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Physio-chemical effects of different CO2 level in recirculating aquaculture systems (RAS) for Atlantic salmon post-smolt.

With focus on heavy metals

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Master of Science	
Submission date:	June 2017
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Acknowledgement

This master thesis is written at the department of chemistry at NTNU, within the study of environmental- and analytical chemistry. The master thesis is a part of the CO2 RAS project within the Ctrl AQUA centre for research-based innovation.

I lived in Sunndalsøra for 4 weeks, where the collection of water samples started in at the 16th November 2016 and continued to the 10th February 2017. The responsibility of the sample collection was shared between me and Kamilla Jansson Grindedal, who is also a master student at NTNU. During my stay at Nofima I got the opportunity to participate in two fish samplings and was given the opportunity to learn new analytical methods through the CO2 RAS project.

I took other courses a long with the master thesis both in autumn 2016 and in spring 2017. This master thesis marks the end of my 5 years of study at NTNU.

I want to thank my supervisor Øyvind Mikkelsen for taking time to answer all of my questions. I also want to thank my two supervisors at Nofima, Vasco Mota and Bendik Fyhn Terjesen for helping me with the thesis and for a nice time at Sunndalsøra. I would like to thank Dorien Dunnebier for reviewing the thesis and I would like to thank Shazia Aslam for helping me with the statistical analysis. Thanks to Kamilla Jansson Grindedal for a good collaboration with the sample collections and tanks to Syvering Lierhagen for the execution of the element analysis with the ICP-MS.

I would like to thank my fellow students for all the nice moments during our 5 years at NTNU.

Gisle Roel Bye

Gisle Roel Bye
Trondheim, 08.06.2017

Abstract

In this master thesis, effects of different carbon dioxide (CO_2) concentrations in a recirculating aquaculture system (RAS) have been studied, with focus in different effects on the different physio-chemical parameters and selected heavy metals. The changes in pH, redox potential, temperature, salinity, conductivity and turbidity have been studied. Other water quality parameters, such as water hardness and alkalinity is also discussed. The changes in the physio-chemical parameters affects heavy metals in water. In this thesis, 8 elements have been selected to be studied. The selected elements are Al, As, Cd, Cr, Cu, Fe, Mn, Ni and Zn. These were chosen because of interesting results, previous research in accumulation of heavy metals in RAS and because of possible toxic effects on the fish.

The sample collection was done in a period of 12 weeks, where 8 different sampling dates were conducted. Water samples was sent to an element analysis by the ICP-MS and analysis of Total organic carbon (TOC) performed by a combustion technique, using a NDIR detector.

The results from the analysis was analysed by an analysis of variance (ANOVA), both by a one-way and a two-way analysis. The purpose of these analysis is to see if there is a statistically significant difference in the obtained values from the element analysis between the different treatments of CO_2 throughout the experiment.

The results shows that iron has a statistical difference between the different CO_2-treatments over time. None of the other elements showed a statistical difference over time. All of the elements had an increase in concentration through the experiment. The elements with the largest increase in concentration over time are Fe, Zn, As and Cd, while the other elements only had a small total increase in concentration.

Today the concentration limit of CO_2 in RAS is debated. This master thesis is a small part of the CO2 RAS project, where the main goal is to determine the effect of dissolved CO_2 (5-40 mg/l) in Atlantic salmon post-smolt (100 – 600g) performance, health and welfare in brackish water recirculating aquaculture systems (RAS).

Sammendrag

I denne masteroppgaven har effekter av ulike karbondioksid (CO_2) konsentrasjoner i et resirkulerende akvakultur system (RAS) blitt undersøkt med fokus på de effektene CO_2 har på ulike fysio-kjemiske parametere og utvalgte tungmetaller. CO_2 i vann påvirker de ulike fysio-kjemiske parameterne, som pH, redokspotensial, temperatur, salinitet, konduktivitet og turbiditet. Andre vannkvalitetsparametere som er blitt undersøkt er alkalinitet og hardhet av vannet. De fysio-kjemiske parameterne har stor innflytelse på tungmetaller i vann. I denne undersøkelsen er 8 grunnstoff blitt plukket ut som viktige elementer å undersøke. De utvalgte grunnstoffene er Al, As, Cd, Cr, Cu, Fe, Mn, Ni og Zn. Disse ble valgt ut fra interessante resultater og tidligere undersøkelser i like systemer som RAS, samt potensiell giftighet.

Prøveinnsamlingen ble gjort i en periode på 12 uker, hvor det ble tatt prøver fra 8 ulike datoer. Vannprøver ble tatt for elementanalyse ved ICP-MS og totalt organisk karbon (TOC) ved bruk av en forbrenningsteknikk og deteksjon ved bruk av en NDIR detektor.

Ut fra disse resultatene ble det gjennomført to ulike variansanalyser (ANOVA), både en en-veis og to-veis analyse. Formålet med disse analysene er å se om det er en statistisk signifikant forskjell i verdiene for elementene og parameterne på grunn av ulike CO_2 behandlinger gjennom hele forsøksperioden.

Resultatene viser at jern har en statistisk signifikant forskjell mellom de ulike CO_2 behandlingene over tid. De andre metallene viste ingen statistisk signifikant forskjell over tid. Det var for alle metallene en økning i konsentrasjon over tid. Metallene med størst økning i konsentrasjon er Fe, Zn, As og Cd, mens de andre elementene har en mindre økning.

I dag er grenseverdien for CO_2 i RAS diskutert. Denne masteroppgaven er en liten del av prosjektet CO2 RAS, hvor formålet er å fastslå effekten av oppløst CO_2 (5-40 mg/l) ved sjekk av helse og velferd hos laksepostsmolt (100-600g) i RAS med brakkvann.

Abbreviations

CCS: Closed Containment System

RAS: Recirculating Aquaculture System

µg/l: microgram per litre

HR-ICP-MS: Høyoppløselig induktivt koblet plasma massespektrometri

ICP-MS: Induktivt koblet plasma massespektrometri

TOC: Total Organic Carbon

Ppb: parts per billion

Ppt: parts per trillion

ms/cm: millisiemens per centimetre

°C: degrees Celsius

mV: millivolt

Rsd: Relative standard deviation

Std: Standard deviation

ANOVA: Analysis of Variance

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Introduction

Salmon production is one of the most important industries in Norway. The production costs of recent years have increased due to higher feeding costs, and the treatment and prevention of sea lice (Nofima, 2015). Researchers at Nofima estimated in 2014 that the total cost of controlling sea lice is NOK 3-4 billion (Nofima, 2015). A second study, performed by SINTEF, Trondheim, confirms that sea lice is one of the most important challenges the salmon industry faces, both in Norway and globally (Liu and vanhauwaer Bjelland, 2014). Salmon aquaculture is under a great pressure to control the spread of sea lice to wild fish and the cost of controlling this could become a considerably limiting factor on both production and profitability (Liu and vanhauwaer Bjelland, 2014).

A growth in salmon production is not only a growth in population of salmon but also a growth of the population of sea lice; the more fish, the higher the reproduction of sea lice (Miljødirektoratet, 2015). An increasing growth in sea lice on farmed fish will also affect the wild population of fish near the net sea cages. The process of this impact starts with attachment of sea lice on the fish. After the sea lice attach to the salmon, they releases their larvae. Since the commercially used net cages are open to the environment, the larvae follows the water stream and eventually reaches wild salmon or other wild fish, like the rainbow trout. When attached to the fish the larvae eats the skin and saliva of the fish (Miljødirektoratet, 2015). According to Miljødirektoratet, (Miljødirektoratet, 2015) six sea lice per fish could decrease the fish growth. In Norway, the population of wild salmon and trout has decreased along the coast of Norway from Rogaland to Nordland. An expected increase in sea temperature, which benefits the sea lice, could also influence the wild fish populations in Troms and Finmark (Miljødirektoratet, 2015).

1.1 Technology design

The industry is trying to find several solutions for the sea lice problem. The use of chemicals and wrasse is now widely used. These methods can hold the population of sea lice down, but since the total production of salmon is so high it is difficult to hold the level of sea lice down. The lice also seem to get resistant to many of the chemicals over time (Miljødirektoratet, 2015).

The industry is looking at other solutions for fish production. One possibility is the use of closed containment systems (CCS). CCS is a widely used term used to describe different production systems with an impermeable barrier to isolate the produced culture to the surrounding ecosystems (Ayer and Tyedmers, 2009). These systems, which can be build both on land and at sea, control the environment in a better way than the commercial fish cages (Thorarensen and Farrell, 2011). Minimized fish escapes, less predator interactions, reduced decease transmission, lower feed inputs, higher stocking densities and improved waste water management are some of the most important potential advantages closed containment systems can offer (Ayer and Tyedmers, 2009). Water quality is controlled and the systems have a flow through system, which controls the input and output of water(Ayer and Tyedmers, 2009), and on land there is a possibility to use a recirculating system. This will be discussed later in the introduction.

The industry has a lot of ongoing research on the subject in hope of designing a good commercial design for closed containment systems both at land and sea. An example of a sea-based system is a construction that looks like an egg. Hauge Aqua, in cooperation with Marin Harvest, stands behind the design of the system. Around 90 % of the "egg" is under sea level. The rest acts as a platform above sea level. Because of the structure, the egg is fully enclosed. Sea lice will not enter the tank and fish will not escape. Water enters at the bottom of the egg and two pumps suck the water from below 20 meters, where there are no sea lice. The water circulates upward where it exits 4 meters below the surface (Aqua, 2017).

Another possibility of producing salmon in seawater is the marine donut, designed by OPD. The "marine donut" is fully enclosed and has an escape proof construction. Here the producers wants to control the environment, keep the salmon free of sea lice and collect the nutritious feed. An advantage the donut has is that it is possible to build both in Norwegian fjords and in more exposed areas (OPD, 2017).

The possibility of building closed containment systems at sea in more exposed area has provided other ideas as well. SINTEF has been working with Salmar, Ocean Farming, on constructing offshore sea cages by using technology from the offshore industry. Standard fish cages follow the movement of the waves. Doing so, the net changes its structure with the current. This is a large reason for fish escape and repair costs. The offshore sea cages are more robust and have the technology to be relatively unaffected by the waves. The system receives clean water with no sea lice from the depths (Sintef, 2016).

When it comes to inland production of fish, there are now several possibilities for commercial production. At Fredrikstad, Norway, a new inland closed containment system is under development. Such a containment system will have the possibilities, if sited properly, of controlling the inlet water from groundwater and seawater, which will give a good possibility to control the water quality and the fish welfare. There will be no problems with sea lice, or fish escape. The water recirculates in a system called recirculating aquaculture systems (RAS) (Aquafarms, 2015).

1.2 Recirculating water system (RAS)

A land-based CCS is dependent on a system that can clean the water and recirculate it through the system. A recirculating water system (RAS) recirculates the water and has the proper means to clean it. By using mechanical and biological filtration, the water quality is assured. Mechanical filtration cleans the water as organic particles are removed from the water. Conditions for nitrification are stable so the biofilter does not clog and the effects on the biofiltration processes is stabilized. The biological filter removes the remaining organic matter after the mechanical filter. The finest particles and dissolved compounds, such as phosphate and nitrogen, will pass through the mechanical filter. Phosphate is not a problem in RAS as it is an inert substance. Nitrogen however, when in free ammonia (NH_3), is toxic and is removed from the water. The biofilter transform ammonia to harmless nitrogen (Bregnballe, 2015).

Indoor fish production using RAS is infinitely expandable with up to 99 % of the water reserved and only 1 % released into the environment, making the impact on the local ecology small. RASs have several advantages when it comes to control of the environment in the system and of the water quality. Temperature, salinity, pH, alkalinity, chemical composition, and oxygen are all monitored and continuously controlled (Timmons, 2013). This is a huge advantage compared to commercial sea cages, where, for instance, the temperature can vary greatly due to seasonal changes.

Using a recirculating system also has its challenges. Accumulation of substances is one of the most important issues related to RAS. For instance, carbon dioxide (CO_2) can accumulate in the system and exceed the recommended concentration (Fivelstad, 2013). The most effective way to avoid accumulation is to change the water regularly. However, one of the reasons for using RAS is to keep a low water discharge to the environment (Bregnballe, 2015). The water needs cleaning, hence having mechanical and biological filter, and means to control the level of carbon dioxide, by using carbon dioxide stripping and measuring the concentration of CO_2 on a daily basis (Bregnballe, 2015).

This master thesis is part of a larger research activity within the CtrlAQUA centre for research-based innovation. The main goal within the CtrlAQUA (SFI) is to develop technological and biological innovations that will make closed systems a reliable and economically viable technology. This master thesis focus on the effects of CO_2 on water quality and heavy metals in land-based closed system with RAS focusing on Atlantic salmon post smolt production.

1.3 Research question and hypothesis

The purpose of this thesis is to look at how changes in physio-chemical parameters, because of different levels of CO_2, affects the water quality over time, how these parameters affect each other and how that affects the concentration, speciation and possible accumulation of some selected heavy metals in the RAS. Statistical tests will be performed to see if there is any statistical difference between the different treatments of CO_2. The effect total organic carbon have on concentrations of the heavy metals will also be discussed.

To test the hypothesis if there is any statistical significant difference in different treatments of CO_2, an analysis of variance (ANOVA) is performed to look statistically differences in the data set. The mean concentration in each replicate tanks will be tested. The hypothesis is written like this:

H_0: the mean of the treatments are equal
H_a: the mean of at least to treatments are unequal

The second aim is to look at the role of TOC. The hypothesis is written like this:

Do low and high concentration of TOC influence the heavy metals in RAS?
H_0 a: There is no effect of low TOC on heavy metals in RAS.
H_0 b: There is no effect of high TOC on heavy metals in RAS.

2. Theory

The theory will focus on the important factors regarding water quality, CO_2 in water, important physio-chemical parameters, accumulation of heavy metals in RAS and the general chemistry of heavy metals. The first part will introduce the experimental design and some theory behind it.

2.1 Experimental design

The research design of the experiment is of upmost importance. The main object of the study is physiological requirements of fish in RAS environment at tank level and the effects of changing water quality (Terjesen et al., 2013). With treatments replicated at tank level, using one RAS provides a proper experimental design (figure 1). A single RAS will have the possibility to provide the same basal water quality to all tanks.

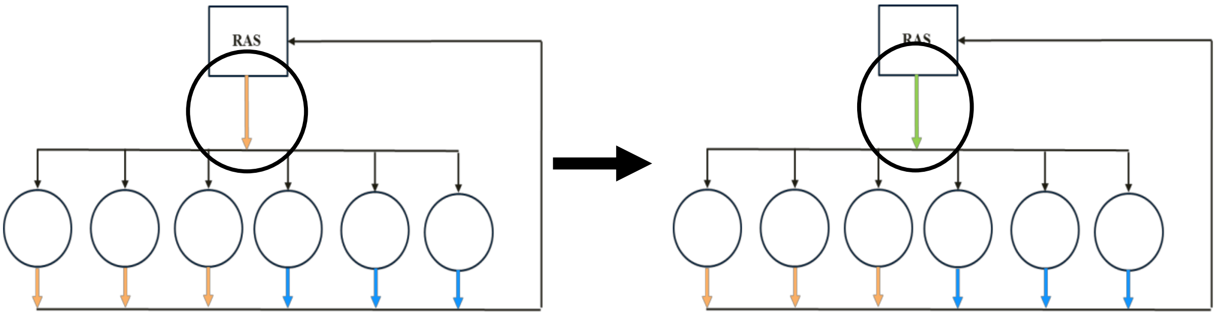


Figure 1: Fish tank level. Recirculation of water and the possibility of accumulation of unknown substances (green) from the control groups (orange) to the treatment groups (blue) (Mota, 2017a).

The design (figure 1) shows that there are several tanks per RAS. The design gives an opportunity to look at what effects the different CO_2 levels have on water quality parameters and heavy metals in the recirculating water. These results can say something about the possible effects the heavy metals have on the fish. If the design were to be different, for instance with one RAS per tank, the focus would have been more on the system and not on the fish (figure 2). Figure 1 will be presented further in the materials and methods chapter.

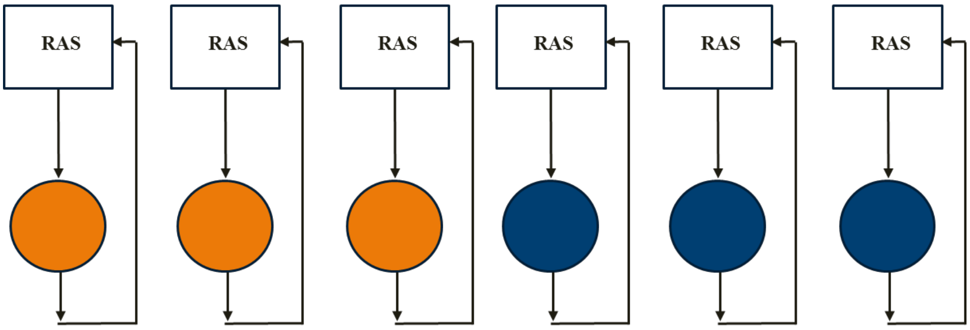


Figure 2: System level. One RAS per tank (Mota, 2017a).

A set up as shown in figure 2 will have some RAS – to – RAS variation (Terjesen et al., 2013). The variability could be higher and it could be difficult to see the exact effects different CO_2 levels will have on the water quality and the fish. In a cost perspective, having several RAS is also more expensive than using one.

In a commercial perspective, using one RAS per tank will in some cases, give a better overall control. It gives a good control in case of spreading diseases by blocking the transfer of disease from one tank to another (Dekhtyarev, 2014).

In this experiment, the tanks are connected to each other through the water, because of the recirculation and water reuse. For this reason, it is interesting to look more into whether substances in the water can accumulate over time. The theory of pseudoreplication is considered as a main factor in this specific experimental design. Pseudoreplication is defined as: *the use of inferential statistics to test for treatment effects with data from experiments where either treatments are not replicated or replicates are not statistically independent* (Hurlbert, 1984). A replicate is more than one experimental unit with the same treatment, in this case 3 replicates per treatment. In this experimental design (figure 1), pseudoreplication is a part of the design, since the tanks are not independent of each other. The question is if the RAS is capable of controlling the water quality and if there is any accumulation of substances over time from one of the treatments, which could have a negative effect on the fish.

2.2 Water quality

Water quality is an important factor to consider in closed containment systems. Environmental monitoring and testing is important to give the produced fish a good environment (Fondriest environmental, 2013b). Monitoring of the environment helps the producers and researchers to predict and learn from the processes that are happening in the water. The results can also assist in new projects and ensure that environmental standards are met. The properties of interest when monitoring the water quality can be physical, chemical or biological. This master thesis will focus on the physical and chemical properties of water. The physical properties that are included are temperature and turbidity, while the chemical properties involve parameters like pH, redox potential, salinity, alkalinity and hardness of the water, which is correlated to the concentration of calcium carbonate ($CaCO_3$) (Fondriest environmental, 2013b).

According to the Norwegian Food Safety Authority (Mattilsynet) and the Directorate of Fisheries (Fiskeridirektoratet), regulations are needed to control water quality in fish hatcheries. Water quality is specified and applies to all fish in land-based aquaculture. In recirculating water, the danger of recirculating infectious diseases is possible. Physio-chemical parameters are therefore necessary to measure and control. (Fiskeridirektoratet, 2003).

For a reuse system, there are two critical criteria for water quality according to Colt (2006). Criteria for the culture species and criteria for the operation of unit processes and unit operations. The second criteria is necessary only for a recirculating system and not for a flow through system (Colt, 2006), and will be of focus in this thesis, with the criteria for culture species in mind. Much of the given information on water quality criteria in past research focus mainly on temperature, dissolved oxygen, total gas pressure, ammonia and nitrite. Further research, according to Colt (2006), is needed on carbon dioxide, heavy metals and other environmental and chemical aspects for reuse systems where substances could accumulate over time.

2.3 CO_2 as a water quality standard

In RAS (recirculating aquaculture system), CO_2 is a crucial parameter to control. In situations with enough water exchange and with no addition of oxygen, oxygen becomes the limiting factor. In a production with addition of oxygen and reduced water consumption, as it is in this project, carbon dioxide (CO_2) is the next critical parameter (VKM, 2008).

When CO_2 reacts with water, it forms carbonic acid (H_2CO_3) (equation1).

$$CO_2\ (aq) + H_2O\ (l) \leftrightarrow H_2CO_3 \tag{1}$$

Because H_2CO_3 is a weak acid, it dissociates to hydrogen ion (H^+) and bicarbonate (HCO_3^-) (equation 2), causing a reduction in pH, due to higher concentration of hydrogen ions (Stumm, 1996).

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^- \tag{2}$$

, where H^+ is written instead of H_3O^+.

An increasing pH can dissociate HCO_3^- to another hydrogen ion and carbonate (CO_3^-) (Nivaldi,J.T, 2011).

$$HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \tag{3}$$

The solubility constant of H_2CO_3 is quite low, however, the constant adding of CO_2 to the tanks will increase the amount of dissolved CO2, which will cause the equilibrium to go left (Fondriest environmental, 2013a).

If the conditions are such that the carbonate is in the form of CO_3^{2-}, the carbonate will form a heavily soluble salt with calcium (S.E.Manahan, 2009). The relation between pH and the carbonate system will be discussed further later in the chapter.

Since plasma PCO_2, which describes the partial pressure of CO_2 in the blood plasma, in the fish increases with increasing levels of PCO_2 in water (Fivelstad, 2013), CO_2 has a direct physiological effect on the fish. High levels of CO_2 do not only affect the fish directly, but also indirectly by changing the pH (equation 2) and other physio-chemical parameters, and by changing the chemistry of heavy metals in the water (Fivelstad, 1998). These changes could affect the fish as a food product. Because of this, standards for CO_2 levels are necessary. According to Norwegian standards (Fivelstad, 2013), the CO_2 concentration in the water should be below 15 mg/l. However, in aquaculture systems, with recirculating water, CO_2 can accumulate, exceeding the recommended concentration. The level CO_2 of for salmon in RAS debated, and some work indicates that even levels as high as 20mg/l do not show any effect on the fish (Fivelstad, 2013).

One study concluded that producing fish in closed systems could reduce the risk of fish contamination from polluted environments (Martins et al., 2011). The trend however, is to reduce water consumption and water discharge in land-based aquaculture. Decreasing the level of water consumption per kg feed will increase the recycling percentage, which is a good thing for the environment around the industry, since the environmental impact will be smaller because of less discharge to the nearby environment. Such trends offer further concern regarding accumulation of substances that could harm the fish and reduce its welfare. One possible hypothesis from this concept is feed-related substance (heavy metals) accumulation, where substances in feed are released into the water (Martins et al., 2010).

2.4 Accumulation of heavy metals in RAS

When it comes to accumulation of substances in a RAS, accumulation of heavy metals is found the be higher in low exchange rate water than in high exchange water (Martins et al., 2011), suggesting an accumulation of heavy metals in RAS when operating at lower water exchange rates. In recirculating systems with low water exchange, studies have shown that there is a potential build-up of heavy metals and metalloids. As, Cu, Ni and Zn were among many metals studied, where the concentration was found to be significantly higher in a low water exchange system than in a high water exchange system (Colt, 2006). In water reuse systems, the source of some metals (Cd, Cu, Zn), can be corrosion of pipes and fitting, or metals added to the feed as part of the vitamin premix (Martins et al., 2009) or from the make-up water (Davidson et al., 2009).

The accumulation of substances depends on the physio-chemical properties of the water. In aquatic environments, metal toxicity and eventually the bioavailability, can be influenced by various physio-chemical factors; such as pH, water hardness and alkalinity (Adhikari, 2006). The pH contributes to control the speciation and solubility of metals, whilst a high alkalinity and water hardness could control the toxicity of many metals, such as Cu (Fivelstad, 1998).

The physiochemical properties of the water affects the heavy metals in water in many ways. In the next chapter, different parameters are enlightened with focus on how CO_2 affects each parameter and how the different parameters affect each other and heavy metals.

2.5 Water quality parameters

As mentioned in the previous paragraph, physio-chemical parameters can affect heavy metals in water. This sub-chapter will look more into the role these and other parameters have on heavy metals. Table 1 shows the different physio-chemical parameters and gives a short explanation of their importance.

Table 1: Different physio-chemical parameters that was measured in the fish tanks, with a short explanation of their importance.

Parameter	**Importance**
pH	Acid-base relationship. Affects the solubility and speciation of heavy metals.
Redox potential (mV)	Redox reactions, reducing and oxidising environment. Affects the solubility and speciation of heavy metals.
Temperature (°C)	Impact on other physio-chemical parameters, mobility of heavy metals and the fish metabolism.
Salinity (ppt)	Measure of major ions in water.
Conductivity (ms/cm)	Measure of waters ability to transfer heat and electricity. Used as a tool for measuring general water quality.
Turbidity (NTU)	Measure of total amount of particles in the water. Gives a general measure on water quality.
Alkalinity	The capacity water has to neutralize an acid. In correlation with water hardness and stabilizes the pH in water.
Water hardness (°dH)	A measure of total [$CaCO_3$]. Gives a general measure on water quality. In correlation with alkalinity. Given in German standards (°dH)

In the next subchapters are presentations of each physio-chemical parameter.

2.5.1 pH and the carbonate system

The pH is not a physical parameter that can be measured as a quantity. It is determined on a defined, logarithmic scale between 0 and 14 that describes the acid-base relationship in water and is described as an intensity factor (Fondriest environmental, 2013a). The value of pH classifies the water: if the $pH < 7$, the water is referred to as an acid solution. A $pH > 7$ is referred to as a basic solution and a $pH = 7$ is referred to as a neutral solution. These values are at 25°C and at 1atm pressure. A change in temperature changes the pH. A decrease in temperature will increase the pH. This means that one can argue that a pH of 7 at 0°C is slightly acidic, because its pH is a bit lower than the neutral value of 7.47 at this temperature. Hence, there is an excess of H^+ ions vs. OH^- ions. (Ayres et al., 1994). The pH is as said logarithmic, meaning a drop in pH of 0, 1 increases the acidity with 30 %.

The pH is dependent on the total amount of hydrogen ions (H^+) in water. The relation between pH and hydrogen ions (H^+) is shown in equation 4, where the pH is explained to be the negative logarithm of the H^+ concentration (Ayres et al., 1994).

$$pH = -\log [H^+] \tag{4}$$

As explained in the chapter about carbon dioxide, higher concentrations of CO_2 results in a higher concentration of hydrogen ions in the water (Equation 1). This gives a decrease in pH in water. When it comes to pH as a master variable, it controls several phenomena in water chemistry, such as the speciation and solubility of metals (Stumm, 1996).

The carbonate system is responsible for determining the pH of most natural waters (Stumm, 1996), as is explained in equations 1, 2 and 3. The carbonate system consists of the dissolved forms of carbon dioxide (CO_2), carbonic acid (H_2CO_3), bicarbonate ion (HCO_3) and the carbonate ion (CO_3^{2-}). The total amount of dissolved CO_2 in water is normally considered to be the same as the [H_2CO_3], since only a small fraction of the CO_2 is hydrated to H_2CO_3. It is also analytically difficult to distinguish between H_2CO_3 and CO2 (Stumm, 1996)

Figure 3 shows the distribution of the carbonate system in different pH. At Nofima the system is not fully closed, however, the impact from the partial pressure of CO_2 from air to water will in this case be small compared to the addition of CO_2 in the water. A closed system will describe the reactions good enough in a simpler way.

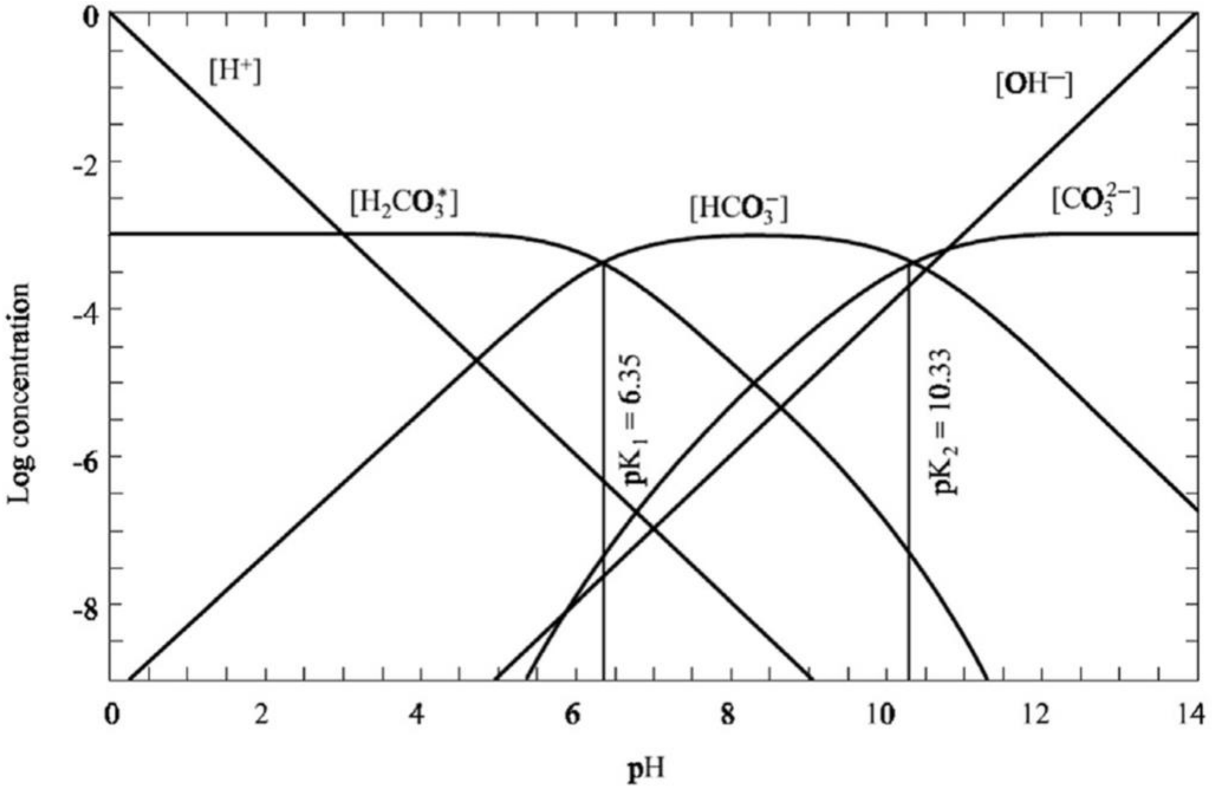


Figure 3: The distribution of CO_2 in a closed system. At low pH CO_2 is in H_2CO_3* (the analytically sum of CO_2 and H_2CO_3). As pH increases CO_2 form HCO_3^- and at even higher pH CO_3^{2-} (Park et al., 2015).

At pH = 6,35, which is the pK_1, is an equilibrium between H_2CO_3/HCO_3^-, where the concentrations of these molecules are equal. A pH > 6,35 will have higher concentration of bicarbonate. When adding carbon dioxide in a system with a pH > 6,35 < 10, the carbon dioxide will be dominate as bicarbonate. Bicarbonate works as a buffer in the water, controlling the pH (Stumm, 1996). In a recirculating system, the pH is quite easy to regulate by addition of bicarbonate (Fivelstad, 2013).

Aquatic animals, such as fish, are generally good at adapting to new environments. Fish can adapt to changes in pH to a long extent. However, large changes could affect the fish in a negative way (Fondriest environmental, 2013a). The pH in aquaculture systems should be between 6,8-7,2 in order to give a good water quality (appendix 1).

2.5.2 Redox potential

Redox reactions are along with acid-base reactions and precipitation reactions, one of the most important chemical reactions in nature. A redox reaction is a reaction that involves transfer of electrons between two species (Nivaldi, 2011). The word redox comes from the words reduction and oxidation, which respectively are the uptake and loss of electrons. In other words this means that the oxidation number is either increasing or reducing for the two atoms/molecules in the two half reactions that forms the redox reaction (Nivaldi, 2011). The resulting oxidation state of the substance is a representation of a hypothetical charge that an atom has if the ion or molecule were to dissociate (Stumm, 1996).

The redox conditions are measured in redox potential (pE), which explains whether the environment is reducing or oxidizing. The potential is an electrical measurement that shows the tendency of a solution to transfer electrons to or from a reference electrode. This measurement can estimate the conditions in the soil. An oxidizing environment gives a high redox potential, higher oxidation states and a low electron activity, whilst a reducing environment will give a low redox potential, lower oxidation states and higher electron activities. A reducing environment is often not favourable because of the formation of toxic substances and low oxygen content (S.E.Manahan, 2009). These conditions decides the form of an element or molecule, affect the mobility, solubility and toxicity of metals (S.E.Manahan, 2009). In rivers and lakes the redox potential values are normally between 300-500mV (S.E.Manahan, 2009).

According to Stumm (Stumm, 1996), the oxidation state is important when it comes to toxicity of metals. For instance, Fe, when in Fe^{2+} (reduced form, low redox potential, soluble) will be more toxic than when in Fe^{3+} (oxidised form, high redox potential, insoluble). The conditions in the water will decide which state the iron has.

The redox potential does not decide the oxidation state alone. The pH is contributing, which can be observed in the figure 4 in a pH-pE diagram. A pH-pE diagram is a simplified model of the reality, which helps with understanding complex reactions in nature (W.Stumm & J.J. Morgan, 1996).

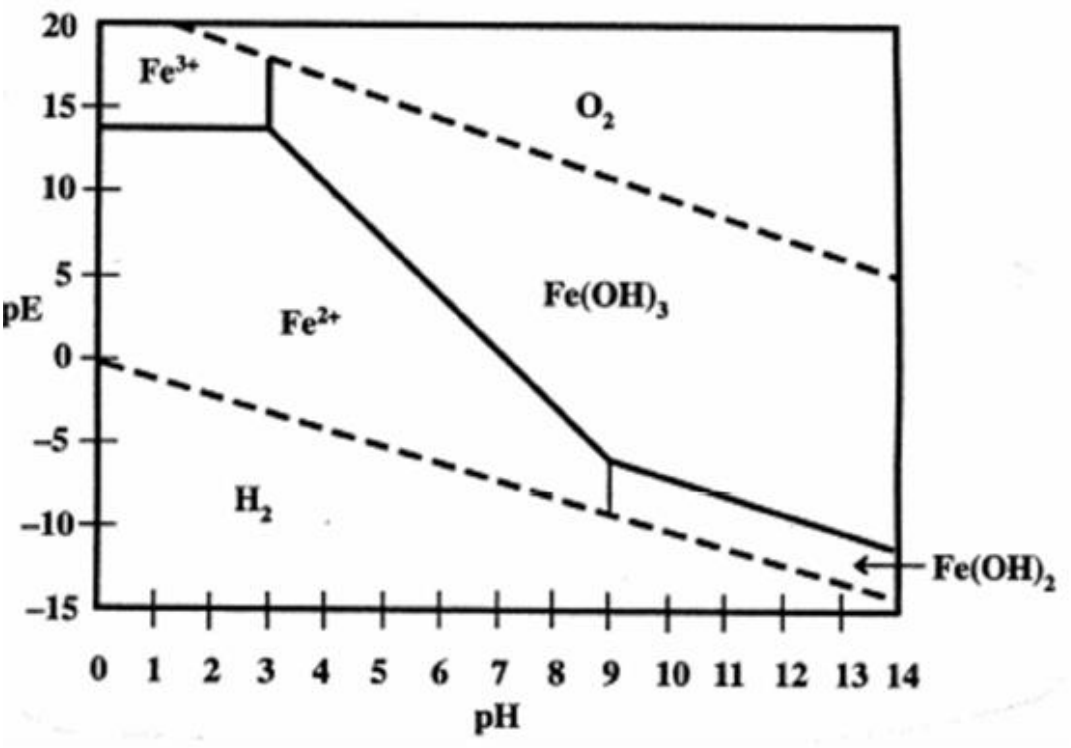


Figure 4: Distribution of Fe in different pH and pE (redox potential) conditions (simplified) (Stumm, 1996).

Figure 4 shows the distribution between different speciation of iron. The different species are dependent on the pH-value and the redox potential in the environenment. The pH-value describes the acid-base relation in the environment, while the redox potential describes wheter the environment is reducing or oxidizing. The degree of reduction and oxidizing is dependent on the atoms/molecules that are in the redox reaction. The pH-pE diagramn helps with explaining the conditions in which an element is reduces or oxidised. Iron is in a reduced form as Fe^{2+}. When the redox potential is very high or the pH is increased, iron will be oxidized and be in an oxidised form (Fe^{3+}, $Fe(OH)_3$). The dotted lines describe what is natural water and when water is either reduced or oxidized. In all natural water the relation between redox potential and pH is inside the two dotted lines.

2.5.3 Temperature

Temperature is a physical property and is used to express the average thermal energy of a substance, described as the kinetic energy of atoms and molecules (Fondriest environmental, 2014b). This kind of energy is transferable to the surroundings. For water, heat transfer is mainly from light, adjacent water sources and thermal pollution (Fondriest environmental, 2014b).

Temperature affects the water in many ways. An increase in temperature increases the rate of chemical reactions and decreasing the solubility of gases in water, such as O_2 and CO_2 (Chapman, 1996). Since the metabolism of fish is affected by the surrounding temperatures the temperature of the water in CCS is very important (VKM, 2008). A decrease or increase in temperature affects the fish metabolism. An increase in temperature over the recommended limit, could affect the fish, causing thermal stress (VKM, 2008). The temperature in aquaculture systems is to be set between 12-13 °C (Mota, 2017b). An increase in temperature increases the oxygen consumption, which leads to more decomposition of organic matter and a higher turbidity (Chapman, 1996). Higher oxygen consumption can lead to higher entry of dissolved substances in the fish, because the fish has a higher metabolic rate. This could lead to higher concentrations of heavy metals entering the gills of the fish. A rise in temperature could increase the concentration of Cu, Cd and Zn in water bodies (Khan et al., 2006). These metals will be discussed later in the theory.

2.5.4 Salinity and conductivity

Salinity is a measure of all salts dissolved in water. As salts dissolve in water, they form ionic particles, each with a positive or a negative charge, giving salinity an effect on the conductivity. The conductivity is the ability water has to transmit heat and electricity (Lenntech, 2017c). As the salinity increases, there are more ions in the water, and there is an increase in conductivity.

Several salts in water contribute to the salinity, the major ones are chloride, sulphate, sodium, magnesium, bicarbonate, bromide and calcium (Fondriest environmental, 2014a). Most of these ions are present in both seawater and freshwater. In freshwater however the concentrations of many ions are much lower, especially sodium and chloride. Freshwater sources have higher concentrations of bicarbonate, alkali and alkaline metals (Fondriest environmental, 2014a). In rivers and lakes the average salinity is about 0,5 ppt or less (EPA). When it comes to groundwater, there could be some differences depending on the soil, which could have high concentrations of salts (Water, 2015). In RAS the use of brackish water is increased, and the salinity in aquaculture systems is to be between 11,5-12,5 ppt (Mota, 2017b).

Salinity contributes to the uptake and accumulation of metals in fish. Uptake of copper seems to be lower in seawater adapted fish than in freshwater fish (Witeska, 2006). Other parameters affects the salinity and conductivity. As the temperature increases, the water viscosity decreases, making the ions more mobile, which then increases the ability the ions have to transmit heat and electricity, giving an increase in conductivity (Fondriest environmental, 2014a). A sudden change in conductivity often indicates some sort of pollution (Fondriest environmental, 2014a). An example could be increased concentrations of nitrate, phosphate and chloride, which could be because of local release of sewage in the make-up water. Conductivity measurements give an indication of the water quality and monitoring of conductivity could help detect pollution over time. (Fondriest environmental, 2014a).

2.5.5 Alkalinity and Water hardness

The alkalinity is the ability water has to buffer or neutralize an acid (Stumm, 1996). Alkalinity in water works as a buffer, in such a way that high inputs of acids do not change the pH in a high scale. When CO_2 is added to water, the pH is reduced (Stumm, 1996). A high change in pH can also affect the alkalinity, as changes in pH also affects the buffer capacity. In water with high amounts of carbonate rich soils, the alkalinity is naturally higher (Fondriest environmental, 2013a). In fish tanks, respiration from the fish can reduce the pH in the water. These effects are often so small that the alkalinity can control the pH (VKM, 2008).

Alkalinity is especially dependent on water hardness, which is a measure of how much magnesium and calcium it is in the water, since the alkalinity in a high degree depends on the total concentration of calcium. A high concentration of calcium increases the buffering capacity, or the alkalinity, of the water (Oram, 2017b). The hardness of the water is directly influenced by the concentration of calcium and other elements, especially magnesium. The main source is usually carbonate rocks, which consists mostly of $CaCO_3$. The amount of $CaCO_3$ is often recognised to be equal to the alkalinity when $CaCO_3$ accounts for most of the alkalinity (WHO, 2011c). In the experiment described in this thesis, there is a high amount of bicarbonate added to the system and this effect could be minor. Still, the water hardness will contribute to some extent the alkalinity of the system. With the introduction of fresh water in the water system, the concentration of calcium and magnesium are often higher than in seawater. This gives an increase in the alkalinity and water hardness.

A decrease in water hardness often increases the toxicity of some metals. Copper toxicity is dependent on water hardness and alkalinity where a decrease in water hardness and alkalinity increases the toxicity of copper. (Miller and Mackay, 1980).

Hardness is calculated by the total permanent water hardness that is equivalent to the concentration of $CaCO_3$, with the formula (Lenntech, 2017d).

$$[CaCO_3] = 2{,}5[Ca^{2+}] + 4{,}1[Mg^{2+}] \tag{5}$$

Using an online calculator at SWM.com calculates the concentration of $CaCO_3$ to a German water hardness scale (°dH).

Table 2: Characterisation of water hardness in German standards (°dH) (SWM, 2017).

Water hardness (°dH)	**Indication**
0 to 8,4	Soft
8,4 to 14	Medium
> 14	Hard

2.5.6 Turbidity

Turbidity is the measure of water clarity, the optical determination of particles in the water. The optical measurement is based on the amount of light scattered from the particles in the water. The more particles in the water the higher scatter of light and turbidity. Turbidity is a tool that can be used to see differences of total amount of particles in water over time (Fondriest environmental, 2014a). Turbidity is measured in NTU, where a turbidity < 5 NTU appears clear, while a reading of 55 NTU will look cloudy. A turbidity > 500 NTU appears as dark (Fondriest environmental, 2014a). Turbidity is a measurement like conductivity used as a tool to explain the water quality.

A change in pH gives a change in solubility of many metals and ions, for instance phosphor and other nutrients, which can give an enhanced growth in organic materials, plant growth and higher turbidity measurements (Fondriest environmental, 2013a). If there is a high amount of organic matter, this is often equivalent to a reducing environment (S.E.Manahan, 2009).

2.5.7 Total organic carbon (TOC)

Total organic carbon (TOC) is the amount of organically bound carbon in water. When analysed, the inorganic carbon is removed leaving only the organically bound carbon. TOC is a measurement that helps with understanding the purity of water. No matter how pure water is, there will be some amounts of carbon materials in the water (Tekmar, 2014b). High amounts of organic carbon can indicate presence of metals bound to organic substances (GeoNorge, 2017).

2.6 Heavy metals

The term heavy metals is used for metals and metalloids with a relatively high atomic mass (>5 g/cm^{-3}) in soils and that could cause toxic effects by exposure (Alloway, 2010). Heavy metals are distributed in the earth crust and are of both natural and anthropogenic sources (Alloway, 2010). Rainwater dissolves rocks, ores, and transport materials, including heavy metals, to rivers, oceans and groundwater. In nature, heavy metals form cations in biological systems by losing one or more electrons. When concentrated by humans or chemical form is altered, heavy metals can be toxic (Klaassen, 2013).

Heavy metals do not only cause toxic effects, but are also essential in several biological processes. Iron for example is very important for the uptake of oxygen in the blood cells, and is described as an essential metal. Deficiency of iron leads to anaemia, which reduces the uptake of oxygen. On the other hand, too much iron could lead to an iron overload. Neither of the options is good for the health of both humans and animals (Klaassen, 2013).

Figure 5 shows the importance of concentration, in this case potential, of metals in biological systems. As we can see from the figure, the example of iron explains this very well. Low concentrations of iron lead to anaemia and too much iron leads to an overload of iron in the system. Either of the options is not good the quality of health. At what concentration that gives a good potential is different for each essential metal.

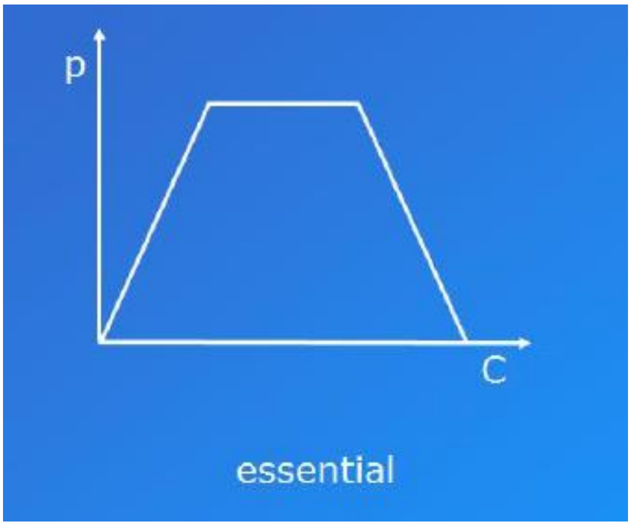


Figure 5: Quality of health given in potential (p) and the concentration of the essential metal (c) as a dose-response diagram (Klaassen, 2013).

For other heavy metals, metals that are not essential for biological systems, a different figure could describe this process in a similar way. Figure 6 shows that at low concentrations the heavy metals gives a good quality of health, while at higher concentration the heavy metal starts to affect the biological system in a negative way. This threshold is different for each element.

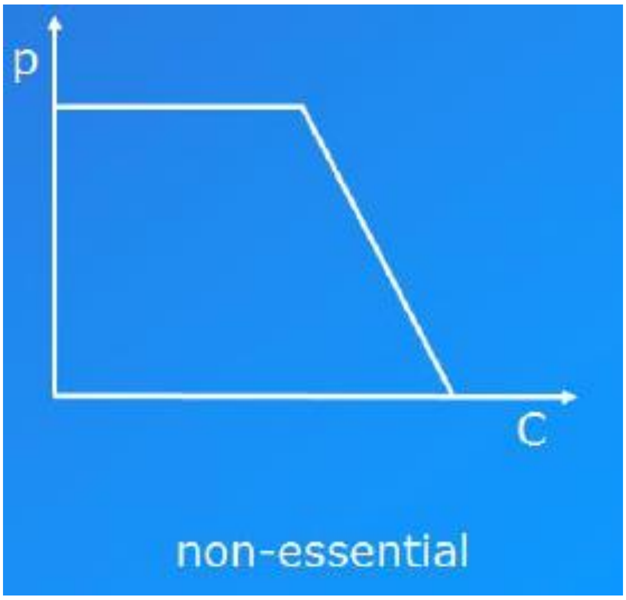


Figure 6: Quality of health given in potential (p) and the concentration of the non-essential metal (c) as a dose response diagram (Klaassen, 2013).

Heavy metals affect fish in many ways, as described previously heavy metals can be both essential and non-essential for organism including humans and fish. The general mechanisms for metal toxicity are binding to biomolecules, disturbing metal homeostasis, and formation of reactive oxygen (ROS). Non-essential metals can mimic essential metals, in the sense that non-essential metals can take the essential metals place in for example proteins and DNA. This can inhibit several cellular processes in the fish (Klaassen, 2013). Metal homeostasis is a process in cells where there is a regulation of essential metals in cells, exposure to metals can interfere with this homeostasis by inactivating transport through the cellular membrane or compete with other metals. The forming of ROS affects both lipids, DNA and proteins. The oxidative species forms when oxygen takes up an extra electron. An example is the Fenton reaction, where iron reacts with hydrogen peroxide and forms a radical hydroxyl, causing oxidative stress. These are some examples of how metals can disturb cellular processes in organisms, such as fish and humans (Klaassen, 2013).

When free metals are dissolved in water they become hydrolyzed by water molecules. When metals are bound to water molecules the acidity of the metals increases. How metals react in water is highly dependent on their speciation. The speciation explains in which form a molecule or ion occurs in water. This gives both information and a better understanding of the functions and distribution of metals in natural waters (Stumm, 1996). The mobility of metals is affected by the solubility of the metals. Figure 7 shows the solubility of some metals versus the pH. Concentrations of free metals ions are in equilibrium with oxides and hydroxides.

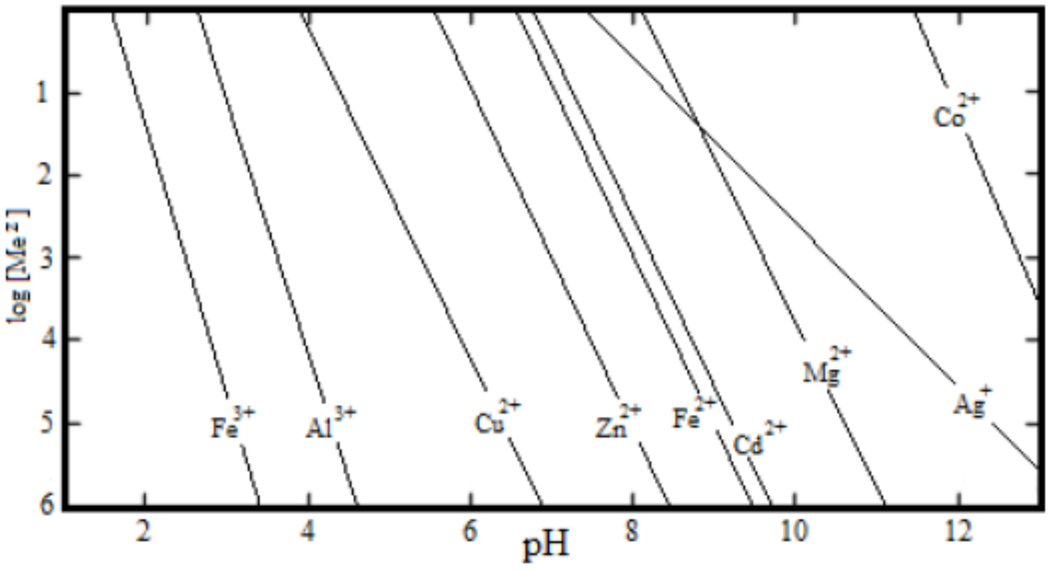


Figure 7: Solubility of oxides and hydroxides (Stumm, 1996).

The relation depicted in figure 7 does not fully describe the solubility of oxides and hydroxides, but gives a good picture of the solubility of some metals and the importance of size and oxidation number. As pH increases the solubility of all the metals decreases to some extent. Multiple-charged ions are in general less soluble than single-charges ions, smaller ions are often more soluble than larger ions (Stumm, 1996).

A low pH can encourage the solubility of heavy metals. The solubility is affected by the concentration of hydrogen ions in the water, and as this concentration increases, metal cations such as aluminium, iron and copper do not adsorb to the sediment. They are released into the water and the concentration of these metals increases. As concentration rises the potential toxicity of these metals increases as well (Fondriest environmental, 2013a).

In the next sub-chapter, the most important heavy metals, both essential and non-essential will be presented. The focus will be on how physio-chemical parameters affect the metals and how they can be toxic to the fish. General concentrations in seawater and groundwater is given because the brackish water used at Nofima, is a mix of both.

2.6.1 Important heavy metals

Water quality criteria in aquaculture are necessary for the industry in order to have control of the toxic effects various heavy metals can have. The concentration limits for the selected heavy metals are important to follow and monitor. Table 3 shows the given limits of some important, selected metals. The limits are based on communication with Nofima, Sunndalsøra. For comparison of limit values and results from the water samples, see the result chapter.

Table 3: Concentration limits of selected metals in RAS (Timmons, 2013).

Element	**Concentration µg/l**
Al	< 10
As	< 50
Cu	< 30
Fe	< 150
Mn	< 10
Ni	< 100
Zn	< 5
Cd	< 5

These metals were selected because they have a tendency to accumulate in recirculating systems as shown in previous research or because of possible toxic effects on the fish. These elements are shown in the periodic table below (8). Cr did not have a given concentration limit.

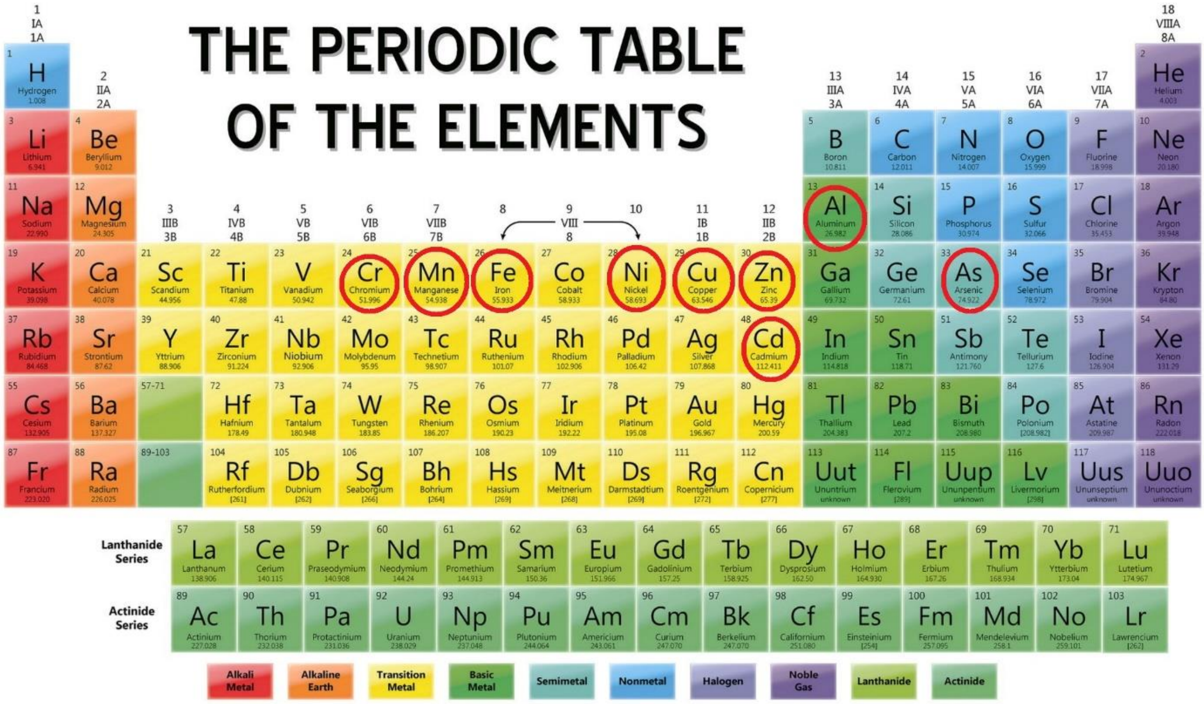


Figure 8: The periodic table with the selected elements (Cards22.com, 2017).

2.6.2 Arsenic (As)

Arsenic (As) is a toxic and carcinogenic metalloid (Klaassen, 2013) that exists in oxidation states of -3, 0, 3 and 5 and is widely distributed in nature through dissolution of rocks, ores and minerals (Alloway, 2010). Arsenic is highly depended on pH and redox conditions, since these parameters affect the speciation of the metal (Nordstrøm, 2002). Arsenic is most likely to be present as arsenate, with an oxidation state of 5 in oxygenated water. In a reducing environment (<200 mV), often in groundwater, arsenic is more likely to be present as arsenite, with an oxidation state of 3 (WHO, 2011a). Arsenite is a toxic form of arsenic, and could be harmful. However, the dominant form of arsenic in seawater and brackish water is arsenate (Neff, 1997).

In natural water, the concentration of arsenic generally rates from 1 – 2 µg/l (WHO, 2011a). The concentrations can be different in areas with volcanic rock and sulfide mineral deposits

(WHO, 2011a). Accumulation of arsenic has been seen in research on accumulation of arsenic in freshwater species (Takatsu et al., 1999). Accumulation of arsenic in RAS has been seen in low exchange systems (Colt, 2006). It is important to control the concentrations of arsenic in both seawater and groundwater, due to seasonal changes and local contamination (Neff, 1997).

The solubility of arsenic is dependent on the pH, where a decrease in pH can increase the concentrations of dissolved arsenic in water. This increases the concentration of arsenic in water and the toxicity of arsenic to marine organisms (Stumm, 1996).

2.6.3 Aluminium (Al)

Aluminium (Al) is the third most abundant element in the earth's crust after oxygen and silicon. Chemical compounds of aluminium occur as Al^{3+} in acidic conditions (Klaassen, 2013). Toxic responses are observed for fish in water with $pH < 5,5$, where labile, inorganic bound speciation occurs. A $pH > 5,5$ gives a stabile speciation with organically bound aluminium (S.E.Manahan, 2009). Controlling the concentration of aluminium and pH in aquaculture systems is necessary in order to control the amount of inorganic, labile aluminium.

The concentration of aluminium in seawater varies between 0,3 and 5 µg/l. The Atlantic Ocean has in general a higher concentration than in the Pacific (Lenntech, 2017a) In groundwater, concentrations could be as high as 400 µg/l, due to presence of insoluble hydroxides in soils. Under highly acidic conditions ($pH < 4,5$) the solubility increases, causing the concentration to elevate, (Lenntech, 2017a)

2.6.4 Cadmium (Cd)

Cadmium (Cd) is a non-essential, toxic transition metal (Klaassen, 2013). The metal is in an oxidation state of + 2 and is in many ways chemically similar to zinc. They are also naturally occurring together in sulphide ores (WHO, 2011b). Most of cadmium in the environment is of anthropogenic sources, for example as a by-product of refining zinc and copper (Hem, 1972). Concentrations in natural waters are usually below 1 µg/l, but can be higher in certain areas, because of anthropogenic sources, as the one mentioned above (Friberg, 1986).

Cadmium is highly dependent on pH (WHO, 2011b). According to World Health Organisation (WHO, 2011b), an increase in acidity can dissolve suspended or sediment-bound cadmium. In natural water however, the pH is too high for dissolving cadmium (Friberg, 1986). A pH below

8 will increase the solubility of cadmium and release Cd^{2+} into the water. Cadmium is a carcinogenic metal that could bind to the DNA and cause carcinogenic effects after exposure of high concentrations over time (Klaassen, 2013).

Contamination in water systems often occur due to presence of cadmium as an impurity in the zinc of galvanized pipes or due to fittings, water heaters, water coolers and taps containing cadmium. In areas with acidic soil, shallow wells can be acidified and the concentration of cadmium increases because of dissolved cadmium from sediments. (WHO, 2011b).

2.6.5 Chromium (Cr)

Chromium (Cr) is an abundant element in the earth's crust, usually in small concentrations, mostly in the trivalent state, but can exist in oxidation state of +2 to +6. (WHO, 2003a). This is an essential trace nutrient important for glucose metabolism in animals (Klaassen, 2013). As for many elements, the speciation is dependent on the redox potential and the pH. In water, Cr (II) is a positive ion that forms hydroxides and complexes, and is adsorbed to sediments at relatively high pH values. In general Cr (VI) salts are more soluble than those of Cr (III) making Cr (VI) relatively mobile (WHO, 2003a).

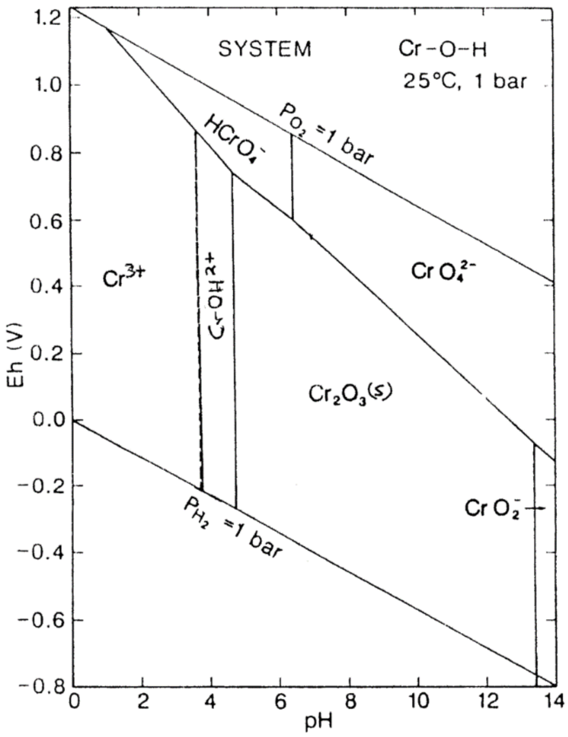


Figure 9: Speciation of chromium in different redox potential (V) and pH values (Oke and Vermeulen, 2017).

Figure 9 shows that a pH higher than 4,3 in an oxidizing environment can result in high amounts of Cr(III) oxides, which are slightly water soluble components (Stumm, 1996). Concentrations are therefore usually low in natural waters. Cr(III) is not considered to be highly toxic to organisms. The most toxic specie of chromium is the hexavalent chromium and toxicity is dependent on the pH (Klaassen, 2013).

Concentrations of chromium in seawater are in general low, where the highest concentration has been found in the North Sea, with a concentration of 0,7 µg/l. In average, the concentration in seawater is 0,04 - 0,5 µg/l. In groundwater, concentrations are generally low, with an average of < 0,1 µg/l. Areas with local industry has often higher concentrations, due to emissions and runoff from local industry (EPA, 2017).

2.6.6 Manganese (Mn)

Manganese (Mn) is an essential metal used in many metabolic and cellular reactions and contributes in reactions by being a cofactor in enzymatic processes (Klaassen, 2013). The essential metal can exist in up to 11 oxidation states, and the most important states in an environmental perspective are Mn^{2+}, Mn^{4+} or Mn^{7+} (Klaassen, 2013).

Manganese is dependent on the pH and the redox potential. These factors control the solubility of the metal. Anaerobic groundwater often contains high concentrations of dissolved manganese. In water with pH of 4-7, the Mn^{2+} dominates. In higher pH, higher oxidising states can occur (WHO, 2011d). The divalent form of manganese is very soluble and mobile in the water. Mn^{2+} can replace iron as a mechanism through mimicry and decrease iron concentrations in the blood (Klaassen, 2013).

According to the World Health Organisation, manganese has an impact on water pipes, where concentrations as low as 0,02mg/l can form coatings of precipitates under oxidising conditions (WHO, 2011d). This is often in correlation with iron rich water, where these metals can reduce the water pressure in the pipes, causing an economical problem over time (Oram, 2017a).

2.6.7 Copper (Cu)

Copper (Cu) is an abundant element in soil, with concentrations depending on the geology. Concentrations vary between 2 and 50 mg/kg in soil. Copper is abundant not only through geology, but also through atmospheric deposition and agricultural practice (fertilisers, pesticides, sewage sludge etc.). The main oxidation state of copper is +2, which has a high affinity for binding to organic matter. In water, copper is very dependent on physio-chemical parameters, the pH and redox potential are highly controlling the speciation of copper in water. A pH up to 6 in natural waters gives a high fraction of Cu^{2+}, while at a pH between 6-9,3 $CuCO_3$ is the dominant species in oxidizing water. $CuCO_3$ is insoluble in water, whereas Cu^{2+} is soluble and more mobile in the water. The general copper concentration in surface water are often between 0,5 to 1000 µg/l in more polluted areas (WHO, 2004).

Copper is used in fish feed, which can accumulate in recirculating systems (Martins et al., 2009). An accumulation of copper could increase the toxicity of copper to the fish. An overload of copper can create free radicals, which accumulates in the tissue (Klaassen, 2013). The toxicity of copper seems to be reversed linearly with the hardness of the water, where an increase in water hardness decreases the general toxicity of copper(Erickson et al., 1996), same as with pH (Davidson et al., 2009).

The pH is not the only parameter that affects the solubility of copper. Temperature has proven to be an important factor when it comes to solubility of copper, particularly in neutral to acidic pH of water (Dortwegt and Maughan, 2001), when higher temperatures decreases the solubility of copper. Salinity affects the toxicity as well as described earlier, where higher salinity decreases the uptake of copper in fish (Witeska, 2006).

2.6.8 Iron (Fe)

Iron (Fe) is, as it was explained previously, an essential nutrient for marine microorganisms. Most of the iron comes into the natural water through the atmosphere, which, in turn, receives it from dust. In the atmosphere, water droplets have a significant fraction of Fe(III), which becomes solubilized and reduces to Fe(II). In seawater however, the thermodynamically stable oxidation state is Fe(III) (Stumm, 1996). In other water bodies, speciation of iron is affected by physio-chemical parameters. The concentrations of iron in natural water are reported to be around 700 µg/l. In anaerobic groundwater, whereas explained, the iron is in the divalent form, the concentrations are usually 500 to 1000 µg/l, but in anaerobic groundwater the concentration of Fe(II) could be very high (up to 5000 µg/l) (WHO, 2003b)

Several important processes control the speciation of iron in water. Redox reactions and pH are two of many important processes that affect iron. In groundwater, the redox potential and pH have a crucial part in speciation of iron, as shown in the figure 10.

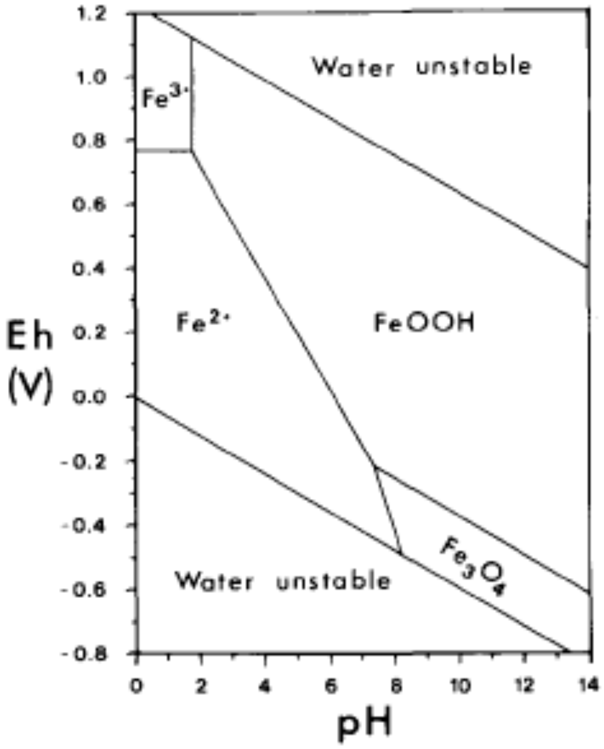


Figure 10: Speciation of iron in groundwater as a function of pH and redox potential (V) (Stumm, 1996).

The solubility of iron is important to understand when predicting the oxidation state of iron in water. The trend in iron solubility by pH is that the lower pH, the higher solubility of iron. Dissolution of iron can occur as a result of oxidation and a decrease in pH. From figure 10, Fe^{2+} is soluble in natural water, while Fe^{3+} and FeOOH is not. High amount of organic matter can stabilize Fe^{2+} and slow the oxidation to Fe^{3+}. Soluble Fe^{2+} is taken up through water and is the most toxic specie of iron to marine organisms. The toxic effect is directly correlated to pH, since Fe^{2+} dominates in acidic water (Vuori, 1995).

An example of toxicity caused by iron is the fenton reaction. Equation 6 describes a reaction in animal cells where Fe^{2+} contributes to create hydroxyl radicals (OH*) (Klaassen, 2013).

$$HOOH + Fe^{2+} \rightarrow Fe^{3+} + OH^{-} + OH^{*} \qquad (6)$$

The radical can contribute to oxidative stress, which could cause inflammation (Klaassen, 2013). It is important to reduce the amount of Fe (II) in the water and control possible accumulation of iron in RAS.

When microorganisms are exposed to high concentrations of CO_2, the iron consumption by the microorganisms is reduced (Gaut, 2005). According to this study, less iron is taken up by the microorganisms, which could lead to an increase in labile iron. These labile forms of iron could go through complexation, increasing the solubility and the total dissolved concentration of iron in the water (Gaut, 2005).

2.6.9 Zink (Zn)

Zink is an abundant essential element, small concentrations are essential in animal metabolism (Hem, 1972). The concentration of zinc in natural surface water is usually below 10 µg/l, in groundwater the concentration is usually between 10-40 µg/l. Concentrations of zinc in water can be altered by local and atmospheric pollution from anthropogenic sources such as atmospheric deposition, fertilization and sewage sludge (Alloway, 2010). In water systems, the concentration of zinc could be higher due to leaching from piping and fittings. A low pH and a high CO_2 concentration could enhance the leaching of zinc into the water (WHO, 2003c), and possible accumulation of zinc in recirculating water systems.

The solubility of zinc depends on the pH. The solubility increases with increasing acidity. At a pH above neutral, zinc is insoluble in water, but when the pH is lower, zinc dissolves in water as Zn^{2+} (Lenntech, 2017e). At a pH below about 7,3-7,5, Zn^{2+} becomes the dominating form, as shown in the figure 11. Zn^{2+} in high concentrations is taken up by the body and could cause neurotoxic effects on animals (Klaassen, 2013).

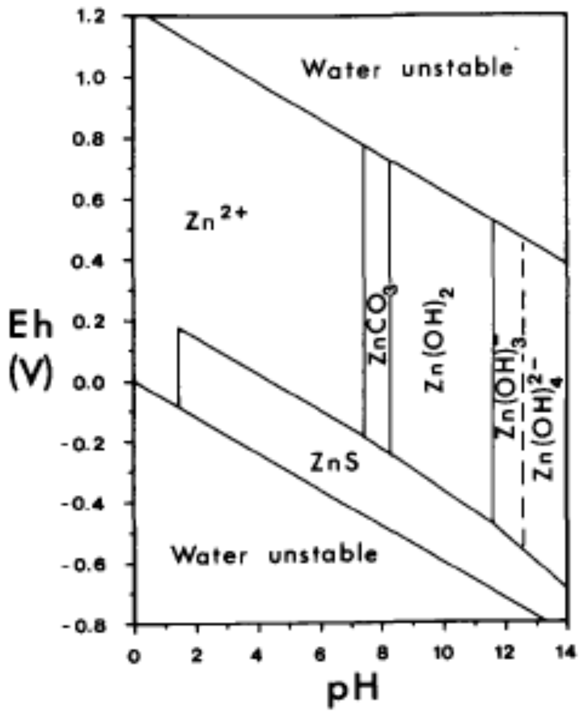


Figure 11: Speciation of zinc at different redox potential and pH values (Stumm, 1996).

2.6.10 Nickel (Ni)

Nickel is a metal that exists mostly in the divalent form in the biosystems. Nickel occurs in water as Ni^{2+} and sometimes as $NiCO_3$. Seawater and freshwater normally contain low values of nickel, whereas seawater contains approximately 0,5-2 µg/L and rivers 0,3 µg/l. The sources of nickel in water are mainly from geological and anthropogenic sources, dominantly from emission and agriculture fertilizers (Lenntech, 2017b). As a non-essential metal, high concentrations of nickel can cause a negative effect on marine organisms. Carcinogenic effects, caused by nickel, can be a result of nickel replacing zinc and magnesium ions in the DNA (Klaassen, 2013).

3. Analytical Methods and statistics

Analytical methods that have been used in the thesis, are the inductively coupled plasma mass spectroscopy (ICP-MS) for element analysis, analysis of total organic carbon (TOC) using a combustion technique and NDIR, and for measuring parameters two multimeters with three different electrodes.

3.1 ICP-MS

ICP-MS (Inductively coupled plasma mass spectroscopy) is a widely used analytical instrument for multi-element analysis. The instrument is especially suitable for detection of cations, and is therefore a good analytical instrument for the analysis of heavy metals.

As for many spectroscopic methods, the sample needs to be atomized (Skoog, 2004). The sample, often in a liquid form, is pumped into the system (Thomas, 2001). By the help of an atomizer, the sample transforms into small drops that is called aerosols (Thomas, 2001). The sample is after this exposed to a high-temperature plasma, where the purpose is to generate positive charged ions (Thomas, 2001). The plasma contains a mix of gas, both cations and electrons, normally argon ions and electrons (Skoog, 2004). By transportation through the hot plasma, the sample is transformed from a liquid aerosol into a solid particle before it is transformed into a gaseous form (Thomas, 2001). The plasma is now converted from aerosols into atoms, and further into ions. These ions are now sent to the mass spectrometer. The spectrometers task is to separate the ions. An electrostatic quadrupole mass filter is used to separate the ions (Thomas, 2001). In the mass filter, where the temperature will reach up to 10000 K, the ions will separate due to different mass/charge-relationship (Skoog, 2004). The separated ions will now reach the detector and convert to an electrical pulse, where the size of the pulse corresponds to the concentration of an element in the sample (Thomas, 2001).

An analytical instrument will have a detection limit, which gives information about the lowest concentration of an element within a certain confidence interval (Skoog, 2004). This limit will vary for each individual element. See appendix 5 for detection limits for the selected elements. In general, for metals, the detection limit is below ppb for ICP-MS and down to a ppt level for a HRICP-MS (High resolution ICP-MS), due to low background levels (Skoog, 2004).

There are some errors to consider when using ICP-MS. The most important errors to consider are development of matrix, drift and interference (Jenner et al., 1990). For ICP-MS the errors are divided in two groups, spectroscopic interference and matrix effects (Skoog, 2004). Spectroscopic interference will happen if the mass/charge relationship is equal between an analysed ion and an ion in the plasma (Skoog, 2004). To reduce or avoid such an interference, HR-ICP-MS is an option. If the concentration in a sample is too high, it could lead to matrix effects that could affect the analysed signal (Skoog, 2004). This effect is stopped by diluting the sample or change the procedure of introducing the sample. A sample should not have more than 0,2 % total dissolved substances if an optimal stability and maximum performance of the system is to be achieved (Thomas, 2001). Over time, the response of the system will change. Drift in the system can develop because of matrix effects. This can inhibit the sensitivity of the instrument (Cheatham et al., 1993). Drift in the system can also affect the surrounding temperature in the system, leading to less stability of electronic circuits. Another reason for drift is if there are any deposited salt in the slot opening of the apparatus because of analysis of samples with a lot of dissolved material (Cheatham et al., 1993).

In general, error sources associated to analytical research are due to systematic or random errors. These errors can lead back to the method, the instrument and human errors (Skoog, 2004). By calibrating, working accurate, using standards and blank samples, systematic errors will reduce (Skoog, 2004). To make sure of keeping the random errors at an acceptable level, it is important to control the different variables affecting the measurements.

3.2 Total organic carbon (TOC)

Total organic carbon (TOC) is a measure of the amount of organic carbon in a water sample. The principle behind TOC is the removal of inorganic carbon from the sample and subsequently oxidising all the remaining carbon within the sample and measure the amount of produced CO_2 (Bisutti et al., 2004).

The procedure of oxidation is a high temperature combustion technique (Tekmar, 2014a). The method is based in a thermal oxidation of the organic carbon and the thermal decomposition of inorganic carbon (Bisutti et al., 2004). This instrument is considered to be an accurate method, accurate and reliable for determination of organic carbon (Bisutti et al., 2004).

The produced CO_2 is measured by a NDIR (non-dispersive infrared spectrometry), which measures CO_2 directly (Bisutti et al., 2004). As the CO_2 is swept through the detector, the

absorption of the infrared light is measured over time. The result correlates to a peak, which is integrated and correlated to a concentration (Tekmar, 2009).
An advantage this instrument has is that it can analyse salt samples, which in this thesis is important because of the salt values in the samples.

3.3 Multimeter

Two multimeters were used to measure pH, redox potential, temperature, conductivity and salinity. These are called Multi 350i and Multi 3430 and are produced by WTW. The Multi 350i was used for salinity and conductivity measurements, while the Multi 3430 was used for pH, redox potential and temperature measurements. Three different sensors were used for measuring the different parameters.

The pH sensor is a pH electrode called sensor Sentix 940 Combined IDS with a gel electrolyte. This electrode do not require any maintenance besides cleaning after use. The electrode have an integrated temperature sensor (WTW, 2017c), which was used in the measurements. The measuring range is 0 – 14 pH. For measuring redox potential, a sensor called Sensolyt ORP electrode 900-p was used. This is an electrode that delivers a sensitive voltage signal from reactions in the water. These reactions react with the platina metal surface on the electrode. The measuring range is $\pm 1200mV \pm 0{,}2mV$ (WTW, 2017b).

The conductivity measurements was performend by a conductivity meter calles ConOX, produced by WTW. The measurement of conductivity is a measure of electrical resistance, where two similar electrodes interact. Voltage applied to one of the electrodes causes ions in the water to migrate to the other electrode. This flow is measured by using Ohm's law and converted to ms/cm, which is the conductivity. This sensor is used in measuring salinity (WTW, 2017a).

These handheld instruments are easily used both in the lab and in the field. They give a fast and reliable measure. Possible errors with the use of these sensors are dried out or dirty electrode heads and too high/low values. If that is the issue, an error code should come on the screen, so that the manual could explain the current problem and how to fix it (GmbH, 2005).

For turbidity measurements, a Turbiquant 1100 IR instrument was used. The light scattered at 90 degrees is measured, and is measured in nephelometric turbidity units (NTU), as was mentioned in the theory. This is a portable instrument, which is easy to use both in lab and in the field (VWr, 2017).

3.4 Statistical review

To treat the data from the ICP-MS measurements several statistical calculations is necessary. The calculations are presented below:

Mean values

To calculate the mean value the presented formula must be used:

$$\overline{x} = \frac{\sum_{i=1}^{n} xi}{n} \tag{4}$$

where x_i is each value in the data set and n is the total values in the data set (Skoog, 2004).

Standard deviation and the relative standard deviation

The standard deviation explains how far away each value is from the mean value. In other words the spread in the data sheet. A low standard deviation refers to a small spread in the data sheet. The formula for standard deviation is:

$$S = \sqrt{\frac{\sum(xi-\overline{x}^2}{n-1}} \tag{5}$$

where x_i is each value in the data set, $\overline{x}$ is the mean value of the values in the data set and n is the number of the total values in the data set (Skoog, 2004).

The relative standard deviation (RSD) is an expression of standard deviation in percentage, and is given by this formula:

$$rsd = \frac{s}{\overline{x}} \text{ x } 100 \tag{6}$$

Where s is the standard deviation of the sample, $\overline{x}$ is the mean value of the values in the data set (Skoog, 2004).

By using ICP-MS for element analysis, the samples can be analysed several times and the RSD-values can be calculated for each analysis. RSD will then tell the precision of the data analysis, by showing the spread of the different parallels each sample. The RSD-values should be below 10 % for trace elements and under 5 % for macroelements. A larger percentage indicates insecurity of the results (Liu, 2008).

Variance

Variance is a measure of spread in the test values, and is calculated by this formula:

$$Var(x) = \frac{\sum_{i=1}^{n}(xi-\overline{x}\,)^2}{n-1} \tag{7}$$

Where x_i is each value in the data set, $\overline{x}$ is the mean value of all values in the data set and n is the total number of values in the data set (Skoog, 2004).

The relative variance is an expression in percent, and is given by this formula:

$$\frac{s^2}{\overline{x}} \text{ x } 100 \tag{8}$$

Correlation

The presence of metals in a sample can correlate to each other. Correlation could describe an increase in concentration of one metal that is connected to an increase of another metal. The correlation is expressed by a correlation factor (R^2). The closer this value is to one, the more does the metals correlate. A correlation factor equals 1 gives a linear correlation between the variables.

When using the trend analysis in Excel the R^2 – value is calculated by this formula:

$$R^2 = \frac{\sum(x-\overline{x})(y-\overline{y})}{\sum(x-\overline{x})^2} \tag{9}$$

The formula is calculated from the equation of the trend line, which is given by a linear equation: $y = ax + b$, where a is the slope and b is the intersection of the y-axis.

Analysis of variance (ANOVA)

The ANOVA test is used to compare variances in a data set, more specifically variances in I population means, μ1, μ2, μ3, ... μI, where the null hypothesis is of the form (Skoog, 2004):

H0: μ1 = μ2 = μ3 = …. = μI

The alternative hypothesis Ha is:
Ha: at least two of the μi's are different.

To state a confidence interval is the most normal procedure to quantify the degree of uncertainty (Marx, 2013). A confidence interval gives the mean of a data set where the true value S (mean of the population) is in a given probability (Skoog, 2004). A probability have to be chosen for an ANOVA-test, where the most common is to choose a confidence interval of 95 %. This level represents the probability to find S in a given interval. The significance level is set to be $p = 0{,}05$, which means that there is a 5 % chance interpreting that there is a significant difference, when there is none, where a lower p-value gives an increased probability for difference between the data sets (Skoog, 2004). If p-values form the ANOVA tests is $< 0{,}05$, then the null hypothesis is reject and there is at least two of the population means are different (SPSS, 2017b). A problem with the two-way ANOVA is that some variables could affect the others or each other, that they are not independent, and that a factor can influence the results of the test (SPSS, 2017b). This will be discussed later in the subchapter about data analysis.

4. Materials and methods

The first part in this chapter presents the used equipment and material, which have been used for the experimental work in this thesis. The second part presents the experimental design. The rest of the chapter will present the collection and treatments of samples, and the preparations for analysis. At the end of the chapter, there will be a presentation of the quality assurance of the data and the data analysis.

4.1 Equipment and materials used in the thesis

The table below shows the different equipment and materials used in this master thesis.

Table 4: Used equipment and materials, which have been involved with the water sampling.

Component	**Type**	**Supplier**	**Properties**
Filter	Art. No 514-0074	VWR International	25mm syringe filter w/ 0,45 µm Polyethersulfone membrane
Glass tubes		VWR International	Sterile and metal free
MQ Water	Ion-exchanged	BioNordika Bergmann	Cleansed water
Nitric Acid	5 M		Dissolves heavy metals
ICP-MS tubes	Art.No 525-0461	VWR International	Sterile and metal free
CO_2 sensor	Franatech	Franatech GMBH	CO_2 measure
Multimeter	WTW 350i and 4340	WTW	pH, redox potential, conductivity, salinity, temperature
Turbidity sensor	TurbiQuant 1100 IR	Merc	Portable, waterproof

4.2 Experimental design

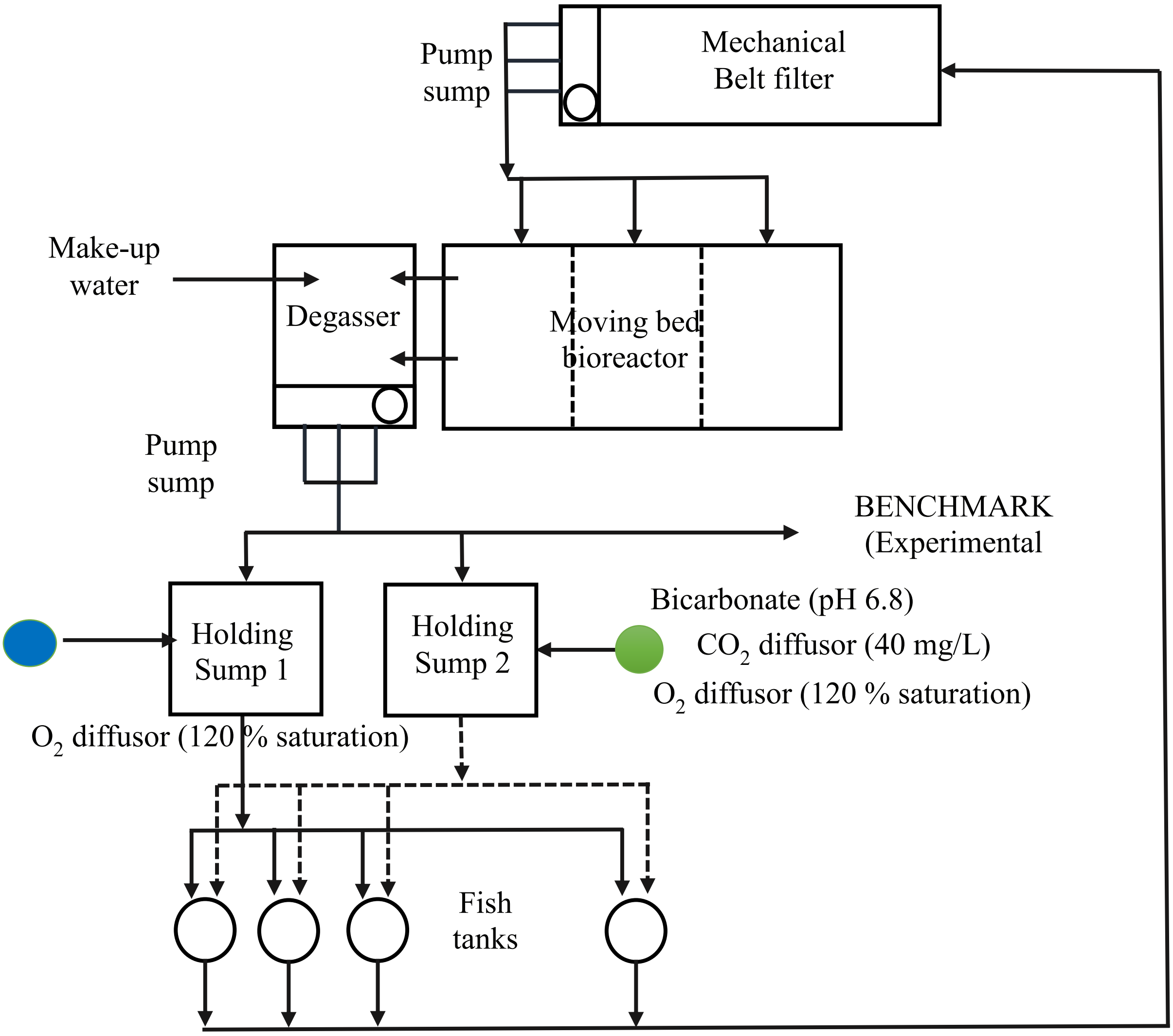


Figure 12 : Complete experimental setup, with mechanical and biological filter, degasser and two holding tanks (Mota, 2017a).

Figure 12 shows the total experimental setup. The water follows the arrows in a circle through the mechanical filter, MBBR (biological filter), degasser and eventaully to the holding tanks (601 and 602), where CO_2 and bicarbonate is added to only one of the tanks (601). Both of the holding tanks is added with saturated oksygen. Water from the holding tanks is mixed and comes into the 18 fish tanks. The water goes back to the mechanical filter after the fish tanks.

Below is the figure describing the experimental design and is the same as presented in the theory (figure 1).

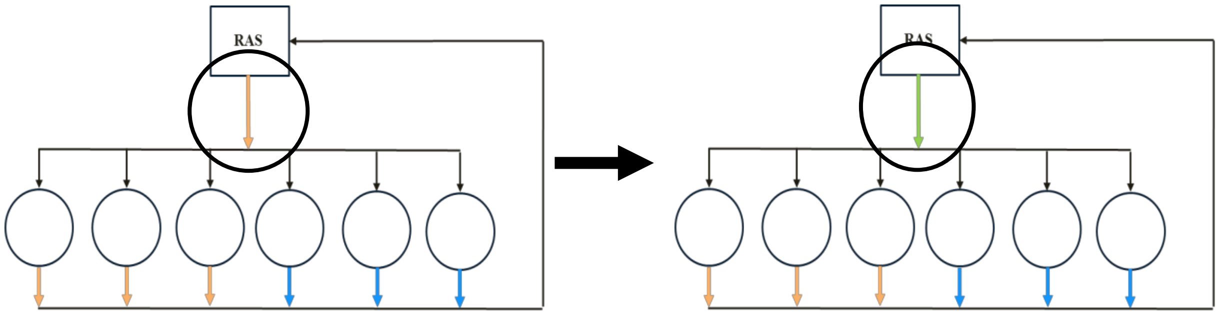


Figure 13: Fish tank level. Recirculation of water and the possibility of accumulation of unknown substances (green) from the control groups (orange) to the treatment groups (blue) (Mota, 2017a).

The CO_2 in all the tanks was controlled each day throughout the experiment. The amount of CO_2 is controlled by two holding tanks (601 and 602), which is called the inlet water. One holding tank had no added CO_2 (602), whilst the other was added with CO_2 (601). The water flow of each tank was calculated to give the correct amount of CO2 and the concentration of each tank was controlled by using three CO_2 sensors (appendix B), produced by Franatech, Germany (Table 1).

The experimental design is built on a mono-factorial design where six CO_2 levels will be tested, using three replicates per treatment for 12 weeks. The CO_2 levels are <5 mg/L (negative control), 12 mg/L, 19 mg/L, 26 mg/L, 33 mg/L and 40 mg/L (positive control). The control groups, 5 mg/l and 40 mg/l has water supply from one holding tank prior to the fish tanks, respectively 602 and 601. The rest is a mix between the two holding tanks where the water flow from each holding tank controls the total CO_2 in the fish tanks. For an overview of tank number and CO_2-levels, see appendix C.

4.3 Sampling and treatment of samples

In order to give the correct answers for the given hypothesis, the location of where the water samples are collected is essential. The water samples was collected from both the inlet water (601 and 602) and the outlet water from all tanks. This means that both the water before and after the tanks containing fish was analysed, as is described in figure 13 about the experimental design. From the picture in figure 14, 601 and 602 delivers water from above the tanks from two different pipes. The outlet water is the one tap at the side of the tanks, where the water comes out, and feed is collected.

Figure 14: Picture of six of the tanks at Nofima, Sunndalsøra. The rest of the tanks are behind or beside the picture. Two plastic pipes with water goes into the tanks. These contain water from holding tanks 601 and 602 (Bye, 2017).

Nine different periods of sampling were completed from 16.11.2016 to 10.02.2017. For time and dates, see appendix D. The thought behind taking samples through the whole period was to look at differences in water quality over time.

Samples for three different analysis were collected from the 18 tanks, both from the outlet water and the two inlet waters (601 and 602). The order of sampling was randomized for each date to avoid systematic errors (Skoog, 2004). One sampling was for trace analysis with ICP-MS at NTNU, another for analysis of total carbon, and the last one for a different master project in the CO2 RAS project with focus on organic substances. For ICP-MS, each sample was collected in 15 ml polyethylene tubes, with volumes up to 15 ml. For total carbon, 50 ml glass or polyethylene tubes were used, with volumes between 15ml – 40 ml. Some of the sampling dates experienced fast clogging of filters. The tubes were washed with sample water three times and the filter was washed through with a small amount of the sample water before filtrating into the tubes.

Parameters (pH, redox potential, temperature, salinity, conductivity and turbidity) were also measured in both inlet water and in every tank at the same time as the water sampling. The measuring was completed as shown in the picture below in figure 15 by two multimeters, with three different electrodes. Turbidity measurements were collected form the outlet water, which is sown down to the left in figure 15.

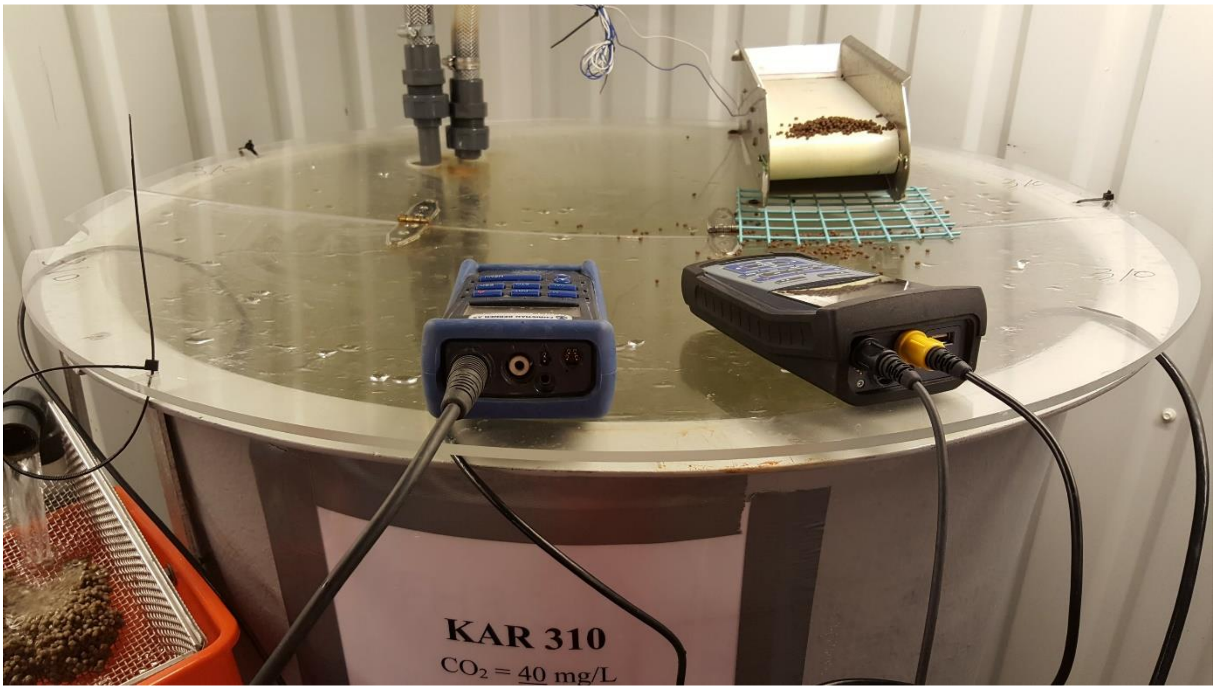


Figure 15: The picture shows how the three electrodes were placed in the water. The electrodes were placed in the upper layer of the water (Bye, 2017).

The measurement of parameters were randomized, meaning the order of tanks were changed for each time. This was done to avoid systematic errors (Skoog, 2004). Water samples from the make-up water, both freshwater and seawater were collected at the 18th of January, in the middle of the project period (Appendix D). The time used in every measurement was between 1-2 minutes. The given parameter values for RAS is shown in the appendix A (Mota, 2017b).

The sampling followed the guidelines of the ISO-method for correct collection and treatment of samples. The samples was filtrated with a syringe filter with a 0,45 µm polyethersulfone membrane. There are several advantages using this type of filter. For analysis of dissolved heavy metals with an analytical instrument, it is recommended to filtrate the water prior to the analysis in order to remove particles in the samples. Precise analysis of many heavy metals, including lead and mercury, depend on not introducing any interference into the samples. Water often contains a large amount of particulate matter, which can offer a challenge for filtrating due to quick clogging of the filter (VWR).

Filtration of water using a 0,45 µm polyethersulfone membrane takes away particles larger than 0,45 µm. Some colloids with low particle size can however, transfer through the membrane. When filtrating with such a filter, it is not always easy to know exactly what you have in the water sample. Metals can bind to colloids or natural organic matter, or are in a free aqueous form, where they are hydrated with water molecules (Stumm, 1996). However, when using this filter, it is said that what is sampled is in a diluted form (S.E.Manahan, 2009).

The samples for ICP-MS was digested with nitric acid (5 M) directly after the sampling in order to dissolve the metals in the sample. Aluminium and chromium does not dissolve, but becomes passive to this reagent owing to oxide formation (Skoog, 2004). The samples were transported in boxes with ice to NTNU, Trondheim, accordingly to the ISO-method, where the samples were stored in a refrigerator before sent for analyses performed by an ICP-MS. Samples for total organic carbon were not treated with acid, but frozen down in a cooler. Samples from 11.01.17 were not filtrated at Nofima, but a few months later at NTNU, Trondheim (appendix D).

4.4 Preparation for analysis

Four blank samples were filtered with milli-q water, using the same filter and procedure as the samples. The reason for having blank samples is to correct the raw analytical response with eventual concentrations of elements from the filter (Skoog, 2004). The samples were not diluted before analysed with ICP-MS, when this was not necessary.

Before sending samples for analysis, the samples were randomized. At first, in discussion with the engineer in charge of the ICP-MS, it was agreed that the samples were to be in order from smallest value of conductivity to highest. In aftermath, this was not necessary, but the samples were either way sent in randomised.

For TOC analysis the samples was diluted 1:10, where 3 ml of sample was diluted with 27 ml of mili-q water. One sample from CO2-treatments 5, 19 and 40 on every sampling date was analysed, giving a total of 18 samples. The thought behind the selection was to give an opportunity to look at the trends of TOC in a low, medium and high treatment of CO_2 over time.

4.5 Quality assurance of data

An analysis should show good precision, both in-series precision and between-series precision. Low precision indicates random errors and a spread in the results (Skoog, 2004). To examine the precision in the analysis the same sample is analysed several times, so called duplicates. Table 5 presents the data for two repeating tests for sample 5 and 10 for selected metals. The repeating tests completed right after each other, which is appropriate since the signal change during an analysis process. From table 5 the values show a small difference between the repeating tests, which implies good precision of the analysis.

Table 5: Concentration (µg/l) in two repeating tests for sample 5 and 10 for the selected metals.

Metal	Sample 5	Sample 5	Sample 10	Sample 10
	Test 1	Test 2	Test 1	Test2
Zn	5,64	5,80	6,52	6,34
Cu	2,28	2,39	2,49	2,15
Cr	0,370	0,460	0,290	0,390
Mn	0,675	0,671	0,555	0,557
As	0,850	0,880	0,820	0,830
Fe	8,92	9,01	7,26	7,46
Ni	0,560	0,450	0,410	0,520

Including in tests of the instruments precision, the concentrations of each metal was read three times, giving three different rsd-values. The blank samples are used for discovering any contamination of the samples during the sampling and during the analysis. During treatment of data, the dataset was correlated for the blank samples. For elements with low concentrations, the blank sample will be a limiting factor for the analysis. By decision of the detection limit for the elements in the analysis the value of the blank test is used with a number of three times the standard deviation of the blank sample (Beauchemin, 2010). See appendix E for detection limits for the selected elements. The spread of the data set increases closer to the detection limit, which gives high RSD-values (Appendix F). This means that the results from the analysis where metals with concentrations above the detection limit is more precise. Among the selected metals, all are above the detection limit.

4.6 Data analysis

The data analysis was done using several programs. The figures are made in Jupyter and Excel, and the statistical analysis was performed by SPSS statistics.
Jupyter is a software program, which uses python as a programming language. Here the excel file was uploaded, so that the figures in the results could be made. It was necessary to save the Excel file as a .csv file. There are also figures made from Excel were the mean values have been calculated and the correlation between concentration and the different CO_2-treatments are shown.

The statistical analysis was performed by both a one-way ANOVA and a two-way ANOVA using SPSS. The reason behind using these two statistical tests is to see differences between the concentrations of elements and values from parameters because of different CO_2-treatments. The one-way analysis of variance (ANOVA) is a good statistical tool to determine if there is a significant difference between the means of three or more independent groups. These groups need to be unrelated or independent from each other (SPSS, 2017a). These independent factors can for example be time or different concentrations, such as different CO_2 concentrations. Three assumptions have to be managed before a test can be performed (SPSS, 2017a). The first is that the dependent variable has to be normally distributed in each group that is being compared. The second assumption is that there is homogeneity of variances, which means that the population variances in each group are equal. The last assumption is that there is an independence of observation (SPSS, 2017a). In this thesis, the two independent variables are concentration/values of elements or physio-chemical parameters and the different CO_2-treatments. The purpose is to see if there is any difference in concentration/value between the different CO_2-treatments.

A two-way ANOVA compares the mean difference between groups that are divided into two independent variables called factors. A two-way ANOVA test is looking at the interaction between the two factors on the dependent variable (SPSS, 2017b). In this thesis, these factors are the different CO_2-treatments and time, which is the different sampling dates. In SPSS, the sampling dates are put as weeks, where week 1 is the first sampling date and since there is three weeks until the next sampling date, the next one is called week 4 and so on. The dependent variable is either the concentration of an element or the values of a physio-chemical parameter. The purpose is to understand whether there is an interaction between CO_2 and time on the concentration of elements or the values of physio-chemical parameters. The two-way ANOVA test can also explain how much the impact of each factor have on the dependent variable (SPSS, 2017b).

When the one-way ANOVA is performed in SPSS, the first thing to do is select the input data from both groups. The next step in the SPSS program is go to Analysis →Compare Means → One-Way ANOVA. Then put concentrations/values in the dependent list and CO_2-treatments in the factor list. Click on post Hoc and Tuckey test. This is a test that determines which specific group differed from each other (SPSS, 2017a). Next, go to options and click on Descriptive and and Fixed and Random effects. These gives the mean, max, min and standard deviation values of the concentrations/values (SPSS, 2017a).

A two-way ANOVA is a bit more complicated. The two independent variable groups and the dependent group needs to be put in the data file. Click on Analyze → General Linear Model →Univariate. Put the depended group in the Depended variable and the two independent groups in Fixed Factors. Go to Plots and put one group to the Horizontal line and the other to Separate Lines. Add these together and press Continue. As with the one-way ANOVA the post Hoc test and the descriptive statistics is necessary. The most important table from the two-way ANOVA test is the "Tests Between-Subjects Effects". This table gives the Degree of Freedom (df) and the F- and p-values of both the independent groups and the F- and p-values of their interaction. The values from the interaction is interesting, when they can statistically say if there is a significant difference in the dependent group because of the independent groups. Addition to this is the Partial Eta Squared, which informs of the relative impact each independent group has on the dependent group (SPSS, 2017a).

Six assumptions have to be managed before performing a two-way ANOVA. Firstly, the dependent variable must be continuous. This means that the independent variable have to be a continuous variable, such as time. The second assumption is that the two independent variables should each consist of two or more categorical, independent groups. In this thesis, both time and different CO_2-treatments go as independent groups. The third assumption is that all observations must be independent. This means that there is no relationship between the observations in each group or between the groups. The fourth assumption is that there are no outliers. The fifth assumption is that the dependent variable is approximately normally distributed for each combination of the groups of the two independent variables. The last assumption is that there needs to be homogeneity of variances for each combination of the groups of the two independent variables. The last three assumptions can be tested in SPSS (SPSS, 2017b). There are no indications that the data sets in this thesis did not pass these assumptions. Both one-way and two-way ANOVAs could be performed.

Calculations of water hardness was calculated by using an online calculator at Lenntech.com, which immediately transform the concentration of $CaCO_3$ to a hardness value given in German degree of water hardness, °dH.

5. Results and discussion

The aim of the results and discussion chapter is to elaborate about differences in values between the different CO_2 treatments, possible accumulation of heavy metals and to give a general comment on the water quality.

A one-way ANOVA test is performed on every parameter and heavy metal, with the intention to see if there is a statistical significant difference in concentration/values of the parameters and heavy metals in different treatments of CO_2. A two-way ANOVA test is performed to see if there is a statistical significant difference between the treatments of CO_2 over time. All obtained values from these tests are in a confidence interval of 95 %, which gives a significance level of $p<0,05$ (5%).

5.1 pH in water samples

The pH values in each treatment of CO_2 are shown in figure 16. This figure shows the correlation between pH and the different CO_2-treatments.

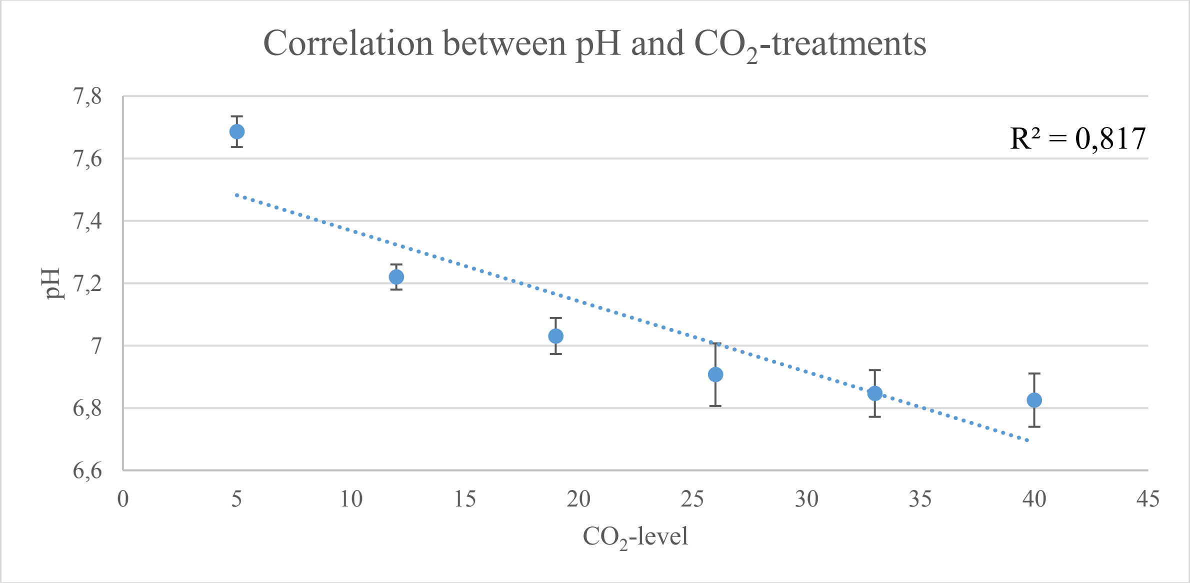


Figure 16: Correlation between pH and the different treatments of CO_2. Standard deviation (±) is marked and the correlation (R^2) is shown in the upper right corner.

A one-way ANOVA test was performed at the $p<0,05$ level showing a statistically significant difference between the treatments of CO_2 ($F_{5,102} = 349$, $p = 0,000$). The pH is highest in the tanks with 5 mg/l and lowest in the tanks with 40 mg/l. This can be explained by equation 1, which describes how CO_2 in water forms H_2CO_3, which dissociates to H^+ and HCO_3^-, causing a reduction in pH because of higher concentration of hydrogen ions.

A two-way ANOVA-test was performed at the $p<0,05$ level, which showed a statistical significant difference between the treatment groups over time ($F_{25,72} = 3,54$, $p = 0,000$). The correlation factor ($R^2 = 0,817$) shows a high correlation between the CO_2-treatments and the pH values, which supports the results from the two-way ANOVA test. There is a large difference in pH between 5mg/l and the 40 mg/l tanks. The total mean difference is 0,905 ± 0,016. This gives a large increase in acidity between the low and high treatment of CO_2, which can affect the speciation of heavy metals in the 40 mg/l tanks. The changes in pH through the experimental period is shown in figure 17.

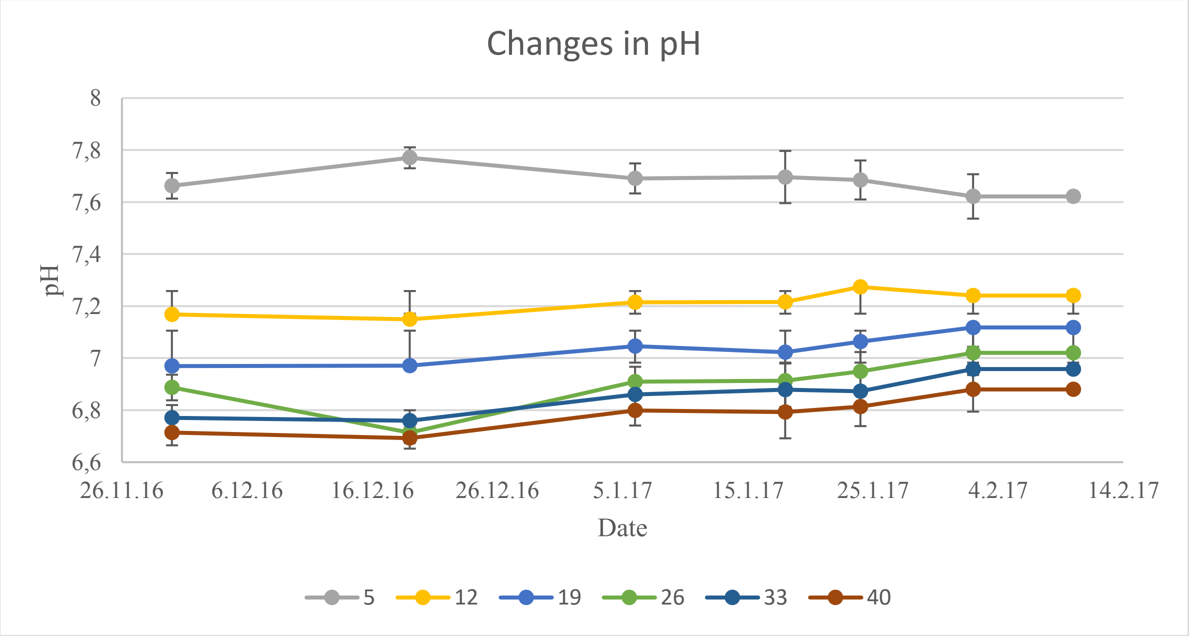


Figure 17: Changes in the mean values of pH in each treatment group. The colours describe each treatment and are represented below the figure.

During the experiment, at the 19th of December, the mean value of the 26 mg/l tanks were at the same pH level as the tanks with 40 mg/l. This could be an error in measuring or maybe some issues regarding the control of the flow into the tanks. It could be that prior to the measurement the flows and mixing of water from the holding tanks were inaccurate, releasing more CO_2 in the water for a short period. The standard deviations are low, giving low variance in the data set.

A possible explanation behind the fact that the 5 mg/l treatments have a larger pH than the rest could be because the 5 mg/l tanks get water directly from the holding tank without added CO_2. This water is not mixed as it is with the other treatments. The 40 mg/l tanks get water from the other holding tank with CO_2. There could be some changes when the water is mixed (see redox potential).

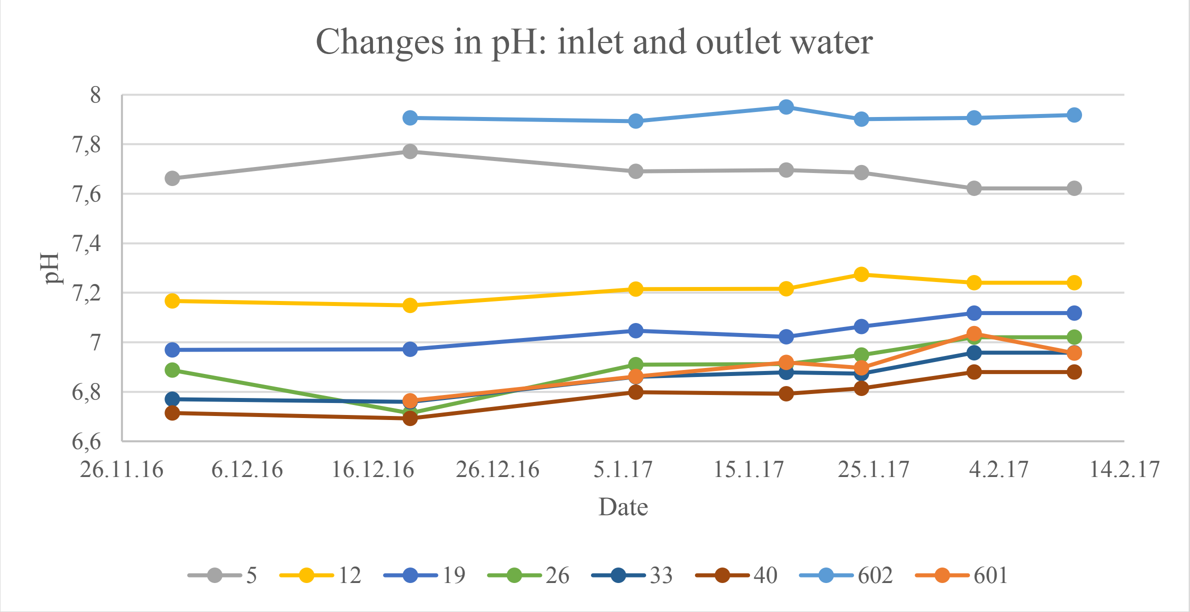


Figure 18: Changes in pH in both inlet and outlet water (601 and 602) in each treatment group.

In figure 18, the 5 mg/l tanks and holding tank 602 has a higher value than the rest. The reason for this could be the mixing of water as previously mentioned, but it also could be because of the respiration of the fish. Since there is a high amount of bicarbonate, this effect should be minor because of the strong alkalinity effect. The other holding tank (601), with added CO_2 has a higher concentration than the 40 mg/l treatments even when this is the same water before and after the fish tanks. The reason behind this is uncertain. A paired t-test was considered to be performed on the difference before and after the fish tanks. Only one measuring from the outlet water was performed each sampling date, giving no mean values to calculate the t-test from.

According to the two-way ANOVA test, the difference in pH over time at the $p<0,05$ level is statistical significant (0,113 ± 0,016) from beginning to the end of the experiment. The CO_2-levels in every tank was measured and controlled approximately every day during the experiment. This should have given a stable pH trough the experiment. The flow system had a tendency to clog, decreased the water flow, and the amount of CO_2 in the tanks either decreased or increased for some period. This could be an explanation of why the pH had a statistical significant difference over time.

The given limit for pH In RAS is 6,8-7,2. For the whole experiment, only fish tank with CO_2-treatment of 19mg/l have a consistent pH between these limits. The low pH in the fish tanks with highest levels of CO_2 can increase the amount of free metals in the water. Solubility and speciation of heavy metals are highly dependent on the pH. Conditions in fish tanks with high CO_2 values could affect the solubility and speciation of some heavy metals mentioned in the theory, such as Cd, Mn, Fe and Zn. These elements will be presented an discussed late in the chapter.

5.2 Redox potential (mV) in water samples

Figure 19 shows the correlation in redox potential between each treatments of CO_2.

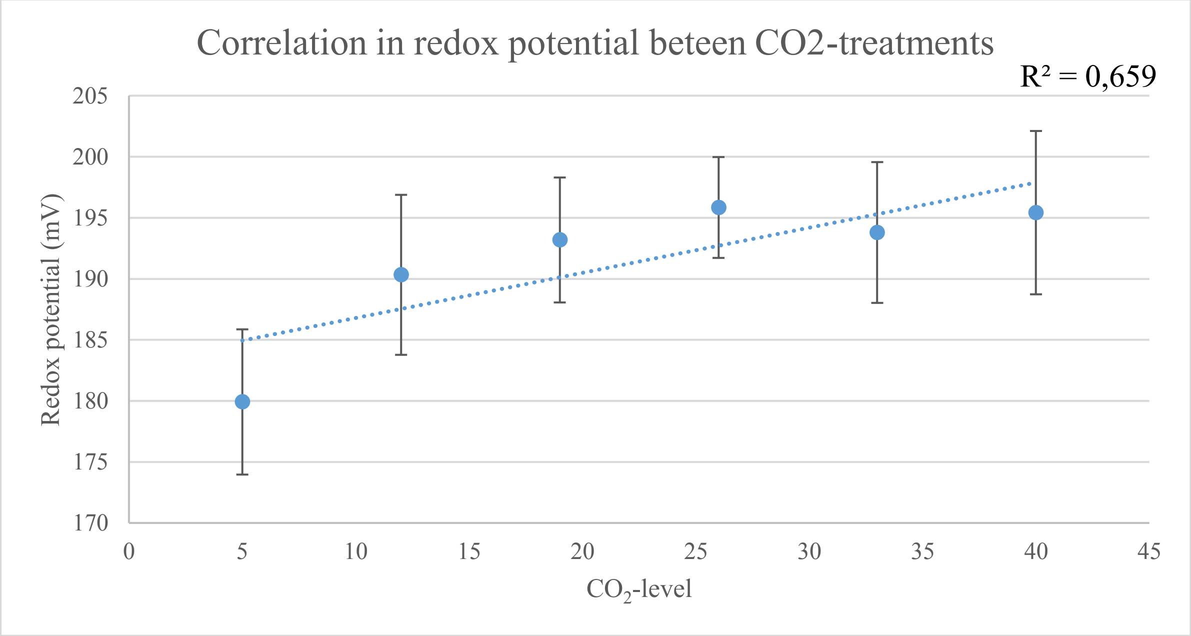


Figure 19: Correlation in redox potential in each treatment of CO2.

A one-way ANOVA test was performed at the $p<0,05$ level, showing a statistical significant difference ($F_{5,102} = 14,5$, $p = 0,000$) between the treatments of CO_2. The redox potential is in general lower for the 5mg/l control compared to the rest of the treatments. A one-way ANOVA test was performed at a $p<0,05$ level on all treatments except 5 mg/l and showed no statistical significant difference ($F_{4,85} = 1,90$, $p = 0,118$) between the treatments of CO_2. A two-way

ANOVA-test was performed at the p<0,05 level and showed no statistical significant difference ($F_{25,72}$ = 1,18, p = 0,000) between the different treatments of CO_2 over time. This could imply that there is only a statistical significant difference between the 5 mg/l tanks and the rest, and that there is no statistical significant difference between any of the other treatments. The correlation (R^2 = 0,659) between the treatments is not high, but there seems to some correlation between the treatments and the different treatments of CO_2.

During measurement of redox potential (mV), the values was not stable at the time the results was written down, which could give a larger standard deviation. It could be that the electrode got dirty during the measurement. Regardless of this, the results are quite stable over time and the values are between 172-208 mV, as seen in figures 19 and 20. These are not high values when compared to rivers and lakes with values between 300-500 mV. The lowest values could have an impact on the speciation of some heavy metals, such as As, which in an environment with redox potential < 200 mV could have a dominate speciation of toxic arsenite.

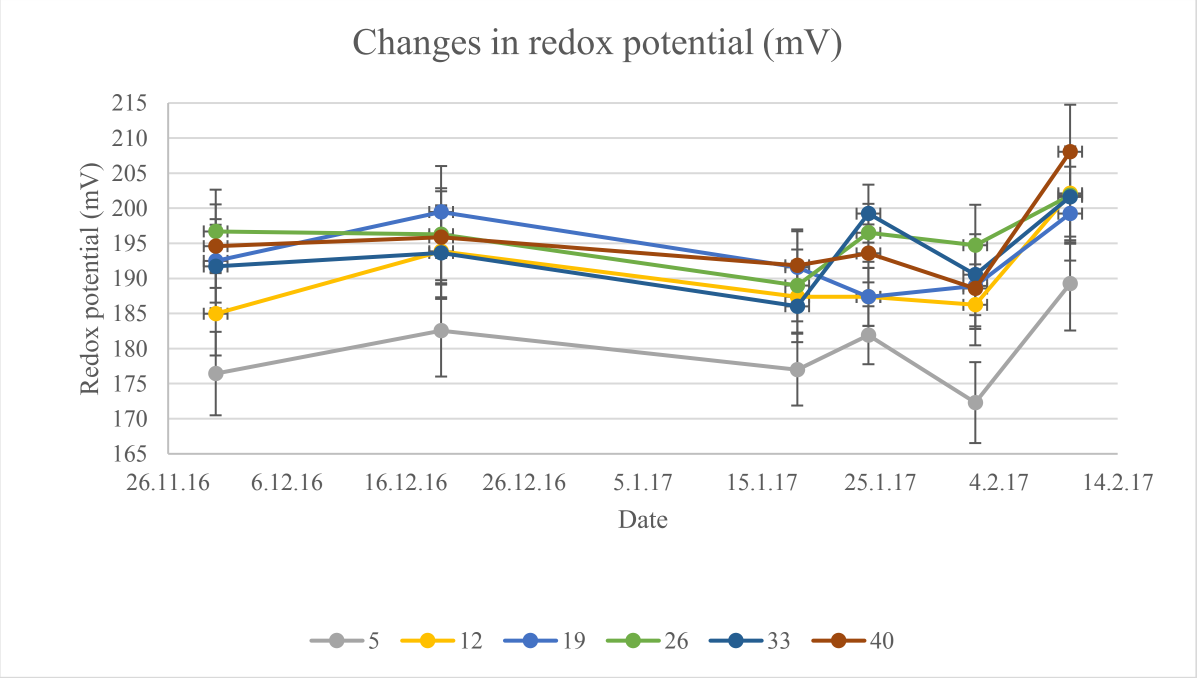


Figure 20: Mean values of redox potential (mV) through the sampling period.

There are no large differences in redox potential over time. There is an increase at the last sampling date, giving a total increase in redox potential. The total mean difference between the first and the last sampling date is 10,8mV ± 1,56.

As shown in figure 21, the mg/l tanks have a lower redox potential than the rest of the tanks.

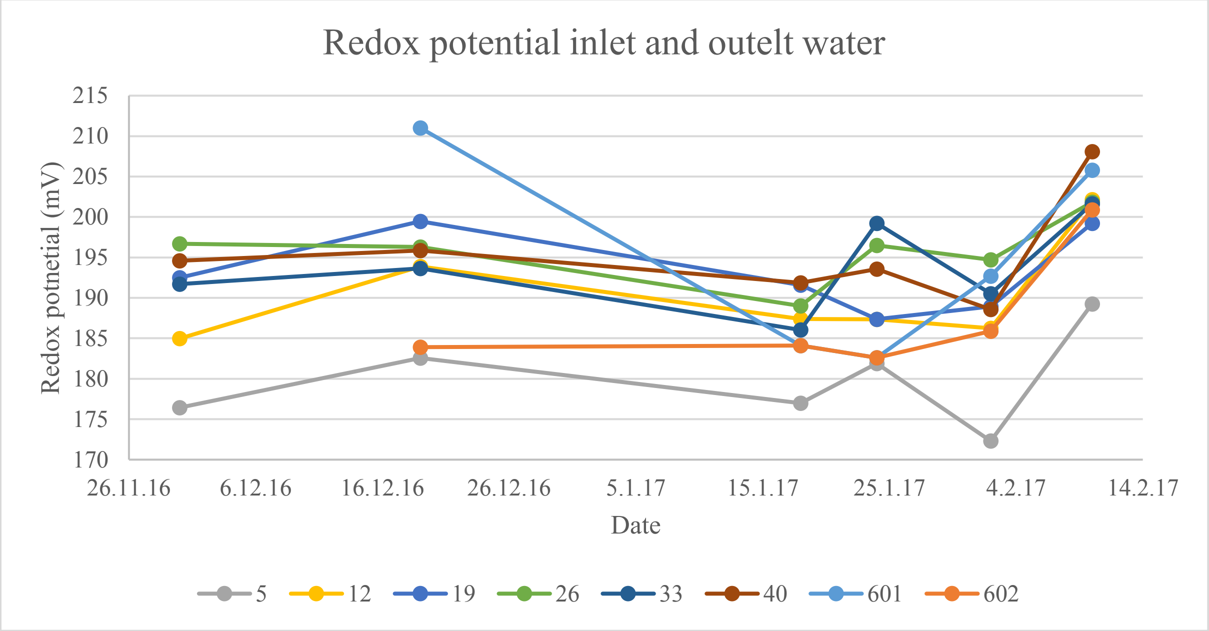


Figure 21: Changes in redox potential in both inlet and outlet water.

The 5 mg/l tanks receives water from holding tank 602, with no addition of CO_2. The other tanks, except 40 mg/l, receives a mix of water from both holding tanks. The mixing of water could have changed the redox potential for the rest of the treatments and not for the control groups. The values from 601 have a large drop from the first measuring to the next, which is not expected. This is most likely an error in the measurement, since the overall trend is that water with high CO_2 values have a high redox potential. A samples t-test was not performed due to lack of mean values from outlet waters. This test could have explained differences between the outlet and inlet water.

5.3 Temperature (°C) in water samples

Figure 22 shows the correlation between temperature measurements and the different CO_2-treatments.

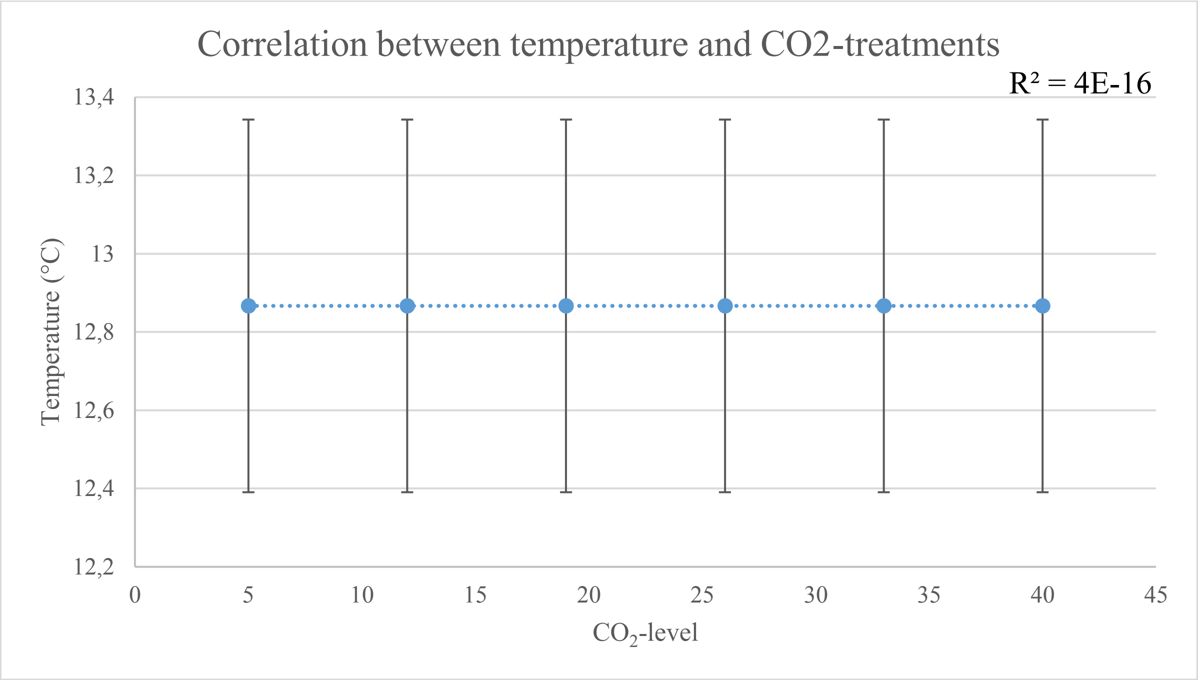


Figure 22: Correlation in temperature in each treatment of CO2.

A one-way ANOVA was performed at the $p<0,05$ level showing no statistical significant difference ($F_{5,102} = 0,005$, $p = 1,00$) between the treatments. A two-way ANOVA-test was performed at the $p<0,05$ level, which showed that there is no statistical significant difference between the CO_2 treatments over time. The correlation between temperature and the different CO_2 treatments is low ($R^2 = 4*10^{-16}$). The statistical data strongly suggests that there is no statistical significant difference between the treatments of CO_2 over time.

Figure 23 shows the mean temperature and the changes over the experimental period.

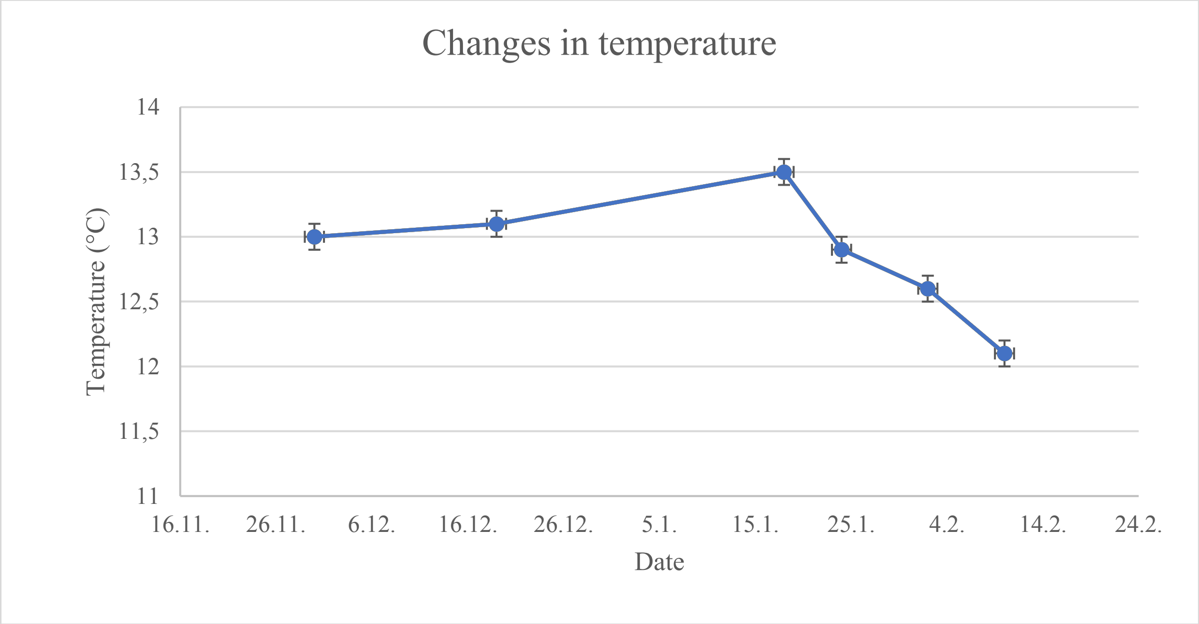


Figure 23: The mean temperature over time. All of the treatment values are equal and are shown as one line.

Figure 23 shows how the temperature for all treatments increases at the beginning of the experiment, before it decreases with over 1 °C. The mean difference between the first and the last sampling date is -0,844°C ± 0,005. The mean value for all the sampling periods is 12,9°C ± 0,476.

The temperature in RAS is set to be 12-13°C. Figure 23 shows that the mean temperature is slightly over this limit for almost two months. An increase in temperature increases the metabolic rate of the fish and could increase the mobility of heavy metals, such as Cu, Cd and Zn. The question however, is if this increase in temperature is large enough. The temperature is only approximately 0,5 °C over the limit, and should not be large enough to be an issue for the fish metabolism or mobilisation of metals in the water. Although, this difference is not positive and should be taken into account.

5.4 Conductivity (ms/cm) in water samples

Figure 24 shows the correlation between conductivity and the different CO_2-treatments.

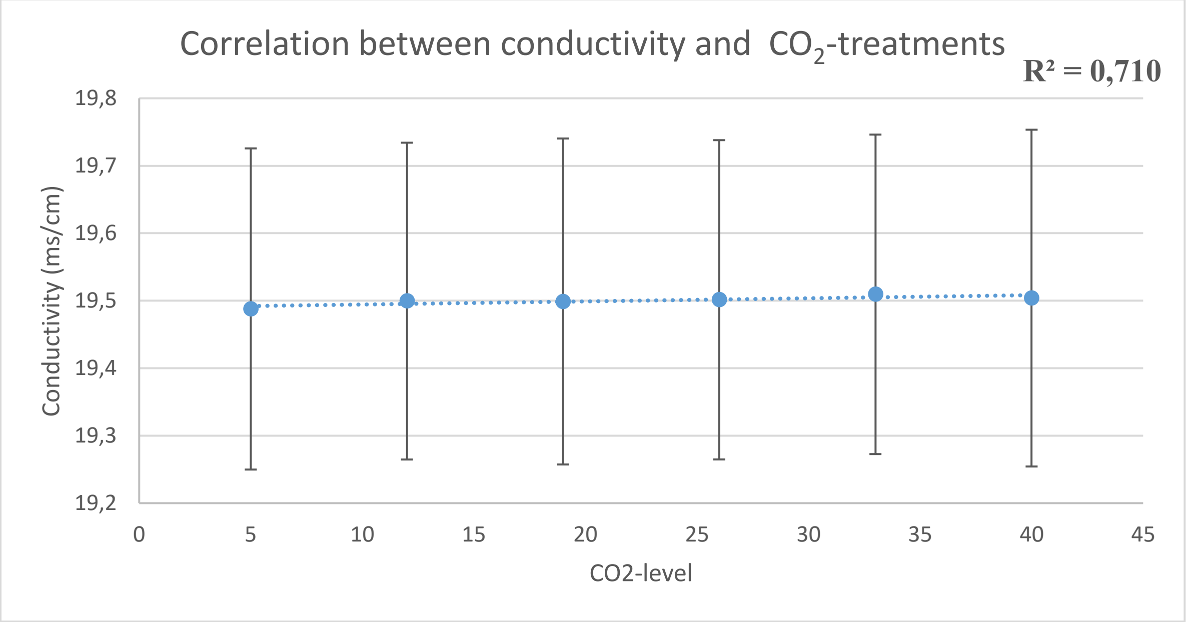


Figure 24: Changes in conductivity in each treatment of CO2.

A one-way ANOVA test was performed at the p<0,05 level, showing no statistical significant difference ($F_{5,102} = 0,018$, p = 1,000) between the treatments of CO2. A two-way ANOVA test was performed at the p<0,05 level, which showed that there is a statistical significant difference between the 5 mg/l treatment and the 26 (p = 0,012), 33 (p = 0,000) and 40 mg/l (p = 0,003) treatments. There is no statistical significant difference between any of the other treatments. The p-values and the R^2-value could imply that there are some differences over time, but not for all treatments. The fact that there is no statistical significant difference between the treatments, but that it is a different over time, could imply that the difference over time has a larger impact on the difference than the CO_2 treatments. From the two-way ANOVA test, the results show that relative impact on statistical significant difference in the data is 3 times larger from time (week) than from CO_2 treatments. This is a strong indication that the difference over time has a larger impact on the difference between the treatments over time than the actual CO_2 treatments, which according to the one-way ANOVA test, did not have a statistical significant difference.

Since data values from the first sampling date could affect the results of the next sampling date a two-way ANOVA test on the three last sampling dates were performed at the p<0,05 level, removing the first three sampling dates, the conductivity showed a statistical significant difference ($F_{10,36} = 3{,}31$, $p = 0{,}004$) between the treatments of CO_2 over time. This strengthens the test, since the values from the previous dates do not affect these values, and strengthens the theory that conductivity is statistical significant different between the treatments of CO_2 over time, where time may have a stronger influence than the different CO_2 levels.

The total mean difference between the low treatment of CO_2 and the high treatment of CO_2 is 0,061ms/cm ± 0,221. This is not a large difference, which is shown in figure 25, but large enough to give a statistical significant difference between the different treatments of CO_2.

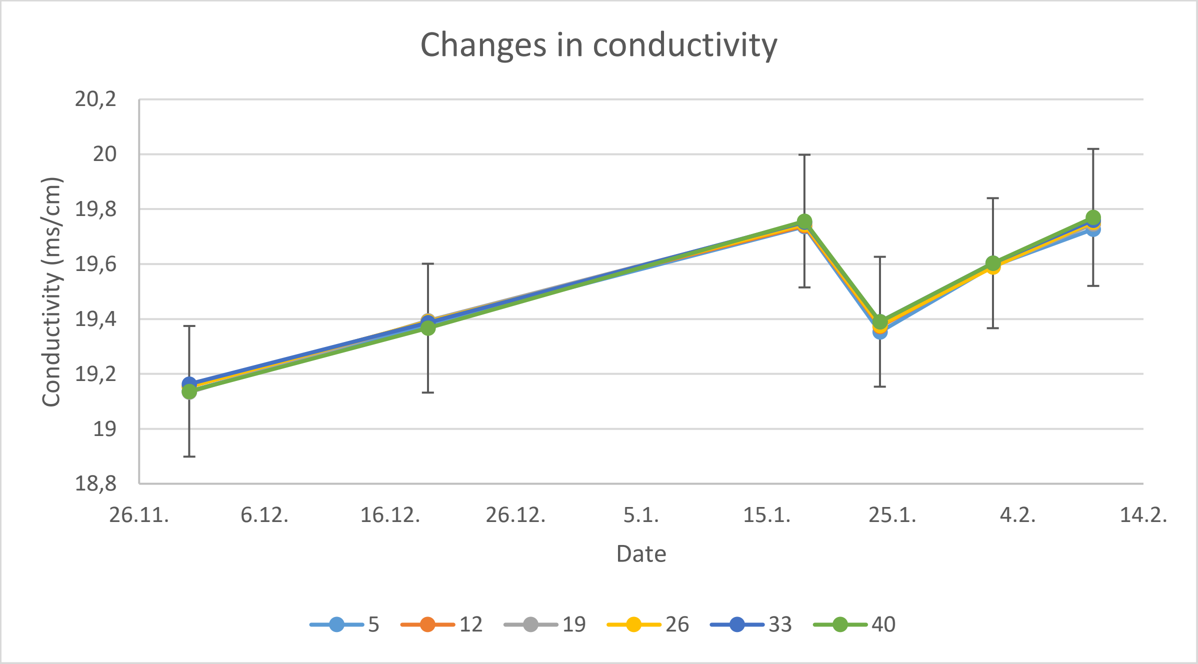


Figure 25: Mean conductivity values in each CO2 treatment over time.

As figure 25 shows, the conductivity increases with approximately 0,6ms/cm, the first 1 ½ month before it drops for a period and then rises again the last weeks of the experiment. The mean difference between the first and the last sampling date is 0,604ms/cm ± 0,004. These values are reflected by the values of salinity. Further discussion will be presented with the salinity values.

5.5 Salinity (ppt) in water samples

The figure below shows the correlation between salinity and the different CO_2-treatments of CO_2.

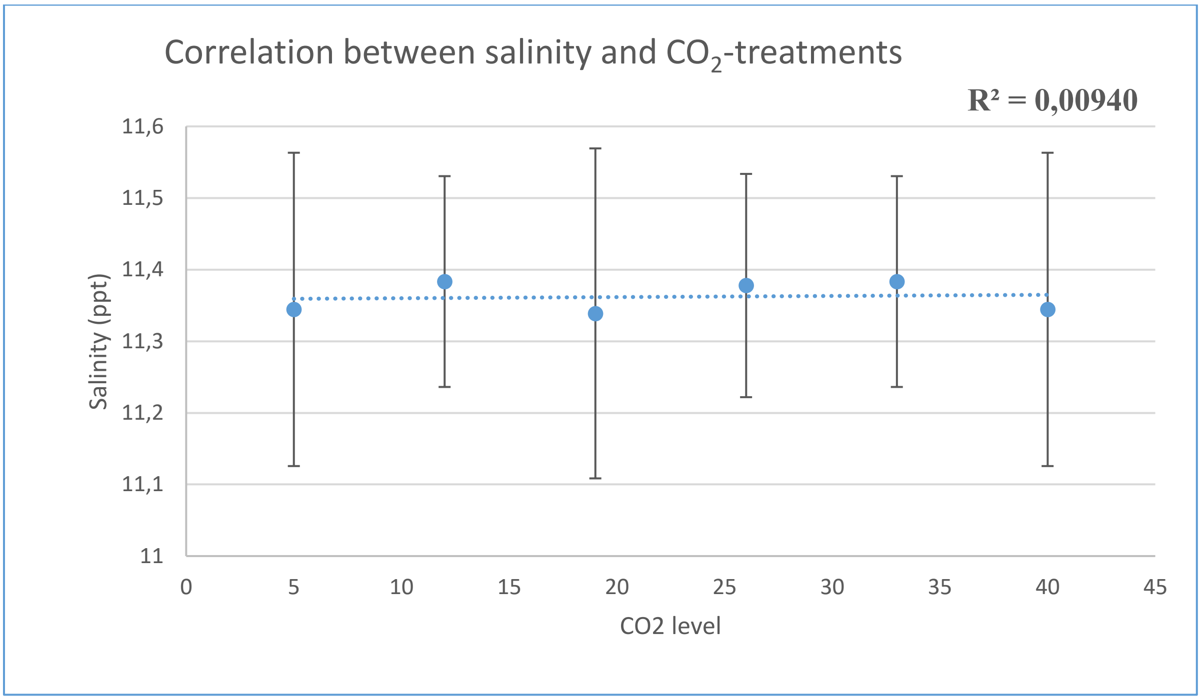


Figure 26: Changes in salinity in each treatment of CO2.

A one-way ANOVA test was performed at the p<0,05 level, which showed no statistical significant difference ($F_{5,102} = 0{,}221$, $p = 0{,}953$) between the treatments over time. A two-way ANOVA test was performed, which showed no statistical significant difference ($F_{25,72} = 1{,}02$, $p = 0{,}454$) between the CO_2-treatments over time. This is in association with the R^2-value (0,009), which shows no correlation between the treatments. The statistical data strongly suggests that there is no statistical significant difference of salinity between the treatments of CO_2 over time.

Figure 27 describes the changes in salinity during the experiment.

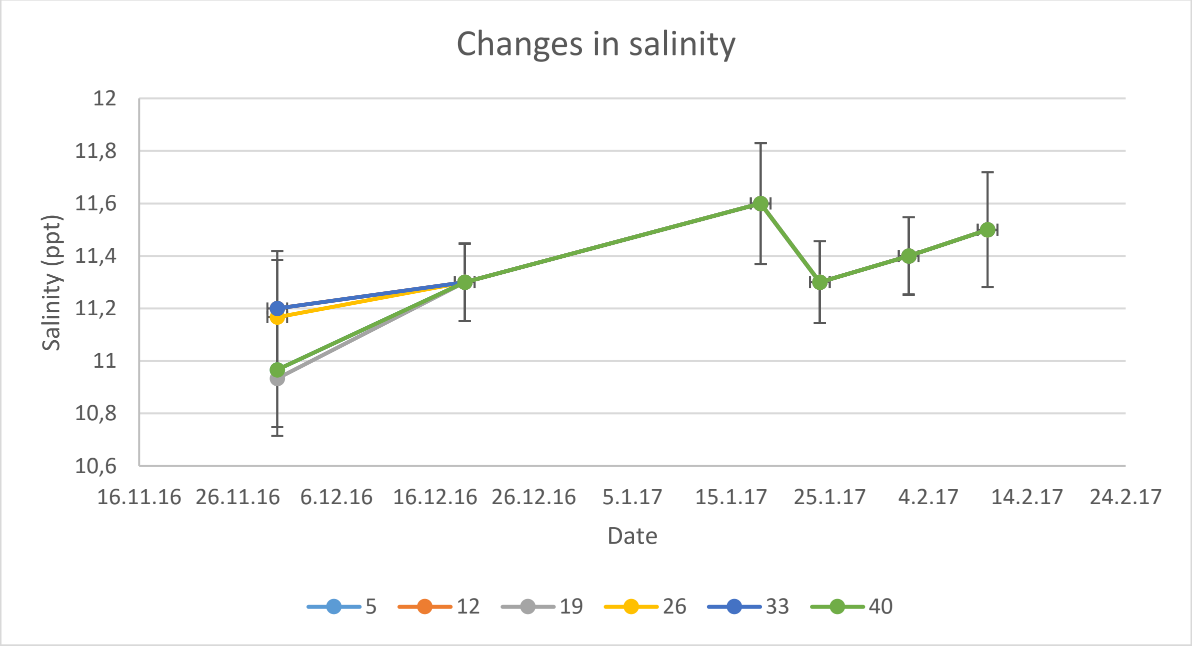


Figure 27: Changes in salinity in each treatment of CO2.

Salinity has the same trend over time as conductivity, which is as expected from the theory, where the amount of ions in water correlates to the ability water has to transfer heat and electricity. However, the correlation ($R^2 < 0,6$) is not large and don't suggest a large similarity.

There is a mean increase of all treatments from the first to the third sampling date of 0,527ppt ± 0,030 ppt and then a decrease of -0,300ppt ± 0,030ppt. At the end of the experiment, there is an increase between the sampling date 4 and 6 with 0,200ppt ± 0,030. The overall mean change from the first to the last sampling date is 0,427ppt ± 0,030.

Both figures (26 and 27) shows that the salinity values are mostly below the limit value of 11,5-12,5ppt, The total mean value of salinity through the whole period is 11,3620ppt ± 0,190, which is under the given concentration limit. How big a difference this is and how it affects possible accumulation of metals in the water and possible toxicity of metals in fish is uncertain. These values will probably not affect these factors and will probably have a bigger impact in a stress test of the fish, where the fish is released in freshwater and seawater.

An increase in total ion concentration could theoretically give an increase in salinity and conductivity. There is a total increase of ions in the water since there is an increase in both salinity and conductivity. An increase in temperature could increase the mobility of ions, increasing the conductivity. It does not seem, however to be a strong correlation between salinity and conductivity and temperature.

5.6 Turbidity

The results of the turbidity measurements were very low, with values down to 2,5-5 NTU. This did not correlate to the fast clogging of the filters, when a turbidity this small made the filter clog after 20 ml of sample filtration. A theory behind the low measurements is that most of the particles in the fish tanks are transparent. This means that the light that should reflect from the particles to the sensor is not. Turbidity measurements with a different turbidity sensor at Nofima, showed in general higher values, between 15-25 NTU. Further research on these particles and the measurement of these in RAS must be a priority.

5.7 Heavy metals

In order to get the best picture of possible accumulation of heavy metals in RAS, 64 elements were analysed by the ICP-MS. The reason behind choosing so many is the unknown accumulation and interaction with different CO_2 levels of heavy metals in RAS. After inspecting every element, it was decided to focus on seven elements. These have either showed some correlation with CO_2 or are important elements in aquaculture systems according to previous research.

After looking at the concentrations of all elements over time, two dates were removed from the data set. A fish sampling was done the first week in January, where many fish were removed and collected. A high volume of make-up water added to the system caused a high drop in concentration for the majority of the elements on the 06.01.2017. This is shown in appendix H, where these figures show a drop in concentration during this period. Because of the high volume of make-up water, the results was not because of the treatment of CO_2, but a result of an extraordinary system dilution. In appendix G, a table shows the make-up water in L/min. At 04.01.2017, the flow of the make-up water was much higher than the period before, diluting the system. The results from 11.01.2017 was also removed, but this was because of strong variance in the data set. These samples were not filtrated at Nofima, but a few months later at NTNU.

During the retention time, something with the chemistry in the samples changed and gave these variable results. This is also visible in the Appendix (H), with the large drops in concentration for some of the fish tanks.

The selected metals are presented in two different figures. The first figure shows the correlation between CO_2-treatments and the metal, with standard deviation. The second one shows the mean concentration value on each sampling date. These figures are have the same focus as with the physio-chemical parameters. A one-way and a two-way ANOVA test was performed on each selected metal, where the focus is if there is a statistical significant difference between the treatments of CO_2 over time.

The next subchapter will present the results of iron. Iron will have an own subchapter, while the rest of the elements will be discussed together.

5.7.1 Iron

Iron has a subchapter by its own because of the interesting results. The element showed an interesting trend when it came to correlation with the different CO_2 treatments.

Figure 28 shows difference between the treatments of CO_2, with a R^2-value that shows a strong correlation between the CO_2 treatments and concentration of iron.

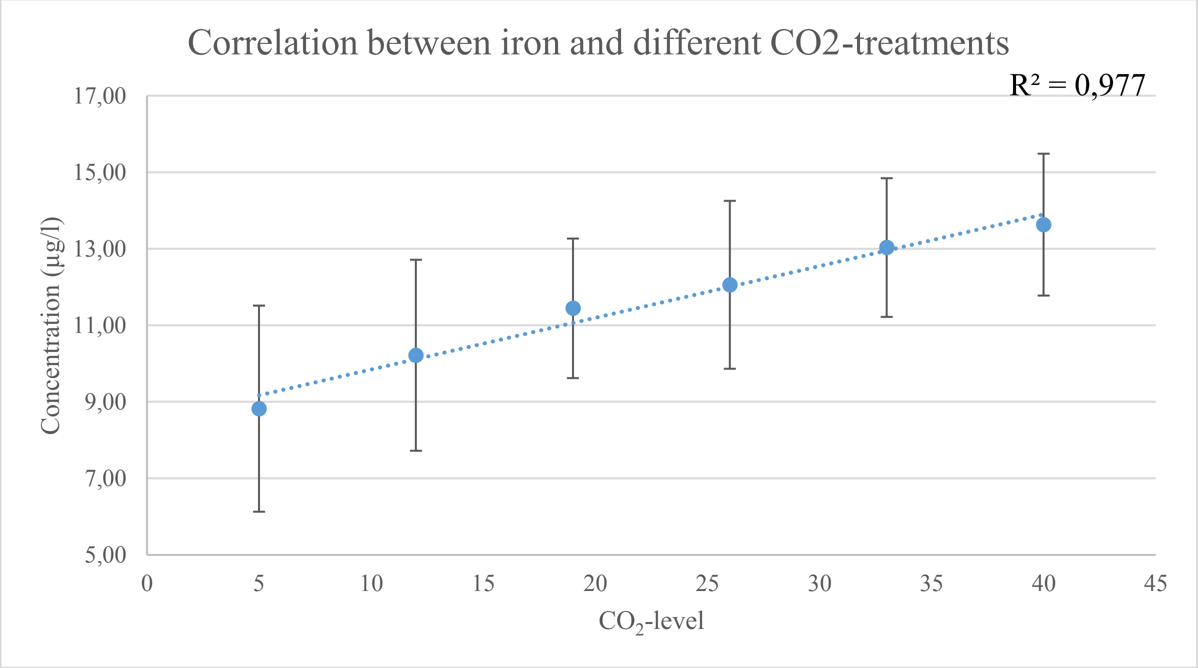


Figure 28: Change in concentration in different CO_2-treatments. The plot shows the positive and negative standard deviation of each data set. The correlation (R^2) is marked.

A two-way ANOVA test was performed at the $p<0,05$ level, which showed a statistical significant difference between the treatments over time ($F_{25,72} = 2,94$, $p = 0,000$). This is supported by the one-way ANOVA test that was performed $p<0,05$ level. This test did not find any difference between the treatments on any sampling date. The assumption is also supported by the correlation value $R^2 = 0,977$. The statistical data shows that there is a statistical significant difference in iron concentration between the different treatments of CO_2 over time.

Figure 29 shows the mean concentration of each treatment as a function of time.

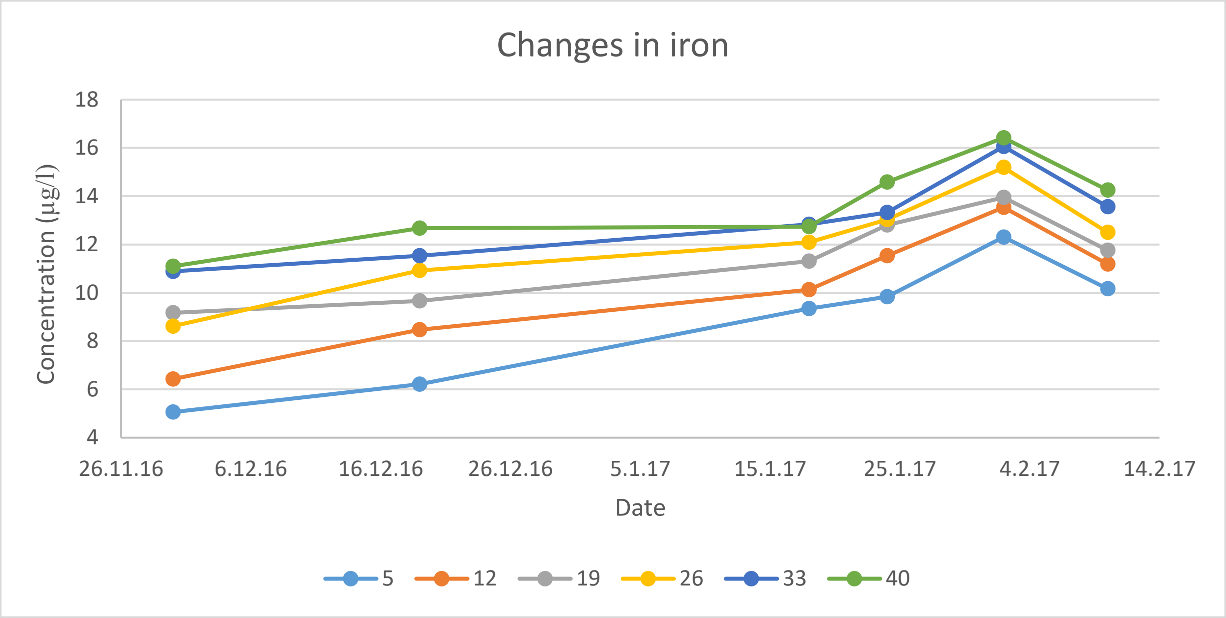


Figure 29: Changes in mean concentration of iron in each treatment of CO2.

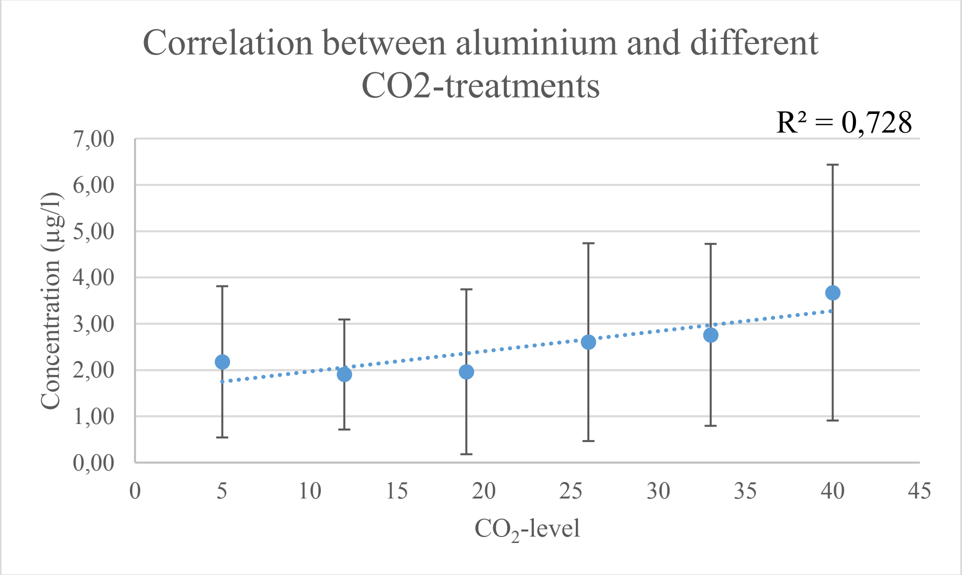
Figure 29 shows a change in concentration because of different CO_2 levels in the fish tanks. The trend is that a higher CO_2 level gives higher concentration in iron. The order in concentration is from lowest to highest treatment of CO_2 5mg/l < 12 mg/l > 19 mg/l > 26 mg/l > 33 mg/l > 40 mg/l.

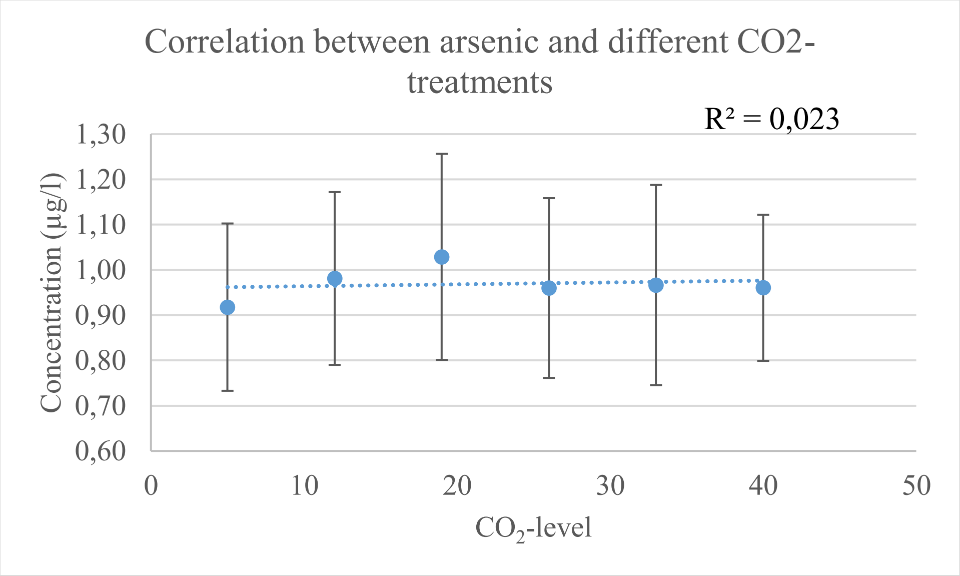
There was a statistical significant increase in concentration over the period of weeks when comparing to the starting sampling point (p = 0,000), however, there was a statistical significant drop (p = 0,000) from the two last sampling dates, giving as total mean decrease of -2,34µg/l ± 0,187. This drop in concentration is visual in figure 29, which could imply that the concentration of iron stabilizes and will not accumulate further. This is just an assumption, when the concentration could have increased again later in the experiment. When looking at the total mean increase in iron concentration from the first sampling date to the last, there is an increase of 3,69µg/l ± 0,187, which is a statistical significant difference according to the two-way ANOVA and a sign of what could be a small accumulation of iron in RAS. The total increase in concentration in percentage is 43,33%.

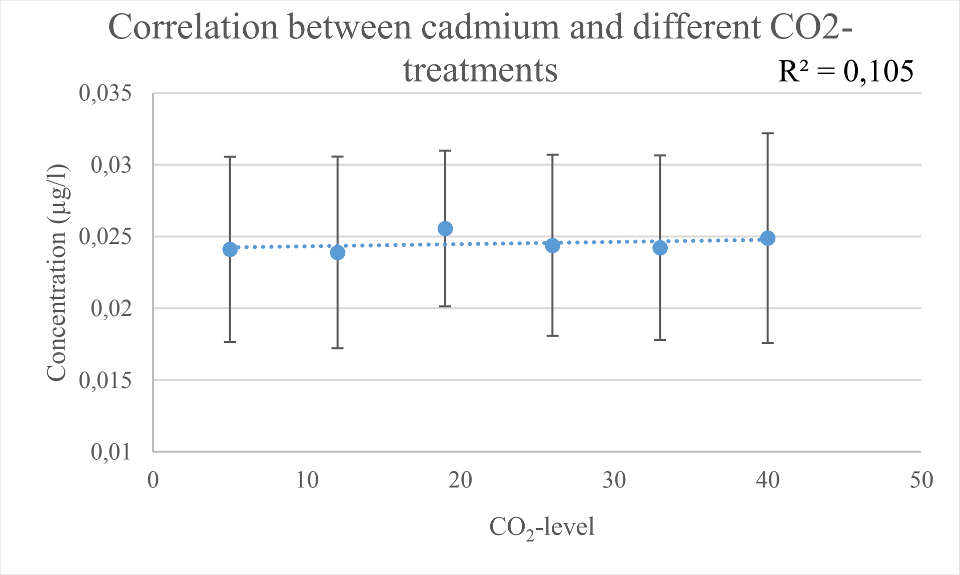
From table 3, the concentration limit in RAS for iron 150 µg/l. The total mean concentration is 11,532µg/l ± 2,623, which is much lower than the concentration limit. It is still a concern that the iron concentration increases with increasing CO_2 concentrations, since CO_2 has a tendency to accumulate in RAS. As mentioned in the theory, as CO_2 levels increases, microorganisms need less iron to consume. This could give more iron in labile form, which can be complexed, making iron more soluble. The redox potential in the tanks with 40 mg/l are around 200mV and the pH is between 6,7 - 6,9. These conditions give, as figure (10) shows a speciation of iron (II). This could increase the dissolved concentration of iron in the water and give a possible higher toxicity to the fish. Since the fish tanks are not independent, this possible speciation of iron could enter the other fish tanks.

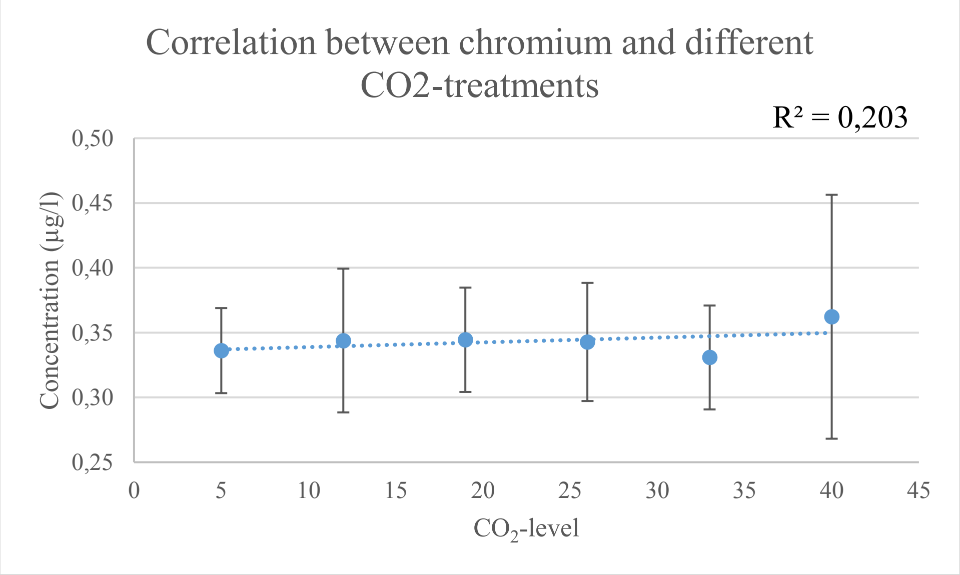
5.7.2 Different CO_2 – treatments on the selected metals

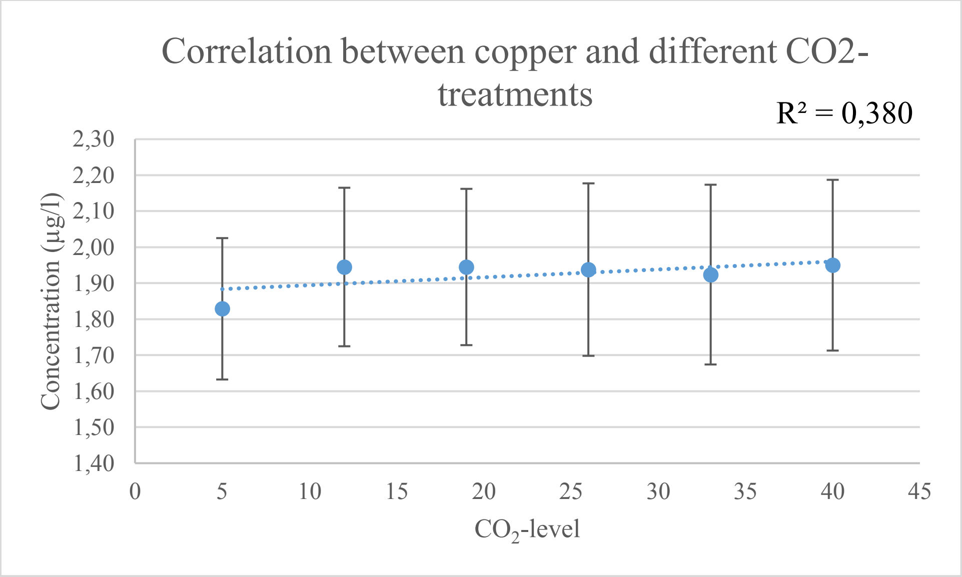
Presented below are figures describing the correlation between concentration of the element and the different treatments of CO_2. The correlation (R^2) is marked in the upper right corner. As with iron, a two-way ANOVA is performed, and will statistically show if concentration of the elements is different between the treatments of CO_2 over time.

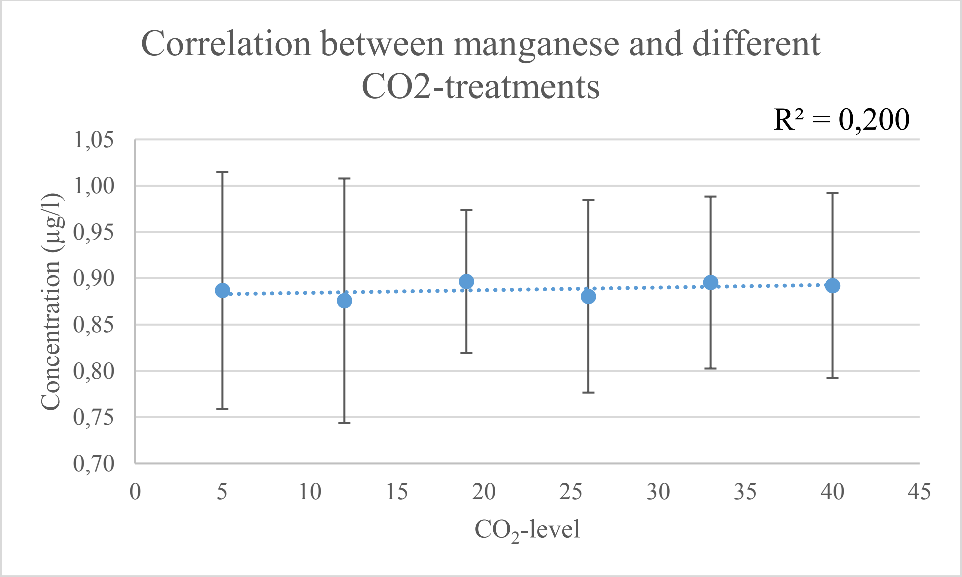


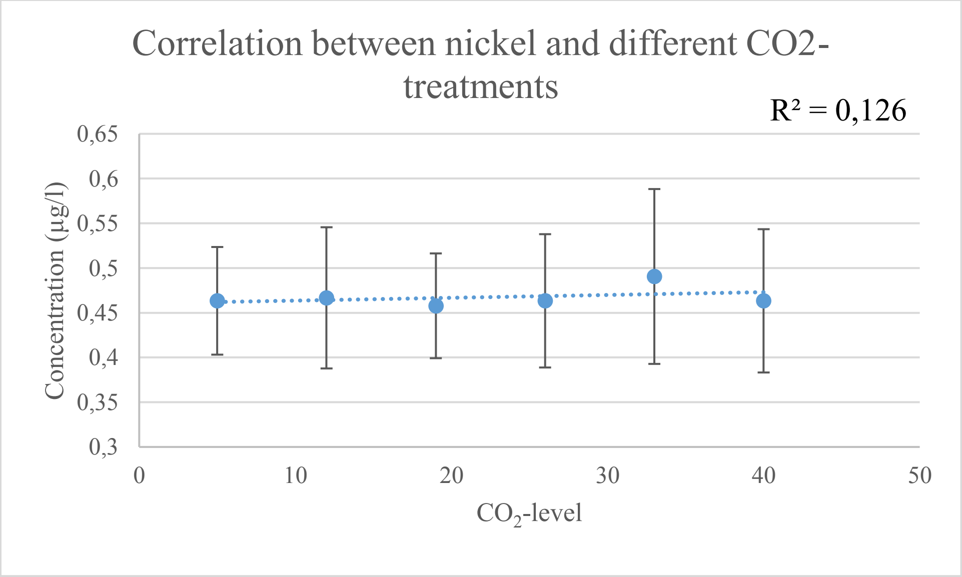

Correlation between arsenic and different CO2-treatments
R² = 0,023
Concentration (µg/l)
1,30
1,20
1,10
1,00
0,90
0,80
0,70
0,60
0
10
20
30
40
50
CO2-level

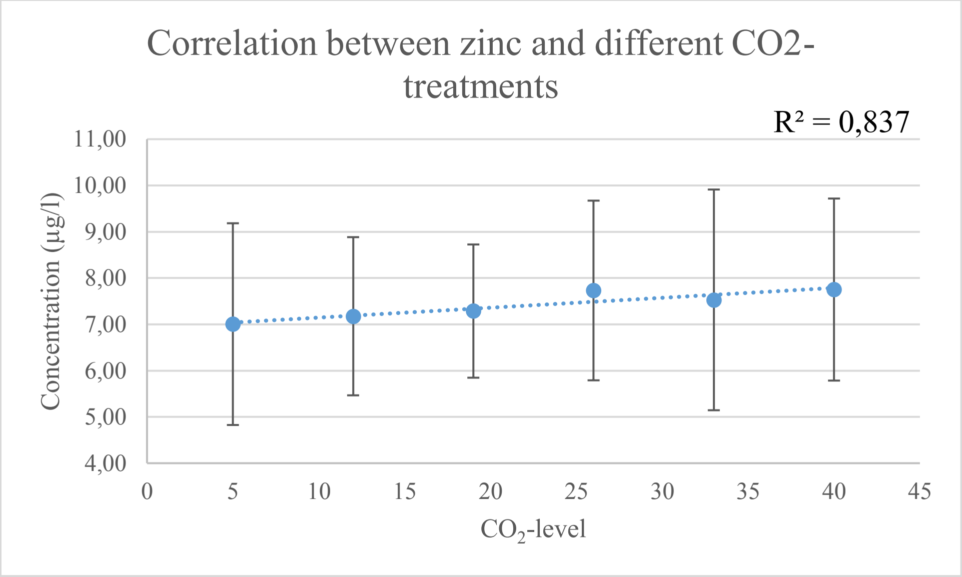

Correlation between cadmium and different CO2-treatments
R² = 0,105
Concentration (µg/l)
0,035
0,03
0,025
0,02
0,015
0,01
0
10
20
30
40
50
CO2-level


Correlation between chromium and different CO2-treatments
R² = 0,203
Concentration (µg/l)
0,50
0,45
0,40
0,35
0,30
0,25
0
5
10
15
20
25
30
35
40
45
CO2-level


Correlation between copper and different CO2-treatments
R² = 0,380
2,30
2,20
2,10
2,00
1,90
1,80
1,70
1,60
1,50
1,40
Concentration (µg/l)
0
5
10
15
20
25
30
35
40
45
CO₂-level


Correlation between manganese and different CO2-treatments
R² = 0,200
1,05
1,00
0,95
0,90
0,85
0,80
0,75
0,70
Concentration (µg/l)
0
5
10
15
20
25
30
35
40
45
CO₂-level


Correlation between nickel and different CO2-treatments
R² = 0,126
0,65
0,6
0,55
0,5
0,45
0,4
0,35
0,3
Concentration (µg/l)
0
10
20
30
40
50
CO₂-level



As the figures show, there is little correlation in the concentrations between each treatment of CO_2 for most of the elements, except for Zn and Al. A two-way ANOVA test was performed at the $p<0,05$ level to see if there was any statistical significant difference in the treatments over time, which is presented in Table 6.

Table 6: Overview of each elements F- and p-value, both for the interaction between week and CO_2 and only for CO_2. Adjusted peta squared for CO_2 and week, and correlation (R^2) are presented. The confidence level is = 0,05 (5%).

Element	**p-value (week*CO_2)**	**F-value (week*CO_2)**	**p-value (CO_2)**	**F-value (CO_2)**	**Adjusted peta squared (CO_2)**	**Adjusted peta squared (week)**	**Correlation (R^2)**
As	0,166	1,35	0,158	1,65	0,103	0,750	0,023
Al	0,514	0,971	0,225	1,43	0,090	0,359	0,729
Cd	0,301	1,17	0,582	0,76	0,050	0,847	0,105
Cr	0,398	1,07	0,848	0,400	0,027	0,267	0,203
Cu	0,228	1,25	0,985	0,132	0,009	0,854	0,380
Mn	0,009(all dates) 0,133 (three dates)	2,07(all dates) 1,66(three dates)	0,914	0,296	0,020	0,733	0,198
Ni	0,028(all dates) 0,307 (three dates)	1,80(all dates) 1,23(three date)	0,451	0,955	0,063	0,697	0,126
Zn	0,582	0,917	0,508	0,867	0,570	0,734	0,837

The values from Table 6 explains if there is any statistical significant difference between the treatments of CO_2 over time. According to the two-way ANOVA test at the $p<0,05$ level there is a statistical significant difference between the treatments over time for manganese ($F_{25,72}$ = 2,07, p = 0,009) and nickel ($F_{25,72}$ = 1,80, p = 0,028). Comparing this value to the correlation (R^2) values from the figures above and in table 6 there is no connection between the values. There is according to the R^2-value, low correlation between the treatments and CO_2.

What could have happened is a "follow-error" in the two-way ANOVA-test, where the values from the previous date affects the next. In table 6, there are two p-values for manganese and nickel. When comparing only the three last sampling dates at the $p<0,05$ level, there is no statistical significant difference between the treatments of CO_2 and manganese ($F_{10,36}$ = 1,66, p = 1,33) and nickel ($F_{10,36}$ = 1,23, p = 0,307). This indicates that there is no statistical significant difference between the CO_2 treatments over time, which is supported by both the F- and p-values for CO_2 excluded from weeks and the adjusted peta squared values in table 6. According to the F- and p-values for CO_2, there is no statistical significant difference between the treatments for manganese ($F_{5,72} = 0,914$, $p = 0,296$) and nickel ($F_{5,72} = 0,451$, $p = 0,955$).

The relative impact of time (week) is much larger than the relative impact of CO_2-treatments over time. For example, CO_2 has a relative impact on manganese of 0,020, while week has a relative impact of 0,733, which is a much larger value. A value of 0,020 is so small that it is negligible. In comparison, iron seems to have a similar relative impact from both time (0,926) and treatments (0,946). These are very similar and high values, meaning they both have a large impact and that they are approximately the same. It is possible that there is no difference between the different CO_2-treatments and that there is a difference of concentration over time. This difference is not because of the CO_2-treatments, but because of other factors.

Zinc ($R^2 = 0,837$) and aluminium ($R^2 = 0,729$) showed a correlation with the treatments of CO_2, but as the values in table 6 shows, the two-way ANOVA test shows that this correlation is not because of the different CO_2-treatments.

5.7.3 Comments on the concentration limit and increase in concentration over time

Table 7 shows the max, min, the total mean concentrations (µg/l) and standard deviation of all the selected elements. These values are compared to the given concentration limit.

Table 7: The table presents the given concentration limits of each element. Max, min, mean and standard deviation of each element is presented. The colour green signals that the values are under the concentration limit, while the colour red signals that the concentration is above the concentration limit.

Element	**Concentration limit (µg/l)**	**Max concentration (µg/l)**	**Min concentration (µg/l)**	**Total mean (µg/l) (N=108)**	**Total standard deviation(N=108)**
As	< 50	1,93	0,58	0,962	2,20
Al	< 10	15,3	-0,007	2,53	2,75
Cd	< 5	0,034	0,013	0,245	0,006
Cr		0,950	0,350	0,343	0,783
Cu	< 30	2,41	1,59	1,94	0,236
Fe	< 150	17,2	4,22	11,5	2,62
Mn	< 10	1,13	0,609	0,888	0,112
Ni	< 100	0,77	0,280	0,468	0,082
Zn	< 5	14,6	3,78	7,42	2,09

Table 7 shows that all of the elements have a total mean value below the concentration limit, except for zinc (7,42 ± 2,09). Aluminium has a maximum value above the concentration limit, but a total mean value below the limit (2,53 ± 2,75). For both of these elements, especially for aluminium, the standard deviation is high, which implies a big variance in the data set. Even so, the few samples with high concentrations of aluminium have an rsd-value < 10, which implies that the high values are certain. The standard deviation of zinc is also high, but since the total mean value is above the limit it is certain to say that most of the values are over the given concentration limit. These values could exceed the total amount of zinc the fish needs and cause a negative effect, and the few samples with high aluminium concentrations causes concern for further accumulation over time.

Concentrations from the make-up water could explain why some elements (Al, Zn) have high concentrations.

Table 8: This table shows the concentrations of selected metals in the make-up water from both seawater and groundwater from 18th of January 2017. The RSD values of each concentration is to the right of the concentration. The seawater was analysed two times.

Element	Seawater test 1	RSD	Seawater test 2	RSD	Ground-water	RSD
Fe	0,030	10,2	0,130	4,70	0,180	6,00
Mn	0,068	15,6	0,054	20,0	10,6	4,70
Ni	0,390	17,3	0,390	19,9	0,180	10,8
Cu	0,400	6,70	0,460	7,50	0,240	15,5
As	1,56	14,4	1,42	9,50	0,060	19,4
Cr	0,220	26,0	0,230	24,4	0,130	32,3
Zn	1,390	8,00	1,04	12,4	0,360	21,8
Al	12,5	4,00	12,6	3,20	14,3	2,80

Table 8 shows that some of the rsd-values are high (> 20). All of the values are in general low, with exception of manganese from the groundwater (10,6 µg/l) and aluminium from both seawater and groundwater. From the given concentration limits, these values are both above the limit of < 10. Since there is only one sample from each, these values will only be indicators and not secure values.

Concentrations in make-water shows that aluminium has a high concentration from both seawater and groundwater. The samples are, as mentioned, collected on the 18th January, and works as an indication for make-up water concentrations, since there is no mean concentration over time. Aluminium have occasionally high concentrations as can be seen in the high standard deviation. A source of these high concentrations of aluminium could be because of changes in concentration in make-up water, since there is probably no aluminium in the fish feed or because of leaching from pipes. From the given conditions in water, there is no evidence that labile, inorganic aluminium will occur. The pH is simply too high (> 6,6), whereas a labile, inorganic form of aluminium demands a pH < 5,5.

Concentrations of zinc in the make-up water are not high and do not explain why zinc concentrations are high. As mentioned in the theory, accumulation of zinc in RAS, could be because of released Zn^{2+} from fish feed. Fish-feed contains zinc because it is an essential nutrient. When the fish feed is in water, zinc can be dissolved and released into the water, increasing the concentration of zinc in the system. This theory is supported by the pH and redox potential in the water. The low pH and redox could increase the amount of Zn^{2+} in water, making it the dominant form of zinc. Zinc concentrations can also be increased by corrosion from piped and fittings, however most of the pipes at Nofima is made of plastic, making this assumption less likely.

The table 9 describes the total mean increase of concentration and the total increase in concentration from the first sampling date to the last in percentage (%). The reason for looking at these values is to see if there is a statistical significant increase of the total concentration of the elements and to discuss the possible reasons for this increase.

Table 9: The table shows the total mean increase in concentration (µg/l and %) for all selected elements from the first to the last sampling date.

Elements	Total mean concentration increase (µg/l) and standard deviation	Total mean increase in concentration (%)
Al	0,998 ± 0,789	26,7
As	0,314 ± 0,314	40,8
Cd	0,009 ± 0,001	40,0
Cr	0,035 ± 0,024	13,8
Cu	0,162 ± 0,036	9,09
Ni	0,046 ± 0,017	8,76
Zn	2,10 ± 0,416	34,8
Mn	0,106 ± 0,021	13,0

In appendix 11, graphs of all the selected metals are presented. These graphs presents the mean concentration of each element in all the different CO_2 treatments. The overall trend in concentrations is either that there is a slight drop in concentration between the first two sample dates or that they are approximately the same, except for manganese, where the concentration increases. For all the elements there is an increase in concentration up to the second last sampling date and a decrease in concentration on the last sampling date.

Table 9 explains the total mean increase of concentration from the first to the last sampling date. The standard deviation is given to see the total variance in the data set. All the elements, except Zn, Cd, As and Fe show a low increase in concentration. The total mean increase of these elements are according to the two-way ANOVA test statistical significant different ($p < 0,05$). The increase in zinc-concentrations could be as a result of zinc from fish-feed. The elements with the highest total increase in concentration (%) have a strong correlation between each other. As showed the lowest correlation with Zn and Cd with a correlation respectively of 0,66 and 0,62. The other elements have a correlation above 0,8. This indicates that the source of the elements could be the same.

Several elements had, as explained earlier, pH and redox potential values in the water that could increase the concentrations of the divalent form of the element. These were, as mentioned, Cd, Mn, Fe, Zn and As. It seems that Fe, Zn, As and Cd could have an effect of these conditions on the accumulation in RAS over time. Mn have a low total increase in concentration, which could be because of other factors such as the make-up water.

5.7.4 TOC and water hardness

The results of the TOC measurements where all under the detection limit, except for two samples, where the concentrations was small. It gives the assumption that TOC plays a smaller part than expected. The reason behind analysing TOC was to study a possible correlation between TOC and the treatments of CO_2, where the theory behind it was that the TOC concentrations would increase with an increase in CO_2 concentrations.

Water hardness was calculated as explained in the theory, by using an online calculator at Lenntech.com. The mean concentrations of calcium and magnesium at each sample date was calculated. The concentrations of magnesium and calcium was not affected by the treatments of CO_2.

Table 10: The table shows the mean concentration of magnesium and calcium (µg/l) in every sample date. Calculations of water hardness in German scale (°dH) is presented.

Date	**Magnesium concentration [µg/l]**	**Calcium concentration [µg/l]**	**Water hardness (°dH German scale)**
30.11.16	393 505	138 047	110
19.12.16	401 891	140 451	112
18.01.17	398 967	138 769	111
24.01.17	398 967	135 380	111
02.02.17	398 564	132 995	110
24.02.17	403 920	132 561	111

The results show a stable trend where the water is of medium hardness. There is little or no difference in the concentrations of magnesium and calcium over the experimental period. This gives a good stability in the hardness of water, indicating no accumulation of magnesium and calcium in RAS. This also indicates a stable alkalinity, since alkalinity is also highly dependent on $CaCO_3$ and from the fact that bicarbonate is added continuously into the system. These values show that there is probably no concern of increased copper toxicity. The copper concentrations were low as well, meaning a low impact on the fish. In addition to the water hardness, there is no data to say that there is a possible copper toxicity.

5.8 Error source

There are some errors concerning the two-way ANOVA test. There are several assumptions that needs to be filled before a test can be performed, as the assumption that there is a similar amount of data in each data set and that each data set is independent of each other. The two-way ANOVA test showed that the data from the previous date had an impact on the next data set and that they are not independent of each other. This could lead to assumptions that are false, because the values from the previous date affects the next, as was seen with Ni and Mn. Comparing the values from the make-up water and inlet water with the concentrations from the outlet water could lead to false assumptions. There was only one sample collected for each dat in the inlet water and make-up water, compared to three replicates from the outlet water. The data from the outlet water is stronger and more reliable than that from the make-up water and inlet water. Some of the values from the outlet water and make-up water also had a high rsd-value, which signals caution using these values.

Another error was dilution of the water in RAS when collecting data during the experiment. One sampling date was removed because of a high dilution of the system. These short periods with dilution of the system could have disturbed some of the other collection dates as well, and possibly decreased the possible accumulation of heavy metals in the system over time.

6. Conclusion

This master thesis has looked at how different treatments of CO_2 have affected the physio-chemical parameters and heavy metals in RAS.

Of the physio-chemical parameters, pH showed a clear statistical significant difference between the treatments of CO_2. Conductivity had a statistically significant difference between the treatments of CO_2, but it seems that the statistical difference is mainly because of the difference over time. The redox potential showed a statistical significant difference between the 5 mg/l treatment and the rest. This could be due to other factors besides CO_2, as the mixing of water between holding tank 1 and 2. When it came to changes in the physio-chemical parameters over time, these where quite stable. Temperature and salinity was at some dates outside the given limit. Redox potential is in general low, which could increase the amount of arsenite in water. The pH is also low in the treatments with high CO_2 (26,33 and 40mg/l). These values along with low redox potential could increase the concentration of free heavy metals.

Of all the elements analysed, iron showed a statistical significant difference between the treatments of CO_2 over time. All of the elements had an overall increase in concentration from the beginning to the end of the experiment. The increase was low for all the elements except for zinc, iron, cadmium and arsenic, where the total increase was over 34 % for all elements.

Zinc concentrations (mean) are over the concentration limit. This could be because of dissolved zinc from fish feed. The conditions give reason to think that released zinc from fish feed is in the divalent form. Aluminium had some values that was higher than the given limit. Conditions does not favour labile, inorganically bound aluminium, but organically bound, less toxic aluminium.

7. Recommended work

This thesis was at the beginning very open, since there have not been many studies on heavy metals in RAS. This has led to a high complexity. Further research could be more narrow, and below are some reflections on such future work.

The reason behind the strong correlation between iron and CO_2 should be studied. The reason behind the accumulation of iron is uncertain and should be investigated. A theory is that the bicarbonate added to the water contains iron. This could increase the concentration of iron in the water. Another theory that should be in focus is the fish-feed related accumulation of heavy metals. Heavy metals as iron and zinc could be a large part of the vitamin premix in the food. These metals could dissolve in the water and increase the concentration of these metals. Another possibility is that some elements could accumulate in the biofilter used in the biological.

The redox potential in the fish tanks are quite low. Such a low potential could increase the concentration of arsenite, but the actual effect of arsenite was not studied in this thesis and could be something of interest in further research. The pH is also low, which could increase the concentration of some elements. Is the given pH limit of 6,8 too low? This could be an interesting question to discuss further. The difference in 5mg/l treatments and the rest could also be studied further. It would be interesting to look at differences in pH and redox potential before and after the fish tanks in order to study differences when water is mixed from the two holding tanks and when fish tanks only get water from one holding tank.

Not all elements were analysed thoroughly in this theses. Elements as mercury could be of interest because the organic bound methylmercury can be very toxic to marine animals.

In order to look at accumulation it could be interesting to study these elements over a longer period of time and without dilution of the system. Would the accumulation of the elements be larger if the experimental period was longer and without dilution of the system?

8. Bibliography

ADHIKARI, S. 2006. Combined effects of water pH and alkalinity on the accumulation of lead, cadmium and chromium to Labeo rohita (Hamilton). 289-296.

ALLOWAY, B. J. 2010. Heavy Metals in Soils. *Springer,* 22.

AQUA, H. 2017. *Technology for sustainable groth in aquaculture* [Online]. Available: http://www.haugeaqua.com/Technology/ [Accessed 26.02 2017].

AQUAFARMS, N. 2015. *Fredrikstad seafoods satser videre* [Online]. Available: http://www.nordicaquafarms.com/portfolio_page/fredrikstad-seafoods-satser-videre/ [Accessed 28.02 2017].

AYER, N. W. & TYEDMERS, P. H. 2009. Assessing alternative aquaculture technologies: life cycle assessment of salmonid culture systems in Canada. *Journal of Cleaner Production,* 17**,** 362-373.

AYRES, D. M., DAVIS, A. P. & GIETKA, P. M. 1994. Removing heavy metals from wastewater. *Engineering Research Centre Report,* 90.

BISUTTI, I., HILKE, I. & RAESSLER, M. 2004. Determination of total organic carbon–an overview of current methods. *TrAC Trends in Analytical Chemistry,* 23**,** 716-726.

BREGNBALLE, J. 2015. *A guide to Recirculation Aquaculture,* FAO and Eurofish, the Food and Agriculture Organization of the United Nations (FAO) and EUROFISH International Organisation.

BYE, G. R. 2017. *RE: Pictures NOFIMA.*

CARDS22.COM. 2017. *Printable images, crads and posters* [Online]. Available: http://cards22.com/printable-periodic-table-of-elements-with-names/ [Accessed 28.05 2017].

CHAPMAN, D. V. 1996. Water quality assessments: a guide to the use of biota, sediments, and water in environmental monitoring.

CHEATHAM, M. M., SANGREY, W. F. & WHITE, W. M. 1993. Sources of error in external calibration ICP-MS analysis of geological samples and an improved non-linear drift correction procedure. *Spectrochimica Acta Part B: Atomic Spectroscopy,* 48**,** 487-506.

COLT, J. 2006. Water quality requirements for reuse systems. *Aquacultural Engineering,* 34**,** 143-156.

DAVIDSON, J., GOOD, C., WELSH, C., BRAZIL, B. & SUMMERFELT, S. 2009. Heavy metal and waste metabolite accumulation and their potential effect on rainbow trout performance in a replicated water reuse system operated at low or high system flushing rates. *Aquacultural engineering,* 41**,** 136-145.

DEKHTYAREV, V. 2014. Comparison of Atlantic salmon net pen and recirculating aquaculture systems: economical, technological and environmental issues. 14-16.

DORTWEGT, R. & MAUGHAN, E. 2001. The chemistry of copper in water and related studies planned at the advanced photon source. Conf. *Proc C,* 106181**,** 1456-1458.

EPA. *Estuarine Science* [Online]. omp.edu. Available: http://omp.gso.uri.edu/ompweb/doee/science/physical/chsal1.htm [Accessed 12.05 2017].

EPA. 2017. *Chromium in drinking water* [Online]. EPA.gov. Available: https://www.epa.gov/dwstandardsregulations/chromium-drinking-water [Accessed 14.05 2017].

ERICKSON, R. J., BENOIT, D. A., MATTSON, V. R., LEONARD, E. N. & NELSON, H. P. 1996. The effects of water chemistry on the toxicity of copper to fathead minnows. *Environmental Toxicology and Chemistry,* 15**,** 181-193.

FISKERIDIREKTORATET 2003. Merknader til Forskrift om drift av akvakulturanlegg (driftsforskriften) Fiskehelse.no: Mattilsynet og Fiskedirektoratet.

FIVELSTAD, S. 1998. Fivelstad Subletahl effects and safe levels of CO2, 1998.pdf. 305-316.

FIVELSTAD, S. 2013. Long-term carbon dioxide experiments with salmonids. *Aquacultural Engineering,* 53**,** 40-48.

FONDRIEST ENVIRONMENTAL, I. 2013a. *pH of water* [Online]. Fondriest.com. Available: http://www.fondriest.com/environmental-measurements/parameters/water-quality/ph/ [Accessed 03.05 2017].

FONDRIEST ENVIRONMENTAL, I. 2013b. *Water quality* [Online]. Fondriest.com. Available: http://www.fondriest.com/environmental-measurements/parameters/water-quality/ [Accessed 02.05 2017].

FONDRIEST ENVIRONMENTAL, I. 2014a. *Conductivity, salinity and total dissolved solids* [Online]. Fondriest.com. Available: http://www.fondriest.com/environmental-measurements/parameters/water-quality/conductivity-salinity-tds/#cond6 [Accessed 10.05 2017].

FONDRIEST ENVIRONMENTAL, I. 2014b. *Water temperature* [Online]. Fondriest.com. Available: http://www.fondriest.com/environmental-measurements/parameters/water-quality/water-temperature/ [Accessed 10.05 2017].

FRIBERG, L. 1986. *Handbook of the toxicology of metals*, Elsevier.

GAUT, S. 2005. Factors influencing microbiological quality of groundwater from potable water supply wells in Norwegian crystalline bedrock aquifers.

GEONORGE. 2017. *TOC - Total Organic Carbon* [Online]. Geonorge.com. Available: https://kartkatalog.geonorge.no/metadata/norges-geologiske-undersokelse/toc-total-organic-carbon/0035d0e4-8c11-4805-9f09-dfe55fd06204 [Accessed 30.05 2017].

GMBH. 2005. *Multi 350i* [Online]. WTW. Available: http://www.geotechenv.com/Manuals/WTW_Manuals/Multi_350i.pdf [Accessed 07.07 2017].

HEM, J. D. 1972. Chemistry and occurrence of cadmium and zinc in surface water and groundwater. *Water Resources Research,* 8**,** 661-679.

HURLBERT, S. H. 1984. Pseudoreplication.pdf. 187-211.

JENNER, G., LONGERICH, H., JACKSON, S. & FRYER, B. 1990. ICP-MS—a powerful tool for high-precision trace-element analysis in earth sciences: evidence from analysis of selected USGS reference samples. *Chemical Geology,* 83**,** 133-148.

KHAN, M., AHMED, S., CATALIN, B., KHODADOUST, A., AJAYI, O. & VAUGHN, M. 2006. Effect of temperature on heavy metal toxicity to juvenile crayfish, Orconectes immunis (Hagen). *Environmental toxicology,* 21**,** 513-520.

KLAASSEN, C. D. 2013. *Casarett & Doull*s Toxicology,* McGraw-Hill Education.

LENNTECH. 2017a. *Aluminum (Al) and water* [Online]. Lenntech.com. Available: http://www.lenntech.com/periodic/water/aluminium/aluminum-and-water.htm [Accessed 10.05 2017].

LENNTECH. 2017b. *Nickel (Ni) and water* [Online]. Lenntech.com. Available: http://www.lenntech.com/periodic/water/nickel/nickel-and-water.htm [Accessed 24.05 2017].

LENNTECH. 2017c. *Water conductivity* [Online]. Lenntech.com. Available: http://www.lenntech.com/applications/ultrapure/conductivity/water-conductivity.htm [Accessed 04.05 2017].

LENNTECH. 2017d. *Water Hardness* [Online]. Available: http://www.lenntech.com/ro/water-hardness.htm [Accessed 26.05 2017].

LENNTECH. 2017e. *Zinc (Zn) and water* [Online]. Lenntech.com. Available: http://www.lenntech.com/periodic/water/zinc/zinc-and-water.htm#ixzz4h4D5SRui [Accessed 24.04 2017].

LIU, Y., HU, Z., GAO, S., GÜNTHER, D., XU, J., GAO, C., & CHEN, H. 2008. In situ analysis of major and trace elements of anhydrous minerals by LA-ICP-MS without applying an internal standard. *Chemical Geology,* 257**,** 34-42.

LIU, Y. & VANHAUWAER BJELLAND, H. 2014. Estimating costs of sea lice control strategy in Norway. *Preventive veterinary medicine,* 117**,** 469-477.

MARTINS, C. I., EDING, E. H. & VERRETH, J. A. 2011. The effect of recirculating aquaculture systems on the concentrations of heavy metals in culture water and tissues of Nile tilapia Oreochromis niloticus. *Food Chemistry,* 126**,** 1001-1005.

MARTINS, C. I. M., EDING, E. H., VERDEGEM, M. C. J., HEINSBROEK, L. T. N., SCHNEIDER, O., BLANCHETON, J. P., D'ORBCASTEL, E. R. & VERRETH, J. A. J. 2010. New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. *Aquacultural Engineering,* 43**,** 83-93.

MARTINS, C. I. M., PISTRIN, M. G., ENDE, S. S. W., EDING, E. H. & VERRETH, J. A. J. 2009. The accumulation of substances in Recirculating Aquaculture Systems (RAS) affects embryonic and larval development in common carp Cyprinus carpio. *Aquaculture,* 291**,** 65-73.

MARX, M. J. L. M. L. 2013. *Introduction to Mathematical Statistics and Its Applications: Pearson New International Edition*, Pearson Education Limited.

MILJØDIREKTORATET. 2015. *Lakselus* [Online]. Available: http://www.miljodirektoratet.no/no/Tema/Arter-og-naturtyper/Villaksportalen/Pavirkninger/Lakselus/ [Accessed 20.02 2017].

MILLER, T. G. & MACKAY, W. 1980. The effects of hardness, alkalinity and pH of test water on the toxicity of copper to rainbow trout (Salmo gairdneri). *Water Research,* 14**,** 129-133.

MOTA, V. 2017a. *RE: Experimental design*

MOTA, V. 2017b. *RE: Water quality standards Aquaculture.*

NEFF, J. M. 1997. Ecotoxicology of arsenic in the marine environment. *Environmental Toxicology and Chemistry,* 16**,** 917-927.

NIVALDI, J. T. 2011. *Chemistry: A molecular approach* Pearson.

NOFIMA. 2015. *High cost in salmon production* [Online]. Available: https://nofima.no/en/forskning/naringsnytte/high-cost-in-salmon-production/ [Accessed 20.02 2017].

NORDSTRØM, D. K. 2002. Worldwide Occurrences of Arsenic in Ground Water. *Sciences compass.*

OKE, S. & VERMEULEN, D. 2017. Geochemical Modeling and Remediation of Heavy Metals and Trace Elements from Artisanal Mines Discharge. *Soil and Sediment Contamination: An International Journal,* 26**,** 84-95.

OPD. 2017. *Marin Dounut - a new technology for closed fish farming* [Online]. [Accessed 27.02 2017].

ORAM, B. 2017a. *Manganese and iron in drinking water* [Online]. Water Research Center. Available: http://www.water-research.net/index.php/manganese [Accessed 14.05 2017].

ORAM, B. 2017b. *The Role of Alkalinity Citizen Monitoring* [Online]. Water Research Center. Available: http://www.water-research.net/index.php/the-role-of-alkalinity-citizen-monitoring [Accessed 23.05 2017].

PARK, H. S., LEE, J. S., HAN, J., PARK, S., PARK, J. & MIN, B. R. 2015. CO2 fixation by membrane separated NaCl electrolysis. *Energies,* 8**,** 8704-8715.

S.E.MANAHAN 2009. *Environmental chemistry,* CRC Press.

SINTEF. 2016. *Offshore salmon fish farming* [Online]. Available: https://www.sintef.no/en/projects/offshore-salmon-fish-farming/ [Accessed 27.02 2017].

SKOOG, D. A. 2004. *Fundamentals of Analytical Chemistry* Brooks/Cole.

SPSS. 2017a. *One-Way ANOVA* [Online]. Statistics.laerd.com. Available: https://statistics.laerd.com/statistical-guides/one-way-anova-statistical-guide.php [Accessed 18.05 2017].

SPSS. 2017b. *Two-way ANOVA in SPSS* [Online]. Statistics.laerd.com. Available: https://statistics.laerd.com/spss-tutorials/two-way-anova-using-spss-statistics.php [Accessed 20.05 2017].

STUMM, J. J. M. W. 1996. *Aquatic Chemistry* Environmental Science and Technology, John Wiley & sons, INC.

SWM. 2017. *Quality of Drinking Water* [Online]. Available: https://www.swm.de/english/m-wasser/quality.html [Accessed 27.05 2017].

TAKATSU, A., KUROIWA, T. & UCHIUMI, A. 1999. Arsenic accumulation in organs of the fresh water fish Tribolodon hakonensis. *Journal of trace elements in medicine and biology,* 13**,** 176-179.

TEKMAR, T. 2009. *Torch combustion TOC analyzer* [Online]. Schientec. Available: http://www.scientech.com.tw/EN/product/sa/teledyne2/torch.asp [Accessed 23.05 2017].

TEKMAR, T. 2014a. *Torch combustion TOC analyzer* [Online]. Teledyntekmar.com. Available: http://www.teledynetekmar.com/Documents/Torch%20Product%20Brochure%202014.pdf [Accessed 23.05 2017].

TEKMAR, T. 2014b. *Understaning Total organic carbon (TOC)* [Online]. TeledynTekmar.com. Available: http://blog.teledynetekmar.com/blog/bid/392322/Understanding-Total-Organic-Carbon-TOC-and-Why-it-should-be-Measured [Accessed 30.05 2017].

TERJESEN, B. F., SUMMERFELT, S. T., NERLAND, S., ULGENES, Y., FJÆRA, S. O., MEGÅRD REITEN, B. K., SELSET, R., KOLAREVIC, J., BRUNSVIK, P., BÆVERFJORD, G., TAKLE, H., KITTELSEN, A. H. & ÅSGÅRD, T. 2013. Design, dimensioning, and performance of a research facility for studies on the requirements of fish in RAS environments. *Aquacultural Engineering,* 54**,** 49-63.

THOMAS, R. 2001. A beginner's guide to ICP-MS. *Spectroscopy,* 16**,** 38-42.

THORARENSEN, H. & FARRELL, A. P. 2011. The biological requirements for post-smolt Atlantic salmon in closed-containment systems. *Aquaculture,* 312**,** 1-14.

TIMMONS, M. B., EBELING, J.M 2013. *Recirculating Aquaculture* Aquaculture.

VKM, V. F. M. 2008. Opinion of the Panel on Animal Health and Welfare of the Norwegian Scientific Committee for Food Safety. *In:* AUTHORITY, T. N. F. S. (ed.).

VUORI, K.-M. Direct and indirect effects of iron on river ecosystems. Annales Zoologici Fennici, 1995. JSTOR, 317-329.

VWR Environmental testing of water. *GE Healthcare.*

VWR. 2017. *Portable turbidity meters, Turbiquant 1100* [Online]. vwr.com. Available: https://uk.vwr.com/store/product/598937/portable-turbidity-meters-turbiquant-1100 [Accessed 06.08 2017].

WATER, G.-M. 2015. Groundwater Salinty. *How to minimize your risks.* g-mwater.com.au.

WHO 2003a. Chromium in Drinking-Water.

WHO 2003b. Iron in Drinking-Water.

WHO 2003c. Zinc in Drinking-Water.

WHO 2004. Copper in Drinking-Water.

WHO 2011a. Arsenic in drinking water. *In:* ET.AL, J. C. (ed.). WHO.

WHO 2011b. Cadmium in Drinking-Water.

WHO 2011c. Hardness in Drinking-Water. World Healt Organisation.

WHO 2011d. Manganese in Drinking-Water. WHO.in.

WITESKA, B. J. M. 2006. THE METAL UPTAKE AND ACCUMULATION IN FISH LIVING IN POLLUTED WATERS. *Springer***,** 107-112.

WTW 2017a. Conductivity meters. wtw.de.

WTW 2017b. IDS ORP electrodes with universal plug head. wtw.com.

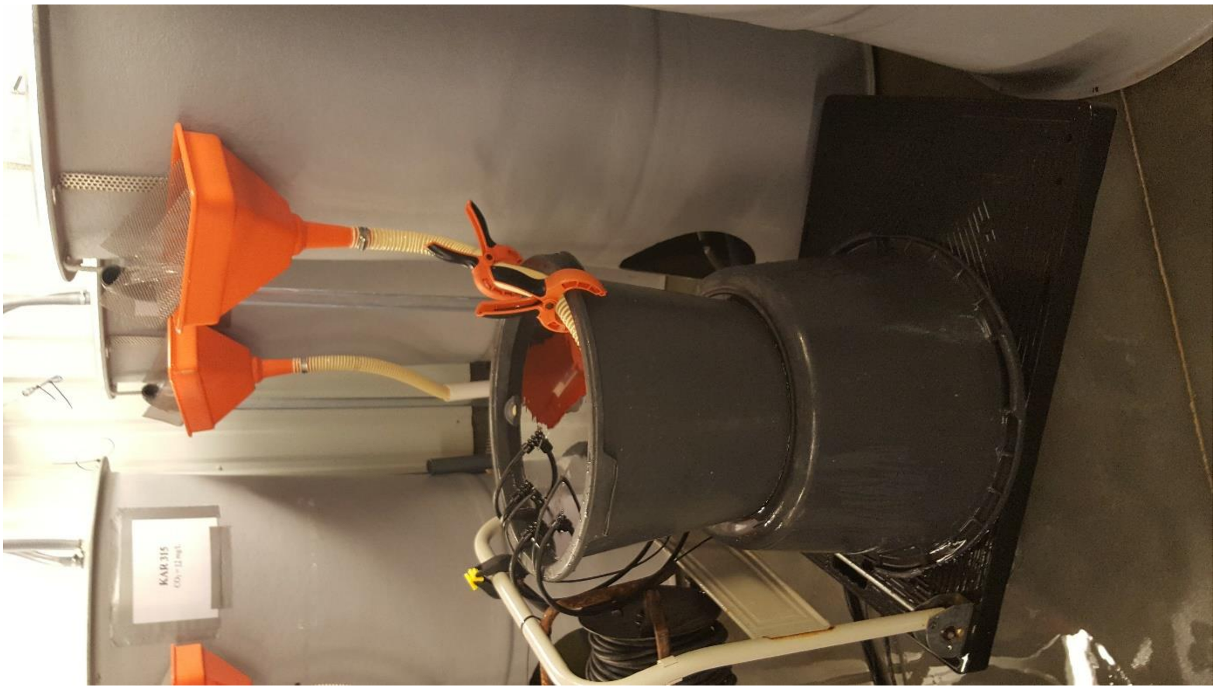
WTW. 2017c. *pH electrodes* [Online]. wtw.com. Available: https://www.wtw.com/en/products/product-categories/sensor-technology/conventional-sensors-lab/ph-electrodes.html [Accessed 08.06 2017].

Appendix

Appendix A: Water quality standards (Mota, 2017b).

Degasser sump (RAS 2)	***min.***	***max.***	***Frequency***
Salinity (ppt)	*11.5*	*12.5*	*daily*
Temperature (^oC)	*12*	*13*	*daily*
pH	*6.8*	*7.2*	*daily*
Alkalinity (mg/L as $CaCO_3$)			*weekly*
TAN (mg/L)	*-*	*0.7*	*weekly*
NO_2-N (mg/L)	*-*	*0.1*	*weekly*
NO_3-N (mg/L)		*<100*	*weekly*
Water exchange rate (% total volume/day)	*-*	*30*	*daily*
Water flow (L/min.)	*790*	*810*	*daily*
Photoperiod	*24L:0D*		

Appendix B: CO_2 measuring using three sensors from Franatech, Germany (Bye, 2017).



Appendix C: CO_2 concentrations of each tank.

Tank	CO2 (mg/l)
301	5
302	40
303	26
304	12
305	33
306	40
307	19
308	33
309	12
310	40
311	19
312	5
313	19
314	26
315	12
316	26
317	33
318	5

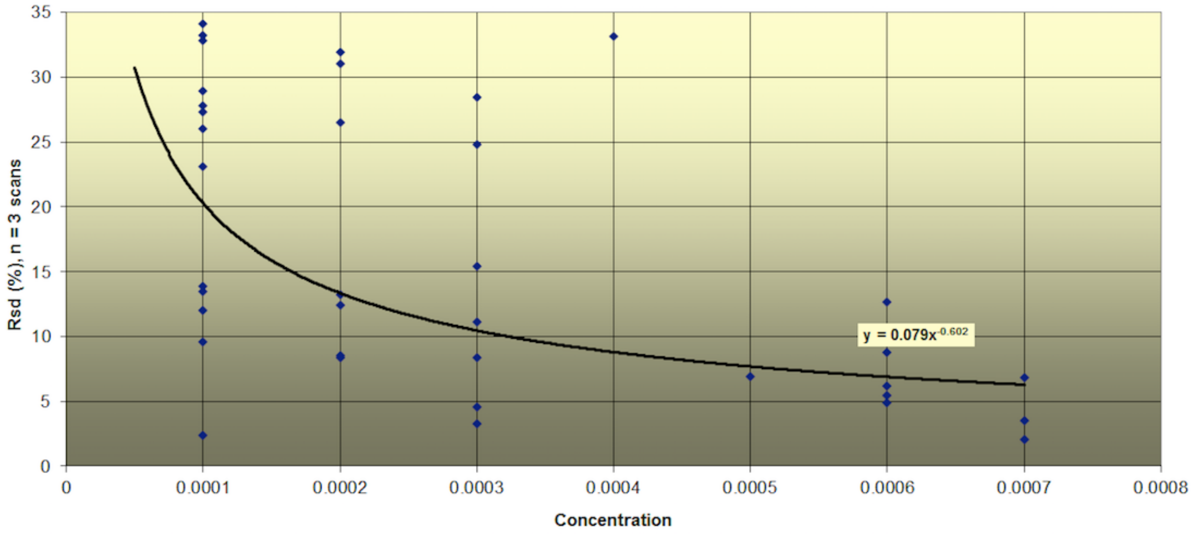
Appendix D: Water sampling dates. X = filtered, U = unfiltered

Tank	**16/11/16**	**30/11/16**	**19/12/16**	**06/01/17**	**11/01/17**	**18/01/17**	**24/01/17**	**02/02/17**	**10/02/17**
601	X	X	X	X	U	X	X	X	X
602	X	X	X	X	U	X	X	X	X
301	X	X	X	X	U	X	X	X	X
302	X	X	X	X	U	X	X	X	X
303	X	X	X	X	U	X	X	X	X
304	X	X	X	X	U	X	X	X	X
305	X	X	X	X	U	X	X	X	X
306	X	X	X	X	U	X	X	X	X
307	X	X	X	X	U	X	X	X	X
308	X	X	X	X	U	X	X	X	X
309	X	X	X	X	U	X	X	X	X
310	X	X	X	X	U	X	X	X	X
311	X	X	X	X	U	X	X	X	X
312	X	X	X	X	U	X	X	X	X
313	X	X	X	X	U	X	X	X	X
314	X	X	X	X	U	X	X	X	X
315	X	X	X	X	U	X	X	X	X
316	X	X	X	X	U	X	X	X	X
317	X	X	X	X	U	X	X	X	X
318	X	X	X	X	U	X	X	X	X
Freshwater		U				X			
Saltwater		U				X			

Appendix E: Detection limits for selected elements.

Element	**Isotope**	**Resolution**	**Detection limit (µg/l)**
Al	27	MR	0,01
As	75	HR	0,015
Cr	52	MR	0,001
Cd	114	LR	0,002
Cu	53	MR	0,012
Fe	56	MR	0,002
Mn	55	MR	0,03
Ni	60	MR	0,005
Zn	66	MR	0,004

Appendix F: RSD as a function of concentration



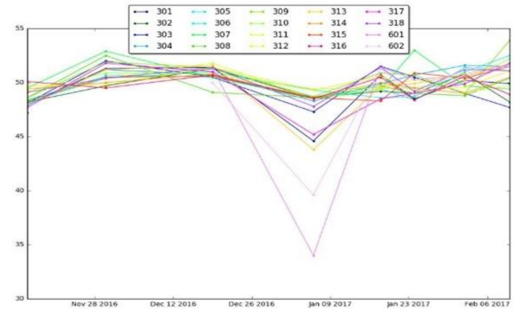
Appendix G: Make-up water (L/min) (Mota, 2017b).

Date	**Experimental day**	**Make-up water (L/min)**
08.11.2016	-14	15
09.11.2016	-13	15
10.11.2016	-12	15
11.11.2016	-11	15
12.11.2016	-10	15
13.11.2016	-9	15
14.11.2016	-8	16
15.11.2016	-7	16
16.11.2016	-6	16
17.11.2016	-5	22
18.11.2016	-4	23
19.11.2016	-3	23
20.11.2016	-2	23
21.11.2016	-1	23
22.11.2016	0	23
23.11.2016	1	23
24.11.2016	2	23
25.11.2016	3	23
26.11.2016	4	23
27.11.2016	5	23
28.11.2016	6	23
29.11.2016	7	22
30.11.2016	8	26
01.12.2016	9	23
02.12.2016	10	23
03.12.2016	11	23
04.12.2016	12	23
05.12.2016	13	23
06.12.2016	14	89
07.12.2016	15	45
08.12.2016	16	85
09.12.2016	17	18
10.12.2016	18	18
11.12.2016	19	18
12.12.2016	20	18
13.12.2016	21	18
14.12.2016	22	18
15.12.2016	23	19
16.12.2016	24	19

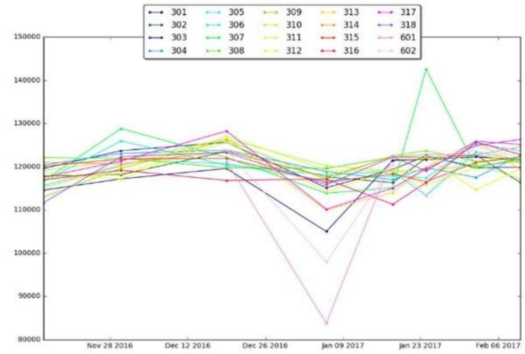
17.12.2016	25	19
18.12.2016	26	19
19.12.2016	27	18
20.12.2016	28	19
21.12.2016	29	18
22.12.2016	30	18
23.12.2016	31	🟨
24.12.2016	32	🟨
25.12.2016	33	🟨
26.12.2016	34	19
27.12.2016	35	19
28.12.2016	36	19
29.12.2016	37	18
30.12.2016	38	18
31.12.2016	39	18
01.01.2017	40	18
02.01.2017	41	19
03.01.2017	42	18
04.01.2017	43	30
05.01.2017	44	18
06.01.2017	45	18
07.01.2017	46	18
08.01.2017	47	18
09.01.2017	48	18
10.01.2017	49	19
11.01.2017	50	19
12.01.2017	51	19
13.01.2017	52	19
14.01.2017	53	19
15.01.2017	54	19
16.01.2017	55	18
17.01.2017	56	19
18.01.2017	57	19
19.01.2017	58	18
20.01.2017	59	18
21.01.2017	60	18
22.01.2017	61	18
23.01.2017	62	18
24.01.2017	63	18
25.01.2017	64	18
26.01.2017	65	19
27.01.2017	66	19
28.01.2017	67	18
29.01.2017	68	18
30.01.2017	69	18

31.01.2017	70	18
01.02.2017	71	18
02.02.2017	72	18
03.02.2017	73	18
04.02.2017	74	18
05.02.2017	75	18
06.02.2017	76	18
07.02.2017	77	18
08.02.2017	78	18
09.02.2017	79	18
10.02.2017	80	18
11.02.2017	81	18
12.02.2017	82	18
13.02.2017	83	19
14.02.2017	84	19
15.02.2017	85	18

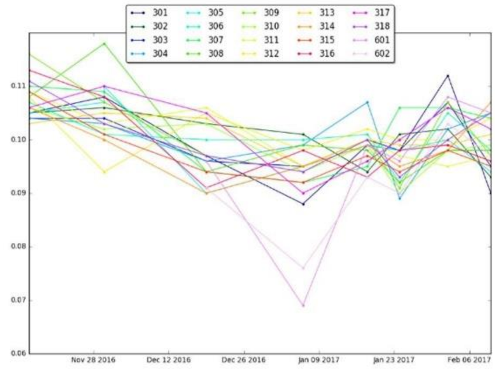
Appendix H: Selection of metals. An overview of the trend.



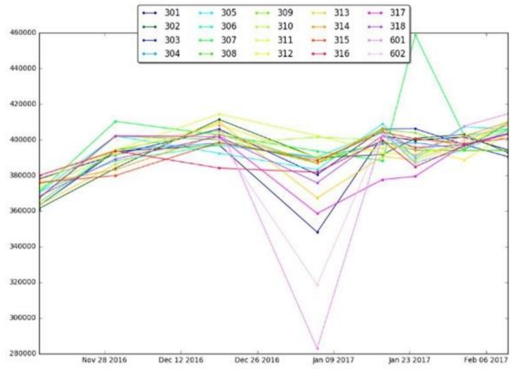
Lithium(Li)



Potassium (K)

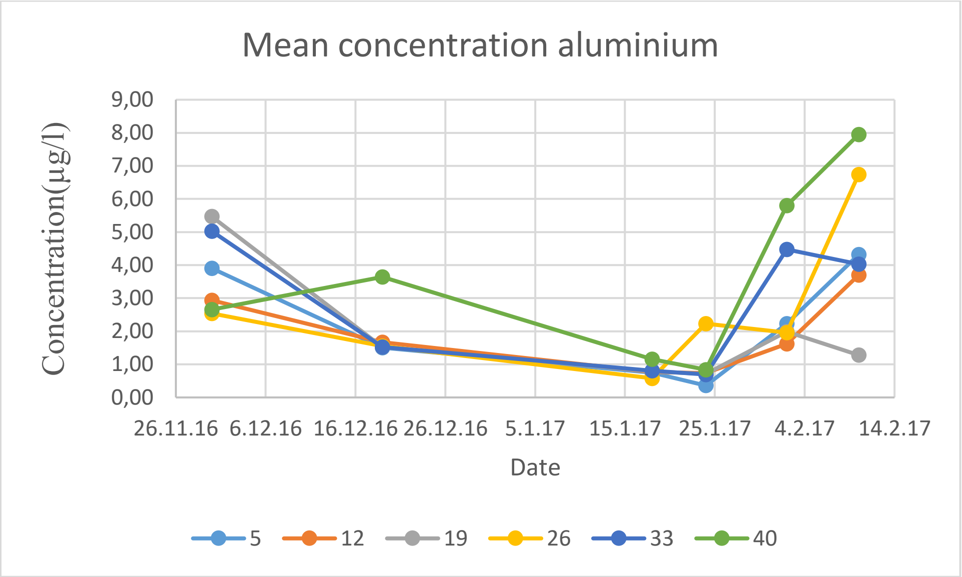


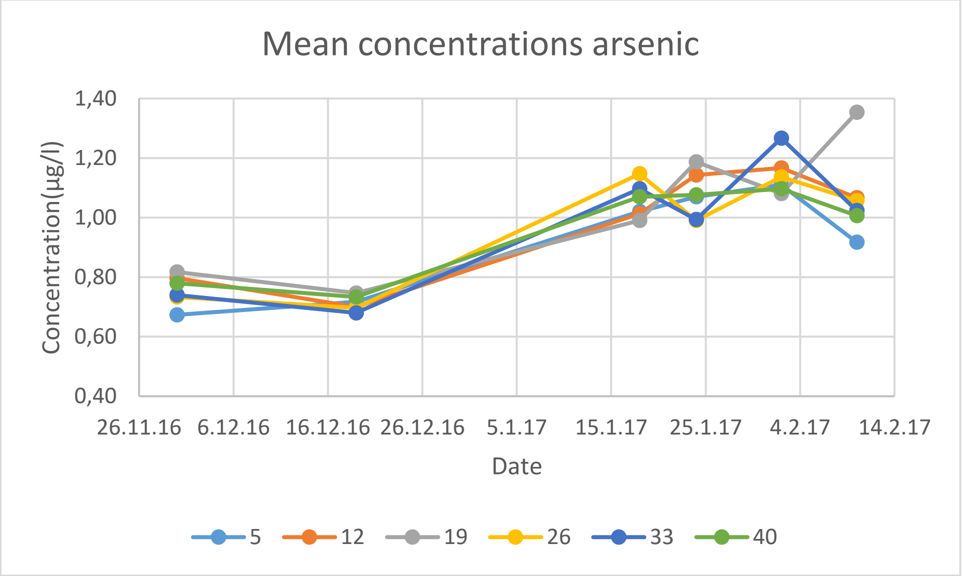
Caesium (Cs)

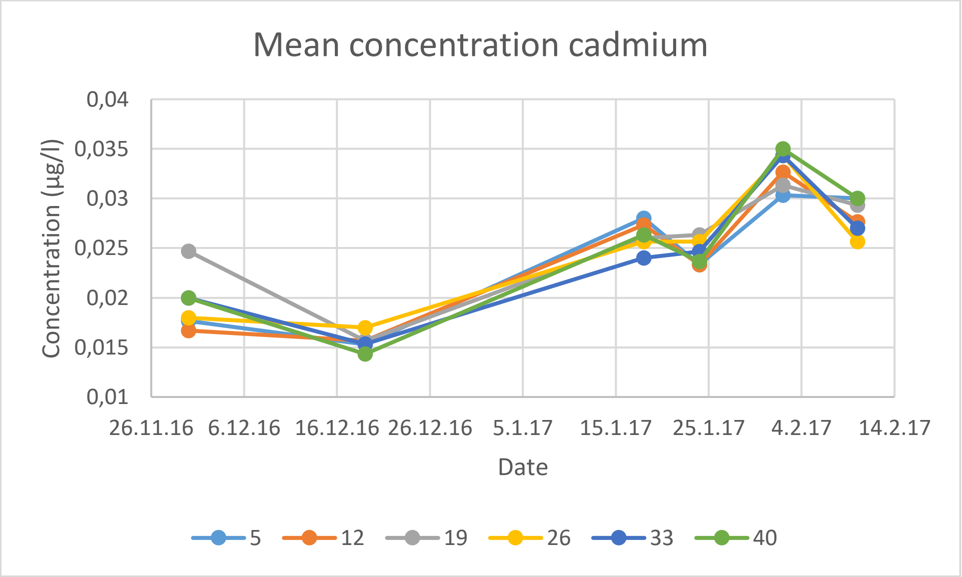


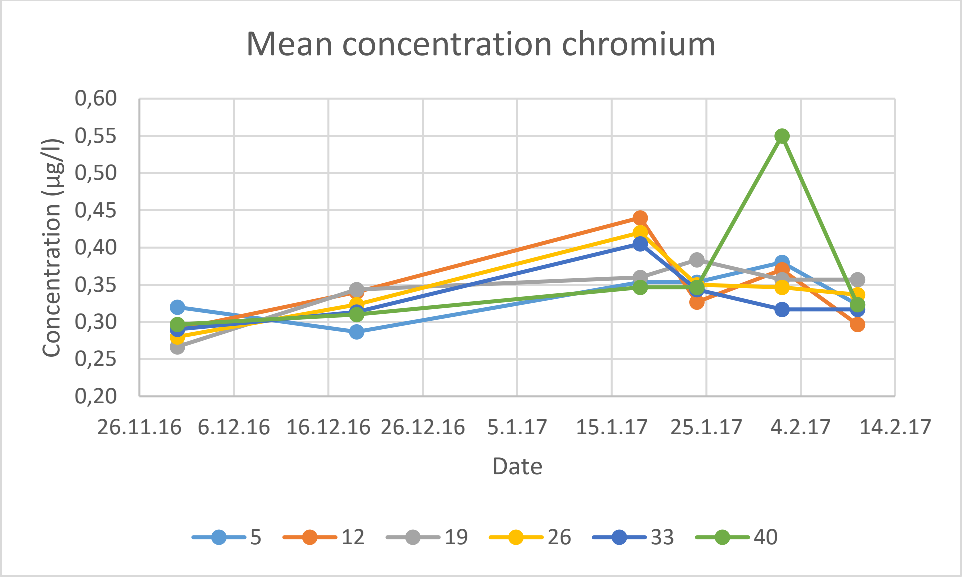
Magnesium (Mg)

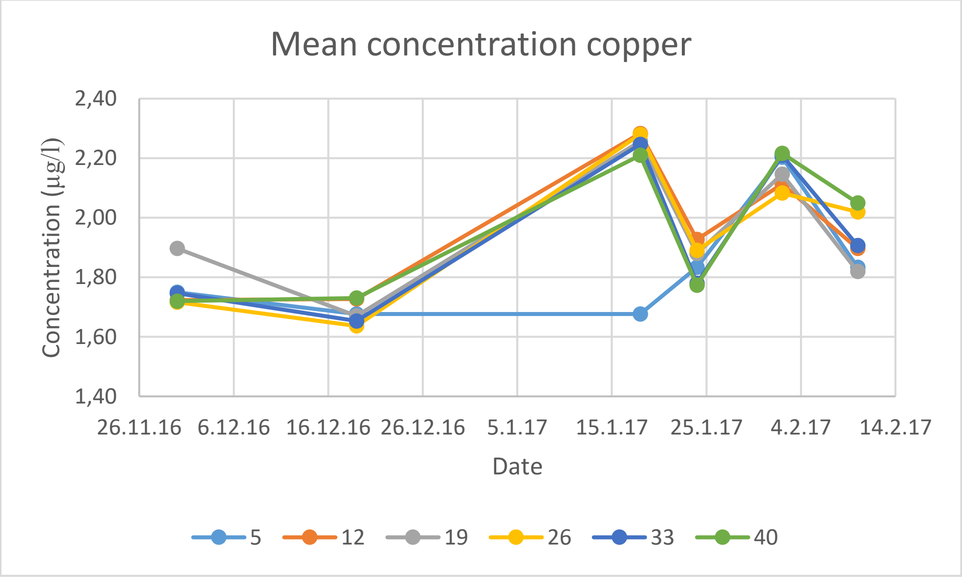
Appendix I: Mean concentration of selected metals over time.

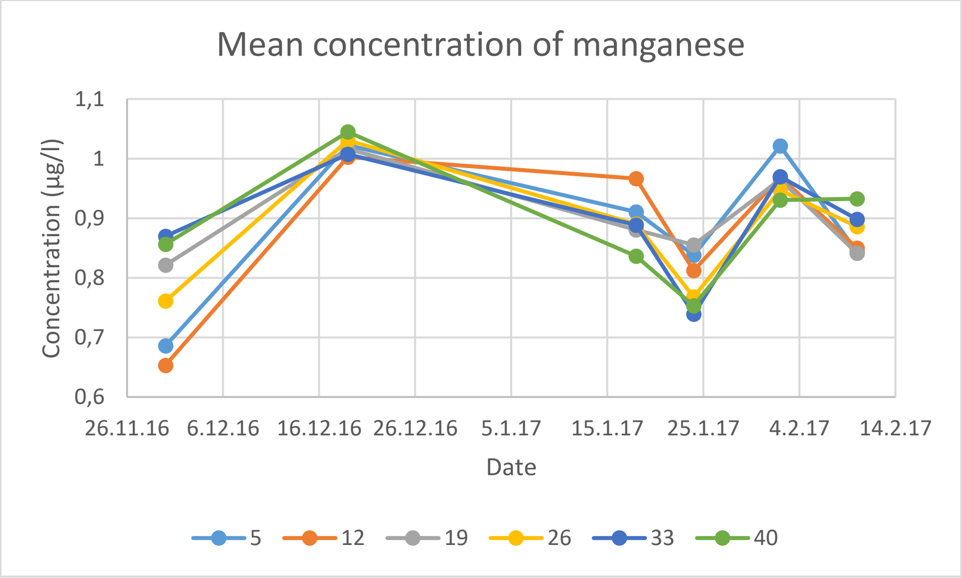


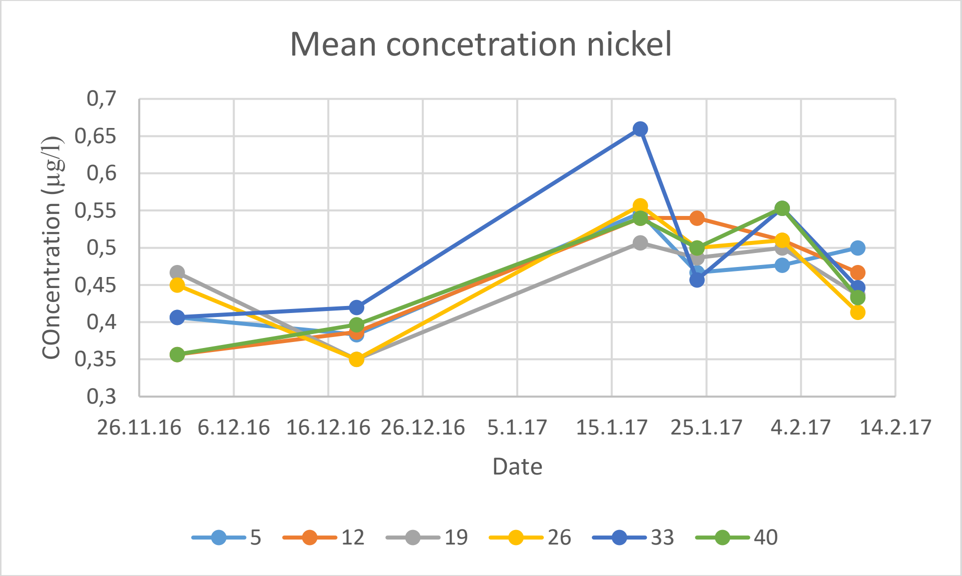


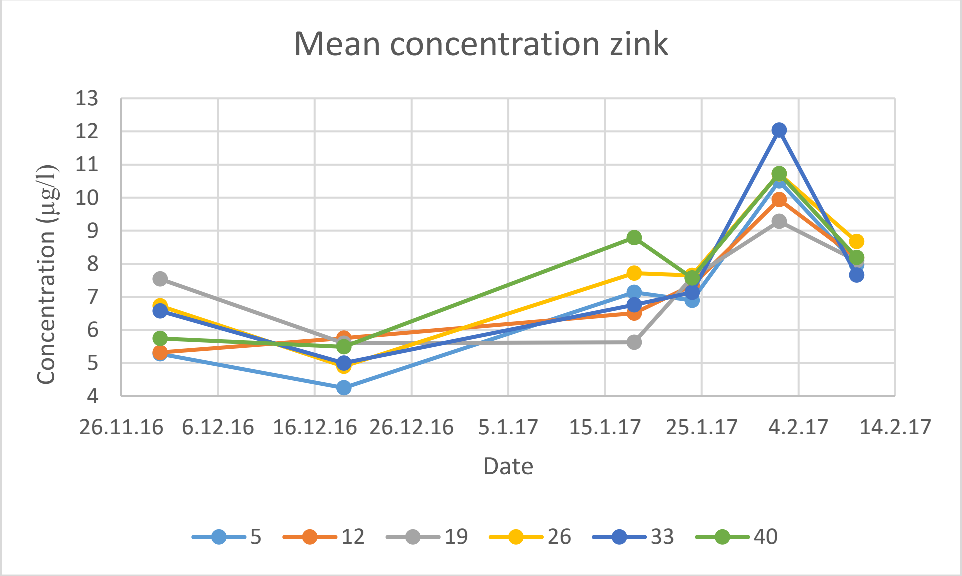



Mean concentration chromium
Concentration (µg/l)
0,60
0,55
0,50
0,45
0,40
0,35
0,30
0,25
0,20
26.11.16
6.12.16
16.12.16
26.12.16
5.1.17
15.1.17
25.1.17
4.2.17
14.2.17
Date
5
12
19
26
33
40


Mean concentration copper
Concentration (µg/l)
2,40
2,20
2,00
1,80
1,60
1,40
26.11.16
6.12.16
16.12.16
26.12.16
5.1.17
15.1.17
25.1.17
4.2.17
14.2.17
Date
5
12
19
26
33
40


Mean concentration of manganese
Concentration (µg/l)
1,1
1
0,9
0,8
0,7
0,6
26.11.16
6.12.16
16.12.16
26.12.16
5.1.17
15.1.17
25.1.17
4.2.17
14.2.17
Date
5
12
19
26
33
40


Mean concetration nickel
COncentration (µg/l)
0,7
0,65
0,6
0,55
0,5
0,45
0,4
0,35
0,3
26.11.16
6.12.16
16.12.16
26.12.16
5.1.17
15.1.17
25.1.17
4.2.17
14.2.17
Date
5
12
19
26
33
40


Mean concentration zink
Concentration (µg/l)
13
12
11
10
9
8
7
6
5
4
26.11.16
6.12.16
16.12.16
26.12.16
5.1.17
15.1.17
25.1.17
4.2.17
14.2.17
Date
5
12
19
26
33
40