Evaluating the Marine Microbiota within the "Living Soil" as Water Quality Control Agents in the TinyOceans Hatchery System

A protocol for monitoring water quality in the TinyOceans and to investigate the potential microbial functional groups present in the "living soil"

Maria Madalena do Canto Brum de Menezes Pena

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Student number: 1772333

Examiner: Jack Middleburg

Supervisor: Michaël Laterveer

Utrecht University

Blue linked

Abstract

Microbial activity is crucial for life on Earth, it ensures cycling of the building blocks of all living organisms (i.e. Nitrogen, Phosphorus, Carbon etc.), allowing life to continue in this closed system. Equally, microbes play an essential role in the marine world. As such, mimicking this nutrient cycling property of these organisms could be extremely helpful when attempting to create a healthy and self-sustaining marine ecosystem on land (mariculture). The TinyOceans project was started with the intent of reducing environmental impact of the aquacultural industry as well as ensuring a healthier environment for the organisms being bred. The system consists of a tank with a "living soil" filled with microorganisms and a propeller mixing the water, mimicking the environment of a natural marine ecosystem on land. In this report, we investigated the efficiency of the microbial community present in a TinyOceans system and predicted the microbial functional groups playing a role in the cycling of the nutrients and maintenance of a healthy water quality. The system filled with adult flat oysters was monitored twice a week for 82 days, and the nutrient content of the water in the system was measured. Samples were taken from just above the living soil, from after the water had been through the living soil and from mid water column, opposite from the living soil. The different samples provided important information on the functioning of the system. When contrasting the nutrient content of the before and after microbial activity, we not only witnessed a balance between N fixation and N loss but also a good balance on the N:P ratio of the water. Both of which suggest that the water input in the system is being properly recycled and maintaining a healthy environment for the marine organisms within. One which could be comparable to natural marine ecosystems. Furthermore, we witnessed a 95% survival rate from the flat oysters and the spawning of same. As such, we concluded the TinyOceans system has the potential of indeed providing a comfortable and healthy environment for the bred organisms whilst being completely water circular.

Layman's Summary

Microbes can recycle the primary elements that make up all living organisms (carbon, phosphorus, nitrogen) and thus ensure life on Earth, a closed system. As such, BlueLinked, when striving to improve the sustainability of breeding marine organisms on land, thought about this microbial property and started the TinyOceans project. The main goal of this project is to mimic the conditions a marine organisms would encounter in their natural habitat, allowing full and balanced ecosystems to flourish within the breeding tanks. Creating a completely circular water system without the need for antibiotics or any other chemicals. The goal of this experiment was to evaluate the efficiency of the system, particularly the role of the microbial communities in ensuring this efficiency in a tank with flat oysters. To investigate this, the nutrient content of the water was monitored. Samples were taken twice a week in three different locations: "dirty", "clean", "control". The comparison between the dirty and clean water provided insight into the ability of the microbes to recycle the harmful nutrients and prevent toxicity. The monitoring of the control provided insight into the conditions the flat oysters were experiencing. Our results not only suggested that the microbial community within the tank was performing well, but they also showed comparable characteristics to those you would encounter in marine ecosystems. More importantly, there was a 95% survival rate from the flat oysters introduced in the tank. As such, the conclusion that the TinyOceans systems have the potential of improving the industries sustainability by being completely water circular whilst maintaining a healthy environment for the marine organisms was drawn.

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1. Introduction

The Earth is a closed system and nutrients are a limited resource, as such, recycling is essential. This recycling is driven by the omnipresent microbiota in the biosphere. Microbes always affect the environment in which they grow. Their functions range from recycling processes, to forming all kinds of close symbiotic relationships with all walks of life. To this day we have witnessed that these effects can be beneficial, harmful, or neutral. However, the most significant effect of the microbes on earth is their ability to recycle the primary elements that make up all living systems, particularly carbon (C), phosphorus (P), and nitrogen (N) (Gupta et al., 2017). There is very likely no naturally occurring organic compound that cannot be degraded by some microbe (Gupta et al., 2017). The microbial communities present in the ocean, within the water column or seabed are no exception. These microorganisms are crucial for a healthy functioning marine ecosystem. Around 40% of the planet's primary productivity is a result of marine microbial communities (Duarte & Cebrián, 1996). They are also responsible for most of the ocean's respiration and key players that drive crucial transformations in marine nutrient cycling.

Nutrient cycling is an innate property of aquatic ecosystems; a property which could be extremely helpful when attempting to create a healthy and self-sustaining marine ecosystem on land (mariculture). Aquaculture has become of dire importance throughout the years as the world tries to feed an ever-growing population. The use of biomimicry could be extremely beneficial for improving and optimising this industry. Biomimicry is innovation inspired by nature, turning to the natural world for answers to the challenges we face. Learning from an entity that has been evolving and perfecting its ways for millions of years (Baumeister et al., 2012). "Life creates conditions conducive to life", a quote by Janine Benyus is the underlining belief of this discipline and dictates its intention. Nature values optimisation of resources as opposed to maximising output and that is why biomimicry has the power of sustainability and growth (Baumeister et al., 2012). A characteristic from which we could greatly benefit for improving the sustainability of the aquacultural industry.

To achieve this however, there needs to be a deep understanding of the biological model used, in this case: the process of nutrient cycling and the role of microbes in maintaining an optimal environment which is conducive to other life and growth.

1.1. Nutrient Cycling

1.1.1. Nitrogen Cycle (N-cycle)

The Nitrogen (N)-cycle is one of the most important biogeochemical cycles due to its direct impact on the ocean's primary productivity and is driven by complex microbial transformation. The microbial processes within this cycle limit the productivity of a marine ecosystem because N availability is one of the main limiting factors for plant biomass production (Gupta et al., 2017).

This cycling is mostly carried out by Bacteria, Archaea, micro-eukaryotes or protists. There are multiple routes by which N can enter the marine section of the N-cycle. Rivers, land waste such as agricultural, sewage or industrial waste and rainfall are the main inputs of N into the oceans. Subsequentially, a series of physical processes will transport nutrients and facilitate their cycling. Each of these physical processes has different consequences and effects (Voss et al., 2013).

The microbially driven biochemical processes governing carbon and nutrient cycles depend greatly on these physical processes and the terrestrial and atmospheric inputs mentioned above due to their influence on oxygen levels and the form of N available in the water. Both of which are of dire importance for the determination of the bacterial communities' dynamics in these locations and the rates at which the cycling happens (Fowler et al., 2013). For instance, in oligotrophic (nutrient poor) environments regeneration occurs around 20 times before the N is exported (out of the euphotic zone). In contrast, in coastal, eutrophicated or upwelled regions this regeneration can happen around once per year. These different processes encompassed within the N-cycle can be divided into three categories: Fixing/gaining, retaining, and losing (**Figure 1.**) (Parsons & Harrison, 1983).

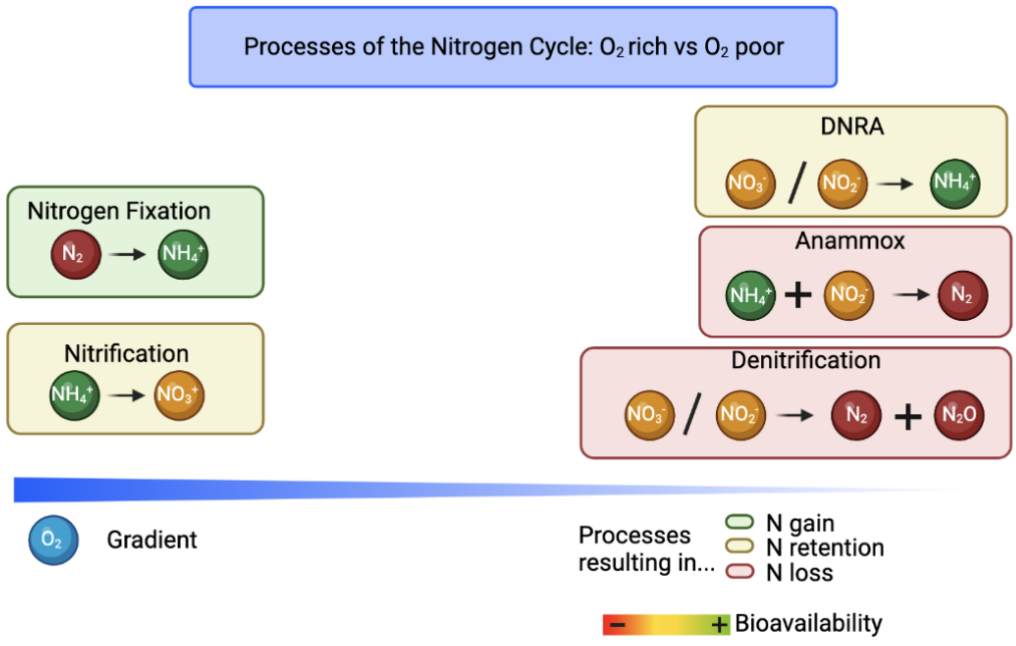

Figure 1. Summary of the different processes within the N-cycle and their interaction with O_2.

Gain

Nitrogen Fixation

The most abundant form of dissolved N in the ocean is dinitrogen (N_2) (95%), however, this form of N is not greatly bioavailable, only being assimilated by N-fixing microbes. For this reason, N-fixation is one of the most crucial steps in the N-cycle. There remains much mystery around this process, especially around its magnitude, however, recent updates have shed some light into these queries (Voss et al., 2013).

N-fixation is the transformation of dinitrogen to ammonia/ ammonium and the process by which N becomes more bioavailable, meaning that a greater diversity of microbial and plant species can assimilate this nutrient without any further transformations or energy expenditure. This process can be carried out by multiple organisms including cyanobacteria (either free-living or in symbioses with some marine diatoms), dinoflagellates, alpha- and gamma-proteobacteria, and some archaea (Mulholland & Lomas, 2008). This is the process that sources the most new N (bioavailable N that enters the ocean from the atmosphere) and is an important source to support biological production in oceanic environments (Bombar et al., 2011).

All N-fixing bacteria use nitrogenase to break down the triple bond between the two N atoms in dinitrogen. Many nitrogen fixers are autotrophic and carry out photosynthesis as a source of energy, which is why they live in oxygen-full waters. However, as this enzyme (nitrogenase) is inhibited by oxygen, N fixing organisms need to adopt strategies to create oxygen-free zones within oxygen-rich waters. Many cyanobacteria synthesize heterocysts, cells that adopt different strategies to slow down oxygen diffusion and ensure the separation of N-fixation from oxygen involving processes. Although not all marine cyanobacteria can synthesize heterocysts. *Trichodesmium spp*., for instance, form diazocytes in place of heterocysts. Diazocytes are groups of nitrogenase-containing cells that play similar roles but lack many of the protective mechanisms of heterocysts (Mulholland & Lomas, 2008). Other strategies involve the performance of N-fixation during the night when photosynthesis is halted and thus O_2 levels drop and allow the nitrogenase reaction to occur. Such strategy has been observed in *Candidatus Atelocyanobacterium thalassa*, a recently discovered cyanobacterium that does not fix CO_2 or perform oxygenic photosynthesis and thus is thought to require an obligate symbiotic relationship with the unicellular haptophyte alga, *Braarudosphaera bigelowiiIn* (Zehr & Capone, 2020).

However, sediment N-fixers such as *Deltaproteobacteria* face a different issue: they require an external source for carbon and energy (Kapili et al., 2020). Environments such as methane seep and whale falls can provide the needed carbon (Dekas et al., 2018) . This carbon source combined with their ability to use multiple terminal electron acceptors, including oxygen, nitrate, iron, sulfur, sulfate, and organic compounds, not only allows the thriving of diazotrophs in (deep-sea) sediment but also suggests that deep-sea diazotrophy is coupled to other biogeochemical cycles. The suggested taxonomic and metabolic flexibility in the diazotroph assemblage has led to the belief that benthic

diazotrophy may be a stable source of fixed N despite changing environmental conditions (Kapili et al., 2020). Leading us to believe we had greatly underestimated the magnitude of N-fixation.

Retention (Transformation):

Nitrification

Nitrification is the process by which ammonia (NH_3)/ ammonium (NH_4^+) is oxidized into nitrate (NO_3^-). This process is carried out by three types of organisms: ammonia oxidising bacteria (AOB) and archaea (AOA), that convert NH_3/ NH_4^+ into nitrite (NO_2^-); nitrite oxidising bacteria (NOBs), that convert NO_2^- into NO_3^-; and some who make the complete ammonia oxidation (comammox) (NH_3/ NH_4^+ into NO_3^-).

NOTE: Ammonium, NH_4^+, is the non-toxic, ionic state of ammonia NH_3. In a healthy environment about 95-99% of the combined concentration should be in the ionic for of NH_4^+.

It is a two-step process, firstly, the AOBs and AOAs oxidise NH_3 into NO_2^-. Previously, the AOA oxidation, like AOB oxidation, was thought to be a two-step process, however, after the ammonia oxidising step, the hydroxylamine is converted into nitric oxide before being finally converted into NO_2^-. The process by which this happens is still unknown, indicating that the functioning of AOAs is not fully understood (Lancaster et al., 2018).

The second step consists of the oxidation of NO_2^- into NO_3^-. This is performed by the NOBs and catalysed by nitrite oxidoreductase. Some marine NOBs (eg.: *Nitrospina*) can produce NH_3 from urea, thus sustain AOBs and AOAs that provide them and the rest of the ocean with NO_2^-.

With regards to comammox, the process was proposed for the first time in 2006 and the bacteria responsible for this, had not yet been isolated (Costa et al., 2006). The recent discovery of bacteria within the genus *Nitrospira* provided some more insight into the process. For instance, the genus was found to have a widespread occurrence and thus thought to play a key role in nitrogen cycling (Sakoula et al., 2021). Nonetheless, much mystery remains behind this process and especially behind the drivers of this process.

Assimilation

NH_4^+ is usually the preferred and thus dominant form of N being taken up by organisms, being taken up by both autotrophs and heterotrophs. This increased bioavailability is due to this form of N's energetic efficiency. NH_4^+ is already reduced and, as a common cellular transient in N metabolism, requires little additional energy for assimilation (Mulholland & Lomas, 2008).

NO_3^- is the most abundant form of bioavailable N, but it requires more energy to assimilate. Its assimilation requires the synthesis of NO_3^- and NO_2^- reductases (NiR), associated active transport systems, and the turnover of cellular ATP and

NADPH. Due to the many requirements for the synthesis of NO_3^- reductase, organisms require genetic adaptations to produce the necessary enzymes and transport systems. Diatoms have traditionally been the primary consumers of NO_3^-, however other examples include the phytoplankton *Synechococcus*, and some heterotrophic bacteria, although their capacity to do so varies according to their genetic make-up, physiological status, and nutrient environment (Mulholland & Lomas, 2008).

NO_2^-, unlike NO_3^-, is generally found in low concentrations, although, on occasion, it accumulates throughout the entire euphotic zone creating a primary NO_2^- maximum. Because the enzyme NiR is required for NO_3^- assimilation, organisms that assimilate NO_3^- can readily assimilate NO_2^-. However, there is a greater variety of microorganisms that can assimilate NO_2^- than those that do NO_3^- (Mulholland & Lomas, 2008).

N reaches higher trophic levels by the feeding and predation of the macrofauna, on other plants and animals.

Dissimilatory nitrate reduction to ammonia (DNRA)

Dissimilatory nitrate reduction to ammonia (DNRA) is an anaerobic process that reduces NO_3^- into NO_2^- and then to NH_4^+. It is mostly carried out by heterotrophic microbes but reduced inorganic compounds such as sulphur and iron can serve as electron donor for autotrophic processes. The heterotrophic DNRA is catalysed by the cytochrome C nitrite reductase, whereas the reduction of NO_2^- to NH_4^+ in the chemolithotrophic DNRA is mainly catalysed by the octaheme tetrathionate reductase. There is enormous diversity of organisms that can carry out DNRA. This process, although producing less energy, is favoured over denitrification within anaerobic and electron-donor- rich environments because it can accept more electrons from a single NO_3^- molecule, making it more efficient in NO_3^- limited contexts. Furthermore, this process feeds nitrification and anammox due to the production of NH_4^+ (Pajares & Ramos, 2019).

Loss:

Denitrification and Anammox

Oxygen concentrations tightly regulate this part of the N-cycle. This is because the presence of oxygen triggers some and inhibits other reactions. Nitrogen fixation has the potential to occur in both O_2 rich and O_2 depletion zones, however, the same cannot be said for other processes. Current estimates say that in oxic conditions ~265– 294 Tg N yr^{-1} is being fixed throughout the entire ocean and becoming more bioavailable, whereas anoxic conditions undo this work and (re)form gaseous nitrogen (dinitrogen) through the process of denitrification or anammox (anaerobic ammonium oxidation), causing a loss of ~275–481 Tg N yr^{-1}. For this reason, the N stock of the ocean largely depends on the balance between N fixation and N loss. A skewed balance towards denitrification and/or anammox, results in lower and potentially limiting N availability, decreasing primary production (Mulholland & Lomas, 2008). As N_2 is unavailable to non-nitrogen fixing phytoplankton, this activity also results in a loss of nitrogen from the cycle within the euphotic zone. Denitrification and anammox occur mainly in sediments but seldomly can also occur within the water column due to low ventilation rates and high oxygen assimilation.

Denitrification is the microbial process of reducing NO_3^- and NO_2^- to gaseous forms of nitrogen, nitrous oxide (N_2O) and dinitrogen (N_2) (Skiba, 2008). This process is carried out by facultative anaerobes of which most are heterotrophs, with the autotrophs also having a role in the sulphur and hydrogen cycles, using H_2 or S compounds as electron donors. This process, unlike anammox, produces N_2O as an intermediate, only to finally reduce it to N_2. N_2O is a gas and therefore may be released before it is consumed, resulting in about 7-35% of N_2O natural oceanic emissions (Pajares & Ramos, 2019).

Anammox is a reaction that oxidizes NH_4^+ to N_2 using NO_2^- as the electron acceptor. This process is carried out by CO_2 fixing bacterial autotrophs all belonging to the same order of *Planctomycetales.* Anammox has been estimated to be responsible for 50% of the marine nitrogen loss (Hu et al., 2011).

Both processes result in the formation of the gaseous form of N (N_2), with denitrification also releasing the strong greenhouse gas (GHG) N_2O. Furthermore, both processes are sometimes dependent on one another. In oxygen depleted zones (ODZs) within the water column this NO_2^- is made available due to denitrification, making anammox bacteria dependant on denitrification. Making denitrification the biggest cause for fixed N loss. In sediments, however, DIN is abundant and therefore, the proportion of anammox can be greater and most loss of N is attributed to anammox (Voss et al., 2013).

As mentioned, DNRA coexists with both these processes in anaerobic conditions, however, their interaction differs. While DNRA competes with denitrification for NO_3^-, reducing the N loss, it provides anammox with NH_4^+, thus favouring anammox over denitrification in conditions of low NO_3^- and increasing the loss of N from the cycle (Jensen et al., 2011). Another factor that defines the competition between anammox and denitrification is availability of organic matter, favouring denitrification when high.

In short, the N cycle is an extremely complex and the governing factors are largely unknown. Although it looks as if the interactions between the different processes and oxygen are well established, there are many more complex interactions occurring. One example is the unexpected increase in denitrification in oxygen-rich zones in the sediment. This occurs due to the almost simultaneous increase in nitrification rates, which results in the formation of nitrate and oxygen depletion, thus making a suitable environment for denitrification. Furthermore, there are very complex couplings of the different processes, many of which are still unclear (Webb & Eyre, 2004). For the sake of this research, the aforementioned biogeochemical processes were summarised to the level of understanding required.

1.1.2. Phosphorus Cycle

The P-cycle was thought to be a relatively simple (because it is not directly involved in redox transformations) and, although it is rapidly recycled by planktonic organism, it is considered an extremely slow biogeochemical cycle. Currently

its complexity, better appreciated than before, remains much of a mystery. The acknowledgement of the increased complexity of the P cycle has risen due to the investigation of the coupling of P with other biogeochemical cycles such as the N, the C and Fe cycles. P is a critical macronutrient for all living cells, as it plays a crucial role in the structural components of cells including DNA and in the transmission of chemical energy by the ATP molecule (Defforey & Paytan, 2018). However, it is a scarce and incredibly bio-limiting nutrient which mainly occurs in only one inorganic form (phosphate).

Due to the lack of a gaseous state, the input of phosphorus into the oceans greatly depends on the weathering of continental material such as apatite minerals (Guidry, 2002). Rivers are the most important provider of phosphorus, however, much of this P tends to be retained immediately within the continental shelf, thus rainfall and other atmospheric deposition pathways also play an important role on open ocean P (Paytan & McLaughlin, 2007). The physical processes playing the greatest role in this cycle would be upwelling, wind-induced mixing and eddies, which, along with seasonal changes, determine the concentration distribution of this nutrient (Dyhrman et al., 2007).

Phosphorus goes through different states in one cycle. Dissolved inorganic and organic phosphate are taken up by autotrophic and heterotrophic marine microorganisms. Around 50% of this uptake is attributed to bacteria (figure 2). Most phytoplankton are not able of up taking dissolved organic phosphate. When in oligotrophic conditions or when organic phosphate is in considerably higher concentration, they produce a membrane-bound or an extracellular enzyme (alkaline phosphatase) that cleaves the phosphate group and releases the phosphate ion which is taken up by the phosphate transport system.

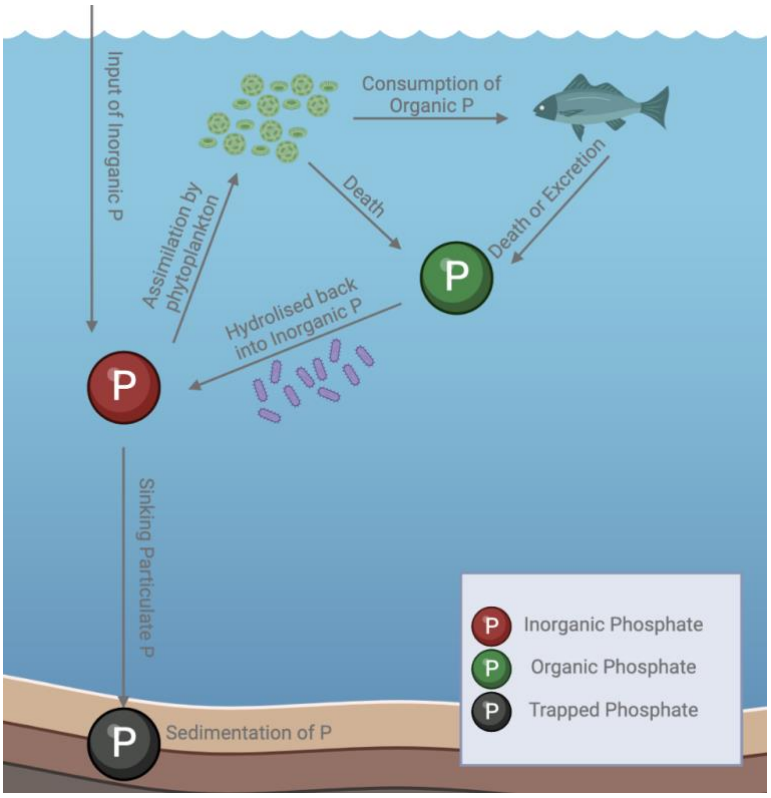

Figure 2. Schematic illustrating a simplified version of the phosphorus cycle.

After the assimilation of P by phytoplankton, the nutrient goes up the food chain in the form of organic matter and can be recycled through excretion, cell lysis and the animals' metabolism. Once P is supplied back into the water column, at oceanic pH, some of it is hydrolysed back to inorganic P, the rest is transformed by free living enzymes (phosphatase). On average, this assimilation and regeneration cycle occurs over two times before phosphate is removed by sinking particles. Oxygen rich environments are often also rich in iron and other metals which react with P forming sinking particulate P. Once it gets delivered to the anoxic sediment, this bond between the P and the metals break and P then forms calcium minerals (Paytan & McLaughlin, 2007).

Similarly, to the N-cycle described above, although there are continual breakthroughs, there are divergent views among scientists. While some describe it as an extremely complex cycle, others describe it as a simple and fast cycle. Perhaps this disagreement arises from the diverse focus within disciplines and coordinating research between the disciplines has the potential to better our collective understanding, resulting in a more complete systems view of the field (Falkowski, 1997; Tyrrell T., 1999; Voss et al., 2013).

Another debate that has been discussed endlessly is the answer to what is the ultimately limiting element for ocean productivity. According to Voss et al. (2013), the consensus seems to be that nitrogen is limiting on short timescales, whereas on geological time scales it seems to be phosphorus. Potentially owing to the higher apparent complexity on N remineralization and the sinking and burial of P in sediment.

As evidenced in the overview of the cycles mentioned above, microbial communities both within the water column and the seabed are crucial for the maintenance of these cycles which ensure healthy marine ecosystems in the world's oceans, including healthy fish and other seafood stocks. The field of microbial ecology rose in the 1970s, and the idea of using microbial communities in aquacultural practices was first considered by Dr. Voeker Rusch in 1977 (Zhou et al., 2009), it is not being investigated enough and thus, the industry might not be fulfilling its potential as a reliable and sustainable source of seafood and in some cases producing lower quality seafood.

1.2. Blue Linked

1.2.1. The company

The aquacultural industry has been growing faster for the past decades while the growth of fishing industry has been on the decline, due to falling wild fish stocks. Consequently, there is a growing pressure for aquaculture to ensure that future generations have access to a source of protein.

BlueLinked is an aquacultural company founded by Michaël Laterveer in 2011, with the intention of not only improving the sustainability of this industry but also recreating conditions analogous to those of the ocean for the breeding of marine organisms on land. This company was created alongside the Oceans at Work Foundation, a charity whose goal

was to establish a “future-proof” source of fish protein by supporting companies like BlueLinked that has the very same goals. Together, and with the help of Wadden Fund, the project “Sea Farm on Land” was underway.

The “Sea Farm on Land” project encompasses many, smaller, individual ventures. Currently, BlueLinked is focused on the establishment of the novel and circular hatchery system - the “TinyOceans”. This is where fish and other marine organisms will be raised from their larval stage up until their juvenile stage, when they would be transferred to an outgrow facility (another one of the ventures of the “Sea Farm on Land” project).

1.2.2. The “TinyOceans” Project

The main goal of this bio-inspired project is to mimic the conditions marine organisms would encounter in their natural habitat, allowing full and balanced ecosystems to flourish within these tanks, and creating a completely circular water system without the need for antibiotics or any other chemicals. Permitting 50% of the production to be used for the restoration of ocean stocks.

The tanks are filled with 6,000l of sea water and the concept relies on a living sand bed, a complete food chain and a bio-inspired mechanical fin which reproduces a natural water movement. The shape of the tank was designed to allow the water flow to be as constant as possible all throughout the water column due to the action of the fin. The angles of the bottom of this tank were designed to allow the guidance of waste and other natural decomposition products to the living sand bed, where organic matter would be remineralized into dissolved inorganic nutrients and made available to the small organisms at the bottom of the food web (autotrophs and some heterotrophs). Thus, this purifying part of the TinyOcean plays a huge role on the connection of all the mentioned components.

Currently, there are four tanks, one of which is operational at BlueLinked and its efficiency is monitored. This is where the research conducted and described in this report comes in. The tank used for the pilot study of the efficiency and water quality monitoring protocol was filled with phytoplankton that has been grown within the Bluelinked facilities and full-grown oysters which were expected to spawn as the temperature and the composition of the water became suitable.

1.3. Intentions

In this thesis, I will shortly discuss the different methods of seafood culturing, the current shortcoming of the industry, and how this novel system by BlueLinked may be an upgrade to a more efficient and sustainable form of culturing seafood. The efficiency of this system will be experimentally evaluated by monitoring the different concentrations of each form of N and P of the tank and analysing the interactions between them during a period of 11 weeks.
Initially, the goal of the experiment was to investigate the role and identity of microbial communities within the living seabed and the water column by monitoring water quality throughout the tank and dissolved oxygen consumption rates within the seabed. However, this soon evolved to become the investigation of the efficiency of this innovation, resulting

also in the development of a competent protocol that BlueLinked could implement long term to continuously monitor the health of their tanks.

To achieve this goal, the questions – How important is the role of microbes in maintaining a healthy water quality? Are the microbial communities within the tank efficient enough for regulating water quality to ensure a healthy environment for the survival of the flat oysters and their larvae? – had to be answered. Additionally, the need for changing current seafood production practices also needs to be evaluated.

The answer to the first question became evident from literature research and thus the expected answer to the second question would be that for the microbes to maintain an optimal water quality, a microbial community would have to be developed. If this community was successfully established, then the "TinyOcean" tanks would be expected to be self-sustainable in terms of water and nutrient cycling just like in nature. The expectation is that the concentration values of each form on N and P will eventually stabilise with time. The stabilising is suggestive of a good job from the microbial community at keeping the water quality good enough for flat oysters and their larvae to survive and thus will provide insight into the efficiency of the novel concept presented in the "TinyOceans" project. Finally, the expectation is that some of the challenges with current practices may be relieved with this innovation.

2. Seafood Production Practices

2.1 Aquaculture and Mariculture

Aquaculture is the farming of aquatic organisms, including fish, molluscs, crustaceans, and aquatic plants (FAO, 1988), while mariculture is a form of aquaculture that focuses solely on the cultivation of marine species as opposed to the full range of aquatic species and it is what is currently practiced at BlueLinked.

As previously mentioned, global aquaculture production has been growing fast, at rates of 8.3% per year, compared to less than 2% of fisheries, and 2.9% of livestock (Martinez-Porchas & Martinez-Cordova, 2012). This industry has been fundamental for improving food security and providing a good source of protein as well as providing millions of people with jobs and further dynamizing global economy: 47% (51 million tons) of the global human fish consumption (Turcios & Papenbrock, 2014). Nonetheless, this production must increase by nearly 60% above its 2018 statistics to supply seafood for the projected 2050 global population. There are multiple deterrents for this growth, some of which will be addressed later in this section.

2.2 Limitations and Progress

Previous fish production strategies were inefficient and were done in monocultures. The growing need for fish protein resulted in the industry's development and intensification. With it, came the use of hormones, antibiotics and the

increased nitrogen and phosphorus waste, causing both lower quality seafood and serious environmental problems. Since then, the sustainability of this industry has improved and some historical practices were incorporated to the new technologies (eg.: parallel production of fish with filter feeders and plants or algae, even in multi-trophic systems). Nonetheless, the production of sludge, the high energy requirement and dependence on frequent maintenance remained an issue (Turcios & Papenbrock, 2014).

In the early 2000s, as another attempt at improvement, a new way of aquaculture was developed: Recirculating aquaculture systems (RASs). RASs were developed with the intention of resolving many of the shortcomings of aquaculture and achieving a high-density intensive method, without increasing its economic cost or environmental harm. This system allowed aquaculture to be less influenced by climate change, to conserve water, to reduce pollution to the environment and having more control over emissions (Xiao et al., 2019). Modern closed RASs can operate with artificial seawater and less than 1% of water renewal per day as well as reduce disease outbreaks and parasites due to the lack of intermediate hosts (Turcios & Papenbrock, 2014).

RASs were initially not a very common practice and not always economically viable, but they were still considered a method of choice under certain conditions. At this stage, dissolved and particulate organic matter (DOM and POM), total suspended solids (TSS), nutrients such as N and P, and compounds such as therapeutants were of great concern due to their environmental impact. Efforts to reduce these emissions included changes in the feed and treatment methods to remove these harmful nutrients and compounds from the wastewater (Piedrahita, 2003).

2.3 Current Practices – Advantages and Remaining challenges

Practices such as seaweed mariculture have been recently regarded as the potential solution to several important Sustainable Development Goals due to the range of ecosystem services it could provide the world with. Currently, this practice accounts for about 50% of global mariculture production with the prospect of great and sustainable growth (Duarte et al., 2022). Seaweed mariculture contributes to the increasing C sinks whilst providing habitats, coastal protection, and provisioning for many industries such as food and medicine. The schematic illustrated in figure 3 perfectly synthesises the potential of the scaling up of Seaweed production (Duarte et al., 2022).

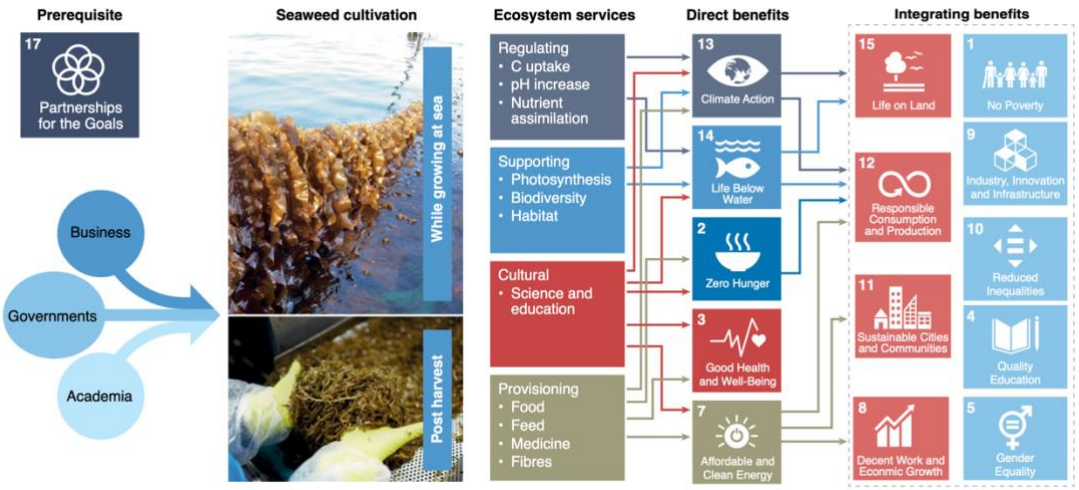

Figure 3. Seaweed production and utilization contributes to advancing several UN SDGs, which provide integrative benefits contributing to additional SDGs (Duarte et al., 2022).

More recently, the contribution of RASs method to seafood production has increased as the system has been developed and so has its complexity. Currently, RASs requires the linking of different devices to perform the different functions of physically filtering suspended solids, biologically filtering harmful levels of ammonia, nitrogen and nitrite and equipment to sterilize, add O_2, remove CO_2 and adjust water temperature (Xiao et al., 2019). Although there are multiple methods to perform each function, they all require the handling of complicated equipment. Furthermore, there is a lack of standardisation, as each piece of equipment requires different handling and assembly and thus furthering the complexity of this system. However, when compared to other, older aquacultural practices, such as open sea cage cultures, this system is less influenced by climate change, conserves water, is more intensive, and less polluting to the environment due to the better control over emissions. This is achieved by a combination of mechanical and biological filters that remove most substances of concern mentioned above (Section 2.2), sterilize the water and re-establish the balance of O_2 to CO_2 resulting in the successful recirculation of 95% of the water daily and improving aquaculture efficiency (Xiao et al., 2019).

However, the use of chemicals remains a problem in this industry. The incorrect use of some therapeutics can result in aquatic pollution, antibiotic resistance and harmful accumulation within human and animal tissues after they have been consumed. In Scotland for example, some parasiticides used were found to be toxic to crustacean or exhibit a range of ecological changes in the benthic ecosystem surrounding fish cages (Bloodworth et al., 2019; Pahl & Opitz, 1999; Vidalis & Nathanailides, 2020). Although this is an example of in situ aquaculture, similar consequences would occur from the discharge of in land fish production waste (Vidalis & Nathanailides, 2020).

Furthermore, this use of chemicals does not completely avert disease emergence and transmission from farmed species to wildlife populations (Mordecai et al., 2021). An example is the appearance of diverse infectious agents (*P. salmonis* and *R. salmoninarum*) of Pacific salmon (*Oncorhynchus spp*.) after the rapid growth of salmon farming in Chile (Figueroa et al., 2019; Mordecai et al., 2021). This has cascading effects for the rest of the food chain, in this case, it also led to the decline of the already endangered killer whales (*Orcinus orca*). Salmon, however, is not the only species being severely affected by diseases brought by fish production (Figueroa et al., 2019).

Finally, the range of species being cultivated is increasing, with about 0.17% of known marine plant species and 0.13% of known marine animal species being cultured. This has consequentially broadened the number of trophic levels being cultured which has allowed for a bigger mariculture output without putting more pressure on wild stocks (Duarte et al., 2007). Nonetheless, the industry is still too dependent on wild fish stocks (about 70% fishmeal used to feed the cultured fish are made from wild catch) and thus, not yet a sustainable protein source (Boyd et al., 2022).

In sum, this industry has been criticised by many due to its negative environmental impact and although many efforts have been put into improving the sustainability of this industry, there is still a long way before this industry can be considered sustainable.

2.4 TinyOcean Project

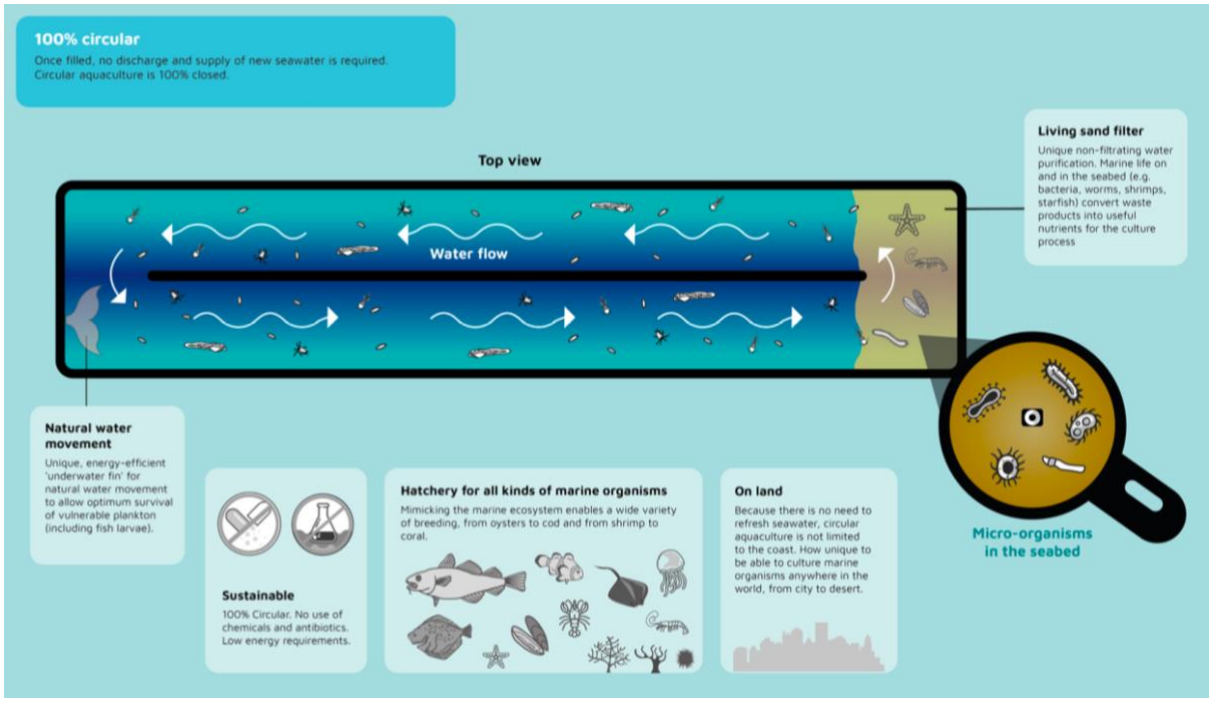

Figure 4. Figure illustrating the concept of the TinyOcean project (Bluelinked, 2022).

The combination of simplicity and efficiency of this TinyOcean system, puts this solution at the top when contrasted with the latest technologies implemented (figure 4). The previously mentioned, RASs although it is a great improvement from previous practices, it is still very complex and both energetically and economically costly. Furthermore, RASs depend on the combination of multiple technologies and machines while the TinyOcean is equipped with everything it needs to have a circular water system. Additionally, this system was estimated to elevate the larval survival from ~30% to least 80-90% (BlueLinked et al., 2022; Oceans at Work Foundation et al., 2022).

At Bluelinked, the culturing of the algae and the breeding of the copepods which are used to feed the larvae are also incorporated in the process, thus having the possibility of becoming a completely self-sustainable and reducing the reliability of wild stocks to feed the larvae and fish fry.

An additional advantage of this system is the significant energy savings since the “living soil” is part of the system, and thus, the TinyOcean requires no mechanical filters or other purification techniques (figure 4).

Finally, as mentioned before, this system does not rely on any therapeutics but instead, relies on the activity of marine microbes to get rid of the previously mentioned concerning properties of wastewater and thus, also removing the need to replace wastewater. Significantly reducing emissions from this industry.

All in all, this system shows great promise as another step towards sustainable seafood production and perhaps bring us closer to achieving some of the previously mentioned SDGs.

3. Materials and methods

The following protocol has been developed to perform the in vitro measurements of the water quality. The measurements in this protocol are aimed to shed light into the microbial processes and biogeochemical cycles within the water system. The measurements of the content of ammonia and ammonium (NH_3 and NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) and phosphate (PO_4^{3-}) in the water will be used to investigate the efficiency of microbial community present in the water tanks and thus, the efficiency of the TinyOceans concept.

Increased levels of N in the different forms can be harmful to the health of phytoplankton, zooplankton, and other marine organisms within the tank (i.e., flat oyster larvae). While NO_2^- and NO_3^- have limited effect on pH, the increase in NH_3 leads to the increase in pH. However, the oxidation of NH_3 to NO_3^-/ NO_2^- can cause proton release and thus acidification. This may have impact on marine organisms.

The phosphate (PO_4^{3-}) content will also be measured as an indicator of the balance between the N content and the P content (Redfield ratio) and to ensure algae are kept in check. The form of phosphate ions (PO_4^{3-}) was the chosen one for measuring also because in nature, this is the most common form of phosphorus. Suboptimal concentrations pf PO_4^{3-}, could prevent the healthy development of algae. In contrast, higher concentrations of PO_4^{3-} may promote the growth of algae and if this growth exceeds a threshold, the algae may limit light incidence for other photosynthetic primary producers, inhibiting and eventually causing their death. Dead cells will eventually sink and the microbial consumers in the sand filter will start decomposing them. For this process dissolved oxygen (DO) is used and a competition for DO begins between bacteria and phytoplankton, zooplankton, and other marine organisms. Therefore, it is imperial that there is a balance between heterotrophic microbial activity and the metabolism of all other organisms within the system.

The dissolved oxygen levels will thus, also be measured to ensure the system is functioning properly. Salinity will also be monitored as very high or very low levels of this abiotic factor can cause sub-optimal growth and development of marine organisms, which can cause deformities and even death. Moreover, many characteristics scale with salinity.

3.1 Setting up the Tanks

The tank was set up in phases. The sediment is made of calcium carbonate ground up in different sizes. Firstly, the smallest grains of the sediment were brewing a microbial culture for a few years by adding waste materials of another aquaculture system (figure 5 a)). This microbial community was thought to be comparable to the North Sea sediment microbiota. The rest of the different sized grains used for the sediment was washed thoroughly to avoid contamination from non-marine microorganisms (figure 5 b)). Once the sediment was added to the system, the tank was filled with 6,000 litres of seawater (~30.5 ppt). The following step was adding the primary consumers to the system: phytoplankton that has been grown at Bluelinked facilities. Finally, the oysters were added to the system and waste products started increasing.

Figure 5. *Pictures depicting the initial procedures for the set-up of the tank. a) the orange arrow shows the movement of the waste produced in the system into the container where the microbiota was being grown. b) the process of washing the gravel.*

3.2 Functioning of the System

As mentioned, the shape of the tank and the movement of the water cause retention of the excrement produced by the oysters and the other living organisms in the system, and the delivery of other natural decomposition products to the "living soil" (figure 6 b)). Within the "living soil", there is a pipe and pump mechanism that encourages the water flow within the tank. The objective of this pipe and pump system is to promote horizontal movement within the sediment and only promote vertical movement when the red valve in figure 6 b) is opened. This would then result in a new batch of wastewater to flow into the sediment and be recycled. Figure 6 a) aids the understanding of this process.

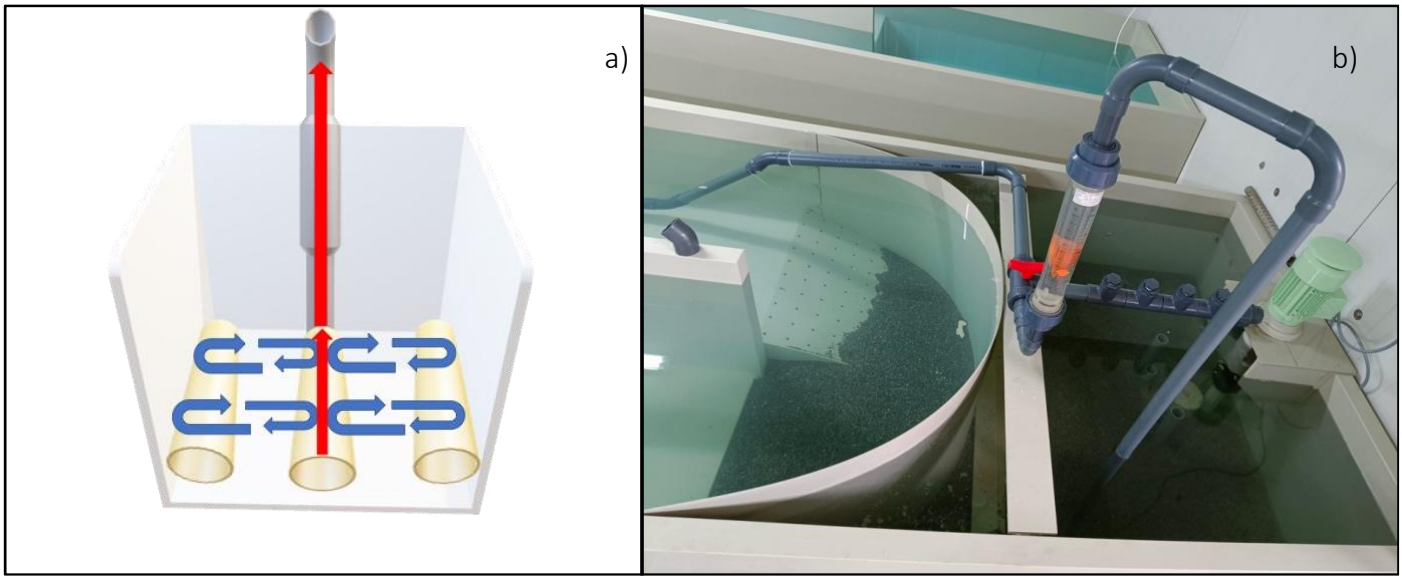

Figure 6. *Pictures of the mechanism within the sediment ("living soil"). a) schematic of the mechanism in an empty system the arrows represent the water movement in the sediment: Blue (horizontal flow within the sediment), red (water that is pumped through the grey pipes seen in b) into the water column); b) picture of the full mechanism filled with the sediment and water.*

3.3 Sampling Methods

The sampling of the tanks started before the oysters were put in the system. This provided a base point to which the samples retrieved over time were compared to. It was also useful for the understanding of the effect of the metabolism of the different organisms added (algae, oysters, and later larvae) on the water quality of the tanks.

The sampling was done every Tuesday and Friday at 1200. To ensure the best accuracy, a routine was put into place on the days of sampling. The solid waste was cleaned and deposited on the surface of the sand filter from 0900 until 1000, the tap was then opened for 15 minutes, allowing this waste to be pulled into the sand bed where the sediment microbial communities carried out their role. The samples were then taken at 1200. As only one system was operating at the time, repetitions were not possible; however, this experiment can pave way for the utilization of this same protocol in similar contexts.

Samples were retrieved from “dirtiest” (S1), the water just above the sand bed; “cleanest” (S2), the water coming out of the tube; and “control” (S3), the surface water in the tank, as demonstrated in figure 7. The sampling locations were chosen because the comparison between S1 and S2 will be valuable to evaluate the functioning of the sand bottom and S3 provides information on how the water quality maintains along the water column and on the environment the oysters are living in. Additionally, the long-term sampling will allow an observation of how water quality changes over time and its progress towards a plateau. The stabilising of the nutrient concentration will be an indication of good functioning of the filter as it suggests the system is being able to cope with the waste products of the living organisms within the system, especially if witnessed in S3. As mentioned, the reference will be the starting point: if fluctuation decreases, this means the quality is good and if it remains it means the filter is doing its job. If not, this means it’s not working.

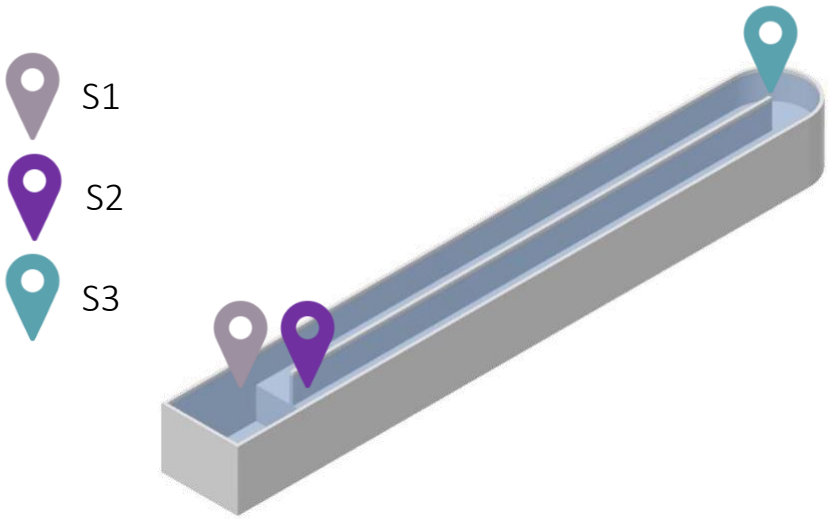

Figure 7. *Simplified diagram of the TinyOcean system depicting the location of the different sampling points. S1: surface of the “living soil”; S2: tube delivering the water from within the sediment to the water column; S3: water column.*

3.4 Analysis Methods

Preparation:

The materials used during the water sample analysis, including pipettes, glass cuvettes and distilled spray bottle were gathered and rinsed three times with distilled water before use. Distilled water is used for this and not tap water to avoid skewed results caused by lime and other undesirable molecules or micro-organisms. Additionally, using the method previously described, the samples were retrieved and labelled accordingly. The blank used was the salt solution used to initially fill up the tanks.

Measurement:

To determine the concentrations of Nitrate, the Hach method described in the DR/890 Data Logging Colorimeter Handbook was used. First, the cuvettes were rinsed 3 times with the distilled water for cleanse, then 10 ml of sample

water (tank water) were used to fill the cuvettes, these were closed, labelled accordingly (Blank, S1 NO_3^-, S2 NO_3^-, S3 NO_3^-) and put back in the rack. Next, the Powder Pillows NitraVer5 (for 10ml) were poured into each cuvette except the blank and swirled properly. The pillows were made with excess powder, as such not all powder was dissolved. The cuvettes were then left to rest for 5 minutes during which a discolouration to different shades of yellow was witnessed in all the cuvettes except the blank. Also, before the 5 minutes are over, the DR/890 colorimeter was set to the correct program (51 and later 54 for Nitrate). Finally, just before the measurements, the outside of the cuvettes was cleaned thoroughly with the paper. For the measurements, first, the blank was inserted in the colorimeter and used to calibrate the program, then the concentration of N in NO_3^- was measured and recorded.

To determine the rest of the nutrients, a similar method was used with only differences in timings, power pillows, colour change and program used on the colorimeter.

For nitrite, the NitriVer3 pillows were used and left to rest for 10 minutes. During the 10 minutes, the sample turned different shades of pink, depending on the concentration of N. The program set on the colorimeter was 60, which measured the concentration N in NO_2^-.

The ammonia measurements required two different reagents: first, the Ammonia Salicylate was added to the cuvettes with the sample water and left to rest for 3 minutes. During this time a discoloration to yellow should already have happened. Next, Ammonia Cyanurate was added to the same cuvettes and left to rest for another 15 minutes during which a further discoloration was observed. After the 18 minutes, the samples were measured using the program 101, delivering a value for the concentration of N in the combined NH_3 and NH_4^+ pool (ΣNH_3).

Finally, to measure the concentration of PO_4^{3-}, the samples had to be diluted 10 times due to the limit of the device (0.9 mg/l) being below the phosphate concentration in the system (0-7 mg/L). This was done by adding 9ml of distilled water to 1ml of sample water. Next, using the same procedure, the PhosVer3 pillows were added to the diluted samples and left to rest for 2 minutes. During this time the colorimeter was set to program 79 that measures the content of PO_4^{3-} and the samples turned different shades of blue. The samples were then measured and multiplied by 10 before entering the database.

Besides recording the measured values of the nutrients, different events that could perhaps affect the nutrient concentrations in the tank were also recorded. This was done to aid the understanding and analysis of the recorded values at a later stage.

3.5 Statistical Work

The first step was to convert the concentrations of the nutrients from ***mg/L*** to ***µmole/L*** to allow for comparisons. This was done by multiplying all values recorded by 1000 to convert ***mg*** into ***g*** and dividing them by 14 for N and by 95 for

P. Secondly, to calculate whether there was a significant change in nutrient content overtime, the difference of the values obtained from the median value was calculated and a linear regression was performed. Next, to facilitate the visualisation of the interaction of the different nutrients, the values were standardised. The standardisation was done by calculating the standard deviation and the mean of the values for each nutrient concentration overtime. This was done to allow the inference of the different processes being performed and to investigate the balance of these processes. Furthermore, it allows the observation of the expected stabilising of the water quality.
Subsequently, a T-Test was performed to compare the values obtained in S1 and S2 to investigate if indeed there is a significant difference between the "dirty" water being treated in the sand bed and the "clean" water coming out of the sand bed. This would shed light into the efficiency of the microbial communities withing the living soil and on the functioning of the system.

Finally, the N:P ratio was calculated to evaluate if the mineralisation of N was being successful. This was done by adding up the moles of N present in the system (combination of N in NO_2^-, NO_3^- and in NH4) and comparing them to the moles of P. The results of this ratio were compared to the Redfield ratio and the criteria used was the closer these values were to the Redfield ratio, the better the health of the tank.

4. Results

4.1 Mole Conversion

As mentioned in the methods section, a colorimeter was used to measure the concentrations of the nutrients in the different locations of the tank. This device produced results in mg/L which made comparisons between different nutrients hard to make. To allow for meaningful comparisons, the mg/L were converted into μmol /L. (table 1).

***Table 1**. Table displaying the converted results for the concentrations of the different nutrients in the different locations in the tank.*

Concentration of NO_2 (μmol /L)																						
Day	0	4	7	11	14	21	25	28	32	35	39	46	49	53	56	60	63	67	70	74	77	82
S1	0,.21	4.14	4.71	6.79	4.79	2.86	3.64	3.64	4.50	1.71	1.86	4.79	1.64	3.21	2.79	3.07	1.79	1.43	1.50	3.86	5.29	3.57
S2	0.21	8.57	1.86	0.71	1.36	3.21	1.57	1.57	1.36	1.14	0.71	1.00	0.36	1.57	1.50	0.79	0.86	2.00	1.57	1.71	1.00	2.21
S3	0.21	0.07	2.21	2.29	2.50	2.43	3.29	3.29	3.00	1.86	1.64	2.43	2.07	3.86	3.50	4.93	1.79	1.36	3.50	4.21	4.79	4.21

Concentration of NO_3 (μmol /L)																						
Day	0	4	7	11	14	21	25	28	32	35	39	46	49	53	56	60	63	67	70	74	77	82
S1	307	707	707	1000	679	377	614	257	621	457	479	1179	479	671	281	143	370	327	146	321	330	383
S2	307	50.0	57.0	---	57.0	220	393	171	286	507	486	314	407	486	521	110	393	330	256	333	301	399
S3	307	86.0	7.00	36.0	36.0	283	300	221	243	550	464	457	507	593	494	71.0	464	339	282	326	317	394

Concentration of NH_3 NH_4^+ (μmol /L)																						
Day	0	4	7	11	14	21	25	28	32	35	39	46	49	53	56	60	63	67	70	74	77	82
S1	2.86	18.6	15.0	17.1	19.3	2.86	18.6	7.14	16.4	4.29	6.43	17.1	6.43	3.57	2.14	5.00	4.29	2.86	5.71	2.86	5.00	5.00
S2	2.86	2.86	2.86	0.71	2.86	2.86	0.71	1.43	2.86	0.71	2.14	0.71	0.71	2.86	0.71	2.14	1.43	2.14	0.71	0.71	2.14	3.57
S3	2.86	2.86	5.71	5.00	2.86	2.86	4.29	5.71	2.86	---	5.00	2.14	1.43	4.29	3.57	3.57	2.86	2.50	2.86	5.00	3.57	4.29

Concentration of PO_4^+ (μmol /L)																						
Day	0	4	7	11	14	21	25	28	32	35	39	46	49	53	56	60	63	67	70	74	77	82
S1	10.4	5.80	4.80	10.4	10.4	---	42.1	34.7	24.2	20.0	21.1	15.8	11.6	21.1	16.8	38.9	28.4	24.2	20.0	17.9	31.6	21.1
S2	10.4	10.4	3.10	7.20	8.80	---	29.5	33.7	11.6	17.9	16.8	28.4	9.50	18.9	54.7	32.6	74.7	28.4	64.2	35.8	58.9	35.8
S3	10.4	2.80	4.30	9.50	10.4	---	16.8	34.7	15.8	17.9	46.3	21.1	8.40	43.2	33.7	18.9	20.0	22.1	23.2	28.4	42.1	14.7

4.2 Reduction of Variance Overtime

The first examination was to investigate whether there was a significant reduction in variance and fluctuation overtime. This was done by calculating the difference of each point from the median value and performing a linear regression analysis of this difference and time. Figure 8 shows the results of this regression analysis for each nutrient in each location. This analysis was performed to investigate whether the changes in fluctuations over time (variance from the median).

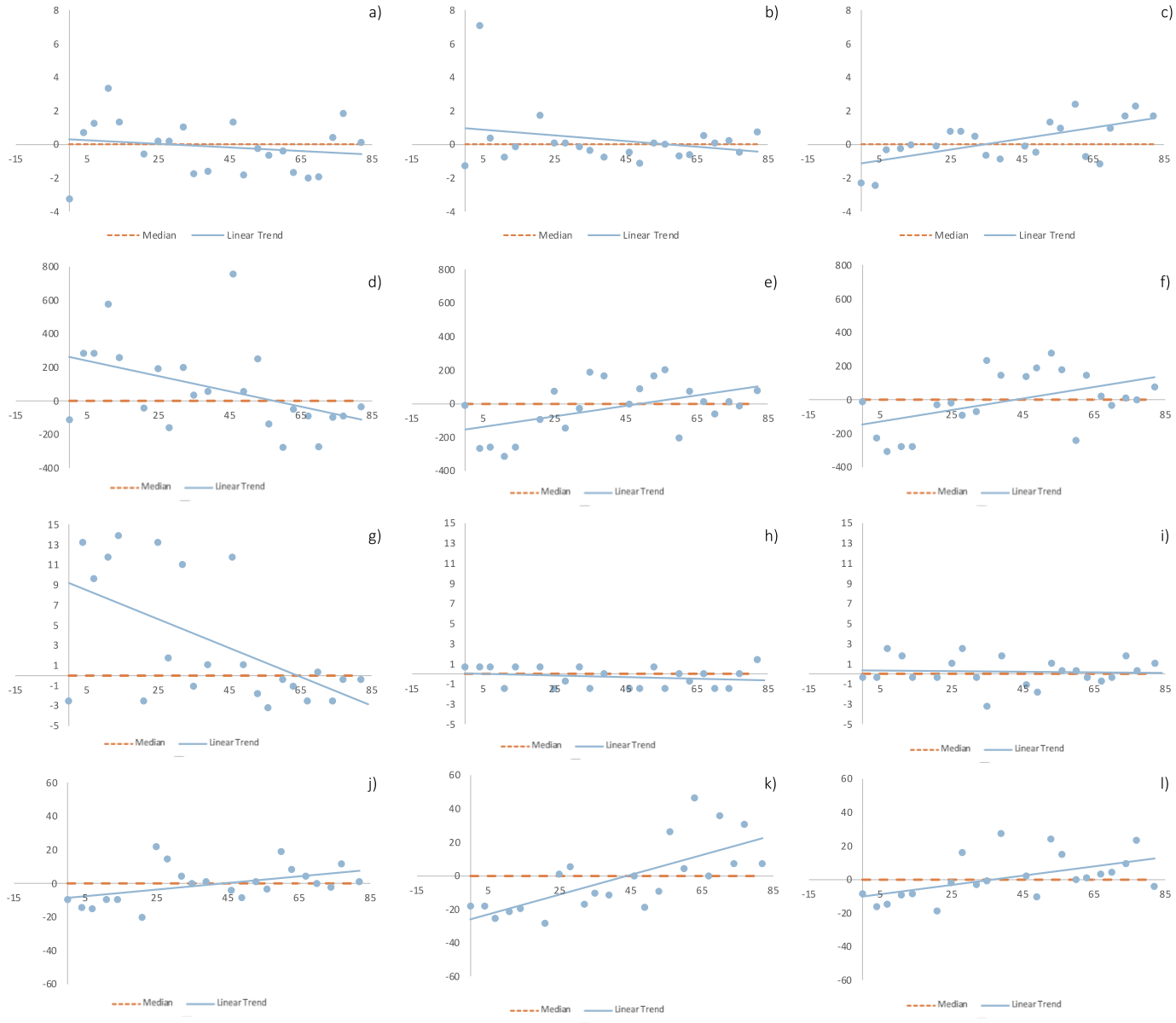

Figure 8. *Scatterplots depicting the linear regression analysis performed. a) NO_2^- in S1, showing no significant change in variance overtime, relatively low fluctuation amplitude, with a slight increase towards the end; b) NO_2^- in S2 showing no significant change in variance overtime, relatively low fluctuation amplitude with a slight decrease towards the end; c) NO_2^- in S3, showing a significant change in variance overtime with increasing fluctuation amplitude; d) NO_3^- in S1, showing a significant change in variance overtime with increasing fluctuation amplitude; e) NO_3^- in S2, showing a significant change in variance overtime with decreasing fluctuation amplitude; f) NO_3^- in S3, showing a significant change in variance overtime with decreasing fluctuation amplitude; g) ΣNH_3 in S1, showing a significant change in variance overtime with decreasing fluctuation amplitude; h)*

ΣNH_3 in S2, showing no significant change in variance overtime and very low fluctuation amplitude throughout; i) ΣNH_3 in S3, showing no significant change in variance overtime and very low fluctuation amplitude throughout; j) PO_4^{3-} in S1, showing no significant change variance overtime and no fluctuation amplitude change; k) PO_4^{3-} in S2, showing a significant change in variance but no change in fluctuation amplitude; l) PO_4^{3-} in S3, showing a significant change in variance and a slight increase in fluctuation amplitude.

The figure is composed of 12 scatterplots in which each rows present the different nutrients, and the different locations are shown in columns. This plot provides information of the change in variance (the scattered dots) and in fluctuation amplitude (the distance of the linear fit to the median line). For example, figure 8 i) on one hand, shows a non-significant change in variance ($pvalue = 0.55$) and a constantly low fluctuation. This is the case when the linear trend line is closer to the median, which in this case is $y = 0$ and the $pvalue$ was not significant ($pvalue = 0.55$). In contrast, figure 8 g), shows both a significant change in variance ($pvalue = 0.00035$) as well as a reduction in fluctuation which can be observed by the fact that the linear trend line becomes closer to the median. Fluctuation amplitude is observed by the distance of the linear trend line to the median line, therefore, at times where the trend line is going up or down, as the distance is the same (just either negative or positive), there is no significant fluctuation change. An example is figure 8 k), showing a significant variance change ($pvalue = 0.00022$) but a stable fluctuation amplitude (~±25points).

With these results it became clear that although in most cases the variance changed significantly, this change did not always affect the fluctuation amplitude. Thus, unlike what we expected, by using this approach, we did not observe an overall stabilisation of the nutrient content in the tank. In most cases the fluctuation amplitude was relatively constant (either low or high) and not significantly different. Nonetheless, the ΣNH_3 is quite uniform in figure 8 h) and i) indicating efficient recycling in the S2 and S3 locations. The same cannot be said for ΣNH_3 in S1 (figure 8 g)), however, this would be expected seeing as the location of S1 is the dirty, untreated (by the microbial community) water.

4.3 Comparison – Location

After the 82 days of sampling the 3 locations in the TinyOceans3 and analysing the content of these samples, the graphs shown in figure 9 were produced to facilitate the visualisation of the differential behaviour of the nutrients in the separate locations.

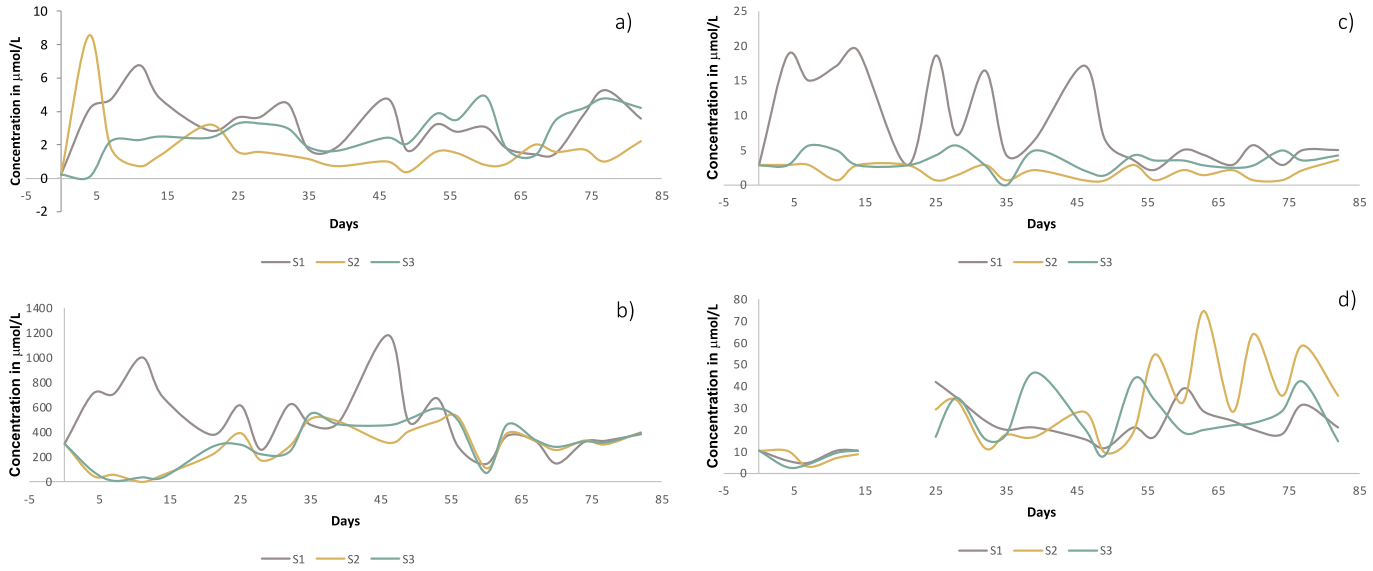

***Figure 9**. Line graph depicting the changes in concentration (in µmol /L) of the different nutrients measured overtime and the contrast between the concentration of this nutrient in the different sample locations. a) concentration of NO_2^-; b) concentration of NO_3^-. NOTE: the scale of the graph reduces the visual difference of the three locations (i.e., day 60: S1 – 143 µmol /L and S2 – 71 µmol /L; c) concentration of ΣNH_3. NOTE: Due to the pH of the water (~7), ~95% of the concentration found in the tank is in the cationic form of NH_4^+; d) concentration of PO_4^{3-}.*

In figure 9, we can see that the concentration of NO_2^- exhibited fluctuations throughout the full length of the experiment, with perhaps the exception of S2. Overall, the variance in NO_2^- concentration overtime was not significant, and fluctuation was found to be higher in S3 than in the other locations (figure 8 – the closer the linear trend line is to the median the lower the overall fluctuation). These results would suggest that the system has perhaps reached a balance. Although in 9 a) there seems to be a lot of fluctuation, the peaks and troughs shown in this graph do not significantly differ from one another (when comparing peak against peak and trough against trough), suggesting that the microbial communities in the tank are indeed keeping the content of NO_2^- values within the range of ~6.5 and 0.5 µmol/L, with the clear exception of the peak in day 4. Finally, one thing to note is that the NO_2^- concentration values in S1 are generally higher than in S2, suggesting that the microbial communities in the sediment are active.

The observations concerning the changes in concentration of NO_3^- again show no sign of stabilisation excluding perhaps the last 20 days (figure 9 b)). Initially, S1 shows an almost opposite trend from that of both S2 and S3 and, was found to exhibit much greater concentrations of NO_3^- than those of the other two locations. Additionally, although the scale of the graph might conceal to some extent the difference between the three locations, the S1 location shows a big

divergence from the water found in the rest of the tank. Again, suggesting that the microbiota in the sediment is performing well. In contrast, S2 and S3 exhibited almost identical trends, with S3 displaying higher concentrations more frequently. Suggesting that the water flow in the tank is efficient and that the oysters are metabolising correctly. Nonetheless, overtime, the concentrations of NO_3^- have converged. This convergence was towards relatively lower concentrations of NO_3^- (ones already experienced in S2 and S3), which is in line with the fat that only S1 showed a significant change in fluctuation overtime (figure 8 d)). This could have been the result of fewer oyster metabolising in the tank and/ or loss of N from the tank due to lack of proper ventilation, creating an imbalance of N-gaining and N-losing processes.

Moreover, unlike the trends encountered for the NO_2^- nutrient, from day 20 similar fluctuation patterns were observed all throughout the tank, apart from the peak around day 45. This was the highest concentration of NO_3^- recorded in the study (1178 μmol/L). This pattern similarity could suggest that nitrification is occurring at similar rates throughout the tank.

The concentrations of ΣNH_3 in S1 were higher in contrast to S2 and S3, as would be expected seeing as similar a pattern was observed for NO_2^- and NO_3. Once again suggesting the right functioning of the sediment microbiota. In contrast, around day 53, the ΣNH_3 concentrations in S1 dropped significantly and so did the fluctuation, showing concentrations that had been witnessed in S2 and S3 from day 0. This can be further explored in figure 8 g) where there is a clear significant change in the concentration of ΣNH_3 overtime in S1 and a significant reduction in fluctuation, unlike in S2 and S3 (figure 8 h) and i)). This change happened to result in the decrease of total ΣNH_3 content of the system overtime (from 24 μmol/L in day 4 to 13 in day 82). Once again this could be attributed to either low ventilation and thus reduction in N fixation or to the fact that less oysters were metabolising in the tank and producing less waste material.

Finally, in figure 9 d), PO_4^{3-} concentration started off quite low rather uniform all throughout the tank, unfortunately there is a big gap of about 10 days which prevents us from observing the changes within that time. This initially low content of PO_4^{3-} is likely to be attributed to the fact that the tank was being set up and primary producers were still being introduced. In a natural context, there are multiple sources of P, however, in the context of this system, the main source of P would be plant decomposition. As such, it could take some time before the concentration of PO_4^{3-} (inorganic form of P) would start increasing.

The PO_4^{3-} concentrations in S2, showed a significant increase in variance overtime and slight increase in fluctuation (figure 8 k) and 9 d)). Interestingly, these fluctuations occurred periodically with peaks exactly 7 days apart. This could be the result of the accumulation of P in the sediment and, due to the dimensions of the tank, this P is probably upwelled every 7 days. The oxygen levels may also play a role in this pattern of P release. Increased DO within the sediment can reduce the release rate of P from the sediments back into the water column (Wang et al., 2008). Unfortunately, with the DO measuring method applied, it is impossible to know with certainty if the DO within the sediment dropped in

such a way to cause these peaks, but it is important to mention that this could have been another reason for the observed behaviour of PO_4^{3-}.

Overall, there were no significant increase in fluctuations in S1 or S3, but PO_4^{3-} was apparently more stable in the start, contrasting with what was observed with the other nutrients. Finally, it seems the concentration of the three locations are not significantly different and that the content of S3 PO_4^{3-} has a constant, relatively high fluctuation. There is no significant difference overtime, but it shows slightly higher fluctuation than S1. This is a pattern that is not observed with the different forms of N (figure 8). This is to be expected because P is mostly recycled within the water column and therefore, depending on the rate of metabolism and death of the phytoplankton and oysters in the water column, there is more or less inorganic phosphorus (PO_4^{3-}) available.

All in all, the patterns observed for the measured nutrient concentrations overtime were in line with what we would have expected to see if the system had an active microbial community. Furthermore, we observed quite big disparities between S1 and S2. As such, their contrast would provide detail into the microbial activity within the sediment, as such, the significance of the difference between these two locations was measured.

4.4 Before and after "living soil"

To evaluate whether the differences we were observing between S1 and S2 in the graphs was significant, a T-test was performed for each measured nutrient.

As predicted, and as is evidenced by figure 10, the N content in the water sampled from the tube coming out of the sediment (S2) significantly lower than in the water sampled above the sediment (S1).

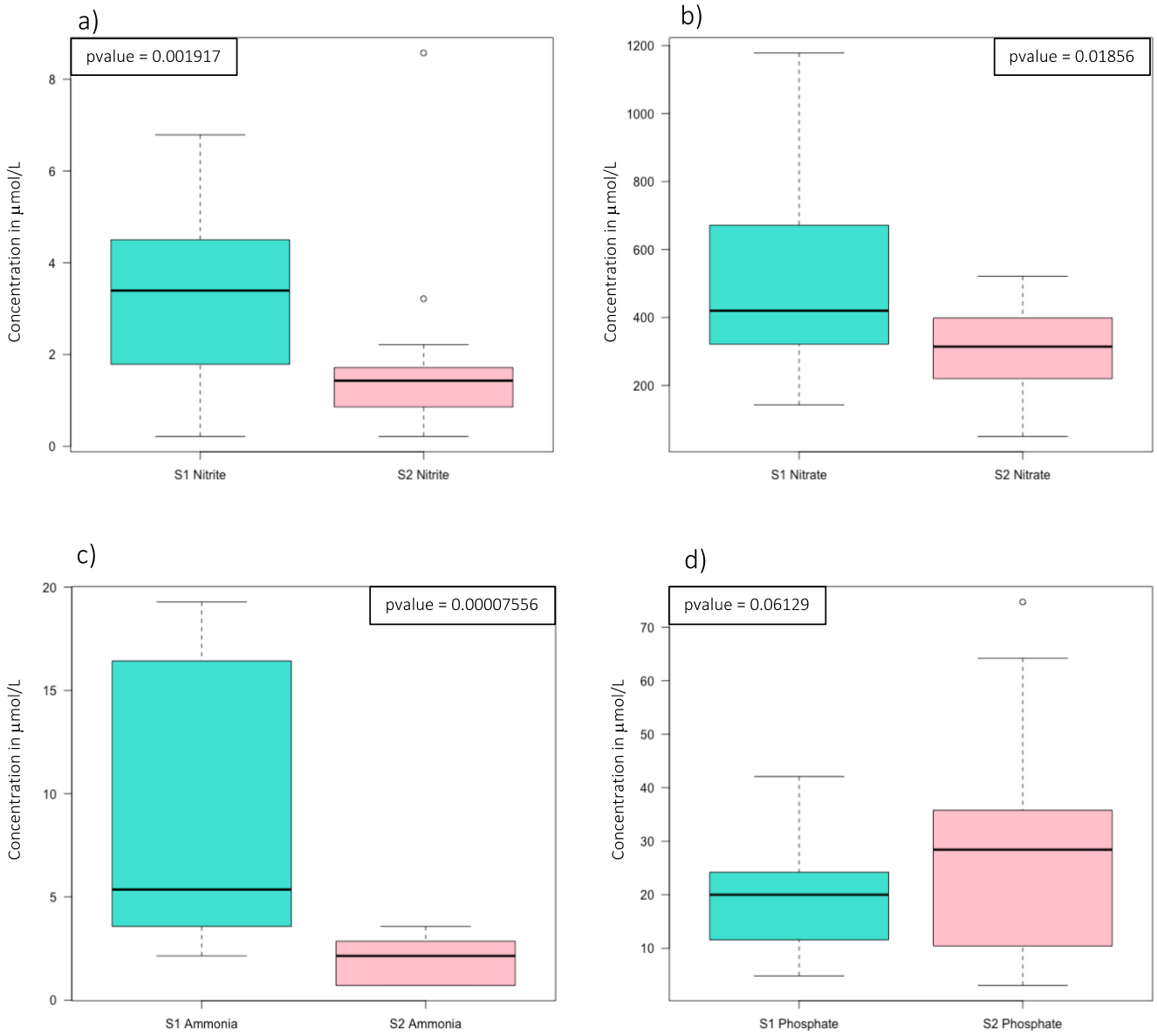

Figure 10. Boxplot illustrating the disparity between the nutrient concentrations found in S1 and S2 and the significance of each comparison.

Overall, the fact that there is a significant decrease in total N content after the water has gone through the system, suggests that anaerobic microbial activity is happening and is resulting in the loss of N from the system. More specifically, from these results we can infer which individual reactions are happening and whether they are happening more than others.

Firstly, the significant decrease in NO_2^- suggests that DRNA, anammox and denitrification could be occurring in the sediment. However, due to the even bigger decrease in ammonia content, it is likely that if DNRA is indeed occurring, it is doing so at a much lower rate than the other reactions such as anammox or denitrification.

Additionally, due to the very high content of ΣNH_3 in S1, anammox does not depend on DNRA for ΣNH_3 As such, it is very likely that another very active microbial functional group would be anammox bacteria.

Moreover, although with less certainty, NO_3^- presenting the least (although still significant) decrease, could mean that aerobic microbial process of nitrification is also taking place in the O_2 rich zones of the sediment. If this is the case, then we could assume the presence of ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA) and Nitrite-oxidizing bacteria (NOB).

Finally, when looking at the difference of PO_4^{3-}, as expected, there is no significant difference between S1 and S2. This would be expected, seeing as the recycling of PO_4^{3-} occurs mainly in the water column. Besides the effect of the DO levels in the sediment and the natural accumulation and release of P in sediment, another thing that could perhaps be inferred from these results is the potential presence of polyphosphate accumulating microorganisms in the sediment. However, to further understand these interactions, the different concentrations of the different nutrients within the same location were compared.

4.5 Comparison – Nutrient

By looking into the interactions of the different nutrients in the same locations in the tank, new information becomes available. The graphs displayed in figure 11 demonstrate the timings where the content of nutrients changes and this detail allows us to again infer the possible biogeochemical reactions occurring in each location of the system at each point in time and provide further insight into the microbial functional groups active in the system, but most importantly this interaction allows us to identify the similarities/differences of processes that we would expect in a natural marine ecosystem.

To build the graphs shown in figure 11, the concentration values were standardised to allow the visualisation of the patterns displayed by each nutrient. This was necessary because the distinct scales of concentrations of each nutrient prevented the pattern of NO_2^- and of NO_3^-, for instance, of being visualised.

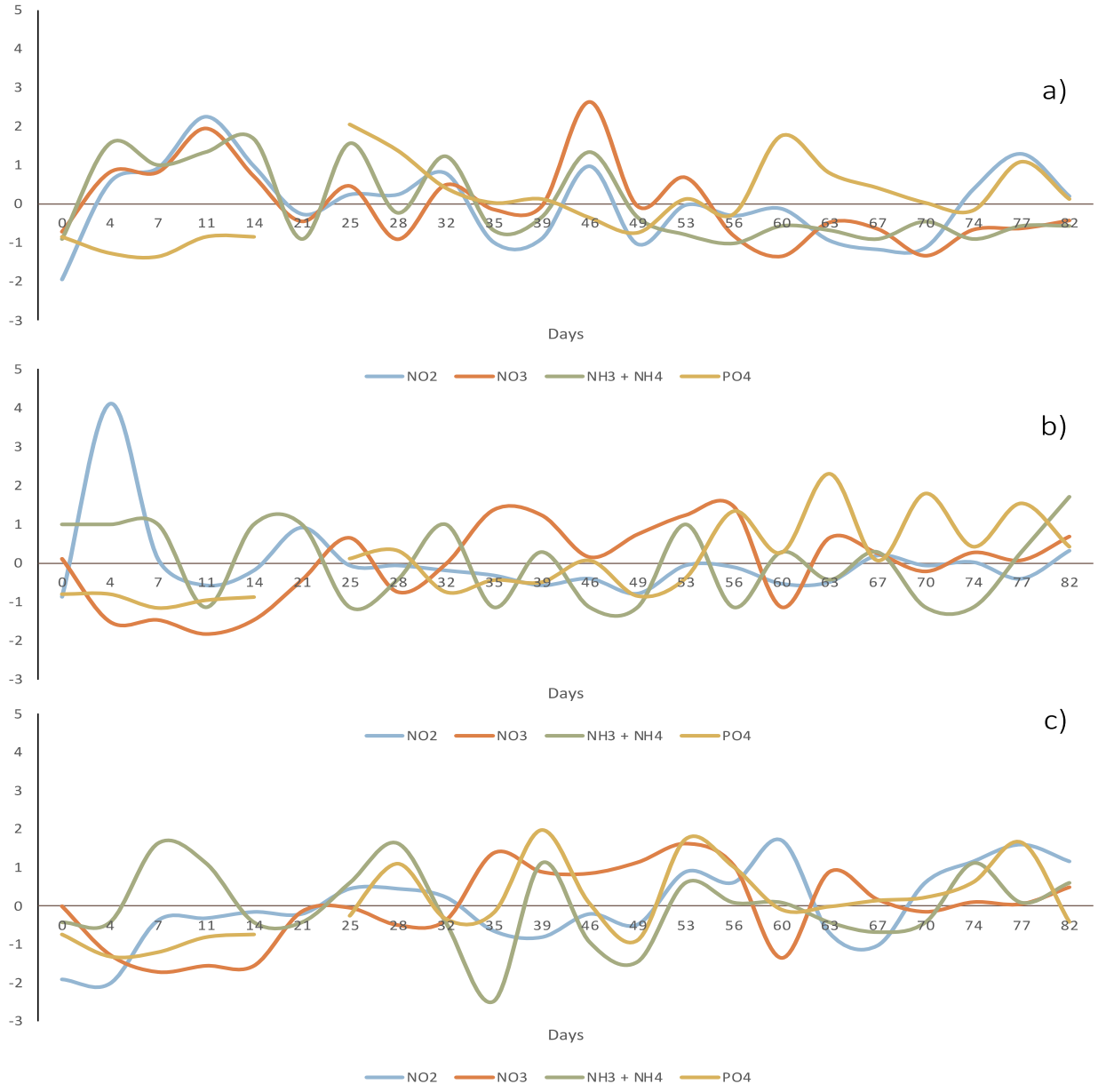

Figure 11. Graphs depicting the nutrient content in each location of the tank. a) S1 location; b) S2 location; c) S3 location

From an initial observation of figure 11 a), we note that, unlike PO_4^{3-}, all forms of N initially follow the same trend in S1, with ΣNH_3 having a slight delay in peaking. In contrast, from day 56 NO_3^- and ΣNH_3 started having opposite trends. In S1, as expected, the content of N is generally high as the solid waste from oysters is being deposited the surface of the sediment before the water is recycled in the living soil. The movement of water through the sediment, into the pipes and out into the water column through the S2 location, can be comparable to the sinking of O_2 rich water and the upwelling of nutrients. As such, we would expect a reduction in the N content and perhaps the retention of some P, which was observed both when looking at the figure 10 and 11. Additionally, we can see PO_4^{3-} increasing overtime in S1, which is likely to be a result of increased cell death in the water; and an even higher concentration is observed overtime in S2, suggesting there is indeed an accumulation of that nutrient in the sediment.
What we can further observe in figure 11 b) though, is the fact that the concentration values for each for of N were more stable in S2 than what was observed in S1. Suggesting once again that the microbial activity in the sediment acting on the nitrogen cycle was constant and reliable. Another observation is that the levels of ΣNH_3 were mostly kept quite constant as well as generally high levels of oxygen. Giving us further indication that nitrification is likely to be occurring within the O_2 rich areas of the sediment and perhaps inside the pipes when oxygenated and thus that ammonia oxidizers (archaea and/or bacteria) and nitrite-oxidizing bacteria are present.

Although the focus of the study is to evaluate the microbial communities in the sediment, when looking into the nutrient interaction in S3 (figure 11 c)), we can also gain insight into the biogeochemical reactions within the water column. From the graph, we can infer that, in the water colunm, the biggest reason for reductions in ΣNH_3 and PO_4^{3-}, would be assimilation by the organisms. We can also see that ΣNH_3 and PO_4^{3-} mostly follow a similar trend, suggesting successful mineralisation and the maintenance of a constant N, and, that the total N is being lost at a similar rate to what is being produced, a balance we want to see in a stable and healthy tank.

All in all, the most important factor to consider is the mineralisation rates. This can be inferred by looking at the N:P ratio. This is an important indicator of both the health of the system, as having a wrong ratio may lead to harmful, toxic algal blooms; and of how similar the water in the system is to what we would encounter in a natural marine ecosystem. As such, further investigation was done on the N:P ratio in the different locations of the system overtime.

4.6 N:P Ratio

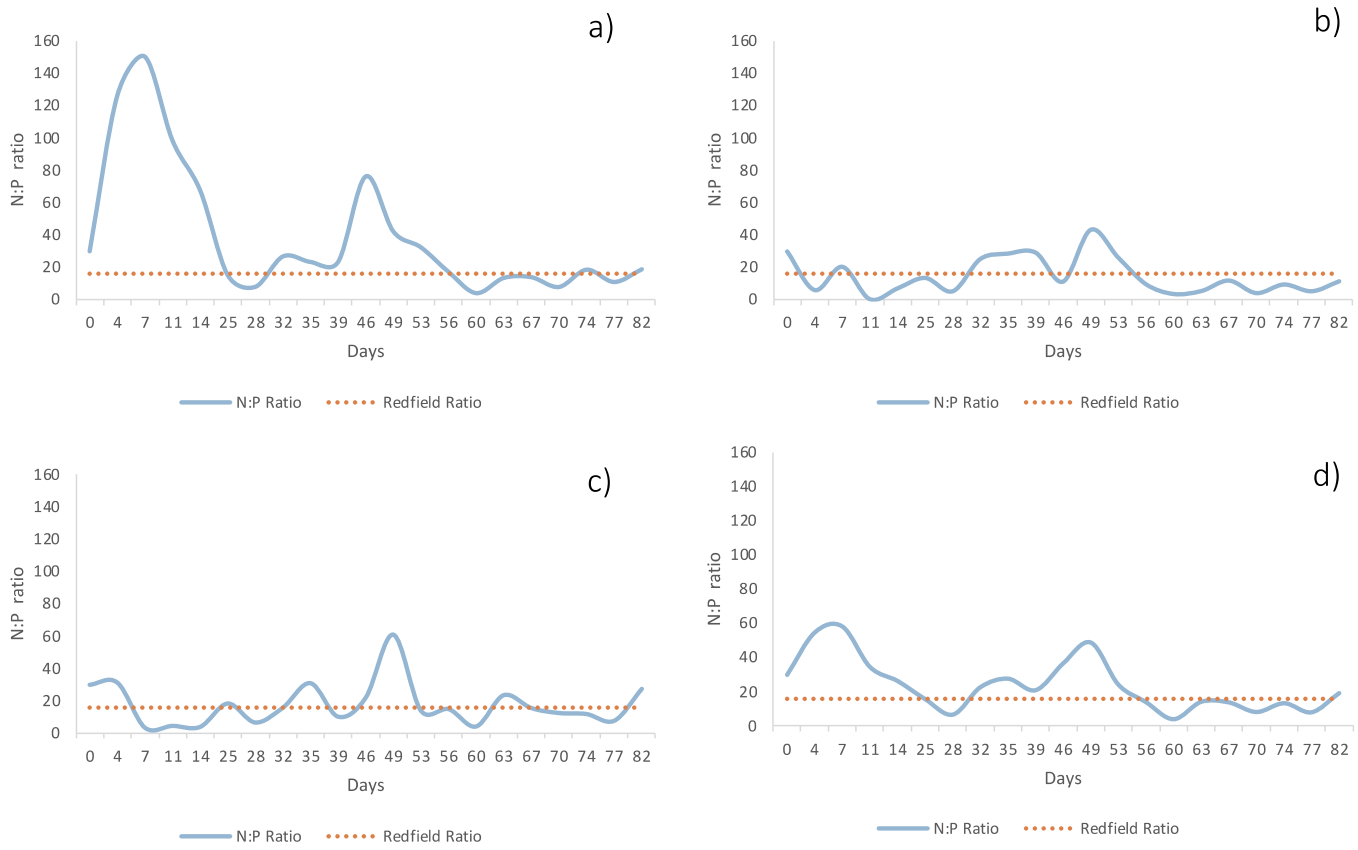

Figure 12. N:P ratio overtime (blue), contrasting with the Redfield ration (red). a) S1 location; b) S2 location; S3 location; d) Average in all the tank.

In S1, initially, was very far from the Redfield ratio which was expected as this location accumulated all the waste from the tank. However, there was a clear approximation of the N:P ratio to the Redfield ratio overtime. This low N:P ratio is characteristic of the deep waters and may be responsible for the observation that nitrogen is commonly the nutrient most limiting for primary production. In contrast, in the S2 and S3 locations, as expected showed a constant fluctuation near the Redfield Ratio. In the S2 samples, (figure 12 b)), we see an overall lower N:P ratio, this could be because the great majority of N is lost during the journey of the water through the sediment (more anoxicity leads to greater N loss) while P is not really being lost from the tank. In the S3 samples, as expected, stays the closest to the Redfield ratio, with an exception of the peak observed on day 49. Furthermore, the fluctuations observed would be expected due to the differencial assimilation rates of the nutrients. Finally, when looking at the overall average on the tank, we can see again an approximation to the Redfield ratio over time. As mentioned before, having an N:P ratio comparable to the values widely considered for the ocean (Redfield ratio) shows that the tank is healthy, that the degradation of organic matter and the recycling of the nutrients is balanced in the tank. Essentially, it suggests that N and P are being successfully cycled, balance between N fixation and N loss and a good enough community of primary producers are being introduced.

With regards to abiotic factors such as pH and salinity, they remained constant throughout the experiment and therefore were not thought to affect the biogeochemical reactions happening in the tank. DO, in contrast, depending on the location was different, experiencing an overall lower DO in the sediment than in the water column. The tank had to be oxygenated at one point but the levels of DO above the oysters (S3) never dropped bellow 80%. This variety though, could explain why perhaps there was a good N:P ratio all throughout the tank overtime because it could suggest that there could be low oxygen pockets within the water column.

5. Discussion

The main observation from the results obtained is that the TinyOceans system shows a lot of promise as an improvement in the sustainability of fish hatcheries.

The main goals of this research were i) to evaluate the system in terms of its capacity to maintain a good water quality for the survival of the bred marine organisms and ii) find an optimized method to apply in the company as a standard water regulation procedure to ensure the well-functioning of the purification system. The initial idea was to measure the efficiency of the "living soil" and make an educated estimate of the functional groups of microbes present in the sediment. Overall, the goals were achieved. However, it was of importance to also have a standard protocol for the company, hence the evaluation of the methods implemented was used to make optimization suggestions for the future implementation of this protocol as a standard practice in the company.

5.1 Microbial Community

When investigating the microbial community in the "living soil" we encountered some difficulties. This is an inherent feature of experimental systems, and in particular within a commercially active context with multiple people working on different projects and with limited resources. Nonetheless, these disturbances did not prevent the investigation of the microbial functional groups present, although they could have had repercussions in the measured concentration values.

As observed in the results, denitrification seems to be occurring within the sediment. This was inferred from the values of total N before and after the recycling. This is in line with what we would expect because DNRA is usually favoured in conditions of low NO_3^-, and, as can be observed by figure 10, this is not the case. Moreover, the previously mentioned unexpected potential for denitrification in oxygen-rich zones in the sediment due to increased nitrification further suggests that indeed this process is happening both within the sediment but also potentially at its surface. The results obtained and the information gathered from literature leads us to believe that one of the most active microbial groups in the sediment would be denitrifying microbes. These could include heterotrophic, potentially some autotrophic, prokaryotes; denitrifying bacteria such as *Bacillus*, *Paracoccus*, and *Pseudomonas*; and eukaryotes such as *foraminifera* (Risgaard-Petersen et al. 2006; Bernhard, 2010; Voss et al. 2013; Lam and Kuypers, 2011).

Another process that could contribute was anammox. Suggesting the presence of anammox bacteria such as the *Candidatus* genera *Brocadia* (Sofia Mayorga, 2022).

The contrasting composition of the water in S1 and S2 also showed potential signs for nitrification. This was inferred because NO_3^- exhibited the least significant reduction from S1 to S2. As such, ammonia-oxidizing bacteria (AOB) belonging to the classes of Betaproteobacteria, Gammaproteobacteria; and ammonia-oxidizing archaea (AOA), including perhaps Nitrosopumilus maritimus and Cenarchaeum symbiosum are likely to be present in and on the "living soil" (Sofia Mayorga, 2022).

As for DNRA, there seems to be little evidence for the occurrence of this reaction. When looking at the transformations in forms of N, it seemed that if this reaction is occurring it is at a very low rate. This process occurs in extremely anoxic environments and is favored over denitrification in nitrate poor waters and under higher carbon-to-nitrogen (C:N) ratio (Liu et al., 2021; Pajares & Ramos, 2019). The only parameter known is the high availability of NO_3^-, suggesting denitrifiers would be more likely to thrive. Unfortunately, neither the anoxia nor the C:N ratio were measured within this experiment. As such, it is hard to know whether DNRA was happening or not.

Finally, the peaks observed in figure 11 b), and the non-significant but slightly higher PO_4^{3-} content in S2 that in S1 (figure 10 d)), could be evidence for Polyphosphate accumulating organisms (PAO). On average the water in the sediment is renewed every hour and as such the peaks observed in figure 11b), spaced exactly 7 days apart are, afterall, not likely to be a result of the time it takes for the water in the system to be totally replaced. When microbes have excess energy, they want to store this energy for a time of scarcity, one of the methods is producing polyphosphate and storing it within their cells. Therefore, the fact that this pattern only started occurring after 49 days suggests that the community of PAOs might have developed as the PO_4^{3-} in the system increased due to the regular addition of primary producers.

The N:P ratio was an important measure of both the rate of remineralisation in the system and the similarity to natural marine ecosystems. Imbalances in the N:P ratio can be caused by multiple factors, in nature, it is mainly caused by anthropogenic activity, whilst in the tank it would likely be due to temperature or the imbalance in microbial functional groups. Nonetheless, the consequences would be similar. Having an imbalance in the ratio could result in either harmful algal blooms (eutrophication) or in decreased primary productivity due to P limitation. The latter was thought to also raise the chance for aerobic methane production via metabolism of phosphonate compounds by P-limited microbes in an oligotrophic lake in Montana (Elser et al., 2022).

Additionally, this information could give us a good overview of the potential primary producer communities present in the system. Diatoms for instance, require relatively nutrient-rich conditions and typically display lower-than-Redfield

N:P (Sharoni & Halevy, 2020). In contrast, dinoflagellates such as *Ceratium furca* and *Ceratium fusus,* also thrive in high N:P nutrient conditions and dominate in situations of P-limitation (Baek et al., 2008).

As phytoplankton are thought to "eat what they can", their cellular N:P matches the N:P in their environment (Sharoni & Halevy, 2020). Allowing the inference that perhaps the presence of dinoflagellates was more dominant during the start of the tank (higher N:P ratio) and diatoms are likely to presently be thriving in the system. Which in congruent with the phytoplankton species being added to the system as food for the oysters. Furthermore, lower N:P ratios are associated with faster protein synthesis and higher growth rates of phytoplankton (Sterner & Elser, 2003), according to the observations this seems to be the case in our system. As such, we can suppose that perhaps the primary producers in the system could potentially replenish themselves, given that the consumption rates of the oysters are slower that the growth rates of the phytoplankton. Finally, with regards to cyanobacteria, they thrive in similar conditions to the ones described for dinoflagellates, however, although they still survive in cold waters, they mostly thrive in waters of about 25ºC, suggesting that perhaps they are less likely to be present in this system (Bertilsson et al., 2003; Giannuzzi, 2019; Sharoni & Halevy, 2020). In contrast, diatoms and dinoflagellates thrive in temperatures of 17-22ºC, these groups are more likely to thrive in the TinyOceans system that remains at about 17-18ºC (Giannuzzi, 2019).

The widely accepted Redfield ratio of 16:1 (N:P) is an important concept in marine biogeochemistry and has been so for centuries, however, even in natural marine ecosystems there are great deviations from this ratio. As such, it would be expected that in our system there would be fluctuations as well. In literature, ratios can range from ~13:1 in cold and nutrient rich waters and 28:1 in warm nutrient limited waters (Sharoni & Halevy, 2020). With this in mind, the results obtain for the N:P ratio observed, except for the observations in S1, are not only in line with these values, but also mimic these fluctuations present in natural marine ecosystems. We could go as far as to say that the fluctuations observed could be representative of the sinking and upwelling of nutrient poor/rich waters.

Finally, the reason for having calculated the N:P ratios and not comparing the individual values experienced in marine ecosystems vs in the system was because the values in the systems would always be greater than those found in the ocean. In marine ecosystems you would expect to see values within the μg/L scale, and in most cases, nutrients can be below analytical detection preventing accurate measurements (Parsons & Harrison, 1983; Tanioka et al., 2022). The ratio therefore allows for a more accurate measure of the nutrient content, since nutrient content in phytoplankton match the content in their environment; and for a more valid comparison, seeing as the values in the tank would always be significantly different (lower) than those in the tank.

In RAS aquaculture units, a currently very promising fish culturing method, biofilters are utilised for cycling the nutrients and the goal of this biofilter is mainly to oxidize NH_3 into NO_3^-, a less toxic form of N. In these units, NH_3 is closely monitored and kept below 0.0125 mg/L (Almeida et al., 2021). When looking at our results, the combination of NH_3 and NH_4^+ the values measured fell between 0.01 mg/L and 0.27 mg/L. Considering the pH remained below 9.5, we can

assume only 1-5% of the total ΣNH_3 concentration is the more toxic form of NH_3, and that the levels of NH_3 stayed between 0.0001 and 0.0135 mg/L. Once again, these numbers propose a good functioning and balance of the microbial communities within the system in maintaining low N toxicity in the tank.

5.2 Protocol

With regards to the methods implemented to carry out the research, there were some issues to consider and improve. However, it provided a good amount of data from which we gathered the information we were hoping to obtain.
Firstly, conducting an experiment in a commercial setting, where others are also running their experiments makes it harder to control many important variables, including the number of individuals in the tank throughout the experiment. This change in number of individuals could also be the reason for significant reduction of ΣNH_3 toward the end of the experiment (figure 9 c)). Regardless, one of the most valuable observations gathered from this experiment was not affected by the lack of control. The comparison made between the water content of S1 and S2 not only provided a lot of information on the potential functional groups present in the sediment but also ensured that the activity we were monitoring was solely the activity of the microbial community of the "living soil".

When measuring the nutrient content in the water samples we also encountered multiple challenges that could easily be avoided in the future and would result in a more accurate assessment of the nutrient content of the different samples. Due to lack of reagents and deregulation of some of the programs of the Hach colorimeter, different methods had to be applied at times. With regards to the lack of reagents, we stored the samples in the freezer up to a maximum of one week before measuring the ammonia content. This was not an issue seeing as according to the Hach handbook, the samples could be stored up to a month before they would be unreliable. As for the case of phosphate, this was not possible, as such, we made use of the reagents available to us. To mitigate this change, the same sample was measured using the previously employed method (one 10ml pillow for 10ml of sample) and the potential future methods (one 5 ml pillow for 5 ml of sample; or 2 pillows for a volume of 10 ml of sample). We realized that using one 5ml pillow in 5ml sample provided similar results to the previously employed method and hence that was the chosen option. Clearly, not an ideal solution but it does not appear to have had a great impact on the observations made. However, for the measurement of nitrate a completely different method was used. The program to measure nitrate in the colorimeter was not well calibrated and thus, after day 56, the Palin test started being used for measuring the nitrate content. We cannot be certain that the differential behavior observed by nitrate content (slight reduction in fluctuation) could have been due to the change in measuring method, however it could have had an impact (figure 9 b) and 11).

S1 samples were initially being taken from within the sediment, however, an improvement was made by changing this location to just above from the sand filter. This alteration prevented the measurements from being skewed due to the turbidity of the water rather than the concentration of nutrients allowing for a more accurate observation of the nutrient content in this location of the system.

During the analysis of the results, multiple statistical tests were carried out. For the linear regression analysis for instance, the median was used as the reference to "stable". The median value was chosen because although it lessens the weight of the peaks and values the frequency of a value, potentially resulting in the higher amplitudes being discredited and less significance to be attributed to a change, it focuses on finding the midpoint of a group. Whereas, using a value such as the average, would result in an even greater loss of significance due to the weight of the high concentrations experienced. Additionally, an issue that all the statistics suffered from was pseudo replication seeing as the 3 samples are taken from the same tank and are compared to each other against time. Conversely, as this is a long-term experiment and only one tank had been set up, true replication was not an option in the time frame of this project. Moreover, this limitation does not take away from the fact that the protocol described, albeit with some improvements, can be carried out by Bluelinked Breeding Centre as a standard water quality assessment protocol for the future.

Finally, although it was out of the scope of the study, it is important to mention that there was a 95% survival rate from the flat oysters introduced in the tank and the spawning of these oysters. This information provides further evidence on the efficiency of the TinyOceans system and the microbiota within, suggesting the provisioning of a comfortable and healthy environment for the species. The survival rate of the oyster larvae was not possible to measure, however, there was evidence of the larvae starting to settle, proposing their survival for about 17-26 days. In the long-term, the survival and development of the larvae will be monitored more closely and thus more information on the well-being of the species will be gathered. However, it is believed that all the larvae died due to the invasion of a copepod species (*Acartia tonsa*) that was larger than the larvae and feeding of these larvae.

Some suggestions to improve the protocol, ensuring a healthy system, and potential future studies include:

i. Evaluate different devices and methods for measuring the concentrations of the different nutrients in the water samples and use this same measuring method throughout – reagents, device, and program (if applicable)
ii. Have a continuous measure of DO within the sediment – allowing for another measure of microbial activity
iii. Investigate the presence of anaerobic pockets – measure the DO in the different layers of the sediment
iv. Further investigate the microbial communities present – perform a DNA analysis of the water samples
v. Evaluate the system for other marine species – use the improved protocol to measure the health of the system
vi. Investigate the other nutrient components of the tank for a fuller overview of the biogeochemistry of the system – i.e. sulphur cycle for instance could further indicate the health of the tank
vii. As P is only lost by the consumption by phototrophs but when they die its back in the system. One thing to consider is ensuring the algae added are live so that the P can be recycled properly (Paytan & McLaughlin, 2007).
viii. Ensure the N:P is calculated and measure DO and pH regularly – N:P should hopefully be within 13:1 and 28:1 is S3; DO in the water column should be above 80% and pH between 7-8.5 to avoid ammonia (NH_3) toxicity (Fernando Kubitza, 2017).

6. Conclusion

The results obtained in this evaluation of the system suggest that the microbial community within the "living soil" and the water column did maintain a healthy water quality for the survival of the flat oysters. They also suggest that, although a few improvements must be made and other studies would be of assistance, the methods implemented, provide a good overview of what reactions are taking place within the system. Evidence on the activity of microbial communities was made available when comparing S1 vs S2. Further evidence could have been provided with continuous measurements of DO within the sediment. Evidence on the conditions being experienced by the oysters was provided when measuring S3. For commercial purposes, more importance is given to the quality of the water within the water column, S3 should be monitored with more frequency. The results also suggest that the conditions experienced in the TinyOceans system, are in line with what you would expect to see in marine ecosystems. All things considered, we can conclude with some certainty that the water input in the system can be properly recycled so to create a healthy environment for the marine organisms within, thus making it completely water circular. As such, with further monitoring and gathering of evidence, this system could be an extremely positive addition to the mariculture, and perhaps in the future to freshwater fish culture as well, not only improving the sustainability of the industry but also rehabilitating the natural stocks of the ocean.

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8. Appendix

Day
0 20/05/22
T 14:30

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	4,3	0,003	U	0,04
S2	4,3	0,003	U	0,04
S3	4,3	0,003	U	0,04

Day
4 24/05/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	9,9	0,058	0,55	0,26
S2	0,7	0,12	LIMIT	0,04
S3	1,2	0,001	0,27	0,04

Day
7 27/05/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	9,9	0,066	0,46	0,21
S2	0,8	0,026	0,29	0,04
S3	0,1	0,031	0,41	0,08

Day
11 31/05/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	14,3	0,095	LIMIT	0,24
S2	0	0,01	0,68	0,01
S3	0,5	0,032	0,9	0,07

DAY NOTES: Ammonia S3 was stored in the fridge due to shortages in the ammonia reagents

nitrate was not okay that day 14,3 --> the nitrate program was not functionning properly (potential outlier)

Day
14 03/06/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	9,5	0,067	LIMIT	0,27
S2	0,8	0,019	0,84	0,04
S3	1,2	0,035	LIMIT	0,04

DAY NOTES: phosphate stored did not give the accurate values (could not store phosphate for longer than 48h)

Day
18 07/06/22

T 12:10

	120	0,137

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	5,72	0,039		0,06
S2	0,4	0,016	0,14	0,05
S3	2,1	0,032		0,05

DAY NOTES:

This is not an accurate measurement, the values kept changing – **day 18 not considered**
Super high measurement - resampled sample one and decided to alter the sample location to the bottom of the tank on plastic to avoid the dirt from the sand filter
Values for S1 nitrate and nitrite

The phosphate measurement will also change slightly, to evaluate the best method we will compare different methods with the original one

The phosphate went limit again so we had to further dilute it; the values for nitrate and nitrite were too high so the samples were repeated (we think it has to do with the initial turbidity of the water samples from S1 position) other sample locations were also higher than usual. this might have to do with the fact that the waste was not hoovered that day or that the tap was not open for the time it usually is.

Changed way we take samples on location of S1: clear water instead of turbid water by sticking syringe into the sandfilter

Day 21 10/06/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	5,28	0,04		0,04
S2	3,08	0,045		0,04
S3	3,96	0,034		0,04

DAY NOTES:

The program used for Nitrate changed to achieve higher accuracy, the previocusly used program was not properly calibrated and produced unreliable results on the last measurements (day 18)

Water was removed from tank 3 to add to drum tank - could explain NO_3 increase

Day 25 14/06/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	8,6	0,051	4	0,26
S2	5,5	0,022	2,8	0,01
S3	4,2	0,046	1,6	0,06

DAY NOTES:

Valve opened at 3h48mins (DO meter)
Phosphate was measured using 5ml pillows - not super accurate but have na idea - repeat when reagents arrive

S3 phosphate used two types, 5 mL with 1 5 mL bag and 10 mL with 2 5 mL bags. 5 mL gave 1,9 and 10 mL gave 1,3 so just using 5 mL with 1 5 mL bag is fine as well.
S1 - diluted 10x due to leakege of container
Increasing due to lack of alive algae?

Day
28 17/06/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	3,6	0,056	3,3	0,1
S2	2,4	0,016	3,2	0,02
S3	3,1	0,042	3,3	0,08

DAY NOTES: ammonia can be valid - according to manual they can be stored in 4º with acid but we stored in freeezer -20 º without acid (does this make them reliable?) and they can be stored for 28 days - ours were stored for 17 days
Phosphate cannot be stored for more than 48h and therefore the values that were not taken on the day will not be counted

Day
32 21/06/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	11,6	0,063	2,3	0,23
S2	5,6	0,019	1,1	0,04
S3	7,6	0,042	1,5	0,04

DAY NOTES: Tap opens every day again
Measurements were repeated

Day 35

T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	6,4	0,024	1,9	0,06
S2	7,1	0,016	1,7	0,01
S3	7,7	0,026	1,7	0

DAY NOTES:

DAY: 24/06/22 Oxygen in filtration system quite low in the morning, 66% when setting up the DO meter for the measurement

Surface DO above oysters is 9 mg/L equals roughly 95%

Surface DO above filter is 8,2 mg/L equals roughly 86,6%

Water added from tank 2

Day 39 28/06/22

T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	6,7	0,026	2	0,09
S2	6,8	0,01	1,6	0,03
S3	6,5	0,023	4,4	0,07

DAY NOTES:

Phosphate in sample from S3 hit limit of 0,9 mg/L even after a 10x dilution so repeated with a 20x dilution

Phosphate after 20x dilution: 0,22 mg/L = undilted 4,4 mg/L

Phosphate could have increased due to the water added on the day 35

Waste split between two tanks

Day
46 05/07/22
T 12:25

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	16,5	0,067	1,5	0,24
S2	4,4	0,014	2,7	0,01
S3	6,4	0,034	2	0,03

DAY NOTES: The opening of the valve was delayed so the sampling was delayed for 25 mins
Also noticed the DO was quite low 60.0% increased to 71,6 after 15 mins of opening valve
Phosphate from tank 2 was measured to check if this could have been the source of the peak in phosphate (20x D) = 1,8
s3 P 20x D
Nitrate and Nitrite values tripled in S1)
Sampling was more turbind than it should have been for S1- explaining the peaks throughout the system)

Day
49 08/07/22
T 12:34

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	6,7	0,023	1,1	0,09
S2	5,7	0,005	0,9	0,01
S3	7,1	0,029	0,8	0,02

Day
53 12/07/22
T 12:34

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	9,4	0,045	2	0,05
S2	6,8	0,022	1,8	0,04
S3	8,3	0,054	4,1	0,06

Day
56 15/07/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	3,9	0,039	1,6	0,03
S2	7,3	0,021	5,2	0,01
S3	6,9	0,049	3,2	0,05

DAY NOTES: S2 N2 pillow looked like it was wet inside

second measurement 0.022

Different test was used - palline test is not super accurate
Waste is split between tanks (explaining why S3 was higher in ammonia than the sand) also 0_2 was extremly low - thus the oxygenating it

Day
60 19/07/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	2	0,043	3,7	0,07
S2	1,54	0,011	3,1	0,03
S3	1	0,069	1,8	0,05

DAY NOTES: Waste was hoovered the day before
Tank has been airated due to a decrease in DO in the water column

NO_2 was stored and measured
NOTE: the decrease in NO_3 and increase in NO_2 could be due to an imabalnce in the bacterial community?

Day
63 22/07/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	5,18	0,025	2,7	0,06
S2	5,5	0,012	7,1	0,02
S3	6,5	0,025	1,9	0,04

DAY NOTES: Waste not added to the filter
Added 20 tiles made of plaster to system and 6 tiles made of plaster + extra
Removed the tiles mentioned above on the 25th of july

Day
70 29/07/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	2,05	0,021	1,9	0,08
S2	3,58	0,022	6,1	0,01
S3	3,95	0,049	2,2	0,04

Day
74 02/08/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	4,5	0,054	1,7	0,04
S2	4,66	0,024	3,4	0,01
S3	4,57	0,059	2,7	0,07

Day
77 05/08/22
T 12:00

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	4,62	0,074	3	0,07
S2	4,22	0,014	5,6	0,03
S3	4,44	0,067	4	0,05

DAY NOTES: Opened valve later, 10:30 ish stayed open for longer as well for about 60 minutes

Day
82 10/08/22
T 12:30

	NO_3 (mg/L)	NO_2 (mg/L)	PO_4^{3-} (mg/L)	NH_3 +NH_4 (mg/L)
S1	5,36	0,05	2	0,07
S2	5,58	0,031	3,4	0,05
S3	5,52	0,059	1,4	0,06

DAY NOTES: Left valve open and running for 1.5 hour from 10:00 to 11:30