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Author: Susanne Bøe	
Programme coordinator: Hannah Hondebrink Supervisor(s): Daniela M. Pampanin	
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PREFACE

The following thesis was written between August 2019 and July 2020 at the Department of Chemistry, Bioscience, and Environmental Engineering, University of Stavanger (UiS). The work was supervised by Professor Daniela M. Pampanin and funded by the Research Council of Norway and the National Research Foundation of South Africa through the South Africa-Norway Research Cooperation on Blue Economy, Climate Change, the Environment, and Sustainable Energy program (SANOCEAN) with project code 287516.

The thesis was unfortunately affected by the SARS-CoV-2 pandemic. Planned laboratory work was reduced due to the closing of the university facilities between March 9th and April 27th, 2020. The continuation of the laboratory work after April 27th aimed to complete ongoing analyses with limited access to laboratories and computer labs within restricted working hours. As a result, the list of target analytes was reduced from over seventy to ten compounds. Planned work on additional extraction methods was also canceled.

ABSTRACT

Active pharmaceutical ingredients (APIs) are increasingly occurring in nature. Their presence is posing a concern about antibiotic resistance in bacteria and other effects on the ecosystem, which are not yet fully understood. Norwegian pollution regulations do not require removal or monitoring of APIs and their transformation products in effluents discharged by wastewater treatment plants (WWTPs). Detection of such emerging contaminants is challenged by low substance concentrations, unknown properties, and underdeveloped methods for detecting the wide variety of APIs from many and diverse therapeutic classes. With the development of increasingly sensitive and selective analytical methods, such as mass spectrometry, detection methods are continuously improved.

This thesis aimed to optimize existing methods for the extraction and quantification of ten selected APIs from seven therapeutic classes:

- Acetaminophen (ACE)
- Atenolol (ATE)
- Atorvastatin (ATV)
- Caffeine (CAF)
- Carbamazepine (CBZ)
- Diclofenac (DCF)
- Ibuprofen (IBU)
- Naproxen (NAP)
- Sulfamethoxazole (SUL)
- Trimethoprim (TMP)

The present study reports the extraction methodology and detection of APIs in sediment and seawater samples collected around the biological WWTP of Stavanger, Sentralrenseanlegget Nord-Jæren (SNJ), as well as in samples of inlet and outlet water from the plant. After collection, sediment samples were freeze-dried and extracted by ultrasonication. Suspended particles were removed from seawater and treatment plant samples by filtration. All extracts and filtrates were further processed by solid-phase extraction and analyzed using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Results were compared to quantification data obtained by ultra-performance LC-MS/MS (UPLC-MS/MS).

The implemented methodology allowed trace amounts of APIs to be detected or quantified. There was a significant difference between the instruments used for analysis, except for CAF in outlet samples. The UPLC-MS/MS detected higher concentrations for all targeted APIs, except for IBU in assessed inlet samples. The differences are assumed to be related to the UPLC-MS/MS´s increased sensitivity compared to HPLC-MS/MS.

In assessed inlet samples, all targeted APIs except CBZ were detected. In outlet samples, all APIs were detected or quantified. The highest concentrations released from the SNJ through outlet water were for ACE, CAF, DCF, IBU and, TMP, respectively at 11.76±1.640, 775.1±1.863, 148.7±6.253, 180.9±16.34, and 250.4±11.77 ng/L.

CAF and DCF were detected below the limit of quantification (<LOQ) in seawater samples, herein also from samples collected from the reference station. ACE and CAF were detected above LOQ in at least one replicate of sediments collected from all marine stations. ACE was also quantified with a maximum concentration of 5.831 ng/g dry weight in one replicate of sediment samples from the marine station closest to the discharge site. No APIs were detected in sediment samples from coastal stations.

The present study demonstrated that all APIs were detectable in the SNJ outlet water; however, because many of the targets could not be detected in environmental samples, their fate remains unclear.

ACKNOWLEDGMENTS

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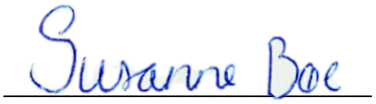

Susanne Bøe, 13.07.2020

ABBREVIATIONS

CE	Collision energy
CV	Cone voltage
CXP	Collision cell exit potential
DREAM	Dose-related Risk and Effects Assessment Model
EP	Entrance potential
ESI	Electrospray ionization
HPLC-MS/MS	High performance liquid chromatography – tandem mass spectrometry
IS	Internal standard
LOD	Limit of detection
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
m/z	Mass-to-charge ratio
OTC	Over the counter (also: non-prescriptive)
R_2	Coefficient of determination
RSD	Relative standard deviation
SD	Standard deviation
t_R	Retention time
TIC	Total ion chromatograms
UAE	Ultrasonic-assisted extraction
UPLC-MS/MS	Ultra-performance liquid chromatography – tandem mass spectrometry
WWTP	Wastewater treatment plant

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1. INTRODUCTION

1.1 OCCURRENCE OF PHARMACEUTICALS

Possible sources of active pharmaceutical ingredients (APIs) entering the aquatic environments includes leaching from agriculture, aquaculture, landfills, or wastewater treatment plant (WWTP) outfalls as a result of undigested or improperly discarded pharmaceuticals [1]–[3]. APIs detected in marine environments depend on the amount of product discarded related to sales amount and user-doses, and the compound´s behavior, for example, transport, ease of degradation, and other bioactive properties. From the attention given to pharmaceuticals and transformation products occurring in freshwater ecosystems, an increased understanding of their release and impact have been obtained [4]. Significantly less attention has been given to APIs entering coastal and marine ecosystems [5].

1.1.1 DISTRIBUTION OF PHARMACEUTICALS

Norway has an accurate reporting system for pharmaceuticals. Reports are available online at the Norwegian Prescription Databases (NorPD). User-doses are defined as average daily user-dose (DDD), according to "Guidelines for ATC classification and DDD assignment 2020" [6].

User-doses are summarized for selected pharmaceuticals in Norway over the period 2014-2018 in Figure 1, presented in the units DDD/1000 inhabitants/day (DID). High turnover of ibuprofen (IBU), diclofenac (DCF), naproxen (NAP), and acetaminophen (ACE) are possibly linked to their accessibility and application. These pharmaceuticals are sold through prescription and non-prescription purchases and are often used to relieve joint pains, inflammation, headaches, menstrual pain, and fevers [7], [8]. There was no significant change in total use of IBU from 2014 to 2018 (43.58 to 41.58 DID), while the total use of DCF decreased slightly (72.37 to 57.70 DID). The DID remained steady for NAP, from 16.77 to 17.69 DID, over the same period. ACE increased slightly regarding the total user-doses from 2014 to 2018 (80.67 to 105.27 DID). The prescribed amount reportedly increased as a treatment for chronic pain [9].

The use of carbamazepine (CBZ), an antiepileptic drug, decrease slightly, from 1.31 DID in 2014 to 1.03 DID in 2018. However, the overall use of antiepileptics was relatively steady in the same period. Beta-blocker atenolol (ATE) is an antihypertensive pharmaceutical used to lower blood pressure by preventing natural chemicals, such as epinephrine, from affecting the

heart and blood vessels [10]. The use of ATE was reportedly decreasing in the period 2014 to 2018, from 4.02 to 2.74 DID, respectively. A similar trend has been found for the total use of beta-blockers. Lipid regulator atorvastatin (ATV) was the most sold statin, a subgroup of cardiological drugs. User-doses have nearly tripled for the past ten years, and, from 2014 to 2018, the doses increased from 63.09 DID to 89.46 DID.

In 2012, it was decided nationally to set an action plan to prevent antibiotic resistance in health service by decreasing the use of antibiotics by 30% (doses) by 2020 [11]. For targeted antibiotics, a reduction in the use of trimethoprim (TMP) was from 0.46 DID in 2014 to 0.34 in 2018. The combination of SUL and TMP decreased slightly, from 0.41 to 0.53 DID.

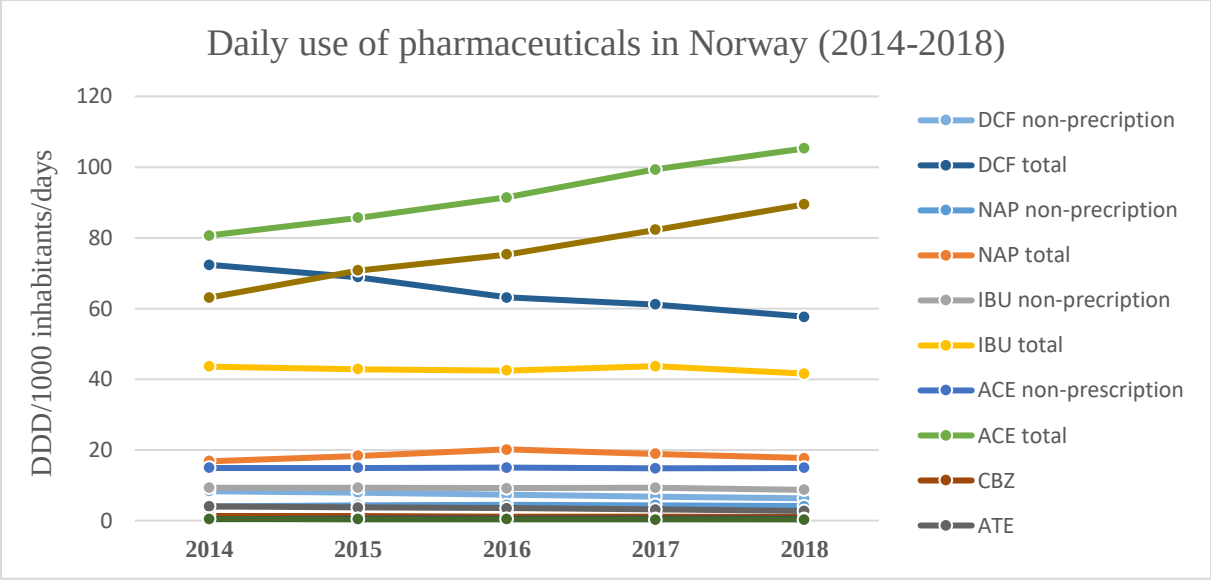

Figure 1 – Use of targeted pharmaceuticals in Norway (2014-2018).

User doses are reported in average daily user doses per 1000 inhabitants per day. Data are from NorPD [9].
ACE = acetaminophen, IBU = ibuprofen, DCF = diclofenac, CBZ = carbamazepine, TMP = trimethoprim, SUL = sulfamethoxazole, ATV = atorvastatin, NAP = naproxen, ATE = atenolol.

One common bioactive compound not mentioned by the NorPD is the natural stimulant found in various drinks and foodstuff: Caffeine (CAF). CAF is not officially regarded as a pharmaceutical. However, being an alkaloid, it has many analgesics, as well as addictive properties [12]–[14]. Natural sources of CAF are reported by Matvareportalen (2019), including coffee (50 - 60 mg/dL), tea (25 mg/dL) and carbonated cola drinks (15 mg per dL) [15]. Norway has one of the highest intakes of coffee globally, with imports reported by Statistics Norway (SSB) (2019) to be stable over time. The imported amount of almost 39 000 tons in 2018, corresponding to approximately 10 kg coffee per Norwegian adult [16]. No reports on total CAF consumption in Norway is available.

1.1.2 PROPERTIES OF PHARMACEUTICALS

The physiochemical behavior of APIs can be utilized to understand or predict their ecological impact. Fate is the pattern of distribution resulting from transport, partitioning, transformation, or degradation. It is influenced by many factors, including abiotic parameters such as temperature, salinity and pH, and inherent physical and chemical properties.

Transport is related to the tendency of a compound to solve in polar and nonpolar phases, i.e., its hydrophobicity [17]. The trait can largely be explained by its ability to dissolve in hexane and water, measured by an octanol-water partitioning coefficient (K_{ow}) (Equation 1). A high K_{ow} relates to lipophilic, i.e., nonpolar properties of the compound, while a low K_{ow} relates to the compound´s polar properties. The K_{ow} affects biological properties, such as toxicity, bioavailability, and bioconcentration [18].

$$log\ K_{ow} = \log\left(\frac{X_{octanol}}{X_{water}}\right)$$

Equation 1 – Hydrophobicity.
Adapted from Walker et al. (2012) [19]

Lipophilic properties tend to favor the transport of contaminants from a polar phase, such as seawater, to a nonpolar phase such as sediments. The chemical properties of contaminants can influence the removal rate at WWTPs, i.e., polar and water-soluble compounds are more likely to be discharged in effluents while lipophilic, while nonpolar compounds tend to accumulate in the sludge. The treatment process of WWTPs is further described in Chapter 1.2.1 and discussed in later sections.

Another means of transportation is described by water solubility (S) [20], [21]. For APIs to be transported to the target organ, water solubility is essential and often a feature added to synthetic pharmaceuticals to improve delivery [22].

The rate of API degradation is often described by environmental or biological half-lives ($t_{1/2}$). Many pharmaceuticals have low persistency and short $t_{1/2}$, as many natural and synthetic drugs are highly bioactive [23]–[26]. Degradation products can have increased bioactivity properties and, if not entirely eradicated in the consumer body, at the WWTP or in the environment. These metabolites are likely pseudo-persistent in the receiving waters due to the constant input into water bodies [27]. The environmental $t_{1/2}$ is highly dependent on media properties and of treatment processing methods applied.

1.2 WASTEWATER TREATMENT PLANTS

A WWTP applies the final processing steps to wastewater and sewage before the treated water is discharged. During the treatment process, the inlet water (also called influent in literature) is filtered by screens and grates, followed by primary (e.g., particle settlement) and secondary/tertiary treatment (e.g., the combination of trickling filter/humus tank or activated sludge/settlement) [28].

For decades, oceans have been the dumping ground for pollutant chemicals, such as runoff from agriculture or from industrial and municipal waste. Starting with the 1972 London Convention (Prevention of Marine Pollution by Dumping of Wastes and Other Matter), a global system was put in place to protect the marine environment by prohibiting the disposal of potentially hazardous material [30].

In the future, the efficiency and capacity of WWTPs are likely to be challenged. Discharge regulations are continuously adapted as more knowledge about contaminant effects are obtained. Sufficient removal of contaminants from wastewater is essential to secure the health of the surrounding environment and aquatic and human populations. The future is predicted to hold global challenges, including extreme weather, rising sea levels, and droughts. Changes in ocean pH and temperature may alter discharged contaminants' properties, while an increasing population near the coast adds pressure to the marine ecosystems [31].

1.2.1 TREATMENT PROCESS

The removal rate of pharmaceuticals by WWTPs range between >10% to close to 100%. The rate depends on the contaminant´s properties (e.g., polarity and solubility), the chemical concentration, and the treatment methods applied [29]. There are many challenges for complete removal of APIs, including low concentrations of contaminants, low sensitivity and high cost of removal methods, fates of APIs not fully understood, and current pollution regulation not requiring monitoring APIs [32].

No single treatment method exists for efficient eradication of APIs in wastewaters. Some methods are associated with high costs and long retention times, such as oxidation by ozone, hydrogen peroxide, chlorine, or UV [33], use of activated carbon (AC) [34], or electrochemical techniques [35]. Tijani et al. (2013) report that physiochemical methods do not eradicate APIs,

and often produce by-products of higher toxicity. The same study reports adequate removal efficiencies when combining biological and physical treatments, finding fewer transformation products [28]. Bolong et al. (2009) agree that physiochemical methods are generally unable to eradicate pharmaceutical compounds [34]. They report AC and oxidation by UV and ozone to remove most organic compounds effectively. However, at neutral pH, acidic compounds (e.g., IBU and DCF) appear to remain in the water phase due to their ionized state. According to Bolong et al. (2009), biological treatment methods can remove many pharmaceuticals, including polar compounds, usually discharged through the outlet water. Many modern WWTPs take advantage of biological treatment steps [19], [20] for removal of most organic compounds, utilizing methods such as high-rate algae ponds and activated sludge [38].

In addition to treatment methods applied by WWTPs, other factors may also affect the eradication rate, herein the properties of the released compounds and the recipient ecosystem. The conditions in receiving environments, including biological (e.g., microbes), chemical (e.g., pH and salts), and photochemical exposure (e.g., sunlight), affect the degradation rate as well as transport of APIs.

1.2.2 SENTRALRENSEANLEGGET FOR NORD-JÆREN

Sentralrensanlegget for Nord-Jæren, the SNJ, is an advanced biological treatment plant with an inlet flow capacity of up to 4000 L/second. The treatment plant is receiving wastewater from both household and industrial sources, including a regional hospital. Sewages and wastewaters from approximately 300 000 households in Rogaland, Norway, are treated at the plant [39].

From the treatment process, solid matter is removed by burning (debris), cleaning and deposition (sand), or by conversion into biogas or fertilizer for agriculture (sludge). Wastewater treatment is regulated according to the Norwegian Pollution Regulation, enforced by the county governor. From the most current version of the regulation (2014), it is required for all WWTPs to remove at least 70% of all degradable biological matter before discharge [40]. The SNJ can reach up to 80% removal.

Treated wastewater is discharged into the ocean, about 1.6 kilometers from shore and at a depth of 80 meters, into Håsteinsfjorden [39]. The monitoring of the recipient area has reported no significant negative impact on the environment due to the SNJ outfalls, according to report 2014.067.I.FMRO by the county governor (Fylkesmannen i Rogaland). The recipient

ecosystem conditions, herein hydrography, plankton, nutrients, bacteria, toxins in sediments and organisms, as well as benthic fauna have been investigated from 1989. The SNJ is controlled every three years by the county governor. However, no assessments of APIs in outlet water have been made [36]. The requirements for wastewater treatment are presented in §14-2, a regulation describing the removal of heavy metals, phosphorus, nitrogen, and other targeted compounds, such as polyaromatic hydrocarbons and bromo-fluoro organic compounds.

1.3 ENVIRONMENTAL MONITORING

Environmental monitoring aims to assess the exposure-related risks to pollution by determining the environmental status. Data are collected for research and to establish a baseline to be able to make decisions about measures to be taken. The Norwegian Environment Agency (Miljødirektoratet, MD) has authority over national monitoring surveys. The observation of the effects of preventive measures applied to exposed areas and protected environments is authorized by the MD at a national level, and the county governor at a regional level [41].

Miljøstatus, the cooperation between multiple agencies and institutes, including MD, reports on the current environmental situation in Norway. Miljøstatus works towards 23 specific goals, herein five goals targeting pollution. Goal 4.1 states that pollution should not harm health and environment [42], and goal 4.2 states that the discharge of compounds hazardous health and environment must be stopped. The goals are related to “goals for sustainability”, set by the United Nations [43].

Environmental monitoring can include both chemical and biological analyses, and it is carried out by applying various analytical techniques [44]. An essential step towards revealing the effects of contaminants is the quantification of pollutants and the exposure routes. To understanding the environmental impact, assessments using sensitive or model species by toxicity assays, biomarkers, biosensors, and models are useful [45], [46].

1.4 SAMPLE DESCRIPTION

In this thesis, samples are the material collected from selected locations. Samples are used for quantitative and qualitative analyses. Inlet water samples wish to describe the load of APIs entering the SNJ, while outlet water samples describe the load of APIs entering the surrounding ecosystem by wastewater discharge. Analyses of seawater and sediment samples aim to contribute to understanding the occurrence and fate of pollutants at selected marine and coastal stations.

1.4.1 TREATMENT PLANT SAMPLES

Inlet water samples are in the present study water samples collected from the SNJ influent after the removal of large objects by grates. Samples are not treated by any chemical or biological means. Inlet water is a non-homogenous flow of sewage, grey-water, and surface runoff water. Because of variations in volume and content entering the plant, the flow will vary on a daily and seasonal basis. Samples collected were grab samples, i.e., aliquots collected within a short period. Because of rapid changes in inlet water content, each sample represents the specific time of collection. All samples are collected within one hour on the same day. Some variations are expected, as water usage in homes is generally high during mornings and evenings and lower in mid-days and nights. A considerable variation could be expected between samples collected in different months and seasons, e.g., the use of pharmaceuticals during flu (winter) or pollen (spring) season.

Outlet water samples are in this thesis samples collected from effluents before discharge into Håsteinsfjorden. Wastewater is treated by multistep filtering and biological treatment at the SNJ, as described by their public website [36]. Because the inlet flow and content are non-homogenous, the same can be expected from the outlet water.

1.4.2 SEAWATER SAMPLES

Seawater samples are water collected from marine stations. The seawater compartment is the most extensive water collection on earth, constituting the seas and oceans covering the planet. Water molecules are highly polar and seawater, therefore an excellent polar solvent. As a result, seawater has a high salinity, generally about 2.5%, from dissolved minerals carried into the sea by rivers and streams, but also released from underwater volcanic activity. The buffer system made up of the dissolved ions and dissolved gases, such as O_2, CO_2, and N_2, keeps the seawater at a stable pH, between 7.5 and 8.5. Temperature is affected by currents distributing water heated near the equator and cold water from the poles in set paths [47]–[49].

Compared to constrained water bodies, such as rivers and lakes, the dilution factor of pollutions entering oceans is much higher. Therefore, contaminants can be distributed over great distances and become highly diluted, dependent on parameters such as volume, currents, weather, and climate. However, the dilution is not uniform, but follow paths set by an intricate pattern of various parameters such as currents, weather, and plume flow [18].

1.4.3 SEDIMENT SAMPLES

Sediments are samples collected from beaches and the sea bottom, and consist of a deposited mixture of sand, silt, organic and inorganic material. Sediments consist of multiple layers, built up by deposits over time [50]. Coastal sediments are often coarse and subject to a rapid change of content. Waves and currents carry fine particles out to calmer seas, where the sediments are generally more uniform [51].

Sediments will, over time, become anoxic by the build-up of new layers, while in the upper layer, aerobe conditions remain. Deep-sea sediments may be exposed to less oxygen-rich seawater, and sediments located in the intertidal zone may be subject to significant variations in oxygen content, experienced with the changing tides. The transformation of compounds is highly dependent on oxygen content, with a high level promoting oxidative transformation and anaerobe conditions promoting reductive transformations. Other influences on the binding of pollutants to sediments include pH, salinity, and temperature [52]. Upon changes in surrounding pH and salinity, sediments can release adsorbed pollutants back into the seawater [31].

2. OBJECTIVES

This master project´s objective was to develop methods for the detection and quantification of APIs in sediments, seawater, and treatment plant samples by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Results are contributing to the understanding of the occurrence of pharmaceuticals in the SNJ and its recipient environment.

Specific goals:

Develop a method for:

- API extraction from seawater, sediments, and treatment plant samples.
- Optimization of analytical parameters for the detection of APIs by HPLC-MS/MS.
- Recovering of APIs in water and sediment samples.

Evaluation of contaminants released from the SNJ by:

- Quantification and detection by HPLC-MS/MS of targeted pharmaceuticals in samples.
- Adjustment of quantification-data concentrations using recovery rates.
- Comparison of quantification data obtained from HPLC-MS/MS and ultra-performance LC-MS/MS (UPLC-MS/MS).

3. METHODS

3.1 CHEMICALS

Organic solvents used were of the highest available grade. HPLC grade methanol (MeOH), FID/GC grade ethyl acetate, and 99-100% formic acid were obtained from VWR Chemicals. ASC reagent ammonium hydroxide solution (28.0-30.0%) was purchased from Sigma Aldrich. Milli-Q water was produced from de-ionized water using an ultrapure water purification system (Sartorius, Germany). Buffer solutions were made fresh weekly before use.

A total of ten APIs were assessed. Available information, including CAS number, molar masses, purity, and concentration of stock solutions, is enclosed in Appendix 1. Appendix 2 describes compound properties, including pK_a, K_{ow}, and (S), and Appendix 3 describes the chemical structures of all compounds. NAP ((S)-(+)-(6-Methoxy-2-naphthylproprionic acid, IBU (4-isobutyl-α-methylphenylacetic acid, ACE (4-acetamidophenol, SUL, and (±)-ATE were purchased from Alfa Aesar. CBZ**,** CAF**,** TMP**,** DCF (sodium salt) were purchased from Sigma Aldrich. ATV (calcium salt) was purchased from Cayman.

Individual stock solutions (0.05-0.2 mg/mL) were prepared for targeted pharmaceutical compounds by dissolving 1-4 mg of analyte into 20 mL of HPLC grade MeOH (VWR, Poland). The stock solutions were wrapped in aluminum foil and stored at 4°C/-20°C.

3.2 SAMPLE COLLECTION

Environmental samples, i.e., sediments and seawater, were collected in proximity to the SNJ discharge site and selected reference stations during September and October of 2019. The locations were selected according to a model of the discharge plume (Figure 2).

Sediments and seawater samples were collected from up to three marine stations. One station was located close to the discharge site, and will hereafter be referred to as the discharge station (DS). Two reference stations were chosen, one station close to Kvitsøy (K) and one in Boknafjorden (BF). Marine samples were collected using a small working boat (MS Scallop) operated Kvitsøy Sjøtjenester AS. Additional sediment samples were collected from three coastal stations: Randabergbukta (RB), Sande Beach (SA) and the reference station Sola Beach (SO). Wastewater samples were provided by the SNJ operator IVAR IKS. The location of all stations is described in Table 1 and Figure 3.

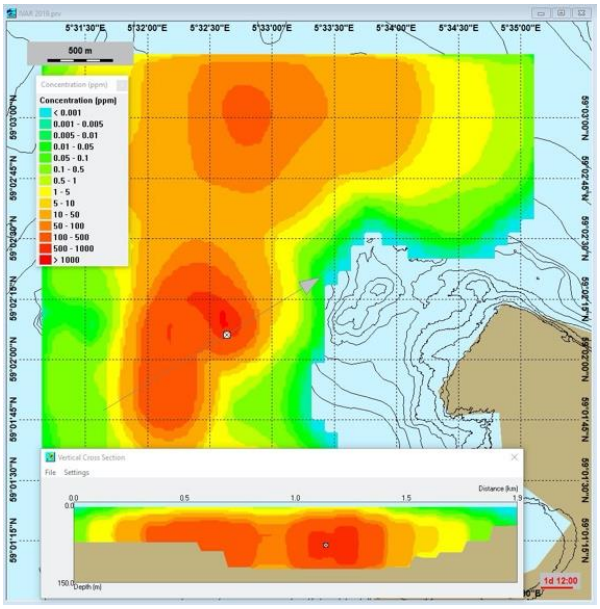

Figure 2 – DREAM model of the plume. Predicted concentration (ppm) of the plume in 3D is from September 25th at 10:00. GPS-coordinates of the discharge site was 59.035, 5.544, and the depth was 80 meters. The plume discharge rate was based on normal flow rates during dry weather (60000 m3/day). Plume components were defined by log Kow, biodegradation, and toxicity characteristics. In the initial single component model parameters were set to concentration = 1 million ppm, biodegradation = 0. Model depths have been manually modified with data from the Norwegian Mapping Authority (kartverket.no), from GeoNorge. Currents have been simulated using coastal currents from the Norwegian Meteorological Institute's website (thredds.met.no).

Figure 3 – Sampling locations. Map of locations sampled for seawater and sediments. Red star = the SNJ discharge site, blue = coastal station RB and SA, purple = coastal station SO, orange = marine station DS, yellow = marine reference station K and BF. RB = Randabergbukta, SA = Sande Beach, SO = Sola Beach, DS = discharge station, K = Kvitsøy, BF =Boknafjorden. (Google Earth 2020)

Table 1 – Summary of sample collection locations.

Station:	**Date:**	**GPS-coordinates:**	**Collected samples:**
Sola beach (SO)	17.09.2019	58.8929360, 5.5933684	Sediments
Sande beach (SA)	18.09.2019	59.018675, 5.590694	Sediments
Randabergbukta (RB)	18.09.2019	59.023727, 5.606794	Sediments
Discharge site (DS)	31.10.2019	59.01836, 5.33075	Seawater, sediments
Kvitsøy (K)	25.10.2020	59.01743, 5.32666	Sediments
Boknafjorden (BF)	31.10.2019	59.10624, 5.39604	Sediments
The SNJ	17-18.10.2019	-	Outlet water
The SNJ	06.11.2019	-	Inlet, outlet water

3.2.1 TREATMENT PLANT SAMPLES

A total of four samples of outlet water and three samples of inlet water were analyzed. Outlet sample 1 was collected over 24 hours. This composite sample wishes to represent the average content of APIs in outlet water over the sampling period. The sample was transported in a 10 L plastic bottle and transferred to 2 L glass bottles upon arrival at the university laboratory. Glass bottles were stored in the freezer at -20°C. The remaining outlet (2-3) and inlet (1-3) samples were grab samples, collected in 1 L glass bottles and stored at -20°C. The content of these samples corresponds to the time of sample collection.

3.2.2 SEAWATER SAMPLES

Seawater samples were collected from two marine stations (DS and BF), from approximately 80 to 200 meters depth using a water sampler (12 L, Niskin). During transportation to the laboratory, samples were stored in glass containers and on ice. No filtration was done before the storage of samples at -20°C.

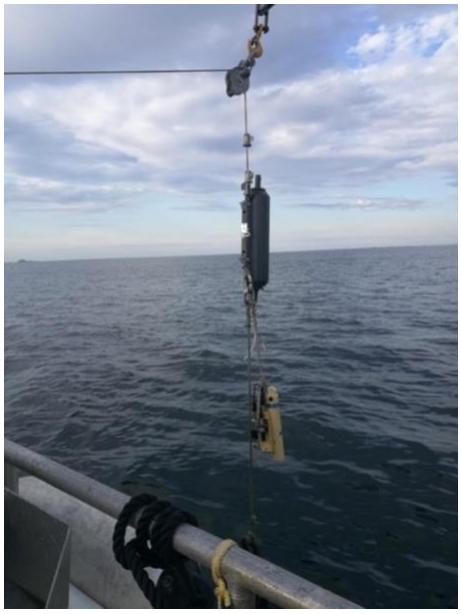

Figure 4 – Seawater sampling. A volume of 12 L seawater was collected near sea bottom from marine stations using a Niskin water sampler.

3.2.3 SEDIMENT SAMPLES

Sediment samples were collected from the intertidal zone of three coastal stations, SO, SA and RB, and three marine stations, DS, K, and BF. For each station, three spots (>50 meters apart) were sampled for top sediments (< 2 cm depth) and pooled into one sample.

Sediments from coastal stations were collected using a core sampler (Figure 5, left). The core sampler was borrowed from the University of Stavanger and did not state the model or manufacturer. Marine samples were collected from the sea bottom by a Van Veen grab [53] (Figure 6). Sediment samples were collected into clean glass bottles. The content was mixed to homogenize the samples. Samples were stored on ice during transport and in a laboratory freezer (-20°C) until further analyses.

Figure 5 – Sediment sampling from a coastal station. Sediment samples assessed by core sampler (left) and transferred into a clean glass bottle (right).

Figure 6 – Sediment sampling from a marine station. Samples obtained by Van Veen grab, which was operated on a hydraulic line. Samples were collected from the sea bottom at 80 – 200 meters depth.

3.3 SAMPLE PREPARATION

All samples were treated before analysis to concentrate target compounds and to remove unwanted sample content. An important aspect of sample preparation is the possible loss, degradation, or transformation of the analyte. Recoveries of target analytes were tested in water and sediment samples to account for potential loss of product during preparation steps. The recovery studies are described in Chapter 3.5.1.

In the following sections, the used methods are described. The scheme is summarized in Figure 7.

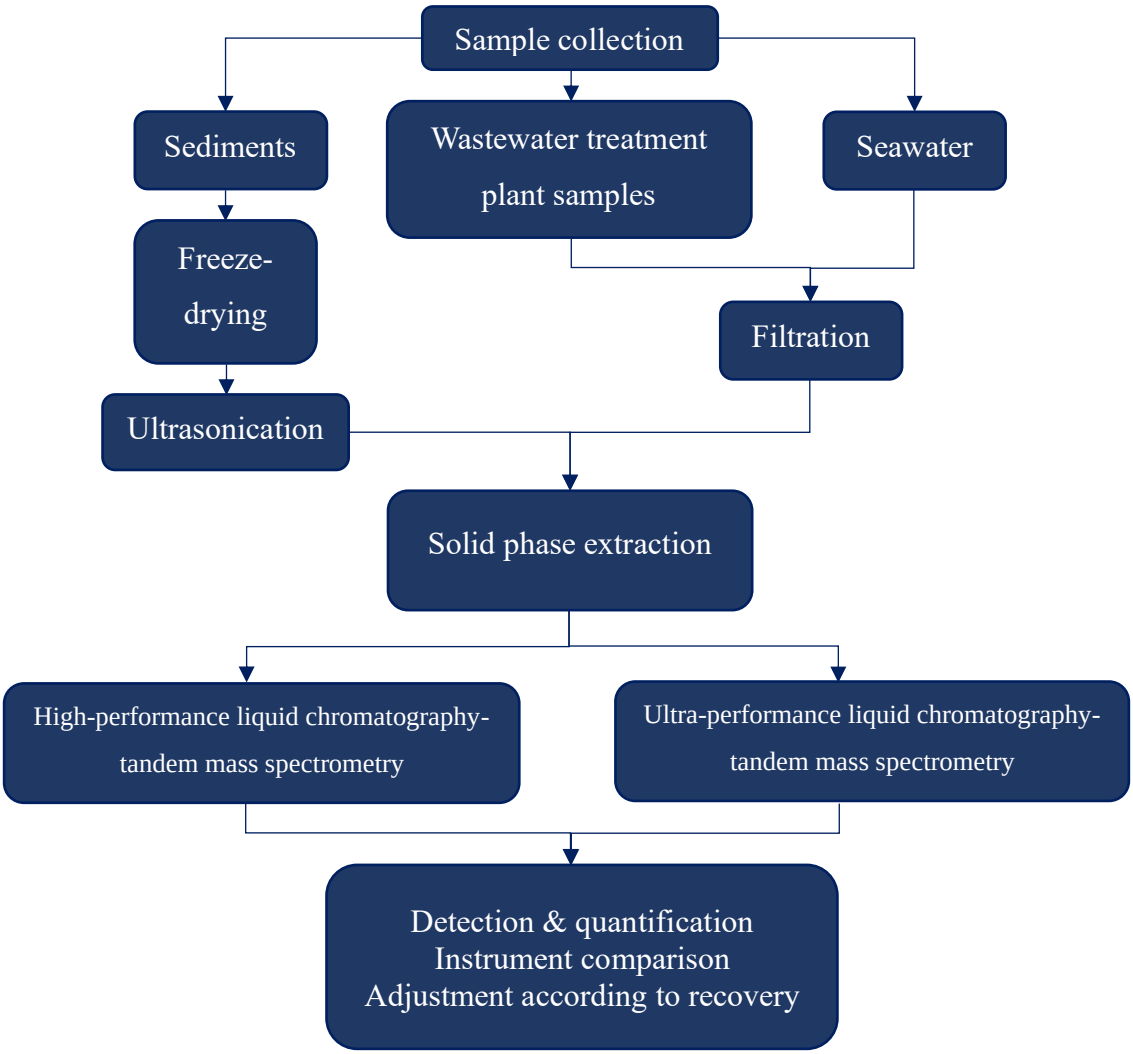

Figure 7 – Scheme of sample preparation and analysis.

3.3.1 FILTRATION OF WATER SAMPLES

Filtration is the process for the removal of particulate solids from a liquid [54]. Two seawater samples, four outlet water samples, and three inlet water samples were individually filtered using filter paper. Due to high amounts of suspended particles in some samples, filters had to be exchanged upon clogging (i.e., filter cake).

Samples were thawed at 4°C and filtered using glass microfiber filters (VWR, Sweden) with decreasing pores sizes (20-25 µm and 2.5 µm) using a vacuum pump. The filtration process is illustrated in Figure 8. The filtration unit was an adapted Sterifil® aseptic system (Merck KGaA, Darmstadt, Germany). The unit was washed with MeOH and Milli-Q water before use and between different samples. The unit enabled the filtration of volumes of up to 250 mL at a time. A liquid trap was added to prevent the filtrate from entering the vacuum pump (Vacuubrandt, Germany). The valve of a vacuum chamber controlled the pressure. At all times, the pump operated at <60 mBar to prevent damage to filter paper and avoid the liquid from entering the trap. As a finalizing step, the filtrate was passed through a 0.2 µm polypropylene syringe filter membrane (VWR international, China).

Filtered water samples of 600 mL were collected into pre-cleaned glass bottles and stored at 4°C until extraction and clean-up by solid-phase extraction (SPE). The pH of samples was not measured nor adjusted during sample preparation and storage.

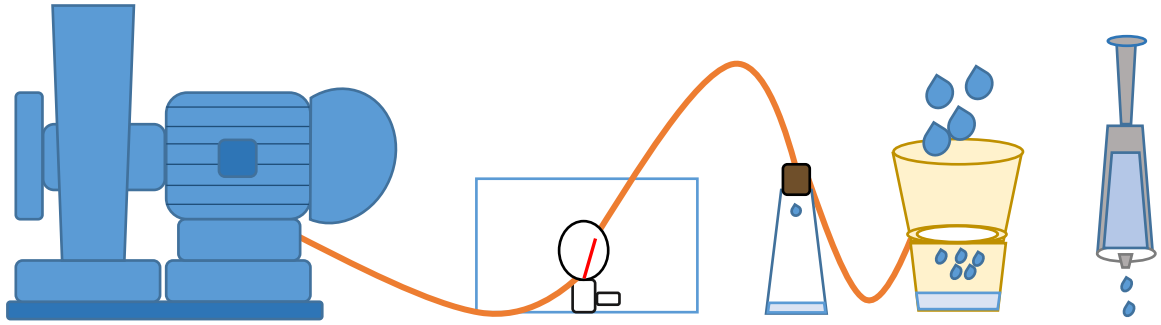

Figure 8 – Illustration of the filtration process. From the left: vacuum pump, ball valve with attached pressure gauge, liquid trap, filtering unit with filter paper inserts (>25 and 2.5 µm), and syringe filter (0.2 µm).

3.3.2 FREEZE-DRYING OF SEDIMENT SAMPLES

Freeze-drying, or lyophilization, is a gentle method used for water removal in solid samples. This step aimed to ensure that further sample preparation steps used the same amount of sediments, i.e., sample mass was not influenced by variation in water content. Six sediment samples, three from coastal stations (SA, RB, and SO) and three from marine stations (DS, K and, BF), were freeze-dried using a MAXI Dry Lyo (Heto-Holten AS, Denmark). The vacuum was obtained by a vacuum pump (Vaccubrand, Germany). The freeze-dryer vacuum-unit displayed 1-10 mBar during operation, and the cooling unit displayed -110°C.

The full process is illustrated in Figure 9. For each sample, a wet mass of ~10 g was transferred into Fast-Freeze® Flasks (Labconco, Kansas City, MO, USA). Samples were kept deep-frozen by immersion into liquid nitrogen for 30-60 seconds every 30 minutes. When close to dryness, samples were for a short time dried at room temperature (RT, 20°C) to accelerate the evaporation of remaining moisture. The duration of the drying was 4-8.5 hours per sample. The relative water loss was calculated from Equation 2. Dry samples were homogenized by mortar and pestle and stored in a closed container at -20°C until further processing.

$$\text{Water loss (\%)} = \frac{mass(wet) - mass(dry)}{mass\ wet} \times 100\% \qquad \text{Equation 2 – Relative water loss}$$

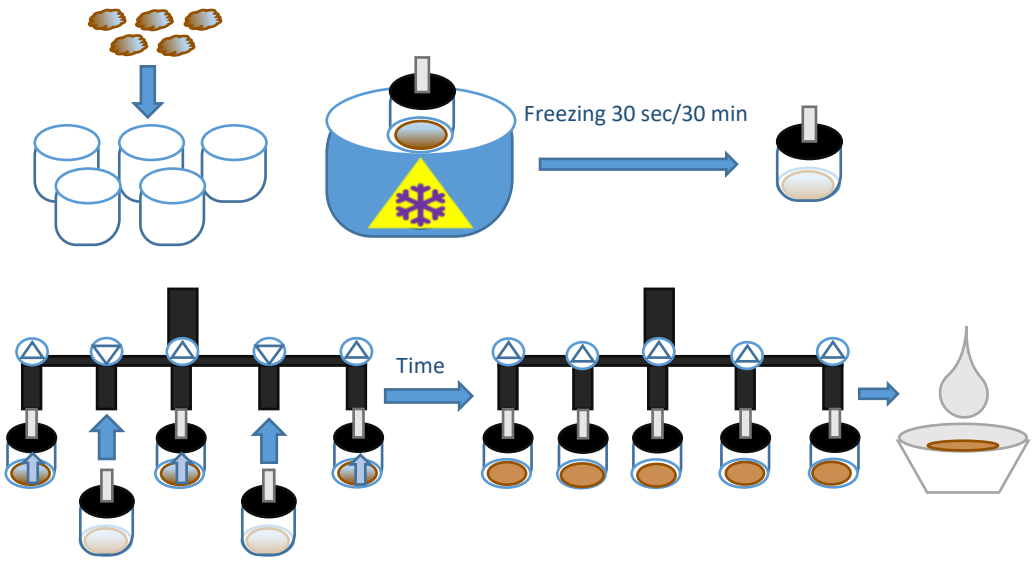

Figure 9 – Illustration of the freeze-drying process. Sediment samples were added to flasks and cooled by immersion into liquid nitrogen. Re-freezing was done for 30 seconds every 30 minutes. Flasks were connected to freeze dryer, and water evaporated under vacuum until complete dryness achieved. Dry sediments were homogenized by a mortar and pestle.

3.3.3 ULTRASONICATION OF SEDIMENTS

Ultrasonic-assisted extraction (UAE) is the application of ultrasonic waves (>20 kHz) to increasing the contact between solvent and solute, thereby releasing APIs from suspended solids into an extraction liquid [55]. Six samples of freeze-dried top sediments, from DS, BF, K, SA, RB, and SO, were extracted and resuspended in Milli-Q water. For each sample, three replicates were prepared.

The extraction process by UAE is illustrated in Figure 10. For each replicate, 1.000±0.002 gram freeze-dried sediments were added into a conical centrifuge tube (15 mL, Genebio Systems). A total of 10 mL solvents was added: a) 5 mL (1:1, v/v) Milli-Q water/HPLC grade MeOH with 1% ammonia, and b) 5 mL (1:1, v/v) Milli-Q water/HPLC grade MeOH with 1% formic acid. Samples were vortexed by a Digital Vortex-Genie2 (Scientific Industries, USA) at 2200 RPM and for 2 minutes and transferred to a ULTRAsonik™ (NEY dental Inc., Bloomfield, CT, USA) for ultrasonication. The ultrasonic bath was operated at RT and 75% power for 15 minutes. After ultrasonication, samples were centrifuged by a 5804 R centrifuge (Eppendorf AG, Hamburg, Germany) operated at 2 000G, 5 minutes, and 4°C. Supernatants (i.e., extracts) were transferred into scintillation vials and air-dried in a working cabinet at RT until volumes were reduced to <1 mL. Extracts were resuspended into a total of 600 mL Milli-Q water and stored at 4°C until extraction by SPE.

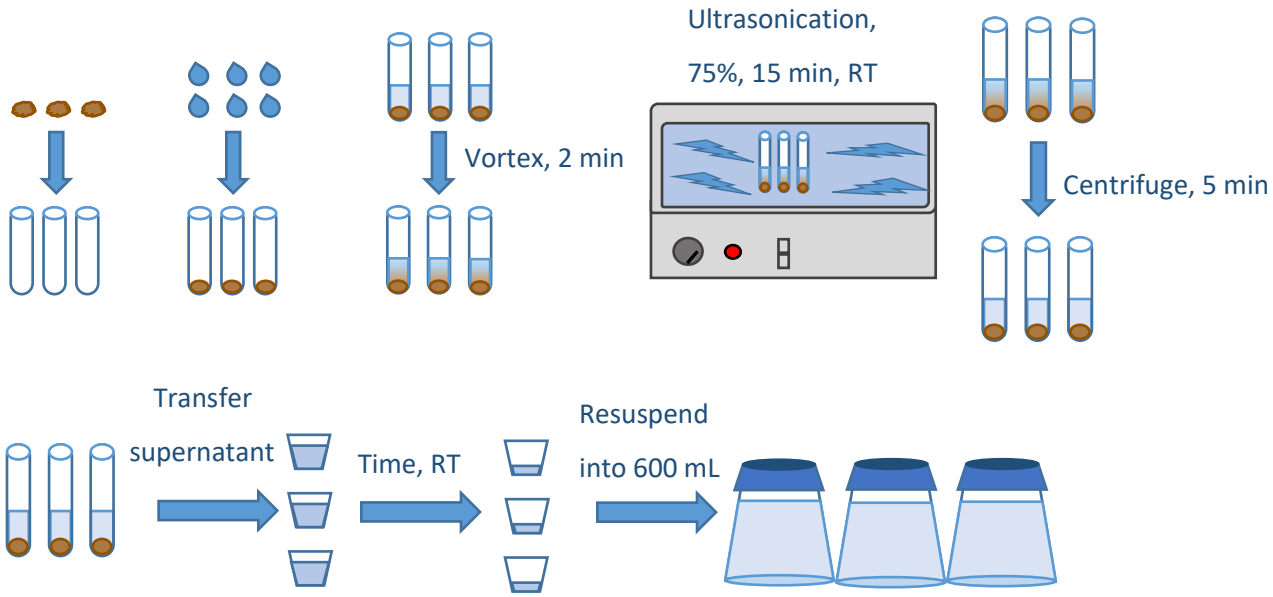

Figure 10 – Illustration ultrasonication process of sediment samples

Freeze-dried sediments were added to centrifuge tubes, and extraction liquids added. Samples were vortexed and ultrasonicated. By centrifugation, the extracts were separated from the solids. Supernatants were decanted into scintillation vials. The extracts were reduced to <1 mL before resuspension into 600 mL Milli-Q water. RT = room temperature.

3.3.4 SOLID PHASE EXTRACTION

SPE is the separation of compounds based on their chemical and physical properties. SPEs were performed as a clean-up step, using the same extract conditions for all samples, i.e., inlet, outlet, and seawater filtrates, and sediment extracts. Samples were extracted using Oasis hydrophilic-lipophilic balance (HLB) cartridges (500 mg, 6 mL, Waters, Ireland). Oasis HLB is a water-wettable, reversed-phase sorbent. The sorbent contains two monomers: hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene. The cartridges are ideal for analytes of all charges, zwitterions, and noncharged compounds, and are stable over pH range 1-14 [56].

The clean-up process is illustrated in Figure 11. For each sample, triplicates with volumes of 600 mL were extracted. The method was modified from *Oasis HLB Cartridges and 96-Well Plates Care and Use Manual* (Waters Corporation, 2014). Each cartridge was conditioned and rinsed with 6 mL MeOH, followed by 6 mL Milli-Q water at a flow rate of 3-5 mL/min. Samples were loaded at a rate of 5-10 mL/min. After loading, cartridges were rinsed with 6 mL Milli-Q water and vacuum-dried for 20 minutes. Cartridges were transferred to a Visiprep™ manifold (Supelco, Inc., Bellefonte, PA, USA) and analytes eluted by addition of 6 mL MeOH/ethyl acetate (1:1, v/v) containing 2% ammonia followed by 6 ml MeOH/ethyl acetate (1:1, v/v) containing 2% formic acid. The elusion flow rate was 5 mL/min. Eluents were evaporated by a stream of nitrogen and resuspended in 600 µL of HPLC grade MeOH, i.e., to a concentration factor of 1000. Before storage, extracts were filtered using 0.2 µm polypropylene membranes (VWR International, China) into 2 mL glass vials (Supelco, USA). All vials were sealed with parafilm and stored at -20ºC.

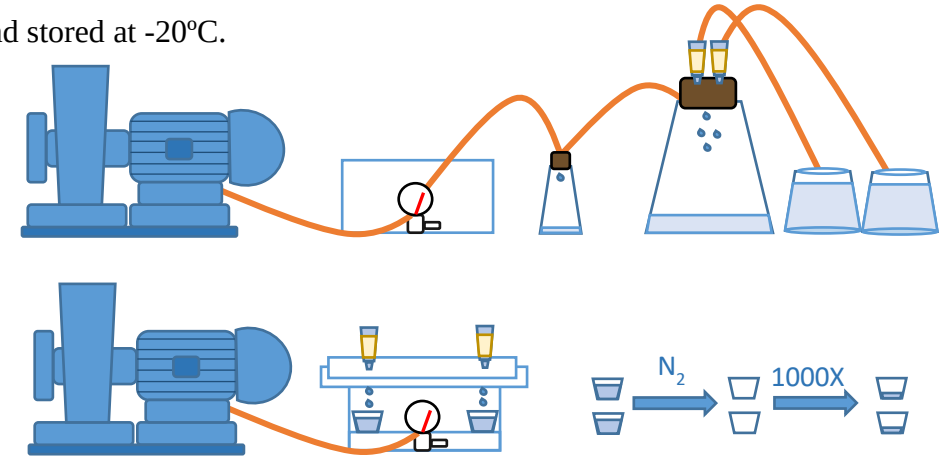

Figure 11 – Illustration of clean-up step by the solid-phase extraction. Cartridges were conditioned and rinsed before loading the sample. After loading, cartridges were rinsed, and vacuum dried. Eluents were released from cartridges by the addition of elution buffer and dried by nitrogen (N_2). Dried extracts were resuspended 1000X.

3.4 QUANTITATIVE ANALYSIS

3.4.1 TUNING OF STANDARDS

Targeted APIs were tuned before analysis to detect strong analyte signals. Tuning was performed in MassLynx (version 4.1), in MS Tune Mode. Standard working solutions (0.05 - 0.30 mg/mL) were infused through a syringe pump at a flow rate of 10 - 25 µL/min. Electrospray ionization (ESI) mode, precursor ions, fragments, collision energy (CE) and cone voltage (CV) were determined for each analyte by adjusting the entrance potential (EP), collision cell exit potential (CXP) and the collision gas flow, according to Quattro Premier Mass Spectrometer Operator´s Guide (2005). Values obtained were added to a multiple reaction monitoring (MRM) MS Method File and are described in Table 2.

Table 2 – Optimized parameters for analysis by high-performance liquid chromatography-tandem mass spectrometry.

Parameters for assessed pharmaceutical compounds are listed in alphabetic order. Ions used for detection and quantification are given in mass-to-charge ratio (*m/z*). Precursor ion and fragment transitions are described by an arrow. Electrospray is in positive (+) ionization mode for all compounds except negative (-) mode for ibuprofen. Voltages of CV and CE are given in volt (V) and electron volt (eV).

Compound	**Ions *(m/z)***	**ESI mode**	**CV (V)**	**CE (eV)**
Acetaminophen	151.9 → 109.8	+	35	15
Atenolol	267.3 → 73.90	+	35	20
Atorvastatin	559.35 → 440.5	+	40	23
Caffeine	195.0 → 137.9	+	35	17
Carbamazepine	237.1 → 194.1	+	35	35
Diclofenac	296 → 214.1	+	25	28
Ibuprofen	207.2 → 161.1	-	17	8
Naproxen	231.1 → 170.0	+	25	15
Sulfamethoxazole	254.1 → 155.9	+	30	15
Trimethoprim	291.2 → 122.9	+	38	25

3.4.2 HPLC GRADIENT PROGRAMS

The aim of testing various HPLC gradient programs was to confirm the suitability of various parameters, such as inlet flow rate and the ratio of mobile phases, with the targeted APIs. Well-adapted gradient programs result in sharp, symmetric peaks for precursor ions $[M + H]^+$/ $[M - H]^-$ and fragments for all analytes [57]. The most abundant peaks with best shape and resolution were selected for the determination of analytes in samples from the detected fragments.

Samples assessed were working solutions of all target analytes, diluted to approximately 100X in HPLC-grade MeOH. Three gradient programs were created to ensure the retention time (T_R) of all APIs were within a detectable range. From here, programs are referred to as HPLC_PI_A1B1, HPLC_PI_A2B1, and HPLC_NI_A2B1. Programs are abbreviated according to "[instrument]_[positive/negative ionization]_[mobile phase composition A1/2+B1]". Programs, described in Table 3, were supplied with mobile phase from a binary solvent system, where mobile phase A1 was 0.2% (v/v) formic acid, A2 was 0.2% (v/v) ammonium hydroxide, and B1 was HPLC-grade MeOH. Mobile phase B2 (acetonitrile) was not implemented in any program. Retention times for all compounds were obtained from chromatograms in Masslynx (Table 4). Sample dilution in 0, 20, and 50% Milli-Q water were assessed to evaluate matrix effects and improvement in peak detection. Trials are not described further, but it was decided to use 50% dilution of extracts for sample analysis.

Table 3 – Description of gradient programs.

The total run time for gradient programs A) HPLC_PI_A1B1 and B) HPLC_P/NI_A2B1 was 11 minutes. The mobile phase flow was constantly at 0.2 mL/min. The ratio of mobile phases is given as a percentage (%). Other parameters remained the same for both gradient programs. A1 = 0.2% (v/v) formic acid, A2 = 0.2% (v/v) ammonium hydroxide and B1 = HPLC grade methanol.

A) Time, min	**Flow (mL/min)**	**A1, %**	**B1, %**
0	0.2	95	5
5	0.2	1	99
7	0.2	1	99
8	0.2	95	5
11	0.2	95	5

B) Time, min	**Flow (mL/min)**	**A2, %**	**B1, %**
0	0.2	95	5
5	0.2	1	99
7	0.2	1	99
8	0.2	95	5
11	0.2	95	5

Table 4 – Retention times of targeted pharmaceuticals.

Retention times (T_R) for assessed pharmaceutical compounds are listed in alphabetic order. T_R is given in minutes for pharmaceuticals after analysis by three gradient programs, HPLC_PI_A1B1, HPLC_PI_A2B1, and HPLC_NI_A2B1. The T_R is shifted for all compounds except carbamazepine due to the change in mobile phase pH. Mobile phases in HPLC_PI_A1B1 were A1 (0.2% (v/v) formic acid) and B1 (HPLC grade MeOH). Mobile phases in HPLC_PI_A2B1 and HPLC_NI_A2B1 were A2 (0.2% (v/v) ammonia) and B1.

Compound	**T_R (min)**		
	HPLC_PI_A1B1	**HPLC_PI_A2B1**	**HPLC_NI_A2B1**
Acetaminophen	3.45	1.54	-
Atenolol	3.39	5.02	-
Atorvastatin	6.59	5.35	-
Caffeine	4.21	4.21	-
Carbamazepine	5.88	5.88	-
Diclofenac	6.86	4.88	-
Ibuprofen	6.95	4.90	5.11
Naproxen	6.36	4.32	-
Sulfamethoxazole	4.44	1.77	-
Trimethoprim	4.04	4.90	-

3.4.3 STANDARD CALIBRATION

Standard calibration solutions were prepared from working solutions with concentrations listed in Appendix 1. Solutions were prepared within one week of analysis and stored at -20ºC to ensure minimal degradation of analytes during storage. All standard compounds were of the highest purity obtainable, as described in the same appendix.

Calibration curves were prepared from serial dilution (2X) of standard solutions at ten concentration levels equivalent to 1.95 - 1000 ng/L or 1.17-600 ng/g dry weight when taking into account the pre-concentration factor applied along with the sample preparation steps (500X). All standard calibration solutions were analyzed the same way as environmental and treatment plant samples, i.e., in HPLC_PI_A1B1, HPLC_P1_A2B1, and HPLC_NI_A2B1. Standard calibration curves were plotted with instrument output on the y-axis and analyte concentration on the x-axis.

3.4.4 INSTRUMENTAL ANALYSIS

Extracts were prepared by dilution into 50% Milli-Q water in 2 mL amber vials (Supelco, USA) with 300 µL pull point inserts (Thermo Scientific, Germany). The instrumentals analysis was performed in MRM mode on an ACQUITY UPLC System (Waters, Manchester, UK)) coupled to a Micromass Quattro Premier XE Mass Spectrometer and equipped with an electrospray ionization source in positive and negative mode. The separation of analytes was achieved chromatographically on an ACQUITY UPLC BEH C18 Column (130Å, 1.7 µm, 2.1 mm*100 mm, Waters) analytical column. Samples were loaded (5 µL) onto the trap column with appropriate mobile phases at a flow rate of 0.2 mL/min. Mobile phase gradient programs (HPLC_PI_A1B1, HPLC_PI_A2B1, and HPLC_NI_A2B1) are described in Chapter 3.4.2. The total run time was 11 min, with initial conditions restored from 8 to 11 minutes to allow the system to equilibrate. The detection was performed between 0.74 and 11 minutes.

The output was analyzed in Masslynx/Targetlynx (version 4.2). Identification and quantification were based on records of precursor ions and the most abundant fragment at expected retention times. Peaks and retention times were checked manually in TargetLynx to ensure that correct peaks were included. An output file containing signal-to-noise ratios (S/N ratio), retention times, and peak areas, was exported to Excel for further analysis.

3.5 STATISTICAL ANALYSIS EXCEL/TARGETLYNX

The statistical analyses of results obtained from the instrumental analyses of standard calibration solutions by HPLC-MS/MS were in Excel (version 16.36, Microsoft 2020). Regression models of standard calibration curves were prepared using an integrated Excel add-in data analysis-tool for regression analysis. Calibration curves were constructed by up to ten points of known concentrations within a suitable range according to predicted sample concentrations.

Assumptions for the regression models were according to suggestions by the LGC Group´s Valid Analytical Measurements (VAM) bulletin from 2003 [58]. Assumptions are as follows: errors in x-values are insignificant compared to errors in y-values, observations are normally distributed, and all numbers are continuous. Residuals, i.e., the difference between the value obtained from the regression line and the observation, should have a constant magnitude, measured by a constant relative standard deviation (RSD). Residuals are assumed unbiased. The regression model linearity is calculated using the least-square principle. The closeness of the residuals to the fitted line is the coefficient of determination (R2), calculated as a ratio of explained variation to the total variation in the model. The y-intercepts were forced through zero, an assumption applied because blank values were of low S/N ratios and with peaks lower than those detected for the lowest standard solution concentrations assessed.

Limits of detection (LOD) and limits of quantification (LOQ) were calculated from the standard error of the calibration-curve response (y-intercept) and the slope of a linear model. Methods were recommended by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guidelines, and are described in Equation 3 and Equation 4.

$$LOQ = \frac{Standard\ error}{slope} \times 10$$ Equation 3 – Limit of quantification

$$LOD = \frac{Standard\ error}{slope} \times 3.3$$ Equation 4 – Limit of detection

3.5.1 METHOD VALIDATION

Validation of methods was performed by assessments of analyte recoveries. A near 100% recovery of spiked samples indicate a conserved mass balance over the sample preparation steps and help explain potential losses or transformations of analytes. Recoveries also indicate a correct alignment to calibration curves.

The accuracy of the method, i.e., the filtration process of water samples, extraction of sediment samples by UAE, and clean-up by SPE of all samples, were assessed. For each sample, five replicates were prepared. Milli-Q water was spiked to three concentration levels, 60, 800, and 2 000 ng/L of APIs (except IBU) before filtration. Concentrations of IBU were 8, 80, and 800 ng/L. Samples of freeze-dried sediments were spiked to three concentration levels, 60, 800, and 2 000 ng/g dry weight for all targeted APIs, except IBU, which was spiked to 8, 80 800 ng/g dry weight, before extraction by UAE. Sediment samples were exposed to spike solution (analytes dissolved in 1 mL MeOH) for 24 hours and at 4ºC before extraction. Spiked samples were prepared in the same manner as environmental and treatment plant samples before analysis by HPLC-MS/MS. Calculations of recoveries are described by Equation 5. The assessed sediments were assumed free from target analytes, i.e., the $C_A = 0$. The method precision is determined by the RSD to ensure correct quantification, as recommended by ICH Guidelines, and described in Equation 6.

$$\text{Recovery} = \frac{C_B - C_A}{\Delta C} \times 100\% \qquad \text{Equation 5 – Recovery}$$

C_A: Analyte concentration measured in the sample
C_B: Analyte concentration measured in the spiked sample
ΔC: Known concentration of the spiked sample

$$RSD = \frac{standard\ deviation\ of\ samples}{mean\ of\ samples} \times 100\% \qquad \text{Equation 6 – Relative standard deviation}$$

3.5.1 COMPARISON OF HPLC-MS/MS AND UPLC-MS/MS

A selection of samples was analyzed at the University of California Riverside (UCR) in February 2020, herein inlet sample 1-3b[1] (n=1), outlet sample 1 (n=6), outlet samples 2-4b[2] (n=1), seawater samples from two marine stations, the discharge station (DS) (n=3) and the reference station in Boknafjorden (BF) (n=3), and three sediment samples from stations DS (n=2), BF (n=2), and K (n=3). The analyzed samples were aliquots of extracts, i.e., identical samples (with two exceptions) to the ones which in previous sections are presented after analysis by HPLC-MS/MS.

Nine APIs (ACE, ATE, CAF, CBZ, DCF, IBU, NAP, SUL, and TMP) were targeted. No standard solution of ATV was available for analysis. ATV was, therefore, not included in this comparison study.

The instrument used was an ACQUITY UPLC-MS/MS system (Waters, Milford, MA) with a binary solvent manager, autosampler manager, and automatic thermostatic column oven. Chromatographic separation of compounds was performed using an ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 m particle size, Waters). Analytes were determined using a Waters Micromass triple quadrupole detector equipped with an ESI source. Data acquisition was performed in both positive and negative ESI modes with optimized MS parameters.

Quantitative analysis was performed in the MRM mode. All data were acquired and processed using the MassLynx 4.1 software. Target analyte ESI mode and transition ions are described in Table 5Calibration curves of the other analytes were in the range of 1.95-1000 ng/L or 1.17-600 ng/g dry weight. The calculated slope, linearity (R^2), LOD, and LOQ of regression models are described in Table 6.

Comparison of quantification by HPLC-MS/MS and UPLC-MS/MS was performed for target analytes detected <LOD for identical samples by a student t-test using an integrated Excel add-in data analysis-tool for t-tests.

[1] Inlet sample 3 from HPLC-MS/MS results ≠ inlet sample 3b from UPLC-MS/MS results.

[2] Outlet sample 4 from HPLC-MS/MS results ≠ inlet sample 4b from UPLC-MS/MS results.

Table 5 – Ultra-performance liquid chromatography-tandem mass spectrometry parameters for targeted analytes. Ion transitions used for quantification are listed in mass-to-charge ratio (*m/z*). Electrospray is either positive (+) or negative (-) ionization mode.

Class	**Compound**	**ESI mode**	**Transition ions *(m/z)***
Analgesic	Acetaminophen	+	152→110
Antibiotics	Sulfamethoxazole	+	245→156
	Trimethoprim	+	291→ 230
Antiepileptics	Carbamazepine	+	237→194
Beta blockers	Atenolol	+	267→145
Non-steroidal anti-inflammatory drugs (NSAIDs)	Diclofenac	-	294→250
	Ibuprofen	-	205→161
	Naproxen	-	229→170
Stimulant	Caffeine	+	195→138

Table 6 – Calibration curve parameters for ultra-performance liquid chromatography-tandem mass spectrometry. Regression model linearity is described by the coefficient of determination (R_2), and are based on n observations. Limits of detection (LOD) and quantification (LOQ) are given in ng/L. The slope describes the regression line used for the calculation of sample concentrations.

Compound	**Slope**	**R_2**	**Observations, n**	**LOD, ng/L**	**LOQ, ng/L**
Acetaminophen	210.8	0.991	10	109.67	332.3
Sulfamethoxazole	271.3	0.993	10	19.62	59.46
Trimethoprim	1276	0.985	10	22.32	67.63
Carbamazepine	649.9	0.999	10	17.26	52.29
Atenolol	397.1	0.997	10	18.36	55.63
Diclofenac	12.46	0.999	10	0.68	2.05
Ibuprofen	38.11	0.989	8	3.86	11.70
Naproxen	194.8	0.988	10	17.73	53.73
Caffeine	921.8	0.999	9	22.44	68.01

4. RESULTS

4.1. SAMPLE PREPARATION

4.1.1 WATER REMOVAL FROM SEDIMENT SAMPLES

Relative water losses in sediment samples are calculated using Equation 2 and summarized in Table 7. It was not possible to confirm a complete dryness of samples. However, the process obtained a relative mass reduction of 21.61 - 38.23% when dried sediments were compared to the wet weight of samples. The calculated water loss did not decrease further upon extension of dying time nor increase of operation temperature, hence complete dryness was assumed.

Table 7 – Relative water-loss after freeze-drying of sediment samples.

Relative water loss (%) range (min/max) and mean after the freeze-drying process of top sediments. The time of operation is reported in hours. The wet mass was approx. 10 g for all samples (n = 2-5). Marine stations: DS = discharge station, K = Kvitsøy, BF = Boknafjorden. Coastal stations: SA = Sande Beach, RB = Randabergbukta, SO = Sola beach.

Sample	**Location**	**Water loss (%)**			**Time of operation (hrs)**
		Min	Max	Mean	
Top sediment	DS	29.21	37.93	32.44	8.5
Top sediment	K	28.32	38.23	33.02	4-8
Top sediment	BF	31.00	37.81	35.41	4-8
Top sediment	SA	22.41	24.99	23.70	4-8
Top sediment	RB	22.01	25.31	23.66	4-8
Top sediment	SO	21.61	30.29	25.95	4-8

4.2 CALIBRATION CURVES

In this section, the concentrations will only be reported in the units ng/L. For the concentration of sediment samples, the units can be converted into ng/g dry weight by multiplicating concentrations in ng/L with 0.6 L/g dry weight. Standard curve parameters, i.e., calibration curve slope, R^2, LOD, and LOQ, are described in Appendix 4.

Figure 12 displays chromatograms of one target analyte, CBZ, obtained from the analysis of ten calibration solutions in HPLC_PI_A1B1 and HPLC_PI_A2B1. Peak areas detected are proportional to the concentration of an analyte, in the figure increasing from top to bottom MRMs from 1.95 ng/L to 1000 ng/L.

The linearity of calibration curves analyzed in HPLC_PI_A1B1 was found by the inclusion of up to ten data points in the linear model. The linearity was qualified by the coefficient of determination, R^2. The concentration range of calibration curves was detectable for concentrations equivalent to 1.95–1000 ng/L for CBZ, SUL, ATV, CAF, DCF (n = 10, R^2-values >0.99). For some compounds, the peak signals were not detectable at the lowest concentration levels. Peaks were excluded if S/N ratios were < 10. ATE, ACE and TMP (n = 9, R^2 >0.98), were detectable from 3.91 ng/L. The lowest detectable concentrations were for NAP 15.6 ng/L (n = 7, R^2 >0.99) and, giving poor linearity, 31.3 ng/L for IBU (n = 6, R^2 = 0.92).

In HPLC_PI_A2B1, CBZ, SUL, ATV, CAF, DCF, TMP and NAP showed variable linearity over the full concentration range (n = 10, R^2>0.97). Only CBZ, CAF, TMP and NAP had R^2 > 0.99. Curves obtained for ATE and ACE yielded a good fit to the regression line in the range 3.91 - 1000 ng/L (n = 9, R^2>0.98), and IBU poor fit (n = 7, R^2 = 0.88) over the range 15.6 - 1000 ng/L.

IBU was reanalyzed in negative ESI to improve the calibration model. The improved model had a concentration range of 3.91-1000 ng/L and a linearity of R^2 = 0.991. The results discussed further are for IBU obtained from gradient program HPLC_NI_A2B1 only.

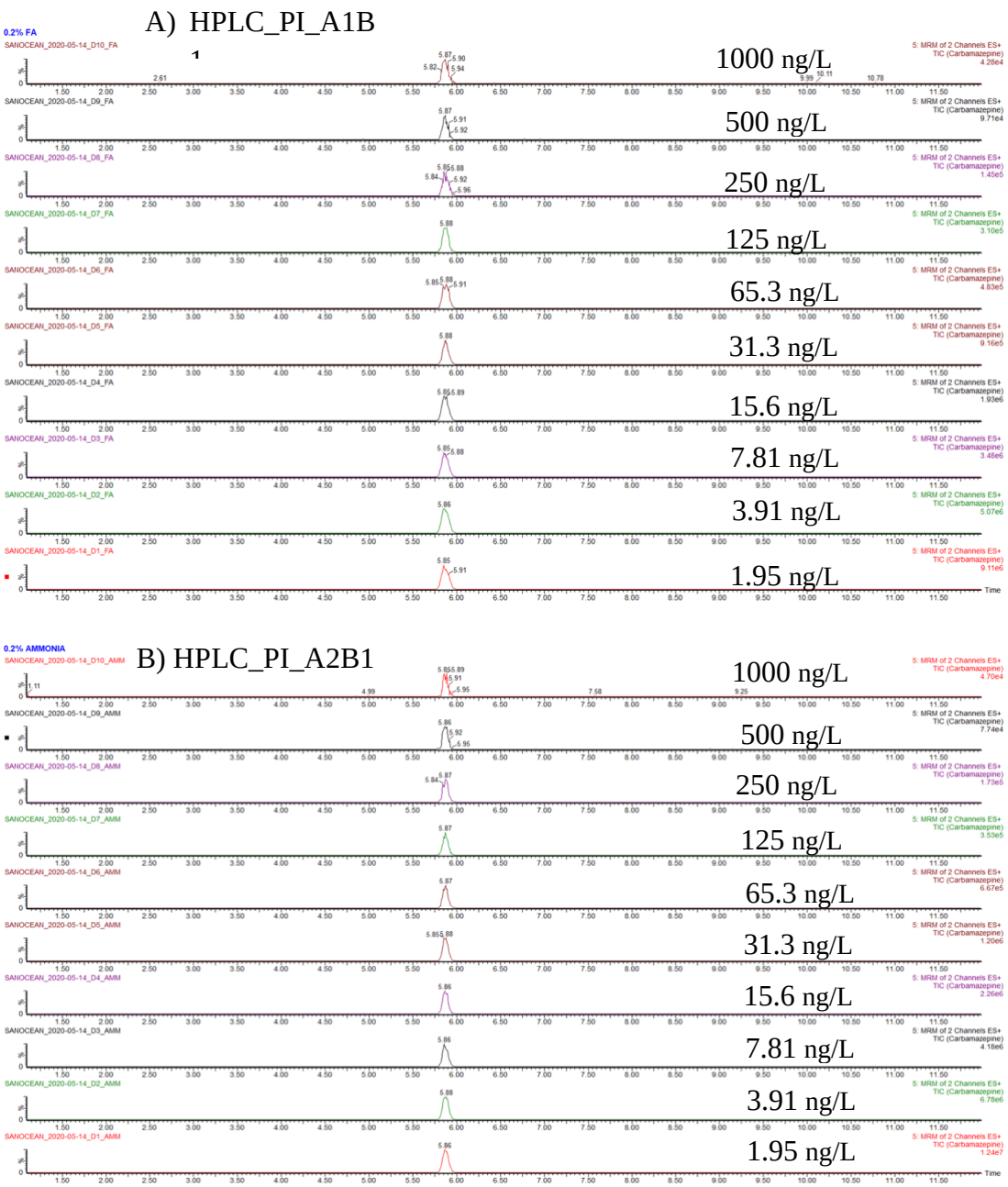

Figure 12 – Chromatograms of carbamazepine in standard calibration solutions.

Total ion current (TIC) of carbamazepine in calibration solution samples was analyzed using the two high-performance liquid chromatogram gradient programs described in Table 3: A) HPLC_PI_A1B1 and B) HPLC_PI_A2B1. The analyte was detected based on ion-transition (237.1→194.1 *m/z*) and retention time (T_R = 5.88±0.2 min). From top: Peak area of calibration solutions samples are equivalent to concentrations of 1.95-1000 ng/L. Chromatograms were obtained from Masslynx version 4.2.

4.3 OCCURRENCE OF PHARMACEUTICALS

Occurrence-data will be presented according to sample type, i.e., inlet, outlet, and seawater samples (in ng/L) and sediment samples (in ng/g dry weight or ng/g). From evaluations of limits of detections/quantification obtained from calibration curves, ACE, ATE, ATV, DCF, NAP, and CAF will be reported from analyses performed in HPLC_PI_A1B1 and CBZ, TMP, and SUL from HPLC_PI_A2B1. Both gradient programs were operated in positive ESI mode. IBU was analyzed in negative ESI mode using mobile phase A2, from here HPLC_NI_A2B1.

Analyses of samples are described in Figure 13. The quantification data of inlet samples were obtained from technical replicates. i.e., one biological replicate was extracted and analyzed by reading this sample three times to obtain an average reading and a standard deviation. Results from assessed outlet samples were obtained from biological and technical replicates. For the composite sample, outlet 1, each replicate was prepared by individual extractions of outlet water collected over 24 hours. Outlet samples 2-4 were extracted separately and analyzed by reading each sample three times to obtain an average reading and a standard deviation. Quantification data was also obtained from biological replicates of seawater samples from marine stations, DS and BF, and biological replicates of sediment samples from marine (DS, K, and BF) and coastal (SA, RB, and SO) stations.

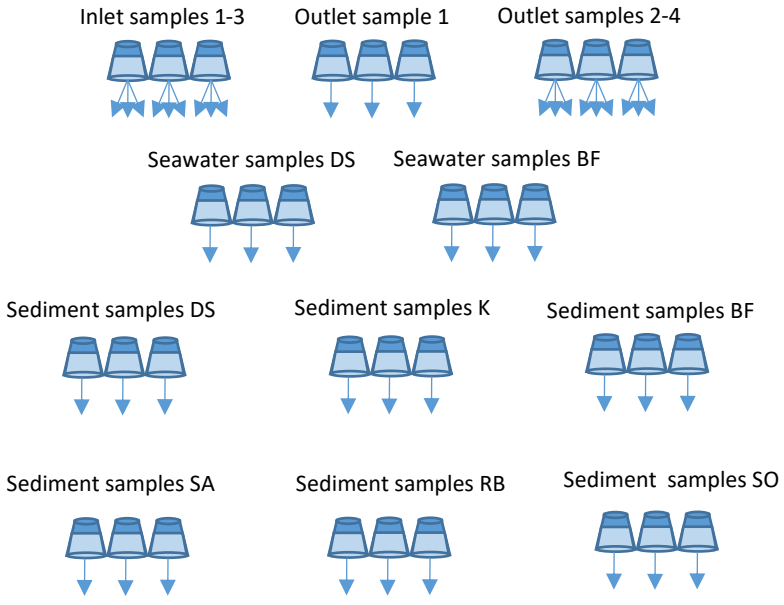

Figure 13 – Instrumental analysis of samples

Arrows indicate sample analysis by high-performance liquid chromatography-tandem mass spectrometry. Biological replicates are samples analyzed one time per replicate, technical replicates are from reanalyzing the same sample extract. DS = discharge station, BF = marine reference station in Boknafjorden, K = marine reference station near Kvitsøy, SA = Sande Beach, RB = Randabergbukta and SO = coastal reference station on Sola Beach.

4.3.1 QUANTIFICATION OF INLET WATER SAMPLES

All targeted APIs except CBZ were detected in at least one sample of inlet water. A summary of all inlet samples is presented in Table 8.

From analyses of inlet samples in HPLC_PI_A1B1 ACE, ATE, ATV, DCF, NAP, and CAF were detected or quantified in at least one sample. Generally, analytes were detected or quantified in inlet samples 1 and 2, but not, or at significantly lower concentrations, in inlet sample 3. ACE was quantified at a high concentration in two out of three samples, with mean values of 10 210, 7 549, and 18.49 ng/L, respectively, and with detection frequency of 100% in all samples. ATE was detected in all samples (100%), and at concentrations of 71.83, 77.11, and 62.21 ng/L respective to samples 1-3. ATV was detected above LOD in samples 1 (42.38 ng/L, 100%) and 2 (48.37 ng/L, 100%), but not in sample 3 (<LOD, 0%). DCF was detected at a frequency of 100% and concentration <LOQ in all samples assessed. NAP was quantified with a detection frequency of 100% in sample 1 and 2 (182.8 and 171.3 ng/L) and <LOQ in sample 3. CAF was detected at high levels in samples 1 and 2, at 5 173 and 4 882 ng/L, but a significantly lower concentration in sample 3, at 89.28 ng/L. CAF was detected with a frequency of 100% in all samples.

From the analysis of inlet samples in HPLC_PI_A2B1, results for SUL and TMP are reported. SUL was detected with a frequency of 100% and concentrations >LOQ in all samples and TMP with a mean concentration of 170.7 ng/L in sample 1 and >LOQ in samples 2 and 3.

IBU was analyzed using the gradient program HPLC_NI_A2B1. The detection frequency was 67% in all inlet samples, and, similar to ACE and CAF, the concentrations were higher in samples 1 and 2 compared to samples 3. The maximum concentration quantified was 10 887, 9 573, and 751.6 ng/L in samples 1, 2, and 3.

Table 8 – Occurrence-data from inlet water samples.

Range (minimum and maximum) and mean concentration in ng/L for target pharmaceuticals in inlet water samples. LOD =limit of detection, LOQ = limit of quantification, frequency = percentage of samples < LOD. NSAIDs = non-steroidal anti-inflammatory drugs, ACE = acetaminophen, SUL = sulfamethoxazole, TMP = trimethoprim, CBZ = carbamazepine, ATE = atenolol, ATV = atorvastatin, DCF = diclofenac, NAP = naproxen, IBU= ibuprofen and CAF = caffeine.

Group	Compound	LOD (ng/L)	LOQ (ng/L)	Min (ng/L)			Max (ng/L)			Mean (ng/L)			Frequency (%)		
				Inlet 1	**Inlet 2**	**Inlet 3**	**Inlet 1**	**Inlet 2**	**Inlet 3**	**Inlet 1**	**Inlet 2**	**Inlet 3**	**Inlet 1**	**Inlet 2**	**Inlet 3**
Analgesic	ACE	0.752	2.279	9 734	7 124	13.13	10 464	8 132	27.04	10 210	7 549	18.49	100	100	100
Antibiotics	SUL	97.72	296.1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	100	100	100
	TMP	33.25	100.8	165.1	<LOQ	<LOQ	174.3	<LOQ	<LOQ	170.7	<LOQ	<LOQ	100	100	100
Antiepileptics	CBZ	25.36	76.86	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
Beta blockers	ATE	20.63	62.53	79.70	68.39	50.53	86.17	91.83	68.37	71.83	77.11	62.21	100	100	100
Lipid regulators	ATV	12.46	37.76	39.25	44.89	<LOD	47.04	50.12	<LOD	42.38	48.37	<LOD	100	100	0
NSAIDs	DCF	11.54	34.98	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	100	100	100
	NAP	25.83	78.29	178.0	169.6	<LOQ	192.0	174.2	<LOQ	182.8	171.3	<LOQ	100	100	100
	IBU	46.47	140.84	<LOD	<LOD	<LOD	10 887	9 573	751.6	5 731	5 960	486.8	67	67	67
Stimulant	CAF	10.99	33.31	4 949	4 573	77.47	5 341	5 394	101.9	5 173	4 882	89.28	100	100	100

4.3.2 QUANTIFICATION IN OUTLET WATER SAMPLES

All target compounds were detected in at least one sample of the SNJ outlet water. A summary of all outlet samples is presented in Table 9.

ACE, TMP, ATE, ATV, DCF, NAP, and CAF were detected or quantified in at least one sample after analysis of outlet samples in HPLC_PI_A1B1. ACE was detected in all samples (67-100%) with means ranging between 2.468-11.79 ng/L. ATE was detected above LOD in all samples (100%) and above LOQ for sample 3, at 85.79 ng/L. ATV was detected (100%) at concentrations <LOQ in sample 2 and 3. DCF was detected in all samples (100%), but only <LOQ. NAP was detected with a frequency of 100% in all samples. Quantification was possible in samples 2 and 3, at 81.76 and 148.7 ng/L, respectively. CAF was detected in all samples (100%) and with mean concentrations of 64.58, 719.4, 775.1, and 79.87 ng/L in samples 1-4.

Analysis in HPLC_PI_A2B1 quantified or detected three target pharmaceuticals. SUL was detected at a frequency of 33% in sample 1; here, the mean concentration was <LOQ. In sample 3, SUL was detected in all replicates, also <LOQ. TMP was detected (100%) in all outlet samples, and >LOQ for samples 3 and 4, at 250.4 and 238.8 ng/L, respectively. CBZ was detected with a frequency of 100% in two samples of outlet water; samples 2 and 3. The concentration of CBZ in these samples was <LOQ.

In HPLC_NI_A2B1, IBU was detected in all samples with a frequency of >75%. Inlet sample 3 had a mean concentration of 180.9±16.34 ng/L, while in the other samples, IBU was detected with a mean concentration <LOQ.

Table 9 – Occurrence-data from outlet water samples. Range (minimum and maximum) and mean concentration in ng/L for target pharmaceuticals in outlet water samples. LOD =limit of detection, LOQ = limit of quantification, frequency = percentage of samples < LOD. NSAIDs = non-steroidal anti-inflammatory drugs, ACE = acetaminophen, SUL = sulfamethoxazole, TMP = trimethoprim, CBZ = carbamazepine, ATE = atenolol, ATV = atorvastatin, DCF = diclofenac, NAP = naproxen, IBU= ibuprofen and CAF = caffeine.

Group	Compound	LOD (ng/L)	LOQ (ng/L)	Min (ng/L)				Max (ng/L)				Mean (ng/L)				Frequency (%)			
				Outlet 1	**Outlet 2**	**Outlet 3**	**Outlet 4**	**Outlet 1**	**Outlet 2**	**Outlet 3**	**Outlet 4**	**Outlet 1**	**Outlet 2**	**Outlet 3**	**Outlet 4**	**Outlet 1**	**Outlet 2**	**Outlet 3**	**Outlet 4**
Analgesic	ACE	0.75	2.3	<LOD	10.139	7.100	<LOD	7.129	13.418	10.884	7.059	4.124	11.785	9.318	2.468	67	100	100	67
Antibiotics	SUL	97.72	296.1	<LOD	<LOD	<LOQ	<LOD	<LOQ	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOQ	<LOD	33	0	100	0
	TMP	33.25	100.8	<LOQ	<LOQ	242.9	231.7	<LOQ	<LOQ	264.0	250.0	<LOQ	<LOQ	250.4	238.8	100	100	100	100
Antiepileptics	CBZ	25.36	76.86	<LOD	<LOQ	<LOQ	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOD	<LOQ	<LOQ	<LOD	0	100	100	0
Beta blockers	ATE	20.63	62.5	<LOQ	<LOQ	84.24	<LOQ	<LOQ	<LOQ	86.72	<LOQ	<LOQ	<LOQ	85.79	<LOQ	100	100	100	100
Lipid regulators	ATV	12.46	37.8	<LOD	<LOQ	<LOQ	<LOD	<LOD	<LOQ	38.64	<LOD	<LOD	<LOQ	<LOQ	<LOD	0	100	100	0
NSAIDs	DCF	11.54	35.0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	40.08	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	100	100	100	100
	NAP	25.83	78.3	<LOQ	<LOQ	139.9	<LOQ	<LOQ	90.47	156.77	78.96	<LOQ	81.76	148.7	<LOQ	100	100	100	100
	IBU	0.75	2.3	<LOQ	<LOQ	<LOQ	<LOD	<LOQ	155.4	191.0	<LOQ	<LOQ	<LOQ	180.9	<LOD	100	100	100	75
Stimulant	CAF	10.99	33.3	57.31	701.6	773.0	72.60	75.64	732.7	776.4	87.10	64.58	719.4	775.1	79.87	100	100	100	100

4.3.3 OCCURRENCE IN SEAWATER SAMPLES.

Analyses of seawater samples in HPLC_PI_A1B1 detected two target APIs in both samples assessed (DS and BF). DCF was detected at a frequency of 33% in samples from both stations. Both detected sample concentration levels were <LOQ. Similarly, CAF was detected in all seawater samples collected from DS and BF, with respective frequencies of 100% and 67%. Concentrations of CAF detected were also <LOQ. Other target APIs were not detected in assessed seawater samples. In HPLC_NI_A2B1, IBU was <LOD in all assessed samples.

Results of the analysis of seawater samples are summarized in with the detection range (min-max), mean, LOD, and LOQ and frequency of detection for all targets in Appendix 5.

4.3.4 OCCURRENCE IN SEDIMENT SAMPLES.

From each sediment sample collected from marine stations, ACE was detected in at least one replicate. In samples collected from DS, a 5.831 ng/g dry weight was detected in one replicate. The other two replicates were <LOD. For samples collected at K and BF, the frequency of detection was 100%, and the mean concentrations were 5.232±4.655 and 2.093±1.506 ng/L, respectively. CAF was detected <LOQ. The detection frequency was 67%, 67%, and 33% for samples collected from DS, K, and BF. No other pharmaceuticals were above LOD in samples of sediment samples collected from either marine or coastal stations and with either HPLC gradient programs. In HPLC_NI_A2B1, IBU was <LOD in marine samples. IBU was not targeted in coastal samples. The results are described in Appendix 6 and Appendix 7.

4.4 VALIDATION

Two recovery studies were performed over sample preparation, and sample analysis in water and sediments spiked with targeted pharmaceuticals at three concentration levels. Five replicates were prepared for each sample. Samples were analyzed by two HPLC-MS/MS gradient programs: HPLC_PI_A1B1 and HPLC_PI_A2B1. IBU was validated using HPLC_NI_A2B1. Recovery accuracy was measured by the average recovery of five replicates and precision by the RSD (Equation 6).

Examples of chromatograms for ATE in water samples, spiked to 60, 800, and 2 000 ng/L in HPLC_PI_A1B1, are displayed in Figure 14. The peak area is proportional to the analyte concentration and calculated from the calibration curve for ATE.

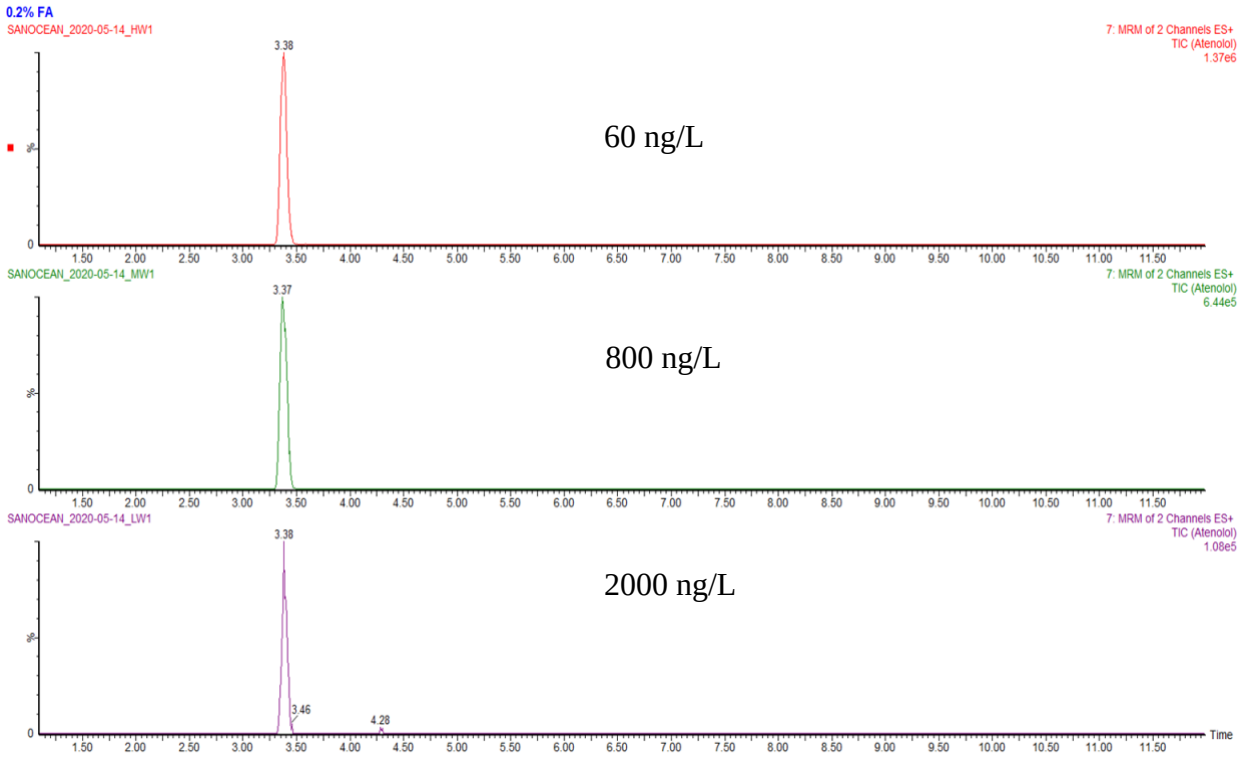

Figure 14 – Chromatograms of atenolol in spiked water samples.

Total ion chromatograms of atenolol in one replicate of water sample spiked to 60, 800, and 2 000 ng/L. Atenolol signal was detected based on in ion-transition (267.3→73.9 *m/z*) and retention time (T_R = 3.39±0.2 min).

4.4.1 RECOVERY OF SPIKED SEDIMENT SAMPLES

Recovery-adjusted concentrations could not be calculated for assessed sediment samples from coastal and marine stations as no APIs were detected above the quantification level in any samples. Recoveries of sediment samples spiked to three concentrations of target pharmaceuticals are presented graphically in Figure 15. ATE had a recovery of >50% for all sample concentrations when analyzed in HPLC_PI_A1B1. The recovery of the lowest concentration, 600 ng/g, of ATE was >100%. ATV has a recovery of <5% for all concentration levels. The remaining target pharmaceuticals were <50% recovered for all concentrations assessed. CBZ, ACE, SUK, CAF, DCF, and NAP had 16 - 47% recoveries. The recoveries were consistent for all replicates where the peak could be detected, with a method precision described by RSD of < 15%, except for NAP (<24%), ATV (<120%) and IBU (12.34-75.38%). The RSD was calculated by Equation 6. A summary table of sample precisions and accuracies is found in Appendix 8.

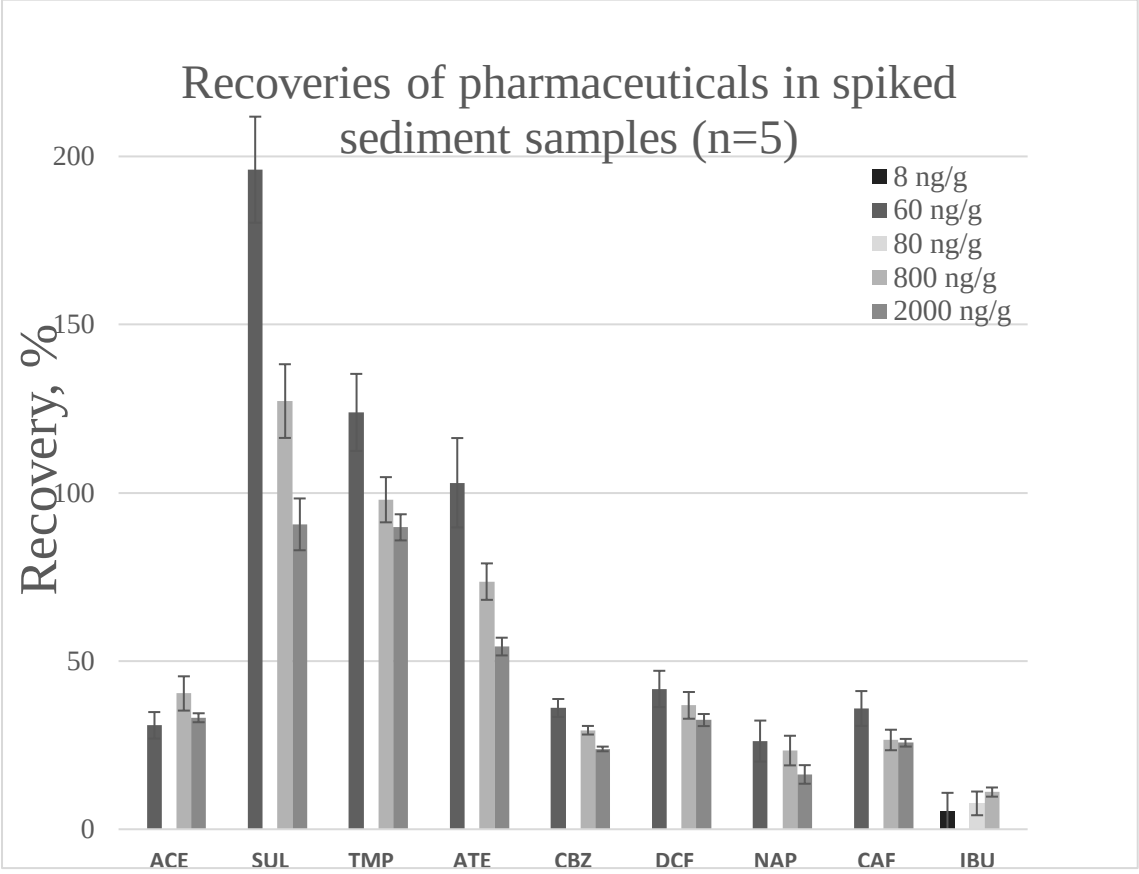

Figure 15 – Recoveries of target pharmaceuticals from analysis of in spiked sediment samples.

Average recoveries of acetaminophen (ACE), sulfamethoxazole, (SUL), trimethoprim (TMP), atenolol (ATE), carbamazepine (CBZ), diclofenac (DCF), naproxen (NAP), caffeine (CAF) and ibuprofen (IBU) obtained by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The recoveries are calculated as a percentage of observed concentration over known concentration. Atorvastatin was excluded from the chart due to poor recovery (<5%).

4.4.2 RECOVERY OF SPIKED WATER SAMPLES

Recovery-adjusted concentrations are described in Table 10 for quantified water samples. Only treatment plant samples were adjusted due to few cases of detection and quantification in seawater samples. The adjustment was made using recovery-percentage of the recovery-level closest to the concentration initially quantified, and only for targets with RSD <30%. For this reason, ATE and IBU were excluded from adjustment.

Recoveries of water samples spiked with target pharmaceuticals are illustrated graphically in Figure 16. From recoveries of pharmaceuticals assessed in HPLC_PI_A2B1, ATE, CAF, and DCF were >50% recovered at the lowest concentration level assessed. Medium and high concentration levels were detected at 19-47% recoveries. ACE and NAP were <35% recovered at all concentrations. ATV had a recovery of >2% for all concentrations. The recoveries were consistent for all replicates where the peak could be detected. The RSD was <10% for ATE, CBZ, SUL, and TMP, <30% for ACE, CAF, DCF and NAP, and <50% for ATV. A summary of mean values and SD of spiked water samples is in Appendix 9.

Table 10 – Recovery-adjusted concentrations in A) inlet sample and B) outlet samples. Adjustment of concentrations was done according to recovery of analytes at similar concentration level. Samples with concentrations <LOD and <LOQ were not adjusted. ACE = Acetaminophen, ATE = Atenolol, NAP = naproxen, CAF = caffeine, TMP = trimethoprim. IBU = ibuprofen.

A) Compound	Before adjustment, ng/L			After adjustment, ng/L		
	Inlet 1	**Inlet 2**	**Inlet 3**	**Inlet1**	**Inlet 2**	**Inlet 3**
ACE	10 210[3]	7 549[3]	18.49[1]	14 190	10 492	19.41
ATE	71.83[1]	77.11[1]	62.21[1]	13.71	14.72	11.88
NAP	182.8[1]	171.3[1]	<LOQ	294.3	275,8	-
CAF	5 173[3]	4 882[3]	89.28[1]	8 578	8 096	108.0
TMP	170.7[1]	<LOD	<LOD	145.1	-	-
IBU	5 731[2]	5 973[2]	486.8[2]	46 279	48 122	3 931

B) Compound	Before correction, ng/L				After correction, ng/L			
	Outlet 1	**Outlet 2**	**Outlet 3**	**Outlet 4**	**Outlet 1**	**Outlet 2**	**Outlet 3**	**Outlet 4**
ACE	4.124[1]	11.79[1]	9.318[1]	2.468[1]	4.326	12.37	9.780	2.590
NAP	<LOQ	81.76[1]	148,7[1]	<LOQ	-	15.62	28.40	-
CAF	64.58[1]	719.4[2]	775.1[2]	79.87[1]	78.10	1091	1176	96.59
IBU	<LOQ	<LOQ	180.9[2]	<LOQ	-	-	1 461	-

[1] Adjusted using the mean of 60 ng/L recovery sample
[2] Adjusted using the mean of 800 ng/L recovery sample
[3] Adjusted using the mean of 2 000 ng/L recovery sample

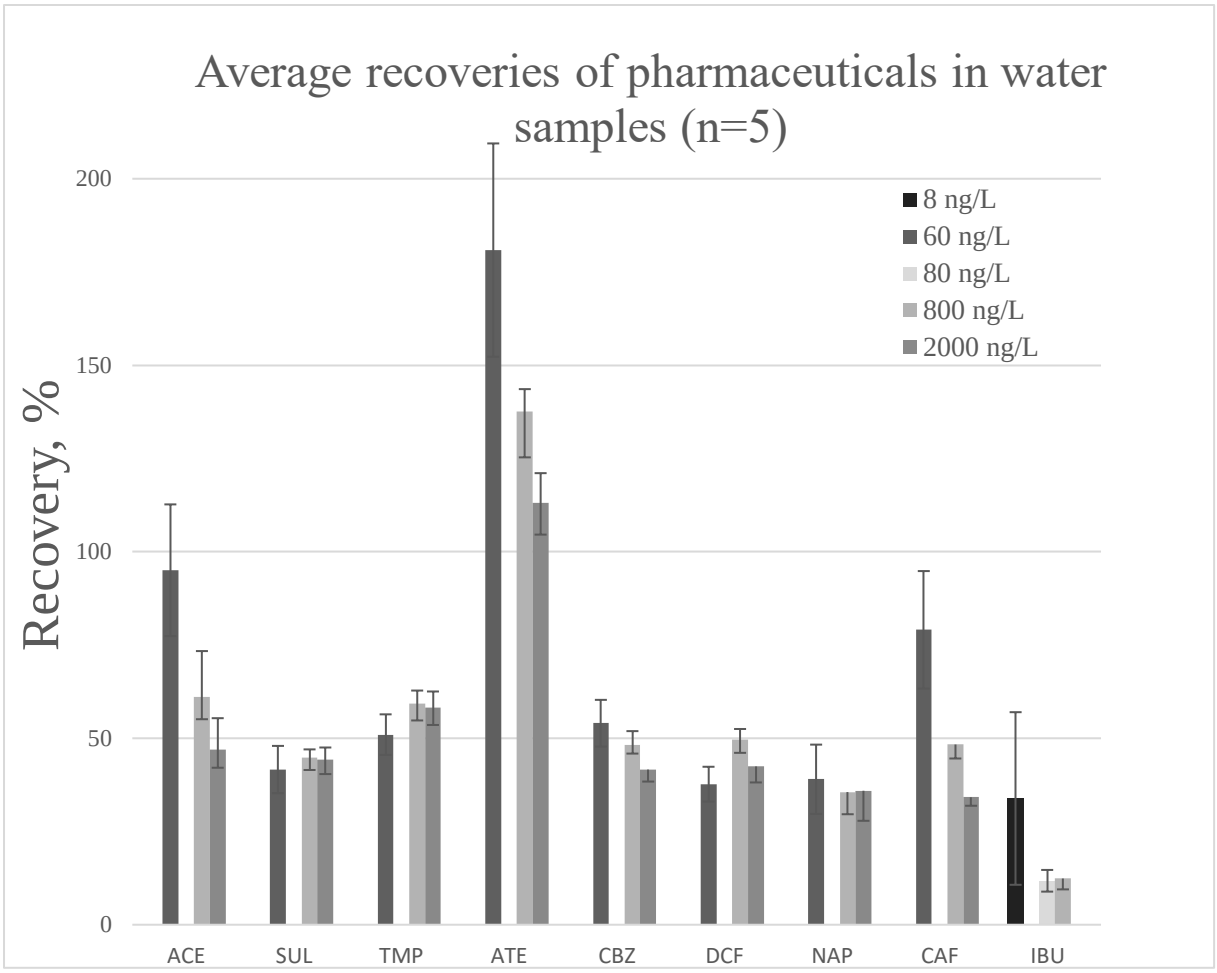

Figure 16 – Recoveries of target pharmaceuticals from analysis of in spiked water samples.

Average recoveries of acetaminophen (ACE), sulfamethoxazole, (SUL), trimethoprim (TMP), atenolol (ATE), carbamazepine (CBZ), diclofenac (DCF), naproxen (NAP), caffeine (CAF) and ibuprofen (IBU) obtained by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The recoveries are calculated as a percentage of observed concentration over known concentration. Atorvastatin was excluded due to poor recovery (<5%).

4.4.3 COMPARISON BY HPLC-MS/MS AND UPLC-MS/MS

Quantification data obtained for samples using UPLC-MS/MS are presented in Appendix 10 (treatment plant samples), Appendix 11 (seawater samples), and Appendix 12 (sediment samples), with quantities and uncertainties calculated from respective calibration curves.

Quantification data obtained by HPLC-MS/MS and UPLC-MS/MS for identical samples with concentrations >LOQ were compared by a two-sided, paired student t-test (Table 11). The following samples and target analytes were included in the t-test: inlet sample 1-2 (ACE, NAP, CAF, DCF, IBU), and outlet samples 2 - 3 (CAF and IBU). The t-test assumed equal variances, two sides, and $\alpha/2 = 0.025$, i.e., there was a significant difference between the samples if the p-value was $<\alpha/2$. There was a significant statistical difference between all samples tested except for CAF quantified in outlet sample 2-3 ($p = 0.073$). The quantification by UPLC-MS/MS yielded higher concentrations in samples compared to quantification by HPLC-MS/MS for the other samples tested. One exception was IBU in inlet samples 1-2, where the concentration was higher after analysis by HPLC-MS/MS.

Table 11 – Statistical comparison of quantification data.

A comparison of samples is described for four analytes in treatment plant samples. T is the critical value calculated from observations with six degrees of freedom (df), and t is the rejection value. The p-value is the probability p(T<=t). Significantly different values, $p< \alpha/2 = 0.025$, are marked with *. In1-3 = inlet samples 1-3, out 2-4 = outlet samples 2-4.

Analyte:	***Sample***	***T /t***	***df***	***p-value, p(T<=t)***
Acetaminophen	**In1-2*	*2.447/-6.736*	*6*	*$5.213*10^{-4}$*
Ibuprofen	**In1-2*	*2.77/4.27*	*4*	*0.013*
Naproxen	**In1-2*	*2.447/-22.57*	*6*	*$4.948*10^{-7}$*
Caffeine	**In1-2*	*2,447/-17.993*	*6*	*$1.896*10^{-6}$*
	Out 2-3	*2.447/-2.167*	*6*	*0.073*

5. DISCUSSION

5.1 METHOD DEVELOPMENT

The preparation of samples was initially performed following existing protocols [59], [60], Protocols were developed to extract seawater samples [60] and have also been used for the extraction of sediments and biota samples [59]. Some modifications were made, such as the use of UAE for the API extraction from top sediments.

Final protocols proved successful for the extraction of all targeted APIs except ATV. Low recoveries experienced were likely the result of a reduced sorption efficiency in the SPE step or the signal suppression by matrix impurities [61]. Recoveries were tested in matrices different from the actual wastewater and environmental samples. Hence, sample matrix effects are not considered in this thesis. However, this could represent a limitation to electrospray ionization HPLC-MS/MS due to co-elution of matrix impurities [61]–[63].

The optimization of extraction methods by changing parameters, e.g., solvents and medium pH, and analysis of additional pharmaceuticals in both negative and positive ESI mode was planned, but not carried out in time for this thesis submission due to the closing of university facilities during the initial phases of the SARS-COV-2 pandemic. Evaluations and suggested improvements in preparation steps are discussed hereafter.

5.1.1 FREEZE DRYING OF SEDIMENTS

Freeze-drying sediment samples aimed to equalize the sample mass, i.e., to ensure that variations in wet mass did not affect the amount of sediment used for the extraction. By operating the freeze-dryer at the lowest obtainable temperature and pressure, loss or damage of potentially unstable or volatile APIs was minimalized. For most APIs, the degradation resulting from freeze-drying is assumed to be low and insignificant with regards to the recovery [64]. The used bottles (Fast-Freeze® Flasks) were of borosilicate and designed to maximize heat transfer during drying and to withstand a high vacuum. The flat bottom allowed a large sample surface [65]. The potential loss of analytes over the freeze-drying process was not assessed. However, it is highly recommended for future studies.

5.1.2 FILTRATION OF WATER SAMPLES

Inlet, outlet, and seawater samples were filtered to remove impurities and prevent the clogging of SPE cartridges and the UPLC column. Filtration of samples has been demonstrated to reduce chromatographic noise [57]. Loss of analytes due to the filtration step was assumed minimal, as most target APIs were polar and, therefore, likely present in the water phase rather than adsorbed to suspended particles. The use of a centrifuge was briefly tested for the separation of particulate solids. It was discontinued due to poor efficiency. The trial of utilizing centrifugation is not described further.

The turbidity of matrices of liquid samples varied. Seawater samples had low turbidity and could easily pass the filtration step (<0.2 µm). On the other hand, inlet and outlet water samples were visibly more turbid. Inlet samples, being sewage and wastewater collected after minimal pre-filtration, had the highest turbidity and required frequent changing of filter-paper due to clogging. Some discoloration was observed in both filtered inlet and outlet samples, despite filtering at 0.2 µm pore size before analysis by HPLC/UPLC-MS/MS.

Vasskog et al. (2008) found that the addition of NaOH to increase the pH of seawater and wastewater effluents resulted in the precipitation of insoluble salts [66]. The removal of these salts was reportedly beneficial for the prevention of SPE clogging and also improved the detection by HPLC-MS/MS. The improved detection was, assumedly, due to the removal of potential ion-suppressants and did not influence the recovery negatively. For our water samples, no clogging of SPE cartridges or UPLC column were experienced. However, some precipitation effects were observed in storage vials for inlet and outlet despite filtering extracts (0.2 µm) before storage. No precipitation was observed in seawater or sediment extracts.

5.1.3 ULTRASONICATION OF SEDIMENT SAMPLES

The UAE has previously been used to extract various pharmaceuticals from solid matrices, including soil [67], sediment [68] [69], and biological matter [70]. The advantages of this method include the extraction of compounds attached to particles in an environmental sample in a fast, cost-effective and straightforward manner without using large volumes of solvents, in contrast to other extraction methods (e.g., Soxhlet).

The ultrasonication can be carried out using either a bath or a probe. An advantage of using a bath is the simple and fast procedure. The distribution of ultrasonic waves into the entire bath allows the simultaneous extraction of multiple samples. However, the bath extraction has reportedly low reproducible due to variations in the intensity [71]. The use of an ultrasonic probe ensures precise exposure yet require one sample to be extracted at a time. There is also a risk of overheating or production of byproducts in the sample because the exposure is more focused [71], [72]. Water, suspended gasses, or organic solvents can be transformed damaging agents, e.g., radicals, by the energy generated [67], [72].

Solvents used with the ultrasonic bath were MeOH and Milli-Q water with 1% formic acid or 1% ammonia. The use of water and MeOH was assessed in a study on sewage sludge by Gago-Ferrero et al. (2015) [73], yielding satisfactory recoveries for most assessed APIs. Water and MeOH of varying pH´s have also been assessed in similar studies, including MeOH (pH 8) for antibiotics [74], [75] and MeOH and water (pH 11) for analgesics such as NAP, ACE, IBU, CAF and DCF, antibiotic SUL and antihypertensive ATE [75]. An increase in the solvent pH by NaOH or ammonia has been demonstrated to increase the yield by opening cells in biological tissue without noticeable degradation of target analytes [70]. Al-Khazrajy and Boxall (2017) successfully extracted pharmaceuticals from sediment samples by a stepwise extraction, using both low (formic acid) and high (ammonia) pH of solvents, i.e., water, ethyl acetate, acetone and MeOH [68]. Variations in pH could affect the extraction of pharmaceuticals because of the change in binding properties to the solid matter. Due to the variation of APIs' chemical structure, the use of one method could compromise some compounds' recovery. The use of several extraction designs will secure the extraction of a broad range of targets and is proposed as a measure for further method optimization [76], [77].

5.1.4 SOLID-PHASE EXTRACTION OF SAMPLES

The aim of the extraction by SPE was to remove interfering components from the samples and to concentrate target analytes. A challenge for the extraction of APIs is to target both acidic, neutral, and alkaline compounds of different therapeutic groups. Oasis HLB cartridges have been preferred over others, e.g., C_{18}-silica, because of their robustness for high and low pH and their load capacity [78], [79]. Homogenous packed beds of HLB have advantages with regards to flow capacity and reproducible characteristics [78]. Another advantage of HLB cartridges is the capability for drying out. In silica-based sorbent, the drying out significantly reduces the recovery, while polymeric sorbets are more resistant to running dry. However, a conditioned sorbet has been recommended to secure an even flow rate. Kasprzyk-Hordern et al. (2008) found low recovery by SPE, possibly due to HLB´s ability to absorb matrix impurities, and that the error potentially leads to ion suppression by ionization before detection by mass spectrometers [61].

The pH of assessed samples was not adjusted before extraction, as performed in other studies [60], [80]. Initially, more than 70 compounds from more than 20 different therapeutic groups were targeted, and selected SPE conditions were foreseen to not be optimal for all targets in this preliminary extraction. For future multi-target assessments, optimization taking into account target properties will likely improve the recovery. The medium pH largely affects the binding of analytes to particles their retention in the sorbent. Targeted APIs were generally acidic, except for the alkaline compounds CBZ and ATE and of alkaline-neutral TMP. In addition to acidity, the solubility of compounds could have affected the compatibility with the extraction method. Generally, target analytes were highly water-soluble, except ATV. CAF, ACE, and ATE are water-soluble compounds (>10000 mg/L), TMP, and SUL relatively soluble (4 - 600 mg/L) and the others slightly soluble (<20 mg/L). ATV is the least soluble (0.001 mg/L). For targeted APIs, ATV was the only non-polar analyte and also the API with the lowest recovery, demonstrating the need for further optimization.

Extraction methods similar to those described in the present study have used various solvents, e.g., MeOH, water, and ethyl acetate [81]. Reportedly, the pH has been adjusted by the addition of acid for the extraction of polar APIs. For example, Valdez et al. (2004) [60] found that the extraction by SPE yielded the highest recoveries when the pH was adjusted to 6. Because only one SPE condition was tested in this thesis, no comparison of recoveries has been made.

5.2 INSTRUMENTAL ANALYSIS

One of the most useful LC-MS/MS applications is the study of micropollutants´ occurrence and fate [82]. With the increased sensitivity of analytical instruments such as HPLC- and UPLC-MS/MS, the detection of trace amounts (ng/L) of APIs has been made possible [76]. APIs are found highly diluted in nature, yet many are assumed bioactive even at such low concentrations [83]. APIs found in the environment are generally of too low concentrations to pose any acute toxicity. However, chronic effects exerted on non-target organisms, and the potential synergetic effects of mixtures are of concern [84]. Without regulated monitoring, detection and quantification are limited to few studies [85].

In the following sections, instrumental analysis and analytical limitations will be discussed. Also, detected APIs will be presented according to therapeutic groups and a discussion of findings in the context of removal efficiency and concerns regarding potential environmental impacts.

5.2.1 ANALYSIS BY HPLC-MS/MS

Simultaneous analyses of APIs from diverse groups require a compromise in the experimental conditions and may reduce the recovery and detection of some targets [86]. To improve detection by HPLC-MS/MS, targeted APIs were analyzed under various conditions, as described in Chapter 3.4. Calibration curves yielded relatively low detection limits. Analyses successfully quantify a range of APIs in inlet and outlet samples from the SNJ and detected others in both WWTP and environmental samples.

Results obtained from the comparison of identical samples by HPLC-MS/MS and UPLC-MS/MS revealed that there was a significant difference ($p<0.025$) for all samples, except for the quantification of CAF in outlet samples ($p = 0.07$). The two instruments had similar LOD, yet the UPLC-MS/MS demonstrated higher sensitivity for detection for all compared APIs, except IBU [57], [61], [63], [87], hence more APIs were detected above LOD and LOQ.

HPLC-MS/MS standard calibration curves were prepared as external standard calibration curves. The advantage of the external calibration curve includes easy preparation and fast analysis. However, there are weaknesses when using external standard calibration compared to calibration curves by more time-consuming or costly calibrations, e.g., such as standard addition or internal standard calibration [62]. For external calibration, errors in sample preparation and instrumental

analysis are being transferred to the calibration curve. No IS could be obtained for our study. Internal standard calibration could, therefore, not be performed. The primary purpose of utilizing an IS is to improve the accuracy and precision of the quantification, as there is a constant relationship between IS and target analyte peaks. The use of stable isotopically labeled ISs ensures that the IS does not already exist in the sample. However, it possesses the same chemical and physical properties as the target analyte, e.g., T_R and *m/z* ratio of precursor ion and fragments [88]. The use of an IS should be added as early as possible in the sample preparation method. The addition of IS´s can also correct for the loss of analytes during the preparation process.

A challenge for the calibration curve design was to achieve both a low LOD/LOQ, in order to assess the low concentrations, but at the same time to include a suitable range for analytes. The obtained instrument LODs and LOQs resulted in APIs occurring below >20-50 not being detectable. Other limitations of calibration curves were that some APIs were detected outside the calibration curve test range. Therefore, the linearity of these concentrations was not qualified. However, from the recovery samples spiked to 2 000 ng/L or 2 000 ng/g. There were no apparent deviations in the recovery measured compared to lower concentrations (60 and 800 ng/g), restoring some faith in the extrapolated calibration curve. APIs quantified at high concentration levels, such as CAF (>5000 ng/L) and ACE (7 000 - 10 000 ng/L) in inlet samples, were outside the calibration curve range. In other samples (outlet, seawater, and sediments), no APIs exceeded 800 ng/L. A separate, extended calibration curve is proposed for improving the detection of APIs expected to appear at high concentrations in inlet samples. The recovery of targeted APIs indicated a higher concentration being present in samples than what was detected. As discussed in previous chapters, improved sample preparation methods, which lead to higher target recoveries, enable detection of more APIs above LOD and LOQ in future samples.

5.2.2 FINDINGS

Targeted APIs were expected in treatment plant samples based on reported removal frequencies and environmental concentrations detected in similar studies [4], [28], [34]. However, considerable variation between treatment plants is also usual. A remark for the interpretation of results is that differences between compared inlet and outlet samples in the present study cannot be directly translated into the removal efficiency of the SNJ.

API concentrations detected are often at levels considered too low to pose an acute risk for humans. However, it is still unknown whether receptors in non-target organisms, e.g., aquatic organisms, are sensitive to pharmaceutical residues or mixtures. Combinations of pharmaceutical compounds may exert a more substantial toxic effect than each compound individually [89]. When pharmaceuticals are introduced continuously to aquatic ecosystems, organisms may experience exposures similar to those of traditional contaminants [81].

Regarding the selection of APIs in this study, only parent compounds were targeted. As a proposition for future assessments, many metabolites are of great concern due to their increased polarity and are suspected to be present in the water phase of effluents [83]. Transformation products are assumed to have higher persistency and sometimes also higher toxicological properties. Therefore, they represent an essential part of discharge [91]. Müller et al. (2013) point out the unfortunate tendency in the literature of reporting only the elimination of parent pharmaceuticals, rather than biotransformation of the parent into transformation products, which gives a more precise image of the contaminant fates [92].

5.2.2.1 ANTIBIOTICS

Today, one of the most relevant global concerns is the threat of antibiotic-resistant and multi-resistant bacteria. In turn, antibiotic resistance can lead to ineffective treatments of infections, and possibly fatal outcomes [93]. The constant addition of antibiotics into natural environments may facilitate the development of bacteria's resistance by selective removal of non-resistant bacteria, multiplication of the resistant strains, and horizontal transfer of resistance gene [94], [95]. Therefore, finding of antibiotics, i.e., SUL and TMP in the SNJ outlet water samples, of up to <LOQ and 238.8±9.820 ng/L respectively, could be of concern. The fate of these compounds remains unknown, as the assessed sediment and seawater samples did not contain any traces of the antibiotics above detectable levels.

TMP and SUL were quantified at low concentrations in the outlet water samples compared to other studies, which report up to 2 000 ng/L SUL and 660 ng/L TMP in WWTP effluents [96], [97]. Only a fraction of SUL is reportedly excreted unmodified (5 - 15%) [92], [96]. However, some of its metabolites are readily cleaved back to the original compound after processing WWTP steps. SUL has reportedly low removal efficiency from conventional WWTPs, about 47% [98]. TMP excreted approximately 60% unchanged structure [96], and is removed with a reportedly higher efficiency [92], [99], [100].

Kümmerer (2003) reports that most antibiotics are persistent in the environment due to inadequate WWTP removal [84]. In a later study, Kümmerer (2009) describes that a critical pathway for the elimination of antibiotics is by sorption [101]. Further, he reports that many antibiotics tend to bind to particles and that this property may affect their transport and elimination. Antibiotics are not necessarily removed by biological or photochemical degradation. This conclusion is often assumed to explain the disappearance of many APIs when released into the environment. Millić et al. (2012) report that although the uptake of antibiotics by biota is not proven, there is a close relationship between organisms and the surrounding environment with the potential of transferring harmful contaminants [98].

The Norwegian Prescription Database reports that the county of Rogaland uses <10% of antibiotics distributed in Norway, equivalent to approximately 2.3 million DDD in 2018. TMP and SUL are often consumed together [10], [98], yet TMP was in the present study detected at a higher frequency and higher concentrations when compared to SUL. The input of antibiotics into natural environments related to agriculture and aquaculture is a small contribution to the overall use. The Norwegian Seafood councils report that the Norwegian salmon is free from antibiotics and that in 2015 only 0.5% of the antibiotics were related to aquaculture, 10.2% to agriculture, and 89.3% of antibiotics were related for human use [102]. From the 2016 report from the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC), Norway reportedly has among the lowest uses of antibiotics in agriculture. Already, actions are taken towards limiting the use of antibiotics, suggested by, e.g., WHO [103] and the Norwegian Directorate of Health [12].

5.2.2.2 ANTIEPILEPTIC

CBZ was detected above LOQ in outlet samples after analysis by HPLC-MS/MS and quantified or detected in all inlet and outlet samples by UPLC-MS/MS (Appendix 10). The highest concentration quantified was 82.10 ng/L in an outlet sample. The improved detection by UPLC-MS/MS confirms the improved sensitivity of this instrument. Detected concentrations in the present study were significantly lower than in other studies, where CBZ has been considered a persistent marker of wastewater effluents in the environment [80], [104], [105], Concentrations of CBZ in effluents from a German WWTP effluents were reportedly up to 6300 ng/L [25], [83]. Zhang et al. (2008) report that, due to CBZ being persistent and hardly biodegradable at low concentrations in the acidic effluents, less than 10% of residues are removed before discharge [25]. In the SNJ samples assessed, higher concentrations were detected in the outlet water samples than inlet water samples. Similar to other APIs targeted, CBZ is excreted mainly metabolized (99%), which during the treatment process is cleaved back into the parent compound, resulting in an elevated concentration in outlet water and environmental concentrations [25], [83], [99], [106].

CBZ has been demonstrated to exert toxic effects on aquatic organisms only at relatively high concentrations. However, Ferrari et al. (2003) reported predicted no-effect concentrations of 0.42 µg/L [107]. Quinn et al. (2008) classified CBZ as harmful using EU directive 93/67/EEC, with its effect being "harmful to aquatic organisms and may cause long term adverse effects in the aquatic environment". However, they did also state the need for further risk assessment of chronic effects [108].

Regarding the output from the HPLC-MS/MS, a significant splitting of the CBZ peak into two equal-sized peaks with the same ion transition (237.1→194.1 *m/z*) was consistently observed in inlet and outlet samples. The double peak was not observed in the calibration curve samples nor in the recovery samples. The splitting is illustrated by a comparison of the TIC of one calibration solution and one inlet sample in Figure 17. The cause of the double peak was not confirmed but is hypothesized to be a result of interference in the complex WWTP sample matrix. The splitting also occurred in the UPLC-MS/MS analysis of SNJ inlet and outlet samples and a similar study by Gago Ferrero et al. (2015) [73]. Possible errors due to the peak splitting are proposedly lower detection of CBZ than the actual amounts present in samples.

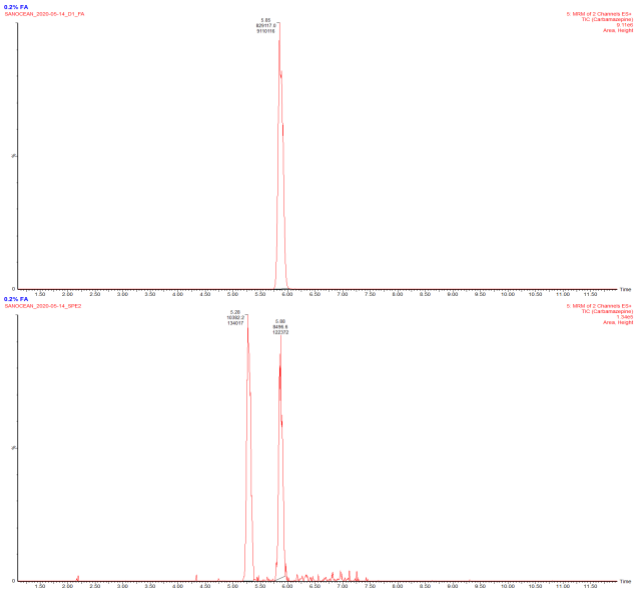

Figure 17 – Splitting of carbamazepine. Peak splitting of carbamazepine was observed in all treatment plant samples assessed, as illustrated in a calibration solution sample (top) compared to an inlet sample (bottom). The retention time of the main peak was 5.88 min, while the second peak has a retention time of 5.28 min.

5.2.2.3 ANTIHYPERTENSIVE

ATE has been ubiquitous in treatment plant samples for the past decades [4], [109], with detection in wastewater effluents of up to 840 - 2870 ng/L [99], [106]. Samples assessed in the present study were significantly lower, with concentrations quantified to a mean value of 73.40±12.22 ng/L in inlet samples and ranging from <LOQ to 86.72 ng/L in outlet samples. The removal of ATE has been proven inadequate. Reported efficiencies vary between <10 and 55% [60], [99], [106], consistent with findings from the SNJ. Poor elimination from WWTP effluents is proposed by Maurer et al. (2007) to be a result of the low adsorption to sludge under treatment conditions, due to their protonated state (pK_a = 9.60) and low K_{ow} (log K_{ow} = 0.16) [109]. ATE has been suggested as a poor representative for the therapeutic group. Its properties widely deviate from other beta-blockers, such as metoprolol, oxprenolol, and propranolol [110].

ATE was not detected in any environmental samples of the present study, and, therefore, its fate remains unclear. ATE is classified as a beta-blocker, and as non-target aquatic have been characterized with similar receptors, there are concerns regarding their non-target effects, as reported by Küster et al. (2010) [110]. However, Maurer et al. (2007) report a low bioaccumulation potential for ATE [109]. This is in agreement with Valdéz et al. (2014), which found the bioaccumulation of ATE to be dose-dependent [60], and that concentrations >µg/L, i.e., concentrations several magnitudes higher than those detected from the present SNJ samples, are not within the range of risk of toxicity.

5.2.2.4 ANTILIPIDEMIC

ATV is reported with high user-doses, comparable to those of conventional non-prescriptive pharmaceuticals, such as ACE, DCF, IBU, and NAP [10]. ATV is, therefore, an API of interest. In previous studies, concentrations of 76±3 ng/L in inlet water samples and 37±2 ng/L in outlet water samples have been quantified [111]. Similarly, the SNJ inlet samples' concentrations of ATV were 48.37±3.01 ng/L and below LOD for outlet samples. Findings are consistent with the removal efficiency, which is reportedly high, e.g., up to 99% [112].

ATV is mostly removed by biodegradation and has been reported to exert no harm to aquatic life at concentrations found [113], [114]. Ottmar et al. (2012) report that effluent levels ranging from 100 to 300 ng/L is several magnitudes less than toxic values, but suggest possible transformation products as future targets of interest given the high percentage of removal due to biodegradation (90%) [114].

A remark for the extraction of ATV was that recoveries were very poor (<5%) for ATV in both matrices assessed, with high RSDs (up to 120%). Therefore, precautions must be taken before making any adjustments to ATE concentrations quantified in samples. One possible cause for the low recovery is the interaction between the carboxylic side-group of ATE and the alcohol solvent (MeOH), resulting in ester-formation, as described by Miao and Metcalfe (2003). However, this interaction has not been observed in the recovery and standard calibration solutions, and MeOH has also been the used solvent for similar studies [115], [116].

5.2.2.5 ANALGESICS

Reduced concentrations of ACE found in treatment plant samples from the SNJ agree with the efficient removal by WWTPs reported regardless of the type of treatment applied., e.g., 96 -100% [62], [83], [99], [104], [117]. Gómez et al. (2007) reported high concentrations of ACE in WWTP influent samples, up to 346 µg/L, while ACE was often not detected in effluent samples [117]. Similarly, Aymerich et al. (2016) report on high influent concentrations, up to 18.52±13.12 ng/L, and significantly reduced effluent concentrations (31±46 ng/L), with a load decreased by more than 90% [118]. Comparatively, in samples from the SNJ, ACE was detected at high levels in inlet water samples, with the highest mean concentration of 10 210±412.7 ng/L, and concentrations ranging between <LOD and 13.42 ng/L in outlet water samples. High concentrations of ACE occurring in inlet water samples is likely related to the high consumption of non-prescriptive drugs

[119]. Even though ACE is one of the most consumed drugs globally, limited research has been done on its occurrence in marine environments.

ACE was not detected in any seawater samples. However, it was detected in marine sediments at concentrations up to 5.831 ng/g in samples collected from DS. In samples collected from BF and K, concentrations were <LOD. K. Löffler et al. (2005) argue that the transport of ACE into sediments is not explained by its chemical properties, which does not readily favor lipophilicity. However, the occurrence in sediments is possibly linked to the transformation of ACE in contact with particles and an increased binding by transformation products [120].

Oliveria et al. (2015) report a medium to high risk for aquatic organisms exposed to ACE, even after removal of ACE from WWTP effluents with reportedly good efficiencies [62]. On the other hand, Grung et al. (2008) report that ACE is readily biodegradable with no persistence in the environment [121]. Chronic effects related to ACE in high doses (mg/L range) suggest, according to Kim et al. (2014), that "potential ecological risks and ecotoxicological assessment of environmentally relevant levels of pharmaceuticals for long-term exposure is needed to more realistically characterize the ecological significance of pharmaceutical contamination in the environment" [112].

5.2.2.6 STIMULANT

The natural origin of CAF is primarily tropical (i.e., coffee and tea) and, therefore, findings in non-tropical areas are related to treatment plant effluents and discharge of domestic waste [13]. High concentrations in wastewater samples are in agreement with the high intake through caffeinated beverages consumed. In Norway, Weigel et al. (2004) assessed raw sewage and were able to quantify up to 293 000 ng/L CAF. They also reported a reduction in CAF after the wastewater treatment in agreement with findings in the present study. The removal rate is in agreement with Nödler et al. (2014), which report removal efficiencies up to 90% [104].

Despite high removal rates, substantial concentrations remain in outlet samples. CAF has a high water-solubility and is expected to be detected in the water phase, e.g., in WWTP effluents rather than adsorbed to sludge. CAF is assumed to pose little risk to the aquatic environment [108]. However, CAF has been a measure of exposure to untreated sewage, which can be harmful due to, e.g., harmful algae blooms or coliform bacteria (>400 ng/L) [104].

CAF has been detected in remote locations, proposing that CAF is a useful tracer for domestic waste in marine environments [26], [104], [122]. In the present study, CAF was detected at concentrations <LOQ in seawater samples from both marine stations, and in all marine sediment samples. In seawater samples, concentrations were similar for samples collected close to the discharge point and at a selected reference site.

5.2.2.7 NSAIDs

In all treatment plant samples, NAP and DCF were detected with a frequency of 100% and IBU with a 66-100% frequency. The rate of detection of NSAIDs in the SNJ samples is consistent with literature reporting frequent detection of these commonly used drugs in samples from WWTPs [83]. Effluents have been reported with concentrations as high as 2 600 – 5 700 ng/L for IBU [3], 1 800 – 4 600 ng/L for NAP [3], and 140 - 1 600 [83], [123] for DCF. The concentrations detected in the present study were lower than what has been reported in other publications.

Carballa et al. (2004) reported a 50 - 55% reduction of NAP and a 70% reduction in IBU by a biological WWTP [3]. However, our assessments indicate a much more variable difference between inlet and outlet samples. NAP was quantified to a maximum of 182.8±7.931 ng/L in an inlet sample and ranged between <LOQ to 148.7 ng/L in outlet samples. The mean concentration of IBU varied in the SNJ samples, but a much lower concentration was always found when comparing inlet and outlet samples. In inlet samples, concentrations of IBU was from 486.8 to 5 960 ng/L and from <LOQ to 180.9 ng/L in outlet samples. The removal of NAP and IBU was mainly a result of the biological treatment step. However, the removal of these contaminants by WWTPs is often inadequate as NSAIDs are generally acidic and with little affinity for adsorption to sediments [80]. However, with decreased pH, e.g., ocean acidification due to global environmental changes, the trend may turn due to their pH-dependent ionization state [3], [124]. DCF has a low removal efficiency reportedly from WWTP because of low adsorption to sludge [99], [125]. In the present study, DCF was detected <LOQ in all inlet samples and mean values ranging from <LOQ to 34.85±6.253 in outlet samples. The concentration of DCF increased in the treated wastewater, likely due to the transformation of metabolites into the precursor compound.

In seawater, DCF was detected at concentrations up to 15 ng/L and a frequency of 33% in samples collected from DS and BF. Neither NAP nor IBU was detected in seawater, and no NSAIDs were detected in sediment samples. IBU was not targeted in coastal samples (SA, RB, and SO). The

detection of low concentration of the API at the reference site BF could also indicate another source of contamination.

DCF has now been recognized priority substance and is included in a dynamic Watch List for monitoring in the marine environment, by Directive 2008/105/EC (the Environmental Quality Standards Directive, EQSD). DCF has previously been linked to bioaccumulation in vultures and the decrease in several species in India and Pakistan, as a result of use as a veterinary drug [126]. Quinn et al. (2009) classified IBU and NAP as toxic according to EU directive 93/67/EEC [108] with regards to both chronic and acute toxicity, in agreement to similar studies on NSAIDs [96], [107], [127]. The levels of toxicity were higher than the rates often found in WWTP effluents. However, the synergetic acute toxicity effects of mixtures of NAP, DCF, and IBU have been demonstrated, e.g., by Cleuvers (2004) [128].

5.3 PERSPECTIVES AND PROPOSALS FOR THE FUTURE

Samples described in this thesis were collected in the fall of 2019. Seasonal variations with regards to findings of APIs have been previously observed in similar studies. For example, Vieno et al. (2005) found that the levels of pharmaceuticals detected in inlet and outlet water samples during winter seasons were up to five times higher for targeted pharmaceuticals, herein DCF, IBU, and NAP [129]. The same study also found that the transport of effluents in river waters was profoundly impacted by seasons. The transport of pharmaceuticals was increased as a result of, e.g., snow-melting and lower temperatures decreased the biological activity in oceans, hence the biological degradation [130]. Therefore, it is proposed that additional assessments of the area recipient of the SNJ effluents could reveal seasonal variations for the exposed area and indicate the persistence of APIs over time.

The detection of targeted pharmaceuticals (CAF and DCF) in seawater from both DS and reference station BF, as well as CAF in sediments from all marine stations, indicate possible alternative sources of contamination. These sources remain unknown. It has also been purported that the pseudo-persistency of compounds can lead to an increased total concentration in the recipient area. The contaminants may also be detected further from the discharge site [131].

The SNJ discharge recipient, Håsteinsfjorden, has been subjected to several reports on the environmental status for the past decades. However, no assessments of APIs or other emerging contaminants have been done. The present study´s findings demonstrate a need for further monitoring and new measures to prevent further contamination.

6. CONCLUSION

The sample preparation methods used herein successfully extracted target analytes with a recovery yield acceptable for all target analytes, except for ATV. However, further method developments are necessary for improved extraction by further optimizing sample preparation steps regarding the targets´ physio-chemical properties. Such improvements may increase the rate of detection of low-concentration targets. The HPLC-MS/MS was advantageous in determining target compounds used to analyze a broad selection of samples in a short time. Now, more samples can more easily be quantified.

The assessed stations were representative of the contamination status of the area. However, additional efforts are underway to determine the presence and the potential biological effects of these pharmaceuticals in the marine environment.

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APPENDICES

Appendix 1 – Details of purchased analytes. Description of therapeutic classes, product information (CAS), purities, and solution concentrations.

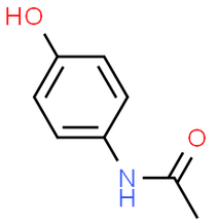
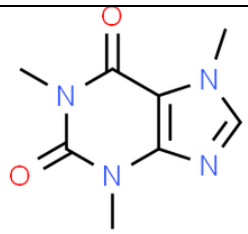
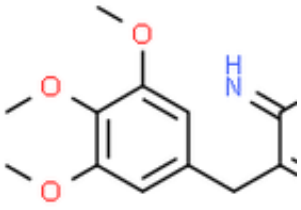
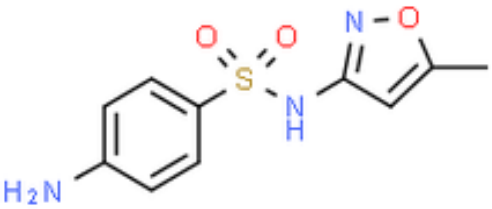
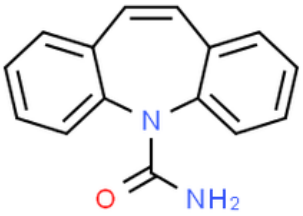
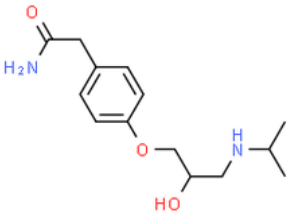
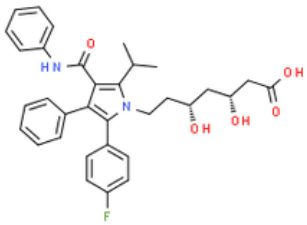
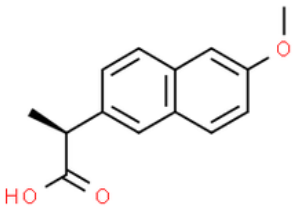
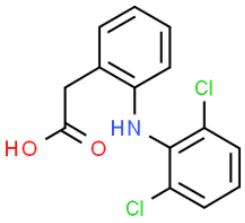
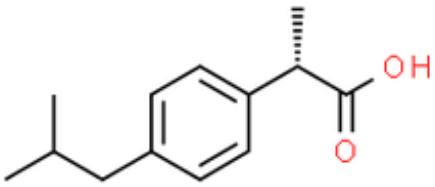
Compound	**Therapeutic class**	**CAS**	**Formula**	**Purity, %**	**Concentration (mg/mL)**
Acetaminophen	Analgesic	103-90-2	$C_8H_9NO_2$	98 %	0.135
Sulfamethoxazole	Antibiotic[2]	723-46-6	$C_{10}H_{11}N_3O_3S$	98 %	0.178
Trimethoprim	Antibiotic[1]	738-70-5	$C_{14}H_{18}N_4O3$	PSS[3]	0.063
Carbamazepine	Antiepileptic	298-46-5	$C_{15}H_{12}N_2O$	PSS[3]	0.187
Atenolol	Antihypertensive	29122-68-7	$C_{14}H_{22}N_2O_3$	N/A	0.127
Atorvastatin	Antilipidemic	134523-00-5	$C_{33}H_{35}FN_2O_5$	N/A	0.077
Diclofenac	NSAIDs	15307-79-6	$C_{14}H_{10}Cl_2NO_2Na$	AS[4]	0.073
Ibuprofen	NSAIDs	15687-27-1	$C_{13}H_{18}O_2$	99 %	0.178
Naproxen	NSAIDs	22204-53-1	$C_{14}H_{14}O_3$	99 %	0.059
Caffeine	Stimulant	58-08-2	$C_8H_{10}N_4O_2$	PSS[3]	0.057

1 Bacteriostatic
2 Sulfonamides
3 Pharmaceutical secondary standard
4 Analytical standard

Appendix 2 – Properties of target pharmaceuticals. Description of chemical properties. Log Kow is the measure of hydrophobicity and (S) is the water-solubility at pH 7.

Compound	**Molecular weight, amu**	**pK_{a1}**	**pK_{a2}**	**Log K_{ow}**	**(S) (mg/L)**
Acetaminophen	151.16	-	9.38	0.46	14000
Sulfamethoxazole	253.28	1.60	5.70	0.89	610
Trimethoprim	290.32	7.12	-	0.91	400
Carbamazepine	236.27	13.90	-	2.45	17.7
Atenolol	266.34	9.60	-	0.16	13300
Atorvastatin	558.60	4.30	14.90	6.36	0.00112
Diclofenac	318.10	4.15	-	4.51	2.37
Ibuprofen	206.28	5.30	-	3.97	21
Naproxen	230.26	4.15	4.15	3.18	15.9
Caffeine	194.19	-	14.00	-0.07	21600

Appendix 3 – Structures of target pharmaceuticals. Chemical structures obtained from Chemspider, 2020.

	Acetaminophen		Caffeine
	Trimethoprim		Sulfamethoxazole
	Carbamazepine		Atenolol
	Atorvastatin		Naproxen
	Diclofenac		Ibuprofen

Appendix 4 – Summary regression model of standard curves. Concentrations are only given in ng/L for the limits of detection (LOD) and quantification (LOQ). Linearity is given by coefficient of determination (R_2). Three gradient programs were tested; PI = positive ionization, NI = negative ionization, 1 = mobile phase A1 = 0.1% (v/v) formic acid and 2 = mobile phase A2 = 0.1% (v/v) ammonium.

Compound	LOD, ng/L		LOQ, ng/L		R_2			Observations, n			Slope		
					HPLC_P1_A1B1	**HPLC_P1_A2B1**	**HPLC_N1_A2B1**	**HPLC_P1_A1B1**	**HPLC_P1_A2B1**	**HPLC_N1_A2B1**	**HPLC_P1_A1B1**	**HPLC_P1_A2B1**	**HPLC_N1_A2B1**
Acetaminophen	0.752	19.34	2.28	58.60	0.984	0.998	-	9	9	-	78.64	196.0	-
Sulfamethoxazole	25.10	97.72	76.06	296.1	0.996	0.978	-	10	10	-	107.7	54.50	-
Trimethoprim	56.42	33.25	171.0	100.8	0.985	0.994	-	9	10	-	73.98	132.9	-
Carbamazepine	55.34	25.36	167.7	76.86	0.991	0.995	-	10	10	-	854.2	1116	-
Atenolol	20.63	44.34	62.53	134.4	0.998	0.989	-	9	9	-	27.67	152.6	-
Atorvastatin	12.46	40.09	37.76	121.5	0.999	0.978	-	10	10	-	175.3	268.7	-
Diclofenac	11.54	69.26	34.98	209.88	0.999	0.971	-	10	10	-	109.0	104.9	-
Ibuprofen	209.8	222.2	635.7	673.2	0.925	0.882	0.991	6	7	9	4.457	2.190	18.71
Naproxen	25.83	37.55	78.29	113.8	0.998	0.992	-	7	10	-	70.93	62.60	-
Caffeine	10.99	31.91	33.31	96.70	0.999	0.994	-	10	10	-	130.6	158.2	-

Appendix 5 – Occurrence-data from seawater samples. Range (minimum and maximum) and mean concentration in ng/L for targeted pharmaceutical compounds in seawater samples. LOD = limit of detection, LOQ = limit of quantification, frequency = percentage of samples <LOD. DS = discharge station, BF = Boknafjorden.

A) Group	Compound	LOD (ng/L)	LOQ (ng/L)	Min (ng/L)		Max (ng/L)		Mean (ng/L)		Frequency (%)	
				Seawater DS	**Seawater BF**	**Seawater DS**	**Seawater BF**	**Seawater DS**	**Seawater BF**	**Seawater DS**	**Seawater BF**
Analgesic	Acetaminophen	0.752	2.279	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
Antibiotics	Sulfamethoxazole	97.72	296.1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
	Trimethoprim	33.25	100.8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
Antiepileptics	Carbamazepine	25.36	76.86	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
Beta blockers	Atenolol	20.63	62.53	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
Lipid regulators	Atorvastatin	12.46	37.76	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
NSAIDs	Diclofenac	11.54	34.98	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOD	33	33
	Naproxen	25.83	78.29	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
	Ibuprofen	46.48	140.8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
Stimulant	Caffeine	10.99	33.31	<LOQ	<LOD	<LOQ	<LOQ	<LOQ	<LOQ	100	66

Appendix 6 – Occurrence-data for sediment samples from coastal stations.

Range (minimum and maximum) and mean concentration in ng/L for targeted pharmaceutical compounds in sediment samples. LOD = limit of detection, LOQ = limit of quantification, frequency = percentage of samples <LOD. DS = discharge station, BF = Boknafjorden.

Group	Compound	LOD (ng/g)	LOQ (ng/g)	Min (ng/g)			Max (ng/g)			Mean (ng/g)			Frequency (%)		
				Sed SA	**Sed RB**	**Sed SO**	**Sed SA**	**Sed RB**	**Sed SO**	**Sed SA**	**Sed RB**	**Sed SO**	**Sed SA**	**Sed RB**	**Sed SO**
Analgesic	Acetaminophen	0.451	1.367	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
Antibiotics	Sulfamethoxazole	58.63	177.67	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
	Trimethoprim	19.95	60.45	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
Antiepileptics	Carbamazepine	15.22	46.11	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
Beta blockers	Atenolol	12.38	37.52	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
Lipid regulators	Atorvastatin	7.476	22.65	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
NSAIDs	Diclofenac	6.925	20.99	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
	Naproxen	15.50	46.97	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
Stimulant	Caffeine	6.595	19.98	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0

Appendix 7 – Occurrence data for sediment samples from marine stations.

Range (minimum and maximum) and mean concentration in ng/L for targeted pharmaceutical compounds in seawater samples. LOD = limit of detection, LOQ = limit of quantification, frequency = percentage of samples <LOD. DS = discharge station, BF = Boknafjorden.

Group	Compound	LOD (ng/g)	LOQ (ng/g)	Min (ng/g)			Max (ng/g)			Mean (ng/g)			Frequency (%)		
				Sed DS	**Sed K**	**Sed BF**	**Sed DS**	**Sed K**	**Sed BF**	**Sed DS**	**Sed K**	**Sed BF**	**Sed DS**	**Sed K**	**Sed BF**
Analgesic	Acetaminophen	0.451	1.367	<LOD	<LOQ	1.57	5.831	10.20	4.584	1.944	5.253	3.093	33	100	100
Antibiotics	Sulfamethoxazole	58.63	177.67	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
	Trimethoprim	19.95	60.45	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
Antiepileptics	Carbamazepine	15.22	46.11	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
Beta blockers	Atenolol	12.38	37.52	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
Lipid regulators	Atorvastatin	7.476	22.65	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
NSAIDs	Diclofenac	6.925	20.99	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
	Naproxen	15.50	46.97	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
	Ibuprofen	27.89	84.51	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0	0
Stimulant	Caffeine	6.595	19.98	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	67	67	33

Appendix 8 – Recoveries (%) of spiked sediment samples.

Recovery study over ultrasonication, solid-phase extraction and high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) procedure. Recoveries (%), calculated from Equation 5, of ten pharmaceuticals in sediment samples (n=5) spiked at three concentration levels (60, 800 and 2 000 ng/g or 8, 80 and 800 ng/g) are listed as means, standard deviation (SD) and relative SD (RSD) of replicates analyzed by three gradient programs; HPLC_PI_A1B1 and 2 and HPLC_NI_1. PI = positive ionization, NI = negative ionization, 1 = mobile phase A1 = 0.1% (v/v) formic acid and 2 = mobile phase A2 = 0.1% (v/v) ammonium. ATE = atenolol, CBZ = carbamazepine, ACE = acetaminophen, SUL = sulfamethoxazole, ATV = atorvastatin, CAF = caffeine, DCF = diclofenac, TMP = trimethoprim, IBU = ibuprofen, NAP = naproxen.

Analyte, ng/g	**HPLC_PI_A1B1**			**HPLC_PI_A2B1**			**HPLC_NI_A2B1**		
	Mean (%)	**SD (%)**	**RDS (%)**	**Mean (%)**	**SD (%)**	**RSD (%)**	**Mean (%)**	**SD (%)**	**RSD (%)**
ATE									
60	103.0	13.28	12.89	57.61	3.176	5.512	-	-	-
800	73.61	5.420	7.363	47.55	2.947	6.198	-	-	-
2 000	54.30	2.636	4.854	37.68	2.579	6.846	-	-	-
CBZ									
60	36.05	2.668	7.402	52.85	3.656	6.918	-	-	-
800	29.43	1.280	4.347	45.35	3.185	7.024	-	-	-
2 000	23.87	0.694	2.908	38.72	1.489	3.845	-	-	-
ACE									
60	30.88	3.951	12.79	31.95	5.668	17.74	-	-	-
800	40.36	5.092	12.62	22.92	1.840	8.027	-	-	-
2 000	33.14	1.332	4.017	17.49	0.428	2.446	-	-	-

SUL									
60	29.79	3.267	10.97	196.1	15.76	8.037	-	-	-
800	32.68	2.586	7.914	127.3	10.96	8.607	-	-	-
2 000	30.40	0.881	2.897	90.63	7.700	8.496	-	-	-
ATV									
60	0.229*	0.275	120.0	0.214	0.105	49.19	-	-	-
800	0.355	0.116	32.59	0.295	0.045	15.44	-	-	-
2 000	0.267	0.053	19.91	0.283	0.033	11.84	-	-	-
CAF									
60	35.90	5.174	14.41	53.11	10.19	19.19	-	-	-
800	26.53	3.051	11.50	26.92	6.856	25.47	-	-	-
2 000	25.71	1.139	4.429	23.68	5.226	22.07	-	-	-
DCF									
60	41.71	5.388	12.92	49.48	7.979	16.12	-	-	-
800	36.82	3.972	10.79	28.20	7.664	27.18	-	-	-
2 000	32.46	1.795	5.530	19.09	3.928	20.57	-	-	-
TMP									
60	46.62	6.243	13.39	123.9	11.45	9.241	-	-	-
800	44.35	1.808	4.076	97.95	6.72	6.857	-	-	-

2 000	41.70	3.085	7.400	89.74	3.88	4.327	-	-	-
NAP									
60	26.21	6.097	23.26	33.93	6.546	19.29	-	-	-
800	23.38	4.404	18.83	25.83	5.472	21.18	-	-	-
2 000	16.29	2.762	16.96	18.80	3.448	18.34	-	-	-
IBU	-	-	-	-	-	-			
8	-	-	-	-	-	-	6.154**	4.639	75.38
80	-	-	-	-	-	-	7.688	3.525	45.85
800							11.05	1.364	12.34

* n=3

** n=4

Appendix 9 – Recoveries (%) of water samples.

Recovery study over ultrasonication, solid-phase extraction and high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) procedure. Recoveries (%), calculated from Equation 5, of ten pharmaceuticals in water samples (n=5) spiked at three concentration levels (60, 800 and 2 000 ng/g or 8, 80 and 800 ng/L) are listed as means, standard deviation (SD) and relative SD (RSD) of replicates analyzed by three gradient programs; HPLC_PI_A1B1 and 2 and HPLC_NI_1. PI = positive ionization, NI = negative ionization, 1 = mobile phase A1 = 0.1% (v/v) formic acid and 2 = mobile phase A2 = 0.1% (v/v) ammonium. ATE = atenolol, CBZ = carbamazepine, ACE = acetaminophen, SUL = sulfamethoxazole, ATV = atorvastatin, CAF = caffeine, DCF = diclofenac, TMP = trimethoprim, IBU = ibuprofen, NAP = naproxen.

Analyte, ng/L	**HPLC_PI_A1B1**			**HPLC_PI_A2B1**			**HPLC_NI_A2B1**		
	Mean (%)	**SD (%)**	**RDS (%)**	**Mean (%)**	**SD (%)**	**RSD (%)**	**Mean (%)**	**SD (%)**	**RSD (%)**
ATE									
60	180.9	28.59	5.369	56.11	3.012	5.37	-	-	-
800	137.65	12.35	9.073	65.72	5.963	9.07	-	-	-
2 000	113.0	8.425	6.790	67.49	4.583	6.79	-	-	-
CBZ									
60	54.04	6.254	9.711	65.89	6.399	9.71	-	-	-
800	48.16	2.271	13.89	61.41	8.531	13.89	-	-	-
2 000	41.59	3.212	22.27	50.68	11.29	22.27	-	-	-
ACE									
60	95.04*	17.65	-	54.89**	-	-	-	-	-
800	61.02	5.926	9.200	33.50	3.082	9.20	-	-	-
2 000	46.95	4.864	9.430	28.10	2.650	9.43	-	-	-

SUL									
60	41.61	6.335	12.01	268.8	32.27	12.01	-	-	-
800	44.73	3.247	7.391	181.3	13.40	7.39	-	-	-
2 000	44.30	3.912	7.615	154.2	11.74	7.62	-	-	-
ATV									
60	0.346***	0.326	41.12	0.170***	0.070	41.12	-	-	-
800	1.968	2.004	97.25	1.470	1.429	97.25	-	-	-
2 000	3.991	3.843	105.3	3.473	3.655	105.3	-	-	-
CAF									
60	79.06	15.75	19.00	89.54	17.01	19.00	-	-	-
800	48.32	3.747	15.25	50.79	7.747	15.25	-	-	-
2 000	34.17	2.274	10.29	35.74	3.678	10.29	-	-	-
DCF									
60	37.68	4.672	18.77	33.14	6.221	18.77	-	-	-
800	49.57	3.482	16.67	35.22	5.872	16.67	-	-	-
2 000	42.43	4.280	15.15	27.65	4.189	15.15	-	-	-
TMP									
60	50.97	5.432	6.826	127.1	8.677	6.83	-	-	-
800	59.30	4.536	10.97	120.3	13.20	10.97	-	-	-

2 000	58.25	4.692	7.909	115.0	9.096	7.91	-	-	-
NAP									
60	39.00	9.292	22.10	37.36	8.257	22.10	-	-	-
800	35.57	5.943	11.49	36.50	4.192	11.49	-	-	-
2 000	35.92	8.052	14.99	27.38	4.104	14.99	-	-	-
IBU									
8	-	-	-	-	-	-	33.85****	23.14	68.36
80	-	-	-	-	-	-	11.78	3.32	28.17
800	-	-	-	-	-	-	12.38	2.91	23.49

* n=4
** n=2
*** n=3
**** n=4

Appendix 10 – Occurrence-data from wastewater treatment plant samples analyzed by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) in positive and negative electrospray ionization mode.

Range (minimum and maximum) and mean concentration in ng/L for target pharmaceutical compounds in inlet and outlet water samples collected from the SNJ. LOD = limit of detection, LOQ = limit of quantification, frequency = percentage of samples <LOD. Outlet sample 1 was collected over 24 hours (composite).

A) Group	Compound	LOD (ng/g)	LOQ (ng/g)	Min (ng/g)			Max (ng/g)			Mean (ng/g)			Frequency (%)		
				Inlet 1-3	**Outlet 1**	**Outlet 2-4**	**Inlet 1-3**	**Outlet 1**	**Outlet 2-4**	**Inlet 1-3**	**Outlet 1**	**Outlet 2-4**	**Inlet 1-3**	**Outlet 1**	**Outlet 2-4**
Analgesic	Acetaminophen	109.67	332.3	18 661	<LOD	<LOD	25 422.20	<LOD	<LOD	22 201	<LOD	<LOD	100	0	0
Antibiotics	Sulfamethoxazole	19.62	59.46	73.93	<LOQ	<LOQ	96.60	<LOQ	89.73	88.87	<LOQ	78.56	100	100	100
	Trimethoprim	22.32	67.63	<LOQ	<LOD	<LOD	78.59	<LOQ	105.01	75.07	<LOQ	95.36	100	50	67
Antiepileptics	Carbamazepine	17.26	52.29	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	82.10	<LOQ	<LOQ	<LOQ	100	100	100
Beta blockers	Atenolol	18.36	55.63	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	100	50	67
NSAIDs	Diclofenac	0.68	2.05	146.94	58.66	83.14	165.24	148.8	170.0	158.90	96.52	130.5	100	100	100
	Naproxen	17.73	53.73	2 033	96.55	226.5	2 417.54	745.3	1 644	2 267	423.5	1 087	100	100	100
	Ibuprofen	3.86	11.70	2 117	14.53	12.98	2 728.15	175.1	365.8	2 410	92.59	219.8	100	100	100
Stimulant	Caffeine	22.44	68.01	13 826	<LOQ	761.9	15 861.63	332.0	2 421	14 830	181.8	1 700	100	100	100

Appendix 11 – Occurrence data from seawater samples analyzed at by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) in positive and negative electrospray ionization mode. Range (minimum and maximum) and mean concentration in ng/L for target pharmaceutical compounds in seawater samples, collected from the discharge site (DS) and the reference station in Boknafjorden (BF). LOD = limit of detection, LOQ = limit of quantification, frequency = percentage of samples <LOD.

Group	Compound	LOD (ng/L)	LOQ (ng/L)	Min (ng/L)		Max (ng/L)		Mean (ng/L)		Frequency (%)	
				Seawater DS	**Seawater BF**	**Seawater DS**	**Seawater BF**	**Seawater DS**	**Seawater BF**	**Seawater DS**	**Seawater BF**
Analgesic	Acetaminophen	109.67	332.3	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
Antibiotics	Sulfamethoxazole	19.62	59.46	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
	Trimethoprim	22.32	67.63	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
Antiepileptics	Carbamazepine	17.26	52.29	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
Beta blockers	Atenolol	18.36	55.63	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
NSAIDs	Diclofenac	0.68	2.05	<LOD	<LOD	35.87	37.88	35.87	37.88	33	33
	Naproxen	17.73	53.73	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
	Ibuprofen	3.86	11.70	25.86	<LOD	36.74	43.54	32.01	43.54	100	67
Stimulant	Caffeine	22.44	68.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	100	67

Appendix 12 – Occurrence data from sediment samples analyzed at by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) in positive and negative electrospray ionization mode. Range (minimum and maximum) and mean concentration in ng/L for target pharmaceutical compounds in sediment samples, collected from the discharge site (DS) and the reference station in Boknafjorden (BF). LOD = limit of detection, LOQ = limit of quantification, frequency = percentage of samples <LOD.

Group	Compound	LOD (ng/g)	LOQ (ng/g)	Min (ng/g)		Max (ng/g)		Mean (ng/g)		Frequency (%)	
				Sediments DS	**Sediments BF**	**Sediments DS**	**Sediments BF**	**Sediments DS**	**Sediments BF**	**Sediments DS**	**Sediments BF**
Analgesic	Acetaminophen	65.80	199.4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
Antibiotics	Sulfamethoxazole	11.77	35.68	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
	Trimethoprim	13.39	40.58	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
Antiepileptics	Carbamazepine	10.36	31.37	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
Beta blockers	Atenolol	11.02	33.38	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
NSAIDs	Diclofenac	0.408	1.230	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
	Naproxen	10.64	32.24	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
	Ibuprofen	2.316	7.020	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0	0
Stimulant	Caffeine	13.46	40.81	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	100	100