Bachelor project

Using juvenile flatfish as indicators of sea bed quality – in connection with a citizen science habitat restoration project

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1 Abstract

In this project the possibility of using juvenile flatfish as bio-indicators of seabed quality in coastal zones, and if nursery habitats can be identified in terms of sediment grain size, -colour, and hydrogen sulphide were investigated. This was done as the coastal zones are habitat for many different species such as flatfish. However, habitat loss caused by environmental disturbances is a major challenge in large parts of the world, including Denmark. One environmental disturbance is an increase in the natural production of hydrogen sulphide which is lethal to oxygen respiring organisms. The production of hydrogen sulphide occurs when high amounts of dissolved organic matter is degraded in the seabed. In this project juvenile flounder (*Platichthys flesus*) and plaice (*Pleuronectes platessa*) were used in a bio-indicator experiment to investigate the behavioural responses of juvenile flatfish when exposed to sand containing hydrogen sulphide. Based on the conducted experiment, it was concluded that under the tested experimental conditions, the juvenile flatfish did not show any change in behavioural response when exposed to sand containing hydrogen sulphide. Only 2 out of 20 juvenile flatfish chose to flee from the sand containing hydrogen sulphide. Furthermore, oxygen profiles were measured in the sand containing hydrogen sulphide both in the morning and in the afternoon which was done to investigate if a change in the amount of hydrogen sulphide had occurred with time. The measured profiles showed that the amount of dissolved oxygen depleted rapidly in a depth from 1,400 μm to 8,000 μm in the morning and in a depth from 1,400 μm to 8,400 μm in the afternoon. Furthermore, core samples of the sand containing hydrogen sulphide were collected and analyzed for iron. The ratio between Fe^{+2} and Fe^{+3} was measured and it showed that the sand containing hydrogen sulphide was dominated by Fe^{+2} which means that the sand was reduced and therefore a production of hydrogen sulphide was present. Prior to the performed bio-indicator experiment, two pre-experiments were conducted to investigate the preference of substrate grain size and -colour for juvenile flatfish which allowed for a differentiation between behavioural responses caused by hydrogen sulphide and behavioural responses caused by grain sizes and colours. The results from the conducted grain size experiment showed that juvenile flatfish had a preference for the finest sediment when given a choice. Furthermore, no clear preference between black- and white sand and black- and light grey plastic sheets was found from the conducted colour selection experiment. Due to the conducted bio-indicator experiment and the two pre-experiments, it was concluded that in terms of grain size juvenile flatfish can be considered to be a possible bio-indicator as they showed clear behavioural responses to sediment changes. Further, it was concluded that since the juvenile flatfish did not show any behavioural responses to the tested colours they cannot be considered as a bio-indicator in terms of colour. From the bio-indicator experiment, it was concluded that juvenile flatfish is considered as a poor bio-indicator when exposed to concentrations of Fe^{+2} that is 80.987 times greater than Fe^{+3} or below, as they did not show any change in behaviour even when exposed to higher amounts of hydrogen sulphide compared to the amounts found in Bellevue Beach where they were caught. Further, a suggestion was made to investigate the effect of a longer time frame for each trial and to examine the maximum tolerance of hydrogen sulphide for juvenile flatfish which would allow for a more adequate conclusion on the possibility of using juvenile flatfish as bio-indicators. Lastly, Horsens Fjord was investigated to examine if it could be a potential nursery area for juvenile flatfish. However, it is unclear whether Horsens Fjord could be a suitable habitat for juvenile flatfish, as the sand from Horsens Fjord had larger grain sizes than the sand from Bellevue Beach which was anticipated to be a nursery area, and due to the fact that the collected core samples from Horsens Fjord could not be analyzed for iron.

2 Preface

This report is a result of a 20 ECTS points bachelor project at the Technical University of Denmark (DTU). The project ran from 1st of February to 17th of May and was main supervised by Mikael van Deurs from the National Institute of Aquatic Resources (DTU Aqua) and co-supervised by Jane Behrens, Ole Henriksen, and Fletcher Thompson from DTU Aqua. Furtermore, the project was co-supervised by Ronnie Glud from the University of Southern Denmark (SDU). The theme of the report is self-chosen within the area of environmental engineering and all members of the group contributed equally to the work done in the project and in the writing of the this report. The report is written by two engineering students on their 6th semester of the Bachelor of Science in Environmental Engineering:

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We wish to thank the supervisors for their time and help during the project. Furthermore, a big thanks to Annie Glud (SDU) for the assistance in the laboratory. Lastly, thanks to Erik Haar Nielsen and Jan Karnøe from the project Kysthjælper for help and guidance during field work in Horsens Fjord.

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3 Introduction

Habitat loss is a major challenge in the coastal zone in large parts of the world, including Danish waters and the adjacent Baltic Sea, causing environmental disturbances (Findling-Rottem, 2021). Denmark is surrounded by sea and has a coastline of around 7,300 km which is divided into 109 different coastal zones (Jensen, 2022), however only five are defined as being in a good ecological state (Findling-Rottem, 2021). Different organizations and agencies are already focusing on eliminating and mitigating these issues, and frameworks have been put in place, in order to obtain a healthier marine environment. These range from large EU based organs, such as the EU Water Framework Directive to smaller national interest groups, such as the Danish Association for Sport Fishing (DASF) that recently started the project Kysthjælper, with the intentions of bringing together volunteer human resources to give the sea a push in the right direction (Sportsfiskerforbund, 2021).

Examples of environmental disturbances in the coastal zones are overfishing, sand extraction, shipping, and discharge of nutrients from land. The latter can cause an increase in the natural production of hydrogen sulphide in the seabed as the nutrients will result in an excessive bloom of biomass resulting in an increase in the amount of dissolved organic matter (DOM) in the seabed (Madigan et al., 2019). The production of hydrogen sulphide due to the degradation of DOM occurs in anoxic conditions and will result in a darkening of the sediment (Moodley et al., 1998; Christensen et al., 2002).

In some locations a combination of hydrogen sulphide in the seabed and low oxygen concentrations in the bottom water can lead to formation of the bacteria *Beggiatoa* covering the top layer of the seabed. In the most severe cases, the production of hydrogen sulphide can lead to the release of free sulphur to the water column which is lethal for fish and invertebrates (Christensen et al., 2002). However, even before the formation of *Beggiatoa* on the sediment surface, the ongoing production of hydrogen sulphide within the sediment may still disturb the cryptic fish species that hide in the surface layer of the sediment. For example juvenile flatfish (flounder, plaice; *Platichthys flesus, Pleuronectes platessa*) may be sensitive to an increased production of hydrogen sulphide since they rely on the sand to hide from predators. As described above, the hydrogen sulphide production takes place only in anoxic conditions but research has shown that in most sediments oxygen is fully depleted just a few millimetres into the sediment (Christensen et al., 2002). Hence, since juvenile flatfish perform cutaneous respiration (Burton, 2002), they might be exposed to hydrogen sulphide. Alternatively, flatfish may have evolved natural behavioural responses to avoid getting caught in areas with potentially lethal levels of hydrogen sulphide reaching the sediment surface.

In this project the possibility of using juvenile flatfish as bio-indicators of seabed quality in coastal zones is investigated. A bio-indicator is defined as an organism that reflects the state of the environment and indicates the impact of environmental changes on a given habitat (McGeoch et al., 2002; Kuklina et al., 2013). A bio-indicator experiment was conducted to investigate the behavioural responses of juvenile flatfish (plaice and flounder) when exposed to sediment containing hydrogen sulphide. This has not been investigated before in previous studies. The behaviour of the flatfish was observed by pictures taken with a GoPro camera, and the final choice of sediment with and without hydrogen sulphide was recorded. The hypothesis was that juvenile flatfish can sense the presence of hydrogen sulphide in the sediment and that a change in their behaviour would occur.

Further, it is investigated if nursery habitats for juvenile flatfish can be identified in terms of sediment grain size, sediment colour, and hydrogen sulphide which is done to pin-point potential nursery habitats for juvenile flatfish. Therefore, prior to testing the response to hydrogen sulphide, the habitat type preferred by the two species studied were identified. As described by Moles and Norcross (1995) and Gibson and Robb (2000), especially the grain size of the sediment can determine the distribution of good potential nursery areas. Furthermore, based on the fact that the hydrogen sulphide production dyes the sediment black, the distribution might also be influenced by the sediment colour. The influence of sediment colour on juvenile flatfish was investigated in a study by Ryer et al. (2008). Therefore, knowledge about grain size and colour preference allows for a differentiation between behavioural responses caused by hydrogen sulphide and behavioural responses caused by grain sizes and colours. To make this differentiation, two simple experiments were performed, testing the effect of sediment in terms of colour and grain size. Based on the results obtained by Moles and Norcross, Gibson and Robb, and Ryer et al. it was expected that the largest proportion of juvenile flatfish would be found on the sediment with the finest grain size and the largest proportion of flatfish would be found on the lightest sediment. Lastly, to make a proof of concept, core- and surface samples were collected from Horsens Fjord and Bellevue Beach to determine the suitability of these locations as potentially nursery habitats.

4 Background

To understand and investigate the stated in section 3, the needed background knowledge is provided in the following section. They contain knowledge about degradation in the seabed and the consequences of formation of hydrogen sulphide. Furthermore, knowledge about the current state of the Danish coastal waters, relevant legislation, and the use of flatfish as bio-indicators is presented.

4.1 Degradation and respiration in the seabed

The sea is teeming with particles and a majority of these particles will eventually sink towards the bottom. Some of the particles are organic material ($(CH_2O)_{106}(NH_3)_{16}(H_3PO_4)$), and already before the material reaches the seabed, the degradation has begun (Christensen et al., 2002). Degradation happens through a variety of processes (figure 1), and results in the release of numerous by-products, including nutrients and carbon dioxide, which are important for the ecological state of the sea (Christensen et al., 2002). Organisms living on the seabed or within the sediment such as sand worms, mussels, and flatfish are called benthic organisms (Thomas and Bowers, 2012). They depend on the organic material reaching the seabed (Kingston, 2019). More specifically the benthic organisms ingest the organic material that reaches the seabed to support their energy demand, and the subsequent waste products are led back into the environment. These benthic organisms are therefore a very important step in the degradation of organic material (Christensen et al., 2002).

The final degradation of the organic material is done by microorganisms. The species composition of bacteria in the seabed is determined by the amount of available oxygen, nitrate, iron, manganese, and sulphate, which are all used for respiration. The largest energy yield for the bacteria is obtained by respiration with oxygen and the yield decreases with nitrate, manganese, iron, and sulphate, respectively (Christensen et al., 2002). In the first few millimetres of the seabed, aerobic bacteria uses oxygen for respiration. The depth of the layers with aerobic respiration, see figure 1, is typically around 5-8 mm during winter and 1-3 mm during summer in the coastal zones of Denmark. Even though aerobic respiration only occurs in the first few millimetres of the top layers in the sediment, it accounts for around 50% of the overall yearly degradation of the organic material in the seabed (Christensen et al., 2002).

Just below the oxygenated top layer, anaerobic respiration with nitrate (denitrification) occurs, see figure 1. Denitrification is responsible for less than 10% of the degradation of organic material in the seabed (Christensen et al., 2002). However, denitrification is important for the removal of fixed nitrogen from the sediment which among other things regulates plant growth (Madigan et al., 2019). Below the layer with denitrification, manganese reduction, iron reduction, and sulphate reduction occur, see figure 1. When all the available sulphate has been respired, methane is produced. The production of methane can result in the formation of large methane bubbles within the sediment which can cause rapid vertical transport of methane through the sediment ("bundvendinger"). The different respiratory processes are not always clearly separated down through the sediment column and it is possible to find sulphate reduction at the same depth as iron reduction (Christensen et al., 2002).

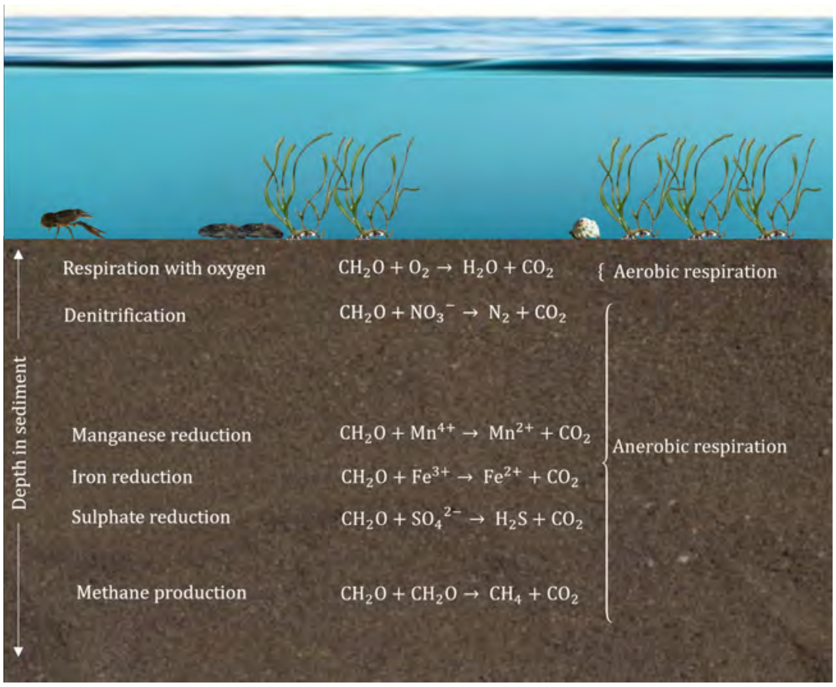


Figure 1: Respiratory processes within the seabed. For simplicity organic material is abbreviated as CH_2O. The figure is modified from (Christensen et al., 2002).

4.1.1 Formation of hydrogen sulphide

Sulphate reduction produce hydrogen sulphide (H_2S) as a residue product which is toxic for oxygen respiring organism living in the sediment. In a healthy marine environment with ample of oxygen, oxygen will be taken up by the organisms and in the blood it binds with iron atoms in the hemoglobin, an oxygen-carrying protein in the blood of the organisms which transport oxygen around the body to be used for energy generation (Oeschger and Storey, 1993). However, in the presence of hydrogen sulphide, oxygen will be replaced by hydrogen sulphide in the binding with iron and this is fatal (Moodley et al., 1998; Christensen et al., 2002). The reduced hydrogen sulphide from the sulphate reduction will eventually be oxidized further up in the sediment. Hydrogen sulphide is rarely oxidized directly by oxygen which is due to the content of oxidized iron (FeOOH) in the seabed. Before hydrogen sulphide reaches the oxygenated layer of the sediment, it will bind with the oxidized iron according to equation 1 and form iron sulphide (FeS) which precipitates and dyes the sediment black (Christensen et al., 2002; Moodley et al., 1998).

$$FeOOH + H_2S \rightarrow FeS \tag{1}$$

The formed iron sulphide will eventually be oxidized by oxygen and restored in the seabed as oxidized iron. The oxidized iron pool in the seabed accumulates and withhold large amounts of hydrogen sulphide delaying and decreasing the usage of oxygen which has a great influence on the development of coastal deoxygenation zones (Christensen et al., 2002). The largest production of hydrogen sulphide occurs in late summer due to higher temperatures and greater amounts of organic material are added to the seabed. When the entire iron pool has been used to oxidize hydrogen sulphide, hydrogen sulphide is oxidized directly by oxygen. To maintain a large iron pool and thereby a healthy seabed, benthic organisms are of great importance as their activities result in the addition of more oxygenated water within the sediment. In a seabed where the supply of oxygen is too low and all the iron has been used, hydrogen sulphide will penetrate the sediment and move towards the surface of the seabed. Under these conditions, bacteria like *Beggiatoa* that obtain energy by the oxidation of hydrogen sulphide can form (Madigan et al., 2019). The bacteria are arranged in biofilm on the surface of the seabed and they are the final barrier to prevent hydrogen sulphide from seeping out into the seawater (Christensen et al., 2002). The formation of *Beggiatoa* is a sign of a very unhealthy and imbalanced seabed. If hydrogen sulphide escapes the biofilm and is released to the water column it will result in the removal of oxygen and the formation of free sulphur which is lethal for oxygen respiring organisms living in the water column (Christensen et al., 2002; Moodley et al., 1998; Oeschger and Storey, 1993).

4.2 Initiatives to achieve a healthy marine coastal zone in Denmark

The coastal waters of Denmark contain a vast animal- and plant life, which is important to protect. To ensure this a healthy marine environment is crucial (Jensen, 2022). However, currently many coastal zones are challenged by environmental disturbances such as the production of hydrogen sulphide in the seabed as mentioned in section 3. This may have a huge influence on the organisms in the areas and result in a decline in the biodiversity and fish stocks (Rafferty, 2019). The EU Water Framework Directive and the interest group the Danish Association for Sport Fishing already focus on eliminating and mitigating these issues, which is described further in the following sections.

4.2.1 The EU Water Framework Directive

The EU Water Framework Directive was established the 22nd of December in 2000 (Miljøstyrelsen, 2022). The key aims of the directive are to expand water protection by limiting emissions and set quality standards. Furthermore, the directive aims to streamline water legislation in EU and get the citizens more involved in water protection (European Commision, 2022). The deadline for each country to fulfill the directive was in 2015 but has been postponed until 2027 (Naturfredningsforening, 2022). Point 17 in the directive is especially related to coastal zones stating that coastal aquatic ecosystems should be taken into account in the water policy as they are vulnerably to the discharge of the inland water. Additionally, it states that protection of river basins will result in protection of fish populations, which also include coastal fish populations that overall will provide an economic benefit (European Commision, 2000). To accomplish the criteria set by the EU Water Framework Directive in Denmark, the Danish Government has made a plan which are governed by the Ministry of the Environment. It is however the municipalities who have the responsibility for the execution of the actual goals described in the plan (Naturfredningsforening, 2022).

4.2.2 Project Kysthjælper

The Danish Association for Sport Fishing founded in 1926 is an interest group for anglers in Denmark (Danmarks Sportsfiskerforbund, 2022). DASF began the project Kysthjælper in 2021 as a part of their mission to give the natural reconstructions a push in the right direction. The project will run until 2025 and has two main goals. The first goal is to promote a more personal and active relationship between the Danish citizen and the marine environment. This will spread more awareness and create a sense of ownership of the marine environment which will ensure that they treat it with respect. The other goal is to improve the marine environment through marine restoration projects. Through the two goals, the project is working in alignment with FN's sustainable development goals (SDG) 13 and 14: climate action and life below water. Especially goal 14 focus on strengthening the resilience but also on restorations of the marine environment to obtain a healthy environment (United Nations, 2022).

The long-term goal is for all of Denmark to have an improved coastal marine environment. However, as a start four local pilot marine restoration projects have been chosen to start in Limfjorden, Århus Bugten, Horsens Fjord, and near Assens. Through these projects knowledge and inspiration will be gained to get a better understanding of the processes happening in the coastal zones. The experiences from these projects and the theoretical knowledge will be gathered in an online toolbox which will be developed in connection with the Kysthjælper project. This toolbox will e.g. contain advice and guidance for future marine restoration projects which can be carried out by volunteers and people who are involved in these kinds of projects (Sportsfiskerforbund, 2021). To reach the goals of the project, DASF is collaborating with a variety of Danish NGO's and professional groups including the University of Southern Denmark (SDU) and the National Institute of Aquatic Resources at the Technical University of Denmark, and the project is financed by the VELUX foundation (Sportsfiskerforbund, 2021).

4.2.3 Ecological state in Horsens Fjord

As mentioned in section 4.2.2, Horsens Fjord is one of the four areas project Kysthjælper has started local pilot marine restoration projects in. In this project the seabed of Horsens Fjord has been investigated, which is described in section 5.5. The ecological state in the inner- and outer fjord in Horsens is in bad conditions. This is among other caused by contamination from the surrounding agriculture and aquaculture, but also from the sailing and mussel scraping which affects the coastal areas of the fjord (Miljøministeriet, 2011). Some restorations have already been made in Horsens Fjord, including planting of eelgrass and placing stones on the seabed to create diffuse reefs. An overview map of the implementations made by project Kysthjælper can be found in appendix A.5.

4.3 Flatfish as bio-indicators

A bio-indicator is defined as an organism that reflects the state of the environment and indicates the impact of environmental changes on a given habitat (McGeoch et al., 2002; Kuklina et al., 2013). However, the right selection of the indicator specie is important especially when it comes to the investigation of ecological integrity. Many different organisms can be used as bio-indicators but the most common are fish and benthic invertebrates which both have advantages and disadvantages when used (Kuklina et al., 2013). According to Kuklina et al. (2013), bio-indicators should preferably have the following characteristics:

- A high ecological relevance
- Sensitive to stressors both in the laboratory and in the field
- A broad geographic distribution
- Easy to maintain in the laboratory
- A high reproductive rate and the ability to give reproductive data under controlled laboratory conditions

In the present project, the seabed integrity is investigated by the possible use of the flatfish species plaice and flounder as bio-indicators. Flounder and plaice are both common flatfish in Danish coastal waters and they share some of the same traits. Especially, as juvenile flatfish, the plaice and flounder are difficult to distinguish, but the differences between the species become more significant as they mature. One of the significant differences between the mature flounder and plaice is the smooth surface that is characteristic for the plaice, whereas the flounder has a rough surface due to bone knots. Furthermore, both plaice and flounder have red and orange dots on the surface, but this trait is more significantly visible on the plaice (Hansen, 2019; DTU Aqua, 2017). It is however possible to distinguish between juvenile plaice and flounder by their dorsal, anal, and caudal fins. The amount of fin rays for each specie is found in figure 2.

	Plaice	Flounder
Dorsal	65-79	52-67
Anal	43-61	34-46
Caudal	19-22	18

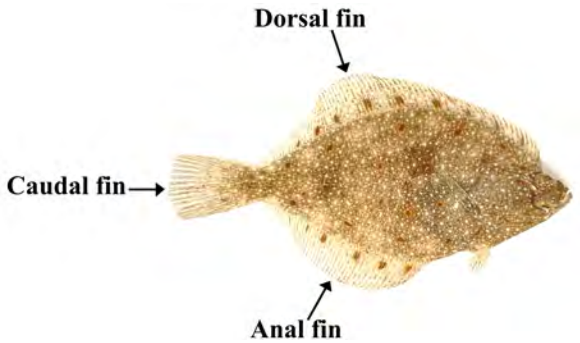


Figure 2: The table on the left shows the amount of dorsal, anal, and caudal fin rays for a plaice and a flounder. The table is modified from Munk and Nielsen (2005). The figure on the right shows where the fins are placed on the flatfish.

Plaice and flounder are spawning at water depths of around 20-100 meters. The current will transport most of the eggs and larvaes to the nursery areas, which are located near the coast. At the nursery areas and sometimes already during the transport, the metamorphosis begins (Hansen, 2019; DTU Aqua, 2017). Both species starts as larvae, which do not differ greatly from other symmetrical fish that swim with an upright posture. However, as they metamorphose from larvae into juvenile flatfish, the body gets more flattened to a laterally compressed body with dramatic internal and external asymmetries and one eye migrate closer to the other eye (Schreiber, 2013). Flatfish will either become dextral or sinistral which means that both eyes are located on the right or left side, respectively. The plaice is dextral, while the flounder is polymorphic which means that they can become both dextral or sinistral, although they are mostly dextral (DTU Aqua, 2017; Schreiber, 2013). After the metamorphose plaice and flounder become benthic fish instead of pelagic, as their new habitat becomes the seabed. Through adaptive colour change they can match the colour and texture of the sediment by darkening and paling of the skin. This is due to the dermal and epidermal chromatophores that are pigment-containing cells. For the colour change to happen, regulatory factors such as hormones, neurotransmitters, and paracrine agents are involved (Burton, 2002). The period of the colour change varies from species to species, however, it can take days or weeks before a final colour adaptation has occurred due to movement and additional production of hormones and neurotransmitters. A part of the reason for the flatfish to adapt to the seabed colour is to avoid predators. If they do not match the sediment, they are more visible and hence become more vulnerable for predation and are therefore more likely to be eaten. To minimize the risk of predation flatfish are active during the night and are mostly found buried in the sediment during the day (Ryer et al., 2008).

5 Materials and Methods

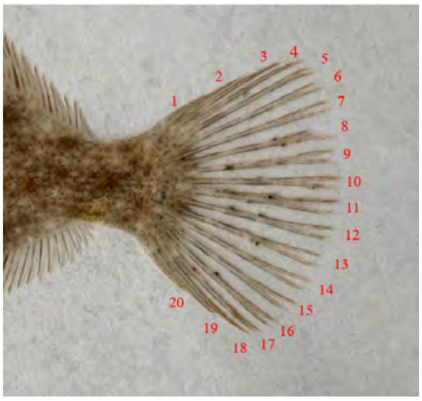
As mentioned in section 3, one bio-indicator experiment was performed to investigate the behaviour of juvenile flatfish when exposed to sand containing hydrogen sulphide. Furthermore, to differentiate between behavioural responses caused by hydrogen sulphide or sediment characteristics, two pre-experiments were conducted to test grain size and colour preference, respectively, for juvenile flatfish. The particle sizes of the used sediments were analysed after all experiments were terminated and a description of the performed grain size analysis can be found in appendix A.2. Furthermore, information about the different sediments bought for the experiments can be found in appendix A.6. In the following sections, the materials, and methods used are described and an overview of the usage of fish in each conducted experiment can be found in table 2.

Table 2: An overview of the usage of fish for all conducted experiments. It is important to note that the time between round 1 and round 2 of the experiment was three weeks.

Experiment	Number of trials	Number of fish	Repeated use of fish	Time before reuse of fish	Number of dead fish
Grain size - round 1	10	25	2 times	-	3
Grain size - round 2	16	38	2 times	-	21
Colour selection - stage 1	16	17	4-5 times	Min. 24 hours	1
Colour selection - stage 2	8	16	2-3 times	Min. 24 hours	-
Hydrogen sulphide	20	16	2-3 times	Min. 24 hours	-

5.1 Fish collection and species determination

All juvenile flatfish used in the experiments were caught with seine nets at Bellevue Beach in September 2021. The 17 flatfish used in the colour selection experiment (section 5.3) were identified to species level and the same species distribution was assumed to apply for the flatfish used in hydrogen sulphide experiment (section 5.4). As described in section 4.3 the species of juvenile flatfish can be distinguished by their fin rays. To determine the species of the juvenile flatfish used in the experiments, a picture was taken of the caudal fin for each flatfish and the number of fin rays were counted from the picture, see figure 3.



(a)

(b)

Figure 3: In **(a)** and **(b)** the caudal fin rays for two different flatfish are shown. **(a)**: 20 caudal fin rays could be counted from the picture and due to this the flatfish was identified as a plaice. **(b)**: 18 caudal fin rays could be counted from the picture and the flatfish was identified as a flounder.

All flatfish were categorized according to table 2, section 4.3, and the species distribution of juvenile flatfish was determined as; 3 flounder with 18 caudal fin rays and 14 plaice with caudal fin rays in the range of 19-21. This method was performed because the flatfish used in the experiments had other future purposes, preventing the possibility of terminating the lives of the flatfish, and as it is difficult to count fin rays on living flatfish.

5.2 Grain size experiment

5.2.1 Test protocol

The aim of the grain size experiment was to investigate the grain size preference of juvenile flatfish when given a choice between three different sediments. Based on the results obtained by Moles and Norcross (1995) and Gibson and Robb (2000), it was assumed that the largest number of juvenile flatfish would be found on the sediment with the finest grain size (<600 μm). Note, that the experiment was conducted by our supervisor Mikael van Deurs and a student assistant, prior to the beginning of this project, but all data analysis was done by us. The experiment was conducted twice; first with gravel, coarse- and fine sand (round 1) as the three different sediments (figure 4a) and second with coarse-, beach- and fine sand (round 2) (figure 4b). The range of all sediment particle sizes can be found in table 3.

Table 3: Range of all sediment particle sizes used in the grain size experiment. In round 1 of the experiment fine sand, coarse sand and gravel were used. In round 2 of the experiment, fine-, beach- and coarse sand were used.

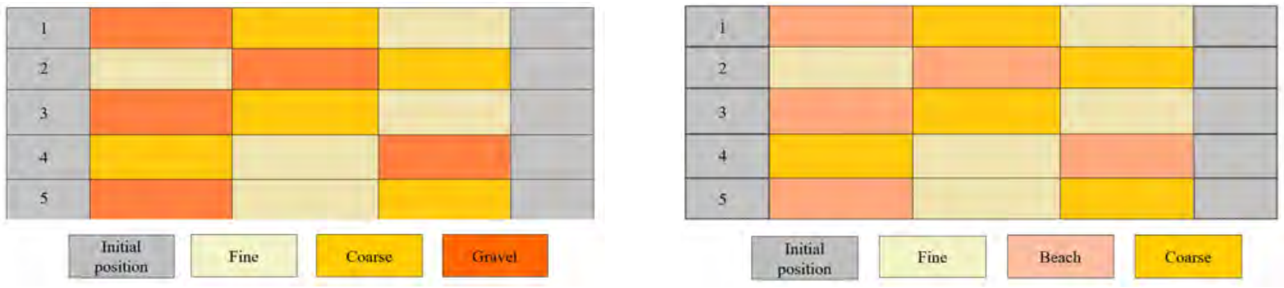
Particle size range (mm)	% Particle size fraction by weight			
	Fine	Beach	Coarse	Gravel
>2.00	0.00	0.49	0.00	88.61
1.00-2.00	0.49	0.49	55.12	8.91
0.600-1.00	0.97	51.72	39.12	1.98
0.355-0.600	56.31	43.84	3.90	0.50
0.250-0.355	38.83	1.97	0.98	0.00
0.180-0.250	2.91	0.49	0.49	0.00
0.125-0.180	0.00	0.49	0.00	0.00
0.090-0.125	0.00	0.00	0.00	0.00
0.063-0.090	0.00	0.00	0.00	0.00
<0.063	0.49	0.49	0.49	0.00

(a)

(b)

Figure 4: (a): The sediments used in round 1 of the experiment. From the left; fine sand, coarse sand, and gravel. **(b)**: The sediments used for round 2 of the experiment. From the left; fine sand, beach sand, and coarse sand.

The experiment was carried out in an arena (figure 5) (description of the arena can be found in appendix A.1) and all five areas within each lane in the arena were used for the experiment. The different sediments used in the experiment were placed in a way to ensure that as many combinations as possible were tested.



(a) (b)

Figure 5: (a): The arena used for the round 1 of the grain size experiment. The grey areas are the initial positions of the fish, white areas represent fine sand, yellow areas represent coarse sand, and orange areas represent gravel. **(b)**: The arena used for round 2 of the grain size experiment. The grey areas are the initial position of the fish, white areas represent fine sand, pink areas represent beach sand and yellow areas represent coarse sand. The arena consists of five lanes which are numbered from 1-5 in figure **(a)** and **(b)**.

Both rounds the experiment was conducted each experimental fish had two consecutive trials before it had a period of rest. The number of fish used in experiments can be found in table 2. The placement of each fish happened at noon and each fish were left undisturbed for 24 hours, after which it was replaced in the initial position and left for another 24 hours. After every 24 hours, the final sediment choice was recorded. If the fish had not moved out of the initial position (grey areas in figure 5), the choice was considered as invalid and not included in the final results. The period of 24 hours was set as it was observed beforehand that the flatfish had not moved out of the initial position until 24 hours had passed. The placement of fish was randomized, and each fish was placed on either the right or left side of the arena. All fish were randomly selected from the group of caught fish and were acclimated to either gravel, coarse sand, or fine sand.

5.2.2 Data analysis

For round 1 and 2 of the experiment, the number of fish on each sediment was tested against the null hypothesis of equal distribution using a logistic regression model. To establish the final model, the additional variables: length of fish, day of testing, distance from the initial position of fish placement, and acclimated sediment were tested with the parameter 'sediment type' one at a time as a function of choice according to equation 2 by the use of the logistic regression model.

$$\text{choice} = \text{sediment type} + \text{x} \tag{2}$$

In equation 2, the parameter 'choice' represents the final sediment choice. The parameter 'sediment type' represents one of the tested sediments, and x is one of the presented additional variables. Only additional variables which had a p-value below 0.05 was included in the final logistic regression model. The final model and results are described in section 6.1.

5.3 Colour selection experiment

5.3.1 Test protocol

The aim of the colour selection experiment was to investigate the colour preference for juvenile flatfish when exposed to dark and light substrates. This was investigated because as described in section 4.1.1, when hydrogen sulphide binds with Fe^{+3} it forms FeS which precipitates and dyes the sediment black. It was expected that the largest number of juvenile flatfish would be found on the substrates with the lightest colour due to their acclimation history. This expectation was based on the results obtained by Ryer et al. (2008), where the majority of the juvenile flatfish chose light sediment over dark when acclimated to light sediment. The colour selection experiment was conducted in two stages to determine whether colour of the substrate or grain size of the substrate have the greatest influence on the choice of sediment. In the first stage, the juvenile flatfish were given a choice between two different sand colours, black and white, with comparable grain sizes. The first stage of the experiment was conducted twice and the second time it was conducted the positioning of the coloured sediment was mirrored (figure 7). This was done to test if the position of the sediment had an influence on sediment choice. In the second stage of the experiment, the substrate was changed from sand to plastic and the potential preference for a specific colour (black or light grey) was investigated (figure 9). Only fish above a length of 8.2 cm used in the experiment to rule out any potential effect of size. Prior to every trial, each fish was photographed for species determination. Using the method suggested by Moles and Norcross (1995), all flatfish were fed 24 hours before every trail to remove hunger as a variable in the experiment. Both stages of the experiment were conducted in the arena (see description, picture, and conditions of the arena in appendix A.1). The three areas in the middle of the arena were used with black dividing plates confining the desired area. The start position in the middle of the three areas contained the same coarse sand the fish were acclimated to beforehand. It was considered to acclimate half of the juvenile flatfish to black sand and the other half to white sand, which was done in the study by Ryer et al. (2008). However, as acclimation can take several weeks and due to the time frame of this project, it was not a possibility. Hence, the coarse sand was chosen. All fish were placed facing the side of the arena with one substrate on each side to prevent affecting their choice, see figure 7 and 9. All experiments were initiated at noon, and the fish were left undisturbed for 24 hours after which the final colour selection was recorded. If the fish had not moved out of the start position, the choice was considered invalid and therefore not included in the results. During the first stage of the experiment one fish died. Further details about the usage of fish can be found in table 2.

5.3.2 Stage 1: Sand colour selection

In the first stage of the experiment, the substrate was three types of sand; black sand, white sand, and coarse sand, see figure 6 for the colours. The range of all sand particle sizes can be found in table 4. The sand was rinsed in freshwater before the experiment to remove unwanted organic material. The grain size of the sand used in the experiment was chosen according to the results obtained from the grain size experiment described above in section 5.2. The setup for the two times the experiment was run can be seen in figure 7.

Table 4: Range of all particles sizes of the sediments used in stage 1 of the colour selection experiment (section 5.3.2).

Particle size range (mm)	% Particle size fraction by weight		
	Black	White	Coarse
>2.00	0.00	0.48	0.00
1.00-2.00	0.97	0.96	55.12
0.600-1.00	1.46	3.35	39.12
0.355-0.600	46.12	79.43	3.90
0.250-0.355	33.50	12.44	0.98
0.180-0.250	11.17	1.91	0.49
0.125-0.180	4.85	0.48	0.00
0.090-0.125	0.97	0.00	0.00
0.063-0.090	0.49	0.00	0.00
<0.063	0.49	0.96	0.49

Figure 6: The colour of the sand used in the experiment. The white sand is seen on the left hand side, coarse sand in the middle, and black sand is on the right hand side.

(a) **(b)**

Figure 7: The arena used for stage 1 of the colour selection experiment. The arena consists of five lanes with three cells each - one for each of the types of sediment. The depicted flatfish show the direction and placement of fish for every trial. The difference between the setup seen in **(a)** and **(b)** is the placement of black- and fine sand which is mirrored.

5.3.3 Stage 2: Plastic colour selection

For stage 2 of the colour selection experiment, the substrate was changed from sand to plastic. Ten plastic plates were cut to fit the spaces in the arena. Half of the plastic plates were covered in mat, black foil, and the rest were left in a light grey, see figure 8.

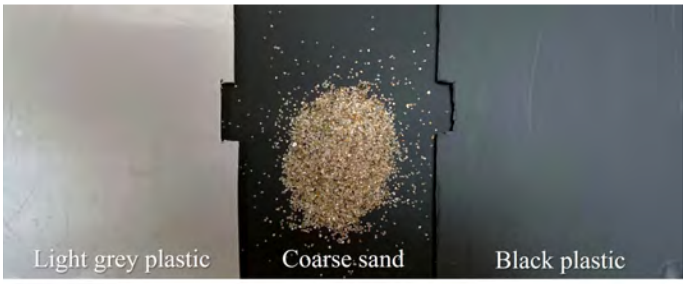


Figure 8: The colour of the coarse sand and the plastic sheets used in stage 2 of the colour selection experiment (section 5.3.3). The light grey plastic is seen on the left hand side, coarse sand in the middle, and the black plastic sheet is on the right hand side.

As in stage 1 of the experiment, the start position was coarse sand. The particle size range can be found in table 4 and the experimental set up for stage 2 can be seen in figure 9.

Figure 9: The arena used for stage 2 of the colour selection experiment where the fish had a choice between black- and white plastic. The arena consists of five lanes which are numbered from 1-5 in the figure. The depicted flatfish show the direction and placement of the fish for every trial.

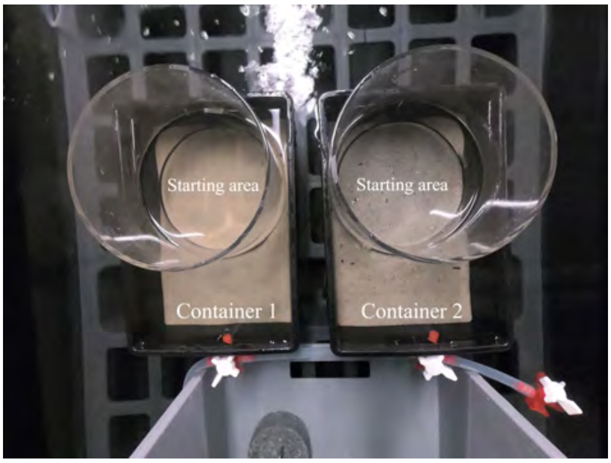
5.3.4 Data analysis

For the colour selection experiment, the number of fish on each sand- and plastic colour was tested against the null hypothesis of equal distribution using a one sample proportions test. All results can be found in section 6.2.

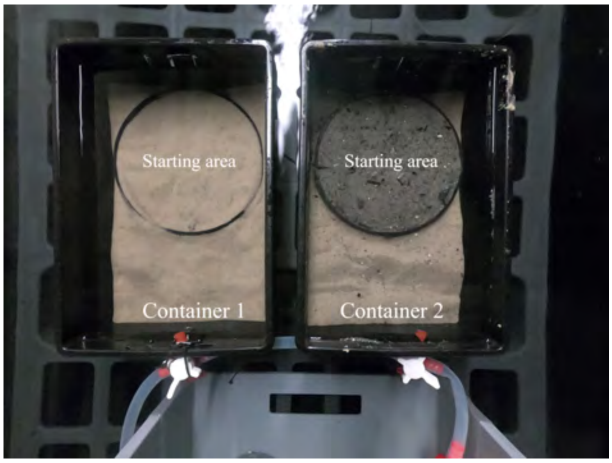
5.4 Hydrogen sulphide experiment

5.4.1 Test protocol

The aim of the hydrogen sulphide experiment was to investigate the behavioural responses of juvenile flatfish when exposed to sand containing hydrogen sulphide. As described in section 4.1.1 hydrogen sulphide is toxic for oxygen respiring organisms living in the sediment and due to this it was predicted that the juvenile flatfish would flee from areas with sand containing hydrogen sulphide. The hydrogen sulphide experiment was performed in a tub filled with flowing water where two smaller black containers were placed next to each other (conditions of the tub can be found in appendix A.4). Water from the tub were lead into the two containers and to decrease the water's retention time, a pump was placed in each container. Furthermore, two air stones were added to the tub to ensure that the water was fully oxygenated.



(a)



(b)

Figure 10: The experimental set-up for the hydrogen sulphide experiment. Both pictures are taken with the GoPro camera used in the experiment. **(a)**: Two cylinders were placed on top of the starting area to prevent sand from mixing when the containers were refilled with water. The same cylinders were used prior to every trial, when a flatfish was released to the starting area. **(b)**: Container 1 (control) contained fine sand in the starting area and in the surrounding area. In container 2 the starting area was filled with sand containing hydrogen sulphide, and the surrounding area contained fine sand. One pump was placed in each container to decrease the retention time of water.

In each container a circular, replaceable area which was used as the starting position for each fish in every trial, was placed, see figure 10. The circular area in container 1 and the surrounding area was filled with the same fine sand used in the grain size experiment (described in section 5.2), making this container a control for every trial. The fine sand was rinsed in freshwater before the experiment to remove unwanted organic material. The starting area in container 2 was filled with sand containing hydrogen sulphide collected from Nivå Bay and the surrounding area was filled with the same fine sand as in container 1. The sand from Nivå Bay was collected in March by digging into the surface layer of the coastal line until the presence of hydrogen sulphide could be determined by the smell and darkening of the sand. The colour of the used sand can be seen in figure 11 and particle sizes can be found in table 5.

Table 5: Range of the sand particle sizes used in the hydrogen sulphide experiment.

Particle size range (mm)	% Particle size fraction by weight	
	Nivå Bay	Fine
>2.00	1.97	0.00
1.00-2.00	3.45	0.49
0.600-1.00	4.43	0.97
0.355-0.600	8.87	56.31
0.250-0.355	30.54	38.83
0.180-0.250	45.81	2.91
0.125-0.180	0.99	0.00
0.090-0.125	2.46	0.00
0.063-0.090	0.49	0.00
<0.063	0.99	0.49

Figure 11: The two types of sand used in the hydrogen sulphide experiment. From the left; Sand from Nivå bay, fine sand.

The sand containing hydrogen sulphide in container 2 was refilled every morning due to oxygenation of the top layer. Further, a tall hollow cylinder was placed on the starting area to prevent all the sand from mixing when the container was refilled with water, see figure 10a. Moreover, the same cylinders were placed when releasing one juvenile flatfish in each container to ensure that both flatfish started the trial in the starting area. The duration of each trial was one hour and to document the behaviour of the flatfish, a GoPro camera was used in time-lapse mode taking pictures of both containers every 60 seconds. The final sand choice was recorded after each trial and when the daily trials had been conducted, all flatfish were fed to remove hunger as a variable before the next trial. Further details of the fish used can be found in table 2.

After all trials had been conducted oxygen profiles were measured in the starting area of the containers. In container 1, oxygen profiles were measured once while in container 2 oxygen profiles were measured right after the sand had been changed in the morning and again in the afternoon. All profiles were measured by the use of sensor technology. An optical oxygen sensor was connected to a FirestingO2, which is a multi-channel PC-operated fiber-optic oxygen meter (PyroScience GmbH, 2022). Furthermore, the sensor was attached to a motor leading it vertically down through the sand and the amount of dissolved oxygen (DO) was measured every 100 μm. The data was logged in PyroScience's software Profix throughout the cross section creating a vertical oxygen profile. In order to measure the oxygen profiles, chemistry was added to the end of the oxygen sensor. The chemistry is responsible for the signal to measure the DO in the sand however, due to the fact that it fell off during the measurements leading to an inefficient signal, it became a limitation on how many accurate oxygen profiles there could be measured. Based on that, it was prioritized to measure the oxygen profiles only in the starting area of the two containers.

Furthermore, core samples of the sand were collected to investigate the distribution of oxidized (Fe^{+3}) and reduced (Fe^{+2}) iron in the sediment. This was investigated due to the importance of Fe^{+3} in the seabed as hydrogen sulphide binds to Fe^{+3}, preventing the release of free sulphur to the water column. In the reaction with hydrogen sulphide, Fe^{+3} is reduced to Fe^{+2} according to equation 1 (described in section 4.1.1). In equation 1, Fe^{+3} corresponds to FeOOH and Fe^{+2} corresponds to FeS. Hence, the ratio of Fe^{+2} and Fe^{+3} indicates the extent of the production of hydrogen sulphide in the sediment as sediment layers dominated by Fe^{+2} often have high contents of hydrogen sulphide. Therefore, the higher ratio, the higher production of hydrogen sulphide. The core samples were taken as triplets in the starting area of both containers. In container 1, the samples were collected once whereas in container 2, the samples were collected right after the sand had been changed in the morning and again in the afternoon. This was done to investigate if a change in the distribution of the amount of Fe^{+3} and Fe^{+2} had occurred with time. The determination of iron in the core samples was done at SDU by the laboratory technician intern Rikke Schleemann Hadow. To determine the iron in the layers of the sediment, the Ferrozin method was used. The core samples were cut in intervals of one centimeter and the iron in each slice was extracted with HCl and centrifuged before adding Ferrozin to the supernatant for the measuring of the Fe^{+2} concentration. Ferrozin is a reagent, that reacts with Fe^{+2} and forms a stable, red colour intense, ferrous complex which can be detected trough spectrophotometry. Furthermore, the total concentration of iron (Fe^{+2}+Fe^{+3}) is determined by adding hydroxylamine to the supernatant, which reduces the Fe^{+3} to Fe^{+2} and thereafter Ferrozin is added. The concentration of Fe^{+3} is found as the difference in the concentration of Fe^{+2} and the total concentraion of iron (Stookey, 1970; Lovley and Phillips, 1987).

5.4.2 Data analysis

The distribution of juvenile flatfish on the sand in the starting area and the sand in the surrounding area was tested individually for each container against the null hypothesis of equal distribution using a one sample proportions test. Results can be found in section 6.3. Further, it was investigated if the final choice was influenced by whether the trials were conducted in container 1 or 2. This was tested according to equation 3 by the use of the logistic regression model.

$$\text{choice} = \text{container} \tag{3}$$

In equation 3, the parameter 'choice' represents the final sand choice. The parameter 'container' represents either container 1 or container 2.

5.5 Sediment collection and comparison of substrates

As a proof of concept and to determine if juvenile flatfish can be used as bio-indicators of seabed quality in terms of grain size, colour, and hydrogen sulphide, a comparison was made between the results obtained from the laboratory experiments and seabed conditions in Bellevue Beach. Bellevue Beach is anticipated to be a potential nursery area as this is where the juvenile flatfish used in the experiments were caught. To examine potential nursery habitats for juvenile flatfish an area of Horsens Fjord where marine restorations by the project Kysthjælper have been made was investigated. As described in section 4.2.3, the ecological state of the Fjord is in bad conditions which might prevent this area from being a potential nursery area for juvenile flatfish. In both Horsens Fjord and Bellevue Beach, surface sediment samples were collected to compare the grain sizes with all sediments used in the experiments. Furthermore, core samples were collected to compare the distribution of Fe^{+3} and Fe^{+2} in the sediment from the two areas with the sand from Nivå Bay used in the hydrogen sulphide experiment (figure 12). Unfortunately, it was later discovered that the layers of the sediment in the core samples collected from Horsens Fjord were mixed and therefore the distribution of Fe^{+3} and Fe^{+2} was not determined.

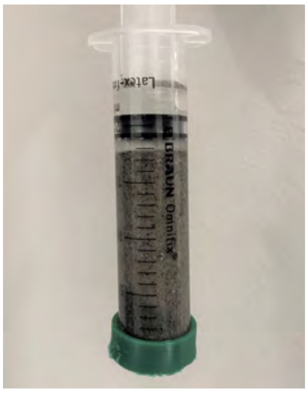


Figure 12: Core sample collected at Bellevue Beach.

In addition, a comparison was made of the colour for the substrate used in the colour selection experiment (section 5.3) and the sediments collected from Nivå Bay, Bellevue Beach, and Horsens Fjord 13.

(a)

(b)

Figure 13: Comparison of the substrate colour from the substrates used in both stages of the colour selection experiment with sediments from Nivå Bay, Horsens Fjord, and Bellevue Beach. **(a)**: From the left; white sand (colour selection experiment), sand from Nivå Bay (hydrogen sulphide experiment), sand from Bellevue Beach, sand from Horsens Fjord, and black sand (colour selection experiment). **(b)**: From the left; light grey plastic (colour selection experiment), sand from Nivå Bay (hydrogen sulphide experiment), sand from Bellevue Beach, sand from Horsens Fjord, black plastic (colour selection experiment).

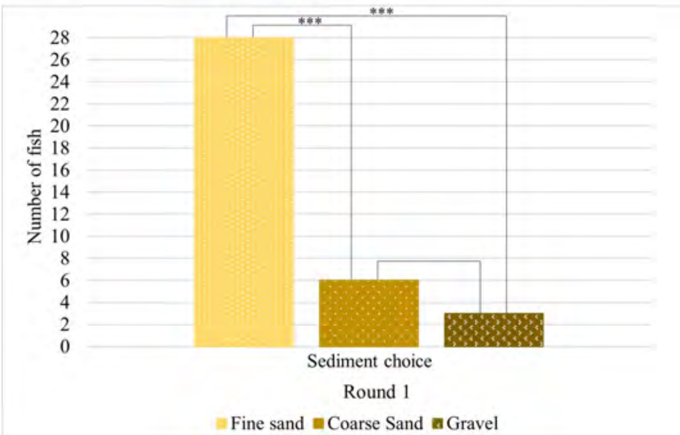
6 Results

6.1 Grain size experiment

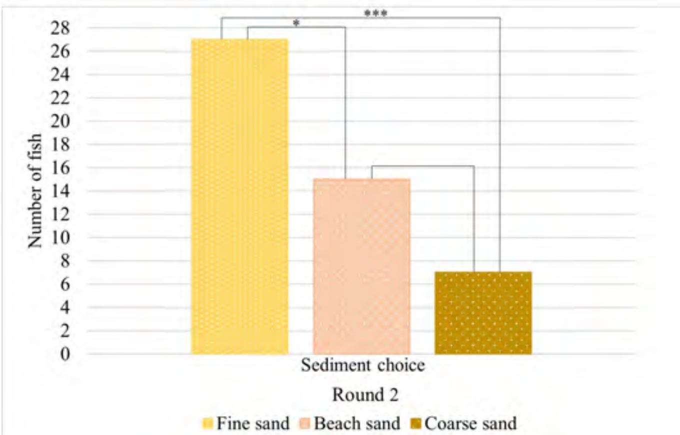
To test the null hypothesis described in section 5.2.2, additional variables was tested, and the final model can be seen in equation 4. For round 1 of the experiment, sediment type and the distance from the initial position of fish placement had an influence on the final sediment choice. However, all results remained unchanged having a model that included the distance from the initial position as the only variable. For round 2 of the experiment only the sediment type had an influence on sediment choice. Based on this, none of the additional variables were included in the final model and the only parameter included was the sediment type.

$$\text{choice} = \text{sediment type} \tag{4}$$

For round 1 and round 2 of the experiment a significant difference ($p<0.05$) between the fine sand and all other sand types were found, see table 6 and 7. This means that the majority of the juvenile flatfish chose the fine sand (figure 14). Due to the obtained results, the null hypothesis of equal distribution was rejected. For round 1 the number of invalid choices was 13 out of 50 tested fish and for round 2 the number of invalid choices was 27 out of 76 tested fish.



(a)



(b)

Figure 14: The sediment choices in the round 1 and 2 in the grain size experiment. **(a)**: In round 1, the used sediments were; fine sand, coarse sand, and gravel. **(b)**: In round 2, the used sediments were; fine sand, beach sand, and coarse sand. Significance codes in the figure are given as; $p<=0.05$ '*', $p<=0.01$ '**' and $p<=0.001$ '***'.

Table 6: The obtained p-values from round 1 of the grain size experiment. All p-values have been corrected using the Bonferroni correction. Significance codes in the figure are given as; p<=0.05 '*', p<=0.01 '**' and p<=0.001 '***'.

	Fine sand	Coarse sand	Gravel
Fine sand	-	$1.44 \cdot 10^{-4}$ ***	$3.56 \cdot 10^{-5}$ ***
Coarse sand	$1.44 \cdot 10^{-4}$ ***	-	0.24005
Gravel	$3.56 \cdot 10^{-5}$ ***	0.24005	-

Table 7: The obtained p-values from round 2 of the grain size experiment. All p-values have been corrected using the Bonferroni correction. Significance codes in the figure are given as; p<=0.05 '*', p<=0.01 '**' and p<=0.001 '***'.

	Fine sand	Beach sand	Coarse sand
Fine sand	-	0.031*	$1.3 \cdot 10^{-4}$ ***
Beach sand	0.031*	-	0.1151
Coarse sand	$1.3 \cdot 10^{-4}$ ***	0.031	-

6.2 Colour selection experiment

6.2.1 Stage 1: Sand colour selection

The distribution of juvenile flatfish on white- and black sand was almost the same which can be seen in figure 15 (data can be found in appendix A.3, table 11a). Based on the one sample proportions test a p-value of $p = 0.793$ for the distribution of juvenile flatfish on white- and black sand was obtained and therefore the null hypothesis of equal distribution was accepted.

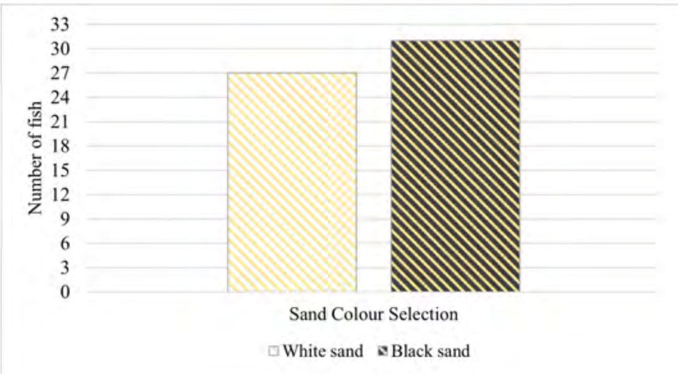


Figure 15: The distribution of sand colour choice for juvenile flatfish. The number of flatfish found on white sand was 27 and the number of flatfish found on black sand was 31. Number of invalid choices was 22.

The total number of invalid choices, for each of the two consecutive runs of stage 1 of the experiment, was 5 out of the 40 fish and 17 out of the 40 fish, respectively (initial versus mirrored set-up) (data can be found in appendix A.3, table 11a).

6.2.2 Stage 2: Plastic colour selection

In stage 2 of the colour selection experiment, the choice of the juvenile flatfish was equally distributed on the black- and light grey plastic sheets, see figure 16 (data can be found in appendix A.3, table 11b). Based on this, the null hypothesis was accepted.

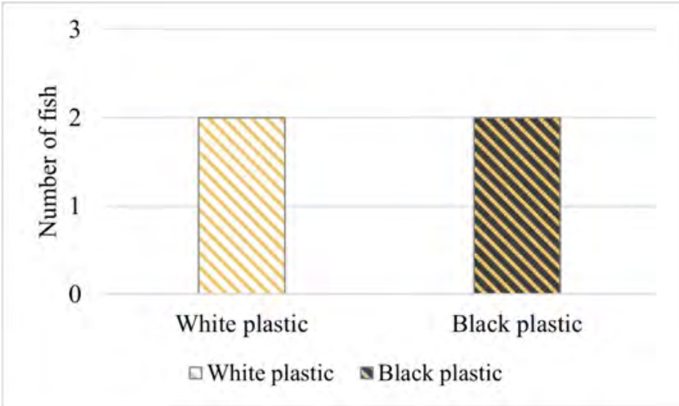
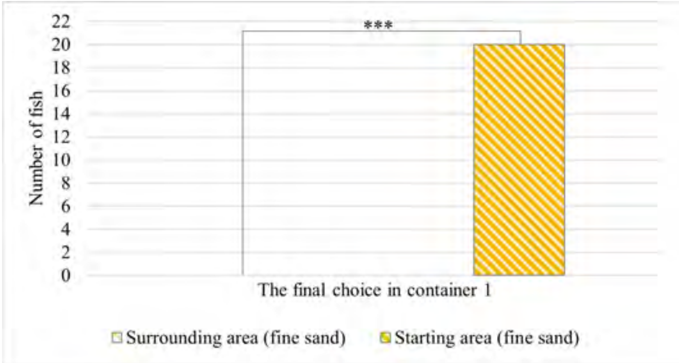


Figure 16: Distribution of plastic colour selection for juvenile flatfish.

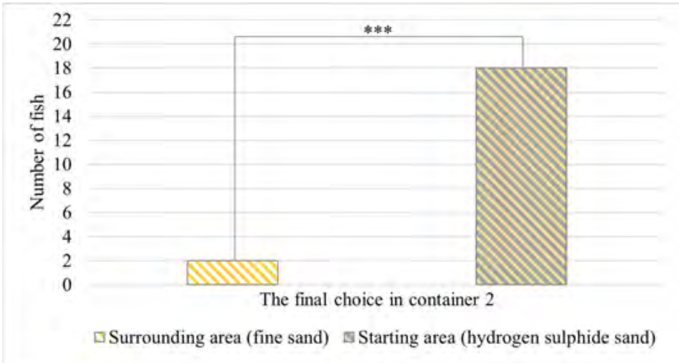
However, it is noticeable from figure 16 that only 4 fish out of the 40 tested fish, the plastic sheets were chosen. For the rest of the trials, the flatfish chose to stay in the acclimated coarse sand, which means that the total number of invalid choices was 36.

6.3 Hydrogen sulphide experiment

To test the null hypothesis described in section 5.4, the influence of whether the trials were conducted in container 1 or 2 was tested using the logistic regression model and based on the conducted test no significant difference was found ($p = 0.9963$). The largest proportion of juvenile flatfish was found in the sand of the starting area in both containers (figure 17). For container 1, the starting area was the final choice in all trials out of ten (figure 17a) and based on a one sample proportion test a significant difference was found between the position in the starting area and the position in the surrounding area ($p = 7.744 \cdot 10^{-6}$ ***). For container 2, 18 out of 20 trials, the final choice was a position in the starting area which contained sand with hydrogen sulphide (figure 17b). Based on the performed one sample proportions test for container 2, a significant difference was found between the position in the starting area and the position in the surrounding area ($p = 3.466 \cdot 10^{-4}$ ***). Hence, the null hypothesis of equal distribution was rejected.



(a)



(b)

Figure 17: (a): For container 1, the final choice of sand in the 10 conducted trials was the fine sand in the starting area. **(b)**: For container 2, the surrounding fine sand was chosen as the final choice for 2 fish, and for the rest of the 18 fish, the sand containing hydrogen sulphide in the starting area was the final choice. Significance codes in the figure are given as; p<=0.05 '*', p<=0.01 '**', and p<=0.001 '***'.

Furthermore, the pictures taken with the GoPro camera did not reveal a noticeable difference between the behaviour of the juvenile flatfish in container 1 and container 2 during the one-hour trials. It was observed that the flatfish showed the same tendency of burying behaviour regardless of grain composition and hydrogen sulphide levels.

As described in section 5.4, oxygen profiles were measured in the starting area of both containers. Oxygen solubility was considered to be 100% at the amount of 329 μm/L dissolved oxygen. The profiles show a difference in the amount of DO (μm) throughout the depth between the control sand (container 1) and the sand containing hydrogen sulphide (container 2 - morning and container 2 - afternoon), see figure 18. The oxygen profiles are calculated as the mean of the two measured oxygen profiles in the starting area of the two containers. The figures show the profiles in a range of -2,000 μm to 18,000 μm. The range from -2,000 to 0 μm is in the water phase to the surface of the sediment, and the range from 0 μm to 18,000 μm is from the surface down through the sediment.

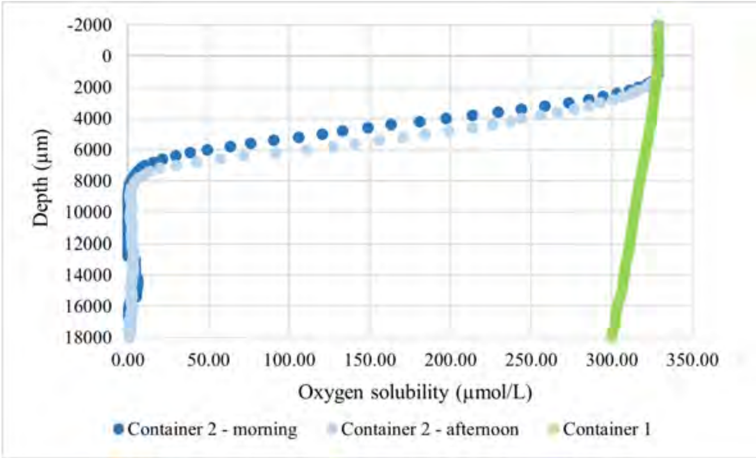


Figure 18: The three oxygen profiles are given as the mean of the oxygen profiles measured in the control sand in the starting area of container 1 (container 1) and in the sand containing hydrogen sulphide (container 2 - morning and container 2 - afternoon). The oxygen profiles measured in container 2 in the morning (container 2 - morning) was after the starting area was refilled with sand containing hydrogen sulphide. The oxygen profiles measured in container 2 in the afternoon (container 2 - afternoon) was in the same sand containing hydrogen sulphide from the morning.

The measured oxygen profile 'container 1' showed a slight decrease in the amount of DO throughout the control sand with a decrease of approx. 329 μmol/L at depth 0 μm to approx. 300 μmol/L at depth 18,000 μm. The measured DO in container 2 for both morning and afternoon was similar to container 1 with approx. 328 μmol/L in the upper surface layer until the profiles reached a depth of 1,400 μm. A rapid decrease of approx. 328 μmol/L to 2 μmol/L in DO was observed from a depth of 1,400 μm to 8,000 μm in oxygen profile 'container 2 - morning' and from a depth of 1,400 μm to 8,400 μm. Thereafter full oxygen depletion (i.e. anoxia) was reached.

6.4 Analysis of iron in sediment

The core samples collected in the starting areas in container 1 with fine sand as the control sand, container 2 both morning and afternoon with sand containing hydrogen sulphide, and *in situ* observation from Bellevue Beach were analysed for oxidized (Fe^{+3}) and reduced (Fe^{+2}) irons (table 8). This included a total mean concentration of [Fe^{+2}] + [Fe^{+3}], the mean concentration of [Fe^{+2}], and the mean concentration of [Fe^{+3}]. Table 8 shows the concentrations at a depth of 1 and 2 cm in the sediment. Furthermore, the ratio between the mean concentration of [Fe^{+2}] and the mean concentration of [Fe^{+3}] was calculated.

Table 8: Core samples collected from the starting areas in container 1 (control sand), container 2 both morning and afternoon (sand containing hydrogen sulphide), and from Bellevue Beach. In the table the mean value of the total concentrations of [Fe^{+3}] + [Fe^{+2}] and the distribution of the mean concentrations of [Fe^{+3}] and [Fe^{+2}] are given. The concentrations are found in a depth of 1 cm and a depth of 2 cm.

Sample	Depth (cm)	Conc. Fe^{+2}+Fe^{+3} (μM)	Conc. Fe^{+2} (μM)	Conc. Fe^{+3} (μM)	Ratio $\frac{Fe^{+2}}{Fe^{+3}}$
Container 1	1	9.713	7.77	1.943	3.999
	2	9.324	5.828	3.497	1.67
Container 2 - morning	1	21.756	19.814	1.943	10.198
	2	19.425	19.425	0.00	-
Container 2 - afternoon	1	31.857	31.469	0.389	80.897
	2	29.526	29.526	0.00	-
Bellevue Beach	1	51.282	36.519	14.763	2.474
	2	54.779	39.239	15.540	2.525

7 Discussion

7.1 Grain size preference for juvenile flatfish

The results presented in section 6.1 indicate that juvenile flatfish have a preference regarding grain sizes. The majority of the juvenile flatfish were found on the finest of the investigated sediments which is in agreement with what was predicted and the results obtained by Moles and Norcross (1995) and Gibson and Robb (2000). However, for both rounds of the grain size experiment only three sediments were tested at a time. A test between sediments with a particle size range closer to each other would allow for an investigation of the sensitivity of the preferred grain size choice for juvenile flatfish. Nonetheless, the results obtained from round 2 of the experiment showed that even when the sediment was changed from gravel to beach sand which have a particle size range between the fine- and coarse sand, only a slight significance was found and the fine sand remained the preferred choice. Further, the grain size experiment showed that the least preferred sediment was gravel which could be due to the fact that the flatfish were less able to bury themselves. An investigation of the ability of the flatfish to bury in the sediments would help clarify its influence on the final sediment choice. According to a study made by Gibson and Robb (1992), juvenile plaice were less able to bury in sediments with comparable grain sizes with the gravel used in this experiment which supports that only three of the experimental fish chose gravel.

The grain size preference of the juvenile flatfish was recorded as the final sediment choice after 24 hours. This time frame was chosen because it was observed prior to the experiment that the flatfish had not moved out of the initial position until the 24 hours had passed. However, this time frame was set without any further investigation of the activity cycle of the flatfish. Consequently, it is possible that the obtained results would have differed depending on whether the final recording was made in a phase where the flatfish were more or less active. It could be argued that a more precise estimate of the preferred sediment choice could be done by an observation of the activity of the flatfish during the experiment which would allow for a determination of the extent to which each tested sediment was inhabited. Based on this, the preferred choice could be measured as the largest amount of time spent in one sediment. In a study by Gibson and Robb (2000), the final choice and the largest amount of time spent in one sediment was investigated, and it was found that the final choice and the most inhabited sediment was in agreement as the preferred sediment on 72% of occasions. Based on these results, the final sediment choice in this experiment was considered as the accurate preferred sediment.

7.2 Colour preference for juvenile flatfish

The results obtained from stage 1 and 2 of the colour selection experiment (section 6.2), indicate that juvenile flatfish have no clear preference between sand colour when comparing white- and black sand and when comparing light grey- and black plastic. Further, the results obtained from stage 2 of the experiment showed that only 4 out of the 40 fish tested chose to move out of the start position when the substrate was plastic sheets, which suggests that the influence of substrate is greater than colour. Hence, the juvenile flatfish prefer substrates, they can bury in. The obtained results are not in accordance with what was predicted or the results found in the study by Ryer et al. (2008). In the study by Ryer et al. (2008), the flatfish were given a choice between light- and dark sand and all flatfish were acclimated to either light- or dark sand for four to six weeks. In the study, a preference for light sand was found for the flatfish, which had been acclimated to light sand whereas no clear preference between light- and dark sand was found for the flatfish acclimated to dark sand. The results from the study by Ryer et al. (2008) and from the present experiment could indicate that juvenile flatfish have no inherent colour preference and that it is possible to change their preferences when acclimating them to sand colours ranging from light- to dark sand.

In the conducted colour selection experiment only preference between black- and white/light grey substrate was tested and the number of fish found in the coarse sand in the start position counted as an invalid choice. The first time stage 1 of the experiment was conducted, it was observed that the black- and white sand were chosen more frequent than the coarse sand as only 5 out of the 40 fish tested chose the coarse sand. As the particle sizes of the black- and white sand were smaller than the coarse sand this is in accordance with the results obtained from the grain size experiment which showed that juvenile flatfish prefer the sediment with the smallest grain size. However, the second time stage 1 of the experiment was conducted the positions of the coloured sand were mirrored and it was observed that a larger number of flatfish were found in the coarse sand as 17 out of the 40 fish tested chose the coarse sand. This somewhat contradicts the obtained results from the grain size experiment which might suggest that other factors were of influence such as the time spent in captivity for the flatfish or that the flatfish were weary after being reused in the experiment over a long period of time. A difference in behavioral response was found between wild and cultured juvenile winter flounder in a study by Fairchild and Howell (2004) which indicates how difficult it is to conduct behavioural experiments with fish. It can therefore be questioned how reliable results from behavioural experiments really are. However, as the effect of coarse sand was not tested in this project, it can not be concluded whether the observed tendencies really are of any significance. To avoid the flatfish from choosing the coarse sand in the start position, the coarse sand could be replaced with gravel which was found to be the least preferred sediment type in the grain size experiment. This change would most likely force the flatfish in a higher extent to chose between the black- and white sand.

7.3 Behavioural responses of juvenile flatfish to hydrogen sulphide

It was expected that the juvenile flatfish would flee from the sand containing hydrogen sulphide. However, the results obtained from the experiment (section 6.3) showed that in only 2 out of the 20 trials did the flatfish flee from the sand containing hydrogen sulphide (figure 17b). A significant difference was found between the starting area and the surrounding area for both containers, which indicates that the sand containing hydrogen sulphide did not cause any change in the behavioural response of the juvenile flatfish. Based on the results from the grain size experiment, the grain sizes of the two sediments used in the hydrogen sulphide experiment could be considered as an influencing factor on the final choice. In addition, the obtained results from the colour selection experiment indicate that the flatfish had no preference for sediment colours and due to this, the colours of the sediments used are not considered as an influencing factor. The sand containing hydrogen sulphide collected from Nivå Bay has overall slightly smaller particle sizes compared to the fine sand (appendix A.2, table 10). This might lead to the suggestion that the juvenile flatfish choose the sand containing hydrogen sulphide due to the ease of burying themselves despite of the possible effect from hydrogen sulphide. As described (section 7.1), a further investigation of grain size preference for juvenile flatfish would allow for a greater differentiation of the final choice between the fine sand and the sand containing hydrogen sulphide.

When comparing the measured oxygen profiles in the starting area for both containers in figure 18, the fine sand ('container 1') which is the control sand appear as a more suitable habitat due to the slight change in DO from approx. 329 μmol/L to 300 μmol/L through a depth of 18,000 μm. The slight change in DO is properly a result of the thorough rinsing of the control sand leading to almost no organic material consuming the oxygen. The oxygen profiles measured in the starting area with sand containing hydrogen sulphide, show rapid changes in the amount of DO only few millimeters into the sand. From a depth of 1,400 μm to a depth of 8,000 μm in the refilled sand in the morning, the amount of DO is almost completely depleted ('container 2 - morning'). It could be expected from the results that the flatfish would be less likely to bury themselves into the sand containing hydrogen sulphide due to the possibility of suffocation. However, the GoPro camera revealed that the flatfish in both containers showed the same tendency to bury themselves whether it was in the control sand or in the sand containing hydrogen sulphide. This could suggest, that when the flatfish bury in the sand, the surrounding sand could be additionally oxygenated to an extent to which the flatfish needs for DO is fulfilled and therefore it is not affected by the low amount of DO in the rest of the sand. Furthermore, a slight difference is seen between the two oxygen profiles measured in the morning and in the afternoon in container 2 ('container 2 - morning' and 'container 2 - afternoon'). In the afternoon, the amount of DO decreases at a slightly lower depth compared to the morning where the amount of DO is almost depleted at a depth of 8,400 μm. This could indicate that an oxygenation of the sand had occurred throughout the day. It can therefore be considered if the oxygenation of the sand could have a potential effect on the final choice of the flatfish when conducting trials in the afternoon. However, as only two flatfish in all the conducted trials chose to flee from the sand containing hydrogen sulphide and the difference being only approx. 400 μm when the DO is almost depleted in the two oxygen profiles, this seems unlikely. Conversely, the difference seen in the two measured oxygen profiles might be due to the described difficulties which evolved during the measurements.

Based on the obtained results (section 6.4) from the analysis of iron in the core samples collected from the starting areas of container 1 and 2, it can be seen that the mean total concentration of iron (Fe^{+2}+Fe^{+3}) is larger in the sand containing hydrogen sulphide than in the control sand. Due to this it can be considered whether the conditions of the control sand is representative for real seabed conditions in the sea. When comparing the ratio between reduced iron (Fe^{+2}) and oxidized iron (Fe^{+3}) in the control sand and the ratio in the sand containing hydrogen sulphide (table 8) it is seen that the sand containing hydrogen sulphide overall have higher ratios which indicates that the respective sand layers are more reduced and therefore as described in section 5.4 it is expected that these layers contain a higher production of hydrogen sulphide. When oxygen is present in the sand, the reduced iron (Fe^{+2}) is rapidly oxidized (Fe^{+3}) and due to this a lower production of hydrogen sulphide in the sand would be expected. The measured oxygen profile 'container 1' shows that oxygen is not depleted within the first 2 cm of the sand layer. This is in agreement with the relatively low ratio found between Fe^{+2} and Fe^{+3} as the sand is not fully reduced due to the presents of oxygen. When comparing oxygen profile 'container 2 - morning' and 'container 2 - afternoon' with the measured ratio between Fe^{+2} and Fe^{+3} it is seen for both oxygen profiles that oxygen is fully depleted within a depth of 2 cm which is in accordance to the relatively high ratios found as it indicates that the hydrogen sulphide is oxidized directly with the oxygen as there is little or no available Fe^{+3} resulting in anoxic conditions (Moodley et al., 1998).

The ratio between Fe^{+2} and Fe^{+3} for the core sample collected from the sand containing hydrogen sulphide in the morning was 10.198 and for the core sample collected from the same sand in the afternoon was 80.897. This indicates that a higher production of hydrogen sulphide had occurred with time. However, this is not in agreement with the measured oxygen profile ('container 2 - afternoon') which suggested that an oxygenation throughout the day might have occurred. If an oxygenation had occurred, Fe^{+2} would have been oxidized to

Fe^{+3} resulting in a lower ratio. A possible reason for the difference between the obtained results from the measured oxygen profile and the core sample could be that the core sample was collected from an area in the sand with an overall higher amount of iron and that no oxygen had yet penetrated through the sand in that area. This could also explain the dissimilarity between the two core samples collected in the sand containing hydrogen sulphide. In relation to this, it can be considered if the amount of hydrogen sulphide in the sand was high enough to cause an effect on the juvenile flatfish. The sand was as mentioned collected from Nivå Bay in the beginning of March. As described by Christensen et al. (2002), the amount of hydrogen sulphide is lowest during winter and highest in late summer due to an increase in the amount of DOM in the seabed. Therefore, it would have been more ideal to collect the sand in late summer. However, this was not a possibility as this project ran from the start of February to mid-May.

In the hydrogen sulphide experiment, the duration of each trial was one hour and it can be considered whether this period was long enough. It is possible that the flatfish were still experiencing stress due to the rapid change in habitat within the one hour trial. Therefore, it can be considered if a longer time frame would ensure adaptation to the new habitat before recording the final choice of the juvenile flatfish. However, a longer time frame could result in an oxygenation of the top layer of the sand containing hydrogen sulphide, leading to a decrease in the potential effect of hydrogen sulphide on the juvenile flatfish.

7.4 Potential nursery areas in terms of grain size, colour, and hydrogen sulphide

Bellevue Beach is anticipated to be a potential nursery habitat due to the fact that it is where the juvenile flatfish were caught. When comparing the particle size ranges of the surface sand collected at Bellevue Beach with the fine sand, the juvenile flatfish had a preference for in the grain size experiment, it is seen that the sand from Bellevue Beach has larger grain sizes (appendix A.2, table 10). This indicates that even though juvenile flatfish have a preference for the fine sand, they still live in habitats with larger grain sizes which was also seen in the grain size experiment were a small number of flatfish chose beach sand, coarse sand, and gravel instead of the fine sand. The same arguments can be applied when comparing the surface sand collected at Horsens Fjord with the fine sand. However, the sand from Horsens Fjord has larger grain sizes than the sand from Bellevue Beach which might indicate that Horsens Fjord seems less likely as a suitable nursery habitat regarding grain sizes. On a further note, only one surface sample from Horsens Fjord and Bellevue Beach were collected. It can be argued that a collection of multiple samples would allow for a more accurate investigation of the two areas as potential nursery habitats for juvenile flatfish in terms of grain sizes. When comparing the colour of the collected sand from Bellevue Beach and Horsens Fjord (figure 13), it is seen that the colours are in the range between the white- and black sand tested in the colour selection experiment. As the flatfish showed no clear preference between the tested white- and black sand (6.2), the colours of the sand from Bellevue Beach and Horsens Fjord are not expected to be of any influence for the choice of nursery area.

It is unclear whether Horsens Fjord is a suitable habitat for juvenile flatfish when only considering the grain size of the sediment. Therefore, an investigation of the extent to which Horsens Fjord is affected by the production of hydrogen sulphide would have been ideal. However, as the core samples collected in Horsens Fjord were mixed, a determination of the distribution of the concentrations of Fe^{+3} and Fe^{+2} was not possible. Furthermore, it is known that Horsens Fjord is in a bad ecological state, and therefore an investigation of additional factors such as water quality would be ideal to determine the possibility of Horsens Fjord as a nursery area. However, it is possible that, the marine restorations done by project Kysthjælper (appendix A.5) and other future restorations might ensure that Horsens Fjord will become a potential nursery habitat in the future.

It was possible to determine the concentration of iron in the samples from Bellevue Beach. It is seen that the amount of Fe^{+2} at Bellevue Beach (table 8) is approx. 2.5 times greater than the concentrations of Fe^{+3} which means that hydrogen sulphide is present in the sediment. However, the amount of hydrogen sulphide might not be the deciding factor for whether Bellevue Beach is a potential nursery area or not. Furthermore, it can be considered that since the core samples from Bellevue Beach was collected in early spring the production of hydrogen sulphide was at its lowest and therefore it is only representative for the conditions at Bellevue Beach in winter/early spring. However, it is expected that the production of hydrogen sulphide will increase during summer due to a higher degradation of DOM in the seabed which might challenge Bellevue Beach as a potential nursery area. Conversely, table 8 shows that a high amount of Fe^{+3} is still available in the sediment at Bellevue Beach which can be bound to the hydrogen sulphide if an increase should occur.

7.5 Juvenile flatfish as bio-indicators of seabed quality

Based on the results obtained from all conducted experiments and the investigated surface- and core samples, a consideration of the possibility of using juvenile flatfish as bio-indicators of sea bed quality can be made. The results from the grain size experiment (section 6.1) showed that the juvenile flatfish had a clear grain size preference and due to this juvenile flatfish can be considered as a satisfying bio-indicator as they showed behavioural responses to the tested environmental changes in terms of grain size. Further, the results from the colour selection experiment (section 6.2) showed that juvenile flatfish had no preference between the black- and white sand or the black- and light grey plastic. Therefore, it can be argued that juvenile flatfish is an inadequate bio-indicator in terms of substrate colour as it had no impact on their behaviour. However, the results obtained from stage 2 of the colour experiment (section 6.2) showed that only 4 out of the 40 fish tested chose to move out of the coarse sand when the substrate was changed from sand to plastic which further supports that juvenile flatfish can be considered as a satisfying bio-indicator regarding substrate type. This would also seem plausible due to the fact that juvenile flatfish live within the sediment of the seabed.

When comparing the ratio between the amount of Fe^{+2} and Fe^{+3} in the sand from Bellevue Beach with the sand containing hydrogen sulphide from Nivå Bay (section 6.4), it is seen that the ratio is greater in the sand containing hydrogen sulphide than in the sand from Bellevue Beach which presumes that the juvenile flatfish were exposed to larger amounts of hydrogen sulphide in the laboratory than at Bellevue Beach.

The results gained from hydrogen sulphide experiment (section 6.3) showed no change in the behaviour of the juvenile flatfish when exposed to sand containing hydrogen sulphide. However, when comparing the ratio between the amount of Fe^{+2} and Fe^{+3} in the sand containing hydrogen sulphide from Nivå Bay (section 6.4) with the sand from Bellevue Beach, it is seen that the juvenile flatfish were exposed to larger amounts of hydrogen sulphide in the laboratory as the ratios are greater in the sand containing hydrogen sulphide. As the juvenile flatfish did not show any change in the behavioral response, it indicates that the juvenile flatfish have a high tolerance to hydrogen sulphide. However, it can be argued if the core samples from Bellevue Beach can be compared to the core samples from the sand containing hydrogen sulphide as the core samples from Bellevue Beach is collected directly from the top layers of the seabed whereas the sand containing hydrogen sulphide is dug from the sediment in Nivå Bay. But since they did not respond to the amount of hydrogen sulphide in the experiment, juvenile flatfish can be considered as a poor bio-indicator in terms of hydrogen sulphide when the ratio between Fe^{+2} and Fe^{+3} is on or below 80.897. However, a further investigation of the exact maximum concentration level of hydrogen sulphide which juvenile flatfish can tolerate before any behavioural responses are observed is needed to fully conclude upon this.

As described by Kuklina et al. (2013) and McGeoch et al. (2002), the right selection of the bio-indicator species is important particularly when investigating ecological integrity. Therefore, it can be considered in terms of hydrogen sulphide whether another bio-indicator specie more sensitive to hydrogen sulphide than juvenile flatfish should be used in future investigations.

8 Conclusion

The main aim of this project was to investigate the possibility of using juvenile flatfish as bio-indicators of seabed quality in coastal zones, and to investigate if nursery habitats can be identified in terms of sediment grain size, -colour, and hydrogen sulphide to pin-point potential nursery habitats for juvenile flatfish. In this project, juvenile flounder (*Platichthys flesus*) and plaice (*Pleuronectes platessa*) were used in a bio-indicator experiment referred to as the hydrogen sulphide experiment, to investigate the behavioural responses of juvenile flatfish when exposed to sand containing hydrogen sulphide, which is toxic for oxygen respiring organisms. Prior to the hydrogen sulphide experiment, two experiments that investigated the preference of sediment characteristics, i.e. grain size and colouration were conducted. Thus, allowing a more thorough interpretation of the behavioural responses caused by hydrogen sulphide. The results from the grain size experiment showed that juvenile flatfish prefer the finest sediment below 0.5 mm when given a choice. Furthermore, no preference for colouration was apparent, i.e. between black- and white sand and black- and light grey plastic sheets. Therefore, it can be assumed that colour is not considered of any influence, whereas grain size could have been an influencing factor in the hydrogen sulphide experiment. In the hydrogen sulphide experiment, the habitat of juvenile flatfish was simulated, where flatfish were exposed to hydrogen sulphide. Based on the results obtained from the experiment, it can be concluded that the juvenile flatfish did not show any change in behavioural response when exposed to sand containing hydrogen sulphide compared to the juvenile flatfish in the control trails. Only few juvenile flatfish chose to flee from the sand containing hydrogen sulphide. This was despite that the sand was almost fully reduced as it was dominated by Fe^{+2} indicating a presence of hydrogen sulphide which was also supported by the measured oxygen profiles showing that the amount of dissolved oxygen was rapidly depleted in a depth from 1,400 μm to 8,000 μm in the morning and from 1,400 μm to 8,400 μm in the afternoon.

Based on the hydrogen sulphide experiment and the pre-experiments, the possibility of using juvenile flatfish as bio-indicators of seabed quality in coastal zones was investigated. In terms of grain size, juvenile flatfish is considered as a possible bio-indicator since they showed clear behavioural responses to sediment changes. However, in terms of colour, juvenile flatfish is not considered as a bio-indicator as they did not show any behavioural responses to the tested colours. When it comes to the hydrogen sulphide experiment, the juvenile flatfish is considered as a poor bio-indicator for conditions were the concentration of Fe^{+2} is 80.897 times greater than the concentration of Fe^{+2} and ratios below this. This is due to no change in the behavioural responses even when the ratio indicates a higher amount of hydrogen sulphide compared to the amounts found in Bellevue Beach where they were caught. To fully conclude on the possibility of using juvenile flatfish as bio-indicators it is suggested that further investigations are needed in terms of a longer time frame for each trial in the experiment and an examination of the maximum tolerance of hydrogen sulphide for juvenile flatfish.

For the examination of a potential nursery habitat Horsens Fjord was investigated. The sand from Horsens Fjord has larger grain sizes than the sand from Bellevue Beach, which is anticipated to be a nursery area. This might indicate that Horsens Fjord seems less likely as a potential nursery area in terms of grain sizes. Furthermore, as the collected core samples from Horsens Fjord could not be analysed for iron, it is unclear whether Horsens Fjord is a suitable habitat for juvenile flatfish when only considering grain size. However, it is possible that, future marine restorations both by the project Kysthjælper and other organisations could enhance the possibility of Horsens Fjord becoming a nursery habitat for juvenile flatfish.

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A Appendix

A.1 Appendix 1 - Conditions of the arena

The grain size experiment (described in section 5.2) and the colour selection experiment (described in section 5.3) were conducted in an arena which contains five separated lanes that is divided into five spaces. Each lane consists of two areas in each end of the arena (24cm times 16cm) and three bigger areas in the middle (32cm times 16cm), see figure 19. To confine specific areas in the arena black dividing plates can be placed within the arena.

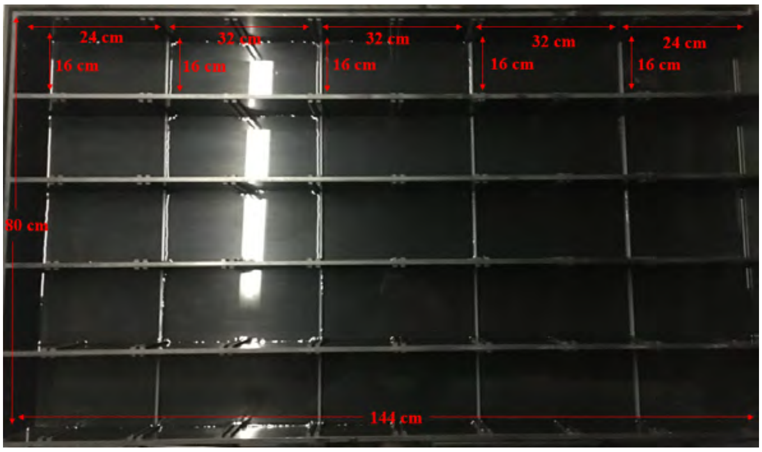

Figure 19: The figure shows the arena without any substrata. All measurements given in the figure are the internal dimensions of the arena.

Furthermore, water was continuously flowing through the arena, and table 9 shows the temperature, salinity, and pH of the water.

Table 9: Temperature, salinity, and pH of the water flowing through the arena.

Water temperature [°C]	Salinity [ppt]	pH
10.2	10.56	7.86

A.2 Appendix 2 - Grain size analysis of sediment

The grain size analysis were made after all experiments were terminated and a total of 10 sediment samples were analyzed, all sediments and the results from the grain size analysis can be found in table 10. Before the grain size analysis was performed each sample of sediment were dried in a 60° warm oven for 48 hours and then cooled for a couple of days. The set of sieves used in the grain size analysis had mesh sizes of 2.0-1.0-0.6-0.355-0.250-0.180-0.125-0.090 and 0.063 mm and the weight of all sieves were measured before the beginning of the analysis. For the analysis the sieves were stacked on top of each other and then placed on the sieving machine, see figure 20a. In the grain size analysis 100 g of each sample was used and each sample was transferred to the top sieve which were covered and fasten in the sieving machine. All samples were sieved for 15 minutes at an amplitude of 1.50 after which the weight of all sieves were measured again and the mass percentages was determined using equation A.2.

$$\text{Mass percentages } = \frac{\text{Weight of particle fraction in each sieve}}{\text{Total weight of particle fraction}} \cdot 100\%$$

(a)

(b)

Figure 20: (a): Sieving machine with the sieves stacked on top of each other. **(b)**: The set of used sieves, each containing different grain sizes after 15 minutes of sieving. The sample in the sieves is from Horsens Fjord.

Table 10: Results from the grain size analysis performed for all sediments used. It is important to notice that for the performed hydrogen sulphide experiment (section 5.4) both sand from Nivå Bay and fine sand were used and the results of the grain size analysis for the fine sand is found in the column for the grain size experiment (section 5.2) as the fine sand used was the same in both experiments.

Particle size range (mm)	**% Particle size fraction by weight**									
	Use of sediment									
	Grain size experiment				***Colour selection experiment***			***Hydrogen Sulphide Experiment***	***Sediment collection***	
	Fine	Beach	Coarse	Gravel	Black	White	Coarse	Nivå Bay	Bellevue Beach	Horsens Fjord
>2.00	0.00	0.49	0.00	88.61	0.00	0.48	0.00	1.97	2.93	15.46
1.00-2.00	0.49	0.49	55.12	8.91	0.97	0.96	55.12	3.45	8.29	7.73
0.600-1.00	0.97	51.72	39.12	1.98	1.46	3.35	39.12	4.43	40.00	14.49
0.355-0.600	56.31	43.84	3.90	0.50	46.12	79.43	3.90	8.87	35.61	30.92
0.250-0.355	38.83	1.97	0.98	0.00	33.50	12.44	0.98	30.54	6.83	14.98
0.180-0.250	2.91	0.49	0.49	0.00	11.17	1.91	0.49	45.81	4.88	8.70
0.125-0.180	0.00	0.49	0.00	0.00	4.85	0.48	0.00	0.99	0.98	5.31
0.090-0.125	0.00	0.00	0.00	0.00	0.97	0.00	0.00	2.46	0.00	0.97
0.063-0.090	0.00	0.00	0.00	0.00	0.49	0.00	0.00	0.49	0.00	0.48
<0.063	0.49	0.49	0.49	0.00	0.49	0.96	0.49	0.99	0.49	0.97

A.3 Appendix 3 - Colour selection experiment

The two following tables show the results from stage 1 and 2 of the colour selection experiment.

Table 11: (a): Stage 1: Sand colour selection. **(b)**: Stage 2: Plastic colour selection.

(a)

	Sand colour		
Trial	White	Black	Coarse
1	3	5	2
2	3	6	1
3	3	4	3
4	1	6	3
5	5	2	3
6	4	3	3
7	2	3	5
8	6	2	2
Total	27	31	22

(b)

	Plastic colour		
Trial	Light grey	Black	Coarse
1	0	1	4
2	0	1	4
3	0	0	5
4	0	0	5
5	0	0	5
6	1	5	4
7	0	0	5
8	1	0	4
Total	2	2	36

A.4 Appendix 4 - Conditions of the set up for the hydrogen sulphide experiment

The hydrogen sulphide experiment (described in section 5.4) were conducted in a tub with the dimensions 159.4cm times 99.7cm containing two containers with each container having a dimension of 34 cm times 23.3 cm, see figure 21.

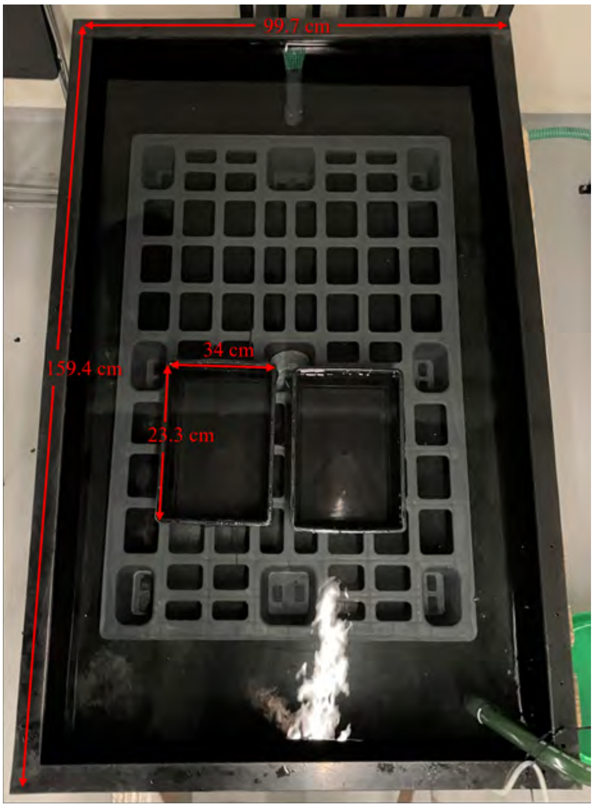

Figure 21: The figure shows the dimensions of the tub and the two containers.

A circular area used as the starting area were placed in each container, see figure 22. The area is 314.16 cm^2.

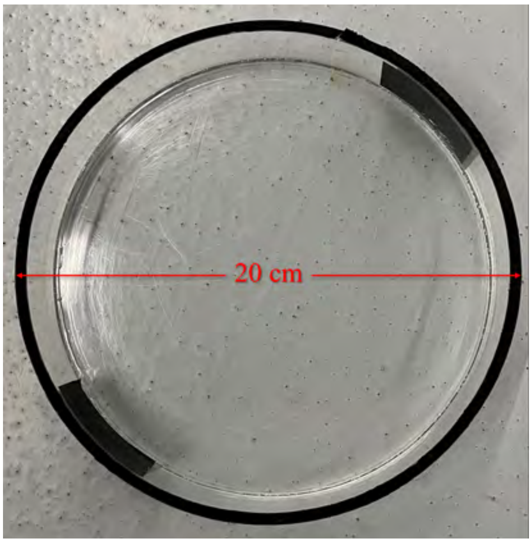

Figure 22: The circular starting area with a diameter on 20cm.

The water flowing continuously in the tub and in the two containers had the pH, temperature, and salinity found in table 12.

Table 12: Temperature, salinity, and pH of the water flowing through the tub and the two containers.

Water temperature [°C]	Salinity [ppt]	pH
10.2	10.56	7.86

A.5 Appendix 5 - Overview map of areas for planting of eelgrass and location of rock reefs

Figure 23: The overview map shows areas with existing eelgrass (red lines), and where project Kysthjælper has planted eelgrass (green boxes) and placed stones to create stone reefs (purple boxes). At area Ho 3 and O2 the planting of eelgrass did not happen (Kysthjælper, 2019).

A.6 Appendix 6 - Information on bought sediments

Table 13: The product names of the bought sediments used in the experiments. The table includes the grain size and the product numbers. *Beach sand is a mixture of fine sand and coarse sand.

Experiment	Experimental name	Product name	Grain size (mm)	Product number	Store
Grain size - round 1	Fine sand	Flod sand	0.1-0.5	15070	MiniZoo
	Coarse sand	Flod sand	0.7-1.2	15072	MiniZoo
	Gravel	Flod grus	2-3.5	15074	MiniZoo
Grain size - round 2	Fine sand	Flod sand	0.1-0.5	15070	MiniZoo
	Beach sand*	-	0.1-1.2	-	MiniZoo
	Coarse sand	Flod sand	0.7-1.2	15072	MiniZoo
Colour selection - stage 1	White sand	Lys flod sand	0.1-0.5	15069	MiniZoo
	Black sand	Akvariesand sort	0.2-0.6	151646	Maxi Zoo
	Coarse sand	Flod sand	0.7-1.2	15072	MiniZoo
Colour selection - stage 2	Coarse sand	Flod sand	0.7-1.2	15072	MiniZoo
Hydrogen sulphide	Fine sand	Flod sand	0.1-0.5	15070	MiniZoo