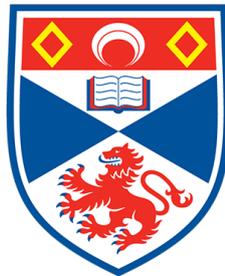


Informing the conservation and restoration of a
keystone species: the larval behaviour of the
European oyster *Ostrea edulis*

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University of
St Andrews

This thesis is submitted in partial fulfilment for the degree of

Doctor of Philosophy (PhD)

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September 2019

Abstract

The European oyster *Ostrea edulis* is a keystone species that is internationally recognised as ‘threatened and declining’ in the North-East Atlantic and several nations have adopted strategies for its conservation and restoration. The overall goal of the present work was to inform conservation and restoration efforts. The purpose of this thesis, therefore, was to study the larval behaviour and ecology of *O. edulis* in as much as is relevant to the dispersal of this species. Specifically, the larvae’s vertical distribution, swimming speeds, settlement preferences and pelagic duration were studied in laboratory experiments. Most larvae concentrated at the bottom of the aquarium, independently of the developmental stage, light, food or temperature. In addition, larvae behaved actively in ~50% of all bottom observations, indicating a behavioural function other than resting. Advection close to the seabed is known to be slower than in any other part of the water column. The observed demersal behaviour would therefore most likely reduce dispersal from natal populations and enhance self-recruitment. At the surface, larvae frequently formed aggregations. In the water column, larvae swam with high vertical directionality and their distribution was homogenous. Swimming speeds ranged from 0.001 mm/s to 9.07 mm/s. *O. edulis* larvae settled preferentially among conspecifics (100% in < 24h), and if conspecifics were absent, larvae also settled in response to habitat-associated biofilms (81% of settlement after a 45h delay). Sterile shells and terrestrial stones did not induce more settlement than control treatments (0-14% settlement). Pelagic duration was strongly dependent on temperature, food and a suitable settlement cue. In the absence of an appropriate settlement cue, 80% of larvae delayed metamorphosis for up to 14 days, when the experiment was terminated. In contrast, 95-100% of larvae delaying their metamorphosis settled when presented with a conspecific. Such a delay in metamorphosis enhances the risk of predation and, ultimately, of losing most larvae to mortality if target habitats are absent. The results of this thesis provide strong evidence that *O. edulis* larvae are targeting their own beds, and that the behaviour of larvae plays a crucial role in their dispersal and successful recruitment. Restoring European oyster beds at a scale that is large and dense enough to promote the retention of larvae may be crucial to the success of restoration efforts.

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Acronym list

AIC	Akaike's Information Criterion
DSC	Danish Shellfish Centre
FAO	Food and Agriculture Organization of the United Nations
FSW	Filtered Sea Water
IQR	Interquartile range
LR	Likelihood Ratio
NGDR	Net-to-Gross Displacement Ratio
NORA	Native Oyster Restoration Alliance
OSPAR	Oslo-Paris Convention for the Protection of the Marine Environment of the North-East Atlantic
PLD	Pelagic Larval Duration
PMF	Priority Marine Feature
USW	Unfiltered Sea Water

Chapter 1. General introduction

1.1. Historical importance and decline

The European native flat oyster (*Ostrea edulis* Linnaeus, 1758; hereafter referred to as European oyster) once covered large areas of Europe's coastline (Fig. 1-1). Its natural distribution range stretched along the western European coast from the Norwegian Sea down to the Atlantic coast of Morocco, in the Mediterranean Sea and in the Black Sea (Perry, F. & Jackson, 2017). For millennia, *O. edulis* constituted a reliable food resource for humans, which coastal populations could harvest year-round. This food-security enticed communities to settle near to beds, which represented up to 32% of their food intake (Kristensen, 1997). Both ancient Greeks and Romans regarded *O. edulis* as a delicacy (Airoldi and Beck, 2007). Romans imported this species from all over Europe and started cultivating it as early as 100 BC to satisfy their increasing demand (Iversen, 1968; Airoldi and Beck, 2007; Gercken and Schmidt, 2014). In the 13th century, *O. edulis* was one of the first commercially operated fisheries (Lotze, 2007) and in the 14th century, it could be used as a monetary equivalent for rent and tax payments (Young 1886 in Low *et al.*, 2007). At its peak production in the mid-1800, the trade with *O. edulis* spurred emerging economies of coastal European countries: for instance, 700 million European oysters were consumed annually in London alone (Philpots, 1891) and 120,000 men, i.e. approximately 1% of the male population (Office for National Statistics, 2015), were engaged in dredging oysters in Britain (Edwards, 1997). The scale of the harvest underlines the productivity of the beds (Beck *et al.*, 2011). This immense demand, together with increasingly more efficient fishing techniques led to the decline of *O. edulis* throughout its distribution range. Similar fates occurred to oyster species all around the world, and today 85% of the world's oyster populations have been lost (Beck *et al.*, 2011).

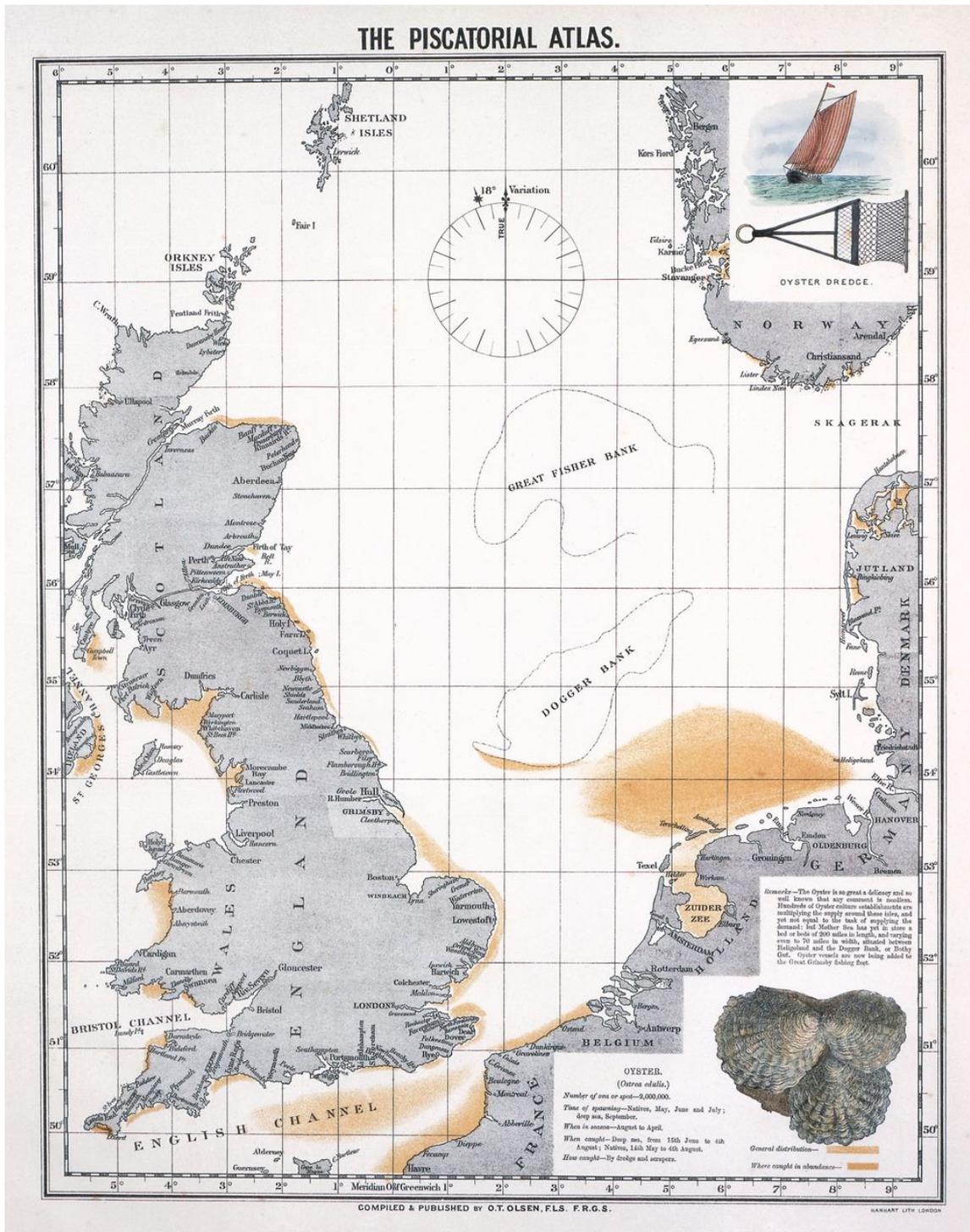


Figure 1-1. Distribution of *O. edulis* in the North-East Atlantic in 1883 (Olsen, 1883)

For millennia Europe's natural oyster beds had been gathered by hand or using bucket and tongs (Yonge, 1960), and their exploitation was limited by the ability of individual persons to reach oysters in the easily accessible lower intertidal zone (Gercken and Schmidt, 2014). However, improving fishing techniques and a developing market drove the commercial exploitation of these beds, initiating the decline of the fishery (Low *et al.*, 2007). Oyster started to be fished with single-masted sailing vessels and iron dredges in

the 13th century (Seaman and Ruth, 1997), but it is not until the mid-17th century that fishing with sail- or oar-powered boats and dredges became the main harvesting method, replacing the oyster gathering by hand (Yonge, 1960). In the 19th century, with the advent of steamboats and the introduction of railways as a route to market, commercial fisheries further expanded. These inventions facilitated exploitation over greater distances and allowed commerce with a wider geographical market (Low *et al.*, 2007). As a result, unsustainable levels of exploitation occurred throughout Europe.

In Germany, extreme winters exacerbated the impacts of overfishing and individual oyster beds were ruined as early as 1695 (Gercken and Schmidt, 2014). One century later, in 1778, oyster stocks also declined in Spain (Pazo 1987 in Laing, Walker and Areal, 2005). The first large-scale fishery collapse occurred in France. In the early 19th century 100 million oysters were landed, but by the middle of the century local populations had been extirpated to the point that commercial harvesting was no longer profitable (Yonge, 1960). Annual oyster production in the UK fell from 700 million oysters in 1864 (Philpots, 1891) to 40 million in 1920. By the end of the decade only 3 million oysters were landed (Edwards, 1997). Oyster stocks also continued to decrease in Germany and Spain. In 1927 the German beds were decimated (Gercken and Schmidt, 2014), and in 1960 oyster exploitation was also no longer profitable in Spain (Laing, Walker and Areal, 2005). In Scotland, production of one of the most prolific oyster fisheries of Europe, the Firth of Forth, fell more than 99% over a period of 60 years in the late 19th century, resulting in the eventual extinction of the population (Low *et al.*, 2007; Thurstan *et al.*, 2013).

Several countries tried to restrain the decline of *O. edulis* through systematic management. In Germany, harvesting bans were implemented in 1703-1706 and in 1882-1891. From 1709 on it was forbidden to sell undersized oysters and to fish during the reproductive season (Seaman and Ruth, 1997). The latter restrictions were also adopted by Great Britain, who granted public fishing rights to regulate fisheries in the same manner (Edwards, 1997).

Restoration was attempted as an additional measure to halt the decline: tonnes of imported breeding oysters and shell cultch, as settlement substrate for larvae, were laid into nearly depleted fishing grounds (Yonge, 1960). These human induced translocations continued for centuries (Beaumont *et al.*, 2006; Gercken and Schmidt, 2014) and diluted many of the natural genetic population structures of *O. edulis*, resulting in low genetic differentiation, especially in the Atlantic populations (Saavedra *et al.*, 1993; Launey *et*

al., 2002; Sobolewska and Beaumont, 2005; Lallias *et al.*, 2010). However, restocking with foreign broodstock did not prevent the collapse of the heavily exploited fisheries and contributed to the depletion of the foreign donor populations (Yonge, 1960; Low *et al.*, 2007; Gercken and Schmidt, 2014). In addition, it provided a pathway for the introduction of diseases *Bonamia* and *Marteilia*, which further diminished the stocks (Laing, Walker and Areal, 2005).

Populations of *O. edulis* also suffered impacts resulting from coastal development, such as coastal degradation and water pollution (Airoldi and Beck, 2007; Thurstan *et al.*, 2013), and some declined drastically despite not being harvested. For example, in Bulgaria a mixture of hypoxia events, increased sedimentation due to coastal development, pathogens and competitive species are thought to have caused the virtual extinction of *O. edulis* (Todorova, Micu and Klisurov, 2009).

The main reason for the loss of most of *O. edulis* populations, however, was overfishing to the point when exploitation was no longer economically viable. The harvesting of adult oysters resulted in both a reduction in larvae and a loss of shell-matrix, which is the larvae's preferred settlement substrate and offers protection from predators and sedimentation. Temporary closures were consequently not able to revive the fisheries and the stocks became more vulnerable to other stressors, such as harsh winters, diseases or sedimentation (Gercken and Schmidt, 2014).

Today, European oyster beds are extirpated throughout most of their natural range: *O. edulis* is now extremely rare in the North Sea, in the Dutch Oosterschelde, in Belgian waters and in the Wadden Sea, where the species is considered to be under immediate threat of extinction (Haelters and Kerckhof, 2009). Local extinction has been documented off the Scottish East coast, in German Helgoland, the German deep sea oyster ground and in the English Channel (Laing, Walker and Areal, 2005; Airoldi and Beck, 2007; Low *et al.*, 2007; Haelters and Kerckhof, 2009; Thurstan *et al.*, 2013; Gercken and Schmidt, 2014). Formerly large populations off the English Wash, North Spain, the Mediterranean and the Black Sea are also gone (Cano and Rocamora, 1996; Laing, Walker and Areal, 2005; van den Berg *et al.*, 2005; Airoldi and Beck, 2007). Although some small populations still persist, they are generally in a poor condition or functionally extinct (Beck *et al.*, 2011).

1.2. *O. edulis*, a keystone species

The concept of a keystone species was coined by Paine (1969) and originally referred to those species that maintained the stability of an ecosystem not because of their abundance but due to the ecological effect they have on other species. Paine (1969) used the starfish *Pisaster ochraceus*, the top predator of the local system, as an example. However, in subsequent papers the term has been applied more broadly with definitions involving 1) a strong influence on ecosystem integrity, 2) a disproportionate effect relative to its abundance, and 3) a disproportionate effect relative to its biomass (reviewed in Cottee-Jones and Whittaker, 2012). In an attempt to reduce ambiguity around the concept, Cottee-Jones and Whittaker (2012) suggested to define a keystone species as ‘a species that is of demonstrable importance for ecosystem function’, arguing that this definition would be in line with the way the term is used in much of the literature and aligned with biodiversity conservation and functional resilience goals. In contrast, the idea of proportionality, where a keystone species is defined as having a disproportional effect relative to its abundance or biomass, introduces uncertainty into the concept according to some authors (Mills, Soulé and Doak, 1993; Cottee-Jones and Whittaker, 2012).

In this thesis, *O. edulis* is defined as a keystone species following Cottee-Jones and Whittaker's (2012) definition of a species that is of high importance for ecosystem functioning. However, it must be stressed that oysters probably do not act as keystone species, following this definition, when they occur in low numbers and densities. The following paragraphs will outline the role former *O. edulis* beds must have played on the ecology of Europe's coastlines given their extensive distribution.

Oyster beds provide three-dimensional hard substrate in an otherwise sedimentary habitat, which provides habitat, refuge and foraging ground for many species, both resident and transient (Coen *et al.*, 2007). Through their filter-feeding behaviour, they also mediate the flux of planktonic primary production and nutrients to the seafloor, which plays an important role in benthic-pelagic coupling. This supports benthic secondary production, accelerating the rate of nutrient turnover and enhancing production of the system (Perry and Tyler-Walters, 2016). 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 known for their conspicuous species richness (Möbius, 1877; Caspers, 1950; Korringa, 1954). Scholars frequently visited the German oyster beds to study the abundant epibenthic community, and these species were subsequently a valuable asset for university classes and museum's collections (Caspers, 1950). Furthermore, it was the species abundance on these beds which inspired the zoologist Karl Möbius (1877) to introduce the term 'biotic community', or 'biocenosis' to ecology, using the native oyster bed as an example – over 50 years before A. G. Tansley (1935) coined the word 'ecosystem'. According to Möbius (1877) the beds were the richest part of the seabed, inhabited by numerous invertebrates, fish and rays (see Annex 1 for an English translation).

Former *O. edulis* beds measured up to 20,000–25,000 km² in the North Sea oyster ground (Berghahn and Ruth, 2005; Christianen *et al.*, 2018) and 166 km² in the Firth of Forth on Scotland's east coast (Low *et al.*, 2007). These large expanses of oyster beds would have had a substantial impact on enhancing water clarity through the filter-feeding behaviour (Cressman *et al.*, 2003; Grabowski and Peterson, 2007). For instance, the oyster population in Chesapeake Bay (USA) may have been able to filter the entire volume of the bay in about 3 days prior to their industrial exploitation in 1870, while it would now take the reduced population about 325 days to perform the same function after nearly a century of harvesting (Newell, 1988; Coen and Luckenbach, 2000).

The filtering capacity of oysters would have also contributed to removing excessive nutrients from the water column, counteracting eutrophication and hypoxia. For example, the planktonic to benthic diatom ratio, which is a proxy for eutrophication, was relatively constant at about 1:1 in Chesapeake Bay prior to the late 18th century. With increased runoff of sediments and nutrients from intensified agriculture after 1750, the ratio increased to about 3:1 and remained stable, until it increased dramatically to 8:1 when oyster populations in Chesapeake Bay crashed in the early 20th century. This suggests that the oysters were able to limit the eutrophication (Jackson *et al.*, 2001). In addition, hypoxia events, associated with large quantities of nutrients entering coastal ecosystems, have afflicted former oyster grounds, leading to mass mortalities of demersal fish and benthos (Diaz and Rosenberg, 2008). If abundant oyster populations were still present and able to counteract eutrophication (Jackson *et al.*, 2001), they may have prevented those hypoxia events.

Finally, the layers of living and dead oyster shells would have had a major effect on stabilising the sediment and reducing turbidity in the water column (Piazza, Banks and La Peyre, 2005). Old oyster beds are now characterised by sedimentary environments (e.g. Korringa, 1940; Caspers, 1950), which can re-suspend into the water column, affecting light penetration (Kamermans et al. 2018). Kilometre long oyster beds would have markedly reduced resuspension, increased water quality and enhanced light penetration in the now rather turbid North Sea (Grabowski and Peterson, 2007; Kamermans *et al.*, 2018). Enhanced light penetration, in turn, favours the development of other habitats, such as the ones formed by seagrasses (Beck *et al.*, 2009). For example, seagrasses in the Dutch Wadden Sea became virtually extinct in the 1930s, coinciding with the population crashes of *O. edulis* in that area. Despite restoration attempts, the seagrasses have never recovered and the lack of recovery is attributed to the high turbidity of suspended sediment (Suding, 2011).

The loss of *O. edulis* is thus likely to have caused a substantial transformation of the European coastal habitat, resulting in a flat and homogenised bottom, and afflicting its diversity and productivity (Airoldi and Beck, 2007). Today, most people do not remember the existence of the once plentiful oyster beds: the gradual deterioration of our coastal habitats has passed almost unnoticed in a collective societal amnesia (Roberts, 2010). An ecological baseline has been lost and expectations for food, economic value and ecosystem services from coastal waters have consequently changed (Alleway and Connell, 2015).

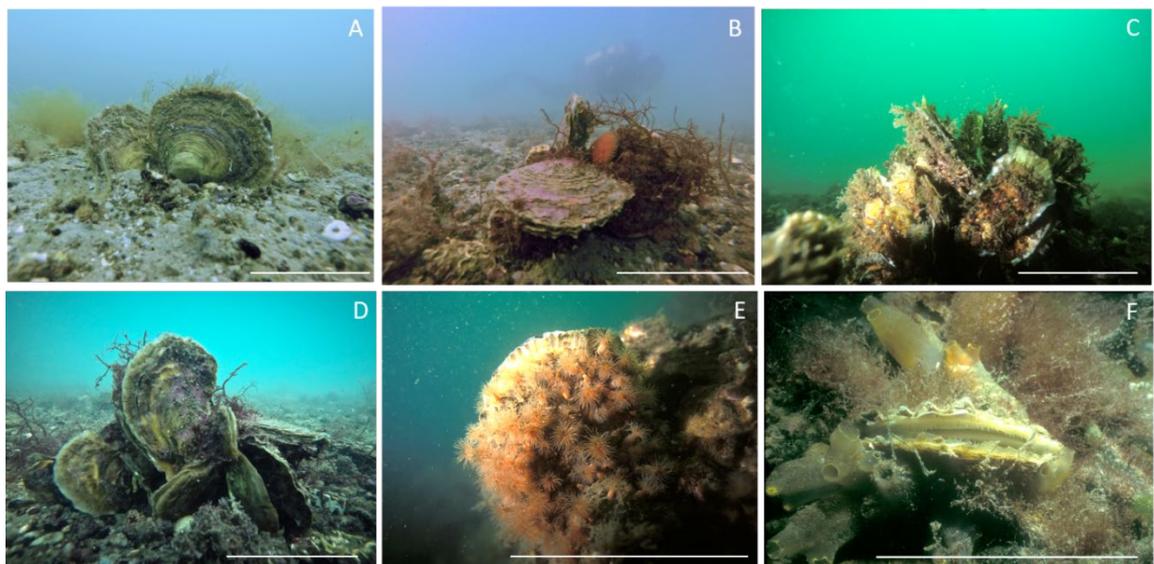


Figure 1-2. *Ostrea edulis* in the wild. Scale: 10 cm. Copyright: (A,B & D) Pouvreau (2017); (C, E & F) Saxifraga foundation (2019)

1.3. Current status, conservation and restoration efforts

Most of the remaining native oyster populations are regulated fisheries or managed for hatchery and aquaculture purposes and their existence is often threatened by legal and illegal use (Gercken and Schmidt, 2014; FAO, 2016). Remains of wild *O. edulis* beds occur on the west coast of Sweden (Gercken and Schmidt, 2014), in southern Norway (Bodvin *et al.*, 2011), in the Grevelingen lake of the Netherlands (Smaal *et al.*, 2015; Christianen *et al.*, 2018), in the Mar Menor of Spain (Cano and Rocamora, 1996), in the Black Sea (Gomoiu *et al.*, 2016) and in the Adriatic Sea (Airoldi and Beck, 2007). In England, there are commercially harvested remnant stocks along the Thames Estuary, the county of Essex and the south coast of Cornwall. Furthermore, populations still occur along the south coast of Wales, in Northern Ireland (in Lough Strangford and in the commercially harvested Lough Foyle), and in the west coast of Scotland, where Loch Ryan supports the only commercially managed fishery for European oyster in Scotland (Laing, Walker and Areal, 2005; Low *et al.*, 2007). One of the largest populations left is located in Denmark, in the Limfjord, where oysters are currently harvested, and the fishery is the first of its kind that has been certified as sustainable by the Marine Stewardship Council (Gercken and Schmidt, 2014).

Most remnant populations are much smaller than the one in the Limfjord, and they are often functionally extinct, i.e. they do not perform any significant ecosystem role (Beck *et al.*, 2011). *O. edulis* has therefore been listed as a ‘Threatened and Declining species’ by the Oslo-Paris Convention (OSPAR) for the Protection of the Marine Environment of the North-East Atlantic (Haelters and Kerckhof, 2009). OSPAR member nations have consequently adopted national legislation and policies for the protection and conservation of *O. edulis*, with the broader aim to achieve biodiversity goals, restore ecosystem functions and enhance ecosystem 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, Manson and Macdonald, 2016; Perry and Jackson, 2017; JNCC, 2018). The measures to conserve *O. edulis* also contribute to environmental targets of the EU Habitats Directive (1992/43/EEC) and the EU Marine Strategy Framework Directive (2008/56/EC).

OSPAR member nations are following two recommendations: 1) protecting, maintaining and expanding remnant *O. edulis* populations, and 2) restoring *O. edulis* to areas they once occupied (Haelters and Kerckhof, 2009). In Scotland, for instance, there are both

remnant and extinct populations. Most remaining *O. edulis* populations are located in the west coast of Scotland, which is regarded as a UK stronghold (Low *et al.*, 2007), while populations of the east coast are virtually extinct (Fig. 1-3; Laing, Walker and Areal, 2005; Low *et al.*, 2007; Thurstan *et al.*, 2013). The extant populations in the west coast are small, dispersed and patchy in distribution, with oyster density generally lower than one per m² (Low *et al.*, 2007), which is below the minimum density of five per m² recommended by OSPAR (Haelters and Kerckhof, 2009). Unlawful exploitation of *O. edulis* is one of the greatest threats to remaining wild populations in Scotland (Donnan, 2003). Scotland has taken actions to conserve the remaining *O. edulis* populations by listing the species as a priority marine feature (PMF), including the populations in the Marine Protected Area Network and tackling illegal harvesting through campaign actions (Donnan, Manson and Macdonald, 2016; Scottish Government, 2019). In addition, Scotland is supporting and investing in restoration efforts (e.g. the Dornoch Environmental Enhancement Project) on the east coast.

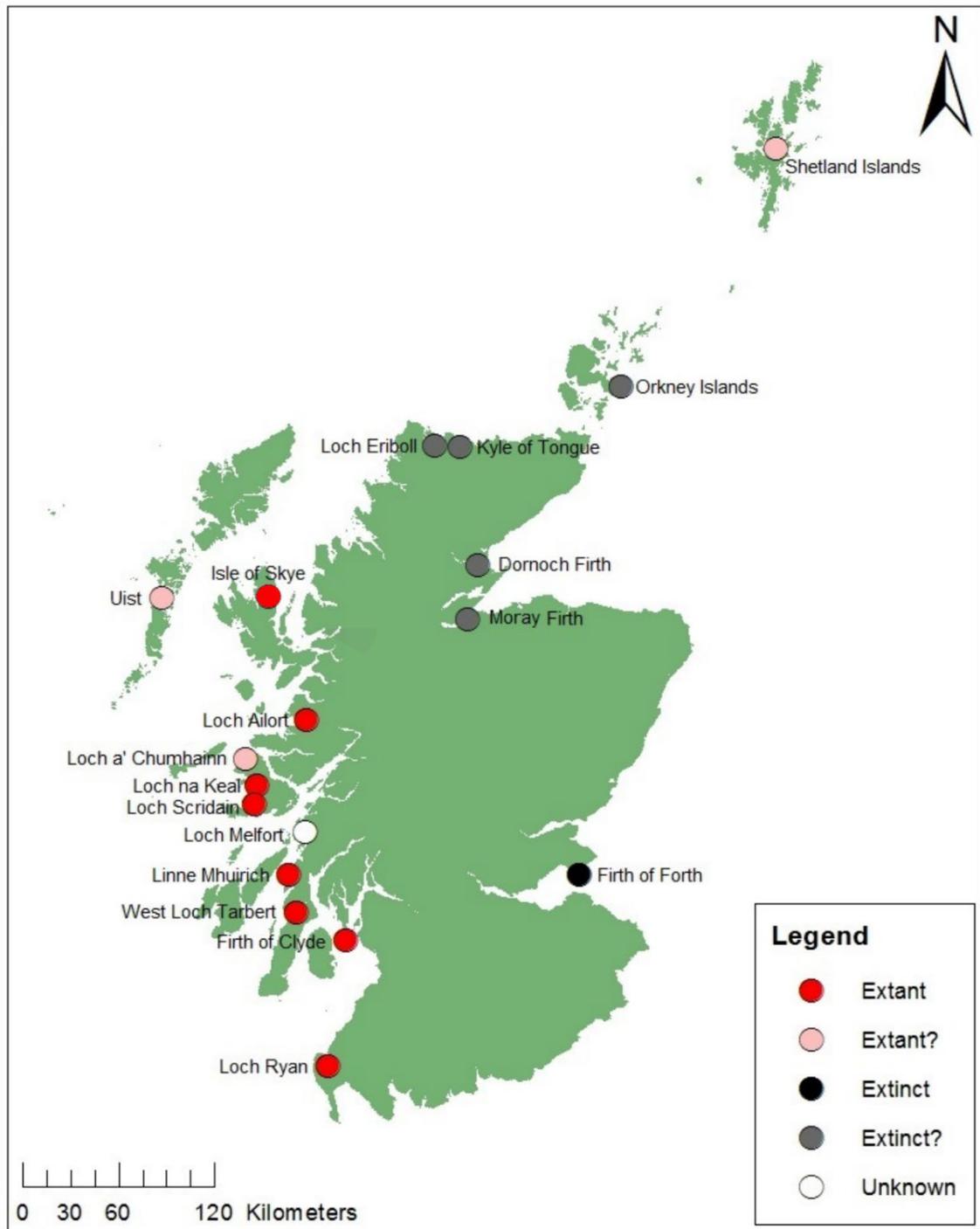


Figure 1-3. Map of Scotland illustrating the current status of *O. edulis* at sites where populations had been recorded in the past (based on records in Laing, Walker and Areal, 2005 and Low *et al.*, 2007)

Restoration projects are not only taking place in Scotland, but all over Europe: in England, Germany, the Netherlands, France, Ireland, Wales, Sweden and the Mediterranean region (Pogoda *et al.*, 2019). The list of countries is still expanding. For instance, Belgium is hoping to start soon too (J. Vanaverbeke, pers. comm.). In 2017, the Native Oyster Restoration Alliance (NORA) was formed to facilitate knowledge and technology transfer

between these countries. Current membership of NORA includes governmental agencies, scientist, non-governmental organizations, as well as oyster growers and other private enterprises. In addition, NORA benefits from the collaboration of US partners, who have gathered extensive experience in oyster restoration through their own restoration projects including Chesapeake Bay. The aspiration of most of these European projects is to restore *O. edulis* on an ecologically meaningful (large) scale. Thus, in order to succeed with their objectives, most countries will address the following tasks (Pogoda, 2019) during their restoration efforts:

- i. Developing a regulatory framework for species reintroduction
- ii. Providing evidence on growth and fitness of oysters in the field
- iii. Identification of oyster seed suppliers for long-term restoration projects
- iv. Hydrodynamic modelling of larval drift: Understanding of larval dispersal for future site connectivity
- v. Recommendations on appropriate sites
- vi. Recommendations on appropriate technologies: substrate, reef design, and scale
- vii. Building skills and experience in restoration methods
- viii. Learning from terrestrial restoration initiatives
- ix. Quantification and evaluation of ecosystem services from restored oyster habitats
- x. Building awareness, capacity, and confidence that key degraded coastal and marine habitats can be repaired

This thesis aims to contribute to the knowledge needed to perform task (iv): understanding larval dispersal for future site connectivity, which is relevant both to conservation and restoration efforts.

1.4. Larval dispersal and population connectivity

1.4.1. Factors driving dispersal

Benthic marine invertebrates rely on pelagic larval recruitment for their populations to persist. This can be either self-recruitment, i.e. the larvae settles in the parental population, or recruitment of larvae originating from geographically distant populations (Robins *et al.*, 2013). How much of each type of recruitment occurs defines the amount of gene flow and connectivity within meta-populations. Understanding larval dispersal is therefore key for ensuring the persistence and connectivity of restored *O. edulis* beds. The two fundamental questions to be answered are: where do larvae come from (i.e. the source populations of settling larvae) and where do they go to (i.e. the settlement sites or sink

locations of dispersing larvae) (Levin, 2006; Pineda, Hare and Sponaugle, 2007). Understanding the biological and environmental processes affecting larval dispersal remains a fundamental challenge, especially because these processes vary at different temporal and spatial scales (Pineda, Hare and Sponaugle, 2007). The main factors driving larval dispersal in the marine environment are reviewed below.

(i) Hydrodynamic features

Oceanographic currents transport larvae by advective and diffusive processes. ‘Advection’ is the mean transport of a group of particles through currents, while ‘diffusion’ represents the differences in transport of individual particles due to turbulent eddy motions (Largier, 2003). Large-scale circulation patterns tend to disperse pelagic larvae, while small-scale processes and interactions of currents with the bathymetry may retain larvae locally and limit horizontal advection and diffusion (Paris and Cowen, 2004). Processes facilitating retention near land masses include eddies, tidal bores, topographically steered currents and vertically stratified waters with different residence times (Paris and Cowen, 2004; Pineda, Hare and Sponaugle, 2007). For example, areas characterised by eddies have been found to harbour greater abundance of oyster spat (Korringa, 1940).

Flow patterns influencing larval dispersal in coastal shallow waters are complex and therefore less understood than in deeper waters (Pineda, Hare and Sponaugle, 2007). Several processes shape the flow in nearshore waters including surface gravity waves, wind-forcing, internal stratification and waves driven by differences in water buoyancy, surface and internal tides, and boundary-layer effects. In addition, flows are broken by coastline topographic features and have smaller spatial coherence (Pineda, Hare and Sponaugle, 2007). The relative importance of these processes in shaping flow patterns decreases with depth, distance from shore and varies intra-annually (Pineda, Hare and Sponaugle, 2007).

Close to the seabed flow velocities decrease in a logarithmic fashion due to bottom friction, and current velocities can become nearly still at the bottom boundary layer (Fig. 1-4; Caldwell and Chriss, 1979; Butman, 1986). A boundary layer may be laminar or turbulent (Butman, 1986), and the turbulent characteristics can differ significantly with different roughness geometries (Papanicolaou *et al.*, 2001). The flow fields above highly irregular bed surfaces are complex and difficult to characterise (Robert, Roy and De Serres, 1996), and they are therefore still not fully understood (Nikora *et al.*, 2004). For instance, it is not clear how flow velocities distribute over rough elements near the seabed,

especially in the layer below the top of the element (Nikora *et al.*, 2004). Model studies suggest that larvae staying close to the bottom would considerably reduce dispersal distances through decreased current velocities (North *et al.*, 2008; Sundelöf and Jonsson, 2012; Puckett *et al.*, 2014). However, we still have limited knowledge on how the geometrical structure of the seabed may influence flow characteristics and thus larval transport and retention.

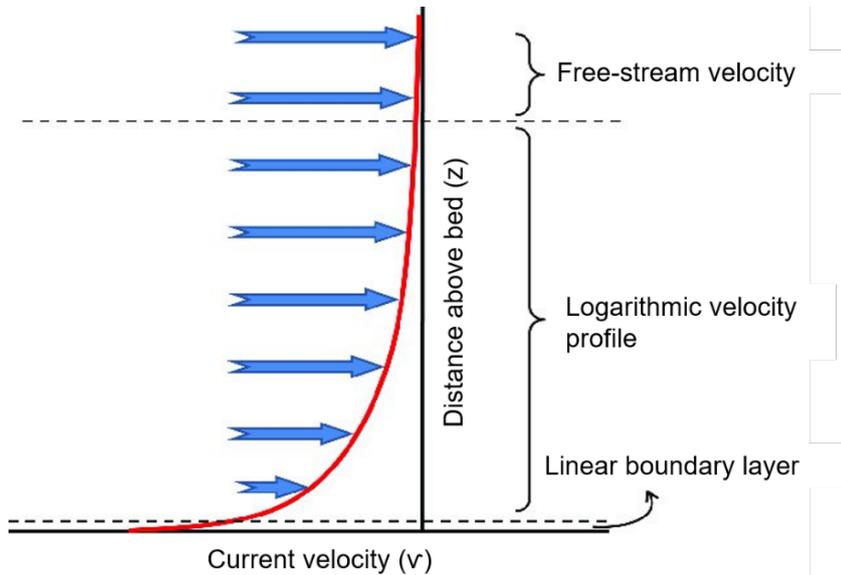


Figure 1-4. Current velocity profile close to the seabed composed of three layers: the free-stream current velocity, the logarithmically decreasing velocity and the linear benthic boundary layer (Montserrat, 2011).

Inter-annual variations in large-scale physical processes such as El Niño or coastal upwelling can modulate smaller-scale processes enhancing or suppressing larval transport (Pineda, Hare and Sponaugle, 2007). For example, a thermocline caused by El Niño may restrict larval transport for vertical diel migrations (Pineda, Hare and Sponaugle, 2007) or allow larvae to control horizontal dispersal by exploiting vertically stratified currents (Paris and Cowen, 2004).

Because larvae have to interact with these physical processes throughout their life history, hydrodynamic features have the potential to influence a range of behaviours (Paris and Cowen, 2004), such as the timing of adult spawning (Huggett *et al.*, 2003), the timing of larval setting (Korringa, 1940) and vertical migration strategies during larval ontogeny (Paris and Cowen, 2004).

(ii) Spawning

Spawning time and location in relation to hydrodynamic features and suitable settlement sites determines dispersal and the finding of suitable habitats. Marine species may

therefore adapt their spawning strategy to the local circulation patterns to enhance larval transport success (Huggett *et al.*, 2003). For example, the release of larvae by the Olympia oyster (*Ostrea lurida*) seems to be synchronised with the period when water residence time is at its maximum due to high temperatures and low levels of stratification, thus optimising larval retention (Peteiro and Shanks, 2015).

In addition, factors affecting spawning and fertilization success such as age and condition of the spawners influence the number of larvae released and thus larval dispersal and population connectivity patterns (Pineda, Hare and Sponaugle, 2007).

(iii) Larval swimming behaviour

Despite weak swimming velocities of planktonic invertebrate larvae compared to ocean current velocities, larvae can control their passive transport through vertical migrations and influence the intensity and direction of their dispersal (North *et al.*, 2008). For example, in stratified flows, larvae can position themselves in a water mass which moves away from the shore or in one that enables onshore transport (Kingsford *et al.*, 2002). In bays where stratification is weak, shear created by bottom friction can also affect horizontal transport (Manuel *et al.*, 1996).

Larval vertical migration is enabled by slow vertical current velocities relative to larval swimming and sinking capabilities (Dekshenieks, Hofmann and Powell, 1993). Larvae modify their vertical position in the water column both to feed and to avoid predators (Wheeler *et al.*, 2015). On the other hand, it is thought that larvae orient themselves in the water column in such a way that there is a higher probability of survival through physical transport to a suitable environment. Inclusion of larval vertical behaviour in larval dispersal models shows that larval behaviour can significantly alter the intensity and direction of their transport (North *et al.*, 2008; Sundelöf and Jonsson, 2012).

(iv) Larval settlement

Once the larvae is competent to metamorphose, it has to make contact with the substratum and evaluate the potential settlement site (Eckman, 1996). Soluble, hydrodynamic and potentially acoustic cues bring larvae in contact with surfaces and trigger the stereotypical settlement behaviour (Fitt *et al.*, 1990; Whitman and Reidenbach, 2012). The larvae may contact the substratum through a combination of passive sinking, active swimming and turbulent advection created by bed roughness (Crimaldi *et al.*, 2002; Whitman and Reidenbach, 2012). In addition, microcurrents from filter feeders can bring larvae in contact with the substratum (Campbell *et al.*, 2011). Upon contacting a potential

settlement site, the larvae will either reject the site and resume the planktonic state, or accept it and metamorphose into the benthic life form (Eckman, 1996). To complete the larval dispersal loop it is therefore important to predict the probability of larvae attaching to the substratum and entering the benthic state (Eckman, 1996).

(v) Larval mortality

Spatial heterogeneity in mortality shapes patterns in species abundance, distribution and demographics (Pineda, Hare and Sponaugle, 2007). For instance, higher mortality rates caused a significant reduction in the numbers of retained and settled larvae (Young *et al.*, 1998). Mortality is seldom accounted for in larval dispersal studies. To assess connectivity between populations, post settlement survival has to be taken into account (Pineda, Hare and Sponaugle, 2007).

(vi) Pelagic larval duration

The period of time during which larvae are in the water column before settlement is called pelagic larval duration (PLD). Planktotrophic larvae, such as *O. edulis*, feed whilst in the plankton, while lecithotrophic larvae obtain their energy from egg yolk reserves supplied by the mother. PLD of planktotrophic larvae is therefore longer than of lecithotrophic larvae (Pawlik, 1992), and may reflect the time larvae need to obtain sufficient energy and nutrients to undergo the growth and anatomical changes required as precursors to settlement. PLD is often taken to be a proxy of dispersal ability and this metric is thus one of the fundamental components examined in the study of dispersal probability and population connectivity (Cowen and Sponaugle, 2009; Leis, 2015). However, while a species with short PLD will inevitably have a short dispersal, species with long PLD do not necessarily disperse widely (Shanks, 2009). This is because larval behaviour can contribute to retention or return to natal sites (Sponaugle *et al.*, 2002), thus breaking the otherwise direct relationship between PLD and dispersal distance (Pineda, Hare and Sponaugle, 2007). Longer PLD yield higher cumulative mortalities than shorter PLD, since the larvae are exposed for longer time to predation and other factors controlling mortality (Korringa, 1940; Pineda, Hare and Sponaugle, 2007).

1.4.2. Biophysical models

Biophysical modelling, i.e. combining hydrodynamic models with particle tracking models, which mimic biological traits, has emerged as a powerful tool to simulate and predict larval dispersal. All the above-mentioned variables, which affect dispersal (hydrodynamic features, spawning time or larval release, larval vertical distribution,

swimming speeds, settlement, PLD and mortality), can be included in biophysical models, if the data are available. Biophysical models have been used to simulate larval dispersal and inform conservation measures in several bivalve species. For instance, in Pamlico Sound (USA) for the Eastern oyster *Crassostrea virginica* (Puckett *et al.*, 2014), in Strangford Lough (Ireland) for *O. edulis* (Smyth *et al.*, 2016), and in the Irish Sea for the common cockle *Cerastoderma edule* (Coscia *et al.*, 2013) and the horse mussel *Modiolus modiolus* (Gormley *et al.*, 2015). The number of biological parameters included in each biophysical model varies depending on the availability of data. For instance, most studies include spawning time, spawning location and PLD, but only some include larval swimming behaviour (e.g. North *et al.*, 2008; Kim *et al.*, 2010; Thomas *et al.*, 2012; Coscia *et al.*, 2013; Robins *et al.*, 2013) or settlement (e.g. North *et al.*, 2008; Coscia *et al.*, 2013; Robins *et al.*, 2013). The more biological information is included, the more accurate the model prediction becomes. The ambition of current *O. edulis* restoration and conservation efforts is to be able to predict larval dispersal with biophysical models. There is, however, a knowledge gap on the larval behaviour and ecology of *O. edulis*, which this thesis aims to address.

1.5. Biology and ecology of *O. edulis* relevant to its dispersal

1.5.1. Habitat

O. edulis is an euryhaline species, able to colonize estuaries and coastal waters exposed to freshwater influence (Yonge, 1960), but optimal growth occurs in fully marine areas (Hutchinson and Hawkins, 1992). It is found in the lower intertidal and deeper sublittoral regions, with the intertidal zone generally being less populated, presumably because of the stress caused by fluctuating environmental factors (Laing, Walker and Areal, 2005; Gercken and Schmidt, 2014). In the sublittoral regions *O. edulis* beds can occur down to a depth of approximately 80 m, e.g. the ‘deep-sea oysters’ of the German Bight (Gercken and Schmidt, 2014), but they were most commonly found at depths of up to 30 m (Laing, Walker and Areal, 2005).

O. edulis can be found in a variety of sites, from exposed to very sheltered (Perry and Tyler-Walters, 2016). The optimal current intensity and exposure level may depend on the substrate composition in each site, since *O. edulis* is sensitive to prolonged high turbidity levels. Thus, higher current velocities may only be suitable in combination with a substrate which is not easily eroded. For example, *O. edulis* beds on shallow sublittoral muddy mixed sediment are characterised by very weak to weak tidal streams (< 50cm/s),

and are in extremely sheltered to very sheltered sites (Perry and Tyler-Walters, 2016). In contrast, the former so called ‘current beds’, located in the German Wadden Sea, were exposed to strong tidal currents and located on hard substrate (Gercken and Schmidt, 2014). *O. edulis* can form beds in up to a medium turbidity level (Perry and Jackson, 2017), but optimal growth requires clear water (Korringa, 1952). While short periods of relatively high suspended sediment quantities can be tolerated (Laing, Walker and Areal, 2005), in the long term it hampers growth by reducing the oysters filtration rate (Korringa, 1952), increasing pseudofeces production (Korringa, 1952), and restricting spatfall (Moore, 1977) and therefore recruitment.

1.5.2. Reproduction and growth

O. edulis is a sessile bivalve mollusc and a rhythmical consecutive hermaphrodite: young individuals mature first as males and after a few years they alternate between male and female sexual phases (Millar, 1964). The time it takes individuals to start reproducing as a female and the subsequent rate of sex change is strongly influenced by sea water temperature and thus the latitude at which the oysters occur (Spärck, 1922; Korringa, 1940). For example, in the Limfjord (Denmark), the first sex change occurs when the oyster is three or four years old (Spärck, 1922) and individuals form only one gender per year, while in the Bay of Biscay and the Mediterranean, gender changes may occur several times per year (Gercken and Schmidt, 2014). In the UK, oysters start reproducing as a female at about two years of age and thereafter they usually function once as each gender per season (Cole, 1942). At about four years of age, individuals at UK latitudes are regarded as adults (Cole, 1941).

O. edulis is a viviparous species, which spawns in the summer months, and subsequently broods the larvae inside the mother oyster before releasing them into the water column. According to Korringa (1957) spawning coincides with spring tides of both new and full moon. Spawning generally requires a minimum temperature of about 15°C (Korringa, 1940), although the exact temperature varies with area and local adaptation of the population (Korringa, 1957). For example, the former population of *O. edulis* in the Firth of Forth seldom experienced temperatures above 15°C, and thus temperatures below 15°C presumably triggered spawning. However, in the warm sea water ‘polls’ in Norway, which are sheltered ponds that communicate to a fjord and are heated by the greenhouse effect of a freshwater layer, spawning did not occur below 20°C (Korringa, 1957). Sas *et al.* (2019, and references therein) estimated a threshold temperature of 7°C and a subsequent temperature sum of 593 degree-days for the first larval peak (i.e. larval release

after spawning) in the Dutch Voordelta. It is not known whether there are genetically triggered differences in spawning (Korringa, 1957), but individuals translocated from France to Loch Ryan in Scotland were able to adapt to local environmental conditions, and after a few years they spawned almost in synchrony with local individuals (Low *et al.*, 2007). Once the oysters have started to spawn, breeding is thought to continue as long as the temperature remains above the threshold value (Korringa, 1940). Thus, the span of the reproductive season can vary in length (Table 1-1) and depends on the local climatological conditions (Korringa, 1940).

Table 1-1. Breeding season of *O. edulis* by location

Location	May	June	July	August	September	Source
Scotland	[Bar from May to August]					Low <i>et al.</i> (2007)
Northern Ireland		[Bar from June to September]				Kennedy and Roberts (1999)
Dutch Oosterschelde		[Bar from June to September]				Korringa (1952)
German Wadden Sea		[Bar from June to September]				Möbius (1877)
Danish Limfjord	[Bar from May to August]					Personal observation

Functionally mature males produce sperm in packets, which break apart only upon contact with seawater. This is probably a mechanism to avoid self-fertilization (Orton, 1927), since ripe sperm packets may remain after sex change in the functional females (Korringa, 1952). Egg production in females increases with age and size from about 90,000 eggs in one-year old oysters to up to 1.5 million eggs in adult females (Cole, 1941; Walne, 1964). In addition, adults which mature as females from the onset of the season produce twice as many eggs than the ones functioning first as males and in the second half of the season as females (Cole, 1941). Functional females transport sperm via the inhalant siphon into the mantle cavity, where eggs are fertilized (Orton, 1927). The larvae are retained in the mantle cavity and brooded for about 7 to 10 days from the date of spawning, although brooding time may exceed these values at temperatures below 15°C (Korringa, 1940). Subsequently the approximately 170-180 µm sized larvae are released into the plankton (Korringa, 1940).

O. edulis larvae are released from the mantle cavity of the mother oyster in the ‘D-stage’ (or veliger stage). As they continue to grow from the ‘D-stage’, they pass through ‘early umbo’ and ‘umbo’ stages, before reaching the ‘pediveliger’ stage, in which they are about ~290-300 µm large and ready to settle (see Table 1-2 and Fig. 1-5 for a description of the stages). The larvae are planktotrophic, which means that they have to feed on plankton to continue their larval development until they are ready to settle.

Table 1-2. Description of *O. edulis* stages, adapted from Acarli and Lok (2009)

Stage	Description
D-stage	When newly released from the pallial cavity of the adult oysters. During this stage, larvae are semi-transparent, and they are slightly 'D' shaped.
Early umbo	The umbo (the top of each half of the shells) becomes slightly oval and the shape of the larvae resembles more a ball. The inside of the larvae is slightly less transparent.
Umbo	The umbo is fully developed and protrudes distinctively. The inside becomes darker.
Pediveliger	In addition to a fully developed umbo, larvae have an eyespot and foot. The eyespot is distinctly visible on both sides of their shell, whilst the foot is generally inside their shell and therefore only visible when protruded during settlement searching behaviour. In this stage, larvae are ready to settle and metamorphose into a young spat.

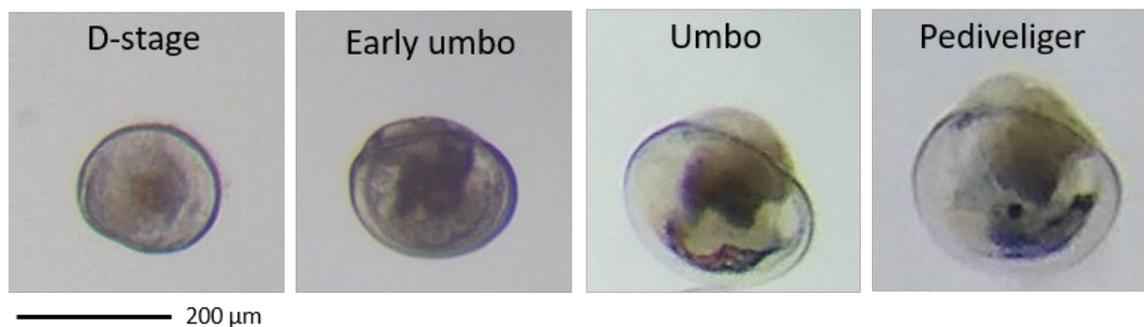


Figure 1-5. Larval development stages of *O. edulis* (stages after Helm, 2004; Acarli and Lok, 2009; photos: Ana Rodriguez Perez). Image illustrates the shell shape at each developmental stage; the inner colouration when still alive is only visible for umbo and pediveliger stages.

Once the larvae is competent to settle, they start to search for suitable settlement substrates by swimming with the foot protruded between the shells (Cole and Knight-Jones, 1939). At his point the sensory organs and strength of the larvae are at their highest development, reflecting the importance of the settling period (Cole and Knight-Jones, 1949). The actual settling seems to be preceded in all cases by an exploratory phase, during which the larvae crawl over the substratum with introverted velum and the foot extended in front. Repeated contractions of the foot drag the body forward. If no suitable substratum for attachment is present, crawling alternating with normal swimming can extend over several days. If the environment is appropriate, larvae will cement permanently to the substratum and metamorphose into juvenile oyster, called spat (Cole and Knight-Jones, 1939; Coon, Bonar and Weiner, 1985).

1.6. Objectives and thesis structure

The aim of this thesis was to study the larval behaviour and ecology of *O. edulis* in so much as it is relevant to the dispersal and settlement of this species, with the goal of informing restoration and conservation efforts. The thesis focuses on the following parameters that are critically important to larval dispersal and can be incorporated into biophysical models: (i) vertical distribution, (ii) swimming speeds, (iii) pelagic larval duration and (iv) settlement preferences.

Chapter 2 quantifies the vertical distribution of *O. edulis* in relation to food, light and temperature throughout the larval life history. In addition, swimming speeds and stereotypical behaviours of larvae in the water are analysed. This chapter also presents a new inexpensive method, to visualise larvae in the water column.

Chapter 3 investigates the settlement preferences of *O. edulis* in the context of its natural habitat. The efficiency of a range of treatments is investigated, based on the hypothesis that habitat-associated chemical cues may be critical in inducing settlement.

Chapter 4 studies the pelagic larval duration (PLD) of *O. edulis* at three different temperatures, relevant to its distribution range. It also investigates the potential of larvae to delay metamorphosis if suitable settlement sites are absent, therefore prolonging their PLD.

Chapter 5 discusses the dispersal of *O. edulis* larvae and population connectivity in depth, considering the results of this thesis, as well as previous literature. It ends by addressing the wider implications for conservation and restoration efforts and provides suggestions on how to maximise recruitment and connectivity.

Chapter 2. Larval behaviour in the water column

2.1. Overview

This chapter quantifies the vertical distribution and swimming speeds of *O. edulis* larvae in relation to larval life history, food, light and temperature. The aim of this study was to obtain relevant parameters, which can be incorporated into biophysical models to predict dispersal of *O. edulis* larvae. The chapter also describes typical behaviours of larvae in the water column and presents a novel and inexpensive method to visualise larvae.

2.2. Introduction

Understanding larval dispersal is key to the management and conservation of marine populations (Jones *et al.*, 1999; Cowen and Sponaugle, 2009; Sundelöf and Jonsson, 2012). Larval dispersal and recruitment determine the distribution and persistence of marine populations (Cowen and Sponaugle, 2009; Ottmann *et al.*, 2016). This can be either self-recruitment, i.e. the larvae settles in the parental population, or recruitment of larvae originating from geographically distant populations (Robins *et al.*, 2013). Until the end of the 1990s the near unanimous view was that larvae were dispersed passively by currents over large spatial scales, leading to open populations (Caley *et al.*, 1996; Roberts, 1997; Jones *et al.*, 1999; Leis, 2015). This traditional view was supported by the fact that pelagic environments are highly dynamic (Ottmann *et al.*, 2016), while larvae of many taxa have weak swimming capabilities (North *et al.*, 2008; Hata *et al.*, 2017). Dispersal was therefore thought to be dominated by currents (Ottmann *et al.*, 2016), with weak dispersal barriers (Giller *et al.*, 2004) and open marine populations, potentially over hundreds to thousands of kilometres (Cowen and Sponaugle, 2009).

Although larvae have the potential for long-distance dispersal (Scheltema, 1986; Cowen, Paris and Srinivasan, 2006) and currents are a major force in larval dispersal, evidence is mounting that retention is much more common than predicted by simple advection models (Cowen, Paris and Srinivasan, 2006; Gerlach *et al.*, 2007). For instance, up to 60% of self-recruitment has been shown in coral reef fish populations using tagging and re-capture techniques (Jones *et al.*, 1999; Almany *et al.*, 2007), demonstrating that larvae

can be retained in their natal environment despite a pelagic dispersal phase (Gerlach *et al.*, 2007). These studies show that larvae of many species, particularly fish and crabs, have the behavioural and sensory capabilities to strongly influence their dispersal trajectories (see Kingsford *et al.*, 2002 for a review). For instance, fish larvae can swim at speeds that are often comparable to the currents of waters in which they live, and they use a range of olfactory, auditory and visual cues to guide them towards their settlement habitat (Leis, 2015). Most marine invertebrate larvae, such as oysters, have much weaker swimming capabilities and it is less clear to what extent they are able to influence their fate in the water column (e.g. compare Korrynga, 1940; Wood and Hargis, 1971; North *et al.*, 2008; Hata *et al.*, 2017). Passive transport is thus often still considered to be a reasonable approximation to their dispersal (Cowen and Sponaugle, 2009; Smyth *et al.*, 2016; Hata *et al.*, 2017).

Marine invertebrate larvae have a well-developed sensory capacity (Kingsford *et al.*, 2002; Lillis, Eggleston and Bohnenstiehl, 2013; Hata *et al.*, 2017) and the evolution of such senses suggests that they are not behaving passively in the water column. Larvae can perform vertical migrations, provided vertical current velocities do not exceed larval swimming capabilities (e.g. Wood and Hargis, 1971; Mileikovsky, 1973), and such vertical migrations can have profound effects on the intensity and direction of their dispersal (North *et al.*, 2008; Sundelöf and Jonsson, 2012). Larvae modify their vertical position in the water column to feed and avoid predators, and it is thought that they orient themselves in the water column in such a way that there is a higher probability of survival through physical transport to a suitable environment (Kingsford *et al.*, 2002; Paris and Cowen, 2004; Moksnes *et al.*, 2014). For instance, in stratified flows larvae can position themselves in a water mass which moves away from the shore or in one that enables onshore transport (Kingsford *et al.*, 2002); while in bays where stratification is weak, shear created by bottom friction results in different horizontal transport velocities, which larvae can also exploit through vertical migrations (Manuel *et al.*, 1996). Systematic behaviours in vertical distribution are therefore expected to have strong effects on dispersal (Sundelöf and Jonsson, 2012). Vertical migration is often based on a combination of responses to external cues, such as pressure, temperature, food, salinity and light (Kingsford *et al.*, 2002) and the larvae's response can vary with ontogenetic stage (Kunkle, 1957; North *et al.*, 2008; Cohen *et al.*, 2015).

In this chapter, the vertical distribution, swimming speeds and behaviour of *O. edulis* were investigated throughout its larval life history and under the different environmental

scenarios of light/ darkness, food/ no food, and temperature. The aim was to elucidate whether (1) *O. edulis* larvae show any systematic behaviour that could affect their dispersal in the water column, and (2) whether this behaviour changes with larval life history or in response to the different environmental variables.

2.3. Materials and methods

2.3.1. Larval cultures

Adult *O. edulis* individuals, obtained from the Limfjord (Denmark), spawned repeatedly at the Danish Shellfish Centre (DSC) throughout the summer spawning season. Newly released larval batches were transferred into 15 L flow-through tanks with 1 µm filtered sea water at an approximate concentration of 10 larvae/ml. The larvae were raised at 25°C and fed daily a microalgae mixture consisting of *Chaetoceros muelleri*, *Tisochrysis lutea* and *Pavlova gyraus* with a drip feed system that maintained a continuous concentration of circa 100 cells/µl. Within approximately 7 days larvae developed from D-stage to mature pediveliger that were ready to settle.

2.3.2. Experimental design and procedure

Two experiments were carried out to study the vertical distribution of *O. edulis* larvae: In the first experiment, the distribution of around 20 larvae was quantified in 50 cm tall aquaria throughout the larval life history and under the different scenarios of light/darkness, food/ no food and two contrasting temperatures. In the second experiment, around 1,000 larvae were observed in small glass beakers and their distribution was estimated at regular time intervals and throughout the larval life history.

Experiment 1:

Experiment 1 tested the influence of larval life history, light, food and temperature on the vertical distribution of larvae and their swimming speeds. Age, length and morphological stage were used as proxies for larval life history. In total 1,170 larvae were observed in 102 aquaria over 13 days. The experiment was carried out in a temperature control room that had a daylight simulating lightbulb installed (model: Philips TL-D 36W Super 80 MASTER). The first half of the experiment was carried out at 24°C and the second part at 13°C with an acclimation step (see below).

Each day, 3-6 aquaria (height x width x depth: 50 x 4 x 1 cm) were set-up with 1 µm filtered sea water (of 25.9 ppt average salinity) and without aeration. Treatment food/ no food was randomly allocated to each aquarium. Aquariums with food received a

microalgae mixture of *Chaetoceros muelleri*, *Tisochrysis lutea* and *Pavlova gyrams* (volume ratio 5:1:1) at a concentration of 100 cells/ μ l. On most days, only one batch of larvae was available, and all aquaria were therefore allocated larvae of the same age class, which were in a similar developmental stage. However, when two larval batches were available, age class was also randomly assigned to each aquarium. Aquaria were observed in light, darkness or consecutively in light and darkness, the order of the latter being randomly determined. When aquaria were observed both in light and darkness, larvae were left to acclimatise for at least 30 min to the new treatment level before observation (Table 2-1 and Fig. 2-1).

Table 2-1. Summary of treatment conditions and number of replicated aquaria per day.

Date	Larval batch [release date]	Larval age (days)	Temp.	Food		Light [order]		Total no. of aquaria
				No. of aquaria with food	No. of aquaria with no food	No. of aquaria with light	No. of aquaria in darkness	
29/06/17	ID1 [28/06]	1	24°C	6	0	6	0	6
30/06/17	ID1	2	24°C	1	2	0	3	3
01/07/17	ID1	3	24°C	2	1	3	0	3
03/07/17	ID1	5	24°C	3	3	6 [1 st]	6 [2 nd]	6
05/07/17	ID1	7	24°C	3	3	6 [2 nd]	6 [1 st]	6
08/07/17	ID1	10	24°C	0	3	3 [1 st]	3 [2 nd]	3
08/07/17	ID1	10	13°C	0	3	3	0	3
12/07/17	ID2 [11/07]	1	13°C	3	3	6	0	6
14/07/17	ID3 [13/07]	1	13°C	3	3	3 [2 nd]	3 [1 st]	6
15/07/17	ID3	2	13°C	0	6	6	0	6
17/07/17	ID3	4	13°C	2	4	6 [1 st]	4 [2 nd]	6
18/07/17	ID3	5	13°C	0	3	3 [2 nd]	3 [1 st]	3
19/07/17	ID3 & ID4 [18/07]	6 & 1	13°C	0	6	6	0	6
20/07/17	ID3 & ID4	7 & 2	13°C	4	0	4	0	4

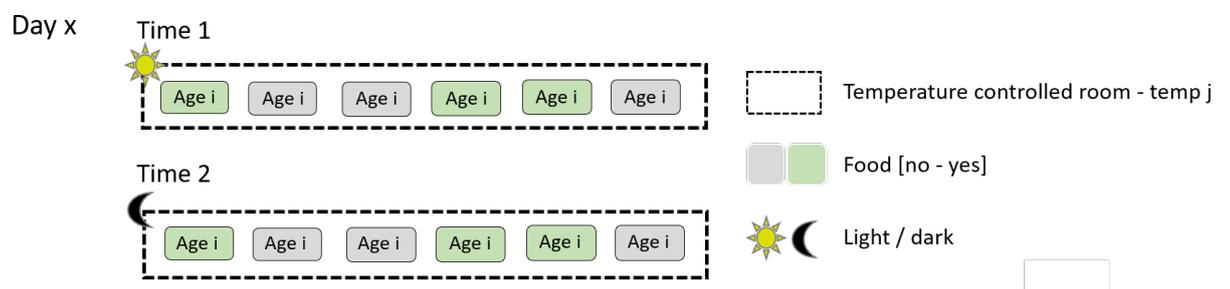


Figure 2-1. Schematic representation of experimental set-up.

An average of 18 larvae (range: 6 - 58) were pipetted out from the holding tanks and into the top of each experimental aquarium. To avoid adding food to the aquaria with 'no food' treatment, larvae were first pipetted into a small 80 μ m sieve (which was partly held in

seawater), then carefully washed with a squeeze bottle from the sieve into a well plate and subsequently pipetted from the well plate into the aquarium. Larvae were left to acclimatise to the aquariums for at least 30 min at 24°C, and overnight (13-18h) at 13°C. The latter time span allowed larvae to acclimatise to the temperature drop from their holding tanks at 25°C to the experimental temperature of 13°C.

Every aquarium was subsequently video recorded with a USB microscope, supported by a retort stand, and connected to a computer for visualisation. Aquaria were supported by a wooden frame, and a graticule behind each aquarium marked the height of the aquarium in cm and mm resolution (Fig. 2-2, see Appendix 2 for a detailed description of the methodology). The order of the recording (i.e. top to bottom or bottom to top) was randomly assigned to each aquarium. A red filter (filtering light to 590-730 nm, Appendix 2 Figure A2-4) was used on the USB microscope in darkness trials to minimise light disturbance to the larvae. Recording of all aquaria lasted between 1 and 7 h per day, depending on the number of aquaria and treatment levels (Table 2-1), with an average time of 5 h per day and 21 min per aquarium. After all aquaria had been filmed, larvae were filtered out onto an 80 µm sieve and preserved in a well-plate with seawater and ethanol. All larvae were subsequently (i) counted and assigned to the predominant morphological state with a binocular microscope, and (ii) measured with a computer imaging system (Nikon Digital Sight DS-U3, imaging software: NIS-Elements BR).

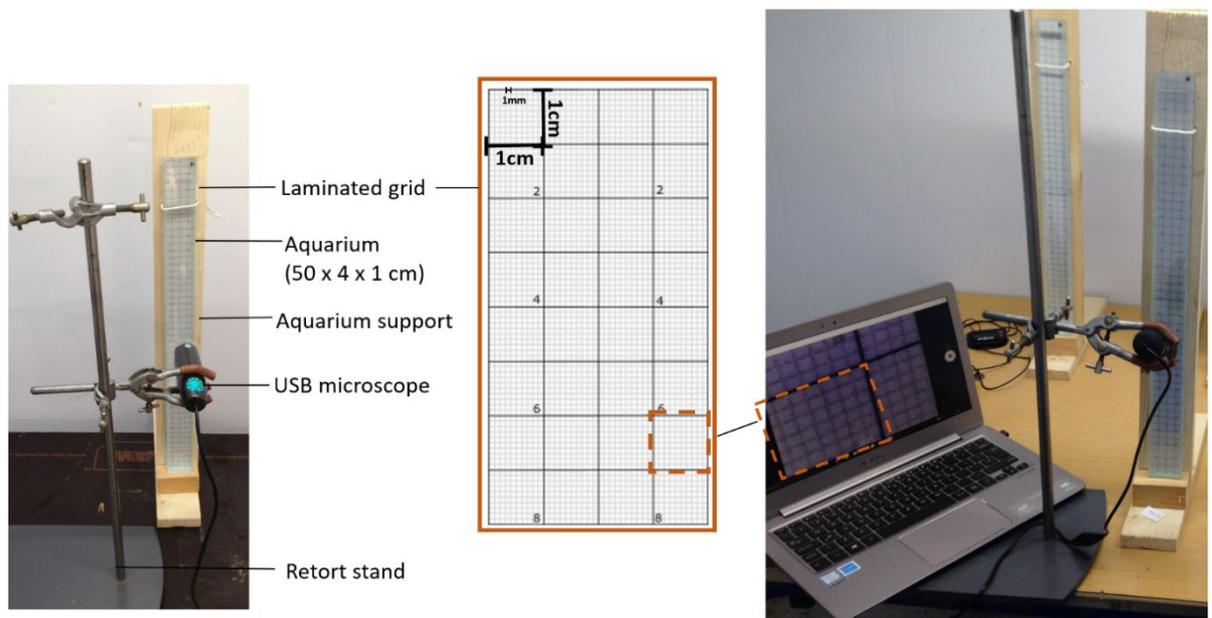


Figure 2-2. Illustration of method used to quantify vertical distribution of larvae in the water column.

Experiment 2:

In experiment 2, an average of 1,276 larvae (range 133 to 3,700) were observed in 150 ml beakers without magnification. Observation by eye was possible due to the high concentration of larvae in the beakers, particularly for the smallest size classes: only the largest size classes, which are ~ 0.3 mm large and thus well visible by eye, were concentrated at $N < 800$ individuals. Each round, two replicate beakers were filled to 80 ml with sea water-, food- and larvae-mixture from the holding tanks. The number of larvae in each beaker was estimated by gently mixing the beaker and counting the number of larvae in three replicate 1 ml samples. The beakers were held under natural day-light cycles and at an average room temperature of 23.6°C. Larvae were monitored for at least 28 h at regular time intervals (on average every 101 min, excluding night time). In each observation, the quantity of larvae at the surface, inside the water column and at the bottom were estimated and assigned to one of the following categories: ‘none’ (0%), ‘very few’ (<5%), ‘few’ (~10%), ‘some’ (~20%), ‘several’ (~30%), ‘many’ (~40%), ‘most’ (>50%), ‘all’ (100%). After 1.5-3 days, larvae were filtered out of both beakers and preserved in ethanol diluted with water. All larvae were subsequently measured with a computer imaging system (Nikon Digital Sight DS-U3, imaging software: NIS-Elements BR) and the predominant morphological state was noted. The whole procedure was repeated seven times, each round with a new group of larvae from the holding tanks, so that all larval states were observed by the end of the experiment.

2.3.3. Analysis

Vertical distribution

All videos from experiment 1 were carefully examined, and the vertical position and behaviour of every larvae was noted. Positions were recorded with millimetre resolution, with 1 being the water surface and 500 the bottom of the aquarium.

To test differences in vertical distribution, the water column was divided into three positions: surface, column and bottom. These positions were identified as the most distinct areas based on visual examination of larval distribution. Surface included up to 1 cm below the surface (mm 1-10), bottom up to 1 cm above the aquarium floor (mm 491-500) and column the remaining water column of 48 cm (mm 11-490). Larval position was subsequently regressed on the predictors: larval life history (age/ size/ morphological stage – all three tested), food (yes, no), light (light, darkness) and temperature (13, 24°C) using ordinal logistic regression. Models of decreasing complexity were compared with the full model including all predictors and the null model with none of the predictors. The

model with the lowest value of Akaike's Information Criterion (AIC) was selected. When model differences were weak (AIC difference < 2) a likelihood ratio test (LR) was performed to test for significant differences. The analysis was conducted in R 3.4.0 (R Core Team, 2017) with the *MASS* package (Venables and Ripley, 2002) and its function 'polr' for ordinal logistic regression.

Swimming speeds

Larval swimming speeds were analysed with the software Tracker 5.0.6 (Brown, 2018), a freely available video analysis tool. A total of 316 larval paths were analysed: larvae's position relative to a calibrated coordinate system were manually marked every 5-20 frames (video: 30 frames/sec), depending on the larvae's swimming speed. Larger frame intervals were selected for slower swimming larvae to avoid overestimating their travel path. The software generated the variables t (time in sec), y (vertical displacement distance in mm) and L (path length in mm), which were used to calculate (i) swimming speeds (Formula 1) and (ii) net-to-gross displacement ratios (NGDR) (Formula 2). NGDR is a measure of how twisted or straight larval paths are, with 0 being a loop and 1 a perfect line (Tamburri, Zimmer-Faust and Tamplin, 1992).

Formula 1: Swimming speed

$$v = y/t$$

Formula 2: Net-to-gross-displacement ratio (NGDR)

$$NGDR = y/L$$

A multiple linear regression was used to test for significant differences in swimming speeds with the variables: larval life history, food, light and temperature. Swimming speed values were square root transformed to comply with model assumptions of normality and homogeneity of variance. The full model, containing all predictors, was compared with each of the three alternatives of larval life history (age, size and morphological stage) and the model with the lowest AIC was selected. A backward stepwise selection procedure was subsequently used with the package *car* (Fox and Weisberg, 2018) to find the model with the fewest predictors possible based and the smallest AIC value.

NGDR values were analysed with a beta-regression. Models containing the variables larval life history, food, light and temperature as individual predictor were compared to the full model and the null model, and the model with the lowest AIC was selected.

Compliance with model assumptions was examined visually through model diagnostics. All analysis was conducted in R 3.4.0 (R Core Team, 2017).

2.4. Results

2.4.1. Vertical distribution

O. edulis larvae showed a marked preference for the bottom of the aquarium, which was independent of larval life history, food, light or temperature (Fig. 2-3 and 2-4), and consistent across both experiments (Fig. 2-3 and 2-6) and over time (Fig. 2-6). On average 61 – 65% of larvae were at the bottom 1 cm, 27 – 29% were spread throughout the water column (48 cm) and 7 – 11% were at the surface (top 1 cm) of the aquarium (experiment 1). Larval size class and food explained best, the proportion of larvae in each position (Table 2-2 and 2-3). Within larval life history, size class (average size rounded to the nearest 10 μm) was a significantly better predictor (lowest AIC, Table 2-2) than larval stage (LR = 12.97, df = 6, $p = 0.04$), age (LR = 138.96, df = 10, $p < 0.001$) or mean size (LR = 146.99, df = 10, $p < 0.001$).

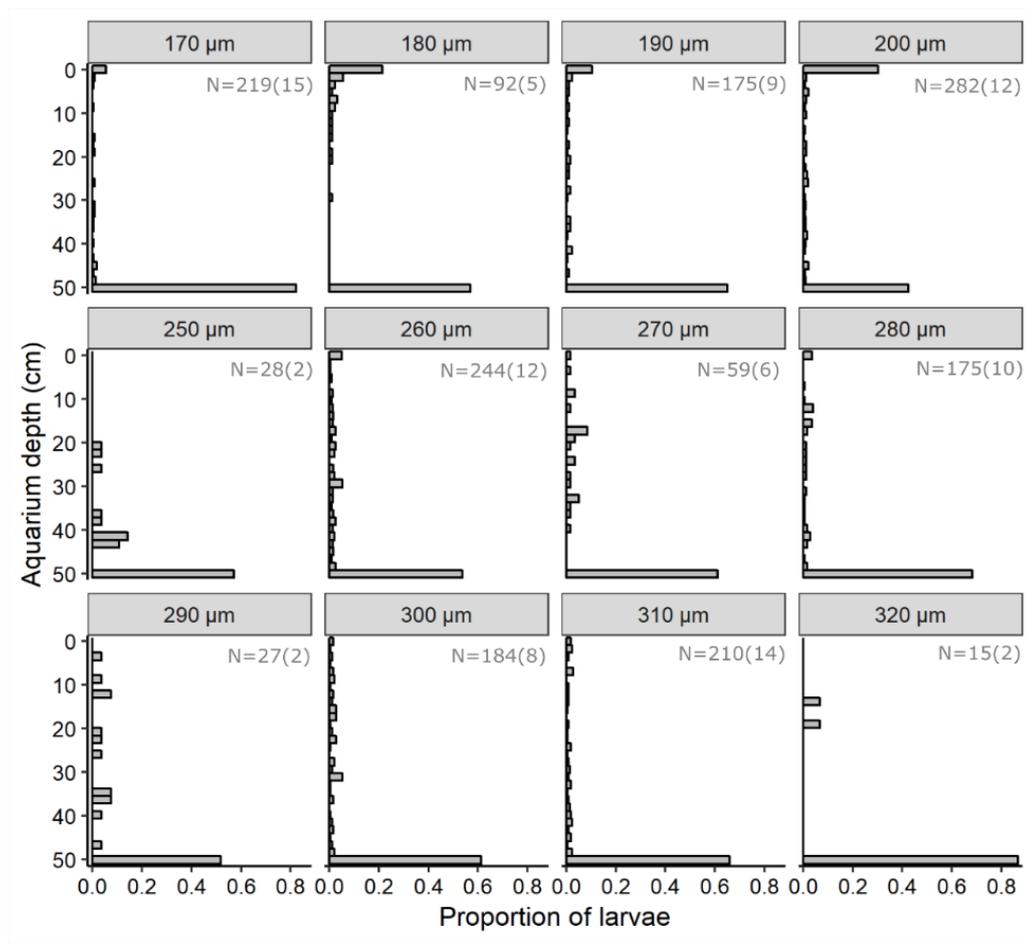


Figure 2-3. Vertical distribution of larvae by size class. N is the number of observed larvae; aquarium number is indicated in brackets.

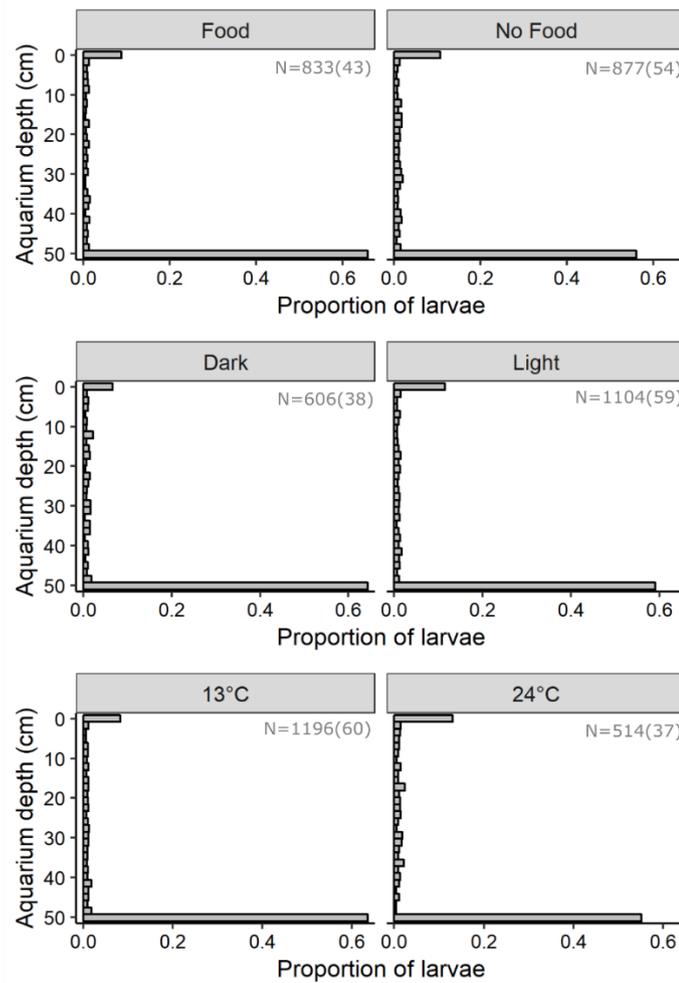


Figure 2-4. Vertical distribution of larvae in response to food/ no food, light/ darkness and temperature (13 and 24°C). N is the number of observed larvae; aquarium number is indicated in brackets.

The largest variability of larvae at the bottom was found within size class. A minimum of 42% of larvae was observed for size class 200 μm and a maximum of 87% for the largest size class of 320 μm (Fig. 2-3 and Appendix 3 Table A3-1). The model predicted a decreasing probability of finding larvae at the bottom from the smallest size class (170 μm) up to size class 200 μm , and a subsequent overall increasing probability until size class 320 μm (Table 2-2 and Fig. 2-5). Except for size class 200 μm and in the presence of food, the predicted probability of finding larvae at the bottom was above 50% throughout the larval life cycle (Fig. 2-5). The categorical observations of experiment 2 supported these numbers, since ‘most’ larvae (> 50%) were at the bottom during nearly all observations (Fig. 2-6). The only observations in which ‘many’/ ‘several’ larvae were observed at the bottom (i.e. < 50%) corresponds to an average larval size of 213 and 218

μm (Fig. 2-6). This matched the observations of experiment 1, in which size class 200 μm was the only size class for which a proportion $<50\%$ was observed.

Larvae also concentrated at the surface, although at much lower proportions than at the bottom (Fig. 2-3 and 2-4). For most size classes, only 0-6% of larvae were at the surface, but this proportion increased during a short developmental period: 180 – 200 μm . 21% of larvae were at the surface at the size of 180 μm , 12% at 190 μm and a maximum of 31% at 200 μm (Fig. 2-3 and Appendix 3 Table A3-1). Similarly, in experiment 2, the only time in which ‘many’ larvae were observed at the surface (the second largest category after ‘most’) was when larvae were 213 and 218 μm large. An estimated 30 – 40% of all larvae were at the surface, which is a similar number to the proportion recorded in experiment 1. At a slightly larger size (220 and 226 μm), there was no concentration of larvae at the surface (Fig. 2-6).

A proportion of the larvae were always in the water column, regardless of treatment. The smallest proportion was 11% for size class 170 μm and the largest 48% in size class 290 μm (Appendix 3 Table A3-1). However, unlike the bottom or the surface, larvae were evenly distributed and did not concentrate at any location of the 48 cm long water column (Fig. 2-3 and 2-4).

The absence of food resulted in significantly less larvae at the bottom and more in the water column and surface (Table 2-2 and Appendix 3 Table A3-1). The effect was particularly pronounced for size class 200 μm , in which the proportion of larvae at the bottom decreased without food and larvae concentrated in turn at the surface (Appendix 3 Fig. A3-1). The model predicted for larvae of size class 200 μm at the bottom a minimum of 42% with food, but only 25% in the absence of food (Fig. 2-5 and Appendix 3 Table A3-2). Similarly, in the absence of food, it also predicted less larvae at the bottom and more in the surface and water column for all other size classes (Fig. 2-5 and Table 2-2).

Table 2-2. Results of ordinal logistic regression showing the model selection process and the coefficients of the selected model. Coefficients are relative to reference classes, i.e. size class 170 in size class and food - yes for food; cat = categorical, con = continuous variable.

NULL Model			AIC = 3118.80
Model 1: Stage [cat] + Light [cat] + Food [cat] + Temperature [cat]			AIC = 2956.33
Model 2: Size class [cat] + Light + Food + Temperature			AIC = 2955.36
Model 3: Age [con] + Light + Food + Temperature			AIC = 3074.32
Model 4: Mean size [con] + Light + Food + Temperature			AIC = 3082.36
Model 5: Size class + Food			AIC = 2951.88
Model 6: Size class + Light			AIC = 2994.75
Model 7: Size class + Temp			AIC = 2991.27
Predictor of Model 5	Coefficient (SE)	<i>t</i> -value	<i>P</i>
Size class: 180	-1.47 (0.28)	-5.24	< 0.001 ***
Size class: 190	-1.45 (0.25)	-5.78	< 0.001 ***
Size class: 200	-2.31 (0.22)	-10.74	< 0.001 ***
Size class: 250	-1.56 (0.41)	-3.79	< 0.001 ***
Size class: 260	-1.20 (0.22)	-5.55	< 0.001 ***
Size class: 270	-0.82 (0.32)	-2.58	0.01 *
Size class: 280	-0.63 (0.24)	-2.66	0.008 **
Size class: 290	-1.73 (0.41)	-4.24	< 0.001 ***
Size class: 300	-1.14 (0.24)	-4.87	< 0.001 ***
Size class: 310	-0.74 (0.23)	-3.26	0.001 **
Size class: 320	0.66 (0.78)	0.86	0.393
Food: No	-0.77 (0.12)	-6.45	< 0.001 ***
Intercept: surface column	-3.94 (0.22)	-18.34	< 0.001 ***
Intercept: column bottom	-2.00 (0.20)	-10.18	< 0.001 ***

Table 2-3. Likelihood ratio (LR) Chi-square test evaluating the significance of each factor in the ordinal logistic regression model: Size class + Light + Food + Temperature

	LR Chi-square	df	<i>P</i>
Size class	154.59	11	< 0.001 ***
Light	0.16	1	0.6905
Food	39.77	1	< 0.001 ***
Temperature	0.30	1	0.5851

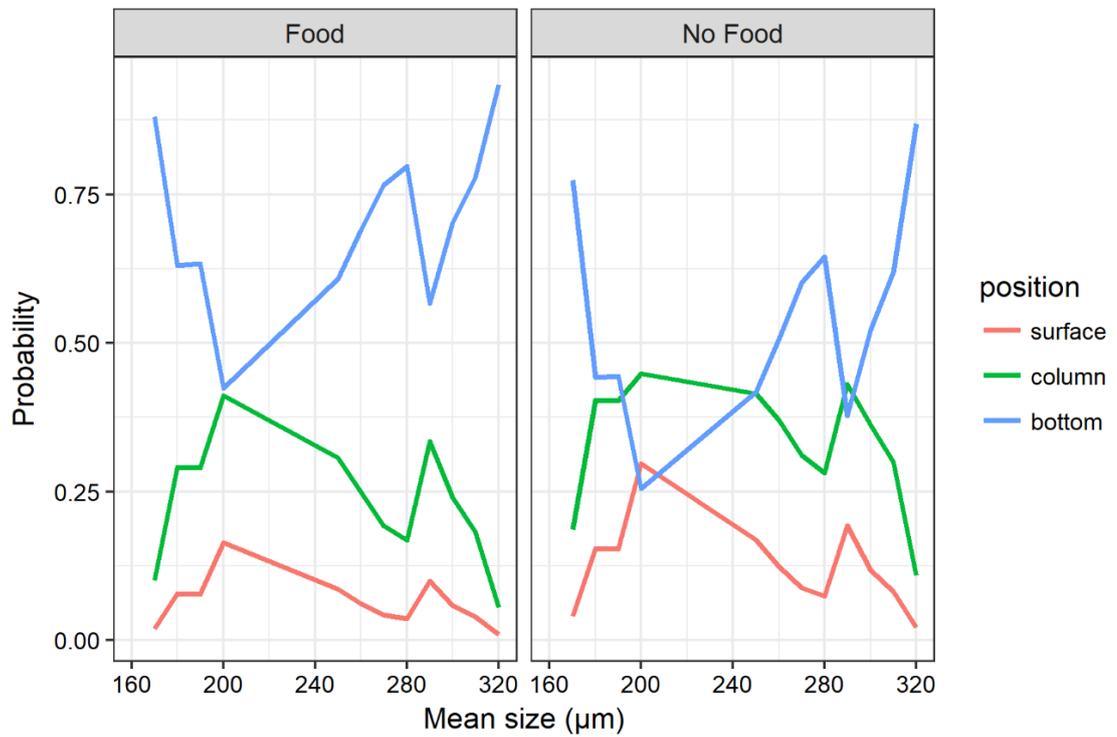


Figure 2-5. Probability of larval positions (surface, column and bottom) with increasing population mean size and the presence of food. Probabilities were calculated with estimates of best fitting model.

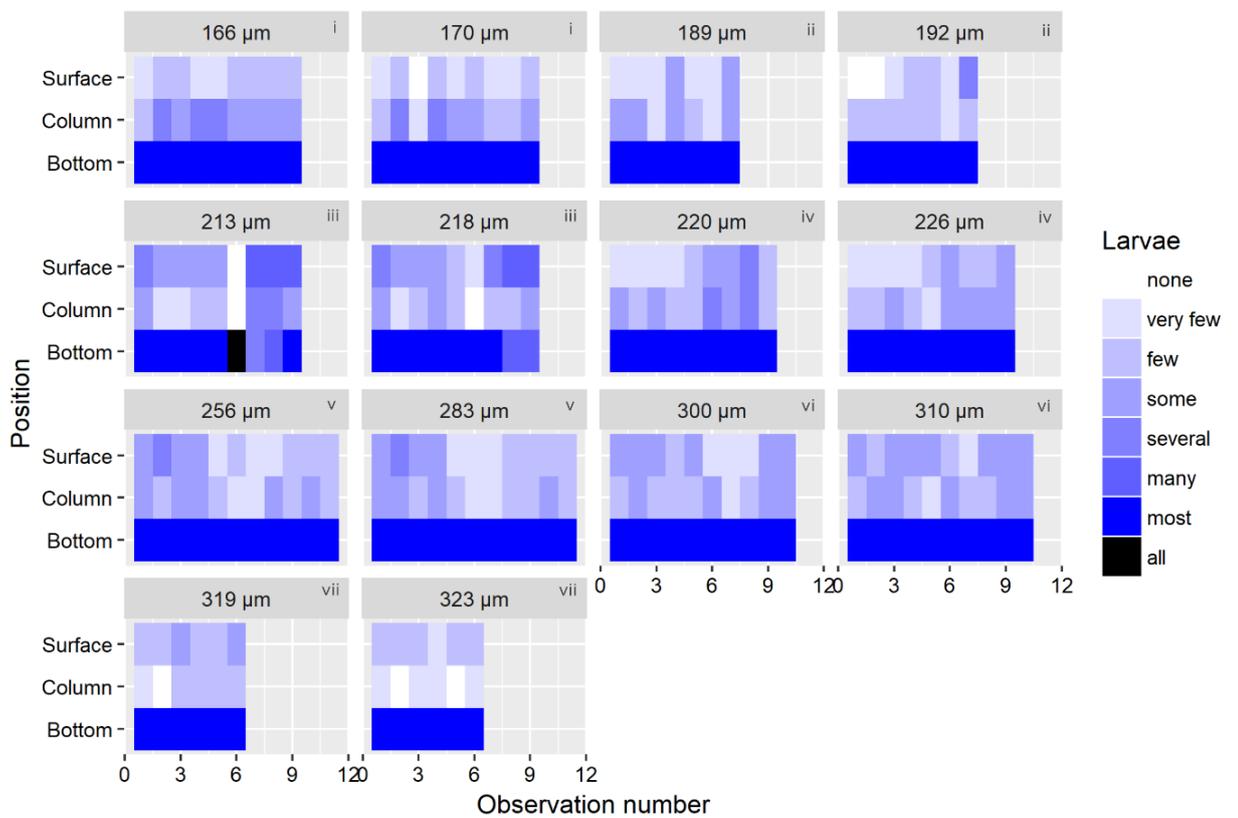


Figure 2-6. Vertical distribution of approximate 1,000 larvae in a 150 ml beaker over a 2-3-day time period. Replicates share the same Roman number.

2.4.2. Behaviour

The behaviour of *O. edulis* larvae was strongly influenced by the positions: ‘surface’, ‘column’ and ‘bottom’. In the water column, larvae generally swam individually but they were also seen swimming in pairs and sometimes in groups of three. At the surface, larvae swam either individually or forming aggregations, and in the bottom larvae were not homogeneously distributed but consistently favoured certain locations (see Table 2-4 for a summary of behaviours by position).

At the bottom, larvae were active in 47% of all observations (Fig. 2-7): most active larvae ‘hovered’, with little or no directional displacement (Fig. 2-8 F & H), but a smaller fraction (16%) moved in circles of about 1 mm diameter. In both cases, larvae had the velum extended with beating cilia, and they were in very close proximity to the bottom (generally <2 mm, e.g. Fig. 2-8 F). Non-active larvae were not moving, and although not discernible through the USB microscope, it was assumed from previous observations with a microscope that most of these larvae were resting on the bottom, with the velum withdrawn and the valves open or closed (e.g. Fig. 2-8 G).

In the water column, larvae were swimming in 77% of all observations (Fig. 2-7). Although they were moving in all directions, most commonly they swam upwards or downwards. One percent of larvae swam horizontal, 2.6% up-diagonal, 3.8% down-diagonal and 5.1% were swimming in circles of about 2-3 mm. In contrast, 48.4% of larvae swam upwards and 39.1% downwards in a highly directional manner: the average net-to-gross displacement ratio (NGDR) of all larval paths was 0.76, indicating trajectories which approximate a straight vertical line (see Annex 2 Fig. A3-2 for example of larval tracks representing NGDR values from 0 to 1). The null model fitted NGDR values best (lowest AIC), indicating that none of the variables (larval life history, food, light or temperature) had a significant effect on larvae’s swimming trajectories. In 6% of all observations, larvae ‘hovered’ in the water column, i.e. there was no visible movement along the x- or y-axis, and they interacted with the aquarium walls or sealant in 16% of the observations (Fig. 2-7).

At the surface, larvae most commonly (64%) swam horizontally while maintaining contact with the surface film (Fig. 2-8 A). They were also observed to ‘bounce’ (Fig. 2-7). Bouncing was defined as repeated loops of contacting the water surface followed by a short sinking of a few mm and a subsequent upwards swimming, touching the surface film anew at a nearby location. This behaviour was only observed in larvae of smaller

size classes (180, 190 and 200 μm). Larvae arriving at the surface from the water column were often observed to immediately sink back into the water column. In contrast, larvae moving from the water column to the bottom, generally remained at the bottom.

Interactions between larvae were common throughout the larval life cycle. At the surface, larvae formed aggregations of a few to up to hundreds of individuals (e.g. Fig. 2-8 B & C). In experiment 1, surface aggregations were present in 15% of all observations, but only for the size classes with highest proportion of larvae at the surface (180 and 200 μm). However, in experiment 2 (where larval densities were considerably higher), aggregations at the surface occurred throughout all life stages (192, 220, 226, 256, 283, 300, 310 μm). Larvae were also seen to interact in the water column, termed as ‘gregarious swimming’. They swam down in pairs or triples, circling each other and intermittently making contact with their velums, or swam up with the velum of one larva attached to the umbo of another. In experiment 1, gregarious swimming was only observed for size class 200 μm , but in experiment 2 it was observed for a much wider range of sizes: 213, 283, 300, 310, 319 μm . Larvae also interacted at the bottom, where they were often close to each other and occasionally touched one another while moving.

Table 2-4. Summary of observed behaviours on *O. edulis* larvae

Position	Behaviour	Description
Surface	Swimming along surface	Slow horizontal displacement along surface. Individually or aggregated. Filtering: cilia continuously in contact with surface.
	Bouncing	Repeatedly touching the surface, sinking for <1 cm and swimming up again.
Column	Swimming	Swimming up, down, diagonal, horizontal or in circles. Filtering, except when ‘sinking’ with closed valves. In general, individually, but sometimes 2-3 larvae gregariously, intermittently contacting each other with their velums.
	Hovering	Within water column, no directional displacement. Larvae possibly touching aquarium front or back wall. Filtering.
	Touching wall	In contact with aquarium side wall/ glue.
Bottom	Hovering/moving	Larvae on bottom or near it (<2 mm). No or little directional displacement. Cilia out and filtering.
	Circling	Swimming in circles of about 1 mm diameter near the bottom. Filtering.
	Not moving	On bottom and not moving. Generally, not filtering with introverted velum. Valves open or closed.

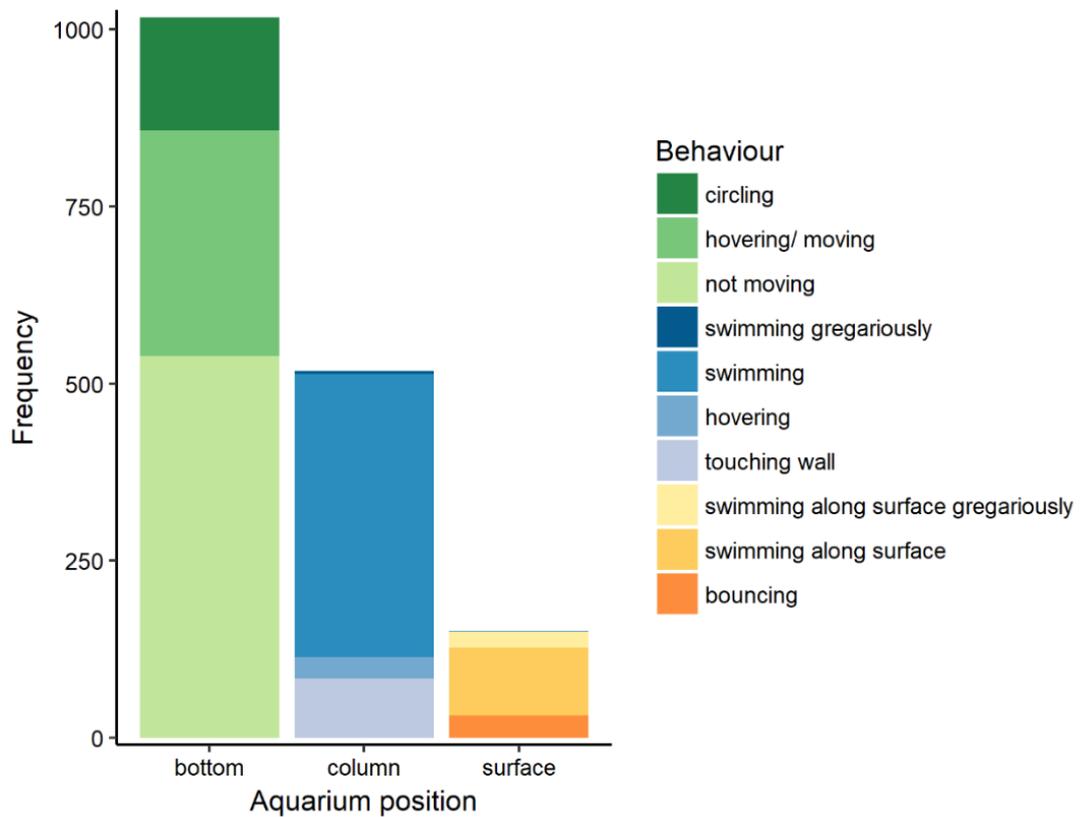


Figure 2-7. Frequency of observed larval behaviours by aquarium position.

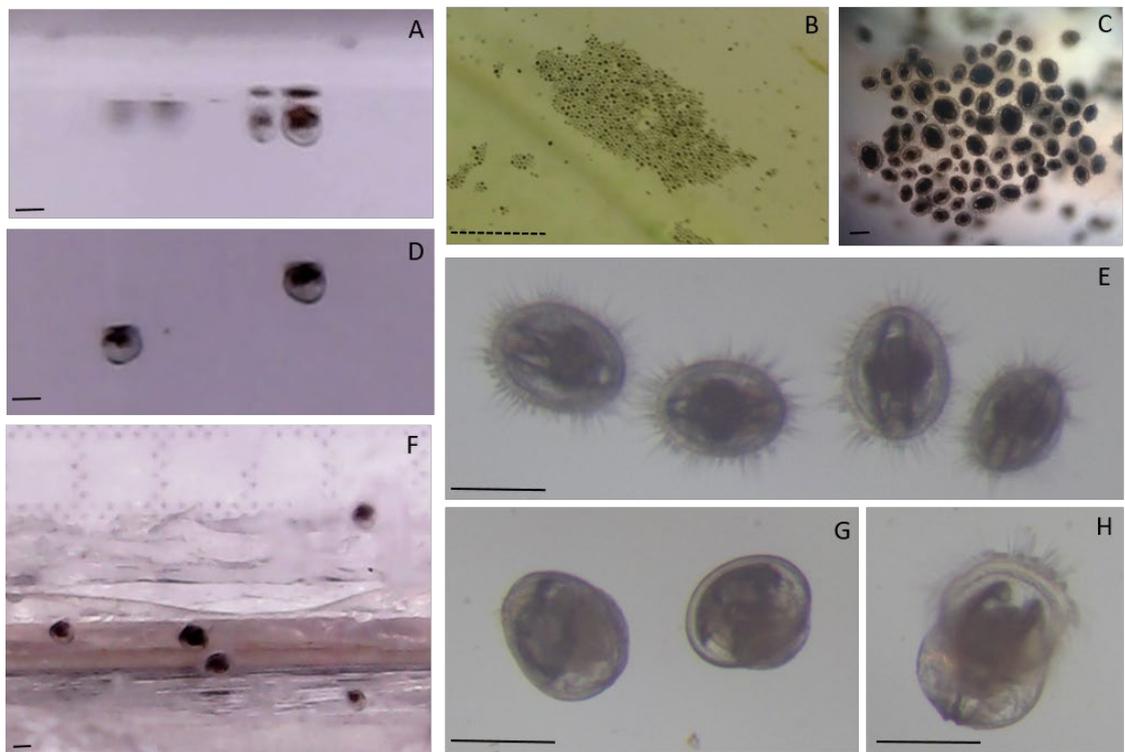


Figure 2-8. Larvae displaying different behaviours: (A & E) swimming along the surface (E: view from the top). (B & C) Swimming along the surface aggregated; both images show top view and a mix of larvae from different size classes. (D) Swimming up in the water column. (F & H) Hovering over the bottom. F: Aquarium bottom visible, dotted quadrats in background indicate 1 x 1 mm. (G) On bottom with closed valves and introverted velum. Scale bars: full indicates 200 μ m, dashed 10 cm

The distribution of the larvae at the bottom was not random and highly restricted to certain locations. In experiment 2, where larval containers had a considerably larger bottom area than in experiment 1 (28 vs 4 cm²), *O. edulis* larvae were consistently more abundant along the bottom edges of the beakers. The disposition of the aggregations varied from covering the whole circle of the bottom edges to just part of it, and could form a narrow band tightly restricted to the bottom edges or a wider band, with sometimes several larvae spread also throughout the middle of the bottom (Fig. 2-9). However, whatever the arrangement, it was generally mirrored in both replicated beakers, and larvae went back to their initial arrangement after interference. For example, if larvae were aggregated only on one half of the bottom edges, and the beakers were turned 180°, most larvae ended up on the same geographical location on which they had been before the beakers were turned around. If the water was gently mixed, so that larvae were transported to the centre of the bottom by circular currents, most larvae returned to bottom edges of the vessel within hours.

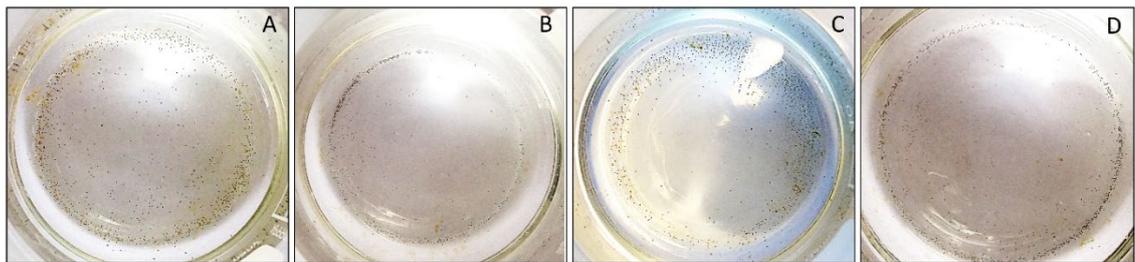


Figure 2-9. (A-D) Examples of larvae aggregating predominantly at the bottom edges of beakers in experiment 2, and their different dispositions. Disposition of the aggregations varied from covering the whole circle of the bottom edges to just part of it, and they could form a narrow band tightly restricted to the bottom edges or a wider band, with sometimes several larvae spread also throughout the middle of the bottom. Picture shows top view of beaker and larvae are the small black dots at the bottom.

2.4.3. Swimming speeds

Larvae generally swam at speeds between 0.35 mm/s and 1.36 mm/s (IQR = 50% of all observations), with a range of 0.001 mm/s to 9.07 mm/s (Fig. 2-10). Observed swimming speeds were best explained through the variables larval size (as a continuous variable), temperature and direction of swimming (model with lowest AIC). The model predicted increasing swimming speeds with larval size and temperature, with a larger effect of temperature than size (0.025 vs 0.001 increase per unit, Table 2-5). At 13°C median swimming speeds increased with size and ontogenetic life stage from 0.56 to 0.60 mm/s, while at 24°C swimming speeds increased from 0.81 to 1.7 mm/s (Table 2-6). There were no significant differences in speed of upwards and downwards swimming larvae, but

horizontally larvae swam significantly slower (Table 2-5 and Fig. 2-11). Although the model was significant, it only explained 13% of the variability observed in swimming speeds (R^2 , Table 2-5).

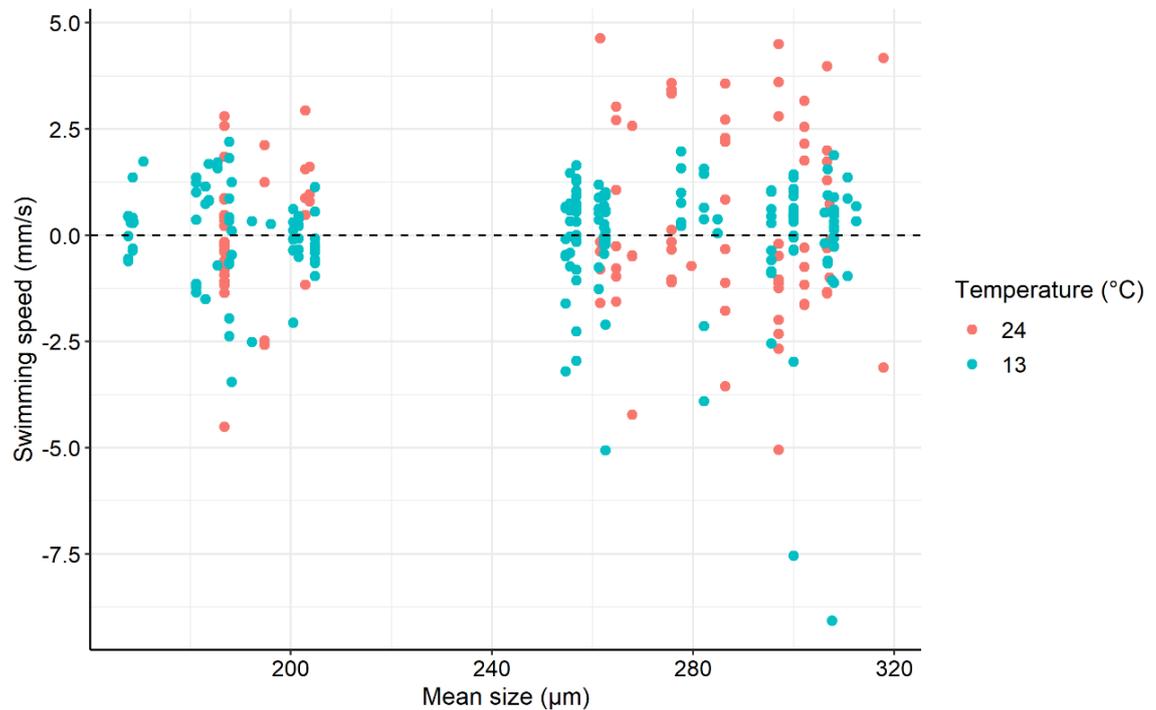


Figure 2-10. Swimming speeds by temperature and size of larvae. Upwards and downwards displacements are represented by positive and negative swimming speed values, respectively.

Table 2-5: Regression estimates for square root transformed values of swimming speed (mm/s). Multiple linear regression, $R^2= 0.131$, $F= 10.97$, $df= 4,291$, $P < 0.001$

Predictor of model	Coefficient (SE)	<i>t</i> -value	<i>P</i>
(Intercept)	0.155 (0.156)	0.933	0.321
Mean size	0.001 (0.001)	2.867	0.004 **
Temperature	0.025 (0.005)	5.152	< 0.001 ***
Direction: horizontal	-0.712 (0.252)	-2.852	0.005 **
Direction: up	0.063 (0.052)	1.224	0.222

Table 2-6. Swimming speeds (V) of *O. edulis* by temperature and size class. IQR = interquartile range.

Temp.	Size class (µm)	Approximate stage	Median V [IQR]	Max V up	Max V down	Analysed paths
13°C	170 – 200	D-stage	0.56 [0.34-1.17]	2.21	-3.44	72
	250 – 280	Umbo	0.61 [0.22-1.02]	1.98	-5.05	68
	280 – 320	Pediveliger	0.60 [0.34-1.06]	1.89	-9.07	65
24°C	170 – 200	D-stage	0.81 [0.43- 1.35]	2.94	-4.50	41
	250 – 280	Umbo	1.00 [0.48- 2.68]	4.64	-4.22	26
	280 – 320	Pediveliger	1.70 [0.96- 2.68]	4.51	-5.05	44

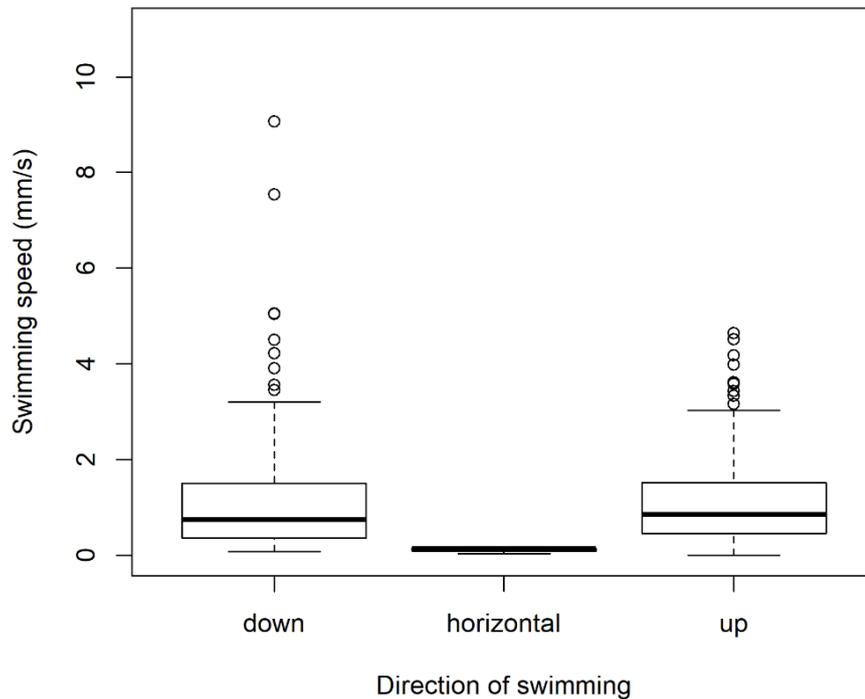


Figure 2-11. Swimming speeds by direction of movement.

2.5. Discussion

This chapter examined the vertical distribution, behaviour and swimming speeds of *O. edulis* larvae in the water column, with the aim of elucidating any systematic behaviour that could affect their dispersal. The bottom, column and surface of the aquarium emerged as the three most distinct positions in shaping the distribution and behaviour. Most larvae concentrated at the bottom of the aquarium, independently of the developmental stage, light, food or temperature. The distribution of larvae at the bottom was not homogenous, and often highly restricted to the container edges. Larvae also concentrated at the surface, although at much lower proportions, and frequently formed aggregations. In the water column, larvae swam with high vertical directionality and their distribution was homogenous.

2.5.1. Vertical distribution and behaviour

Bottom

The bottom of the aquarium was consistently the position with most *O. edulis* larvae: 42-87% of larvae were at the bottom throughout the larval life cycle and all treatments. The preference and proportion of larvae at the bottom was highly consistent over time and across both experiments: neither experimental container nor larval density therefore

influenced the bottom preference. In addition, the proportion of larvae at the bottom matches initial observations from laboratory experiments with larvae from the west coast of Scotland, where 53-62% were observed at the bottom (Appendix 3, Fig. A3-3).

Larvae at the bottom behaved actively in ~50% of all observations: they were either hovering or circling over the bottom, indicating that the bottom-dwelling behaviour did not have resting as the sole function. Active larvae generally moved within an area comprising the first 2 mm over the bottom. In the open sea, this region would lie within the boundary layer: a region of depressed flow in the benthic-pelagic interface, where flow velocity decreases logarithmically due to bottom friction until becoming nearly still at the seabed (Caldwell and Chriss, 1979; Butman, 1986). Advection of larvae so close to the seabed is slower than in any other part of the water column, and thus highly effective in reducing dispersal distances (Sundelöf and Jonsson, 2012). For instance, maximum dispersal distances of simulated surface- and bottom-bound particles differed by ~10 km in well-mixed systems (Sundelöf and Jonsson, 2012; Puckett *et al.*, 2014), and ~100 km in partially-mixed systems (North *et al.*, 2008). If *O. edulis* larvae behaved in the same manner in the sea, it would markedly reduce their dispersal away from natal beds. This behaviour appears to be genetically conserved, since *O. edulis* populations from both the west coast of Scotland and Denmark, concentrated in very similar proportions at the bottom in laboratory experiments (compare Appendix 3, Fig. A3-3 for Scottish populations).

The distribution of larvae at the bottom was not homogenous and highly restricted to the edges of the container (Fig. 2-9). Larvae sometimes aggregated throughout the beaker's bottom edge, while other times they covered only part of the circumference. Their position on a certain side was highly consistent: both beakers generally mirrored the same distribution, and if the beakers were turned 180°, most larvae ended up on the same geographical side. Preliminary observations from this study suggest that larvae may prefer locations close to edges and in the shadow. Staying close to edges may offer larvae additional protection from resuspension by currents in the sea. For instance, when larvae in a beaker were gently mixed through circular movements, it was not possible to resuspend the larvae, which were staying close to a circular protuberance in the middle of the beaker (Fig. 2-12). The preference for particular bottom locations, may further contribute to minimise resuspension and advection by currents, and potentially reduce exposure to predation. However, it is important to note that the observations on the heterogeneous distribution of larvae at the bottom were only preliminary. No thorough

experiments with controls were conducted on these observations. For instance, the bottom of the beaker may have been slightly concave, resulting in larvae slowly moving towards the edges due to gravity. Therefore, the possibility remains that the observed distribution was a result of physical characteristics of the beakers (i.e. ‘tank-effects’) instead of larval behaviour. Thorough experiments could be conducted with larvae and inanimate particles of the same density as controls, as well as with different-shaped aquaria. Moreover, small objects could be placed on the bottom to observe whether larvae also stay close to their edges. Future studies may elucidate whether there are external factors, such as edges, which control the spatial distribution of larvae at the bottom, and whether such a heterogeneous distribution would also occur on the seabed.



Figure 2-12. Screenshot of a video in which larvae are mixed with circular movements of a pipette in a beaker. Image shows larvae along the margin of a circular protuberance in the middle (appearing as a dotted black and curved line), which are not re-suspended by the movement.

The idea of larvae being predominantly at the bottom is counterintuitive. Most previous studies investigating the vertical distribution of larvae have therefore not sampled the bottom, which limits the number of *in situ* studies with which these results could be compared. However, some planktotrophic larvae are known to draw close to the seabed during the latter part of their life cycle (Andrews, 1983; Welch, Forward Jr and Howd, 1999; Baker, 2003), and non-feeding (lecithotrophic) *Balanophyllia elegans* coral larvae are demersal throughout their development (Gerrodette, 1981). In addition, there is one study investigating the distribution of oyster larvae (unspecified species, but probably *C.*

virginica) in Milford Harbor (Connecticut, USA), which sampled the bottom, and found many larvae (Prytherch, 1929). The author of this study wondered about the scarcity of swimming larvae, in an area with abundant recruitment each year. He postulated that larvae may be at the bottom to counteract dispersal and found an average of 149 larvae/m² at the bottom, as opposed to an average of 18 larvae per 227 litres of filtered seawater throughout the tidal cycle (Prytherch, 1929). The number of larvae obtained from the bottom was still small compared to the number of spat found in nearby areas, and the author hypothesised that this could be due to the samples being taken from clean and smooth areas, without shells or other objects that could obstruct the currents (Prytherch, 1929). This hypothesis matches the observations of this study, in which larvae were not randomly distributed throughout the bottom, but highly restricted to edges and protrusion of the container.

Empirical evidence also suggest that shellfish dispersal can be very limited. For instance, most successful mussel recruits appeared within <5 km of the parent population for *Mytilus galloprovincialis*, and within <10 km for *Perna perna*, despite absolute dispersal distances reaching 100-200 km (McQuaid and Phillips, 2000). Similarly, the majority of oyster larvae in Milford Harbor (USA) settled within a radius of ~270 m from the centre of the spawning bed (Prytherch, 1929). Although dispersal is advantageous because it reduces density-dependent mortality and inbreeding (Ottmann *et al.*, 2016), over-dispersal from parental beds can be a major problem leading to larval loss (Manuel *et al.*, 1996). Passive larvae will almost certainly be carried away from their natal bed with greater chances of inappropriate benthic habitat (Manuel *et al.*, 1996; Robins *et al.*, 2013). Thus, in the absence of larval supply from another population, reducing dispersal away from their natal habitat to facilitate self-recruitment may be one of the most efficient larval strategies to ensure successful settlement and persistence of a population (e.g. Paris and Cowen, 2004).

Surface

Larvae concentrated at the surface-film, albeit at much smaller proportions than at the bottom, and frequently formed aggregations. The likelihood of aggregation could be related to the number of larvae at the surface: in experiment 1 larval densities were low and aggregations were observed only for the size classes with highest proportion of larvae at the surface, while in experiment 2 larval densities were considerably higher and aggregations occurred throughout all life stages. Scallop larvae (*Placopecten magellanicus*) were also reported to aggregate at the surface of the water column in large

numbers (Gallager *et al.*, 1996). Aggregation could help generating refuge from predation, such as in copepods, which are thought to swarm to minimise predation risk (Hamner and Carleton, 1979). On the other hand, it could be evolutionary advantageous staying close to conspecifics during dispersal: if larvae were dispersed to a location devoid of adult conspecifics, settling as a group would allow them to start a new population and reproduce, while a larva settling individually, would not be able to reproduce. This strategy would not compromise genetic diversity if larvae in the water column originated from several mother oysters within a large bed. In support of this hypothesis, *O. edulis* larvae are known to form ‘rafts’ when they are ready to settle, in which a group of larvae at the surface will collectively sink to the bottom, without losing touch of one another and resembling a sinking raft. *O. edulis* larvae have only been observed to sink collectively in this manner when they are ready to settle (pers. observations verified by hatchery employees), and in hatcheries it is a sign that larvae should be transferred into settlement tanks. If *O. edulis* larvae behaved in the same way in the wild, it may allow a group of larvae at the surface to settle close-by. However, the ‘rafting’ behaviour may also be an artefact of the high larval density in hatchery holding tanks (~10 larvae/ml) and it may therefore not happen in the wild.

If there is an evolutionary advantage in staying close to conspecifics during dispersal, the surface-film and the bottom are likely to be the spatial domains in which the probability of encountering other larvae is highest: the two-dimensional planes of the surface and bottom are minimal compared to the three-dimensional water column (Moláček, Denny and Bush, 2012). This strategy is used by some gametes, which maximise their chance of fertilisation by being constrained to the surface or bottom, through positive or negative buoyancy (Moláček, Denny and Bush, 2012). Similarly, one of the reasons why larvae seek the surface may be a higher encounter rate with conspecifics. For instance, green sea urchin larvae (*Strongylocentrotus droebachiensis*) were highly abundant at the surface and also ‘bounced’ in the same way as *O. edulis* larvae were observed to bounce at the surface (Daigle and Metaxas, 2012). It could be that the bouncing behaviour increases the chances of encountering conspecifics, by repeatedly touching the surface-film at nearby locations. Alternatively, ‘bouncing’ could have a different function, such as increasing the chances of passing through discontinuity layers, which can inhibit larval movement (Vazquez and Young, 1996). The fact that this behaviour was observed in taxa as different as a sea urchin and oyster larvae, suggests that it is an important behaviour that may be

common to larvae of a wide range of species. It would therefore be interesting to further our understanding of its purpose.

Water column

Larvae were distributed homogeneously within the water column and did not concentrate at any height. This coincides with the distribution of *O. edulis* larvae in the Dutch Oosterschelde: samples collected from ~30 cm below the surface and ~50 cm above the bottom showed a homogenous distribution of larvae (Korringa, 1940). However, the Oosterschelde is a turbulent and well-mixed sea. In partially-mixed systems, discontinuity layers can act as barriers for larval movement (Vazquez and Young, 1996). For instance, larvae of the sea star *Asterias rubens*, the sea urchin *S. droebachiensis* and the mussel *Mytilus edulis* all aggregated in laboratory experiments below the halocline, with the upper low salinity layer acting as a barrier for larval movement. The aggregations were more pronounced with increasing strength of the vertical gradient and the specific salinity, which inhibited the crossing, varied for the three species (Sameoto and Metaxas, 2008). *Crassostrea virginica* oyster larvae from Barnegat Bay (New Jersey, USA) also frequently aggregated along the halocline (Korringa, 1940). In a partially mixed environment, *O. edulis* larvae may therefore aggregate along the halo- or thermocline instead of at the surface, provided the vertical gradient is strong enough to inhibit their crossing.

The most common behaviour (88%) of larvae within the water column was to swim up or down with high vertical directionality. Larval paths often approximated straight lines, independent of any of the variables tested, including the presence of food. Moreover, speeds of upward or downward swimming larvae were significantly faster than horizontally swimming larvae. This indicates that *O. edulis* larvae usually travel up and down through the water column. They do not generally 'hover' or swim horizontally, behaviours by which horizontal currents would advect them without vertical displacement. In the sea, the directional swimming behaviour is probably the main feeding mechanism of larvae. Although *O. edulis* larvae may also be able to feed at the bottom, when they are active and their cilia are moving, the nutritional quality of microalgae within the water column (and at the surface) is likely to be higher. Indeed, a proportion of larvae (11-48%) were always within the water column: larvae swam up or down through the water column for some time, and then stayed at the surface-film or, most commonly, at the bottom. In turn, other larvae would lift from the bottom and swim through the water column.

Larvae occasionally interacted both with the aquarium sides and with each other in the water column. In 16% of the observations, larvae interacted with the aquarium sidewalls or sealant, and they may have been touching the front or back aquarium wall when they were ‘hovering’ (6%) within the water column. *O. edulis* larvae in the wild may thus also interact with structures and protrusions extending into the water column in the sea. Additionally, larvae may interact with each other and swim gregariously, as observed in this study, provided larval densities are high enough. These interactions point to a complex larval behaviour in the water column, as well as to the likely ability of *O. edulis* larvae to detect one another, possibly through chemo- or mechanoreceptors, which is how other planktonic species detect each other (Yen, Weissburg and Doall, 1998).

2.5.2. Factors influencing vertical distribution

Larval life history

Size class was the most significant factor influencing vertical distribution. The model predicted a decrease of larvae at the bottom (and a concomitant increase of larvae at the column and surface) from 88% in the smallest size class (170 μm) to 42% in size class 200 μm , and a subsequent overall increase to 93% in the largest size class (320 μm). A bottom preference was therefore apparent throughout the larval life cycle, despite differences in size classes: the probability of larvae being at the bottom was >50% for all size classes, except for size class 200 μm . These values also fit observations of experiment 2, in which only larvae of 213 and 218 μm average size were at <50% at the bottom.

The observed trend could reflect nutritional needs of larvae, as they may need to spend a different amount of time feeding in the water column and surface depending on developmental stage. For instance, newly spawned larvae appear to have reserves from the time spent within the mother oyster: during the first days after release microalgae ingestion of *O. edulis* larvae was low and shell length increased steadily, regardless of food density (Robert, Vignier and Petton, 2017). Umbo larvae (~270 μm) developed to mature pediveliger without any food in the water (pers. observation), which also indicates nutritional reserves from previous feeding. It may therefore be that the most critical feeding time of *O. edulis* larvae is some days after being released from the mother oyster, when their maternal reserves are depleted. In our experimental conditions, this seemed to be around 200-220 μm , when most *O. edulis* larvae were consistently observed in the water column and surface. Feeding success following maternal reserve depletion is also thought to be critical for other species, and it may determine the number of larvae surviving at the end of the planktonic stage (Hare and Cowen, 1997).

A paper by Acarli and Lok (2009) describes differences in larval behaviour with ontogenetic stage that differ from the observations in this study: D-stage larvae swam near the surface of the tank, early umbo were less mobile and swam through the water column, umbo larvae swam more slowly than in earlier stages and pediveliger congregated deeper in the water column. These behaviours and swimming speeds are based on visual observations in 180-L tanks and it is not reported whether the authors were able to see the bottom of the tanks. Moreover, the sizes of larvae do not match larval sizes reported in previous work and measured in this study. For instance, D-stage larvae were on average 147 ± 5 μm long, instead of 170-180 μm (Korringa, 1940; Davis and Calabrese, 1969; Helm, 2004, Appendix 5). It may be that there are differences in size and behaviour with geographic distant populations, since larvae from Acarli and Lok, (2009) originated from Turkey. However, until the observations are corroborated by accurate quantification of vertical distribution and swimming speeds, they should be taken with caution. The results of this study highlight the critical importance of including the bottom in any future study on the vertical distribution of larvae.

Food

Food had a significant effect on vertical distribution in this study: in the absence of food, the model predicted significantly less larvae at the bottom, and more in the water column and surface. This may reflect hungry larvae, which had an urge to feed; particularly at 13°C, where larvae had been acclimatised for 13-18h without food. While no food scenarios are unrealistic in the sea, these results indicate that if larvae develop under food-limitation, the proportion of larvae in the column and surface is likely to be higher than in an environment with enough food. Release of benthic invertebrate larvae, such as *O. edulis*, generally coincides with large phytoplankton blooms in spring and late summer to maximise food availability. Ingestion rates are therefore often saturated, such as with *C. virginica* larvae in Chesapeake Bay, USA (Baldwin and Newell, 1995). Nevertheless, food limitations can occur, and modelling studies have shown that bivalve larvae in north western European waters are likely to be food-limited for most of their lives (Bos *et al.*, 2006). Food limited growth during the summer probably also applies for polychaete larvae (Hansen, 1999).

Preliminary experiments showed a remarkably strong reaction of *O. edulis* larvae to food input under food-limitation: within seconds of the microalgae arriving at the bottom (visible as a green shadow in the water column), most larvae lifted from the bottom into the water column to feed. However, larvae that were not food-limited had a considerably

weaker reaction and mostly stayed at the bottom. These observations provide further evidence that food can alter the vertical distribution of *O. edulis* larvae, particularly when larvae are not fully satiated. In addition, they provide evidence that larvae can sense food. Sea urchin larvae can detect both the quantity and quality of the food, probably through a chemosensory mechanisms (Burdett-Coutts and Metaxas, 2004), and it is likely that *O. edulis* larvae can detect microalgae in the same manner. In the sea, phytoplankton patchiness (Martin, 2003; Durham *et al.*, 2013) may therefore shift the distribution of *O. edulis* larvae towards the water column areas with most food, as observed for sea urchin *Echinometra lucunter* larvae in the lab (Metaxas and Young, 1998) and bivalve larvae in the field (Thomas, Garen, *et al.*, 2012).

Larvae may also obtain nutrition close to the bottom, by feeding on particles other than phytoplankton. Bivalve species can have diets that include a wide range of particles from bacteria to detritus (e.g. Davenport *et al.*, 2011; Kent *et al.*, 2017). Growth experiments with *O. edulis* in offshore locations near Helgoland (Germany) also indicate that spat and adults may be obtaining their nutrition from sources other than phytoplankton (V. Merk 2019, manuscript in preparation). *O. edulis* larvae may therefore not only rely on phytoplankton for their development.

Light

This study found no effect of light on the vertical distribution of *O. edulis* larvae. This is in agreement with previous studies in both the field and the lab. Korringa (1940) found no difference in the Oosterschelde (Netherlands) between the number of *O. edulis* larvae in the surface- and bottom-layers of the water column during day or night. Moreover, he cites other authors, who did not observe any phototactic movements in the lab (Hagmeier 1932 and Mazzarelli 1922 in Korringa, 1940). For many zooplankton species, light is the most important external factor that regulates diel vertical migrations to minimise predation risk (Forward, 1988). This is particularly true for holoplankton species, which spend their entire life cycle in the plankton. In contrast, meroplankton species such as *O. edulis*, spend only part of their life cycle in the water column. Their primary evolutionary drive is to settle in an appropriate habitat, where they will survive as adults and reproduce. For these species, diel vertical migrations may therefore not necessarily represent the best strategy to find optimal settlement habitats.

The best vertical migration strategy often depends on the hydrodynamic regime. For instance, *Carcinus maenas* crab larvae in the Wadden Sea performed tidal migrations but no diel migrations, and a biophysical model showed that tidal migrating larvae had two

times higher settlement success than larvae with a diel behaviour. In contrast, no difference between the two behaviours was found for the microtidal Skagerrak, and *C. maenas* larvae of this population displayed nocturnal migration to the surface, but no tidal migration (Moksnes *et al.*, 2014). Scallop and snail larvae from different populations of the same species also displayed different vertical migration strategies, which were adapted to their particular hydrodynamic regime (Manuel *et al.*, 1996; Fuchs *et al.*, 2018). The same might therefore apply to *O. edulis* larvae, and some populations may perform diel migrations, despite an absence of evidence so far.

Temperature

There was no effect of temperature on the vertical distribution of *O. edulis* larvae. This agrees with previous studies, which found no correlation between temperature and vertical distribution of *O. edulis* larvae, when temperature was homogenous (~1-2°C variation) throughout the basin (Korringa, 1940 and references therein). Yet, larger temperature variations within a basin may influence the larvae's distribution. For instance, vertical distribution of bivalve larvae in French Polynesia was positively correlated with temperature (Thomas, Garen, *et al.*, 2012). Bivalve larvae may have evolved to actively seek the warmest areas, because larval development is positively correlated with temperature (O'Connor *et al.*, 2007; Robert, Vignier and Petton, 2017), and a faster development reduces the risk of mortality (Korringa, 1940; Pineda, Hare and Sponaugle, 2007). In addition, thermoclines can act, just as haloclines, as a barrier to larval movement (Daigle and Metaxas, 2011). *O. edulis* larvae may therefore aggregate along the thermocline, instead of at the surface. This behaviour was observed in scallop larvae (*P. magellanicus*) in the northwest Atlantic, where peak concentrations of larvae were associated with the occurrence of a thermocline (Tremblay and Sinclair, 1988). If a thermocline persists over an extended period, larvae could be captive in the water mass in which they were spawned throughout their development (Vazquez and Young, 1996), limiting their vertical migrations to that water mass.

Other factors: tides, currents, bottom rugosity and predation

This study analysed the behaviour and vertical distribution of *O. edulis* larvae under the confinement of an aquarium. Factors such as tides, currents, bottom type and predation, could alter the observed behaviours in the sea. For instance, larvae may lift from the bottom to perform tidal migrations, known as 'selective-tidal-stream transport'. This behaviour has strong empirical support for estuarine crab postlarvae, which will swim almost exclusively during flood tides and remain at or near the bottom at other times,

resulting in a net upwards transport within the estuary (Welch, Forward Jr and Howd, 1999; Moksnes *et al.*, 2014). *C. virginica* oyster larvae are also thought to perform selective-tidal-stream transport, particularly at the mature pediveliger stage (Kunkle, 1957; Wood and Hargis, 1971). The rise of oyster larvae into the water column during flood tide is probably stimulated by an increase in salinity (e.g. Wood and Hargis, 1971; Hidu and Haskin, 1978). *O. edulis* larvae may therefore respond similarly to incoming tides, particularly since they would sense the heavier high-salinity water flowing up along the bottom during flood tide (Korringa, 1940) if they were on the bottom. Alternatively, they may sense incoming tides through pressure differences: in laboratory conditions, *O. edulis* larvae swam up with an increase in pressure, and down following a pressure decrease (Cragg and Gruffydd, 1975). To date, only one study has investigated the distribution of *O. edulis* along a tidal cycle: large larval concentrations were observed at or shortly after maximum low water in the Dutch Oosterschelde, yet the author ascribed these to larvae being moved passively with the tide, instead of active larval behaviour (Korringa, 1940). Future studies may elucidate whether *O. edulis* larvae respond to tidal cycles. If a tidal migration pattern is discovered, it could be specific to estuarine populations, as is the case for *C. maenas* crab larvae (Moksnes *et al.*, 2014).

Strong currents are likely to resuspend larvae from the bottom into the water column, altering the observed bottom preference. In a flume tank, larvae of the cockle *Cerastoderma edule* and of the oyster *C. virginica* remained within the boundary layer (1 mm to < 1 cm above the bottom) at moderate flows (5-10 cm/s), drifting slowly in streamwise direction (Jonsson, André and Lindegarth, 1991; Finelli and Wetthey, 2003). However, at flow velocities exceeding 15 cm/s, larvae tumbled along the bottom with high probability of resuspension and bed-load transport (Jonsson, André and Lindegarth, 1991). These experiments suggest that the observed bottom preference of *O. edulis* is likely to persist under the influence of slow currents, but that stronger currents will override the larvae's behaviour.

The critical speed at which larvae are resuspended into the water column probably depends on bottom type. Near-bed flow over a relatively smooth, flat bottom (e.g. sand, mud) is considerably faster than over large rough elements, such as rocks, dense canopy or reefs (Butman, 1986; Whitman and Reidenbach, 2012). The critical speed to resuspend larvae over a three-dimensional oyster bed is probably considerably higher than over muddy or sandy bottoms. In the sea, *O. edulis* larvae may therefore be more frequently at the bottom on rough and three-dimensional seabed types than on flat bottoms. Larvae on

soft bottoms may also lift into the water column in response to bed-load siltation caused by strong currents, which is likely to be detrimental to larvae (Andrews, 1983). Finally, larvae may lift from the bottom when they sense microcurrents generated by filter-feeding organisms and predators to avoid ingestion. Future studies will elucidate to which degree and under which circumstances larvae are able to display their bottom preference in the field.

2.5.3. Swimming speeds

Swimming speeds of *O. edulis* larvae ranged from 0.001 mm/s (i.e. almost no directional displacement) to 9 mm/s. However, most frequently, larvae swam between 0.35 mm/s and 1.36 mm/s (IQR, 50% of observations). This indicates that although larvae are capable of swimming fast, their most commonly employed swimming speeds are well below maximum values – probably to save metabolic cost. The swimming speeds observed in this study are consistent with literature values: invertebrate marine larvae typically swim between 1-10 mm/s in still water (Mileikovsky, 1973; Chia, Buckland-Nicks and Young, 1984; Hata *et al.*, 2017), with a maximum of 10 mm/s observed for *C. virginica* oyster larvae (Wood and Hargis, 1971). However, under flow and turbulence, invertebrate marine larvae may be able to swim considerably faster for short time periods. For instance, *C. virginica* oyster larvae reached swimming speeds of 18 mm/s (Hubbard and Reidenbach, 2015) and 30 mm/s (Fuchs *et al.*, 2013) under strong turbulence. Similarly, the upward swimming velocity of barnacle larvae *Semibalanus balanoides* was as high as 72 mm/s for short time periods in a down-welling flume (DiBacco *et al.*, 2011). Maximum short-term swimming speeds of *O. edulis* larvae under turbulence are therefore likely to be considerably faster than the ones observed in this study.

The observed speeds should allow larvae to control their vertical position in the water column in many cases. For instance, in James River (USA) the speed of vertical water displacement was 0.1 mm/s, and therefore well below the larvae's swimming capability (Wood and Hargis, 1971). Mileikovsky (1973) also concluded that all types of bottom invertebrate larvae, with speeds > 0.17 mm/s should be able to perform active vertical movement with tidal currents of moderate speed. However, when current speeds surpass certain thresholds, vertical larval movements are likely to be compromised. For instance, currents > 50 cm/s overcame the swimming behaviour of *Ostrea lurida* oyster larvae (Peteiro and Shanks, 2015). This matches estimates of Finelli and Wethey (2003), who calculated that if *C. virginica* oyster larvae sank at 4.8 mm/s over smooth bottoms, they would be able to alter their passive larval dispersal at freestream flow velocities from 10

– 31 cm/s, while if the larvae sank at 8 mm/s they would be able to control their vertical position in flows from 17 – 52 cm/s. The cited sinking velocities are within the range observed in this study, indicating that *O. edulis* larvae may be able to control their vertical position in flow stream velocities of up to 50 cm/s.

Factors influencing swimming speeds

Temperature and larval size had a significant effect on the swimming speeds of *O. edulis* larvae, but not light and food. This is consistent with previous studies, which also reported an effect of temperature (Hidu and Haskin, 1978; Bolton and Havenhand, 1997; Daigle and Metaxas, 2011) and size (Bolton and Havenhand, 1997; North *et al.*, 2008) on swimming speeds of marine invertebrate larvae. The greatest effect was induced by temperature (0.025 vs 0.001 increase per unit), which inversely relates to viscosity, so the higher the viscosity the more effort larvae need to swim through it (Bolton and Havenhand, 1997). Lower temperatures may thus reduce swimming speeds through an increased viscosity of the water (Bolton and Havenhand, 1997). In addition, the rate of metabolisms decreases with temperature (Schulte, 2015), which may further contribute to a decrease in swimming speeds. Although the model with temperature and size was significant, it only explained 13% of the variability observed in swimming speeds. Most of the variability may come from the larvae themselves, i.e. some larvae swim fast and others slow at a particular moment, regardless of temperature or size.

2.6. Conclusion

Increasing evidence suggest that there is high self-recruitment in marine populations, shifting the traditional view of predominantly ‘open’ populations with extended larval exchange (e.g. Cowen *et al.*, 2000; Sponaugle *et al.*, 2002; Levin, 2006). While this has been clearly demonstrated for fish larvae (e.g. Jones *et al.*, 1999; Almany *et al.*, 2007), evidence for benthic marine invertebrates is scarcer, and they are still often believed to have little control over their dispersal. The results of this study suggest that *O. edulis* larval behaviour matters and that it is tailored to reduce dispersal away from parent populations. The results provide an account of the complexity of larval behaviour, which includes aggregations and interactions throughout the larval life cycle that may well reflect life history strategy. This study also highlights the importance of including bottom samples in larval vertical distribution studies. Future studies will elucidate to which degree and under which circumstances *O. edulis* larvae are able to display the observed behaviours in the field. Although it is easy and tempting to make simple assumptions of passive larval transport in hydrodynamic models, the present results indicate that such

models will overestimate down-stream dispersal and that they will not predict the long-term behaviour of populations (Jones *et al.*, 1999), particularly in low energy environments. Our increasing understanding of larval behaviour highlights how easy it is to underestimate the larvae's capacity to influence their fate, particularly when larvae are so small that it is difficult to study them. Addressing such knowledge gaps on larval behaviour is key for an effective conservation and management of marine species (Ottmann *et al.*, 2016).

Chapter 3. Settlement preferences

3.1. Overview

This chapter studies settlement of *O. edulis* larvae from a conservation and restoration scenario point of view: the importance of habitat-associated chemical cues is tested. The study builds on knowledge gained from previous settlement experiments, which often aimed at enhancing commercial production of *O. edulis*. The work presented in this chapter was published in *Marine Pollution Bulletin* (Rodriguez-Perez *et al.*, 2019). The author of this thesis conceived the initial idea, conducted the experiment and wrote the main paper. The co-authors advised on experimental design and analysis, and provided inputs to the manuscript.

3.2. Introduction

Understanding the settlement requirements of *O. edulis* larvae is crucial to the conservation and restorations of European 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 Knight-Jones, 1939; Coon, Fitt and Bonar, 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 Knight-Jones, 1939). A preference for shell substrate has also been repeatedly cited (e.g. Cole and Jones, 1939; Laing, Walker and Areal, 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.

Marine habitats are thought to have unique chemical signatures (Kingsford *et al.*, 2002). Therefore, many marine invertebrate larvae are chemotactic and respond to small variations in water chemistry, especially close to settlement sites where decisions are made on a scale of less than a few meters (Kingsford *et al.*, 2002). Larvae may recognize the chemical cues of unique species assemblages from their natal habitats or respond to the pheromones of their own species and population (Gerlach *et al.*, 2007). In some cases, larvae have to contact the substrate before being able to respond to the inducer and if appropriate metamorphose (Fitt *et al.*, 1990; Kingsford *et al.*, 2002). However, larvae may also be able to recognise chemical cues of their preferred settlement site from further away and use smell as a cue to select favourable water masses. For example, fish larvae preferred water masses with chemical cues of their home reef, and this preference may allow them to select currents that return them to their home reef (Gerlach *et al.*, 2007).

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, Walker and Areal, 2005; and Low *et al.*, 2007). However, *O. edulis* was noted to settle preferentially on collectors which already bore some spat of their own species (Cole and Knight-Jones, 1949; Bayne, 1969), but if the spat were killed the larvae that subsequently settled showed no preference for these collectors (Cole and Knight-Jones, 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 chapter was to study the settlement preferences of *O. edulis* larval settlement guided by natural habitat conservation and restoration scenarios: comparing

the efficacy of a range of treatments that could be used. The selection of treatments was based on previous settlement studies and 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. The hypothesis was that the treatments would differ in their ability and speed with which they would induce metamorphosis, thereby reflecting settlement preferences.

3.3. Materials and methods

3.3.1. Larval cultures

Adult oysters (*O. edulis*) were obtained from the Limfjord (Fig. 3-1) 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.

3.3.2. Experimental design and procedure

Pediveliger larvae were subjected to eight treatments (Table 3-1). 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. 3-2). 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 4, Table A3-1). 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 Knight-Jones, 1939) (Fig. 3-3 a), 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 shell growth (Fig. 3-3 c-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.

Table 3-1. Treatments used to study settlement prefers 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 ± 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 Ovive). 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 <i>Nykøbing Bugt</i> (Fig. 3-1) at ~ 0.5 m depth where oysters occurred (mainly <i>Crassostrea gigas</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. 3-1), 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).

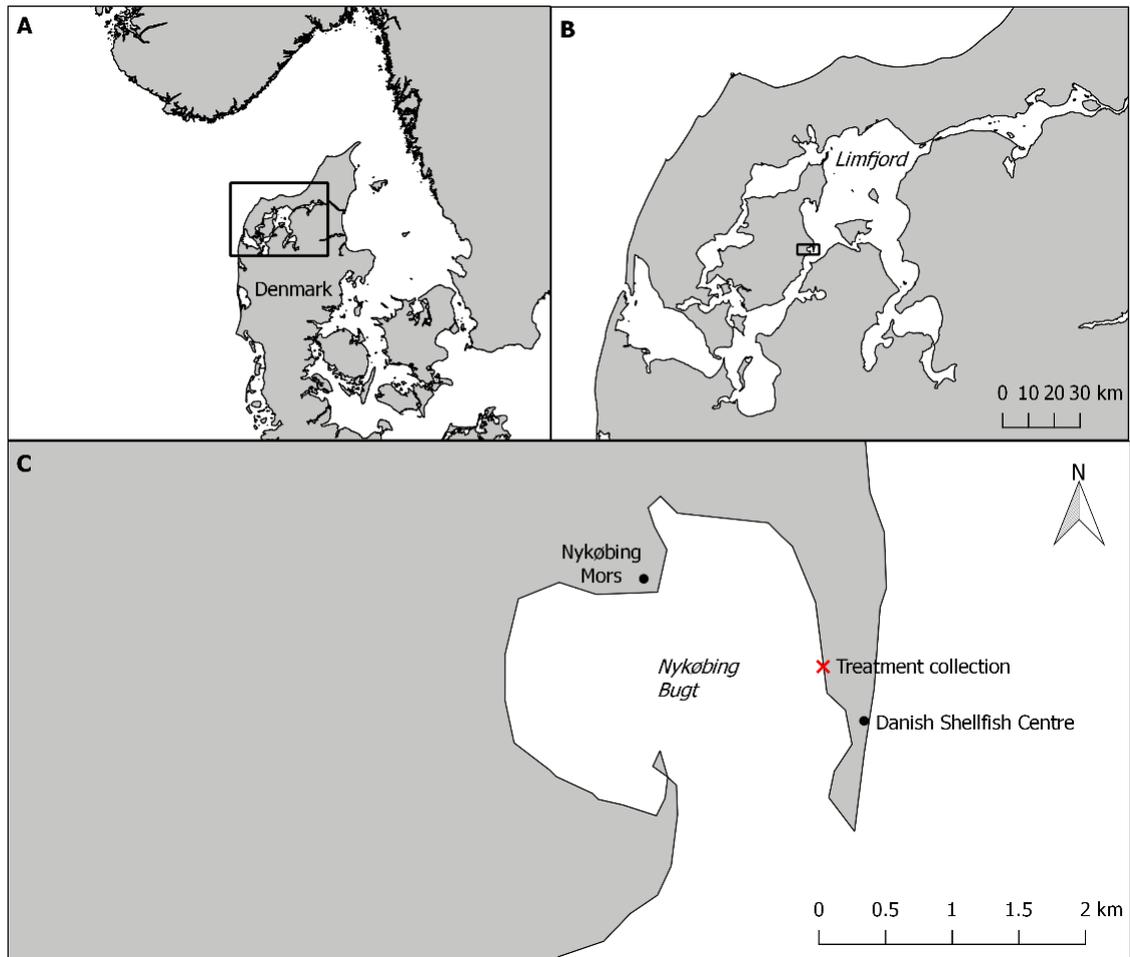


Figure 3-1. Collection site of ‘Stone’ and ‘Biofilm stone’ treatments. (A) Location of the Limfjord within Denmark; (B) Map showing the Limfjord with the collection area surrounded by a black box; (C) Close-up of treatment collection site

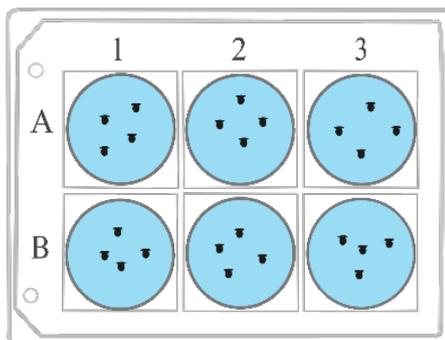


Figure 3-2. Diagram of a 6-well culture plate with four larvae per well. Each well had one randomly allocated treatment. Image not to scale.

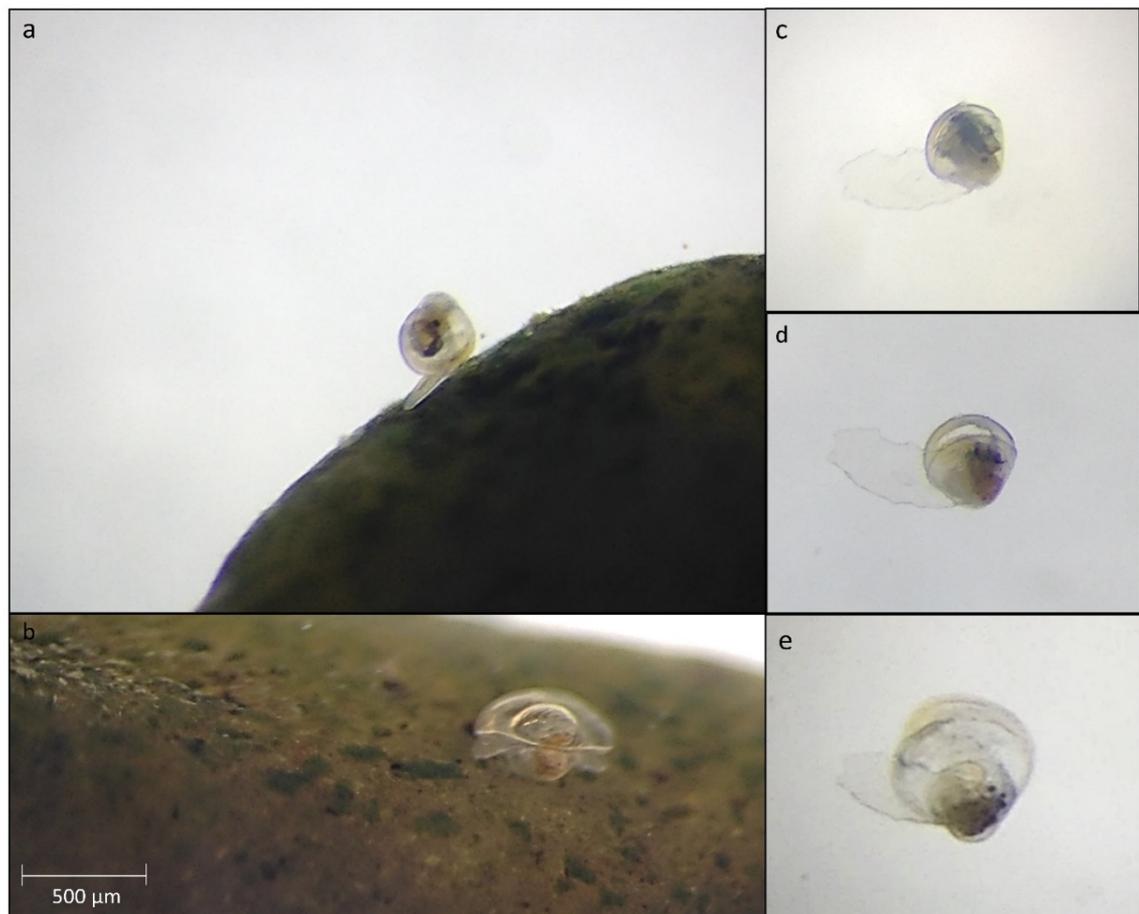


Figure 3-3. a) Larvae displaying stereotypical searching behaviour with extended foot on the biofilm stone treatment; b) metamorphosed larvae with secondary shell growth and open valves (filtering water) on the biofilm stone treatment; c-e) time series of an attached larvae metamorphosing to a small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

3.3.3. Data analysis

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 ‘settled’ at that time point too.

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).

3.4. 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 4, Table A3-2). 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. 3-4).

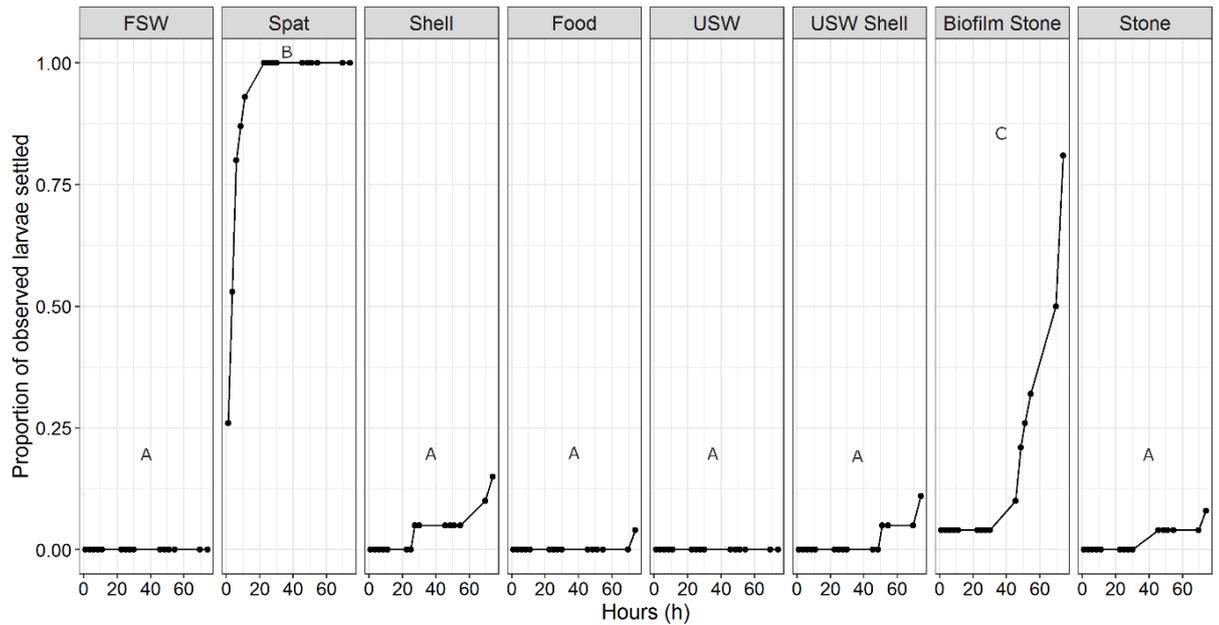


Figure 3-4. Cumulative proportion of larvae settled over the 74 h of experimental duration in each treatment. All treatments were in FSW, with exception of the ones explicitly labelled with USW. Treatments that differ in A-C notations were significantly different ($p < 0.001$). Note that the graph depicts the proportion of observed larvae (see Appendix 4, Fig. A3-1 for number of larvae not observed at each time point and treatment).

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. 3-4 and Appendix 4, Fig. A3-2). 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 3-2).

Table 3-2. 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
<i>FSW Spat</i>			
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
<i>FSW Shell</i>			
27.5	4.5	0	12.9
69.5	9.3	0	20.8
74.0	14.4	0	28.2
<i>FSW Food</i>			
74.0	4.2	0	11.8
<i>USW Shell</i>			
51	5.0	0	14.1
74	10.6	0	23.5
<i>Biofilm Stone</i>			
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
<i>Stone</i>			
45.5	4.2	0	11.8
54.5	8.3	0	18.7

Settlement in the biofilm treatment resembled an exponential response curve – rising sharply after 45 h (Fig. 3-4). 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 3-2).

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. 3-3a) 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 less than 3% in all remaining treatments (Fig. 3-5).

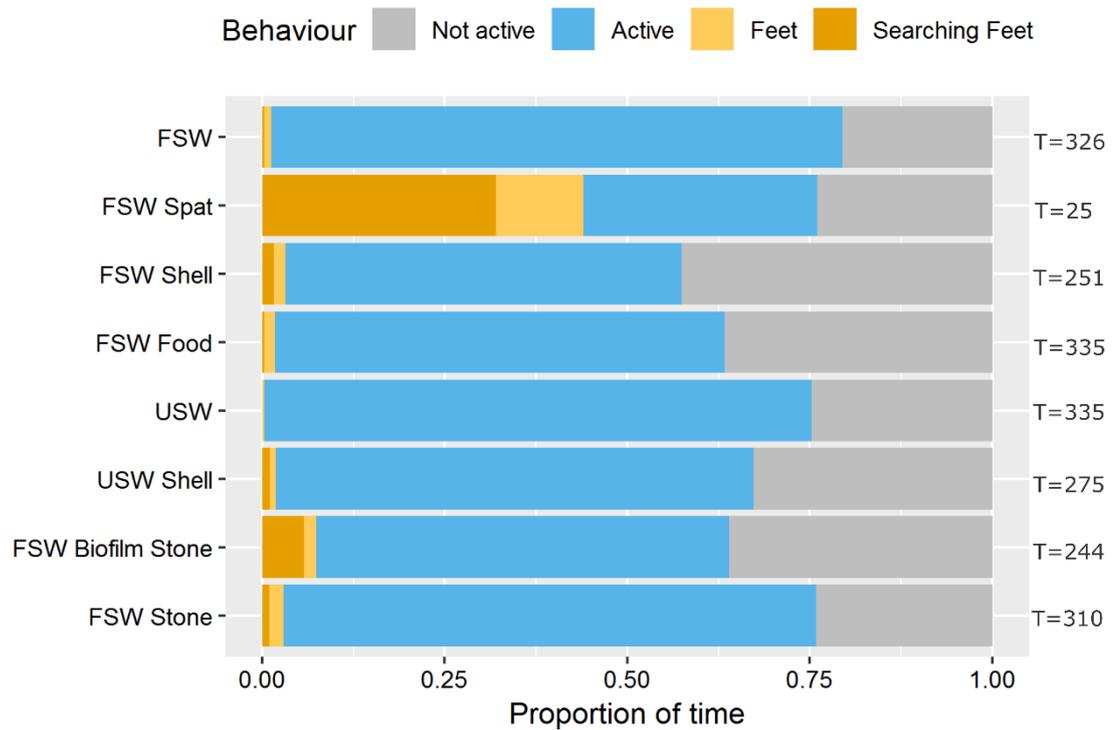


Figure 3-5. Proportion of time larvae were observed displaying each behaviour. T = total number of behaviour observations in each treatment. Behavioural observations were only possible if larvae had not settled and if they were seen in that observation round. Category ‘searching feet’ refers to the stereotypical settlement searching behaviour in which larvae crawl on a surface with extended feet, while ‘feet’ stands for other behaviour in which larvae protruded their feet without searching.

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 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. 3-6).

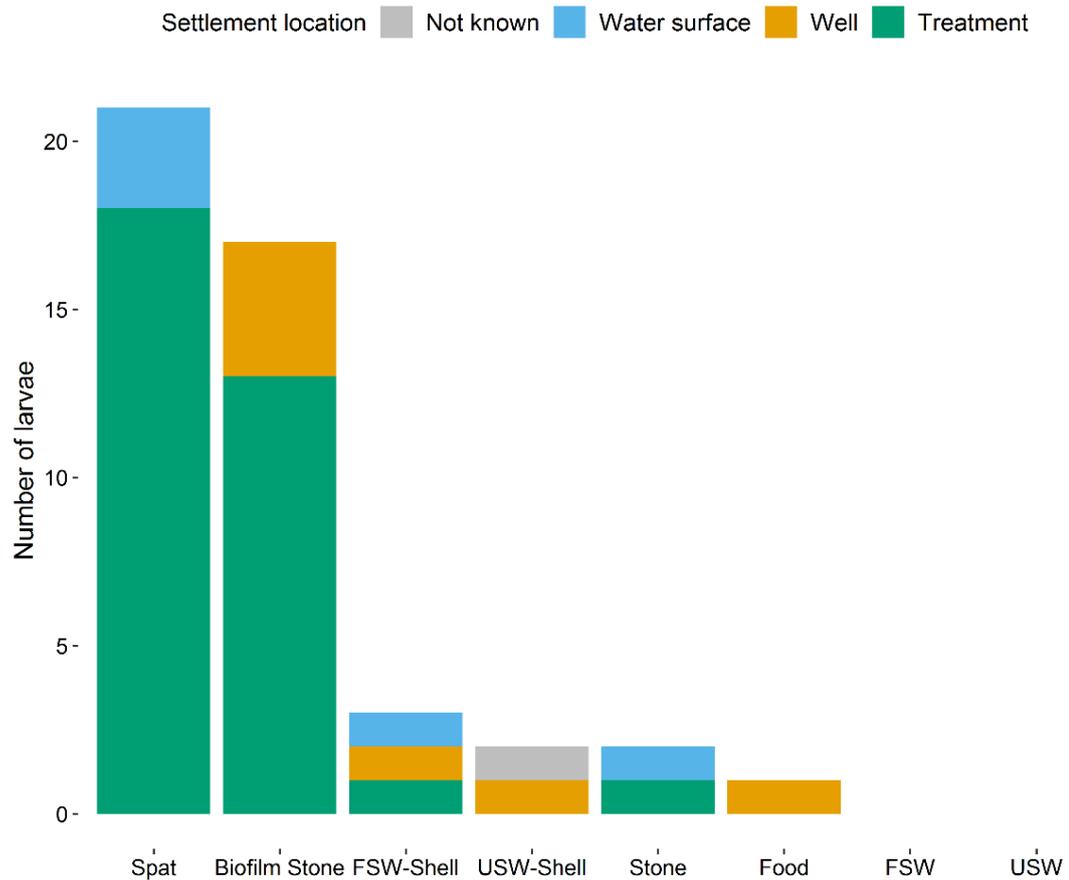


Figure 3-6. Settlement locations after 74 h. Location ‘treatment’ refers to the spat, stone or shell surface of the respective treatment. ‘Well’ is the experimental container where larvae were observed.

At the end of the experiment, most larvae that had not settled were still alive (Fig. 3-7). 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. 3-8).

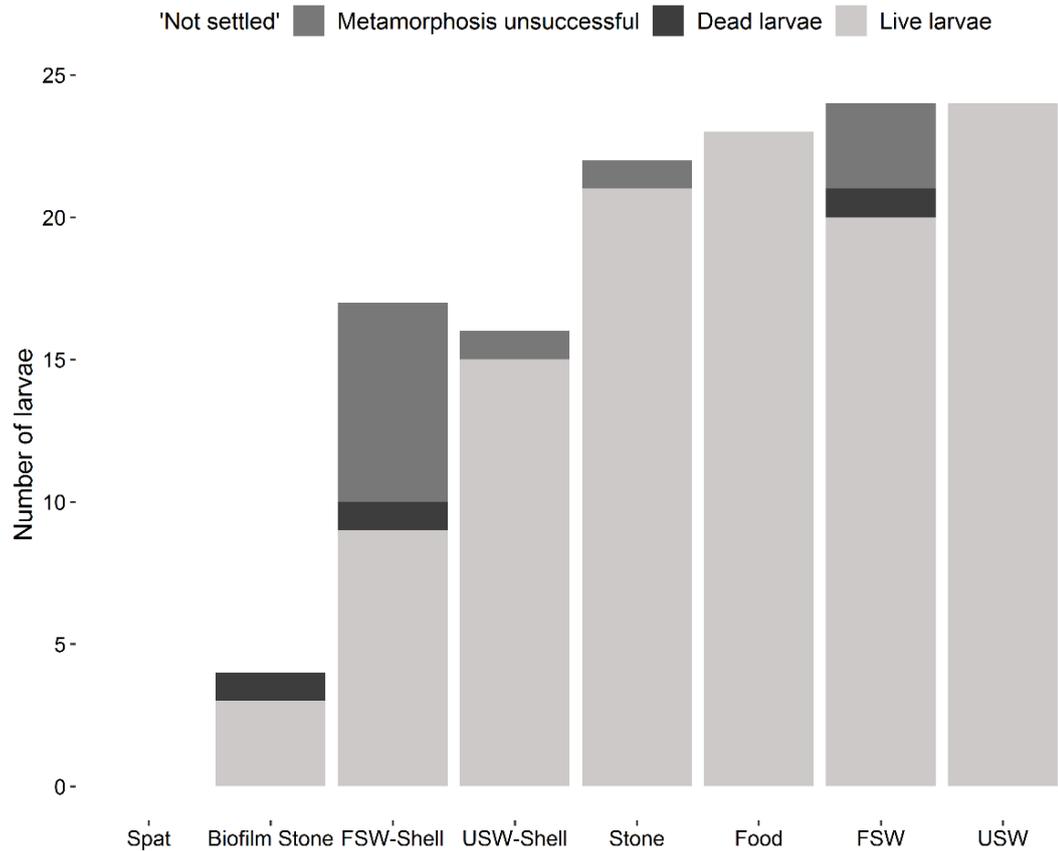


Figure 3-7. Number of larvae per treatment that had not settled after 74 h and their respective state at the end of the experiment. Category ‘metamorphosis unsuccessful’ was defined as larvae that died while trying to metamorphose or after completion of metamorphosis.

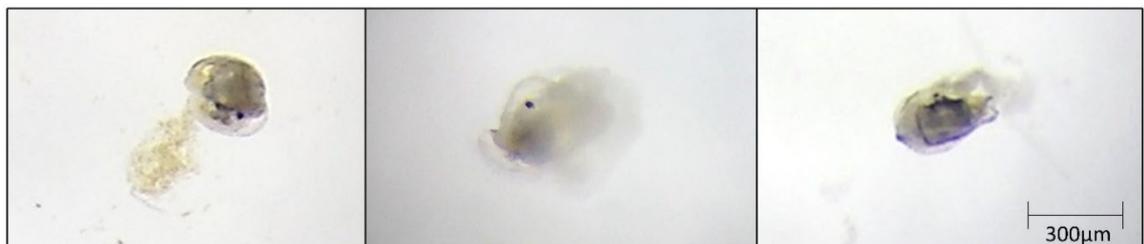


Figure 3-8. Examples of larvae classified into ‘metamorphosis unsuccessful’.

3.5. 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 one hour, 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 less than 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 Simith, Abrunhosa and Diele, 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 and Knight-Jones, 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 conspecific 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, Zimmer-Faust and Tamplin, 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 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 Simith, Abrunhosa and Diele, 2017). The biofilms tested in this experiment were collected from a habitat in which oysters (*C. gigas*) 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 (Mesías-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, Bonar and Weiner, 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, Zimmer-Faust and Tamplin, 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 Knight-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, Hare and Sponaugle,

2007). It is thus a trade-off which has to be carefully balanced. With the large-scale disappearance of most *O. edulis* beds in less than 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 Knight-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 Knight-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. 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, Eggleston and Bohnenstiehl, 2013). It is therefore recommended 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.

3.6. 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. In the open sea, the 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, Smyth and Roberts, 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 upon mature hard substrata, provided it is colonised by an appropriate biofilm.

Chapter 4. Pelagic larval duration

4.1. Overview

This chapter examines the pelagic larval duration (PLD) of *O. edulis* larvae under the influence of (i) three temperatures, spanning the biogeographical range of *O. edulis*, and (ii) the absence of a suitable settlement cue.

4.2. Introduction

The amount of time larvae stay in the water before settlement – i.e. their pelagic larval duration (PLD) – has an important effect on the distance larvae are dispersed (Cowen and Sponaugle, 2009). However, while a species with short PLD will inevitably have a short dispersal, species with long PLD do not necessarily disperse more widely (Shanks, 2009). This is because larval behaviour can contribute to retention or return to natal sites (Sponaugle *et al.*, 2002; D'Aloia *et al.*, 2015); thus breaking the otherwise direct relationship between PLD and dispersal distance (Pineda, Hare and Sponaugle, 2007). Although PLD and realised dispersal distances are only weak to moderately correlated in many species (Macpherson and Raventós, 2006; Shanks, 2009), PLD can provide indications of dispersal ability or population connectivity in an ecological and evolutionary context (Leis, 2015). Despite its limitations, PLD remains the most widely used proxy of dispersal potential in marine species (e.g. Macpherson and Raventós, 2006; Shanks, 2009; Selkoe and Toonen, 2011), and it is a fundamental parameter in biophysical models to predict larval dispersal.

PLD is composed of two components: a period of obligatory dispersal before larvae become competent to metamorphose, and a possible extension of that period until metamorphosis is triggered by a cue (Pechenik, 1990; Pawlik, 1992). The attainment of competency may occur within minutes to days in lecithotrophic larvae or require weeks to months for most planktotrophic larvae (Pawlik, 1992). Moreover, it can vary considerably within a single species (Korringa, 1940; O'Connor *et al.*, 2007). This is because PLD is determined by the growth of larvae, which in turn is affected by environmental factors and is therefore location and season specific (Deksheniaks, Hofmann and Powell, 1993; Cowen and Sponaugle, 2009). The main factors affecting larval development are temperature and food (Korringa, 1957; Deksheniaks, Hofmann

and Powell, 1993; Deksheniaks *et al.*, 1996). Higher temperatures result in faster larval development and a reduced PLD. The population of a species at lower latitudes will therefore have a shorter PLD than a population occurring at higher latitudes (Deksheniaks, Hofmann and Powell, 1993). Inadequate food quantity and/or quality also significantly delays development (Deksheniaks *et al.*, 1996; Robert, Vignier and Petton, 2017). However, variations in food in coastal environments may not be large enough to notably influence development (Korringa, 1940).

Once larvae are mature to settle, PLD can be prolonged if suitable settlement sites are absent: metamorphosis is delayed, while maintaining the capacity to metamorphose (Pechenik, 1990). Planktotrophic larvae may remain competent for extended periods, depending primarily on nutritional availability (Pawlik, 1992), but possibly also on the temperature at which they developed (Pechenik, 1990). The capability of competent larvae to delay metamorphosis in the absence of adequate cues increases the likelihood of being transported into a more suitable habitat for survival and reproduction (Pechenik, 1990).

PLD described in literature for *O. edulis* range from around 6 days at 22°C to 16-17 days at 15-16°C (Korringa, 1940 and references therein; Davis and Calabrese, 1969; Robert, Vignier and Petton, 2017). Perry and Jackson (2017) reported a maximum larval pelagic length of 30 days, but with no associated temperature. Most previous studies were aimed at optimising hatchery production of *O. edulis*. Therefore, they have been conducted at temperatures where growth is satisfactory and relevant for shellfish culture. However, in the northern distribution range of *O. edulis*, such as Scotland, summer temperatures can be on average 13°C (World Sea Temperature, 2018) and larval development time at those temperatures has not been studied. In addition, experiments aimed at enhancing aquaculture production have only investigated development time from swarming (larval release from mother oyster) to maturity. Evidence suggest that *O. edulis* larvae can delay metamorphosis for at least a couple of days (Cole and Knight-Jones, 1939; and results of chapter 3), but the extent to which larvae are capable of delaying metamorphosis has not been studied.

The aim of this chapter was to study the PLD of *O. edulis* larvae relevant to natural dispersal scenarios and its restoration. Larval development from swarming to competency was studied at three temperatures spanning the biogeographical range of *O. edulis*. Moreover, the larvae's ability to delay metamorphosis, while maintaining the capacity to metamorphose and survive as a spat, was examined over two weeks.

4.3. Materials and methods

Larvae were obtained from Danish Limfjord oysters (*O. edulis*), which were induced to spawn at the Danish Shellfish Centre (DSC) following FAO guidelines (Helm, 2004). Both experiments were carried out at the DSC.

4.3.1 Larval development at different temperatures

Larval development time was investigated at three temperatures: 13, 18 and 25°C. The latter temperatures served as control for the 13°C treatment, as they could be compared with data from literature. Temperature-controlled water baths were prepared in large 150 L boxes: the 13°C bath was regulated by a cooler (Aquamedic Titan 1500), while the 18 and 25°C baths, were warmed by submersible heaters (Hidom Aquarium Heater 50w HT-2050). A temperature control system (TMP-REG instrument - Pt100 probe, Loligo System ApS), pumped cold water into the 18 and 25°C baths, when temperature rose above the set value. Each water bath was equipped with a submersible pump (flow rate 300 L/h) to homogenise water temperature, and a calibrated USB temperature data logger (Elitech RC-51), which measured temperature every 5 min.

At the start of the experiment, nine 3 L aquarium glass jars were filled with 0.2 µm-filtered seawater (FSW) at 22°C. A batch of newly spawned *O. edulis* larvae (at 22°C) was divided into each jar at an approximate concentration of 1.5 larvae/ml and three replicated jars were randomly allocated into each temperature treatment. The water within each jar was left to cool/ heat naturally to the temperature of each water bath and fitted with slow aeration (air filtered to 0.2 µm). Three additional jars containing 0.2 µm FSW were placed into each water bath as reserve water for future water changes. Larvae were fed daily a microalgae mixture consisting of *Chaetoceros muelleri*, *Tisochrysis lutea* and *Pavlova gyrans* (volume ratio 5:1:1) at a concentration of 100 cells/ µl.

Every 2-3 days, the larvae's water was changed (Helm, 2004) and samples were collected. Larvae were poured gently onto a partially submerged 80-µm sieve, and then washed with a squeeze bottle into a 1 L beaker, prefilled with 0.2 µm FSW. Larval samples were collected from the beaker with a pipette after gently mixing the larvae. At least 3 x 0.5 ml were collected, but when larval concentration was low, this procedure was repeated until a minimum of 10 living larvae had been sampled (average: N=19). In each sample, living and dead larvae were counted, and the number of living larvae in the jar was calculated (Formula 3). The sampled larvae were preserved in sea water and ethanol, and the remaining larvae were poured into a clean jar with reserve water (pre-heated/cooled in

the water bath to the appropriate temperature). Larvae were fed their daily microalgae mixture and returned into their corresponding temperature bath. This procedure was repeated until all nine jars had been sampled and the water changed. Any settled spat on the empty jars was counted and all jars were subsequently cleaned with hydrogen-peroxide and fresh water. The clean jars were filled with 0.2 μm FSW and placed back into the water baths as reserve water. The average seawater salinity throughout the experiment was 26.5 ppt. Larvae of all temperature treatments were cultured until > 50% had reached pediveliger stage or until cultivation was no longer viable due to mortality.

Preserved larvae were inspected for maturity to settle (i.e. eyespot in the pediveliger stage, see Appendix 5) with a binocular microscope and additional lights providing side-illumination. All larvae, which were alive during sampling (i.e. full shells), were measured with a computer imaging system (Nikon Digital Sight DS-U3, imaging software: NIS-Elements BR). Percentage of pediveliger larvae and mortality per day and temperature were calculated with Formula 4.

Formula 3: Living larvae in jar

$$N(\text{jar}|\text{alive}) = n(\text{sample}|\text{alive}) * \frac{\text{vol beaker (ml)}}{\text{vol pipetted (ml)}}$$

Formula 4: Per cent of x larvae per temperature and day (e.g. x = dead/ pediveliger)

$$\% x \text{ larvae} = \frac{N x \text{ larvae } [\text{sample1} + \text{sample2} + \text{sample3}]}{N \text{ total larvae } [\text{sample1} + \text{sample2} + \text{sample3}]} * 100$$

Statistics

A Von Bertalanffy Growth model was fitted to the length by age data of each temperature treatment (Formula 5), where L_{inf} represents the population's asymptotic average length, L is the length of the larvae at time t , K is a growth rate coefficient, and t_0 is a modelling artefact, representing the time when the average length was zero (Derek, 2018).

Formula 5: Von Bertalanffy Growth equation

$$E[L|t] = L_{inf} * (1 - \exp(-K * (t - t_0)))$$

The effect of temperature on larval development was also evaluated through a two-way analysis of variance (ANOVA), with day and temperature as explanatory variables, and larval length as dependent variable. Model diagnostics were visually inspected to check for model assumptions. All analysis was conducted in R 3.4.0 (R Core Team, 2017).

4.3.2 Delay of metamorphosis

Newly spawned *O. edulis* larvae were transferred into 15 L flow-through holding tanks with 1 μm FSW at 25° C. They were raised at an approximate concentration of 10 larvae/ml and 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 . Larvae were monitored every 1-2 days for developmental stage. After 9 days, 60% of larvae had developed to pediveliger stage.

A subsample of larvae was examined under a binocular microscope, and pediveliger larvae (N=153) were selected for experimentation. Larvae were divided into three 250 ml beakers covered by a lid and fitted with slow aeration (filtered to 0.2 μm). They were maintained in 0.2 μm FSW at an average room temperature of 24.2°C and fed daily 100 cells/ larvae of the microalgae mixture prepared for the holding tanks (see above). Cell concentrations were determined from flourometer readings and a regression line providing microalgae cell counts for each flourometer value. Water was changed every 3 days: larvae were sieved into a small 80 μm sieve, washed with a squeeze bottle into a well and pipetted back into a clean beaker with FSW. Dead and settled larvae were counted and removed from the culture.

Settlement viability was examined on day 0, 4, 7 and 11 after selection of pediveliger. In every settlement round, 41-48 larvae from the beaker culture were subjected to two treatments (Table 4-1), which had been previously determined to be highly suitable and not suitable for settlement, respectively (chapter 3). Each treatment was replicated six times and the replicates were randomly assigned into a well of two 6-well culture plates. Four larvae were assigned into each well (filled to 3 ml), with 24 larvae per treatment (except for the last settlement round, where remaining larvae (N=41), where divided between two plates). Larvae were left to settle at an average ambient room temperature of 24.2°C. After three days, the status of each larvae ('alive'/ 'dead', 'settled'/ 'not settled') was examined with a binocular microscope. Larvae, which did not settle and were still alive, were mixed back into the beakers for future settlement rounds. Settled spat were measured and placed into a growing tray from the DSC. After three days, the spat's survival and growth was noted.

Table 4-1. Treatments used to study delay of metamorphosis in *O. edulis* larvae

Treatment	Description
Spat	1 μm filtered seawater (FSW) with a living spat (length: 1-2 cm) and food: 100 cell/ μl of <i>Chaetoceros muelleri</i> , <i>Tisochrisys lutea</i> and <i>Pavlova gyrans</i> at a volume ratio of 5:1:1
Control	1 μm FSW with food as in treatment above

Statistics

Fisher's Exact test was used to test for significant differences between the number of living larvae, which had settled or not settled in each settlement round and treatment. Significant differences in mortality per settlement round and treatment were also tested with Fisher's Exact test.

4.4. Results

4.4.1. Larval development at different temperatures

The average (\pm sd) temperatures of the baths were 13.04°C (\pm 0.30), 18.38°C (\pm 0.14) and 24.76°C (\pm 0.25) respectively. Larval growth and development differed significantly between the three temperature treatments ($F_{3,2315} = 811.9$, $p < 0.001$, Fig. 4-1). At 25°C, 1.6% of larvae reached pediveliger stage after 5 days. The number of pediveliger subsequently increased continuously, reaching 50% between day 8 and 9, and 73% at day 11, when the treatment was closed (Fig. 4-1, Table 4-2). Until day 7, larval growth was between 12-20 μm per day (average: 14.74 $\mu\text{m day}^{-1}$), but it decreased to 7 μm on day 9, and ended with no additional growth on day 11 (Fig. 4-2). Larval mortality was <10% throughout the 25°C treatment (Fig. 4-3).

At 18°C, the first larvae (1.5%) reached pediveliger stage after 13 days. The subsequent increase in pediveliger was much slower: at day 20, 37% of larvae were in the pediveliger stage, but over the next eight days, the proportion of pediveliger only increased to 42.42% (Fig. 4-1, Table 4-2). Larval growth at 18°C was on average 7.34 $\mu\text{m day}^{-1}$ until day 13, decreasing to an average of 2.2 $\mu\text{m day}^{-1}$ between day 15 and 22, and with no additional growth on the last two sampling points (Fig. 4-2). Mortality remained <10% until day 20, but it subsequently increased exponentially to 44.5% at day 28 (Fig. 4-3). When the treatment was terminated at day 28, larvae of at least one replicate tank seemed to be infected with bacteria (such as *vibrio* spp.), displaying reduced feeding rates, a faded colouration of their inner organs and sometimes erratic swimming behaviours.

At 13°C, the first pediveliger larvae (6%) were detected at day 25. However, no additional pediveliger were sampled on any consecutive day (Fig. 4-1). Average larval growth was 2.2 $\mu\text{m day}^{-1}$ during the whole time period (range: 0.3-3.5 $\mu\text{m day}^{-1}$), except for two days, in which average larval size did not increase (Fig. 4-2). Mortality was <10% until day 13, but it increased exponentially afterwards, particularly from day 18 on (Fig. 4-3). The treatment was stopped at day 30 (51% of mortality) due to larvae appearing infected with bacteria such as *vibrio* spp (symptoms: reduced feeding rates, a faded colouration of their inner organs and high mortality). Within the next three days most larvae died.

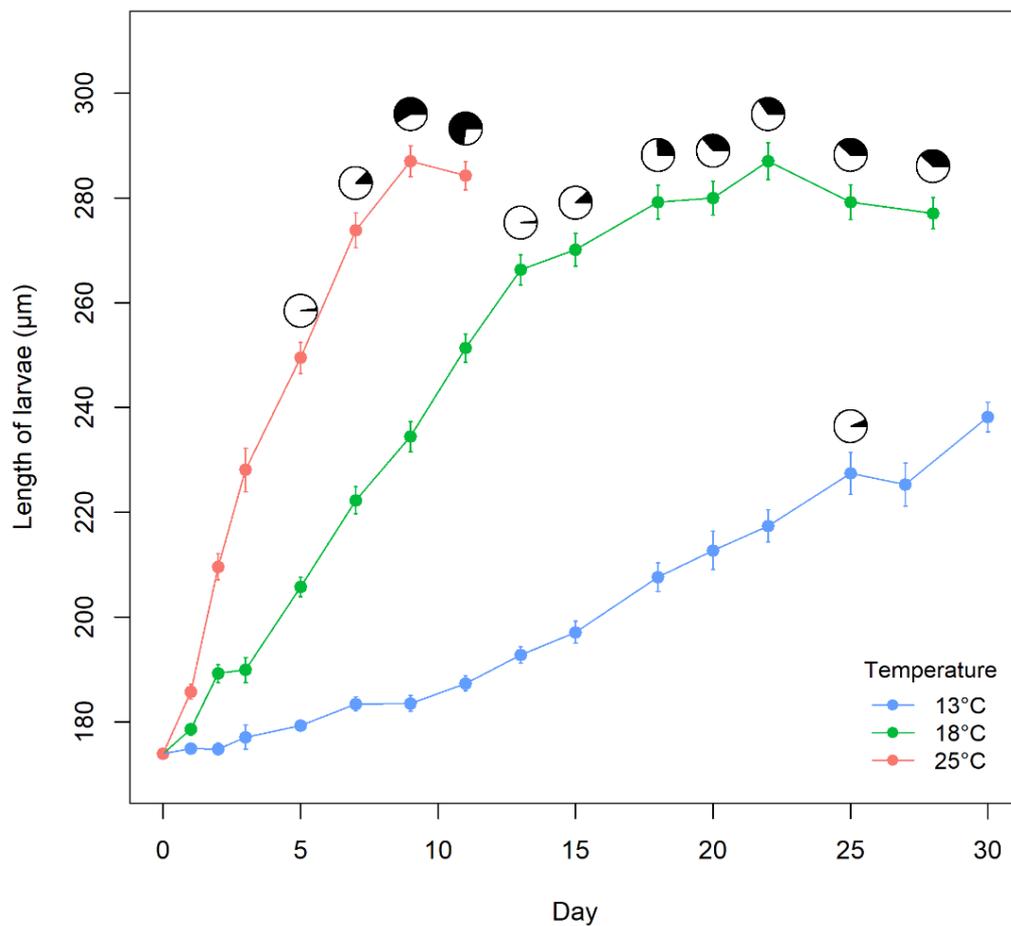


Figure 4-1. Effect of temperature on the growth and development of *O. edulis* larvae. Values are mean \pm se larval length (μm). Pie charts indicate proportion of pediveliger larvae (i.e. mature to settle, in black) on each day. When pie charts are absent, no pediveliger larvae were sampled.

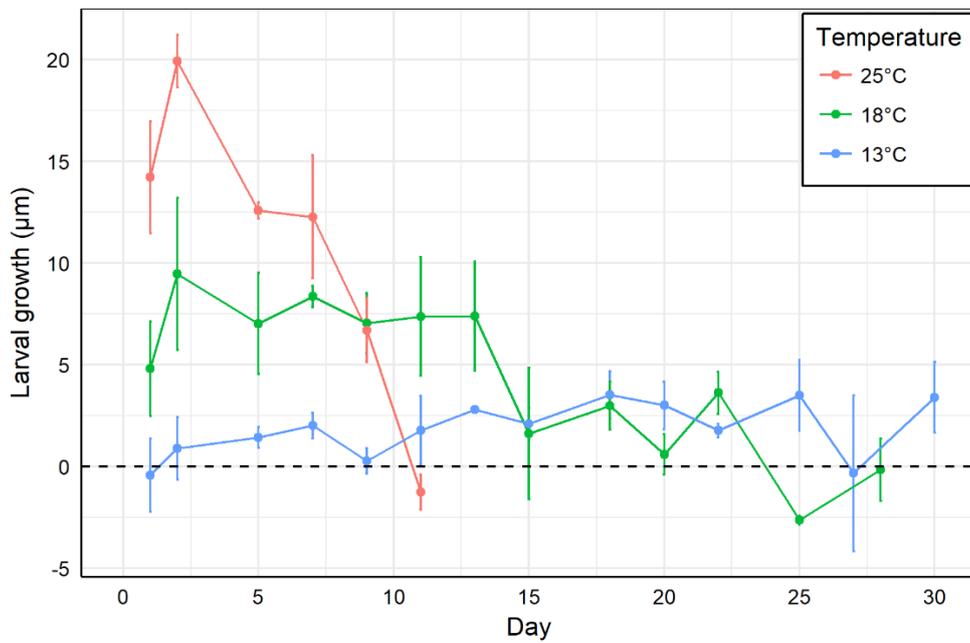


Figure 4-2. Mean \pm se growth of larvae per day and temperature. Negative values indicate a smaller average larval size than on previous sampling day.

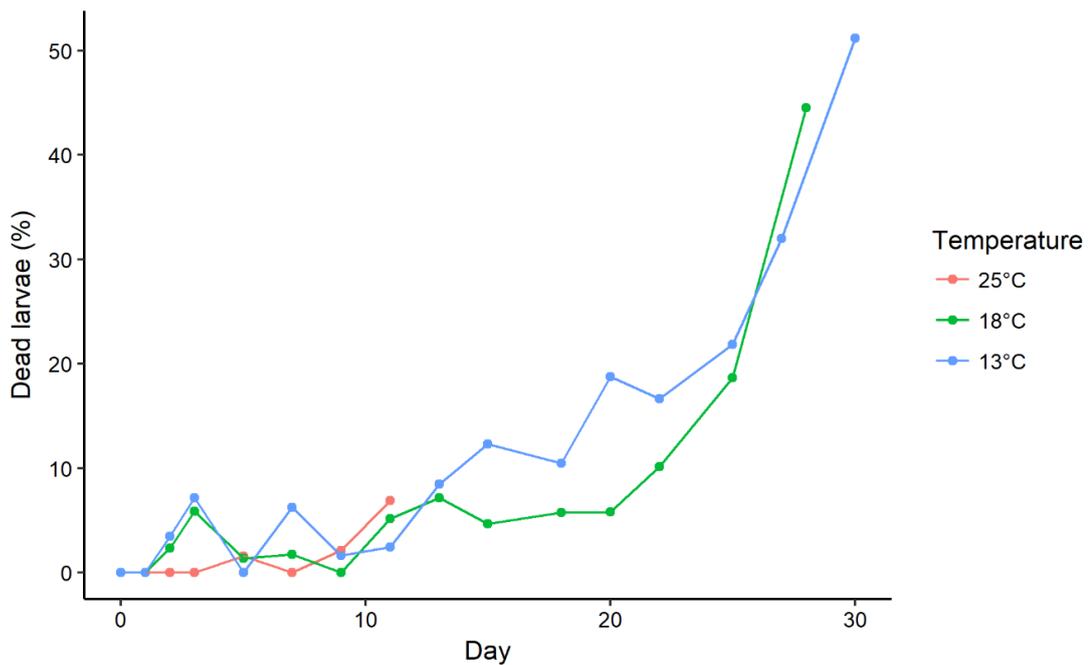


Figure 4-3. Percent of dead larvae sampled per day and temperature treatment.

Many larvae did not grow at 13°C, despite being still alive: although the upper length limit of sampled larvae increased continuously, the lower length limit remained approximately constant (Fig. 4-4). In contrast, at 18 and 25°C the whole cohort of larvae grew steadily with age (Fig. 4-4). Larvae were on average 173.94 (\pm 4.82 SD) μ m long after swarming, when the experiment started, and they grew to an average size of 286.77 (\pm 4.1 SD) μ m when pediveliger was the predominant state (i.e. > 50% of pediveliger in

a sample). This is close to the estimated mean maximal length values of the Von Bertalanffy Growth model (L_{inf} parameter), which ranged from 295 to 310 μm (Fig. 4-4).

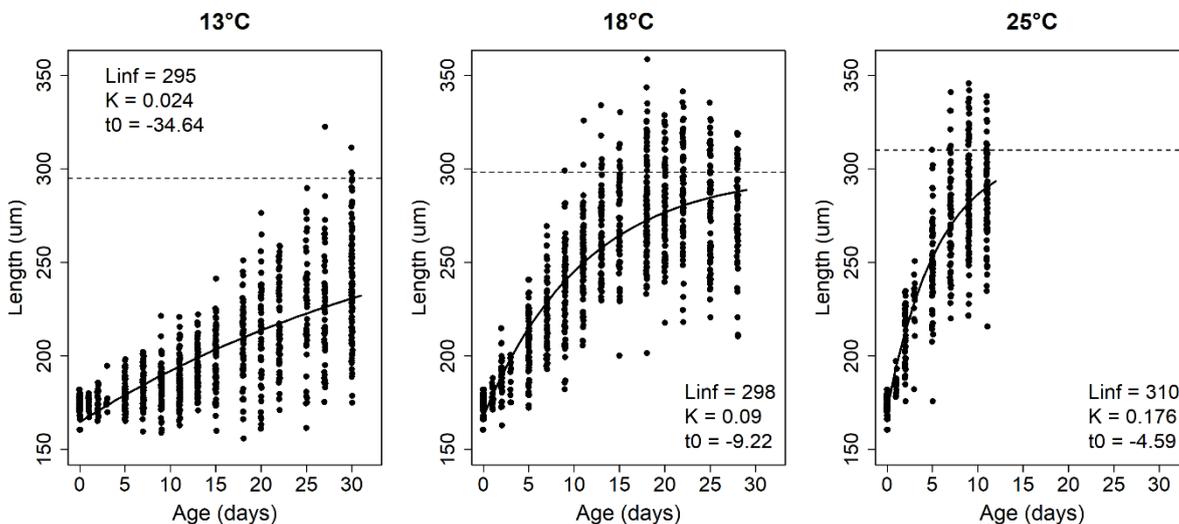


Figure 4-4. Larval length by age with fitted Von Bertalanffy Growth Curves. Estimated L_{inf} , K and t_0 parameters are given for each temperature. Dashed lines mark L_{inf} values (i.e. estimated mean maximal length of larvae).

Only a small proportion of larvae settled on the aquarium containers, despite larvae being mature to settle. Settlement in the aquaria only occurred when the proportion of pediveliger was $> 35\%$, and only 0.07 to 9.25% of the mature pediveliger larvae settled in the aquarium. The largest proportion of settlement (9.25% of pediveliger) was observed when $\sim 73\%$ of larvae were in the pediveliger stage (Table 4-2).

Table 4-2. Percent of larvae in pediveliger stage and settled in holding tanks per day and temperature treatment. Values are average of the three replicate tanks.

Temperature	Day	Mean size (μm)	% pediveliger	% pediveliger settled in holding tank
25°C	5	250	1.59	0
25°C	7	274	11.94	0
25°C	9	287	58.70	0.07
25°C	11	284	72.84	9.25
18°C	13	266	1.54	0
18°C	15	270	11.48	0
18°C	18	279	26.83	0
18°C	20	280	36.92	0.87
18°C	22	287	35.48	6.17
18°C	25	279	40.54	3.35
18°C	28	277	42.42	7.80
13°C	25	227	6.00	0

4.4.2. Delay of metamorphosis

O. edulis larvae were able to delay their settlement for at least 11-14 days: 95% of larvae (19/20), presented with the spat treatment on day 11 and still alive by day 14, had metamorphosed successfully to a spat. In contrast, only 11% (2/18) of living larvae had settled in the control treatment after 14 days (Fig. 4-5). 80% of larvae (16/20) from the control treatment, were thus still alive and had not metamorphosed 14 days after reaching maturity to settle, when the experiment was terminated (Table 4-3). The number of settled larvae versus not settled larvae did not differ significantly between days in the spat treatment (Fisher's Exact test, $df = 3$, $p = 0.60$) nor in the control treatment (Fisher's Exact test, $df = 3$, $p = 0.32$). Overall, 95-100% of larvae settled in the spat treatment, 0-11% in the control treatment (Fig. 4-5) and 0-9% in the beakers with larvae for future settlement rounds (Table 4-4).

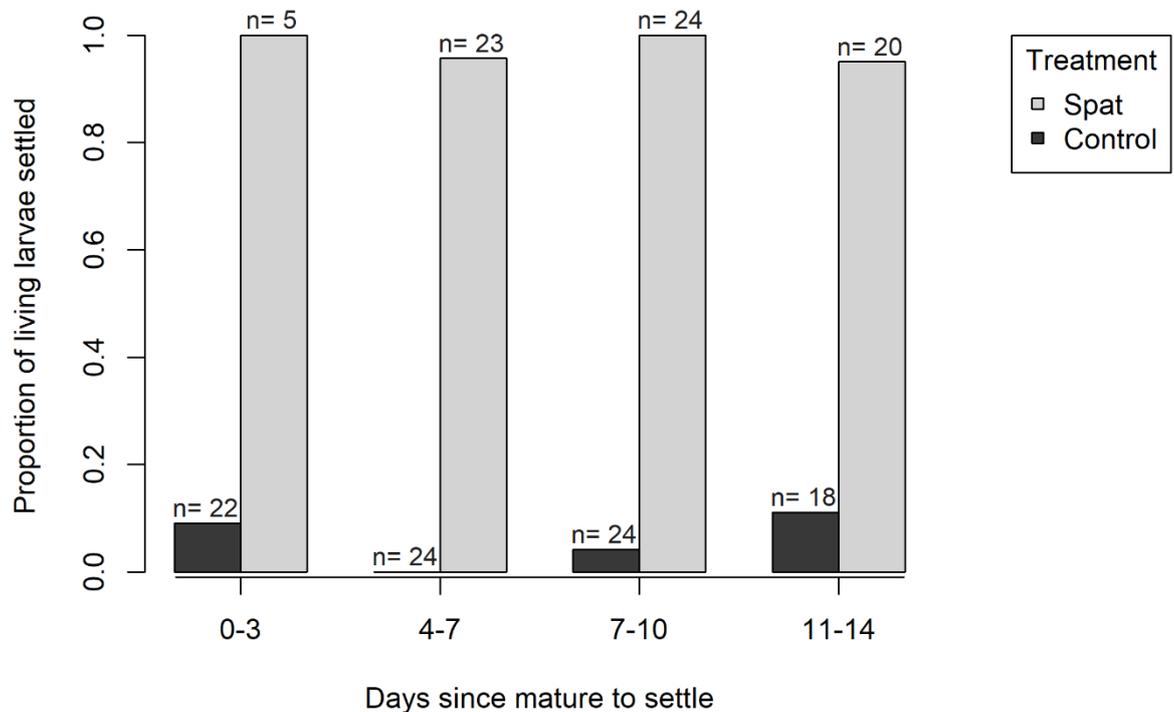


Figure 4-5. Proportion of living larvae settled in each experimental round after three days of exposure to the treatment. N is number of larvae that were observed and still alive at the end of each experimental round (see Table 4-3 for total number of larvae in each round). All larvae were mature to settle from day 0 (start of the experiment).

The number of dead versus living individuals (both settled and not settled) was significantly different between settlement rounds within the spat (Fisher's Exact test, $df = 3$, $p < 0.001$) and the control treatment (Fisher's Exact test, $df = 3$, $p = 0.047$). In the spat treatment, 64.3% of larvae died in the first settlement round (day 0-3), while only

4.3% died in the control. No further dead larvae were found in any of the subsequent spat treatments. In the control treatment, there were no further dead larvae either until the last settlement round (day 11-14), when 10% of larvae died (Table 4-3, Figure 4-6). Only dead larvae were found at the end of each experimental round, all settled spat were alive. Mortality of larvae in reserve beakers was <4% until the end (day 11), when all larvae had been put to settle (Table 4-4).

Table 4-3. Total number of larvae at the start of the experiment and observed number of individuals (dead/ alive) after each settlement round. Only ‘not-settled larvae’ were dead, all settled spat were alive.

Settlement round	Days since start	Treatment	Total	Observed	Alive	Dead
1	3	Control	24	23	22	1
1	3	Spat	24	14	5	9
2	7	Control	24	24	24	0
2	7	Spat	24	23	23	0
3	10	Control	24	24	24	0
3	10	Spat	24	24	24	0
4	14	Control	20	20	18	2
4	14	Spat	21	20	20	0

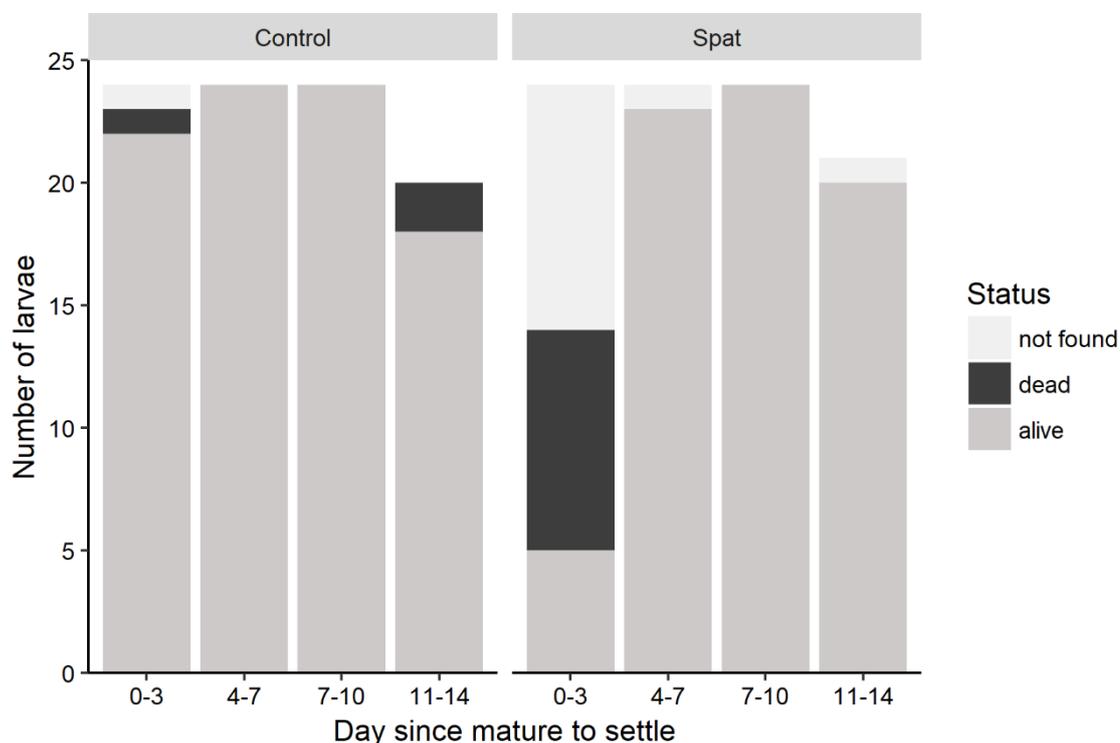


Figure 4-6. Number of individuals per treatment alive, dead and not found after each settlement round.

Table 4-4. Percent of settled, living and dead larvae in beakers with larvae for future settlement rounds

Days since start	% alive	% dead	% settled	N
0	100.00	0.00	0.00	153
3	97.87	2.13	6.38	94
6	96.23	3.77	9.43	53
7	100.00	0.00	0.00	42
11	100.00	0.00	0.00	21

Survival of settled spat was 96 – 100% within the three days of monitoring. The spat grew from average \pm se length x width of $458 \pm 2.9 \times 459 \pm 3.8 \mu\text{m}$ to $625 \pm 12 \times 582 \pm 12.9 \mu\text{m}$. Spat survival and growth was not compromised with increasing delay of metamorphosis (Table 4-5).

Table 4-5. Mean size of spat after each settlement round, as well as their growth and survival three days later. NA indicates that no measurements were taken.

	Day 3	Day 7	Day 10	Day 14
Initial size [μm]	NA	NA	428 x 381	487 x 510
Size [μm] 3 days later	NA	649 x 599	608 x 578	613 x 568
Survival (alive/ total) 3 days later	NA	21/22	19/19	19/19

4.5. Discussion

4.5.1. Larval development time

The obligatory period of dispersal for *O. edulis* larvae encompasses the time from swarming until larvae develop to mature pediveliger. The speed at which larvae develop is highly dependent on both temperature and food. Temperature affects growth through an increase in metabolic rate (Brockington and Clarke, 2001; O'Connor *et al.*, 2007) and possibly through a higher metabolic cost of swimming in colder and more viscous water (Bolton and Havenhand, 1997). As a result, larvae ingest considerably more microalgae cells at higher temperatures, which results in faster development. For instance, at 25°C *O. edulis* larvae ingested between 30 000 to > 100 000 cells day⁻¹ with increasing size, while at 15°C ingestions remained < 30 000 cells day⁻¹ throughout their development (Robert, Vignier and Petton, 2017).

In this experiment, growth was fastest until a large proportion of larvae had reached pediveliger stage, and it differed markedly between the three treatments: on average, 14.74 $\mu\text{m day}^{-1}$ at 25°C, 7.34 $\mu\text{m day}^{-1}$ at 18°C, and 2.2 $\mu\text{m day}^{-1}$ at 13°C. In previous

experiments, growth rate was also fastest at temperatures between 17.5 and 30°C, very slow at temperatures below 15°C or above 32.5°C, and growth increased steadily from 12.5°C (Davis and Calabrese, 1969). Although the mean larval size of this experiment did increase steadily at 13°C, some larvae appeared not to grow (Fig. 4-4). This may result from 13°C being close to *O. edulis* no-growth threshold, which is probably around 10°C, where virtually no larval growth was observed (Davis and Calabrese, 1969).

Growth and development of *O. edulis* larvae were slower than in previous experiments. For instance, at 25°C, 59% of larvae reached pediveliger stage after 9 days, while development (89%) occurred within 7 days in another lab experiment (Robert, Vignier and Petton, 2017, Fig. 4-7). Development of larvae in the colder treatments was even further from previously reported values: at 18°C larvae (37%) attained pediveliger stage after 20 days, while in the field, *O. edulis* developed within 12 days at 17-19°C (Korringa, 1940, Fig. 4-7). The slower development time in this experiment was probably caused by a nutrient deficit. Nutrition can be the largest factor influencing larval growth (Dekshenieks *et al.*, 1996; Marshall, McKinley and Pearce, 2010; Robert, Vignier and Petton, 2017) and larvae require a mixture of biochemical components including protein, lipid, carbohydrate and essential fatty acids for growth and survival (Marshall, McKinley and Pearce, 2010). In the sea, required nutrients are generally attained readily, but in the lab nutrition must be optimised through provision of mixed algal diets (Helm, 2004). In this experiment, larvae were fed three microalgae species (*Chaetoceros muelleri*, *Tisochrysis lutea* and *Pavlova gyrans*) according to the hatchery procedure, which has provided good larval growth in the past. However, although food quantity was constant (100 cells/ μ l), the quality is likely to have varied, since *Tisochrysis lutea* and *Pavlova gyrans* microalgae cell densities in culture bags collapsed a few times during the experiment. Larvae are therefore likely to have received a larger proportion of *C. muelleri* and fewer *T. lutea* and *P. gyrans* microalgae cells during their development, resulting in a potential suboptimal nutrient combination. This may have resulted in the observed longer developmental times. Davis and Calabrese (1969) also reported variable food quality, and their development times were consequently slower (Fig. 4-7). The longest developmental time was recorded in a batch of *O. edulis* larvae reared in Ardtoe (Scotland): 37 days at 21°C (personal observation, Fig. 4-7). These larvae had been underfed throughout their development, illustrating how strongly nutrition can influence development time in the lab.

In the sea variations in food quality are probably not large enough to notably influence development (Korringa, 1940), as long as larvae remain in coastal waters which are highly productive and rich in nutrient inputs (Swearer *et al.*, 1999). Temperature therefore appears to be the main driver influencing development time of *O. edulis* larvae under natural conditions (Korringa, 1940). Development time of *O. edulis* larvae in the coastal Oosterschelde (Korringa, 1940) were among the fastest recorded regardless of temperature (Fig. 4-7), indicating that larvae obtain a high-quality nutrition from the sea, which is at least equal, but often better, than the one provided in the lab. If larvae were to disperse to nutrient-depleted oceanic waters, their development would probably be slower than in the nutrient-rich coastal waters (Swearer *et al.*, 1999).

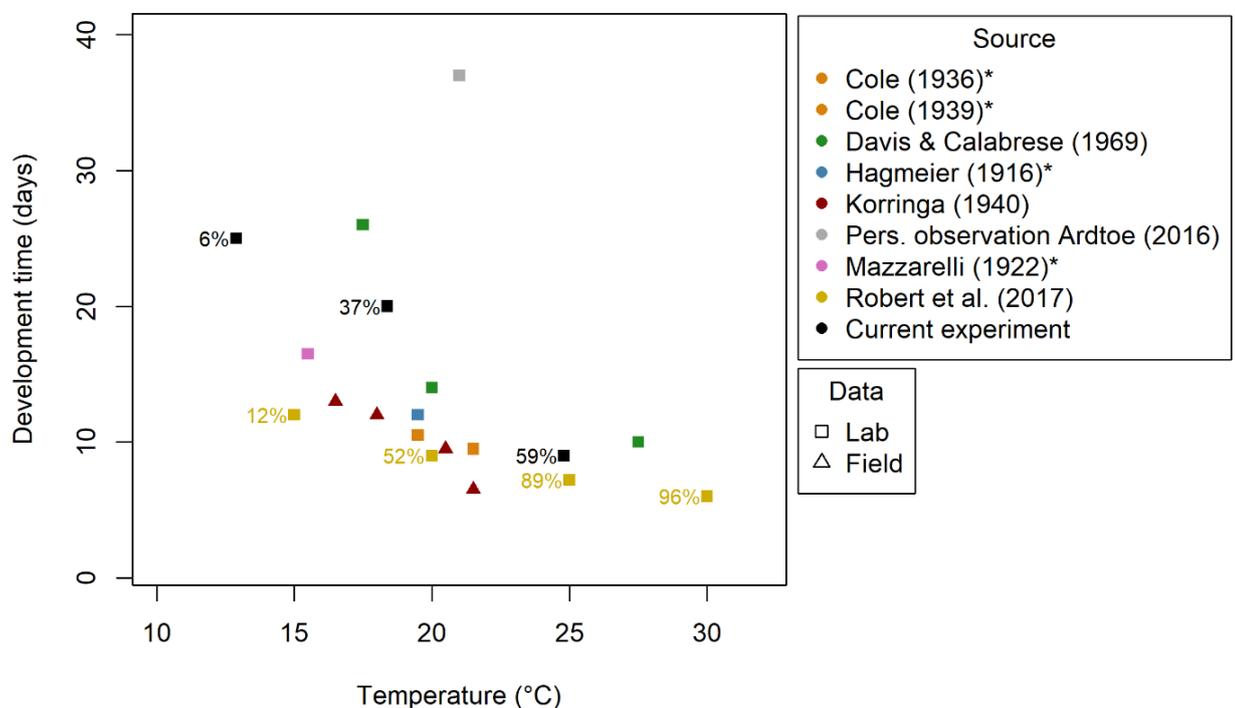


Figure 4-7. Development time of newly spawned *O. edulis* larvae to mature pediveliger with temperature. Graph depicts values reported in literature (both lab and field data), as well as the results of this experiment and a personal observation in a hatchery in Ardtoe (Scotland). Percent values are larvae in pediveliger stage when given (it is assumed that for the remaining points at least 50% of larvae were in pediveliger stage). Sources marked with an asterisk are cited in Korringa (1940).

Larval survival was high (~90%) until day 20, when mortality increased exponentially, probably due to a bacterial infection (such as *vibrio* spp.). When the 18°C and 13°C treatments were closed, most larvae were dead or had faded colours of their inner organs and they were barely moving. If there was an incubation time of the infection, it may have also been the reason why larvae at 18°C did not develop > 42% to pediveliger despite a

proportion of larvae being in pediveliger stage since > 2 weeks. It is unlikely that temperature itself was causing mortality, since larvae survived well until day 20. Moreover, previous experiments found a high survival (>70%) of *O. edulis* larvae at temperatures between 12.5 and 25°C (Davis and Calabrese, 1969). Larval survival was, however, reduced at 10°C and 30°C (~50%) and seriously hampered at 32.5°C (Davis and Calabrese, 1969).

One of the main aims of this experiment was to obtain a developmental time for larvae in Scottish waters, i.e. at ~13°C. Some pediveliger larvae (6%) were sampled on day 25, but no further pediveliger were sampled on any consecutive days, indicating that the actual proportion of pediveliger may have been lower. All larvae subsequently died before being able to fully develop to pediveliger. However, even if larvae had developed to pediveliger at 13°C, the development time would have likely been an overestimation, since development of larvae in this experiment was overall slower than literature values (Fig. 4-7).

Nevertheless, an estimation of development time at 13°C can be obtained: Robert, Vignier and Petton (2017) developed an equation (Formula 6) based on their experiments ranging from 15°C to 30°C, with which growth rates of *O. edulis* can be calculated (Formula 7). According to the equation, following growth rates would apply to the temperatures studied in this experiment: 15.1 $\mu\text{m day}^{-1}$ at 25°C, 7.7 $\mu\text{m day}^{-1}$ at 18°C, and 4.66 $\mu\text{m day}^{-1}$ at 13°C. These theoretical growth rates are not far from the averages observed in this experiment: 14.74 $\mu\text{m day}^{-1}$ at 25°C, 7.34 $\mu\text{m day}^{-1}$ at 18°C and 2.2 $\mu\text{m day}^{-1}$ at 13°C. *O. edulis* larvae must grow approximately 110 μm in the water column before attaining the competency to settle: from an average 170-180 μm at swarming (Appendix 5, Table A5-2; Korringa, 1940; Helm, 2004) to an average of 280-290 μm when $\geq 50\%$ of the population is mature to settle (Appendix 5, Table A5-2; Robert, Vignier and Petton, 2017). If 110 μm is divided by the daily growth rate (Formula 8), an estimated development time per temperature can be obtained: 7.3 days at 25°C, 14.3 days at 18°C and 23.6 days at 13°C.

Formula 6 (Robert, Vignier and Petton, 2017):

$$\ln(\text{growth rate}) = -8354.9 x + 30.737$$

where x is temperature in Kelvin⁻¹.

Formula 7 (adapted from Formula 6):

$$growth\ rate = \exp(-8354.9 * \frac{1}{x + 273.15} + 30.737)$$

where x is temperature in degree Celsius.

Formula 8:

$$development\ time\ (days) = \frac{110\ (\mu m)}{growth\ rate\ (\mu m/days)}$$

These values are based on laboratory experiments, where nutrition is not optimal. Development is therefore likely to be somewhat faster in the sea: for instance, *O. edulis* larvae developed within 12 days at 17-19°C in the Dutch Oosterschelde (Korringa, 1940, Fig. 4-7), instead of at the predicted 14.3 days by the formula. Moreover, the equation is based on an experiment in which the lowest temperature treatment was 15°C. Extrapolation to values < 15°C should therefore be treated with caution. For instance, the 13°C growth rate of the equation is more than double the one observed in this experiment (4.66 vs 2.2 $\mu m\ day^{-1}$), while the other two temperatures growth rates match well (15.1 vs 14.74 $\mu m\ day^{-1}$ and 7.7 vs 7.34 $\mu m\ day^{-1}$). Nevertheless, the equation provides a good framework with which to obtain a first estimate of developmental time. Considering the equation, and *O. edulis* field development times (Korringa, 1940, triangles in Fig. 4-7), it may be that development of *O. edulis* in Scottish waters is somewhere around 20 days at 13°C.

4.5.2. Delay of metamorphosis

O. edulis larvae were able to delay metamorphosis for at least 11-14 days: 95% of larvae presented with a conspecific on day 11 had metamorphosed successfully to a spat by day 14. The proportion of larvae metamorphosing in response to the spat treatment (95-100%) did not differ significantly over the weeks and it was similar to settlement observed in chapter 2 with conspecifics (100%). The settled spat survived to 96-100% during the three days of monitoring. Since spat mortality is highest during the first days after settlement (Searcy and Sponaugle, 2001; Shima and Findlay, 2002; McCormick and Hoey, 2004), it is likely that the spat would have continued growing healthily, despite a delay in metamorphosis.

At day 14, 80% of larvae in the control treatment were still alive and had not settled, indicating that *O. edulis* larvae would be able to delay metamorphosis even further. The ability to delay metamorphosis varies widely among species, from hours to months, and it is thought to depend on the rate of development: the longer the pre-competent period, the greater the capability of delaying metamorphosis (Pechenik, 1990). For instance, the blue mussel *Mytilus edulis* was able to delay metamorphosis for 28-46 days at a rearing temperature of 10-13°C and for 2-25 days at 16-21°C (Bayne 1965 cited in Pechenik, 1990). However, in another experiment at 16°C *M. edulis* was also able to delay metamorphosis for at least 45 days (Pechenik *et al.*, 1990). The pacific oyster, *Crassostrea gigas* (*Magallana gigas*), was able to delay metamorphosis for at least 30 days at rearing temperature of 23°C, without losing the ability to metamorphose (Coon, Fitt and Bonar, 1990). Since *O. edulis* is closely related to these two species it is likely that its larvae will also be able to delay metamorphosis for a similar time span. The capability to delay metamorphosis may depend on the rate at which larvae developed, as previously suggested (Coon, Fitt and Bonar, 1990; Pechenik, 1990), or it may be fairly constant – which could be investigated in future experiments.

The time larvae delay their metamorphosis is strongly correlated to their specific substrate and habitat requirements (Coon, Fitt and Bonar, 1990). As long as they do not encounter a suitable settlement cue, they will continue to delay metamorphosis. Some larvae may also postpone their metamorphosis if they sense the presence of dominant competitors (Pechenik, 1990) or predators (Welch *et al.*, 1997) on examined substrates. However, as metamorphosis is delayed, larvae become more sensitive to environmental stimuli, which trigger metamorphosis (Coon, Fitt and Bonar, 1990; Pechenik, 1990 and references therein). For instance, in chapter 2, *O. edulis* larvae settled immediately in response to conspecifics, but it took them almost two days to start settling in response to the biofilm – which suggest, that they became more sensitive to this environmental stimulus over time. In contrast, when a habitat-associated chemical stimulus was absent, the proportion of larvae settling was remarkably constant both through time and across experiments: ~10% maximum (see Table 4-6 for a summary).

Table 4-6. Summary of *O. edulis* larval settlement in response to treatments, which did not include habitat-associated chemical cues.

Chapter	Experiment	Treatment	Settlement
Chapter 4	Delay in metamorphosis	Control	0-11%
Chapter 4	Delay in metamorphosis	Reserve larvae in beaker	0-9%
Chapter 4	Development with temperature	Larvae in jars	0-9%
Chapter 3	Settlement experiment	All treatments, except spat and biofilm	0-14 %

There were significant differences in mortality between both treatments. During the first settlement round (day 0-3), many larvae died in the spat treatment (38%), but not in the control treatment (4%). This indicates contamination originating from the spat (e.g. their faeces), which may have been exacerbated by hot temperatures (~24°C). In addition, a large proportion of larvae from the spat treatment (58%) were not found at the end of that settlement round. These larvae may have also died and subsequently been ingested by the spat through its filtering-feeding behaviour. No further mortality was observed in the spat treatment, nor in control treatment – until day 14, when 10% of control larvae died. Natural mortality was thus low throughout the 14 days of observation. However, if the experiment had continued, *O. edulis* larvae would have eventually died or metamorphosed spontaneously. Future experiments may investigate where that balance lies, and whether, in the absence of suitable settlement cues, most *O. edulis* larvae would eventually die, or whether they would spontaneously metamorphose post the consistently observed ~10%.

The immediate benefits of being able to delay metamorphosis are clear: it increases the larvae's chance of finding a suitable settlement location, which will support survival and reproduction (Pechenik, 1990). However, as larvae delay metamorphosis, the risk of predation in the water column increases (Korringa, 1940), and if larvae do not find a suitable settlement location, many may eventually die without metamorphosing (Coon, Fitt and Bonar, 1990; Diele and Smith, 2007).

4.5.3. Importance of PLD for dispersal of *O. edulis*

Pelagic duration as a proxy for dispersal distance requires the assumption that flow is reasonably uniform and that larvae are behaving largely passively (Selkoe and Toonen, 2011). However, larvae behave actively and their behaviour can have a greater impact on dispersal than their PLD (Shanks, 2009). For instance, maximum dispersal distance of reef fish larvae were 16.4 km, despite having an average larval phase of 26 days (D'Aloia

et al., 2015). Similarly, ~60% of fish larvae self-recruited to their natal reef, independent of short (< 2 weeks) or long (> 1 month) pelagic duration (Almany *et al.*, 2007). The predominantly demersal behaviour of *O. edulis* larvae (chapter 2) is likely to result in a weak correlation between PLD and dispersal distances, as long as current velocities and other factors, such as predators and filter feeders, do not override *O. edulis* larval behaviour.

However, PLD may determine maximum dispersal potential, as well connectivity with other areas. For instance, storms tend to advect larvae further and faster than normal, and they generally overwhelm larval behaviours that resist long-distance dispersal (Shanks, 2009; Selkoe and Toonen, 2011). The annual abundance of new recruits would thus be negatively correlated with extreme weather events (Shanks, 2009). Since, the probability of extreme weather events is correlated to PLD, PLD may determine maximum dispersal potential and long-term genetic averages (Shanks, 2009; Selkoe and Toonen, 2011). In addition, if beds were exposed to higher current velocities, PLD would determine connectivity with other areas, as larval behaviour may not be able to counteract advection.

The direct relationship of PLD to seawater temperature may also influence dispersal and recruitment success of larvae. For instance, if summer temperatures were particularly cold, the obligatory development time of *O. edulis* would be prolonged, potentially causing larvae to disperse past their natal bed – particularly if beds were not large enough. This may result in good recruitment occurring during warm summers and poor recruitment during colder summers, as recorded for *O. edulis* beds in the Oosterschelde and Limfjord (Korringa, 1940; Spärck, 1951). Warming seawater temperatures associated with climate change may also lead to enhanced recruitment success of *O. edulis* in the future.

Finally, PLD is likely to have another critical implication for *O. edulis* larvae: it determines the time larvae are exposed to sources of mortality (O'Connor *et al.*, 2007). For instance, in the Dutch Oosterschelde, around 14% of *O. edulis* larvae were lost per tidal cycle: ~10% due to predation and ~ 4% due to advection by currents (Korringa, 1946). Mortality was also correlated with sea temperature and thus with an increase in PLD: 10% at 22°C, 5% at 20°C and 2% at 18°C (Korringa, 1946). The proportion of larvae lost from every spawning batch may thus be correlated to their PLD.

4.6. Conclusion

PLD of *O. edulis* larvae can vary considerably, depending on seawater temperature, food and availability of suitable settlement substrate. Larval obligatory development time may range from < 6 days to potentially ~30 days when temperatures are close to *O. edulis* no-growth threshold. Once larvae are competent to settle, they can prolong their pelagic duration for at least two weeks, and, judging from closely related species, possibly for \geq 1 month. Larvae can therefore remain pelagic for considerable time, regardless of seawater temperature, if suitable settlement sites are absent. Prolonging PLD enhances larval mortality, which can drastically affect recruitment. The absence of a suitable settlement habitat may result in most larvae dying instead of metamorphosing. If *O. edulis* larval behaviour is not overridden by factors in the field, PLD is likely to be a poor proxy of average dispersal distances. However, when beds are subject to strong currents, PLD may reflect maximum dispersal potential and explain connectivity patterns among populations. In addition, PLD appears to be strongly correlated to larval mortality and it may explain enhanced recruitment success during warmer summers, particularly when beds do not reach a critical size to retain larvae.

Chapter 5. General discussion

The persistence of restored *O. edulis* populations depends on the supply and settlement of larvae. Understanding larval dispersal is therefore key to the success of European oyster restoration. The purpose of this thesis was to study the larval behaviour and ecology of *O. edulis* in as much as is relevant to the dispersal of this species, with the goal of informing conservation and restoration efforts. The research presented in this thesis consistently showed larval behaviours that would cause them to target their own beds. Larvae were predominantly demersal throughout their development, which in the sea would reduce dispersal from natal populations and enhance self-recruitment. This retentive effect would be enhanced if, as preliminary observations suggest, larvae are also seeking crevice/ cryptic sub-habitats within the bed. Larvae settled preferentially among conspecifics, and, if conspecifics were absent, they also settled in response to habitat-associated biofilms. In the absence of an appropriate settlement cue, larvae delayed metamorphosis for weeks. Such a delay in metamorphosis increases the risk of predation and, therefore larval mortality rates, if target habitats are absent. The results of this thesis provide strong evidence that the behaviour of *O. edulis* larvae plays a crucial role in their dispersal and illustrate the importance of conspecific association in the overall life history strategy of *O. edulis*. The following general discussion will address: (i) the degree to which *O. edulis* beds may be open or closed populations, (ii) the habitat requirements of *O. edulis* larvae at settlement, and (iii) the implications for conservation and restoration efforts.

5.1. Open versus closed populations

Marine populations can range from fully open to fully closed (Cowen and Sponaugle, 2009). A species life history and larval capabilities (e.g. spawning time, larval behaviour and PLD) generally influence self-recruitment, while local hydrodynamics regulate larval exchange between populations (Cowen, Paris and Srinivasan, 2006). The interplay between both determine where populations lie in the open to closed continuum.

O. edulis life history traits clearly favour retention. Spawning and swarming of *O. edulis*, occurs in spring and summer (Korringa, 1940), when temperature and phytoplankton productivities are highest (Macpherson and Raventós, 2006). This ensures a fast growth of larvae and reduces their obligatory dispersal time (chapter 4). The viviparous behaviour

of *O. edulis* also reduces the larvae's obligatory dispersal time, since initial development occurs within the mother oyster. In addition, release of *O. edulis* larvae from the mother oyster may be matched with predictable hydrographic features that favour larval retention, as is the case for the closely related Olympia oyster *Ostrea lurida* (Peteiro and Shanks, 2015). Finally, the demersal behaviour of *O. edulis* larvae may strongly increase the likelihood of self-recruitment (chapter 2), with larvae being able to control their position in the water column from the moment they are released from the mother oyster in the 'D-stage'. In contrast, larvae of species which are fertilised externally cannot influence their vertical distribution until they have developed cilia to swim.

Although a species life history, and particularly larval behaviour, can greatly reduce dispersal distance (Shanks, 2009), greater larval dispersal may be inevitable when beds are exposed to stronger currents. For instance, the former 'current beds' in the Wadden Sea were located on exposed tidal channels, and they were subjected to strong tidal currents (Gercken and Schmidt, 2014). It is suspected that these stocks were supported by larval supply from the 'deep-sea oysters' in the southern North Sea (Berghahn and Ruth, 2005; Gercken and Schmidt, 2014; Fig. 5-1). The North Sea oyster beds in turn, were probably supported by occasional mass invasion of larvae from regions south of the English Channel caused by climatic variations (Berghahn and Ruth, 2005). In contrast, the shallower 'flat' and 'internal' beds of the Wadden Sea only had a small amount of water exchange with reduced larval dispersal (Gercken and Schmidt, 2014; Fig. 5-1). Although these beds in the inner regions of the Wadden Sea may have benefited from some of the larvae produced in the exposed current beds (Gercken and Schmidt, 2014), the beds are thought to have been predominantly self-recruiting (Berghahn and Ruth, 2005). In the Oosterschelde, larvae were probably also self-recruiting: only ~4% of larvae were lost each tidal cycle due to a high degree of water retention and larvae settled in their natal population (Korringa, 1940, 1952, Fig. 5-1). Former *O. edulis* populations are therefore likely to have ranged from closed to nearly open, and many populations may have been interlinked.

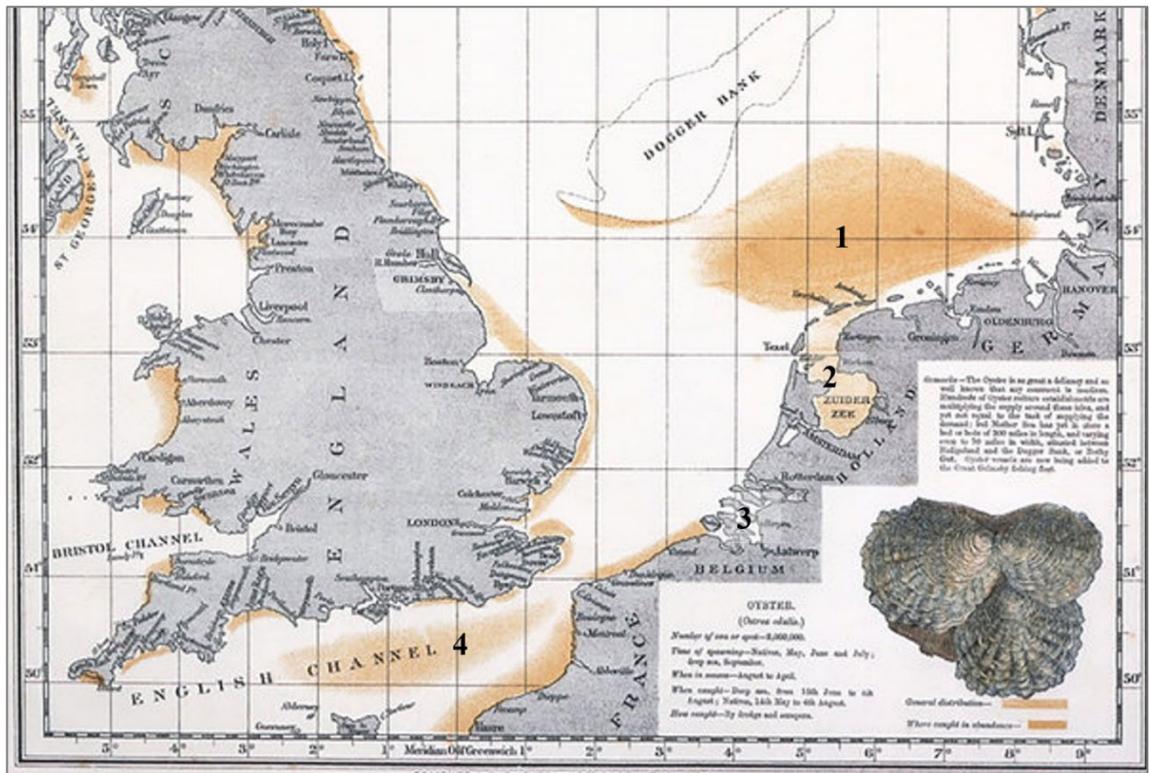


Figure 5-1. Extract from Olsen's (1883) piscatorial map, illustrating the location of discussed *O. edulis* beds: (1) 'deep-sea oysters' in the southern North Sea; (2) Wadden Sea with exposed 'current beds' and the shallower 'flat beds' and 'internal beds'; (3) Oosterschelde bed (not highlighted on the original map); (4) English Channel bed.

Populations can be closed on scales of <10 km or on scales of hundreds of kilometres, and the scale at which populations are closed will vary geographically and possibly with time (McQuaid and Phillips, 2000). Many of the former *O. edulis* populations are likely to have been closed on large scales. For instance, the former 'deep-sea oyster' beds in the North Sea (Fig. 5-1 (1)) measured up to 20,000–25,000 km² (Berghahn and Ruth, 2005; Christianen *et al.*, 2018), while the Firth of Forth oyster bed in Scotland covered an area 32.2 km long and 9.7 km wide (Thurstan *et al.*, 2013). Larvae originating from these beds would have been able to drift considerable distances and still settle on their natal bed. In many populations, the size of the bed may have thus been a crucial factor for the retention of larvae, particularly in relatively tidally exposed populations, such as the 'deep-sea oysters'. Beds in low current velocities, such as the Oosterschelde (Fig. 5-1 (3)), may have been self-recruiting on smaller scales. Self-recruitment in *C. virginica* oyster larvae was also directly related to reef area: reef patches ranging from ~7,000 – 9,000 m² experienced 26-37% of self-recruitment, while in a reef patch of ~18,000 m² there was 81% of self-recruitment (Hubbard and Reidenbach, 2015).

In the west coast of Scotland, remnant *O. edulis* occur in small, scattered and patchy populations, fringing sea lochs (Low *et al.*, 2007; see Fig. 1-3). These populations tend to be in areas of low current velocity (Scottish Natural Heritage records, D. Donnan, pers. communication). Larvae may therefore be self-recruiting, with potential occasional connectivity between populations during stormy weather (cf. genetic studies on Scottish oyster populations by Beaumont *et al.*, 2006). However, the low population density (generally $<1 \text{ m}^2$) and low population size ($\sim 1,000 - 20,000$, except Loch Ryan) is likely to have a negative effect on the reproductive success of these populations (Low *et al.*, 2007), thereby reducing recruitment success. In addition, unlawful gathering of oysters in the west coast of Scotland is causing significant reductions in population sizes (Donnan, 2003; Low *et al.*, 2007), which further reduces reproduction and recruitment success. In contrast, the west coast population of Loch Ryan, which is the largest known wild population of Scotland with ~ 5.7 million adults (Low *et al.*, 2007), may have a considerably higher proportion of self-recruitment.

A critical question for the restoration of *O. edulis* in the Dornoch Firth (east coast of Scotland) is whether larvae will be able to recruit into their natal population, or whether they may disperse into the wider Moray Firth (Fig. 5-2). The Dornoch Firth has strong tidal beds, with a mean current velocity of 40 cm/s (Fig. 5-3). Previous work suggest that oyster larvae are not able to control their vertical position in the water column at current speeds $> 50 \text{ cm/s}$ (Peteiro and Shanks, 2015), and that flow speeds $> 15 \text{ cm/s}$ frequently resuspend cockle larvae from the boundary layer on a smooth bottom (Jonsson, André and Lindegarth, 1991). Considering the velocity profile of the Dornoch Firth, in which current speeds $< 15 \text{ cm/s}$ are rare (Fig. 5-3), it seems unlikely that *O. edulis* larvae would be able to counteract dispersal by currents. However, with ongoing restoration efforts, a larger and denser *O. edulis* bed may slow down the near-bed hydrodynamics (Butman, 1986; Whitman and Reidenbach, 2012), and provide a larger natal bed expanse for larvae to settle. Thus, in the future some larvae may be able to recruit into their natal populations. Future studies may investigate how flow-speed and bottom complexity affect the self-recruiting potential of *O. edulis* larvae.

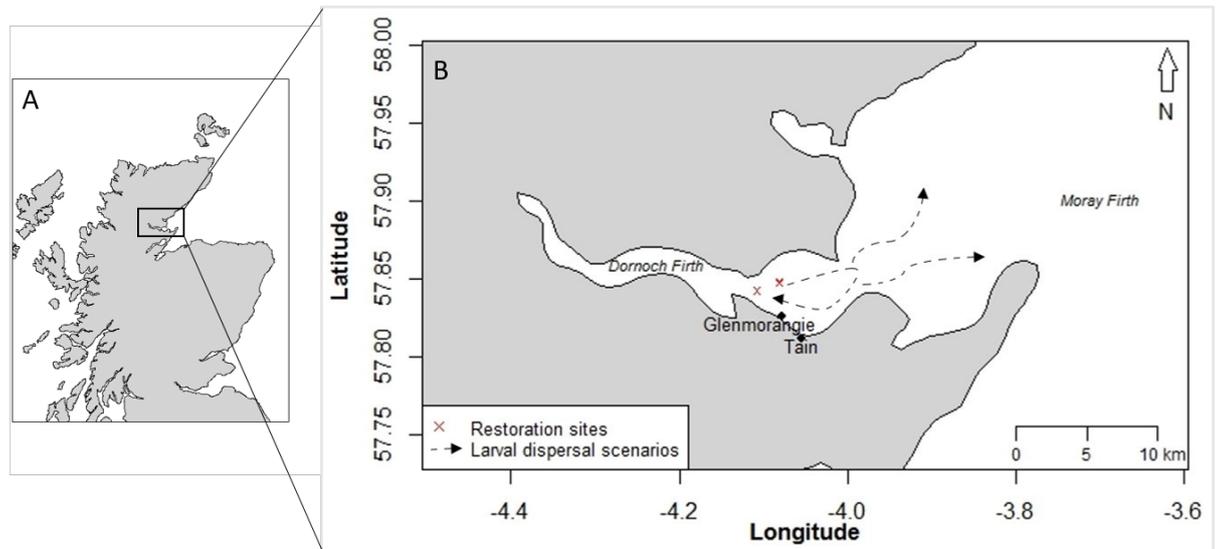


Figure 5-2. Larval dispersal scenarios for *O. edulis* populations restored into the Dornoch Firth as part of the Dornoch Environmental Enhancement Project (DEEP). A: map of Scotland with the Dornoch Firth area surrounded by a black box. B: Close up of the Dornoch Firth, illustrating the initial restoration sites and schematic larval dispersal scenarios for self-recruitment versus dispersal into the Moray Firth.

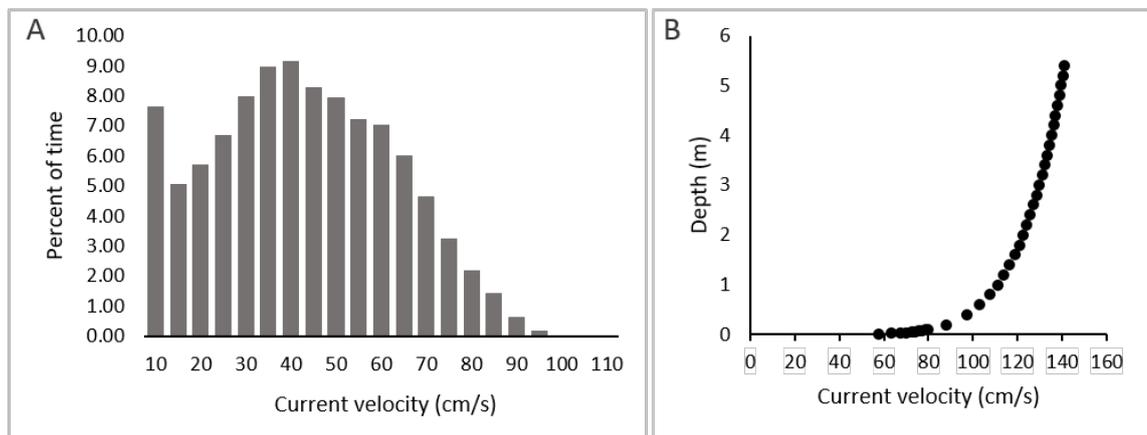


Figure 5-3. Current velocities in the Dornoch Firth recorded at ‘west site’ (see Figure 5-2; A. Cordingley, R. OH. Murray and A. Gallego, unpublished data). A: Histogram of current velocities over 31 months. B: Modelled current velocity profile by depth at the highest recorded speed (102 cm/s).

In summary, although *O. edulis* has life history traits which favour retention, larval loss seems inevitable when populations are exposed to strong currents. Larvae released from a population at a site with low current velocity, are likely to show lower dispersal distances and a higher probability of self-recruitment, while larvae released from less sheltered environments with higher velocities will probably disperse more (Robins *et al.*, 2013; Hubbard and Reidenbach, 2015). Populations in exposed areas may therefore receive comparably little of their own larvae and depend more on connectivity with other areas (Hubbard and Reidenbach, 2015), particularly when beds do not reach a minimum

size, density and structural complexity to retain larvae. The extent of a bed is a critical parameter in determining the proportion of self-recruitment: the larger the bed, the more self-recruitment will occur (cf. Hubbard and Reidenbach, 2015). The minimum surface area and structural complexity of a bed necessary to retain larvae will probably depend on the current velocities in each environment.

5.2. Habitat requirements at settlement

Some species have a restricted target habitat, while other species have an extensive target habitat. For instance, the megalopae crab larvae of *Cancer magister* settle almost anywhere on the benthos along the coast in less than 80 m deep water, and intertidal barnacles settle on any hard surface in the intertidal zone (Shanks, 2009). Generalist species may respond to bacteria in biofilms which are common to many substrata, while specialist settlers, i.e. those settling in response to conspecifics, prey or specific living species, settle in response to cues specifically indicative of surfaces where those species occur (Hadfield and Paul, 2001).

The results of this thesis, in agreement with the literature, indicate that *O. edulis* is a specialist settler. The species' main target seems to be their own beds: competent larvae settled fastest and to 100% when conspecifics were present (chapter 3). These findings were corroborated in chapter 4: 95-100% of larvae settled with conspecifics throughout every settlement round and independent of the time larvae had delayed their metamorphosis. After delaying metamorphosis for over two days, 85% of *O. edulis* larvae settled in response to biofilms formed in a habitat where oysters occur (chapter 3). However, *O. edulis* larvae do not appear to settle indiscriminately in response to biofilms: larvae did not settle in response to a biofilm collected in a marina (pers. observation, chapter 3), and in the Dutch Oosterschelde only 1% of mature larvae settled despite oyster farmers laying 6,000,000 limed tiles and 4,000 m³ of mussel-shells as collectors (Korringa, 1946), which should have been rapidly colonised by biofilm forming microorganisms (Salta *et al.*, 2013). The Oosterschelde is an enclosed bay with high water retention (Korringa, 1940). Considering the bottom-dwelling behaviour of *O. edulis* larvae (chapter 2), it seems unlikely that most larvae were washed away into the open ocean. Instead, larvae may have been dispersed away from the mother bed and delayed metamorphosis due to a lack of adequate cue (chapter 4). The lack of adequate settlement cues in what seems to be a specialist settler may result in many *O. edulis* larvae falling to predation or natural mortality without metamorphosing when target habitats are absent.

In the sea, attachment of *O. edulis* is very mollusc-focused, with a particular emphasis on bivalves. For instance, in the recently discovered shellfish reef in the Dutch North Sea, which is composed of European oysters, Pacific oysters (*Crassostrea gigas/ Magallana gigas*) and blue mussels (*M. edulis*), *O. edulis* settled predominantly on the shell or shell fragments of bivalves: 81% on *C. gigas* and 12% on other bivalves, such as blue mussels, common cockle and native oysters (Christianen *et al.*, 2018). Subfossils of *O. edulis* found in the same area were also attached to shells of molluscs, including *Buccinum undatum*, *Neptunea antiqua* and *O. edulis* (Christianen *et al.*, 2018). Similarly, in Ireland, *O. edulis* settled predominantly on conspecifics and shells of other molluscs (45.1%), including those of the common periwinkle *Littorina littorea*, the mussel *Mytilus edulis* and the oyster *C. gigas*. Molluscs, other than oysters, were common on former *O. edulis* beds (Möbius, 1877; Caspers, 1950) and *O. edulis* may have evolved to metamorphose in response to these habitat-associated species. Some mollusc species may be more suitable than others at inducing settlement; yet the likelihood of *O. edulis* larvae attaching to a given species may depend on the species frequency in the area (e.g. compare Christianen *et al.*, 2018 to Smyth *et al.*, 2018).

The preference of *O. edulis* to settle on shell material in the sea has been noted in previous studies (e.g. Möbius, 1877; Cole and Knight-Jones, 1939; Christianen *et al.*, 2018; Smyth *et al.*, 2018). However, a critical question, which has received little attention, is whether larvae may be settling in response to living or recently dead shells, as opposed to an abiotic shell preference over other materials. The metabolites and microbiota associated with living or recently dead species are different to the ones on animals which have been dead for a longer time (Caffrey, Hollibaugh and Mortazavi, 2016; Pechal *et al.*, 2018; Preiswerk, Walser and Ebert, 2018). In addition, in the sea, newly grown shell-edges of bivalves are often the only hard and clean surfaces available for larvae to settle (Korringa, 1946; see Fig. 5-4). *O. edulis* larvae may have therefore evolved to settle predominantly in response to living bivalve cues, which are likely to provide a more precise and advantageous habitat signal than dead shells. For instance, in an undisturbed oyster bed in Helford River, Cornwall, *O. edulis* settled at their highest density on living *M. edulis*, followed by recently dead *M. edulis* shells, despite an abundance of old shell (Cole and Knight-Jones, 1939). Similarly, in the shellfish reef of the Dutch North Sea, *O. edulis* were often attached to living bivalves (Christianen *et al.*, 2018). If *O. edulis* do target predominantly living bivalves, it may not be enough to spread large quantities of dead shells to encourage settlement of larvae. These dead shells would depend on the

colonisation of an appropriate biofilm to be suitable for settlement (chapter 3) – just as any other non-living substrate. However, in many cases dead shells may not be colonised by the appropriate bacteria, particularly if the environment is turbulent and the shells are covered with silt and sediment – such as in the Oosterschelde, where almost none of the mature larvae settled, despite the presence of large quantities of dead mussel shell as collectors (Korringa, 1946).

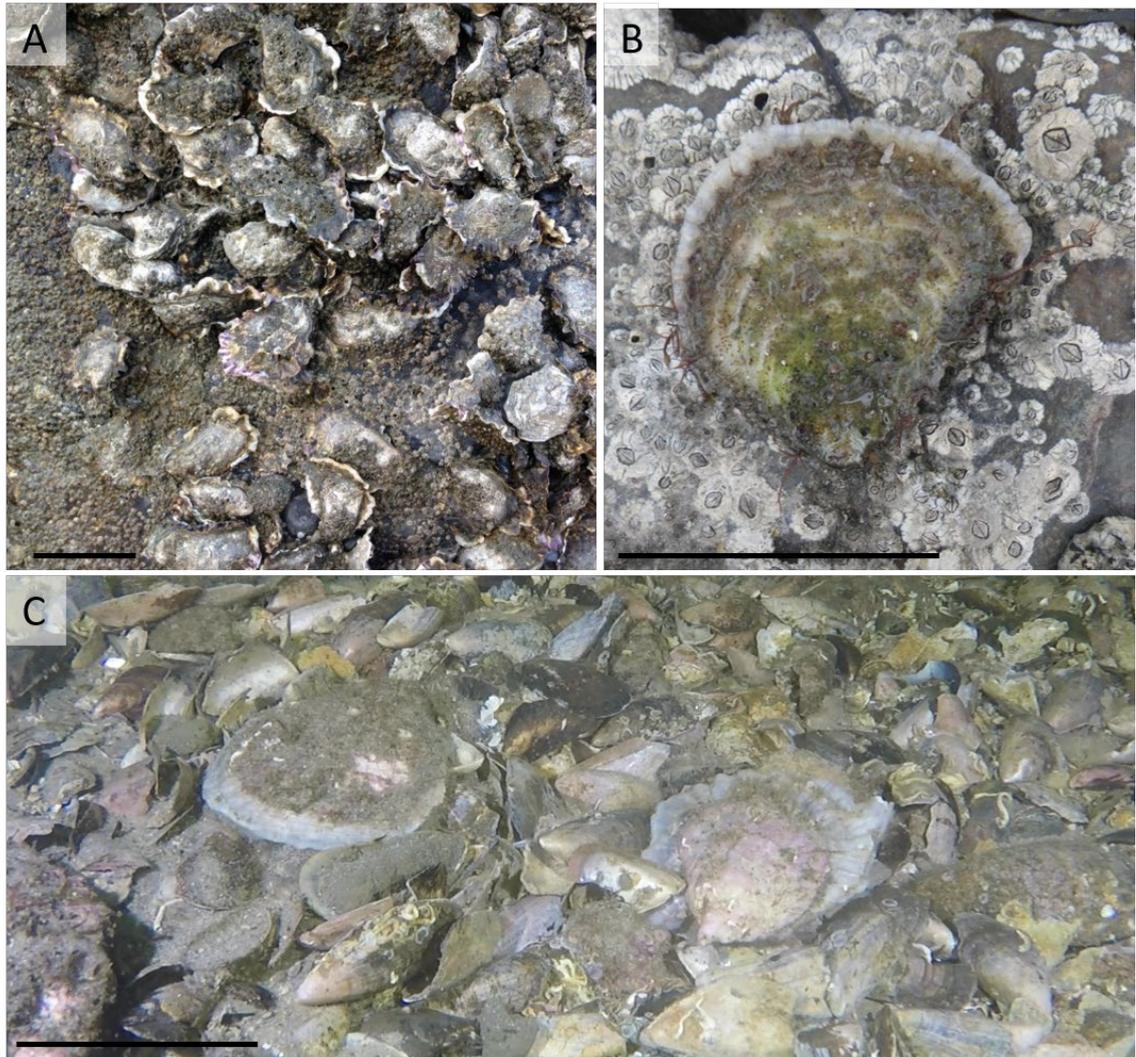


Figure 5-4. Example of oysters in the wild with their newly grown shell-edges being the only clean surface to settle on. A: *C. gigas* oysters attached to a wall in Oostende harbour (Belgium). B: A single *O. edulis* oyster from an inlet in Loch Ailort (Scotland), which harbours a small wild *O. edulis* population. C: Restored *O. edulis* oysters in the Dornoch Firth (at ‘east side’, Fig. 5-2). Scale bar: 10 cm. Photo A & B: A. Rodriguez-Perez, photo C: W.G. Sanderson.

The presence of nearby bivalves, and particularly conspecifics, may be enough to make *O. edulis* larvae settle, because many of the cues inducing settlement in oysters seem to be soluble in water (Coon, Fitt and Bonar, 1990). For instance, settlement of *O. edulis* was enhanced if collectors were merely soaked in water containing *O. edulis* tissue

(Bayne, 1969), and a spat can induce oyster larvae metamorphosis, even when separated by a filter (Coon, Fitt and Bonar, 1990). Biofilms immersed in water containing conspecifics have been shown to be able to ‘store’ the conspecific odour, enhancing settlement in crabs (de Brito Simith, Abrunhosa and Diele, 2017). *O. edulis* larvae may therefore be induced to settle in the sea by the odour of nearby conspecifics and other bivalves, without settling on those individuals themselves. In this thesis, the biofilms collected for settlement in chapter 3 may have had the odour of nearby oysters (*C. gigas*) impregnated in them. This may have enhanced the speed and proportion of larvae settling in that treatment, as compared to the possible effect of a biofilm formed in the same habitat but without oysters. Similarly, dead shells may be suitable for settlement, as long as there are nearby conspecifics or other bivalves, which impregnate them with their odour. In the sea, it may be more advantageous for larvae to settle when conspecifics are close enough that their metabolites are impregnating the surroundings, instead of attempting to find the conspecific to settle gregariously (cf. chapter 3 and 4), particularly when larvae have already delayed metamorphosis and are more sensitive to stimuli (Coon, Fitt and Bonar, 1990; Pechenik, 1990). The degree to which *O. edulis* settles gregariously in natural banks is unknown, and confounded with the effect of century long fishing activities. Möbius (1877) reports that *O. edulis* settles gregariously in the sea, but that this species is found more often individually, while (Korringa, 1946) states that on natural banks the oysters are frequently found in clusters instead of single.

When only a subset of habitats provide a suitable settlement cue, larvae will metamorphose at low rates on surfaces which provide a habitat that is neither optimal nor deleterious for juveniles (Johnson and Sutton, 1994). In this thesis, settlement of *O. edulis* larvae without an adequate cue was consistently around 10% (chapter 3 & chapter 4), even after having delayed their metamorphosis for two weeks (chapter 4). This means that in the sea most *O. edulis* larvae may not metamorphose, if their settlement preferences are not fulfilled. If mortality due to predation and other sources is added (Korringa, 1940), the proportion of settling larvae may be very low under non-optimal conditions (Korringa, 1946).

In hatcheries and ponds, *O. edulis* larvae often settle in larger numbers, despite using non-optimal substrates, such as sterile shells (chapter 3). However, hatcheries put several 10,000s of larvae to settle in small settlement tanks and leave them for about one week. A small proportion of larvae are thus likely to settle, as observed in this thesis, and this settled spat may in turn induce more larvae to settle. Such abundance of larvae is unlikely

to be observed at sea in current circumstances of either extant populations or current restoration scenarios. Many hatcheries use recirculating water, in which the oyster ‘smell’ is most likely impregnated. This water is likely to provide a good settlement cue for larvae (see above). Similarly, the ponds, in which larvae are sometimes spawned and reared, are filled with hundreds of mother oysters at the bottom, which permeate the whole pond with their odour. Although larvae settle preferentially on the clean mussel shells laid as spat collectors within the pond (Legg, 2019), the shells had time to build a small biofilm impregnated with the smell of conspecifics. In addition, the large number of larvae in the ponds may also be influencing each other to settle. Research on *O. edulis* larval settlement in hatchery and pond conditions may therefore not accurately reflect the settlement behaviour of wild populations, which is relevant in the conservation and restoration context.

Overall, the weight of evidence indicates that *O. edulis* is a specialist settler with restricted habitat requirements. In contrast, *C. gigas* oyster larvae may be more generalist than *O. edulis*. This could explain *C. gigas* invasion success (e.g. Zwerschke *et al.*, 2017), despite active measures trying to halt their spread (Guy and Roberts, 2010). For instance, in an area where *O. edulis* and *C. gigas* co-occur, *O. edulis* settled predominantly on bivalves (45.1%) and to a lesser extent on boulders (19.4%). In contrast, only 21% of *C. gigas* were attached to bivalves, while the majority (69.4%) was attached to boulders (Zwerschke *et al.*, 2017). Similarly, in the recently discovered shellfish reef of the Netherlands, large boulders scattered over the floor where colonised by *C. gigas* and *M. edulis*, but not by *O. edulis* (Christianen *et al.*, 2018). Settling predominantly on boulders – a common substrate – may facilitate the spread of *C. gigas*. In contrast, the likely restricted settlement specificity of *O. edulis* larvae for a habitat, which has been largely lost, may explain why, so far, this species has not been able to recover from extensive overfishing, despite restoration attempts in the past (Korringa, 1946).

Furthering our understanding of the settlement requirements of *O. edulis* and its target habitat(s) is thus paramount to the success of current restoration efforts. Future settlement experiments may investigate the following questions:

- (1) The importance of living/ recently dead shell compared to dead shell.
- (2) Which (living) bivalve species – or potentially other habitat associated species – induce settlement of *O. edulis*.

- (3) The minimum distance of conspecifics (or other molluscs) necessary to induce settlement of *O. edulis* larvae, i.e. the minimum dilution of their 'smell', and the degree to which different materials can retain that odour.
- (4) Which bacteria (or bacterial assemblages) induce settlement and how these bacteria relate to the different habitats/ substrates.
- (5) Comparison of the settlement preferences between *C. gigas* and *O. edulis* to elucidate differences in their specific requirements for settlement.
- (6) The sensitivity of *O. edulis* larvae to different cues, the longer larvae delay their metamorphosis.
- (7) The maximum time *O. edulis* larvae can delay metamorphosis and whether this depends on their (temperature-dependent) obligatory development time.
- (8) The current velocities which inhibit settlement and how this relates to three-dimensional seabed complexity.

5.3. Implications for restoration and conservation

Reviving European oyster beds is not an easy endeavour (Korringa, 1946). Yet, this species has the potential to build up large and stable populations when the right conditions are met (Berghahn and Ruth, 2005). For instance, the population of the Limfjord established themselves in 1825, after a severe storm broke the barrier between the North Sea and the Limfjord; within a short time period *O. edulis* landings reached up to 7 million a year (Berghahn and Ruth, 2005). Another example comes from Lake Grevelingen (Netherlands): although the lake harboured a long-standing *O. edulis* population, recruitment was sporadic (Berghahn and Ruth, 2005). In 1971, the lake was closed off from the remaining estuary to prevent the southwestern part of the Netherlands from flooding. After the lake was ponded, the oysters produced a very strong recruitment, despite the number of mother oysters remaining low (Korringa, 1980; Berghahn and Ruth, 2005).

In both examples, successful and large recruitments occurred when current velocities and seston loads were low (Berghahn and Ruth, 2005). For instance, after Lake Grevelingen was closed, it was excluded from the influence of tides, and current velocities decreased from 0-100 cm/s to < 10 cm/s. Moreover, the Secchi disc transparency increased from 0.5-2.5 m to 1-7 m (Nienhuis, 1978). These two conditions probably allowed abundant recruitment despite a small number of mother oysters: the low current velocities (< 15 cm/s) would have not overcome the larvae's demersal behaviour (cf. Jonsson, André and Lindegarth, 1991), resulting in limited dispersal and larvae potentially being able to settle

on the mother oyster bed; while the low seston loads, may have allowed suitable biofilms to form without being covered by organic matter, which may have further encouraged settlement of larvae (chapters 2&3).

In most cases, however, current velocities and seston loads are likely to be well above the ideal conditions for a strong recruitment if the oyster bed is small. In those cases, the only viable solution may be to restore *O. edulis* either at a large enough scale to ensure larvae do (1) not disperse past the natal bed, or (2) disperse into another restored bed. The critical surface area and density of a self-sustaining *O. edulis* bed is a key knowledge gap in restoration efforts (Sas *et al.*, 2019). Korringa (1946) believed that in the sheltered Oosterschelde 10,000,000 oysters was the minimum number necessary to have successful recruitment after deploying cultch, and that a restoration attempt in the German Wadden Sea with only 500,000 oysters would fail. However, if a more efficient settlement substrate was placed, such as the proposed ‘spat-on-shell’ (Sas *et al.*, 2019), a stronger recruitment may be obtained with a smaller number of oysters. In Orkney (Scotland), 800,000 English oysters and cultch were laid on an exhausted bed in the early 20th century, yielding the greatest annual landing: 15,300 oysters, three years later (Low *et al.*, 2007). The extent of former *O. edulis* beds and records of annual landings may provide indications as to the necessary scale to ensure at least some level of self-recruitment.

Although the extent and landings of former beds can be indicative of the scale necessary for a self-sustaining bed, the conditions that once allowed an oyster bed to persist may have changed in some locations. For instance, man-made changes to hydrography in the Dutch Wadden Sea, such as embankments and dams from the mainland to the islands, are suspected to have compromised the self-recruitment of oyster beds in the inner regions of the Wadden Sea (Fig. 5-1 (2); Berghahn and Ruth, 2005). Beds restored to those areas at the historical scale would therefore probably not be able to sustain themselves anymore under the present conditions (Berghahn and Ruth, 2005).

Biophysical models are an excellent tool to obtain insight into the scale of larval drift in a certain location under present hydrodynamical conditions. These models can inform (1) the necessary extent of a bed to ensure self-recruitment, or (2) where to place connected populations to recruit ‘lost larvae’ (cf. Gormley *et al.*, 2015). However, in the case of *O. edulis*, the hydrodynamic model should have a high resolution at the boundary layer and resolve the decreasing speed appropriately, if they are to provide adequate dispersal estimates. Hydrodynamic models, without larval behaviour, may provide an indication of the flow-velocity characteristics of a site considered for restoration, and whether self-

recruitment is likely (in sheltered, slower moving waters), or whether a large degree of larval loss may occur (in exposed, high-velocity areas; Hubbard and Reidenbach, 2015). However, if hydrodynamic models are used with passive larval dispersal, they are likely to overestimate the dispersal of *O. edulis* larvae and underestimate the importance of maintaining natal habitats at sufficient scale to help promote retention of larvae.

Biophysical models can also aid in the conservation of remnant wild *O. edulis* populations. For instance, biophysical models can provide insight into the likelihood of self-recruitment in each population, as well as in understanding larval flow patterns and which populations may act as sinks and sources. This information can provide the basis for future campaigns and conservation efforts, such as tackling unlawful gathering in a specific district. In the west coast of Scotland, for instance, people confuse the legal right to gather most shellfish (all except mussels and oysters) with a right to gather all shellfish (Low *et al.*, 2007). The effects of unlawful gathering on the populations are probably large, since hundreds of oysters are sometimes gathered at once, and it is the largest oysters that are targeted (Low *et al.*, 2007), which contribute most to reproduction (Cole, 1941). Recruitment is still occurring in remnant wild *O. edulis* Scottish populations, yet at low levels (Low *et al.*, 2007). Without the pressure of unlawful gathering, remnant *O. edulis* populations may increase their population sizes in time.

Restoration efforts of *O. edulis* are benefitting and learning from the pioneering work done on *Crassostrea virginica* oyster restoration in the US (e.g. Chesapeake Bay Program). These initiatives are restoring *C. virginica* on a large scale and placing large amounts of shell cultch as settlement substrate. Similar procedures are currently being developed for *O. edulis* (e.g. Pogoda *et al.*, 2019). Although these measures have proven to be successful for *C. virginica*, it does not necessarily imply that they will work to the same degree for *O. edulis*. For instance, in the past, oyster farmers laid large amounts of shell cultch as collectors (about 6,000,000 limed tiles and 4,000 m³ of mussel-shells) at a time point deemed suitable by research, but only 1% of mature larvae settled (Korringa, 1946). The above section provided evidence that *C. gigas* may be less selective at settlement than *O. edulis*. Thus, the settlement requirements of *C. gigas* may be more easily fulfilled in the current North-East Atlantic conditions. *C. virginica* belongs to the same genus/ subfamily and shares a similar life history than *C. gigas* (e.g. broad-cast spawners, intertidal species); the settlement requirements of *C. virginica* may thus be much closer aligned to *C. gigas* than to *O. edulis*. In addition, species of *Crassostreinae* subfamily produce considerably more offspring than the viviparous *O. edulis*: ~ 500,000

to 1,000,000 vs. 1,000,000 to 50,000,000 eggs (Cole, 1941; Korringa, 1952; Cox and Mann, 1992). A higher recruitment success in *Crassostrea* species may therefore also be due to the total number of offspring. Overall, *C. virginica* may be an easier species to restore than *O. edulis*, as it may be less selective at settlement and it produces substantially more offspring.

Restoring *O. edulis* will require considerable monetary investment, persistence and work on a large scale (Korringa, 1946), yet the positive effect of *O. edulis* on the ecosystem is expected to outweigh investment costs in the long run (Sas *et al.*, 2019). Ensuring successful recruitment will be critical, and for this purpose, it may be necessary to restore *O. edulis* at a scale which is large enough to ensure larval settlement within its own beds. Placing large amounts of shells as collectors may not be as efficient in inducing settlement of *O. edulis* than with *C. virginica* oyster larvae, yet this practice may still be crucial to increase the structural complexity of the bottom. The degree of structure of a seabed will probably play an important role in larval retention, by slowing down near-bed hydrodynamics (Whitman and Reidenbach, 2012). Laying down large quantities of shell during restoration efforts, next to conspecifics, may therefore contribute in creating a structural complexity, which enhances larval retention. Large and dense beds would also increase fertilisation success (Guy, Smyth and Roberts, 2018), which would further enhance recruitment. A better understanding of both *O. edulis* larval ecology and structural retention mechanisms will help designing restoration efforts in a way that maximises recruitment success, and it can guide conservation efforts of remnant wild populations.

5.4. Conclusion

Each species has a unique set of constraints and selective pressures on its dispersal, and evolution has shaped life-history traits to these constraints to maximise survival and reproduction (Shanks, 2009). *O. edulis* evolved in a setting, where, over millions of years, it was a dominant benthic species, with large beds spreading along much of Europe's coast lines and subtidal regions (Olsen, 1883; Korringa, 1946). The results of this thesis indicate that *O. edulis* larvae have evolved to minimise dispersal and target their own beds, and that evolution may have shaped the larvae into being very selective at settlement. Such a high selectivity may have been suitable under historical conditions, but it makes recruitment and recovery of *O. edulis* populations more difficult. Once the species target habitat is lost, larvae may die predominantly, instead of metamorphosing

in sub-optimal conditions. Dispersal of *O. edulis* larvae appears inevitable when currents reach a certain speed threshold, despite the observed bottom-dwelling preference. Future studies may elucidate the speed threshold necessary to resuspend *O. edulis* larvae from the bottom, and how resuspension is affected by structural complexity. Restoring European oyster beds at sufficient scale and density to help promote retention of larvae may be crucial to successful recruitment and a long-term persistence of restored beds. Future work may elucidate the critical surface area and density of a self-sustaining bed (Sas *et al.*, 2019), and how it varies with local hydrodynamics. The results of this thesis highlight the importance of considering *O. edulis* larval behaviour in restoration efforts.

Appendix 1. An oyster bed is a biocenosis or a biotic community

Translated extract from: Möbius (1877, pp. 72-77)

The history of the impoverishment of the French oyster beds is very instructive. When the oyster beds of Cancale were almost fully stripped of oysters due to overfishing, cockles (*Cardium edule*) [*Cerastoderma edule*] took their place, and swarms of blue mussels (*Mytilus edulis*) appeared on the exhausted beds of Rochefort, Marennnes and Ile d'Oleron.

Oyster beds are not inhabited only by oysters. In the Wadden Sea of Schleswig-Holstein [Germany] and in the estuaries of English rivers, the oyster beds are the parts of the seabed with greatest animal richness, as I have observed myself. Whenever the oyster fishermen empty a full net onto the deck of the ship, agile green shore crabs (*Carcinus maenas*) and slow spidercrabs (*Hyas aranea*) [*Hyas araneus*] can be seen working their way out of the pile of oyster shells and living oysters, in search of water. Abraded snail shells are set in motion when the hermit crabs (*Pagurus bernhardus*) [*Pagurus bernhardus*], which have occupied them as their home, try to crawl away with their shelter. Whelks (*Buccinum undatum*) protrude from their shells as much as they can, twisting their body with all their strength from side to side to roll back into the water. Red starfishes with five broad arms (*Asteracanthion rubens*) [*Asterias rubens*] lay flat on the ground, unable to move, despite setting in motion their hundreds of tube-like feet. Sea urchins the size of small apples and fully covered with greenish spikes (*Echinus miliaris*) [*Psammechinus miliaris*] rest motionless on the pile. Here and there a blueish shimmering ringed worm (*Nereis pelagica*) slips from underneath the partly dead, partly living bulk. Black-shaded blue mussels (*Mytilus edulis*) and white cockles (*Cardium edule*) [*Cerastoderma edule*] lay closed tightly, like the oysters. Even the shells of the living oysters are inhabited. Sometimes the entire surface of one of their valves is covered by barnacles (*Balanus crenatus*), with tent-shaped lime shells and rank-shaped feet. They are also frequently draped with small yellowish tassels, each consisting of a community of thousand small gelatinous moss animals (*Alcyonidium gelatinosum*), or they are covered with a yellowish sponge (*Halichondria panicea*), whose soft tissue contains fine siliceous spicula. On some beds they are burdened with thick clumps of sand, knitted by the skin mucus of small polychaetes, the so-called honeycomb worms (*Sabellaria anglica*) [*Sabellaria*

alveolata], in an organ-pipe-shape, to have a hard substrate on which they can live sociably next to one another.

On some beds at the south tip of the island of Sylt [Germany], where the best tasting oysters of our Wadden Sea grow, tube worms (*Pomatoceros triqueter*) grow on the oyster shells, their white, three-edged lime tubes often bent like a large Latin S. Here, the shells also often carry so-called dead man's fingers (*Alcyonium digitatum*), which are white or yellow polyp lobes, the size and shape of a clunky glove.

The oyster shells are also often covered with brown turf, which is made of tree-shaped polyps (*Eudendrium rameum* und *Sertularia pumila* [*Dynamena pumila*]), or with clusters of yellowish, almost finger-long tubes, with reddish polyps on their ends (*Tubularia indivisa*). Overhanging these polyps, long tree-shaped structures protrude, which shine yellowish or brownish and carry polyp chalices (*Sertularia argentea*).

Animals even settle inside the calcareous shell. The shells are frequently perforated by a boring sponge (*Clione celata*) from the outer to the innermost layer, on which the mantle of the living oyster lies; and in the interstices between the shell layers of an old oyster hides a green-brown polychaete worm (*Dodecaceraea concharum*), with twelve long feeling-filaments in its neck. I counted once each animal sitting on two oysters individually. On one lived 104 and on the other I counted 221 animals of three different species.

Every now and then, the oyster trawl also brings up fishes, although it is not very suitable for catching them. Plaices (*Platessa vulgaris*) [*Pleuronectes platessa*], trying to save themselves back into the water by leaping up, or hooknoses (*Aspidophorus cataphractus*) [*Agonus cataphractus*] and thornback rays (*Raja clavata*), striking their tails, are all frequent on oyster beds.

Besides the quoted animals, many larger species live their too, but they are caught less abundantly by the nets, and hidden between the large ones, are many small animals, which can only be seen through the optical enlargement of a magnifying glass or microscope.

Plants grow little on the oyster beds. Seagrass (*Zostera marina*) only spreads over a single bed of the Wadden Sea over the oyster's reservoir. On some beds it is possible to find red-brown algae (Florideae). In the water streaming over the oyster beds, there are microscopic green algae (Desmidiaceae) and diatoms, which serve as food to the oyster.

If the trawl is dragged along an area of the seabed of the Wadden Sea where there is no oyster bed, considerably fewer animals are caught, and one retrieves different species from muddy than from sandy areas.

In a way, each oyster bank is a community of living beings, a selection of species and a sum of individuals, which find all necessary conditions for their development and sustenance at this given spot, i.e. the appropriate ground, enough food, proper salinity levels and temperatures which are endurable and favour development. [...]

Science does not yet have a word for such a community of living beings, for a selection and number of species and individuals which overlap in their average environmental living conditions, and which depend on each other and sustain themselves on a certain spot over time through reproduction. I name such a community biocenosis or biotic community.

Each change of any contributory factor causes a change of other factors within a biocenosis. When any external living condition deviates over a prolonged period from its previous state, the whole biocenosis changes; however, it also changes if the number of individuals of a species belonging to the biocenosis decreases or increases in response to human interference, or if a species disappears completely, or if a new species enters the community.

When large quantities of oysters had been removed from the rich beds off Cancale, Rochefort, Marennes and d'Oleron, more living space and food became available for the offspring of the cockles and blue mussels living there, which therefore succeeded to mature at higher numbers than previously. Overfishing therefore completely changed the biocenosis of those French oyster beds. The oysters will not be able to establish themselves in the same quantities as before, until the numbers of cockles and blue mussels are not reduced to their former abundance, as the seabed is already occupied, and other shellfish abstract their food.

Appendix 2. An inexpensive method for larval visualisation

A new method was developed as part of this PhD to visualise larvae in the water column. The method is more cost-effective than previously used methodologies and it facilitates accurate observation and quantification of larvae in the water column, yielding their vertical distribution, behaviour and swimming speeds.

The method consists of: (i) a USB microscope, supported by a retort stand, which is connected to a computer for visualisation and recording, (ii) a thin glass aquarium, supported by a wooden frame, and (iii) a plasticised graticule in the background to mark the larvae's vertical position and measure swimming speeds (Fig. A2-1).

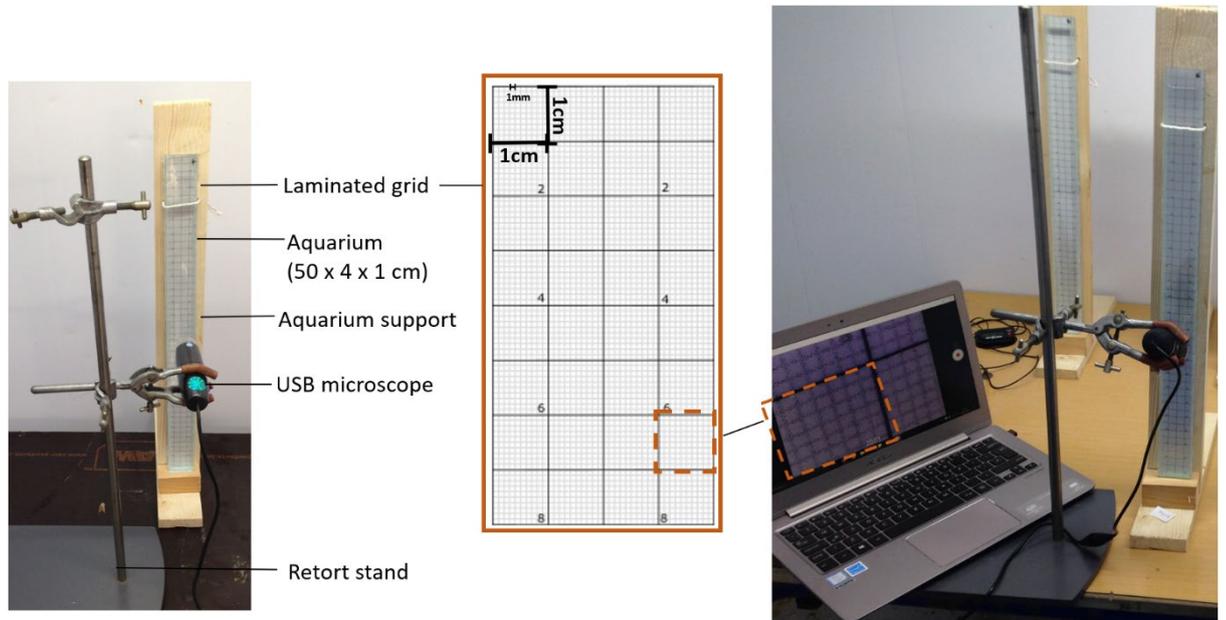


Figure A2-1. Illustration of the method developed as part of this PhD developed to visualise larvae in the water column

Method development

Aquarium

Two aquarium sizes were tested, 50 x 4 x 1 cm and a 50 x 4 x 4 cm. The 4 cm deep aquarium was too deep for the USB microscope to focus throughout its depth; hence, the 50 x 4 x 1 cm aquarium was selected for experimentation. For future experiments an aquarium of 50 x 4 x 2 cm may also be appropriate. The glass used to build the aquaria was 3 mm thick, optically clear and bespoke. The aquarium sides were bonded and sealed

using a specialist non-toxic aquarium sealant. Excess sealant on the aquarium edges was cleaned with a scalpel, as the opaqueness impedes visualisation.

Aquarium support

The aquarium support was built from long wooden tiles, which were cut to the necessary size and screw-fixed together.

Grid

A 1 mm grey graticule was obtained from the internet and marked with black lines every 1 cm. In addition, the vertical height of the graticule was marked numerically every 2 cm along both edges (Fig. A2-1). These markings allowed to discern the position of larvae in the water column with mm resolution. The graticule was printed (with printer setting set on 'real size'), cut to match the aquarium dimensions, laminated and glued.

USB microscope

The initial intention was to use a USB microscope with near-infrared (NIR) light. However, the model purchased (MiView USB Microscope Model MV200UM850IR by GMM) did not have enough magnification to visualise newly spawned larvae, which are ~170 μm large and very transparent. In addition, the NIR-light of the USB microscope was not strong enough to visualise larvae in darkness, not even if addition NIR-light sources were added.

A regular USB microscope with white LED lights was therefore used (Model eBoTrade 2MP 800X). This USB microscope was cheaper (~£15-30 versus > £100), had a stronger magnification (20X-800X), and it allowed to visualise and record *O. edulis* larvae throughout their life history (video: 30 frames/sec). The light emitted by this USB microscope was bimodal with a peak around 450 nm corresponding to blue light and a stronger peak at 600 nm equivalent to yellow light (Fig. A2-2):

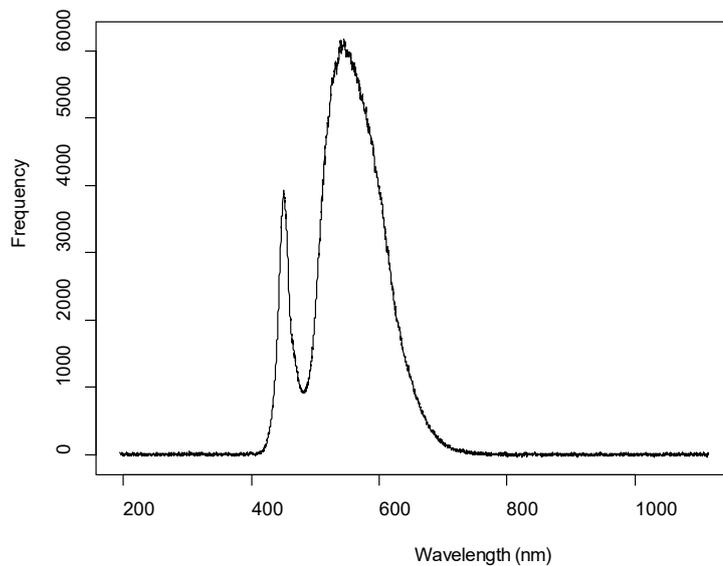


Figure A2-2. Light emitted by the LED-light USB microscope, with the lights turned on to maximum strength. Light spectrum was obtained with a spectrometer.

Preliminary tests indicated that *O. edulis* larvae do not react to the LED light of the USB microscope (no change in vertical distribution, swimming speed or behaviour). However, the light may influence other species: for instance, *Artemia* shrimps consistently swam towards the light of the microscope. In such cases, the USB microscope can be used with its light turned off (provided there is background light), yet, the image loses some of its sharpness and contrast.

A red-filter was created for the LED-light USB microscope to reduce light disturbance when larvae were observed in darkness. The filter was made from transparent plastic material, which was cut to match the shape of the USB microscope, and painted red with a dark-red permanent marker (Fig. A2-3). Several materials were tested as potential red filters and the light spectrum they produced were analysed with a spectrometer. The conclusion was that the filter had to have two qualities: (1) the red or red-painted material could not be opaque, and (2) the image produced by the USB microscope with red filter had to look red to the eye: if the image looked pinkish rather than red, the light spectrum had additional peaks in the blue- and green-wavelength spectrum. The selected filter produced a light between 590-730 nm, with a peak around 620 nm, corresponding to orange and red light (Fig. A2-4).



Figure A2-3. Illustration of the red filter created for the USB microscope for trials in darkness.

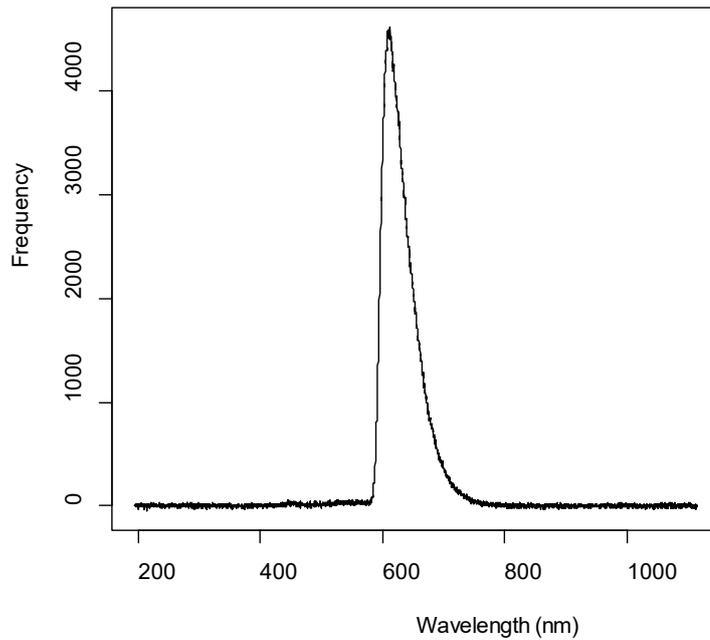


Figure A2-4. Light emitted by the LED-light USB microscope using a red filter. Light spectrum was obtained with a spectrometer.

The USB microscope with red filter was well suited for observing larvae in the dark, as it was capable of visualising them without background light. Preliminary trials showed that *O. edulis* larvae do not react to the red light. This was anticipated, since most zooplankton species do not, or only barely, perceive red light: their sensitivity maxima is generally at about 460-530 nm (Forward, 1988). This is because red light is the light within the visible spectrum that is first absorbed by the sea, entering only to a depth of ~0.1 m (Gühmann *et al.*, 2015).

Retort stand

The USB microscope had to be supported by a stable framework to be able to focus. The retort stand was selected as the best choice: the USB microscope could be secured by one of the clamps and moved along the x- and y-axis of the aquarium without disturbing the larvae through vibration or noise.

Video recording software

An accurate representation of time in the videos filmed by a USB microscope is critical for swimming speed estimation. The video recording software that came with the USB microscopes were therefore tested by filming a stopwatch. The videos showed that the software of both the NIR- and LED light- USB microscope consistently distorted time, with videos being shorter than the actual time it had taken to film then. In contrast, any of the inbuilt video recording programmes of Microsoft or Apple (i.e. Windows Movie

Maker, Windows Camera Roll or iMovie) did not distort time and the USB microscope worked well with those programmes. All videos were therefore filmed using Windows Camera Roll.

Method assessment: *O. edulis* case study

The USB microscope method was well suited in providing data on the vertical distribution, behaviour and swimming speeds of *O. edulis*. The accuracy of the method was assessed by comparing the number of larvae counted in the videos versus the number of larvae in the aquarium (Formula 9). The latter was obtained by filtering larvae out of the aquarium after visualisation and counting them under a microscope. The USB microscope count of larvae was highly accurate, with an average value of 102.8%, i.e. 2.8% overestimation of larvae (N=91 aquaria). The largest sample overestimation was 176.5% (i.e. almost double as many larvae counted in the video than the number of larvae in the aquarium), and the largest underestimation was 25% (i.e. only ¼ of the larvae in the aquarium found with the USB microscope). Larvae may have been over-counted if they were swimming through the water column faster than the movement of the recording USB microscope, and they may have been overseen if they were close to the aquarium edges and not moving, or if the USB microscope was slightly out of focus (i.e. not covering the whole depth of the water column). Smaller larvae (size class 170 and 180 µm) were more frequently underestimated, while larger larvae (particularly size class 290 and 300 µm) were more commonly overestimated (Fig. A2-5). This is probably due to smaller size classes being transparent and more difficult to see, while larger size classes had the capacity to move faster through the water column. Overall, the method was fairly accurate, with 50% of all observations underestimating the number of larvae no more than 12.1% and overestimating it no more than 19%.

Formula 9:

$$Accuracy (\%) = \frac{N_{video}}{N_{aquarium}} * 100$$

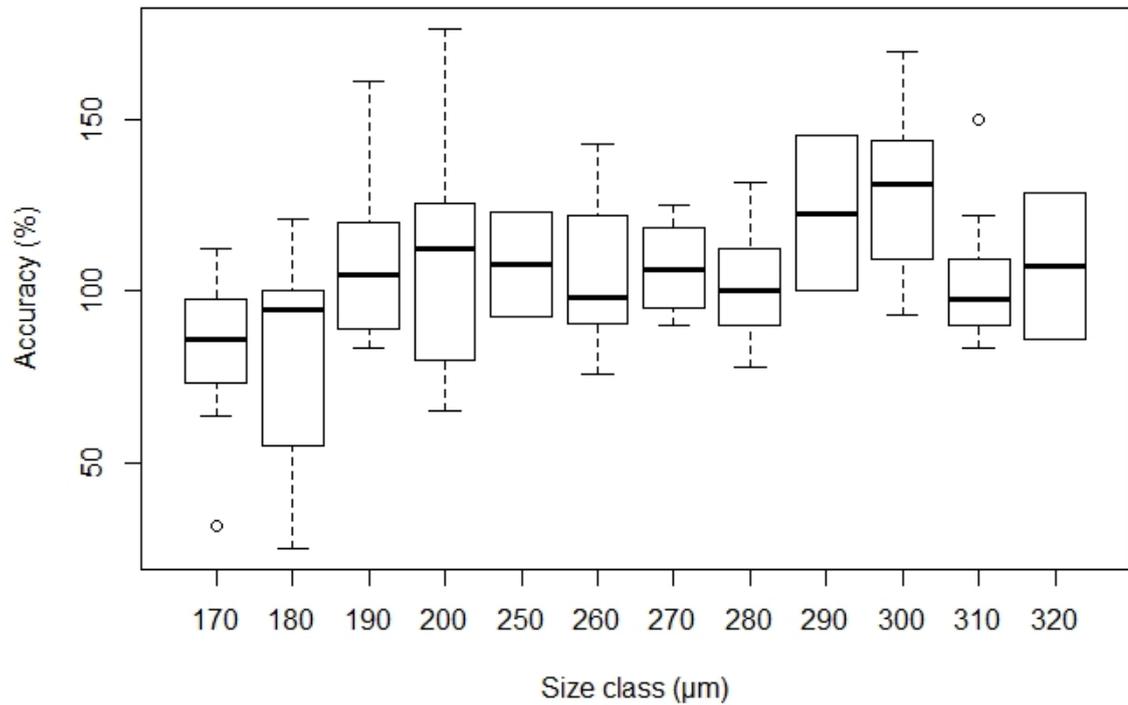


Figure A2-5. Accuracy of USB microscope method in visualising *O. edulis* larvae of different size classes (Formula 9). Values <100% indicate underestimation and values >100% are overestimation of larvae.

Generally, larvae were seen clearly with the USB microscope (e.g. Fig. A2-6), and it was therefore possible to study their behaviour. For instance, most *O. edulis* larvae were at the bottom; but without the addition of the USB microscope image, it would have not become apparent that in ~50% of the cases they were active and at approximately 2 mm from the aquarium floor. The addition of this active behaviour at the bottom leads to a different interpretation than if all larvae were resting passively. Studying behaviour is thus an important addition to vertical distribution, facilitated by this method.

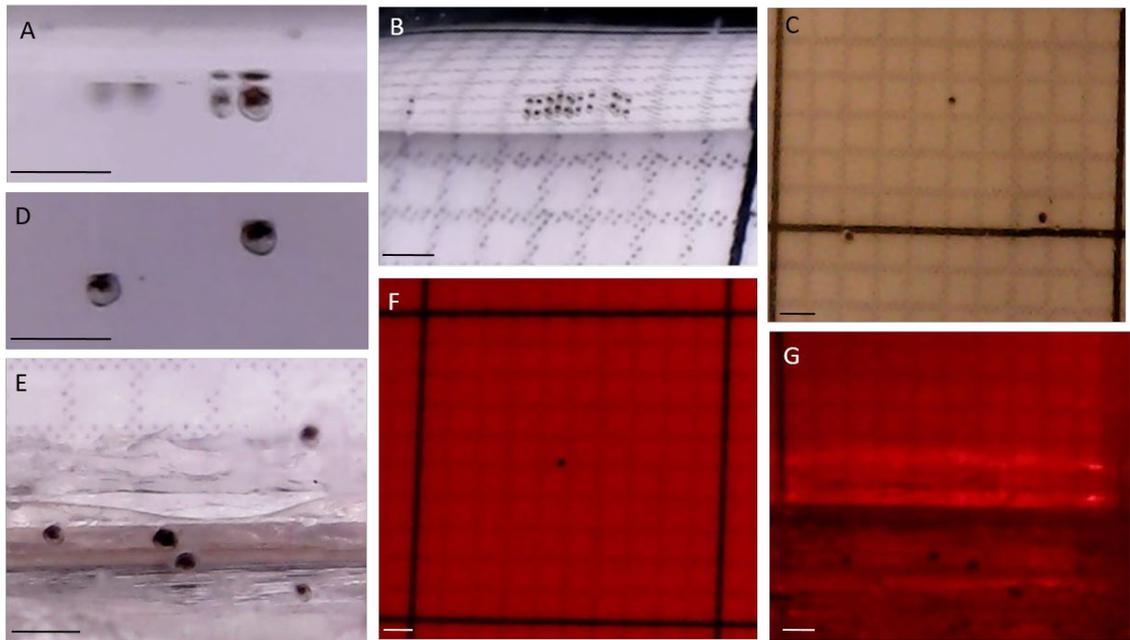


Figure A2-6: *O. edulis* larvae as seen through the USB microscope (video screenshots). (A & B) Larvae swimming along the surface; in (B) larvae are aggregated and their reflection on the water surface is visible (the graticule marking every 1 mm distance appear as dotted lines due to the magnification). (C, D & F) Larvae swimming through the water column, in (C) and (F) larvae appear as black dots with the graticule in the background. (E & G) Larvae at the bottom, with the graticule visible in the background. In (G) four larvae appear as black dots over the bottom. (F & G) Filmed with a red filter, (C) filmed with the LED-light of the USB microscope turned off. Scale bars: 1 mm.

Swimming speeds are an essential component of biophysical models and they can be obtained with this methodology. Although the method may somewhat underestimate swimming speeds due to wall-induced drag ('wall effect') of the narrow aquarium, the difference to swimming speeds in an infinite medium is expected to be small. For instance, the sinking velocity of a barnacle larvae in a column of 3.15 cm radius and 74 cm length was only 0.04 mm/s slower than the actual sinking speed (Miller, 2013). Swimming speeds can be calculated either manually with the background grid, or more accurately with a video analysis program, such as Tracker (Brown, 2018), which is freely available. Programs, such as tracker, can also provide additional information, such as the net-to-gross displacement ratio (NGDR), which is a measure for how twisted or straight larval paths are, with 0 being a perfect loop, and 1 a straight line (Tamburri, Zimmer-Faust and Tamplin, 1992).

There are a few limitations, which have to be taken into consideration, if this method is to be used with another species: (1) the aquarium has to be thin (1-2 cm) and made out of thin glass (~3 mm) for the USB microscope to be able to focus. (2) The focus of the USB

microscope requires fine-tuned adjustment to visualise larvae well, and the focus has to be readjusted when the microscope is moved along the aquarium. (3) Organisms have to be of a minimum size to be able to be visualised with the USB microscope. For *O. edulis* it was possible to visualise the larvae throughout the larval life cycle, because *O. edulis* is a viviparous species and its fertilisation occurs within the mother oyster. When larvae are spawned, they are already $\sim 170 \mu\text{m}$ large, and large enough to be seen with the USB microscope. However, for most other marine invertebrates, fertilisation occurs externally, and larvae are thus initially much smaller. It may therefore not be possible to visualise the earliest life stages of such species (e.g. *Crassostrea gigas*, *Mytilus edulis* and *Pecten maximus*) with this methodology. (4) If temperature is to be held constant or different to room temperature, a temperature-controlled room is needed. This methodology (without modification) cannot be combined with a water bath since the USB microscope is not capable of focusing through the water or double glass.

Despite these limitations, the method has many advantages. It is very cost-effective, and much data can be recorded in a short time and analysed retrospectively. For instance, the average time to record a whole aquarium was 21 min, with most videos being between 16 and 25 min (range: 8-37 min). Next, the distribution of nearly the whole population rather than just a sample can be quantified, and any organism larger than the threshold ($\sim 150 \mu\text{m}$) can be visualised. Finally, a wide array of data (distribution, behaviour and speeds) can be collected with this method; these data supplement each other and, in combination, provides a holistic picture of larvae in the water column.

Appendix 3. Supplementary material for chapter 2

Table A3-1. Observed proportion of larvae per variable and position. Bottom and surface are the bottom and top 1 cm of the aquarium respectively; column is remaining water column (48 cm length).

Factor	Level	Bottom	Column	Surface
Size class (μm)	170	0.83	0.11	0.06
	180	0.56	0.23	0.21
	190	0.70	0.18	0.12
	200	0.42	0.27	0.31
	250	0.64	0.36	0.00
	260	0.56	0.39	0.05
	270	0.63	0.36	0.02
	280	0.70	0.26	0.04
	290	0.52	0.48	0.00
	300	0.63	0.35	0.02
	310	0.68	0.30	0.02
	320	0.87	0.13	0.00
Food	Food	0.67	0.23	0.09
	No Food	0.58	0.31	0.11
Light	Light	0.60	0.28	0.12
	Dark	0.67	0.26	0.07
Temperature ($^{\circ}\text{C}$)	13	0.65	0.26	0.09
	25	0.57	0.30	0.13

Table A3-2: Predicted proportion of larvae at each position (surface, column and bottom) given their size class and the presence food. Values calculated from ordinal logistic regression estimates.

Size class (μm)	Food	Bottom	Column	Surface
170	Yes	0.88	0.10	0.02
	No	0.77	0.19	0.04
180	Yes	0.63	0.29	0.08
	No	0.44	0.40	0.15
190	Yes	0.63	0.29	0.08
	No	0.44	0.40	0.15
200	Yes	0.42	0.41	0.16
	No	0.25	0.45	0.30
250	Yes	0.61	0.31	0.09
	No	0.42	0.41	0.17
260	Yes	0.69	0.25	0.06
	No	0.51	0.37	0.12
270	Yes	0.77	0.19	0.04
	No	0.60	0.31	0.09
280	Yes	0.80	0.17	0.04
	No	0.64	0.28	0.07
290	Yes	0.57	0.33	0.10
	No	0.38	0.43	0.19
300	Yes	0.70	0.24	0.06
	No	0.52	0.36	0.12
310	Yes	0.78	0.18	0.04
	No	0.62	0.30	0.08
320	Yes	0.93	0.06	0.01
	No	0.87	0.11	0.02

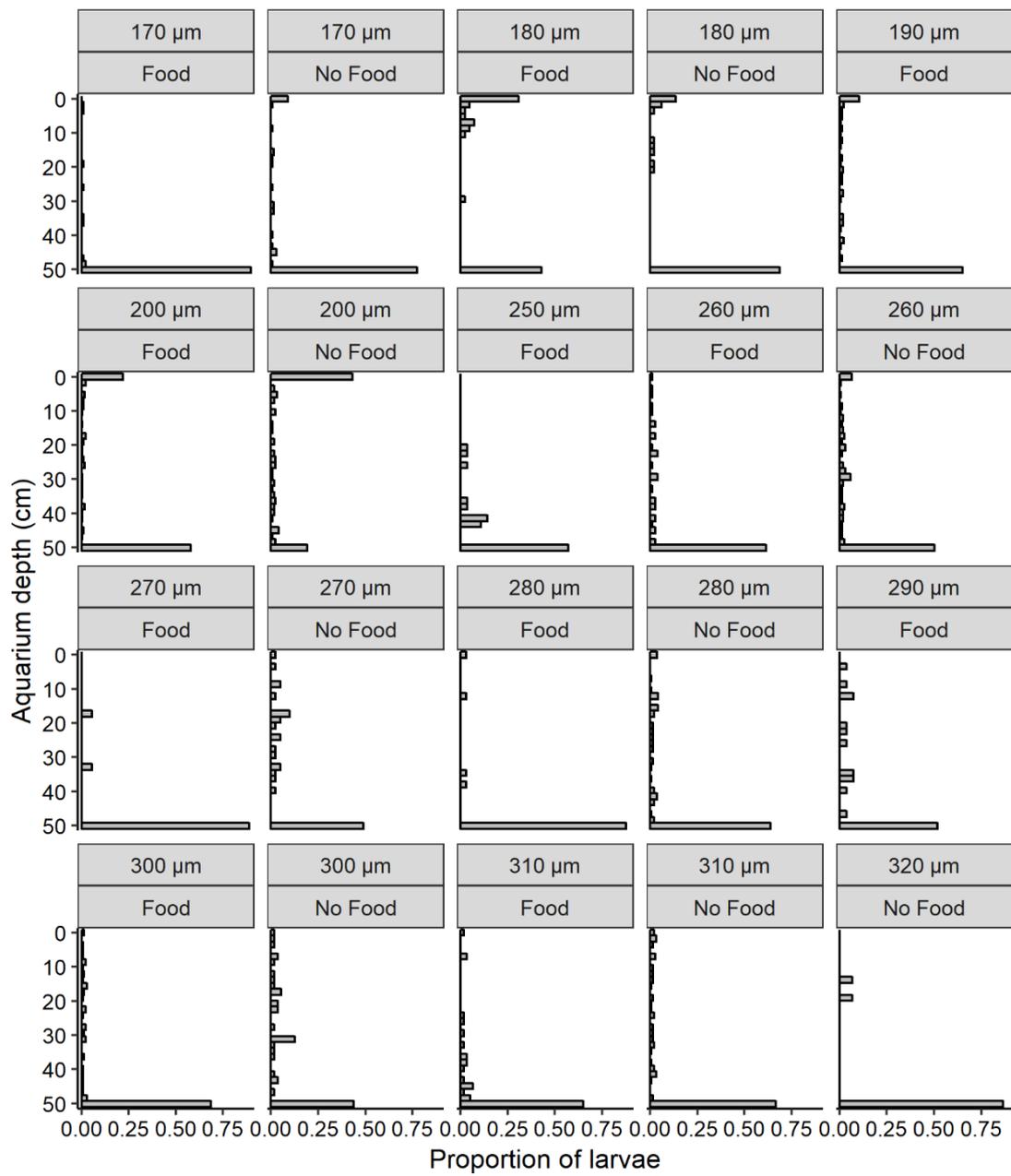


Figure A3-1. Observed vertical distribution of larvae by size class and food.

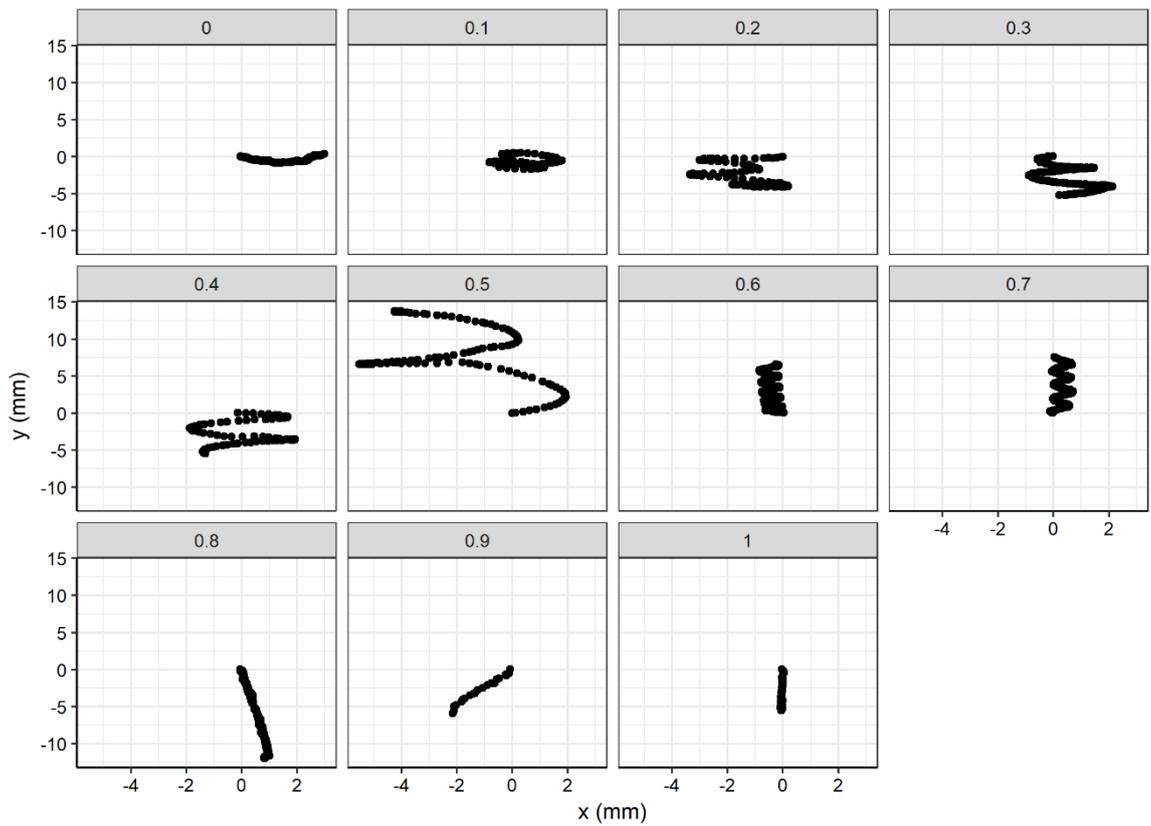


Figure A3-2: Example of larvae tracks along an x- and y-axis representing net-to-gross displacement (NGDR) values from 0 to 1

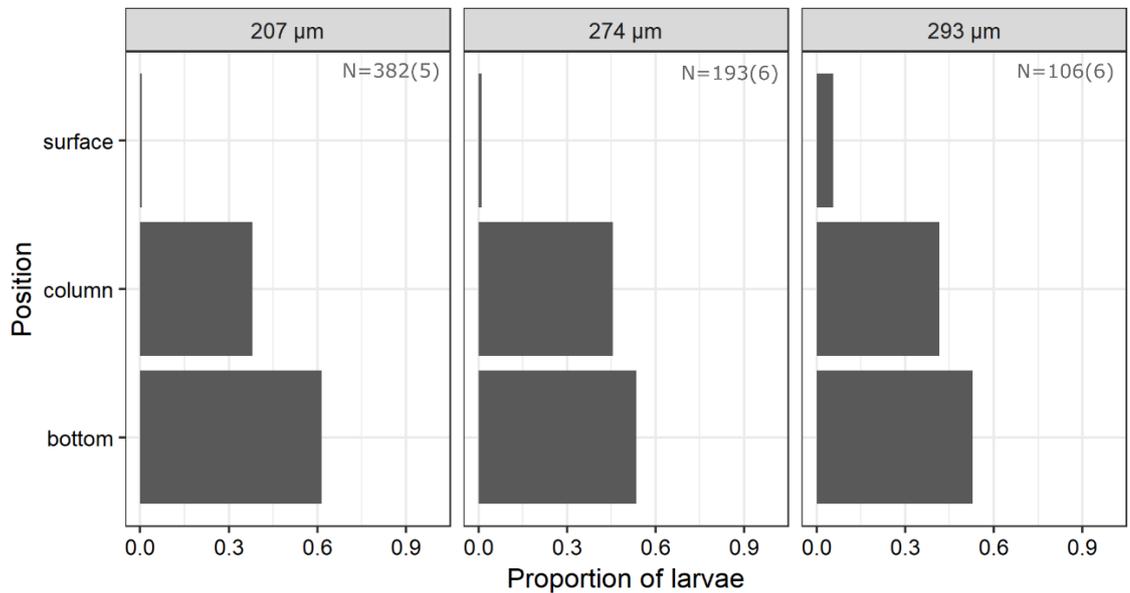


Figure A3-3. Vertical distribution of larvae of three size classes (mean size indicated above each plot) in a preliminary laboratory experiment in Ardtoe (west coast of Scotland). N is number of observed larvae, with aquarium replicates indicated in brackets.

Appendix 4. Supplementary material for chapter 3

Table A3-1. 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 observation [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 A3-2. Log-rank test results evaluating if the settlement distribution differed between treatments. N=188 (4 observations deleted due to missing event times). $\chi^2=297$, df=7, $p < 0.001$

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

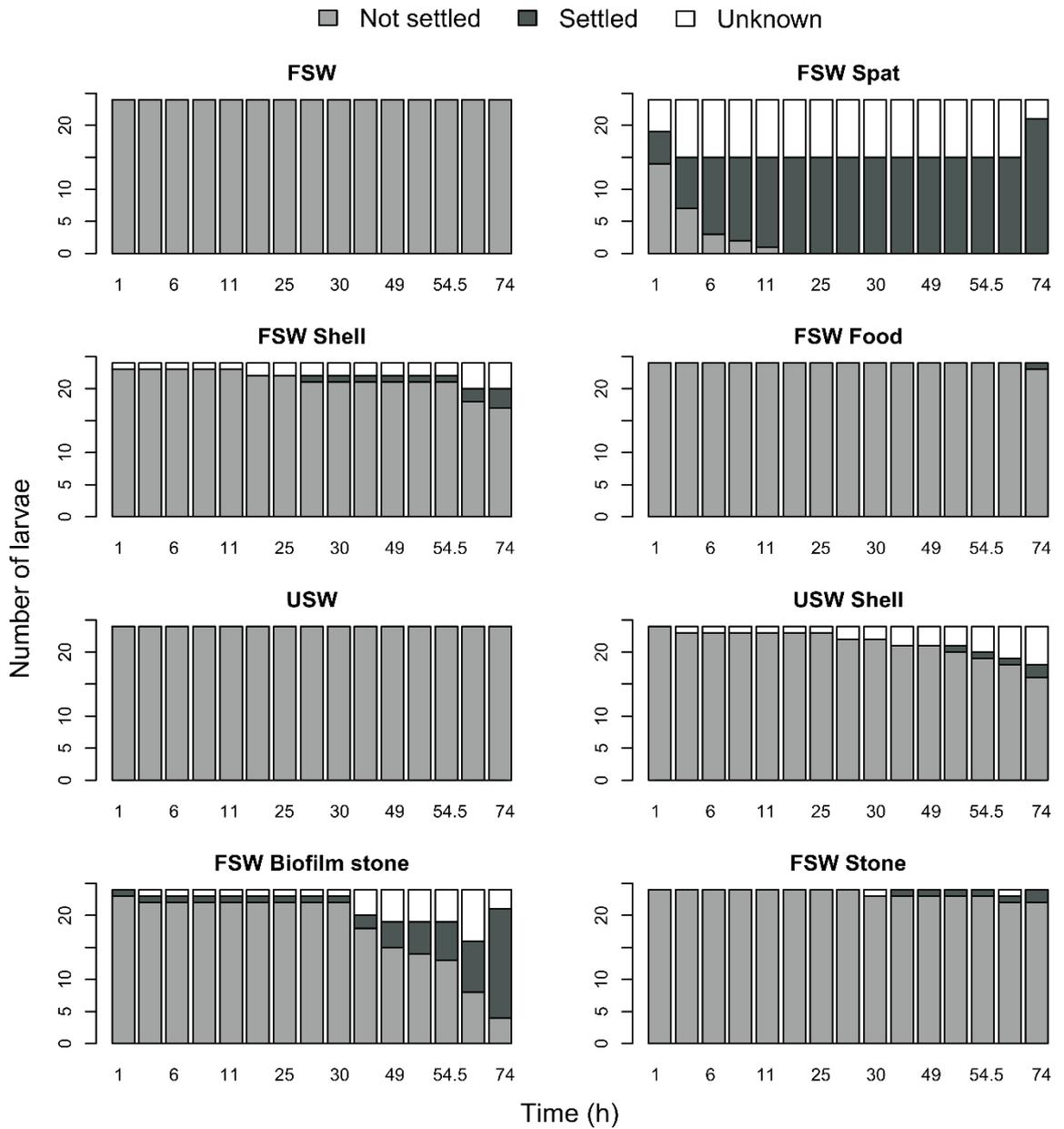


Figure A3-1. Number of larvae settled, not settled and not observed per hour and treatment. At the end of the experiment (74h) all treatment media were inspected for larvae that may have settled on surfaces not visible during the experiment.

Appendix 5. Morphological stages of *O. edulis* larvae

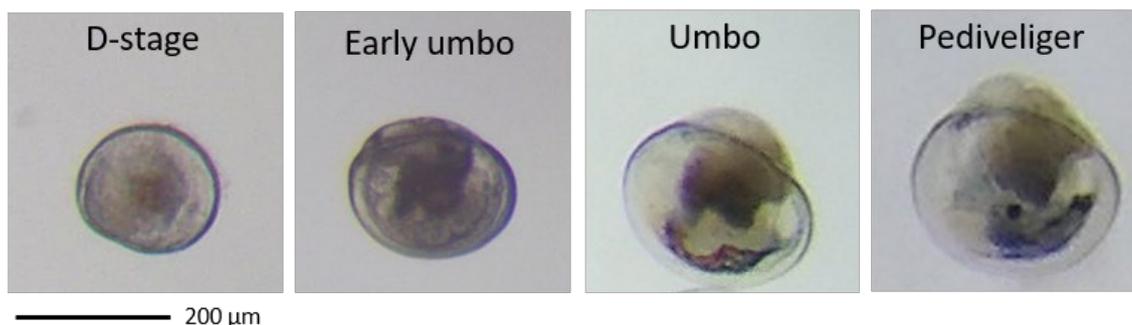


Figure A5-1. Larval development stages of *O. edulis* (stages after Helm, 2004; Acarli and Lok, 2009; photos: Ana Rodriguez-Perez).

Table A5-1. Description of *O. edulis* stages, adapted after Acarli and Lok (2009)

Stage	Description
D-stage	When newly released from the pallial cavity of the adult oysters. During this stage, larvae are semi-transparent and they are slightly 'D' shaped.
Early umbo	The umbo (the top of each half of the shells) becomes slightly oval and the shape of the larvae resembles more a ball. The inside of the larvae is slightly less transparent.
Umbo	The umbo is fully developed and protrudes distinctively. The inside becomes darker.
Pediveliger	In addition to a fully developed umbo, larvae have an eyespot and foot. The eyespot is distinctly visible on both sides of their shell, whilst the foot is generally inside their shell and therefore only visible when protruded during settlement searching behaviour. In this stage, larvae are ready to settle and metamorphose to a young spat.

Table A5-2. Summary of mean \pm sd (μm) *O. edulis* larval size by stage. Table presents data from chapter 2 (vertical distribution) and chapter 4 (development with temperature).

Stage	Chapter 2	Chapter 4 – 25°C	Chapter 4 – 18°C	Chapter 4 – 13°C
D-stage	177.71 (\pm 10.1)	178.60 (\pm 6.5)	176.33 (\pm 3.7)	178.43 (\pm 4.4)
D-stage/ early umbo	201.41 (\pm 3.8)	NA	189.34 (\pm 2.3)	188.68 (\pm 1.3)
Early umbo	NA	215.45 (\pm 11.1)	214.92 (\pm 13.2)	206.60 (\pm 9.6)
Early umbo & umbo	255.73 (\pm 1.5)	NA	218.42 (\pm NA)	224.19 (\pm 6.5)
Umbo	272.67 (\pm 13.6)	264.78 (\pm 16.2)	269.96 (\pm 13.1)	235.29 (\pm 9.8)
Umbo & pediveliger (1:1)	290.86 (\pm 15.9)	NA	NA	NA
Pediveliger (>50%)	307.97 (\pm 7.9)	285.64 (\pm 3.9)	288.66 (\pm 4.6)	NA

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