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Abbreviations

BCF	Bioconcentration factor
CE	Collision energy
CV	Cone voltage
ESI	Electrospray-ionization
LC MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
NSAID	Non-steroidal anti-inflammatory drug
SANOCEAN	South Africa-Norway research cooperation on blue economy, climate change, the environment and sustainable energy program
SPE	Solid phase extraction
UPLC	Ultra-performance liquid chromatography
WWTP	Wastewater treatment plant

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1: Abstract

The presence of antidepressants and other pharmaceuticals in micro concentrations in the marine environment have, in recent years, become a focal area for ecotoxicology. These compounds might have unexpected impacts on fish and other aquatic animals despite their seemingly scant presence. The negative consequences caused by bioaccumulation and biomagnification are two notable concerns. In 2019, the SANOCEAN team from the University of Stavanger identified a cocktail of contaminants in a region surrounding the outlet waters of a wastewater treatment plant. One of the compounds found in the sediment samples was from the tricyclic antidepressant amitriptyline. As research had not been done on the bioaccumulation potential of this compound in benthic animals, this thesis was designed around investigating that question. This was done through a 28-day exposure study using the polychaete benthic worm *Nereis virens*. Three different environmental sediment samples, two laboratory amitriptyline spiked samples (3.057µg/g-sediment and 30.57µg/g-sediment), and one negative control sample were used for the exposure. At the end of the study the concentration of amitriptyline in biota, sediment, and water was analyzed. After the 28 days of exposure, there was a clear indication of accumulation in the biota. In the sediment samples collected from a reference area (assumed to be a clean location) the level of amitriptyline in the worms was below the limit of detection. In the spiked sediment samples, there was a detectable amitriptyline level with a mean average of 9.5ng/g-biota and 56.5ng/g-biota for the low and high spiked concentration respectively. This is indicative of bioaccumulation. The speed and level of this accumulation as well as the impact that it could have on the food chain and aquatic environment are further research suggestions as well as amitriptyline's biological effects on *Nereis virens*.

2: Introduction and Goal of the Thesis

The modern world is full of amazing advancements in science, technology and chemistry. One of the greatest of these categories are the many discoveries in the field of medicine and pharmacology. The society we live in today is improved incalculably by the presence and usage of these drugs. However as with all things nothing is without consequence, and one of the main consequences of the production of these products is their escape, or deliberate dumping, into the environment. Pharmaceuticals enter our environment through a variety of sources the biggest of which is wastewater/sewage effluent from households, hospitals, clinics, and occasionally industrial drug companies (Kolpin et al. 2002; Harrison et al. 2006). Once there they disperse into different phases of the aquatic ecosystem they can cause adverse effects to both wildlife and humans. Pharmaceutical compounds have already been detected in sediment, water column, and marine organisms. Many pharmaceuticals are also lipophilic, meaning they have a chance to bioaccumulate in the food chain, and this has brought the conversation to the world of human public food health and safety. The study utilized the marine polychaetae nereis virens (figure 4.3-1) to evaluate the bioaccumulation potential of the anti-depressant pharmaceutical amitriptyline. The scope of this introduction and following theory section is to give a general overview of the current status of research regarding these pollutants with a focus on wastewater treatment plants as a source with a brief comment on typical methodologies employed in the field.

3: Theory

3.1 Sources of pharmaceuticals in the aquatic environment

3.1.1 Wastewater and sewage

Wastewater treatment plant (WWTP) effluents in combination with runoffs of sewer overflow are large contributors to pharmaceuticals entering the aquatic environment (Kolpin et al. 2002; Harrison et al. 2006). The source of the pharmaceuticals entering WWTP plants can be traced to three major players, household usage, hospital discharge, and sometimes from industrial pharmaceutical production (Daughton & Ternes. 1999). The most significant of these is household/at home patient use which combines both drugs administered to the body correctly and those that are improperly disposed of via sinks, toilets, etc. (Fabbri, 2015). Once reaching a WWTP the contaminated water is potentially treated in a variety of different ways, or not at all such as in the case of the marine receiving environment near Victoria Canada (Krogh et al. 2017). Current processing of wastewater does not have any formal direction for the removal of micropollutants such as pharmaceuticals. Thus, the removal rates are highly dependent on the physical and chemical properties of the compound in question as well as the treatment in question. This can result in anywhere from less than 10% to 100%, or complete removal, of the pollutant (Kümmerer, 2009). Recent research has shown promising results for combined treatment processes (activated sludge, microfiltration, and reverse osmosis combination) that may remove up to 97% of certain pharmaceuticals under polluted water concentrations of 0.1μg/L (Al-Rifai et al. 2011) and much research has been done on advanced oxidation processes (AOPs) as well (Méndez-Arriaga et al. 2008). While these research advancements are being made at this time many places around the world have not invested in the upgrades. Many are still unsure about the added value and for developing nations the technology is often prohibitively expensive. Regardless, the wastewater effluent is often deposited directly into a nearby water body and serves as a practically continuous source of chronic exposure to pharmaceutical pollutants. It should be noted that the rate of discharge can change however and depends on the surrounding environment and human activities (Cantwell et al 2016).

3.1.2 Agriculture and Aquaculture

Aquaculture provides a direct source of pharmaceutical pollution to marine environments. Almost all (90%) of aquaculture is stationed in Asia (Sapkota et al. 2008) with approximately 80% being in China alone (Chen et al. 2015). The industry employs a large amount of antibiotics and other veterinary medicines to their stock on a regular basis. In addition to the exposure to the farmed fish directly, up to 75% of the applied pharmaceuticals can be wasted to the environment. This happens through methods such as the escape of improperly/non ingested medicine, excretion of unmetabolized drugs, and waste (fecal or renal) of product metabolites (Grigorakis and Rigos 2011). The drugs and metabolites lost in these ways find their way into the environment and contaminate the surrounding ecosystem.

3.1.3 Leachate

A smaller but still notable source of contamination is leachate from landfills and other waste disposals (Rodríguez-Navas et al., 2013) These wastes come mainly directly from households in modern times but can also come from hospitals, veterinarian centers, and clinics. The pharmaceutical waste in this instance is deposited on land but through the action of the water system the contaminants leach into the soil and either run off to a larger water body or sink into the ground and potentially contaminate groundwater.

3.2 Major classes of pharmaceuticals entering the aquatic environment

Each year new pharmaceuticals are developed, tested, and released to the market. Trends in human health, economics, and accessibility will change which pharmaceuticals are primarily discharged. Currently the main categorical groups of pharmaceuticals found in water bodies are analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), neuroactive drugs, steroid hormones, antibiotics, lipid regulators, neuroactive drugs, and cardioactive drugs (Fabbri, 2015). Some specific drugs are listed under their given categories in figure 3.2-1. Which of these is the

most prominent depends on the location around the globe (Fabbri, 2015), the proximity to urban developments, and the physical environment itself that the chemicals are deposited.

Antibiotics	Lipid Regulators	Steroid Hormones
Amoxicillin Ciprofloxacin Erythromycin Ofloxacin Neomycin Norfloxacin Trimethoprim Roxithromycin Sulfadiazine Sulfadimidine Sulfamethoxazole Tetracycline Trimethoprim	Bezafibrate Clofibric Acid Gemfibrozil	17-ß estradiol 17-α ethinylestradiol Estrone Testosterone
NSAID/Analgesics	**Neuroactive Drugs**	**Cardioactive drugs**
Acetamoxifen Aspirin Diclofenac Ketoprofen Ibuprofen Indomethacin Naproxen Piroxicam	Antiepileptic Carbamazepine Antidepressant Fluoxetine Tranquilizer Diazepam	Atenolol Betasolol Metoprolol Ouabain Propranolol Sotalol Verapamil

Figure 3.2-1. Examples of pharmaceuticals commonly detected in the aquatic environment (Fabbri, 2015) (Gaw et al. 2014)

3.3 Temporary and permanent sinks for pharmaceuticals in the aquatic environment

Humans have, for much of history, viewed large bodies of water or fast flowing rivers as locations of purification. Dumping wastes was common practice and it was thought that the waters were so deep (or moving so quickly away from their source) that whatever impurity was introduced would be forever lost in its' body. A modern equivalent phrase would be the ubiquitous "the solution to pollution is dilution" that many learned in their youth. Unfortunately, this thinking is deeply flawed.

The aquatic environment can be thought of generally in three different categories, the water column, the sediment, and the members of the biotic community. These groups interact with chemicals, including pharmaceuticals and other pollutants in very different ways based on their physical attributes. This also means that scientific monitoring and research can be quite complex and require different strategies and methodologies.

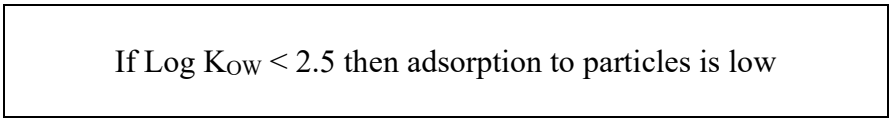
3.3.1 Water column

The water column is what most think of when they hear the term aquatic environment. It is also, primarily, the first of the three categories that an errant pharmaceutical will come in contact with when entering the ecosystem. In this thesis, the focus will be on sea waters. Much reporting has been done on freshwater interactions with pharmaceuticals and there is an overlap in knowledge, but there are also impacts that diverge with the introduction of salts. Many processes happen in the ocean that move and concentrate chemicals. For example, hydro dynamism has been hypothesized to play a large role in their dissemination around coastal regions. Bayen et al. (2013) discovered in Singapore, using 3D computer models, that the main predictive factor for pollutant distribution was not the distance from the point source but was instead based heavily on the various mixing patterns of the coastal waters and their residence times. This resulted in findings such as higher pollutant levels at sites where the flushing potential of the water was at its lowest. Summer thermoclines, a typical seasonal phenomenon, were shown to reduce the movement of certain pharmaceuticals from the bottom of the water column to the top as well (Fenet et al. 2014). These physical properties of water bodies have seen some recent attention as modeling technology improves for hydrodynamics. Salt and pH are two factors that directly affect the electrostatic aspects of pharmaceuticals. Especially when considering the various functional groups and their acid dissociation constants. Sea water, compared to freshwater, has a much higher and a slightly more basic pH (around 8). These differences can cause initially counter intuitive changes ranging from increased lipophilicity (Owen et al. 2009) to a loss of solubility to the point of precipitation out of solution at the freshwater to saltwater outfall (Togola and Buszinki 2007). Overall salinity and pH must be considered when discussing the fate of pharmaceuticals entering the aquatic environment. The potential synergistic effects and unpredictability, caused from the lack of testing most drug products in seawater prior to public release, cannot be overstated.

3.3.2 Sediment

Medical drugs are designed not to degrade until they have completed their mode of action, interacting with their site of action, so absent performing this task many of these chemicals persist and can sink to the bottom of the water column (Tijani et al. 2013). The sediment location can exacerbate this residence time by protecting the compounds from biodegradation they would receive more easily in suspension, and also other forms of physical degradation such as photodegradations (Szymonik et al. 2017). Overtime these pollutants can accumulate and instead of staying on the bottom they are often resuspended through movement of the sediment or through biotic means. Flora and fauna living in the marine sediment can consume detectable concentrations of these chemicals either through direct high concentration deposition, or from bioaccumulative factors, and often both (Krogh et al. 2017)(Fabbri and Franzellitti 2015). Actions that bring the pharmaceutical waste back into bioavailability for the rest of the aquatic ecosystem.

Determining if a compound will separate from the water and join the sediment involves a few key factors as we understand it today. The first is hydrophilicity. The more hydrophilic a compound is the lower its' K_{ow} and the less likely it is to be in particulate form. Most pharmaceuticals are hydrophilic due to their high polarities (Tijani et al. 2013) and thus water soluble. K_{OC} is another factor and measures the chemical's likelihood to adhere to organic carbon. The higher this value the more it will adsorb onto carbon based suspended solids and settle. figure 3.3-1 shows a very basic method for determining if a given pharmaceutical will adsorb onto particulate matter and possibly enter the sediment. However, it should be noted that research was done on the breakout of pharmaceuticals found in aquatic environments and their various sinks and there are some exceptions to the generalization. In Cantwell et al. 2016 it was found that it was amine functional groups controlling which pharmaceuticals would be present in the particulate. The drugs included here were all the antihypertensives tested and 3 of the beta blockers. This went directly against their predicted solubility and emphasizes the need to include a chemical's functional groups into account when determining the fate of a pollutant.



If 2.5 < Log K_{OW} < 4.0 then adsorption to particles is mediocre
If log K_{OW} > 4.0 then adsorption to particles is high

Figure 3.3-1. Quantitative prediction method to determine adsorption potential of a chemical in an aquatic environment to particles in the sediment or wastewater sludge (Jones-Lepp and Stevens 2007) (Caliman and Gavrilescu 2009)

3.3.3 Biological organisms as storage and transport of pharmaceuticals

As organisms come into contact with pharmaceuticals they can act as transformation systems, taking in the chemical and outputting potentially toxic metabolites. Their biodegradation abilities are a large part of the cleanup associated with the drugs, and without their activity the concentrations of harmful chemicals can increase. For example, in the surface of the seawater in winter lower temperatures and decreased biodegradation resulted in more pharmaceutical pollutants present than in summer (Choong et al. 2006). And lastly living organism's ability to bioconcentrate pollutants which then bioaccumulate in the food web is a large issue. Not only does this harm the individual but it makes these drugs bioavailable in dangerous concentrations to higher trophic levels.

3.4 Repercussions of exposure to errant pharmaceuticals

3.4.1 Effect on biota

The effect of pharmaceuticals on living organisms in the environment, particularly fauna, have been shown to be potentially huge. The different types of effects are quite variant, and don't always follow the drugs intended modes of action. One branch of research is into antibiotic resistances. A growing global concern as these resistant bacteria eventually find their way into human populations and are, in fact, already wreaking havoc on populations of humans today (Lin et al. 2008). When it comes to aquatic environments this issue is of particular note not just due to the volume of antibiotic exposure, but also because marine bacteria have been found to have high rates of horizontal gene transfer (McDaniel et al. 2010). This means antibiotic resistances can

grow quicker among their ranks. Another research area is in the vein of endocrine disrupting effects. While the magnitude of these effects is still disputed the existence of the effects is apparent. As of 2013 there were more than 87,000 new chemicals entering circulation untested for endocrine disrupting capabilities (Snyder et al. 2007) and 38,000 compounds that had been designated potentially as such (Tijani et al. 2013). The types of effects seen in endocrine disruption depend on the hormone that the compound is mimicking or the hormone receptor with which it is interfering. In many studies, effects were seen on secondary sex characteristics and had a directly adverse effect on the reproductive abilities of marine animals (Walker et al. 2012). However, the hormones present in a living organism go beyond ones that control sexual development and there are a lot of potential undiscovered effects researchers have yet to uncover.

Another series of effects on aquatic wildlife would be, for example, the negative effects of analgesics. Such effects are immune reaction alterations (Solé et al. 2010), decreased feeding behaviors (Solé et al. 2010), decreased strength in mussels (Ericson et al. 2010), and overall population survival rate drops (Guler and Ford, 2010). As shown the number of negative effects on aquatic organisms is robust and more research is needed on pharmaceuticals currently on the market, and before they enter the market, in order to begin mitigating this growing problem.

3.4.2 Effect on human life

Humans, similar to other animals, are contaminated with errant pharmaceuticals through their water, food, or sometimes absorption through the skin (Olujimi et al. 2010). Currently the primary concerning source for human exposure is through drinking water. There are however growing worries over food contaminations in both aquaculture (see previous sections on antibiotic resistance and bioaccumulation) and in agriculture as the use of recycled wastewater for farmlands increases and drugs find their way into the soil (Rodriguez-Navas et al. 2013). Also, farms may use wastewater sludge as fertilizer and this sludge often contains high levels of lipophilic chemicals, which pharmaceuticals can be a member (Tijani et al. 2013).

3.5 Example of current methodology for research and monitoring

3.5.1 Environmental study process for wild ecological data

The current methodology for monitoring the presence of pharmaceuticals (and other personal care products) in the marine environment can be split into three main parts: field sampling, chemical extraction and determination, and data analyzation.

Field sampling starts with determining the type of samples and the locations where they will be taken. The three primary sample types are sediment, water, and biotic tissue. When selecting sites previous data on the surrounding environment should be used to optimize the results. For example, in Long et al. (2013) previous data on Puget Sound contaminations in conjunction with a probabilistic design for the Bellingham Bay was used to select 40 total sites for sampling with 10 of those sites set aside as long term monitoring locations. Another factor might be included as well, such as salinity (Bowen et al. 2015) or ocean currents (Krogh et al. 2017). In addition, if taking biological samples species must also be a consideration. Timing between sampling and length of the study are also important as seasonal and long-term temporal variations could be of considerable effect as was found near Victoria Canada (Krogh et al. 2017).

Chemical extraction analysis is nowadays mainly done through LC-MS/MS techniques (Long et al. 2013). First samples are treated and brought to the correct pH. In some instances, water measured samples are filtered before analysis with the filtrate only being tested (Krogh et al. 2017). Data analysis on the samples can be done through different statistical systems, but the usage of R for large data sets is a good standard (Bowen et al. 2015).

3.5.2 Exposure studies for bioaccumulation of pharmaceuticals in marine organisms

To gain insight on the bioaccumulation potential of a given pharmaceutical, exposure studies are run on various organisms. This data can then be used to predict the environmental impact on the specific organism and potentially their food web when they are exposed to the same chemicals in the wild. For benthic animals, a common choice for aquatic research, these studies usually follow a similar pattern. Generally, after setup and acclimation is complete benthic

animals are added to a series of test jars with various levels of spiked sediment. They are then allowed to live in these jars for several days so they can be exposed to the pharmaceutical that was added. Then the specimens are analyzed for chemical content within their bodies and data is gathered from there (McLesse et al. 1980; Bennett et al. 2011; Wang et al. 2014). These studies are typically done under as exact of a condition as can be reasonably executed and vary slightly in their starting points depending on what natural environmental conditions are being studied.

3.6 *Nereis virens*

For this thesis the benthic polychaete *Nereis virens* has been selected as a test organism. *N. virens* is a marine worm that is the prey of many different aquatic animals in its ecosystem including but not limited to, crabs, skates, and fish. They are common in the north Atlantic waters along both the European coast as well as the North American coast (Elise, 2015; Cabi, 2021). This means that a form of their typical water temperature, salinity, and pH is consistent with that of the North Atlantic, especially for the worms employed for this thesis. They are also adapted to salinity variation common in estuary environments (Elise, 2015). *N. virens* has also been used by fisherman as bait for many years and in certain regions became over harvested (Watson et al., 2007) (Watson et al. 2016). Now artificial cultivation of these animals is a common practice to provide as bait or grind into feed pellets especially for shrimp producers (Oddsen, 2014; Cabi, 2021). They are in fact a leading source of feed for the aquaculture industry overall (Oddsen, 2014; Cabi 2021). As they are re-workers of the sediment, they are exposed to any chemicals that may accumulate or settle in the marine floor. This makes them particularly susceptible to contamination and particularly important to the potential bioaccumulation chain of those compounds.

4: Methods and Materials

4.1 Chemical list

Chemical	Producer
Amitriptyline	Sigma Aldrich
D-Amitriptyline	Sigma Aldrich
Dichloromethane with 0.002% 2-methyl-2-butene	VWR Chemicals
Ethyl Acetate 99.5% purity	Alfa Aesar
Formic Acid	VWR Chemicals
Methanol 99.9% purity	VWR Chemicals
Sodium Azide Extra Pure	Merck
Sulfuric Acid 90-91%	Sigma Aldrich

4.2 Sediment sample collection

The collection of sediment was done by the SANOCEAN team in September and October 2019. The marine samples used in the study originated from three different locations near the Stavanger peninsula, Kvitsøy, Boknafjorden, and a discharge point for the IVAR WWTP. All three sediments were characterized previously by the SANOCEAN team, and it is this data that forms the basis for the objective of this thesis. In addition, Bore beach sand was used for the initial acclimation period of the worms. The exact dates and locations the various sediment samples were taken are presented in table 4.2-1 and figure 4.2-1.

Table 4.2-1: Sediment sample dates and locations

Sample	Date	GPS-Coordinate
Kvitsøy	October 25th 2019	59.02452, 5.32826
Boknafjorden	October 31st 2019	59.18323, 5.66035
IVAR	October 31st 2019	59.02998, 5.54416

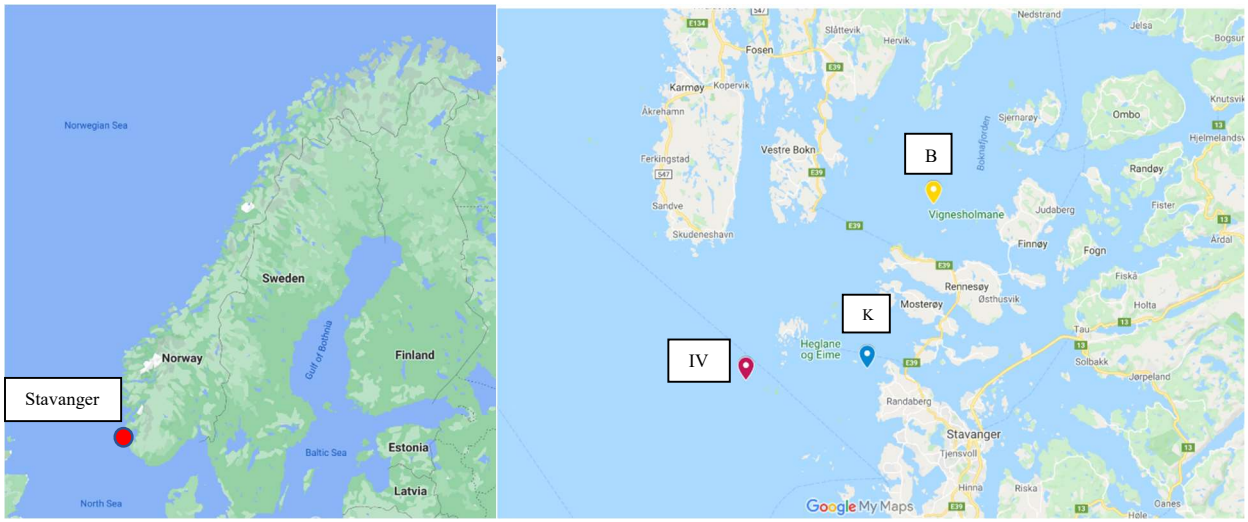


Figure 4.2-1: Map locations of sediment samples. IV= IVAR (Red), K= Kvitsøy(Blue), B=Boknafjorden (Yellow)(GoogleMaps, 2021)

Three sites at each location spaced 50 meters apart received a sampling. These three site sediments were mixed together into a single sample. The sediment was stored in clean glass bottles (figure 4.2-2) at -20°C in the laboratory at the Department of Chemistry, Bioscience and Environmental Engineering, University of Stavanger. A Van Veen grab, seen in figure 4.2-3, was employed as collection tool. The depth of the water where the collection occurred ranged from 80 to 200 meters. For the organism acclimation, top layer sand was obtained at Bore beach.

Figure 4.2-2: Amber glass bottles used to store sediment (Note: This is 2020 Sampling)

Figure 4.2-3: Van Veen Grab being used to collect sediment samples aboard the MS Scallop. (Note: This is 2020 Sampling).

4.3 Pre-Exposure Study Period

Nereis virens individuals were purchased from Topsy Baits Zeeaas Kwekerij/polychaete farm located in the Netherlands and were transported for no more than 24h before arrival in Stavanger.

Figure 4.3-1: Nereis virens worm from Topsy Baits farm.

At least 24h prior 16 cleaned and autoclaved glass beakers were filled with 300ml of the dried sand from Bore beach. Sterile seawater was then added to each and all beakers were placed into an incubator set to 13°C and allowed to rest for the next 24h. During this period a pump system was established using four Eheim air400 aquarium air pumps, of the 200 and 400 series. Once arrived at the lab, 7 typical sized worms were added to 15 different beakers and any left over

organisms were kept in a larger container of beach sand sediment as an additional control. The setup of the holding space described can be seen in (figure 4.3-2).

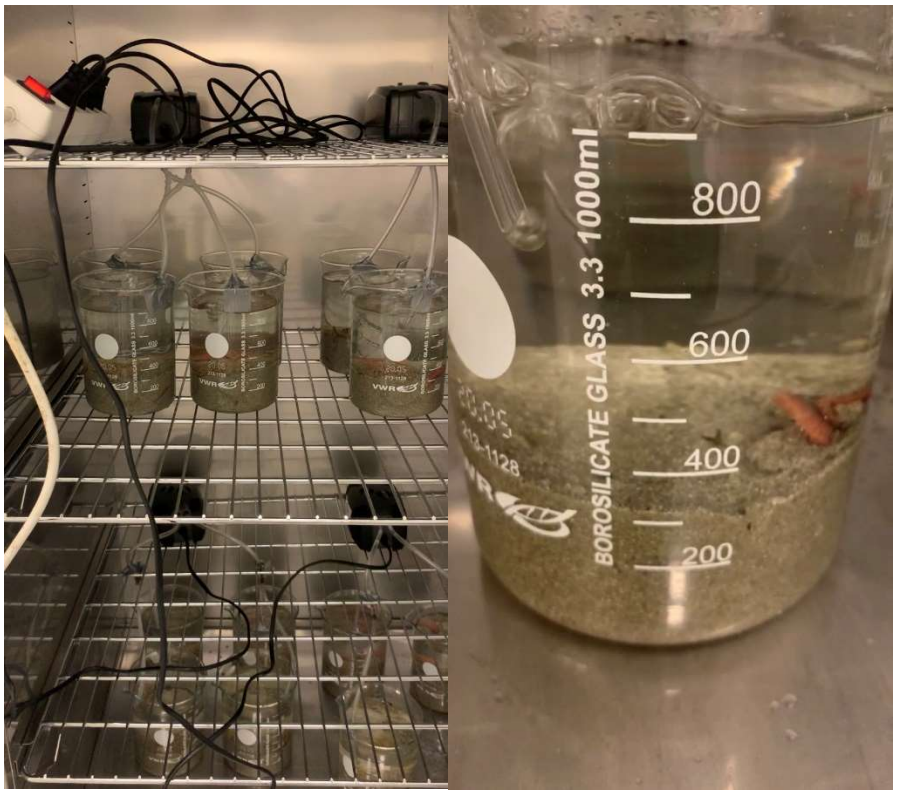


Figure 4.3-2: Pre-Exposure set up before clips (left) and tube aeration bubbling process (right).

The acclimation period lasted approximately 8 days. During this period, the overlying seawater was changed daily. Typically, farmed bait worms are very well fed so a feeding schedule was important to their continued survival. Live sea angling bait from Topsy Baits was provided every other day to the worms in the form of 2 pellets per worm that were dropped into each beaker after the water change. The food pellets were stored in a fridge in the lab at 4°C.

4.4 Sediment spiking

Two of the five exposure trials (See section 4.5) involved sediment spiked with amitriptyline. The lower concentration was 3.057µg/g and the higher concentration was set as a positive control at 30.57µg/g. Both concentrations were below the LD_{50} for this pharmaceutical (PubChem, 2021). The chemical structure of amitriptyline can be seen in figure 4.4-1. It can be dissolved well in water, so water became the chosen solvent for the spiking. This was also a good choice because by not using, for example, alcohol as a solvent, the amount of potential chemical contamination stress in the exposure study was reduced. The Kvitsøy reference sample sediment was used as the base for the spiked solution. The amitriptyline was then weighed and mixed with 5ml of the solvent water and added to the sediment samples and well incorporated with the use of a sterile metal spoon. These samples were then set into an incubator at the exposure study temperature of 13°C with magnet spinners and allowed to mix for 24 hours (Klosterhaus et al. 2011). The sediment spiking procedure is represented graphically in figure 4.4-2.

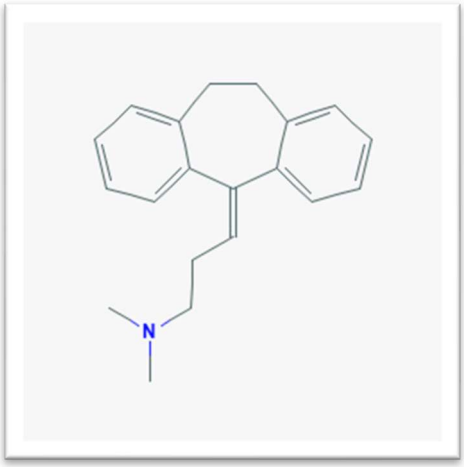


Figure 4.4-1: Amitriptyline chemical structure (PubChem, 2021)

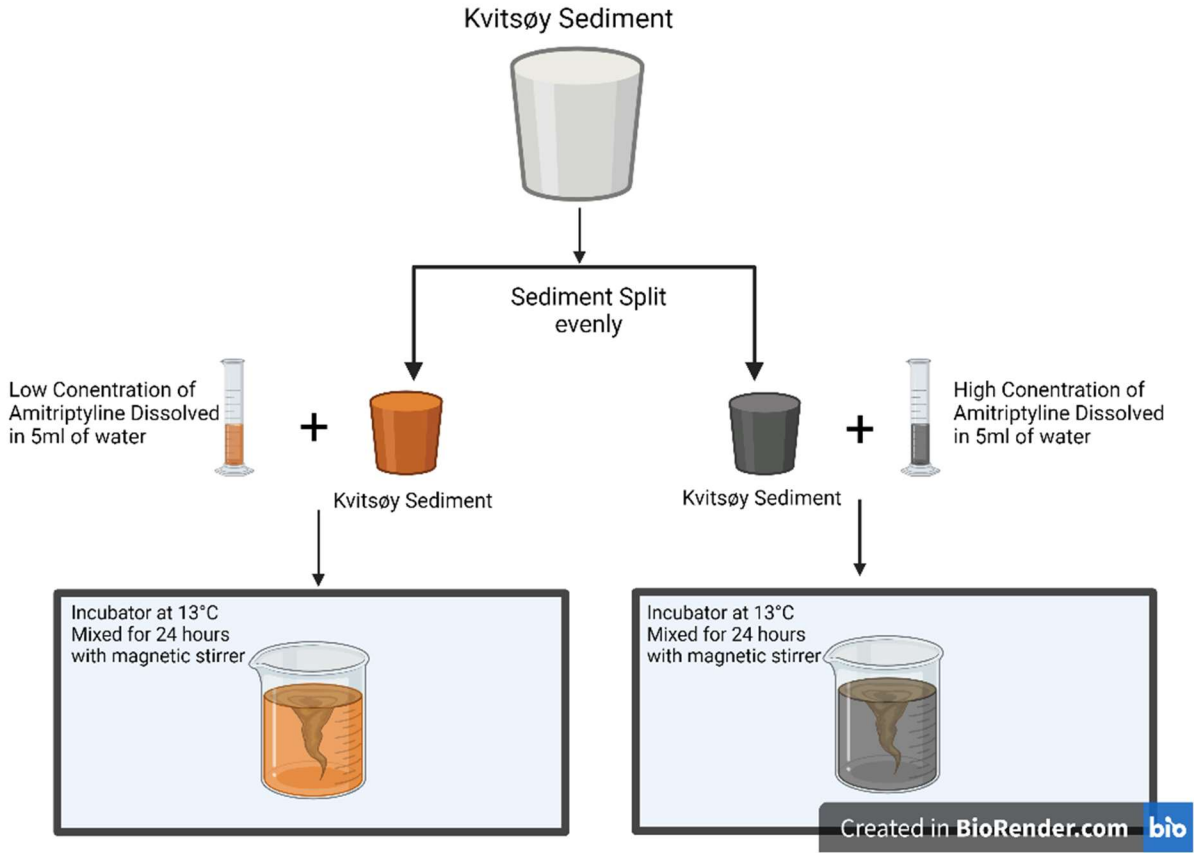


Figure 4.4-2: Sediment spiking procedures used in this project. This figure was created in BioRender (BioRender, 2021).

4.5 Exposure study

The exposure study lasted 28 days starting November 25th, 2020. An exposure enclosure was set up in a third incubator using tin foil to create isolated chambers to reduce the risk of cross contamination between any of the samples. This is especially important given the water solubility of amitriptyline and the open container nature of the beakers. 15 beakers were arranged as shown in (figure 4.5-1) with 5 different trials each with three replicates. They were also arranged by contamination level with the most contaminated being placed at the bottom and the least contaminated at the top. A graphical showcase of the exposure system set up is seen in figure 4.5-2.

Figure 4.5-1: Exposure study setup.

Each sample trial had a volume of sediment between 200 and 250ml. Sterile seawater at 13°C was added to a final volume of 900ml and 7 individuals were placed in each beaker. The temperature of the exposure was 13°C and a cycle of light to dark hours was set up at 8-16 respectively. Throughout the exposure the worms were fed 2 pellets per individual of the live sea angling bait from Topsy Baits on Tuesdays and Fridays. 300 ml of water was also changed for 300ml of fresh sterile seawater in each beaker twice a week. This helped to regulate water quality and keep the salinity consistent (can be seen in figure 4.2-2). Before storage, the

following chemicals were added to the sediment and water samples to prevent the microbial degradation during the interim. For the sediment 1gr of sodium azide was added immediately after collection to each bottle. In the water samples each bottle was treated with 50mL of methanol and 100µL of 4M sulfuric acid. Worms were removed from the sediment rinsed and placed into fresh sea water to ensure that they were fully cleaned of sediment material before they were divided and added to 5ml clear glass vials and put in the -20°C freezer. Throughout the exposure study a detailed journal was kept that included daily recorded salinity, oxygen, pH, and temperature levels starting on December 2nd. This data is presented in appendix A.1.

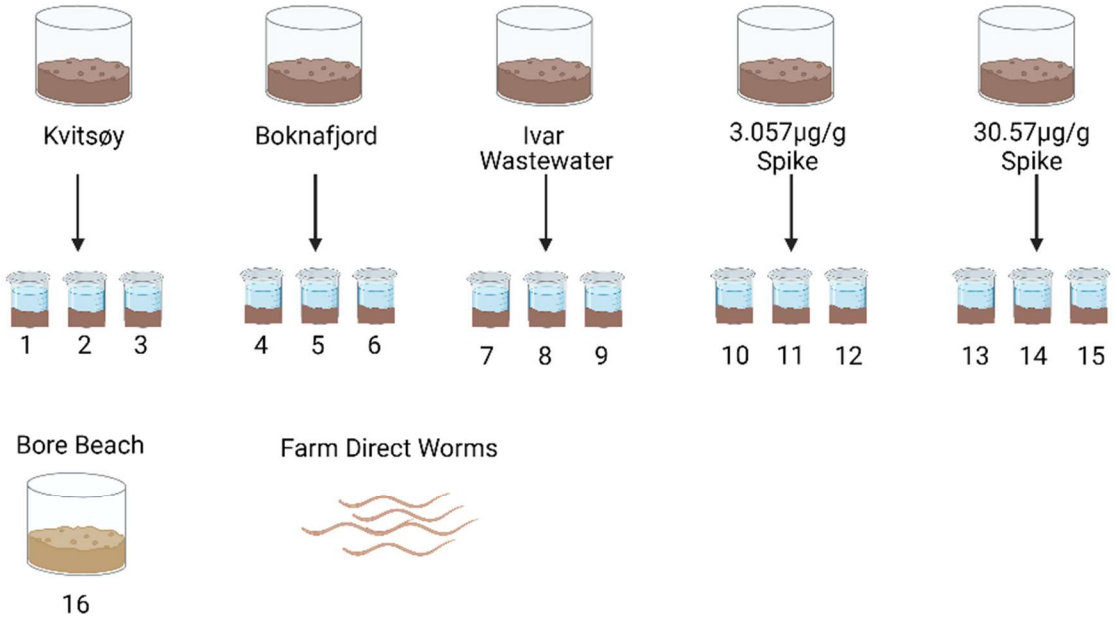


Figure 4.5-2: Graphical explanation of the exposure trials to be evaluated in the study. 15 total exposure trials were done that consisted of 5 different exposure conditions. In addition a sediment control, Bore beach sediment, and a biological control, Nereis virens directly from the farm were also evaluated. Where trial numbers are referred to this is their association. When chemical analysis of the samples were done each numbered group was split into four more replicate trials for error mitigation. This figure was created using BioRender (BioRender,2021).

4.6 Biota and Sediment Extraction Process

4.6.1 Biota and Sediment Freeze-Drying

Freeze-drying is a method that allows for the removal of water in a way that is conducive with retaining adequate chemical extract quality by reducing the damage that could be done using heat added methods of dehydration. In the process ice is automatically converted to water vapor gas before being removed by a vacuum. This means that the liquid phase in the evaporation process is bypassed. In industry this process is also referred to as lyophilization (de Castro and Garcia, 2002). The reason for including this step was to ensure that the amount of sample that was to be extracted would remain consistent across all trials. Without a form of advanced drying, it would not have been possible to be certain of the weight of each sample as simple evaporation does not remove enough water due to the lack of contact between water and air under normal circumstances.

For this study *N. virens* individuals as well as sediment from each beaker used in the exposure study (1-15) as well as material from the pre-exposure farm delivered worms and Bore beach sand were freeze-dried for a total of 16 samples in both cases. A MAXI Dry Lyo machine was used for the process in conjunction with a vacuum pump. The freeze-drying steps are laid out in figure 4.6-1, and described verbally, below.

For *N. virens* processing worms were removed from the beakers and washed by sea water to ensure the removal of any sediments before being stored and frozen at -20°C. Farm direct samples were frozen before the pre-exposure study and individuals were stored long term in the -80°C freezer. Then the samples were thawed when the process of freeze-drying began. Seven individuals of approximately the same size (except for exposure trial 2 which had six individuals at the end of the study) were homogenized with a OMNI international homogenizer and then placed into Labconco 120 ml beakers that were cleaned and autoclaved between each use. Four Labconco beakers of sediment were dried at a time and after being weighed were placed in the MAXI Dry Lyo system. Before the vacuum was put onto them, the samples were flash frozen using liquid nitrogen. The vacuum was then allowed to enter into the chambers and the drying process began. After drying samples were reweighed and the water loss was calculated using equation 4.6-1, these values can be found in table 4.6-1 and table 4.6-2. Finally, the samples

were homogenized to a fine dust in a mortar and pestle and added to 15ml glass vials for storage at -20°C. A flow chart showing all steps included in the freeze-drying can be seen simplified below (figure 4.6-1).

For the sediment an appropriate weight of wet sediment sample was measured and placed into Labconco beakers. The amount of wet sediment chosen was between 15g and 22g as at least 8g would be required from each beaker to complete all replicates for the sediment extraction and this would ensure at least 10g of dried sediment would be present. The process was the same for the sediment as the biota above. After these too were stored in 15ml glass vials at -20°C until further extraction.

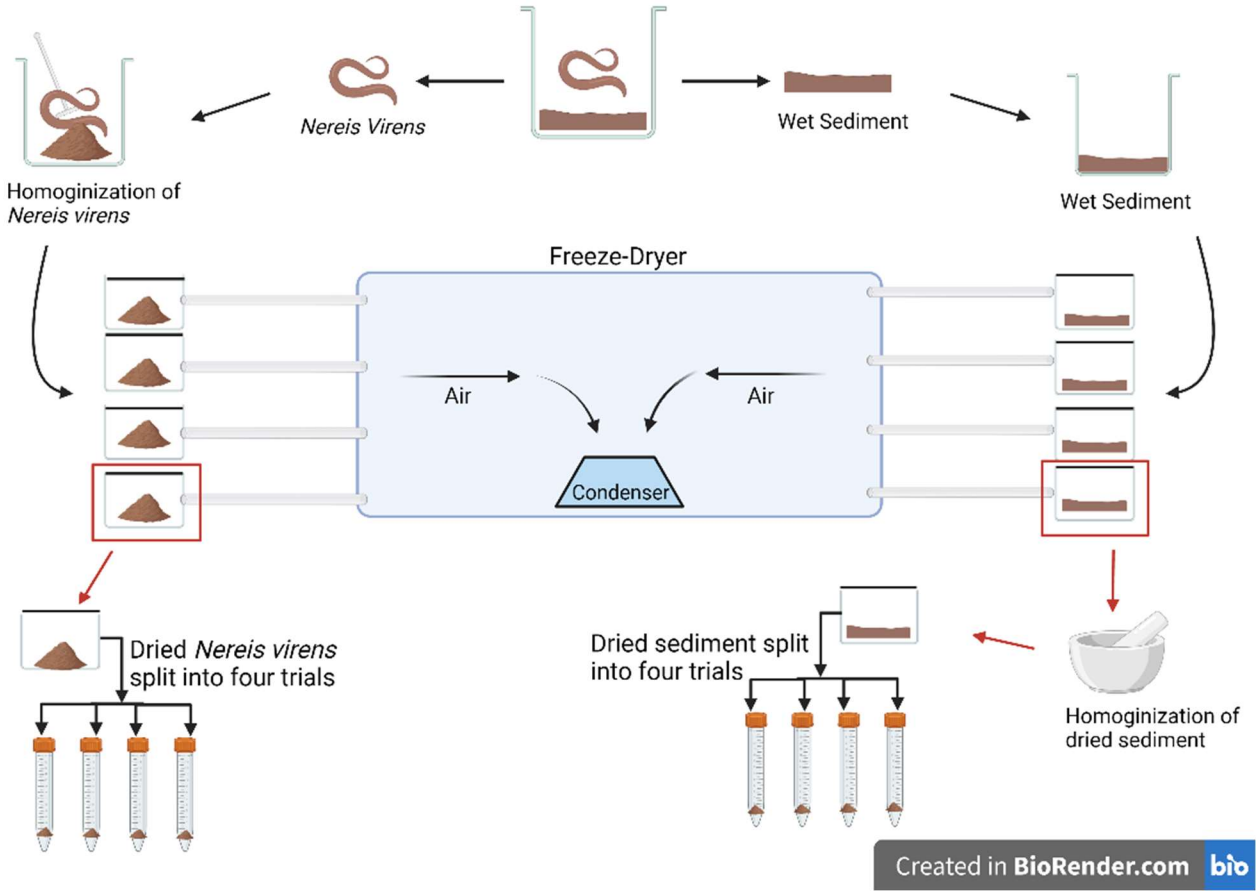


Figure 4.6-1: Steps of the freeze-drying process as described for the Nereis virens biological samples as well as the sediment samples. Figure was created using BioRender. (BioRender,2021).

Total drying time was approximately 6-8 hours and was tested by an extended 10 hour drying of a first set of samples to gauge how much water loss would be feasibly possible from the remaining samples. Since after this period no more loss was being recorded each hour, it can be proven that near complete dehydration had occurred. The water loss data of both the sediment samples and the biota can be seen in table 4.6-1 and table 4.6.21. This was calculated via the water loss equation 4.6-1.

$$Waterloss\ \% = 1 - \frac{Wet\ mass\ -\ Dry\ mass}{Wet\ mass} X100 \qquad (4.6-1)$$

Table 4.6-1: Water loss calculated using equation 4.6-1 for sediment samples under freeze-drying procedures. Trial number corresponds to the exposure tank and the source of the sediment used in said tank is listed in the far-left column.

Sediment Source	Water Loss % for Sediment Samples	Trial Number
Kvitsøy	33.3	1
	33.3	2
	29.4	3
Boknafjord	29.4	4
	30.0	5
	33.3	6
Wastewater Discharge Point	30.0	7
	33.3	8
	28.6	9
Spiked (corrected) 3.057µg/g	31.8	10
	28.6	11
	33.3	12
Spiked (corrected) 30.57µg/g	30.0	13
	30.0	14
	28.6	15
Bore Beach Sand	20.0	16

Table 4.6-2: Water loss calculated using equation 4.6-1 for Nereis virens biota samples under freeze-drying procedures. Trial number corresponds to the exposure tank that housed the individuals and the source of the sediment used in said tank is listed in the far-left column.

Sediment Source	Water Loss % for Biota Samples	Trial Number
Kvitsøy	54.5	1
	57.1	2
	60.0	3
Boknafjord	60.0	4
	55.6	5
	54.5	6
Wastewater Discharge Point	53.8	7
	55.6	8
	58.3	9
Spiked (corrected) 3.057µg/g	57.1	10
	56.3	11
	57.1	12
Spiked (corrected) 30.57µg/g	58.8	13
	58.3	14
	55.6	15
Farm Direct Worms	57.1	16

4.6.2 Biota and sediment standard spiking and ultrasonication

In order to properly evaluate the quality of the extraction procedure performed samples had to be spiked with a standard that could be tracked throughout the reading. The recovery of the standard could be used to estimate the recovery quality of the other components within the sample. A deuterated version of the pharmaceutical in question, amitriptyline, was selected.

From each of the freeze-dried 16 biota samples and 16 sediment samples 0.5g of biota or 2g of sediment were placed into 4 replicate individual 15mL tubes for each trial group. In each of these individual sample containing tubes 100ng of the internal standard was added. The standard came from Sigma Aldrich as 100µg deuterated amitriptyline/mL MeOH, suspended in methanol as the solvent. Then each tube was mixed by hand to ensure all the solvent was removed from the sample and that the sample was also well mixed with regards to the standard. The samples were then added to the -20°C freezer for storage except for the ones to be immediately used for extraction which were placed in the 4°C fridge for no more than 24 hours.

Extraction begins with ultrasonication and the removal of the compounds from the dehydrated material (biota or sediment) itself into a solvent medium. This is done first by the addition of 10mL of methanol to each sample and vortexing each for at least 30 seconds. Then the tube was placed in the UltraSonik ultrasonicator for 15 minutes at 75% power. Samples were then removed and put into the Eppendorf 5804R centrifuge for 10 minutes at 2800rpm. The supernatant from the top of the tubes was removed and added to a new 1L glass flask. This process was then repeated. Next the same basic procedure was used but with 10mL methanol with 0.1% (v/v) formic acid in Milli-Q water (5:5 v/v). 300ml of miliQ water and 30µl of sulfuric acid was added to each 1L amber flask to reduce the percentage of methanol to under 10% and prepared for the next step of the extraction (section 4.6.4) the solid phase extraction (SPE). (Chen et al., 2012; Liu et al., 2017) This can be seen graphically in figure 4.6-2.

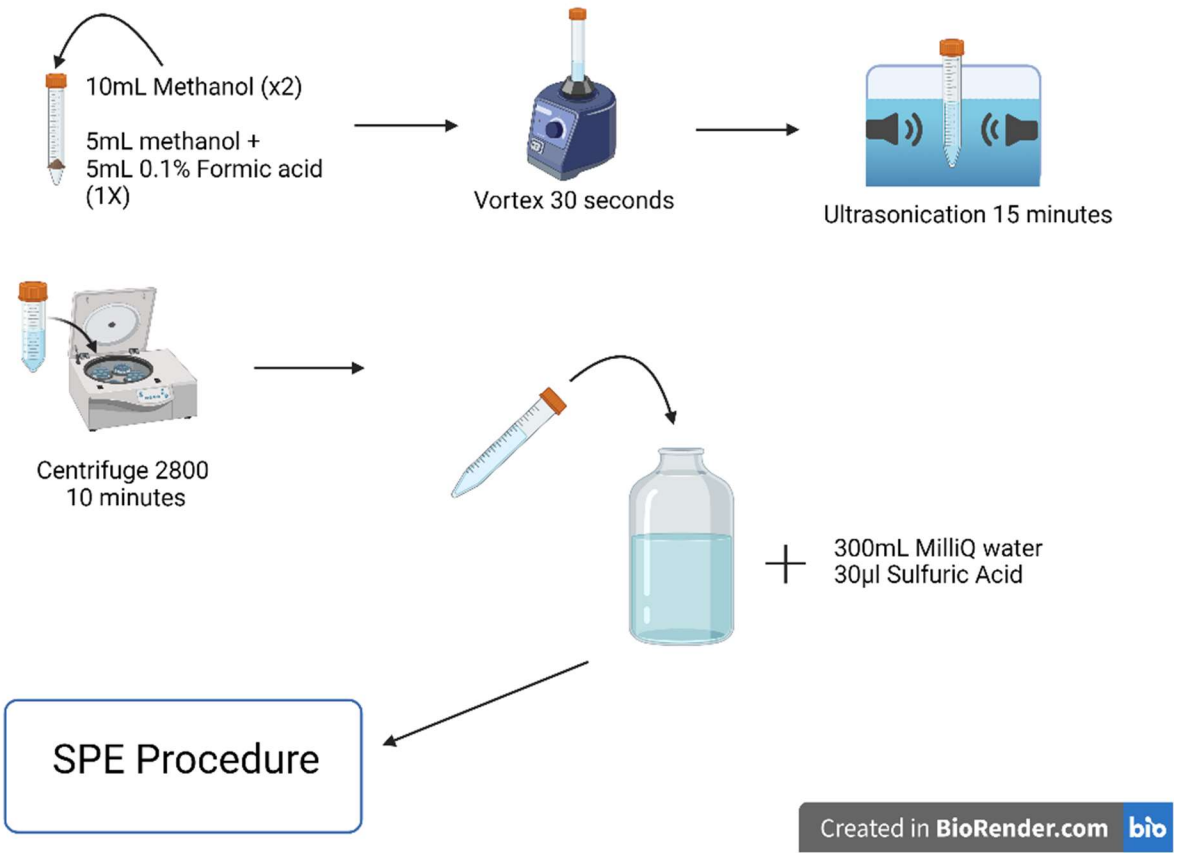


Figure 4.6-2: Extraction procedure through ultrasonication and centrifuge described above. This figure was created in BioRender (BioRender, 2021).

4.6.3 Water sample extraction and preparation for SPE

Water samples were collected throughout the exposure study two days a week. Two of these water collection series were selected for preparation for extraction and preparation for SPE. This process included filtering each 900ml sample through a series of three filters of decreasing pore size. The first 25µm, the second 4.7µm, and the third 2.5µm in size. This filtered water was then moved to the next step in the process SPE and the procedures remained the same for that of the biota and sediment samples.

4.6.4 SPE- Solid phase extraction and final sample preparation

Solid phase extraction (SPE) is a process used to remove a large number of particulates and unwanted large compounds from a sample. A diagram showing the process of SPE can be seen in figure 4.6-3. The SPE process was completed through the use of 6mL, 500mg, Oasis hydrophilic lipophilic balance cartridges (HLB). These cartridges separated the desired extracts from other compounds by their physiochemical properties (Waters, 2015).

Before the SPE process on the sample began each cartridge was conditioned with 10mL of methanol followed by 10mL of Milli-Q water. Then rinsed and cleaned SPE tubes were attached to the tops of the labeled Oasis cartridges with the opposite end of the tubes placed into their respective sample from the previous section. Samples were passed through the cartridges at approximately 10mL/min. After the sample was finished 50mL of 5%(v/v) methanol and Milli-Q waster was added to the containers and allowed to pass through the oasis tubes to ensure all of the extract was properly removed from the initial glass jar. The samples were then dried with the vacuum for 1.5 hours. The above procedure was obtained from the Waters Corporation manuals for use of their Oasis HLB extraction cartridges as well as from the study on the determination of biocides via high performance liquid chromatography by Chen et al. (2012) (Waters, 2015).

After drying the SPE samples had to be removed from the cartridges and pulled into a liquid form. The process used to eluate the extracts was obtained from (Jia et al., 2020) and was as follows. First 5mL of methanol was passed through the oasis tubes followed immediately by 4mL of Ethyl Acetate and 3mL of dichloromethane. This 12mL of chemical as well as the desired compounds trapped by the Oasis tube were collected in small 20mL glass vials placed directly under the oasis tubes. After samples were stored in -20°C freezer before moving on to

the drying step. Drying of the samples was done under a nitrogen stream and remaining was immediately resuspended in methanol. After resuspension, the sample was filtered through a 0.22µm filter that was activated first with a bit of methanol. It was then stored in 2mL glass vials and kept at -20°C until analysis through liquid chromatography tandem mass spectrometry (LC-MS/MS) technology. ……..

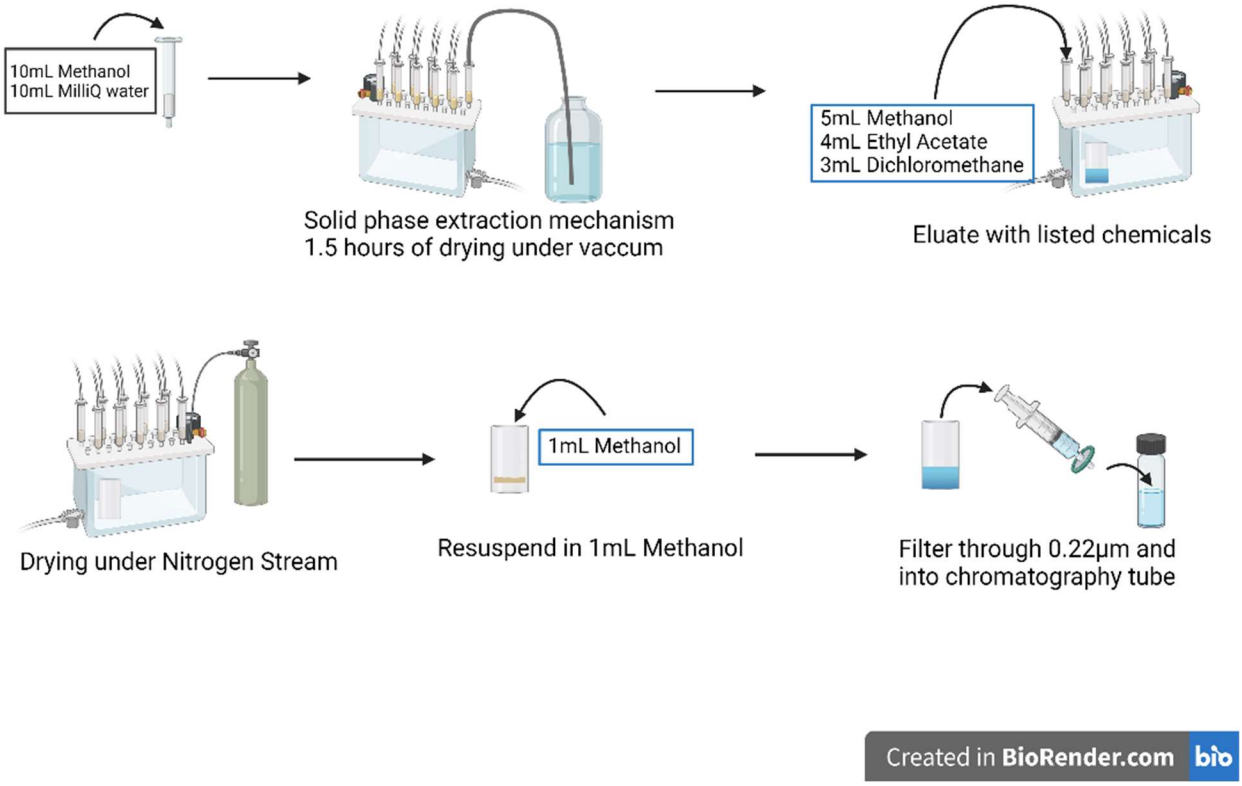


Figure 4.6-3: SPE extraction process and preparation for LC-MS/MS evaluation. This image was created using BioRender (BioRender, 2021).

4.7 LC-MS/MS

4.7.1 Tuning

Detection of amitriptyline was done through LC-MS/MS procedures. This instrumental analysis was done with a Waters Aquity Ultra-Performance Liquid Chromatography system and Quattro Premier XE tandem Quadrupole Mass spectrometer with electrospray-ionization (ESI). Tuning for both amitriptyline and the deuterated amitriptyline that was used for recovery calculations

was done starting with previously established parameters from the previous SANOCEAN sediment, water, and biota evaluations. From these values more specific tuning was completed within the MassLynx software program as to retain a comparative capability between this study and the prior. For deuterated amitriptyline a standard with a concentration of 0.1mg/ml in MeOH was fed through with an infusion rate of 2µl/min after it was determined the signal strength was too high with higher infusion rates. For the LC mobile phase, a two part solvent system was utilized labeled A1 and B1. A1 consisted of 0.2% formic acid and B1 contained MeOH, the flow rate was 200µl/min. The cone voltage (CV) was used to detect the *m/z* signal and the collision energy (CE) was adjusted to produce desired fragmentation. These values as well as other settings for the compound can be seen displayed in table 4.7.411. The process was run through the multiple reaction monitoring (MRM) software system.

Table 4.7-1: Tuning parameters for LC-MS/MS evaluation of amitriptyline and deuterated amitriptyline. Both compounds used electrospray in the positive ionization mode (ES+).

Amitriptyline	
Formula	C20H23N
Molecular Weight	278.2
A1	0.2% Formic Acid
B1	MeOH
Mobile phase flow	200µl/min
Infusion rate	2µl/min

	m/z	Cone Voltage (V)	Collision Energy (V)	Capilary (V)	
Parent Ion	278.2	33	23	3	
Fragment 1	90.9	33	23	3	
Fragment 2	104.95	33	23	3	

D. Amitriptyline	
Formula	C20D3H20N
Molecular Weight	281.21
A1	0.2% Formic Acid

B1	MeOH
Mobile phase flow	200µl/min
Infusion rate	2µl/min

	m/z	Cone Voltage (V)	Collision Energy (V)	Capilary (V)	
Parent Ion	281.1	33	23	3	
Fragment 1	90.9	33	23	3	
Fragment 2	104.9	33	23	3	

4.7.2 Analysis of Samples

For all analytics in this thesis positive mode was exclusively used. 10µl of sample to process was injected at a time from each source and the temperature was kept cool around 5°C. The mobile phase consisted of 0.2% Formic Acid in distilled MillQ water (A1) and MeOH (B1). The process consisted of a starting block of 95% A1 5% B1 followed by B1 at 99% and then a return to the initial conditions for a reset of the column in the last 3 minutes of the program. Each sample was run for 13 minutes, and amitriptyline had a retention time of around 6.33 minutes. The two fragments which can be seen in table 4.7-1 were used for quantification, the most abundant fragment, and qualification, the second most abundant fragment. The column used was an AQUITY UPLC BEH C18 (100mm x 2.1 mm, 1.7µm particle size). The software Targetlynx and Masslynx were used in obtaining the raw data which was then processed manually through excel. This included both automated computerized integration of peaks as well as manual correction for any peak errors.

4.8 Method validation

4.8.1 Exposure study conditions

The conditions surrounding the exposure study were monitored and regulated to ensure the consistency of environmental parameters throughout the 28 day period. The habitat specifications were selected to mimic North Atlantic and North Sea conditions where *N. Virens*

are active. Prior to arrival temperature, salinity, pH, and oxygen were stabilized and *N. Virens* were allowed to acclimate for 8 days which also doubled as a trial period to confirm that conditions set were appropriate for survival. Water samples were also obtained, stored, and tested, to monitor a potential exit point of amitriptyline from the system and understand its water solubility. This put the exposure study survival rate at 99% with tunneling and eating as normal observed.

4.8.2 Analyte extraction and LC-MS/MS analysis

Method validation for the extraction and LC-MS/MS analysis process procedures were done through a series of recovery tests. For this method, an average of 30% deuterated (D) amitriptyline was calculated from a series of spikes in each sample. For each trial 100ng of deuterated amitriptyline suspended in MeOH was added prior to extraction procedures from a standard solution (Sigma Aldrich). This concentration was followed up in the analytical analysis by determining how much D. amitriptyline remained after the procedure was complete. In this way a recovery could be calculated for the protonated amitriptyline in the sample and the error associated with this could be accounted. The equation used to calculate the recovery is seen below as equation 4.8-1. In addition, prior to this study the techniques utilized in this method have been validated through both the studies done by Chen et al. (2012), Liu et al. (2017) and through previous Sanocean sediment/biota analysis. The later was done with a simple series of spike and recoveries where sediment was spiked with varying concentrations of target analytes and the recovery analyzed. In this way it is similar to the validation performed specifically for this study by the previous team however it was not repeated in this experiment and serves only as additional theoretical support of the processes chosen.

$$\%Recovery = \left(1 - \frac{D.Amitriptyline\ added\ -\ D.Amitriptyline\ measured}{Amount\ of\ D.Amitriptyline\ added}\right) X100 (4.8-1)$$

4.8.3 Standard Curve Calibration

For standard calibrations, a series of dilutions were made in accordance with the range of potential analyte concentration. For the typical amitriptyline, a ½ dilution set was prepared starting with 150ng/ml-0.586ng/ml and ending with a blank at 0 ng/ml consisting of 10 level sets. As deuterated amitriptyline was used for recovery standards and validation each series set was provided with the same amount of standard as was added to each sample prior to extraction. An additional curve was produced to process sediment samples from beakers 10-15. The concentrations of analytes in these beakers were higher than anticipated and required a higher scaled standard curve. This curve consisted of 13 level sets also separated by a ½ dilution series starting from 10,000ng/ml – 2.441ng/ml. The graphs of the standard curves were set up through linear regression with the signal response of the LC-MS/MS on the y-axis and the standard solution concentration on the x-axis. These graphs can be seen in figure 4.81 and figure 4.82. The carrier liquid used was MeOH which is the same as the carrier for the analyte samples.

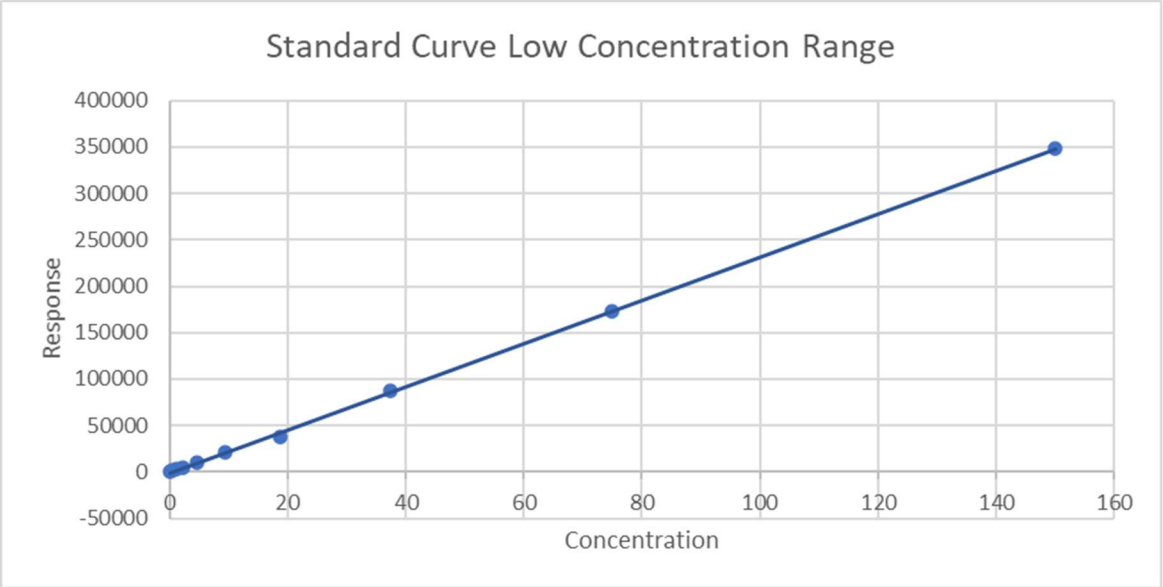


Figure 4.81: The graph in this figure represents the standard calibration curve span covering the lower range of concentrations and representative of all replicate trials except for the two spiked sediment trials. The r^2 value was 0.9997.

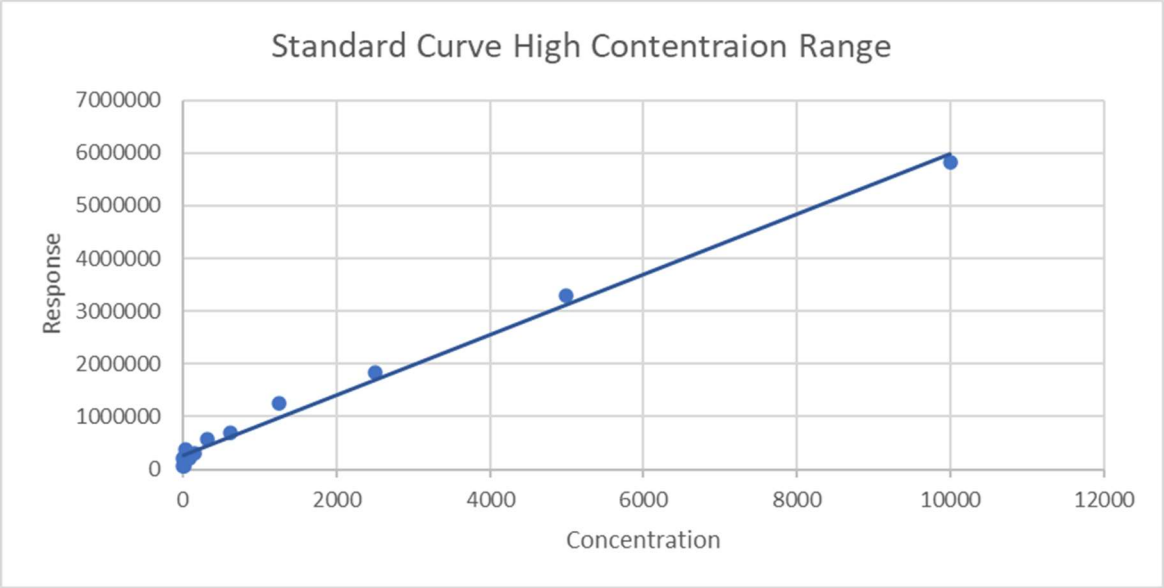


Figure 4.82: The graph in this figure represents the standard calibration curve span covering the higher range of concentrations and is representative of the two spiked sediment samples. The r^2 value was 0.9915.

4.8.4 Standard Error LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the standard curve deviation error (y-intercept) in correlation with the regression model slope. The theoretical definition of LOD is any response with a three times greater value compared with the standard deviation of the response from the blank sample. As for LOQ this same determination can be applied however with a ten times greater response. What this means is that in order to obtain qualitative data, or to confirm that substance is in fact present within the compound it must be above the LOD and to evaluate it for statistically sound quantitative data it must land above the LOQ. The equations used for calculation of LOD and LOQ are equation 4.8-2 and equation 4.8-3 respectively (Lister, 2005). The standard error and slope of the standard curve was calculated using linear regression analysis through excel.

$$LOD\ (ng/mL) = \frac{3\ X\ Standard\ Error\ (Noise\ to\ signal\ ratio)}{Slope\ of\ Calibration\ Curve} \qquad (4.8-2)$$

$$LOQ\ (ng/mL) = \frac{10\ X\ Standard\ Error\ (Noise\ to\ signal\ ratio)}{Slope\ of\ the\ Calibration\ Curve} \qquad (4.8-3)$$

For the standard curves, the Pearson's correlation coefficient (r^2) was 0.994 for amitriptyline in the lower range. An additional curve was run due to higher range results than expected that were not properly addressed by the previous calibration standards, the r^2 for this curve was 0.992. The LOD was found to be 3.5mg/ml and the LOQ was found to be 10.6mg/ml. The standard curves used can be seen in figure 4.81 and figure 4.82 and an example chromatogram can be seen in appendix A.2 figure A.2-1.

5: Results

5.1 Exposure study results

During the exposure study regular readings of pH, temperature, oxygen, and salinity were recorded and their values stabilized to the typical environment of *Nereis virens* in the North Atlantic/North Sea region (Elise, 2015; Cabi, 2021). An overview of these values can be seen in table 5.1-1 and a full read out of each jar's readings can be seen in appendix A.1 (tables A.1-1-A.1-4). In addition to the stability of the abiotic factors the survival rate of the worms was high, 99% for the exposure study (table 5.1-2). Water tests were also conducted to record any pharmaceutical leaving the system via the water as well as to record potential sediment re-worker effects on availability. The results of this validation can be seen below and in the results section 5.4.

Table 5.1-1: Environmental conditions throughout the exposure study on Nereis Virens, an average of all jars across all trials

Average exposure study environmental parameters in all jars

Average pH	8.32
Average Oxygen (mg/L)	9.02
Average Salinity (ppt)	36.08
Average Temperature (C°)	12.57

Table 5.1-2: Survival rate of Nereis Virens throughout the study seen as total number of deaths and survival rate as a percent of all subjects.

Survival Rate of *Nereis Virens*

	# of Deaths	Survival Rate
Acclimation Period	4	96.60%
Exposure Study	1	99%

5.2 Analysis results of amitriptyline in *Nereis virens*

The dried biological material was separated by the exposure (or non-exposure) the original worms were involved in which resulted in 16 groups consisting of the 15 exposure tank conditions and one farm control group. The water loss and weights associated with the freeze-drying step can be seen in table 4.6-1. These were then divided into 4 trials for each exposure. A graphical representation of this can be seen in appendix A.3 figure A.3-1. The total weight of each trial for the *Nereis virens* samples was 0.5g. The data presented below is organized into table 5.2-1 and table 5.2-2 and figure 5.2-1 at the end of section 5.2.

The first exposure trial consisted of samples from Kvitsøy, one of two chosen environmental reference sites for the study. The location can be seen in the map in figure 4.2-1 marked 'K' in the BLANK section of this thesis. Kvitsøy reference samples refer to exposure beakers labeled 1-3. Worms from all three exposures registered as below the limit of detection (3.51ng/ml) for the experiment.

The second exposure trial was the remaining environmental reference site, Boknafjord. In figure 4.2-1 above this is marked as 'B'. Beakers labeled 4-6 correspond to the Boknafjord sediments. Trial 4 and trial 6 both reported concentrations below the LOD. Trial 5 showed a result of 8.5ng/g(biota) with a standard deviation of 2.5.

The third group consisted of sediment from the Ivar wastewater discharge point. This sediment and water was tested prior to the thesis experiment for various pharmaceuticals. The location of the discharge point is marked IV on figure 4.2-1. Ivar discharge point sediment exposures correspond to trials 7-9. All three trials registered below the LOD.

The final two exposures were spiked laboratory experiments. Both used Kvitsøy reference sediment as their base and had different concentrations of amitriptyline added manually. The first of these trials, represented by beaker numbers 10-12 were spiked at 3.057µg/g-sediment and 100% of the biota samples in this set had concentrations above the LOD. The limit of quantification from this curve was calculated to be around 10.6ng/mL the values for the biota here were very close so these values are reported in the table and presented in the text as follows. Beaker trial 10's biota had a concentration average of 9.2ng/g-biota with a standard deviation of 0.6. Trial 11's biota had a concentration average of 9.7ng/g-biota and a standard deviation of 1.

The concentration average of the last trial in this spike concentration, beaker 12, was also 9.7ng/g-biota also with a standard deviation of 1. Thus, the average concentration of the biota in this exposure concentration was 9.5ng/g-biota with a standard deviation of 0.24.

The last exposure set was in beakers 13-15 and as mentioned above were created by taking Kvitsøy reference sediment and set as a 10X increase from group four at 30.57µg/g-sediment. As in the lower spiked concentration 100% of the worm biota samples in this trial contained detectable concentrations of pharmaceutical. The value also exceeded the LOQ. In beaker trial 13 an average of 53.9ng/g-biota was found with a standard deviation of 13.2. In trial 14 an average of 59.4ng/g-biota was detected with a standard deviation of 16.8. Finally in exposure group 15 an average of 56.6ng/g-biota was discovered with a standard deviation of 9. This meant that the average concentration of amitriptyline in biota exposed to this level of contamination was 56.6ng/g-biota and a standard deviation of 2.25.

Table 5.2-1: Average concentration of amitriptyline in ng/g-biota found in LC-MSMS analysis of Nereis virens samples from exposure study tanks and direct from farm control. Each trial number's average consisted of two-four separate replicates from the same samples and the standard deviation of that average is found on the rightmost column of this table. Sediment sources used in the exposure tanks are listed on the leftmost column.

Sediment Source	Average Concentration (ng/g-Biota)/Trial	Trial Number	Standard Deviation
Kvitsøy	< LOD	1	
	< LOD	2	
	< LOD	3	
Boknafjord	< LOD	4	
	8.5	5	2.5
	< LOD	6	
Wastewater Discharge Point	< LOD	7	
	< LOD	8	
	< LOD	9	
Spiked (corrected) 3.057µg/g	9.2	10	0.6
	9.7	11	1
	9.7	12	1
Spiked (corrected)	53.9	13	13.2

30.57µg/g	59.4	14	16.8
	56.6	15	9
Farm Direct Worms	< LOD		

Table 5.2-2: Average concentration of amitriptyline in ng/g-biota found in LC-MSMS analysis of Nereis virens samples from each exposure tank and direct from farm control group. Each trial number's average consisted of two-four separate replicates from the same samples and then the average of those sample trials is displayed below. The standard deviation for each exposure trial that combined to form the average is found on the rightmost column of this table. Sediment sources used in the exposure tanks are listed on the leftmost column. For samples where the analyte concentration was below the LOD in a sample set where others were above the LOD the result was replaced with the LOD/2 for statistical analysis (Bailey and Michelson, 2013)(Cohen and Ryan, 1989).

Sediment Source	**Average Concentration (ng/g-Biota) / Sediment Source**	**Standard Deviation for Biota Average of Sediment Source**
Kvitsøy	< LOD	
Boknafjord	5.2	2.8
Wastewater Discharge Point	< LOD	
Spiked (corrected) 3.057µg/g	9.5	0.2
Spiked (corrected) 30.57µg/g	56.6	2.2
Farm Fresh Worms	< LOD	< LOD

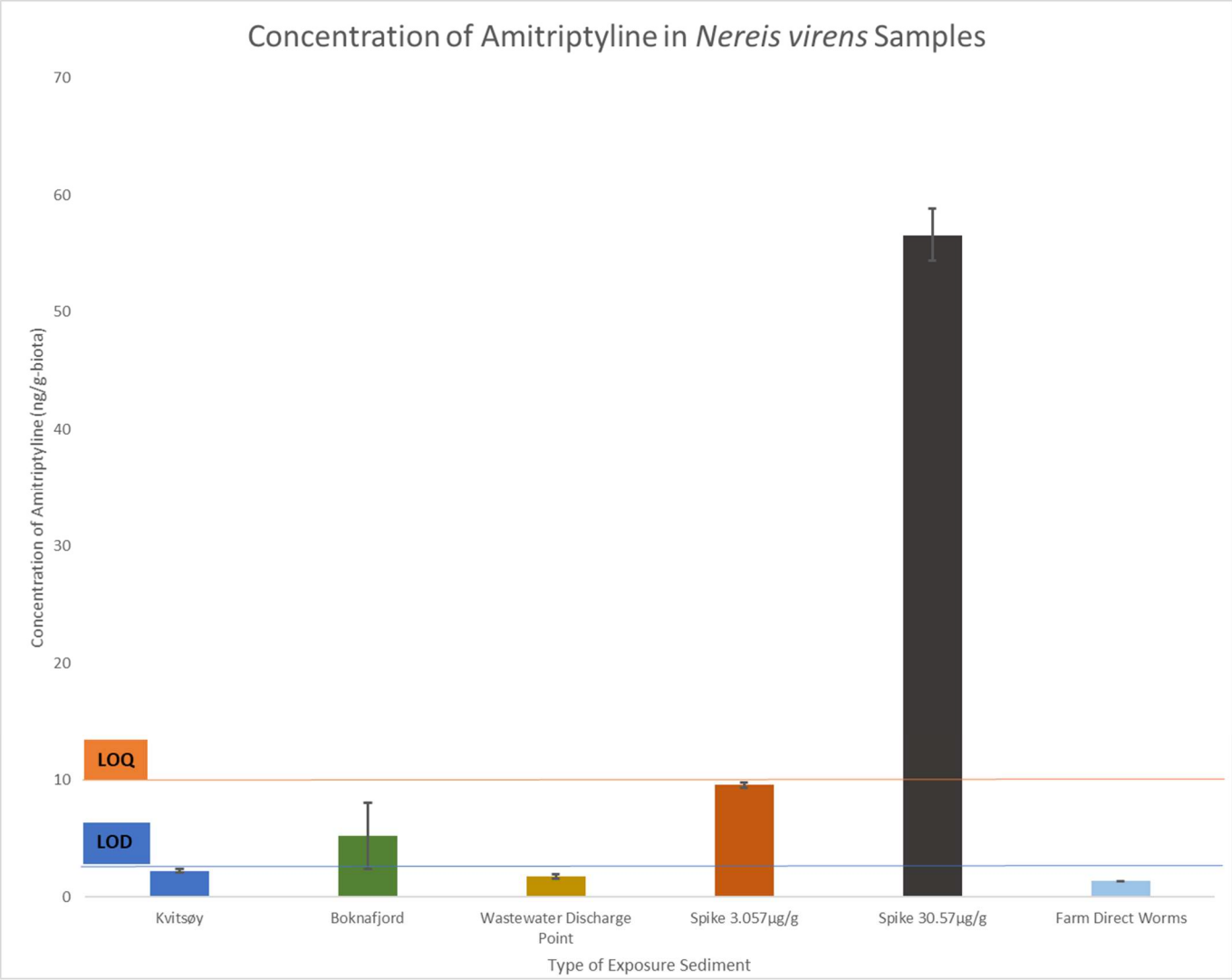


Figure 5.2-1: A graphical representation of the average concentration of amitriptyline in ng/g-biota found in LC-MSMS analysis of Nereis virens samples from each exposure tank and direct from farm control group. Each trial number's average consisted of two-four separate replicates from the same samples and then the average of those sample trials is displayed above. The standard deviation for each exposure trial that combined to form the average is presented as error bars seen on each sample. Sediment sources used in the exposure tanks are listed on the x-axis as a label under each bar. For samples where the analyte concentration was below the LOD in a sample set where others were above the LOD the result was replaced with the LOD/2 for statistical analysis of standard deviation (Bailey and Michelson, 2013)(Cohen and Ryan, 1989) The LOD and LOQ are represented by a blue and orange line respectively.

5.3 Analysis results of amitriptyline in exposure sediment

The sediment was collected for testing at the end of the 28-day period and a subset of each was freeze-dried. The water loss and weights of this can be seen in table 4.6-2. In the same manner as the biological material the sediment was separated into the different exposure condition containers (1-15) and the Bore beach sand (16). This was split into four different trials for each container and extracted using the process described in the procedure. The averages of each trial from the four replicates can be seen in figure A.3-2 in appendix A.3. The testing of these sediments was done as a control and checkpoint for understanding the effect on the biota samples analyzed. This data is presented in table 5.3-1, table 5.3-2 and figure 5.3-1.

The first sediments corresponded to Kvitsøy reference sediment (marked K in the map of figure 4.2-1) and are from exposure groups 1-3. The average of group one was 20.6ng/g-sediment with a standard deviation of 17.55. For exposure two the average was 15.6ng/g-sediment with a standard deviation of 11.45. The final trial was below the limit of detection.

The second sediment group was from Boknajord (marked B in the map of figure 4.2-1) and represented trials 4-6. The average of trial 4 was 32.5ng/g-sediment with a standard deviation of 29.15. Trial 5 had an average below the limit of detection. Trial 6 had an average of 12.5ng/g-sediment and a standard deviation of 10.85.

The third sediment group was from the Ivar wastewater discharge point (marked IV in the map of figure 4.2-1) and corresponded to tanks 7-9. The average for group 7 was 6.5ng/g-sediment with a standard deviation of 1.2. Group 8 was below the limit of detection. Trial 9 had an average of 11.2ng/g-sediment and a standard deviation of 8.75.

The fourth group and first spike using Kvitsøy sediment was set to 3.057µg/g-sediment. The first of this group exposure represented by beaker 10 had an average of 377.5ng/g-sediment with a standard deviation of 38.5. The next trial 11 had an average of 441ng/g-sediment and a standard deviation of 50.5. Trial 12, the final in this exposure set, had an average of 453ng/g-sediment with a standard deviation of 5.75. This gave an overall average for the first spiked samples of 423.83ng/g-sediment and a standard deviation of 33.1.

The fifth group was the second spike using Kvitsøy sediment and was set as a 10X increase from group four at 30.57µg/g-sediment. Beaker 13 was the first for this exposure trial set and had an

average of 943.55ng/g-sediment with a standard deviation of 108.55. Beaker 14 was the second for this exposure trial and had an average of 841.4ng/g-sediment with a standard deviation of 41.7. 15 finished off this set with an average of 513.7ng/g-sediment and a standard deviation of 47.8. The average then for this trial set was 763.2ng/g-sediment with a standard deviation of 180.5.

The final group was another control using sand from costal Bore beach and had been collected on foot (compared to the oceanic ship collection methods used for the other sediments). This was represented by trial number 16 and had amitriptyline levels below the limit of detection.

Table 5.3-1: Average concentration of amitriptyline in ng/g-sediment found in LC-MSMS analysis of sediment samples from exposure study tanks. Each trial number's average consisted of four separate replicates from the same samples and the standard deviation of that average is found on the rightmost column of this table. Sediment sources are listed on the leftmost column.

Sediment Source	**Average Concentration (ng/g-sediment)/Trial**	**Trial Number**	**Standard Deviation**
Kvitsøy	20.6	1	17.55
	15.6	2	11.45
	< LOD	3	
Boknafjord	32.5	4	29.15
	< LOD	5	
	12.5	6	10.85
Wastewater Discharge Point	6.5	7	1.2
	< LOD	8	
	11.2	9	8.75
Spiked (corrected) 3.057µg/g	377.5	10	38.5
	441	11	50.5
	453	12	5.75
Spiked (corrected) 30.57µg/g	934.55	13	108.55
	841.4	14	41.7
	513.7	15	47.8
Bore Beach Sand	< LOD	16	

Table 5.3-2: Average concentration of amitriptyline in ng/g-sediment found in LC-MSMS analysis of sediment samples from each sediment source. Each trial number's average consisted of four separate replicates from the same samples and then the average of those sample trials is displayed below. The standard deviation for each exposure trial that combined to form the average is found on the rightmost column of this table. Sediment sources are listed on the leftmost column. For samples where the analyte

concentration was below the LOD in a sample set where others were above the LOD the result was replaced with the LOD/2 for statistical analysis (Bailey and Michelson, 2013)(Cohen and Ryan, 1989).

Sediment Source	Average Concentration (ng/g-sediment) / Sediment Source	Standard Deviation for Average of Sediment Source
Kvitsøy	12.7	8
Boknafjord	15.6	12.7
Wastewater Discharge Point	6.5	3.9
Spiked (corrected) 3.057µg/g	423.83	33.1
Spiked (corrected) 30.57µg/g	763.2	180.5
Bore Beach Sand	< LOD	< LOD

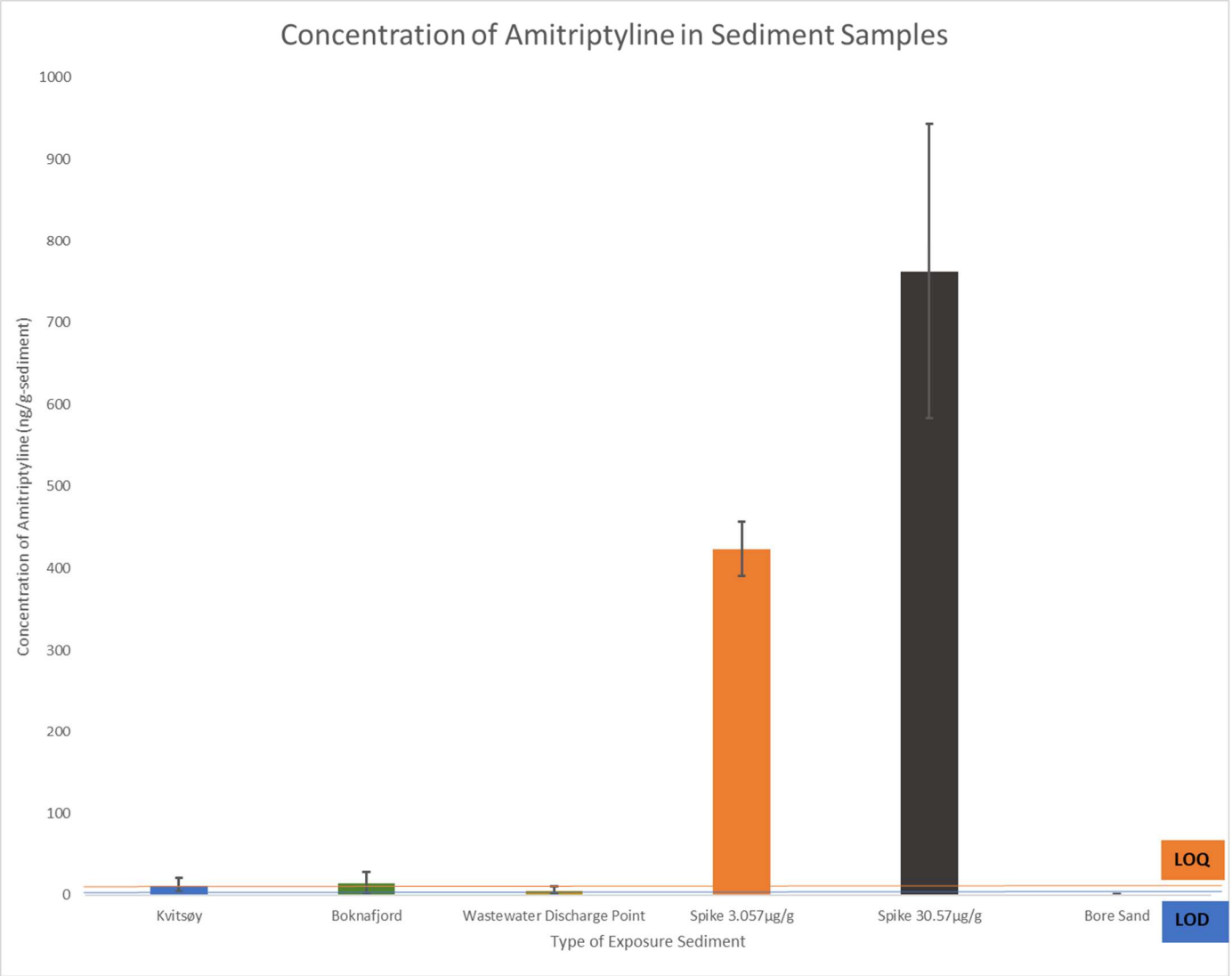


Figure 5.3-1: A graphical representation of the average concentration of amitriptyline in ng/g-sediment found in LC-MSMS analysis of sediment samples from each exposure tank after 28 days and a bore beach sand control sample. Each trial number's average consisted of two-four separate replicates from the same samples and then the average of those sample trials is displayed above. The standard deviation for each exposure trial that combined to form the average is presented as error bars seen on each sample. Sediment sources used in the exposure tanks are listed on the x-axis as a label under each bar. For samples where the analyte concentration was below the LOD in a sample set where others were above the LOD the result was replaced with the LOD/2 for statistical analysis of standard deviation (Bailey and Michelson, 2013)(Cohen and Ryan, 1989) The LOD and LOQ are represented by a blue and orange line respectively.

5.4 Analysis results of amitriptyline in exposure water

. The water from two of those dates (December 1st and 18th, 2020) was processed and data analyzed with LC-MS/MS procedures. The concentrations are as follows and are displayed in table 5.4-1.

For December 1st, 2020 trial 1 (Kvitsøy reference sediment, beakers 1-3) the concentration in the original was 0.0062ng/ml. For trial 2 (Boknafjord reference sediment, beakers 4-6) the concentration was 0.0037ng/ml. Trial 3 (Ivar wastewater discharge point sediment, beakers 7-9) the concentration was 0.03ng/ml. For Trial 4 (Spiked 3.057µg/g Kvitsøy sediment, beakers 10-12) the concentration was 0.44ng/ml. Finally for trial 5 (spiked 30.57µg/g Kvitsøy sediment, beakers 13-15) the concentration was 3.52ng/g.

For December 18th, 2020 trial 1 (Kvitsøy reference sediment, beakers 1-3) the concentration was 0.03ng/ml. Trial 2 (Boknafjord reference sediment, beakers 4-6) had a concentration of 0.002ng/ml. The third trial (Ivar wastewater discharge point sediment, beakers 7-9) registered a concentration of 0.009ng/g. The first spike trial 4 (spiked 3.057µg/g Kvitsøy sediment, beakers 10-12) the concentration was 0.72ng/ml. Last trial 5 (spiked 30.57µg/g Kvitsøy sediment) had a concentration of 2.83ng/ml.

Table 5.4-1: Average concentration of amitriptyline in ng/ml-water found in LC-MSMS analysis of 900ml of water sampled from each exposure study trial (consisting of three tanks per well mixed) on two dates, December 1st 2020 and December 18th 2020. Sediment sources are listed on the leftmost column.

Sediment Source	**DECEMBER 1st, 2020 Average Concentration ng/ml water after adjusting for magnification**	**DECEMBER 18th, 2020 Average Concentration ng/ml water after adjusting for magnification**
Kvitsøy	0.0062	0.03
Boknafjord	0.0037	0.002
Wastewater Discharge Point	0.03	0.009

Spiked (corrected) 3.057µg/g	0.44	0.72
Spiked (corrected) 30.57µg/g	3.52	2.83

6: Discussion

Concentrations of pharmaceutical waste in the environment is an area of scientific research largely still unknown, but rapidly becoming incredibly important. Through a 28 day exposure study this thesis sought to evaluate changes in the concentration of amitriptyline in the benthic polychaete *N. virens* as well as collect data on the sediment and water in that period. An evaluation of the results of this study and their implications will be presented in this section below.

6.1 Exposure study

The average water temperature was 12.5°C, average salinity was 36.1ppt, average pH was 8.3, and average oxygen was 9.0mg/L. These parameters were previously selected as ideal to replicate quality living conditions for the worms to be tested (Elise, 2015; Cabi, 2021). During the exposure study only one individual was lost immediately after transfer. Due to the proximity to relocation as well as the exposure group itself (group one, trial two) not being either of the laboratory spikes it can be reasonably assumed the death was due either to random mortality, or stress associated with habitat change. All other worms in this group remained healthy and active throughout the experiment. This high rate of survival as well as the observed consistent activity rate is evidence of correctly selected parameters. In addition, it serves to prove the selected spiking conditions for the laboratory samples remained well enough below the LD^{50} to not impact the mortality of the individuals in the study (RxList, 2019; PubChem, 2021). A well oxygenated aquatic environment also serves to help minimize the incidence of bacterial growth and their potential bio degradational impacts to the amitriptyline (Baez and Shiloach, 2014).

6.2 Biota samples

The concentrations of amitriptyline in the worms that underwent the exposure study was determined through LC-MS/MS after undergoing extraction via freeze-drying and homogenization.

The environmental sediment samples used for the experiment came from three different sources. Kvitsøy, which was a supposed reverence site, Boknafjord another reference, and sediment outside of a wastewater discharge plant. All three locations can be seen on the map in figure 4.2-1 in section 4.2. All three sites had averages either below or near to the LOD (3.51ng/ml).

Boknafjord was the only site where the *Nereis virens* had an average above that at 5.2ng/g-biota. However, with a standard deviation of 2.8 this number could be, due to error, also below the LOD. Worms taken directly from the farm also indicated levels far below the LOD. However, while it is understood that these numbers fall below the LOQ (10.6ng/ml) a T-test was performed between all environmental samples to seek out any potential significant difference. None was found between the environmental exposure animals so it can be considered that all three samples were within a close enough range to be considered statistically similar with the level of sensitivity of the machine. However, when compared to the farm direct worms that were never in any environmental sample Kvitsøy and wastewater discharge site exposed worms both indicated a significant difference. Boknafjord did not show a statistic difference, however this is most likely due to the effect of the standard deviation of the sample and a more stable consistent reading would be needed to confirm this parameter. What this could indicate is that there is a possibility that all three environmental sites harbor some level of amitriptyline contamination that is resulting in a difference between the biota exposed to them. All of this however is tempered heavily by the fact that the LOD and LOQ were both below so analysis is done in a hypothetical to explore possibilities related to future studies.

While one spike was above environmental micropollutant concentrations they both are a good representation of the potential for accumulation of amitriptyline in the biota. Both samples showed that the pharmaceutical did in fact collect to a certain capacity in the bodies of the worms reworking the contaminated sediment. The lower spiked soil produced individuals with an average of 9.5ng/g-biota, a value that is just below the threshold of the limit of quantification but well above the limit of detection. Due to the nature of the determination of the LOD and LOQ this value could be considered by some to be quantifiable (Bailey and Michelson, 2013) (Cohen and Ryan, 1989), however in the parameters set by this study this value is seen as evidence of only qualitative means. As before with the environmental samples this number will still be given statistical treatments and comparative analysis, but the arranged limitations should be remembered. When comparing this spike to the three environmental samples and the blank farm sample using the same T-test parameters as before it can be seen that in all cases but Boknafjord there is a significant difference between the concentrations. The situation with the

Boknafjord sample remains with the standard deviation, the average value is certainly similar to the average value of the spiked sample, but the error conditions make it difficult to say for sure where it falls statistically. This indicates that even at lower spiked concentrations there is a significant change in the presence of the amitriptyline. In the higher spike condition, the accruing of the pharmaceutical is even more obvious. All values for this trial were over both the LOD and LOQ and so can be quantified and qualified without doubt. The average for this set was 56.6ng/g-biota and was significantly different from all other trials. When comparing the value for the first spike and the second spike we see that the concentration of the worms in the second spike is 5.96X or approximately 6X more than the concentration in the first spike. This is of note because the magnification difference between the spiked sediments was set to be a 10X difference. There are many potential reasons for this not least of all the limitation surrounding accurately spiked sediments (Northcott and Jones 2000; Amelug, 2007) which is discussed further in section 6.3. However, it is important to note that there was a quantifiable difference that shows correlation with higher sediment concentrations.

When the question of qualifying if a bioaccumulation process involving amitriptyline is taking place is considered, the experiment would indicate this to be true. However, the question of quantifying through bioconcentration calculations is more complex. The equation for bioconcentration factor is seen in equation 6.2-1 (European Chemicals Agency, 2017; ChemSafetyPro, 2021).

$$Bioconcentration\ Factor(BCF) = \frac{Concentration\ in\ biota}{Concentration\ in\ surrounding\ media} \qquad (6.2-1)$$

This value is interpreted in varying ways by varying locations. In the United States a BCF below 1000 indicates something is not bioaccumulative, 1000-5000 is bioaccumulative, and anything above 5000 is very bioaccumulative. The EU REACH however sets the limit for bioaccumulation at a BCF above 2000 and very accumulative at factor above 5000 (CehmSafetyPro, 2021). The major factor affecting this thesis, however, is the equilibrium conditions required to properly assess the BCF. In order to utilize this equation the living organism must be in equilibrium with its environment in relation to the toxin in question (Wang, 2016). There is no indication within the scope of this experiment that the individuals in the study ever reached equilibrium, there is only an indication that amitriptyline was absorbed into their

biomass. Other studies utilizing *Nereis virens* for different chemical bioaccumulations over longer periods of time noted that by approximately the 24 day mark a linear accumulation was still occurring (Ray et al., 1980). This time scale of equilibrium is dependent on many factors, and one study cannot be used to justify the timeline of another absolutely, but it can provide some level of guidance. Needless to say, more data points on different days would be needed to affirm an equilibrium for certain. However crudely it can be preliminarily assessed that a BCF of over 1000 is not apparent for either group at this time and seems unlikely to be surpassed if the same exposure trend were to occur. Regardless of the BCF, an accumulation of pharmaceutical is occurring in correlation with increasing sediment concentration, and this has potential environmentally disruptive capabilities.

6.3 Environmental and laboratory sediment samples

As mentioned, within the thesis there were three environmental samples used in the exposure study, one environmental sample used as a control, and two laboratory spiked samples.

All environmental samples tested above the LOD, however when the lower end of the standard deviation is considered then Kvitsøy is the only environmental sample consistently above the LOD at 12.7ng/g-sediment. Boknafjord (15.6ng/g-sediment) and the wastewater discharge point (6.5ng/g-sediment) sediment can, through error, fall below the LOD. Bore beach sand is safely under the limit of detection. Similar to the biological samples not all sediments registered above the LOQ but are evaluated statistically regardless in significant comparisons with the caveat that the quantifications are below where this study has chosen to affirm quantitative data. With this in mind none of the environmental samples could be evaluated as significantly different from the others at a 95% confidence. This is most likely due to the error variances having much overlap which adds uncertainty to data. Unlike in the biological data where there was a significant difference between the control farm group and environmental sample exposed individuals, the Bore beach sand control appears to have a much lower value than the other sediments but due to the standard deviation and the number of replicates it cannot be determined statistically different. This all indicates there is likely indication of contamination within the three (Kvitsøy,

Boknafjord, and the discharge point) exposure samples and likely not contamination in the Bore beach sand qualitatively, but quantitatively comparisons require further research and additional data to make clearer.

At the end of the study it was quantified that the lower spiked sediment contained an average of 423.83ng/g-sediment and the higher spiked sediment contained 763.2ng/g-sediment. Both of these sediments were significantly different from all other samples, each other and all environmental tests. The timeline mentioned previously is important in evaluating these results as many factors can influence and change the concentration of contaminant in the sample throughout an exposure study. The simplest example, a removal of dissolved chemical during the twice weekly water change. Water data showcasing this effect is presented both in the section 5.4 in table 5.4-1 and discussed in the following section 6.4. Amitriptyline is known to be water soluble (RxList, 2019; PubChem, 2021) and so escape via this route was expected. Another more complex process is related to the biological activity of the worms. As was discussed prior *N. virens* did begin accumulating amitriptyline into their biomass throughout the 28-day study. This as well as their processing of the sediment and excrement of unincorporated pharmaceutical can pull amitriptyline from the soil and into a more bioavailable form (Ray et al., 1980; Klosterhaus et al., 2011). Typical abiotic degradation could have played a role in the removal of amitriptyline from the sediment through the period. As the exposure tanks were oxygenated and exposed to a daily light level for 8 hours over a 24h period it may be expected that the chemical will breakdown through the study. However, amitriptyline was found to have very good resilience in the environment and seems to have a very limited photodegradation ruling this factor out (Chen et al., 2017). Finally, the act of spiking sediment itself is very complex and can be unreliable (Northcott and Jones 2000; Amelung et al., 2007), this limitation will be discussed in more detail in section 6.6. However, it should be noted that at the end of the study the higher spike was 2X the lower spiked sediment, which is interesting because regardless of what occurred during the exposure the difference between the *Nereis virens* samples at the end of the study in those same exposures was 6X. Further research and replicates tests with varying spike conditions would need to be done to quantify this comparison for certain, understand the effect, and

eliminate/minimize the variables listed above that were largely left unregulated in this exploratory experiment but within the scope of this study the difference is there.

6.4 Water samples

It is known that amitriptyline is a water-soluble chemical (Pubchem, 2021), for this reason monitoring the water within the 28-days of the experiment as a control was valuable. A minimal loss of amitriptyline from the soil in all groups that seemed to correlate with the original concentrations. The loss appeared to be steady over the course of the study if these two data points are to be extrapolated but of course it is possible that during the first few days the concentration was much greater and there was an equilibrium balanced reached by the time the first week had passed. What this indicates, however, is that amitriptyline does in fact enter the water column near the marine floor from the sediment when benthic workers are present and active. It should also be noted that the water samples came from the top half of the exposure tank, while the tanks were aerated with oxygen bubblers it is still entirely possible that the concentrations in the water column are higher again above the surface of the sediment compared to the top of the water. It can be determined that the loss is not sufficient to change the outcome of the accumulation experiment drastically when these two data points are considered, testing the water on the very first day of sampling would provide stronger evidence to this claim.

6.5 Discussion of key result implications

Bioaccumulation potential for micropollutants is of course the main question being asked by an exposure study such as this one. Prior research in the SANOCEAN project revealed potential amitriptyline contamination in the wastewater outlet stream sediment. This triggered the discussion surrounding testing the potential for this anti-depressant to enter the bodies of animals living within said sediments. The data surveyed in this study shows that the drug does have the capacity for accumulation within the bodies of *N. virens*. Bioaccumulation of antidepressants at any trophic level has a host of repercussions, and when it comes to benthic animals low on the food chain, the problem comes from the bottom up. As *Nereis virens* are a common food source for many marine fish and are harvested for use frequently as bait and aquaculture fish feed, the pollutants present in their bodies have a direct impact on these animals in the trophic level above them (Oddsen, 2014; Elise, 2015; Cabi, 2021). The effect of certain antidepressants on fish has been studied in the past. In one study done on zebrafish larvae, exposure to environmental

concentrations caused an increase in the number of abnormal larvae and embryo developments. The hatching time was also decreased which had a direct impact on the number of fish born (Nowakowska et al., 2020). Another example of micro concentration effects on marine organisms is the multigenerational impact seen in zebrafish from fluoxetine (FLX) which was shown to impact three generations after an initial ancestral contamination (Vera-Chang et al., 2018) It is data like this that brings into light the impact that exposure to these types of drugs can have, even at very low concentrations. In addition to simply passing this chemical to the animals that consume them, *Nereis virens* specifically is known to remobilize compounds from the sediment allowing them to enter the water column as well as the aquatic food web more readily than they would have otherwise (Klosterhaus et al., 2011). This means that even if amitriptyline were to come out of a wastewater discharge outlet in lower concentrations as to be buried by the sediment continuously, *Nereis virens* has the potential to dig them up. As they incorporate this into their bodies over time they can retain even initially micro concentrations of the pharmaceutical moving it into the more available upper level of the ecosystem. Another recently researched topic relevant to this discussion is the additional magnifying effect of microplastics on organic pollutants. Studies have shown that many of these chemicals such as polycyclic aromatic hydrocarbons (PAH), dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyles (PCBs), as well as antidepressants such as venlafaxine (VFX) are concentrated by the presence of microplastics (Hirai et al., 2011; Van et al., 2012; Rochman et al., 2013; Qu et al., 2018). This enhancing effect can double down on the work already being done by sediment reworkers bringing even more of these pollutants into the food chain. Studies in this area show the complexity of a moving ecosystem and how seemingly small alterations, such as accumulations of micropollutants in the ng/g range, can have unforeseen long reaching impacts on many aspects of the environment.

This study has not explored how much of these accumulations passes into the next trophic level, and it has not looked in depth at the effect this drug's increasing concentrations have on *Nereis virens* itself, beyond daily sight checks and mortality rates. However, it has shown that amitriptyline has the capability to accrue within the bodies of these specific benthic worms that are very important members of the marine ecosystem and pillars at the base of a complex food web. It is not hard to imagine or extrapolate that the fate of this antidepressant could follow the paths and impacts of other previously researched pharmaceuticals, of a similar chemical nature.

6.6 Limitations of the study

While overall the study was successful it is none the less important to address the relevant limitations that impacted its' quality. Firstly, the environment itself is a dynamic system. Samples taken are often uneven and unpredictable in their concentrations and purity. This makes testing natural sediments tricky when one is relying on a sample to be the reference or control. For example, Kvitsøy in this study was set to be a reference, as was Boknafjord, but as the results showed there seemed to have been potential contaminations present in both that showed in both the analysis of the sediment and the analysis of the biota. In the biota the difference between the individuals in the exposures and the ones that came directly from the farm was significant, so that indicates some level of contamination in the control sediment that was used to make the spiked sediments. Although small it can still offset the totals at the end of the spiking. In relation to the spiking, correctly spiking sediment is known to be very difficult (Northcott and Jones 2000; Amelung, 2007) for a variety of compounds and drugs, amitriptyline is no exception. Although this project followed a spiking procedure used before for other similar compounds (Chen et al., 2012; Liu et al., 2017) it is difficult without quantification to prove it worked. A solution to this could have been to take a sample of the sediment before the exposure started and test to ensure that the spiking was at the correct level before proceeding. However due to time allowances this was not a feasible option for the thesis. Another sediment related limitation was the high standard deviation of the environmental samples. The source of this variance is not well understood, but subsequent testing and retesting of the exposure sediment would yield a clearer picture if the deviation was due to laboratory error, colloidal interactions between the drug and the particles in the sediment making the homogenization worse, or open the door to other explanations. Another important note is that the photodegradation of amitriptyline was not found to be very robust (Chen et al., 2017) so it can be assumed natural biodegradations from the UV light system was not a strong factor in the change of amitriptyline concentrations throughout the study. In the processing of the *Nereis virens* samples an issue of quantity appeared. Due to the high water content and lower weight of the worms the amount of processible sample at the end of the freeze-drying was quite low. This meant that each replicate could only have 0.5g of biota vs perhaps a more reasonable 1g. The low replicate weight meant a lower final concentration that is harder for the machine to read accurately. A higher quantity of starting sample before the extraction process would have meant perhaps results from the

environmental samples that reached above the LOD or LOQ and could give stronger confirmations about the differences between those samples. The extraction itself was also a limitation as the procedure was vetted by the studies done by Chen et al. (2012) and Liu et al. (2017), but each compound, sediment, and biological material is unique and processes that have been developed and perfected for different samples can yield variable results when extrapolated out. This uncertainty is increased with the variable particles of material extracted. Meaning, the sediment has a very particular particle type that seems largely uniform but can have many different chemical interactions, the biota on the other side when dried and homogenized again was more flakey in shape and weight compared to round. These factors all impact the extraction technique's capabilities and can cause variations and losses. A solution to this would be to repeat the extraction multiple times with various techniques from multiple papers and then decide which of those techniques worked best for the task at hand. The extractions used in this study were a variant of ones used in previous SANOCEAN projects and were maintained as they were validated prior and for comparison purposes. However, the previous studies sought to extract and study many different chemicals so the process was not specialized for amitriptyline specifically. This was also true for the LC-MS/MS tuning processes as they were kept within a similar range to those used prior in order to maintain consistency across the studies. Finally, a note on the calculation of the LOD and LOQ. The determination of LOD and LOQ is a statistical choice made by the researcher in any given study and can be done in a variety of different ways. The calculations used in this thesis are the most typical calculations and are generally seen as conservative (Lister, 2005) however there is much contention surrounding which to use and what is most accurate (Cohen and Ryan, 1989; Bailey and Michelson, 2013). This means that while this study viewed certain numbers as qualitatively or quantitatively valid, another study could include or exclude those same values depending on what the researcher used to calculate them. While not a direct impact to the laboratory practice itself it is just as important to understand the data, analysis processes and the limitations of the chosen mathematical models and statistics surrounding their implementation.

7: Next Steps and Future Research

The results obtained in this study open up the possibility for many interesting future experiments and questions. The most imperative of these surround the bioaccumulating impact of amitriptyline. As the qualification of if amitriptyline could accumulate in benthic worms was identified the logical next step would be to better understand that process. A timed study where the contamination of the biota was recorded over time, compared to only once at a 28-day mark, would give an indication of when the equilibrium point was reached. This is necessary to properly quantify the BCF (Wang, 2016) and thus understand how much exactly can be expected to gather in the *Nereis virens*' bodies over time. Still looking at the worms themselves, investigating the excretion rate of this pharmaceutical is also of interest. A suggestion might be a study exposing the worms to high levels of the drug and allowing it to accumulate before placing them in clean sediment. Then monitoring the water, sediment, and biota levels overtime in order to understand how quickly, or if at all, amitriptyline leaves their mass and how. This is important because, as mentioned previously, the environment is a variable place and it is often true that chemicals can be moved from one place to another by organisms; there could be pockets of high concentrations of chemicals near areas with a low concentration. Questions such as, can these animals bring those chemicals to the cleaner sediments and in what volume, alongside a question of the health impact on the organisms if they are not able to remove it at all come to mind. This brings into light another potential study on the presence of metabolites of amitriptyline in the biota and surrounding sediments. That experiment could help explain how *Nereis virens* processes the chemicals, if at all, and could present new potential issues in the environment if the metabolites (such as nortriptyline) were somehow toxic to the surrounding flora and fauna and produced in high quantities (Chen et al, 2017). It would also impact the level of amitriptyline in the sediment and ecosystem and is important to understanding how it is processed.

Examining the food chain impacts is also of immediate concern. Since *Nereis virens* are a common food source for many aquatic animals, used as bait, and is a leading worm farmed for aquaculture fish feed, any toxin accumulating in their bodies could lift into said predators (Oddsen, 2014; Cabi, 2021). Raised earlier, as even small concentrations of certain pharmaceuticals can impact fish health and ecosystem health heavily, understanding this effect is

important. A study could be conducted allowing contaminated worms to be consumed by fish for varying levels of time and then testing if any accumulation occurred in the fish and where in the body that accumulation happened. Since fish have a different metabolic system than *Nereis virens* this study might also include a search for metabolites or an impact on the metabolic defenses of the chosen animal. As very little is understood fully about amitriptyline's movements within the ecosystem there is a lot of work left to do to completely understand the repercussions and options available for this micropollutant.

8: Conclusion

The aim of this study was to evaluate the accumulation potential of amitriptyline in the benthic polychaete *Nereis virens*. There is very little understanding of the accumulation potential of amitriptyline, but other antidepressants have been known to cause problems in the marine environment (Choong et al., 2006; Guler and Ford, 2010; Qu et al., 2018) and as amitriptyline is a commonly prescribed antidepressant (Chang et al., 2021) understanding its impact became of great importance to the SANOCEAN team. It was found that while 28 days was possibly not enough to reach the equilibrium required to test for a bioconcentration factor (BCF) there was accumulation in the tissues of the worms within the study whose increase correlated with an increase in sediment contamination levels. This finding could have far reaching consequences for both the marine ecosystem and the aquaculture food industry. Understanding the level of impact this could have will require future research into the timeline involved with *Nereis virens* accumulations, food chain effects, metabolic processes of various marine animals involved with said chain, and the power of these benthic re-workers to continuously remobilize amitriptyline in the sediment and water column.

The fates, behaviors, impacts, problems, and solutions regarding the topic are exceedingly variant and every bit of added data to this void is crucial. As medicines are a cornerstone of modern societies elevation of the quality of life for all humans, reducing their usage due to environmental effects is unlikely to occur outside of alternative product developments. This means the waste products associated with drug consumption and creation must be monitored, researched, and dealt with the same as any other environmental pollutant. As other researchers are already attempting to solve ways to remove this micropollutant from the environment/prevent it from entering, studies such as this can aid in the conversation surrounding when and how to implement those solutions (Chang et al., 2021).

9: References

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Appendix

Appendix A.1 – Exposure study environment data; pH, oxygen, salinity, temperature.

Table A.1-1: Average pH of jars through the exposure study.

Jar	Jar 1	Jar2	Jar3	Jar4	Jar5	Jar 6	Jar 7	Jar 8	Jar 9	Jar 10	Jar 11	Jar 12	Jar 13	Jar 14	Jar 15
Average pH	8.347619	8.354762	8.39	8.270952	8.269048	8.331905	8.32619	8.348095	8.131905	8.366667	8.316667	8.35381	8.35381	8.26619	8.379524
Standard Deviation	0.112076	0.065873	0.059682	0.093345	0.095014	0.04905	0.044237	0.047771	0.952504	0.035501	0.087795	0.049133	0.046545	0.17724	0.058836

Average pH of all Jars	8.320476
Stardard Deviation	0.062787

Table A.1-2: Average oxygen of jars through the exposure study in mg/L.

Jar	Jar 1	Jar2	Jar3	Jar4	Jar5	Jar 6	Jar 7	Jar 8	Jar 9	Jar 10	Jar 11	Jar 12	Jar 13	Jar 14	Jar 15
Average Oxygen mg/L	8.180724	8.228025	8.270562	7.835501	7.939139	8.399801	8.442627	8.398644	8.531016	8.907312	8.170777	8.28599	8.425849	8.273406	8.725823
Standard Deviation	2.182329	2.215201	2.225878	2.10393	2.110923	2.262631	2.276584	2.261928	2.239754	2.420863	2.234157	2.158733	2.244435	2.201043	2.343027

Average Oxygen of all Jars	9.020952
Stardard Deviation	0.312727

Table A.1-3: Average salinity of jars through the exposure study in ppt.

Jar	Jar 1	Jar2	Jar3	Jar4	Jar5	Jar 6	Jar 7	Jar 8	Jar 9	Jar 10	Jar 11	Jar 12	Jar 13	Jar 14	Jar 15
Average Salinity ppt	33.5668	33.9514	34.03808	33.90388	33.80322	34.01071	33.88439	33.95891	33.85216	33.6853	32.78464	33.8373	33.07179	33.45384	33.77845
Standard Deviation	8.841541	8.916543	8.93135	8.892995	8.860559	8.859842	8.883026	8.88741	8.838167	8.85829	8.574235	8.88306	8.720921	8.80618	8.861855

Average Salinity of all Jars	36.08254
Stardard Deviation	0.433105

Table A.1-4: Average temperature of jars through the exposure study in degrees Celsius

Jar	Jar 1	Jar2	Jar3	Jar4	Jar5	Jar 6	Jar 7	Jar 8	Jar 9	Jar 10	Jar 11	Jar 12	Jar 13	Jar 14	Jar 15
Average Temperature C°	12.6819	12.31951	12.28271	12.61442	12.46959	12.44845	12.01301	12.00056	11.95004	11.3379	11.23211	11.57424	11.50372	11.27219	11.49952
Standard Deviation	3.283571	3.201825	3.195851	3.273026	3.237144	3.227986	3.106764	3.114378	3.097049	2.926617	2.904137	2.990654	2.959453	2.913771	2.973115

Average Temperature of all Jars	12.56984
Stardard Deviation	0.070427

Appendix A.2: Standard Curve Chromatogram example

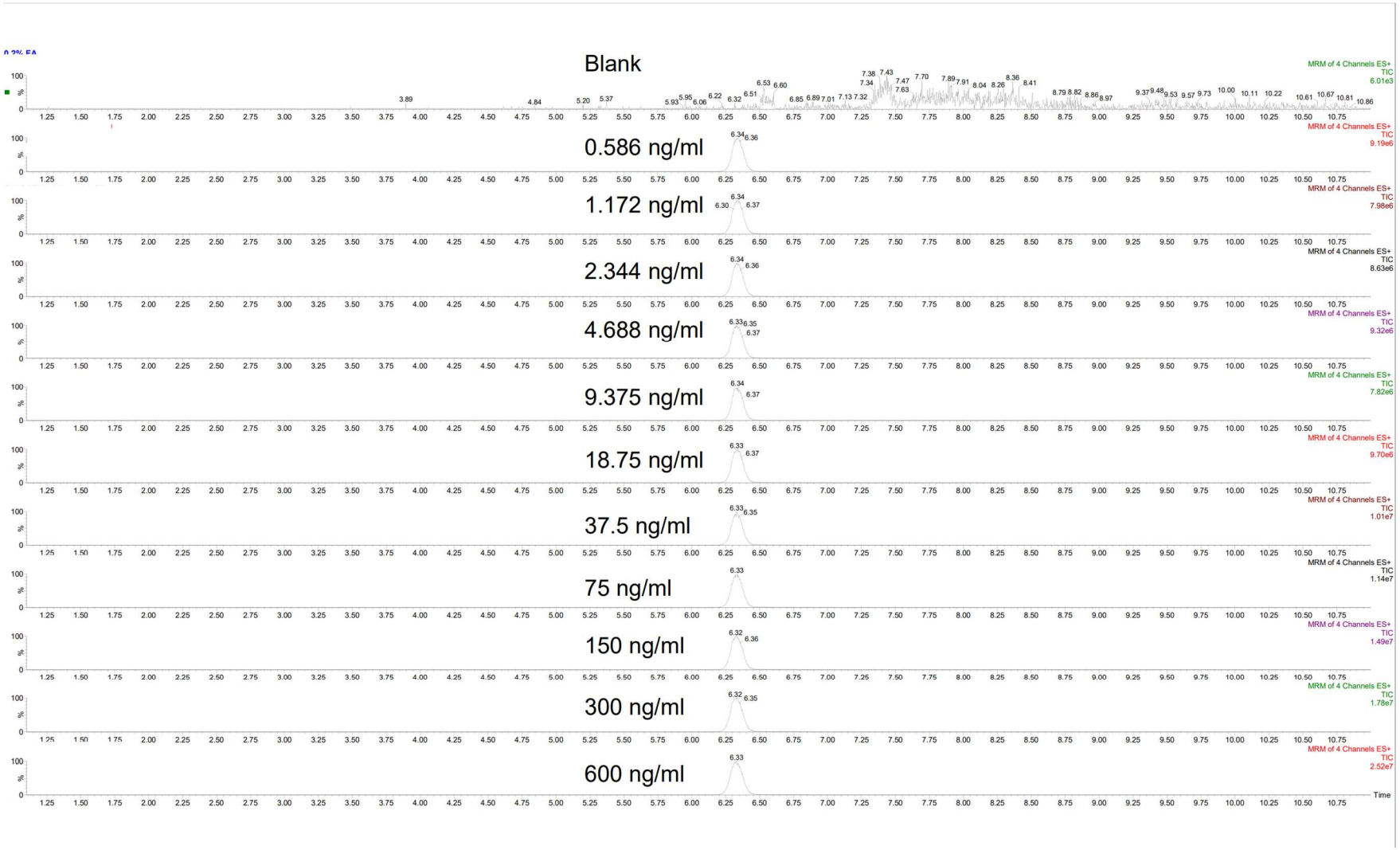

Figure A.2-1: Example of total ion count (TIC) chromatogram from the lower concentration standard curve to showcase strong clear peaks and signal strength seen throughout the study.

Appendix A.3: Analyte data by replication trial

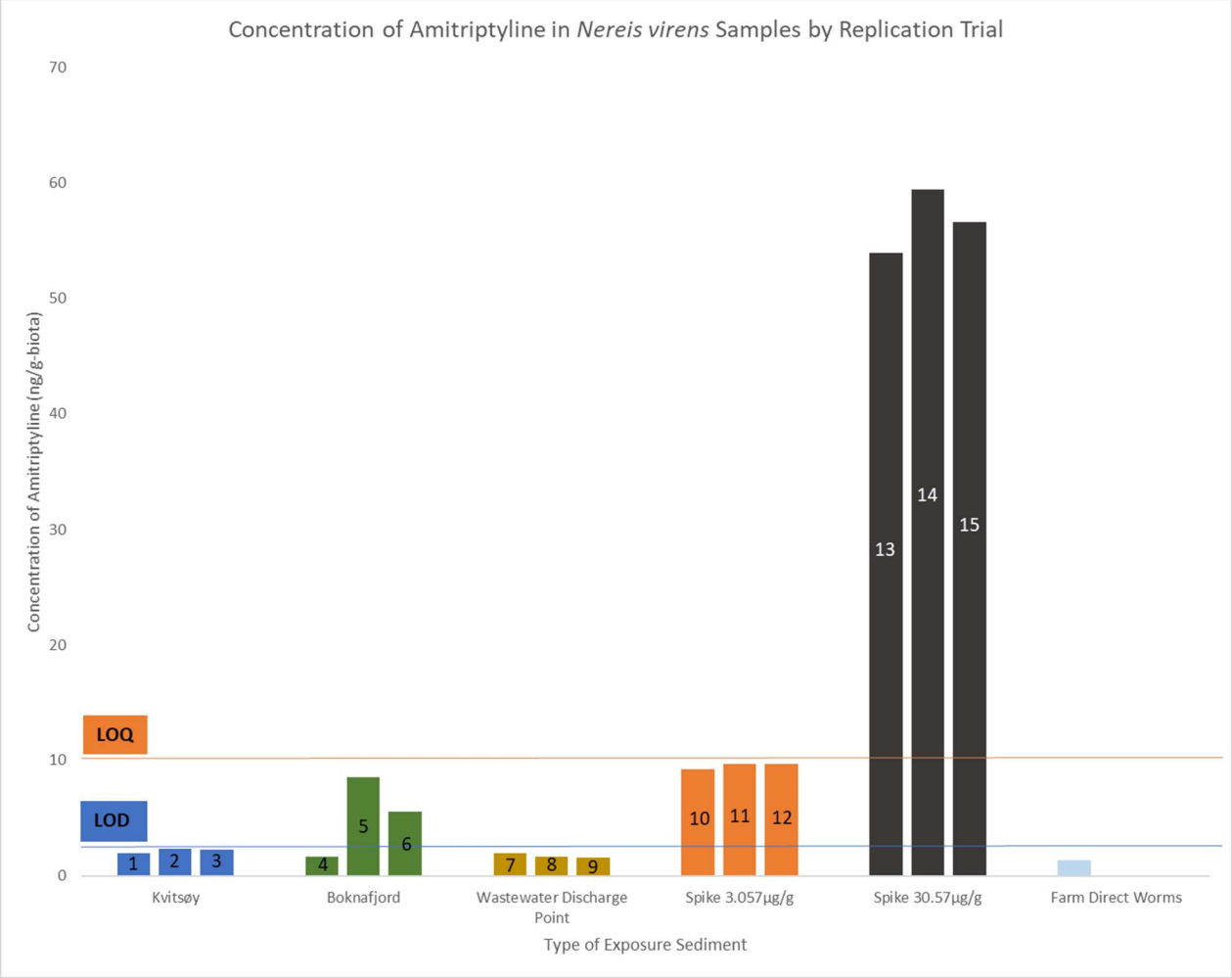

Figure A.3-1: A graphical representation of the concentration of amitriptyline in ng/g-biota found in LC-MSMS analysis of Nereis virens samples from each exposure tank replicate and direct from farm control group. Each trial number's average consisted of two-four separate replicates from the same samples. Sediment sources used in the exposure tanks are listed on the x-axis as a label under each bar. For samples where the analyte concentration was below the LOD in a sample set where others were above the LOD the result was replaced with the LOD/2 for statistical analysis of standard deviation (Bailey and Michelson, 2013)(Cohen and Ryan, 1989) The LOD and LOQ are represented by a blue and orange line respectively.

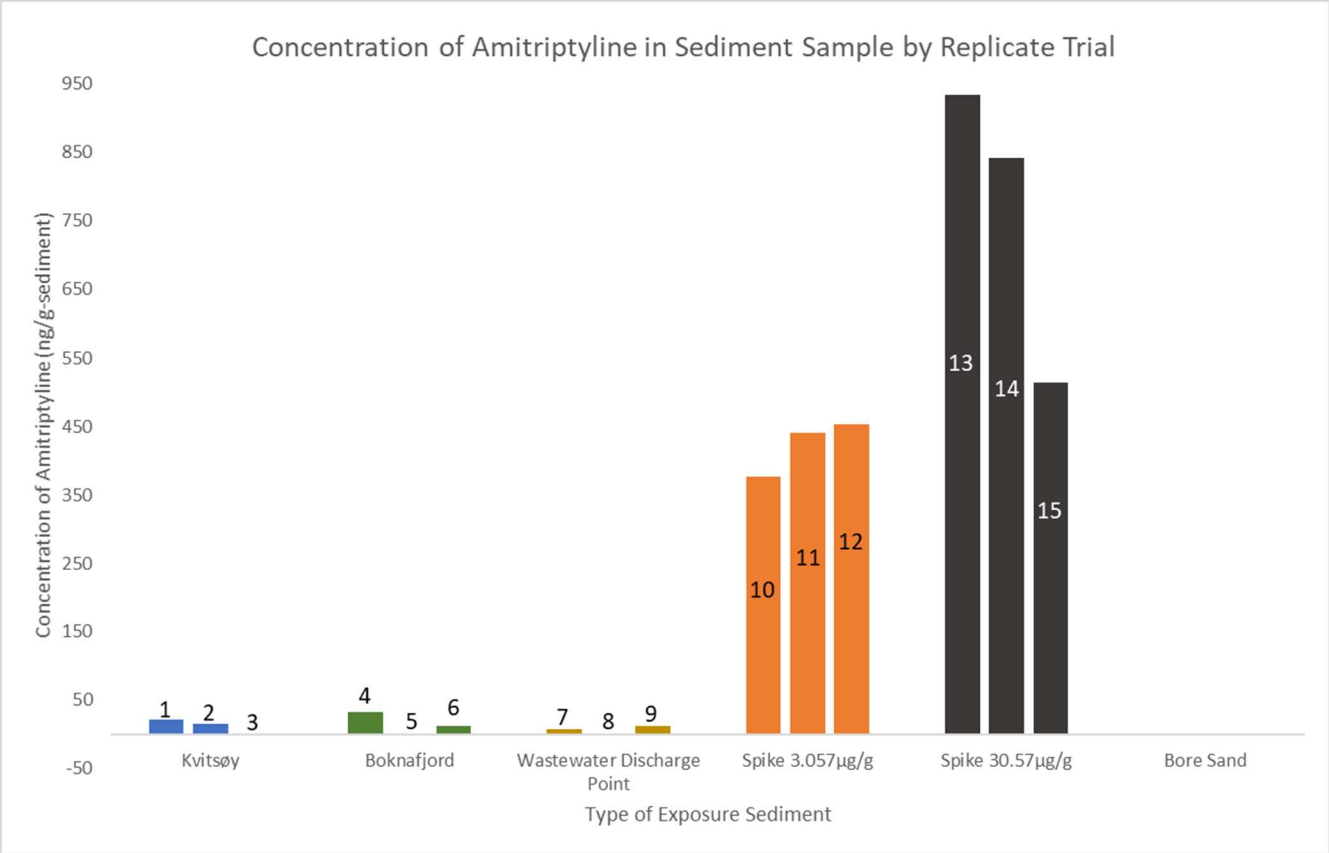

Figure A.3-2: A graphical representation of the concentration of amitriptyline in ng/g-sediment found in LC-MSMS analysis of sediment samples from each exposure tank replicate and a Bore sand control group. Each trial number's average consisted of two-four separate replicates from the same samples. Sediment sources used in the exposure tanks are listed on the x-axis as a label under each bar. For samples where the analyte concentration was below the LOD in a sample set where others were above the LOD the result was replaced with the LOD/2 for statistical analysis of standard deviation (Bailey and Michelson, 2013)(Cohen and Ryan, 1989).