DEPARTMENT OF PHARMACY

Stability of Pharmaceuticals in a Simulated Marine Environment

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Acknowledgements

Abstract

In the past few decades there has been an increasing awareness of pharmaceuticals in the environment and pharmaceuticals as environmental pollutants. Pharmaceuticals find their way to the aquatic environment mostly through sewage treatment plants. The potential toxicity of pharmaceuticals is of great interest as they have been discovered in air, water, soil, sediment and biota.

The aim of the thesis was to investigate the stability of selective serotonin reuptake inhibitors (SSRIs) in seawater. This was achieved by looking at the degradation of SSRIs in filtered seawater containing the marine diatoms *S. marinoi* and *A. longicornis* in monocultures. It was desirable to have a natural environment, and this was achieved by regulating parameters including light and temperature.

Liquid-phase microextraction (LPME) was used to extract and quantify the SSRIs, while ultra performance liquid chromatography coupled with a tandem mass spectrometry (UPLC-MS/MS) was used to analyze the samples.

This thesis showed that there was a difference in degradation of the SSRIs between the two monocultures of *S. marinoi* and *A. longicornis*. The experiment containing the diatom *S. marinoi* had a higher decrease in the average measured concentration for fluoxetine, fluvoxamine, paroxetine and citalopram than the *A. longicornis* experiments. Fluoxetine and fluvoxamine were the least stable SSRIs. For the first 7 days of the *S. marinoi* experiment, fluoxetine had a decrease of 95%, while fluvoxamine had a decrease of 99.5%, while fluvoxamine had a decrease of 87% from day 2 to day 9, in the *A. longicornis* experiment.

The Stockholm County Council classifies Sertraline as a moderate environmental risk, which is the highest environmental risk given to any of the SSRIs. In this study sertraline had a slower degradation in the average measured concentration compared to fluoxetine and fluvoxamine, but it was not as stable as Citalopram.

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Abbreviations

°C	Degrees centigrade
5-HT	Serotonin
A. longicornis	*Attheya longicornis*
AMAP	Arctic Monitoring and Assessment Program
Ar	Argon
ATC	Anatomical therapeutic chemical
AUC	Area under the curve
Chl *a*	Chlorophyll *a*
DEET	N,N-dietyl-meta-toluamid
DHE	Dihexyl ether
DNA	Deoxyribonucleic acid
ECOSAR	Ecological structure– activity relationship
EPA	United States Environmental Protection Agency
eV	Electron voltage
FDA	United States Food and Drug Administration
g	Gravity
IS AUC	Internal standard area under the curve
kg	kilogram
L	Liter
LC	Liquid-phase chromatography
LPME	Liquid-phase microextraction
m/z	Mass/charge ratio
Mg	Magnesium
mg	Milligrams
mL	Milliliters
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
NaOH	Sodiumhydroxide
NOK	Norwegian kroners
OECD	The Organization for Economic Co-operation and Development
PAH	Polyaromatic hydrocarbons
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration
POP	Persistent organic pollutant
PPCP	Pharmaceuticals and personal care products
R^2	Regression constant, expression for linearity
REACH	Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals
S. marinoi	*Skeletonema marioni*
S/N	Signal-to-noise ratio
SLM	Supported liquid membrane
SSRI	Selective serotonin reuptake inhibitors
STP	Sewage treatment plant
UPLC	Ultra performance liquid chromatography
UPLC-MS/MS	Ultra performance liquid chromatography coupled with a tandem mass spectrometer
UV	Ultra violet
μg	Micrograms
μL	Microliters

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1. Introduction

1.1 Pharmaceuticals in the Environment

1.1.1 Pharmaceuticals in the Environment – *a Perspective*

Reports from the Arctic Monitoring and Assessment Program (AMAP), the Stockholm Convention on Persistent Organic Pollutants, the European Unions "Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals" (REACH), and the United States Environmental Protection Agency (EPA) recognize several pollutants as a global problem. Persistent organic pollutants (POPs), polyaromatic hydrocarbons (PAHs), pharmaceuticals and personal care products (PPCP), metals and radioactive substances are a few groups of these pollutants which are viewed as problematic and need to be investigated.

Tabak and Bunch (1) expressed a concern for hormones, both natural and synthetic, not being easily biodegradable in the environment already in 1970. The Food and Drug Administration (FDA) has since 1980 required an assessment on the environmental risks for veterinary medicine. In the late 1990s the European Union set requirements for ecotoxicology testing of pharmaceuticals (2, 3).

Human pharmaceuticals make their way to the environment in different ways, and the most common way is through excretion after normal usage where both the drug and its metabolites may be excreted. Unused drugs might be flushed down the drain or the toilet, or thrown away with household waste. Through these ways pharmaceuticals then may enter the sewage system and end up in the aquatic environment (2, 4, 5). Figure 1 (6) gives an overview of how pharmaceuticals may enter the environment and be distributed to air, water, soil and sediment (3).

In a report from 2010 published by the Norwegian Ministry of Environment (now: Ministry of Climate and Environment) (7) acknowledging the problem of pharmaceuticals as en environmental pollutant. They state that there are gaps in the current knowledge concerning pharmaceuticals in the environment, and that environmental risk assessments would not be crucial for the benefit/risk assessment of pharmaceuticals.

Sweden started their environmental classification of pharmaceuticals in 2004, and the first results were published in 2005. The environmental classification is based on toxicity, bioaccumulation of a given pharmaceutical and a ratio between the predicted environmental concentration (PEC) and the predicted no effect concentration (PNEC) for that pharmaceutical. The PNEC is individual for the species in question. A pharmaceutical with an insignificant risk for the environment has a PEC/PNEC ratio of less than 0.1, while a pharmaceutical with a high environmental risk factor has a ratio larger than 10 (8). The Organization for Economic Co-operation and Development (OECD) has a number of test guidelines that may be used when looking at the environmental risk of chemicals and pharmaceuticals, the criteria of which the Swedish environmental classifications use (9).

Pharmaceuticals are made to produce a biological effect and have properties, for example being lipophilic and being able to bind to other molecules and yield cellular effects, which make them susceptible to bioaccumulation in the environment and possible harmful effects (10, 11). The general focuses of the environmental toxicology studies are the potentially negative effects that the pharmaceuticals might have on non-target organisms, for example algae, zooplankton or fish (2, 3, 8). Most of the studies say something about the acute toxicology of the pharmaceuticals and has a focus on mortality as an endpoint for the environmental risk assessments, and the concentrations in these studies are often higher than the concentrations found in the environment. As there is a continuous release of low concentrations of pharmaceuticals into the environment, the risk of acute toxicity is low, but the pharmaceuticals may be present at all times. Both aquatic and terrestrial organisms may be exposed to these low concentrations over longer periods of time (2, 3).

Webb (12) conducted a meta-analysis (2001) where he listed environmental risk assessments, both acute and chronic, of pharmaceuticals on aquatic organisms. He suggests that algae are the most sensitive of the marine species when exposed to pharmaceuticals in acute toxicology studies. Antidepressants were mentioned as one of the most ecotoxic pharmaceuticals. Among the pharmaceuticals that had acute effects below 1 mg/L were fluoxetine and fluvoxamine. Fluoxetine had a median effective concentration of 0.031 mg/L for unspecified green algae, while fluvoxamine had a lowest observed effective concentration of 0.003 mg/L for the fresh water clam *Sphaerium striatinum*. In the chronic ecotoxicology data from this study, a no-observed effect concentration of 0.001 mg/L for fluoxetine and 31 mg/L for fluvoxamine was found for unspecified green algae. He concluded that there was a focus on the acute endpoint lethality, and there were only 20 pharmaceuticals with chronic risk assessments. One does not yet know a lot about the chronic effects pharmaceuticals might have, as studies on multigenerational lifecycles are rarely conducted.

Another weakness of the environmental risk studies on pharmaceuticals is that they manly focus on just one pharmaceutical and its effect on organisms, even though all pharmaceuticals that are distributed through a waste water treatment plant will always come as a mixture, and may create a "cocktail effect" in aquatic organisms (13). Brooks et al (14) found fluoxetine, sertraline, and their metabolites norfluoxetine and desmethylsertraline in brain, liver and muscle tissues of several types of fish in the Pecan Creek and Clear Creek streams in Texas,

United States. This indicates that the "cocktail effect" of pharmaceuticals in the environment is a fact and it should be investigated further in addition to the chronic effects of pharmaceuticals.

Even though pharmaceuticals have been released to the environment for a long time, it is only in the recent years, due to the continuous improvement of knowledge, measurement and analyzing methods, that one has been able to quantify them from environmental samples. The quantification of pharmaceuticals in the environment is usually done with liquid-phase chromatography (LC) and tandem mass spectrometry (MS/MS) (2, 3).

1.1.2 Pharmaceuticals in the Norwegian Environment

Northern Europe has been suggested to be among the more sensitive areas in Europe towards exposure of man-made pollutants, including pharmaceuticals, due to daylight and temperature conditions, and demographics. Because of the seasonal daylight conditions, with light for up to 24 hours in the summer and the absence of daylight in winter, the photochemical degradation of some pollutants will necessarily be impacted. There are overall lower temperatures in northern Europe than in central Europe, which may lead to lower biodegradation and extended half lives for some pharmaceuticals. Typical for Scandinavian demographics are the smaller, scattered settlements where modern sewage treatment plants (STP) are not affordable. This results in the release of certain pharmaceuticals in to the environment without the necessary sewage treatment being performed (15).

A study conducted in Tromsø by Weigel et al (16) in 2004 showed high amounts of several pharmaceuticals, caffeine and the insect repellent N,N-dietyl-meta-toluamid (DEET) in sewage samples and seawater samples. They found traces of ibuprofen and its metabolites hydroxyl-ibuprofen and carboxy-ibuprofen, diclofenak, triclosan, metoprolol, propranolol and SSRIs. Traces of SSRIs have been found in STPs in Longyearbyen, Tromsø and Oslo (17, 18).

Schlabach et al (19) did a non-target screening in 2013 for several types of pollutants from different environmental samples from Norway. This resulted in the detection of 4395 substances where 1476 of these were identified. Among these were several phthalates, PAHs and PPCPs such as diethylhexylphthalate, fluoranthene and lovastatine respectively.

1.1.3 General Use of Pharmaceuticals in Norway

Since 1977 the Norwegian Institute of Public Health has published statistics of drug consumption in Norway (20). Data of drug consumption in Norway from 2004 has been available for the public in an online database, "Reseptregisteret", since 2004. Very few countries have publications or databases that can give statistics on drug consumption, and therefore there are no available data on the total amount of pharmaceuticals used in the world, and the consumption of pharmaceuticals differ from country to country (21).

For pharmaceuticals with marketing authorization sold from pharmacies, hospitals, nursing homes and non-pharmacy outlets in Norway in 2013 totaled 13.6 billion Norwegian kroners (NOK) with an estimated retail cost of about 20.0 billion NOK (20). From 2004 to 2013 the sales of pharmaceuticals, excluding veterinary medicine, have increased with 39%, from 1.62 billion defined daily dosages (DDD) to 2.25 billion DDD in Norway (20). The amount of pharmaceuticals sold may give us an indication of the amount of pharmaceuticals in the environment.

For antidepressants (N06A), the total sale was 288 million NOK, or about 98 million defined daily dosages (DDD) in 2013 (22). Today there are a number of antidepressants on the Norwegian market. Table 1 summarizes the different groups of antidepressants by the anatomical therapeutic chemical (ATC) classification system and the DDD per 1000 inhabitants per day sold in 2013. Of the antidepressants, the SSRIs were the most sold antidepressants based on DDD per 1000 inhabitants per day. The total sales of SSRIs (N06A B) in NOK was 150 million, or 65 million DDD in 2013 (23). The World Health Organization (WHO) defines DDD as "*the assumed average maintenance dose per day for a drug used for its main indication in adults*" (24).

Table 1: The DDD per 1000 inhabitants per day sold of antidepressants based on anatomical therapeutic chemical (ATC)-code in 2013 in Norway (20).

ATC	Antidepressants	DDD/1000 inhabitants/day
N06AA	Non-selective monoamine reuptake inhibitors	3.48
N06AB	SSRI	37.47
N06AG	Monoamine oxidase inhibitors	0.15
N06AX	Other antidepressants	15.55

Figure 2 gives an overview of the number of users of SSRIs per 1000 inhabitants per year from 2004 until 2013. From this illustration one can see that the use of SSRIs has been stable since 2004 when it comes to users per 1000 inhabitants per year.
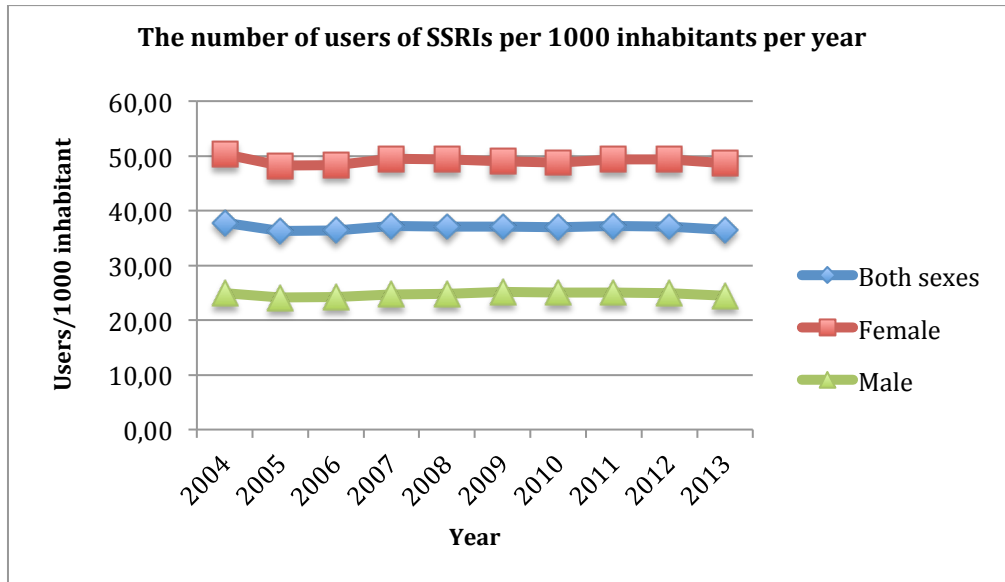
Figure 2: The number of users of all ages of SSRIs (N06A B) in Norway (25).

1.1.4 Selective Serotonin Reuptake Inhibitors

Of the affective disorders, depression is one of the most common. The main theory behind depression is the monoamine theory presented by Schildkraut in 1965 (11). It is based on the effect that some drugs have on monoamines. For example, reserpine may cause depression by reducing the amount of noradrenaline and serotonin (5-HT) in the brain. Antidepressants such as tricyclic antidepressants (TCAs) and other non-selective monoamine reuptake inhibitors block the reuptake of these substances which in turn increases the amount of these in the brain (11, 26). Although the actions and effects of these drugs support the theory, there are no pharmacological evidence that can draw a distinct line between the lack of monoamines and depression after biochemical studies in depressed patients (11).

SSRIs work by selectively inhibiting the serotonin (5-HT) uptake in the nerve synapse in the brain. This selectivity to one monoamine, unlike for example TCA, gives less severe adverse effects and a lower risk of overdose (11).

Today there are six SSRIs on the Norwegian market. The first SSRI on the Norwegian market was fluvoxamine (1990). Paroxetine followed in 1993, and then citalopram (1995), sertraline (1996), fluoxetine (1997) and escitalopram, the S-enantiomer of citalopram, (2002) were marketed. The marketing dates may be found in the summary of product characteristics (SPC) for each respective SSRI on the webpages for the Norwegian Medicines Agency (www.legemiddelverket.no). Table 2 summarizes the sales of these SSRIs in NOK, DDD and kilograms (kg) in 2013 in Norway (27).

Table 2: The sales in NOK, DDD and for all the SSRIs individually based on ATC-code in 2013 in Norway (27).

ATC	SSRI	Sales in NOK	Sales in DDD	Total sale kg
N06AB03	Fluoxetine	15,463,494	3,790,935	76
N06AB04	Citalopram	16,570,791	8,152,222	163
N06AB05	Paroxetine	14,261,411	5,297,402	106
N06AB06	Sertraline	29,464,176	12,594,441	630
N06AB08	Fluvoxamine	1,009,849	171,014	17
N06AB10	Escitalopram	73,437,376	35,096,512	702

The Stockholm County Council (28) has made a list of the environmental risks of the pharmaceuticals they have assessed so far. The environmental risk assessment is based on bioaccumulation, persistence and possible toxicity for aquatic organisms. For the SSRIs they state that sertraline has a moderate environmental risk, while fluoxetine has a low environmental risk. Fluvoxamine has an insignificant environmental risk, and for paroxetine, citalopram and escitalopram an environmental risk cannot be excluded because the documentation is insufficient. These risk assessments are based on data from Sweden, and they might not apply for other countries. Different consumption patterns for pharmaceuticals, different types of STPs (biodegradation or sewage sludge), and where in the environment the pharmaceuticals end up will differ from country to country.

Johnson et al (29) looked at the toxicity of the SSRIs citalopram, paroxetine, sertraline, fluoxetine and fluvoxamine for the algae *Pseudokirchneriella subcapitata, Chlorella vulgaris, Scenedesmus acutus*, and *S. quadricauda*. For the growth inhibition sturdy they looked at PEC and PNEC to determine the acute growth inhibition toxicity in 96 hours. Sertraline turned out to be the most toxic of the SSRIs with growth inhibition occurring at 4.57 μg/L, closely followed by fluoxetine (31.34 μg/L) and fluvoxamine (1662.91μg/L).

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1.1.5 Diatoms

Microalgae are important primary producers and from the foundation of marine food webs. Diatoms are eukaryotic, unicellular microalgae, and dominate the marine phytoplankton communities (30-32). There are two main shapes of diatoms: Centrics and Pennates. The main difference between them is the sculpting of the frustule, a highly evolved silicate cell wall, which consists of two valves, one overlapping the other. Diatoms have enzymatic pathways for uptake of nutrients such as nitrogen, phosphorus and iron; and they also use endocytosis and exocytosis for uptake of organic matter. One distinct feature of diatoms is their large vacuole, where they may store nutrients or use to regulate buoyancy. Diatoms also have a biochemical defense mechanism. They are capable of releasing allelopathic compounds such as fatty acids that are enzymatically turned into unsaturated aldehydes (33).

Diatoms may be found in a number of aquatic habitats for example marine, fresh water, polar or tropic habitats, and even in soil. This is due to their ability to acquire new traits for the different habitats. Morphology has been the main method of identifying diatoms, but in later years, the possibility, and the improvement of techniques, of looking at deoxyribonucleic acid (DNA) has given the indication that there is large species diversity, and that most diatoms are more likely not to be cosmopolitan, but confined to their habitat (33, 34). There are discussions on the number of species of diatoms, and Guiry (35) bluntly estimated in 2012 that there are about 12,000 discovered species of diatoms and about 8,000 species that remains to be discovered. Others estimate that there are about 200,000 different species of diatoms (34, 35).

Through asexual reproduction by cell division, some species may divide up to twice per day, producing up to 4 million cells in 3 weeks. Favorable conditions for diatom growth include the presence of nutrients and light (36). When conditions are unfavorable for the diatoms, they form resting spores that can be germinated when conditions are favorable again (37). Such life history strategies are common in temperate and arctic waters (38).

During the winter months in the northern seas there are almost no diatoms present (39). A low phytoplankton biomass was observed by Degerlund and Eilertsen (40) during the pre-bloom. When the day length increases in spring and optimal light conditions are achieved as well as an up-welling of resting spores from the sediment and the mixing of nutrients, a spring bloom

is initiated. In northern waters, the spring bloom of phytoplankton take place in March-April, with a peak bloom in mid-April as the daylight length increases (37, 38, 41). Eilertsen and Frantzen (42) showed a significant linear correlation between spring bloom biomass and day length.

Skeletonema marinoi (*S. marinoi*) and *Attheya longicornis* (*A. longicornis*) are both centric diatom species. The *S. marinoi* (Figure 3) cells are about 2-12 μm in diameter, and each cell may contain 1 or 2 chloroplasts. They have external projections with flared ends. These can overlap with the external projections of other *S. marinoi*, forming chains (43). *S. marinoi* is found along the northern European coast from April-July (44), and blooms in March and April (40). *A. longicornis* cells are about 4-6 μm in diameter with 1-2 chloroplasts in each cell. They have four horn-like setae that are about 8-10 times their cell length. *A. longicornis* is found in northern cold to temperate waters and in the Sea of Japan, though the biogeographical information relating to this species is limited (45, 46).

Figure 3: *Skeletonema marinoi.* **Photo used with permission by the Plankton lab at the Institute for Arctic and Marine Biology.**
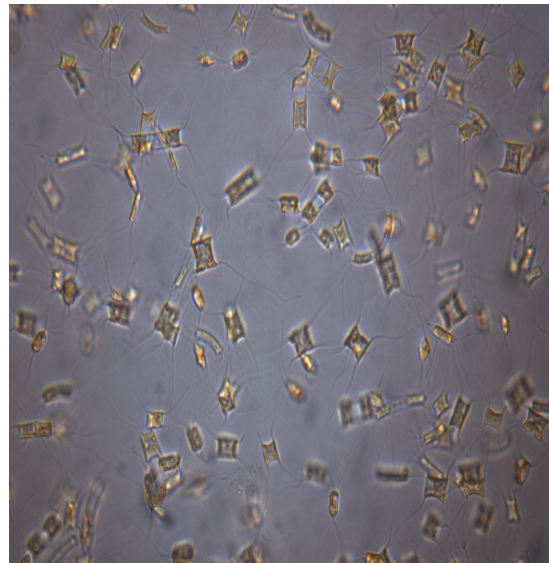
Figure 4: *Attheya longicornis.* **Photo used with permission by the Plankton lab at the Institute for Arctic and Marine Biology.**

1.2 Extraction-, Separation, and Detection Techniques

1.2.1 Liquid-Phase Microextraction

The hollow fiber liquid-phase microextraction (LPME) developed by Pedersen-Bjergaard and Rasmussen (47) in 1999 is based on two techniques: the first LPME technique and the supported liquid membrane (SLM) technique. The first LPME technique involved the passive diffusion of the analyte from the aqueous sample in to a drop of organic solvent. The SLM technique was based on the extraction of the aqueous phase with the analyte through a sheet of supported liquid membrane with the acceptor phase on the other side of the membrane. LPME has since then been developed and may now be applied to numerous analytes (48).

LPME is the extraction of an analyte from an aqueous sample, through an organic phase and into the acceptor phase. If the analyte is either acidic or basic, the aqueous solution is pH-adjusted so that the analyte is not ionized. The hollow fiber is dipped into an organic solution that fills the pores of the fiber wall and forms a SLM. The lumen of the fiber is then filled with an acceptor phase (48).

LPME may be done in either a 2-phase or a 3-phase extraction. In a 2-phase extraction both the pores and the lumen of the hollow fiber are filled with an organic solvent. In a 3-phase extraction the pores of the hollow fiber are filled with an organic solvent, while the lumen is filled with an aqueous solution. The aqueous solution that fills the lumen is pH-adjusted and may be either acidic, if the analyte is basic, or alkaline, if the analyte is acidic. This is to ionize the analyte so that it is trapped in the acceptor phase. The sample is also pH-ajusted so that the analyte is neutral. The fiber is then stirred in the aqueous solution containing the analyte. The analyte passes through the SLM and into the lumen of the hollow fiber through passive diffusion (48). This is illustrated in Figure 5.
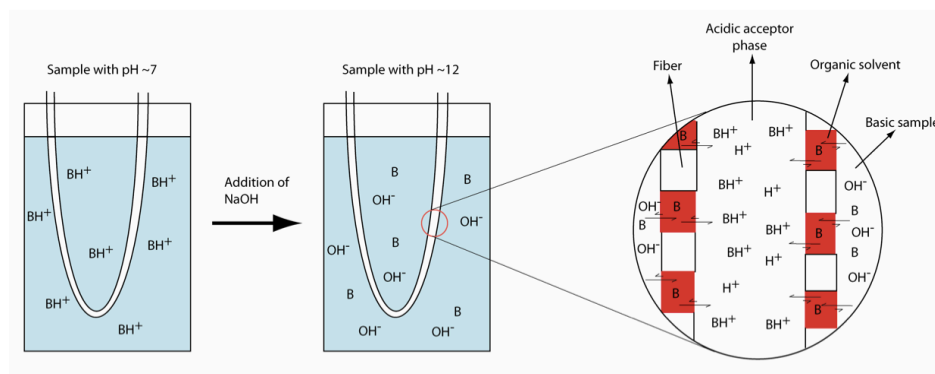
Figure 5: This figure illustrates the LPME method described above with an alkaline analyte. The illustration is used with permission by Terje Vasskog (49).

1.2.2 Ultra Performance Liquid Chromatography

Chromatography is a method for separating analytes. The analytes are separated based on their interaction with a stationary and a mobile phase, and identified by using one or more of several identification techniques like UV-absorbance, retention time and/or mass spectrometer. The analytes that have a high affinity to the mobile phase will have a shorter retention time than those that have more affinity to the stationary phase (50).

In high performance liquid chromatography (HPLC) the analytes are injected into to the liquid mobile phase and then pumped through a column that is packed with a stationary phase. The analyte interacts with the stationary phase, and will thereafter be detected by an appropriate detector (50).

Ultra performance liquid chromatography (UPLC) is a further development of HPLC. The UPLC uses a smaller particle size in the stationary phase, and the UPLC column often has a smaller inner diameter than the HPLC column. This means that the pumps that pump the mobile phase through the column must be able to withstand a higher pressure, which is true for the UPLC. The smaller particle size and the smaller inner diameter in the UPLC columns gives better resolution than what is normal for HPLC (51, 52).

1.2.3 Electrospray Ionization

Electrospray ionization (ESI) is an atmospheric ionization technique often used with UPLC coupled with mass spectrometry as a detector.

The mobile phase from the UPLC goes through a steel capillary to which voltage is applied. This forms an aerosol spray where the analytes in the aerosol droplets are ionized. A nebulizer gas (usually N_2) evaporates the liquid in the droplets. Depending on whether the ionization is positive or negative, protonated $[M+H]^+$ or deprotonated $[M-H]^-$ analytes are formed. Depending on the analyte and matrix other ions might also be formed, such as sodium adducts in the positive ionization $[M+Na]^+$. The charged analytes are then guided to the mass spectrometer by different ion guides for separation and detection. ESI is a soft ionization technique, which means that the ions formed are rarely fragmented (53, 54).

1.2.4 Tandem Mass Spectrometer

The mass spectrometer is a detection method that separates ions by making use of the ions mass/charge ratio (*m/z*). The tandem mass spectrometer (MS/MS) consists of two quadrupoles with a collision cell between them. One quadrupole consists of four metal rods that are placed parallel to one another and given an electrical field with a combination of alternating current (AC) and direct current (DC). Because of the combination of AC and DC and the fact that these may be set to particular values with alternating voltages, only ions with specific *m/z* ratios may pass through (53-55).

The collision cell may consist of hexa – or octapoles, or other types of ion guides such as a T-wave collision cell found in some newer instruments from Waters. In the collision cell a gas, often N_2 or Ar, fragments the precursor ion by increasing the internal energy of the precursor ion through collision until the weakest bonds brake in the precursor ion and product ions are formed (53, 54).

Figure 6 gives an overview of an ESI-MS/MS instrument.
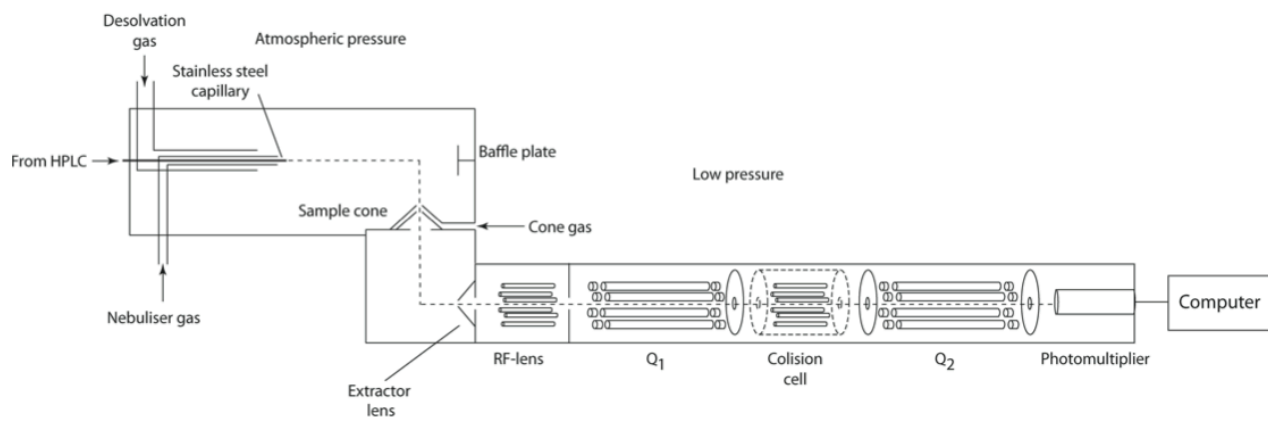
Figure 6: An overview of an ESI coupled with a MS/MS. The illustration is used with permission by Terje Vasskog (56).

1.3 Aim of the Thesis

The aim of this thesis was to investigate the stability of selective serotonin reuptake inhibitors (SSRIs) in seawater. This was achieved by looking at the degradation of SSRIs in filtered seawater containing the marine diatoms *S. marinoi* and *A. longicornis* in monocultures. It was desirable to have a natural environment, and this was achieved by regulating parameters including light and temperature.

Liquid-phase microextraction (LPME) was used to extract and quantify the SSRIs, while ultra performance liquid chromatography coupled with a tandem mass spectrometry (UPLC-MS/MS) was used to analyze the samples.

2. Materials and Methods

2.1 Chemicals

The selective serotonin reuptake inhibitors (SSRI) citalopram *(1-[3-(dimetylamino)propyl]-1-4-fluorphenyl)-1,3-dihydro-5-isobenzofurancarbonitril),* fluoxetine *((±)-N-methyl-γ-[4(trifluoromethyl)phenoxy]benzenepropanamine),* fluvoxamine *(5-methoxy-1-[4-(trifluoromethyl)-penyl]-1-pentanone-O-(2-aminoethyl)oxime),* paroxetine *(trans-(-)-3-[(1,3-benzo-dioxol-5-yloxy)methyl]benzoenepropanamine),* sertraline *((1S-cis)-4-(3,4-dichlorphenyl)-1,2,3,4-tetrahydro-N-methyl-1-naphytalamine)* were purchased from Toronto Research chemicals (TRC, Toronto, ON, Canada). The metabolites and the internal standards (IS) desmethylcitalopram *(1-(4-fluorphenyl)-1,3-dihydro-1-[3-(methylamino)propyl]-5-isobenzofurancarbonitril),* didesmethylcitalopram *(1-(3-aminopropyl)-1-(-4-fluorophenyl)-1,3-dihydro-5-isobenzofurancarbonitril),* norfluoxetine *(γ-[4-(trifluoromethyl)phenoxy]-benzenepropanamine),* desmetylsertraline *(4-(3,4-dichlorphenyl)—1,2,3,4-tetrahydro-(1S,4S)-1-naphthalenamine)* and citalopram-D6, fluoxetine-D5, rac-trans-paroxetine-D4 and sertraline-D3 were also purchased from TRC. Figure 7 shows the structures, Pka-values and the monoisotopic mass for the SSRIs and their metabolites.

Attheya longicornis (*A. longicornis*) was isolated 09.03.07 from sediment samples collected in Rjipfjorden under the first spring bloom. The Art ID used at the Institute for Arctic and Marine Biology (AMB) is AMB 20.2. *Skeletonema marioni* (*S. marioni*) was isolated 01.12.08 from water samples collected in Håkøybotn. The Art ID for *S. marinoi* used at AMB is AMB 86. They were both used in monocultures and cultivated in a silica mass-cultivating medium at AMB at the University of Tromsø. 1 liter (L) of the silica mass-cultivating medium contained 0.25 mL Substral from Scotts Celaflor GmbH &Co. KG (Mainz, Tyskland), 1 mL silicate solution and 1 μL earth extract both made at the laboratory at the Institute for Arctic and Marine Biology at the University of Tromsø (see Appendix 1 for the composition of the silicate solution and the earth extract).

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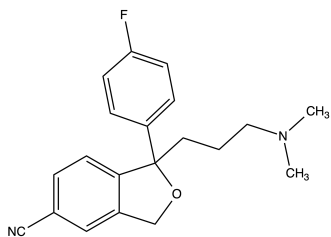
Citalopram
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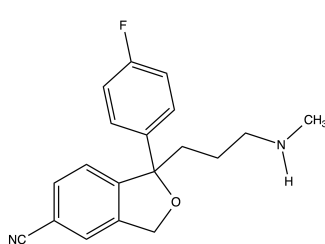
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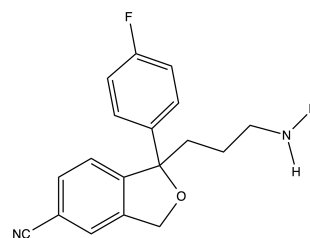
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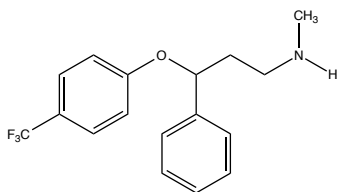
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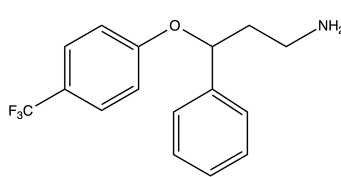
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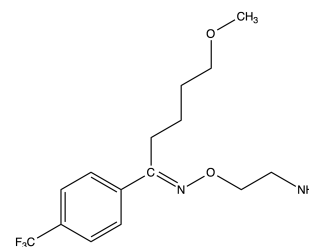
Fluvoxamine
Monoisotopic mass= 318.16
Pk_a= 9.39
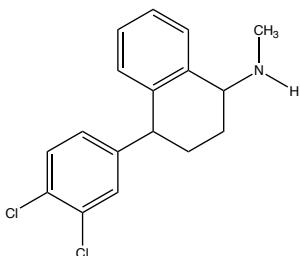
Sertraline
Monoisotopic mass= 305.7
Pk_a= 9.47
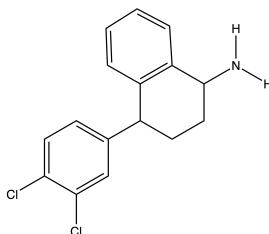
Desmethylsertraline
Monoisotopic mass= 291.06
Pk_a= 9.41
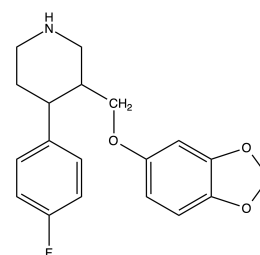
Paroxetine
Monoisotopic mass= 329.14
Pk_a= 10.32

Figure 7: Structures, Pka-values (18) and monoisotopic mass of the SSRIs and their metabolites. The structures are drawn in ChemDraw. Monoisotopic mass was calculated by using a mass calculator by Christoph Gohlke(57).

Two different mixtures of growth medium were used. The growth medium used in the glass beakers with *A. longicornis* was 0.25 mL/L Substral from Scotts Celaflor GmbH & Co. (Mainz, Germany) and 1 mL/L silica mass-cultivating medium as mentioned before. For the glass beakers containing *S. marioni,* the growth medium used was 4 mL/L Guillard´s f/2 marine saltwater enrichment from Sigma-Aldrich (St. Louis, MO, USA), and 1 mL/L silica

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mass-cultivating medium from Sigma-Aldrich as well.

In the fluorometer method, ethanol 70% and HCl 10% was used, both purchased from Sigma-Aldrich.

For the LPME method ethanol absolute from VWR BDH Prolabo (Leuven, Belgium) sodium hydroxide pellets from VWR BDH Prolabo and Merck KGaA (Darmstadt, Germany), dihexylether purum ≥ 97% (GC) from Fluka Sigma-Aldrich Chemie GmBH (DHE, Steinheim, Germany), formic acid 98-100% for analysis from Merck, and water obtained from a Rios 100 MilliQ purification unit from EMD Millipore Corporation (Billerica, MA, USA) were used.

Mobile phases used for UPLC were acetonitrile hyper grade for LC-MS from Merck KGaA, acetonitrile LC-MS grade for HPLC from VWR BDH Prolabo, formic acid 98-100% for analysis from Merck and MilliQ water. Argon (Ar) 4.0 from AGA (Oslo, Norway) was used as collision gas in the mass spectrometer. The nebulizer gas used was nitrogen (N_2) from a NM32LA generator from Peak Scientific (North Billerica, Ma, USA).

2.2 Materials and Method Development

Figure 8 is an illustration of the method set up for this thesis. The algae used are the diatoms *S. marinoi* and *A. longicornis*. Chl a analysis and extractions were conducted for each experiment, but not simultaneously. The experiment was conducted at AMB and Norut.
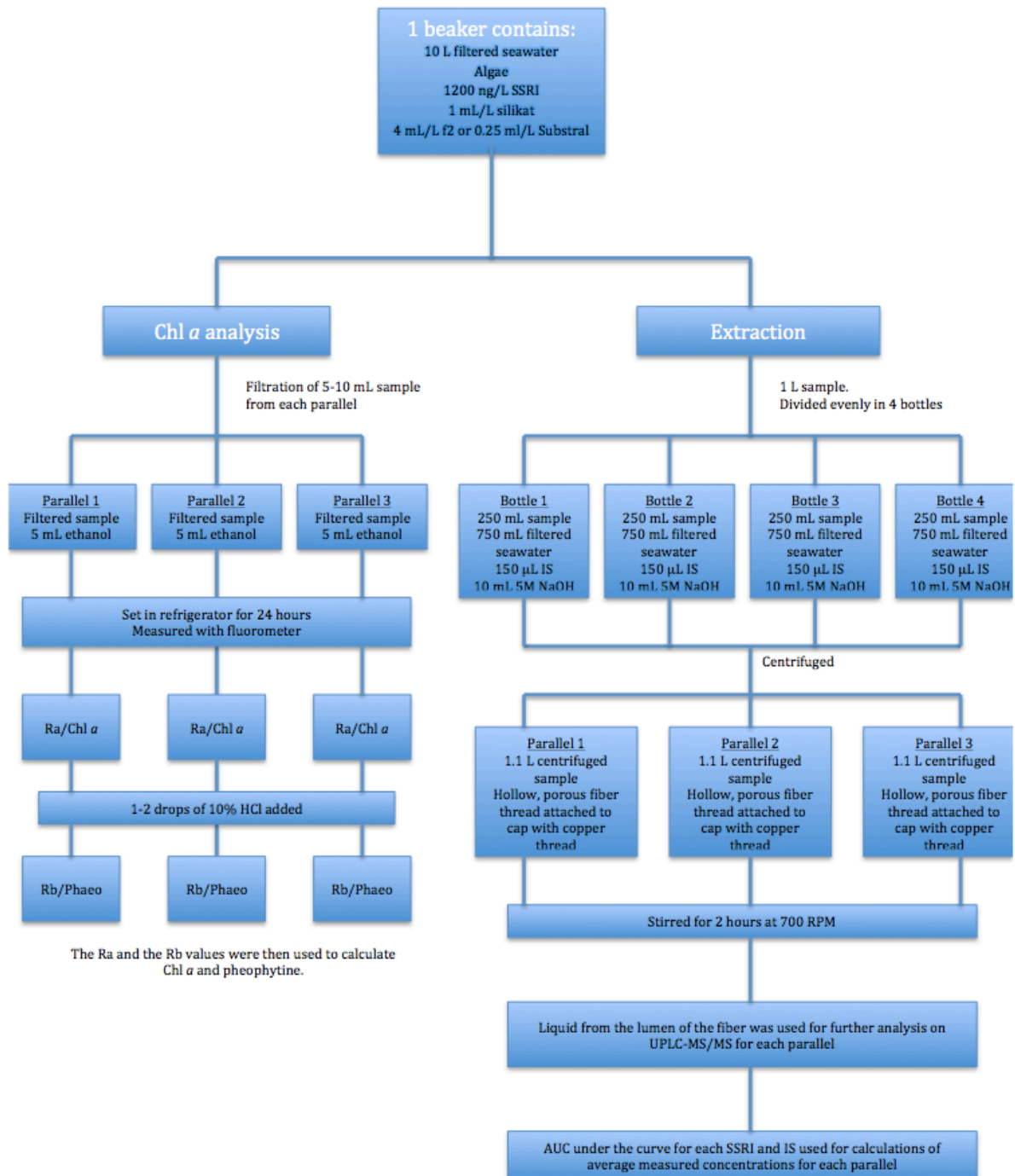
Figure 8: Schematic illustration of the method proceedings of Chl *a* analysis and extraction. "Beaker" refers to one parallel in one experiment, i.e. there are 3 10 L glass beakers for each experiment.

2.2.1 Set Up for Samples with Algae and SSRIs

This part of the experiment was carried out at AMB.

To establish the concentration of algae in each culture 2 mL four-well Nunc counting chambers from Apogent (Roskilde, Danmark) and a Zeiss Primo Vert microscope from Carl Zeiss AG (Oberkochen, Germany) were used. The algae were counted and the concentration in cells per liter was calculated. The appropriate amount of algae was then added to each beaker (Table 3).

Table 3: The amount of algae added to each experiment given in cells per liter (L).

Algae experiment	Cells/L
S. marinoi	419,522
A. longicornis, first experiment	1,285,182
A. longicornis, second experiment	1,285,182

Seawater for the experiment was filtered with a filtering system consisting of a NVAS UF 4040 membrane filter, a UV-C light, and a coal filter from Nordisk Vannteknikk AS (Drammen, Norway). This filtration system insures that 99.9% of all bacteria and viruses are filtered from the sample. The filtered seawater was placed in 3 10 L glass beakers in a cold room that held a temperature of about 4°C. The light in the room was set to imitate circadian rhythms with 14 hours of daylight and 10 hours of darkness. This was to optimize the conditions for algae bloom. A computer controlled the parameters for light and temperature.

SSRI standards of sertraline, fluoxetine, fluvoxamine, paroxetine and citalopram were added to each glass beaker so that the concentration was 1200 ng/L.

To assure constant mixing of the samples through turbulence, air under pressure was applied to the glass beakers through plastic tubing and aquarium rocks in the experiment with *S. marinoi* and in the second experiment with *A. longicornis* (as illustrated in Figure 9). In the first experiment with *A. longicornis*, air was not applied, as illustrated in Figure 10.
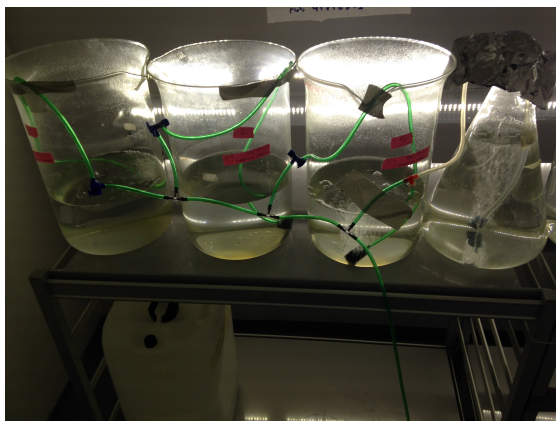
Figure 9: Set up of *S. marinoi* (photo by Kine Smellror).
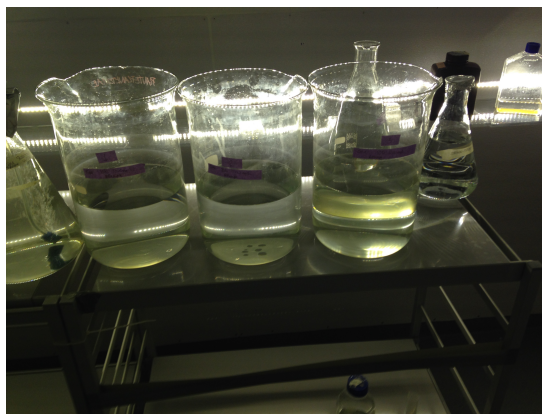
Figure 10: The first set up of *A. longicornis* (photo by Kine Smellror).

For each experiment, a control in a 5 L glass beaker containing the same concentration of algae as the actual experiment (Table 3) was set up. This was done to look at the over all and daily cell growth with the same parameters of light and temperature as the experiment.

To ensure that the growth medium or other experimental parameters did not break down or react with the SSRIs, one control for each growth medium, f/2 and Substral, was set up (with out algae). These controls were set up in 5 L glass beakers with a concentration of 1200 ng/L of the SSRIs as mentioned earlier in this chapter. The glass beakers were manually stirred after adding all the ingredients so that the mixture would be homogeneous.

It was decided that a desired concentration for the SSRIs of 300 ng/L for each sample would be sufficient because the concentration was in the linear area of the calibration curve. See chapter 2.2.6 Calibration Curve for the method development and concentrations of reference standards in the calibration curve.

As there were no containers large enough available to hold the amount of seawater needed to take out the exact amount of sample (1L*4) for each sampling during the course of the experiment with the desired concentration of SSRIs at 300 ng/L, the concentration of SSRIs had to be increased to 1200 ng/L so that a volume of 10 L would be more than sufficient to take out the samples needed. 1 L of sample was taken from each glass beaker each sampling day. The 1 L of sample was then divided into four bottles with 250 mL each and diluted to 1 L by adding 750 mL of filtered sea water as described further on in *2.2.3 Centrifuge Set Up*. This is illustrated under "Extraction" in Figure 8.

2.2.2 Chlorophyll *a* Analysis

Chlorophyll is the green pigment found in plants and has an important role in their photosynthesis as it transforms the energy in photons into sugar. Chlorophyll *a* (Chl *a*) is found in all algae. Chlorophyll emits fluorescent light and can therefore be measured in a fluorometer, the amount of emitted light is directly proportional to the amount of Chl *a* in the cells. Phaeophytin is formed by a degradation of Chl *a*, with the result in a loss of magnesium (Mg) from the porphyrine ring. This compound emits fluorescent light at a different wave length than Chl *a* (58).

Chl *a* is used in this thesis to give an estimation of the biomass of *S. marinoi* and *A. longicornis.* A schematic illustration of the Chl *a* analysis is given in Figure 8.

To determine the amount of Chl *a* in each glass beaker, samples were taken from them and filtered with 25 mm circle GF/C filters from Whatman (GE healthcare, Little Chalfont, United Kingdom) in a 12-cylinder filtration device with a vacuum pump, both from EMD Millipore Corporation.

The filter papers were put in test tubes and 5 mL of the extractant, ethanol, was then added to each test tube. The samples were covered with parafilm to avoid evaporation, and aluminum foil to prevent photodegradation, and then set in the refrigerator (4°C) for ca 24 hours. The samples were taken out of the refrigerator and added to the cuvettes, and measured when they were at room temperature.

To analyze the samples, the fluorometers TD-700 and Trilogy, both from Turner Design (Sunnyvale, CA, USA), were used. In the TD-700 fluorometer quarts cuvettes were used, while in the Trilogy fluorometer disposable cuvettes of plastic (2.5 mL, 12.5 x 12.5 x 45 mm) from Brand GmBH (Wertheim, Germany) were used.

In the TD-700 fluorometer, the Ra-values (the fluorometer reading before acidity) were measured first. One drop of 10% HCl was then added to the samples and the Rb-values (the fluorometer reading after acidity) were measured. HCl was added to break down the Chl a to phaeophytin by removing Mg from the porphyrine ring. The difference in fluorescence reading before and after HCl addition is used to infere the amount of active and inactive Chl a (59).

The average of the Ra- and Rb-values were used to calculate the amount of Chl a with the formula:

$$\mu g\ Chl\ a\ L^{-1} = \frac{F\ x\ (Ra-Rb)}{V} \qquad (2.1)$$

Ra and Rb denotes the fluorescence reading before and after HCl, V is volume and F is a constant (0.003439) that is adjusted by calibration of the instrument.

Phaeophytin is calculated with the formula:

$$\mu g\ Phaeo\ L^{-1} = \frac{(F\ x\ (2.11xRb))-Ra}{V} \qquad (2.2)$$

where V is the amount of filtered water in liters (L).

The amount of Chl a was used to calculate the overall growth of the cells (μ) whit the formula:

$$\mu = \frac{\ln B/B_0}{t-t_0} \qquad (2.3)$$

B_0 is the amount of chlorophyll at day one (t_0); while B is the amount of chlorophyll at day t. μ was used further on to calculate the daily growth of the cells (k) by using the formula:

$$k = 1.443\mu \qquad (2.4)$$

For the Trilogy fluorometer the Chl a value and the phaeophytin value was calculated directly by the instrument, so the average of the Chl a values were used to calculate the overall growth of the cells and daily growth of the cells by using formulas 2.3 and 2.4 respectively.

2.2.3 Centrifuge Set Up

This part of the experiment and onwards was conducted at Norut.

Before centrifugation, the samples of 250 mL were diluted to 1 L with 750 mL of filtered seawater. 10 mL of a 5M NaOH-solution and 150 ng/L of the internal standards were added, as illustrated in Figure 8.

Each 1 L sample was centrifuged at 8000 rounds per minute (RPM) for 10 minutes with either a JLA-8.1000 or a JLA-9.1000 rotor in a Beckman Coulter Avanti Centrifuge J-26 XP (Brea, CA, USA). The relative centrifugal force (RCF) may be found in Table 4. This was done to remove salt that precipitates from the samples when NaOH is added.

Table 4: Shows the radius of the rotors (mm), the maximum rotor speed and the rotor speed used (RPM) and the relative centrifugal force (RCF) average and maximum (g = the gravitational force of the rotor).

	Rotor	
	JLA-8.1000	**JLA-9.1000**
Rotor radius minimum	119 mm	82 mm
Rotor radius maximum	222.8 mm	185 mm
Maximum rotor speed	8000 RPM	9000 RPM
Rotor speed used	8000 RPM	8000 RPM
RCF (average)	12250 g	9569 g
RCF (maximum)	15970 g	13261 g

2.2.4 Liquid-Phase Microextraction

A porous hollow fiber with an inner diameter of 330 μm from Membrana GmBH (Wuppertal, Germany) was submerged in dihexylether (DHE) for about 10 seconds. The excess DHE was removed using a 3510 ultrasonic bath from Branson Ultrasonics (Danbury, CT, USA) for about 3 seconds. The lumen of the hollow fiber was then filled with the acceptor phase, MilliQ water adjusted to a pH of about 2 with formic acid. The ends of the hollow fiber were then closed with a thin copper thread. The same type of copper thread was then used to hold the fiber in the middle of the extraction bottle during the extraction.

The extraction bottles were filled with 1.1 L of the already centrifuged samples. The samples were stirred for two hours at 700 RPM with a magnetic stirrer as illustrated in Figure 11 and

Figure 12. The liquid inside the lumen of the fibers were transferred to vials for further analysis in Ultra Performance Liquid Chromatography (UPLC-MS/MS).
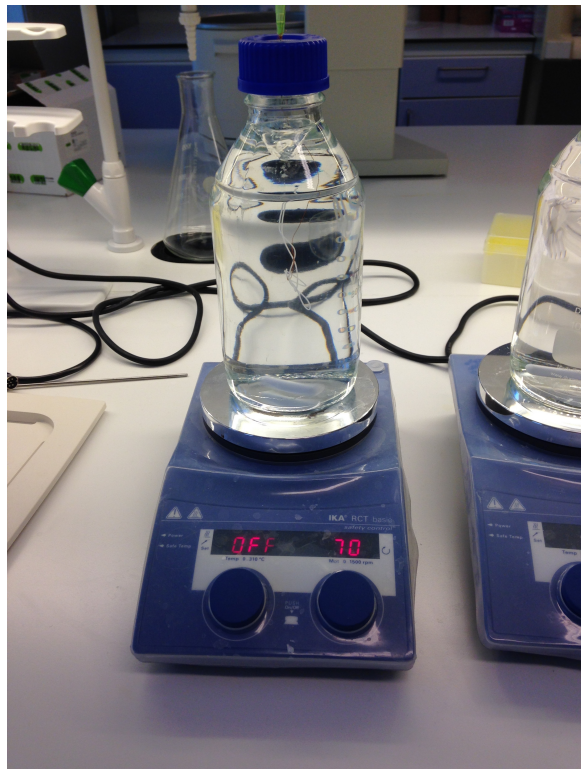
Figure 11: Photo of the LPME set up (photo by Kine Smellror).
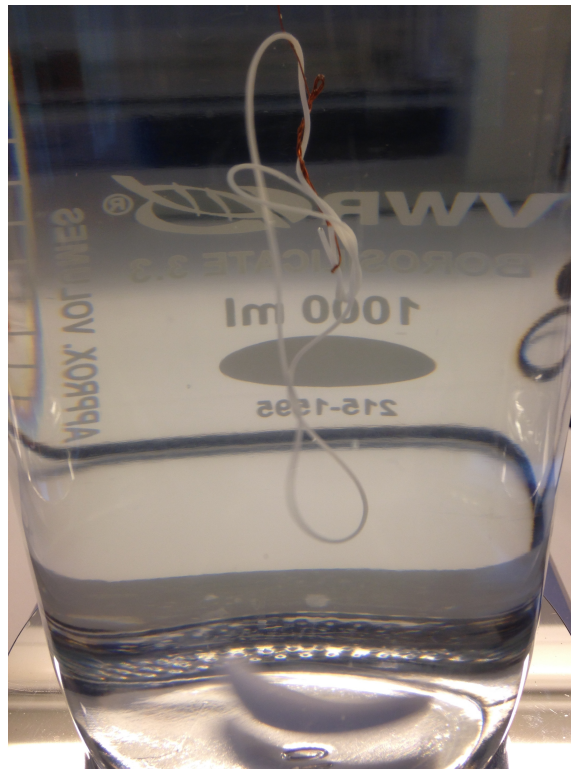
Figure 12: Photo of the fiber thread during LPME (photo by Kine Smellror).

2.2.5 Ultra Performance Liquid Chromatography and Mass Spectrometry Method Development

All the SSRIs mentioned in Table 5 were infused into the tandem quadrupole (Xevo TQ-MS from Waters Inc.). This was done in order to find the ratio between the mass (m) and the charge (z) at which each precursor ion of the SSRIs was detected and at what cone voltage the signal for each protonated molecular ion was most intense. To find this signal, a full scan in the Tune-page of the MassLynx program from Waters Inc was used. The Tune-page was also used to find the product ions. The measured precursor ions were set, one by one, in a product ion scan mode. The electron voltage (eV) of the collision energy (Ar as a collision gas) was adjusted to obtain the highest intensity for each of the product ions.

The observed precursor ions and product ions, the cone voltage and the collision energy are summarized in Table 5.

Table 5: Monoisotopic mass, observed protonated molecular ion and product ions, cone voltage (CV) and collision energy (CE).

Compound	Monoisotopic mass	[M+H]$^+$ (m/z)	Product ion 1 (m/z)	Product ion 2 (m/z)	CV (V)	CE (eV)
Citalopram	324.16	325.35	109.05	262.20	35	23
Citalopram D6	330.20	331.30	109.30	262.40	38	24
Desmethylcitalopram	310.15	311.30	109.05	262.20	34	20
Didesmethylcitalopram	296.13	297.30	109.05	262.20	26	21
Sertraline	305.07	306.25	275.15		18	10
Sertraline D3	308.09	309.20	275.35		18	11
Rac-cis-N-desmethylsertraline	291.06	292.20	275.15		16	15
Fluoxetine	309.13	310.35	44.05		22	11
Fluoxetine D5	314.17	315.25	44.30		22	11
Norfluoxetine	295.12	296.30	134.15		15	8
Fluvoxamine	318.16	319.35	71.05		19	13
Fluvoxamine	318.16	319.35	226.20		19	21
Paroxetine	329.14	330.30	192.20		37	25
Rac-trans-paroxetine D4	333.17	334.25	196.40		37	25

Detailed parameters for the mass spectrometric method are given in Appendix 2.

For some of the SSRIs, two product ions were found. This was because there was a high intensity for both signals. For quantitative analysis only the product ion with the highest intensity was used ("Product ion 1"). The results in Table 5Table 5.

Table 5 were used to set up a multiple reaction monitoring (MRM) method used in the quantification method. In a MRM method, the first quadrupole will let through the selected precursor ion, while the last quadrupole will let through the selected product ion. When the ions have passed both quadrupoles an appropriate detector will identify them. This gives advantages such as high specificity and high signal-to-noise ratio (S/N). When there is a high specificity, one is able to detect the analyte even if there are other substances present in the sample. S/N is an important citeria for detection, i.e. the amount of analyte compared to the baseline noise. For quantification, a detection limit of S/N=10 is typical (60).

2.2.6 Calibration Curve

For the development of the method and the calibration curve, tap water was used instead of filtered seawater. The extraction bottles were filled with 1.1 L tap water, a given amount of the different standard SSRIs and internal standards (IS), and 10 mL of 5M NaOH-solution. 1.1 L tap water was used for the samples since this filled the bottles to a high enough level to avoid vortex and bubble formation, which could have lead to the LPME fiber being only partly submerged in the sample and giving lower extraction efficiency.

In this experiment the calibration curve compares the area of the peaks of a reference standard with that of an internal standard in a ratio plotted against a concentration gradient. The calibration curves are then used for quantification of the SSRIs.

The concentrations of the reference standards were 0.91 ng/L, 9.09 ng/L, 45.45 ng/L, 136.36 ng/L, 272.73ng/L, 545.45 ng/L and 818.18 ng/L. The internal standards were added at the same concentration to all samples, 136.36 ng/L. The LPME method was then used, and the samples were analyzed by UPLC-MS/MS.

The peaks given in a chromatogram was used to calculate a peak area ratio between the reference standards and the internal standards. The peak area ratios were then plotted as a function of the concentrations. The regression lines from these plots were then used to calculate the concentrations in the experiments. Collecting data for the calibration curve was done over four days.

2.2.7 Ultra Performance Liquid Chromatography and Tandem Mass Spectrometry

The samples were analyzed with ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) with the tandem MS in MRM mode. This was done on an Acquity Ultra Performance LC with a Xevo TQ-MS from Waters Corp. (Milford, MA, USA). The separation was conducted with a Waters Aquity CSH C_{18}-column (2.1 x 100 mm) with a particle size of 1,7 μm, also from Waters Corp. ESI was set to positive mode. Temperature on the column was set to 50°C.

The optimal elution gradient used in this analysis is shown in Table 6. Both solution A and B were made acidic by adding formic acid. The injection volume for these elution gradients was 5 μL.

Table 6: Optimal elution gradient. Solution A contains MilliQ water with 0.1% formic acid. Solution B contains acetonitrile with 0.1% formic acid.

Time (min)	Flow (mL/min)	Solution A (%)	Solution B (%)
0.00	0.6	80	20
1.00	0.6	80	20
8.00	0.6	78.7	21. 3

2.2.8 TargetLynx Method

TargetLynx is a softeare addition to MassLynx and may be used to automatically process large amounts of data. In this thesis, to process the results, a TargetLynx method was used. This method gave the retention time, height of the curve, area under the curve and the date and time for which each sample was run on the UPLC-MS/MS. The AUC for each substance from all the samples was compared to the area under the curve for the respective IS.

The use of the deuterated fluoxetine as the IS for fluvoxamine was determined by comparing all the IS to fluvoxamine to see which gave the most stable values at a given concentration, and looking at the linearity. The linearity (R^2) for the fluvoxamine/fluoxetine D5 calibration curve was 0.9945. Bergersen et al (61) described a method where they also used fluoxetine D5 for the calculations of the fluvoxamine calibration curve with a R^2-value of 0.9662. As the R^2-value for the calibration curve in this experiment was higher, it was decided that the AUC for fluvoxamine was to be compared with the IS AUC for fluoxetine.

For parameters for the target lynx method, see Appendix 3.

2.2.9 Software

For drawing the SSRIs, ChemDraw for iPad version 2.0.1 by PerkinElmer was used. The Molecular Mass Calculator by Christoph Golhke was used for the calculation of the monoisotopic mass (57). To calculate the RCF for the rotors used, the Beckman Coulter Rotor Calculations calculator was used (62).

The UPLC-MS/MS was operated by MassLynx version 4.1 SCN810, copyright © 2010 Waters Inc. (Milford, MA, USA). TargetLynx version 4.1 SCN810, copyright © 2010 Waters Inc. (Milford, MA, USA) was used to collect data from the chromatograms. To analyze the data from the TargetLynx software, Microsoct Excel 2011 for Mac was used.

3. Results and Discussion

Pollutants in the environment is an increasing problem, and since 1970 (1) one has been aware of pharmaceuticals as pollutants. It has taken some time to establish standard environmental risk assessments for pharmaceuticals, and the question of safe disposal and removal of pharmaceuticals from sewage treatment plants through sewage sludge and biodegradations are being discussed (4, 5).

The environment in the northern parts of Europe has overall lower temperatures and differences in daylight with almost no daylight in the winter season, and daylight for up to 24 hours of daylight during the summer season. This may have an effect on the photochemical degradation and the biodegradation of pharmaceuticals, causing them to be more persistent than in warmer climates (15). Several studies have proved that SSRIs are found in the environment (14, 16-18), and there are also studies that look at the toxicity of the SSRIs (12, 13, 29).

In this thesis the degradation of SSRIs in monocultures of *S. marinoi* and *A. Longicornis* in filtered seawater was investigated. Chl *a* analysis was used to look at the biomass of the diatoms. LPME was used for the extraction and up-concentration of the SSRIs, while UPLC-MS/MS was used for detection and quantification.

3.1 Chlorophyll Analysis

As there were little or no algae in the ocean outside of Tromsø in the seasonal time when conducting this thesis (40), the degradation of SSRIs was looked upon in filtered seawater with the presence of the algae *S. marinoi* and *A. longicornis* in monocultures. A computer controlled the light and temperature parameters, and they were set to optimize algae bloom with a daylight length of 14 hours and a temperature of 4°C. If the parameters are favorable for a bloom for the diatoms, which may duplicate from 1 cell to 1 million daughter cells in less than three weeks (36).

A control containing only filtered seawater and the same concentration of cells as the experiments was set up for all the experiments. Chl *a* were taken the 1st, 7th and 14th day, and measured the 2nd, 8th and 15th day for each experiment and for the control. This was to measure the biomass. A schematic illustration of this part of the experiment is shown in Figure 8 under "Chl *a* analysis". The negative values for the growth rate indicate that there are fewer algae in the water from one measurement to another, and that the growth rate has therefore decreased.

In the *S. marinoi* experiment, there is a negative overall and daily growth rate for "Beaker 1" between the first and the last measurement (Table 7). There is a positive overall and daily growth rate between the first and the second measurement, and a negative overall and daily growth rate between the second and last measurement. For "Beaker 2" there was a negative overall and daily growth rate between the second and the last measurement. For "Beaker 3" and the control there was a positive overall and daily growth rate for all the measurements. In this experiment the largest daily and overall growth was between the first and the second measurements for all the beakers.

Table 7 shows the calculated overall (μ) and daily (k) growth rate for the *S. marinoi* experiment.

Table 7: The calculated overall (μ) and daily (k) growth rate for the *S. marinoi* experiment. The measurements are given in μg/L. For the Ra- and Rb-values used to calculate the overall- and daily growth rate see Appendix 10. Formulas 2.1-2.4 were used for these calculations.

Growth rate		Measurements		
		First and last measurement(μg/L)	**First and second measurement(μg/L)**	**Second and last measurement(μg/L)**
Beaker 1	μ	-0,01	0,18	-0,20
	k	-0,01	0,26	-0,29
Beaker 2	μ	0,07	0,16	-0,02
	k	0,10	0,23	-0,03
Beaker 3	μ	0,13	0,20	0,07
	k	0,19	0,29	0,10
Control	μ	0,26	-0,01	0,53
	k	0,37	-0,02	0,76

In the first experiment with *A. longicornis*, as shown in Table 8, there was an overall and daily positive growth rate for the cells between the first and the last measurement for all the beakers and the control. The negative growth between the first and the second measurement might be due to *A. longicornis* algae sticking to the glass walls because of not stirring or having air under pressure added to the glass beaker. From day ten manual stirring was started, and between the second and the last measurement there was a positive growth rate both overall and daily for all the beakers and the control. In this experiment the largest daily and overall growth was between the second and the last measurements for all the beakers.

Table 8 shows the calculated overall (μ) and daily (k) growth rate for the first *A. longicornis* experiment.

Table 8: The calculated overall (μ) and daily (k) growth rate for the first *A.longicornis* experiment. The measurements are given in μg/L. For the Ra- and Rb-values used to calculate the overall- and daily growth rate see Appendix 11. Formulas 2.1-2.4 were used for these calculations.

Growth rate		Measurements		
		First and last measurement(μg/L)	**First and second measurement(μg/L)**	**Second and last measurement(μg/L)**
Beaker 1	μ	0,21	-0,08	0,50
	k	0,31	-0,11	0,72
Beaker 2	μ	0,21	-0,12	0,53
	k	0,30	-0,17	0,77
Beaker 3	μ	0,20	-0,08	0,49
	k	0,29	-0,11	0,70
Control	μ	0,27	-0,11	0,65
	k	0,39	-0,16	0,94

In the second *A. longicornis* experiment there was an overall and daily positive growth rate for the cells between all the measurements. For "Beaker 1", "Beaker 2" and "Beaker 3" the largest growth rate was between the second and the last measurement. For the control, the largest growth rate was between the first and the second measurement.

The values in Table 9 are calculated from the values given in Appendix 12 using formulas 2.1-2.4.

Table 9 shows the calculated overall (μ) and daily (k) growth rate for the second *A. longicornis* experiment.

Table 9: The calculated overall (μ) and daily (k) growth rate for the second *A.longicornis* experiment. The measurements are given in µg/L. For the Chl *a* values used to calculate the overall- and daily growth rate see Appendix 12.

Growth rate		Measurements		
		First and last measurement(µg/L)	First and second measurement(µg/L)	Second and last measurement(µg/L)
Beaker 1	μ	0.11	0.07	0.15
	k	0.16	0.11	0.21
Beaker 2	μ	0.12	0.09	0.14
	k	0.17	0.13	0.21
Beaker 3	μ	0.12	0.09	0.14
	k	0.17	0.14	0.20
Control	μ	0.11	0.12	0.11
	k	0.17	0.18	0.15

3.2 Method development

To optimize the elution gradient, a number of gradients were tried. The mobile phase consisted of MilliQ-water with 0.1% formic acid (Solution A) and acetonitrile with 0.1% formic acid (Solution B). The first elution gradient was tried because it was used for a similar experiment in a master thesis. The three first elution gradients tried are listed in Table 11. They were not optimal elution gradients as all the analytes were eluted after less than 2 minutes, and some analytes co-eluted.

Table 10: Elution gradients tried.

Time (min)	Flow (mL/min)	The first elution gradient		The second elution gradient		The third elution gradient	
		Solution A (%)	Solution B (%)	Solution A (%)	Solution B (%)	Solution A (%)	Solution B (%)
0.00	0.6	60	40	80	20	70	30
1.00	0.6	60	40	80	20	70	30
7.00	0.6	55	45	70	30	60	40
12.00	0.6	50	50	60	40	55	45

Another elution gradient that was tried is shown in Table 11. For this elution the analytes did not elute within the wanted time set for each sample.

Table 11: Elution gradient tried.

Time (min)	Flow (mL/min)	Solution A (%)	Solution B (%)
0.00	0.6	90	10
1.00	0.6	90	10
7.00	0.6	85	15
12.00	0.6	80	20

The elution gradient used in this thesis is listed in Table 6. This is an optimal elution gradient since all the analytes eluted between 1 and 8 minutes, the first being didesmethylcitalopram at around 1.35 minutes. There was a good enough separation for the purpose of this thesis. This is illustrated in the chromatograms that follow in this chapter.

There are more than one analyte in the same time window of elution, which is not optimal, but good enough for this thesis. The best solution would be if there were single time windows of elution for each analyte. This would increase the sensitivity of the method.

3.3 Calibration Curve

The calibration curve is a quantified expression for the correlation between the known concentration of reference standard and the known concentration of an internal standard.

The calibration curve is in this experiment used for quantification of the SSRIs and their metabolites by the correlation between concentration of the analytes and the AUC of their respective peaks. The lowest concentration on the curve is 0.91 ng/L while the highest concentration is 818.18 ng/L. The sampling for the calibration curve was conducted over four days, with two parallels each day.

The calibration curve yields a regression line with the formula:

$$y=mx+b \tag{2.5}$$

where m is the slope, and b is the intercept. One wishes the linearity (R^2) of the curve to approach 1, so that y and x would be proportional, which in turn will give a straight line and linearity. As seen in Table 12, the R^2-values are approaching 1. Desmethylcitalopram, with R^2=0.9425, and didesmethylcitalopram, with R^2=0.8603, are the lowest R^2-values, and are therefore less linear than the other SSRIs. There is a weaker correlation between the concentration and the signal then for the other SSRIs.

The formulas for the regression lines given by the calibration curves are applied in the calculations of the concentrations and can be seen in Table 12.

SSRI	y=mx+b	R^2
Sertraline	y=0.0127x	0.9986
Desmethylsertraline	y=0.0051x	0.9953
Fluoxetine	y=0.0042x	0.9966
Norfluoxetine	y=0.007x	0.9975
Fluvoxamine	y=0.0101x	0.9945
Paroxetine	y=0.0051x	0.9987
Citalopram	y=0.0045x	0.9991
Desmethylcitalopram	y=0.001x	0.9425
Didesmethylcitalopram	y=0.0007x	0.8603

To confirm and remove any outliers in the calibration curves a q-test was used. The formula applied for this purpose was:

$$q = \frac{gap}{range} \tag{2.6}$$

Gap is the absolute difference between the value one wants to test as an outlier and the value closest to it, while range is the absolute difference between the minimum and maximum values in the dataset. The q-value calculated is then compared to a given value, Q, in a table corresponding to the sample size and the confidence level. If $q > Q$, then the value in question is rejected.

There are many metabolites for the different SSRIs, but the only standards available for this study were desmethylsertraline, norfluoxetine, desmethylcitalopram and didesmethylcitalopram. This in turn implies that other metabolites for the SSRIs would not be detected through the method used.

Since, in this thesis, one did not work with low concentrations, limit of detection and limit of quantification was not tested. Some of the average measured concentrations were below the lowest concentration of the standard curve, but this is not of importance since it was the decrease in concentration over time that was of interest.

Examples of chromatograms of the calibration curve for the concentration 272.73 ng/L for all the precursor ions of the SSRIs and their fragment ions detected with an MRM-method are shown in Figure 13 and Figure 14.
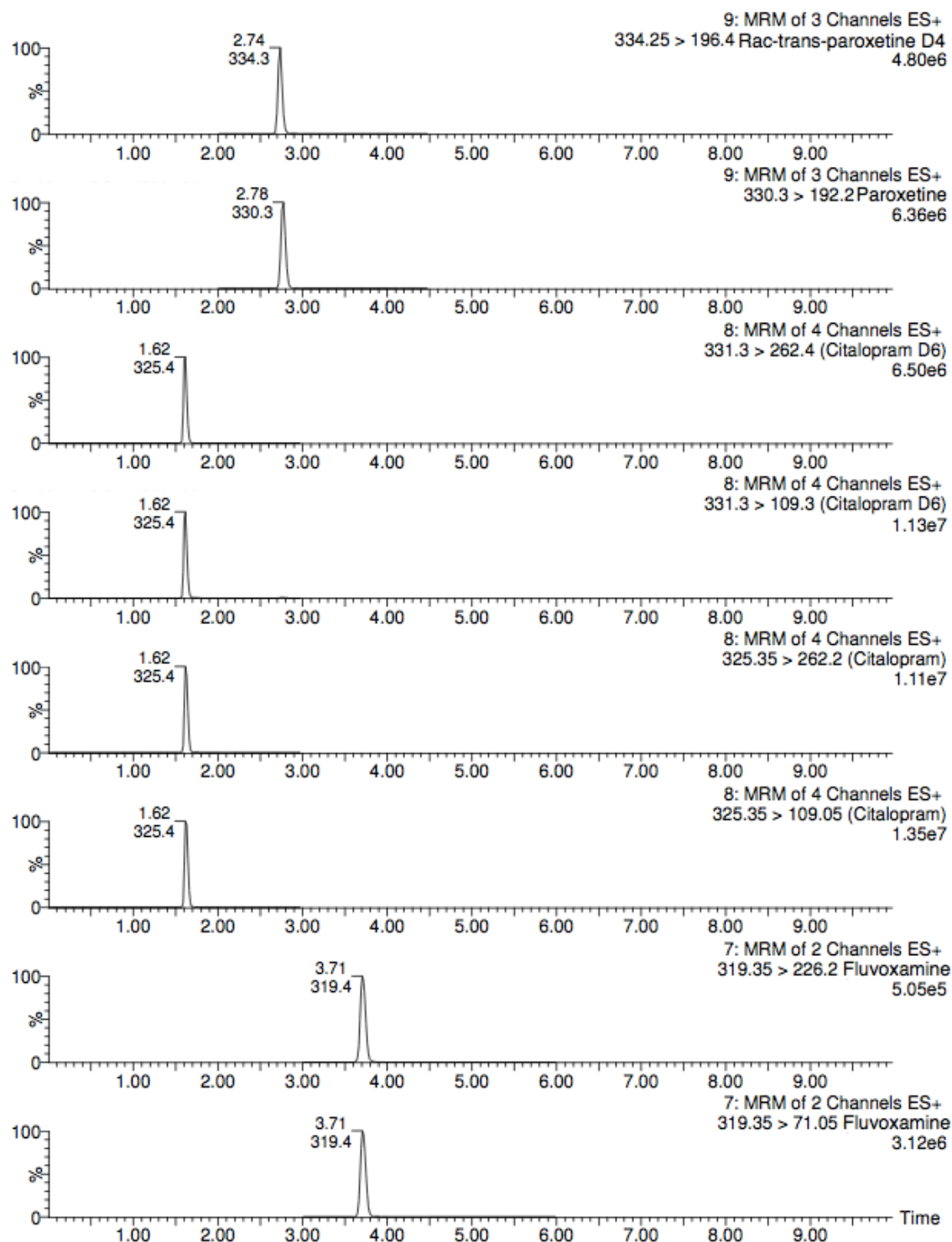
Figure 13: Chromatogram of the calibration curve (272.73 ng/L) for all the ions for rac-trans-paroxetine D4, paroxetine, citalopram D6, citalopram and fluvoxamine.
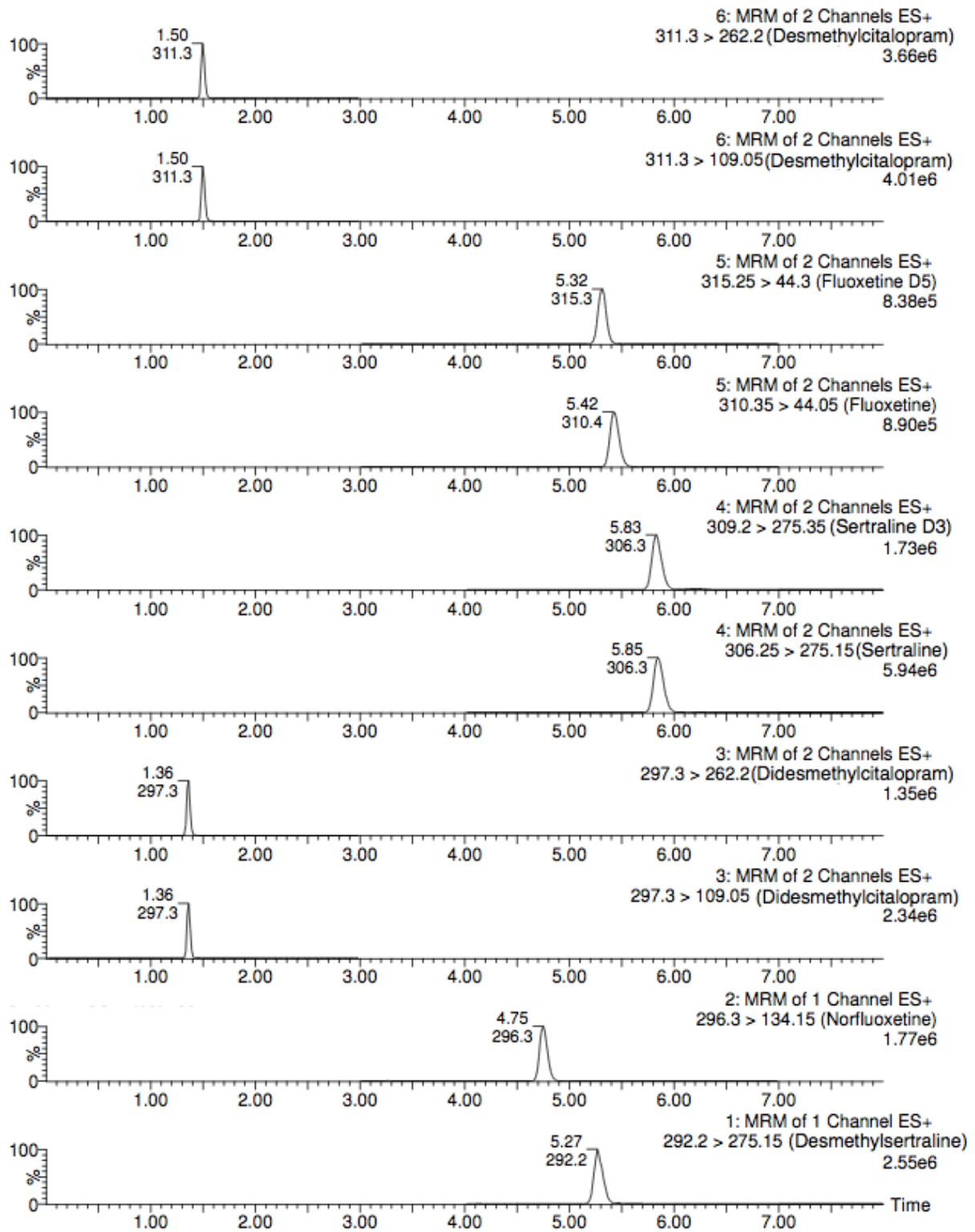
Figure 14: Chromatogram of the calibration curve (272. ng/L) for all the ions for desmethylcitalopram, fluoxetine D5, fluoxetine, sertraline D3, sertraline, didesmetylciralopram, norfluoxetine and desmethylsertraline.

3.4 Extraction of Blank Samples

Examples of chromatograms of a blank sample (containing just MilliQ-water) for all the precursor ions of the SSRIs and the fragment ions used in the TargetLynx-method detected with an MRM-method are shown in Figure 15 and Figure 16.
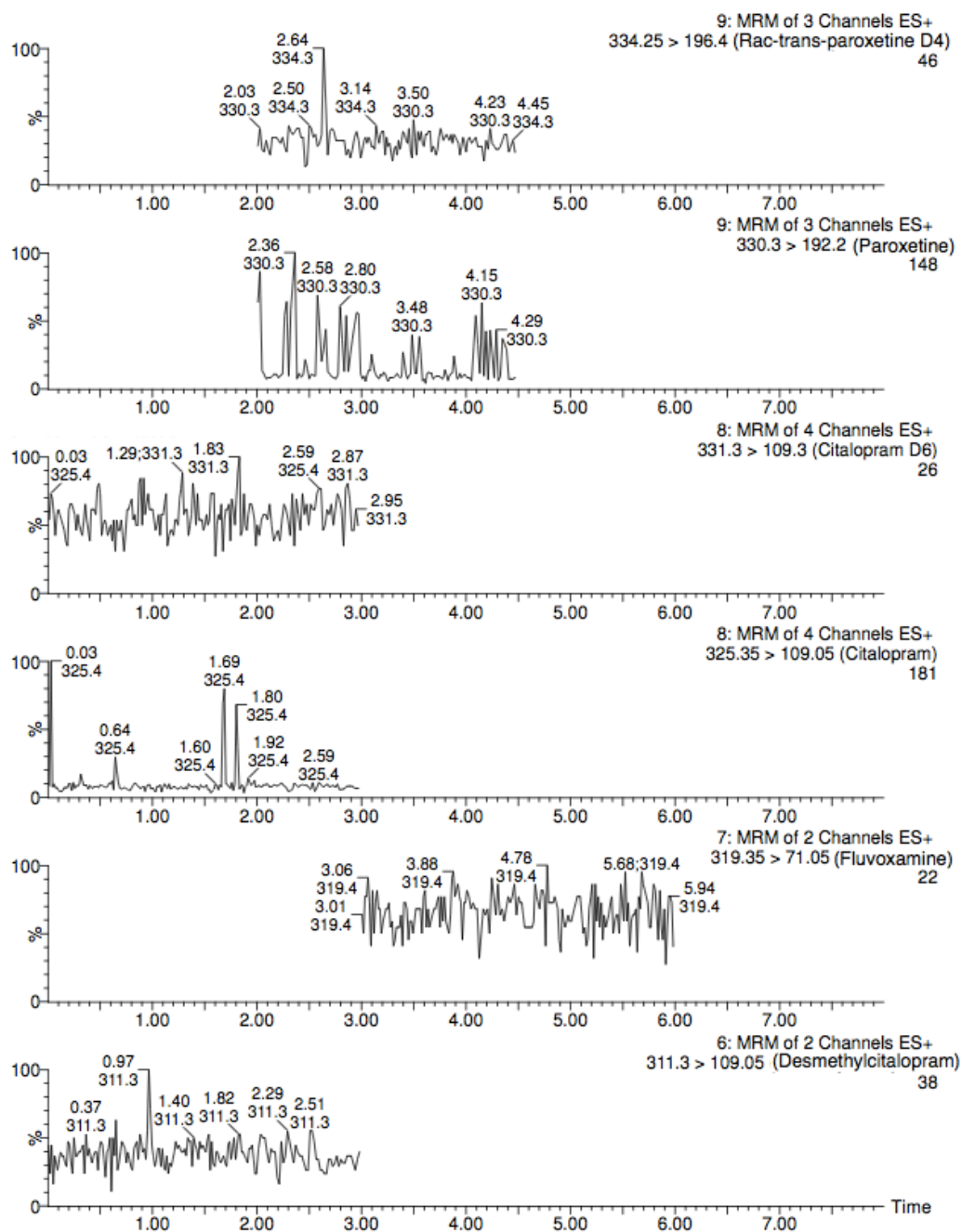
Figure 15: Chromatogram of a blank sample for rac-trans-paroxetine D4, paroxetine, citalopram D6, citalopram, fluvoxamine and desmethylcitalopram.
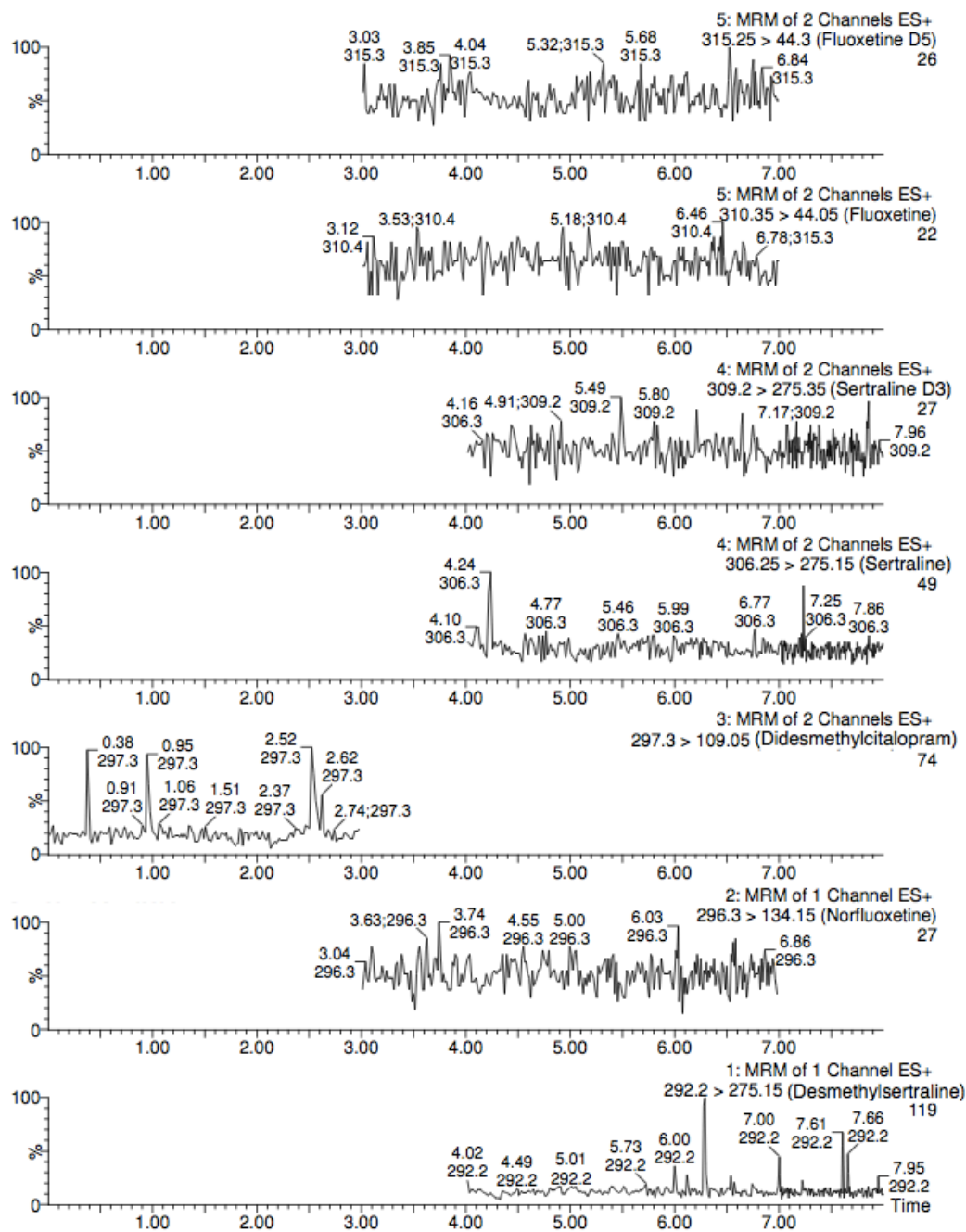
Figure 16: Chromatogram of a blank sample for fluoxetine D5, fluoxetine, sertraline D3, sertraline, didesmetylciralopram, norfluoxetine and desmethylsertraline.

Some blank samples that were extracted had "carry-over" effects with analytes like citalopram and sertraline, i.e. that the analyte was eluted with the blank samples. This happened following a sample with a high concentration. The AUC of for example citalopram in the blank sample would be about 1/1000 of the AUC in the actual samples. This has no significance for the calculations of the average measured concentrations.

3.5 Control Experiment Without Diatoms

To ensure that the growth medium (f/2 and Substral) or the other parameters in the experiment (light conditions and temperature) did not influence the measured SSRI concentrations, a control experiment was set up. The set up for both the controls were identical to the set ups for the experiments with the algae, except for not applying air under pressure. This was because constant mixing of the samples did not seem necessary as the SSRIs and the growth medium would be homogenous after manually stirring them once.

Exact values for average concentrations, standard deviations, and relative standard deviations (RSD) for each SSRI are found in Appendix 6. For each sampling, three parallels were run. An overview is given in Figure 15 to Figure 23.

There were no measurements taken on the first day. The decrease or increase in average measured concentration between day 6 and day 14 is therefore used to calculate the average decrease or increase per day.

There is a decrease in the average measured concentrations for both growth mediums for all the SSRIs. For sertraline (Figure 17) the decrease in average measured concentration for the f/2 growth medium was 8% from day 6 to day 14, with an average decrease per day of 1.00%. The decrease for Substral during the same period was 3%, with an average decrease of 0.38% per day.

For fluoxetine (Figure 18), the f/2 growth medium the average measured concentration from day 6 to day 14 decreased with 11%, and for the Substral growth medium there was a decrease of 9% from day 6 to day 14. The average decrease per day was 1.38% for the f/2 medium and 1.13% for Substral.
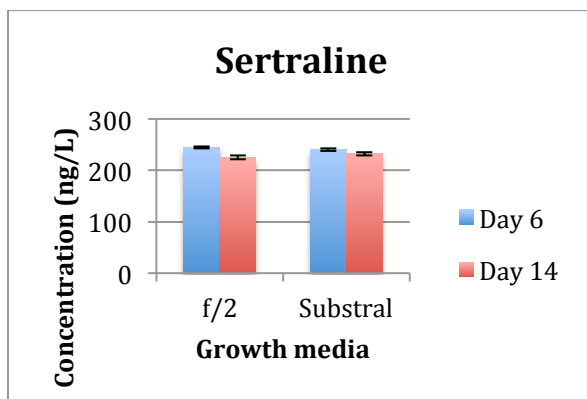
Sertraline

Figure 17: The average measured concentrations for sertraline for each growth medium for each sampling day. The standard deviation for each growth medium is also shown.
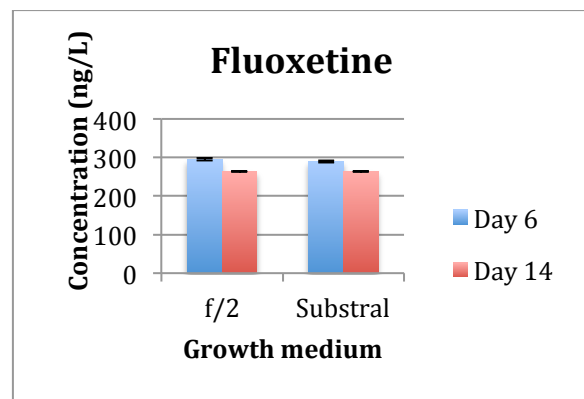
Fluoxetine

Figure 18: The average measured concentrations for fluoxetine for each growth medium for each sampling day. The standard deviation for each growth medium is also shown.

For fluvoxamine (Figure 19) the decrease in the average measured concentration of the f/2 medium for day 6 to day 14 was 9%, which corresponds to a daily decrease of 1.13%. For the Substral medium the decrease in average measured concentration for day 6 to day 14 was 6%, with a daily decrease of 0.75%.
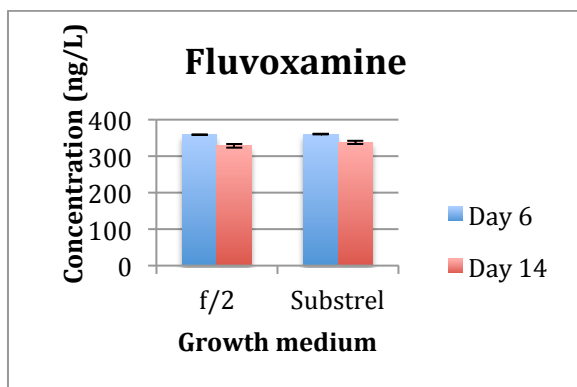
Fluvoxamine

Figure 19: The average measured concentrations for fluvoxamine for each growth medium for each sampling day. The standard deviation for each growth medium is also shown.

Paroxetine (Figure 20) had the highest decrease in the average measured concentration of the Substral medium from day 6 to day 14 (24%). This gives a daily decrease of 3.00%. The decrease in f/2 medium for the average measured concentration in the same period was 13%, which corresponds to a daily decrease of 1.63%.

Citalopram (Figure 21) had a lower decrease with 6% for the average measured concentration for the f/2 growth medium, and 3% for the Substral growth medium from day 6 to day 14. The daily decrease was 0.75% and 0.38% for f/2 and Substral respectively.
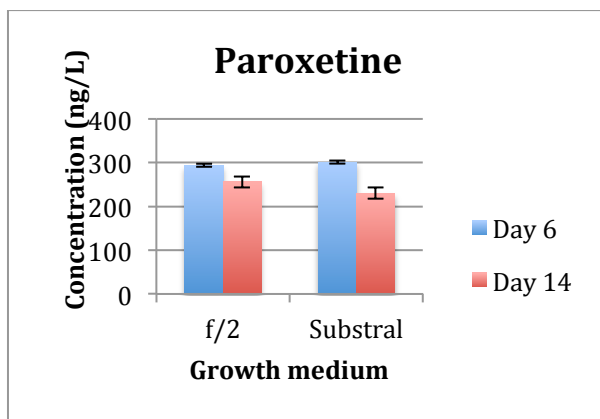
Figure 20: The average measured concentrations for paroxetine for each growth medium for each sampling day. The standard deviation for each growth medium is also shown.
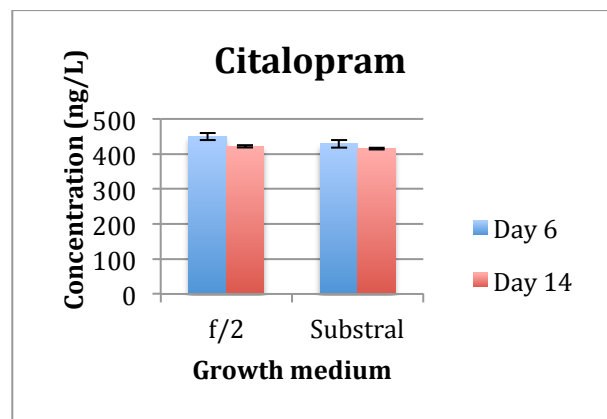
Figure 21: The average measured concentrations for citalopram for each growth medium for each sampling day. The standard deviation for each growth medium is also shown

The average measured concentrations for desmethylsertraline (Figure 22), norfluoxetine (Figure 23) and didesmethylcitalopram (Figure 24) are below the lowest concentration in the calibration curve, hence the average measured concentrations are uncertain compared to those that are within the calibration curve.

Desmethylsertraline had an increase in the average measured concentration for the f/2 growth medium of 21% and 21% for the Substral medium. This gives a daily increase of 9.63% for f/2 and 2.63% for Substral.

For norfluoxetine the increase in the average measured concentrations from day 6 to day 14 was 23% for the f/2 medium and 61% for the Substral growth medium, which correspond to a daily increase of 7.25% for the f/2 medium and 7.63% for Substral.
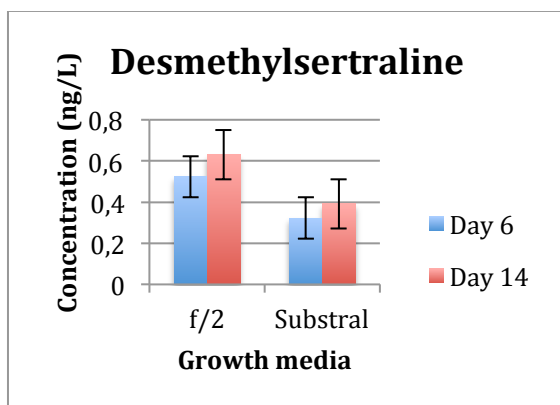
Figure 22: The average measured concentrations for desmethylsertraline for each growth medium for each sampling day. The standard deviation for each growth medium is also shown.
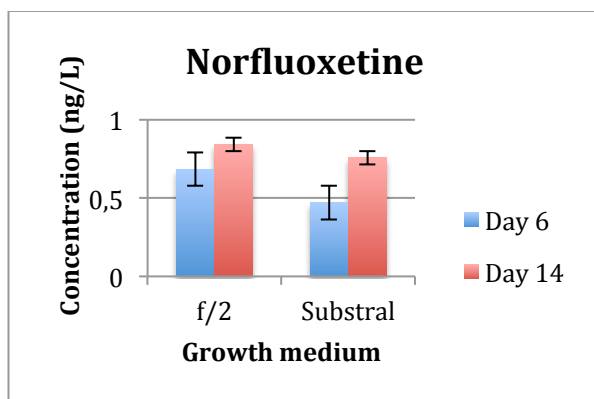
Figure 23: The average measured concentrations for norfluoxetine for each growth medium for each sampling day. The standard deviation for each growth medium is also shown.

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Didesmethylcitalopram had a decrease in the average measured concentration from day 6 to day 14 of 65% for the f/2 growth medium. This gives a daily decrease of 8.13%. For the Substral growth medium there was a 55% decrease from day 6 to day 14, with a daily decrease of 6.88%.

Desmethylcitalopram (Figure 25) is the only one of the metabolites analyzed that has average measured concentrations above the lowest concentration on the standard curve. For the f/2 growth medium the decrease in the average measured concentration for day 6 to day 14 was 1.6%, and for the Substral growth medium an increase in 2%. This gives a daily increase of 0.20% for the f/2 medium and a no significant daily increase for Substral (0.25%).
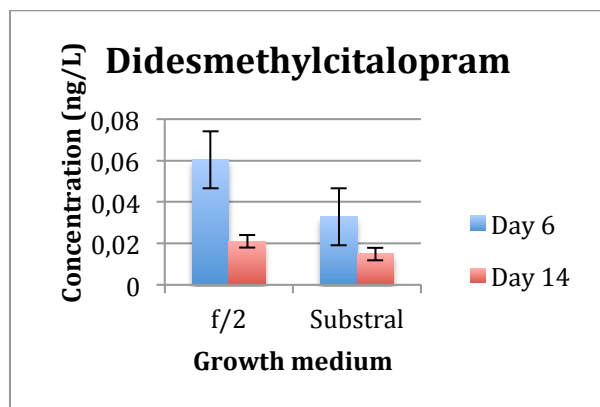
Figure 24: The average measured concentrations for didesmethylcitalopram for each growth medium for each sampling day. The standard deviation for each growth medium is also shown.
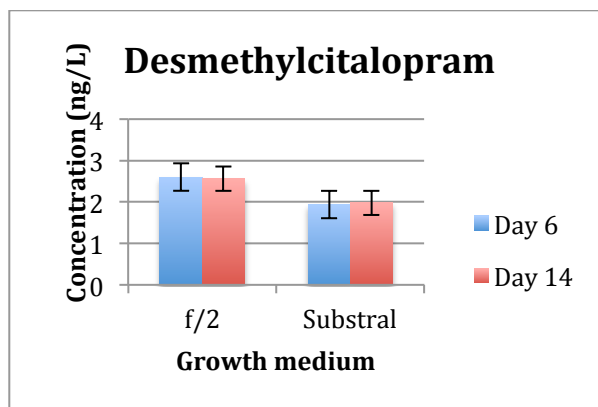
Figure 25: The average measured concentrations for desmethylcitalopram for each growth medium for each sampling day. The standard deviation for each growth medium is also shown.

In the control experiment that contained the growth mediums and the SSRIs there seems to be little lowering of the average measured concentrations for sertraline, fluoxetine, fluvoxamine, citalopram. The slight decrease in concentration might indicate that the growth mediums, light settings, temperature and filtered seawater do not have a big impact on these SSRIs in general. The decrease in sertraline and fluoxetine indicates that a small amount of sertraline and fluoxetine is degraded to desmethylsertraline and norfluoxetine respectably. This may indicate that sertraline and fluoxetine is degraded by light or other parameters. Paroxetine had a decrease of 24% for the average measured concentration of Substral, which might indicate that the growth medium, light settings or filtered seawater might have had an impact on the degradation.

There were different average measured concentrations for the two growth mediums for all the samples taken. In theory, the average measured concentrations should be the same as the concentrations of the SSRIs added to the glass beakers. Substral had a constant lower average measured concentration, except for paroxetine. The lower measurements in Substral indicate the presence of some compounds with a slight effect on the extraction efficiency or the stability of the SSRIs in the samples.

Examples of chromatograms of the control experiment for all the SSRI ions used in the quantification method are shown in Figure 26 and Figure 27. Examples of chromatograms of the Substral growth medium may be found in Appendix 5.

In Figure 27, one can clearly see that there is a lower signal for didesmethylcitalopram than for the other analytes, and the S/N ratio is lower than 10, which means that it is below the quantification limit and the measured concentration is highly uncertain. For desmethylsertraline and norfluoxetine, the signal is 10^3 lower than for the other analytes. These low signals reflect their average measured concentration.
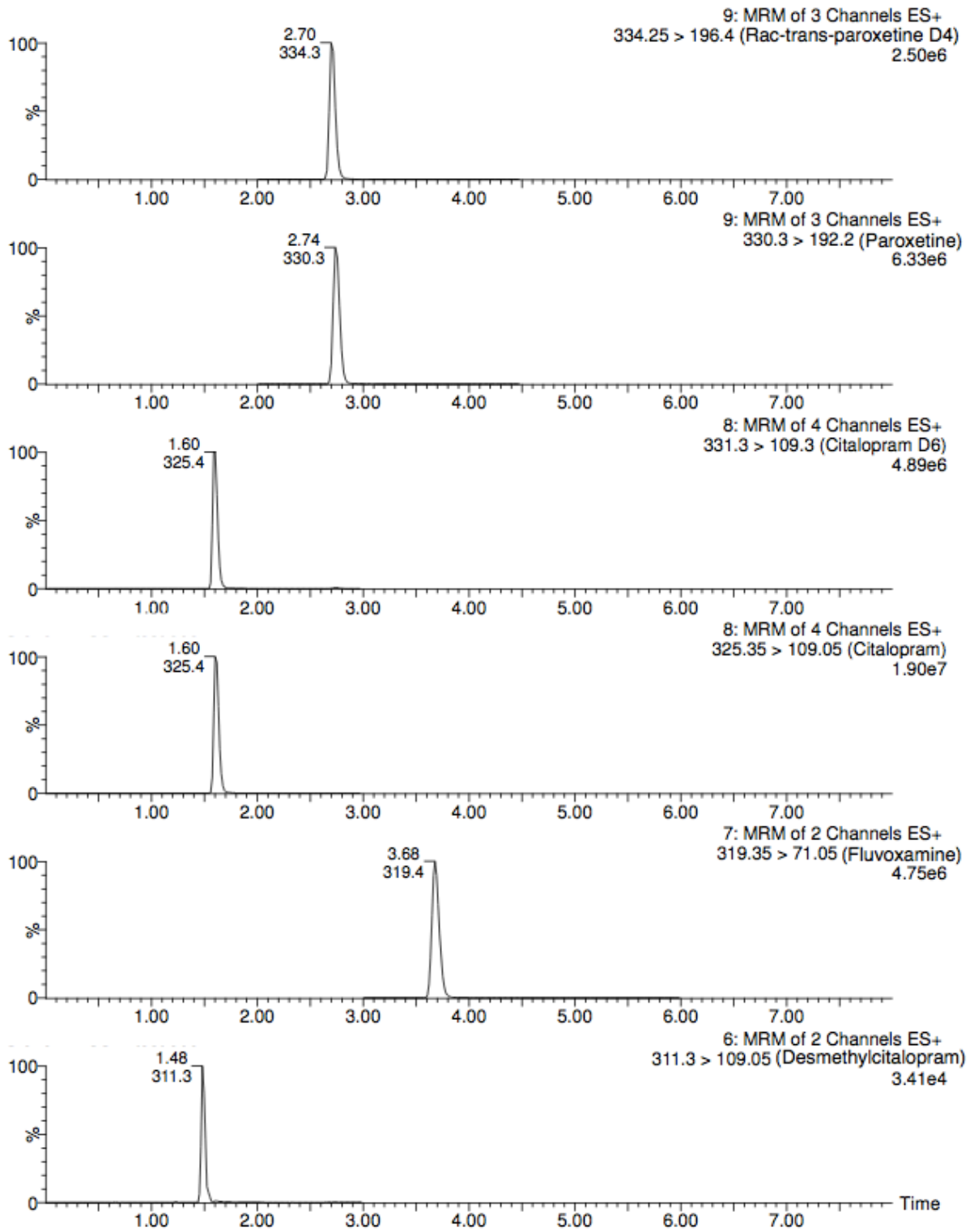
Figure 26: Chromatogram of the f2 growth medium for rac-trans-paroxetine D4, paroxetine, citalopram D6, citalopram, fluvoxamine and desmethylcitalopram.
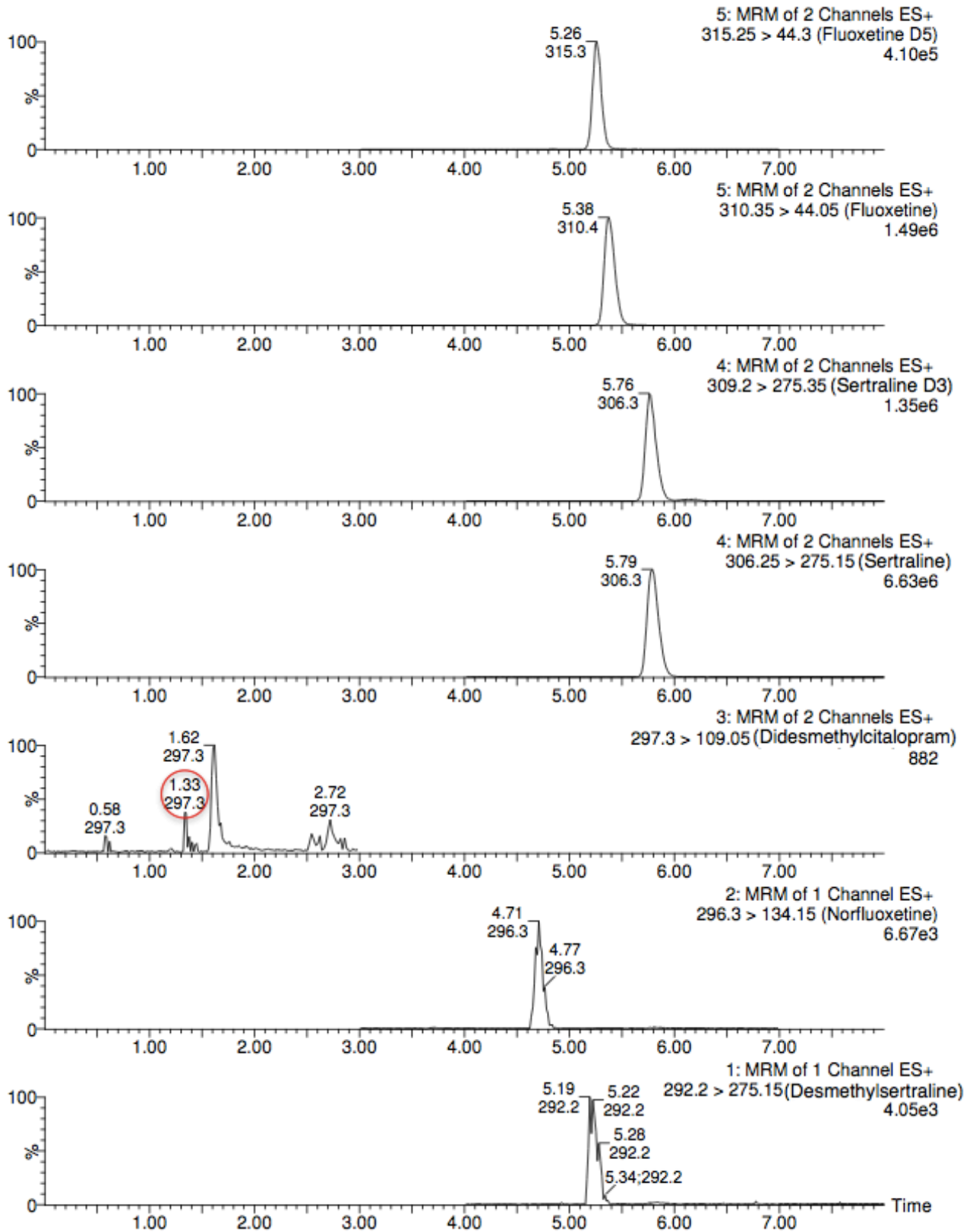
Figure 27: Chromatogram of the f2 growth medium for fluoxetine D5, fluoxetine, sertraline D3, sertraline, didesmetylciralopram, norfluoxetine and desmethylsertraline. The red circle indicates the peak for didesmethylcitalopram.

3.6 Concentrations of Selective Serotonin Reuptake Inhibitors

Three experiments were conducted in total for this thesis. The two diatoms *S. marinoi* and *A. longicornis* were used in the experiments in monocultures. The first experiment included *S. marinoi*, while the two others included *A. longicornis*. For each experiment 3 10L glass beakers were set up. Figure 8 illustrates one of these 3 glass beakers and the tests conducted ("Chl *a* analysis" and "Extraction"). For each of the sampling days, three parallels were run for the "Extraction"-part of the experiment for each "beaker".

In the graphs "Parallel 1" refers to beaker number 1, "Parallel 2" is beaker number 2 and "Parallel 3" is beaker number 3. In the graphs each column is the average measured concentration for the three parallels from each experiment as shown in Figure 8.

3.6.1 Concentrations of Selective Serotonin Reuptake Inhibitors in the *S. marinoi* Experiment

The experiment with *S. marinoi* was the first experiment conducted. *S. marinoi* forming agglomerates at the bottom of the glass beakers was observed from day 2, even though there was added air under pressure to create turbulence .

Detailed measured average concentrations, SD and RSD % for this experiment are found in Appendix 7, while an overview is given in Figure 28 to Figure 36.

For sertraline (Figure 28) there was a decrease in the average measured concentration of 39% from day 1 to day 7 for the parallels combined. From day 7 to day 14 there was a decrease of 27%, while the overall decrease from day 1 to 14 was 55% in the average measured concentration. The daily decrease from day 1 to day 14 was 3.93%.
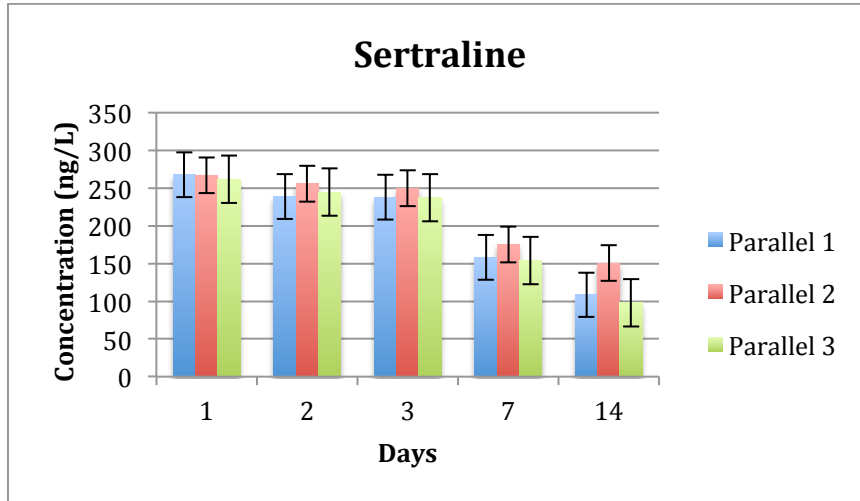
Figure 28: The average measured concentrations of sertraline for each parallel experiment for each sampling day in the *S. marinoi* experiment. The standard deviation for each parallel experiment is also shown.

Fluoxetine (Figure 29) has a decrease in the average measured concentration of 95% from day 1 to day 7 for the parallels combined, and a decrease of 48% from day 7 to day 14. The majority of the decrease happened in the duration of the first 7 days, making the decrease in the last 7 days irrelevant. The overall total decrease from day 1 to day 14 was 98%, with a daily decrease of 7%. From day 1 to day 3 there was 70% decrease. This gives a daily decrease of 23.33% for the first 3 days.

Figure 29: The average measured concentrations of fluoxetine for each parallel experiment for each sampling day in the *S. marinoi* experiment. The standard deviation for each parallel experiment is also shown.

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Fluvoxamine (Figure 30) had the fastest decline in average measured concentration of all the SSRIs in this experiment. The decrease in average measured concentration for all the parallels was 99.5% from day 1 to day 7. From day 7 to day 14 the decrease was 20%, this decrease is irrelevant as the majority of the decrease happened in the first 7 days. The overall decrease from day 1 to day 14 was 99.6%. This shows that fluvoxamine is completely broken down already after 7 days, more so than fluoxetine. The daily decrease in the average measured concentration was 7.11%. From day 1 to day 3 there was 81% decrease. This gives a daily decrease of 27.00% for the first 3 days.
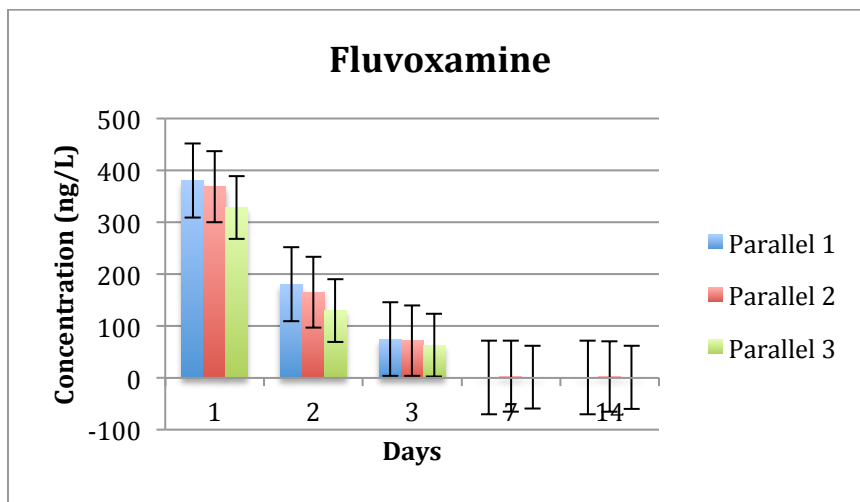
Figure 30: The average measured concentrations of fluvoxamine for each parallel experiment for each sampling day in the *S. marinoi* experiment. The standard deviation for each parallel experiment is also shown.

For paroxetine (Figure 31) the decrease in the average measured concentration from day 1 to day 7 for all the parallels combined was 48%, the same decrease was measured from day 7 to day 14. The overall decrease in the average measured concentration from all the parallels combined from day 1 to day 14 was 73%, which corresponds to a daily decrease of 5.21%.
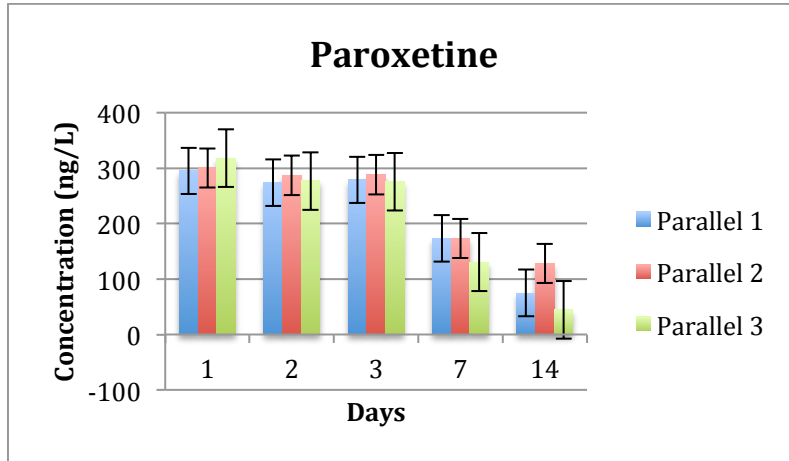
Figure 31: The average measured concentrations of paroxetine for each parallel experiment for each sampling day in the *S. marinoi* experiment. The standard deviation for each parallel experiment is also shown.

Citalopram (Figure 32) had the lowest decrease in the average measured concentration of all the SSRIs in this experiment. For all the parallels combined, there was a decrease in 6% from day 1 to day 7. From day 7 to day 14 the decrease was only 2%, while the overall decrease from day 1 to day 14 was 8%. The daily decrease from day 1 to day 14 was 0.57%.
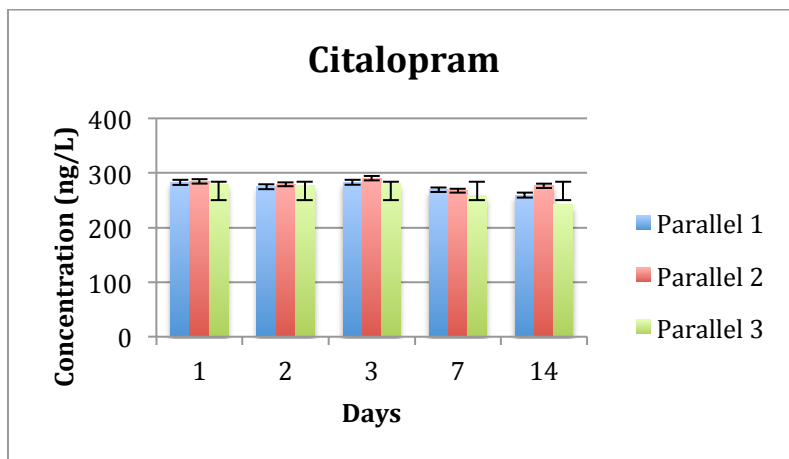
Figure 32: The average measured concentrations of citalopram for each parallel experiment for each sampling day in the *S. marinoi* experiment. The standard deviation for each parallel experiment is also shown.

Desmethylsertraline (Figure 33), norfluoxetine (Figure 34), and didesmethylcitalopram (Figure 35) have average measured concentrations below the lowest concentration in the calibration curve, so the average measured concentrations are uncertain compared to those that are within the calibration curve.

For desmethylsertraline there is an increase in the average measured concentration from day 1 to day 7 of 181%, and 58.72% from day 7 to day 14. The increase in the average measured concentration from day 1 to day 14 is 347%. This gives a daily increase of 19.28%. There is a weak trend in the increasing concentration of desmethylsertraline that indicates that sertraline is degraded to desmethylsertraline, but the concentrations measured of desmethylsertraline are nowhere near of being equivalent to what is degraded from the sertraline concentration. This might indicate that other metabolites are formed or that sertraline accumulate in the diatoms.

Figure 33: The average measured concentrations of desmethylsertraline for each parallel experiment for each sampling day in the *S. marinoi* experiment. The standard deviation for each parallel experiment is also shown.

For norfluoxetine there is an increase from day 1 to day 3, but a decrease in the average measured concentration from day 1 to day 7 of 9% and from day 7 to day 14 of 25.57%. The overall decrease in the average measured concentration was 25.57% from day 1 to day 14. This gives a daily decrease of 1.83%. The concentrations are lower than what would be expected given the degradation of fluoxetine, indicating that norfluoxetine show no trend of being formed during the experiments.

Figure 34: The average measured concentrations of norfluoxetine for each parallel experiment for each sampling day in the *S. marinoi* experiment. The standard deviation for each parallel experiment is also shown.

Didesmethylcitalopram has an increase in average measured concentration of 380% the first 7 days, and a decrease of 72% from day 7 to day 14. There is an overall increase from day 1 to day 14 of 35%. The low concentrations of didesmethylcitalopram indicate that there is no trend of it being formed during the experiment.

Figure 35: The average measured concentrations of didesmethylcitalopram for each parallel experiment for each sampling day in the *S. marinoi* experiment. The standard deviation for each parallel experiment is also shown.

Desmethylcitalopram (Figure 36) is the only one of the metabolites analyzed that has average measured concentrations above the lowest concentration on the standard curve. Desmethylcitalopram had an increase in the average measured concentration for all the parallels combined of 178% from day 1 to day 7. From day 7 to day 14 the increase was 133%, while the overall increase from day 1 to day 14 was 546%. This gives a daily increase of 39.00%. The average measured concentrations for desmethylcitalopram are low, but degradation from citalopram to desmethylcitalopram is indicated. This trend is not visible for didesmethylcitalopram.

Figure 36: The average measured concentrations of desmethylcitalopram for each parallel experiment for each sampling day in the *S. marinoi* experimen. The standard deviation for each parallel experiment is also shown.

Examples of chromatograms of the *S. marinoi* experiment for all the SSRI ions used in the quantification method are shown in Figure 37 and Figure 38.
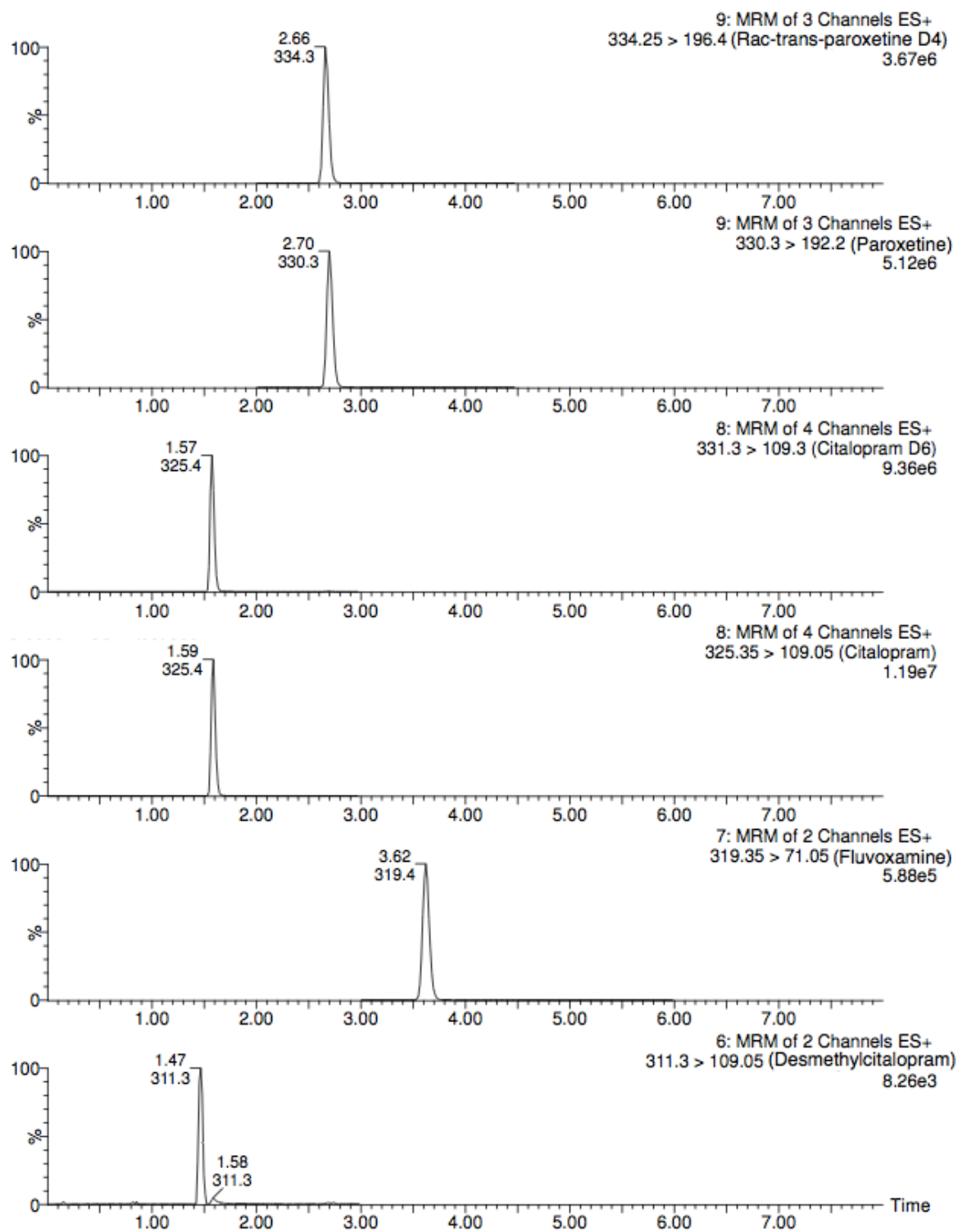
Figure 37: Chromatogram of the *S. marinoi* experiment for rac-trans-paroxetine D4, paroxetine, citalopram D6, citalopram, fluvoxamine and desmethylcitalopram for day 3 of the experiment.
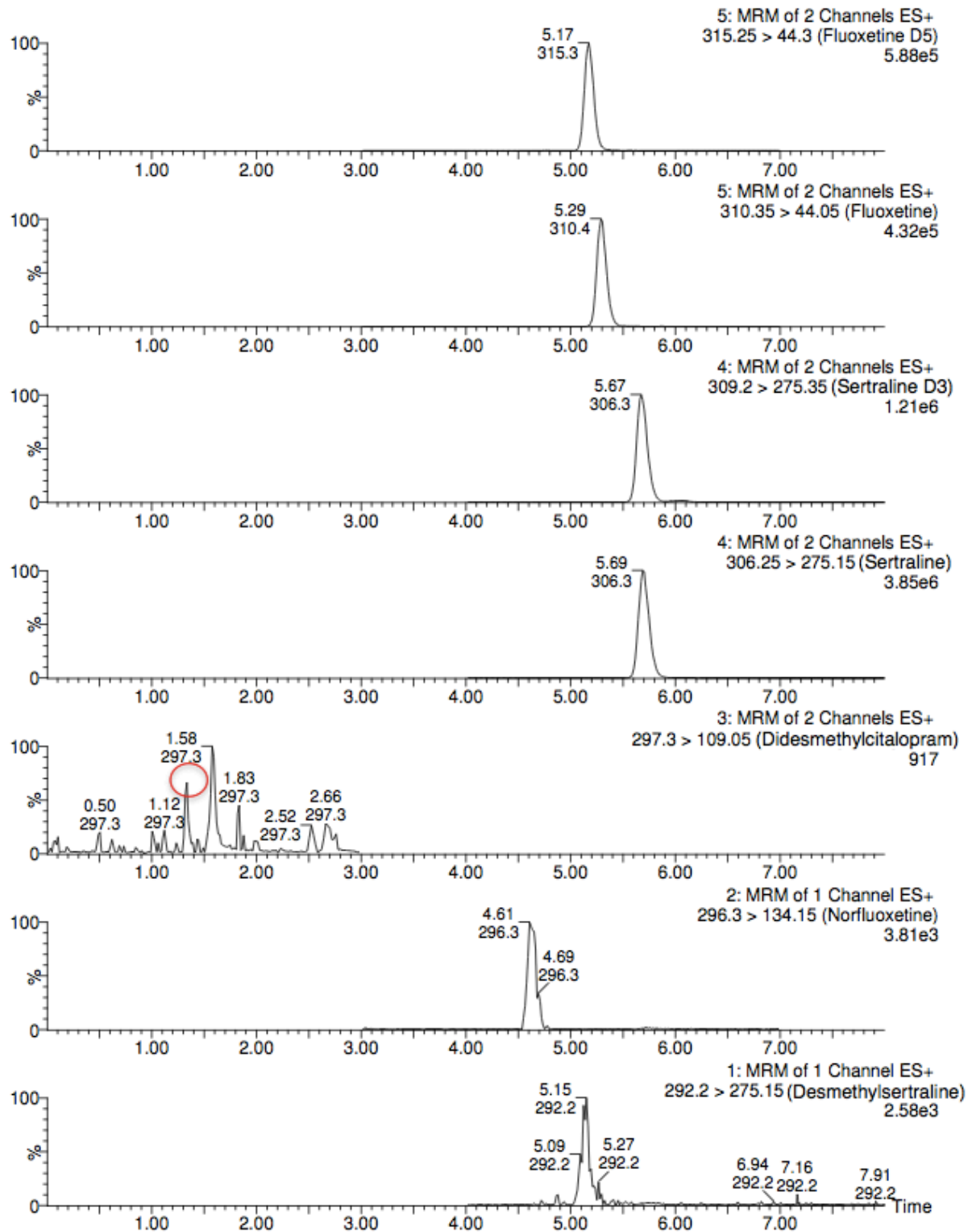
Figure 38: Chromatogram of the *S. marinoi* experiment for fluoxetine D5, fluoxetine, sertraline D3, sertraline, didesmetylciralopram, norfluoxetine and desmethylsertraline for day 3 of the experiment. The red circle indicates the peak for didesmethylcitalopram.

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3.6.2 Concentrations of Selective Serotonin Reuptake Inhibitors in the First *A. longicornis* Experiment

In this experiment there was no added air under pressure to the three glass beakers. The *A. longicornis*, drifted down and sedimented at the bottom of the beakers. This was not discovered until day 8 in the experiment because of the monitoring of cell growth, and from that day of the experiment all three beakers were manually stirred every day except for day 11 and 12. This has most likely had an impact on the average measured concentrations, and will be discussed later.

Detailed measured average concentrations, SD and RSD % for this experiment are found in Appendix 8, while an overview is given in Figure 39 to Figure 47.

Sertraline, fluoxetine, paroxetine and citalopram had an increase in concentration during the first days of the experiment. An increase in concentration should not occur, and the cause for this increase might be that there is something with these samples that make the extraction of sertraline more efficient or it might be due to variations in the quantification method.

Sertraline (Figure 39) had an increase in the average measured concentration for all the parallels combined of 11% from day 1 to day 7. There was a decrease of 26% in average measured concentration for all the parallels combined from day 7 to day 14, and an overall decrease from day 1 to day 14 of 18%. This gives a daily decrease of 1.29%. An increase in the concentration is not possible and is probably due to the extraction or quantification method. It is most likely that the decrease in average measured concentration did not occur until manual stirring of the glass beakers was initiated.

Figure 39: The average measured concentrations of sertraline for each parallel experiment for each sampling day in the first *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

Fluoxetine (Figure 40) had an increase in the average measured concentration of 6% from day 1 to day 2 for all the parallels combined. From day 1 to day 7, however, there was a decrease of 0.8%. From day 7 to day 14 there was a decrease of 9%. There was a total decrease in the average measured concentration of 10% from day 1 to day 14 for all the parallels combined, giving a daily decrease of 0.71%.

Figure 40: The average measured concentrations of fluoxetine for each parallel experiment for each sampling day in the first *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

Fluvoxamine (Figure 41) had an increase in average measured concentration from day 1 to day 7 of 3% for all the parallels combined. From day 7 to day 14 there was a decrease in the average measured concentration of 5%, with an overall decrease from day 1 to day 14 of 3% for all the parallels combined. There was a daily decrease of 0.21% from day 1 to day 14. Just as for sertraline, an increase in the concentration is not possible and is probably due to the extraction or quantification method. It is also most likely that the decrease in average measured concentration did not occur until manual stirring of the glass beakers was initiated.
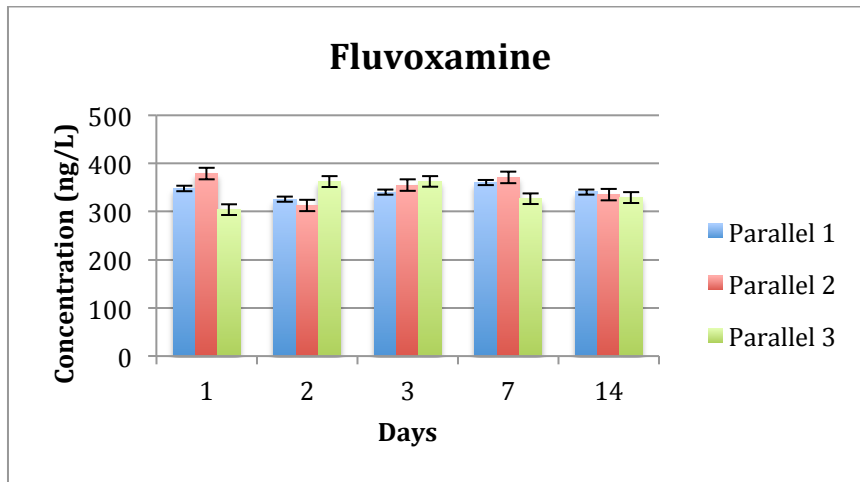
Figure 41: The average measured concentrations of fluvoxamine for each parallel experiment for each sampling day in the first *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

Paroxetine (Figure 42) had a decrease of 7% from day 1 to day 7 in the average measured concentration for all the parallels combined. From day 7 to day 14 the decrease was 21%, and from day 1 to day 14 the decrease was 27%, which corresponds to a daily decrease of 1.93%.
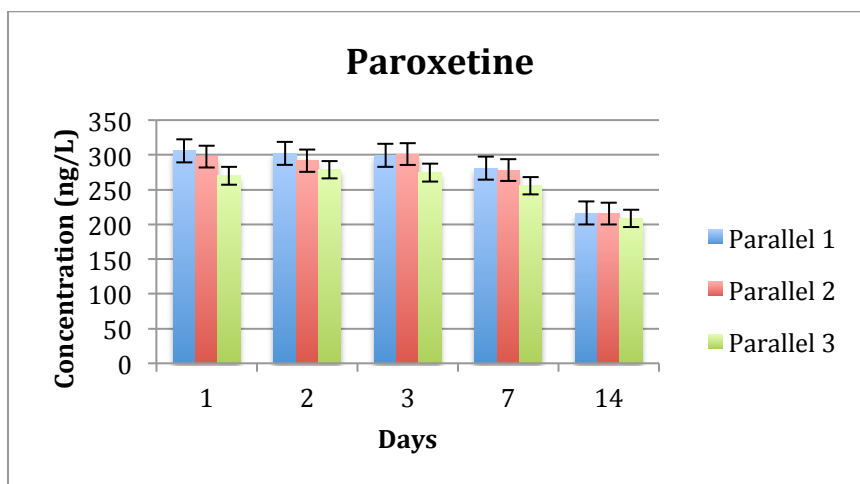
Figure 42: The average measured concentrations of paroxetine for each parallel experiment for each sampling day in the first *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

Citalopram (Figure 43) had an increase of 7% from day 1 to day 7 in the averaged measured concentration for all parallels combined. The increase from day 7 to day 14 was 3%, while the overall increase from day 1 to day 14 was 10%. This gives a daily increase of 0.71%. Just as with sertraline and fluvoxamine, it is most likely that the decrease in average measured concentration did not occur until manual stirring of the glass beakers was initiated. Just as for sertraline and fluyvoxamine, an increase in the concentration is not possible and is probably due to the extraction or quantification method. It is also most likely that the decrease in average measured concentration did not occur until manual stirring of the glass beakers was initiated.
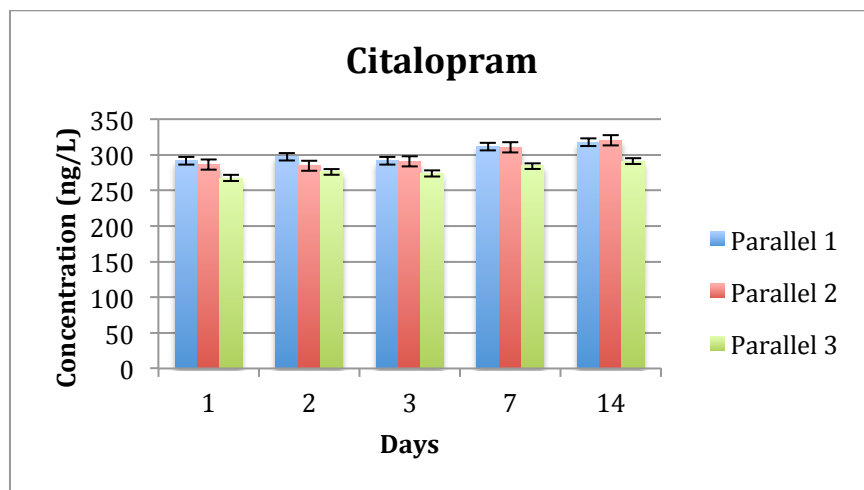
Figure 43: The average measured concentrations of citalopram for each parallel experiment for each sampling day in the first *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

Desmethylsertraline (Figure 44) and didesmethylcitalopram (Figure 45) had average measured concentrations below the lowest concentration in the calibration curve, so the average measured concentrations are uncertain compared to those that are within the calibration curve. The low concentrations of these metabolites are reflected in the chromatogram in Figure 49.

For desmethylsertraline there was an increase in the average measured concentration from day 1 to day 7 of 207%, and from day 7 to day 14 of 34%. The overall increase from day 1 to day 14 was 310%, giving a daily increase of 22.14%. Even though the concentrations are low, there is still an increasing trend indicating that sertraline is degraded to desmethylsertraline.
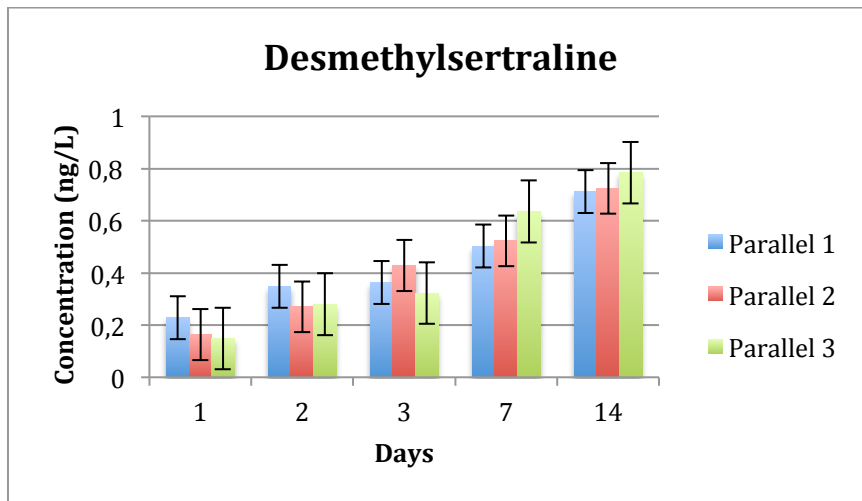
Figure 44: The average measured concentrations of desmethylsertraline for each parallel experiment for each sampling day in the first *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

Didesmethylcitalopram had an increase in the average measured concentration from day 1 to day 7 of 140%. From day 7 to day 14 there was a decrease of 12%, and an overall increase in the average measured concentration from day 1 to day 14 of 112%. This gives a daily increase of 8.00%. There is a considerable variation in the measurements for didesmethylcitalopram, and this makes it difficult to predict a trend.
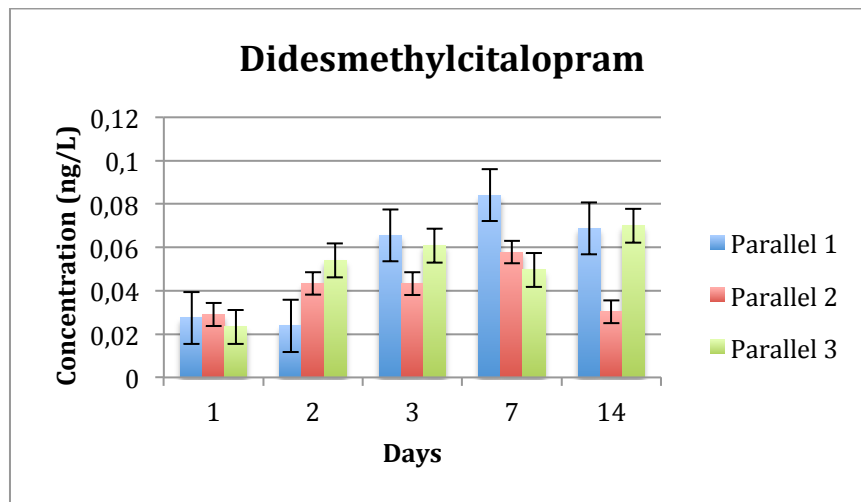
Figure 45: The average measured concentrations of didesmethylcitalopram for each parallel experiment for each sampling day in the first *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

The first three measurements for norfluoxetine (Figure 46) and desmethylcitalopram (Figure 47) are below the lowest concentration of the calibration curve. For norfluoxetine, the increase in average measured concentrations for all parallels was184% from day 1 to day 7, and 79% between day 7 and 14. The overall increase in average measured concentration was 410% from day 1 to day 14, giving a daily increase of 29.29%. The concentrations are low, but never the less, there is a trend indicating that fluoxetine is being degraded to norfluoxetine.
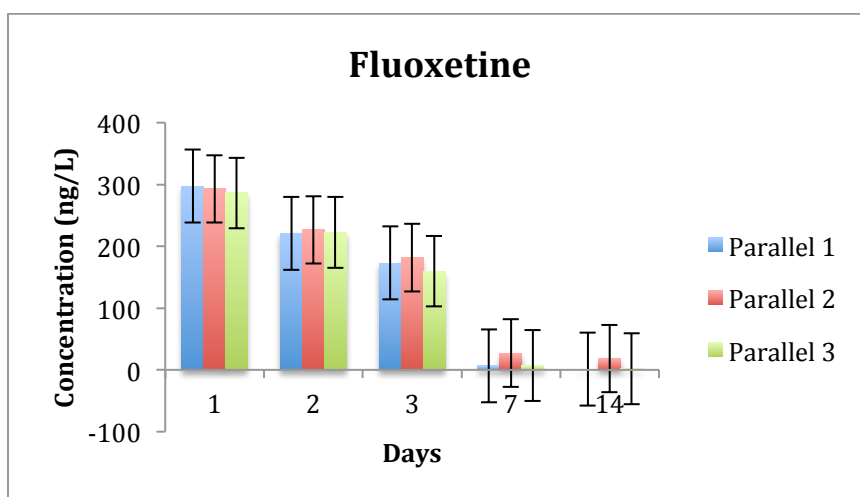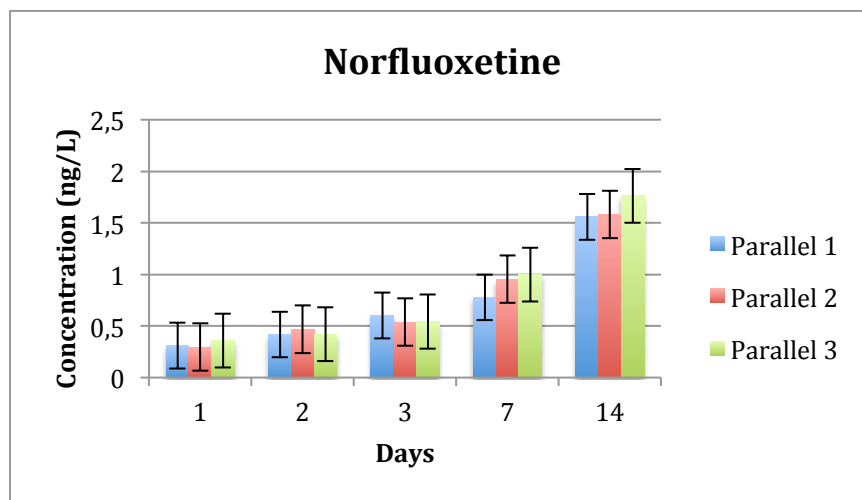

Figure 46: The average measured concentrations of norfluoxetine for each parallel experiment for each sampling day in the first *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

Desmethylcitalopram had an increase in the average measured concentration of 151% from day 1 to day 7. There was a decrease in the average measured concentration of 11% from day 7 to day 14. From day 1 to day 14 the increase was 124%, which corresponds with a daily increase of 8.86%. The average measured concentrations for desmethylcitalopram are low, but they still indicate a degradation of citalopram to desmethylcitalopram.
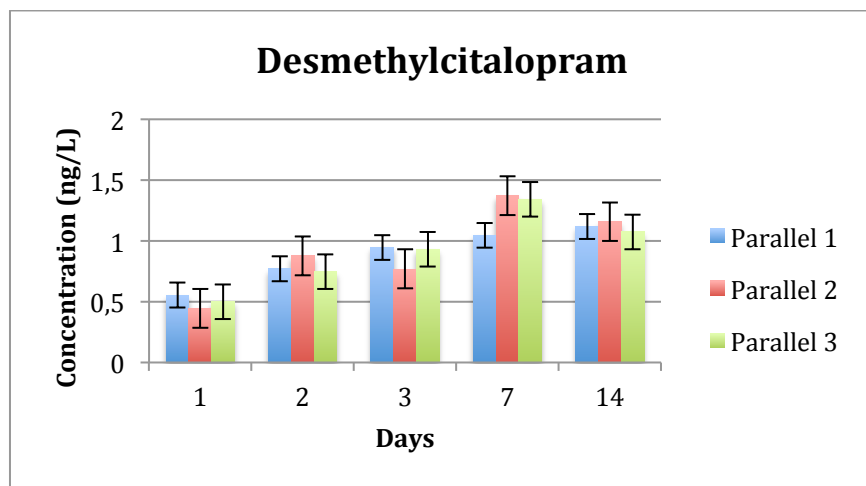
Figure 47: The average measured concentrations of desmethylcitalopram for each parallel experiment for each sampling day in the first *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

Examples of chromatograms of the first *A. longicornis* experiment for all the SSRI ions in the quantification method are shown in Figure 48 and Figure 49.
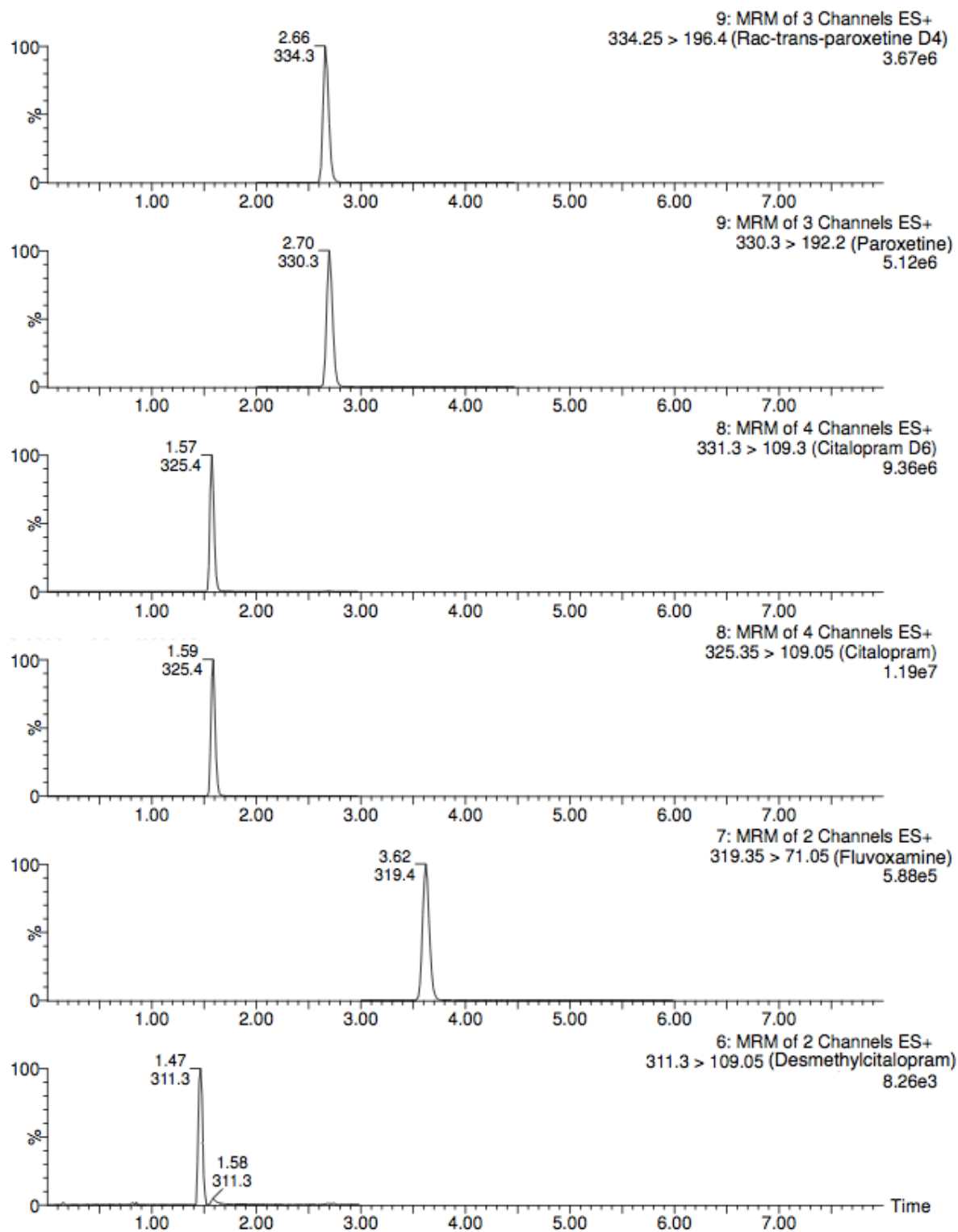
Figure 48: Chromatogram of the first *A. longicornis* experiment for rac-trans-paroxetine D4, paroxetine, citalopram D6, citalopram, fluvoxamine and desmethylcitalopram for day 3 of the experiment.

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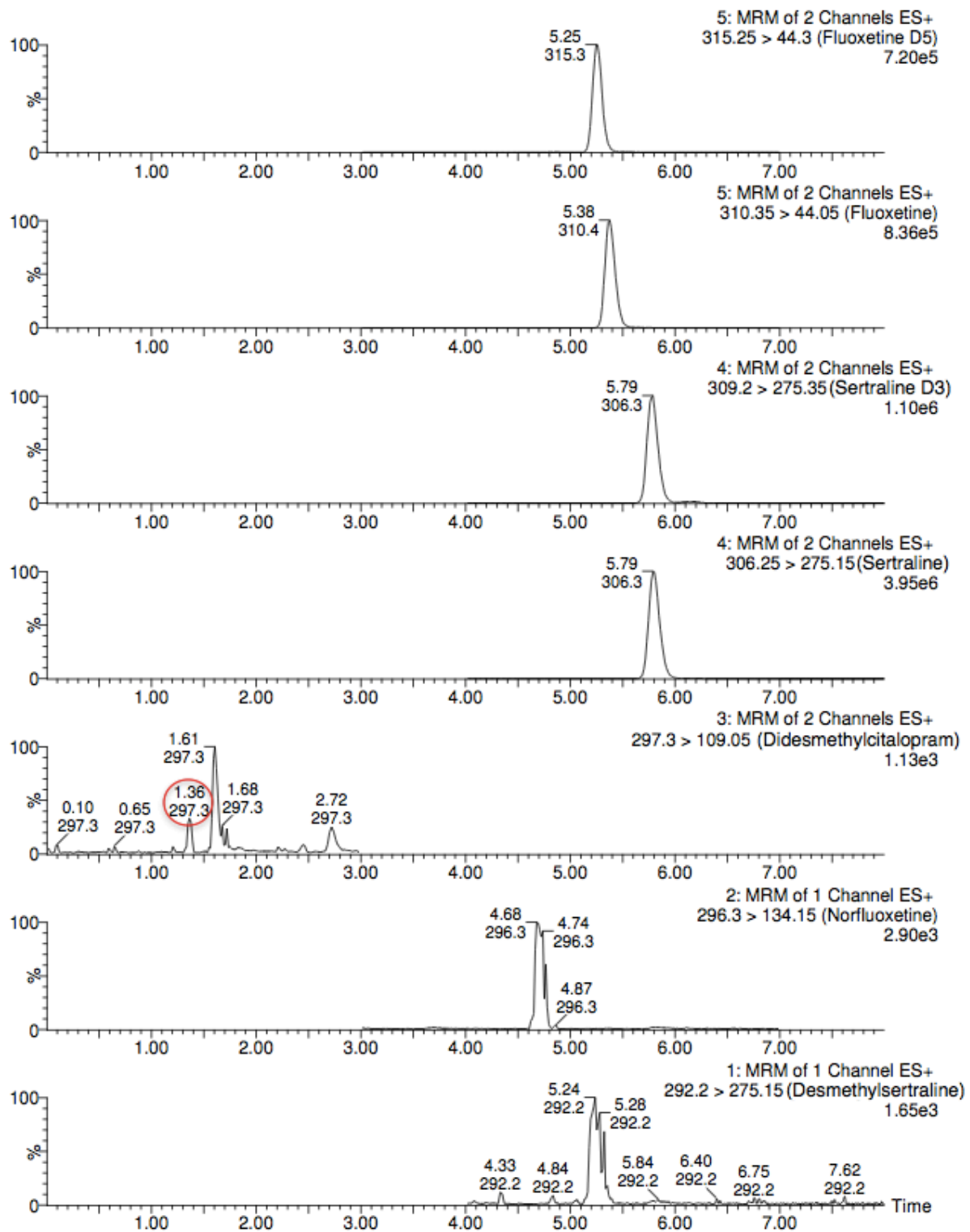
Figure 49: Chromatogram of the first *A. longicornis* experiment for fluoxetine D5, fluoxetine, sertraline D3, sertraline, didesmetylciralopram, norfluoxetine and desmethylsertraline for day 3 of the experiment. The red circle indicates the peak for didesmethylcitalopram.

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3.6.3 Concentrations of Selective Serotonin Reuptake Inhibitors in the Second *A. longicornis* Experiment

Since it was discovered that stirring in the samples might have an impact on the results in the first *A. longicornis* experiment, air under pressure was added to all three glass beakers, giving turbulence so that the A. longicornis would be suspended in the sample. To begin with, there was manually stirring as well, except for days 5 and 6, until day 9. At day 10 there was discovered a bacterial contamination in "Parallel 3", and at day 11 in "Parallel 2". At day 14 the bacterial contamination was present in "Parallel 1" as well. This is illustrated in Figure 50. "Parallel 2" and "Parallel 3" have an opalescent color, which indicates a bacterial contamination.
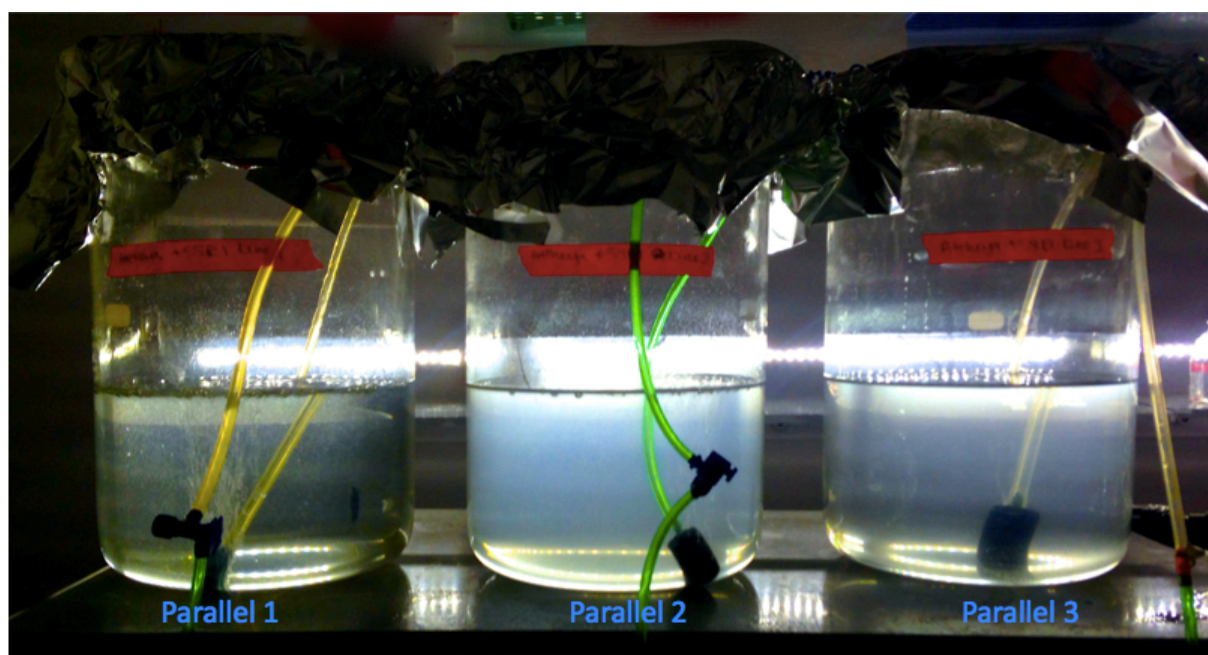
Figure 50: Set up of the second *A. longicornis* experiment. "Parallel 2" and "Parallel 3" are opalescent, which indicates a bacterial contamination. (Picture taken by: Kine Smellror)

Detailed measured average concentrations, SD and RSD % for this experiment are found in Appendix 9, while an overview is given in Figure 28 to Figure 36.

For sertraline (Figure 51) there was a decrease in the average measured concentration of 67% for all parallels combined from day 1 to 9. From day 9 to day 16 the decrease was 32%, while the overall decrease from day 1 to day 16 was 78%, which corresponds to a daily decrease of 4.88%. From day 2, "Parallel 2" had a higher average measured concentration than the two other parallels.
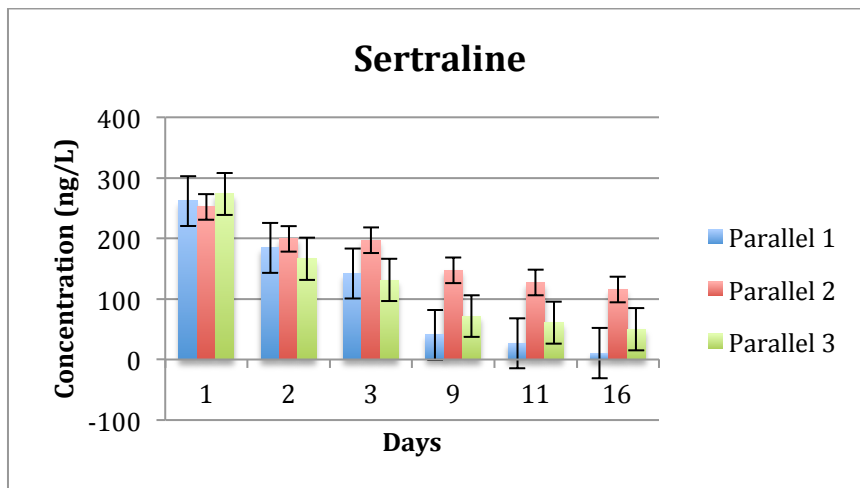
Figure 51: The average measured concentrations of sertraline for each parallel experiment for each sampling day in the second *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

Fluoxetine (Figure 52) has an increase in the average measured concentration from day 1 to day 2 of 31% for all the parallels combined. This increase in concentration should not occur, and as mentioned before, this might be because of the efficiency of the extraction or the quantification method. From day 2 to day 9 there was a decrease of 68%. A 58% decrease was observed from day 1 to day 9, and from day 9 to day 16 there is a decrease of 48% for the average measured concentration for all parallels combined. The overall decrease from day 1 to day 16 was 78%. This gives a daily decrease of 4.88%. From day 3, "Parallel 3" shows a higher concentration than the other two parallels. "Parallel 1" and "Parallel 2" combined show a decrease of 93% from day 1 to day 16, while "Parallel 3" only had a 50% decrease in that same period.
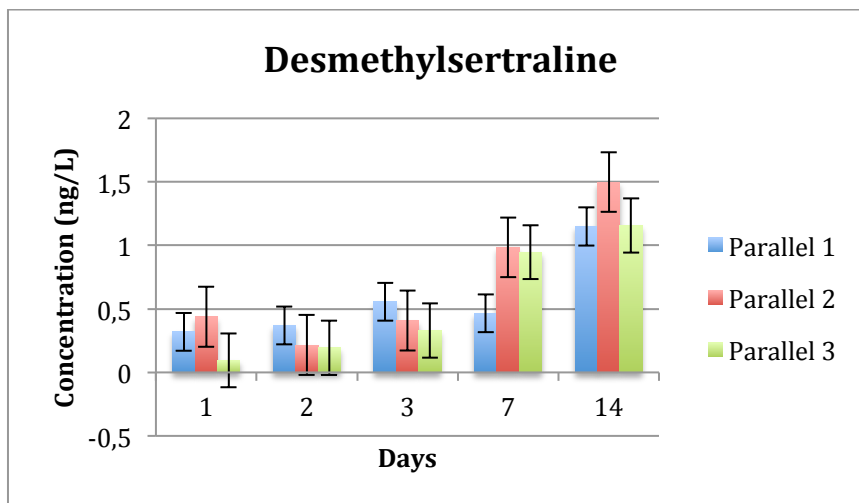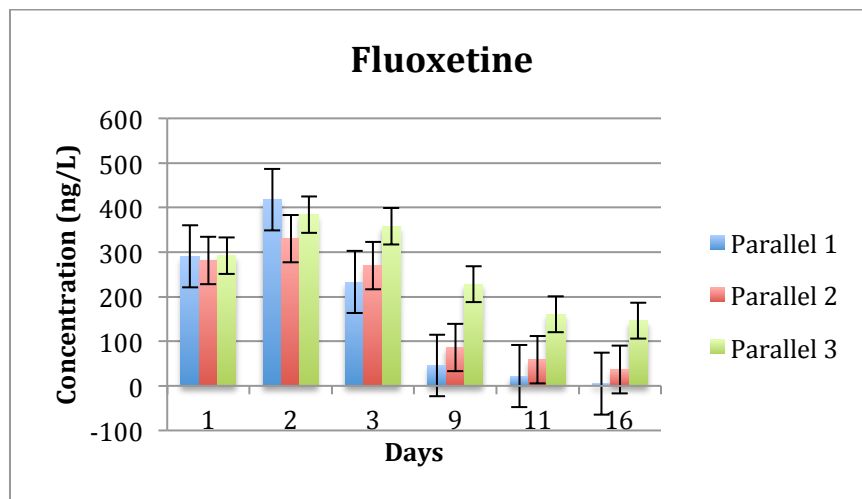

Figure 52: The average measured concentrations of fluoxetine for each parallel experiment for each sampling day in the second *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

For fluvoxamine (Figure 53), when combining all the parallels, there is a decrease in the average measured concentration of 87% from day 1 to day 9, 43% from day 9 to day 16, and 93% from day 1 to day 16, giving a daily decrease of 5.81%. "Parallel 3" shows a higher concentration from day 3, just as in fluoxetine. "Parallel 1" and "Parallel 2" combined show a decrease of 99% from day 1 to day 16, while "Parallel 3" only had a 80% decrease in that same period.
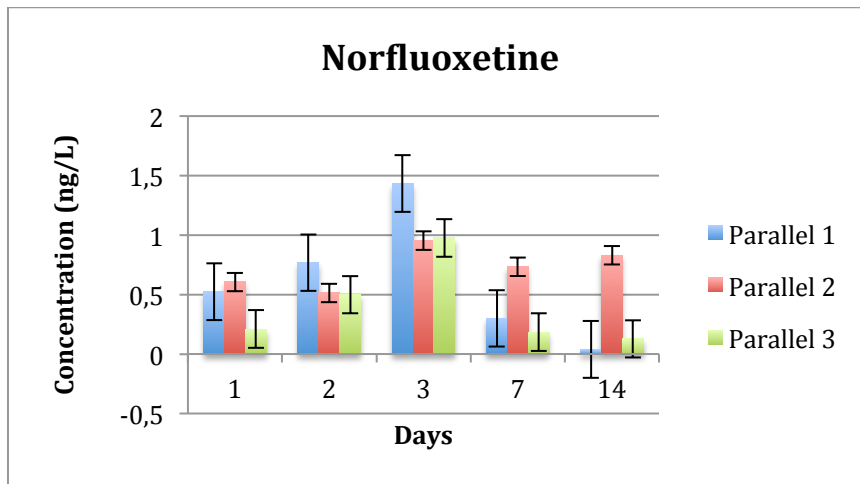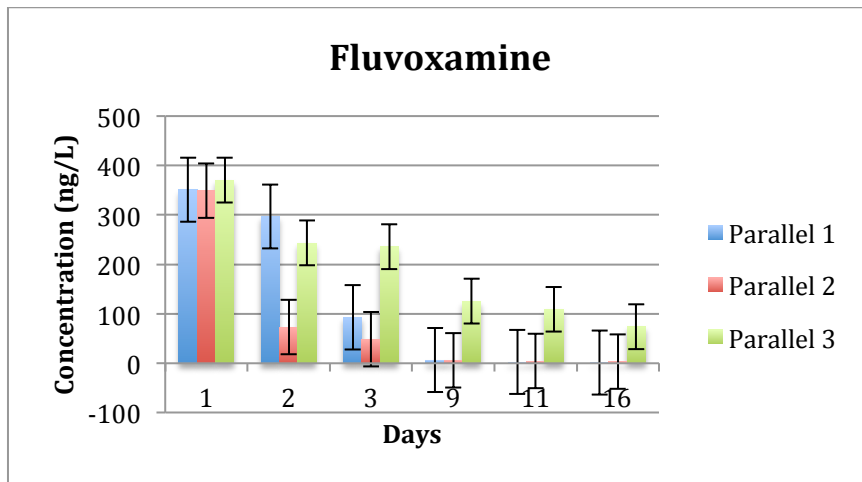

Figure 53: The average measured concentrations of fluvoxamine for each parallel experiment for each sampling day in the second *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

For paroxetine (Figure 54) there was a decrease in the average measured concentration for all the parallels combined of 44% from day 1 to day 9 and 42% from day 9 to day 16. The overall decrease in the average measured concentration from day 1 to day 16 was 47%. This gives a daily decrease of 2.94%. "Parallel 3" has a higher average measured concentration from day 3.
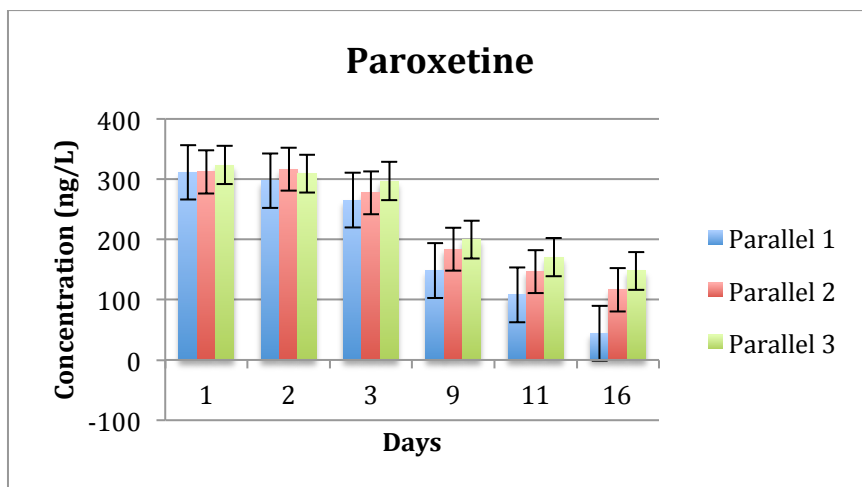
Figure 54: The average measured concentrations of paroxetine for each parallel experiment for each sampling day in the second *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

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Citalopram (Figure 55) has an increase in the average measured concentration of 33% for all the parallels combined from day 1 to day 2. There cannot be an actual increase in concentration, but rather indicating an uncertainty in the extraction or quantification method. It is more likely to be the quantification method. The values from day 2 to day 16 give a more realistic view, and will be used from this point onwards. From day 2 to day 9 there is an increase in the average measured concentration of 1%, but from day 9 to day 16 there is a decrease of 5%. From day 2 to day 16 there is a decrease in the average measured concentration of 4%, which corresponds to a daily decrease of 0.27%.
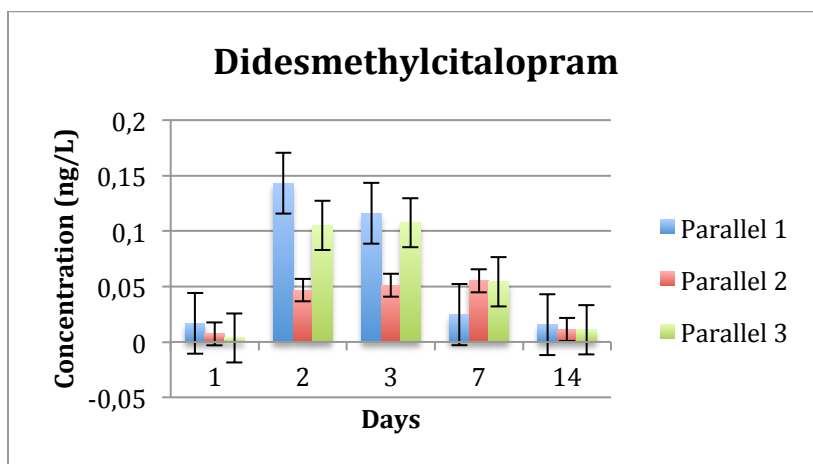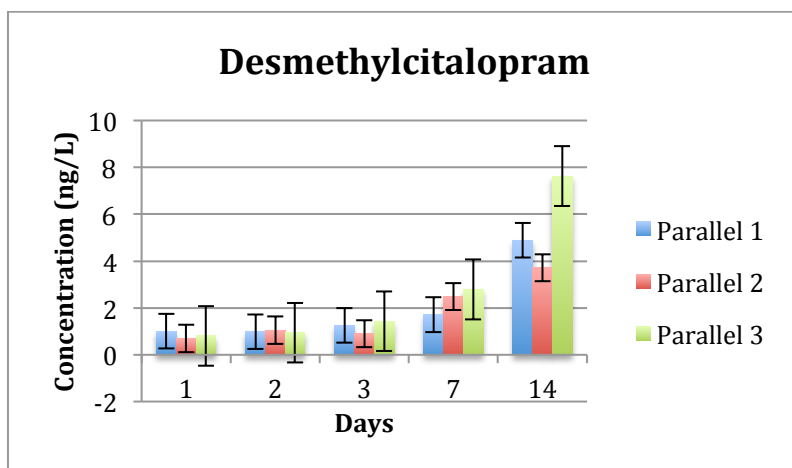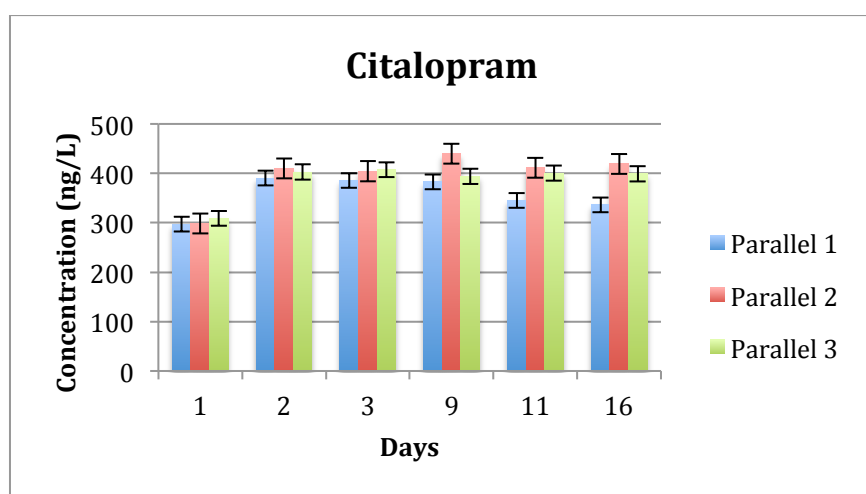

Figure 55: The average measured concentrations of citalopram for each parallel experiment for each sampling day in the second *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

Desmethylsertraline (Figure 56) and didesmethylcitalopram (Figure 57) had average measured concentrations below the lowest concentration in the calibration curve, so the average measured concentrations are uncertain compared to those that are within the calibration curve. Desmethylsertraline had an increase in the average measured concentration from day 1 to day 9 of 6%. From day 9 to day 16 the increase was 22%, and the overall increase from day 1 to day 16 was 19%, giving a daily increase of 1.19%. Desmethylsertraline have a higher concentration of "Parallel 2", just like sertraline. The average measured concentrations do not indicate the degradation of sertraline to desmethylsertraline.
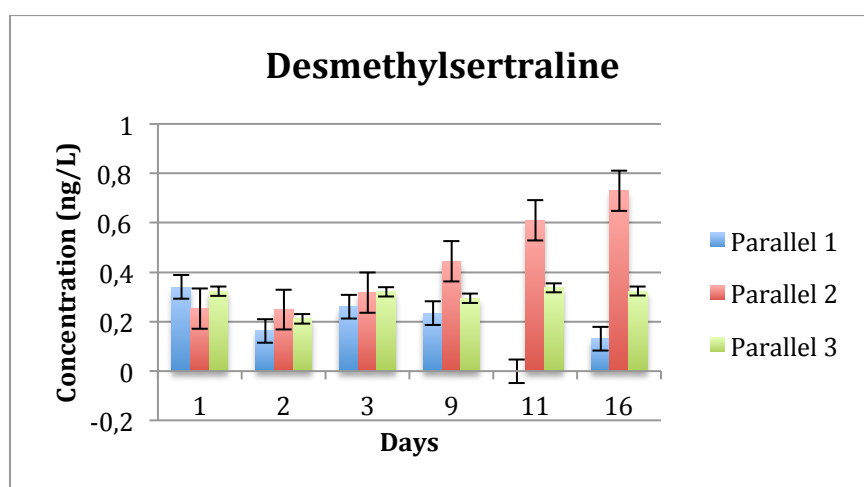
Figure 56: The average measured concentrations of desmethylsertraline for each parallel experiment for each sampling day in the second *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

For didesmethylcitalopram there is an increase in the average measured concentration from day 1 to day 9 of 71%, while from day 9 to day 16 there is a decrease of 35%. This gives an overall increase in the average measured concentration of 10% from day 1 to day 16, with a daily increase of 0.63%. There is a considerable variation in the measurements for didesmethylcitalopram, and this makes it difficult to predict a trend.
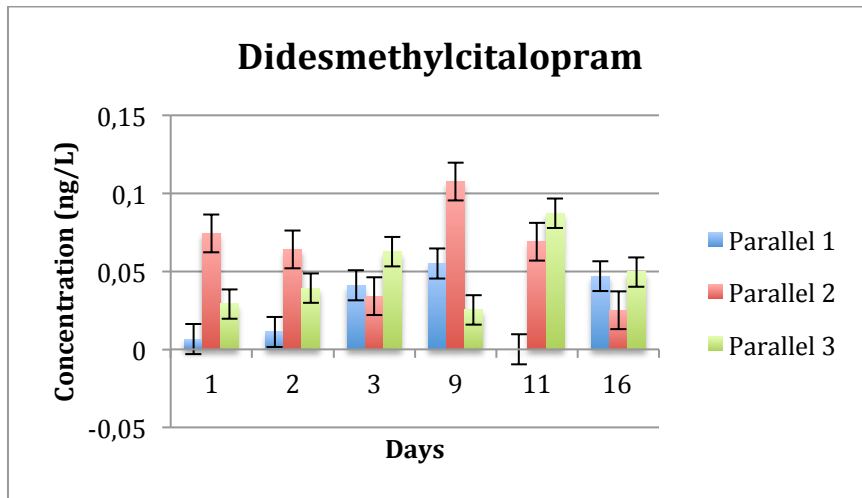
Figure 57: The average measured concentrations of didesmethylcitalopram for each parallel experiment for each sampling day in the second *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

For norfluoxetine (Figure 58) the average measured concentrations are above the lowest concentration for the calibration curve from day 2 for "Parallel 2" and "Parallel 3", and "Parallel 1" from day 3. The average measured concentration for "Parallel 1" increases with 191% from day 1 to day 3, and decreases with 80% from day 3 to day 16. The other two parallels increase with 287% from day 1 to day 9, and decrease with 15% from day 9 to day 16. There is an overall increase in the average measured concentration of 228% from day 1 to day 16, which corresponds to a daily increase of 14.25%. This increase in the average measured concentration, even though the concentrations are low, indicates that fluoxetine degrades to norfluoxetine.
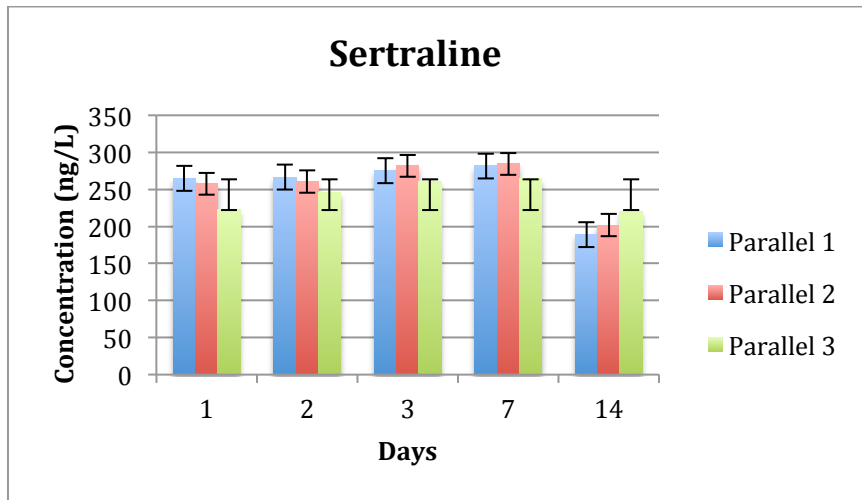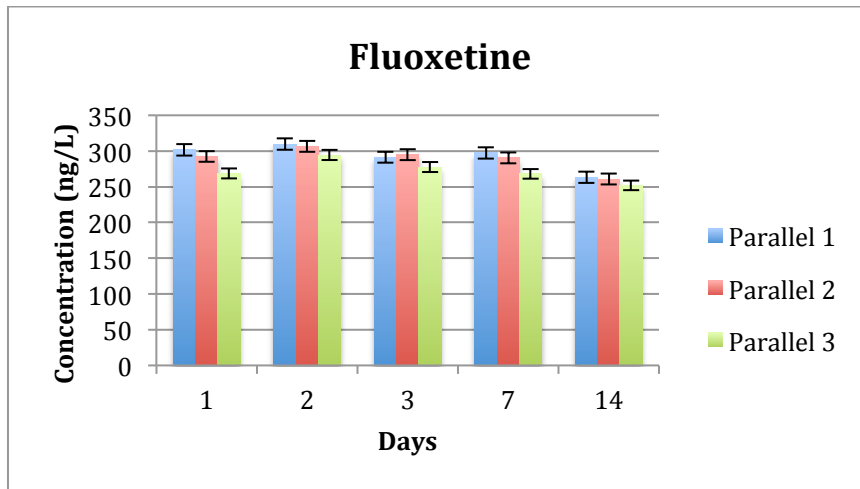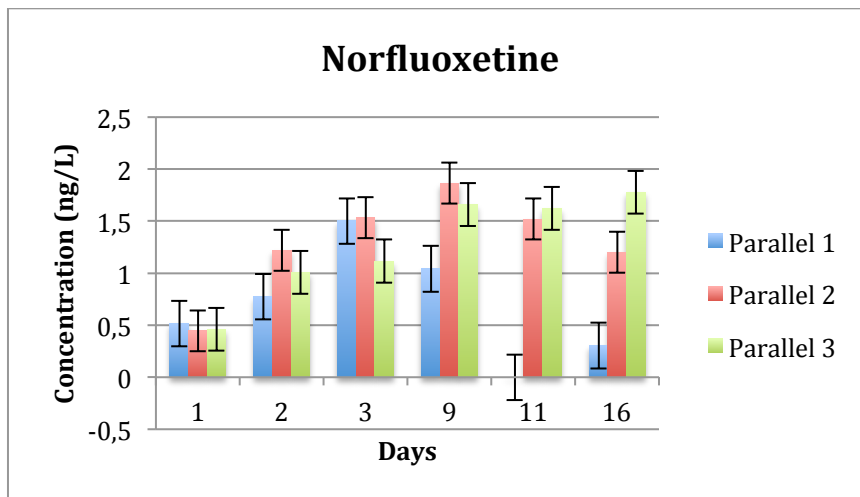

Figure 58: The average measured concentrations of norfluoxetine for each parallel experiment for each sampling day in the second *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

For desmethylcitalopram (Figure 59) the average measured concentrations are above the lowest concentration for the calibration curve from day 2. From day 1 to day 9 there was an increase of 480%, while from day 2 to day 9 there was an increase of 231% for all parallels combined. From day 9 to day 16 there was a low increase of 14%, and the overall increase from day 1 to day 16 was 561%. This gives a daily increase of 35.06%. Citalopram has a measured (not real) increase in concentration from day 1 to day 9, especially from day 1 to day 2. There is a decrease in the citalopram average measured concentration from day 9 to day 16, which may indicate that citalopram is degraded to desmethylcitalopram.
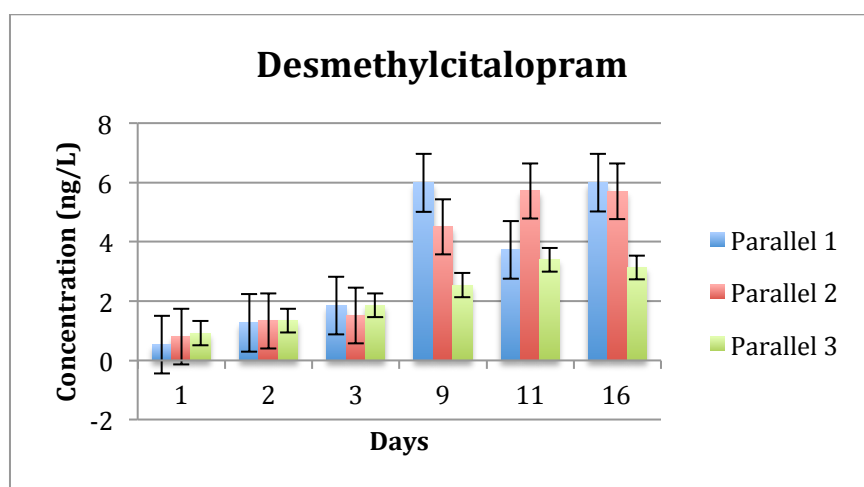
Figure 59: The average measured concentrations of desmethylcitalopram for each parallel experiment for each sampling day in the second *A. longicornis* experiment. The standard deviation for each parallel experiment is also shown.

Fluoxetine, fluvoxamine and paroxetine had a higher average measured concentration in "Parallel 3" than in the other parallels. There was observed less air under pressure in "Parallel 3". This could have had an effect on the average measured concentration, especially since manual stirring stopped after day 9 due to bacterial contamination. The bacterial contamination might also have affected the average measured concentration. The bacterial contamination was observed in "Parallel 3" first. This may have affected the average measured concentrations for fluoxetine, fluvoxamine and paroxetine as well.

Examples of chromatograms of the second *A. longicornis* experiment for all the SSRI ions in the quantification method are shown in Figure 60 and Figure 61.
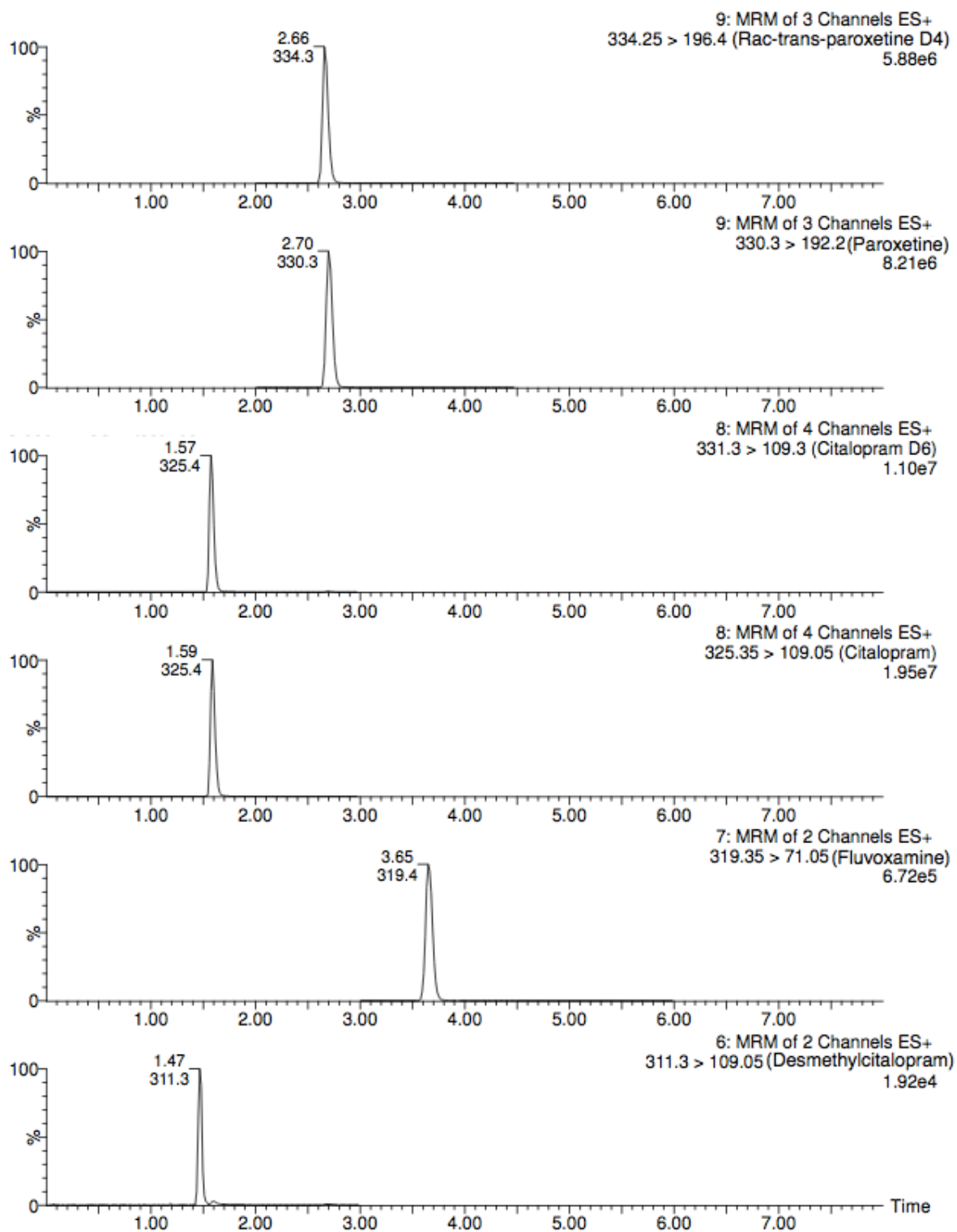
Figure 60: Chromatogram of the second *A. longicornis* experiment for rac-trans-paroxetine D4, paroxetine, citalopram D6, citalopram, fluvoxamine and desmethylcitalopram for day 3 of the experiment.
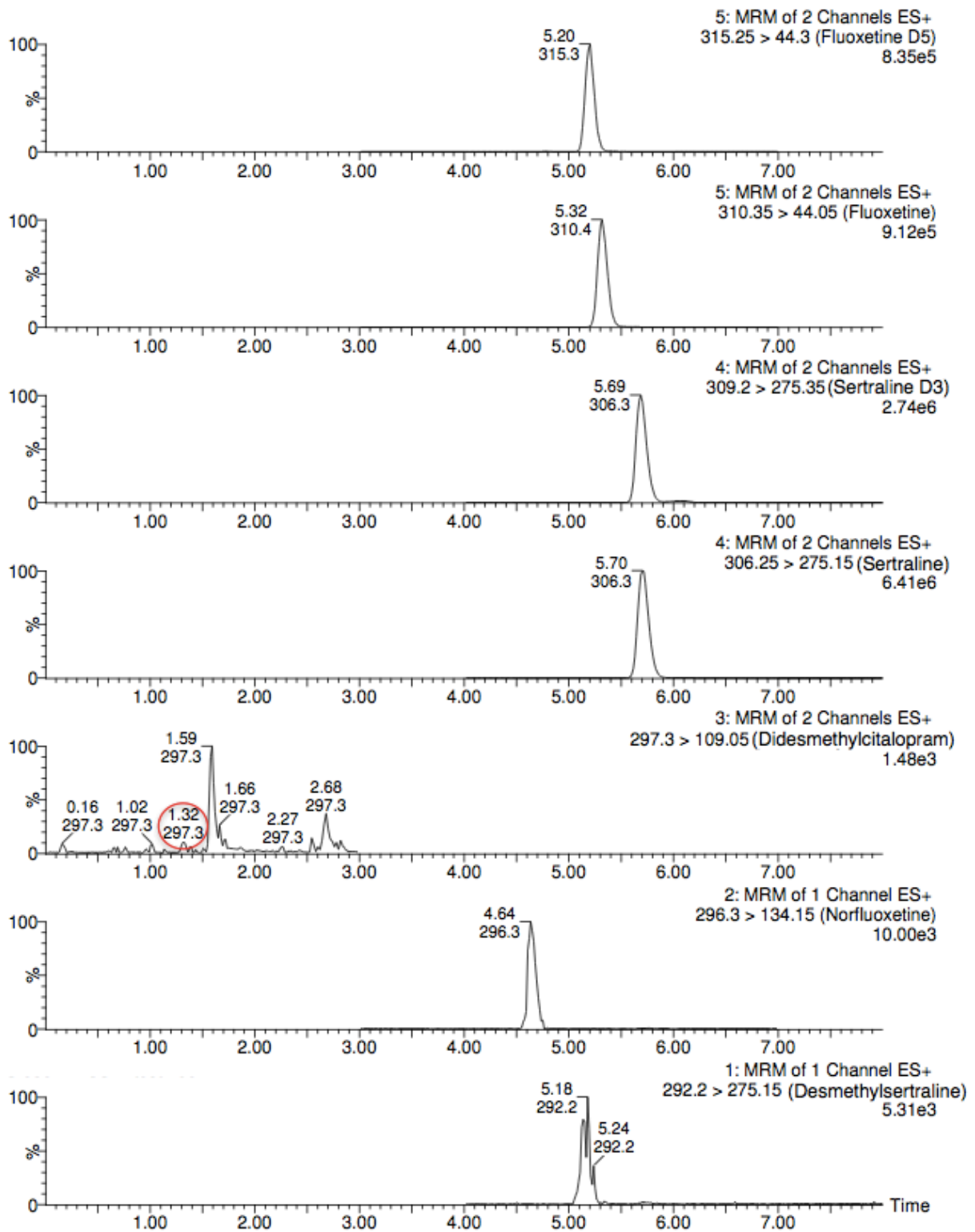
Figure 61: Chromatogram of the second *A. longicornis* experiment for fluoxetine D5, fluoxetine, sertraline D3, sertraline, didesmetylciralopram, norfluoxetine and desmethylsertraline for day 3 of the experiment. The red circle indicates the peak for didesmethylcitalopram.

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3.7 Comparing the Experiments

In the S. marinoi experiment the largest daily and overall growth rate was between the first and second measurements, i.e. between day 1 and day 7. The largest decrease in the average measured concentrations for fluoxetine and fluvoxamine was between day 1 and day 7 in the experiment, while the other SSRIs, sertraline, paroxetine and citalopram, had a larger decrease from day 7 to day 14.

In the first *A. longicornis* experiment the largest daily and overall growth rate was between day 7 and day 14. Sertraline, fluoxetine, fluvoxamine and paroxetine had the largest decreases in the average measured concentrations in this period compared to the first 7 days. This might have been caused by the lack of stirring and turbulence from added air under pressure the first 8 days.

The second *A. longicornis* experiment also had the largest daily and overall growth rate was between day 7 and day 14. This was to be expected. The decreases in the average measured concentrations from day 1 to day 9 were largest for sertraline, fluoxetine, fluvoxamine and paroxetine.

There is an overall constant decrease of the average measured concentration of the SSRIs, but over time it seems that the concentrations do not have an effect on the algae growth.

The first *A. longicornis* experiment has a lower decrease and increase in average measured concentrations than the second *A. longicornis* experiment. This is probably due to the lack of turbulence in the glass beakers because air under pressure was not applied. This was not discovered until day 8 of the experiment. The *S. marioni* experiment will therefor be compared to the second *A. longicornis* experiment from now on.

Table 13 shows the decrease of average measured concentrations of all the experiments in % between the first and the last day of the experiments. The *S.marinoi* experiment and the *A. longicornis* experiment were conducted over 14 days, while the second *A.longicornis* experiment was conducted over 16 days. This might have had an effect on the decrease and increase in average measured concentrations.

The *S. marinoi* experiment had a large decrease in average measured concentrations for fluoxetine, fluvoxamine, paroxetine and citalopram compared to the decrease in average measured concentrations of *A. longicornis*, while *A. longicornis* had a larger decrease than *S. marinoi* in average measured concentration for sertraline.

Table 13: The decrease of the average measured concentration in % for each SSRI from the first to the last day of each experiment. *A. Longicornis* 1 refers to the first *A. Longicornis* experiment, while *A. Longicornis* 2 refers to the second *A. Longicornis* experiment.

	Experiment		
SSRI	*S. marinoi*	*A. longicornis* 1	*A. longicornis* 2
Sertraline	55%	18%	78%
Fluoxetine	98% (95%)*	10%	78%
Fluvoxamine	99.6% (99.5%)*	4%	93% (87%)*
Paroxetine	73%	27%	47%
Citalopram	8%	0	4%

*Degradation from day 1 to day 7 given in parenthesis.

From Table 13 one sees that in the *S. marinoi* experiment fluoxetine and fluvoxamine had a higher decrease in the average measured concentrations compared the other SSRIs. Fluoxetine and fluvoxamine had a decrease of 98% and 99.6% respectably from day 1 to day 14, but already after 7 days fluoxetine had decreased with 95% and fluvoxamine had decreased with 99.5%. In the second *A. longicornis* experiment fluvoxamine had the highest decrease in the average measured concentration compared to the other SSRIs with a 93% decrease from day 1 to day 14, and a decrease of 87% the first 7 days. Fluoxetine had a decrease of 78% from day 1 to day 14. This shows that fluoxetine and fluvoxamine were the least stable SSRIs.

Citalopram had a stable average measured concentration in both experiments, with low degradation, and sertraline had a decrease of 55% in the *S. marinoi* experiment and 78% in the second *A. longicornis* experiment.

Table 14 shows the daily decrease of average measured concentrations of all the experiments in % between the first and the last day of the experiments.

Table 14: The decrease of the daily average measured concentration in % for each SSRI from the first to the last day of each experiment. *A. Longicornis* 1 refers to the first *A. Longicornis* experiment, while *A. Longicornis* 2 refers to the second *A. Longicornis* experiment.

	Control			Experiment	
SSRI	**f/2**	**Substral**	***S. marinoi***	***A. longicornis* 1**	***A. longicornis* 2**
Sertraline	1.00%	0.38%	3.93%	1.29%	4.88%
Fluoxetine	1.38%	1.13%	7.00% (13.57%)*	0.71%	4.88%
Fluvoxamine	1.13%	0.75%	7.11% (14.21%)*	0.21%	5.81% (9.67%)*
Paroxetine	1.63%	3.00%	5.21%	1.93%	2.94%
Citalopram	0.75%	0.38%	0.57%	0	0.27%

* Degradation from day 1 to day 7 given in parenthesis.

For the average measured concentrations in the *S. marinoi* experiment, both fluoxetine (7.00%) and fluvoxamine (7.11%) have a higher daily decrease in concentration than the other SSRIs from day 1 to day 14. Fluvoxamine had a daily decrease of 13.57% the first seven days, while fluoxetine hade a daily decrease of 14.21% in the same period. In the second *A. longicornis* experiment, there was a high daily decrease of fluvoxamine (5.81%) compared to the other SSRIs from day 1 to day 16, with daily decrease of 9.67% the first 9 days.

Citalopram shows a low daily decrease in both the *S. marinoi* and the second *A. longicornis* experiment in the average measured concentrations of 0.57% (day 1 to day 14) and 0.27% (day 2 to day 16) respectably. Sertraline had a decrease in the average measured concentration of 3.93% in the *S. marinoi* experiment and 4.88% in the second *A. longicornis* experiment.

In a mete-analysis conducted by Webb et al (12) it was stated that fluoxetine and fluvoxamine were 10 of the most acute toxic pharmaceuticals. The Stockholm County Council (9) concludes with fluoxetine being a low environmental risk and fluoxetine being an insignificant environmental risk in the aquatic environment. In this study there is an almost complete degradation of both fluoxetine and fluvoxamine in both experiments, which may indicate that they have a low or an insignificant environmental risk in the aquatic environment even though they have been shown to have acute toxic effects.

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Citalopram had a stable average measured concentration in both experiments, with low degradation. The Stockholm County Council (9) does not have sufficient documentation to draw a conclusion about the environmental risk for citalopram in the aquatic environment. One cannot conclude from this study any environmental risk factor for citalopram.

In an environmental risk assessment study conducted by Styrishave et al (13) they looked at cocktail effect exposure of sertraline, citalopram and fluoxetine. It was concluded with sertraline being the most toxic of the three SSRIs, and was the most likely to contribute to a cocktail effect after STP degradation. The Stockholm County Council (9) concludes with sertraline being a moderate environmental risk. The degradation of sertraline is not complete for any of the experiments in this study, so one can only assume that the same grade of degradation is a possible scenario in a marine environment. In that case, there is a possibility of sertraline being a moderate environmental risk. It would be of interest to investigate this closer.

Diatoms may release allelopathic compounds as a biochemical defense mechanism. They are also in need of nutrients and have pores and enzymatic pathways in order to retrieve the nutrients the need. It might be possible that either these allelopathic compounds or that they may have used the SSRIs as nutrients may have been factors in the decreasing average measured concentrations of the SSRIs.

An interesting perspective is the fact that Norway is traditionally a maritime nation, where export of fish is a large, important industry for the country. It is important that we, through our own research, keep an international credibility in our knowledge about the influence pharmaceuticals and other pollutants have on the environment. We need to be able to confirm or disprove the claims that international press make from time to time, that will directly affect or export market.

4. Conclusion and Further Perspectives

This thesis showed that there was a difference in degradation of the SSRIs between the two monocultures of *S. marinoi* and *A. longicornis*. The experiment containing the diatom *S. marinoi* had a higher decrease in the average measured concentration for fluoxetine, fluvoxamine, paroxetine and citalopram than the *A. longicornis* experiments. Fluoxetine and fluvoxamine were the least stable SSRIs. For the first 7 days of the *S. marinoi* experiment, fluoxetine had a decrease of 95%, while fluvoxamine had a decrease of 99.5%, while fluvoxamine had a decrease of 87% from day 2 to day 9, in the *A. longicornis* experiment.

The Stockholm County Council classifies Sertraline as a moderate environmental risk, which is the highest environmental risk given to any of the SSRIs. In this study sertraline had a slower degradation in the average measured concentration compared to fluoxetine and fluvoxamine, but it was not as stable as Citalopram.

LPME was used for the extraction and up concentration of the SSRIs, while UPLC-MS/MS with an MRM method was used for detection and quantification. These methods are well suited for the extraction and quantification of SSRIs from seawater containing algae and growth media.

For a further perspective, one should optimize the MRM method used in this experiment by giving the SSRIs and metabolites their own "time window" in the method. This would increase the sensitivity of the method. One should also analyze the samples on a UPLC coupled to a quadrupole time of flight mass spectrometer. This would help identify more compounds in the sample, for example other metabolites and degradation products.

In order to asses the consequences pharmaceuticals have on diatoms, it would be interesting to look at how diatoms initially react to pharmaceuticals, if they consume them in any way, and if so, to also look at possible metabolizing pathways. But since there is an estimate of more than 200,000 different species of diatoms, this will be difficult to for each and every one.

There is a need for studies that look at degradation of pharmaceuticals and their metabolites in the environment, and the possible uptake of these in aquatic organisms. Not only for separate pharmaceuticals, but one should also take into account the possibility for "cocktail effects".

Bioaccumulation and biomagnification of pharmaceuticals in food webs should also be looked into.

Very few countries have publications or databases that can give statistics on drug consumption, and therefore there are no available data on the total amount of pharmaceuticals used in the world, and the consumption of pharmaceuticals differ form country to country. A model for worldwide pharmaceutical use and how these pharmaceuticals end up in the marine environment is needed.

5. References

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Appendix

Appendix 1

The composition of earth extract and silicate solution.

Table 15: The composition of earth extract

Earth extract	Amount
Earth, dried in the oven at about $50^{o}C$	300 mL
MilliQ water	600 mL

Table 16: The composition of silicate solution

Silicate solutin	Amount
Sodium meta-silicate nonahydrate	3,5 g
MilliQ water	1000 mL
HCl (37%)	20 mL

Appendix 2

Table 17 shows the parameters used in the mass spectrometer.

Table 17: Parameters for the mass spectrometer.

Type	Start Mass	End Mass	Set Mass
MS2 Scan	2.00	598.00	

Source (ES-)	Settings	Readbacks
Capillary (kV)	1.50	1.54
Cone (V)	30.00	-35.62
Extractor (V)	3.00	-5.86
Source Temperature (°C)	150	150
Desolvation Temperature (°C)	350	350
Cone Gas Flow (L/Hr)	OFF	
Desolvation Gas Flow (L/Hr)	800	797
Collision Gas Flow (mL/Min)	0.15	0.14

Analyser	Settings	Readbacks
LM 1 Resolution	3.0	
HM 1 Resolution	15.0	
Ion Energy 1	0.5	
MS Mode Collision Energy	2.00	
MSMS Mode Collision Energy	20.00	
MS Mode Entrance	0.50	
MS Mode Exit	0.50	
Gas On MS Mode Entrance	0.50	
Gas On MS Mode Exit	0.50	
Gas On MSMS Mode Entrance	0.50	
Gas On MSMS Mode Exit	0.50	
Gas Off MS Mode Entrance	30.00	
Gas Off MS Mode Exit	30.00	
Gas Off MSMS Mode Entrance	30.00	
Gas Off MSMS Mode Exit	30.00	
ScanWave MS Mode Entrance	0.50	
ScanWave MS Mode Exit	0.50	
ScanWave MSMS Mode Entrance	0.50	
ScanWave MSMS Mode Exit	0.50	
LM 2 Resolution	3.0	
HM 2 Resolution	15.0	
Ion Energy 2	0.5	
Gain	1.00	
Multiplier	529.43	
Active Reservoir	Wash	

Pressure Gauges		
Collision Cell Pressure (mbar)	$2.424462 \times 10^{-003}$	

Instrument Configuration		
MS Inter-scan delay (secs)	0.005	
Polarity/Mode switch Inter-scan delay (secs)		0.020
Enhanced switch Inter-scan delay (secs)		0.020
Inter-channel delay (secs)	0.005	

Appendix 3

Parameters for the TargetLynx method are shown in Table 18 through Table 34.

Table 18: Compound name and quantification trace for the TargetLynx method.

	Compound Name	CAS Number	Compound Type	Quantification Trace	Include Quan Trace in Response?
1	Desmetylsertralin (1)			292.2 > 275.15	YES
2	Norfluoxetine			296.3 > 134.15	YES
3	Didesmetylcitalopram			297.3 > 109.05	YES
4	Sertralin			306.25 > 275.15	YES
5	Sertralin D3			309.2 > 275.35	YES
6	Fluoxetine			310.35 > 44.05	YES
7	Fluoxetine D5			315.25 > 44.3	YES
8	Desmetylcitalopram			311.3 > 109.05	YES
9	Fluvoxamin			319.35 > 71.05	YES
10	Citalopram			325.35 > 109.05	YES
11	Citalopram D6			331.3 > 109.3	YES
12	Paroxetin			330.3 > 192.2	YES
13	Race-trans paroxetin D4			334.25 > 196.4	YES

Table 19: Parameters for the chromatogram mass window for the TargetLynx method.

	Use absolute mass win...	Chro Mass Window (Da)	Chro Mass Window (ppm)	Locate Peak Using	Propagate RT?
1	YES	0.0200	10.0000	Retention Time	NO
2	YES	0.0200	10.0000	Retention Time	NO
3	YES	0.0200	10.0000	Retention Time	NO
4	YES	0.0200	10.0000	Retention Time	NO
5	YES	0.0200	10.0000	Retention Time	NO
6	YES	0.0200	10.0000	Retention Time	NO
7	YES	0.0200	10.0000	Retention Time	NO
8	YES	0.0200	10.0000	Retention Time	NO
9	YES	0.0200	10.0000	Retention Time	NO
10	YES	0.0200	10.0000	Retention Time	NO
11	YES	0.0200	10.0000	Retention Time	NO
12	YES	0.0200	10.0000	Retention Time	NO
13	YES	0.0200	10.0000	Retention Time	NO

Table 20: Retention time parameters for the compounds named in Table 18.

	Predicted RT (mins)	Predicted RRT	RRT Reference	RT Window (mins) ±	RT Upper Tol (%)	RT Lower Tol (%)
1	5.3000	1.0000	None	1.0000	0.00	0.00
2	4.7200	1.0000	None	1.0000	0.00	0.00
3	1.3800	1.0000	None	1.0000	0.00	0.00
4	5.9800	1.0000	None	2.0000	0.00	0.00
5	5.8000	1.0000	None	1.0000	0.00	0.00
6	5.5000	1.0000	None	1.0000	0.00	0.00
7	5.3000	1.0000	None	1.0000	0.00	0.00
8	1.5100	1.0000	None	1.0000	0.00	0.00
9	3.7200	1.0000	None	1.0000	0.00	0.00
10	1.6400	1.0000	None	1.0000	0.00	0.00
11	1.6200	1.0000	None	1.0000	0.00	0.00
12	2.7000	1.0000	None	1.0000	0.00	0.00
13	2.7400	1.0000	None	1.0000	0.00	0.00

Table 21: Parameters for the TargetLynx metod.

	Propagate Accur...	Qual Data Win	Max i-FIT	Flag i-FIT?	Max i-FIT Norm	Flag i-FIT Norm?	Min i-FIT Conf
1	NO	0.0200	100.0	NO	0.0	NO	95.0
2	NO	0.0200	100.0	NO	0.0	NO	95.0
3	NO	0.0200	100.0	NO	0.0	NO	95.0
4	NO	0.0200	100.0	NO	0.0	NO	95.0
5	NO	0.0200	100.0	NO	0.0	NO	95.0
6	NO	0.0200	100.0	NO	0.0	NO	95.0
7	NO	0.0200	100.0	NO	0.0	NO	95.0
8	NO	0.0200	100.0	NO	0.0	NO	95.0
9	NO	0.0200	100.0	NO	0.0	NO	95.0
10	NO	0.0200	100.0	NO	0.0	NO	95.0
11	NO	0.0200	100.0	NO	0.0	NO	95.0
12	NO	0.0200	100.0	NO	0.0	NO	95.0
13	NO	0.0200	100.0	NO	0.0	NO	95.0

Table 22: Symmetry thresholds and calibration references for the compounds used.

	Totals Group	Totals Include	Symmetry Thresh (%)	Always Print?	Multiply Traces?	Calibration Ref Compound
1		All	90.0000	YES	NO	1: Desmetylsertralin (1)
2		All	90.0000	YES	NO	2: Norfluoxetine
3		All	90.0000	YES	NO	3: Didesmetylcitalopram
4		All	90.0000	YES	NO	4: Sertralin
5		All	90.0000	YES	NO	5: Sertralin D3
6		All	90.0000	YES	NO	6: Fluoxetine
7		All	90.0000	YES	NO	7: Fluoxetine D5
8		All	90.0000	YES	NO	8: Desmetylcitalopram
9		All	90.0000	YES	NO	9: Fluvoxamin
10		All	90.0000	YES	NO	10: Citalopram
11		All	90.0000	YES	NO	11: Citalopram D6
12		All	90.0000	YES	NO	12: Paroxetin
13		All	90.0000	YES	NO	13: Race-trans paroxetin D4

Table 23: The Response type and uses, polynomial type, calibration origin, weight function and axis transformation.

	Response Type	Response Uses	Polynomial Type	Calibration Origin	Weighting Function	Axis Transformation	Concentration Units
1	External (absolute ...	Area	Linear	Exclude	1/X	None	
2	External (absolute ...	Area	Linear	Exclude	1/X	None	
3	External (absolute ...	Area	Linear	Exclude	1/X	None	
4	External (absolute ...	Area	Linear	Exclude	1/X	None	
5	External (absolute ...	Area	Linear	Exclude	1/X	None	
6	External (absolute ...	Area	Linear	Exclude	1/X	None	
7	External (absolute ...	Area	Linear	Exclude	1/X	None	
8	External (absolute ...	Area	Linear	Exclude	1/X	None	
9	External (absolute ...	Area	Linear	Exclude	1/X	None	
10	External (absolute ...	Area	Linear	Exclude	1/X	None	
11	External (absolute ...	Area	Linear	Exclude	1/X	None	
12	External (absolute ...	Area	Linear	Exclude	1/X	None	
13	External (absolute ...	Area	Linear	Exclude	1/X	None	

Table 24: Smoothing enabling and smoothing method used.

	User RF Mode	User RF Value	Ignore Zero Stds?	Ignore Zero QCs?	Propagate Cal?	Smooth Enabled?	Smooth Method
1	NO	0.0000000000	NO	NO	NO	YES	Mean
2	NO	0.0000000000	NO	NO	NO	YES	Mean
3	NO	0.0000000000	NO	NO	NO	YES	Mean
4	NO	0.0000000000	NO	NO	NO	YES	Mean
5	NO	0.0000000000	NO	NO	NO	YES	Mean
6	NO	0.0000000000	NO	NO	NO	YES	Mean
7	NO	0.0000000000	NO	NO	NO	YES	Mean
8	NO	0.0000000000	NO	NO	NO	YES	Mean
9	NO	0.0000000000	NO	NO	NO	YES	Mean
10	NO	0.0000000000	NO	NO	NO	YES	Mean
11	NO	0.0000000000	NO	NO	NO	YES	Mean
12	NO	0.0000000000	NO	NO	NO	YES	Mean
13	NO	0.0000000000	NO	NO	NO	YES	Mean

Table 25: Parameters for smoothing and baseline noise.

	Smooth Iterations	Smooth Width	Apex Track Enabled?	Apex params from Ref Peaks?	PK to PK Baseline Noise
1	1	2	YES	NO	10.0000
2	1	2	YES	NO	10.0000
3	1	2	YES	NO	10.0000
4	1	2	YES	NO	10.0000
5	1	2	YES	NO	10.0000
6	1	2	YES	NO	10.0000
7	1	2	YES	NO	10.0000
8	1	2	YES	NO	10.0000
9	1	2	YES	NO	10.0000
10	1	2	YES	NO	10.0000
11	1	2	YES	NO	10.0000
12	1	2	YES	NO	10.0000
13	1	2	YES	NO	10.0000

Table 26: Baseline and peak width parameters.

	Auto PK to PK Baseline Noise?	Peak Width at 5% Height (mins)	Auto Selection of Peak Width?	Baseline Start Thresh %
1	YES	30.0000	YES	0.10
2	YES	30.0000	YES	0.10
3	YES	30.0000	YES	0.10
4	YES	30.0000	YES	0.10
5	YES	30.0000	YES	0.10
6	YES	30.0000	YES	0.10
7	YES	30.0000	YES	0.10
8	YES	30.0000	YES	0.10
9	YES	30.0000	YES	0.10
10	YES	30.0000	YES	0.10
11	YES	30.0000	YES	0.10
12	YES	30.0000	YES	0.10
13	YES	30.0000	YES	0.10

Table 27: Baseline and noise parameters.

	Baseline End Thresh %	Detect Apex Track Shldr Peaks?	PK to PK Noise Amplitude	Auto Noise Measurement?	Balance
1	0.40	NO	100	YES	70.00
2	0.40	NO	100	YES	70.00
3	0.40	NO	100	YES	70.00
4	0.40	NO	100	YES	70.00
5	0.40	NO	100	YES	70.00
6	0.40	NO	100	YES	70.00
7	0.40	NO	100	YES	70.00
8	0.40	NO	100	YES	70.00
9	0.40	NO	100	YES	70.00
10	0.40	NO	100	YES	70.00
11	0.40	NO	100	YES	70.00
12	0.40	NO	100	YES	70.00
13	0.40	NO	100	YES	70.00

Table 28: Shows splitting, detected standard shoulder peaks and threshold, reduced tail and reduced height.

	Splitting	Detect Standard Shldr Peaks?	Detect Shldr Peaks Thresh%	Reduce Tail	Reduce Height
1	20.00	YES	30.00	50.00	5.00
2	20.00	YES	30.00	50.00	5.00
3	20.00	YES	30.00	50.00	5.00
4	20.00	YES	30.00	50.00	5.00
5	20.00	YES	30.00	50.00	5.00
6	20.00	YES	30.00	50.00	5.00
7	20.00	YES	30.00	50.00	5.00
8	20.00	YES	30.00	50.00	5.00
9	20.00	YES	30.00	50.00	5.00
10	20.00	YES	30.00	50.00	5.00
11	20.00	YES	30.00	50.00	5.00
12	20.00	YES	30.00	50.00	5.00
13	20.00	YES	30.00	50.00	5.00

Table 29: Threshold parameters

	Resp Thresh Use Relative Height?	Resp Thresh Relative Height	Resp Thresh Use Absolute Height?	Resp Thresh Absolute Height
1	NO	3.00	YES	0
2	NO	3.00	YES	0
3	NO	3.00	YES	0
4	NO	3.00	YES	0
5	NO	3.00	YES	0
6	NO	3.00	YES	0
7	NO	3.00	YES	0
8	NO	3.00	YES	0
9	NO	3.00	YES	0
10	NO	3.00	YES	0
11	NO	3.00	YES	0
12	NO	3.00	YES	0
13	NO	3.00	YES	0

Table 30: Threshold parameters.

	Resp Thresh Use Relative Area?	Resp Thresh Relative Area	Resp Thresh Use Absolute Area?	Resp Thresh Absolute Area
1	NO	400.00	YES	20
2	NO	400.00	YES	20
3	NO	400.00	YES	20
4	NO	400.00	YES	20
5	NO	400.00	YES	20
6	NO	400.00	YES	20
7	NO	400.00	YES	20
8	NO	400.00	YES	20
9	NO	400.00	YES	20
10	NO	400.00	YES	20
11	NO	400.00	YES	20
12	NO	400.00	YES	20
13	NO	400.00	YES	20

Table 31: Integration and signal to noise parameters.

	Integration Window Extent	Propagate Integration Params?	Min Sig/Noise Ratio	Flag Sig/Noise Ratio?
1	5.00	YES	2.00	NO
2	5.00	YES	2.00	NO
3	5.00	YES	2.00	NO
4	5.00	YES	2.00	NO
5	5.00	YES	2.00	NO
6	5.00	YES	2.00	NO
7	5.00	YES	2.00	NO
8	5.00	YES	2.00	NO
9	5.00	YES	2.00	NO
10	5.00	YES	2.00	NO
11	5.00	YES	2.00	NO
12	5.00	YES	2.00	NO
13	5.00	YES	2.00	NO

Table 32: Target ion ration method and ion parameters.

	Calculate Ion Ratio Tolerance As	Target Ion Ratio Method	1:Ion Trace	1:Ion Ratio	1:Ion Ratio Win (%) ±
1	Ratio	Quan/Target		0.0000	0
2	Ratio	Quan/Target		0.0000	0
3	Ratio	Quan/Target	297.3 > 262.2	0.0000	0
4	Ratio	Quan/Target		0.0000	0
5	Ratio	Quan/Target		0.0000	0
6	Ratio	Quan/Target		0.0000	0
7	Ratio	Quan/Target		0.0000	0
8	Ratio	Quan/Target	311.3 > 262.2	0.0000	0
9	Ratio	Quan/Target	319.35 > 226.2	0.0000	0
10	Ratio	Quan/Target	325.35 > 262.2	0.0000	0
11	Ratio	Quan/Target	331.3 > 262.4	0.0000	0
12	Ratio	Quan/Target		0.0000	0
13	Ratio	Quan/Target	334.25 > 74.35	0.0000	0

Table 33: Noise parameters and signal level measure.

	Signal-to-Noise method	Noise Calculation Factor	Noise Window Start (min)	Noise Window End (min)	Measure peak signal level from
1	RMS	3.00	0.0000	0.0000	Peak Baseline
2	RMS	3.00	0.0000	0.0000	Peak Baseline
3	RMS	3.00	0.0000	0.0000	Peak Baseline
4	RMS	3.00	0.0000	0.0000	Peak Baseline
5	RMS	3.00	0.0000	0.0000	Peak Baseline
6	RMS	3.00	0.0000	0.0000	Peak Baseline
7	RMS	3.00	0.0000	0.0000	Peak Baseline
8	RMS	3.00	0.0000	0.0000	Peak Baseline
9	RMS	3.00	0.0000	0.0000	Peak Baseline
10	RMS	3.00	0.0000	0.0000	Peak Baseline
11	RMS	3.00	0.0000	0.0000	Peak Baseline
12	RMS	3.00	0.0000	0.0000	Peak Baseline
13	RMS	3.00	0.0000	0.0000	Peak Baseline

Table 34: Predicted retention time parameters.

	Measure Peak Quality?	PQ Predicted RT (mins)	PQ Flag Predicted RT?	PQ Predicted RT Window ...	PQ Predicted RRT
1	NO	5.3000	NO	5.00	1.0000
2	NO	4.7200	NO	5.00	1.0000
3	NO	1.3800	NO	5.00	1.0000
4	NO	5.9800	NO	5.00	1.0000
5	NO	5.8000	NO	5.00	1.0000
6	NO	5.5000	NO	5.00	1.0000
7	NO	5.3000	NO	5.00	1.0000
8	NO	1.5100	NO	5.00	1.0000
9	NO	3.7200	NO	5.00	1.0000
10	NO	1.6400	NO	5.00	1.0000
11	NO	1.6200	NO	5.00	1.0000
12	NO	2.7000	NO	5.00	1.0000
13	NO	2.7400	NO	5.00	1.0000

Appendix 4

Calibration curves for each SSRI is shown in Figure 62-Figure 70. The peak area ratio between the reference standards (AUC) and the internal standards (IS AUC) is given on the y-axis, while the concentration (ng/L) is given on the x-axis. The formula for the regression line (y=mx+b) and the regression constant (R^2) is also shown.
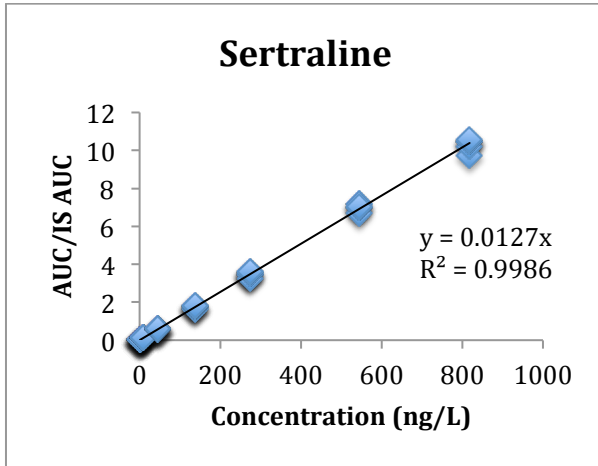
Figure 62: The calibration curve for Sertraline.
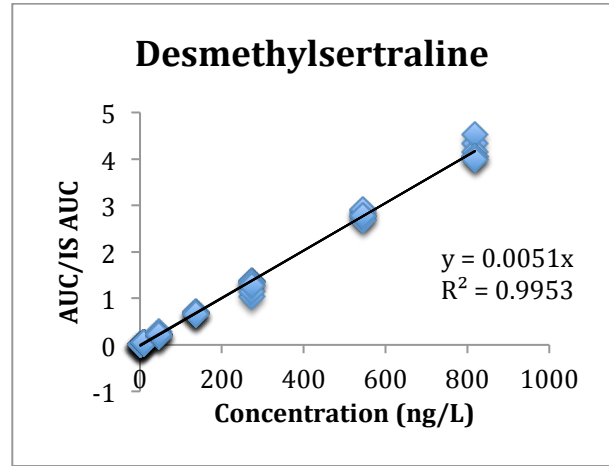
Figure 63: The calibration curve for Desmethylsertraline.
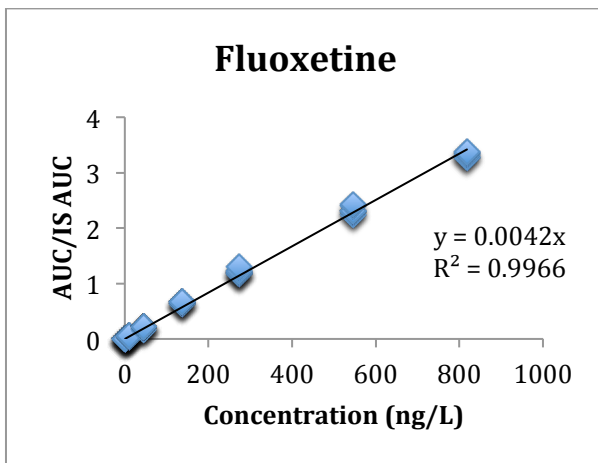
Figure 64: The calibration curve for Fluoxetine
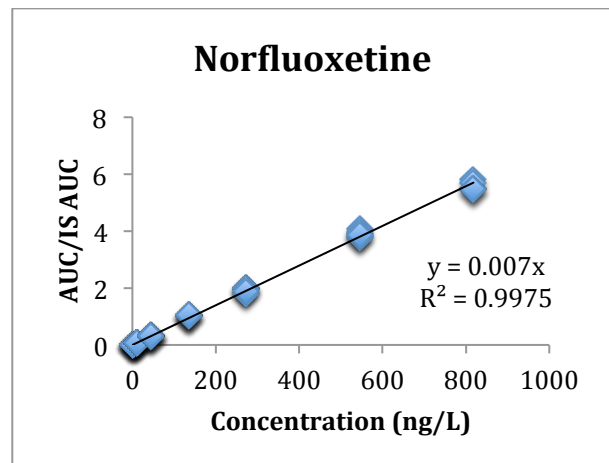
Figure 65: The calibration curve for Norfluoxetine.
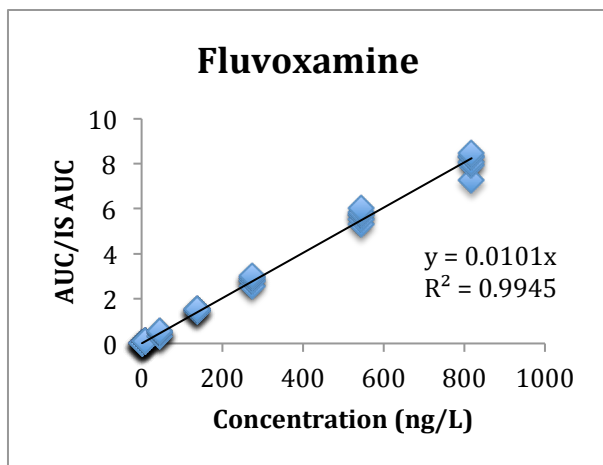
Figure 66: The calibration curve for Fluvoxamine.
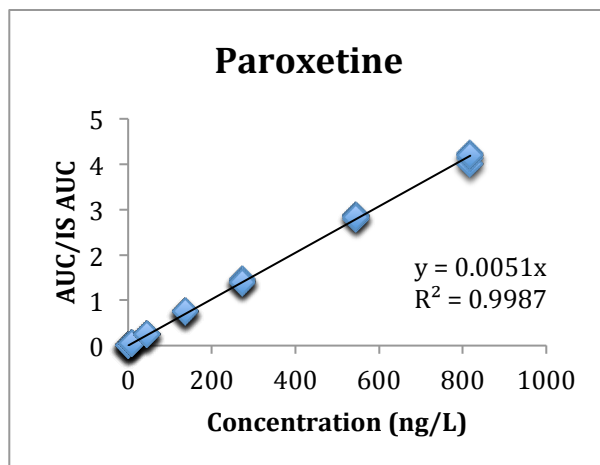
Figure 67: The calibration curve for Psroxetine.
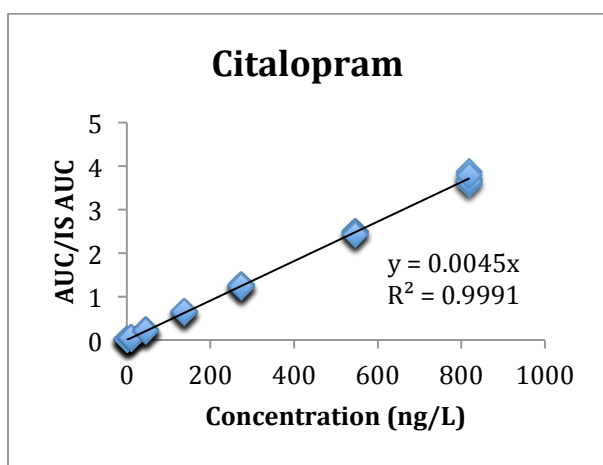
Figure 68: The calibration curve for Citalopram
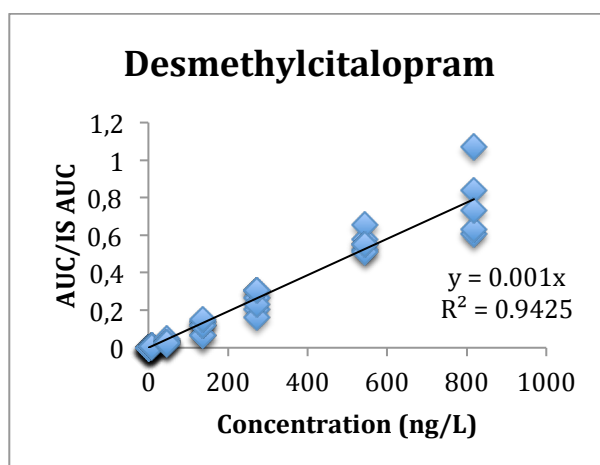
Figure 69: The calibration curve for Desmethylcitalopram.
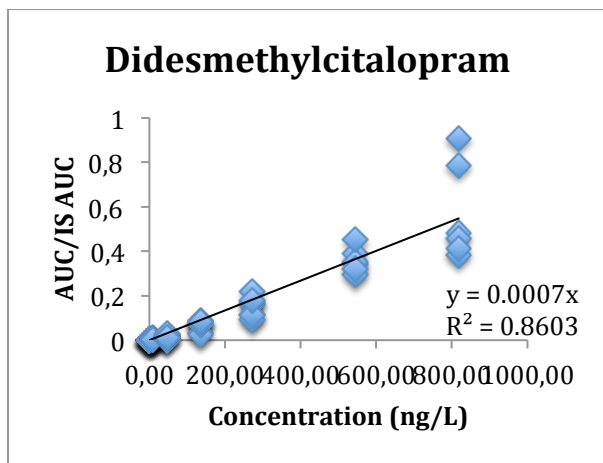
Figure 70: The calibration curve for DidesmethylcitalopramAppendix 5

Appendix 5

Examples of chromatograms of the substral growth medium for all the precursor ions of the SSRIs and the fragment ions used in the TargetLynx-method detected with an MRM-method are shown in
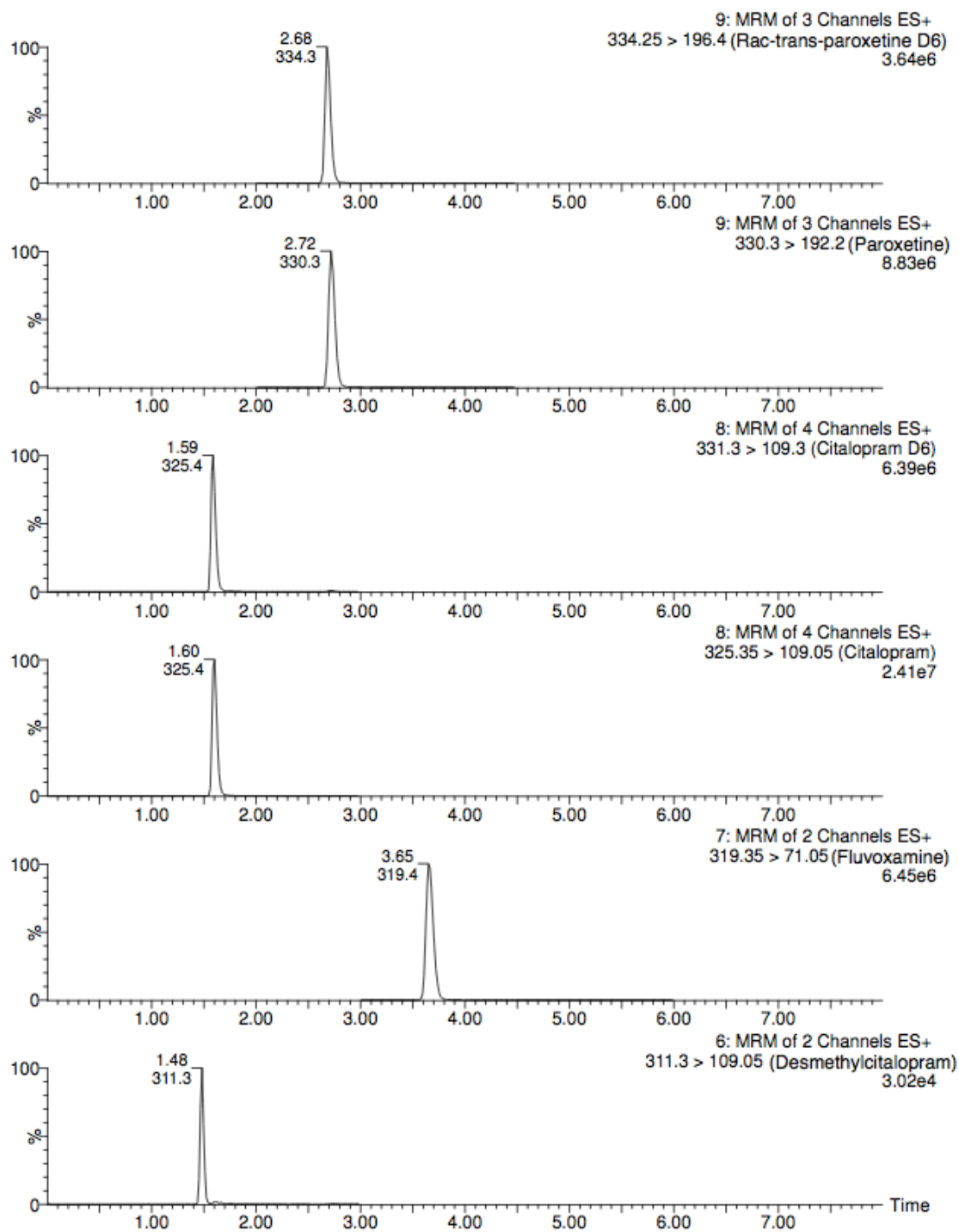
Figure 71: Chromatogram of the substral growth medium for rac-trans-paroxetine D4, paroxetine, citalopram D6, citalopram, fluvoxamine and desmethylcitalopram.

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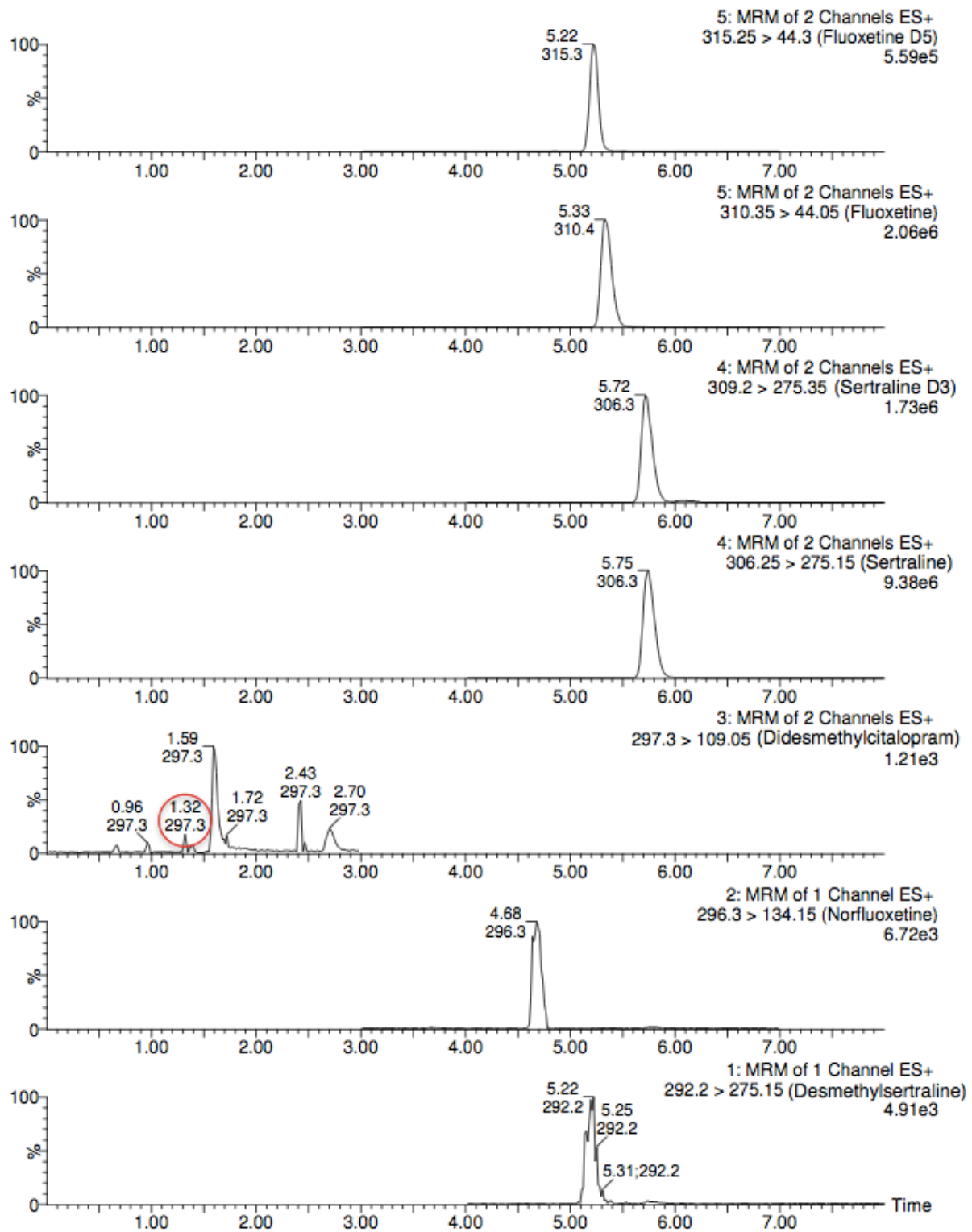
Figure 72: Chromatogram of the substral growth medium for fluoxetine D5, fluoxetine, sertraline D3, sertraline, didesmetylciralopram, norfluoxetine and desmethylsertraline. The red circle indicates the peak for didesmethylcitalopram.

Appendix 6

Table 35 shows the SSRIs and their average measured concentrations, standard deviation (SD), and relative standard deviation (RSD) by growth medium in the control experiment.

Table 35: The average measured concentrations for each day test were taken, standard deviation (SD), and relative standard deviation (RSD) for each average.

SSRI	Growth media	Average day 6 (ng/L)	Average day 14 (ng/L)	SD day 6 (ng/L)	SD day 14 (ng/L)	RSD % day 6	RSD % day 14
Sertraline	F2	245	225	4,9315	7,8778	2,016	3,502
	Substral	240	232	1,4440	7,0858	0,601	3,055
Desmethylsertraline	F2	0,52	0,63	0,0433	0,0829	8,281	13,152
	Substral	0,32	0,3912	0,0850	0,0003	26,364	0,070
Fluoxetine	F2	295	264	14,2547	5,3247	4,826	2,019
	Substral	289	264	8,8856	16,9331	3,071	6,423
Norfluoxetine	F2	0,68	0,84	0,0771	0,0615	11,273	7,306
	Substral	0,5	0,8	0,2151	0,1731	45,741	22,857
Fluvoxamine	F2	359	328	8,0374	5,3496	2,241	1,631
	Substral	360	337	13,3621	14,7469	3,713	4,370
Paroxetine	F2	294	256	2,3892	1,4262	0,812	0,557
	Substral	302	231	3,1741	5,5943	1,053	2,426
Citalopram	F2	449	421	6,9566	4,0210	1,548	0,954
	Substral	428	416	5,3821	3,3350	1,256	0,802
Desmethylcitalopram	F2	2,6	3	0,6943	1,0944	26,683	42,746
	Substral	1,9	2,0	0,1676	0,1578	8,642	7,977
Didesmethylcitalopram	F2	0,06	0,02	0,0403	0,0263	66,774	124,696
	Substral	0,03	0,015	0,0246	0,0053	74,783	35,422

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Appendix 7

Table 36 shows the average measured concentrations (ng/L) for each SSRI for each sampling day in the *S. marinoi* experiment. Standard deviation (SD) and realtive standard deviation (RSD) in % are also given.

Table 36: The average measured concentrations (ng/L) for each SSRI for each sampling day in the *S. marinoi* experiment. Standard deviation (SD) and relative standard deviation (RSD) in % is also given.[1]

SSRI	Parallel	Average day 1 (ng/L)	Average day 2 (ng/L)	Average day 3 (ng/L)	Average day 7 (ng/L)	Average day 14 (ng/L)	SD day 1 (ng/L)	SD day 2 (ng/L)	SD day 3 (ng/L)	SD day 7 (ng/L)	SD day 14 (ng/L)	RSD % day 1	RSD % day 2	RSD % day 3	RSD % day 7	RSD % day 14
Sertraline	Parallel 1	268	239	237,6	158	109	-	13,0600	0,1208	2,8232	1,3841	-	5,473	0,051	1,787	1,276
	Parallel 2	267	256	250	175,1	151	2,0901	4,1007	3,4264	0,6134	3,6966	0,783	1,605	1,372	0,350	2,453
	Parallel 3	261	244	237	153,9	98	42,0703	4,3649	9,3545	0,3139	2,7470	16,097	1,786	3,941	0,204	2,812
Desmethylsertraline	Parallel 1	0,3	0,37	0,6	0,5	1,1	-	0,0227	0,1216	0,2249	0,1368	-	6,150	21,913	48,205	11,916
	Parallel 2	0,44	0,2	0,41	0,98	1,50	0,0594	0,1868	0,0813	0,0220	0,0653	13,598	86,717	19,932	2,240	4,356
	Parallel 3	0,1	0,2	0,3	0,9	1,2	0,1640	0,2728	0,2954	0,1302	0,3454	173,205	141,421	89,768	13,763	29,875
Fluoxetine	Parallel 1	297	221	172,9	6,5	1,1	-	9,8552	0,9096	0,7165	0,1631	-	4,464	0,526	11,054	14,438
	Parallel 2	293	226	181	27,1	18,5	1,3549	5,4334	1,8242	0,6931	0,1099	0,463	2,399	1,005	2,559	0,595
	Parallel 3	286	222	160	6,87	1,6	9,2224	7,7463	8,7467	0,0271	0,1097	3,222	3,486	5,470	0,394	6,750
Norfluoxetine	Parallel 1	0,5	0,77	1,43	0,30	0,04	-	0,6661	0,0261	0,0704	0,0656	-	86,880	1,825	23,450	173,205
	Parallel 2	0,6	0,5	1,0	0,73	0,83	0,1210	0,4463	0,1508	0,0400	0,0119	19,973	86,854	15,847	5,451	1,432
	Parallel 3	0,2	0,5	1,0	0,2	0,13	0,3621	0,7058	0,8451	0,2600	0,0327	173,205	141,421	86,652	141,421	25,650
Fluvoxamine	Parallel 1	380	181	74,72	0,836	0,66	-	14,0225	4,8980	0,0047	0,0926	-	7,761	6,555	0,565	14,036
	Parallel 2	368	165	71,8	3,3	2,649	12,0227	13,9207	2,6749	0,1232	0,0032	3,264	8,422	3,727	3,716	0,121
	Parallel 3	328	130	62,90	1,25	1,0	43,3567	42,8340	12,8461	0,0832	0,1014	13,211	33,025	20,425	6,667	10,045
Paroxetine	Parallel 1	295	274	279	173,4	75	-	23,1228	2,0096	0,5967	1,0721	-	8,446	0,720	0,344	1,434
	Parallel 2	300	287	288	172,99	128	1,8878	8,4791	1,6098	1,3660	1,0336	0,628	2,956	0,559	0,790	0,809
	Parallel 3	318	277	276	130,4	44	21,6571	9,8905	1,6699	0,7018	2,7486	6,815	3,573	0,606	0,538	6,210
Citalopram	Parallel 1	283	275	283	269,0	259	-	8,8517	2,1057	0,8204	5,6647	-	3,222	0,745	0,305	2,185
	Parallel 2	284	279	290	268	276	1,3383	4,1754	1,4724	2,2233	3,3194	0,471	1,495	0,507	0,831	1,202
	Parallel 3	279	276,4	282	258	242	14,1951	0,1490	2,8126	1,0988	3,1504	5,087	0,054	0,999	0,426	1,304
Desmethylcitalopram	Parallel 1	1,01	1,0	1,26	1,7	4,9	-	0,4332	0,0548	0,1728	0,7821	-	43,718	4,351	10,110	16,002
	Parallel 2	0,69	1,0	0,89	2,48	3,7	0,0493	0,2938	0,0446	0,1147	0,1812	7,115	28,091	2,504	1,796	3,089
	Parallel 3	0,81	0,9	1,4	2,8	7,6	*	*	0,1812	0,4917	0,4831	*	*	12,658	17,640	6,334
Didesmethylcitalopram	Parallel 1	0,017	0,14	0,116	0,025	0,02	-	0,0476	0,0078	0,0006	0,0122	-	33,272	6,765	2,504	79,574
	Parallel 2	0,007	0,047	0,05	0,05	0,0112	0,0023	0,0099	0,0166	0,0223	0,0002	31,660	21,210	32,471	40,589	1,665
	Parallel 3	0,004	0,11	0,108	0,05	0,011	*	*	0,0012	*	0,0026	*	*	1,069	*	23,842

[1] -: no liquid from the fiber thread
 *: no signal from the MS/MS

Appendix 8

Table 37 shows the average measured concentrations for each SSRI for each sampling dag and parallel in the first *A. longicornis* experiment. Standard deviation (SD) and relative standard deviation (RSD) is also given

Table 37: The average measured concentrations for each SSRI for each sampling day in the first *A.longicornis* experiment. Standard deviation (SD) and relative standard deviation (RSD) in % is also given.

SSRI	Parallel	Average day 1 (ng/L)	Average day 2 (ng/L)	Average day 3 (ng/L)	Average day 7 (ng/L)	Average day 14 (ng/L)	SD day 1 (ng/L)	SD day 2 (ng/L)	SD day 3 (ng/L)	SD day 7 (ng/L)	SD day 14 (ng/L)	RSD % day 1	RSD % day 2	RSD % day 3	RSD % day 7	RSD % day 14
Sertraline	Parallel 1	265	266	275	282	189	1,2683	4,2863	2,0488	1,2125	2,5143	0,479	1,610	0,744	0,430	1,330
	Parallel 2	257,7	260	282	285	202	0,8879	5,2608	1,4613	5,5624	2,9461	0,345	2,020	0,519	1,955	1,460
	Parallel 3	223	246	261	264	220	28,2324	4,5762	4,9691	3,0750	5,9847	12,682	1,858	1,906	1,164	2,720
Desmethylsertraline	Parallel 1	0,23	0,35	0,36	0,50	0,71	0,0658	0,0386	0,0656	0,0311	0,0211	28,737	11,080	18,071	6,183	2,965
	Parallel 2	0,16	0,27	0,43	0,52	0,7	0,0863	0,0979	0,0987	0,1595	0,0618	52,708	36,271	23,047	30,478	8,535
	Parallel 3	0,15	0,28	0,32	0,64	0,78	0,0374	0,0748	0,0747	0,0820	0,1226	25,117	26,706	23,164	12,914	15,621
Fluoxetine	Parallel 1	302	310	291	297	263	3,4729	3,3507	6,7192	2,2992	2,7779	1,152	1,082	2,306	0,773	1,055
	Parallel 2	293	306	295	290	261	4,8755	3,2487	3,1189	2,7430	5,2185	1,667	1,061	1,059	0,945	2,002
	Parallel 3	269	294	278	268	252	17,4180	10,9617	2,5944	2,0807	3,9826	6,485	3,725	0,935	0,776	1,580
Norfluoxetine	Parallel 1	0,31	0,42	0,6	0,78	1,6	0,0854	0,0235	0,1140	0,0888	0,1166	27,661	5,646	18,976	11,401	7,492
	Parallel 2	0,29	0,5	0,54	0,95	1,6	0,0144	0,1669	0,0980	0,0270	0,2383	4,895	35,702	18,218	2,832	15,082
	Parallel 3	0,36	0,4	0,54	1,00	1,8	0,0252	0,1542	0,0883	0,0158	0,2327	7,041	36,580	16,273	1,582	13,200
Fluvoxamine	Parallel 1	348	326	340	360	341	14,2915	34,4531	8,5028	16,8898	15,2150	4,108	10,569	2,498	4,691	4,465
	Parallel 2	379	313	355	371	335	34,4531	64,2117	35,8069	35,4705	6,3252	9,091	20,515	10,099	9,572	1,886
	Parallel 3	304	362	363	327	329	14,0682	63,0465	38,0258	5,5831	2,8432	4,624	17,407	10,476	1,707	0,864
Paroxetine	Parallel 1	306	302	299	281	216	2,2294	4,9531	4,4266	6,1081	2,3946	0,729	1,638	1,479	2,174	1,107
	Parallel 2	298	291	301	278	215	4,9531	4,4664	3,8905	2,1601	1,6977	1,665	1,533	1,292	0,777	0,788
	Parallel 3	270	279	274	256	209	21,7723	10,0270	2,8684	2,7723	2,7246	8,070	3,600	1,046	1,085	1,306
Citalopram	Parallel 1	291	297	291	312	317	2,3903	1,6214	2,2612	2,3809	3,9688	0,820	0,545	0,776	0,764	1,251
	Parallel 2	286	285	291	310	320	1,6214	3,7135	6,0224	1,6297	2,7122	0,566	1,305	2,071	0,525	0,847
	Parallel 3	267	276	274	284	291	9,5119	1,4439	2,0673	2,7310	1,4854	3,558	0,523	0,756	0,961	0,510
Desmethylcitalopram	Parallel 1	0,55	0,77	0,9	1,0	1,1	0,0176	0,0840	0,2992	0,3396	0,1713	3,177	10,913	31,642	32,468	15,331
	Parallel 2	0,45	0,9	0,8	1,4	1,2	0,0840	0,1076	0,1084	0,1524	0,2295	18,878	12,267	14,096	11,107	19,835
	Parallel 3	0,5	0,7	0,9	1,34	1,1	0,1004	0,1161	0,1379	0,0313	0,1583	20,097	15,559	14,812	2,330	14,748
Didesmethylcitalopram	Parallel 1	0,03	0,02	0,07	0,08	0,07	0,0195	0,0206	0,0480	0,0104	0,0396	70,989	86,728	73,124	12,332	57,582
	Parallel 2	0,03	0,04	0,043	0,06	0,03	0,0206	0,0192	0,0074	0,0137	0,0241	71,032	44,236	17,109	23,638	79,693
	Parallel 3	0,023	0,054	0,061	0,0496	0,07	0,0076	0,0457	0,0070	0,0061	0,0327	32,733	84,641	11,570	12,280	46,693

Appendix 9

Table 38 shows the average concentrations for each SSRI for each sampling dag and parallel in the second *A. longicornis* experiment. Standard deviation (SD) and relative standard deviation (RSD) is also given.[2]

Table 38: The average concentrations for each SSRI for each sampling day in the second *A.longicornis* experiment. Standard deviation (SD) and relative standard deviation (RSD) in % is also given.

SSRI	Parallel	Average day 1 (ng/L)	Average day 2 (ng/L)	Average day 3 (ng/L)	Averag e day 9 (ng/L)	Average day 11 (ng/L)	Average day 16 (ng/L)	SD day 1 (ng/L)	SD day 2 (ng/L)	SD day 3 (ng/L)	SD day 9 (ng/L)	SD day 11 (ng/L)	SD day 16 (ng/L)	RSD % day 1	RSD % day 2	RSD % day 3	RSD % day 9	RSD % day11	RSD % day16
	Parallel 1	262	184,2	142	40,6	26,7	10,4	1,9205	0,8211	1,5882	-	0,6688	0,2050	0,734	0,446	1,121	#	2,509	1,971
Sertraline	Parallel 2	252	199	197	147,2	127	116	7,6479	10,1738	6,1834	-	1,0381	5,5413	3,034	5,101	3,145	#	0,817	4,795
	Parallel 3	274	166	131	71,8	61,0	49,8	2,8803	2,5886	3,2898	0,2143	0,8182	0,1201	1,053	1,558	2,507	0,299	1,341	0,241
	Parallel 1	0,3	0,16	0,26	0,23	0,00	0,131	0,1429	0,0407	0,0434	-	*/-	0,0058	42,017	24,932	16,675	#	#	4,416
Desmethyl-sertraline	Parallel 2	0,25	0,25	0,32	0,44	0,61	0,73	0,0993	0,0845	*/-	-	0,0206	0,0556	39,296	33,957	#	#	3,383	7,614
	Parallel 3	0,3	0,21	0,321	0,29	0,34	0,32	0,1182	0,0667	0,0032	0,0838	*/-	0,0383	36,555	31,604	0,986	28,418	#	11,814
	Parallel 1	290	418	233	46	22	4,9	6,1514	3,1951	2,6465	-	2,9510	0,2122	2,118	0,765	1,136	#	13,535	4,341
Fluoxetine	Parallel 2	281	330	270	86	58	36	10,5042	61,5134	11,3483	-	1,4760	1,7826	3,740	18,617	4,207	#	2,528	4,898
	Parallel 3	292	384	358	228	161	146,5	3,6129	8,8987	50,0240	1,0308	29,4876	0,5216	1,239	2,318	13,980	0,452	18,348	0,356
	Parallel 1	0,5	0,78	1,5	1,04	0,0	0,30	0,3231	0,0572	0,1043	-	*/-	*	62,514	7,378	6,943	#	#	#
Nor-fluoxetine	Parallel 2	0,45	1,2	1,5	1,86	1,5	1,20	0,0851	0,2624	*/-	-	0,1794	0,0790	19,031	21,552	#	#	11,809	6,581
	Parallel 3	0,46	1,008	1,12	1,66	1,6	1,78	0,0802	0,0073	0,0820	0,0808	*/-	0,0386	17,366	0,727	7,351	4,875	#	2,169
	Parallel 1	351	297	93	6	2,5	1,1	15,7400	7,3103	1,6437	-	0,8284	0,1132	4,490	2,460	1,773	#	33,086	10,152
Fluvoxamine	Parallel 2	349	73	49	5	4,2	2,82	5,0204	141,8316	17,7810	-	0,2511	0,0967	1,439	194,962	36,559	#	6,046	3,422
	Parallel 3	370	243	236	126	109	73,8	11,0751	51,5235	10,6523	44,4061	32,7315	0,5607	2,989	21,193	4,523	35,359	29,908	0,760
	Parallel 1	312	297	265	148	108	44	4,3409	2,9881	5,4107	-	5,0003	1,4397	1,393	1,006	2,040	#	4,637	3,264
Paroxetine	Parallel 2	312	316	278	184	147	116	1,8554	11,1674	26,1644	-	3,7126	6,2040	0,595	3,529	9,426	#	2,532	5,339
	Parallel 3	323	309	297	200	171	148	6,8562	11,2154	3,1522	6,5062	21,1432	1,1444	2,119	3,631	1,062	3,255	12,399	0,774
	Parallel 1	297	390,6	386	383	345	336	5,6845	0,4247	6,5648	-	3,9671	7,2871	1,913	0,109	1,702	#	1,150	2,167
Citalopram	Parallel 2	298	410	404	440	411	419	7,5814	14,9383	6,7538	-	9,0533	16,0314	2,541	3,646	1,671	#	2,203	3,825
	Parallel 3	309	402	407	394	401	399	4,4206	17,8779	9,6724	8,9401	2,7359	7,0345	1,432	4,442	2,374	2,270	0,683	1,763
	Parallel 1	0,5	1,3	1,85	6,0	3,7	6,0	0,1391	0,3929	0,3633	-	0,7719	0,7773	26,425	30,978	19,667	#	20,729	12,966
Desmethyl-citalopram	Parallel 2	0,8	1,3	1,51	4,5	6	5,7	0,2481	0,3343	*/-	-	1,2235	0,4720	30,896	25,141	#	#	21,419	8,278
	Parallel 3	0,9	1,3	1,86	2,5	3	3,1	0,1479	0,1762	0,3921	0,7730	*/-	0,2408	16,166	13,161	21,077	30,469	#	7,678
	Parallel 1	0,007	0,01	0,04	0,055	0,00	0,0470	0,0029	0,0124	0,0312	-	*/-	0,0003	43,874	111,155	76,114	#	#	0,538
Didesmethyl-citalopram	Parallel 2	0,07	0,06	0,03	0,107	0,07	0,025	0,0193	0,0543	*/-	-	0,0510	0,0087	25,988	84,899	#	#	73,832	34,676
	Parallel 3	0,03	0,04	0,062	0,025	0,09	0,050	0,0130	0,0226	0,0024	0,0011	*/-	0,0024	44,701	57,435	3,812	4,401	#	4,784

[2] -: no liquid from the fiber thread
*: no signal from the MS/MS
#: not able to calculate RSD % beacuse of no SD avilable

Appendix 10

Table 39 shows the measured Ra- and Rb-values for the *S. marinoi* experiment. These values are used further on to calculate the Chl *a* and the phaeophytine values in using formulas 2.1 and 2.2.

Table 39: Measured Ra- and Rb-values for the *S. marinoi* experiment[3].

Date samples taken	03.03.14	10.03.14	17.03.14
Date samples measured	04.03.14	11.03.14	18.03.14
Ra-values	µg/L	µg/L	µg/L
Beaker 1. Parallel 1	8.80	65.90	8.60
Beaker 1. Parallel 2	7.90	58.50	9.70
Beaker 1. Parallel 3	7.10	47.10	10.40
Average. Beaker 1	7.93	57.17	9.57
Beaker 2. Parallel 1	8.70	47.60	-
Beaker 2. Parallel 2	7.80	-	34.30
Beaker 2. Parallel 3	6.60	50.30	22.10
Average. Beaker 2	7.70	48.95	28.20
Beaker 3. Parallel 1	6.10	58.00	42.30
Beaker 3. Parallel 2	6.40	49.60	34.60
Beaker 3. Parallel 3	6.30	49.70	50.30
Average. Beaker 3	6.27	52.43	42.40
Rb-values	µg/L	µg/L	µg/L
Beaker 1. Parallel 1	5.10	36.10	6.20
Beaker 1. Parallel 2	4.40	34.80	6.90
Beaker 1. Parallel 3	4.10	29.10	6.70
Average. Beaker 1	4.53	33.33	6.60
Beaker 2. Parallel 1	4.90	28.90	21.20
Beaker 2. Parallel 2	4.60	28.80	21.90
Beaker 2. Parallel 3	3.80	29.70	15.30
Average. Beaker 2	4.43	29.13	19.47
Beaker 3. Parallel 1	3.60	35.30	28.40
Beaker 3. Parallel 2	3.70	29.50	22.40
Beaker 3. Parallel 3	3.90	30.80	27.30
Average. Beaker 3	3.73	31.87	26.03
Control			
Date samples taken	09.03.14	16.03.14	20.03.14
Date samples measured	10.03.14	17.03.14	21.03.14
Ra-values	µg/L	µg/L	µg/L
Control. Parallel 1	8.30	6.40	148.40
Control. Parallel 2	6.60	10.30	142.90
Control. Parallel 3	5.70	8.20	144.80
Average	6.87	8.30	145.37
Rb-values	µg/L	µg/L	µg/L
Control. Parallel 1	6.20	5.40	80.00
Control. Parallel 2	4.80	7.70	80.30
Control. Parallel 3	4.30	6.90	81.50
Average	5.10	6.67	80.60

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[3] -: Did not measure Ra before adding 10% HCl.

Table 40: The calculated Chl *a* and phaeopytine values for the *S. marinoi* experiment.

Date samples taken	03.03.14	10.03.14	17.03.14
Date samples measured	04.03.14	11.03.14	18.03.14
Beaker 1			
μg Chla/L	2.34	8.20	2.04
μg Phaeo/L	1.12	4.53	2.99
Beaker 2			
μg Chla/L	2.25	6.81	6.01
μg Phaeo/L	1.14	4.31	8.86
Beaker 3			
μg Chla/L	1.74	7.07	11.26
μg Phaeo/L	1.11	5.09	8.62
Control			
Date samples taken	09.03.14	16.03.14	20.03.14
Date samples measured	10.03.14	17.03.14	21.03.14
μg Chla/L	1.22	1.12	44.55
μg Phaeo/L	0.89	3.97	16.99

Appendix 11

Table 41 shows the measured Ra- and Rb-values for the first *A. longicornis* experiment. These values are used further on to calculate the Chl *a* and the phaeophytine values in Table 42 using formulas 2.1 and 2.2.

Table 41: Measured Ra- and Rb-values for the first *A. longicornis* experiment.

Date samples taken	11.03.14	18.03.14	24.03.14
Date samples measured	**12.03.14**	**19.03.14**	**25.03.14**
Ra-values	**µg/L**	**µg/L**	**µg/L**
Beaker 1, Parallel 1	22.50	5.10	173.70
Beaker 1, Parallel 2	10.70	5.60	179.40
Beaker 1, Parallel 3	19.80	5.80	154.50
Average, Beaker 1	**17.67**	**5.50**	**169.20**
Beaker 2, Parallel 1	20.90	4.80	158.20
Beaker 2, Parallel 2	19.00	4.10	177.00
Beaker 2, Parallel 3	21.40	4.50	167.80
Average, Beaker 2	**20.43**	**4.47**	**167.67**
Beaker 3, Parallel 1	17.20	5.10	145.60
Beaker 3, Parallel 2	17.90	5.70	148.10
Beaker 3, Parallel 3	16.80	4.90	143.00
Average, Beaker 3	**17.30**	**5.23**	**145.57**
Control, Parallel 1	14.50	4.20	63.40
Control, Parallel 2	12.80	4.60	58.40
Control, Parallel 3	14.90	3.50	59.90
Average, Control	**14.07**	**4.10**	**60.57**
Rb-values	**µg/L**	**µg/L**	**µg/L**
Beaker 1, Parallel 1	12.20	2.90	91.50
Beaker 1, Parallel 2	5.30	3.20	93.10
Beaker 1, Parallel 3	10.80	3.10	80.80
Average, Beaker 1	**9.43**	**3.07**	**88.47**
Beaker 2, Parallel 1	12.00	2.70	82.80
Beaker 2, Parallel 2	10.60	2.30	91.30
Beaker 2, Parallel 3	12.30	2.70	91.40
Average, Beaker 2	**11.63**	**2.57**	**88.50**
Beaker 3, Parallel 1	9.40	2.90	77.00
Beaker 3, Parallel 2	9.70	3.30	76.60
Beaker 3, Parallel 3	9.50	2.70	79.30
Average, Beaker 3	**9.53**	**2.97**	**77.63**
Control, Parallel 1	7.30	2.60	32.00
Control, Parallel 2	7.00	2.80	29.30
Control, Parallel 3	7.70	2.30	30.80
Average, Control	**7.33**	**2.57**	**30.70**

Table 42: The calculated Chl *a* and phaeopytine values for the first *A. longicornis* experiment.

Date samples taken	11.03.14	18.03.14	24.03.14
Date samples measured	**12.03.14**	**19.03.14**	**25.03.14**
Beaker 1			
μg Chl*a* L^{-1}	2.83	1.67	55.53
μg Phaeo L^{-1}	0.77	0.67	12.01
Beaker 2			
μg Chl*a* L^{-1}	3.03	1.31	54.45
μg Phaeo L^{-1}	1.41	0.65	13.12
Beaker 3			
μg Chl*a* L^{-1}	2.67	1.56	46.72
μg Phaeo L^{-1}	0.97	0.71	12.55
Control			
μg Chl*a* L^{-1}	2.32	1.05	102.71
μg Phaeo L^{-1}	0.48	0.90	14.48

Appendix 12

Table 43 shows the measured Chl *a* and phaephytine values for the second *A. longicornis* experiment.

Table 43: Measured Chl *a* and phaeophytine values for the second A. Longicornis experiment[4].

Date samples taken	01.04.14	07.04.14	15.04.14
Date samples measured	**02.04.14**	**08.04.14**	**16.04.14**
Chl a	**µg/L**	**µg/L**	**µg/L**
Beaker 1, Parallel 1	2.21	3.70	9.56
Beaker 1, Parallel 2	2.20	3.87	9.36
Beaker 1, Parallel 3	2.20	3.53	11.84
Average, Beaker 1	**2.20**	**3.70**	**10.25**
Beaker 2, Parallel 1	1.70	3.50	7.37
Beaker 2, Parallel 2	1.65	3.90	10.44
Beaker 2, Parallel 3	2.51	3.66	12.14
Average, Beaker 2	**1.95**	**3.69**	**9.98**
Beaker 3, Parallel 1	2.54	5.16	16.62
Beaker 3, Parallel 2	1.95	4.48	13.67
Beaker 3, Parallel 3	2.93	4.71	8.41
Average, Beaker 3	**2.47**	**4.78**	**12.90**
Control, Parallel 1	3.21	6.54	15.68
Control, Parallel 2	2.69	6.61	16.09
Control, Parallel 3	2.66	7.13	10.93
Average, Control	**2.85**	**6.76**	**14.23**
Phaephytine	**µg/L**	**µg/L**	**µg/L**
Beaker 1, Parallel 1	*	1.63	0.40
Beaker 1, Parallel 2	0.10	0.84	2.21
Beaker 1, Parallel 3	0.12	0.24	0.33
Average, Beaker 1	**0.11**	**0.90**	**0.98**
Beaker 2. Parallel 1	0.13	*	*
Beaker 2. Parallel 2	0.70	*	1.02
Beaker 2. Parallel 3	*	*	2.88
Average. Beaker 2	**0.42**	**0**	**1.95**
Beaker 3, Parallel 1	*	0.59	*
Beaker 3, Parallel 2	*	*	*
Beaker 3, Parallel 3	*	*	*
Average, Beaker 3	**0**	**0.59**	**0**
Control, Parallel 1	*	0.37	0.89
Control, Parallel 2	*	*	*
Control, Parallel 3	0.24	*	3.71
Average, Control	**0.24**	**0.37**	**2.30**

[4] *: Negative values for the phaephytine measurents. This means that the cells are in the beginning of a growth-phase.