assay was developed using three different sediment qualities obtained from natural sediment from tidal flats, natural, sterile and ashed sediment. Settlement assays were performed and high settlement rates (75-95 %) were found on response to natural sediment for both species. On sterile sediment *P. cornuta* settled to 40-50 % and *S. benedicti* to 75-90 %. Ashing of natural sediment significantly decreased settlement for both species 5-50 %. Differences in settlement response to sediment treated by sterilization or ashing may result from a variety of factors such as sediment fabric, grain size distribution and quantity of adsorbed organic matter. The indirect role of microorganisms as mediators of larval settlement in *S. benedicti* cannot completely ruled out, since bacteria and benthic diatoms largely contribute to the TOC pool in the upper sediment layer due to their secretion of extracellular polymers. To investigate the potential role of microorganisms and microbial metabolites as mediators for larval settlement, ashed sediment was inoculated with viable microorganisms derived from natural sediment. The percentage of larval settlement of both species in all inoculated sediment treatments was significantly higher than in the ashed sediment treatments. This suggested that at last partly larval settlement was mediated by the presence of microorganisms or metabolites associated on the sediment.

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Sediment-associated cues for larval settlement of the infaunal spionid polychaetes *Polydora cornuta* and *Streblospio benedicti*

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Abstract

Do patchy distribution patterns of infaunal polychaetes result from active site selection of larvae influenced by sediment-associated microbial cues? This hypothesis was tested with still-water laboratory settlement assays revealing the acceptance or rejection of polychaete larvae to qualitatively different sediments. Laboratory brood cultures of the spionid polychaetes *Polydora cornuta* and *Streblospio benedicti* yielded a sufficient number of larvae with planktotrophic development for bioassays. High settlement rates (75–95%) of test larvae were observed in response to natural sediment. Sterilization of natural sediment significantly decreased settlement of *P. cornuta* (25–55%) while combustion of sediment significantly decreased the settlement rate in both species (5–50%). Differences in settlement responses to sediments treated by sterilization or combustion most likely resulted from a variety of factors such as modified sediment fabric, grain size distribution and quantity of adsorbed organic matter. To experimentally address the potential role of microorganisms and microbial metabolites as mediators of larval settlement, ashed sediment was inoculated with viable microorganisms obtained from natural sediment. In both polychaete species, this treatment significantly increased larval settlement in comparison to the control of ashed sediment indicating that larval settlement was at least partially mediated by the presence of microorganisms associated with sediment.

Keywords: Larvae; Polychaeta; *Polydora cornuta*; Sediment; Settlement; *Streblospio benedicti*

1. Introduction

Many sessile and hemisessile marine invertebrates, such as oysters, barnacles and tube-building polychaetes, display life cycles with a dispersive larval phase followed by benthic juvenile and adult life stages. During the pelagic phase, which lasts in a species-specific pattern from a few hours to several months, invertebrate larvae distribute into new habitats. Active site selection is commonly observed on small spatial scales while at large scales hydrodynamic processes dominate distribution patterns (Keough and Downes, 1982; Mullineaux and Butman, 1991; Desroy et al., 1997; Harvey and Bourget, 1997; Zimmer and Butman, 2000; Pernet et al., 2003). Habitat selection is mediated by both positive and negative settlement cues (Woodin, 1991; Snelgrove and Butman, 1994). For organisms settling on hard substratum laboratory experiments have confirmed that exploring larvae respond to physical factors (Mullineaux and Butman, 1991; Maida et al., 1994; Thiyagarajan et al., 2003) and chemical cues of different origin (Crisp and Meadows, 1962; Lambert and Todd, 1994; Kirchman et al., 1981; Wieczorek et al., 1995; Wieczorek and Todd, 1998).

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In contrast to settlers on hard substratum the limited mobility of infaunal organisms is not as strict since juveniles and adults are able to migrate within small spatial scales (Cummings et al., 1995; Whitlatch et al., 1998; Snelgrove et al., 1999; Sarda et al., 2000; Norkko et al., 2001; Stocks, 2002; de Montaudouin et al., 2003). Although post-settlement processes influence the spatial distribution of infaunal invertebrates, habitat selection by larvae may set initial distribution patterns (Snelgrove et al., 1999; Snelgrove et al., 2001a; Armonies and Reise, 2003; de Montaudouin et al., 2003). Different parameters have been identified as positive or negative cues of settlement processes of larvae, postlarvae and juveniles, such as the organic content of the sediment (Grassle et al., 1992), sediment disturbance (Woodin et al., 1998; Marinelli and Woodin, 2004), sediment grain size distribution (Pinedo et al., 2000), presence of conspecific juveniles or adults (Olivier et al., 1996; Hardege et al., 1998; Snelgrove et al., 2001b), haloaromatic metabolites of sympatric organisms (Woodin et al., 1993; Hardege et al., 1998) and the presence of bacteria. Although the influence of microorganisms on sediment grains on larval settlement of infaunal animals has been hypothesized in earlier studies (Jaegerstein, 1940; Wilson, 1955; Scheltema, 1961, Gray, 1967), no follow-up works identifying the role of sediment-associated microorganisms on infaunal recruitment patterns have been performed since.

Contrary to infaunal species occupying soft sediments, in hard substrate settlers marine biofilms (agglomerates of attached bacteria, benthic diatoms, fungi and protozoa) have been intensively investigated and demonstrated to elicit a highly specific larval response with respect to their different origin and/or growth phase (Wieczorek and Todd, 1997; Qian et al., 2003; Lau et al., 2005).

The analysis of sediment cores along transects in tidal flats of the Wadden Sea (Germany) revealed distinctive patterns of abundance in the spionid polychaetes *Polydora cornuta* and *Streblospio benedicti* (Stamm, 2000). The observed patterns were neither correlated with sediment characteristics such as the mud content nor the organic carbon and nitrogen content (Stamm, 2000). Since the populations of *P. cornuta* and *S. benedicti* in our study area, i.e., the backbarrier tidal flat of Spiekeroog island, display planktotrophic larval development, we hypothesized that the observed distribution patterns resulted from active larval site selection triggered by factors others than mud, organic carbon and nitrogen content. The main emphasis of this investigation was placed on the potential role of sediment-associated microbial cues as triggers of larval settlement.

To test this hypothesis, we investigated different larval development stages of *P. cornuta* and *S. benedicti* for their ability to actively accept or reject sediments in still-water laboratory assays. Sediments under investigation were natural sediment, sterilized natural sediment, ashed sediment and ashed sediment inoculated with microbial communities obtained from natural sediments. These works comprised the development of laboratory-based spionid cultures, the design of larval settlement bioassays, and the temporal correlation of larval morphology and ability to settle.

2. Materials and methods

2.1. S. benedicti and P. cornuta

The tube-building, infaunal spionid polychaetes *S. benedicti* (Webster, 1879) and *P. cornuta* (Bosc, 1802) (Annelida, Polychaeta) are widely abundant in muddy sands of tidal flats (Levin, 1984; Hartmann-Schröder, 1996). These temperate, hemisessile species occupy the upper sediment layer. With their ciliated tentacles they feed on surface deposits and suspended organic matter (George and Hartmann-Schröder, 1985). *P. cornuta* reproduces by internal fertilization and releases three setiger planktotrophic larvae (Blake, 1969). The mode of larval development in the poecilogonous species *S. benedicti* has been reported to be either lecithotrophic or planktotrophic (Levin, 1984). Spionid settlement is characterized by burrowing activity and metamorphosis into juveniles which build mucoid tubes within a few hours.

2.2. Collection of worms

Adult polychaetes were collected during low tide in mudflats of the Wadden Sea (53°38′31″N, 8°04′55″E) (Hooksiel, Germany). The top sediment layer (3 cm) containing adult polychaetes was transported to the laboratory at 15 °C in covered plastic containers within 1 h after collection. Sediment was dispersed in plastic trays and polychaetes were carefully picked with forceps and sorted under a stereomicroscope according to morphological features, such as shape, number of setigers, presence and number of tentacles and characteristics of the pygidium. Fixed specimens were identified to species level by Hernandez Guevara (Alfred Wegener Institute, Sylt, Germany) and Vöge (Senckenberg Institute, Wilhelmshaven, Germany).

2.3. Collection and treatment of sediment samples

Sediment was repeatedly collected during low tide throughout the year. Surface sediment samples (top 3 mm) were characterized by particle size distribution

(Fritsch Particle Sizer Analysette 22, Germany), total organic carbon and nitrogen content (NA 2000, Fisons Instruments, Germany) and bacterial abundance (Lunau et al., 2005). For the setup of polychaete cultures and bioassays sediment aliquots were processed as follows:

1) Sediment was sieved over 1 mm, washed twice with tap water, frozen at −20 °C for 2 days and defrosted (in the following referred to as "defaunated sediment").
2) Newly collected sediment was stored in the darkness in plastic containers at 4 °C for no longer than 1 week (in the following referred to as "natural sediment").
3) Natural sediment was sterilized by autoclaving immediately before bioassays (in the following referred to as "sterilized sediment").
4) Sediment was ashed at 600 °C for 4 h in a muffle kiln. Before usage in the bioassay, ashed sediment was covered with sterile filtered seawater and autoclaved (in the following referred to as "ashed sediment").
5) Ashed sediment was inoculated with the detachable fraction of microorganisms obtained from natural sediment (in the following referred to as "inoculated sediment"). To obtain these microorganisms 25 g of natural sediment were combined with 100 ml of sterile-filtered seawater and gently shaken overhead for 1 h. After the removal of coarse suspended particles by centrifugation (500×*g*, 1 min) the supernatant was filtered (1.0 μm pore size, Sartorius, Germany) and suspended microorganisms were harvested by centrifugation (6000×*g*, 20 min). The pellet was resuspended in 20 ml sterile seawater and incubated with aliquots of 2.5 g ashed sediment for 1, 2 and 3 days. Non-attached bacteria were removed by washing with sterile seawater. The magnitude of recolonization of ashed sediment by inoculated microorganisms (mainly bacteria) was determined by the relative fluorescence intensity of sediment samples after exposure to fluorescein diacetate (FDA, Sigma, USA) for 1 h (490/525 nm, Fluostar Optima, BMG Labtech, Germany). Specifically, 1 g of inoculated sediment was transferred into sterile 15 ml Corning tubes (Nunc, USA), diluted with 9 ml of sterile seawater and shaken for 1 h (150 rpm). Coarse suspended particles were pelletized at 500×*g* for 1 min. A volume of 200 μl of the supernatant was pipetted into each well of a 96-well plate and incubated with 50 μl FDA working solution (f.c. 0.4 mg ml^{-1}) for 1 h. Every treatment was triplicated and measured four times each. To compare the microbial abundance in these treatments with that in natural sediment the fluorescence intensity of natural sediment in different dilutions was determined accordingly. Autoclaved ashed sediment was used as a negative control to ensure that treatments were not contaminated during the experimental period of 3 days.

2.4. Laboratory maintenance of polychaetes

Culture techniques of polychaetes were adopted from Irvine and Martindale (1999) with modifications. Culture vessels (210 × 160 × 100 mm) were filled with defaunated sediment (3 cm high) and 2 l of filtered (25 μm) natural seawater (FSW) at 30 ppt.

Polychaetes colonized the sediment within a few hours and built new tubes at a surface density of 1–2 (*P. cornuta*) and 5–10 (*S. benedicti*) worms cm^{-2}, respectively. The total culture vessel surface was 2300 cm^2 for *P. cornuta* and 660 cm^2 for *S. benedicti*. Cultures were maintained at constant temperature (18 °C) under 12 h/12 h photoperiod conditions and aeration. Biweekly, adults were fed with ground fish food (Tetra Marin) suspended in FSW. The seawater in the culture vessel was changed daily. With each water change newly hatched polychaete larvae were sieved out (50 μm mesh) and rinsed into aerated 1 l culture vessels. Owing to this procedure, the larval age distribution with the same batch differed by 24 h at maximum. Larval cultures were maintained under constant temperature (18 °C) and photoperiod conditions (12 h/12 h); the water was changed biweekly. Larvae were fed a mixture of the unicellular algae *Isochrysis galbana*, *Dunaliella tertiolecta* and the chryptomonad *Rhodomonas spec*. Algal stock cultures were obtained from the Culture Collection of Algae (University of Göttingen, Germany). The unicellular algae were cultured in 7-l Perspex tubes, *Rhodomonas spec.* was grown in 2-l glass beakers with cotton stoppers. Algae were cultured in f3/4 medium in FSW (Guillard and Ryther, 1962) at 16 °C under aeration and permanent fluorescent light exposure. Algal cells were harvested by centrifugation and resuspended in FSW. Larvae of *S. benedicti* and *P. cornuta* were cultured for 6–8 days and 14–21 days, respectively.

2.5. Larval development and behavior at settlement

During larval development, morphological characteristics such as body length and number of setigers were determined in both species by measurements of digital images (Carl Zeiss AxioCam MRm) taken under the stereomicroscope (Carl Zeiss Stemi SV 11). For this purpose, ca. 100 larvae at different developmental stages were pooled and randomly analyzed. Larval locomotion was traced in a small test chamber (12 × 10 × 35 mm) with a digital camera under the stereomicroscope.

2.6. Settlement bioassays

Settlement assays were carried out with (a) larvae of different age and size, and (b) with different sediment treatments in sterile 12-well microplates (3.8 cm^2 well surface area, Corning, USA). For the assay, 2.5 g (wet weight) of sediment was transferred into each well resulting in a 7 mm sediment layer. The sediment was overlayed with 1.75 ml sterile-filtered seawater and 10 larvae randomly picked with a pipette under the stereomicroscope were added. Experiments were conducted for 1 h with replication ($n=6$). The well plates were maintained under ambient photoperiod conditions. After 45 min, the non-toxic vital stain Neutral Red (Sigma, USA) was added into each well at the final concentration of 10 ng ml^{-1}. After 1 h, the number of swimming (not settled) larvae was counted under the stereomicroscope. Stained larvae on the sediment surface without burrowing activity were interpreted as not settled. Additionally, 50 larvae were randomly picked from the same batch under investigation to determine the body length and numbers of setigers in order to correlate the settlement response with the developmental stage of larvae.

Bioassays were performed as follows:

1) The development-dependent ability of larvae to settle was investigated by observing their response to natural sediment in three independent assay series. On consecutive days, larvae originating from the same batch were used in settlement assays utilizing aliquots of the same sediment sample. The three assay series utilized sediments from samples taken at different times at the same sampling site.
2) To investigate the ability of larvae for habitat acceptance or rejection natural, sterile and ashed sediments were assayed simultaneously with the same batch of larvae of both spionid species with replication ($n=3$).
3) To test the effect of microorganisms associated with the sediment on larval settlement ashed sediment was recolonized with a natural microbial community detached from natural sediment and assayed for both spionid species with replication ($n=2$). To verify that inoculated ashed sediment samples contained a sufficient microbial abundance compared to the control, the fluorescence intensity between treatment and control was measured daily. Once the fluorescence intensity in the treatment differed significantly (Student's *t*-test), the treatment was used in the settlement bioassay.

2.7. Assessment of potential experimental artifacts

To assess the validity of the bioassay results the potential influence of larval staining on settlement was tested. Unstained larvae were simultaneously assayed with larvae stained after 45 min with replication ($n=2$). To test the potential influence of the leachate of ashed sediment on larval settlement ashed sediment was washed twice vigorously with sterile seawater. The larval settlement response to untreated and rinsed ashed sediment was assayed simultaneously. In addition, the supernatant of rinsed ashed sediment added to natural sediment was compared with sterile seawater in the bioassay ($n=2$).

2.8. Statistical analysis

All settlement bioassays were performed with 10 larvae in 6 replicates. The rates of larval settlement were expressed in percentage and tested for normal distribution (Shapiro–Wilk's W-test). Since settlement data were generally not normally distributed, they were rank transformed. After rank transformation, settlement data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test (Conover and Iman, 1980). The data presented in the figures are not transformed.

3. Results

3.1. Sediment characteristics

The number of bacteria associated with natural sediment from Hooksiel ranged around 10^7 cells g^{-1} sediment throughout the year. The mean particle size distribution of this sediment was <20 μm: 30%; 20–63 μm: 15.0%; 63–200 μm: 30%; 200–500 μm: 25%. The organic carbon and nitrogen content in natural sediment was 0.9% and 0.01%, respectively.

3.2. Maintenance of polychaetes under laboratory conditions

The polychaetes *P. cornuta* and *S. benedicti* were successfully cultured with nearly constant larval supply throughout an entire year. Daily yield was 0.7–1.4 and 0.3–0.4 larvae cm^{-2} culture vessel surface for *S. benedicti* and *P. cornuta* resulting in 1000–2000 and 800–1000 larvae, respectively. Both species revealed strict planktotrophic larval development. Larval growth was highly dependent on food quality and larval density in the culture vessel. Optimum culture conditions were achieved with 1 larva ml^{-1} FSW.

3.3. Larval development and settlement behavior

P. cornuta: The pelagic phase lasted for 14–21 days. The number of setigers was significantly correlated with

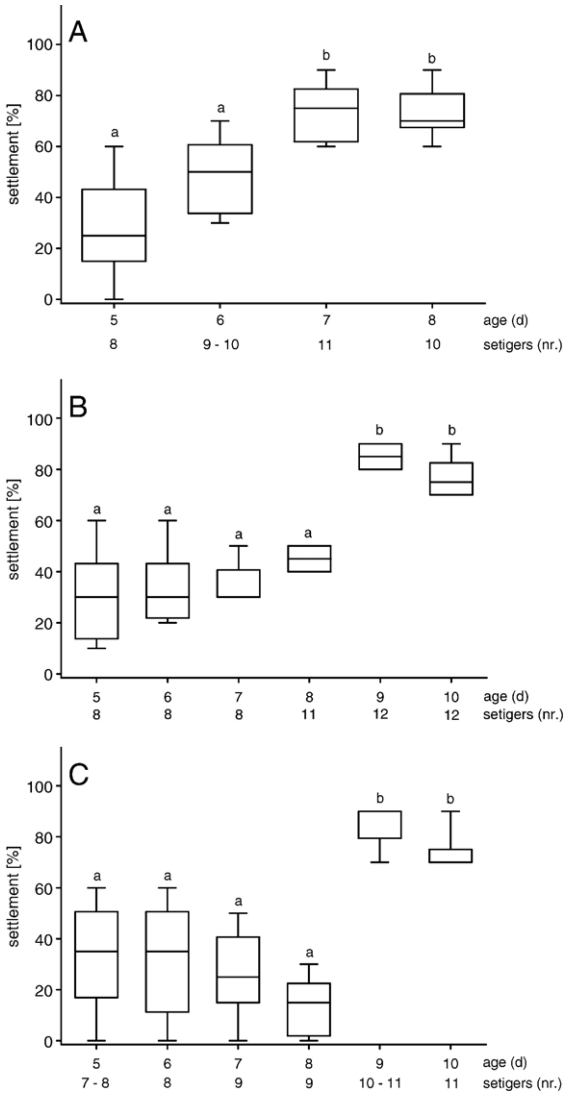

Fig. 1. *S. benedicti.* Percentage of larval settlement after 1 h in response to natural sediment. The bioassay utilized larvae of the same batch but at different developmental stage as indicated by age and number of setigers. The figures in (A–C) present the results of three assays obtained with different batches of larvae. Statistically significant different settlement rates of larvae of different developmental stage are indicated by different letters ($\alpha = 0.05$, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of six replicates shown in a box-and-whisker diagram.

the larval body length ($p < 0.001$, $R^2 = 0.77$). The size variance of 27±5% S.D. among larvae of the same age (±12 h) was high. The maximum number of setigers observed was 18.

S. benedicti: The pelagic phase lasted for 6–8 days. The number of setigers was significantly correlated with the larval body length ($p < 0.001$, $R^2 = 0.90$). The size variance of 18±7% S.D. among larvae of the same age (±12 h) was lower than in *P. cornuta*. The maximum number of setigers observed was 14.

The settlement behavior was characterized as follows: Larvae of *P. cornuta* repeatedly contacted the sediment with the ventral body part during a constant up-and-down movement. The sediment contact lasted from a few seconds to several minutes and was sometimes combined with crawling on the sediment surface. Whilst larvae of *S. benedicti* similarly contacted the sediment, no crawling behavior was observed. Instead, larvae swam near to the sediment surface and touched it repeatedly. Exploratory sediment contact lasted from a few seconds to several minutes in both species. In both species, the actual burrowing activity only lasted a few seconds followed by tube building. Overall, the pre-settlement behavior of larvae was the same on both natural and ashed sediment. The behavior of larvae prior to settlement did not indicate subsequent settlement responses.

3.4. Settlement bioassays

3.4.1. Development-dependent settlement

In *S. benedicti* the magnitude of larval settlement in natural sediment significantly increased with later developmental stage (Fig. 1A–C). In all three repeats, there was a significant difference in larval settlement between larvae of 0.59 and 0.71 mm mean size ($p < 0.05$, Fig. 1A). Generally, larvae displayed high settlement rates after 6–8 days at the 11 setiger stage and an average length

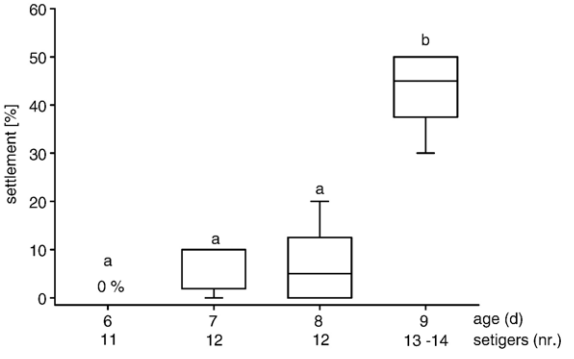

Fig. 2. *S. benedicti.* Percentage of larval settlement after 1 h in response to ashed sediment. The bioassay utilized larvae of a single batch on consecutive days and therefore at different developmental stage as indicated by age and number of setigers. Statistical differences are indicated by different letters above the boxes ($\alpha = 0.05$, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of six replicates shown in a box-and-whisker diagram.

of 0.7 mm. Larvae with more than 12 setigers revealed lower selectivity in assays settling in high percentage even in unfavorable sediment treatments (Fig. 2).

Contrary, larval settlement of *P. cornuta* increased constantly over the developmental phase (Fig. 3A–C). Due to the loss of selectivity at the 16–17 setiger stage in assays with unfavorable sediment treatments (Fig. 4),

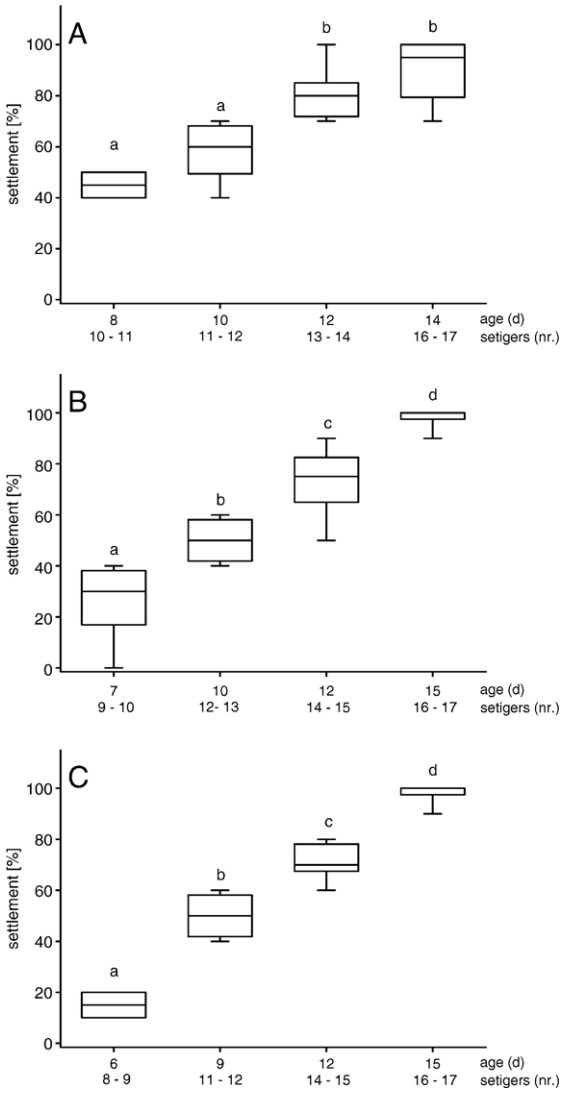

Fig. 3. *P. cornuta*. Percentage of larval settlement after 1 h in response to natural sediment. The bioassay utilized larvae of the same batch but at different developmental stage as indicated by age and number of setigers. The figures in (A–C) present the results of three assays obtained with different batches of larvae. Statistically significant different settlement rates of larvae of different developmental stage are indicated by different letters ($\alpha = 0.05$, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of six replicates shown in a box-and-whisker diagram.

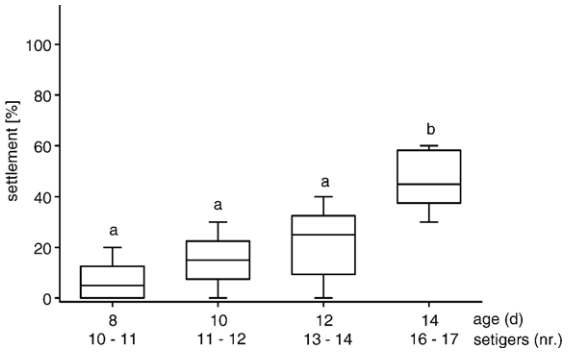

Fig. 4. *P. cornuta*. Percentage of larval settlement after 1 h in response to ashed sediment. The bioassay utilized larvae of a single batch on consecutive days and therefore at different developmental stage as indicated by age and number of setigers. Statistical differences are indicated by different letters above the boxes ($\alpha = 0.05$, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of six replicates shown in a box-and-whisker diagram.

larvae with 14–15 setigers and 1.0–1.1 mm length were used in the subsequent assays.

3.4.2. Larval response to different sediment treatments

The percentage of larval settlement of *S. benedicti* was significantly lower in ashed than in natural sediment (Tukey's test, $p < 0.001$, Fig. 5A–C). Among three experimental repeats, the larval response to natural and sterile sediment treatments differed significantly but no clear trend was observed (Fig. 5A–C). In two repeats, the larval response to natural sediment was higher than in sterile sediment, whilst the opposite was the case in the third repeat.

The percentage of larval settlement of *P. cornuta* was significantly lower in ashed than in natural sediment (Tukey's test, $p < 0.001$, Fig. 6A–C). The larval response in all the sterile sediment treatments was significantly lower than in the natural sediments (Tukey's test, $p < 0.005$, Fig. 6). Among three experimental repeats, the larval response to sterile and ashed sediment treatments did not reveal a clear statistical trend (Fig. 6A–C). In two repeats, the larval response to sterile sediment was the same as in ashed sediment, whilst settlement in sterile sediment was significantly higher than the ashed treatment in the third repeat.

3.4.3. Larval response to ashed sediment inoculated with viable microorganisms

After 3 days of inoculation with viable microorganisms obtained from natural sediment this sediment treatment showed a relative fluorescence significantly different from the control (Student's *t*-test, $p < 0.05$).

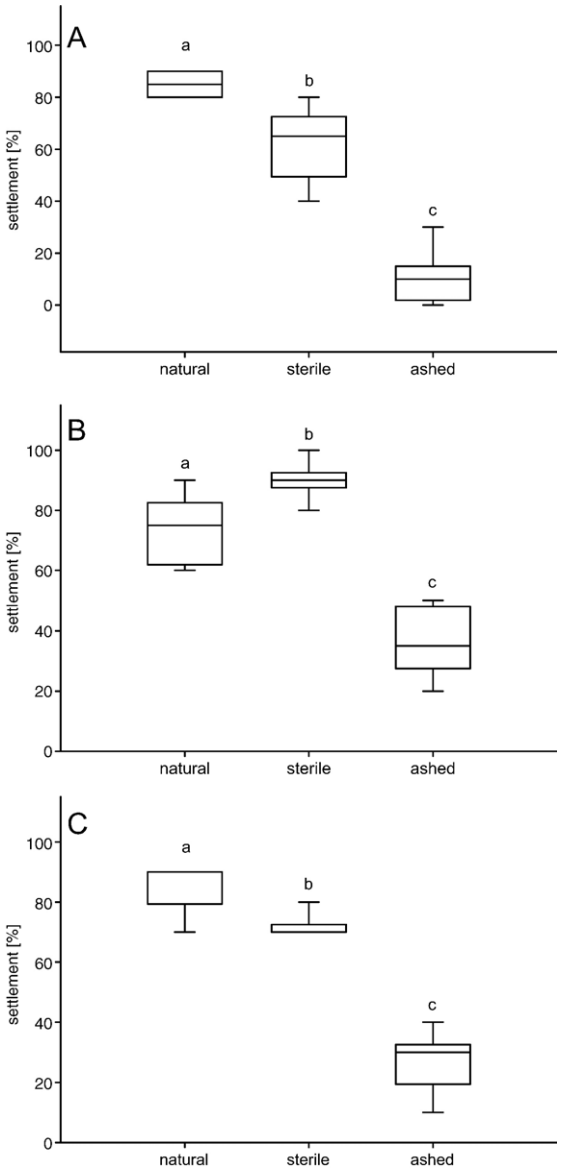

Fig. 5. *S. benedicti.* Mean percentage of larval settlement of test larvae of three different larval batches ((A) 0.78±0.11 mm body length, 11–12 setigers, (B) 0.70±0.11 mm body length, 11 setigers, and (C) 0.66±0.09 mm body length, 10–11 setigers) after 1 h in response to three different sediment treatments, i.e., natural, sterile and ashed. Statistical differences are indicated by different letters above the boxes (α=0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of six replicates shown in a box-and-whisker diagram.

The percentage of larval settlement of both species in all inoculated sediment treatments was significantly higher than in the ashed sediments (Tukey's test, $p<0.05$, Figs. 7 and 8).

3.5. Examination for potential artifacts

There was no effect at the significance level of 95% (Tukey's test) on larval settlement due to staining with the dye neutral red. Similarly, the potential dissolution

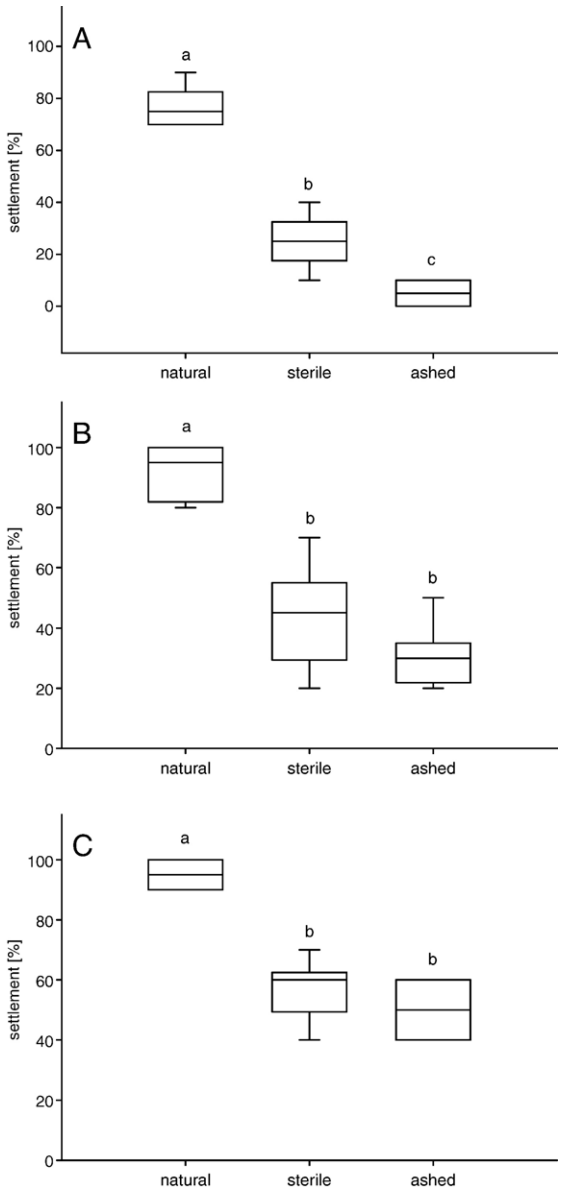

Fig. 6. *P. cornuta.* Percentage of larval settlement of test larvae of three different larval batches ((A) 1.02±0.23 mm body length, 15 setigers, (B) 1.16±0.18 mm body length, 16 setigers, and (C) 1.05±0.15 mm body length, 15 setigers in average) after 1 h in response to three different sediment treatments, i.e., natural, sterile and ashed. Statistical differences are indicated by different letters above the boxes (α=0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of six replicates shown in a box-and-whisker diagram.

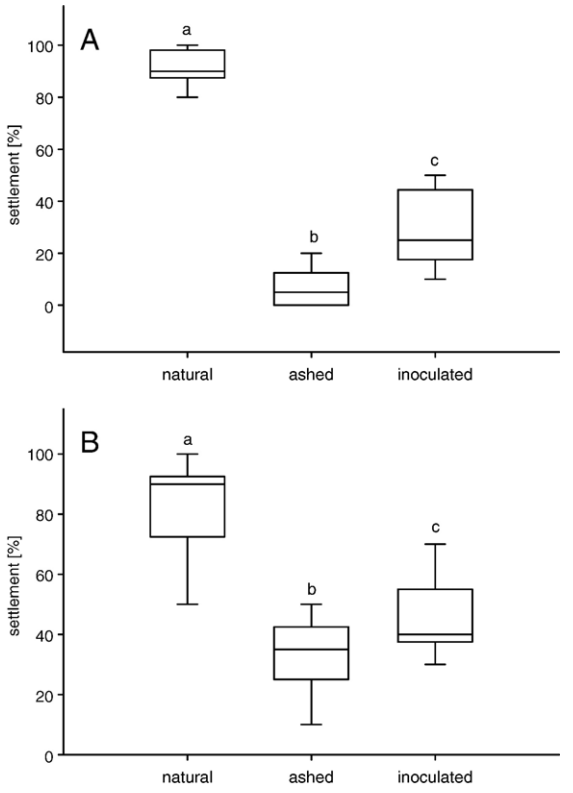

Fig. 7. *S. benedicti.* Percentage of larval settlement of test larvae of two different larval batches ((A) 0.63±0.11 mm body length, 10–11 setigers and (B) 1.02±0.10 mm body length, 13 setigers) after 1 h in response to three different sediment treatments, i.e., natural, ashed and inoculated. Statistical differences are indicated by different letters above the boxes ($\alpha = 0.05$, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of six replicates shown in a box-and-whisker diagram.

of substances from ashed sediment did not influence the settlement of larvae (Tukey's test, $p < 0.05$).

4. Discussion

In this study, laboratory brood cultures of the spionid polychaetes *S. benedicti* and *P. cornuta* were established. The larve obtained from these cultures were used to investigate their ability to actively accept or reject different sediment qualities in laboratory settlement assays.

So far, laboratory studies with larvae of infaunal invertebrates have mainly been carried out with bivalves (Bachelet et al., 1992; Snelgrove et al., 1993; Turner et al., 1994; Snelgrove et al., 1998; Dunn et al., 1999; Cummings and Thrush, 2004) and polychaetes (Jaegerstein, 1940; Wilson, 1955; Gray, 1967; Thiebaut et al., 1998; Cha and Bhaud, 2000). Among the polychaetes, special emphasis has been devoted to *Capitella* sp. I, which in turn has become a standard assay organism for laboratory studies (Grassle, 1980; Cuomo, 1985; Dubilier, 1988; Pechenik and Cerulli, 1991; Biggers and Laufer, 1992; Butman and Grassle, 1992; Snelgrove et al., 1993; Cohen and Pechenik, 1999; Snelgrove et al., 2001b; Marinelli and Woodin, 2004; Thiyagarajan et al., 2005). The larvae of the *S. benedicti* have been used to study the impact of pollutants (endosulfan and PAHs) in marine sediments (Chandler and Scott, 1991; Chandler et al., 1997). So far, the utilization of *P. cornuta* larvae to study active site selection in laboratory bioassays has not been described.

The cultures provided a year-round larval supply for bioassay purposes. In comparison to *P. cornuta*, the culture of *S. benedicti* was characterized by higher larval yield (0.3–0.4 vs. 0.7–1.4 larvae cm^{-2} vessel surface area) and lower size variance (27±5 vs. 18±7% S.D.) among larval cohorts of the same age (±12 h).

Generally, settlement assays with pelagic larvae require clear criteria of larval competence to warrant

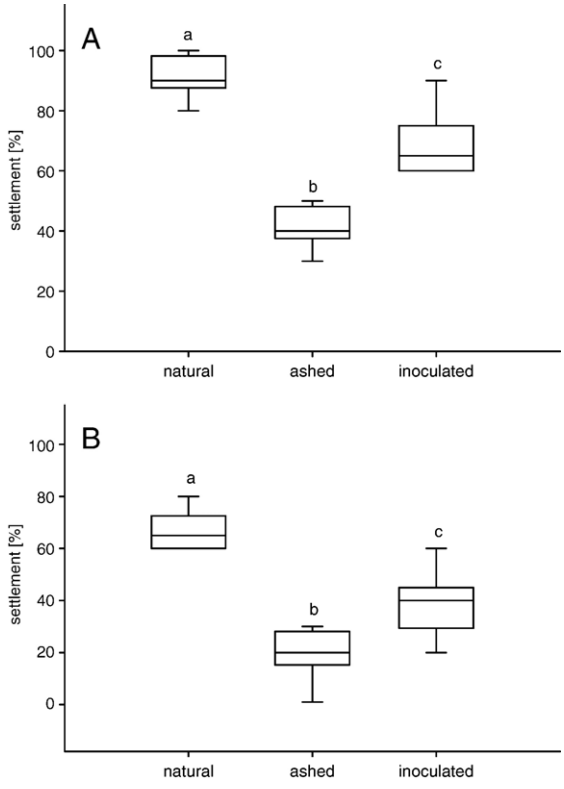

Fig. 8. *P. cornuta.* Percentage of larval settlement of test larvae of two different larval batches ((A) 1.07±0.26 mm body length, 15 setigers and (B) 1.15±0.21 mm body length, 16 setigers) after 1 h in response to three different sediment treatments, i.e., natural, ashed and inoculated. Statistical differences are indicated by different letters above the boxes ($\alpha = 0.05$, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of six replicates shown in a box-and-whisker diagram.

reproducibility and comparability of settlement data obtained under laboratory conditions. In all settlement bioassays performed, we observed a rate of ca. 30±15% settlement in early larval developmental stages of both species on natural sediment, questioning the definition of larval competence (Hadfield et al., 2001) for these polychaetes. However, in *S. benedicti*, there was a reproducible significant increase in larval settlement at later developmental stages (Fig. 1). Beyond this stage, larvae lost their ability to reject formerly unfavorable sediments, such as in ashed sediment (Fig. 2). This phenomenon may have resulted from decreased selectivity due to delayed settlement of aged or "desperate" larvae (see "desperate larva theory" in Toonen and Pawlik, 2001). This observation highlighted the necessity to control the actual development stage of the larvae by photographic analysis and include accurate controls, i.e., natural (positive control) and ashed (negative control) sediment to utilize larvae that displayed maximum selectivity at high settlement rate (in the following referred to as "test larvae").

The larval age was no reliable predictor to estimate the suitability of larvae as test larvae. Their suitability was dependent on the number of setigers, which in turn were significantly correlated with the larval length in both species. For practicability, the larval length could be more easily and quickly determined under the stereomicroscope as the number of setigers. Therefore, the parameter "larval body length" was used as a proxy of the suitability of larvae as test larvae. The required length of *S. benedicti* and *P. cornuta* larvae was at an average 0.7 and 1.0 mm, respectively. At this length, larval settlement on natural sediment ranged between 75% and 95% in both species (Figs. 5 and 6).

The pre-settlement behavior of test larvae was the same on natural and ashed sediment. Just based on the larval behavior prior to settlement it was not possible to deduce subsequent settlement responses. Therefore, observations of larval behavior prior to settlement did not substitute the necessity of settlement bioassays. For the simplicity of the evaluation of settlement bioassays, larval settlement was defined as the number of absent larvae in the water column of the test vessel after 1 h.

Overall, the still-water laboratory assays with three experimental treatments of sediment, i.e., natural, sterile and ashed, revealed clear differences in the larval settlement response in both species. Whilst natural sediment clearly induced settlement in both species, sterilization and combustion of natural sediment significantly decreased the settlement rate of *P. cornuta* (Figs. 5 and 6). This was in contrast to *S. benedicti*, where the inductive effect on larval settlement remained after sterilization of sediment. The ashing of sediment significantly decreased the settlement rate of *S. benedicti* (Fig. 5). In the ashed sediment treatment dissolution of material from the sediment into the water column might have occurred and negatively influenced water quality and, thus, the fitness of the larvae. We addressed this potential artifact with two different experiments and ruled out that the rejection of ashed sediment was influenced by changes in water quality.

In assays with *P. cornuta* the pronounced decrease of larval settlement in sterile in comparison to natural sediment indicated that the larval response was influenced by sediment properties susceptible to autoclaving, such as modified sediment fabric and structure and/or the elimination of microbial viability. Contrary, in assays with *S. benedicti*, the observation of a similar settlement rate in sterile and natural sediment indicated that heat-labile sediment properties and microbial viability were no decisive factors for larval settlement behavior. However, the low settlement rate of both species in ashed sediment indicated that the removal of organic material seemed to influence the suitability of such sediment samples, although other processes such as modified grain size distribution due to fracturing of sand grains might have caused deviations in settlement responses as well. To experimentally address the potential role of microorganisms or their metabolites as mediators of sediment suitability artificially manipulated sediments of the same quality (i.e., ashed sediment) were inoculated with natural communities of viable microorganisms obtained from natural sediment. These treatments evoked a pronounced increase of larval settlement in comparison to the control of ashed sediment (Figs. 7 and 8) indicating that larval settlement of both species was at least partially mediated by the presence of microorganisms. Given the results of the inoculation experiment, the data obtained might be cautiously interpreted as follows: In assays with *P. cornuta* heat and pressure labile microbial constituents act as a positive settlement cue, whilst such constituents are no decisive factors of sediment suitability in *S. benedicti.* However, the low settlement rate of both species in ashed sediment may indicate the requirement of organic carbon associated with the sediment (TOC content) for larval settlement. This interpretation would agree with larval preferences observed in *Capitella* sp. I for high TOC contents (Cohen and Pechenik, 1999).

It remains speculative whether the organic carbon acts as a direct cue or may trigger larval settlement indirectly by influencing biogeochemical sediment parameters, such as cohesion, compaction and pelletization. At this stage, the indirect role of microorganisms as mediators of larval settlement in *S. benedicti* cannot be completely ruled out, since bacteria and benthic diatoms largely

contribute to the TOC pool in the upper sediment layer due to their secretion of extracellular polymers.

A concept of the role of sediment-associated microorganisms as partial mediators of sediment suitability in soft sediment settling organisms would comply with the induction of larval settlement by microbial films in hard substrate settling organisms where both small organic metabolites (Fitt et al., 1990; Taniguchi et al., 1994; Harder et al., 2002) and macromolecular extracellular polymers have been identified as settlement cues (Maki et al., 1990; Szewzyk et al., 1991; Lau et al., 2003; Lam et al., 2005).

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3 MONOSPECIES BACTERIAL FILMS ON SEDIMENT INDUCE LARVAL SETTLEMENT OF THE INFAUNAL SPIONID *POLYCHAETE POLYDORA* CORNUTA AND *STREBLOSPIO BENEDICTI*

SUMMARY

Larval settlement of *P. cornuta* and *S. benedicti* was mediated by microorganisms naturally associated on the sediment surface. The hypothesis was tested if there was a correlation between the phylogenetic affiliation of bacterial isolates and their ability to influence larval settlement. To experimentally address the potential role of single bacteria or their metabolites as mediators for larval settlement, 15 bacteria isolates obtained from sediment of tidal flats in the German Wadden Sea were cultivated in the laboratory and screened for their ability to induce larval settlement. Sterile and ashed sediments were recolonized with bacteria isolates and larval settlement was performed in still water no-choice and in multiple-choice assays. Recolonization of ashed sediment with bacteria isolates resulted in low bacterial cell densities at which none of the 15 isolates triggered larval settlement of both spionids. Recolonization of sterile sediment resulted in higher bacterial densities one magnitude lower than in natural sediments. In *P. cornuta* larval settlement was induced in two out of 15 isolates; DF11 (α-*Proteobacteria*, *Roseobacter clade*) and strain 54 (*Flavobacteria*) in a no-choice assay. The multiple-choice assay with natural, sterile and two treatments with different sediment quantity and quality of bacterial isolates revealed clear differences in larval settlement of both spionids. At bacterial cell densities of 2.0 to 9.0 x 10^8 g^{-1} sediment strain DF11 and 54 induced similar rates of settlement as the control of natural sediment. Strain DF16 (α-*Proteobacteria*, *Roseobacter clade*) did not trigger larval settlement at any of bacteria densities under investigation. Larval settlement on sediment recolonized with strain 54 was higher at low cell densities, indicating that settlement induction is not always positively correlated with bacterial densities. Dead or suspended cells as well as water soluble products of strain DF11 did not trigger larval settlement. The settlement cue for *P. cornuta* and *S. benedicti* may be located in bacterial origin but not related to a unique bacterial genus and associated with the sediment.

MONOSPECIES BACTERIAL FILMS ON SEDIMENT INDUCE LARVAL SETTLEMENT OF THE INFAUNAL SPIONID POLYCHAETES *POLYDORA CORNUTA* AND *STREBLOSPIO BENEDICTI*

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ABSTRACT

Larval settlement of the spionid polychaetes *Polydora cornuta* and *Streblospio benedicti* is induced by microorganisms naturally associated with surface sediment. In the present study, 15 bacterial isolates obtained from the habitat of adult spionids belonging to 5 phylogenetic classes i.e. α-*Proteobacteria* (4), γ-*Proteobacteria* (3), *Bacilli* (3), *Flavobacteria* (3) and *Sphingobacteria* (2), were screened for their ability to induce larval settlement in *P. cornuta* and *S. benedicti*. Manipulated sediments, i.e. ashed and sterilized natural sediments, were recolonized with bacterial isolates and used in still-water laboratory settlement bioassays with and without choice option for larvae. Recolonization of ashed sediment with bacteria resulted in low bacterial cell densities (< 10^5 cells g^{-1} sediment) at which none of the 15 isolates triggered settlement of spionid larvae. Recolonization of sterilized natural sediment resulted in bacterial densities between 10^7-10^8 cells g^{-1} sediment, i.e. one order of magnitude lower than in natural sediments. In single-choice assays with *P. cornuta* on recolonized sediments two out of 15 isolates, i.e. strain DF11 (α-*Proteobacteria*, *Roseobacter*-clade) and strain 54 (*Flavobacteria*), triggered a significant settlement response. Both inductive isolates and one non-inductive isolate strain DF16 (α-*Proteobacteria*, *Roseobacter-clade*) were further tested in multiple-choice assays using four parallel experimental treatments of sediment, i.e. natural and sterile sediment and two sediment treatments of different quantity and quality of bacteria. The multiple-choice assays revealed clear differences in larval settlement of both spionids. Generally, natural sediment triggered high rates of settlement while sterile sediment evoked significantly lower rates of settlement. At bacterial cell densities of 2.0 to 9.0 x 10^8 g^{-1} sediment strain DF11 and 54 induced similar rates of settlement as the control of natural sediment. Strain DF16 did not trigger larval settlement at any of the densities (2.0 x 10^6 to 5.0 x 10^7 g^{-1}) under investigation. Dead or suspended cells as well as water soluble products of strain DF11 did not induce larval settlement either. The results of this study suggest that the settlement cue of *S. benedicti* and *P. cornuta* is of bacterial origin but not related to a unique bacterial genus, heat labile and associated with the sediment.

Keywords: Bacteria, Biofilm, Larval settlement; *Polydora cornuta*; Sediment; *Streblospio benedicti.*

INTRODUCTION

Marine biofilms, agglomerates of surface-attached bacteria, benthic diatoms, fungi and protozoa, have been shown to stimulate or inhibit larval settlement of benthic invertebrates settling on hard substratum (reviewed by Wieczorek and Todd 1998, Steinberg, 2002). Differential larval settlement patterns on surfaces covered with biofilms of different origin or growth under different environmental and physiological conditions indicated a highly specific larval response towards biofilm-derived cues (Wieczorek et al., 1995; Wieczorek and Tood 1997; Olivier et al., 2000; Hamer et al., 2001; Qian et al., 2003).

Contrary, studies on settlement cues of infaunal organisms are rare and date back to early studies of Jägersten (1940) and Wilson (1955). The archiannelid *Protodrilus rubropharyngeus* metamorphoses in the presence of shells or gravels obtained from its original habitat (Jägersten, 1940). The settlement cue was hypothesized to be an inorganic, resistant material produced by microorganisms associated with shells and gravels. The first systematic study of microorganisms as potential candidates for larval settlement cues of infaunal organisms investigated the influence of the presence and quality of sediments for the bivalve *Ophelia bicornis* (Wilson, 1948, 1953a, 1953b, 1954, 1955). In the search for the "attractive factor" for larval settlement sediments of different attractiveness were mixed (Wilson, 1953) and acid-washed sediment was soaked in natural and filtered seawater (Wilson, 1954, 1955). In 1955, Wilson concluded that the presence of living organisms on sand grains, such as bacteria in certain densities is the most active factor for the induction of larval metamorphosis in *O. bicornis*. Follow up works by Gray (1966, 1967) studying substrate selection in *Protodrilus symbioticus* and *P. rubropharyngeus* (Polychaeta) demonstrated that sterilized sediment recolonized with both natural bacterial communities and bacterial isolates increased the attractiveness of the substrate to larvae. Larval settlement was increased in sediment treatments with *Pseudomonas* sp. and *Flavobacterium* sp. compared to treatments with bacteria obtained from natural sand. Since then, only Herrmann (1975, 1995) studied metamorphic cues of *Phoronis mülleri* (Tentaculata, an infaunal invertebrate) using bacterial isolates suspended in seawater. Metamorphosis of *P. mülleri* was induced in the water column and was dependent on the growth phase and density of the bacteria.

These experiments provided sufficient evidence to further investigate the hypothesis that sediment-associated microorganisms may be involved in site

selection and larval settlement of infaunal organisms. We have previously shown that the sterilization of natural sediment significantly decreased settlement of *P. cornuta* larvae in single-choice assays while combustion of sediment significantly decreased the settlement rate in *P. cornuta* and *S. benedicti* (Sebesvari et al., 2006). The recolonization of ashed sediment with microorganisms obtained from natural sediment enhanced larval settlement indicating that settlement was at least partially mediated by the presence of microorganisms (Sebesvari et al., 2006).

In this study, we revisited the phenomenon of bacterially influenced settlement responses in Polydora cornuta and Streblospio benedicti and tested the hypothesis of a correlation between the phylogenetic affiliation of bacterial isolates and their ability to stimulate larval settlement. In total, 15 isolates from intertidal surface sediments of the Wadden Sea were recolonized on ashed and autoclaved sediment and screened for larval settlement of both spionids in still water assays without choice. Subsequently, selected bacterial isolates were further investigated in a series of still water multiple-choice assays at different cell densities of bacteria.

MATERIAL AND METHODS

TEST ORGANISMS

The tube-building, infaunal spionids *Streblospio benedicti* (Webster, 1879) and *Polydora cornuta* (Bosc, 1802) (Annelida, Polychaeta) are widely abundant in muddy sands of intertidal flats (Levin, 1984; Hartmann-Schröder, 1996). Both spionids reproduce by internal fertilization and display indirect development.

Females of *Polydora cornuta* release planktotrophic larvae with 3 to 4 setigers and 260 to 280 µm length (Blake, 1969; Hartmann-Schröder, 1996; Radashevsky, 2005). The planktotrophic phase of larvae lasts 2 to 3 weeks (Anger et al., 1986). After larvae reach 1.200 – 1.300 µm and 17 to 18 setigers they are able to settle and metamorphose (Hartmann-Schröder, 1971).

The mode of larval development in the poecilogonous species *Streblospio benedicti* is either lecithotrophic or planktotrophic (Levin, 1984). Planktotrophic larvae from small eggs hatch with 3 to 5 setigers and 220 - 230 µm body length (Levin, 1986). Planktonic development ranges from 2 to 3 weeks (Levin, 1984) to 7 - 45 days (Blake and Arnofsky, 1999). Lecithotrophic larvae leave the maternal

tube with ca. 640 µm length and 10 to 12 setigers, immediately attain competence to settle (Levin and Creed, 1986) and may remain pelagic up to one week (Levin, 1984). *Streblospio benedicti* larvae settle at the 10 to 13 setiger stage (Blake and Arnofsky, 1999).

LARVAL CULTURE

Larvae of *Polydora cornuta* and *Streblospio benedicti* were obtained from our laboratory brood stock (Sebesvari et al., 2006). Briefly, defaunated sediment was colonized by spionids at average surface density of 1 to 2 (*Polydora cornuta*) and 5 to 10 (*Streblospio benedicti*) worms cm^{-2}, respectively. Cultures were maintained at constant temperature (18 °C) under 12h/12h photoperiod conditions and aeration. Biweekly, adults were fed ground Tetra Marin (Tetra, Germany) fish food suspended in filtered seawater (FSW). The culture medium (FSW, 30 ppt salinity) was changed biweekly. Every day, newly hatched larvae were sieved out (50 µm mesh) and rinsed into aerated culture vessels. The larval age distribution within the same batch differed by 24 h at maximum. Larval cultures were maintained under constant temperature (18 °C) and photoperiodic conditions (12h/12h); the water was changed biweekly. Larvae were fed a mixture of the unicellular algae *Isochrysis galbana*, *Dunaliella tertiolecta* and the chryptomonad *Rhodomonas spec.* Larvae of *S. benedicti* and *P. cornuta* reached competence after 6 to 8 d and 14 to 21 d, respectively. *S. benedicti* displayed planktotrophic larval development. Larval competence was estimated according to larval body length and number of setigers and recorded by length measurement. In single-choice assays, where the larval quality strongly influenced the assay results, additional tests were carried out to ensure larval competence and selectivity:

1) An aliquot of test larvae was subjected to a positive control known to trigger settlement, i.e. fresh natural sediment from the habitat of adult polychaetes (Sebesvari et al., 2006).

2) An aliquot of test larvae was subjected to a negative control, i.e. ashed sediment from the habitat of the adult polychaetes, known to be rejected by larvae (Sebesvari et al., 2006). Statistically significant lower larval settlement in the negative control than in the positive control indicated appropriate selectivity of test larvae.

COLLECTION OF SEDIMENT

Sediment samples were collected in intertidal mud flats of the Wadden Sea, Hooksiel, Germany. For assay purposes sediment was repeatedly collected during low tide throughout the year.

ISOLATION OF BACTERIA AND PHYLOGENETIC ANALYSES

11 of the 15 bacterial strains used in this study were obtained from intertidal surface sediment at Hooksiel. For detachment of bacteria from sediment triplicate samples of 1 g fresh surface sediment (topmost 1 - 2 mm) were suspended in 9 ml 0.001 % (vol/vol) Tween 80 in seawater and shaken for 1 h. From each sediment suspension a serial dilution was prepared and 100 µl aliquots were spread onto marine nutrient agar (0.5 %, peptone, 0.3 % yeast extract, 1.5 % agar in seawater) in triplicates. Agar plates were incubated at 24 ˚C for 72 h. Different colony types were isolated and regrown at least 3 - 5 times on nutrient agar. To establish stock cultures, the isolates were grown to the stationary phase in nutrient broth (0.5 %, peptone, 0.3 % yeast extract in seawater), mixed with an equal volume of autoclaved glycerol and stored at – 80 °C in 1 ml aliquots. *Phaeobacter inhibens* strain T5, an antibiotic producing bacterium was obtained from a bulk water sample taken from the German Wadden Sea, and strain T5-3 was a spontaneous mutant of *P. inhibens* without antibiotic production (Brinkhoff et al., 2004). Strain DF11 and strain DF16 were isolated from an intertidal surface sediment sample taken from the German Wadden Sea near Neuharlingersiel. Aliquots (ca. 100 µl) of the fresh sediment were spread on agar plates prepared with natural seawater (from the same location), containing 10% sediment, 0.1% yeast extract and 1.5% agar. Plates were incubated at 15°C in the dark. Different types of colonies were selected and transferred at least three times until considered as pure. After isolation strains were transferred for routine cultivation on marine agar 2216 (Difco, USA). Purified PCR amplicons of bacterial 16S rRNA genes were sequenced bidirectionally using an ABI PRISM™ big-dye terminator cycle-sequencing ready-reaction kit (Applied Biosystems). Obtained 16S rRNA gene sequences of each isolate were compared to sequences in the non-redundant nucleotide database in GenBank using BLAST (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/blast).

TREATMENT OF SEDIMENT SAMPLES

For bioassays sediment samples were processed as follows:

1) Newly collected sediment was stored in the darkness in plastic containers at 4 °C for no longer than 2 weeks (in the following referred to as “natural sediment”).

2) Natural sediment was sterilized by autoclaving immediately before use (in the following referred to as “sterilized sediment”).

3). Sediment was ashed at 600°C for 4 h in a muffle kiln. Before usage in the bioassay, ashed sediment was washed with sterile seawater twice and autoclaved (in the following referred to as “ashed sediment”).

4) To recolonize sediment with bacteria, ashed or sterile sediment was inoculated with bacterial isolates (in the following referred to as “recolonized sediment”). For recolonization, bacterial colonies were picked from agar plates and grown in marine broth to the stationary phase (0.5 %, peptone, 0.3 % yeast extract in seawater) at 24 °C. 20 ml of the bacterial suspension were pipetted into sterile 50 ml tubes (Nunc, USA) and bacteria were harvested by centrifugation (5000 g, 20 min). The bacterial pellet was resuspended in 10 ml of sterile seawater. From the bacterial suspension 3 ml aliquots were used to inoculate 20 g of ashed or sterile sediment. Inoculation was carried out in sterile 50 ml tubes over night at 24 °C. Following inoculation, the sediment was centrifuged at 800 x g for 2 min and the supernatant was decanted.

Aliquots of recolonized sediments were further treated to influence either the bacterial density through washing (in the following referred to as “washed recolonized sediment”) or the bacterial viability through heating (in the following referred to as “heated recolonized sediment”).

For "washed recolonized sediment", recolonized sediment was centrifuged at 800 x g for 2 min to pelletize the sediment. The supernatant was removed and 10 ml of sterile seawater were added. After vigorous shaking, the sediment was centrifuged at 800 x g for 2 min. The procedure was repeated twice.

For "heated recolonized sediment", recolonized sediment was placed in a water bath for 2 h at 60 °C. During heat exposure, the bulk of bacteria were killed. This less invasive methodology was preferred over autoclaving due to its presumably weaker modification of sediment properties and bacterial exopolymers.

MONITORING OF TREATMENT EFFICIENCIES

The magnitude of sediment-recolonization by inoculated bacteria and the success of treatments, such as washing and heating, were measured before settlement assays using the relative fluorescence intensity of sediment samples after exposure to the viability stain fluorescein diacetate (FDA, Sigma, USA). Whilst this method was used as a fast screening before usage of recolonized sediment in settlement assays, bacterial cell densities were determined subsequently by counting the number of colony forming units using the dilution plate count technique.

Specifically, 1 g of sediment was transferred into sterile 15 ml tubes (Nunc, Wiesbaden, Germany), diluted with 9 ml of 0.001 % SDS in sterile seawater and shaken for 1 h (150 rpm). Coarse suspended particles were pelletized at 800 x g for 1 min. For analysis, 200 µl of the supernatant were dispensed into black 96-well microplates (Fluoronunc F96, Nunc, Germany) and incubated with 50 µl FDA working solution (f. c. 0.4 mg ml^{-1}) for 40 min in the darkness. In living bacterial cells FDA is transformed into fluorescein by intracellular hydrolysis. Fluorescence intensity was determined in a microplate reader (Fluorostar Optima, BMG Labtechnologies, Germany) at 485/520 nm. All measurements were carried out in three reading cycles with integration of 10 flashes and 0.2 s delay between plate movement and readings. Every treatment was duplicated or triplicated and measured 4 times each. To compare the microbial abundance in these treatments with that in natural sediment the fluorescence intensity of natural sediment in different dilutions was determined accordingly. Fluorescence intensities of the dilution series were compiled into a calibration curve, which expressed the bacterial density of sediment samples as the percentage of the microbial density in natural sediment. The temporal and spatial variability in abundance of bacteria on natural Wadden Sea sediment is usually low (Köpke et al., 2005), which makes natural sediment a useful control to "calibrate" the relative bacterial density of treated sediment samples. Recolonized sediment treatments (before washing or heating) with lower fluorescence intensity than 0.01 diluted natural sediments were not used in settlement assays.

The bacterial cell density in recolonized sediment treatments was determined with the dilution plate count technique as follows. After detachment of bacteria from sediment by shaking in 0.001 % SDS solution for 1 h, a dilution series was

prepared. 100 µl aliquots of different dilutions were spread on agar plates in triplicates and incubated upside down for 1 to 4 days at 24 ˚C.

LARVAL SETTLEMENT RESPONSES TOWARDS MONO-SPECIES BACTERIAL SEDIMENT TREATMENTS (SINGLE-CHOICE ASSAYS)

Single-choice settlement assays were carried out with different sediment treatments in sterile 12-well microplates (3.8 cm^2 well surface area, Corning, USA). 2.5 g (wet weight) of sediment was transferred into each well resulting in a 7 mm sediment layer. The sediment was overlaid with 1.75 ml sterile-filtered seawater and 10 larvae randomly picked with a pipette under the stereo microscope were added. Experiments were conducted for 1 h with replication (n = 6). The well plates were maintained under ambient photoperiod conditions. After 45 min, the vital stain Neutral Red (Sigma, USA) was added into each well at the final concentration of 10 ng ml^{-1}. After 1 h, the number of swimming (not settled) larvae was counted under the stereo microscope. Stained larvae on the sediment surface without burrowing activity were interpreted as not settled. Additionally, 50 larvae were randomly picked from the same batch under investigation to determine the body length and numbers of setigers in order to record the developmental stage of larvae.

Ashed and sterile sediment was recolonized with 15 different isolates and assayed with either both spionid species and *Polydora cornuta* only with replication (n = 2). In both assay series, due to the limited number of larvae available on the same day, usually 2 to 4 isolates were assayed together with controls of natural (positive), and sterile and ashed (negative) sediments with larvae from the same batch.

EFFECT OF BACTERIAL CELL DENSITY AND VIABILITY ON LARVAL SETTLEMENT (MULTIPLE-CHOICE ASSAY)

Multiple-choice settlement assays were carried out with four different sediment treatments in sterile cylindrical Plexiglas containers (∅ 17 cm, 1.5 cm high) with a removable Plexiglas bottom and sixteen cylindrical slots (∅ 2 cm, 0.3 cm depth). The slots were arranged in four by four rows and columns separated by 1-cm bars. In a 4 x 4 Latin-square design four replicates of four different sediment treatments were placed in the container. Every treatment was placed exactly

once per row and column. In the following, the 16 sediment-filled slots are referred to as "sites". The sediment was overlaid with 200 ml of sterile-filtered seawater and 300 - 500 and 600 - 700 larvae of *Polydora cornuta* and *Streblospio benedicti*, respectively, were added. Experiments were conducted for 20 h in darkness. After 20 h, the Plexiglas bottom was removed and the sediment treatments were separated in glass plates. Neutral Red (Sigma, USA) was added to each plate at the final concentration of 10 ng ml^{-1} and the number of settled larvae in each treatment was counted under the stereo microscope. Settlement rates were related to the total number of settled larvae (100 %) discounting all larvae still swimming or lying on the sediment surface without burrowing activity. The results were presented as percentage of average settlement per site of each treatment.

To investigate the ability of larvae to actively choose between different sediment treatments, such as natural, sterile, and sediment with different bacterial qualities and quantities recolonized sediments were assayed in multiple-choice assays. For the multiple-choice assays only selected isolates were used to carry out the following assays:

Assay 1: sediment treatments were a) sterile sediment, b) natural sediment, c) sterile sediment recolonized with strain DF11 and d) sterile sediment recolonized with strain DF11 followed by washing.

Assay 2: sediment treatments were a) sterile sediment, b) natural sediment, c) sterile sediment recolonized with strain 54 and d) sediment recolonized with strain 54 followed by washing.

Assay 3: sediment treatments were a) sterile sediment, b) natural sediment, c) sterile sediment recolonized with strain DF16 and d) sediment recolonized with strain DF16 followed by washing.

Assay 4: sediment treatments were a) sterile sediment, b) natural sediment, c) sterile sediment recolonized with strain DF11 and d) sterile sediment recolonized with strain DF11 followed by heating at 60˚C in the water bath for 2 h.

Assay 5: sediment treatments were a) sterile sediment, b) natural sediment, c) sterile sediment recolonized with strain 54 and d) sterile sediment recolonized with strain 54 followed by heating at 60˚C in the water bath for 2 h.

Assay 6: all sediment treatments were recolonized with strain DF11. Bacterial suspensions used for recolonization were either undiluted or 10^{-2}, 10^{-4} and 10^{-6} diluted. A summary of all performed assays is given in Tab. 1.

Tab. 1 List of performed multiple-choice assays.

Assay no.	Species	Treatment			
		1	2	3	4
Assay 1 a	*S. benedicti*	sterile	natural	DF11	DF11 washed
Assay 1 b	*P. cornuta*	sterile	natural	DF11	DF11 washed
Assay 2 a	*S. benedicti*	sterile	natural	54	54 washed
Assay 2 b	*P. cornuta*	sterile	natural	54	54 washed
Assay 3 a	*S. benedicti*	sterile	natural	DF16	DF16 washed
Assay 3 b	*P. cornuta*	sterile	natural	DF16	DF16 washed
Assay 4 a	*S. benedicti*	sterile	natural	DF11	DF11, 60℃
Assay 4 b	*P. cornuta*	sterile	natural	DF11	DF11, 60℃
Assay 5 a	*S. benedicti*	sterile	natural	54	54, 60℃
Assay 5 b	*P. cornuta*	sterile	natural	54	54, 60℃
Assay 6	*S. benedicti*	DF11	DF11, dil. 10^{-2}	DF11, dil. 10^{-4}	DF11, dil. 10^{-6}

INVESTIGATION OF WATER SOLUBLE BACTERIAL PRODUCTS ON LARVAL SETTLEMENT

An assay was designed to determine whether soluble products from strain DF11 induced larval settlement of Polydora cornuta. Bacterial colonies were picked from agar plates and grown either in marine broth or on sterile sediment suspended in sterile filtered seawater to stationary phase at 24 °C. Both cultures were maintained either stationary or under shaking conditions to enrich marine broth and seawater with water soluble bacterial metabolites. At stationary phase, 20 ml of the bacterial suspension or bacterial/sediment suspension were pipetted into sterile 50 ml tubes (Nunc, USA) and bacteria and sediment particles were pelletized by centrifugation (5000 g, 20 min). The supernatant was sterile filtered (0.22 µm) and used to cover sterile sediment in single-choice assays. Settlement on sterile sediment covered with supernatant was compared with settlement on natural and sterile sediment covered with sterile filtered seawater. Two assay-series were carried out with 5 different sediment types being assayed simultaneously. In the first assay-series sediment treatments were natural sediment, sterile sediment covered with sterile marine broth, sterile sediment covered with sterile filtered marine broth enriched with bacterial metabolites maintained either stationary or by shaking and sterile sediment recolonized with strain DF11 (n = 2). In the second assay series, instead of marine broth a sediment/seawater suspension was used as a food source for the bacteria.

Sediment treatments were natural sediment, sterile sediment covered with sterile filtered seawater, sterile sediment covered with sterile filtered seawater enriched with bacterial metabolites maintained either stationary or by shaking and sterile sediment recolonized with strain DF11 (n = 2).

INVESTIGATION OF SUSPENDED BACTERIAL CELLS ON LARVAL SETTLEMENT

An assay was designed to determine whether suspended cells of strain DF11 induced larval settlement of *Polydora cornuta*. Cells of strain DF11 were harvested from marine broth by centrifugation. The pellet was resuspended and diluted in sterile filtered seawater. The bacterial suspension was used immediately in settlement assay. Three arbitrarily chosen cell densities were used in assays and afterwards quantified by the dilution plate count technique.
Sediment treatments used in this assay were natural sediment, sterile sediment covered with sterile filtered seawater, and sterile sediment covered with bacterial suspension at different densities (n = 2).

STATISTICAL ANALYSIS

All single-choice assays were performed with 10 larvae in 6 replicates. The rates of larval settlement were expressed as percentage and tested for normal distribution (Shapiro-Wilk's W-test). Since settlement data are generally not Gaussian distributed they were rank-transformed prior to further statistical analyses. After rank transformation, settlement data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test (Conover and Iman, 1980).
For the analysis of the multiple-choice assay, normal distribution of the settlement response in populations from which the samples were drawn was assumed. The number of counted larvae was log(x+1) transformed to stabilize their variance. Levene's test was employed to check the assumption of homogeneity. Where significant heterogeneity of variance could not be removed by transformation, a lower significance level ($p = 0.01$ instead of $p = 0.05$) was used (Underwood, 1997). Effects of row, column, treatment (fixed factors) on larval metamorphosis were analyzed with main-effect ANOVA for each experiment. Experiments with significant row or column effects were not considered in the results (this was the case in one experiment). If the treatment effect was significant, Tukey's multiple comparison tests were used to locate the

differences identified by ANOVA (α = 0.05). Nucleotide sequence accession numbers: The 16S rRNA gene sequences of strain DF11 and DF16 obtained in this study are available from GenBank under accession numbers EF127894 and EF127895, respectively.

RESULTS

ISOLATION OF BACTERIA AND PHYLOGENETIC ANALYSIS

Thirty-nine bacteria were isolated from natural sediment at Hooksiel. The isolates were affiliated to 22 genera, including *Bacillus* (11), *Vibrio* (4), *Pseudoalteromonas* (3), *Cytophaga* (3), *Shewanella*, *Marinobacter*, *Tenacibaculum*, *Phaeobacter* (2 isolates each) and *Alteromonas*, *Halomonas*, *Halobacillus*, *Aestuariibacter*, *Marinobacterium*, *Flexibacter*, *Salegentibacter*, *Flavobacterium*, *Cellulophaga*, *Psychroflexus, Zooshikella, Algoriphagus, and Loktanella* (1 isolate each). Due to low sequence similarities (≤ 95%) to 16S rRNA gene sequences of described species strain DF16 could not be clearly assigned to an existing genus. The isolates distributed over 5 phylogenetic classes: α-*Proteobacteria,* γ-*Proteobacteria*, *Bacilli*, *Flavobacteria* and *Sphingobacteria*. For bioassay purposes 15 strains belonging to different classes were selected. The nucleotide sequence accession numbers of the closest published match and phylogenetic assignment of the selected strains are given in Tab. 2.

Tab. 2. Phylogenetic affiliation of selected strains and accession numbers of closest published match in GenBank. Strains were isolated from Wadden Sea sediment.

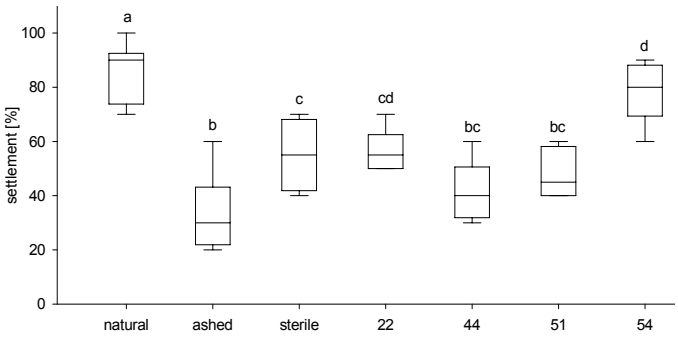
Nr.	Class	Closest published match at GenBank	Acc. nr.	Similarity [%]
13	Bacilli	*Halobacillus sp.* MO50	AY553121	99%
30	Bacilli	*Bacillus pumilus*	AB211228	98%
43	Bacilli	*Bacillus cereus*	AF290554	100%
DF11	α-Proteobacteria	*Loktanella* sp. strain DF11	EF127894	100%
DF16	α-Proteobacteria	*Rhodobacteraceae bacterium* strain DF16	EF127895	100%
T5	α-Proteobacteria	*Phaeobacter inhibens*	AY177712	100%
T5-3	α-Proteobacteria	*Phaeobacter inhibens,* mutante	AY177713	100%
22	γ-Proteobacteria	*Vibrio pacinii*	AJ316194	99%
24	γ-Proteobacteria	*Shewanella baltica*	AF173966	98%
44	γ-Proteobacteria	*Zooshikella ganghwensis*	AY130994	98%
16	Flavobacteria	*Salegentibacter sp. DPA2*	DQ344850	98%
53	Flavobacteria	*Cellulophaga lytica*	AB032511	99%
54	Flavobacteria	*Psychroflexus tropicus*	AF513434	98%
32	Sphingobacteria	*Gramella echinicola*	AY608409	99%
51	Sphingobacteria	*Algoriphagus ratkowskyi*	AJ608641	98%

TREATMENT EFFICIENCY ON ASHED AND STERILE SEDIMENT

Generally, recolonization of ashed sediment with bacterial isolates resulted in low bacterial densities (< 10^5 cells g^{-1} sediment wet weight). Contrary, the recolonization of sterile sediment with bacterial isolates was successful since a sufficient amount of bacteria (~10^7 – 10^8 cells g^{-1} sediment wet weight) attached on sediment grains. The recolonization yields are summarized in the figures legend of each assay.

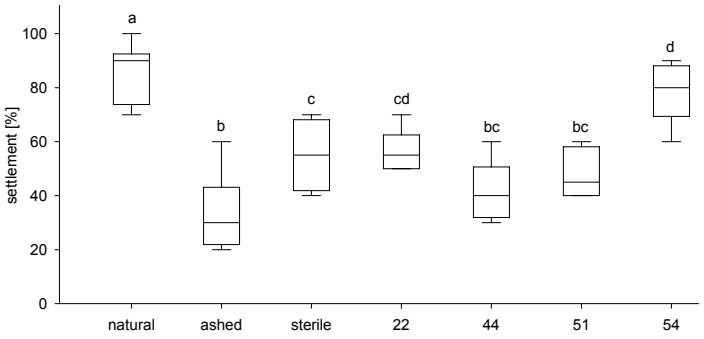
LARVAL SETTLEMENT RESPONSES TOWARDS MONO-SPECIES BACTERIAL SEDIMENT TREATMENTS (SINGLE-CHOICE ASSAYS)

Although recolonization of ashed sediment yielded low bacterial densities, the treatments were utilized in settlement assays for both species. At the given bacterial cell densities on ashed sediment no treatment evoked statistically significantly settlement responses compared to the negative control (1-w-ANOVA, $\alpha = 0.05$). Larval settlement of *Polydora cornuta* was significantly higher in sediment treatments recolonized with strain 54 and strain DF11 compared to the sterile negative control, but significantly lower than in the natural sediment (Tukey's test, $p < 0.05$). All other isolates under investigation did not trigger larval settlement different from the sterile control. Due to the high number of figures required to present all the data (15 isolates assayed in duplicates at different combinations) the results of assay series with inductive isolates are exemplified in Fig. 1 – Fig. 4.



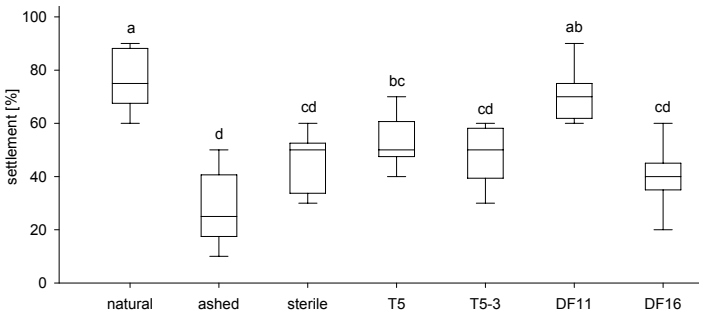
Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 6 replicates shown in a box-and-whisker diagram.

Fig. 1. *P. cornuta*. Percentage of larval settlement of test larvae of a single larval batch (0.96 ± 0.12 mm body length, 14-15 setigers) in single-choice assays after 1 h in response to 7 different sediment treatments, i.e. natural, ashed, sterile and sediment recolonized with strain 30, 44, 51 and 54, respectively.



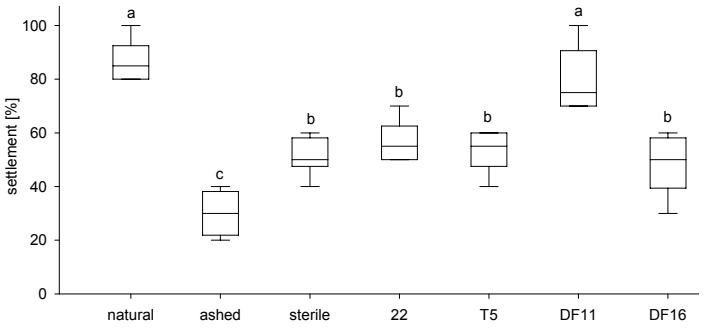
Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 6 replicates shown in a box-and-whisker diagram.

Fig. 2. *P. cornuta*. Percentage of larval settlement of test larvae of a single larval batch (0.93 ± 0.14 mm body length, 14 setigers) in single-choice assays after 1 h in response to 7 different sediment treatments, i.e. natural, ashed, sterile and sediment recolonized with strain 22, 44, 51 and 54, respectively.



Statistical differences are indicated by different letters above the boxes (= 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 6 replicates shown in a box-and-whisker diagram.

Fig. 3. *P. cornuta*. Percentage of larval settlement of test larvae of a single larval batch (0.94 ± 0.12 mm body length, 14 setigers) in single-choice assays after 1 h in response to 7 different sediment treatments i.e. natural, ashed, sterile and sediment recolonized with strain T5, T5-3, DF11 and DF16, respectively.



Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 6 replicates shown in a box-and-whisker diagram.

Fig. 4. *P. cornuta*. Percentage of larval settlement of test larvae of a single larval batch (0.99 ± 0.10 mm body length, 15 setigers) in single-choice assays after 1 h in response to 7 different sediment treatments i.e. natural, ashed, sterile and sediment recolonized with strain 22, T5, DF11 and DF16, respectively.

EFFECT OF BACTERIAL CELL DENSITY AND VIABILITY ON LARVAL SETTLEMENT (MULTIPLE-CHOICE ASSAY)

Due to the settlement results obtained with strain DF11 and strain 54, these strains were used for further settlement studies in multiple-choice assays. Additionally, the strain DF16, which did not trigger enhanced settlement in the single-choice assay, was selected as a control. In the following, the results of 6 different assay types are summarized:

Assay 1a: sediment treatments were sterile sediment, natural sediment, sterile sediment recolonized with strain DF11, sterile sediment recolonized with strain DF11 followed by washing.

In *S. benedicti* larval settlement was significantly higher in both DF11-treatments compared to the control of sterile sediment (Tukey's test, $p < 0.05$, Fig. 5) and did not differ from the settlement response towards natural sediment ($p = 0.56$).

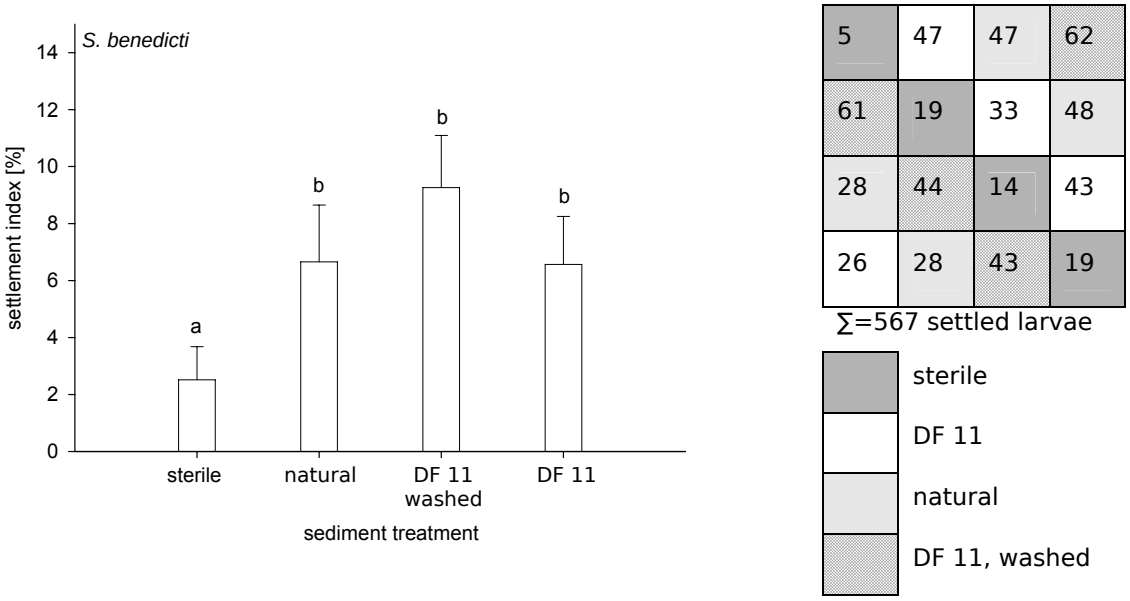


Fig. 5. *S. benedicti,* Assay 1a. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment and sediments recolonized with different densities of the strain DF11. Bacterial cell densities were 9.4×10^8 cells g^{-1} in the "strain DF11" treatment and 4.4×10^8 cells g^{-1} sediment in the "strain DF11, washed" treatment. Statistical differences are indicated by different letters above the bars ($\alpha = 0.05$, Tukey's test).

Assay 1b: Due to the high standard deviations in settlement indices larval settlement of *P. cornuta* did not differ among the four different sediment treatments (Tukey's test, $p = 0.182$, Fig. 6).

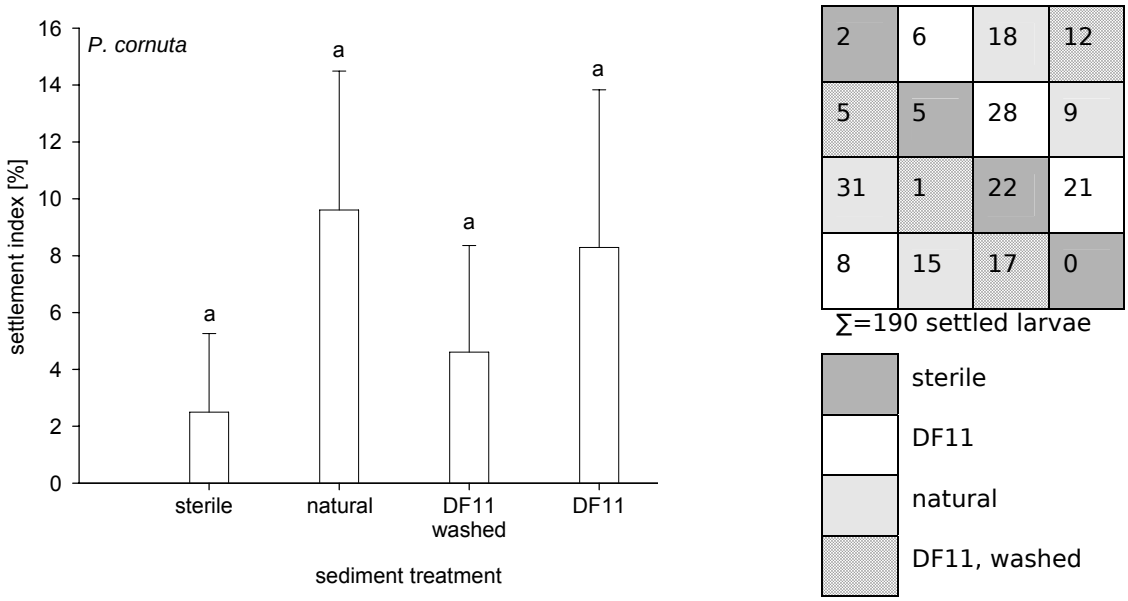


Fig. 6. *P. cornuta*, Assay 1b. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment and sediments recolonized with different densities of the strain DF11. Bacterial cell densities were 7×10^8 cells g^{-1} in the "strain DF11" treatment and 2.2×10^8 cells g^{-1} sediment in the "strain DF11, washed" treatment. Statistical similarities are indicated by same letters above the bars ($\alpha = 0.05$, Tukey's test).

Assay 2a: sediment treatments were sterile sediment, natural sediment, sterile sediment recolonized with strain 54 and sediment recolonized with strain 54 followed by washing.

In *S. benedicti* larval settlement was significantly higher in sediment treated with strain 54 than in the control of sterile sediment (Tukey's test, $p < 0.05$, Fig. 7). Whilst settlement in the washed recolonized sediment did not differ from the control of natural sediment ($p = 0.14$), settlement in the recolonized sediment without washing was significantly lower (Tukey's test, $p < 0.005$) and higher (Tukey's test, $p < 0.05$) than in natural sediment and sterile sediment, respectively.

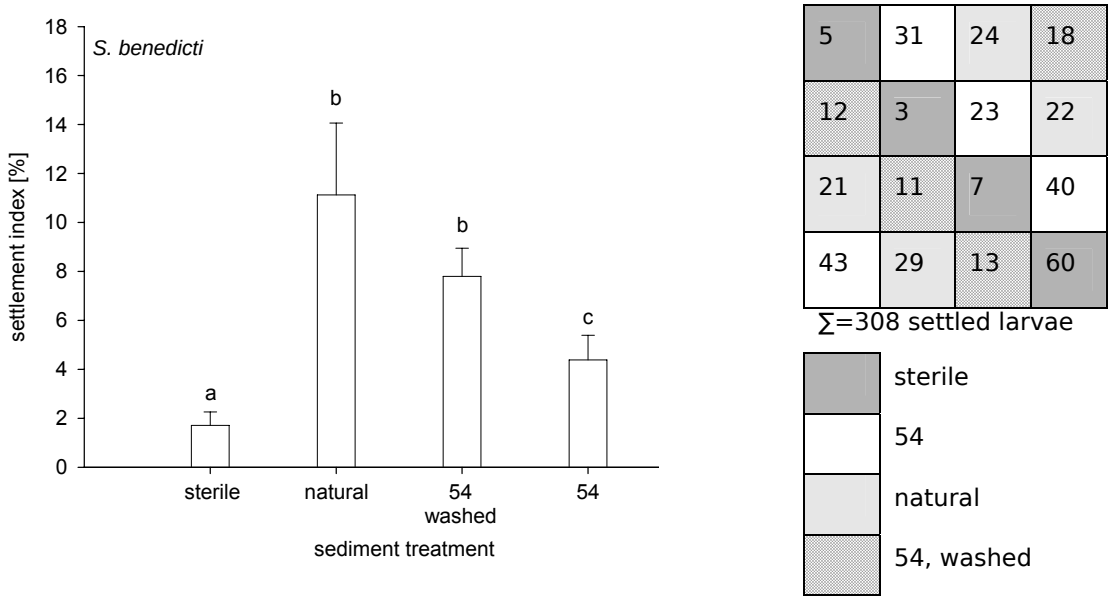


Fig. 7. *S. benedicti,* Assay 2a. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment and sediments recolonized with different densities of the strain 54. Bacterial cell densities were 7.7 X 10^8 cells in the "strain 54" treatment and 2 X 10^8 cells / g^{-1} sediment in the "strain 54, washed" treatment. Statistical differences are indicated by different letters above the bars (α = 0.05, Tukey's test).

Assay 2b: Similarly, in *P. cornuta* larval settlement was significantly higher in the washed sediment treatments of strain 54 than in the control of sterile sediment (Tukey's test, $p < 0.05$, Fig. 8) and did not differ statistically from the natural sediment control ($p = 0.98$). The recolonized treatment without washing did not trigger larval settlement ($p = 0.99$).

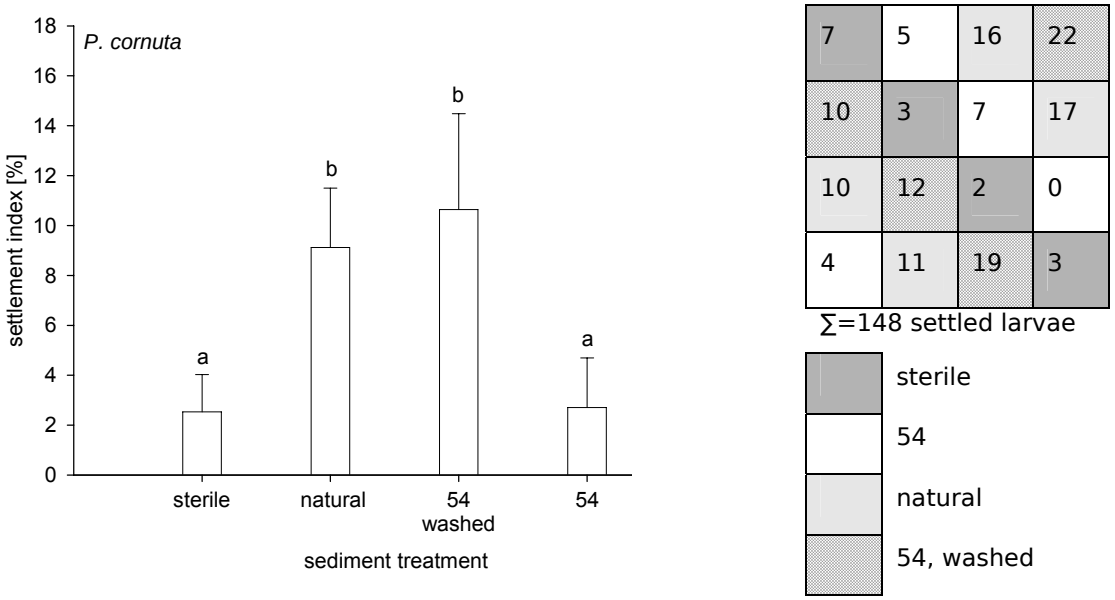


Fig. 8. *P. cornuta*, Assay 2b. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment and sediments recolonized with different densities of the strain 54. Bacterial cell densities were 5.8×10^8 cells in the "strain 54" treatment and 3.3×10^8 cells / g^{-1} in the "strain 54 washed" treatment. Statistical differences are indicated by different letters above the bars ($\alpha = 0.05$, Tukey's test).

Assay 3a: sediment treatments were sterile sediment, natural sediment, sterile sediment recolonized with strain DF16 and sediment recolonized with strain DF16 followed by washing.

In *S. benedicti* larval settlement was significantly higher in natural sediment treatment than in all other sediment treatments (Tukey's test, $p < 0.05$, Fig. 9). Recolonization with strain DF16 did not trigger settlement in comparison to sterile sediment control ($p = 0.06$).

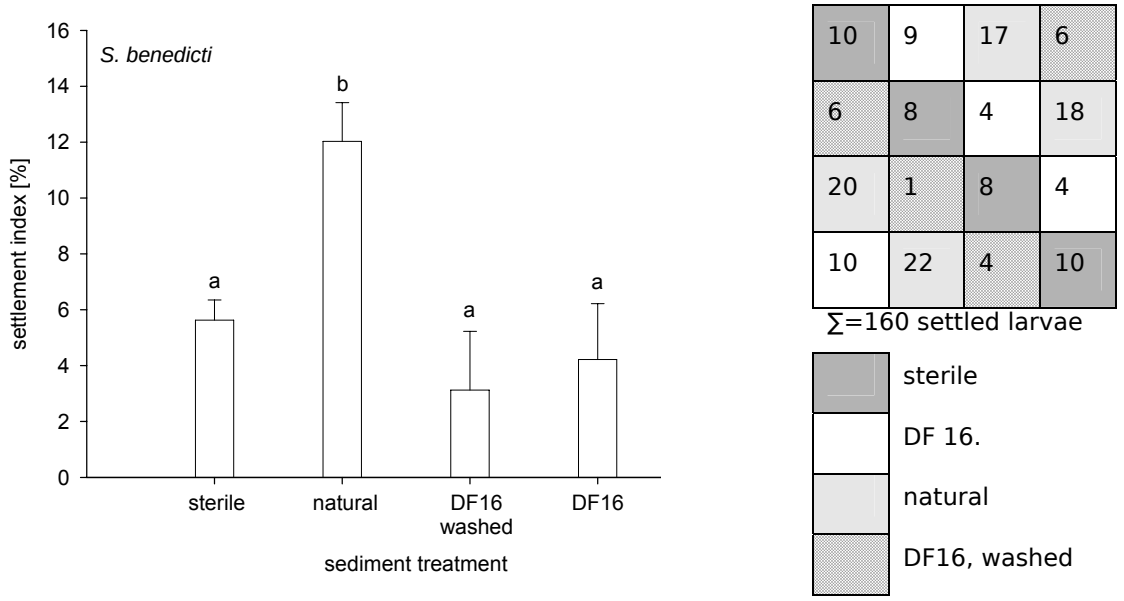


Fig. 9. *S. benedicti*, Assay 3a. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment and sediments recolonized with different densities of the strain DF16. Bacterial cell densities were 5.3×10^7 cells in the "DF16" treatment and 2×10^6 cells / g^{-1} sediment in the "DF16, washed" treatment. Statistical differences are indicated by different letters above the bars ($\alpha = 0.05$, Tukey's test).

Assay 3b: In *P. cornuta* larval settlement was significantly higher in natural sediment than in all other sediment treatments (Tukey's test, $p < 0.05$, Fig. 10). Recolonization with strain DF16 did not trigger more settlement than the sterile sediment control (Tukey's test, $p = 0.15$).

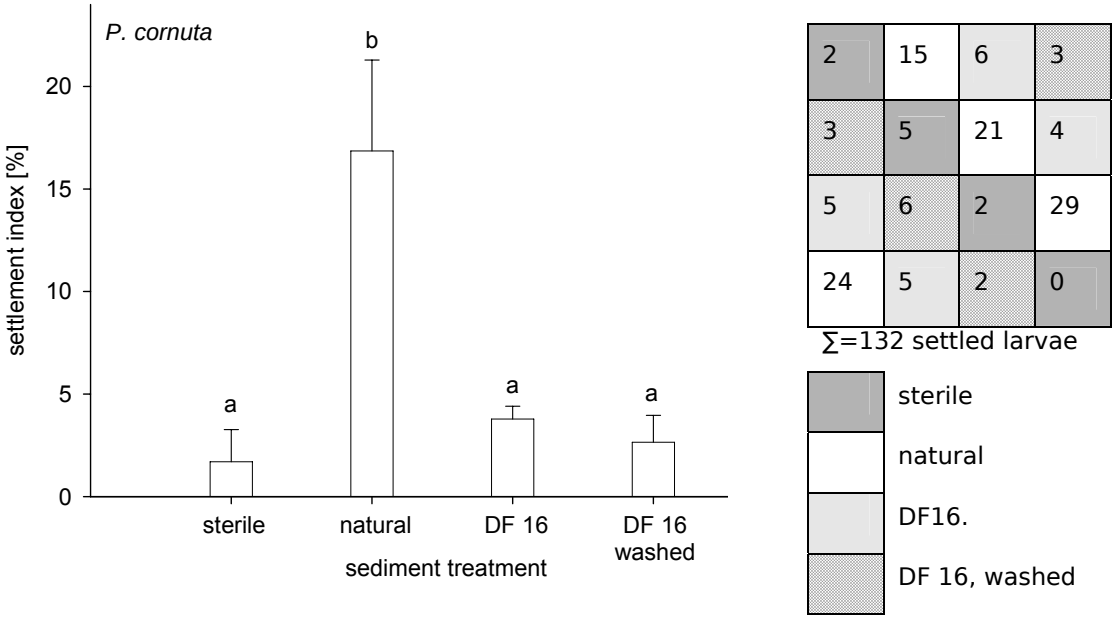


Fig. 10. *P. cornuta*, Assay 3b. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment and sediments recolonized with different densities of the strain DF16. Bacterial cell densities were 1.9 X 10^7 cells in the "strain DF16." treatment and 3.3 X 10^6 cells / g^{-1} sediment in the "strain DF16, washed" treatment. Statistical differences are indicated by different letters above the bars (α = 0.05, Tukey's test).

Assay 4: sediment treatments were sterile sediment, natural sediment, sterile sediment recolonized with strain DF11, sterile sediment recolonized with strain DF11 followed by heating at 60˚C in the water bath for 2 h.

Similarly to the results obtained in Assay 1, larval settlement of *S. benedicti* was significantly higher in the sediment treatments recolonized with strain DF11 than in the sterile sediment control (Tukey's test, $p < 0.05$, Fig. 11) and did not differ statistically from natural sediment ($p = 0.99$). Contrary, settlement in the sediment which was colonized with strain DF11 and heated at 60˚C was significantly lower than in the unheated counterpart and did not differ from sterile sediment ($p < 0.01$).

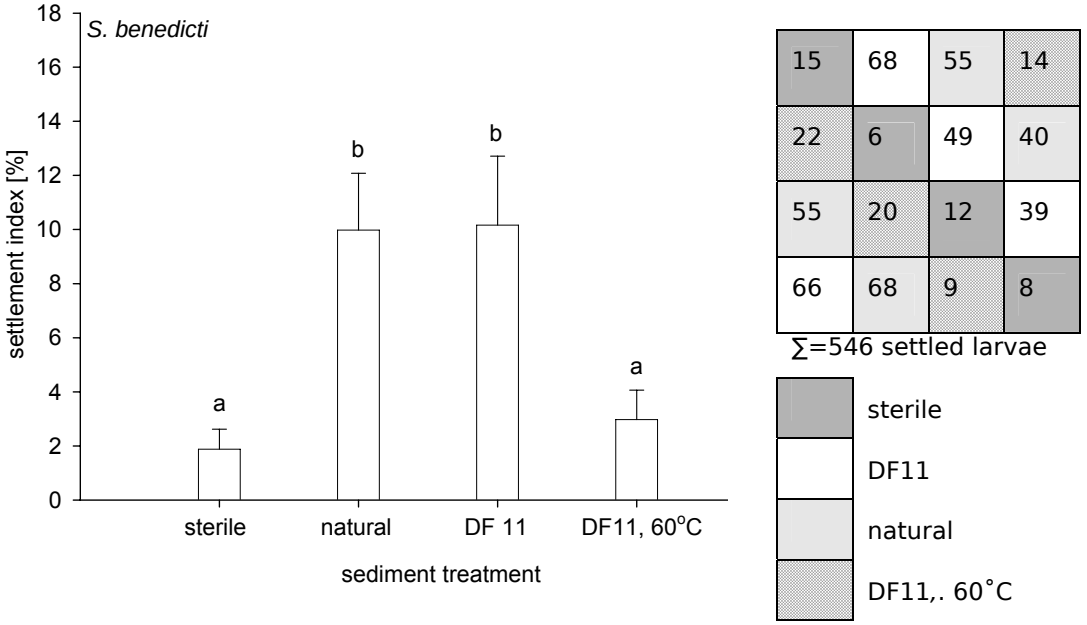


Fig. 11. *S. benedicti*, Assay 4a. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment, sediment recolonized with strain DF11 and heated sediment formerly recolonized with strain DF11. Bacterial cell densities were 9.2 X 10^8 cells in the "strain DF11" treatment and <1000 cells / g^{-1} sediment in the "strain DF11, 60˚C" treatment. Statistical similarities are indicated by same letters above the bars (α = 0.05, Tukey's test).

Assay 4b: Larval settlement of *P. cornuta* was significantly higher in sediment recolonized with strain DF11 than in the sterile sediment control (Tukey's test, $p < 0.05$, Fig. 12) and did not differ from the natural sediment control ($p = 0.92$).

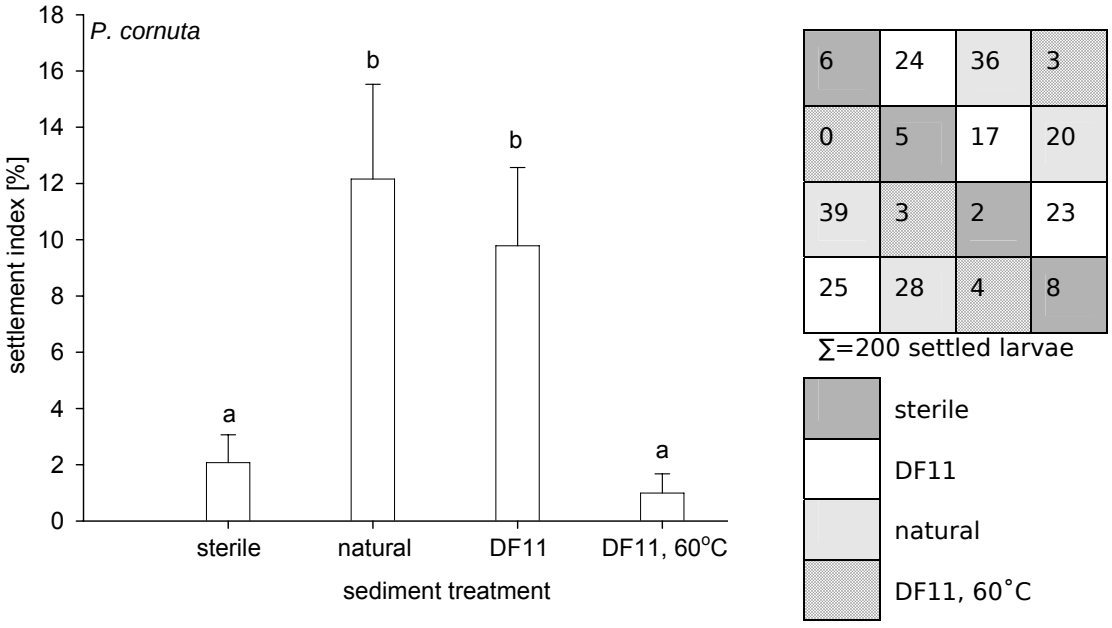


Fig. 12. *P. cornuta*, Assay 4b. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment, sediment recolonized with strain DF11 and heated sediment formerly recolonized with strain DF11. Bacterial cell densities were 5.2 X 10^8 cells in the "strain DF11" treatment and 4.3 X 10^3 cells / g^{-1} sediment in the "strain DF11, 60˚C" treatment. Statistical similarities are indicated by same letters above the bars (α = 0.05, Tukey's test).

Contrary, settlement in sediment recolonized with strain DF11 and exposed to heat was significantly lower than in the unheated counterpart ($p < 0.005$) and did not differ from the sterile sediment control ($p = 0.28$).

Assay 5a: Similarly to Assay 4, larval settlement of *S. benedicti* was significantly higher in the sediment recolonized with strain 54 successfully than in the sterile sediment control (Tukey's test, $p < 0.005$, Fig. 13) and did not differ from the natural sediment control ($p = 0.99$). Contrary, settlement in sediment recolonized with strain 54 and exposed to heat was significantly lower than in the unheated counterpart $p < 0.05$) and did not differ from the sterile sediment control ($p = 0.08$).

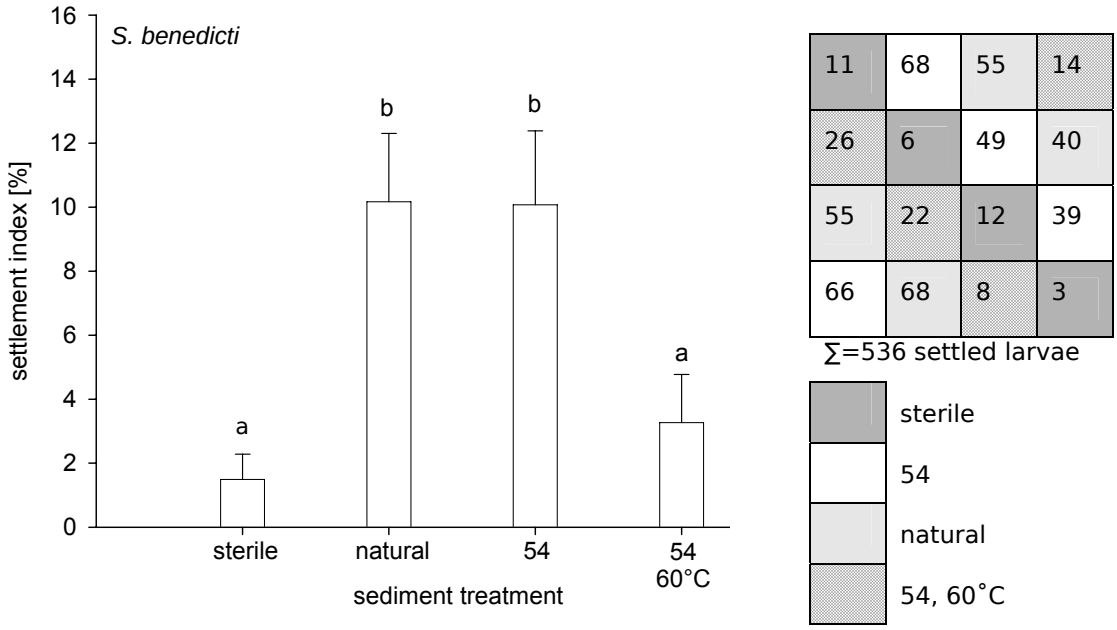


Fig. 13. *S. benedicti*, Assay 5a. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment, sediment recolonized with strain 54 and heated sediment formerly recolonized with strain 54. Bacterial cell densities were 2.7 X 10^8 cells in the "strain 54" treatment and <1000 cells / g^{-1} sediment in the "strain 54, 60˚C" treatment. Statistical differences are indicated by different letters above the bars (α = 0.05, Tukey's test).

Assay 5b: Larval settlement of *P. cornuta* in sediment recolonized with strain 54 was the same as in the sterile and in the natural sediment control (Tukey's test, $p = 0.24$, Fig. 14). Statistical significant differences were recorded between the natural and sterile sediment and the natural and heat treated recolonized sediment ($p < 0.05$).

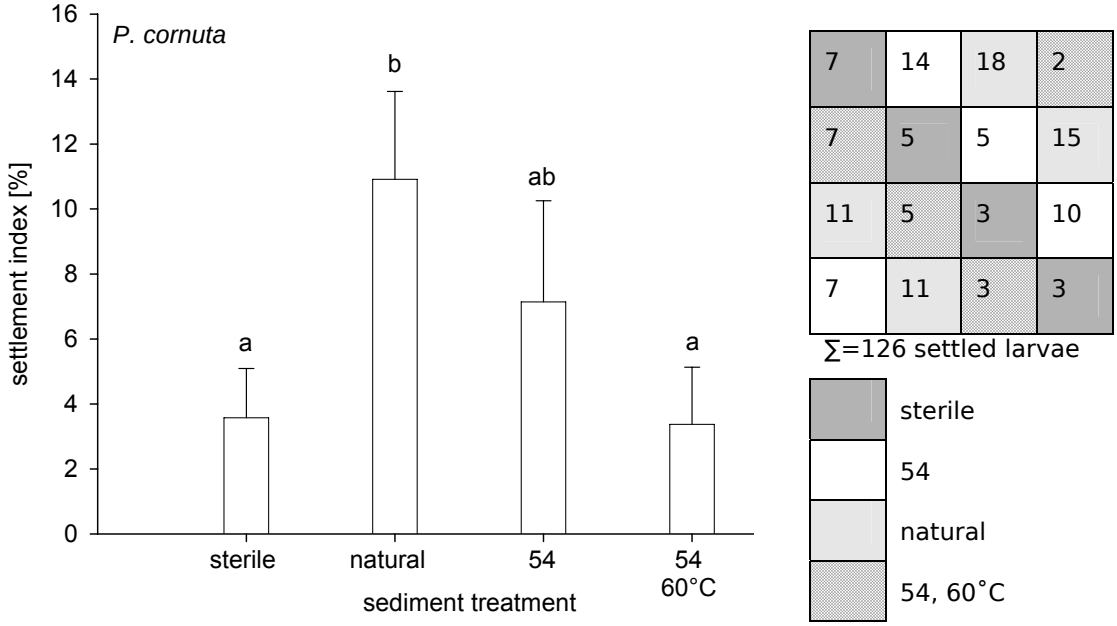


Fig. 14. *P. cornuta*, Assay 5b. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment, sediment recolonized with strain 54 and heated sediment formerly recolonized with strain 54. Bacterial cell densities were 2.5 X 10^8 cells in the "strain 54" treatment and <1000 cells / g^{-1} sediment in the "strain 54, 60˚C" treatment. Statistical differences are indicated by different letters above the bars ($\alpha = 0.05$, Tukey's test).

Assay 6: sterile sediments were recolonized with strain DF11. The bacterial suspension was used at original concentration and in dilutions of 10^{-2}, 10^{-4} and 10^{-6} fold. Recolonization experiments at different cell densities resulted in different cell densities on the sediment: 1.8 x 10^9 cells g^{-1} at original concentration, 5 x 10^7 cells g^{-1} at 10^{-2} dilution, 8 x 10^4 cells g^{-1} at 10^{-4} dilution and less than 10^3 cells g^{-1} sediment at 10^{-6} dilution.

Larval settlement of *S. benedicti* in recolonized sediments was dependent on bacterial density. Settlement in response to treatments at original concentration and 10^{-2} dilution were the same (Tukey's test, $p = 0.19$, Fig. 15) and differed significantly from the higher dilutions ($p < 0.05$).

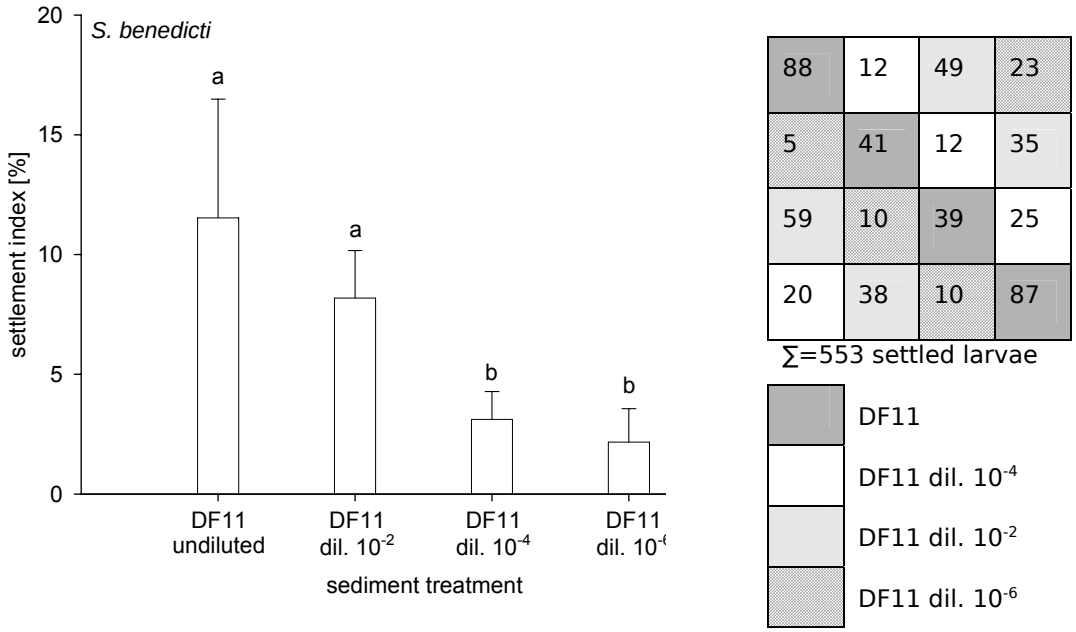


Fig. 15. *S. benedicti*, Assay 6. Percentage of settlement index per site in a multiple-choice assay. Larval response to sediments recolonized with different densities of the strain DF11. Bacterial cell densities were 1.8 X 10^9 cells in the "strain DF11" treatment, 5 X 10^7 cells in the "strain DF11 10^{-2} diluted" treatment, 8 X 10^4 cells in the "strain DF11 10^{-4} diluted" treatment and less than 10^3 cells / g^{-1} sediment in the "strain DF11 10^{-6} diluted" treatment. Statistical differences are indicated by different letters above the bars (α = 0.05, Tukey's test).

THE EFFECT OF WATER SOLUBLE BACTERIAL PRODUCTS ON LARVAL SETTLEMENT

Waterborne bacterial products of strain DF11 did not influence larval settlement at the significance level of $\alpha = 0.05$ (one-way ANOVA). There was no effect of bacterial culture conditions (marine broth vs. sediment/seawater suspension and shaking vs. stationary conditions) on larval settlement.

THE EFFECT OF SUSPENDED BACTERIA ON LARVAL SETTLEMENT

The presence of suspended bacterial cells in three different cell densities (3 x 10^9, 6 x 10^7 and 5 x 10^5 cells ml^{-1} seawater) did not change the settlement response in comparison to sterile filtered seawater at the $\alpha = 0.05$ level (one-way ANOVA).

DISCUSSION

In this study thirty-nine bacterial strains were isolated from intertidal surface sediment at Hooksiel and Neuharlingersiel and identified by comparison of 16S rRNA gene sequences in GenBank. The comparison of the cultivable bacterial community from Hooksiel to communities obtained from other Wadden Sea sites such as Neuharlingersieler Nacken (sandy-site) and Groninger Plate (sandy-muddy site) by Köpke et al. (2005) revealed clear differences in cultivable bacterial community patterns among these sites. From 26 isolates obtained at Neuharlingersieler Nacken and Gröninger Plate only two were identical at both sites, whereas none of the isolates matched with those ones obtained at Hooksiel, highlighting that on scales of kilometers bacterial community compositions of surface sediment differ remarkably in intertidal flats of the Wadden Sea. Analogous conclusions on small scale (i.e. mm to cm) cannot be drawn since the bacterial community composition in intertidal flats has not yet been investigated. However, in intertidal flats localized environmental conditions, such as organic content and redox conditions may differ strongly even on the scale of few centimeters. The organic content may vary due to local hotspots influenced by fecal pellets and troughs between ripple crests, which are typically rich in organic material (D'Andrea et al., 2002; Yager et al., 1993). Moreover, redox conditions in the upper sediment layer are strongly influenced by bioturbation of infaunal organisms. Thus, patchiness of sediment microhabitats is likely reflected in clear differences in microbial community patterns and may deliver integrated information for the sediment surface exploring larvae.

The recolonization of sterilized natural sediment with bacterial strains proved to be a practical approach to study the influence of mono-species biofilms on larval settlement. Typically, recolonization-yields on sterilized natural sediment were 10^7 to 10^8 cells g^{-1} sediment, i.e. one order of magnitude lower than in natural sediments. On the contrary, recolonization of ashed sediment with bacteria yielded in low bacterial cell densities ($< 10^5$ cells g^{-1} sediment) at which none of the 15 isolates triggered settlement of spionid larvae. Low recolonization yields on ashed sediment were most likely due to the lack of suitable nutrients, since many bacteria preferentially form biofilms in a nutrient-rich environment (O'Toole et al., 2000).

Due to the ineffectiveness of bacterial recolonization on ashed sediment we were obligated to base our experiments on sterilized natural sediment. Since larvae of *Streblospio benedicti* display high settlement rates in sterile sediment in the single-choice assay (Sebesvari et al., 2006) this spionid was not a suitable test organism in single-choice recolonization-assays. Consequently, single-choice assays were performed only with larvae of *Polydora cornuta*.

The 15 bacterial isolates used in subsequent assays belonged to 5 phylogenetic classes α-*Proteobacteria* (4) γ-*Proteobacteria* (3), *Bacilli* (3), *Flavobacteria* (3) and *Sphingobacteria* (2). In the single-choice assay, 2 out of these 15 isolates triggered larval settlement, i.e. strain DF11 (α-*Proteobacteria*, *Roseobacter*-clade) and strain 54 (*Flavobacteria*). Thus, the phylogenetic affiliations of these bacterial species alone were not indicative of their capacity to induce larval settlement. Similar observations were made in the polychaete *Hydroides elegans* where larval settlement was induced by a range of phylogenetically different bacteria including *Pseudoalteromonas, Cytophaga*, *Bacillus*, *Brevibacterium*, *Micrococcus, Staphylococcus, Alteromonas*, *Pseudoalteromonas* and *Vibrio* (Lau and Qian, 2002; Huang and Hadfield, 2003).

The two inductive strains belong to phyla which are highly abundant in marine environments and reported to actively produce secondary metabolites and acylated homoserine lactones (AHL), i.e. potential signaling compounds for larval settlement (Llobet-Brossa et al., 1998, Gram et al., 2002, Kirchman, 2002, Buchan et al., 2005, Martens et al., 2006).

After screening 15 bacterial strains in the single-choice assay, both inductive isolates and one of the non-inductive strains, DF16, were further tested in multiple-choice assays. Due to the ability of *Streblospio benedicti* larvae to distinguish between sterile and natural sediment if offered simultaneously in a multiple-choice assay (Sebesvari, unpubl.) larval settlement of both spionids was investigated with this assay design. The multiple-choice assays with four parallel experimental treatments of sediment, i.e. natural, sterile and two treatments with different quantity and quality of bacterial isolates revealed clear differences in the larval settlement response in both species. Generally, natural sediment was the strongest trigger for larval settlement, while sterile sediment evoked significantly less settlement (Figs. 5 - 14). Some bacterial treatments evoked similar rates of settlement as the natural sediment control. These treatments resulted from addition of strain DF11 (with *S. benedicti* also the washed

treatment) and washed sediment previously recolonized with strain 54. In these treatments bacterial densities ranged from 4.4 x 10^8 to 9.4 x 10^8 cells g^{-1} (strain DF11*)* and 2.5 x 10^8 to 7.7 x 10^8 cells g^{-1} sediment (strain 54*)*. These densities were in the range observed in natural intertidal surface sediments from the Wadden Sea (Köpke et al., 2005).

The strain DF16 was chosen for comparative purposes because it did not trigger larval settlement at any given bacterial densities different from the sterile sediment control (Figs. 9 – 10). In the DF16-treatments, cell densities ranged from 2 x 10^6 to 5.3 x 10^7 cells g^{-1} sediment, i.e. with this bacterium lower cell densities were obtained after recolonization than with strain DF11 and strain 54. At this stage, it remains unclear if DF16 generally did not trigger larval settlement or whether a certain required threshold density to induce larval settlement was not achieved in our treatments. In principle, this statement had to be extended to all non-inductive strains tested in the single-choice assay. This objection was supported by the results obtained with strain DF11 and 54 in the multiple-choice assay, which demonstrated a clear correlation between the bacterial cell density on sediment and larval settlement (Figs. 7, 8, 15). Interestingly, larval settlement on sediment recolonized with strain 54 was higher at low cell densities, indicating that settlement induction is not always positively correlated with bacterial densities. Possibly, the larval settlement response to some mono-specific bacterial films follows a saturation curve with a species-specific optimum and negative effects at higher cell concentrations. However, washing might have lead to the leaching of accumulated bacterial metabolites, which in turn may have otherwise affected larval settlement in a concentration dependent fashion.

The potential effect of bacterial density on larval settlement induction has rarely been investigated so far. In the fouling polychaete *Hydroides elegans,* metamorphosis was positively correlated with bacterial density in either natural biofilms or biofilms composed of a single bacterial species (Huang and Hadfield, 2003; Lau et al., 2005) whereas larval settlement of *Balanus amphitrite* and *Balanus trigonus* did neither correlate with the biomass nor the bacterial density in biofilms (Lau et al., 2005). In the soft sediment settler *Protodrilus rubropharyngeus* (Polychaeta) Gray (1967) reported a density dependent larval response to four mono-specific biofilms.

Bacterial threshold densities in induction of larval settlement may be strongly species-specific. In previous studies, certain bacterial strains were strongly inductive at densities much lower than in natural biofilms whilst others did not induce settlement even at unrealistically high cell densities (Unabia and Hadfield, 1999; Lau and Qian, 2001; 2002; Huang and Hadfield, 2003).

The heat exposure of sediments recolonized with strain DF11 and 54 to 60˚C for 2 h significantly decreased bacterial density and larval settlement on these treatments, indicating that bacterial viability was necessary to evoke settlement in both spionid species. Therefore, the settlement cue was likely composed of heat labile microbial constituents. Furthermore, neither water soluble bacterial products nor suspended cells of strain DF11 had any effect on larval settlement *of Polydora cornuta*. Settlement was also not influenced by growth conditions (different nutrient conditions and shaking or stagnation) of strain DF11. Similarly, settlement in the presence of suspended bacteria was independent of the cell density in seawater. Evidently, only living attached cells of strain DF11 were able to evoke settlement in *Polydora cornuta* larvae. Therefore, the settlement cue of strain DF11 is likely insoluble in seawater and associated with the biofilms surrounding sand grains. This hypothesis was supported by the observation, that exploring larvae repeatedly contacted the sediment surface prior to settlement and comply with the induction of larval settlement by microbial films in hard substrate settling organisms where both bacterial cells and water soluble chemical have been identified as settlement cues (Maki et al., 1990; Szewzyk et al., 1991; Harder et al., 2002; Lau et al., 2003; Lam et al., 2005).

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4 MATERIAL AND METHODS

4.1 INVESTIGATION OF DIATOMS AS POTENTIAL SETTLEMENT CUES

4.1.1 NATURAL SEDIMENT TREATED WITH ALGICIDE AND ANTIBIOTICS

Natural sediment was treated with antibiotics (penicillin and streptomycin) and algicide (cycloheximide) to disengage on the one hand the bacterial component of the biofilm and on the other hand the diatom fraction on the sediment surface. Algicides and antibiotics were used after their degree of efficiency. Penicillin referred to a group of β-lactam antibiotics which worked by inhibiting the formation of peptidoglycan cross links in the bacterial cell wall. The β-lactam moiety of penicillin bind to the enzyme (transpeptidase) that links the peptidoglycan molecules in bacteria, and this weakens the cell wall of the bacterium when it multiplies. In addition, the build-up of peptidoglycan precursors triggers the activation of bacterial cell wall hydrolases which further digest the bacteria's existing peptidoglycan (Williams 1962; Williams and Wilson 1995). In addition the quadraceptics of mechanics, the bacterial wall can break down. Streptomycin stopped bacterial growth by damaging cell membranes and inhibiting protein synthesis rapidly and irreversibly without disturbing the architecture of the bacterial cells (Davis 1987). Specifically, it binds to the 16S rRNA of the bacterial ribosome, which prevents the release of the growing protein (polypeptide chain). Cycloheximide was used as an inhibitor of protein biosynthesis in eukaryotic organisms by acting on the translation process at the 80S ribosomes present in the cytoplasm, but chloroplast and mitochondrial functions remain unaffected (Galling 1982). In no prokaryotic cells the protein biosynthesis on 70S ribosome were affected.

4.1.1.1 PREPARATION OF SEDIMENT TREATMENTS

Sediment samples (upper 2 mm layer) were collected from undisturbed natural surface sediment without infauna, stones or other physical irregularities on the inertial area of the Wadden Sea (53°38'31''N, 8°04'55''E) (Hooksiel, Germany) at low tide. Sediment samples were collected in June 2005 during the daytime emersion using Petri dishes. 35 g sediment per treatment was placed into plastic

centrifuge cap (50 ml, Corning USA). 20 ml of sterile filtered (0.02 µm) seawater were used and algicide or/and antibiotics were added. Cycloheximide was used in single-choice assays in two different concentrations to adjust and optimize the suitable concentration, the first treatment utilized 10 mg g^{-1}, and the second treatment utilized 1 mg g^{-1} according to Raubuch and Beese (1998) and Raubuch et al. (2006). Penicillin was utilized in concentration of 37.2 µg ml^{-1} and streptomycin in concentrations of 364 µg ml^{-1} according to Harder et al. (2002). The mixture was given onto the sediment and shaked over head for 12 h or 36 h (ASSAY 1-4, Tab. 1) on a rotor shaker. After incubation the plastic centrifuge caps were centrifuged (2000 g, 2 min) and washed twice with sterile filtered seawater. Natural sediment was treated as described above except the addition of algicide and antibiotics and used as control in settlement assays.

4.1.1.2 SETTLEMENT ASSAYS

Settlement assays were carried out in the single-choice assay design (Sebesvari et al. 2006). Natural, ashed and sterile sediments were assayed simultaneously in well-plates. Accordingly natural sediment treated with either algicides, antibiotics, or both were added into each of 6 wells (Tab. 1). 2.5 g (wet weight) of sediment was transferred into each well, resulting in a 7 mm sediment layer. The sediment was overlaid with sterile filtered seawater up to 1 cm and 10 *Streblospio benedicti* larvae (ASSAY 1) and *Polydora cornuta* larvae (ASSAY 2-4) were randomly picked from a homogenous control batch and added onto the sediment. The single-choice settlement experiment was processed as described in chapter 2.

Tab. 1: Larval single-choice test on natural sediment in ASSAY 1 treated with algicide and antibiotic, performed with *S. benedicti* and in ASSAY 2 A+B treated with algicide and antibiotic and in illumination performed with *P. cornuta* larvae

		ASSAY 1	ASSAY 2A	ASSAY 2 B
treatment	**sediment**		**conditioning**	
1	natural		no condition (positive control)	
2	ashed		800 °C (negative control)	
3	sterile		autoclaving	
4	natural	antibiotics (12 h)	antibiotics (36h)	antibiotics (36h)
5	natural	algicide (10 mg g^{-1}, 12 h)	algicide (10 mg g^{-1}, 36h)	algicide (10 mg g^{-1}, 36h)
6	natural	algicide (1 mg g^{-1}, 12 h)	antibiotic + algicide (10 mg g^{-1}, 36h)	antibiotic + algicide (10 mg g^{-1}, 36h)
7	natural	incubation; 12h	incubation; 36h	incubation; 36h
8	natural	———	light (4h)	———

4.1.1.3 ENUMERATION OF BACTERIA AND DIATOMS ON NATURAL AND TREATED SEDIMENTS

Enumeration of **BACTERIA** was conducted with sediment treatments used in ASSAY 1, conditioned with algicide, antibiotics and the control (natural) sediment. Sediment conditioned with algicide and/or antibiotics (1 g) was mixed with 10 ml Tris-HCl-buffer (10 mM, pH 8), than 100 µl Tween-80 solution (0.5 %, sterile filtered) was added. After ultrasonication (5*30 sec.) 5 µl were added to 1 ml Tris-HCl-buffer and mixed gently, than 10 µl SyberGreen I solution (1:200 diluted with Tris HCl-buffer, 10mM, pH 8, sterile filtered), and 15 µl propidium iodine solution (1mg ml^{-1} Tris-HCl-buffer, 10 mM, pH 8) were added respectively and incubated in darkness for 10 min. 1000 µl of the solution was filtered through a black 0.2 µm polycarbonate filter (Nuclepore black, 25 mm diameter, shiny side up) and washed twice with 4-6 ml Tris-HCl-buffer (sterile filtered, pH 8). The dried filter was fixed with 20 µl glycerin and Tris-HCl-buffer (1:1) solution and stored at room temperature. SyberGreen I (Molecular Probes, Eugene, Oregon, USA) and propidium iodine intercalates DNA helices and stains DNA, providing a UV fluorescent stain. SyberGreen I has been used to stain bacteria and viruses in samples from aquatic environments for epifluorescence microscopy because of its high specificity of dsDNA and the low background fluorescence (Noble and Fuhrman 1998; Weinbauer et al. 1998; Lunau et al. 2005). It stains life and dead bacteria cells providing a fluorescent green stain. Propidium iodine stained as a result of its charge only dead cells without intact membrane and stains these orange. The cells will be visible under Fluorescent Microscopy (450 - 490 nm), (Boulos et al. 1999). Bacteria were counted with a Zeiss Axiolab microscope at 1000x magnification (lamp: HBO 50, filter set: Zeiss, Ex 450-490, FT 510, LP 515). Per sediment treatment three replicates were performed. Per filter the bacteria cells of 10 microscopic fields (squares) were randomly counted. Numbers of bacteria in treatments where antibiotics were used were parted in dead and life cells.

DIATOMS were enumerated by direct light microscopy (ASSAY 1-5). Sediment conditioned with algicide and/or antibiotics, kept in illumination or in darkness and sterile or ashed sediment inoculated with diatom suspension used in ASSAY 4 was weighed (1 g) in 20 ml plastic tubes and 10 ml sterile filtered seawater were added and mixed gently. This mixture was diluted 1:1000 with sterile filtered seawater and 3 ml were added into cylindrical 2.6 cm in diameter plastic tubes.

Microscopically enumeration of diatoms was processed through a cover slip thick bottom using an inverse microscope (Axiovert 10). Per sediment treatment three replicates were used to determine the amount and mobility of diatoms after algicide/antibiotic treatment. The documentation was processed in number of diatoms g^{-1} sediment for each treatment. The number of diatoms were determined using a grid inserted in the microscope objective split into 10 to 10 small fields, the mean amount of diatoms per one grid (sum of all 10 to 10 grids) multiplied with the area of the cylindrical tube (54.54 mm^2) multiplied with dilution factor 1000, multiplied with the net weight and divided through the amount of used solution (3 ml) and the outcome of this was the number of diatoms per mm^2.

4.1.2 NATURAL SEDIMENT IN ILLUMINATION AND DARKNESS

Natural untreated sediment (2.5 g) was placed into each well of the 6 well sterile microplates and overlaid with sterile-filtered seawater to a water layer of 2 cm. One treatment was placed in illumination (white light, 10.0 µmol $m^{-2}s^{-1}$) for 4 h or 2 days and the other one in complete darkness as performed in ASSAY 3, 4, (Tab. 2). DIATOMS were enumerated by direct light microscopy as described above.

Tab. 2: Larval single-choice test on natural sediment in ASSAY 3 in illumination and darkness and ASSAY 4 A+B treated with algicide, antibiotic, in illumination and darkness; performed with *P. cornuta* larvae

		ASSAY 3	ASSAY 4 A	ASSAY 4 B
treatment	**sediment**		**conditioning**	
1	natural		no condition (positive control)	
2	ashed		800 °C (negative control)	
3	sterile		autoclaving	
4	natural	———	algicide (10 mg g^{-1}, 36h)	algicide (10 mg g^{-1}, 36h)
5	natural	———	incubation; 36h	antibiotics (36h)
6	natural	———	light (48h)	incubation; 36h
7 (4)	natural	light (2d)	dark (48h)	light (48h)
8 (5)	natural	dark (2d)	———	dark (48h)

4.1.2.1 SETTLEMENT ASSAYS

The single-choice assay was performed with 2.5 g (wet weight) of sediment transferred into each well, resulting in a 7 mm sediment layer. After incubation time the sediment was overlaid with sterile filtered seawater up to 1 cm and 10 *Polydora cornuta* larvae were randomly picked from a homogenous control batch

and added onto the sediment. The single-choice settlement experiment was processed as described in chapter 2.

4.1.3 APPLICATION OF STERILE AND ASHED SEDIMENT WITH VIABLE DIATOMS

Natural sediment was obtained at low tide from the field using Petri dishes. In the laboratory lens tissue paper (1.5*1.5 cm) was placed on the top of the sediment and captured for 12h at 20°C in light (10.0 µmol $m^{-2}s^{-1}$). Diatoms got caught in the lens tissue paper according to the lens tissue technique of Eaton and Moss 1966. After 12h 3 filter papers (for each treatment) were removed from the sediment surface and rinsed with sterile filtered seawater into one plastic centrifuge cap (50 ml, Corning USA) and centrifuged (500 g, 5 min) to concentrate the diatom suspension. After decanting the supernatant 12 ml sterile filtered seawater was added. The diatom solution was treated in different ways; the first treatment was contaminated with antibiotics penicillin (37.2 µg ml^{-1}) and streptomycin (364 µg ml^{-1}) and incubated for 36 h in darkness. The second treatment was used as control and contains only the diatom solution without antibiotics. The quantification of diatoms in sterile and ashed sediment treatments was performed as described in the chapter above. After 36h the plastic centrifuge caps with the antibiotics and the control solution were centrifuged (500 g, 5 min) and diluted with 12 ml sterile filtered seawater.

4.1.3.1 SETTLEMENT ASSAYS

For the assay, 2.5 g (wet weight) of sediment (ashed and sterile) was placed into each of 6 wells of a single-choice assay (Tab. 3), resulting in a 7 mm sediment layer. 1.75 ml of diatom solution with or without antibiotics was used to inoculate ashed or sterile sediment. After incubation (5 h) the suspension was removed and the sediment was overlaid with sterile filtered seawater up to 1 cm. 10 *Polydora cornuta* larvae were randomly picked from a homogenous control batch and added onto the sediment. The single-choice settlement experiment was processed as described in chapter 2. Diatoms were enumerated by direct light microscopy as described above.

Tab. 3: Larval single-choice test (ASSAY 5) on sterile and ashed sediment conditioned with diatom suspension (with or without antibiotics) performed with *P. cornuta* larvae

	ASSAY 5	
treatment	**sediment**	**conditioning**
1	natural	no condition (positive control)
2	ashed	800 °C (negative control)
3	sterile	autoclaving
4	sterile	diatoms (no antibiotics)
5	sterile	diatoms + antibiotics (36h)
6	ashed	diatoms (no antibiotics)
7	ashed	diatoms + antibiotics (36h)

4.1.4 DETERMINATION OF THE DIFFUSIVE BOUNDARY LAYER IN THREE SEDIMENT TYPES

Experiments were done at room temperature (20 - 22°C) by illumination (white light, 10.0 µmol $m^{-2}s^{-1}$). Natural, sterile and ashed sediment was transferred into glass vials (5 cm in diameter) resulting in a 1 cm sediment layer and overlaid with 2 ml sterile filtered seawater under constant aeration. Water movement was created by direction a gentle air stream over the water surface via Pasteur pipette connected to an air pump. Depth profiles of oxygen (dissolved O_2) concentration were measured by a Clark-type oxygen microelectrode (Max-Planck Institute, Germany) with a guard cathode (Revsbech 1989) mounted on a micromanipulator (Märzhäuser, Germany) and connected to a picoammeter and a stripchart recorder. Surface positioning of the microsensors was done visually. The electrodes had a tip diameter of 2-10 µm, a stirring sensitivity of 1-2 % and a 90 % response time of 0.2-0.4 s. Linear calibration of electrodes was done from electrode readings in air-saturated water above the biofilm and in the anoxic part of the biofilm.

A potentiometric oxygen sensor with a sensing cathode and an internal AG/AgCl reference anode submerged in an aqueous electrolyte chamber. Oxygen is a strong oxidant and will react with two electrons per O-atom, if a suitable reductant is present: $O_2 + 4e^- \rightarrow 4OH^-$. Oxygen diffusing through a silicone membrane at the electrode tip is reduced at the cathode, which is polarized at -0.8 V. The current produced is proportional to the oxygen partial pressure of the medium which the sensor is immersed.

Calculations: Vertical solute profiles are in steady state when conversion rates equal transport rates. Assuming diffusion as the only transport mechanism the activity of microorganisms, can be determined by applying a one-dimensional

diffusion reaction model (Revsbech and Jorgensen 1986) on a steady state microprofiels. This model based on Fick's first law calculating the flux, i.e. the rate of mass transfer per unit area, for each data point of the concentration profile:

$$J = D^e \frac{dC(z)}{dz}$$

Where J is the flux [mmol m^{-2} h^{-1}], D_e is the apparent diffusion coefficient in sediment as calculated from the diffusion coefficient in water D_0 and the porosity Φ of the sediment ($D_s = D_O * \Phi$) [m^2 h^{-1}], and δc/x is the concentration gradient between two depth x_1 and x_2. Diffusion coefficient for O_2 was 2.1 x 10^{-5} cm^2 s^{-1} in water and was found in the literature (Broecker and Peng 1974) with a porosity of 1. Microprofiles were obtained at randomly chosen locations (n=5) in a single sediment treatment (glass vial).

4.2 APPLICATION OF EXOPOLYMERS, ENZYMES AND LECTINES ON SEDIMENT

4.2.1 ISOLATION OF EPS FROM NATURAL SEDIMENTS

The uppermost (5 mm) surface layer of sediments was collected from sediments (Hooksiel, Germany) for EPS extraction and measurement of EPS (carbohydrate) concentration. Natural EPS was extracted from sediments according to Decho et al. (2003). 5 g of wet sediment were put into a plastic centrifuge cap (50 ml, Corning, USA), mixed with 20 ml (4mM) EDTA (ethylene diaminetetraacetic acid) in sterile filtrated seawater, vortexed gently for 1 min and heated in an ultrasonication bath (40 ± 1°C, 15 min). The suspension contained cells, detritus and EPS; it was centrifuged (10000 X g, 6 min) to pelletize cells and particulate detritus. The resulting supernatant, contained EPS, was removed by pipette, mixed with 70% (final concentration) cold (4°C) ethanol, and precipitated for 8 h in the fridge (4°C). The pellet and remaining sediment were re-extracted using EDTA and heated as described above. This latter procedure was repeated 3 times for a total of four extractions. Six replicates were used for each treatment. After 8 h the precipitated material from the supernatant was collected by centrifugation (3200 X g, 10 min). The EPS pellet was washed (2 times) with 20 ml 96% ethanol (4°C), vortexed (1 min) and centrifuged (3200 X g, 10 min). The EPS was lyophilized and stored at -70°C prior to analysis. The sediment from each sample was washed with deionized water to remove salts and dried at 70°C for 24 h to determine the dry weight.

4.2.2 QUANTIFICATION OF EPS

The quantification of EPS material was conducted according to ration of the phenol-sulfuric acid method Underwood et al. (1995) and Smith and Underwood (1998). The phenol-sulfuric acid method measured total carbohydrate concentration of sediment directly in sediment samples that had not been subjected to any extraction procedure; therefore, all carbohydrates within detection capacity of the assay, including intracellular, extracellular, and particle-bound material, was hydrolyzed and measured.

Total EPS concentrations of extracted pelletized EPS were measured by adding 2 ml of distilled water to a known amount of sediment, followed by 1 ml 5% aqueous phenol (wt/vol) and 5 ml of concentrated H_2SO_4. The resulting hydrolization products were measured spectrophotometrically against a reagent blank at 485 nm (U-3000, Hitachi). Calibration was via a standard curve of absorption vs. glucose concentration to give results in microgram glucose equivalents (glc. eq.).

Natural sediment was centrifuged (4000 x g, 20°C, 1 min) to separate pore water and sediment to determine directly the EPS concentration without sediment extraction as mentioned above. 40 mg of sediment were weighed into a plastic centrifuge cap (50 ml, Corning, USA) and 2 ml of distilled water, 1 ml 5% aqueous phenol (wt/vol) and 5 ml of concentrated H_2SO_4 were added. The viscous solution was carefully shaken and centrifuged (3500 X g, 1 min, 20°C). The supernatant was put into a glass cuvette (2 ml) and the absorption was measured against a reagent blank at 485 nm (U-3000, Hitachi). Sediment aliquots were dried at 70°C for 24 h to determine the dry weight and to calculate the EPS concentration.

4.2.3 APPLICATION OF EPS ON ASHED SEDIMENT AND LARVAL SETTLEMENT ASSAYS OF EPS TREATED SEDIMENTS

Ashed sediment (15 mg) was weighed into a plastic centrifuge cap (50 ml, Corning, USA) and 2 ml EPS solution (500 µg EPS^{-1} dissolved in sterile filtered seawater) were added and shaken gently for 1 min. Sediment was incubated at 8°C for 24 h (ASSAY 1A) and 18 h (ASSAY 1B, Tab. 4). 1 g sediment was used to determine the EPS concentration ashed sediments inoculated with EPS suspension in use of the phenol sulfuric acid method. In a third assay ashed sediment was inoculated with EPS solution for 7 days and EPS concentration was determined, in that case no additional larval settlement assay was performed.

4.2.3.1 SETTLEMENT ASSAYS

After incubation the EPS suspension was removed and the sediment was centrifuged (4000 X g, 1 min, and 20°C). The EPS concentration was determined using the phenol-sulfuric acid method. 2.5 g of sediment was weighed into each well of the sterile 6 well sterile microplates and overlaid with sterile filtered seawater up to 1 cm and 10 *Polydora cornuta* larvae were randomly picked from

a homogenous control batch and added onto the sediment. The single-choice settlement experiment was processed as described in chapter 2.

Tab. 4: ASSAY 1 A, B: Larval single-choice test on ashed sediment conditioned with EPS solution for 18 h and 24 h performed with larvae of *P. cornuta*

		ASSAY 1A	ASSAY 1B
treatment	**sediment**	**conditioning**	
1	natural	no condition (positive control)	
2	ashed	800 °C (negative control)	
3	sterile	autoclaving	
4	ashed	EPS solution (18h)	EPS solution (24h)

4.2.4 ENZYMATICAL, CHEMICAL AND MECHANICAL DEGRADATION OF NATURAL SEDIMENT AND PERFORMANCE OF LARVAL SETTLEMENT ASSAYS

A mixture of five different enzymes were chosen with consideration of their effects on EPS degradation on natural sediment according to Böckelmann et al. (2003), Wolfaardt et al. (1997) and Kirchman et al. (1982b). The enzyme mixture contained β-glucuronidase (Sigma), it cuts side chains of carbohydrates of proteins and showed endo- and exoglycoside activity. β-glucosidase from baker's yeast (pH 6.0, 25°C, Sigma) was chosen for the cleavage of β –D-glucoside residues. Trypsin (Sigma) hydrolyzed polypeptide on the carboxy side of arginin and lysine residue, it belongs to the endopeptidases which were characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain. β-galactosidase from *Aspergillus oryzane* (pH 4.5, 30°C, Sigma) was selected to hydrolyze β-galactosidic bonds and lipase from porcine pancreas (pH 7.7, 37°C, Sigma) was added to the enzyme mixture as lipids represented a considerable part (1-40%) of the bacterial EPS (Flemming and Wingender 2001).

Enzyme concentrations were calculated for 20 g (wet weight) natural sediment and 400 mg (2000 units) β-glucuronidase, 20 ml (2000 units) β-glucosidase, 1 mg ml^{-1} trypsin, 400 mg (2000 units) β-galactosidase and 300 mg (9600 units) lipase were added to 20 ml 1M Tris Na_2HPO_4 buffer (pH 5.6), 2 mM $MgCl_2$, further referred to as "enzyme-mix".

Natural sediment (20 mg) was weighed into a plastic centrifuge cap (50 ml, Corning, USA) and washed with 30 ml [1M Tris Na_2HPO_4 buffer (pH 5.6)] and centrifuged for 3 min at 1700 g for three times. After short spin the supernatant was discarded and buffer (10 ml, 1M Tris Na_2HPO_4, pH 5.6, 2 mM $MgCl_2$) and

enzyme mixture (20 ml) were added onto the sediment. Samples were incubated for 15 h on a rotor shaker at 30 °C and 30 ml 5 mM EDTA (ethylenediamineteraacetic acid) and 0.1 % sodium dodecylsulfate (SDS) solution was added and mixed gently and treated in an ultrasonic water bath for 30 min. After short centrifugation (2000 g) the supernatant was discarded and 30 ml 5 mM EDTA and 0.1 % SDS solution was added, this was processed for three times. After last centrifugation the sediment treatments were washed with sterile natural seawater (32‰) twice.
Additionally, three different treatments were compounded by adding EDTA (5 mM), EDTA and ultrasonication and only Tris buffer as control onto natural sediment.

4.2.4.1 SETTLEMENT ASSAYS

2.5 g sediment of each treatment (Tab. 5) was weighed into each well (n = 12) and overlaid with sterile filtered seawater up to 1 cm and 10 *S. benedicti* larvae were randomly picked from a homogenous control batch and added onto the sediment. The single-choice settlement experiment was processed as described in chapter 2.

Tab. 5: Larval single-choice test on sediment treated with enzymes, EDTA and ultrasonication performed with larvae of *S. benedicti*

treatment	sediment	conditioning
1	natural	no condition (positive control)
2	ashed	800 °C (negative control)
3	sterile	autoclaving
4	natural	EDTA + ultrasonication
5	natural	washing with Tris buffer (control)
6	natural	enzyme mix + EDTA
7	natural	EDTA

4.2.5 TREATMENT OF SEDIMENT WITH LECTINS AND PERFORMANCE OF LARVAL SETTLEMENT ASSAYS

Lectins of different sugar specificities and concentrations were used and based on experiments of Heller (1979); Kirchman et al. (1982b); Hood and Schmidt (1996); Matsumura et al. (1998) and Khandeparker et al. (2003). Concanavalin A (conA) a lectin specific to glucose and mannose moieties of larger

glycoconjugates and peanut lectin with an affinity for galactose (Goldstein and Hayes 1978) was used in combination to treat sterile sediment.
Concanavalin A (Sigma Type I) (2.0 mg/ml) and peanut lectin (Sigma) (0.082 mg/ml) isolated from *Arachis hyogaea* were dissolved in 20 ml 3.0 % (w/v) NaCl and mixed gently. 25 g of sterile sediment were transferred into a centrifuge cap (50 ml, Corning, USA) and 20 ml of combined lectin solution was added, mixed permanently and incubated at 18°C for 3 h. Thereafter the sediment was centrifuged (1000 g, 5 min) and the supernatant was discarded. The sediment was washed once with sterile filtered seawater (0.2 µm), centrifuged (1000 g, 5 min).

4.2.5.1 SETTLEMENT ASSAYS

1.75 g of lectin conditioned sediment was weighed into each well (n = 12) of the sterile microplates and overlaid with sterile filtered seawater up to 1 cm. 10 *S. benedicti* were randomly picked from a homogenous control batch and added onto the sediment treatments (Tab. 6). The single-choice settlement experiment was processed as described in chapter 2.

Tab. 6: Larval single-choice test (ASSAY 1) on sterile sediment conditioned with lectins performed with larvae of *S. benedicti*

		ASSAY 1
treatment	**sediment**	**conditioning**
1	natural	no condition (positive control)
2	ashed	800 °C (negative control)
3	sterile	autoclaving
4	sterile	lectin mixture

4.2.6 TREATMENT OF LARVAE WITH LECTINS AND PERFORMANCE OF LARVAL SETTLEMENT ASSAYS

20 ml of combined lectin solution (conA and peanut lectin) was filled into a Petri dish and larvae of *S. benedicti* were added and incubated for 1 h at room temperature. 2.5 g sterile sediment was weighed into each well (n = 12) of the sterile microplates and overlaid with sterile filtered seawater up to 1 cm and 10 *S. benedicti* were put on the sediment per well. The single-choice settlement experiment was processed as described in chapter 2.

4.2.7 TREATMENT OF SEDIMENT WITH MONOSACCHARIDES AND PERFORMANCE OF LARVAL SETTLEMENT ASSAYS

Processes mediated by lectins are inhibited by simple sugars for that purpose sterile sediment was treated with four different types of monosaccharides; D-Glucose, D-Mannose, D-Fructose and D-Galactose, all obtained from Sigma, Germany. Each of which was diluted in sterile filtered seawater (0.2 µm) at a concentration of 50 mM and 200 mM respectively (Tab. 7). A combined monosaccharide solution (200 mM) was prepared of all four monosaccharides diluted in sterile seawater.

4.2.7.1 SETTLEMENT ASSAYS

2.5 g of sterile sediment was weighed into each well of the sterile 12 well microplates and overlaid with each monosaccharide solution or monosaccharide mixture up to 1 cm and directly 10 *S. benedicti* larvae were randomly picked from a homogenous control batch and added onto the sediment of each well (n = 12). The single-choice settlement experiment was processed as described in chapter 2.

Tab.7: Larval single-choice test (ASSAY 2, 3) on sterile sediment conditioned with lectins and monosaccharides (50 mM) and ASSAY 4 on sterile and ashed sediment conditioned with monosaccharides and sugar mixture (200mM) performed with larvae of *S. benedicti*

		ASSAY 2	ASSAY 3	ASSAY 4
treatment	**sediment**	**conditioning**		
1	natural	no condition (positive control)		
2	ashed	800 °C (negative control)		
3	sterile	autoclaving		
4	sterile	lectin mixture		D-glucose 200 mM
5	sterile	larvae in lectin mixture		D-mannose 200 mM
6	sterile	D-glucose 50 mM		D-fructose 200 mM
7	sterile	D-mannose 50 mM		D-galactose 200 mM
8	sterile	D-fructose 50 mM		———
9	sterile	D-galactose 50 mM		sugar monomer mix 50 mM
10	sterile	———	sugar monomer mix 200 mM	
11	sterile	———	control	———
12	ashed	———	———	D-glucose 200 mM
13	ashed	———	———	D-mannose 200 mM
14	ashed	———	———	D-fructose 200 mM
15	ashed	———	———	D-galactose 200 mM
16	ashed	———	———	sugar monomer mix 200 mM
17	ashed	———	———	sugar monomer mix 50 mM

4.3 APPLICATION OF HALOMETABOLITES ON SEDIMENT

4.3.1 ADULT BROOD STOCKS AND LARVAL TEST ORGANISMS

Test organisms in this study were the tube-building, infaunal spionid polychaete *Streblospio benedicti* obtained from mud flats at Hooksiel (53°38’31’’N, 8°04’55’’E) and maintained in the laboratory according to Sebesvari et al. (2006) and *Capitella* sp.I. At the sampling site *S. benedicti* occurs in densities of 5000 individuals per m^2 (Vöge, Senckenberg Institue, Wilhelmshaven, Germany) in intertidal flats of the western part of the Jadebusen but the interannual fluctuation was very high, in particular not a single individuum has been observed in the years 2000 and 2001 (pers. observation). The protocols for larval cultures and selection of test larvae for bioassays were adopted from the same study (Sebesvari et al. 2006). The larvae from this brood stock exhibit a planktotrophic mode of larval development. Laboratory stock cultures of adult *Capitella* sp.I were established in our laboratory in September 2004 with worms obtained from JM Rosario (Institute of Marine and Coastal Sciences, Rutgers State University, New Jersey, USA) and reared according to Grassle and Grassle (1976) and Butman and Grassle (1992).

4.3.2 SEDIMENT PREPARATION

Sediment was collected during low tide in mudflats of the Wadden Sea (53°38’31’’N, 8°04’55’’E) (Hooksiel, Germany) throughout summer month of 2006. Newly collected sediment was stored in the darkness in plastic containers at 4 °C for no longer than 1 week (in the following referred to as “natural sediment”). Natural sediment was sterilized by autoclaving immediately before bioassays (in the following referred to as “sterile sediment”). Natural sediment was ashed at 600°C for 4 h in a muffle kiln. Before usage in the bioassay, ashed sediment was covered with sterile filtered seawater and autoclaved (in the following referred to as “ashed sediment”).

4.3.3 LARVAL SETTLEMENT ASSAYS

Single-choice settlement assays were performed with different sediment treatments in sterile 12-well microplates (3.8 cm^2 well surface area, Corning,

USA). Natural, ashed and sterile sediments were assayed simultaneously in well-plates. 2.5 g (wet weight) of sediment was transferred into each well resulting in a 7 mm sediment layer. The sediment was covered with 1.75 ml of sterile-filtered seawater (i.e. filtered through 0.22 µm membranes) and 10 *S. benedicti* larvae randomly picked from a homogenous control batch and added onto the sediment. The single-choice settlement experiment was processed according to Sebesvari et al. (2006), chapter 2.

Multiple choice settlement assays were performed with four different sediment treatments in sterile cylindrical Plexiglas plates (17 x 1.5 cm) containing 16 cylindrical holes (2 x 0.3 cm) and equipped with a removable bottom (chapter 3). The wells were arranged in four by a rows and columns separated by 1 cm. In a 4 x 4 Latin-square design four replicates of different sediment treatments were transferred into wells, i.e. every sample was placed exactly once per row and column. The plate was filled with 200 ml of sterile-filtered seawater and ca. 1000 larvae were added. After 20 h, the water was poured off and the plexiglas bottom was removed. Individual sediment samples were transferred onto glass plates. Settled larvae were stained as above and counted under the stereo microscope. The settlement index in replicated samples was calculated based on the total number of settled larvae (100 %) discounting all larvae still swimming or lying on the sediment surface without burrowing activity.

4.3.4 SPECIMEN CLLECTION AND COMPOUND EXTRACTION

Adult worms were pooled from laboratory brood stocks. The average length and width (n = 10) of adult specimen was measured under the binocular and the tissue volume was calculated assuming a cylindrical shape of worms. The extraction consisted of placing pooled worms (60 individuals of *S. benedicti* and 40 individuals of *Capitella* sp.I) in 600µl of GC-MS grade acetone (*S. benedicti*) or methanol (*Capitella* sp.I). Samples were stored for 48h at 4°C. Insoluble tissue material was spun down and the supernatant was dried in vacuo. The dry extract residue was dissolved in 50 µl of acetone or methanol for quantification.

4.3.5 QUANTIFICATION OF HALOMETABOLITES IN WORM TISSUE

The dry extracted residue of *S. benedicti* and *Capitella* sp.I were analyzed using coupled gas chromatography-mass spectrometry analysis (Varian 3900, MS-

Varian Saturn 2100T, USA). All samples were centrifuged prior to analysis to remove particular matter. The GC thermal parameters (Tab. 8) were used for the analyses of halometabolites in adults of *S. benedicti* and *Capitella* sp.I. Aliquots of 1 µl tissue extract were injected and separated on WCOT VF-5ms (Varian, USA) capillary column (30 m x 0.25 mm x 0.25 µm film thickness) using a constant pressure of 72 kPa helium.

Tab. 8: mass-spectrometer acquisition-parameters to analyze adults of *S. benedicti* and *Capitella* sp. I, temperature program and MS-method editor

temperature program

Streblospio benedicti				*Capitella sp.I*			
Temp. (°C)	**rate (C/min)**	**hold (min)**	**total (min)**	**Temp. (°C)**	**rate (C/min)**	**hold (min)**	**total (min)**
70		3.00	3.00	90		3.00	3.00
210	5.00	3.00	34.00	150	3.00	0.00	23.00
320	100.00	1.00	36.10	320	20.00	0.00	31.00

MS-Method-Editor

low mass (m/z)	high mass (m/z)	ionization mode	low mass (m/z)	high mass (m/z)	ionization mode
40	350	EI Auto	50	300	EI Auto

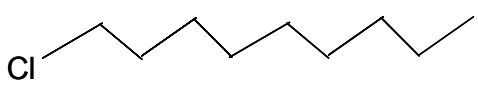
The electron impact ion-spectra of volatile tissue extract components were compared with entries in the NIST mass spectral library (NIST 05).

A calibration of halogenated proxy compounds was performed with 1-chlorononane and 2,6-dibromophenole (Sigma Aldrich, Germany). The average concentrations of halogenated proxies were calculated from the mean of three injections of each sample. Following this protocol the resulting average amount of the sum of chlorononane isomers (for *S. benedicti*) and dibromophenoles (for *Capitella* sp. I) was 70.57 ± 0.08 ng mm^{-3} and 20.71 ± 1.65 ng mm^{-3} tissue volume, respectively. These halometabolite concentrations were used as the basis for spiking experiments with sterile and natural sediments.

4.3.6 SPIKING, EXTRACTION AND QUANTIFICATION OF HALOMETABOLITES IN SPIKED SEDIMENTS

All single-choice and multiple-choice settlement assays were performed with commercially available 1-chlorononane and 2,6-dibromophenole (Sigma Aldrich, Germany) (Fig 4).

1-chlorononane; $C_9H_{19}Cl$



2,6-dibromophenole;
$C_6 H_4 Br_2 O$

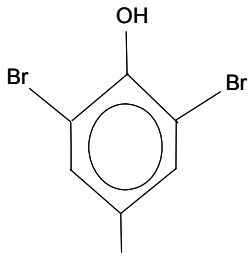


Fig. 4: 1-chlorononane and 2,6-dibromophenol used in larval single-choice and multiple-choice assays

The amount of halogenated proxies used to spike sediment aliquots of 2.5 g each (see bioassays) was equivalent to the concentration of chlorononane isomers and dibromophenoles determined in 20 adults (in the following referred to as 1x concentration). This final concentration reflected a homogeneous distribution of halometabolites provided their complete dissolution in the sediment. Under the assumption that halometabolites were more patchily distributed in spots of high concentration (e.g. worm burrows), a ten fold elevated concentration of halogenated proxies was applied in bioassays (in the following referred to as 10x concentration). Stock solutions of the halogenated proxies 1-chlorononane and 2,6-dibromophenole were prepared in 100 µl of acetone and methanol, respectively. Aliquots of the stock solutions were diluted with 5 ml of sterile filtered seawater and mixed with sterile or natural sediment. The final concentrations in sediment treatments for bioassay purposes were 0.56 and 5.6 µg 1-chlorononane g^{-1} sediment, and 0.165 and 1.65 µg 2,6-dibromophenole g^{-1} sediment. Blank controls were prepared accordingly by adding 100 µl of methanol or acetone to sediment treatments.

To determine the actual concentration of halogenated proxies in spiked sediments, sediment aliquots were extracted. Additionally 1 g of treated sediment sample was taken for bacterial DNA extraction (method described below). Briefly, 5 g of spiked sediment with either 1-chlorononane or 2,6-dibromophenole were put into a plastic centrifuge cap (50 ml, Corning, USA) and centrifuged (3900 g, 10 min) to discard the pore water containing dissolved proxy compounds. Sediments were extracted with 3 ml of dichloromethane, shaken overhead for 1 h on a rotor shaker and subsequently centrifuged (3900 g, 10 min). After centrifugation the supernatant was collected and transferred into a round bottom flask (25 ml). The extraction was repeated and pooled supernatants were concentrated by rotor evaporation (700 mbar, 35°C) followed

by solid phase extraction on silica cardiges (top Ø 0.8 cm, end Ø 0.2 cm, column-length 6 cm, silica fill-height 2 cm) equilibrated with dichloromethane. The column was eluted with 3 ml of dichloromethane.

The extraction was repeated and pooled supernatants were concentrated by rotor evaporation, followed by solid phase extraction on silica cartridges. The dichloromethane eluate was reduced to 100 µl by rotor evaporation (700 mbar, 35°C). 1 µl of the sample was injected into the GC-MS. The concentration of halogenated proxies was determined by GC-MS as described in Tab. 9.

Tab. 9: mass-spectrometer acquisition-parameters to analyze sediment spiked with halometabolites, temperature program and MS-method editor

temperature program

spiked sediment treatments

Temp. (°C)	rate (C/min)	hold (min)	total (min)
70		3.00	3.00
210	5.00	0.00	31.00
300	50.00	3.00	35.80

MS-Method-Editor

low mass (m/z)	high mass (m/z)	ionization mode
40	400	EI Auto

4.3.7 SETTLEMENT BIOASSAYS

4.3.7.1 DIRECT EFFECT OF HALOGENATED PROXIES ON LARVAL SETTLEMENT

In a replicated single-choice assay (n = 2), the halogenated proxies 1-chlorononane and 2,6-dibromophenole were assayed at both spiking concentrations together with natural sediment (positive control), ashed and autoclaved sediments (negative controls), and the solvent (acetone and MeOH) treatments (blank control). The single-choice settlement experiment (Tab. 10) was processed as described in chapter 2.

Tab. 10: Direct effect of 1-chlorononane and 2,6-dibromophenole at 1x and 10x concentration and acetone or MeOH spiked on sterile sediment performed in a single-choice assay (ASSAY 1, 2,) with *S. benedicti* larvae

treatment	sediment	conditioning
1	natural	no condition (positive control)
2	ashed	800 °C (negative control)
3	sterile	autoclaving
4	sterile	dibromophenole (0.165 µg g^{-1})
5	sterile	dibromophenole (1.65 µg g^{-1})
6	sterile	1-chlorononane (0.56 µg g^{-1})
7	sterile	1-chlorononane (5.6 µg g^{-1})
8	sterile	MeOH (assay 1+2)
9	sterile	acetone (assay 2)

The same sediment treatments were investigated in 3 multiple choice assays with replication (n = 3) in different combinations of sediment treatments (Tab. 11, ASSAY 1-3), testing the larval choice between the negative control, the blank control and one halogenated porxy at 10x and 1x concentration, and both halogenated proxies at 10x concentration together with the positive and negative control. The multiple-choice test was performed as desctibed in (Sebesvari et al. 2007).

Tab. 11: Direct effect of halometabolites (1-chlorononane, 2,6-dibromophenole) at 1x and 10x concentration and acetone or MeOH spiked on sterile sediment performed in a multiple-choice settlement ASSAY 1-3 with *S. benedicti* larvae

		ASSAY 1	ASSAY 2
treatment	**sediment**	**conditioning**	
1	sterile	autoclaving	autoclaving
2	sterile	acetone	MeOH
3	sterile	1-chlorononane (0.56 µg g^{-1})	dibromophenole (0.165 µg g^{-1})
4	sterile	1-chlorononane (5.6 µg g^{-1})	dibromophenole (1.65 µg g^{-1})

ASSAY 3	
sediment	**conditioning**
sterile	autoclaving
natural	no condition (positive control)
sterile	dibromophenole (1.65 µg g^{-1})
sterile	1-chlorononane (5.6 µg g^{-1})

4.3.7 2 INDIRECT EFFECT OF ADULT WORMS AND HALOGENATED PROXIES ON LARVAL SETTLEMENT

Aliquots of 2.5 g (wet weight) natural sediment was populated with 20 live adults of each worm species per well (in a single-choice microplate or multiple-choice

test chamber). In parallel, natural sediment was spiked with halogenated proxies at 10x concentration. The sediment treatments (Tab. 12) were transferred into each well of a single-choice or multiple-choice test chambers in 4 x 4 Latin square manner as described in chapter 3 (Sebesvari et al. 2007) together with natural sediment as positive control and incubated in natural seawater (32 ppt, 16°C) for 7 days under a 12 h dark: 12 h light photo cycle. Additionally no choice microplates were incubated for 6, 7, 8, 12, 14, 23, 27 and 29 days (Tab. 12).

Tab. 12: Indirect effect of adult worms and halometabolites (1-chlorononane, 2,6-dibromophenole) on larval settlement of *S. benedicti* in natural sediment incubated in seawater for 6-29 days (single-choice settlement ASSAY 1-8) and multiple-choice assays for 7 days

no choice ASSAY

treatment	sediment	conditioning
1	natural	no condition (positive control)
2	ashed	800 °C (negative control)
3	sterile	autoclaving
4	natural	adult Capitella
5	natural	adult Streblospio
6	natural	control no adults

multiple-choice		ASSAY 4	ASSAY 5
treatment	sediment	conditioning	
1	sterile	autoclaving (after 7 days)	autoclaving (after 7 days)
2	natural	7 days incubation	7 days incubation
3	natural	adults of Capitella (7 days incubation)	DBP (7 days, incubation)
4	natural	adults of Streblospio (7 days incubation)	1CIN (7 days incubation)

After individual incubation times, test chambers were carefully retrieved and the natural see water was removed. The remaining test wells were filled with sterile sediment and bioassays were carried out as described above. Aliquots of 1 g of each sediment treatment were sampled to determine the concentration of halogenated proxies at day 0 and day 7 and for molecular biological analyses (method described below).

4.3.8 MOLECULAR BIOLOGICAL ANALYSIS OF SEDIMENT ASSOCIATED BACTERIAL COMMUNITIES

4.3.8.1 EXTRACTION OF BACTERIAL GENOMIC DNA ON NATURAL SEDIMENT

To extract bacterial community DNA from different sediment treatments aliquots of 1 g sediment were sampled and mixed with 1 ml of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM Na_2-EDTA [pH 8.0], 100 mM sodium phosphate [pH

8.0] 1.5 M sodium chloride, 1% cetyltrimethylammonium bromide, 2 % sodium dodecylsulfate [pH 7.5], in sterile 2 ml screw caps and stored at -20°C. Samples were repeatedly frozen in liquid nitrogen and defrosted in warm water (65°C). Bacterial cells were enzymatically lysed by addition of 20 µl proteinase K (20 mg ml^{-1}) and incubation for 30 min at 37°C under gentle shaking. Subsequently, 100 µl of sterile filtered sodium dodecylsulfate (20 %) was added and the samples were incubated for 2 h at 65°C. After centrifugation the supernatants were mixed with equal volume of chloroform-isoamyl alcohol (24:1, v/v) for 15 min. The DNA in the aqueous phase was precipitated by addition of 0.6 volumes of isopropanol and storage at 4°C over night. Crude DNA was pelleted by centrifugation washed twice with cold 70 % ethanol and dissolved in 50 µl of sterile deionized water. Aliquots of crude community DNA were stored at -20°C until usage.

4.3.8.2 PCR AMPLIFICATION OF 16S RRNA GENE FRAGMENTS

16S-rRNA genes in bacterial community DNA were amplified by polymerase chain reaction (PCR) in a total volume of 50 µl containing 1 µl of DNA template, 20.5 µl sterile deionized water, 5 µl PCR-buffer, 7.5 µl of each dNTP, 7.5 µl bovine serum albumin (BSA), 5 µl $MgCl_2$ (20 mM), and 2 µl of each universal primer: 341F (5´-ATT ACC GCG GCT GCT GG-´3) and 534R_GC (5´- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG-3´) (Muyzer et al. 1993), and 0.5 µl taq polymerase (peqlab, Germany). After the initial denaturing cycle at 95°C for 3 min, samples were amplified with a touchdown PCR protocol of 2 cycles at 94°C for 30 s, 64°C for 30 s and 72°C for 1 min and 9 cycles of 94°C for 30 s, 63 °C – 1°C per cycle for 30s and 72°C for 1 min and 25 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 1 min, followed by a final extension step at 72°C for 10 min. A negative control containing the PCR master mix without template DNA was run in parallel. After the amplification 4 µl of each PCR amplicon was electrophoresed along with a 1000 bps ladder and a positive control prepared from 7 bacterial isolates and a negative control on a 1.0 % agarose gel in 1x TAE at 70V for 30 min. The resulting PCR-product of 193 bps was stained with ethidium bromide for 30 min and evaluated under the UV-table (Alpha-Imager, Biozyme Scientific GmbH, Germany) and photographed in use of an digital camera (Olympus) mounted on the UV table.

4.3.8.3 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) ANALYSIS OF PCR PRODUCTS

For denaturant gel electrophoresis (DGGE) 30 µl of PCR product were loaded on a 6.5% [w/v] polyacrylamide gel. An optimal separation was achieved with a gradient of 40 to 75% denaturant from top to bottom (100% denaturant was defined as 7M urea and 40% [v/v] formamide. DGGE was performed with a PhorU-System (Ingeny, Netherlands) in 1 x TAE running buffer at 60°C for 18 h at 100V. Gels were stained for 30 min in SybrGold nucleic acid stain (Molecular Probes, Netherlands) according to the manufacturers protocol.

The gel banding patterns of different samples were subject to cluster analysis. Bray Curtis similarities were used to produce a similarity matrix based on the total number of bands found in all samples and the presence or absence of these bands in individual samples. For the construction of a dendrogram depicting the similarity of microbial communities in different samples group average linkage and hierarchical agglomerative clustering was performed using the PRIMER v. 5 computer program (Plymouth Marine Laboratory, UK).

4.4 STATISTICAL ANALYSIS

LARVAL ASSAYS

The single-choice larval settlement experiments were performed with 10 larvae in 6 or 12 replicates. The rates of larval settlement were expressed in percentage and tested for normal distribution (Shapiro-Wilk's W-test). Since settlement data are generally not normally distributed they were rank-transformed prior to further statistical analyses. After rank transformation, settlement data were analyzed by one-way ANOVA followed by Tukey's multiple comparison Test (Conover and Iman 1980).

All multiple-choice assays were performed with ~1000 *S. benedicti* larvae, normal distribution of the settlement response in populations from which the samples were drawn was assumed. The number of counted larvae was log(x+1) transformed to stabilize their variance. Levene's test was employed to check the assumption of homogeneity. Where significant heterogeneity of variance could not be removed by transformation, a lower significance level ($p = 0.01$ instead of $p = 0.05$) was used (Underwood 1997). Effects of row, column, treatment (fixed factors) on larval metamorphosis were analyzed with main-effect ANOVA for each experiment. If the treatment effect was significant, Tukey's multiple comparison tests were used to locate the differences identified by ANOVA ($\alpha = 0.05$).

CLUSTER ANALYSIS

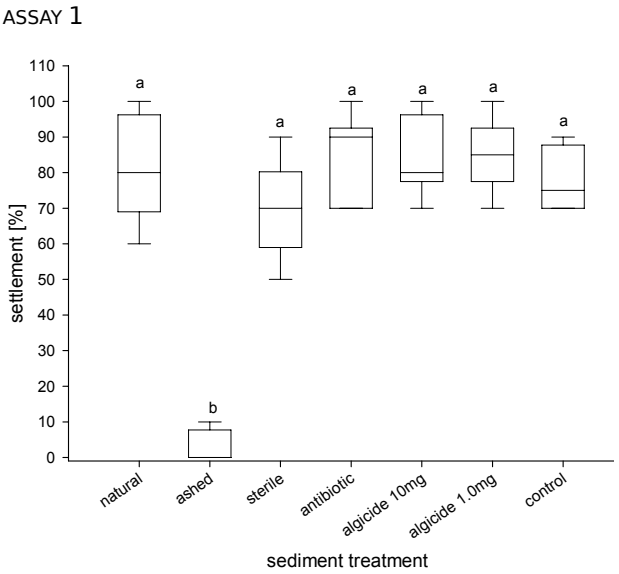
The DGGE banding patterns of different bacterial community DNA samples of all treatments were subjected to cluster analysis. Bray Curtis similarities were used to produce a similarity matrix based on the total number of bands found in all samples and the presence or absence of these bands in individual samples. For the construction of a dendrogram demarcating the similarity of microbial communities on the gels, group average linkage in the hierarchical, agglomerative clustering algorithm was performed using the PRIMER v. 5 computer program (Plymouth Marine Laboratory, UK).

5 RESULTS

5.1 ROLE OF DIATOMS AS POTENTIAL SETTLEMENT CUES

5.1.1 ROLE OF ALGICIDE AND ANTIBIOTICS ON DIATOM VIABILITY IN NATURAL SEDIMENT ON LARVAL RESPONSE

The percentage of larval settlement of *Streblospio benedicti* was significantly lower in ashed sediments than in natural, sterile and natural sediments treated with antibiotics (streptomycin, penicillin), cycloheximide (1, 10 mg g^{-1}) and natural sediment as control (1-way ANOVA, $p < 0.05$, Tukey's test, $p < 0.001$, Fig 5, ASSAY 1). Larval settlement on natural sediment treated with antibiotics, or algicide at 1 or 10 mg g^{-1} was the same (Tukey's test, $p = 0.99$).



Larval response to natural, ashed, sterile sediment; and sediment conditioned for 12 h with antibiotics (streptomycin 364.0 µg ml^{-1}, penicillin 37.2 µg ml^{-1}) and algicide (cycloheximide at 1 or 10 mg g^{-1}) and natural (control) sediment. Statistical differences are indicated by different letters above the boxes ($\alpha = 0.05$, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 6 replicates shown in a box-and-whisker diagram.

Fig. 5: ASSAY 1: Percentage of larval settlement of *Streblospio benedicti* (0.70 ± 0.11 mm body length, 11 setigers) per treatment in a single-choice assay after 1 h.

Diatom counts on sediment treated with cycloheximide either at 1 or 10 mg g^{-1} were significantly lower in comparison to sediment treated with antibiotics and natural sediment (control) (Student's *t*-test, $p < 0.05$). The antibiotic treatment had no influence on diatom abundance (Student's *t*-test, $p < 0.05$, Tab. 13).

Tab. 13: Diatom cell numbers (mm^{-2}) of natural sediment treated with antibiotics (streptomycin 364.0 µg ml^{-1}, penicillin 37.2 µg ml^{-1}) and algicide (cycloheximide) 1 and 10 mg g^{-1} sediment and natural (control) sediment (12 h) used in ASSAY 1

ASSAY1 treatment	mean cell numbers of diatoms (mm^{-2}) sediment	SD
antibiotic (12h)	$5.9 * 10^5$	$1.1 * 10^3$
algicide 10mg g^{-1} (12h)	$1.2 * 10^4$	$0.4 * 10^3$
algicide 1.0mg g^{-1} (12h)	$1.7 * 10^5$	$0.8 * 10^3$
control (12h)	$6.2 * 10^5$	$5.5 * 10^3$

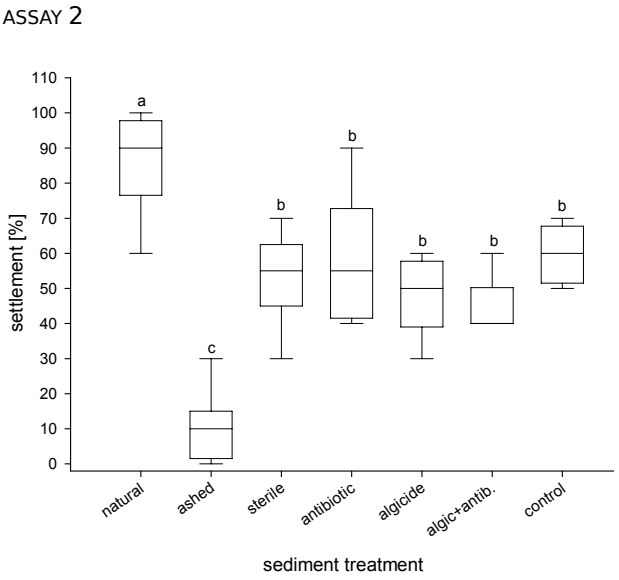
Number of bacteria cells in natural sediment treated with antibiotics were lower ($0.4*10^5 \pm 3.1*10^4$ g^{-1}) than in natural sediment treated with algicide ($2.1*10^7 \pm 1.5*10^4$ g^{-1}) or in natural sediment ($2.5*10^7 \pm 2.0*10^4$ g^{-1}). The bacterial abundance was not significantly affected in algicide treatments (Student's *t*-test, $p < 0.05$, Tab. 14)

Tab. 14: Bacterial cell counts (g^{-1}) in natural sediment treated with antibiotics (streptomycin 364.0 µg ml^{-1}, penicillin 37.2 µg ml^{-1}) and algicide (cycloheximide) 10 mg g^{-1} (12 h) used in ASSAY 1.

treatment	bacteria cell counts g^{-1} sediment	SD
antibiotic (all cells)	$1.7 * 10^7$	$4.0 * 10^4$
antibiotic (viable cells)	$0.4 * 10^5$	$3.1 * 10^4$
antibiotic (dead cells)	$1.3 * 10^7$	$2.2 * 10^4$
algicide (all cells)	$2.1 * 10^7$	$1.5 * 10^4$
natural (all cells)	$2.5 * 10^7$	$2.0 * 10^4$

The percentage of larval settlement of *Polydora cornuta* (ASSAY 2, Fig 6) in natural sediment was significantly higher (Tukey's test, $p < 0.05$) than in all other sediment treatments. The percentage of larval settlement of *P. cornuta* was significantly lower in ashed sediment than in natural, sterile and sediments treated with antibiotics (36 h), algicide (10 mg g^{-1}, 36 h), and algicide/antibiotics (36 h) and the control (natural) sediment (Tukey's test, $p < 0.05$).

Diatom counts in sediment used in ASSAY 2 treated with cycloheximide (10 mg g^{-1}) and the combination of cycloheximide and antibiotics were lower ($< 1.2*10^4 \pm 1.2*10^3$ mm^{-2}) than in sediment treated with antibiotics and natural sediment (control) ($> 5.5*10^5 \pm 0.6*10^3$ mm^{-2}) (Student's *t*-test, $p < 0.05$, Tab. 15).



Larval response to natural, ashed, sterile sediment; and sediment conditioned for 36 h with antibiotics (streptomycin 364.0 µg ml^{-1}, penicillin 37.2 µg ml^{-1}) and algicide (cycloheximide 10 mg g^{-1}), antibiotics / algicide, and control (natural) sediment. Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 6 replicates shown in a box-and-whisker diagram.

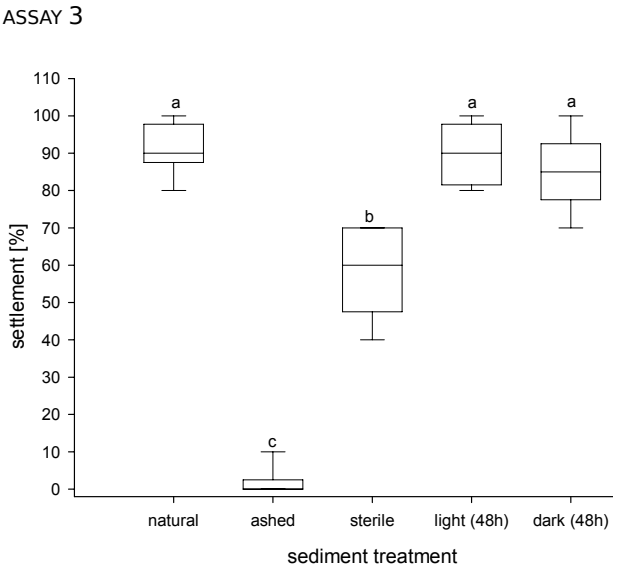
Fig. 6: ASSAY 2: Percentage of larval settlement of *Polydora cornuta* (0.99 ± 0.10 mm body length, 15 setigers) per treatment in a single-choice assay after 1 h.

Tab. 15: Diatom cell numbers (mm^{-2}) of natural sediment treated with antibiotics (streptomycin 364.0 µg ml^{-1}, penicillin 37.2 µg ml^{-1}) and algicide (cycloheximide) 10 mg g^{-1} and algicide/antibiotic and natural (control) sediment (36 h) used in ASSAY 2

ASSAY 2 treatment	mean cell numbers of diatoms (mm^{-2}) sediment	SD
antibiotic (36h)	$5.5 * 10^5$	$0.6 * 10^3$
algicide 10mg g^{-1} (36h)	$0.9 * 10^4$	$0.3 * 10^3$
algicide+antibiotic (36h)	$1.2 * 10^4$	$1.2 * 10^3$
control (36h)	$6.2 * 10^5$	$1.7 * 10^3$

5.1.2 ROLE OF VIABLE DIATOMS IN NATURAL SEDIMENT UNDER ILLUMINATION AND DARKNESS ON LARVAL RESPONSE

The percentage of larval settlement of *Polydora cornuta* (ASSAY 3, Fig 7) was significantly lower in ashed sediment (0 %) than in natural (91.7 %), sterile (58.3 %) and sediment under constant illumination (90.0 %) or in darkness (85.0 %) for 48 h (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.001$). Larval settlement in sediment under illumination and darkness was the same (Tukey's test, $p = 0.93$).



Larval response to natural, ashed, sterile sediment; and sediment under illumination and darkness (48 h). Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 6 replicates shown in a box-and-whisker diagram.

Fig. 7: ASSAY 3: Percentage of larval settlement of *Polydora cornuta* (0.93 ± 0.12 mm body length, 14 setigers) per treatment in a single-choice assay after 1 h.

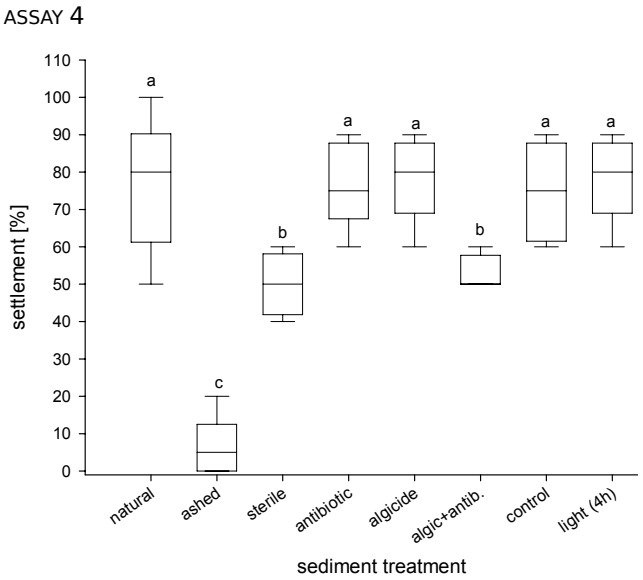
The numbers of diatoms in illuminated sediments were significantly higher than in sediments kept in totally darkness (Student's *t*-test, $p < 0.05$, Tab. 16).

Tab. 16: Diatom cell numbers (mm^{-2}) of natural sediment under constant illumination or darkness (48 h) (ASSAY 3)

	mean cell numbers of diatoms (mm^{-2}) sediment	SD
illumination	$7.7 * 10^5$	$1.6 * 10^3$
darkness	$3.1 * 10^4$	$0.8 * 10^3$

5.1.3 ROLE OF ALGICIDE AND ANTIBIOTICS AND NATURAL SEDIMENT UNDER ILLUMINATION OR DARKNESS ON DIATOM VIABILITY ON LARVAL RESPONSE

The percentage of larval settlement of *Polydora cornuta* (ASSAY 4, Fig 8) was significantly lower in ashed sediment than in natural, sterile and sediments treated with antibiotics (36 h), algicide (10 mg g^{-1}, 36 h), natural (control) sediment and natural sediment in illumination (4 h) (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.05$). The percentage of larval settlement of *P. cornuta* in sterile sediment treated with antibiotics and algicide (36 h) was significantly higher ($p < 0.05$) than in ashed and significantly lower ($p < 0.05$) than in all other sediment treatments and the same in sterile sediment ($p = 0.99$).



Larval response to natural, ashed, sterile sediment; and sediment conditioned for 36 h with antibiotics (streptomycin 364.0 µg ml^{-1}, penicillin 37.2 µg ml^{-1}) and algicide (cycloheximide, 10mg g^{-1}), antibiotics / algicide, sediment under constant illumination (4 h) and control (natural) sediment. Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 6 replicates shown in a box-and-whisker diagram.

Fig. 8: ASSAY 4: Percentage of larval settlement of *Polydora cornuta* (0.99 ± 0.10 mm body length, 15 setigers) per treatment in a single-choice assay after 1 h.

Diatom counts in sediment used in ASSAY 4 treated with cycloheximide (10 mg g^{-1}) and the combination of cycloheximide and antibiotics were lower (< $1.7*10^4$ ± $0.6*10^3$ mm^{-2}) than sediment treated with antibiotics and natural sediment (control) or under illumination (4 h) (> $5.1*10^5$ ± $1.3*10^3$ mm^{-2}) (Student's *t*-test, $p < 0.05$, Tab. 17). Sediments treated with algiciede showed no viable diatoms.

Tab. 17: Diatom cell numbers (mm^{-2}) of natural sediment treated with antibiotics (streptomycin 364.0 µg ml^{-1}, penicillin 37.2 µg ml^{-1}) and algicide (cycloheximide 10 mg g^{-1}) and algicide/antibiotic and natural (control) sediment (36 h) used in ASSAY 4 and sediment under illumination (4 h)

ASSAY4 treatment	mean cell numbers of diatoms (mm^{-2}) sediment	SD
antibiotic (36h)	$5.1 * 10^5$	$1.3 * 10^3$
algicide 10mg g^{-1} (36h)	$1.7 * 10^4$	$0.6 * 10^3$
algicide+antibiotic (36h)	$1.6 * 10^4$	$0.4 * 10^3$
control (36h)	$5.8 * 10^5$	$0.7 * 10^3$
light (4h)	$6.3 * 10^5$	$1.5 * 10^3$

The percentage of larval settlement of *Polydora cornuta* (ASSAY 5 A, B, Fig 9) was significantly lower in ashed and sterile sediment than in natural sediment treated with algicide or antibiotics or a combination of both and sediment under illumination or darkness (85.0 %) for 48 h (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.05$). In both assays, larval settlement in ashed sediment was significantly lower than in sterile sediment (Tukey's test, $p < 0.05$). Larval settlement under

constant illumination, darkness and in sediment treated with algicide was the same (Tukey's test, p = 0.46 and 0.99, Fig. 9, ASSAY 5 A, B).

The abundance of viable and non viable diatoms in the sediment treatments were expressed as a percentage of total density mm^{-2} sediment (Tab. 18). Sediment treated without algicide and under constant illumination showed significantly higher numbers of diatoms (Student's *t*-test, $p < 0.05$, ASSAY 5A, B) than sediments treated with algicide or exposed to constant darkness. No viable diatoms were found in sediment treated with algicide.

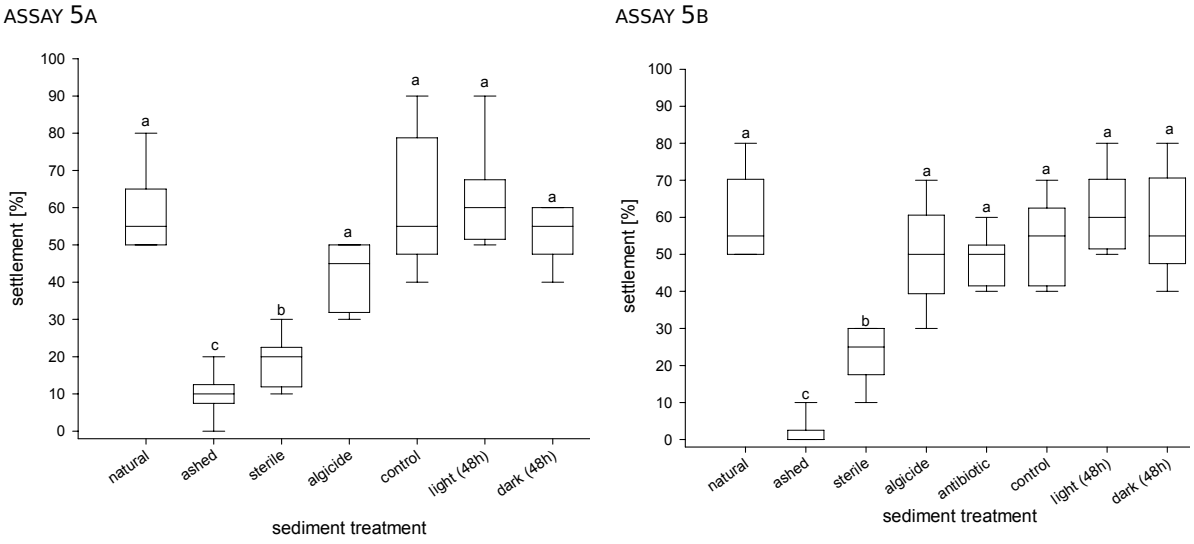


Fig. 9: ASSAY 5 A, B: Percentage of larval settlement of *Polydora cornuta* (ASSAY 5A: 0.96 ± 0.14 mm body length, 15 setigers; ASSAY 5B: 0.90 ± 0.12 mm body length, 15 setigers) per treatment in a single-choice assay after 1 h. Larval response to natural, ashed, sterile sediment; and sediment conditioned (36 h) with algicide (cycloheximide, ASSAY 5A, B), antibiotics (streptomycin 364.0 µg ml^{-1}, penicillin 37.2 µg ml^{-1}, ASSAY 5B), and natural (control) sediment, and sediment under constant illumination or darkness (48 h). Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 6 replicates shown in a box-and-whisker diagram.

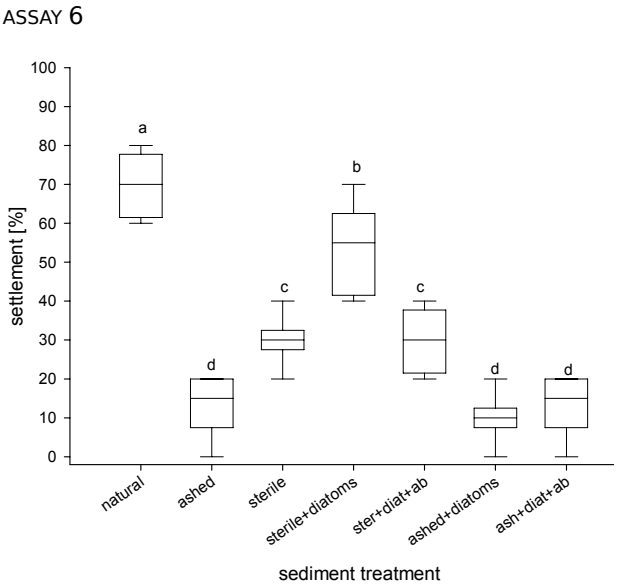
Tab. 18: Diatom cell numbers (mm^{-2}) of untreated natural sediment, natural sediment with algicide (cycloheximide 10 mg g^{-1}) and natural sediment under illumination or darkness (48 h), percentage of non-viable and viable diatoms (ASSAY 5A, B)

ASSAY 5A	total number of diatoms mm^{-2} sediment	SD	% non-viable	% viable
no algicide	$6.3 * 10^5$	$1.0 * 10^3$	31,17	68,83
algicide	$4.6 * 10^4$	$1.8 * 10^3$	100	0
white light	$6.8 * 10^5$	$0.9 * 10^3$	11,2	88,8
darkness	$3.6 * 10^5$	$1.3 * 10^3$	58,56	41,44

ASSAY 5B	total number of diatoms mm^{-2} sediment	SD	% non-viable	% viable
no algicide	$6.6 * 10^5$	$1.3 * 10^3$	29	71
algicide	$3.9 * 10^4$	$1.5 * 10^3$	100	0
white light	$6.2 * 10^5$	$1.0 * 10^3$	11,8	88,2
darkness	$3.1 * 10^5$	$1.1 * 10^3$	55	45

5.1.4 ROLE OF STERILE AND ASHED SEDIMENT INOCULATED WITH DIATOM SUSPENSION ON LARVAL RESPONSE

The percentage of larval settlement of *Polydora cornuta* (ASSAY 6, Fig 10) was significantly higher in natural than in sterile or ashed sediments treated with or without diatoms or antibiotics (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.05$). Larval settlement on sterile sediment inoculated with diatom suspension was higher and significantly different from sterile sediment (Tukey's test, $p < 0.05$). Larval settlement on sterile sediment and sterile sediment inoculated with diatom suspension treated with antibiotics were not significant different (Tukey's test, $p = 0.03$). Larval settlement on ashed sediment and ashed sediment inoculated with diatom suspension either with or without antibiotics was the same (Tukey's test, $p = 0.01$).



Larval response to natural, ashed ("ash"), sterile; and sterile sediment conditioned with diatoms or with diatoms ("diat") and antibiotics ("ab", streptomycin 364.0 µg ml^{-1}, penicillin 37.2 µg ml^{-1}), and ashed sediment conditioned with diatoms or conditioned with diatoms and antibiotics. Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 6 replicates shown in a box-and-whisker diagram. Number of diatoms in treated sediment samples.

Fig. 10: ASSAY 6: Percentage of larval settlement of *Polydora cornuta* (0.96 ± 0.14 mm body length, 15 setigers) per treatment in a single-choice assay after 1 h.

The numbers of diatoms in sterile sediment treated with or without antibiotics were higher ($3.6*10^5 \pm 0.4*10^3$ mm^{-2} to $2.9*10^5 \pm 0.9*10^5$ mm^{-2}) than in ashed sediment samples inoculated with diatoms suspension with or without antibiotics

($1.4*10^5 \pm 1.2*10^3$ mm^{-2} to $1.4*10^5 \pm 0.7*10^5$ mm^{-2}, Student's *t*-test, $p < 0.05$, Tab. 19). Generally, the recolonization of ashed sediment with viable diatoms was rarely successful due to low nutrient availability, only a low amount of diatoms was attached on the sediment surface after the experiment.

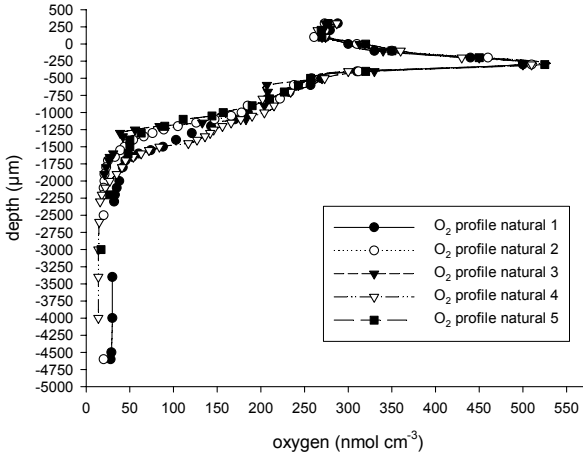
Tab. 19: Diatom cell numbers (mm^{-2}) of untreated sterile and ashed sediment inoculated with diatom suspension and sterile and ashed sediment treated with antibiotics and inoculated with diatom suspension (ASSAY 6)

ASSAY 6 **treatment**	**mean cell numbers of diatoms (mm^{-2}) sediment**	**SD**
sterile diatoms	$3.6 * 10^5$	$0.4 * 10^3$
sterile diatoms + antibiotics	$2.9 * 10^5$	$0.9 * 10^3$
ashed diatoms	$1.6 * 10^5$	$0.7 * 10^3$
ashed diatoms + antibiotics	$1.4 * 10^5$	$1.2 * 10^3$

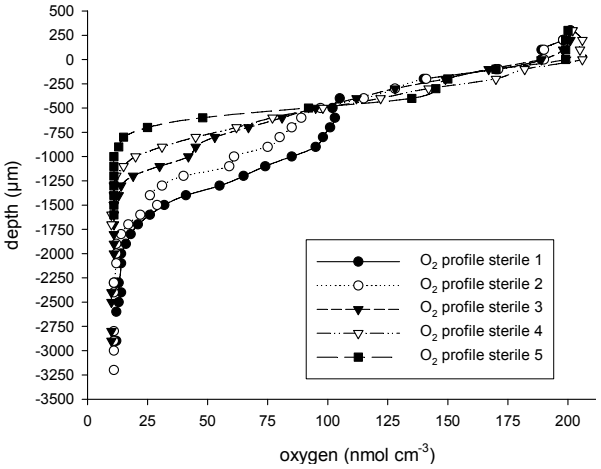
5.1.5 SURFACE BOUNDARY LAYER ON TREATED SEDIMENTS

Oxygen profiles of natural, natural sterile and ashed sediment, with aerated water surface (O_2 saturation) under illumination at 20°C, were conducted in repetition (5 times) and specified in Fig. 11, Tab. 20. All of the three investigated sediment treatments showed oxygen saturation at the sediment surface. In natural sediment the photosynthesis in the surface layers caused high oxygen concentrations up to 550 nmol cm^{-3} and a decrease to <50 nmol cm^{-3} in 2000 µm depth. The surface boundary layer in natural sediment was 750 µm thick. In the oxygen concentration on the sediment surface in sterile sediment was 200 nmol cm^{-3} and 100 nmol cm^{-3} in 500 µm depth. It decreased to 15 nmol cm^{-3} in < 1000 µm depth. In ashed sediment the oxygen concentration differed little from the surface layer with 260 nmol cm^{-2} to 190 nmol cm^{-2} at 250 µm depth. The surface boundary layer in sterile and ashed sediments was > 250 µm and > 50 µm (Tab. 20).

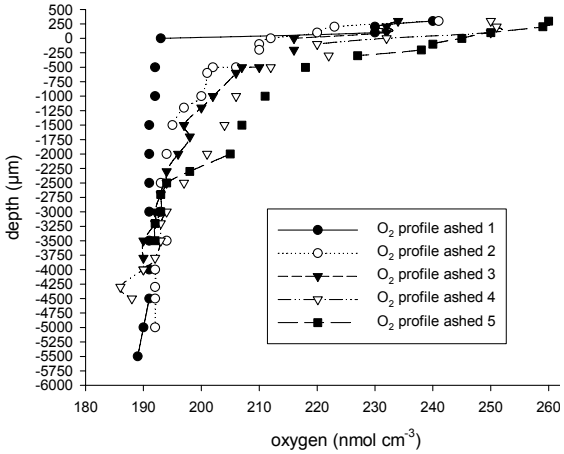
natural sediment



sterile sediment



ashed sediment



Tab. 20: surface boundary layer thickness in natural, sterile and ashed sediment under constant aeration, mean O_2 mA (nmol cm^3) per sediment treatment

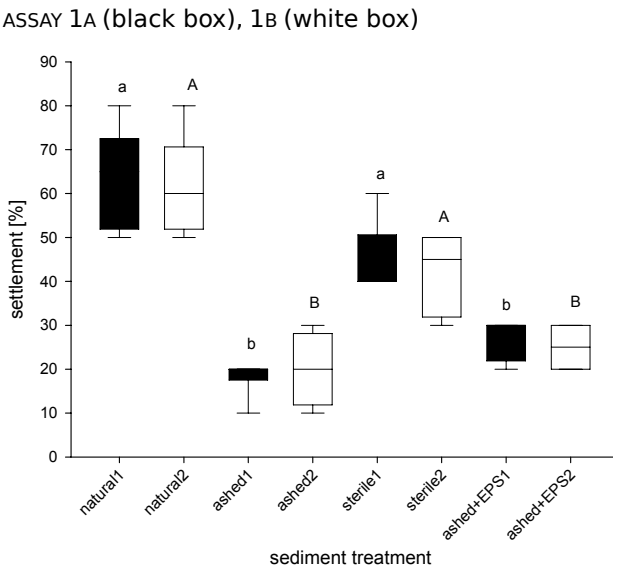
sediment treatment	surface boundary layer thickness (µm)	higest O_2 mA (nmol/cm³)
natural	750	550
sterile	> 250	200
ashed	> 50	260

Fig. 11: Oxygen (µM O_2 nmol cm^{-3}) profiles of natural, natural sterile and ashed sediment, with aerated water surface (O_2 saturation) and illumination at 20°C, in repetition (5 times)

5.2 ROLE OF EXOPOLYMERS, ENZYMES AND LECTINS AS POTENTIAL SETTLEMENT CUES

5.2.1 ROLE OF ASHED SEDIMENT INOCULATED WITH NATURAL EPS-SOLUTION ON LARVAL SETTLEMENT

The percentage of larval settlement of *Polydora cornuta* (ASSAY 1A, Fig 12) was significantly higher in natural than in sterile and ashed sediment with or without inoculation (24 h) of EPS solution obtained from natural sediment (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.014$, ASSAY 1A). Larval settlement in ashed sediment showed no difference to ashed sediment inoculated with EPS solution (500 µg EPS g^{-1} sediment) (Tukey's test, $p = 0.479$). The percentage of larval settlement of *P. cornuta* (ASSAY 1B, Fig 12) in natural and sterile sediment was significantly higher than in ashed sediment inoculated with or without EPS solution for 18 h (Tukey's test $p < 0.001$). None of the ashed sediment treatments inoculated with EPS solution elicited a larval response comparable to the positive control.



Treatments were natural, ashed, sterile sediment; and ashed sediment inoculated with EPS solution (500 µg EPS g^{-1} sediment) for 24 h (ASSAY 1A, BLACK) and 18 h (ASSAY 1B, WHITHE). Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test) by using upper and lower case letters for different assays. Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 6 replicates shown in a box-and-whisker diagram.

Fig. 12: ASSAY 1 A, B: Percentage of larval settlement of *Polydora cornuta* (ASSAY 1A BLACK: 0.93 ± 0.11 mm body length, 13-14 setigers; ASSAY 1B WHITHE: 0.90 ± 0.10 mm body length, 13 setigers); per treatment in a single-choice assay after 1 h.

The amounts of glucose equivalents g^{-1} and carbohydrates in natural sediment were given in Tab. 21. EPS content of natural sediment (determined by 3 fold extraction) was between 14.31 ± 1.09 and 22.13 ± 5.02 µg glucose eq. g^{-1}

sediment (Tab. 21). In contrast the carbohydrate concentration measured from the same sediment using the phenol-sulfuric acid method showed around 15 fold higher values than the pooled EPS from all extractions 1-4 (Tab. 21).

Tab. 21: Extraction of EPS from natural sediment: results in microgram glucose equivalent g^{-1} sediment, sum of four sediment extractions and values of total carbohydrate concentration in the same sediment in use of phenol-sulfuric acid method, mean and SD (3 replicates)

sample replic.	**extraction 1-4** µg gluc. eq. / g sediment	**carbohydrate conc. in sediment** µg gluc. eq. / g sediment
1	21.58 ± 1.53	352.98 ± 05.85
2	17.84 ± 3.75	369.30 ± 12.76
3	14.31 ± 1.09	400.00 ± 32.26
4	16.80 ± 1.44	530.06 ± 12.13
5	22.13 ± 5.02	421.56 ± 22.44
6	19.23 ± 1.12	272.46 ± 10.06

The carbohydrate concentration in ashed sediment inoculated for 24 h with 500 µg EPS g^{-1} sediment revealed low values of microgram glucose eq. g^{-1} sediment, partly under the detection limit (Tab. 22). Ashed sediment inoculated for 7 days with 500 µg EPS g^{-1} sediment revealed similar values of 137.08 ± 8.00 to 180.53 ± 8.00 µg EPS g^{-1} sediment than sediment inoculated for 24 h (Tab 22).

Tab. 22: Extraction of EPS from ashed sediment inoculated with 2 ml EPS solution (500 µg ml^{-1} sterile filtered seawater) after 24 h and 7 days, mean and SD of three measurements per sample, results in microgram glucose equivalent g^{-1} sediment

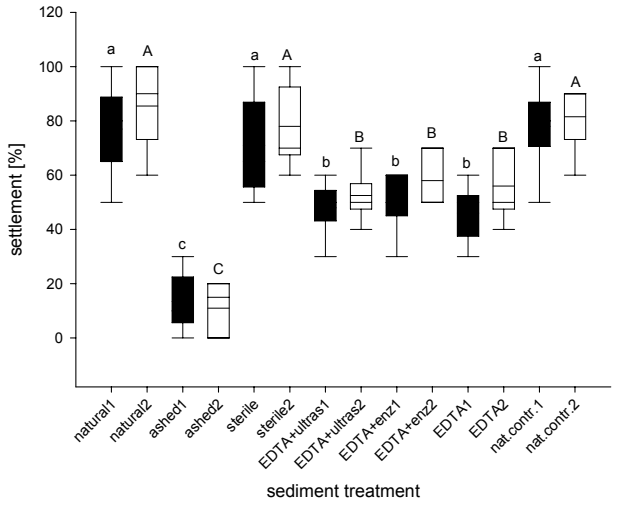
sample replic.	**ashed sediment (cond. 24 h)** µg gluc. eq. / g sediment	**ashed sediment (cond. 7d)** µg gluc. eq. / g sediment
1	< limit of detection	180.53 ± 8.00
2	< limit of detection	< limit of detection
3	< limit of detection	162.54 ± 6.63
4	140.34 ± 11.20	< limit of detection
5	< limit of detection	< limit of detection
6	< limit of detection	137.08 ± 8.00

5.2.2 DETACHMENT OF EPS WITH ENZYMES, EDTA, ULTRASONICATION FROM NATURAL SEDIMENT AND LARVAL RESPONSE

The percentage of larval settlement of *S. benedicti* (ASSAY 1A, Fig. 13) was significantly higher in natural, sterile and natural (control) sediment than in ashed sediment or natural sediment treated with enzymes, EDTA or ultrasonication (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.009$, ASSAY 1A, B). Natural sediment treated with enzymes or both enzymes and EDTA showed no difference in larval settlement to sediment treated with EDTA and ultrasonication (Tukey's test, $p = 0.227$, ASSAY 1A). Larval settlement was also the same in

natural sediment treated with EDTA and ultrasonication or only EDTA (Tukey's test, p = 0.545, ASSAY 1B, Fig. 13).

ASSAY 1A (black box), 1B (white box)



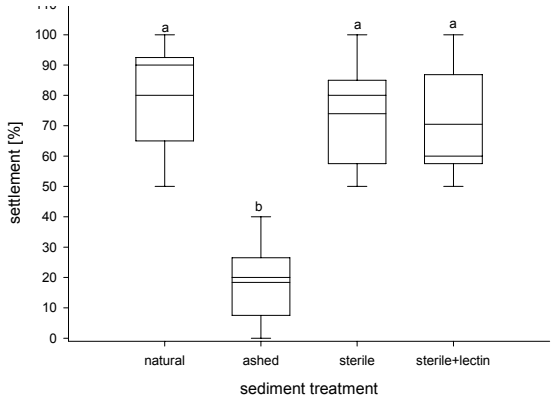
Treatments were natural, sterile, ashed, natural sediment treated with EDTA + ultrasonication, natural sediment treated with EDTA and an enzyme-mix, natural sediment treated with EDTA and natural (control) sediment. Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test) by using upper and lower case letters for different assays. Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 12 replicates shown in a box-and-whisker diagram.

Fig. 13: ASSAY 1A, B: Percentage of larval settlement of *S. benedicti* (ASSAY 1A: 0.78 ± 0.11 mm body length, 15 setigers; ASSAY 1B: 0.81 ± 0.12 mm body length, 15-16 setigers); per treatment in a single-choice assay after 1 h.

5.2.3 ROLE OF LECTINS AND MONOSACCHARIDES IN STERILE OR ASHED SEDIMENT ON LARVAL RESPONSE

The percentage of larval settlement of *S. benedicti* (ASSAY 1) was significantly higher in natural, sterile and sterile sediment treated with lectins (ConA 2.0 mg ml^{-1}, peanut lectin 0.082 mg ml^{-1}) (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.05$, Fig. 14) than in ashed sediment.

ASSAY 1



Larval response to natural, ashed, sterile and sterile sediment conditioned with lectin solution for 3 h. Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 12 replicates shown in a box-and-whisker diagram.

Fig. 14: ASSAY 1: Percentage of larval settlement of *S. benedicti* (0.80 ± 0.13 mm body length, 15 setigers) per treatment in a single-choice assay after 1 h.

No statistical difference in larval settlement was observed between sterile and sterile sediment treated with lectins (Tukey's test, p = 0.3, Fig 14). Larval settlement of *S. benedicti* was significantly lower in ashed sediment than in all other sediment treatments (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.019$, Fig. 15). The percentage of larval settlement in sterile (control) sediment was the same than in sterile sediment conditioned with lectin solution or all of four monosaccharides at 50 mM or larvae which were incubated for 1 h in lectin solution (Tukey's test, p = 0.112). Sediment conditioned with lectines or monosaccharides were statistically the same (Tukey's test, p = 0.206 Fig. 15, ASSAY 2).

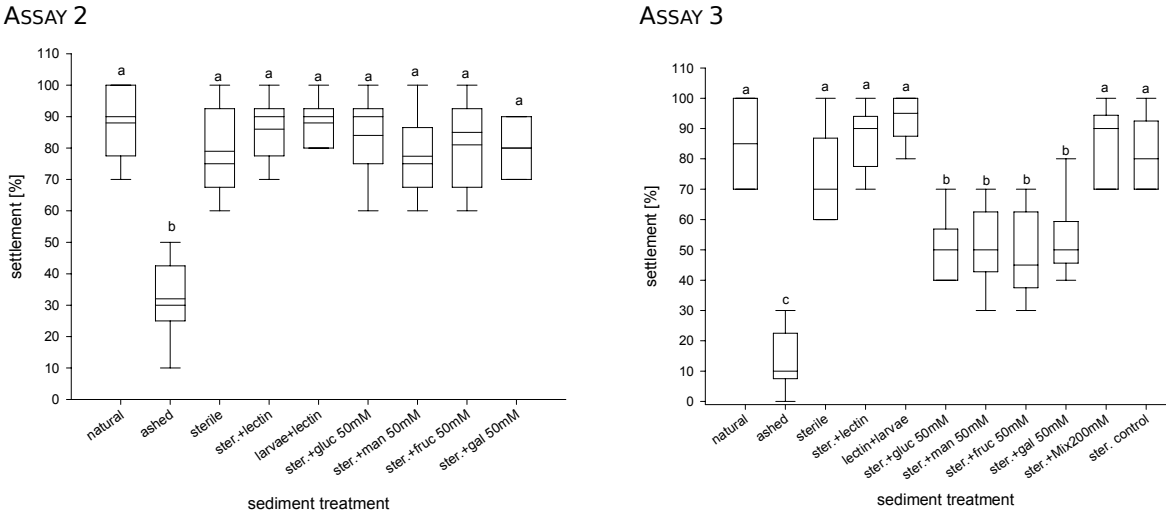
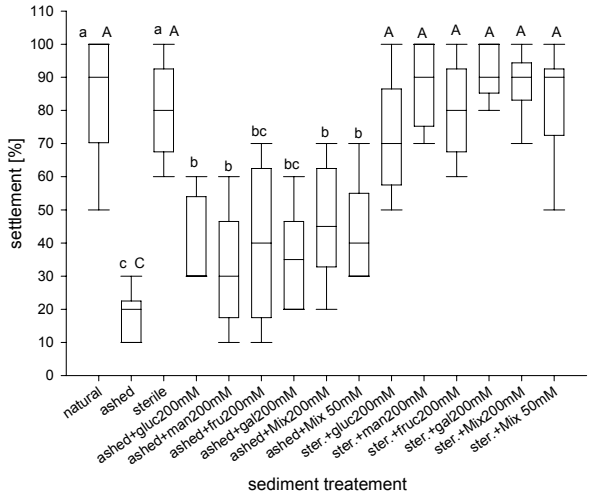


Fig. 15: ASSAY 2 + 3: Percentage of larval settlement of *S. benedicti* (ASSAY 2: 0.79 ± 0.12 mm body length, 15 setigers, ASSAY 3: 0.83 ± 0.11 mm body length, 16 setigers) per treatment in a single-choice assay after 1 h. ASSAY 2+3 were performed with natural, ashed, sterile and sterile sediment conditioned with lectin solution (ConA and peanut lectin) for 3 h, larvae incubated in lectin solution (1 h) (ASSAY 2) and sterile sediment conditioned with monosaccharides (50 mM, D-glucose, mannose, fructose, galactose), sterile sediment conditioned with a mixture of all four monosaccharides (200 mM, ASSAY 3) and control (sterile) sediment. Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). The upper and lower case letters indicate separate statistical analysis between sterile sediment treated with lectins or monosaccharides, ASSAY 3. Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 12 replicates shown in a box-and-whisker diagram.

Larval settlement of *S. benedicti* was significantly lower in ashed sediment than in all other sediment treatments (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.014$, Fig. 15, ASSAY 3). The settlement response of larvae (ASSAY 3) in sterile sediment conditioned with lectins (3 h) and larvae conditioned in lectin solution (1 h) was the same than in natural and sterile sediment (Tukey's test, p = 0.88)

but significant higher than in sterile sediment conditioned with all monosaccharides (Tukey's test, $p < 0.05$). The percentage of larval settlement in sediment treated with lectins were significant higher than in sterile sediment conditioned with each of four monosaccharides (Tukey's test, $p < 0.03$, ASSAY 3, Fig. 15). None of the sterile sediment conditioned with each of four monosaccharides elicited a larval response comparable to the natural control (Tukey's test, $p = 0.086$). Larval settlement on sterile sediment treated with a combination of all four monosaccharides at 200 mM was significantly higher than in sterile sediment conditioned with monosaccharides at 50 mM (Tukey's test, $p < 0.05$) but similar to either natural or lectin treatments (Tukey's test, $p = 0.99$, Fig. 15). Larval settlement of *S. benedicti* (ASSAY 4) was conducted with monosaccharides and a combination of all four monosaccharides on sterile and ashed sediment (Fig. 16).

ASSAY 4



Larval response to natural, ashed and sterile sediment and ashed or sterile sediment conditioned with monosaccharides (D-glucose, mannose, fructose, galactose, 50 mM), a mixture of all monosaccharides (200 mM, 50 mM). Statistical differences are indicated by different letters above the boxes ($\alpha = 0.05$, Tukey's test). The upper and lower case letters indicate separate statistical analysis between ashed and sterile sediment treated with monosaccharides. Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 12 replicates shown in a box-and-whisker diagram.

Fig. 16: ASSAY 4: Percentage of larval settlement of *S. benedicti* (0.78 ± 0.10 mm body length, 14 setigers) per treatment in a single-choice assay after 1 h.

The percentage of larval settlement of *S. benedicti* was significantly higher in ashed sediment conditioned with each of four monosaccharides (200 mM) or a mixture of monosaccharides (200 mM, 50 mM) than in ashed sediment (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.05$). Larval settlement was the same on sterile sediment conditioned with monosaccharides and a combination of all monosaccharides at 200 mM and 50 mM in comparison to natural or sterile sediment (Tukey's test, $p = 0.05$) but settlement was significant higher on ashed sediment (Tukey's test, $p < 0.05$).

5.3 ROLE OF HALOMETABOLITES AS POTENTIAL SETTLEMENT CUES

5.3.1 IDENTIFICATION AND QUANTIFICATION OF HALOMETABOLITES IN ADULT WORM SPECIES

The chemical analysis of adult *S. benedicti* tissue following extraction showed three chlorinated and one brominated compounds. Two unknown isomers of chlorononane were identified according to characteristic diagnostic ions and mass spectroscopic fragmentation patterns and calibrated with 1-chlorononane ($y = 32792x – 7981.5$; $R^2 = 0.9967$) (Tab. 23). The amount of 70.57 ± 0.08 ng chlorononane mm^{-3} in tissue volume was used to adjust the concentration of the halogenated proxy compound 1-chlorononane. The tissue extract of *Capitella* sp. I revealed three brominated aromatic compounds, they were calibrated with 2,6-dibromophenole ($y = 2*10^7x – 118541$; $R^2 = 0.9945$).

The amount of 20.71 ± 1.65 ng dibrominated phenolic compounds mm^{-3} in tissue volume was used to adjust the concentration of the halogenated proxy compound 2,6-dibromophenole. The proposed elemental compositions of tissue extracts of adult *Streblospio benedicti* and *Capitella* sp. I are described in Tab. 23. Representative GC profiles for selected halometabolites of tissue extract of S. benedicti and Capitella sp. I is described in Fig. 17.

Tab. 23: GC-MS analyses of tissue extracts of adult *Streblospio benedicti* and *Capitella* sp. I. Where possible *compounds* and *formulas* were identified by comparison with the mass spectral database (NIST 05), otherwise detected compounds are labeled as unidentified halogenated hydrocarbons. *Characteristic ions* list dominant mass fragments. The concentration of identified compounds is given in ng mm^{-3} for selected compounds. (nd = not determined)

Compounds	**Characteristic ions**	**Formula**	**Retention time (min)**	**Concentration (ng mm^{-3})**
Streblospio benedicti				
dichlorinated hydrocarbon	91, 93, 119, 121	$C_nH_nCl_2$	8,48	nd
brominated hydrocarbon	135, 137, 149, 151	C_nH_nBr	10,75	nd
unidentified chlorononane isomer	91, 93, 119, 121, 147, 149	$C_9H_{19}Cl_1$	11,36	19.59 ± 7.7
unidentified chlorononane isomer	91, 93, 119, 121, 147, 149	$C_9H_{19}Cl_1$	14,22	50.98 ± 8.6
***Capitella* sp.I**				
2,6-dibromophenol	162, 164, 250, 252, 254	$C_6H_4Br_2O$	15,01	18.11 ± 4.5
2,6-dibromo-4-methyl-phenol	263, 265, 267, 252	$C_7H_6Br_2O$	17,79	2.6 ± 1.2
2,4-dibromo-6-(bromomethyl)phenol	215, 217, 263, 265, 267	$C_7H_5Br_3O$	25,87	nd

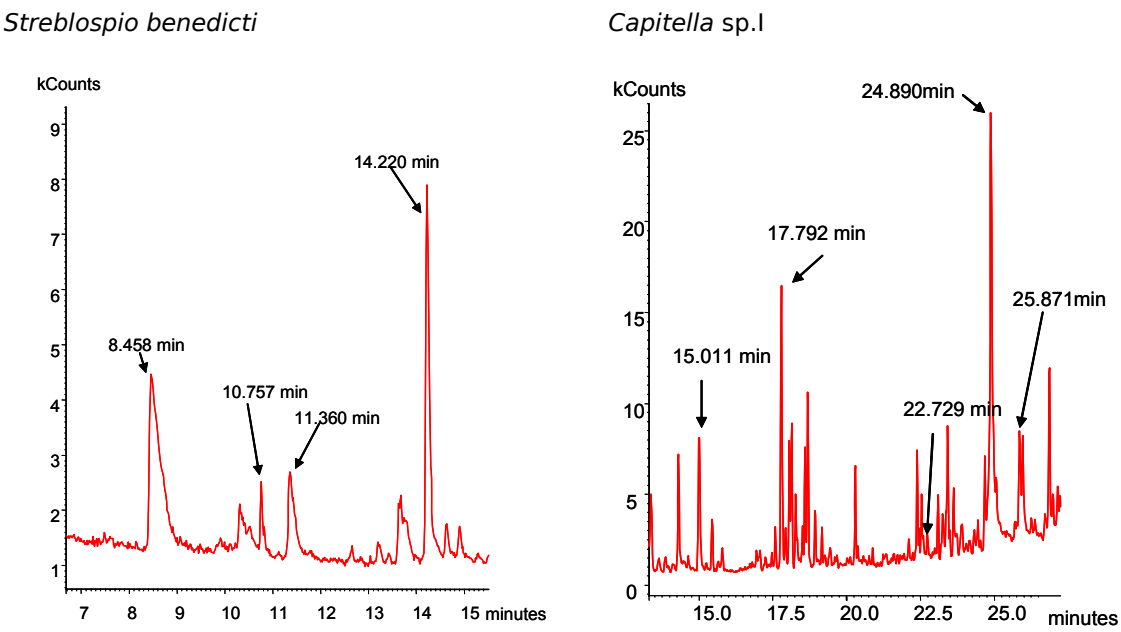


Fig. 17: Representative GC profile for selected halometabolites from *S. benedicti* and *Capitella* sp. I tissue showed peaks of multiple brominated and chlorinated compounds

5.3.2 QUANTIFICATION OF HALOGENATED PROXIES IN SPIKED SEDIMENT TREATMENTS

The recovery yields of halogenated proxies differed significantly with regard to the compound and to the residence time of compounds in sediments (Tab. 24). Whilst 1-chlorononane could be recovered to 50-60% immediately after the spiking procedure the concentration was below the detection limit of 10 µg ml^{-1} after 7 d of incubation in both natural and sterile sediment. Spiked sediments were calculated due to the calibration curve of 1-chlorononane (y = 149217x – 791093; R^2 = 0.9989). The recovery of 2,6-dibromophenole was below 10% after immediate extraction and could not be detected in aged sediment treatments. Sediments were calibrated with 2,6-dibromophenole (y = 4476x – 98471; R^2 = 0.9925). The background concentration of 1-chlorononane and 2,6-dibromophenole in natural and sterilized sediment was below the detection limit of 10 µg ml^{-1}.

Tab. 24: Mean recovery yields and concentrations of 1-chlorononane (CLN) and 2,6-dibromophenole (DBP) measured after extraction and GC-MS analysis in different sediment treatments after zero and seven days of incubation. Natural and sterile sediment was spiked with 5.64 µg CLN and 1.65 µg DBP g^{-1} sediment.

Incubation (days)	Treatment	Mean recovery yield (%)	Concentration (µg g^{-1} sediment)
	natural + CLN	52.06 ± 10.15	2.94 ± 0.70
0	sterile + CLN	62.04 ± 10.64	3.499 ± 0.73
	natural + CLN	0	0
7	sterile + CLN	0	0
	natural + DBP	2.75 ± 1.95	0.045 ± 0.023
0	sterile + DBP	11.93 ± 7.71	0.196 ± 0.14
	natural + DBP	0	0
7	sterile + DBP	0	0
	natural (control)	0	0
0	sterile (control)	0	0
	natural (control)	0	0
7	sterile (control)	0	0

5.3.3 DIRECT EFFEDCT OF HALOGENATED PROXIES ON LARVAL SETTLEMENT IN SINGLE-CHOICE ASSAYS

In both experimental repeats of the single-choice assay (Fig. 18, ASSAY 1, 2), no statistical differences in percentage larval settlement index were observed among spiked sediment treatments with both halogenated proxies regardless of the concentration used. These treatments evoked the same settlement response as the positive control of natural sediment. Only the negative control of ashed sediment significantly reduced larval settlement (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.05$). The organic solvent controls did not interfere with larval settlement. Larval settlement in ashed sediment treatment was lower and significant different to all sediment treatments (Tukey's test, $p < 0.05$, Fig. 18).

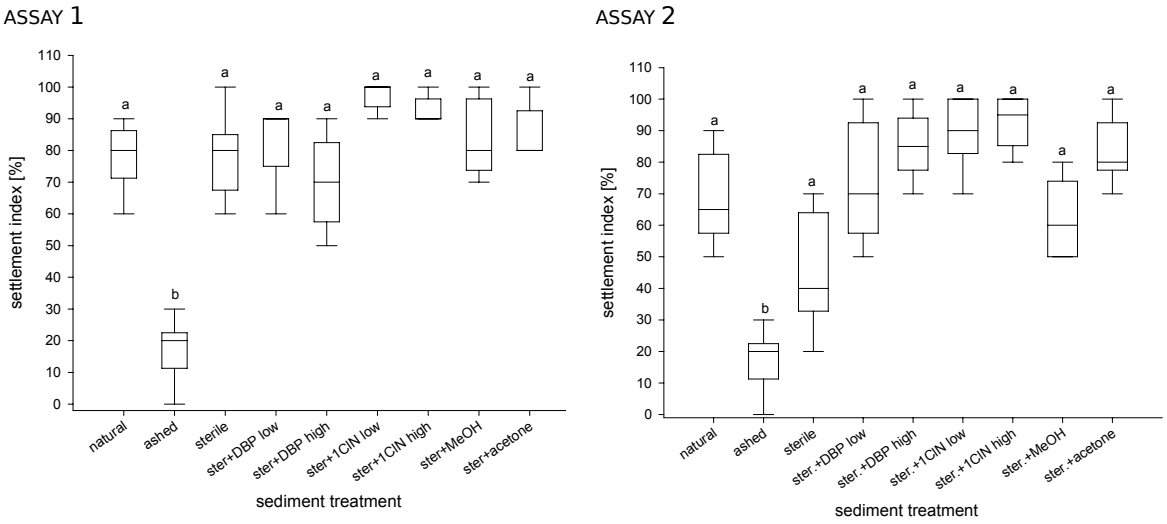
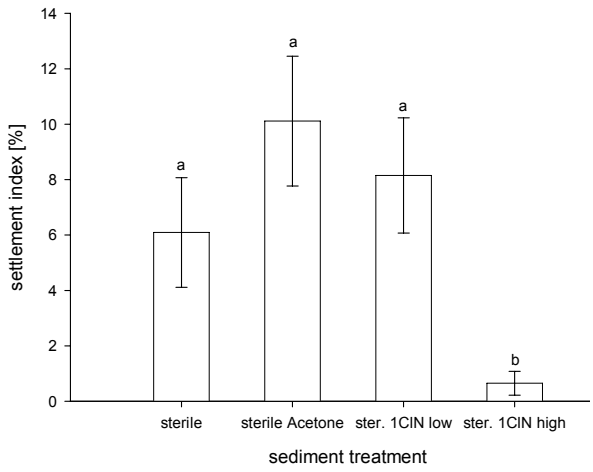


Fig. 18: ASSAY 1, 2: Percentage of larval settlement of *Streblospio benedicti* of two experimental repeats (ASSAY 1: 0.72 ± 0.12 mm body length; ASSAY 2: 12 setigers, 0.80 ± 0.13 mm body length, 14 setigers) of single-choice assays after 1 h testing the direct effect of spiked sterile sediments with 2,6-dibromphenole (DBP) at 0.8 (low, 1x) and 8.0 µg g^{-1} sediment (high, 10x), and sediment spiked with 1-chlorononane (1ClN) at 6.93 (low, 1x) and 69.3 µg g^{-1} sediment (high, 10x) concentration, and sterile sediment treated with MeOH or acetone. Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 12 replicates shown in a box-and-whisker diagram.

5.3.4 DIRECT EFFEDCT OF 1-CHLORONONANE ON LARVAL SETTLEMENT IN MULTIPLE-CHOICE ASSAYS

A different result of a direct influence of halogenated proxies on larval settlement was observed when spiked sediment treatments were investigated in multiple choice assays. In all three experimental repeats, larvae significantly rejected the 1-chlorononane treatment at 10x (high) concentration compared to the 1x concentrated treatment, the solvent control and the negative control of sterile sediment (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.001$, Fig. 19). No row or column effects were observed (Tukey's test, $p = 0.88$). The number of settled larvae was 220 for ASSAY 1A and 842 for ASSAY 1B (Fig 19).

ASSAY 1A

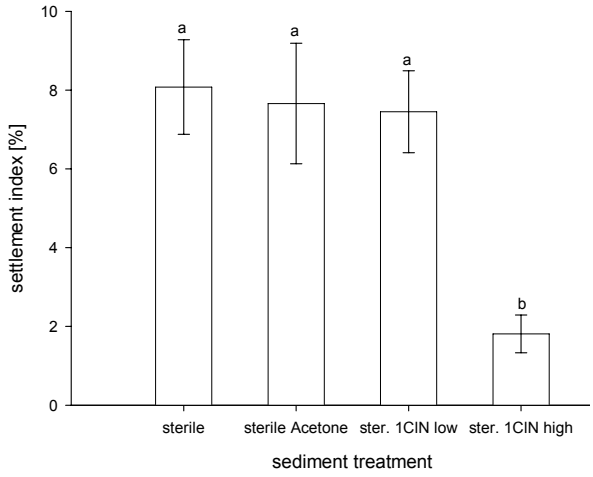


ASSAY 1A

11	30	25	1
1	20	20	15
20	3	15	18
25	15	1	10

∑ = 220 settled larvae

ASSAY 1B



ASSAY 1B

67	76	57	15
12	58	72	75
63	13	65	63
47	56	21	82

∑ = 842 settled larvae

sterile

sterile acetone

sterile + 1-chlorononane

sterile + 1-chlorononane

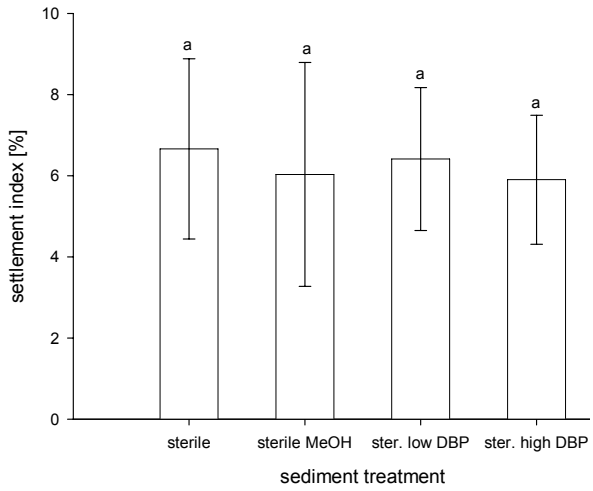
Fig. 19: ASSAY 1 A, B: Larval settlement index [%] of *Streblospio benedicti* of two experimental repeats of multiple-choice assays testing the direct effect of sterile sediment spiked with 0.56 (low, 1x) and 5.56 µg g^{-1} sediment (high, 10x) 1-chlorononane (1ClN) with a solvent control (acetone) and a negative control of sterile sediment. Statistical differences are indicated by different letters above the bars (α = 0.05, Tukey's test). The table depicts the assay design and states the number of settled larvae in individual wells for ASSAY 1 A, B.

5.3.5 DIRECT EFFEDCT OF 2,6-DIBROMOPHENOLE ON LARVAL SETTLEMENT IN MULTIPLE-CHOICE ASSAYS

A similar experiment testing the effect of 2,6-dibromophenole at 10x and 1x concentration did not reveal any differences in percentage larval settlement between all sediment treatments (1-way ANOVA, $p < 0.05$; Tukey's test, $p <$

0.001, ASSAY 2 A, B Fig. 20). No row or column effects were observed (Tukey's test, p = 0.59). The number of settled larvae was 774 for ASSAY 2A and 368 for ASSAY 2B.

ASSAY 2A

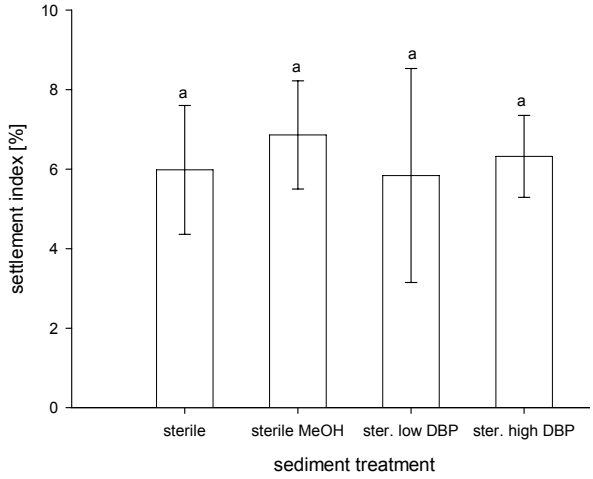


ASSAY 2A

21	56	39	52
35	67	73	47
46	38	55	40
22	71	62	50

∑ = 774 settled larvae

ASSAY 2B



ASSAY 2B

29	29	19	28
22	15	22	36
17	19	24	30
20	14	24	20

∑ = 368 settled larvae

Fig. 20: ASSAY 2 A, B: Larval settlement index [%] of *Streblospio benedicti* of two experimental repeats of multiple-choice assays testing the direct effect of sterile sediment spiked with 0.165 (low, 1x) and 1.65 µg g^{-1} sediment (high, 10x) 2,6-dibromophenole together with a solvent control (methanol) and a negative control of sterile sediment. Statistical differences are indicated by different letters above the bars (α = 0.05, Tukey's test).The table depicts the assay design and states the number of settled larvae in individual wells for ASSAY 2 A, B.

sterile

sterile + MeOH

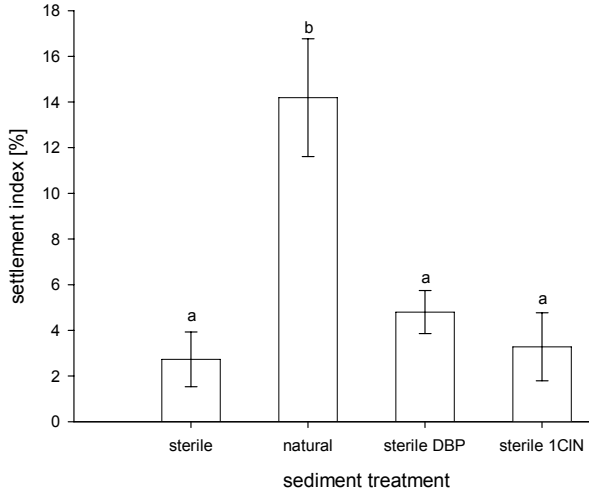
sterile + 2,6-dibromophenole

sterile + 2,6-dibromophenole

5.3.6 DIRECT EFFEDCT OF 2,6-DIBROMOPHENOLE AND 1-CHLORONONANE ON LARVAL SETTLEMENT IN MULTIPLE-CHOICE ASSAYS

When both halogenated proxies were assayed at 10x concentration together with the positive control of natural sediment and a negative control of sterile sediment, the percentage of larval settlement in ASSAY 3 A-C in natural sediment was higher and significantly different to all other treatments (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.01$, ASSAY 3 A-C, Fig. 28). No statistical difference in percentage larval settlement was observed between sterile (control) sediment and sterile sediment spiked with halometabolites (Tukey's test, $p = 0.15$, ASSAY 3 A-C, Fig. 21). No row or column effects were observed (Tukey's test, $p = 0.8$). General, either 2,6-dibromophenole or 1-chlorononane did affect the larval selectivity (Fig. 21 , ASSAY 3 A-C). The number of settled larvae was 229 for ASSAY 3A, 209 for ASSAY 3B and 568 for ASSAY 3C.

ASSAY 3A

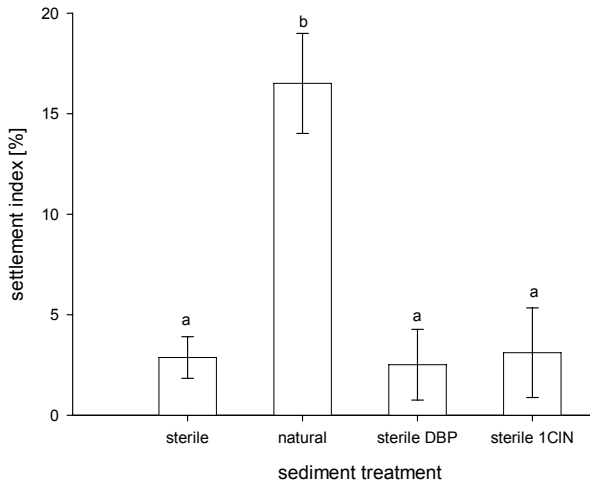


ASSAY 3A

12	11	5	38
26	11	9	8
3	29	8	7
3	9	37	13

∑ = 229 settled larvae

ASSAY 3B

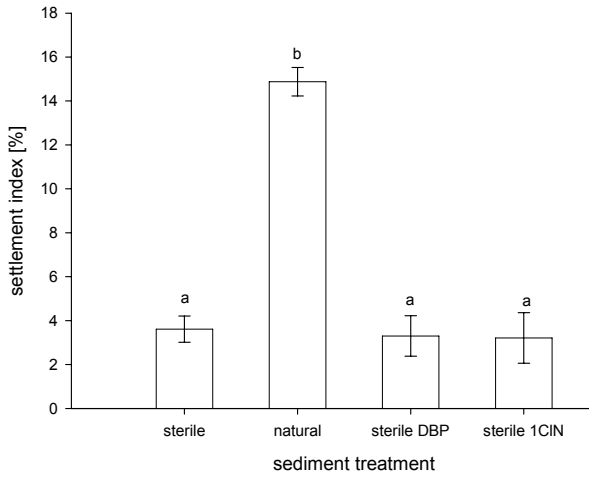


ASSAY 3B

5	1	6	38
27	10	5	3
8	35	5	8
12	7	38	1

∑ = 209 settled larvae

ASSAY 3C



ASSAY 3C

14	14	83	17
19	26	25	88
87	25	19	28
16	80	21	16

∑ = 568 settled larvae

sterile + 2,6-dibromophenole

sterile + 1-chlorononane

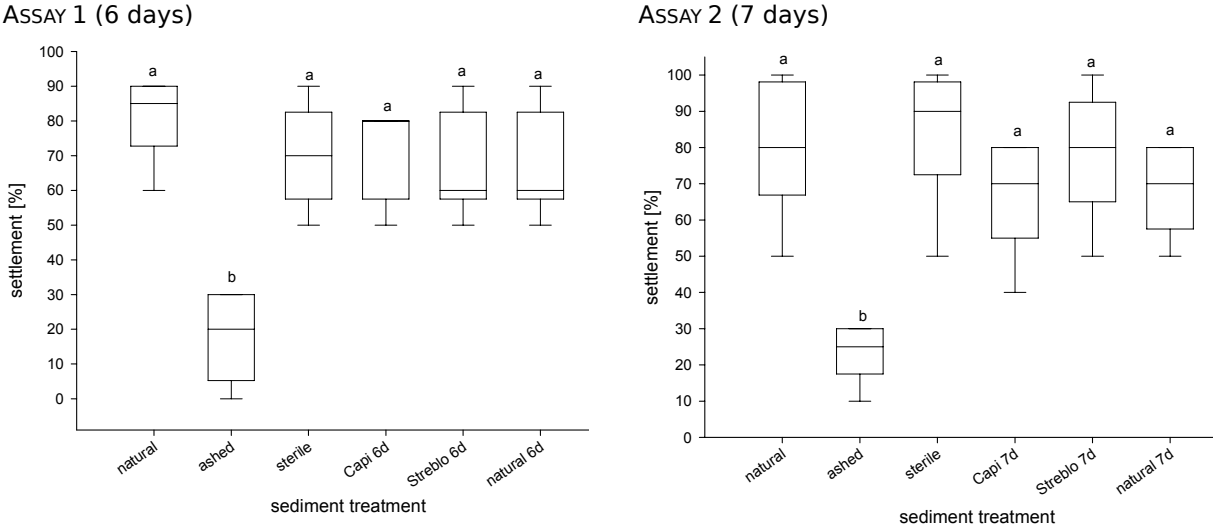
sterile

natural

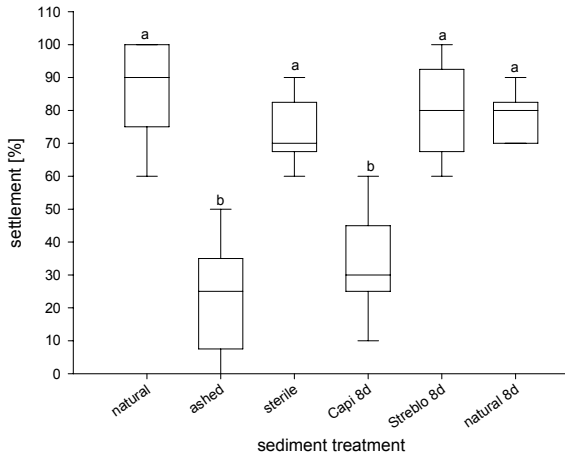
Fig. 21: ASSAY 3 A-C, Larval settlement index [%] of *Streblospio benedicti* of three experimental repeats of multiple-choice assays testing the direct effect of sterile sediment spiked with 1.65 µg g^{-1} 2,6-dibromophenole (10x) and 5.6 µg g^{-1} sediment 1-chlorononane (10x) together with a positive control of natural sediment and a negative control of sterile sediment. Statistical differences are indicated by different letters above the bars (α = 0.05, Tukey's test). The table depicts the assay design and states the number of settled larvae in individual wells for ASSAY 3 A-C.

5.3.7 INDIRECT EFFEDCT OF METABOLITES PRODUCED OF ADULT WORMS IN SITU CONDITIONED ON NATURAL SEDIMENT ON LARVAL SETTLEMENT IN SINGLE-CHOICE ASSAYS

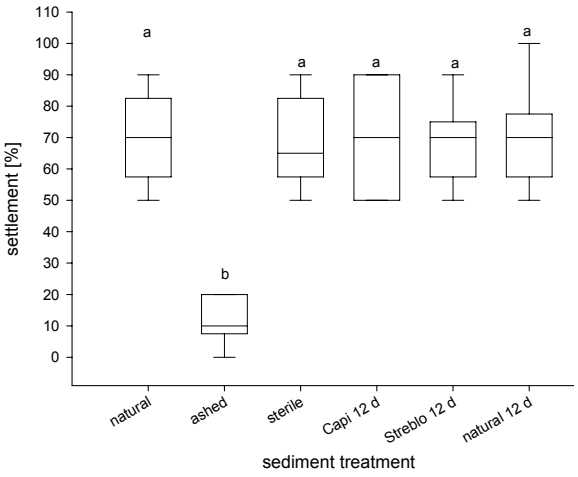
None of the sediments treated with adult worm species neither *S. benedicti* nor *Capitella* sp.I elicited a larval response statistical different to natural sediment (Tukey's test, $p = 0.98$, Fig. 22). The percentage of larval settlement of *S. benedicti* was significantly lower in ashed sediment in all assays than in natural sediment treated with adults or the control (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.05$). Larval settlement in sediments conditioned in situ with adult metabolites produced of adult worms for 6 days was the same than in sediments conditioned for at least 29 days (Tukey's test, $p = 0.88$), except sediment conditioned with adults of *Capitella* sp.I in ASSAY 3 after 8 days and in ASSAY 8 after 29 days, was lower and significant different to natural sediment conditioned with *S. benedicti* and the control (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.05$, Fig. 22).



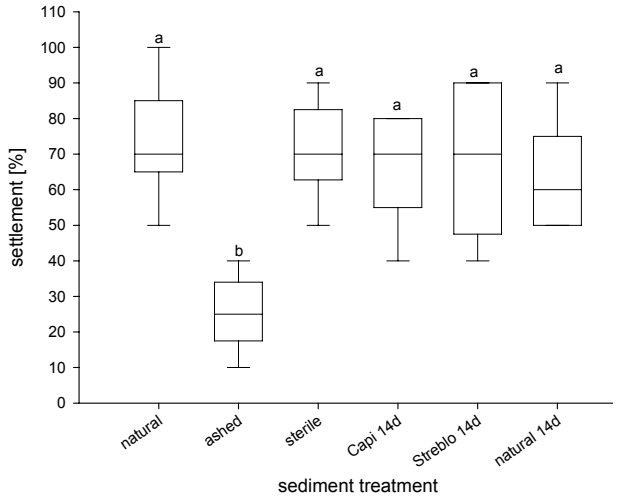
ASSAY 3 (8 days)



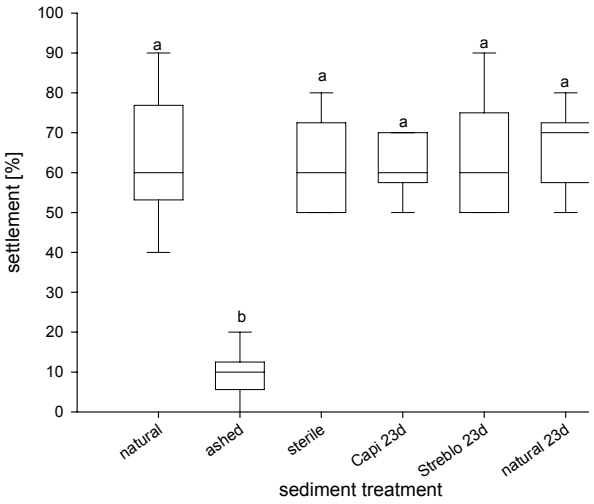
ASSAY 4 (12 days)



ASSAY 5 (14 days)



ASSAY 6 (23 days)



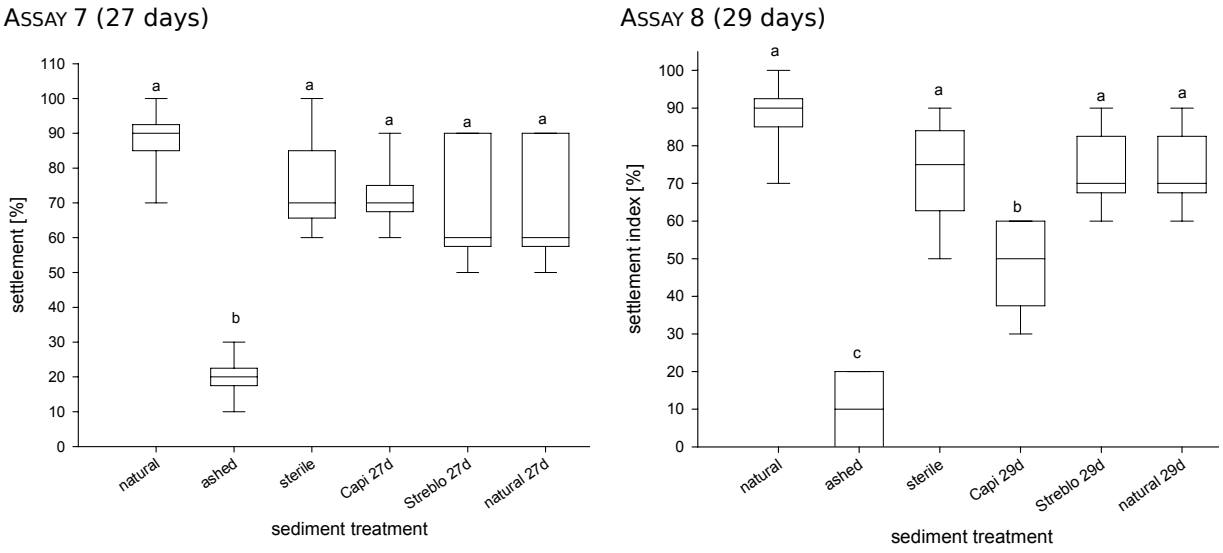


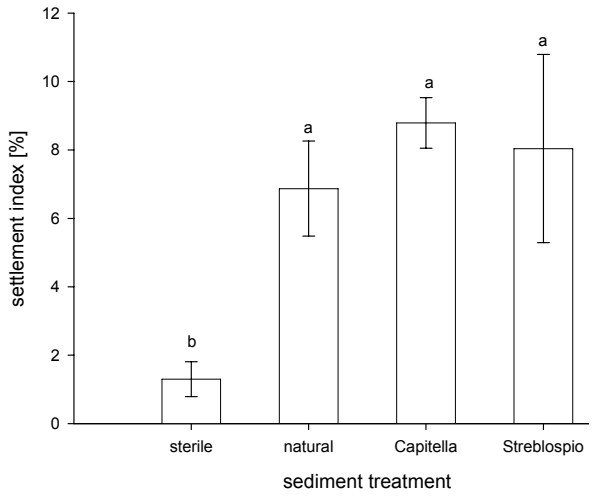
Fig. 22: ASSAY 1-8: Percentage of larval settlement of *Streblospio benedicti* (0.72 ± 0.12 mm body length, 12 setigers, 0.80 ± 0.13 mm body length, 14 setigers, 0.69 ± 0.10 mm body length, 10 setigers, 0.75 ± 0.14 mm body length, 12 setigers, 0.79 ± 0.13 mm body length, 13 setigers, 0.81 ± 0.12 mm body length, 14 setigers, 0.70 ± 0.11 mm body length, 11 setigers, 0.77 ± 0.12 mm body length, 13 setigers, ASSAY 1-8 respectively) in a single-choice assay after 1 h testing the indirect effect of natural sediment conditioned with either adult *Capitella* or *S. benedicti* per well and natural sediment as control for experimental time of 6, 7, 8, 12, 14, 23, 27, 29 days in natural seawater and sterile sediment as negative control added just before starting the experiment. Statistical differences are indicated by different letters above the boxes (α = 0.05, Tukey's test). Data plotted are the five-number summary (minimum, lower quartile, median, upper quartile and maximum) of 12 replicates shown in a box-and-whisker diagram.

5.3.8 INDIRECT EFFEDCT OF METABOLITES PRODUCED OF ADULT WORMS IN SITU CONDITIONED ON NATURAL SEDIMENT ON LARVAL SETTLEMENT IN MULTIPLE-CHOICE ASSAYS

The indirect effect of halometabolites on larval settlement of *S. benedicti* larvae was assayed in 3 experimental repeats in a multiple-choice assay on natural sediment populated with adult specimen of *S. benedicti* and *Capitella* sp.I respectively for 7 days in natural seawater. The percentage of larval settlement in sterile (control) sediment was lower and significantly different to all other treatments (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.05$, ASSAY 4 A-C, Fig. 23). No statistical difference in percentage larval settlement was observed between natural sediment and natural sediment conditioned with adults of *S. benedicti* or *Capitella* sp.I, (Tukey's test, $p = 0.11$, ASSAY 4A; $p = 0.78$, ASSAY 4B; $p = 0.1$, ASSAY 4C; Fig. 23). No statistical differences in row or column effects were observed

(Tukey's test, p = 0.7). Generally, neither adults of *S. benedicti* nor *Capitella* sp.I conditioned in natural sediment did affect the larval settlement after 7 days incubation in natural seawater. The number of settled larvae was 404 for ASSAY 4A, 474 for ASSAY 4B and 713 for ASSAY 4C.

ASSAY 4A

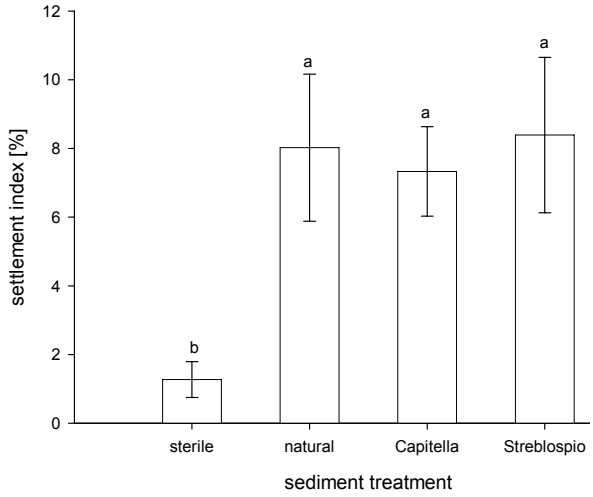


ASSAY 4A

37	47	35	4
7	39	32	29
25	7	33	20
31	22	3	33

∑ = 404 settled larvae

ASSAY 4B

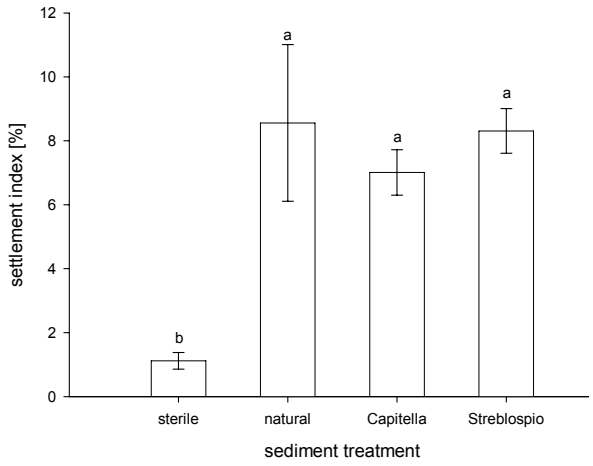


ASSAY 4B

46	36	31	4
7	49	34	39
30	9	39	42
27	52	4	25

∑ = 474 settled larvae

ASSAY 4C



ASSAY 4C

58	43	46	9
6	59	51	84
65	10	54	55
51	49	7	66

∑ = 713 settled larvae

natural + S. *benedicti*

natural + *Capitella*

natural

sterile

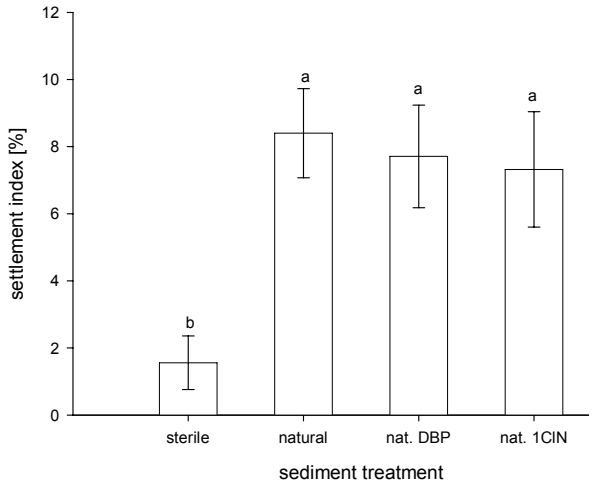
Fig. 23: ASSAY 4A-C, Larval settlement index [%] of *Streblospio benedicti* of three experimental repeats of multiple-choice assays testing the indirect effect of natural sediment in situ conditioned with adults of *Capitella* sp. I and *S. benedicti* (20 per well) respectively, together with natural sediment (positive control) in natural seawater for 7 days. Sterile sediment was added as negative control after experimental time. Statistical differences are indicated by different letters above the bars (α = 0.05, Tukey's test). The table depicts the assay design and states the number of settled larvae in individual wells for ASSAY 4 A-C.

5.3.9 INDIRECT EFFEDCT OF 2,6-DIBROMOPHENOLE AND 1-CHLORONONANE ON NATURAL SEDIMENT ON LARVAL SETTLEMENT IN MULTIPLE-CHOICE ASSAYS

In 3 experimental repeats (ASSAYS 5 A-C) of the multiple-choice assay larval settlement in natural sediment incubated for 7 days with halogenated proxies at 10x concentration was the same as in the positive control of natural sediment. Only the negative control of sterile sediment evoked a lower settlement index than the other treatments (1-way ANOVA, $p < 0.05$; $p < 0.01$, Tukey's test, Fig. 24). No statistical difference in percentage larval settlement was observed in all sediment treatments in ASSAY 5 A-C (Fig. 24) between natural sediment and natural sediment spiked with halometabolites, (Tukey's test, $p = 0.1$, ASSAY 5A; $p = 0.7$, ASSAY 5B; $p = 0.11$, ASSAY 5C, Fig. 24, ASSAY 5 A-C). No statistical differences in row or column effects were observed (Tukey's test, $p = 0.9$). General, neither 2,6-dibromophenole nor 1-chlorononane spiked on natural sediment affected the

larval settlement after 7 days incubation in natural seawater. The number of settled larvae was 512 for ASSAY 5A, 312 for ASSAY 5B and 514 for ASSAY 5C.

ASSAY 5A

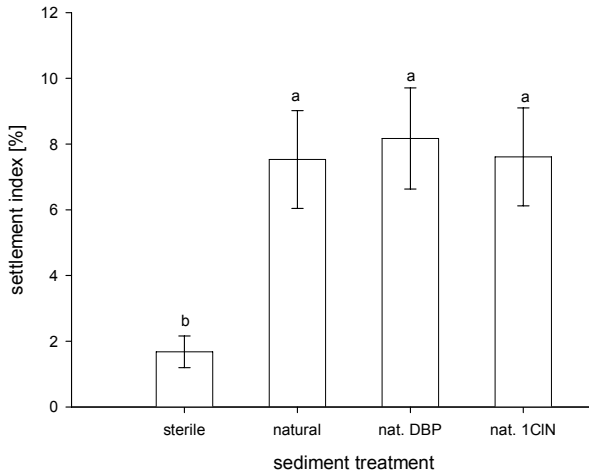


ASSAY 5A

45	40	50	11
5	28	27	42
34	4	41	35
48	46	12	44

∑ = 512 settled larvae

ASSAY 5B



ASSAY 5B

30	27	30	6
4	28	22	22
23	4	19	28
18	19	7	25

∑ = 312 settled larvae

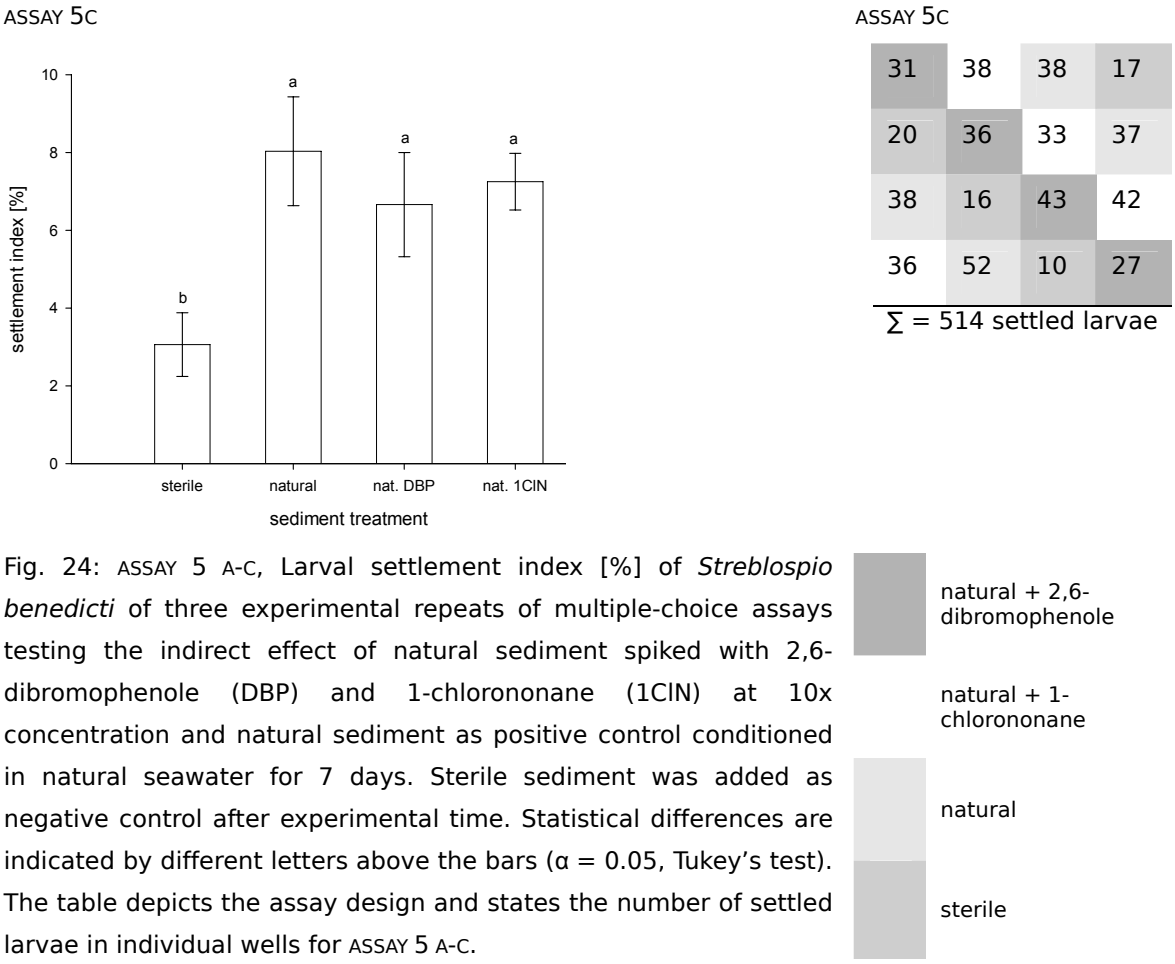


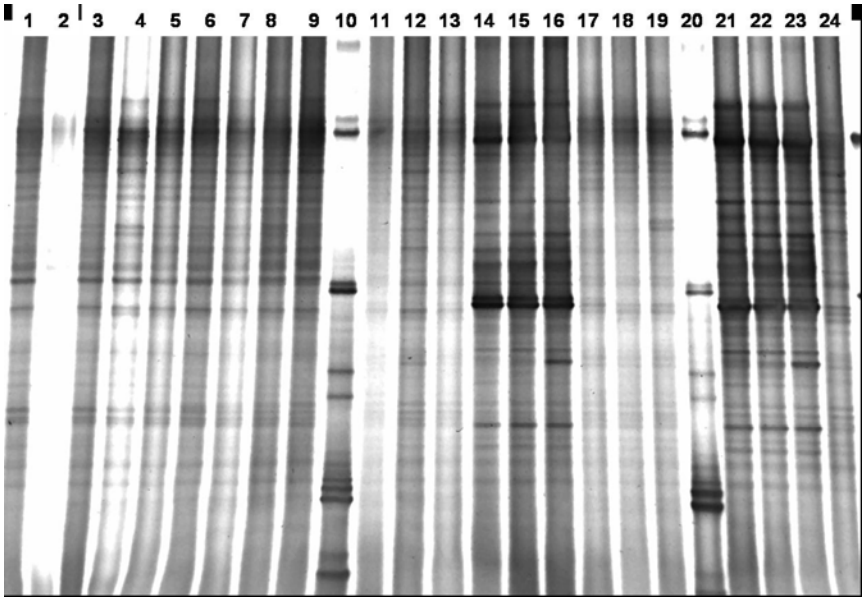
Fig. 24: ASSAY 5 A-C, Larval settlement index [%] of *Streblospio benedicti* of three experimental repeats of multiple-choice assays testing the indirect effect of natural sediment spiked with 2,6-dibromophenole (DBP) and 1-chlorononane (1ClN) at 10x concentration and natural sediment as positive control conditioned in natural seawater for 7 days. Sterile sediment was added as negative control after experimental time. Statistical differences are indicated by different letters above the bars (α = 0.05, Tukey's test). The table depicts the assay design and states the number of settled larvae in individual wells for ASSAY 5 A-C.

5.3.10 BACTERIAL COMMUNITY PROFILE IN NATURAL AND SPIKED SEDIMENT TREATMENTS

The one week incubation experiments served to provide virgin and aged sediment samples (n = 3) of natural sediment, natural sediment spiked with halogenated proxies, and sediment populated with adult worms of both species. Two sediments populated with *Streblospio benedicti* were lost during the experiment. PCR amplicons obtained from these samples were developed on a single denaturant gradient gel (Fig. 25).

The bacterial communities obtained from spiked sediment treatments were characterized by the absence of bands in comparison to the control of natural sediment, which supposedly reflected a community of chemically unaffected bacteria. In sediments populated with worms additional bands were observed in comparison to the control of natural sediment indicating a contamination of

sediments with bacterial phylotypes originating from worms. These additional bands were omitted in the analysis of bacterial community patterns.



legend of bands	
1	natural T0
2	natural T0
3	natural T0
4	1ClN T0
5	1ClN T0
6	1ClN T0
7	DBP T0
8	DBP T0
9	DBP T0
10	pos
11	natural T7
12	natural T7
13	natural T7
14	1ClN T7
15	1ClN T7
16	1ClN T7
17	DBP T7
18	DBP T7
19	DBP T7
20	pos
21	Capi T7
22	Capi T7
23	Capi T7
24	S.bened T7

Fig. 25: Inverted image of DGGE banding patterns of PCR amplified 16S rRNA gene fragments of sediment associated bacterial communities in natural sediment (natural) and sediment treated with 1-chlorononane (CLN) or 2,6-dibromophenole (DBP) and natural sediment treated with adults of *S. benedicti* (S. bened.) and *Capitella* sp.I. (Capi) at day 0 (T0) and after 7 days (T7) in natural seawater; pos = positive control, natural sediment)

Under the combination of PCR primers the number of discernible gel bands obtained from sediment treatments and controls ranged from 14 to 23. In total, 40 bands were used to create a similarity matrix based on Boolean character sets (1 or 0) corresponding to the presence or absence of a given band in a gel lane. The analysis of similarity (ANOSIM) of given bands per treatment produced a global R-value of 0.822 at the significance level of 0.1 %. The pairwise comparisons of bacterial community richness in different sediment treatments resulted in no differences among all virgin sediment treatments at day 0. Contrary, all pairwise comparisons of virgin and aged sediment treatments were significantly different, with the exception of the pair 2,6-dibromophenole at day 0 and natural sediment populated with live *S. benedicti* for 7 days. Whilst bacterial community richness in sediments populated with live worms for 7 days was identical, it differed in the corresponding sediment treatments spiked with species-specific halogenated proxies for 7 days (Tab. 25).

Tab. 25: Analysis of similarity (ANOSIM) of DGGE-patterns of bacterial community DNA samples obtained from 3 replicates of natural sediment at day 0 and 7 (N-0, N-7) natural sediment spiked with 1-chlorononane at day 0 and 7 (CLN-0, CLN-7), natural sediment spiked with 2,6-dibromophenole at day 0 and 7 (DBP-0, DBP-7) and natural sediment populated with adults of *Capitella* sp. I and *S. benedicti* at day 7 (Cap-7, Streb-7). Calculated R-values stem from the analysis of present and absent gel bands per treatment. The global R-value was 0.822. The asterisk marks statistical significance (α = 0.05).

	N-0	**N-7**	**DBP-0**	**DBP-7**	**CLN-0**	**CLN-7**	**Cap-7**	**Streb-7**
N-0	-							
N-7	0.833*	-						
DBP-0	0.741	1.0*	-					
DBP-7	1.0*	1.0*	1.0*	-				
CLN-0	0.426	0.889*	0.37	1.0*	-			
CLN-7	1.0*	1.0*	1.0*	1.0*	1.0*	-		
Cap-7	1.0*	1.0*	1.0*	1.0*	1.0*	1.0*	-	
Streb-7	0.833*	0.611	0.556	0.889*	0.889*	0.889*	0.352	-

On the basis of these findings, the bacterial community profiles associated with different sediment treatments and controls were subject to cluster analysis. The cluster analysis of bacterial community richness associated with different sediment treatments and controls resulted in four subclusters (Fig. 26). The bacterial community richness associated with replicate sediment treatments revealed high similarities (data not shown) and further virgin and aged natural sediment, as well as virgin spiked sediments showed high compositional similarities of >70% (Fig. 26).

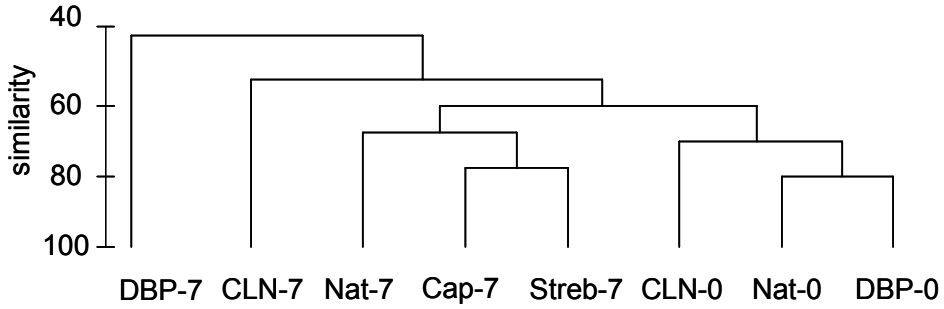


Fig. 26: Dendrogram showing the relatedness of sediment-associated bacterial communities in virgin natural sediment at day 0 and 7 (N-0, N-7) natural sediment spiked with 1-chlorononane (10x) at day 0 and 7 (CLN-0, CLN-7), natural sediment spiked with 2,6-dibromophenole (10x) at day 0 and 7 (DBP-0, DBP-7) and natural sediment populated with adults (n = 20) of *Capitella* sp. I and *S. benedicti* at day 7 (Cap-7, Streb-7).The dendrograms were constructed by using the similarity matrix determined Bray-Curtis coefficients.

6 DISCUSSION

6.1 ROLE OF DIATOMS AS POTENTIAL SETTLEMENT CUES

A mature biofilm represents an organized community of e.g. bacteria and diatoms and may act as a cue for surface exploring larvae. In this study I investigated the relevance of diatoms as larval settlement cue for soft sediment settlers using three different ways. The first attempt was to compare the role of diatoms and bacteria in biofilms on natural sediments by eliminating diatoms or bacteria on the sediment surface to test the effect of the remaining group of microorganisms as meditative settlement cue. The second attempt was to influence the number of diatom cells on natural sediment under illumination or kept under constant darkness. The third attempt was to inoculate ashed or sterile sediment with viable diatom suspension obtained from natural sediments.

Bacteria and diatoms were selectively eliminated with algicides (cycloheximide) and antibiotics (streptomycin, penicillin). The efficiency of algicides in inserted concentration (10 mg g^{-1}) in natural sediment was 10^4 non-viable diatom cells mm^2 sediment, i.e. one order of magnitude lower than viable diatom cells in natural sediments or sediments treated with antibiotics. Low bacteria densities ($< 10^5$ cells g^{-1} sediment) were found in natural sediments treated with antibiotics i.e. two orders of magnitude lower than in natural sediment or sediment treated with algicides.

However, in all five single-choice settlement assays either with *S. benedicti* or *P. cornuta* the percentage of larval settlement on sediment treated either with algicide or antibiotics was not significant different to natural (control) sediment. The mediative effect of vital diatoms as hypothesized must be negated because larvae settled in the same range as in natural sediment. Due to the settlement behavior of larvae in multiple-choice assays and their differentiation between attractive (natural, sterile) and unattractive (ashed, acid washed) sediments larvae may distinguish sediments either with viable diatoms or no (dead) diatoms on the sediment surface. Considering the effect of antibiotics on bacteria cells or algicide on diatoms; the viable bacteria or diatom numbers on natural sediment depleted but their constituents e.g. cell wall, chemical compounds (metabolites) or extracellular polymers, such as polysaccharides, proteins and glycoproteins still remained and as such they may influence larval settlement. The involvement

of polysaccharides as meditative settlement cues has previously been addressed to other invertebrate phyla such as polychaetes, tunicates, hydroids and barnacles (Kirchman et al. 1982a, 1982b, Szewzyk et al. 1991, Leitz and Wagner 1993, Maki 1999).

The second attempt of this study was to investigate larval settlement on pretreated sediments under illumination or sediments exposed to constant darkness. Due to the migratory behavior of diatoms to light I assumed that high and low densities were found in illuminated and darkened sediments. Increasing light intensities led to increases in cell numbers on the sediment surface and maybe resulted in variations in species composition migrating to the sediment surface, whereas in darkness they stayed within the sediment bed. Sediment under constant darkness showed 10^4 cells mm^2 one magnitude lower than in sediment under illumination. My results comply to Palmer and Round (1967) and Sauer et al. (2002) where darkening of formerly light exposed sediments led to a reduction in cell density, while exposing formerly darkened sediment to light led to increased cell density. However, larval settlement of *P. cornuta* was not significant different in illuminated sediments in comparison to sediment kept in darkness investigated in four single-choice assays. This suggests that the settlement cue may not found as hypothesized in the surface abundance of benthic diatoms on natural sediment. However, in natural sediments under illumination or exposed to constant darkness bacteria and/or their epipolysaccharides would not be influenced in a high degree and can act as a meditative settlement cue. Further more in sediment exposed to constant darkness not all diatoms migrated into the sediment and on surface remaining diatom cells may also act as larval settlement cue.

Thirdly I addressed the possible effect of ashed sediment to regain attractiveness for larvae by inoculation with viable diatoms suspensions obtained from natural sediment. The recolonization of ashed or sterile sediment was rarely successful and only a low amount of diatoms was attached on the sediment surface on ashed and to a greater extend on sterile sediment. Nor ashed sediment inoculated with diatom suspension either ashed sediment and the additionally treatment of antibiotics exhibit a higher percentage of larval settlement in comparison to pure ashed sediment. This could be due to the moderate success of the attachment of diatoms on ashed sediment or in the absence of epipolysaccharides on the sediment surface. The hypothesis that ashed sediment

inoculated with diatoms gain in attractiveness can be negated. Patil and Anil (2005) referred that larval metamorphosis of *Balanus amphitrite* was density and species depended and Lam et al. (2003) described that the magnitude of larval settlement of *Hydroides elegans* correlated with the density of diatoms in biofilms. These observations supported my assumption that the low percentage of larvae on ashed inoculated sediments was partly founded in low diatom densities. Further sterile sediment was inoculated with diatom suspension and in a second treatment antibiotics were added. Only sterile sediment inoculated with viable diatom suspension increased larval settlement of *P. cornuta* in comparison to only sterile sediment. In this case sterile sediment could achieve higher attractiveness for larvae of *P. cornuta.* This may be due to the presence of viable bacteria or diatoms on the sediment surface or the presence of epipolysaccharides of diatom or bacteria origin. A biofilm comprising much diatom EPS may give weak to strong positive cues for the incoming larvae. Quian et al. (2003) hypothesized that changes in the microbial community lead to changes in the recruitment pattern of marine invertebrates at microscopic scale. This can also be a reason and a further explanation to low settlement rates in these experiments. After the application of diatoms suspension with antibiotics for 36 h and the following inoculation time (5 h) the microorganism composition can shift and only dominant groups would exist.

In conclusion, larval single-choice assays revealed that the benthic diatoms do not effect larval settlement. Nor in natural sediments without the viable bacteria component, natural sediments in illumination or darkness with high or low diatom numbers or ashed and sterile sediments inoculated with diatom suspension with or without the viable bacteria component showed an inhibition in the percentage of larval settlement. These investigations showed that besides of diatoms bacteria may play an important role not only in increasing of EPS production but also in providing settlement cues to invertebrate larvae.

The idea to investigate sediments in application to use microelectrode measurements was to investigate surface boundary layer (SBL) thickness as a meditative trigger for larval settlement. Larvae which explore the sediment surface recognize this layer and could be influenced in their choice to accept or reject the sediment. In this study the oxygen concentration was used as parameter to determine the SBL thickness. Three sediment treatments, natural, sterile and ashed were performed. Oxygen was found in all sediments in a depth

of up to 500 µm in saturation. In natural sediment the SBL was 750 µm thick in fact of natural photosynthesis which resulted in a higher oxygen concentration. The SBL in sterile or ashed sediment was thin (> 250 µm, > 50 µm) this may be due to the absence of viable microorganisms. The SBL thickness in sterile sediment was not a meditative settlement cue for *S. benedicti* because the percentage of larval settlement in sterile sediment was similar than on natural sediment. Whereas larvae of *P. cornuta* settled in low numbers on sterile and ashed sediment and in higher numbers on natural sediment, in that case the SBL thickness may be a meditative cue for larvae of *P. cornuta*.

6.2 ROLE OF EXOPOLYMERS AS POTENTIAL SETTLEMENT CUES

The objective of this study was to investigate the influence of epipolysaccharides on larval settlement of *Polydora cornuta* or *Streblospio benedicti*. This was investigated in five different ways; firstly I extracted EPS from natural sediment and inoculate ashed sediment with natural EPS solution. Secondly the EPS on natural sediment was enzymatically degraded and thirdly natural sediment was mechanically or chemically treated to destroy natural EPS. Fourthly carbohydrate binding proteins (lectins) were used to mask potentially settlement cues e.g. monosaccharides as a component of a biofilm to test the effect on larval settlement. Fifthly different monosaccharides were given on sediment because larvae may have binding sides for special monosaccharides which can influence larval settlement. Larval settlement of *Polydora cornuta* regularly was significantly lower on ashed sediment than on sterile or natural sediment. Further more, reestablishment of microorganisms on ashed sediment significantly triggered larval settlement (Sebesvari et al. 2006). However larval settlement on ashed sediment reapplied with EPS solution showed no significant differences to ashed sediment without any treatment. It seems that there was no direct effect of EPS solution on larval settlement. Due to experimental difficulties to achieve considerable amounts of EPS from natural sediment to inoculate ashed sediment the non-inductive effect of EPS in larval settlement can not be stated.

Firstly I assumed that the obtained EPS either did not attach on ashed sediment or if doing so than the EPS composition was not representative to the EPS found on natural sediment. Sutherland (2001) detailed that exopolymers synthesized by microbial cells vary greatly in their composition and they will only represent a single “snapshot” of the EPS composition. Studies of Zhou et al. (1998) described that the ratios of specific sugar monomers (e.g., rhamnose and fucose) may be important in the cohesive properties of the EPS, and their ability to form aggregates. EPS polymers often contain hydrophobic and surfactant moieties, which may greatly alter the chemical properties of the polymers (Neu 1996). These compounds influence the surface characteristics or the polymer and may affect the subsequent colonization of other bacteria (Decho 2000). A non-attached EPS or an EPS with altered chemical, physical properties may not elicit in the same larval response than the natural EPS.

Secondly I argue that the amount of extracted EPS from natural sediment was to low to inoculate a high amount of ashed sediment which was required to perform single-choice assays for an adequate number of replicates. This assumption was supported by the measurement of the concentration of reextracted EPS from ashed sediment with 140.34 µg gluc.eq g^{-1} sediment in comparison to natural sediment with a highest value of 530.06 gluc.eq g^{-1} sediment. The phenol-sulfuric acid method was used as a comparison method to determine the overall concentration of carbohydrates in natural sediment (Underwood et al. 1995). Four consecutive extractions were sufficient to remove all EPS from the sediment. This was similar to preliminary experiments of Decho et al. (2003). Some other methods can be used to detect the amount of EPS e.g. the measurement of abundances of uronic acid in exopolymers (Fazio et al. 1982), rather than general measurements of carbohydrates in application of the phenol sulfuric acid method. The methods most commonly used for quantitative determination of uronic acids have the disadvantage that neutral sugars interfere with their specificity (Blumenkranz and Asboehan 1973). Uhlinger and White (1983) described that the percentage of uronic acids in EPS fractions decreased from 26 % in cultures for bacteria to 5 % and 3.2 % in extractions from marine sand and muds. These findings were the reason not to conduct these methods for a closer determination of EPS in marine sediments.

Due to the difficulties to acquire high amounts of EPS from natural sediments I investigated EPS obtained from one bacterial monoculture of *Bacillus pumilus* (AB098578) to inoculate ashed sediment and tested in a larval single-choice assay. The advantage to take one single bacteria strain was to maintain higher amounts of EPS than obtained from natural sediment. Another advantage was that EPS originated from a known bacteria culture and less other factors e.g. humic acids (derived from natural sediments) may influence larval settlement. The percentage of larval settlement on ashed sediment inoculated with EPS obtained from one bacterium monoculture was the same as on pure ashed sediment. EPS obtained from *B. pumilus* may not be inductive for *P. cornuta* larvae and larval settlement may be mediated by specific molecular components in the extracellular polymer. However these results were not representative because the experiment was performed with a lower number of replicates of larvae due to larval culturing difficulties. One year after my experiments Sebesvari et al. (2007) detailed that *B. pumilus* as biofilm (i.e. bacteria cells and

EPS) recolonized on sterile sediment did not trigger larval settlement of *P. cornuta* different from the sterile control sediment. These data can be related to my experiments using EPS as a mediative cue. It may be reasonable that in focus of a non-inductive bacteria strain the EPS also may non-inductive on larval settlement in regard to use the EPS obtained from this strain inoculated on ashed sediment.

The second attempt in this study was to damage the biofilm structure on natural sediment in a different manner either enzymatically, chemically or mechanically. This was done in use of a mixture of enzymes, EDTA, a combination of EDTA and ultrasonication applied on natural sediment and tested in single-choice assays with larvae of *S. benedicti*. I hypothesized, if larval settlement was mediated by EPS than a remove of EPS may inhibit larval settlement. The percentage of larval settlement on natural sediment treated with different enzymes, EDTA, EDTA and enzymes or EDTA and ultrasonication were significant lower than on natural and sterile sediment. I assume that larvae settled on enzymatically, mechanically or chemically treated natural sediments in less amounts in comparison to natural sediment because of the degradation of EPS. The mediative cue to influence larval settlement may be found in EPS derived from the viable bacteria or diatom components on natural treated sediments. The technique combining enzymatic treatment and ultrasonication was a very efficient method for sloughing off bacterial biofilms from sediment surfaces and was used for soil samples (Böckelmann et al. 2003). A mixture of enzymes, such as proteases (Aldridge et al. 1994), polysaccharide lyases (Sutherland 1995; Johansen et al. 1997) was used with partial success for the inhibition and removal of model single species biofilms. In contrast to this, I used a defined mixture of enzyme activities for the detachment of unknown bacterial communities in a sediment sample. The enzymes used in my methods were selected in sufficient concentrations in regard to the criteria for an adequate destabilization of the biofilm and degradation of EPS. Additionally the ultrasonic treatment of natural sediment samples was performed for 30-min to damage mechanically the biofilm structure. Regarding to this I assume that the used methods were effective and transferable to natural sediments used in single-choice assays. A 3-min treatment seemed to be a reasonable minimum, probably destroying only a minority of the indigenous cells, whereas an 18-min treatment resulted in a loss of 20-40 % of intact cells (Lindahl and Bakken 1995). In conclusion *S. benedicti* larvae were influenced negatively in

their settlement behavior in single-choice assays. Additionally multiple-choice assays can give more evidence in regard of the influence of degraded or dissolved microbial EPS on natural sediment as mediative settlement cues.

Fourthly in this study I assumed to block the lectin-polysaccharide bond with artificial lectins that compete with the larval lectin for receptor sites on the surface. Sterile sediments probed with ConA which binds specifically to glucose and its derivatives mannose and peanut lectin with an affinity for galactose negated the promontory effect. In single-choice assays the percentage of larval settlement on sterile sediment inoculated with lectins was equal to sterile or natural sediments. However these carbohydrate moieties were supposed to block the “lectin-polysaccharide” receptor of the larvae. If the larvae carried a lectin receptor, I would expect their larval settlement behavior was inhibited on sediment treated with lectins. No investigation has been carried out to investigate larval settlement of *S. benedicti* in regard to the lectin-polysaccharide binding sites. An other explanation of the settlement behavior of larvae on sterile sediments treated with lectins in comparison to natural sediments is the fact that the larvae do not carry a lectin receptor. Bacterial films were composed of a variety of carbohydrates in this experimental design only glucose, mannose and galactose were blocked with artificial lectins. The use of various lectins with different binding sides e.g. *Lens culinaris* agglutinin (LCA) bound to mannose, glucose, N-acetyl-D-glucosamin, *Wheat germ* agglutinin (WGA) bound to N-acetyl-D-glucosamine, *Liumulus polypehemus* (Limulin) bound to N-acetylneuraminic acid, glucorunic acid and phosphorycholine analogs, Gly max (Glycine max) bound to N-acetyle-D-galactosamine may be useful to influence larval settlement. These carbohydrate-binding proteins mediate cell-cell interactions in other systems that influence the regulation of intracellular biochemical events which are coupled with developmental processes. For example, there are lectins found on the surface of mammalian liver cells that specifically recognize galactose residues. It is believed that these cell-surface receptors are responsible for the removal of certain glycoproteins from the circulatory system (Ni Y and Tizard I 1996). Lectins were also known to play important roles in the immune system by recognizing carbohydrates that were found exclusively on pathogens, or that were inaccessible on host cells (Ni Y and Tizard I 1996). Lectins appeared to regulate the species-specific aggregation of slime mold cells by mediating cell contact, an important signal in the morphogenesis of *Janua brasiliensis* (Frazier

and Glaser, 1979). Two experiments have been carried out to inoculate *S. benedicti* larvae in a mixture of ConA and peanut lectin. I assumed to converse the lectin-polysaccharide model to block carbohydrates on the surface of the larvae and further to test larval settlement on sterile sediments. Larvae conditioned in lectin solution settled similar on sterile sediment as larvae without lectin condition. The presumed inhibitory effect of lectins could be negated. Larval settlement on sediments treated with lectins was contrarious to experiments of Kirchman and Mitchell (1981). They investigated the settlement of *J. brasiliensis* and described no settlement on films treated with ConA.

Fifthly I assumed that monosaccharides added to sterile or ashed sediment acted as carbohydrate binding site for *S. benedicti* larvae. In this study four sugar monosaccharides or a mixture of all were added to ashed and sterile sediment and larval settlement significantly increased in comparison to only ashed sediment. This indicated that larvae may respond positively because of the existence of carbohydrate receptors on the larval-surface and the affinity to the binding properties of sugars on ashed sediments. On the other hand, Kirchman et al (1982a, 1982b) reported that in the polychaete *J. brasiliensis* glucose blocked the settlement and metamorphosis of treated bacterial films. Further Neal and Yule (1996) described an inhibition of D-glucose and D-glucuronic acid in regard to the temporary adhesion of barnacle cyprids. They suggested that the chemical identity of bacterial exopolymers, particularly in respect of their pentose:hexose and neutral sugar:uronic acid rations, may be an important factor in determining the strength temporary adhesion shown by barnacle cyprids towards bacterial films. My results may suggest that involvement of sugars mediate larval settlement of *S. benedicti* because of specific lectin-sugar interactions. In this experimental stage it was likely that the settlement cue for *S. benedicti* may be found in a source derived from bacteria. Combined all results obtained from single-choice assays on 1.) ashed sediment inoculated with EPS obtained from natural sediment; 2.) natural sediment treated with enzymes, EDTA or ultrasonication; 3.) sterile sediment treated with lectins or 4.) sterile or ashed sediment treated with monosaccharides; the promontory effect of EPS was only found on natural sediment treated with enzymes, EDTA or ultrasonication. The hypothesis on EPS guided settlement was supported in one case and multiple-choice assays may provide detailed findings in regard of EPS as a mediative settlement cue.

6.3 ROLE OF HALOMETABOLITES AS POTENTIAL SETTLEMENT CUES

Halogenated metabolites widely occur in a variety of infaunal polychaetes (Ashworth and Cormier 1967; Higa and Scheurer 1975a, b; Sheikh and Djerassi 1975; King 1986; Woodin et al. 1987; Steward et al. 1996; Fielman et al. 1999). Surface-associated mircroorganisms have long been demonstrated to act as local signpods and larval settlement cues in a wide variety of benthic invertebrate taxa settling on hard substrata (Pawlik 1992; Leitz and Wagner 1993; Hadfield et al. 1994; Bryan et al. 1997; Lau and Qian 1997; Harder and Quian 1999; Harder et al. 2002). Previously we studied whether a similar concept might also operate in infaunal organisms guiding burrowing behavior, metamorphosis and settlement. Utilizing the spionid polychaete *Streblospio benedicti* we demonstrated that competent larvae, whilst significantly rejecting experimental sediment lacking organic carbon, were stimulated to settle if this sediment was re-infected with viable microorganisms dissociated from natural sediment (Sebesvari et al. 2006). Considering that a variety of infaunal polychaetes produce and release halogenated metabolites into the surrounding sediment (Woodin et al. 1987; Steward et al. 1992; Fielman et al. 1999) I tested the hypothesis that polychaete-derived halometabolites may either pose a direct negative effect on larvae or alter the bacterial richness in surface sediments of densely populated sediments, and thus indirectly influence larval settlement. Such an indirect interaction would represent a hitherto unrecognized role of biogenic halometabolites in inter- and intraspecific competition during recruitment.

To investigate this hypothesis I raised planktotrophic larvae of *Streblospio benedicti* in the laboratory and tested their settlement response in single- and multiple choice assays of experimental sediments spiked with halogenated compounds. Halogenated compounds for spiking experiments were selected based on their occurrence and predominance in adult specimen of *S. benedicti* and *Capitella* sp. I. Firstly, *Capitella* sp. I was chosen because of its production of bromophenoles and as a complement which was not found in *S. benedicti*. And secondly, *Capitella capitata* (Faibricius) has occurred 1995 in low amounts in the Ems and Weser (Germany) estuary during a BfG monitoring program (Nehring and Leuchs 1999) but was not found next to *S. benedicti* in my observation area. The concentrations measured in adult worm species were used as a point of reference to spike natural or sterile sediment with each synthetic halometabolite.

The total tissue concentrations of unidentified chlorononane isomers and dibrominated phenols in adult *S. benedicti* and *Capitella* sp. I obtained from our laboratory cultures was 70.57 ± 0.08 and 20.71 ± 1.65 ng mm^{-3}, respectively. These values fall into the same order of magnitude of total halogenated metabolites determined in adult *S. benedicti* obtained from intertidal mudflats in South Carolina (USA) and laboratory cultures of *Capitella* sp. I (Cowart et al. 2000). For the setup of experimental sediments the content of dominant, partially unidentified halometabolites was mimicked with commercially available compounds, such as 1-chlorononane as a proxy for chlorononane isomers and 2,6-dibromophenole as a proxy for dibrominated phenoles.

For bioassays the concentrations of halogenated proxies in spiked experimental sediment aliquots of 2.5 g and 2 cm^2 surface area were adjusted equivalent to the total concentration of halometabolites present in 20 adult worms. This calculation overestimated the actual population density of both species at the sampling site (ca. 5000 individuals m^{-2}) roughly twenty fold. However, the resulting sediment concentration of 0.165 µg g^{-1} 2,6-dibromophenole was well within the range reported for a relatively large bromophenole producing polychaete, *Notomastus lobatus* (Steward et al. 1992, Lovell et al. 1999). To the best of my knowledge, comparable quantifications of biogenic chlorinated hydrocarbons in surface sediments do not exist in the literature. Given the small size of the study organisms, I did not determine the concentrations of halometabolites in burrow linings and in close proximity. I purposely mimicked a homogenous distribution of the supposedly high concentration of halometabolites present in direct proximity to worm burrows in the entire experimental sediment in order to test the effect of halometabolites on sediment-associated bacteria in the topmost sediment layer. The rationale for this setup was based on previous observations that competent polychaete larvae repeatedly touched the sediment surface with their ventral body parts prior to burrowing and settlement (Sebesvari et al. 2006).

To verify to which extent spiked halogenated proxies were bound to particulate organic matter in sediments and thus bioavailable to bacterial biofilms in the sediment, halogenated proxies in sediments were extracted and quantified. The recovery yields were surprisingly low and differed significantly between the two compounds, indicating that either the extraction efficiency was low, as previously demonstrated by Steward et al. (1992) i.e. 4-bromophenole underestimated the

true values by approximately 40 % but this underestimation was consistent for all samples analyzed and/or that a large proportion of spiked proxies did not adsorb to the sediment. Sterile and natural sediments spiked with each of both halometabolites before and after experimental time showed a strong odor which suggest that presumably 1-chlorononane or 2,6-dibromophenole was present after 7 days incubation in natural seawater. In order to compensate for potential losses, the spiking concentration of halogenated proxies in multiple choice-assays was elevated ten fold resulting in final recovery yields of ca. 3 µg 1-chlorononane g^{-1} sediment and 0.05 - 0.2 µg 2,6-dibromophenole g^{-1} sediment (Tab. 24). Given these concentrations of halogenated proxies in experimental sediment treatments the hypotheses raised in this study were answered as follows:

My first hypothesis of a direct influence of halogenated proxies on larval settlement of *Streblospio benedicti* strongly depended on the assay design. In sterile sediment the microbial component was inactivated therefore decomposition would not effect synthetic halomgenated proxies spiked on sediment. The percentage of larval settlement of *S. benedicti* larvae in single-choice assays on sterile sediment spiked with 2,6-dibromophenole or 1-chlorononane in both concentrations were the same to untreated natural and sterile sediment (Fig. 18). This displayed that the given halometabolites at both concentrations did not inhibit larval settlement and further that these halometabolites were not toxic to surface exploring larvae.

However, in multiple-choice assays the percentage of larval settlement of *S. benedicti* was significantly lower on sterile sediment spiked with the 10x concentration 1-chlorononane in comparison to sterile sediment or sterile sediment spiked with 1x concentration or acetone (Fig. 19). Whereas a similar effect was not observed with the other halogenated proxy 2,6-dibromophenole and the percentage of larval settlement was the same in sterile sediments spiked either with 2,6-dibromophenole in 1x or 10x concentration and MeOH or only sterile sediment (Fig. 20). When larvae were offered both proxies spiked on sterile sediment at high concentrations together with the positive alternative of untreated natural sediment, the spiked treatments were significantly rejected (Fig. 21). This indicated that both halometabolites did not influence larval settlement of *S. benedicti*. In contrary to this single-choice assays showed a high percentage of larval settlement in sterile sediment spiked with both

halometabolites. Given that larvae inspect the sediment surface repeatedly on small spatial scales of several cm^2 (Sebesvari et al. 2006; personal observation) these results indicate a potential direct effect of halometabolites on larval settlement provided that surface properties differ within the investigated sediment patch. The single-choice assay seemed inappropriate to resolve these preferences because larvae may loose their selectivity and comply with a moderately acceptable sediment quality after repeated exposure to the same negative stimulus.

My second hypothesis of an indirect influence of halogenated proxies on larval settlement was clearly rejected. Experiments were performed on natural sediments to investigate the indirect influence of halometabolites on larval settlement of *S. benedicti*. King (1986) reported that bromophenoles inhibit microbial activity in marine sediments. I hypothesized that the assumed antimicrobial effect of halometabolites may influence larval settlement indirectly by changing the microbial community pattern. Two different experimental designs were chosen to address this question.

First adults of *Capitella* sp. I and *S. benedicti* were separately transferred in natural sediments and incubated in natural seawater for 6-29 days. In single-choice assays, the percentage of larval settlement of *S. benedicti* in sediments with adults either *Capitella* sp. I or *S. benedicti* was the same in comparison to natural or sterile sediment after nearly any of the conditioning periods (Fig. 22). Except at day 8 and day 29 were *S. benedicti* larvae settled on natural sediment conditioned with adults of *Capitella* sp.I lower than on natural, sterile and natural sediment conditioned with adults of *S. benedicti*. This may be due to microbial changes in the surface sediment treatments but there is no clear evidence to support this assumption.

In 3 experimental repeats of a multiple-choice assay testing the effect of 7 day incubations of natural sediments conditioned with adult specimen of both adults similar results were obtained as in single-choice tests (Fig. 23). When live specimen were incubated in natural sediment for the same duration, allowing the sediment to be contaminated with biogenic halometabolites, there was no difference in larval settlement between sediments populated with live worms and the unpopulated positive control of natural sediment (Fig. 23). Further after 7 days of incubation of natural sediments spiked with 1-chlorononane or 2,6-dibromophenol at 10x concentration and the natural control sediment the

percentage of larval settlement was the same in all treatments but significant different to sterile sediment (Fig. 24). These results suggested that a.) the overall inductive effect of viable bacteria on larval settlement as observed in the control treatments of natural sediment was unchanged in the presence or after the utilization of halometabolites, and b.) that potential changes in bacterial abundance and/or community richness as a consequence of long term exposure to halometabolites or microbial utilization did not change the suitability of these sediments for settling larvae. A direct effect of halogenated proxies on larval settlement as outlined above could be ruled out since the recovery yields for these compounds were below the detection limit after 7 days of incubation, indicating a complete microbial turnover of halogenated compounds (Tab. 24).

In order to evaluate the potential changes in bacterial community richness between virgin and the corresponding aged sediment treatments, the banding patterns obtained by denaturant gradient gel electrophoresis (DGGE) were subject to statistical similarity analysis. Since DGGE of PCR amplicons does not provide quantitative information of the bacterial community the similarity analysis was based on the presence and absence of bacterial phylotypes (Muyzer et al. 1993, Murray et al. 1996, Rochelle 2001). The analysis of similarity revealed a significant change in bacterial community richness between virgin and the corresponding aged sediment treatments. In the treatment of natural sediment out of 21 discernible gel bands at day 0, four bacterial phylotypes were absent after 7 days of incubation in natural seawater. These phylotypes were likely eliminated due to the lack of nutrients or bacterial dynamics in complex microbial assemblages (Costerton et al. 1995). Two of the phylotypes eliminated in aged natural sediment were also absent in aged sediment treatments spiked with halogenated proxies. Moreover, four identical bacterial phylotypes were eliminated in these sediment treatments. In addition, nine bacterial phylotypes were eliminated in the aged sediment treatment spiked with 2,6-dibromophenole. These results indicated a pronounced limiting effect of 2,6-dibromophenole on the bacterial community richness present in natural sediment, supporting the earlier notion of strong bactericidal effects of bromometabolites (King 1986). Interestingly, 11 and 4 additional phylotypes were observed in aged sediment treatments spiked with 1-chlorononane and 2,6-dibromophenole, respectively, in comparison to natural sediment. Whilst these bacterial types were presumably present already in natural sediment, their lack of detection may be explained

with their low abundance in untreated sediment. In order to profile a bacterial constituent within a complex population by DGGE analysis of PCR-amplified genes coding for 16S rRNA the minimum abundance must exceed 1 % of the total population (Muyzer et al. 1993). It is possible that the additional phylotypes observed in sediment incubations treated with halogenated proxies increased in abundance due to the utilization of these compounds or metabolic byproducts of other bacteria within the population capable to remediate halogenated compounds. Contrary to these findings, there was no bactericidal effect of halometabolites released by live worms in the populated sediment treatments. The bacterial community richness in these samples was the same as in the aged treatment of natural sediment.

Studies employing intact sediment cores (Steward et al. 1992, Steward and Lovell 1997, Lovell et al. 1999) or examining burrow linings that were not homogenized or incubated with a halogenated substrate (Alongi 1985, Jensen et al. 1992, Steward et al. 1996) have shown no inhibition of sediment microbiota by infaunal brominated metabolites. The accumulation of biogenic bromophenoles in wormbeds has been suggested to be limited by bacterial remediation (Steward and Lovell 1997). Our study complies with the latter suggestion, since recovery yields of halogenated compounds after short incubation times were low or no longer detectable. However, this study clearly demonstrates that halogenated compounds reduce the bacterial community richness in surface sediments and can be utilized by sediment associated bacteria. Moreover, my results suggest a significantly more pronounced limiting effect of biogenic bromophenoles than chlorinated hydrocarbons. These findings were supported through Steward et al. (1995). They described that high concentration of bromometabolites external of adult worms were found in sediment lining the burrows. These burrows support substantial and complex microbial communities, implying adaptation of burrow-lining microbial communities to long term exposure of these compounds. The shift in microbial community in sediment treated with 2,6-dibromophenole may exhibit a good source for debrominating bacteria which were more persistent at day 7 than at day 0. The inhibition of sediment microflora in proximity to worm burrows and linings has been suggested to restrict the availability of microbially derived sources for carbon and nitrogen for worms (Newell 1965, Cammen 1980), alter sediment texture by reduced production of exopolymers and thus change sediment biogeochemical processes (Decho 2000).

Contrary to my findings, Woodin et al. (1997) described that the addition of bromophenoles to sediments had a significantly negative effect on acceptance of the sediment by recently settled juveniles of *Arenicola cristata*, *Mercenaria mercenaria* or *Mulinia lateralis*. Further bromophenoles of *Notomastus lobatus* may also serve as negative cues for recruitment of the planktonic larvae of other infaunal species which is detailed in Woodin (1991). Newell (1965) and Cammen (1980) described that an inhibition of sediment microflora around the worm burrows could restrict potential sources of carbon and nitrogen for the worms, alter sediment texture by reducing production of diatom exopolymers and substantially alter sediment biogeochemical processes. These impacts would be detrimental to the bromometabolite-producing worm species. Infauna like spionid polychaetes were often subject to predation, where any body part extending above the sediment surface can be bitten off by predators. Worm bromometabolites may instead function as antipredator compounds as described in Woodin et al. (1987) or Woodin and Marinelli (1991).

In regard to experiments utilizing adults of *S. benedicti* and *Capitella* sp. I in situ conditioned on natural sediment it has to be shortly discussed if gregarious settlement behaviour may exist. Interspecific interactions at settlement may occur among settling larvae, between larvae and adults and between larvae and juveniles and result in inhibition, tolerance or promotion. Laboratory and field studies have demonstrated that barnacle cyprids prefer to metamorphose on or near conspecifics, a process termed gregariousness, and this feature has been related i.e. to arthropodin (settlement factor), a glycoprotein present in the adults (Knight-Jones and Crisp 1953). The presence of adult *Arenicola marina* negatively influences the settlement of juveniles whereas the presence of juvenile conspecifics positively influences the settlement (Hardege et al. 1998). The findings of multiple-choice experiments suggested that larval settlement of *Streblospio benedicti* was not influenced by gregariousness because the percentage of larval settlement did not differ in sediments conditioned with adults of both species in comparison to natural sediment.

In conclusion, larval settlement was not directly influenced in presence of 1-chloronaonane or 2,6-dibromophenole on sterile sediment. Additionally either adults of *Capitella* sp. I or *S. benedicti* or natural sediment spiked with 1-chlorononane or 2,6-dibromophenole on natural sediments did not effect larval settlement of *S. benedicti* larvae. But the analysis of PCR amplified 16S rRNA

gene fragments of sediment associated bacterial communities in all applications after 7 days showed differences in the bacterial community richness. These observations supported my assumption that halometabolites influenced the bacterial community. However in this study, the potential modified sediment quality as a result of a shift in bacterial community richness was not recognized by larvae of the polychaete *Streblospio benedicti*, indicating that biogenic halometabolites did not serve as indirect larval settlement cues in this species.

7 CONCLUSION

In summary, the results obtained in this thesis to the major objectives raised initially are given below:

- Do benthic diatoms affect larval settlement of *Polydora cornuta* and *Streblospio benedicti*?

Natural sediment was treated in three different ways to influence the viable diatom fraction on biofilms. 1) Natural sediments were treated with algicides to inactivate the “diatom part”. 2) Further natural sediment was kept in illumination or under constant darkness and further more 3) ashed and sterile sediment was inoculated with a viable diatom suspension obtained from natural sediment. All single-choice assays showed that larval settlement of *S. benedicti* and *P. cornuta* was not inhibited on natural sediments treated with algicides. Further larval settlement of *P. cornuta* in natural sediments kept in darkness or illumination was the same. Diatom counts on natural sediments treated with algicides or in natural sediment exposed to constant darkness revealed depletion in the abundance of diatoms. Diatoms obtained from natural sediment and reapplied on ashed or sterile sediment did not lead to a higher percentage of settlement of *P. cornuta*. Diatoms may not act as a potential settlement cue for larvae of *S. benedicti* or *P. cornuta*. I assumed that the settlement cue may found in other components of biofilms i.e. the existence of exopolymers. The single choice assay seemed inappropriate to resolve these preferences because larvae may loose their selectivity and comply with a moderately acceptable sediment quality after repeated exposure to the same stimulus. However, multiple-choice assays may clearly indicate their selectivity behavior.

- Do bacteria and diatom EPS on natural sediment influence larval settlement?

In *Polydora cornuta* no enhancement in larval settlement was observed in ashed sediment inoculated with EPS obtained from natural sediment. However, qualitative and quantititative reestablishment of EPS on sediment was a challenge, which may significantly influence the outcomes of the settlement assays. The next step was to influence EPS on natural sediments in use of different EPS degrading enzymes, EDTA or ultrasonication. Larval settlement of *Streblospio benedicti* in single-choice assays on natural sediments treated with

enzymes, EDTA or ultrasonication revealed in lower percentage of larval settlement in comparison to sterile or natural sediment. This experiment showed the only case where EPS degradation on natural sediments inhibited larval settlement of *S. benedicti*. The investigation of EPS as a mediative cue obtained from one bacteria monoculture and inoculated on ashed sediment did not elicit higher larval settlement than on pure ashed sediment. The assumption that EPS may influence larval choice can be drawn for one case but multiple-choice assays may differentiate larval settlement behaviour of *S. benedicti* on sediments treated in a different manner.

- Do lectins and monosaccharides inoculated on natural sediment influence larval settlement?

1) In *Streblospio benedicti* larval settlement was not influenced on sterile sediment treated with lectins. In all single-choice assays larvae settled in similar percentage on sterile sediments treated with lectins in comparison to natural or sterile sediment. My assumption that lectins may influence larval settlement must be negated. This can be due to a.) *S. benedicti* larvae did not carry as assumed the lectin-polysaccharide receptor or b.) larval settlement of *S. benedicti* may not be guided of EPS as potential settlement cue.
2) Larval settlement of *S. benedicti* on sterile sediment conditioned with four different monosaccharides or a combination of all was the same as on natural or sterile sediment. Whereas ashed sediment inoculated with monosaccharides or a combination of all revealed an increase in larval settlement in comparison to pure ashed sediment. This indicated that individual treatments of i.e. glucose, mannose, fructose or galactose as a potential component of natural EPS lead to an attractiveness for surface exploring larvae on treated ashed sediments.
The attempt utilizing lectins as mask for larval receptors which bind on polysaccharides associated on sediments was negatively in regard to the larval settlement response on treated sediments. But monosaccharides conditioned on ashed sediment gave an incidence and reveal in a higher settlement of *S. benedicti* larvae as on pure ashed sediment.

- Do secondary halometabolites at environmental concentrations influence larval settlement of *S. benedicti* larvae directly or indirectly?

In multiple-choice assays I have shown that larval settlement of *S. benedicti* was only influenced directly in one sediment treatment spiked with 1-chlorononane at

10x concentration. Larval settlement was the same in sterile sediment in comparison to sediment spiked with 2,6-dibromophenole. However 1-chlorononane and 2,6-dibromophenole spiked on sterile sediment and assayed against each other was lower and significantly different to natural sediment. In that case sterile sediment spiked with halometabolites inhibit larval settlement and directly effect larval settlement, larvae prefer untreated natural sediment.
The indirect influence of halometabolites was assayed on natural sediment spiked with halometabolites at concentrations mimicking those found in adult worm specimen. In multiple-choice assays natural sediment spiked either with 1-chlorononane or 2,6-dibromophenole were tested against each other and the percentage of larval settlement was the same in comparison to natural sediment and significantly different to sterile sediment. This indicates that halometabolites did not effect larval settlement indirectly. However the microbial community richness changed in natural sediments spiked with halometabolites between day 0 and day 7. Moreover the concentration of halometabolites after 7 days was no longer detectable. I assume this was due to microbial decomposition.

- Do halometabolites released form adult worms inhabiting natural sediments influence larval settlement of *S. benedicti*?

Halometabolites produced from adult worm species either of *Capitella* sp.I or *S. benedicti* and their release into the surrounding sediment did not influence larval settlement of *S. benedicti*. *S. benedicti* larval settlement was the same in natural sediment conditioned with each of both adult worm and natural sediment and significant different to sterile sediment. The bacterial community changed from day 0 to day 7 but did not inhibit larval settlement. Gregarious behavior in *S. benedicti* larvae does not influence larval settlement.

In summary the spionid polychaetes *Polydora cornuta* and *Streblospio benedicti* were suitable for settlement bioassays. In regard to their selectivity in single-choice assays on different sediments *P. cornuta* was more selective because of the rejection of ashed and the low percentage of settlement on sterile sediment in comparison to natural untreated sediments. *S. benedicti* was less selective and accepted even less attractive sediments e.g. sterile sediment in single-choice assays. However, in multiple-choice assays *S. benedicti* decided actively between attractive (natural) with higher percentage of larval settlement and unattractive

(sterile) sediments resulting in lower larval settlement, similar to the selectivity observed in *P. cornuta*.

In ecological studies field-experiments were indispensable and have to be undertaken in future experiments. Generally all my experiments were processed in the laboratory under equal conditions i.e. temperature, light, still water and constant salinity. Studying the effect of diatoms or bacterial cues at laboratory conditions may result in an unrepresentative species composition as found in the field. However dominant species may remain in the laboratory and the analyses of amplified 16S rRNA gene fragments of sediment associated bacterial communities only give a "snap shot". The bacteria community structure in the field is more sensitive and immediately reacted to environmental factors.

During my experimental work in regard to investigate biofilms particularly different component such as diatoms, EPS or monosaccharides as potential settlement cues I ended up in several dead ends. No attempt revealed in a clear clue which guided to a definite larval settlement behavior of *Streblospio benedicti* or *Polydora cornuta*.

The final experimental design to investigate the direct or indirect effect of halogenated proxies i.e. produced from adult species *Capitella* sp.I or *S. benedicti* released into the sediment or artificial halogenated proxies spiked on natural or sterile sediment posses a wider range to investigate larval settlement of *S. benedicti*. The quantification of halometabolites in spiked natural sediments at day zero and day seven and the investigation of the effect of halogenated proxies on the microbial community on treated sediments were used in analogy to larval settlement assays. However *S. benedicti* accepted natural sediment either spiked with 1-chlorononane or 2,6-dibromophenole or in situ conditioned with adult worm species in comparison to natural or sterile sediment and larval settlement was the same in all sediment treatments. Further the observed shift of bacterial community richness on natural sediments at day zero and day 7 as a result of inserted halometabolites showed no clear indices as hypothesized to the larval settlement behavior of *S. benedicti*.

I assume that larval settlement of investigated spionid polychaetes may be influenced by various interacting parameters i.e. grain size, water flow, surface boundary layer thickness, and biofilms comprised of various microorganisms (bacteria, diatoms) their epipolysaccharides or secondary metabolites.

Additionally assemblies of soft sediment may alter rapidly through i.e. water current or inbenthic bioturbating organisms.

In future experimental designs it would be interesting to combine microelectrode measurements with larval settlement assays, e.g. single bacteria strains inoculated on ashed or sterile sediment may reveal differences of larval settlement and as a comparative tool the thickness of surface boundary layer could be used as an additional parameter to investigate larval settlement. One aspect would be interesting considering EPS of single bacteria strains which induce larval settlement. This shows that EPS synthesized of respective bacteria mediates larval settlement of spionid larvae. Additionally in examination of biofilms on soft sediments it will be interesting to use the application of light microscopy, e.g. confocal scanning laser microscopy (CLSM) in combination of molecular biology tools to get a deeper insight into structure/function relationships in microbial communities. Cell-to cell and cell-to substratum adhesion are processes whereby cells recognize and adhere to each other in crucial and complicated events. Further studies of marine sediment surfaces could characterize specific receptor molecules and assess the relationships of glycoconjugates with cells as well as characterize receptors on settling larvae.

The understanding in larval settlement behavior in genraral may important because of the fact that in marine systems increasingly overfishing, eutrophication and destruction of fisheries habitats occurred. Knowledge of specific settlement keys for i.e. organisms which maybe important for human food supply may be important as basis to design marine protected areas. Further global warming may change the duration of larvae in the plankton which may effect the surviving of adult species.

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ERKLÄRUNG

Hiermit bestätige ich, dass ich die vorliegende Dissertation selbstständig verfasst, keine anderen als die angegebenen Hilfsmittel und Quellen benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.
Ferner versichere ich, dass diese Arbeit weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung in einem Promotionsverfahren vorliegt oder vorgelegen hat.

Oldenburg, 24. Mai 2007

APPENDIX

DIRECT AND INDIRECT EFFECT OF HALOGENATED METABOLITES FROM INFAUNAL POLYCHAETES ON LARVAL SETTLEMENT OF THE SPIONID POLYCHAETE *STREBLOSPIO BENEDICTI*

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ABSTRACT

Previously we observed that the polychaete *Streblospio benedicti*, whilst significantly rejecting sediment lacking organic carbon, was stimulated to settle if this sediment was re-infected with viable bacteria dissociated from natural sediment. Considering that a variety of infaunal polychaetes release halogenated metabolites with potent bactericidal effects into the surrounding sediment we tested the hypothesis that polychaete-derived halometabolites may either pose a direct negative effect on larvae or alter the bacterial community in surface sediments, and thus indirectly influence larval settlement. Commercially available halogenated compounds identical or similar to the dominant halometabolites in *S. benedicti* and *Capitella* sp. I served as experimental proxies in multiple-choice settlement assays with larvae of *S. benedicti*. The hypothesis of a direct influence of halogenated proxies on larval settlement was verified at concentrations of 3.5 µg 1-chlorononane g^{-1} sediment. A similar effect was not observed with 2,6-dibromophenole. The indirect influence of halogenated proxies on larval settlement was clearly rejected. However, the analysis of similarity of PCR-amplified bacterial genes coding for 16S rRNA revealed a significant change in bacterial community richness between untreated and natural sediment spiked with halogenated proxies after 7 days of incubation. This change was characterized by the elimination of up to 13 and the additional occurrence of up to 11 phylotypes. The increased abundance of new phylotypes is likely due to the bacterial turnover of halogenated proxies or metabolic byproducts of other bacteria within the population capable to remediate the halogenated proxies. The shift in bacterial community richness of sediments exposed to halogenated proxies was not recognized by larvae, indicating that biogenic halometabolites may not serve as indirect larval settlement cues in *S. benedicti*.

KEYWORDS: Halometabolites, Larval settlement, Polychaetes, Infauna, Bacteria, Sediment, *Streblospio benedicti*

INTRODUCTION

The occurrence of natural halogenated organic compounds among temperate marine infauna is widespread (Woodin et al. 1987, Fielman et al. 2001). A variety of infaunal polychaetes and hemichordates contain high concentrations of halogenated noxious secondary metabolites which are released into the sediment in proximity of worm burrows (Ashworth & Cormier 1967; Higa & Scheuer 1975a, 1975b; King 1986; Woodin et al. 1987; Steward et al. 1992). In biological systems the specific incorporation of halogens into organic molecules is regulated by a haloperoxidase enzyme (Fenical 1979). In adults of many soft-bodied infaunal taxa such as hemichordates and polychaetes 2-3% of the total protein content is comprised of haloperoxidase enzymes and used in the biosynthesis of halogenated compounds (Chen et al. 1991).

In previous studies the effect of halometabolites on microorganisms (Zsolnai 1960, Higa & Scheuer 1975b, Sheikh & Djerassi 1975, King 1988, Reineke 2003), fish (Casillas & Myers 1989, Malins et al., 1987) and fish eggs (Reineke 2003) was generally toxic. The potent deterrent effect of halometabolites on epibenthic predation by fishes and crabs at environmentally realistic sediment concentrations in the nano- to micromolar range indicated a potential ecological role of these compounds in sediments populated by infaunal organisms (King 1986, 1988, Reineke 2003, Cowart et al. 2000). However, thus far the role of halometabolites as biogenic antimicrobials has not been unequivocally identified. For example, whilst bromophenols were assumed to selectively target microbial activity in worm burrows (King 1986, 1988) other studies did not detect any significant antimicrobial effect of this compound class (Jensen 1992, Steward et al. 1992, 1996, Steward & Lovell 1997, Lovell et al. 1999). Besides their antimicrobial effect biogenic haloaromatic metabolites have been demonstrated reduce recruitment of heterospecific, non- producing infauna and deter predators via allelopathy (Woodin et al. 1993, 1997, Yoon et al. 1994, Fielman & Targett 1995, Steward & Lovell 1997). The general hypothesis in all these studies was based on the

assumption that the production of halometabolites serves as a defense strategy against microorganisms, predators and interspecific competition.

In addition to the direct role of biogenic halometabolites as negative settlement cues for infaunal polychaetes (Woodin et al. 1993, 1997), in this study we tested the hypothesis that halometabolites may indirectly influence larval settlement of an infaunal polychaete *Streblospio benedicti*. The hypothesis was inspired by our previous finding that competent larvae of *S. benedicti*, whilst significantly rejecting ashed sediment in comparison to natural sediment, were stimulated to settle in ashed sediment that was reinfected with viable microorganisms obtained from natural sediment (Sebesvari et al. 2006). Thus, we raised the question whether polychaete-derived halometabolites may alter the microbial abundance and richness in surface sediments and in turn indirectly affect settlement of polychaete larvae.

We experimentally addressed this hypothesis with two infaunal polychaetes, *Streblospio benedicti* and *Capitella* sp. I. The adult organisms qualitatively differ in their halometabolite contents; *S. benedicti* contains at least 11 chlorinated and brominated alkylhalides (Fielman et al. 1999) while *Capitella* sp. I contains 3 brominated aromatic compounds (Cowart et al. 2000). For both species the predominant halometabolites in worm tissues were determined and quantified by coupled gas chromatography-mass spectrometry. Commercially available halogenated compounds identical or similar to the dominant halometabolites in worm tissue served as experimental proxies in laboratory settlement assays with larvae of *S. benedicti*. In accordance with Cowart et al. (2000), *S. benedicti* mainly contained an unidentified isomer of chlorononane that was approximated with 1-chlorononane, *Capitella* sp. I mainly contained 2,6-dibromophenol which was commercially available as such.

To test the direct effect of halogenated proxies on larval settlement sterilized sediment, i.e. without viable microorganisms, was mixed with individual compounds at different concentrations and subject to larval settlement assays after different time intervals. The indirect effect was tested accordingly but with natural sediment, i.e. sediment containing viable microorganisms. The assays were accompanied with positive and negative controls of

natural and sterile sediments, respectively. The concentration of added proxies was simultaneously measured when assays were evaluated for settlement rates of larvae. In a more ecologically realistic set up, halogenated proxies were replaced with adult specimen of *Streblospio benedicti* or *Capitella* sp. I to contaminate natural sediments with halometabolites and possibly modify the microbial community composition. To visualize and statistically compare the effect of halogenated proxies on the community composition of bacteria associated with the treated sediment and untreated controls we applied the molecular fingerprinting tool of denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA bacterial gene sequences. The aim of this approach was to test whether the qualitative modification of the bacterial community associated with sediment due to exposure to halogenated metabolites resulted in a less attractive sediment for larval settlement. The verification of this hypothesis would indicate a new ecological role of halometabolites released by infaunal polychaetes on subsequent colonization of sediment patches already inhabited by adult polychaetes.

MATERIAL AND METHODS

Adult brood stocks and larval test organisms

Laboratory brood stocks of *Streblospio benedicti* were obtained from mud flats at Hooksiel (53°38’31’’N, 8°04’55’’E) and maintained in the laboratory according to Sebesvari et al. (2006). At the sampling site *S. benedicti* occurs at densities of 5000 individuals m^{-2} (pers. observation). The protocols for larval cultures and the selection of test larvae for bioassays were adopted from Sebesvari et al. (2006). The larvae exhibited a planktotrophic mode of larval development. Laboratory brood stocks of *Capitella* sp. I were obtained from JM Rosario (Institute of Marine and Coastal Sciences, Rutgers State University, New Jersey, USA). Larvae were obtained and reared according to Grassle & Grassle (1976) and Butman & Grassle (1992).

Sediment

Sediment was collected during low tide in mudflats of Hooksiel, Germany (53°38'31''N, 8°04'55''E) throughout the summer of 2006. Newly collected sediment was stored in the darkness in plastic containers at 4 °C for no longer than 1 week (in the following referred to as "natural sediment"). Natural sediment was sterilized by autoclaving prior to bioassays (in the following referred to as "sterile sediment"). Natural sediment was ashed at 600°C for 4 h in a muffle kiln. Before usage in the bioassay, ashed sediment was covered with sterile filtered seawater and autoclaved (in the following referred to as "ashed sediment").

Larval settlement assays

Single-choice: Settlement assays with single choice for larvae were performed with different sediment treatments in sterile 12-well microplates (3.8 cm^2 well surface area, Corning, USA). 2.5 g (wet weight) of sediment was transferred into each well resulting in a 7 mm sediment layer. The sediment was covered with 1.75 ml of sterile-filtered seawater (i.e. filtered through 0.22 µm membranes) and 10 larvae were added into each well. Experimental dishes were incubated for 1 h with replication (n = 12). Afterwards swimming larvae were stained with the vital stain Neutral Red (Sigma, USA) at 10 ng ml^{-1} for 1 h. Stained larvae were counted under the stereo microscope. Stained larvae without burrowing activity on the sediment surface were interpreted as not settled. Sediment treatments were assayed together with natural (positive control) and sterile or ashed sediment (negative control). Assays were always performed with larvae obtained from the same batch.

Multiple choice: Multiple choice settlement assays were performed with four different sediment treatments in sterile cylindrical plexiglas plates (17 x 1.5 cm) containing 16 cylindrical holes (2 x 0.3 cm) and equipped with a removable bottom. The wells were arranged in four by a rows and columns separated by 1 cm. In a 4 x 4 Latin-square design four replicates of different sediment treatments were transferred into wells, i.e. every sample was placed exactly once per row and column. The plate was filled with 200 ml of sterile-

filtered seawater and ca. 1000 larvae were added. After 20 h, the water was poured off and the bottom was removed. Individual sediment samples were transferred onto glass plates. Settled larvae were stained as above and counted under the stereo microscope. The settlement index in replicated samples was calculated based on the total number of settled larvae (100 %) discounting all larvae still swimming or lying on the sediment surface without burrowing activity.

Specimen collection and compound extraction

Adult worms were pooled from laboratory brood stocks. The average length and width (n = 10) of adult specimen was measured under the binocular and the tissue volume was calculated assuming a cylindrical shape of worms. The extraction consisted of placing pooled worms (60 individuals of *Streblospio benedicti* and 40 individuals of *Capitella* sp. I) in 600 µl of GC-MS grade acetone (*S. benedicti*) or methanol (*Capitella* sp. I). Samples were stored for 48h at 4°C. Insoluble tissue material was spun down and the supernatant was dried *in vacuo*. The dry extract residue was dissolved in 50 µl of acetone or methanol for quantification.

Sample analysis

Samples were analyzed using coupled gas chromatography-mass spectrometry. All samples were centrifuged prior to analysis to remove particular matter. A WCOT VF- 5ms capillary column (Varian, USA) (30 m x 0.25 mm x 0.25 µm film thickness) was used on a Varian 3900 gas chromatograph equipped with a Saturn 2100T (Varian, USA) ion trap mass selective detector. Samples were injected in splitless mode with an inlet pressure of 72 kPa. The injection port and the interface were held at 270 °C. The gas chromatograph was held at 70 °C for 3 min and ramped at 5 °C min^{-1} to 210 °C (for *Streblospio benedicti* and spiked sediment) or 150 °C (for *Capitella* sp. I and spiked sediment) then at 50 °C min^{-1} to 320 °C and held for 3 min. Helium was used as the carrier gas. The mass selective detector was

operated in scan mode (*m/z* 40-350). The electron impact ion-spectra of volatile tissue extract components were compared with entries in the NIST mass spectral library (NIST 05).

A calibration of halogenated proxies was performed with 1-chlorononane and 2,6-dibromophenole (Sigma Aldrich, Germany). The average concentrations of halogenated proxies were calculated from the mean of 3 injections of each sample. Following this protocol the resulting average amount of the sum of chlorononane isomers (for *Streblospio benedicti*) and dibromophenoles (for *Capitella* sp. I). was 70.57 ± 0.08 ng mm^{-3} and 20.71 ± 1.65 ng mm^{-3} tissue volume, respectively (Table 1).

Spiking of sediment with halogenated proxies

The amount of halogenated proxies used to spike sediment aliquots of 2.5 g each (see Bioassays) was equivalent to the concentration of chlorononane isomers and dibromophenoles determined in 20 adults (in the following referred to as 1x concentration). This final concentration reflected a homogeneous distribution of halometabolites provided their complete dissolution in the sediment. Under the assumption that halometabolites were more patchily distributed in spots of high concentration (e.g. worm burrows), a ten fold elevated concentration of halogenated proxies was applied in bioassays (in the following referred to as 10x concentration). Stock solutions of the halogenated proxies 1-chlorononane and 2,6-dibromophenole were prepared in 100 µl of acetone and methanol, respectively. Aliquots of the stock solutions were diluted with 5 ml of sterile filtered seawater and mixed with sterile or natural sediment. The final concentrations in sediment treatments for bioassay purposes were 0.56 and 5.6 µg 1-chlorononane g^{-1} sediment, and 0.165 and 1.65 µg 2,6-dibromophenole g^{-1} sediment. Blank controls were prepared accordingly by adding 100 µl of methanol or acetone to sediment treatments.

To determine the actual concentration of halogenated proxies in spiked sediments, sediment aliquots were extracted. Briefly, 5 g of spiked sediment were centrifuged at 3900 x g for 10 min to discard the pore water containing dissolved proxies. Sediments were extracted with 3

ml of dichloromethane for 1 h on a rotor shaker. After centrifugation the supernatant was collected. The extraction was repeated and pooled supernatants were concentrated by rotor evaporation, followed by solid phase extraction on silica cartridges. The dichloromethane eluate was reduced to 100 µl and used for quantification of halogenated proxies by GC-MS as described above.

Bioassays

1. Direct effect of halogenated proxies on larval settlement

In a replicated single-choice assay (n = 2), the halogenated proxies 1-chlorononane and 2,6-dibromophenole were assayed at both spiking concentrations together with natural sediment (positive control), ashed and autoclaved sediments (negative controls), and the solvent treatments (blank controls). The same sediment treatments were investigated in independent multiple choice assays with replication (n = 3) in different combinations of sediment treatments, testing the larval choice between the negative control, the blank control and one halogenated proxy at both concentrations. Alternatively, both halogenated proxies were assayed at 10x concentration together with the positive and negative control.

2. Indirect effect of adult worms and halogenated proxies on larval settlement

Aliquots of 2.5 g (wet weight) natural sediment were populated with 20 adult worms of either species. In parallel, natural sediment was spiked with halogenated proxies at 10x concentration. The sediment treatments were transferred into multiple choice test chambers together with natural sediment and incubated for 7 days in an aerated aquarium filled with natural seawater (32 ppt, 16° C) under a 12 h dark: 12 h light photo cycle. After 7 d, test chambers were carefully retrieved and the seawater in wells was removed. The remaining test wells were filled with sterile sediment and bioassays were carried out as described above. Aliquots of 1 g of each sediment treatment were sampled to determine the

concentration of halogenated proxies at the beginning and the end of the experiment and for molecular biological analyses.

Bacterial community analysis in sediment treatments

To extract bacterial community DNA from different sediment treatments aliquots of 1 g sediment were sampled and mixed with 1 ml of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM Na_2-EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0] 1.5 M sodium chloride, 1% cetyltrimethylammonium bromide, 2 % sodium dodecylsulfate [pH 7.5], in sterile 2 ml screw caps and stored at -20°C. Samples were repeatedly frozen in liquid nitrogen and defrosted in warm water (65°C). Bacterial cells were enzymatically lysed by addition of 20 µl proteinase K (20 mg ml^{-1}) and incubation for 30 min at 37°C under gentle shaking. Subsequently, 100 µl of sterile filtered sodium dodecylsulfate (20 %) was added and the samples were incubated for 2 h at 65°C. After centrifugation the supernatants were mixed with equal volume of chloroform-isoamyl alcohol (24:1, v/v) for 15 min. The DNA in the aqueous phase was precipitated by addition of 0.6 volumes of isopropanol and storage at 4°C over night. Crude DNA was pelleted by centrifugation washed twice with cold 70 % ethanol and dissolved in 50 µl of sterile deionized water. Aliquots of crude community DNA were stored at -20°C until usage.

16S-rRNA genes in bacterial community DNA were amplified by polymerase chain reaction (PCR) in a total volume of 50 µl containing 1 µl of DNA template, 20.5 µl sterile deionized water, 5 µl PCR-buffer, 7.5 µl of each dNTP, 7.5 µl bovine serum albumin (BSA), 5 µl $MgCl_2$ (20 mM), and 2 µl of each universal primer: 341F (5´-ATT ACC GCG GCT GCT GG-´3) and 534R_GC (5´- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG-3´) (Muyzer et al. 1993), and 0.5 µl taq polymerase (peqlab, Germany). After the initial denaturing cycle at 95°C for 3 min, samples were amplified with a touchdown PCR protocol of 2 cycles at 94°C for 30 s, 64°C for 30 s and 72°C for 1 min and 9 cycles of 94°C for 30 s, 63 °C – 1°C per cycle for 30s and 72°C for 1 min and 25 cycles of

94°C for 30 s, 54°C for 30 s and 72°C for 1 min, followed by a final extension step at 72°C for 10 min. A negative control containing the PCR master mix without template DNA was run in parallel. After the amplification 4 µl of each PCR amplicon was electrophoresed along with a 1000 bps ladder and a positive control prepared from 7 bacterial isolates and a negative control on a 1.0 % agarose gel in 1x TAE at 70V for 30 min. The resulting PCR-product of 193 bps was stained with ethidium bromide.

For denaturant gel electrophoresis (DGGE) 30 µl of PCR product were loaded on a 6.5% [w/v] polyacrylamide gel. An optimal separation was achieved with a gradient of 40 to 75% denaturant from top to bottom (100% denaturant was defined as 7M urea and 40% [v/v] formamide. DGGE was performed with a PhorU-System (Ingeny, Netherlands) in 1 x TAE running buffer at 60°C for 18 h at 100V. Gels were stained for 30 min in SybrGold nucleic acid stain (Molecular Probes, Netherlands) according to the manufacturers protocol.

The gel banding patterns of different samples were subject to cluster analysis. Bray Curtis similarities were used to produce a similarity matrix based on the total number of bands found in all samples and the presence or absence of these bands in individual samples. For the construction of a dendrogram depicting the similarity of microbial communities in different samples group average linkage and hierarchical agglomerative clustering was performed using the PRIMER v. 5 computer program (Plymouth Marine Laboratory, UK).

Statistical analysis

In single-choice assays the rates of larval settlement were expressed in percentage and tested for normal distribution (Shapiro-Wilk's W-test). Since settlement data were not normally distributed they were rank-transformed prior to further statistical analyses. After rank transformation, settlement data were analyzed by one-way ANOVA followed by Tukey's multiple comparison Test (Conover & Iman 1980).

In multiple-choice assays the number of counted larvae was log(x+1) transformed. Levene's test was employed to check the assumption of homogeneity. Where significant heterogeneity

of variance could not be removed by transformation, a lower significance level ($p = 0.01$ instead of $p = 0.05$) was used (Underwood 1997). Fixed factor effects of row, column, and treatment on larval settlement were analyzed by main-effect ANOVA. If the treatment effect was significant, Tukey's multiple comparison test was used to identify differences.

RESULTS

Identification and quantification of halometabolites in tissue extracts

The chemical analysis of *Streblospio benedicti* tissue revealed 3 chlorinated and 1 brominated compound (Table 1). Two unknown isomers of chlorononane were identified according to characteristic diagnostic ions and mass spectroscopic fragmentation patterns and calibrated with 1-chlorononane.

Table. 1: GC-MS analysis of tissue extracts of adult Streblospio benedicti and Capitella sp. I. Where possible compounds and formulas were identified by comparison with the mass spectral database (NIST 05), otherwise detected compounds are labelled as unidentified halogenated hydrocarbons. Characteristic ions list dominant mass fragments. The concentration of identified compounds is given in ng mm^{-3} for selected compounds. (nd = not determined)

Compounds	Characteristic ions	Formula	Retention time (min)	Concentration (ng mm^{-3})
Streblospio benedicti				
dichlorinated hydro-carbon	91, 93, 119, 121	$C_nH_nCl_2$	8,48	nd
brominated hydrocarbon	135, 137, 149, 151	C_nH_nBr	10,75	nd
unidentified chlorononane isomer	91, 93, 119, 121, 147, 149	$C_9H_{19}Cl_1$	11,36	19.59 ± 7.7
unidentified chlorononane isomer	91, 93, 119, 121, 147, 149	$C_9H_{19}Cl_1$	14,22	50.98 ± 8.6
Capitella sp.I				
2,6-dibromophenol	162, 164, 250, 252, 254	$C_6H_4Br_2O$	15,01	18.11 ± 4.5
2,6-dibromo-4-methyl-phenol	263, 265, 267, 252	$C_7H_6Br_2O$	17,79	2.6 ± 1.2
2,4-dibromo-6-(bromomethyl)phenol	215, 217, 263, 265, 267	$C_7H_5Br_3O$	25,87	nd

The amount of 70.57 ± 0.08 ng chlorononane mm^{-3} tissue volume was used to adjust the concentration of the halogenated proxy compound 1-chlorononane. The amount of 20.71 ± 1.65 ng dibrominated phenolic compounds mm^{-3} tissue volume was used to adjust the concentration of the halogenated proxy 2,6-dibromophenole.

Quantification of halogenated proxies in spiked sediment treatments

The recovery yields of halogenated proxies differed significantly with regard to the compound and to the residence time of compounds in sediments (Table 2). Whilst 1-chlorononane could be recovered to 50-60 % immediately after the spiking procedure the concentration was below the detection limit of 10 µg ml^{-1} after 7 d of incubation in both natural and sterile sediment. The recovery yield of 2,6-dibromophenole was below 10% after immediate extraction and could not be detected in aged sediment treatments. The background concentration of 1-chlorononane and 2,6-dibromophenole in natural and sterilized sediment was below the detection limit.

Table 2. Mean recovery yields and concentrations of 1-chlorononane (CLN) and 2,6-dibromophenole (DBP) measured after extraction and GC-MS analysis in different sediment treatments after zero and seven days of incubation. Natural and sterile sediment was spiked with 5.64 µg CLN and 1.65 µg DBP g^{-1} sediment.

Incubation (days)	Treatment	Mean recovery yield (%)	Concentration (µg g^{-1} sediment)
0	natural + CLN	52.06 ± 10.15	2.94 ± 0.70
	sterile + CLN	62.04 ± 10.64	3.499 ± 0.73
7	natural + CLN	0	0
	sterile + CLN	0	0
0	natural + DBP	2.75 ± 1.95	0.045 ± 0.023
	sterile + DBP	11.93 ± 7.71	0.196 ± 0.14
7	natural + DBP	0	0
	sterile + DBP	0	0
0	natural (control)	0	0
	sterile (control)	0	0
7	natural (control)	0	0
	sterile (control)	0	0

Bioassays

1. Direct effect of halogenated proxies on larval settlement

In both experimental repeats of the single-choice assay, no statistical differences in percentage larval settlement were observed among sediment treatments spiked with both halogenated proxies regardless of the concentration used. These treatments evoked the same settlement response as the positive control of natural sediment. Only the negative control of ashed sediment significantly reduced larval settlement (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.05$). The organic solvent controls did not interfere with larval settlement (Figure 1).

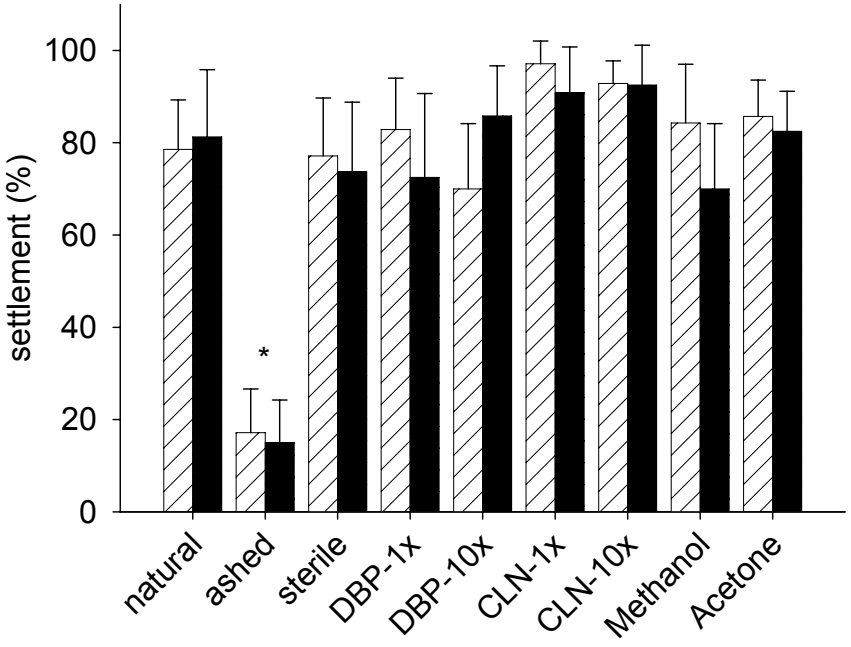
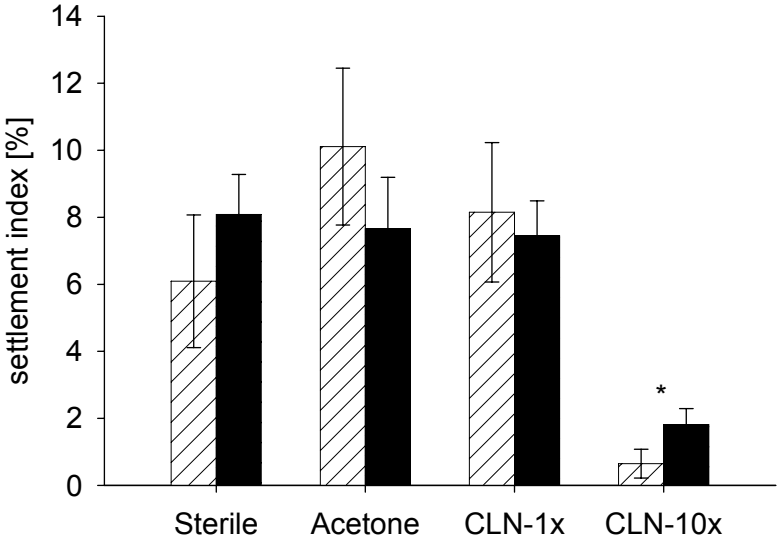


Fig. 1: Percentage of larval settlement of *Streblospio benedicti* of two experimental repeats of single choice assays (hatched and black bars) testing the direct effect of sterile sediments spiked with 1-chlorononane (CLN) or 2,6-dibromophenole (DBP) at 10x and 1x concentrations. Controls were natural, sterile, and ashed sediment and the solvent controls of acetone and methanol. Statistical differences are indicated by asterisks above bars (α = 0.05, Tukey's test). Data plotted are means ± standard deviation.

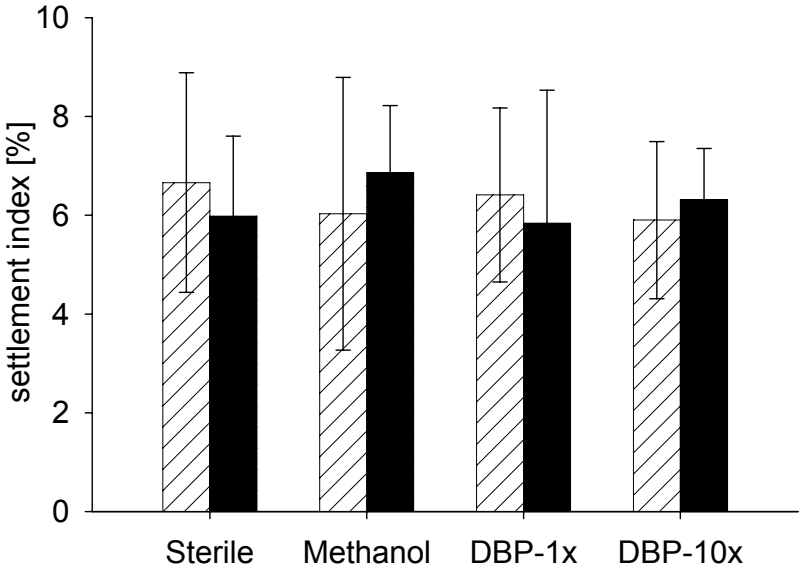
A different result of a direct influence of halogenated proxies on larval settlement was observed when spiked sediment treatments were investigated in multiple choice assays. In all three experimental repeats, larvae significantly rejected the 1-chlorononane treatment at 10x concentration compared to the 1x concentrated treatment, the solvent control and the negative control of sterile sediment (1-way ANOVA, $p < 0.05$; Tukey's test, $p < 0.001$, Figure 2).



Replicate 1				Replicate 2				Treatment
11	30	25	1	67	76	57	15	sterile
1	20	20	15	12	58	72	75	sterile Acetone
20	3	15	18	63	13	65	63	ster. CLN-1x
25	15	1	10	47	56	21	82	ster. CLN-10x
Σ 230				Σ 842				

Fig. 2: Larval settlement index (%) of *Streblospio benedicti* of two experimental repeats (black and hatched) of multiple choice assays testing the direct effect of sterile sediment spiked with 1-chlorononane (CLN) at 1x and 10x concentration together with a solvent control (acetone) and a negative control of sterile sediment. Statistical differences are indicated by asterisks above bars (α = 0.05, Tukey's test). Data plotted are means ± standard deviation. The table depicts the assay design and the total and individual number of settled larvae in individual wells.

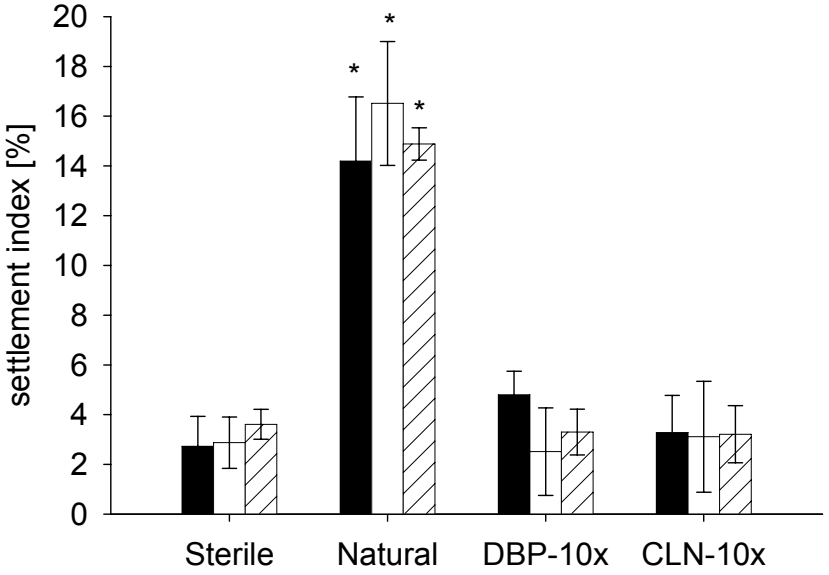
A similar experiment testing the effect of 2,6-dibromophenole at 10x and 1x concentration did not reveal any differences among spiked sediment treatments, solvent control and the negative of sterile sediment (ANOVA, p = 0.977, Figure 3).



Replicate 1				Replicate 2				Treatment
21	56	39	52	29	29	19	28	sterile
35	67	73	47	22	15	22	36	sterile MeOH
46	38	55	40	17	19	24	30	ster. DBP-1x
22	71	62	50	20	14	24	20	ster. DBP-10x
Σ 774				Σ 368				

Fig. 3: Larval settlement index (%) of *Streblospio benedicti* of two experimental repeats (black and hatched) of multiple choice assays testing the direct effect of sterile sediment spiked with 2,6-dibromophenole (DBP) at 1x and 10x concentration together with a solvent control (methanol) and a negative control of sterile sediment. Data plotted are means ± standard deviation. The table depicts the assay design and the total and individual number of settled larvae in individual wells.

When both halogenated proxies were assayed at 10x concentration together with the positive control of natural sediment and a negative control of sterile sediment, larvae significantly preferred natural sediment in three experimental repeats (1-way ANOVA, $p < 0.05$; $p < 0.01$, Tukey's test, Figure 4).



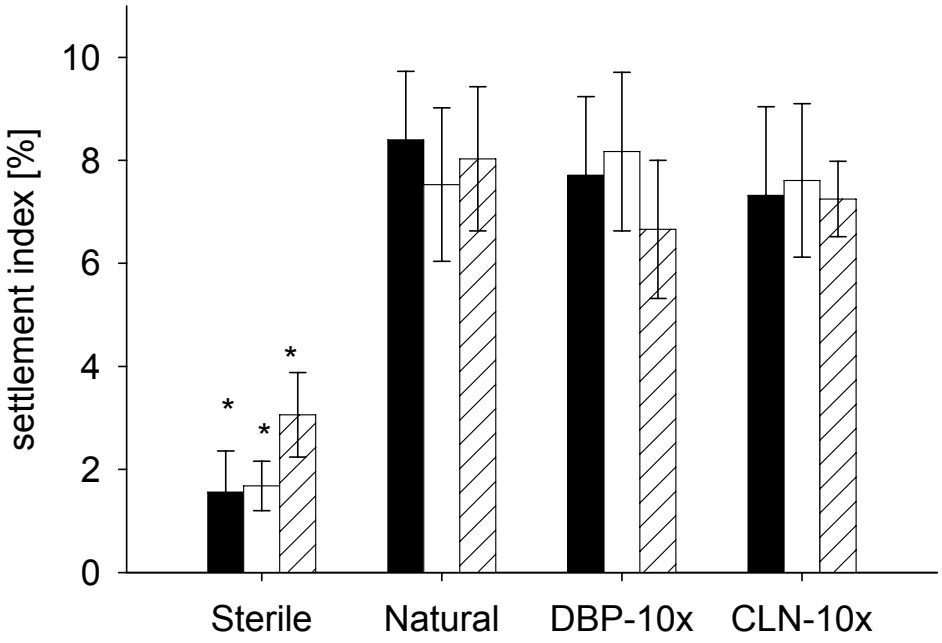
Replicate 1				Replicate 2				Replicate 3				Treatment
12	11	5	38	5	1	6	38	14	14	21	88	sterile
26	11	9	8	27	10	5	3	87	26	15	17	natural
3	29	8	7	8	35	5	8	19	25	19	28	ster DBP-10x
3	9	37	13	12	7	38	1	16	80	83	16	ster. CLN-10x
Σ 229				Σ 209				Σ 568				

Fig. 4: Larval settlement index (%) of *Streblospio benedicti* of three experimental repeats (black, white, hatched) of multiple choice assays testing the direct effect of sterile sediment spiked with 2,6-dibromophenole (DBP) and 1-chlorononane (CLN) at 10x concentration together with a positive control of natural sediment and a negative control of sterile sediment. Statistical differences are indicated by asterisks above bars (α = 0.05, Tukey's test). Data plotted are means ± standard deviation. The table depicts the assay design and the total and individual number of settled larvae in individual wells.

2. Indirect effect of adult worms and halogenated proxies on larval settlement

In 3 experimental repeats of the multiple-choice assay larval settlement in natural sediment incubated for 7 days with halogenated proxies at 10x concentration was the same as in the positive control of natural sediment. Only the negative control of sterile sediment evoked a

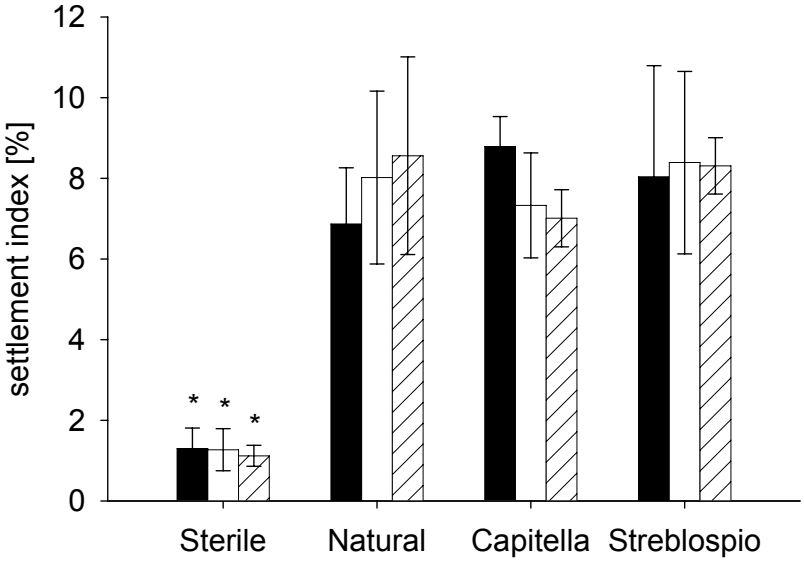
lower settlement index than the other treatments (1-way ANOVA, $p < 0.05$; $p < 0.01$, Tukey's test, Figure 5).



Replicate 1				Replicate 2				Replicate 3				Treatment
45	40	50	11	30	27	30	6	31	38	38	17	sterile
5	28	27	42	4	28	22	22	20	36	33	37	natural
34	4	41	35	23	4	19	28	38	16	43	42	nat. DBP-10x
48	46	12	44	18	19	7	25	36	52	10	27	nat. CLN-10x
Σ 512				Σ 312				Σ 514				

Fig. 5: Larval settlement index (%) of *Streblospio benedicti* of three experimental repeats (black, white, hatched) of multiple choice assays testing the indirect effect of natural sediment spiked with 2,6-dibromophenole (DBP) and 1-chlorononane (CLN) at 10x concentration and incubated in natural seawater for 7 days prior to the settlement assay. The positive control was virgin natural sediment, the negative control was sterile sediment. Statistical differences are indicated by asterisks above bars (α = 0.05, Tukey's test). Data plotted are means ± standard deviation. The table depicts the assay design and the total and individual number of settled larvae in individual wells.

A similar result was obtained after natural sediment was populated with adult specimen of *Streblospio benedicti* and *Capitella* sp. I for 7 days. In 3 experimental repeats of the multiple-choice assay larval settlement was statistically the same in natural sediment and in populated sediments. Only the negative control of sterile sediment evoked a lower settlement index than the other treatments (1 way-ANOVA, $p < 0.001$, Tukey's test $p < 0.001$, Figure 6).



Replicate 1				Replicate 2				Replicate 3				Treatment
37	47	35	4	46	36	31	4	58	43	46	9	sterile
7	39	32	29	7	49	34	39	6	59	51	84	natural
25	7	33	20	30	9	39	42	65	10	54	55	nat. Capitella
31	22	3	33	27	52	4	25	51	49	7	66	nat. Streblosp.
Σ 404				Σ 474				Σ 713				

Fig. 6: Larval settlement index (%) of *Streblospio benedicti* of three experimental repeats (black, white, hatched) of multiple choice assays testing the indirect effect of natural sediment populated with live specimen of *Streblospio benedicti* and *Capitella* sp.I, followed by 7 days of incubation in natural seawater. The positive control was virgin natural sediment, the negative control was sterile sediment. Statistical differences are indicated by asterisks above bars (α = 0.05, Tukey's test). Data plotted are means ± standard deviation. The table depicts the assay design and the total and individual number of settled larvae in individual wells.

Bacterial community analysis in natural, spiked and sediments populated with adult worms

The one week incubation experiments served to provide virgin and aged sediment samples (n = 3) of natural sediment, natural sediment spiked with halogenated proxies, and sediment populated with adult worms of both species. Two sediments populated with *Streblospio benedicti* were lost during the experiment. PCR amplicons obtained from these samples were developed on a single denaturant gradient gel. The bacterial communities obtained from spiked sediment treatments were characterized by the absence of bands in comparison to the control of natural sediment, which supposedly reflected a community of chemically unaffected bacteria. In sediments populated with worms additional bands were observed in comparison to the control of natural sediment indicating a contamination of sediments with bacterial phylotypes originating from worms. These additional bands were omitted in the analysis of bacterial community patterns. Under the combination of PCR primers the number of discernible gel bands obtained from sediment treatments and controls ranged from 14 to 23. In total, 40 bands were used to create a similarity matrix based on Boolean character sets (1 or 0) corresponding to the presence or absence of a given band in a gel lane. The analysis of similarity (ANOSIM) of given bands per treatment produced a global R-value of 0.822 at the significance level of 0.1 %. The pairwise comparisons of bacterial community richness in different sediment treatments resulted in no differences among all virgin sediment treatments at day 0. Contrary, all pairwise comparisons of virgin and aged sediment treatments were significantly different, with the exception of the pair 2,6-dibromophenole at day 0 and natural sediment populated with live *S. benedicti* for 7 days. Whilst bacterial community richness in sediments populated with live worms for 7 days was identical, it differed in the corresponding sediment treatments spiked with species-specific halogenated proxies for 7 days (Table 3).

Table 3. Analysis of similarity (ANOSIM) of DGGE-patterns of bacterial community DNA (n = 3) in natural sediment at day 0 and 7 (N-0, N-7) natural sediment spiked with 1-chlorononane at day 0 and 7 (CLN-0, CLN-7), natural sediment spiked with 2,6-dibromophenole at day 0 and 7 (DBP-0, DBP-7) and natural sediment populated with adults of *Capitella* sp. I or *Streblospipo benedicti* after 7 days (Cap-7, Streb-7). Calculated R-values stem from the analysis of present and absent gel bands per treatment. The global R-value was 0.822. The asterisk marks statistical significance (α = 0.05).

	N-0	N-7	DBP-0	DBP-7	CLN-0	CLN-7	Cap-7	Streb-7
N-0	--							
N-7	0.833*	--						
DBP-0	0.741	1.0*	--					
DBP-7	1.0*	1.0*	1.0*	--				
CLN-0	0.426	0.889*	0.37	1.0*	--			
CLN-7	1.0*	1.0*	1.0*	1.0*	1.0*	--		
Cap-7	1.0*	1.0*	1.0*	1.0*	1.0*	1.0*	--	
Streb-7	0.833*	0.611	0.556	0.889*	0.889*	0.889*	0.352	--

The cluster analysis of bacterial community richness associated with different sediment treatments and controls resulted in 4 subclusters (Figure 7). The bacterial community richness associated with replicate sediment treatments revealed high similarities (data not shown).

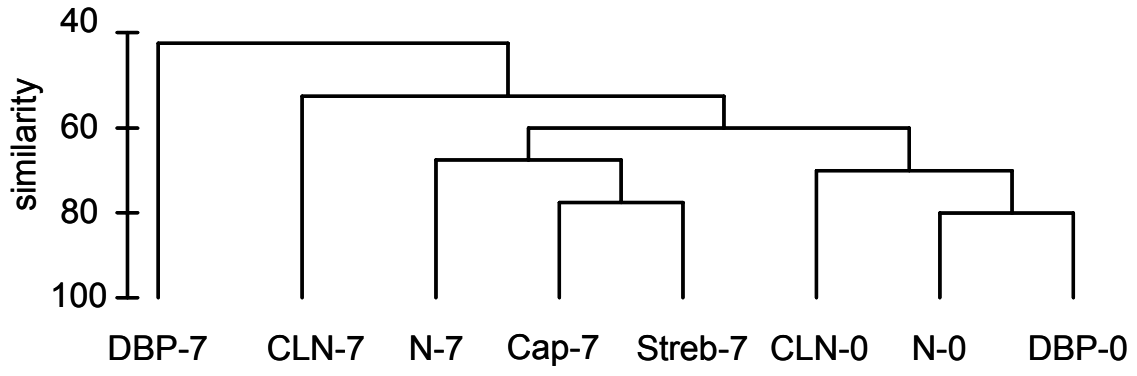


Fig. 7: Dendrogram showing the relatedness of sediment-associated bacterial communities in virgin natural sediment at day 0 and 7 (N-0, N-7), natural sediment spiked with 1-chlorononane (10x) at day 0 and 7 (CLN-0, CLN-7), natural sediment spiked with 2,6-dibromophenole (10x) at day 0 and 7 (DBP-0, DBP-7) and natural sediment populated with adults (n = 20) of *Capitella* sp. I and *S. benedicti* for 7 days (Cap-7, Streb-7).

DISCUSSION

Surface-associated microorganisms have been demonstrated to act as local signposts and larval settlement cues in a wide variety of benthic invertebrate taxa settling on hard substrata (Pawlik 1992, Hadfield et al. 1994; Harder et al. 2002). Previously we studied whether a similar concept might also operate in infaunal organisms guiding burrowing behavior, metamorphosis and settlement. Utilizing the spionid polychaete *Streblospio benedicti* we demonstrated that competent larvae, whilst significantly rejecting experimental sediment lacking organic carbon, were stimulated to settle if this sediment was re-infected with viable microorganisms dissociated from natural sediment (Sebesvari et al. 2006). Considering that a variety of infaunal polychaetes produce and release halogenated metabolites into the surrounding sediment (Woodin et al. 1987; Steward et al. 1992; Fielman et al. 1999) we tested the hypothesis that polychaete-derived halometabolites may either pose a direct negative effect on larvae or alter the bacterial richness in surface sediments of densely populated sediments, and thus indirectly influence larval settlement. Such an indirect interaction would represent a hitherto unrecognized role of biogenic halometabolites in inter- and intraspecific competition during recruitment.

To investigate this hypothesis we raised planktotrophic larvae of *Streblospio benedicti* in the laboratory and tested their settlement response in single- and multiple choice assays of experimental sediments spiked with halogenated compounds. Halogenated compounds for spiking experiments were selected based on their occurrence and predominance in adult specimen of *S. benedicti* and *Capitella* sp. I. The total tissue concentrations of unidentified chlorononane isomers and dibrominated phenols in adult *S. benedicti* and *Capitella* sp. I obtained from our laboratory cultures was 70.57 ± 0.08 and 20.71 ± 1.65 ng mm^{-3}, respectively. These values fall into the same order of magnitude of total halogenated metabolites determined in adult *S. benedicti* obtained from intertidal mudflats in South Carolina (USA) and laboratory cultures of *Capitella* sp. I (Cowart et al. 2000). For the setup of experimental sediments the content of dominant, partially unidentified halometabolites was

mimicked with commercially available compounds, such as 1-chlorononane as a proxy for chlorononane isomers and 2,6-dibromophenole as a proxy for dibrominated phenoles.

For bioassays the concentrations of halogenated proxies in spiked experimental sediment aliquots of 2.5 g and 2 cm^2 surface area were adjusted equivalent to the total concentration of halometabolites present in 20 adult worms. This calculation overestimated the actual population density of both species at the sampling site (ca. 5000 individuals m^{-2}) roughly twenty fold. However, the resulting sediment concentration of 0.165 µg g^{-1} 2,6-dibromophenole was well within the range reported for a relatively large bromophenole producing polychaete, *Notomastus lobatus* (Steward et al. 1992, Lovell et al. 1999). To the best of our knowledge, comparable quantifications of biogenic chlorinated hydrocarbons in surface sediments do not exist in the literature. Given the small size of our study organisms, we did not determine the concentrations of halometabolites in burrow linings and in close proximity. We purposely mimicked a homogenous distribution of the supposedly high concentration of halometabolites present in direct proximity to worm burrows in the entire experimental sediment in order to test the effect of halometabolites on sediment-associated bacteria in the topmost sediment layer. The rationale for this setup was based on our previous observations that competent polychaete larvae repeatedly touched the sediment surface with their ventral body parts prior to burrowing and settlement (Sebesvari et al. 2006).

To verify to which extent spiked halogenated proxies were bound to particulate organic matter in sediments and thus bioavailable to bacterial biofilms in the sediment, halogenated proxies in sediments were extracted and quantified. The recovery yields were surprisingly low and differed significantly between the two compounds, indicating that either the extraction efficiency was low, as previously demonstrated by Steward et al. (1992), and/or that a large proportion of spiked proxies did not adsorb to the sediment. In order to compensate for potential losses, the spiking concentration of halogenated proxies in multiple choice-assays was elevated ten fold resulting in final recovery yields of ca. 3 µg g^{-1} 1-

chlorononane and 0.05 - 0.2 µg g^{-1} 2,6-dibromophenole (Table 2). Given these concentrations of halogenated proxies in experimental sediment treatments the hypotheses raised in this study were answered as follows:

Our first hypothesis of a direct influence of halogenated proxies on larval settlement of *Streblospio benedicti* strongly depended on the assay design. In the single-choice assay settlement in sediment spiked with halogenated proxies was the same as in controls of natural and sterile sediment (Figure 1). However, when larvae were given a choice between experimental treatments spiked with 1-chlorononane at different concentrations, there was a clear direct inhibitory effect of the high spiking concentration on larval settlement (Figure 2). A similar effect was not observed with the other halogenated proxy 2,6-dibromophenole (Figure 3). When larvae were offered both proxies at high concentrations together with the positive alternative of untreated natural sediment, the spiked treatments were significantly rejected (Figure 4). Given that larvae inspect the sediment surface repeatedly on small spatial scales of several cm^2 (Sebesvari et al. 2006; personal observation) these results indicate a potential direct effect of halometabolites on larval settlement provided that surface properties differ within the investigated sediment patch. The single-choice assay seemed inappropriate to resolve these preferences because larvae may loose their selectivity and comply with a moderately acceptable sediment quality after repeated exposure to the same negative stimulus.

Our second hypothesis of an indirect influence of halogenated proxies on larval settlement was clearly rejected. In 3 experimental repeats of a multiple-choice assay testing the effect of 7 day incubations of natural sediments spiked with halogenated proxies larval settlement was the same as in the positive control of natural sediment (Figure 5). A direct effect of halogenated proxies on larval settlement as outlined above could be ruled out since the recovery yields for these compounds were below the detection limit after 7 days of incubation, indicating a complete microbial turnover of halogenated compounds (Table 2). When live specimen were incubated in natural sediment for the same duration, allowing the

sediment to be contaminated with biogenic halometabolites, there was no difference in larval settlement between sediments populated with live worms and the unpopulated positive control of natural sediment (Figure 6). These results suggested that a.) the overall inductive effect of viable bacteria on larval settlement as observed in the control treatments of natural sediment was unchanged in the presence or after the utilization of halometabolites, and b.) that potential changes in bacterial abundance and/or community richness as a consequence of long term exposure to halometabolites or microbial utilization did not change the suitability of these sediments for settling larvae.

In order to evaluate the potential changes in bacterial community richness between virgin and the corresponding aged sediment treatments, the banding patterns obtained by denaturant gradient gel electrophoresis (DGGE) were subject to statistical similarity analysis. Since DGGE of PCR amplicons does not provide quantitative information of the bacterial community the similarity analysis was based on the presence and absence of bacterial phylotypes (Muyzer et al. 1993, Murray et al. 1996, Rochelle 2001). The analysis of similarity revealed a significant change in bacterial community richness between virgin and the corresponding aged sediment treatments. In the treatment of natural sediment out of 21 discernible gel bands at day 0, four bacterial phylotypes were absent after 7 days of incubation in natural seawater. These phylotypes were likely eliminated due to the lack of nutrients or bacterial dynamics in complex microbial assemblages (Costerton et al. 1995). Two of the phylotypes eliminated in aged natural sediment were also absent in aged sediment treatments spiked with halogenated proxies. Moreover, four identical bacterial phylotypes were eliminated in these sediment treatments. In addition, nine bacterial phylotypes were eliminated in the aged sediment treatment spiked with 2,6-dibromophenole. These results indicated a pronounced limiting effect of 2,6-dibromophenole on the bacterial community richness present in natural sediment, supporting the earlier notion of strong bactericidal effects of bromometabolites (King 1986). Interestingly, 11 and 4 additional phylotypes were observed in aged sediment treatments spiked with 1-chlorononane and 2,6-

dibromophenole, respectively, in comparison to natural sediment. Whilst these bacterial types were presumably present already in natural sediment, their lack of detection may be explained with their low abundance in untreated sediment. In order to profile a bacterial constituent within a complex population by DGGE analysis of PCR-amplified genes coding for 16S rRNA the minimum abundance must exceed 1 % of the total population (Muyzer et al. 1993). It is possible that the additional phylotypes observed in sediment incubations treated with halogenated proxies increased in abundance due to the utilization of these compounds or metabolic byproducts of other bacteria within the population capable to remediate halogenated compounds. Contrary to these findings, there was no bactericidal effect of halometabolites released by live worms in the populated sediment treatments. The bacterial community richness in these samples was the same as in the aged treatment of natural sediment.

Studies employing intact sediment cores (Steward et al. 1992, Steward & Lovell 1997, Lovell et al. 1999) or examining burrow linings that were not homogenized or incubated with a halogenated substrate (Alongi 1985, Jensen et al. 1992, Steward et al. 1996) have shown no inhibition of sediment microbiota by infaunal brominated metabolites. The accumulation of biogenic bromophenoles in wormbeds has been suggested to be limited by bacterial remediation (Steward & Lovell 1997). Our study complies with the latter suggestion, since recovery yields of halogenated compounds after short incubation times were low or no longer detectable. However, this study clearly demonstrates that halogenated compounds reduce the bacterial community richness in surface sediments and can be utilized by sediment associated bacteria. Moreover, our results suggest a significantly more pronounced limiting effect of biogenic bromophenoles than chlorinated hydrocarbons. The inhibition of sediment microflora in proximity to worm burrows and linings has been suggested to restrict the availability of microbially derived sources for carbon and nitrogen for worms (Newell 1965, Cammen 1980), alter sediment texture by reduced production of exopolymers and thus change sediment biogeochemical processes (Decho 2000). However in this study, the

potential modified sediment quality as a result of a shift in bacterial community richness was not recognized by larvae of the polychaete *Streblospio benedicti*, indicating that biogenic halometabolites did not serve as indirect larval settlement cues in this species.

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