

Article

Optimized Screening Methods for Investigation of the Larval Settlement of *Lanice conchilega* on Artificial Substrates

Alice D'Hurlaborde ^{1,2,*}, Alexia Semeraro ¹, Thomas Sterckx ³ and Gert Van Hoey ¹

¹ Department Animal Science / ILVO-marine, ILVO, Institute for Agricultural and Fisheries Research, Ankerstraat 1, 8400 Oostende, Belgium

² International Master of Science in Marine Biological Resources, IMBRSea, Ghent University, Krijgslaan 281/S8, 9000 Ghent, Belgium

³ Department Benelux Division, Dredging International DEME Group, Scheldedijk 30, 2070 Zwijndrecht, Belgium

* Correspondence: alice.dhurlaborde@imbrsea.eu

Abstract: The Belgium sandy coastline is very vulnerable to erosion; therefore, development of sustainable and nature-based coastal protection solutions is important. Enhancing the settlement of the ecosystem engineer *Lanice conchilega* (Pallas, 1766) which stabilises the sediment bed, is a possible solution. In order to enhance larval settlement by artificial substrates in the field, efficient methodologies are required to screen a wide range of artificial substrates and measure how they influence currents and larval settlement. Therefore, in this study, we describe the development of innovative artificial substrate screening methodologies using an optimised recirculating aquaculture system (RAS) by: (1) analysing the capture rate of passively floating plastic particles, (2) measuring current velocity by means of an acoustic doppler velocimeter and (3) monitoring settlement of living *L. conchilega* larvae. Of the eight substrates evaluated, one was proven to significantly enhance the settlement of *L. conchilega*, namely Geotextile 3D knitted fabric with PES knit, PA spacers and wood sticks mounted at a density of 680 sticks/m². The results of this study show that controlled lab conditions, in conjunction with innovative methods, allowed for successful screening of a number of substrates in a short time in terms of their ability to enhance larvae settlement.

Keywords: coastal erosion; restoration; ecosystem engineer; Aulophora; recirculation aquaculture system; acoustic velocity metre; artificial substrate; geotextiles; distribution of particles

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1. Introduction

Coastal areas represent less than 15% of Earth's land surface [1] but host 67% of the world's population [2], as well as 15 of the 20 megacities of the world [1,3]. Increasing anthropogenic activities (e.g., construction of buildings, ports and marinas) have led to intense modification of these areas over the last few decades. In Europe, the result is that 50% of the shoreline has been modified by construction [2].

Under the current climate change scenario, estimates shows that, within the next 50 years, 30% of residences situated on low coastlines and within 200 m of the sea worldwide may be severely affected by property losses due to erosion [4,5]. This number takes a different dimension in Belgium, as 85% of the coastal zone is located below 5 metres TWA of elevation, making it very vulnerable to erosion, especially with the added effect of sea-level rise and an increasing number of storms [6–8].

To face the erosion threat, societies across the world have relied on engineering and hard coastal protection solutions, such as groynes, dykes, breakwaters, jetties or sea walls [9,10]. These solutions are becoming unsuitable due to their costly and constant maintenance requirements, as well as their rigidity to adapt to the increasing erosion risk [5]. Additionally, they alter the natural adaptive capacity of the coastline to the relative sea-

level rise [11]. The world needs intelligent coastal protection strategies that are sustainable, multifunctional and economically viable to help solve immediate and projected coastal erosion and flooding problems [11,12], as well as to enhance ecosystem functioning [13,14]. Mindful of the holistic approach of marine ecosystem services, new solutions based on nature-induced design have been proposed, with the creation and restoration of existing coastal ecosystems, which naturally provide coastal protection and have a capacity for self-repair and recovery [15].

The Coastbuster project allies research and industry to selectively strengthen a part of the Belgium coastline through the use of artificially enhanced biogenic reefs [16]. The common tube-dwelling polychaete *Lanice conchilega* can be considered an ecosystem engineer, colonising intertidal and subtidal sediments to depths of 1900 m [12,17–19]. The physiology, tube structure [20,21] and occurrence of *L. conchilega* aggregations [22,23], as well as their feeding habits [24], have already been described at length. Aggregations of *L. conchilega* can reach densities of thousands of individuals per m² [17,24] and have the ability to alter sediment properties (grain size composition or porosity), modify the hydrodynamic regime [25,26], offer refuge from predation [27], increase the stability of the habitat and oxygen supply [28] and improve the availability of attachment surfaces for larvae and small organisms [29]. Thus, the faunal community has a higher abundance and richness in areas with *L. conchilega* tubes than free bare sand [18,30].

The success of the artificial enhancement of *L. conchilega* aggregation is largely dependent on their life cycle. *L. conchilega* is an iteroparous free spawner [31]. Egg fertilisation happens within the water column and leads to trochophore larvae, which undergo a short benthic transition into the pelagic phase as aulophore larvae [32]. Finally, the aulophore larvae search for a substrate to settle on, which marks the final transition from free pelagic larvae to the sessile benthic juvenile stage [33]. As a result, the key question with respect to artificial enhancement of *L. conchilega* beds that remains partially unanswered is what kind of substrate can trigger the settlement of aulophore larvae. Selected studies have addressed this question through in situ and laboratory assessments of different types of epibenthic holdfast structures, including plastic straws [34], 3D epibenthic structure resembling macroalgae [35], metal tubes [30,36], concrete, polystyrene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polycarbonate (PC) [36], sticks of wood [2,36] and geotextiles [37,38].

These field studies showed that *L. conchilega* larvae can primarily use any epibenthic structure that reduces the near-bottom flow rate as a means of settlement. Enhancing larval settlement is therefore possible, although more research is needed to determine the optimal strategy and artificial substrate design. However, field trials are expensive and time-consuming, and the potential substrate candidates are numerous. Reliable estimations on settlement enhancement and reef formation would require high-frequency sampling performed with a constant methodology over short and long periods in different locations [36,38]. Laboratory conditions allow for the testing of a wide range of innovative substrate solutions in a fast and controlled manner. To optimise this testing, efficient methodologies are required to screen this wide range of artificial substrates and to measure how they influence currents and the ability to capture passively floating particles (larvae). Characterising the influence of the substrate on both hydrodynamics and capture rate is important because velocity can influence settlement [39], and the capture capacity is an essential tool for colonization of defaunated substrates [40]. Arganda-Carreras et al. [37] attempted to develop laboratory experiments to study the settlement of *L. conchilega* larvae on artificial substrates, but no significant preference was found between the tested substrates, emphasising the difficulties in screening substrates using live organisms in a laboratory context.

The objective of this study was to find innovative experimental lab conditions to compare a large range of substrates (eight were tested in this study) in terms of their ability to enhance the settlement of *L. conchilega* larvae. The first experimental method verified the ability of the substrates to trap passively floating plastic particles mimicking larvae

density and size. The second experimental method consisted of measuring, with an acoustic Doppler velocimeter, the ability of a substrate to affect the near-bottom current velocity. Finally, the settlement rate of living larvae of *L. conchilega* was compared for the most promising substrates used in the previous tests.

2. Materials and Methods

2.1. Experimental Setup Design

The experiments were executed in a rearing tank (Figure 1) consisting of a two-compartment closed recirculating aquaculture system (RAS, based on the Kreisel principle [41]). This design has many advantages for rearing planktonic organisms. A laminar flow of water along one of the tank's walls allows a circular flow [42], and its shape avoids aggregation in corners. A fine net (90 μm pore size, Plansifter SEFAR NYTAL® PA, sourced from: SEFAR, Heiden, Switzerland) at the water exiting site protects the organisms from any damaging suction effect [41]. However, previous work [37] revealed that the experimental setup (RAS) needs to be optimised in order to achieve homogenisation of flow conditions. The critical aspects of the setup to be addressed were rearing tanks, inlet pipes, flowmeters and controlled water condition.

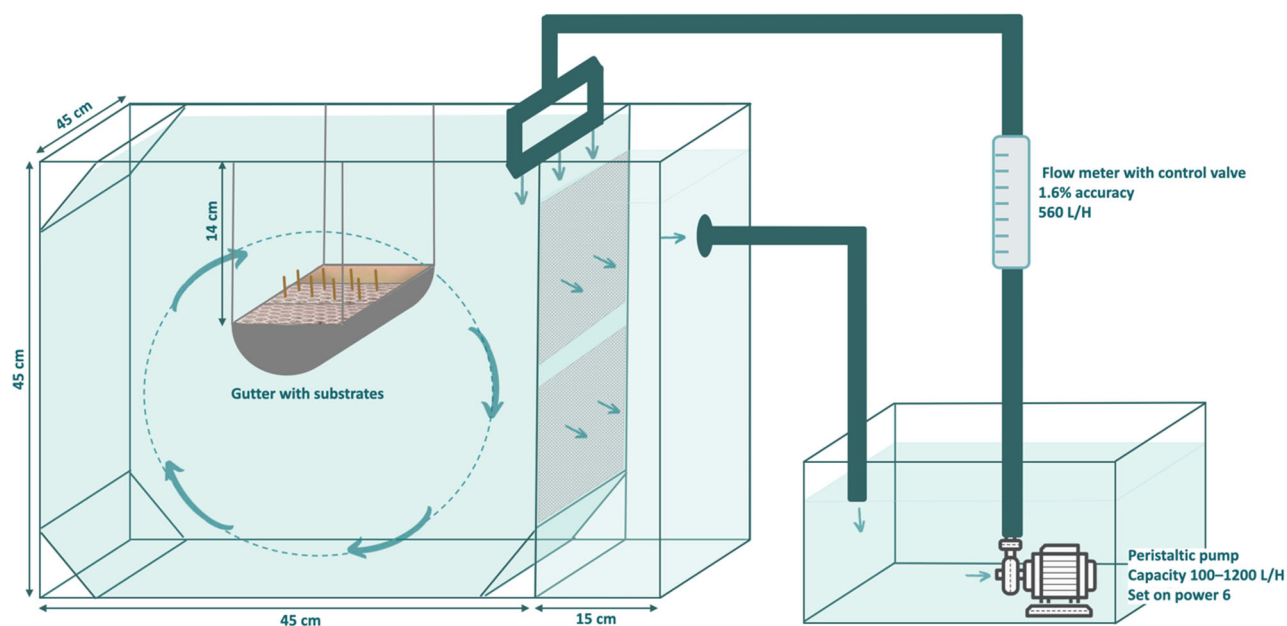


Figure 1. Illustration of the experimental design (larvae settlement experiment) and its components. The arrows represent the direction of water flow.

Observations and hydraulic calculations (pressure difference between the pipe's holes) showed that the shape of the inlet pipe used in previous experiments [37] caused variation in water flow and a lack of repeatability. The optimised water inlet system (Figure 2a) consists of two water entry points on each side of the inlet pipe with 19 3 mm holes distributed every 2 cm along the full length of the tank. The angle of the inlet pipe was carefully adjusted to achieve maximal flow homogeneity. Optimizations were based on observations and empirical trials using picture analysis [43] of the top view of the tanks containing moving plastic pellets (1 mm). The combination of a newly designed inlet pipe and its adjusted position led to suitable homogeneous water distribution in the tank (Figure 2b), providing the best experimental condition for repeatability and reproducibility within the tank and between different tanks.

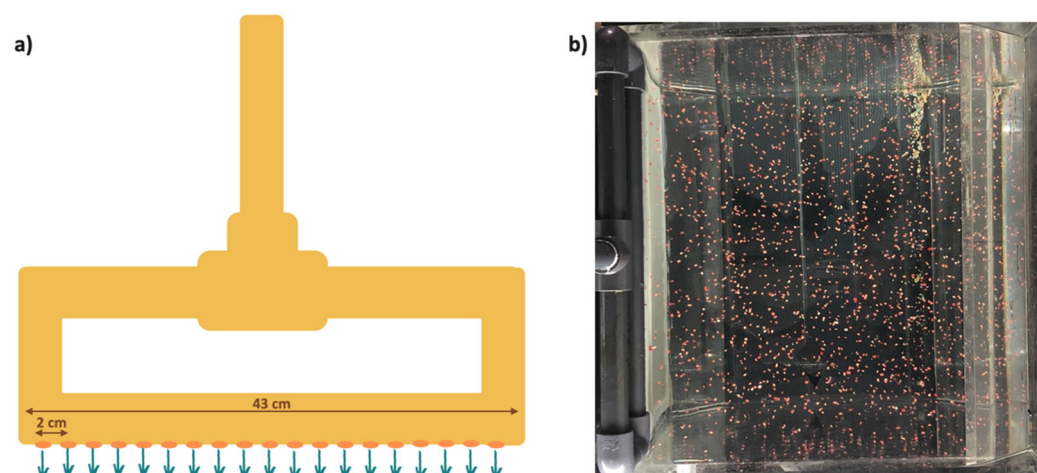


Figure 2. Optimised inlet pipe design (a); top view picture of the tank under optimised conditions (b).

In each tank, the water was pumped by a peristaltic pump (Jecod DCS-1200 pump with controller: 8 power settings, sourced from: Ocean Store, Dordrecht, Nederland) in an enclosed system. The addition of a variable-area flowmeter (Series 2000 TechFluid, 100–1000 L/H, with a reading accuracy of 1.6%, control valve, sourced from: Techfluid, Barcelona, Spain) allowed for standardization of the water injection between tanks and to monitor the flow rate during the length of the study. Optimal flow rate was identified based on the homogenous distribution of moving plastic pellets (shown by picture analysis) and based on flow stability over time. All the experiments presented in the study were performed at a flow rate of 560 L/H corresponding to the pump setting of 5 or 6.

The water quality was monitored during the full length of the study. The mean salinity (± 1 SD) was 34 (salinity refractometer). The temperature was 14 ± 2 °C (thermometer). Continuous bubbling maintained dissolved oxygen (DO) at ~100% saturation (>8 mg/L DO). These parameters were set to match the water conditions of the coast of the North Sea, where *L. conchilega* settlement occurs [44]. The water conditions were kept constant throughout the experiments (with or without living animals).

A gutter for placing the substrates was hung inside the tank at a height of 14 cm from the top of the tank (Figure 1). In the screening tests without living larvae, the gutter was filled with shell fragments ((E) control), and only one artificial substrate was placed in the gutter at a time. In the screening test with living larvae, the gutter was divided into 3 sections with different substrates (Figure 1). The substrates had different structures, thicknesses and weaving and represented three categories: mats, sticks and a combination of mats and sticks (Figure 3). Four types of mats were tested: (A) geotextile (220 g/m² 3D knitted fabric (10 mm thickness) based on PES knit and PA spacers), (B) non-woven geotextile Kena260 black (260 g/m²), (C) 3 layers of non-woven geotextile Kena260 black (260 g/m²) and (D) non-woven geotextile NW170 white (170 g/m²). The mats were provided by Sioen industries. One type of stick was tested: (F) wooden sticks with a diameter of 5 mm and length of 5 cm (out of substrate) and positioned with a density of 680 tubes/m². The sizing of the wood sticks used in this experiment mimicked adult *L. conchilega* [15,45,46]. Two types of mat/stick combinations were tested: (G) substrate A with sticks and (H) substrate B with sticks.

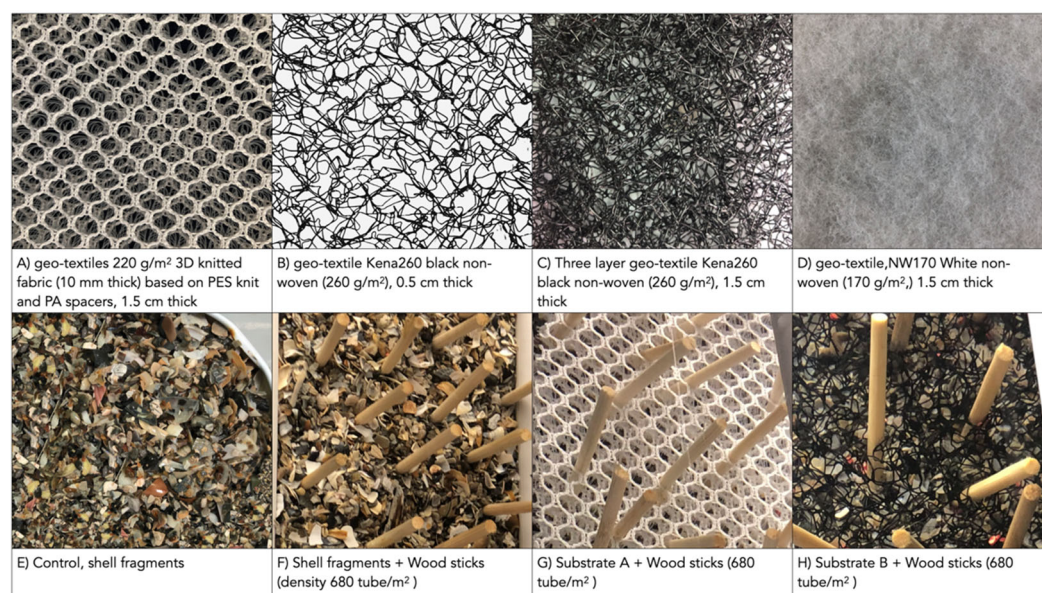


Figure 3. Pictures and descriptions of all artificial substrates tested.

2.2. Artificially Screening Substrates Based on Capture Rate

Artificial particles have been successfully used in the past to study settlement and flow movement [25,35]. In order to choose an artificial particle to mimic *L. conchilega* larvae, we first had to characterise the aulophore in terms of size, shape and density. The aulophore planktonic larval stage measures 2–3 mm [32,47], with a rectangular shape and an estimated density of 1.029 g/m³. The density of the larvae was calculated according the Stoke Law:

$$v = \frac{gd^2(p - m)}{18\mu}$$

where v is the terminal velocity of a spherical particle; g is the gravitational acceleration—for Earth, equal to 9.80665 m/s²; d is the particle diameter; p is the density of the particle; m is the density of the fluid; and μ is the dynamic viscosity of the fluid. The terminal velocity of the particle and the salinity and temperature of the water during the experiment were taken from an article by Bhaud et al. [47]. The density and the dynamic viscosity of the fluid were deduced from the salinity and temperature.

The chosen artificial particles mimicking larvae were red ABS pellets, with a density of 1.05 g/m³, a length of 4 mm and a diameter of 3 mm. They were chopped into smaller pieces around 1/4 of the original size, which made them closer to the aulophore larvae population (in size and heterogeneity). In this study, pellets were quantified by volume using a 25 mL measuring cylinder.

In order to compare the capture properties of the tested substrates, the decrease in the amount of chopped plastic pellets in suspension over time was measured. A known volume of pellets (25 mL) was added to the tank in front of the inlet pipe to ensure quick dispersion. Every 5 min for a period of 20 min, all pellets still in suspension were fished out using a hand net and quantified before being reintroduced in the tank. A linear regression model was applied to the data (volume~time), and the value of the slope was used as a measurement of capture rate (R, version 4.0.2.). Five replicates were performed for each substrate candidate. This protocol was established based on trial runs (Figure 4), showing that maximal pellet capture occurs for all substrates within the first 20 min and that resuspension did not occur, regardless of the substrate. A longer measuring period would be time-consuming, providing no added value.

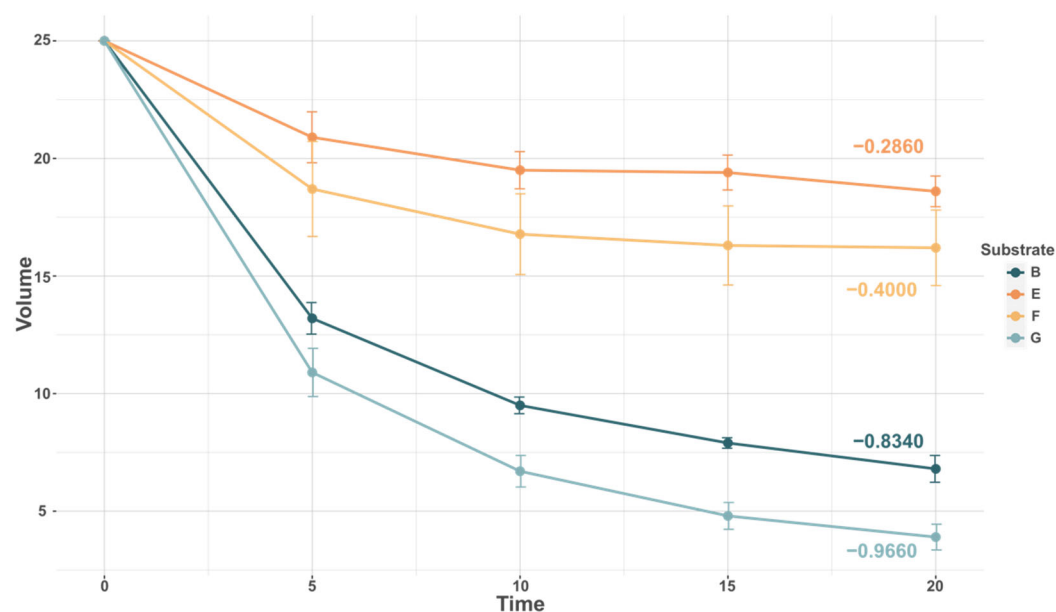


Figure 4. Regression of the floating plastic pellet volume over time due to settlement in the substrate present in the gutter. Four substrate types were tested (B: non-woven geotextile Kena260 black; E: control, shell fragments; F: shell fragments with wood sticks; G: geotextile 3D knitted fabric based on PES knit and PA spacers with wood sticks), with four replicates each. The value of the slope shown in the figure is extracted from a linear regression model.

2.3. Screening Substrates Based on Their Ability to Affect Current Velocity

Previous studies showed that the larval settlement rate is affected by current velocity, as well as bed topography [39], and that the presence of polychaete tubes affects the surrounding current velocity [25]. In this study, we were interested in characterising the effect of different artificial substrates on the overlaying current velocity. An acoustic Doppler velocimeter (ADV) can be used to characterise current velocity in a Kreisel tank [42]. In this study, our hypothesis was that an ADV could be used to measure variation of the velocity by moving the probe over the length of the gutter. A Nortek acoustic Doppler velocity profiler (Nortek Vectrino Profiler, 15 MHz, sourced from: Nortek Scientific Acoustic Development Group Inc., Boston, USA) from Flanders Hydraulics Research was used for the period of the experiment. The Vectrino Profiler sensor is a high-precision instrument that generates a short pulse of sound at a known frequency that is reflected by fine particles dissolved in water. In this study, we used artificial seeding (Polyamide (PA) beads of 60 μm) dispersed in the tank. The ADV was mounted on a tripod, the focus area was defined above the gutter in terms of height and length and always at its centre in terms of width. Each measurement lasted 1 min, with velocity profile readings taken every 0.066 s based on Nortek supplier recommendations [48]. The following data handling process was used for each test. The first step consisted of averaging the magnitude of the X, Y and Z velocity components for each depth and replicate during each measurement. The velocity magnitude (Vmag) in the 3D plane was then calculated for every depth in the sampling volume and replicated using vector addition, $V_{mag} = \sqrt{V_x^2 + V_y^2 + V_z^2}$. Entries with a Vmag values of less than 0.001 m/s were removed from the analysis. Velocity values below the ADV detection threshold of 0.001 m/s are either due to the signal of the bottom (null velocity) or are unreliable measurements. The first and third centimetres of the sampling volume were removed from the analysis to focus on a sampling interval of only 1 cm (highlighted in orange in Figure 5a) in order to avoid the effect of vertical velocity variability present in the Kreisel tank [42]. Finally, a quality check was performed on the data using two parameters: the small noise-to-noise ratio (SNR), with a threshold value of 15 dB; and the correlation, with a threshold value of 70% based on literature recommendations [48–50].

To the best of our knowledge, this was the first time that ADV was used to measure small-current velocity above a gutter in a Kreisel tank. As a result, the optimal measurement condition had to be characterised to ensure an appropriate level of data quality. The height at which the probe was placed above the gutter was carefully identified based on data quality tests. Four height levels were tested: 101, 85, 76, 67 and 54 mm (Figure 6a) with between 3 and 5 replicates for each level (with added replicas in more sensitive positions (101 mm, 85 mm and 54 mm) due to potential bottom and surface disturbance). A quality level was attributed to each velocity measurement of the sampling volume based on the quality check criteria found in the literature (Figure 6b). Data in the category “Off limit” had a $V_{mag} < 0.001\text{m/s}$. Data in the category “Poor quality” had an $\text{SNR} \geq 15$ or a correlation ≥ 70 . Finally, all data deemed of good quality were labelled “Conform”. The gutter was in the sampling volume of the ADV at distances of 54, 67 and 76 mm (highlighted in yellow in Figure 5a), which explains the considerable number of off-limits and poor quality values resulting from the measured null velocities (83, 70 and 50%, respectively). At distances of 101 mm and 85 mm, the quality of the data was more optimal (71 and 78% of conform data, respectively). The probe depth with the least data loss in relation to data quality was a distance of 85 mm from the gutter, which also allowed the velocity to be measured just above the substrate. Further velocity measurements in this study were performed at this depth.

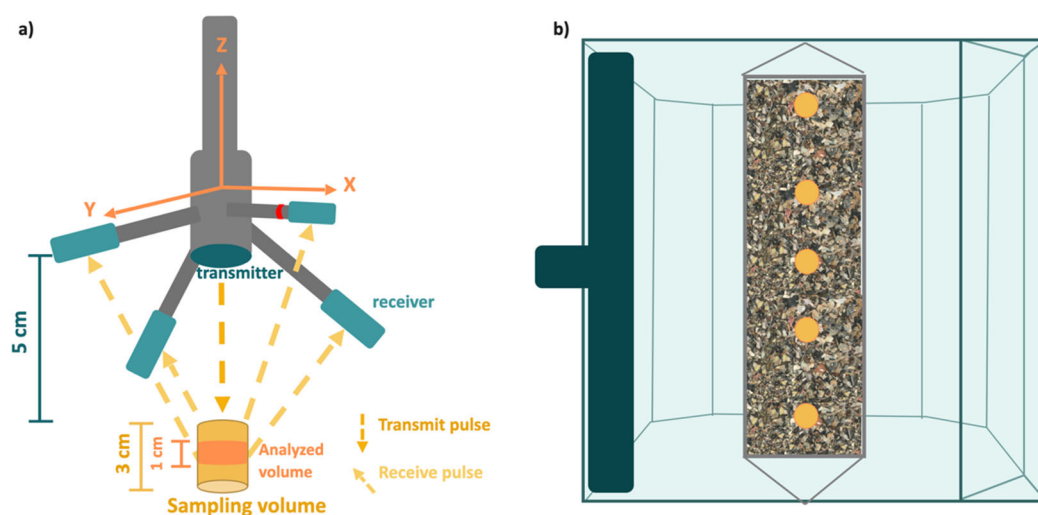


Figure 5. Screening substrates based on their ability to affect current velocity. **(a)** Illustration of Nortek Vectrino profiler probe head with the process of 3 D velocity measurement. The position of the probe sampling volume is highlighted in yellow, and the position of the analysed volume in the screening test is highlighted in orange. **(b)** Top-view illustration of the tank with the five ADV probe positions above the gutter indicated by yellow circles (5, 14, 23, 31 and 40 cm).

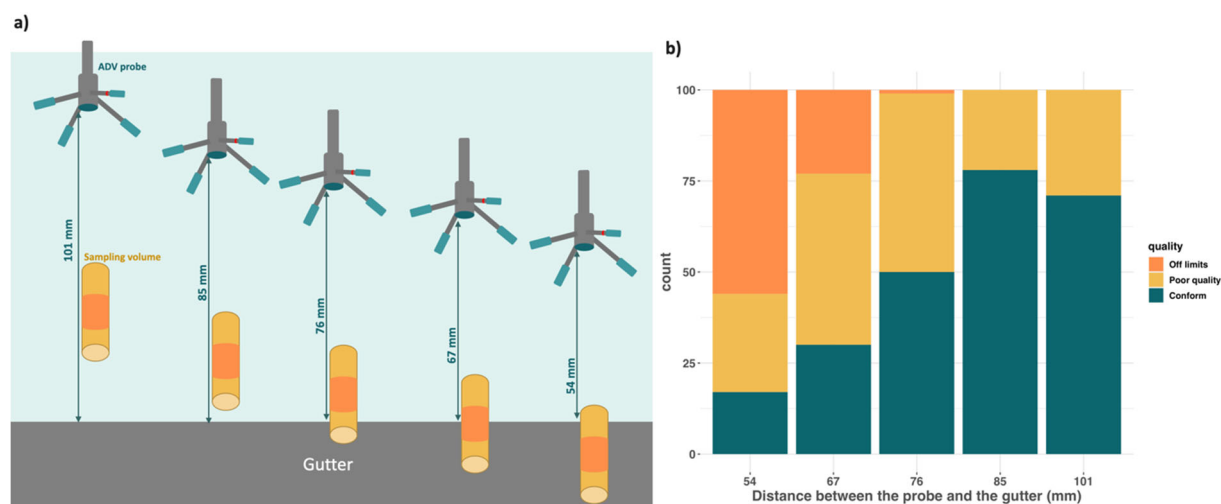


Figure 6. Probe height test. (a) Illustration of the probe position in relation to the gutter and the associated sampling volume (yellow). (b) ADV data quality, expressed in percentage, based on the probe height above the gutter.

The current velocity above the gutter was measured at a fixed height (85 mm) along the length of the gutter at five positions: 5, 14, 23, 31 and 40 cm, with 4 replicates for each position (Figure 5b). Substrate candidates were placed one at a time to cover the whole gutter surface. The average velocity for all positions was calculated for each replicate to obtain an accurate measurement of velocity, taking into account the velocity heterogeneity in the tank.

2.4. Screening Substrates Based on Larval Settlement

L. conchilega is not listed as an endangered or vulnerable species under IUCN, nor named in any international nature conservation legislation or convention. Therefore, no specific licence is required to capture the larvae. All the methodologies carried out in the field and lab work for this project were carefully regulated to ensure minimal disturbance to the animal.

The aulophore larvae were collected in the Belgian part of the North Sea (BPNS) at eight different stations on 12 April 2021 and on 30 April 2021, with the Research Vessel Simon Stevin (Vlaams Instituut voor de Zee (VLIZ)): Nieuwpoortbank 1 (51°013'12"; 20°44'24"), Nieuwpoortbank 2 (51°012'36" ; 20°41'24"), Nieuwpoortbank 3 (51°010'48" ; 20°37'48"), West Diep 1 (51°09'1" ; 20°37'11"), West Diep 2 (51°09'18" 20°38'36"), West Diep 3 (51°09'40" ; 20°40'5"), West Diep 4 (51°010'39" ; 20°39'44") and West Diep 5 (51°011'9" ; 20°39'4"). Aulophore larvae were sampled using a vertical haul WP2 plankton net with 200 µm mesh size. The sample was retrieved from the net and filtered through a 1 mm sieve to remove jellyfish and large organisms and through a 200 µm sieve to reduce the volume of water. The samples were stored in a sealed bucket filled with seawater.

Plankton samples were processed as described in [37]. The buckets with the samples were kept oxygenated until use. The content of each bucket was sorted under a magnifying glass using fractions of 200 mL. Aulophores were identified according to [32] (Figure 7a) and isolated from the sample and moved to an oxygenated 5 L beaker using a pipette.

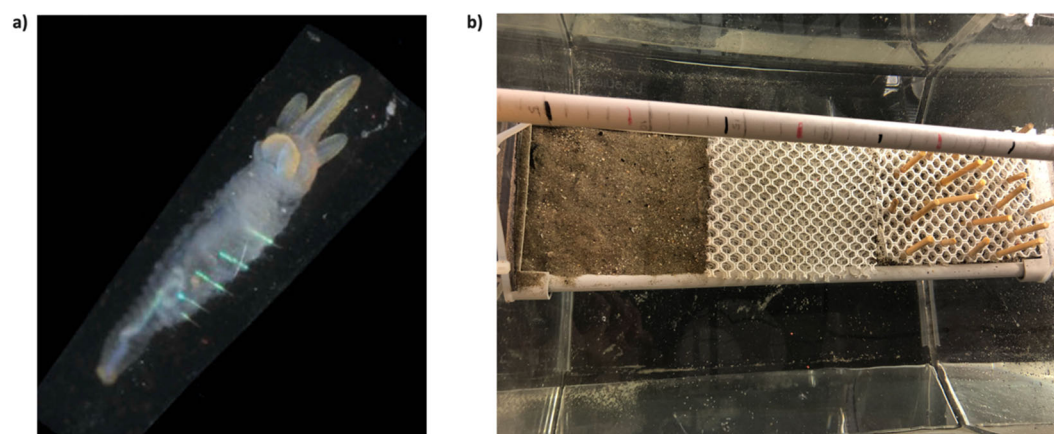


Figure 7. Larvae experiment. (a) *L. conchilega* aulophore; (b) organisation of the 3 substrates in the gutter.

Larvae were introduced in front of the inlet pipe to ensure quick dispersion, and the experiment lasted 10 days. The larvae were fed three times a week with 200 mL of a mixed culture of *Nannocloropsis* and *Tetraselmis* [37,51]. The gutter was filled with fine sand, which is the natural habitat of *L. conchilega* beds [18]. In order to compare the settlement rate of larvae in each substrate, the gutter was divided into 3 sections: control (fine sand), substrate A and substrate G (Figure 7b). Three replicates were performed, and the position of the substrate in the gutter differed for each replicate. The first replicate was performed using the larvae collected on the first sampling day (100 larvae/tank), and the two other replicates were performed using larvae from the second sampling day (80 larvae/tank).

On day 10, the gutter was carefully removed from the tank, and visual observation allowed quantification of aulophores and juveniles in the substrate in the first layer of sand and in the water. The survival rate was quantified for each trial (initial number of individuals/retrieved individuals). Different initial numbers of individuals were introduced into the tanks for the first ($N = 110$) and second ($N = 85$) trials. Therefore, the distribution of individuals (aulophores and juveniles) in each of the substrates was expressed as a percentage of the retrieved population.

2.5. Statistical Tests

A one-way analysis of variance (ANOVA) was conducted to examine the effect of the substrate on each of the screening parameters (catchability, mean velocity and distribution of larvae). Residual analysis was performed to test for the assumptions of the one-way ANOVA. Outliers were assessed by box plot method normality was assessed using the Shapiro–Wilk normality test and homogeneity of variances was assessed by Levene’s test. In the instance of a significant result, one-way ANOVA was followed by a Tuckey HSD test to allow for subsequent pairwise comparison tests.

An additional two-way ANOVA was conducted with a subset of the data (removing substrate D) for two screening parameters: catchability and mean velocity. The assumptions of the two-way ANOVA were met. Consequently, an analysis of simple main effects for the wooden sticks was performed with statistical significance receiving a Bonferroni adjustment. All analyses were performed using R (version 4.0.2).

3. Results

3.1. Screening of Substrate Based on Capture Rate

The benefit of using artificial substrate in the restoration of *L. conchilega* beds lies in its ability to capture pelagic larvae. Thus, the first screening phase of this study targeted the capture property of each substrate (Figure 8). The presence of an artificial substrate

(A, B, C, G or H) in the gutter significantly increased the capture of the particles compared to the control with (F) or without (E) wood sticks ($p < 0.05$ for all substrates). The thickness of the geotextile appeared to be a key factor with respect to increasing the capture rate. A significant difference was found between an identical geotextile tested with different thicknesses (B and C, $p = 2.30 \times 10^{-4}$), and no significant difference was found between two different geotextiles of similar thickness (A and C, $p = 0.99$). Substrate D was removed from the analysis because it was inefficient in capturing pellets (data not shown).

The two-way ANOVA showed a significant effect of the presence/absence of sticks ($p = 6.58 \times 10^{-5}$) and the substrate type ($p < 2 \times 10^{-16}$) on the capture rate. A significant interaction was also shown between the sticks and the substrate ($p = 0.0254$). The analysis of simple main effect for the wooden sticks showed, when the significant threshold was $p < 0.16$, a positive effect of the presence of sticks paired with either the control ($p = 2.7 \times 10^{-5}$), geotextile 3D knitted ($p = 0.098$) or geotextile Kena260 ($p = 0.16$).

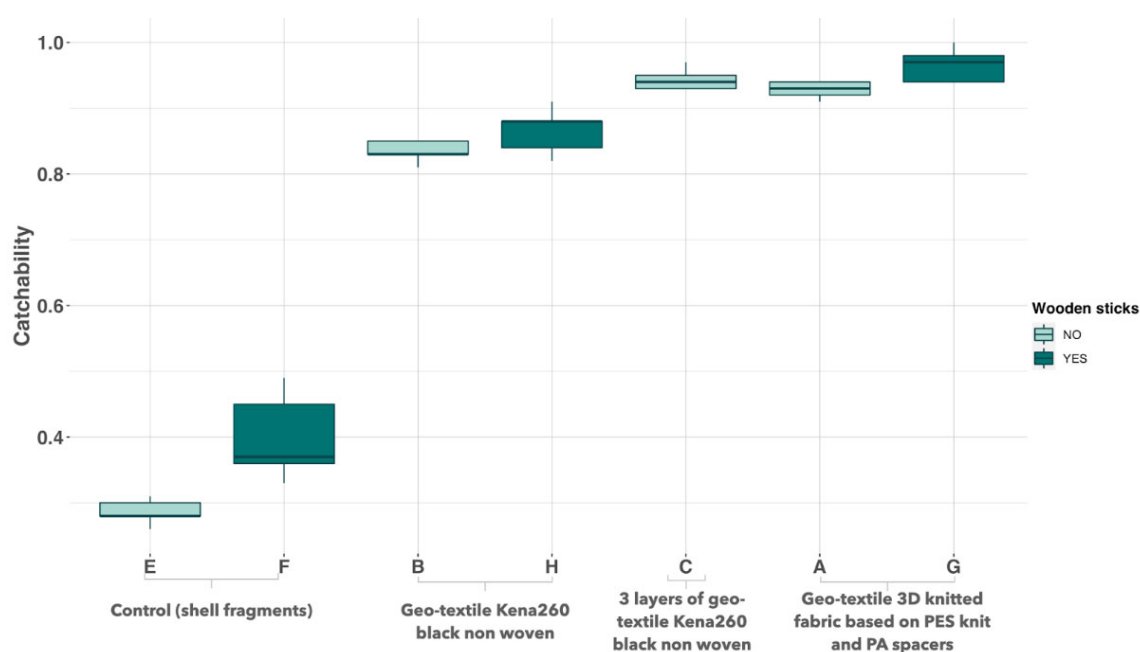


Figure 8. Box plot of the capture properties of each substrate based on plastic pellet catch rate (slope of the linear model volume of particle-time), with four replicates for each treatment. Colour represents the presence/absence of wooden sticks on the substrate.

3.2. Screening of Substrates Based on Velocity

The average flow velocity overlaying the different substrates was measured (Figure 9) and showed significant differences between substrates. The average velocity above the gutter in the presence of wood sticks only (F) was significantly lower than the control (E) and all other tested substrates ($p < 4.92 \times 10^{-4}$). The presence of substrate (G) in the gutter leads to a significant increase in the measured velocity compared to the control (E) ($p < 1.37 \times 10^{-2}$). The substrate (B) also appears to increase the velocity ($p = 5.77 \times 10^{-2}$). The presence of a geotextile ((A) and (C)) does not induce a significant difference in the average velocity above the gutter in comparison to the control.

The two-way ANOVA showed a significant effect of the presence/absence of sticks ($p = 1.07 \times 10^{-8}$) and the substrate type ($p = 1.14 \times 10^{-4}$) on the velocity. A significant interaction was also shown between the sticks and the substrate ($p = 1.23 \times 10^{-5}$). The analysis of simple main effects for the wooden sticks showed significant difference in mean velocity in both the presence and the absence of wooden sticks paired with either control ($p = 1.96 \times 10^{-6}$), geotextile 3D knitted ($p = 2.72 \times 10^{-2}$) and geotextile Kena260 ($p = 8.05 \times 10^{-4}$). The impact of wooden sticks on the velocity depended on the substrate with which it was

paired; a decrease in average velocity is visible for the control and geotextile Kena260, whereas an increase is visible with geotextile 3D knitted.

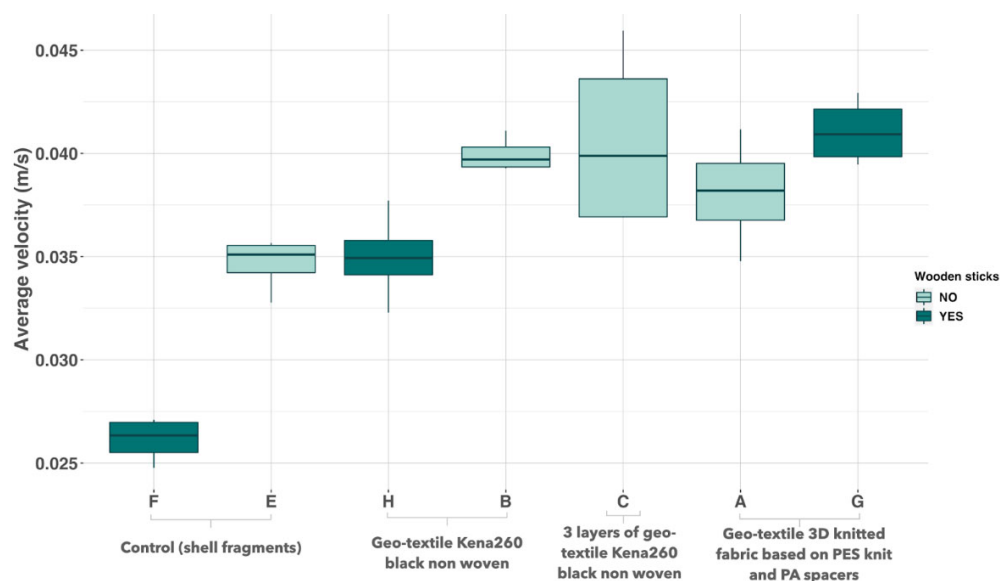


Figure 9. Box plot of the average velocity (m/s) above the gutter containing different substrate types. Four replicates were performed for each treatment, for which the average velocity is based on velocity measurement at five points along a horizontal transect above the gutter (Figure 5b). Colour represents the presence/absence of wooden sticks on the substrate.

3.3. Screening of Substrates Based on Larval Settlement of *L. Conchilega*

The first two screening phases showed that substrate G (geotextile 3D knitted fabric based on PES knit and PA spacers with added wood sticks) significantly differed from the control in terms of capture rate and average velocity; therefore, it was selected for the *L. conchilega* experiment and compared with substrate A (geotextile 3D knitted fabric based on PES knit and PA spacers without added wood sticks) and substrate E (fine sand), the control.

Living aulophore larvae were added to tanks containing a gutter with substrates G, A and E in parallel (Figure 10). After 10 days, the average mortality was $88.4\% \pm 1.8$. Among the accounted alive individuals, a few were found outside of the substrates (residuals: juveniles = 11.9 ± 11.2 ; aulophores = 19.3 ± 5.1). For both life stages of *L. conchilega*, no significant difference was shown between the control and the residual ($p = 0.28$). The distribution of the juveniles was significantly higher ($p < 5.08 \times 10^{-3}$) in substrate G (58.5 ± 7.1) and substrate A (29.6 ± 6.4) compared to the control (no juveniles in natural substrate). Wooden sticks in substrate G captured almost twice as many juveniles as substrate A ($p = 5.91 \times 10^{-3}$). The same trend was observed with the aulophores, although not significant: G (18.5 ± 23.1) > A (7.4 ± 12.8) > control (2.2 ± 3.8).

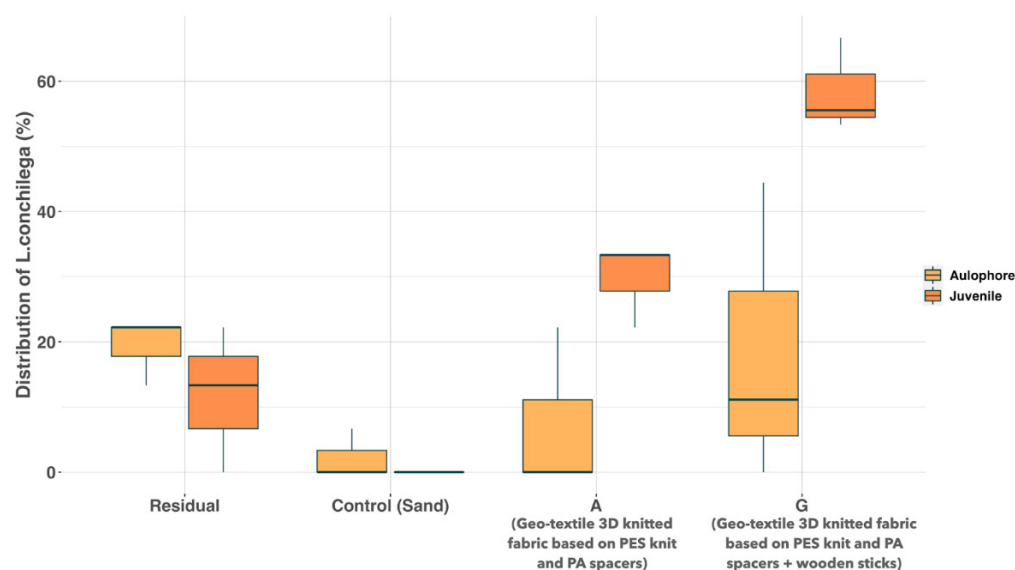


Figure 10. Box plot of distribution of *L. conchilega* juveniles (dark orange) and aulophores (light orange) in each fraction of the gutter and in the residual (water column and plastic part of the gutter). Three replicates were performed for each treatment. The distribution of individuals in each of the substrates is expressed as a percentage of the retrieved population.

4. Discussion

The objective of this study was to find innovative experimental lab conditions to compare the accuracy a wide range of substrates in terms of their ability to enhance the settlement of *L. conchilega* larvae. As field experiments are costly and time-consuming, optimal lab procedures should be developed to allow for fast and accurate screening of artificial substrates in terms of a variety of criteria, including tests with living animals. In this study, the experimental design was optimised to ensure adequate water mixing and stable flow conditions for different experiments. These conditions were not met in a previous study using the same RAS system, preventing accurate ranking of substrates [37]. The conjunction of the new design of the inlet pipe and the precise control of the water supply rate was critical in the obtention of homogeneous flow conditions in the different tanks. This design allowed for successful testing of living larvae settlement behaviour with different substrates. The optimised RAS system was also used to develop two new methods to characterise the substrates in terms of their ability to trap passively floating particles and measure their effect on overlaying current velocity.

4.1. Methodological Development

Characterising substrates in terms of their particle capture capacity is important, as they are essential tools for colonisation of defaunated substrates [40]. The method (kinetics of the number of floating particles) developed in this study allowed for accurate measurement of substrate capture properties in a relatively short amount of time, enabling successful characterisation and ranking of the tested substrates. This method could be applied to investigate the capture properties of a substrate when facing fluctuating flow rate conditions or varying particle concentrations in the water column. Improvements could still be made through the replacement of manual pellet quantification with imagery techniques, specifically multiple particle tracking (MPT) [52], which would enable a higher number of replicates and test capacity.

Characterising the influence of the substrates on hydrodynamics is important because velocity can influence settlement in two ways: the encounter rate and the attachment rate [39]. In this study, we demonstrated, for the first time, the relevance of using an ADV to measure velocity in a Kreisel tank. Nevertheless, four challenges were encountered in the use of the ADV in our system.

The first challenge concerned the density of suspended particles in the water column. Several studies demonstrate recurrent problems associated with a lack of suspended particles, leading to weak ADV signals (low correlation and low noise-to-noise ratio) [53,54]. To counteract this, in the present study, seeding was performed regularly in the main compartment in order to preserve the data quality standards.

The second challenge is associated with the probe position in terms of height. It was important to avoid impact of vertical heterogeneity above the gutter due to the circular flow pattern of the Kreisel tank [42]; secondly, the probe need to be placed as far as possible from the solid boundary layer [50]. As a result, the probe was positioned at 85 mm above the gutter to ensure optimal data quality. We tested three substrate thicknesses: 0, 5 and 15 mm. The analysed sampling volume, located 15–25 mm above the gutter, was therefore closer to the surface of the thicker substrates, which might have had an effect on the results. Further experiments should consider this parameter.

The third challenge concerned data analysis. In this study, the ADV data were converted to ASCII format, which allowed for analysis in R, which is an appropriate way to achieve the output needed in this study. Nevertheless, the use of the MAT format, the other ADV output format, should be considered if more in-depth data analyses are needed [49]. The fourth challenge concerned the measured velocity range. Our velocity measurement oscillated between 0.0011 m.s^{-1} and 0.0780 m.s^{-1} , which is close to the ADV detection limit and low in comparison to other studies measuring flow velocity under lab conditions, in which the velocity oscillated between 0.01 m.s^{-1} and 0.25 m.s^{-1} [25,35,36,40]. Comparison with another method (such as laser Doppler velocimetry (LDV)) could be an interesting way to definitively validate the use of the ADV in low flow conditions [55], as the present study was conducted close to the ADV detection limit. The use of ADV to efficiently compare substrates should be further investigated, especially to define turbulent motion around the substrates. Nevertheless, the use of an ADV is a cost-efficient, fast way to get a rough insight into flow velocity changes caused by different substrates and an appropriate technique for rapid screening of multiple substrates. In conclusion, the laboratory setup and methods used in this study could be applied to other substrates, as well as different types of planktonic larvae to test capture rates and changes in flow velocities.

The aim of the present study was to test the retention efficiency of substrates with living specimens as a necessary step before recommending one particular substrate for in situ deployment. Therefore, we opted to perform tests with *L. conchilega* as a means to validate the hypothesis emerging from results obtained in the previous ranking tests. Thicker substrates with loose mesh size and 3D structure were found to be suitable candidates to enhance larval settlement. Nevertheless, working with real organisms is not straightforward, as many aspects influence success. First, the process of catching and sorting larvae did not allow for the collection of a large number of individuals and affected the choice of the number of individuals per tank. Second, *L. conchilega* larvae are very fragile organisms with a naturally high mortality rate [56], and their maintenance under lab conditions is a complex process, as illustrated by high mortality during the sorting and experimental period, as similarly reported in a previous study [37]. During our sampling, the abundance per sample was rather low compared to that reported in [37], resulting in a longer sorting time (up to 2 days). Additionally, the high mortality observed during the handling of the samples was probably due to lower oxygen levels combined with the dense presence of microalgae in the samples. Important algae blooms are frequent during the month of April in the North Sea [57], and high chlorophyll-a concentrations were measured close to the Belgian coastline on both sampling days (European Organisation for the Exploitation of Meteorological Satellites, OLCI Level 2 CHL Concentration Daily Accumulated—Sentinel-3, on 11 April 2021 and 30 April 2021). Ideally, the sorting and start of the experiment should be done on the sampling day itself. The practical difficulties encountered during this work led to only three replicates with one performed 18 days earlier than the two others. The larvae of the second sampling campaign were, on average,

larger in size, possibly indicating a difference in maturity and stage of the larvae [32], which could have an effect on their ability to settle [58]. Despite these practical challenges, a test with living animals is an important part of the substrate screening process.

4.2. Substrate Ranking

A strong understanding of the key parameters allowing for optimal settlement of larvae is essential for a successful restoration plan.

In this study, the control (shells or sand) had a pellet capture rate close to zero, with an average overlaying velocity of 3.46 cm/s, and no juveniles were found, suggesting that larval settlement on bare sand is unlikely to occur. These results are supported by the abundant literature describing the weak settlement of larvae on bare sand in comparison to artificial substrate, dead shells or adult tubes [2,30,34,36].

If the absence of a substrate is not an option, some substrates can also be easily rejected. The high density of the weave of the geotextile NW170 White non-woven (D) made it ineffective in terms of particle capture, as also observed in a previous study with living larvae [37]. Additionally, a dense mesh substrate could lead to anoxic conditions [38]. The mat density has to allow the particles/aulophore larvae to pass through. The identical capture rates of substrates A and B (with differing mesh structures: geotextile 220 g/m² 3D knitted fabric (10 mm thick) based on PES knit and PA spacers and geotextile Kena260 black non-woven (260 g/m²), respectively), showed that as long as the mesh size allows particles/aulophore larvae to pass through it, the substrate can be considered an artificial substrate candidate.

Our comparative study showed that one of the most discriminant criteria was the three-dimensional (3D) aspect of the substrate. This 3D aspect can be expressed on two different levels: the thickness of the geotextile and the presence of wood sticks. Our results showed that the geotextile's thickness is positively related to the pellet capture rate. Thicker geotextiles (1.5 cm), as well as multiple layers of a thin geotextile (3 × 0.5 cm), showed a significantly higher capture rate. In nature, adult tubes provide the 3D aspect of reefs, with a diameter of 5 mm and a length (out of sediment) that can reach 4 cm [15,45,46]. In this study, wooden sticks were used as a mimic. Our results showed that the addition of sticks increased the capture rate in all cases, confirming the results of previous studies [2,36]. As a result, elevation, created either by thickness or by sticks, seems to be a key factor. The best performance was observed with substrate G (1.5 cm thick + 4 cm wooden sticks), also achieving the highest capture rate, velocity and juvenile settlement rate. This 3D selection process is in accordance with the natural environment, as elevation relative to surrounding sediment is a characteristic of *L. conchilega* beds, and the high density of individuals influences hydrodynamics and can lead to seabed elevation from 10 to 40 cm [18,59,60].

Our comparative study showed that another discriminant criterion is the ability to create an appropriate hydrodynamic regime surrounding the area of intended settlement. The velocity measured above the substrate combined with wood sticks showed a significant reduction in the average velocity in the case of the control and the geotextile Kena260. This result is supported by the literature; the flow perturbation induced by the presence of adult tubes (or sticks) creates areas of reduced flow velocity and chaotic motion, enhancing the capture of particles [25,40]. The opposite result was observed with geotextiles, which tended to increase the overlaying flow velocity. The increase in velocity could be due to a change in the bed roughness [61]; however, further research is needed to validate this hypothesis. In the context of a turbulent system, this increase in velocity would lead to an increase in encounter rate between the pellet/larvae and the substrate [39], promoting a higher pellet/larvae capture rate. However, high velocity could also have a negative impact on the attachment rate of the pellet/larvae via mobilising forces (drag, lift and acceleration reaction [39]), which have to be considered but could not be studied in the context of this work. Along the Belgian coastline, the presence of sand mason worm aggregates is higher on the lee side of sand banks than on the exposed side [59]. The exposed

side is characterised by an increase in near-bed velocities and a decrease in turbulence, while the lee side is characterised by reversed flow with high turbulence intensity [62]. These field observations substantiate our hypothesis of a higher settlement rate in turbulent systems.

In our study, 30 wooden sticks were placed in the gutter for substrates F, G and H, which represents a density of 680 tubes/m². The natural presence of *L. conchilega* in a density superior to 500 individuals/m² can be considered as an aggregation [36]. Further studies are required to optimise the density of sticks and better understand the interaction between the geotextile and the sticks. Higher flow disturbance and settlement rates of larvae with higher densities (>1000 tubes/m²) have been reported in previous studies [15,25,40]. Based on our results, we hypothesise that the presence of tube-like structures in addition to a geotextile could counterbalance the negative effect of velocity on attachment rate by locally reducing the flow velocity and increasing turbulence in a higher water layer while maintaining an optimal encounter rate.

The substrate combining the geotextile 3D knitted fabric based on PES knit and PA spacers with wood sticks (G) achieved the most promising results in all the substrate screening tests. It had the highest capture rate, with a significant increase in velocity (compared to control) and the highest presence of juveniles after 10 days. These results raise the question of the role of behaviour in the settlement of larvae. The addition of wooden sticks to the substrate considerably increased the settlement of larvae and their growth into juveniles in the experiment with living animals. Previous studies proved the hydrodynamic influence of polychaete adult tubes (or mimics, such as wooden tubes) on particle capture [25,40], with larvae observed to use the tube structure to attach and crawl down to settle in the sand. We hypothesised that substrate G would perform better because it combines the effects of a potential increase in flow velocity by the geotextile, which increases the encounter rate and the potential local reduction in velocity, and behavioural advantage due to the wood sticks, which increases the attachment rate. An additional test comparing the settlement rate of the larvae between substrate F (control + sticks) and substrate G (3D knitted mat + sticks) would further validate the positive effect of the combination of geotextile and wood sticks. In this study, one geotextile type was tested with the larvae (with and without sticks). However, geotextile Kena260 black non-woven (C) obtained a similar capture rate of pellet as the 3D knitted mat (A), with a similar effect on hydrodynamics. A comparison of the settlement rate of these two geotextiles would provide information on the possible effect of behaviour on the choice of geotextile.

5. Conclusions and Future Prospects

This work provides encouraging results with respect to the use of plastic pellet capture rate and ADV measurement as a low-cost and efficient methodology to screen artificial substrates for the enhancement of *L. conchilega* settlement. We also optimised an experimental design [37], which can be used for artificial substrate screening in a time- and cost-efficient way. We provided a list of recommendations concerning key characteristics to take into account for the development of an appropriate artificial substrate for field application. The optimal artificial substrate should have a specific structure, including a mat-type base with a loose mesh size (>5 mm diameter) and a relatively high thickness (>1.5 cm) or/and a tube-like structure rising above the mat (>5 cm length and ~5 mm diameter). This optimal artificial substrate should be able to create a dynamic flow, increasing the encounter rate between larvae and the substrate while also locally reducing the velocity in order to favour the attachment rate of the larvae on the substrate. The methodology developed in the context of this work will allow for further testing of innovative substrates in order to complete this list of characteristics to allow for the production/engineering of an adequate substrate in the form of a biodegradable material [63] that is non-destructive for the future established reef.

The efficiency of an optimal artificial substrate when facing the stress of natural hydrodynamic conditions, such as waves or tides, should be tested in a larger-scale

laboratory design (e.g., flume tanks to allow for the description of turbulent currents above the substrate with varying flow velocities and wave conditions [64], as well as the optimal anchoring of the substrate. Finally, laboratory conditions would allow for the study of settlement enhancement in future ocean conditions (physical and environmental), which will make settlement even more challenging for species with planktotrophic, calcifying or weakly swimming larvae with specialised adult habitat [65]. However it is impossible to fully reproduce natural conditions in laboratory experiments, risking results with low ecological validity. Therefore, the small-scale behaviours observed and theorised under laboratory conditions must be validated within the larger, more dynamic framework of the Belgian coastal ecosystem. Under such conditions, much larger-scale hydrodynamic mechanisms strongly impact the settlement of pelagic larvae. These mechanisms can include waves, currents, fronts, coastal boundary layers and tides [64,66]. Therefore, field trials are key, as they allow for the testing of the substrates under natural conditions, with no alteration of variables and external influence on the environment. Previous field trials have elaborated on the specific techniques used to placing substrates in the field at the low waterline according to specific design to keep the substrates as close as possible to the sediment [2,34–36,38]. Preliminary trials using the same 3D geotextiles used in this study reported interesting results, opening the possibility of effectively applying this technique in the field [38].

Therefore, the research process of finding optimal substrates should follow a three-step approach. First, the capture rate and effect on current velocity of a large number of substrates can be tested with small lab experiments, such as those described in this study. In a secondary step, promising candidates can be further tested under larger-scale lab conditions to verify their performance under various hydrodynamic conditions in order to better simulate natural situations. Finally, field tests are necessary to validate the tested substrate in practice for application on an industrial scale.

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