ROSKILDE UNIVERSITY

MASTER THESIS

Biotransformation of the polycyclic aromatic hydrocarbon 9-nitroanthracene in the marine benthic polychaete *Alitta virens*

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PREFACE AND ACKNOWLEGEMENTS

This is a 60 ECTS interdisciplinary Master Thesis in Chemistry and Environmental biology. The following study was performed at the Department of Science and Environment, at Roskilde University. The duration of this study was from September 2016 to June 2017. The Supervisors of this thesis are Professors Poul Erik Hansen and Annemette Palmqvist.

I would like to thank my two supervisors for their continuous help and scientific guidance throughout this thesis. Many thanks to the lab technicians who helped and guided me throughout any struggles I faced. A special thank you goes to my family who have supported me throughout the past year and tried to boost my self- confidence, whenever I felt stressed or uncertain about anything. Finally, I would like to thank Roskilde University for allowing its students to do a combination project, as it enlightens us to try to merge both subjects in a beautiful way.

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ABSTRACT

Alitta virens is a polychaete that is found to inhabit the intertidal zones of marine and brackish waters in Europe. The worm is found to be present in areas that are heavily polluted with contaminants, such as, PAHs. The PAH, 9-NA is found to be abundant in the vehicle exhaust systems, and thus contribute greatly to the pollution in the air. The vehicle systems emit PAHs that are washed to the aquatic environment. These contaminants bind to the sediment and are subsequently assimilated by the benthic fauna. *A. virens* is a sediment dwelling organisms that ingests sediment particle. Therefore, *A. virens*, is one of the many aquatic animals that are affected by the release of these contaminants into the aquatic waters. We investigated the bioaccumulation of 9-NA by, *A. virens*, by exposing the worms to different concentrations of sediments spiked with 9-NA. The parent compound and its metabolites were analyzed using GC/MS. The contaminant was assimilated by the worms and the BAF was estimated among the different treatments. The control sediment was found out to be contaminated with 9-NA. The worms metabolized 9-NA to form the 9-NO_2-dihydrodiol and anthraquinone metabolites. The concentration of 9-NA in the guts of the worms was found to be more in the 7 days exposure period compared to the 14 days exposure period. In addition, the 9-NO_2-dihydrodiol and anthraquinone metabolites were found to be more abundant in the guts of the worms at the 7 days exposure to the contaminant compared to the 14 days exposure period.

ABBREVIATIONS

BA	Benz(a)anthracene
BAF	Bioaccumulation factor
B(a)P	Benzo(a)pyrene
CYP	Cytochrome P450 enzymes
DEP	Diesel exhaust particles
DD	Dihydrodiol dehydrogenase
GC/MS	Gas chromatography/mass spectrometry
HPLC	High pressure liquid chromatography
K_{ow}	Octanol-water partition coefficient
9, 10-AQ	9, 10-anthraquinone
9,10-DNA	9, 10-dinitroanthracene
2-NA	2-nitroanthracene
9-NA	9-Nitroanthracene
Nitro-PAHs	nitro polycyclic aromatic hydrocarbons
PAHs	Polycyclic aromatic hydrocarbons
VOCs	Volatile organic compounds
NO_x	Nitrogen oxides
PM	Particulate matter
SCR	Selective catalytic reduction
K_m	Michaelis- Menton constant
NATs	N-acetyltransferases
PAPS	Phosphoadenosyl phosphosulfate.

UDPGA UDP-glucuronic acid

UGT Uridinediphosphateglucuronyl transferase

SIM Selected ion monitoring

S_N2 Nucleophilic substitution bimolecular reaction

SULTS Sulfotransferases

(trans-1,2-dihydrodiol), trans-1,2-dihydroxy-9-nitro- 1,2-dihydroanthracene

(trans- 3,4-dihydrodiol), trans-3,4-dihydroxy-9-nitro- 3,4-dihydroanthracene

(1,2,3,4-tetrahydrodiol), 1,2,3,4-tetrahydroxy-9-nitro-1,2,3,4- tetrahydroanthracene

(7-NO_2-BA), 7-nitrobenz(a)anthracene

INTRODUCTION

The nitro groups of polycyclic aromatic hydrocarbons (nitro-PAHs) are formed through different routes. They can be formed either through combustion processes or by the reaction of PAHs with the surrounding atmosphere. These nitro PAHs are mutagens and can cause cancer as well (Möller, 1994; Rosenkranz and Mermelste, 1983). The most abundant nitro-PAH emitted from diesel fuel systems is 1-nitropyrene, followed by 9-nitroanthracene (9-NA). The reduction of the nitro group (-NO_2) by the enzymes nitroreductases is responsible for the mutagenic and carcinogenic activities of these nitro-PAHs. For instance, the enzymatic nitroreduction of 9-NA, results in the formation of the active N-hydroxyaminoanthracene (Fu et al., 1985; Howard et al., 1983). In addition, the oxidation of 9-NA, results in the production of the biologically active 9-NO_2-dihydrodiol epoxide (Fu et al., 1985). In the aquatic systems, these nitro-PAHs emitted, bind to suspended particles due to their hydrophobic character. The particles then sink to the bottom of the waters and becomes part of the sediment, which allows easier access to particle associated contaminants by the benthic fauna (Ferguson & Chandler, 1998). Therefore, it is very important to study the effect of the uptake of these PAHs by the benthic fauna, and the different biotransformation processes that take place. It is essential to understand the transfer of these PAHs from one trophic level to the next (McElroy, 1990).

Polychaetes are among the groups that are being excessively used to study the effect of toxicants on them. They contribute to a considerable percentage of the marine invertebrate species. Irrespective of the depth of the ocean, polychaetes contribute with 35%- 50% of the total marine invertebrate species. This makes them an important species to consider when targeting toxicological testing (Reish & Gerlinger, 1997) . Therefore, the polychaete, *Alitta virens* (*A. virens*) is the organism that was used in my study.

Biotransformation is a process that is driven by a number of enzymes that are mainly present in the liver of the vertebrate species and in the tissues where food is utilized in invertebrates. The enzymatic processes are similar in vertebrates and invertebrates. Biotransformation simply converts the hydrophobic PAH into a more water- soluble compound by increasing its hydrophilicity. The first phase of this biotransformation is to convert the PAH into a polar metabolite. This is done using different enzymes such as cytochrome P450 monooxygenase. Phase two then allows the phase I metabolite to conjugate with a large polar group, which then allows the resulting metabolite to be more easily excreted out of the organism's body. The process of biotranformation affects the

fate and sometimes the toxic potency of the parent compound. This means that the metabolites can either be less active than the parent compound or more active than the parent compound. The amount and type of cytochrome P450 monooxygenase present varies from one species to the other. It is found to be present in larger amount in fish compared to aquatic invertebrates (Livingstone, 1998). It is also found to be present in polychaetes (Jørgensen et al., 2005).

Polycyclic aromatic hydrocarbons

The incomplete combustion of organic compounds results in the formation of the persistent PAHs. These PAHs are ubiquitous in the surrounding atmosphere and are complex fused organic compounds consisting of the atoms carbon and hydrogen. The simplest PAH consists of a minimum of two fused benzene rings. Some of the PAHs have been found to be carcinogenic. As a rule of thumb, the carcinogenicity of PAHs is associated with increasing molecular weight of the PAH. Therefore, a PAH with a larger molecular weight has a higher tendency to be carcinogenic than one with a lower molecular weight. Pyrosynthesis is the process by which low molecular hydrocarbons produce PAHs. A simple molecular weight molecule such as ethane is heated to a very high temperature. Upon heating, the molecule converts into radicals which then react with acetylene. Following this, the molecule produced condenses and forms the PAH, which is unable to be broken down upon heating. Sediments that are deprived of oxygen can act as sources of producing PAHs as well through the diagenesis of the organic matter present in them (Ravindra et. al, 2008). Therefore, studying the effect of PAHs present in the sediment on dwelling benthic organisms is essential.

Vehicle exhaust systems

Molecules that are exposed to vehicle exhaust systems have been proven to contribute greatly to the formation of PAHs in the atmosphere. The motor present in the exhaust system emits particulate matter. These particulate matters are constituted of fine particles that can be easily inhaled from the surrounding atmosphere (Huiru et al, 2014). Therefore, these PAHs can cause severe health effects on humans, and thus studying their effects would be of a great advantage to us. The tailpipe can release PAHs at different rates, which are dependent on the type of fuel that is used, type of engine and the quality of the fuel (Huiru et al, 2014; Ravindra et al., 2008). The distribution of PAHs in the environment varies as well depending on location. For instance, areas with dense traffic consisting of car vehicles are prone to have a higher percentage of PAHs than other urban areas. A study was performed by Huiru and coworkers where they studied the presence of different PAHs in three

parking lots of a metropolitan area. The presence of these PAHs varied among the different areas studied, however it was confirmed that the exhaust systems contributed greatly to the presence of these PAHs (Huiru et al, 2014).

Nitro PAHs

The surrounding atmosphere is affected in a negative way through the emission of different substances such as, volatile organic compounds (VOCs), different nitrogen oxides (NO_x) and the emission of fine particulate matter (PM) (Inomata et al., 2015; LIoyd and Cakette, 2001). These substances are emitted from diesel engines, and thus are responsible for negatively affecting the quality of the air. There are different chemical contaminants found in the diesel exhaust particles (DEP), such as PAHs and the substituted PAHS, including oxygenated and nitro- polycyclic aromatic hydrocarbons (Inomata et al., 2015; Levsen, 1988). The combustion process that takes place in the diesel engine results in the nitration of PAHs to form the nitro-PAHs. The emission of exhaust gases including the NO_x and DEP can be decreased using different after-treatments. Selective catalytic reduction (SCR) is used to decrease the NO_x emissions by simply reducing the NO_x present using a selective catalyst. The PM emission is reduced through the addition of a diesel particle filters (DPF).

Inomata et al., (2015) conducted an experiment to measure the emissions of 4-nitrophenol, 1-nitropyrene and 9-nitroanthracene from diesel exhaust systems. GC/MS and LC/MS were used to measure the concentration of the emitted nitro-organics. They found that 4-nitrophenol had the highest emission compared to the other two nitro-organics in the DEP. Hu and coworkers (2013) performed an experiment to determine the emission of PAHs, after using the after-treatments in diesel engines. Their results concluded that the addition of the after-treatments reduced the total PAHs emitted. This confirms that the after-treatments are effective in reducing the emission of PAHs. However, despite after-treatments, nitro PAHs are clearly found in the car exhaust systems and thus studying their effect on the environment is essential. The aquatic environment is negatively affected by these nitro-PAHs, since they may deposit on water surfaces or get washed to the aquatic systems after land depositions, thus becoming available to aquatic organisms. The nitro-PAH chosen for my experiment is 9-nitroanthracene (9-NA). 9-NA ($C_{14}H_9NO_2$) has a molecular weight of 223.23 g/mole. 9-NA has a low volatility and is saturated under low vapor pressure, and is therefore not expected to be found in the gas phase for longer time or in considerable amounts (Inomata et al., 2015). The crystals of 9-NA are found to be bright yellow in color.

There are several important factors that determine the fate of a PAH in a species. These factors include the bioavailability of the PAH in the environment, the uptake route of the toxicant, the biotransformation processes that results in the formation of different metabolites and subsequent excretion. These factors are discussed in more details throughout the paper.

Nitration of PAHs

Nitro PAHs are produced through the nitration of PAHs. Amy and coworkers have studied the nitration of a PAH using the catalyst, sulfuric acid, which is supported on a silica-gel (Smith et al., 1999). Several PAHs were nitrated using this method, and the yield of the corresponding nitro derivative PAH was reported. Naphthalene, pyrene, chrysene, fluorine, fluoranthene and most importantly anthracene was included in their study. Their method revolved around mixing the sulfuric acid on silica-gel catalyst with the various PAH, using dichloromethane as the solvent. Nitric acid was added as well in stoichiometric amounts at room temperature. What was interesting about this method is that it produced quantitative yields and regioselectivity in most of the reactions was achieved as well. Some of the arenes such as pyrene and chrysene produced single nitro derivatives. The main product that was produced by anthracene was 9-NA and it was the only monosubstituted nitro derivative that was obtained in 45%. The reason behind this low yield is that anthraquinone (9,10-AQ) was produced as a side product throughout the reaction, which resulted in lowering the overall yield of the 9-NA that was isolated (Smith et al., 1999). This study highlighted that nitro PAHs can be synthesized in an effective method that forms pure isomeric compounds, which can be further used as substrates in animal testing for instance. However, an interesting finding that I can use in my own synthesis of 9-NA is that the nitration of anthracene formed anthraquinone as a side product. Therefore, it was important that I aimed to form a pure 9-NA, and subsequently look for methods to remove any traces of anthraquinone if it is formed as a side product.

PAHs are prone to react with different oxidants that are present in the atmosphere, such as: oxygen (O_2), hydroxides (-OH) and nitrogen oxides (NO_x). The reaction of these PAHs with the atmospheric oxidants produces the nitrogen and oxygen derivative PAHs. These compounds that are produced are thought to have more adverse mutagenic and carcinogenic effects on the human health.

The heterogeneous reaction of anthracene adsorbed on sodium chloride particles with nitrogen dioxide was investigated by Chen and his coworker Zhu (Chen and Zhu, 2014). They examined the heterogeneous reaction of the PAH, anthracene adsorbed on sodium chloride particle with NO_2, since it is one of the main pollutants produced from car exhaust systems. The heterogeneous reaction between anthracene adsorbed on NaCl with NO_2 produced several nitro-PAHs. The products that were produced are 9,10-AQ, 9-NA, 2-nitroanthracene (2-NA) and 9, 10-dinitroanthracene (9, 10-DNA). Gas chromatography- mass spectrometry (GC/MS) was the analytical method that was used to identify the products that were formed during the reaction procedure. The major products that were further investigated were 9-NA and 9, 10- DNA. They wanted to investigate the effect of humidity on the formation of 9-NA and 9, 10- DNA. Anthracene adsorbed on NaCl was subjected to different concentrations of NO_2. For instance, at a relative humidity of 0, 10 and 20%, the concentration of 9, 10- AQ was higher than the concentration of 9-NA in particle throughout the whole experimental time. On the other hand, the concentration of 9, 10- AQ decreased at a higher relative humidity of 40% and above. 9- NA, however, showed different results. Increasing the relative humidity showed to significantly decrease the concentration of 9-NA in particle (Chen & Zhu, 2014). This concludes that relative humidity is an important factor to take into consideration when determining the concentration of products in particles in a heterogeneous reaction.

Bioavailability, bioaccumulation and biotransformation

Bioavailability is an important factor that determines the overall fate of the organic pollutant in an organism. This is because bioavailability determines how accessible an organic pollutant is by the exposed organisms. The lipophilic contaminant binds to the organic phase of the sediment (Rand, 1995). The pollutant can subsequently be assimilated by sediment feeding organisms, which in some cases might result in the toxicity of the organism.

The age of the organic pollutant in the sediment is an important factor when considering the bioavailability of the compound for uptake by organisms. The empirical relationship behind this is that a contaminant is less taken up by an organism once it has been placed longer in the sediment or soil. This means that the bioavailability of the toxicant decreases with increased aging of the contaminant in the sediment or soil (Alexander, 2000). This means that although a compound may be present in the sediment, only a percentage of it is available to be assimilated by benthic organisms (Vinturella et al. 2004). Knowing how much of the organic pollutant is available by the organism is important, since it gives us a better insight on whether the organic pollutant is likely to be accumulated in the organism. Therefore, the bioavailability and bioaccumulation of a contaminant are interlinked, because if a contaminant is very available for uptake, then the chances of it being accumulated by the organism is high.

The concentration and distribution of a contaminant in the environment is important for determining the accumulation in an organism from different exposure routes (Meador et al., 1997). The way an organic contaminant partitions in the aquatic environment (log K_{ow}) is important, since, it provides a better insight on the behavior of that contaminant. Some studies showed that the presence of sediment may reduce the bioaccumulation of a contaminant in an organism. The reason behind this, is that the sediment acts as a sink to these contaminants making them less available for uptake by the organisms (Meador et al., 1997, Knezovich & Harrison, 1988). However, this is not the case for sediment dwelling organisms such as the polychaete *A. virens*, which feeds by ingesting sediment.

The bioaccumulation of a contaminant varies from one organism to the next. Christensen et al., 2002, investigated the bioaccumulation and elimination of pyrene in two polychaetes, *Nereis diversicolor* (*N. diversicolor*) *and Arenicola marina*. The results showed that they both were able to accumulate pyrene and subsequently eliminate it at a fast rate.

An experiment was performed by Rust and coworkers where they examined the relationship between the metabolism and bioaccumulation of a PAH, benzo(*a*)pyrene (BaP) in several benthic invertebrates including, *A. virens*. *A. viren*s was able to metabolize the PAH by as much as 70% after being exposed to a spiked sediment of the PAH for 7 consecutive days (Rust et al , 2004). This is consistent with other studies that showed that *N. diversicolor*, which is another species that belongs to the nereidae family is able to metabolize BaP and eliminate it at a faster rate compared to other polychaetes (Driscoll and McElroy, 1997). Both of these studies conclude that the Nereidae family of polychaetes are indeed efficient at metabolizing PAHs. This is interesting to compare with my current study to see whether the worms will be efficient at metabolizing 9-NA.

McElroy studied the effect of the PAH, BA on the polychaete *A. virens*. Different exposure routes to the contaminant were used and *A. virens* was able to biotransform the PAH by metabolizing it into a series of different forms of metabolites. The different metabolites that were formed were classified under being either: polar, organic soluble metabolites; conjugate metabolites and finally bound materials that cannot be extracted. The conjugate and bound metabolites were the highest throughout all exposure routes (McElroy, 1990). This study clearly demonstrated that *A. virens* is capable of taking up and biotransforming the PAH efficiently.

Several parameters are important to take into consideration when investigating the bioaccumulation of a contaminant from the environment. The characteristics of a chemical substance is essential is determining the accumulation of a contaminant from the sediment. For instance, the octanol-water partition coefficient (K_{ow}) is a ratio that defines the concentration of a chemical in the water phase compared to the octanol phase. This ratio can be used to measure the hydrophobic character of contaminants. Chemical substances that have a higher hydrophobic character tend to bind more to the sediment than contaminants that are less hydrophobic.

$$Kow = \frac{[Octanol]}{[Water]}$$

Toxicokinetics of an organism is of great significance when describing the accumulation of contaminants. The uptake of a contaminant from the environment is driven by a series of physiochemical processes, whereas the metabolism of a contaminant is preceded through a series of enzymes (Livingstone, 1998). Another important factor that affects the accumulation of a contaminant from the environment is the biaccumulation factor (BAF), which is a ratio that

basically expresses the concentration of a contaminant in the organism over the concentration of a contaminant in the sediment.

$$BAF = \frac{[organism]}{[sediment]}$$

The accumulation of sediment that contains PAHs results in a BAF that lies between 1.2 to approximately 10 (Landrum, 1989). The relationship between the BAF and the log K_{ow} results in the formation of a bell- shaped graph. Landum (1989) investigated the bioaccumulation of several PAHs and his results showed that the chemical substances that have a low K_{ow} factor have a low BAF. The reason behind this is that the elimination of a contaminant is more easily performed by an organism if the contaminant has a low K_{ow} ratio, thus resulting in a low BAF. In addition, chemical substances that have a high K_{ow} value have a low BAF as well. This is because chemical substances with a high K_{ow} ratio are strongly adsorbed to the sediments, resulting in a lower accumulation in the organism. The reason behind this, is that the more a chemical is adsorbed to the sediment the higher the desorption rate of this chemical, resulting in a lower accumulation in the organism. However, compounds with a log K_{ow} of around 5 show the highest BAF. 9-NA has been experimentally found to have a log K_{ow} of 4.78 (Debnath, A. K. and Hansch, C., 1992). Therefore, 9-NA is an ideal candidate to study when investigating toxicological studies, due to it being easily taken up by organisms.

Several benthic invertebrates are able to biotransform the parent compound into a series of different metabolites, with the aim of allowing the PAH to be easily excreted out of the organism's body. However, in general, some of the PAHs produce metabolites that might be more toxic than the parent compound, which can cause adverse effects on the organism. The biotransformation of xenobiotics like PAHs comprises a series of enzymes in phase I and phase II reactions (James, 1987).

Phase I metabolism

The initial phase of biotransformation, phase I, includes different processes such as oxidation, reduction and hydrolysis (Remmel et al., 2008; Guengerich 2008). These processes are carried using the cytochrome enzymes. Cytochrome P450 (CYPs) are a class of hemoproteins, and the name is derived from the absorption peak that was observed in the carbon monoxide binding form at a wavelength of 450 nm. There are different families and subfamilies of the P450 enzyme (Nelson, 1998). The main function of the enzyme is to mono-oxygenate the substrate, which is

performed by utilizing molecular oxygen. This process is mediated using NAD(P)H or NADH, which act as reducing equivalents (Omura, 1999). It has been found that in some cases the CYP enzyme results in the activation of the xenobiotic (Werck-Reichart and Feyereisen 2000). They are important catalysts in the transformation of both endogenous and exogenous compounds (Hlavica and Lewis, 2001). These CYPs are either found in the microsomes of the endoplastic reticulum or in the mitochondria (Omura, 1999). Lee and Singer, (1980) examined the mixed-function oxygenase system (MFO; P450 enzymes) in the ploychaetes, *A. virens* and *Capitella capitata.* The pharynx, esophagus, upper and lower parts of the intestines of *A.virens,* were analyzed for the activity of the MFO enzymes. The results showed that the enzyme is found to be more present in the microsomes of the lower part of the intestine. However, no enzyme activity was detected in the pharynx and the esophagus. The polychaete, *Capitella capitata* showed that there was no enzyme activity of the MFO detected in the worms that were not exposed to the contaminant. The same result is apparent in the polychaete, *A. virens.* However, the activity of the CYP and MFO enzymes increased when the polychaete was exposed to BA. This concludes that the exposure of the worms to the different contaminants, such as PAHs, induces the enzymes' activity.

The CYP involves a catalytic cycle with the initial binding of the substrate to the active site of the enzyme. Following this, the reductase molecule transfers an electron to the enzyme- substrate complex, which results in the reduction of the Fe^{3+} heme group to form Fe^{2+}. An oxygen molecule then binds to the reduced heme group to form Fe^{2+} -O_2. This molecule is then converted into a Fe^{3+}-O_2^- which is a more stable. The complex then undergoes a second reduction to convert the ferric iron back to its ferrous state. The O-O bond is cleaved, by the reaction of the O_2^{2-} anion with two protons, subsequently forming a water molecule. Finally, the oxygen in the $(Fe\text{-}O)^{3+}$ complex is transferred to the initial substrate, resulting in the formation of the hydroxyl group on the substrate (Lewis, 2003). An example of this hydroxylation is the production of 9-NO_2-dihydrodiol metabolites from the parent compound 9-NA, in rat liver microsomes (Fu et. Al, 1985).

Phase II biotransformation

Phase II reactions involve transforming the product produced from phase I to a more polar compound. Phase II metabolism involves conjugation reactions, which then allows the compounds to be more easily excreted from the organism's body (Clemens et al., 2014; James, 1987). Glycosylation is an important phase II reaction in which many organic molecules can undergo this type of reaction. Other important phase II reactions that take place in aquatic species are: sulfation,

the formation of mercaputuric acid and the conjugation reaction with amino acids (James, 1987). Glutathione—S-transferases, uridinediphosphateglucuronyl transferase (UGT) and sulfotransferases are the important enzymes that mediate the metabolism of phase II reactions (De Knecht et at., 2001).

Glucuridation and glucosidation are the two glycosylation reactions catalyzed using the UGT enzymes, that are found in the endoplasmic reticulum. The catalytic reaction of the UGT enzyme is essential in the production of the lipophilic glucuronides (Tukey and Strassburg, 2000). The co-substrate for this reaction is UDP-glucuronic acid (UDPGA) (Kaivosaari et al., 2011; Burchell and Coughtrie 1989; Miners and Mackenzie 1991; Tukey and Strassburg 2000). The glucurinidation is a nucleophilic substitution bimolecular reaction (S_N2). When the active site of the enzyme is bound to both UDPGA and the substrate, the heteroatom of the aglycone substrate attacks the first carbon of the glucuronic acid. This results in the inversion of configuration at the invasion site of C1 from an α to a β-configuration (Kaivosaari et al., 2011). The co-substrate for the glucosidation reactions is UDP-glucose. It has been apparent that marine invertebrate species use UDP-glucose, whereas vertebrate species use UDPGA as the co-substrate for the UGT enzymes (James, 1987).

Sulfation is another phase II pathway that results in the formation of sulfate conjugates. There are two enzymes involved in the sulfation pathway: the sulfotransferases (SULTS) located in the cytoplasm, and the sulfatases located in the lysosomes or the endoplasmic reticulum. The molecule that donates the sulfate group to SULTS is called phosphoadenosyl phosphosulfate (PAPS). The availability of the substrate is important in the sulfation pathway. The reason behind this is that there are other enzymes that compete for the substrate supply such as the UGT and the N-acetyltransferases (NATs) (Coughtrie et al., 1998).

Jørgensen et al., (2005) investigated the biotransformation of the PAH pyrene, in the polychaete *A. virens*. The polychaetes were exposed to pyrene for 5 days and the metabolites formed were identified using HPLC. Biotransformation of pyrene by CYP produced 4 % of 1-hydroxypyrene. This metabolite was then conjugated using phase II enzymes to form 65% of pyrene-1-glucuronide, 12% of pyrene-1-sulfate and 2% of pyrene -1-glucoside (Giessing et al., 2003). It was found that 1-hydroxypyrene glucuronide is the major phase II conjugated metabolite that forms when the polychaete *N. diversicolor* is exposed to pyrene. The Nereid polychaetes have glucuronide conjugation as the major phase II metabolic pathway, whereas, the *Capitella spI* and *Arenicola* were found to use the sulfate and the glucoside conjugation pathways. This shows that there are

differences that arise between the biotransformation paths of contaminants within the same class of animals (Giessing at al., 2003).

As mentioned previously, there are different enzymes that catalyze phase I and phase II reactions. It is therefore important to measure the efficiency of the metabolism of these PAHs and other xenobiotics by these enzymes. The rate at which a drug is metabolized is calculated using the ratio between the maximum rate of the metabolism by the enzyme and the Michaelis- Menton constant ($\frac{Vmax}{km}$) (Houston, 1994). A substantial number of CYP enzymes that catalyze the monooxygenation of the substrate follow the hyperbolic enzyme kinetic model derived by Michaelis Menten (Ainslie et al., 1972).

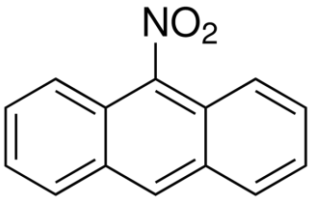
Toxicity of nitro- PAHs.

The assimilated contaminant can be biotransformed to different metabolites, but in many cases these contaminants can be activated to different metabolites that can bind to functional biomolecules such as proteins and nucleic acids (Livingstone 1993). The activated metabolites are believed to be highly toxic and can sometimes cause the contaminant to be carcinogenic (Penning, 1993). The reason behind this is that the metabolic activation of some PAHs results in the formation of diol epoxides, which results in the production of DNA adducts. However, the carcinogenicity of certain PAHs can be reduced using the enzyme dihydrodiol dehydrogenase (DD), which reduces the amount of anti-diol-epoxides present (Penning, 1993).

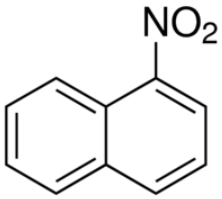
Several studies have highlighted the toxic effects of the exposure to nitro-PAHs. For instance, Dihl and coworkers investigated the genotoxicity of four nitro polycyclic aromatic hydrocarbons: 1,5-dinitronaphthalene, 9-NA, 1-nitronaphthalene, 2- nitrofluorene in Drosophila melanogaster (Dihl et al., 2008). The molecular structure of the four nitro-PAHs is provided in figure 1. The different nitro-PAHs produced a wing spot mutation in different degrees. For instance, 9-NA produced this mutation by 75%, whereas 1-nitronaphthalene produced it by a 100%. This confirms that these nitro-PAHs induce genotoxic effects, thereby emphasizing on the toxicity of 9-NA (Dihl et al., 2008).

Another study examined the toxic effects of the nitro-PAH, 2-nitrofluorene. The exposure to 2-nitrofluorene through either the inhalation of the contaminant or the ingestion of the contaminant through contaminated food, resulted in the formation of metabolites that are highly toxic. These metabolites have been found to be mutagens and can cause genotoxicity on the organisms tested. Rats that have been continuously fed with contaminated food of 2-nitrofluorene have been found to develop several tumors in the liver and other organs (Möller, 1994). This emphasizes on the toxicity of the nitro-PAHs, thereby highlighting the risks of being exposed to PAHs.

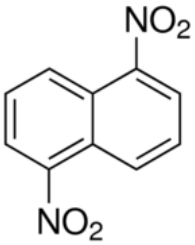
9-nitroanthracene



1-nitronaphthalene



1,5-dinitronaphthalene



2-nitrofluorene

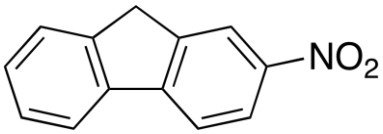


Figure 1. The molecular structure of 9-NA, 1,5-dinitronaphthalene, 1-nitronaphthalene and 2-nitrofluorene

Metabolism of 9- NA

Fu et al., (1985) studied the metabolism of 9-NA by uninduced rat liver microsomes, and the metabolites that were formed were identified, and their mutagenic activity was tested. The metabolites formed are trans-1,2-dihydroxy-9-nitro- 1,2-dihydroanthracene (trans-1,2-dihydrodiol), trans-3,4-dihydroxy-9-nitro- 3,4-dihydroanthracene (trans- 3,4-dihydrodiol), 1,2,3,4-tetrahydroxy-9-nitro-1,2,3,4- tetrahydroanthracene (1,2,3,4-tetrahydrodiol) and anthraquinone (9,10-AQ). Figure 2 represents the metabolic pathway of 9-NA to form the different metabolites mentioned. The CYP enzyme metabolizes 9-NA to form 9,10-AQ and the intermediates 1,2 and 3,4 epoxides. The further metabolism of the epoxides by epoxide hydrolase forms the 1,2 and 3,4-dihydrodiols. 3,4-dihydrodiol can be further metabolized by the CYP enzymes to form the 1,2,3,4-tetrahydrodiol.

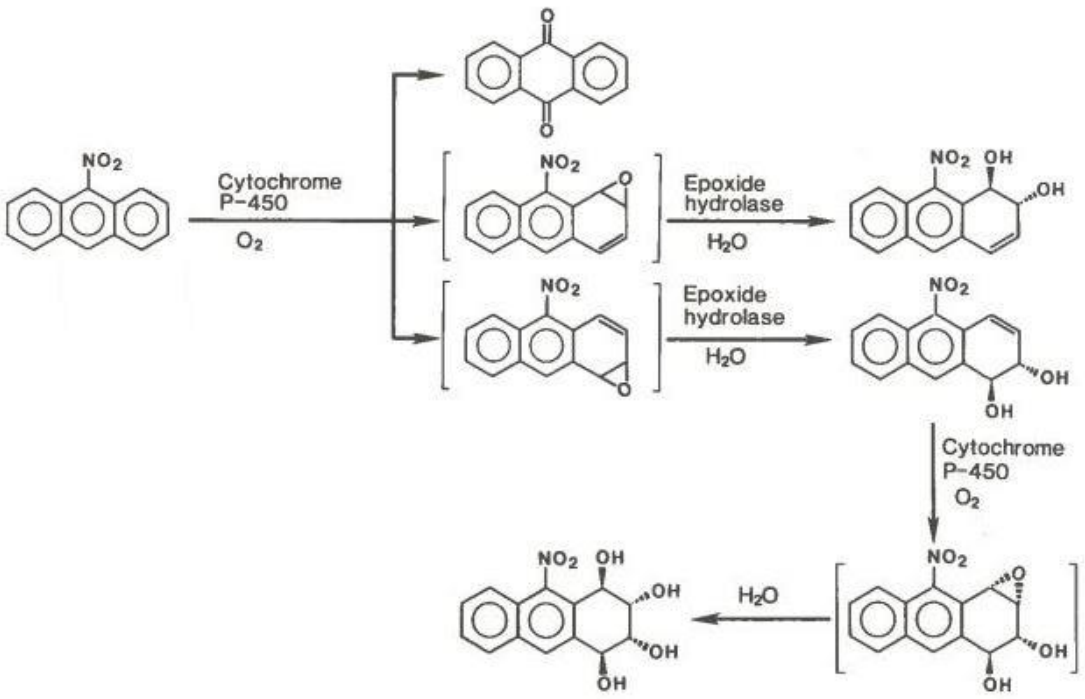


Figure 2. The metabolic pathway of 9-NA to form 9,10-AQ, 1,2-dihydrodiol, 3,4-dihydrodiol and 1,2,3,4-tetrahydrodiol. Fu et al., (1985).

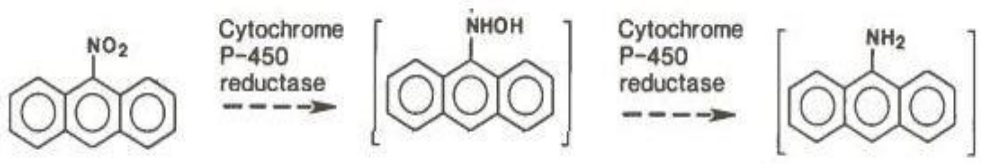


Figure 3. The metabolic pathway of 9-NA to form 9-aminoanthracene. Fu et al., (1985).

Figure 3 shows the metabolic pathway that converts 9-NA to 9-aminoanthracene. The cytochrome P-450 reductase enzymes reduces the 9-NA to form the active intermediate, N-hydroxyaminoanthracene, which is metabolized further to form the 9-aminoanthracene. The reduction of the nitro group to form the 9-aminoanthracene was not observed in the rat liver microsomes. This explains the weak mutagenic activity of 9-NA, since the formation of the active intermediate N-hydroxyaminoanthracene, is responsible for the mutagenic activity of the nitro PAHs. The experiment performed by Fu and coworkers is connected to my present study. It is very interesting to find whether the metabolites detected will be the same. Although the rats and worms are very different animals and use different CYP families for phase I metabolism, the metabolites formed by the rat liver microsomes can be used as a first guide to which metabolites to look for in the worm extracts.

Information on *A.Virens*

A. virens is a polychaete species that is found to populate the intertidal zone of both marine and brakish waters in Europe. In addition, they are also found to inhabit estuaries. There are several factors that govern the abundance and thereby distribution of the polychaete. Examples include, abiotic factors such as : temperature, salinity and the organic content of the sediment (Hutchings, 1998). The polychaete lives in burrows that are U-shaped (Neilsen et al., 1995). The polychaete ingests the uppermost layer of the sediment which contains dead particulate organic material and microbenthic algae that help nourish them (Wells and Dales, 1951). The metabolism of the sediment is stimulated by the bioturbation process that is carried by many polychaetes (Lopez and Levinton, 1987). In fact, the worms cope with the anoxic conditions by ventilating their burrows to carry respiratory and other important processes (Kristensen et al., 2012).

Caron et al., (2004) studied the feeding behavior of the polychaete *A.virens* and found out that the juveniles are detritus feeders. On the other hand, the adults of these worms can be carnivorous or omnivorous. The polychaete feeds using its powerful jaws (Gross 1921, Copeland & Wieman 1924, Tenore & Gopalan 1974, Heip & Herman 1979, Ronn et al. 1988). Deposit feeders such *A. virens* fulfill their nutritional needs by ingesting sediment particles which have organic matter dissolved in them (Lopez and Levinton, 1987). Deposit feeders show a selective preference towards small sediment particles (Self and Jumars, 1988). Therefore, this behavior exposes deposit-feeders to hydrophobic chemicals associated with the sediment particles and in particularly with the smaller sediment size fraction, because this has a larger surface to volume ratio and contains a high proportion of organic matter.

A.virens is widley used in ecotoxicological testing due to it being an important species in the food web (Copeland and Wieman, 1924). The worm is an important source of food for other species such as fish, crustaceans, and birds as well (Catalano et al., 2012; Masero et al, 1999). It is found to be dominant in marine sediments and thus contribute greatly to the biomass in the marine environment (Reish & Gerlinger, 1997). They are commonly found in polluted areas and thus are important to consider when evaluating the toxic effects of different contaminants (Eriksen et al., 1988). Mclroy and Sisson (1989) showed that BaP metabolites can be transferred from the polychaete *A. virens* to the Winter flounder, *Pdeudopleuronectes americanus*. The biological and ecological aspects of the worm have been well acknowledged which makes it an easier species to target in ecotoxicological

testing (Catalano et al., 2012). *A. virens* have been extensively used due to them being easily cultivated in the lab (Goerke, 1979).

Figure 4. *A. virens*

AIM OF STUDY

PAHs may have negative effects on benthic marine species such as, *A.virens*. Therefore, it is essential to measure the bioaccumulation of these contaminants, thus allowing us to better understand the fate of these contaminants. The two targeted aims of this thesis are to:

1) Measure the bioaccumulation of the PAH, 9-NA on the benthic marine species *A. virens.*
2) Identify the metabolites as a result of the uptake of 9-NA by *A.virens.*

METHODS

Synthesis of 9-nitroanthracene

The synthesis of 9-NA was carried out as described by Braun et al., 2003. Briefly, 10 grams of anthracene were added to 40 ml of glacial acetic acid. 4 ml of nitric acid was added drop wise to the mixture with continuous stirring. The flask was placed in a water bath to control the temperature of the reaction. The solution was stirred for 30 more minutes, after all the nitric acid was added. The mixture was filtered to ensure that there are no traces of anthracene in the reaction flask. Following this, 25 ml of concentrated hydrochloric acid and 25 ml of glacial acetic acid were added and the pale-yellow precipitate (9-nitro-10-chloro—9, 10-dihydroanthracene) was filtered and washed twice with 12.5 ml glacial acetic acid and then with distilled water until the pH paper confirmed that the washings were neutral. The product was grand using a pestle and mortar and 30 ml of 10% of warm sodium hydroxide was added. 9-NA was separated by suction filtration. Following this, four portions of 20 ml 10% sodium hydroxide solution was added to the product. The product of 9-NA was then washed with warm distilled water, until the litmus paper is purple. The crude product of 9-NA was air dried and purified by re-crystallizing it from glacial acetic acid. The recrystallization process required 10 ml of glacial acetic acid for every gram of 9-NA produced. To prevent the formation of 9,10-AQ as a contaminant, it was essential that the acetic acid was boiled first before adding the 9-NA in small amounts.

Worm culturing

The worms were cultured in aquaria with around 7 cm of sediment and seawater aerated with air-pumps. The aquaria were set up at least 4 days before adding the worms. 60 worms of *A. virens* bought from the fishing shop "Jan & Bo's Lystfiskershop" in Roskilde, were placed in the aquaria with 30 promille seawater for four days. Twenty worms were placed in each aquarium. Salinity was changed gradually every two days and after a salinity of 17 promille was reached, the worms were left for a minimum of a week to acclimatize before proceeding with my experiments.

Sediment preparations

Sediments were collected in shallow water at the bottom of Isefjorden next to the Munkholmbro, by scaping off the first 5 cm of the sediment. The sediments were sieved to 1 mm and 0.5 mm in distilled water. The sieved sediment was then allowed to settle down completely before removing the overlaying water and washed three times with 17 promille sea water. The 1 mm sieved sediment was used to keep cultures and the 0.5 mm sieved sediment was used for experimental purposes. The sediments were kept in the freezer before being used. The wet weight to dry weight ratio of the 0.5 mm sieved sediment was determined in order to calculate the amount of wet sediment to add during spiking.

Three empty crucibles were placed at 105 °C overnight- subsequently cooled down in a dessicator, marked and weighed. The sediment was homogenized by stirring it using a large plastic spoon and a small amount of sediment was placed in each of the crucibles, which were weighed. The samples were placed in the oven at a temperature of 105 °C overnight. The dry samples were cooled down and weighed, and the percent of dry weight was calculated according to the equation:

$$\frac{(\text{Mass of crucible} + \text{dry sediment}) - (\text{Mass of empty crucible})}{(\text{Mass of crucible} + \text{wet sediment}) - (\text{Mass of empty crucible})} \times 100$$

The sediment for the screening experiment was spiked with 9-NA and the concentrations of 9-NA per gram of dry sediment were: 2232.3 [5], 223.23 [4], 22.323 [3], 2.2323 [2], and 0.22323 µg/g dry weight sediment [1]. An overview of the different treatments applied is present in table 1. The sediments for the screening experiment were spiked by preparing a stock solution to spike the sediment of the highest concentrations, which was then diluted to the nominal concentrations. This was done by dissolving 0.67 grams of 9-NA in acetone in a 20 ml volumetric flask (to prepare for the [5] concentration). 2 ml of this solution was placed in a 10 ml volumetric flask with acetone to prepare for the [4] concentration. Following this, 1 ml was taken from solution [4] to prepare the lower concentration, [3]. Then 1 ml was transferred from [3] to a 10 ml volumetric flask to form the [2]. The same was done to form the [1] solution, since there is a factor of 10 between each nominal concentration. For the highest concentration [5], 16 ml of the prepared solution was placed in a 1 liter beaker. 8 ml of the other solutions and 8 ml of acetone was placed in each of the other 1 liter beakers. 16 ml of pure acetone was added to the control beaker. After all the acetone evaporated, 240 grams of dry sediment was added to every beaker. The wet weight to dry weight ratio of the

sediment was 1.54. For the time and concentration dependent accumulation exposure experiment, the sediment was spiked with 2232.3 [5], 223.23 [4] µg/g dry weight sediment, and a control. The 2232.3 µg/g dry weight sediment [5], was spiked by weighing 1.7 grams of 9-NA into a 100 ml volumetric flask. Acetone was added to the mark and the solution was homogenized. Then 6 ml was added to the 600 ml beakers and the acetone was left to evaporate. Following this, 45 grams of dry sediment was added to each of the beakers. The same procedure was used to prepare the 223.23 µg/g dry weight sediment [4], but, instead, 0.17 grams of 9-NA in a 100 ml acetone was used. The control beakers were prepared by adding 6 ml of pure acetone to every beaker. The beakers were covered with aluminum foil to prevent the photo-oxidation of 9-NA and were placed on a shaking table for 48 hours.

Screening experiment

100 grams of the spiked sediment (2232.3 [5], 223.23 [4], 22.323 [3], 2.2323 [2], and 0.22323µg/g dry weight sediment [1] and the control) were added to 600 ml beakers- after which 300 ml of 17 promille water was added to the different beakers. The sediment was allowed to settle for 4 hours and the beakers were aerated before adding the worms. Four replicates were used in each treatment, thus making a total of 24 worms used in the screening experiment. The worms were weighed before adding them to the different concentrations. This ensured that the worms were evenly divided among the different treatments. In addition, weighing the worms would give extra endpoint in terms of the growth or de-growth of the different worms. The initial, final and percent de-growth of the worms are provided in Appendix A. The whole experiment was kept in the dark to prevent the photooxidation of the 9-NA. The exposure duration was over a period of 18 days.

Table 1. Overview of the different treatments applied.

Different concentrations	**[1]**	**[2]**	**[3]**	**[4]**	**[5]**
Con. in g/g dry wt	2.2323 $\times 10^{-7}$	2.2323$\times 10^{-6}$	2.2323$\times$ 10^{-5}	2.2323$\times$ 10^{-4}	2.2323$\times$ 10^{-3}
Grams of 9-NA needed in the 1L beaker	0.0000 536	0.000 536	0. 00536	0. 0536	0. 536
stock solution concentration (g/ml)	0.0000 066969	0.000066969	0.00066969	0.0066969	0.066969

Time and concentration dependent accumulation exposure experiment

300 ml of 17 promille water was added to the different beakers. In total 48 worms were used. The number of replicates at each treatment at each time frame was four. The worms were taken out for analysis of the concentration of 9-NA in their guts and remaining tissues after 6 hours, 24 hours, 7 days and 14 days of exposure. The metabolites formed were identified and the response of the detected metabolites was measured. The response is an arbitrary unit for the area of the current peak in the chromatogram. The whole experiment was kept in the dark to prevent the photooxidation of the 9-NA. The initial, final and percent de-growth of the worms are provided in Appendix A.

Sediment, gut and tissue extractions and analysis

The worms were placed in 250 ml beakers with 17 promille sea water, to allow them to depurate the guts overnight. The worms were placed on a frozen Oasis block and a pin was placed on the peristomium of the worm, which is the second segment that is located on the anterior body end. Another pin was placed on the posterior end, which prevented the worm from moving during the dissection process. The gut was extracted by cutting along the dorsal blood vessel using a scissors. This opened the coelom which allowed the gut to be extracted. The gut of the first half of the worm was removed. In addition, the first 2 cm of tissue behind the peristomium were removed. All the samples were kept in the freezer for further analysis. For the screening experiment, 9-NA and the formed metabolites in the guts and tissues of the worms were extracted using 1 ml of acetonitrile. 9-NA in the sediment before exposure was extracted using 5 ml of acetonitrile. For the time and concentration dependent accumulation exposure experiment (second exposure experiment), 9-NA and the metabolites in the guts were extracted using 0.5 ml of acetone and 0.5 ml ethyl acetate. The tissues were extracted using, 2 ml of acetone and 2 ml of ethyl acetate. 9-NA in the sediment before exposure was extracted using 4 ml of acetonitrile. 30 µL of phenanthrene d-10 (100 mg/ 100ml) as the internal standard was added to each of the samples. The samples were placed on ultrasound for 30 minutes, and then centrifuged for 10 minutes at an angular velocity of 4000 revolutions per minute. Following this, the supernatant was removed and placed in a GC vial. The samples were analyzed using a GC/MS.

GC/MS model and control parameters:

The GC/MS was used to measure the concentration of 9-NA in the gut, tissue and sediment samples. The formed metabolites were detected and the response of theses metabolites was measured. The name of the GC model is "6890N Network GC system" and the MS model is "Agilent technologies 5975 inert mass selective detector". The GC column produced by the Phenomenex company is called "Zebron capillary" and the model number is: ZB-semivolatiles. The dimensions of the column are: 30 m in length, 0.25 mm internal diameter and a film thickness of 0.25 µm. The samples were injected and the initial temperature of the oven was 70°C and the final temperature of the oven reached was 300 °C The initial starting time was 3 minutes. The rate of temperature increase was 12°C/ minute and the overall run time of every sample analyzed was 24.17 minutes.

Rat liver experiment

The procedure was carried out as described by Fu et al., (1985). Briefly, 10 mg of microsomes of male Sprague-Dawley rats were purchased from Sigma. The microsomes were added to a 500 ml beaker, and the following were added: a solution of 10 ml of 40 µmole 9-NA in acetone, 25 mmol of tris(hydroxymethyl)aminomethane HCL buffer (the pH required is 7.5). 1.5 mmole of magnesium chloride, 100 units of the enzyme glucose-6-phosphate dehydrogenase, 48 mg of the reducing equivalent $NADP^+$, and finally 0.28 grams of glucose-6-phosphate. The mixture was incubated at a temperature of 37°C for 1 hour. 400 ml acetone, followed by 800 ml ethyl acetate were used to extract 9-NA and the formed metabolites. The organic layer was extracted and any traces of water were removed by using anhydrous sodium sulfate. The extracted mixture was placed on a rotary evaporator. The residues formed were dissolved in 20 ml acetone. The solution was then centrifuged and dissolved in 1.5 ml of methanol, and placed in a GC vial for further analysis.

Statistical analysis

The statistical package for social sciences (SPSS) was used to perform statistical measures on my data. The data was tested for normal distribution and equal variances, which are the two criterion required for performing a parametric test. If the p- values obtained for the normality tests and equal variances were greater then 0.05, then an analysis of variance test (ANOVA) was performed. This statistical tool enabled us to see whether there is a significant difference between the treatments. An

ANOVA test with a p-values less than 0.05 indicated that the differences observed are statistically significant. A post hoc test (tukey test) was performed to see which groups are significantly different from each other. When the ANOVA assumptions were not fulfilled, a box plot was constructed to visualize which treatments were significantly different from each other. A 2- way ANOVA test was used to measure whether there was a significant interaction term between the time dependency in the uptake of 9-NA and the different exposure concentrations. However, the 2-way ANOVA is a robust statistical analysis tool and was performed, even though the assumptions of a normal distribution and equal variances were not fully met.

RESULTS

Synthesis and purification of 9-NA

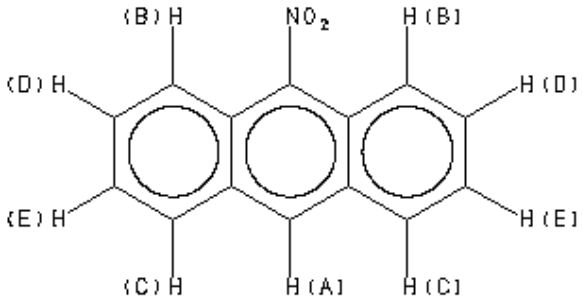


Figure 5. The molecular structure of 9-NA with labeling of the different protons present.

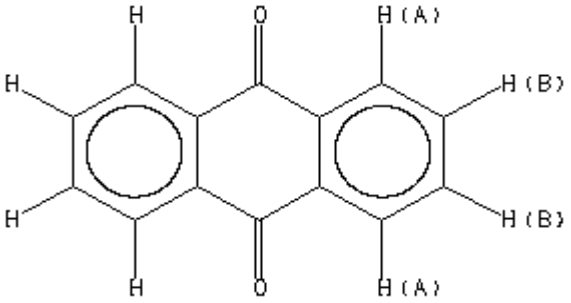
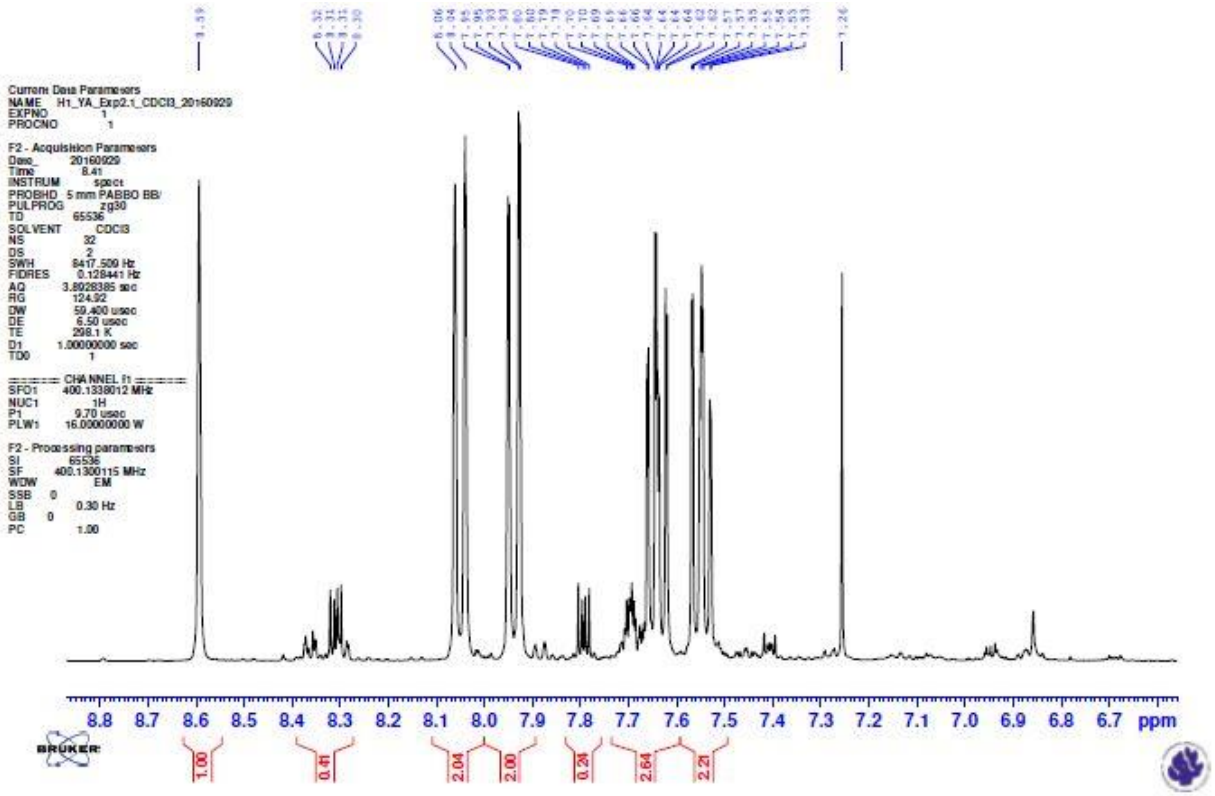


Figure 6. The molecular structure of 9,10-AQ with labeling of the different protons present.

Figure 5 shows the molecular structure of 9-NA and the protons labeled A to E will be used in the description of the NMR peaks present in figure 7. Figure 6 shows the molecular structure of 9,10-AQ and the protons labeled A and B will be used in the description of the NMR peaks present in figure 7. Figure 7 shows the NMR spectrums labeled A to D. Spectrum A shows that the peaks from 9-NA is as the following: there is a single peak at 8.59 ppm, which corresponds to the proton at position (A). The protons at position (B) correspond to a doublet at 8.06 and 8.04 ppm. There is another doublet peak between 7.95 and 7.93 ppm, which correspond to the protons at position (C). In addition, there is a triplet peak between 7.66 and 7.62 ppm. These peaks are due to the protons present at position (D). There is triplet peak between 7.57 and 7.53 ppm. These peaks are due to the protons, which are present at positions (E). Spectrum A shows that there are traces of 9,10-AQ, which is the contaminant that was produced throughout the reaction. The peak that is around 8.32 ppm corresponds to the protons present at positions (A) in figure 6. The chemical shift that is around 7.8 ppm is due to the protons that are present at position (B). The numbers in (red) present below the graphs represent the integration of the peaks. Using spectrum A, the ratio of 9-NA to the ratio of 9,10-AQ present can be calculated from these integral values. The integral value of the singlet proton in 9-NA is 1.00 ppm. This indicates that one hydrogen equals 1.00 ppm. The integral value of the protons that are present at position (A) in anthraquinone is 0.41. Since there are four protons that have an integral value of 0.41, then every proton has a value of (0.41/4 = 0.1025). This

means that the ratio of every proton of 9,10-AQ at that position is equal to 0.1025. Therefore, the ratio of 9-NA to 9,10-AQ is 1: 0.1025. In order to purify the 9-NA, recrystallization of my raw product was necessary to remove any traces of the 9,10-AQ contaminant. The amount of 9,10-AQ present was reduced significantly after the first re-crystallization in spectrum B. Spectrums C and D show the NMR spectrums of the second and third re-crystallizations. The peaks from the contaminant, 9,10-AQ disappeared.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SOLVENT CDCl3
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FIDRES 0.128441 Hz
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DW 59.400 usec
DE 6.50 usec
TE 298.1 K
D1 1.00000000 sec
TD0 1
CHANNEL f1
SFO1 400.1338012 MHz
NUC1 1H
P1 9.70 usec
PLW1 16.00000000 W
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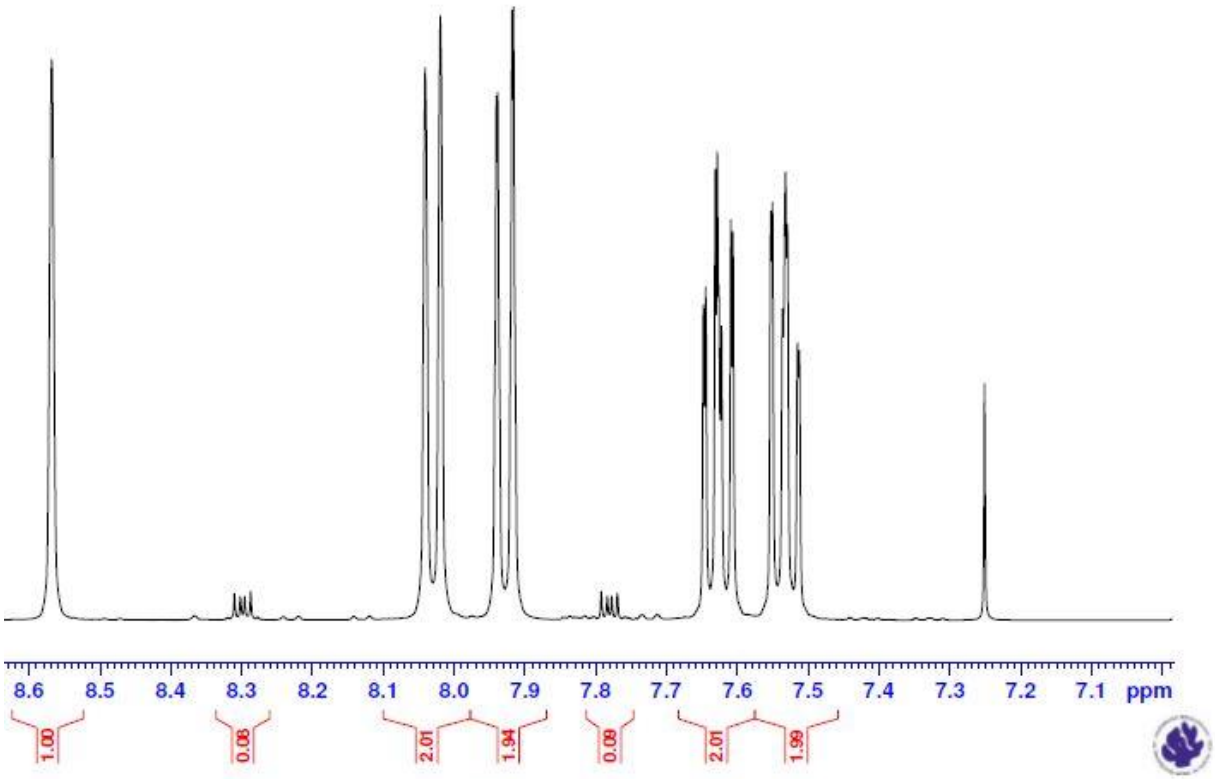
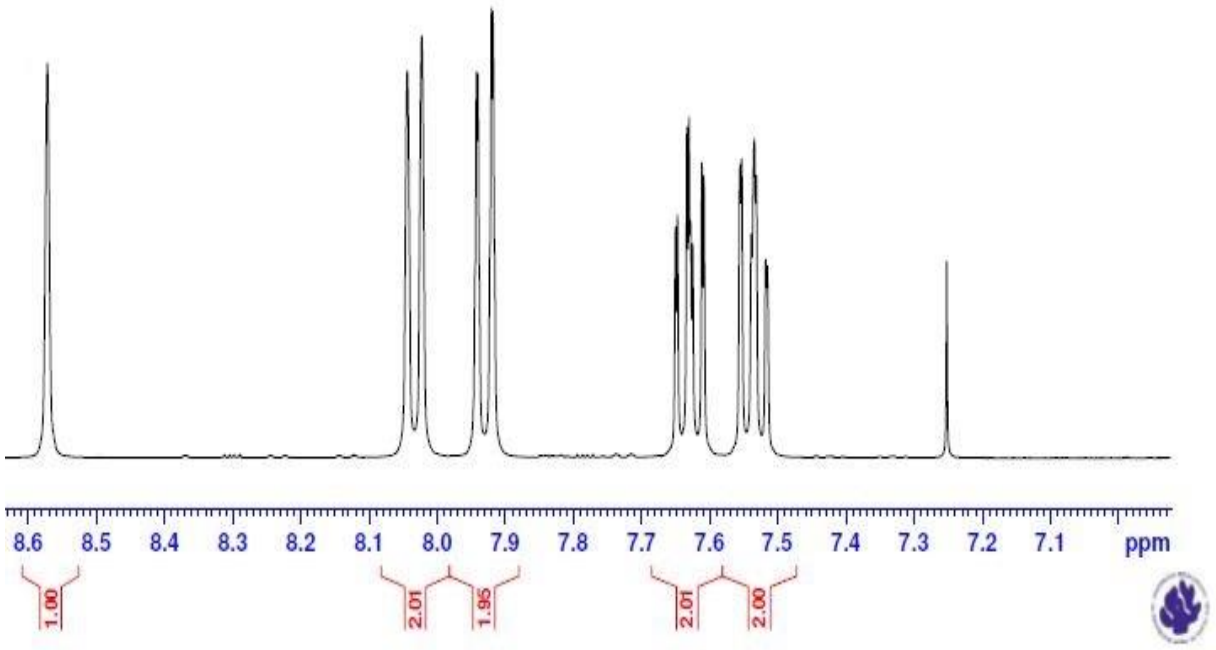
SF 400.1300115 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
8.8 8.7 8.6 8.5 8.4 8.3 8.2 8.1 8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7.0 6.9 6.8 6.7 ppm
1.00 0.41 2.04 2.00 0.24 2.64 2.21

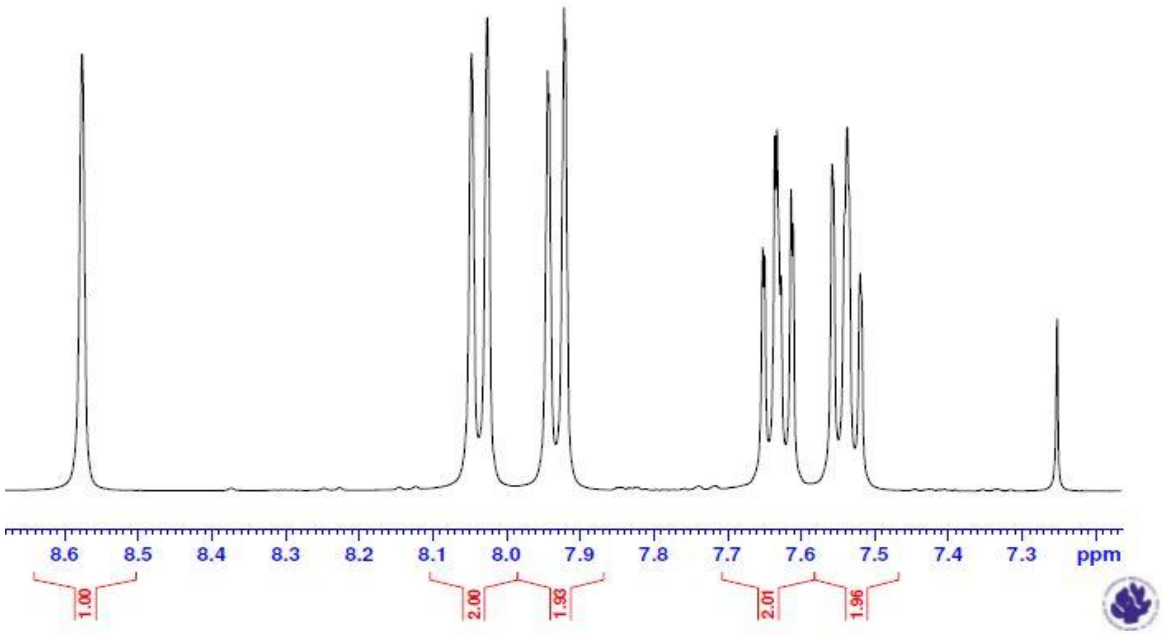
A


8.6 8.5 8.4 8.3 8.2 8.1 8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 ppm
1.00 0.06 2.01 1.94 0.09 2.01 1.99

B



C



D

Figure 7. The NMR spectrum of the synthesized 9-NA (A), the NMR spectrum after the first recrystallization (B), the NMR spectrum after the second recrystallization (C) and NMR spectrum after the third recrystallization (D).

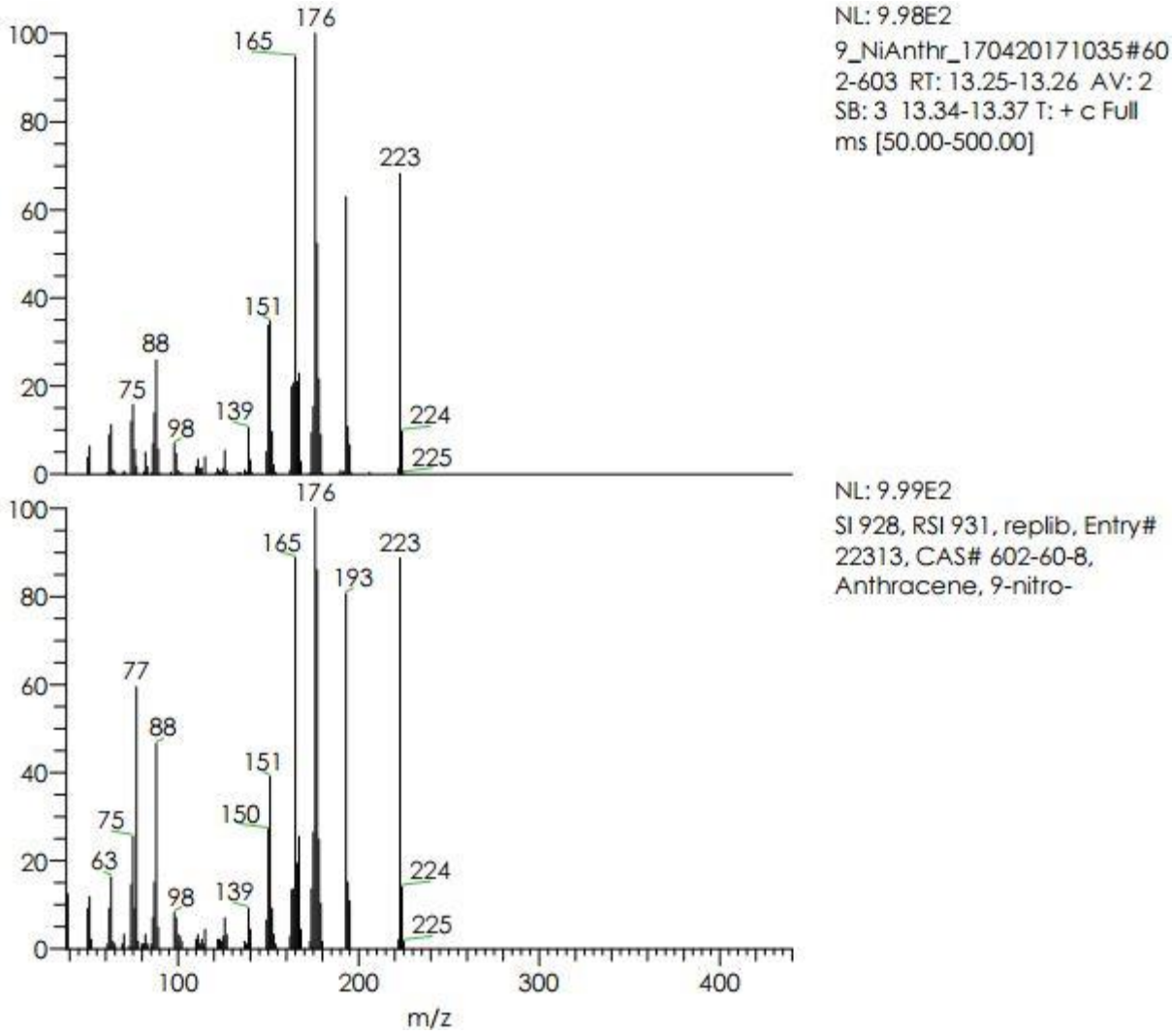


Figure 8. Mass spectrum of the synthesized 9-NA (top figure) in comparison to the mass spectrum obtained from the library collection (below figure). X-axis represents the mass to charge ratio, while the y-axis represents the relative abundance of the peaks.

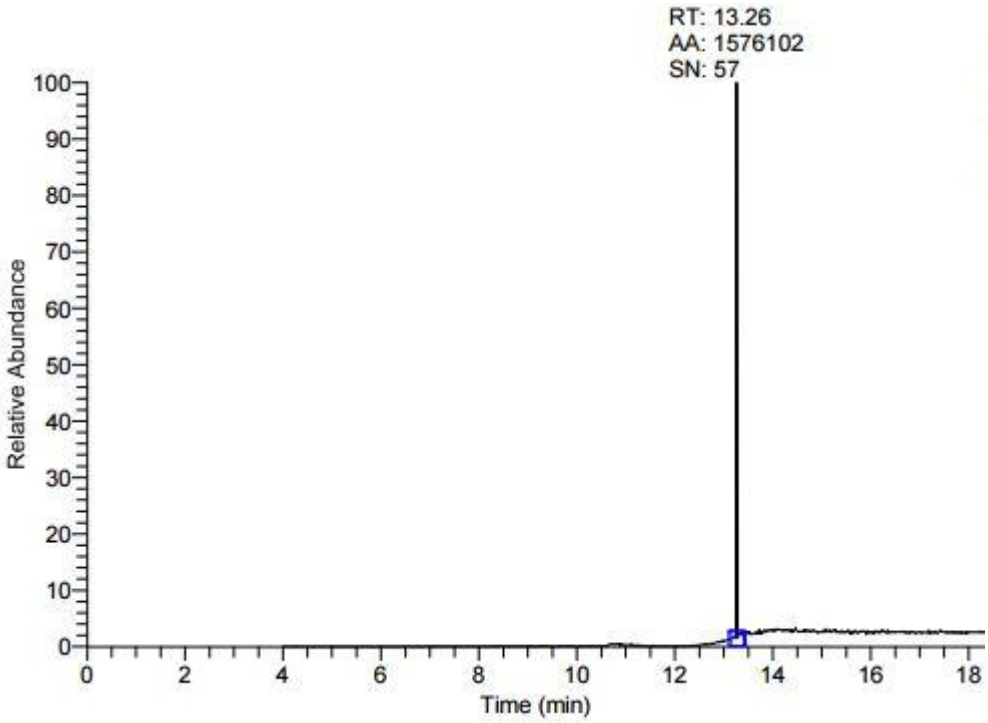


Figure 9. The retention time of the synthesis product at 13.26 minutes.

Figure 8 represents the mass spectrum of the synthesized 9-NA that has a similar mass spectrum when compared to the library. This confirms the presence of a pure 9-NA. There were no traces of 9,10-AQ present when analyzing the components present in my compound. This confirms that the recrystallization of 9-NA using glacial acetic acid, resulted in the production of a pure 9-NA, removing any traces of 9,10-AQ. The retention time of 9-NA is 13.26 minutes (figure 9).

Results of the screening experiment:

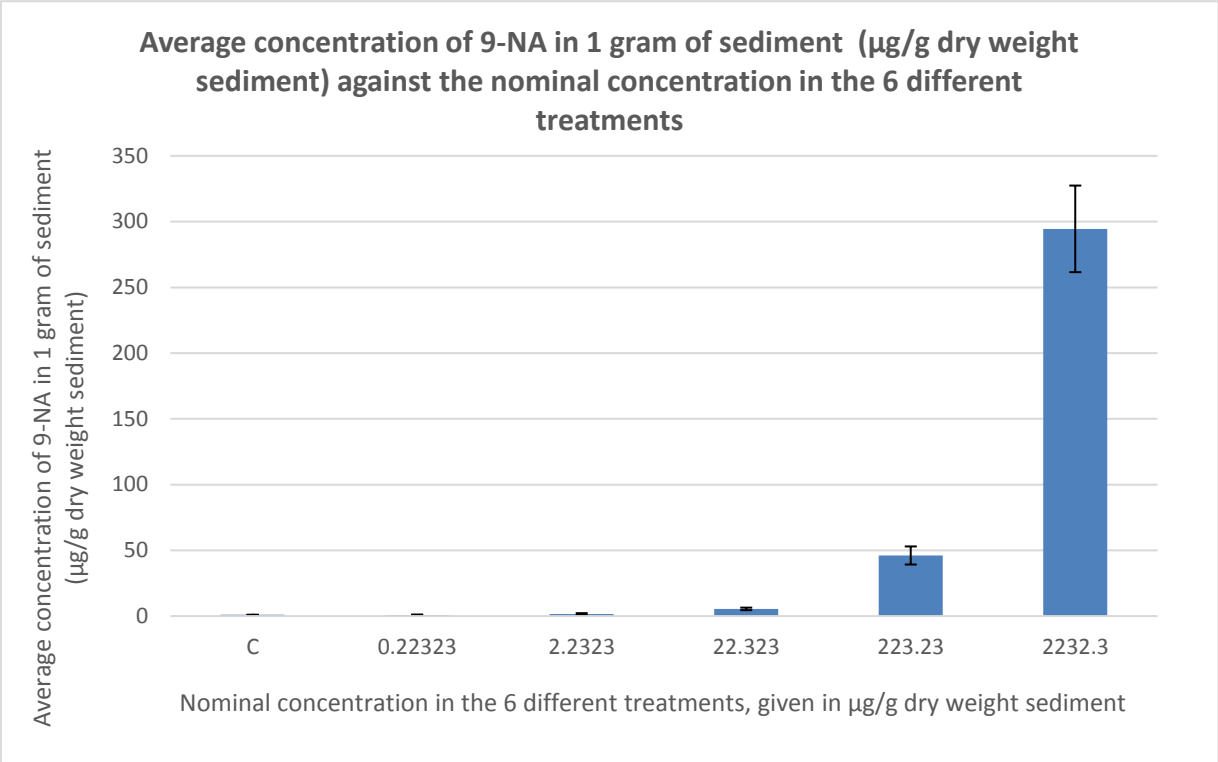


Figure 10. The average concentration of 9-NA in 1 gram of sediment, given in (µg/g dry weight sediment) against the nominal concentrations in the 6 different treatments, given in µg/g dry weight sediment. The data is expressed as the mean ± Standard deviations.

Figure 10 shows the average concentration of 9-NA in 1 gram of sediment, given in (µg/g dry weight sediment) against the nominal concentrations in the 6 different treatments, given in µg/g dry weight sediment. The measured average concentration in the sediment increases, however it is not the same as expected. The measured average concentration of 9-NA per gram of dry sediment is 0.93 µg/g sed for [1], 1.48 µg/g sed for [2], 5.47 µg/g sed for [3], 46.1 µg/g sed for [4] and 294.49 µg/g sed for [5]. The control sediment had 9-NA as well with an average concentration of 0.94 µg/g sed. Figure 11 shows the presence of 9-NA in the control treatment when analyzed using GC/MS, at a retention time of 18.216 minutes. A post hoc test (tukey test) showed that, there was no significant difference between the control treatment and concentrations [1] and [2]. There is no significant difference between teatments [1] and [2]. However, treatments [3], [4] and [5] differed significantly from all of the other treatments.

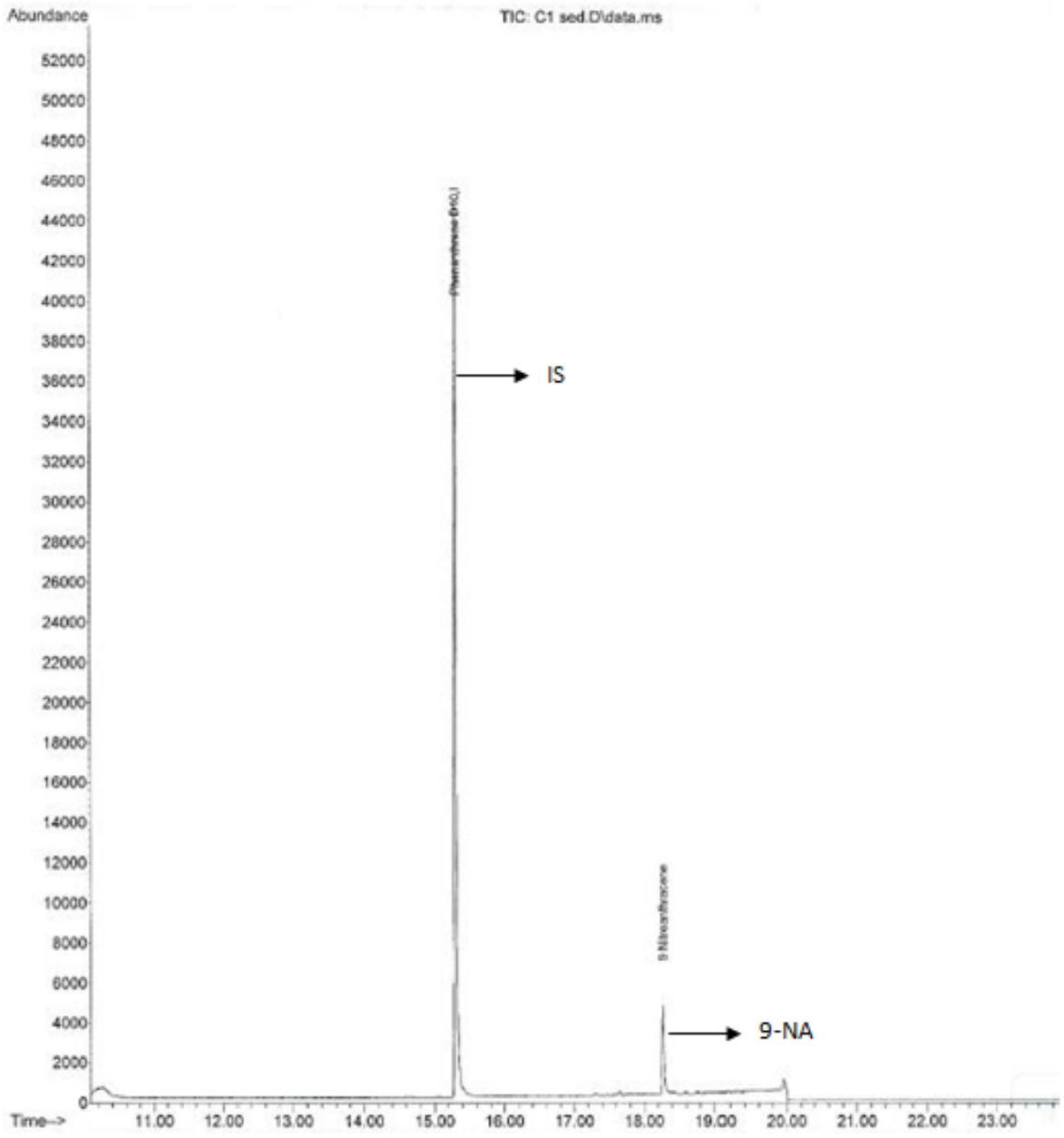
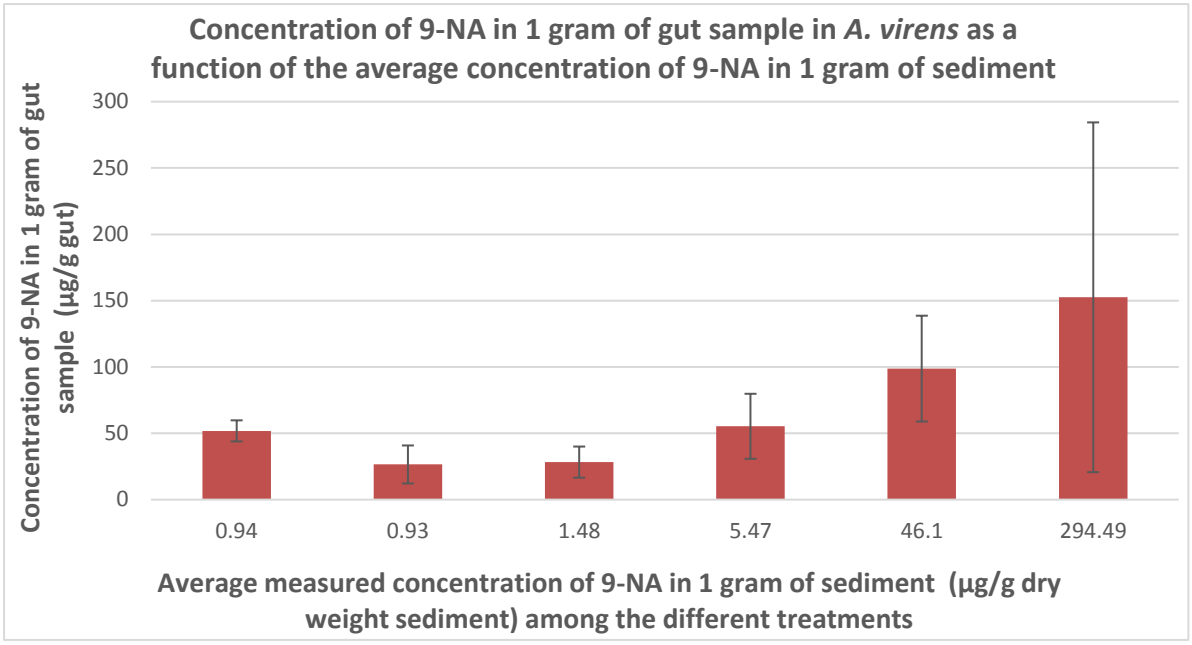
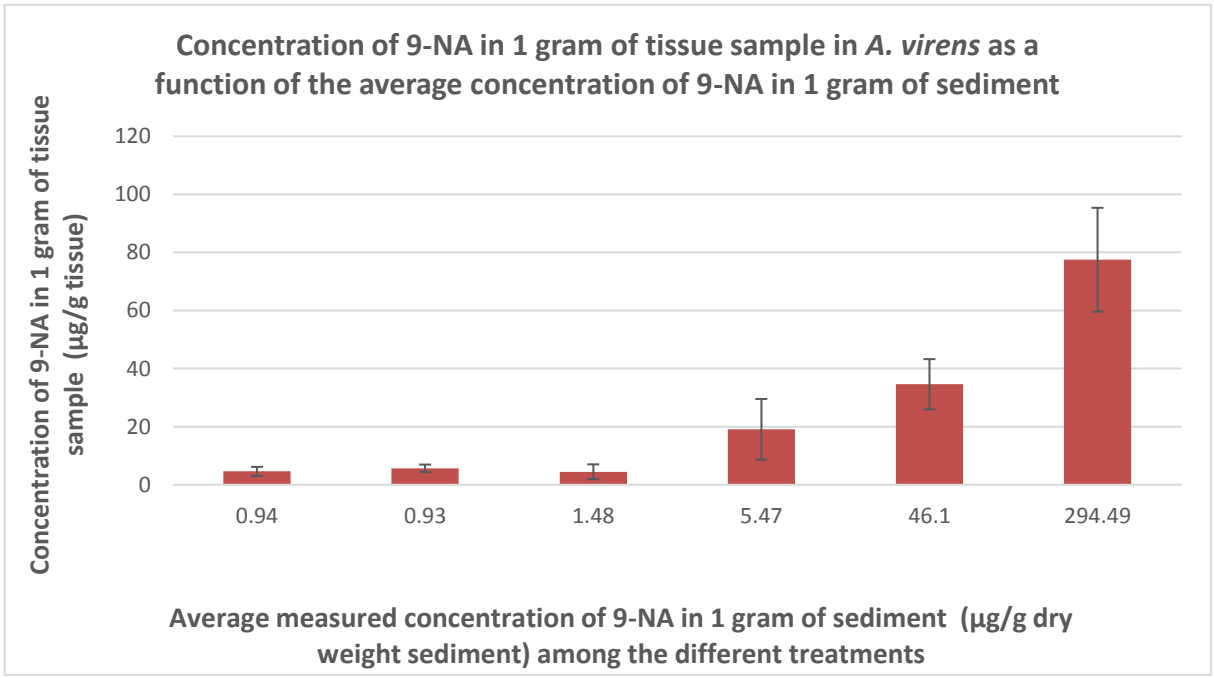


Figure 11. The chromatogram of the sediment control sample. The internal standard (IS) has a retention time of 15.30 minutes. 9-NA has a retention time of 18.216 minutes.



A



B

Figure 12. The average concentration of 9-NA in 1 gram of gut sample (A) and tissue sample (B) in *A.virens*, given in (µg/g) against the average concentrations of 9-NA in the 6 different treatments, given in µg/g dry weight sed. The data is expressed as the mean ± SDs.

Figure 12 represents the average concentration of 9-NA in 1 gram of gut and tissue samples against the different treatments. The concentration of 9-NA increases in the guts and tissues of the worms with increasing the concentration of 9-NA in the sediment. The average concentration of 9-NA is higher in the guts of the worms than the tissues in all the treatments. The data from the first figure (A) did not fulfill the requirments for parametric testing; however, using 95% confidence intervals indicated that the worms exposed to the highest sediment concentration had significantly higher gut concentrations compared to worms exposed to control and the two lowest sediment concentrations. The data from the second graph (B) fulfilled the requirments for parametric testing after log transformation. The test showed that there is a significant difference between at least two of the treatments applied. A tukey test was then chosen to make a pairwise comparison between the treatments. The results showed that there is a significant difference between the control and the three highest concentrations. In addition, there is a significant difference between the highest concentration and all the treatments, except [4]. There is a significant difference between [4] and the two lowest treatments and control. In addition, there is a significant difference between between [3] and treatments [1], [2],[5] and the control.

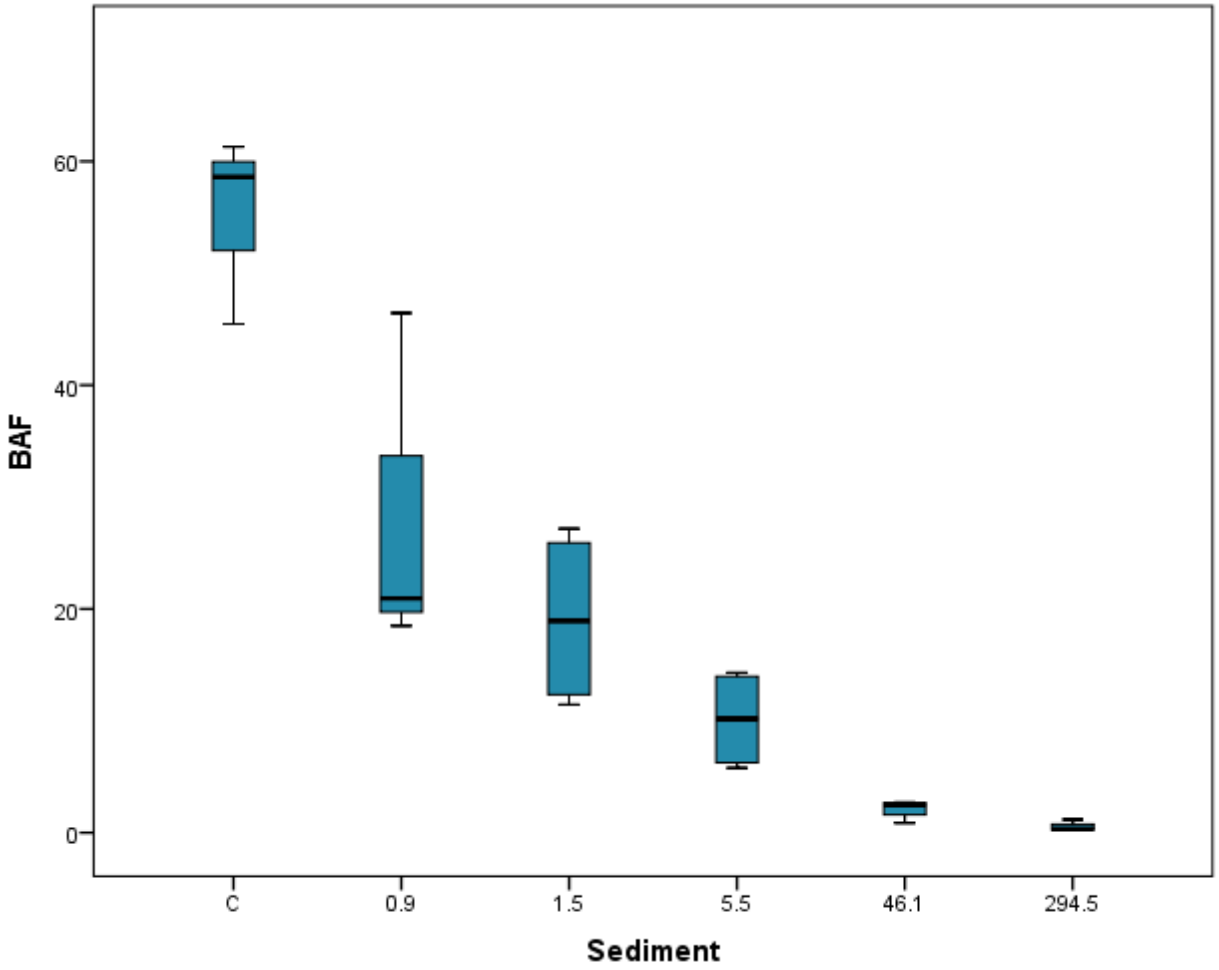


Figure 13. A box plot of the BAF against the average concentration of 9-NA in the different treatments, given in in µg/g dry weight sediment.

The data did not fulfill the requirements of parametric testing, and therefore a box plot (figure 13) was chosen to see whether there is a significant difference in the BAF among the different treatments. The box plot indicates that there is a significant difference between the control and treatments [2], [3], [4] and [5]. In addition, there is a significant difference between [1] and treatments [3], [4] and [5]. The bars show that there is a significant difference between [2] and treatments [4] and [5]. Additionally, there is a difference between [3] and treatments [4] and [5]. There is no significant difference in the BAF between treatments [4] and [5]. The possible metabolites formed were investigated, and the results are present in the next section.

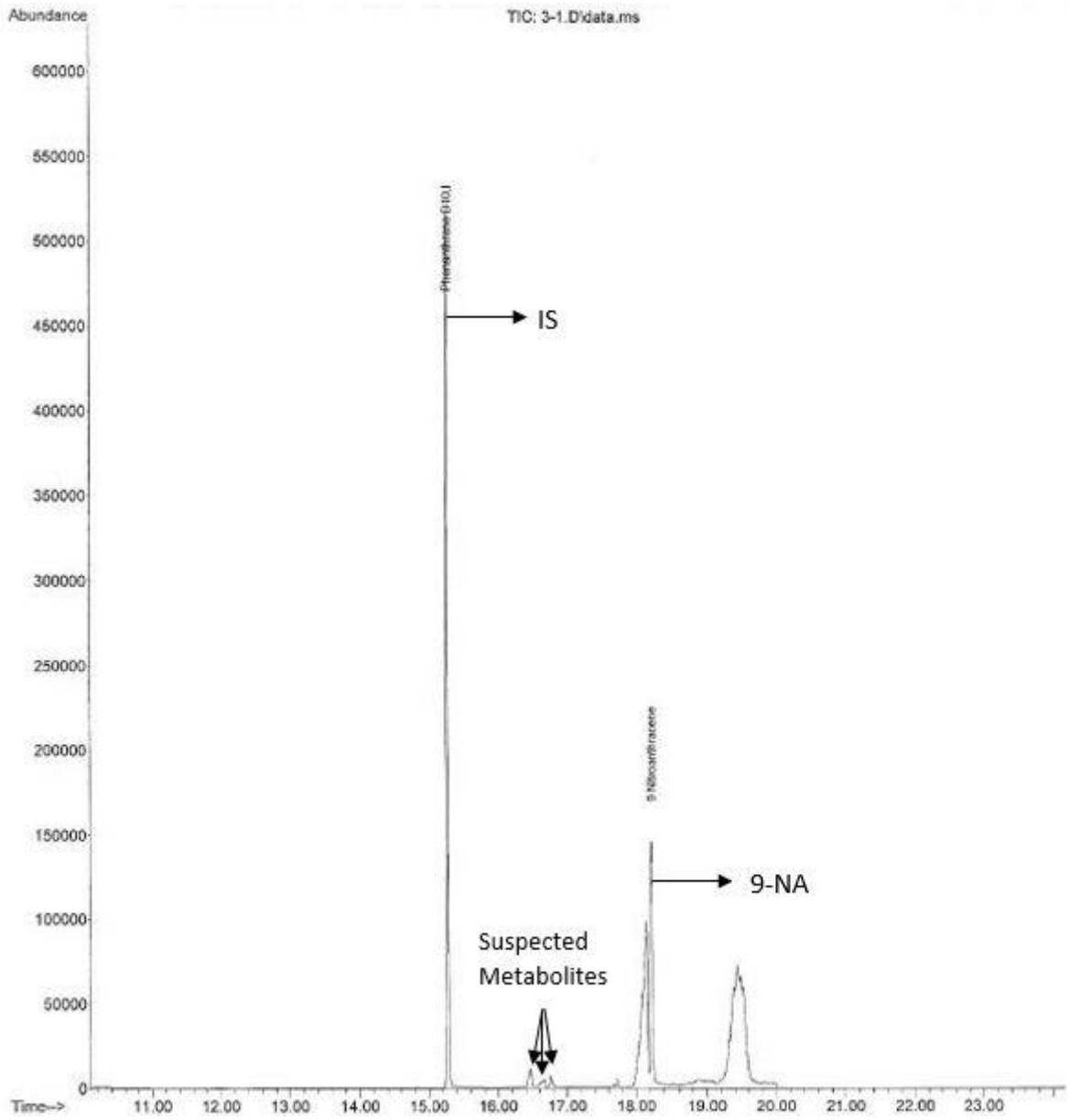


Figure 14. The chromatogram of the [3]1 gut sample obtained. The IS has a retention time of 15.3 minutes. 9-NA has a retention time of 18.216 minutes. The small peaks present at 16.419, 16.585 and 16.756 minutes are suspected to be the possible metabolites formed, since these peaks appeared with increasing concentrations of 9-NA.

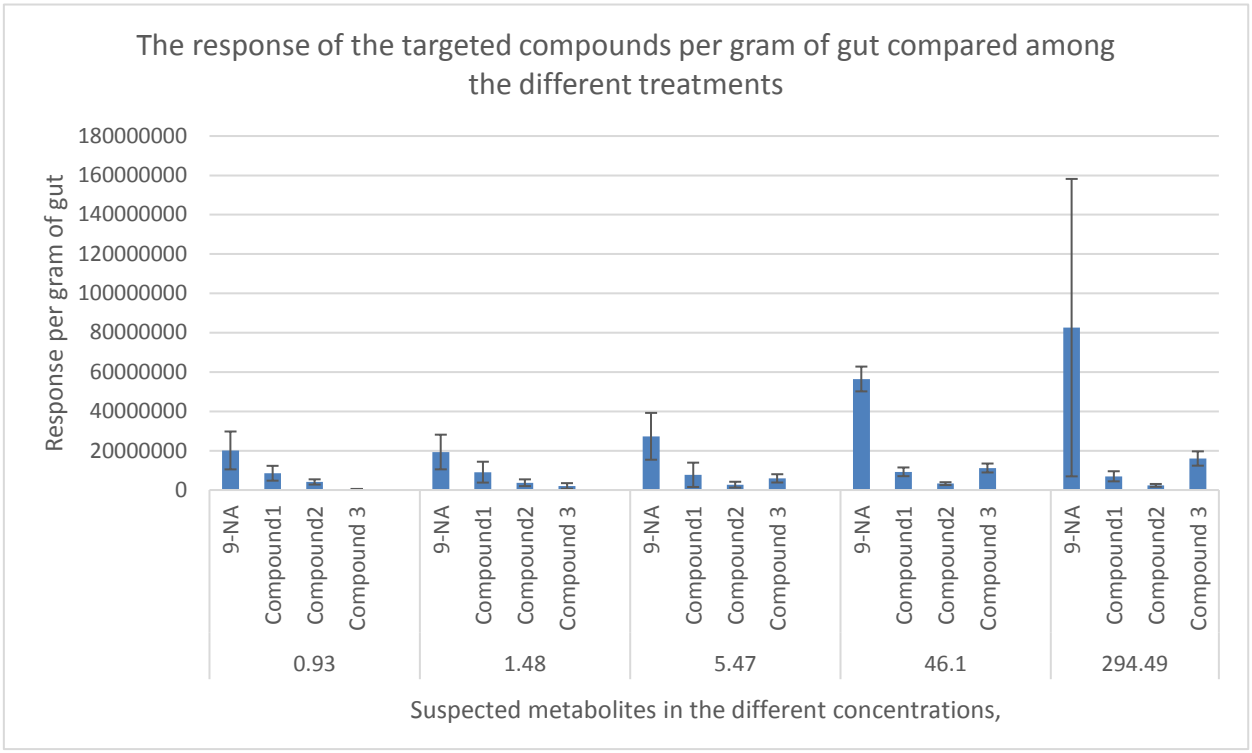
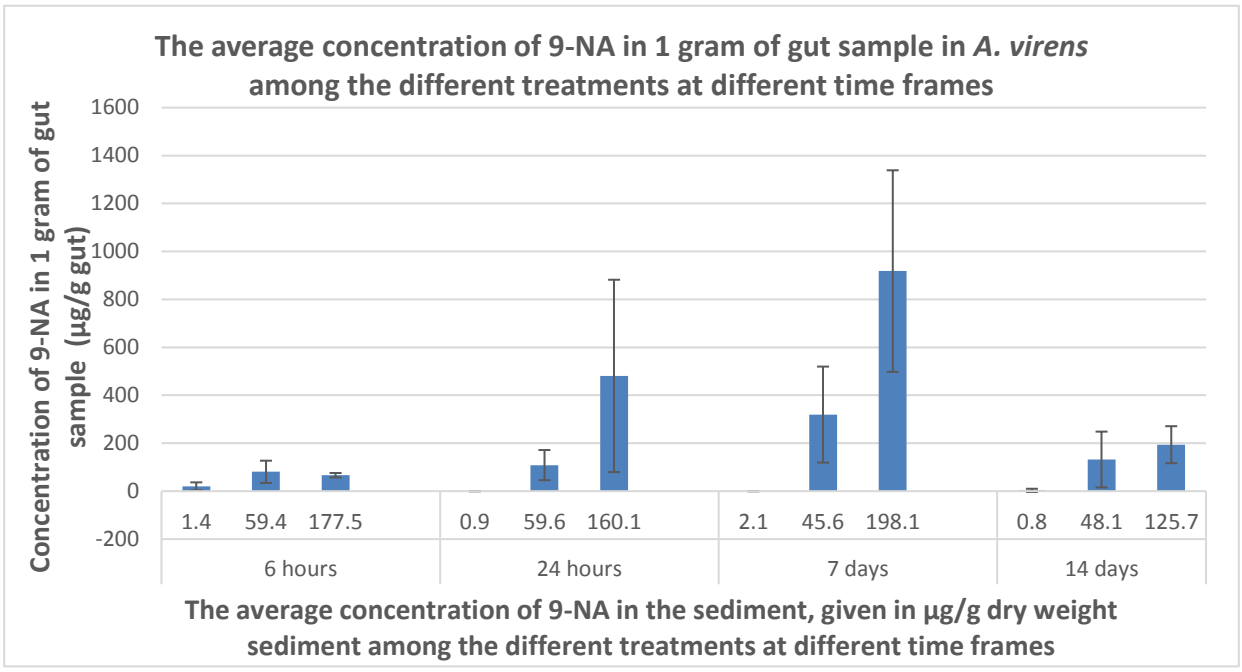


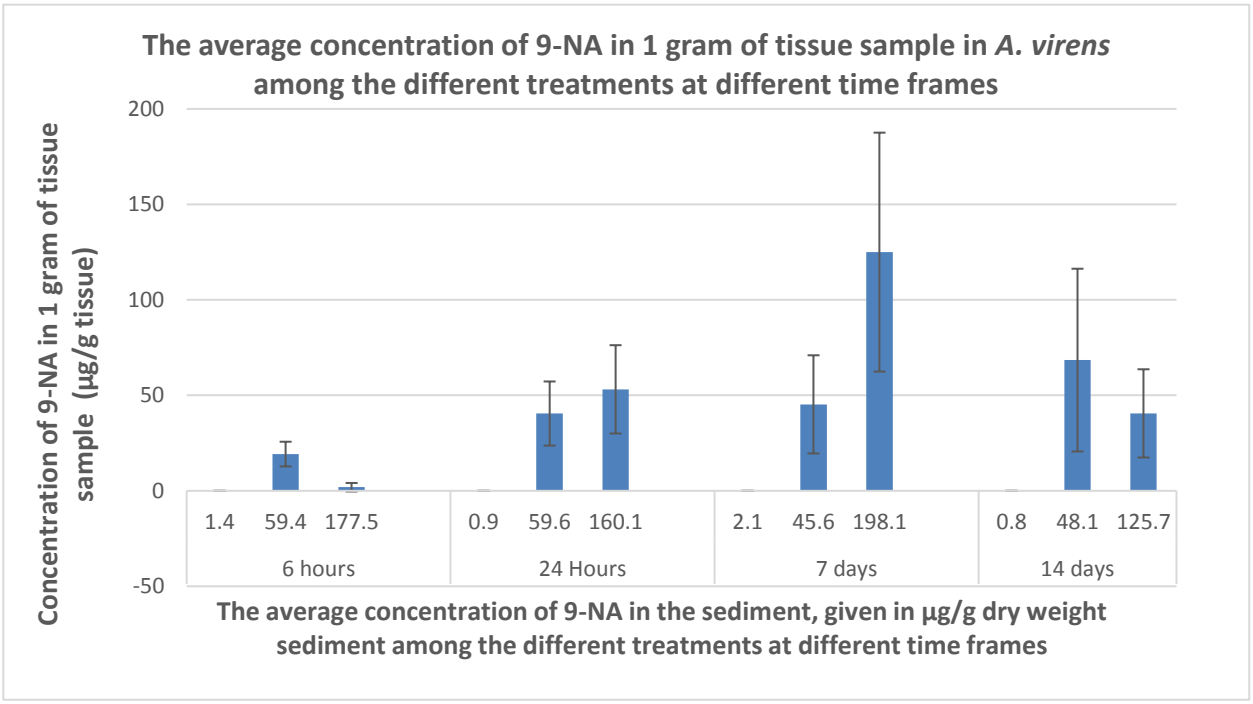
Figure 15. The response of the targeted compounds per gram of gut compared between the different treatments, ±SDs. 0.93, 1.48, 5.47, 46.1 and 294.49 µg/g dry weight sediment represent the concentration of 9-NA in these treatments [1], [2], [3], [4] and [5] respectively. The suspected metabolites (compound 1,2 and 3) have retention times of 16.419, 16.585 and 16.756 minutes respectively. The retention time of 9-NA is 18.216 minutes. Compound 3 represents 9,10-AQ.

Figure 14 represents the chromatogram of the 3[1] gut sample, highlighting the suspected metabolites. Figure 15 illustrates the response per gram of the suspected metabolites among the different treatments. The suspected metabolites were examined using a selected-ion monitoring (SIM) mode to see whether the suspected metabolites are indeed what we believe they are. The following compounds were selected for analysis: 9,10-AQ, dihydroxy-9-nitro-dihydroanthracene (9-NO_2-dihydrodiol), 1,2,3,4-tetrahydrodiol and 9-aminoanthracene. These are the metabolites that were investigated by Fu et al., 1985. The only metabolite identified to be present was 9,10-AQ, at a retention time of 16.756 minutes.

Results of the second exposure experiment:



A



B

Figure 16. The average concentration of 9-NA in 1 gram of gut sample (A) and tissue sample (B) in *A.virens*, given in (µg/g) against the average concentrations of 9-NA in the different treatments, given in µg/g dry weight sediment at the different time frames. The data is expressed as the mean ± standard deviations.

Figure 16 (A) represents the average concentration of 9-NA in the gut of the worms among the different treatments at the various times of exposure. The graphs representing the concentration of 9-NA in the sediment before exposure at each time frame for every replicate is present in Appendix B. The nominal concentrations were 2232.3 µg/g dry weight sediment [5] and 223.23 µg/g dry weight sediment [4]. The average measured concentrations were however different from the nominal concentrations. The measured [4] concentrations for the 6 hours, 24 hours, 7 days and 14 days were 59.4, 59.6, 45.6 and 48.1 µg/g dry weight sediment respectively. The measured [5] concentrations for the 6 hours, 24 hours, 7 days and 14 days were 177.5, 160.1, 198.1 and 125.7 µg/g dry weight sediment respectively. The control sediments had 9-NA as well. The measured concentrations of 9-NA in the control treatments at the 6 hours, 24 hours, 7 days and 14 days were 1.4, 0.9, 2.1 and 0.8 µg/g dry weight sediment respectively. The graphs representing the concentration of 9-NA in the guts and tissues of the worms among the different treatments, at each time frame is present in Appendix B. Figure 16 (A and B) shows that the concentration of 9-NA in the guts and tissues of the worms, depend on the increase in concentration of 9-NA in the sediment, except after 6 hours, which shows that the concentration in the gut and tissue is higher in the [4] treatment than the [5]. In addition, there is a general decrease in the body burden after 14 days of exposure, except for the control. The tissues of the worms showed that after 14 days of exposure, the average concentration of 9-NA is higher in the [4] treatment compared to the [5] treatment. The concentration of 9-NA in the guts of the worm is higher than the concentrations in the tissues. The 2- way ANOVA test is a robust statistical analysis tool and therefore can be performed, even though the criterions for parametric testing were not completely fulfilled. The results of the 2- way ANOVA indicated that there is a significant interaction term, which shows that the time dependency in the uptake of 9-NA, depends on the exposure concentrations.

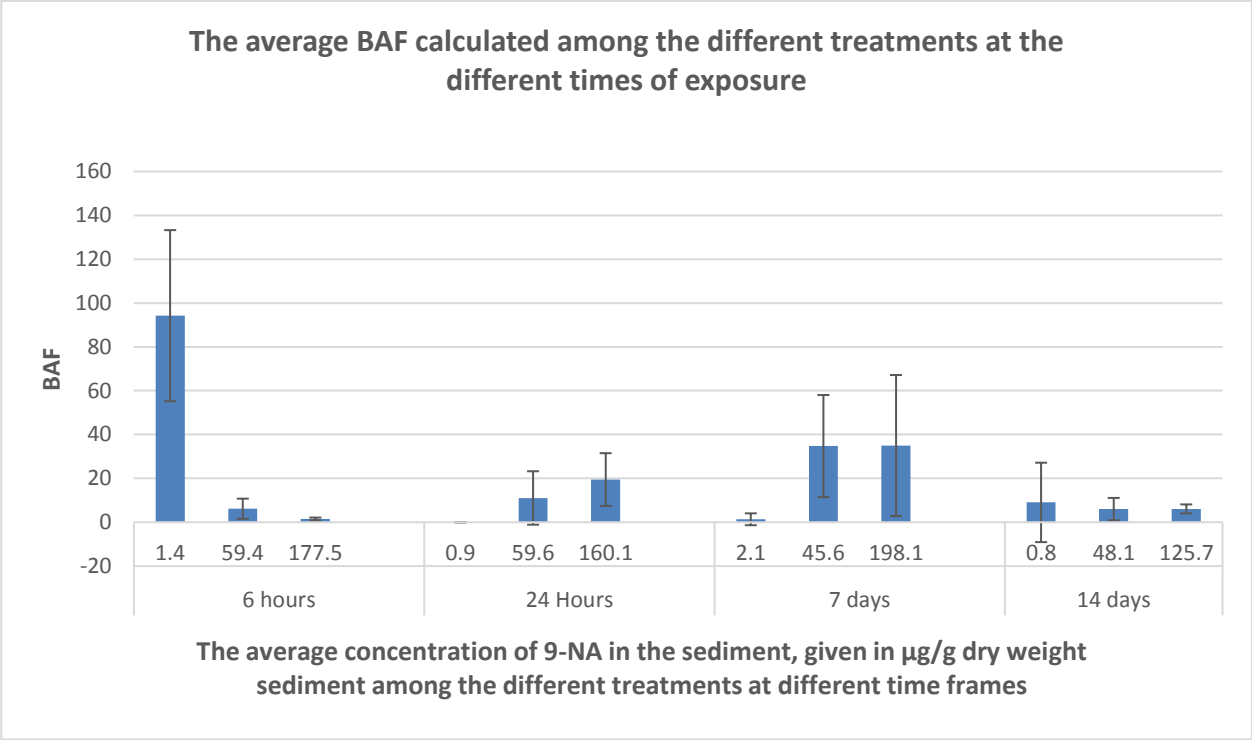


Figure 17. The average BAF in *A. virens* among the different treatments at different time frames, given in ±SDs.

Figure 17 represents the average BAF among the different treatments at the various times of exposure. The graphs representing the BAF among the different treatments at each time frame is present in Appendix B. The BAF is highest in the control treatment at the 6-hour exposure period. The BAF increases with increasing the exposure duration. However, the BAF decreases after 14 days of exposure. This is because the concentration in the gut is less at 14 days of exposure, when compared to 7 days of exposure. A 2- way ANOVA test was used even though a normal distribution was not met. The results indicated that there is an interaction term, which show that the BAF depends on the concentration of 9-NA in the sediment and the exposure duration.

Identification of metabolites using GC/MS

The samples were analyzed using the selected ion monitoring (SIM) mode in order to identify the suspected metabolites formed. The gut samples were examined for these metabolites: 9,10-AQ, 9-NO_2-dihydrodiol, 1,2,3,4-tetrahydrodiol, 9-aminoanthracene and epoxide compounds. The two metabolites found are 9-NO_2-dihydrodiol and 9,10-AQ. The SIM mode was chosen, because it gives a better response in comparison to scanning the samples.

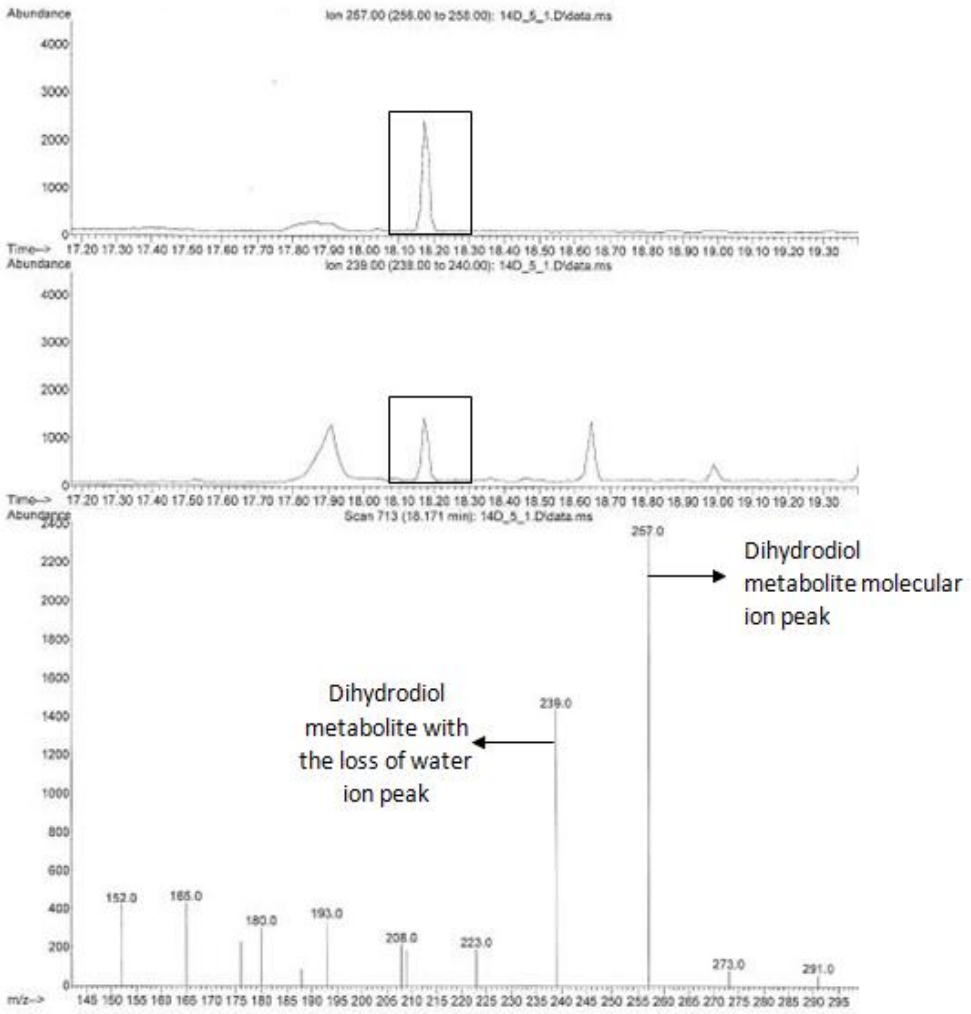


Figure 18. The mass spectrum of the 9-NO_2-dihydrodiol metabolite detected at a retention time of 18.171 minutes for sample [5] replicate 1 at 14 days of exposure, and the highlighted boxes represent the ions of the 9-NO_2-dihydrodiol metabolite that were selected for analysis using the SIM mode.

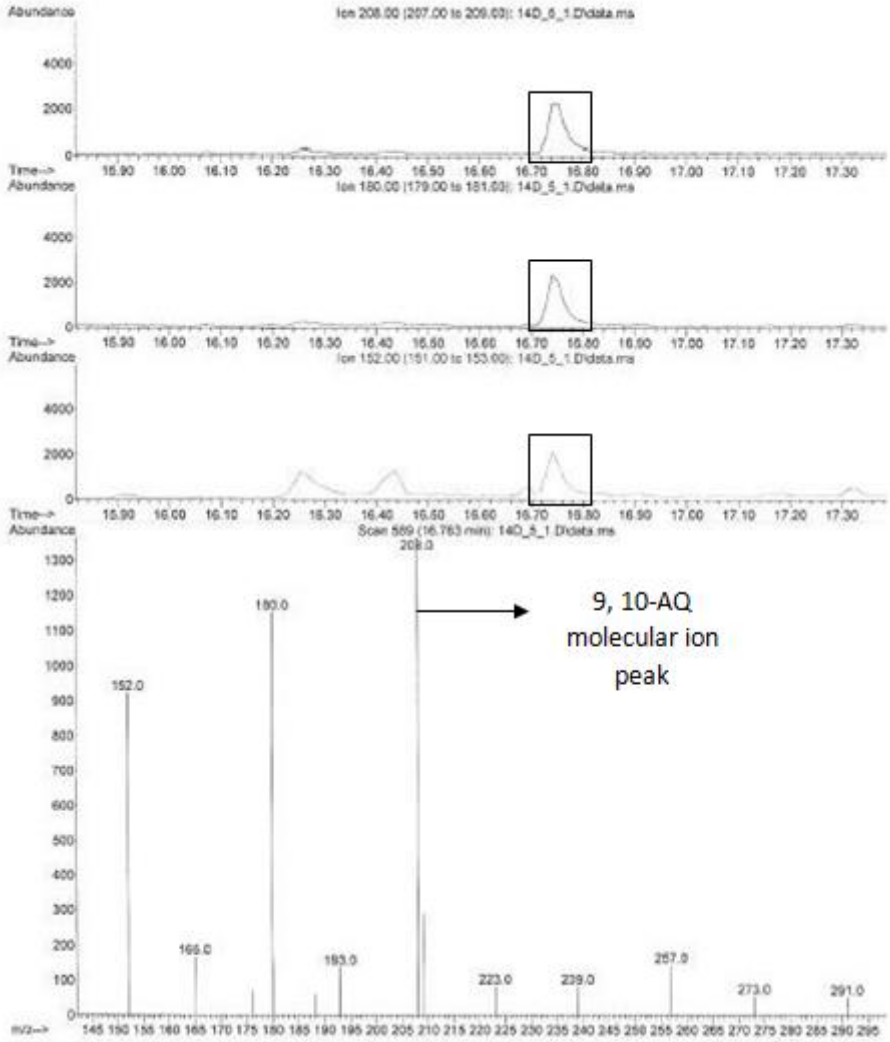
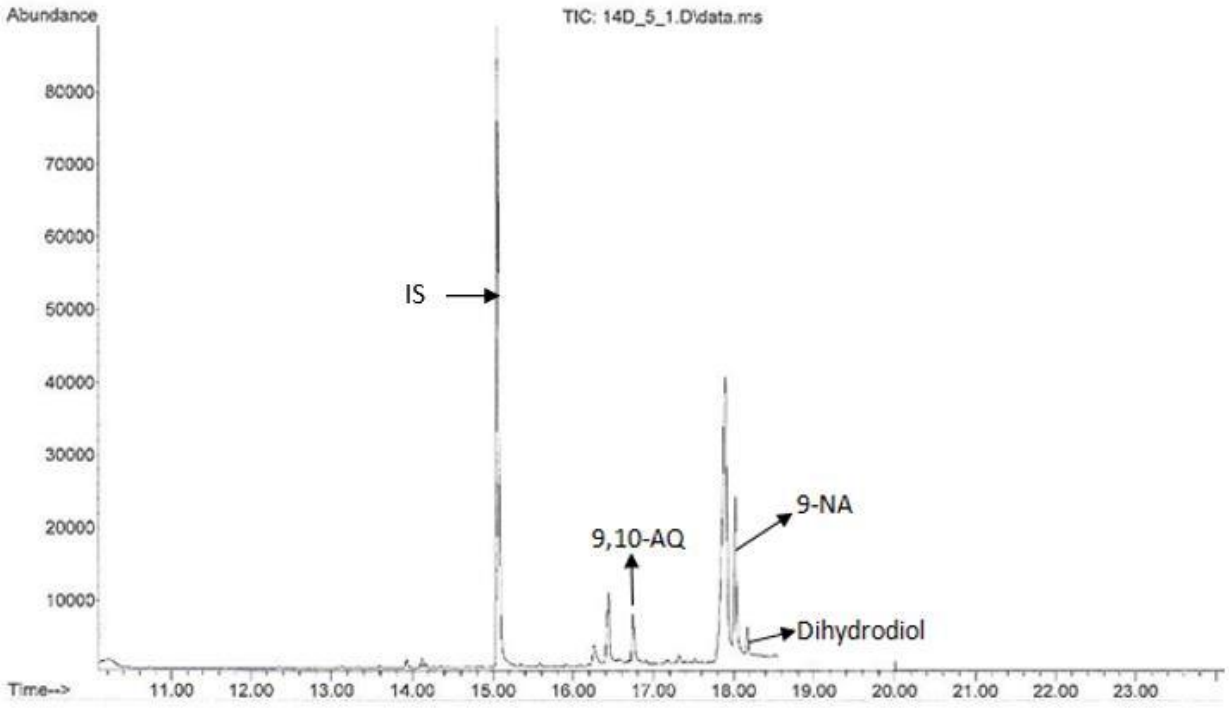
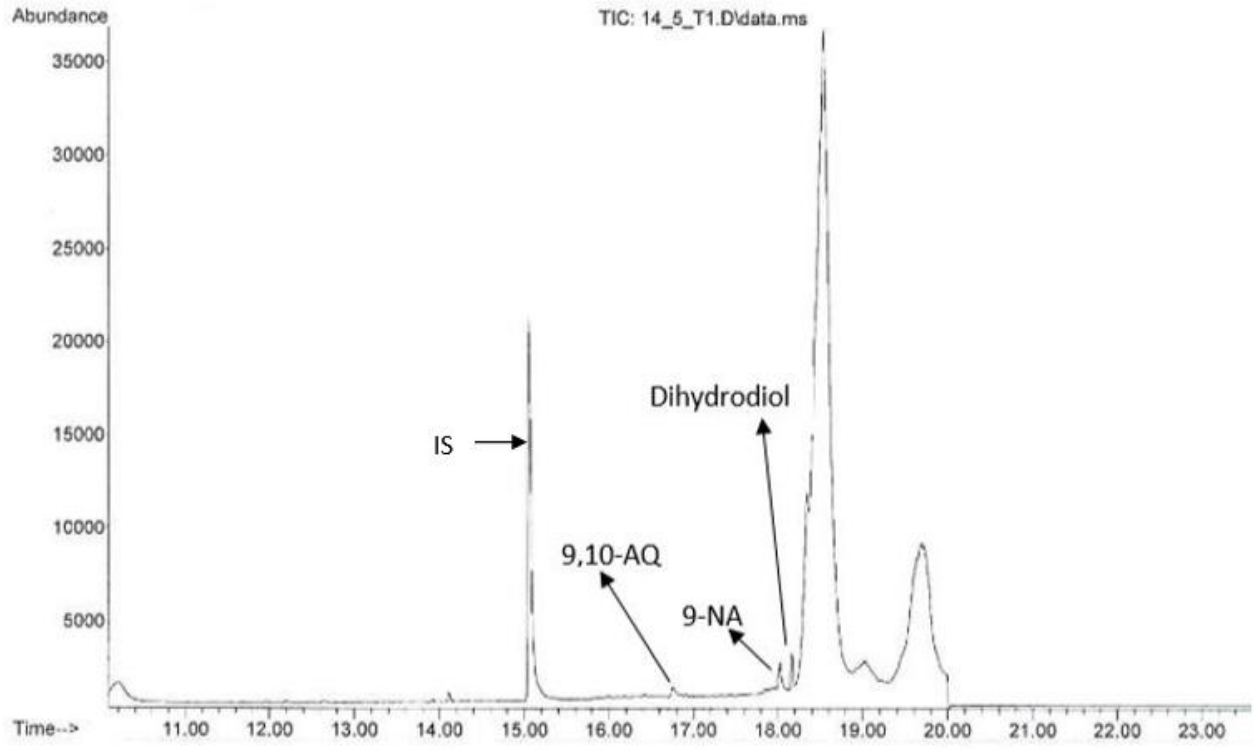


Figure 19. The mass spectrum of 9,10-AQ metabolite detected at a retention time of 16.783 minutes and the highlighted boxes represent the ions of the 9,10-AQ metabolite that were selected for analysis using the SIM mode.

Figure 18 shows the mass spectrum obtained when the gut sample [5] replicate 1, at 14 days of exposure was tested using GC/MS for the presence of the 9-NO_2-dihydrodiol metabolite. Similar peaks can be found in spectras from other samples. The highlighted boxes in figure 18 represent the ions that were selected for analysis using the SIM mode. The molecular ion peak of the 9-NO_2-dihydrodiol metabolite is 257.0 m/z and the other peak, 239 m/z indicates the loss of a water molecule. Figure 19 shows the mass spectrum obtained for the same gut sample, when it was analyzed for the presence of 9,10-AQ. The highlighted boxes in figure 19 represent the ions that were selected for analysis using the SIM mode. The molecular ion peak of the 9,10-AQ metabolite is 208 m/z.



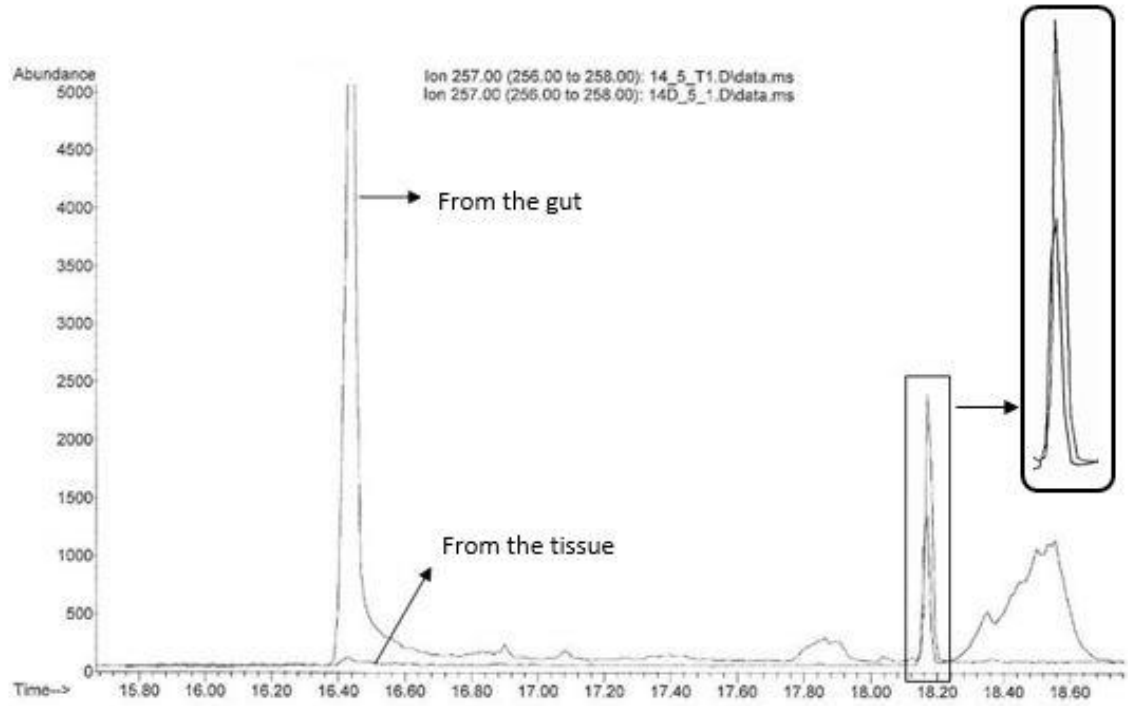
A



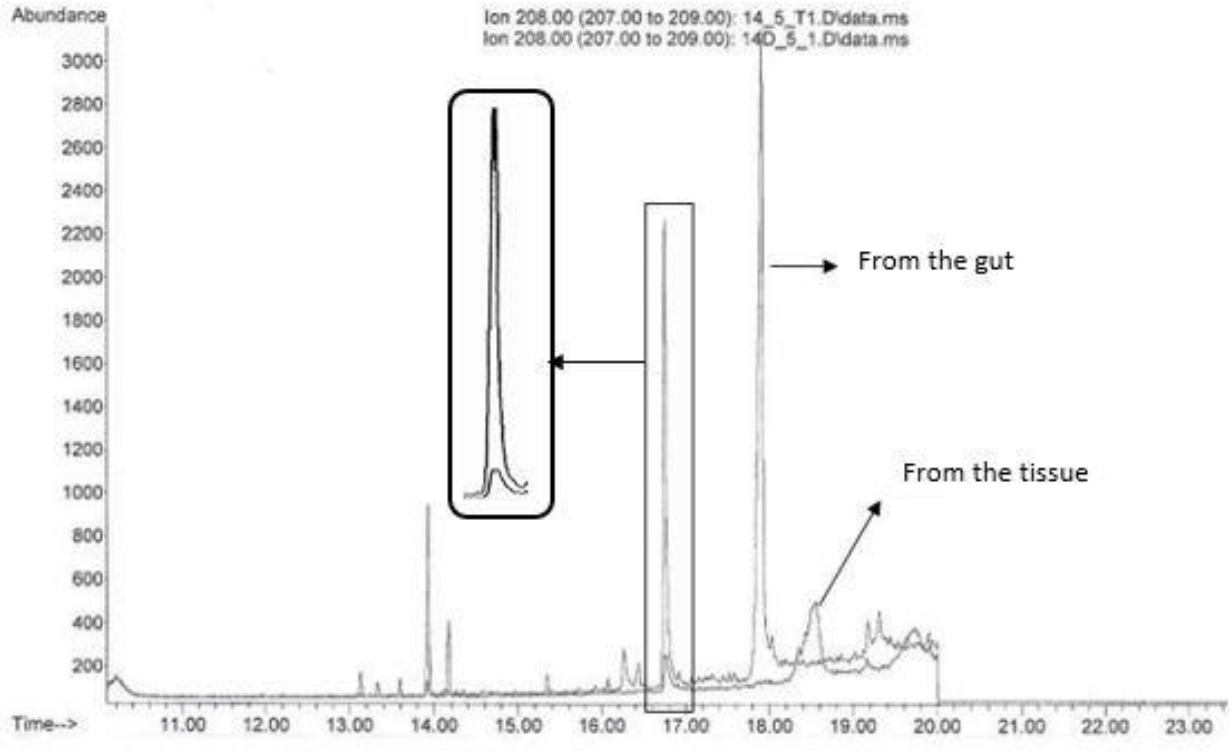
B

Figure 20. The chromatogram obtained for the gut (A) and the tissue (B) for the sample [5] replicate 1 at 14 days of exposure.

Figure 20 represents the chromatogram obtained for the gut and tissue of one of the replicate worms exposed to the [5] concentration at 14 days. The retention time for 9,10-AQ, 9-NA and 9-NO_2-dihydrodiol is 16.733, 18.0 and 18.171 minutes respectively. The internal standard (IS) has a retention time of 15 minutes. All the detected compounds in the gut and tissue samples are labelled in figure 20.



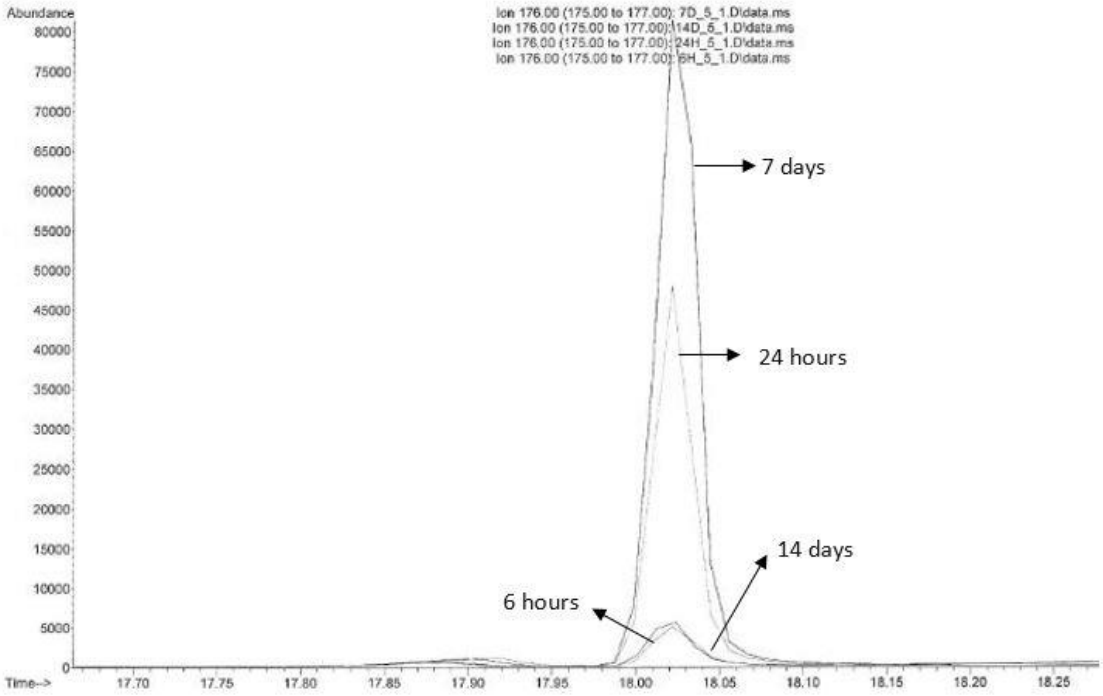
A



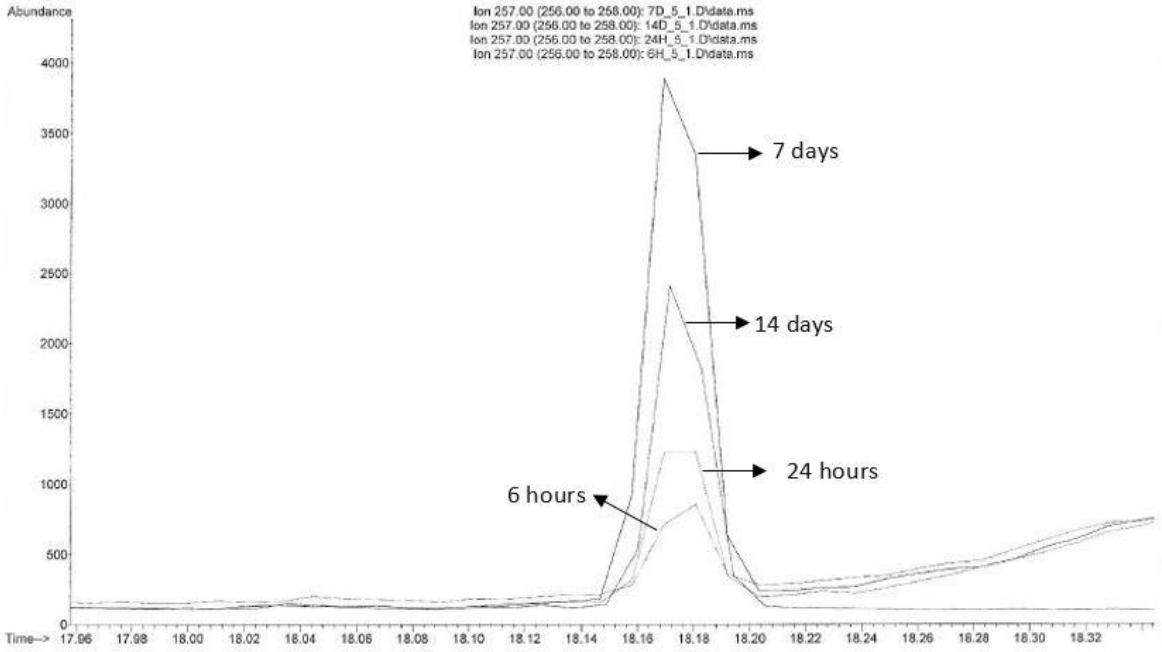
B

Figure 21. Overlay of the ion 257 m/z (9-NO_2-dihydrodiol) chromatogram between the gut and the tissue samples of the [5] worm, at 14 days of exposure (A). Overlay of the ion 208 m/z (9,10-AQ) chromatogram between the gut and the tissue samples of the [5] worm, at 14 days of exposure (B).

Figure 21 (A) shows that the 9-NO_2-dihydrodiol metabolite is present in the gut and remaining tissue of the worms. However, it is apparently more abundant in the gut. The second panel (B) shows that that the presence of the 9,10-AQ metabolite is also more abundant in the gut tissues. However, it is important to keep in mind that the chromatograms are not adjusted for potential differences in weights of gut and remaining tissue.


Abundance
Ion 176.00 (175.00 to 177.00): 7D_5_1.D\data.ms
Ion 176.00 (175.00 to 177.00): 14D_5_1.D\data.ms
Ion 176.00 (175.00 to 177.00): 24H_5_1.D\data.ms
Ion 176.00 (175.00 to 177.00): 6H_5_1.D\data.ms
7 days
24 hours
14 days
6 hours
Time-->

A


Abundance
Ion 257.00 (256.00 to 258.00): 7D_5_1.D\data.ms
Ion 257.00 (256.00 to 258.00): 14D_5_1.D\data.ms
Ion 257.00 (256.00 to 258.00): 24H_5_1.D\data.ms
Ion 257.00 (256.00 to 258.00): 6H_5_1.D\data.ms
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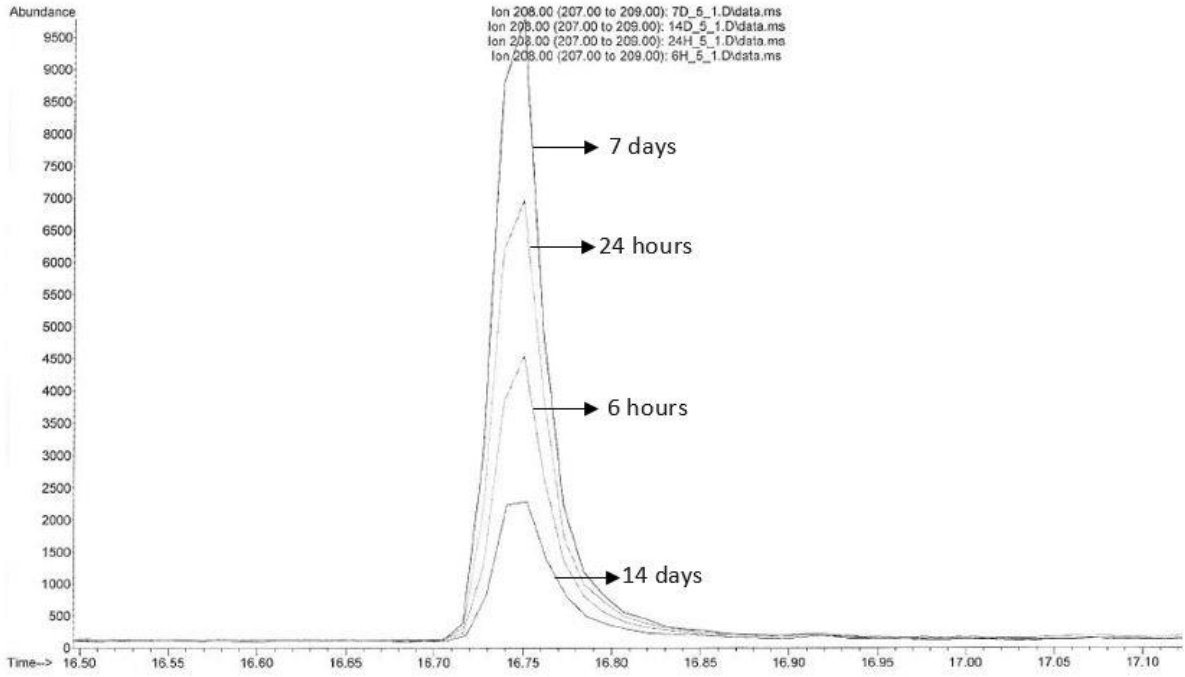
14 days
24 hours
6 hours
Time-->

B



C

Figure 22. Overlay of 9-NA (A), 9-NO_2-dihydrodiol (B) and 9,10-AQ (C) chromatograms between the gut of the [5] replicate 1 worms at every time of exposure.

Figure 22 (A) shows that the concentration of 9-NA in the gut increases with increasing the duration of exposure. However, the highest concentration is found to be at the 7 days of exposure. The concentration of 9-NA decreases when analyzed at the 14 days of exposure. Panels B and C show that the biotransformation of the parent 9-NA, into the 9-NO_2-dihydrodiol and 9,10-AQ metabolites increases with increasing the time of exposure. However, the highest detection of these metabolites is found at the 7 days exposure period. The presence of the 9-NO_2-dihydrodiol metabolite in the gut of the worm at 14 days of exposure, is lower than the presence of this metabolite at the 7- day exposure period. The presence of 9,10-AQ in the gut of the worm at 14 days of exposure, is even lower than the presence of this metabolite at the 6- hour exposure period.

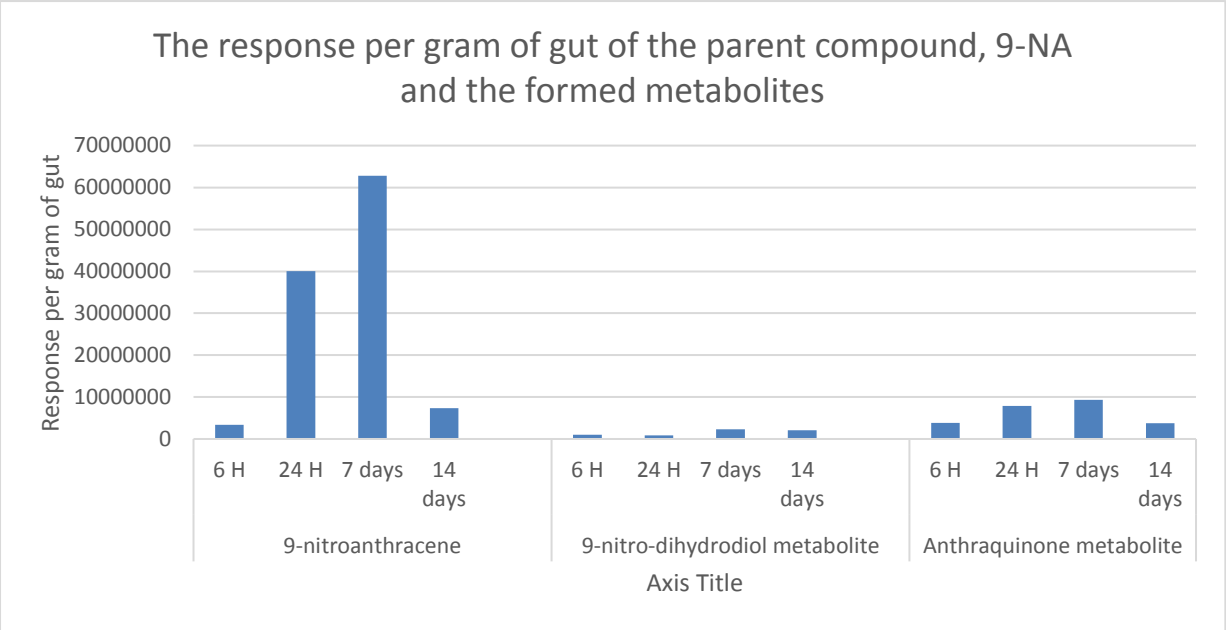


Figure 23. The response of 9-NA, 9-NO_2-dihydrodiol and anthraquinone per gram of gut compared between the [5] replicate 1 treatments at every time of exposure.

Figure 23 represents the measured metabolites in one of the replicates for one of the treatments at all the exposure times. The first replicates of the worms that were exposed to the highest concentration of 9-NA in the sediment were analyzed for the different metabolites formed. The highest treatment was used, since it would be interesting to see the response of the formed metabolites over time in worms exposed to high concentrations. Consistent with what was seen previously, the concentration of 9-NA in the gut increases with increasing the concentration of 9-NA in the sediment. There was a tendency that the concentration of 9-NA in the gut decreases after 14 days of exposure. The response of the different metabolites per gram of gut is shown in figure 23. The response from the 9-NO_2-dihydrodiol and 9,10-AQ metabolites increases with increasing the exposure duration. It is found to be highest at the 7 days period and decreases after the 14 days exposure period. Since only one replicate per time interval was analyzed, this observation is only a trend, and the rest of the replicates should be analyzed to determine whether this is a general pattern, but this is not possible within the time frame of the project.

Results of the rat liver microsome experiment:

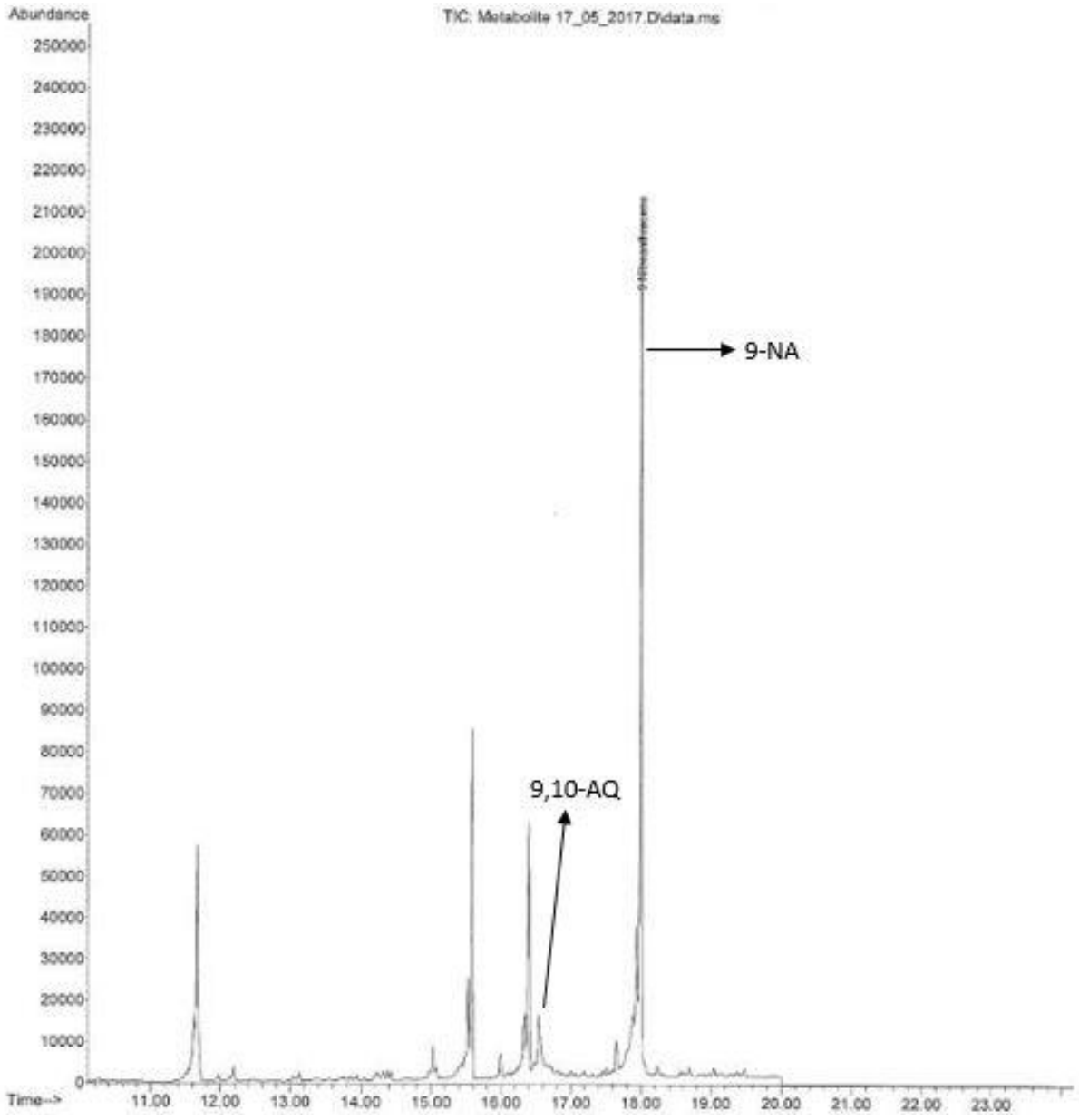


Figure 24. The chromatogram of the rat liver microsomes after the exposure experiment. The chromatogram shows peaks of 9-NA and the metabolite 9,10-AQ.

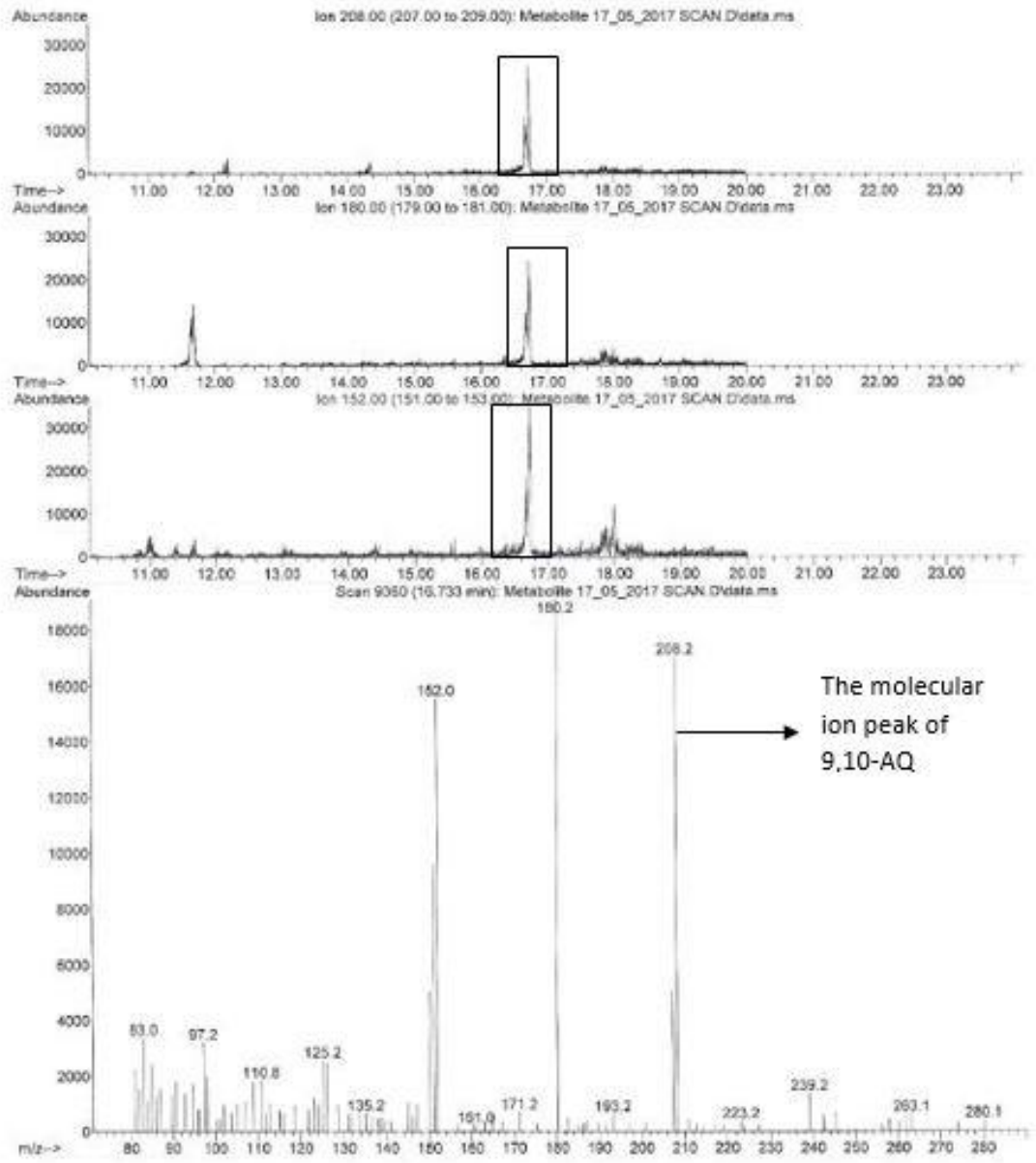


Figure 25. The mass spectrum of 9,10-AQ metabolite detected at a retention time of 16.783 minutes and the highlighted boxes represent the ions of the 9,10-AQ metabolite that were selected for analysis using the SIM mode.

Figure 24 represents the chromatogram after the exposure experiment. The chromatogram shows peaks of 9-NA and the metabolite 9,10-AQ. The peak at a retention time of 18.0 represents 9-NA that was not transformed into metabolites by the rat liver microsomes. The mass spectrum (figure 25) represents the metabolite formed, 9,10-AQ, at a retention time of 16.733 minutes. There were no traces of the other metabolites formed.

DISCUSSION

The sediment was collected in shallow water at the bottom of Isefjorden next to the Munkholmbro, the bridge is located on the southeastern side of Holbæk. The sediment was found to be contaminated with the PAH, 9-NA. The results of the control experiments confirmed the presence of 9-NA in the sediment. I therefore, wanted to reassure this conclusion by taking four samples from the bucket of sediment collected and tested it using GC/MS. The results showed that the average concentration of 9-NA in the sediment is 0.95 µg/g sediment. The reason behind this is that many vehicles pass by the bridge daily, depositing various contaminants such as 9-NA. When the rain falls, these contaminants are washed off the road and sinks to the waters. This then allows the contaminant to bind to the sediment, allowing easier access of these contaminants to the benthic fauna. This confirms that the after-treatments applied to the vehicle exhaust systems are not 100% efficient. This is consistent with the results by Hu and coworkers, since they found out that the after-treatments reduced the emissions of the PAHs, but, did not eliminate the emissions of them completely (Hu et al., 2013). This means that the after-treatments indeed reduce the emissions of PAHs, but fail to completely eradicate them. It has been found that some of the DPF actually produce 9-NA. In addition, the emissions of some nitro-PAHs increased after the use of the catalytic DPF (Heeb et al., 2008)

The synthesis of 9-NA resulted in the formation of 9,10-AQ as a side product. However, the re-crystallization of 9-NA using glacial acetic acid formed a pure 9-NA. This is supported by the NMR spectrums that show how the peaks from the 9,10-AQ contaminant gradually decrease until they completely disappear. The purity of the synthesized 9-NA is also supported by the GC/MS results that show how the mass spectrum of the synthesized 9-NA is consistent to the mass spectrum obtained from the library database.

The measured concentrations in the spiked sediment, determined by GC/MS, did not match the nominal concentrations. This could be related to the spiking procedure, for example that too little of the compound that was spiked onto the glass surface actually moved into the sediment. Previous studies have showed successful results in the spiking method for PAHs (Palmqvist et al., 2006), however, it seems that the spiking method does not work well for 9-NA.

Despite this, a general trend was seen in the screening experiment that illustrates that increasing the concentration of the contaminant in the sediment, results in an increase in the concentration of 9-NA in the guts of the worms. Therefore, the second exposure experiment was spiked with only two high concentrations of 9-NA: 2232.3 µg/g dry weight sediment [5], 223.23 µg/g dry weight sediment [4] and a control. These were chosen because the statistical results showed that there is no significant difference between the concentration of 9-NA in the gut of the worms between the control, [1], [2] and [3] treatments in the first exposure experiment. However, there was a significant difference in the results between the control and the [4] and [5] treatments. Therefore, this is the reason why only two high concentrations were chosen for the second exposure experiment.

The worms were efficient at accumulating 9-NA from the sediment, and were able to biotransform this compound within as early as 6 hours of exposure. This confirms the findings by previous studies, such as McElroy (1990), that the polychaete, *A. virens* is efficient at metabolizing PAHs. The first exposure experiment was conducted over an 18-day period. The results showed an increase in the concentration of 9-NA in the gut of the worms with increasing the concentration of the contaminant in the sediment. The bioaccumulation factor, however, showed a decrease in value with increasing the concentration of 9-NA in the sediment. One likely reason behind this is that 9-NA was biotransformed, which results in lower accumulation in the organisms. The major assumption for calculating a BAF is that the system is in steady state, when the uptake of 9-NA over time had stabilized, however, it was not certain the worms in my exposure experiments reached the steady state.

The second exposure experiment was performed to examine the temporal differences in bioaccumulation of 9-NA by the worms. The results showed that the concentration of 9-NA in the gut increased with increasing the duration of exposure. The highest concentration in the gut of the worm is observed at the 7- day exposure period. However, at 14 days of exposure, the concentration of 9-NA in the guts of the worms decreased significantly. One likely reason behind this is that the worms become better at metabolizing and transforming 9-NA to the different metabolites. Therefore, more of the 9-NA is metabolized resulting in the formation of water soluble metabolites, and subsequently excretion of these.

Another possible explanation is that the worms become dissatisfied and fed up with the taste of the sediment having 9-NA. The worms have sensed the presence of the yellow contaminant and

disliked it after a period of continuous exposure to the same sediment. Therefore, the worms might have decreased their intake of the sediment, after a period of time. However, if this is true, then the greatest loss in weight will be between 7 and 14 days of exposure. The results show that the percent degrowth in the weight of the worms exposed to the highest concentration at 7 days and 14 days is almost the same. This means that this explanation does not explain why the concentration of 9-NA in the gut at 14 days is lower than at 7 days of exposure. Overall, the worms decreased in body weight.

The rat liver microsome experiment was performed to produce the possible metabolites. However, only 9,10-AQ was detected as the metabolite formed, whereas the experiment by Fu et al., 1985, found more metabolites. Despite the fact, that the other metabolites were not produced by the rat liver microsome experiment performed, the extraction of the gut and the tissues of the worms using acetone and ethyl acetate resulted in the detection of the metabolites in the worms. Even though I did not find any 9-NO_2-dihydrodiols with the rat liver microsome experiment, I did so with the new extractions of the worm tissues.

The gut and tissues of the worms in the first exposure experiment were extracted using acetonitrile. GC/MS was used to analyze the samples and detect the presence of different possible metabolites formed. Whereas Fu and coworkers (1985), detected several different metabolites in a rat liver microsome experiment, 9,10-AQ was the only metabolite detected in the worm tissues in the first of the present exposure experiments. A possible explanation could be that the extraction with acetonitrile was insufficient to extract the more polar metabolites seen in the rat liver microsome experiment.

The guts and the tissues of the second exposure experiment were extracted using acetone and ethyl acetate. This combination of extracting solvents was used, because the rat liver microsomes were extracted using this method. Therefore, this was the optimal method to extract the suspected metabolites formed. The GC/MS results of the gut and tissue of the worm that was exposed to the highest concentration of 9-NA for a period of 14 days, showed that 9-NO_2-dihydrodiol and 9,10-AQ are the metabolites formed. The worms therefore, followed a similar path to the rat microsomes in metabolizing 9-NA. A probable guess would be that the CYP enzyme metabolizes 9-NA to form the intermediates 1, 2 and 3, 4 epoxides. The further metabolism of the epoxides by epoxide hydrolase forms the 1, 2 and 3, 4 dihydrodiols. It was not possible to determine whether the trans-3, 4-dihydrodiol or the trans-1, 2-dihydrodiol was the 9-NO_2-dihydrodiol that was formed. However, it

is expected that both were produced. The experiment by Fu et al, 1985, confirmed the metabolism of 9-NA formed the trans-3, 4-dihydrodiol in a larger percentage than the trans-1, 2-dihydrodiol. The results by Fu et al., 1985, confirms that the nitro group at position 9 does not inhibit the formation of the 1,2-dihydrodiol that is peri to the nitro group. This could indicate that there may be some steric inhibition by the nitro group, although the metabolite can still be formed. This is consistent to the findings by Fu and Yang which showed that rat liver microsomes metabolized 7-nitrobenz(a)anthracene (7-NO_2-BA) to form the corresponding 7-NO_2-BA trans-8,9-dihydroiol. The other metabolite formed is the 7-NO_2-3, 4-dihydrodiol, which is peri to the nitro group located on the seventh position. This confirms that the nitro group does not always inhibit the oxidation at the peri position (Fu and Yang, 1983).

The possible metabolite, 9-aminoanthracene was not found to be present in the guts or tissues of the worms. This is consistent with the rat liver microsome experiment (Fu et al., 1985). The lack of 9-aminoanthracene indicates that the reduction 9-NA to form the active intermediate, N-hydroxyaminoanthracene pathway was not activated.

The overlaying chromatograms of the molecular ion 208 m/z of 9,10-AQ show that the metabolites are present in a larger degree in the guts of the worm, compared to the tissues of the worms. In addition, the overlaying chromatogram of the molecular ion of 257 m/z of 9-NO_2-dihydrodiol confirms that the metabolites are more abundant in the guts of the worms. This confirms the findings of Lee and Singer, 1980, that the CYP enzyme is present to a larger extent in the gut tissue. The uptake is higher in the gut compared to the remaining tissue, since the gut is what meets most of the sediment associated contaminants, and therefore the metabolites are also more abundant. Therefore, 9-NA is biotransformed to a different extent in the two tissues.

The 9-NO_2-dihydrodiol metabolite is found to be more present in the 7 days exposure period, compared to the 14 days exposure period. This suggests that the worms either excrete more of the metabolite at the 14 days of exposure or the worms stop feeding after a specific period of time. This explains why the uptake of 9-NA is less at 14 days compared to 7 days of exposure.

The overlaying chromatogram of the 208 m/z (9,10-AQ) ion shows that the highest abundance of this metabolite is found at the 7 days exposure period, followed by the 24 hours exposure period. 9,10-AQ is found to be least abundant in the gut of the worm at 14 days exposure period. The concentration of 9-NA in at the 14 days exposure period is lower than at the 7 days exposure period.

This shows that as the concentration of the parent 9-NA decreases, more of the 9-NO_2-dihydrodiol and anthraquinone metabolites are formed.

The worms were able to assimilate 9-NA from the sediment, subsequently biotransforming it to different metabolites. The screening experiment showed that 9,10-AQ was the only metabolite formed, however, the extraction with the acetone and ethyl acetate suggested by, Fu et al., 1985, was successful in detecting the 9-NO_2-dihydrodiol metabolite.

CONCLUSION

The overall aim of the study was to estimate the BAF in the polychaete, *A. virens* and to identify the metabolites formed as a result of the uptake of 9-NA by the worms. The experiments performed were successful in illustrating a trend in the bioaccumulation of the contaminant, with increasing the concentration of 9-NA in the sediment. The first exposure experiment was a screening experiment where no other metabolites apart from 9,10-AQ were detected. However, the second exposure experiment was performed over a time scale and was indeed successful in identifying a 9-NO_2-dihydrodiol metabolite and 9,10-AQ. The polychaete was able to biotransform 9-NA as early as 6 hours after exposure to the contaminant. This paper produces consistent results to previous studies, as it illustrates the efficiency of *A. virens* in metabolizing different contaminants. This study showed that 9-NA is emitted from vehicle exhaust systems and is found in the sediment of aquatic environments, which is an interesting discovery. There were some limitations when analyzing the chromatograms of the guts and tissues of the worms. In the first exposure experiment, three peaks were observed at retention times 16.419, 16.585 and 16.756 minutes (9,10-AQ) respectively. At the beginning, it was thought that these peaks are probably the metabolites formed. However, the response from the peaks at 16.419 and 16.585 minutes do not respond to changes in the concentration of 9-NA. Therefore, since the gut and tissue samples were not cleaned prior to the extraction step, many peaks were observed. These peaks were not part of the metabolic conversion of 9-NA. However, they were gut components consisting of DNA, fatty acids and other things. It is therefore recommended to clean the samples prior to the extraction, if someone wants to repeat the experiment and improve the outcome of the results. This will subsequently produce better peaks removing the noise and allowing easier detection of the different metabolites.

Further work could be performed in the future, by analyzing the sediment after exposure to the contaminant. This will be interesting as it allows us to measure the rate of disappearance of 9-NA in the sediment along time. The sediment after exposure could be analyzed to detect the presence of the 9-NO_2-dihydrodiol and 9,10-AQ metabolite.

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Appendix A

Initial, final and % degrowth of the worms in the screening experiment

Table 1. Weight of the worms before the exposure experiment

	Replicate 1	Replicate2	Replicate 3	Replicate 4
Control	3.0695 grams	6.3701 grams	7.1337 grams	5.8818 grams
Concentration 1	6.2271 grams	5.2763 grams	8.7469 grams	1.7133 grams
Concentration 2	4.0205 grams	1.8766 grams	4.0118 grams	8.0480 grams
Concentration 3	7.8989 grams	5.3917 grams	6.2651 grams	2.1905 grams
Concentration 4	6.4636 grams	5.8621 grams	6.52593	3.7096 grams
Concentration 5	9.0121 grams	5.0370 grams	3.6019 grams	5.6672 grams

Table 2. Weight of the worms at the end of the exposure experiment.

(-) indicates that the worm died.

	Replicate 1	Replicate2	Replicate 3	Replicate 4
Control	2.4 grams	5.9 grams	-	4.4 grams
Concentration 1	4.8 grams	3.7 grams	-	1.6 grams
Concentration 2	2.8 grams	1.3 grams	3.7 grams	5.5 grams
Concentration 3	6.0 grams	3.8 grams	4.6 grams	1.6 grams
Concentration 4	5.2 grams	4.8 grams	5.8 grams	3.1 grams
Concentration 5	6.3 grams	3.5 grams	2.7 grams	4.2 grams

The weights of the worms decreased upon the end of the experiment. The percentage of de-growth is calculated upon the following equation:

$$\%\, De - growth = \left(\frac{Initial\ wt\ of\ worm - final\ wt\ of\ worm}{Initial\ wt\ of\ worm}\right) \times 100$$

Table 3. Percent de-growth among the different worms at the end of the exposure experiment.

	Replicate 1	Replicate2	Replicate 3	Replicate 4
Control	22.6 %	7.8 %	-	25.4 %
Concentration 1	22.6 %	30.2 %	-	5.6 %
Concentration 2	30.0 %	31.6 %	7.5 %	31.25 %
Concentration 3	24.1 %	30.0 %	27.0 %	27.3 %
Concentration 4	20 %	18.6 %	10.8 %	16.2 %
Concentration 5	30 %	30 %	25 %	26.3 %

Initial, final and % degrowth of the worms in the second exposure experiment

Table 4. The initial weight of the worms at the 6H exposure period:

Treatments	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Control	6.773	3.574	4.481	3.412
223.23 µg/g	7.839	5.537	2.517	3.499
2232.3 µg/g	6.421	5.941	3.439	3.173

Table 5. The initial weight of the worms at the 24H exposure period. (-) indicates that the worm died.

Treatments	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Control	3.211	4.7096	5.587	-
223.23 µg/g	2.717	5.345	4.495	7.543
2232.3 µg/g	2.32	4.178	1.88	8.383

Table 6. The final weight of the worms at the end of the 24H exposure period. (-) indicates no worm.

Treatments	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Control	2.947	4.307	5.215	-
223.23 µg/g	2.717	4.66	4.742	6.945
2232.3 µg/g	2.021	4.105	1.86	7.747

Table 7. Percentage de-growth among the different worms at the end of the 24 H exposure period.

Treatments	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Control	8.22%	8.55%	6.66%	-
223.23 µg/g	0%	12.82%	5.49%	7.93%
2232.3 µg/g	12.89%	1.75%	1.06%	7.59%

Table 8. The initial weight of the worms at the 7 days exposure period:

Treatments	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Control	3.951	4.331	2.351	3.899
223.23 µg/g	3.164	4.824	2.224	3.805
2232.3 µg/g	3.582	4.666	2.582	5.831

Table 9. The final weight of the worms at the end of the 7 days exposure period:

Treatments	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Control	3.569	4.11	1.787	3.015
223.23 µg/g	2.695	4.471	1.554	3.175
2232.3 µg/g	2.962	3.704	2.276	4.487

Table 10. Percentage de-growth among the different worms at the end of the 7 days exposure period.

Treatments	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Control	9.67%	5.10%	23.99%	22.67%
223.23 µg/g	14.82%	7.32%	30.13%	16.56%
2232.3 µg/g	17.31%	20.62%	11.85%	16.87%

Table 11. The initial weight of the worms at the 14 days exposure period.

Treatments	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Control	2.846	3.602	4.406	3.416
223.23 µg/g	2.533	4.594	3.149	3.609
2232.3 µg/g	3.353	5.131	4.678	3.863

Table 12. The final weight of the worm at the end of the 14 days exposure period.

Treatments	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Control	2.231	3.100	3.695	2.525
223.23 µg/g	1.866	3.617	2.338	3.215
2232.3 µg/g	2.713	4.409	-	4.409

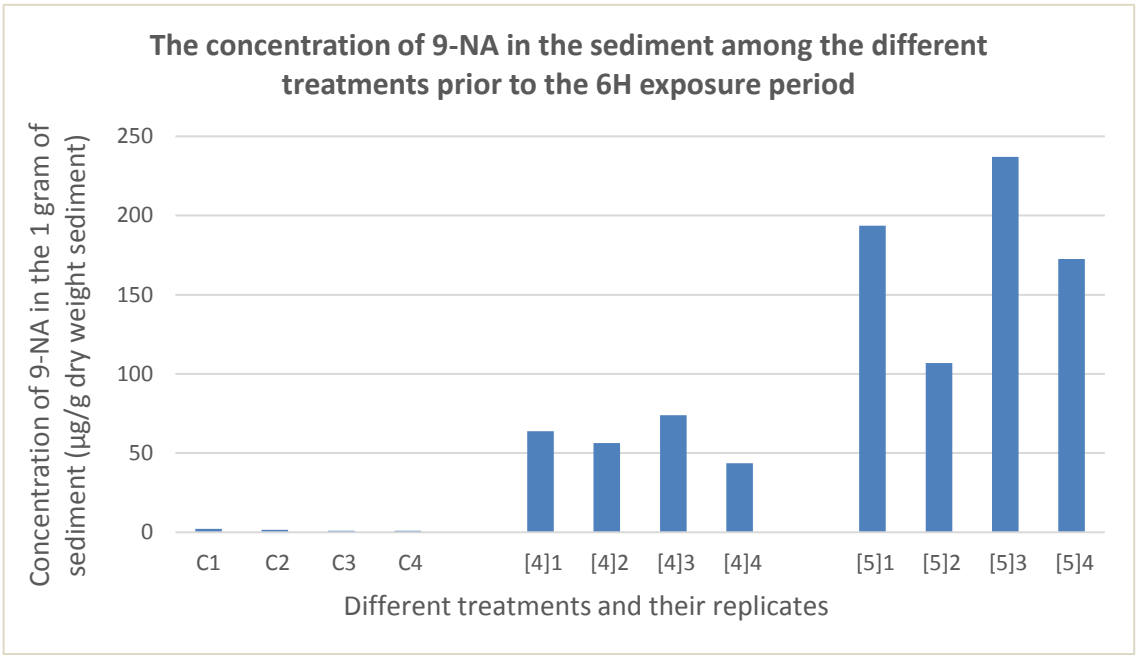
Table 13. Percentage de-growth among the different worms at the end of the 14 days exposure period. (-) represents the worm died.

Treatments	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Control	21.61%	13.94%	16.14%	26.08%
223.23 µg/g	26.33%	21.27%	25.75%	10.92%
2232.3 µg/g	19.09%	14.07%	-	16.65%

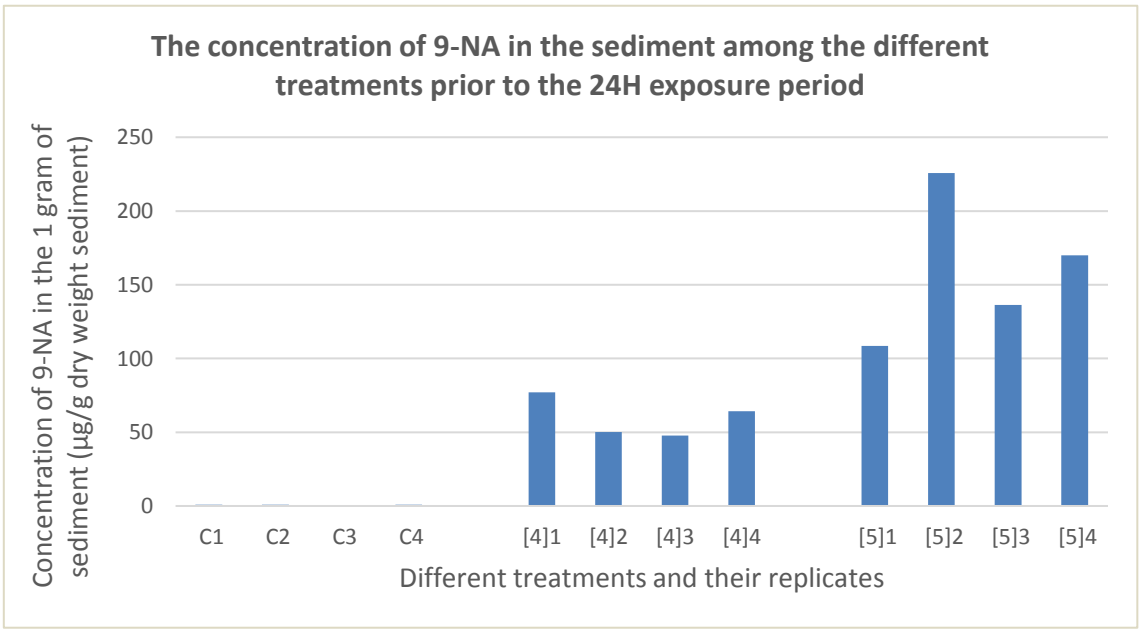
Appendix B

Experiment 2:

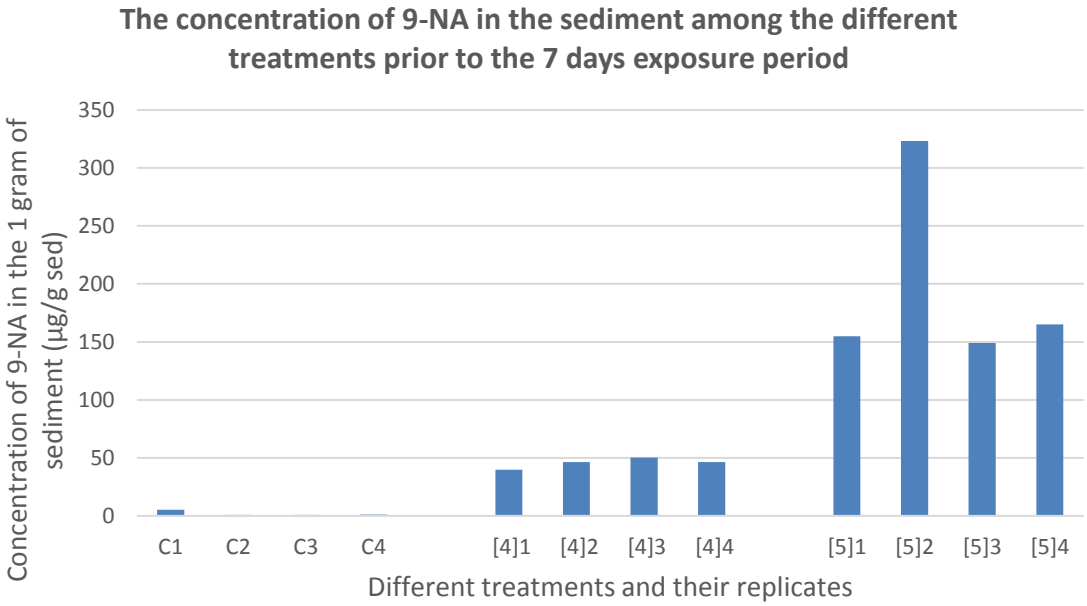
Analysis of 9-NA in the sediment before exposure experiment



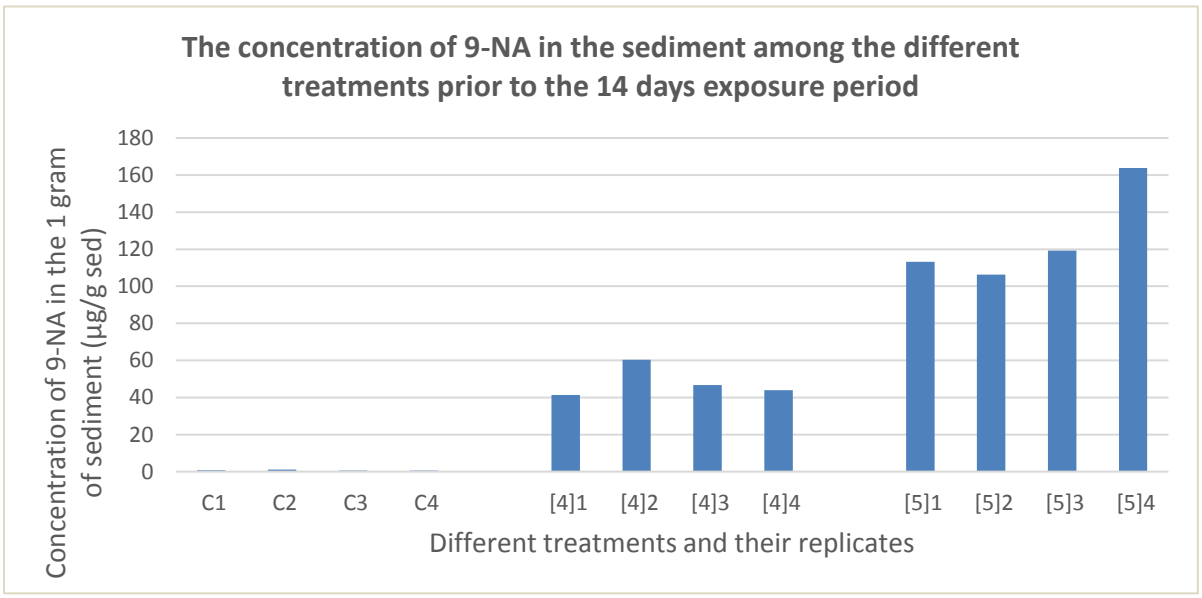
A



B



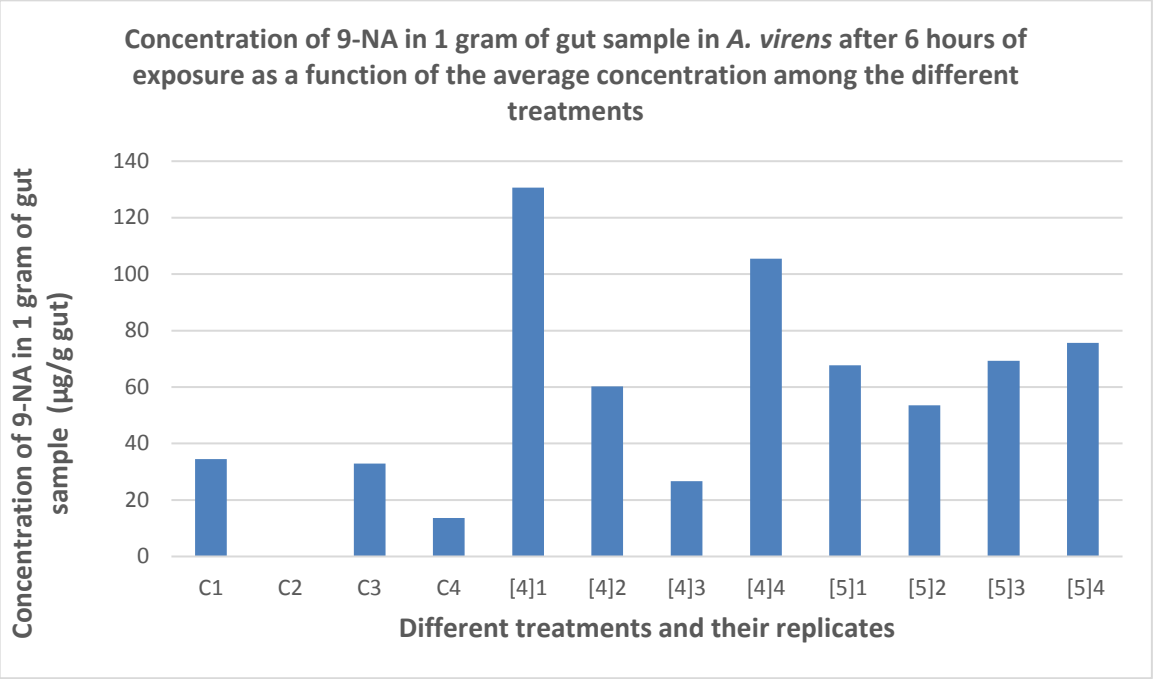
C



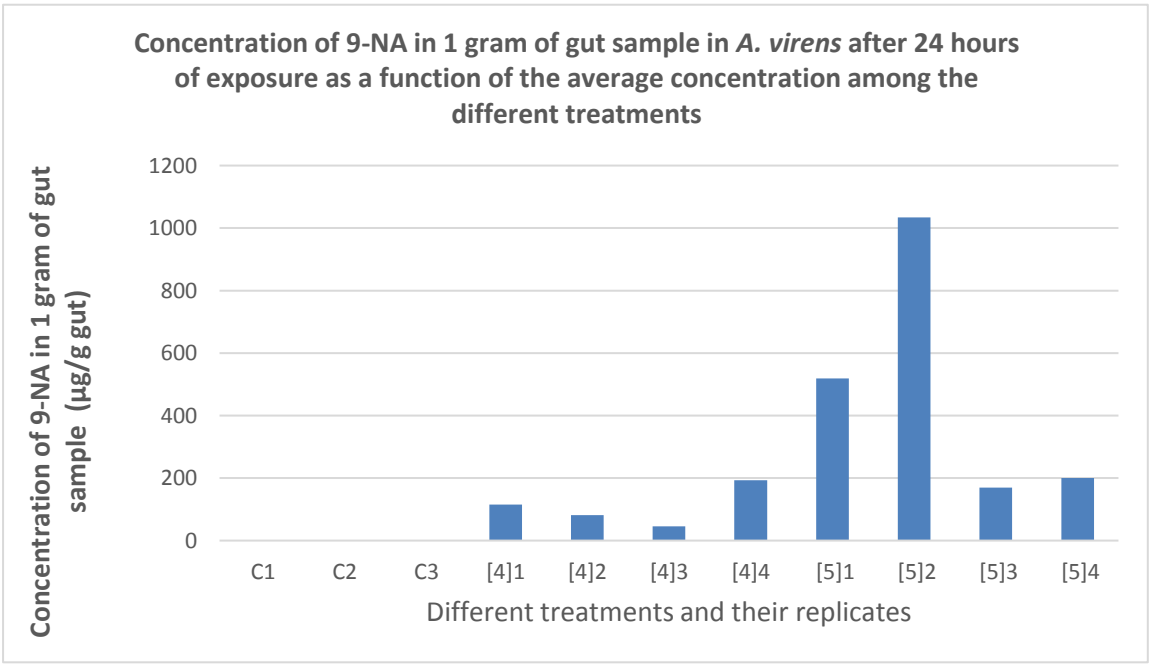
D

Figure 1. The concentration of 9-NA in the sediment before exposure among the different treatments at 6 hours (A), 24 hours (B), 7 days (C) and 14 days (D) given in µg/g dry weight sediment. C represents the control, whereas [4] and [5] represent the concentration of the spiked 9-NA sediment in increasing order. The number next to the concentration represents the different replicates.

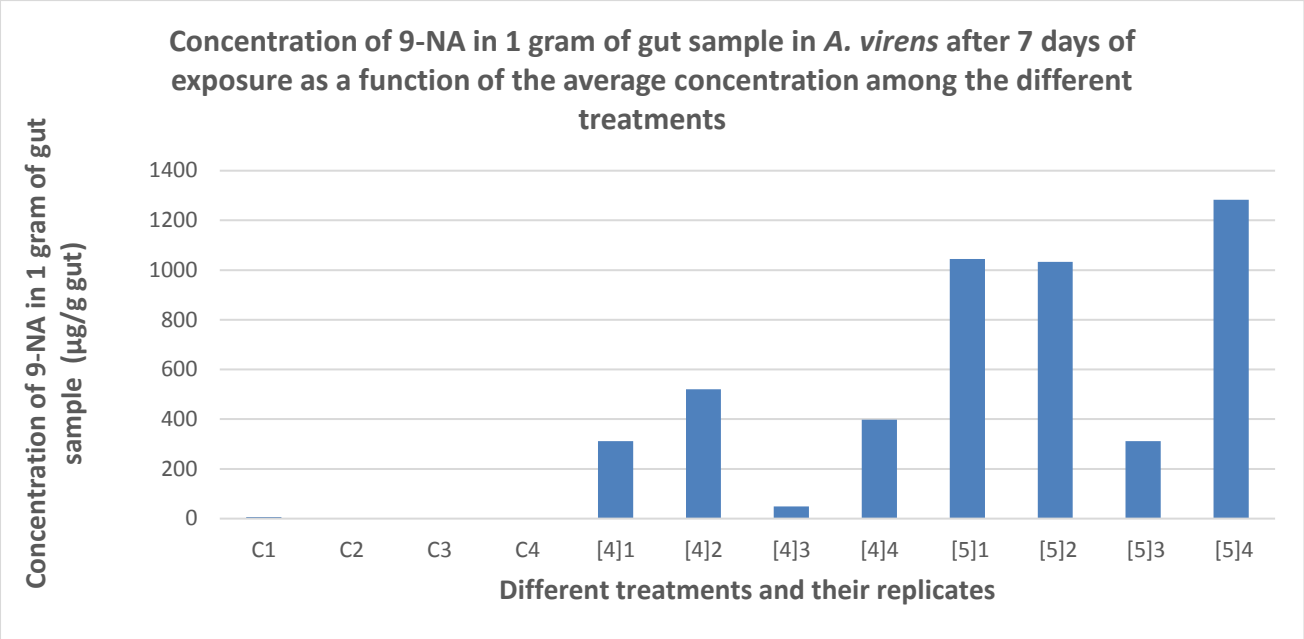
Analysis of 9-NA in the gut content of *A. virens* at the times of exposure



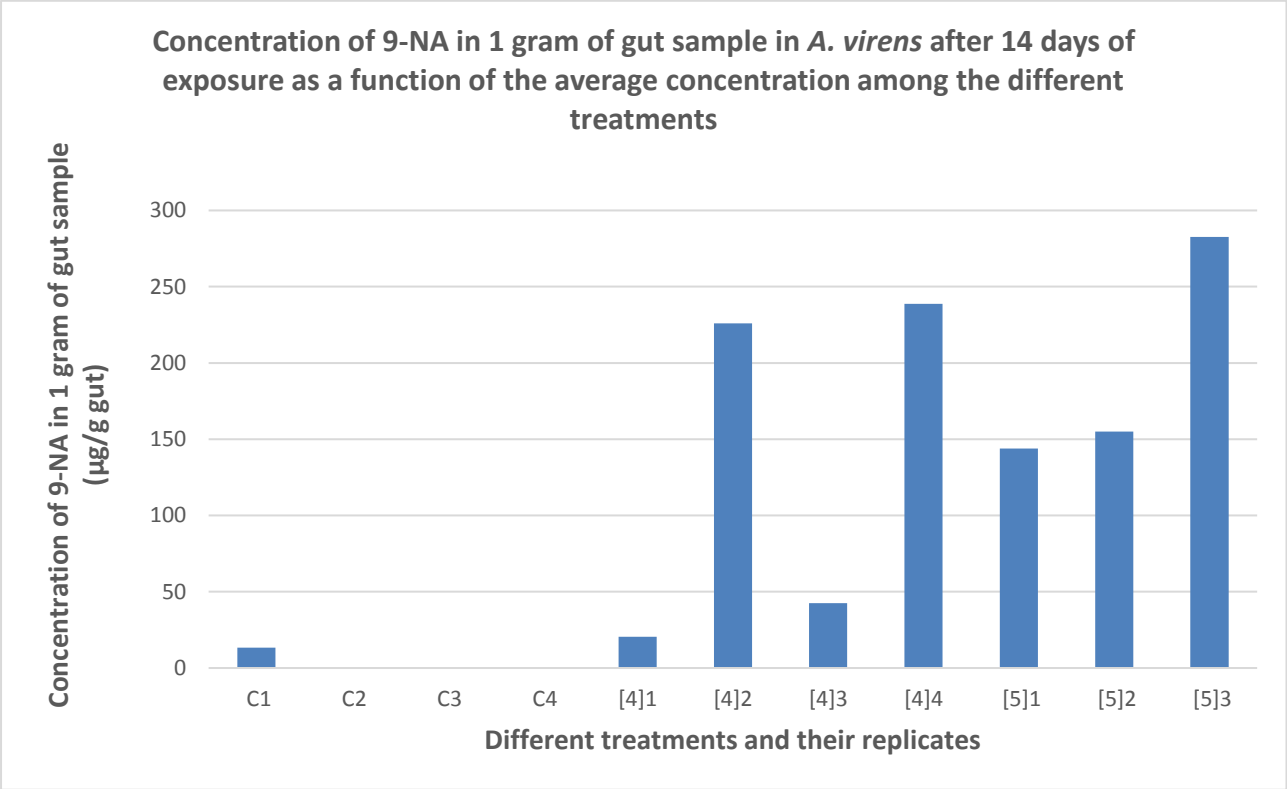
A



B



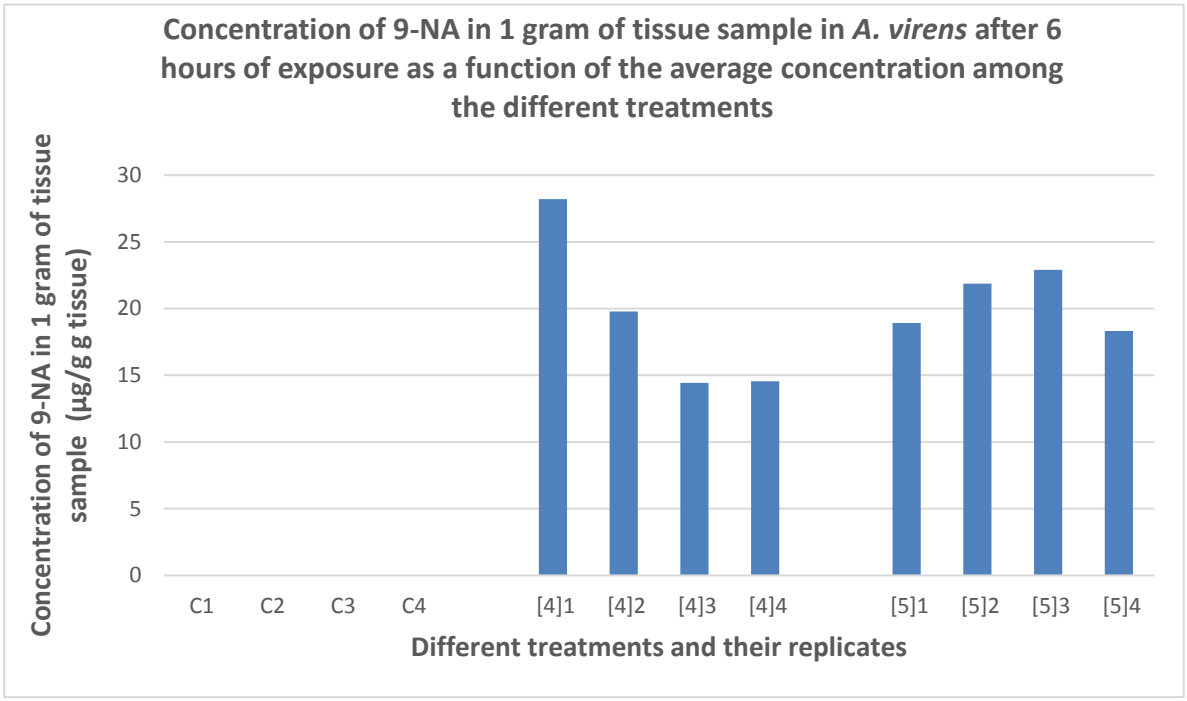
C



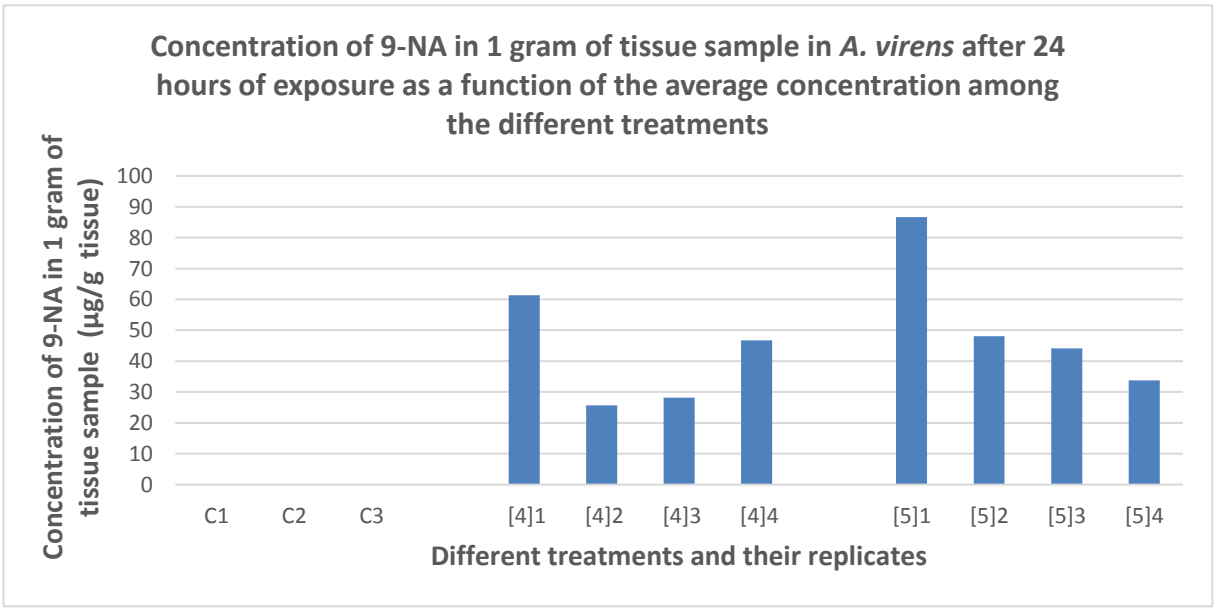
D

Figure 2. The concentration of 9-NA in 1 gram of gut sample in *A. virens* after 6 hours of exposure (A), 24 hours of exposure (B), 7 days of exposure (C) and 14 days of exposure (D) as a function of the different treatments, given in µg/g gut. C represents the control, whereas [4] and [5] represent the concentration of the spiked 9-NA sediment in increasing order. The number next to the concentrations represent the different replicates.

Analysis of 9-NA in the tissues of *A. virens* at the times of exposure



A



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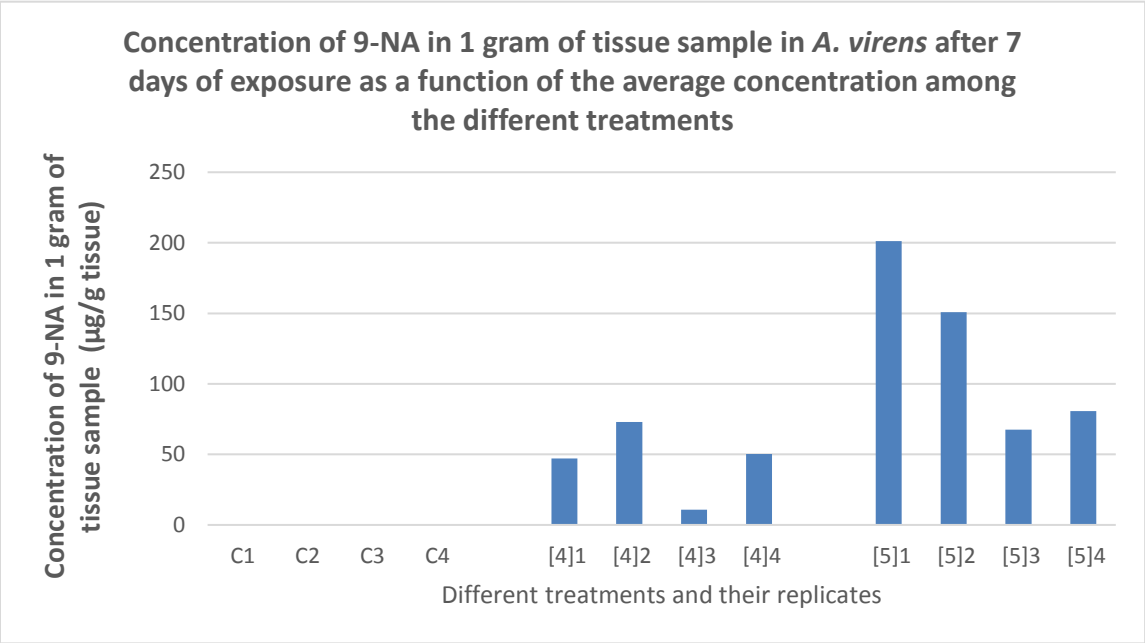

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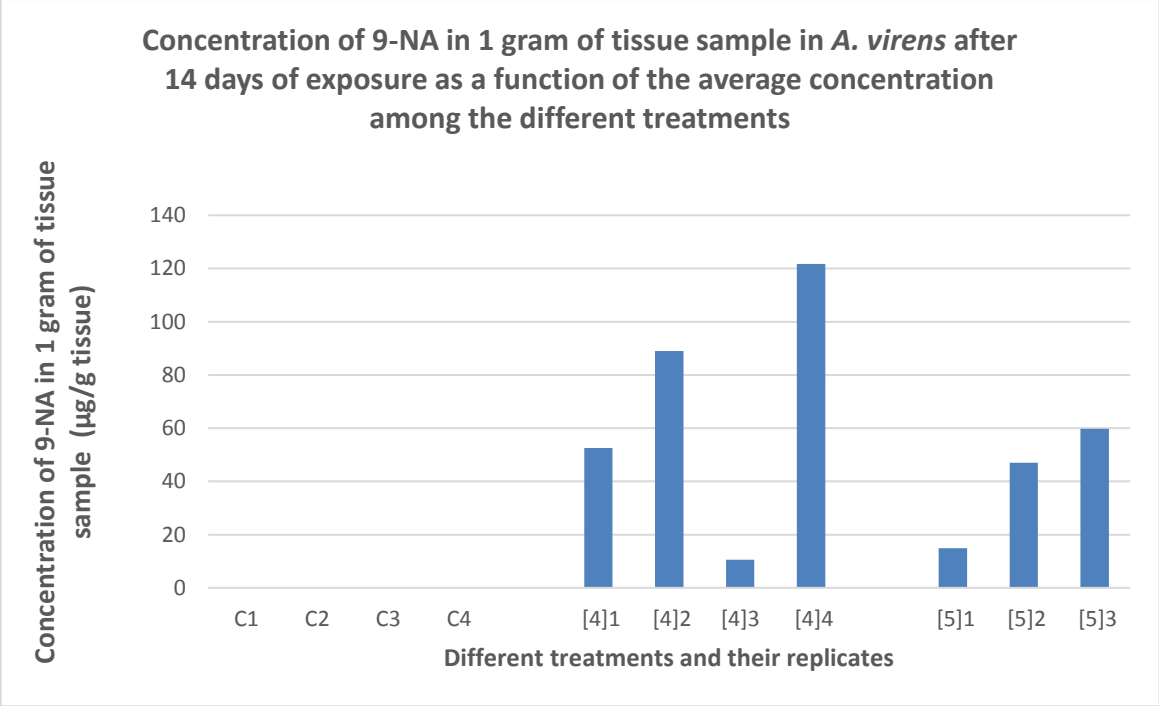
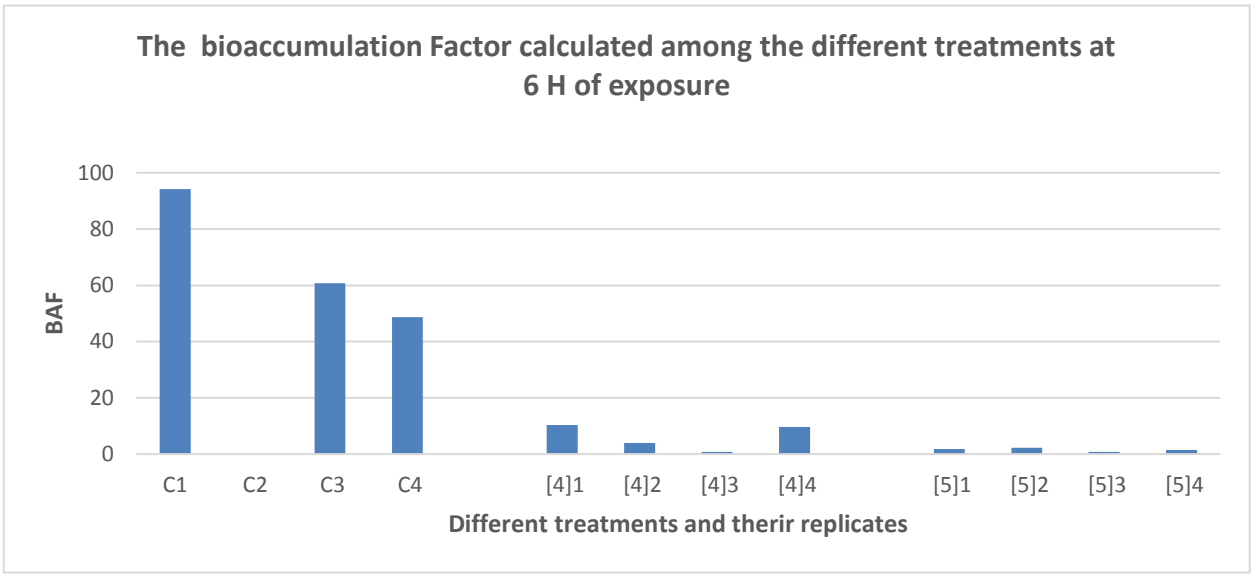


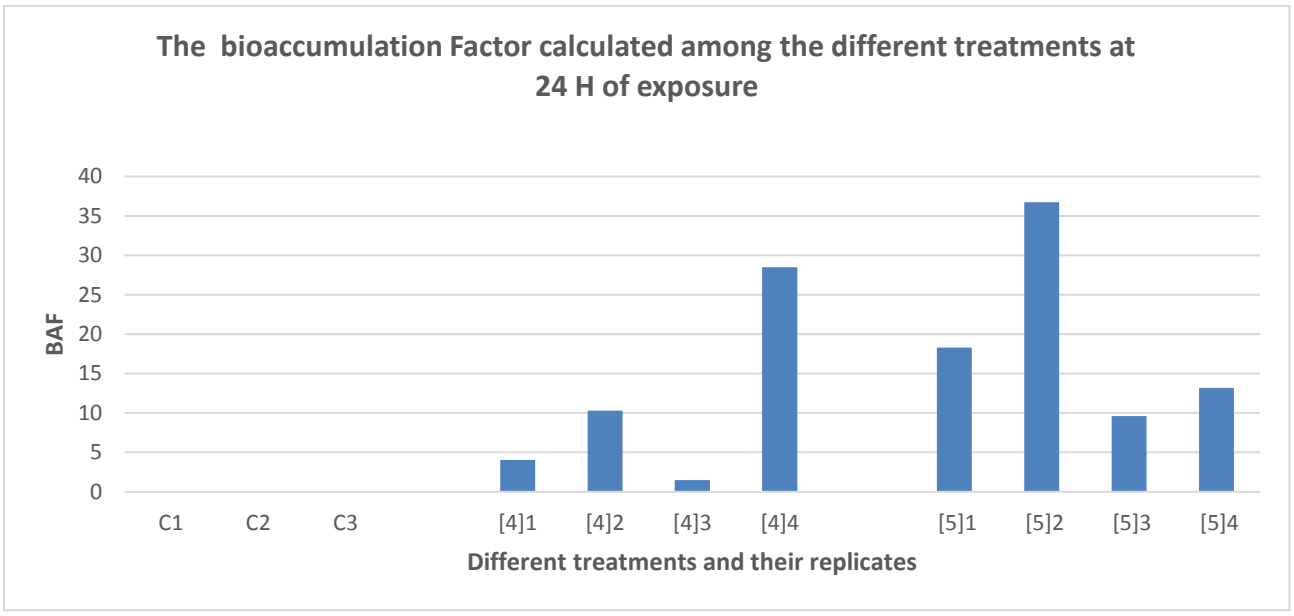
D

Figure 3. The concentration of 9-NA in 1 gram of tissue sample in *A. virens* after 6 hours of exposure (A), 24 hours of exposure (B), 7 days of exposure (C) and 14 days of exposure (D), as a function of the different treatments, given in µg/g tissue. C represents the control, whereas [4] and [5] represent the concentration of the spiked 9-NA sediment in increasing order. The number next to the concentration represents the different replicates.

Analysis of the bioaccumulation factor



A



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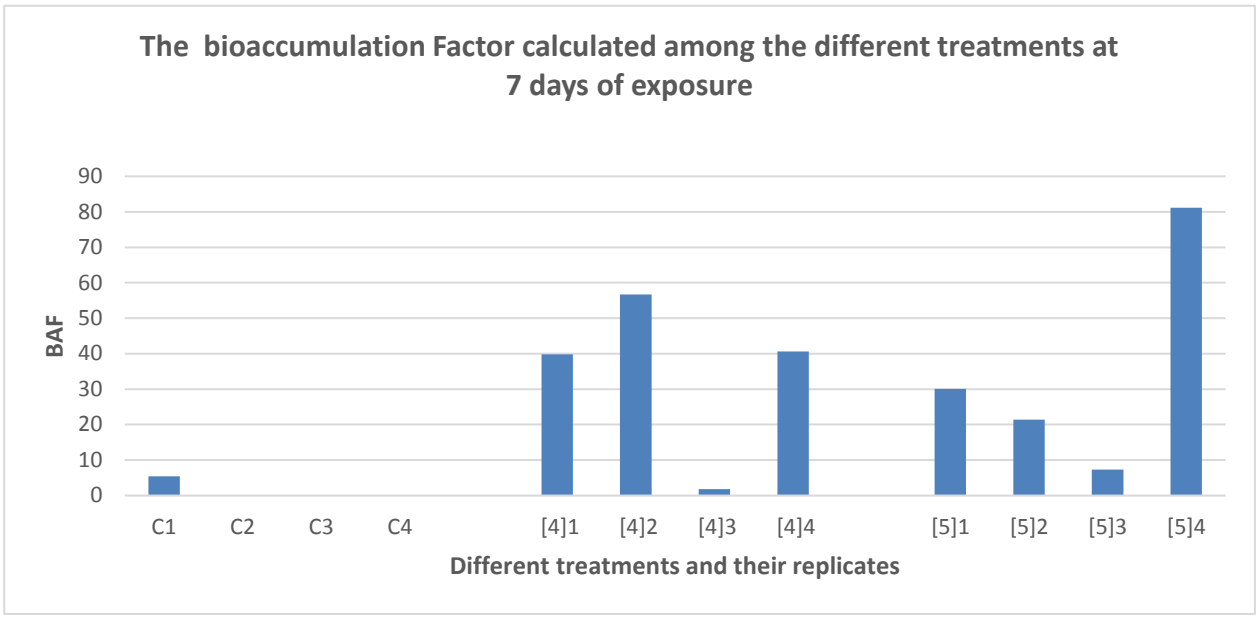

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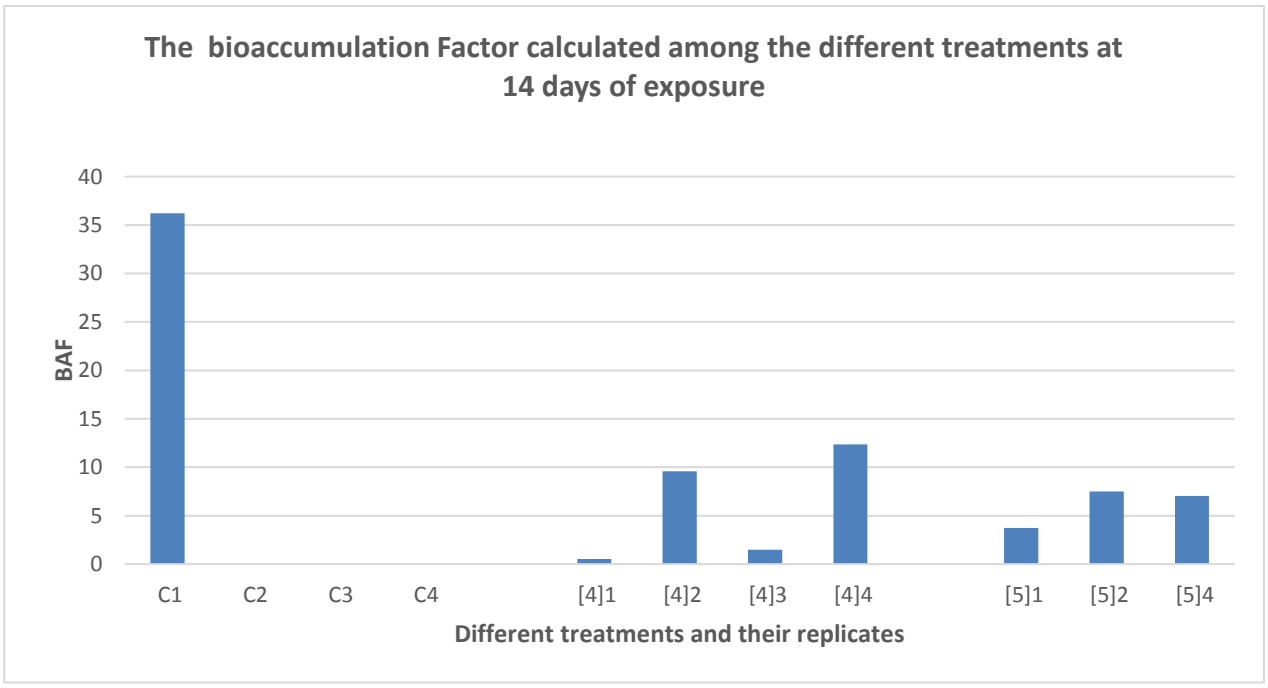

D

Figure 4. The BAF of the different treatments and their replicate at 6 hours of exposure (A), 24 hours of exposure (B), 7 days of exposure (C) and 14 days of exposure (D). C represents the control, whereas [4] and [5] represent the concentration of the spiked 9-NA sediment in increasing order. The number next to the concentration represents the different replicates.