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Master

Biomonitoring of contaminants in blue mussels (*Mytilus edulis*) from the city fjord in Flekkefjord

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Preface

This thesis is written in collaboration with the University of Agder and the University of Milan. The project is overseen by forskningsmobilisering Agder and Flekkefjord Municipality.

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Abstract

The Norwegian government has issued a statement where they express concern for high levels of contamination in the fjords of Norway. To establish an overview over the contaminants in the city fjord of Flekkefjord and to monitor the trends of contaminants over a 6-month period of time, an active biomonitoring survey was performed. Blue mussels (*Mytilus edulis, Linnaeus 1758*) were transplanted at two different depths (5 m and 15 m depth) in five different locations within the fjord. The trace elements arsenic (As), manganese (Mn), aluminium (Al), iron (Fe), titanium (Ti) copper (Cu), lead (Pb), cadmium (Cd), chromium (Cr), zinc (Zn), nickel (Ni) and mercury (Hg), were investigated in blue mussels transplanted in the five locations. To complete the overview of the contamination in the city fjord the contamination pattern of legacy contaminants and emerging pollutants was reported. The levels of some trace elements, such as manganese, chromium and zinc changed over the study period, mainly because of an undersea landslide causing an upwhirl of contaminated sediments. The landslide happened during dredging activity in the fjord, showing how human activity and restoration effort may impact fjord biota. The results obtained confirm that active biomonitoring using blue mussels is an excellent approach to assess both the status and the trend of inorganic and organic contaminants in marine ecosystems.

Sammendrag

Den norske regjeringen har utrykket bekymring over høye forurensningsnivåer i fjordene i Norge. For å etablere en oversikt over forurensningene i byfjorden i Flekkefjord og for å overvåke trender for forurensninger over en 6-måneders periode, ble en aktiv biomonitoringsundersøkelse utført. Blåskjell (Mytilus edulis) ble satt ut på to forskjellige dybder (5 m og 15 m dybde) på fem forskjellige steder i byfjorden. Sporelementene arsen (As), mangan (Mn), aluminium (Al), jern (Fe), titan (Ti) kobber (Cu), bly (Pb), kadmium (Cd), krom (Cr), sink (Zn), nikkel (Ni) og kvikksølv (Hg), ble undersøkt i blåskjell som var satt ut på de fem lokasjonene. For å fullføre oversikten over forurensningen i byfjorden ble forurensningsmønsteret av vedvarende og fremvoksende organiske forurensninger undersøkt. Nivået på noen sporstoffer, blant annet mangan, krom og sink, endres under undersøkelsesperioden, hovedsakelig på grunn av et undersjøisk skred som forårsaket en hvirvel av forurensede sedimenter. Mens monitoreringen pågikk, var det mudringsaktivitiet i områdene rundt lokasjonene som gav noe innsikt i hvordan menneskelig aktivitet og restaureringsinnsats kan påvirke biota i fjorden. Aktiv bioovervåking ved bruk av blåskjell har vist seg til å være en utmerket tilnærming for å vurdere både status og trenden for uorganiske og organiske forurensninger i marine økosystemer.

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1. Introduction

Blue mussels *(Mytilus edulis, Linnaeus 1758*) are bivalves commonly found along the coast in European waters (Brooks et al., 2011). They represent a popular food source (Gosling, 1992), and can also be used for biomonitoring (Beyer et al., 2017). In this study blue mussels are shown to be valuable sentinels for monitoring (Haker, 2011; Misund, 2012) the contaminant levels in biota of the city fjord in Flekkefjord.

1.1 Investigated contaminants

Trace elements are substances that are a natural part of our ecosystem, in addition to some being developed as a consequence of anthropogenic processes and pressures (Richir & Gobert, 2016). Trace elements differ in atomic mass and properties, by definition any element having an average concentration of less than 100 ppma could be referred to as a trace element (Franco et al., 2015). While the generic term "heavy metals" has often been used for trace metals, this term is being discussed (Richir & Gobert, 2016). Because some elements are not considered "heavy" (e.g., Al and Ni) while others are not considered metals (e.g., As) (Richir & Gobert, 2016). Trace elements with higher atomic mass such as Lead, Cadmium and Mercury are regularly referred to as heavy metals (Azizi et al., 2018). The trace elements with lower atomic mass such as titanium, iron and aluminium are referred to as metals. They are often necessary for the organism, but can be toxic if they are absorbed in larger quantities (Azizi et al., 2018). Many trace elements are known to be toxic if they are ingested and consumed in large doses (Richir & Gobert, 2016), some even in small quantities (cadmium, lead, mercury, arsenic) (Aras & Ataman, 2007). However many of them are vital and have essential roles in diverse biological and natural processes (zinc, copper, iron) or have positive effects when ingested in small quantities (manganese, nickel, iron) (Aras & Ataman, 2007).

Trace elements are non-biodegradable, which causes them to be difficult to remove when they have entered an ecosystem (Richir & Gobert, 2016). They are substances that are present in the crust of the earth and will be released when activity in the crust occur. Such disturbances can occur naturally due to the flow of water (Azizi et al., 2018). They can also be released due to anthropogenic activities such as mining, sewage and waste from anthropogenic sources (Brooks et al., 2015). Some trace elements can accumulate along the trophic chain (Burger &

Gochfeld, 2004) which may affect the ecosystem as a whole when the trace elements enters the chain. The accumulation of trace elements can be a major environmental stressor for several species (Muszyńska & Labudda, 2019; Richir & Gobert, 2016). Trace elements are known to induce physiological responses resulting in negative fitness (Cyr & Romero, 2007; Romero, 2004), which will impact population dynamics (Wikelski & Cooke, 2006).

Organic contaminants are often referred to as persistent organic pollutants (POPs) and in Europe, legacy contaminants (Hutchinson et al., 2013). Legacy contaminants are known to bioaccumulate in organisms and they have the ability to affect organisms negatively (Wania & Mackay, 1996). They are difficult to break down in the environment, hence persistent, and have shown to be moving among different trophic levels when they are accumulated (Jones & De Voogt, 1999; Schöne & Krause Jr, 2016). Bioaccumulation, coupled with their danger to humans and biota, makes them of interest for research and removal (Jones & De Voogt, 1999).

Emerging pollutants are a group of "new" substances that have been produced within the last decades (Thomaidis et al., 2012). They are now common in aquatic environments, although their full effect on biota is still unknown to researchers (Thomaidis et al., 2012) and still more types of emerging pollutants and their properties are being discovered.
The legacy contaminants that will be discussed in this thesis include: Polychlorinated biphenyls (PCBs), Polycyclic aromatic hydrocarbons (PAHs), Organochlorine pesticides (OCPs), and Organophosphate pesticides (OPs), while emerging contaminants included polybrominated diphenyl ethers (PBDEs), and some relevant per – and polyfluoroalkyl substances (PFASs).

PCBs ($C_{12}H_{10-x}Cl_x$) are a group of 209 congeners that were previously widely used as a coolant, as dielectric fluid, in electrical units and in heat transfer fluids (Griesbaum et al., 1989). The used of PCBs was discontinued because the compound has large impacts on ecological environments and biological life (Hutchinson et al., 2013). PCBs are known to cause cancer and other diseases if exposed to for a longer period of time (Griesbaum et al., 1989).

PAHs are compounds often found in tar and coal deposits, or produced by the burning of some types of organic matter (Griesbaum et al., 1989). A PAH itself has often no effect since it is extremely abundant in ecosystems (Griesbaum et al., 1989), and the issue here is more often linked to long term exposure or in combination with other PAHs. In this case it has shown to be linked to growth of cancers and other diseases.

OCPs often reach the marine environment from landfill runoff, wastewater discharge or surface runoff (Chiesa et al., 2018). Most OCPs are prohibited, however, there are still traces of them in biota, the coastal line, and sediments surrounding farmland as well as in lakes and coastal waters (Richardson and Zheng 1999).

OPs are used as insecticides, applied as flame retardants and as gas for warfare (Walker et al., 2016). Their level of toxicity is high, and they are easier to break down than the OCPs, but as they are usually present for a shorter amount of time, more acute toxicology is caused, which can be difficult to measure. OPs are known to cause behavioural effects in the organisms that accumulate them (Walker et al., 2016). Today they are not used as often as previously, because of danger they can cause (Kjetil Sagerup, 2011). Despite this, OPs are still used as insecticides in some countries as well as applied as flame retardants in certain products (Sidhu et al., 2019). OPs have been confirmed to an airborne pollutant in Norway, in addition to being found in sediments and animals, according to the Norwegian environment agency (Kjetil Sagerup, 2011).

PBDEs are a group of emerging contaminants which were previously widely used as flame retardants, but have as of recently been banned in most countries as they are a danger to both health and the environment (Rahman et al., 2001). They have been shown to dissolve in aquatic environmental sediments and in general behave very similarly to PCBs and OCPs due to their similar chemical structure (Rahman et al., 2001). PBDEs bioaccumulate well and can be transported far by air and water (Chiesa et al., 2018).

PFASs are a group of emerging contaminants that have been used in various man-made products over the last 50 years or so, both in industrial and commercial products (Sammut et al., 2019). PFAS are relatively new emerging contaminants and pollutants that are known through recent research to cause environmental problems and are toxic for biota. It has also been found that they are prone to bioaccumulate in nature through water and organic digestible material (Sznajder-Katarzyńska et al., 2019). PFAS has shown to have high absorption levels in biota and it has also been found difficult to eliminate them, as they are often accumulated in the body (Sznajder-Katarzyńska et al., 2019).

1.2 Blue mussels and biomonitoring

Blue mussels, commonly known as mussels or common mussels, are of the class Bivalvia. This is a class that includes species of clams, oysters, scallops, and mussels (Gosling, 1992). Blue mussels are common in most temperate waters near the coast, usually located near the surface of the water (Brooks et al., 2011). They are a popular product for seafood in aquaculture since they are easy to grow and farm. They are also used in research as a model organism in several projects (Gosling, 1992). Mussels are well dispersed through pelagic larval stages if they have good living conditions and this is one of the many reasons why they are being used in biomonitoring research (Beyer et al., 2017).

Mussel shells are divided by two halves split in the middle. They are fastened together by a mid-dorsal line, and muscles draw the two halves tightly together, which will fasten and protect them (Gosling, 1992). When the shell is open, the bivalve may use its foot for anchoring or digging (Saba, 2012). The mantle cavity of a bivalve contains gills that are used for filter-feeding and respiration (Gosling, 1992). When they feed, they trap fine particles in mucus that cover and coats the gills and cilia and then convey the particles to the mouth (Von Moos et al., 2012) Being suspension-feeders, bivalves are mostly sessile organisms and have no need for much movement (Beyer et al., 2017).

Blue mussels have been used in biomonitoring through the mussel watch programme with success since 1981 in Norway (Bråte et al., 2018). Mussel watch is important for monitoring hazardous contaminants, both organic and non-organic. The most common area to use mussels are in coastal areas, where they are found naturally since their biological traits are suited for this area (Bråte et al., 2018). Their suitable criteria were further approved by the OSPAR (Oslo-Paris) commission (Beyer et al., 2017).

An active biomonitoring process requires employing a common mussel, often local, that works as a sentinel species in the area of contamination (Beyer et al., 2017). These mussels are then placed, often in round baskets (Kljaković-Gašpić et al., 2006) on different depths (Beyer et al., 2013). In these cages, they will acclimate, accumulate toxins, and later be sampled for analysis.

Active biomonitoring means transporting a material, in this case Blue mussels (*Mytulus eduilis, Linnaeus 1758.*), from an unpolluted site, to the site of the study, and thereby exposing them to substances that they will absorb (Phillips & Rainbow, 1998). Using farmed mussels allows us to ensure that they are as comparable as possible in sex, age, health, size, and background concentrations of contaminants because they will be grown and released at the same time. It is important to ensure that the project and trends are as precise as possible. The mussels filter large amounts of water which causes them to quickly ingest contaminants through feeding if they are present in the body of water. The mussels can also be helpful in giving an overview over the environment in the ecosystem because they are consumed by several predators including fish and crustaceans as well as humans. This makes using blue mussels beneficial, compared to many other species.

1.3 Project background

Flekkefjord is both a city and a municipality on the Norwegian southern coast, located in the Agder region, at the mouth of a medium long fjord (Fig. 1.2). Flekkefjord has a long history as a shipping and trading post, as well as an industrial background in many different areas including shipbuilding. The extensive traffic in the city fjord of Flekkefjord has emitted chemicals from the ships, and also from the coastal industry in the fjords, as described in previous ground work performed by agencies hired by the Municipality (Haker, 2011). In these reports, samples were taken of sediment and water in which there was found contaminants (Fig. 1.3). The contamination issue was discussed recently, and the Municipality expressed concern about how the implications may have an effect on the conditions of the fishery and aquaculture industries. These industries lie just outside the city fjord and are directly impacted by the water quality in the fjord. There are also concerns on

Figure 1.2: The location of Flekkefjord (red pin) in Norway.

how the contaminated waters may have implications on how the people use the fjord because of how this affects the species that live there.

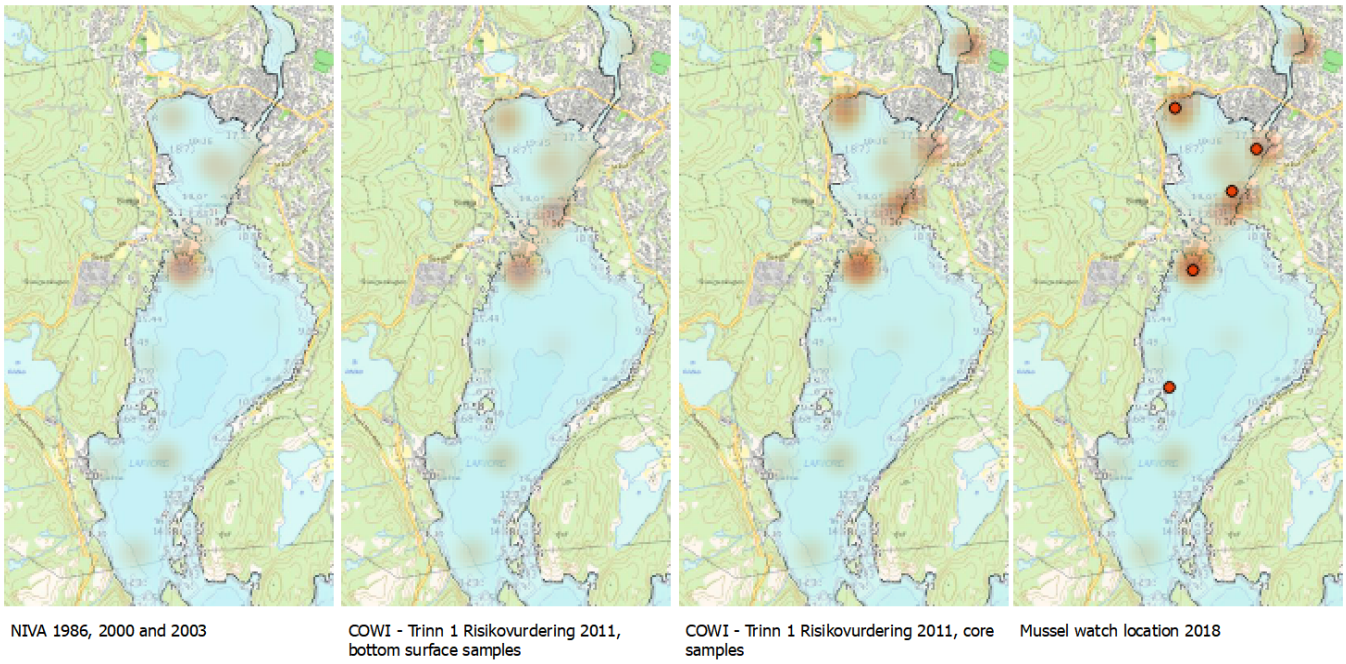

Figure 1.3: Level of contaminants (organic and trace elements) in sediments measured in the city fjord throughout the years. Contaminants historically comes from industry around the city fjord but also from wastewater and recent activities. Figure retrieved from diver Federico Haaland Gaeta.

In 2001, the Norwegian government declared in their report "Stortingsmelding nr.12" (2001-2002) and "Stortingsmelding nr.14" (2006-2007) that a number of Norwegian fjords have contaminated sediments, and were in need for action to remove or cover sediments that could potentially put human health at risk by restoration activities and dredging of the sediment. The biomonitoring initiative in Flekkefjord will be a response to this concern by collectively monitor the contamination levels in the city fjord but also by monitoring the potential leakage and success of the restoration activities, as previous studies has suggested that dredging may result in increased uptake of PAHs, PCBs and trace elements in mussel species (Bellas et al., 2007; Bocchetti et al., 2008). Routine analysis was conducted of the sediment, sea water, and samples of the biota of the different parts of the fjord (Haker, 2011). These analyses revealed large portions of PCBs, as well as heavy metals, in samples taken from the sediment. This led Flekkefjord Municipality to request and effort to cover the sediment layer, for the safety of the inhabitants. The goal was that covering the sediment layer would cause the levels of contamination in the fjord to decrease.

Bottom sediments are usually a sink for contaminants that enter fjord systems, since the contaminants are often heavier than the water, and because the cycling of water in fjords is slow (Alvarez et al., 2012). Activities in fjords will often cause the sediment to be disturbed. This enhances the risk of the contaminants interacting with marine biota as well as humans, which can negatively affect their health (Ghrefat & Yusuf, 2006).

Contaminants vary in molecular structure and composition which determines how they may behave in water (Loska & Wiechuła, 2003). Various factors can impact the sediment layer including pH, and alterations in temperature and salinity, which changes with seasonal variation from the freshwater and seawater input (Perillo, 1995). The sediment layer can also be altered through movement caused by external factors such as waves, boat activity such as anchoring, currents, disposal, dredging and bioturbation (Walling, 2006). This sort of impact on the sediment can cause it to fluctuate and spread into the water masses, as well as travel and interact with biological material through animals taking up the particulate contaminants (Meador et al., 1995; Weber et al., 2013). In some cases, polluted sediments being covered up by human activity is the sole source of the enhancements of organic contaminants and emerging pollutants, as well as heavy metals in organisms, which is shown in Bocchetti et al. (2008) and Bellas et al. (2007). Despite the issues that follow such actions, these activities are necessary to hinder the potential spread of contaminants at a larger scale.

The reason why biomonitoring is a solid solution to measuring how contaminants may impact human health, is that it uses organic material that are closer related to how humans may react to contaminants, as well as organisms that humans may consume. In addition, it measures the waves of exposure before, during, and after restoration efforts. This shows how effective these measures are, not only when it comes to water samples, but also in general with biological species that are present when the events take place. Biomonitoring will reveal the base values of contamination as well as providing a trend over time, which gives a warning if the levels are above the health risk limit. This can provide a chance to limit and halt any potential danger and damage these substances can cause if they are released.

1.4 Significance of the study

This thesis aims to monitor the levels of contamination in blue mussels in the city fjord in Flekkefjord over a period of 196 days, and to examine if the contaminant levels in the fjord are acceptable in regard to biota and human health. Bioaccumulation of trace elements as well as legacy and emerging organic pollutants was measured through an active monitoring approach. At the same time, the effect of sediment restoration activities on the monitored blue mussels was measured to study the effects of contaminants during restoration efforts. This study can contribute in the understanding of how POPs and trace elements bioaccumulate in Flekkefjord, and what risks are involved when we excavate fjords for environmental beneficial purposes.

2. Materials and methods

2.1 Experimental design

The mussels were purchased from a mussel farm located in Kaldvellfjord in Lillesand county, 155 km from Flekkefjord. They were grown close to the shore and reproduced naturally, with the seasons, before being transported to Flekkefjord in a cooling box within 2 hours of being removed from the water. On location they were released, then seeded in cages that were put at the five sampling sites (location 1- location 5) (Fig 2.1).

Figure 2.1: The locations of the experimental sites in the city fjord (Location 2-5) and Lafjorden (location 1) in Flekkefjord (city red pin). Location 1 is close to the city fjord but far enough to be a reference location.

The five sites in Flekkefjord, were chosen based on previous measures done in the sediments (Haker, 2011) on organic chemicals and heavy metal pollution. Cage site 1 (location 1, L1) is set as a reference site outside of Flekkefjord city bay. (58° 16' 30.0" N - 6° 39' 12.9" E) (fig 2.1). The fjord in which location side 1 is placed may have runoff contaminants from the city fjord but it is expected to be cleaner than the other locations.

Cage sites 2-5 (location 2-5, L2-L5) were placed in the inner fjord close to the town. Location 2 is placed closed to a shipping industry that has measured levels of contaminants in the sediment in the past. Amongst these were copper, PAHs and PCBs. (58° 17' 02.7" N - 6° 39' 15.6" E) (fig 2.1), Location 3 is placed right next to Slippen which was an old industrial site where amongst other things there were building of ships. Outside this area there were measured PCBs and PAHs in the sediment in the past. (58° 17' 23.0" N - 6° 39' 30.9" E) (fig 2.1), Location 4 is right by the canal that runs through the city as well as being in the eye of the stream that runs from the upper fjord and down into the city fjord. Next to this site there was also a old garbage disposal area which may have runoff of metals and other contaminants. (58° 17' 33.8" N - 6° 39' 41.3" E) (fig 2.1). Location 5 is placed next to an old tannery as well as being placed close to the town and road. From this area there has been efforts to remove contaminated sediments in the past (58° 17' 43.3" N - 6° 39' 12.5" E) (fig 2.1). Locations 3, 4 and 5 were the locations that were expected to be higher in contaminant levels than the other locations, as they were characterized by more operative human activity. Locations 1 and 2 were closer to the middle of the fjord where there were more currents and a higher potential for clean water.

Table 2.1: Dates which the sampling was done. The cages of blue mussels were deployed at time 0, the 27th of June 2018, with the last sampling in 2018 being at t=196. For year 2 (2019) the first cages were deployed at time 0, the 11th of January and the last sampling was done at t=247 (15th of September). Under status there is an overview over which samples are included in the thesis.

Date	Time	Sample nr	Year	Status
27.06.2018	0	0	1	Included
27.07.2018	t=30	1	1	Included
10.10.2018	t=135	2	1	Included
15.11.2018	t=166	3	1	Included
15.12.2018	t=196	4	1	Included
11.01.2019	0	0	2	Not included
06.03.2019	t=54	1	2	Not included
16.05.2019	t=125	2	2	Not included
14.07.2019	t=184	3	2	Not included
15.09.2019	t=247	4	2	Not included

At each location, two cages each containing appx. 300 blue mussels were placed at separate depths 5 m and15 m to account for any difference in the distribution of contaminants within the water column. The cages were inspected by scuba divers to monitor if they were in good condition. Since the monitoring started the 27th of June 2018 and had its first proper sampling the 27th of July (t=30) (table 2.1), the mussels had been placed in good time as the restoration

activities started in August of 2018, which began the dredging of sediments from the inner fjord. This allowed us to have some background information about the contamination and conditions of the fjord from before the operations in the fjord started. Sample 2 was collected 10.10.2018 (t=135), sample 3 on 15.11.2018 (t=166), and sample 4 on 15.12.2018 (t=196). In 2019 the samples were deployed on 11.01.2019. Sample 1 was collected 06.03.2019 (t=54), sample 2 was collected 16.05.2019 (t=125), sample 3 on 14.07.2019 (t=184) and sample 4 on 15.09.2019 (t=247). These samples from 2019 could not be included in this thesis unfortunately, this because of time limits and limitations during the analyses process (Table 2.1). The dredging was finished July of 2019. From 2018 to 2019 the mussels were replaced to keep them unrelated to each other. Because of this the mussels were not sampled until March so the new mussels could reach the steady state with contaminant levels within the water column.

2.2 Data collection and extraction

Around 30 to 50 mussels were collected from each location and each depth. The collection was done on the same day and in a short timeframe to avoid potential mussel death. After the mussels were collected, they were swiftly transported by boat and stored at -20^{o}C. The mussels were kept frozen until the contaminant extraction procedure.

From the frozen stage, 10 mussels were collected to measure the weight and the length of the mussels from each depth and location. This was done to allow a correct comparison between the data; the goal was to have mussels that were the same size because filtration rate and contaminant accumulation can differ between small and big mussels. After the measurements, the soft tissue was extracted from the rest of the mussels to be used for measuring the levels of organic contaminants. About 200-300g of soft tissue was extracted from each sample of mussels to make sure that there was enough to sample for the analysis. The soft tissue was then wrapped in tinfoil, marked and frozen down to -20 ^{o}C until it was ready to be shipped to the laboratory at the University of Milan.

T=166 and t=196 in Location 1 and Location 2 in 2018 were unfortunately lost because of storms, rough seas, and winds, which destroyed the cages with the mussels (Table 2.2). This

meant that these samples could not be collected at this time, with the exception of the last sample in location 2, 5m.

T=135, Location 2, 15m were also all lost because of mass mussel death possibly due to the landslide that happened around this time (Table 2.2). The mussels during t=196 in location 5, 15m did not survive due to possible toxicity.

Table 2.2: Sampling overview of 2018 and which samples is included. List of dates and status of samplings. The samples marked in grey and with x are the samples that were lost for various reasons.

Sample	Location	Depth (m)	Condition
t=0 (Sample 1)	1	5	v
		15	v
	2	5	v
		15	v
	3	5	v
		15	v
	4	5	v
		15	v
	5	5	v
		15	v
t=30 (sample 2)	1	5	v
		15	v
	2	5	v
		15	v
	3	5	v
		15	v
	4	5	v
		15	v
	5	5	v
		15	v
t=135 (sample 3)	1	5	v
		15	v
	2	5	v
		15	X
	3	5	v
		15	v
	4	5	v
		15	v
	5	5	v
		15	v
t=166 (sample 4)	1	5	X
		15	X
	2	5	X
		15	X
	3	5	v
		15	v
	4	5	v
		15	v
	5	5	v
		15	v
t=196 (sample 5)	1	5	X
		15	X
	2	5	v
		15	X
	3	5	v
		15	v
	4	5	v
		15	v
	5	5	v
		15	X

2.3 Homogenization and lyophilization

Samples were homogenized and separated into two groups from each sample at ~10g, one for analysis of organic contaminants and one for heavy metal analysis. The samples were then frozen down again at -20°C to await further procedures. The sample dedicated to heavy metal analysis were then lyophilized (freeze-dried) to eliminate the water from the meat of the mussels. To do this, a freeze-dry machine was used to shock freeze the samples at -40°C and then adding heat for a longer time (from 1 up to 2 days) at 20°C to allow the frozen water to sublimate from ice to gas. The freeze-drying process worked in two stages: the frozen stage and the drying stage. The freezing stage was started as the samples were all deep frozen before the lyophilize process. The drying process, or the sublimation process, lowered the pressure and added heat, which caused the water to sublimate. Lastly, a vacuum collected the condensed water. After this process was done, most of the moisture in the mussels was gone and the dry weight of the product remained. The samples for heavy metal analysis were then grinded down using a normal kitchen grinder, until the fibres were small and in a powdery state. From this they were further broken down into fine powder by a grinder device, which was necessary for it to burn more easily.

To analyse the levels of organic contaminants about 3g of frozen samples, of the 10g previously collected, were randomly selected and put in clear, clean falcon tubes (QuEChERS extraction) to then be transported for lab analysis.

2.4 Chemical analysis

The chemical analysis of the organic contaminants and the trace elements from 2018 was performed at the laboratories of the University of Milan by Marco Parolini, Sara Panseri and their teams according to published methods (Chiesa et al., 2018; Chiesa et al., 2019; Parolini et al., 2020). For continuity, the method was replicated in this thesis. I was involved in the analyses of the samples from 2019 during a research stay at the University of Milan where the same exact methods were used, but the analyses were not finished before the university had to close down due to the COVID-19 pandemic of 2020.

2.4.1 Trace element analysis

The trace element analysis was performed on the following metals: Ca (calcium), K (potassium), Mn (manganese), P (phosphorous), Mg (magnesium), Na (sodium), Fe (iron), Zn (zinc), Sr (strontium), Cu (copper), Ti (titanium), Pb (lead), Cr (chromium), Al (aluminium), Ni (nickel), As (arsenic), Cd (cadmium), and Hg (mercury). The freeze-dried samples were mineralized in a microwave system using 3 ml nitric acid SpA (Chiesa et al., 2019). The samples were diluted to 50 ml by addition of bi- distilled water. Elemental analysis was performed by inductively coupled plasma-atomic emission spectrophotometer. Nebulizing and auxiliary gas flows were 12 L min^{-1} and the radiofrequency power was set at 1000 W. The limit of detection (LOD) and limit of qualifications (LOQ) were calculated as three and ten times, respectively; the standard deviation of a blank solution with 10 repetitions. The LOQs ranged from 0.007 to 0.020. Quality controls were carried out based on the recovery percentage study obtained with the certified Reference Materials under reproducible conditions in order to verify the accuracy of the analytical procedure. The obtained concentrations were in good agreement with a 95% confidence limit with the certified concentrations; relative standard deviation (RSD) values were lower than 10%.

2.4.2 Chemicals and reagents that were used in the extraction of organic compounds

A mixed solution of PCB congeners (CB-28; CB-52; CB-101; CB-138; CB-153 and CB-180), CB-209 (internal standard [IS] for PCBs and PAHs), a mixed solution of PBDEs (BDE-28; BDE-33; BDE-47; BDE-99; BDE-100; BDE-153, and BDE-154 numbered according to the IUPAC nomenclature) and fluorobromodiphenyl ether (FBDE), as well as the internal standard (IS) for flame retardants were purchased from AccuStandard.

A standard solution of 15 organochlorine compounds (OCPs), and their metabolites (α–HCH; hexachlorobenzene; β-HCH; lindane; heptachlor; aldrin; heptachlor epoxide; trans chlordane; 4,4′-dichlorodiphenyldichloroethylene [4,4′-DDE]; endosulfan I; endosulfan II, endosulfan sulfate; endrin, 4,4′-dichlorodiphenyldichloroethane [4,4′-DDD], 2,4′-dichlorodiphenyltrichloroethane [2,4′-DDT]), six organophosphate compounds (OPs – i.e., demeton, disulfoton, diazinon, phorate, mevinphos, ethoprophos), and a standard solution of

four polycyclic aromatic hydrocarbons (i.e., chrysene, benzo(α)anthracene, benzo(β)fluoranthene and benzo(α)pyrene) were purchased from Restek.

The 17 per- and polyfluoroalkyl substances (PFASs) examined were perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluorobutane sulphonic acid (PFBS), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorohexane sulphonate (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluorooctane sulfonic acid (PFOS), perfluorododecanoic acid (PFDoA), perfluoroundecanoic acid (PFUnDA), sodium perfluoro-1-decanesulfonate (PFDS), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), perfluorohexadecanoic acid (PFHxDA), and perfluorooctadecanoic acid (PFODA). All of these compounds and the two 13C-labeled internal standards (ISs), MPFNA, and MPFOS were purchased from Fluka (Sigma Aldrich, St. Louis, MO, USA).

2.4.3 Analytical preparation standards

Stock solutions (10 µg mL^{-1} in hexane) of OCPs, OPs, PCBs, PBDEs, and PAHs were used to prepare the working solutions by serial dilutions. Mixed compound calibration solution, in hexane, was prepared daily, and the proper volume was used as a spiking solution as well. Stock solutions of PFASs (1 mg mL^{-1}) were dissolved in methanol, from which working solutions at the concentrations of 10 and 100 ng mL^{-1} were prepared during each analytical session. All the standard solutions were stored at -20 °C.

2.4.4 Extraction procedure for OCPs, OPs, PCBs, PBDEs and PAHs

The extraction of PCBs, PBDEs, OCPs, Ops, and PAHs from mussels were performed using the QuEChERS approach method was done according to the validated method described by Chiesa et al. (2018). The 2g of mussels in the falcon tubes were numbered according to their sample, location, and depth first. Then the internal standard was added. The internal standards contain raw product of 25µL PCB 209, 20µL FBDE, and 100µL 4-n Nonyl phenol which are compounds that are used to ensure that the methods will work. They are not present in the fjord therefore they would not have occurred naturally. 10mL of a mixture of hexane/acetone was then added as extraction solvent; the tube was shaken for 2-3 min to mix all the solvents together, and then centrifuged for 10 minutes at 5,000rpm at 4 °C. Then, the supernatant was

transferred to a QuEChERS purification tube, shaken, and centrifuged at the same conditions. The extract was transferred into a flask and evaporated in a centrifuge evaporator. The residue of this was dissolved in 1mL of hexane and analysed by GC/MS-MS.

2.4.5 GC-MS/MS analyses

A blank sample without contaminants were used to compare with the contaminated mussels. These blank samples were bought in a local market, homogenized fresh from the supermarket, and placed directly into falcon tubes before they were frozen and sent to the lab for the same analysis procedure as previously described.

Triple quadrupole mass spectrometry (QqQ) in electronic impact (EI) mode was used for the detection, identification, and quantification of compounds. A mass spectrometry works by generating ions from the organic contaminants and separating them by their mass-to-charge ratio (m/z) (Gross, 2006). Then, a standard calibration curve will be created adding five standard concentration of the five contaminants (PCB, PBDE, PAH, OCPs, and OPs) that will allow to create a calibration curve for each contaminant. The five groups of contaminants also have five different calibration curves for each contaminant. Each curve is made considering the concentration that is expected to be found (in relationship with previous analyses). The five classes include:

1 PBDE = 0.5 ng/g – 50 ng/g
2 PCB = 0.5 ng/g – 100 ng/g
3 PAH = 0.5 ng/g – 100 ng/g
4 OCPs = 1 ng/g – 500 ng/g
5 OPs = 1 ng/g – 500 ng/g

In order to prepare the standard calibration curve, we will extract all the samples with the QuEChERS extraction protocol. With this, a standard calibration curve will be created, with the concentration on the X axis, the A/A on the Y axis, and the concentration Z as the sample. A/A is calculated as: Area of Analysis and area of Internal Standard.

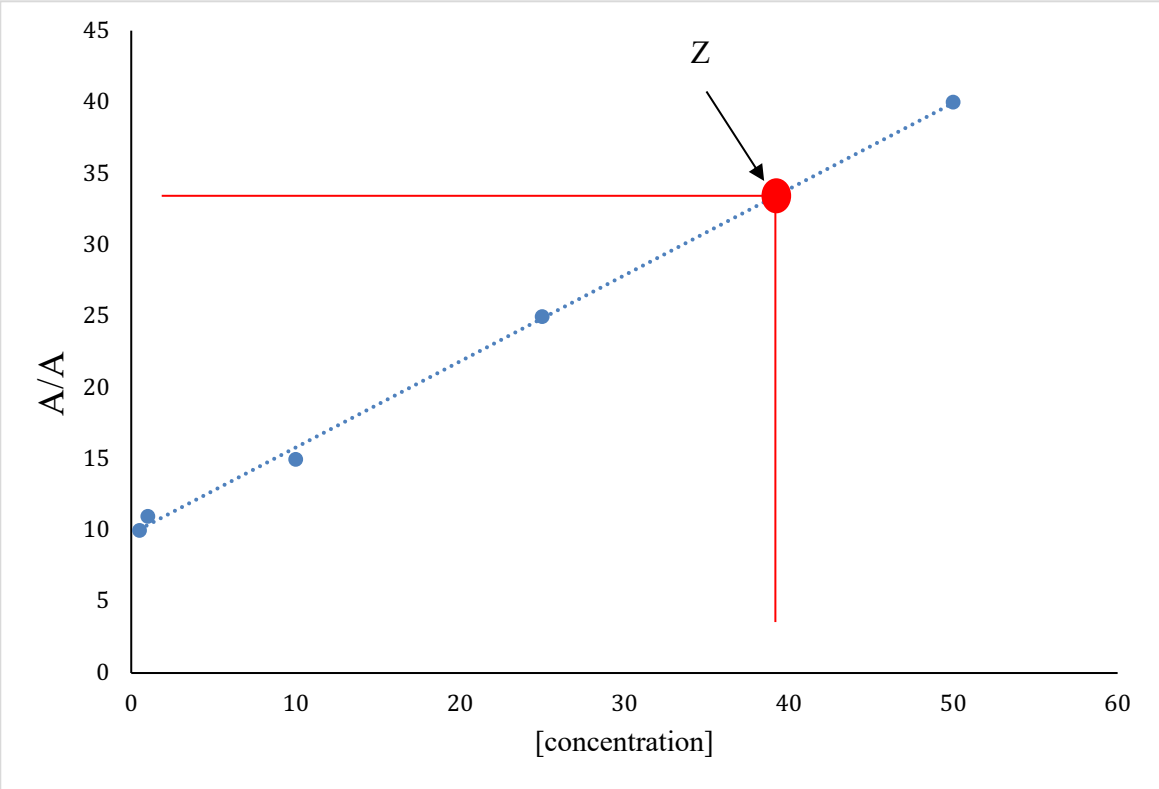

Figure 2.3: Calculation of the calibration curve of organic contaminants. This provides exact concentration of each compound following mass spectrometry.

By the interpolation of the sample Z (of unknown concentration) on the standard calibration curve, the concentration of contaminants in the sample will be obtained. This will deduce the concentration of Z from the A/A.

The QqQ mass spectrometer was operated in selected reaction monitoring mode (SRM), detecting two to three transitions per analyte. Identification of POPs were carried out by comparing sample peak relative retention times, with those obtained for standards under the same conditions, and the MS/MS fragmentation spectra obtained for each compound. As a result of this a list over the present compounds of each site, location, and depth were eventually obtained.

2.4.6 Extraction procedure for PFASs

The analysis of PFASs in mussel tissues was only done for the 2018 organic samples. No major contamination was detected and because of this combined with unnecessary laboratory costs,

a decision was made to exclude this from the 2019 samples. 2g of sample were spiked with the 2 internal standards at the concentration of 5 µg/mL, and 10 mL of acetonitrile were added for extraction and protein precipitation. The sample was then vortexed and sonicated for 15 min. After centrifugation (2,500 × g, 4 °C for 10 min), the supernatant was evaporated in a rotary vacuum evaporator at 40 °C.

The extract was suspended in 10 mL of water and purified by SPE Oasis WAX Cartridges under vacuum. The SPE cartridges were preconditioned with 3 mL of 0.5% ammonium hydroxide in methanol, 3 mL of methanol, and 3 mL of Milli-Q water. After sample loading, the cartridges were washed with 3 mL of 25 mM acetate buffer pH 4.5 to minimize interferences, followed by 2 mL of methanol. The elution was done with 3 mL of 0.5% ammonium hydroxide in methanol and the eluate was dried and then suspended in 100 µL of methanol: ammonium formate 20 mM (10:90 v/v).

2.4.7 LC-HRMS analyses

The HPLC system was coupled to a QExactive Orbitrap, equipped with a heated electrospray ionization (HESI) source, operating in negative mode. A “Synergi Hydro-RP” reversephase HPLC column, with a C18 guard column was used for the chromatographic separation. Stainless steel capillary tubes were used for minimizing PFAS background contamination in the system. Moreover, since PFOA and PFOS were always present in the chromatographic system, a small Megabond WR C18 column was introduced between pump and injector, allowing us to delay elution of the contaminants of the system by 2 min relative to the analytes present in the samples. The mobile phases were: Solvents A (aqueous ammonium formate, 20 mM), and B (MeOH).

2.5 Statistical analysis

The same GLM procedure described in Parolini et al., (2020) analysis of PCBs and PAHs was completed using RStudio software, the same procedure was similarly used on all the investigated trace elements. For the trace elements Microsoft Excel was used to perform the raw data percentages and mean, while RStudio was used to visualize the data (RStudio Team, 2016). The reason for the use of different software’s was the availability of the software while the work was being performed and what was found to be useful for the task they were needed for.

3. Results

3.1 Status of mussels

The survival rate of the caged mussels during the 6-month biomonitoring period of Flekkefjord was good, although there was some mortality at both depths. The health status of mussels in location 1 (L1) and location 2 (L2) after the third sampling (t = 166 days) could not be monitored because the cages were lost due to harsh weather. The cage placed at 15 m depth in location 3 was confirmed by divers to have been robbed by crabs after the third sampling (t = 166 days), so fewer than 50 mussels were collected at the fourth and fifth sampling. Full mortality of mussels was noted at t =166 days in the cage placed at 15 m depth in location 5, meaning that for the rest of the period we did not obtain data from this location.

3.2 Visualisation of data

Information was obtained from all trace elements. Most elements, however, had low concentrations. The raw data for the trace elements can be found in Appendix 1. The focus will be on the trace elements that are either considered to be toxic or who showed relevant or interesting patterns in the raw data. The rest of the trace elements are excluded in this section but are included under Appendix 2. In detail, the trace element data that are presented include copper, manganese, lead, chromium, zinc, aluminium, nickel, arsenic, titanium, iron and mercury. For the trace elements that are considered highly toxic there is a limit in the graph that represents the value of where the limit of contamination for people lies according to SFT (2011) (Norwegian environmental agency). This is anything above baseline values, however, contamination above the baseline values in this does not mean there is an immediate health concern. This is simply done for comparison purposes. For element Ti, Fe, Mn and Al there is no indicator in the figure due to there not being any maximum contamination limit for these trace elements (SFT, 2011).

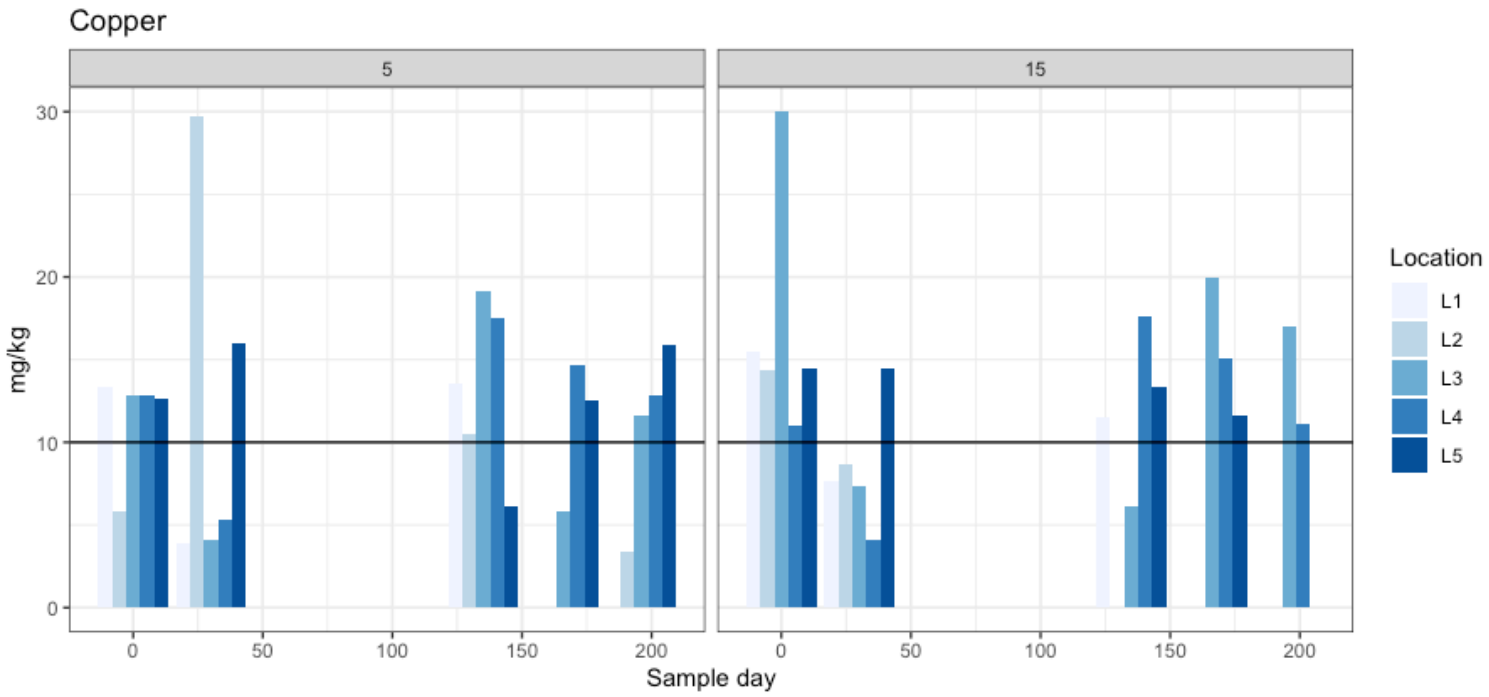

Figure 3.1: Distribution of copper found in samples at 5 m depth (left) and 15 m depth (right). Concentrations above the horizontal black line at 10 mg/kg are considered above baseline levels according to SFT (2011). Concentrations are expressed in mg/kg dry weight.

The mussel samples where copper was measured ranged from 3,39 to 30 mg/kg dry weight (d.w) (Fig. 3.1). The average was 12,12 mg/kg at 5 m and 13,07 mg/kg at 15m. There was an increase in the 5 m sample at location 2 from 5,79 mg/kg in t=0 to 29,76 mg/kg in t=30 after which followed an abrupt decrease at t=135. The other locations also had fluctuating levels, but to a smaller degree. In the 15 m sample there was a larger concentration during t=0 than in the 5m sample. The mean copper levels are above the background level >10 mg/kg d.w (Table 3.1). The mean location value were close in concentrations (Table 3.1), all with values above the baseline limit (background concentration).

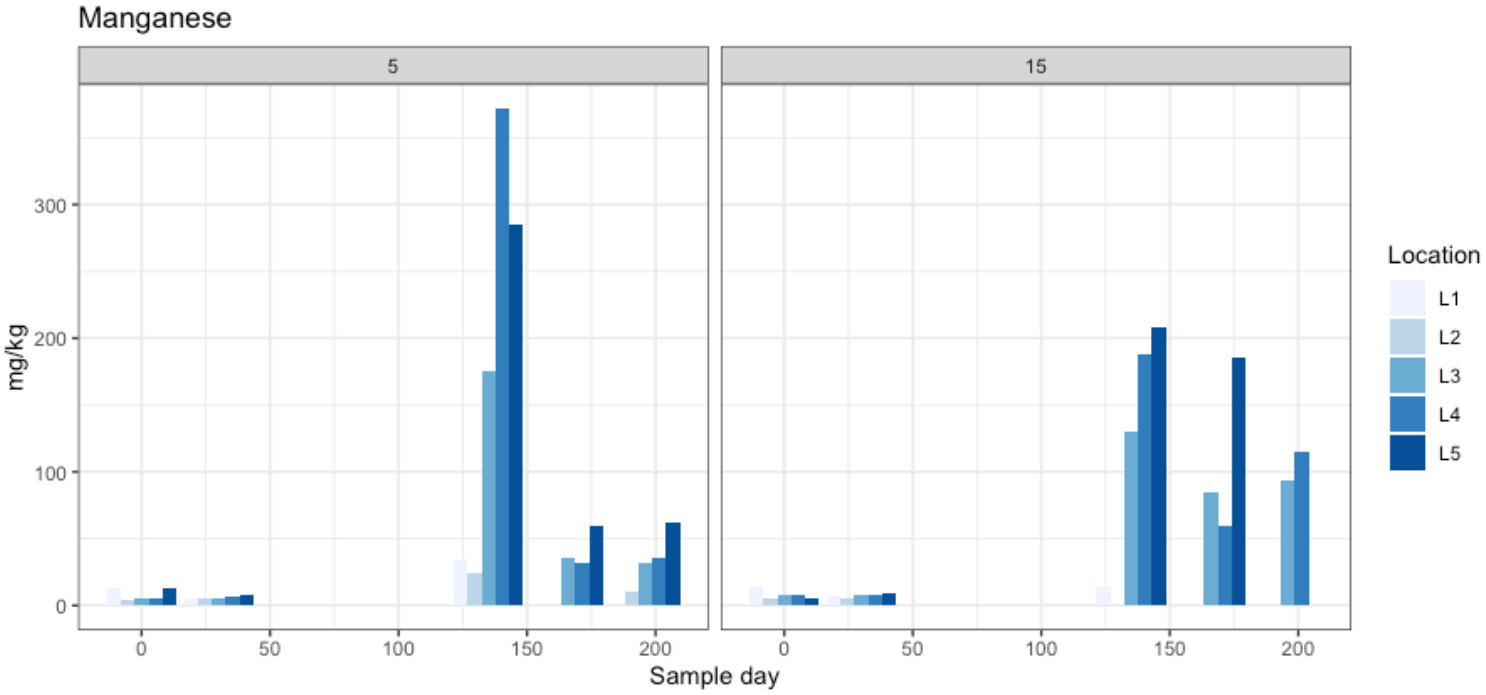

Figure 3.2: Distribution of Manganese found in samples at 5m depth (left) and 15m depth (right). Data are expressed in mg/kg dry weight.

The concentration of manganese measured in the mussels varied from 4,38 mg/kg to 371,62 mg/kg, with an average of 53,27 mg/kg at 5 m and an average of 57,90 mg/kg at 15 m depth, respectively. There was a distinct increase in the mussel manganese concenstrations at t=135 for both depths in mussels transplanted to location 3-5 (Fig. 3.2), while the sample for 5 m had a higher concentration compared to the other locations. The concentration seemed to linger for a longer period at 15 m, particularly at location 5.

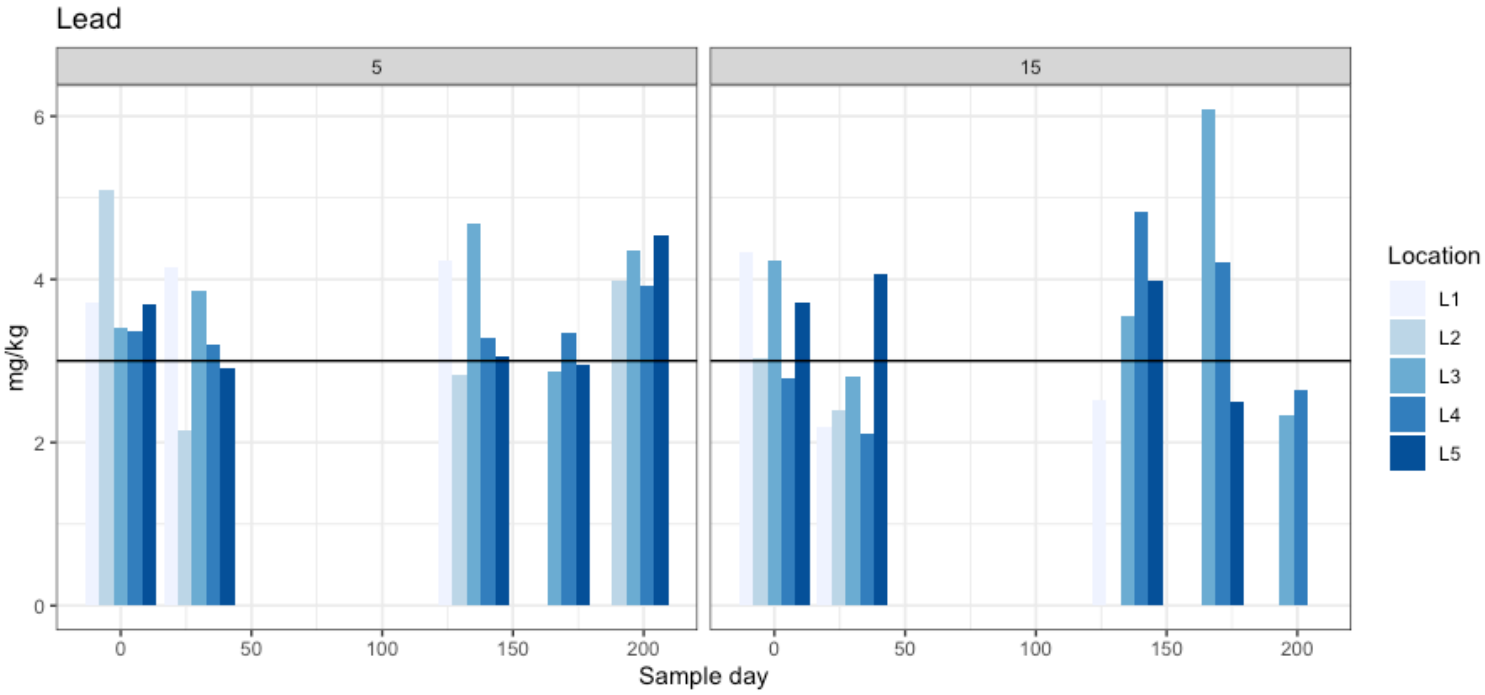

Figure 3.3: Distribution of lead found in samples at 5m depth (left) and 15m depth (right). Concentrations above the horizontal black line at 10 mg/kg are considered above baseline levels according to SFT (2011). Concentrations are expressed in mg/kg dry weight.

The concentration of lead measured in the mussels (Fig. 3.3) varied from 2,11 mg/kg to 6,08 mg/kg and had an average of 3,62 mg/kg at 5 m and an average of 3,38 mg/kg at 15 m. Mean lead values values have been above the background limit the whole period with a few increases in several of the samples at both meters. At 15 m there was a gradual increase after t=135 before a decrease for the final sample. At 5 m the concentration rises for the final sample. The concentration mean is very similar for all the different locations.

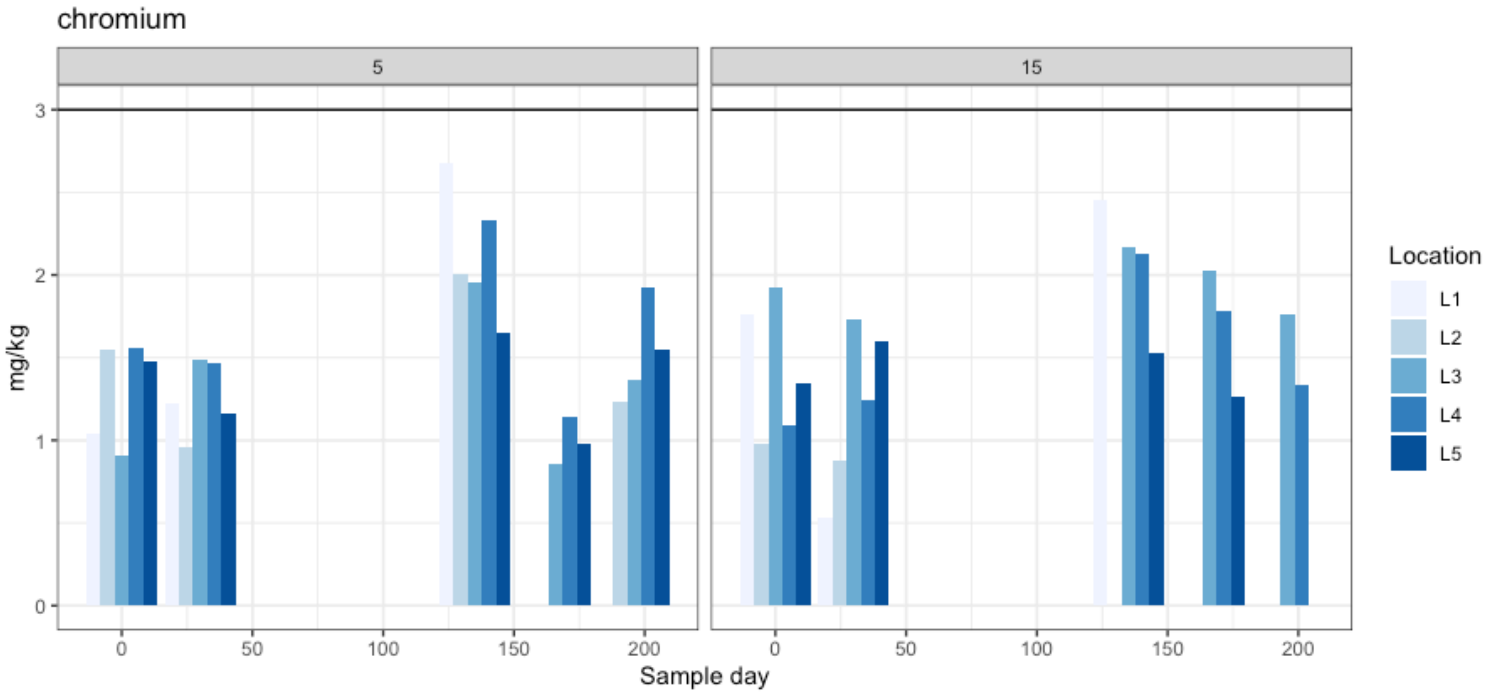

Figure 3.4: Distribution of chromium found in samples at 5m depth (left) and 15m depth (right). Concentration above the horizontal black line would according to SFT (2011) be concidered hazardous to human health. Data are expressed in mg/kg dry weight.

The concentration of chromium measured in the mussels (Fig 3.4) varied from 0,53 mg/kg to 2,68 mg/kg and had an average of 1,48 mg/kg at 5 m and an average of 1,55 mg/kg at 15 m. Chromium had an increase of concentration on t=135 at both depths, with an immediate decrease at 5 m and with a slow decrease at 15 m. There is not much noticable difference between the locations and all samples are concidered background levels.

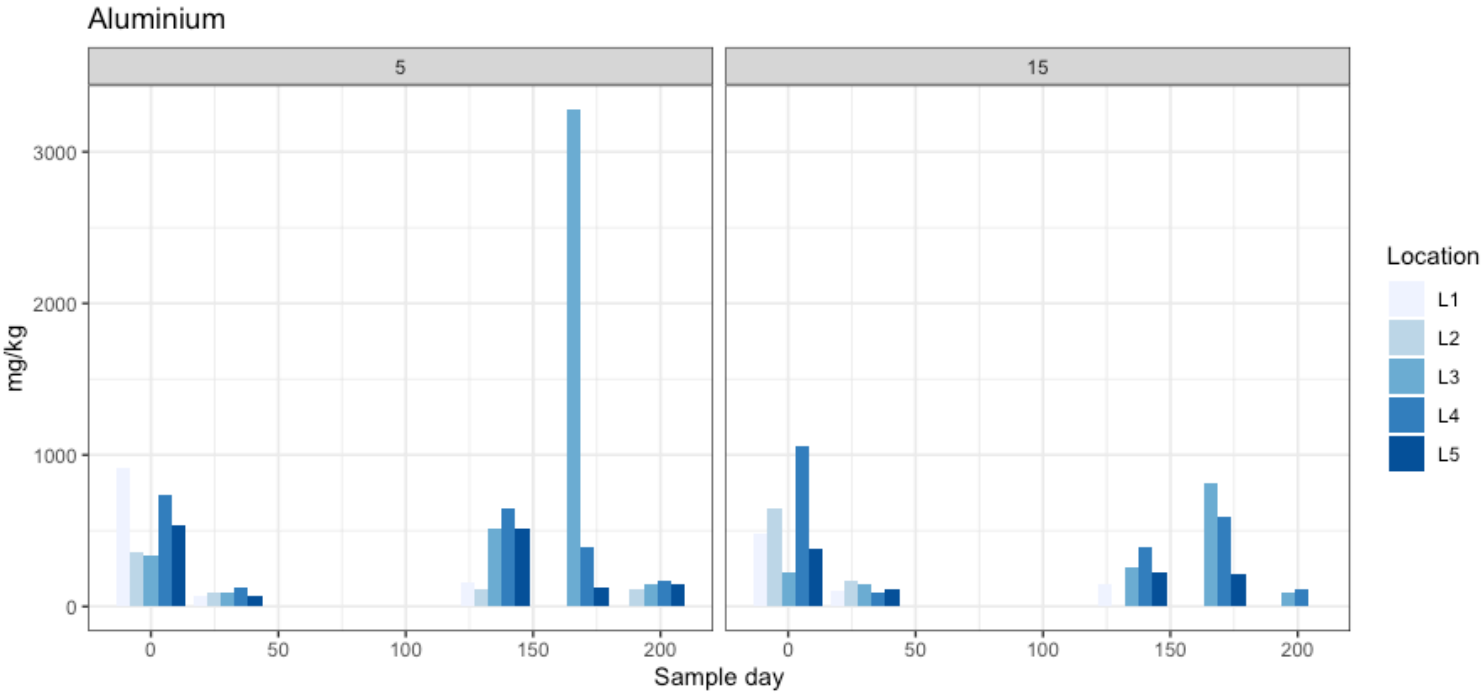

Figure 3.5: Distribution of aluminium found in samples at 5m depth (left) and 15m depth (right). Data are expressed in mg/kg dry weight.

The concentration of Aluminium measured in the mussels (Fig 3.5) varied from 69,21 mg/kg to 3276,80 mg/kg and had an average of 439,00 mg/kg at 5 m and an average of 329,65 mg/kg at 15 m. Aluminium had an increase of concentration on t=166 at location 3 at 5 m. Compared to this increase the levels of aluminium was low for the other samples. There was a slight increase of contamnination for the same sample at 15 m but with not as much value compared to the increase at 5 m. Location 3 and 4 had the largest levels of aluminium.

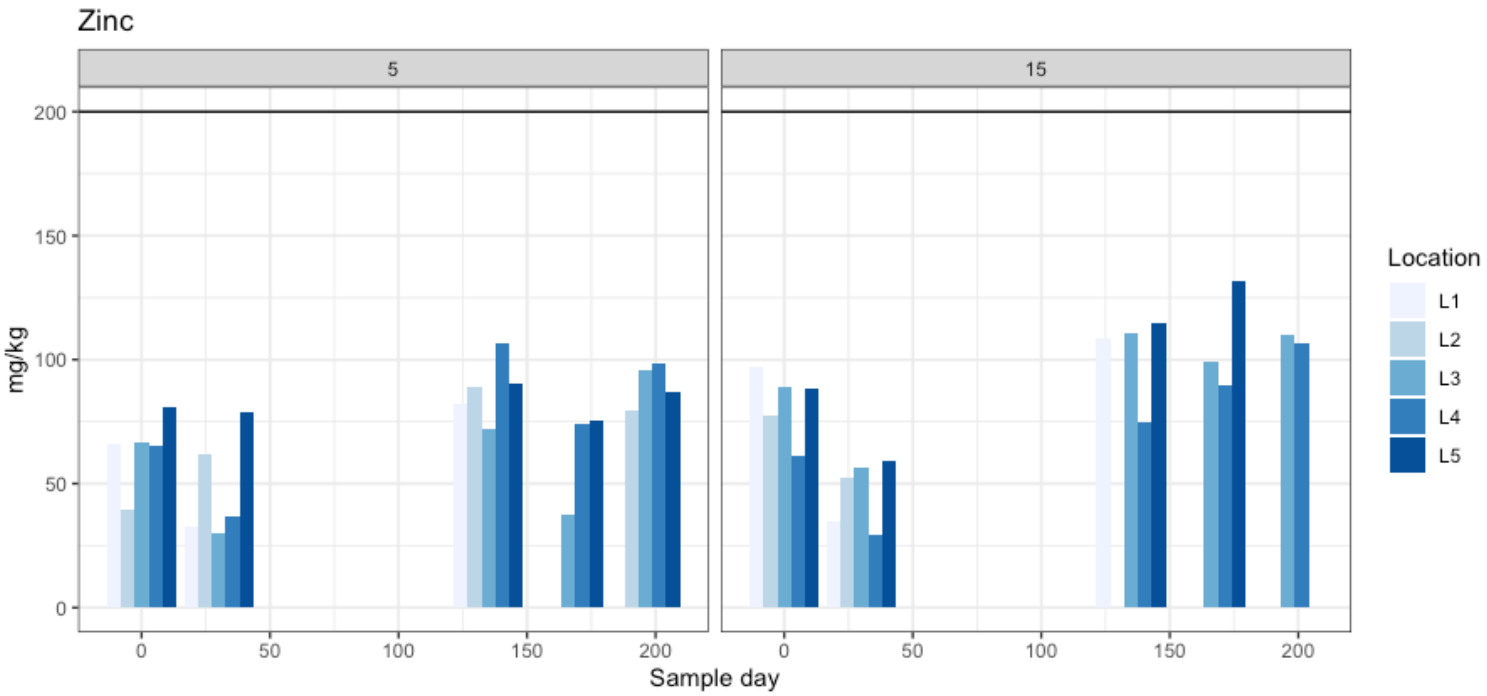

Figure 3.6: Distribution of zinc found in samples at 5m depth (left) and 15m depth (right). Concentration above the horizontal black line would according to SFT (2011) be concidered hazardous to human health. Data are expressed in mg/kg dry weight.

The concentration of Zinc measured in the mussels (Fig 3.6) varied from 29,50 mg/kg to 131,58 mg/kg and had an average of 69,38 mg/kg at 5 m and an average of 83,34 mg/kg at 15 m. Zinc values was fluctuating but have a steady low throughout the sampling. The concentration of zinc at 15m for location 5 had increased from sample t=30 to t=135 and t=166. The highest concentration mean is found at location 5 (Table 3.1).

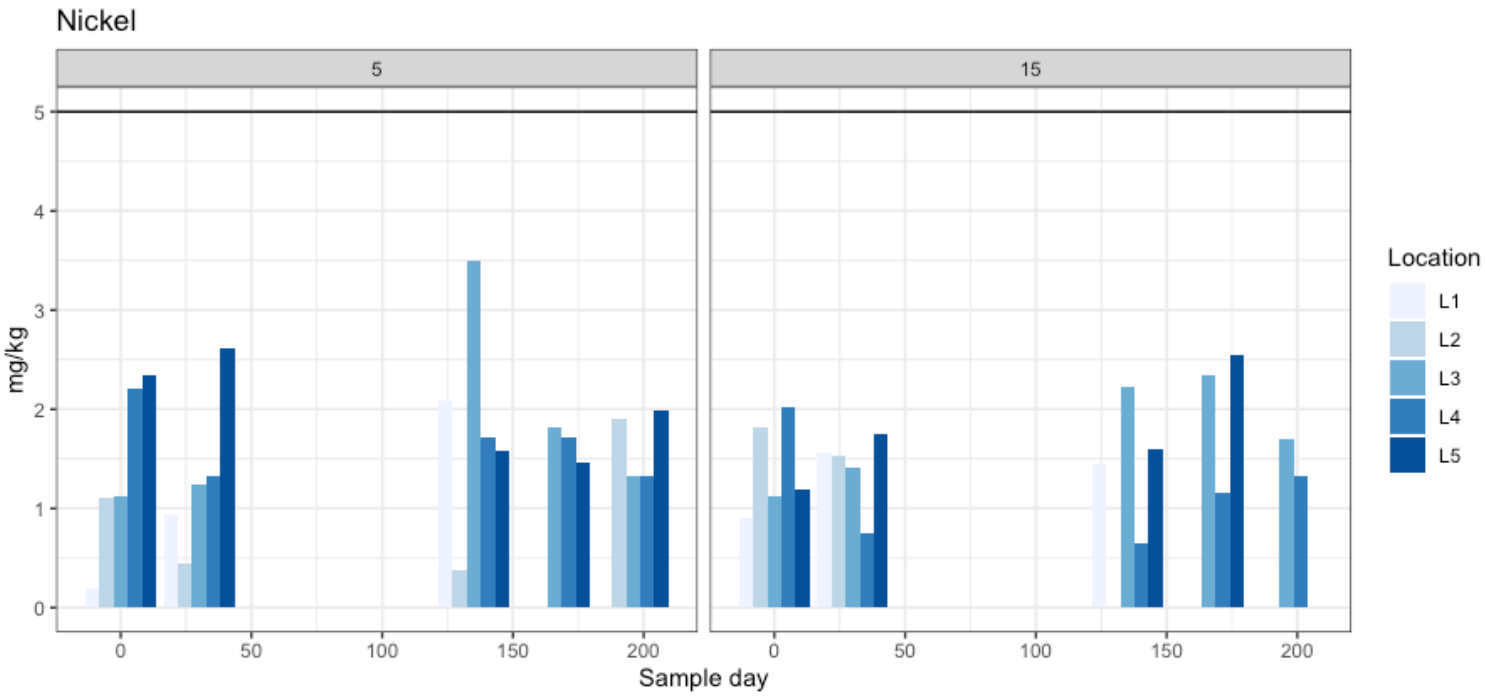

Figure 3.7: Distribution of nickel found in samples at 5m depth (left) and 15m depth (right). Concentration above the horizontal black line would according to SFT (2011) be concidered hazardous to human health. Unit of measurement is mg/kg

The concentration of nickel measured in the mussels (Fig 3.7) varied from 0,19 mg/kg to 3,49 mg/kg and had an average of 1,56 mg/kg at 5 m and an average of 1,53 mg/kg at 15 m. In t=145 location 3 at 5 m there is an increase, as well as in the same location for the same and next sample at 15 m. Highest mean was found in location 3 (Table 3.1).

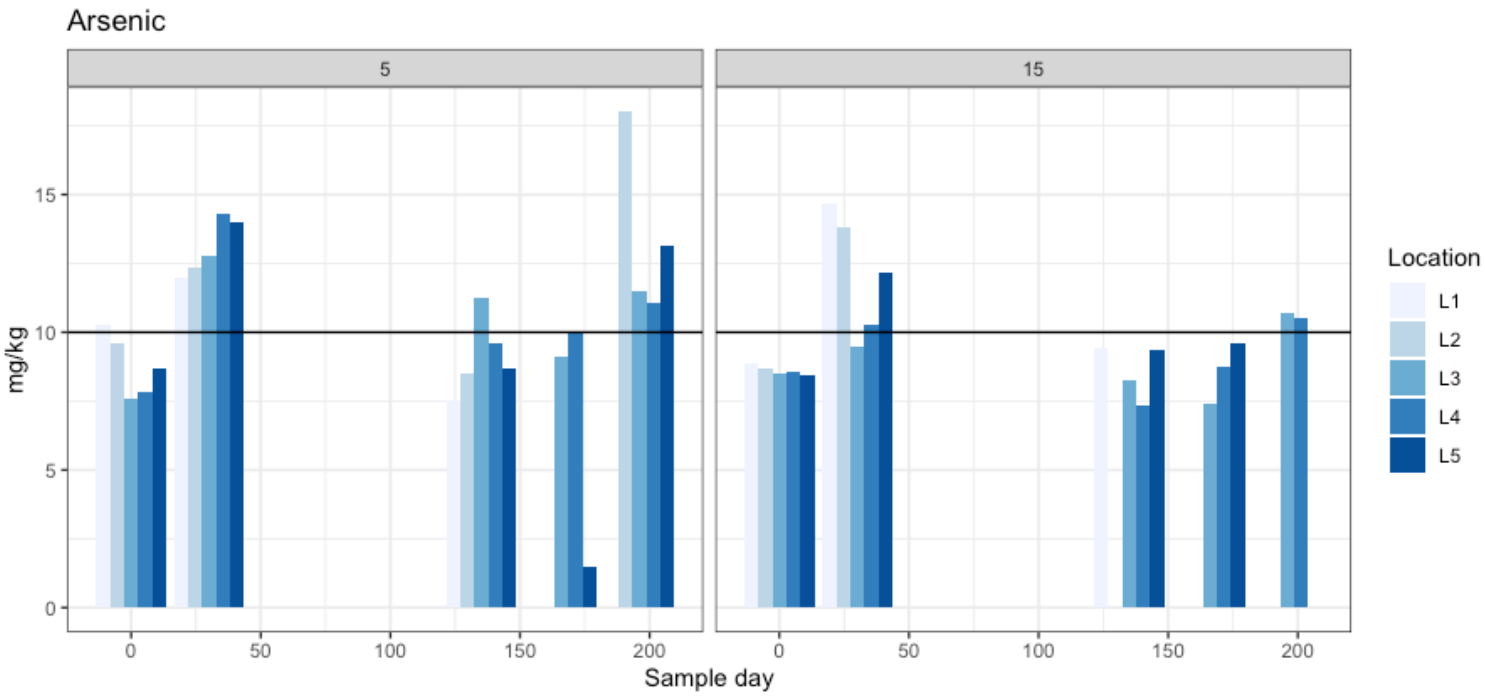

Figure 3.8: Distribution of arsenic found in samples at 5m depth (left) and 15m depth (right). Concentrations above the horizontal black line at 10 mg/kg are considered above baseline levels according to SFT (2011). Unit of measurement is mg/kg

The concentration of arsenic measured in the mussels (Fig. 3.8) varied from 1,46 mg/kg to 18,01 mg/kg had an average of 10,43 mg/kg at 5 m and an average of 9,73 mg/kg at 15 m. Arsenic values have differences between the levels and are generally higher than the baseline level of contamination, samples of note are t=30 and t=196 days. The highest mean was found in location 2 (Table 3.1).

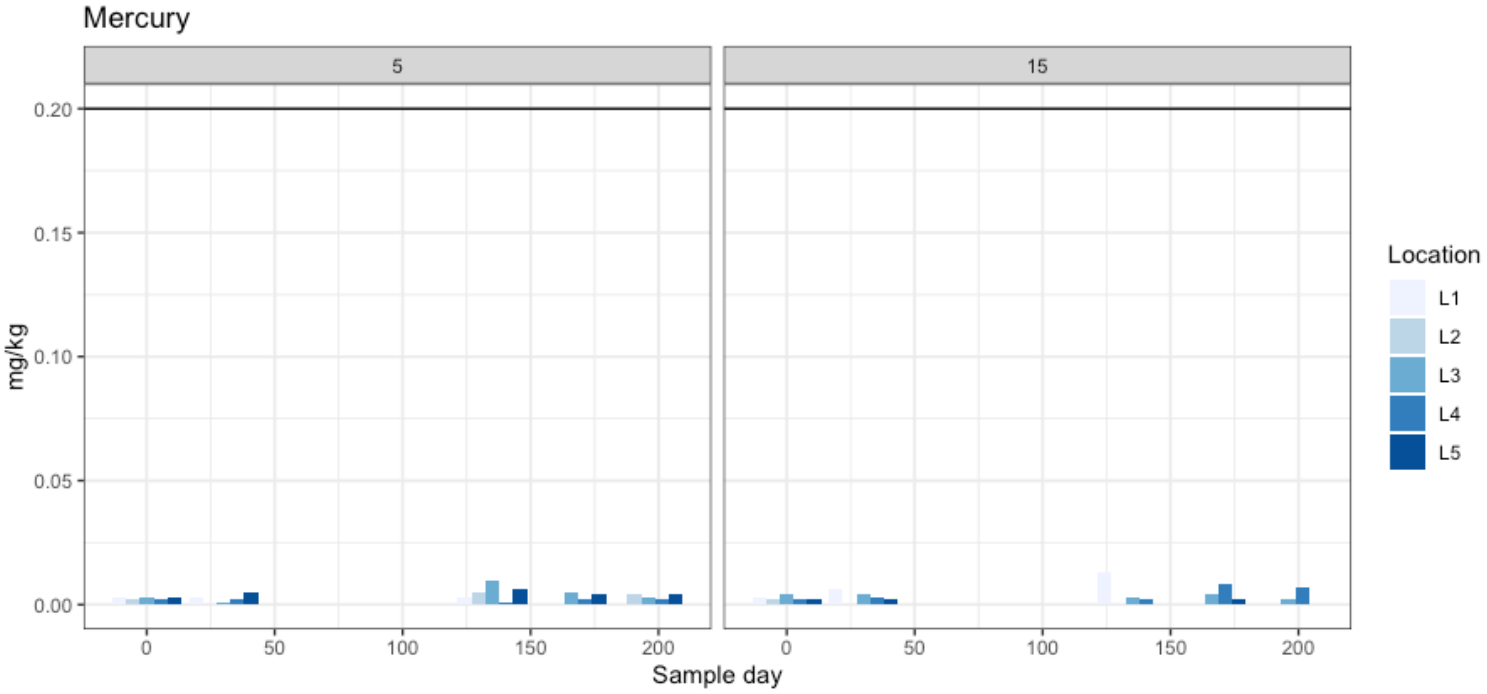

Figure 3.9: Distribution of mercury found in samples at 5m depth (left) and 15m depth (right). Concentration above the horizontal black line would according to SFT (2011) be concidered hazardous to human health. Unit of measurement is mg/kg

The concentration of mercury measured in the mussels (Fig 3.9) varied from 0 to 0,013 mg/kg and there was an average of 0,003 mg/kg at 5 m and an average of 0,004 mg/kg at 15 m. Mercury had relativly low values to the point where the value was close to zero and very far from the standard limits.

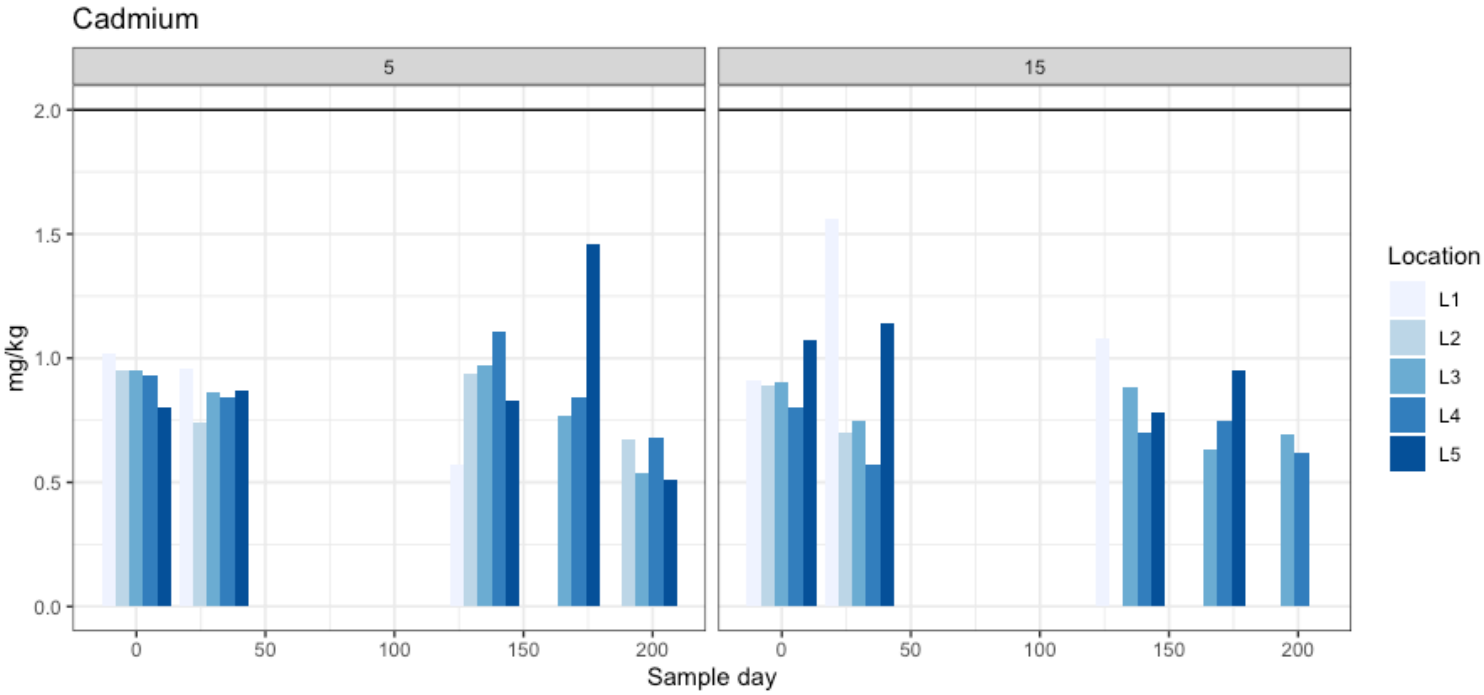

Figure 3.10: Distribution of Cadimum found in samples 5m depth (left) and 15m depth (right). Concentration above the horizontal black line would according to SFT (2011) be concidered hazardous to human health. Unit of measurement is mg/kg.

The concentration of cadmium measured in the mussels (Fig. 3.10) varied from 0,51 mg/kg to 1,56 mg/kg and had an average of 0,85 mg/kg at 5 m and an average of 0,86 mg/kg at 15 m. Cadimum had low values below limit. There was an increase in t=166 location 5 for 5 m as well as in t=30 in Location 1 for 15 m. Aside from this the values generally decreased with time.

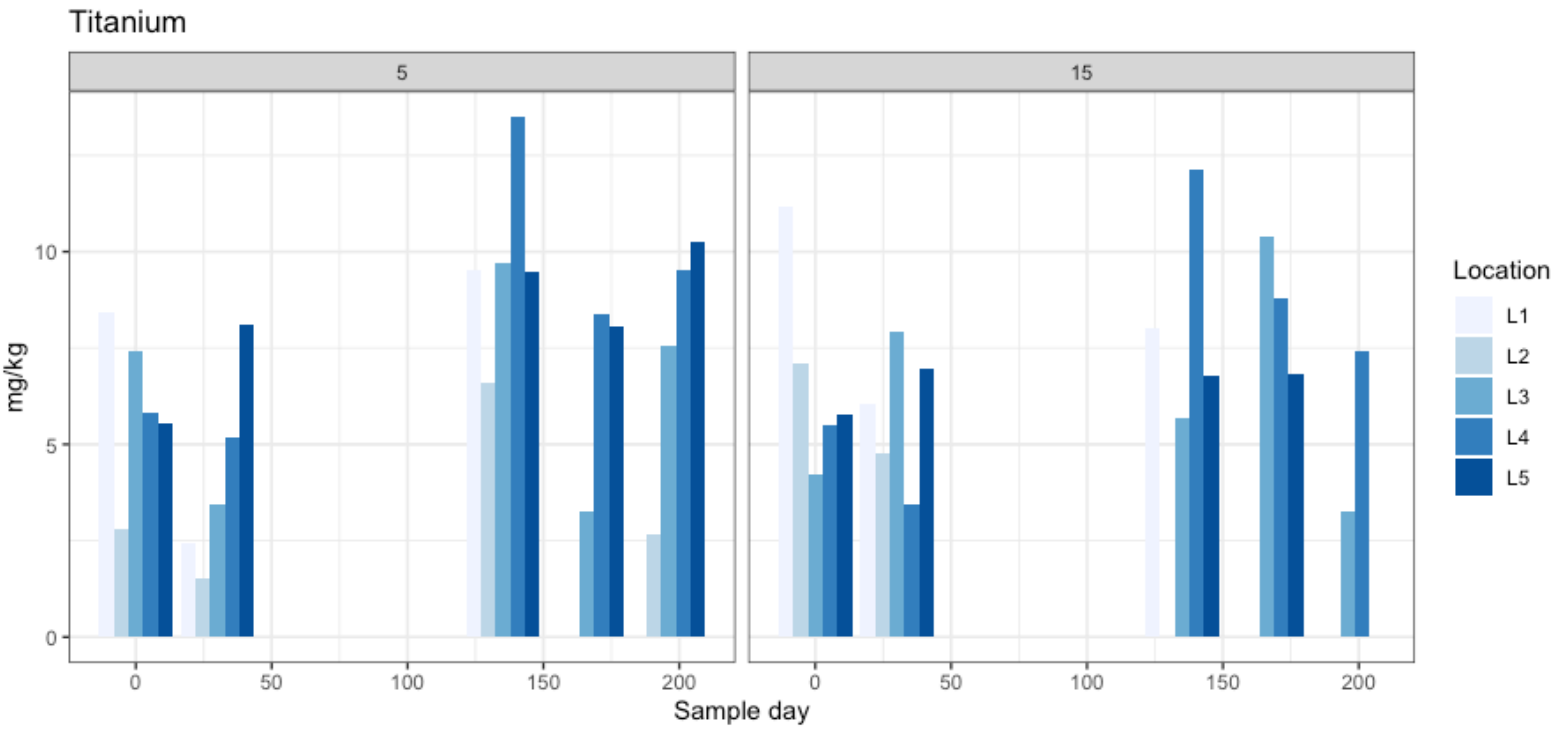

Figure 3.10: Distribution of Titanium found in samples 5m depth (left) and 15m depth (right). Unit of measurement is mg/kg.

The concentration of titanium measured in the mussels (Fig. 3.10) varied from 1,52 mg/kg to 13,49 mg/kg and had an average of 6,78 mg/kg at 5 m and an average of 6,96 mg/kg at 15 m. There is a visible growth of contamination from the frist two samples and the t=135 sample.

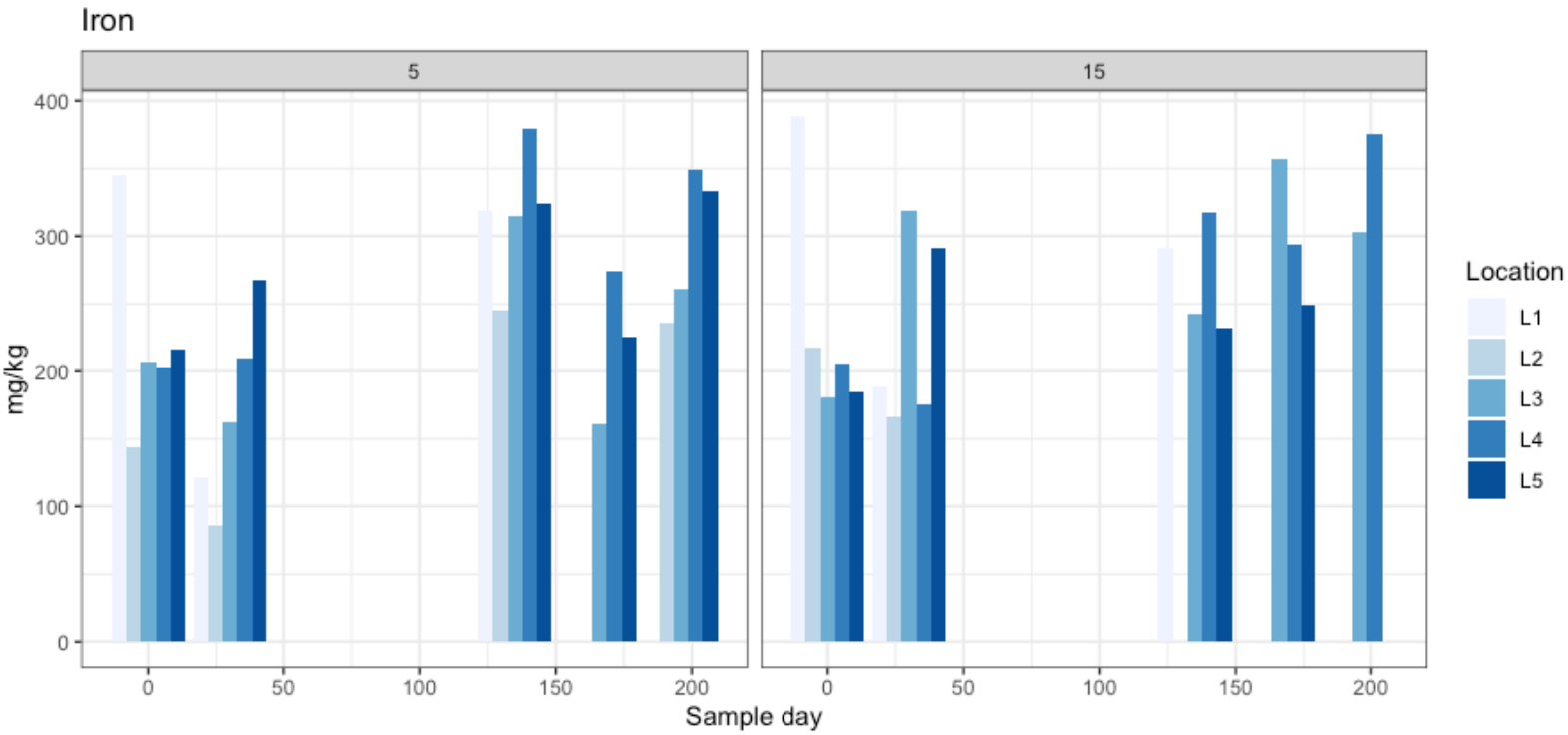

Figure 3.10: Distribution of Iron found in samples 5m depth (left) and 15m depth (right). Unit of measurement is mg/kg.

The concentration of iron measured in the mussels (Fig. 3.10) varied from 85,72 mg/kg to 388,26 mg/kg and had an average of 244, 76 mg/kg at 5 m and an average of 260,05 mg/kg at 15m.

Table 3.1: Mean values for trace elements (mg/kg d.w) at each location

	Location 1	Location 2	Location 3	Location 4	Location 5	Mean total
Cu	10,9 ± 4,34	12,08 ± 8,49	13,4 ± 8,18	12,21 ± 4,57	13 ± 2,99	12,595 ± 5,98
Mn	14,58 ± 10,57	9,11 ± 7,54	57,69 ± 60,40	82,94 ± 117,38	92,76 ± 105,55	51,416 ± 85,08
Pb	3,52 ± 0,94	3,25 ± 1,10	3,81 ± 1,10	3,37 ± 0,80	3,49 ± 0,67	3,50 ± 0,89
Cr	1,61 ± 0,840	1,27 ± 0,440	1,62 ± 0,457	1,60 ± 0,426	1,40 ± 0,226	1,51 ± 0,48
Al	313,17 ± 329,44	250,68 ± 219,44	590,26 ± 970,17	431,3 ± 323,88	258,05 ± 175,78	368,692 ± 528,50
Zn	70,34 ± 31,77	66,60 ± 18,50	76,69 ± 28,95	74,34 ± 27,04	84,85 ± 14,90	74,564 ± 25,98
Ni	1,19 ± 0,66	1,19 ± 0,67	1,78 ± 0,74	1,42 ± 0,50	1,40 ± 0,51	1,54 ± 0,65
As	10,47 ± 2,541	11,83 ± 3,700	9,67 ± 1,812	9,83 ± 1,987	9,51 ± 3,671	10,43 ± 2,73
Hg	0,005 ± 0,0041	0,002 ± 0,0020	0,004 ± 0,0026	0,003 ± 0,0023	0,003 ± 0,0017	0,003 ± 0,003
Cd	1,02 ± 0,323	0,81 ± 0,128	0,79 ± 0,144	0,78 ± 0,159	0,94 ± 0,268	0,86 ± 0,22
Ti	7,60 ± 3,034	4,23 ± 2,277	6,29 ± 2,694	7,97 ± 3,170	7,54 ± 1,593	6,86 ± 2,795
Fe	275,56 ± 101,17	182,28 ± 61,83	250,84 ± 71,41	278,43 ± 76,46	258,18 ± 50,39	252,77 ± 75,65

3.3 Trace elements standard limits

Comparison of the samples to the limits of the surroundings is done with numbers from the Norwegian environmental agency who have converted the values set by the EU to dry weight limits for blue mussels (SFT, 2011). These are appropriate to compare with since other projects have also used this limitation data to compare with in the future, and to potentially find a more common trend in the fjords on a country-wide basis this will then be easier to match with other data. A maximum value and a mean value of trace elements detected was found across all samples as included in Table 3.2 and Table 3.3. The limits were retrieved from a report done by formally named the pollution control agency in Norway (SFT) in 2011 (Norwegian environmental agency). As of now (2020) there is no official limit to the release of manganese and aluminium so this data could not be included.

Table 3.2: List of quality standard in mussel d.w based off the Norwegian environmental agency (previously SFT) and converted biota limits from the EU. As of now there is no presented upper recommended limit for aluminium and manganese. Values are expressed in mg/kg dry weight (d.w).

Compound	Limits of contaminants in biota Baseline/Good/moderate/poor/maximum (mg/kg d.w)	Reference	Maximum	Mean
Copper (Cu)	<10/ 10-30/ 30-100/ 10-200/ >200	(SFT, 2011)	30	12,595
Manganese (Mn)	-	-	371.62	51,416
Lead (Pb)	<3/ 3-15/ 15-40/ 40-100/ >100	(SFT, 2011)	6.08	3,50
Chrominum (Cr)	<3/ 3-10/ 10-30/ 30-60 />60	(SFT, 2011)	2.68	1,51
Aluminium (Al)	-	-	3276.80	368,68
Nickel (Ni)	>5/ 5-25/ 25-50/ 50-100/ >100	(SFT, 2011)	3.49	1,54
Arsenic (As)	<10/ 10-30/ 30-100/ 10-200/ >200	(SFT, 2011)	18.01	10,43
Mercury (Hg)	<0,2/ 0,2-0,5/ 0,5-1,5/ 1,5-4/ >4	(SFT, 2011)	0.013	0,003
Zinc (Zn)	<200/ 200-400/ 400-1000/ 1000-2500/ >2500	(SFT, 2011)	131.58	74,564
Cadimum (cd)	<2/ 2-5/ 5-20/ 20-40/ >40	(SFT, 2011)	1.56	0,85
Iron (Fe)	-	-	388,26	252,77
Titanium (Ti)	-	-	13,49	6,86

Table 3.3: Concentrations (in mg/kg) of selected metals and heavy metals classified according to the limits set by the Norwegian environmental agency (SFT, 2011). Blue colour means concentrations is below the background limit, green colour means there is pollution but it is to not immediate concern and there are low to no health risks, yellow colour means there are moderate levels of pollution and there may be toxic effects to humans if exposed to for a long period. Grey in this table means there is no data. White gaps mean that the data was lost.

Sample	Location	depth	Aluminum (Al)	Manganese (Mn)	Arsenic (As)	Chrominum (Cr)	Copper (Cu)	Mercury (Hg	Nickel (Ni)	Zinc (Zn)	Lead (Pb)	Cardimum (cd)	Iron (Fe)	Titanium (Ti)
t=0	Location 1	5	916,84	12,94	10,28	1,04	13,36	0,003	0,19	66,25	3,72	1,02	345,48	8,41
t=0	Location 1	15	475,45	13,84	8,89	1,76	15,50	0,003	0,91	97,31	4,34	0,91	388,26	11,15
t=0	Location 2	5	362,38	4,38	9,63	1,55	5,79	0,002	1,10	39,55	5,10	0,95	143,57	2,78
t=0	Location 2	15	649,82	5,75	8,66	0,98	14,34	0,002	1,82	77,41	3,04	0,89	217,23	7,08
t=0	Location 3	5	337,72	5,00	7,60	0,91	12,86	0,003	1,12	66,73	3,40	0,95	206,83	7,41
t=0	Location 3	15	221,39	7,83	8,53	1,92	30,00	0,004	1,13	89,29	4,23	0,90	180,28	4,24
t=0	Location 4	5	733,28	5,09	7,85	1,56	12,81	0,002	2,20	65,38	3,36	0,93	202,72	5,80
t=0	Location 3	15	1063,06	8,02	8,55	1,09	11,04	0,002	2,02	61,12	2,79	0,80	205,89	5,49
t=0	Location 5	5	533,22	12,38	8,68	1,48	12,59	0,003	2,35	80,97	3,70	0,80	216,57	5,53
t=0	Location 5	15	378,41	5,46	8,46	1,34	14,51	0,002	1,19	88,64	3,71	1,07	184,30	5,79
t=30	Location 1	5	71,76	5,01	11,98	1,22	3,88	0,003	0,93	32,62	4,14	0,96	120,76	2,44
t=30	Location 1	15	102,86	6,41	14,69	0,53	7,68	0,006	1,57	34,86	2,18	1,56	188,95	6,07
t=30	Location 2	5	89,02	4,93	12,36	0,96	29,76	0,000	0,44	62,11	2,15	0,74	85,72	1,52
t=30	Location 2	15	172,74	5,23	13,81	0,88	8,71	0,000	1,53	52,60	2,40	0,70	166,20	4,75
t=30	Location 3	5	95,37	5,61	12,78	1,49	4,09	0,001	1,24	29,71	3,85	0,86	161,77	3,45
t=30	Location 3	15	148,24	8,07	9,48	1,73	7,33	0,004	1,41	56,37	2,80	0,75	319,09	7,91
t=30	Location 4	5	130,02	6,63	14,32	1,47	5,32	0,002	1,33	36,86	3,20	0,84	209,92	5,17
t=30	Location 3	15	90,19	8,19	10,28	1,24	4,09	0,003	0,75	29,50	2,11	0,57	175,83	3,46
t=30	Location 5	5	69,21	7,88	14,00	1,16	15,96	0,005	2,62	79,00	2,92	0,87	267,91	8,11
t=30	Location 5	15	110,71	9,65	12,16	1,60	14,49	0,002	1,75	59,40	4,06	1,14	290,67	6,96
t=135	Location 1	5	160,78	34,50	7,55	2,68	13,50	0,003	2,09	82,18	4,23	0,57	318,94	9,50
t=135	Location 1	15	151,35	14,77	9,41	2,45	11,47	0,013	1,45	108,83	2,52	1,08	290,98	8,03
t=135	Location 2	5	115,33	23,82	8,49	2,01	10,46	0,005	0,37	88,74	2,83	0,94	244,57	6,59
t=135	Location 2	15												
t=135	Location 3	5	512,28	175,89	11,27	1,96	19,17	0,010	3,49	72,34	4,68	0,97	314,65	9,72
t=135	Location 3	15	258,80	130,16	8,25	2,17	6,09	0,003	2,22	110,59	3,54	0,88	242,86	5,67
t=135	Location 4	5	641,99	371,62	9,63	2,33	17,50	0,001	1,71	106,58	3,29	1,11	379,17	13,49
t=135	Location 3	15	395,62	188,03	7,33	2,13	17,64	0,002	0,64	74,76	4,83	0,70	317,28	12,12
t=135	Location 5	5	516,42	284,91	8,71	1,65	6,11	0,006	1,58	90,20	3,05	0,83	324,62	9,48
t=135	Location 5	15	228,39	208,43	9,35	1,53	13,39	0,000	1,59	114,85	3,98	0,78	231,65	6,80
t=166	Location 1	5												
t=166	Location 1	15												
t=166	Location 2	5												
t=166	Location 2	15												
t=166	Location 3	5	3276,80	35,18	9,14	0,86	5,85	0,005	1,81	37,42	2,86	0,77	161,33	3,27
t=166	Location 3	15	814,95	84,36	7,43	2,03	20,00	0,004	2,34	99,05	6,08	0,63	357,51	10,41
t=166	Location 4	5	389,88	31,56	9,94	1,14	14,66	0,002	1,72	74,35	3,34	0,84	274,18	8,39
t=166	Location 3	15	592,33	59,67	8,75	1,78	15,04	0,008	1,15	89,57	4,21	0,75	294,30	8,81
t=166	Location 5	5	120,90	59,25	1,46	0,98	12,50	0,004	1,46	75,59	2,96	1,46	225,36	8,08
t=166	Location 5	15	212,37	185,19	9,57	1,26	11,60	0,002	2,55	131,58	2,49	0,95	248,92	6,83
t=196	Location 1	5												
t=196	Location 1	15												
t=196	Location 2	5	114,82	10,56	18,01	1,23	3,39	0,004	1,90	79,16	3,98	0,67	236,42	2,65
t=196	Location 2	15												
t=196	Location 3	5	149,93	31,42	11,52	1,37	11,62	0,003	1,32	95,64	4,36	0,54	260,56	7,57
t=196	Location 3	15	87,15	93,34	10,67	1,76	16,98	0,002	1,70	109,77	2,33	0,69	303,50	3,24
t=196	Location 4	5	167,18	35,07	11,09	1,92	12,84	0,002	1,32	98,36	3,92	0,68	349,52	9,54
t=196	Location 3	15	109,45	115,55	10,54	1,33	11,11	0,007	1,32	106,88	2,64	0,62	375,49	7,40
t=196	Location 5	5	152,82	61,70	13,17	1,55	15,84	0,004	1,99	86,71	4,54	0,51	333,63	10,26
t=196	Location 5	15												

3.4 Trace Element data analysis

Table 3.3: Comparison of significant P-values based on location, time, depth. GLM test was done on trace elements and tested for correlations between time, locations and depth on the trace element levels measured in the mussels. Interaction between location and time, location and depth and time and depth was also analysed. Statistically significant results are marked in **bold**. F values and df values are included in appendix 3.

	Location (loc)	Time	Depth	Loc*Time	Loc*Depth	Time*Depth
Ca	0.517	0.221	0.936	0.343	0.143	0.353
K	0.2888	0.1966	0.5466	0.0815	0.2712	**0.0417**
Mn	**0.000406**	**2.36e-06**	0.381311	**0.001475**	0.791168	**0.002519**
Mg	0.7811	**0.0443**	0.6065	0.1382	0.5143	0.1003
Na	0.5421	0.3797	0.8402	0.1854	0.6307	0.0904
P	0.9769	0.0563	0.6059	0.4029	0.9534	0.6398
Fe	**0.004269**	**0.000589**	0.110137	**0.015570**	0.117072	**0.023882**
Zn	**0.02813**	**3.98e-05**	**0.00116**	0.26769	0.06995	0.03571
Sr	0.3777	0.0684	0.7660	0.1274	0.7589	0.0818
Cu	0.945	0.675	0.502	0.356	0.608	0.491
Ti	**0.0257**	**0.0193**	0.8784	0.1876	0.2452	0.1288
Pb	0.848	0.560	0.485	0.857	0.731	0.430
Cr	0.18099	**0.00108**	0.43109	0.36444	0.08908	0.22750
Al	0.4796	**0.0357**	0.2814	0.2323	0.5310	0.5924
Ni	**0.0265**	0.5354	0.8072	**0.0352**	0.1628	0.1559
As	0.21716	**0.00119**	0.49167	0.40488	0.33050	0.50064
Cd	0.127	0.132	0.795	0.330	0.171	0.613
Hg	0.427	0.325	0.645	0.642	0.131	0.787

Statistical analysis was done to test the two-way interactions for correlations between time, locations and depth on the trace element levels measured in the mussels. No significant results were noted on the levels measured in mussels transplanted to the city fjord in Flekkefjord for trace elements Hg, Pb, Cd, Cu, Sr, Ca, K, Na and P (Table 3.3).

Independently of sampling time and depth, manganese levels significantly differed among the sampling locations. Manganese levels measured in L1 were lower than those measured in mussels from L4 ($P = 0.005$ and L5 ($P = 0.002$), while L2 levels were significantly lower than those measured in mussels from L3 ($P = 0.041$), L4 ($P = 0.003$) and L5 ($P = 0.001$). No differences were observed among L3, L4 and L5 levels of manganese in the mussels. An effect was observed with time, with an increase in manganese levels measured in mussels at t = 0

compared to those at t = 30 and t = 135 ($P < 0.001$ in both samples), independently of sampling site and depth. However, a decrease in manganese levels was measured at t = 166 days and t = 196 days compared to t = 135 days ($P < 0.002$ in both samples). In addition, manganese levels measured in mussels transplanted in L3, L4 and L5 at t = 135 days were higher than those measured in the same sites at previous time points ($P < 0.01$ in all the cases).

Similar results as manganese were obtained for iron levels. Levels measured in mussels transplanted in L2 had significantly lower concentration from those of all the other sampling sites ($P < 0.027$ in all the cases), independently of sampling time and depth. Overall, Fe levels measured in mussels at t = 135 days and t=196 days were significantly higher in concentration from those measures at t = 0 day ($P < 0.030$ in both the cases) and t = 30 days ($P < 0.002$ in both the cases). Despite significant site and time interaction, no clear trend was noted.

Overall, Zinc levels measured in mussels transplanted in L5 were significantly higher than those measured in L2 ($P = 0.027$) but did not differ compared to the other sampling sites. Zinc levels measured at t = 0 days significantly differed from those recorded in the other samples ($P < 0.00446$ in all the cases), independently of sampling time and depth. However, the levels of Zinc measured in mussels transplanted at 15 m depth were higher than those measured in mussels transplanted at 5 m depth ($P = 0.001$), independently of sampling site and time.

Titanium levels measured in mussels transplanted in L2 were significantly lower than those measured in L4 ($P = 0.023$) and L5 ($P = 0.050$) but did not differ from the mussels measured in other sampling sites. In addition, titanium levels measured at t = 135 days were significantly higher than those recorded at t = 30 days ($P = 0.010$), independently of sampling site and depth.

Overall, Cr levels measured in transplanted mussels at t = 135 days, were significantly higher compared to those measured at t = 0 ($P = 0.0035$) and t = 30 days ($P = 0.001$), but were significantly lower than those measured at t = 166 ($P = 0.005$) and t = 196 days ($P = 0.035$), independently of sampling site and depth.

A significant effect of sampling time on Mg, Cr, Al, Ni and As was noted, independently of sampling site and depth. Mg levels measured in transplanted mussels at t = 0 days were significantly lower than those at t = 30 days ($P = 0.043$), but no other differences in comparisons occurred. Al levels measured in transplanted mussels at t = 166 days were

significantly higher than those measured at t = 30 days (P = 0.054), independently of sampling site and depth. A similar result was obtained also for As (P = 0.009), while As levels measured at t = 0 were significantly different compared to those at t = 30 (P = 0.0066) and t = 166 days (P = 0.0138), independently of sampling site and depth. Levels of nickel measured in mussels transplanted in L2 were significantly lower from those recorded in L4 and L5 ($P < 0.05$ in both the cases), independently of sampling time and depth. Despite of significant site and time interaction, no clear trend was noted.

3.5. Organic contaminants

Data from analyses of the organic contaminant samples from 2018 were published by Parolini et al. (2020). Because the analyses of the samples from 2019 were not finished due to the COVID-19 pandemic's outbreak in Northern Italy, the results are discussed also in this thesis. For the sake of the thesis completion, the methods and preparation used to sample and prepare the mussels for analyses of the contaminants are included. Briefly, Parolini et al. (2020) found that the levels of contamination at t=0 was generally low, and the OCPs and OPs were low for the whole period. PCBs and PAHs were detected in 90%-100% of the 2018 samples from t=30 to t=196 days. PCB concentrations were much higher at 15 m depth compared to 5 m depth, and the concentration increased with time. PAH concentration followed the same pattern, particularly with Benzo(ß)fluoranthene and benzo(a)pyrene, however these did not differ from 5 m and 15 m depth (Parolini et al. 2020).

4. Discussion

The approach of this thesis was to monitor the concentrations of trace elements and organic contaminants in blue mussels in the city fjord of Flekkefjord. Elements which had significant results from the analysis were manganese, iron, zinc, titanium, chromium, aluminium and nickel. Trace element contaminants that were above the background level for contamination were arsenic, copper and lead. Several trace elements did not show significant results, which indicates that the levels were stable for the transplantation period. The legacy contaminants that were present in the fjord to a great degree were some PAHs and PCBs. According to the data, locations 3, 4 and 5 seemed more contaminated than locations 1 and 2.

4.1 Content of trace elements in mussels

The biomonitoring initiative of this thesis was ongoing in parallel with restoration activities to cover contaminated sediments n the fjord. In September of 2018, when the dredging actives had been going on for three months, there was an incident on the industrial site (location 5; Fig. 2.1) which resulted in a major slide of rocks and sand into the fjord. This disturbed the sediment close to locations used in the project. Because this happened while in the monitoring process, right before t=135, this caused some of the results from surrounding locations to be affected. This may also have affected the survival of the mussels in later samples, because of the toxins coming from the sediment and the upwhirl. The implications of the slide may be most prominent in the mussel samples representing Mn and Al (Fig. 3.2; Fig. 3.5). For all samples, some contamination was detected which was expected due to previous analyses (Haker, 2011; Misund, 2012) as well as the general presence of trace elements in the natural ecosystem. High concentrations at 15 m may indicate that the concentrations are caused by leaking from sediments where trace elements are already natural and present in the ecosystem (Richir & Gobert, 2016). As of now trace elements presented no significant indication that the 15 m samples were decreasing compared to the 5 m samples (Table 3.3). This is important to monitor further due to the dredging efforts.

High and fluctuating levels of copper could be due to leakage of contaminated sediments which contains fragments of old ship production. A study done in Bergen, a city on the west coast of Norway, in 2004 (Airas et al., 2004) indicated that while the copper contamination in general was low, the areas with known contamination from shipping had slightly higher levels. While

in the Flekkefjord study there were no significant effects, there could still be some fragments that affected the concentration. In the same Bergen study there were similarly high levels of zinc in areas with heavy shipping traffic, particularly in 15 m samples closer to the sediment. This is very likely caused by the same old shipping and boating traffic.

A high level of arsenic were found in location 1 during t=30 where values increased higher than the other locations (Fig 3.8). Arsenic had significant values depending on time, so this element could be affected by seasonality to some degree. Unfortunately, since arsenic had a higher uptake during t=30 and t=166 which is samples from two different seasons this could be difficult to determine. However there is knowledge that mussel species may filter arsenic to a larger degree during winter and spring months due to less nutrients available (Klarić et al., 2004). Copper, lead and arsenic was higher than other trace elements, albeit not to an immediate worrisome degree. These contaminants are all in the "good" category, according to (SFT, 2011). This category indicates that exposure should not be a concern as long as the levels remain this low, however there are clear signs of the trace elements presents in the ecosystem. Iron levels were significantly lower for location 2 than for any other location, and levels t=135 days and t=196 days were significantly higher than during the other days.

Chromium levels reported at t=135 days were significantly higher compared to earlier samples and they were significantly lower than those reported in the final two samples meaning something have triggered chromium to be released at this time. In a study in Farsund (Øxnevad et al., 2018), a neighbor municipality, there was reported high levels of chromium in water samples that was believed to be coming from contaminated water circulated in the fjord. Haker (2011) stated that high levels of chromium were reported in blue mussels in the past but since then, levels have gone down to baseline levels. In the raw data the levels are found to be baseline, but the significant levels of the mussels during the landslide may indicate that there are still contaminants in the sediment.

Manganese became bioavailable during t=135 at 5m location 4. This may be due to manganese's ability to become bioavailable in hypoxic conditions (Ochs et al., 2020) since in previous tests of water and sediment, poor oxygen conditions were found, which leads to hypoxia (Haker, 2011) . An overexposure of Mn has been reported to cause severe effects on some species nervous system and has caused damage to the immune system of lobsters (Ochs et al., 2020). It is still unclear what the source of manganese is, but it is an essential element

that is used for various forms of industry, so it is likely that it originates from industry or from the dredging process since we see that the significant results of manganese are all high for locations 3, 4 and 5 which are the locations close to where the slide-out occurred. The samples that followed then decreased in concentration.

In Schøyen et al. (2017) the innermost harbour location in Kristiansand had high levels of nickel concentration due to a nickel processing plant that was located in the area and the area being an active shipping location. In the Flekkefjord study higher concentrations of nickel were also found in the inner fjord close to areas that has been contaminated from the shipping industry. Nickel measured in water samples in the neighbouring fjord (Øxnevad et al., 2018) was suggested to be due to currents, which may also be a possibility due to the significant results being independent of depth.

Titanium concentration was significantly high in locations 4 and 5. It was also reported that this trace element had high levels at t=135. This could be due to the slide-out but this is unclear. Why the titanium levels are high in these areas are unknown since there had not been much reported contamination of this element in the past. It was also difficult to find comparative data on titanium from similar studies in the area.

4.2 Content of legacy contaminants in mussels

The data discussed below are from Parolini et al. (2020). In the paper, organic samples from 2018 presents results with PCBs and PAHs. Levels of OCPs, OPs, PBDEs and PFASs were either not detected or had levels that were negligible in relation to this project. PCBs and PAHs levels were low before the restoration efforts began, however, after t=166 at location 3, 4 and 5 there was an increase in concentration (Fig 4.1). In Schøyen et al. (2017), PCB-7 exceeded the environmental quality standards (EQS) in all stations, and the same is true in the mussel samples in this study. The levels of PCB demonstrated can be a serious risk for the health of mussel predators and, for the most part they were at the equal level of the mussels monitored in Schøyen et al. (2017). This level was above the limits of EU health regulations for food safety (Parolini et al., 2020). Another worry with this high level of PCB is the dangers of secondary predators carrying the contaminants further up in the food web, which can cause

serious health and safety concerns. Doing further tracking of contamination here is vital to ensure public health before consuming local mussels.

It is also worth noting that the levels of four PAHs in locations 3 and 4 exceeded the threshold at t = 196 days, especially for benzo(a)pyrene which exceeded threshold for all samples. This can be because PAH have a tendency to build up in concentration with exposure to contaminants instead of flushing it out. Nonetheless, it may be important to measure native mussels to have a clear overview over the contaminant situation.

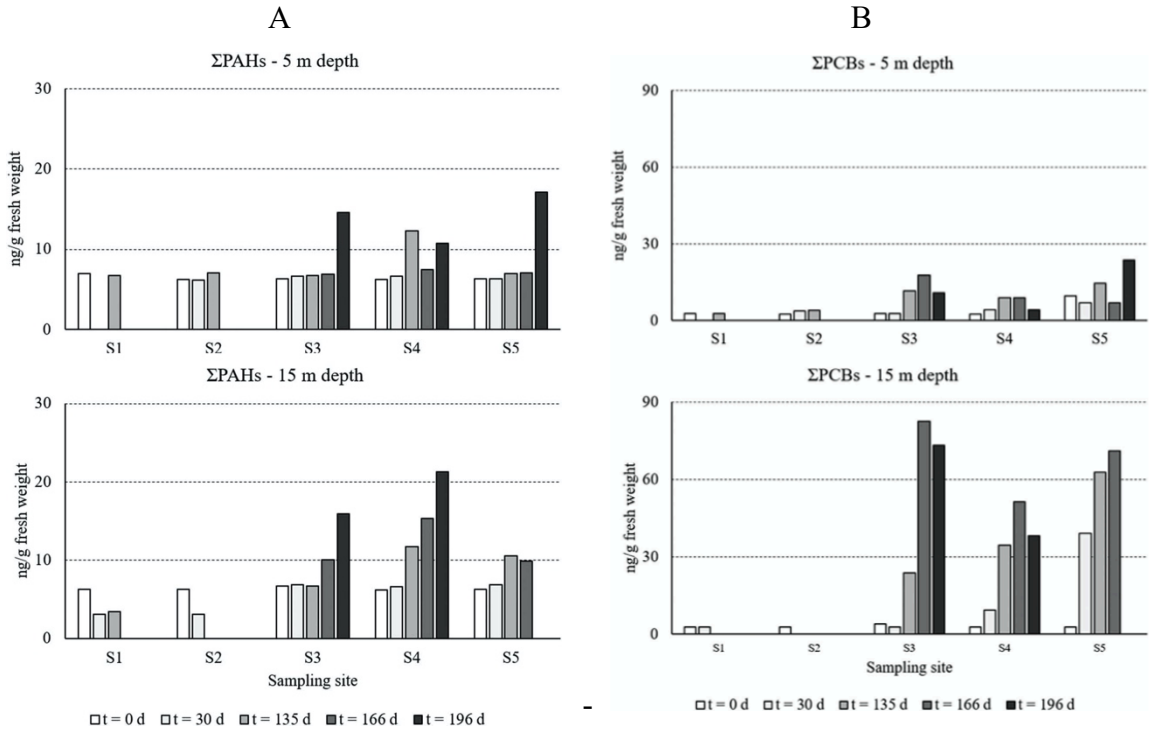

Figure 4.1: ∑PAHs (A) and ∑PCBs (B) contaminants measured in 2018. 5m measurements is on top, while 15m measurements is at the bottom. Figures and data presented by Parolini et al. (2020).

In the 15m depth samples, however, there is a clear difference between the monitoring depths (Fig 4.1). The concentration here was higher than the Kristiansand harbour, which was expected due to previous monitoring studies on sediment in these areas that showed high concentrations (Arve Misund, 2014; Haker, 2011). This paper also showed that the PCB congeners measured at 5m depth were low-chlorinated congeners while the mussels measured at 15m depth were with high-chlorinated congeners. This could indicate that certain types of PCBs may be bioavailable at different depths.

It is predicted that the legacy contaminants will decline. This is mainly because the sedimentation process from the dredging efforts will cause the contaminants to be removed from the sediment, which in turn reduces the bioavailability. Nonetheless, of the contaminants that are the cause for worry is the PCBs and PAHs which both had increased in value in the final samplings as well as being far above the recommended limit for several of the locations.

4.3 Differences between locations

Lead in location 1 fluctuated between values while remaining fairly high together with some other trace elements. There were, however, no significant values of any trace elements. It is important to study this location further to ensure the contaminants remain low considering this was a reference site and the contaminants were expected to be at baseline levels. Location 2 had generally high levels of copper, arsenic and lead but no significant values of any of these.

Location 3 was one of the areas where there was an expectation of a higher level of copper due to previous background studies (Haker, 2011; Misund, 2012) and high activity around the location in the past. However, this location did not show any significant values of this element (Table 3.3). This location had one of the highest measured levels of PCBs, especially in the latest samples. It also had a growing level of PAHs. There was also a significant result of manganese where the measures reported were significantly higher than those reported at locations 1 and 2. The sample t = 135 was higher than those reported in different samples (Fig 3.2).

In location 4 the concentration to note is manganese which had a very high concentration, which was significantly higher than the other locations, especially after the slide-out. Location 4 also had significantly high levels of nickel compared to location 2, and it had high levels of contamination from PCBs and some PAHs including in the last samples which is a worrying trend. Therefore, according to this data, this location is considered to be one of the highest contaminated areas.

Location 5 had high levels of copper, which also matched well with sediment samples where they measured high levels (Haker, 2011; Misund, 2012). This location had significant results of manganese where the measures reported was significantly higher than those reported at L1 and L2, especially after the slide-out. This location also had high levels of lead at the 5m depth for the final sample at t=196 which could indicate a rise in concentration. High levels of lead

can be caused by constant urban run-off from roads (Airas et al., 2004) which can be a cause for this since this location is close to roads and houses. Location 5 had the highest levels of PCBs, particularly just after the slide-out, and high levels of zinc and nickel.

4.4 Limitations of research / evaluation of method

A limitation concerning the samples is that in one of the copper samples (location 3, sample t=0 (Fig 3.1)) the measurement is almost twice as high as the trend of the rest of the samples. This could be because of the solution being applied twice, which would give a higher measurement. While copper has an increase in later samples at t=166 and t=196 the concentration is never as high as in the first sample meaning that since the trend is decreasing. This is also an interesting find since in the paper written by Schøyen et al. (2017) they have documented the strange contaminant level of copper in one of their first samples. This may be a coincidence but they have also received their mussels from the same farm as in this project so this may be an error from the manufacturers.

The mussel death which caused less samples from certain locations has also affected the complete overview of the location. This, however, did not decrease the power of the dataset since the data that exists still contain much information since there is still a good total overview of the locations and contaminants. This issue could be solved by reinforcing the mussel cages in the future or having the cages further from the ground to hinder predators, but this will also change how the project is operated. All in all, the project was well researched, and while some limitations were expected, they did not cause the final product to have less value.

Schøyen et al (2017) discusses that a limit to their study being seasonal fluctuations whereas measuring mussels at the location where the mussels originated from would be a way to assess the influence of seasonal fluctuations. This is equally a limit to this study. Mussels are biological creatures and their digestive system may fluctuate based on season and temperature which also varies how much they filter. Other abiotic factors such as salinity, primary production and other seasonal cycles could also potentially affect the mussels (Franco et al., 2015). The reasons for this not being performed in this study is simply because of time and costs, but it is something to be considered for the future of this project.

4.5 Passive biomonitoring

An important factor for having a total overview of the fjord for the sake of evaluating the risk for human health is to monitor the species that are consumed locally from the fjord. This is also related to the aquaculture industries that are close to the area. This form of monitoring is passive biomonitoring where the target species are natural local species in the ecosystem such as crabs, fish, mussels, other bivalves and kelp that are collected directly from the environment (Besse et al., 2012). Passive monitoring will give a good overview over the natural occurring contamination that exist for the local species that are resident in the fjord at all times. Monitoring done with fish liver or fillet has been proven to show different results compared to in mussels (Nesto et al., 2007) as fish feed differently and have a different living pattern than mussels. However, when considering the use of passive biomonitoring there are other factors that should be considered before doing such tests. Biotic factors that are easier to control with active biomonitoring such as age, size and soft tissue weight become important factors to consider when sampling (Franco et al., 2015) as well as when doing analysis. As suggested by Schøyen et al. (2017) further studies that can be used to compare active and passive biomonitoring are needed to broaden our perspective on how we can do biomonitoring in the most effective way.

5. Concluding remarks

The results from this thesis contribute to a better understanding of how we can monitor the contaminants of fjords in the southern region of Agder. The study is a continuation of an ongoing effort of mapping out contaminants and can help to keep the fjord ecosystems healthy. This thesis shows that the general values of trace elements is below the threshold of immediate concern for human and biota health, however this will be further examined together with the finalisation of the dredging efforts and the results from 2019 which is the next stage of the project. Data for 2019 has already been collected and is being processed for analysis. There is an expectation for these samples that the contamination will be lower than in the 2018 samples which will follow the dredging operations and the capping of contaminants. Some trace elements discussed were higher than what may be desired for long term health reasons, and these should be focused on for the next group of samples. PCBs and some PAHs had concentrations that are too high which could potentially be a health risk. PCBs are toxic for both humans and for biota and biodiversity when consumed in large quantities. Efforts to measure native mussels should be considered in order to give a better overview of the health risks. Other studies in the area with passive monitoring on local fish, mussels and crabs is highly recommended since it will give an insight on the impact of contamination on local biota which benefits the residents along the fjord.

This project is an important contribution towards creating a proper overview over how contaminants may affect and accumulate in biological species in the city fjord of Flekkefjord, as well as the rate of how contaminants bioaccumulate differently from how they accumulate in sediments. If it is only known how much is freed in the water or in the sediments, there is a risk of having an incomplete overview and understanding of how humans can be affected by contaminants. Having a direct understanding on how contaminants may accumulate in the biota that is used, consumed and cultivated along the fjord strengthens the understanding of how connected the environment is with public health and can further the interest and protection of the local environment.

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Appendix

App 1: Table of metal contamination content

App 2: Other trace elements

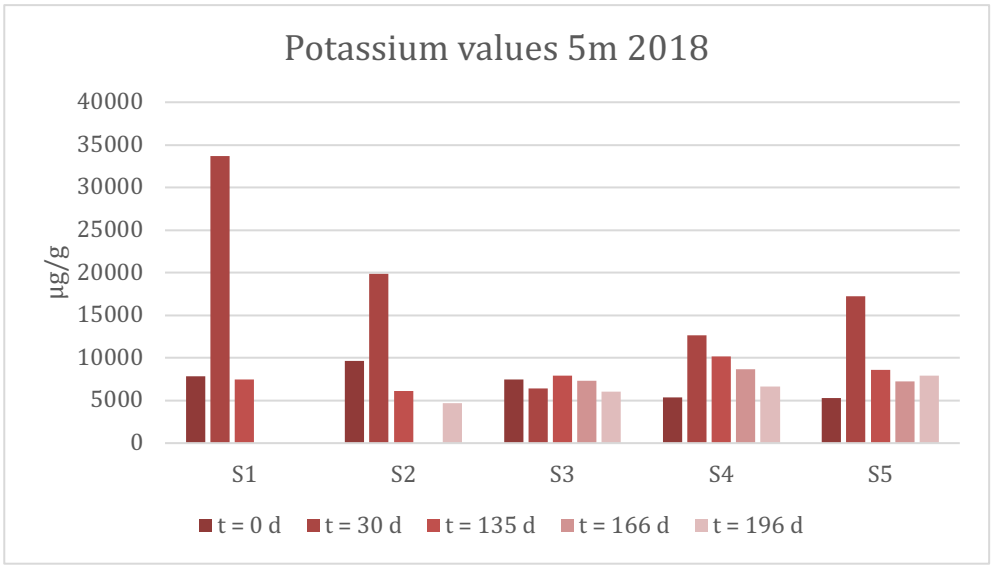
App 3: GLM analysis of data

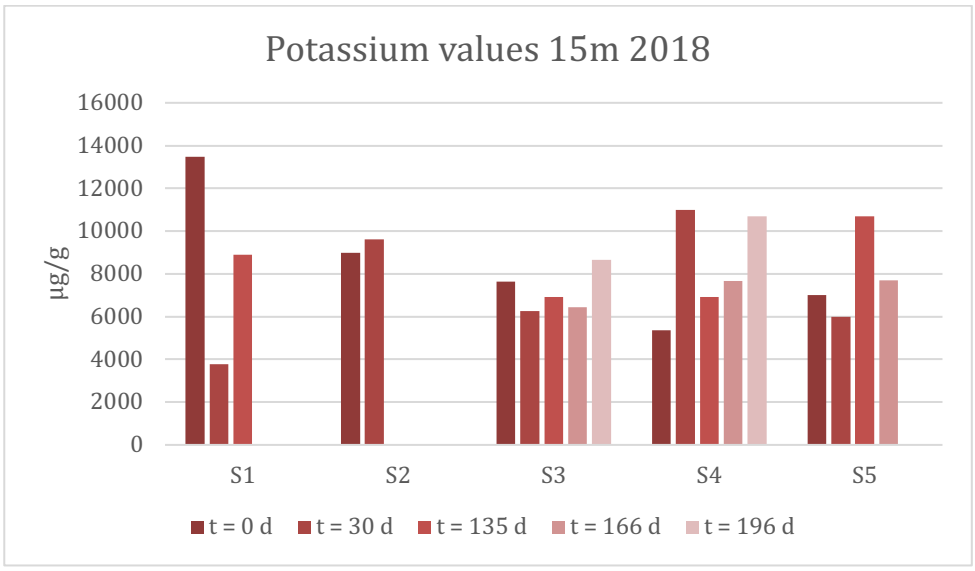
Appendix 1: Raw data for Trace Elements 2018.

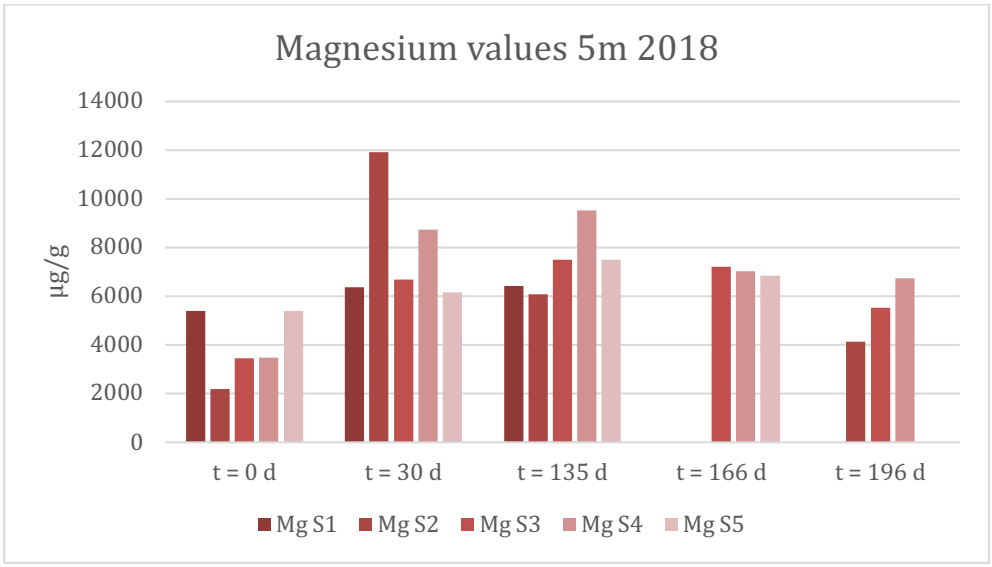
Units = mg/kg or µg/g

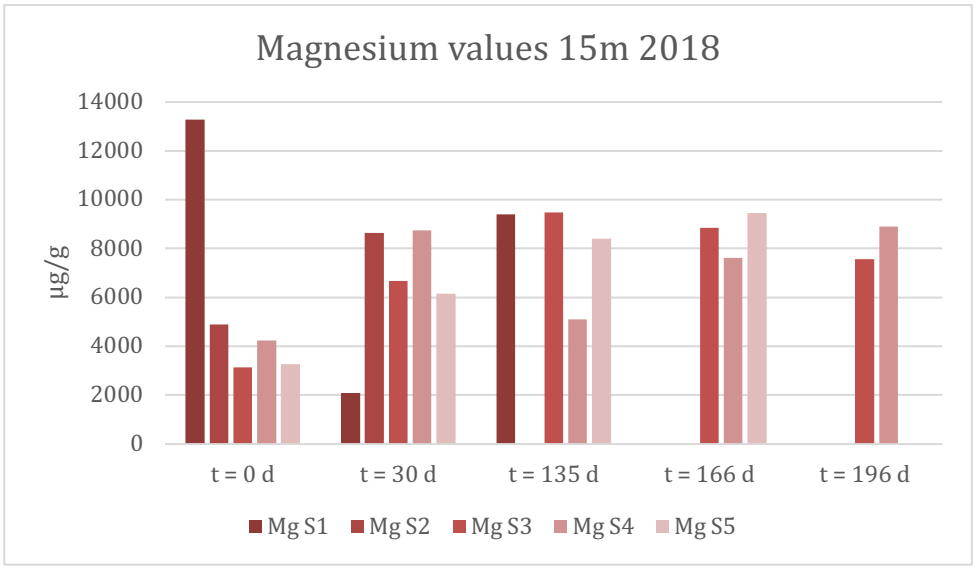
id	sampling ti	site	depth	Ca	K	Mg	Na	P	Fe	Zn	Sr	Cu	Mn	Ti	Pb	Cr	Al	Ni	As	Cd	Hg
1	control	s1	5	4681	8030	5396	21418	6141	345,48	66,25	44,04	13,36	12,94	8,41	3,72	1,04	916,84	0,19	10,28	1,02	0,003
2	control	s1	15	12139	13474	13267	49768	11233	388,26	97,31	74,32	15,50	13,84	11,15	4,34	1,76	475,45	0,91	8,89	0,91	0,003
3	control	s2	5	2417	9647	2195	20383	5169	143,57	39,55	33,02	5,79	4,38	2,78	5,10	1,55	362,38	1,10	9,63	0,95	0,002
4	control	s2	15	12254	8981	4882	26741	4485	217,23	77,41	56,29	14,34	5,75	7,08	3,04	0,98	649,82	1,82	8,66	0,89	0,002
5	control	s3	5	2837	7469	3455	18649	3992	206,83	66,73	30,10	12,86	5,00	7,41	3,40	0,91	337,72	1,12	7,60	0,95	0,003
6	control	s3	15	12619	7639	3129	19171	4140	180,28	89,29		30,00	7,83	4,24	4,23	1,92	221,39	1,13	8,53	0,90	0,004
7	control	s4	5	4570	5399	3474	13481	4670	202,72	65,38	35,73	12,81	5,09	5,80	3,36	1,56	733,28	2,20	7,85	0,93	0,002
8	control	s4	15	7439	5370	4241	16178	4870	205,89	61,12	50,68	11,04	8,02	5,49	2,79	1,09	1063,06	2,02	8,55	0,80	0,002
9	control	s5	5	35843	5270	5390	17028	5209	216,57	80,97	0,00	12,59	12,38	5,53	3,70	1,48	533,22	2,35	8,68	0,80	0,003
10	control	s5	15	8157	7020	3270	15359	4763	184,30	88,64	43,26	14,51	5,46	5,79	3,71	1,34	378,41	1,19	8,46	1,07	0,002
11	cmp1	s1	5	4741	7834	6360	21168	6926	120,76	32,62	54,36	3,88	5,01	2,44	4,14	1,22	71,76	0,93	11,98	0,96	0,003
12	cmp1	s1	15	2238	3789	2096	8249	2899	188,95	34,86	22,97	7,68	6,41	6,07	2,18	0,53	102,86	1,57	14,69	1,56	0,006
13	cmp1	s2	5	3480	19844	11915	59118	5758	85,72	62,11		29,76	4,93	1,52	2,15	0,96	89,02	0,44	12,36	0,74	0,000
14	cmp1	s2	15	4079	9627	8643	36297	6971	166,20	52,60	41,87	8,71	5,23	4,75	2,40	0,88	172,74	1,53	13,81	0,70	0,000
15	cmp1	s3	5	3704	6418	6137	18182	6788	161,77	29,71	63,48	4,09	5,61	3,45	3,85	1,49	95,37	1,24	12,78	0,86	0,001
16	cmp1	s3	15	4412	6260	6682	22568	7137	319,09	56,37	45,23	7,33	8,07	7,91	2,80	1,73	148,24	1,41	9,48	0,75	0,004
17	cmp1	s4	5	4362	12669	10367	40609	6640	209,92	36,86	60,51	5,32	6,63	5,17	3,20	1,47	130,02	1,33	14,32	0,84	0,002
18	cmp1	s4	15	4484	11000	8736	32483	6964	175,83	29,50	34,83	4,09	8,19	3,46	2,11	1,24	90,19	0,75	10,28	0,57	0,003
19	cmp1	s5	5	9858	17250	13328	52369	5854	267,91	79,00	100,18	15,96	7,88	8,11	2,92	1,16	69,21	2,62	14,00	0,87	0,005
20	cmp1	s5	15	7613	5988	6149	14732	7568	290,67	59,40	66,25	14,49	9,65	6,96	4,06	1,60	110,71	1,75	12,16	1,14	0,002
21	cmp2	s1	5	33676	7471	6433	25820	5730	318,94	82,18	106,70	13,50	34,50	9,50	4,23	2,68	160,78	2,09	7,55	0,57	0,003
22	cmp2	s1	15	12129	8898	9394	32886	6113	290,98	108,83	71,36	11,47	14,77	8,03	2,52	2,45	151,35	1,45	9,41	1,08	0,013
23	cmp2	s2	5	5352	6097	6083	20224	6046	244,57	88,74	43,00	10,46	23,82	6,59	2,83	2,01	115,33	0,37	8,49	0,94	0,005
24	cmp2	s2	15																		
25	cmp2	s3	5	16532	7937	7488	21517	7226	314,65	72,34	139,13	19,17	175,89	9,72	4,68	1,96	512,28	3,49	11,27	0,97	0,010
26	cmp2	s3	15	21689	6929	9491	23881	7022	242,86	110,59		6,09	130,16	5,67	3,54	2,17	258,80	2,22	8,25	0,88	0,003
27	cmp2	s4	5	15057	10165	9511	29450	5804	379,17	106,58	103,98	17,50	371,62	13,49	3,29	2,33	641,99	1,71	9,63	1,11	0,001
28	cmp2	s4	15	9072	6906	5100	13728	7279	317,28	74,76	65,23	17,64	188,03	12,12	4,83	2,13	395,62	0,64	7,33	0,70	0,002
29	cmp2	s5	5	6127	8631	7486	25632	6275	324,62	90,20	58,64	6,11	284,91	9,48	3,05	1,65	516,42	1,58	8,71	0,83	0,006
30	cmp2	s5	15	4533	10681	8393	35421	6016	231,65	114,85	50,13	13,39	208,43	6,80	3,98	1,53	228,39	1,59	9,35	0,78	0,000
31	cmp3	s1	5																		
32	cmp3	s1	15																		
33	cmp3	s2	5																		
34	cmp3	s2	15																		
35	cmp3	s3	5	4587	7284	7219	24273	6863	161,33	37,42	46,00	5,85	35,18	3,27	2,86	0,86	3276,80	1,81	9,14	0,77	0,005
36	cmp3	s3	15	33433	6442	8850	16271	7519	357,51	99,05		20,00	84,36	10,41	6,08	2,03	814,95	2,34	7,43	0,63	0,004
37	cmp3	s4	5	6314	8685	7029	23555	6465	274,18	74,35	54,98	14,66	31,56	8,39	3,34	1,14	389,88	1,72	9,94	0,84	0,002
38	cmp3	s4	15	14671	7666	7617	19263	8280	294,30	89,57	122,88	15,04	59,67	8,81	4,21	1,78	592,33	1,15	8,75	0,75	0,008
39	cmp3	s5	5	11003	7216	6853	25565	6354	225,36	75,59	57,31	12,50	59,25	8,08	2,96	0,98	120,90	1,46	1,46	1,46	0,004
40	cmp3	s5	15	4903	7706	9462	29604	7689	248,92	131,58	50,14	11,60	185,19	6,83	2,49	1,26	212,37	2,55	9,57	0,95	0,002
41	cmp4	s1	5																		
42	cmp4	s1	15																		
43	cmp4	s2	5	3647	4673	4137	14874	9199	236,42	79,16	37,40	3,39	10,56	2,65	3,98	1,23	114,82	1,90	18,01	0,67	0,004
44	cmp4	s2	15																		
45	cmp4	s3	5	4681	6045	5517	21149	9643	260,56	95,64	49,13	11,62	31,42	7,57	4,36	1,37	149,93	1,32	11,52	0,54	0,003
46	cmp4	s3	15	8124	8658	7555	29342	7056	303,50	109,77		16,98	93,34	3,24	2,33	1,76	87,15	1,70	10,67	0,69	0,002
47	cmp4	s4	5	18266	6618	6743	14025	7775	349,52	98,36		12,84	35,07	9,54	3,92	1,92	167,18	1,32	11,09	0,68	0,002
48	cmp4	s4	15	8679	10681	8916	37482	7126	375,49	106,88	65,15	11,11	115,55	7,40	2,64	1,33	109,45	1,32	10,54	0,62	0,007
49	cmp4	s5	5	13892	7890	7242	20806	7997	333,63	86,71	109,50	15,84	61,70	10,26	4,54	1,55	152,82	1,99	13,17	0,51	0,004
50	cmp4	s5	15																		

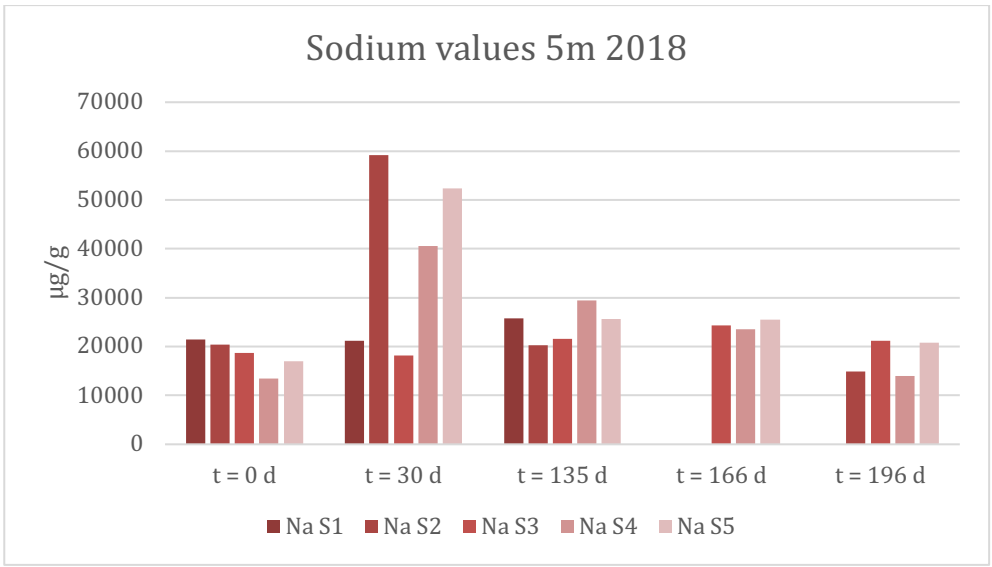
Appendix 2: Distribution of other trace elements, namely K, Mg, Na, P and Sr, found in samples at 5 m depth (left) and 15 m depth (right). Unit of measurement is mg/kg dw.

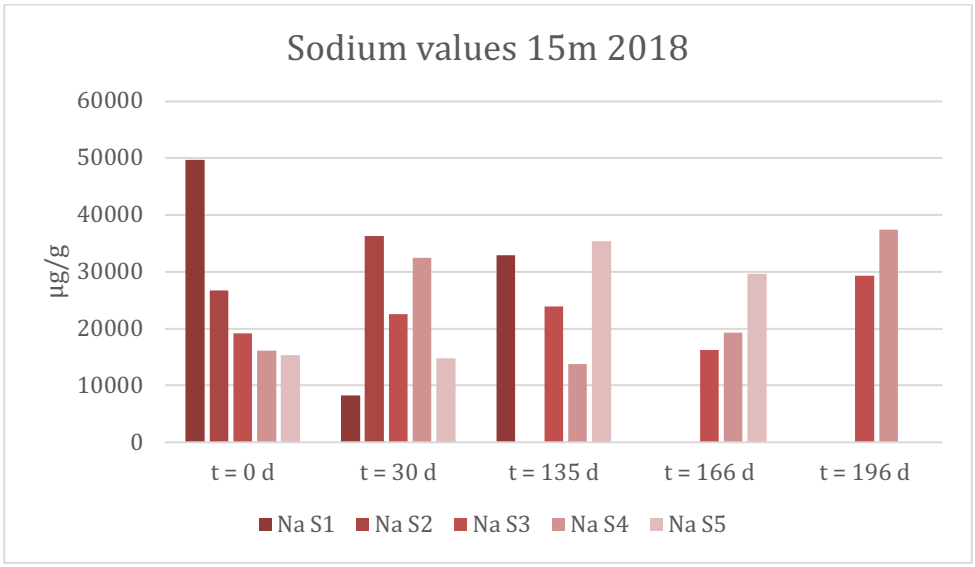


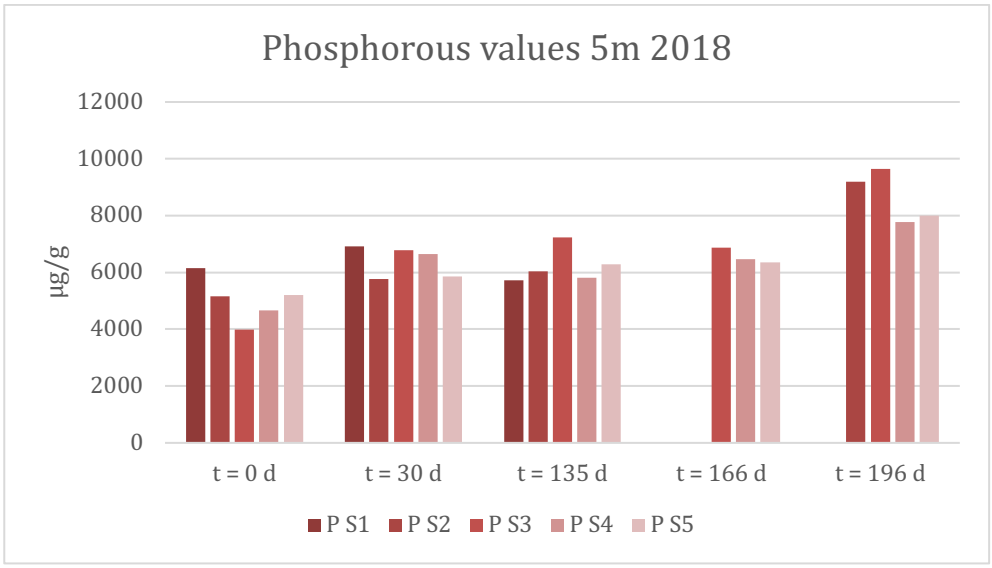


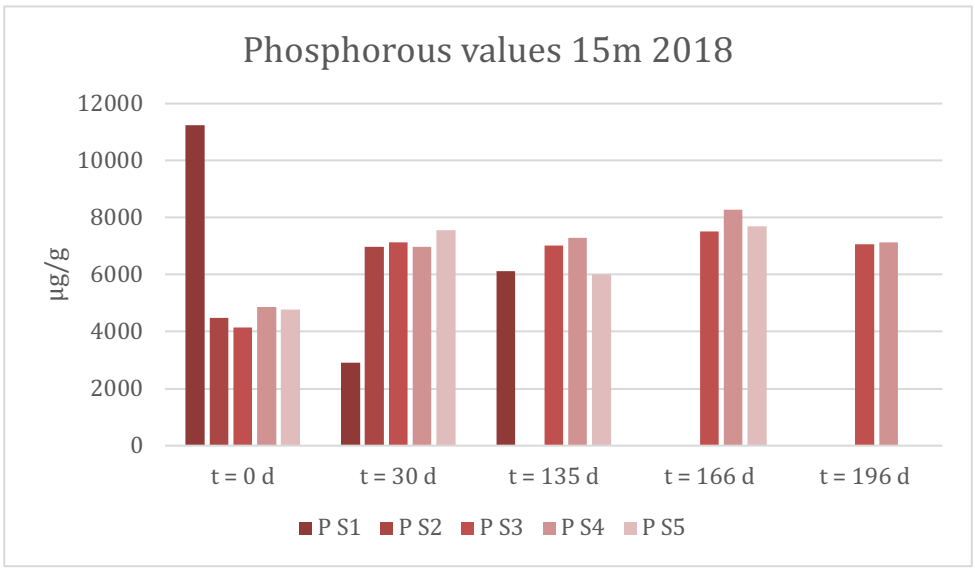

Magnesium values 5m 2018
14000
12000
10000
8000
6000
4000
2000
0
g/gtl
t = 0 d
t = 30 d
t = 135 d
t = 166 d
t = 196 d
Mg S1
Mg S2
Mg S3
Mg S4
Mg S5

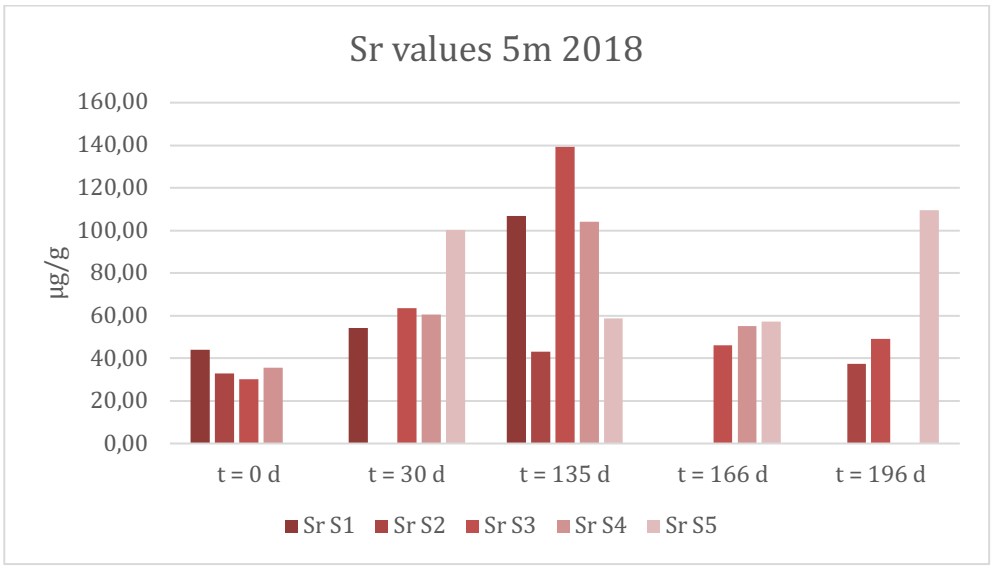

Magnesium values 15m 2018
14000
12000
10000
8000
6000
4000
2000
0
g/gtl
t = 0 d
t = 30 d
t = 135 d
t = 166 d
t = 196 d
Mg S1
Mg S2
Mg S3
Mg S4
Mg S5

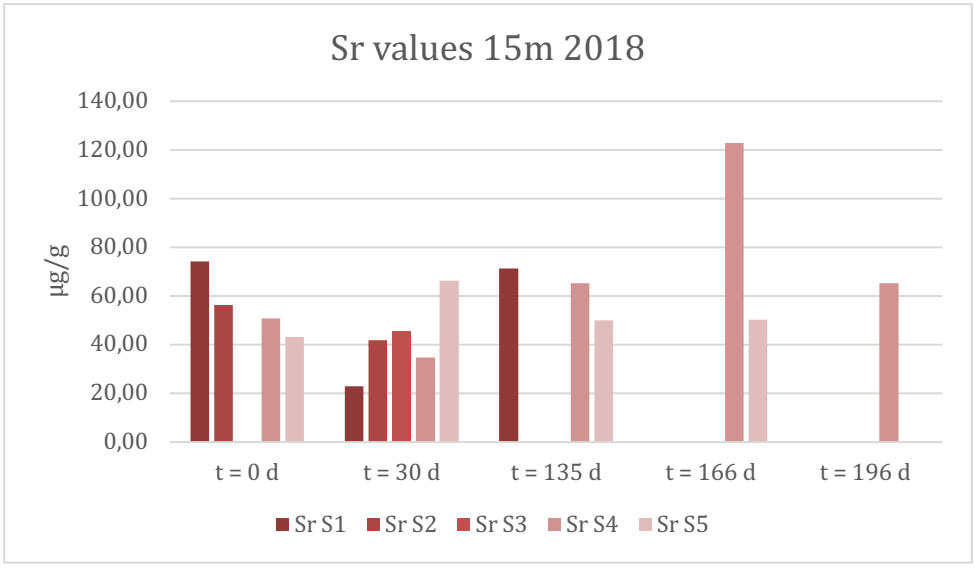

Sodium values 5m 2018
70000
60000
50000
40000
30000
20000
10000
0
g/gtl
t = 0 d
t = 30 d
t = 135 d
t = 166 d
t = 196 d
Na S1
Na S2
Na S3
Na S4
Na S5


Sodium values 15m 2018
60000
50000
40000
30000
20000
10000
0
µg/gd
t = 0 d
t = 30 d
t = 135 d
t = 166 d
t = 196 d
Na S1
Na S2
Na S3
Na S4
Na S5


Phosphorous values 5m 2018
12000
10000
8000
6000
4000
2000
0
µg/gd
t = 0 d
t = 30 d
t = 135 d
t = 166 d
t = 196 d
P S1
P S2
P S3
P S4
P S5


Phosphorous values 15m 2018
12000
10000
8000
6000
4000
2000
0
µg/gd
t = 0 d
t = 30 d
t = 135 d
t = 166 d
t = 196 d
P S1
P S2
P S3
P S4
P S5


Sr values 5m 2018
160,00
140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
µg/gtl
t = 0 d
t = 30 d
t = 135 d
t = 166 d
t = 196 d
Sr S1
Sr S2
Sr S3
Sr S4
Sr S5


Sr values 15m 2018
140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
µg/gtl
t = 0 d
t = 30 d
t = 135 d
t = 166 d
t = 196 d
Sr S1
Sr S2
Sr S3
Sr S4
Sr S5

Appendix 3 – GLM analysis of data

In columns it was reported typical statistical indicators for General Linear Models (GLM), namely F = F distribution; df = degrees of freedom; P = P-value representing the likelihood of observing significant results outside the mean, assuming that the null hypothesis is correct. In the df column we reported the values of degrees of freedom at the numerator and denominator, respectively.

	F	df	P
Ca			
Location	0.866	4,40	0.517
Time	1.725	4,40	0.221
Depth	0.007	1,40	0.936
Location*Time	1.301	13,40	0.343
Location*Depth	2.195	4,40	0.143
Time*Depth	1.245	4,40	0.353
K			
Location	1.447	4,40	0.2888
Time	1.847	4,40	0.1966
Depth	0.389	1,40	0.5466
Location*Time	2.445	13,40	0.0815
Location*Depth	1.511	4,40	0.2712
Time*Depth	3.726	4,40	**0.0417**
Mn			
Location	14.108	4,40	**0.000406**
Time	44.806	4,40	**2.36e-06**
Depth	0.839	1,40	0.381311
Location*Time	7.583	13,40	**0.001475**
Location*Depth	0.420	4,40	0.791168
Time*Depth	8.870	4,40	**0.002519**
Mg			
Location	0.434	4,40	0.7811
Time	3.640	4,40	**0.0443**
Depth	0.283	1,40	0.6065
Location*Time	2.002	13,40	0.1382
Location*Depth	0.871	4,40	0.5143
Time*Depth	2.602	4,40	0.1003
Na			
Location	0.818	4,40	0.5421
Time	1.172	4,40	0.3797
Depth	0.043	1,40	0.8402
Location*Time	1.769	13,40	0.1854
Location*Depth	0.665	4,40	0.6307
Time*Depth	2.726	4,40	0.0904
P			

Location	0.108	4,40	0.9769
Time	3.321	4,40	0.0563
Depth	0.284	1,40	0.6059
Location*Time	1.181	13,40	0.4029
Location*Depth	0.161	4,40	0.9534
Time*Depth	0.650 0	4,40	0.6398
Fe			
Location	7.677	4,40	**0.004269**
Time	12.877	4,40	**0.000589**
Depth	3.073	1,40	0.110137
Location*Time	4.112	13,40	**0.015570**
Location*Depth	2.421	4,40	0.117072
Time*Depth	4.538	4,40	**0.023882**
Zn			
Location	4.291	4,40	**0.02813**
Time	24.196	4,40	**3.98e-05**
Depth	20.176	1,40	**0.00116**
Location*Time	1.488	13,40	0.26769
Location*Depth	3.042	4,40	0.06995
Time*Depth	3.943	4,40	0.03571
Sr			
Location	1.317	4,40	0.3777
Time	4.379	4,40	0.0684
Depth	0.099	1,40	0.7660
Location*Time	2.850	13,40	0.1274
Location*Depth	0.468	4,40	0.7589
Time*Depth	4.089	4,40	0.0818
Cu			
Location	0.177	4,40	0.945
Time	0.595	4,40	0.675
Depth	0.485	1,40	0.502
Location*Time	1.274	13,40	0.356
Location*Depth	0.702	4,40	0.608
Time*Depth	0.918	4,40	0.491
Ti			
Location	4.428	4,40	**0.0257**
Time	4.871	4,40	**0.0193**
Depth	0.025	1,40	0.8784
Location*Time	1.760	13,40	0.1876
Location*Depth	1.615	4,40	0.2452
Time*Depth	2.312	4,40	0.1288
Pb			
Location	0.336	4,40	0.848

Time	0.785	4,40	0.560
Depth	0.526	1,40	0.485
Location*Time	0.533	13,40	0.857
Location*Depth	0.509	4,40	0.731
Time*Depth	1.050	4,40	0.430
Cr			
Location	1.936	4,40	0.18099
Time	11.067	4,40	**0.00108**
Depth	0.673	1,40	0.43109
Location*Time	1.256	13,40	0.36444
Location*Depth	2.744	4,40	0.08908
Time*Depth	1.693	4,40	0.22750
Al			
Location	0.940	4,40	0.4796
Time	3.942	4,40	**0.0357**
Depth	1.296	1,40	0.2814
Location*Time	1.596	13,40	0.2323
Location*Depth	0.839	4,40	0.5310
Time*Depth	0.729	4,40	0.5924
Ni			
Location	4.382	4,40	**0.0265**
Time	0.831	4,40	0.5354
Depth	0.063	1,40	0.8072
Location*Time	3.228	13,40	**0.0352**
Location*Depth	2.051	4,40	0.1628
Time*Depth	2.098	4,40	0.1559
As			
Location	1.742	4,40	0.21716
Time	10.788	4,40	**0.00119**
Depth	0.510	1,40	0.49167
Location*Time	1.177	13,40	0.40488
Location*Depth	1.311	4,40	0.33050
Time*Depth	0.898	4,40	0.50064
Cd			
Location	2.327	4,40	0.127
Time	2.281	4,40	0.132
Depth	0.071	1,40	0.795
Location*Time	1.330	13,40	0.330
Location*Depth	1.997	4,40	0.171
Time*Depth	0.694	4,40	0.613
Hg			
Location	1.056	4,40	0.427
Time	1.329	4,40	0.325
Depth	0.225	1,40	0.645

Location*Time	0.815	13,40	0.642
Location*Depth	2.295	4,40	0.131
Time*Depth	0.425	4,40	0.787