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Water quality in closed-containment aquaculture systems (CCS) for Atlantic salmon post-smolt

Non-target screening of organic substances
using UPLC-MS/MS

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Environmental Toxicology and Chemistry
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Preface

Nofima's research station at Sunndalsøra has a range of strategic projects ongoing and they were appointed the coordinator role for the CtrlAQUA centre by Norway's Research Council in 2015. From 2015 until 2023 CtrlAQUA will be the Norwegian centre for research-based innovations in controlled-environment aquacultures. The target is to form the basis for future fish farming concepts. The work is financed 50% by Norway's Research Council and 50% by major national and international fish industrial players.

This master project is part of the larger research activity within the CtrlAQUA center 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. The target of the project is to optimize all aspects of the production process in closed production facilities, especially focusing on the post-smolt phase, which is the most vulnerable period of the salmon production cycle. In closed containment systems, the degree of innovation is very high and the water quality is essential for the fish health.

This study is conducted as a sub project to the CtrlAQUA: CO2RAS project by Nofima. In this context, this study will clarify how water quality will be affected by the different levels of carbon dioxide.

Acknowledgement

My work with this master thesis has been performed in three phases:

Phase I: Sample collections at NOFIMA's research station at Sunndalsøra
Phase II: Sample extractions and preparations for analysis
Phase III: Compilation, analyses, discussion and reporting

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Abstract

Organic substances may be released in a recirculating aquaculture system (RAS) from a variety of sources such as fish, feed, and bacteria among other. Due to continuous re-use of the water, compounds can potentially accumulate in the system and impact the water quality negatively. Water samples have been collected from RAS farming Atlantic salmon (*salmo salar*) post-smolt over a 12-week time period at Nofimas's research station at Sunndalsøra. A novel non-target screening was performed to characterize organic compounds produced in aquaculture in response to various concentrations of carbon dioxide (CO_2 mg/l). Physico-chemical parameters included are pH, redox potential, temperature, salinity, conductivity and turbidity. In total the study included 35 samples from fish tanks treated with concentration levels ranging from 5 to 40 mg/l CO_2. A liquid-liquid extraction (LLE) and liquid chromatography (UPLC) method was developed and optimized for the water in RAS. Ethyl Acetate (EtAc) stood out as the most suitable solvent for liquid-liquid extraction of the sample matrix followed by Dichloromethane (DCM).

Elevated CO_2 concentrations led to significant decrease in pH and increased Redox potential. Despite the significant decrease in pH, the levels were kept within safe recommended levels for farming Atlantic salmon (*salmo salar*). Twelve organic compounds were tentatively identified and assigned 8 different classes of chemical compounds. The classes which are Organophosphorus compound (OP), Carbohydrate, Amino acid, Ester, Alcohol, Steroid hormone, Ketone and unknowns respectively. Comparison between CO_2 treatments investigated (5, 12, 26, 40 mg/l) revealed that the relative level of organic compounds did not differ significantly among treatments over the course of the study, where the majority had a declining trend. Steroid hormones and two compounds of unknown classes were likely metabolites released by fish during acclimatization to the new environment. Increasing levels of these compounds indicated structurally similar compounds produced within the system at the end, potentially toxic to the salmon post-smolt. One compound, Organophosphorus compound, showed signs of accumulation due to high levels detected at the end of the experiment.

Sammendrag

Organiske stoffer kan lekke ut i et resirkulerende akvakultur anlegg (RAS) fra ulike kilder som fisk, fôr, bakterier blant andre. På grunn av kontinuerlig gjenbruk av vannet er det risiko knyttet til at stoffer akkumuleres i systemet og kan potensielt redusere vannkvaliteten. Vannprøver har blitt tatt fra et RAS-akvakulturanlegg på Nofima sitt forskningssenter i Sunndalsøra, Midt-Norge. En «non-target» analyse har blitt benyttet til å karakterisere organiske stoffer i RAS som en respons til ulike nivåer av karbondioksid (CO_2 mg/l) i vannet. Fysiske-kjemiske parametere har blitt målt over tid og inkluderer pH, redoks potensiale, temperatur, salinitet, konduktivitet og turbiditet. Totalt omfattet studien 35 vannprøver fra fisketanker behandlet med ulike CO_2 nivåer i intervallet 5 til 40 mg/l CO_2. En væske-væske-ekstraksjons metode og en væskekromatogafi metode (UPLC) har blitt utviklet og optimalisert for analyse av vannet i RAS. Etyl acetat (EtAc) utpekte seg som det mest egnede løsningsmiddelet for væske-væske-ekstraksjon av vannprøvene.

Forhøyede CO2-konsentrasjoner førte til en signifikant reduksjon i pH og økt redoks potensial over tid. Til tross for en signifikant reduksjon i pH, så holdt nivåene seg innenfor de anbefalte nivåene for oppdrett av atlantisk laks (salmo salar). Tolv organiske stoffer ble tentativt identifisert. Stoffene bestod av 8 forskjellige klasser av kjemiske forbindelser, henholdsvis organofosforforbindelse (OP), karbohydrat, aminosyre, ester, alkohol, steroidhormoner, keton og gruppe ukjente stoffer. Sammenligning av fire ulike CO_2-konsentrasjoner (5, 12, 26, 40 mg / l) viste at det relative nivået av organiske forbindelser ikke var vesentlig forskjellig ved de ulike CO_2 nivåene. Et flertall av de organiske stoffene viste en nedgående trend fra start til slutt. Steroidhormonene og to ukjente forbindelser var sannsynligvis metabolitter produsert av fisken under akklimatiseringsfasen til det nye miljøet. De målte økende nivåene på disse forbindelsene indikerer at lignende kjemiske forbindelser lakk ut fra-/ble produsert i systemet, og kan ha potensielt skadelige effekter på fisk. Ett stoff, en organofosforforbindelse, viste en tendens til akkumulering ved slutten av eksperimentet.

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Acronyms

LLE: Liquid-Liquid extraction

EtAc: Ethyl Actetate

DCM: Dichloromethane

UPLC – MS/MS : Ultra Performance Liquid Chromatography Tandem Mass Spectrometry

HPLC: High Performance Liquid Chromatography

MS: Mass Spectrometry

NTNU: Norges Teknisk-Naturvitenskapelige Universitet (Norwegian University of Science and Technology.

PCA: Principle Component Analysis

XIC: Extracted ion-chromatogram

BIP: Base-peak chromatogram

m/z: Mass-to-charge ratio

RT: retention time

EDC: Endocrine

1 INTRODUCTION

1.1 Aquaculture

Aquaculture is one of the fastest growing food sectors globally [1] and is responsible for the remarkable growth of fish available for human consumption today. While production rates within wild capture fisheries have been relatively stable since 1980, the aquaculture sector has grown, making up 47% of global fish production in 2016 [1]. This has brought many benefits, like greater availability for humans to consume nutritious and healthy diets, since fish are an important source of animal protein, essential amino acids, unsaturated fats and vitamins [2], while also alleviating harvesting pressure from wild populations. However, due to high pollution levels there is a growing public demand for more sustainable solutions within aquaculture. Current conventional technologies employ open sea-cages and flow through (FT) systems for fish farming. In flow through systems, the culture water makes one pass and further goes to waste. These techniques can cause serious impact on the environment by, e.g. releasing organic waste products, having escaping fish or spreading diseases to natural populations [3]. In addition, lack of space, seasonal variations and limited fresh water supply represent additional obstacles to expansion of current conventional methods [4].

Recirculating Aquaculture Systems (RAS) has emerged as one of the solutions to further development within aquaculture. The RAS are land-based closed-containment systems (CCS), which means that there is a physical barrier between the rearing water (culturing water) and the surrounding environment. In RAS, culture water is re-used after undergoing water treatment processes for waste removal. In general, these systems are referred to as intensive systems due to high stocking density/feed load and low water exchange rates [5]. RAS technology offer advantages in terms of better waste and disease management and more stable environmental conditions through control over essential water parameters (pH, dissolved oxygen, carbon dioxide, salinity, ammonia, suspended solids and more)[6]. In addition, high intensity systems can allow for as much as 100-fold reduction in makeup-water use [7].

In order to keep the production feasible, intensive fish production rates are required to cover investment and operational costs and generate required returns. High fish stocking densities

may in turn pose a threat to the water quality due to the low water exchange rates. Previous studies have found that RAS farming Atlantic salmon (*Salmo salar*), as well as for other fish species [8][9], results in accumulation of steroids released by fish into the water, which may be detected by fish as pheromones and interact with maturation, even at low concentrations[8]. These studies and experiments have targeted mainly steroid concentrations- and accumulation in re-used water.

In this study, a novel non-targeted analysis have been applied to screen a broader range of organic substances in combination with the effect of different CO_2 (mg/l) concentration levels in RAS.

1.2 Recirculating Aquaculture Systems (RAS)

In a recirculating aquaculture system the effluent from culture tanks undergoes water treatment processes and is returned to the tanks for re-use. The term make-up water is a common term used to describe recirculated, treated water. The basic components in RAS (figure 1.1) are: culture units, particle filtration, biological filtration, CO_2 removal, disinfection and oxygen supply [10]. It is an energy demanding process as the water is circulated through the units by pumps [11].

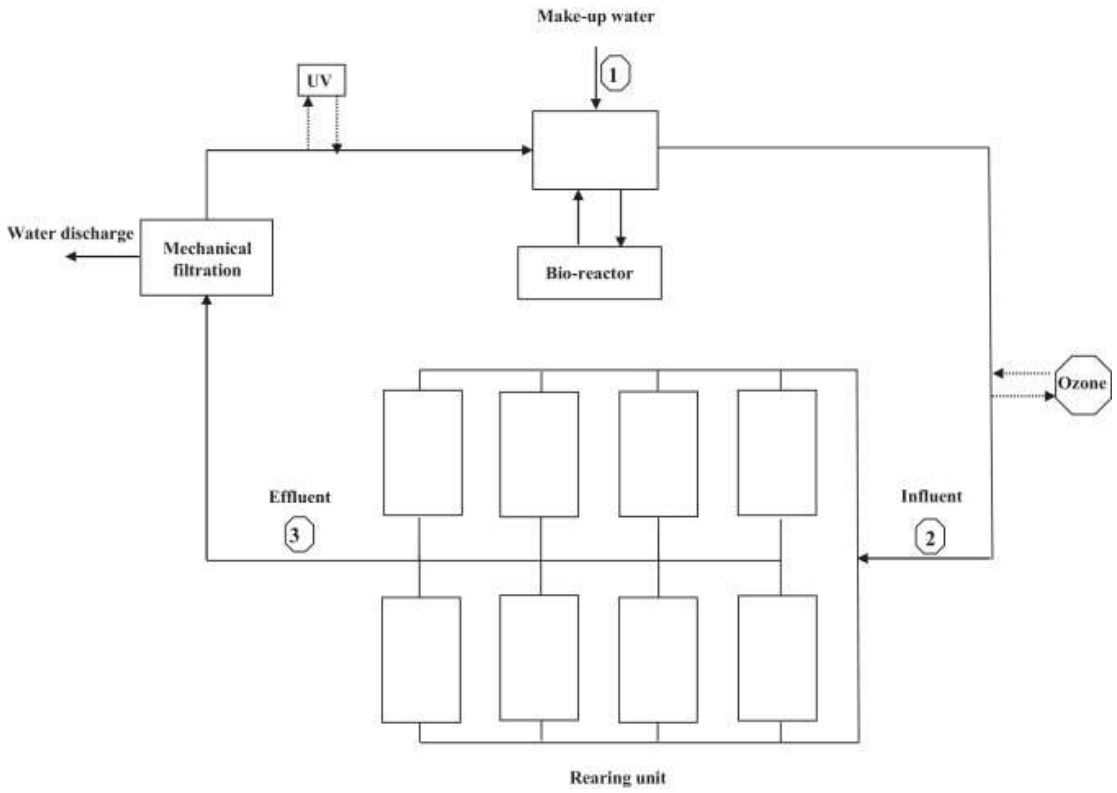


Figure 1.1 Schematic figure of a recirculating aquaculture system (RAS). Source: [32]

Removal of solids in the culture water is the first part of the process. Sources of these solids could be uneaten feed, faeces released by fish, algae and slough off from biofilms during biological filtration [10]. Quick removal is essential to avoid particulates to fragment into smaller dissolvable particles which accumulate within the system. There are two ways solids can be removed; within the tank itself and by an external unit following the tanks that filter particles based on particle size [10]. The first step is sedimentation of particles to the tank

bottom. Octagonal and circular tanks are used a lot as their tank geometry promote mixing of the water and rapid settlement of particles which then can be removed [12]. External units to further treat the effluent have varying options, however mechanical filtration is traditionally used [13].

In order to control nitrogenous waste, biofilters which houses the nitrifying bacteria are used in RAS [14]. Toxic ammonia (NH_3 and NH_4^+) is oxidized to nitrate (NO_3^-), which is relatively non-toxic to aquatic organisms [14]. Carbon dioxide is primarily removed by degassers in intensive RAS. The water is passed through a stripping unit where the gas diffuses from the water into the air [15]. Disinfection, as UV or ozone treatment or a combination of both, are included to prevent accumulation of pathogens in the system [16]. Lastly, oxygen is added in pure form to the inlet water for the system to be able to sustain a high fish biomass [17] [3].

1.3 Carbon dioxide in RAS

Carbon dioxide is considered a critical water quality parameter in recirculating aquaculture systems [18]. The common effects of long-term exposure to high concentrations of carbon dioxide in Atlantic salmon are seen as growth inhibition, low feed conversion efficiency and nephrocalcinosis [3][19][20]. Other effects include reduced oxygen binding capacity and increase in blood acidity [17] The listed effects have been observed at 20 mg/l. Thereby, a safe upper limit of dissolved carbon dioxide for smolt production in Norway is currently set to 15 mg/l [21]. However, the safe criterion used is also dependent on the cultured species, life stage and the characteristics of water, such as pH, alkalinity and dissolved oxygen [17]. In low alkalinity water and in water with a high content of dissolved metals, it is suggested that the limit should be further reduced [21]. The main effect from increasing carbon dioxide concentrations is more acidic conditions due to a drop in pH-level. How well the system cope with the pH drop, and the magnitude of it, is determined by the alkalinity, i.e. capacity to neutralize acid.

Carbon dioxide is highly soluble in water, up to 40 times more soluble than oxygen [22]. When carbon dioxide is released into water, the majority will remain as CO_2 (aq), while a small fraction transform into carbonic acid (H_2CO_3) via the equilibrium reaction (Eq.1). The concentration of dissolved CO_2 is referred to as $H_2CO_3^*$, which represent the sum of CO_2 and H_2CO_3, as it is difficult to analytically to distinguish between the two components. Carbonic acid is a weak acid with low water solubility and therefore readily dissociates into free hydrogen ions (H^+) and bicarbonate (HCO_3^-) ions [22]. Bicarbonate (HCO_3^-) further dissociate into carbonate (CO_3^2) along with H^+-ions[3]. The process is described by the equilibrium reactions (Eq.1-3) [19]:

$$CO_2\,(aq) + H_2O \leftrightarrow H_2CO_3^* \quad (1)$$

$$H_2CO_3^* \leftrightarrow H^+ + HCO_3^- \quad (2)$$

$$HCO_3^- \leftrightarrow H^+ + CO_3^{2-} \quad (3)$$

When CO_2 is dissolved at high concentrations in the water the effect is a shift in the equilibrium equations, causing a greater amount of H^+-ions to be dissolved in the water which results in acidified conditions in the water (lower pH) [22]. How well the system copes with the pH-drop is dependent on alkalinity, i.e capacity to neutralise acid. Alkalinity is measured in terms of $CaCO_3$ (mg/l), adjusted by adding sodium bicarbonate to the system ($NaHCO_3$).

It is essential to maintain control of dissolved carbon dioxide in intensive water reuse systems where volume and water exchange rate can be low [3]. The latter is defined by the rate of makeup-water flow per biomass of fish in the system. When oxygen is saturated, water exchange rates can be lowered and carbon dioxide may become the next limiting factor. Input of carbon dioxide to the system occur naturally *via* aerobic metabolism in fish where molecular CO_2 diffuse through the gills. According to earlier studies, a considerable amount of CO_2 is released by fish in RAS where it accumulates during recirculation [23].

1.4 Water quality parameters

Physicochemical parameters such as are pH, redox potential, salinity, conductivity, temperature and turbidity are important to consider in RAS as poor water quality can increase fish susceptibility to diseases.

pH is a measure of the acid balance in a solution [22]. It is an important parameter to study in aquatic systems as it can affect solubility and speciation of compounds present in water. In, addition low and high pH levels leads to slower nitrification processes in RAS, leading to higher concentration-levels of toxic un-ionized ammonia (NH_3) [18]. In operating, commercial scale RAS the pH level is kept within 6.8 – 7.3 [6].

Redox potential is used to describe a system's oxidation and reduction capacity. Redox processes play an important role regulating reactions in biological systems [24]. Oxidation occurs when organic matter is broken down by microorganisms such as bacteria and fungi. Redox potential can be an indication of the specific system has a tendency to be oxidising or reducing. This in turn can affect chemical speciation. For instance, a water saturated with oxygen is a relatively oxidizing medium with a high redox potential. The chemical species present will be in their oxidized forms [25], e.g. CO_2, NO_3^-, SO_4^-, MnO_2 and $Fe(OH)_3$. Reducing

systems, on the other hand, contain the reduced forms of these compounds, such as CH_4, NH_4^+,H_2S and $Fe(H_2O)_6^{2+}$, along with various organic substrates.

Salinity represent the total concentration of inorganic salts dissolved in water, as gram salt per kilogram water [22] [26]. The particular RAS-system used in this study utilize brackish water, a mixture of freshwater and seawater, at 12 ppt salinity. Recent research suggests that brackish water RAS operated at 10-22 ppt can be more optimal for fish before transfer to sea-water, seen from better growth performance and survival rates [27].

Conductivity is directly related to salinity, where an increasing abundance of ions will increase conductivity. However, it is not only controlled by the abundance of ions, but also by the degree of dissociation, charge of each ion, ion mobility and temperature [22]. It is commonly used in pollution monitoring, measured as micro-siemens per centimetre (μs/cm).

Temperature influence several other physico-chemical parameters and is therefore important when considering water quality. It affects the solubility and reaction rates of chemicals present in the water. Elevated temperatures can increase solubility of toxic compounds, e.g. ammonia. Also, It can lead to increased solubility of salts in the water, hence lead to an increase in salinity and conductivity. Higher temperatures leads to lower solubility of dissolved gases, e.g. dissolved oxygen levels and also CO_2 [22]. High CO_2 levels combined with low temperatures (5°C) have shown to inhibit growth of Atlantic salmon compared to high CO_2 at high temperatures (15 °C) [3]. In general, temperature levels in RAS for Atlantic salmon lie within 12-14°C [6]

Turbidity is a measurement of the transparency of water, controlled by the amount of particles, suspended solids, present [22]. It is measured using nephelometry, a technique which uses light scattering by the particles present. The amount of light scattered is dependent on particle geometry, concentration of particles and the optical properties of the suspension. This makes turbidity a parameter that can vary greatly between different water bodies. In aquaculture, it is considered a parameter of great importance as it can have adverse effects on the water quality and fish health [13]. Turbulence in RAS can cause suspended particles to fragment into smaller particles which can accumulate within the system [13] [28].

1.5 Accumulation of organic substances in RAS

Due to the continuous re-use of the water in RAS there are risks associated with the accumulation of substances within the system. There are three primary sources; excretions from fish, uneaten feed and bacteria and fungi [18] In addition, substances may leach from the materials used within the system [29].

Substances excreted by fish

There are two main ways chemicals are released from fish; diffusion through gills and excretion of waste products (urine and faeces). Steroid hormones are one group of substances which have been found to accumulate within RAS [9]. These can be released as "free" steroids (lipid-soluble) or as conjugated (lipid-insoluble) with sulfonate and glucuronide groups, depending on where they are excreted from [8][30]. One frequently measured steroid is cortisol, a stress hormone released by fish as a response to external stress factors (e.g. poor water quality, pathogens, fish handling, etc.) [31]. Other reported steroids include a group of sex steroids such as testosterone, 11-ketotestosterone, 17,20β-dihydroxypregn-4-en-3-one [32]. The basic structure of these steroids include a 4-carbon ring system and carbon skeleton with variations related to functional groups on C3 and C17. Sex steroids have been identified to act as pheromones in fish [33]. Pheromones play an important role regarding communication and may also impact maturation [18]. Other pheromones that may be released from fish include prostaglandins, bile salts and amino acids [33]. Prostaglandins are lipids consisting of a 5-carbon ring and 20 carbon atoms. Their functions and structural components can vary greatly. Bile salts are characterized by carbon skeleton containing 24-27 carbon atoms and hydroxylation at C3, C7 and C12 [33]. Similarly, they are quite diverse in functionality and structure. Amino acids consists of amine, carboxylic acid and a variety of side chain groups. They can range from basic, neutral and acidic in nature depending on presence of functional groups.

In addition, nutrients are introduced into the water as waste products from fish. Particulate P and N are excreted *via* faecal waste, with phosphorus being particularly abundant [34]. The majority of nitrogen is excreted as (inorganic) dissolved ammonia from protein metabolism, accompanied by a smaller fraction excreted as urea (about 10% of total N for salmonids) [35].

It is readily degradable, converted into CO_2 and ammonia in the presence of free urease or by bacteria that are able to express urease.

Substances leaching from feed

To maximize growth and performance high loadings of feed are added to the culturing water. However, this can generate a large amount of waste if the feed is not efficiently utilized by the fish [34]. Apart from a high content of phosphorus and nitrogen, fish feed contains complex nutrients such as proteins, lipids, fibre, ash and carbohydrates [36][37]. Moreover, due to lower availability of fish meal from capture fisheries plant based diets are becoming increasingly common [38]. These diets need to be supplemented with essential amino acids, required for reproduction, growth and immune response [39][40]. Some of those are amino acids from the glutamate family, i.e. arginine, glutamine and glutamate. Another important supplement is Taurine, a sulfonic acid with abundant concentrations in animal tissue. The high load of feed pellets entering the water may cause these substances to leach out and ultimately affect the water quality.

Substances produced by bacteria and fungi

Two common off-flavour chemicals in recirculating aquaculture systems are Geosmin and 2-methylisoborneol (MIB) [41][42]. They are semi-volatile, terpenoids with a lipophilic character. and can cause problems with off-flavour even at low concentrations (15-18 ng/L) in both water and fish [43]. Production of these compounds are mainly attributed to cyanobacteria, fungi and actinomycetes [41]. In addition, microbial degradation of complex organic compounds leads to production of humic acids [18][44]. These may originate from degradation of feed, faeces and dead bacteria in the system [45]. In intensive aquaculture systems, humic acids may comprise as much as 90% of dissolved organic compounds, giving the water a typical yellow-brownish appearance[46].

Substances leaching from the system

Fiberglass and plastics are used in re-use systems [10] in large amounts (tanks, pipe systems etc.) and may leach out endocrine disrupting chemicals (EDC's) [18]. In general, these chemicals comprise a wide variety of compounds such as plastic components, natural and synthetic hormones, pesticides, plant constituents. Plastic components, and their additives

(plasticizers, fungicides, flame retardants, release agents) can leach out from the materials and dissolve in the water column, potentially impacting the endocrine system of fish [18].

1.6 Aim

Stressful events such as elevated levels of dissolved CO_2 will lead to physiological changes in fish and the release of organic molecules (such as cortisol) in the water. Organic substances may be released into a recirculating aquaculture system (RAS) from other sources as well (such as fish feed and system). As the water is recirculated within the system, compounds can potentially accumulate over time and have a negative impact on the water quality. This study aim to:

- Determine the compounds released by fish under stressful environmental conditions i.e. high CO_2 and low pH

- Determine the occurrence of organic substances in RAS, which can have detrimental effect on fish welfare, under a range of CO_2 concentrations.

- Detect accumulation of organic substances under a range of CO_2 concentrations over the experimental period.

1.7 Objectives

This study has three main objectives:

1. To develop and optimize a liquid-liquid extraction method for water samples collected

2. To perform a non-target screening of organic substances in water samples collected from RAS containing Atlantic salmon post-smolt using a UPLC-MS/MS instrument.

3. Determine the impact of range of CO_2 concentration on organic substances in RAS.

2 THEORETICAL BACKGROUND

2.1 Ultra Performance Liquid Chromatography

2.1.1 Introduction

Ultra Performance Liquid Chromatography (UPLC) was commercially introduced in 2004 [47]. The common term to describe this technology is *Ultra High Performance Liquid* Chromatography. Principles of UPLC follow High Performance Liquid Chromatography (HPLC) with enhanced resolution, speed and sensitivity. A higher performance level than conventional HPLC techniques is achieved through the use of column packing's with smaller particles (sub-2 µm) and a higher operating pressure (up to 1000-1500 bar) [48]–[50]. Coupling UPLC with tandem mass spectrometry (MS/MS) detection can provide a powerful tool in the analysis of complex sample matrices where compounds are present at low concentrations [51]. The methodology is described below and illustrated in figure 2.1.

2.1.2 Methodology of UPLC

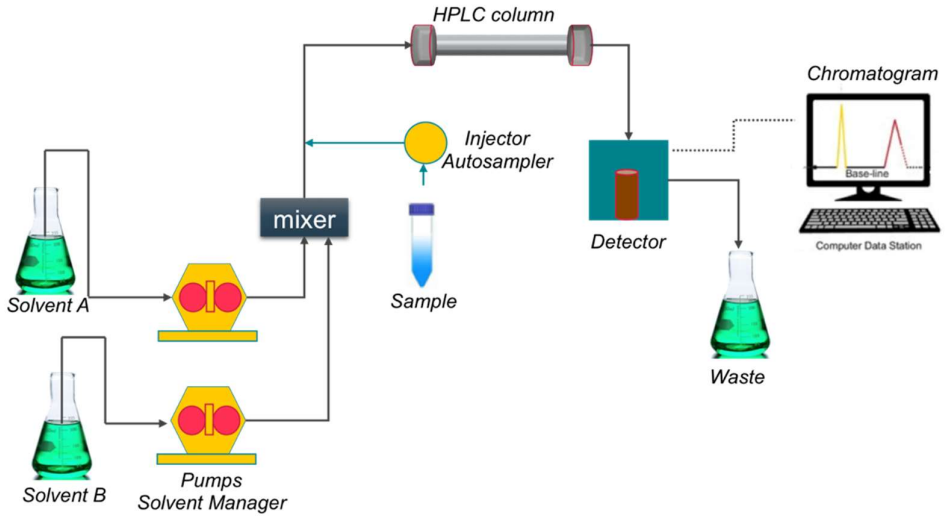


Figure 2.1 Schematic diagram of a Ultra Performance Liquid Chromatography System (UPLC) with a binary pump system. Source: [52]

2.2 Detection system

2.2.1 Electrospray ionization (ESI)

For detection, tandem mass spectrometry (MS/MS) with an electrospray ionization (ESI) source was used. ESI has become an important ionization technique for on-line coupling of liquid chromatography with mass spectrometry [55]. ESI generates detectable ions of non-volatile molecules of various sizes directly from a liquid chromatography effluent. A common constraint of ESI is particularly ion suppression of analytes which can lead to false or absent response due to competition from matrix components [56][57].

The ionization mechanism is shown in figure 2.2 and follow three steps: (1) Production of charged droplets from nebulization of sample solution (2) liberation of ions from repeated evaporation of solvent and (3) transportation of ions in gas phase to a mass spectrometer.

The liquid sample from the chromatograph enter the ESI spray needle at a low flow rate (0.1-10µl/min). A high voltage (2-5 kV) is applied to the needle to provide an electric field gradient, either positively or negatively charged. From the electric field between the needle and counter electrode, an excess of positive charge is produced on the surface of the cone. Formation of charged droplets occur from the tip of the cone which evaporate when they move towards the mass spectrometer. Evaporation of the droplets leads to production of free, charged analyte molecules in vapour phase which can be detected by a mass spectrometer [56].

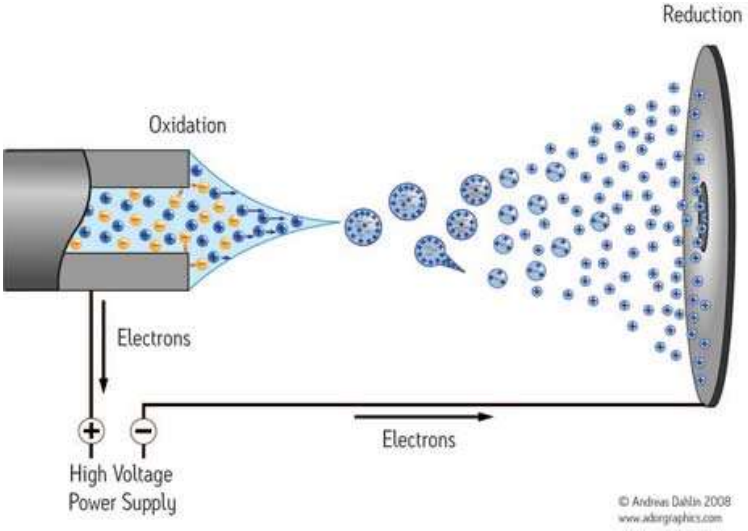


Figure 2.2 Electrospray ionization in positive mode (ESI+). Source: [58].

2.2.2 Tandem mass spectrometry (MS/MS)

The detection method involves two stages of mass analysis (measurement of mass-to-charge ratios (m/z) of ions). Two mass analyzers are combined: a Quadrupole and a Time-of-flight instrument, giving the generic term Q-TOF. Between the two components is a collision cell to promote fragmentation of ions. The use of two mass analyzers for analysis of unknown compounds is particularly valuable as it can provide detailed fragmentation ion data needed for structure elucidation [59]. From accurate mass detection, elemental composition can be determined and analyte concentrations can be measured within ppm range [60].

The basic concept of MS/MS involves two stages: Isolation of precursor ions and secondly fragmentation of precursor ions (m_p^+) into product-ions (m_f^+) and neutral fragments (m_n). Reaction mechanism:

$$m_p^+ \rightarrow m_f^+ + m_n$$

From the ESI-source, precursor ions in gas phase are separated and selected based on their m/z ratios by the first mass analyzer, the Quadrupole. From there, selected ions enter a collision cell to undergo fragmentation by collisions with an inert gas, a principle called collision-induced-dissociation (CID). This process can be considered as an 'energy transfer' needed for ions to produce fragments. Lastly, the resulting fragments are separated by their m/z ratios in the second mass analyzer, generating spectra of precursor ions and their product ions [61]. The mass spectra obtained is a graphical representation of the relative intensity of the ion signal (y-axis) as a function of the m/z value (x-axis).

3 MATERIALS AND METHODS

3.1 Experimental design

3.1.1 Experimental systems

The experiment was conducted at the Nofima Centre for Recirculation in Aquaculture (NCRA) in Sunndalsøra, Norway. The facility accommodate 6 experimental sections with access to freshwater and seawater [62]. The experimental section utilized for this study consisted of a RAS-system of 18 culture tanks (0.5 m^3) and 2 holding tanks (Fig, Holding sump 1 and 2) connected to a water treatment system. Recirculation of water from the culture tanks follows a loop of mechanical belt filter, moving bed bioreactor, CO_2 degasser and oxygen enrichment in holding tanks. Finally, oxygen rich water is returned to the culture tanks from outlets of the holding tanks. Details of the RAS-system is illustrated in figure 3.1.

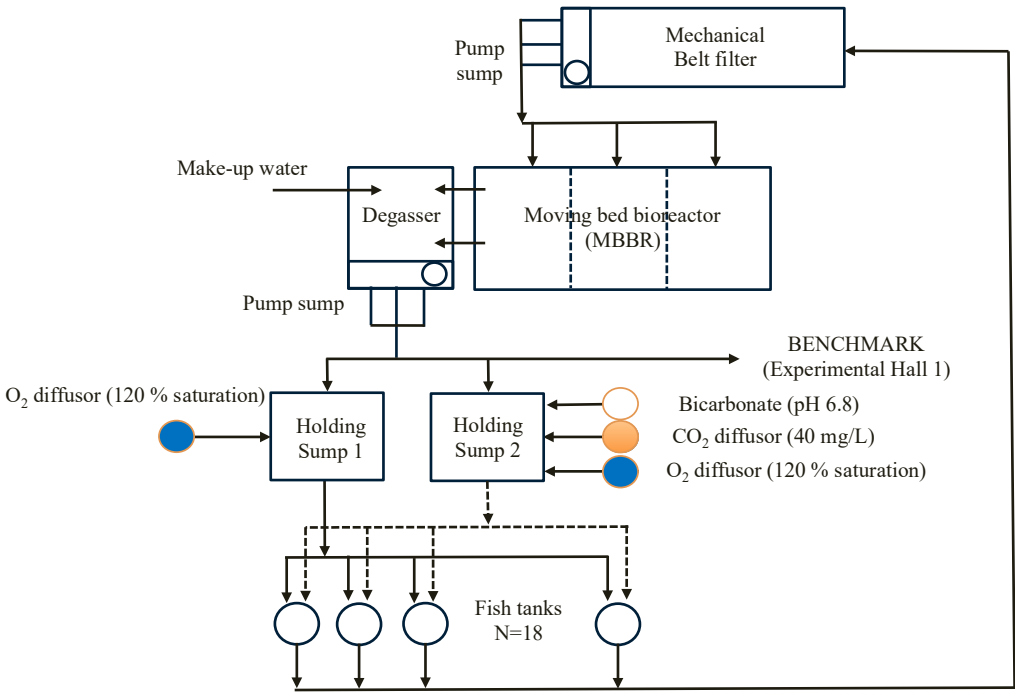


Figure 3.1 Experimental set-up of the recirculation aquaculture system (RAS) in Nofima Centre for Recirculation in Aquaculture (NCRA). Source: [63].

3.1.2 CO_2 experiment

This experiment consisted of culturing Atlantic salmon post-smolt in brackish water (ppt) RAS to six concentration-levels of CO_2 over a 12-week time period. The concentration levels included 5, 12, 19, 26, 33 and 40 mg/l CO_2 in a mono-factorial design with 3 replicates per treatment. The different carbon dioxide concentrations in each fish tank were obtained from the specific mix of two water inlets (Fig): holding sump 1 (CO_2 = 5 mg/l) and holding sump 2 (CO_2 = 40 mg/l). The experimental period lasted from November 22nd 2016 until 10th of February 2017. Stocking of Atlantic salmon occurred 3 weeks prior to start-up of the experiment at a density of 50 fish weighing 71 (± 9 g) per tank. During these weeks, the fish was adapted to the environment and no CO_2 was added. The adaption period lasted from November 3rd until November 22nd. From day 1 of the experiment, CO_2-increase was gradual at a rate of 5 mg/L per day until the desired concentration level was achieved for each tank. Details of carbon dioxide increase design (mg/l) can be found in appendix B.

3.2 Sample collection and measurements

3.2.1 Water samples

Effluent water was collected from tank outlets of 18 replicated 0.5 m^3 fish tanks (figures 3.2 and 3.3) containing Atlantic salmon post-smolt. Sampling was performed on 8 occasions in total; once during the adaption period (no CO_2 added) and the other 7 times during the experimental period.

Figure 3.2 Experimental hall 3 utilized for the experiment. at Nofima Centre for Recirculation in Aquaculture (NCRA).

In addition, 2 samples were collected from makeup-water (holding Sump 1 (numbered as 602) and holding Sump 2 (numbered as 601). Makeup-water was not included in sampling during the adaption period (see Appendix A). Sampling intervals followed ± 2 ½ weeks until January and ± 1 week from January, until the end of the experiment. Vials and syringes were thoroughly rinsed with distilled water prior to sampling.

Water samples were filtered with 0.45 μm polyethersulfone membrane filter using polypropylene syringe (20 ml capacity) into a vial. Two sets of water samples were collected (1) 25-30 ml, filtered water for analysis of dissolved organic compounds and (2) 15 ml, filtered and acidified (5 M HNO3) water for trace elemental analysis (Data presented by [63]).

Samples for analysis of dissolved organic compounds, were collected in glass vials (30 ml capacity) and polypropylene falcon tubes (45 ml capacity), depending on the availability of glass vials.

Samples were transported to NTNU, Trondheim on ice in an isolated box. Upon arrival to laboratory samples were stored at -20°C until analysed. Sample collection (ISO 5667-24) and storage (ISO 5667-3) was done in accordance to the standard methods.

In total, 176 water samples were collected. To achieve a representable overview of the presence of organic compounds during the experimental period, 3 sampling times were selected: (1) prior to the experiment (16.11.2016), (2) a month of CO_2 exposure (19.12.2016) and (3) at the end of the experiment (10.02.2017). The first date was included to compare the natural condition of the water with its condition affected by altered CO_2 concentrations. Four CO_2 concentration (5, 12, 26 and 40 mg/l) with 3 replicates of each were included.

Figure 3.3 Sampling point, effluent water from a fish tank outlet.

3.2.2 Physico-chemical measurements

Parameters measured at each sampling date included pH, temperature, turbidity, redox potential, conductivity and salinity. Equipment used for measurement are listed in table 3.1. A complete list of water quality parameters maintained at the facility are presented in appendix B.

Table 3.1 Physico-chemical parameters and measured during sampling.

Parameter	Value	Equipment
pH		WTW Multi 3430
Redox potential	mV	As above
Temperature	°C	As above
Salinity	ppt	WTW Multi 350i
Conductivity	mS/cm	As above
Turbidity	NTU	Turbiquant ® 1100 IR turbidimeter.
CO2	mg/l	Franatec ® sensors (SP2, SP3 and PP3)

Temperature, pH, oxygen and salinity were measured inside the fish tank due to operational reasons. Briefly, electrodes of portable multimeters (table 1.1) were placed in the fish tank and the parameters (pH, redox potential, temperature, salinity and conductivity) were observed after 1-2 minutes. Turbidity was measured from the tank outlets using a turbidimeter. Water was collected from outlets in plastic bottles (1 L) and measurements were taken by keeping the electrodes in there for 2-5 minutes. The supplementing glass vial was rinsed with water from the outlet of the tank followed by measurement. The order of water quality measurements was

randomised at each date. CO_2 (mg/l) was measured daily during the experiment using 3 Franatech ®sensors, SP2, SP3 and PP3.

3.3 Method development

3.3.1 Chemicals

n-Hexane (HPLC grade, 98%), Ethyl Acetate (HPLC grade, 98%), Dichloromethane (GC grade, 99.9%), Methanol (HPLC grade, 99.8%), Water (LC-MS grade) and anhydrous sodium sulphate (98.5-101%) were purchased from VWR Chemicals (VWR International, Norway). Acetonitrile, Formic acid, Acetone was technical grade and were also purchased from VWR Chemicals (VWR International, Norway). High-purity grade Silica gel was supplied by Sigma-Aldrich.

3.3.2 Materials

All glassware used during sample preparation was made of borosilicate glass. Nitrile gloves were used during all stages of sample handling, often disposed to avoid cross contamination. Transfer of small sample volumes (≤ 5 ml) was made with disposable glass pipets supplied by VWR Chemicals. Before use, all glassware was washed twice with distilled. The steps followed washing with distilled water (18.2 MΩ, MilliQ) followed by acetone and was left to dry in a clean fume hood.

3.3.3 Method development

Liquid-liquid extraction and concentration

Liquid-liquid extraction (LLE) was initially performed using Hexane, Ethyl Acetate (EtAc) and Dichloromethane (DCM). Extracts were prepared from a 1L 'test sample' collected from a fish tank (40 mg/L CO_2) at the end of the experiment. Each extraction was repeated three times with 200 ml of sample and organic solvent. The three organic layers were combined and dried with 2-3 spatulas of Na_2SO_4. The drying agent was removed with a 125 mm VWR® Grade 415 Filter Paper. Organic extracts were concentrated to 3-5 ml at reduced pressure using a rotary evaporator (VARIO® PC 2001). During concentration the water bath was kept at 35°C and the

mechanical rotation was held at a low speed. The pressure was adjusted to the boiling point of the solvent used e.g. EtAc was evaporated at 300-200 mbar while DCM was evaporated at 500-400 mbar. The rotary evaporator was flushed with acetone and the solvent used for extraction in between sample concentrations to minimize cross-contamination of samples. Method blanks and solvent blanks were prepared along with samples. Quality controls were made by transferring an aliquot (5-10 µl) of each sample into a HPLC vial.

Fractionation using Flash Chromatography

A silica gel column was used to purify and further separate organic components in extracts based on polarity (Figure 3.4). Glass column (100 ml) with sintered glass filter and a bottom valve was packed with a slurry of high-purity grade silica gel (25 g, pore size 60 Å, mesh 200-400, Sigma-Aldrich) with Hexane (60 ml). Column was allowed to settle before sample was loaded. Once the column was settled, concentrated samples were loaded on column with glass pipettes. Fractionation was accomplished initially with non-polar solvent (hexane) and the polarity was increased then stepwise. The extract made with EtAc were fractionated with Hexane (100%), Hexane/EtAc (80:20, v/v), Hexane/EtAc (40:60, v/v) and EtAc (100%). DCM sample extract was also fractionated in similar manner using the same ratios of Hexane and DCM i.e. 100:00, 80:20, 40:60 and 100:00 of Hexane:DCM. The sample extract of Hexane was eluted with 100 ml of Hexane only. A new column was prepared for each sample extract. Method blanks were prepared for each fraction where 50 ml of the solvent mixture used was passed through the column. The fractions collected were reduced to 1-2 ml using rotary evaporator. The samples were then transferred to test tubes and 0.25 ml of LC-MS grade water was added to it. Samples were then concentrated under gentle flow of N_2 at 35°C (Biotage TurboVap LV) until the volume was reduced to 0.25 ml. Finally the volume was adjusted to 0.5 ml by adding 0.25 ml of Methanol. Solvent blanks were prepared by evaporating 1 ml of the solvent until dryness under N_2 and reconstitution with MeOH/Water (50:50). The samples were stored (-20°C) in HPLC vials until analysed. An overview of the procedure is shown in figure 3.4.

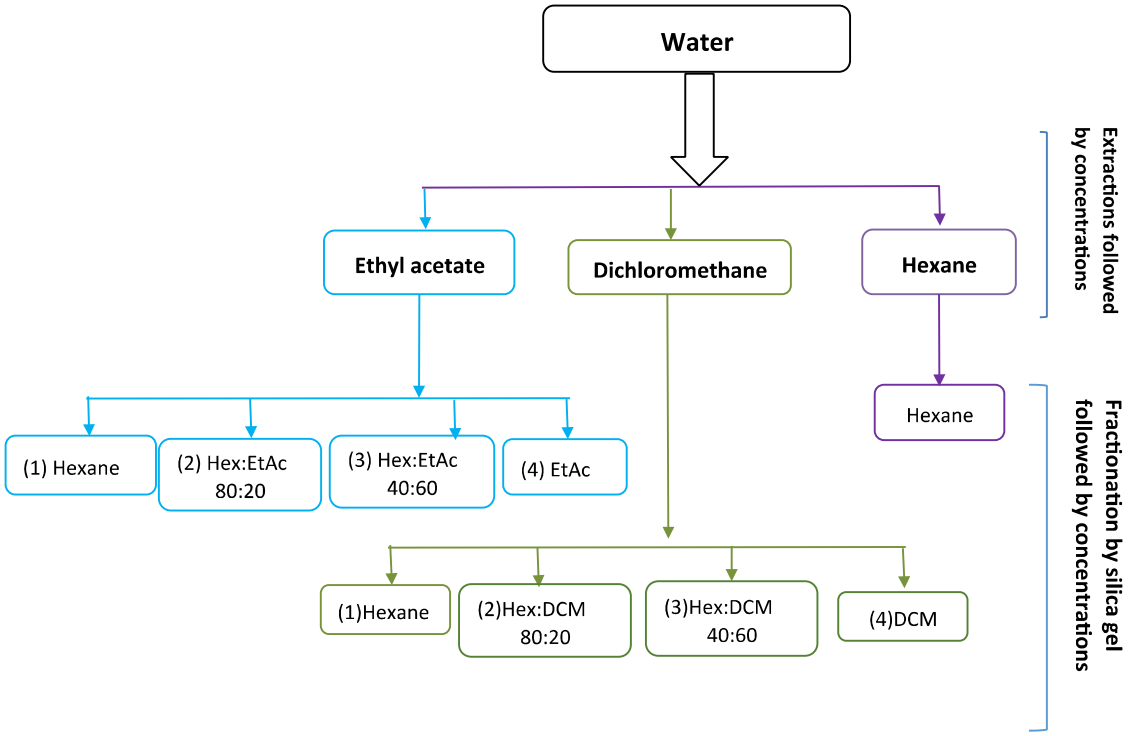


Figure 3.4 Schematic diagram of extraction and fractionation procedure.

3.3.4 Preparation of dual extract – Standard extraction procedure

The procedure described under this section was used as the standard procedure for extraction of organic compounds from all the samples collected during this study.

Based on results from the analysis of fractions, EtAc and DCM were selected as the suitable solvents for extraction. A combined extract of the two solvents was prepared as the next step in optimization of liquid-liquid extraction. Approximately 30 ml from the 1L "test sample" was filtered and extracted twice with separately first with EtAc and then with DCM. EtAc extract was first reduced to 0.5 ml and then DCM extract was added to it. Combined extracts were then reduced to 0.5 ml using rotary evaporator. As described above, 0.25 ml of LC-MS grade water was added to it, and the volume was reduced to 0.25ml and then methanol (0.25 ml) was added to it to constitute the final volume to 0.5 ml. Samples were stored (-20°C) in HPLC vials until analysis.

3.3.5 Formulas for calculation

Given below is the formula used to calculate the final volume of extracts needed to obtain an equal relative amount per ml sample for UPLC. . The original volume of water in each sample was multiplied by this factor to achieve the total volume (ml) of MeOH/water needed.

$$\frac{V_{\text{MeOH/Water}}}{V_{\text{sample}}} = \frac{0.5\text{ ml}}{30\text{ ml}} = 0.0167$$

For 30 ml of water sample extracted, 0.5 ml of 50:50 MeOH/Water was added.

3.4 UPLC-MS/MS analysis

3.4.1 Instrumentation

The UPLC analysis was carried out using an AQUITY UPLC system connected to a Synapt G2-Si Mass Spectrometry detector. The Synapt was equipped with an electron ionization source operating in positive (ESI+) and negative (ESI-) mode. Two chromatographic columns were utilized during analysis; an Aquity UPLC HSS T3 column (2.1 x 100mm, 1.8 μm) and an Aquity UPLC BEH C18 column (2.1 x 100 mm, 1.7 μm) supplied by Waters. The latter was only included during method development.

3.4.2 Method development

UPLC-MS/MS conditions

The temperature of the columns and autosampler was 30°C and 10°C respectively. Injection volume was 5 μl. The flow rate was kept at 0.5 mL/min.

MS/MS detection was carried out in positive (ESI+) and negative (ESI-) modes. Capillary voltage was 2.5 kV. Desolvation temperature was 500°C and desolvation flow 1000 l/h. Source block temperature 120°C. Cone voltage was set to 3 kV and cone gas flow 100 l/h. Spray voltage was Collision energy was set to 4.0. Acquisition mass range was 50 – 1500 m/z. MS scan time was 1.0/sec.

The gradient elution was adjusted with different proportions of solvent A and B to obtain the best resolution of compounds on the column used. The initial % of Water + 0.1%FA was 95% (Gradient elution programme 1, table 3.2) and later changed to 70% (elution programme 2, table 3.2). Sample run time was initially set to 22 min and later reduced to 15 min as peaks were observed within this time window.

Table 3.2 Gradient elution programs used during method development.

Gradient elution	Time (min)	% A	% B
1. Elution program	Initial	95	5
	18	5	95
	20	0	100
	22	95	5
2. Elution program	Initial	70	30
	10	5	95
	13	0	100
	15	70	30

3.5 Identification analysis

The chromatographic data was analysed using two softwares: Masslynx V4.1 and Progenesis QI V2.3. Masslynx was used to visually inspect each chromatogram and to determine the peaks that likely represented actual substances originating from water samples. This information was further taken into account during compound identification using Progenesis QI.

The raw data obtained from the UPLC-MS/MS analysis was first imported to Progenesis QI. Each run (sample) were then aligned to a reference run to compensate for drifts in retention time between the runs. The alignment of each sample was shown as a 2D-ion intensity map where areas marked as green indicated good quality and areas marked as red indicated with poor quality. It is a critical step in the data processing as the quality affects the accuracy of peak detection. When this section was considered sufficient, the next step was to set up an experiment design. The two options for this were between-subject design and within-subject design. In this

study, only the former was used. The samples were grouped according to their condition (either CO_2 treatment or sampling date in this study) depending on what comparisons needed to be made. The experiment design could easily be adjusted by creating new designs or adjusting existing designs. The next step was peak picking. Ion intensity was set to > 1000. Retention time limits were set to 0.5 to 15 min. The possible adducts were then selected. For the analysis in ESI+ the following adducts were included: [M+H] [M+2H] [M+H-2H20] [M+H-H20] [M+NH4] [M+Na] [M+CH3OH+H] [M+K] [M+ACN+H] [2M+H] [2M+NH4] [2M+Na]. For the analysis in ESI-, the following adducts were included: [M-H] [M-2H] [M-CO2-H] [M+Na-2H] [M+Cl] [M+FA-H] [2M-H]. Further, the deconvolution was reviewed. In this process, ions were grouped into compounds. Ions were either added to or rejected from a compound depending on matching chromatographic profile and mass spectra. In general, ions that actually belonged to a compound would have very similar profiles of the two. As such, the deconvolution ensured validity in each compounds composition. The next step in the process was identification of compounds. In this study, Elemental composition, Chemspider and Metlin MS/MS Library search methods were used, although several other were available from the software. Elements included were H, C, N, O, P, S, Cl. Elemental composition search was performed at the following limits: 5 ppm precursor tolerance and isotopic similarity at 90%. Chemspider search was performed at 5 ppm precursor tolerance, isotope similarity at 95% and theoretical fragmentation at 5 ppm tolerance. Within the Chemspider database a wide variety of data sources are available, however the ones included in this experiment were NIST, NIST Spectra, NIST Chemistry WebBook, KEGG and PubChem. Metlin MS/MS search was performed at 5 ppm and 12 ppm precursor tolerance and fragment tolerance.

The next step in identification was to review the list of possible matches given to each compound. To do such, parameters obtained from the search methods were evaluated. These were: Mass error (-5 to 5 ppm), Isotope similarity (0-100%) and Fragmentation score (0-100). The overall score (0-100) given an identification was calculated from the mean value of these, where each parameter can contribute with a maximum value of 20. Hence, the overall score of a possible match was limited to 0-60. In general, identifications were accepted based on the match having the highest score and isotope similarity, as it would give the most likely identification. Whenever a high fragmentation score was obtained (> 80%), this was taken into account as well. Mass error was considered to a less degree. If very unlikely identifications

occurred, e.g. unusual isotopes (C^{13} and N^{15}) and doubly charged compounds, these were removed from the list of possible matches to reduce the risk of false identifications.

3.6 Statistical approach

3.6.1 Physico-chemical parameters and organic compounds

Normality of data was tested using Shapiro-Wilk test in R with the function shapiro.test(). Since dataset did not follow the normality assumption so non parametric tests for used for comparison of different variables. Kruskalis-Wallis H-test was used to study the impact of CO_2 levels on physicochemical parameters (pH, redox potential, turbidity, salinity, conductivity and temperature) and organic compounds. Kruskalis-Wallis H-test was applied in R with the function kruskal.test(). Spearman's rank correlation coefficient analysis was used to study relationship between CO_2 levels and physicochemical parameters.

3.6.2 Principal Component Analysis (PCA)

Principle component analysis was performed to determine underlying relationships and patterns within the dataset. The variables used were physico-chemical parameters, trace elements and organic substances. A loading plot was created to visualize correlations among all the variables. A biplot compounds was created to visualize how organic compounds related to the samples.

Trace elements included were Al, As, Cd, Cr, Cu, Fe, Mn, Ni and Zn based on results from trace metal analysis (data presented by Bye, 2017). Physico-chemical parameters are the parameters measured along with water sampling (pH, temperature, salinity, redox potential and conductivity. Turbidity was excluded from due to missing values at sampling 16.11.16. The organic substances included are the 12 substances listed in table 4.2. Chromatographic measurements of organic substances are collected from the software Progenesis QI. These contain the abundance of every organic compound in each sample.

4 Results

4.1 Physico-chemical parameters

The water quality parameters (pH, redox potential, temperature, salinity, conductivity and turbidity) measured during the course of the study are shown Figures 4.1 to 4.3. The measurements included 8 time points over a 12-week time period (16.11.2016 -10.02.2017).

Overall, pH values ranged from 6.5 – 7.8 with lowest pH-level detected in 40 mg/l CO_2 and highest pH level detected in 5 mg/l CO_2. pH was significantly reduced over the time period (Kruskal-Wallis, $\chi^2_{(5)}$ 95.611, p <0.001). Within the respective CO_2 treatments, pH-levels remained unaltered over the course of the study (figure 4.1). Redox potential was recorded from 171. 5 – 221.3 mV and the highest level was detected in 40 mg/l CO_2 and the lowest level in 5 mg/l (figure 4.2). Fluctuations were observed for redox potential which increased significantly over the time period (Kruskal-Wallis, $\chi^2_{(5)}$ 44.845, p <0.001). The lowest and highest temperature recorded was 12.1 and 13.8 °C respectively. Salinity varied from 10.6 – 11.6 ppt. Conductivity was measured within from 18.3 – 19.7 (mS/cm). Turbidity was measured from 1.8 - 9 NTU. Temperature, salinity, conductivity and turbidity was maintained at a similar level among CO_2 treatments over the time period (figure 4.2-4.3).

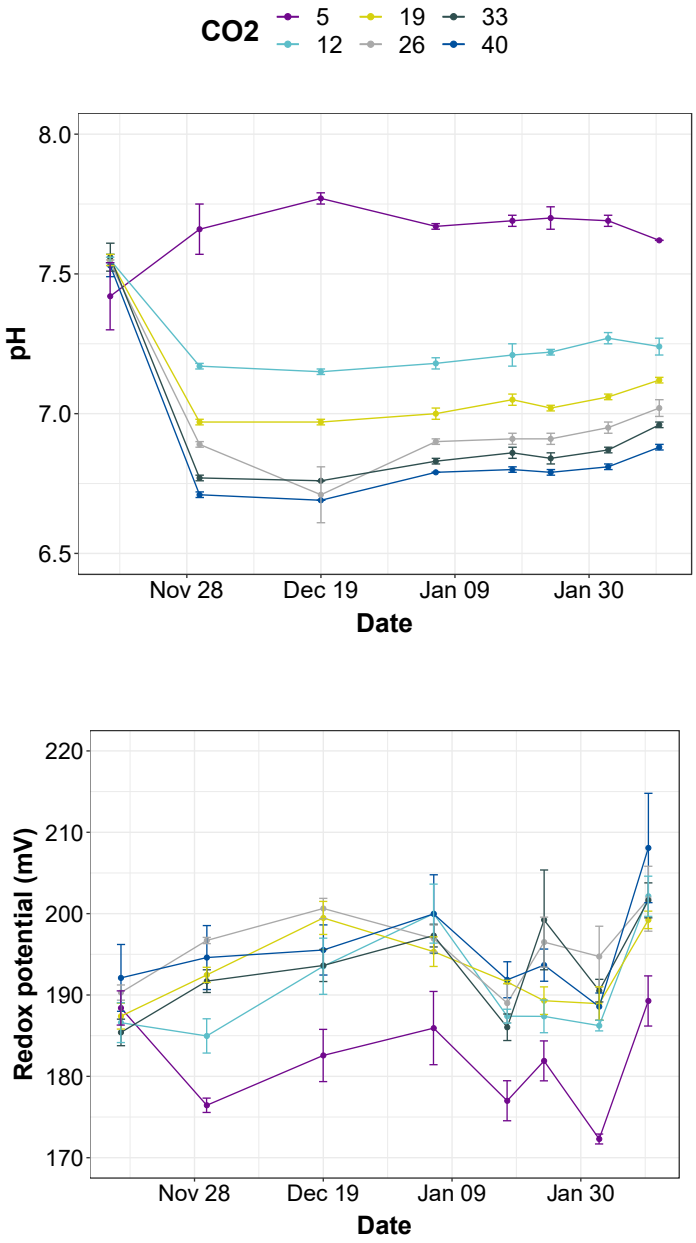


Figure 4.1 Variations in pH and redox potential (mV) from start (16.11.16) to end (10.02.17). Each point represent mean values of 3 replicates □ standard error (n=3), within 6 CO2 concentration levels (5, 12, 19, 26 33, 40 mg/l).

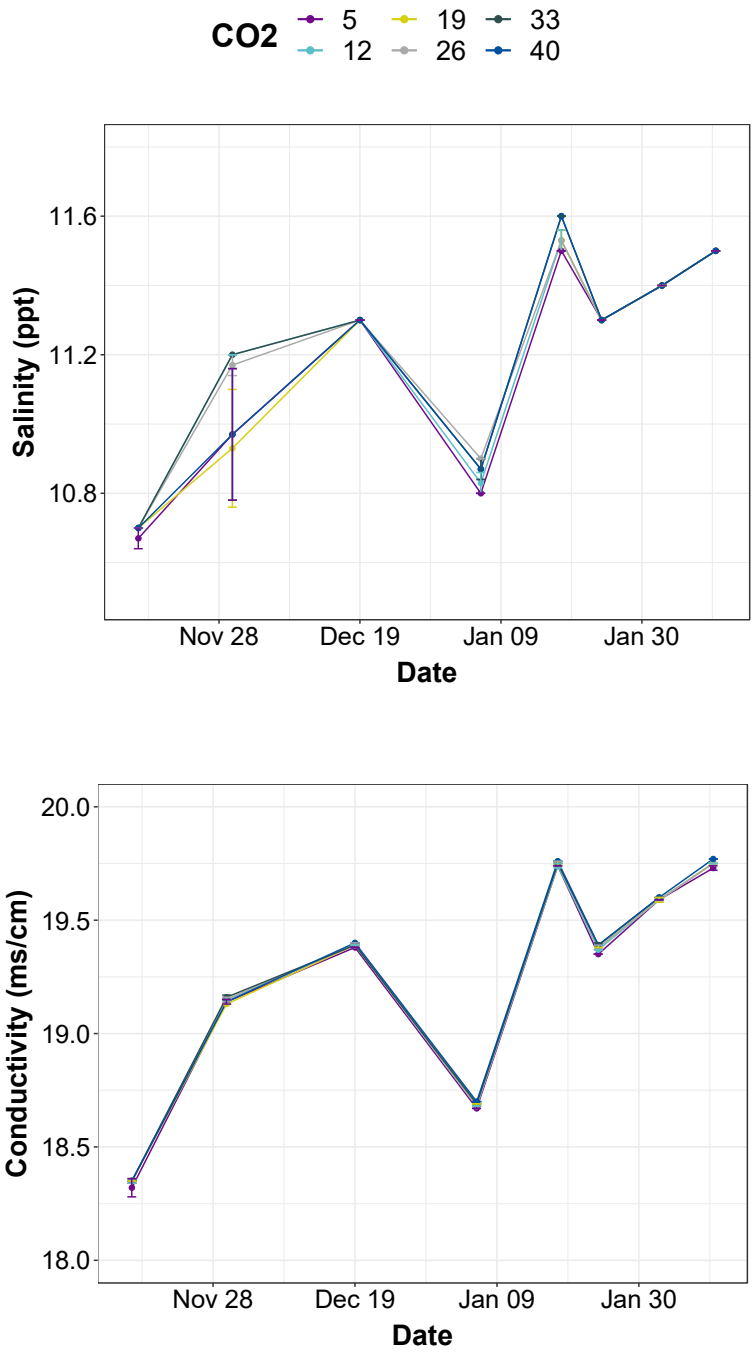


Figure 4.2 Variations in salinity (ppt) and Conductivity (mS/cm) from start (16.11.16) to end (10.02.17). Each point represent mean values of 3 replicates standard error (n=3), within 6 CO2 concentration levels (5, 12, 19, 26 33, 40 mg/l).

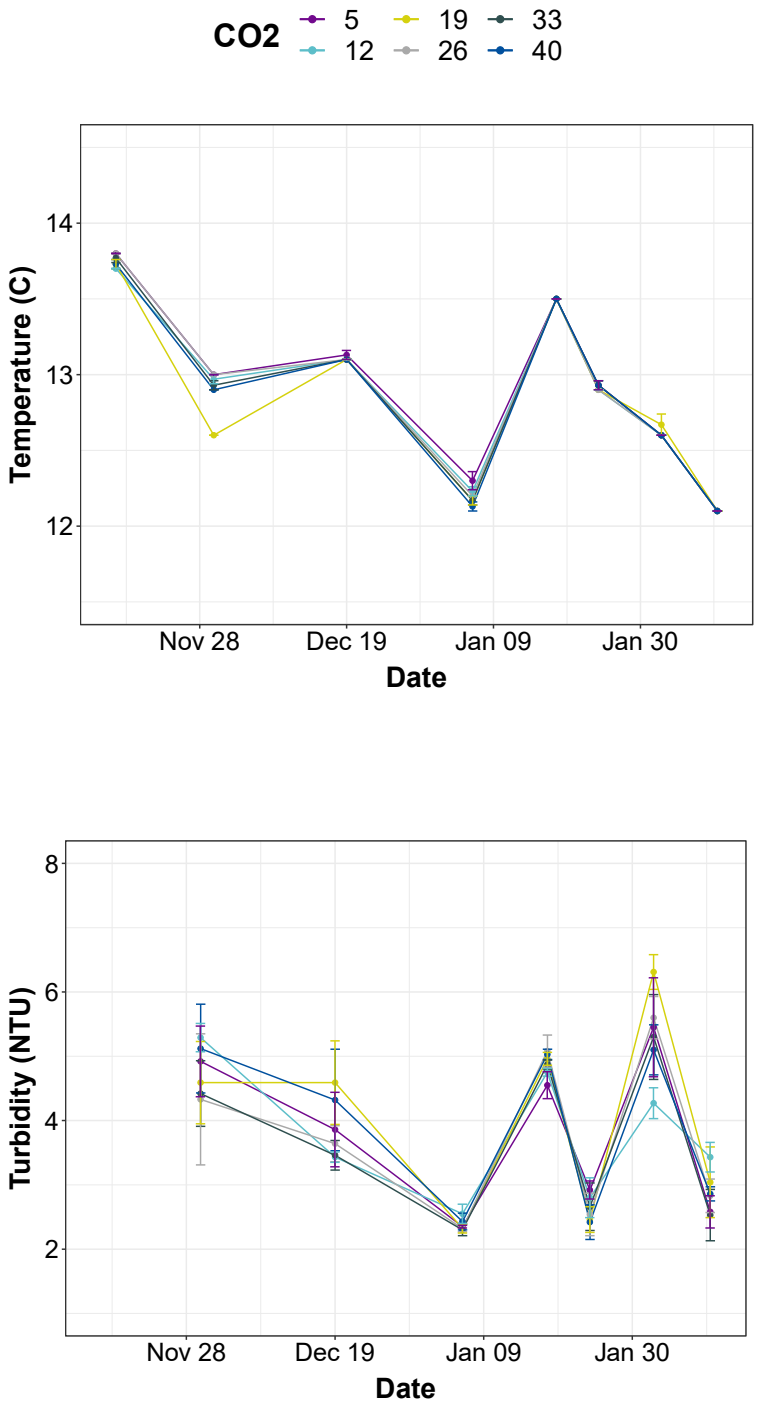


Figure 4.3 Variations in temperature (°C) and turbidity (NTU) from start (16.11.16) to end (10.02.17). Each point represent mean values of 3 replicates ± standard error (n=3), within 6 CO2 concentration levels (5, 12, 19, 26 33, 40 mg/l).

4.2 Relationship between parameters and CO_2

The relationship between physico-chemical parameters and CO_2 (mg/l) are shown in figure 4.4. pH and CO_2 was significantly correlated with a negative relationship (Spearman's p <0.001). Redox potential and CO_2 was significantly correlated with a positive relationship (Spearman's p <0.001). Salinity, conductivity, turbidity and temperature were not significantly correlated with CO_2. Correlation plot for salinity, conductivity, temperature and turbidity versus CO_2 are plotted as well to illustrate the stability of the system under different CO_2 levels (figure 4.4).

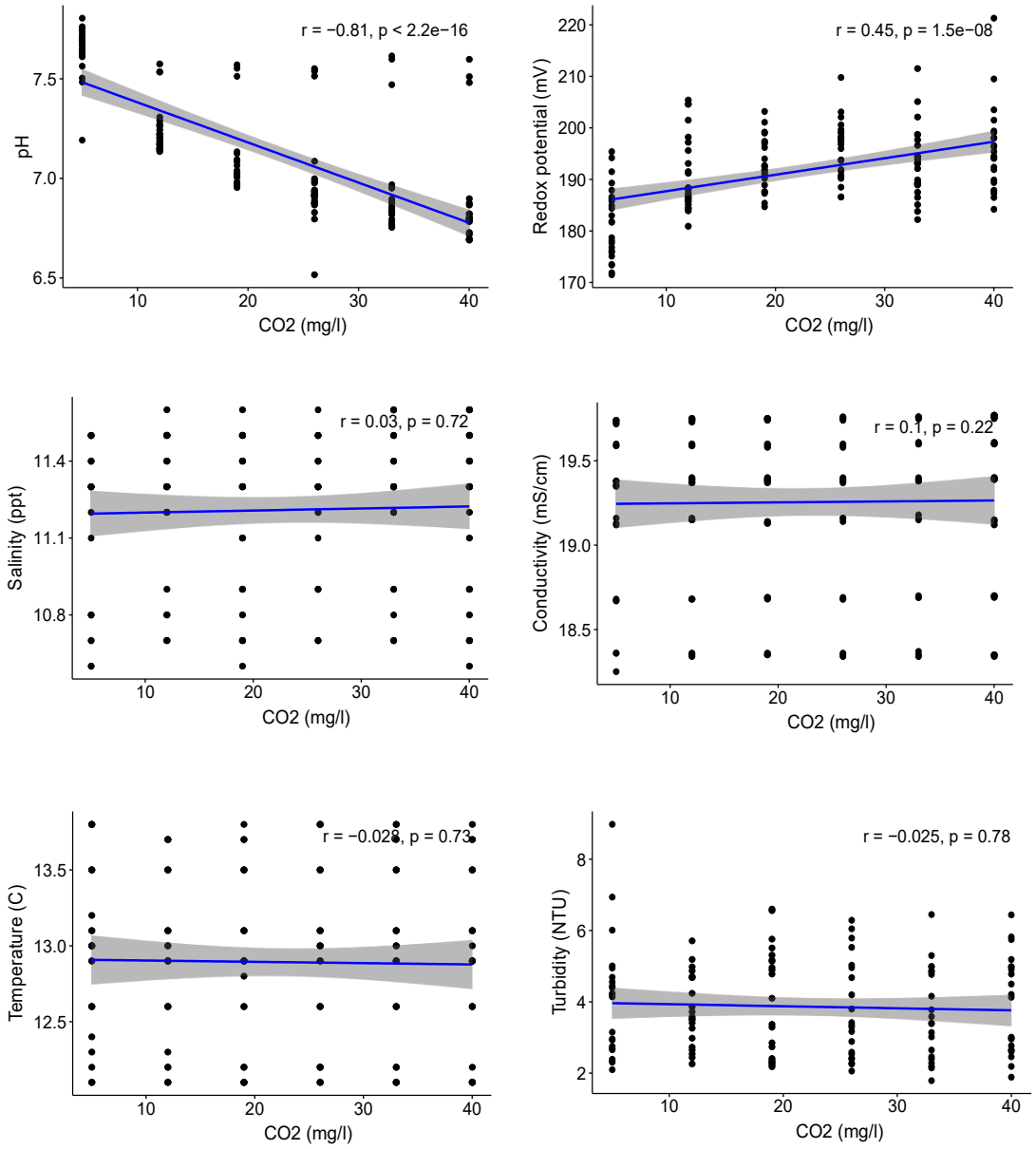


Figure 4.4 Plot showing the correlations for pH, Redox potential, salinity, conductivity, temperature, turbidity versus concentration levels of CO2 (5, 12, 19, 26, 33 40 mg/l). Data points represent residuals from 8 time points, within each CO_2 level. The blue line represent a linear regression through the residuals. The light grey area represent 95% confidence interval. Spearman correlation coefficients (r) are displayed on each

4.3 Method development

4.3.1 Selection of suitable solvent system for extraction

In an attempt to optimize the liquid-liquid extraction method for analysis of organic substances, three solvents were tested at the start of the study (Hexane, Ethyl Acetate and Dichloromethane). Flash chromatography was applied to fractionate the extracts further according to polarity. Chromatograms from UPLC-Q-TOF analysis of fractions are shown in figures 4.5 - 4.6. Visual inspection and comparison with blank samples indicated inefficient extractions using Hexane. The chromatographic pattern was equal to blank samples, in both ionisation modes.

Visual inspection and comparison with blank samples indicated a high abundance of peaks in chromatograms of EtAc and DCM extracts, particularly positive mode analysis (ESI+) (figure 4.5) fractionated with ≥ 60-100% of polar solvent (F3, F4). The highest number of peaks were detected in EtAc extract (eluted with 100% EtAc, 14 peaks) and followed by DCM extracts (eluted with 100% DCM, 8 peaks). DCM extracts analysed in negative mode (ESI-) showed mostly unresolved peaks (figure 4.6). Fraction F3 (Hexane:EtAc 20:80 v/v) from EtAc extract is not shown below as the sample was excluded from analysis due to contamination. The chromatographic profile differed slightly among fractions of EtAc and DCM extracts, therefore both extraction solvents were included in further sample preparation steps.

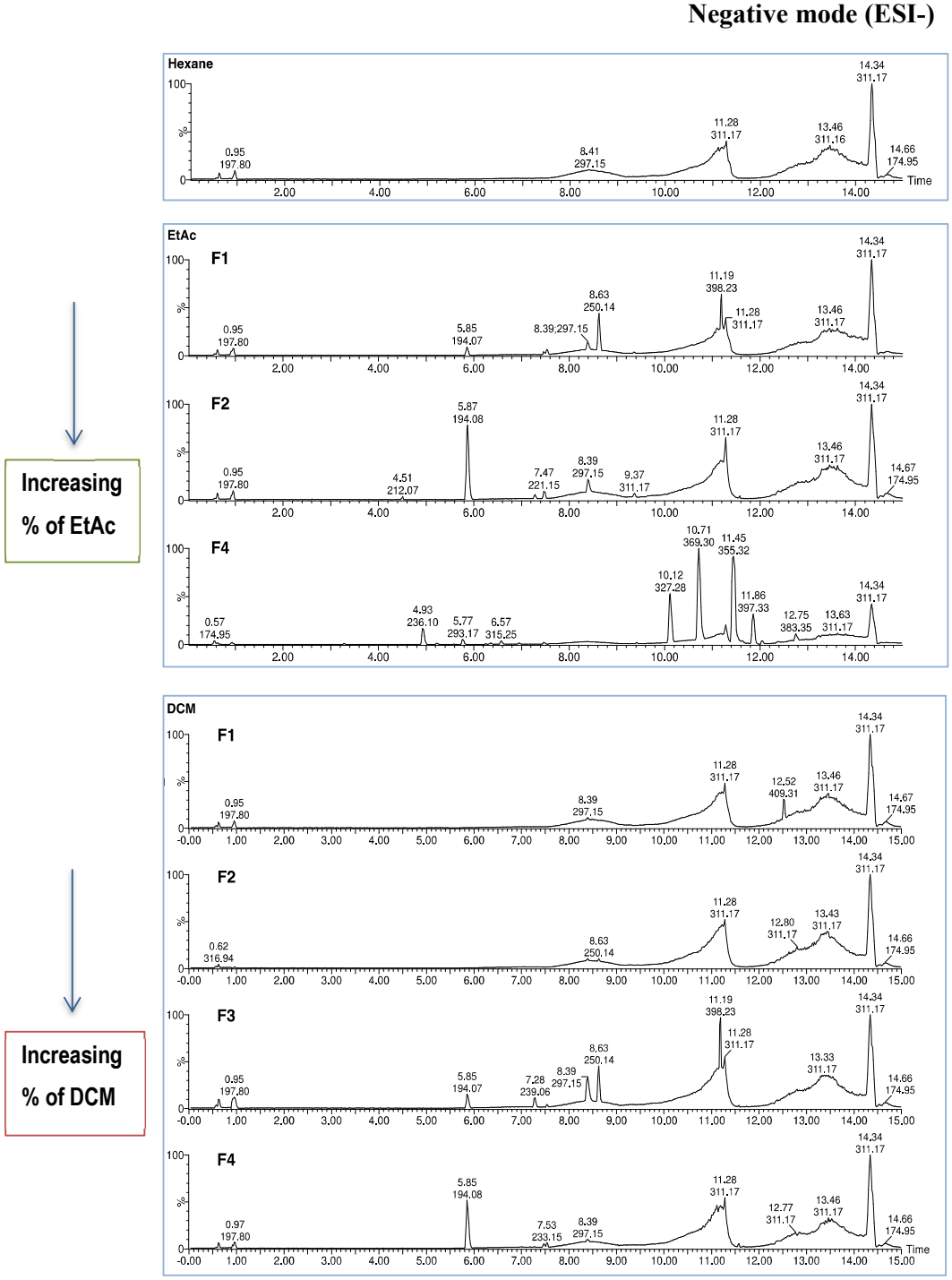


Figure 4.5 UPLC-Q-TOF chromatograms obtained after chromatographic separation on the HSS T3 column.. Chromatograms showing fractionated extracts of Hexane, Ethyl Acetate (F1, F2,F4) and Dichloromethane (F1-F4) analysed in negative mode. Fractions F1, F2 and F3 represent 100:00, 80:20, 40:60 and 100:00 of Hexane:DCM or EtAc.

Positive mode (ESI+)

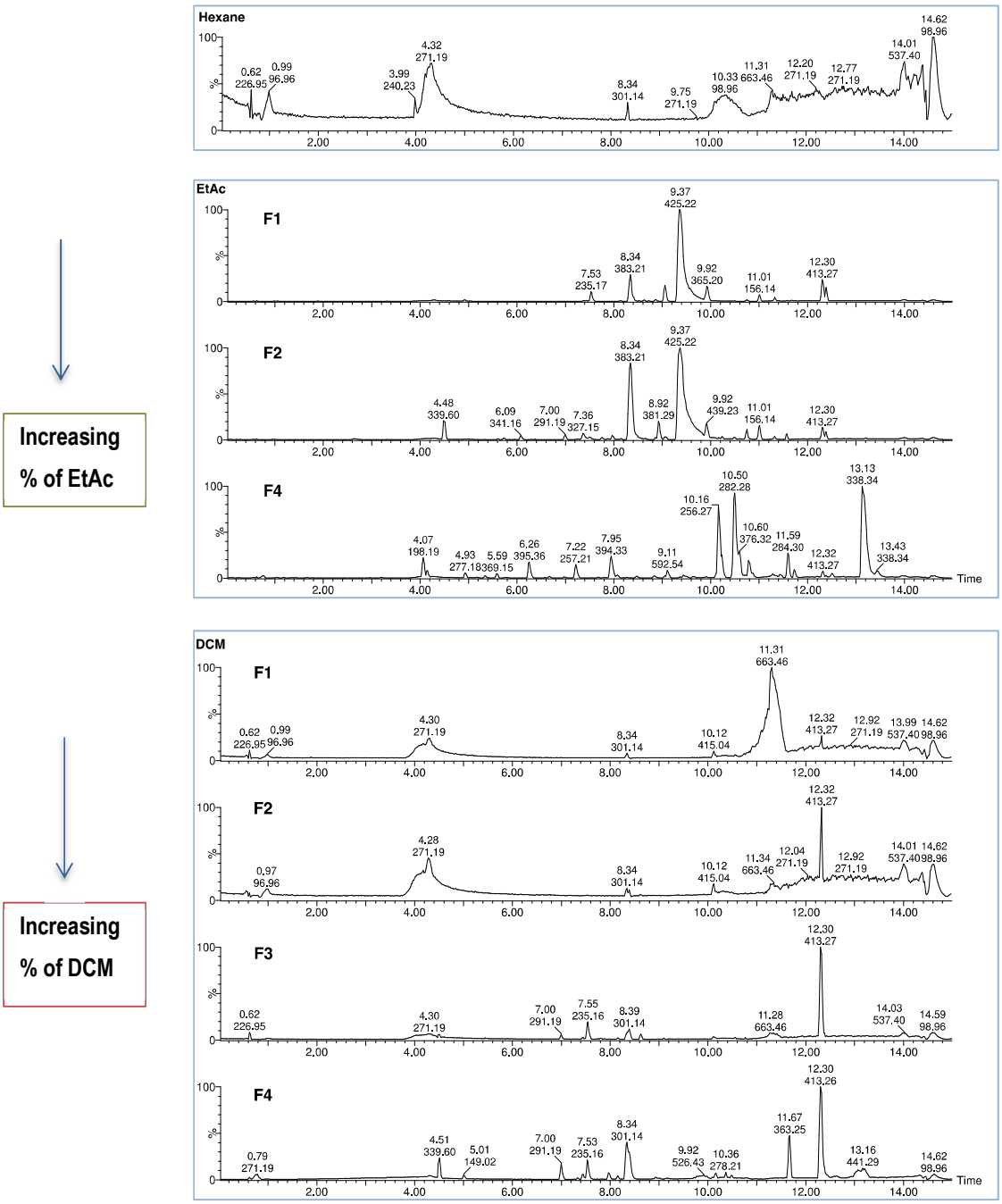


Figure 4.6 UPLC-Q-TOF chromatograms obtained after chromatographic separation on the HSS T3 column.. Chromatograms showing fractionated extracts of Hexane, Ethyl Acetate (F1, F2,F4) and Dichloromethane (F1-F4) analysed in positive mode. Fractions F1, F2 and F3 represent 100:00, 80:20, 40:60 and 100:00 of hexane:DCM or EtAc

4.3.2 Selection of suitable UPLC column

Two chromatographic columns were tested and evaluated for the analysis of the combined extract of EtAc and DCM. The chromatograms obtained are shown below in figure 4.7. The extract was first tested on the column used for analysis of fractions, HSS T3, and later on a BEH C18 column. The chromatographic profiles obtained were similar, however the resolution was improved on the HSS T3 column, due to better baseline separation and sharper peaks. This column was selected for further analysis of samples.

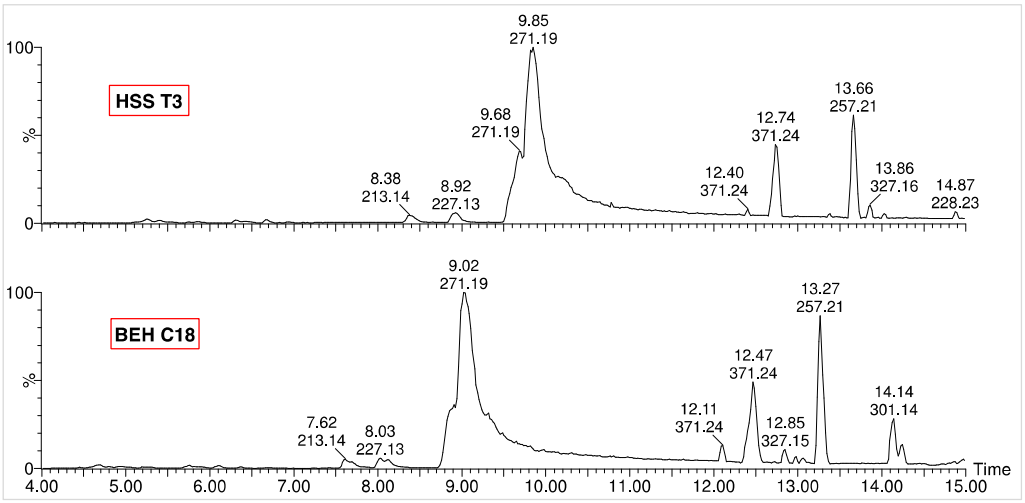


Figure 4.7 Base-peak-chromatograms obtained from chromatographic separation of combined extract (DCM and EtAC) on two columns, HSS T3 and BEH C18.

4.4 Chromatogram analysis

The chromatographic profile of samples were visually compared with respective solvent blanks and method blanks in both ionization modes (ESI-/ESI+) to identify compounds that could originate from the RAS water. Further, chromatograms were compared across sampling dates to investigate if there were tendencies to accumulation among the observed compounds.

4.4.1 Analysis in negative mode

Figure 4.8 shows two chromatograms of samples collected from a fish tank with 40 mg/l CO_2 at the start and end of the experiment. The chromatographic profile analysed in negative mode was overall similar for all the samples studied here including method blanks. Therefore, results from the analysis in negative mode were therefore not included in identification analysis.

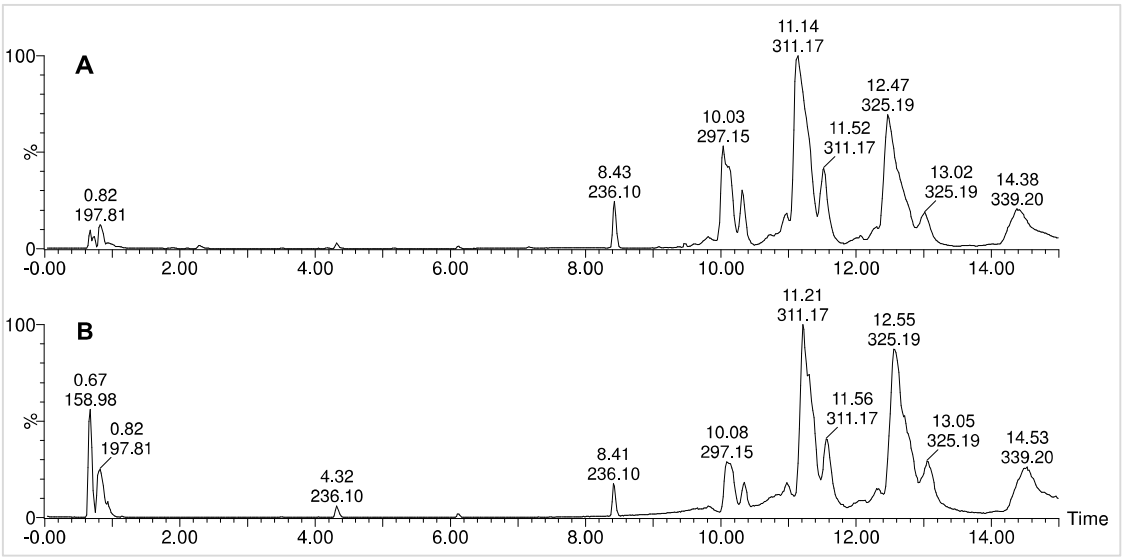


Figure 4.8 Example showing the overall chromatographic profile obtained from samples analysed in negative mode (ESI-). Base-peak chromatogram showing retention time (min) versus peak intensity (%). A represent a sample collected prior to the CO2 experiment (16.11.16), B represent a sample collected at the end (10.02.17). The samples were collected from the same tank treated with 40 mg/l CO2.

4.4.2 Analysis in positive mode

Identification analysis

Based on conclusions from inspection of chromatograms, the identification process was narrowed down to focus on compounds measured within 9-12 min in positive ionization mode (ESI+). All identifications made in this study are referred to as tentative as they were not confirmed by analysis of reference standard.

Data analysis by Progenesis QI

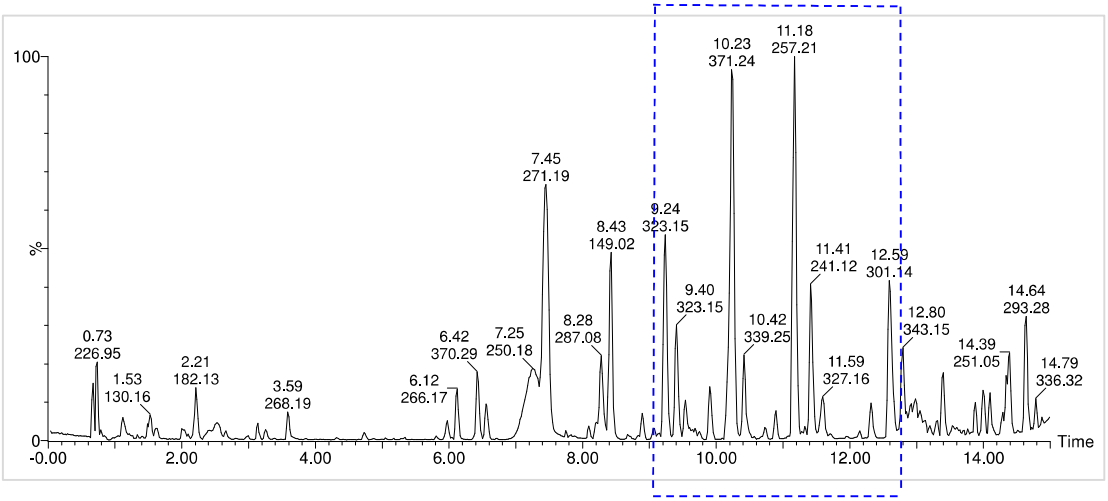
Tentative identifications were made by using the software Progenesis QI. Raw data obtained from UPLC-Q-TOF was used during peak picking and yielded 521 detected ions in positive

mode and 375 ions in negative mode. Overall, in positive mode, 309 of 411 compounds were attributed possible identifications using the available databases within the software. Identifications were made for 10 of the 12 observed peaks eluting within 9-12 min. Ions detected as m/z 323.15 (RT 9.54) and m/z 257.21 (RT 10.90) were not identified by Prog. QI. However, the total number of compounds was12 as some ions were observed at several retention times in chromatograms (m/z 241.12 and m/z 327.15) and were therefore accounted for during identification analysis. Elemental composition search produced elemental formulas for each compound with elements of C,H,N,O,P. Chemical structures were obtained from database search with Chemspider and Metlin MS/MS. Theoretical fragmentation was found for 4 of the compounds eluting within 11.41-11.59 min with (fragmentation score > 85%).

Organic compounds from different CO_2 treatments

Figure 4.9 A shows a UPLC-Q-TOF chromatogram obtained from analysis of a sample in positive mode. The highlighted area indicate 11 peaks that were not detected in blanks (figure 4.9 A-B). A few peaks were persistent in chromatograms throughout the study (figure 4.9 C). These were detected as m/z 323.15 (RT 9.24) m/z 323.15 (RT 9.40) and m/z 327.15 (RT 11.41). One important observation was a compound at 9.15 min, 378 m/z that appeared in chromatograms from last set of samples collected during CO_2 RAS experiment (figure 4.11D).

A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-0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
Time
0.73
226.95
1.53
130.16
2.21
182.13
3.59
268.19
6.12
266.17
6.42
370.29
7.25
250.18
7.45
271.19
8.28
287.08
8.43
149.02
9.24
323.15
9.40
323.15
10.23
371.24
10.42
339.25
11.18
257.21
11.41
241.12
11.59
327.16
12.59
301.14
12.80
343.15
14.39
251.05
14.64
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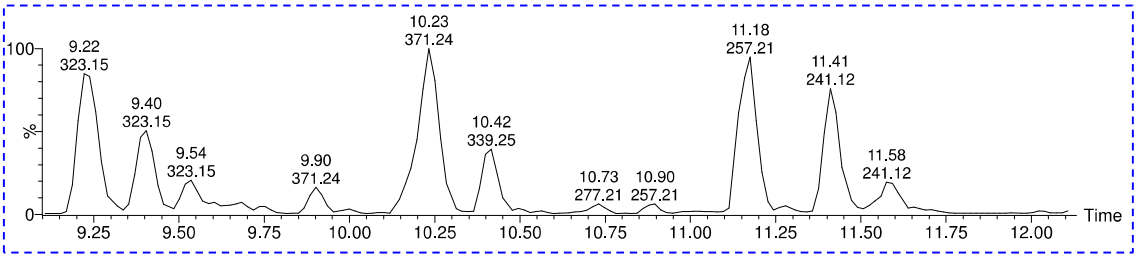
14.79
336.32

B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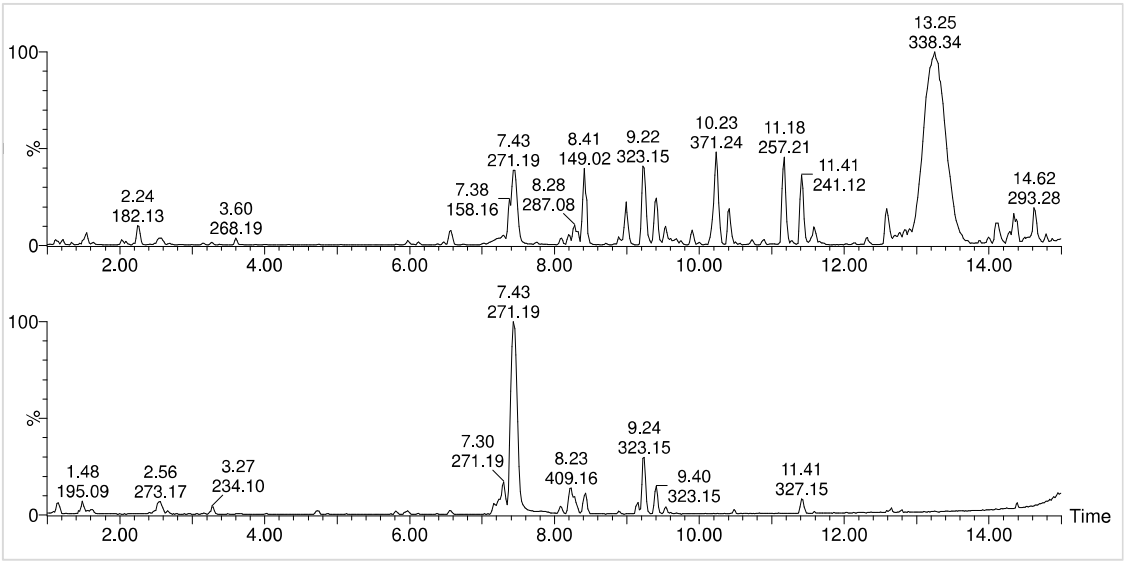
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271.19
8.28
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323.15
10.23
371.24
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11.41
241.12
13.25
338.34
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293.28
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195.09
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234.10
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D

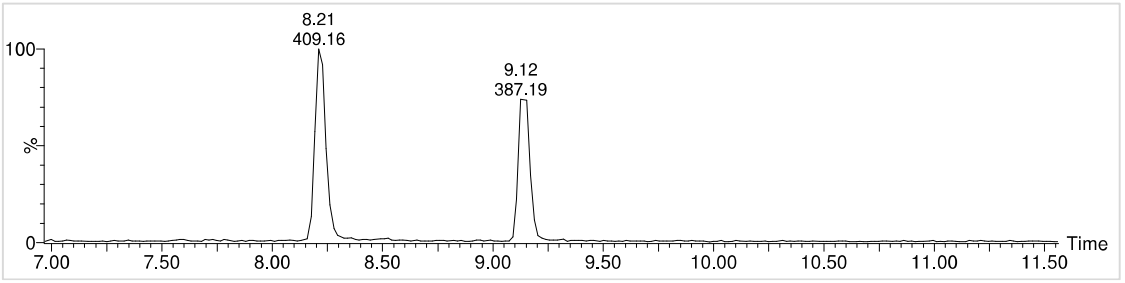


Figure 4.9 UPLC-Q-TOF chromatograms obtained after chromatographic separation on the HSS T3 column. A) Chromatogram of a sample collected before the CO2 treatment had started (16.11.16). Retention time versus peak intensity (%) is shown. The blue area indicate compounds that were not detected in blanks. B) Compounds eluting within 9-12 min, highlighted in figure A. C) Example of chromatograms showing a decline in peaks present from the start until the end of the study. The chromatogram below show 3 persistent compounds (RT 9.24, 9.40, 11.41). D) Extracted-ion-chromatogram (XIC) of the peak occurring late in the experiment, (10.02.17) detected at m/z 387.

The MS/MS spectrum (figure 4.10) revealed that the peaks at 9.24 and 9.40 could be the same compound, as the precursor of m/z 323.15 fragmented into similar product-ions (163, 135, 107, 91 and 79 m/z). As for the ion 323.15 at 9.54 min, the fragmentation pattern differed from the first two. Similarly, the ions detected at m/z 241 and m/z 327.15 shared the same fragmentation pattern and were present in their respective MS/MS spectra. Fragmentation pattern of m/z 241 and 327 are presented in appendix D.

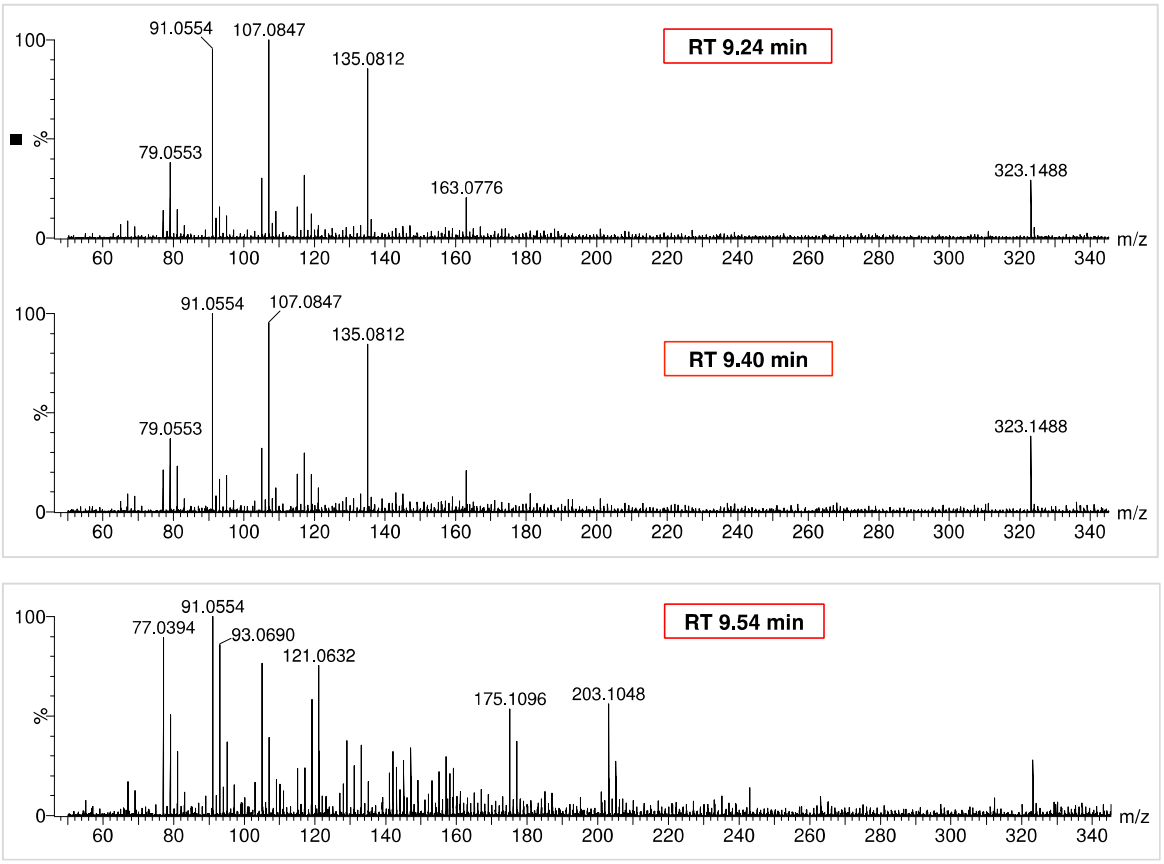


Figure 4.10 MS/MS spectrum of ion m/z 323.15 detected at 9.24, 9.40 and 9.54 min.

Changes in the profile of organic compounds from different CO_2 treatments during the course of study. Overall, higher number of peaks were observed in the chromatogram of samples collected at the start of experiment (16.11.16) in all CO_2 treatments (Figure 4.11). In total 11 distinct peaks detected in 5, 12 and 26 mg/l CO_2, whereas 8 peaks were detected in 40 mg/l CO_2 at the start of this study. However, afterward there was a decrease in the number of peaks detected in all CO_2 treatments.

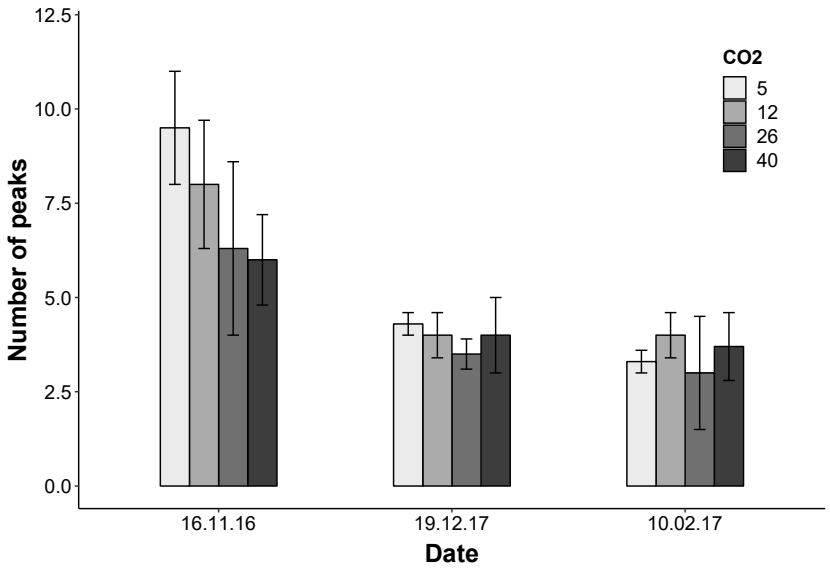


Figure 4.11 Number of peaks in chromatograms not observed in blank samples(16.11, 19.12, 10.02). Each bar represent the mean of 3 replicates within CO2 group. Error bars show the standard error of the mean (n=3). CO2 516.11 and CO2 26 at19.12.17 represent the mean of 2 replicates (n=2). (see table 4.1 for compound information).

Figure 4.12-4.13. show changes in relative abundance of 12 identified compounds (table 4.1) in CO_2 treatments from start to end of the study. Each bar graph show the variations measured within one CO_2 treatment (5, 12, 26, 40 mg/l CO_2 respectively). Each compound shows abundance for three sampling dates. Compounds 2-12 show a decreasing trend over the 12-week time period (16.11.2016 - 10.02.2017). Most noticeable change was observed in compound 1, its abundance increased 5 –and 22-fold in 26 and 40 mg/l CO_2 respectively towards the end of the experiment. In holding tank 601 all compounds had increasing levels towards the end, while in holding tank 601, the trend was similar to fish tanks. Measurements from makeup-water holding tank 601 (40 mg/l CO_2) and 602 (5 mg/l CO_2) are presented in appendix E.

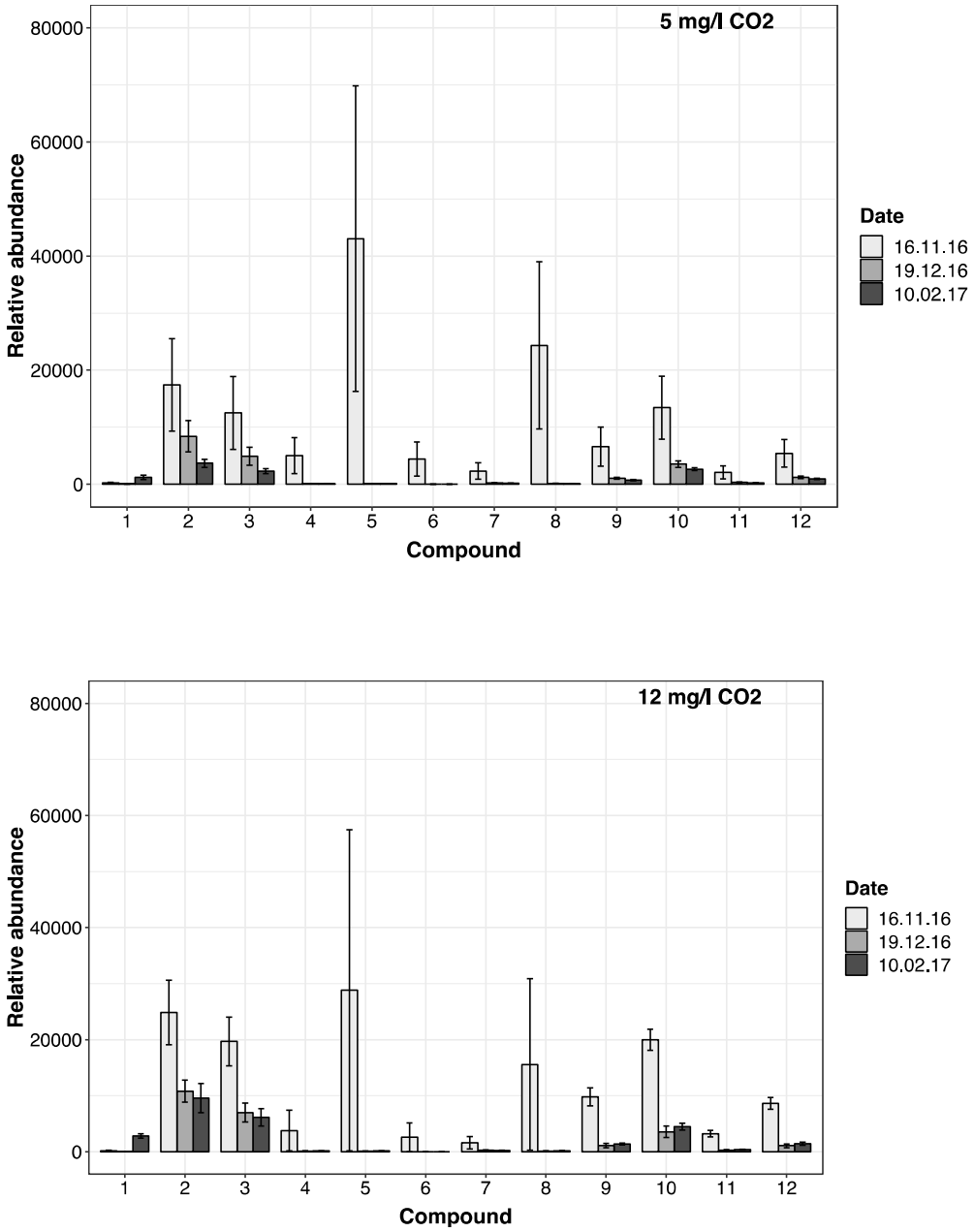


Figure 4.12 Mean relative abundance of compound 1-12 (table 4.1) within treatments of 5 and 12 mg/l CO2, from start to end of the study. Each bar represent mean of 3 replicates ± standard error (n=3). (See table 4.1 for compound information)

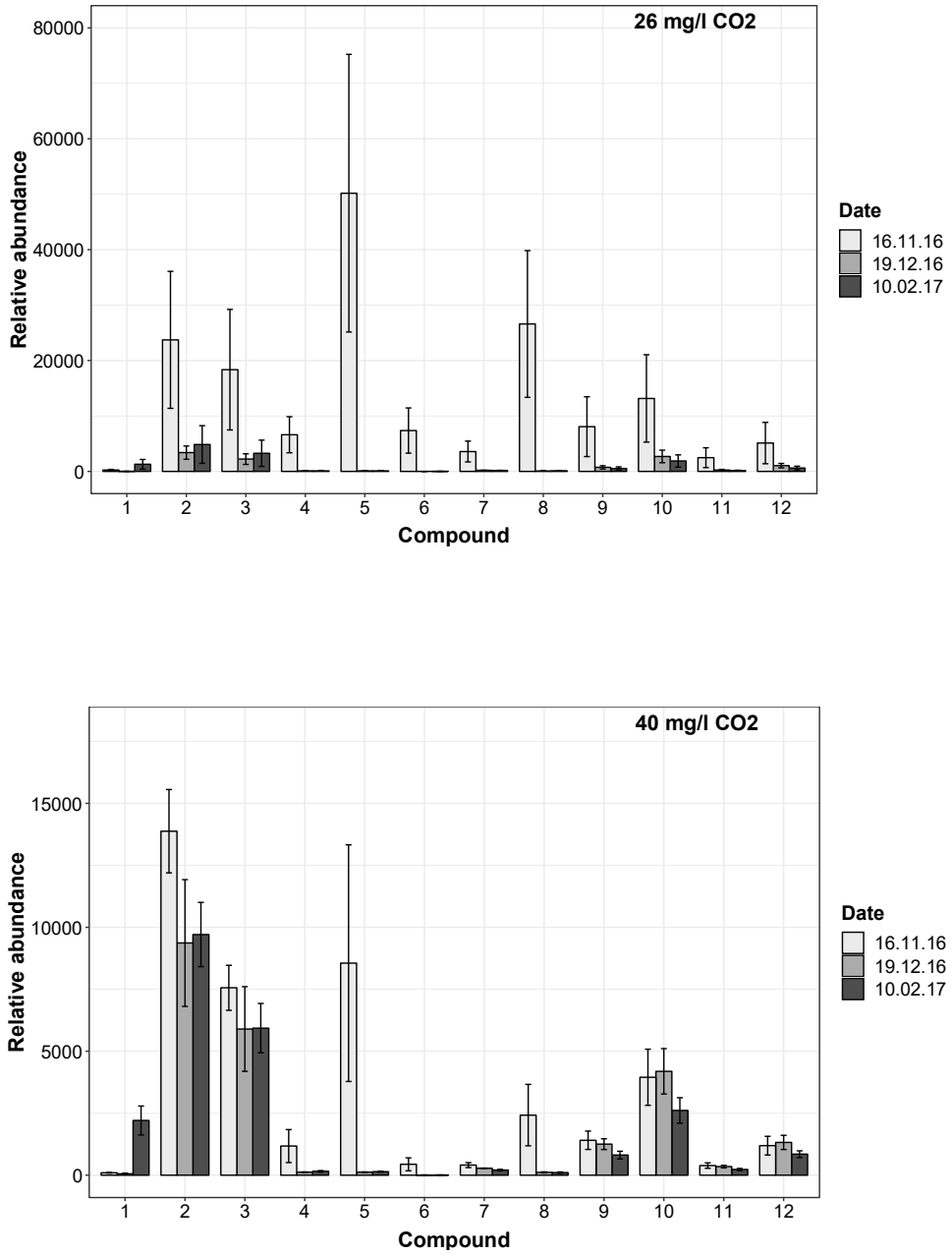


Figure 4.13 Mean relative abundance of compound 1-12 (table 4.1) within treatments of 26 and 40 mg/l CO2, from start to end of the study. Each bar represent mean of 3 rep replicates ± standard standard error (n=3). (See table 4.1 for compound information).

Figure 4.14 shows box plots total change in relative abundance over the 12-week period for compound 1 and compound 10 (see table 4.1 for compound information). Box plot of compound 1 is shown, as in contrast to compound 2-12, this compound had an increasing trend over the 12-week period. Box plot of compound 10 is illustrated, as it shows the overall pattern observed for compounds 2-12. The negative response value indicates a decreasing trend. The total change in relative abundance of compounds 1-12 (table 4.1) over the course of the study was not significantly different between CO_2 treatments. As seen from figure 4.16, the greatest change was observed in 26 mg/l CO_2 for compound 2-12, over the 12-week time period. In addition, the smallest change after 12 weeks is observed for 40 mg/l CO_2.

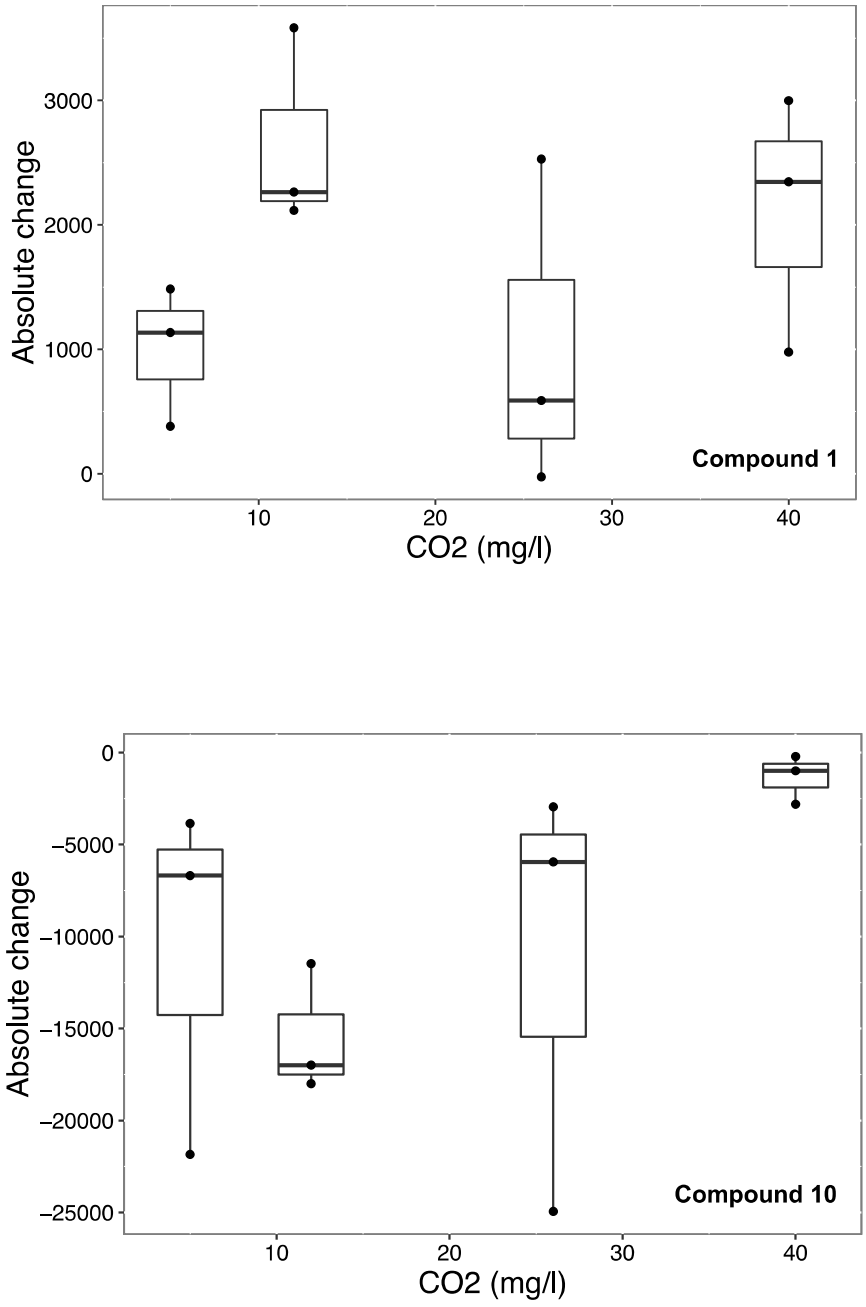


Figure 4.14 Box plot showing the change in compound abundance versus concentration levels of CO2 (5, 12, 26, 40 mg/l), over a 12-week time period. Illustrated are (a) Compound 1 (Organophosphorus compound) increasing over time (b) compound 10 (Steroid hormone), decreasing over time.

The identified compounds are listed below in table 4.1. Identified compounds were assigned a chemical class to get an overview of the predominant groups of compounds. The identified compounds are listed below in table 4.1. According the software used for compounds identification, compounds assigned fragmentation match can achieve a total score of maximum 60, while compounds given only elemental formula and chemical structure can achieve a total score of maximum 40. Compounds where a suitable match was not found from the database were classified as unknowns.

Table 4.1 Identifications of substances in water samples analysed in (ESI+) mode.

Nr	Compound class	Elemental formula	RT (min)	m/z	Adduct	Search hits	Isotopic sim(%)	Score
1	Organophosphorus compound (OP)	$C_{15}H_{25}O_7P$	9.15	387.1930	M+H-H2O, M+K	12	97.08	39.3
2	Unknown[a]	$C_{10}H_{21}N_8OP$	9.24	323.1476	M+Na, M+K	850	99.13	39.2
3	Unknown[a]	$C_{11}H_{23}N_4O_5P$	9.40	323.1467	M+H	913	98.58	38.8
4	Unknown	$C_{14}H_{32}N_6O_4$	9.90	371.2400	M+H-H2O, M+Na	3	96.38	38.7
5	Carbohydrate	$C_{18}H_{36}O_6$	1.23	371.2408	M+H-H2O, M+Na	27	95.84	39.1
6	Unknown	$C_{14}H_{30}N_2O_5$	10.42	339.2502	M+CH3OH+H	364	97.84	38.6
7	Amino acid	$C_{13}H_{25}NO_4$	10.73	277.2111	M+NH4	306	98.16	38.7
8	Ester	$C_{15}H_{30}O_4$	11.18	257.2107	M+H-H2O, M+Na	42	96.32	38.9
9	Alcohol	$C_{14}H_{18}O$	11.41	241.1227	M+K	512	97.66	58.3
10	Steroid hormone[a]	$C_{18}H_{24}O_4$	11.41	327.1572	M+Na	2361	96.53	55.9
11	Ketone	$C_{16}H_{18}O_3$	11.58	241.1225	M+H-H2O	1251	95.09	57.7
12	Steroid hormone	$C_{18}H_{24}O_4$	11.59	327.1570	M+Na	2083	95.75	55.8

[a] Compounds found to be persistent in samples.

4.4.3 Relationship of organic compounds detected with CO_2 concentration

Principle component analysis was performed to visualize potential relationships between organic compounds and CO_2 concentrations (Figure 4.16). The biplot (Figure 4.16) displays the scores of each sample, and loadings of organic compounds and CO_2, on principle component 1 and 2. Principle component 1 explain 73% while principal component 2 explains 9.5% variation in the data set. CO_2 had little impact to the model, however it is the most influential variable on principle component 2. Samples were clustered in two distinct groups on the PCA plot. The samples collected at the start of experiments (16.11.2016) characterised with high concentration of most of organic compounds (except OP) clustered on one site of PCA. Whereas the samples collected on two other occasions (19.12.2016 and 10.02.2017) had high concentrations of OP clustered on other site of PCA plot. The samples collected towards the end of seemed to be influenced by CO_2 concentrations.

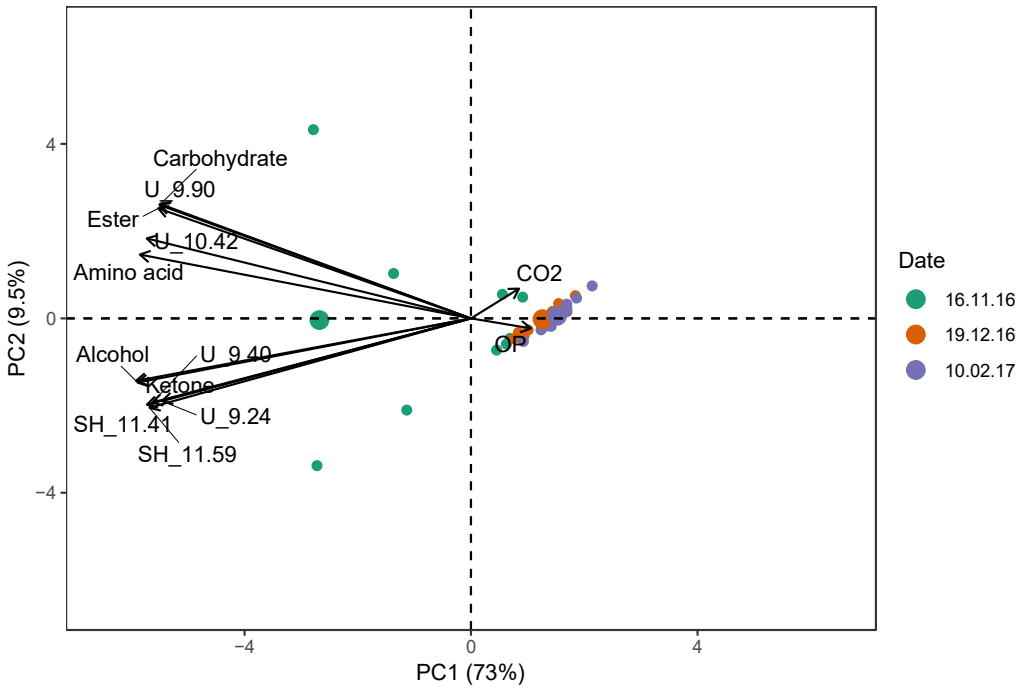


Figure 4.15 Biplot with loadings represented by organic compounds and CO2 and scores represented by observations (samples). A few compounds are described by their respective retention time to distinguish compounds given the same chemical class. These are compound 2 (U_9.24), 3 (U.9.40), 4 (U_9.90), 6 (U_10.42), 10 (SH_ 11.41) and compound 12 (11.59).

4.4.4 Relationship of variables in the dataset

PCA analysis was performed to study the relationship between Organic compounds and physicochemical parameters (CO_2, pH, temperature, salinity, redox potential, conductivity). The biplot plot in figure 4.15 displays the projection of variables and scores of samples on principle component 1 and 2. The resulting principle component 1 and 2 in the plot explain 44.3 % 17.3% respectively. The biplot shows a separation of organic compounds, collected during the adaption period (before CO_2 was added to the system, 16.11.2016). In addition, the compounds were positively correlated with elements Cu and

Cr, and less with Ni. Noticeably, samples collected at the end of the experiment (10.02.2017) characterised with high concentration of Organophosphorus compound (OP) clustered on one side of the PCA plot. This compound (Organophosphorus compound) was correlated with CO_2 and the group of elements Cd, As, Fe, Mn, and Al. The cluster of organic compounds had a high, negative contribution to principle component 1, indicated by the vector distance from the origin (magnitude of the loading). The cluster of elements (Zn, Fe, As, Cd, Al) had a strong positive contribution to principle component 2. In contrast, CO_2 and Mn were the least influential variables on the dataset.

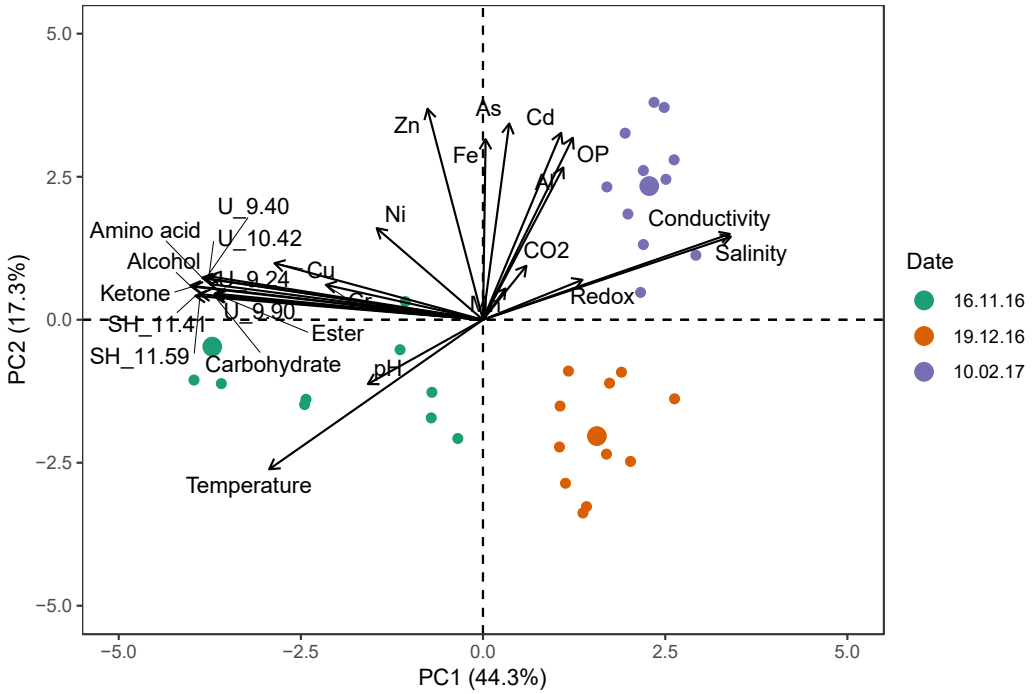


Figure 4.16 Loadings of organic compounds, trace elements and physico-chemical parameters on principle component 1 and 2. A few compounds are described by their respective retentio time to distinguish compounds given the same chemical class. These are compound 2 (U_9.24), 3 (U.9.40), 4 (U_9.90) 6 (U_10.42), 10 (SH_ 11.41) and compound 12 (11.59).

5 Discussion

5.1 Physico-chemical parameters

CO_2 was found to cause significant changes in the water's chemistry. First, increasing CO_2 concentrations caused reductions in pH levels [19] [21]. Lowest pH values were measured in fish tanks treated with 40 mg/l CO_2 which is explained by the strong, inverse correlation between pH and CO_2 (p=2.2e-16, r=-0.81). Correspondingly, the treatment with the greatest CO_2 concentration, 40 mg/l, yielded a pH around 6.5, while the lowest treatment, 5 mg/l, remained more alkaline, above pH 7.5. However, pH levels were maintained within recommended levels (7.7 - 6.8) for salmonids [64] within the study.

In contrast, redox potential increased significantly with increasing CO_2 concentration, which was evident from the positive correlation between CO_2 and redox potential (p = 1.5e-08, r=0.45). Other physicochemical parameters studied here (salinity, temperature, conductivity) were almost at constant level throughout the study in all CO_2 treatments. In the hunt for optimized rearing conditions, recent studies on Atlantic salmon post-smolt in RAS have found 12 ppt to be a advantageous over freshwater as seen from better fish performance and survival, and therefore supports the operated salinity level used in the system [27]. The operating temperature (12°C) agrees with a previous study on farming Atlantic salmon post-smolt in RAS [7].

5.2 Method development

Previous studies have focused on analysis of steroids released by fish in RAS [8] [9][32], in which Radioimmunoassay (RIA) has been employed for analysis steroids in combination with solid phase extraction (SPE) using Ethyl Acetate [31][65], Ethanol [8] or Methanol [66] as eluents. In contrast, this study has used non-target analysis for the determination of trace organic substances present in the culturing water.

The novelty of this study is the implementation of a non-target analysis specifically in the field of aquaculture. The main goal of liquid-liquid-extraction (LLE) method optimization has been to create a broad, generic method to maximize the coverage of organic compounds present in the sample matrix. The complex nature of the water in RAS has been relatively unknown, particularly under given conditions of long-term altered CO_2 levels (low pH stress).

However, it is a matrix assumed to have a high fat content, along with various other interfering substances which might be co-extracted with compounds of interest [67]. For non-target analysis, sample extraction methods that allow for minimal analyte selection are preferred to avoid any compound discrimination. Compared to LLE used in this study, solid-phase extraction (SPE) have been the widely used sample pre-treatment technique for isolation of semi-polar to polar organic compounds in effluent and surface water samples [68] as well as in non-target screening methods [69][70]. SPE has been favourable due to advantages such as reduced organic solvent consumption, improved sample clean-up, among other [71]. LLE is a robust method which can be used to produce clean sample extracts [71]. Compared to SPE, LLE is a less analyte selective extraction method [72] and as such might be more suitable for non-

target analysis. However, chances of pre-concentrating matrix constituents along with analytes are generally higher in LLE. Another challenge with LLE is the formation of emulsions [73] which may trap some of the analytes present as well as making it difficult to separate the liquid layers. The clean-up process involving multiple steps of sample volume reduction and sample transfer may increase error [71]. During sample preparation, emulsions occurred frequently during extractions with DCM, however this was mostly resolved by letting the extract settle for some time. In a few cases when emulsions did not resolve after time, 1-2 droplets of Methanol was added.

In the present study using LLE, the best overall results were achieved through use of EtAc and DCM as extraction solvents. Fractions analysed in positive mode purified with 100% of DCM and 40-100% of EtAc showed a high abundance of peaks, ranging from 8 to 14. Noticeably, EtAc extract eluted with 60:40, Hex:EtAc had a three-fold higher number of peaks compared to DCM extract eluted with 60:40 of Hex:DCM. Similarly, EtAc extract eluted with 100% EtAc had a nearly two-fold higher amount of peaks compared to DCM extract eluted with 100% DCM. As such EtAc might have been an overall more effective solvent for this particular matrix. However, the number of peaks observed in the DCM fraction (a total of 8) eluted with 100% DCM was not negligible, and DCM was therefore combined with EtAc for liquid-liquid extraction of the set of samples collected over the 12-week period.

The chromatographic conditions utilized in this study agreed well with previous studies [74] which have implemented non-target analysis on environmental matrices. The UPLC conditions employed in this study agreed with a recent collaborative study with 17 projects performing non-target screening for multiresidue analyses of organic compounds from aqueous

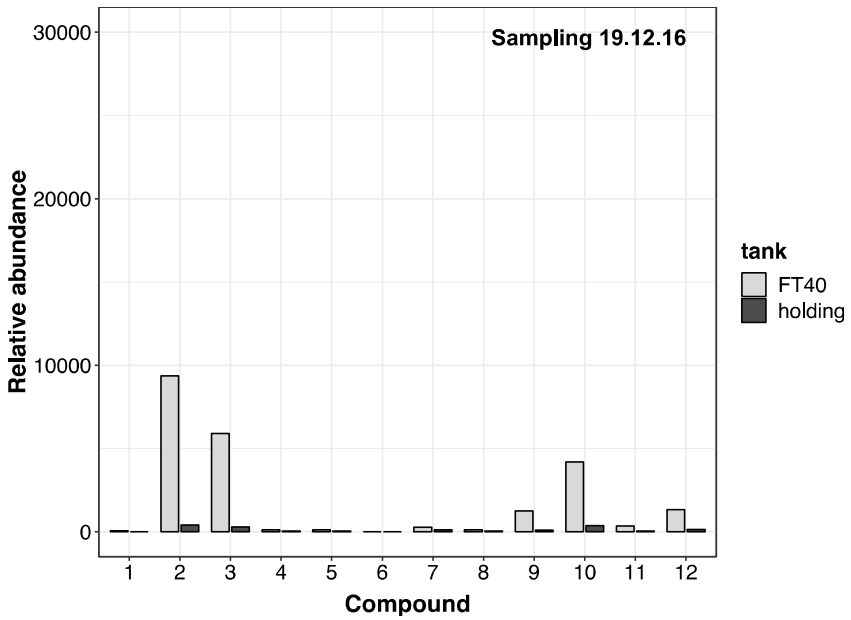
sample medium. In that study, all but one participant used Reversed-phase chromatographic separation (C18 column for separation), ESI (+/-) for ionization and either water-methanol or water-acetonitrile for gradient elution, providing a generic method of detection.

The present work demonstrated the possibilities of discovering unknown compounds form water samples collected from a RAS-system using the developed extraction method combined with a non-target UPLC-MS/MS analysis.

5.3 Impact of CO2 on production and accumulation of organic compounds

The results from the initial screening tentatively identified 12 organic compounds present in the particular fish tanks farming Atlantic salmon (*Salmo salar*). Further, the majority (11 of 12) of compounds did not show signs of accumulation in fish tanks over the 12-week period for any of the CO2 concentration levels investigated (5, 12 , 26, 40 mg/l CO_2). One observation was that the level of substances converged towards the same level for all compounds at the end of the sampling period in all CO2 treatments. One of the 12 compounds showed tendencies to accumulation in fish tanks and makeup-water at the end of the experiment. In contrast, 7 of the 12 compounds had increasing levels in one holding tank (makeup-water) at the end of the experiment, which suggests that these compounds were produced by the system or was a result of inadequate cleaning.

Compound levels in holding tank 601 (40 mg/l CO_2) and 602 (5 mg/l CO_2) showed very different results (Appendix E). As samples from makeup-water were not collected during the adaption period (16.11.16), levels could not be compared with initial levels in fish tanks. While all the 12 compounds were completely absent in holding tank 601 (40 mg/l CO_2) at the second date (19.12.16), 7 of the compounds increased towards the last date (10.02.17) as shown in the figure below 5.1 where holding tank 601 (40 mg/l CO_2) is compared to mean value of fish tank 40 mg/l CO_2). Firstly, there are too few samples for the observations to be conclusive. One possible explanation may be inadequate cleaning of the water, as a result compounds accumulated over time. In contrast, fish tanks of CO_2 treatments and holding tank 602 (5 mg/l CO2) had very similar levels showing a declining trend over the 12-week time period.



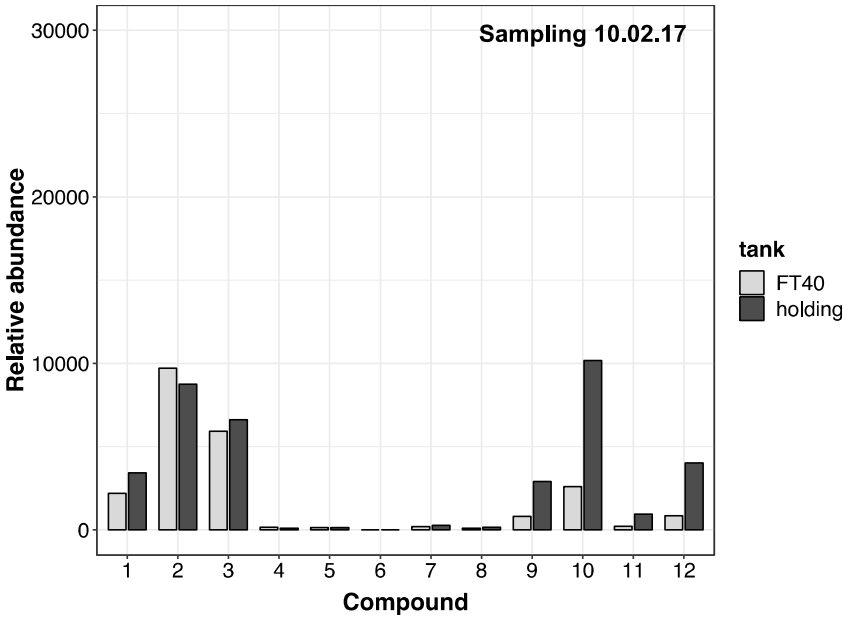


Figure 5.1 Bar plot showing mean values (n=3) of fish tanks treated with 40 mg/l CO2 (FT40) compared to makeup-water from 1 measurement of holding tank 601 (n=1) (40 mg/l CO2)

Accumulation of substances have been a topic of concern in RAS systems due to continuous re-use of water. Particularly, accumulation of steroids released by fish in RAS have been investigated under different set of conditions [8][32]. A recent study investigated the effect of steroid accumulation at low pH (5.8) versus high pH (7.3) over a 70 day period [32]. The study found that steroid hormones (in particular testosterone and cortisol) accumulated at higher levels in low pH (5.8) RAS possibly as a stress response to reduced pH level. In contrast, this study did not detect a similar trend for the detected steroid hormones (compound 10 and 12) in fish tanks in RAS at low pH level. The likely explanation may be the fact that the lowest measured pH level in the fish tanks was 6.5. The presence of compound 10 appears to have been persistent in the water throughout the sampling period and stabilizing at lower levels towards the end. This may indicate a slow, continuous release from fish over

the time period. Higher level at the first sampling (16.11.16) following the adaption period from 03.11.2016 to 22.11.2016, can be an indication to the post-smolt acclimatizing to the new environment and thus releasing metabolites into the water at higher rates in the beginning. The levels of steroid hormones increased in holding tank 601 (40 mg/l CO_2). This suggests that initially compounds were released by fish and naturally degraded, while over time compounds were produced by the system. A possible explanation may be leaching of endocrine disruptors (ED's) structurally similar to the steroid hormones, i.e. estrogenic xenobiotics[18] . Based on structural similarities such compounds have the ability to mimic the behaviour of natural steroids and interfere with reproduction of fish[75]. Structural similarity could have resulted in poor separation during chromatographic analysis (co-eluting substances) and which was unable to separate the natural from the synthetic compounds. The finding suggests that the steroid hormones were produced partly by fish and partly by the system, which might have been unable to remove the compounds during treatment processes.

In addition, the assigned identifications suggest that the two steroids are the same compound. The compounds, which were detected as m/z 327.15 observed at different retention times (RT 11.41 and 11.59 min) were assigned nearly exact same identification properties such as formula ($C_{18}H_{24}O_{24}$), structure, fragmentation pattern and match (85%) and total score (55.8, 55.9 respectively). The ketone and alcohol, detected with a lower mass at m/z 241, with similar retention time variations (RT 11.41 and 11.58 min) fragmented at similar m/z values as the steroid hormones above, at m/z 157, 143, 128 and 91 respectively. Therefore, it is likely that the alcohol and ketone are product-ions of the steroid hormones.

Compound 2 and 3, of classes of unknowns, assigned formula $C_{10}H_{21}N_8OP$ and $C_{11}H_{23}N_4O_5P$ respectively, followed the same pattern as the identified steroid hormones described above. However, it was not possible to assign these compounds to a chemical class as they were only provided with elemental formulas and no structural information. There was no indication that these compounds are related to hormones. The presence of these compound appeared also to be persistent in the water throughout the sampling period and stabilizing towards the end. This may indicate an immediate release from fish at the beginning and degrading over time. Alternatively, it could have been an indication of two compounds present in the water at the outset. As the observations follow those of the steroid hormones, it may suggest that these compounds were produced by the system over time as well.

An important observation was that compound 1 (Organophosphorus compound, OP) appeared in the last set of samples collected for all CO_2 concentrations. Notably, this was observed in both fish tanks and makeup water (holding tank 601 and 602). However this compound did not seem to be related with CO_2, as this compound was present at similar levels in both low and high pH conditions. Organophosphorus compounds occur naturally [76][77] produced by bacteria. Many Organophosphorus compounds are synthetic compounds used as flame retardants or plasticizers to enhance properties of plastics among other materials [78]. As previously mentioned, these compounds can potentially leach out from the system components used in RAS (tanks, pipe systems etc.) and interfere with endocrine system of fish[18]. Additionally, Organophosphorus compounds can be attributed to pesticides, which are previously detected in commercial fish feeds [67] specifically in plant ingredients [79]. However, since this emerged at the end of the experiment (10.02.17) it is not likely a result from the diet continuously

added to the fish tanks. Further, it is possible that the compound might have been released earlier in the experiment in the period between 19.12.16 until 10.02.17. On the other hand it may be a result of bacterial metabolism under low pH stress. Due to equal levels observed in fish tanks and makeup-water the identified Organophosphorus compound was likely a substance leaching from the components in the system and potentially accumulating.

Compounds that were found to be not-persistent showed a very similar trend in fish tanks and makeup-water, namely compound 5-8 (Amino acid, carbohydrate, ester, unknown) . Apart from the unknown compounds, the other compounds are closely linked to feed ingredients used in aquaculture. On the other hand, compounds, in particular amino acid and carbohydrate, could have been produced by micro biota from degradation of particulate matter [80]. From second (19.12.16) to last date (10.02.17) compound 5-8 were absent in all tanks. Due to absent levels over time, these substances, which were attributed to classes of amino acid, carbohydrate, ester and unknown, either occurred at the beginning as a stress response (by bacteria or fish) or were already present in the water at the outset.

5.4 Limitations of non-targeted screening

The applicability of non-target analysis of environmental matrices a challenge since proper quantitation cannot be obtained in many cases [81]. As organic compounds were tentatively identified, quantification through the use of reference standard was not implemented in the study and therefore concentration levels of compounds were not obtained. Thus, it can not be concluded if the observed levels of organic compounds may be harmful to the

post-smolt or not. Secondly, levels of compounds can not be directly compared with previous knowledge on e.g. steroid hormone accumulation in RAS systems, since measurements used in this study are semi-quantitative as compared to quantitative measurements in the literature. On the other hand, the consistent trends observed in chromatograms answers the questions related to relative levels of compounds in samples as well as signs of accumulation within the given time frame, which were also part of the objectives in the study.

6 Conclusion

The study aim to identify organic compounds produced in aquaculture in response to various concentrations of carbon dioxide (CO_2). Initially methods were optimized for efficient extraction of organic compounds from water using three different solvents with liquid-liquid extraction. Screening of the compounds was done with a novel non-target screening analysis. Of the solvents tested, Ethyl Acetate (EtAc) stood out as the most suitable solvent for liquid-liquid extraction of the culture water in RAS, followed by dichloromethane (DCM).

Elevated CO_2 concentrations led to significant decrease in pH ($p < 0.001$) and increased Redox potential ($p < 0.001$). Redox potential ($p < 0.001$ r=0.45) and pH ($p < 0.001$, r=-0.81) correlated significantly with CO_2 (mg/l). Although there was a significant decrease in pH levels in high CO_2 concentration treatments, but the levels were still within safe recommended levels for farming Atlantic salmon (*salmo salar*) RAS.

The results from the non-target analysis tentatively identified 12 organic compounds in water samples collected at the RAS farming Atlantic salmon (*Salmo salar*) post-smolt. The compounds were assigned 8 different classes of chemical compounds which are Organophosphorus compound (OP), Carbohydrate, Amino acid, Ester, Alcohol, Steroid hormone, Ketone and unknowns respectively. Compounds assigned classes of Ketone and alcohol were found to be related to, and likely product-ions, of steroid hormones due to identical fragmentation pattern obtained from mass spectra analysis. Comparison between CO_2 treatments (5,12, 26, 40 mg/l) revealed that the

relative level of organic compounds did not differ significantly among treatments over the course of the study. The majority of compounds had a declining level in fish tanks towards the end of the experiment.

Steroid hormones and two unknown compounds were likely metabolites released by fish at the start of the experiment perhaps as a possible stress response during acclimatization. Similarly, a group of 5 compounds, Carbohydrate, Amino acid, Ester, two unknowns, completely disappeared towards the end.

Increasing compound levels of steroid hormones in one out of two holding tanks suggest that structurally related compounds more likely potential endocrine disruptors (ED's) were produced within the system, as seen from high levels in one holding tank (with high concentration of CO_2) and were not adequately removed within the RAS treatment units. These compounds can be harmful to fish due to interaction with fish reproductive system. The OP had increasing levels in makeup-water and fish tanks at the end of the experiment, which indicates an accumulating substance leaching from the system (i.e. flame retardant, plasticiser). Although the non-targeted technique has successfully identified a range of compounds in this study, however the future work is required for complete structural characterization of these compounds such as NMR etc.

7 Future work

The non-targeted screening have successfully identified a number of compounds in water samples, however these identifications were tentative. Thus, it would be advisable to investigate the identified classes of compounds in RAS by performing a targeted analysis in order to clarify the findings in this study. In that case, a full structural characterization of these compounds is required. This would require purification of each compound using a suitable technique like preparative HPLC followed by a suitable technique for structural characterization. Nuclear Magnetic Resonance (NMR) would be very useful to give a complete structural characterization of organic compounds.

In addition, since the fraction of organic compounds bound to particulates in RAS were not covered in this study, it is recommended to further look into that to compare with dissolved organic compounds. Since there are speculations on whether a few of the compounds were released by bacteria or fish, it could be useful to investigate the bacterial activity under similar low and high pH conditions in RAS.

Most importantly, it would be advisable to obtain quantifications of compounds in order to determine if the actual levels of organic compounds provide healthy and optimal environment for the salmon post-smolt., since several of the compounds suggested present in RAS are potentially toxic to fish.

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Appendix A: Sample collection

Table A.1 : Collection of water samples including dates and tank number. X = filtered water, U = unfiltered water.

	Sampling date							
Tank	**16.11.16**	**30.11.16**	**19.12.16**	**06.01.17**	**18.01.17**	**21.01.17**	**02.02.17**	**10.02.17**
601	X	X	X	X	X	X	X	X
602	X	X	X	X	X	X	X	X
301	X	X	X	X	X	X	X	X
302	X	X	X	X	X	X	X	X
303	X	X	X	X	X	X	X	X
304	X	X	X	X	X	X	X	X
305	X	X	X	X	X	X	X	X
306	X	X	X	X	X	X	X	X
307	X	X	X	X	X	X	X	X
308	X	X	X	X	X	X	X	X
309	X	X	X	X	X	X	X	X
310	X	X	X	X	X	X	X	X
311	X	X	X	X	X	X	X	X
312	X	X	X	X	X	X	X	X
313	X	X	X	X	X	X	X	X
314	X	X	X	X	X	X	X	X
315	X	X	X	X	X	X	X	X
316	X	X	X	X	X	X	X	X
317	X	X	X	X	X	X	X	X
318	X	X	X	X	X	X	X	X

Appendix B: CO2 RAS details

Table B.1: Details of specie and fish diet

Specie	Atlantic salmon Salmo salar post smolt
Number of fish/tank	50
Initial body size	100g
Commercial diet	3-4 mm pellets Skretting Nutra Olymic, Stavanger
Feed load	Continously 24h, overfed (120%)

Table B.2: CO_2 increase design in holding tank 601

Date	**CO_2 (mg/l) in 601**
22.11.16	2
23.11.16	10
24.11.16	15
25.11.16	20
26.11.16	25
27.11.16	30
28.11.16	35
29.11.16	40
30.11.16	40

Table: B.3 Inlet water flow for 4.5 kg biomass. Flows were adjusted to variations in fish biomass.

		Water flow (L/min)	
Treatment (CO_2 mg/l)	**Fish tank**	**601**	**602**
5	301, 312,318	0	11
12	304, 309, 315	3	8
19	307, 311, 313	5	6
26	303, 314, 316	7	4
33	305, 308, 317	9	2
40	302, 306, 310	11	0

Table B.4: Water quality parameters and frequency of measurements at Nofima.

Unit	Parameter	Value	min	max	Frequency
Degasser sump	Salinity	ppt	11.5	12.5	daily
	Temperature	°C	12	13	daily
	pH		6.7	7.5	daily
	NH4	mg/l	-	0.7	1x month
	NO2	mg/l	-	0.1	1x month
	NO3	mg/l	-	<100	1x month
	Water exchange rate	l/min	-	25	daily
Holding tank 601 (602 except CO2 < 5 mg/l	Oxygen saturation	%	100	120	daily
	Salinity	ppt	11.5	12.5	3x week
	Temperature	°C	12	13	3x week
	pH		6.7	7.5	daily
	CO2	mg/l	48	52	daily
	Water flow	l/min	105	110	3x week
	TIC	mg/l	-	-	2x month
	Alkalinity	mg/l	-	-	1x month
	TSS		-	-	1x month
	NH4	mg/l	-	-	1x month
	NO2	mg/l	-	-	1x month
	NO3	mg/l	-	-	1x month
Fish tank outlet	Oxygen saturation	%	85	100	daily
	Salinity	ppt	11.5	12.5	6 tanks/day
	Temperature	°C	12	13	6 tanks/day
	pH		6.7	7.2	6 tanks/day
	CO2	mg/l	variable	variable	6 tanks/day
	Water flow	l/min	11	11	6 tanks/day
	TIC	mg/l	-	-	2 x month
	Alkalinity (CaCO3)	mg/l	-	-	1 x month (6 tanks)
	TSS		-	-	1 x month (6 tanks)
	NH4	mg/l	-	-	1 x month (6 tanks)
	NO2	mg/l	-	-	1 x month (6 tanks)
	NO3	mg/l	-	-	1 x month (6 tanks)
	Photoperiod		24L:0D		

Appendix C: Chromatographic data

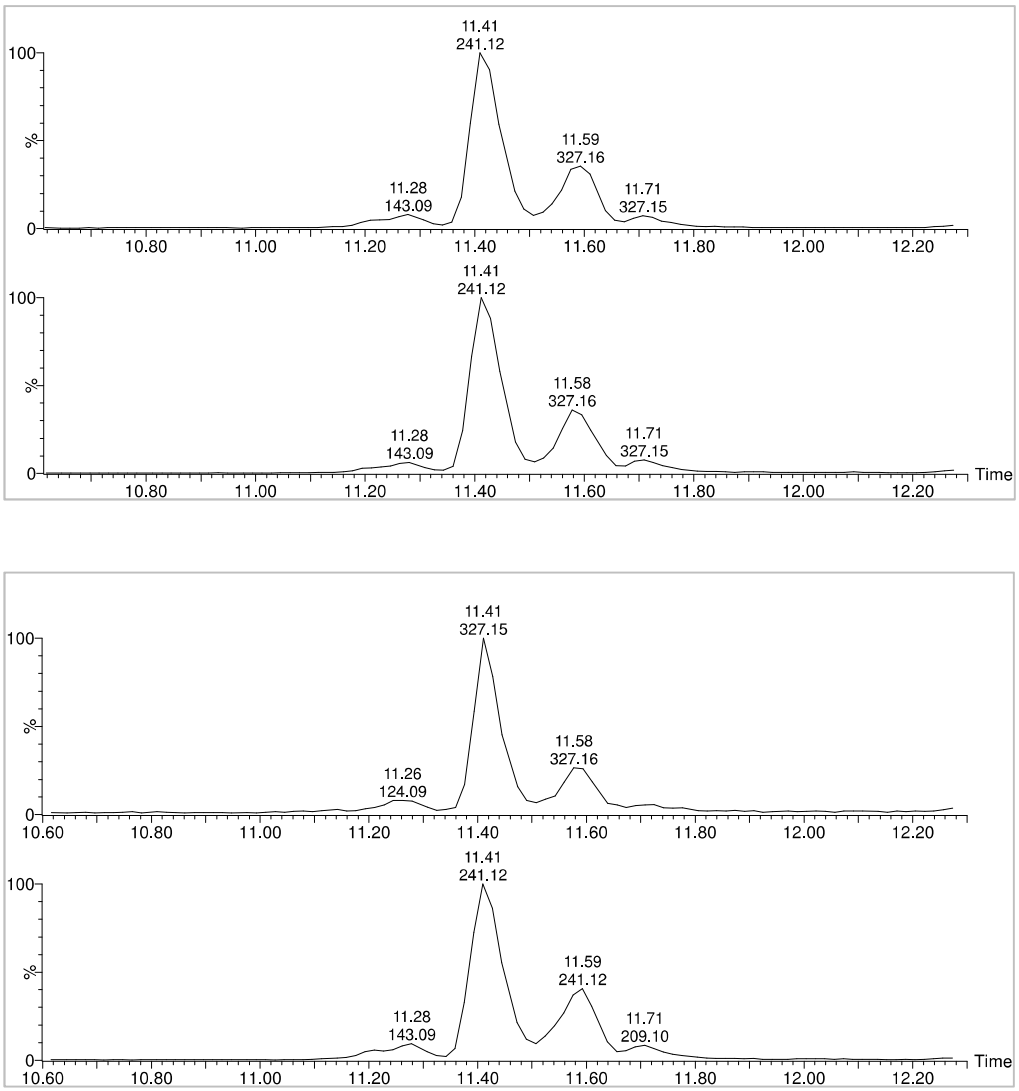


Figure C.1: Extracted-ion-chromatograms (EIC) of ion detected at m/z 327.16. Sample 1, 11, 3, 42 Illustrated.

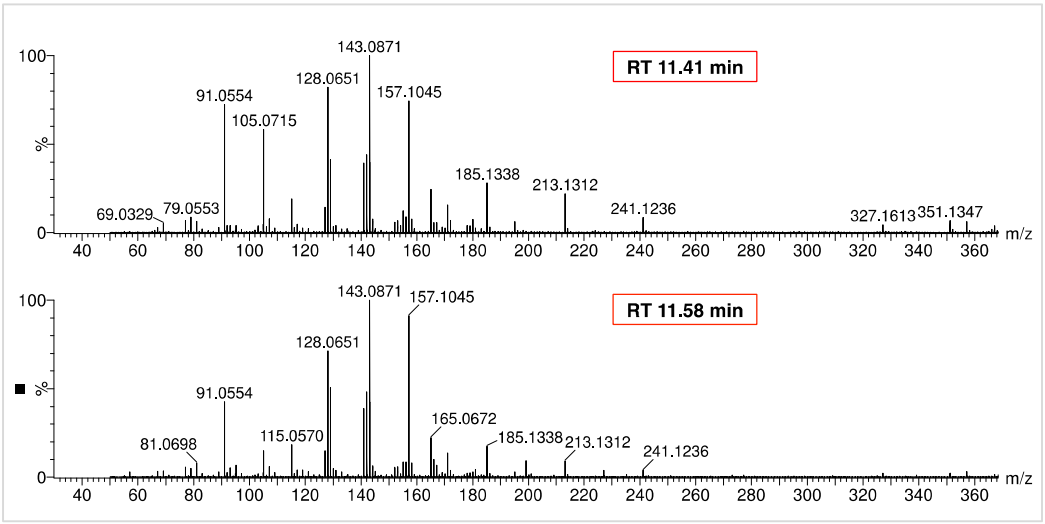


Figure C.2: MS/MS spectra of ion detected at m/z 241 (top) and ion detected at m/z 327 (bottom).

Appendix D: UPLC-MS/MS analysis

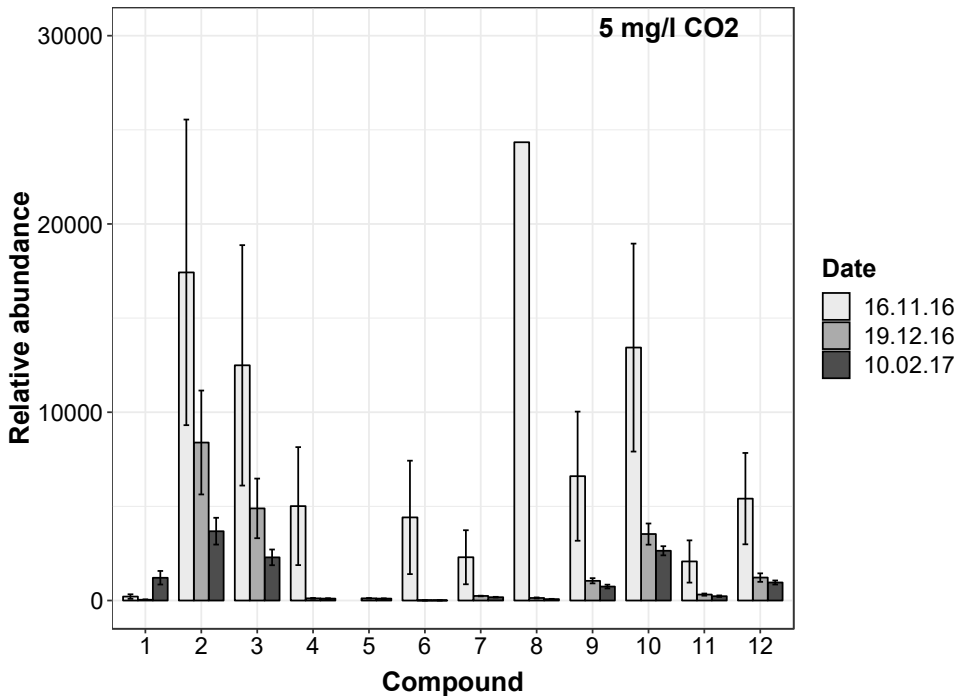
Table D.1: Sample sequence for analysis of samples

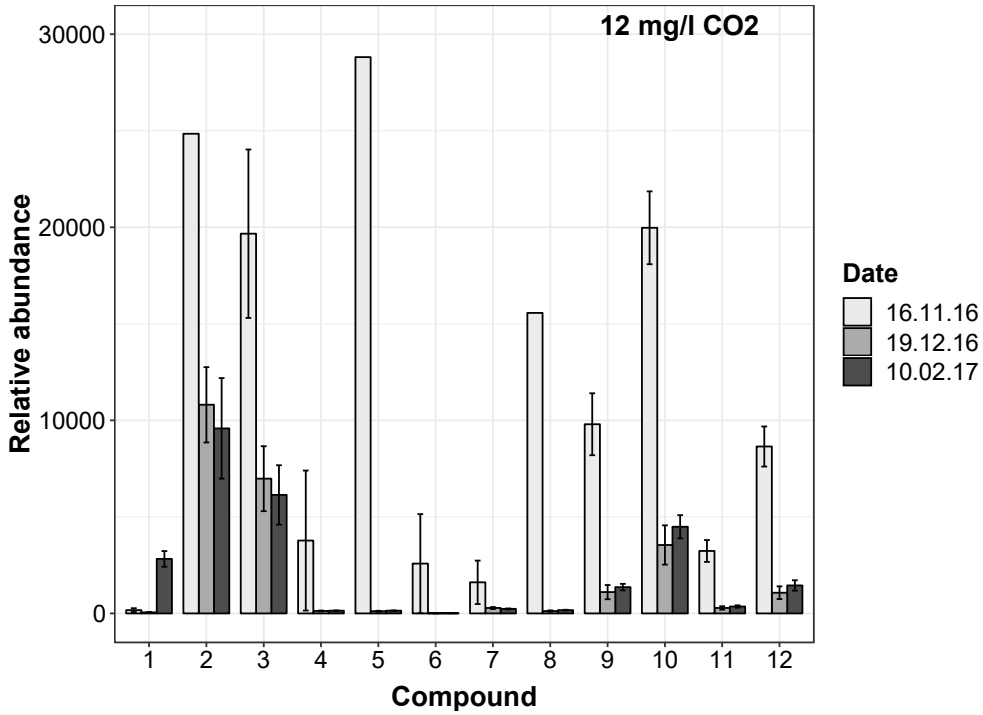
Sampling date	Sample nr	Tank	CO2 concentration (mg/l)	Vsample (ml)	Vtotal H20/MeOH (ml)
16.11.16	1	301	5	27	0,45
	2	302	40	24	0,4
	3	303	26	23	0,383
	4	304	12	25,5	0,425
	5	306	40	23	0,383
	6	309	12	17	0,283
	7	MB1		15	0,25
	8	310	40	16	0,267
	9	312	5	16	0,267
	10	314	26	16	0,267
	11	315	12	18	0,3
	12	316	26	9,5	0,158
	13	318	5	18	0,3
	14	MB2		15	0,25
19.12.16	15	301	5	28,5	0,475
	16	302	40	26,5	0,442
		303	-	-	-
	17	304	12	27	0,45
	18	306	40	27,5	0,458
	19	309	12	29	0,483
	20	MB1		10	0,167
	21	310	40	28	0,467
	22	312	5	28,5	0,475
	23	314	26	28,5	0,475
	24	315	12	26,5	0,442
	25	316	26	26,5	0,442
	26	318	5	29	0,483
	27	601		27	0,45
	28	602		28	0,45
	29	MB2		15	0,25
10.02.17	30	301	5	23	0,383
	31	302	40	24,5	0,408
	32	303	26	23,5	0,392
	33	304	12	23,5	0,392
	34	306	40	23	0,383
	35	309	12	22,5	0,375
	36	MB1		15	0,25
	37	310	40	23,5	0,392
	38	312	5	21,5	0,358
	39	314	26	23	0,433
	40	315	12	22	0,2
	41	316	26	22	0,367
	42	318	5	22	0,367
	43	601		23	0,383
	44	602		24,5	0,408
	45	MB2		15	0,25

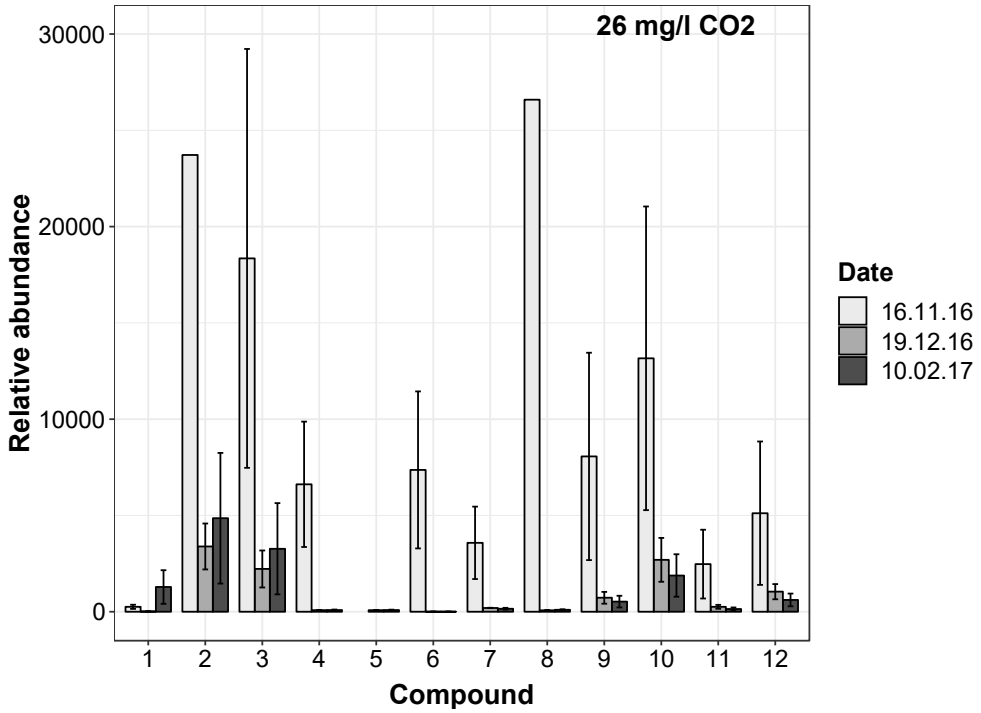
Appendix E: Measurements of organic compounds

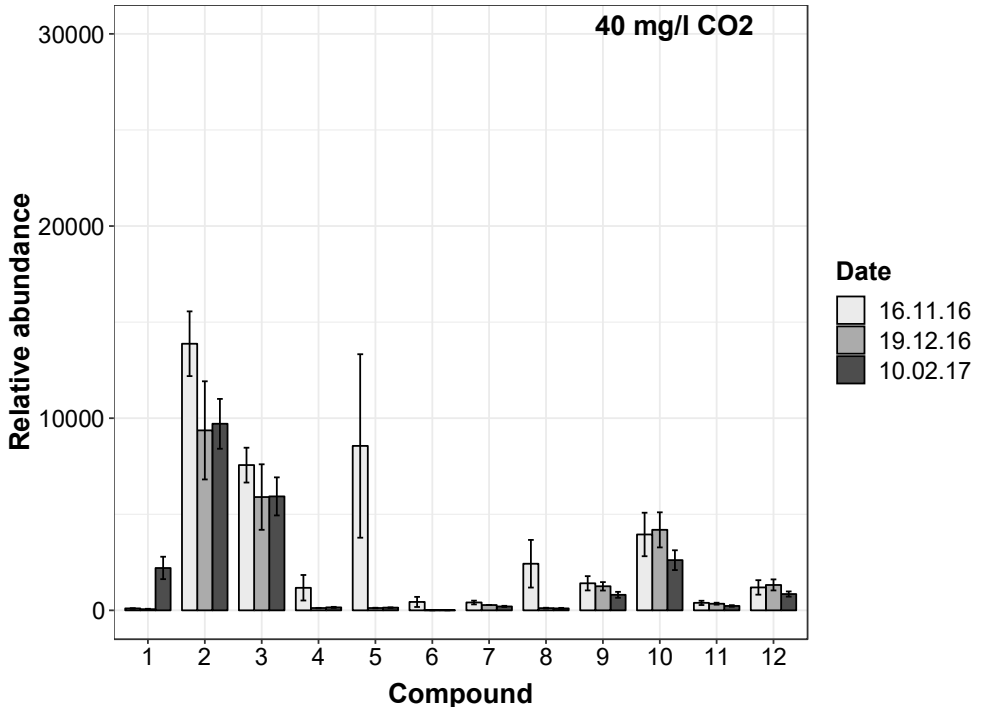
Fish tanks

These figures are used for comparison. Comp 5

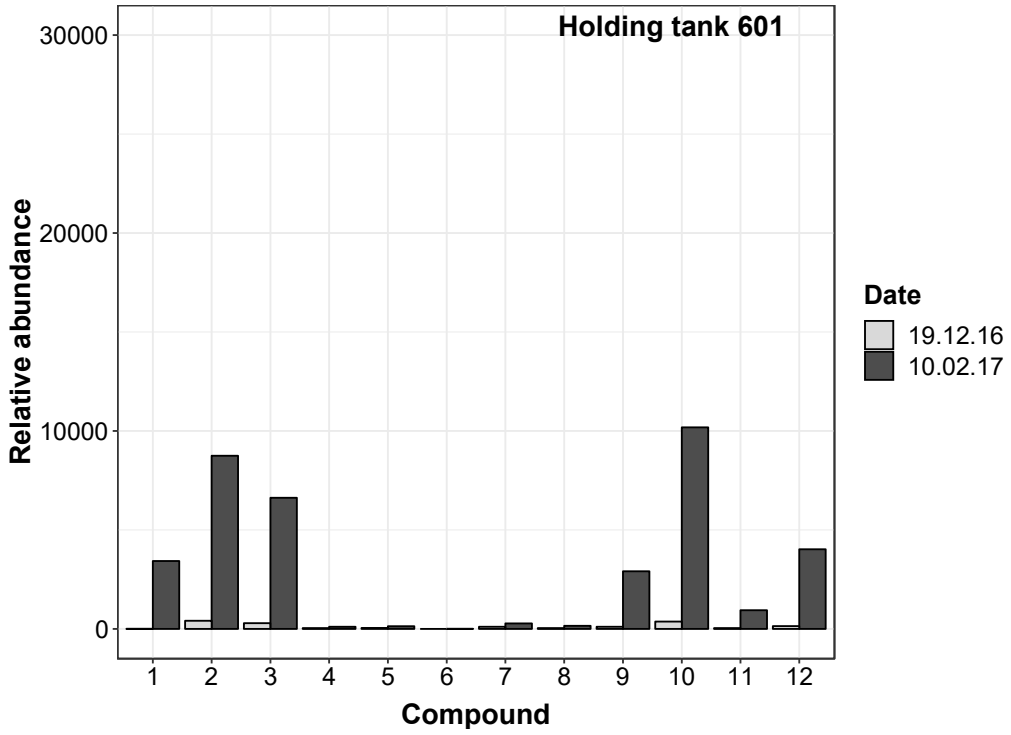



12 mg/l CO2
Relative abundance
30000
20000
10000
0
1
2
3
4
5
6
7
8
9
10
11
12
Compound
Date
16.11.16
19.12.16
10.02.17

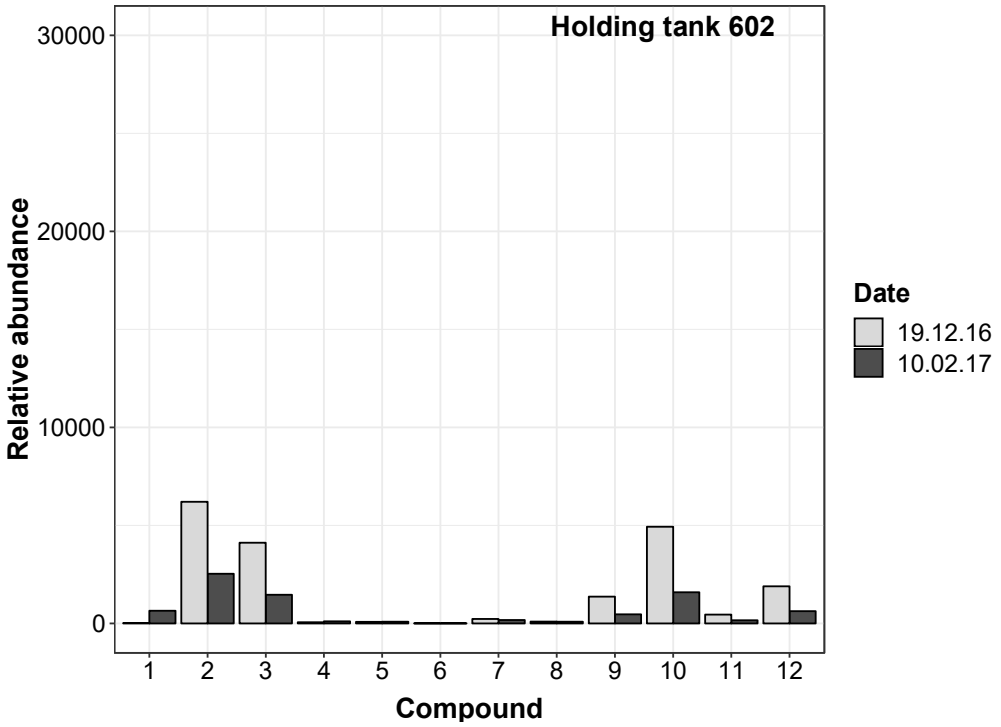

26 mg/l CO2
Relative abundance
30000
20000
10000
0
1
2
3
4
5
6
7
8
9
10
11
12
Compound
Date
16.11.16
19.12.16
10.02.17


40 mg/l CO2
Relative abundance
30000
20000
10000
0
1
2
3
4
5
6
7
8
9
10
11
12
Compound
Date
16.11.16
19.12.16
10.02.17

Makeup-water



40 mg/l CO2


Holding tank 602
Relative abundance
30000
20000
10000
0
1
2
3
4
5
6
7
8
9
10
11
12
Compound
Date
19.12.16
10.02.17

5 mg/l CO2