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Conservation and restoration of a keystone species: Understanding the settlement preferences of the European oyster (*Ostrea edulis*)

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ABSTRACT

The European oyster *Ostrea edulis* is a keystone species that is internationally recognised as ‘threatened and declining’ in the NE Atlantic by OSPAR and several nations have consequently adopted strategies for its conservation and restoration. Understanding the settlement behaviour of *O. edulis* larvae is crucial to inform these strategies. We compared the efficiency of several treatments in triggering settlement. The most effective settlement occurred with the presence of conspecifics: 100% settled in < 23 h. Marine stones with habitat-associated biofilms induced 81% settlement that started after a 45 h delay. Sterile shells and terrestrial stones did not induce more settlement than control treatments. These results indicate that *O. edulis* larvae are gregarious and finely-tuned to settle in response to cues which are indicative of their adult habitat requirements. The role of chemical cues in mediating settlement, and the importance of this to restoration, are discussed.

1. Introduction

The European native oyster *Ostrea edulis* (Linnaeus, 1758) once formed extensive beds along Europe's coastline that constituted a highly significant resource for coastal populations and were probably a dominant ecological component (Thurstan et al., 2013; Gercken and Schmidt, 2014; Kent et al., 2016; Fariñas-Franco et al., 2018). Shellfish, such as oysters, can be keystone species that provide habitat, refuge and foraging ground for many invertebrates and vertebrates, some with commercial value, while their filter-feeding behaviour contributes to benthopelagic coupling (Coen et al., 2007; Kent et al., 2017a; Kent et al., 2017b). As a result, oyster beds greatly increase biodiversity and trophic complexity and induce a shift from an ecosystem dominated by microbial and planktonic organisms to predominantly benthic flora and fauna (Grabowski and Peterson, 2007). *O. edulis* beds were recognised for their species richness (Möbius, 1877; Caspers, 1950; Korringa, 1954) in an otherwise sedimentary environment (e.g. Korringa, 1940; Caspers, 1950) and this biodiversity probably mediated effective coastal ecosystem functioning and productivity (Worm et al., 2006; Heip et al., 2009). Moreover, the abundant oyster populations would have enhanced water clarity through their filter-feeding behaviour

(Cressman et al., 2003; Grabowski and Peterson, 2007).

Harvesting of *O. edulis* contributed to food security since prehistoric times (Kristensen, 1997; Gercken and Schmidt, 2014), and in the 13th century it was one of the first commercially operated fisheries in Europe (Lotze, 2007). At its peak production in the 19th century, 700 million European oysters were consumed annually in London alone (Philpots, 1891); underlining the former scale of these beds and the importance of *O. edulis* to local economies. However, by the mid-20th century demand for *O. edulis*, combined with coastal degradation, water pollution and disease outbreaks, led to the decline of this species throughout its distribution range (Airoldi and Beck, 2007; Thurstan et al., 2013; Gercken and Schmidt, 2014).

Today, *O. edulis* beds are rare or absent in most of their natural range (Laing et al., 2005; Airoldi and Beck, 2007; Low et al., 2007; Haelters and Kerckhof, 2009; Gercken and Schmidt, 2014). *O. edulis* has therefore been listed as a ‘Threatened and Declining species’ by the OSPAR convention for the Protection of the Marine Environment of the North-East Atlantic (Haelters and Kerckhof, 2009). OSPAR member nations have adopted national legislation and policies for the protection and conservation of *O. edulis*, with the broader aim of achieving biodiversity goals, restoring ecosystem functions and enhancing ecosystem

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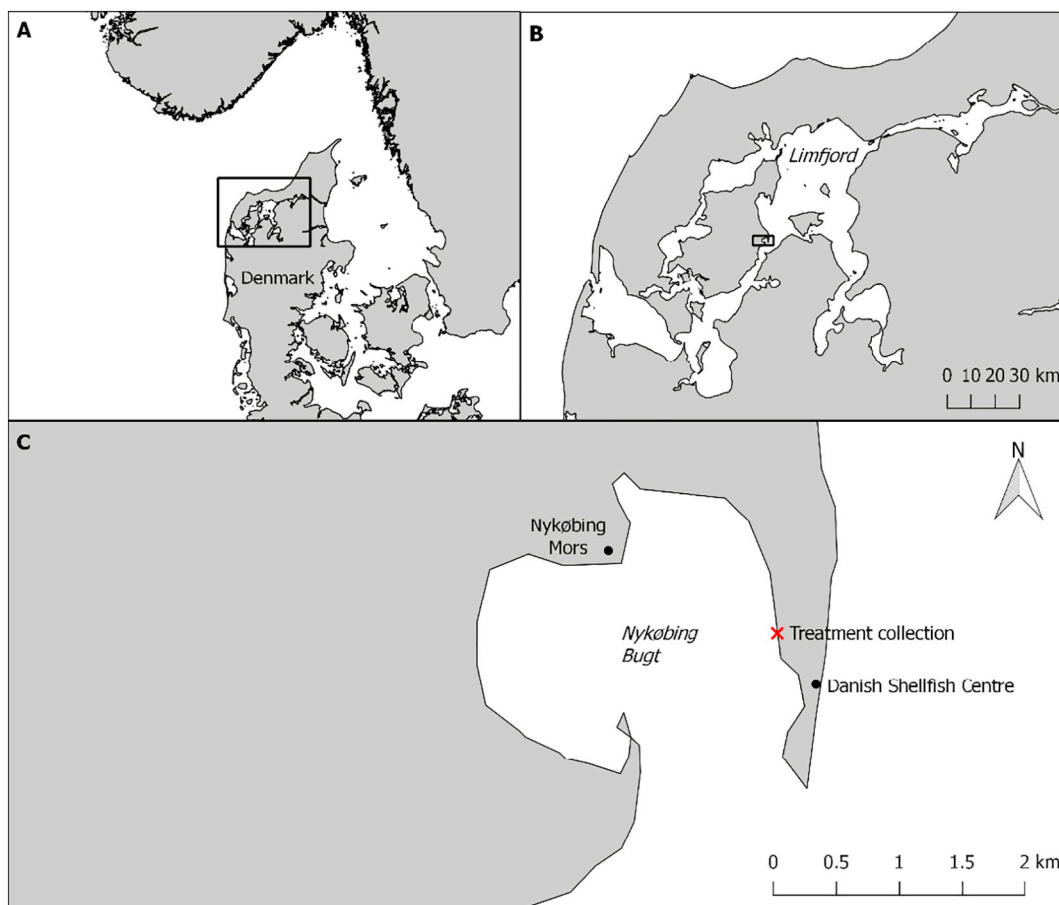


Fig. 1. Collection site of 'Stone' and 'Biofilm stone' treatments. (A) Location of the Limfjord within Denmark; (B) Map showing the Limfjord with the collection area surrounded by a black box; (C) Close-up of treatment collection site.

services (e.g. Smaal et al., 2015). In the UK, *O. edulis* has been included in the UK Biodiversity Action plan, it is a protected feature of the UK Marine Protected Area Network, a species of principal importance in England and Wales, and a priority marine feature (PMF) in Scotland (Donnan et al., 2016; Perry and Jackson, 2017; JNCC, 2018). To conserve *O. edulis*, OSPAR member nations are following two recommendations (Haelters and Kerckhof, 2009): 1) protecting, maintaining and expanding remnant *O. edulis* populations (e.g. Low et al., 2007; Shelmerdine and Leslie, 2009; Donnan et al., 2016; Kerckhof et al., 2018); and 2) restoring *O. edulis* to areas they once occupied (e.g. Gercken and Schmidt, 2014; Smaal et al., 2015; Fariñas-Franco et al., 2018).

Understanding the settlement of *O. edulis* larvae is crucial in developing strategies for protecting extant populations and restoring former oyster beds. Availability of suitable settlement substrate is considered to be one of the principal factors governing recruitment success of oyster populations (Möbius, 1877; Korringa, 1946; Low et al., 2007; Smyth et al., 2018), and its lack may constrain the expansion of natural or restored oyster beds (Möbius, 1877; Korringa, 1946). Knowledge of settlement cues is therefore critical to providing adequate settlement substrate. Understanding larval settlement is also important to model larval connectivity between beds, which is key to informing the design and management of protected area networks and restoration sites. This is because oyster larvae can delay metamorphosis if suitable settlement cues are absent (Cole and Jones, 1939; Coon et al., 1990), thereby altering dispersal and connectivity between populations.

The settlement preferences of *O. edulis* larvae have been extensively studied, in the 20th century, in an effort to revive commercial oyster culture (reviewed in Korringa, 1952). These studies found that,

although larvae were able to attach to a wide range of hard substrates, certain surfaces such as shells, tiles coated with lime, or lime and sand mixture performed better, while smooth surfaces such as glass and seaweed, were intrinsically unsuitable (Cole and Jones, 1939). A preference for shell substrate has also been repeatedly cited (e.g. Cole and Jones, 1939; Laing et al., 2005; Low et al., 2007; Smyth et al., 2018), and commercial hatcheries have therefore developed small shell fragments as their prime substrate to promote settlement in *O. edulis*. Most of these studies were aimed at enhancing commercial production of individually settled oysters, and many of these results are only partially applicable to natural restoration and conservation scenarios.

The influence of habitat-associated chemical cues on the settlement of *O. edulis* larvae has been neglected. Most studies have focused on non-chemical properties such as substrate type, colour, inclination or light (reviewed in Cole and Jones, 1939; Korringa, 1940; Laing et al., 2005; and Low et al., 2007). However, *O. edulis* was noted to settled preferentially on collectors which already bore some spat of their own species (Cole, 1949; Bayne, 1969), but if the spat were killed the larvae that subsequently settled showed no preference for these collectors (Cole, 1949). Enhanced settlement was also noted if collectors were soaked in water containing *O. edulis* tissue (Bayne, 1969). Biofilms are likely to be another critical chemical cue for *O. edulis* larvae, since they are an excellent indication of habitat type (Unabia and Hadfield, 1999) and known to promote larval settlement in many marine invertebrate taxa (Hadfield, 2011), including other species of oyster (e.g. Tritar, 1992; Campbell et al., 2011). To date only one study has investigated settlement of *O. edulis* larvae in response to biofilms and specifically in response to the bacterium *Shewanella colwelliana* (Tritar, 1992).

The aim of this study was to further our understanding of *O. edulis*

larval settlement guided by natural habitat conservation and restoration scenarios: comparing the efficiency of a range of treatments that could be used. We selected treatments based on previous settlement studies and based on the hypothesis that habitat-associated chemical cues may be critical in inducing settlement. The treatments included juvenile *O. edulis* spat, biofilms formed in a relevant benthic habitat and shell fragments devoid of an appropriate chemical cue. We hypothesised that the treatments would differ in their ability and the speed with which they would induce metamorphosis, thereby reflecting settlement preferences.

2. Materials and methods

2.1. Larval cultures

Adult oysters (*O. edulis*) were obtained from the Limfjord (Fig. 1) and induced to spawn at the Danish Shellfish Centre (DSC) following FAO guidelines (Helm, 2004). Larvae were transferred into 15 L flow-through tanks at an approximate concentration of 10 larvae/mL and raised at 25 °C in 1 µm filtered seawater. They were fed daily a microalgae mixture consisting of *Chaetoceros muelleri*, *Tisochrysis lutea* and *Pavlova gyrans* (volume ratio 5:1:1) at a concentration of circa 100 cells/µL. After approximately 7 days, larvae reached the mature pediveliger stage with eyespot and foot, indicating that they are competent to settle and metamorphose to a spat.

2.2. Experimental design and procedure

Pediveliger larvae were subjected to eight treatments (Table 1). Each treatment was replicated six times and the replicates were randomly assigned into a 16 mL well of eight 6-well culture plates. Four larvae were assigned into each well, with a total of 24 larvae per treatment (Fig. 2). The sea water in each well was not changed for the duration of the experiment, nor was additional food added. Larvae were kept at a room temperature of circa 22 °C and under natural day-night cycles. The behaviour of each larvae was monitored with a binocular microscope for 74 h, starting 1 h after experimental set-up and then approximately every 2.5 h, except during night breaks where intervals were longer (Appendix Table A.1). At each observation, it was noted whether larvae had settled or not, as well as the behaviour of those larvae that had not settled. Behaviours were categorised into 'active', 'not active', 'searching feet' and 'feet'. Category 'searching feet' referred to the stereotypical settlement searching behaviour in which larvae crawled on a surface with extended foot (Cole and Jones, 1939) (Fig. 3a), while 'feet' was when larvae extended their foot without searching. If larvae had settled, it was noted if they were attached, in the process of metamorphosing or fully metamorphosed with secondary

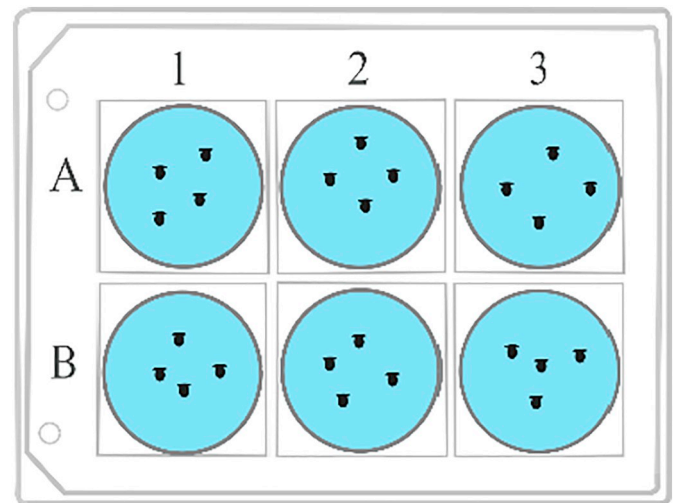


Fig. 2. Diagram of a 6-well culture plate with four larvae per well. Each well had one randomly allocated treatment. Image not to scale.

shell growth (Fig. 3c–e). Sometimes, larvae failed to metamorphose and died after attachment or metamorphosis, in which case they were recorded as 'metamorphosis unsuccessful'. If larvae or metamorphosed spat were not found during an observation round, they were assigned to 'unknown'.

At the end of the experiment, all treatments involving hard structures were lifted and carefully inspected for hidden spat that had settled underneath the settlement media. All spat were measured and their settlement location was noted. Dead larvae were distinguished from 'none active' larvae by a prolonged immobility and faded colour of their inner organs. In those cases where larvae had attached during one of the last observation rounds, metamorphosis was verified 48 h after completion of the experiment.

2.3. Data cleaning

The 'settled' status of larvae were retrospectively validated, and only maintained if larvae metamorphosed, with secondary shell and were still alive at the end of the experiment. In addition, the 'unknown' status was retrospectively reassigned to a 'not settled' or 'settled' status if prior and subsequent observation supported this reallocation. For instance, if a larva was observed to be 'not settled' at a given time point, it could be inferred that all previously recorded 'unknown' statuses were also 'not settled'. If a spat had been observed to be 'settled' before and after it was assigned to 'unknown', it could be inferred that it was

Table 1
Treatments used to study settlement preferences of *O. edulis* larvae.

Treatment	Description
Filtered Sea Water (FSW)	Natural sea water of the Limfjord, filtered to 1 µm and with a salinity of 25.9 ppt. This treatment also served as a control for all other treatments in which FSW was used.
Unfiltered Sea Water (USW)	Natural sea water from the Limfjord. Microalgae concentration in USW was 135 ± 25 cells/µl (mean \pm se, estimated using a Haemocytometer Neubauer counting chamber). Salinity 25.0 ppt. This treatment also served as a control for another treatment in which USW was used.
FSW Food	FSW with microalgae at an initial concentration of circa 100 cells/µl: <i>Chaetoceros muelleri</i> , <i>Tisochrysis lutea</i> and <i>Pavlova gyrans</i> at a volume ratio of 5:1:1.
FSW Shell	FSW with 300–400 µm shell pieces covering bottom of well (product name: 'Microbrisure 300/400 µ' from Ovine). Shells predominantly from <i>Crassostrea gigas</i> , and sterilised at 500 °C, dried, crushed and sieved. Substrate typically used in hatcheries to induce settlement of <i>O. edulis</i> larvae.
USW Shell	Same treatment as 'FSW Shell' but with USW instead of FSW.
FSW Biofilm Stone	FSW and a marine stone of 1–2 cm diameter with a natural biofilm. The stones were collected from Nykøbing Bugt (Fig. 1) at ~0.5 m depth where oysters occurred (mainly <i>Crassostrea virginica</i> , but <i>O. edulis</i> was also present in slightly deeper water). Only stones with a clear green biofilm were selected. To avoid any damage to the biofilm, stones were carried to the experimental facility in natural sea water.
FSW Stone	FSW with a terrestrial stone of 1–2 cm diameter. The stones were collected 10 m from where the marine biofilm stones were collected (Fig. 1), and above the intertidal zone. This treatment also served as a control for the 'biofilm stone' treatment.
FSW Spat	FSW with a living, juvenile <i>O. edulis</i> spat. The spat were obtained from the DSC, where they had previously settled and grown to a length x width of $12 \pm 0.4 \times 9 \pm 0.8$ mm (mean \pm se).

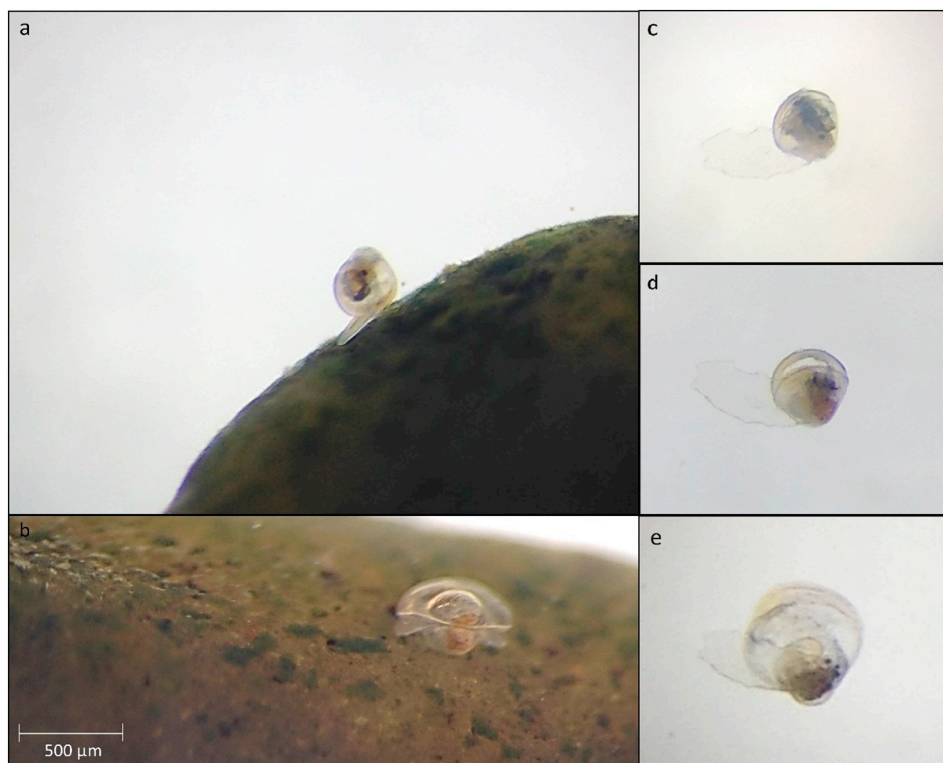


Fig. 3. a) Larvae displaying stereotypical searching behaviour with extended foot on the biofilm stone treatment, b) metamorphosed larvae with secondary shell growth and open valves (filtering water) on the biofilm stone treatment, c–e) time series of an attached larvae metamorphosing to a small spat with secondary shell growth.

‘settled’ at that time point too.

2.4. Statistical analysis

Larval settlement times were analysed via survival analysis, a collection of statistical procedures to analyse how long it takes for a certain event to occur. To perform the analysis, an observed settlement time point was allocated to every larva that settled. In the few uncertain cases (2/24 in ‘FSW Biofilm stone’ and 6/24 in ‘FSW Spat’) when the larvae had settled where they were not visible until the end of the experiment, settlement time was taken as the time point after the larvae’s last observation. The assumption being that if the larvae had not settled, they would have been observed during a later observation round; this approach was also aligned with their measured sizes at the end of the experiment. Larvae that did not settle by the end of the experiment were marked as ‘censored’ at 74 h, and those that were lost to observation during the experiment were ‘censored’ at the time of their last observation (Clark et al., 2003).

The non-parametric Kaplan-Meier-Estimator was subsequently used to construct a survival function $S(t)$ for each treatment based on the observed event times (both censored and non-censored). The survival function describes the probability that the event of interest does not occur within time t . To obtain the opposite cumulative event incidence (cumulative settlement probability) we calculated $1-S(t)$ (Clark et al., 2003). Survival curves were compared for significant differences via logrank test and pairwise post hoc comparisons between curves were performed using logrank test with adjusted p -values following the Benjamini & Hochberg procedure. All survival analysis was performed in R v.3.4.0 (R Core Team, 2017) with the packages *survival* (Therneau, 2015) and *survminer* (Kassambara and Kosinski, 2018).

3. Results

There were marked differences in the cumulative number of larvae that settled between treatments (Log-rank test, $\chi^2 = 297$, $df = 7$, $p < 0.0001$; see Appendix Table A.2). No larvae were observed to

settle in filtered sea water (FSW) nor in unfiltered sea water (USW). Three larvae settled in ‘FSW Shell’, two in ‘USW Shell’, two in the ‘FSW Stone’ and one in ‘FSW plus Food’. None of these treatments were statistically different to the treatments that elicited no settlement (all $p > 0.1$, Fig. 4).

In contrast, the spat and biofilm treatments prompted clear settlement responses. The fastest and greatest response was observed in the spat treatment. Here settlement approximated to a logarithmic curve, with cumulative settlement rising quickly from the first hour of observation until all observable larvae had settled at 22.5 h (Fig. 4 and Appendix Fig. A.1). At the end of the experiment, when all spat were lifted and inspected for settled larvae, 21 of the 24 original larvae were found, all having settled. The fitted Kaplan-Meier function estimated that in the presence of an *O. edulis* spat 50% of the larval population would settle after 3.5 h, with a 95% confidence interval of between 1 h to 6 h (Table 2).

Settlement in the biofilm treatment resembled an exponential response curve – rising sharply after 45 h (Fig. 4). By the end of the experiment 17 (80.8% of 21 found) larvae had settled. The fitted function estimated with a 95% confidence interval that half of an *O. edulis* larval population would settle after 54.5 h to 74 h of exposure to such a biofilm (Table 2).

The settlement response that each treatment elicited was also reflected in the amount of time larvae displayed settlement searching behaviour along a surface (e.g. Fig. 3a) or protruded their feet into the water (both summarised as “feet events”). Spat treatment prompted searching behaviour of the longest duration with 44% of all behavioural observations being “feet events”. In all remaining treatments, “feet events” constituted a considerably smaller proportion of the larval behaviour, with 7% of “feet events” observed in the biofilm treatment, and < 3% in all remaining treatments (Fig. 5).

Settlement location was highly specific in the significant treatments (spat and biofilm): most of the larvae settled on the treatment surface and not randomly in the experimental well. For instance, 18 of the 21 settled larvae in the spat treatment settled onto the spat. The remaining three larvae settled on the water surface, two of which were visibly

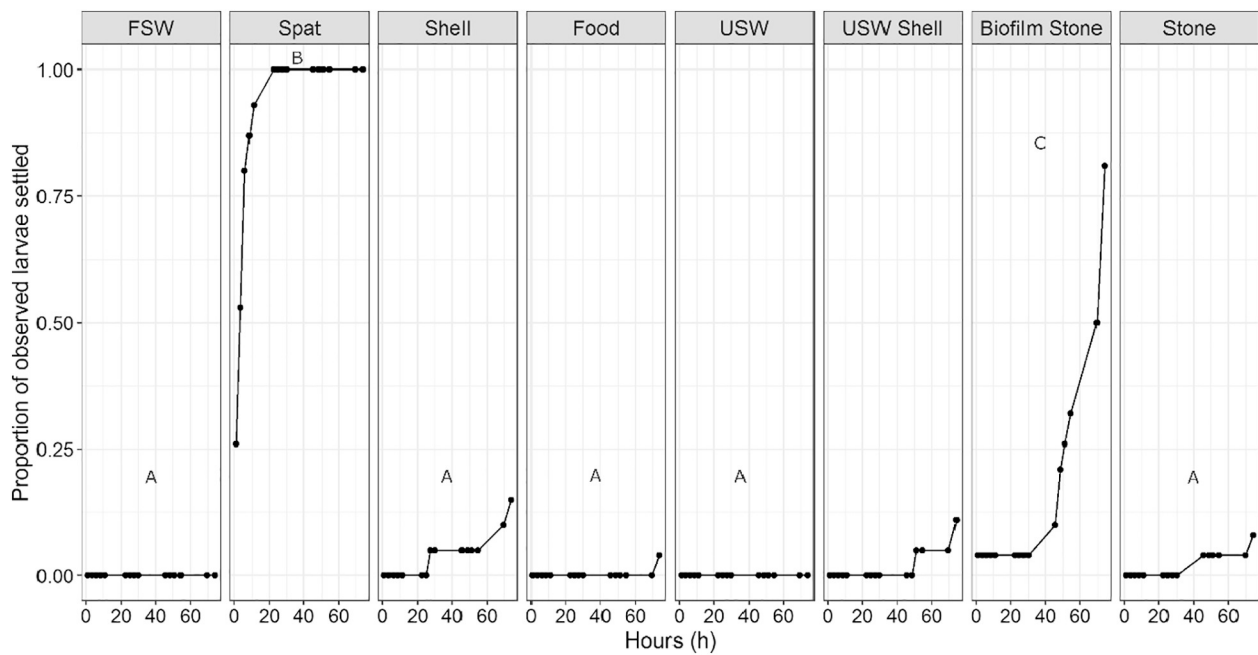


Fig. 4. Cumulative proportion of larvae settled over the 74 h of experimental duration in each treatment. All treatments were run in FSW, with exception of the ones explicitly labelled with USW. Treatments that differ in A–C notations were significantly different ($p < 0.0001$). Note that the graph depicts the proportion of observed larvae (see Appendix Fig. A.1 for number of larvae not observed at each time point and treatment).

Table 2

Estimated values of cumulative settlement probability and their 95% confidence interval (CI) for all treatments in which at least one settlement event was observed. Values calculated using Kaplan-Meier survival estimator.

Time (h)	Cumulative settlement (%)	Lower 95% CI	Upper 95% CI
FSW Spat			
1.0	36.4	12.7	53.6
3.5	59.1	32.4	75.2
6.0	77.3	50.9	89.5
8.5	81.8	55.9	92.5
11.0	87.9	60.0	96.3
22.5	100	NA	NA
FSW Shell			
27.5	4.5	0	12.9
69.5	9.3	0	20.8
74.0	14.4	0	28.2
FSW Food			
74.0	4.2	0	11.8
USW Shell			
51	5.0	0	14.1
74	10.6	0	23.5
Biofilm Stone			
1.0	4.2	0	11.8
45.5	9.0	0	20.1
48.5	23.3	3.1	39.4
51.0	28.1	6.2	44.9
54.5	32.9	9.6	50.2
69.5	47.3	21.0	64.9
74.0	80.8	53.8	92.1
Stone			
45.5	4.2	0	11.8
54.5	8.3	0	18.7

attached to a piece of spat shell that was floating on the surface. Similarly, in the biofilm treatment 13 of the 17 settled larvae settled on the surface of the biofilm covered stone, while only four larvae settled on the plastic surface of the well plate (Fig. 6).

At the end of the experiment, most larvae that had not settled were still alive (Fig. 7). All larvae that were classified into ‘metamorphosis

unsuccessful’ appeared to be dead. They ranged from not metamorphosed to fully metamorphosed but had often deformed features and were generally surrounded by a grey substance (e.g. Fig. 8).

4. Discussion

The aim of this study was to further our understanding of *O. edulis* larval settlement cues in potential natural benthic habitats, and to evaluate whether habitat-associated chemical cues could play an important role in inducing settlement. Larvae only settled significantly in response to treatments which involved what are presumed to be habitat-associated chemical stimuli, namely their own spat and a relevant biofilm. Hard surfaces on their own, such as shell and terrestrial stone, without a right chemical cue, did not induce more settlement than the control filtered sea water. Likewise, the potential stimulus of food did not result in more settlement than the control sea water. However, for the two treatments that prompted significant settlement, time was also a critical factor: one-third of larvae settled on the spat treatment after 1 h, but it took over two days (54 h) for a similar proportion of larvae to settle on the biofilm treatment.

The *O. edulis* spat treatment elicited the most effective settlement response. There was 100% of settlement in < 24 h, of which 86% was gregariously on the spat. Although this study tested the settlement effect of young oysters, extracts of adult *O. edulis* have also been shown to promote larval settlement (Bayne, 1969), indicating a general conspecific effect (see also de Brito Smith et al., 2013). Gregarious settlement has been documented for a large number of benthic sedentary organism (e.g. Knight-Jones, 1953; Hidu, 1969; Scheltema et al., 1981; Burke, 1986), including *O. edulis* (Cole, 1949; Bayne, 1969); yet this study is the first to document the relative speed and intensity at which larvae settled on conspecifics compared to other options. Gregarious settlement in the Eastern oyster *Crassostrea virginica* is triggered by a glycoprotein produced on shells of living conspecifics (Vasquez et al., 2014). A shell-bound molecule is also likely to be involved in mediating settlement of *O. edulis* larvae, since two of the three larvae that did not settle gregariously attached to a broken piece of spat shell. However, extracts of *O. edulis* tissue have also been found to promote larval settlement (Bayne, 1969), which suggests that there may be several

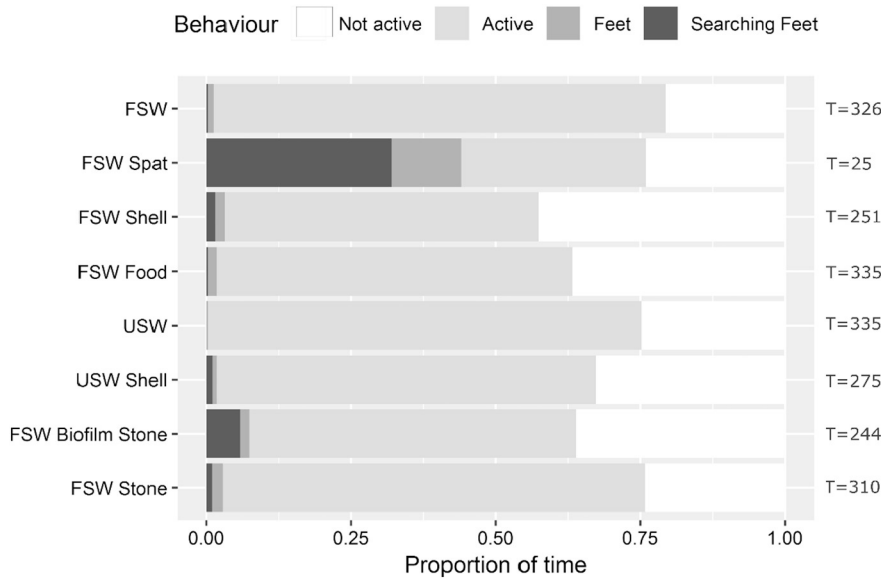


Fig. 5. Proportion of time larvae were observed displaying each behaviour. T = total number of behavioural observations in each treatment. Behavioural observations were only possible if larvae had not settled and if they were seen in that observation round. Category “searching feet” refers to the stereotypical settlement searching behaviour in which larvae crawl on a surface with extended feet, while “feet” stands for other behaviours in which larvae protruded their feet without searching.

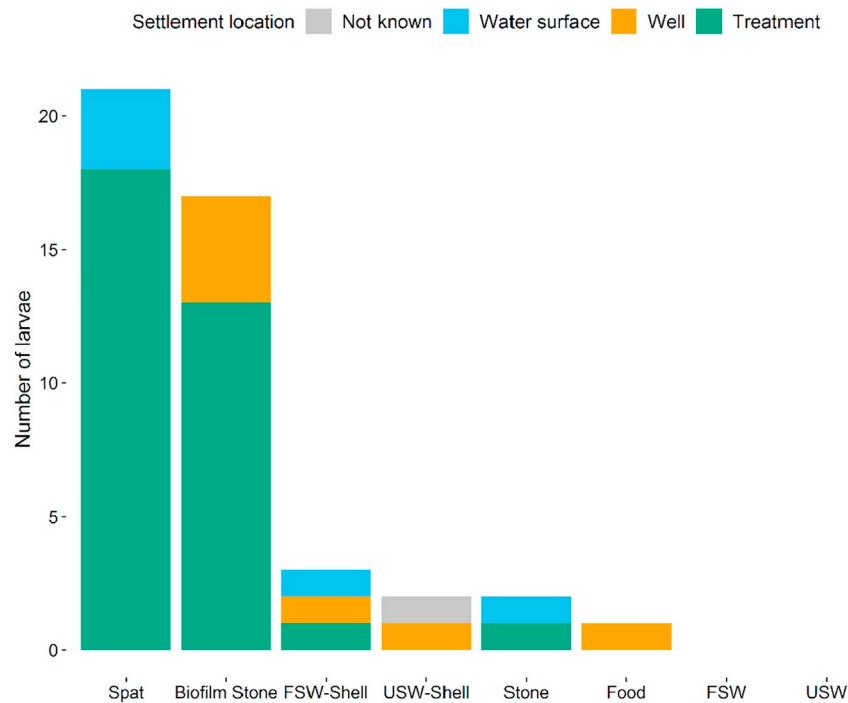


Fig. 6. Settlement locations after 74 h. Location ‘treatment’ refers to the spat, stone or shell surface of the respective treatments. ‘Well’ is the experimental container where larvae were observed.

conspicuous cues to which larvae respond. This potential richness in conspecific settlement cues, combined with the speed at which larvae settled, is indicative of the importance of adult conspecific aggregations on the reproductive success of *O. edulis*. Being a viviparous sedentary organism, its reproduction relies on sperm reaching female individuals. A minimum population density is therefore required, and gregarious settlement can be critical to achieve this. Gregarious settlement may moreover enhance filter-feeding efficiency, while the resulting shell matrix can offer larvae protection from sedimentation and predation (Tamburri et al., 1992; Whitman and Reidenbach, 2012; Gercken and Schmidt, 2014). A preferential metamorphosis in response to conspecifics is thus likely to have represented a strong evolutionary advantage, particularly considering the former, pre-exploitation widespread distribution of *O. edulis* which increased the likelihood of mature

larvae finding conspecifics. Indeed, the degree of settlement behaviour displayed in the spat treatment relative to other treatments, as well as the subsequent speed and percentage of metamorphosis, indicates that *O. edulis* larvae are finely tuned to settle preferentially in association with living conspecifics.

The biofilm was the only other treatment that prompted significant settlement, albeit over a much longer time span. In total 81% of the larvae settled, but settlement may have increased further if the experiment had lasted longer, since larvae were still settling when the experiment ended. An extensive body of literature has investigated the effects of biofilms in inducing metamorphosis of marine invertebrate larvae, and a near universality of biofilm stimulation has emerged in numerous phyla including corals, echinoderms, bivalve molluscs, bryozoan, barnacles, ascidians and crabs (Hadfield, 2011, and

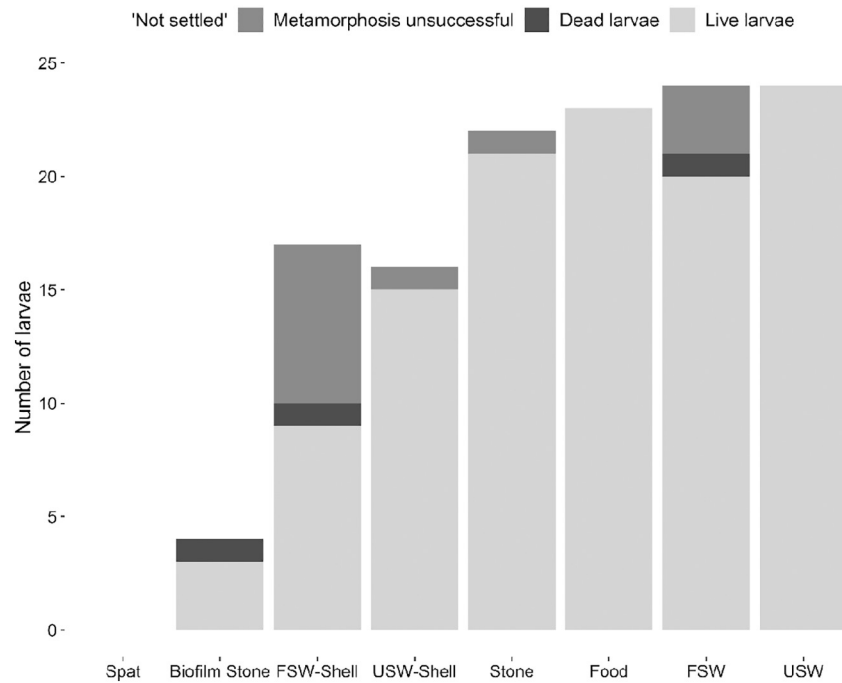


Fig. 7. Number of larvae per treatment that had not settled after 74 h and their respective state at the end of the experiment. Category “metamorphosis unsuccessful” was defined as larvae that died while trying to metamorphose or after completion of metamorphosis.

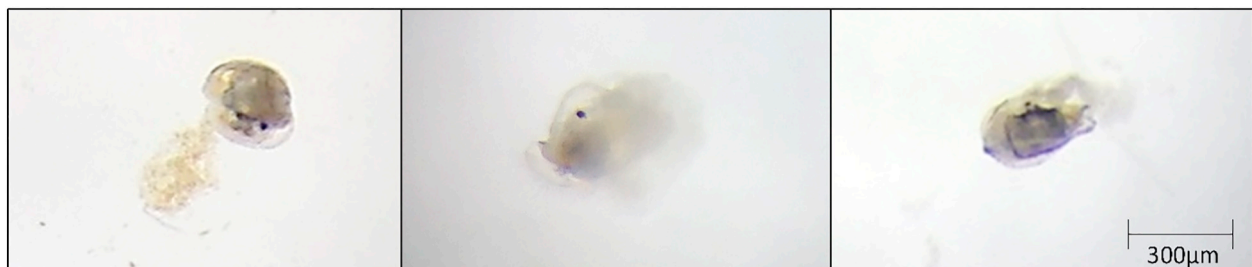


Fig. 8. Examples of larvae classified into ‘metamorphosis unsuccessful’. This category was defined as larvae that died during or after metamorphosis.

references therein). Although biofilms are a complex assemblage of microorganisms, which includes bacteria, diatoms, fungi and protozoa, the cue seems to be produced only by living bacteria (Unabia and Hadfield, 1999; Bao et al., 2007; Hadfield, 2011). It is thought that the receptors for bacterial cues developed very early in metazoan history (Hadfield, 2011), probably as an adaptation to a sea which had already been colonised by bacteria for over 2.5 billion years (Marshall, 2019) when the first metazoans evolved (Yong, 2016). The presence of a bacterial biofilm can signal that food is present, and that a surface is neither toxic nor temporary (Unabia and Hadfield, 1999). Surface permanence is specifically indicated by mature biofilm communities, and recruitment of sessile invertebrates, including oysters, was consistently positively correlated with biofilm age (Hadfield and Paul, 2001; Bao et al., 2007; Campbell et al., 2011). The bacterial community of biofilms is also an accurate reflection of ecological conditions, and larvae respond only to those bacteria relevant to their adult habitat (e.g. Lau et al., 2005; Bao et al., 2007; de Brito Smith et al., 2017). The biofilms tested in this experiment were collected from a habitat in which oysters (*C. virginica*) occur and they were presumably mature biofilm communities. They were therefore anticipated to be relevant to *O. edulis* larvae. However, if the biofilms had been collected from the slightly deeper areas in which *O. edulis* occur, the bacterial community may have represented *O. edulis* habitat requirements more accurately and the settlement response may have been quicker. Biofilms formed on ropes in the water column of a marina were also tested in preliminary

experiments; but no larvae settled, corroborating the importance of habitat specificity in *O. edulis* settlement response to biofilms.

Despite the importance of biofilms to the settlement of *O. edulis* larvae very little has been studied. The American Eastern oyster *C. virginica* was found to settle only if specific bacteria taxa were present, which was also correlated with biofilm age (Campbell et al., 2011). The only bacterium that is known to trigger settlement in *O. edulis* larvae is *Shewanella colwelliana* (Tritar, 1992). However, a number of chemical compounds (e.g. GABA, L-DOPA, epinephrine, norepinephrine) are known to induce metamorphosis or increase settlement rates of *O. edulis* larvae (Mesias-Gansbiller et al., 2013), and all these compounds are related to bacterial products. For instance, GABA is an analogue of a compound which is produced by cyanobacteria, while L-DOPA is produced by *S. colwelliana* when fixed on a substratum (Tritar, 1992), and it is also a precursor of epinephrine and norepinephrine (Coon et al., 1985). Increasing our knowledge of bacterial biofilm communities, together with specific bacteria that trigger settlement could allow us to predict whether oyster larvae will settle or not (Campbell et al., 2011).

Only 8–14% of larvae settled on the three treatments involving shell fragments and terrestrial stone. These treatments represented substrates traditionally regarded as suitable for *O. edulis* larval settlement, but they were devoid of any relevant chemical cue. The proportion of larvae that settled on these treatments was minor compared to the settlement elicited by the spat and biofilm treatment, and it was statistically not different to the 0–4% settlement in the control filtered sea water (FSW)

and sea water with food treatments. Similar results were observed for the oyster larvae of *C. virginica*: settlement on oyster shell devoid of its natural biofilm did not differ significantly from the sea water control (Tamburri et al., 1992). Although this study tested shells that originated predominantly from *C. gigas*, preliminary experiments conducted with sterile *O. edulis* shell fragments did not result in any larval settlement either, indicating that the species of oyster shell would have not altered the outcome. A number of larvae, particularly in the FSW shell and FSW treatment, appeared to have died after attachment and they were often surrounded by a grey or yellowish substance. No such incomplete metamorphosis or dead spat were observed in the biofilm and spat treatment. It may be that the larvae were lacking an appropriate stimulus for completing the metamorphosis successfully, or that they were subject to a bacterial infection or had become energetically compromised. Most larvae that did not attach were however still alive, which provides further evidence that in the absence of adequate cues, *O. edulis* larvae can delay metamorphosis (Cole and Jones, 1939). A delay in metamorphosis increases the larvae's chances of finding a suitable substratum elsewhere (Pawlik, 1992); however, it also increases the risk of mortality, since larvae are exposed for longer time to predation and other factors controlling mortality (Korringa, 1940; Pineda et al., 2007). It is thus a trade-off which has to be carefully balanced. With the large-scale disappearance of most *O. edulis* beds in < 100 years, it is likely that *O. edulis* larvae did not have time to evolve to the new conditions, shifting the balance to larvae dying predominantly rather than metamorphosing. For instance, in the Dutch Oosterschelde only 1% of larvae succeeded to metamorphose despite oyster farmers laying vast quantities of lime tiles and mussel shells as collectors (Korringa, 1946), which traditionally have been thought to be highly suitable settlement materials (Cole and Jones, 1939). This underlines the necessity of carefully understanding the settlement requirements of *O. edulis* larvae if recruitment is to be maximised.

The results of this study indicate that chemical compounds on substrates, such as the ones produced by biofilms, are more important in triggering settlement of *O. edulis* larvae than the material itself. This could explain the often observed location-specific substrate settlement preferences of *O. edulis* in the wild (e.g. Low et al., 2007; Smyth et al., 2018). However, some substrates may be intrinsically more suitable than others. For instance settlement of *O. edulis* larvae was greatest on substrates with highest rugosity, particularly microscopically rough (Korringa, 1940) while smooth surfaces were inherently unsuitable (Cole and Jones, 1939). It may be that microscopic roughness provides a more sheltered and adequate environment for bacterial colonisation than smooth surfaces, particularly under stronger hydrodynamic regimes. Similarly, shells may be intrinsically more suitable for bacterial colonisation than stones, due to, for instance, more interstitial spaces or their shape in relation to hydrodynamics, and they may therefore provide a more effective settlement substrate in the wild – which could be addressed by more nuanced additional experimentation. In addition, three-dimensional shaped settlement structure can increase oyster larval settlement because shear stress is markedly reduced in the

interstitial spaces (Whitman and Reidenbach, 2012), and oyster larvae are not able to settle in strong currents (Korringa, 1940). Finally, while chemical cues appear critical to the settlement of *O. edulis* larvae, acoustic cues related to their adult habitat are likely to increase settlement too, since *C. virginica* oyster larvae settled in response to habitat-associated underwater sounds (Lillis et al., 2013). We therefore recommend that future experiments take a more multidimensional approach to settlement, in which not only habitat- and substrate-specific biofilm formation and their settlement-inducing effect is considered, but also other potentially critical factors such as the local hydrodynamics and underwater acoustics.

In conclusion, *O. edulis* larvae appear to be finely-tuned to settle in response to cues which are indicative of their adult habitat requirements, and chemical cues appear to play a critical function in mediating this response. The most effective settlement cue originates from conspecifics, and this settlement preference was probably shaped by millions of years of evolution in which settling on conspecifics was both advantageous and viable due to the once widespread distribution of *O. edulis*. Biofilms representative of an adequate habitat were also effective in promoting settlement, but after a longer time interval. In the open sea, this delay in settlement on a biofilm may have once been a suitable strategy to increase the chance of finding a conspecific for settlement. This settlement strategy would appear to be predicated upon relatively high oyster densities which may help explain why remnant low density and isolated populations are sensitive to decreased reproductive success (Low et al., 2007; Guy et al., 2018). The likelihood of successful settlement may be dramatically reduced without a robust oyster population of sufficient scale. Advancing our knowledge of habitat and substrate specific biofilm formation and their settlement-inducing effect is critical to understanding and predicting *O. edulis* larval settlement under natural scenarios. In a restoration context, populations of adult conspecifics could be positioned as 'recruiters' in locations predicted to receive large amounts of mature larvae by hydrodynamic models (see also Gormley et al., 2015). If *O. edulis* larvae do reach those locations, and there is no other factor impeding attachment, they will probably settle most readily in response to their conspecifics and to some extent upon mature hard substrata, provided it is colonised by an appropriate biofilm.

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Appendix A

Table A.1
Larvae observation time points and time intervals between observations.

Observation ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Time since start [h]	1	3.5	6	8.5	11	22.5	27.5	30	45.5	48.5	51	54	69.5	74
Time since last observations [h]	NA	2.5	2.5	2.5	2.5	10.5	5	2.5	15.5	3	2.5	3	15.5	4.5

Table A.2

Log-rank test results evaluating if the settlement distributions differed between treatments. $N = 188$ (4 observations were deleted due to missing event times). $\chi^2 = 297$, $df = 7$, $p < 0.0001$.

Treatment	N	Observed settlement	Expected settlement	(O-E) ² /E	(O-E) ² /V
FSW	24	0	6.87	6.86	8.34
FSW Spat	22	21	1.72	216.57	247.69
FSW Shell	23	3	6.00	1.50	1.78
FSW Food	24	1	6.86	5.01	6.08
USW	24	0	6.86	6.86	8.34
USW Shell	24	2	5.88	2.56	3.02
FSW Biofilm Stone	24	17	5.20	26.74	31.15
FSW Stone	24	2	6.62	3.22	3.89

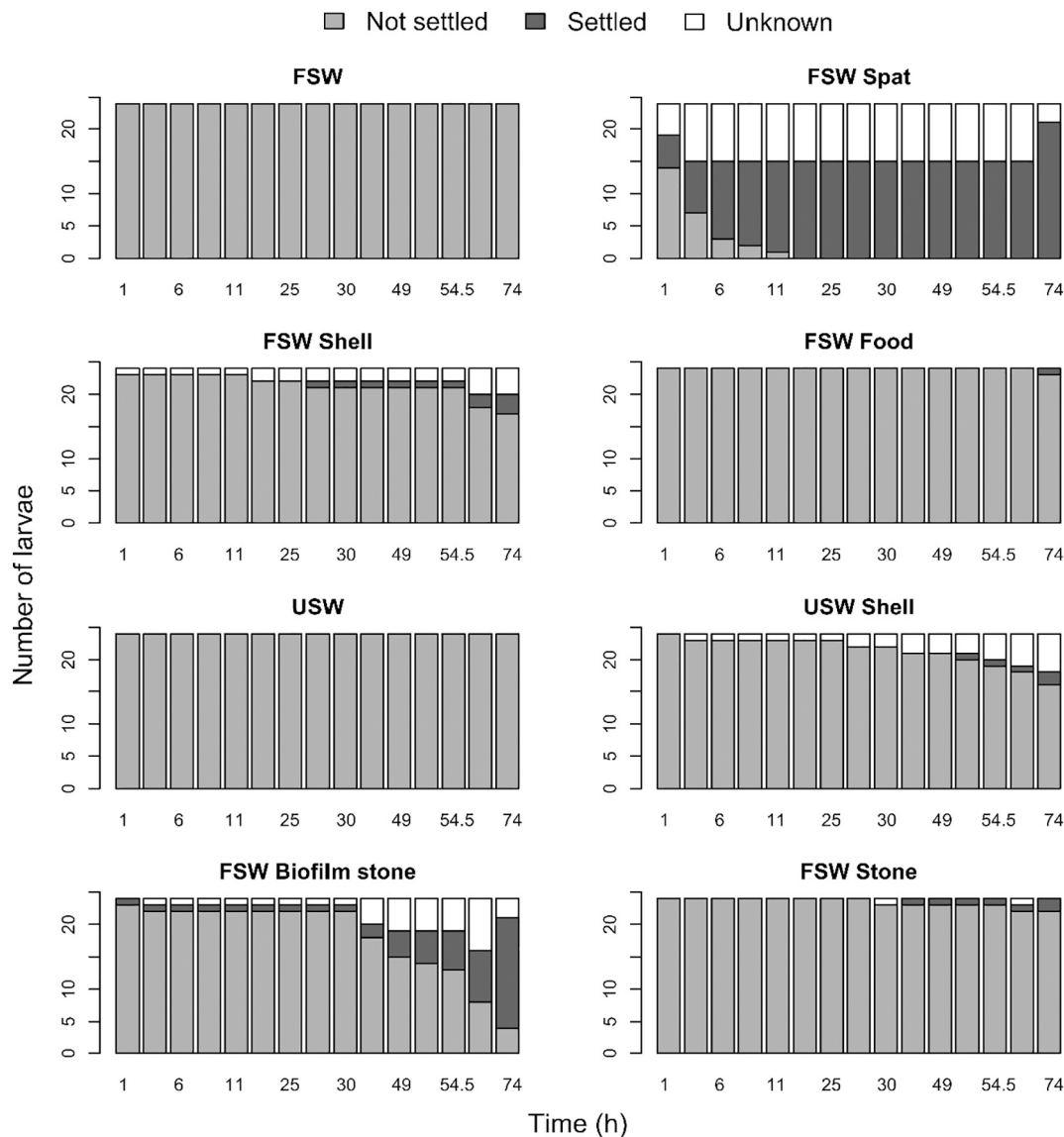


Fig. A.1. Number of larvae settled, not settled and not observed per hour and treatment. At the end of the experiment (74 h) all treatment media were inspected for larvae that may have settled on surfaces not visible during the experiment.

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