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Assessment of Geosmin Level and Nutrient measurements (ammonia, nitrite, nitrate) in Recirculated Aquaculture Systems (RAS). A case study from African Catfish (*Clarias gariepinus*) production in Sweden

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Abstract

Recirculating aquaculture systems (RAS) offer a promising method of producing fish, but problems such as the presence of geosmin, a compound known for its earthy taste and odour, can affect the quality of the fish This study investigates African catfish (*Clarias gariepinus*) farmed at the RAS facility in Floda, Sweden, where geosmin-related taste issues have been sporadically observed. Despite the effectiveness of RAS, questions remain regarding it environmental and economic sustainability.

The presence of geosmine-producing bacteria in various parts of the RAS system was analysed using analysis of the geosmine coding gene through PCR. The levels of key water quality nutrient parameters - ammonia, nitrite, and nitrate - in the RAS environment were analysed using spectrophotometric methods.

The results demonstrated the stability of the water recirculation system and the efficiency of biofiltration. Ammonium, nitrite, and nitrate levels remained below the threshold values and were stable during sample. The used “Cyc” primer was unable to detect the *GeoA* gene of geosmin-producing bacteria. Further studies using alternative primers and optimization of PCR thermocycling conditions are necessary.

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Abstrakt

Återcirkulerande vattenbrukssystem (RAS) erbjuder en lovande metod för att producera fisk, men problem som förekomsten av geosmin, en förening som är känd för sin jordnära smak och lukt, kan påverka kvaliteten på fisken. Denna studie undersöker odlad afrikansk havskatt (Clarias gariepinus) vid RAS-anläggningen i Floda, Sverige, där geosminrelaterade smakproblem sporadiskt har observerats. Trots effektiviteten hos RAS kvarstår frågor om den miljömässiga och ekonomiska hållbarheten.

Förekomsten av geosminproducerande bakterier i olika delar av RAS-systemet analyserades med analys av den geosminkodande genen genom PCR. Nivåerna av viktiga näringsparametrar för vattenkvalitet - ammoniak, nitrit och nitrat - i RAS-miljön analyserades med spektrofotometriska metoder.

Resultaten visade stabiliteten hos vattenåtercirkulationssystemet och effektiviteten av biofiltrering. Ammonium-, nitrit- och nitratnivåerna förblev under tröskelvärdena och var stabila under provet. Den använda "Cyc"-primern kunde inte detektera GeoA-genen från geosminproducerande bakterier. Ytterligare studier med användning av alternativa primers och optimering av PCR-termocykliska förhållanden är nödvändiga.

Contents

Introduction 5
Recirculating aquaculture system (RAS) 6
Main components of RAS: 6
Water Quality: Key to Fish Health 7
Steps of nitrification process 7
Catfish (Clarias gariepinus) farming in RAS 8
Geosmin presence in RAS 8
Aims and Objectives 9
Methods 10
Operation and maintenance of the studied RAS system 10
Sampling strategy 12
Laboratory analysis 12
Nutrient measurements 12
Geosmin analysis 13
DNA extraction 13
RNA extraction 13
cDNA synthesis 13
Amplification and cloning of *geoA* by quantitative PCR 13
16S PCR normalization 14
GeoA positive control testing 14
Results 14
Cleaning service 14
Nutrient flow measurements 15
Ammonia measurements 15
Nitrite measurements 18
Nitrate measurements 20
Comparative Nutrient Analysis: colour scale vs. spectrophotometry 22
geoA PCR-analysis 23
16S rRNA Gene Expression for qPCR Normalization 23
geoA primer qPCR analysis 24
GeoA positive control testing 26
Discussion 27
Nutrient flow assessment 27
GeoA PCR analysis 29
References 31

Introduction

The growing global population, projected to reach 8.5 billion by 2030 and 9.7 billion by 2050, is driving growing demand for food, including from marine as well as land-based sources (FAO, 2022). However, relying on wild fish stocks to meet this demand in a sustainable way is no longer possible (ref). To feed this expanding population, a significant expansion in food production, especially in the aquaculture sector, will be necessary (Grealis et al., 2017). The importance of the aquaculture industry has increased significantly over recent decades, today it is a significant part of the global food system and the fastest growing food sector in the world (Puszkarski and Śniadach, 2022). There are numerous interpretations of the concept of aquaculture, the Food and Agriculture Organization (FAO) offers the following definition: "Aquaculture is the farming of aquatic organisms including fish, molluscs, crustaceans, and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc." (FAO, n.d.). The implementation of aquaculture processes occurs in different environments and on all continents, except for Antarctica, and a total of 622 aquatic species are grown in aquaculture conditions (FAO, 2020). In 2017, 67.7% of all seafood was of aquaculture origin (Ahmad et al., 2022).

Such high demand and pressure on industry productivity have led to the development of intensive aquaculture practices, which span various aquatic environments, and resulting in intense competition for basic resources pivotal to its growth, including water, feed, and land. The development of intensive aquaculture practices has led to negative consequences and severe pressure on the environment. An example is the occurrence of eutrophication, caused by excess nutrients in fish farms such as nitrogen and phosphorus, can lead to excessive growth of algae and other aquatic plants. This excessive growth of vegetation can reduce oxygen levels in the water, harming marine ecosystems and creating environmental problems (Primavera, 2006). Another major problem is loss or alteration of the natural environment. A global example is the conversion of mangrove forests to fish and shrimp farming. Over the past 20 years, nearly a third of the world's mangrove forests have disappeared due to intensive aquaculture activities. What leads to the loss of ecosystem, reduction of biodiversity, soil erosion, floods. Moreover, the cultivation of aquatic organisms in open environments poses a significant threat by contributing to the spread of diseases among wild populations. Operating under conditions of high density, common in aquaculture, not only facilitates pathogen transmission but also induces stress in farm organisms, consequently diminishing their resistance to infections (Ahmed and Glaser, 2016). Furthermore, intensive aquaculture practices are the heavy dependence on wild fish stocks for food. Which is paradoxical, since one of the main tasks of aquaculture is precisely to contribute to the conservation of wild aquatic reserves (Primavera, 2006). However, despite the reduction in the share of fish products in fish feed and its replacement with vegetable protein, fish products still make up a significant part of the fish feed (Tacon and Metian, 2005). Given these multifaceted challenges, there is a need for ongoing development of the aquaculture sector. This development should be guided by sustainable production processes and a concerted effort to transition away from intensive aquaculture practices. This approach is vital not only to meet the demands of a growing population but also to ensure the long-term health and resilience of aquatic ecosystems. Sustainable aquaculture is a concept or philosophy that involves the rational use of natural resources, avoiding environmental degradation while at the same time maintaining economically viable production (Valentini et al., 2016). Sustainability of processes, including the aquaculture sector, should be based on three key concepts: economic sustainability: the production process must be financially viable and demonstrate consistent profitability, social

sustainability: public approval of the processes, promoting positive relationships with local communities and stakeholders, and environmental sustainability – the critical aspect, highlights the need to conduct production processes in a manner that protects the environment and ensures that they are carried out without causing damage to ecological systems. (Boyd et al., 2020). Sustainable aquaculture is a key tool for achieving the United Nations global sustainable development goals (SDGs; ref). Responsible aquaculture can reduce the gap between supply and demand, reduce pressure on wild fish stocks and provide populations with a healthy source of nutrients including omega-3 fatty acids. Aquaculture has the potential to stimulate economic growth by creating jobs and generating income. Sustainable aquaculture activities can contribute to livelihoods, helping to reduce poverty and improve living conditions. Responsible aquaculture practices can help reduce overfishing and habitat destruction, helping to conserve marine biodiversity. Bringing aquaculture into line with the SDGs includes introducing sustainable practices such as the appropriate use of recirculating aquaculture systems (RAS), integrated multi-trophic aquaculture (IMTA), reducing dependence on wild fish in fish feed, implementing stewardship and certification schemes (Stead, 2019). The next section will focus on RAS, water quality parameters,affecting fish health, and the production of high-quality food products.

Recirculating aquaculture system (RAS)

Transferring places for farming aquatic organisms to land-based closed systems protected from external factors, is one of the alternative and promising directions for the further development of aquaculture such as RAS systems (Boyd et al., 2020). RAS allows precise control over different water parameters, including temperature, pH, dissolved oxygen levels, and water quality. This control ensures that the aquatic organisms are kept in optimal conditions, promoting their health and growth. Any deviations in water quality can be quickly identified and addressed, reducing stress on the fish, and minimizing the risk of disease outbreaks. With such a controlled environment, the risk of diseases from external sources is minimized. The closed nature of RAS reduces the environmental impact of aquaculture by minimizing water use and preventing nutrient-rich wastewater from entering natural ecosystems. It also avoids habitat destruction, making it an environmentally conscious approach to fish farming. A key aspect of RAS is water reuse, which is achieved through a combination of mechanical and biological filtration coupled with efficient oxygenation processes. This filtration system allows RAS reuse up to 99% of the water (Tetreault et al., 2023). RAS plants can operate year-round, allowing fish production even in regions with extreme weather conditions where traditional open water aquaculture may be limited by seasonal factors (Ahmed and Turchini, 2021).

Main components of RAS:

While the specific configuration of RAS components may vary, the fundamental principles remain consistent. Fish are kept in tanks, and the design and size of the fish containers can differ depending on the scale of the RAS and the species being cultured. Water from the fish tank flows into a mechanical filter - the component responsible for the continuous removal of suspended particles. During the cleaning process, solid particles are captured by the micro-screen of the drum filter, which significantly reduces the turbidity of the water. This mechanical filtration process is important for removing organic particles and preparing water for the next treatment step - biological filtration (Ahmed and Turchini, 2021). The biofilter plays a crucial role in maintaining the water quality in the RAS. The biofilter provides a stable water environment and prevents the accumulation of harmful compounds. (Bartelme et al., 2017). To ensure water circulation between all RAS components, water pumps are necessary. In addition to the fundamental components listed above, the specific configuration of RAS may incorporate additional elements, such as oxygenation elements, ultraviolet units, monitoring

elements. These additional components are designed to optimize the efficiency and functionality of the RAS.

Water Quality: Key to Fish Health

The main sources of nitrogenous compounds in the RAS are the metabolism of fish. Fish consume food containing proteins that are broken down during digestion. Metabolism of these proteins results in the formation of nitrogenous waste compounds such as ammonia and urea (Figure 1). Uneaten food and the accumulation of organic matter in water also contribute to the formation of nitrogenous compounds. The efficiency of nitrogen assimilation by fish is an important indicator that influences water quality and the productivity of the RAS system, research shows that the range of nitrogen recovery is between 11 - 36%, the rest of the nitrogen is excreted with waste (Hargreaves, 1998). Although nitrogen is an essential nutrient for all living organisms, it is required in relatively small quantities to meet physiological needs. Excess amounts quickly turn into nitrogenous waste that requires disposal. Nitrogen compounds that accumulate in recirculating aquaculture systems have detrimental effects on aquatic organisms, especially when they are reared in high-density conditions. Even with frequent water changes in systems, ammonia and nitrite levels increase exponentially (Timmons et al., 2018, p. 38-39). Both ammonia and nitrite are toxic to aquatic organisms in low concentrations. It is lead to the a variety of physiological problems, including decreased blood pH caused by the accumulation of acidic byproducts, gill damage, and water and mineral imbalances (ref). Studies have shown that ammonia exposure can affect blood transaminase levels by interfering with fish metabolism, cardiac function, liver lysosome functionality in rainbow trout, and ATP levels (ref). Nitrite's primary impact on fish is the induction of methemoglobinemia, a condition extensively researched. Upon entry into fish red blood cells via the gills, nitrite oxidizes the iron atom within haemoglobin. Consequently, haemoglobin transforms into methaemoglobin, losing its ability to effectively bind oxygen, consequently giving the blood a brownish colour (Tomasso, 2008).

Ammonia accumulates in RAS water because of fish excrement and excess feed. Ammonia deposition to toxic levels in closed systems, as opposed to open systems, is a significant problem that must be addressed to achieve successful production and welfare of fish. Ammonia occurs in two distinct forms: unionized (NH_3-N) and ionized (NH_4^+-N) (ammonium). The equilibrium between these two forms is highly dependent on the pH, salinity, and temperature. The combined concentration of both NH_4^+ and NH_3 is commonly referred to as Total Ammonia Nitrogen (TAN). Due to its ability to penetrate cell membranes, the most toxic form is unionized ammonia (Timmons et al., 2018, p. 39-40). As was mentioned above, such a parameter as water pH can influence on ammonia toxicity. Usually, optimal pH value should be between 7-8. Increasing the pH parameter values contributes to increase in the toxic form of ammonia (unionized) (Bartelme et al., 2017).

Steps of nitrification process

The first nitrification process is the oxidation of ammonia to nitrite (NO_2) by bacteria such as. *Nitrosomonas sp*., *Nitrosococcus sp*., *Nitrospira sp*. etc. Nitrite is an intermediate product of the nitrification process. It is less toxic than ammonia but also becomes unsafe for fish if the nitrite level in the water is higher than 2 mg/l. The threshold value may vary, this value applies to African catfish (Roques et al., 2015). In this case, nitrite from the water begins to accumulate in the fish's blood and interferes with the transfer of oxygen in the blood. The second step of the nitrification process is the oxidation of nitrites by nitrite-oxidizing bacteria that convert nitrite to nitrate (NO_3), a compound that is relatively non-toxic to fish (Figure 1) (Bregnballe, 2022).

Process of nitrification: $NH_4 + 1.5\ O_2 \rightarrow NO_2 + H_20 + 2H^+ + 2e \rightarrow NO_2 + 0.5\ O_2 \rightarrow NO_3 + e$

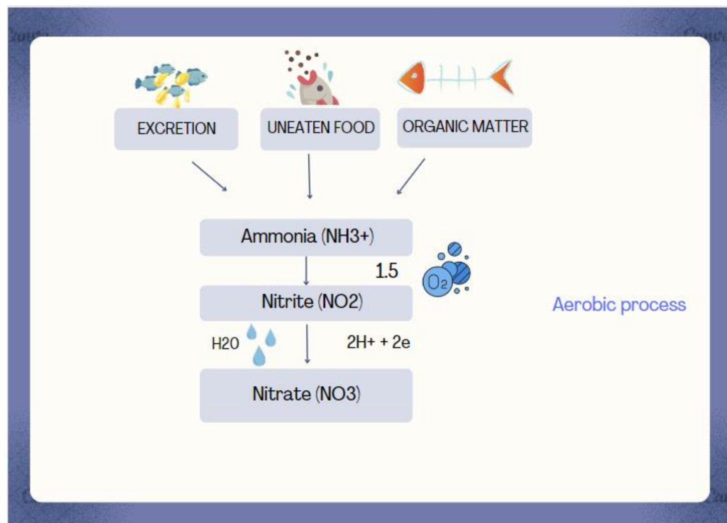

Figure 1: Process of nitrification in RAS

As can be seen from the process described above, ammonia is primarily produced by fish as a by-product of protein metabolism, and it is excreted through the gills, as well as in the urine. In addition, uneaten food and decomposing organic matter contribute to increased ammonia levels in the water. Ammonia and the subsequent product of nitrification (nitrite) are important parameters that must be strictly controlled to ensure the welfare of fish in the aquatic recirculation system.

Catfish (Clarias gariepinus) farming in RAS

Aquaculture production in Europe has historically centred around predatory fish, constituting 66% of the total output. However, a discernible shift in this trend is observed, marked by a growing emphasis on farming fish at lower trophic levels. This transition aligns with global initiatives aimed at bolstering the ecological sustainability of fish farming and diminishing reliance on species occupying higher trophic levels (Gyalog et al., 2022).

African Catfish (*Clarias gariepinus)* are considered good choices for growing in closed water systems for several reasons. Catfish has remarkable endurance, enabling them to adapt and thrive under many abiotic water conditions. One of the important advantages of Catfish compared to others is that they show tolerance to high density, good welfare of fish can be maintained in conditions of high concentration of individuals. Catfish be grown at a stocking density of up to 500 $kg/m^{3.}$ (Nieuwegiessen et al., 2009). At the same time, they are quite resistant to infectious diseases, even at high concentrations. A further advantage lies in the adaptability of African catfish to simplified feeding strategies. As omnivores, catfish exhibit versatility in accepting various types of food, maintaining a commendable growth rate. This adaptability aligns with the practicalities of aquaculture management. (Ekawati et al., 2021). In addition to the mentioned advantages, catfish is recognized for its high taste qualities. The tender fillet with a mild taste is well-received by consumers, contributing to the market appeal of catfish products (Boyd et al., 2020).

Geosmin presence in RAS

One of the limitations of using freshwater systems, including RAS, is the occurrence of undesirable tastes and odours. Among the numerous components responsible for these discomforts, geosmin and 2-methylisoborneol (MIB) are the most common. Geosmin stands out as a common component. Even small amounts of geosmin in aquacultured fish can lead to negative consumer preferences and affect the value of fish products. This compound is

secondary metabolite that is produced by various groups of bacteria, including Actinobacteria, Cyanobacteria and Proteobacteria (Hooper et al., 2023). Research on off-flavours in RAS indicates that geosmin production in RAS is primarily associated with freshwater cyanobacteria and actinomycetes, particularly the genus Streptomyces. These gram-positive spore-forming bacteria, belonging to this order, are filamentous organisms primarily functioning as facultative anaerobes. They possess distinctive traits, including the capacity to produce a wide array of complex terpene secondary metabolites such as geosmin and MIB (Lukassen et al., 2017).

Fish absorb geosmin through the gills, but possibly also from the intestinal tract due to ingested geosmin-producing bacteria. Geosmin then accumulates in adipose tissue, therefore fish with a high lipid content are most susceptible to geosmin accumulation (Houle et al., 2010). Geosmin is quickly taken in by fish and builds up in their systems. Removing this compound from fish is a gradual process that takes several days to decrease to levels undetectable by humans, usually falling below 250 ng/kg of fish. The processes required to decontaminate fish of these lipophilic compounds, such as extended purging times in clean water or the use of advanced filtration systems, are energy-intensive and thus decrease the ecological sustainability of the fish farming operation. These additional processing steps increase the operational costs and, consequently, the market price of the fish (Dupre et al., 2023).

Since geosmin production originates from various bacterial groups, molecular identification has predominantly relied on the geosmin synthase, the enzyme responsible for the biosynthesis of geosmin. The *geoA* gene is responsible for encoding the geosmin synthase enzyme that (Lindholm-Lehto and Vielma, 2018). In contrast to developed molecular detection methods for geosmin producers, there is a notable absence of such tools for MIB producers in RAS. This gap in knowledge about these bacteria is likely due to the limited diversity observed in the sequences of the MIB synthesis gene (Lukassen et al., 2017).

Currently, a dominant method used for the mitigation of geosmin level in fish tissue is purging technique, which represents the pre-slaughter transfer of fish to tanks with freshwater. This transitional phase, can last from several hours to several weeks, necessitates a period of fasting for the fish, coupled with the daily renewal of water. During this transitional phase, geosmin, being a dissolved substance, tends to move from an area of higher concentration, within the fish's tissues, to an area of lower concentration in the surrounding water through a semi-permeable membrane – this process called osmosis. The gradual reduction of geosmin levels in the fish is achieved through this osmotic process (Podduturi et al., 2020). However, such a technology is not only unsustainable due to high water consumption, but also economically unprofitable - due to high resource consumption and lost fish mass.

Aims and Objectives

The work aims to improve understanding of water quality in recirculating aquaculture systems (RAS) by identifying bacterial contamination hotspots and assessing key nutrient parameters:

1. To identify “hot spots” in the RAS components that host geosmin-producing bacteria
1. To evaluate water quality of the RAS with respect to ammoniacal nitrogen (TAN), nitrite (NO_2), and nitrate (NO_3).
2. To examine the relationship between nutrient indicators: ammonia (TAN), nitrite (NO_2-N), and nitrate (NO_3-N) and the system maintenance stages (before/after cleaning).

The findings of this research, conducted in collaboration with “Pond: Fish&Green” company, are expected to enhance the understanding of RAS processes. Furthermore, they are also

expected to contribute to the development of strategies to improve the quality of their products (*Clarias gariepinus*).

Methods

Operation and maintenance of the studied RAS system

The studied recirculating aquaculture system is a rearing facility for indoor production of African catfish (*Clarias gariepinus*), located in Floda, western Sweden. The facility includes 4 tanks for fish, the volume of each tank is 3,16 m^3 (Figure 2). Each fish tank contains about 150-220 fish. The fish are sorted by size at regular intervals to prevent intense intraspecific competition within the fish, which can occur due to size differences.

The water in the tanks of the RAS is maintained at a constant temperature of 25-26 degrees Celsius. This temperature range is tailored to the special needs of the African catfish, providing an optimal environment for its growth and well-being. Additionally, it is imperative to sustain a consistent pH level within the prescribed range of 7.4-7.6. This pH range is particularly significant as it influences the chemical speciation of ammonia. Within the system, the pH range of 7.4-7.6 promotes the predominance of the less toxic ammonium ion (NH_4^+). This not only ensures the health of the fish but also creates an environment conducive to increased activity of nitrifying bacteria, thereby enhancing the efficiency of the nitrogen cycle - a critical aspect of system functionality.

The short water retention time of approximately 45 minutes in each tank is designed to optimize water quality and nutrient cycling within the recirculating aquaculture system. This rapid turnover ensures efficient removal of waste by-products and promotes a well-maintained environment. However, regular maintenance of the interior surfaces of the fish tanks is necessary as they tend to accumulate residue, consisting mainly of uneaten food and fish waste by-products. Once a week, the inner walls of the fish tanks are cleaned with a brush without draining the water. Once every month, during the maintenance of the fish tanks, the water level in it is reduced to as low as possible, so that it is possible to clean the internal walls of the tank, but at the same time the fish can survive without much stress. The walls are cleaned with a sponge and water. Sometimes a water pressure machine is added to the cleaning procedure. On an annual basis, the fish tanks undergo a comprehensive cleaning procedure, during which the fish are temporarily relocated to an alternate container and the fish tank is completely cleaned with a chlorinated agent and dried.

The drumfilter is a physical filter in the RAS, the size of the screen in the filter can vary and in the study system it is 60 microns. The drum filter requires constant maintenance and is cleaned once a week with a brush and water. After the water has undergone mechanical purification through a drum filter, it enters biofilters. The studied RAS has two moving bed biofilm reactor (MBBR) biofilters, each filled with 'HXF13KLL' plastic carriers - they create an extensive surface to host bacteria in biofilms (Figure 3). The biofilters are equipped with an aeration system that ensures the movement of plastic media and sludge. In the studied system, the biofilters are not usually cleaned, but bottom water is occasionally drained. Situated at the lowest point within the system, the pool serves as the nexus where all components converge through a network of outlets and inlets. This component needs maintenance. As part of a routine maintenance regimen, the pool undergoes weekly cleaning, involving the use of a brush and a high-pressure water machine.

Routine system maintenance includes the daily monitoring of crucial water parameters, such a temperature, pH, the levels of ammonia, nitrite, and nitrate. Ammonia ($NH3/NH_4^+$), nitrite

(NO_2^-) and nitrate (NO_3^-) levels are measured using ready-made kits and colour scales. To ensure the well-being of the fish, it is necessary to establish a constant feeding regime. Fish should be fed five times a day at three-hour intervals, starting with the first feeding at 8 a.m. and ending with the last feeding at 8 p.m. The feeding dose is 400 grams, which are gradually poured out of the feeder over 300 seconds. "Meerval 4.5. Skretting" specially developed for catfish is used for feeding. Catfish are omnivorous animals that can adapt to different diets. Used feeding has a sufficiently high protein content – 41-43% and is and supplied with omega-3 fatty acids.

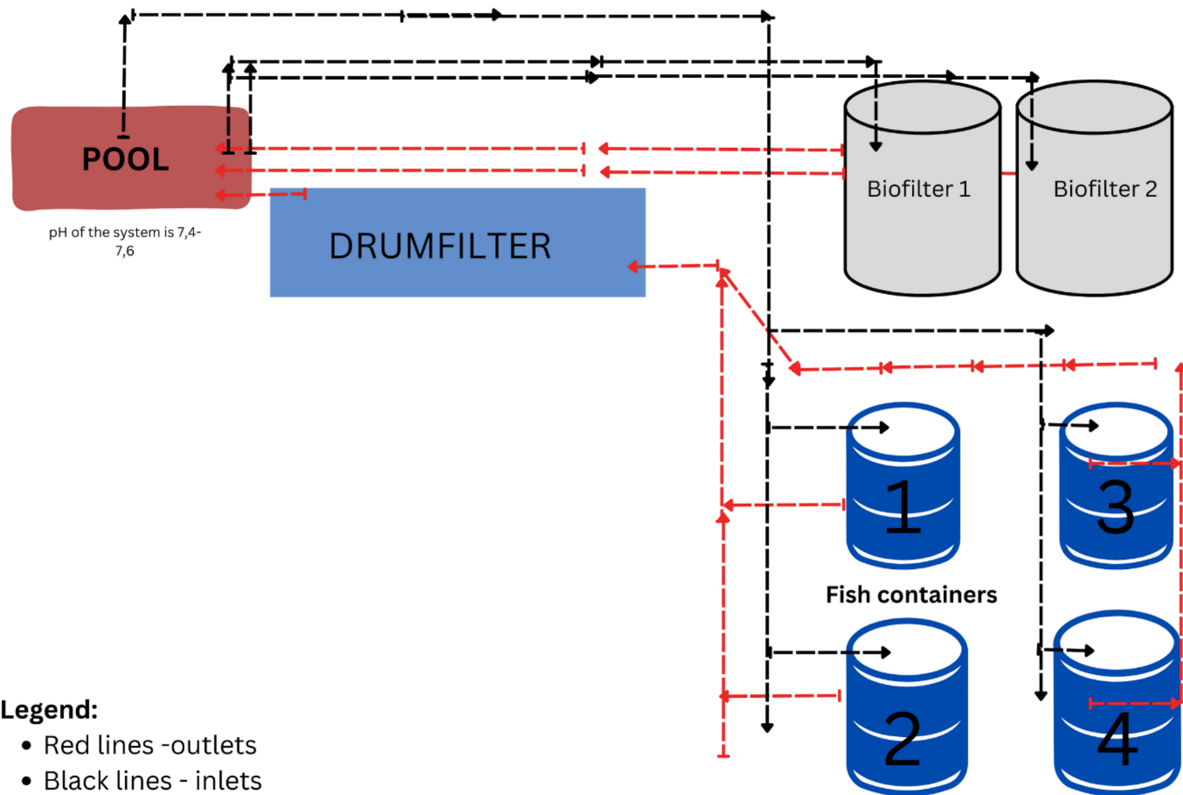

Figure 2. The recirculating aquaculture system scheme at "Pond: Fish&Greens" in Floda, where the study was conducted)

Figure 3: Plastic carries of the biofilters and biofilter of the RAS

Sampling strategy

Intensive sampling was carried out in the RAS during Autumn 2023 and Winter 2024. The sampling period was divided on cycles, each cycle was performed for one week, every three cycles was period of analysing in the laboratory.

There were three types of samples: water samples, biofilm, and fish samples. All samplings were done in the first half of the day when feeding occurred. Water samples were from each fish tank and from the pool. Approximately 50 ml of water sample were taken into dry, clean centrifuge tubes. Biofilm samples were taken from each fish tanks, from the pool, from biofilters, and from the drum filter. Due to differences in the servicing status (cleanliness) of these RAS sections, the volume of collected biofilm samples varied, from 1 ml to 2-3 ml. In some cases, sample collection was hampered by insufficient material availability, usually occurring immediately after element cleanup (Figure 4). Every week of a cycle also samples were taken fish from random selected fish. These samples included mucosal samples extracted from the skin, mucus samples from the intestine and gill arch samples. Water samples and biofilm were kept at -20 ºC, fish samples - at -80 ºC Celsius until further analysis in the laboratory.

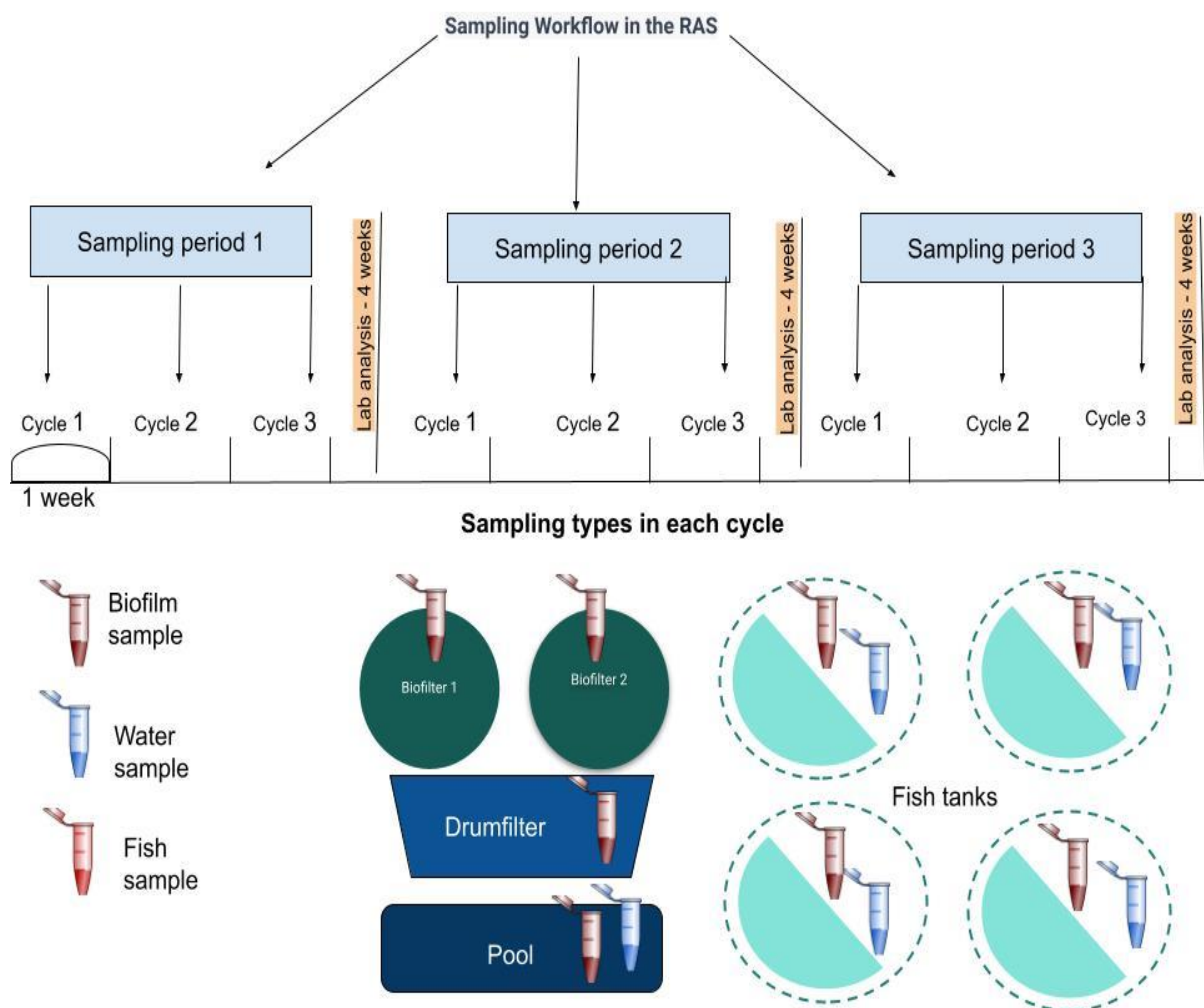

Figure 4: Sampling workflow

Laboratory analysis

Nutrient measurements

Total ammonia nitrogen concentration (NH_3-N) the sum of NH_3 and NH_4^+. , nitrite (NO_2^--N) and nitrate (NO_3^--N) measured. These measurements contribute to a holistic understanding of

nitrogen dynamics in the recirculating aquaculture system. The method employed for analysing nutrient concentrations in this study was spectrophotometry. The HACH reagent kit was used for measurement these parameters according to protocols provided with kit.

Calculation of the unionized form of ammonia

The non-ionized form of ammonia using the equation proposed below:

$NH_3 = TAN/\ 1 + 10^{(pKa - pH)}$

where pKα is the negative logarithm (base 10) of the acid dissociation constant (Kα) for ammonia, which is approximately 9.25 at 25°C

Geosmin analysis

DNA extraction

The DNA extraction process involved the utilization of biofilm specimens obtained from various sources within the Recirculating Aquaculture System (RAS), including fish tanks, the pool, outlets of the biofilters, biofilter 1 and biofilter 2, and the drum filter. Before the DNA extraction procedure, the biofilm samples were thawed, followed by the removal of excess water. The DNA extraction itself was conducted using the DNeasy PowerSoil Pro Kit, adhering to the protocol provided with the kit (Qiagen, 2023). Ater extraction, purified DNA from biofilm samples were stored at –20ºC temperature.

RNA extraction

For RNA extraction fish samples were used: mucus from the skin, mucus from the intestine, arch gills. For RNA extraction were used about 30 mg. of each sample. The procedure of RNA extraction was conducted using RNeasy Plus Kits adhering to the protocol provided with the kit. This kit is recognized for its efficacy in isolating high-quality RNA, particularly suited for challenging sample types such as those obtained from mucosal surfaces and gill tissues. After extraction, purified RNA from samples were stored at –80ºC temperature. This stringent storage condition was chosen to safeguard the RNA's stability and integrity.

cDNA synthesis

Reverse transcription was performed using a commercially available iScript cDNA synthesis kit (Bio-Rad). The required volume of nuclease-free water along with the original RNA sample was carefully determined to achieve a standardized amount of RNA of 1000 ng per sample. This calculation was based on the concentration of the original RNA samples. Each sample was brought to a volume of 20 µL, including 15 µL of the original RNA sample and nuclease free water in calculated ratios, as well as 1 µL of reverse transcriptase and 4 µL of the reaction mixture obtained from the kit. The reactions underwent an incubation period of 5 minutes at 25°C for priming, followed by 20 minutes at 46°C for reverse transcriptions and 1 minute at 95°C for ferment inactivation.

Amplification and cloning of *geoA* by quantitative PCR

Fragments of the *geoA* gene, widespread among geosmin-producing bacteria, were targeted for amplification via quantitative polymerase chain reaction (qPCR) using the Cyc primers: CycFW and CycRW (sequences provided below) (Lukassen et al., 2017).

Sequence 5′-3′ for Cyc FW: TGGTAYGTITGGGTITTYTTYTTYGAYGAYCAYTT

Sequence 5′-3′ for Cyc RW: CATRTGCCAYTCRTGICCICCISWYTGCCARTCYTG

The qPCR reactions were performed in a 10 µL reaction mixture containing: 4 µL of template DNA, 0.5 µL of each primer: Cyc FW and Cyc RW, 5 µL of SYBR Green Supermix (Bio-Rad). QPCR reactions were carried out on CFX Connect Real-Time PCR Detection System. For qPCR reactions were tested two different conditions: a hot start activation at 95°C for 3

minutes, followed by 40 cycles 95°C for 10 second (denaturation) and 60°C for 30 second (annealing and extension) followed by melting curve analysis spanning from 65°C to 95°C. For the second condition: a hot start activation at 95°C for 5 minutes, followed by 30 cycles 95°C for 1minute (denaturation) and 50°C for 1 minute (annealing and extension) followed by final extension 72°C for 5 minutes and melting curve 65°C to 95°C.

For efficiency testing, different amounts of DNA were evaluated in the reaction mixture: 100 ng, 50 ng, 25 ng, 12.5 ng, 6.25 ng, and 3.125 ng.

16S PCR normalization
To detect the 16S rRNA gene, primers with next nucleotide sequences (5'-CCTACGGGAGGCAGCAG-3′′) and (5'- ATTACCGCGGCTGCTGG -3′) were used.

Quantitative PCR was carried out on cDNA. The qPCR reaction was performed in a 10 μL reaction mixture containing: 4 μL of template cDNA, 0.5 μL of each primer, 5 μL of SYBR Green Supermix (Bio-Rad). The following conditions were used: a hot start activation at 95°C for 3 minutes, followed by 40 cycles 95°C for 10 second (denaturation) and 60°C for 30 second (annealing and extension) followed by melting curve analysis spanning from 65°C to 95°C.

For DNA samples from biofilms and water conventional PCR was carried out due to it is cost-effectiveness. The PCR reaction was performed in a 25 μL reaction mixture containing: 4 μL of template DNA, 0.75 μL of each primer, 12.5 μL KAPA HiFi HotStart ReadyMix (Roche) and 7 μL Milli-Q water. The conventional PCR reaction started with an initial denaturation step at 98°C for 5 minutes. Subsequently, following 30 cycles, denaturation was performed at 95°C for 1 minute, followed by annealing at 57°C for 1 minute. Extension of the DNA fragments occurred at 72°C for 1 minute and 30 seconds, a final extension was carried out at 72°C for 5 minutes.

GeoA positive control testing
A positive control was obtained from colleagues from Denmark. This control consisted of bacterial colonies containing the *geoA* gene. DNA extraction was initially performed from these bacterial colonies using the same methodology and kit as described previously. Subsequently, the isolated DNA was tested using 16S primers to confirm the presence of bacterial DNA in the samples. Conventional PCR was used for this purpose using the same PCR conditions and reaction mixtures used to test original DNA template with 16S primers. To optimize assay conditions, different amounts of DNA (50 ng, 25 ng, and 12.5 ng) were assessed in the reaction mixture.

After confirming the presence of bacterial DNA in positive control samples, a PCR test was performed using Cyc primers (CycFw and CycRw) targeting *geoA*. For this analysis, conventional PCR was again used with an initial denaturation step at 98°C for 5 minutes. Subsequently, 30 cycles were performed consisting of denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute and 30 seconds. Final extension was performed at 72°C for 5 minutes. Similarly, different amounts of DNA (50 ng, 25 ng, and 12.5 ng) were assessed in the reaction mixture to optimize assay conditions.

All PCR products obtained after conventional PCR were subjected to electrophoresis in a 1% agarose gel at 90 V for 45-60 minutes to evaluate amplification.

Results

Cleaning service
One of the aims of this study was to trace the dynamics of the nutrient concentrations between the stages when the RAS parts have undergone a scheduled cleaning service and before the

weekly cleaning of the RAS parts. The results outlined in the Table 1, display the adherence to routine weekly cleaning for parts within the RAS. From the table it can been seen that during the sampling period, certain weeks the cleaning was skipped (for example sampling week 5), or cleaning of RAS sections was conducted selectively.

Table 1. Summary of Maintenance Cleaning Services for RAS Elements.

Sampling week	Sampling date	Was cleaned	Fish tank 1	Fish tank 2	Fish tank 3	Fish tank 4	The pool	Drum filter
1	06.10.23	NOT				√		No info
		YES	√	√	√		√	
2	12.10.23	NOT	√	√	√			√
		YES				√	√	
3	19.10.23	NOT	√	√	√	√		√
		YES					√	
4	24.11.23	NOT	√		√		√	√
		YES		√		√		
5	30.11.23	NOT	√	√	√	√	√	√
		YES						
6	07.12.23	NOT						
		YES	√	√	√	√	√	√
7	13.12.23	NOT						
		YES	√	√	√	√	√	√
8	18.12.23	NOT	√	√	√	√		√
		YES					√	
9	05.01.24	NOT	√	√	√	√		√
		YES					√	

Nutrient flow measurements

Ammonia measurements

Total ammonia concentrations (TAN) did not differ between “before” and “after” cleaning (Fig. 5 and 6).

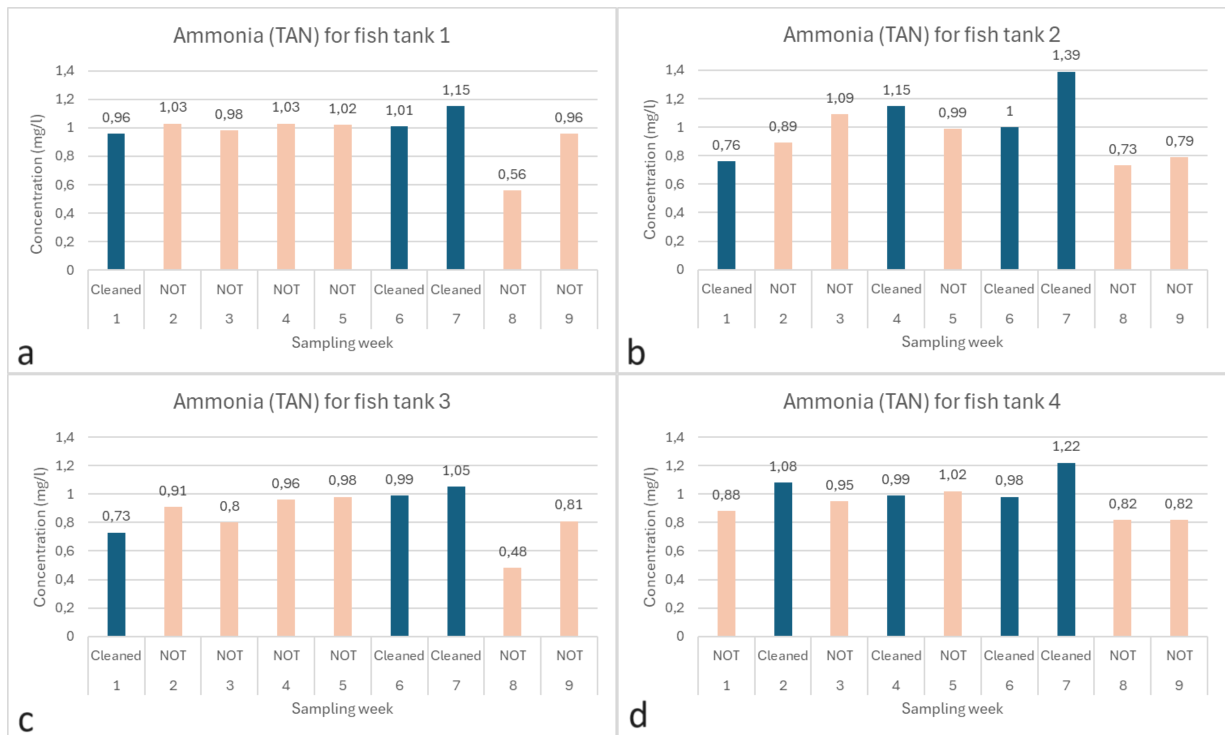

Figure 5. Total ammonia nitrogen (TAN) concentration in fish tank 1 (a) fish tank 2 (b) fish tank 3 (c) fish tank 4 (d). Values highlighted in orange indicate the ammonia measurements, taken before service cleaning of the element. Values highlighted in blue indicate the ammonia measurements, taken after service cleaning.

Notably, the concentration of total amount ammonia in the "Pool" RAS element is lower than in the fish tanks. This difference arises due to the composition of the "Pool" water, which includes water from both: fish tanks, as well as water subjected to mechanical and biological filtration processes.

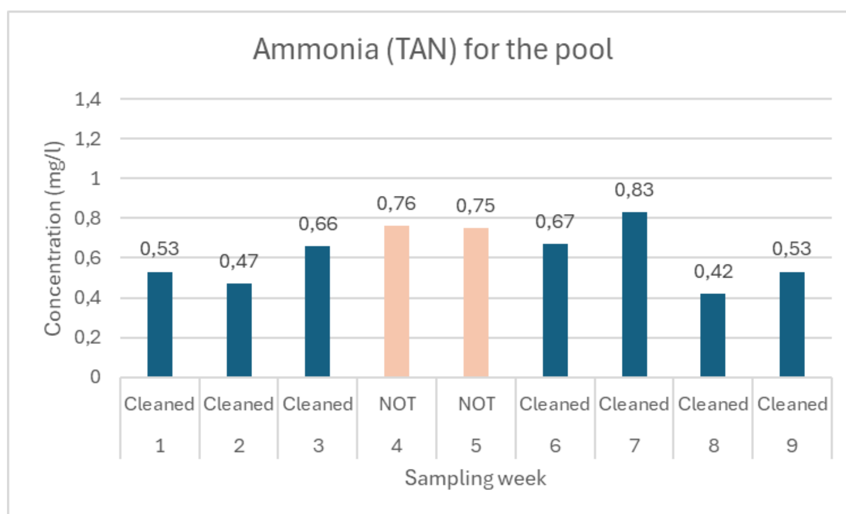

Figure 6. Total ammonia nitrogen (TAN) concentration for the pool

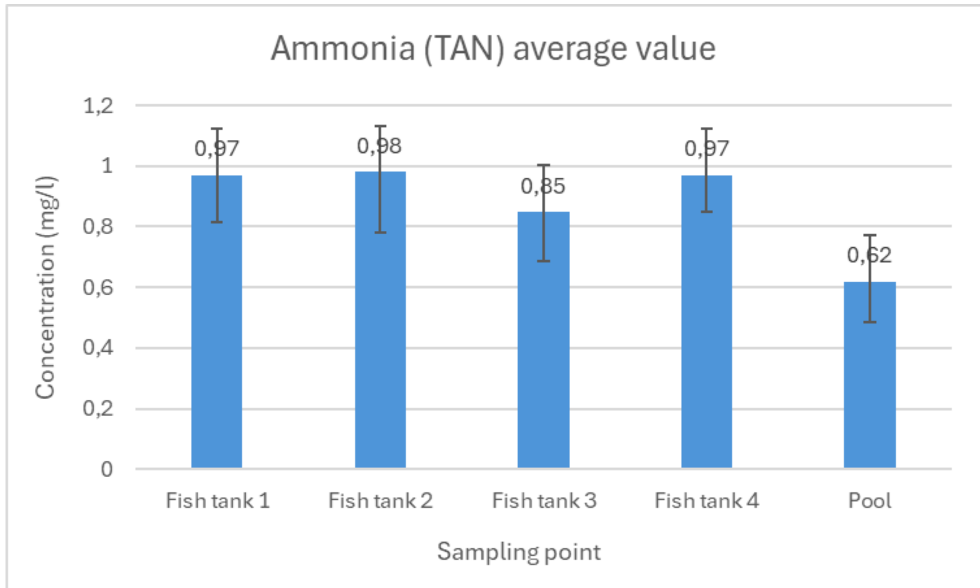

Figure 7. Average values of total ammonia nitrogen (TAN) concentration for fish tanks and "Pool" in the RAS. Error bars on a chart represent the variability data in measurements.

A calculation was conducted to determine the values of the unionized form of ammonia (NH3–N). Figure 7 illustrates the trend in the concentration of unionized ammonia over the course of the 9-week sampling period.

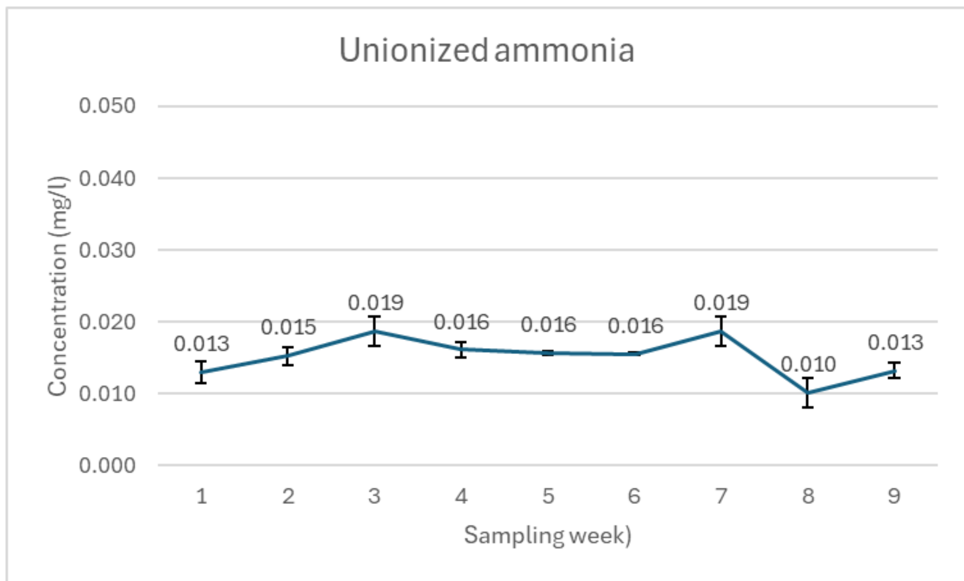

Figure 8. Concentration of unionized ammonia (average value of four fish tanks). Error bars on a chart represent the variability data in measurements.

Considering the irregularity of service cleaning of RAS, it became possible to develop a dependences grid of the concentrations of ammonium, nitrites, and nitrates on the number of days since the last cleaning. Figure 9 give an idea of the dynamics of ammonia concentration (TAN). There is no clear relationship between the concentration of total ammonia and the distance from the day of cleaning.

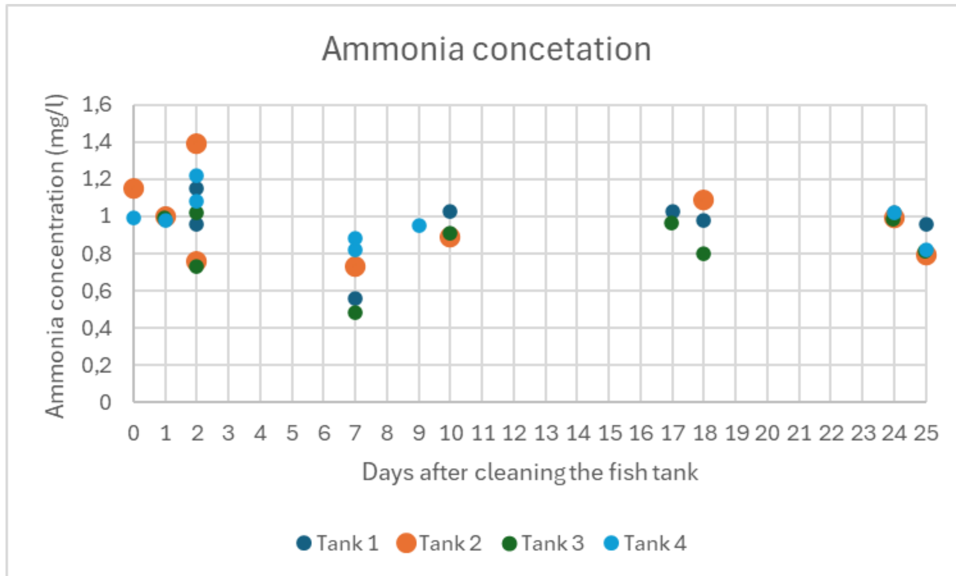

Figure 9. Ammonium cconcentration (TAN) Grid by Days Since Cleaning

Nitrite measurements

Figures 10, 11 shows the nitrite measurements in water samples for the RAS elements. It is notable that during the 8th and 9th weeks was a decline in nitrite levels in all sample points of RAS.

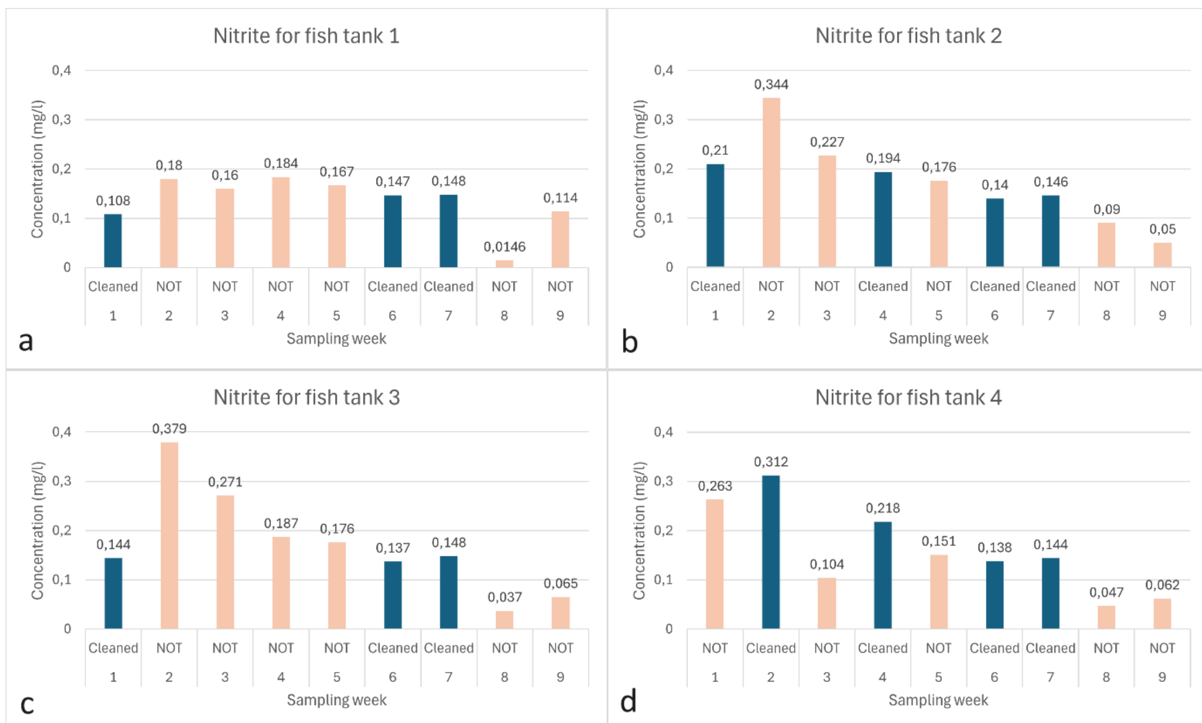

Figure 10. Nitrite concentration in (a) fish tank 1, (b) fish tank 2, (c) fish tank 3, (d) fish tank 4. Values highlighted in orange indicate the nitrite measurements, taken before service cleaning of the element. Values highlighted in blue indicate the nitrite measurements, taken after service cleaning.

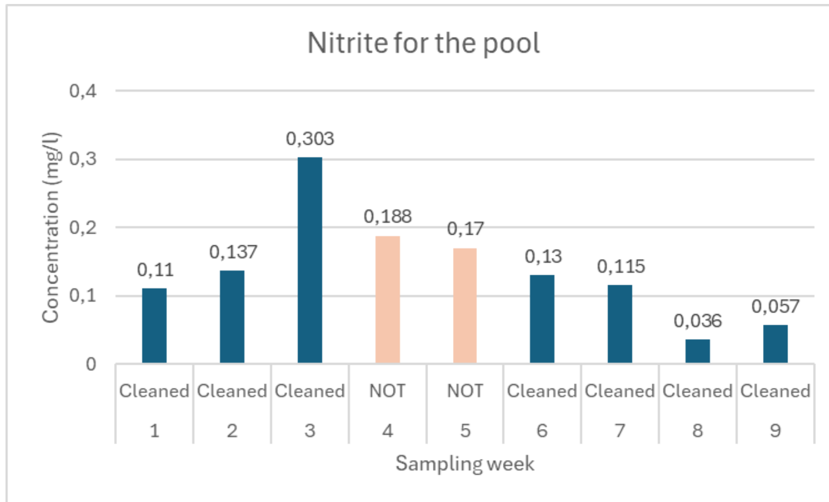

Figure 11. Nitrite concentration for the pool

Nitrite concentrations are in proximity between Fish tanks 3 and 4. Fish tank 1 has a lower nitrite concentration, recording a decrease of 17.23% and 18.68% compared to Fish tanks 2 and 3 respectively. In addition, in Fish tank 4 there is a similar concentration of nitrites, close to Fish tank 3, 4

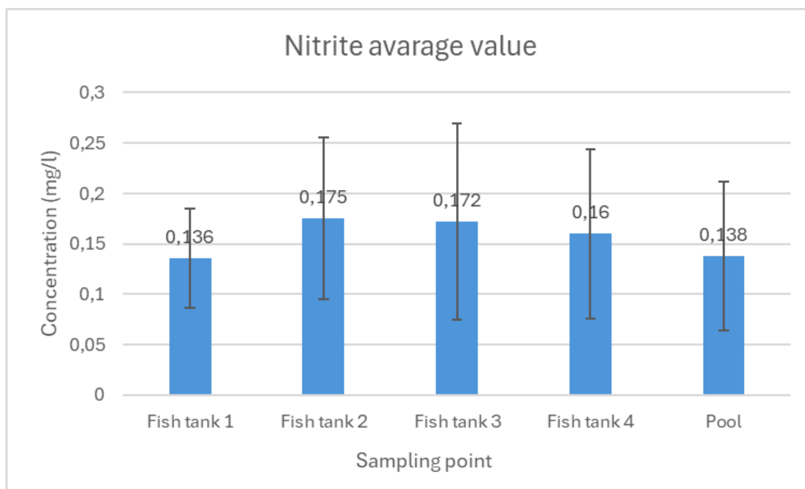

Figure 12. Average values of Nitrite concentration for fish tanks and "Pool" int the RAS. Error bars on a chart represent the variability data in measurements.

A grid of dependencies for nitrite concentration was also developed; the result is presented in Figure 13. There was no clear development in nitrate concentration after the tanks had been cleaned.

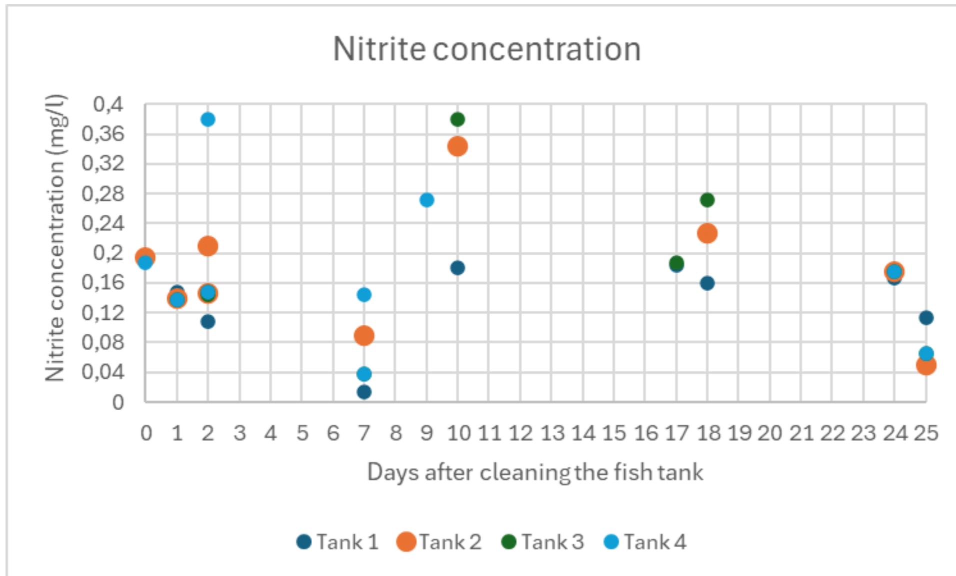

Figure 13. Nitrite concentration Grid by Days Since Cleaning

Nitrate measurements

In figures 14, 15 the outcomes of nitrate measurements in water samples for the RAS elements are presented. The results were obtained from the second week of the sampling period due to insufficient water collection for analysis in the first week. During the 8th and 9th weeks, a decline in nitrate levels was observed across all sample points of RAS.

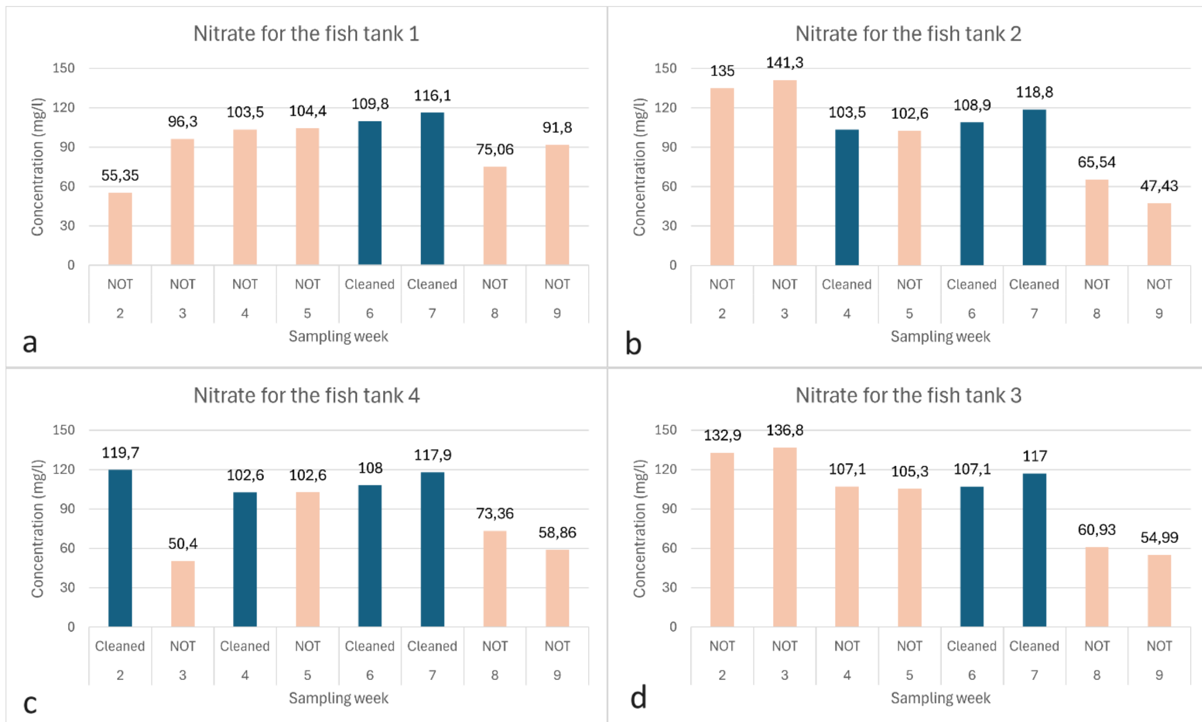

Figure 14. Nitrate concentration in fish tank 1 (a) fish tank 2 (b) fish tank 3 (c) fish tank 4 (d). Values highlighted in orange indicate the nitrate measurements, taken before service cleaning of the element. Values highlighted in orange indicate the nitrate measurements, taken before service cleaning of the element.

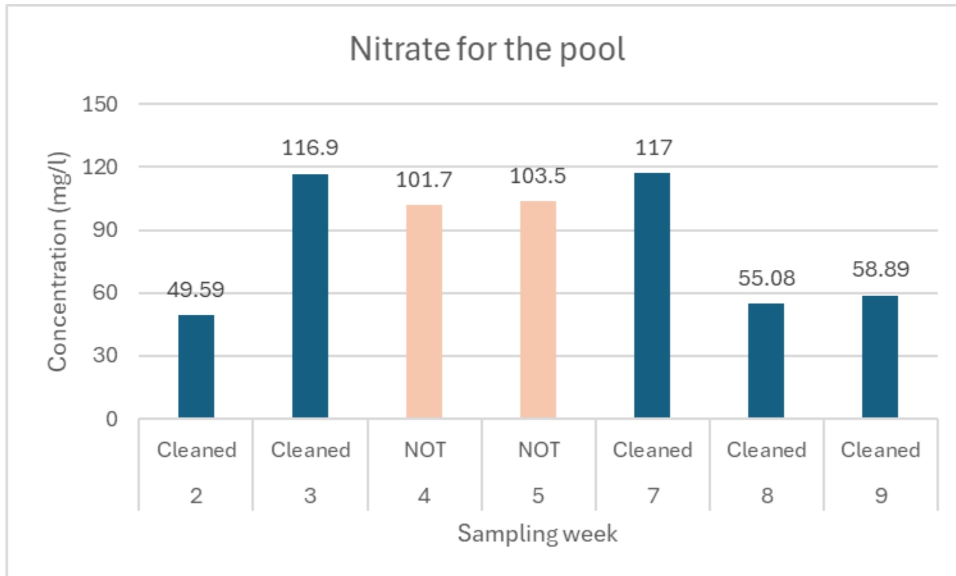

Figure 15. Nitrate concentration in the pool

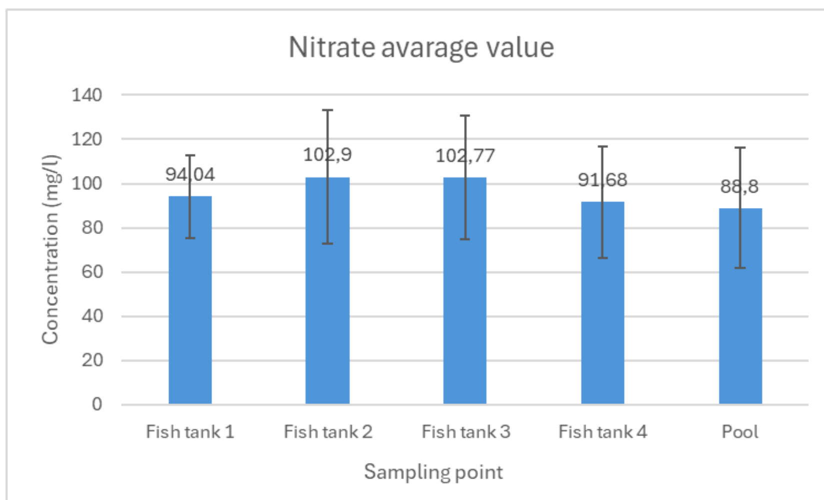

Figure 16. Average values of Nitrate concentration for fish tanks and “Pool” in the RAS. Error bars on a chart represent the variability data in measurements

A figure 17 present the the dependence grid for nitrate concentration. The distance from the cleaning date did not show a correlation with the nitrate concentration values.

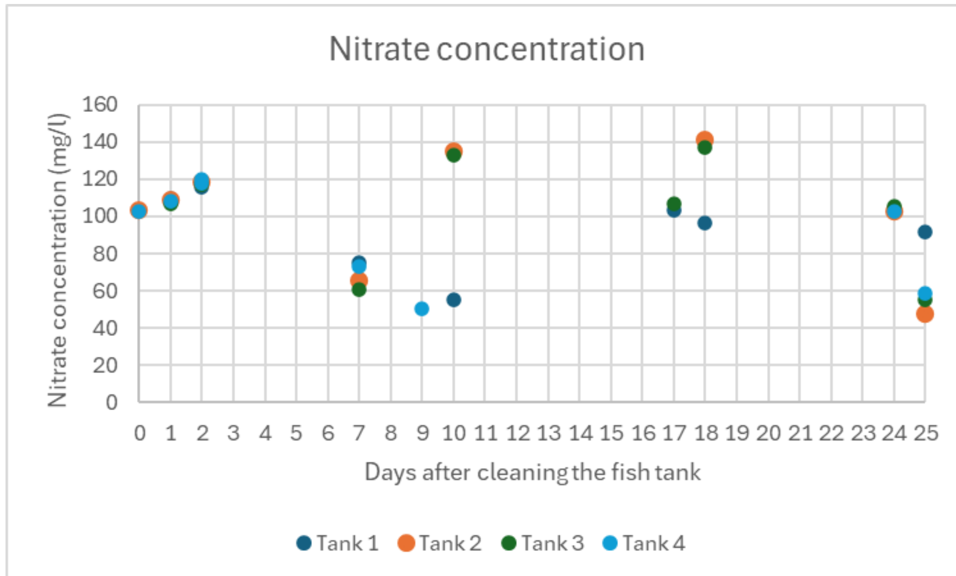

Figure 17. Nitrate concentration Grid by Days Since Cleaning

Comparative Nutrient Analysis: colour scale vs. spectrophotometry

Given that ammonium, nitrites, and nitrates are routinely monitored on the fish farm using the colour scale method, a comparative analysis between the results obtained from spectrophotometric analysis and colour scale analysis was conducted. Figures 18, 19, and 20 displays the comparative results. The findings reflect the significant difference in the values obtained by spectrophotometric analysis and the values obtained by colour scale analysis for all three studied parameters: ammonia, nitrite, nitrate.

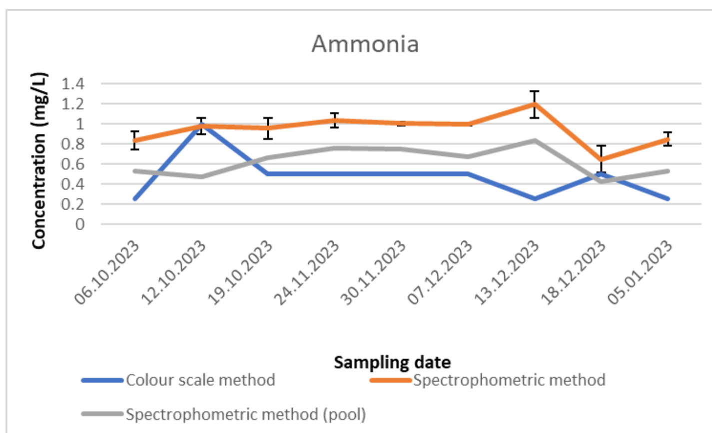

Figure 18. Comparative ammonia (TAN) analysis: Colour scale vs. spectrophotometry method. Spectrophotometric values represent the average values obtained from four tanks on each day of sampling. Error bars on a chart represent the variability data in measurements.

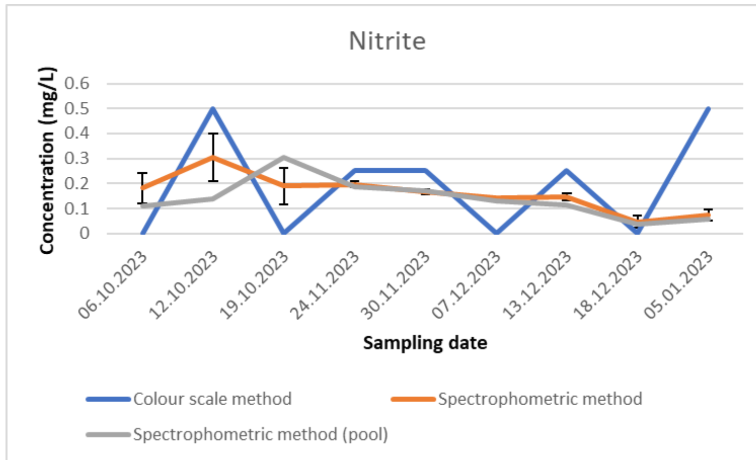

Figure 19. Comparative nitrite analysis: Colour scale vs. spectrophotometry method. Error bars on a chart represent the variability data in measurements.

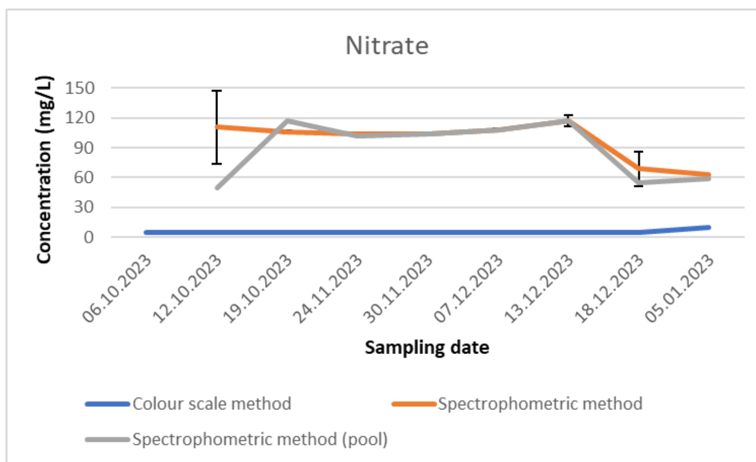

Figure 20. Comparative nitrate analysis: Colour scale vs. spectrophotometry method. Error bars on a chart represent the variability data in measurements.

geoA PCR-analysis

16S rRNA Gene Expression for qPCR Normalization

To establish amplification efficiency in DNA samples and confirm the functionality of SYBR Green Supermix used in qPCR, a 16S qPCR analysis was performed. This step was necessary to verify the presence of bacterial DNA in the samples, given that the 16S target is widely accepted as a typical marker for the presence of bacteria. Amplification of cDNA samples is displayed in Figure 18, where the amplification curves exhibited an S-shaped pattern. The figure shows that the amplification signals began to rise around cycle 20, indicative of exponential amplification, where the amount of amplified cDNA is increasing exponentially, as expected in a successful qPCR reaction.

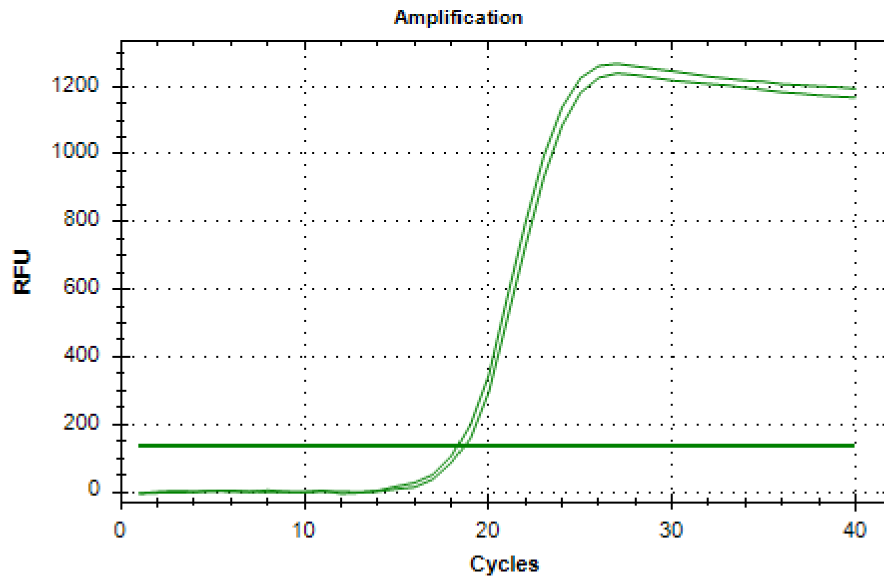

Figure 21 . qPCR amplification of cDNA samples with 16S primer

DNA samples with 16S rRNA primers – were tested with conventional PCR. The resulting amplicons were subsequently visualized via electrophoresis, and the results are shown in Figure 19. Various amounts of DNA template were tested in duplicate, including amounts of 100 ng, 50 ng, 25 ng, 12.5 ng. The results of gel electrophoresis show distinct bands corresponding to amplified DNA products at various amounts of the tested DNA templates. The approximate size of the target product, approximately 200 base pairs, is determined based on DNA ladder markers,

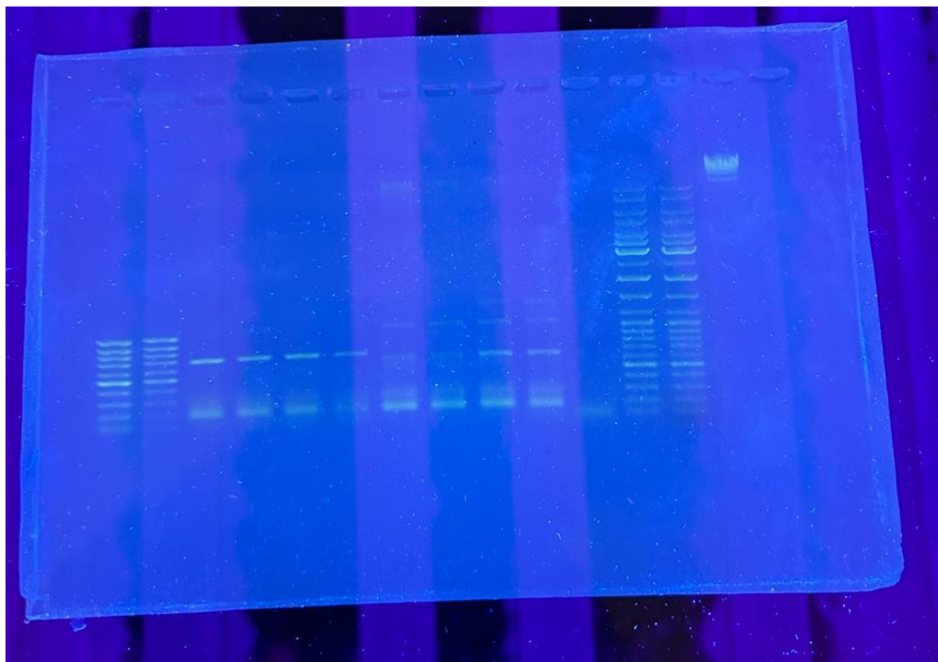

Figure 22. cPCR amplification of DNA samples with 16S primer

geoA primer qPCR analysis

Next, DNA templates were tested with the Cyc primer. As stated in the previous section, two different thermocycling conditions were used. For cDNA templates the following conditions were used: a hot start activation at 95°C for 3 minutes, followed by 40 cycles 95°C for 10

second (denaturation) and 60°C for 30 second (annealing and extension) followed by melting curve analysis spanning from 65°C to 95°C. The cDNA templates used in this analysis were extracted from the arch gills and intestine of fish. Despite employing stringent thermocycling conditions, Figure 20 illustrates an absence of the expected amplification signal for the Cyc primer and its corresponding target gene.

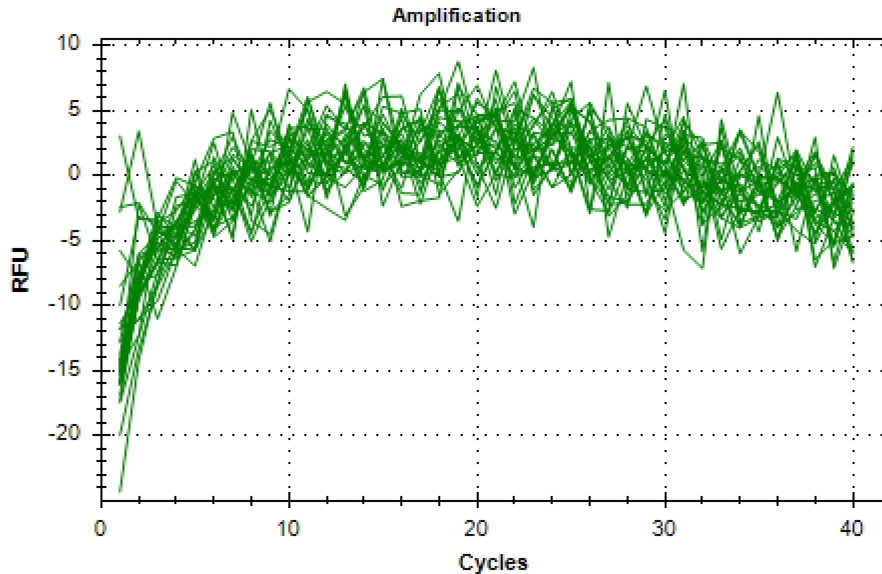

Figure 23: Result of qPCR testing cDNA samples with Cyc primer

For qPCR testing, an alternative set of thermocycling conditions was employed: a hot start activation at 95°C for 5 minutes, followed by 30 cycles 95°C for 1minute (denaturation) and 50°C for 1 minute (annealing and extension) followed by final extension 72°C for 5 minutes and melting curve 65°C to 95°C. These conditions were selected based on their demonstrated efficacy in prior research and their compatibility with the Cyc primer. (Lukassen et al., 2017). DNA templates were extracted from "Pool", "Drumfilter", "Fish tank 1", "Biofilter" were used for testing another thermocycling conditions. Despite the adoption of these optimized thermocycling conditions and the use of diverse DNA templates, Figure 21 displays a continued absence of amplification with the Cyc primer.

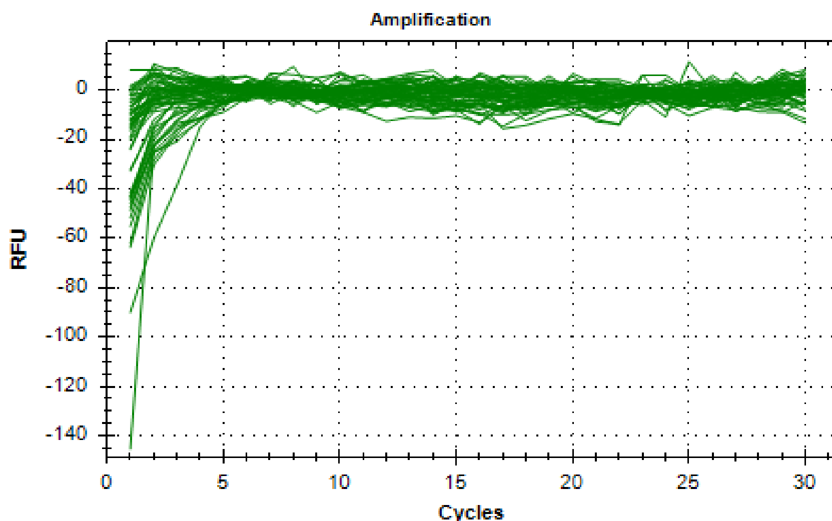

Figure 24: Result of qPCR testing DNA samples with Cyc primer

PCR analysis using the 16S and Cyc primers displays differences in their amplification efficiency and target specificity. The 16S primer set demonstrated robust amplification, as evidenced by the distinct bands observed on gel electrophoresis (Figure 19) and the strong qPCR amplification signal (Figure 18). This successful amplification confirms the presence of bacterial DNA in the samples, consistent with the established role of the 16S gene as a marker of bacterial presence.

In contrast, the Cyc primer set revealed problems with target gene amplification despite the use of optimized thermal cycling conditions. Both sets of thermal cycling conditions failed to produce the expected amplification signal for the Cyc primer, as shown in Figures 20 and 21.

GeoA positive control testing

Following the absence of amplification in the study samples during PCR testing, the study moved to introduce positive control testing. To ensure the specificity and efficiency of primers Cyc FW and Cyc RW in amplifying the geoA gene. This phase involved the use of DNA templates known to express the target gene geoA. DNA templates were initially tested with 16S primers to establish the bacterial identity of the extracted DNA template. Subsequently, the results of gel electrophoresis are presented in Figure 22: which show bands corresponding to amplified DNA product.

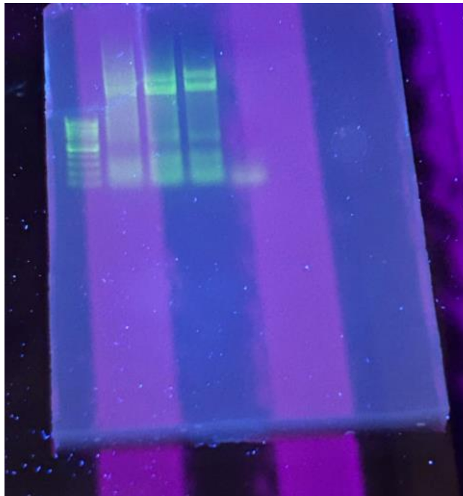

Figure 25: Positive control testing of DNA samples with 16S primer

After confirming the bacterial identity of the DNA samples, the positive control phase became pivotal, involving testing with a Cyc primer. The electrophoresis results presented in Figure 23 reveal the outcome of this testing, indicating the absence of primer bindings with the target gene from the positive control sample.

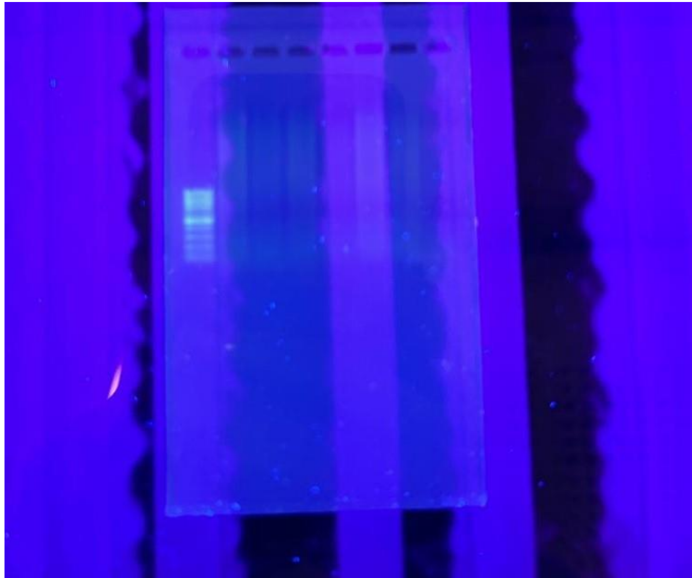

Figure 26: Positive control testing of DNA samples with Cyc primer

The testing was conducted twice to minimize the likelihood of human error.

Discussion

Nutrient flow assessment

The nitrogen cycle plays a critical role in the functioning of Recirculating Aquaculture System, influencing the dynamics of ammonia, nitrite, and nitrate within the RAS. The studied system efficiently converts ammonia to nitrate, as evidenced by stable levels of total ammonia nitrogen (TAN) and the presence of nitrites and nitrates within acceptable limits throughout the all-sampling period. The effectiveness of the biofilter is confirmed by the significantly lower concentration of total ammonium in the "Pool" RAS element compared to individual fish tanks, which demonstrate the successful removal of waste nitrogenous compounds. There is also a consistent downward trend in ammonia and nitrite and nitrate downstream of the biofilter, indicating the conversion of ammonia to nitrite and ultimately to nitrate in RAS system. This trend corresponds with the expected sequence of nitrogen transformations in a well-functioning RAS environment. The results indicate that the studied RAS system effectively controls nitrogen dynamics.

The introduction of water quality management into the functioning of the RAS is important to ensure the well-being and health of the fish. Even though the RAS system under study has round tanks installed, which have the advantage of self-cleaning, due to the constant rotation of water in the tank, fish tanks, like other RAS elements, tend to become contaminated (Timmons et al., 2018, p. 102). Studies indicate that a substantial portion of the feed provided to fish, up to half in some cases, is excreted as solid waste, with not all feed being fully consumed. Consequently, the accumulation of uneaten feed remnants and faeces within fish tanks fosters the formation of biofilms on various RAS components. These biofilms not only serve as reservoirs for nutrient accumulation, thereby compromising water quality, but also contain potential pathogens (Malone, 2013). Due to limited labour resources at the fish farm, records of routine maintenance of the RAS system collected during the research show that systematic cleaning has gaps and the planned cleaning was not carried out every week, deeper cleaning procedures, slated for periodic intervals, were overlooked as well. It is noteworthy that on cleaning days, biofilm samples were retrievable from RAS components. The absence of comprehensive records detailing the cleaning history of individual components impedes a

thorough assessment of the impact of regular cleaning practices on nutrient dynamics within the RAS, including the concentrations of ammonium, nitrite, and nitrate.

Despite the lack of regular cleaning management of RAS elements, the main indicators of nutrient flow: ammonia, nitrite, nitrate showed stability throughout the entire sampling period. Average total ammonia nitrogen (TAN) values for fish tanks ranged from 0.85-0.97 mg/L. There are differences in average total ammonium values between individual fish tanks and the "Pool" RAS element, where the "Pool" has a significantly lower concentration of 0.62 mg/L. This difference serves as an indicator of the effectiveness of the biofilter elements. Considering that biologically filtered water enters the "Pool", the reduced TAN concentration emphasizes the successful removal of nitrogenous waste compounds by the biofilter.

A previous study, evaluating the effects of ammonia on the physiology, growth, and feed intake of African catfish, showed that at a constant TAN concentration of 2.5 mg/L, no serious physiological changes except for observed gill morphology abnormalities, but TAN concentrations above 1.5 mg/L can reduce feed intake and reduce the growth rate of fish (Schram et al., 2010). Comparing these findings with our research, it appears that TAN values are acceptably below the threshold values and should not negatively affect the growth, physiology, and welfare of fish. The values of the non-ionized form of ammonia were determined since it is the most toxic form of ammonia and able to penetrate cell membranes. The value of this parameter should not exceed more than 0.05 mg/L (Timmons et al., 2018, p. 39). In our study, the reported values of non-ionized ammonia consistently fell below this critical threshold, ranging from 0.01 to 0.019 mg/L.

The main mechanism of nitrite toxicity is the conversion of hemoglobin into methemoglobin, which is unable to deliver oxygen to tissues. Previous research has shown that the African catfish is able to adapt to even high nitrite levels such as 10.49 mg/L. This is due to the ability of African catfish to internally detoxify nitrites to nitrates. However, to preserve fish welfare and avoid negative impacts, the recommended nitrite threshold should not exceed 1.98 mg/L (Roques et al., 2015). In our research, nitrite values for fish tanks ranged from 0.136 to 0,175 mg/L, it appears that nitrite values are acceptably below the threshold values.

Direct evidence of nitrate toxicity on fish health is limited, it is assumed that nitrate toxicity has a similar mechanism to nitrite toxicity. African catfish show tolerance to high levels of nitrate concentrations - previous studies have shown that even chronic exposure to 1674.27 mg/L showed no significant physiological change, but suggests that, given the possible negative effects of feed intake and growth of African catfish, it is not recommended to exceed nitrate concentrations in water more than 140 mg/L (Schram et al., 2014). In our research, nitrate values for fish tanks ranged from 88.9 to 102.9 mg/L. Although our study observed nitrate values nearing this threshold on third sampling week: nitrate values were 136.88 mg/l (for fish tank 4) and 141.3 (for fish tank 2), overall nitrate levels remained within acceptable limits, indicating minimal risk to fish health for African catfish.

In the "Recirculating Aquaculture Guide", the Food and Agriculture Organization (FAO) advocates for regular checking and cleaning of RAS elements. This practice is important to mitigate the accumulation of organic waste, which can otherwise lead to the additional release of ammonium and serve as a substrate for pathogenic microorganisms. By adhering to these recommendations, can increase effectivity manage water quality of RAS (Bregnballe, 2022). One of the primaries aims of this study was to investigate the association between regular cleaning practices and the levels of nutrients (ammonia, nitrite, nitrate) within the RAS. However, the absence of systematic cleaning management resulted in potentially unequal groups within the sampling period. Furthermore, the relatively small sample size further

complicates the analysis and interpretation of the data. These limitations underscore the challenges inherent in assessing the impact of cleaning routines on ammonia, nitrite, and nitrate concentrations in the water.

The observed variability in nutrient concentrations may be influenced by numerous factors, including the timing of sample collection. Fish feed and feed load are key factors in determining the ammonia removal capacity of a RAS. TAN production in fish depends on feeding interval and can increase if the amino acid profile of the fish feed is not balanced correctly (Lindholm-Lehto, 2023). As the concentrations of ammonia, nitrite, and nitrate exhibit significant fluctuations following fish feeding. The exact time of sample collection during the sampling period, though not recorded, typically occurred between the first and second feeding sessions at 8 am and 11 am, respectively. Following fish feeding, a notable increase in ammonia concentrations often occurs as fish begin metabolizing food and excreting waste. This rise in ammonia levels may subsequently lead to an increase in nitrite concentrations, as certain bacteria within the system convert ammonia into nitrite via nitrification processes. Over time, nitrite levels may diminish as nitrite-oxidizing bacteria convert nitrite to nitrate (Lindholm-Lehto, 2023). To establish any potential relationship between cleaning procedures and nutrient levels, future studies should not only establish strict cleaning protocols and increase sample sizes, but also consider changes in nutrient concentrations after feeding. In addition, sampling during non-feeding periods, such as at night, will provide insight into baseline nutrient levels and their fluctuations. Therefore, this study is initial research, further studies incorporating these considerations are needed to comprehensively identify the interactions between cleaning routine and nutrient dynamics in RAS environments.

Another direction of nutrient research was the comparative analysis of the spectrophotometric method and the colour scale method, which has been implemented for monitoring nutrients in studied fish farms. It is noteworthy that regular monitoring of ammonium, nitrite and nitrate levels is carried out almost daily by the employees. Samples for analysis are taken from one random fish tank, most often from fish tank number two. Water samples for nutrient analysis are collected in the morning, between the first and second feedings, although the exact time was not recorded.

The results of our comparative analysis revealed differences between the values obtained by the spectrophotometric analysis method and the values obtained by the colour scale method (Figures 18, 19 and 20). Particularly noticeable differences were observed nitrate values, where the colour scale method gave a concentration 5 mg/L while the spectrophotometric average values ranged from 68.7 to 118.5 mg/L. Variations were also noticeable in ammonia and nitrite measurements. Thus, on certain sampling days, the colour scale method gave zero nitrite values. Such results are considered impossible in the context of a functioning fish farm, where a certain level of nitrite presence is typically expected. This discrepancy calls into question the reliability and accuracy of the colour scale method, especially for consistent and accurate monitoring of critical indicators such as ammonium, nitrite, and nitrate. In conclusion, despite the obvious advantages in terms of cost-effectiveness and ease of use, the colour scale method appears to lack the reliability required for routine monitoring of key nutrient parameters in RAS settings.

GeoA PCR analysis

The PCR analysis conducted in this study aimed to detect the presence of GeoA-encoding bacteria using the Cyc primer. The choice of the Cyc primer for PCR analysis was based on its ability to target all bacteria encoding the GeoA gene (Lukassen et al., 2017) Thus, Cyc primer should have provided the opportunity to cover a wide range of bacterial taxa that carry the *GeoA* gene, providing a comprehensive assessment of microbial diversity. Initially, the

effectiveness of the primer and the PCR reagents (SYBR Green and KAPA) was confirmed through testing with prepared cDNA and DNA templates, employing primer 16S to prove the bacterial origin of the extracted DNA and RNA products. However, despite these preliminary validations, amplification of the target gene was not observed during reactions with the DNA templates under investigation.

The subsequent phase involved employing a positive control, representing a bacterial population expected to express *GeoA*. However, conventional PCR testing of DNA templates, extracted from positive control bacterial populations using the selected primer gave negative results, since the binding of the Cyc primer to the target gene could not be detected (Figure 23).

The inability to detect *GeoA*-encoding bacteria raises several important considerations for future studies. Firstly, it prompts a re-evaluate of the primer design and selection, as the lack of amplification suggests potential limitations in primer specificity or efficiency. Moreover, alternative primer sets targeting different regions of the target could be evaluated to increase sensitivity. However, alternative primers may lack the broad applicability of the Cyc primer, leading to challenges in elucidating the microbial diversity of geosmin-producing bacteria in RAS. Other primers targets specific groups of *geoA,* while the Cyc primer targets all *geoA* groups.

Secondly, variations in PCR conditions, such as annealing temperature and cycling parameters, should be explored to optimize amplification efficiency. As stated above, 2 different types of thermocycling conditions were used in this study. The second condition of thermocycling reaction described “Materials and Methods” in the sections was adopted from previous study (Lukassen et al., 2017). However, further experimentation with the primer and adjustment of thermal cycling conditions may be required to improve its efficiency. In addition, testing using alternative reaction mixtures can provide information on optimizing PCR performance.

Moreover, the discrepancy between the expected expression of *GeoA* in the positive control and the observed negative results highlights the need to validate the positive control. It is important to offer updated information on the geosmin problem in the RAS system. The last recorded occurrence of musty odour and taste in fish dates to December 2022. Following this observation, no off-flavour testing was conducted on fish taken directly from the fish tank. Thus, there is a possibility that the studied samples do not actually express *GeoA*

In conclusion, PCR analysis using the Cyc primer to detect bacteria encoding *GeoA* did not produce the expected results, indicating potential limitations in primer specificity or efficiency. Future studies should focus on re-evaluating primer design and selection, exploring alternative primer sets, and optimizing PCR conditions to improve amplification efficiency. Additionally, stringent positive control testing and ongoing monitoring of geosmin-related problems in studied RAS systems are important to improving water quality management practices.

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