Sediment-associated cues for larval settlement of *Polydora cornuta* and *Streblospio benedicti* (Polychaeta, Spionidae)

Sediment-assoziierte Signale für die larvale Ansiedlung von *Polydora cornuta* und *Streblospio benedicti* (Polychaeta, Spionidae)

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The most exciting phrase to hear in science,
the one that heralds new discoveries,
is not “Eureka!” but “That’s funny... ”

Isaac Asimov

ERKLÄRUNG

VERÖFFENTLICHUNGEN

Teilergebnisse dieser Arbeit sind als Beitrage in Fachzeitschriften erschienen (Kapitel 3), als Manuskript eingereicht worden (Kapitel 4) oder in der Diplomarbeit von Justus Lodemann verwendet worden (Teile von Kapitel 3). Mein Beitrag an der Erstellung der Arbeiten wird im Folgenden erläutert:

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SEBESVARI, Z., ETTWIG, K.F., EMONS, H. 2005. Biomonitoring of tin and arsenic in different compartments of a limnic ecosystem with emphasis on *Corbicula fluminea* and *Dikerogammarus villosus*. J. Environ. Mon. 7, 203-207.

EMONS, H., SEBESVARI, Z., FALK, K., KRACHLER, M. 2004. Occurrence and speciation of arsenic, antimony and tin in specimens used for environmental biomonitoring of limnic ecosystems. In: Hirner, A.V., Emons, H. (eds.) Organic metal and metalloid species in the environment: Analysis, distribution, processeses and toxicological evaluation. Springer, Berlin, Heidelberg, New York.

AZAIZEH, H.A., SALHANI, N., SEBESVARI, Z., EMONS, H. 2003. The potential of rhizosphere microbes isolated from a constructed wetland to biomethylate selenium. J. Environ. Qual. 32, 55-62.

ABSTRACT

Patchy distribution of infaunal polychaetes may result from active site selection of larvae guided by sediment associated microbial cues. This hypothesis was tested in still water laboratory settlement assays with and without choice option for larvae of two spionid polychaetes with indirect development: *Polydora cornuta* and *Streblospio benedicti*. Laboratory brood cultures of these spionids were established and yielded a sufficient number of larvae with planktotrophic development for bioassays. Larvae were obviously able to accept or reject attractive and unattractive sediment qualities in no-choice assays in a species specific manner. In both species larvae displayed high settlement (75 to 95 %) in response to natural sediment and significantly low settlement (5 to 50 %) in ashed sediments, whilst in case of *P. cornuta* significantly low settlement (25 to 55 %) resulted in sterile sediment. Low settlement in 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 a mixture of viable microorganisms detached from natural sediment of adult habitats. The presence of viable microorganisms 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. Subsequently, 15 bacterial isolates obtained from natural sediment of adult habitats belonging to 5 phylogenetic classes i.e. *α-Proteobacteria* (4), *γ-Proteobacteria* (3), *Bacillales* (3), *Flavobacteria* (3) and *Sphingobacteria* (2), were screened for their ability to induce larval settlement in *P. cornuta* and *S. benedicti*. Recolonization of ashed sediment with bacteria resulted in low bacterial cell densities (< 10^5 cells g^{-1} sediment). At these densities 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 sediment. In no-choice assays two out of 15 isolates, i.e. *Loktanella* sp. DF11 strain (*α-Proteobacteria*, *Roseobacter*-clade) and strain 54 (*Flavobacteria,* closest match at GenBank *Psychroflexus tropicus*) significantly triggered settlement of *P. cornuta* larvae. There was no correlation between the

phylogenetic affiliation of bacteria and their effect on larval settlement. Both inductive isolates and the non-inductive isolate *Rhodobacteraceae bacterium* strain DF16 (*α-Proteobacteria*, *Roseobacter* clade) were further tested in multiple-choice assays using four parallel experimental treatments of sediment, i.e. natural, sterile and two sediment treatments of different quantity and quality of bacteria. 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. 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 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. Furthermore, the settlement of *P. cornuta* is influenced by gregarious behavior and the signal is mainly harboured in sediments formerly inhabited by conspecific adults. In *S. benedicti* gregarious behavior does not influence larval settlement.

ZUSAMMENFASSUNG

Aggregiertes Vorkommen von inbenthischen Polychaeten im Habitat kann durch eine von sediment-assoziierten mikrobiellen Signalen geleitete aktive Substratwahl der Larven erklärt werden. Diese Hypothese wurde durch laborbasierten larvalen Ansiedlungstests für zwei Spioniden mit indirekter Entwicklung, *Polydora cornuta* und *Streblospio benedicti,* getestet. Für die beiden Polychaetenarten wurden Laborkulturen etabliert, um einen ganzjährigen Zugang zu planktonischen Larven zu gewährleisten. In Testreihen ohne Auswahlmöglichkeit waren die Larven offensichtlich in der Lage aktiv eine Siedlungsentscheidung zu treffen. Beide Arten zeigten hohe Siedlungsraten in natürlichem Sediment (zwischen 75% und 95 %) und niedrige im bei 600 ˚C veraschten Sediment (zwischen 5% und 50 %). Im durch Autoklavieren sterilisierten Sediment siedelte *S. benedicti* in gleich hohen Raten wie im natürlichen Sediment, während die Siedlung von *P. cornuta* im sterilen Sediment signifikant niedriger war als im natürlichen Sediment (zwischen 25% und 55 %). Niedrige Siedlungsraten im veraschten bzw. sterilen Sediment können jedoch durch eine Vielzahl von Parametern wie die veränderte Struktur, Korngrößenverteilung und Qualität bzw. Quantität des organischen Materials verursacht werden. Um die potenzielle Rolle der Mikroorganismen und deren Metaboliten in der larvalen Ansiedlung zu untersuchen, wurde veraschtes Sediment mit lebendigen Mikroorganismen beimpft, die durch Abschütteln von natürlichem Sediment aus dem Habitat der adulten Tiere gewonnen wurden. Die Anwesenheit dieser Mikroorganismen führte zu signifikant erhöhten Siedlungsraten der Larven beider Arten im Vergleich zu den unbeimpften Negativkontrollen, ein Hinweis darauf, dass die larvale Ansiedlung dieser Spioniden - zumindest teilweise - von sediment-assoziierten Mikroorganismen geleitet wird. Um Auslöser des positiven Siedlungssignals zu identifizieren, wurden 15 bakterielle Isolate des natürlichen Lebensraumes beider Spioniden auf die Fähigkeit larvale Ansiedlungen zu beeinflussen getestet. Die Isolate gehörten zu 5 phylogenetischen Klassen, namentlich zu *α-Proteobacteria* (4), *γ-Proteobacteria* (3), *Bacillales* (3), *Flavobacteria* (3) und zu *Sphingobacteria* (2). Das Beimpfen von veraschtem Sediment führte regelmäßig zu Zellzahlen von < 10^5 Zellen g^{-1} Sediment. In dieser Zelldichte vermochte keine der 15 Isolate die larvale Ansiedlung zu beeinflussen. Das Beimpfen von sterilem Sediment führte

zu Zellzahlen zwischen 10^7 und 10^8 Zellen g^{-1} Sediment. Damit sind diese Zelldichten etwa um eine Größenordnung niedriger als die natürlicher mariner Sedimente. In larvalen Ansiedlungstests ohne Auswahlmöglichkeit haben zwei der fünfzehn Isolate, DF11 (*α-Proteobacteria*, *Roseobacter*-Stamm) und Isolat 54 (*Flavobacteria*), die Siedlung der Larven beider Arten signifikant gefördert. Die beiden siedlungs-induktiven Isolate und ein nicht-induktives Isolat *Rhodobacteraceae bacterium* strain DF16 (*α-Proteobacteria*, *Roseobacter* clade) wurden mittels Ansiedlungstest mit Vierfachauswahl näher untersucht. Dabei wurden natürliches, steriles sowie zwei mit Isolaten verschiedener Zelldichte oder Qualität beimpftes Sediment angeboten. Die Ansiedlungstests mit Vierfachauswahl haben ein klares Siedlungsmuster beider Arten gezeigt. Im Allgemeinen war natürliches Sediment sehr attraktiv für die larvale Ansiedlung, während steriles Sediment von den Larven signifikant seltener gewählt wurde. Die beiden induktiven Isolate DF11 und 54 haben bei Zelldichten von 2.0 bis 9.0 x 10^8 g^{-1} Sediment gleich hohe Siedlungraten gezeigt wie das natürliche Sediment. DF16 hat die larvale Ansiedlung bei Dichten, die mit diesem Isolat erzielbar waren (2.0 x 10^6 bis 5.0 x 10^7 g^{-1} Sediment) nicht beeinflusst. Durch Hitze abgetötete oder lebendige aber suspendierte Zellen sowie wasserlösliche Metabolite des Isolates DF11 haben die larvale Ansiedlung ebenfalls nicht beeinflusst.

Die Ergebnisse dieser Arbeit weisen darauf hin, dass die Siedlungssignale für die Spioniden *S. benedicti* und *P. cornuta* zumindest teilweise bakterieller Herkunft sind, aber nicht auf eine einzelne bakterielle Gattung zurückzuführen sind. Die signalgebenden chemischen Verbindungen sind hitzelabil und sedimentassoziiert. Desweiteren konnte gezeigt werden, dass die Siedlung von *P. cornuta* durch das Vorhandensein von Individuen der gleichen Art positiv beeinflusst wird. Dabei ist besonders Sediment, in dem bereits vorher adulte *P. cornuta* lebten, attraktiv für die larvale Ansiedlung, während die Adulten selbst und deren leere Röhren keine oder nur geringe Siedlungsinduktion zeigten. Bei der larvalen Ansiedlung von *S. benedicti* scheinen die eigenen Artgenossen keinen nachweisbaren Einfluss zu haben.

CONTENTS

1 GENERAL INTRODUCTION

Many sessile benthic marine invertebrates, such as oysters, barnacles and polychaetes, develop via a pelagic larval phase followed by benthic juvenile and adult existence. Advantages of larval dispersal are genetic exchange between different populations, avoidance of competition for resources with adults, fast (re)colonization of new habitats and increased ability to withstand local extinction (Levin 1984, Pawlik 1992, Pechenik 1999). Possible disadvantages include dispersal away from favorable habitats or conspecifics, greater vulnerability to environmental stresses and predation (Thorson 1950, Pechenik 1999). The early life-history of marine organisms is commonly divided into a series of functional or behavioral stages, such as passive dispersal, development, achievement of competence for settlement and metamorphosis, active substrate exploration, initial settlement, metamorphosis and survival of the newly settled individuals (Rodríguez et al. 1993). This thesis focuses on active larval choice at initial settlement in two spionid polychaetes with indirect development, *Polydora cornuta* and *Streblospio benedicti*. Since there are many inconsistencies in the literature about the relative importance of passive and active dispersal, the existence and role of active larval choice and the terminology of competence, settlement, metamorphosis and recruitment; these issues are briefly summarized in the following.

1.1 DISPERSAL OF PLANKTONIC LARVAE

During the pelagic phase, which lasts in a species-specific pattern from a few hours to several months, invertebrate larvae disperse over various spatial scales. On large scale (< 10s of kilometers) larval dispersion is driven by hydrodynamic processes such as currents, tides and stagnations. Maximal dispersion capacity of larvae is related to the length of their planktonic life and to the rate and direction of prevailing currents (Scheltema 1986). Whereas large scale distribution is commonly accepted to be driven by hydrodynamic processes, larval fate shortly prior to settlement is discussed controversively. The hypotheses that larvae ready to settle sink passively through the water column like "*seeds in the wind*" was formulated very early (e.g. Yonge 1937) and is still discussed in the literature. In a well-balanced study Butman investigated the relationship between small scale hydrodynamic processes and patterns of initial larval settlement (Butman 1989).

She concluded that in most species passive deposition determines where larvae initially encounter the sea bed. However, she pointed out that after encountering the surface active or passive redistribution of larvae takes place. Redistribution after sediment contact may happen through crawling or hopping (Butman 1989), active swimming above the bottom or passive redistribution (Eckman 1979, Palmer 1988). Butman (1989) hypothesized that "*active selection mechanisms probably operate at the time the larva first reaches the bottom, such that the organisms can opt to remain or leave the depositional locale. The larva is not, however, "free to choose" in the sense of freely inspecting all the options*." On this note, active site selection of larvae is commonly observed on small spatial scales (Keough and Downes 1982, Woodin 1986, Butman et al. 1992, Desroy et al. 1997, Harvey and Bourget 1997, Pernet et al. 2003).

1.2 CHARACTER AND PERCEPTION OF SETTLEMENT SIGNALS

A critical phase in the developmental mode via pelagic larvae is the detection of a suitable substratum for subsequent juvenile and/or adult life. 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 (reviewed by Pawlik 1992, Qian 1999, Steinberg et al. 2002). A frequently studied biotic source of cues was biofilms (agglomerates of attached bacteria, benthic diatoms, fungi and protozoa). Biofilms have been shown to elicit a highly specific larval response with respect to their different origin and/or growth phase in hard substrate settlers (ZoBell et al. 1935, Wieczorek and Todd 1997, Qian et al. 2003, Lau et al. 2005). Both, small organic metabolites and macromolecular extracellular polymers have been identified and partially purified as larval settlement cues in major biofilm components, i.e. bacteria (Fitt et al. 1990, Zimmer-Faust and Tamburri 1994, Harder et al. 2002b) bacterial extracellular polymers (EPS) (Kirchman et al. 1982, Maki et al. 1990, Szewzyk et al. 1991, Lau et al. 2003), diatoms (LeTourneux and Bourget 1988, Harder et al. 2002a) and diatom EPS (Lam et al. 2003/2005).

Although in soft sediment habitats less effort was spent to identify the cues influencing larval settlement than in hard substrata (Woodin 1986), several parameters were discussed or identified in this regard, such as the organic content of the sediment (Butman and Grassle 1992, Grassle et al. 1992, Zettler 1996), 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 conspecific juveniles or adults in the habitat (called gregarious settlement) (Highsmith 1982, Olivier et al. 1996, Hardege et al. 1998) and haloaromatic metabolites of sympatric organisms (Woodin et al. 1993, Hardege et al. 1998). Similar to hard substrate settling organisms, the role of microorganisms has been suggested for infaunal organisms (Wilson 1955, Gray 1966, 1967) but only few follow-up works identifying the role of sediment-associated microorganisms on infaunal recruitment patterns have been performed since (Hadl et. al 1970, Hermann 1975 and 1995).

The perception of settlement cues is determined by their nature and chemical properties. Whilst physical parameters sich as sediment grain size or surface texture have to be explored by direct surface contact of larvae chemical cues may also be detected in the water column or in close proximity to surfaces, such as the surface boundary layer (SBL, a few hundred µm thin layer of water near the surface in which the velocity changes from zero at the surface to the free stream value away from the surface). Under flow conditions water-soluble chemical cues are likeky perceived at the SBL at best, where flow-speed is low and chemical substances may accumulate (Pawlik 1992). The perception of water soluble cues above the SBL by the larvae is limited due to fast dilution. Under flow conditions a realistic model of cue perception might thus function by signal detection near the surface accompanied by controlled behavioral surface exploration like crawling, walking (e.g. barnacle cyprids, once attached, use their antennae to walk along the substrate surface), drifting with the water like a "*balloonist*" (passive bedload transport) or a "*ping pong ball*" (active change of the vertical position) (Abelson 1997). Furthermore, Hadfield (2004) reported that larvae of the nudibranch *Phestilla sibogae* rapidly responded to dissolved settlement cues by sinking which enhanced their transport to the substratum, even in wave-driven turbulent flows. In areas or time slots of low flow-velocities water soluble cues may be perceivedin the water column and induce larval settlement (Coon et al. 1990, Highsmith 1982). However, in most cases larvae

have been reported to detect chemical settlement signals through direct contact with the surface or in the SBL (Pawlik 1992, Morse and Morse 1984, Pearce und Scheibling 1990).

1.3 SETTLEMENT, METAMORPHOSIS AND RECRUITMENT

Burke (1983) defined settlement as the termination of the pelagic life and considered it as a behavioral, reversible trait, whereas metamorphosis is a developmental process expressed in morphological and physiological changes, which is irreversible and occurs only once during the life cycle. Unfortunately the distinction between settlement and metamorphosis has not been made this clearly in the relevant literature. Rodríguez et al. (1993) considered settlement as "*the passage from a pelagic way of life to a benthic way of life*" and distinguished two phases of the settlement process: a behavioral phase of exploration for suitable substrate and a phase of permanent residence or attachment to the substrate, which triggers metamorphosis. Although both, settlement and metamorphosis are often interlinked, in some cases they can be triggered independently (Coon et al. 1990). Moreover, in soft sediment habitats Rodriguez's requirement of "*permanent residence*" in the substrate can not applied since individuals often leave the sediment after initial settlement as larvae, post-larvae or juveniles (Woodin 1991, Duchêne 2004). Additionally, many species metamorphose gradually causing difficulties in accurately determing the "event" of metamorphosis in settlement assays. For example, *Pectinaria koreni* (Polychaeta, Pectinariidae) displays an intermediate stage between metatrochophore larvae and juveniles, called aulophore larvae or post-larvae. Post-larvae have lost metatrochophore attributes but miss some juvenile traits (Thiébaut et al. 1998).

The term recruitment is usually defined as the entry into the benthic population and also includes the survival of the individual up to a specific size after settlement (Fraschetti et al. 2003). Recruitment is also 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).

In this thesis settlement assays were carried out to evaluate initial larval settlement. In this regard settlement was considered and defined as a behavioral, repeatable trait.

1.4 POST-SETTLEMENT EVENTS

Once settled and metamorphosed local disturbances, predation or differences in the habitat requirements of juvenile and adults may cause dislocation of juveniles and adults. These post-settlement processes may subsequently change initially established species-specific distribution patterns. The relative importance of pre- and post-settlement processes at the formation of spatial patterns differs between hard and soft-substrates and among species (Stoner 1990, Ólafsson et al. 1994, Fraschetti et al. 2003). Generally, in soft sediment settlers the limitation of mobility is by far not as strict as in hard substrate settlers (Cummings et al. 1995, Whitlatch et al. 1998, Snelgrove et al. 1999b, Sarda et al. 2000, Norkko et al. 2001, Stocks 2002, de Montaudouin et al. 2003, Hernandez Guevara 2004). Studies investigating the ability of juveniles and adults for active settlement choice have been rare so far. In settlement experiments with juveniles instead of larvae Woodin (1993, 1995) observed high selectivity at settlement. Adult polychaetes used by Gray (1967) to test the attractiveness of different sediments displayed active substrate choice.

1.5 OBJECTIVES

The analysis of sediment cores along transects in tidal flats of the Wadden Sea (Germany) revealed distinctive patterns of abundance in spionid polychaetes with larval development (Stamm 2000). The observed patterns were neither correlated with sediment characteristics such as the silt content nor the organic carbon and nitrogen content (Stamm 2000). Therefore, I hypothezised that the distribution patterns may have resulted from active site selection of larvae triggered by factors others than silt, organic carbon and nitrogen content. In particular, I focused on the role of bacteria associated with the sediment surface as mediators in the settlement process.

To test this hypothesis, I chose two spionid species with pelagic larval development, *Polydora cornuta* Bosc, 1802 and *Streblospio benedicti* Webster, 1879 and raised the following questions:

- Do larvae of *P. cornuta* and *S. benedicti* actively accept or reject different sediment types? Is there any behavioral evidence that patchy distribution in the field is the result of larval choice?

To test this hypothesis, I investigated different larval developmental 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 untreated or sterilized, ashed or acid washed sediment. If larvae, as hypothesized, did actively select sediment quality, different preferences would be displayed in the presence of clearly differentiated sediment types (Chapter 3).

➧ Do larvae respond to signals derived from bacteria associated with sediment?

The main emphasis of this approach was to focus on the role of sediment-associated microbial cues as potential triggers of larval settlement in *P. cornuta* and *S. benedicti*. Microorganisms from natural sediment and different bacterial isolates from the habitat of adult polychaetes were reestablished on sterile sediment to test if different bacterial isolates and their abundance on the sediment influenced larval settlement (Chapter 4).

➧ Do larvae detect settlement signals in the water column or on the sediment surface?

The signal triggering larval settlement may be detected by the larva either on the surface or in the overlaying water. A series of screening experiments were performed to localize the settlement cue (Chapter 4).

➧ Does the presence of conspecifics influence settlement of larvae? Do the larvae behave gregariously at settlement?

Species specific patterns may be caused by gregarious larval settlement, i.e. larvae prefer to settle in substrates with already settled conspecifics (Knight-Jones 1953). Therefore the settlement of *P. cornuta* larvae in presence of conspecific adults, juveniles, empty tubes and culture sediment was investigated and compared with larval settlement in sediment inhabited by *S. benedicti* and culture sediment of *S. benedicti,* respectively (Chapter 5).

To investigate larval settlement in spionid polychaetes it was necessary to develop and set up spionid cultures under laboratory conditions, design and compare different settlement bioassays, study the competence-progression of larvae by temporal correlation of larval morphology as well as their ability to settle. These works are outlined in Chapter 2 and 3.

2 GETTING STARTED: SELECTION AND CULTURING OF TEST POLYCHAETES

2.1 SELECTION OF SUITABLE TEST POLYCHAETES

To test larval settlement, the maintenance of laboratory based brood cultures is advantageous. 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 (Jägersten 1940, Wilson 1937, Gray 1967, Cha and Bhaud 2000). Among the polychaetes, special emphasis has been devoted to *Capitella* sp. I, which in turn has become a standard assay species for laboratory studies all over the world (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). Spionids, one of the largest polychaete families, have been barely investigated concerning larval settlement so far. Among spionid polychaetes only *Streblospio benedicti* was used in larval settlement assays. Here, the impact of pollutants (endosulfan and PAHs) on larval of *S. benedicti* was tested (Chandler and Scott 1991, Chandler et al. 1997). Spionids seemed to be suitable organisms to study larval substrate selection, because many of them disperse via planktonic larvae (Rouse and Pleijel 2001) and species specific distribution patterns of spionids commonly occur in the field (e.g. Stamm 2000). Many spionid polychaetes are particularly easy to obtain due to their high abundance in beach and estuarine habitats. They live in burrows or tubes (Rouse and Pleijel 2001) with typical abundances of several thousand individuals per square meter (Zajac 1991). Spionids are recognized by a pair of elongated grooved palps extending from the head. 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).

The following criteria were used to select suitable test species among spionids:

1) occurrence in the Wadden Sea (logistics),

2) development via planktotrophic larvae to ensure natural dispersion potential and ecological relevance of the assay,

3) cultivable adults and larvae in the laboratory, with a realistic purpose of year-round larval supply in the laboratory culture,
4) known and described distribution patterns in the field,
5) worldwide occurrence to ensure adaptability of the assay.

In the North Sea, ca. 60 spionid species belonging to 15 different genera were reported (listed e.g. in the database of the "North Sea Benthos Project 2000"). Initially criteria 2 and 3 were applied to narrow down the number of possible candidates followed by evaluation of criteria 4 and 5.

A member of the *Polydora* group (the species-richest spionid genus in the North Sea), *Polydora cornuta* has been previously cultured in the laboratory by Anger et al. (1986) and Irvine and Martindale (1999). In both studies continuous supply of planktotrophic larvae was achieved at optimal culture conditions. *P. cornuta* is widely abundant in muddy sands of tidal flats in the temperate zone (Hartmann-Schröder 1996) and occurs in the Wadden Sea (Dankers 1981). Stamm (2000) reported distinctive patterns of abundance of *P. cornuta*. Similar observation of patchy distribution of *P. cornuta* was also reported in the Atlantic at Eastern Passage, Halifax, Canada (Snelgrove et al. 1999a).

P. cornuta was collected during low tide in mudflats near Hooksiel (53° 38' 31" N, 8° 04' 55" E, Wadden Sea, Germany) in August 2003. The characteristic vertical living tubes constructed by *P. cornuta* helped to localize adult sampling sites. Suitable laboratory culture methods were developed or adopted from Irvine and Martindale (1999), respectively. Following establishment of the culture by settlement of all newly hatched larvae continuous larval supply was achieved at the beginning of 2004. Detailed information on species characteristics and culture conditions are given in Chapter 2.4.

Streblospio benedicti (Webster 1879), the only spionid already used in larval settlement assays prior to this study (Chandler and Scott 1991, Chandler et al. 1997) was reported to be cultivable in the laboratory (Lewin 1986, Bridgess and Heppell 1996, Chandler et al. 1997). Earlier cultivation attempts of this species were mainly motivated by the intraspecific variation in its developmental mode (poecilogony), which predestines *S. benedicti* for studies of different developmental and maternal effects. Although *S. benedicti* occurs in the Wadden Sea, it is less abundant than *Polydora cornuta*. In the first year of my study, *S. benedicti* could not be found at the sampling site in Hooksiel. However in summer 2004, the species occurred in Hooksiel and a polychaete culture was

successfully established in the laboratory. Only females with planktotrophic larval development mode fulfilled selection criterion 2. Therefore, females of *S. benedicti* were preliminary cultured to ascertain planktotrophic development of larval broods. Due to the experiences in culture techniques of *P. cornuta*, cultures of *S. benedicti* were established quickly. Sufficient larval supply for bioassay purposes was achieved in September 2004. Detailed species and culture information is given in Chapter 2.4.

2.2 POLYDORA CORNUTA

Polydora cornuta Bosc, 1802 (formerly also *Polydora ligni* Webster, 1880) is a member of the polydorid species complex (Blake 1969). Adults are up to 32 mm long, 1.5 mm wide and have up to 90 setigers (Radashevsky 2005). It colonizes sandy and muddy habitats. *P. cornuta* tolerates high organic contents and is an early colonizer of defaunated sites (Dauer 1984). It builds U-shaped tubes up to 40 mm in length and 1 mm width (Hartmann-Schröder 1996). The tubes protrude from the sand and form funnel-like vents easily visible by the naked eye. *P. cornuta* feeds with palps as long as 15 - 35 setigers on planktonic organisms and detritus either from water or from the sediment surface (Stamm 2000). While recently settled juveniles of *P. cornuta* almost exclusively show suspension feeding, larger juveniles and adults mainly feed on deposit unless the flow exceeds ~10 cm/s (Hentschel 1999). *P. cornuta* reproduces by internal fertilization. Females deposit spherical eggs (∅ 100 - 110 µm) in capsules. Egg capsules are attached to the inside wall of the tube and contain up to 30 eggs. The eggs in broods develop synchronously into larvae, which enter the plankton with 3 - 4 setigers and approximately 260 - 280 µm length (Blake 1969, Hartmann-Schröder 1996, Radashevsky 2005). Released larvae spend 2 - 3 weeks in the water (Anger et al. 1986) feeding on plankton and swimming actively. A maximum of 85,000 larvae per cubic meter sea water was reported by Orth (1971). New segments develop one by one in the growth zone in front of the disc-like pygidium. 1,200 – 1,300 µm long 17 – 18 setiger larvae are able to settle and undergo metamorphosis. Maximum length of larvae found in plankton was about 1,700 µm long with 25 setigers (Radashevsky 2005). Additionally, Mackay and Gibson (1999) reported adelphophagic (feeding on the nurse eggs) larval development in *P. cornuta*. Adelphophagic females produced broods in which

95 % of the eggs were non-developing nurse eggs, which were ingested by the developing offspring. Nurse egg ingestion leads to the release of larvae of a wide range of sizes (3 - 11 setiger) at hatching and a corresponding decrease in the duration of the planktonic period.

Settlement of *Polydora cornuta* is followed by metamorphosis characterized by loss of larval features such as trochs (except nototrochs) and long bristles and elongation of their palps. Newly settled individuals build small tubes. In a recent work by Radashevsky (2005) smallest settled juveniles were described as about 1,200 µm long with 18 setigers; adults developed gametes one or two weeks after settlement. The smallest described mature male had 22 setigers, whereas the smallest mature female had 30 setigers. The period from settlement and metamorphosis to first hatching of larvae of the following generation lasted at least one month (Anger et al. 1986). Benthic life-spans may reach up to several years in the laboratory. In the field, where average temperatures are lower and predation occurs, life-span is probably between one and two years (Anger et al. 1986).

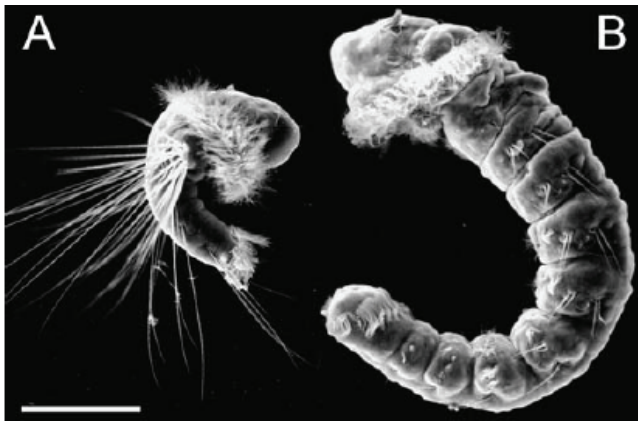
Polydora cornuta is common in tidal and shallow subtidal regions of the Wadden Sea (Dankers 1981). In several consecutive years Dekker studied the macrozoobenthos on twelve trawls in the Wadden Sea and the Eems-Dollard (Dekker 1995). In those years he reported the occurrence of *P. cornuta* in this area. In the Otzumer Balje (near Spiekeroog Island) low abundances were recorded in 1998 (Reiß 1999). On the Swinnplate near the island of Spiekeroog intermittently high abundances of *P. cornuta* were reported 1994 (Stamm 2000). Stamm investigated six sites along a 2 km transect with increasing distance from a *Mytilus* bed. Occurrence of *P. cornuta* was highly site- and time-dependent, with high abundances on two sites (site 4 and 5, ~ 150,000 ind. m^{-2}) in July and August 1994. In August 1994, *P. cornuta* supplied 19 % of all recorded individuals. The two sites with *P. cornuta* occurrence displayed intermediate sediment characteristics concerning the mud-, TOC- and N-content in comparison to the other four sites *(*mud content: 21 - 23 % w/w, TOC 0.79 - 0.9 %, N: 0.05 - 0.06 %) and were characterized by *Lanice* (site 4) and *Lanice* mixed with a young *Mytilus* bad (site 5). Larvae were found in the seawater throughout the whole year except of December and January. Highest larval abundances were recorded in June and August with 6 - 12 larvae per liter seawater. *P. cornuta* larvae were found to be more abundant in the ebb tide stream then in flood tide stream. Therefore,

Stamm (2000) concluded that most of the larvae were produced in the Wadden Sea and exported with the ebb flood stream. Rodriguez Valencia (2003) investigated horizontal and vertical distribution patterns of meroplanktonic larvae from the List tidal basin in the eastern North Sea. During his study benthic stages of *P. cornuta* occurred inside the basin and larvae were very common from April to October. Abundances of *P. cornuta* did not significantly fluctuate during the study period (1996 - 2001). Markert (2006) studied the associated macrofauna of a mussel bed in the backbarrier tidal flat of Juist island. Samples were taken outside of the mussel bed, from sites dominated by *Crassostrea gigas*, from sites dominated by *Mytilus edulis*, from sites with mixed population and finally from sites within the mussel bed but without mussels on the sediment surface. Occurrence of *P. cornuta* was strongly correlated with them of *C. gigas*.

2.3 STREBLOSPIO BENEDICTI

Streblospio benedicti Webster, 1879 is a small (< 20 mm, up to 50 setigers) tube-dwelling spionid. It colonizes mainly muddy sediments in poly- and oligohalin intertidal and subtidal areas in the North Pacific, North Atlantic, Black Sea, North Sea and Baltic Sea (Hartmann-Schröder 1996). It builds fragile mucus-tubes incorporating fine mud and sand grains (García-Arberas und Rallo 2004). Typically *S. benedicti* can be found in the upper 4 cm of sediment; only 1 % of adults and juveniles were found in deeper sediment areas (McCann und Levin 1989). With its two long palps *S. benedicti* feeds on plankton and detritus either from the water column or from the sediment surface. Juveniles and adults of *S. benedicti* are highly selective for food particles (Mahon and Dauer 2005). They tolerate high organic contents and settle in new habitats as a pioneer organism (García-Arberas and Rallo 2004). Fertilization is internal; embryos and larvae are brooded in dorsal pouches during early development. Females can produce several broods during their lifetime (Levin et al. 1987). Some females produce small eggs (60 - 70 µm in diameter) and release from the maternal brood pouch planktotrophic (obligatory feeding) larvae. Other females produce large eggs (100 - 200 µm) that are released as obligatory non-feeding lecithotrophs (Levin 1984) or facultative feeding planktotrophic larvae (recently discussed by Pernet and McArthur 2006). Such intraspecific variation in egg size and developmental mode is a specific kind of "poecilogony" (Chia et al. 1996). Adults with different reproductive modes may co-occur in the same habitat (Pernet and McArthur

2006). Obligatory planktotrophic larvae from small eggs hatch with 3 - 5 setigers, 220 - 230 µm body lengths and long provisional chaetae on the first setiger (Levin 1986). One brood contains between 100 and 600 larvae (Blake and Arnofsky 1999). Data on planktonic development span from 2 - 3 weeks (Levin 1984) to 7 - 45 days (Blake und Arnofsky 1999). Larvae are good swimmers and react positively phototactic (Levin 1984). Larvae hatched from big eggs (facultative feeding planktotrophic larvae) leave the maternal tube with ~ 640 µm length and 10 - 12 segments and are immediately competent to settle (Levin and Creed 1986). In this developmental mode 10 - 100 larvae hatch per brood (Blake and Arnofsky 1999), which remain pelagic for one week at maximum (Levin 1984).



Larvae shown to same scale (scale bar, 100 µm).
A: Right lateral view of a larva that developed from a small egg, with prominent larval chaetae arising from the first setiger.
B: Left lateral view of a larva that developed from a large egg.

Fig. 1: *S. benedicti*. Scanning electron micrographs of larvae immediately after release from the maternal brood pouch (Pernet and McArthur 2006, with permission, © Springer).

Streblospio benedicti larvae settle during the 10 - 13 setigers stadium (Blake and Arnofsky 1999). Settlement is delayed by up to 14 days in the absence of suitable substrates (Pechenik 1990, Levin 1984). Larvae of the same brood may display different length at the competent developmental stage (Levin 1984). Settled larvae undergo raoid metamorphosis with competent larvae developing thickened palps and branchiae, but still retaining cilia until the first mucous tube is constructed (Blake and Arnofsky 1999). Juveniles produce gametes at a body length of ~5 mm (Hentschel and Larson 2005). The body size does not increase after the attainment of sexual maturity (Bridgess and Heppell 1996) but the length and setigers number of females is positively correlated with fecundity traits such as number of ova and brood size (Levin and Creed 1986).
Concerning the distribution of in the Wadden Sea only few published data exists. In the theses of Rodriguez Valencia (2003) and Hernandez Guevara (2004) the

abundances of *S. benedicti* and *S. shrubsolii* in the List tidal basin have been observed, but the species affiliation has not been distinguished. They reported low abundances in some of the stations of the basin. Vöge (Senckenberg Institute, Wilhelmshaven, Germany) recorded up to 4,000 individuals m^{-2} in intertidal sand 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. comm.).

2.4 CULTURING OF ADULT POLYCHAETES

Culturing techniques of polychaetes have been adopted from Irvine and Martindale (1999) and Anger et al. (1986) with modifications. Culture vessels (210 x 160 x 100 mm) have been filled with sediment (3 cm high) and 2 l of filtered natural sea water (FSW, (50 µm). Culture sediment was obtained from the adult sampling site (Hooksiel, Germany), washed twice, sieved (1 mm mesh) and frozen for a minimum of 1 day at -20 °C. Natural seawater was collected from the North Sea at Wilhelmshaven, stored in 20 l containers at 14 °C, filtered (50 µm) and adjusted to 30 - 31 psu before use.

Polychaetes colonized the sediment within a few hours and built new tubes at a surface density of 1 - 2 (*Polydora cornuta*) and 5 - 10 (*Streblospio benedicti*) worms cm^{-2}, respectively. The total culture vessel surface was ca. 2,300 cm^2 for *P. cornuta* and 660 cm^2 for *S. benedicti* at average over three years of culturing. Cultures were maintained at constant temperature (18 °C) under 12h/12h photoperiod conditions and constant aeration. Biweekly, adults were fed with ground fish food (Tetra Marin) suspended in FSW and the seawater in the culture vessel was changed.

2.5 LARVAL CULTURE TECHNIQUES

Every day, newly hatched larvae were sieved out (50 µm mesh) and rinsed into aerated 1-liter culture vessels. Owing to this procedure, the larval age distribution within the same batch differed by 24 h at maximum (except the Monday-batch). Larval cultures were maintained under constant temperature (18 °C) and photoperiod conditions (12h/12h); the water was changed biweekly. Larvae were fed a mixture of the live unicellular algae *Dunaliella tertiolecta* (∅ ~ 8 µm),

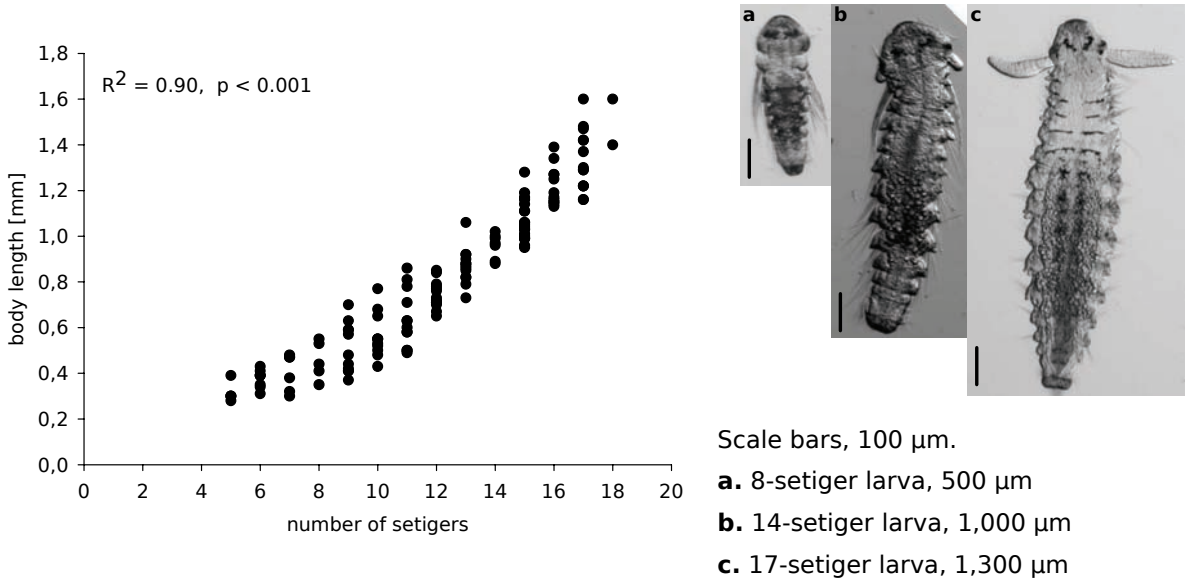
Isochrysis galbana (∅ ~ 5 - 6 µm) and the chryptomonad *Rhodomonas* sp. (∅~ 5 - 10 µm).

Algal stock cultures were obtained from the Culture Collection of Algae (University of Göttingen, Germany). The unicellular algae were cultured in 7-liter Perspex tubes, *Rhodomonas* sp. was grown in 2-liter glass beakers with cotton stoppers. Algae were cultured in f ¾ 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 *Streblospio benedicti* and *Polydora cornuta* were cultured for 6 - 8 days and 14 - 21 days, respectively.

The polychaetes *Polydora cornuta* and *Streblospio benedicti* were successfully cultured with nearly constant larval supply throughout an entire year. Daily yields ranged between 0.7 - 1.4 and 0.3 - 0.4 larvae cm^{-2} culture vessel surface for *S. benedicti* and *P. cornuta* resulting in 1,000 - 2,000 and 800 - 1,000 larvae, respectively. In this culture, *S. benedicti* displayed planktotrophic development because larvae hatched with 3 - 4 setiger, possessed provisional setae, and fed on phytoplankton after release. Larval growth was highly dependent on temperature, food quality and larval density in the culture vessel. Optimum culture conditions were achieved at 16 ˚C with 1 larva ml^{-1} FSW.

2.6 LARVAL DEVELOPMENT AND SETTLEMENT BEHAVIOR

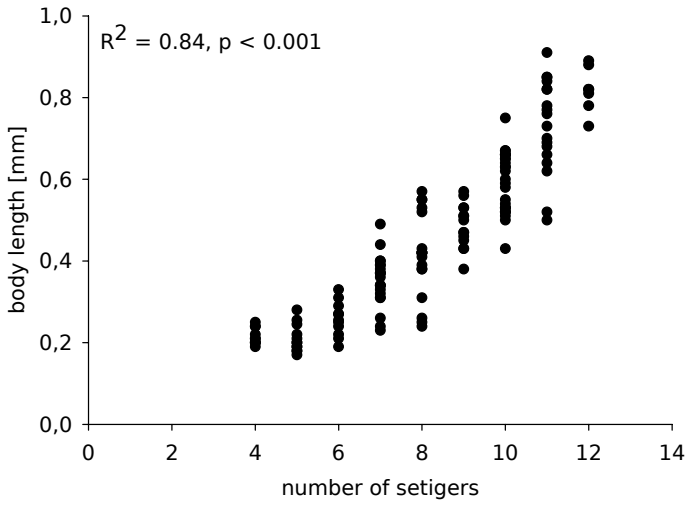
During larval development, body length and the number of setigers were monitored. For this purpose, 150 larvae at different developmental stages were pooled, placed on microscope slides and photographed using a stereo microscope (Carl Zeiss Stemi SV 11) and a digital camera (Carl Zeiss AxioCam MRm). Larval length was determined from these images using the program AxioVision (Carl Zeiss Vision GmbH). Body length was measured with a curved line drawn from the tip of the larval episphere to the tip of the pygidium through the centre of the body using the "curve spline" tool in AxioVision. The maximum error of measurement was less than 2 % of the mean of repeated measures of the same individuals. Measurement errors were mainly due to larvae lying not completely flush on the glass slide. Additionally, the number of setigers was counted. Results are shown in Fig. 2 and Fig. 3.

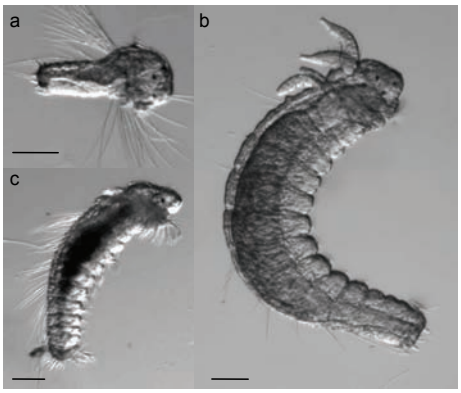


Scale bars, 100 µm.
a. 8-setiger larva, 500 µm
b. 14-setiger larva, 1,000 µm
c. 17-setiger larva, 1,300 µm

Fig. 2. *P. cornuta*. Correlation of body length (in mm) and number of setigers in larvae (n = 140)

In *Polydora cornuta* the pelagic phase in the lab culture lasted for 14 - 21 days. The number of setigers was significantly correlated with the larval body length ($p < 0.001$, $R^2 = 0.90$). The maximum number of setigers observed was 18.
In *Streblospio benedicti* the pelagic phase lasted for 6 - 8 days. The number of setigers was also significantly correlated with the larval body length ($p < 0.001$, $R^2 = 0.90$). The maximum number of setigers observed in the culture was 14.
The settlement behavior was characterized as follows: Larvae of *Polydora 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. Both species burrowed only a few seconds and built tubes subsequently. 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.





Scale bars, 100 µm.

a. 4-setiger larva, 300 µm

b. 12-setiger larva, 600 µm

c. 14-setiger larva, 1,000 µm

Fig. 3. *S. benedicti*. Correlation of body length (in mm) and number of setigers in larvae (n = 140)

3 DEVELOPMENT OF SETTLEMENT ASSAYS TO STUDY ACTIVE SETTLEMENT CHOICE IN POLYCHAETE LARVAE

3.1 INTRODUCTION

The ability of *Streblospio benedicti* to accept or reject sediment was previously reported utilizing a settlement assay without choice (Chandler and Scott 1991, Chandler et al. 1997). So far, corresponding studies with *Polydora cornuta* larvae are lacking. The first objective of this study was to provide experimental evidence that the spionid polychaetes under investigation indeed performed active choice during surface exploration.

To address this objective, both no-choice and multiple-choice assays were developed in the course of this study. No-choice assays were supposed to demonstrate the mere ability of larvae to accept or reject different sediment qualities actively, whilst multiple-choice assays were suggested to assess the ability of both species for active settlement choice.

To assure comparable and reproducible assay conditions, larval behavior and morphogenetic development had to be studied first. Traits and factors under investigation were the attainability of larval competence to settle, the potential loss of substrate selectivity in aged larvae, and the potential influence of larval phototaxis (light guided larval orientation) on the outcome of assay results. Brief sections discussing the concept and potential consequences of competence, delayed metamorphosis and larval phototaxis on larval settlement are given in the following chapters 3.1.1, 3.1.2 and 3.1.3.

3.1.1 LARVAL COMPETENCE

Scheltema (1967) divided the planktonic developmental phase into two periods: a developmental period with growth and differentiation of the larva and a delay period during which the larva is capable of settlement and metamorphosis. This capability was termed "competence" and requires the ability of larvae to respond to appropriate settlement cues (Coon et al. 1990). However, competence to settle and competence to metamorphose may develop gradually, causing confusion in the terminology. Coon et al. (1990) suggested the term "behavioral competence" if the larva was able to display settlement behavior in response to an appropriate

signal and "morphogenetic competence" if it was able to metamorphose. In this thesis the term competence was used always as a synonym for "behavioral competence".

Larvae reach competence in a species-specific time slot (Davis 1994), which is influenced by environmental conditions, such as temperature (Bachelet et al. 1992, Zimmerman and Pechenik 1991), salinity (Zimmerman and Pechenik 1991) and in the case of feeding larvae nutrient availability (Davis 1994, Hadfield and Strathmann 1996). Larvae with lecithotrophic development usually gain competence immediately after hatching. Planktotrophic larvae often need several weeks or months to develop into competent larvae (Pawlik 1992). The duration of the competent phase is species-specific and varies in a wide range from few hours to several months (Coon et al. 1990, Pechenik 1990). The beginning of the competent stage is usually not related to larval age or size and competence usually does not cause morphological changes (Pechenik 1990, Davis 1994, Zimmerman and Pechenik 1991). Even larvae of the same species from one culture batch may develop competence at markedly different ages (Pechenik 1990). Therefore, in most cases a combination of age, size, behavior and morphological features are needed to estimate competence (Davis 1994). Pechenik (1990) summarized that the only way to determine competence is to successfully trigger their metamorphosis. Therefore in practice, competence of a larval batch is usually determined by subjecting a subsample of the batch to a settlement cue known to trigger settlement (positive control).

Due to the lack of information about the beginning and the duration of competence in larvae of *Streblospio benedicti* and *Polydora cornuta* I characterized the development-dependent settlement ability of this species in natural sediment from the adult habitat as positive controls. The ability of larvae to settle was observed in three independent assay-series for both species.

3.1.2 LARVAL SELECTIVITY AT SETTLEMENT VERSUS DELAYED METAMORPHOSIS

In the absence of appropriate settlement cues the duration of larval competence phase may be extended (Thorson 1950, Wilson 1952, Pechenik 1990, Hadfield and Strathmann 1996). Bayne and Pechenik proved that the time at which a larva may delay metamorphosis is correlated with the specificity of the substrate and other habitat requirements of this species (Bayne 1965, Pechenik 1980). Larvae with short competent phase are mainly generalists, i.e. their metamorphosis may

be triggered by manifold signals (Davis 1994). For most species studied in the laboratory, metamorphosis can not be postponed indefinitely (Scheltema 1961, Pechenik 1980). With increasing time, larvae either die or metamorphose on less suitable substrates (Chia 1978, Pechenik 1980). Larvae accepting a less favorable substrate due to delayed metamorphosis have been called "desperate larvae" by Knight-Jones (1953a). According to this terminology, the phenomenon of "desperate larvae" has been revisited by several authors reporting both the occurrence and appearance of larval desperation in different species (Toonen and Pawlik 2001, Marshall and Keough 2003, Botello and Krug 2006, Gribben et. al 2006).

In settlement assays, the use of larvae with low substrate selectivity would strongly influence the results. Therefore, I firstly studied the occurrence of this phenomenon in *Polydora cornuta* and *Streblospio benedicti* larvae. The aim of my study was to disqualify larvae from settlement assays, which possibly had delayed metamorphosis and might have contributed to less selective assay results.

3.1.3 INFLUENCE OF LIGHT ON LARVAL SETTLEMENT

Phototaxis is an important property for larvae to control and direct their position in the water column (Sulkin 1990). Thorson (1964) reviewed the literature on larval responses to light (phototaxis) for 141 invertebrate species and concluded that light was the main cue for swimming behavior. According to Thorson, 82 % percent of larval species responded positively to light, 12 % were indifferent, and 6 % responded negatively to light. He noted that larval responses to light generally differ between intertidal and subtidal species. The larvae of the few intertidal species examined were photopositive throughout their planktonic period. Thorson suggested that this behavior would position larvae in the upper water column where the chance of encountering shallow habitats at the end of larval life is high. In contrast, larvae of most subtidal species were initially photopositive, hence promoting dispersal, but they became photonegative prior to settlement, presumably to enhance their chances of encountering subtidal adult habitats.

Larvae of the intertidal species *Streblospio benedicti* and *Polydora cornuta* revealed positive phototactic behavior during larval development. Since the photopositive larvae in multiple-choice assays under ambient light conditions

would aggregate on the bright (illuminated) area of the assay chamber, falsifying settlement results, multiple-choice assays were carried out in the darkness. Since larvae with positive phototactic behavior may delay metamorphosis in absence of light (Thorson 1964), the influence of light and darkness on larval settlement was investigated.

3.2 MATERIAL AND METHODS

3.2.1 DEVELOPMENT OF SETTLEMENT ASSAYS

Generally, there are two basic designs for a comparative laboratory experiment: the repeated-measures design, also known as a within-subjects design and the between-subjects design. In a within-subjects design, each individual is tested under each condition. In a between-subjects design, each individual is tested under exactly one condition.

3.2.1.1 NO-CHOICE ASSAY

In this study, the no-choice assay was developed based on the between-subjects design, i.e. one group of larvae was tested for one treatment and the other group for the second treatment. In this type of assay differences in competence and selectivity of larvae may strongly influenced the results since all larvae incorrectly estimated as competent and used in the assay decreased the observed settlement rate by false negative settlement records. Contrary, larvae delaying metamorphosis may display lower selectivity at settlement influencing the significance and comparability of assays. Therefore differences in larval quality had to be minimized prior to the utilization of larvae in assays.

Settlement assays were carried out in sterile 12-well microplates (3.8 cm^2 well surface area, Corning, USA, Fig. 4). For the assay, 2.5 g (wet weight) of sediment was transferred into each well resulting in a 7 mm thick sediment layer. The sediment was overlaid with 1.75 ml of sterile-filtered seawater and 10 larvae were randomly picked from a homogenous control batch were added. All experiments were conducted with replication (n = 6). The optimal duration and evaluation of the assay was ascertained after 1 h. 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 stereo microscope. Stained larvae on the sediment surface without burrowing activity were interpreted as not settled.

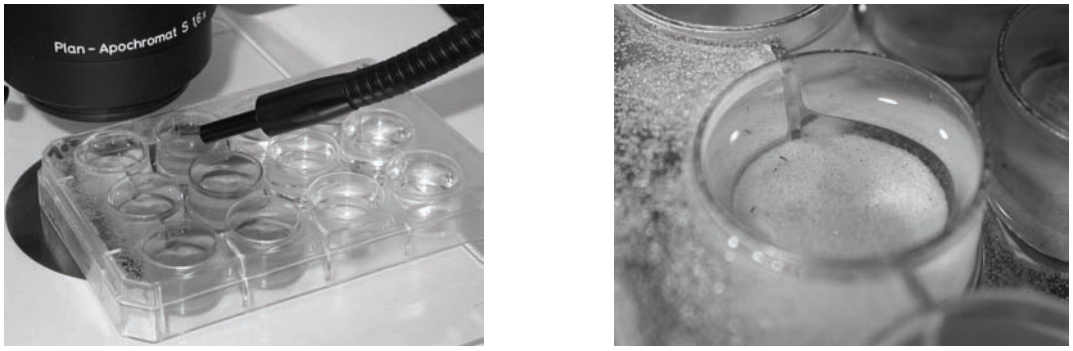


Fig. 4. No-choice assay. Sterile 12-well microplates are filled with different sediments, assayed with 10 larvae with replicates (n = 6).

3.2.1.2 MULTIPLE-CHOICE ASSAY

In the multiple-choice assay each larva was tested simultaneously against multiple qualitatively different treatments. However, in a multiple-choice assay larvae are not tested successively. Therefore, this design was not a classic within-subjects design. Successive assays for larvae in a settlement assay were not possible because already settled larvae could not be used again.

In his master thesis, Lodemann developed six different multiple-choice assay designs and tested different sizes, formats and numbers of treatments (Lodemann 2005). Assays suffered from small size of the assay chamber and insufficient size of the sediment patches offered to the larvae. Based on these experiences, I developed a new multiple-choice assay with Latin-square design to test the larval response to different sediment treatments. The Latin-square is an n × n array with n distinct cases such that each case appears exactly once in each row and column.

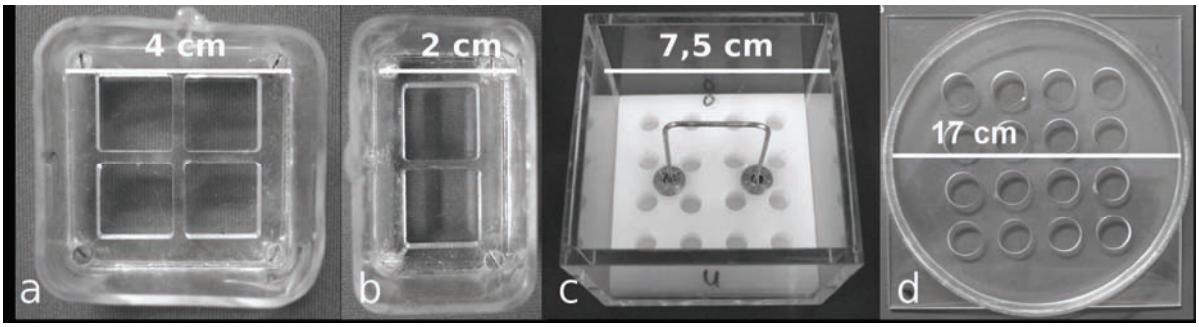


Fig. 5. Four different multiple-choice assay designs. a, b and c are preliminary designs (Lodemann 2005), whereas the design d was used in subsequent settlement assays.

In detail, cylindrical Plexiglas containers (∅ 17 cm, 1.5 cm high) with a removable Plexiglas bottom and sixteen cylindrical slots (∅ 2 cm, 0.3 cm depth) were used. The slots were arranged in four rows and four columns and separated by 1-cm bars. In a 4 x 4 Latin-square four replicates of four different sediment treatments were placed in the container. Every treatment was placed exactly once per row and once per column. In the following the 16 sediment-filled slots are referred as "sites". The container was transferred to a climate chamber held at 18 °C. 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 agar plates. The non-toxic vital stain 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 (settlement index) discounting all larvae still swimming or lying on the sediment surface without burrowing activity. The results were presented as percentage of average settlement index per site of each treatment.

3.2.2 COLLECTION AND TREATMENT OF NATURAL SEDIMENT SAMPLES

Sediment was repeatedly collected during low tide throughout the year. For the setup of polychaete cultures and bioassays sediment aliquots 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 bioassays (in the following referred to as “sterile sediment”).

3) 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) Natural sediment was washed with 5 M hydrochloric acid for 2 h. Supernatant was removed; sediment was neutralized by 5 M NaOH and washed twice with sterile filtered seawater (in the following referred to as “acid-washed sediment”).

3.2.3 SETUP OF SETTLEMENT ASSAYS

Settlement assays were performed as follows:

1) Settlement in different sediment qualities without choice

To investigate the ability of larvae for habitat acceptance or rejection natural, sterile and ashed sediments were assayed simultaneously in the no-choice assay with the same batch of larvae of both spionid species with replication (n = 3).

2) Settlement in different sediment qualities with multiple-choice

To investigate the ability of larvae for active sediment choice natural, sterile, ashed and acid-washed sediments were assayed in a multiple-choice assay with the same batch of larvae of both spionid species with replication (n = 2).

3) Determination of larval competence

The development dependent ability of larvae for settlement (competence) settlement in attractive sediment (determined by the first experiment) was assayed. 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.

4) Testing the selectivity of aged larvae

Development dependent changes in the selectivity of larvae were assayed by investigating the settlement rates in unattractive sediment (determined by the first experiment). On consecutive days, larvae originating from the same batch were used in settlement assays utilizing aliquots of the same sediment sample. No-choice assays were done for both spionid species with replication (n = 3).

5) Testing the influence of light on larval settlement

To investigate the influence of light on larval settlement the response to sterile sediment was assayed under ambient light conditions and in darkness in a no-choice assay with the same batch of larvae in both spionid species with replication (n = 2).

3.2.4 ASSESSMENT OF POTENTIAL EXPERIMENTAL ARTIFACTS

3.2.4.1 INFLUENCE OF THE LIVE STAIN NEUTRAL RED ON THE SETTLEMENT

The influence of the live stain Neutral Red on larval behavior and settlement was observed by recording larval exploration behavior in the presence and absence of the dye. Influence of the dye on survival was controlled by dividing larval batches

in two parallel batches and controlling the survival rates for two consecutive days (n = 2). In cooperation with Lodemann (2005), the possible influence of larval staining on settlement was tested in no-choice assays with replicates (n = 3).
1) Larvae were stained 15 min before usage with Neutral Red (f. c. 10 ng ml^{-1}) and assayed on natural sterile sediment in well-plates.
2) Unstained larvae were assayed on natural sterile sediment in well-plates. After 45 min assay duration larvae were stained with Neutral Red (f. c. 10 ng ml^{-1}).
A comparison with completely unstained larvae could not be carried out because of the low recovery rates of unstained larvae once settled from natural sediment.

3.2.4.2 INFLUENCE OF LEACHATE OF ASHED SEDIMENT ON SETTLEMENT

Presumably, ashed sediment released potentially toxic or harmful leachates which might have negatively influenced larval fitness and settlement. To test this hypothesis ashed sediment was washed three times vigorously with sterile filtered seawater (Lodemann 2005). The larval settlement response to following treatments was assayed:
1) Washed, ashed sediment overlaid with fresh sterile filtered sea water.
2) Sterile sediment overlaid with the first wash-water (leachate) of ashed sediment.
3) Ashed sediment (without washing) overlaid with fresh sterile filtered seawater.
4) Sterile sediment overlaid with fresh sterile filtered seawater.

3.2.4.3 INFLUENCE OF THE MULTIPLE-CHOICE ASSAY DESIGN ON LARVAL SETTLEMENT

Ideally, in multiple-choice assays larvae distribute and settle uniformly over all 16 sites provided all sites are loaded with the sediment of the same quality. However, larval behavior and settlement may be influenced by the shape of the chamber, e.g. larvae may prefer to settle in the middle of the chamber. Furthermore, already settled larvae may influence the choice of exploring larvae autoinducing subsequent settlers. To test these potential artifacts on larval settlement in a multiple-choice assay each well was filled with natural sediment, overlaid with sterile filtered sea water and assayed for larval settlement.

3.2.5 STATISTICAL ANALYSES

All no-choice assays 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 are generally not distributed Gaussian 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, I assumed normal distribution of the settlement response in populations from which the samples were drawn. Data were analyzed as counts and were 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 (one experiment). If the treatment effect was significant, Tukey's multiple comparison test was used to locate the differences identified by ANOVA (α=0.05).

3.3 RESULTS

3.3.1 SETTLEMENT IN DIFFERENT SEDIMENT QUALITIES (NO-CHOICE ASSAYS)

The percentage of larval settlement of *Streblospio benedicti* was significantly lower in ashed than in natural sediment (Tukey's test, $p < 0.001$, Fig. 6 A, B, C). Among three experimental repeats, the larval response to natural and sterile sediment treatments differed significantly but no clear trend was observed. In two repeats, the larval response to natural sediment was higher than in sterile sediment, while the opposite was the case in the third repeat.
The percentage of larval settlement of *Polydora cornuta* was significantly lower in ashed than in natural sediment. The larval response in all the sterile sediment treatments was significantly lower than in the natural sediments (Tukey's test, $p < 0.001$, Fig. 7 A, B, C).
Among 3 experimental repeats, the larval response to sterile and ashed sediment treatments did not reveal a clear statistical trend (Fig. 7 A, B, C). In 2 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.

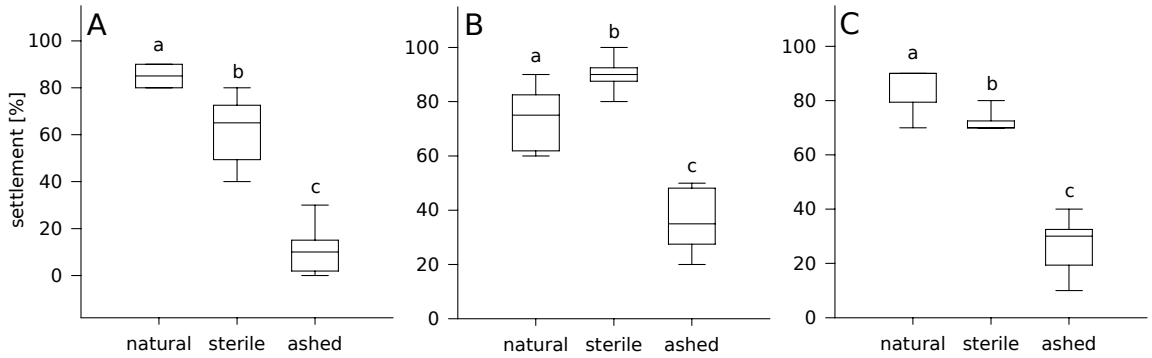


Fig. 6. *S. benedicti*. Mean percentage of larval settlement of 3 different larval batches.
A: 0.78 ± 0.11 mm body length, 11 - 12 setigers, B: 0.70 ± 0.11 mm body length, 11 setigers, and C: 0.66 ± 0.09 mm body length, 10 - 11 setigers after 1 h in response to 3 different sediment treatments, i.e. natural, 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 6 replicates shown in a box-and-whisker diagram.

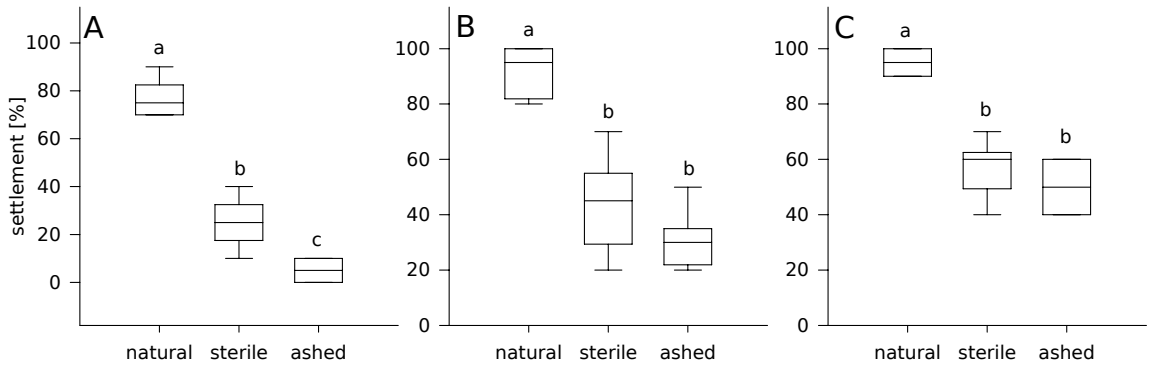
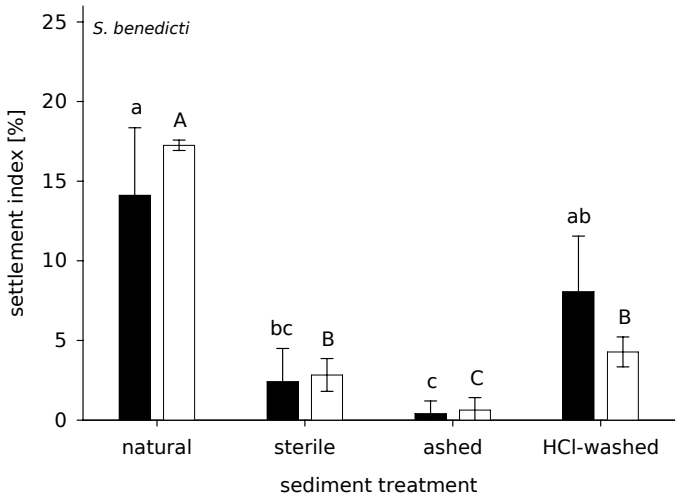


Fig. 7. *P. cornuta*. Percentage of larval settlement of 3 different larval batches.
A: 1.02 ± 0.23 mm body length, 15 setigers, B: 1.16 ± 0.18 mm body length, 16 setigers, and C: 1.05 ± 0.15 mm body length, 15 setigers in average after 1 h in response to 3 different sediment treatments, i.e. natural, 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 6 replicates shown in a box-and-whisker diagram.

3.3.2 SETTLEMENT IN DIFFERENT SEDIMENT QUALITIES (MULTIPLE-CHOICE ASSAYS)

Streblospio benedicti: the percentage of larval settlement was significantly higher in natural than in sterile and ashed sediment treatments (Tukey's test, $p < 0.001$, Fig. 8, Tab. 2).



Average settlement per site and treatment after 20 h in response to natural, sterile, ashed and acid-washed sediment in two different assays. Statistical differences are indicated by different letters above the bars ($\alpha = 0.05$, Tukey's test) by using upper and lower case letters for different assays. Total number of settled larvae in both assays was 124 (black bars) and 397 (white bars), respectively.

Fig. 8. *S. benedicti*. Percentage of settlement index per site in a multiple-choice assay

Ashed sediment triggered only a very low amount of larval settlement. Sediment, washed with 5M hydrochloric acid for two hours received intermediate settlement rates. Among the two experimental repeats, the larval response to the different sediment treatments was comparable; the first assay with lower number of total settled larvae displayed higher standard deviations of the settlement rates.

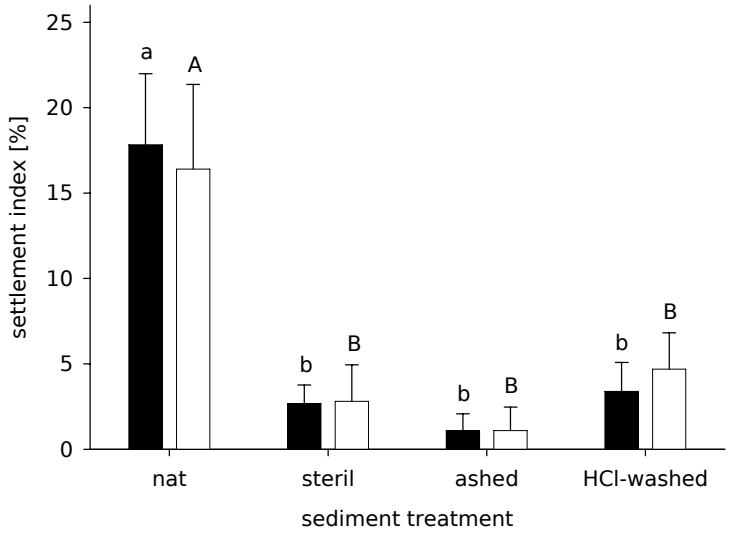
Tab. 1. Assay design and number of settled larvae of *S. benedicti* in the multiple-choice assay. Data for Assay 1 are related to the black bars and Assay 2 to the white bars of Fig. 8.

Assay 1 (black bars)			
0	0	20	12
10	6	0	22
18	4	2	0
2	10	14	4
∑ settled larvae = 124			

Assay 2 (white bars)			
12	2	67	17
16	6	0	69
70	13	11	1
7	68	22	16
∑ settled larvae = 397			

Sterile
Ashed
Natural
HCl-washed

Polydora cornuta: the percentage of larval settlement was significantly higher in natural than in all other sediment treatments (Tukey's test, $p < 0.001$, Fig. 9, Tab. 2).



Average settlement per site and treatment after 20 h in response to natural, sterile, 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) by using upper and lower case letters for different assays. Total number of settled larvae was 317 (black bars) and 160 (white bars), respectively.

Fig. 9. *P. cornuta*. Percentage of settlement index per site in a multiple-choice assay.

Tab. 2. Assay design and number of settled larvae of *P. cornuta* in the multiple-choice assay. Data for assay 1 are related to the black bars and assay 2 to the white bars of Fig. 9.

Assay 1 (black bars)

9	2	59	17
12	7	3	57
61	10	13	1
8	39	4	5

∑ settled larvae = 317

Assay 2 (white bars)

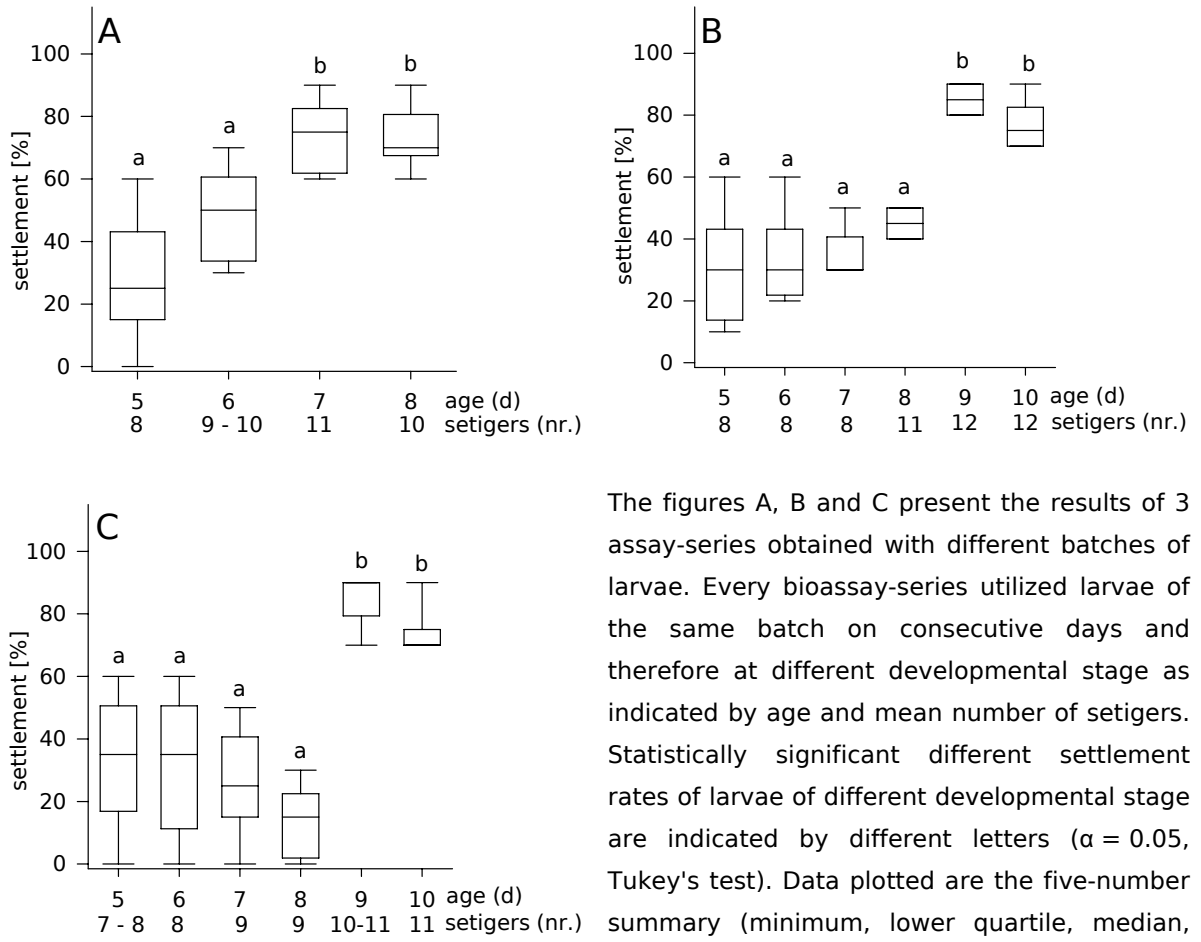
1	0	24	7
9	9	1	28
17	3	3	1
5	36	11	5

∑ settled larvae = 160

sterile
ashed
natural
HCl-washed

3.3.3 LARVAL COMPETENCE

In *Streblospio benedicti* the magnitude of larval settlement in natural sediment significantly increased in later developmental larval stages (Fig. 10 A, B, C).

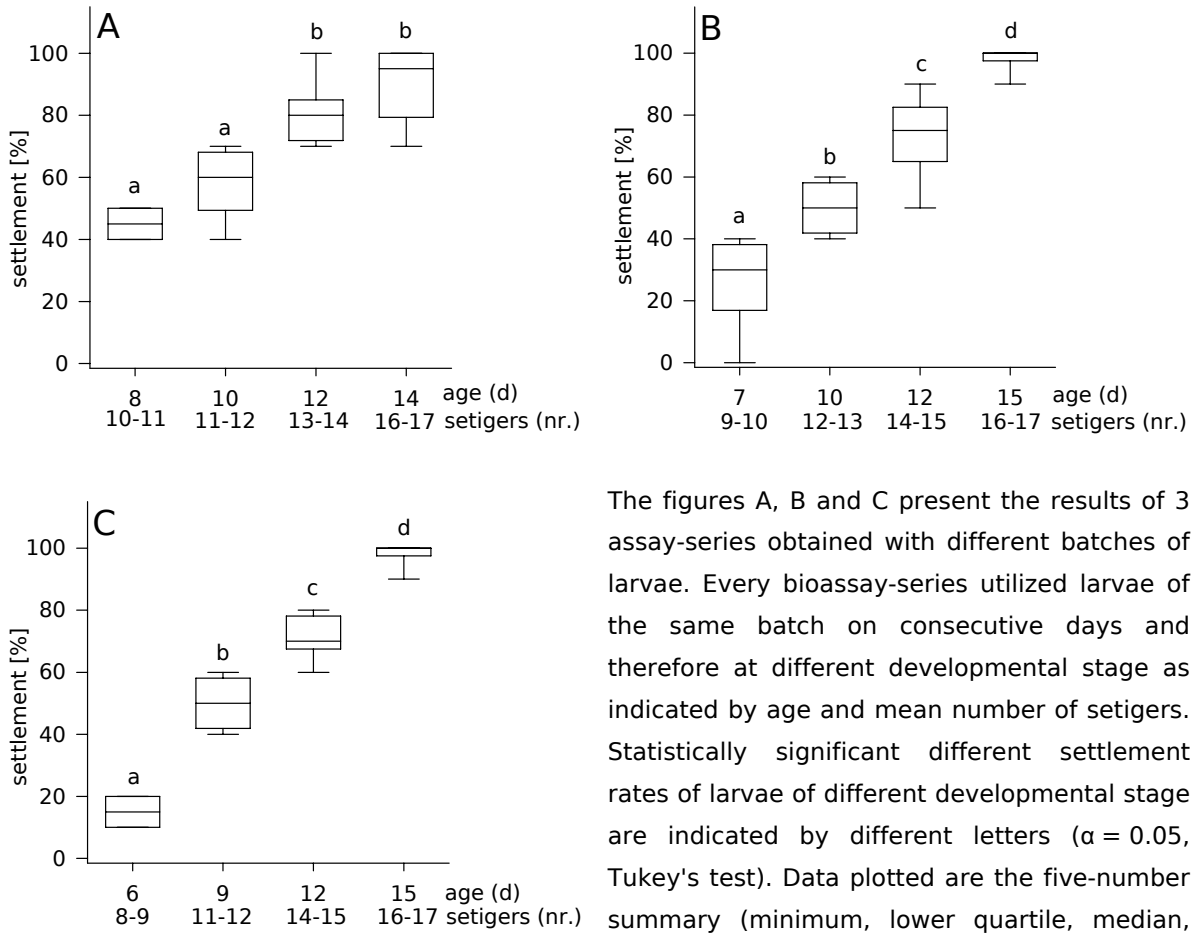


The figures A, B and C present the results of 3 assay-series obtained with different batches of larvae. Every bioassay-series utilized larvae of the same batch on consecutive days and therefore at different developmental stage as indicated by age and mean number of setigers. Statistically significant 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 6 replicates shown in a box-and-whisker diagram.

Fig. 10. *S. benedicti*. Percentage of larval settlement after 1 h assay duration in response to natural sediment.

In all 3 repeats, there was a significant difference in larval settlement between larvae of 0.59 and 0.71 mm mean size ($p < 0.05$, A, B, C), indicating that many larvae reach competence in this development stage. Generally, larvae displayed high settlement rates after 6 – 8 d at the 11-setiger stage and an average length of 0.7 mm.

Contrary, larval settlement of *Polydora cornuta* increased staedily throughout development (Fig. 11 A, B, C).



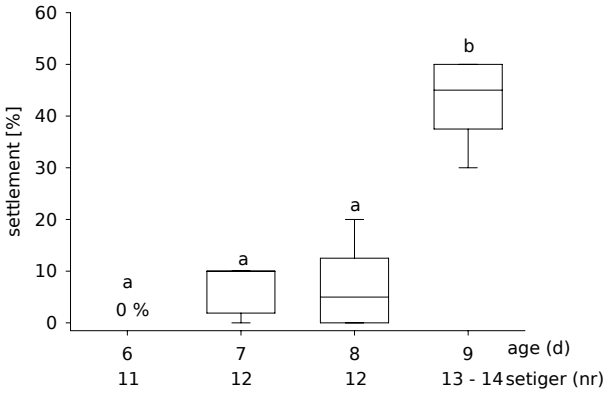
The figures A, B and C present the results of 3 assay-series obtained with different batches of larvae. Every bioassay-series utilized larvae of the same batch on consecutive days and therefore at different developmental stage as indicated by age and mean number of setigers. Statistically significant 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 6 replicates shown in a box-and-whisker diagram.

Fig. 11. *P. cornuta*. Percentage of larval settlement after 1 h assay duration in response to natural sediment.

3.3.4 LARVAL SELECTIVITY AT SETTLEMENT VERSUS DELAYED METAMORPHOSIS

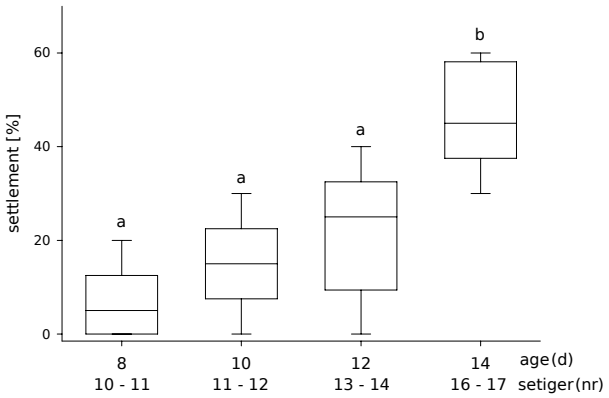
In *Streblospio benedicti* larvae with more than 12 setigers revealed lower selectivity in assays settling in high percentage even in unfavorable sediment treatment (Fig. 12). Since larvae at the 11 setiger stage and an average length of 0.7 mm displayed high settlement rates in natural sediment with high selectivity this stage was used in the subsequent assays.

In *Polydora cornuta* a decrease of selectivity at the 16 - 17-setiger stage was recorded (Fig. 13), therefore larvae with 14 - 15 setigers and 1.0 - 1.1 mm length were used in the subsequent assays.



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 (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. 12. *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 (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. 13. *P. cornuta*. Percentage of larval settlement after 1 h in response to ashed sediment.

3.3.5 TESTING THE INFLUENCE OF LIGHT ON LARVAL SETTLEMENT

In all three assays with *Streblospio benedicti* and in two assays with *Polydora cornuta* there was no effect on larval settlement due to light (main effect ANOVA, $\alpha = 0.05$, Fig. 14 A, B, C, E, F). In one assay with *P. cornuta* the percentage of larval settlement was significantly lower in darkness than under ambient light condition (Tukey's test, $p < 0.001$, Fig. 14 D).

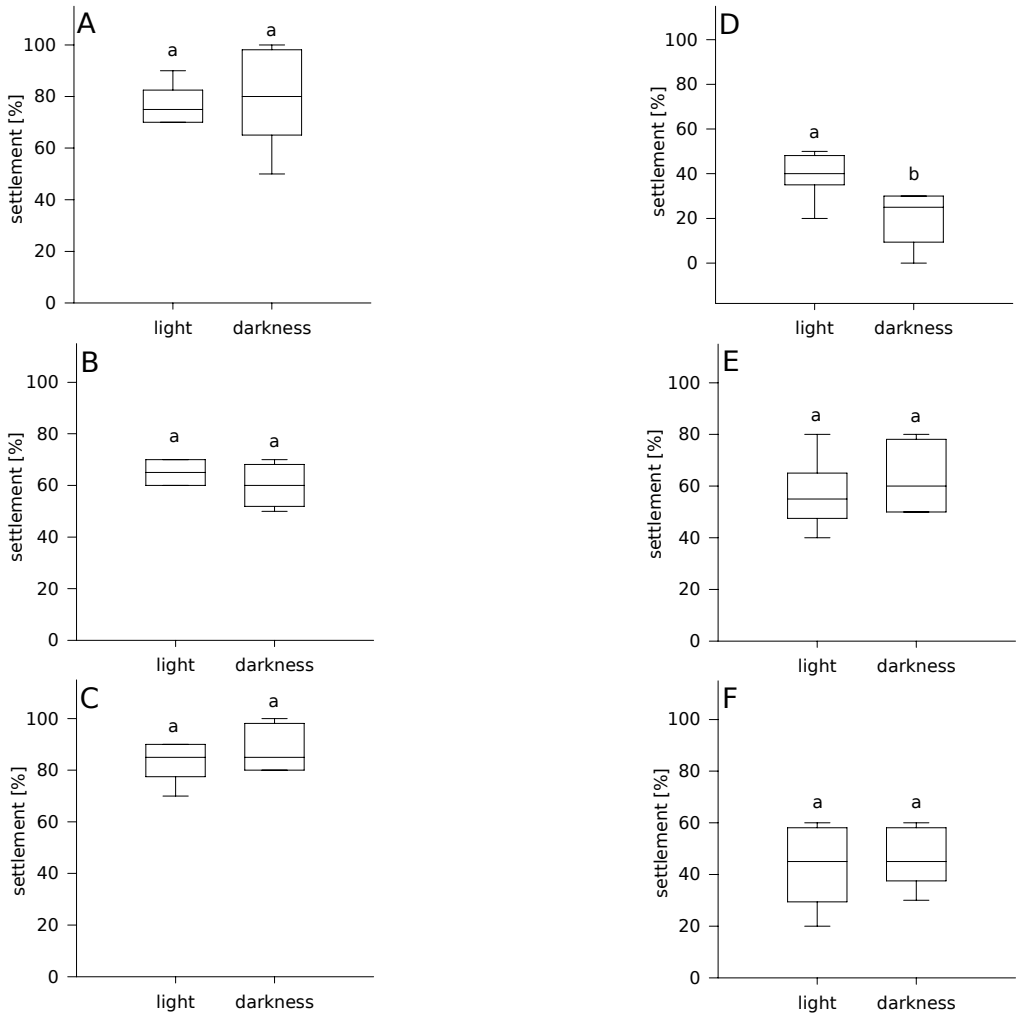


Fig. 14. *S. benedicti* (A, B, C) and *P. cornuta* (D, E, F). Influence of light on settlement. Percentage of larval settlement after 1 h assay duration in response to sterile sediment. Modified from Lodemann (2005).

3.3.6 ASSESSMENT OF POTENTIAL EXPERIMENTAL ARTIFACTS

3.3.6.1 INFLUENCE OF THE LIVE STAIN NEUTRAL RED ON SETTLEMENT

In the assays investigating larval settlement rates of pre-stained and post-stained larvae in all repeats with *Streblospio benedicti* and in two repeats with *Polydora cornuta* larval settlement due to staining did not differ (main effect ANOVA, $\alpha = 0.05$, Fig. 15 A, B, C, D, E,). In one assay with *P. cornuta* the percentage of larval settlement was significantly lower in post stained larvae (Tukey's test, $p < 0.001$, Fig. 15 F).

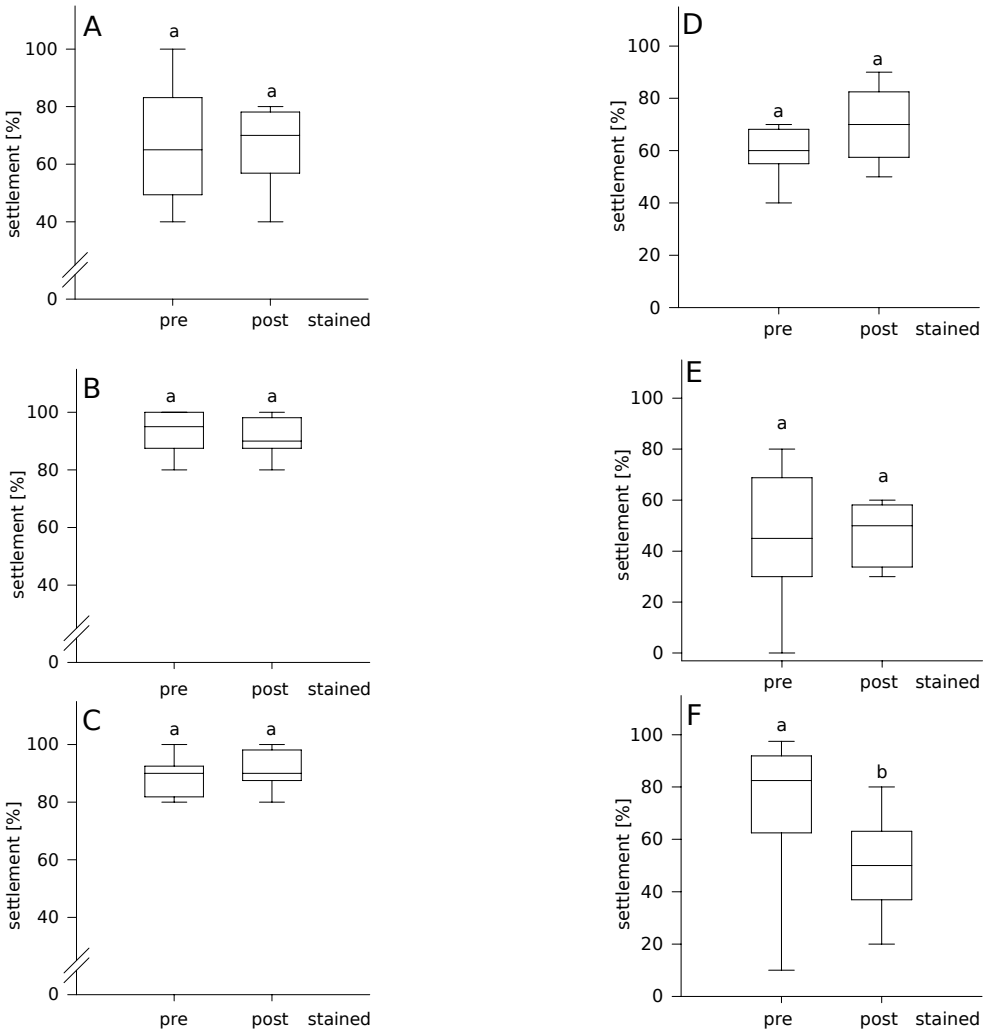


Fig. 15. *S. benedicti* (A, B, C) and *P. cornuta* (D, E, F). Influence of staining with Neutral Red on settlement. Percentage of larval settlement after 1 h assay duration in response to sterile sediment. Modified from Lodemann (2005).

Influence of the stain on survival was controlled by dividing larval batches in two parallel batches and controlling the survival rates for three consecutive days (n = 2). Survival rates in the batches with and without Neutral Red were the same (1-w-ANOVA, $\alpha = 0.05$). Larval behavior was investigated qualitatively by observing the larvae. At a stain concentrations of 10 ng ml $^{-1}$ no abnormal larval movement or settlement behavior was observed.

3.3.6.2 INFLUENCE OF LEACHATE OF ASHED SEDIMENT ON SETTLEMENT

The potential dissolution of substances from ashed sediment did not influence larval settlement (main effect ANOVA, $\alpha = 0.05$, Fig. 16 A, B, C, D). Settlement in ashed sediment and in three times washed ashed sediment was in all cases

statistically the same. Furthermore, settlement in sterile sediment covered with sterile seawater did not differ from the settlement in sterile sediment covered with the leachate of ashed sediment.

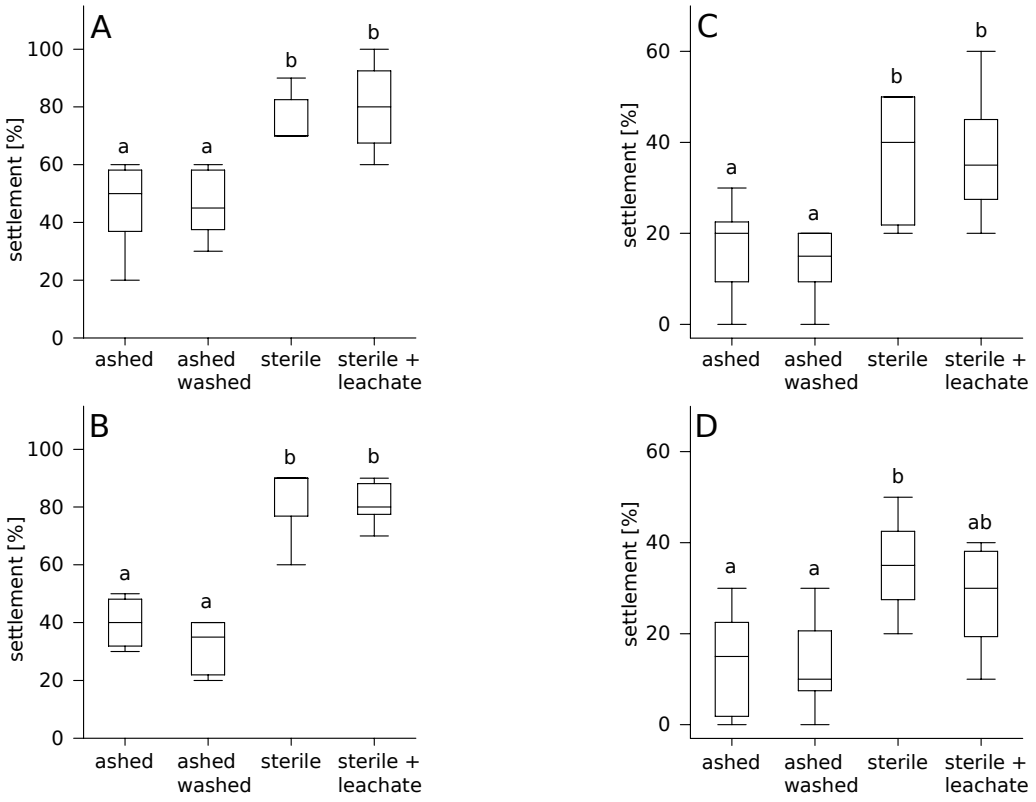


Fig. 16. *S. benedicti* (A, B) and *P. cornuta* (C, D). Influence of leachates of ashed sediment on settlement. Percentage of larval settlement after 1 h assay duration in response to sterile sediment. A & B modified from Lodemann (2005).

3.3.6.3 INFLUENCE OF THE MULTIPLE-CHOICE ASSAY DESIGN ON SETTLEMENT

In *Streblospio benedicti* average larval settlement per site was 41 ± 8 and 31 ± 6 in natural sediment (Tab. 3). No row or column effects were found due to main effect ANOVA at the $\alpha = 0.05$ level.

In *Polydora cornuta* average larval settlement per site was 16 ± 9 and 12 ± 7 in natural sediment (Tab. 4). No row or column effects were observed due to main effect ANOVA at the $\alpha = 0.05$ level. However, the standard deviation of settlement indices was higher than in *Streblospio benedicti*.

Tab. 3. Assay design and number of settled larvae of *S. benedicti* in the multiple-choice assay in response to natural sediment.

Assay 1 (natural sed.)				Assay 2 (natural sed.)				
33	45	40	23	31	30	30	34	natural
47	43	42	34	38	23	34	21	
38	40	40	45	35	43	29	22	
38	56	56	53	30	29	32	42	
∑ settled larvae = 659				∑ settled larvae = 503				

Tab. 4. Assay design and number of settled larvae of *P. cornuta* in the multiple-choice assay in response to natural sediment.

Assay 1 (natural sed.)				Assay 2 (natural sed.)				
19	26	16	14	23	8	6	15	natural
21	4	23	16	1	18	4	6	
4	24	5	7	18	4	17	12	
25	10	5	30	18	24	10	11	
∑ settled larvae = 242				∑ settled larvae = 195				

3.4 DISCUSSION

3.4.1 LARVAL SETTLEMENT IN DIFFERENT SEDIMENT QUALITIES

The no-choice assays with three parallel experimental treatments of sediment, i.e. natural, sterile and ashed, revealed clear differences in larval settlement of both species. Whilst natural sediment clearly induced settlement in both species, sterilization and ashing of natural sediment significantly decreased the settlement rate of *Polydora cornuta* (Fig. 6, Fig. 7). This was in contrast to *S. benedicti,* where the inductive effect on larval settlement remained after sterilization of sediment whereas ashing of sediment significantly decreased the settlement rate of *S. benedicti* (Fig. 6). Contrary to the results of the no-choice

assay, sterile sediment did not trigger settlement of *S. benedicti* in the multiple-choice assay (Fig. 8). Obviously, *S. benedicti* larvae rejected settlement in sterile sediment when they were simultaneously offered natural sediment. In *P. cornuta* the results of the multiple-choice assay corresponded to the ones obtained with the no-choice assay (Fig. 9).

In both assay types, low settlement rates of both species in ashed sediment indicated that complete removal of organic material influenced the attractiveness of such sediments dramatically, 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 figure out if in the ashed sediment treatment dissolution of material from the sediment into the water column negatively influenced water quality and thus the fitness of the larvae, I addressed this potential artifact with two different experiments. I showed that the rejection of ashed sediment was not influenced by changes in water quality (Fig. 16) and therefore must have arised from changes in sediment quality.

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. The fact that *Streblospio benedicti* larvae accepted this sediment in absence of an attractive sediment quality corresponded with the opportunistic character of this species.

In the multiple-choice assay a fourth sediment treatment was introduced. Larvae of both species settled significantly less frequently in acid-washed sediment than in natural sediment (Fig. 8, Fig. 9). In the first experiment with *S. benedicti*, the settlement rate in acid-washed sediment ranged between sterile and natural sediment, in all other cases acid-washed sediments evoked the same range of settlement rates as the sterile sediment. Acid washing of the sediment killed the microflora and –fauna and changed various physical and chemical sediment properties.

In summary, larvae of both species were able to accept or reject sediment and chose actively between different sediment qualities. At this stage, it appeared likely that the loss of sediment-attractiveness due to autoclaving might have been caused at least partially due the removal of sediment-associated microbial viability. To experimentally address this hypothesis a new experimental series was designed (see Chapter 4).

3.4.2 LARVAL COMPETENCE AND SELECTIVITY ISSUES

In *Streblospio benedicti,* larvae displayed high settlement rates after 6 - 8 d pelagic phase at the 11 setiger stage and an average length of 0.7 mm (Fig. 10). Larvae with more than 12 setigers revealed lower selectivity in assays settling in high percentage even in unfavorable sediment treatments (Fig. 12). Therefore, larvae with 11 setigers and 0.7 mm length were used in the subsequent assays. Contrary, larval settlement of *Polydora cornuta* increased constantly over the developmental phase (Fig. 11). Due to the loss of selectivity at the 16 - 17 setiger stage in assays with unfavorable sediment treatments (Fig. 15), larvae with 14 - 15 setigers and 1.0 - 1.1 mm length were used in the subsequent assays.
Fig. 10 and Fig. 11 show that in both species a remarkable percentage of "small" larvae has been found in the sediment, and hence was estimated as settled. This kind of settlement might have been caused by larvae searching for a shelter in the unevenness of the sediment surface (Duchêne 2004). On the other hand, competence in this species could be a gradual process, which may lead to some rates of competence even in small larvae.

3.4.2.1 CONSEQUENCES FOR THE ASSAY PROCEDURE

Due to the sensitivity of the no-choice assay to larval competence and selectivity issues (Fig. 10 - Fig. 13) the larval quality was carefully controlled. The following assay procedure was established to choose competent and selective larvae for the no-choice assay (in the following referred to as "test larvae"):
Larvae were checked under the stereo microscope. Based on experience, for *Streblospio benedicti* the 11 setiger stage with an average length of 0.7 mm and for *Polydora cornuta* the 14 - 15 setiger stage with an average length of 1.0 - 1.1 mm was sorted with a pipette. To verify and protocol the developmental stage of the sorted test larvae, 50 larvae were randomly picked and larval length was documented by photography using a stereo microscope and a digital camera as described in Chapter 2.6. The Number of setigers was calculated on the basis of Fig. 2 and Fig. 3.
In addition to the assay of interest subsequent concomitant no-choice assays were carried out and utilized as positive and negative controls:
1) A subsample of test larvae was subjected to a sediment quality usually known to trigger settlement, and used as positive control. The positive control was always carried out with fresh natural sediment from the habitat of the adult

polychaetes. Sufficient (> 70 %) larval settlement in the positive control documents the competence of test larvae.
2) A subsample of test larvae was subjected to a sediment quality usually known to be rejected by the larvae, and used as negative control. The negative control was always carried out with ashed sediment from the habitat of the adult polychaetes. Low (< 30 %) larval settlement in the negative control documents the selectivity of test larvae.

3.4.3 SUITABILITY OF DIFFERENT ASSAY DESIGNS TO TEST LARVAL SETTLEMENT

Generally, the assays developed in the course of this study have been shown to be suitable to investigate larval settlement. Light conditions and staining of larvae did not influence larvae. The multiple-choice assay was more sensitive than the no-choice assay, clearly revealingthe rejection of sterile sediment by *Streblospio benedicti* in the presence of natural sediment. Additionally, this assay was less sensitive to larval quality as discussed above. However, the evaluation of larval settlement in the multiple-choice assay was time consuming since every larva had to be recovered from the sediment. Due to their small size and tiny tubes they were difficult to be tracked. Thus, for screening purposes no-choice assays are recommended. Additionally, the multiple-choice assay may bear other sources for errors:
1) Interaction between different treatments may occur, e. g. bacteria from natural sediment sites may "contaminate" the sterile site during assay duration.
2) Assays deliver only a relative settlement index in comparison to the other three treatments of the assay. The same treatment assayed with other treatments may meet higher or lower larval acceptance. Therefore, the relativity of the results had to be taken into account.
3) Uneven spatial distribution of settled larvae within the four replicates of one treatment leads to high standard deviations of the data (settlement per site and treatment) and thus to difficulties of the statistical analysis. Uneven or patchy settlement within the same treatment may occur if the assay design influences larval settlement (e.g. larvae prefer to settle in the middle of the chamber) or if settlement of larvae does not occur independently from each other but rather autoinduces or catalyses the settlement of subsequent exploring larvae. It was not possible to test these two cases independently. I carried out two independent

assay series with the same sediment quality in each 16 wells of the multiple-choice assay for both species. While *Streblospio benedicti* settled evenly in the 16 wells, *Polydora cornuta* larvae tended to settle patchier (Tab. 3 and Tab. 4). However, patchiness did not follow a distinct pattern related to the position within the chamber. This observation lead to the hypothesis that gregariousness might be an important property in this spionid (refer to Chapter 5).

4 THE ROLE OF SEDIMENT-ASSOCIATED BACTERIA IN THE SETTLEMENT OF INFAUNAL POLYCHAETES

4.1 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 Todd 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 polychaete *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 1953a) 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 Hermann (1975, 1995) studied metamorphic cues of *Phoronis mülleri* (Tentaculata, an infaunal invertebrate) using bacterial isolates

suspended in sea water. 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 no-choice assays while combustion of sediment significantly decreased the settlement rate in *P. cornuta* and *S. benedicti* (Chapter 3), I investigated how the presence of bacteria on sediment influences larval settlement in *Polydora cornuta* and *Streblospio benedicti*. Firstly, the influence of a natural bacterial community on settlement was investigated. Subsequently, I 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.

4.2 MATERIAL AND METHODS

4.2.1 TESTING THE INFLUENCE OF NATURAL BACTERIAL COMMUNITIES ON LARVAL SETTLEMENT

To test the effect of microorganisms associated with natural sediment on larval settlement ashed sediment was recolonized with the detachable fraction of microorganisms obtained from natural sediment (in the following referred to as “recolonized ashed sediment”).

4.2.1.1 PREPARATION OF SEDIMENT TREATMENTS

To obtain these microorganisms, 25 g of natural sediment were combined with 100 ml of sterile-filtered seawater and gently shaken overhead for 1h. After the removal of coarse suspended particles by centrifugation (500 x g, 1 min) and filtration (Filter papers 3hw, Sartorius, Germany) the microorganisms in the filtrate were harvested by centrifugation (6000 x g, 20 min). The pellet was resuspended in 20 ml of sterile seawater and incubated with aliquots of 2.5 g of

ashed sediment for 1, 2 and 3 days. Non-attached bacteria were removed by washing. For this purpose 20 ml of sterile seawater was shaken gently with the sediment followed by pelletization (600 x g, 1 min) and removal of the supernatant. To verify that inoculated ashed sediment samples contained a sufficient microbial abundance compared to the control (sterile, ashed sediment), the bacterial density of the treatment and control was recorded daily. Once the bacterial density in the treatment differed significantly (Student's *t*-test) in comparison to the negative control (similarly treated ashed sediment), the treatment was used in the settlement bioassay. For a detailed description of the method of bacterial density control refer to chapter 4.2.3.

4.2.1.2 SETTLEMENT ASSAYS

Settlement assays were carried out in the no-choice assay design. Natural, ashed and inoculated sediments were assayed simultaneously in well-plates with the same batch of larvae of both spionid species with replication (n = 3).

4.2.2 TESTING THE INFLUENCE OF BACTERIAL ISOLATES ON LARVAL SETTLEMENT

4.2.2.1 ISOLATION OF BACTERIA AND PHYLOGENETIC ANALYSES

Bacteria used in this study were obtained from the natural habitat of *Streblospio benedicti* and *Polydora cornuta*. 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 25 ˚C for 72 h. The bacterial colonies that grew on the agar plates were examined under a dissecting microscope for morphological characteristics such as color, shape, size and surface topography. Conspicuous 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. Purified PCR amplicons of bacterial DNA were sequenced bidirectionally using an ABI PRISM™ big-dye terminator cycle-

sequencing ready-reaction kit (Applied Biosystems). Obtained rDNA sequences of each isolate were compared to the DNA sequences in the non-redundant nucleotide database in GenBank using BLAST (Basic Local Alignment Search Tool).

Another four bacterial strains used in this thesis were obtained from the Group Aquatic Microbial Ecology (ICBM, Oldenburg). *Phaeobacter inhibens* strain T5, an antibiotic producing bacterium was isolated 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, 0.05% peptone, 0.05% sodium thiosulfate 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).

4.2.2.2 PREPARATION OF SEDIMENT TREATMENTS

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.

4.2.3 MONITORING THE 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 25 ˚C.

4.2.3.1 LARVAL SETTLEMENT RESPONSES TOWARDS MONO-SPECIES BACTERIAL SEDIMENT TREATMENTS (NO-CHOICE ASSAYS)

No-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 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 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 or *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.

4.2.3.2 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 at 18 °C. After 20 h, the Plexiglas bottom was removed and the sediment treatments were separated in glass plates. The non-toxic vital stain 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 diluted 10^{-2}, 10^{-4} and 10^{-6}. A summary of all performed assays is given in Tab. 5.

Tab. 5. List of the 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}

4.2.3.3 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 no-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).

4.2.3.4 INVESTIGATION OF SUSPENDED BACTERIAL CELLS ON LARVAL SETTLEMENT

An assay was designed to determine whether suspended cells of the strain DF11 induced larval settlement of *Polydora cornuta*. Cells of the 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).

4.2.3.5 STATISTICAL ANALYSIS

Statistical analysis was performed similarly to the methods described in Chapter 3.2.5.

4.3 RESULTS

4.3.1 ISOLATION OF BACTERIA AND PHYLOGENETIC ANALYSIS

Thirty-nine bacteria were isolated from natural sediment and identified by comparison of 16S rDNA sequences to the DNA sequences in the GenBank database. The phylogenetic affiliation of the isolates is given in Tab. 6. 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. These genera distribute over 5 phylogenetic classes: *α-Proteobacteria*, *γ-Proteobacteria*, *Bacillales*, *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 isolated strains are given in (Tab. 6).

Tab. 6: Phylogenetic affiliation of isolated strains and accession numbers of published closest match in GenBank. Strains DF11, DF16, T5 and T5-3 were obtained from the Group Aquatic Microbial Ecology, ICBM. These strains are registered at GenBank with the listed accession numbers. 15 strains were used (in the list marked with*).

Nr.	Class	Strain (closest match at GenBank)	Acc. nr.	Similarity [%]
10	Bacillales	*Bacillus licheniformis*	AY162134	98%
12	Bacillales	*Bacillus aquimaris*	AF483625	98%
13 *	Bacillales	*Halobacillus sp. MO50*	AY553121	99%
21	Bacillales	*Bacillus* sp. YY	AF414443	99%
23	Bacillales	*Bacillus pumilus* strain CC-2U5-1	AY315434	99%
26	Bacillales	*Bacillus* sp. 2216.25.2	AB094471	99%
29	Bacillales	*Bacillus mycoides*	Z84583	96%
30 *	Bacillales	*Bacillus pumilus*	AB211228	98%
41	Bacillales	*Bacillus* sp. UST020129-005	AY241452	100%
43 *	Bacillales	*Bacillus cereus*	AF290554	100%
60	Bacillales	*Bacillus* sp. TP1.	AF440439	97%
68	Bacillales	*Bacillus* sp. MB-3	AF326362	96%
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%
1	γ-Proteobacteria	*Halomonas taeanensis*	AY671975	96%
4	γ-Proteobacteria	*Glaciecola* sp. 27III/A02/218	AY576759	93%
7	γ-Proteobacteria	*Vibrio sp.* NAP-4	AF064637	98%
8	γ-Proteobacteria	*Shewanella marisflavi*	AY485224	99%
14	γ-Proteobacteria	*Vibrio fortis*	AJ514914	99%
18	γ-Proteobacteria	*Vibrio* sp. Gp-MBA-3	AJ849367	98%
22 *	γ-Proteobacteria	*Vibrio pacinii*	AJ316194	99%
24 *	γ-Proteobacteria	*Shewanella baltica*	AF173966	98%
25	γ-Proteobacteria	*Pseudoalteromonas* sp. LOB-15	DQ412067	98%
33	γ-Proteobacteria	*Pseudoalteromonas* sp. UST020129-007	AY241429	100%
38	γ-Proteobacteria	*Pseudoalteromonas* sp. SM9913	AY305857	99%
39	γ-Proteobacteria	*Marinobacterium jannaschii*	AB006765	93%
40	γ-Proteobacteria	*Alteromonas marina*	AF529060	96%
42	γ-Proteobacteria	*Marinobacter* sp. GPM2541	AJ871938	98%
44 *	γ-Proteobacteria	*Zooshikella ganghwensis*	AY130994	98%
63	γ-Proteobacteria	*Marinobacter flavimaris*	AY517632	99%
15	Flavobacteria	*Polaribacter dokdonensis*	DQ004686	96%
16 *	Flavobacteria	*Salegentibacter sp. DPA2*	DQ344850	98%
27	Flavobacteria	*Tenacibaculum mesophilum*	AB032504	97%
34	Flavobacteria	*Flavobacterium* sp. V4.MS.12	AJ244703	98%
53 *	Flavobacteria	*Cellulophaga lytica*	AB032511	99%
54 *	Flavobacteria	*Psychroflexus tropicus*	AF513434	98%
64	Flavobacteria	*Tenacibaculum mesophilum*	AB032501	97%
32 *	Sphingobacteria	*Gramella echinicola*	AY608409	99%
35	Sphingobacteria	*Cytophaga* sp. I-377	AB073588	96%
51 *	Sphingobacteria	*Algoriphagus ratkowskyi*	AJ608641	98%
34	Sphingobacteria	*Cytophaga marinoflava*	AY167315	98%

4.3.2 LARVAL RESPONSE TO NATURAL BACTERIAL COMMUNITY

4.3.2.1 DEPLOYMENT-SUCCESS OF NATURAL BACTERIAL COMMUNITY ON ASHED SEDIMENT

Generally, the recolonization of ashed sediment with viable microorganisms was rarely successful due to low nutrient availability: regularly only a low amount of bacteria was attached on the sediment surface after the sediment treatment. In two experimental series, which have been carried out with microorganisms obtained from natural sediment collected in summer, a moderate attachment success was achieved. These sediment treatments showed a relative fluorescence significantly higher than the negative control (Student's *t*-test, $p < 0.05$) and were used for further experiments.

4.3.2.2 LARVAL SETTLEMENT ON ASHED SEDIMENT WITH REESTABLISHED NATURAL BACTERIAL COMMUNITY (INOCULATED SEDIMENT)

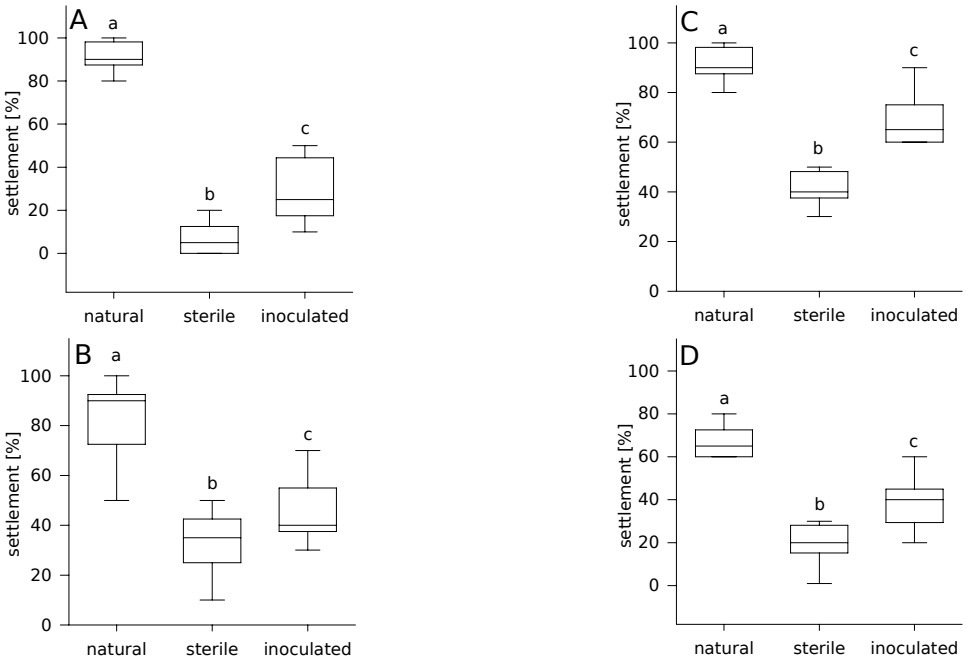


Fig. 17. *S. benedicti* (A, B) and *P. cornuta* (C, D). Percentage of larval settlement of test larvae of 2 different larval batches (A: 0.63 ± 0.11 mm body length, 10 - 11 setigers, B: 1.02 ± 0.10 mm body length, 13 setigers, C: 1.07 ± 0.26 mm body length, 15 setigers and D: 1.15 ± 0.21 mm body length, 16 setigers) after 1 h in response to 3 different sediment treatments, i.e. natural, ashed and ashed inoculated with natural bacterial community. 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.

The percentage of larval settlement of both species in both sediment treatments with successfully reestablished natural bacterial community was significantly higher than in the ashed sediments but significantly lower than in the natural sediment (Tukey's test, $p < 0.05$, Fig. 17).

4.3.3 LARVAL RESPONSE TO SEDIMENT INOCULATED WITH BACTERIAL ISOLATES

4.3.3.1 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 ($\sim 10^7 - 10^8$ cells g^{-1} sediment wet weight) attached on sediment grains. The recolonization yields are summarized in the figures legend of each assay.

4.3.3.2 LARVAL SETTLEMENT RESPONSE TOWARDS MONO-SPECIES BACTERIAL SEDIMENT TREATMENTS

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 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. 18 to Fig. 21.

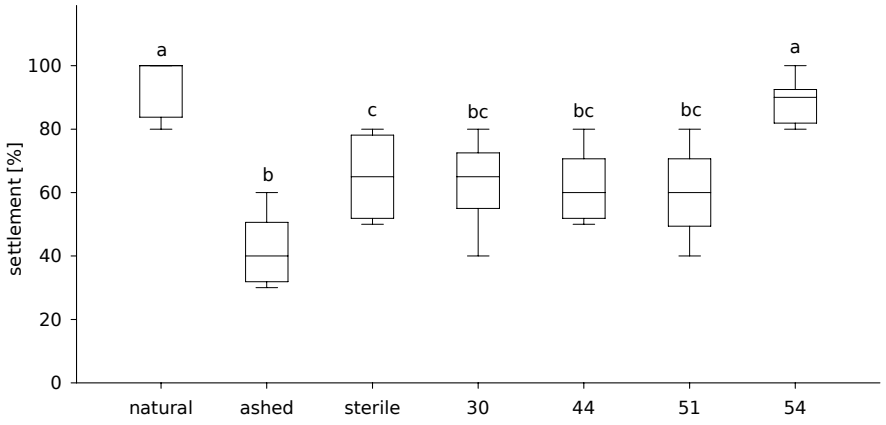


Fig. 18. *P. cornuta*. Percentage of larval settlement of test larvae of a single larval batch (0.96 ± 0.12 mm body length, 14 - 15 setigers) in no-choice assays after 1 h in response to 7 different sediment treatments, i.e. natural, ashed, sterile and sterile natural sediment recolonized with strain 30, 44, 51 and 54. 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.

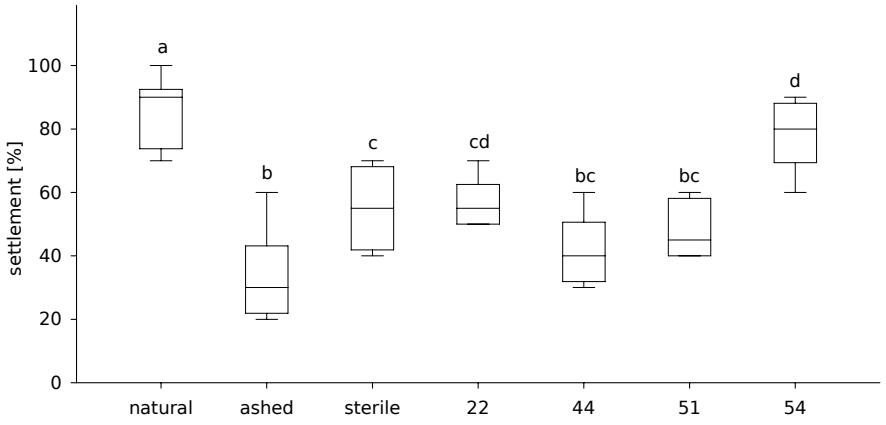


Fig. 19. *P. cornuta*. Percentage of larval settlement of test larvae of a single larval batch (0.93 ± 0.14 mm body length, 14 setigers) in no-choice assays after 1 h in response to 7 different sediment treatments, i.e. natural, ashed, sterile and sediment recolonized with strain 22, 44, 51 and 54, respectively. Statistical differences are indicated by different letters above the boxes (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.

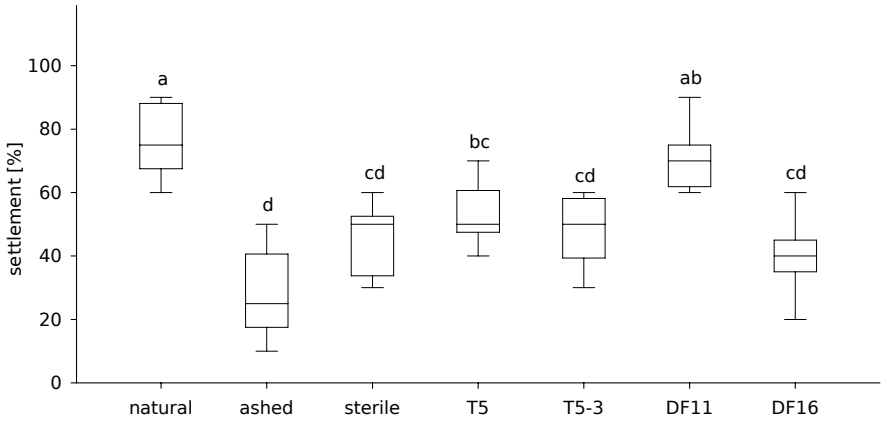


Fig. 20. *P. cornuta*. Percentage of larval settlement of test larvae of a single larval batch (0.94 ± 0.12 mm body length, 14 setigers) in no-choice assays after 1 h in response to 7 different sediment treatments i.e. natural, ashed, sterile and sediment recolonized with strain T5, T5-3, DF11 and DF16, 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.

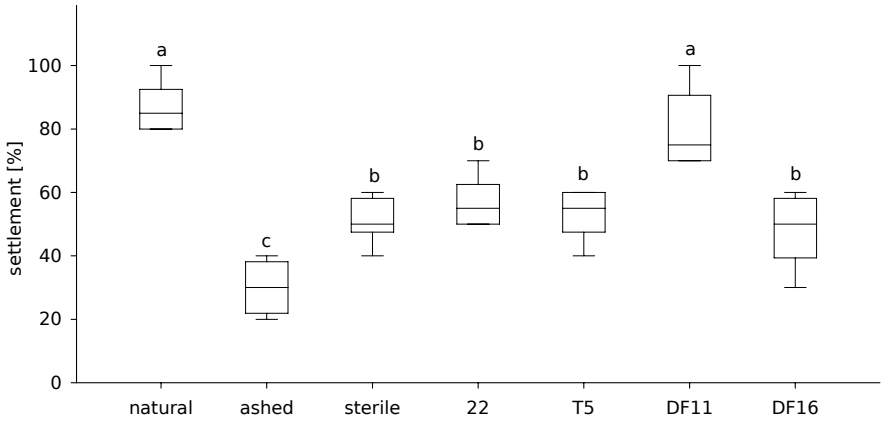


Fig. 21. *P. cornuta*. Percentage of larval settlement of test larvae of a single larval batch (0.99 ± 0.10 mm body length, 15 setigers) in no-choice assays after 1 h in response to 7 different sediment treatments i.e. natural, ashed, sterile and sediment recolonized with strain 22, T5, DF11 and DF16, respectively. 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.

4.3.4 EFFECT OF BACTERIAL CELL DENSITY AND VIABILITY ON LARVAL SETTLEMENT

Due to the settlement results obtained with strain DF11 and 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 no-choice assay, was selected as a control. In the following, the results of 6 different assay types are summarized:

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 1a In *S. benedicti* larval settlement was significantly higher in both sediments treated with DF11 compared to the control of sterile sediment (Tukey's test, $p < 0.05$, Fig. 22) and did not differ from the settlement response towards natural sediment ($p = 0.56$).

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. 23).

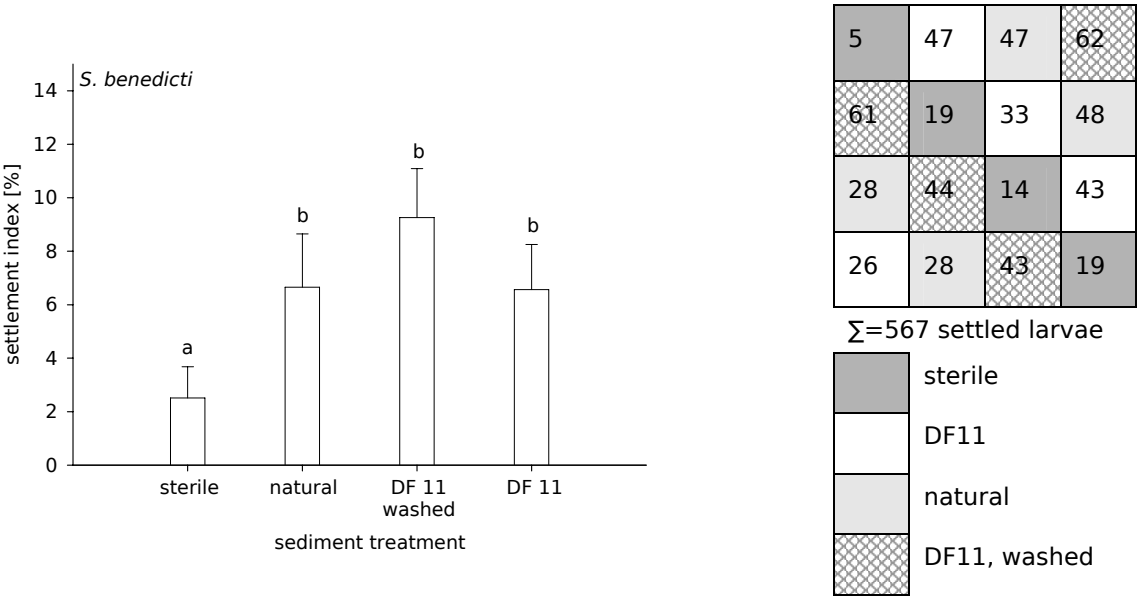


Fig. 22. *S. benedicti,* Assay 1a. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural, sterile and sterile sediments recolonized with different densities of the strain DF11. Bacterial cell densities were 9.4×10^8 cells g^{-1} in the "strain DF11" and 4.4×10^8 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).

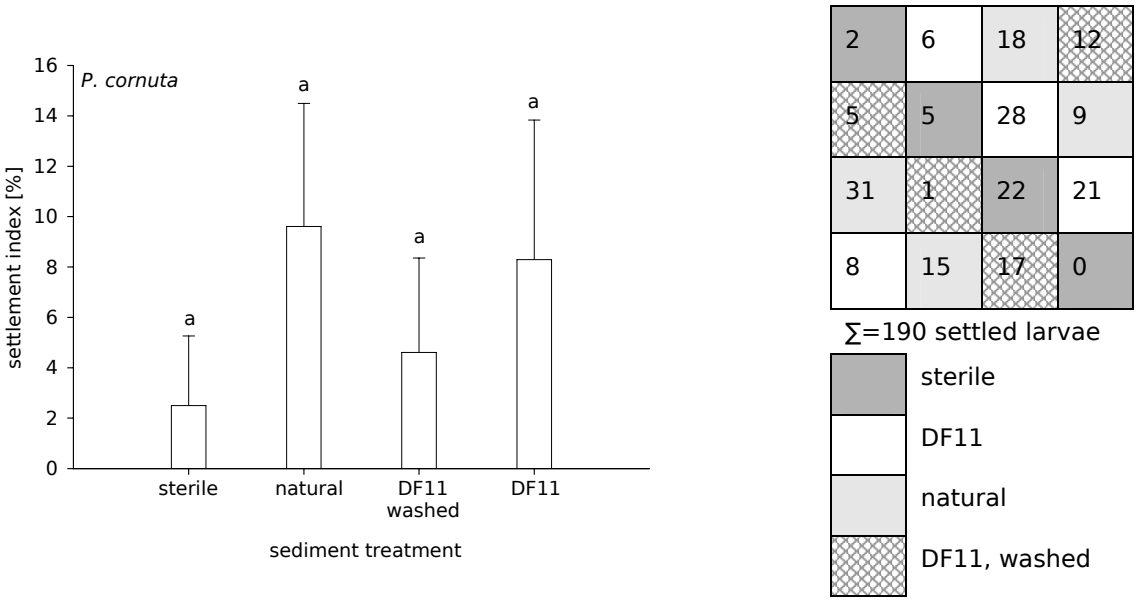


Fig. 23. *P. cornuta*, Assay 1b. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment and sediments recolonized with different densities of the strain DF11. Bacterial cell densities were 7 X 10^8 cells g^{-1} in the "strain DF11" and 2.2 x 10^8 cells g^{-1} sediment in the "strain DF11, washed" treatment. Statistical similarities are indicated by same letters above the bars (α = 0.05, Tukey's test)

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 2a: 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. 24). 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.

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. 25) and did not differ statistically from the natural sediment control ($p = 0.98$). The recolonized treatment without washing did not trigger larval settlement ($p = 0.99$).

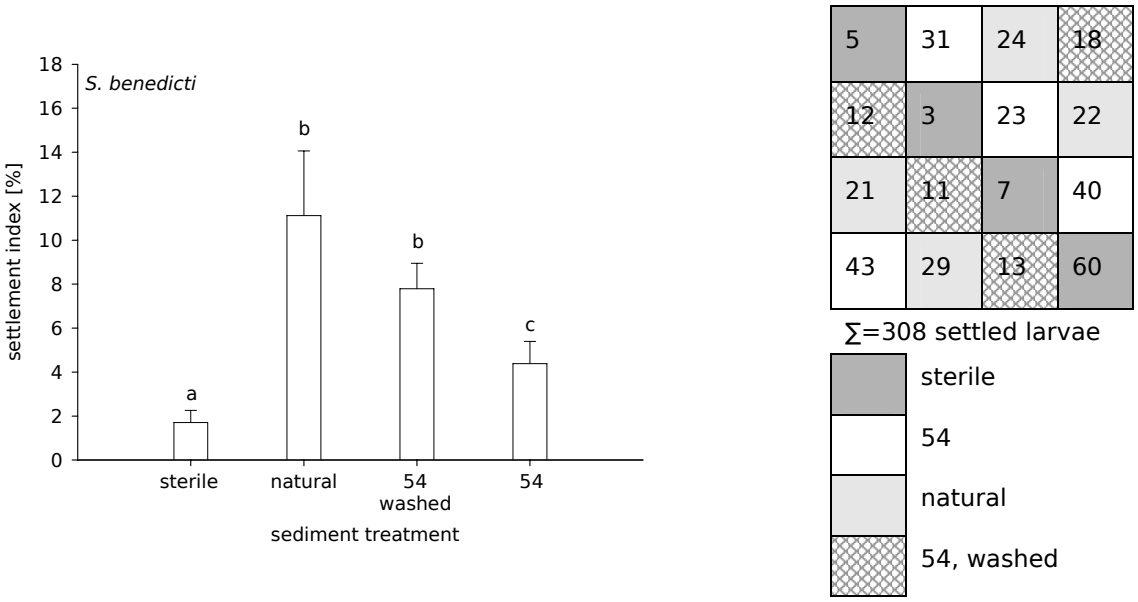


Fig. 24. *S. benedicti,* Assay 2a. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment and sediments recolonized with different densities of the strain 54. Bacterial cell densities were 7.7×10^8 cells in the "strain 54" treatment and 2×10^8 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).

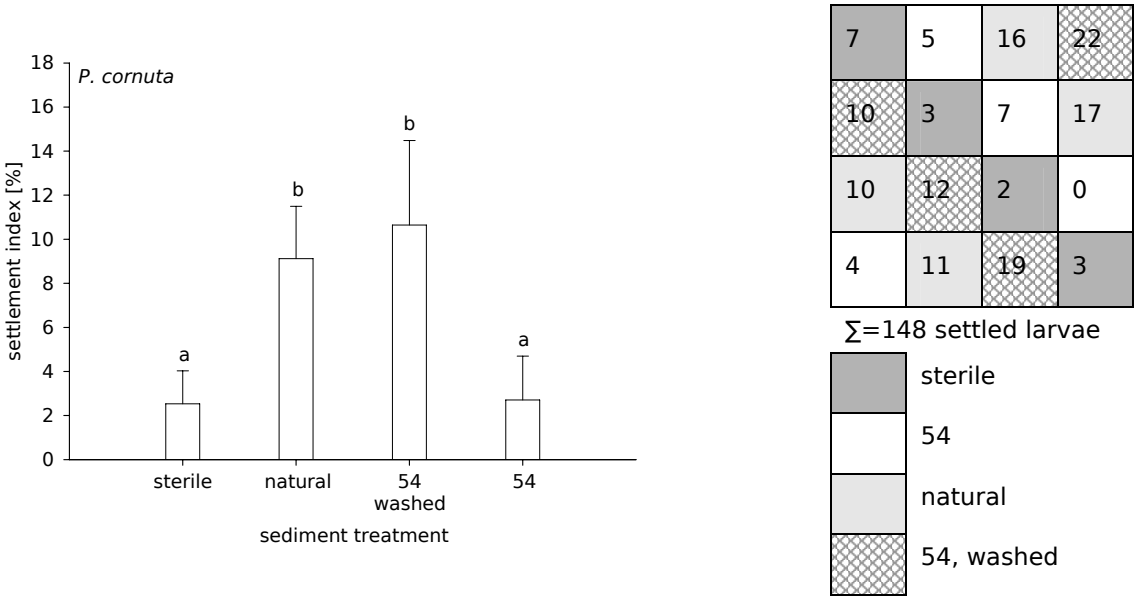


Fig. 25. *P. cornuta*, Assay 2b. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment and sediments recolonized with different densities of the strain 54. Bacterial cell densities were 5.8×10^8 cells in the "strain 54" treatment and 3.3×10^8 cells / g^{-1} in the "strain 54 washed" treatment. Statistical differences are indicated by different letters above the bars (α = 0.05, Tukey's test).

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 3a: 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. 26). Recolonization with strain DF16 did not trigger settlement in comparison to sterile sediment control ($p = 0.06$).

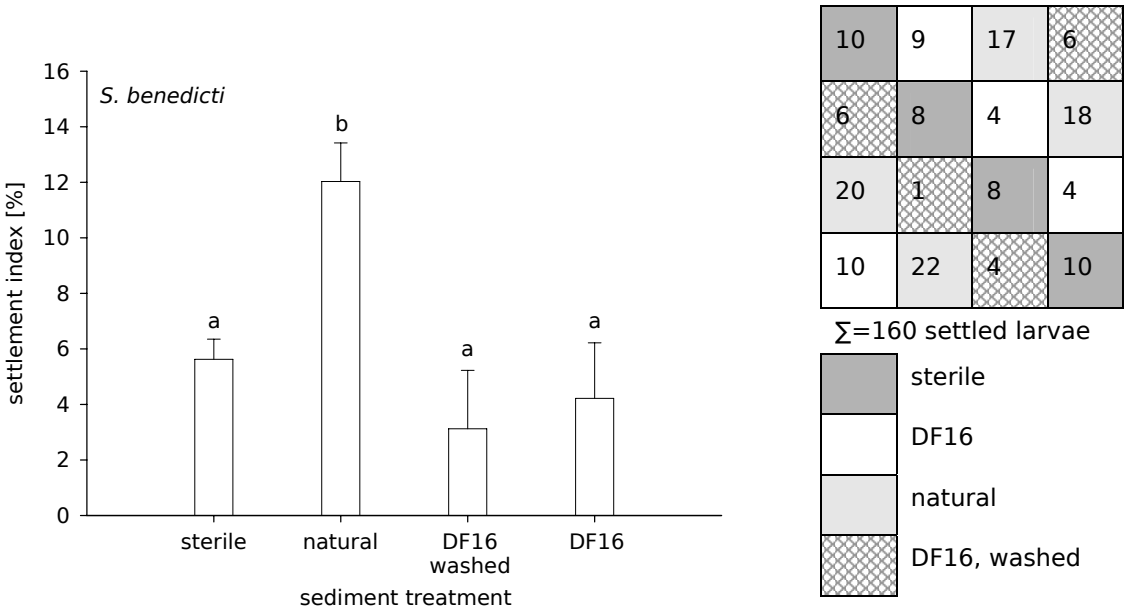


Fig. 26. *S. benedicti*, Assay 3a. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment and sediments recolonized with different densities of the strain DF16. Bacterial cell densities were 5.3 X 10^7 cells in the "DF16" treatment and 2 X 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. 27). Recolonization with strain DF16 did not trigger more settlement than the sterile sediment control (Tukey's test, $p = 0.15$).

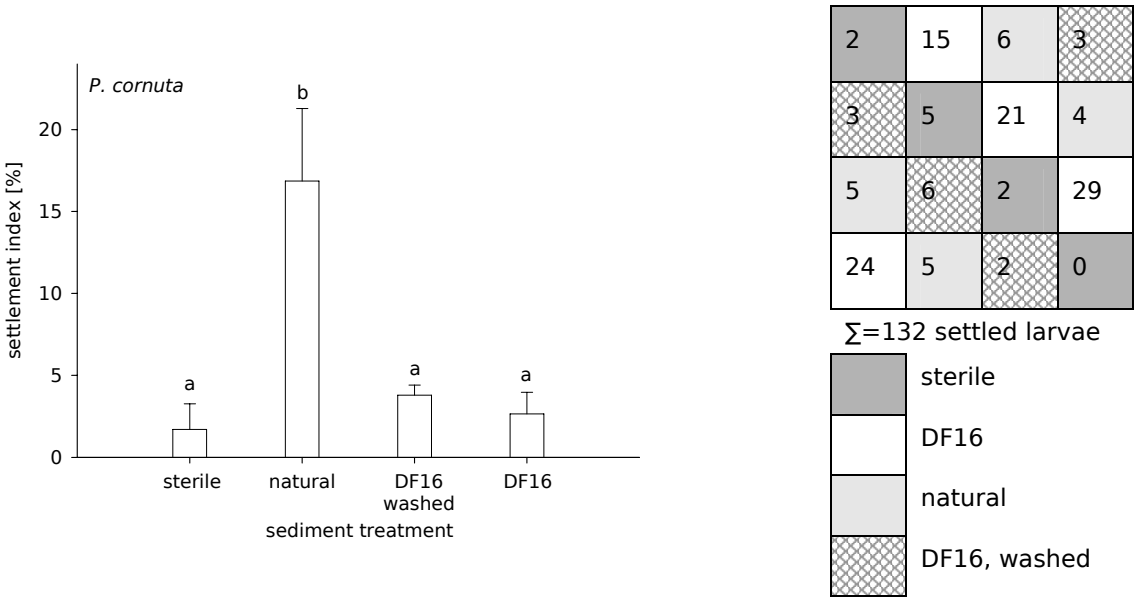


Fig. 27. *P. cornuta*, Assay 3b. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment and sediments recolonized with different densities of the strain DF16. Bacterial cell densities were 1.9 X 10^7 cells in the "strain DF16." treatment and 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 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 4a: 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. 28) 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 ($p < 0.01$).

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. 29) and did not differ from the natural sediment control ($p = 0.92$). 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 ($p = 0.28$).

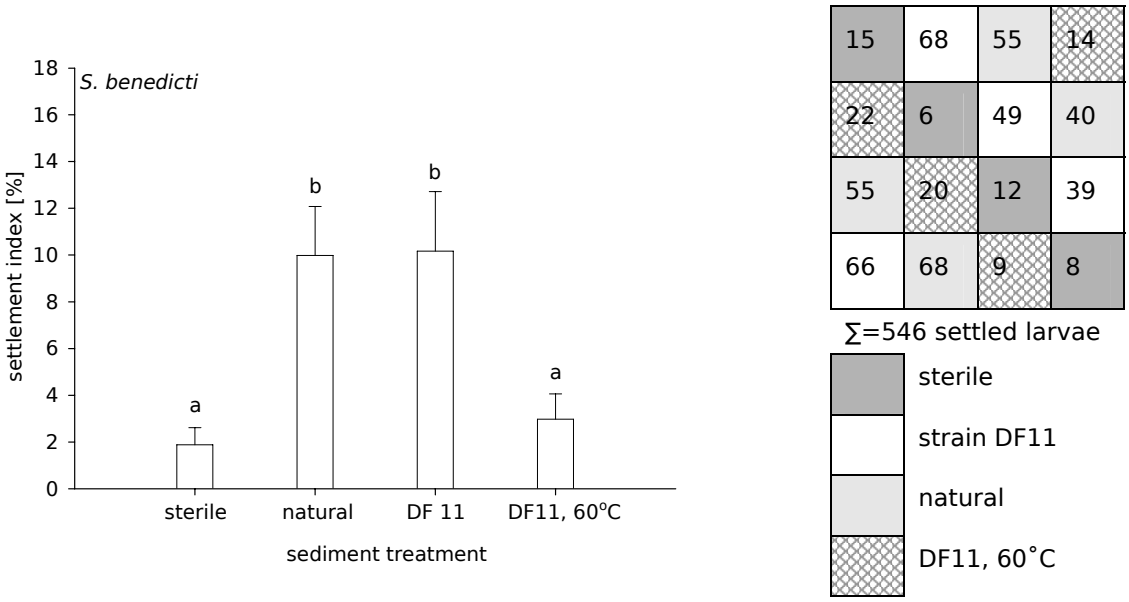


Fig. 28. *S. benedicti*, Assay 4a. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment, sediment recolonized with strain DF11 and heated sediment formerly recolonized with strain DF11. Bacterial cell densities were 9.2 X 10^8 cells in the "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).

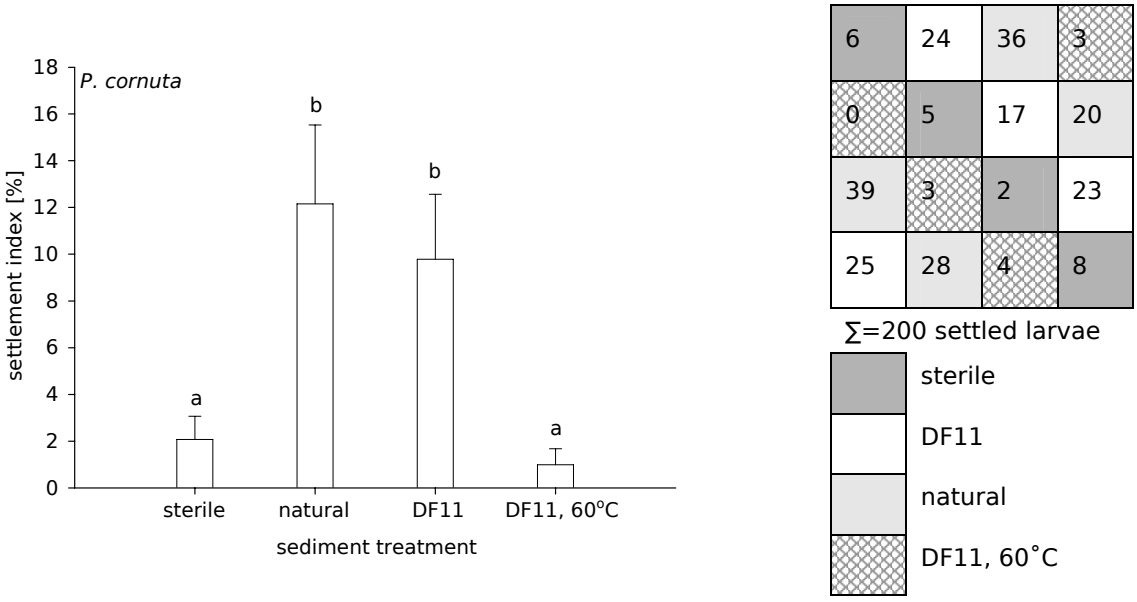


Fig. 29. *P. cornuta*, Assay 4b. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment, sediment recolonized with strain DF11 and heated sediment formerly recolonized with strain DF11. Bacterial cell densities were 5.2 X 10^8 cells in the "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).

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 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. 30) 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 ($p = 0.08$).

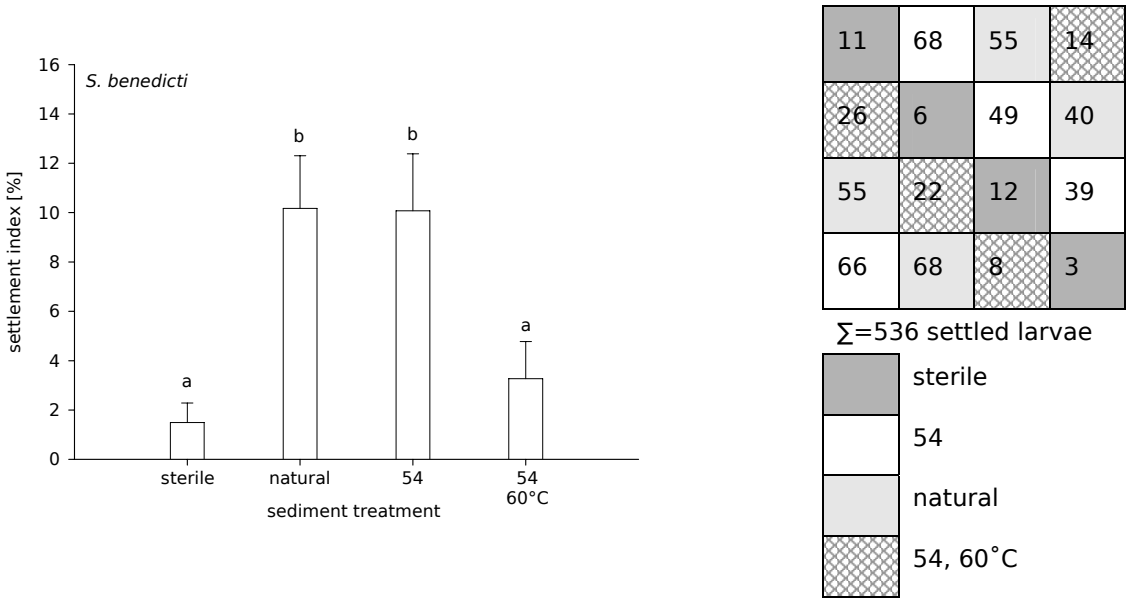


Fig. 30. *S. benedicti*, Assay 5a. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment, sediment recolonized with strain 54 and heated sediment formerly recolonized with strain 54. Bacterial cell densities were 2.7 X 10^8 cells in the "strain 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. 31). Statistical significant differences were recorded between the natural and sterile sediment and the natural and heat treated recolonized sediment ($p < 0.05$).

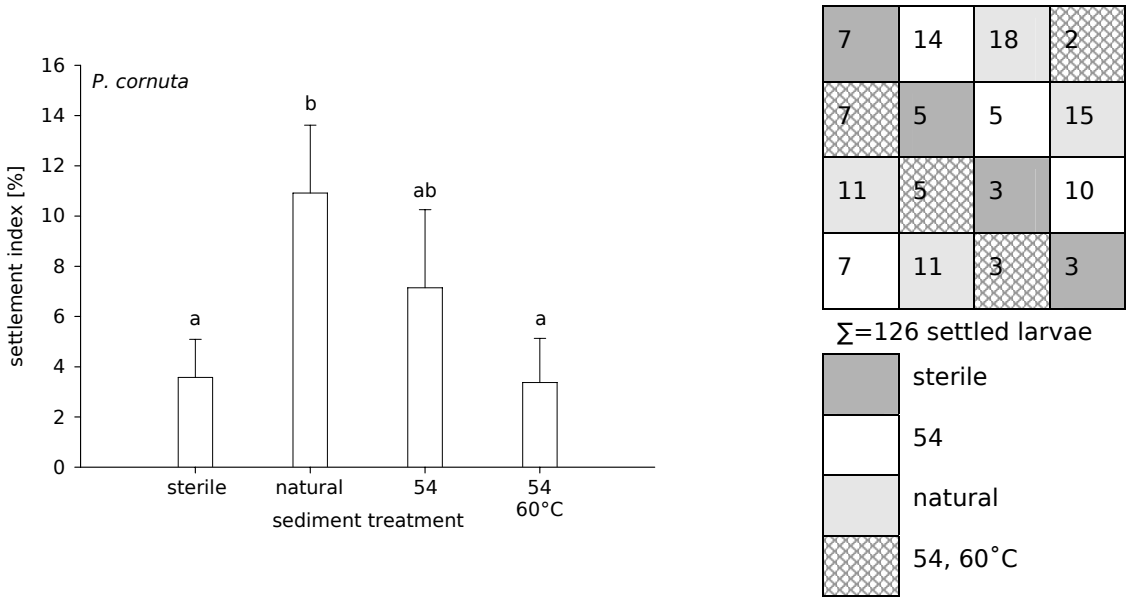


Fig. 31. *P. cornuta*, Assay 5b. Percentage of settlement index per site in a multiple-choice assay. Larval response to natural and sterile sediment, sediment recolonized with strain 54 and heated sediment formerly recolonized with strain 54. Bacterial cell densities were 2.5 X 10^8 cells in the "strain 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 (α = 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 pro gram cells at original concentration, 5 x 10^7 at 10^{-2} dilution, 8 x 10^4 cells 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. 32) and differed significantly from the higher dilutions ($p < 0.05$).

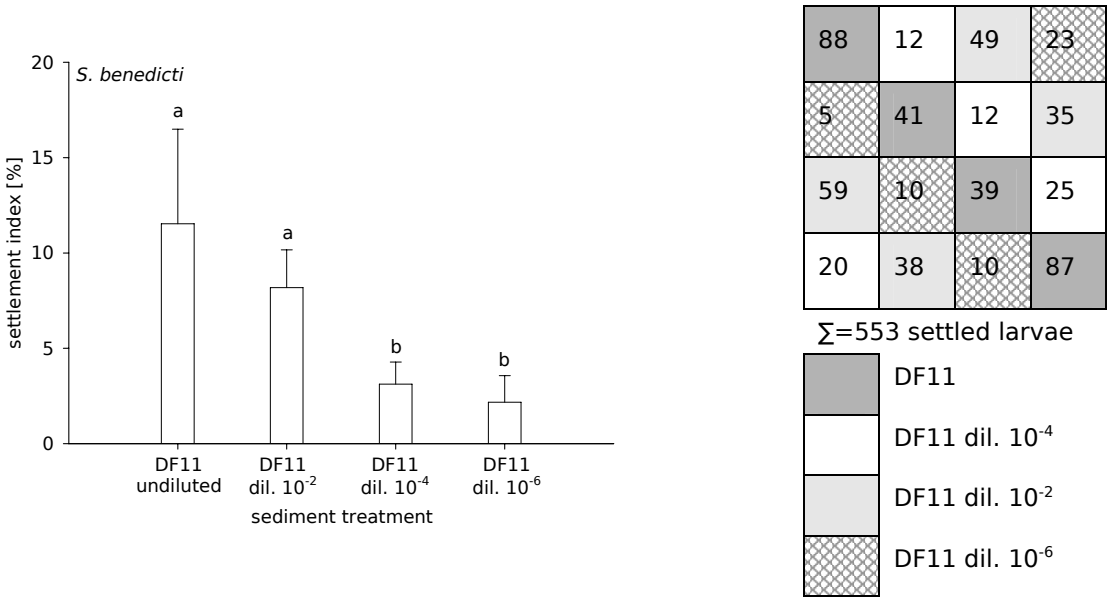


Fig. 32. *S. benedicti*, Assay 6. Percentage of settlement index per site in a multiple-choice assay. Larval response to sediments recolonized with different densities of the strain DF11. Bacterial cell densities were 1.8 X 10^9 cells in the "strain DF11" treatment, 5 X 10^7 cells in the "strain DF11 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).

4.3.5 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 α = 0.05 (1-w-ANOVA). There was no effect of bacterial culture conditions (marine broth vs. sediment/seawater suspension and shaking vs. stationary conditions) on larval settlement.

4.3.6 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 α = 0.05 level (one-way ANOVA).

4.4 DISCUSSION

The recolonization of ashed sediment with viable microorganisms obtained from natural sediment was rarely successful: regularly only a low amount of bacteria was attached on the sediment surface after the sediment treatment. In two

experimental series, which have been carried out with microorganisms obtained from natural sediment collected in summer, a moderate attachment success was achieved. These treatments evoked a pronounced increase of larval settlement in comparison to the control of ashed sediment in *Polydora cornuta* and *Streblospio benedicti* (Fig. 17) indicating that larval settlement of both species was at least partially mediated by the presence of microorganisms. To investigate the phenomenon of bacterially influenced settlement in these spionids closer I isolated bacteria from the habitat of adult spionids and recolonized ashed and sterile sediments with single bacterial species.

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 I were obligated to base the experiments on sterilized natural sediment. Since larvae of *Streblospio benedicti* display high settlement rates in sterile sediment in the no-choice assay (Chapter 3.3.1) this spionid was not a suitable test organism in no-choice recolonization-assays. Consequently, no-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 no-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 no-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 (Chapter 3.3.2) 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 (Fig. 22 - Fig. 31). 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 (Fig. 26 - Fig. 27). 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 no-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. 24, 25 and 32). 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).

5 DO CONSPECIFICS TRIGGER LARVAL SETTLEMENT?

5.1 INTRODUCTION

In Chapter 2, uneven spatial distribution in larval settlement of *Polydora cornuta* within four replicates of the same treatment was reported in multiple choice assays. At that stage I hypothised that uneven or patchy settlement among the replicates of the same treatment was due to a potential interaction between settling individuals of *P. cornuta*. Generaly, 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. In the case of tube dwelling organisms mostly tolerance and promotion was reported (Gallagher et al. 1983, McCann and Levin 1989). The observed settlement patterns in *P. cornuta* may be due to the promotion of interspecific settlement, which is often referred to as gregariousness. Gregarious settlement was firstly described by Knight-Jones (1953b) in the barnacle *Balanus balanoides*. He observed that "*isolated bare surfaces collect abnormally undiscriminating pioneer settlers, which are soon followed by gregarious individuals.*" This observation was later revisited by several authors reporting gregarious settlement in various hard-substrate settlers e.g. in the polychaetes *Phragmatopoma californica* (Jensen and Morse 1984, 1990), *Hydroides dianthus* (Scheltema et al. 1981, Toonen and Pawlik 1996), *Hydroides ezoensis* (Okamoto et al. 1998) and in the slipper limpet *Crepidula onyx* (Zhao and Qian 2002). Gregariousness was also reported in infaunal species e.g. in the sand dollar *Dendraster excentricus* (Highsmith 1982, Burke 1984), in the echiuran *Urechis caupo* (Suer and Phillips 1983) and in the polychaete *Pectinaria koreni* (Olivier et al. 1996).

Cues for gregarious settlement may be either waterborne or surface associated. In some tube building polychaetes like *Phragmatopoma californica* (Sabellariidae) and *Hydroides ezoensis* (Serpulidae) the cue was associated with tubes from conspecifics. The cue for *P. californica* was heat labile and recognized by the larvae at direct contact with the tube (Jensen and Morse 1984, Okamoto et al. 1998). Contrary, larvae of the serpulid polychaete *Hydroides dianthus* settle gregariously in presence of a waterborne compound (Toonen and Pawlik 1996). The water-soluble cue was not associated with the tube, but rather with the body of live adults. Settlement in response to live conspecific adults and their

amputated tentacular crowns was significantly higher than settlement in response to dead worms or empty tubes. A single adult was equally capable to elicit settlement as were five or 25 conspecifics (Toonen and Pawlik 1996).

In soft sediments, in addition to conspecific organisms and their tubes the sediment substrate may accumulate and harbor chemical substances inducing larval settlement. Sediment obtained from adult fiddler crab (*Uca pugnax*) habitats stimulates conspecific megalopae to metamorphose (molt). Already short incubation of non-inductive sediments by adult crabs enhanced the molting rate of megalopae significantly. Obviously, adult crabs release chemical cues that are retained by sediments and stimulate molting of megalopae (O´Connor and Van 2006). Similarly, sediment becomes attractive to the larvae of *Dendraster excentricus*, *Urechis caupo* and *Golfingia misakiana* after exposure to adults (Highsmith 1982, Suer and Phillips 1983, Burke 1984). Contrary, in *Arenicola marina* the presence of adults negatively influences the settlement of juveniles, whereas the presence of juvenile conspecifics positively influenced the settlement (Hardege et al. 1998). Obviously, early recruits, juveniles and adults may elicit different larval response at settlement. Keough (1998) measured the settlement onto experimental substrata using known densities of recruits of nine taxa as potential settlement cues. These residents had weak effects on subsequent settlement processes. Newly settled juveniles of *Hydroides dianthus* began to induce gregarious settlement of conspecific larvae after approximately 96 h (Toonen and Pawlik 1996). However, the influence of early recruits on larval settlement is almost completely unexplored.

In this chapter I revisited my hypothesis that patchy distribution of settled larvae of *Polydora cornuta* within treatment of same quality in the multiple choice assay was due to gregarious larval settlement. In particular I focused on the role of conspecific adults and of different constituents of the adult habitat such as sediment, tubes and adult worms on the settlement rate.

5.2 MATERIAL AND METHODS

Larval maintenance, sediment treatments and all assay procedures are in accordance to the methodology described in Chapter 2 and 3 with the following additions: in one of the assays sediment from the sandy intertidal flat Janssand (southwest of Spiekeroog Island) was used. In addition to the spionids the capitellid polychaete *Capitella* sp. I was employed in some assays. *Capitella* sp. I

was obtained from the Institute of Marine and Coastal Sciences at Rutgers State University, New Jersey, USA. The polychaete cultures were kept at 16 ˚C in aerated, filtered natural seawater with a salinity of approximately 32 psu. Adults were feed with sterilized mud collected at Hooksiel.

Assays were carried out to address the following objectives:

1) Larval response to the presence of conspecific adults.

To investigate the influence of the presence of conspecific adults on larval settlement the response to four different treatments, i.e. sterile sediment, sterile sediment with 3 adults of *Polydora cornuta*, sterile sediment with 10 adults of *Capitella* sp. or *Streblospio benedicti* were investigated in the multiple choice assay with the same batch of larvae of both spionid species with replication (n = 2). Different numbers of adults were choosen according to size variances among the three polychaete species.

2) Larval response to the presence of different constituents of the adult habitat e.g. adult worms, empty tubes and sediment.

To investigate the influence of different constituents of the adult habitat on larval sediment the response to four different treatments e.g. sterile sediment, sterile sediment with 3 adults of *Polydora cornuta*, sterile sediment with 3 empty tubes from *P. cornuta* and sediment formerly inhabited by *P. cornuta* in the laboratory polychaete culture was assayed in the multiple choice assay with the same batch of *P. cornuta* larvae. Empty tubes and sediment was freshly obtained from the laboratory polychaete culture. Sediment was sieved (mesh 250 µm) bevore usage to exclude juveniles and adults.

3) Larval response to natural sediment from the field vs. laboratory culture sediment formerly inhabited by adults of *P. cornuta.*

To investigate if larvae preferred fresh sediment from the field over laboratory culture sediment formerly inhabited by adult polychaetes the response to four different treatments, i.e. fresh natural sediment from Hooksiel or Janssand and laboratory culture sediment formerly inhabited by adults of *P. cornuta* or *Capitella* sp. was assayed in the multiple choice assay with the same batch of *P. cornuta* larvae.

4) Larval response to conspecific adults vs. juveniles.

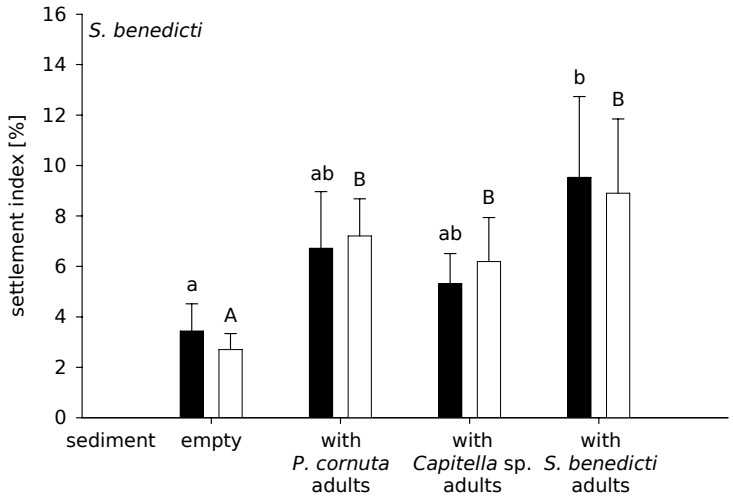
To investigate if larvae preferred habitats inhabited by juveniles or adults the response to four different treatments, i.e. sterile sediment, sterile sediment with 3 adults of *Polydora cornuta*, sterile sediment with 10 juveniles of *P. cornuta* and

sterile sediment with 3 empty tubes from adults of *P. cornuta* (freshly obtained from the lab culture) were assayed in the multiple choice assay with the same batch of *P. cornuta* larvae.

5.3 RESULTS

5.3.1 LARVAL RESPONSE TO CONSPECIFIC ADULTS

In four out of six cases larval settlement of *Streblospio benedicti* in sediments inhabited by adults was higher than in sterile sediment void of conspecific adults ($p < 0.05$, Tukey's test, Fig. 33, Tab. 7).



Average settlement per site and treatment after 20 h in response to four different treatments i.e. empty sterile, sterile with adults of *P. cornuta*, *Capitella* sp. and *S. benedicti*. Statistically significant differences are indicated by different letters above the bars (α = 0.05, Tukey's test) by using upper and lower case letters for different assays. Total numbers of settled larvae in the assays were 160 (black bars) and 222 (white bars).

Fig. 33. *S. benedicti*. Percentage of settlement index per site in a multiple-choice assay.

Larval settlement of *S. benedicti* in sediment inhabited by conspecific adults did not differ statistically from sediments inhabited by adult *Capitella* sp. or *Polydora cornuta* (Tukey's test, $p = 0.21$).

Tab. 7. Assay design and number of settled larvae of *S. benedicti* in the multiple-choice assay. Data for Assay 1 are related to the black bars and Assay 2 to the white bars of Fig. 33.

Assay 1 (black bars)

7	16	11	12
10	6	9	9
7	18	3	10
8	7	21	6

∑ settled larvae = 160

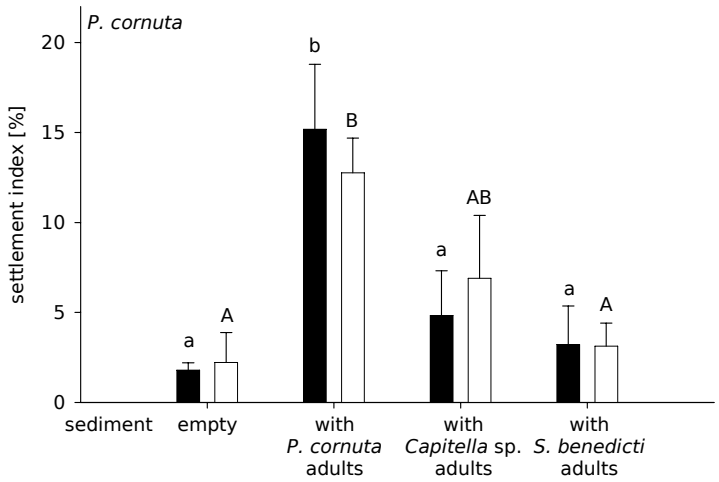
Assay 2 (white bars)

6	20	18	15
22	7	16	16
10	28	7	12
16	11	14	4

∑ settled larvae = 222

Empty sediment
adult *P. cornuta*
Adult *Capitella* sp.
adult *S. benedicti*

In both assay series larval settlement of *Polydora cornuta* was higher in sediment inhabited by conspecific adults than in all other treatments (Tukey's test, $p < 0.05$, Fig. 34, Tab. 8) except of the sediment inhabited by *Capitella* sp. in the second assay (Tukey's test, $p = 0.34$).



Average settlement per site and treatment after 20 h in response to four different treatments i.e. empty sterile, sterile with adults of *P. cornuta*, *Capitella* sp. and *S. benedicti*.
Statistically significant differences are indicated by different letters above the bars (α = 0.05, Tukey's test) by using upper and lower case letters for different assays. Total numbers of settled larvae in the both assays were 140 (black bars) and 192 (white bars).

Fig. 34. *P. cornuta*. Percentage of settlement index per site in a multiple-choice assay.

Tab. 8. Assay design and number of settled larvae of *P. cornuta* in the multiple-choice assay. Data for assay 1 are related to the black bars and assay 2 to the white bars of Fig. 34.

Assay 1 (black bars)

2	23	11	9
3	2	20	8
3	3	3	15
27	5	3	3

∑ settled larvae = 140

Assay 2 (white bars)

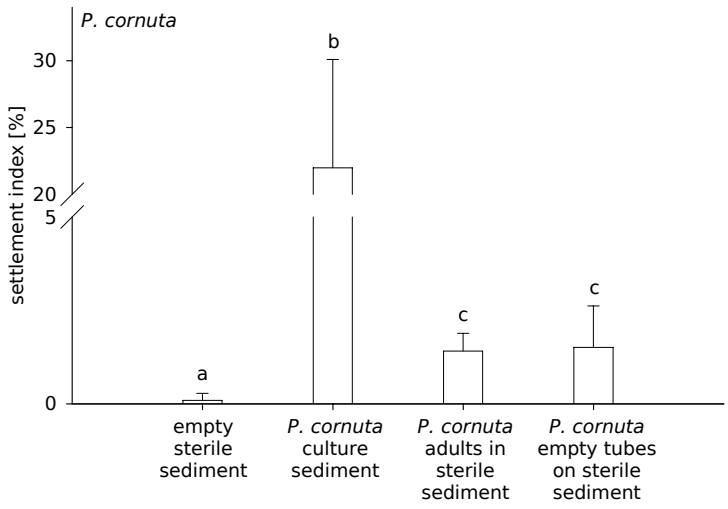
3	23	20	4
9	3	20	8
7	7	2	28
27	18	4	9

∑ settled larvae = 192

empty sediment
adult P. cornuta
Adult Capitella sp.
adult S. benedicti

5.3.2 LARVAL RESPONSE TO DIFFERENT CONSTITUENTS OF THE ADULT HABITAT

In *Polydora cornuta* larval settlement in sediment formerly inhabited by conspecific adults was significantly higher than in all other treatments i.e. sterile sediment, sterile sediment with three conspecific adults and sterile sediment with three empty tubes of conspecific adults ($p < 0.001$, Tukey's test, Fig. 35).



1	54	4	7
4	0	31	5
4	0	0	66
82	2	5	0

∑= 265 settled larvae

sterile sed.
culture sed.
adults
empty tubes

Fig. 35. *P. cornuta*. Percentage of settlement index per site in a multiple-choice assay. Average settlement per site and treatment after 20 h in response to four different treatments i.e. empty sterile sediment, sediment formerly inhabited by cultured *P. cornuta*, sterile sediment with adults of *P. cornuta* and sterile sediment with empty tubes of *P. cornuta.* Statistical differences are indicated by different letters above the bars ($\alpha = 0.05$, Tukey's test). Total number of settled larvae in the both assays was 265.

Settlement rates in sediments containing adults or empty tubes did not differ from each other (p = 0.96, Tukey's test) but were significantly higher than in sterile sediment (p < 0.05, Tukey's test).

Larval settlement in the sediment formerly inhabited by adults was higher than in all other treatments i.e. natural sediment from Hooksiel and Janssand and sediment formerly inhabited bei *Capitella* sp. I (p < 0.05, Tukey's test, Fig. 36). Settlement rates in the latter three treatments did not differ from each other (p = 0.95, Tukey's test).

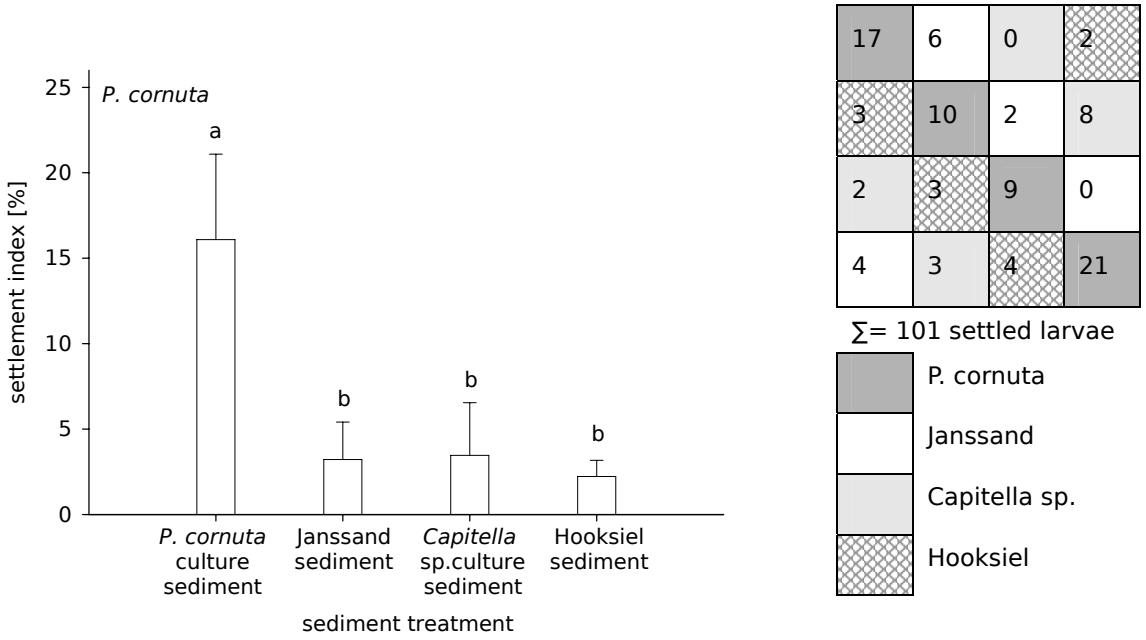


Fig. 36. *P. cornuta*. Percentage of settlement index per site in a multiple-choice assay. Average settlement per site and treatment after 20 h in response to four different treatments e.g. sediment formerly inhabited by cultured *P. cornuta* or *Capitella* sp., natural sediment from Hooksiel or Janssand. Statistical differences are indicated by different letters above the bars (α = 0.05, Tukey's test). The total number of settled larvae was 101.

5.3.3 LARVAL RESPONSE TO CONSPECIFIC ADULTS AND JUVENILES

In this assay Levene's test for homogeneity of variances revealed heterogeneity of the data (p = 0.048), therefore a lower significance level (p=0.01 instead of p=0.05) was used. At this significance level settlement in all treatments were statistically the same. Larval settlement tended to be higher in the sediment containing adults than juveniles and to be higher in these both treatments than in sterile sediment or sediment with empty tubes.

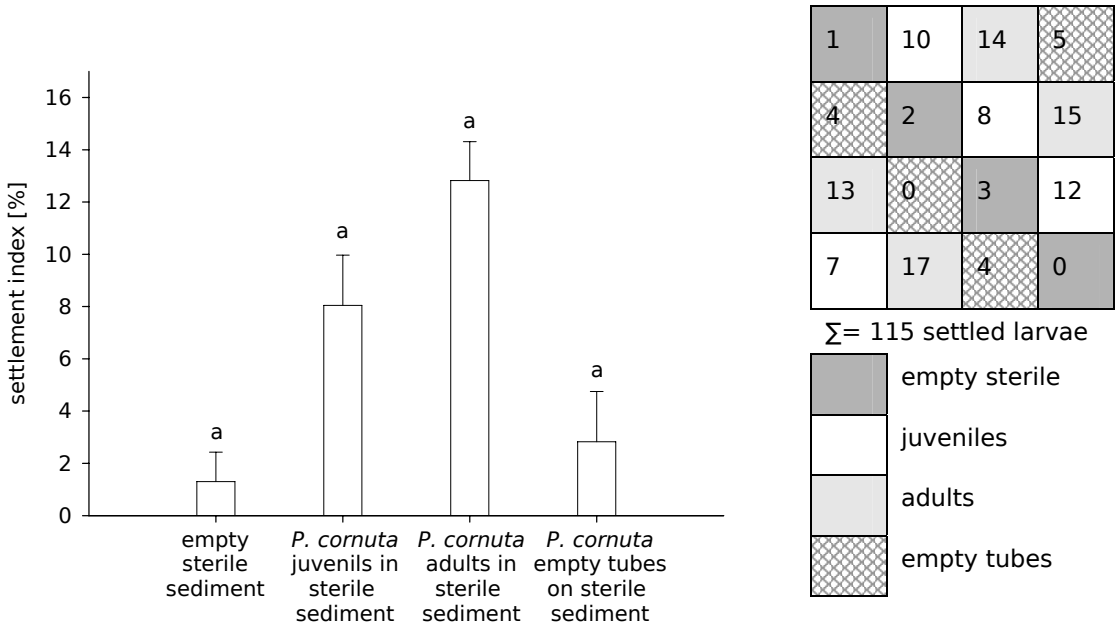


Fig. 37. *P. cornuta*. Percentage of settlement index per site in a multiple-choice assay. Average settlement per site and treatment after 20 h in response to four different treatments e.g. empty sterile sediment, sterile sediment with juveniles or adults of *P. cornuta* and sterile sediment with empty tubes of *P. cornuta.* Statistical differences are indicated by different letters above the bars (α = 0.01, Tukey's test). Total number of settled larvae was 115.

5.4 DISCUSSION

To experimentally address whether gregarious settlement of *Polydora cornuta* influenced the assay results sediments without adults were assayed against sediments containing either conspecific adults or adults of *Capitella* sp. I and *Streblospio benedicti*, respectively. Sediment inhabited by adult *P. cornuta* triggered significantly higher settlement than any of the other treatments (Fig. 34). Contrary, in a similarly designed assay larvae of *S. benedicti* did not prefer sites with conspecific adults over sites with adults of *P. cornuta* or *Capitella* sp. I (Fig. 33). Comparing the influence of conspecific adults and juveniles on larval settlement of *P. cornuta*, there was a slight trend of higher settlement in sites inhabited by adults but this conclusion is not backed stastically.

Given the results of these experiments, the settlement response in *P. cornuta* larvae to different constituents of the adult habitat such as live adult worms, empty tubes and sediments conditioned by adults was investigated. Larval settlement in the sediment formerly inhabited by adults was significantly higher than in all other treatments whereas the presence of adults or empty tubes

enhanced larval settlement only slightly in comparison to the negative control sterile sediment (Fig. 35). Sediment conditioned with conspecific adults was highly inductive for larval settlement even in comparison to fresh natural sediment from two different sampling sites (Fig. 36). Furthermore, *P. cornuta* larvae did not prefer sediment formerly inhabited by *Capitella* sp. I (Fig. 36). This opportunistic, deposit-feeding polychaete is numerically dominant in disturbed and enriched sediments and rarely co-occurs with other abundant mud-dwelling macrofauna (Grassle et al. 1992). In my observations, adults were able to pelletize the sediment almost completely, leading to the depletion of organic matter. Individuals of *Capitella* sp. I were able to quickly take over a culture chamber formerly inhabited by *Streblospio benedicti*, if larvae were accidentally mixed up, whereas in the culture chambers inhabited by *P. cornuta* the assertiveness of *Capitella* sp. I was much lower.

These results suggest that larval settlement of *Polydora cornuta* is influenced by gregariousness and that the settlement signal is mainly harbored in sediments formerly inhabited by conspecific adults. Such a strong response to formerly adult-associated sediments has been shown in *Uca pugnax*, in *Dendraster excentricus*, *Urechis caupo* and *Golfingia misakiana* (Highsmith 1982, Suer and Phillips 1983, Burke 1984, O´Connor and Van 2006).

The presence of juveniles also evoked higher settlement than the negative control, but here the heterogeneity of the data caused a loss of statistical power and therefore a definitive conclusion could not be drawn. At this stage, it remained unclear if and how the presence of conspecific larvae might have influenced the outcomes of the settlement assays of my study. To address this issue in more detail bioassays with different numbers of participating larvae have to be performed. Head et al. (2003) quantified the effect of gregariousness within settlement assays with barnacle cyprids by using different container sizes and cyprid numbers. They detected significant gregarious effects with more than 5 cyprids in a single well. However, I argue that by using comparable conditions among different settlement assays regarding the size of the assay-chamber and the numbers of participating larvae, the multiple-choice assay used in my study is a valuable tool for larval settlement studies.

6 CONCLUSION

In summary, the results to the individual objectives raised initially are given below:

➧ Do larvae of *Polydora cornuta* and *Streblospio benedicti* actively accept or reject different sediment types? Is there any behavioral evidence that the patchy distribution in field is caused by larval choice?

I have shown that larvae of both species were able to accept or reject sediments in no-choice assays and choose actively between different sediment qualities in the multiple-choice assay. Under flow regimes in the field larval settlement will be strongly influenced by hydrodynamic forces. However, it appears likely that on small scale larval settlement of these polychaetes is under behavioral control.

➧ Do larvae respond to signals derived from bacteria associated with sediment?

In both spionid species sediment associated bacteria act as a positive settlement cue. Out of 15 pure isolates from the habitat of adult polychaetes, the strains strain DF11 and 54 have been demonstrated to trigger larval settlement. 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. The inductive effect was influenced by the cell density of bacteria in a species specific manner.

➧ Do larvae detect settlement signals in the water column or on the sediment surface?

In case of strain DF11 the signal triggering larval settlement was detected on the sediment surface and likely originated from insoluble, surface-associated and heat labile microbial constituents.

➧ Does the presence of conspecifics influence larval settlement? Do larvae behave gregariously at settlement?

My results suggest that the settlement of *Polydora cornuta* is influenced by gregarious behavior and that the signal is mainly harbored in sediments formerly inhabited by conspecific adults. In *Streblospio benedicti* gregarious behavior does not influence larval settlement.

In summary, the spionid polychaetes *Polydora cornuta* and *Streblospio benedicti* have been demonstrated to be suitable for settlement bioassays. Both species reproduce in the laboratory, release planktotrophic larvae and thus allow experiments throughout the year. Comparing the suitability of both species,

S. benedicti was less selective at settlement by accepting even less attractive sediments (e.g. sterile sediment) in no-choice assays. However, if *S. benedicti* was allowed to choose between different sediment qualities, selectivity of this spionid did not fall behind the selectivity observed in *P. cornuta*. On the other hand, the use of *P. cornuta* larvae harbors some disadvantages. Firstly, the yield of competent larvae is relatively low due to high larval mortality during a long developmental phase of three weeks resulting in small numbers of test larvae and low frequencies of assay-events. Secondly, the patchy settlement of *P. cornuta* larvae within replicates of the same sediment treatment causes high standard deviations and thus weakens the statistical significance of the achieved results. Therefore, the usage of *S. benedicti* larvae in a manner of multiple choice assays would be preferable in the future.

Comparing the two assay types, while the no-choice assay allows a fast screening of numerous treatments, the multiple-choice assay is highly time-consuming but more powerful to reveal minute differences among treatments. A clear disadvantage of the no-choice assay is its high sensitivity to differences in larval competence among the same batch of test larvae, i.e. larvae estimated wrongly as competent and used in this assay will falsify the results. However, the different settlement behavior of *S. benedicti* larvae in sterile sediment in both assay types points to the necessity of different experimental approaches and thus choice in assays designs. Given the feasibility of larval settlement assays with *P. cornuta* and *S. benedicti* laboratory experiments under flow conditions would be adventageous in the future to approximate hydrodynamic conditions in the field at larval settlement.

Although generally the use of field experiments is highly recommended in ecological studies, I used exclusively laboratory assays in my study. This decision derived from my specific objectives namely initial larval settlement due to bacterial cues. In the field, differential mortality after settlement and post-settlement events may mask the actual initial settlement choice of exploring larvae making larval settlement and recruitment inseparable. In the laboratory assay, differential mortality and post-settlement events can be ruled out. Studying the effect of bacterial cues only laboratory assays on sediments filmed with a single bacterial species can lead to the identification of specific components of a biofilm that may be an important cue to larval settlement in the field. However, I admit that laboratory experiments are inappropriate if direct

inferences on larval responses to biofilms under natural condition are to be drawn. For example, the analyses of bacterial community profiles of inhabited versus not inhabited sites by a specific polychaete will obviously lead to the "Chicken or the egg" dilemma due to obligate changes in the microbial community of inhabited sites as a consequence of settlement. A possible strategy to circumvent this disparity could be to assay adequate but not colonized habitats (called empty habitats, Armonies and Reise 2003) for larval settlement. Some of the empty habitats are not colonized due to the lack of larval supply or disturbances after settlement whilst others have been rejected by exploring larvae. By analyzing bacterial communitiy patterns of empty sites which are accepted or rejected by larvae in the laboratory, a theoretical bacterial fingerprint of a repellent or inductive site may be deduced.

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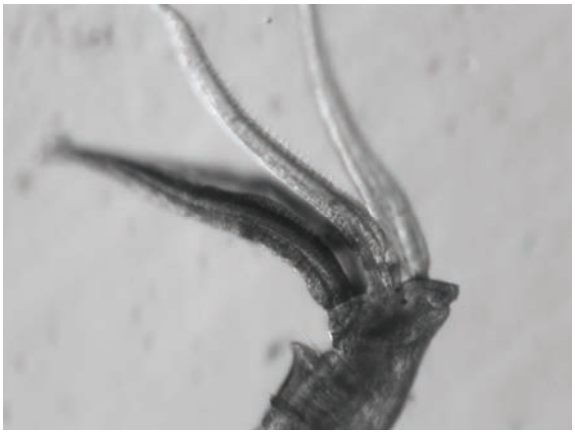

So long, and thanks for all the worms!

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ERKLÄRUNG

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