Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
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Early Warning Signs and Dynamics of Hydrogen Sulfide Production in Recirculating Aquaculture System (RAS)

With Nitrate and Iron (III) Addition

Master's thesis in Health Management in Aquaculture (AquaH - Erasmus Mundus Joint Master Degree Programme)
Supervisor: Murat Van Ardelan
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Abstract

Aquaculture sector is adopting more Recirculating Aquaculture System (RAS) for fish production. In Norway, RAS was earlier used only for smolt production in freshwater, but now industry is slowly shifting post-smolt production and even full production cycle to marine RAS. Such a production system is claimed to be a better sustainable alternative method for fish farming. The use of seawater in RAS increases the risk of production of hydrogen sulfide (H_2S), due to high sulfate concentration. H_2S is extremely toxic for the fish and events of H_2S induced mortality is being reported from several RAS facilities across Norway. Previous studies reported that H_2S is produced through sulfate reduction by action of Sulfate Reducing Bacteria (SRB) and the redox reaction takes place in the order O_2, NO_3^-, Mn (IV), Fe (III) oxides and oxy-hydroxides, SO_4^{2-} and CO_2. It is evident from earlier studies that addition of Nitrate (NO_3^-) delays the H_2S production. Here in this thesis we are checking the ability of Fe (III) to delay H_2S production and how effectively we can use Fe (II) as an early warning detection for H_2S production.

An experiment was designed where sludge from RAS was mixed with seawater and incubated in 33 screw cap bottles for a period of 19 days. The bottles were equally divided among control, nitrate-added treatment (NAT) and iron-added treatment (FAT). The control was with sludge and seawater. In addition to sludge and seawater to NAT, NO_3^- was added at a concentration of 6 mM. In FAT bottles, Fe (III) was added at a concentration of 0.4 mM. Samples were drawn from each of the groups following a fixed schedule and analyzed for H_2S, Fe (II) and nutrients. Results shows NO_3^- delayed the H_2S production by 8 days while Fe (III) additions suppressed the H_2S production for about 5 days. In FAT, there was a delay of 5 days between increase in concentrations of Fe (II) and H_2S. Here lies the possibility of using Fe (II) as an early warning sign for H_2S production in RAS.

Preface

The experiment described in this thesis was performed at the Department of Chemistry, NTNU, under the guidance Murat Van Ardelan as my supervisor and Mathew Kuttivadakkethil Avarachen as my co-supervisor, in accordance with the established Health, Safety, and Environment (HSE) protocols. The RAS sludge was provided by Nofima's RAS facility at Sundalsøra and seawater was collected from Trondheim Biological Station (TBS). The analysis was carried out at the Department of Chemistry (NTNU), and the Trondheim Biological Station (TBS).

Acknowledgement

I would like to begin by expressing my sincere gratitude to the almighty for giving me strength and inspiration during the challenging phases of my master thesis. I am immensely indebted to my supervisor, Murat Van Ardelan, whose exceptional guidance, expertise, and encouragement have been instrumental in shaping this thesis. Your profound knowledge, patience, and dedication to research have been an endless source of inspiration for me.

I would also like to extend my heartfelt appreciation to my co-supervisor, Mathew Kuttivadakkethil Avarachen, for his valuable insights, constructive feedback, support and constant motivation throughout this thesis. I would also like to thank Andre Meriac from Nofima for sending the RAS sludge, Heindrick Petero from Pure Salmon for providing me with field level informations, Anica Simic for helping me to prepare and deliver ICP-MS samples, Siv Anina Etter for helping me in nutrient analysis and Stephen Grant for providing me with probes to measure water quality parameters. I am also grateful to my parents, family, friends and loved ones for their unwavering support and words of motivation.

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List of Abbreviations

CO_2	Carbon dioxide
DNRA	Dissimilatory nitrate reduction to ammonia
DO	Dissolved Oxygen
DOM	Dissolved organic matter
E_h	Redox potential
FAT	Iron (III) added treatment
Fe	Iron
FeS	Iron sulfide
H_2S	Hydrogen sulfide
HCl	Hydrochloric acid
UP HNO_3	Ultra-Pure Nitric acid
HS^-	Bisulfide
ICP-MS	Inductively coupled plasma mass spectrometry

LC50	Lethal concentration 50
Mn	Manganese
MAB	Maximum Allowable Biomass
NAT	Nitrate-added treatment
N_2	Nitrogen gas
N_2O	Nitrous oxide
NH_4^+	Ammonium ion
NO_3^-	Nitrate
NO_2^-	Nitrite
NOB	Nitrite oxidizing bacteria
O_2	Oxygen
PO_4-P	Phosphate-phosphorous
RAS	Recirculating aquaculture system
Redox	Reduction-oxidation
S^{2-}	Sulfide
SO_4^{2-}	Sulfate
SRB	Sulfate reducing bacteria
TAN	Total ammonia nitrogen

1. Introduction

Global demand for seafood is increasing with needs of growing population. Increased aquaculture production to meet the increased demand has environmental concerns and impacts. Aquaculture is also vulnerable to the effects of climate change especially global warming and other environmental variables of fish production including availability of ambient quality water. Fish farming in open system has many consequences like release of nutrients and chemicals into the marine environment (Ahmed and Turchini, 2021). The fish that escape from cages compete with the wild stock for resources, may transfer diseases and also interbreed which may reduce the fitness of wild stock and dilute the natural gene pool (Rosamond Naylor et al., 2015). An alternative strategy is the implementation of Recirculating Aquaculture Systems (RAS) in which fish is farmed on land in a closed system and the culture water is recycled and reused. RAS has the ability to effectively manage collect and treat the waste that accumulate during the fish growth which makes it an environment friendly fish production system. All these contributed to the development of RAS as a mainstream fish production system (Piedrahita, 2003). Developments in the RAS technology has been accelerated in the last two decades and it became popular in these years mainly in countries that invest more into aquaculture (Goddek et al., 2020). It's being increasingly used for marine fish production in Mediterranean region and salmonid production cycle, especially for juvenile stages before moving out to sea (Bostock et al., 2016). In North America and Europe RAS was developed even as an alternative to open water cage culture.

In Norway, traditional freshwater RAS system has been operational for many years for growing salmon juveniles called parr. Smoltification is the transformation of salmonids from parr to smolts which is marked by the transition from life in freshwater to sea water. Usually after smoltification, the smolts are moved to sea cages. To check the possibilities to minimize the

growth time period in sea, salmon farming industry has introduced marine RAS for the production of post smolts. This strategy will increase the production stability because of less exposure to parasites, sea lice and diseases in addition to reducing the risk of escapes that affects natural gene pool. In Norway there is a maximum allowable biomass (MAB) in salmon farming which regulates the quantity of fish that is legally permitted to farm in an area. It is decided based on regulations, environmental conditions, and farming practices. It aims to maintain fish welfare, water quality, and ecosystem health. Post smolt production in RAS favors better utilization of maximum allowable biomass and reduces permit charges for salmon farming (Ytrestøyl et al., 2020). Technologically improved RAS systems significantly reduce the water consumption and nutrient outlet concentration through high degree of water recycling and proper waste accumulation facilitating later removal. In these systems all water quality parameters like temperature, pH, dissolved oxygen, nitrite, nitrate, ammonia and salinity can be controlled to provide good rearing conditions for better feed utilization and optimized growth (Dalsgaard et al., 2013).

Although RAS has several advantages compared to open sea cages there are also some challenges associated with it. Since fishes are stocked at high densities in a RAS system there can be chances of accumulation of harmful metabolites from fish like ammonia and nitrite as well as bacterial load during recirculation. If the water treatment units like biofilter, oxygenation, temperature control etc. are not working properly it can ultimately result in suboptimal conditions for the fish (Fjellheim et al., 2016). One of the major challenges that may critically affect the survival of fish stocks in the RAS system is the production of Hydrogen Sulfide. Hydrogen Sulfide is a highly toxic gas which cause significant threat to fish health and welfare. H_2S can be produced in any aquaculture system but it is more severe in closed aquaculture system like RAS where fishes are grown in confined space at high

densities. H_2S is produced mainly because of two factors: a) High supply of labile organic matter as electron donor, and quick consumption of basic available electron acceptors, such as O_2 and NO_3^-. b) Availability of amble SO_4^{2-} as electron acceptor especially in case of marine RAS (Letelier-Gordo et al., 2020). The H_2S production RAS is emerging as a serious issue as the industry is adopting more marine RAS systems for land-based farming of salmon post-smolts.

The production of H_2S is the result of a redox reaction which involves exchange of electrons between dissolved sulfate in water and organic matter in the absence of oxygen. Anaerobic sulfate reducing bacteria is the main player which utilize sulfate (SO_4^{2-}) as an electron acceptor for the decomposition of organic matter (Harada et al., 1994). The type of bacteria available for decomposing organic matter is based the availability of electron acceptors O_2, NO_3^-, Mn (IV), Fe (III), SO_4^{2-}, and CO_2. The highest to lowest energy derived by the bacteria by the decomposition of organic matter by using these species as electron acceptor follows the same order. When oxygen is not available NO_3^- is the next preferred electron acceptor and then proceeds for Mn (IV) and Fe (III) before using SO_4^{2-}. Based on these preferences, in addition to general preventive measures for H_2S production like good system design and regular cleaning practices there is a practice of maintaining high nitrate concentration so that there won't be any sulfate reduction (Letelier-Gordo et al., 2020). Though nitrate is less toxic than ammonia and nitrite, excess nitrate accumulation can cause chronic health and welfare impacts for the fish (Davidson et al., 2014). Addition of nitrate into RAS also pose a risk of production of ammonia by direct nitrate reduction to ammonia (DNRA pathway) i.e., by the reduction of NO_3^- to NO_2^- and further reduction to NH_3 (Kamp et al., 2015). Both NO_2^- and NH_3 are toxic for the fishes (Thurston et al., 1981). In this master thesis work we are trying to improve our understanding on how addition of Fe (III) affects the redox reactions and

ultimately H_2S production from a RAS sludge. When Fe (III) is used as an electron acceptor it reduces to Fe (II) and then upon oxidation it again turns to Fe (III). Thus, it self-replenishes and enter into the redox process again as an electron acceptor. We are checking if this Fe (III)-Fe (II) shuttle process can postpone the H_2S production in RAS system.

2. Theory

2.1 Recirculating Aquaculture System (RAS)

In RAS, the fishes are reared in tanks and when the conditions of the water become unsuitable for the fish it is taken for various treatments to make it again optimum for fish before being pumped back into the tank. The types of treatment and its order varies from system to system but the ultimate aim is to remove leftover feed, faeces and metabolic wastes to avoid their concentrations reaching a level that is harmful for the fish. Ammonia (NH_3) and CO_2 are released into the water during fish metabolism (Robert R. Stickney, 1994). In addition to that, heterotrophic bacteria in RAS also contribute to oxygen consumption, production of CO_2 and NH_3 (Fjellheim et al., 2016). Ammonia is highly toxic to the fish and the toxicity of ammonia depends on various other factors like chemical form of ammonia (NH_3 or NH_4^+), pH, temperature and length of exposure. Ammonia affects the gill physiology and may lead to acute toxicity causing damage to the central nervous system. As per the Norwegian Food Safety Authority (NFSA) the Total Ammonia Nitrogen (TAN ie; $NH_3 + NH_4^+$) should be <2 ppm.

In RAS fishes are grown in tanks that are designed for efficient waste removal. The water from the fish tank first goes for solids removal in a mechanical filter where larger particles (>20 um) are removed (Figure 2.1). Removal of particles increases the efficiency of the water treatment system(van Rijn, 2013). From there the water goes to biofilter where the ammonia in the water is converted via nitrite (NO_2^-) to nitrate (NO_3^-). This is mediated by nitrifying bacteria mainly ammonia oxidizing *Nitrosomonas* and nitrite oxidizing

Nitrobacter which grows on the substrate in the biofilter. Nitrate is comparatively less toxic for the fish. Studies in Atlantic salmon post smolt did not show any significant health effects at NO_3^- concentration up to 100 mg/L (Davidson et al., 2017). But it can't be left unchecked because excess nitrate can affect the health and welfare of fish (Davidson et al., 2014). The nitrate accumulation in RAS is generally controlled by water exchange or by incorporating an anaerobic denitrification unit where facultative anaerobic bacteria convert nitrate to nitrogen gas (van Rijn et al., 2006).

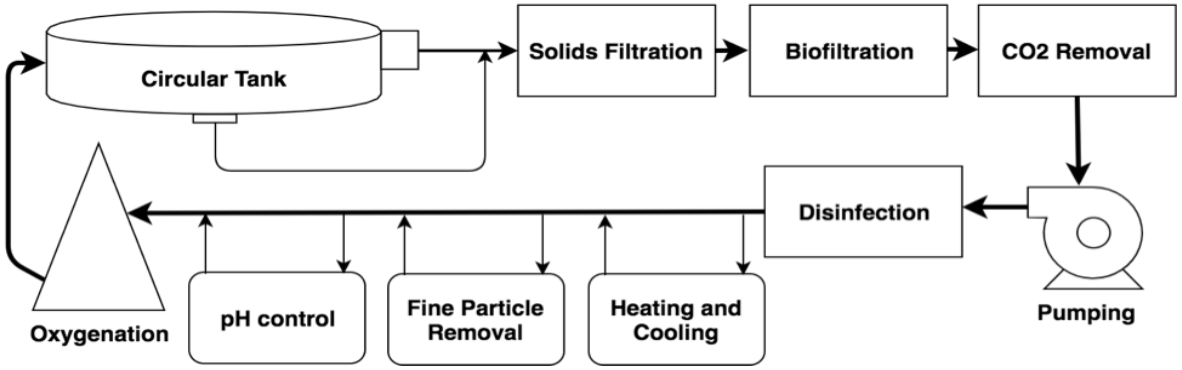

Figure 2.1 A flow chart for the various water treatment processes in RAS (Andyy Paradise, 2018)

Water from the biofilter goes to degasser where CO_2 is removed. At high CO_2 concentrations the fish's capacity for oxygen uptake and acid/base regulation is reduced, high amount of CO_2 in blood lowers the blood pH and cuts down the oxygen binding capacity of hemoglobin (Fjellheim et al., 2016; Ishimatsu et al., 2004). Then the water goes to the protein skimmer where smaller particles are removed from the water column by capturing it in foam. Use of ozone increases the efficiency of protein skimmer (Ranjan et al., 2019). Since there will be significant reduction in the amount of dissolved oxygen in the water because of fish consumption and bacterial activities, the water is oxygenated before it is pumped back into the rearing tank(Fjellheim et al., 2016). All the water quality parameters can be constantly monitored and controlled in a RAS to make the conditions optimum for the growth of fishes.

2.2 Redox reactions in RAS

Reduction-oxidation (redox) reaction involves transfer of one or more electrons, oxygen atoms or hydrogen atoms between chemical reactants. Most often these redox reactions are mediated by bacteria and other prokaryotic microorganisms which act as biological catalysts and they derive metabolic energy from these chemical reactions (Burgin et al., 2011). In marine sediments, these reactions involve organic carbon as electron donors from which electrons are transferred to the electron acceptors or oxidants, it ultimately results in the mineralization of the organic matter(Jørgensen, 2000). The typical electron acceptors or oxidants in marine sediments are O_2, NO_3^-, Mn (IV), Fe (III) oxides and oxy-hydroxides, SO_4^{2-} and CO_2. The preference of these electron acceptors follows the same order and is based on the highest to lowest energy released by reduction of these species which corresponds to gradual decrease in the redox potential of these oxidants. The value of redox potential is more negative if less energy is released during the redox reaction (Jørgensen, 2000; Weiner, 2007). The change in the free energy of metabolic process in prokaryotic organisms while using different oxidants are given in the set of equations below

Pathway and stoichiometry of reaction	ΔG° (KJ/mol)	pε
Aerobic Respiration:		
$CH_2O + \mathbf{O_2} \rightarrow CO_2 + H_2O$	-479	+13.75
Denitrification:		
$5CH_2O + \mathbf{4NO_3^-} \rightarrow 2N_2 + 4HCO_3^- + CO_2 + 3H_2O$	-453	+12.65
Mn (IV) Reduction:		
$CH_2O + 3CO_2 + H_2O + \mathbf{2MnO_2} \rightarrow 2Mn^{2+} + 4HCO_3^-$	-349	+8.9
Fe (III) reduction:		
$CH_2O + 7CO_2 + \mathbf{4Fe(OH)_3} \rightarrow 4Fe^{2+} + 8HCO_3^- + 3H_2O$	-144	-0.8
Sulfate reduction:		
$2CH_2O + \mathbf{SO_4^{2-}} \rightarrow H_2S + 2HCO^{3-}$	-77	-4.13
Methanogenesis:		
$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	-28	-8.20

Table 2.1 Pathways of organic matter oxidation, their standard free energy yields (ΔG°) and pε values (Jørgensen, 2000; Stumm et al., 1996)

In marine sediments the consecutive reduction of these oxidants depends on the depth and availability of oxygen. Water column can be characterized by three zones oxic, sub-oxic and anoxic. In oxic zones oxygen will be the dominant oxidant and other electron acceptors will be also present in oxidized states. If the oxygen consumption by the microbial activity exceeds, the water turns to sub-oxic, at this point NO_3, Mn (IV) and Fe (III) will be used as electron acceptors. If the amount of labile organic matter is very high it results in anoxic condition and reduction of SO_4^{2-} occurs leading to the production of H_2S.

Recirculation of water mainly aims to replenish water with oxygen and to remove organic matter and nutrients as much as possible. If in case one or more treatment system fails or if there is some faulty system design or operational error there can be accumulation of organic matter and sub-oxic condition in the system. In this scenario NO_3^-, Mn (IV) and Fe (III) will be reduced and if the oxygen is not replenished there the redox potential will proceed with SO_4^{2-} reduction leading to the production of H_2S, which is lethal for fishes (Roman et al., 2019).

2.3 H_2S production in marine RAS

H_2S is colorless, toxic, flammable (at higher concentrations) gas with characteristic 'rotten egg odor' (Harbison et al., 2015). Because of high density than air it tends to accumulate in bottom areas of anoxic environment. In aqueous solutions H_2S is present in equilibrium with its anions sulfide (HS^-) and bisulfide (S^{2-}) which is shown in the equation 2.1. (Li and Moore, 2008)

$$H2S_{(aq)} \rightleftharpoons HS + H^+ \rightleftharpoons S^2 + 2\ H^+ \qquad 2.1$$

H_2S is a weak acid that can exist in equilibrium with the sulfide ion (HS^-) depending on the pH of the water. When pH increases, the concentration of

$H2S_{(aq)}$ will be reduced significantly as it will get dissociated into HS^- and S^{2-} as shown in Figure 2.2 (Holmer and Hasler-Sheetal, 2014). At pH 7 both HS^- and S^{2-} will be present in equal proportions. At low pH values, H_2S predominates, while at higher pH values, HS^- becomes the dominant form. While both H2S and HS- can be toxic to aquatic organisms, H_2S is more toxic due to its ability to easily penetrate cell membranes and disrupt cellular functions (Smith Jr and Oseid, 1974). RAS are generally operated between pH 6-8, at this range H_2S mainly exist as HS^- which is less toxic (Yongsiri et al., 2004).

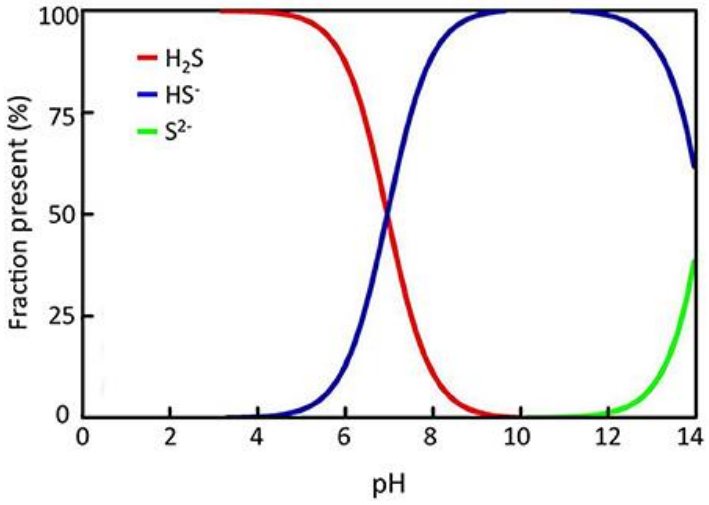

Figure 2.2 Sulfide solubility chart showing the relative fraction of each sulfide species at different pH (Holmer and Hasler-Sheetal, 2014)

High level of biosecurity and better control over the environmental conditions motivates the aquaculture industry to adopt more land-based marine recirculating aquaculture systems (Martins et al., 2010). These marine land-based RAS are facing a great challenge in the risk of production of H_2S in the system. When other oxidants are depleted the anaerobic sulfate reducing bacteria (SRB) utilize sulfate as electron acceptors for the decomposition of organic matter resulting in H_2S production. The risk of H_2S production is high in marine RAS as compared to freshwater RAS because of the high abundance of sulfate in seawater (Nazaroff and Alvarez-Cohen, 2001). Seawater contains

28.1 mM of sulfate on average meanwhile the sulfate level in fresh water is only 0.05-0.5 mM (Tanudjaja, 2021). Apart from the sulfate concentration, the sulfide production rates of SRB depends on pH, temperature and organic matter bioavailability (Muyzer and Stams, 2008; Plugge et al., 2011).

H_2S is extremely toxic for the fish, it can cause mass fish mortality and severe odor problems in the surrounding areas. Incidents of H_2S accidents are increasingly being reported from land-based marine RAS (Dalsgaard et al., 2013). In fishes H_2S prevents binding of oxygen to cytochrome c oxidase through competitive inhibition, generating cellular anoxia ultimately preventing ATP production (Kiemer et al., 1995). The LC50 value represents the concentration of a substance that causes 50% mortality in a test population within a specified timeframe. Study carried out in eight freshwater species showed LC50 values of H_2S is between 0.5 - 1.6 µM (Smith and Oseid, 1974) and for marine fish species it is between 1.5 – 15 µM (Boyd, 2021). Study by Kiemer et al., 1995 reported that Atlantic salmon (*Salmo salar*) is more tolerant for H_2S and significant damage was not observed during periodic exposure of H_2S until 18 days to a concentration of 7.9 µM. the same study also reported that a single acute dose of H_2S between 22.5 to 29 µM would lead to considerable stress and damage to gill tissues which further led to progressive liver damage, reduced growth and greater susceptibility to diseases.

2.4 Tracking and Controlling H_2S production in RAS

Since H_2S is extremely toxic for the fish and events of H_2S production ends up in huge economic loss, it is important to track the events leading to the production of H_2S and prevent H_2S production. Once H_2S is produced a quick mitigation measure is water exchange which is also stressful for the fish (Kidder III et al., 2006). So, it is better to prevent H_2S from being produced. The best way to reduce the incidence of H_2S production is to employ a good system design that effectively flush out waste from the system and with less

dead pockets. Solid waste accumulated in the system can cause continuous production of H_2S, thus the fish will be exposed to sub-lethal concentration for long-term (Rojas-Tirado et al., 2021). Biofilters are another area of concern which is a hotspot for the production of H_2S. If the biofilm in bio-media is thick, the lower layers become anoxic and favor the growth of SRB and produce large amount of H_2S in a short time. So optimal operation of biofilter with proper mixing and cleaning will reduce the risk of H_2S production (Rojas-Tirado et al., 2021).

Another viable option to prevent H_2S production in RAS is to maintain high nitrate concentration in the system, thus not allowing sulfate (SO_4^2) from getting reduced. Nitrate also scavenges the H_2S if it is produced in the system. Sudden drop in nitrate level is observed in RAS where H_2S induced mortality occurred (Dalsgaard et al., 2013). So, care should be taken during the initial days of stocking and also during event of starving the fish towards the harvest. During these periods there will not be enough nitrate production from the biofilter since there is less ammonia available in the system for the nitrifying bacteria to feed on (Sunde et al., 2004).

Apart from all these preventive measures, early detection of episodes that produce of H_2S is possible by focusing on the redox reactions occurring in the RAS system (Tanudjaja, 2021). Before sulfate (SO_4^2) reduction, Mn (IV) will reduce to Mn (II) followed by reduction of Fe (III) to Fe (II). Both Mn (II) and Fe (II) can be used a warning indicator for H_2S formation (Tanudjaja, 2021).

2.5 Nitrogen Cycle in RAS

Another challenge in RAS is the accumulation of nitrogenous wastes and removal of these nitrogenous wastes is one of the crucial processes occurring in a RAS. Nitrogenous compounds accumulated in aquaculture systems have lethal effects on fishes especially in RAS where they are reared at higher stocking densities in closed environment (Kuhn et al., 2010). Major nitrogenous waste is ammonia and it is produced in fish as an end-product of

protein catabolism and are excreted as un-ionized ammonia (NH_3) across gills (Ebeling and Timmons, 2010). Ammonia is also released during degradation of nitrogen containing organic matter by microbes. Nitrification and denitrification are the major remedies to resolve the nitrogenous toxicity in RAS (Preena et al., 2021).

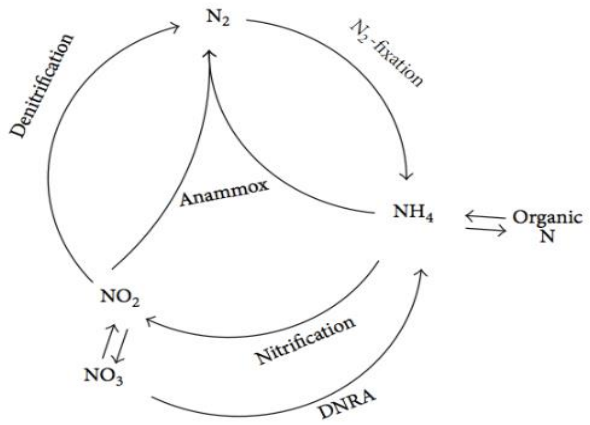

Figure 2.3 Dagramatic representation of Nitrogen Cycle

Nitrification process occurs in aerobic biofilters where ammonia is oxidized to nitrate via nitrite. This chemolithoautotrophic oxidation is performed in several steps. First ammonia (NH_4^+) is oxidized to hydroxylamine (equation 2.2) and then to nitrite (NO_2^-) (equation 2.3) by ammonia oxidizing bacteria (AOB) like *Nitrosomonas*. The nitrite (NO_2^-) is converted to nitrate (NO_3^-) by nitrate oxidizers (equation 2.4) like *Nitrobacter* (Preena et al., 2021). The overall reaction is shown in equation 2.5. Recent studies have reported that the enzymes responsible for both ammonia oxidation and nitrite oxidation may be present in a single group of microorganisms. For eg. *Nitrospira* can mediate the whole nitrification process (Bartelme et al., 2017). The formed nitrate (NO_3^-) is less toxic as compared to nitrite (NO_2^-) and ammonia (NH_4^+), but at higher concentration it can be toxic for the fishes. So, it is important to avoid excessive accumulation of Nitrate (NO_3^-) in the RAS system. It is achieved either by water exchange or by including anaerobic denitrifying biofilter in the recycling loop. There can be also anaerobic nitrification of ammonia to

nitrogen gas (annamox) by autotropic nitrifying bacteria. These are obligate anaerobic bacteria so the process takes place in the oxygen depleted areas of the biofilter (Preena et al., 2021).

$$NH_3 + 2H^+ + O_2 + 2e \rightarrow NH_2OH + H_2O \quad 2.2$$

$$NH_2OH + H_2O \rightarrow NO_2 + 5H^+ + 4e \quad 2.3$$

$$NO_2 + H_2O \rightarrow NO_3 + 2H^+ + 2e \quad 2.4$$

$$NH_3 + 2H^+ + O_2 + H_2O + 2e \rightarrow NO_3 + 7H^+ + 6e \quad 2.5$$

In anoxic conditions nitrate is preferred oxidant and is reduced to nitrite by denitrifying bacteria. The nitrate reduction can be assimilatory or dissimilatory based on the type of nitrate reductase catalyzing the reduction process (Zumft, 1997). Nitrite (NO_2^-) yielded from assimilatory nitrate reduction is used in biosynthesis of amino acids, nucleotides, and other essential biomolecules. It is very unlikely to occur in the presence of ammonium or organic nitrogen. In dissimilatory nitrate reduction, the nitrite (NO_2^-) formed is reduced to nitric oxide (NO) then to nitrous oxide (N_2O) and finally to dinitrogen gas (N_2) catalyzed by four different metalloenzymes present in denitrifying microorganisms. These metalloenzymes contain iron (Fe), molybdenum (Mo) or Copper (Cu) as metal co-factors, which is cruicial for its enzymatic activity (Knowles, 1982; Philippot, 2002; Zumft, 1997). Individual reactions are presented in equations 2.6-2.9 and overall reaction is shown in equation 2.10. Complete removal of nitrate (NO_3^-) accumulated in RAS is possible with this dissimilatory nitrate reduction carried out in an anaerobic denitrification biofilter. Since nitrate is the preferred electron acceptor in anoxic condition, a safe nitrate level is maintained in RAS to prevent sulfate reduction.

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \quad (2.6)$$

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \quad (2.7)$$

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O \quad (2.8)$$

$$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O \quad (2.9)$$

$$2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O \quad (2.10)$$

The nitrite (NO_2^-) formed during dissimilatory nitrate reduction can also follow other pathways apart from denitrification. It can be directly reduced to ammonia without forming any nitrogen intermediates. This reaction called Dissimilatory Nitrate Reduction to Ammonia (DNRA) catalyzed by the enzyme cytochrome-c nitrate reductase (Einsle et al., 1999). DNRA reaction is not preferred in RAS since it results in the accumulation of toxic ammonia in the system. Here lies the risk of maintaining high nitrate concentrations in RAS, since there is excess organic carbon there can be production of ammonia through DNRA (Gottschalk, 1986).

2.6 Use of iron oxides to control H2S

Iron is an essential element for bacteria, plants and animals. It is the fourth most abundant element on the earth crust but the concentration of iron is less in oceans and surface waters. Iron occurs in two valence states as oxidized ferric iron Fe (III), and reduced ferrous iron Fe (II). Iron (III) oxides and hydroxides generally have low solubility in water. The limited solubility arises from the tendency of iron (III) compounds to form stable, insoluble precipitates, such as hematite (Fe_2O_3) and goethite (FeOOH), under ambient conditions (Cornell et al., 2003).

In seawater, iron (III) is primarily present as colloidal form (Öztürk and Bizsel, 2003). The concentration of dissolved Fe (III) in seawater is typically low due to its very low solubility. Iron (II) compounds, such as ferrous sulfate ($FeSO_4$)

or ferrous chloride ($FeCl_2$), generally exhibit higher solubility in water compared to iron (III) compounds (Wu and Luther, 2016). The increased solubility of Fe (II) arises from its weaker bonding and higher reactivity compared to Fe (III) (Stumm and Morgan, 1996). Iron (II) is indeed prone to oxidation in the presence of oxygen (oxic conditions). Upon exposure to oxygen, Fe (II) can be oxidized to Fe (III), which may subsequently precipitate as iron (III) oxides and hydroxides (Eric Viollier et al., 2000).

Two principal biological process are important in connection with iron cycle. Assimilation process in which microorganisms such as magneto-tactic bacteria or phytoplankton depend on the uptake of iron as a pre-requisite for their cell growth. The other one is dissimilation process in which microorganisms conserve energy to maintain their physiology by the reduction of Fe (III). In latter case Fe (III) act as an electron acceptor which is also termed as oxidant. These processes occur in marine sediments along with several other abiotic reactions depending on the thermodynamic and kinetic conditions (Haese, 2000).

Apart from iron (III) there are many electron acceptors in marine sediments, one such is SO_4^{2-} and reduction of this results in the production of toxic H_2S. Study by Froelich et al., 1979 revealed succession of electron acceptors used by dissimilatory bacteria according to energy gain and it follows the order NO_3^- , Mn (IV), Fe (III), SO_4^{2-} as discussed in section 2.2. In RAS availability of other oxidants in the system delays the reduction of SO_4^{2-} and iron (III) is one such oxidant. Studies shows that ferric (III) iron of iron oxides as well as sheet silicates can be used by dissimilatory iron reducing bacteria (Haese, 2000). A detailed review of microorganisms reducing Fe (III), the respective electron donors are given by Lovley, (1997) and Lovley et al., (1987).

Soluble ferric ions or amorphous ferric oxides has been used as an oxidizing agent to reduce the sulfide effect in sewage systems (Lahav et al., 2004). Iron

species are also used in biogas plant for the removal of H_2S (Li and Ebrahimi, 2003; Pagella and De Faveri, 2000). Study by Connell and Patrick, (1969) shows a reduction in the quantity of H_2S in soils during addition of freshly grounded Fe_2O_3. Generation of H2S through biological sulfate reduction in marine sediments and anoxic paddy soils by SRB can be inhibited by FeOOH powders and Fe (III) salts (Achtnich et al., 1995). Coming to aquaculture, a study by Poulton et al., (2002) showed that ferrihydrite coated zeolite is efficient in removal of hydrogen sulfide in marine flow through systems. Also, all hematite compounds have the capacity to remove significant amount of sulfide from the system through a combined effect of oxidation and FeS precipitation.

Recent studies developed a granular iron-cycling technology (Fe (III) - Fe (II) shuttle) for the in-situ control of biogenic hydrogen sulfide in the sediment systems (Sun et al., 2019, 2014, 2013). It has been demonstrated that ferric hydroxides (FeOOH) in granular form, such as ferric hydroxide (GFH), granular ferric oxide (GFO) and rusted iron granules containing FeOOH, persistently retain in the sediments and effectively control the biogenic hydrogen sulfide slowly generated, with nearly no iron loss into the water phase at near-neutral pH. More importantly, the used FeOOH granules can be regenerated via oxidizing the surface Fe II products using oxygen. This granular iron-cycling technology is a long-lasting, renewable, and chemical-saving alternative for the control of biogenic hydrogen sulfide in the sediments of polluted waters in sewage networks and treatment plants (Cao et al., 2019; Ganigue et al., 2011; Jiang et al., 2015). The above-mentioned Fe (III) - Fe (II) shuttle can be also applied in a RAS system for controlling H_2S and the formed Fe (II) gives an early warning sign for the H_2S production. Here the toxicity of iron for fishes should be also taken to consideration.

3. Objectives

a. The primary objective of this project is to study the redox reactions in waste from RAS system called sludge with special focus on H_2S development.
b. To compare how Fe (III) and NO_3 addition delays the H_2S production in RAS.
c. To check if Fe (II) can be used as an early warning sign for H_2S production
d. To study the Fe (III)-Fe (II) shuttle and Fe (II) precipitation within the system and see if Fe (III) addition can effectively delay H_2S production in RAS.
e. To follow the nutrient levels in samples and see if this can be related to H_2S production.

4. Hypothesis

a. Addition of Fe (III) delays the H_2S production in RAS sludge same as NO_3 addition.
b. Fe (II) can be used as an early warning sign for H_2S production in RAS.
c. Formed Fe (II) reacts with S^{2-} and precipitate as Ferrous Sulfide ($FeS_{(S)}$) which further reduces the H_2S concentration in iron-adedd treatment (FAT).

5. Materials and Methods

5.1 Materials

The experiment was performed in Department of Chemistry, Norwegian University of Science and Technology (NTNU), Trondheim. The sludge used for the experiment was collected from the RAS facility of Nofima AS (Akva Sunndalsøra).

5.2 Fish sludge and Seawater

Fish sludge waste for this experiment originated from the Atlantic Salmon (*Salmo salar*) reared in Recirculating Aquaculture System (RAS) at Nofima Sunndalsøra. The waste was collected from the bottom outlet of the swirl separators in two tanks in grown out hall 3. Each tank has a volume of 100 m^3 and average weight of the fish in the tank was 11 kg. Total biomass in the system was 6000 kg with a density of 30 kg/m^3. The fishes were fed at a rate of 14Kg feed per day. 45% of the water in the whole system was exchanged on daily basis. The system was maintained at a temperature of 12°C. The sludge was received in a frozen condition and it was stored in freezer until the start of experiment. The sludge was transferred to a refrigerator for thawing one day before and it was homogenized well before transferring to bottles.

Seawater used in the experiment was collected from Trondheim Biological Station (TBS). It was pumped from a depth of 80 meters from Trondheim fjord to a cistern located above the main TBS building. From there it was collected in 100L drum which was already acid washed and transported to Department of Chemistry, NTNU. Necessary measures were taken while collecting and transporting sea water to avoid any possible contamination.

5.3 Acid Washing and Conditioning

All bottles and materials used in this experiment were acid washed prior to use. First washing was carried out with 1M HNO_3 and kept for two days. Then it was rinsed 3 times with Milli-Q water. Rinsing was carried out in a gradually increasing fashion. That is first rinsing was carried out by filling very little water and volume of water used for rinsing was gradually increased in subsequent rinsing. It was done to prevent the sudden pH rise which can cause re-adsorption of these metals back to the walls of the bottles. The next washing was carried out using 0.1 M UP HNO_3. The bottles were filled with acid and kept for 5 days. Final rinsing was performed within the clean lab. All bottles were rinsed five times using Milli-Q water in same gradually increasing

fashion starting with small volume of water as explained above. The day before the start of the experiment the bottles were filled with sea water for conditioning.

5.4 Experimental design

The experiment had control and two treatments; control was with sludge and seawater. The two treatments were accordingly one with nitrate addition (NAT) in the form of a $NaNO_3$ and other with Fe III addition (FAT) in the form of $FeCl_3$. The experiment was carried out in 33 amber glass screw cap bottles with maximum volume of 595mL. 11 bottles were assigned for each of the treatments as well as the control. The bottles were acid washed prior to use with final washing carried out within the clean lab.

30 mL (5% bottle volume) of well mixed sludge was added to each of the bottles. To the nitrate-added treatment (NAT) 2mL of 1.75 M $NaNO_3$ was added. The addition was aimed to attain a final NO_3-N concentration of ~ 6mM (82 mg/L) in each of the bottles, which is below the maximum allowed level of NO_3-N in salmon farms (100 mg/L). To the iron treatment (FAT) bottles 0.5 mL of 0.5 M $FeCl_3$ solution was added which results in a final Fe(III) concentration of 0.4 mM (22.3 mg/L) in each bottle. This eventually forms amorphous colloidal $Fe(OH)_{3(s)}$ and $FeOOH_{(s)}$. Fe (III) can be toxic for the aquatic organism and a previous study reported safe limit of iron for zooplankton *Daphnia longispina* is 30.2 µM (1.6 mg/L) (Randall et al., 1999). In this experiment the toxicity of Fe to the fish is not considered and the amount of Fe(III) added is calculated based on stoichiometry of the oxidation of organic matter by FeOOH. The bottles were then filled up to the rim with seawater and were incubated in cold lab at 12°C in dark.

The bottles were shaken twice daily at 09:00 and 16:00 to prevent formation of anoxic pockets and to keep the homogeneity in solution. On each sampling day one bottle each from the three groups were sacrificed to collect the samples. Sampling days in each of the treatments were finalised by checking

the trends in H_2S production and details of sampling days are given in Appendix 1. Sampling is carried out after about an hour after shaking the bottles allowing the suspended matter to settle down so there may not be any bias due to particulate matter in the samples. One sample and a technical replicate were drawn from each bottle for each of the analysis. It is done by siphoning with syringe and tube to prevent the mixing of oxygen. There were separate tubes and syringes for each treatment and they were acid washed prior to use. In this study, all experimental procedures were performed in accordance with the established Health, Safety, and Environment (HSE) protocols. By strictly adhering to these guidelines, potential hazards were identified and mitigated, minimizing any risks associated with the experiment.

5.5 Chemical Analysis

5.5.1 Hydrogen Sulfide

Hydrogen sulphide analysis was performed following methylene blue method by Letelier-Gordo et al., (2020). Diamine reagent was prepared by dissolving 0.8 g N,N-di-methyl-p-phenyldiamine and 1.2g $FeCl_3.6H_2O$ in 200mL dilute HCl (100 mL 37% HCl in 100 mL Milli-Q Water). The reagent was prepared prior to the experiment and stored in refrigerator at 4°C in dark bottles. This reagent forms methylene blue when combined with hydrogen sulfide.

During sampling 45 mL of the supernatant was transferred into 50 mL plastic centrifuge tubes and they were centrifuged at 4000 rpm for 15 minutes. After centrifuging 1.6 mL of diamine reagent was added to 20 mL of the supernatant and it was kept in dark for 30 minutes for colour development. Another 20 mL of the supernatant was taken to measure the background noise. 1.6 mL of diamine reagent was added to 20 mL Milli-Q Water to measure the background absorbance by the reagent. All the absorbances were measured at 665 nm using a 5cm cuvette in a Jenway 6715 UV-VIS spectrometer using Milli-Q water as blank. When the intensity of the colour was beyond the measuring range, each sample was diluted with Milli-Q to obtain 1/10, 1/100, 1/1000

dilutions. To get 1/10 dilution 18mL Milli-Q Water was added to 2mL of the initial mixture. 1/100 and 1/1000 dilutions were made from 1/10 and 1/100 dilutions respectively.

The concentration was calculated from the absorbance using the equation of the standard curve plotted with known concentrations of Sodium sulfide nonahydrate ($Na_2S.9H_2O$), y = 0.0191x - 0.0204 (R^2 = 0.999) where y is the concentration of S^{2-} in µM and x if the final absorbance after subtracting all background noises (Appendix 2).

5.5.2 Fe (II) – Ferrozine Method

Fe(II) levels in the samples were measured using the modified ferrozine technique (E Viollier et al., 2000). Since we were interested in only Fe (II) and not the total Fe, the reduction step in the ferrozine method which convert Fe (III) in sample to Fe (II) was not performed. There can be some interference of Fe (III) in the measured absorbance (E Viollier et al., 2000). Here in this work we are neglecting the interference of Fe (III) since the main focus of the work is to follow the trend in Fe(II) production and not to quantify the amount of Fe (II) formed.

For the ferrozine method 300 mL 0.01M Ferrozine solution was prepared in 0.1M ammonium acetate solution. It was made by dissolving 2.312g of Ammonium acetate in 300mL Milli-Q Water and to which 1.48g of Ferrozine reagent (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate) was added. The regent solution was prepared prior to the experiment and stored in a refrigerator at 4°C in dark bottles. The ferrozine reagent reacts with divalent iron to form a stable magenta coloured complex with maximum absorbance at 562 nm (Stookey, 1970).

45mL of sample was transferred into a 50mL centrifuge tube. Soon after sample collection the pH was measured and reduced to 1.7-2 by adding 3.6 M Ultra-Pure HNO_3. This preserves the Fe (II) in reduced and soluble phase.

It was then centrifuged at 4000 rpm for 15 minutes. After centrifuging the sample was filtered through 0.2 µm Sartoban-Sartorious filtering cartridge which helps in removing the possible interferences by other metal forms. (USA. Department of Environment and Natural Resources Division of Water Resources., 2015). Separate filtering cartridges were used for each of the three treatment conditions and the cartridges were stored in a refrigerator when it is not in use. 2mL of ferrozine regent solution is added to 20 mL of the filtrate. Immediately after ferrozine addition, 75µL 3.6 M NH_4OH was added to increase the pH to 4.5 – 5 for effective colour formation (Virginia A. Elrod, 1991).

In Jenway 6715 UV-VIS spectrophotometer absorbance at 562 nm was measured using a 5cm cuvette. Milli-Q water was used to blank zero the spectrophotometer. Another 20 mL of the filtrate was taken to record the background noise. The absorbance of the reagent was captured by adding ferrozine solution to 20 mL Milli-Q water and absorbance was measured. The final absorbance was obtained by subtracting background noises. It was then converted to concentration by using the equation of the standard curve created with known concentrations of Ferrous (II) ammonium sulfate hexahydrate ($Fe(NH_4)_2(SO_4)_2.6H_2O$): $y = 0.0014x - 0.0034$ ($R^2 = 0.9973$); where y is the concentration of Fe (II) in µM and x is final absorbance (Appendix 2).

5.5.3 Fe (II+III) – ICP-MS

Total Fe (Fe II+ Fe (III)) in the samples were measured using ICP-MS. From the sample taken for Fe (II) analysis 13 mL filtrate was transferred into 15 mL centrifuge tubes. This filtrate was with pH 1.7-2 which keeps Fe in soluble phase, so there won't be any adsorption of Fe to the walls of the centrifuge tubes or precipitation as hydroxides (Fitzsimmons and Boyle, 2012).

The analysis was performed using an Agilent 8800 Triple Quadrupole ICP-MS instrument. Sample introduction was performed using an integrated sample

introduction system (ISIS) and an SPS4 autosampler from Agilent Technologies. Additionally, a standard introduction system was utilized, which consisted of a glass concentric nebulizer for creating a fine mist, a quartz double pass spray chamber, a quartz torch with a 2.5 mm internal diameter, and standard nickel cones. System parameters for the ICP-MS are given in Table 5.1.

Parameter	Value
RF Power	1550 W
RF Matching	1.80 V
Sample depth	8.0 mm
Nebulizer Gas Flow	1.05 L/min
Option Gas Flow	0.0 L/min
Make Up Gas Flow	0.0 L/min
Nebulizer Pump	0.1 rps
S/C Temp	2°C
Cell Tuning modes	No Gas and O2
O2 Flow Rate	30%
Scan Type	MS/MS
Replicate/peak pattern/sweeps	4/3/30

Table 5.1 Agilent 8800 Series Triple Quadrupole ICP-MS System parameters

5.5.4 Nutrients (NO_3-N, NO_2-N and PO_4-P)

Samples were drawn from bottles for analysis of NO_3-N, NO_2-N and PO_4-P. It was then centrifuged at 4000 rpm for 15 minutes. After centrifuging the supernatant was transferred into 15 mL centrifuge tubes and they were stored in a freezer for later analysis.

The frozen samples were taken to Trondheim Biological Station (TBS) and thawed before analysis. The samples were filtered through a 0.45 µm syringe filter and it was diluted with saline as per the table 5.2 to get the concentration within the linear range. Linear range is where the concentration will increase

linearly with absorbance limited by lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ). Outside this linear range the concentration and absorbance are no longer linear and quantification is not possible. The expected concertation in sample is obtained from a similar experiment and is shown in table 5.2.

The analysis was performed on a Flow Solution IV (O.I. Analytical) with an autosampler using standard calibration curves. Concentrations of NO_2-N + NO_3-N, NO_2-N and PO_4-P were analysed separately. After calculating the concentration of N-NO_2, to the same diluted sample the analyser adds reducing agent that convert the NO_3 present in sample to NO_2 and this on further analysis gives the concentration of NO_2-N + NO_3-N. Later NO_3-N concentration was calculated by subtracting N-NO_2 concentration from total concentration of N-NO_2+ NO_3-N. The protocol used for the determination of NO_3-N and NO_2-N was NS4745 (NS4745, 1991), for PO_4-P it was NS-EN-ISO 6878 (NS-EN ISO 6878, 2004).

Analyte	Expected concentration	Linear Range		Dilution		
		ULOQ (µg/L)	LLOQ (µg/L)	Control	NAT	FAT
NO_2-N+ NO_3-N	82.4 mg/L	2	250	1:50	1:500	1:50
NO_2-N	8.2 mg/L	2	250	1:50	1:500	1:50
PO_4-P	60 mg/L	0.6	50	1:1500	1:1500	1:1500

Table 5.2 Dilutions, expected concentrations and the linear range for nutrient analysis

5.5.5 pH, ORP and Dissolved Oxygen (DO)

Small amount of sample was transferred into a beaker. pH, ORP and Dissolved Oxygen (DO) were measured using appropriate probes connected to an Arduino Nano Every board and the values are taken using Adruino IDE software in a computer.

6. Results

6.1 Hydrogen Sulfide (H_2S)

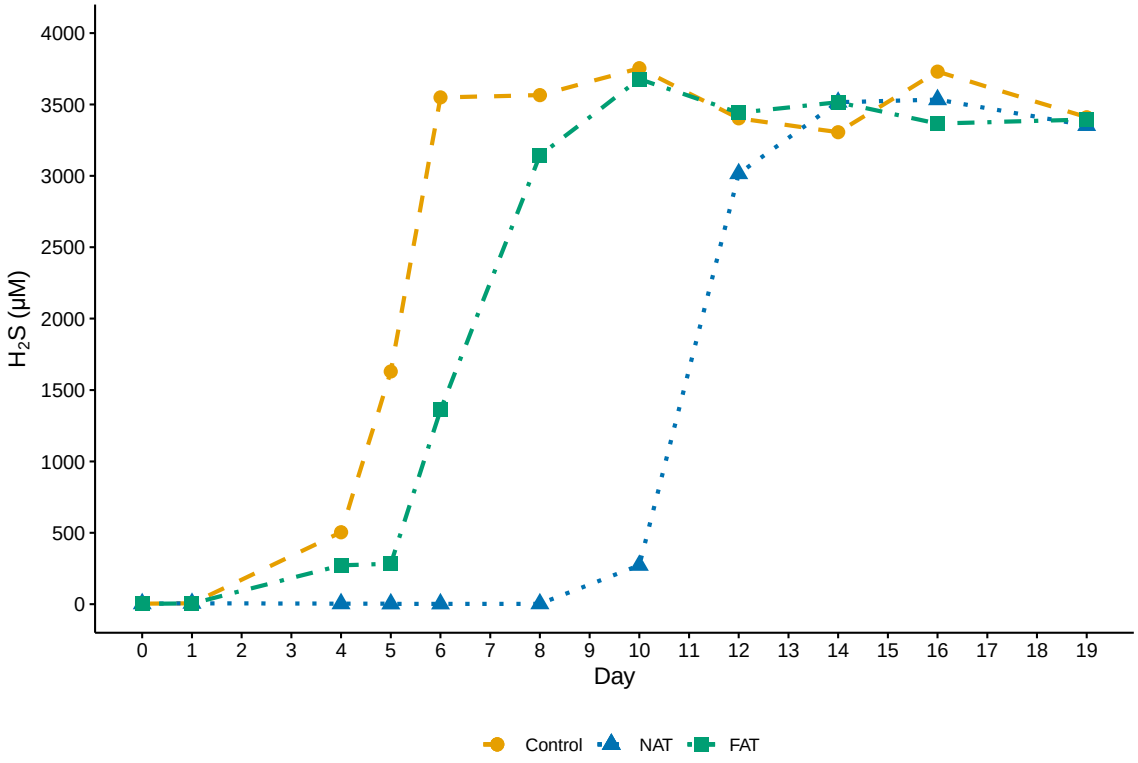

Figure 6.1 H_2S development in control, NAT, and FAT

The production of H_2S in three treatments control, NAT and FAT is shown in figure 6.1. In control, H_2S started developing slowly from day 1 to day 4. The increase was steep from day 4 to day 6 and reached at maximum concentration of 3.7 mM on day 10. The H_2S concentration remained more or less equal from day 10 until the end of experiment in day 19. In NAT, there were no visible H_2S formation until day 8. First increase in H_2S concentration was observed on day 10 and it was 0.2mM. From day 10 H_2S concentration started to increase steeply and reached a maximum concentration of 3.5 mM on day 14. After day 14 H_2S concentration was more or less stabilized until the end of the experiment on day 19.

In FAT, H_2S started developing slowly from the start of experiment until day 5, but the concentration remained less than that in control. A steep increase

in H_2S concentration was seen from day 5 until day 10. The maximum H_2S concentration 3.6mM is observed on day 10 and there was a small drop in H_2S concentration from day 10 until the end of the experiment in day 19.

When comparing the H_2S concentration among the treatments the H_2S level in control always stayed higher than other two treatments except on day 14. Though there was H_2S development in FAT from day 1, the concentration remained much less than that in control. On day 6 when the H_2S concentration reached 3.5 mM in control it was 1.3 mM in FAT. It is worth to note down that though concentration of H_2S in FAT were lower than in control during development, both reached the peak concentration on day 10 and the concentrations were nearly equal i.e. 3.7 mM in control and 3.6 mM FAT. H_2S concentration on day 6 in control (3.54 mM) was comparable to H_2S concentration on day 14 (3.51 mM) in NAT. Also, in FAT the H_2S concentration reached 2.7 mM on day 5 and same level of H_2S is formed in NAT only on day10.

6.2 Fe (II) – Ferrozine Method

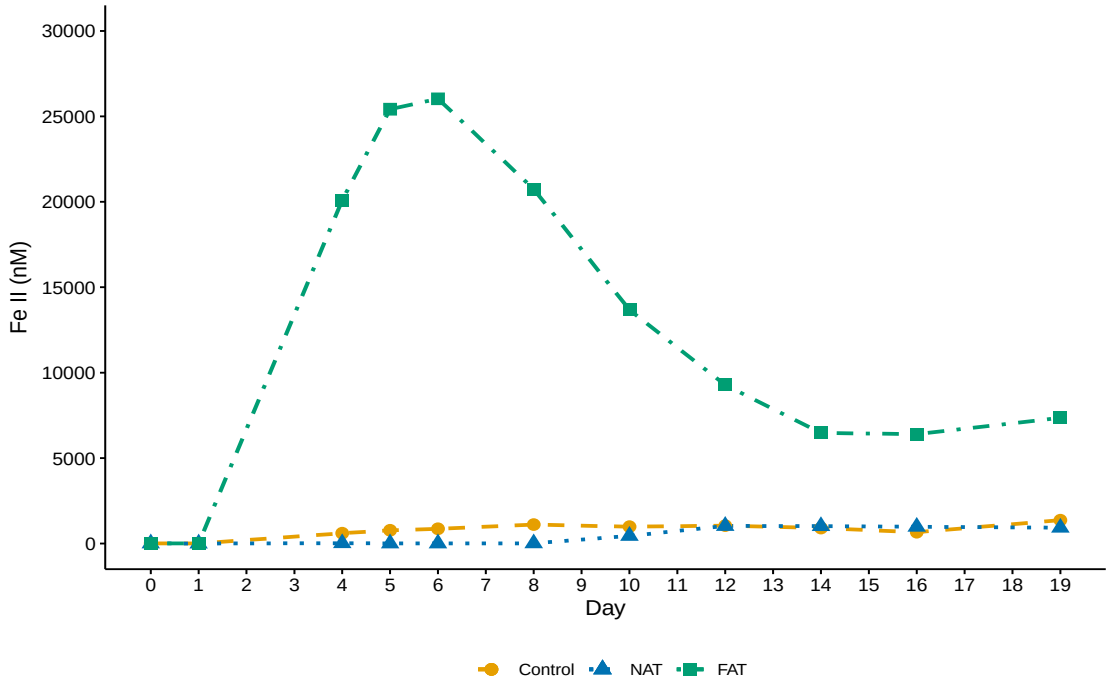

Figure 6.2 Fe (II) formation in control, NAT, and FAT

Fe(II) formation as a result of Fe (III) reduction in three treatments control, NAT and FAT is shown in figure 6.2. In control, considerable increase in Fe (II) was observed from day 4 and reaching peak value of 1.11 µM in day 8. In NAT no significant Fe (II) formation was observed until day 8. Fe (II) started forming from day 8 and reached peak value of 1.03 µM on day 12. Starting from day 12, Fe(II) levels were more or less equal in control and NAT. In FAT to which 0.4 mM Fe (III) was added, Fe (II) started forming from day 1 onwards and reached the peak value of 26.02 µM on day 6. After day 6 Fe (II) concentration stated dropping and towards the end of the experiment the curve got flattened.

6.3 Comparing H_2S and Fe (II) development in control, NAT, and FAT

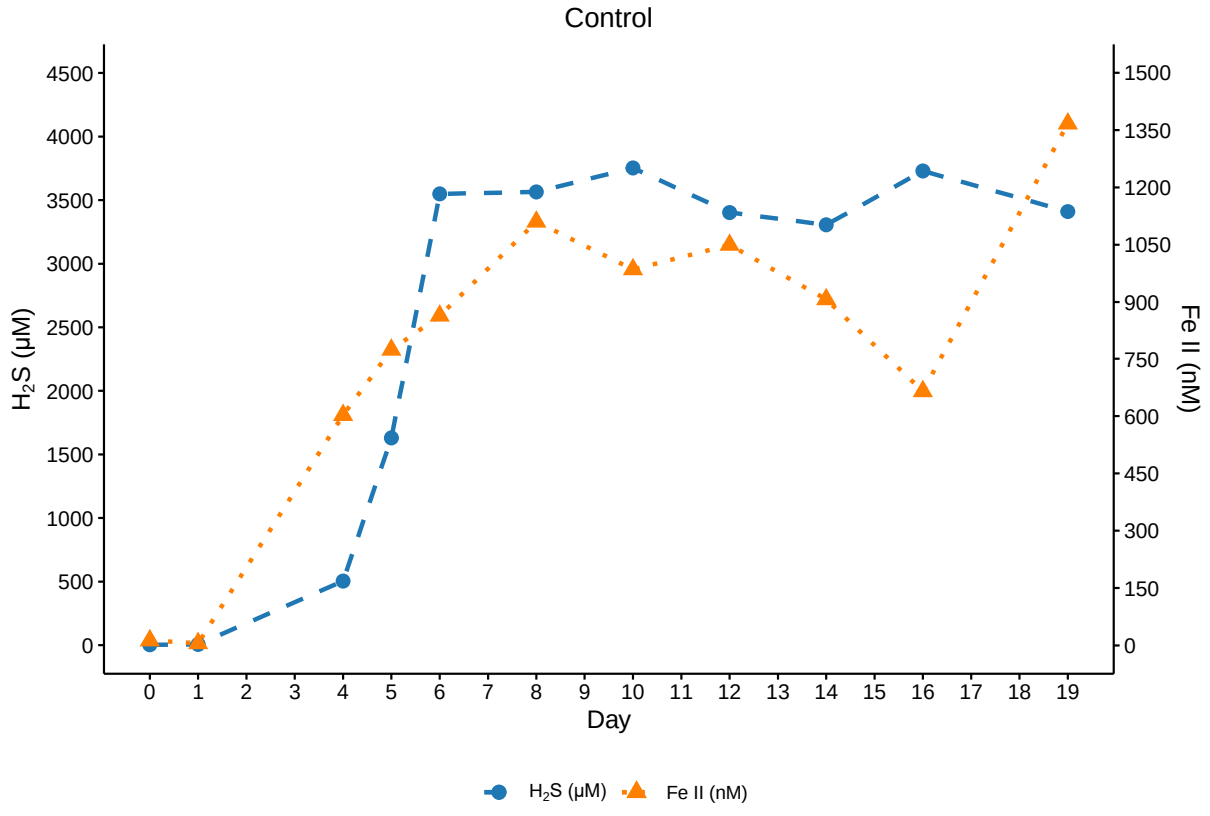

Figure 6.3 H_2S and Fe (II) development in Control

H_2S and Fe (II) formation in control is shown in figure 6.3. Fe(II) started forming after day 1. From day 1 until day 4 Fe(II) increases and from day 4

onwards Fe (II) fluctuates without any sudden increase or drop. In case of H_2S, rapid increase in concentration was observed only after day 4. When comparing Fe (II) and H_2S development, During the period when the concentration was increasing, from day 1 to day 4 the slope of Fe (II) curve is more than H_2S curve i.e. the rate of formation of Fe (II) is higher than the rate of development of H_2S. An opposite trend was seen after day 4, where rate of H_2S development was higher than Fe(II) formation which is clear from the steeper slope of H_2S curve. After reaching the peak maximum concentration both Fe (II) and H_2S concentration follows similar trend by fluctuating around the peak value until the end of experiment on day 19.

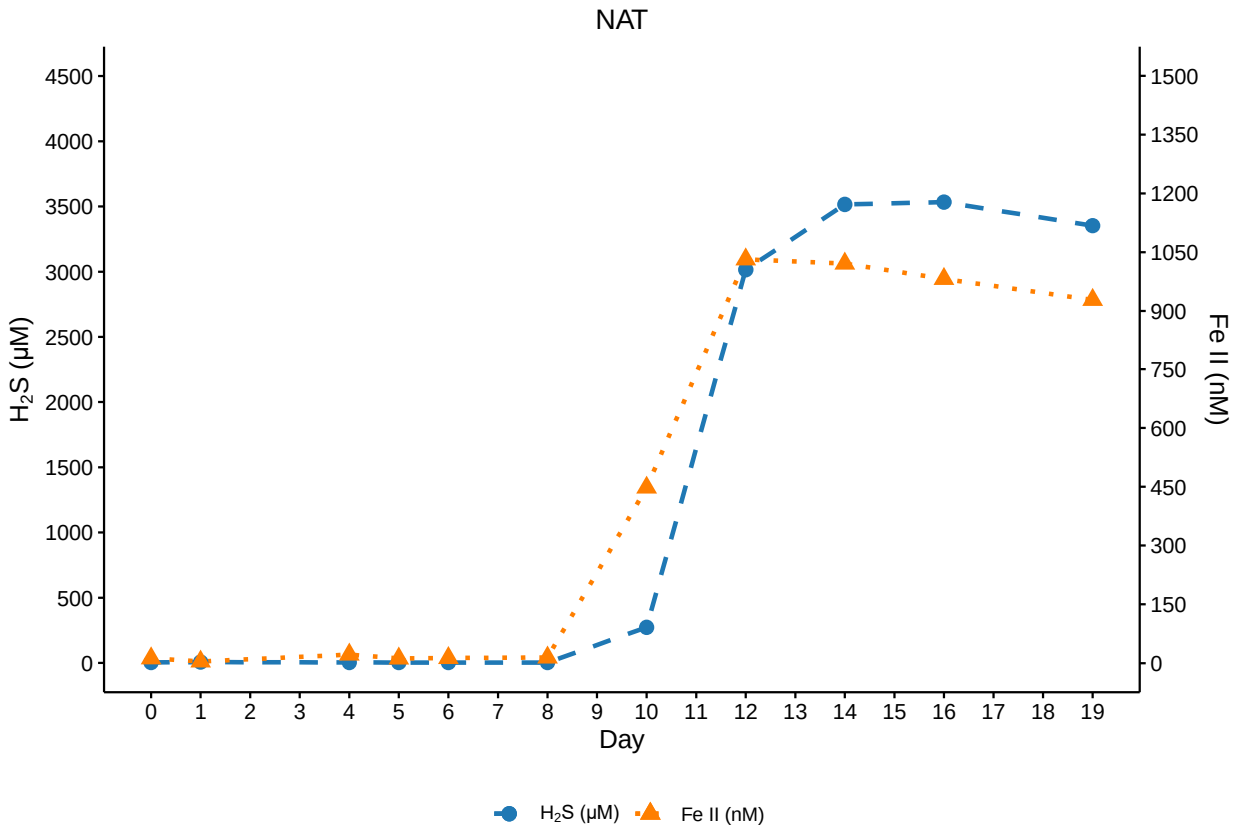

Figure 6.4 H_2S and Fe (II) development in Nitrate-added treatment (NAT)

Figure 6.4 compares H_2S and Fe (II) formation in NAT. No Fe(II) formation was observed until day 8. Significant amount of Fe (II) was observed only on day 10 and it reached the maximum concentration of 1.03 µM on day 12. H_2S

also started forming after day 8 and reached the peak value of 3.5 mM on day 16. On comparing the slopes of both curves, it is clear that though H_2S and Fe (II) started forming after day 8, the rate of Fe (II) formation was higher than H_2S development at beginning from day 8 to day 10. This trend got reversed from day 10 to day 12, where rate of formation of H_2S is higher than Fe(II) which is clear from the steeper curve of H_2S. This trend was similar to that observed in control. In the above result there is no visible gap between the development of Fe (II) and H_2S since both started increasing on same day. It can be also because there was no sampling carried out on day 9, so we are not sure whether H_2S started forming from day 8 or from day 9.

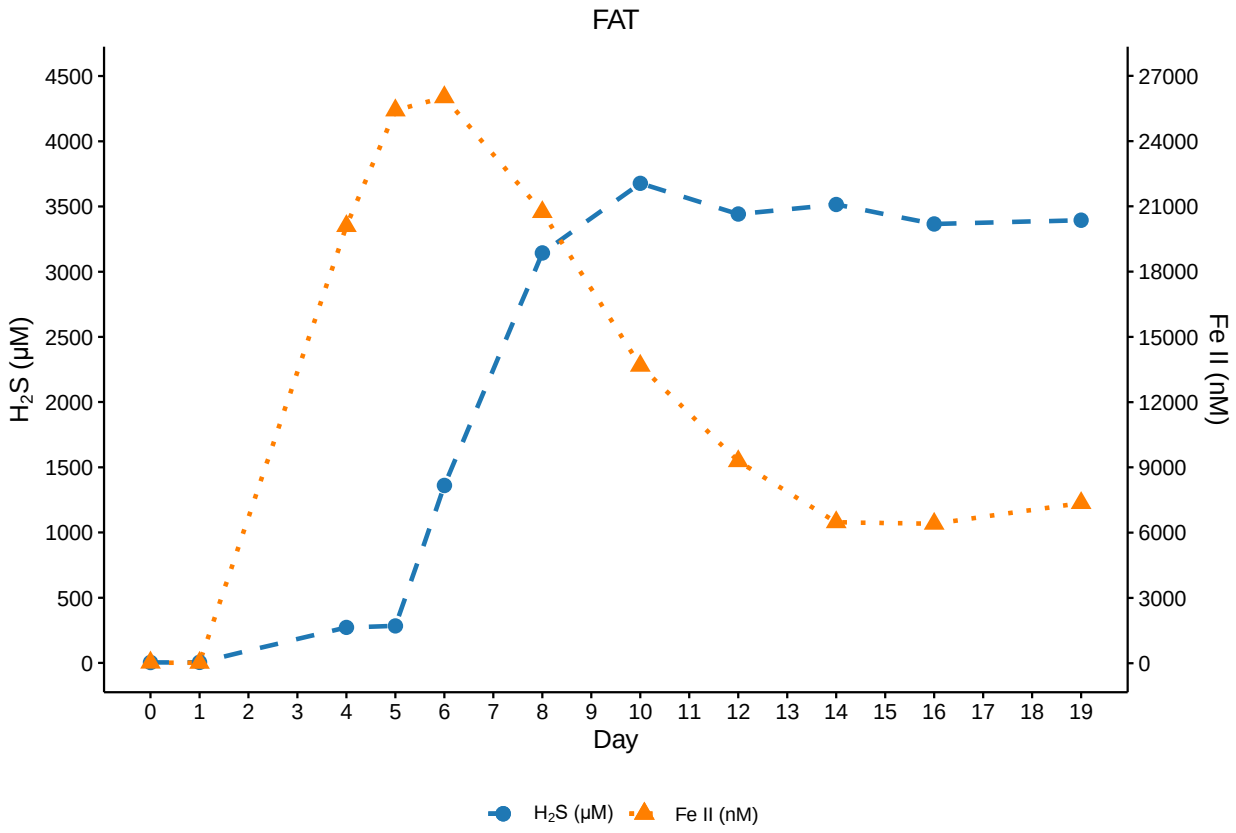

Figure 6.5 H_2S and Fe (II) development in Fe (III)-added treatment (FAT)

Figure 6.5 compares the formation of Fe (II) and H_2S in FAT. Fe (II) formation starts from day 1 onwards reaching its peak value of 26.02 µM on day 6. After day 6 the Fe (II) level gradually drops and get stabilized from day 14 until the

end of experiment. Small amount of H_2S development was there from the start of experiment but an increase in H_2S concentration was seen only after day 5. Unlike the control and NAT, here an increase in the H_2S concentration is observed after Fe (II) reached its highest value. The peaks of both curves can be separately seen. Fe (II) reached its highest value on day 6 on the other hand highest H_2S concentration was observed only on day 10 i.e. 4 days after getting the Fe (II) peak. Also, here in FAT the Fe (II) level was gradually decreasing after reaching the highest concentration at the same time the H_2S was more or less stabilized around the peak value.

6.4 Total Fe (II+III) ICP-MS

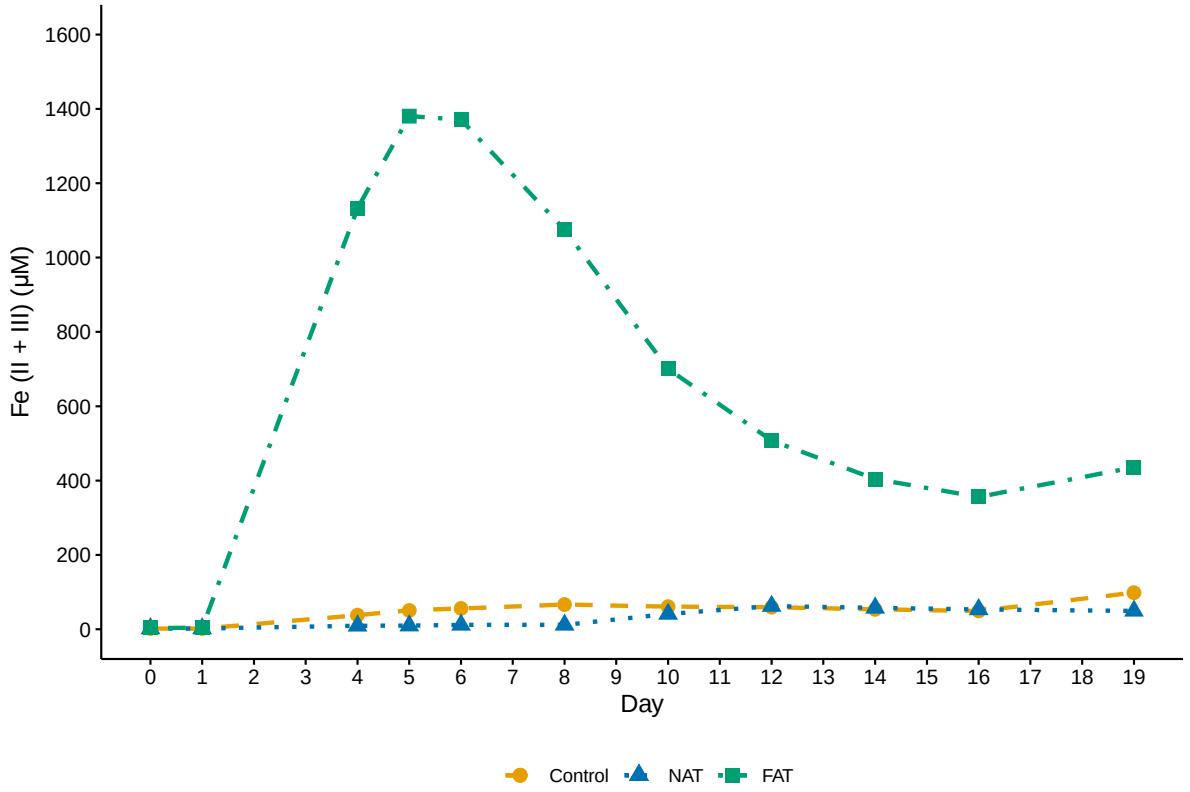

Figure 6.6 ICP-MS result of total Fe (II +III) in three treatments

Figure 6.6 shows the total Fe (II + III) in control, NAT and FAT measured with ICP-MS. In FAT, total Fe started to increase from day 1 reaching a peak value of 1.3 mM on day 6 and then it started to decrease gradually and reached 0.3

mM on day 16. Until day 8 total Fe in both control and NAT followed the same trend and from day 8 total Fe in NAT increased slightly. On day 19 an increase in Fe was observed in control, NAT and FAT as compared to previous the sampling day.

Figures 6.7 - 6.9 compares total Fe measured with ICP-MS and Fe (II) measured with ferrozine technique in control, NAT, and FAT. As expected, both followed the same trend in control and two treatments.

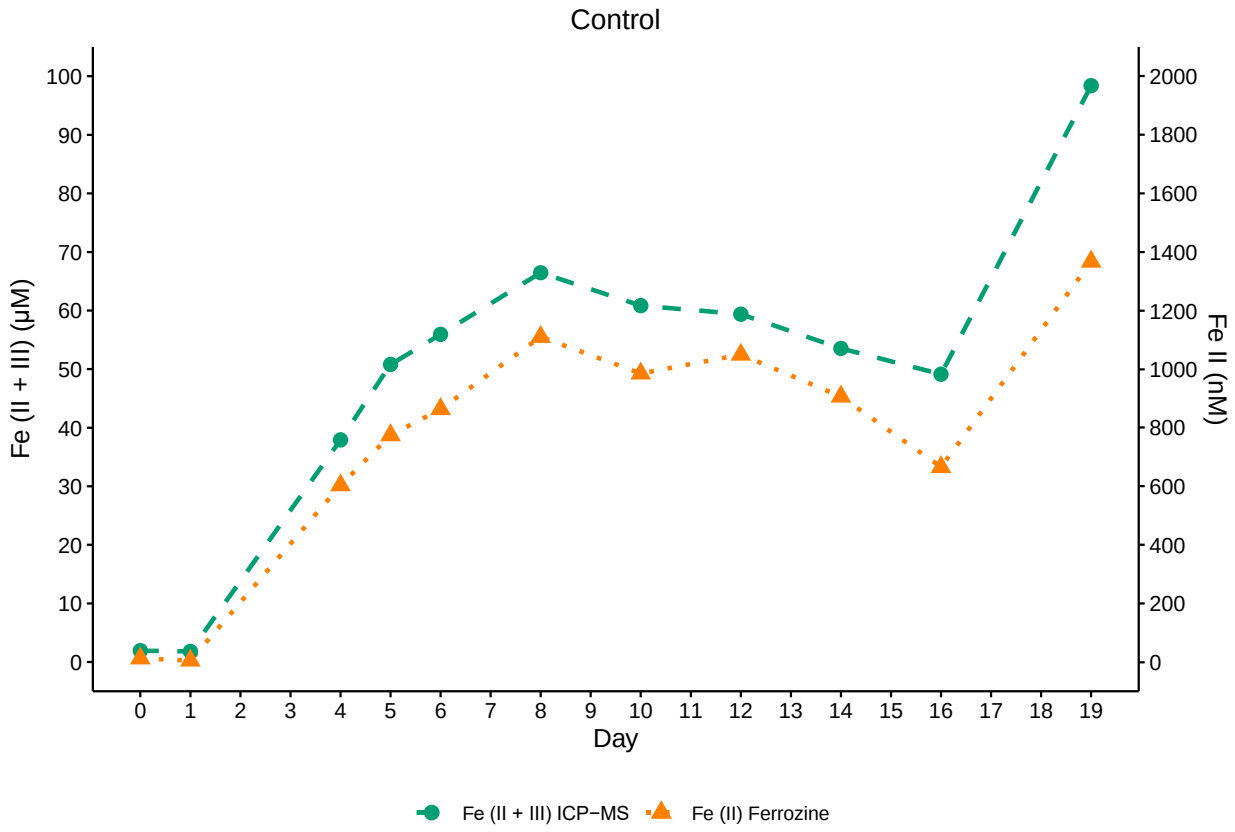

Figure 6.7 Total Fe (ICP-MS) and Fe (II) (Ferrozine method) in control

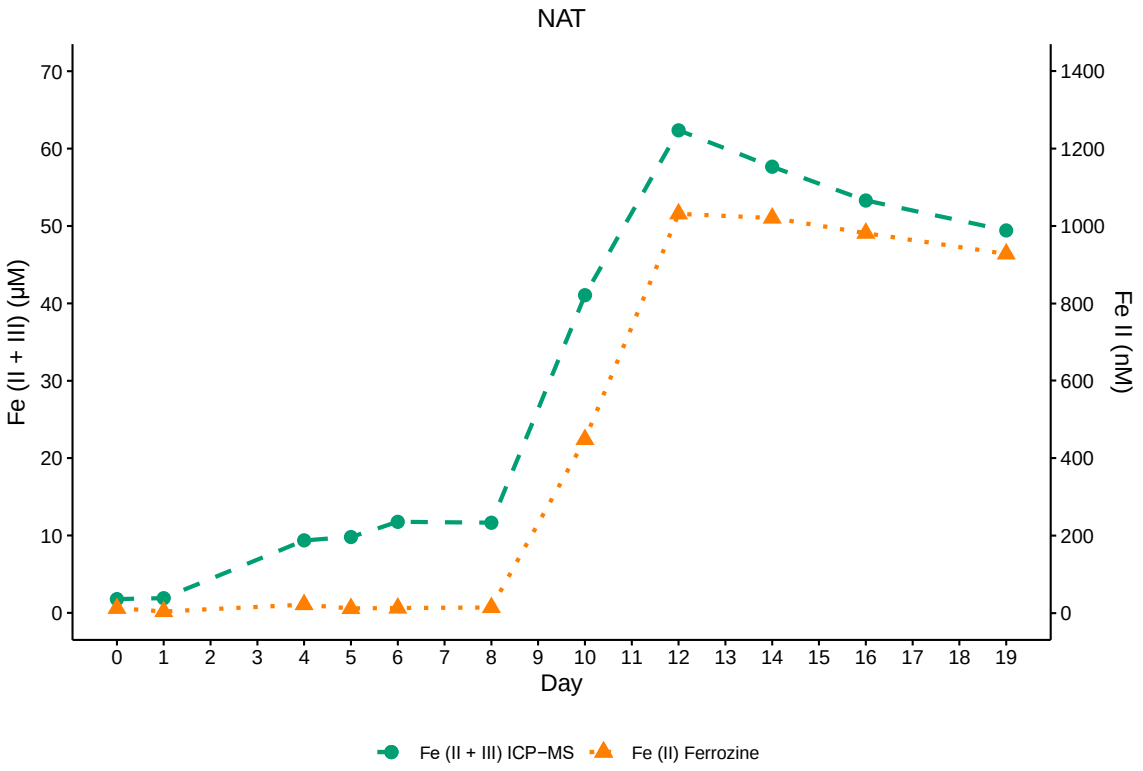

Figure 6.8 Total Fe (ICP-MS) and Fe (II) (Ferrozine method) in nitrate-added treatment (NAT)

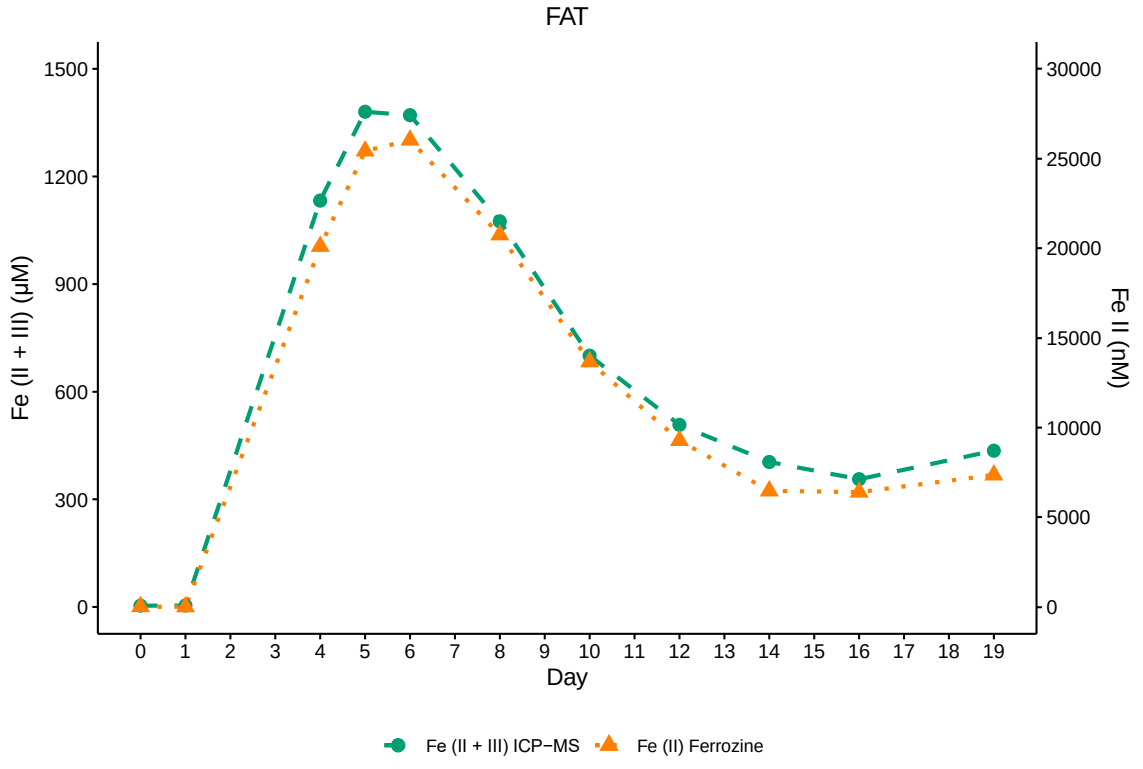

Figure 6.9 Total Fe (ICP-MS) and Fe (II) (Ferrozine method) in iron-added treatment (FAT)

6.5 Nutrient Analysis (NO_2^- and PO_4^{3-})

6.5.1 Nitrite (NO_2^-)

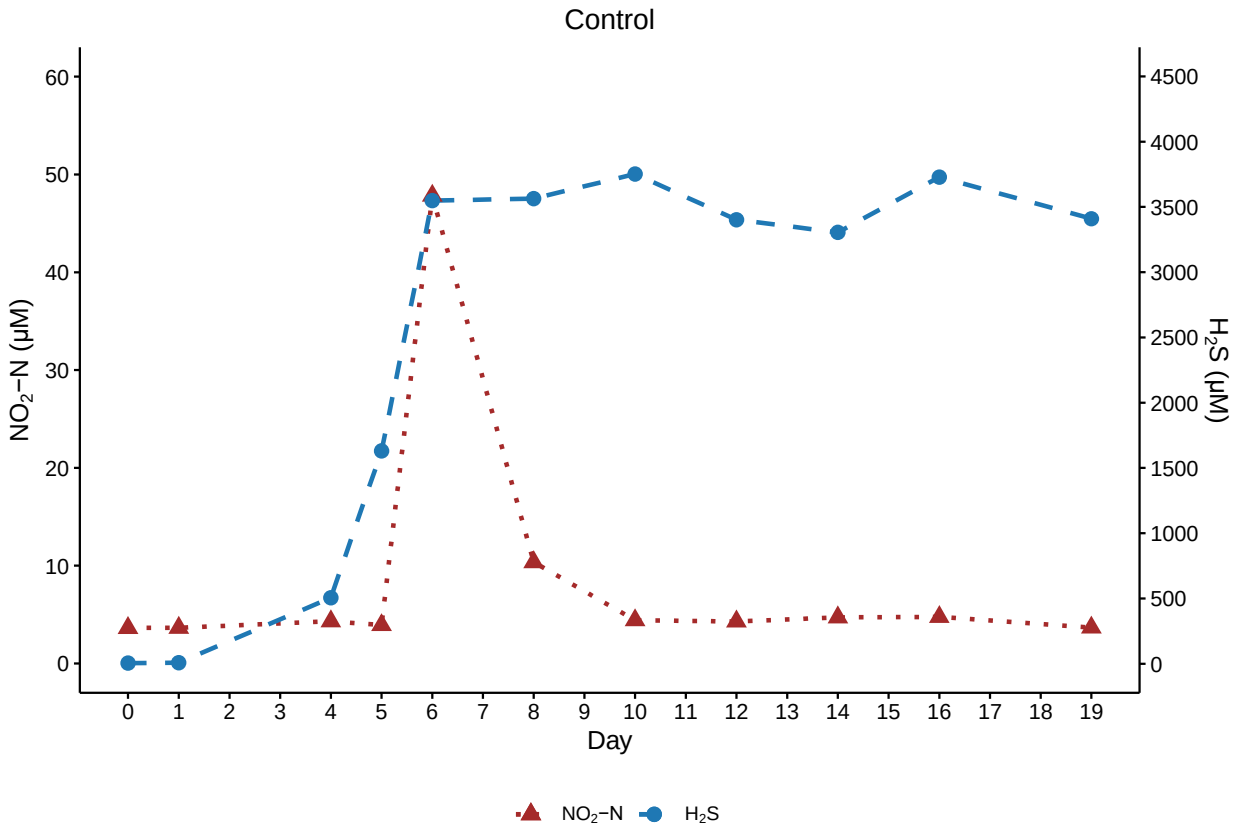

Figure 6.10 NO_2-N and H_2S development in control

Figure 6.10 shows the nitrite (NO_2-N) and H_2S development in the control. An increase in the amount of nitrite-N was seen after day 5 and reached maximum concentration of 47.8 µM on day 6. Nitrite-N concentration started to decrease after day 6 and on day 10 the nitrite-N level reached back to the initial range and continued same level until the end of the experiment. H_2S also started to go up during the same time as nitrite-N and reached maximum concentration of 3.7 mM on day 10.

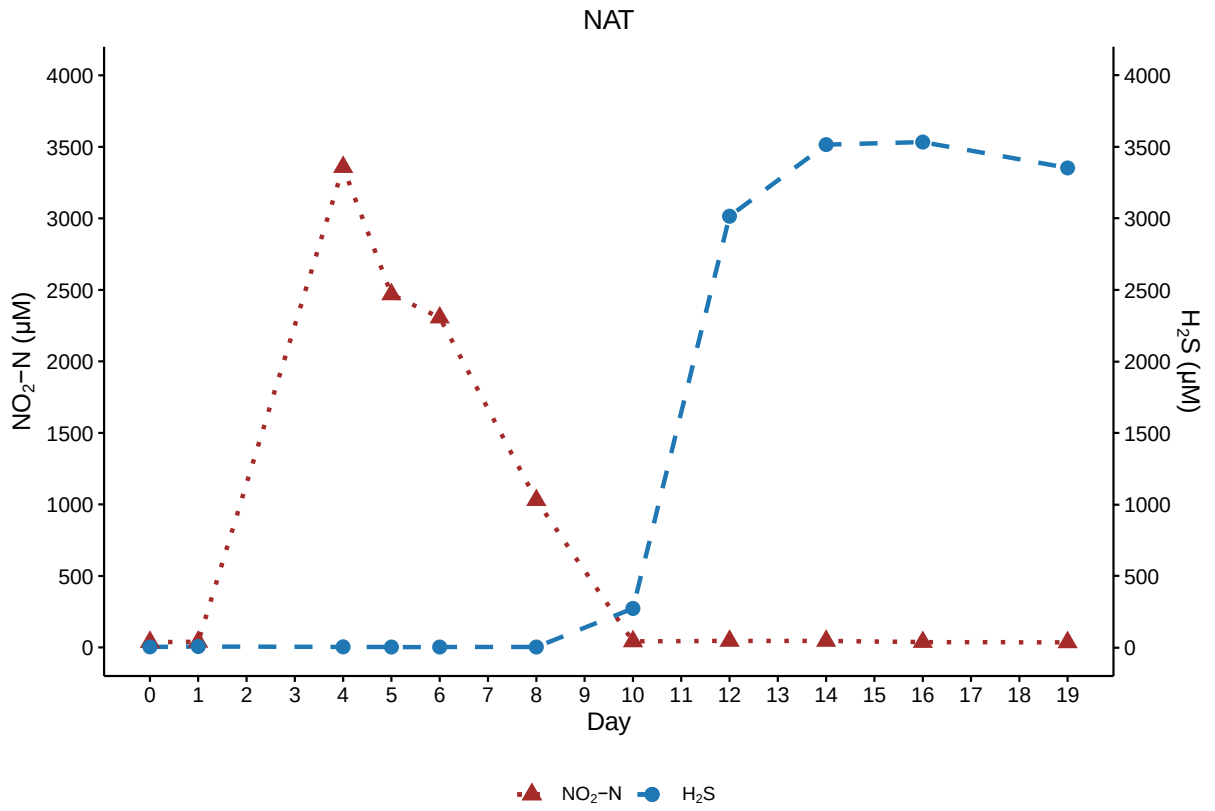

Figure 6.11 NO_2-N and H_2S development in nitrate-added treatment (NAT)

Variation of nitrite-N with H_2S development in NAT is shown in figure 6.11. The nitrite-N formation and H_2S formation can be seen as a mutually exclusive events in this treatment. Nitrite-N started to form after day 1 and reached a concentration of 3.3 mM on day 2. The nitrite-N level started dropping after day 2 and reached back to initial level on day 10. When nitrite-N started dropping H_2S started to develop slowly from day 8 and rapid H_2S increase was observed on day 12 after nitrite-N returned to its initial low value.

In FAT, the trend in the development of nitrite is similar to that in control. Figure 6.12 shows increase in nitrite-N and H_2S occurred simultaneously. Nitrate-N reached its maximum value of 11.2 µM on day 8.

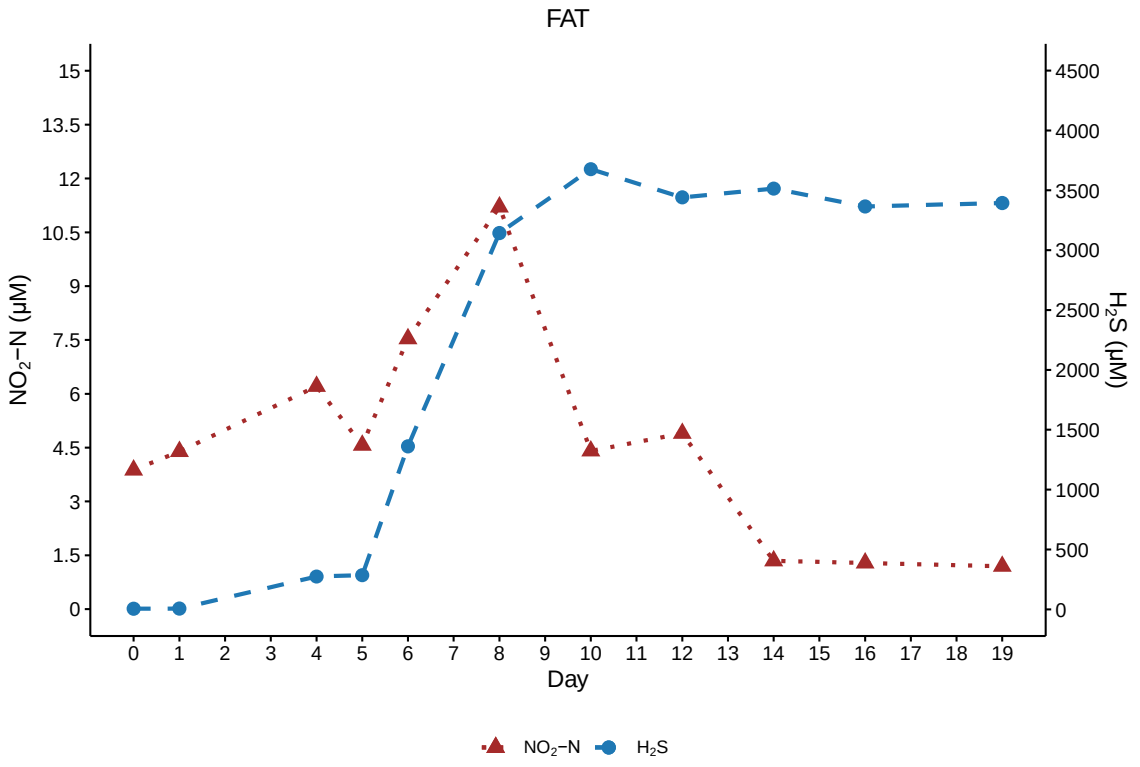

Figure 6.12 NO_2-N and H_2S development in iron (III)-added treatment (FAT)

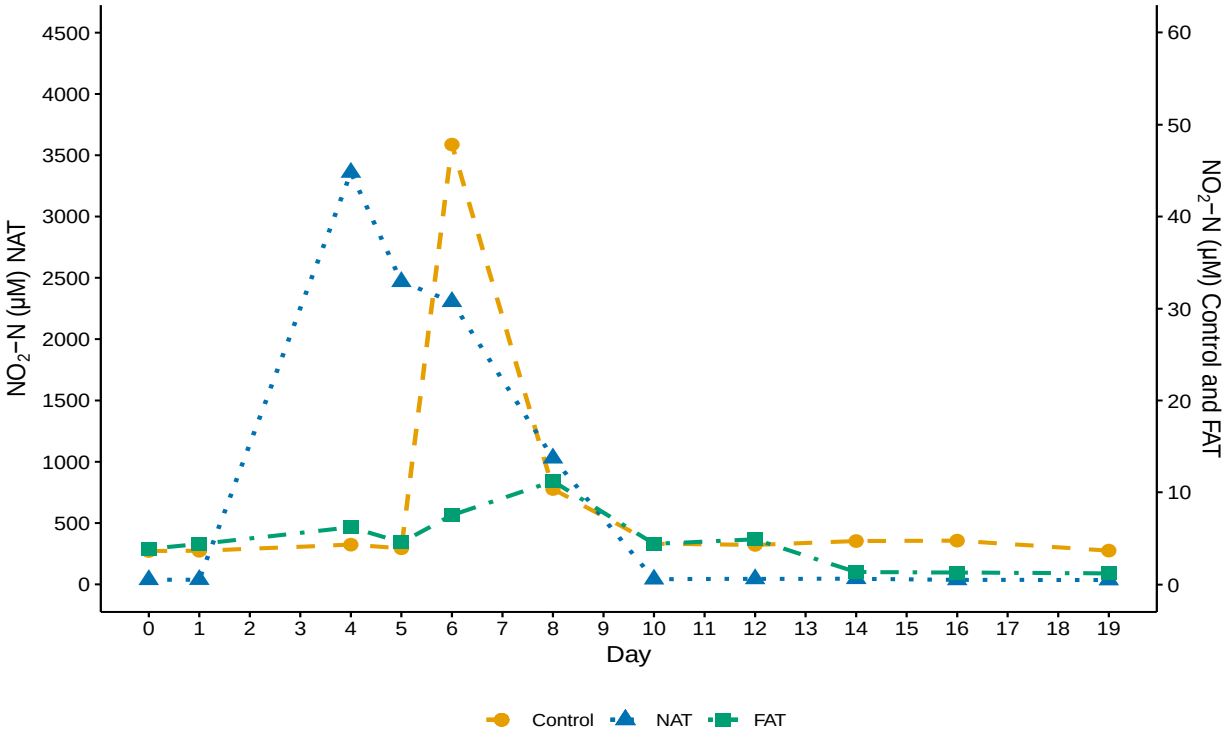

Figure 6.13 NO_2-N development in control, NAT, and FAT

Figure 6.13 compares the nitrite development in control, NAT and FAT. In control and FAT NO_2-N follows similar trend except during the period when H_2S was going up in FAT. The NO_2-N formed in FAT during this time was less than that in control. On day 6 in control amount NO_2-N is 47.8 µM while on the same day in FAT amount of NO_2-N is 7.5 µM which was less than one-fifth of the NO_2-N in control. NO_2 development in NAT behaved quite differently from the other two treatments. In NAT NO_2 formed in large amounts before H_2S development.

6.5.2 Phosphate (PO_4^{3-})

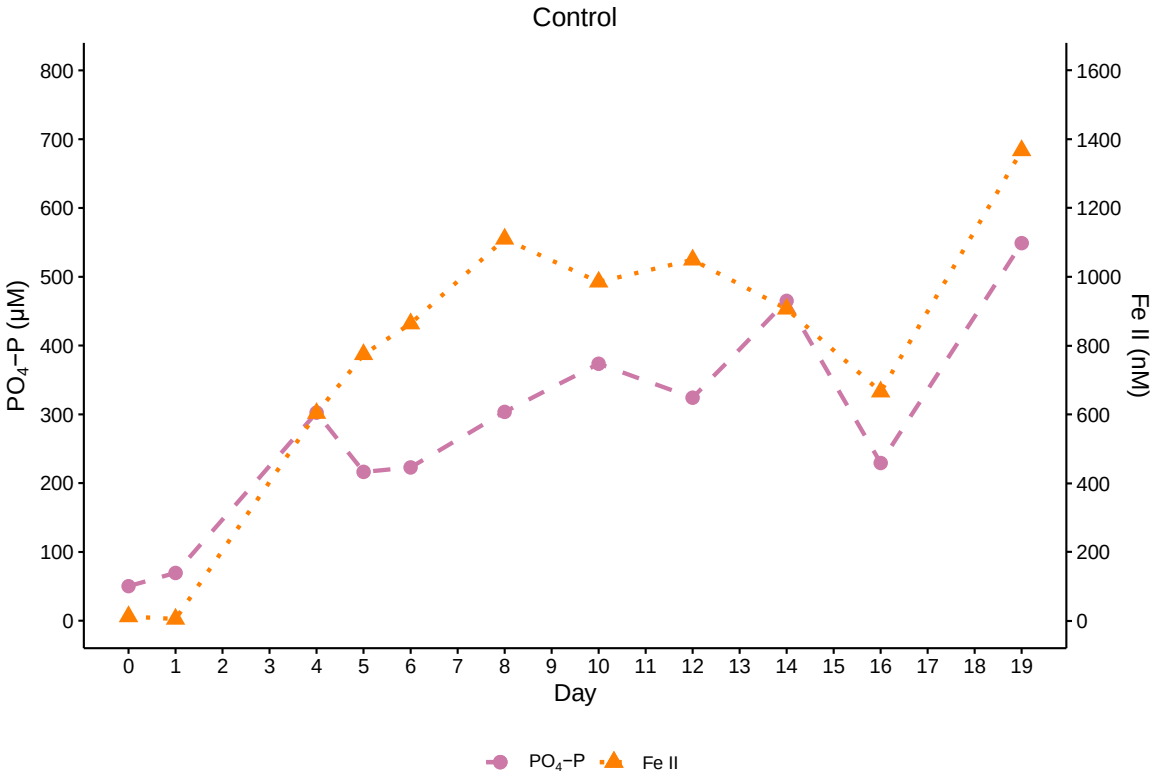

Figure 6.14 Fe (II) and PO_4-P in control

Figure 6.14 shows the amount of PO_4-P and Fe (II) formed in the control. Here both PO_4-P and Fe (II) follows the same trend. When Fe (II) was increasing PO_4-P also increases and when amount of Fe (II) goes down PO_4-P also behaves in same way.

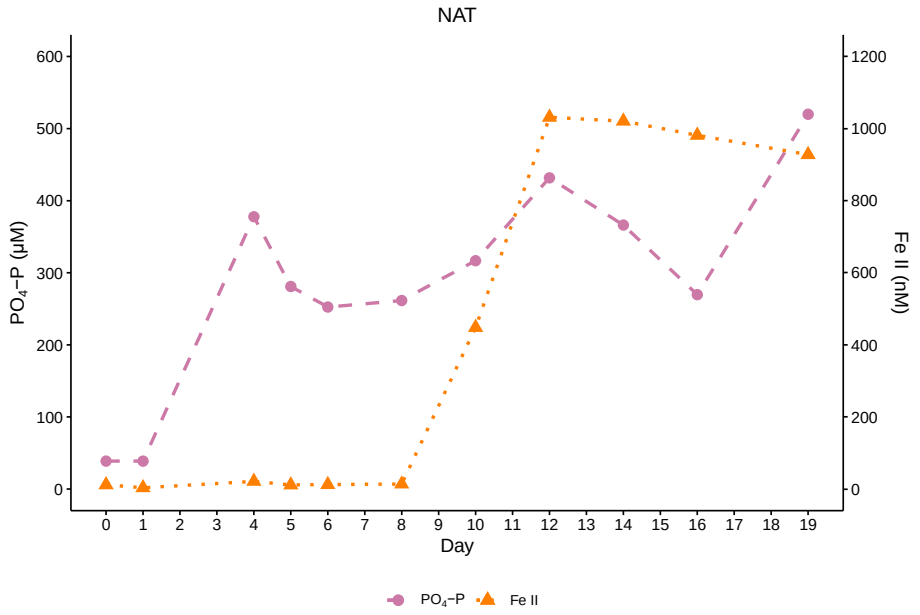

Figure 6.15 Fe (II) and PO_4-P in nitrate-added treatment (NAT)

PO_4-P in nitrate added treatment shows a sudden increase after day one reaching a concentration of 377.7 µM then it started decreasing gradually until day 6. After day 6 PO_4-P started to increase again until day 12. Fe (II) also showed same trend. After day 12 both PO_4-P and Fe (II) decreases gradually but after day 16, PO_4-P shows a sudden increase to 519.85 µM.

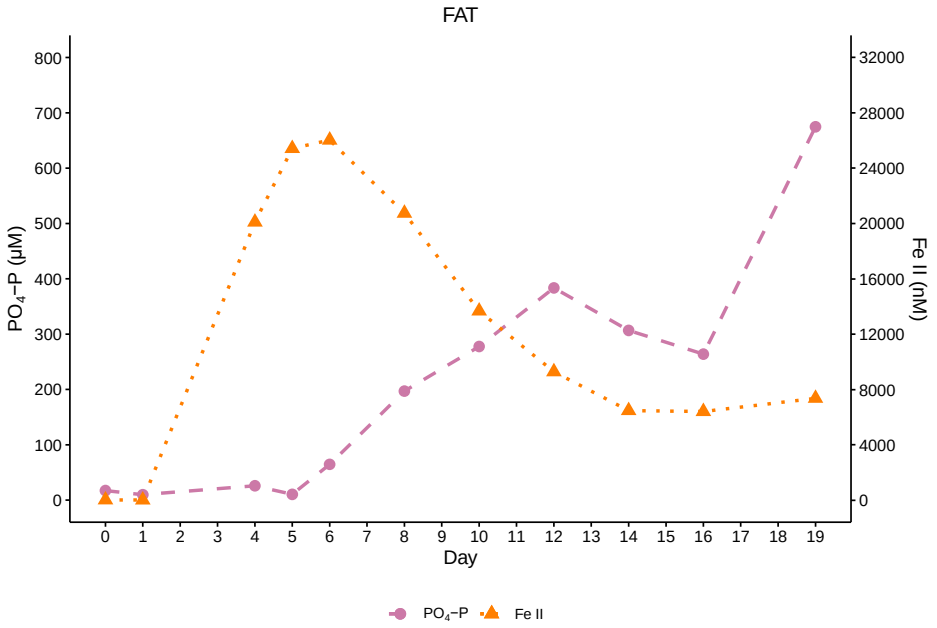

Figure 6.16 Fe (II) and PO_4-P in iron-added treatment (FAT)

Figure 6.16 compares PO_4-P and Fe (II) in FAT. When Fe (II) started to increase the PO_4-P also increases and this increasing trend continued until day 12 even though Fe (II) started to drop after day 6. After day 12 the PO_4-P also started to drop down reaching 263.8 µM on day 16. After day 16 PO_4-P again increases and reached 674.8 µM on day 19.

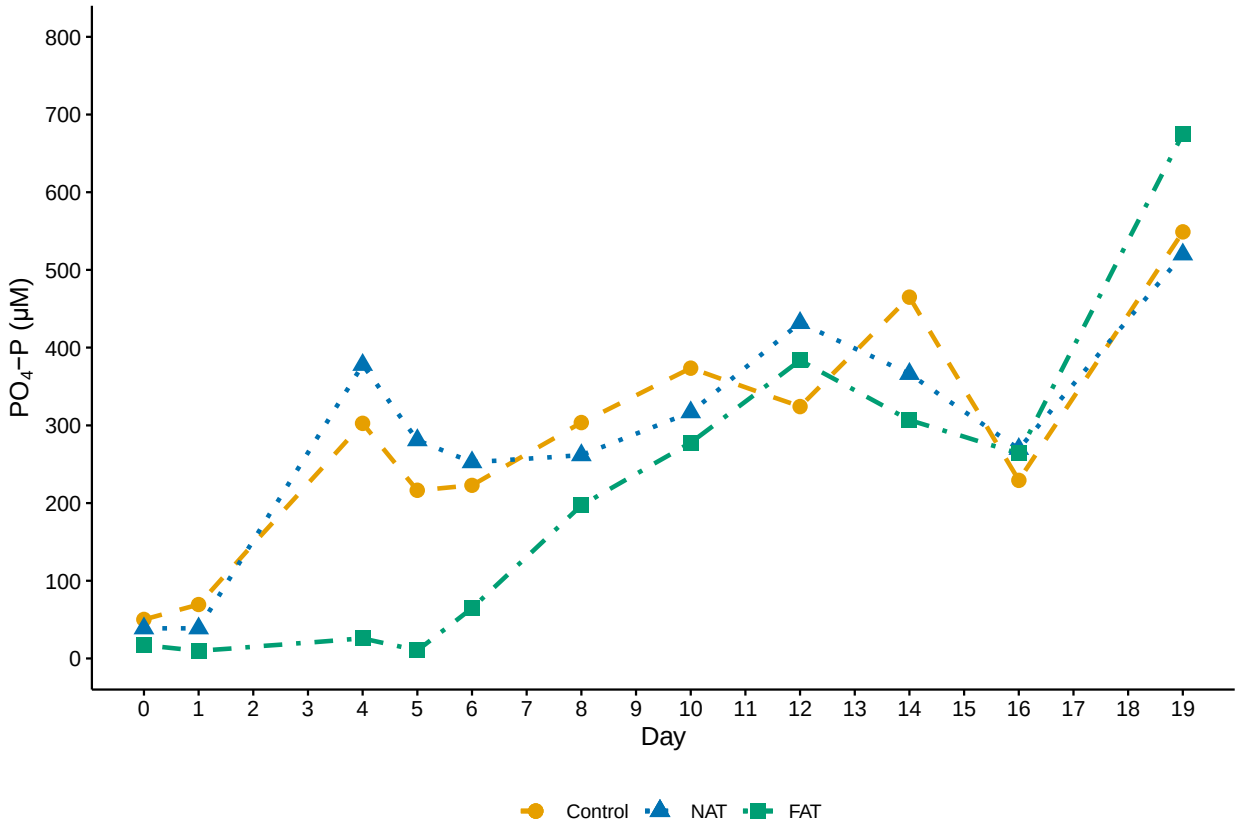

Figure 6.17 PO_4-P in control, NAT, and FAT

The change in PO_4-P in control, NAT and FAT are shown in Fig. 6.17. PO_4-P follows same trend in both control and nitrate-added treatments. A notable difference is seen only after day 10 when PO_4-P in control started to decrease while PO_4-P in NAT started to increase. This different trend continued until day 14. On the other hand, PO_4-P in FAT remained less than that in control and NAT throughout the whole experiment. PO_4-P in FAT reached near levels of control and NAT only on day 8.

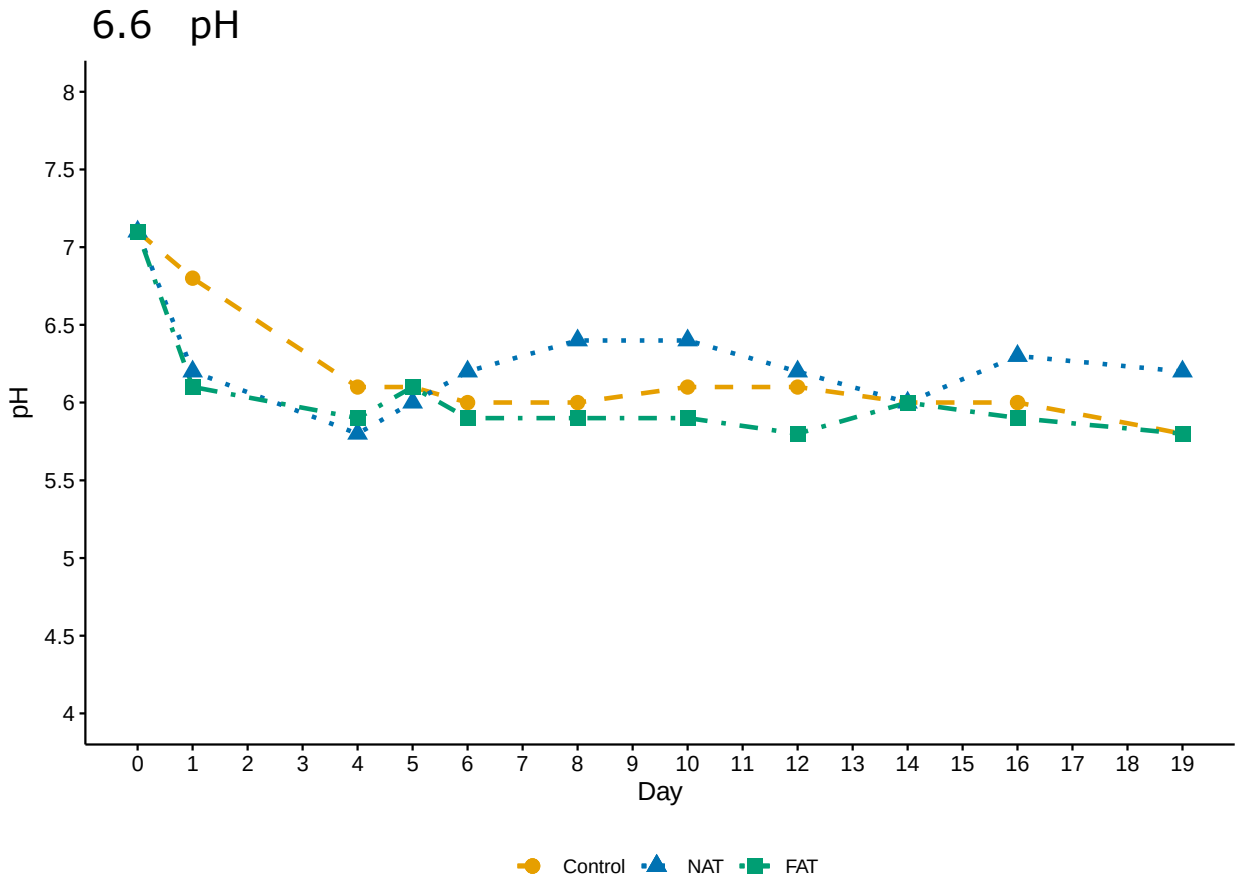

Figure 6.18 pH changes in control, NAT, and FAT throughout experiment

pH is an important parameter that changes during the course of a redox reaction. Maintaining pH level is also essential for well-being of fish in a RAS system. The variation of pH in control, NAT and FAT are shown in figure 6.18. From the figure it is clear that the pH variation was more or less comparable in the three treatments. At start the pH was 7.1 in the three treatments. It started dropping until day 4 reaching a value of 6.1, 5.8 and 5.9 in control, NAT and FAT respectively. After day 4 a small increase in pH was observed in NAT. It rises up to 6.4 on day 10 when pH in the control and FAT were at 6.1 and 5.9 respectively. When comparing the three treatments, the FAT recorded lower pH value during the experiment. At the end of the experiment on day 19 the pH values were 5.8 in control and FAT and 6.2 in NAT.

6.7 Dissolved Oxygen

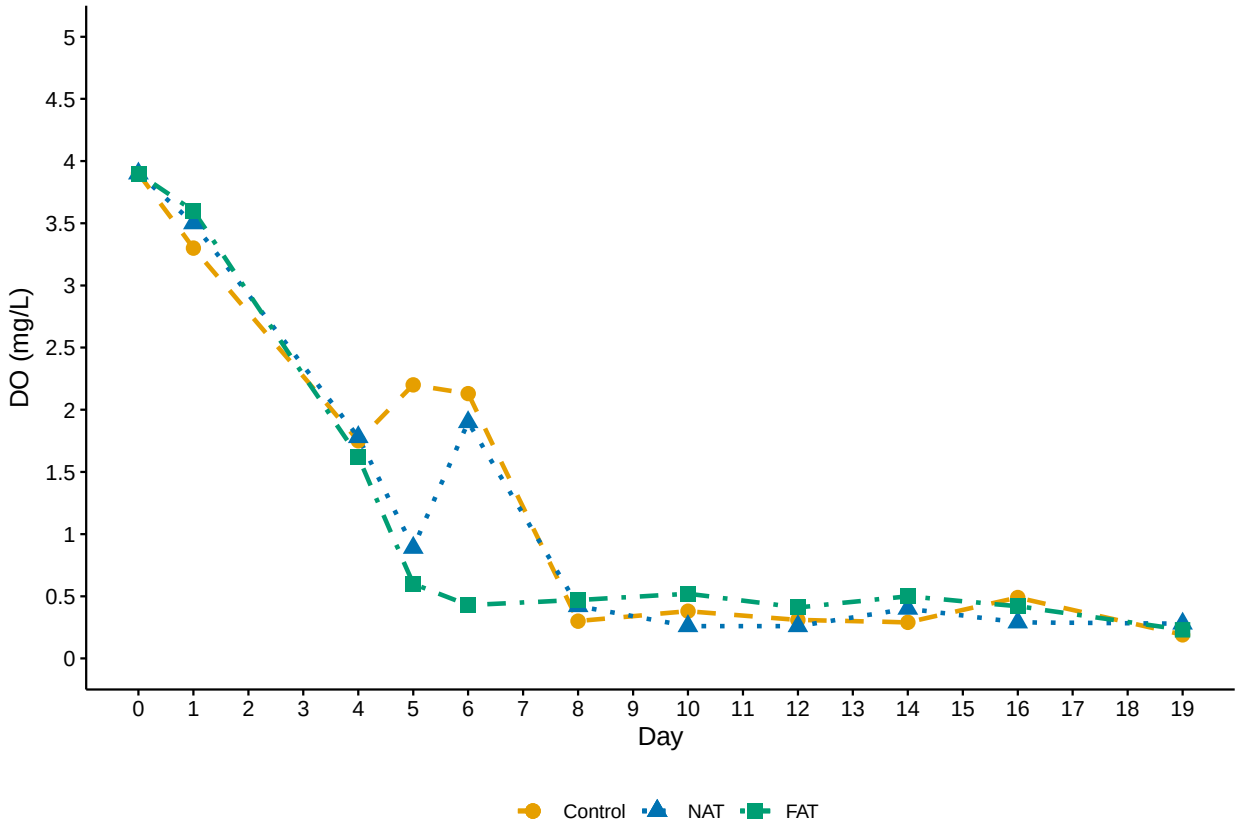

Figure 6.19 Change in the level of Dissolved oxygen in control, NAT and FAT

The amount of oxygen dissolved in the water is a major deciding factor for choosing the oxidants during a redox reaction. As explained in section 2.2 bacteria will utilize lower oxidants only when oxygen is depleted from the system. Here the dissolved oxygen measurement was not carried out in an oxygen minimal environment. It was measured by taking the water sample in a beaker and atmospheric oxygen might have dissolved in the sample. But the general decreasing trend of dissolved oxygen is evident from the results starting from day 1 until the end of experiment on day 19. A steep decrease in DO level was observed in three treatments. On day 6 in NAT and control, the recorded DO shows small increase when compared to previous day, which may be because of the incorporation of atmospheric oxygen during the procedure. By day 8 the dissolved oxygen level reached sub-zero values i.e. 0.3, 0.42 and 0.47 mg/L in control, NAT and FAT.

6.8 Redox Potential (E_h)

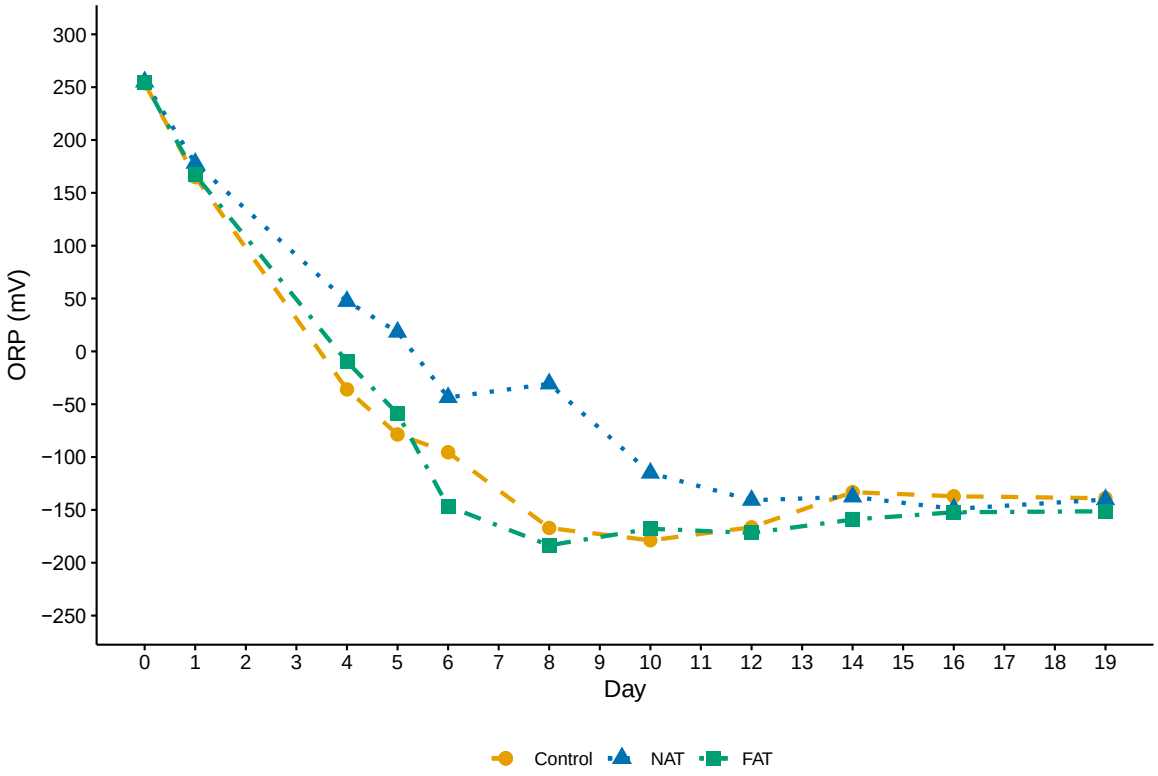

Figure 6.20 Change in Oxidation Reduction Potential in Control, NAT and FAT

Figure 6.20 shows changes in the redox potential (E_h) throughout the experiment in three treatments. Redox potential is correlated with energy released during the redox reaction. If less energy is released during the redox reaction the redox potential is more negative. The value of E_h also says which electron accepter was used in the chemical reaction. In this experiment a general decreasing trend was observed in control as well as in NAT and FAT. In all the three treatment the decrease was steep until day six, then a gradual dropping was observed from day 6 to day 12 and become stabilized towards the end of the experiment.

In control and FAT, the change in the redox potential was more or less similar. In all the three treatments the E_h was 255 ± 2 mV on day 0. Starting from day 2 the NAT showed significant deviations from the other two treatments

and followed a less steep curve. In control and FAT, the E_h decreased continuously from day 0 to day 8 reaching value of -167.1 mV and -183.5 mV respectively in both. From day 8 until the end of experiment, the change in the E_h was very little and it showed slight increase in the value to -138.9 mV and -151.3 mV on day 19 in control and FAT respectively. In NAT the E_h became similar to the other two treatments only from day 12. Until then E_h remained higher than other two treatments.

6.9 Comparing Redox Potential (E_h) and H_2S development in control, NAT and FAT

Figure 6.21 – 6.23 compares redox potential and H_2S development in control, NAT and FAT. In all three conditions H_2S started developing when E_h is dropping down. In control and FAT, E_h and H_2S follows similar trend. While in NAT an increase in H_2S is observed after E_h reached its lowest value which is on day 12.

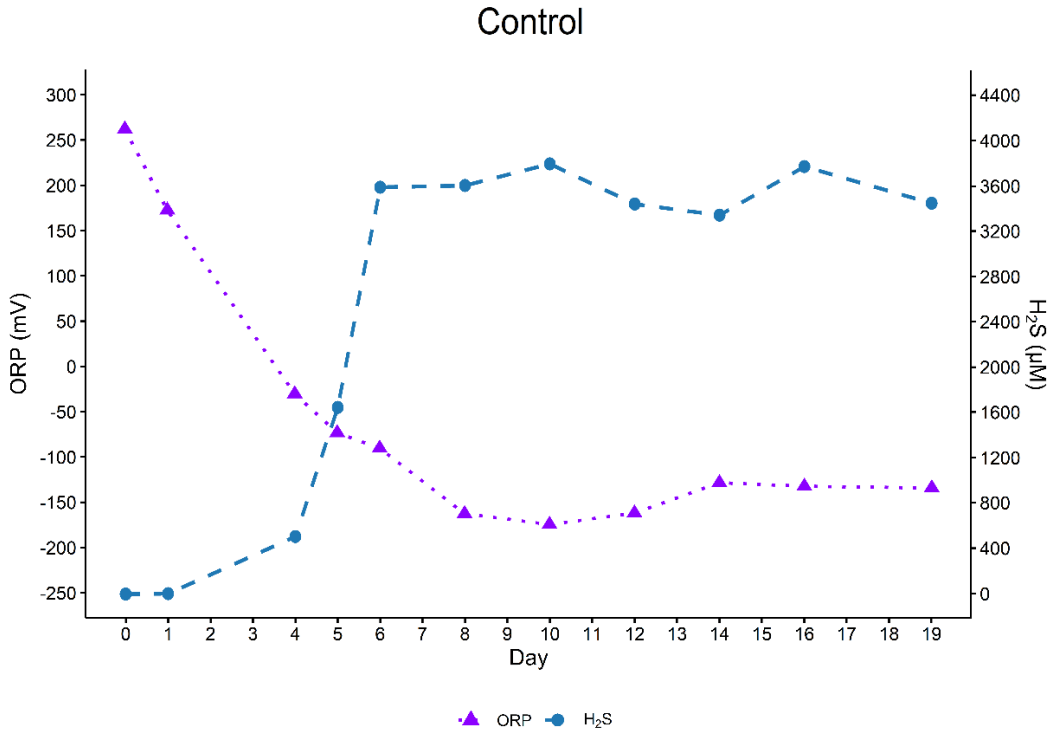

Figure 6.21 ORP and H_2S development in Control

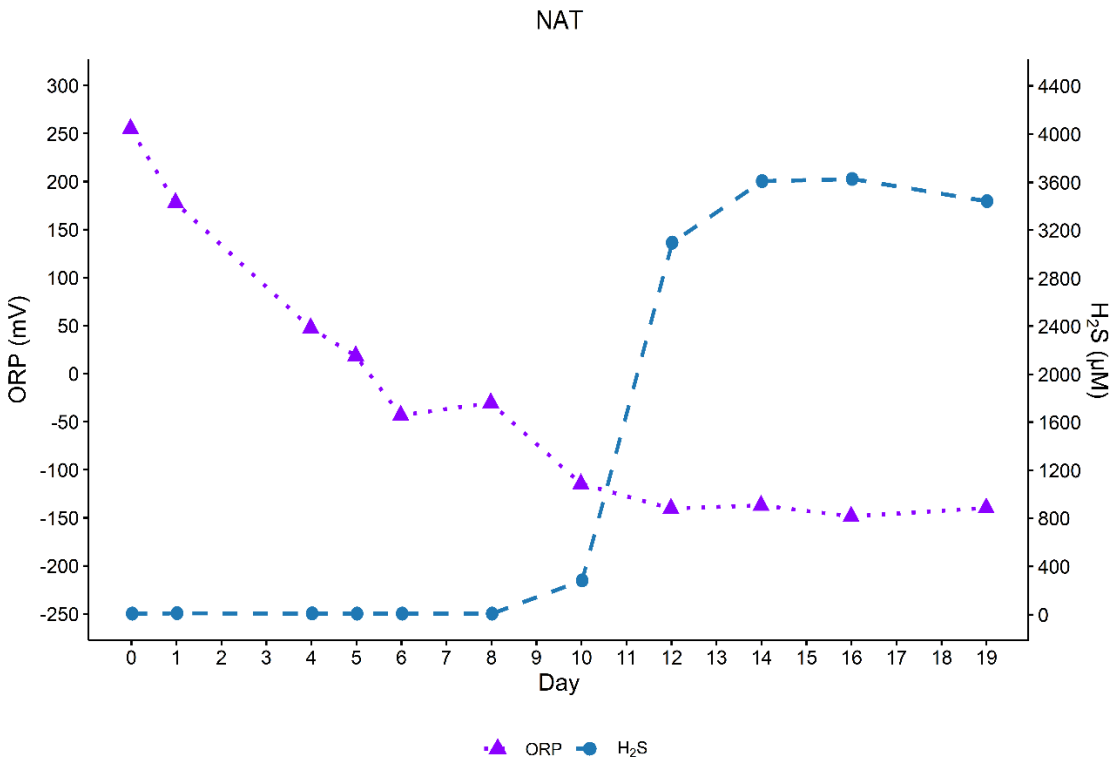

Figure 6.22 ORP and H_2S development in Nitrate-added treatment (NAT)

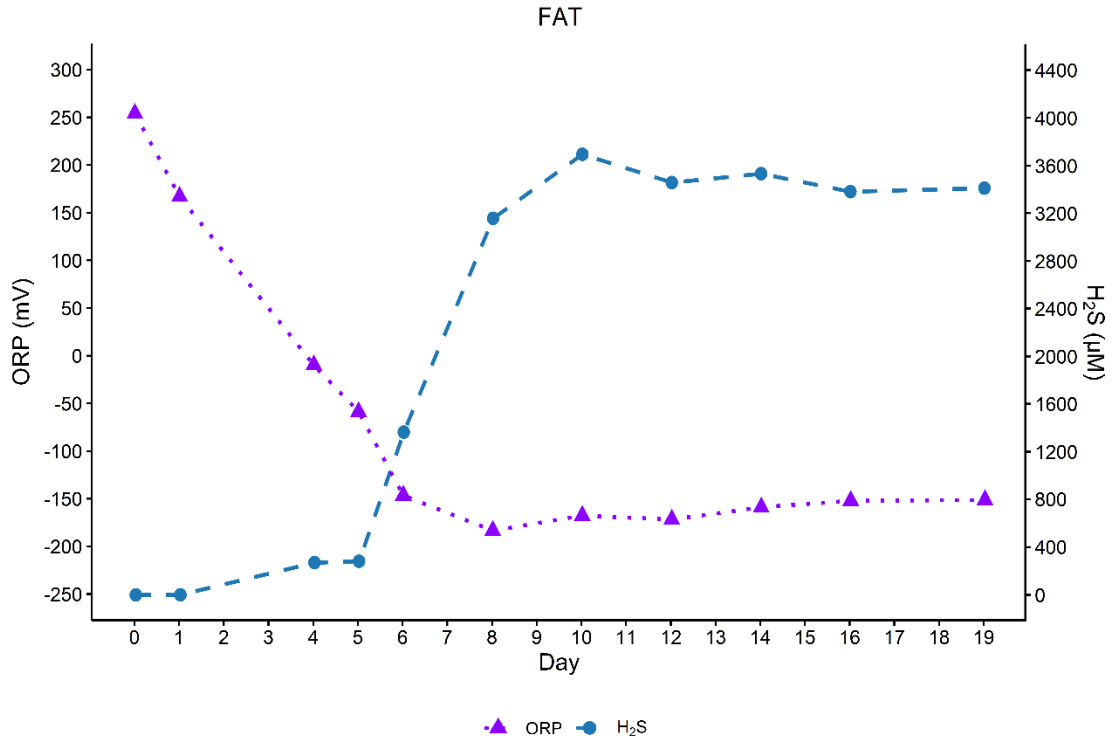

Figure 6.23 ORP and H_2S development in Iron-added treatment (FAT)

7. Discussion

7.1 H_2S Development

Results from the experiment shows addition of nitrate and iron (Fe III) in RAS sludge delayed the H_2S production. In NAT nitrate was added at a concentration of 6 mM, which delayed the H_2S production for about 8 days. The results are comparable to the previous studies that showed nitrate addition delays H_2S development either by inhibiting sulfate (SO_4^{2-}) reduction (Estensen, 2021; Mohanakrishnan et al., 2009; Tanudjaja, 2021). In the NAT, H_2S started developing after day 8. On the other hand, in control H_2S was there starting from the first day of the experiment.

In FAT where Fe (III) was added at a concentration of 0.4 mM, H_2S started forming after day 1 but the H_2S concentration remained lower than that in control. This observation is in line with the results of studies by Lahav et al., (2004) and Poulton et al., (2002) where they showed the potential of iron oxides in controlling the H_2S production in aqueous systems. It is important to note that, Fe (III) was added to the treatment at a concentration less than 7 % of the nitrate addition in NAT. Though Fe (III) was added at a very low concentration, it was able to reduce the concentration of H_2S produced in the system. On day 6 when H_2S concentration was 3.5 mM in control, it was just 1.3 mM in Fe (III) added treatment. This potential of iron oxides in controlling H_2S production is also reported by Sun et al. (2020), where they showed that the manually dosed or naturally occurring Fe (III) control biogenic hydrogen sulfide.

In FAT, there were significant deposition of black substance which is Iron (II) Sulfide (FeS). FeS is formed as a result of reaction between Fe^{2+} formed by microbial iron reduction and the sulfide S^{2-} which is formed by microbial sulfate reduction. This removes biogenic hydrogen sulfide from the system (Sun et al., 2020) which can also be a reason for lower H_2S concentration in FAT. All these points that Fe (III) can be employed to control the H_2S production in

RAS system. Since there is toxicity associated with use of Fe (III) at high concentration it can only be used in combination with existing method like nitrate addition which enhances the effectiveness.

Here in this experiment H_2S started developing in NAT after day 8 and first significant amount of H_2S was recorded on day 10. But in a similar experiment done by Tanudjaja, (2021) and Estensen, (2021) under same conditions, H_2S started forming only after day 15. This shows that the potential of nitrate to suspend the sulfate reduction also depends on the composition of sludge in the system. Sludge composition can vary depending on species, feed inputs, and management practices. So, we may not be able to make a generalization on how long the nitrate can suspend H_2S formation in a RAS system. It varies from system to system.

7.2 Fe (II) Development

When following the development of Fe (II) in three treatments we can see that Fe (II) started developing before H_2S production in all the three treatments. This is in accordance with the theory of redox reaction sequence that states that the reduction of Mn (IV) and Fe (III) precedes the reduction of sulfate (Weiner, 2007). In control and FAT Fe (II) started developing from day 1. In control H_2S reached its peak value even before Fe (II) reached its peak value. While the result from FAT is interesting since H_2S started to increase significantly only after Fe (II) has reached its peak value (Figure 6.3 and 6.5). Fe (II) reached the highest value of 26.02 µM on day 6 and only after 4 days H_2S reached the peak value (3.6 mM). Here addition of iron (III) has influenced the redox reaction in the system which suppressed and delayed the sudden increase in H_2S concentration. Since there is a 4 days gap between Fe (II) development and H_2S formation, looking at increasing Fe(II) level can be used as an early warning sign for H_2S production and preventive measures can be taken in advance.

In NAT, Fe (II) started to develop immediately after all nitrate has been used up (Figure 6.4). This indicates reduction of Fe (III) has started after the reduction of NO_3 which is in line with the observations form other studies (Weiner, 2007). H_2S started to increase along with increase in Fe (II) concentration showing that both redox reactions are occurring parallelly. The nitrate can also oxidize the Fe (II) back to Fe (III) in the presence of organisms that are capable of oxidizing Fe (II) which retains iron in its oxidized form (Weber et al., 2006).

In FAT, after reaching the highest value of 26.02 µM, the Fe (II) concentration started to decrease gradually reaching a concentration of 6.47 µM and stayed stable until the end of the experiment. This gradual decrease in concentration of Ferrous iron may be because of the precipitation reaction between S^{2-} and Fe^{2+} that produce black coloured FeS (Haese, 2000) which is evident from the black deposits found in the bottles in iron treatment. So, addition of iron also controls the H_2S production through precipitation reaction by removing sulfide S^2 from the system (Poulton et al., 2002). There can be also Fe (III) – Fe (II) shuttle occurred in the system in which Fe (III) is reduced to Fe (II) and subsequently it might have oxidized back to Fe (III). This might have also suppressed the H_2S production.

The total Fe (II+III) results from ICP-MS followed same trend as Fe (II) in control, NAT and FAT. Also amount of Fe (II + III) measured in ICP-MS always stayed higher than Fe (II). This validates the correctness of Fe (II) measured with ferrozine method.

7.3 Nutrient Analysis (NO_2-N and PO_4-P)

It is clear from the nutrient analysis results that in control and FAT, NO_2 forms simultaneously with H_2S development. This shows sulfate reduction and nitrate reduction is happening hand-in-hand in these two treatments. This was probably because less amount of nitrate is present in these two bottles and SRB started to thrive. The situation is entirely different in NAT where NO_2

started to develop an reached its peak value much before H_2S started to form. This shows that nitrate reduction and sulfate reduction are mutually exclusive events in a nitrate added system. The addition of nitrate might have suppressed the growth of SRB. This is in line with the study by (Kamarisima et al., 2018). Sulfate reduction started only after all nitrate were used up.

When comparing the NO_2-N three treatments, the peak value of NO_2 formed in FAT is less than that in control. This shows addition of Fe (III) has significantly suppressed nitrate reduction keeping peak value of NO_2-N at 11.2 µM in FAT. At the same time in control NO_2-N went up to 47.8 µM. During nutrient analysis the results of NO_3-N were overestimated thus giving negative values. This was most probably due to over dilution of samples used for nitrate (NO_3-N) analysis.

Studies by Ruttenberg and Sulak, (2011) and Chen et al., (2022) reported DP get adsorbed on to colloids formed by iron (III) oxyhydroxides and Mn (IV) oxides. During sub-oxic and anoxic conditions these get reduced to Fe (II) and Mn (II) and the adsorbed phosphate is released back into the water which is very evident when comparing the PO_4-P in control, NAT and FAT. In control and NAT, PO_4-P is higher than in FAT because when Mn (IV) gets reduced PO_4 adsorbed on its surface get released. At the same time in FAT the released PO_4 might have adsorbed into FeOOH and released again after reduction of Fe (III). That is why there is a delay in PO_4-P release in FAT. So, the phosphate availability depends considerably on these reduction process and it occurs prior to reduction of sulfate.

7.4 Redox Potential (E_h)

The initial E_h values in the experiment is 254.2 mV (average in three treatments) is similar to most of the studies in the redox chemistry. E_h is determined by a number of factors including amount of organic matter and number of electron acceptors. Presence of more organic matter and electron acceptors lowers E_h (Gardiner and James, 2012). This can be a reason for the

initial Eh value in the experiment lower than typical E_h under aerobic condition 300-700 mV (DeLaune and Reddy, 2005). A low E_h value suggests a reduced environment with limited oxygen availability. In this case, the conditions are favorable for sulfate-reducing bacteria to thrive, leading to the production of H2S. It is more evident in nitrate added treatment where H_2S suddenly shoot up from 0.2 mM to 3.0 mM when E_h approached to -140.8 mV. So, dropping of E_h is also an excellent indicator of conditions that produce H_2S.

When comparing the Redox Potential (E_h) and H_2S development it is evident that H_2S started to shoot up after E_h has dropped down in all the three treatments. E_h dropped gradually during the initial days of the experiment followed by a constant E_h towards the end of the experiment. This redox trend is same in three treatments but in nitrate treatment the E_h became stable 4 days later than control and iron added treatments. This is also observed in a similar experiment by Bailey and Beauchamb, (1971) where they observed a decrease in E_h in the first days of the experiment followed by a constant E_h of about -300 mV towards the end the experiment.

8. Conclusions

Addition of nitrate at a concentration of 6 mM to 5% RAS sludge delayed H_2S production by 8 days compared to its controls. At the same time, Fe (III) addition at a concentration of 0.4 mM reduced the H_2S evolution by about 82.6 % for 5 days compared to control. The Fe (III) additions in FAT bottles were 15 times lower than the concentrations of NO_3^- added to NAT bottles. This result shows the potential of Fe (III) in controlling hydrogen sulfide production in RAS. Due to the toxicity associated with Fe it is not the most ideal substitute for NO_3^-, however a combination of Fe (III) and NO_3^- might give an additive effect in controlling H_2S production. As there was a delay of 5 days between Fe (II) production and increase in H_2S concentration in FAT, Fe (II) can be successfully used as an early warning sign for H_2S production in RAS. In addition to this, the nutrients analysis results showed an increase in PO_4-P with Mn (IV) reduction that precedes the SO_4^{2-} reduction.

9. Future Perspectives

From this work it is clear that addition of Fe (III) reduces the H_2S production in RAS sludge even it is added 15 times lower concentration than nitrate. Due to the toxicity associated with iron, it is not practical to increase the concentration too much. So, a combination of electron acceptors like Nitrate + Fe(III) or Nitrate + Mn(IV) or Mn(IV) + Fe(III) at different proportions may be tested to find optimal ratio for controlling H_2S production. In this experiment, in treatment where iron (III) was added, there is significant gap between the production of Fe (II) and increase in H_2S concentration (Figure 6.5). This opens a way for developing sensors for giving an early warning sign of H_2S production in RAS.

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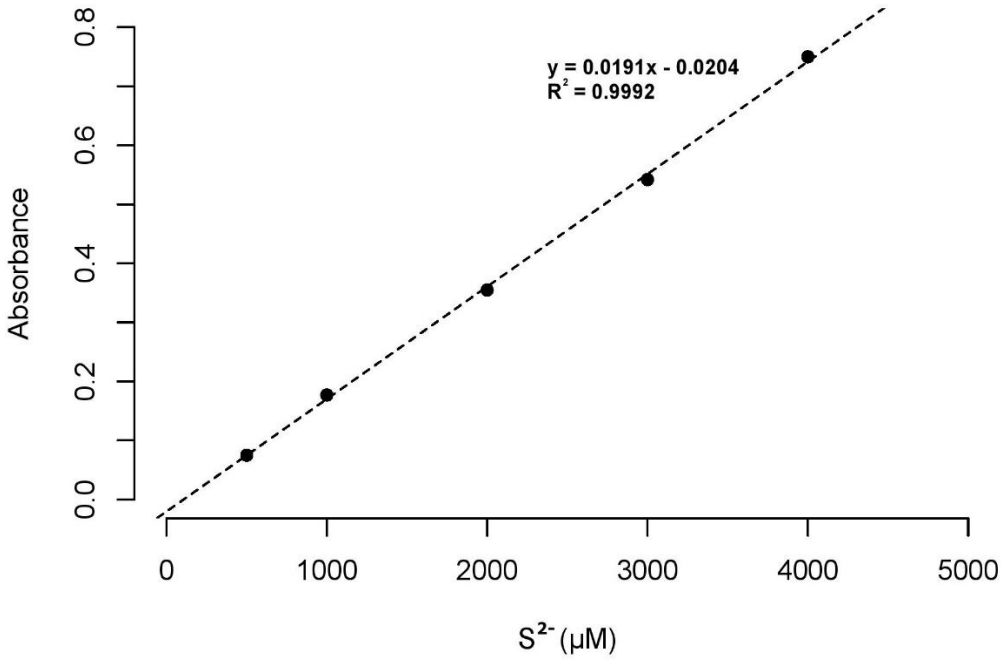
Appendices

Appendix 1 : Sampling Days

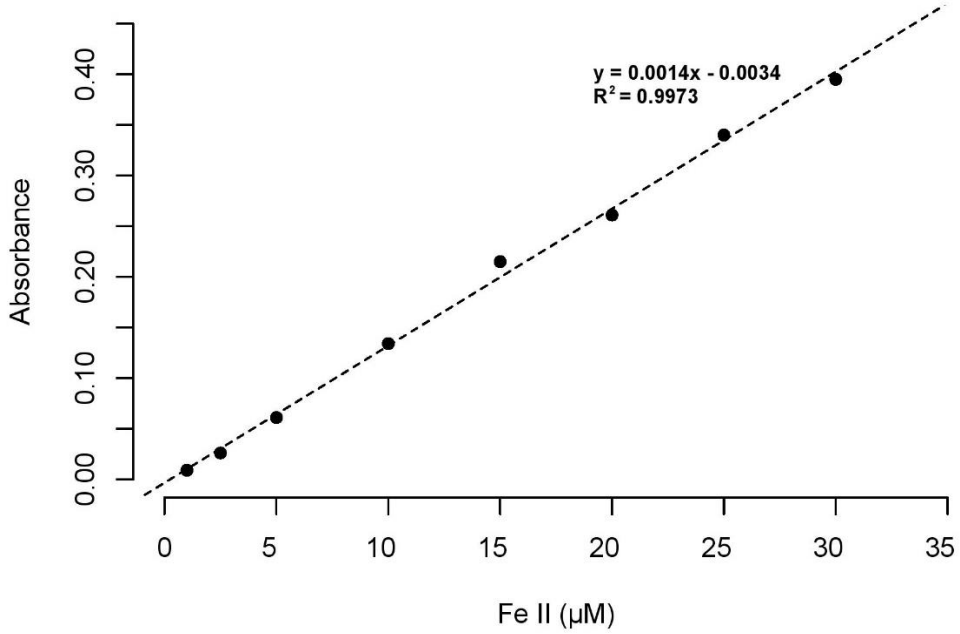
Experiment Day	Date	Sampling Day	Control (C)	NAT	FAT
0	23/03/2023	0	X	X	X
1	24/03/2023	1	X	X	X
2	25/03/2023				
3	26/03/2023				
4	27/03/2023	2	X	X	X
5	28/03/2023	3	X	X	X
6	29/03/2023	4	X	X	X
7	30/03/2023				
8	31/03/2023	5	X	X	X
9	01/04/2023				
10	02/04/2023	6	X	X	X
11	03/04/2023				
12	04/04/2023	7	X	X	X
13	05/04/2023				
14	06/04/2023	8	X	X	X
15	07/04/2023				
16	08/04/2023	9	X	X	X
17	09/04/2023				
18	10/04/2023				
19	11/04/2023	10	X	X	X

Appendix 2 : S^{2-} and Fe (II) Standard curve

S^{2-} Standard curve



Fe (II) Standard curve



Appendix 3 : H_2S Measurements

Treatment	Exp Day	Sampling day	Dilution	Absorbance	Background (MQW + Diamine)	Background (Sample)	Final Absorbance	H_2S (in µM)
C1	0	0	1	0.208	0.164	0.002	0.042	3.27
C2	0	0	1	0.202	0.164	0.002	0.036	2.95
N1	0	0	0	0	0		0	0.00
N2	0	0	1	0.211	0.164	0.002	0.045	3.42
F1	0	0	1	0.213	0.164	0.002	0.047	3.53
F2	0	0	1	0.21	0.164	0.002	0.044	3.37
C1	1	1	1	0.268	0.164	0.003	0.101	6.36
C2	1	1	1	0.253	0.164	0.005	0.084	5.47
N1	1	1	1	0.259	0.164	0.008	0.087	5.62
N2	1	1	1	0.276	0.164	0.002	0.11	6.83
F1	1	1	1	0.236	0.164	0.004	0.068	4.63
F2	1	1	1	0.228	0.164	0.005	0.059	4.16
C1	4	2	10	0.988	0.02	0.019	0.949	507.54
C2	4	2	10	0.985	0.02	0.03	0.935	500.21
N1	4	2	1	0.405	0.164	0.2	0.041	3.21
N2	4	2	1	0.388	0.164	0.171	0.053	3.84
F1	4	2	10	0.275	0.02	0.03	0.225	128.48
F2	4	2	10	0.308	0.02	0.028	0.26	146.81
C1	5	3	100	0.293	0.003	0.005	0.285	1,598.95
C2	5	3	100	0.305	0.003	0.006	0.296	1,656.54
N1	5	3	1	0.454	0.182	0.24	0.032	2.74
N2	5	3	1	0.45	0.182	0.236	0.032	2.74
F1	5	3	10	0.586	0.02	0.045	0.521	283.46
F2	5	3	10	0.582	0.02	0.041	0.521	283.46
C1	6	4	100	0.667	0.002	0.003	0.662	3,572.77
C2	6	4	100	0.658	0.002	0.003	0.653	3,525.65
N1	6	4	1	0.489	0.149	0.308	0.032	2.74
N2	6	4	1	0.478	0.149	0.296	0.033	2.80
F1	6	4	100	0.271	0.002	0.017	0.252	1,426.18
F2	6	4	100	0.245	0.002	0.016	0.227	1,295.29
C1	8	5	100	0.665	0	0.007	0.658	3,551.83
C2	8	5	100	0.669	0	0.006	0.663	3,578.01
N1	8	5	1	0.538	0.149	0.355	0.034	2.85
N2	8	5	1	0.536	0.149	0.354	0.033	2.80
F1	8	5	100	0.621	0	0.024	0.597	3,232.46
F2	8	5	100	0.588	0	0.025	0.563	3,054.45
C1	10	6	100	0.773	0.003	0.011	0.759	4,080.63

C2	10	6	100	0.648	0.003	0.011	0.634	3,426.18
N1	10	6	10	0.594	0.019	0.033	0.542	294.45
N2	10	6	10	0.51	0.019	0.033	0.458	250.47
F1	10	6	100	0.732	0.003	0.017	0.712	3,834.55
F2	10	6	100	0.672	0.003	0.017	0.652	3,520.42
C1	12	7	100	0.639	0.005	0.012	0.622	3,363.35
C2	12	7	100	0.654	0.005	0.012	0.637	3,441.88
N1	12	7	100	0.59	0.005	0.007	0.578	3,132.98
N2	12	7	100	0.545	0.005	0.007	0.533	2,897.38
F1	12	7	100	0.667	0.005	0.014	0.648	3,499.48
F2	12	7	100	0.645	0.005	0.014	0.626	3,384.29
C1	14	8	100	0.615	0.005	0.017	0.593	3,211.52
C2	14	8	100	0.651	0.005	0.017	0.629	3,400.00
N1	14	8	100	0.64	0.005	0.012	0.623	3,368.59
N2	14	8	100	0.697	0.005	0.012	0.68	3,667.02
F1	14	8	100	0.664	0.005	0.013	0.646	3,489.01
F2	14	8	100	0.674	0.005	0.013	0.656	3,541.36
C1	16	9	100	0.726	0.004	0.011	0.711	3,829.32
C2	16	9	100	0.688	0.004	0.011	0.673	3,630.37
N1	16	9	100	0.638	0.004	0.01	0.624	3,373.82
N2	16	9	100	0.699	0.004	0.01	0.685	3,693.19
F1	16	9	100	0.613	0.004	0.012	0.597	3,232.46
F2	16	9	100	0.664	0.004	0.012	0.648	3,499.48
C1	19	10	100	0.625	0.004	0.015	0.606	3,279.58
C2	19	10	100	0.675	0.004	0.015	0.656	3,541.36
N1	19	10	100	0.669	0.004	0.012	0.653	3,525.65
N2	19	10	100	0.603	0.004	0.012	0.587	3,180.10
F1	19	10	100	0.662	0.004	0.013	0.645	3,483.77
F2	19	10	100	0.628	0.004	0.013	0.611	3,305.76

Appendix 4 : Fe (II) Measurements

Treatment	Exp Day	Sampling Day	Dilution	Absorbance	Background (Sample)	Background (MQW+ Ferrozine)	Final Abs	Fe II (µM)
C1	0	0	1	0.045	0.001	0.034	0.01	0.10
C2	0	0	1	0.045	0.001	0.027	0.017	0.15
N1	0	0	1	0.039	0.001	0.027	0.011	0.10
N2	0	0	1	0.043	0.001	0.027	0.015	0.13
F1	0	0	1	0.045	0.002	0.027	0.016	0.14
F2	0	0	0			0	0	0.00
C1	1	1	1	0.032	0.001	0.027	0.004	0.05
C2	1	1	1	0.031	0.001	0.027	0.003	0.05
N1	1	1	1	0.03	0.001	0.027	0.002	0.04
N2	1	1	1	0.029	0.001	0.027	0.001	0.03
F1	1	1	1	0.029	0.001	0.027	0.001	0.03
F2	1	1	1	0.03	0.001	0.027	0.002	0.04
C1	4	2	1	0.824	0.004	0.034	0.786	5.64
C2	4	2	1	0.937	0.007	0.034	0.896	6.42
N1	4	2	1	0.091	0.006	0.034	0.051	0.39
N2	4	2	1	0.045	0.009	0.034	0.002	0.04
F1	4	2	100	0.288	0	0	0.288	208.14
F2	4	2	100	0.268	0	0	0.268	193.86
C1	5	3	10	0.106	0.002	0	0.104	7.67
C2	5	3	10	0.107	0.001	0	0.106	7.81
N1	5	3	1	0.047	0.007	0.027	0.013	0.12
N2	5	3	1	0.047	0.008	0.027	0.012	0.11
F1	5	3	100	0.344	0.002	0	0.342	246.71
F2	5	3	100	0.365	0.002	0	0.363	261.71
C1	6	4	10	0.124	-0.002	0.001	0.125	9.17
C2	6	4	10	0.109	-0.002	0.001	0.11	8.10
N1	6	4	1	0.043	0.004	0.027	0.012	0.11
N2	6	4	1	0.047	0.004	0.027	0.016	0.14
F1	6	4	100	0.358	-0.003	0.004	0.357	257.43
F2	6	4	100	0.366	-0.003	0.004	0.365	263.14
C1	8	5	10	0.149	0.001	0	0.148	10.81
C2	8	5	10	0.157	0.001	0	0.156	11.39
N1	8	5	1	0.021	0.007	0.001	0.013	0.12
N2	8	5	1	0.027	0.007	0.001	0.019	0.16
F1	8	5	100	0.284	0.001	0	0.283	204.57
F2	8	5	100	0.292	0.001	0	0.291	210.29
C1	10	6	10	0.131	0.011	0.001	0.119	8.74

C2	10	6	10	0.162	0.011	0.001	0.15	10.96
N1	10	6	1	0.633	0.012	0.006	0.615	4.42
N2	10	6	1	0.658	0.019	0.006	0.633	4.55
F1	10	6	100	0.185	0.003	0	0.182	132.43
F2	10	6	100	0.197	0.003	0	0.194	141.00
C1	12	7	10	0.132	0.007	0	0.125	9.17
C2	12	7	10	0.169	0.007	0	0.162	11.81
N1	12	7	10	0.15	0.005	0	0.145	10.60
N2	12	7	10	0.142	0.005	0	0.137	10.03
F1	12	7	100	0.123	0.005	0	0.118	86.71
F2	12	7	100	0.14	0.005	0	0.135	98.86
C1	14	8	10	0.139	0.005	0.001	0.133	9.74
C2	14	8	10	0.12	0.005	0.001	0.114	8.39
N1	14	8	10	0.136	0.006	0.001	0.129	9.46
N2	14	8	10	0.157	0.006	0.001	0.15	10.96
F1	14	8	10	0.894	0.006	0.001	0.887	63.60
F2	14	8	10	0.925	0.006	0.001	0.918	65.81
C1	16	9	1	0.915	0.006	0.001	0.908	6.51
C2	16	9	1	0.956	0.006	0.001	0.949	6.80
N1	16	9	10	0.134	0.001	0.001	0.132	9.67
N2	16	9	10	0.138	0.001	0.001	0.136	9.96
F1	16	9	10	0.908	0.001	0.001	0.906	64.96
F2	16	9	10	0.882	0.001	0.001	0.88	63.10
C1	19	10	10	0.148	0.006	0.001	0.141	10.31
C2	19	10	10	0.242	0.006	0.001	0.235	17.03
N1	19	10	10	0.132	0.006	0.001	0.125	9.17
N2	19	10	10	0.135	0.006	0.001	0.128	9.39
F1	19	10	10	0.432	0.006	0.001	0.425	30.60
F2	19	10	100	0.162	0.002	0	0.16	116.71

Appendix 5 : Total Fe ICP-MS

Sample No	Sampling Day	Sample	Date (Exp day)	Fe (II+III) measured (µg/L)	Dilution	Fe (II+III) in sample (µg/L)
1	0	IC1	23/03/23 (0)	1.803	50	90.1448
2	0	IC2	23/03/23 (0)	2.483	50	124.1552
3	1	IC1	24/03/23 (1)	2.288	50	114.3882
4	1	IC2	24/03/23 (1)	1.859	50	92.9345
5	2	IC1	27/03/23 (4)	43.186	50	2159.318
6	2	IC2	27/03/23 (4)	41.493	50	2074.63
7	3	IC1	28/03/23 (5)	59.510	50	2975.484
8	3	IC2	28/03/23 (5)	53.934	50	2696.685
9	4	IC1	29/03/23 (6)	68.800	50	3440.016
10	4	IC2	29/03/23 (6)	56.126	50	2806.294
11	5	IC1	31/03/23 (8)	76.252	50	3812.579
12	5	IC2	31/03/23 (8)	72.207	50	3610.339
13	6	IC1	02/04/23 (10)	61.970	50	3098.502
14	6	IC2	02/04/23 (10)	73.927	50	3696.363
15	7	IC1	04/04/23 (12)	52.596	50	2629.792
16	7	IC2	04/04/23 (12)	80.034	50	4001.692
17	8	IC1	06/04/23 (14)	60.858	50	3042.892
18	8	IC2	06/04/23 (14)	58.714	50	2935.693
19	9	IC2	08/04/23 (16)	54.877	50	2743.856
20	10	IC1	11/04/23 (19)	82.922	50	4146.099
21	10	IC2	11/04/23 (19)	136.882	50	6844.081
22	0	IN1	23/03/23 (0)	1.855	50	92.75
23	0	IN2	23/03/23 (0)	2.094	50	104.6784
24	1	IN1	24/03/23 (1)	2.368	50	118.3783
25	1	IN2	24/03/23 (1)	1.885	50	94.22635
26	2	IN1	27/03/23 (4)	11.037	50	551.8679
27	2	IN2	27/03/23 (4)	9.902	50	495.0758
28	3	IN1	28/03/23 (5)	11.480	50	573.9987
29	3	IN2	28/03/23 (5)	10.403	50	520.1379
30	4	IN1	29/03/23 (6)	14.045	50	702.2482
31	4	IN2	29/03/23 (6)	12.253	50	612.6716
32	5	IN1	31/03/23 (8)	12.779	50	638.9275
33	5	IN2	31/03/23 (8)	13.260	50	662.9853
34	6	IN1	02/04/23 (10)	45.858	50	2292.915
35	7	IN1	04/04/23 (12)	71.554	50	3577.703
36	7	IN2	04/04/23 (12)	67.757	50	3387.855
37	8	IN1	06/04/23 (14)	58.443	50	2922.134
38	8	IN2	06/04/23 (14)	70.353	50	3517.658

39	9	IN1	08/04/23 (16)	66.857	50	3342.846
40	9	IN2	08/04/23 (16)	52.207	50	2610.362
41	10	IN1	11/04/23 (19)	57.367	50	2868.365
42	10	IN2	11/04/23 (19)	53.019	50	2650.953
43	0	IF1	23/03/23 (0)	4.016	50	200.7872
44	0	IF2	23/03/23 (0)	3.777	50	188.8322
45	1	IF1	24/03/23 (1)	3.922	50	196.0961
46	1	IF2	24/03/23 (1)	4.812	50	240.6236
47	2	IF1	27/03/23 (4)	1232.727	50	61636.33
48	2	IF2	27/03/23 (4)	1297.523	50	64876.13
49	3	IF1	28/03/23 (5)	1567.657	50	78382.84
50	3	IF2	28/03/23 (5)	1515.738	50	75786.9
51	4	IF1	29/03/23 (6)	1514.916	50	75745.82
52	4	IF2	29/03/23 (6)	1547.230	50	77361.51
53	5	IF1	31/03/23 (8)	1120.222	50	56011.08
54	5	IF2	31/03/23 (8)	1280.738	50	64036.92
55	6	IF1	02/04/23 (10)	818.562	50	40928.12
56	6	IF2	02/04/23 (10)	746.042	50	37302.08
57	7	IF1	04/04/23 (12)	574.532	50	28726.61
58	7	IF2	04/04/23 (12)	559.335	50	27966.73
59	8	IF1	06/04/23 (14)	390.509	50	19525.45
60	8	IF2	06/04/23 (14)	512.107	50	25605.37
61	9	IF1	08/04/23 (16)	424.669	50	21233.47
62	9	IF2	08/04/23 (16)	370.647	50	18532.34
63	10	IF1	11/04/23 (19)	173.108	50	8655.42
64	10	IF2	11/04/23 (19)	839.867	50	41993.34

Appendix 6 : NO_2-N Analysis Results

Sample No	Day	Date	Name	N-NO_2 (µg/L)	Dilution	N-NO_2 (ug/L)
1	0	23/03/23 (0)	PC1	1.055	50	52.75
2	0	23/03/23 (0)	PC2	0.98	50	49
3	1	24/03/23 (1)	PC1	0.986	50	49.3
4	1	24/03/23 (1)	PC2	1.057	50	52.85
5	2	27/03/23 (4)	PC1	0.989	50	49.45
6	2	27/03/23 (4)	PC2	1.429	50	71.45
7	3	28/03/23 (5)	PC1	1.085	50	54.25
8	3	28/03/23 (5)	PC2	1.112	50	55.6
9	4	29/03/23 (6)	PC1	13.421	50	671.05
10	4	29/03/23 (6)	PC2	13.363	50	668.15
11	5	31/03/23 (8)	PC1	3.005	50	150.25
12	5	31/03/23 (8)	PC2	2.797	50	139.85
13	6	02/04/23 (10)	PC1	1.399	50	69.95
14	6	02/04/23 (10)	PC2	1.076	50	53.8
15	7	04/04/23 (12)	PC1	1.206	50	60.3
16	7	04/04/23 (12)	PC2	1.198	50	59.9
17	8	06/04/23 (14)	PC1	1.304	50	65.2
18	8	06/04/23 (14)	PC2	1.335	50	66.75
19	9	06/04/23 (14)	PC1	1.318	50	65.9
20	9	08/04/23 (16)	PC2	1.346	50	67.3
21	10	11/04/23 (19)	PC1	1.062	50	53.1
22	10	11/04/23 (19)	PC2	0.994	50	49.7
23	0	23/03/23 (0)	PN1	0.988	500	494
24	0	23/03/23 (0)	PN2	1.117	500	558.5
25	1	24/03/23 (1)	PN1	1.092	500	546
26	1	24/03/23 (1)	PN2	1.075	500	537.5
27	2	27/03/23 (4)	PN1	123.512	500	61756
28	2	27/03/23 (4)	PN2	64.585	500	32292.5
29	3	28/03/23 (5)	PN1	52.933	500	26466.5
30	3	28/03/23 (5)	PN2	85.3	500	42650
31	4	29/03/23 (6)	PN1	42.781	500	21390.5
32	4	29/03/23 (6)	PN2	86.356	500	43178
33	5	31/03/23 (8)	PN1	33.208	500	16604
34	5	31/03/23 (8)	PN2	24.439	500	12219.5
35	6	02/04/23 (10)	PN1	1.157	500	578.5
36	6	02/04/23 (10)	PN2	1.213	500	606.5
37	7	04/04/23 (12)	PN1	1.287	500	643.5
38	7	04/04/23 (12)	PN2	1.262	500	631

39	8	06/04/23 (14)	PN1	1.263	500	631.5
40	8	06/04/23 (14)	PN2	1.297	500	648.5
41	9	08/04/23 (16)	PN1	1.076	500	538
42	9	08/04/23 (16)	PN2	1.009	500	504.5
43	10	11/04/23 (19)	PN1	0.953	500	476.5
44	10	11/04/23 (19)	PN2	1.023	500	511.5
45	0	23/03/23 (0)	PF1	1.083	50	54.15
46	0	23/03/23 (0)	PF2	1.088	50	54.4
47	1	24/03/23 (1)	PF1	1.159	50	57.95
48	1	24/03/23 (1)	PF2	1.3	50	65
49	2	27/03/23 (4)	PF1	2.123	50	106.15
50	2	27/03/23 (4)	PF2	1.353	50	67.65
51	3	28/03/23 (5)	PF1	1.186	50	59.3
52	3	28/03/23 (5)	PF2	1.372	50	68.6
53	4	29/03/23 (6)	PF1	1.224	50	61.2
54	4	29/03/23 (6)	PF2	2.995	50	149.75
55	5	31/03/23 (8)	PF1	2.292	50	114.6
56	5	31/03/23 (8)	PF2	3.983	50	199.15
57	6	02/04/23 (10)	PF1	1.245	50	62.25
58	6	02/04/23 (10)	PF2	1.223	50	61.15
59	7	04/04/23 (12)	PF1	1.415	50	70.75
60	7	04/04/23 (12)	PF2	1.328	50	66.4
61	8	06/04/23 (14)	PF1	0.404	50	20.2
62	8	06/04/23 (14)	PF2	0.349	50	17.45
63	9	08/04/23 (16)	PF1	0.392	50	19.6
64	9	08/04/23 (16)	PF2	0.328	50	16.4
65	10	11/04/23 (19)	PF1	0.349	50	17.45
66	10	11/04/23 (19)	PF2	0.319	50	15.95

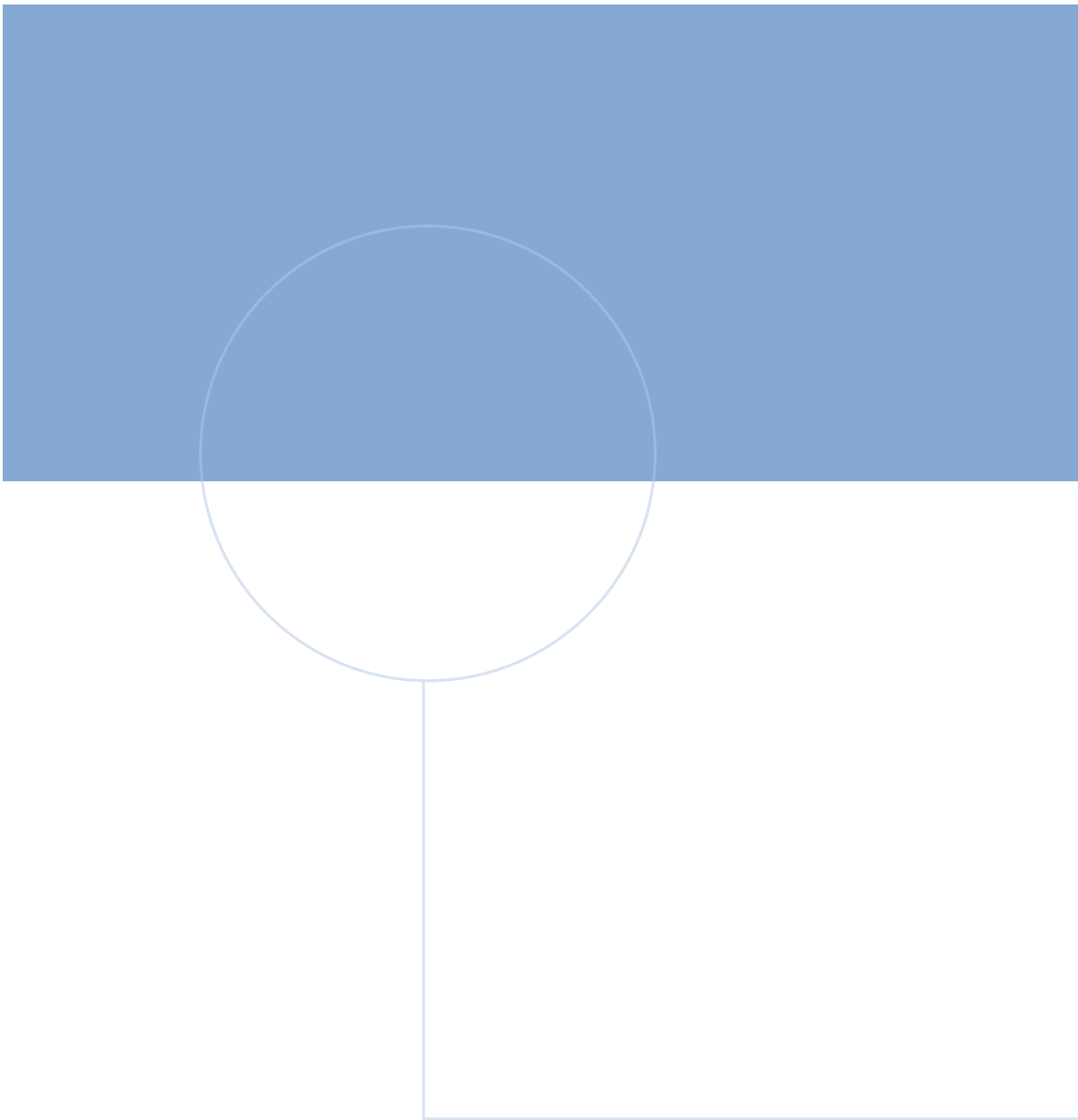
Appendix 7: PO_4-P Analysis Results

Sample No	Day	Date	Name	Dilution	PO_4 (µg/L)	PO_4 (mg/L)
1	0	23/03/23 (0)	PC1	1500	0.892	1.338
2	0	23/03/23 (0)	PC2	1500	1.18	1.77
3	1	24/03/23 (1)	PC1	1500	1.559	2.3385
4	1	24/03/23 (1)	PC2	1500	1.313	1.9695
5	2	27/03/23 (4)	PC1	1500	7.705	11.5575
6	2	27/03/23 (4)	PC2	1500	4.8	7.2
7	3	28/03/23 (5)	PC1	1500	4.977	7.4655
8	3	28/03/23 (5)	PC2	1500	4.084	6.126
9	4	29/03/23 (6)	PC1	1500	4.851	7.2765
10	4	29/03/23 (6)	PC2	1500	4.482	6.723
11	5	31/03/23 (8)	PC1	1500	6.395	9.5925
12	5	31/03/23 (8)	PC2	1500	6.241	9.3615
13	6	02/04/23 (10)	PC1	1500	8.26	12.39
14	6	02/04/23 (10)	PC2	1500	7.168	10.752
15	7	04/04/23 (12)	PC1	1500	8.107	12.1605
16	7	04/04/23 (12)	PC2	1500	5.281	7.9215
17	8	06/04/23 (14)	PC1	1500	10.389	15.5835
18	8	06/04/23 (14)	PC2	1500	8.941	13.4115
19	9	06/04/23 (14)	PC1	1500	4.422	6.633
20	9	08/04/23 (16)	PC2	1500	5.175	7.7625
21	10	11/04/23 (19)	PC1	1500	12.016	18.024
22	10	11/04/23 (19)	PC2	1500	10.673	16.0095
23	0	23/03/23 (0)	PN1	1500	0.815	1.2225
24	0	23/03/23 (0)	PN2	1500	0.903	1.3545
25	1	24/03/23 (1)	PN1	1500	1.161	1.7415
26	1	24/03/23 (1)	PN2	1500	0.565	0.8475
27	2	27/03/23 (4)	PN1	1500	9.494	14.241
28	2	27/03/23 (4)	PN2	1500	6.171	9.2565
29	3	28/03/23 (5)	PN1	1500	6.555	9.8325
30	3	28/03/23 (5)	PN2	1500	5.106	7.659
31	4	29/03/23 (6)	PN1	1500	4.584	6.876
32	4	29/03/23 (6)	PN2	1500	5.843	8.7645
33	5	31/03/23 (8)	PN1	1500	6.291	9.4365
34	5	31/03/23 (8)	PN2	1500	4.545	6.8175
35	6	02/04/23 (10)	PN1	1500	6.795	10.1925
36	6	02/04/23 (10)	PN2	1500	6.298	9.447
37	7	04/04/23 (12)	PN1	1500	9.25	13.875
38	7	04/04/23 (12)	PN2	1500	8.581	12.8715
39	8	06/04/23 (14)	PN1	1500	7.555	11.3325

40	8	06/04/23 (14)	PN2	1500	7.577	11.3655
41	9	08/04/23 (16)	PN1	1500	6.368	9.552
42	9	08/04/23 (16)	PN2	1500	4.771	7.1565
43	10	11/04/23 (19)	PN1	1500	11.749	17.6235
44	10	11/04/23 (19)	PN2	1500	9.797	14.6955
45	0	23/03/23 (0)	PF1	1500	-0.483	-0.7245
46	0	23/03/23 (0)	PF2	1500	-0.225	-0.3375
47	1	24/03/23 (1)	PF1	1500	-0.242	-0.363
48	1	24/03/23 (1)	PF2	1500	-0.229	-0.3435
49	2	27/03/23 (4)	PF1	1500	0.369	0.5535
50	2	27/03/23 (4)	PF2	1500	0.768	1.152
51	3	28/03/23 (5)	PF1	1500	-0.15	-0.225
52	3	28/03/23 (5)	PF2	1500	-0.282	-0.423
53	4	29/03/23 (6)	PF1	1500	1.668	2.502
54	4	29/03/23 (6)	PF2	1500	1	1.5
55	5	31/03/23 (8)	PF1	1500	5.285	7.9275
56	5	31/03/23 (8)	PF2	1500	2.878	4.317
57	6	02/04/23 (10)	PF1	1500	6.763	10.1445
58	6	02/04/23 (10)	PF2	1500	4.706	7.059
59	7	04/04/23 (12)	PF1	1500	9.021	13.5315
60	7	04/04/23 (12)	PF2	1500	6.828	10.242
61	8	06/04/23 (14)	PF1	1500	7.85	11.775
62	8	06/04/23 (14)	PF2	1500	4.883	7.3245
63	9	08/04/23 (16)	PF1	1500	5.538	8.307
64	9	08/04/23 (16)	PF2	1500	5.362	8.043
65	10	11/04/23 (19)	PF1	1500	7.971	11.9565
66	10	11/04/23 (19)	PF2	1500	19.954	29.931

Appendix 8: pH, ORP and DO Measurements

Treatment	Exp. Day	Sampling Day	pH	ORP (mV)	DO (mg/L)
C	0	0	7.1	253.6	3.9
N	0	0	7.1	255	3.9
F	0	0	7.1	254	3.9
C	1	1	6.8	165	3.3
N	1	1	6.2	178	3.5
F	1	1	6.1	167	3.6
C	4	2	6.1	-36	1.75
N	4	2	5.8	47.4	1.78
F	4	2	5.9	-9.8	1.62
C	5	3	6.1	-78.6	2.2
N	5	3	6	18.3	0.89
F	5	3	6.1	-58.9	0.6
C	6	4	6	-95.4	2.13
N	6	4	6.2	-43.6	1.9
F	6	4	5.9	-146.6	0.43
C	8	5	6	-167.1	0.3
N	8	5	6.4	-30.7	0.42
F	8	5	5.9	-183.5	0.47
C	10	6	6.1	-178.8	0.38
N	10	6	6.4	-115.1	0.26
F	10	6	5.9	-167.8	0.52
C	12	7	6.1	-166.4	0.31
N	12	7	6.2	-140.8	0.26
F	12	7	5.8	-171.8	0.41
C	14	8	6	-133.2	0.29
N	14	8	6	-137.6	0.4
F	14	8	6	-159.1	0.5
C	16	9	6	-137.1	0.49
N	16	9	6.3	-149	0.29
F	16	9	5.9	-152.2	0.42
C	19	10	5.8	-138.9	0.19
N	19	10	6.2	-140.2	0.28
F	19	10	5.8	-151.3	0.23



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