# Early colonization by marine ecosystem engineers: settlement and metamorphosis of the Pacific oyster (*Magallana gigas*) in a complex sensory landscape of chemical, tactile and sound cues

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# Glossary

**Alarm cue** - Chemical signal released by injured conspecifics that can be perceived as a warning to other conspecific organisms.

**Attachment (for bivalve larvae)** - The stage during which a larva uses its foot to temporarily fix itself to a substrate, but has not yet completed metamorphosis.

**Attachment point theory** - The theory that marine larvae prefer surfaces with maximum contact points relative to their body size.

**Benthic boundary layer** - The layer of water flow immediately above the seabed, where friction between the moving water and substrates on the seabed cause a reduction in water flow velocity.

**Biofilm** - A microbial layer that develops on submerged surfaces, composed of colonizing bacteria, algae, fungi, and protozoa. Biofilms continue to develop over time as these microorganisms interact and produce an extracellular polymeric substance (EPS) matrix.

**Bioreceptive concrete** - Engineered concrete designed to encourage colonization by marine life, often through surface texture or chemistry modifications.

**Biphasic** - A life history with two distinct phases: a pelagic, free-swimming larval stage and a benthic, bottom-dwelling juvenile/adult stage.

**Broadcast spawning** - Reproductive strategy in which eggs and sperm are released into the water column for external fertilization. Spawning is often synchronized by environmental cues (e.g., temperature, salinity, lunar cycles, photoperiod, or currents) and often cascade once some individuals begin.

**Brooding species** - Species of invertebrates (for example oysters from the genus *Ostrea)* where larvae are internally fertilized and retained within the female until they are released.

**Chemoreception** - The physiological process by which organisms detect and respond to chemical stimuli.

**Cilia** - Hair-like cells found on larvae, cilia are used for larvae movement, feeding, and sensing.

**Competency (in larvae)** -The developmental stage, in bivalves this is the pediveliger stage, where a larva can recognize suitable habitat, attach, and initiate metamorphosis.

**Conspecific** - of the same species.

**Cue (for invertebrate larvae)** - A cue is a physical or chemical signal that larvae can interpret as an indicator of habitat suitability. Cues may originate from a wide range of sources and can elicit positive or negative responses. Unlike general environmental factors that merely create favorable conditions for survival, cues actively inform or guide larval behavior, such as navigation settlement, or metamorphosis.

**Dive bombing (for larvae)** - Rapid downward swimming bursts used by larvae to reach the deeper waters. In diving acceleration of the larvae is greater than passive sinking.

**Dissipation rate (ε)** - A measure of how quickly turbulent energy is lost.

**Essential fatty acids (EFA)** - long-chain polyunsaturated fatty acids, essential for animals that must be obtained from food. EFAs can be used as indicators of food quality in plankton and biofilms.

**Extracellular polymeric substances (EPS) -** Product of biofilms which increase as the biofilm matures, and the microbial species of the biofilm interact among themselves and their environment. The EPS is mainly composed of proteins, polysaccharides, and lipids and acts as a protection and glue for the biofilm cells.

**Eyespot** - a light-sensing organ that forms in bivalves in their pediveliger stage.

**Filter feeding** - the feeding strategy used by many marine invertebrates, including mussels and oysters, in which suspended particles such as plankton, detritus, or organic matter are removed from the water column to be used as food. Both adults and larvae employ this feeding strategy. In larvae, the velum is the main feeding organ is used to trap food, cilia on the velum moves to produce currents in which to trap microalgae and move it to the mouth. In adults, gills are the primary organ, which filter suspended particles, which are then transported to the labial palps surrounding the mouth. Bivalves are also capable of selective feeding, as unwanted particles can be sorted out by the palps and expelled as pseudofaeces.

**Geotaxis** – Movement of an organism in response to gravity.

**Glycoprotein (in context of biofilms)** – A protein molecule covalently bonded to one or more carbohydrate chains which is a component of the biofilm EPS and contributes to cell adhesion, and structural stability.

**Helical swimming** - A downward spiral swimming pattern observed in some larvae species including oysters.

**Heterospecific** - Belonging to a different species.

**Kairomone** - A chemical cue released by one species that is detected by another species and used to the receiver's advantage. Commonly associated with predator cues.

**Hertz (Hz)** - Unit of sound frequency.

**Lecithotrophic (larvae)** - Larvae that rely on yolk reserves rather than feeding in the plankton.

**Lux** - Unit measure of light intensity or brightness.

**Mechanosensation -** The biological process by which organisms detect and respond to mechanical stimuli such as touch, pressure, vibration, stretch, or fluid flow.

**Metamorphosis -** (in context of bivalve larvae) The irreversible developmental process in which larvae transform into juveniles this process is typically triggered following settlement and attachment onto a substrate surface. The process of metamorphosis involves structural changes to a larva body including apoptosis of larval tissues and development of adult structures.

**Microtopography** - Small-scale variations in a substrate surface (micrometer to millimeter range).

**Near-infrared (NIR)** - Light wavelengths beyond visible light measured in nanometers (700–1100 nm).

**Non-consumptive predator effects (NCEs)** - Predator cues that change prey behavior without direct predation. These cues are perceived chemically by bivalve larvae.

**Particle motion** - The movement of particles within a medium as a sound wave passes through it. In the case of underwater acoustics, these are water particles.

**Pediveliger** - The last stage of development where marine invertebrate larvae enter the period where they can complete metamorphosis. In bivalve larvae including mussels and oysters this stage is marked by the development of an eyespot and a larval foot.

**Photoperiod** - The daily light- dark cycles.

**Phototaxis** - Movement of an organism in response to light.

**Planktotrophic (larvae)** - Larvae that feed on plankton during their free swimming pelagic period.

**Pseudofeces** - Particles rejected by bivalves during feeding that are expelled without passing through the digestive tract.

**Quorum sensing** - Chemical communication between microorganisms (mainly bacteria) in response to fluctuations in cell-population density, allowing organisms to coordinates behaviors, including biofilm formation.

**Roughness / Rugosity** - Measures of substrate surface topography.

**Settlement (in context of bivalve larvae)** - The process in which competent larvae descend through the water column and attach to a substrate surface. Settlement involves an initial descent that combines passive sinking, and active swimming. Upon reaching the substrate, larvae engage in exploratory crawling, using the sensory capabilities of their foot to detect suitable microhabitats. Throughout settlement, larvae detect environmental cues that influence their behaviors (swimming, attachment).

**Sound pressure level (SPL)** - Measure of sound intensity, expressed in decibels (dB re 1 µPa).

**Soundscape** - The full mixture of sounds in a given location (similar to landscape).

**Statocyst** - A sensory organ found in many invertebrates (including bivalves) that detects gravity, orientation, and movement. Statocysts function similarly to the vertebrate inner ear and consist of a fluid-filled cavity lined with sensory hair cells containing statoliths (hard particles). As the animal's position changes, the statolith shifts, stimulating the sensory cells and allowing the organism to detect its orientation.

**Trophic -** Relating to feeding and nutrition.

**Turbulence** - Chaotic water movement characterized by rapid fluctuations in velocity.

**Ultraviolet (UV)** - Light wavelengths shorter than visible light measured in nanometers (300–400 nm).

**Velum** - Ciliated organ used by bivalve larvae for swimming and feeding.

**Vorticity** - a measure of the rotation of a fluid particle in flow.

**Wettability** - The degree to which a liquid can maintain contact with a solid surface, described as a property of that surface. It is quantified by the contact angle formed between a liquid droplet and the surface.

# Chapter 1: Introduction & Outline for the Thesis

Bivalve mollusks have played a pivotal role in marine ecosystems for hundreds of millions of years, emerging in the fossil record during the Cambrian period around 620 Ma (Dame, 2011; Ponder et al., 2019)

Although early bivalve mollusks likely originated in shallow coastal waters, they have since expanded to inhabit nearly all marine and freshwater environments. Bivalves are found in various ecosystems, including intertidal and shallow subtidal zones, estuaries, marshes, and even deep-sea hydrothermal vents (Dame, 2011). Within the class Bivalvia, some of the most ecologically, economically, and culturally important species are those in the order Mytilidae and Ostreidae, which include all species of true mussels and true oysters (van der Schatte Olivier et al., 2020). Mussels and oysters are sister species, both members of the subclass Pteriomorphia (Lemer et al., 2016). The most notable common traits of these species are their filter-feeding mechanisms and shell formation processes; these traits, in particular, are responsible for the unique position that mussels and oysters play in the ecosystems they reside, helping create and sustain unique marine reef environments. Mussels and oysters differ in several key traits. As adults mussel shells are generally thinner, elongated, and smooth, whereas oyster shells are thicker, irregular, and rough (Gosling., 2008). Mussels attach themselves to surfaces using byssal threads and can move their position slightly throughout their life (Bayne, 1964a). In contrast oysters undergo a single, irreversible metamorphosis, permanently fixing themselves to one location on a hard substrate (Bonar et al., 1990; Tamburri et al., 2008).

**Bivalves as ecosystem engineers**

Mussels and oysters have been designated the role of ecosystem engineers, a term used to describe a species that modulates the resources available to other species through a physical state change in the environment they occupy (Jones et al., 1994 Grabowski & Peterson., 2007; Borthagaray & Carranza., 2007). In their natural state, both mussels and oysters form reefs, although in the case of mussels these are more often referred to as ‘beds’.These reefs are composed of individuals aggregated together, creating densely

packed 3D biogenic habitats (Ysebaert et al., 2019). The presence of these reefs alters the physical, biological, and chemical functioning of their ecosystem. The physical structure of reefs creates habitats for other species by providing new hard substrates for the attachment of sessile species, and refuge from predators to others (Ysebaert et al., 2019). The attraction of new species creates feeding grounds for other species, and bivalves themselves are key prey for a number of other species, including fish, mammals, crustaceans, and birds (zu Ermgassen et al., 2016; Bateman & Bishop., 2017; Craeymeersch & Jansen., 2019). Bivalves filter water, controlling the transport of particles and solutes in the benthic environment, which can stabilize sediments and also increase the light penetration into coastal areas, which in turn affects benthic macroalgal communities (Karlson et al., 2016). The accumulation of organic particles through biodeposition by bivalves enhances the surrounding sediments, increasing nitrogen fixation and removal rates (zu Ermgassen et al., 2016).

In addition to ecosystem benefits aiding the sustainment of a unique and biodiverse ecosystem, bivalve reefs have been highlighted for their potential to be used in nature-based solutions for coastal management, as the physical structure of the reefs can reduce harsh wave actions that contribute to coastal erosion (de Paiva et al., 2018). Bivalves are also extensively farmed, accounting for about 14% of global marine production (Wijsman et al., 2019), with mussels and oysters among the most economically profitable species (Rees et al., 2010; van der Schatte et al., 2020). These species are both harvested from wild populations and deliberately cultured in hatcheries or through mixed hatchery wild grow-out approaches (Helm et al., 2004). Currently, the majority of marine bivalve production (89%) comes from aquaculture, compared to 11% from wild harvests (Wijsman et al., 2019). Overall, the ecological and economic importance of bivalves and their reef structures is considerable, underscoring the need to better understand their ecology and the processes required to sustain healthy reef systems.

**Distribution of mussels and oysters**

Mussels and oysters are found worldwide, with a strong dominance in temperate and subtropical regions, occurring on every continent except Antarctica (Beck et al., 2011; Kasoar et al., 2015). Both groups inhabit intertidal and subtidal zones, with species-specific preferences. In addition to forming established reefs and beds, they can also be found on hard substrates such as rocky shores and man-made coastal marine structures (Bulleri & Chapman., 2010; de Gibert et al., 2012).

Globally, mussel and oyster reefs have sharply declined from their historical distributions, and the current extent of remaining populations is difficult to assess (Beck et al., 2011; Kasoar et al., 2015). Kasoar et al. (2015) reviewed available evidence and found documented reefs in North America, Europe, and Oceania, with fewer reports from Asia and Africa, though this could reflect limited sampling rather than true absence. Some of the best-studied bivalve species for their role as reefs builders include oysters such as *Ostrea edulis* (European flat oyster), *Magallana gigas* (Pacific oyster), *Crassostrea virginica* (eastern oyster), *Ostrea lurida* (Olympia oyster), and *Ostrea angasi* (Australian flat oyster), as well as mussels such as *Mytilus edulis* (blue mussel), *Mytilus galloprovincialis* (Mediterranean mussel), *Mytilus californianus* (California mussel), and *Perna canaliculus* (green-lipped mussel) (Smith et al., 2006; Beck et al., 2011; de Paoli et al., 2015; Howie et al., 2021; Sea et al., 2022).

**Larval life history**

*Spawning*

The life cycle of most marine invertebrates, including mussels and oysters, is biphasic, starting in a pelagic larval stage before metamorphosing into adults (Bayne., 2017). Mussels and oysters are predominantly broadcast spawners, and adults release both male and female gametes in the water column, where larvae will remain feeding on phytoplankton until they reach their pediveliger stage and are ready to settle (Gosling et al., 2008) (see Figure 1.1). There are a few deviations from this form of reproduction in the order Mytilidae and Ostreidae, most notably, oysters from the genus *Ostrea* are brooding species, and male gametes are released into the water column, but fertilization of female gametes takes place internally, where larvae remain for a few weeks before beginning their pelagic period (Gray et al., 2019). *Ostrea* larvae employ a mixed feeding strategy and are lecithotrophic during brooding, then planktotrophic after release (Gray et al., 2019). These early life strategies of *Ostrea* remain important for future life stages, as the proximity of opposite sex individuals will be especially important for these species.

*Planktonic*

The length of time that bivalve larvae spend free-living typically lasts from a few days to a few weeks (depending on the species). While in their dispersal phase, larvae have two main functions: feed and avoid predators. Bivalve larvae therefore use light and gravity as cues to keep closer to the plankton-rich surface waters. As larvae approach settlement competency, these cues switch, and larvae move away from surface waters towards the benthos (Thorson., 1946; Kennedy., 1996). While larvae are planktonic, water currents

and local turbulence are primarily responsible for their transport. As larvae near competency and descend the water column, the influence of water flow conditions changes. As larvae approach the seabed, they enter the lower regions of the benthic boundary layer, including the viscous and logarithmic layers. In these areas, turbulence decreases and flow becomes less intense (Crimaldi et al., 2002; Reidenbach & Hume., 2013). These conditions allow chemical signals from environmental settlement cues to concentrate rather than rapidly disperse, allowing larvae to detect these cues (Reidenbach & Hume., 2013).

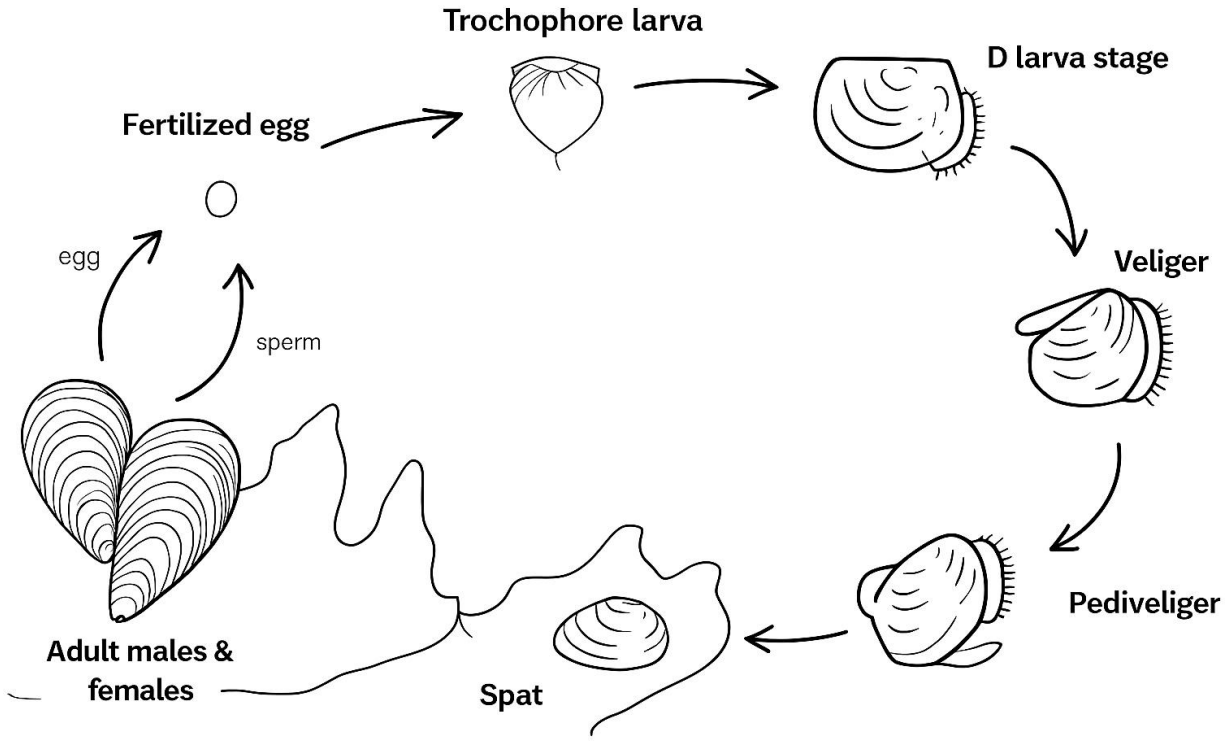


Figure 1.1. Following fertilization, mussel and oyster larvae undergo rapid cell division to reach their trochophore stage, at which point bivalves are ciliated, free-swimming larvae. The trochophore stage is short-lived, transitioning typically within the first 24 hours to D-stage larvae (species dependent), marked by the development of a calcium carbonate shell. Following this, larvae enter their veliger stage, characterized by the formation of the velum, an organ used for swimming and feeding on plankton. Finally, larvae become competent to settle and enter their pediveliger stage, during which they develop a more complex sensory system, a functional foot, and an eye spot, allowing the larva to detect environmental cues and suitable hard substrates for metamorphosis.

*Settlement and Metamorphosis*

For most marine invertebrates that undergo settlement including bivalves, there are two distinct phases: first, a searching/settlement phase, during which cues are used to locate appropriate sites. Followed by metamorphosis, which is an irreversible developmental process involving apoptosis of larval structures and the differentiation of juvenile/adult tissues (Hadfield., 2000; Hadfield & Paul., 2001). During settlement, bivalve larvae descend through the water column using a combination of passive sinking, active swimming, and diving via their ciliated velum and helical swimming movements (Mileikovsky., 1973; Wheeler et al., 2015). Upon reaching the substrate, they begin exploratory behavior, crawling along the surface using the sensory capabilities of their larval foot to detect suitable microhabitats for metamorphosis (Hadfield & Paul., 2001). Each stage of this process is guided by environmental cues, to which larvae have evolved complex reactions.

It is likely that both settlement and metamorphosis are regulated by independent neuroendocrine pathways, but specific characterization of these processes in bivalves is not yet well understood (Joyce & Vogeler., 2018). In the literature, this entire process from initiation of larval descent to the substratum to the attachment by their larval foot and following metamorphosis is sometimes referred to solely as “settlement” (Rodriguez et al., 1993).

**Importance of settlement cues**

A biphasic life cycle allows these species the chance to drift from their location of origin, ensuring that reefs continue to expand and promote genetic diversity for future populations. However, the larval period is also where the populations see the most mortality, with the vast majority of larvae spawned dying before they can settle (Pechenik., 1999). Early research demonstrated that predation represents a major cause of mortality during early developmental stages, estimates of larval mortality are highly variable, between 2% and 100% of the population , making the “decision” of where and when to settle and if then to complete metamorphosis one of great consequence (Young & Chia., 1987; Rumrill., 1990; Morgan., 2020; Allan et al., 1976).

To maximize the chances of survival and continuation of the species, larvae have evolved sophisticated mechanisms to perceive ideal (or nonideal) environments. In early research, invertebrate larvae were thought to be passive particles, with their final settlement location dictated by the currents on which they were carried (Carrier et al., 2018). However, the continued study of larval ecology has revealed that these

settlement locations are the result of evolved responses to signals or cues associated with preferred habitats, which guide larvae to species-specific locations optimal for their survival. Evidence of this is the discrepancy between larval supply and settlement, while the two are inevitably related (no larvae, no settlement), a greater supply of larvae does not always result in increased settlement. In reviewing this decoupling, Pineda et al. (2010) found that the behavior of larvae, their response to settlement and metamorphic cues, mainly accounts for these differences, suggesting that the behavior of the larvae is just as important to survival as supply, if not more so.

Invertebrates can use the same or different cues for settlement and metamorphosis, but identifying which cues trigger which response is not thoroughly understood. While cues associated with substrates (such as substrate surface microtopography) cannot initiate settlement, if cues experienced while larvae are in the water column (such as sound) prior to attachment to substrates, which are known to induce settlement, also work to initiate metamorphosis is not known for the vast number of cues and species. How cues are utilized and for what step of the process is not consistent in all larvae or all cue origins, even among evolutionarily similar species; therefore, it is a difficult task to extrapolate conclusions made from one species.

**Settlement optimization under a complex sensory landscape**

Larvae use a range of cues for an accurate discernment of their environment originating from both chemical and physical sources, but the identity of these cues is highly species-specific, with some larvae utilizing only a few or one cue while some utilize a combination of many environmentalcues (Hadfield., & Paul 2001). The study of environment-larvae relationships has been well characterized in terms of reactions to individual cues, but overall, the strategies larvae use to integrate cue information multimodally are lacking. Specifically there is a need for more assessments of the impact of environmental signals in a dynamic marine environment, where larvae will likely experience multiple sources of information simultaneously (see figure 1.2).

It has been considered that invertebrate larvae may respond to different cues in a hierarchical way, first employing broader cues such as turbulence, salinity, and light on a meter-to-kilometer scale and then utilizing cues which would be experienced at smaller scale, (conspecific chemical signals, trophic cues), and finally, cues located on the substrate (biofilms, substrate topography, chemical cues from substrates)

(Hodin et al., 2015; 2018). Responses to cues may also be stage-dependent, where cue sensitivity shifts depending on when it is most useful to larvae (Bishop et al., 2006). These theories, however, need elucidation by examination across different taxa and cue type.

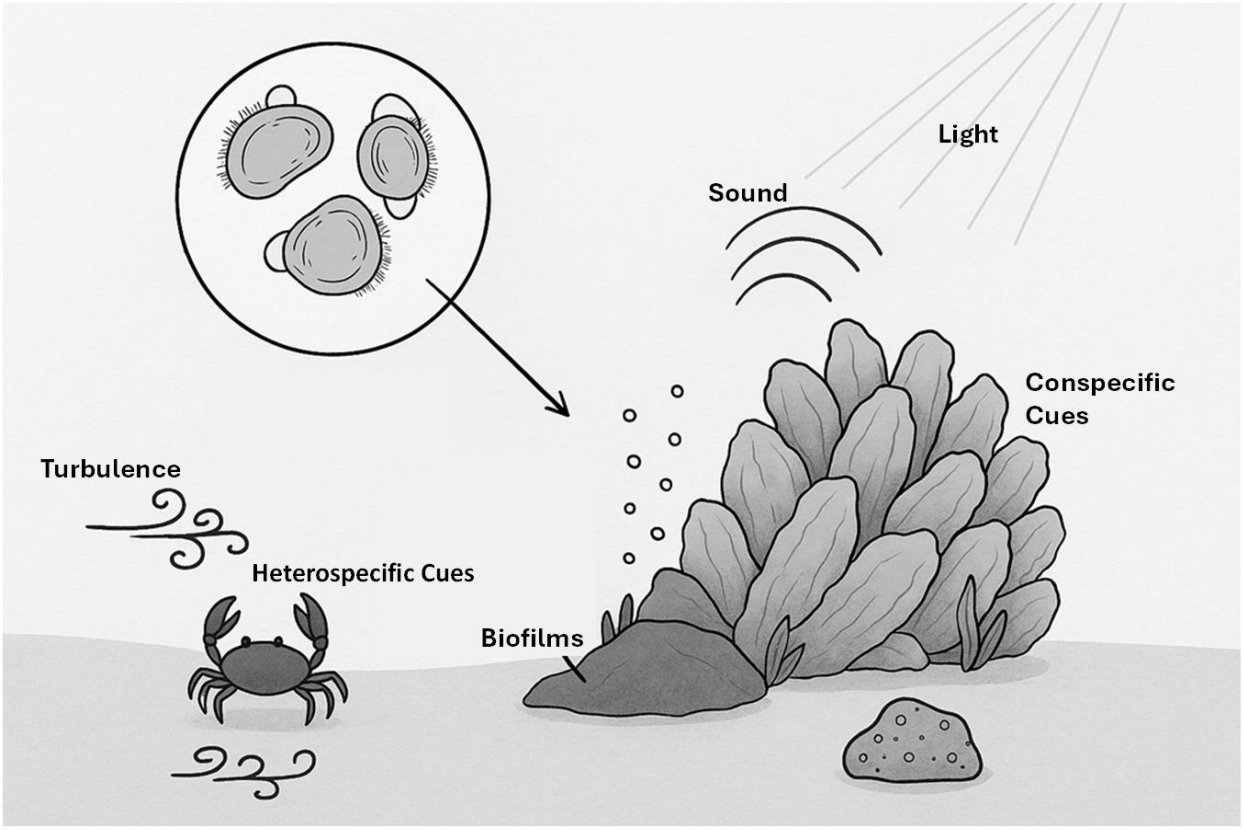


Figure 1.2 Representation of the cue landscapes that bivalve larvae may encounter during settlement and metamorphosis.

**Mussel and oyster global decline and reef restoration**

Bivalve reef disappearance has been well-documented and extensively studied worldwide (Beck et al., 2011; zu Ermgassen et al., 2012; Gillies et al., 2018). While bivalve reefs were once widespread along temperate and tropical coastlines, reefs have declined dramatically due to overharvesting and destructive fishing practices between the 1700s and early 1900s, reducing their habitats today to an estimated 15% of their historical extent (Beck et al., 2011; zu Ermgassen et al., 2012; Gillies et al., 2018; Herbert et al., 2016).

While the intense fishing pressures that originally wiped out these populations have eased, this alone has not led to large-scale reef recovery. It's been suggested that the continued dredge harvesting not only removed live oysters but also stripped away the shell base, leaving behind little hard substrate for new oysters to settle on (Howie & Bishop, 2021). Beyond the loss of hard substrate, the absence of reefs also means the loss of conspecific cues, such as reef sounds, localized turbulence, and chemical signals, that would attract new larval recruits. Even if larvae are present in the water, recruitment may remain low without these habitat-associated cues.

The United Nations has acknowledged the importance of ecosystem recovery by declaring 2021–2030 the Decade on Ecosystem Restoration and including marine and coastal habitat restoration as a target in its Sustainable Development Goals (Stewart-Sinclair et al., 2020; Howie & Bishop., 2021). Reconstruction of lost reefs and mitigation of future losses is at the forefront of conservation goals, with efforts now focused on developing effective restoration techniques (Beck et al., 2011; zu Ermgassen et al., 2020; Howie & Bishop., 2021).

**Reef Restoration**

Bivalve reefs consist of tens of thousands or even millions of individuals. Unlike trees, we cannot just plant them individually. Successful recruitment is critical for restoring these reefs. Some of the earliest restoration work began with the Eastern oyster (*Crassostrea virginica*) along the Atlantic coast of the United States (see Billion Oyster Project (https://www.billionoysterproject.org)) In Europe, restoration has focused mainly on the native flat oyster (*Ostrea edulis*) and the blue mussel (*Mytilus edulis*).

Bivalve reef restoration initiatives have been launched across several European countries, including England, Scotland, Wales, Germany, the Netherlands, France, and Belgium. Projects like Reefcovery (https://www.blauwecluster.be/projecten/reefcovery), UNITED (H2020) (https://www.h2020united.eu), ULTFARMS (Horizon Europe) (https://ultfarms.eu), and BLUE CONNECT (Horizon Europe) (https://submariner-network.eu/blue-connect), exemplify this growing movement. Common restoration interventions include translocating or seeding adult oysters, deploying shell, gravel, or artificial structures to provide hard substrate, and using suspended or seabed cages for predator protection. While many projects have reported successes, attracting new recruits to artificial reefs remains one of the challenges.

**The Pacific Oyster**

The invasive Pacific oyster (*Magallana gigas*, formerly *Crassostrea gigas*) has also received some attention, not necessarily for conservation, but pragmatic acknowledgment that restored reef sites with native species are likely to include invasive individuals as well (Pogoda et al., 2019). The Pacific oyster *M. gigas* is a species native to temperate estuarine and coastal waters from latitudes roughly between 30°N and 48°N (Japan, Korea, China), (FAO 2016). The species is considered highly invasive in some areas of the world including and has been introduced and is established in almost all continents aside from its native range: Europe, Africa, North America, South America, and Oceania (Herbert et al., 2016). The Pacific oyster is a complicated case of an invasive species. Total elimination of *M. gigas* is neither feasible or even recommended, due to its high population densities, large-scale eradication or control efforts for this species are likely to harm other components of the native ecosystem, such as the native mussel beds, with which *M.gigas* are often found together (Reise et al. 2005). Additionally, continued settlement from remaining oysters is likely even with extensive species removal (Herbert et al., 2016). While co-existing individuals of *O.edulis* and *M.gigas* are found, there is a threat of competition from the oysters on a long-term scale. In the Dutch Wadden Sea, for example, long-term monitoring showed a decline in mussel bed coverage concurrent with oyster expansion (Troost., 2010). And mixed reefs tend to be dominated by oysters (Markert et al., 2010; Reise et al., 2005). Due to the loss of mussel beds, it has been suggested that *M. gigas* reefs may partly compensate in an ecologically relevant role, for the loss of these beds by providing similar ecosystem functions, including filtration, habitat structure, erosion control, and coastal protection (Borsje et al., 2011; Herbert et al., 2012).

Between invasive and native oysters in Europe, *C. gigas* and *O. edulis,* the tendency of the oysters to occupy different vertical zones reduces potential competition. *O. edulis* is sublittoral, and *C. gigas* intertidal. In the Adriatic Sea, a stable coexistence between *O. edulis* and *C. gigas* has been reported, yet even though they occupy different niches, *O. edulis* remains rare and *M. gigas* prolific (Stagličić et al., 2020). Overall, while this invasive species is considered a pest, it is also not likely to be eradicated, especially in Europe, where populations are firmly established. Embracing, or at least anticipating the presence of this species when designing restoration projects for native mussels and oysters is necessary.

Especially as predicted climate change-related habitat shifts will favor *M. gigas* in the long term (King et al., 2021).

### ***Magallana gigas*** **biology**

*Magallana gigas* is the study organism utilized for the experimental component of this thesis. *M. gigas* are broadcast spawner and spawning is largely controlled by water temperatures and food availability, *M.gigas* becomes mature and induces spawning when water temperatures range between 17–21C in a single spawning event (Lango-Reynoso et al., 2006). Following fertilization, D-larvae form within approximately 48 hours (Helm et al., 2004; personal observation). The larvae are planktotrophic and typically spend 2–3 weeks in the water column feeding on microalgae before reaching the pediveliger stage see figure 1.3 (Helm et al., 2004). *M. gigas* are generalists and respond to a range of environmental cues during settlement and metamorphosis. Following metamorphosis, the juveniles continue to grow and can reach adulthood (defined by reproductive maturity) within 1–2 years (Helm et al., 2004).

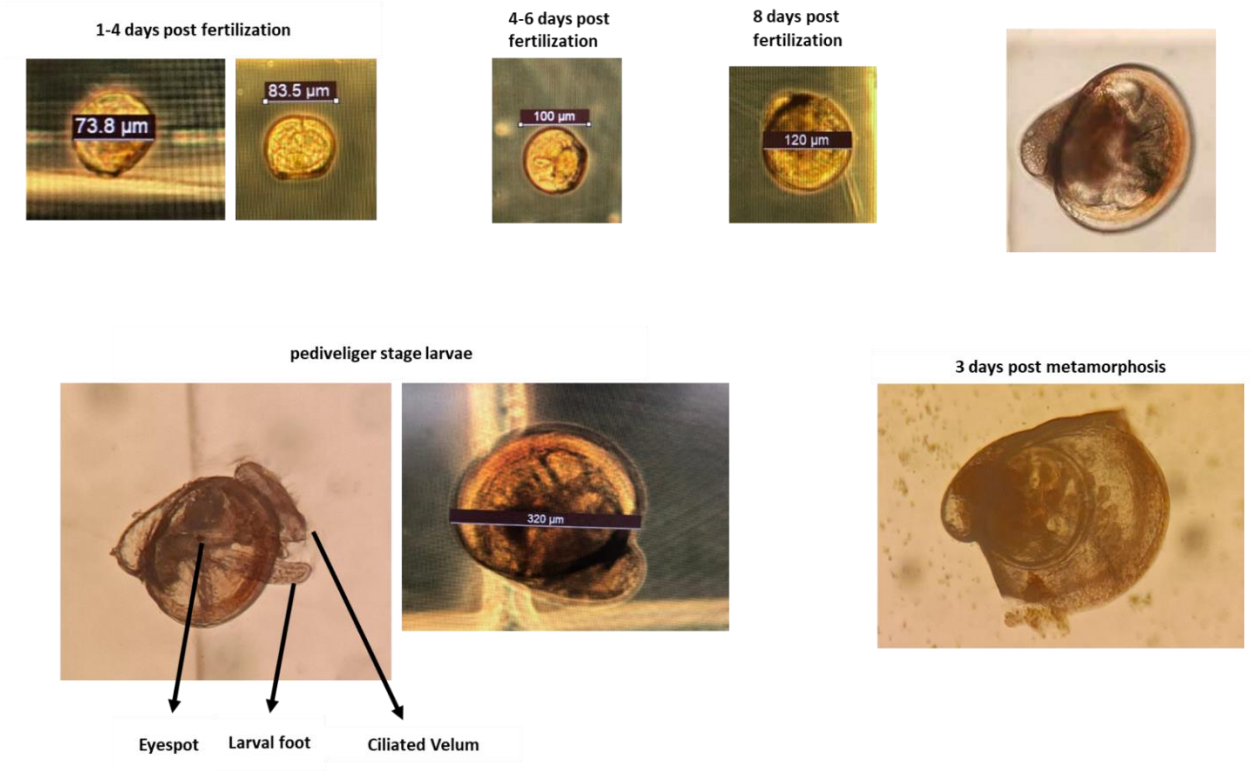


Figure 1.3. Photos from different developmental stages of larvae of the pacific oyster (*Magallana gigas*).

# Thesis chapters

Within this thesis, I present four chapters describing the results of a literature review, five laboratory behavioral experiments, and one field experiment. While the literature review focuses on all reef-building bivalve species, laboratory and field experiments use the oyster *Magallana gigas* as the study species. The overall aim of the PhD was to highlight key gaps in the literature regarding bivalve larval settlement and metamorphosis, and to contribute toward filling these gaps, specifically focusing on the mechanisms by which larvae respond to scenarios involving multiple cues. I further focused my work on two understudied types of cues, acoustic-associated sounds and microtopography, both of which are relevant for incorporating into artificial reef restoration design.

## Chapter 2

In the first chapter, I conducted a literature review aimed at providing a comprehensive and accessible overview of the current research on the environmental cues that reef-building bivalves, specifically mussels and oysters, use during settlement and metamorphosis. By compiling this knowledge into a single source, my goal was to create a resource that can serve both experts and non-experts seeking to better understand these fundamental life history processes. This review also helped identify key gaps in the literature. While existing reviews often focus on a single cue type, offering valuable insights into these mechanisms across a broad range of invertebrates, this approach can obscure species-specific or family-specific differences. By synthesizing studies covering all cue types (chemical + physical, and waterborne + substrate-bound) for reef-building bivalves, my aim was to provide a clearer picture of the complexity of larval-environment interactions and to support interests in fundamental larval ecology and applied ecology alike. The gaps identified in this review helped in shaping the direction of the subsequent chapters,

particularly in highlighting which cues remain poorly understood in these species and the limited knowledge of how multiple, and sometimes conflicting, cues interact to influence larval behavior. In the following chapters, I present foundational experimental work aimed at addressing these understudied areas.

# Chapter 3

In the second chapter, I investigate how larvae respond to multiple and potentially conflicting chemical cues acting in different environmental contexts, originating from conspecific adults, predators, and biofilms. While research suggests that invertebrate larvae are capable of processing multiple types of information simultaneously (Morello & Yund et al., 2016; Birch & Plachetzki, 2023), a mechanistic understanding of how larvae integrate these cues during settlement and metamorphosis, remains limited especially bivalve larvae, as these species are not often used as model organisms (likely due to their small size and long developmental periods). In this chapter, I aim to explore how larval behavioral responses shift when additional cues are introduced, with a particular focus on the interaction between waterborne and substrate-bound cues.

# Chapter 4

In chapter three, we explore how cues from marine soundscapes can influence larvae settlement and metamorphosis. The ability of larvae to use acoustic cues during settlement is a relatively recent discovery in larval ecology research. Evidence that larvae can use reef sounds to locate suitable habitats or conspecifics remains limited to a small number of species (Solé et al., 2023; Pysanczyn et al., 2023). In this chapter, we explore whether our study species, *Magallana gigas*, responds to reef-associated sounds. Additionally, given that coastal soundscapes are increasingly dominated by anthropogenic noise (Bittencourt et al., 2020; Wilson et al., 2023), we examine how vessel noise affects settlement rates and whether it has the potential to modulate the influence of reef sounds.

# Chapter 5

Finally, the last chapter of this thesis focuses on another poorly understood cue in bivalve larval settlement: substrate surface microtopography. At the final stage of the settlement process, after larvae have attached to a substrate, specific topographical features can play a crucial role in initiating metamorphosis. While some studies have explored how certain features may inhibit metamorphosis, there is still little consensus on which microtopographical characteristics promote optimal settlement. In real-world environments, these surface features are not encountered in isolation; they naturally interact with biofilm development. Therefore, in this chapter, we also examine how microtopography and biofilm growth interact to influence larval settlement and metamorphosis.

# Chapter 2: Settlement and Metamorphic Cues Influencing Reef-Building Bivalves (Mytilidae & Ostreidae)

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# Abstract

Reef-building bivalves (families Ostreidae and Mytilidae) are ecosystem engineers of high ecological and economic importance, capable of creating biogenic structures that enhance biodiversity, filter water, and stabilize sediments. Over the past century, these reefs have declined globally, prompting extensive restoration initiatives. Additionally, these species are major contributors to global aquaculture, being cultivated extensively for food production. The success of both wild and cultivated populations depends, in part, on understanding how their pelagic larvae select suitable habitats for settlement and metamorphosis, processes that ultimately determine population establishment and reef persistence.

We performed a systematic literature review to synthesize current knowledge on the environmental cues utilized by larvae during their competency period to guide settlement and metamorphosis. This review encompasses all studies that experimentally quantified settlement and/or metamorphic responses in true mussels and oysters. Identified cues include physical cues from light, hydrodynamics, sound, and substrate topography, and chemical cues from conspecifics, biofilms, algae, predators, and other heterospecific interactions.

# Introduction

### Importance of reef-building bivalves: ecological and economic

Reef-building bivalves are some of the most ecologically as well as economically important species in the marine environment, with habitats distributed across the globe (Figure 2.1). While bivalves as a whole occupy many habitats, dense reefs and beds are only formed by brackish and marine species from the families Mytilidae, encompassing all true mussels, and Ostreidae, encompassing all true oysters (Beck et al. 2011; Huang et al., 2023; Richardson et al. 2022). Mussels and oysters are considered to be ecosystem engineers due to their ability to modify their environment in both a biological and abiotic way, modulating the habitat quality and availability of resources to other species, increasing biodiversity (Jones et al.,1994; Ysebaert et al., 2019).

Despite their important role within the ecosystem, bivalve reefs have suffered major losses worldwide compared to their historical abundances (Beck et al., 2011; Fariñas-Franco et al., 2018; Pogoda et al., 2019; Sampaio et al., 2022; Thurstan et al., 2025). In the past 100 years, it is estimated that there has been an 85% loss of native oyster reef ecosystems globally; their recent abundances are so low that, as an ecosystem, they are essentially extinct as they cannot play the role they once did (Baggett et al., 2015; Beck., 2011). Human activities are responsible for these losses, in particular the physical impacts from fisheries gear disturbing the sea floor, organic pollution, ocean acidification, and overharvesting (Hall-Spencer et al., 2010; Beck et al., 2011; Sampaio et al., 2022). Future reefs are additionally threatened by temperature rises, and in climate change prediction scenarios, oyster reefs and mussel beds are anticipated to be negatively affected, although the degree of this effect is difficult to calculate (Zippay & Helmuth., 2012).

In addition to their status as ecosystem engineers, many bivalves also hold significant value in global aquaculture, where they are cultivated extensively for human consumption. Bivalves account for approximately 16 million tonnes of aquaculture product per year and are estimated to have a market value of $23.9 billion (Van der Schatte Olivier et al., 2020). Nonprovisioning services from bivalves, including pearls, shell, nutrient removal, etc, are also economically important, with one assessment concluding that the global, non-food bivalve aquaculture services are worth $6.47 billion per annum (Van der Schatte Olivier et al., 2020).

The ecological and economic interest of these species extends to their removal as well. Presence of bivalves can be considered a nuisance, invasive species, such as Pacific oysters (*Magallana gigas*), which were introduced through aquaculture and have since established widespread populations across Europe and the Americas, can outcompete native species and alter local ecosystems (Escapa, et al., 2004; Schmidt et al., 2008). Additionally, bivalves contribute to biofouling on marine infrastructure; their accumulation on surfaces can clog systems, increase hydrodynamic drag on ships, and reduce vessel maneuverability, which imposes significant costs on marine industry (Chambers et al., 2006).

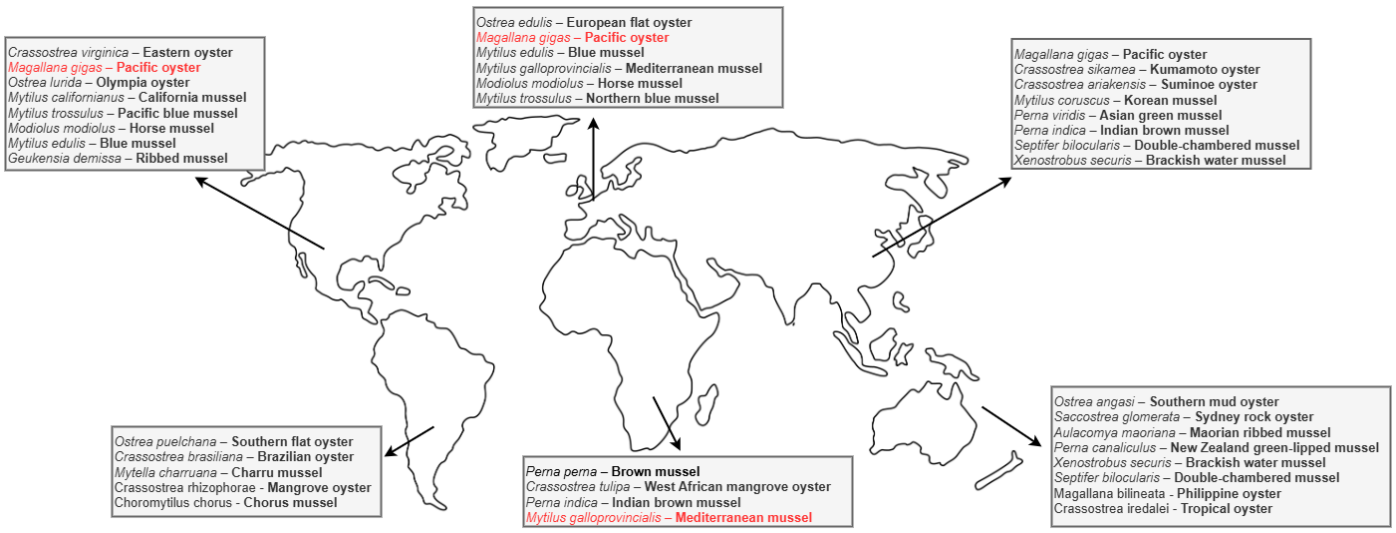


Figure 2.1. Species of oyster and mussel that were identified in this review, grouped by continent. Lists include both native and some invasive species that have established populations. Invasive species are highlighted in red.

**Reproduction and larval settlement**

In order to support these environmental and commercial goals, whether this be to encourage or remove reef-building bivalves, a solid scientific foundation of their life history and reproductive strategies must be understood. Mussels and oysters have distinct life histories. Like most aquatic invertebrates, they begin with a pelagic larval stage ending in metamorphosis, where a permanent settlement location is established (Widdows., 1991; Bayne., 2017). This period, during which larvae settle and metamorphose, is controlled by complex biotic and abiotic interactions, which determine the final location of metamorphosed larvae. At the beginning of the 20th century, the popular opinion in invertebrate ecology was that larval distribution was mainly influenced by larval supply and patterns of prevailing currents (Colman, 1933; Petersen, 1913). However, advances in research revealed that larvae were not completely at the mercy of hydrodynamic forces and instead actively participate in substrate site selection (Pineda et al., 2010). Pineda et al. (2010) found that the behavior of larvae, including their response to environmental cues, mainly accounts for the differences in larval supply and presence of recruits, suggesting that this behavior of the larvae is just as important to survival as supply, if not more so. Further exemplifying this point, Morgan (2014) reviewed larval transport in highly advective coastal upwelling systems and found that, contrary to predictions from passive dispersal models, larvae are not typically transported far offshore. Instead, species-specific larval behaviors mediate transport, often retaining larvae near coastal

zones and adult populations. Today, a considerable amount of literature has been devoted to the study of these settlement cues, which regulate larval settlement and metamorphosis.

**Settlement vs Metamorphosis**

Settlement and metamorphosis in bivalves are two distinct processes often included together under the term 'settlement' in scientific literature (Abdul Wahab et al., 2023). However, settlement can also purely refer to the movement and attachment of competent larvae from the water column onto the substratum, which immediately precedes metamorphosis. Metamorphosis is instead marked by complex physiological changes, including degeneration of the velum and retraction of the larval foot, which in oysters regresses entirely after cementation; in mussels, the larval foot adapts and attaches to a surface by secretion of byssal threads. Following metamorphosis, these bivalve larvae continue physiological changes, oysters begin secreting calcium carbonate to reinforce their shells, and mussels continue production of byssal threads and shell growth (Baker & Mann 1994). Molluscan shells are composed primarily of calcium carbonate. In true oysters, the shells are almost entirely calcitic (Bai et al., 2023). In contrast, true mussels produce shells with an outer calcitic prismatic layer and an inner aragonitic nacreous layer (Naik & Hayes., 2019).

It remains unclear whether both the process of settlement and metamorphosis in bivalves can be triggered by the same cues or if different and distinct ones are necessary to initiate each of these steps. While it can be fairly certain that cues encountered at the substrate level, such as surface topography, likely initiate metamorphosis alone. In contrast, it is not well understood whether cues detected in the water column, solely trigger the initial descent toward the substrate or also contribute to initiating metamorphosis (Bonar., 1990; Hadfield & Paul., 2001; Bao et al., 2007; Grasso et al., 2011).

**Differences between the settlement strategies of mussels & oysters**

Mussels and oysters differ fundamentally in their settlement strategies. Mussel larvae typically prefer to settle first onto a filamentous substance (filamentous algae in the wild, or the byssal threads of other adult mussels) and then later may go through a secondary settlement where they will detach from the filament and attach more permanently to hard substrates releasing an adhesive plaque and byssal threads in their final metamorphosis (Silvermann & Francisco., 2010). This primary/secondary settlement theory is challenged in the literature, and individuals may repeatedly settle and relocate over time, sometimes

due to factors outside of behavioral choice (hydrodynamic conditions) (Navarrete et al., 2015). It is known that mussels can also change their location slightly throughout their adult life, first settling in small crevices and moving outward as they age (Bayne 1964a). In contrast, oyster larvae, after finding a suitable substrate, will settle and then permanently metamorphose, secreting a cement-like substance from their pedal glands to fix themselves to the substratum (Bonar et al., 1990; Tamburri et al., 2008). Before this permanent fixation, oysters can, at times, attach to a surface with their foot and later release if the substrate is not ideal (Bayne, 2017).

**Cues for bivalve settlement and metamorphosis**

Larvae encounter both physical and chemical cues that influence their behavior in the water column and at the substrate surface. In the water column, such cues can affect swimming behavior, particularly vertical positioning, prompting larvae to descend toward the benthic zone. There, they can detect signals from near-bottom environments and, eventually, from the substrate itself, where final metamorphosis may be triggered by additional cues (Hadfield & Paul., 2001). Physical cues include light, turbulence, and sound in the water column, as well as topographical features once larvae reach the substrate. Chemical cues may originate from conspecifics, or other species interactions, most notably biofilms, and algae. Other heterospecific interactions can come from competitors or predators species.

Bivalve larvae can perceive environmental cues across a range of spatial scales, from the substrate surface to several meters away (Hodin et al., 2018). Cues such as light, gravity, and sound operate over large spatial scales from meters to kilometers, providing broad environmental information that may guide larvae swimming and navigation. Turbulence as a habitat associated cue influences bivalve larvae near reefs at intermediate scales from meters to centimeters. Finally, chemical cues from biofilms, conspecifics, or other biological sources act at very fine scales, from the substrate surface to a few centimeters away. becoming increasingly effective under low-flow conditions where chemical signals can accumulate (Wheeler et al ., 2015; Hodin et al., 2018).

**Detection of cues by chemosensory, photosensory, and mechanosensory sensory systems**

Depending on the cue type, mussels and oysters detect environmental signals through either chemosensory or photosensory, or mechanosensory systems. Chemosensory detection (from chemical

stimuli) in bivalves is hypothesized to be mediated by G-protein-coupled receptors (GPCRs), cell surface sensors on the larval body including their apical organ and larval foot (Yurchenko et al., 2019; Xu et al., 2023). The photosensory ability of the larvae is theorized to be perceived via apical organ and the eyespot developed in pediveliger stage larvae (Kim et al., 2021). Mechanosensory detection in bivalves (the detection of mechanical stimuli, such as vibration, water movement, or substrate texture) is thought to occur via ciliated cells on the larval surface that respond to movement and vibration, or through statocyst receptor systems that contribute to equilibrium and geotaxis and may also detect particle motion (Budelmann, 1992; Solé et al., 2023). Specific receptor systems and signaling pathways underlying sensory detection in bivalve larvae remain largely uncharacterized.

**Goal of the review**

The evolved environment-larvae relationships during larvae settlement and metamorphosis is critical in dictating the location and survival of the adult populations (Pineda et al., 2010). It is immensely valuable to know which habitat selection cues are primarily used by which species, and where information is lacking. There is an extensive amount of literature on settlement and metamorphic cues generally, but species or family-specific reviews are rare. Evidence of extreme interspecies differences for environmental cue utilization, even among evolutionary similar species, makes simplifications of these relationships difficult and extrapolations from one group to another potentially inaccurate.This review covers all known environmentally associated settlement and metamorphic cues for reef-building bivalves, excluding synthetic chemicals used in aquaculture. In this review we define cues only as a physical or chemical signal that larvae can interpret as an indicator of habitat suitability, not environmental factors which might simply create favorable conditions. We included scientific manuscripts if they reported either changes to larval attachment and metamorphosis or influences on the behaviors of larvae in the water column. Larval behaviors in the water column, however, were only reported for larvae during their competency period, and reactions of pre-competent larvae to cues were outside the scope of this review.

# Methods

**Paper selection**

Papers were found using the Web of Science databaseusing a variety of combinations of keywords: Both terms “larvae settlement” and, “larvae recruitment” were used combined with each term from this list; light, sounds, marine noise, acoustic, substrate, topography, microtopography, hydrodynamics, flow, chemical cues, predator, conspecific, adults, gregarious, biofilms, bacteria, diatoms, algae, trophic. These searches were further refined by the terms “mussel” and “oyster” (see figure 2.2).

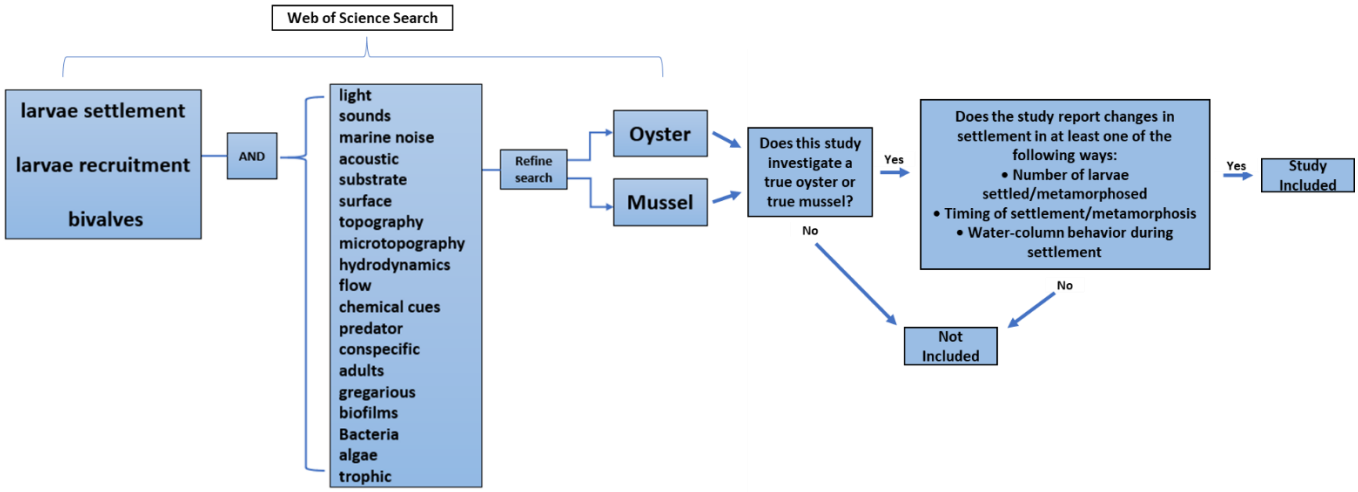


Figure 2.2. Flow chart demonstrating the search terms used to collect papers and the selection process.

Studies included in this review needed to report changes in settlement in at least one of the following ways: (1) the number of larvae settled, (2) settlement timing, (3) water-column behaviors during settlement, or any combination thereof. We excluded studies that did not address at least one of these criteria, for example, those focusing solely on adult behaviors or on larvae after settlement. Modeling studies without direct data collection were also excluded. Eligible papers had to include, either wholly or in part, settlement/metamorphosis experiments conducted in the laboratory or in situ, with larval recruits quantified using settlement collectors in field studies. The literature review was completed in March, 2023. Table 2.1 lists all species covered by the studies reviewed in this paper. In a couple of cases the scientific names of the bivalve species have been updated since the original text publication. These species are referred to as the updated name in this manuscript.

| True Oysters (Family Ostreidae) | True Mussels (Family Mytilidae) |
|---|---|
| ***Magallana gigas*** | *Perna viridis* |
| ***Magallana bilineata*** | *Mytella charruana* |
| ***Ostrea edulis*** | *Perna canaliculus* |
| ***Crassostrea virginica*** | *Perna* |
| ***Crassostrea sikamea*** | *Perna indica* |
| ***Ostrea angasi*** | *Mytilus edulis* |
| ***Crassostrea iredalei*** | *Mytilus galloprovincialis* |
| ***Crassostrea rhizophorae*** | *Mytilus trossulus* |
| ***Crassostrea brasiliana*** | *Mytilus coruscus* |
| ***Crassostrea tulipa*** | *Mytilus californianus* |
| ***Crassostrea ariakensis*** | *Modiolus* |
| ***Saccostrea glomerata*** | *Aulacomya maoriana* |
| ***Ostrea puelchana*** | *Xenostrobus securis* |
| ***Ostrea lurida*** | *Septifer bilocular* |
| | *Geukensia demissa* |
| | *Choromytilus chorus* |

Table 2.1. List of oyster and mussel species involved in the studies collected for this review.

# Findings

**Literature Search**

The results of the literature search, including publication reference, species studied, and cues involved can be found in this public google doc.

**https://docs.google.com/spreadsheets/d/1B8z3AYEkEsHFcGNHsTNf6HHT0m8dOxtuGgi6mkA88fY/edit?usp=sharing**

Overall, 165 papers were included in the literature review. The cues were categorized as either physical or chemical. Physical cues included light, hydrodynamics, topography, and sound. Chemical cues included those from conspecifics, biofilms, algae, and heterospecific interactions, including predators and competitors. Although biofilms and algae are technically heterospecific to bivalves, the abundance of

literature focused on these interactions warranted their classification as separate categories. The papers collected for this literature review show a strong dominance of research focused on a few species of mussels and oysters (see Figure 2.4). This bias is likely driven by the fact that these species are either prioritized for restoration or widely cultivated for aquaculture. Both contexts attract significant scientific attention, resulting in the observed concentration of studies on these particular taxa. This imbalance highlights a research gap in our understanding of settlement and metamorphosis processes across bivalve species generally. There is also a bias for studies which focus on cues from conspecifics and biofilms with these cues also appearing earlier in the timeline of published literature (see figure 2.3 and figure 2.5). In contrast, cues with fewer publications, such as sound, topography, and light, are represented by more recent studies, suggesting that these research gaps are now actively being addressed.

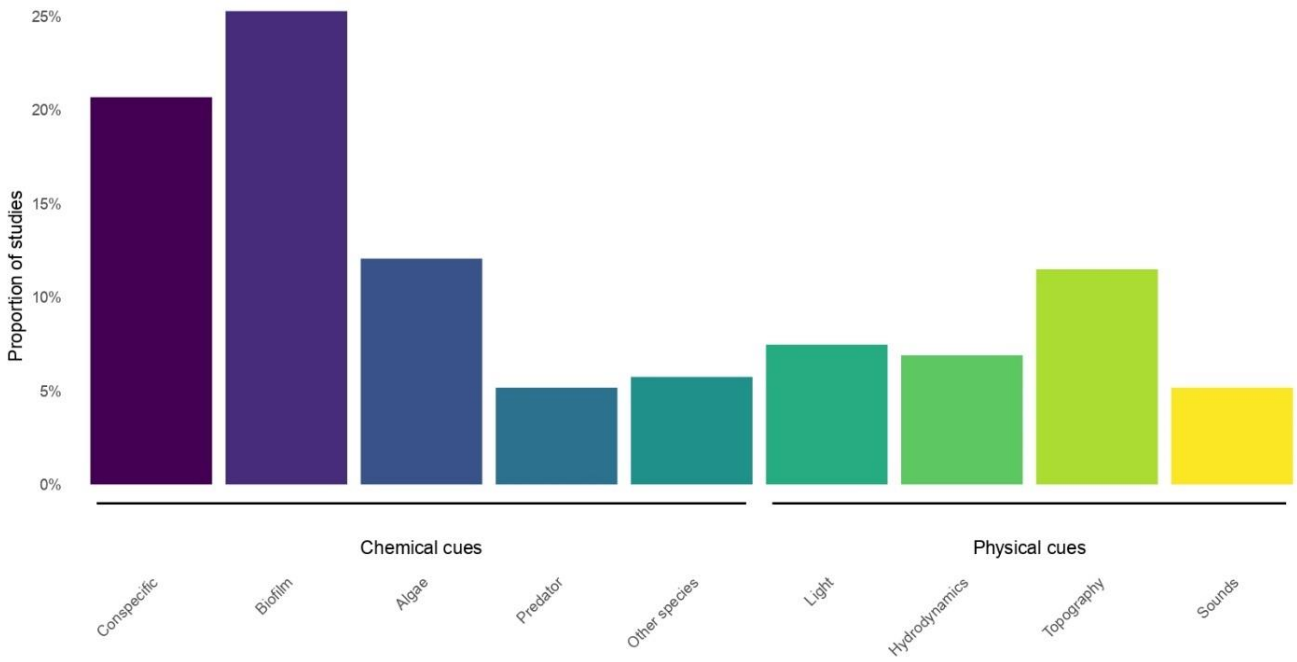


Figure 2.3. Proportion of studies identified in the literature search by cue type, grouped into chemical cues (conspecific, biofilm, algae, predator and other species) and physical cues (light, hydrodynamics, topography, and sound).

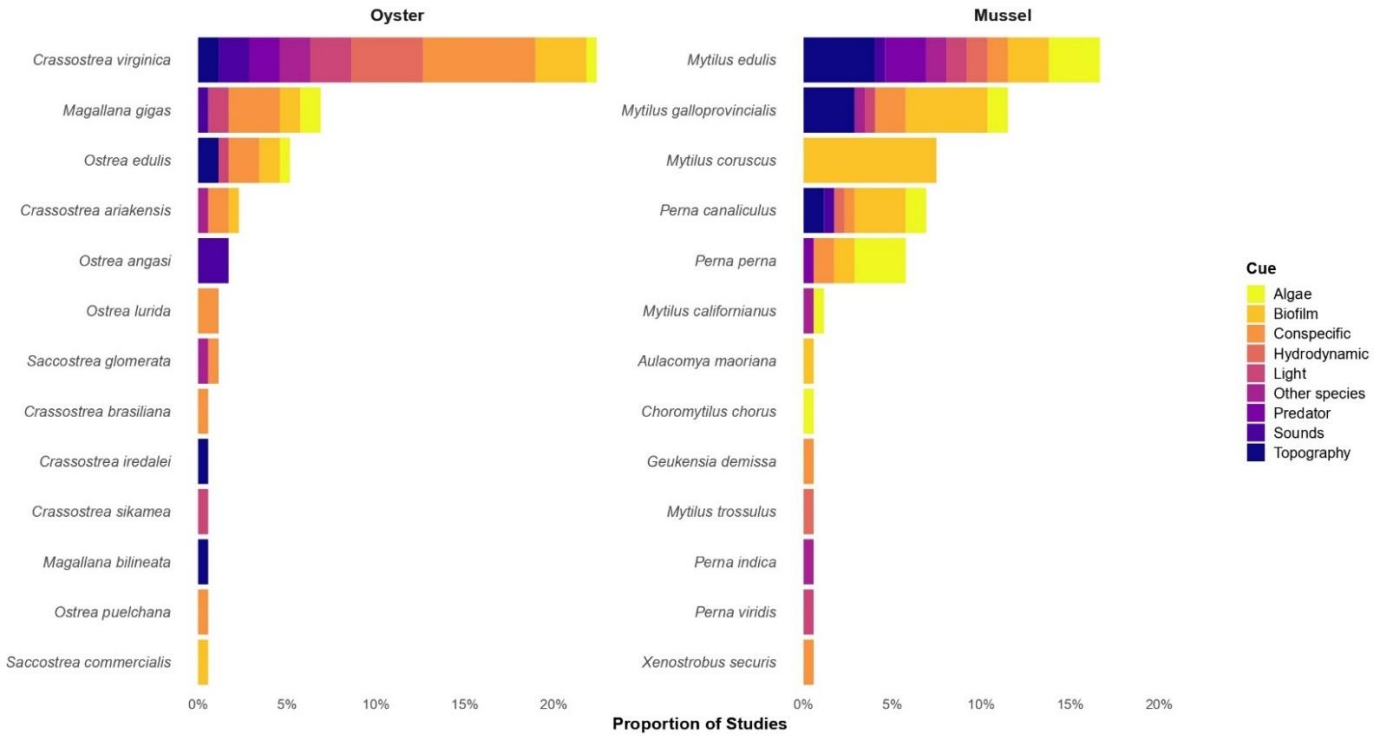


Figure 2.4 Proportion of studies identified in the literature search which investigates each bivalve species and cue type: chemical cues (conspecific, biofilm, algae, predator and other species) and physical cues (light, hydrodynamics, topography, and sound)..

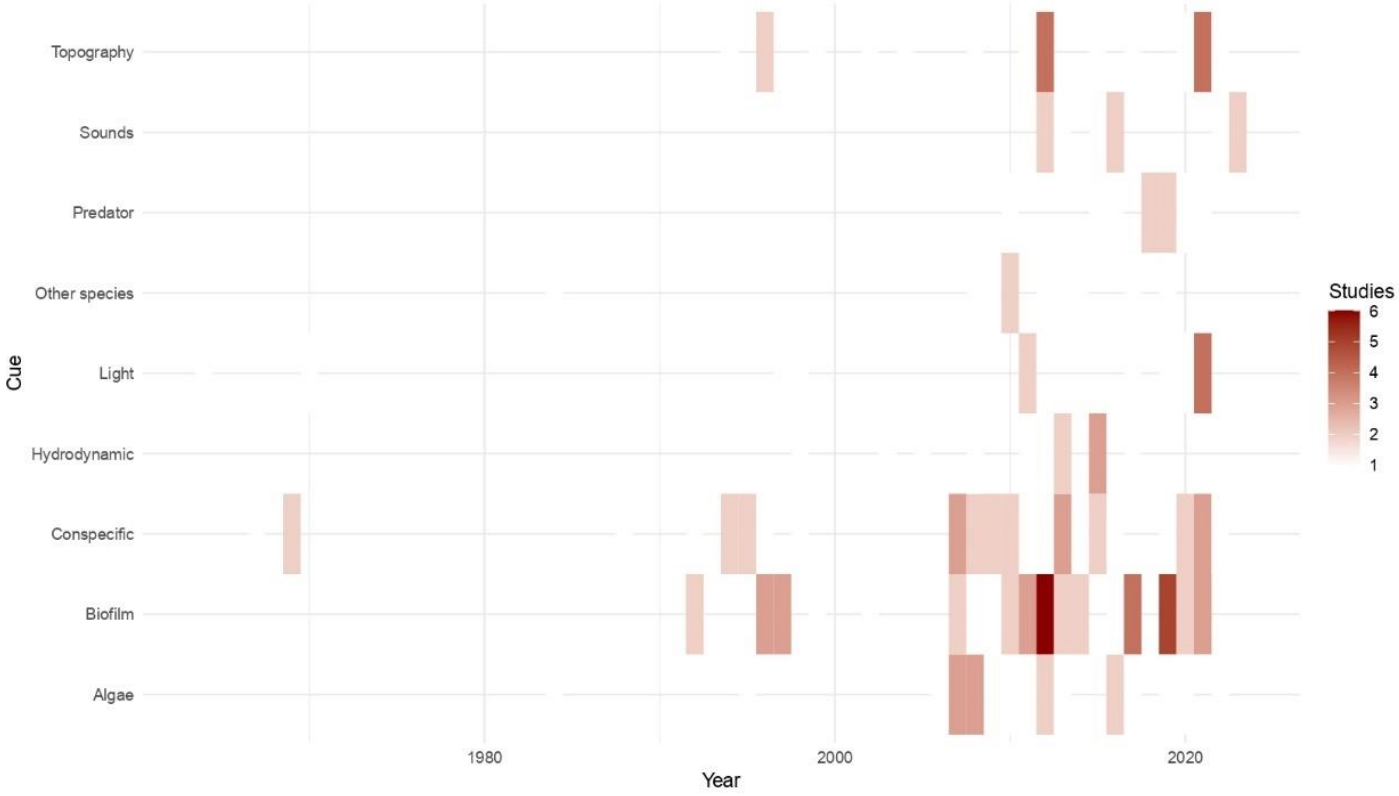


Figure 2.5. Heatmap showing the number of publications per year per cue type: conspecific, biofilm, algae, predator, other species, light, hydrodynamics, topography, and sound.

# Physical cues

## Light

Light is a fundamental environmental signal that regulates numerous biological processes in marine invertebrates. While bivalve larvae lack image-forming vision capabilities, they still perceive light through photoreceptor cells which develop over their larval ontogenetic processes (Cole., 1939; Baker & Mann,1998). Evidence suggests that larvae perceive light stimuli from as early as their trochophore stage, but this detection increases towards the end of their larval period and light sensing capabilities are highest with the development of an "eyespot" in their pediveliger stage (Hasan et al., 2024). It has been theorized that many marine planktotrophic larvae have an ontogenetic switch in their light preferences and young display photopositive preferences, driving them into surface waters to feed, but, as larvae become competent to settle, they transition and instead become more photonegative in order to initiate descent towards the seafloor and substrates in which to settle (Thorson., 1946; Kennedy, 1996). While many studies do confirm this theorized preference for darker environments for settlement in bivalve larvae (Bayne, 1964; Shaw et al., 1970; Baker & Mann., 1998; Siddik & Satheesh., 2021), more complicated relationships might exist between different features of light, such as wavelength, intensity, and photoperiod, and the larval behavioral response.

**Wavelength and Intensity**

Light is an electromagnetic radiation quantified both by wavelength, measured in nm, and intensity, which is expressed as either the total energy of light (including all wavelengths) reaching a surface per unit area measured in W/ $m^2$, or how bright the light appears (illuminance), measured in lux. Both these features determine properties of light and, in turn, elicit specific biological responses for many taxa. Wavelengths between 300nm to 1100nm are particularly important to human and animal biological processes and can be classified into ultraviolet light (300-400nm), visible light (400–700 nm) (determining color), and near-infrared (NIR) 700–1,100 nm. Investigating preference in light wavelength shows that oyster *M. gigas* increased settlement under red (660 nm), near-infrared (735 nm), and similarly oysters *M.gigas* and *C. sikamea* hybrids, increased settlement in red-green light (mix of 700 and 546.1 nm) (Kim et al., 2021; Zhang et al., 2021). In contrast to these preferences, when larvae were given a choice between different light wavelengths, oyster *C. virginica* preferentially settle on the shaded sides of a substrate over white

light (400-700 nm), red light (600-700 nm), green light (450-575nm), and blue light (400-500nm) (Baker and Mann, 1998). Negative responses to certain wavelengths have also been observed including settlement reductions by near ultraviolet (375 nm), white (460, 570 nm) and green (525 nm) light for M. gigas, compared with dark treatments (Kim et al., 2021), and *M. gigas* and *C. sikamea* hybrid larvae decrease settlement and moved directionally away from blue light (435.8 nm) (Zhang et al., 2021).

In addition to the wavelength-specific reactions, it is clear that behavioral reactions to light can also change based on light intensity. While the different experimental methodologies and limited light intensities tested make it hard to set a boundary, light intensities over 80 lux seem disruptive to settlement in both mussel and oysters, with settlement decreasing proportionally as intensity increases (Bayne 1964b; Shaw et al., 1970; Siddik & Satheesh., 2021). In experiments where specific light wavelengths were tested, settlement preferences were again dependent on the intensity of which larvae were exposed (Kim et al., 2021)

Light wavelength and intensity reactions may also work to trigger specific behaviors in the settlement process, for example, some features may trigger directional movement or swimming, and some induce the process of metamorphosis. Competent oyster larvae were shown to move towards green light (546.1 nm) but only increased attachment when red light was also present in addition to the green; red light alone did not increase directional movement, nor did green light increase attachment (Zhang et al., 2021). Additionally, oyster larvae moved towards light intensities of 5–10 lux but away from light above 25 lux (Zhang et al., 2021). Wheeler et al ., (2017) found that specific swimming behaviors of *C. virginica* including helixes and diving, were triggered by light at ~2000 lux, an intensity which was previously identified as disruptive (Kim et al., 2021), however only the proportion of larval helixical swimming and diving were influenced and light induced no change in vertical directional swimming (Wheeler et al ., 2017).

**Photoperiod**

Duration of the day/night cycles is generally an important trigger of biological processes in animals, and highly important for adult bivalves for biological processes including feeding and gamete production (Fabioux et al., 2005; Tran et al., 2011). However, evidence for the photoperiod effect on settlement and metamorphosis in bivalves is thus far contradictory. Mussel *M. galloprovincialis* preferentially settled in 12h light:12 dark photoperiods compared to both 24h light and 24h dark cycles (Carl et al., 2011). In contrast, the same photoperiods (24h light, 12h light:12 dark, and 24h dark) have shown no significant differences in the settlement rate for mussel *P. viridis* (Mero et al., 2019).

**Light vs Geotaxis**

Gravity can also be detected by a number of invertebrates, yet distinguishing between larval responses to light and gravity poses a challenge, as both phototaxis and geotaxis are likely to drive larvae downwards to substrates. In laboratory studies where light can be controlled from gravity, pediveliger larvae of *M. edulis* and *O. edulis* were positively geotactic and move towards the bottoms of tanks, despite no light (Bayne., 1964b; Rodriguez-Perez et al., 2020). The same pattern was seen for *C. virginica* larvae, which settled preferentially on the underside of substrates regardless of lighting conditions (Baker & Mann., 1998). Given this reaction, it is likely that both of these forces represent cues that can be independently perceived by larvae.

Collectively, evidence points to a preference for both mussel and oyster species to settle more readily in the dark, which, as previously stated, can be explained as a larva seeking deeper areas of the water column to find substrates. This driver might explain why larvae avoid blue/green wavelengths. In the ocean, blue and green wavelengths are absorbed least by water and are dominant at deeper depths, yet could still signal that larvae are still in the water column and have not yet reached substrates thus, a high presence of blue/green light could signal a need to continue to keep sinking. However, following this logic, preferences for red and near-infrared wavelengths are surprising, as these wavelengths cannot penetrate into deeper waters and would not provide any indication that larvae are close to the seafloor. An alternative explanation for positive responses to specific wavelengths could be connected to the reflection of different wavelengths from food sources (plankton), but this theory is lacking more evidence.

## Hydrodynamic cues (turbulence cues)

Marine larvae can use the features of local hydrodynamic conditions as more than transport mechanisms, but also to provide information about their environment. Coastal environments are typified by dynamic hydrodynamic conditions and typically experience strong turbulence and higher energy, mainly due to wave actions, tides, currents, and water-seafloor interactions. Within a reef, this complexity increases as the features of the reef create microhabitats that can interact with prevailing flow patterns to induce varying degrees of turbulence, creating hydrodynamically unique environments (Fuch et al., 2015a). Of the cues covered in this paper, hydrodynamic cues have the potential to act on larvae over the largest scale (m to km), potentially impacting the behavior of larvae over large spatial areas. Hydrodynamic

conditions also influence chemical cue detection (Wheeler et al., 2019); however, this section will address the effects of hydrodynamic cues alone.

**Larvae swimming behaviors**

As previously covered, it is typical of bivalve larvae to seek deeper environments when competent, in order to reach substrates on which to settle and complete metamorphosis. To complete this transition, bivalve larvae employ a variety of swimming behaviors by means of their velum, an organ used for both propulsion and feeding. Larvae can sink passively by retracting their velum, or they can actively swim, propelling themselves using coordinated waves generated by rows of concentric cilia on the velum. Both mussel and oyster larvae can either sink passively towards substrates or they can actively adjust both their swimming speed and direction, reaching swimming speeds that surpass normal vertical flow. In response to cues experienced in the water column, larvae can move downward, exerting more effort than passively sinking would require (Wheeler et al., 2015). Oyster, *C. virginica* have been identified to rapidly move themselves downward, a term called 'dive bombing' (Finelli & Wethey., 2003). Wheeler et al., (2015) classified this diving more specifically as a transient response occurring over timescales of approximately one second, where larvae abruptly accelerate downward, reaching speeds up to one cm per second, or approximately fifty body lengths per second. While this diving behavior has been described only for *C. virginica*, it is likely employed by other bivalves as well. While diving or active downward swimming is likely used to reach substrates quickly, larvae may also use helical swimming, or swimming in downward spirals, which is thought to enhance environmental sensing by allowing larvae to sample a larger volume of water (Wheeler et al. 2015). The detection organ for hydrodynamic sensing in larvae was identified to be likely via larvae statocysts rather than cilia by Fuchs et al., (2015b), who exposed oyster larvae to controlled shear flow and solid-body rotation to determine whether strain rate (which deforms cilia) or vorticity (which causes body rotation) triggers the dive response. As vorticity was the only driver of diving behavior, it's likely that statocysts are the main organ used for flow detection.

**Turbulence**

Turbulence can be described as an irregular or chaotic flow pattern in which water moves rapidly with unpredictable velocity fluctuations. Turbulence is classically measured as the Energy Dissipation Rate (ε), which is calculated based on velocity gradients, using models, e.g., $\varepsilon \approx (\Delta u)^3 / L$, where $\Delta u$ is the velocity fluctuation and L is the length scale of flow (Vassilicos., 2015). The bulk of the literature testing responses of bivalves to turbulence indicates that larvae actively induce downward swimming, and eventually

complete metamorphosis in more turbulent waters, with the assumption that an increase in turbulent waters alert larvae to the presence of coastal reef environments (Pernet et al., 2003; Fuchs & DiBacco., 2011; Fuchs et al., 2013; 2015a; 2015b). This preference for turbulence seems to have upper and lower thresholds; below the threshold, larvae would switch from upward swimming to active downward swimming, and above, at the highest turbulence levels, larvae would stop swimming and act as passive particles (Pernet et al., 2003; Fuchs & DiBacco., 2011). Oyster *C. virginica* has been particularly studied for the response of larvae in the water column to turbulence. Fuchs et al. (2013;2015a; 2015b) identified that increases in turbulence would specifically initiate *C. virginica* larvae diving responses, a response assumed to aid settlement by driving larvae towards bottom substrates. Fuchs et al (2015b) found that *C. virginica* would also change their response type depending on the type of flow action. In turbulent waters produced by vertical oscillations, larvae increase diving, but in seiches (standing waves), larvae swim upward. These results were however, contrasted by Wheeler et al. (2013) and Wheeler et al., (2017), who instead found that *C. virginica* displays upward swimming in high turbulence, with downward diving disappearing completely in highly turbulent conditions. Furthermore, by separating different features of turbulence, Wheeler et al., (2015) found that larvae responded to relative acceleration changes, not turbulence intensity alone. The actual acceleration values at which larvae would increase diving varied between turbulence levels. In low turbulence, a small acceleration may be enough to trigger a dive, but in higher turbulence, a stronger acceleration is needed to induce diving. While some evidence remains contradictory, it may be that differences in experimental designs or the availability of other cues could account for the discrepancies observed. It seems likely that different features of turbulent flow can be interpreted by larvae to elicit specific responses. Larvae could respond to turbulence in multiple steps, where first acceleration triggers the initial dive response, and following this, turbulence intensity influences how strongly they continue swimming downward. It is also possible that larvae integrate cues from hydrodynamic conditions with other environmental cues, or even override hydrodynamic cues in the presence of other “more important” cues.

While turbulence in the water column may initiate downward swimming, at the substrate surface larvae actively avoid high shear areas, regions where water flow changes rapidly over short distances,instead choosing to complete metamorphoses in areas with slower smoother water flow and(Pernet et al., 2003; Dobretsov & Wahl., 2008). In mussels, initial attachment is increased in areas of lower shear stress, but later larvae migrate to areas of higher flow (Dobretsov & Wahl., 2008).

# Sounds

## Soundscapes as information

Marine invertebrates interpret characteristics of their soundscapes to assess information on their environment (Simpson et al., 2011; Solé et al., 2023). The overlay of different underwater sounds originating from both biotic and abiotic sources accumulates into a rich acoustic profile, a so-called soundscape. Soundscapes, or specific elements of them, can give away important environmental information such as the presence of potential mates or predators, food availability, etc (Duarte et al., 2021). In the past ten years, it has become known that these underwater soundscapes also influence bivalves during their larval stage and certain sounds can increase settlement/metamorphosis in both oysters and mussels (Lillis et al., 2013, 2015; Eggleston et al., 2016; Williams et al., 2022, 2023; McAfee et al., 2023; Wilkens et al., 2012; Jovilet et al., 2016).

The use of soundscapes to direct planktonic larvae of benthic organisms to ideal habitats has been recognized for decades. Early work in the 1980's and 1990's focused on larvae of fish and crustaceans, guided by physiological studies on sound-perceiving morphological structures such as otoliths in fishes (Rogers & Cox., 1988) and sensory spines and hairs in crustaceans (MacMillan et al., 1992). Aquatic invertebrates, including oysters, sense particle motion rather than sound pressure (Solé et al., 2023). As this particle motion is detected through mechanosensation bivalve larvae are theorized to detect sound in one of two ways. Either through mechanosensory cilia, or through the statocyst structures, developed at the pediveliger stage (Budelmann 1989; Gosling 2008; Solé et al., 2023).

**Reef sounds**

The diversity of bivalve reefs in terms of their biological composition and physical structure in particular makes them distinct as soundscapes(Lillis et al., 2014a; Watson et al., 2025). In healthy reef communities, the structure of the reefs along with their inhabitants produce a high level of sound with distinct characteristics, comparatively much different than that of other habitats such as soft sediment bottoms (Lillis et al., 2014a). Larvae from oysters *C. virginica, O. angasi,* and *M. gigas* have been found to settle/metamorphose more readily when exposed to the sounds of their conspecific reefs rather than sounds from adjacent mud or sandy off-reef areas (Lillis et al., 2013, 2015; Williams et al., 2022; McAfee et al., 2023, Schmidlin et al., 2024(chapter 4, this thesis)). As sound propagates well within water, reef sounds could represent an important component of environmental signaling over distances (Lillis et al., 2014b). Williams et al., (2022) found that not only will larvae settle/metamorphose more readily, but they can also orient and move vertically towards preferred sounds from up to 4 m away.

**Anthropogenic sounds**

Marine soundscapes are not only characterized by biological inhabitants, but many noisy anthropogenically produced sounds, which are particularly present in coastal areas where bivalve reefs are located (Bittencourt et al., 2020). While there is some evidence that the attractive effects of reef noises on oyster larvae may be masked by sounds from anthropogenic sources, such as marine vessels (Williams et al., 2022; Schmidlin et al., 2024), the effect of vessel noises has shown a distinctly different response in mussel larvae. Because vessel hulls are rapidly colonized and overgrown by fouling organisms (which comes at a large expense to the shipping industry), questions about the role of acoustic cues in biofouling have been investigated. For mussels *P. canaliculus* and *M. edulis,* vessel noises increase settlement propensity by significantly reducing the mean time until settlement for *P. canaliculus* (Wilken et al., 2012) and increased settlement/metamorphic success in *M. edulis* (Jolivet et al., 2016).

**Characteristics of larvae-attractive sounds**

It is currently still poorly understood how bivalve larvae perceive sound and to which sound features they respond. While it is known that bivalve larvae can distinguish between natural off-reef and reef sounds, the distinct characteristics that are perceptible to larvae are unknown. Lillis et al., (2014a) showed that subtidal oyster reefs in coastal North Carolina, USA caused an increase in sound levels of up to 30 dB re 1 µPa at frequencies typically 2-23 kHz, and higher measures of acoustic diversity, compared to off-reef areas. In Australian reefs, this was similarly found, and SPL increases were as high as 40 dB re 1 µP at similar frequencies (Williams et al., 2022). North Sea oyster reefs from the Netherlands were louder and more diverse than their off-reef counterparts (Watson et al., 2025), however, Schmidlin et al., (2024) found vessel sounds, which were at times even louder than reef sounds, were not preferred over the sounds of reefs. It can be extracted that larvae prefer diverse and loud acoustic features, but no work has yet completely uncoupled these to see if the larvae response is reliant on one of these features or a combination. These preferences could also be distinctly adapted for each species to their local environment.

Little is known about the acoustic characteristics of mussel beds, and no studies have yet been performed to see if mussels will respond positively to these habitat-associated sounds. As mussel larvae responded positively to vessel sounds, it may be assumed that these sounds mimic those found in natural beds. Wilken et al. (2012) observed that the frequency of underwater vessel sounds (characterized by 100 to 1000 Hz) matched the frequency ranges to which fish, crustaceans, and corals reacted. In both studies where vessel noise induced mussel settlement/metamorphosis, the noises measured between 100 and 1000 Hz at intensities of 100 - 127 dB re 1 µPa, additionally, increasing positive reactions of larvae were found at higher intensities (Wilken et al., 2012; Jolivet et al., 2016).

## Substrate Topography

After the descent from the water column and arrival at a substrate surface, the topographical features of the surface provide larvae with more information about its suitability as a location to complete metamorphosis. Different formations of topographical features on a surface can both attract or repel larvae (Scardino et al. 2006, 2008; Bauer et al., 2024). Microtopography (topography features in the micrometer to millimeter scale) is generally thought to increase metamorphosis rates when larvae have

access to the greatest number of attachment points (the attachment point theory), and according to this theory, ideal topographical features would be equal to the size of the larvae (Callow et al. 2002; Scardino et al. 2008). A maximum number of contact points is hypothesized to signal to larvae protection from hydrodynamic pressures and/or predation (Scyphers & Powers., 2013). However, experimental evidence shows that selection for only features the exact length of the larvae is not found to be true for every species (Scardino et al. 2008; Carve et al. 2019). Larger topography modifications, on the centimeter scale can also effectively influence metamorphosis and are often used to increase the bioreceptivity of substrates (Sanmartín et al. 2021), but microscale larvae more likely experience these modifications as changes in the resulting microhabitats created (water flow patterns, predator protection, light, suspended food particles, and temperatures) rather than as true cues sensed through mechanosensory reception (Bauer et al., 2024). Terminology used in scientific literature regarding topographical differences can be hard to compare. Differences in surface characteristics, including the size, shape, and spatial organization of topographical features, are not often captioned (Erramille & Genzer, 2019). Roughness and rugosity are often used to describe the changes in height of a surface relative to the actual true surface. Roughness can be hard to distinguish as features of the topography can have similar heights but differ in shape, and in turn change the contact angle available to larvae or the flow of water.

**Microtopography**

Relatively few studies have addressed alterations in the metamorphosis of bivalves due to microscale alterations. One of the few was Carl et al., (2012), who tested mussel *M. galloprovincialis* attachment on microtopography features with surface feature heights ranging from 0 -1000 µm. In alignment with the attachment point theory, they found that topographical features smaller than the size of settling larvae decreased attachment, with features in the range of 10, 20, 100, 200, and 250 µm performing significantly worse than those over 300 µm. Features, however, larger than the size of the larvae (max length 260 µm), from 300 µm through 1000 µm, attracted a similar number of larvae. Under field conditions, Kohler et al., (1999) found that features larger than larvae showed to be even more attractive for mussel *M. edulis*, with the largest features 5,000 µm, showing the most attraction compared to 1000 µm,100 µm, and 500 µm. *P. canaliculus* likewise was found to prefer 1000 µm features compared to smooth controls, but, in this study, only one topography treatment was offered (Gribben et al., 2011). For oyster *O. edulis,* Potet et al., (2021) found a clear topographical preference, however, specific features of the topography which dictated these changes could not be identified; the best-performing surfaces in this study contained

topographical features between 3mm -17mm, yet some of the tested topography treatments had features in this range and still performed significantly worse. The surfaces with the least larvae metamorphosed were smooth or with a sandy texture, and an observation was made that surfaces with the highest number of larvae resembled natural surfaces.

**Centimeter-scale topography**

Interventions of larger surface modifications promote more larvae metamorphosis as well, while this is likely owed to the creation of favorable microhabitats, especially favorable flow conditions, these interventions will still be included in this review as it is hard to decouple the effect of the topography and that of the microhabitats (Bauer et al., 2024). Both Whitman & Reidenbach (2012) and Johnson et al. (2017) tested oyster *C. virginica* larvae on different surface configurations. Whitman & Reidenbach (2012) found that triangular concrete structures with a height of 10 cm and spacing of 5 cm (2:1 aspect ratio) yielded the highest number of metamorphosed larvae. Whereas, structures with both height and spacing of 10 cm (1:1 aspect ratio) showed only moderate improvements to flat surfaces, which saw the least larvae. Johnson et al. (2017) used similar size ranges but instead tested concave surface depressions. Finding that surfaces with a depth of 2.5 cm and a width of 12.5 cm (width:depth ratio 5:1) had the highest number of larvae. Shallower depressions, depth 2.5 cm, width 5.0 cm (width:depth ratio 2:1) observed less metamorphosis, and treatments with very wide and shallow depression depth 2.5 cm, width 40.0 cm (width:depth ratio 16:1) had similarly low metamorphosis rates as flat surfaces. In both studies, larvae concentrated on certain areas of the structures. In Whitman & Reidenbach., 2012 the peaks and the valleys of the structures, and in Johnson et al. (2017), the concave depressions exclusively. Both of the flow conditions in the study represented areas that were too still or alternatively, too turbulent, conditions which were also noted as unfavorable in the previous section on hydrodynamic cues. These conditions strike a balance between flow delivery and retention. This preferred intermediate flow was created by either the tight spacing of raised structures (Whitman & Reidenbach, 2012) or moderately concave surface depressions (Johnson et al., 2017). These features slow water movement just enough to allow larvae to remain near the substrate and explore. Not all topographical alterations generated differences in metamorphosis. O'Shaughnessy et al., 2021 altered concretes with parallel ridges and crevices 2.5 cm (height: length) and 5 cm (height: length), finding that the oyster *O. edulis* was significantly more abundant on both alterations compared to smooth controls. Interestingly, the mussel *Mytilus spp.* was unaffected by these topographical modifications.

Topographical alterations are also frequently used in new ecologically based concretes with the aim of increasing their bioreceptivity to a diverse range of species. These alterations often include adding holes and crevices of varying sizes, and some of these new bioreceptive concretes reported huge increases in the number of recruits found (up to 50 times the number as on control concretes) (Perkol-Finkel et al., 2013; Ido & Shimrit 2015). These studies, however, test both modifications to the formula of the concretes used along with topography, making it hard to decouple the effects from topography alone (Perkol-Finkel et al., 2014).

**Mussels prefer ropes for initial settlement**

As some mussel species prefer to first settle on filamentous algae before moving to hard substrates for final metamorphosis, it is common in mussel aquaculture to use filamentous rope for harvesting wild mussel spat, and some research has focused on identifying the best size and formation of rope longlines for improving aquaculture yield. Mussel numbers were generally greater on thicker, more complex rope collectors. Rope collectors with high structural complexity and larger surface area result in the strongest response, whereas those with the lowest complexity/least surface area generally show lower numbers of larvae (Protopopescu & Beal, 2015 ; Aghzar et al., 2012; Carl et al., 2012b). Protopopescu & Beal (2015) found the response of larvae to complex ropes is not slight, but highly significant, showing a nearly 10-fold increase in the number of mussel *M. edulis* from the smooth ropes to those with the highest complexity (looped ropes). While this preference for complexity is generally supported, reports of contradictions exist. Skelton & Jeffs (2020) did not find any differences in the number of the mussel *P. canaliculus* larvae found on different styles of rope collectors.

## Chemical cues

The both the pelagic and benthic period of marine invertebrate larvae development puts them in contact with a cocktail of different chemicals with which larvae have evolved complex behavioral adaptations (Hadfield & Paul 2001; Rodríguez et al. 1993; Pawlik., 1992). Chemical waterborne cues are theorized to be most useful to larvae only in relatively small physical distances, either centimeters above a substrate

surface below where water flow is slow, so chemical cues can accumulate, forming rich cue environments or experienced as surface bound cues directly on the substrate surface. From a larva's perspective, chemical plumes are likely experienced as fine-scale filaments and patches, creating a landscape of different and interacting signals (Koehl & Hadfield., 2010; Wheeler et al., 2019). Above the benthic boundary layer, faster water flow dilutes cues, while larvae may still encounter these cues, they are more intermittent and lower in concentration (Koehl & Hadfield., 2010,). Chemical cues may also be experienced at the substrate surface, including cues from shells of conspecifics and substrate associated biofilms (Hadfield & Paul., 2001). In bivalve larvae, chemoreception has been associated with the apical organ, early peripheral sensory neurons, and the larval foot (Yurchenko et al., 2018; 2019; Yu et al., 2023). In response to chemical cues, larval settlement and metamorphosis might be encouraged or inhibited; additionally, larvae can orient themselves away from cues experienced in the water column. The responses of marine invertebrate larvae generally to chemical cues, especially those from biofilms, have been covered in previous reviews, see Hadfield & Paul (2001), Rodríguez et al. (1993) & Pawlik., (1992), Dobretsov & Rittschof., (2020), but bivalve larvae have yet to be investigated independently.

## Biofilms

Immediately after submersion of new substrates in seawater, members of the planktonic microbial community will begin colonizing these fresh surfaces, eventually forming mature biofilms (Davey and O'Toole, 2000; Salta et al., 2013; Qian et al., 2022). While the composition of species that make up biofilms can vary depending on environmental conditions, usually biofilm succession happens in a similar order with bacteria as the initial colonizing species, followed by diatoms, and other algae as well as fungi, protozoa, and some species of cyanobacteria (Flemming & Wingender., 2010; Salta et al., 2013; Qian et al., 2022). As biofilms mature, these species interact among themselves (quorum sensing) and their environment and form a matrix of extracellular polymeric substances (EPS) (Flemming & Wingender., 2010).

Nearly a century ago, Zobell & Allen (1935) suggested that larvae of many marine invertebrates required a bacterial biofilm to settle and metamorphosize,and research into biofilm and larvae interactions that followed concluded that biofilms can act as a positive cue for bivalve species, acting to attract larvae independent of positive cues from conspecific adults (Tamburri et al., 1992; Bonar et al., 1990). The

evolved relationship between larvae and biofilms has been theorized to have arisen either to indicate that food is present, that an area is not toxic, and/or that a surface has not been only temporally submerged (Unabia & Hadfield., 1999). Since biofilm formation is influenced by the conditions under which it develops, including microbial community composition, hydrodynamic patterns, temperature, and water quality (such as pH and the presence of heavy metals or other toxins), it is probable that biofilm characteristics can provide valuable information about preferred ecological conditions (Bao et al., 2010; Hadfield et al., 2011; Salta et al., 2013; Misic & Harriague., 2019; Dobretsov & Rittschof., 2020; Espinel-Velasco et al., 2021).

**Water-soluble cue vs surface-bound cue**

How bivalve larvae perceive biofilm cues, whether through direct contact with a substrate or via diffusible attractants in the water, has been explored in several species. In the oyster *M. gigas*, biofilms function as both waterborne and substrate-bound cues, and biofilm-conditioned water alone can induce both larval settlement and metamorphosis (Zimmer-Faust & Tamburri, 1994). In the mussels *M. coruscus* and *M. galloprovincialis,* biofilms act through two distinct mechanisms: waterborne cues can induce settlement, but physical contact with the biofilm is required to trigger metamorphosis (Bao et al., 2007; Yang et al., 2013). In the case of mussel *M. coruscus,* this two-step process was later challenged by Chang et al., (2021), who found that some species of bacterial biofilm can stimulate both processes through biofilm-conditioned water alone. It should be noted, however, that Chang et al., (2021) also found that the combination of the waterborne and substrate-bound biofilm cues together has a synergistic effect on settlement. Overall, both surface contact and exposure to water-soluble components of biofilms can stimulate larval settlement and/or metamorphosis, but the nature of the effective cue appears to be highly species-specific, even among closely related taxa. In marine invertebrate larvae beyond bivalves, conclusions also vary, though most studies suggest that biofilms primarily act as surface-bound cues (Hadfield, 2011).

**Drivers of biofilm attraction**

It remains debatable which specific features of the biofilm drive larval attraction. Age is known to influence the response oflarvae, with cue intensity often increasing as the biofilm matures (Campbell et al., 2011; Toupoint et al., 2012a). However, because numerous biofilm characteristics change with age, it is difficult to pinpoint the exact factor responsible for cueing bivalve larvae. Older biofilms commonly exhibit higher densities of bacteria and algae, along with a more diverse community composition, and

these traits have been linked to the induction of larval settlement and metamorphosis in several studies (Bao et al., 2007; Peteiro et al., 2007; Campbell et al., 2011; Wang et al., 2012; Yang et al., 2014). Yet, other factors have also been proposed, such as community structures that develop during specific times of year (Bao et al., 2007), the presence of particular species within the biofilm (Yang et al., 2013; Campbell et al., 2011; Peng et al., 2020; Chang et al., 2021), and even specific components of extracellular polymeric substances (EPS) (Bao et al., 2007; Liang et al., 2020).

**Algae in Biofilms**

Both biofilm bacteria and microbial eukaryotes are known parts of the biofilm that can attract invertebrate larvae. For some species of invertebrates, algae incorporated in the biofilm are known to trigger settlement/metamorphosis in some invertebrates (Harder et al., 2002). However, in bivalves, most of the literature focuses on the bacterial component of biofilms. While it is theorized that algae in the biofilm could provide an important trophic resource for the larvae, it cannot be conclusively stated that the cue from marine biofilms originates from the algal component of the biofilm independently of other biofilm components.

Toupoint et al., (2012a) found that the defining characteristic in older biofilms, which acted as a stronger settlement/metamorphic cue for *M. edilus*, was a shift from bacterial communities to a higher concentration of photosynthetic picoeukaryotes in the biofilm. This would suggest algae in the biofilm as a dominating cue. However, it is also important to note in that study, older biofilm also changed the dominate species of bacteria, which could also explain increased larval attraction. Additionally, the study found that essential fatty acid (EFA) proportions in biofilm did not change as the biofilm aged and thus did not have a relationship with biofilm cue strength. Both Cambell et al., (2011) and Wang et al., (2012) showed no apparent relationship with chlorophyll-a concentration in marine biofilms and increased larvae attraction for oyster *C. virginica* and mussel *M. coruscus,* respectively.

**Bacteria in biofilms**

The bacterial component of biofilms is most typically described as a driving force of invertebrate settlement and metamorphosis. Species from the genus *Pseudoalteromonas* are commonly used in these studies, particularly with mussel larvae, due to their widespread occurrence as surface colonizers (Peng et al., 2020; Liang et al. 2020; Hu et al. 2021).

Bivalve larvae have been shown to exhibit species-specific preferences for bacterial biofilms, settling and metamorphosing in higher numbers in response to some bacterial species over others. These preferences remained consistent even when the bacterial origin changed (Zhao et al., 2003; Peng et al., 2020). Notably, these patterns could not be linked to bacterial phylogeny as closely related species did not necessarily share similar levels of settlement-inducing strength (Yang et al., 2013; Peng et al., 2020). Peng et al. (2020) further demonstrated that specificity exists even within a single genus, testing seven different strains of *Pseudoalteromonas* spp. and finding that each induced *Mytilus coruscus* larvae with varying strengths of attraction.

**Biofilm EPS**

As previously mentioned, mature biofilms form a matrix of extracellular polymeric substances (EPS) which are mainly composed of proteins, polysaccharides, and lipids and act as a protection and glue for the biofilm cells (Dragoš & Kovács., 2017). Since Bao et al., (2007) first identified that the exopolysaccharide or glycoprotein on the biofilm surface was responsible for larval metamorphosis of for mussel *M. galloprovincialis,* it has been theorized that the EPS of the biofilm would play a greater role (Dobretsov & Rittschof., 2020). Ganesan et al., (2012) found that EPS from biofilms formed by *Macrococcus sp*. and *Bacillus sp*. were responsible for the settlement of *P. canaliculus* rather than bacterial cells alone. They also noted this result to be species-specific, as in *Pseudoalteromonas sp.* the EPS exudates were toxic to larvae instead**.** Chang et al., (2021) investigated further the composition of the EPS of several bacterial species and found key differences, indicating that EPS matrix structure may be dependent on the bacterial species. Some of these differences included changes in the composition of extracellular polysaccharides α-polysaccharides and β-polysaccharides, which were higher distributed in bacterial biofilm from *Virgibacillus sp.* than proteins and lipids. In contrast, β-polysaccharides and lipids in the BFs formed by *Pseudoalteromonas marina* were lower than α-polysaccharides and proteins. Biofilms of both these species induced *M. coruscus* larvae settlement and metamorphosis; however, *Virgibacillus sp* had the highest inducing activity. Liang et al. (2020) manipulated *P. marina* by deleting the flagellin gene *fliP*, resulting in biofilms with increased extracellular proteins, reduced β-polysaccharides, unchanged α-polysaccharides and lipids, and enhanced bacterial aggregation. These altered biofilms significantly reduced *Mytilus coruscus* settlement and metamorphosis. This provides further evidence that the structure of the biofilm is responsible for larval attraction, particularly the polysaccharide component of EPS may play a bigger role in biofilm attraction to larvae. Furthering this work, Liang et al., (2021) manipulated the cellulose synthesis gene bcsQ from bacteria *P. marina*, finding that this gene controls the

secretion of exopolysaccharides and biofilm formation, which in turn increased larval settlement/metamorphosis for *M. corcscus.* Hu et al. (2021) furthered this, specifying that bacterial fatty acid metabolism, mediated by the *tesA* gene in biofilms from *P. marina*, plays a crucial regulatory role in *M. coruscus* metamorphosis.

**Negative cue or no effect**

A few examples exist where biofilms induce no reaction or a negative reaction for invertebrate larvae generally (Chang et al. 1996; Labare et al. 1997), but active avoidance of biofilms is not commonly seen in bivalves. Mussel, *A. atra* showed a negative reaction to bacterial biofilms from *Acrococcus spp*, *Aacillus spp*, and *Pseudoalteromonas sp*, which have been shown attract other mussel species. Researchers theorized that the neuronal control of settlement/metamorphic behavior for *A. maoriana* differs from that of other mussel species (Alfaro et al., 2011)

The importance of biofilms, when compared to other cues, is hard to decipher as studies rarely focus on multiple cues at the same time. Tamburri & Zimmer-Faust., 1992 concluded that while biofilms are independent from conspecific cues, conspecific cues will induce more settlement/metamorphosis than biofilms alone for *C. virginica*. Similarly, Rodriguez-Perez et al., (2019) found that oyster *O. edulis*, would metamorphosize in higher numbers in the presence of live conspecifics, followed by the presence of biofilm, whereas trophic cues had no effect.

**Biofilms on different materials**

Changes to substrates can influence the attractive components of biofilms. Surface wettability has been shown to influence biofilms by influencing bacterial and diatom attachment; low surface wettability results in decreasing bacterial abundance and low attractiveness for *M. galloprovincialis* larvae (Yang et al., 2017). Even when biofilms were left to develop over longer periods of time (28 days), the bacterial density on surfaces with less wettability stayed lower and attracted less larvae. Surface wettability did not only change bacterial density but also altered the bacterial community in the biofilm. It is important to note that other studies show conflicting results, with similar bacterial communities found in biofilms regardless of the initial surface wettability (Hung et al., 2008; Huggett et al., 2009). Substrate color has also been suggested to have an effect on biofilm formation and, in turn, attractive properties. Biofilms gown on a variety of colors had differences in community composition, with biofilms grown on black surfaces showing the highest metamorphosis of *M. coruscus* and green showing the least (Li et al., 2017).

## Conspecific cues

Some of the most well researched settlement cues are those produced by members of the same species, these cues tend to show the strongest positive attractive responses in larvae behavior and are a common trait among many marine invertebrate species (Hadfield & Paul., 2001). From the very early days of larval settlement/metamorphosis research, it was suspected that larvae were attracted to the bodies and tissues of conspecific adults, as observations of gregarious behavior in bivalve larvaewere evident by their adult formation of dense reefs with individuals growing on the shells of others (Bayne., 1969; Woodin., 1986). While settling near conspecific adults is a reproductive strategy, the response is a tradeoff for larvae. The presence of adults of the same species indicates that a location is suitable for survival, but adults can cannibalize their own larvae through their filter feeding and also represent competition for food later in their life (Kautsky 1982; Lehane & Davenport., 2004). However, despite competition and predation, it is clear from the attractive cue by conspecifics that being near to other conspecific adults has an evolutionary advantage for bivalves.

Very early on in larval research, it was established that conspecific cues can be experienced multimodally, both in the water column and upon contact with shells. Cole & Knight-Jones., (1939) are often cited as the first to show gregarious behavior of bivalve larvae from the oyster *O. edulis* in response to conspecific cues from shells. Hidu.,1969 confirmed this for C. virginica, finding that waterborne cues could increase settlement independent of shells. Conspecific waterborne cues can also be absorbed into substrates by soaking these with tissue extracts (Crisp., 1967; Bayne., 1969). While larvae preferentially metamorphosed on shell from both living and dead oysters. The attraction to shells can decrease over time, Preston et al., (2020) found that *O. edulis* larvae were twice as likely to metamorphosize on plates made from recently deceased oysters (not containing flesh) than plates made from older oysters (collected in intertidal, with age unknown). These results point to a cue found in the shell of oysters that will dilute over time, although the exact time at which this dilution takes effect was not determined.

**Conspecific cue chemical identity**

The chemical identity of the waterborne cues inducing swimming behavior and larval attachment were established by Zimmer-Faust & Tamburri.,(1992), finding that this was a low molecular weight peptide with arginine at the C-terminal. Later, the identity of the cue from the shell of the oyster *M. gigas* was further characterized as a heat-stable, ~55 kDa glycoprotein (Vasquez et al., 2013; 2014). Building on this work, Sedanza et al. (2022) demonstrated that this cue is part of a macromolecular assembly of shell matrix proteins, which they named the "*Crassostrea gigas* Settlement Pheromone Protein Components" (CGSPPC). This complex system requires an interplay of different amino acid groups, disulfide bonds, glycans, and phosphorylation crosstalk for conspecific recognition. Within this assembly, Gigasin-6 isoforms X1 and X2 were identified as the primary contributors to the settlement-inducing activity.

Vasquez et al. (2013) compared shells from 11 different bivalve mollusk species to assess preferences of *M. gigas* larvae. They found that conspecific shells elicited the strongest metamorphic response. However, shells from other species also contained chemical components capable of inducing metamorphosis, though to a lesser degree. This suggests that the chemical cues involved in metamorphosis may be partially conserved among related species, but the strength of the larval response likely depends on how closely the cue source matches the larvae own species. These patterns, however, appear to be species-specific. *S. glomerata* larvae were found to prefer the shells of *M. gigas* over their own (Wilkie et al., 2013). Similarly, Montes et al. (2021) reported that while *M. galloprovincialis* larvae preferred to settle on conspecific shells, the invasive mussel *X. securis* larvae showed no specific substrate preference.

**Conspecific larvae interactions**

Larvae from *O. edulis* also utilize cues from recently settled/metamorphosed conspecific larvae (Cole & Knight-Jones., 1939; Rodriguez-Perez et al., 2019), and Rodriguez-Perez et al., 2019 found that *O. edulis* metamorphosis among recently metamorphosed conspecific larvae far surpassed cues from microalgae or biofilms. In mussels, the relationships between larvae individuals may be more complex and include an ontogenetic shift in preference. Van der Meden et al., (2010) investigated larvae to early settler interactions with mussels *P. perna* and *M. galloprovincialis* in the field, looking at both primary and secondary settlement events (defined by the size of the settled larvae) on collectors that had been pre-seeded with newly attached larvae 6 days previously. Primary settlement from both species did not show any attraction to the collector with previously settled larvae. Primary settlement was increased by biofilm

presence. But a combination of biofilm and conspecific settlers reduced settlement lower than biofilms alone. Secondary settlement, however, was highest on treatments with both biofilm and settlers present.

### Alarm cues

While conspecific cues usually indicate a preferable location for larvae, when an adult has become injured, the resulting metabolites can act as an "alarm cue" and can affect both adults and larvae behavior (Cheung et al., 2004; 2006). Pruett et al., (2019) tested the effect of alarm cues on *C. virginica* larvae in the field and in the lab. The results in the field showed no preferential settlement/metamorphosis away from injured adults. The results of the laboratory study showed larvae would orient themselves away from the alarm cue. The discrepancy of the results in the field vs lab could be due to hydrodynamic flows diluting cues to the point where there is no effect.

## Algae

Marine algae can be the prey of marine bivalve larvae, a competitor for space on substratum, and also for many mussel species, a location in which to settle and metamorphose (Soares et al., 2008; Cho et al., 2013; Jolivet et al., 2016). Like other cues previously discussed, algae can influence larvae both in the water and, for macroalgae, upon contact with the algae surface. The relationship between bivalve larvae and algae species is very context-dependent and can be both a positive or negative one, depending on the particular species or the mode of delivery (waterborne vs substrate-bound) (Gribben et al., 2011).

### Macroalgae

Filamentous algae and mussels share a well-documented ecological relationship. Many mussel species initially settle on algae during the larval stage before relocating to hard substrates as post-larvae (Yang et al., 2007). However, this model of primary and secondary settlement remains contested. Some evidence suggests that the majority of recruits may bypass algae and attach directly onto hard substrates (Reaugh et al., 2007). Regardless of whether primary settlement on algae is the dominant pathway for most

recruits, an association with large macroalgae is evident in many mussel species. All interactions gathered here on the relationship of macroalgae use mussel larvae rather than oysters. This is intuitive as oysters do not first settle on macroalgae and nor can they undergo a secondary settlement.

Positive larval attraction to macroalgae has largely been attributed to surface-bound chemical exudates (Alfaro et al., 2006; Satuito et al., 2007; Soares et al., 2007; Gribben et al., 2011). Gribben et al. (2011) tested both polar (waterborne) and non-polar (surface-associated) extracts from eight algal species on the settlement of P. canaliculus. They found that surface-associated cues slightly, but not significantly, increased settlement, while waterborne extracts generally reduced it. This attraction to surface-bound cues but inhibitory effect of waterborne cues was also observed by Davis and Moreno (1995). However, responses varied by algal species, suggesting that these interactions are species-specific and not every species are ranked equally. Similarly, Satuito et al. (2007) found that *M. galloprovincialis* larvae showed a preference for certain red algae *(Ceramium tenerrimum, Centroceras clavulatum*), while other species had no effect. Importantly, they noted that the physiological state of the algae affected their attractiveness, and algae under stress or suboptimal culture conditions lost their inductive quality. These findings indicate that larval responses to algal cues are both species-specific and condition-dependent (Davis & Moreno, 1995; Alfaro et al., 2006; Satuito et al., 2007).

Similar to attractive conspecific chemicals, surface-bound algae-associated chemical cues can be transferred to inert substrates and still influence larval behavior. Dobretsov and Wahl (2007) demonstrated that *M. edulis* larvae increased metamorphosis on PVC panels that had been soaked in metabolites from *Cladophora rupestris*. Interestingly, these chemical cues altered larval distribution patterns across the panels, overriding their natural topographical preferences. Whereas larvae typically metamorphose in regions with reduced shear, the presence of algal cues promoted metamorphosis across the entire plate.

Cues from algae can also have negative effects on larval settlement/metamorphosis, which has been explored for its potential use in antifouling (Gama et al., 2008; Plouguerné et al., 2009; Cho., 2013). While positive interactions with larval metamorphosis are associated primarily with surface-bound cues, negative cues from algae can use both modes, Cho (2013) demonstrated that waterborne chemical cues from the brown alga *Sargassum horneri* significantly reduced the settlement of *M. edulis* larvae and were able to identify this compound as chromanols consisting of a polyprenyl chain. Similarly, Gama et al. (2008)

found that *Perna sp*. larvae displayed reduced attachment to red algae *Rhodophyta sp.* and (to a lesser extent) brown algae *Phaeophyta sp.* Interestingly, in some cases, algae needed to be damaged before the antifouling compounds were released, suggesting an inducible defense mechanism. The production and potency of inhibiting negative cues may also be regulated by the condition of the algae. Plouguerné et al. (2009) showed that surface extracts from *Sargassum vulgare* significantly inhibited the attachment of juvenile *P. perna* mussels, but the strength of this inhibitory effect varied depending on the collection site of the algae. Suggesting that local environmental factors, such as light intensity, UV exposure, salinity, hydrodynamics, nutrient availability, and grazing pressure, may influence the algae associated cues in ways that are perceptible to larvae.

**Microalgae**

Microalgae are a prey species for bivalve larvae, with evidence pointing to positive or neutral effects on settlement/metamorphosis, sometimes referred to as trophic cues. *M. edulis* was found to increase settlement/metamorphosis under picoeukaryote species (trophic resource), , but not indiscriminately, and did not respond to picoeukaryote species that were not ingested by the larvae (Jolivet et al., 2016). Interestingly, the effect of the trophic cue had a synergistic relationship with acoustic cues from vessel-associated sound when experienced simultaneously. This relationship was mirrored in Stocks et al., (2012), where oyster *M. gigas* increased swimming behaviors indicative of settlement in the presence of both algae and acoustic cues but not for either cue independently.

Trophic cues, similar to other cues which can be experienced by larvae in the water column beyond the benthic boundary layer, are likely to drive bivalve larvae from the water column down towards bottom substrates. While micro algae did not induce a rapid descent for oyster *C. virginica* (Maciejewskia et al., 2019), oyster *O.edulis* showed food cues induced a tendency to migrate downwards and remain near the bottom of the vessels containing them (Rodriguez-Perez et al., 2020). When presented with a choice to move horizontally towards cues Morello & Yund (2016) found that microalgae can stimulate a positive response with *M. edulis* larvae, moving towards cue-conditioned water, although this study noted the reaction to conspecific cues was stronger. When two cues were presented together in opposite directions, larvae exhibited significantly more no-choice responses.

Associations between the abundance of preferred algal species and high recruitment rates have been reported in field studies on larval recruitment. For example, Androuin et al. (2021) and Toupoint et al. (2012b) found that *M. edulis* recruitment peaked with elevated concentrations of picoeukaryotes, and that recruitment was higher in years when food quality, in particular essential fatty acid content, was improved. Similarly, Lagarde et al. (2017) reported that higher recruitment rates of *Crassostrea spp*. were associated with increased abundances of nanophytoplankton.

## Heterospecific cues

Larvae can evolve relationships to other species, which can alert them to the presence of predators, competitors, or provide information about the habitable suitability.

**Predator cues**

Predator-prey interactions play a large role in species survival and ecological ranges. While direct predation is a major bottleneck limiting species survivability, predator cues, sometimes referred to as nonconsumptive predator effects (NCEs) can also strongly modulate prey demography with an effect which can be as strong as or stronger than predator consumptive effects (Preisser, Bolnick & Benard, 2005; Preisser et al. 2005). In bivalve larvae, cues from predator species can induce multiple effects on mussels and oysters at different life stages, including shell growth, reduced feeding, and strength of attachment to a substrate (Olof & Tedengren, 1996; Rahmat & Rudstam, 2013; Robinson et al., 2014; Bible et al., 2017). In settling larvae, predator presence can modulate larvae behaviors, although less is known about these effects than those for adults.

Bivalve larvae will both decrease metamorphosis and actively move away from associated withpredators (Table 2.2), and there are likely more predator species aside from those in the studies listed here that similarly produce negative cues. Larvae of both mussels and oysters avoid both predators that represent immediate risk, and predators that predate on their adult forms (Morello & Yund., 2016; Bertolini et al., 2019; Pruett & Weissburg., 2019).

Table 2.2. Predators known to impact settlement for a bivalve species through NCE

| **Predator Species** | **Bivalve Species** | **Observed Response** | **Reference** |
|---|---|---|---|
| ***Neanthes succinea* (Clam worm)** | *Crassostrea virginica* | Reduced settlement in response to predator-conditioned water | Barnes et al. (2010) |

| | | | |
|---|---|---|---|
| ***Neanthes succinea* (Clam worm)** | *Crassostrea ariakensis* | No response to predator-conditioned water | Barnes et al. (2010) |
| ***Nucella lapillus* (Dogwhelk)** | *Mytilus edulis*, *Mytilus trossulus* | Reduced settlement in response to predator-conditioned water | Ehlers et al. (2018); Morello & Yund (2016) |
| ***Nucella dubia* (Dogwhelk)** | *Perna Perna*, *Mytilus galloprovincialis* | Reduced settlement at one site; unexpected preference for predator cues at another | von der Meden et al. (2015) |
| ***Asterias rubens* (Starfish)** | *Mytilus edulis* | Reduced recruitment; effect magnified over multiple weeks | Bertolini et al. (2019) |
| ***Panopeus herbstii* (Mud crab)** | *Crassostrea virginica* | Reduced settlement in some field sites; larvae oriented away from predator-conditioned water in lab | Kimbro et al. (2020); Pruett & Weissburg (2019) |
| ***Carcinus maenas* (European green crab)** | *Mytilus edulis* | Larvae move horizontally away from predator-conditioned water | Morello & Yund (2016) |
| **Conch (unspecified)** | *Crassostrea virginica* | Decreased recruitment across all sites of the study | Kimbro et al. (2020) |

The response of *M. edulis* larvae to conflicting cues from both conspecifics and predator *C. maenas* was studied by Morello & Yund (2016). When faced with conflicting cues originating from different physical directions, mussel larvae will move toward positive conspecific cues and away from the negative predator cue. But, when presented with conflicting cues originating from the same direction, larvae will move less towards the cue combination than if there were no predator cue, but still in higher percentages than if there were no positive cue.

Predator avoidance was not always found, and in field experiments, Von der Meden et al. (2015) found that dog whelks *Nucella dubia* presence reduced settlement/metamorphosis for *P. perna* and *M. galloprovincialis* larvae, but this effect was only seen in one experimental location. At another site, larvae unexpectedly preferred predator-associated the predator treatments. The authors speculated this could reflect an adaptive strategy, similar to that seen in some crab species, where larvae group to overwhelm predators (Stevens, 2003).

Kimbro et al. (2020) found that recruitment of the oyster *Crassostrea virginica* decreased in response to chemical cues from conchs across all field sites in their study, while predator cues from crabs reduced larval recruitment at only some locations. This result was surprising, as mud crabs are known to be more voracious predators of juvenile oysters (Grabowski, 2004) than conchs, which typically prey on adults. Although the study demonstrated that predator cues can influence recruitment, it ultimately concluded that the effects of such cues on settlement/metamorphosis were outweighed by direct predation later in life.

The degree to which predator kairomones affected prey can change based on the conditions of the specific predator individual. The diet of the predator can increase the potency of the cue, particularly if the

predator has a diet consisting of conspecific species(Weissburg et al., 2016; Kimbro et al., 2020; Wirsing et al., 2021). Predator cues can also work simultaneously with other cues, for example =crab presence was found to decrease *C. virginica* larvae recruitment only in combination with alarm cues from predated oysters Pruett & Weissburg, (2019) .

**Other species interactions**

Bivalve larvae also respond to cues from species with which they may compete for space or that may signal habitat suitability (Table 2.3). However, not all potential competitors elicit negative responses. For example, barnacles have been shown to either reduce or enhance settlement/metamorphosis, depending on the species involved (Boudreaux et al., 2009; Ank et al., 2009; Barnes et al., 2010). While methodological differences could partly explain these varied outcomes, the responses are also likely to be context-dependent. Bivalve larvae generally respond positively to the presence of other bivalve species, including invasive ones, likely interpreting their presence as an indicator of favorable conditions or because the mechanism of induction might be chemically similar enough to their own conspecific cue (Vasquez et al., 2013; Wilkie et al., 2013).

Table 2.3. Species which are not direct predators of bivalves but have been reported to influence larvae settlement.

| **Species** | **Bivalve Species Affected** | **Observed Response** | **Reference** |
|---|---|---|---|
| ***Schizoporella errata*** **(Bryozoan)** | *Perna perna* | Reduced settlement from exposure to animal | Ank et al. (2009) |
| ***Symplegma rubra*****,** ***Didemnum speciosum*** **(Colonial ascidians)** | *Perna perna* | Reduced settlement from exposure to animal | Ank et al. (2009) |
| ***Balanus improvisus*** **(Barnacle)** | *Crassostrea ariakensis, Crassostrea virginica* | Exposure to animal increased settlement in both oyster species. Barnacle waterborne cue created with a shell did not increase settlement but barnacle waterborne cue without a shell did increase settlement. | Barnes et al. (2010) |
| ***Amphibalanus eburneus*****,** ***Amphibalanus amphitrite*** **(Barnacles)** | *Crassostrea virginica* | Settlement was reduced increasing with increasing density of animal | Boudreaux et al. (2009) |
| ***Balanus trigonus (a barnacle)*** | *Perna perna* | No effect to settlement | Ank et al. (2009) |
| ***Membranipora tenuis*** **(Bryozoan)** | *Crassostrea ariakensis, Crassostrea virginica* | Weak settlement reducing effect for *C. virginica,* no effect for *C. virginica* | Barnes et al. (2010) |
| ***Magallana gigas*** **(Oyster)** | *Saccostrea glomerata* | Increased settlement on *M. gigas* shells, even higher than conspecific shells | Wilkie et al. (2013) |
| ***C. nippona, C. nippona, Ostrea circumpicta, O. denselamellosa, Saccostrea*** | *Magallana gigas* | *M. gigas* larvae settled on the shells of all species tested except on *Patinopecten yessoensis* and *Atrina* | Vasquez et al., (2013) |

| | | | |
|---|---|---|---|
| ***kegaki, S. mordax, Patinopecten yessoensis, Pinctada fucata martensii, Atrina pinnata, Haliotis discus*** **(Mollusks)** | | *pinnata. C. nippona* was the most inductive compared to the other species. | |
| ***Ciliophora sp.*** **(Ciliates in biofilm)** | *Mytilus galloprovincialis* | Reduced settlement when animal was present on biofilm surface | Shimeta et al. (2012) |
| ***Phormidium*** **sp. (Cyanobacterium)** | *Mytilus galloprovincialis* | Reduced settlement, chemical identity of cue was found to be portoamides, a peptides produced naturally by the cyanobacterium | Antunes et al. (2019a) |
| ***Callyspongia*** **sp.,** ***Dysidea granulosa*****,** ***Dysidea herbacea*** **(Sponge)** | *Mytilus edulis* | Reduced settlement in response to chemical exudes from animal | Ortlepp et al. (2008) |
| ***Excoecaria agallocha*** **(Mangrove)** | *Perna indica* | Exudes from leaf, deterred settlement | Ramasubburayan et al. (2017) |

## Concluding remarks

Bivalve larvae are generalists in terms of the cues that can induce both settlement and metamorphosis, having evolved specific relationships with a variety of environmental signals. This body of work highlights the complexity and diversity of these cues and how they act on larvae across different spatial scales, contributing to a more complete understanding of the sensory ecology of bivalve species during their final larval stage. There are several paths that one can take to further this work: 1) identifying factors that may modulate larval responses to cues, 2) Mechanistically characterizing the chemical and physical nature of settlement cues, 3) Investigating how larvae integrate multiple environmental cues during the settlement decision-making process. This effort requires a deeper understanding of the photo-, chemo-, and mechanosensory systems in bivalve larvae, supported by behavioral evidence. As ecologically and commercially important organisms that respond to a multitude of cues, bivalves represent ideal candidates for further research.

# Chapter 3: Planktonic oyster larvae optimize settlement and metamorphotic decisions in complex sensory landscapes

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# Abstract

The settlement and metamorphosis of pelagic larvae constitutes a pivotal phase in the life cycle of benthic aquatic species. The choice of location is critical for recruitment into established populations and the colonization of unoccupied habitats. Our study aims to enhance our comprehension of the underlying principles of how larvae optimize these decisions when exposed to multiple natural chemical cues simultaneously. Through a series of laboratory experiments, we investigate metamorphosis patterns of Pacific oyster *Magallana gigas* larvae when exposed to different combinations of attractant and repellent cues. Our findings reveal additive increases in metamorphosis rates in the presence of attractive cues originating from conspecifics and biofilms. Conversely, attraction by conspecific water-borne cues was reduced in the presence of repellent cues emanating from predators, although this could only be substantiated by a nonsignificant trend. Notably, when repellent predator cues were presented alongside attractant cues linked to substrates (biofilms or shells from conspecific adults), any repellent effect was nullified.

# Introduction

Settlement and metamorphosis are critical steps in the life history of aquatic species with a planktonic larval stage. In sessile organisms, the process consists of settlement from the water column to the substrate surface, attachment, and finally metamorphosis (Morse., 1990; Hadfield & Paul 2001). For organisms with only limited or no relocation ability this process is essential for survival of a population (Crisp 1976, Bayne 2017). Settling larvae, therefore, screen their environment for cues that will give away information about the suitability of a site for settlement and survival (Zimme-Faust & Tamburri 1994, Hadfield & Paul 2001). Such information may include abiotic conditions (e.g. salinity, light, water current, sound; Rittschof et al., 1998; Koehl, 2007; Kim et al., 2021; Williams et al., 2022), the presence of conspecifics (Hadfield & Paul 2001, Dobretsov & Rittschof 2020) or predators (Morello & Yund 2016, Bertolini et al. 2019, Pruett & Weissburg 2019, Scrosati 2021), the composition of biofilms (Hadfield 2011, Campbell et al. 2011, Dobretsov & Rittschof 2020), or the presence of food (Forêt et al. 2018).

Depending on the information they carry, cues can elicit different reactions by being either attractive or repellent. Specific environments may release different cues at once, sending potentially conflicting messages to pelagic larvae. For larvae, the optimal behavioral strategy would be to respond to cues that signal immediate risks and opportunities while also considering (potentially conflicting) cues indicative for future conditions for feeding or mating to maximize survival and fitness (optimality theory (Milinski & Heller 1978, Sih 1980)). Their settlement and metamorphic decision should also depend on the relative importance of the cues (threat sensitivity hypothesis (Sih 1986, Helfman 1989)).

Numerous studies have been conducted, focusing on a wide range ofcues, including for example microtopography (Carl et al. 2012, Potet et al. 2021), presence of conspecifics (Zimme-Faust & Tamburri 1994, Vasquez et al. 2013) or predators (Bertolini, C. et al. 2019, Pruett & Weissburg 2019, Scrosati 2021) biofilms (Hadfield 2011, Campbell et al. 2011, Dobretsov & Rittschof 2020), or sound (Lillis et al. 2013, Williams et al. 2022, McAfee et al. 2023). Most of these studies share a common goal: to isolate a specific cue and quantify its efficacy as an attractant or repellent. However, exposure to a single cue is not a realistic scenario in nature. Gaining insight into the response of larvae to multiple simultaneous cues could help to predict patterns recruitment for reef restoration management. Larvae for example may disregard the negative predator cue in the presence of positive cues emitted by conspecifics. In another hypothetical example, the substrate preference may change depending on the abundance of prey items. It is therefore critical to understand how different cues interact for the decision-making settlement.

In this study, we seek to advance our understanding of how multiple natural chemical cues interact to influence metamorphosis behaviors for pediveliger larvae of the Pacific oyster (*Magallana gigas*). Oysters have been dubbed ecosystem engineers as the hard substrates created by the cumulation of their shells provide crucial ecosystem and economic services. For example, oysters create more biodiverse and complex environments by providing nurseries for species and filtering water, contributing to nutrient cycling (Grabowski & Powers., 2004; Tolley & Volety 2005; Ruesink et al., 2005). Oyster reefs also help to attenuate wave action, which can contribute to coastal erosion (Walles et al. 2016, Ysebaert et al. 2019). Aside from the environmental benefits, their high commercial value also increases socio-economic interest in these species (Grabowski & Peterson 2007, van der Schatte Olivier et al. 2020).

We address specifically in this study, how *M. gigas* larvae behave when facing simultaneous signaling from contradicting attracting (conspecific adults) and repelling (predators) cues. When faced with multiple conflicting cues i.e. those associated with conspecifics and those associated with predators, we

hypotheses that larvae could adopt the following decision rules: (1) if the positive cue is perceived as important, larvae metamorphose when exposed to positive cues regardless of the presence of a negative cue, (2) if the negative cue is perceived as important, larvae may not metamorphose in the presence of negative cues regardless of the addition of positive cue, (3) in the absence of a preference, larvae add up positive and negative cues and the larval response depends on their relative strength. To test these hypotheses, we exposed lab-reared pediveliger larvae to conspecific, predator and biofilm cues in full-factorial experiments.

# Methods

**Overview**

We conducted multiple laboratory experiments to assess the main and interaction effect of various settlement cues on metamorphosis propensity in Pacific oyster larvae. Treatment combinations of different positive and negative cues were applied to larvae (Figure 3.1).

Firstly, we performed a small-scale pilot study to estimate effect sizes and response variation. The results of this experiment are not considered for further analyses as cues were not added in similar volumes as the subsequent experiments. All information related to the pilot study can be found in the supporting information (Table 3.S1 and Figure 3.S1).

In the first main experiment (experiment 1), we subjected larvae to three different cues. These cues were conspecific waterborne cues, conspecific shell cues, and waterborne cues from natural predator *Carcinus maenas*. We replicated each of the treatment combinations in 20 separate Petri dishes (six larvae per dish) over two consecutive days with seven to nine replicates performed each day (Figure 3.1).

Based on the results of experiment 1, we performed a second experiment, where treatments were applied in a fully factorial design (experiment 2) using cues from the predator *C. maenas*, from both waterborne and shell-associated conspecific cues, and from biofilms (see Figure 3.1). We replicated each of the $2^4$ treatment combinations in 24 separate Petri dishes (six larvae per dish) over three consecutive days with seven to nine replicates of each treatment combinations performed each day (see Figure 3.1).

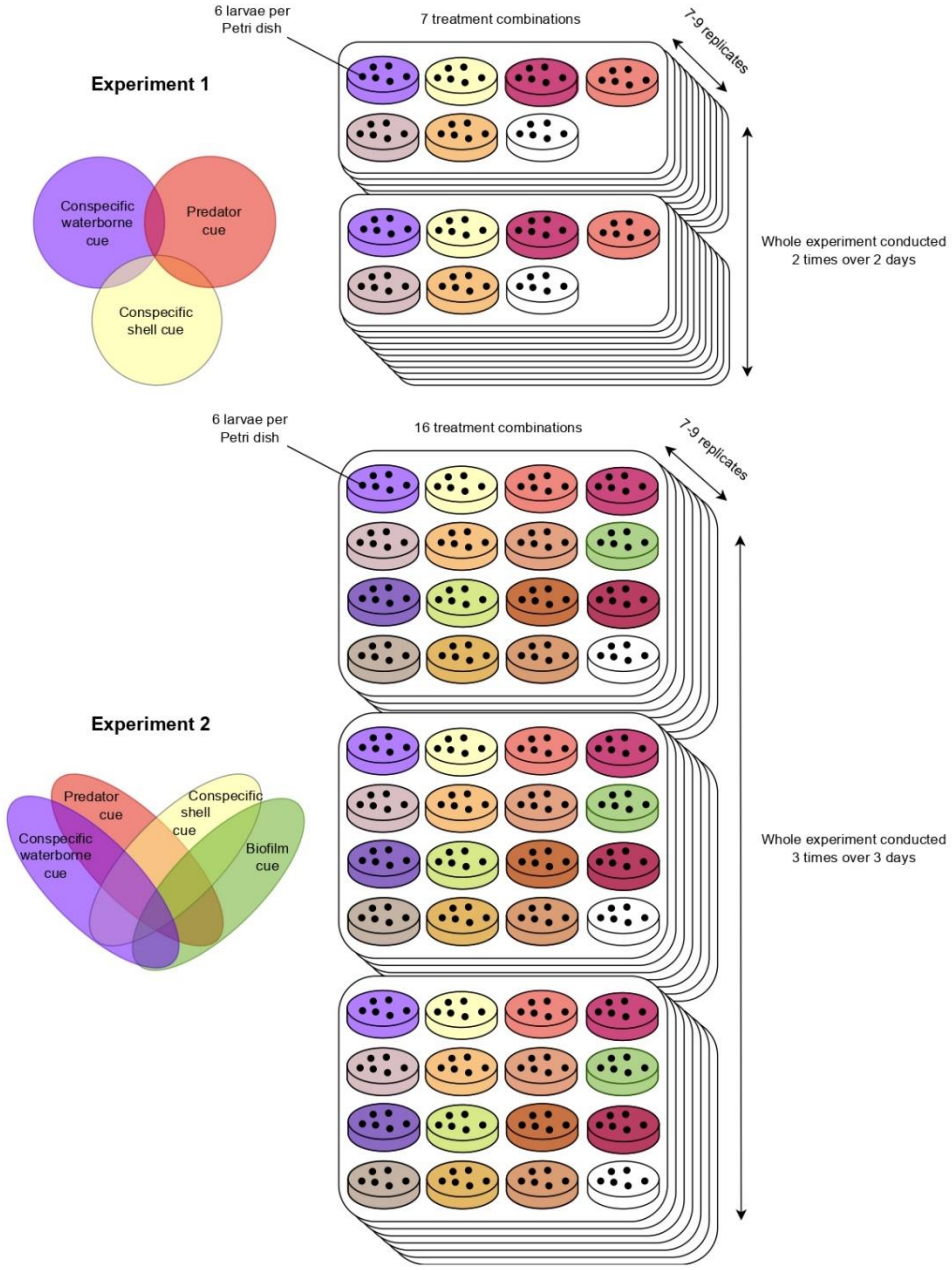


Figure 3.1. Schematic representation of the experimental design, sample sizes, and levels of replication for Experiment 1 (above) and Experiment 2 (below). Each color of Petri dish corresponds to a specific cue treatment combination. In Experiment 1, the experiment was repeated twice over two days, and in Experiment 2, it was repeated three times over three days. Each cue treatment combination was replicated on each day of the experiment, with 7–9 replicates per treatment per day.

## Larvae cultures

The parent oysters used in experiment 1, were purchased from Guernsey Sea Farms Ltd (Guernsey, UK). We fertilized two batches of eggs from these adults (five of each sex) five days apart so that competent larvae were available during all days of the experiment. For experiment 2, we used mature adult oysters (10 of each sex) from the harbor of Ostend (Belgium). The process of rearing larvae was kept the same in both experiments. See supplement for a detailed account. Immediately prior to using larvae in the experiments, larvae were taken from the culture tanks and filtered through two consecutive sieves of 300 and 260 µm. Only larvae retained on the second (260 µm) sieve were used in the experiment. Larvae were allowed to acclimate in 0.1µm filtered sea water (FSW) for an hour in the climate-controlled room where experiments took place.

### Preparation of conspecific waterborne cues

Production of all waterborne cues (predator and conspecific) was performed similarly to the methods from Morello and Yund (2016). For production of conspecific cues, adult oysters were collected (wet weight of soft parts was 7.6 ± 2.96 g) from the harbor of Ostend (Belgium) 3 days prior to the start of the experiments. Adults were scrubbed clean of any biofilms and kept for 24 hours in filtered seawater (0.1µm) at 15 °C. Subsequently, we placed eight adult oysters in 1800 ml of filtered aerated water for 48 hours and then filtered the water through a 30 µm nylon sieve. Water was kept at 15 °C until used in experiments.

### Preparation of Predator cues

To produce predator cues, we collected adult individuals of *C. maenas* from the harbor of Ostend (Belgium) 51.237889° N, 2.924133° E and held them in FSW aerated tanks at 15 °C before use. Predator cue water was created similarly to conspecific cue by placing one crab into 600 ml of FSW for 48 hours. Four crabs were used in the first experiment and three crabs were used in the second. The average wet weight of crabs in the first experiment was 53.2 ± 13 g and measuring 6.05 ± 0.62 cm in max carapace length. In the second experiment crabs weighing 66.8 ± 5g and measuring 6.6 ± 0.60 cm were used and cue water was combined before use in experiments. At 48 hours crabs were removed and cue water was filtered through a 30 µm sieve.

**Preparation of conspecific shell cues**

Conspecific shell chips came from live oysters collected from the Ostend coast. First, we removed the soft parts of the oyster and scrubbed the shells thoroughly. We then crushed the shells using a hammer and sieved them through 1.0 mm and 0.5 mm metal sieves, collecting the shell fragments that were retained by the 0.5 mm mesh screen and drying them in a 30 °C oven. Shells were stored in a freezer at −20 °C until use in experiments (similar to the method used by Vasquez et al., (2013)). To create substrates without an active cue, we heated the shell chips at 300 °C for 3 hours. This has been shown in previous research to eliminate the attractive properties of the cue associated with the shell (Vasquez et al., 2013). The preparation of chips without attraction was necessary so that all treatments would have the same settlement surface topography.

**Preparation of biofilm cues**

Marine biofilms occur naturally on surfaces in seawater. These biofilms consist of a combination of species, including bacteria, diatoms, other algae, fungi, and protozoa. Over time, these diverse organisms interact, eventually forming a matrix of extracellular polymeric substances (Davey and O'Toole, 2000; Flemming & Wingender 2010). It has been well documented that components of marine biofilms induce settlement and metamorphosis of larvae in many marine invertebrates, including oysters (Dobretsov & Rittschof, 2020), and so biofilms were included in this study to represent what we hypothesized to be a positive settlement cue.

Natural biofilms were allowed to develop on shell chips placed submerged in the Ostend harbor. Shell chips were kept in 260 µm nylon mesh, allowing water to flow through to the shell chips, enabling colonization by microbial organisms. Biofilms were left to develop undisturbed for 9 days. Immediately prior to experiments we retrieved the chips with biofilms and transported them with natural seawater to prevent them from drying out.

**Metamorphosis Assessment**

Per replicate, we moved six random pediveliger larvae into 15 ml Petri dishes with shell chip substrate (either substrate with cue or sham chips without cue). Each Petri dish contained 7 ml of one or both of the two types of treatment water and were topped up with FSW for a total of 14 ml. Petri dishes were

made of polystyrene and had a diameter of 35 mm and a maximum volume of 15 ml. New plates were used for each replication. We evaluated the cue treatments by counting the number of individuals that metamorphosed to post-larvae after 30 hours of exposure. In experiment one metamorphosis was assessed after 10, 20, and 30 hours, metamorphosis increased in all treatments overtime and no differences were noted in interactions. Due to the low metamorphosis rates at shorter time allowed , statistical analysis was more difficult, thus 30 hours was chosen as the time to assess metamorphosis (see table 3.S2 in supplement). Microalgae were added to the seawater in each treatment at the same rate as larvae cultures (*Caetoceros muelleri*, and *Isochrysis galbana* at 100,000 cells/ml at a volume ratio of 3:1) and were not limiting throughout the duration of the experiment. All trials happened in a 12 h day, 12 h night environment at 19 (±1) °C in a climate-controlled room.

**Statistical analysis**

For each experiment we created a generalized linearized mixed-effect model using the glmer function of the lme4 package (Bates et al., 2014) in R version 4.1.3 (2022-03-10) (R Core Team, 2021). As the response variable was binary (metamorphose vs. not metamorphose) we fitted a Bernoulli distribution using a logit link function. For each experiment, a base model was established, consisting of each cue treatment (conspecific cue, predator cue, conspecific shell, and biofilm) as fixed effect variables, and the batch from which the larvae originated as a random variable. A forward selection procedure, using the Akaike Information Criterion (AIC), was performed to determine if 1) any interaction effects of cue treatments or 2) the age of the larvae should also be included as fixed effect variables in the model as larvae age has been shown to impact settlement and metamorphosis propensity (Meyer et al., 2018) and larvae aged over the course of a multiday the experiment. A description of the final models can be found in the supporting information. Finally, we performed post-hoc tests using the emmeans function in R (Lenth., 2025). to calculate the marginal means adjusting p-values for multiple comparisons with Tukey's method and used the pairs function to display pairwise comparisons. See supporting information for model descriptions and descriptions of post-hoc comparisons (tables 3.S3 and 3.S4). Results display prediction plots produced by the model using the ggpredict function from the ggeffects package in R (Lüdecke., 2018). The effect of the treatments is given in predicted percent metamorphosized from the model. To compare raw data with model predictions, we calculated larvae metamorphosis as a percentage of the total larvae metamorphosized per petri dish.

# Results

**Experiment 1**

In the first experiment the presence of conspecific waterborne cues and conspecific shell both significantly increased predicted metamorphosis (p= 0.03, and p=1.77*10-10 respectively) (Table 3.1, Figure 3.2). Predator cues did not decrease metamorphosis significantly, however there was a non-significant trend showing a reduction of metamorphosis (p=0.09) (Table 3.1, Figure 3.2). The interaction between predator cue and shell treatments showed a non-significant trend (p=0.058). While the interaction of shell and conspecific cue, and conspecific cue and predator cue did not show any significant interaction (Table 3.1).

**Experiment 2**

In the second experiment, the presence of waterborne conspecific cues, conspecific shells, and biofilms significantly increased the predicted metamorphosis (Table 3.1). Post-hoc tests showed that for all positive cues, metamorphosis was significantly increased with each additional cue (Figure 3.3, table 3.S4 in supplement). There were some significant interactions between cues (conspecific shell and predator cues and conspecific cues and biofilms (Figure 3.3, table 3.1).

Synergistic interactions were defined when there was both a significant interaction effect between two main effects and where the addition of these main effects resulted in more predicted metamorphosis than the anticipated sum of the the effects individually, calculated by subtracting the model output probability of metamorphosis from an expected probability of metamorphosis that is the addition of the probability of each of the two cue. By this definition there was a slightly synergistic effect of conspecific cues and biofilms.

The presence of predator cues did not significantly change the predicted metamorphosis overall (Table 3.1). Post hoc analysis revealed that when waterborne conspecific cues and predator cues were combined there was a non-significant trend for some reduction in metamorphosis, compared to treatments of conspecific cues alone (p= 0.0998) (see table 3.S4 in supplement). This same pattern (reduce metamorphosis when both positive and negative cues were experienced together compared to

treatments of positive cues alone) was not seen with the other positive cues (biofilms and conspecific shell) (see table 3.S4 in supplement).

Table 3.1. Results of the statistical models for each experiment. Significant ($p < 0.05$) are in **bold**, while marginally-significant ($p < 0.1$) are in *italic*.

| Experiment | Predictor Variable | Estimate | Std. error | z-value | p-value |
|---|---|---|---|---|---|
| Exp. 1 | Conspecific cue present | 1.598 | 0.529 | 3.020 | **0.003** |
| | Predator cue present | -1.864 | 1.108 | -1.683 | *0.092* |
| | Shell cue present | 3.285 | 0.515 | 6.380 | **$1.77*10^{-10}$** |
| | Conspecific cue * predator cue | 0.946 | 1.187 | 0.797 | 0.426 |
| | Conspecific cue * shell cue | -0.238 | 0.635 | -0.375 | 0.708 |
| | Predator cue * shell cue | 2.180 | 1.151 | 1.893 | *0.058* |
| Exp. 2 | Conspecific cue present | 1.780 | 0.192 | 9.263 | **$< 2*10^{-16}$** |
| | Predator cue present | 0.208 | 0.188 | 1.104 | 0.270 |
| | Shell cue present | 2.246 | 0.163 | 13.738 | **$< 2*10^{-16}$** |
| | Biofilm present | 1.320 | 0.239 | 5.529 | **$3.22*10^{-8}$** |
| | Conspecific cue * predator cue | -0.426 | 0.213 | -1.997 | **0.046** |
| | Conspecific cue * biofilm | -0.453 | 0.221 | -2.054 | **0.040** |
| | Shell cue * biofilm | -0.403 | 0.223 | -1.812 | *0.070* |
| | Predator cue * biofilm | -0.261 | 0.213 | -1.222 | 0.222 |

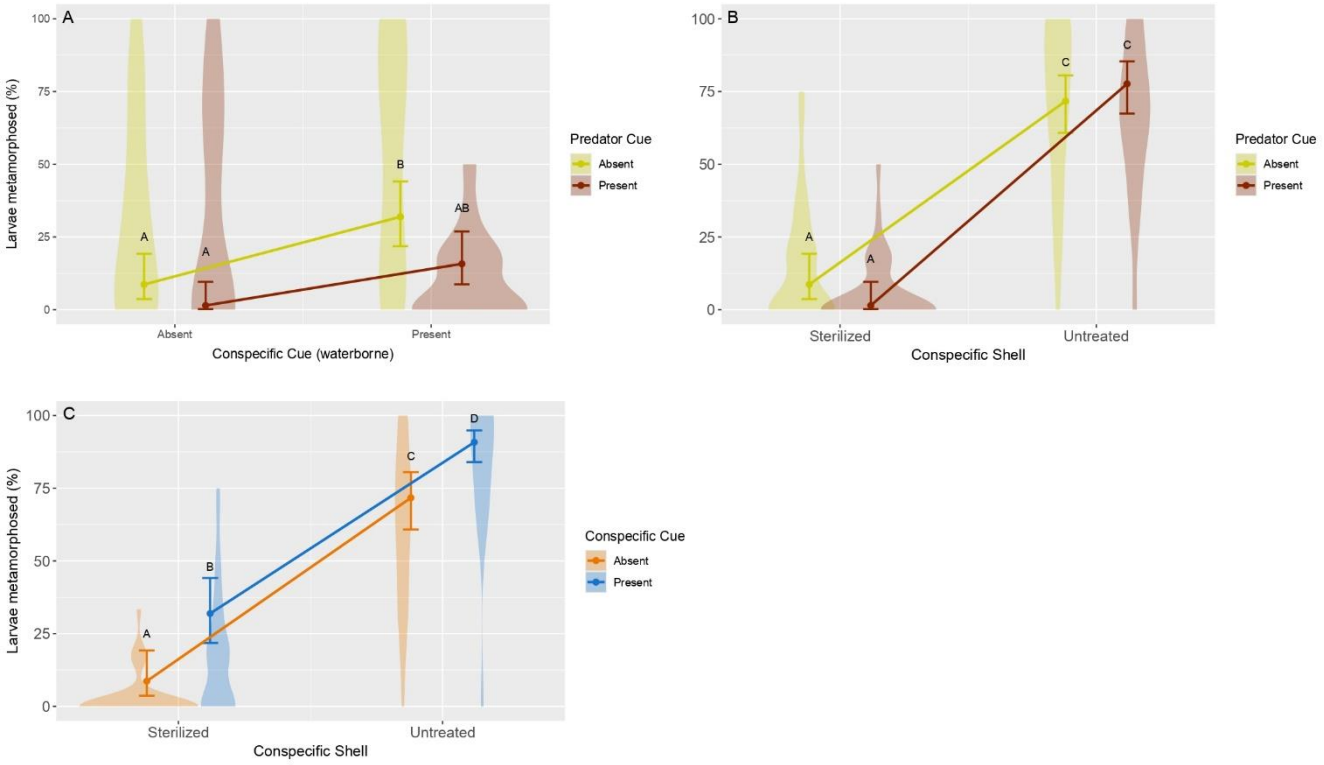


Figure 3.2. Results from experiment 1. Predictions of the generalized linear mixed effect model showing estimated probability of larvae settlement. Error bars represent 95 % confidence intervals of model prediction. Raw data from percentage larvae settled is represented by violin plots. **A)** Predicted larvae settlement when exposed to cues from predator *C. maenas* and conspecific waterborne cues. **B)** Predicted larvae settlement when exposed to cues from predator *C. maenas* and conspecific shells. **C)** Predicted larvae settlement when exposed to cues from waterborne and shell conspecific cues.

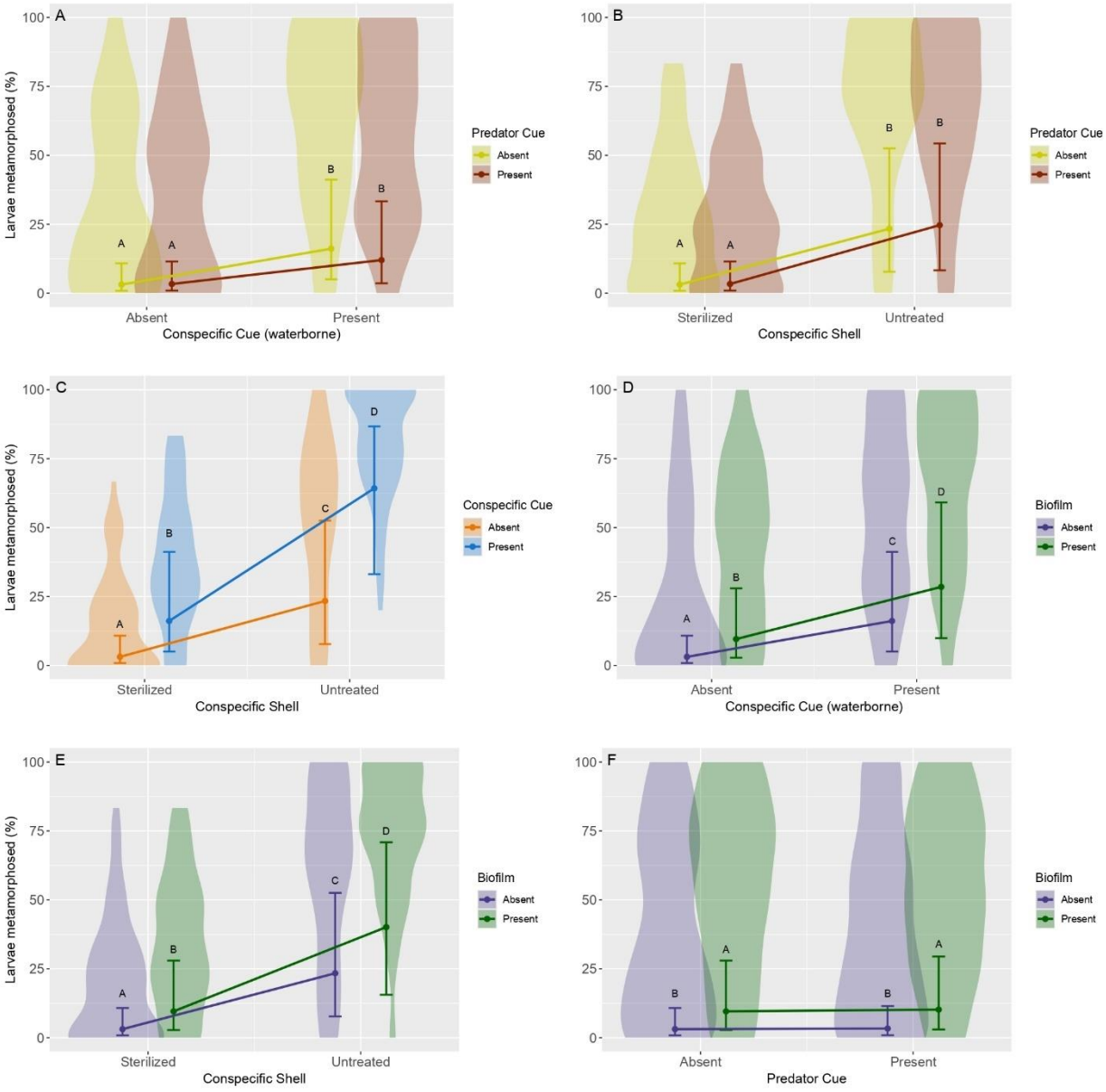


Figure 3.3. Results from experiment 2. Predictions of the generalized linear mixed effect model showing estimated probability of larvae settlement. Error bars represent 95 % confidence intervals of model prediction. Raw data from percentage larvae settled is represented by violin plots. **A)** Model predicted and actual larvae settlement when exposed to cues from predator *C. maenas* and conspecific waterborne cues. **B)** Model predicted and actual larvae settlement when exposed to cues from predator *C. maenas* and conspecific shells. **C)** Model predicted and actual larvae settlement when exposed to cues from waterborne and shell conspecific cues. **D)** Model predicted and actual larvae settlement when exposed to cues from waterborne conspecific cues and biofilms. **E)** Model predicted and actual larvae settlement from conspecific shells and biofilms. **F)** Model predicted and actual larvae settlement from predator cues and biofilms.

# Discussion

Natural environments are complex sensory landscapes. Multiple simultaneous settlement/metamorphosis cues may require the larva's responses to an individual cue to change depending on the prevailing combination of cues. However, little is known about behavioral responses to multiple conflicting cues. In both experiments 1 and 2 the interaction of positive cues led to higher rates of metamorphosis overall with each additional cue raising the probability of metamorphosis higher. Slight synergistic relationships could be seen between some of the positive cues, specifically, between conspecific waterborne cues and biofilm cues in experiment 2.

Responses to predator cues were less clear and changed slightly in experiment 1 and 2. In experiment 1 we found a non-significant trend suggesting that predator cues decreased metamorphosis (*P=0.092*). In experiment 2, there was also a nonsignificant trend (*P=0.0998*) reducing metamorphosis, but only in response to conflicting cues from a combination of waterborne positive conspecific cues and negative predator cues, compared to treatment with only conspecific cues. There are a few ways to interpret these differences between the two experiments.

In experiment 2 overall metamorphosis was generally lower than in experiment 1 metamorphotic rates without any cues present (in the control) or in predator-cue-only treatments were generally very low (~5 %) making it difficult to detect a response. The reason a response could be detected could be that overall metamorphosis was raised enough to see an effect by the addition of conspecific waterbourn cue. Aside from an issue of experimental power, there could be other reasons for the discrepancy. The two experiments had different parental origins of the larvae. In experiment 1, adults were purchased from a hatchery in Guernsey, while in experiment 2 adults were collected from the Ostend harbor. While this predator (*Carcinus maenas*) is present both in Guernsey and in Belgium differences between the frequency of predators or predator exposure of the parents could lead to different response in offspring, such as that seen in marine snails where constant exposure to predators resulting in offspring who display bolder predator responses (Donelan & Trussell., 2018). Overall, interpretation of the effect of the predator cue must be taken with caution. However, as there was some negative effect of predators in both studies (albeit showing non-significant trends) we call for continued investigation of these predator-prey

dynamics between these two species and can lightly interpret this predator effect in the context of this study.

Interestingly in both experiment 1 and 2, we did not observe any reaction to predator kairomones in combination with the presence of positive cues from oyster shells or biofilms. While previous work has identified (or theorized) that environmental cues may be ranked in a hierarchical way (Igulu et al., 2013; Hodin et al., 2018) to the best of our knowledge these are the first results suggesting that hierarchy applies to predator cue interpretation. Discrimination of cues may play an important role in decision making in the presence of multiple conflicting cues.

**Settling larvae may rank conflicting cues**

Our results indicate that when exposed to conflicting cues, *M. gigas* larvae adjust their behavior in a way that is specific to the respective combination of cues. When presented with two conflicting waterborne cues from conspecifics and their predator *Carcinus maenas*, it seems that larvae have evolved the ability to "added up" positive and negative cues and adjust behaviour accordingly. This means that the presence of predator kairomones reduced metamorphosis, but not to the level observed in the absence of the positive cue. But when faced with positive cues associated with the shells of conspecifics or with biofilms, larvae were less risk averse and settled regardless of the presence of a negative cue. This observation could suggest that oyster larvae may perceive cues differently based on their source (waterborne vs substrate-bound). If the positive cue is perceived as stronger or more important than the negative cue, the negative cue will be disregarded (or downweighed), resulting in an optimistic decision for settlement. The cues we found in this study to be high ranking were those associated with conspecific shells and biofilms, both of which are substrate-bound. In the case of a similar ranking, the additive effect of both cues will render the settlement choice balanced according to the relative strength of the cues, as seen in the conflicting waterborne cues in our experiment. Extrapolating, this would imply that for the lowest ranks of positive cues, the larvae use a pessimistic strategy and do not settle in the presence of negative cues. Combinations of multiple positive cues were seen to increase metamorphosis in an additive way: the addition of simultaneous positive cues will provide more confidence of larvae in their choice.

**Waterborne vs substrate-bound cues**

In many marine invertebrate taxa, both settlement (the initial larva descent to a substrate, , and metamorphosis can be induced by a single cue source (Morse and Morse, 1984; McGee and Targett, 1989;

Pearce and Scheibling, 1990). Larvae of other taxa require a combination of waterborne and substrate-bound cues to first settle and then metamorphose (Chia and Koss, 1988). Different signaling pathways may exist in bivalve larvae for perceiving and reacting to different cues (Joyce & Vogeler., 2018). In our study, settlement vs metamorphic cues could not be distinguished therefore, we cannot discriminate between the effect of settlement cues on initiating descent from the water column to the substrate and on metamorphosis, respectively. The scope of acceptable cues for bivalve settlement and metamorphosis tends to be broad, and which combination is needed can be species specific (Bao et al., 2007; Yang et al., 2013). For *M. gigas*, waterborne conspecific cues have been shown to increase settlement propensity (Zimme-Faust & Tamburri, 1994) and, as indicated in our study, also metamorphosis. Chemical waterborne cues from biofilms and conspecific cues can operate on larvae mainly within centimeters of the substrate but before they attach, influencing their swimming behaviors (Hodin et al., 2018). Substrate-bound cues, however, indicate an immediate favorable settlement location. Our study suggests that waterborne cues become irrelevant once larvae sense a substrate-bound cue, and thus predator cues lose importance for the decision to metamorphize. This fits well within the hypothesized theory that hierarchically arranged signaling pathways for which larvae receive cues may be structured in a way that corresponds to the spatial scale over which the cue operates.

**Future perspectives and limitations**

This study provides a proof of concept, and our findings should be substantiated with future research. How larvae respond at different levels of cues was not tested here but is relevant to predicting actual outcomes in the field and incorporating into models predicting larvae behavior. Whether or not predator response in this species depends on the dose or simply presence of the kairomones needs to be established experimentally. In this study we focused on larvae metamorphosis, specifically the reactions to cues once they had reached a settlement location. However, assessing larvae behaviors in the water column in vertical dimensions could complement these results, while predators may be disregarded when larvae have reached shell substrates on which to settle, they may deter more larvae from even reaching this point. Addressing these questions experimentally could also help answer questions about the mechanisms underlying how larvae discriminate and prioritize cues. While little is known about cue integration in bivalve larvae on a physiological level, it is known that in other species, this integration of information happens in a structured way where one cue will activate or repress sensory pathways necessary to perceive other cues. For example, in sea urchin *Strongylocentrotus purpuratus* and sand dollar *Dendraster excentricus*, water turbulence primes larvae to respond to local chemical and substrate-

associated cues (Gaylord et al. 2013; Hodin et al. 2015). While our study theorizes that substrate bound cues may override signals from predators, this theory could be further supported by investigating the specialized sensory pathways or receptor systems in bivalves responsible for detecting different cue types (substrate-bound vs. waterborne). Complementing behavioral experiments such as the one presented in this study with assessment of neurological and physiological mechanisms will provide a more comprehensive understanding of cue integration in larval settlement.

**Implications for pacific oyster reefs**

Marine invertebrate larvae have evolved to make the transition from pelagic to sessile quickly once suitable conditions are met, in contrast to other metamorphosing taxa like insects and amphibians (Hadfield, 2000). This may be especially true for *M. gigas*, which has broader ecological tolerances than other oyster species (Troost K., 2010). For example, oyster *Ostrea edulis* reproduces through internal fertilization (Bayne, 2017), meaning that proximity to conspecific adults may be perceived as more critical than for *M. gigas* which are external broadcast spawners. For generalist species like *M. gigas*, it therefore might not be very advantageous to delay settlement until suitable conditions are met, thus favoring the evolution of optimistic settlement/metamorphic strategies. In the long run, insights from this research may enable tailored ecosystem management practices for designing reef restoration projects, spat collectors for bivalve mariculture, or developing biofouling mitigation strategies.

# Chapter 4: Comparison of the effects of reef and anthropogenic soundscapes on oyster larvae settlement/metamorphosis

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# Abstract

Settlement/metamorphosis is a critical period in the life cycle of marine invertebrates with a planktonic larval stage. For reef-building invertebrates such as oysters and corals, settlement rates are predictive for long-term reef survival. Increasing evidence suggests that marine invertebrates use information from ocean soundscapes to inform settlement decisions. Sessile marine invertebrates with a planktonic stage are particularly reliant on environmental cues to direct them to ideal habitats. As gregarious settlers, oysters prefer to settle amongst members of the same species. It has been hypothesized that oyster larvae from species Crassostrea virginica and Ostrea angasi use distinct conspecific oyster reef sounds to navigate to ideal habitats. In controlled laboratory experiments we exposed Pacific Oyster *Magallana gigas* larvae to anthropogenic sounds from conspecific oyster reefs, vessels, combined reef-vessel sounds as well as off-reef and no speaker controls. Our findings show that sounds recorded at conspecific reefs induced higher percentages of settlement by about 1.44 and 1.64 times compared to off-reef and no speaker controls, respectively. In contrast, the settlement increase compared to the no speaker control was non-significant for vessel sounds (1.21 fold), combined reef-vessel sounds (1.30 fold), and off-reef sounds (1.18 fold). This study serves as a foundational stepping stone for exploring larval sound feature preferences within this species.

# Introduction

Identifying a suitable habitat prior to permanently transitioning to a benthic life stage is critical for future survival, growth, and reproduction in many marine invertebrates with planktonic larvae. These species therefore utilize of a variety of environmental cues, enabling them to identify promising settlement locations (Hadfield & Paul., 2001). Experimental research has shown that in some species, a single cue can induce settlement and subsequent metamorphosis (Burke et al., 1984; Pearce, C. M. & Scheibling., 1990; Hadfield & Paul., 2001). But in many other species larvae may respond to more than one cue, *Crassostrea virginica* larvae for example respond similarly to chemicals released by conspecific adults, and chemicals released from mature biofilms (Tamburri et al., 1992). Cues can have chemical and physical origins, and while some types of cues require contact with a prospective settlement location such as cues associated with shells of conspecifics adults or cues from topographical features of a substrate (Vasquez et al., 2013;

Johnson., 2017), other cues, such as turbulence may act over larger distances to guide larvae to their preferred habitat (Fuchs et al., 2015). More recently, acoustic cues have been identified as drivers of larval settlement (Vermeij et al., 2010; Williams et al., 2022). As sound propagates relatively fast and far underwater, it serves as an efficient signal transmission medium. For many marine species, sounds can convey specific events, such as presences of a predator or a mating opportunity (Hughes et al., 2014; Amorim et al., 2015). But, collectively, soundscapes can also convey overall quality and suitability of an environment for a species (Duarte et al., 2021; Miller et al., 2022). Research on acoustic cues informing larvae about optimal habitats has only been established relatively recently (Vermeij et al., 2010; Williams et al., 2022) In certain invertebrate species with a settlement/metamorphosis stage, including crabs, corals, and bivalves, acoustic cues have been shown to affect larvae swimming direction (Vermeij et al., 2010; Williams et al., 2022), settlement rates(Lillis et al., 2013; Williams et al., 2022), and amount of time a larvae takes from entering competency to completing metamorphosis (Stanley et al., 2010; Wilkens et al., 2012; Pine et al., 2012). In general, it seems that natural environmental sounds can convey information to invertebrate species in that environment (Solé et al., 2023). In coral and bivalve reefs, larvae seem to be attracted to soundscapes from healthier reefs, which produce louder and more acoustically complex sounds compared to less healthy reefs which are much quieter (Lillis et al., 2014a; 2016). However, the particular characteristics of reef soundscapes (e.g. sound pressure level (SPL), specific frequencies, complex mixtures of these or other acoustic characteristics) that elicit settlement behaviors remain unclear.

Anthropogenic sounds may interfere with or mask natural marine soundscapes (Duarte et al., 2021). Vessel noise can mask important sound cues resulting in poorer orientation toward reef sounds for some species of fish (Holles et al., 2013; Wilson et al., 2023), and cause coral larvae (planulae) to delay settlement (Lecchini et al., 2018). Anthropogenic noise can not only disrupt or reduce larval settlement but may also be (mis)interpreted as a cue to settle in some taxa (Wilkens et al., 2012; Pine et al., 2012; Stocks et al., 2012; Jolivet et al., 2016; Gigot et al., 2023). Vessel noises have been shown to increase some larvae settlement, including in mussel *Perna canaliculus* and *Mytilus edulis* (Wilkens et al., 2012; Jolivet et al., 2016). Why anthropogenic noises are interpreted as settlement cues in some taxa but are inhibiting to others is unknown. The reaction to anthropogenic sounds may depend on the acoustic profile of a species' preferred habitat and which features of this profile are responsible for attraction (Erbe., 2008; Pine et al., 2012; Stocks et al., 2012; Li et al., 2015).

The oviparous true oyster *Magallana gigas* is an important reef-building ecosystem engineer (Walles et al., 2016) and a valuable species for aquaculture (Botta et al., 2020). But in many areas it is invasive and considered a biofouling pest that poses a threat to local species and ecosystems (Ruesink et al., 2005; McAfee & Connell., 2021). There is considerable interest in settlement preferences of this species for both bolstering as well as reducing recruitment. In recent years, there has been a global effort to restore oyster reefs, as widespread habitat destruction has left native historical populations decimated (Beck., 2011). Availability of settlement cues is crucial for reef sustainment, with some reports suggesting that these cues may outweigh other recruitment factors such as local hydrodynamics, and larvae supply (Pineda et al., 2010; Koehl & Cooper., 2015). A recent revelation that oyster *Ostrea angasi* not only settle more rapidly but also exhibit horizontal swimming movements toward sound sources underscores the significance of soundscapes as a navigation tool for larvae (Williams et al., 2022). So far, larvae of *M. gigas* have not been studied for their response to acoustic settlement cues (but see Stocks et al, (2012) for an account of swimming activity in response to natural and vessel sounds). *M. gigas* adults have been studied for their sense of hearing, and were found to react by valve closure to pure tones in the range of 10 to 1000 Hz at minimum energy of 122 dBrms re 1 µPa (Charifi et al., 2017). While these adults are studied for their pure tone reactions, the range of hearing of these larvae have not yet been identified. Other true oysters with relevant experimental data are the closely related and also oviparous *C. virginica,* and the more distantly related larviparous *O. angasi*. Experimental studies have shown that both *C. virginica* and *O. angasi* larvae prefer louder reef sounds in frequency ranges 1.5-20kHz over quieter off-reef playbacks or no-sound controls (Lillis et al., 2013; McAfee et al., 2023; Williams et al., 2022).

In this study, we present the results of laboratory-playback based settlement experiments on the role of acoustic cues in settlement and metamorphosis of *Magallana gigas*. Firstly, we were interested in the importance of oyster reef sound compared to off-reef sound. Secondly, we wanted to know whether vessel noise attracts or repels pediveliger larvae. To do so, we exposed larvae to different vessel and reef sounds as well as off-reef and no-speaker controls. Finally, we subjected the larvae to vessel and reef sounds simultaneously to find out whether vessel noise modifies, or masks oyster reef sound cues.

# Methods

We conducted laboratory tank-based playback experiments to investigate whether oyster species *Magallana gigas* larvae alter their settlement response in reaction to sounds emitted by conspecific oyster reefs and vessels. Sound recordings were obtained from two regions within the North Sea, and acoustic spectral features were analyzed based on recordings made within experimental tanks.

### North Sea soundscape measurements

All recordings used during the experiment were recorded in two regions of the North Sea: the Southern Bight near the Belgian coast and in the Dutch Wadden Sea (see Figure 4.1).

In the Dutch Wadden Sea, recordings at subtidal reef sites were collected with hydrophones (SoundTrap 300STD; Ocean Instruments, NZ; sampling rate 24 kHz; see Table S1 for details manufacturer-calibrated; set at low gain). Hydrophones were suspended in PVC frames anchored to the seafloor and a subsurface buoy ensured that the hydrophone was positioned approximately 1 m above the seafloor in water depths ranging from 2 to 5 m. These were deployed from a small boat, and left to record continuously for two weeks or the maximum battery life. Reef sound recordings used for this experiment were taken at two subtidal oyster reefs in the Marsdiep tidal basin of the eastern Wadden Sea (Table S2). An off-reef sand recording used in this experiment was obtained from a control recording of an artificial reef monitoring in the Eierlandse Gat tidal basin, near the island Vlieland.

For the Belgian part of the North Sea, underwater sound data used were recorded as part of the LifeWatch Broadband Acoustic Network (Parcerisas et al., 2021). These data are collected continuously in different fixed locations in the Southern Bight. All locations were close to a shipwreck (<50 m), but there are no oyster reefs present. These data were collected using a RESEA 320 recorder (RTSys, France) together with a hydrophone. For the stations Buitenratel and Grafton, the hydrophone used was a Colmar GP1516M-LP (Colmar, Italy, sensitivity: -168 dB/V re 1 µPa, frequency range -3 dB: 10 Hz to 70 kHz). For the stations Fairplay and Faulbaums, it was a Colmar GP1190M-LP hydrophone (Colmar, Italy, sensitivity: -180 dB/V re 1 µPa, frequency range -3 dB: 10 Hz to 170 kHz). The hydrophone and the acoustic recorder were attached to a stainless-steel structure frame, at 1 m above the sea bed which stood stable on the seafloor. All stations were between 10 and 30 m deep. Deployments lasted between 4 and 6 months.

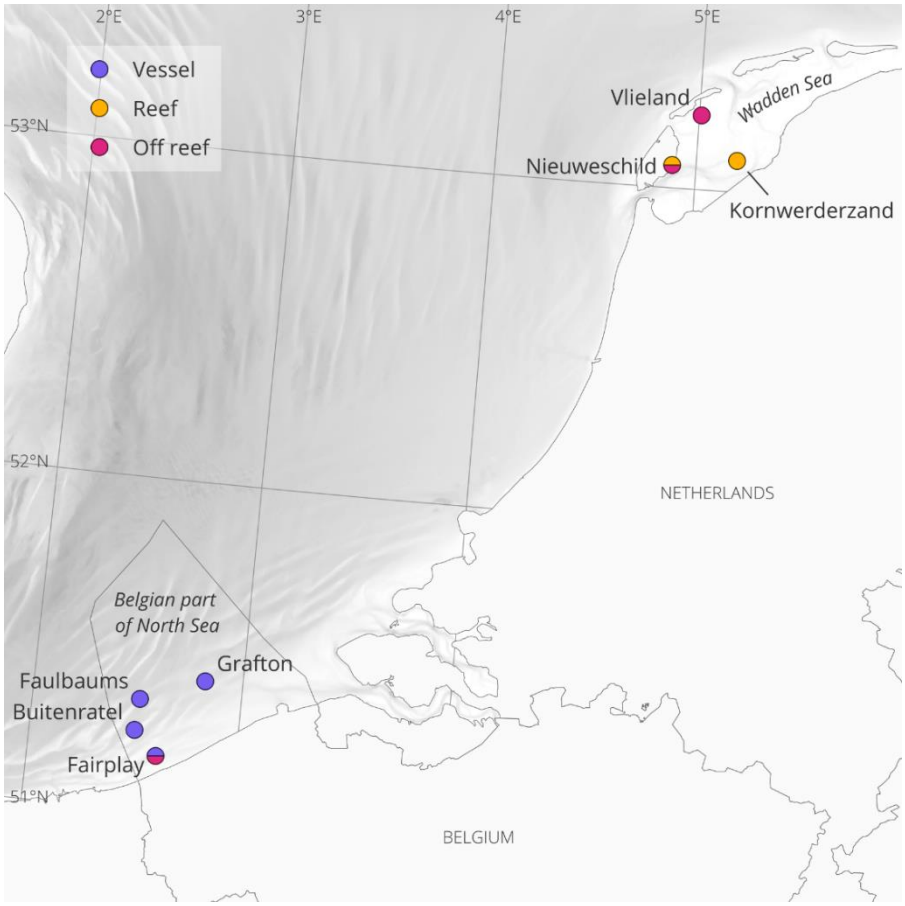


Figure 4.1. Distribution of the locations where underwater sound data were collected. Colors represent which treatment was collected there. Sounds acquired in locations with two colors were used for different treatments. Map made by maps@vliz using QGIS version 3.30.0-'s-Hertogenbosch (https://qgis.org/en/site/).

## Sound treatments and playbacks

Suitable recording files for each treatment were manually selected. Only Belgian recordings from spring and summer were considered, in order to correspond to the recording period of the sounds from the Wadden Sea (reef and off-reef). Reef sounds were only selected when they contained no apparent outside influences (e.g. vessel sounds). For vessel sounds, a fair variability of sounds was selected, from short sounds of distant vessels to longer continuous sounds from vessels operating close by, with no other audible background sounds. All vessel sounds were recorded from locations in the Southern Bight (see Table S2). For reef treatment, sounds used were recorded from the same location in Texel, NL but sound files used during each day of the experiment were selected from different recording dates (see Table S2). For off-reef sounds, two sound files were used recorded from Texel, NL and two sound files were used

recorded from non-reef areas in the Southern Bight off the coast of Belgium (see Table S2). Treatments where vessel sounds and reef sounds were played together were created artificially overlaying reef sound files and vessel sound files. Treatments where vessel sounds and reef sounds were played together were created artificially overlaying reef sound files and vessel sound files.

Selected segments were then combined to create one 1 h file per treatment and day. In some cases, the selection led to files shorter than 1 h, so the segments were repeated and combined by applying crossfading with Audacity (Team A. Audacity., 2023) to create a 1 h file. When enough recordings were available for 1 h or more, segments were not repeated. To deal with differences in sampling rate and minimum recording frequency between the selected files, all files were filtered using a Butterworth bandpass filter (N=4) between 20 Hz and 12 kHz. After filtering, all files were downsampled or upsampled to 48 ksps to match the playback requirements. Details of the selected data are listed in Table S2. In total, 3 recordings of reefs from 2 different locations, 4 vessel recordings with several boats on each recording from 4 different locations, and 4 off-reef recordings from 3 different locations were used to represent our treatments.

Throughout the experiment, each treatment group containing sound playback (“reef” “vessel” “reef+vessel” “off reef”) consisted of a separate recording representing the intended environment. The use of multiple sound files of the same treatment was used to strengthen confidence that the sounds were representative of treatments as a whole, and not of a single event. Employing a series of recordings from various sound sources representing the same treatment enhances the extrapolative capacity of a study (Slabbekoorn & Bouton., 2008; Slabbekoorn., 2013; Hubert et al., 2021).

The playback set-up consisted of five 100L tanks (49x65x50.5 cm), separated 20 cm from each other on a rack. Each tank sat upon a 4 cm layer of polystyrene to isolate it from the rack and an additional layer of acoustically absorbent foam (25mm thick) between the polystyrene and the tank bottom. Acoustic foam was also placed at the tank sides. Four Lubell UW30 Underwater Speakers with custom-made amplifiers, battery-powered to avoid 50 Hz noise or electrical interference from the power grid, were used. Each speaker was connected to one TASCAM playback device which played playback files on repeat. No speaker was placed in the no-sound control (see Figure 4.2). This no speaker treatment was added as a second control (aside from the off-reef control) to establish if there were differences between treatments with sound and normal lab conditions. The speakers were hung in the middle of the tank with ropes so they would not touch the tank walls. Larvae were placed inside 100 ml polystyrene jars and these containers were fixed in the same position in the tank for every day of the experiment. These positions were 12.75

cm far from the closest jar (distance between outer jars and center jar), and 33.5 from the speakers (see Figure 4.2).

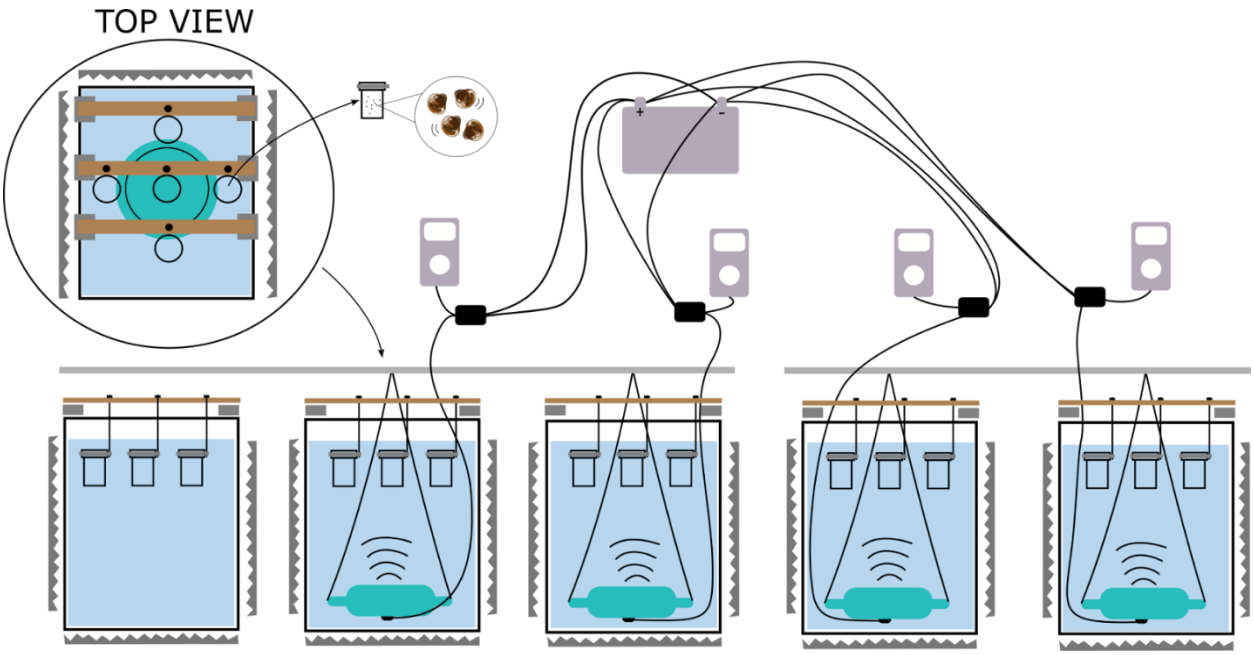


Figure 4.2. Schematic depicting five tanks. Four of these tanks are equipped with speakers, each of which is connected to a playback device. All speakers and playback devices are linked to a DC battery as their power source. Five 100ml jars were securely positioned to hang at the same height above speakers. The speakers were suspended within the tanks in a manner ensuring they did not come into contact with the tank's bottom. Acoustic isolation foam panels were placed around the tanks to help isolate the tanks from each other.

For each treatment file, a playback volume was chosen so exposure power spectral density (PSD) would match the sound levels recorded in field as closely as possible. Sound levels specified in literature as typical of reefs (at 1 m from the seafloor) and off-reefs (at 2 km from the reef) (Lillis et al., 2015; Williams et al., 2022) roughly matched the chosen levels.

This was done separately for each sound exposure treatment by adjusting the volume in an iterative fashion until the recorded sound levels in tanks were similar to the desired ones. For this purpose, each tank setup was recorded using a TASCAM recorder device together with an Aquarian Scientific hydrophone (AS-1). The hydrophone was placed inside a jar without touching the walls, simulating the position where larvae would be during the experiments. The hydrophone cable was taped to the structure supporting the jars. Recorded sound was converted to sound pressure by using the available calibration

data for the hydrophone and the TASCAM recorder. This calibration was cross-checked by comparing it to measurements of the hydrophone used in Hubert et al., (2022).

To measure sound level and acoustic characteristics of each playback received by larvae during the experiment, each treatment was recorded using the chosen playback volume for 1 h (tank recordings) at 48 ksps. This same procedure was used when selecting the playback volume. When recording these 1 h files, all four different sound treatments of that batch were turned on to record possible acoustic crosstalk from the other treatments. Furthermore, the room noise was also recorded using the same protocol when no speaker was active.

For each treatment, several acoustic features were computed for both 1 h recorded tank files and 1 h compiled field files. Acoustic Complexity Index (ACI), Acoustic Evenness Index (AEI), and Acoustic Diversity Index (ADI) were computed using the maad python package (Ulloa et al., 2021), and Power Spectrum Density (PSD) was computed using the scipy python package (Virtanen et al., 2020). The average PSD was computed for three different bands by averaging the spectrum density of all the frequency bins included in the specified frequency band. The parameters used to compute each of the features are summarized in Table S3.

Both ACI and ADI are proxies to quantify acoustic complexity (the higher the number, the more complex), while low values of AEI represent an even sound and higher values represent more uneven sounds. This is not correlated with the ecological concept of evenness, as acoustic evenness refers to an even distribution of sound energy in different frequency bands, and this can be achieved due to a high biodiversity vocalizing at the same time covering all the frequency bands or by constant broadband sounds such as some anthropogenic sounds (Bradfer-Lawrence et al., 2019; Mammides et al., 2020).

## Broodstock and Larvae Culture

Ten mature adult oysters (five females and five males) were purchased from the Guernsey Sea Farms Ltd (Guernsey, UK) and used to produce larvae. Eggs were fertilized by gonad stripping following FAO guidelines (Helm et al., 2004). Fertilized eggs were kept undisturbed in flat bottom tanks for 48 hours at 22 °C at a density of ten eggs per ml of filtered seawater (FSW). All seawater used in this experiment was filtered at 0.1 µm and passed through UV light. After 48 hours larvae were sieved over 70 µl nylon mesh, rinsed, and transferred to rearing tanks with FSW. Tanks were aerated and kept at 22 °C for the entire duration of larvae rearing. Every two days larvae were sieved over mesh corresponding to the average

size of the larvae and the water in the tanks was changed. Larvae were fed a mixture of fresh microalgae mixture consisting of *Chaetoceros muelleri*, and *Isochrysis galbana* (clone T-ISO). For the first 4 days larvae were fed at 40,000 cells/ml water using only *I. galbana* (clone T-ISO). Days 5-12 larvae were fed *C. muelleri*, and *I. galbana* (clone T-ISO) at 100,000 cells/ml at a volume ratio of 1:1. Days 13+ larvae were fed *C. muelleri*, and *I. galbana* (clone T-ISO) at 100,000 cells/ml at a volume ratio of 3:1. Larvae entered their pediveliger stage and became competent to settle at 29 days and were used in settlement experiments starting on this day. Larvae were determined for competence when they had a prominently displayed eyespot and larval foot and were sized at 320-350 µm in diameter.

### Settlement Experiment Design

The experiment aimed at assessing the effect of sound treatment on larval settlement. In the experimental design, we were constrained by having only four underwater speakers at our disposition. We therefore repeated the experiment four times over five consecutive days. In each of the four trials, different treatments were assigned to a unique combination of speaker and tank to account for possible speaker or tank effects. The sound treatment was applied at the tank level, making tank the experimental unit. As the experiment has a binary outcome (settled vs. not settled), many sample units (larvae) are needed to accurately assess the treatment effect. Therefore, ten larvae were placed together in jars and five such jars were placed in each tank (see Figure 4.2). Larvae were not re-used and for each trial, new pediveliger stage larvae were taken randomly for the same stock. As a consequence, larvae were gradually older through the five day experiment.

### Settlement Assays

Experiments took place over five consecutive days (03/03/23 – 07/03/23) using larvae from the same batch. To control for larvae size, on each experiment day some larvae from culture tanks were filtered between 260 µm and 300 µm nylon mesh sieves, only larvae retained on the 260 µm sieve were used in the experiment. 10 larvae were gently pipetted randomly into each of the five 100 ml containers per tank and filled with filtered seawater (FSW), at the bottom of the jars 0.2 grams of oyster shells were placed which could act as a settlement substrate. To get a consistent shell topography, shells were crushed using a hammer and crushed shells were sieved between 1.0 mm and 0.5 mm metal sieve. For each treatment

tank, 5 individual containers were used. As all treatments were repeated over 4 consecutive days, 20 jars were used per treatment in total. All trials were conducted in a dark environment at 20 (±1) °C in a climate-controlled room.

To avoid any air in jars containing larvae, larvae were placed in the jars and the lid was fixed while the jar was fully submerged in FSW. This step was necessary to prevent any distortion of the sounds due to reflection from air bubbles. All FSW used in the experiment had added microalgae *Chaetoceros muelleri*, and *Isochrysis galbana* (clone T-ISO) at 100,000 cells/ml at a volume ratio of 3:1. In a previous study, *M. gigas* larvae increased swimming when exposed to reef sounds, but only if larvae were fed (Stocks et al., 2012), thus microalgae were added to our larvae containers. Microalgae were added at the same concentration as used in larvae rearing tanks and food levels were not limiting for the duration of the experiment.

On top of each tank, jars were attached to a wooden pole sitting horizontally across the tank. Each larvae jar was attached so that it was in a fixed position for the duration of the experiment, the position of the jar was noted so that the effect from placement in the tank could be ruled out. Jars were fully closed so that no water was shared between tanks and jars. The wooden pole was isolated from tank walls with polystyrene to avoid vibration propagation. One jar was located directly above the speaker and the other 4 jars were at the same distance from the center of the speaker (see Figure 4.2).

After 24 hours of exposure, larval metamorphosis was checked using a dissecting microscope and the number of larvae that had cemented themselves to the substrates were counted. Metamorphosis was confirmed by gently blowing water over the larvae with a pipette to ensure that larvae were fixed to the substrate.

### Statistical analyses

A generalized linear mixed-effect model was created using the glmer function of the lmer package (Bates et al., 2015) in R version 4.1.3 (2022-03-10) (R Core Team 2023). As the response variable was binary (settled vs. not settled) we fitted a Bernoulli distribution using a logit link function. The predictor variables that were considered included sound treatment, date, speaker, tank, jar and jar position. First, we established a base model that included treatment and date as fixed effect variables and jars nested in the treatment-tank interaction as a random effect variable. We then included each of the other variables (i.e. speaker, tank and jar position) individually as fixed effects and compared model fit using the Akaike

information criterion (AIC) and visually inspected model prediction plots. As the inclusion of any of those variables did not decrease the AIC value and had little to no effect on the effect sizes, we did not consider them in the final analysis. The assumptions of the model were met. See supplementary information for description of all model used (Table S5). Post hoc tests were performed using the emmeans function of the lsmeans package (Lenth et al., 2016) to calculate the marginal means adjusting p-values for multiple comparisons with Tukey's method and the pairs function was used to display pairwise comparisons.

# Results

### Playback

The recorded sound in the tanks did not perfectly match the spectrum of the sounds recorded in the field due to the technical limitations of the reproduction equipment and the resonances that inevitably occur in tank-based experiments (Figure 4.3). For example, sound levels were amplified at 2 and 7 kHz due to the speaker frequency response, with a dip at 5 kHz. A 50 Hz and its 3rd and 5th harmonic can be observed in the PSD of all the treatments, probably generated in the hydrophone due to electromagnetic interferences from the lab (not coming from the playback system and detectable by the larvae). Despite these limitations, when computing different acoustic metrics in tank and field sound recordings, similar trends were observed (Figure 4.4). For example, ACI was higher for reef treatments compared to all other treatments in both tank and field sound recordings, and the PSD order from loudest to quietest for each batch was the same for field and tank recordings.

When analyzing the tank recordings, some faint cross-talk between tanks could be detected. This occurred only below 200 Hz, and it was most audible in the no speaker tank recording during the loudest moments of the neighboring tank. Nevertheless, the treatments remained very different and recognizable acoustically.

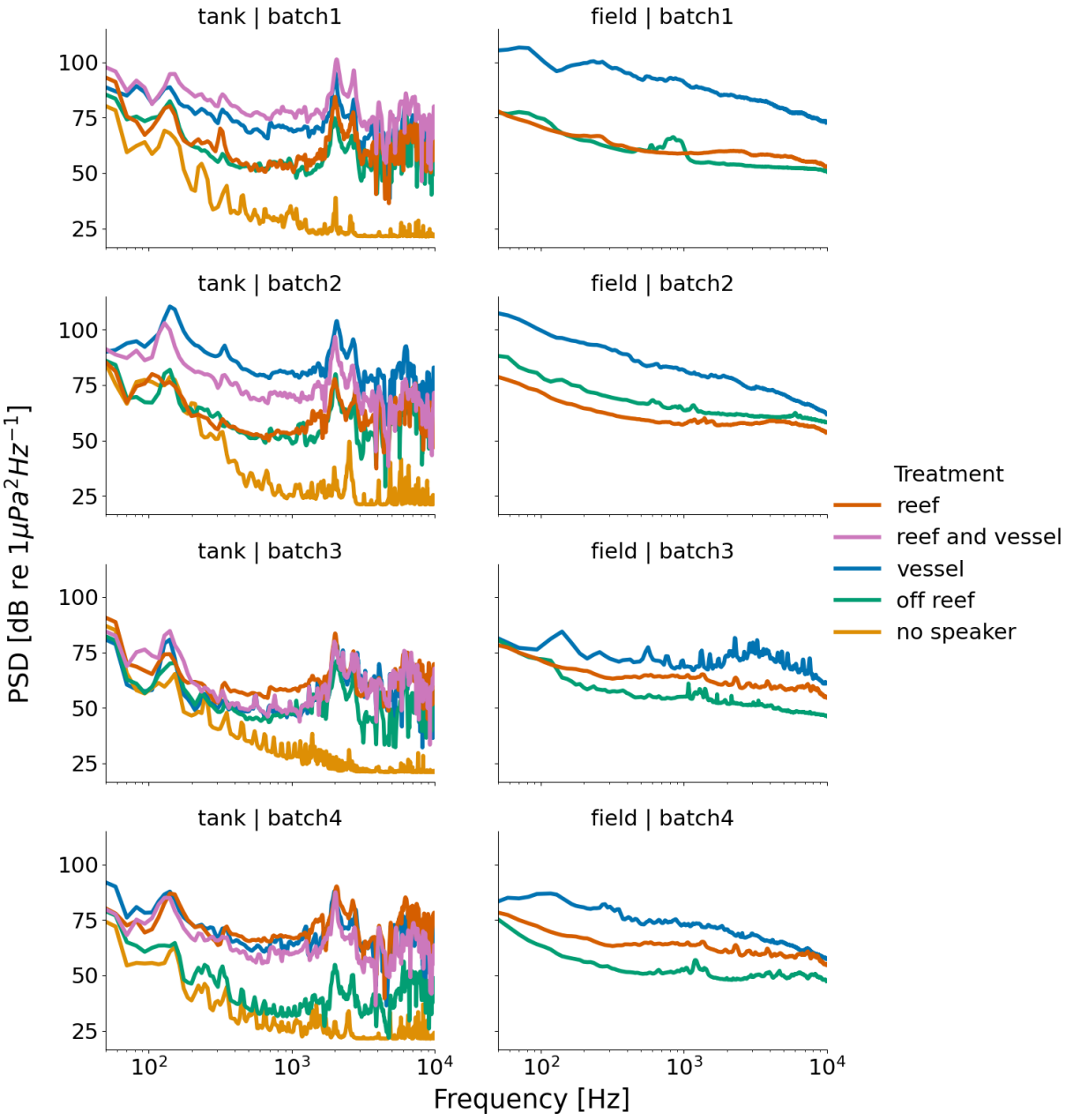


Figure 4.3. Comparison between the field and the tank recorded playback spectrum levels. No speaker refers to the tank recording when all the other playbacks were on (recoded in the tank with no speaker inside).

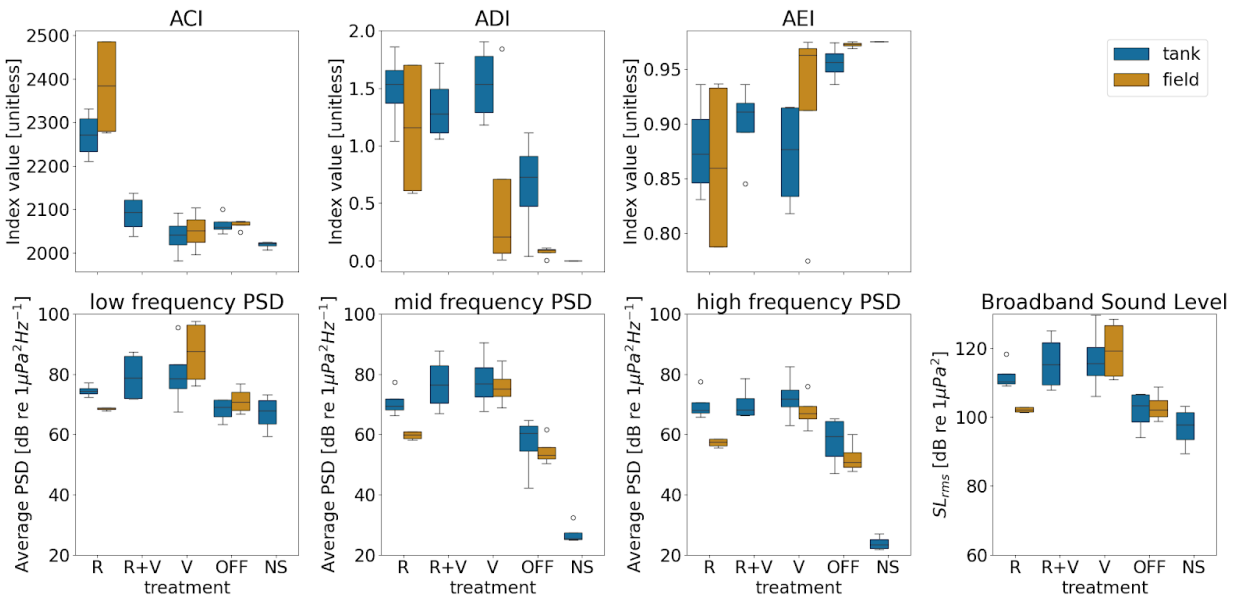


Figure 4.4. Comparison of the obtained acoustic metrics for the tank and the field recordings. The acoustic metrics include the acoustic complexity index (ACI), acoustic diversity index (ADI), and acoustic evenness index (AEI). NS=no speaker, OFF=off reef, R=Reef, R+V=reef and vessel, V=vessel. The number of data points for each treatment is 4. The definition and computation of each of the features are explained in Table S3.

## Settlement rate

Larvae settlement increased significantly in response to reef sound compared to vessel sounds (β = 0.715, SE = 0.260, p = 0.047), compared to off-reef sounds (β = 0.745, SE = 0.261, p = 0.034), and compared to the no speaker treatment (β = 1.015, SE = 0.262, p = 0.0010; Table 1). When vessel sound were added to the reef sound, the settlement rate decreased about 1.29 times compared to the pure reef sound (β = 0.560, SE = 0.259, p=0.193), and was 1.09 times higher than in the vessel-only sound treatment (β = 0.155, SE = 0.261, p = 0.976). Comparisons among other treatments revealed only minor differences (Table 1). Vessels and off-reef sounds had very similar effects on settlement. The lowest settlement rates were observed in a no-sound control treatment. Model predictions are plotted in Figure 4.5.

Table 1. The results of the posthoc of the GLMER model 1 using all data and comparing all treatments. Significant values (p ≤ 0.05) are in **bold**.

| Contrasting treatments | Estimate | SE | df | z.ratio | p.value |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| reef - off reef | 0.7455 | 0.261 | Inf | 2.86 | **0.0344** |
| reef – vessel | 0.7154 | 0.260 | Inf | 2.750 | **0.0471** |
| reef - no speaker | 1.0151 | 0.262 | Inf | 3.879 | **0.0010** |
| reef - (reef + vessel) | 0.5600 | 0.259 | Inf | 2.164 | 0.1934 |
| off reef – vessel | 0.0301 | 0.263 | Inf | 0.115 | 1 |
| off reef - no speaker | 0.2695 | 0.263 | Inf | 1.025 | 0.8441 |
| off reef - (reef + vessel) | 0.1855 | 0.261 | Inf | -0.711 | 0.9541 |
| no speaker - vessel | 0.2997 | 0.264 | Inf | -1.137 | 0.7869 |
| no speaker - (reef + vessel) | 0.4551 | 0.262 | Inf | -1.738 | 0.4103 |
| vessel - (reef + vessel) | 0.1554 | 0.261 | Inf | -0.595 | 0.9758 |

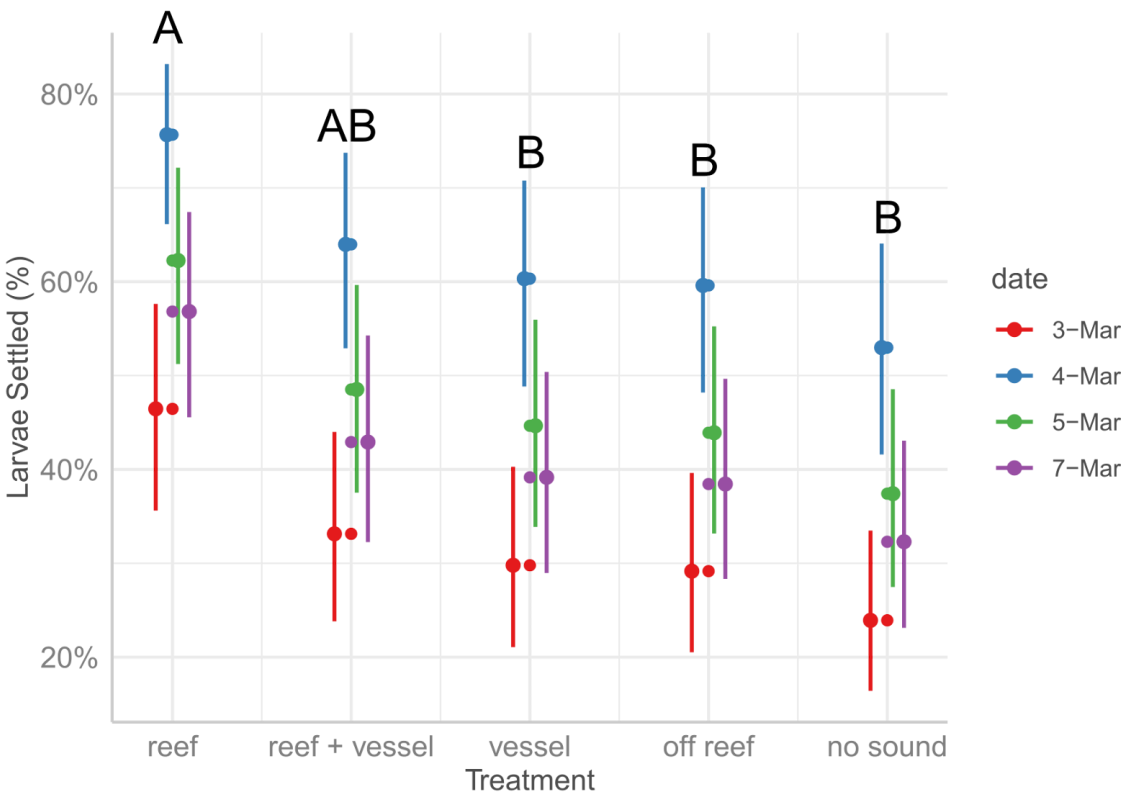


Figure 4.5. Prediction plots comparing predicted settlement across sound treatments. The average settlement from each 100ml jar on each day is represented in circles. Error bars represent 95 % confidence intervals of the model prediction. Letters represent significant differences in the treatment, same letters mean no significant difference between treatments, but different letters indicate a significant difference.

# Discussion

The results of our laboratory experiment showed increased settlement during exposure to sounds of conspecific oyster reefs for larvae of the oyster *Magallana gigas*, with settlement increasing 1.44 and 1.64 times under the oyster reef treatment compared to off-reef and no speaker control treatments, respectively. The settlement of larvae in response to vessel sounds, as well as the combined effects of reef and vessel treatments, did not show statistically significant differences compared to off-reef conditions and controls with no speaker.

### Preference for oyster reef sounds

Larvae of *M. gigas* consistently settle more readily when exposed to sounds of reefs inhabited by conspecifics. Our finding thus corroborates earlier research in fish, corals, and other oyster species, where larvae were found to increase settlement or orient more readily towards playback of reef sounds (Montgomery et al., 2006; Radford et al., 2011; Vermeij et al., 2012; Lillis et al., 2013). Yet the sound features that trigger this response in larvae still remain unidentified. In general, oyster and coral reefs exhibit higher sound levels and greater acoustic diversity than off-reef counterparts, due to increased soniferous biological activity including vocalizations of soniferous fishes and invertebrates, both passive or active, as well as the physical complexity of the reef (Lillis et al., 2014a; Lecchini et al., 2018). It remains undecided in the literature if larvae can distinguish particular sounds from different habitats, or if there is simply a preference for certain acoustic features such as SPL (Lillis et al., 2016). The spectrum of reef sounds recorded for our study followed patterns similar to other oyster reefs (Lillis et al., 2014a; Williams et al., 2022), and were louder than off-reefs in two out of four experiments. They also consistently presented a higher acoustic complexity, and higher evenness (lower AEI value) than off-reef areas. Compared to the vessel sounds, our reef sounds tended to have similar or lower PSD (depending on the vessel). Reef sounds were unique amongst the other treatments in their diversity, with consistently higher ACI and ADI values, and lower AEI values. This indicates that loudness (SPL) alone is not responsible for larval attraction, instead spectro-temporal patterns responsible for a high ACI may play a more important role. This conclusion can be corroborated in other marine invertebrates (Pine et al., 2012; Gigot et al., 2023). Pine et al., (2012) found that crab megalopae reduce metamorphosis (in comparison to natural habitat sounds) when exposed to wind turbine noise, but when the same turbine noises were played back at higher SPL, this did not result in any further changes to crab metamorphosis time. Leading to the conclusion that spectro-temporal characteristics were more relevant feature for megolopae attraction to habitat sounds than volume. Similarly, Gigot et al., (2023) found that scallop larvae reduced metamorphosis rates during drilling sounds, but increased metamorphosis rates when exposed to pile driving sounds. As both sounds were substantially louder than the no control, this further indicates the importance of temporal and spectral composition over simple preference for louder sounds. It would be incorrect to say that louder sounds are not a preferred sound feature for a number of invertebrates. Wilkens et al. (2012) found that when exposed to (the same) vessel sound at increasingly louder SPLs, mussel larvae increased settling at the louder treatments. Lillis et al., (2016) also conclude that louder reefs attract more coral settlers than quieter reefs. Based on the results of this present study and associated literature, a reasonable hypothesized could be made that both of these sound qualities

(loudness and spectro-temporal patterns) are perceptible to *M.gigas* larvae, and the preferences for each may be highly species-specific and could be based on the preferred habit qualities. In comparison to *M.gigas* adults, who have previously been studied for their sense of hearing (Charifi et al., 2017), larvae appear to be able to detect a larger range of acoustic frequencies. Future research should therefore not only collect species specific data on acoustic feature detection, but also from different life stages.

### Vessel noises and larvae settlement

Our results show that exposure to vessel sounds alone did not manifest in any disruptions to settlement compared to off-reef or no speaker controls. This indicates that there may be no intrinsically negative reaction of *M. gigas* larvae to these vessel sounds. In marine invertebrates generally, vessel sounds induce a wide range of physiological and behavioral changes (see Solé et al., (2023) and Murchy et al., (2020) for reviews of vessels on marine invertebrates). Much of the evidence indicates a stress response to vessel noises, but cases where no reaction or a positive reaction to vessel sounds do exist (Solé et al., 2023). In the few cases where vessel noises are specifically tested on invertebrates during their settlement stage, reactions have varied. While corals show settlement reduction (Holles et al., 2013), mussels and sea squirt larvae increase settlement (Jolivet et al., 2016; McDonald et al., 2014).

In this study, while there was no significant difference between the reef treatment and the vessel + reef treatment, there appears to be a trend of reduced settlement in the vessel + reef treatment. The effect size is potentially ecologically relevant, with larvae being 1.29 times less likely to settle when vessel noise is added to the oyster reef sound. Treatments of reef + vessel noises did not differentiate significantly from off-reef and no speaker controls. Although this study does not provide conclusive evidence of habitat sounds being masked by vessel noises, it highlights the need for further investigation in this area. While cases of anthropogenic masking in other invertebrate settlement experiments remains unconfirmed, a recent study by McAfee et al. (2023) found that acoustic enriching experimental *Ostrea angasi* oyster reefs with reef sound was effective in low background noise areas, but ineffective in high background noise environments. However, the specific element of the background noise responsible for these results remains uncertain, as the term 'background noise' in the study encompassed all sounds within the soundscape (anthropogenic, geophysical, or biological). Looking past the biological response of overlaying vessels and reef sounds, acoustic characteristics of the reef sounds appeared to change with the addition

of vessel noises in the recorded files. We observed a steep decrease in ACI, a mild decrease in ADI, and an increase in AEI, when vessel noises were added to reefs, although statistical confirmation is needed.

### Tank experiment limitations and future work

Lab-based sound exposure experiments are key as a first approach to test certain hypotheses, as they can be very controlled. External factors can be isolated, background ambient sound can be removed, and controls can be established from the same batch at the same time.

On the other hand, lab-based sound exposure experiments are incomplete in some aspects. For example, when presenting the whole picture of a soundscape. Reefs will change in their sonorific properties from a myriad of factors (time of day, time of year, etc). Vessels, as well, are not likely to produce sound continuously at one location, as experienced in the playbacks. Nevertheless, to understand the acoustic basis for sound discrimination and use as a cue, it is necessary to use defined and identifiable sources so robust and direct conclusions can be extracted. In future work, once the contribution of acoustic characteristics are better understood, longer exposure experiments in the field should be done with more realistic soundscapes and environmental conditions.

Tank experiments also pose technical challenges in keeping playback sounds true to their original field recordings for several reasons. First, aquatic invertebrates, including oysters, sense particle motion rather than sound pressure (Solé et al., 2023). Particle motion currently remains challenging to quantify, especially in small tanks. In the field, sound pressure and particle motion levels are strongly correlated, but that is not the case when close to the sound source and reflecting and pressure-relieving surfaces. Therefore, in smaller spaces such as a tank, the sound propagation will not necessarily be related to particle motion because the walls and surface will act as pressure release surfaces (Rogers et al., 2016). Hence, sound pressure measurements can be a poor indicator of the particle motion levels, especially close to the tank walls. However, the magnitude and direction of the particle motion are expected to differ substantially from the one the larvae would experience in the field.

Second, cross-talk between tanks is possible, as seen in the results Section. In this study, this cross-talk happened mostly at frequencies below 200 Hz, probably due to vibration propagation instead of air propagation, and it was mostly present during loud periods from the neighboring tank. However, tank

recordings retained the acoustic characteristics necessary to make them distinguishable, as proven by the obtained results and by the manual analysis of the tank recordings.

Last, in tank sound experiments, the sound field can present great variations at small spatial scales. For this reason, the received sound levels were measured at all the jars when the speaker was playing white noise, giving very similar results, so the levels received at all the jars were considered the same treatment. We acknowledge that if the material would be available, doing simultaneously the tank recordings in all the jars would be of increased value, but still because the larvae were in jars of 100 ml, they still could be exposed to different sound fields (jars had a diameter of 4.4cm and a length of 6.5cm). Still, to account for these possible differences, jar position was included as a possible effect in the GML model.

To make causal inference, all parameters should be kept constant throughout and experiment. In the present study, we could not adhere to this principle in two aspects. Firstly, we used a different sound file for each of the treatments in each of the trials. This approach has the advantage that we make inferences about the effect of sound types, rather than specific sound files. Using multiple sounds offers a more realistic insight to each treatment, which enhances the ability to extrapolate, as explained in Section 'Sound Treatments and Playback'. But it comes at the cost of limited power to detect a causal relationship as there is an additional confounding variation coming from the differences among sound files within sound types. Secondly, we had a limited number of speakers at our disposition. The trials were therefore conducted over several consecutive days and larvae could not be randomly assigned to the different trials and varied by age throughout the study. Our statistical analysis revealed that the date of the experiment had a significant effect on settlement. As the number of trials was limited, our design did not allow us to discriminate between the effect of experiment date and the effect of the variation among different sound files among treatment types. More and more extensive studies are needed to investigate which features of a specific sound type elicit the response in larvae settlement we observed in this study.

These limitations do not pose a problem for the current experiment, as our target was a proof-of-concept study into whether the settlement rate presented any differences when exposed to different acoustic stimuli in lab conditions, and to do so a fully controlled environment is necessary. Furthermore, the fluctuations over time at a large enough time scale represented in the ACI values are probably not affected by the dynamics of the tank resonances. Hence this cue remains valid in this experiment. While it necessary for future research to confirm our results under field conditions, proof of concept lab studies such as these, are essential first steps, as in the field, it is currently possible to control added sound, but not possible to remove other background sounds.

### Implications for reef restoration

Classic oyster reef mitigation and restoration projects focus on providing new hard substrates for wild larvae to settle, as well as supplying new adults to reefs, however, the importance of acoustic cues may be overlooked. Recently McAfee et al., (2023) used underwater speakers to enhance soundscapes on constructed reefs resulting in greater initial settlement of the oyster *Ostrea angasi*. If similar acoustic preferences are established for other species, these same techniques could be employed elsewhere. Our results indicate that *M. gigas* also responds to acoustic cues, and thus may respond positively to acoustic enrichment as a restoration strategy.

# Conclusion

We show that *M. gigas* larvae will settle more readily during playback of oyster reef sounds. The reef sounds were unique in being very acoustically diverse (high ACI), while other acoustic features, such as SPL varied among treatments. This suggests that oyster larvae may be able to detect complex spectro-temporal patterns in the soundscape rather than rely solely on SPL. Furthermore, we find that noise from vessels does not inhibit larvae settlement any more than the effect of off-reefs sounds or no speaker controls. We call for more research to replicate our findings in the laboratory in field experiments. More quantitative evidence is needed to determine if vessel noise (or other anthropogenic sounds) may affect oyster recruitment in ecologically realistic settings in the field.

# Chapter 5: Biofilm development alters surface micro-topography preferences of settling Pacific oyster larvae (*Magallana gigas*)

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# Abstract

Larval metamorphosis of benthic invertebrates is guided by environmental cues that signal suitable habitats. Surface microtopography has been shown to strongly influence these metamorphic decisions, yet the specific features preferred by many taxa, including oysters, remain unclear. Microtopography also shapes the development and characteristics of microbial biofilms, which additionally affect larval settlement and metamorphosis. We tested how specific features of microtopography, combined with natural marine biofilms aged 5 or 20 days, affect metamorphosis of the Pacific oyster (*Magallana gigas*) in laboratory behavioral assays conducted over two years. We also evaluated these preferences in a field experiment by quantifying natural recruitment of *M. gigas* in the Belgian part of the North Sea (BPNS). Biofilm effects varied between years: a 20-day biofilm enhanced settlement in one year but was avoided in the other, indicating that larval responses to biofilms are context dependent. The response to biofilms was also dependent on specific microtopographical features. Field experiments also indicate larvae preferences for specific topographical features. These results indicate a need for future work assessing larval metamorphosis under complex scenarios involving multiple cues.

# Introduction

Larvae of benthic marine invertebrates utilize cues from their environment to assess the suitability of a habitat. In marine bivalves, larvae use cues first to direct their settlement which is the searching phase where larvae will descend the water column and attach to substrates. Larvae will continue to use cues from the substrate level to initiate metamorphosis, an irreversible developmental transition characterized by the programmed degeneration of larval structures and the formation of juvenile or adult tissues (Hadfield, 2000; Hadfield & Paul, 2001). These cues become critical for the survival of populations, especially for sessile animals such as oysters, whose metamorphosis results in permanent fixation to the substrate they choose (Hadfield et al., 2001). A wide range of signals from environmental sources are used by larvae to assess habitats, originating from both biotic (conspecific cues, biofilms) and abiotic sources (sounds, light, hydrodynamic changes) experienced both in the water column and at a substrate surface (Tamburri et al., 1992; Vasquez et al., 2013; Fuchs et al., 2015; Wheeler et al., 2017; Williams et al., 2022).

For sessile organisms, responding to cues within the water column is only one half of the challenge; once a larva reaches a substrate surface they can continue to crawl along the surface, in the case of oysters using their larval foot, until they encounter further cues alerting them to the suitability of the surface. In some cases, if the surface is deemed unsuitable, larvae may even resuspend themselves in the water column in search of another location (Krug, 2006).

The topographical features of a substrate surface have long been known to influence the attractiveness of the surface for potential settlers, and the addition of complex topographical features has been used in eco-engineering to encourage greater numbers and diversity of settlers (Strain et al., 2018a). Manipulation of surface topography, including changes to features, such as width, height, spacing, geometry, angles, and spatial arrangement, influence the type of organisms that will be attracted (Schumacher et al., 2007; Carl et al., 2012; Erramilli & Genzer., 2019; Reidenbach et al 2021; Bauer et al., 2024). Arrangements of micro-topography, which include features from the millimeters to centimeter scale have shown a particular influence on larvae of invertebrates, and it has been generally concluded that surface microtopographies nearing the size of a settling larva will attract these larvae, known as the "attachment point theory"(Scardino et al., 2006; 2008). This theory proposes that larvae prefer surfaces with the most contact points. Under this theory, features smaller than the sizes of settling larvae discourage settlement and metamorphosis, while those with features larger than the body of settling larvae still improve settlement over smooth surfaces but not to the extent of the body-sized features (Callow et al. 2002; Scardino et al. 2006, 2008). The biological reasoning for surface preferences is most often assumed to be due to protection from hydrodynamic forces (Scardino et al. 2006, 2008). The presence of topographical features changes prevailing flow patterns, encouraging more complex hydrodynamic conditions (Koehl 2007). Strong flow conditions across a substrate's surface can dislodge larvae and microstructures can help larvae to retain their positions. But the degree to which flow is disturbed in favor of attracting larvae settlement depends substantially on the surface feature characteristics. Features of the same height but with different shapes and spacing can result in very different flow microhabitats (Reidenbach et al 2021; Bauer et al., 2024). Aside from hydrodynamics, predation is also suspected to drive larvae to prefer surface topography (Griffin et al., 2009; Strain et al., 2018b; Bauer et al., 2024). Predation is a major bottleneck in the lifecycle of invertebrate larvae, and refuge among microstructures aids cryptic behavior. This theory is evidenced by the fact that species more vulnerable to predation after settlement have a stronger preference to settle in microstructures than species with lesser predation potential after settlement (Walters & Wethey., 1996).

Actively modifying features of microtopography has been utilized in attempts to both attract more settlement of beneficial organisms and deter undesired fouling species (Sedano et al., 2020). As reef-building species that play a critical role in the functioning of their environment, oysters are a target species for restoration projects worldwide (Smith & Pruett 2024). Optimizing materials for use in oyster reef restoration to attract more settlers has found that adding more features to substrates increases oyster settlement, but studies have so far not made many conclusive observations about specifications that might be important for this observed effect. Potet et al. (2021) investigated how different surface topographies, applied to the same material, influenced settlement in *Ostrea edulis* larvae. They found a strong preference for certain textures, with the most effective surfaces resulting in settlement rates up to five times higher than smooth surfaces (2.1 larvae/$cm^2$ on settlement plates vs 0.45 larvae/$cm^2$). However, the specific topographical features responsible for this increase remain unclear. While the most successful surfaces had roughness between 3 mm and 17 mm, not all surfaces within this range performed well. Surfaces with the lowest settlement were either smooth or had a fine, sandy texture.

While both hydrodynamics and predation might be driving forces influencing larval preferences for microstructures, the attractiveness of microtopography can be altered by biofilms, which quickly cover all new surfaces exposed to seawater and have been shown to critically influence larvae settlement and metamorphosis (Dobretsov & Rittschof., 2020). Marine biofilms grow in succession with the addition of new elements changing the composition of the next layer (Flemming & Wingender., 2010; Salta et al., 2013; Qian et al., 2022). Initially, a new surface will be rapidly colonized by bacteria and archaea (~1 day), which will remain an important component to the biofilm composition as they secrete extracellular material, produced by the organisms themselves (Flemming & Wingender., 2010; Salta et al., 2013; Qian et al., 2022). This matrix, present after 1 day, consists of a conglomeration of different types of biopolymers known as extracellular polymeric substances (EPS) and will continue to develop and mature over the life of the biofilm (Qian et al., 2022). Within 3 to 7 days, the biofilm becomes more complex with the addition of diatoms, fungi, and protozoa (Flemming & Wingender., 2010; Salta et al., 2013; Qian et al., 2022). Diatoms, in particular, are often early colonizers and contribute their own EPS, adding to the matrix's complexity (Salta et al., 2013). In the following days to months, the biofilm will become more heterogeneous in its species composition and structure and will start attracting macrofoulers, including invertebrate larvae (Hadfield, 2011; Antunes et al., 2019b; Flemming & Wingender., 2010). This attractive relationship between biofilm and invertebrate larvae has been thought to indicate to larvae that a surface is not toxic or temporary (Hadfield et al., 2011), evidenced by the fact that while most mature biofilms will actively attract more settlers, in cases where biofilms have been grown under unsuitable conditions,

low pH or in areas where heavy metals are present for example, larvae response to biofilms change (Hadfield et al., 2011; Bao et al., 2010; Dobretsov & Rittschof., 2020; Espinel-Velasco et al., 2021). Larval attraction to biofilms could also arise because biofilms signal food availability, or because they provide stabilizing surfaces for passive attachment on the biofilm extracellular polymeric substances (Van Colen et al., 2009; Dobretsov & Rittschof., 2020). Formation of biofilms, including community composition, density, and EPS structure, is driven by the local microbial species pool as well as biochemical and physical interactions with the environment. The initial biofilm formation can be altered based on the microtopography of the substrate on which it's growing likely because of the secondary effects of substrate microtopography on the immediate microenvironment, including light and small scale hydrodynamics (Kumar et al., 2013; Salta et al., 2013; Misic & Harriague 2019; Krsmanovic et al., 2021). The nature of the exact changes to the biofilms by secondary effects of substrates topography is not well understood (Hadfield & Paul, 2001; Dobretsov et al., 2006; Myan et al., 2013; Carve et al., 2019; Dobretsov & Rittschof., 2020). Alterations to biofilm formation from factors such as water flow or light could determine their signaling effect to macrofoulers. For example, barnacle larvae attach more tightly to biofilms developed under high-shear flow (regions where water flow changes rapidly over short distances) compared to those formed under low-shear conditions (Neal & Yule, 1994; Neal et al.1996).

While some conclusions have been drawn about general preferences for topographical features (attachment point theory), it is unknown whether this can be translated into applied research. Very small and specific modifications to the surface may be impractical to produce large-scale, or could become quickly obscured by macrofoulers other than the target organism. However, well-designed and thoroughly studied topography alterations could increase settlement by a large degree, improving reef design and chances of success. To determine if these alterations are worthwhile for attracting larvae, assessing topographical modifications must include interactions of biofilms, as there is no material used for reef restoration that will not develop a biofilm, and this biofilm/topographical relationship will also have an impact on larval attraction.

In this study, we seek to identify which features of microtopography are preferred by oyster *Magallana gigas* for larval settlement and if the addition of biofilms influences this process. We conducted settlement trials both in the laboratory under controlled conditions and in the field under ecologically realistic conditions. Different features of topography were chosen based on microtopography preferences from similar species, while also keeping in mind features that could be implemented into practical designs for surfaces aimed at restoration. We include small features, near the size of the larvae (micrometers scale),

features of different aspect ratios, and larger features (millimeter scale), and a feature representing a different degree of contact angle. While these are not fully representative of the topographical features that could affect the settlement of this species, the results can be used to make generalizations that can be further refined.

# Methods

### Topography Feature Description

This study combines a laboratory and field experiment where settlement of oyster *Magallana gigas* larvae was assessed for different features of surface microtopography. These topography treatments were arranged on 5 cm x 5 cm concrete tiles on which larvae were allowed to settle. Topographical arrangements were first 3D printed in plastic, and these plastic replicas were used to create silicone molds in which concrete mix was poured. Concrete was used as the material type, as this is the most commonly used substrate for engineering in marine environments, and is often used in reef restoration projects (Bone et al., 2022). CEM II/B-L 32,5 N Portland cement was used to create the tiles, and the cement was mixed at a 0.75 water-to-cement ratio. Both lab and field experiments were conducted using the same surface topographies. Concretes were soaked for 24 hours prior to the experiment in filtered seawater (0.1µm) and scrubbed prior to the start of the experiment. Topographical features were presented as grooves with specified widths and heights, five separate topographical formations were created, referred hereafter as Topography-1 (T-1) through Topography-5 (T-5) (figure 5.1).

Topography 1 - smooth with no added features.

Topography 2 - parallel groove array with a square-shaped cross-section; the grooves and the flat areas between them are 800 µm and equal in width (1:1 aspect ratio).

Topography 3 - has an identical structure to Topography 2 but the grooves and flat areas are 5 mm.

Topography 4 - has the same parallel groove array but with a groove height of 1600 µm and the flat areas of 800 µm (1:2 aspect ratio).

Topography 5 - parallel groove array with a trapezoidal wave profile, consisting of sloped sides and flat tops and bottoms tops and bottoms are 800 µm. The distance between the flat tops and bottoms are 800 µm. The angle between the flat bottom and the sloped side is 120 degrees.

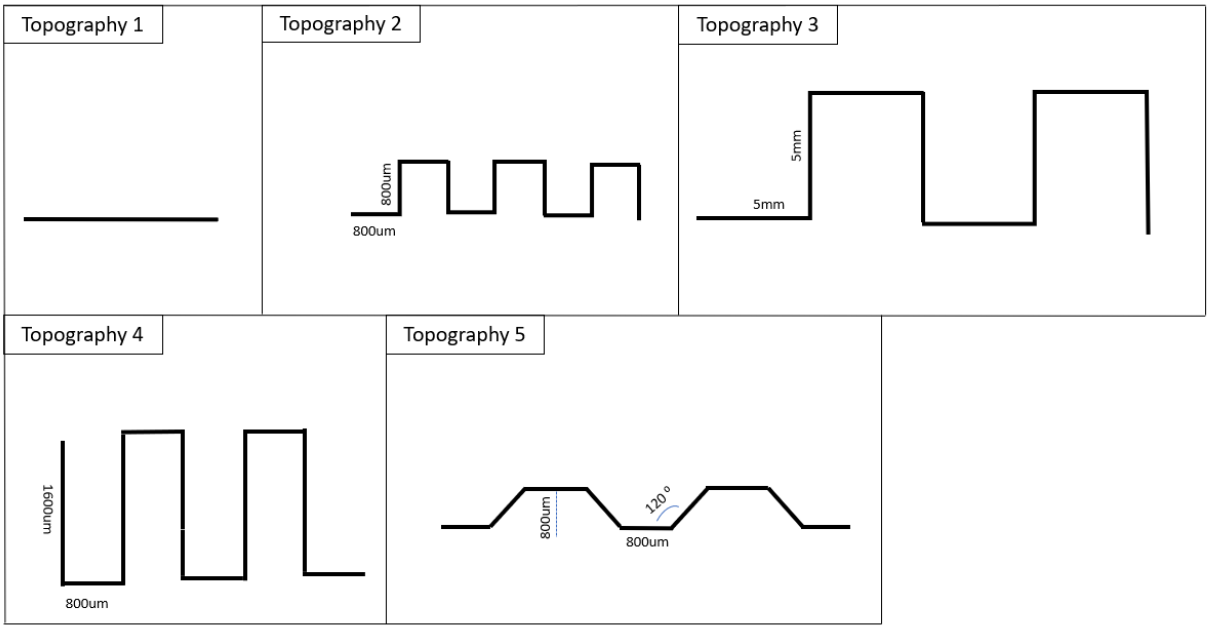


Figure 5.1: Side profile of different topography treatments. Topography 1 is smooth with no added features. Topography 2 has a parallel groove array with a square-shaped cross-section; the grooves and the flat areas between them are 800 µm and equal in width (1:1 aspect ratio). Topography 3 has an identical structure to Topography 2 but the grooves and flat areas are 5 mm. Topography 4 has the same parallel groove array but with a groove height of 1600 µm and the flat areas of 800 µm (1:2 aspect ratio). Topography 5 is a parallel groove array with a trapezoidal wave profile, consisting of sloped sides and flat tops and bottoms tops and bottoms are 800 µm. The distance between the flat tops and bottoms are 800 µm. The angle between the flat bottom and the sloped side is 120 degrees.

## Lab experiment

### Experimental Design

Two lab experiments were conducted with similar experimental designs, one in August 2023 and the other in August 2024, these will be referred to respectively as experiment 1 and experiment 2. Both experiments assessed the metamorphosis of lab-reared oyster larvae on the different topographies. In addition, the interaction between topography and biofilm was assessed. Biofilms were allowed to grow on each of the different topography treatments for 5 or 20 days, respectively. This resulted in a total of 15 unique treatment combinations (5 topographies * 3 biofilm conditions: 0, 5, 20 days). Treatment combinations of topography/biofilm were placed in a 350 ml container (5.5 cm H x 5 cm W) filled with 0.1µm, filtered

sea water (FSW) and 15 pediveliger larvae were added to the tile surface directly. In experiment 1 each treatment was replicated 6 times, with 2 replicates performed each day, over 3 days. In experiment 2 each treatment was replicated 9 times, with 3 replicates performed over 3 days. See figure 5.2 for a schematic of the experimental design.

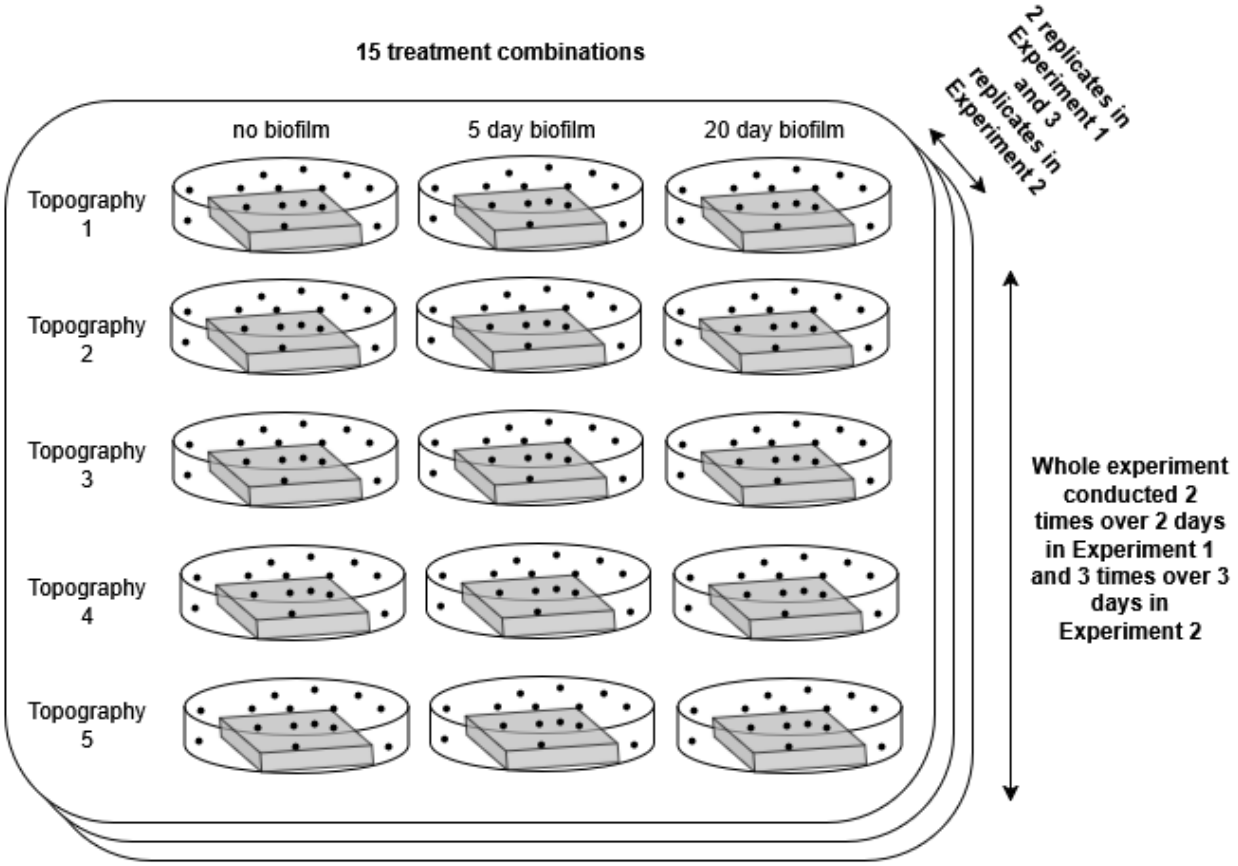


Figure 5.2: Schematic representation of the experimental design, sample sizes and levels of replication.

## Larvae cultures

In experiment 1, M. gigas larvae were reared to their pediveliger stage in the Marine Station Ostend, Belgium. The parent oysters were collected from the harbor of Ostend during natural maturity (August 2023), and gametes from 10 females and 6 males were used to produce fertilized embryos. The process of spawning, fertilization, and larval rearing can be found in chapter 1 of this thesis. Larvae were reared in 10 L conical tanks filled with UV-treated FSW (filtered at 0.1 µm) and kept at 22 °C. Larvae were fed a

microalgae mixture consisting of *Chaetoceros muelleri*, and *Isochrysis galbana* (clone T-ISO) purchased from Proviron Industries NV. During the first four days, larvae were fed exclusively with *Isochrysis galbana* at a concentration of $4*10^4$ cells/ml. From days 5 to 12, the diet consisted of a 1:1 volume ratio of *C. muelleri* and *I. galbana*, provided at a total concentration of $10^5$ cells/ml. From day 13 until the end of the experiment, larvae were fed the same two species at a 3:1 volume ratio (*C. muelleri*:*I. galbana*), maintaining the concentration at $10^5$ cells/ml. Larvae entered their pediveliger stage and became competent to settle between 26-30 days. Larvae were considered competent once they developed a prominent eyespot and larval foot and reached a size of 320–350 µm in diameter 28-30 days post fertilization. In experiment 2, pediveliger oyster larvae were purchased from an aquaculture farm, France Naissain. Immediately after arrival, larvae were placed in 10 L conical tanks in FSW at 22 °C stocked at 10 larvae/ml until use in experiments and fed 3:1 ratio *C. muelleri*:*I. galbana*, at $10^5$ cells/ml. Larvae were used for experiments 24 hours after arrival.

### Preparation of Biofilms

Natural biofilms were allowed to develop on each concrete tile by submerging them in the non-tidal part of the Ostend harbor (51.231685° N, 2.931362° E), where they remained fully submerged throughout their development. The concrete tiles were enclosed in 260 µm nylon mesh, which allowed water to flow through and promote microbial biofilm colonization while preventing macrobiofoulers including wild oyster larvae from natural populations from settling. Biofilms were left to develop undisturbed for either 5 or 20 days prior to the experiment. Deployment of the concrete tiles was staggered so that on each experiment date, the biofilm would be the appropriate age. On the day of each experiment, the concrete tiles were retrieved and transported in natural seawater to prevent drying. All tiles were used in the experiments within one hour of retrieval. In experiment one, the 20-day biofilm concrete tiles were first placed on the 2nd of August 2023, 5-day biofilms were placed on the 17th of August 2024, and the first experiment started on the 22nd of August 2023. In the second experiment, 20-day biofilm concrete tiles were first placed on the 27th of July 2024, 5-day biofilms were placed on the 12th of August 2024, and the experiment started on the 16th of August 2024.

## Settlement assay

Larvae became competent to settle, entering their pediveliger stage between 28-30 days post fertilization. On each day of the experiment, larvae were filtered from the culture tanks between 260 µm and 300 µm sieves; only larvae retained on the 260 µm sieve were used in the experiment. A random sample of 10 larvae was taken from the larvae retained on the sieve each day of the experiment and assessed for competency, determining competency by the presence of an eyespot and a larval foot. Following sieving, larvae were allowed to acclimate in FSW for an hour in a climate-controlled room at 19 ± 1 °C where experiments took place. At the start of each experiment, 15 larvae were gently pipetted into each container containing the topography/biofilm treatment. Larvae were left for 24 hours undisturbed before they were assessed for metamorphosis. After 24 hours, ethanol was added to each container to preserve conditions as they were, and prevent further metamorphosis in the time that it took to inspect each replicate. To assess metamorphosis, each concrete tile was removed from the container and rinsed gently with water. The leftover water from the container was sieved over a 90 µm mesh, and any larvae retained on this sieve were counted and marked as "not metamorphosed". Metamorphosis was assessed on each concrete tile by using a dissection microscope, and only larvae that had cemented themselves to the substrate were counted. To do so, we blew a small amount of water over the larvae with a pipette to check that the larvae were fixed to the substrate. The position of the larvae on the topography feature was also recorded as either middle, side, corner, or top. See figure 5.3 for a visualization of this position designation. T-1, which was a smooth surface, did not get a position description.

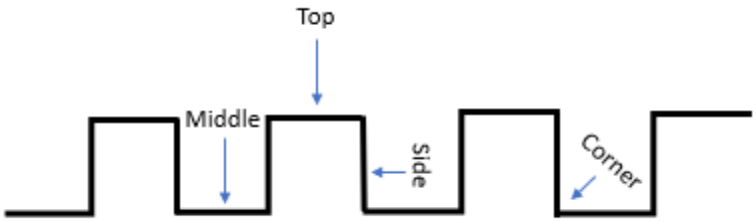


Figure 5.3. Schematic representing the location where larvae settled within a topography arrangement.

## Field Experiment

### Location

Field experiments to evaluate wild *M. gigas* larval settlement on different topographical features were conducted in the harbor close to the Marine Station Ostend in Belgium (51.234635° N, 2.926924° E), this location was 400m from the location where biofilms from laboratory experiment were cultivated. The harbor of Ostend, located at the Southern Bight of the North Sea, is a semi-enclosed, tide-influenced coastal system shaped by freshwater inputs from inland canals and dynamic exchanges with the open sea. This area experiences moderate to strong tidal currents, and is generally well mixed (Vanlede et al., 2012). *M. gigas* are established in the region, colonizing rocky intertidal areas and marine infrastructure within the harbor (personal observation).

### Settlement collectors

To assess natural settlement, experimental frames were attached to harbor walls and suspended 1-2 meters below the surface. The same formation of topographical features was studied in both the lab and the field experiment, and the size and material of the tiles was the same as previously described for the laboratory study. Two types of frames to hold the concrete tiles were deployed: one configuration presented concrete tiles parallel to the water surface , the other frame was hung over the harbor wall, and presented the concrete tiles perpendicular, these will be referred to as ‘parallel frame’ and ‘perpendicular frame’ (see Figure. 5.4). Two perpendicular frames and one parallel frame were installed. The concrete tiles on each perpendicular frame were arranged in three rows (top, middle, bottom), 10 tiles were placed on each row. The tiles were placed with the surface microtopography facing upright. On the parallel frame there were 50 concrete tiles arranged in five rows of ten.

Figure 5.4: Picture of the different frames used to deploy concrete tiles in the field experiment. The top image is the parallel frame, and the bottom image is the perpendicular frames.

### Experiment design

On the parallel frame, each topography treatment was replicated 10 times. On the perpendicular frames, each topography treatment was replicated 6 times. Each topography treatment was placed on the frame in a random pattern. Concrete tiles were deployed on the 9$^{th}$of August 2024 and removed from the water on the 21$^{th}$ of August 2024. After removal, the position of the tiles was noted on the frame, and the tile were held,not submerged in water, in a cold storage room before analysis. Larvae were counted on each tile using a dissecting microscope, and the position of the topography feature of the metamorphosed larvae was noted, similar to the lab experiment.

## Statistical analyses

### Laboratory experiments

For both experiment 1 and experiment 2, generalized linearized models were created using the glmer function of the lme4 package (Bates et al., 2014) in R version 4.1.3 (2022-03-10) (R Core Team, 2021). As the response variable was binary (settled vs. not settled) these models used a Bernoulli distribution using a logit link function. A base model was established consisting of topography treatment and biofilm age as fixed effect variables. We performed a forward selection procedure, using the Akaike Information Criterion (AIC), to determine if the age of the larvae or the interaction of topography treatment and biofilm age should be included as fixed effects in the final model. The age of larvae was included as a potential fixed effect as the experiment was spread out over several days, and larvae may display different behaviors during settlement as they age. The best fitting model in both experiment 1 and experiment 2 (lowest ACI) had fixed effect variables: topography treatment, biofilm age, the interaction of topography treatment and biofilm age, and larvae age. We used the emmeans function in R (Lenth., 2025) to perform post-hoc analysis. This function calculates the marginal means adjusting p-values for multiple comparisons with Tukey's method, finally, we used the pairs function in R to display pairwise comparisons. A description of the model and the post-hoc can be found in the supporting information (table 5.S.1.) 95% confidence intervals and odds Ratios were calculated from the models based on Wald estimates and have been added to the supplement (table 5.S.3). Raw data were compared to model predictions by calculating the percentage larvae metamorphosis per container of larvae this was plotted as a violin plot along with model predicted results.

### Field Experiment

For analysis of the field experiment, we created a negative binomial generalized linear model using the MASS package in R (Venables & Ripley., 2002). This model type was chosen as the data were over dispersed, as determined by a calculation of the dispersion ratio (> 1.5). A base model was established, consisting of topography treatment as the fixed effect variable. A forward selection procedure, using AIC tested if frame type or the interaction of frame type and topography treatment should be included in the model. The forward selection procedure revealed that frame type should be included as a fixed effect in the model. We used the emmeans function in R (Lenth., 2025) to perform post-hoc analysis. A description of the model and the post-hoc can be found in the supporting information.

### Larvae location

For both the lab and field experiments, the location of the larvae on the features of the topography treatments was recorded as either top, middle, corner, or side (see figure 5.3). For each experiment, negative binomial generalized linear models using the MASS package in R (Venables & Ripley., 2002) were created with location and topography type as fixed and interacting effects in the models. For each experiment a base model was established, consisting of topography treatment and larvae position as fixed effect variables. A forward selection procedure, using AIC tested if the interaction of topography treatment and larvae position should also be included as a fixed effect. For the two lab experiments, the addition of the interaction of topography treatment and larvae position did not lower the AIC. For the field experiment, the interaction of these variables did lower the AIC. We used the emmeans function in R (Lenth., 2025) to perform post-hoc analysis. A detailed description of the models and post hoc tests can be found in the supporting information.

# Results

### Lab Experiments

### Experiment 1

In the first experiment in 2023, the presence of biofilm and topography treatments both significantly influence model predicted larvae metamorphosis (Table 5.1). Results of the post hoc analysis showed the 5-day biofilm did not significantly influence metamorphosis compared to the no biofilm treatment (0.313). The 20-day biofilm significantly increased model-predicted metamorphosis overall compared to no biofilm (p=0.002) (Table 5.1). However, the results of the post hoc reveal that only on topography-3 does the 20-day biofilm significantly increase metamorphosis compared to no biofilm and 5-day biofilm (P=0.012) (Table 5.S2 in supplement, Figure 5.S5). While topography-2 and -5 increased predicted settlement compared to topography-1 (the smooth control treatment) in the overall model predictions, the post hoc results revealed that there were no significant differences between each topography treatment within the same biofilm treatment (Table 5.S2 in supplement, Figure 5.S5). The age of larvae significantly influenced settlement; settlement rates decreased in the oldest larvae (age 32 days).

### Experiment 2

In the second experiment in 2024, The presence of the biofilm treatments and the topography treatments significantly influence model predicted metamorphosis (Table 5.1). The presence of the 20-day biofilm significantly decreased model-predicted settlement compared to no biofilm treatment (p=0.019) (Table 5.1). Post-hoc analysis revealed that a significant decrease in settlement from the addition of the 20-day biofilm were only seen on topography-2 and topography-4. While model-predicted settlement showed that all topographies -2 ,-3, -4, -5 increased settlement significantly compared to topography-1 (the smooth control treatment) in the overall model predictions (Table 5.1, Figure 5.5), Post-hoc analysis revealed that on treatments without a biofilm presence, topography-2 and -4 increased settlement compared to topography-1. Topography-2 also significantly increased settlement compared to topography-3 (Table 5.S3 in supplement, figure 5.5). The age of larvae significantly influenced settlement (Table 5.1).

Table 5.1. Results of the statistical models for lab experiments 1 and 2. Significant differences ($p < 0.05$) are in bold, while marginally significant ($p < 0.1$) are in italic.

| Experiment | | | Estimate | Std. Error | z-value | p-value |
|---|---|---|---|---|---|---|
| **1** | **(Intercept)** | | -2.5422 | 0.5331 | -4.769 | **<0.001** |
| | **Topography** | | | | | |
| | Topography1 | | Reference | – | – | – |
| | Topography2 | | 1.7037 | 0.5987 | 2.846 | **0.004** |
| | Topography3 | | 1.1053 | 0.6505 | 1.699 | *0.089* |
| | Topography4 | | -1.4876 | 1.1353 | -1.31 | 0.19 |
| | Topography5 | | 1.5576 | 0.6283 | 2.479 | **0.013** |
| | **Biofilm** | | | | | |
| | Biofilm0 | | Reference | – | – | – |
| | Biofilm5 | | -1.1473 | 1.1375 | -1.009 | 0.313 |
| | Biofilm20 | | 1.9551 | 0.639 | 3.06 | **0.002** |
| | **Age** | | | | | |
| | Age- day1 | | Reference | – | – | – |
| | Age- day2 | | 0.1402 | 0.2377 | 0.59 | 0.555 |
| | Age- day3 | | -0.6553 | 0.3091 | -2.12 | **0.034** |
| | **Topography × Biofilm** | | | | | |
| | Topography2 × Biofilm5 | | 0.1348 | 1.2361 | 0.109 | 0.913 |
| | Topography3 × Biofilm5 | | -1.0692 | 1.5737 | -0.679 | 0.497 |
| | Topography4 × Biofilm5 | | 1.886 | 1.6822 | 1.121 | 0.262 |
| | Topography5 × Biofilm5 | | -1.2561 | 1.3913 | -0.903 | 0.367 |
| | Topography2 × Biofilm20 | | -1.7419 | 0.7671 | -2.271 | **0.023** |
| | Topography3 × Biofilm20 | | -0.5476 | 0.8064 | -0.679 | 0.497 |
| | Topography4 × Biofilm20 | | 1.1695 | 1.2385 | 0.944 | 0.345 |
| | Topography5 × Biofilm20 | | -1.8612 | 0.8178 | -2.276 | **0.023** |
| **Experiment** | **(Intercept)** | | **-2.132** | **0.2885** | **-7.39** | **<0.001 *** ** |
| **2** | **Topography** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| Topography1 | | Reference | – | – | – |
| Topography2 | | 1.5018 | 0.3293 | 4.56 | **<0.001** |
| Topography3 | | 0.6566 | 0.335 | 1.96 | **0.050** |
| Topography4 | | 1.5425 | 0.3221 | 4.789 | **<0.001** |
| Topography5 | | 0.9278 | 0.3246 | 2.858 | **0.004** |
| **Biofilm** | | | | | |
| Biofilm0 | | Reference | – | – | – |
| Biofilm5 | | -0.4376 | 0.399 | -1.097 | 0.273 |
| Biofilm20 | | -1.3526 | 0.5784 | -2.339 | **0.019** |
| **Age** | | | | | |
| Age- day1 | | Reference | – | – | – |
| Age- day2 | | 0.4899 | 0.1478 | 3.315 | **<0.001** |
| Age- day3 | | 0.525 | 0.1642 | 3.198 | **0.001** |
| **Topography × Biofilm** | | | | | |
| Topography2 × Biofilm5 | | -0.8119 | 0.5218 | -1.556 | 0.12 |
| Topography3 × Biofilm5 | | 0.2704 | 0.4862 | 0.556 | 0.578 |
| Topography4 × Biofilm5 | | -1.3029 | 0.5412 | -2.407 | **0.016** |
| Topography5 × Biofilm5 | | -0.1555 | 0.4792 | -0.324 | 0.746 |
| Topography2 × Biofilm20 | | -0.0676 | 0.6481 | -0.104 | 0.917 |
| Topography3 × Biofilm20 | | 0.3824 | 0.6895 | 0.555 | 0.579 |
| Topography4 × Biofilm20 | | -0.9368 | 0.7073 | -1.325 | 0.185 |
| Topography5 × Biofilm20 | | 0.5485 | 0.6777 | 0.809 | 0.418 |

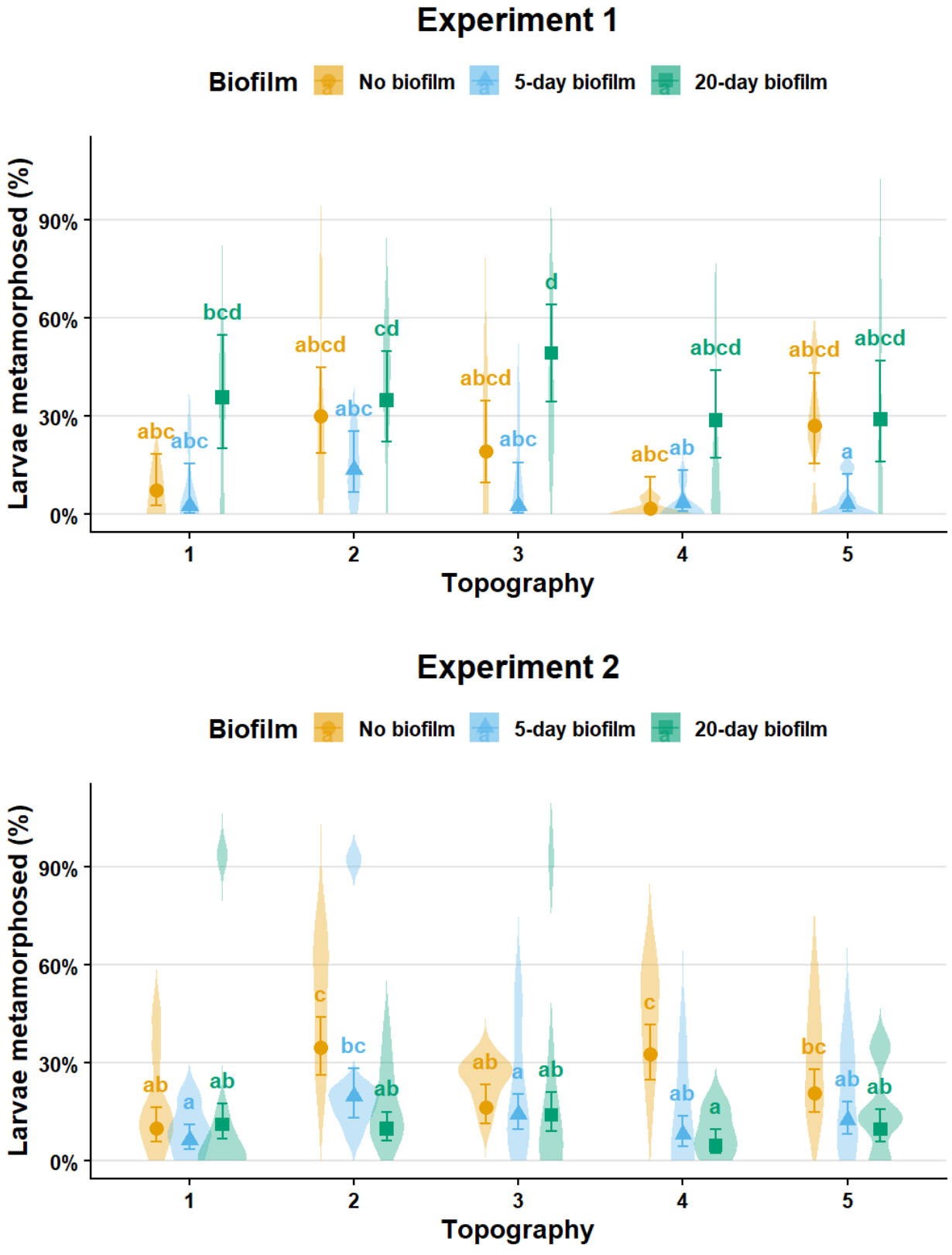


Figure 5.5: A) Predictions of the generalized linear model showing the probability of larvae settlement in experiment 1. Error bars represent 95 % confidence intervals of model predictions. Different letters above points indicate

statistically significant differences ($p < 0.05$) based on post-hoc comparisons using estimated marginal means. Groups sharing the same letter are not significantly different. B) Predictions of the generalized linear model showing the probability of larvae settlement in experiment 2. Error bars represent 95 % confidence intervals of model predictions. Different letters above points indicate statistically significant differences ($p < 0.05$) based on post-hoc comparisons using estimated marginal means. Groups sharing the same letter are not significantly different. Raw data is plotted in the background with violin plots.

## Field experiment

Results of the field experiment showed that the topography treatments -1 -2,-3,-4, and -5 had statistically significant levels of model-predicted settlement (table 5.2. figure 5.6, table 5.S4 in supplement). Topography treatment 4 showed the lowest predicted settlement, while topography treatment 5 showed the highest settlement (table 5.2. figure 5.6,). One factor which contributed to the results seen in the field study was the presence of an amphipod in the genus *jassa* which covered many of the topographical features and in particular were found on topography-2 and topography-4. *Jassa* are tube-dwelling amphipods, which construct small tubes made from detritus and secretions. The tubes which these animals constructed and inhabited completely covered topography features on the tiles they were found, and might have greatly contributed to the number of larvae found on topography treatments 2 and 4. The parallel facing frame had significantly higher settlement than both perpendicular frames. The perpendicular frames were not significantly different from each other (tables 5.2, figure 5.6).

Table 5.2. Results of the statistical model for the field experiment. Significant differences ($p < 0.05$) are in bold.

| | **Estimate** | **Std. Error** | **z-value** | **P-value** |
|---|---|---|---|---|
| (Intercept) | 1.7388 | 0.1324 | 13.132 | **<2.00E-16** |
| **Topography** | | | | |
| Topography1 | Reference | - | - | - |
| Topography2 | 0.5052 | 0.1614 | 3.13 | **0.00175** |
| Topography3 | 1.2602 | 0.1515 | 8.32 | **<2.00E-16** |
| Topography4 | -0.9073 | 0.2166 | -4.188 | **2.81E-05** |
| Topography5 | 1.7199 | 0.148 | 11.617 | **<2.00E-16** |
| **Collector Type** | | | | |
| Parallel collector | Reference | - | - | - |
| Perpendicular Collector 1 | -0.4983 | 0.1148 | -4.342 | **1.41E-05** |
| Perpendicular Collector 2 | -0.2586 | 0.11 | -2.35 | **1.88E-02** |

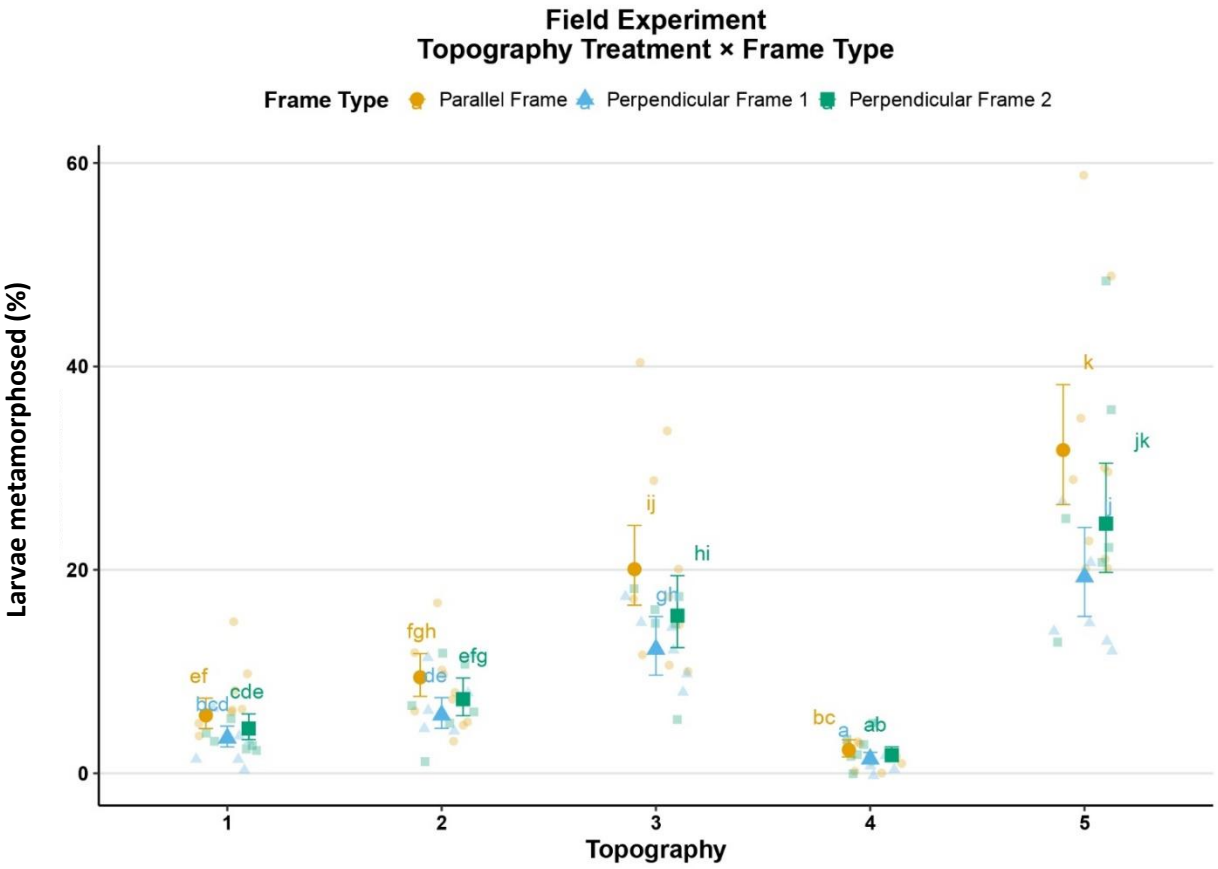


Figure 5.6. Predictions of the negative binomial generalized linear model showing the probability of larvae settlement in the field experiment. Error bars represent 95 % confidence intervals of model predictions. Different letters above points indicate statistically significant differences ($p < 0.05$) based on post-hoc comparisons using estimated marginal means. Groups sharing the same letter are not significantly different. Raw data for the number of larvae metamorphosed per concrete replicate is overlaid in the background.

## Larvae Position

Model predicted metamorphoses from specific locations of the larvae of the different topographical features show that larvae preferentially metamorphose in corners of topographical features in all experiments regardless of the topography treatment, this effect was highly significant in laboratory experiment 2 and the field experiment (see table 5.3, figure 5.7.). While preference for corners was seen in the field, in both lab experiments, the middle of the topographical features was also preferred over tops and sides of topographical features in lab experiment 2 (see table 5.3, figure 5.7).

Table 5.3. Results of the statistical models for larvae position. Significant differences ($p < 0.05$) are in bold.

| Experiment | | Estimate | Std. Error | z-value | P-value |
|---|---|---|---|---|---|
| **Lab- 1** | **(Intercept)** | -0.60864 | 0.35881 | -1.696 | 0.08984 |
| | **Topography** | | | | |
| | Topography2 | Reference | - | - | - |
| | Topography3 | 0.06832 | 0.39251 | 0.174 | 0.86182 |
| | Topography4 | -0.63052 | 0.43221 | -1.459 | 0.14461 |
| | Topography5 | -0.47250 | 0.42123 | -1.122 | 0.26199 |
| | **Position** | | | | |
| | Corner | Reference | - | - | - |
| | Middle | 0.46869 | 0.36992 | 1.267 | 0.20516 |
| | Side | -0.64694 | 0.42427 | -1.525 | 0.12731 |
| | Top | -1.35310 | 0.49508 | 2.733 | **0.00627** |
| | | | | | |
| **Lab- 2** | **(Intercept)** | 0.33270 | 0.17109 | 1.945 | 0.05182 |
| | **Topography** | | | | |
| | Topography2 | Reference | - | - | - |
| | Topography3 | -0.21205 | 0.20898 | -1.015 | 0.31026 |
| | Topography4 | -0.10179 | 0.21195 | -0.480 | 0.63106 |
| | Topography5 | -0.04397 | 0.20426 | -0.215 | 0.82955 |
| | **Position** | | | | |
| | Corner | Reference | - | - | - |
| | Middle | 0.48114 | 0.15101 | 3.186 | **0.00144** |
| | Side | -3.77029 | 0.59274 | -6.361 | **2.01e-10** |
| | Top | -3.48305 | 0.51781 | -6.726 | **1.74e-11** |
| | | | | | |
| **Field** | **(Intercept)** | 1.83621 | 0.13874 | 13.235 | < 2e-16 |
| | **Topography** | | | | |

| | | | | |
|---|---|---|---|---|
| Topography2 | Reference | - | - | - |
| Topography3 | 0.73917 | 0.18631 | 3.967 | **7.26e-05** |
| Topography4 | -1.74090 | 0.27911 | -6.237 | **4.45e-10** |
| Topography5 | 1.33233 | 0.18211 | 7.316 | **2.55e-13** |
| **Position** | | | | |
| Corner | Reference | - | - | - |
| Middle | -2.84781 | 0.39529 | -7.204 | **5.83e-13** |
| Side | -2.15466 | 0.30619 | -7.037 | **1.96e-12** |
| Top | -2.73003 | 0.3773 | -7.236 | **4.64e-13** |

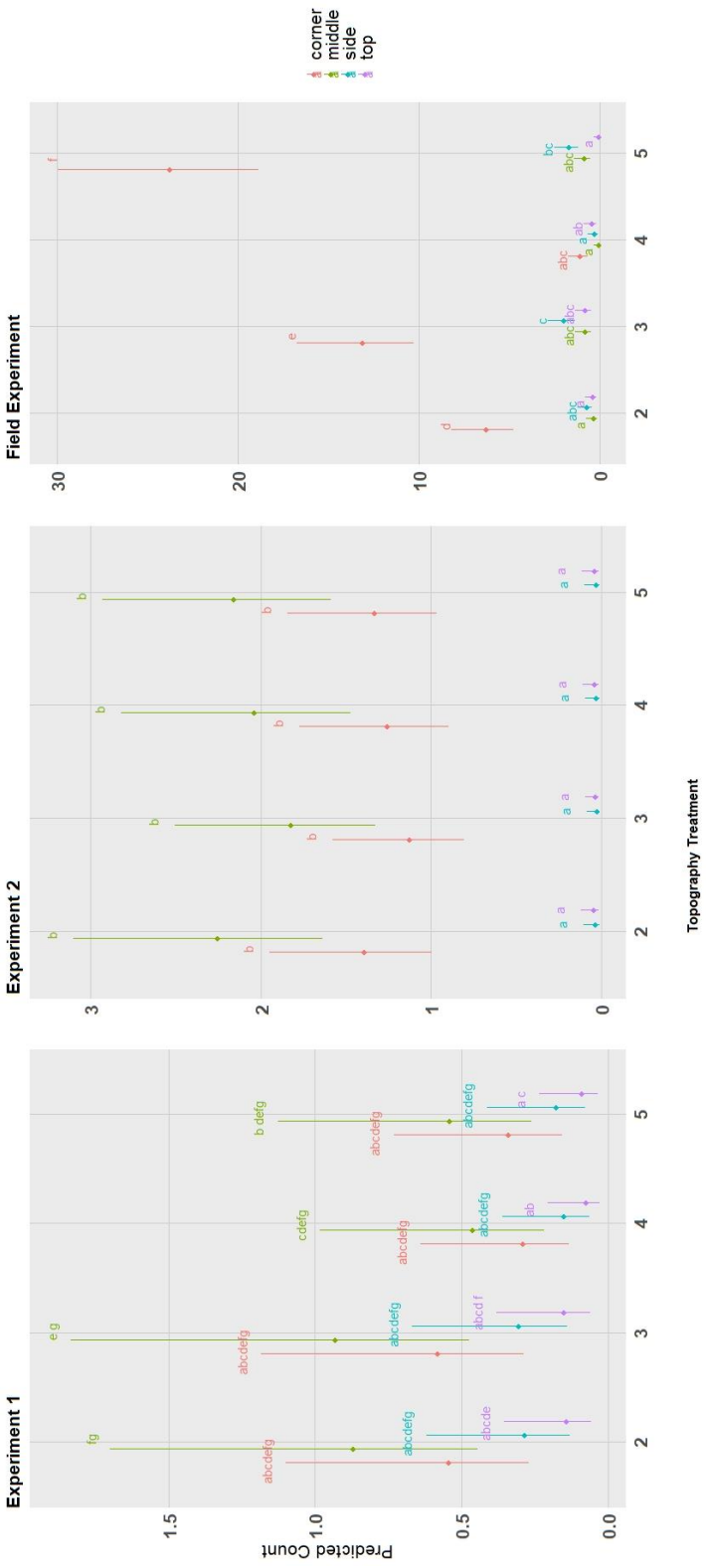


Figure 5.7. Prediction of the negative binomial generalized linear models showing the probability of larvae position, for each experiment. Locations on the topographical features were defined as: “middle”, “corner”, “side”, and “top”.

# Discussion

The presence of the right environmental cues is crucial to determining the type and number of larvae that will settle and complete metamorphosis in an area and should be an important consideration when designing marine restoration projects with a certain species in mind. These larval-environment relationships are often complex, and the intricacies are not fully characterized, even for species with a great ecological importance such as oysters. After an oyster larva descends through the water column and reaches the surface of a substrate, the micro topographical arrangement of that surface influences its subsequent metamorphic decision (Potet et al., 2021). While other substrate characteristics, such as the substrate material type, can affect this metamorphic decision, numerous studies report that topographical modifications to substrates lead to the greatest differences in metamorphic success (Carl et al., 2012; Potet et al., 2021; Erramilli & Genzer.,2019; Bone et al., 2022). The results of this study concur that micro-topography alterations alone can increase the number of larvae found metamorphosed on a substrate and that the addition of biofilms can alter which characteristics are preferred. Importantly, our field and laboratory studies revealed distinct preferences for topographical characteristics, underscoring the need to incorporate both approaches in ecological research.

### Effect of Micro-topography

The most preferred topography in the field was T-5, which featured a large contact angle of 120° between the flat base and sloped side, and a wall spacing of 800 µm. This treatment attracted 3.4 times more predicted metamorphosis than the smooth concrete control and was favored by larva regardless of the substrate's orientation in the water column. In contrast, laboratory experiments produced mixed results. In 2023, no clear preferences for topography alone could be identified. However, when statistical power was increased in 2024, two topographic configurations, T-2 and T-4, were significantly preferred over the smooth control. Both of these had surface features spaced 800 µm apart, though their aspect ratios differed: T-2 had a 1:1 ratio (width = height), while T-4 had a 1:2 ratio (height = 2x width). Despite being preferred in the lab, T-4 showed the lowest settlement rates in the field, and T-2 attracted metamorphosis at levels similar to the smooth control. This discrepancy can be explained (at least in part) by a key

experimental artifact that must be considered when comparing our field and lab outcomes. In the field experiment, the topographical features of treatments T-2 and T-4 were colonized by tube building macrofoulers, amphipods of the genus *Jassa sp.*, whose tubes obstructed the grooves in the substrate. As a result, it remains unclear whether the low metamorphosis on these treatments was due to their intrinsic unattractiveness, or the physical masking of preferred features by *Jassa sp*. colonization. Interestingly, tiles from T-3 and T-5 were not colonized by *Jassa sp.* tubes. The large surface features of T-3 (5 mm × 5 mm) may have been physically unsuitable for *Jassa* colonization. The only difference between T-2 and T-5 was the contact angle and resulting wall slope on T-5, which must have presented some unsuitable for *Jassa* colonization as well. Although not a primary aim of the study, the observed variation in macrofouler colonization among topographies represents a valuable finding that warrants further investigation and demonstrates the importance of considering the interactions of topography and the macrofouling biofilm, as well as the microscopic biofilm. Another strong observation is the preference for larvae to metamorphose in the middle and corners of microtopography features. This observation has previously been noted for oyster *Ostrea edulis* (Potet et al., 2021). In the field experiment this preference for corners especially increased, likely related to the need for larvae to avoid high flow areas or predation. The oyster preferences for corners could be especially useful in designing bio-receptive concretes.

The topographic features selected in this study were designed to represent distinct structural differences, with the aim of identifying specific features of microtopography that influence larval metamorphosis preferences. Much of the previous work on microtopography has focused on antifouling strategies and from this work the attachment point theory was concluded which posits that organisms prefer surfaces with features at least as wide as their own body size, as this maximizes contact points for secure attachment. Supporting this theory, studies have shown that features smaller than the organism often reduce metamorphosis compared to smooth controls or to features equal to or larger than the body length of larvae (Schumacher et al., 2007; Scardino et al., 2008; Carl et al., 2012; Vucko et al., 2014; Erramilli & Genzer, 2019). However, when approaching from the perspective of maximizing metamorphosis rather than preventing it, relatively few studies have examined how larvae respond to topographic features larger than their own body size (Scardino et al., 2008; Carl et al., 2012). In previous studies with bivalve species *Saccostrea glomerata* and *Mytilus galloprovincialis*, while features smaller than the size of thelarvae reduce metamorphosis, after this threshold is crossed, features larger than the size of thelarvae do not influence metamorphosisrates further (Vucko et al., 2014; Carl et al., 2012).

In this study, we tested features larger than the size of *Magallana gigas* larvae at two scales: a micrometer scale (800 µm) and a millimeter scale (5 mm). Features which can be replicated easily in concrete, and create distinct microhabitats for larvae (Bauer et al 2024). From a hydrodynamic standpoint, micrometer-scale features influence the surface boundary layer, providing shelter from shear stress without substantially altering overall flow or food delivery (Koehl, 2007; Reidenbach et al., 2021; Bauer et al., 2024). These features may also offer protection from small grazing predators. In contrast, millimeter-scale features generate localized turbulence and vertical mixing, potentially enhancing particle delivery and increasing access to food (Commito & Rusignuolo, 2000; O'Donnell, 2008; Bauer et al., 2024). However, their capacity to protect larvae from predation may be limited to larger predators.

In our field experiment, the topography treatment with the largest features (T-3, 5 mm × 5 mm) was preferred over the smooth control but not to the same extent as T-5, which also had millimeter-scale features but with a distinct geometry. T-3 outperformed T-2 and T-4, both of which had micrometer-scale features, though field interpretation was complicated by macrofouling, particularly the presence of *Jassa* sp. tubes, which obscured key features on T-2 and T-4. In the second laboratory experiment, all treatments with micrometer-scale features (T-2, T-4, and T-5) were preferred over smooth controls. T-3 also showed improved metamorphosis over the control, though to a lesser extent. Notably, the attractiveness of T-3 increased when paired with a 20-day biofilm. Taken together, these results suggest that oyster larvae may preferentially metamorphose on micrometer-scale topographies but can also interpret larger topographic structures, or the microhabitats they create, as positive cues for metamorphosis.

An additional objective of this study was to determine whether oyster larvae exhibit preferences for different aspect ratios; the proportional relationship between the width and height of topographic features. Reidenbach et al. (2021) modeled flow dynamics over surfaces with varying aspect ratios and found that when the width-to-height ratio (w/h) exceeds 1, coral larvae are subjected to higher shear stress, increasing the likelihood of dislodgement. In our study, the topographical treatments T-2 (800 µm × 800 µm; aspect ratio 1:1) and T-4 (800 µm × 1600 µm; aspect ratio 1:2) elicited no significant differences in larval metamorphosis under laboratory conditions. However, field data indicated a slight preference for T-2, the configuration with equal width and height. This is consistent with findings in mussels, where

larval preference is not influenced by aspect ratio as long as the surface features exceed larval body size (Carl et al., 2012).

### Effect of Biofilm

Research on the relationship between biofilms and larval settlement/metamorphosis has documented inductive, neutral, and inhibitory responses (Bao et al., 2007; Campbell et al., 2011; Hadfield, 2011; Wang et al., 2012; Hadfield et al., 2014; Dobretsov & Rittschof, 2020). The specific characteristics of biofilms that mediate these effects remain poorly understood, making it difficult to predict how larval behavior may vary due to alteration of the biofilms. In oyster larvae, biofilms have generally been shown to induce both settlement and metamorphosis, with inductive effects often increasing with biofilm age, often showing attractive effects after one week maturity (Campbell et al., 2011; Rodriguez-Perez et al., 2019). However, our results were unexpectedly inconsistent between the two years of the study. In 2023, larvae were strongly attracted to the oldest biofilms (20 days), while in 2024, the same treatment was unattractive. These findings suggest that environmental conditions differed between the two years in ways that altered the composition of the biofilms perceptible to larvae. Known stressors including heavy metals, temperature anomalies, and acidification, can modify biofilm communities, reducing their capacity to induce invertebrate larvae settlement (Lau et al., 2005; Bao et al., 2010; Espinel-Velasco et al., 2021). Although the substrates in our study were deployed at the same site and time of year, interannual environmental variability, such as temperature, acidity, presence of heavy metals or other toxins, could have influenced biofilm development in ecologically significant ways. One limitation of this study is that we don't have information on the environmental data from the periods during which the biofilms were developed, which might provide some better explanation for the observed results. Another potential theory is that the larvae's origin influenced their biofilm preference. In Experiment 1, larvae were produced from adults collected along the coast of Ostend, Belgium, whereas in Experiment 2, the larvae were purchased from France, likely originating from hatchery-reared adults. It is possible that the parental origin may have primed the larvae to be more receptive to particular biofilm species.

### Biofilms modulate the attractiveness of specific micro-topographies

While biofilm both increased or decreased metamorphosis depending on biofilm age and the experiment, its presence also tended to homogenize micro-topography, obscuring specific preferences for topography treatments in the laboratory experiments. While larvae showed a moderate or strong preference for topography treatments T-2, T-4 and T5, this preference largely disappeared when the surface was covered with biofilm, rendering different micro-topographies almost equally attractive (or unattractive).

Notably, in Experiment 1, the addition of the 20-day biofilm did not lead to significant differences among the other treatments. It is possible that topographical features less relevant for larvae were overridden by the strong inductive cue provided by the biofilm, prompting metamorphosis regardless of topography type. Experiment 2 showed an overall negative effect of the 20-day biofilm on metamorphosis. The most preferred substrates in the absence of biofilms, T-2 and T-4, exhibited reduced metamorphosis when the biofilm was present. These findings suggest that when a biofilm acts as a deterrent, even substrates that would otherwise be favorable are avoided by the larvae. Extrapolating these results further indicates that oyster larvae may prioritize biofilm-associated cues over topographical features when selecting sites. However, this hypothesis requires additional investigation to be fully substantiated.

In the first experiment conducted in 2023, the addition of a 20-day biofilm generally enhanced larval metamorphosis, but a statistically significant increase was observed only on treatment T-3. This treatment featured the largest surface structures, suggesting that biofilm-mediated modifications, such as altered water flow or light penetration, may have contributed to the observed pattern. Another explanation for these results may be that larvae are integrating multiple environmental cues simultaneously and react on the perceived importance of different cues. When a suboptimal cue is present, larvae may still initiate settlement/metamorphosis if another, more favorable cue is available. A lack of necessary cues such as food availability might also cause larvae to disregard favorable cues.

# Conclusions

In reviewing the concept of bioreceptivity in built structures, originally defined by Guillitte (1995), Sanmartín et al. (2021) highlights intrinsic and extrinsic bioreceptivity of material, noting that these extrinsic factors (biofilms formation, flow patterns, light availability) are often overlooked in research and make the overall bioreceptivity of a substrate very context-driven. Our findings align well with this perspective. Context plays a critical role, particularly when designing substrates intended to enhance

larval metamorphosis of target species for restoration purposes. While our laboratory experiments indicated a general preference for micro-scale topographical features, field trials demonstrated the strongest success only on the treatment that resisted colonization by competing macrofoulers. This highlights the importance of local biotic interactions, which may vary by region depending on the resident fouling community. Restoration projects would benefit from the identification of key competitors for substrate space and whether design modifications can improve outcomes for the target species. The development of a favorable biofilm matrix proved essential for attracting larvae, underscoring the importance of strategic deployment timing and/or preconditioning of restoration materials to maximize metamorphotic success. This study also underscores the need for a deeper understanding of how larvae prioritize different environmental cues during settlement/metamorphosis, both for furthering fundamental knowledge of larvae ecology but also to inform applied restoration efforts.

# Chapter 6: General Discussion

The transition of bivalve larvae from their larval stage into adulthood represents the culmination of countless, meticulously coordinated evolutionary relationships. Just as a human might assess their environment by sight, sound, touch, and smell, larvae similarly use a suite of senses to detect environmental signals that indicate the highest chances of survival as adults. Of course, larvae do not possess decision-making capabilities, and no conscious choices drive their behaviors. Rather, species-specific, evolved mechanisms regulate settlement and metamorphosis.

Research into these environmental signals began in the 20th century (Cole & Jones., 1939; Crisp., 1962; Bayne., 1969; Hidu., 1969), documenting key behavioral patterns, starting with larvae's tendency to settle and metamorphoses among conspecific adults, a phenomenon later termed gregarious settlement. A considerable body of research has followed, identifying which environmental factors larvae can detect. Yet, despite how well some of these relationships are documented, significant knowledge gaps remain. Notably, there is a lack of mechanistic understanding of how multiple, simultaneously experienced cues interact in dynamic environments and which factors can change the influence of these cues. Just as humans assess situations by prioritizing variables based on their relative importance or by integrating independent signals to increase confidence in a choice, larvae have likely evolved analogous systems that remain largely undefined. As more research emerges, the effort to characterize these relationships will become easier and predictions about larvae behaviors in realistic dynamic environments will become more accurate.

### Cue Conflicts and Sensory Integration

One implication of the work presented here is that larvae prioritize conflicting signals during settlement and metamorphosis depending on the nature of the cues involved. In chapter 3 of this thesis, chemical cues associated with predators were disregarded once favorable substrate cues were detected. In chapter

5, cues related to surface microtopography were modulated by the presence of biofilms. These behavioral experiments expand our understanding of how these interactions between cue types manifest in behavioral adaptations. While in chapter 3, there was a distinction between waterborne and substrate bound cues showing that substrate bound cues have a higher priority as signals during metamorphosis, chapter 5 finds that among substrate bound signals, certain priorities can also exist.

Our results imply that despite the fact that bivalve larvae have limited neural complexity, their responses can still be complex. The cue prioritizations observed in this thesis are likely not due to the result of active decision making or comparison, but instead evolutionary tuning that biases sensory pathways toward certain responses over others, depending on which cues have historically led to higher survival value. The logical next step is to investigate these behavioral outcomes in the context of larval sensory physiology.

In invertebrates, settlement and metamorphosis are distinct processes regulated by separate but interconnected physiological mechanisms. Settlement is mediated by fast-acting neural pathways involving neurotransmitters and neuropeptides that respond to external environmental cues. While, metamorphosis is driven by slower-acting, internal neuroactive factors that govern irreversible developmental changes (Coon et al., 1990; Conzelmann et al., 2013; Joyce & Vogeler, 2018). Therefore, cue integration at the behavioral level is likely mirrored at the molecular level, through the coordinated activity of signaling molecules regulating both processes. However, evidence of how environmental cue interactions manifest at the molecular level remains limited, and to the best of my knowledge, no studies have yet fully characterized these processes in bivalve larvae. Other invertebrate species offer useful insight. For instance, Say and Degnan (2020) showed that larvae of the sponge *Amphimedon queenslandica* do not settle in response to chemical cues when exposed to constant light. They found that both nitric oxide (NO) and the receptor ANPR-A play roles in regulating settlement in this species. Light exposure disrupted the normal downregulation of ANPR-A without affecting NO production, indicating that light specifically interferes with this particular signaling pathway, while NO remains activated by a chemical cue. The findings presented in this thesis can, and should, serve as a foundation for future molecular studies aimed at uncovering how bivalve larvae integrate multiple environmental signals. A molecular approach will be essential for fully understanding the complex interplay between environmental stimuli and behavior outcomes.

**Acoustic Cues and Larval Settlement: A New Frontier for Bivalves**

Soundscape cues have only relatively recently been identified as influential in larval settlement, and confirmed acoustic attraction has been demonstrated in only a few species. The work presented in this thesis is the first to show that Pacific oysters (*Magallana gigas*) increase settlement/metamorphosis in response to habitat-associated sounds. This finding aligns *M. gigas* with the other oyster species known to utilize acoustic cues, such as *Ostrea angasi* and *Crassostrea virginica*. Notably, these results are also among the first to use soundscapes from North Sea oyster reefs rather than the tropical or subtropical reef environments typically studied.

Work characterizing the acoustic profile of North Sea reefs is still in its early stages. However, existing evidence points to a distinct and biologically rich soundscape, though the primary soniferous organisms contributing to these sounds have not yet been identified. To the best of my knowledge, our study is one of the first to demonstrate that invertebrates respond behaviorally to the unique acoustic profile of North Sea reefs, suggesting that these soundscapes may serve as ecologically relevant cues, although it's likely that more will follow.

Despite these promising findings, which this thesis has contributed to, much remains unknown about the specific features of reef sounds that elicit settlement responses in larvae. This gap in knowledge applies broadly across taxa known to use reef sounds as cues, including bivalves, corals, and fish larvae (Vermeij et al., 2007; Lillis et al., 2013; Aoki et al., 2024). The work in this thesis points to the conclusion that the acoustic complexity of reef soundscapes, rather than louder volume, is a key driver of larval settlement/metamorphosis behavior. This conclusion is supported by the observation that playback of vessel noise, despite being louder than both off-reef and silent controls, did not significantly affect metamorphosis rates. Although our study could not conclusively show that vessel noise masks reef cues, a non-significant trend in that direction was observed. Moreover, masking effects from anthropogenic noise have been demonstrated in oyster *O. angasi* (Williams et al., 2024), lending support to this concern. Moving forward, a key goal should be to dissect the composition of attractive reef sounds, identifying which acoustic features (e.g., frequency range, temporal patterns, amplitude, complexity) are relevant to oyster larvae.

The relevance of identifying the specific acoustic features that drive settlement/metamorphosis becomes particularly compelling when considering that attraction may be highly species-specific, even among closely related taxa. For example, some mussel species have been shown to use vessel noises as cues (Wilkens et al., 2012; Jolivet et al., 2016), suggesting that mussels and oysters may rely on different acoustic features during habitat selection. If future work can make the connection between acoustic feature preferences and species-specific attraction, this could have important implications for restoration ecology, antifouling technologies, and aquaculture.

Finally, future work on acoustic cues should not view sound in isolation, but rather aim to place its importance in context with other environmental signals, such as chemical or tactile cues. Integrating our understanding of multimodal cue systems will be essential for advancing both fundamental research and applied conservation strategies.

### Divergent Metamorphic Strategies Among Invertebrate Larvae

Not all invertebrate larvae complete metamorphosis in the same way, leading to multiple hypotheses about how different cues are used during settlement/metamorphosis. Differences in ontogeny and the physical requirements of various species can result in distinct cue interpretation strategies. Understanding these differences is essential for placing into context the role and importance of environmental cues in each species.

Bishop et al. (2006) outline several of these hypotheses, including the Desperate Larva Hypothesis, the Variable Retention Hypothesis, and the Death Before Dishonor Hypothesis. The Desperate Larva Hypothesis, originally proposed based on the work of Wilson (1953) and Knight-Jones (1953), suggests that as larvae age, they become less selective about cues due to the energetic cost of maintaining competency (readiness to settle and complete metamorphosis). Eventually, they may settle indiscriminately, even in suboptimal habitats lacking appropriate cues. Desperate Larva behavior is thought to apply mainly to lecithotrophic larvae, which do not feed during the larval phase and rely on finite energy reserves. In contrast, planktotrophic larvae, those that feed, can theoretically extend their larval period to seek more ideal locations. However, planktotrophic larvae may also exhibit increasingly indiscriminate settlement behaviors over time. This leads to the Variable Retention Hypothesis, which proposes that although these larvae are more flexible due to their ability to feed, they may still become

less selective as time passes. This reduced selectivity could stem from food limitations or the increased demand of energy required to sustain new juvenile features. Finally, the 'death before dishonor' hypothesis applies to habitat specialists that will not undergo metamorphosis unless specific environmental cues are present. In the absence of these cues, larvae will eventually die rather than exist in unsuitable habitats.

Larvae of bivalves in the families Mytilidae and Ostreidae are predominantly planktotrophic, though some exceptions exist, such as certain brooding species of *Ostrea*. These families are generally habitat generalists and tend to accept a wider range of cues. Currently, there is insufficient evidence to definitively assign these species to any single hypothesis. However, the Variable Retention Hypothesis appears to be the most likely model (Bishop et al., 2006). Still, the point at which these larvae become less selective, if at all, remains unclear. Likewise, there is no strong evidence that they completely refuse to metamorphose in the absence of cues, making it unlikely that the Death Before Dishonor hypothesis fully applies to them. While this was not the original focus of my research, the nature of my behavioral experiments spanning multiple days and using larvae fertilized on the same day made it possible to observe the effects of larval age on metamorphosis. Statistical analysis revealed that age had a significant effect on metamorphosis rates in every experiment. However, the direction of this effect was inconsistent across chapters (see statistical analysis for Chapters 3, 4, and 5), and did not always show an increase in metamorphosis with age. Moreover, there were no clear interaction effects between larval age and specific cues. These findings suggest that while age may influence settlement/metamorphosis behavior, it does not do so in a uniform or predictable manner, and its interaction with environmental cues remains complex. Meyer et al., (2018) studied how larval age dictates oyster larvae behavior in an attempt to investigate this question, but the results were inconsistent depending on the different methods of assessing a behavioral response (different swimming behaviors). There was a tendency for older larvae in the absence of cues to remain vertically close to substrate bottoms, suggesting increased changes of settling in suboptimal conditions. There is a need for further investigation into how *Mytilidae* and *Ostreidae* larvae use environmental cues as part of their broader metamorphic strategies, and how this aligns with the theories discussed above. If larval age intersects with cue sensitivity, this relationship should be more clearly integrated into our overall understanding of settlement and metamorphic cues.

The behavioral experiments presented in this thesis found that in the absence of positive cues, metamorphosis was very low but not non-existent, suggesting that some larvae are willing to metamorphose in suboptimal habitats. This may reflect individual variation, suggesting that some larvae

may do so regardless of cue presence. These seemingly desperate or bold individuals could be an artifact of the experiment (some larvae reaching competency earlier than others and becoming "desperate"), or they may represent a form of bet-hedging strategy aimed at colonizing new habitats. It has been speculated that within a single clutch, some larvae act as pioneers, settling more indiscriminately and potentially creating a signal-rich environment that later attracts more selective "joiner" larvae. This idea has been proposed by several larval ecologists (Toonen and Pawlik, 1994; Bishop et al., 2006; Clobert et al., 2009), but definitive empirical support for this adaptive strategy is lacking. Investigating this hypothesis would be highly relevant to the field, though developing adequate experimental methods would likely be challenging.

## Studying Larval Settlement and Metamorphosis: Methodological Considerations and Limitations

The methodology for studying larval settlement and metamorphic cues deserves careful consideration. While there are clear limitations to investigating environmental cues under small-scale laboratory conditions, such experiments are necessary when studying multiple and interacting cues. Laboratory settings allow for control over confounding variables, which is especially important when investigating specific cue combinations, as it is challenging to create field conditions devoid of background signals.

Though I believe these controlled experiments are a necessary first step, I also acknowledge their limitations. One of the practical challenges is selecting the appropriate number of larvae. While it may seem advantageous use as many individuals as possible to maximize the power of the experiment, each larva must be individually inspected under a microscope to determine settlement or metamorphosis, which imposes logistical constraints on sample size. Another critical decision is determining the exposure duration. In my work, I selected time points based on relevant literature and my own preliminary experiments. However, I believe future studies could benefit from designs incorporating multiple time points. For example, research on acoustic cues might aim to pinpoint how rapidly larvae respond to sound, especially considering that, in natural conditions, exposure to reef sounds may occur only for a few minutes to hours (Lillis et al., 2014b).

One variable absent from my methodology, but crucial for future work, is water flow. In natural environments, flow shapes how larvae experience cues, particularly waterborne chemical signals and surface microtopography. While adding realistic flow conditions can enhance ecological relevance, it also introduces complexity. As discussed in Chapter 2, turbulence itself may act as an additional cue, complicating interpretation. Moreover, delivering chemical cues in ecologically relevant ways under flow, especially at consistent concentrations is technically challenging. It is very difficult to introduce chemical olfactory cues in ecology-relevant ways, whether there is flow or not. If I had unlimited time and resources, I would have conducted experiments testing multiple cue concentrations under a range of flow conditions. However, the factorial design necessary to test multiple interacting cues already requires a large number of treatments, and adding additional variables such as flow makes the experimental design significantly more demanding. Despite these challenges, careful methodological improvements, particularly those that bridge controlled laboratory work with field experiments, such as my methodology in chapter 5, should be considered best practice.

### Practical application of the results: artificial reef restoration design

Many of the environmental cues investigated in this thesis have direct and practical applications in reef restoration. Several cues are already being integrated into restoration design. For instance, the use of conspecifics, either in the form of shells or live adults, is one of the most common strategies used to promote settlement on restored reefs (Pogoda et al., 2019). As demonstrated in this work, chemical cues produced by live adults, when combined with shell substrate, significantly enhance settlement beyond what shells alone can achieve. When feasible, this combined approach would likely be the most effective intervention.

Acoustic enrichment is another emerging strategy in reef restoration. While this technique has shown clear benefits for coral and fish larval recruitment (Gordon et al., 2019; Aoki, 2024), more recent experiments have also demonstrated promising results for corals and bivalves (McAfee et al., 2023; Pysanczyn et al., 2023; Aoki et al., 2024). In Australia, acoustic enrichment has been used to increase settlement/metamorphosis in the native flat oyster *Ostrea angasi* (McAfee et al., 2023; Williams et al., 2023). There is strong potential for applying soundscape enrichment in other coastal systems, including the North Sea especially as our understanding of local reef acoustics continues to improve.

Surface topography is another commonly used intervention, with textured or roughened substrates incorporated in substrate design to encourage invertebrate recruitment (Perkol-Finkel et al., 2014). However, the specific design of microtopographic features is rarely tailored to the settlement preferences of individual species, largely because these preferences remain poorly understood. Findings from this thesis highlight the importance of refining substrate features with greater species-specificity to improve restoration outcomes. As demonstrated in chapter 5, modifications for surface structures need to also include consideration of the study habitat and how the microscopic and macroscopic biofilm will develop. Prior investigation into the resident fauna should be standard practice when choosing a potential artificial reef restoration site.

Throughout this project, I have explored cues that act on larvae across different spatial scales. Cues like hydrodynamics and reef-associated sounds are likely detectable tens to hundreds of meters away from the reef (depending on environmental conditions), while chemical cues from conspecifics likely only influence larvae within centimeters above the benthos due to rapid dilution and diffusion in the water column (Hodin et al., 2018). Finally, substrate-bound cues such as microtopography, are likely detected at the moment of physical contact, just prior to metamorphosis. This suggests that larvae may encounter multiple decision points or "bottlenecks" as they progress from the water column to the benthos.

Applying this knowledge to restoration could take the form of a multi-pronged strategy, one that offers environmental cues at all relevant spatial and sensory scales. For example, combining acoustic enrichment, live adult conspecifics, and micro-textured surfaces designed for optimal performance, may collectively enhance settlement/metamorphosis rates far more effectively than any single intervention. Multi-pronged restoration approaches are already being used with success. Temmink et al. (2021), for example, enhanced oyster recruitment by combining substrate provision with predation protection. Williams et al. (2023) simultaneously used acoustic enrichment and artificial kelp structures, which suppress competitive species and create a more favorable environment for oyster larvae. A multi-pronged restoration approach aimed at enhancing environmental cues from multiple scales of influence would be a worthwhile approach.

# Concluding Remarks

Reef-building bivalves are critical components of the ecosystems they occupy, earning them the designation of ecosystem engineers (Gutiérrez et al., 2003; Ysebaert et al., 2019). The final larval stage in the development of these species represents a major bottleneck, determining both their settlement/metamorphosis location and likelihood of post-settlement survival and recruitment. The work presented here expands our understanding of the settlement and metamorphic cues for reef-building bivalves in general, and for the oyster *Magallana gigas* specifically. I offer further insight into the specific characteristics of cues to which larvae respond, including reef-associated sounds and substrate surface microtopographic. By investigating multiple and conflicting cues, a theory is proposed to explain how oysters integrate multiple cues encountered simultaneously. I hope to leave the reader with a more holistic understanding and appreciation of the complex processes of settlement and metamorphosis, and of the critical interplay between environmental cues, larval behavior, and the persistence of these species.

# Summary (English)

For marine invertebrates with a larval stage, settlement and metamorphosis represent critical life-history transitions. This irreversible shift from a pelagic to a benthic habitat is triggered by environmental cues that larvae use to identify suitable habitats for their species. Reef-building bivalves, including mussels and oysters, are an important subgroup of marine invertebrates, and their ecological role extends beyond the sustainment of their own populations. The biogenic reefs formed by aggregated adult bivalves improve the local environment in a variety of ways, earning them the title of ecosystem engineers.

Settlement and metamorphosis, though sometimes grouped under the umbrella term "settlement" in the literature, are two distinct processes. For marine bivalves during settlement, larvae descend the water column and attach to a substratum. This attachment is potentially reversible, but metamorphosis, which follows settlement, is a permanent process in which larvae undergo physiological changes and become fixed to the substratum, beginning their sessile adult life. As habitat generalists, bivalves rely on a variety of cues to indicate the suitability of a habitat, including light, sound, hydrodynamics, biofilms, trophic conditions, and signals from conspecifics. Additionally, larvae can detect cues from predators or other organisms to assess habitat suitability.

While a substantial body of research on these cues exists, there are still significant knowledge gaps. Chemical cues are relatively well-studied, but physical cues, such as sound and microtopography, remain less understood. Furthermore, the lack of species-specific data often leads to generalized conclusions across multiple taxa, which may not be entirely accurate. As scientific research disproportionately focuses on a small number of model organisms. There is also a lack of information regarding how the interaction

of multiple environmental cues is interpreted by larvae, or how cue perception may be impacted by anthropogenic changes to marine environment. Understanding the mechanisms underlying these critical processes for reef-building bivalves advances our knowledge of fundamental larval ecology, particularly settlement and metamorphosis as adaptive reproductive strategies. Additionally, as bivalve reefs are rapidly declining and restoration efforts are a priority for conservation, a better understanding of bivalve recruitment can improve the design of reef restoration projects.

In this thesis, I experimentally investigate the settlement and metamorphic cues that influence bivalve larvae, focusing on how larvae respond to interacting cues and how cues can be modulated in ways that can impact their effectiveness. This work covers both chemical and physical cues, focusing especially on those with potential applications to improve recruitment on artificial reefs.

First, I conduct a literature review of all cues influencing the settlement and metamorphosis of reef-building bivalves from the families Mytilidae and Ostreidae. This review serves as a foundation for future research on bivalve settlement and metamorphosis and is intended for both fundamental ecologists and those focusing on applied work, providing context for each cue type for readers less familiar with the topic.

Next, I experimentally test how larvae of the Pacific oyster (*Magallana gigas*) respond to multiple, and conflicting chemical cues at different scales (waterborne and substrate-bound), and from various sources, conspecific adults, predators, and biofilms. We test larvae behaviors in mesocosm experiments, using a fully factorial experiment design. The results of this study indicate that detection of positive cues which are substrate bound, cause larvae to disregard conflicting (negative) waterborne chemical cues. The combination of conflicting (positive and negative) waterborne cues however does result in a trend in reduction of metamorphosis. These results also reveal that larvae do not respond uniformly to positive cues, and the presence of multiple positive cues further enhances metamorphosis success.

In the following chapter, we investigate how marine acoustic environments influence larvae metamorphosis by testing reef-associated sounds, vessel noise, combined reef and vessel noise, and off-reef conditions. This influence on larvae metamorphosis in *M. gigas* is assessed through lab-based acoustic playback lab experiment. This study demonstrates for the first time in this species the use of acoustic cues for settlement/metamorphosis. We find that vessel noise alone does not induce metamorphic rates beyond those observed with the control (off-reef) sounds. While the results do not

show significant evidence of vessel noise masking the attractive reef sounds, a reductive trend suggests that further research into this area is warranted.

In the final chapter, we experimentally test how specific features of surface microtopography influence larvae metamorphosis on concrete substrates, and how the presence of biofilm modulates preferences for these microtopographic features. Microtopographic features, ranging from micrometers to millimeters in scale, were tested in controlled lab experiments and in a field experiment at Ostende Harbor, Belgium. Results from field data show that the response of *M.gigas* larvae to biofilms changed between the two years: in year one, biofilms were generally attractive, while in year two they were repulsive. In the lab, larvae showed a preference for microstructures around 800 µm in size, with no preference observed for different aspect ratios or contact angles. Results from the field experiment further demonstrate how context-dependent surface microtopography can be, as a macrofouling competitor obscured the preferred topographical features identified in the lab. Interestingly, microstructures with the widest contact angles were not colonized by the macrofouler and showed significantly higher metamorphic rates.

# Samenvatting (Nederlandse)

Voor mariene ongewervelden met een larvaal levensstadium vormen vestiging en metamorfose cruciale overgangsfasen in hun levenscyclus. Deze onomkeerbare omschakeling van een pelagisch naar een benthisch habitat wordt getriggerd door omgevingssignalen die larven gebruiken om geschikte soortspecifieke habitats te identificeren. Rifvormende tweekleppigen, waaronder mosselen en oesters, vormen een belangrijke subgroep van mariene ongewervelden en hun ecologische rol reikt verder dan het in stand houden van hun eigen populaties. De biogene riffen die worden gevormd door geaggregeerde volwassen tweekleppigen verbeteren de lokale omgeving op verschillende manieren, wat hen de titel van ecosysteemingenieurs oplevert.

Vestiging en metamorfose worden in de literatuur soms onder de overkoepelende term "vestiging" geschaard, maar het zijn twee afzonderlijke processen. Tijdens de vestiging dalen larven af in de waterkolom en hechten zich vast aan een substraat. Deze vasthechting is potentieel omkeerbaar, maar

de daaropvolgende metamorfose is een onomkeerbaar proces waarbij larven fysiologische veranderingen ondergaan en zich vastzetten op het substraat, waarmee hun sessiele volwassen leven begint. Als habitat generalisten vertrouwen tweekleppigen op een verscheidenheid aan signalen om de geschiktheid van een habitat te bepalen, waaronder licht, geluid, hydrodynamica, biofilms, trofische omstandigheden en signalen van soortgenoten. Daarnaast kunnen larven ook chemische signalen van predatoren of andere organismen detecteren om de geschiktheid van een habitat in te schatten.

Hoewel er veel onderzoek is gedaan naar deze signalen, bestaan er nog steeds aanzienlijke kennishiaten. Chemische signalen zijn relatief goed bestudeerd, maar fysieke signalen, zoals geluid en microtopografie, zijn minder goed begrepen. Bovendien leidt het gebrek aan soortspecifieke gegevens vaak tot algemene conclusies over verschillende taxa, die mogelijk niet volledig accuraat zijn. Er is ook weinig bekend over hoe larven de interactie tussen meerdere omgevingssignalen interpreteren of hoe de waarneming van signalen kan worden beïnvloedt door antropogene veranderingen in het mariene milieu. Inzicht in de mechanismen die aan de basis liggen van deze cruciale processen bij rifvormende tweekleppigen bevordert onze fundamentele kennis van larvale ecologie, met name vestiging en metamorfose als adaptieve voortplantingsstrategieën. Gezien de riffen van tweekleppigen snel achteruitgaan en herstelmaatregelen een prioriteit zijn binnen natuurbescherming, kan beter begrip van de rekrutering van tweekleppigen bijdragen het ontwerp van rifherstelprojecten verbeteren.

In deze thesis onderzoek ik experimenteel de vestigings- en metamorfosesignalen die larven van tweekleppigen beïnvloeden, met een focus op hoe larven reageren op interacterende signalen en hoe signalen gemoduleerd kunnen worden op manieren die hun effectiviteit kunnen beïnvloeden. Dit werk omvat zowel chemische als fysieke signalen, met bijzondere nadruk op signalen die mogelijk toegepast kunnen worden om rekrutering op kunstmatige riffen te bevorderen.

Allereerst heb ik een literatuuronderzoekuitgevoerd naar alle signalen die invloed hebben op de vestiging en metamorfose van rifvormende tweekleppigen uit de families Mytilidae en Ostreidae. Deze studie vormt een basis voor toekomstig onderzoek naar vestiging van tweekleppigen en is bedoeld voor zowel fundamentele ecologen als onderzoekers met een toegepaste focus, waarbij context wordt geboden voor elk type signaal voor lezers die minder vertrouwd zijn met het onderwerp.

Vervolgens testen we experimenteel hoe larven van de Japanse oester (*Magallana gigas*) reageren op meervoudige en conflicterende chemische signalen die op verschillende schalen worden waargenomen (via het water of gebonden aan het substraat) en afkomstig zijn van verschillende bronnen: soortgenoten,

predatoren en biofilms. We testen het gedrag van larven in mesocosmosexperimenten met een factorieel experimentele opzet. De resultaten van deze studie tonen aan dat de detectie van positieve substraatsgebonden signalen ervoor zorgt dat larven conflicterende (negatieve) chemische signalen in het water negeren. De combinatie van tegenstrijdige chemische signalen in het water veroorzaakt echter wel een significante vermindering van de metamorfose. De resultaten tonen ook aan dat larven niet uniform reageren op de aanwezigheid van een positief signaal, en dat de aanwezigheid van meerdere positieve signalen het metamorfosesucces verder verhoogt.

In het volgende hoofdstuk onderzoeken we hoe mariene akoestische omgevingen de vestiging van larven beïnvloeden, door geluiden te testen die geassocieerd zijn met riffen, scheepsgeluid, een combinatie van rif- en scheepsgeluid, en omstandigheden zonder rifgeluid. De invloed op larvale vestiging bij *M. gigas* wordt onderzocht via een akoestisch afspeelexperiment in het laboratorium. Deze studie toont voor het eerst bijdeze soort het gebruik van akoestische signalen voor vestiging en metamorfose aan. We constateren dat scheepsgeluid op zichgeen hogere vestigingspercentages teweeg brengt dan de controle (geluiden buiten het rif). Hoewel de resultaten geen significant bewijs leveren dat scheepsgeluid het aantrekkelijke rifgeluid maskeert, suggereert een neerwaartse trend dat verder onderzoek nodig is.

In het laatste hoofdstuk testen we experimenteel hoe specifieke kenmerken van microtopografie de metamorfose van larven op betonnen substraten beïnvloeden, en hoe de aanwezigheid van een biofilm voorkeuren voor deze microtopografische kenmerken moduleert. Microtopografische kenmerken, variërend van micrometers tot millimeters, werden getest in gecontroleerde labomstandigheden en in een veldexperiment in de haven van Oostende, België. Resultaten van twee jaar veldgegevens tonen aan dat de aard van de biofilm tussen de twee jaren veranderde: in het eerste jaar waren biofilms over het algemeen aantrekkelijk, terwijl ze in het tweede jaar afwerend waren. In het laboratorium toonden larven een voorkeur voor microstructuren met een grootte van ongeveer 800 µm, zonder duidelijke voorkeur voor verschillende aspectverhoudingen of contacthoeken. Resultaten van het veldexperiment tonen bovendien aan hoe contextafhankelijk oppervlaktetopografie kan zijn, aangezien concurrerende macrofouler de in het lab geïdentificeerde voorkeurstopografie verdoezelde. Opvallend was dat microstructuren met de grootste contacthoeken niet werden gekoloniseerd door deze macrofouler en significant hogere vestigingspercentages vertoonden.

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# Supporting information for chapter 3: Planktonic oyster larvae optimize settlement and metamorphotic decisions in complex sensory landscapes

Raw data and statistical analysis are all available on the GitHub repository: https://github.com/sschmidlin/oyster-larvae-settlement-cues.git

***Pilot study***

In a pilot study, performed in March 2022, larvae were subjected to four different treatments of cues and cue combinations; conspecific waterborne cue only, predator waterborne cue from *C. maenas* only, conspecific waterborne cue + predator (*C. maenas)* waterborne cue, and a control treatment without cues. This pilot study was not included in the final analysis as cues were not added in similar volumes in this study. Waterborne conspecific cues and predator cues were added at 14ml to each petri dish in isolated treatments but in treatments where cues were combined there were 7ml of conspecific waterborne cues and 7ml of predator cue. Preparation of the waterborne conspecific cue and the predator cues were the same as those listed in the main experiments.

Statistical analyses were performed similar to the main experiments, a generalized linearized mixed-effect model was used to compare the interaction of conspecific waterborne cue and predator cue (Bates et al., 2015) in R version 4.1.3 (2022-03-10) (R Core Team, 2021).

Table 3.S1 Results of the statistical model for the pilot experiment

| Predictor variable | Estimate | Std. error | z-value | p-value |
|---|---|---|---|---|
| Conspecific cue present | 1.5411 | 0.39488 | 3.903 | **9.51E-05** |
| Predator cue present | 0.48187 | 0.44092 | 1.093 | 0.27445 |
| Interaction conspecific cue - predator cue | -1.28398 | 0.54041 | -2.376 | **0.01750** |

**Figure 3.S1**

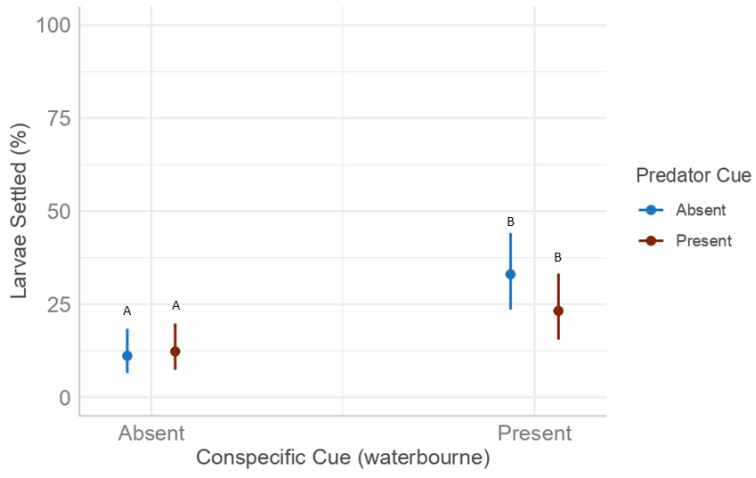


Figure 3.S1.1 Predictions of the generalized linear mixed effect model showing estimated probability of larvae settlement when exposed to predator cues from *C. maenas* and conspecific waterborne cues. Error bars represent 95 % confidence intervals of model prediction. This graph

represents the results of the Pilot study showing interaction of waterborne conspecific cues and predator cues from *C. maenas.*

**Experiment 1 assessment of metamorphosis at different time points**

In experiment 1 metamorphosis was assessed at 10, 20, and 30 hours exposure to treatments. The statistical model used to assess settlement after 10, and 20 hours was the same as the model used for the final analysis at 30 hours described in the statistical analysis section of the manuscript.

Table 3.S2 Experiment 1 results from metamorphosis assessed after 10, 20, and 30 hours.

| Hour assessed | **Predictor Variable** | **Estimate** | **Std. error** | **z-value** | **p-value** |
|---|---|---|---|---|---|
| 30 hrs | Conspecific cue present | 1.59795 | 0.52913 | 3.020 | **0.00253** |
| | Predator cue present | -1.86435 | 1.10798 | -1.683 | *0.09244* |
| | Shell cue present | 3.28470 | 0.51480 | 6.380 | **1.77e-10** |
| | Interaction conspecific cue - predator cue | 0.94550 | 1.18659 | 0.797 | 0.42555 |
| 20hrs | Conspecific cue present | 2.27899 | 0.76780 | 2.968 | **0.003** |
| | Predator cue present | -0.90030 | 1.23592 | -0.728 | 0.466 |
| | Shell cue present | 3.59219 | 0.74970 | 4.792 | **1.66e-06** |

| | | | | | |
|---|---|---|---|---|---|
| | Interaction conspecific cue - predator cue | 0.33131 | 1.30703 | 0.253 | 0.800 |
| 10hrs | Conspecific cue present | 1.75764 | 0.78663 | 2.234 | **0.025458** |
| | Predator cue present | -14.80977 | 124.18193 | -0.119 | 0.905070 |
| | Shell cue present | 2.54814 | 0.75966 | 3.354 | **0.000796** |
| | Interaction conspecific cue - predator cue | 13.88916 | 124.17916 | 0.112 | 0.910944 |

### Larval Rearing

Adult oyster were induced to spawn through gonad stripping, and eggs were fertilized following FAO guidelines (Helm, 2004). Fertilized eggs were kept undisturbed in 5-liter flat bottom tanks for 48 hours at 22 °C at a density of 10 eggs per ml of 0.1 µm filtered seawater (FSW). All seawater was sourced from the water basin at NIOZ Yerseke and filtered at 0.1 µm to remove harmful bacteria and other small microorganisms. After 48 hours larvae were sieved over 30 µm nylon mesh, rinsed, and added larvae to 10-liter conical tanks with FSW. Conical tanks were static but aerated and kept at 22 °C for the entire duration of larvae rearing. Every two days, we sieved the larvae over mesh corresponding to the average size of the larvae and the water in the tanks was changed. Seawater used throughout the larval rearing and experiment maintained a pH of 7.8 ± 0.95 and salinity of 31.4 ± 1.2 in the first experiment and pH of 8.03 ± 0.05, and salinity of 32.7 ± 0.49 in the second experiment. Larvae fed *ad libitum* from a fresh microalgae mixture consisting of *Chaetoceros muelleri*, and *Isochrysis galbana* (clone T-ISO) purchased from Proviron Industries NV. For the first four days larvae were fed at 40,000 cells/ml water using only *I. galbana*. Days 5-12 larvae were fed *C. muelleri*, and *I. galbana* at 100,000 cells/ml at a volume ratio of 1:1. From day 13 until the end of the experiment larvae were fed *C. muelleri*, and *I. galbana* at 100,000 cells/ml at a volume ratio of 3:1. Larvae entered their pediveliger stage and became competent

to settle between 25-28 days. We deemed the larvae competent when they had a prominent eyespot and larval foot and measured 320-350 µm in diameter.

### Description of models used and post hoc results

In experiment 1 the model included interactions effects between conspecific waterbourn and predator cues, between conspecific shells and waterbourn conspecific cues, and between predator cues and conspecific shells (see Table S2). In experiment 2 the model included interactions effects between conspecific waterbourn and predator cues, between conspecific waterbourn cues and biofilms, and between conspecific shells and biofilms (see Table S2). In the combined data, the model included interactions effects between conspecific waterbourn and predator cues, and between conspecific shells and waterbourn conspecific cues (see Table S2.1).

Table 3.S3 Description of the models used for each experiment.

| Experiment | |
|---|---|
| Pilot study | glmer(Settled ~ predator_cue + conspecific_cue + predator_cue:conspecific_cue + Larvae.age + (1\|Larvae.batch), data = data, family = binomial) |
| Experiment 1 | glmer(Settled ~ conspecific_cue + predator_cue + Shell + conspecific_cue:predator_cue + conspecific_cue:Shell + predator_cue:Shell + Larvae.age + (1\|Larvae.batch), data=data, family=binomial) |
| Experiment 2 | glmer(Settled ~ conspecific_cue + Shell + biofilm + conspecific_cue:predator_cue + conspecific_cue:biofilm + Shell:biofilm + Larvae.age + (1\|Batch), data=data_aug, family=binomial) |

| | |
|---|---|
| Combined data | glmer(Settled ~ conspecific_cue + predator_cue + Shell + conspecific_cue:predator_cue + conspecific_cue:Shell + Larvae.age + (1 \| Larvae.batch), data = data_combined, family = binomial) |

Table 3.S4. Results of the pairwise post hoc performed on every model used.

| **Experiment** | **Contrasts** | | | | | **odds.ratio** | **SE** | **df** | **null** | **z.ratio** | **p.value** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Pilot | Conspecific_absent | Predator_absent | / | Conspecific_present | Predator_absent | 0.254 | 0.073 | Inf | 1 | -4.769 | <.0001 |
| | Conspecific_absent | Predator_absent | / | Conspecific_absent | Predator_present | 0.895 | 0.294 | Inf | 1 | -0.339 | 0.9866 |
| | Conspecific_absent | Predator_absent | / | Conspecific_present | Predator_present | 0.419 | 0.125 | Inf | 1 | -2.921 | 0.0183 |
| | Conspecific_present | Predator_absent | / | Conspecific_absent | Predator_present | 3.528 | 0.973 | Inf | 1 | 4.571 | <.0001 |
| | Conspecific_present | Predator_absent | / | Conspecific_present | Predator_present | 1.650 | 0.394 | Inf | 1 | 2.100 | 0.1531 |
| | Conspecific_absent | Predator_present | / | Conspecific_present | Predator_present | 0.468 | 0.134 | Inf | 1 | -2.649 | 0.0402 |
| Experiment 1 | conspecific_absent | predator_absent | / | conspecific_present | predator_absent | 0.2279 | 0.0724 | Inf | 1 | -4.653 | <.0001 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | conspecific_absent | predator_absent | / | conspecific_absent | predator_present | 2.1689 | 1.2482 | Inf | 1 | 1.345 | 0.5340 |
| | conspecific_absent | predator_absent | / | conspecific_present | predator_present | 0.1920 | 0.1308 | Inf | 1 | -2.423 | 0.0728 |
| | conspecific_present | predator_absent | / | conspecific_absent | predator_present | 9.5170 | 5.2264 | Inf | 1 | 4.103 | 0.0002 |
| | conspecific_present | predator_absent | / | conspecific_present | predator_present | 0.8426 | 0.6030 | Inf | 1 | -0.239 | 0.9952 |
| | conspecific_absent | predator_present | / | conspecific_present | predator_present | 0.0885 | 0.0982 | Inf | 1 | -2.187 | 0.1268 |
| Experiment 1 | Sterilized shell | predator_absent | / | Untreated shell | predator_absent | 0.04219 | 0.01375 | Inf | 1 | -9.714 | <.0001 |
| | Sterilized shell | predator_absent | / | Sterilized shell | predator_present | 4.02126 | 2.38773 | Inf | 1 | 2.344 | 0.0884 |
| | Sterilized shell | predator_absent | / | Untreated shell | predator_present | 0.01917 | 0.01266 | Inf | 1 | -5.990 | <.0001 |
| | Untreated shell | predator_absent | / | Sterilized shell | predator_present | 95.31582 | 54.1249 | Inf | 1 | 8.025 | <.0001 |
| | Untreated shell | predator_absent | / | Untreated shell | predator_present | 0.45446 | 0.30478 | Inf | 1 | -1.176 | 0.6421 |
| | Sterilized shell | predator_present | / | Untreated shell | predator_present | 0.00477 | 0.00517 | Inf | 1 | -4.930 | <.0001 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Experiment 1 | sterilized | conspecific_absent | / | Untreated shell | conspecific_absent | 0.01259 | 0.00730 | Inf | 1 | -7.540 | <.0001 |
| | sterilized | conspecific_absent | / | Sterilized shell | conspecific_present | 0.12610 | 0.07482 | Inf | 1 | -3.490 | 0.0027 |
| | sterilized | conspecific_absent | / | Untreated shell | conspecific_present | 0.00201 | 0.00218 | Inf | 1 | -5.738 | <.0001 |
| | untreated | conspecific_absent | / | Sterilized shell | conspecific_present | 10.01535 | 2.73373 | Inf | 1 | 8.441 | <.0001 |
| | untreated | conspecific_absent | / | Untreated shell | conspecific_present | 0.16001 | 0.11035 | Inf | 1 | -2.657 | 0.0394 |
| | sterilized | conspecific_present | / | Untreated shell | conspecific_present | 0.01598 | 0.01111 | Inf | 1 | -5.947 | <.0001 |
| Experiment 2 | conspecific_absent | predator_absent | / | conspecific_present | predator_absent | 0.212 | 0.0332 | Inf | 1 | -9.922 | <.0001 |
| | conspecific_absent | predator_absent | / | conspecific_absent | predator_present | 0.932 | 0.1409 | Inf | 1 | -0.469 | 0.9659 |
| | conspecific_absent | predator_absent | / | conspecific_present | predator_present | 0.300 | 0.0451 | Inf | 1 | -7.998 | <.0001 |
| | conspecific_present | predator_absent | / | conspecific_absent | predator_present | 4.386 | 0.6875 | Inf | 1 | 9.431 | <.0001 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | conspecific_present | predator_absent | / | conspecific_present | predator_present | 1.410 | 0.2114 | Inf | 1 | 2.292 | 0.0998 |
| | conspecific_absent | predator_present | / | conspecific_present | predator_present | 0.322 | 0.0487 | Inf | 1 | -7.493 | <.0001 |
| Experiment 2 | conspecific_absent | biofilm_absent | / | conspecific_present | biofilm_absent | 0.207 | 0.0332 | Inf | 1 | -9.819 | <.0001 |
| | conspecific_absent | biofilm_absent | / | conspecific_absent | biofilm_present | 0.372 | 0.0588 | Inf | 1 | -6.258 | <.0001 |
| | conspecific_absent | biofilm_absent | / | conspecific_present | biofilm_present | 0.123 | 0.0198 | Inf | 1 | -12.999 | <.0001 |
| | conspecific_present | biofilm_absent | / | conspecific_absent | biofilm_present | 1.794 | 0.2656 | Inf | 1 | 3.949 | 0.0005 |
| | conspecific_present | biofilm_absent | / | conspecific_present | biofilm_present | 1.794 | 0.2656 | Inf | 1 | -3.491 | 0.0027 |
| | conspecific_absent | biofilm_present | / | conspecific_present | biofilm_present | 0.591 | 0.0891 | Inf | 1 | -7.281 | <.0001 |
| Experiment 2 | conspecific_absent | Sterilized shell | / | conspecific_present | Sterilized shell | 0.261 | 0.02900 | Inf | 1 | -12.092 | <.0001 |
| | conspecific_absent | Sterilized shell | / | conspecific_absent | Untreated shell | 0.130 | 0.01468 | Inf | 1 | -18.073 | <.0001 |
| | conspecific_absent | Sterilized shell | / | conspecific_present | Untreated shell | 0.034 | 0.00607 | Inf | 1 | -18.934 | <.0001 |
| | conspecific_present | Sterilized shell | / | conspecific_absent | Untreated shell | 0.497 | 0.06713 | Inf | 1 | -5.174 | <.0001 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | conspecific_present | Sterilized shell | / | conspecific_present | Untreated shell | 0.130 | 0.01468 | Inf | 1 | -18.073 | <.0001 |
| | conspecific_absent | Untreated shell | / | conspecific_present | Untreated shell | 0.261 | 0.02900 | Inf | 1 | -12.092 | <.0001 |
| Experiment 2 | predator_absent | biofilm_absent | / | predator_present | biofilm_absent | 1.146 | 0.1220 | Inf | 1 | 1.281 | 0.5749 |
| | predator_absent | biofilm_absent | / | predator_absent | biofilm_present | 0.469 | 0.0507 | Inf | 1 | -7.007 | <.0001 |
| | predator_absent | biofilm_absent | / | predator_present | biofilm_present | 0.537 | 0.0797 | Inf | 1 | -4.188 | 0.0002 |
| | predator_present | biofilm_absent | / | predator_absent | biofilm_present | 0.409 | 0.0634 | Inf | 1 | -5.763 | <.0001 |
| | predator_present | biofilm_absent | / | predator_present | biofilm_present | 0.469 | 0.0507 | Inf | 1 | -7.007 | <.0001 |
| | predator_absent | biofilm_present | / | predator_present | biofilm_present | 1.146 | 0.1220 | Inf | 1 | 1.281 | 0.5749 |
| Experiment 2 | predator_absent | Sterilized shell | / | predator_present | Sterilized shell | 1.146 | 0.1220 | Inf | 1 | 1.281 | 0.5749 |
| | predator_absent | Sterilized shell | / | predator_absent | Untreated shell | 0.130 | 0.0147 | Inf | 1 | -18.073 | <.0001 |
| | predator_absent | Sterilized shell | / | predator_present | Untreated shell | 0.149 | 0.0229 | Inf | 1 | -12.384 | <.0001 |
| | predator_present | Sterilized shell | / | predator_absent | Untreated shell | 0.113 | 0.0178 | Inf | 1 | -13.902 | <.0001 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | predator_present | Sterilized shell | / | predator_present | Untreated shell | 0.130 | 0.0147 | Inf | 1 | -18.073 | <.0001 |
| | predator_absent | Untreated shell | / | predator_present | Untreated shell | 1.146 | 0.1220 | Inf | 1 | 1.281 | 0.5749 |
| Experiment 2 | sterilized | biofilm_absent | / | Untreated shell | biofilm_absent | 0.106 | 0.0174 | Inf | 1 | -13.719 | <.0001 |
| | sterilized | biofilm_absent | / | Sterilized shell | biofilm_present | 0.383 | 0.0618 | Inf | 1 | -5.944 | <.0001 |
| | sterilized | biofilm_absent | / | Untreated shell | biofilm_present | 0.061 | 0.0102 | Inf | 1 | -16.642 | <.0001 |
| | untreated | biofilm_absent | / | Sterilized shell | biofilm_present | 3.607 | 0.5176 | Inf | 1 | 8.940 | <.0001 |
| | untreated | biofilm_absent | / | Untreated shell | biofilm_present | 0.574 | 0.0852 | Inf | 1 | -3.739 | 0.0011 |
| | sterilized | biofilm_present | / | Untreated shell | biofilm_present | 0.159 | 0.0244 | Inf | 1 | -11.990 | <.0001 |

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# Supporting information for chapter 4: Comparison of the effects of reef and anthropogenic soundscapes on oyster larvae settlement/metamorphosis

Raw data and statistical analysis are all available on the GitHub repository: https://github.com/sschmidlin/larvae-and-sound.git

### Instrument Type

Table 4.S1. description of all instruments used to collect acoustic data.

| Instrument type | Treatments | Model | Serial number | End to end sensitivity [dB re. 1 µPa/V] | Sampling rate [kHz] |
|---|---|---|---|---|---|
| SoundTrap | REEF_34 | ST300 STD | 6045 | -176 | 24 |
| SoundTrap | REEF_1, OFF_2 | ST300 STD | 6046 | -176.7 | 24 |
| SoundTrap | REEF_2 | ST300 STD | 6049 | -177.1 | 24 |
| SoundTrap | OFF_1 | ST300 STD | 6042 | -176.8 | 24 |
| RTSys | OFF_3, VESSELS_3 | RTSys RESEA + Colmar GP1190M-LP | EA- SDA14_2003003 GP1190M 134 | -176.98 | 96 |
| RTSys | VESSELS_1 | RTSys RESEA + Colmar GP1190M-LP | EA- SDA14_2003001 GP1190M 130 | -176.98 | 48 |

| | | | | | |
|---|---|---|---|---|---|
| RTSys | VESSELS_2 | RTSys RESEA + Colmar GP1516M-LP | EA- SDA14_2003003 GP1516M 185 | -164.98 | 48 |
| RTSys | VESSELS_4 | RTSys RESEA + Colmar GP1516M-LP | EA- SDA14_2003002 GP1516M 191 | -164.98 | 48 |

Table 4.S2. Different treatments with their corresponding acoustic data collection information. For each day of experiment (Day column in the Table), the reef + vessel combination was don by synthetically mixing the vessel and the reef recordings using Audacity.

| Treatment | Name | Location | Lat | Lon | Depth | Day | Description | Recordings Date times | Day moment |
|---|---|---|---|---|---|---|---|---|---|
| Reef | REEF_1 | Nieuweschild | 53.07 | 4.88 | X | 1 | Two times 10 minutes at different moments of twilight | 07/06/2021 02:40<br>07/06/2021 05:20 | Civil twilight<br>Day |
| Reef | REEF_2 | Kornwerderzand | 53.09 | 5.20 | 1.35 | 2 | 10 minutes | 30/05/2022 20:20 | Civil twilight |
| Reef | REEF_34 | Nieuweschild | 53.07 | 4.88 | X | 3,4 | 20 consecutive minutes | 07/06/2022 19:00 | Day |
| Off reef | OFF_1 | Nieuweschild | 53.07 | 4.88 | X | 1 | Sand area close to Nieuweschild reef. 20 consecutive minutes | 13/04/2021 20:39 | Astronomical twilight |
| Off reef | OFF_2 | Vlieland | 53.22 | 5.01 | 1.34 | 2 | Control recording for experiment of artificial reef. 10 minutes | 29/09/2022 06:19 | Day |
| Off reef | OFF_3 | Fairplay | 51.17 | 2.62 | 14.5 | 3 | 1 h of continuous off reef sound | 06/06/2021 04:19<br>06/06/2021 04:42<br>06/06/2021 04:54 | Day<br>Day<br>Day |
| Off reef | OFF_4 | Fairplay | 51.17 | 2.62 | 14.5 | 4 | 30 min continuous, with one distant vessel removed | 08/06/2021 15:46 | Day |
| Vessels | VESSELS_1 | Faulbaums | 51.33 | 2.51 | 15.2 | 1 | 3 different vessels: passing close by (11min), short trawling event (4min), loud and long anthropogenic sound (20min, trawling) | 08/06/2022 14:34<br>08/06/2022 14:44<br>06/06/2022 17:03 | Day<br>Day Day |
| Vessels | VESSELS_2 | Grafton | 51.41 | 2.82 | 19.14 | 2 | Not repeated, 1h20min of continuous boat | 06/05/2022 14:39<br>06/05/2022 15:31 | Day<br>Day |
| Vessels | VESSELS_3 | Fairplay | 51.17 | 2.62 | 14.5 | 3 | 3 different vessels passing far (2, 5, and 5 min) | 06/06/2021 03:04 | Civil twilight<br>Day<br>Day |
| Vessels | VESSELS_4 | Buitenratel | 51.24 | 2.50 | 6.93 | 4 | Two different boats, one passing by and one close by anchored with constant sound. | 14/05/2022 18:55 | Day<br>Day |

## Acoustic Features

Table 4.S3. Computed acoustic features and their parameters. Broadband refers to all the frequencies from 0 to the Nyquist frequency (24 kHz). nfft stands for the number of Fast Fourier Transforms bins.

| Metric | Description | Frequency band [Hz] | Parameters | Reference |
|---|---|---|---|---|
| SPL | Root mean squared value | broadband | NA | International Organization for Standardization, (2017)[1] |
| PSD | Spectrum | broadband | nfft=4096 | International Organization for Standardization, (2017)[1] |
| Low freq. | Average power spectral density | [0, 1000] | nfft=4096 | International Organization for Standardization, (2017)[1] |
| Mid freq. | Average power spectral density | [1000, 5000] | nfft=4096 | International Organization for Standardization, (2017)[1] |
| High freq. | Average power spectral density | [5000, 10000] | nfft=4096 | International Organization for Standardization, (2017)[1] |
| ACI | Acoustic Complexity Index. Expresses the changes in amplitude in time within a frequency band.<br>Quantifies the acoustic irregularity and variability. | broadband | Hann window, nfft=4096, overlap=0.5 | Pieretti et al., (2011)[2] |
| ADI | Acoustic Diversity Index. | broadband | Hann window, nfft=4096, overlap=0.5 | Villanueva-Rivera et al., (2011)[3] |

| | | | | |
|---|---|---|---|---|
| | Quantifies the evenness across frequency bands. A high value would be given if all the frequency bands have the same level, and a low value if one frequency band concentrates all the energy. | | | |
| AEI | Acoustic Evenness Index.<br><br>The opposite than ADI. Higher values indicate bigger unevenness in spectral distribution. | [0, 20000] | bin_step=500<br>dB_threshold=-50 | Villanueva-Rivera et al., (2011)[3] |

**Speaker Assignment**

The assignment of which speaker and which treatment would be used at each tank per batch was done randomly, resulting in the combinations listed in Table S4

Table 4.S4. Distribution of treatment per tank and speaker. R+V stands for Reef+Vessel

| Day | | Tank1 | Tank2 | Tank3 | Tank4 | Tank5 |
|---|---|---|---|---|---|---|
| 1 | Treatment | NA | Vessel | Off reef | Reef | R+V |
| | Speaker ID | NA | 3 | 2 | 1 | 4 |
| 2 | Treatment | Reef | NA | Vessel | R+V | Off reef |
| | Speaker ID | 2 | NA | 4 | 1 | 3 |
| 3 | Treatment | Vessel | Off reef | R+V | NA | Reef |
| | Speaker ID | 1 | 4 | 2 | NA | 3 |
| 4 | Treatment | R+V | Reef | NA | Off reef | Vessel |
| | Speaker ID | 3 | 4 | NA | 1 | 2 |

**Playback Measurements**

Prior to the experiment, we conducted recordings of white noise at all the jar positions to assess the differences in sound levels received at each jar. The jars were placed in a way that all of them except jar 3 were at the same distance and position from the speaker. The received PSD at each jar is very similar (see Figure 4.S1). Nevertheless, we did not exclude these acoustic differences from having an impact on the settlement and for this reason jar position was investigated using a GLMER model, as described in the statistical analysis section.

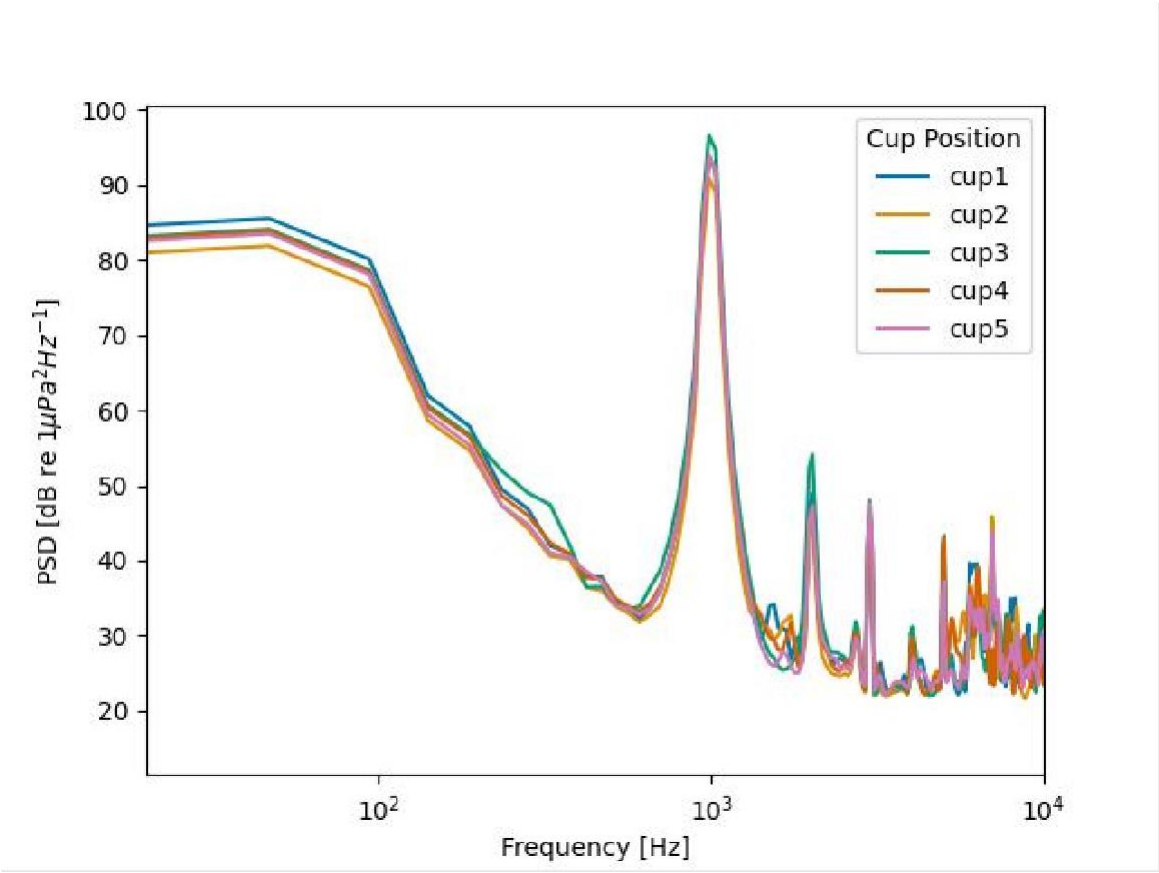


Figure 4.S1. Power spectrum density received at all the jars when playing a white noise sound.

## Statistical Analysis

Table 4.S5. Description and output of statistical models used to determine if random variables had any effect to the base model.

| | Models compared | npar | ACI | BIC | logLik | deviance | Chisq | Df | Pr (>Chisq) |
|---|---|---|---|---|---|---|---|---|---|
| Assessing speaker effect on settlement | Base model | 10 | 960.6 | 1006.5 | -470.3 | 940.6 | | | |
| | Model including speaker | 13 | 964.25 | 1023.9 | -469.13 | 938.25 | 2.3471 | 3 | 0.5036 |
| Assessing tank effect on settlement | Base model | 10 | 1196.8 | 1244.9 | -588.41 | 1176.8 | | | |
| | Model including tank | 14 | 1203.9 | 1271.2 | -587.94 | 1175.9 | 0.9327 | 4 | 0.9198 |
| Assessing Jar position effect on settlement | Base model | 9 | 902.29 | 942.87 | -442.14 | 884.29 | | | |
| | Model including jar position | 13 | 906.11 | 964.73 | -440.06 | 880.11 | 4.1733 | 4 | .3831 |

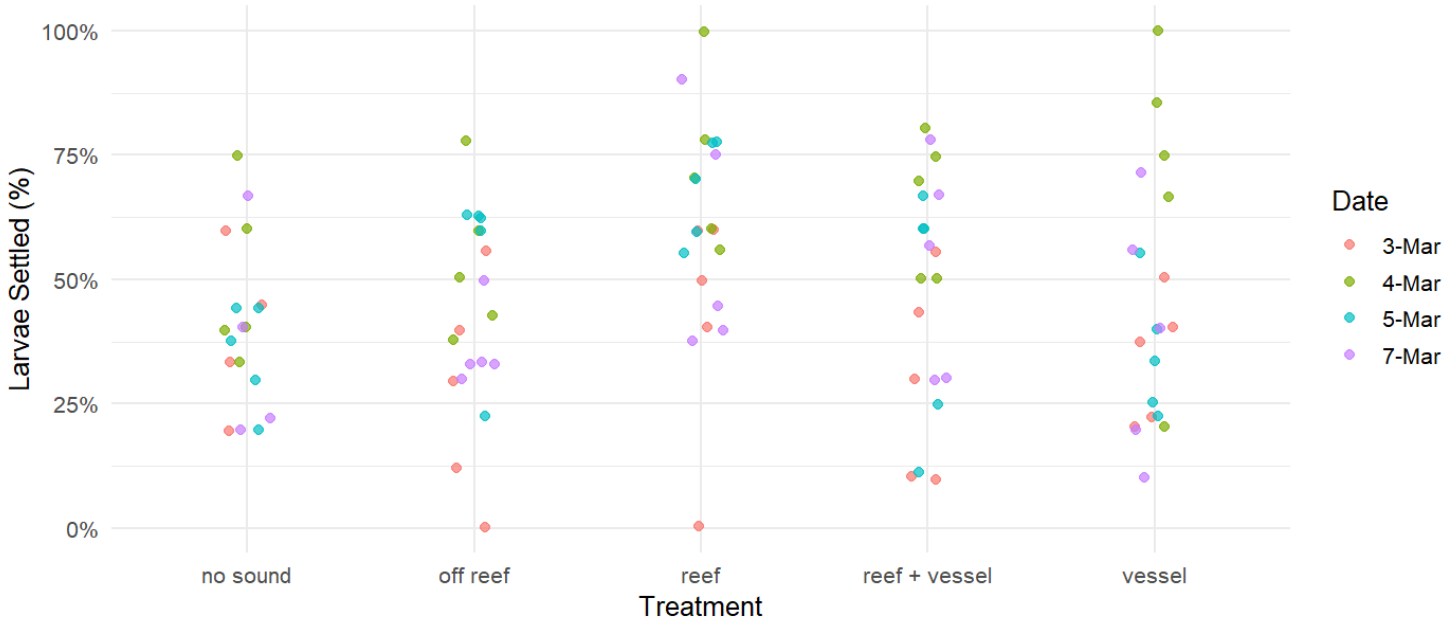


Figure 4.S2 plot of raw data from all experimental dates. Raw data was calculated by the percentage of larvae metamorphosed in each individual jar.

**References**

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Villanueva-Rivera, L. J., Pijanowski, B. C., Doucette, J. & Pekin, B. A primer of acoustic analysis for landscape ecologists. *Landsc. Ecol.* 26, 1233–1246 (2011).

# Supporting information for chapter 5: Biofilm development alters surface micro-topography preferences of settling Pacific oyster larvae (*Magallana gigas*)

Raw data and statistical analysis are all available on the GitHub repository:

https://github.com/sschmidlin/TopographyANDLarvae.git

Table 5.S1. Description of the models used to predict larvae settlement from each experiment.

| **Experiment** | |
|---|---|
| Experiment 1 | glm(Settled ~ Topography * Biofilm + Age, data = data, family = binomial) |
| Experiment 2 | glm(Settled ~ Topography * Biofilm + Age, data = data, family = binomial) |
| Field Experiment | glm.nb(settled ~ topography + collector_type, data = data) |
| Larvae position Experiment 1 | glm.nb(Count ~ Topography + larvae_position, data = data) |
| Larvae position Experiment 2 | glm.nb(Count ~ Topography + larvae_position, data = data) |
| Larvae position field Experiment | glm.nb(Count ~ topography * larvae_position, data = data) |

Table S2. Results of the pairwise post hoc analysis performed for experiment 1. Significant differences ($p < 0.05$) are in bold, while marginally significant ($p < 0.1$) are in italic.

| **Contrasts** | | | | | **odds.ratio** | **SE** | **df** | **null** | **z.ratio** | **p.value** |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography1 | Biofilm0 | / | Topography2 | Biofilm0 | 0.182 | 0.109 | Inf | 1 | -2.846 | 0.2225 |
| Topography1 | Biofilm0 | / | Topography3 | Biofilm0 | 0.3311 | 0.215 | Inf | 1 | -1.699 | 0.9339 |
| Topography1 | Biofilm0 | / | Topography4 | Biofilm0 | 4.4266 | 5.03 | Inf | 1 | 1.31 | 0.9934 |
| Topography1 | Biofilm0 | / | Topography5 | Biofilm0 | 0.2106 | 0.132 | Inf | 1 | -2.479 | 0.4556 |
| Topography1 | Biofilm0 | / | Topography1 | Biofilm5 | 3.1496 | 3.58 | Inf | 1 | 1.009 | 0.9996 |
| Topography1 | Biofilm0 | / | Topography2 | Biofilm5 | 0.5009 | 0.322 | Inf | 1 | -1.074 | 0.9992 |
| Topography1 | Biofilm0 | / | Topography3 | Biofilm5 | 3.038 | 3.46 | Inf | 1 | 0.975 | 0.9997 |
| Topography1 | Biofilm0 | / | Topography4 | Biofilm5 | 2.1146 | 1.88 | Inf | 1 | 0.843 | 1 |
| Topography1 | Biofilm0 | / | Topography5 | Biofilm5 | 2.3298 | 2.07 | Inf | 1 | 0.953 | 0.9998 |
| Topography1 | Biofilm0 | / | Topography1 | Biofilm20 | 0.1416 | 0.0904 | Inf | 1 | -3.06 | 0.1312 |
| Topography1 | Biofilm0 | / | Topography2 | Biofilm20 | 0.1471 | 0.0878 | Inf | 1 | -3.209 | *0.0867* |
| Topography1 | Biofilm0 | / | Topography3 | Biofilm20 | 0.081 | 0.0485 | Inf | 1 | -4.201 | **0.0025** |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography1 | Biofilm0 | / | Topography4 | Biofilm20 | 0.1946 | 0.12 | Inf | 1 | -2.656 | 0.3325 |
| Topography1 | Biofilm0 | / | Topography5 | Biofilm20 | 0.1918 | 0.123 | Inf | 1 | -2.584 | 0.3807 |
| Topography2 | Biofilm0 | / | Topography3 | Biofilm0 | 1.8193 | 0.896 | Inf | 1 | 1.215 | 0.9969 |
| Topography2 | Biofilm0 | / | Topography4 | Biofilm0 | 24.3215 | 25.6 | Inf | 1 | 3.03 | 0.1417 |
| Topography2 | Biofilm0 | / | Topography5 | Biofilm0 | 1.1573 | 0.536 | Inf | 1 | 0.315 | 1 |
| Topography2 | Biofilm0 | / | Topography1 | Biofilm5 | 17.3048 | 18.3 | Inf | 1 | 2.702 | 0.3034 |
| Topography2 | Biofilm0 | / | Topography2 | Biofilm5 | 2.7523 | 1.33 | Inf | 1 | 2.094 | 0.7394 |
| Topography2 | Biofilm0 | / | Topography3 | Biofilm5 | 16.692 | 17.7 | Inf | 1 | 2.662 | 0.3285 |
| Topography2 | Biofilm0 | / | Topography4 | Biofilm5 | 11.6184 | 9.07 | Inf | 1 | 3.143 | 0.1047 |
| Topography2 | Biofilm0 | / | Topography5 | Biofilm5 | 12.8006 | 9.98 | Inf | 1 | 3.269 | *0.0728* |
| Topography2 | Biofilm0 | / | Topography1 | Biofilm20 | 0.7777 | 0.371 | Inf | 1 | -0.527 | 1 |
| Topography2 | Biofilm0 | / | Topography2 | Biofilm20 | 0.808 | 0.34 | Inf | 1 | -0.507 | 1 |
| Topography2 | Biofilm0 | / | Topography3 | Biofilm20 | 0.4453 | 0.188 | Inf | 1 | -1.921 | 0.8429 |
| Topography2 | Biofilm0 | / | Topography4 | Biofilm20 | 1.069 | 0.478 | Inf | 1 | 0.149 | 1 |
| Topography2 | Biofilm0 | / | Topography5 | Biofilm20 | 1.0536 | 0.503 | Inf | 1 | 0.109 | 1 |
| Topography3 | Biofilm0 | / | Topography4 | Biofilm0 | 13.3685 | 14.5 | Inf | 1 | 2.394 | 0.5196 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography3 | Biofilm0 | / | Topography5 | Biofilm0 | 0.6361 | 0.336 | Inf | 1 | -0.856 | 0.9999 |
| Topography3 | Biofilm0 | / | Topography1 | Biofilm5 | 9.5117 | 10.3 | Inf | 1 | 2.075 | 0.7517 |
| Topography3 | Biofilm0 | / | Topography2 | Biofilm5 | 1.5128 | 0.826 | Inf | 1 | 0.758 | 1 |
| Topography3 | Biofilm0 | / | Topography3 | Biofilm5 | 9.1749 | 9.97 | Inf | 1 | 2.039 | 0.7751 |
| Topography3 | Biofilm0 | / | Topography4 | Biofilm5 | 6.3861 | 5.24 | Inf | 1 | 2.26 | 0.6207 |
| Topography3 | Biofilm0 | / | Topography5 | Biofilm5 | 7.0359 | 5.77 | Inf | 1 | 2.379 | 0.5304 |
| Topography3 | Biofilm0 | / | Topography1 | Biofilm20 | 0.4275 | 0.231 | Inf | 1 | -1.576 | 0.964 |
| Topography3 | Biofilm0 | / | Topography2 | Biofilm20 | 0.4441 | 0.218 | Inf | 1 | -1.65 | 0.9474 |
| Topography3 | Biofilm0 | / | Topography3 | Biofilm20 | 0.2447 | 0.12 | Inf | 1 | -2.866 | 0.2123 |
| Topography3 | Biofilm0 | / | Topography4 | Biofilm20 | 0.5876 | 0.302 | Inf | 1 | -1.034 | 0.9995 |
| Topography3 | Biofilm0 | / | Topography5 | Biofilm20 | 0.5791 | 0.313 | Inf | 1 | -1.011 | 0.9996 |
| Topography4 | Biofilm0 | / | Topography5 | Biofilm0 | 0.0476 | 0.0509 | Inf | 1 | -2.849 | 0.2207 |
| Topography4 | Biofilm0 | / | Topography1 | Biofilm5 | 0.7115 | 1.02 | Inf | 1 | -0.238 | 1 |
| Topography4 | Biofilm0 | / | Topography2 | Biofilm5 | 0.1132 | 0.122 | Inf | 1 | -2.021 | 0.7861 |
| Topography4 | Biofilm0 | / | Topography3 | Biofilm5 | 0.6863 | 0.981 | Inf | 1 | -0.263 | 1 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography4 | Biofilm0 | / | Topography4 | Biofilm5 | 0.4777 | 0.592 | Inf | 1 | -0.596 | 1 |
| Topography4 | Biofilm0 | / | Topography5 | Biofilm5 | 0.5263 | 0.652 | Inf | 1 | -0.518 | 1 |
| Topography4 | Biofilm0 | / | Topography1 | Biofilm20 | 0.032 | 0.0345 | Inf | 1 | -3.195 | *0.0904* |
| Topography4 | Biofilm0 | / | Topography2 | Biofilm20 | 0.0332 | 0.0349 | Inf | 1 | -3.237 | *0.0799* |
| Topography4 | Biofilm0 | / | Topography3 | Biofilm20 | 0.0183 | 0.0193 | Inf | 1 | -3.804 | **0.012** |
| Topography4 | Biofilm0 | / | Topography4 | Biofilm20 | 0.044 | 0.0466 | Inf | 1 | -2.945 | 0.1757 |
| Topography4 | Biofilm0 | / | Topography5 | Biofilm20 | 0.0433 | 0.0466 | Inf | 1 | -2.916 | 0.1887 |
| Topography5 | Biofilm0 | / | Topography1 | Biofilm5 | 14.9526 | 16 | Inf | 1 | 2.522 | 0.4241 |
| Topography5 | Biofilm0 | / | Topography2 | Biofilm5 | 2.3782 | 1.23 | Inf | 1 | 1.671 | 0.9418 |
| Topography5 | Biofilm0 | / | Topography3 | Biofilm5 | 14.4231 | 15.5 | Inf | 1 | 2.488 | 0.449 |
| Topography5 | Biofilm0 | / | Topography4 | Biofilm5 | 10.0391 | 8.05 | Inf | 1 | 2.877 | 0.2071 |
| Topography5 | Biofilm0 | / | Topography5 | Biofilm5 | 11.0606 | 8.86 | Inf | 1 | 3.001 | 0.153 |
| Topography5 | Biofilm0 | / | Topography1 | Biofilm20 | 0.672 | 0.346 | Inf | 1 | -0.771 | 1 |
| Topography5 | Biofilm0 | / | Topography2 | Biofilm20 | 0.6981 | 0.322 | Inf | 1 | -0.78 | 1 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography5 | Biofilm0 | / | Topography3 | Biofilm20 | 0.3847 | 0.177 | Inf | 1 | -2.073 | 0.753 |
| Topography5 | Biofilm0 | / | Topography4 | Biofilm20 | 0.9237 | 0.446 | Inf | 1 | -0.164 | 1 |
| Topography5 | Biofilm0 | / | Topography5 | Biofilm20 | 0.9104 | 0.468 | Inf | 1 | -0.183 | 1 |
| Topography1 | Biofilm5 | / | Topography2 | Biofilm5 | 0.159 | 0.172 | Inf | 1 | -1.7 | 0.9336 |
| Topography1 | Biofilm5 | / | Topography3 | Biofilm5 | 0.9646 | 1.38 | Inf | 1 | -0.025 | 1 |
| Topography1 | Biofilm5 | / | Topography4 | Biofilm5 | 0.6714 | 0.834 | Inf | 1 | -0.321 | 1 |
| Topography1 | Biofilm5 | / | Topography5 | Biofilm5 | 0.7397 | 0.919 | Inf | 1 | -0.243 | 1 |
| Topography1 | Biofilm5 | / | Topography1 | Biofilm20 | 0.0449 | 0.0485 | Inf | 1 | -2.877 | 0.207 |
| Topography1 | Biofilm5 | / | Topography2 | Biofilm20 | 0.0467 | 0.0492 | Inf | 1 | -2.906 | 0.1933 |
| Topography1 | Biofilm5 | / | Topography3 | Biofilm20 | 0.0257 | 0.0271 | Inf | 1 | -3.469 | **0.0389** |
| Topography1 | Biofilm5 | / | Topography4 | Biofilm20 | 0.0618 | 0.0658 | Inf | 1 | -2.613 | 0.3608 |
| Topography1 | Biofilm5 | / | Topography5 | Biofilm20 | 0.0609 | 0.0657 | Inf | 1 | -2.595 | 0.3731 |
| Topography2 | Biofilm5 | / | Topography3 | Biofilm5 | 6.0647 | 6.56 | Inf | 1 | 1.666 | 0.9432 |
| Topography2 | Biofilm5 | / | Topography4 | Biofilm5 | 4.2213 | 3.44 | Inf | 1 | 1.769 | 0.9105 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography2 | Biofilm5 | / | Topography5 | Biofilm5 | 4.6508 | 3.78 | Inf | 1 | 1.89 | 0.8586 |
| Topography2 | Biofilm5 | / | Topography1 | Biofilm20 | 0.2826 | 0.15 | Inf | 1 | -2.373 | 0.5348 |
| Topography2 | Biofilm5 | / | Topography2 | Biofilm20 | 0.2936 | 0.142 | Inf | 1 | -2.543 | 0.4094 |
| Topography2 | Biofilm5 | / | Topography3 | Biofilm20 | 0.1618 | 0.0778 | Inf | 1 | -3.787 | **0.0128** |
| Topography2 | Biofilm5 | / | Topography4 | Biofilm20 | 0.3884 | 0.195 | Inf | 1 | -1.88 | 0.8634 |
| Topography2 | Biofilm5 | / | Topography5 | Biofilm20 | 0.3828 | 0.204 | Inf | 1 | -1.805 | 0.8969 |
| Topography3 | Biofilm5 | / | Topography4 | Biofilm5 | 0.696 | 0.865 | Inf | 1 | -0.292 | 1 |
| Topography3 | Biofilm5 | / | Topography5 | Biofilm5 | 0.7669 | 0.952 | Inf | 1 | -0.214 | 1 |
| Topography3 | Biofilm5 | / | Topography1 | Biofilm20 | 0.0466 | 0.0504 | Inf | 1 | -2.836 | 0.2275 |
| Topography3 | Biofilm5 | / | Topography2 | Biofilm20 | 0.0484 | 0.0511 | Inf | 1 | -2.866 | 0.2121 |
| Topography3 | Biofilm5 | / | Topography3 | Biofilm20 | 0.0267 | 0.0281 | Inf | 1 | -3.434 | **0.0436** |
| Topography3 | Biofilm5 | / | Topography4 | Biofilm20 | 0.064 | 0.0682 | Inf | 1 | -2.581 | 0.3824 |
| Topography3 | Biofilm5 | / | Topography5 | Biofilm20 | 0.0631 | 0.0682 | Inf | 1 | -2.557 | 0.3991 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography4 | Biofilm5 | / | Topography5 | Biofilm5 | 1.1018 | 1.12 | Inf | 1 | 0.095 | 1 |
| Topography4 | Biofilm5 | / | Topography1 | Biofilm20 | 0.0669 | 0.0544 | Inf | 1 | -3.329 | *0.0607* |
| Topography4 | Biofilm5 | / | Topography2 | Biofilm20 | 0.0695 | 0.0542 | Inf | 1 | -3.422 | **0.0453** |
| Topography4 | Biofilm5 | / | Topography3 | Biofilm20 | 0.0383 | 0.0298 | Inf | 1 | -4.19 | **0.0026** |
| Topography4 | Biofilm5 | / | Topography4 | Biofilm20 | 0.092 | 0.0728 | Inf | 1 | -3.014 | 0.1477 |
| Topography4 | Biofilm5 | / | Topography5 | Biofilm20 | 0.0907 | 0.0736 | Inf | 1 | -2.958 | 0.1702 |
| Topography5 | Biofilm5 | / | Topography1 | Biofilm20 | 0.0608 | 0.0493 | Inf | 1 | -3.449 | **0.0416** |
| Topography5 | Biofilm5 | / | Topography2 | Biofilm20 | 0.0631 | 0.0491 | Inf | 1 | -3.549 | **0.0298** |
| Topography5 | Biofilm5 | / | Topography3 | Biofilm20 | 0.0348 | 0.027 | Inf | 1 | -4.319 | **0.0015** |
| Topography5 | Biofilm5 | / | Topography4 | Biofilm20 | 0.0835 | 0.066 | Inf | 1 | -3.141 | 0.1052 |
| Topography5 | Biofilm5 | / | Topography5 | Biofilm20 | 0.0823 | 0.0668 | Inf | 1 | -3.079 | 0.1246 |
| Topography1 | Biofilm20 | / | Topography2 | Biofilm20 | 1.0389 | 0.497 | Inf | 1 | 0.08 | 1 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography1 | Biofilm20 | / | Topography3 | Biofilm20 | 0.5725 | 0.272 | Inf | 1 | -1.173 | 0.9979 |
| Topography1 | Biofilm20 | / | Topography4 | Biofilm20 | 1.3745 | 0.69 | Inf | 1 | 0.634 | 1 |
| Topography1 | Biofilm20 | / | Topography5 | Biofilm20 | 1.3547 | 0.711 | Inf | 1 | 0.579 | 1 |
| Topography2 | Biofilm20 | / | Topography3 | Biofilm20 | 0.5511 | 0.232 | Inf | 1 | -1.418 | 0.9859 |
| Topography2 | Biofilm20 | / | Topography4 | Biofilm20 | 1.3231 | 0.587 | Inf | 1 | 0.631 | 1 |
| Topography2 | Biofilm20 | / | Topography5 | Biofilm20 | 1.304 | 0.623 | Inf | 1 | 0.556 | 1 |
| Topography3 | Biofilm20 | / | Topography4 | Biofilm20 | 2.4009 | 1.06 | Inf | 1 | 1.975 | 0.8133 |
| Topography3 | Biofilm20 | / | Topography5 | Biofilm20 | 2.3662 | 1.13 | Inf | 1 | 1.811 | 0.8942 |
| Topography4 | Biofilm20 | / | Topography5 | Biofilm20 | 0.9856 | 0.493 | Inf | 1 | -0.029 | 1 |

Table S3. Results of the pairwise post hoc analysis performed for experiment 2. Significant differences ($p < 0.05$) are in bold.

| **Contrasts** | | | | | **odds. ratio** | **SE** | **df** | **null** | **z.ratio** | **p.value** |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography1 | Biofilm0 | / | Topography2 | Biofilm0 | 0.223 | 0.0733 | Inf | 1 | -4.56 | **0.0005** |
| Topography1 | Biofilm0 | / | Topography3 | Biofilm0 | 0.519 | 0.174 | Inf | 1 | -1.96 | 0.8219 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography1 | Biofilm0 | / | Topography4 | Biofilm0 | 0.214 | 0.0689 | Inf | 1 | -4.789 | **0.0002** |
| Topography1 | Biofilm0 | / | Topography5 | Biofilm0 | 0.395 | 0.128 | Inf | 1 | -2.858 | 0.2162 |
| Topography1 | Biofilm0 | / | Topography1 | Biofilm5 | 1.549 | 0.618 | Inf | 1 | 1.097 | 0.999 |
| Topography1 | Biofilm0 | / | Topography2 | Biofilm5 | 0.777 | 0.301 | Inf | 1 | -0.652 | 1 |
| Topography1 | Biofilm0 | / | Topography3 | Biofilm5 | 0.613 | 0.206 | Inf | 1 | -1.454 | 0.9823 |
| Topography1 | Biofilm0 | / | Topography4 | Biofilm5 | 1.219 | 0.513 | Inf | 1 | 0.47 | 1 |
| Topography1 | Biofilm0 | / | Topography5 | Biofilm5 | 0.716 | 0.241 | Inf | 1 | -0.995 | 0.9997 |
| Topography1 | Biofilm0 | / | Topography1 | Biofilm20 | 3.867 | 2.24 | Inf | 1 | 2.339 | 0.5613 |
| Topography1 | Biofilm0 | / | Topography2 | Biofilm20 | 0.922 | 0.325 | Inf | 1 | -0.232 | 1 |
| Topography1 | Biofilm0 | / | Topography3 | Biofilm20 | 1.368 | 0.573 | Inf | 1 | 0.748 | 1 |
| Topography1 | Biofilm0 | / | Topography4 | Biofilm20 | 2.11 | 0.965 | Inf | 1 | 1.632 | 0.9518 |
| Topography1 | Biofilm0 | / | Topography5 | Biofilm20 | 0.884 | 0.359 | Inf | 1 | -0.304 | 1 |
| Topography2 | Biofilm0 | / | Topography3 | Biofilm0 | 2.328 | 0.631 | Inf | 1 | 3.12 | 0.1115 |
| Topography2 | Biofilm0 | / | Topography4 | Biofilm0 | 0.96 | 0.245 | Inf | 1 | -0.16 | 1 |
| Topography2 | Biofilm0 | / | Topography5 | Biofilm0 | 1.775 | 0.458 | Inf | 1 | 2.225 | 0.6465 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography2 | Biofilm0 | / | Topography1 | Biofilm5 | 6.954 | 2.41 | Inf | 1 | 5.585 | **<.0001** |
| Topography2 | Biofilm0 | / | Topography2 | Biofilm5 | 3.489 | 1.17 | Inf | 1 | 3.733 | **0.0156** |
| Topography2 | Biofilm0 | / | Topography3 | Biofilm5 | 2.752 | 0.751 | Inf | 1 | 3.71 | **0.017** |
| Topography2 | Biofilm0 | / | Topography4 | Biofilm5 | 5.472 | 2.04 | Inf | 1 | 4.563 | **0.0005** |
| Topography2 | Biofilm0 | / | Topography5 | Biofilm5 | 3.213 | 0.876 | Inf | 1 | 4.28 | **0.0018** |
| Topography2 | Biofilm0 | / | Topography1 | Biofilm20 | 17.363 | 9.45 | Inf | 1 | 5.244 | **<.0001** |
| Topography2 | Biofilm0 | / | Topography2 | Biofilm20 | 4.138 | 1.21 | Inf | 1 | 4.86 | **0.0001** |
| Topography2 | Biofilm0 | / | Topography3 | Biofilm20 | 6.143 | 2.28 | Inf | 1 | 4.899 | **0.0001** |
| Topography2 | Biofilm0 | / | Topography4 | Biofilm20 | 9.475 | 3.92 | Inf | 1 | 5.441 | **<.0001** |
| Topography2 | Biofilm0 | / | Topography5 | Biofilm20 | 3.967 | 1.42 | Inf | 1 | 3.861 | **0.0097** |
| Topography3 | Biofilm0 | / | Topography4 | Biofilm0 | 0.412 | 0.107 | Inf | 1 | -3.404 | **0.0481** |
| Topography3 | Biofilm0 | / | Topography5 | Biofilm0 | 0.762 | 0.201 | Inf | 1 | -1.029 | 0.9995 |
| Topography3 | Biofilm0 | / | Topography1 | Biofilm5 | 2.987 | 1.05 | Inf | 1 | 3.109 | 0.1148 |
| Topography3 | Biofilm0 | / | Topography2 | Biofilm5 | 1.498 | 0.511 | Inf | 1 | 1.185 | 0.9977 |
| Topography3 | Biofilm0 | / | Topography3 | Biofilm5 | 1.182 | 0.329 | Inf | 1 | 0.601 | 1 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography3 | Biofilm0 | / | Topography4 | Biofilm5 | 2.35 | 0.885 | Inf | 1 | 2.268 | 0.6144 |
| Topography3 | Biofilm0 | / | Topography5 | Biofilm5 | 1.38 | 0.384 | Inf | 1 | 1.158 | 0.9982 |
| Topography3 | Biofilm0 | / | Topography1 | Biofilm20 | 7.457 | 4.09 | Inf | 1 | 3.667 | **0.0198** |
| Topography3 | Biofilm0 | / | Topography2 | Biofilm20 | 1.777 | 0.528 | Inf | 1 | 1.935 | 0.8358 |
| Topography3 | Biofilm0 | / | Topography3 | Biofilm20 | 2.638 | 0.991 | Inf | 1 | 2.583 | 0.3809 |
| Topography3 | Biofilm0 | / | Topography4 | Biofilm20 | 4.069 | 1.7 | Inf | 1 | 3.362 | *0.0548* |
| Topography3 | Biofilm0 | / | Topography5 | Biofilm20 | 1.704 | 0.618 | Inf | 1 | 1.468 | 0.9806 |
| Topography4 | Biofilm0 | / | Topography5 | Biofilm0 | 1.849 | 0.457 | Inf | 1 | 2.489 | 0.448 |
| Topography4 | Biofilm0 | / | Topography1 | Biofilm5 | 7.243 | 2.46 | Inf | 1 | 5.83 | **<.0001** |
| Topography4 | Biofilm0 | / | Topography2 | Biofilm5 | 3.634 | 1.19 | Inf | 1 | 3.927 | **0.0075** |
| Topography4 | Biofilm0 | / | Topography3 | Biofilm5 | 2.866 | 0.752 | Inf | 1 | 4.015 | **0.0053** |
| Topography4 | Biofilm0 | / | Topography4 | Biofilm5 | 5.7 | 2.08 | Inf | 1 | 4.765 | **0.0002** |
| Topography4 | Biofilm0 | / | Topography5 | Biofilm5 | 3.346 | 0.878 | Inf | 1 | 4.605 | **0.0004** |
| Topography4 | Biofilm0 | / | Topography1 | Biofilm20 | 18.085 | 9.77 | Inf | 1 | 5.361 | **<.0001** |
| Topography4 | Biofilm0 | / | Topography2 | Biofilm20 | 4.31 | 1.22 | Inf | 1 | 5.169 | **<.0001** |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography4 | Biofilm0 | / | Topography3 | Biofilm20 | 6.399 | 2.33 | Inf | 1 | 5.099 | **<.0001** |
| Topography4 | Biofilm0 | / | Topography4 | Biofilm20 | 9.868 | 4.02 | Inf | 1 | 5.622 | **<.0001** |
| Topography4 | Biofilm0 | / | Topography5 | Biofilm20 | 4.132 | 1.45 | Inf | 1 | 4.042 | **0.0048** |
| Topography5 | Biofilm0 | / | Topography1 | Biofilm5 | 3.917 | 1.34 | Inf | 1 | 3.991 | **0.0058** |
| Topography5 | Biofilm0 | / | Topography2 | Biofilm5 | 1.965 | 0.65 | Inf | 1 | 2.041 | 0.7741 |
| Topography5 | Biofilm0 | / | Topography3 | Biofilm5 | 1.55 | 0.412 | Inf | 1 | 1.651 | 0.9472 |
| Topography5 | Biofilm0 | / | Topography4 | Biofilm5 | 3.082 | 1.13 | Inf | 1 | 3.062 | 0.1303 |
| Topography5 | Biofilm0 | / | Topography5 | Biofilm5 | 1.809 | 0.48 | Inf | 1 | 2.234 | 0.6397 |
| Topography5 | Biofilm0 | / | Topography1 | Biofilm20 | 9.78 | 5.3 | Inf | 1 | 4.211 | **0.0024** |
| Topography5 | Biofilm0 | / | Topography2 | Biofilm20 | 2.331 | 0.665 | Inf | 1 | 2.964 | 0.1676 |
| Topography5 | Biofilm0 | / | Topography3 | Biofilm20 | 3.46 | 1.27 | Inf | 1 | 3.389 | *0.0504* |
| Topography5 | Biofilm0 | / | Topography4 | Biofilm20 | 5.337 | 2.18 | Inf | 1 | 4.093 | **0.0039** |
| Topography5 | Biofilm0 | / | Topography5 | Biofilm20 | 2.235 | 0.79 | Inf | 1 | 2.275 | 0.6093 |
| Topography1 | Biofilm5 | / | Topography2 | Biofilm5 | 0.502 | 0.203 | Inf | 1 | -1.709 | 0.9309 |
| Topography1 | Biofilm5 | / | Topography3 | Biofilm5 | 0.396 | 0.14 | Inf | 1 | -2.623 | 0.354 |
| Topography1 | Biofilm5 | / | Topography4 | Biofilm5 | 0.787 | 0.342 | Inf | 1 | -0.551 | 1 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography1 | Biofilm5 | / | Topography5 | Biofilm5 | 0.462 | 0.163 | Inf | 1 | -2.186 | 0.675 |
| Topography1 | Biofilm5 | / | Topography1 | Biofilm20 | 2.497 | 1.47 | Inf | 1 | 1.553 | 0.9682 |
| Topography1 | Biofilm5 | / | Topography2 | Biofilm20 | 0.595 | 0.219 | Inf | 1 | -1.409 | 0.9867 |
| Topography1 | Biofilm5 | / | Topography3 | Biofilm20 | 0.883 | 0.383 | Inf | 1 | -0.286 | 1 |
| Topography1 | Biofilm5 | / | Topography4 | Biofilm20 | 1.362 | 0.641 | Inf | 1 | 0.657 | 1 |
| Topography1 | Biofilm5 | / | Topography5 | Biofilm20 | 0.57 | 0.241 | Inf | 1 | -1.329 | 0.9924 |
| Topography2 | Biofilm5 | / | Topography3 | Biofilm5 | 0.789 | 0.271 | Inf | 1 | -0.691 | 1 |
| Topography2 | Biofilm5 | / | Topography4 | Biofilm5 | 1.569 | 0.668 | Inf | 1 | 1.058 | 0.9993 |
| Topography2 | Biofilm5 | / | Topography5 | Biofilm5 | 0.921 | 0.316 | Inf | 1 | -0.24 | 1 |
| Topography2 | Biofilm5 | / | Topography1 | Biofilm20 | 4.977 | 2.89 | Inf | 1 | 2.762 | 0.2675 |
| Topography2 | Biofilm5 | / | Topography2 | Biofilm20 | 1.186 | 0.426 | Inf | 1 | 0.475 | 1 |
| Topography2 | Biofilm5 | / | Topography3 | Biofilm20 | 1.761 | 0.744 | Inf | 1 | 1.339 | 0.9919 |
| Topography2 | Biofilm5 | / | Topography4 | Biofilm20 | 2.716 | 1.25 | Inf | 1 | 2.164 | 0.6908 |
| Topography2 | Biofilm5 | / | Topography5 | Biofilm20 | 1.137 | 0.465 | Inf | 1 | 0.314 | 1 |
| Topography3 | Biofilm5 | / | Topography4 | Biofilm5 | 1.988 | 0.752 | Inf | 1 | 1.817 | 0.8917 |
| Topography3 | Biofilm5 | / | Topography5 | Biofilm5 | 1.167 | 0.326 | Inf | 1 | 0.553 | 1 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography3 | Biofilm5 | / | Topography1 | Biofilm20 | 6.309 | 3.46 | Inf | 1 | 3.356 | *0.0559* |
| Topography3 | Biofilm5 | / | Topography2 | Biofilm20 | 1.503 | 0.449 | Inf | 1 | 1.366 | 0.9901 |
| Topography3 | Biofilm5 | / | Topography3 | Biofilm20 | 2.232 | 0.842 | Inf | 1 | 2.129 | 0.7155 |
| Topography3 | Biofilm5 | / | Topography4 | Biofilm20 | 3.443 | 1.44 | Inf | 1 | 2.954 | 0.1721 |
| Topography3 | Biofilm5 | / | Topography5 | Biofilm20 | 1.442 | 0.526 | Inf | 1 | 1.002 | 0.9996 |
| Topography4 | Biofilm5 | / | Topography5 | Biofilm5 | 0.587 | 0.222 | Inf | 1 | -1.409 | 0.9868 |
| Topography4 | Biofilm5 | / | Topography1 | Biofilm20 | 3.173 | 1.92 | Inf | 1 | 1.91 | 0.8485 |
| Topography4 | Biofilm5 | / | Topography2 | Biofilm20 | 0.756 | 0.297 | Inf | 1 | -0.712 | 1 |
| Topography4 | Biofilm5 | / | Topography3 | Biofilm20 | 1.123 | 0.51 | Inf | 1 | 0.255 | 1 |
| Topography4 | Biofilm5 | / | Topography4 | Biofilm20 | 1.731 | 0.848 | Inf | 1 | 1.121 | 0.9987 |
| Topography4 | Biofilm5 | / | Topography5 | Biofilm20 | 0.725 | 0.321 | Inf | 1 | -0.726 | 1 |
| Topography5 | Biofilm5 | / | Topography1 | Biofilm20 | 5.405 | 2.97 | Inf | 1 | 3.075 | 0.126 |
| Topography5 | Biofilm5 | / | Topography2 | Biofilm20 | 1.288 | 0.384 | Inf | 1 | 0.848 | 0.9999 |
| Topography5 | Biofilm5 | / | Topography3 | Biofilm20 | 1.912 | 0.721 | Inf | 1 | 1.72 | 0.9275 |
| Topography5 | Biofilm5 | / | Topography4 | Biofilm20 | 2.949 | 1.23 | Inf | 1 | 2.585 | 0.3797 |
| Topography5 | Biofilm5 | / | Topography5 | Biofilm20 | 1.235 | 0.45 | Inf | 1 | 0.579 | 1 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Topography1 | Biofilm20 | / | Topography2 | Biofilm20 | 0.238 | 0.133 | Inf | 1 | -2.567 | 0.3925 |
| Topography1 | Biofilm20 | / | Topography3 | Biofilm20 | 0.354 | 0.213 | Inf | 1 | -1.723 | 0.9263 |
| Topography1 | Biofilm20 | / | Topography4 | Biofilm20 | 0.546 | 0.344 | Inf | 1 | -0.961 | 0.9998 |
| Topography1 | Biofilm20 | / | Topography5 | Biofilm20 | 0.228 | 0.136 | Inf | 1 | -2.485 | 0.4513 |
| Topography2 | Biofilm20 | / | Topography3 | Biofilm20 | 1.485 | 0.581 | Inf | 1 | 1.009 | 0.9996 |
| Topography2 | Biofilm20 | / | Topography4 | Biofilm20 | 2.29 | 0.987 | Inf | 1 | 1.921 | 0.8428 |
| Topography2 | Biofilm20 | / | Topography5 | Biofilm20 | 0.959 | 0.364 | Inf | 1 | -0.111 | 1 |
| Topography3 | Biofilm20 | / | Topography4 | Biofilm20 | 1.542 | 0.753 | Inf | 1 | 0.888 | 0.9999 |
| Topography3 | Biofilm20 | / | Topography5 | Biofilm20 | 0.646 | 0.284 | Inf | 1 | -0.993 | 0.9997 |
| Topography4 | Biofilm20 | / | Topography5 | Biofilm20 | 0.419 | 0.2 | Inf | 1 | -1.821 | 0.8902 |

Table S4. Results of the pairwise post hoc analysis performed for the field experiment. Significant differences ($p < 0.05$) are in bold.

| **Contrasts** | | | | | **ratio** | **SE** | **df** | **null** | **z.ratio** | **p.value** |
|---|---|---|---|---|---|---|---|---|---|---|
| topography1 | Horizontal | / | topography2 | Horizontal | 0.8835 | 0.19 | Inf | 1 | -0.575 | 1 |
| topography1 | Horizontal | / | topography3 | Horizontal | 0.3577 | 0.0708 | Inf | 1 | -5.197 | **<.0001** |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| topography1 | Horizontal | / | topography4 | Horizontal | 4.4 | 1.4 | Inf | 1 | 4.641 | **0.0003** |
| topography1 | Horizontal | / | topography5 | Horizontal | 0.2321 | 0.0449 | Inf | 1 | -7.552 | **<.0001** |
| topography1 | Horizontal | / | topography1 | Vertical_1 | 2.9333 | 0.959 | Inf | 1 | 3.291 | 0.0682 |
| topography1 | Horizontal | / | topography2 | Vertical_1 | 0.9778 | 0.244 | Inf | 1 | -0.09 | 1 |
| topography1 | Horizontal | / | topography3 | Vertical_1 | 0.5789 | 0.134 | Inf | 1 | -2.364 | 0.5418 |
| topography1 | Horizontal | / | topography4 | Vertical_1 | 4.5833 | 1.88 | Inf | 1 | 3.711 | **0.0169** |
| topography1 | Horizontal | / | topography5 | Vertical_1 | 0.4314 | 0.0965 | Inf | 1 | -3.757 | **0.0143** |
| topography1 | Horizontal | / | topography1 | Vertical_2 | 2.3158 | 0.706 | Inf | 1 | 2.755 | 0.2718 |
| topography1 | Horizontal | / | topography2 | Vertical_2 | 1.0476 | 0.265 | Inf | 1 | 0.184 | 1 |
| topography1 | Horizontal | / | topography3 | Vertical_2 | 0.5116 | 0.117 | Inf | 1 | -2.941 | 0.1774 |
| topography1 | Horizontal | / | topography4 | Vertical_2 | 2.9333 | 0.959 | Inf | 1 | 3.291 | 0.0682 |
| topography1 | Horizontal | / | topography5 | Vertical_2 | 0.2667 | 0.0574 | Inf | 1 | -6.14 | **<.0001** |
| topography2 | Horizontal | / | topography3 | Horizontal | 0.4049 | 0.0758 | Inf | 1 | -4.832 | **0.0001** |
| topography2 | Horizontal | / | topography4 | Horizontal | 4.98 | 1.56 | Inf | 1 | 5.133 | **<.0001** |
| topography2 | Horizontal | / | topography5 | Horizontal | 0.2627 | 0.0479 | Inf | 1 | -7.325 | **<.0001** |
| topography2 | Horizontal | / | topography1 | Vertical_1 | 3.32 | 1.06 | Inf | 1 | 3.742 | **0.0151** |
| topography2 | Horizontal | / | topography2 | Vertical_1 | 1.1067 | 0.267 | Inf | 1 | 0.419 | 1 |
| topography2 | Horizontal | / | topography3 | Vertical_1 | 0.6553 | 0.146 | Inf | 1 | -1.903 | 0.8521 |
| topography2 | Horizontal | / | topography4 | Vertical_1 | 5.1875 | 2.1 | Inf | 1 | 4.063 | **0.0044** |
| topography2 | Horizontal | / | topography5 | Vertical_1 | 0.4882 | 0.105 | Inf | 1 | -3.344 | 0.058 |
| topography2 | Horizontal | / | topography1 | Vertical_2 | 2.6211 | 0.781 | Inf | 1 | 3.233 | 0.0808 |
| topography2 | Horizontal | / | topography2 | Vertical_2 | 1.1857 | 0.29 | Inf | 1 | 0.696 | 1 |
| topography2 | Horizontal | / | topography3 | Vertical_2 | 0.5791 | 0.127 | Inf | 1 | -2.499 | 0.4411 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| topography2 | Horizontal | / | topography4 | Vertical_2 | 3.32 | 1.06 | Inf | 1 | 3.742 | **0.0151** |
| topography2 | Horizontal | / | topography5 | Vertical_2 | 0.3018 | 0.062 | Inf | 1 | -5.829 | **<.0001** |
| topography3 | Horizontal | / | topography4 | Horizontal | 12.3 | 3.7 | Inf | 1 | 8.336 | **<.0001** |
| topography3 | Horizontal | / | topography5 | Horizontal | 0.6487 | 0.105 | Inf | 1 | -2.677 | 0.3192 |
| topography3 | Horizontal | / | topography1 | Vertical_1 | 8.2 | 2.54 | Inf | 1 | 6.803 | **<.0001** |
| topography3 | Horizontal | / | topography2 | Vertical_1 | 2.7333 | 0.619 | Inf | 1 | 4.443 | **0.0009** |
| topography3 | Horizontal | / | topography3 | Vertical_1 | 1.6184 | 0.332 | Inf | 1 | 2.345 | 0.5566 |
| topography3 | Horizontal | / | topography4 | Vertical_1 | 12.8125 | 5.08 | Inf | 1 | 6.436 | **<.0001** |
| topography3 | Horizontal | / | topography5 | Vertical_1 | 1.2059 | 0.238 | Inf | 1 | 0.95 | 0.9998 |
| topography3 | Horizontal | / | topography1 | Vertical_2 | 6.4737 | 1.85 | Inf | 1 | 6.537 | **<.0001** |
| topography3 | Horizontal | / | topography2 | Vertical_2 | 2.9286 | 0.673 | Inf | 1 | 4.676 | **0.0003** |
| topography3 | Horizontal | / | topography3 | Vertical_2 | 1.4302 | 0.288 | Inf | 1 | 1.775 | 0.9083 |
| topography3 | Horizontal | / | topography4 | Vertical_2 | 8.2 | 2.54 | Inf | 1 | 6.803 | **<.0001** |
| topography3 | Horizontal | / | topography5 | Vertical_2 | 0.7455 | 0.14 | Inf | 1 | -1.569 | 0.9653 |
| topography4 | Horizontal | / | topography5 | Horizontal | 0.0527 | 0.0157 | Inf | 1 | -9.867 | **<.0001** |
| topography4 | Horizontal | / | topography1 | Vertical_1 | 0.6667 | 0.265 | Inf | 1 | -1.019 | 0.9996 |
| topography4 | Horizontal | / | topography2 | Vertical_1 | 0.2222 | 0.075 | Inf | 1 | -4.454 | **0.0008** |
| topography4 | Horizontal | / | topography3 | Vertical_1 | 0.1316 | 0.0426 | Inf | 1 | -6.26 | **<.0001** |
| topography4 | Horizontal | / | topography4 | Vertical_1 | 1.0417 | 0.488 | Inf | 1 | 0.087 | 1 |
| topography4 | Horizontal | / | topography5 | Vertical_1 | 0.098 | 0.0312 | Inf | 1 | -7.286 | **<.0001** |
| topography4 | Horizontal | / | topography1 | Vertical_2 | 0.5263 | 0.2 | Inf | 1 | -1.689 | 0.9369 |
| topography4 | Horizontal | / | topography2 | Vertical_2 | 0.2381 | 0.081 | Inf | 1 | -4.221 | **0.0023** |
| topography4 | Horizontal | / | topography3 | Vertical_2 | 0.1163 | 0.0374 | Inf | 1 | -6.691 | **<.0001** |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| topography4 | Horizontal | / | topography4 | Vertical_2 | 0.6667 | 0.265 | Inf | 1 | -1.019 | 0.9996 |
| topography4 | Horizontal | / | topography5 | Vertical_2 | 0.0606 | 0.019 | Inf | 1 | -8.961 | **<.0001** |
| topography5 | Horizontal | / | topography1 | Vertical_1 | 12.64 | 3.87 | Inf | 1 | 8.276 | **<.0001** |
| topography5 | Horizontal | / | topography2 | Vertical_1 | 4.2133 | 0.938 | Inf | 1 | 6.464 | **<.0001** |
| topography5 | Horizontal | / | topography3 | Vertical_1 | 2.4947 | 0.502 | Inf | 1 | 4.546 | **0.0005** |
| topography5 | Horizontal | / | topography4 | Vertical_1 | 19.75 | 7.78 | Inf | 1 | 7.57 | **<.0001** |
| topography5 | Horizontal | / | topography5 | Vertical_1 | 1.8588 | 0.358 | Inf | 1 | 3.219 | 0.0843 |
| topography5 | Horizontal | / | topography1 | Vertical_2 | 9.9789 | 2.82 | Inf | 1 | 8.137 | **<.0001** |
| topography5 | Horizontal | / | topography2 | Vertical_2 | 4.5143 | 1.02 | Inf | 1 | 6.668 | **<.0001** |
| topography5 | Horizontal | / | topography3 | Vertical_2 | 2.2047 | 0.435 | Inf | 1 | 4.007 | **0.0055** |
| topography5 | Horizontal | / | topography4 | Vertical_2 | 12.64 | 3.87 | Inf | 1 | 8.276 | **<.0001** |
| topography5 | Horizontal | / | topography5 | Vertical_2 | 1.1491 | 0.21 | Inf | 1 | 0.761 | 1 |
| topography1 | Vertical_1 | / | topography2 | Vertical_1 | 0.3333 | 0.115 | Inf | 1 | -3.184 | 0.0932 |
| topography1 | Vertical_1 | / | topography3 | Vertical_1 | 0.1974 | 0.0655 | Inf | 1 | -4.893 | **0.0001** |
| topography1 | Vertical_1 | / | topography4 | Vertical_1 | 1.5625 | 0.741 | Inf | 1 | 0.941 | 0.9998 |
| topography1 | Vertical_1 | / | topography5 | Vertical_1 | 0.1471 | 0.048 | Inf | 1 | -5.87 | <.0001 |
| topography1 | Vertical_1 | / | topography1 | Vertical_2 | 0.7895 | 0.305 | Inf | 1 | -0.611 | 1 |
| topography1 | Vertical_1 | / | topography2 | Vertical_2 | 0.3571 | 0.124 | Inf | 1 | -2.964 | 0.1675 |
| topography1 | Vertical_1 | / | topography3 | Vertical_2 | 0.1744 | 0.0574 | Inf | 1 | -5.303 | **<.0001** |
| topography1 | Vertical_1 | / | topography4 | Vertical_2 | 1 | 0.404 | Inf | 1 | 0 | 1 |
| topography1 | Vertical_1 | / | topography5 | Vertical_2 | 0.0909 | 0.0292 | Inf | 1 | -7.476 | **<.0001** |
| topography2 | Vertical_1 | / | topography3 | Vertical_1 | 0.5921 | 0.152 | Inf | 1 | -2.047 | 0.7699 |
| topography2 | Vertical_1 | / | topography4 | Vertical_1 | 4.6875 | 1.99 | Inf | 1 | 3.637 | **0.022** |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| topography2 | Vertical_1 | / | topography5 | Vertical_1 | 0.4412 | 0.11 | Inf | 1 | -3.282 | 0.0701 |
| topography2 | Vertical_1 | / | topography1 | Vertical_2 | 2.3684 | 0.767 | Inf | 1 | 2.661 | 0.3293 |
| topography2 | Vertical_1 | / | topography2 | Vertical_2 | 1.0714 | 0.296 | Inf | 1 | 0.25 | 1 |
| topography2 | Vertical_1 | / | topography3 | Vertical_2 | 0.5233 | 0.132 | Inf | 1 | -2.56 | 0.3972 |
| topography2 | Vertical_1 | / | topography4 | Vertical_2 | 3 | 1.04 | Inf | 1 | 3.184 | 0.0932 |
| topography2 | Vertical_1 | / | topography5 | Vertical_2 | 0.2727 | 0.0659 | Inf | 1 | -5.375 | **<.0001** |
| topography3 | Vertical_1 | / | topography4 | Vertical_1 | 7.9167 | 3.28 | Inf | 1 | 4.998 | **0.0001** |
| topography3 | Vertical_1 | / | topography5 | Vertical_1 | 0.7451 | 0.172 | Inf | 1 | -1.277 | 0.9949 |
| topography3 | Vertical_1 | / | topography1 | Vertical_2 | 4 | 1.24 | Inf | 1 | 4.475 | **0.0007** |
| topography3 | Vertical_1 | / | topography2 | Vertical_2 | 1.8095 | 0.469 | Inf | 1 | 2.289 | 0.5988 |
| topography3 | Vertical_1 | / | topography3 | Vertical_2 | 0.8837 | 0.207 | Inf | 1 | -0.527 | 1 |
| topography3 | Vertical_1 | / | topography4 | Vertical_2 | 5.0667 | 1.68 | Inf | 1 | 4.893 | **0.0001** |
| topography3 | Vertical_1 | / | topography5 | Vertical_2 | 0.4606 | 0.102 | Inf | 1 | -3.489 | **0.0365** |
| topography4 | Vertical_1 | / | topography5 | Vertical_1 | 0.0941 | 0.0386 | Inf | 1 | -5.766 | **<.0001** |
| topography4 | Vertical_1 | / | topography1 | Vertical_2 | 0.5053 | 0.232 | Inf | 1 | -1.487 | 0.9783 |
| topography4 | Vertical_1 | / | topography2 | Vertical_2 | 0.2286 | 0.0975 | Inf | 1 | -3.46 | **0.0401** |
| topography4 | Vertical_1 | / | topography3 | Vertical_2 | 0.1116 | 0.046 | Inf | 1 | -5.321 | **<.0001** |
| topography4 | Vertical_1 | / | topography4 | Vertical_2 | 0.64 | 0.303 | Inf | 1 | -0.941 | 0.9998 |
| topography4 | Vertical_1 | / | topography5 | Vertical_2 | 0.0582 | 0.0236 | Inf | 1 | -7.018 | **<.0001** |
| topography5 | Vertical_1 | / | topography1 | Vertical_2 | 5.3684 | 1.63 | Inf | 1 | 5.523 | **<.0001** |
| topography5 | Vertical_1 | / | topography2 | Vertical_2 | 2.4286 | 0.613 | Inf | 1 | 3.514 | **0.0336** |
| topography5 | Vertical_1 | / | topography3 | Vertical_2 | 1.186 | 0.269 | Inf | 1 | 0.751 | 1 |
| topography5 | Vertical_1 | / | topography4 | Vertical_2 | 6.8 | 2.22 | Inf | 1 | 5.87 | **<.0001** |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| topography5 | Vertical_1 | / | topography5 | Vertical_2 | 0.6182 | 0.133 | Inf | 1 | -2.242 | 0.634 |
| topography1 | Vertical_2 | / | topography2 | Vertical_2 | 0.4524 | 0.148 | Inf | 1 | -2.43 | 0.4924 |
| topography1 | Vertical_2 | / | topography3 | Vertical_2 | 0.2209 | 0.0679 | Inf | 1 | -4.914 | **0.0001** |
| topography1 | Vertical_2 | / | topography4 | Vertical_2 | 1.2667 | 0.49 | Inf | 1 | 0.611 | 1 |
| topography1 | Vertical_2 | / | topography5 | Vertical_2 | 0.1152 | 0.0343 | Inf | 1 | -7.252 | **<.0001** |
| topography2 | Vertical_2 | / | topography3 | Vertical_2 | 0.4884 | 0.125 | Inf | 1 | -2.798 | 0.2474 |
| topography2 | Vertical_2 | / | topography4 | Vertical_2 | 2.8 | 0.973 | Inf | 1 | 2.964 | 0.1675 |
| topography2 | Vertical_2 | / | topography5 | Vertical_2 | 0.2545 | 0.0624 | Inf | 1 | -5.585 | **<.0001** |
| topography3 | Vertical_2 | / | topography4 | Vertical_2 | 5.7333 | 1.89 | Inf | 1 | 5.303 | **<.0001** |
| topography3 | Vertical_2 | / | topography5 | Vertical_2 | 0.5212 | 0.114 | Inf | 1 | -2.979 | 0.1616 |
| topography4 | Vertical_2 | / | topography5 | Vertical_2 | 0.0909 | 0.0292 | Inf | 1 | -7.476 | **<.0001** |

Table S5. Results of the pairwise post hoc analysis performed for analysis of the position of larvae on topographical features.

| **Experiment** | **contrast** | | | | | **ratio** | **SE** | **df** | **null** | **z.ratio** | **p.value** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Lab-1** | Topography2 | corner | / | Topography3 | corner | 0.934 | 0.367 | Inf | 1 | -0.174 | 1 |
| | Topography2 | corner | / | Topography4 | corner | 1.879 | 0.812 | Inf | 1 | 1.459 | 0.9868 |
| | Topography2 | corner | / | Topography5 | corner | 1.604 | 0.676 | Inf | 1 | 1.122 | 0.9992 |
| | Topography2 | corner | / | Topography2 | middle | 0.626 | 0.232 | Inf | 1 | -1.267 | 0.997 |
| | Topography2 | corner | / | Topography3 | middle | 0.584 | 0.315 | Inf | 1 | -0.995 | 0.9998 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Topography2 | corner | / | Topography4 | middle | 1.176 | 0.665 | Inf | 1 | 0.286 | 1 |
| | Topography2 | corner | / | Topography5 | middle | 1.004 | 0.561 | Inf | 1 | 0.007 | 1 |
| | Topography2 | corner | / | Topography2 | side | 1.91 | 0.81 | Inf | 1 | 1.525 | 0.9799 |
| | Topography2 | corner | / | Topography3 | side | 1.784 | 1.03 | Inf | 1 | 1.002 | 0.9998 |
| | Topography2 | corner | / | Topography4 | side | 3.588 | 2.19 | Inf | 1 | 2.097 | 0.7676 |
| | Topography2 | corner | / | Topography5 | side | 3.063 | 1.84 | Inf | 1 | 1.864 | 0.8924 |
| | Topography2 | corner | / | Topography2 | top | 3.869 | 1.92 | Inf | 1 | 2.733 | 0.3091 |
| | Topography2 | corner | / | Topography3 | top | 3.614 | 2.28 | Inf | 1 | 2.036 | 0.805 |
| | Topography2 | corner | / | Topography4 | top | 7.269 | 4.82 | Inf | 1 | 2.99 | 0.1729 |
| | Topography2 | corner | / | Topography5 | top | 6.207 | 4.06 | Inf | 1 | 2.787 | 0.2761 |
| | Topography3 | corner | / | Topography4 | corner | 2.011 | 0.874 | Inf | 1 | 1.608 | 0.9674 |
| | Topography3 | corner | / | Topography5 | corner | 1.717 | 0.728 | Inf | 1 | 1.276 | 0.9967 |
| | Topography3 | corner | / | Topography2 | middle | 0.67 | 0.361 | Inf | 1 | -0.743 | 1 |
| | Topography3 | corner | / | Topography3 | middle | 0.626 | 0.232 | Inf | 1 | -1.267 | 0.997 |
| | Topography3 | corner | / | Topography4 | middle | 1.259 | 0.715 | Inf | 1 | 0.405 | 1 |
| | Topography3 | corner | / | Topography5 | middle | 1.075 | 0.602 | Inf | 1 | 0.129 | 1 |
| | Topography3 | corner | / | Topography2 | side | 2.045 | 1.18 | Inf | 1 | 1.237 | 0.9977 |
| | Topography3 | corner | / | Topography3 | side | 1.91 | 0.81 | Inf | 1 | 1.525 | 0.9799 |
| | Topography3 | corner | / | Topography4 | side | 3.841 | 2.35 | Inf | 1 | 2.201 | 0.6967 |
| | Topography3 | corner | / | Topography5 | side | 3.28 | 1.98 | Inf | 1 | 1.97 | 0.8419 |
| | Topography3 | corner | / | Topography2 | top | 4.143 | 2.62 | Inf | 1 | 2.247 | 0.6634 |
| | Topography3 | corner | / | Topography3 | top | 3.869 | 1.92 | Inf | 1 | 2.733 | 0.3091 |
| | Topography3 | corner | / | Topography4 | top | 7.783 | 5.18 | Inf | 1 | 3.082 | 0.1366 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Topography3 | corner | / | Topography5 | top | 6.645 | 4.37 | Inf | 1 | 2.881 | 0.2244 |
| | Topography4 | corner | / | Topography5 | corner | 0.854 | 0.393 | Inf | 1 | -0.343 | 1 |
| | Topography4 | corner | / | Topography2 | middle | 0.333 | 0.19 | Inf | 1 | -1.923 | 0.8659 |
| | Topography4 | corner | / | Topography3 | middle | 0.311 | 0.179 | Inf | 1 | -2.034 | 0.806 |
| | Topography4 | corner | / | Topography4 | middle | 0.626 | 0.232 | Inf | 1 | -1.267 | 0.997 |
| | Topography4 | corner | / | Topography5 | middle | 0.534 | 0.316 | Inf | 1 | -1.06 | 0.9996 |
| | Topography4 | corner | / | Topography2 | side | 1.017 | 0.612 | Inf | 1 | 0.027 | 1 |
| | Topography4 | corner | / | Topography3 | side | 0.949 | 0.573 | Inf | 1 | -0.086 | 1 |
| | Topography4 | corner | / | Topography4 | side | 1.91 | 0.81 | Inf | 1 | 1.525 | 0.9799 |
| | Topography4 | corner | / | Topography5 | side | 1.631 | 1.02 | Inf | 1 | 0.782 | 1 |
| | Topography4 | corner | / | Topography2 | top | 2.06 | 1.34 | Inf | 1 | 1.11 | 0.9993 |
| | Topography4 | corner | / | Topography3 | top | 1.924 | 1.25 | Inf | 1 | 1.004 | 0.9998 |
| | Topography4 | corner | / | Topography4 | top | 3.869 | 1.92 | Inf | 1 | 2.733 | 0.3091 |
| | Topography4 | corner | / | Topography5 | top | 3.304 | 2.23 | Inf | 1 | 1.771 | 0.9267 |
| | Topography5 | corner | / | Topography2 | middle | 0.39 | 0.22 | Inf | 1 | -1.673 | 0.954 |
| | Topography5 | corner | / | Topography3 | middle | 0.364 | 0.206 | Inf | 1 | -1.787 | 0.9214 |
| | Topography5 | corner | / | Topography4 | middle | 0.733 | 0.432 | Inf | 1 | -0.527 | 1 |
| | Topography5 | corner | / | Topography5 | middle | 0.626 | 0.232 | Inf | 1 | -1.267 | 0.997 |
| | Topography5 | corner | / | Topography2 | side | 1.191 | 0.708 | Inf | 1 | 0.293 | 1 |
| | Topography5 | corner | / | Topography3 | side | 1.112 | 0.663 | Inf | 1 | 0.178 | 1 |
| | Topography5 | corner | / | Topography4 | side | 2.237 | 1.4 | Inf | 1 | 1.284 | 0.9965 |
| | Topography5 | corner | / | Topography5 | side | 1.91 | 0.81 | Inf | 1 | 1.525 | 0.9799 |
| | Topography5 | corner | / | Topography2 | top | 2.412 | 1.56 | Inf | 1 | 1.365 | 0.9933 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Topography5 | corner | / | Topography3 | top | 2.253 | 1.46 | Inf | 1 | 1.258 | 0.9972 |
| | Topography5 | corner | / | Topography4 | top | 4.532 | 3.07 | Inf | 1 | 2.231 | 0.6753 |
| | Topography5 | corner | / | Topography5 | top | 3.869 | 1.92 | Inf | 1 | 2.733 | 0.3091 |
| | Topography2 | middle | / | Topography3 | middle | 0.934 | 0.367 | Inf | 1 | -0.174 | 1 |
| | Topography2 | middle | / | Topography4 | middle | 1.879 | 0.812 | Inf | 1 | 1.459 | 0.9868 |
| | Topography2 | middle | / | Topography5 | middle | 1.604 | 0.676 | Inf | 1 | 1.122 | 0.9992 |
| | Topography2 | middle | / | Topography2 | side | 3.051 | 1.25 | Inf | 1 | 2.727 | 0.3131 |
| | Topography2 | middle | / | Topography3 | side | 2.85 | 1.61 | Inf | 1 | 1.849 | 0.8982 |
| | Topography2 | middle | / | Topography4 | side | 5.732 | 3.45 | Inf | 1 | 2.903 | 0.2133 |
| | Topography2 | middle | / | Topography5 | side | 4.895 | 2.9 | Inf | 1 | 2.682 | 0.3418 |
| | Topography2 | middle | / | Topography2 | top | 6.183 | 2.98 | Inf | 1 | 3.778 | 0.0149 |
| | Topography2 | middle | / | Topography3 | top | 5.775 | 3.58 | Inf | 1 | 2.825 | 0.2545 |
| | Topography2 | middle | / | Topography4 | top | 11.615 | 7.62 | Inf | 1 | 3.736 | 0.0174 |
| | Topography2 | middle | / | Topography5 | top | 9.917 | 6.42 | Inf | 1 | 3.545 | 0.0338 |
| | Topography3 | middle | / | Topography4 | middle | 2.011 | 0.874 | Inf | 1 | 1.608 | 0.9674 |
| | Topography3 | middle | / | Topography5 | middle | 1.717 | 0.728 | Inf | 1 | 1.276 | 0.9967 |
| | Topography3 | middle | / | Topography2 | side | 3.267 | 1.85 | Inf | 1 | 2.086 | 0.7748 |
| | Topography3 | middle | / | Topography3 | side | 3.051 | 1.25 | Inf | 1 | 2.727 | 0.3131 |
| | Topography3 | middle | / | Topography4 | side | 6.138 | 3.71 | Inf | 1 | 3.004 | 0.1668 |
| | Topography3 | middle | / | Topography5 | side | 5.241 | 3.12 | Inf | 1 | 2.786 | 0.2769 |
| | Topography3 | middle | / | Topography2 | top | 6.62 | 4.12 | Inf | 1 | 3.034 | 0.1545 |
| | Topography3 | middle | / | Topography3 | top | 6.183 | 2.98 | Inf | 1 | 3.778 | 0.0149 |
| | Topography3 | middle | / | Topography4 | top | 12.436 | 8.2 | Inf | 1 | 3.825 | 0.0125 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Topography3 | middle | / | Topography5 | top | 10.619 | 6.9 | Inf | 1 | 3.636 | 0.0248 |
| | Topography4 | middle | / | Topography5 | middle | 0.854 | 0.393 | Inf | 1 | -0.343 | 1 |
| | Topography4 | middle | / | Topography2 | side | 1.624 | 0.956 | Inf | 1 | 0.824 | 1 |
| | Topography4 | middle | / | Topography3 | side | 1.517 | 0.895 | Inf | 1 | 0.707 | 1 |
| | Topography4 | middle | / | Topography4 | side | 3.051 | 1.25 | Inf | 1 | 2.727 | 0.3131 |
| | Topography4 | middle | / | Topography5 | side | 2.605 | 1.6 | Inf | 1 | 1.558 | 0.9754 |
| | Topography4 | middle | / | Topography2 | top | 3.291 | 2.1 | Inf | 1 | 1.865 | 0.8917 |
| | Topography4 | middle | / | Topography3 | top | 3.074 | 1.97 | Inf | 1 | 1.757 | 0.9313 |
| | Topography4 | middle | / | Topography4 | top | 6.183 | 2.98 | Inf | 1 | 3.778 | 0.0149 |
| | Topography4 | middle | / | Topography5 | top | 5.279 | 3.51 | Inf | 1 | 2.503 | 0.4694 |
| | Topography5 | middle | / | Topography2 | side | 1.902 | 1.11 | Inf | 1 | 1.104 | 0.9994 |
| | Topography5 | middle | / | Topography3 | side | 1.777 | 1.04 | Inf | 1 | 0.985 | 0.9998 |
| | Topography5 | middle | / | Topography4 | side | 3.574 | 2.21 | Inf | 1 | 2.062 | 0.7892 |
| | Topography5 | middle | / | Topography5 | side | 3.051 | 1.25 | Inf | 1 | 2.727 | 0.3131 |
| | Topography5 | middle | / | Topography2 | top | 3.855 | 2.44 | Inf | 1 | 2.13 | 0.7457 |
| | Topography5 | middle | / | Topography3 | top | 3.6 | 2.28 | Inf | 1 | 2.02 | 0.8143 |
| | Topography5 | middle | / | Topography4 | top | 7.241 | 4.84 | Inf | 1 | 2.96 | 0.186 |
| | Topography5 | middle | / | Topography5 | top | 6.183 | 2.98 | Inf | 1 | 3.778 | 0.0149 |
| | Topography2 | side | / | Topography3 | side | 0.934 | 0.367 | Inf | 1 | -0.174 | 1 |
| | Topography2 | side | / | Topography4 | side | 1.879 | 0.812 | Inf | 1 | 1.459 | 0.9868 |
| | Topography2 | side | / | Topography5 | side | 1.604 | 0.676 | Inf | 1 | 1.122 | 0.9992 |
| | Topography2 | side | / | Topography2 | top | 2.026 | 1.06 | Inf | 1 | 1.346 | 0.9942 |
| | Topography2 | side | / | Topography3 | top | 1.892 | 1.24 | Inf | 1 | 0.974 | 0.9999 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Topography2 | side | / | Topography4 | top | 3.806 | 2.6 | Inf | 1 | 1.958 | 0.8484 |
| | Topography2 | side | / | Topography5 | top | 3.25 | 2.19 | Inf | 1 | 1.746 | 0.9347 |
| | Topography3 | side | / | Topography4 | side | 2.011 | 0.874 | Inf | 1 | 1.608 | 0.9674 |
| | Topography3 | side | / | Topography5 | side | 1.717 | 0.728 | Inf | 1 | 1.276 | 0.9967 |
| | Topography3 | side | / | Topography2 | top | 2.169 | 1.42 | Inf | 1 | 1.181 | 0.9986 |
| | Topography3 | side | / | Topography3 | top | 2.026 | 1.06 | Inf | 1 | 1.346 | 0.9942 |
| | Topography3 | side | / | Topography4 | top | 4.076 | 2.79 | Inf | 1 | 2.052 | 0.7955 |
| | Topography3 | side | / | Topography5 | top | 3.48 | 2.36 | Inf | 1 | 1.841 | 0.9015 |
| | Topography4 | side | / | Topography5 | side | 0.854 | 0.393 | Inf | 1 | -0.343 | 1 |
| | Topography4 | side | / | Topography2 | top | 1.079 | 0.73 | Inf | 1 | 0.112 | 1 |
| | Topography4 | side | / | Topography3 | top | 1.007 | 0.683 | Inf | 1 | 0.011 | 1 |
| | Topography4 | side | / | Topography4 | top | 2.026 | 1.06 | Inf | 1 | 1.346 | 0.9942 |
| | Topography4 | side | / | Topography5 | top | 1.73 | 1.21 | Inf | 1 | 0.786 | 1 |
| | Topography5 | side | / | Topography2 | top | 1.263 | 0.847 | Inf | 1 | 0.348 | 1 |
| | Topography5 | side | / | Topography3 | top | 1.18 | 0.793 | Inf | 1 | 0.246 | 1 |
| | Topography5 | side | / | Topography4 | top | 2.373 | 1.66 | Inf | 1 | 1.237 | 0.9977 |
| | Topography5 | side | / | Topography5 | top | 2.026 | 1.06 | Inf | 1 | 1.346 | 0.9942 |
| | Topography2 | top | / | Topography3 | top | 0.934 | 0.367 | Inf | 1 | -0.174 | 1 |
| | Topography2 | top | / | Topography4 | top | 1.879 | 0.812 | Inf | 1 | 1.459 | 0.9868 |
| | Topography2 | top | / | Topography5 | top | 1.604 | 0.676 | Inf | 1 | 1.122 | 0.9992 |
| | Topography3 | top | / | Topography4 | top | 2.011 | 0.874 | Inf | 1 | 1.608 | 0.9674 |
| | Topography3 | top | / | Topography5 | top | 1.717 | 0.728 | Inf | 1 | 1.276 | 0.9967 |
| | Topography4 | top | / | Topography5 | top | 0.854 | 0.393 | Inf | 1 | -0.343 | 1 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Lab-2** | Topography2 | corner | / | Topography3 | corner | 1.236 | 0.258 | Inf | 1 | 1.015 | 0.9998 |
| | Topography2 | corner | / | Topography4 | corner | 1.107 | 0.235 | Inf | 1 | 0.48 | 1 |
| | Topography2 | corner | / | Topography5 | corner | 1.045 | 0.213 | Inf | 1 | 0.215 | 1 |
| | Topography2 | corner | / | Topography2 | middle | 0.618 | 0.0933 | Inf | 1 | -3.186 | 0.1028 |
| | Topography2 | corner | / | Topography3 | middle | 0.764 | 0.197 | Inf | 1 | -1.046 | 0.9997 |
| | Topography2 | corner | / | Topography4 | middle | 0.684 | 0.178 | Inf | 1 | -1.459 | 0.9868 |
| | Topography2 | corner | / | Topography5 | middle | 0.646 | 0.164 | Inf | 1 | -1.722 | 0.9415 |
| | Topography2 | corner | / | Topography2 | side | 43.393 | 25.7 | Inf | 1 | 6.361 | <.0001 |
| | Topography2 | corner | / | Topography3 | side | 53.642 | 33.8 | Inf | 1 | 6.329 | <.0001 |
| | Topography2 | corner | / | Topography4 | side | 48.042 | 30.3 | Inf | 1 | 6.148 | <.0001 |
| | Topography2 | corner | / | Topography5 | side | 45.343 | 28.4 | Inf | 1 | 6.082 | <.0001 |
| | Topography2 | corner | / | Topography2 | top | 32.559 | 16.9 | Inf | 1 | 6.726 | <.0001 |
| | Topography2 | corner | / | Topography3 | top | 40.25 | 22.5 | Inf | 1 | 6.608 | <.0001 |
| | Topography2 | corner | / | Topography4 | top | 36.047 | 20.2 | Inf | 1 | 6.403 | <.0001 |
| | Topography2 | corner | / | Topography5 | top | 34.022 | 18.9 | Inf | 1 | 6.334 | <.0001 |
| | Topography3 | corner | / | Topography4 | corner | 0.896 | 0.19 | Inf | 1 | -0.52 | 1 |
| | Topography3 | corner | / | Topography5 | corner | 0.845 | 0.173 | Inf | 1 | -0.823 | 1 |
| | Topography3 | corner | / | Topography2 | middle | 0.5 | 0.129 | Inf | 1 | -2.683 | 0.3413 |
| | Topography3 | corner | / | Topography3 | middle | 0.618 | 0.0933 | Inf | 1 | -3.186 | 0.1028 |
| | Topography3 | corner | / | Topography4 | middle | 0.554 | 0.144 | Inf | 1 | -2.27 | 0.6459 |
| | Topography3 | corner | / | Topography5 | middle | 0.522 | 0.133 | Inf | 1 | -2.552 | 0.433 |
| | Topography3 | corner | / | Topography2 | side | 35.101 | 22 | Inf | 1 | 5.668 | <.0001 |
| | Topography3 | corner | / | Topography3 | side | 43.393 | 25.7 | Inf | 1 | 6.361 | <.0001 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Topography3 | corner | / | Topography4 | side | 38.862 | 24.4 | Inf | 1 | 5.818 | <.0001 |
| | Topography3 | corner | / | Topography5 | side | 36.679 | 23 | Inf | 1 | 5.751 | <.0001 |
| | Topography3 | corner | / | Topography2 | top | 26.338 | 14.7 | Inf | 1 | 5.866 | <.0001 |
| | Topography3 | corner | / | Topography3 | top | 32.559 | 16.9 | Inf | 1 | 6.726 | <.0001 |
| | Topography3 | corner | / | Topography4 | top | 29.16 | 16.3 | Inf | 1 | 6.032 | <.0001 |
| | Topography3 | corner | / | Topography5 | top | 27.522 | 15.3 | Inf | 1 | 5.962 | <.0001 |
| | Topography4 | corner | / | Topography5 | corner | 0.944 | 0.196 | Inf | 1 | -0.279 | 1 |
| | Topography4 | corner | / | Topography2 | middle | 0.558 | 0.145 | Inf | 1 | -2.238 | 0.6701 |
| | Topography4 | corner | / | Topography3 | middle | 0.69 | 0.179 | Inf | 1 | -1.427 | 0.9894 |
| | Topography4 | corner | / | Topography4 | middle | 0.618 | 0.0933 | Inf | 1 | -3.186 | 0.1028 |
| | Topography4 | corner | / | Topography5 | middle | 0.583 | 0.15 | Inf | 1 | -2.101 | 0.7652 |
| | Topography4 | corner | / | Topography2 | side | 39.193 | 24.7 | Inf | 1 | 5.831 | <.0001 |
| | Topography4 | corner | / | Topography3 | side | 48.451 | 30.5 | Inf | 1 | 6.161 | <.0001 |
| | Topography4 | corner | / | Topography4 | side | 43.393 | 25.7 | Inf | 1 | 6.361 | <.0001 |
| | Topography4 | corner | / | Topography5 | side | 40.955 | 25.7 | Inf | 1 | 5.914 | <.0001 |
| | Topography4 | corner | / | Topography2 | top | 29.408 | 16.4 | Inf | 1 | 6.047 | <.0001 |
| | Topography4 | corner | / | Topography3 | top | 36.354 | 20.4 | Inf | 1 | 6.418 | <.0001 |
| | Topography4 | corner | / | Topography4 | top | 32.559 | 16.9 | Inf | 1 | 6.726 | <.0001 |
| | Topography4 | corner | / | Topography5 | top | 30.73 | 17.1 | Inf | 1 | 6.144 | <.0001 |
| | Topography5 | corner | / | Topography2 | middle | 0.591 | 0.15 | Inf | 1 | -2.066 | 0.7868 |
| | Topography5 | corner | / | Topography3 | middle | 0.731 | 0.185 | Inf | 1 | -1.235 | 0.9977 |
| | Topography5 | corner | / | Topography4 | middle | 0.655 | 0.168 | Inf | 1 | -1.652 | 0.9587 |
| | Topography5 | corner | / | Topography5 | middle | 0.618 | 0.0933 | Inf | 1 | -3.186 | 0.1028 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Topography5 | corner | / | Topography2 | side | 41.526 | 26 | Inf | 1 | 5.945 | <.0001 |
| | Topography5 | corner | / | Topography3 | side | 51.335 | 32.2 | Inf | 1 | 6.277 | <.0001 |
| | Topography5 | corner | / | Topography4 | side | 45.975 | 28.9 | Inf | 1 | 6.095 | <.0001 |
| | Topography5 | corner | / | Topography5 | side | 43.393 | 25.7 | Inf | 1 | 6.361 | <.0001 |
| | Topography5 | corner | / | Topography2 | top | 31.158 | 17.3 | Inf | 1 | 6.18 | <.0001 |
| | Topography5 | corner | / | Topography3 | top | 38.518 | 21.5 | Inf | 1 | 6.552 | <.0001 |
| | Topography5 | corner | / | Topography4 | top | 34.497 | 19.2 | Inf | 1 | 6.346 | <.0001 |
| | Topography5 | corner | / | Topography5 | top | 32.559 | 16.9 | Inf | 1 | 6.726 | <.0001 |
| | Topography2 | middle | / | Topography3 | middle | 1.236 | 0.258 | Inf | 1 | 1.015 | 0.9998 |
| | Topography2 | middle | / | Topography4 | middle | 1.107 | 0.235 | Inf | 1 | 0.48 | 1 |
| | Topography2 | middle | / | Topography5 | middle | 1.045 | 0.213 | Inf | 1 | 0.215 | 1 |
| | Topography2 | middle | / | Topography2 | side | 70.206 | 41.4 | Inf | 1 | 7.203 | <.0001 |
| | Topography2 | middle | / | Topography3 | side | 86.789 | 54.4 | Inf | 1 | 7.118 | <.0001 |
| | Topography2 | middle | / | Topography4 | side | 77.728 | 48.8 | Inf | 1 | 6.936 | <.0001 |
| | Topography2 | middle | / | Topography5 | side | 73.362 | 45.8 | Inf | 1 | 6.875 | <.0001 |
| | Topography2 | middle | / | Topography2 | top | 52.677 | 27.1 | Inf | 1 | 7.698 | <.0001 |
| | Topography2 | middle | / | Topography3 | top | 65.121 | 36.3 | Inf | 1 | 7.501 | <.0001 |
| | Topography2 | middle | / | Topography4 | top | 58.322 | 32.5 | Inf | 1 | 7.295 | <.0001 |
| | Topography2 | middle | / | Topography5 | top | 55.046 | 30.5 | Inf | 1 | 7.232 | <.0001 |
| | Topography3 | middle | / | Topography4 | middle | 0.896 | 0.19 | Inf | 1 | -0.52 | 1 |
| | Topography3 | middle | / | Topography5 | middle | 0.845 | 0.173 | Inf | 1 | -0.823 | 1 |
| | Topography3 | middle | / | Topography2 | side | 56.791 | 35.5 | Inf | 1 | 6.46 | <.0001 |
| | Topography3 | middle | / | Topography3 | side | 70.206 | 41.4 | Inf | 1 | 7.203 | <.0001 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Topography3 | middle | / | Topography4 | side | 62.876 | 39.4 | Inf | 1 | 6.608 | <.0001 |
| | Topography3 | middle | / | Topography5 | side | 59.344 | 37 | Inf | 1 | 6.545 | <.0001 |
| | Topography3 | middle | / | Topography2 | top | 42.612 | 23.6 | Inf | 1 | 6.764 | <.0001 |
| | Topography3 | middle | / | Topography3 | top | 52.677 | 27.1 | Inf | 1 | 7.698 | <.0001 |
| | Topography3 | middle | / | Topography4 | top | 47.178 | 26.2 | Inf | 1 | 6.927 | <.0001 |
| | Topography3 | middle | / | Topography5 | top | 44.528 | 24.6 | Inf | 1 | 6.862 | <.0001 |
| | Topography4 | middle | / | Topography5 | middle | 0.944 | 0.196 | Inf | 1 | -0.279 | 1 |
| | Topography4 | middle | / | Topography2 | side | 63.411 | 39.7 | Inf | 1 | 6.621 | <.0001 |
| | Topography4 | middle | / | Topography3 | side | 78.39 | 49.2 | Inf | 1 | 6.95 | <.0001 |
| | Topography4 | middle | / | Topography4 | side | 70.206 | 41.4 | Inf | 1 | 7.203 | <.0001 |
| | Topography4 | middle | / | Topography5 | side | 66.262 | 41.4 | Inf | 1 | 6.706 | <.0001 |
| | Topography4 | middle | / | Topography2 | top | 47.579 | 26.5 | Inf | 1 | 6.942 | <.0001 |
| | Topography4 | middle | / | Topography3 | top | 58.818 | 32.8 | Inf | 1 | 7.31 | <.0001 |
| | Topography4 | middle | / | Topography4 | top | 52.677 | 27.1 | Inf | 1 | 7.698 | <.0001 |
| | Topography4 | middle | / | Topography5 | top | 49.718 | 27.6 | Inf | 1 | 7.041 | <.0001 |
| | Topography5 | middle | / | Topography2 | side | 67.186 | 42 | Inf | 1 | 6.738 | <.0001 |
| | Topography5 | middle | / | Topography3 | side | 83.056 | 51.9 | Inf | 1 | 7.068 | <.0001 |
| | Topography5 | middle | / | Topography4 | side | 74.384 | 46.6 | Inf | 1 | 6.886 | <.0001 |
| | Topography5 | middle | / | Topography5 | side | 70.206 | 41.4 | Inf | 1 | 7.203 | <.0001 |
| | Topography5 | middle | / | Topography2 | top | 50.411 | 27.9 | Inf | 1 | 7.079 | <.0001 |
| | Topography5 | middle | / | Topography3 | top | 62.319 | 34.6 | Inf | 1 | 7.448 | <.0001 |
| | Topography5 | middle | / | Topography4 | top | 55.813 | 31 | Inf | 1 | 7.242 | <.0001 |
| | Topography5 | middle | / | Topography5 | top | 52.677 | 27.1 | Inf | 1 | 7.698 | <.0001 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Topography2 | side | / | Topography3 | side | 1.236 | 0.258 | Inf | 1 | 1.015 | 0.9998 |
| | Topography2 | side | / | Topography4 | side | 1.107 | 0.235 | Inf | 1 | 0.48 | 1 |
| | Topography2 | side | / | Topography5 | side | 1.045 | 0.213 | Inf | 1 | 0.215 | 1 |
| | Topography2 | side | / | Topography2 | top | 0.75 | 0.578 | Inf | 1 | -0.373 | 1 |
| | Topography2 | side | / | Topography3 | top | 0.928 | 0.741 | Inf | 1 | -0.094 | 1 |
| | Topography2 | side | / | Topography4 | top | 0.831 | 0.664 | Inf | 1 | -0.232 | 1 |
| | Topography2 | side | / | Topography5 | top | 0.784 | 0.625 | Inf | 1 | -0.305 | 1 |
| | Topography3 | side | / | Topography4 | side | 0.896 | 0.19 | Inf | 1 | -0.52 | 1 |
| | Topography3 | side | / | Topography5 | side | 0.845 | 0.173 | Inf | 1 | -0.823 | 1 |
| | Topography3 | side | / | Topography2 | top | 0.607 | 0.485 | Inf | 1 | -0.625 | 1 |
| | Topography3 | side | / | Topography3 | top | 0.75 | 0.578 | Inf | 1 | -0.373 | 1 |
| | Topography3 | side | / | Topography4 | top | 0.672 | 0.537 | Inf | 1 | -0.497 | 1 |
| | Topography3 | side | / | Topography5 | top | 0.634 | 0.506 | Inf | 1 | -0.571 | 1 |
| | Topography4 | side | / | Topography5 | side | 0.944 | 0.196 | Inf | 1 | -0.279 | 1 |
| | Topography4 | side | / | Topography2 | top | 0.678 | 0.542 | Inf | 1 | -0.487 | 1 |
| | Topography4 | side | / | Topography3 | top | 0.838 | 0.67 | Inf | 1 | -0.221 | 1 |
| | Topography4 | side | / | Topography4 | top | 0.75 | 0.578 | Inf | 1 | -0.373 | 1 |
| | Topography4 | side | / | Topography5 | top | 0.708 | 0.565 | Inf | 1 | -0.432 | 1 |
| | Topography5 | side | / | Topography2 | top | 0.718 | 0.572 | Inf | 1 | -0.416 | 1 |
| | Topography5 | side | / | Topography3 | top | 0.888 | 0.708 | Inf | 1 | -0.149 | 1 |
| | Topography5 | side | / | Topography4 | top | 0.795 | 0.634 | Inf | 1 | -0.288 | 1 |
| | Topography5 | side | / | Topography5 | top | 0.75 | 0.578 | Inf | 1 | -0.373 | 1 |
| | Topography2 | top | / | Topography3 | top | 1.236 | 0.258 | Inf | 1 | 1.015 | 0.9998 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Topography2 | top | / | Topography4 | top | 1.107 | 0.235 | Inf | 1 | 0.48 | 1 |
| | Topography2 | top | / | Topography5 | top | 1.045 | 0.213 | Inf | 1 | 0.215 | 1 |
| | Topography3 | top | / | Topography4 | top | 0.896 | 0.19 | Inf | 1 | -0.52 | 1 |
| | Topography3 | top | / | Topography5 | top | 0.845 | 0.173 | Inf | 1 | -0.823 | 1 |
| | Topography4 | top | / | Topography5 | top | 0.944 | 0.196 | Inf | 1 | -0.279 | 1 |
| **Field** | topography2 | corner | / | topography3 | corner | 0.4775 | 0.089 | Inf | 1 | -3.967 | 0.0072 |
| | topography2 | corner | / | topography4 | corner | 5.7025 | 1.59 | Inf | 1 | 6.237 | <.0001 |
| | topography2 | corner | / | topography5 | corner | 0.2639 | 0.0481 | Inf | 1 | -7.316 | <.0001 |
| | topography2 | corner | / | topography2 | middle | 17.25 | 6.82 | Inf | 1 | 7.204 | <.0001 |
| | topography2 | corner | / | topography3 | middle | 7.6667 | 2.26 | Inf | 1 | 6.913 | <.0001 |
| | topography2 | corner | / | topography4 | middle | 125.4545 | 127 | Inf | 1 | 4.755 | 0.0002 |
| | topography2 | corner | / | topography5 | middle | 7.2632 | 2.1 | Inf | 1 | 6.846 | <.0001 |
| | topography2 | corner | / | topography2 | side | 8.625 | 2.64 | Inf | 1 | 7.037 | <.0001 |
| | topography2 | corner | / | topography3 | side | 3.1364 | 0.729 | Inf | 1 | 4.92 | 0.0001 |
| | topography2 | corner | / | topography4 | side | 20.9091 | 9.33 | Inf | 1 | 6.813 | <.0001 |
| | topography2 | corner | / | topography5 | side | 3.6316 | 0.871 | Inf | 1 | 5.375 | <.0001 |
| | topography2 | corner | / | topography2 | top | 15.3333 | 5.79 | Inf | 1 | 7.236 | <.0001 |
| | topography2 | corner | / | topography3 | top | 7.6667 | 2.26 | Inf | 1 | 6.913 | <.0001 |
| | topography2 | corner | / | topography4 | top | 13.9394 | 5.28 | Inf | 1 | 6.954 | <.0001 |
| | topography2 | corner | / | topography5 | top | 138 | 140 | Inf | 1 | 4.852 | 0.0001 |
| | topography3 | corner | / | topography4 | corner | 11.9421 | 3.25 | Inf | 1 | 9.11 | <.0001 |
| | topography3 | corner | / | topography5 | corner | 0.5526 | 0.0947 | Inf | 1 | -3.461 | 0.0447 |
| | topography3 | corner | / | topography2 | middle | 36.125 | 14.1 | Inf | 1 | 9.186 | <.0001 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | topography3 | corner | / | topography3 | middle | 16.0556 | 4.63 | Inf | 1 | 9.635 | <.0001 |
| | topography3 | corner | / | topography4 | middle | 262.7273 | 266 | Inf | 1 | 5.493 | <.0001 |
| | topography3 | corner | / | topography5 | middle | 15.2105 | 4.3 | Inf | 1 | 9.618 | <.0001 |
| | topography3 | corner | / | topography2 | side | 18.0625 | 5.42 | Inf | 1 | 9.648 | <.0001 |
| | topography3 | corner | / | topography3 | side | 6.5682 | 1.47 | Inf | 1 | 8.402 | <.0001 |
| | topography3 | corner | / | topography4 | side | 43.7879 | 19.4 | Inf | 1 | 8.551 | <.0001 |
| | topography3 | corner | / | topography5 | side | 7.6053 | 1.76 | Inf | 1 | 8.749 | <.0001 |
| | topography3 | corner | / | topography2 | top | 32.1111 | 12 | Inf | 1 | 9.319 | <.0001 |
| | topography3 | corner | / | topography3 | top | 16.0556 | 4.63 | Inf | 1 | 9.635 | <.0001 |
| | topography3 | corner | / | topography4 | top | 29.1919 | 10.9 | Inf | 1 | 9.024 | <.0001 |
| | topography3 | corner | / | topography5 | top | 289 | 293 | Inf | 1 | 5.59 | <.0001 |
| | topography4 | corner | / | topography5 | corner | 0.0463 | 0.0125 | Inf | 1 | -11.408 | <.0001 |
| | topography4 | corner | / | topography2 | middle | 3.025 | 1.34 | Inf | 1 | 2.502 | 0.4697 |
| | topography4 | corner | / | topography3 | middle | 1.3444 | 0.478 | Inf | 1 | 0.833 | 1 |
| | topography4 | corner | / | topography4 | middle | 22 | 22.8 | Inf | 1 | 2.986 | 0.1748 |
| | topography4 | corner | / | topography5 | middle | 1.2737 | 0.447 | Inf | 1 | 0.689 | 1 |
| | topography4 | corner | / | topography2 | side | 1.5125 | 0.552 | Inf | 1 | 1.134 | 0.9991 |
| | topography4 | corner | / | topography3 | side | 0.55 | 0.168 | Inf | 1 | -1.956 | 0.8492 |
| | topography4 | corner | / | topography4 | side | 3.6667 | 1.79 | Inf | 1 | 2.66 | 0.3565 |
| | topography4 | corner | / | topography5 | side | 0.6368 | 0.198 | Inf | 1 | -1.449 | 0.9877 |
| | topography4 | corner | / | topography2 | top | 2.6889 | 1.15 | Inf | 1 | 2.32 | 0.6086 |
| | topography4 | corner | / | topography3 | top | 1.3444 | 0.478 | Inf | 1 | 0.833 | 1 |
| | topography4 | corner | / | topography4 | top | 2.4444 | 1.05 | Inf | 1 | 2.09 | 0.7722 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | topography4 | corner | / | topography5 | top | 24.2 | 25 | Inf | 1 | 3.079 | 0.1375 |
| | topography5 | corner | / | topography2 | middle | 65.375 | 25.4 | Inf | 1 | 10.76 | <.0001 |
| | topography5 | corner | / | topography3 | middle | 29.0556 | 8.29 | Inf | 1 | 11.804 | <.0001 |
| | topography5 | corner | / | topography4 | middle | 475.4545 | 482 | Inf | 1 | 6.082 | <.0001 |
| | topography5 | corner | / | topography5 | middle | 27.5263 | 7.71 | Inf | 1 | 11.829 | <.0001 |
| | topography5 | corner | / | topography2 | side | 32.6875 | 9.72 | Inf | 1 | 11.727 | <.0001 |
| | topography5 | corner | / | topography3 | side | 11.8864 | 2.62 | Inf | 1 | 11.224 | <.0001 |
| | topography5 | corner | / | topography4 | side | 79.2424 | 34.9 | Inf | 1 | 9.933 | <.0001 |
| | topography5 | corner | / | topography5 | side | 13.7632 | 3.15 | Inf | 1 | 11.473 | <.0001 |
| | topography5 | corner | / | topography2 | top | 58.1111 | 21.5 | Inf | 1 | 10.974 | <.0001 |
| | topography5 | corner | / | topography3 | top | 29.0556 | 8.29 | Inf | 1 | 11.804 | <.0001 |
| | topography5 | corner | / | topography4 | top | 52.8283 | 19.6 | Inf | 1 | 10.67 | <.0001 |
| | topography5 | corner | / | topography5 | top | 523 | 530 | Inf | 1 | 6.18 | <.0001 |
| | topography2 | middle | / | topography3 | middle | 0.4444 | 0.201 | Inf | 1 | -1.793 | 0.9194 |
| | topography2 | middle | / | topography4 | middle | 7.2727 | 7.8 | Inf | 1 | 1.85 | 0.898 |
| | topography2 | middle | / | topography5 | middle | 0.4211 | 0.189 | Inf | 1 | -1.926 | 0.8641 |
| | topography2 | middle | / | topography2 | side | 0.5 | 0.23 | Inf | 1 | -1.507 | 0.982 |
| | topography2 | middle | / | topography3 | side | 0.1818 | 0.0753 | Inf | 1 | -4.114 | 0.004 |
| | topography2 | middle | / | topography4 | side | 1.2121 | 0.682 | Inf | 1 | 0.342 | 1 |
| | topography2 | middle | / | topography5 | side | 0.2105 | 0.0881 | Inf | 1 | -3.721 | 0.0183 |
| | topography2 | middle | / | topography2 | top | 0.8889 | 0.453 | Inf | 1 | -0.231 | 1 |
| | topography2 | middle | / | topography3 | top | 0.4444 | 0.201 | Inf | 1 | -1.793 | 0.9194 |
| | topography2 | middle | / | topography4 | top | 0.8081 | 0.413 | Inf | 1 | -0.417 | 1 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | topography2 | middle | / | topography5 | top | 8 | 8.58 | Inf | 1 | 1.94 | 0.8574 |
| | topography3 | middle | / | topography4 | middle | 16.3636 | 17 | Inf | 1 | 2.689 | 0.3377 |
| | topography3 | middle | / | topography5 | middle | 0.9474 | 0.344 | Inf | 1 | -0.149 | 1 |
| | topography3 | middle | / | topography2 | side | 1.125 | 0.424 | Inf | 1 | 0.312 | 1 |
| | topography3 | middle | / | topography3 | side | 0.4091 | 0.131 | Inf | 1 | -2.795 | 0.2718 |
| | topography3 | middle | / | topography4 | side | 2.7273 | 1.36 | Inf | 1 | 2.017 | 0.8162 |
| | topography3 | middle | / | topography5 | side | 0.4737 | 0.154 | Inf | 1 | -2.296 | 0.6265 |
| | topography3 | middle | / | topography2 | top | 2 | 0.873 | Inf | 1 | 1.587 | 0.9709 |
| | topography3 | middle | / | topography3 | top | 1 | 0.368 | Inf | 1 | 0 | 1 |
| | topography3 | middle | / | topography4 | top | 1.8182 | 0.796 | Inf | 1 | 1.365 | 0.9933 |
| | topography3 | middle | / | topography5 | top | 18 | 18.7 | Inf | 1 | 2.782 | 0.2793 |
| | topography4 | middle | / | topography5 | middle | 0.0579 | 0.0601 | Inf | 1 | -2.744 | 0.3021 |
| | topography4 | middle | / | topography2 | side | 0.0687 | 0.0717 | Inf | 1 | -2.567 | 0.4219 |
| | topography4 | middle | / | topography3 | side | 0.025 | 0.0256 | Inf | 1 | -3.604 | 0.0278 |
| | topography4 | middle | / | topography4 | side | 0.1667 | 0.182 | Inf | 1 | -1.64 | 0.9611 |
| | topography4 | middle | / | topography5 | side | 0.0289 | 0.0297 | Inf | 1 | -3.454 | 0.0457 |
| | topography4 | middle | / | topography2 | top | 0.1222 | 0.13 | Inf | 1 | -1.972 | 0.8411 |
| | topography4 | middle | / | topography3 | top | 0.0611 | 0.0635 | Inf | 1 | -2.689 | 0.3377 |
| | topography4 | middle | / | topography4 | top | 0.1111 | 0.118 | Inf | 1 | -2.06 | 0.7906 |
| | topography4 | middle | / | topography5 | top | 1.1 | 1.57 | Inf | 1 | 0.067 | 1 |
| | topography5 | middle | / | topography2 | side | 1.1875 | 0.443 | Inf | 1 | 0.461 | 1 |
| | topography5 | middle | / | topography3 | side | 0.4318 | 0.136 | Inf | 1 | -2.664 | 0.354 |
| | topography5 | middle | / | topography4 | side | 2.8788 | 1.42 | Inf | 1 | 2.138 | 0.7402 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | topography5 | middle | / | topography5 | side | 0.5 | 0.16 | Inf | 1 | -2.16 | 0.7253 |
| | topography5 | middle | / | topography2 | top | 2.1111 | 0.915 | Inf | 1 | 1.724 | 0.9408 |
| | topography5 | middle | / | topography3 | top | 1.0556 | 0.384 | Inf | 1 | 0.149 | 1 |
| | topography5 | middle | / | topography4 | top | 1.9192 | 0.834 | Inf | 1 | 1.5 | 0.9828 |
| | topography5 | middle | / | topography5 | top | 19 | 19.7 | Inf | 1 | 2.838 | 0.2474 |
| | topography2 | side | / | topography3 | side | 0.3636 | 0.12 | Inf | 1 | -3.061 | 0.1443 |
| | topography2 | side | / | topography4 | side | 2.4242 | 1.22 | Inf | 1 | 1.756 | 0.9316 |
| | topography2 | side | / | topography5 | side | 0.4211 | 0.141 | Inf | 1 | -2.575 | 0.416 |
| | topography2 | side | / | topography2 | top | 1.7778 | 0.79 | Inf | 1 | 1.294 | 0.9962 |
| | topography2 | side | / | topography3 | top | 0.8889 | 0.335 | Inf | 1 | -0.312 | 1 |
| | topography2 | side | / | topography4 | top | 1.6162 | 0.721 | Inf | 1 | 1.077 | 0.9995 |
| | topography2 | side | / | topography5 | top | 16 | 16.7 | Inf | 1 | 2.66 | 0.3568 |
| | topography3 | side | / | topography4 | side | 6.6667 | 3.09 | Inf | 1 | 4.095 | 0.0043 |
| | topography3 | side | / | topography5 | side | 1.1579 | 0.313 | Inf | 1 | 0.542 | 1 |
| | topography3 | side | / | topography2 | top | 4.8889 | 1.94 | Inf | 1 | 3.994 | 0.0065 |
| | topography3 | side | / | topography3 | top | 2.4444 | 0.782 | Inf | 1 | 2.795 | 0.2718 |
| | topography3 | side | / | topography4 | top | 4.4444 | 1.77 | Inf | 1 | 3.74 | 0.0171 |
| | topography3 | side | / | topography5 | top | 44 | 45 | Inf | 1 | 3.699 | 0.0199 |
| | topography4 | side | / | topography5 | side | 0.1737 | 0.0811 | Inf | 1 | -3.748 | 0.0167 |
| | topography4 | side | / | topography2 | top | 0.7333 | 0.404 | Inf | 1 | -0.563 | 1 |
| | topography4 | side | / | topography3 | top | 0.3667 | 0.182 | Inf | 1 | -2.017 | 0.8162 |
| | topography4 | side | / | topography4 | top | 0.6667 | 0.368 | Inf | 1 | -0.735 | 1 |
| | topography4 | side | / | topography5 | top | 6.6 | 7.21 | Inf | 1 | 1.729 | 0.9396 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | topography5 | side | / | topography2 | top | 4.2222 | 1.7 | Inf | 1 | 3.585 | 0.0296 |
| | topography5 | side | / | topography3 | top | 2.1111 | 0.687 | Inf | 1 | 2.296 | 0.6265 |
| | topography5 | side | / | topography4 | top | 3.8384 | 1.55 | Inf | 1 | 3.335 | 0.0663 |
| | topography5 | side | / | topography5 | top | 38 | 38.9 | Inf | 1 | 3.549 | 0.0334 |
| | topography2 | top | / | topography3 | top | 0.5 | 0.218 | Inf | 1 | -1.587 | 0.9709 |
| | topography2 | top | / | topography4 | top | 0.9091 | 0.452 | Inf | 1 | -0.192 | 1 |
| | topography2 | top | / | topography5 | top | 9 | 9.59 | Inf | 1 | 2.062 | 0.7893 |
| | topography3 | top | / | topography4 | top | 1.8182 | 0.796 | Inf | 1 | 1.365 | 0.9933 |
| | topography3 | top | / | topography5 | Top | 18 | 18.7 | Inf | 1 | 2.782 | 0.2793 |
| | topography4 | top | / | topography5 | Top | 9.9 | 10.6 | Inf | 1 | 2.151 | 0.7319 |

# Field pilot experiment

Prior to the 2024 field experiment a pilot experiment took place in 2023 to test the location and the perpendicular frame. Concrete tiles were created similarly to those used in the main experiment with the same topography treatments (T1-T5). The frame was placed in a the same location as described in the main experiment and was the same design as the perpendicular collector described in the main experiment. Due to many tiles being displaced from the frame the replication ranged from 4- 8 per treatment. Therefore, this experiment was not included in the main analysis.

Statistical analysis

We performed a one-way analysis of variance (ANOVA) with metamorphosis counts as the response variable and topography as the fixed factor. We conducted Tukey's Honestly Significant Difference (HSD) post hoc test to assess pairwise differences between treatments.

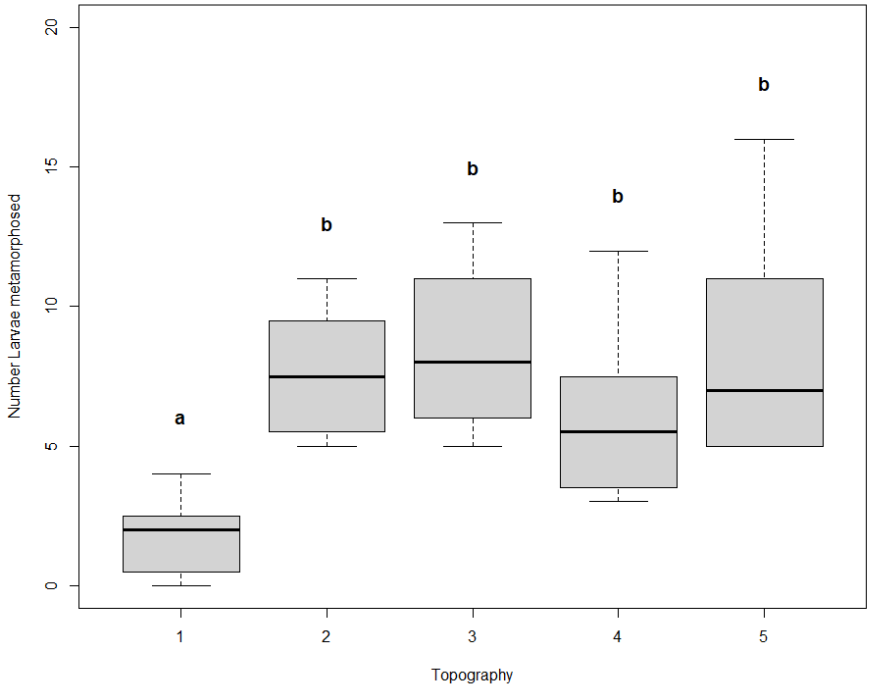


Figure 5.S1. Results of the pilot experiment from summer 2023.

Table 5.S2. the results of the ANOVA for the pilot experiment

| Topography comparison | Difference | Lower (95% CI) | Upper (95% CI) | p-value |
|---|---|---|---|---|
| 2–1 | 5.875 | 1.73 | 10.02 | 0.0022 |
| 3–1 | 6.95 | 3.018 | 10.882 | 0.00012 |
| 4–1 | 4.25 | 0.105 | 8.395 | 0.0422 |
| 5–1 | 6.75 | 2.273 | 11.227 | 0.001 |
| 3–2 | 1.075 | –2.857 | 5.007 | 0.933 |
| 4–2 | –1.625 | –5.770 | 2.52 | 0.7912 |
| 5–2 | 0.875 | –3.602 | 5.352 | 0.9797 |
| 4–3 | –2.700 | –6.632 | 1.232 | 0.2994 |
| 5–3 | –0.200 | –4.481 | 4.081 | 0.9999 |
| 5–4 | 2.5 | –1.977 | 6.977 | 0.504 |

Table 5.S3. 95% confidence intervals and odds Ratios for lab experiments 1 and 2. This has been computed based on Wald estimates.

| | Terms | Odds Ratio | 95% CI (Lower–Upper) | p-value |
|---|---|---|---|---|
| **Experiment 1** | (Intercept) | 0.079 | 0.023 – 0.199 | < 0.001 |
| | Topography 2 | 5.49 | 1.84 – 20.4 | 0.004 |
| | Topography 3 | 3.02 | 0.88 – 12.0 | 0.089 |
| | Topography 4 | 0.226 | 0.011 – 1.59 | 0.19 |
| | Topography 5 | 4.75 | 1.48 – 18.4 | 0.013 |
| | Biofilm 5 | 0.318 | 0.016 – 2.25 | 0.313 |
| | Biofilm 20 | 7.06 | 2.15 – 27.9 | 0.002 |
| | Age 28 | 1.15 | 0.72 – 1.83 | 0.555 |
| | Age 32 | 0.519 | 0.277 – 0.936 | 0.034 |
| | Topography 2 × Biofilm 5 | 1.14 | 0.127 – 25.4 | 0.913 |
| | Topography 3 × Biofilm 5 | 0.343 | 0.011 – 10.7 | 0.497 |
| | Topography 4 × Biofilm 5 | 6.59 | 0.299 – 326.0 | 0.262 |
| | Topography 5 × Biofilm 5 | 0.285 | 0.019 – 7.44 | 0.367 |
| | Topography 2 × Biofilm 20 | 0.175 | 0.036 – 0.753 | 0.023 |
| | Topography 3 × Biofilm 20 | 0.578 | 0.112 – 2.74 | 0.497 |
| | Topography 4 × Biofilm 20 | 3.22 | 0.359 – 71.9 | 0.345 |
| | Topography 5 × Biofilm 20 | 0.155 | 0.029 – 0.742 | 0.023 |

| | | | | |
|---|---|---|---|---|
| | Topography 2 | 4.82 | 2.61 – 9.32 | < 0.001 |
| | Topography 3 | 1.79 | 0.94 – 3.54 | 0.084 |
| | Topography 4 | 4.38 | 2.37 – 8.48 | < 0.001 |
| | Topography 5 | 2.36 | 1.26 – 4.57 | 0.009 |
| | Biofilm 5 | 0.61 | 0.28 – 1.34 | 0.223 |
| | Biofilm 20 | 1.13 | 0.55 – 2.37 | 0.744 |
| | Age 29 | 1.6 | 1.21 – 2.14 | 0.001 |
| | Age 30 | 2.55 | 1.91 – 3.44 | < 0.001 |
| | Topography 2 × Biofilm 5 | 0.75 | 0.29 – 1.98 | 0.556 |
| | Topography 3 × Biofilm 5 | 1.35 | 0.52 – 3.56 | 0.536 |
| | Topography 4 × Biofilm 5 | 0.29 | 0.10 – 0.82 | 0.019 |
| | Topography 5 × Biofilm 5 | 0.88 | 0.34 – 2.29 | 0.799 |
| | Topography 2 × Biofilm 20 | 0.18 | 0.07 – 0.45 | < 0.001 |
| | Topography 3 × Biofilm 20 | 0.73 | 0.28 – 1.88 | 0.518 |
| | Topography 4 × Biofilm 20 | 0.09 | 0.03 – 0.26 | < 0.001 |
| **Experiment 2** | Topography 5 × Biofilm 20 | 0.36 | 0.14 – 0.94 | 0.039 |