DEVELOPMENT OF A HIGH THROUGHPUT SEQUENTIAL PHYTOREMEDIATION SYSTEM FOR SUSTAINABLE WATER PURIFICATION USING ENDEMIC MACROPHYTES

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND

The prevalence of emerging organic contaminants in surface waters because of inadequate wastewater treatment or the inability of conventional wastewater treatment to remove emerging organic contaminants is of great concern. This is not only because of the potential ecotoxicological impact, but also because of the increasing incidence of antibiotic resistance in environmental isolates and the ramifications thereof for therapeutic efficacy of the antibiotics in question. The desirability of low cost, low maintenance and "green" solutions to water treatment has led to many and varied approaches to remove emerging organic contaminants. The work described here is based on the green liver concept described by Sandermann (1994). It is intended as a proof of concept for implementing post-wastewater treatment removal of xenobiotics.

RATIONALE

Overloading of wastewater treatment systems and the inadequate provision of sewerage in many rural areas can result in very poor quality surface waters containing high concentrations of emerging organic contaminants being released from water treatment facilities. These xenobiotics include pharmaceuticals and frequently also cyanotoxins where polishing ponds precede release. The released water typically also contains high levels of nutrients. The reliance on raw water for household use, although diminishing, is still high, and the use of raw water containing xenobiotics or natural toxins for agriculture, aquaculture and recreation also poses certain risks. In addition to these problems, the increased costs required for potable water production from contaminated raw waters are significant, and often beyond the capabilities of smaller water purification plants. The added ecotoxicological concerns and the emergence of antibiotic resistance in environmental samples with the implications for emerging resistance in pathogens indicate an urgent need to address these contaminants.

A low technology, low maintenance and low cost solution is required to treat contaminated raw waters either at source or prior to abstraction. The system should also be suitable for purifying an impoundment *in situ*. A biological system based on the "green liver" systems successfully implemented in China and Brazil by Pflugmacher et al. (2015) is therefore proposed. This type of system has several advantages, namely, using submerged or floating macrophytes prevents damage to the biological component from grazing or other land use, and the biological consortium can be purpose-designed for the particular pollutants. Traditional ponding or wetland systems do not offer such benefits. The limited successes of wetland systems for removing xenobiotics is largely due to the very large footprint, limited flow and the associated requirement for very large areas; inability to easily manipulate populations for specific desired xenobiotic biotransformation or bioaccumulation; and the eventual bioaccumulation of the xenobiotics within the system, which may pass up the food chain. Additionally, damage to the wetland by foot traffic or grazing is common, as is the uncontrolled harvesting of biomass that may have various uses. The potential advantages of the green liver system are the smaller footprint, controlled flow rate, the xenobiotic-driven assembly of appropriate macrophytes, and the lack of obvious use for the biomass coupled with the ease of harvesting and potential added value to the harvested biomass.

OBJECTIVES AND AIMS

The aim of this work was to validate the concept of a consortium of macrophytes in series to remove multiple xenobiotics while protecting those macrophytes that may be harmed by one or more of the xenobiotics. To that end, the following specific objectives constitute the deliverables of the project:

Aim 1

Develop rapid simple methods to quantify selected xenobiotics both in water and in a plant matrix.

Aim 2

Select individual macrophytes based on their response to the selected xenobiotics.

Aim 3

Determine the preferred configuration for xenobiotic removal and evaluate a model system.

METHODOLOGY

Appropriate analytical methods for the xenobiotics, which were selected based on the Umgeni catchment, were developed and optimised. Selected endemic aquatic macrophytes were evaluated for their ability to bioaccumulate and/or biotransform environmental toxins and xenobiotics, and selected as components of a laboratory scale high throughput sequential phytoremediation consortium model based on this data.

The bioaccumulation and biotransformation were evaluated by creating a culture of macrophytes in an appropriate medium at laboratory scale and exposing it to various concentrations and combinations of the relevant xenobiotics. Exposed plants were monitored for uptake, accumulation and depuration of the xenobiotics, and for potentially harmful toxic effects. Ecotoxicology was determined by photosynthetic parameters. Selected macrophytes were tested in various configurations in a sequential exposure model system to evaluate the consortium's potential for removing identified xenobiotics and biological toxins. The optimised model system was evaluated further for determining their tolerance to physicochemical and flow rate variation, and their pollutant load. The collected data form the basis for a proof of the high throughput sequential phytoremediation system (HTSPS) concept to inform the possibility of application at a pilot scale.

RESULTS AND DISCUSSION

Acetaminophen, ampicillin, nevirapine, nalidixic acid, and β-N-methylamino-L-alanine (BMAA) were selected as xenobiotics.

- Ampicillin and nalidixic acid were selected because:

  o They represent both relatively hydrophilic and relatively hydrophobic examples, which may alter their bioaccumulation and biotransformation potential.
  o They are both found often in appreciable concentrations in surface waters, and constitute models for emerging organic contaminants that may result in increased resistance in pathogens to therapeutic pharmaceuticals.

- Nevirapine was selected as a commonly prescribed antiretroviral that is an emerging contaminant in surface waters.
- Acetaminophen is a very commonly used antipyretic and analgesic that is commonly found in surface waters.
- BMAA is a natural toxin produced by most cyanobacteria. It was selected as being representative of a natural toxin that is common in eutrophied waters.

Aim 1

Suitable, sensitive and reproducible liquid chromatography mass spectrometry methods of either derivatised or underivatised analytes were developed with suitable limits of detection and quantification, and appropriate precision and accuracy. These methods allowed for the necessary monitoring of removal, accumulation and biotransformation of the xenobiotics by the selected macrophytes – both for macrophyte screening and for model HTSPS evaluation.

Aim 2

Several macrophytes were selected and evaluated in terms of their tolerance for the xenobiotics, their uptake and depuration kinetics and their ability to biotransform the xenobiotics. This data formed the basis for the configuration of the model HTSPS.

Aim 3

The model HTSPS was constructed and tested in various configurations and at different xenobiotic loadings and flow rates. The results exceeded expectations with complete or almost complete removal of all or most xenobiotics under conditions vastly more taxing than might be encountered in real-world application.

CONCLUSION

The HTSPS is clearly a valid concept that may offer a simple sustainable solution to specific xenobiotic pollution management while also having the potential to create revenue via the biomass production. This would encourage maintenance and care of the system while creating employment.

RECOMMENDATIONS FOR FUTURE RESEARCH

We recommend that the HTSPS concept be tested in a small facility where there are known water quality issues. The system is scalable but we suggest a small-scale implementation where there is a constant flow of contaminated water, there is space for the system to be constructed, and where there is appropriate community involvement to manage the biomass production. We suggest that biomass processing to add value (in the form of fuel pellet production) be investigated prior to construction to ensure that excess biomass does not in itself become a waste problem.

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HTSPS	high throughput sequential phytoremediation system
BMAA	β-N-methylamino-L-alanine
LLOQ	lower limit of quantification
LLOD	lower limit of detection
LC-MS	liquid chromatography mass spectrometry
RSD	relative standard deviation
L-BMAA-4,4,4-d_3,$^{15}N_2$	stable isotope-labelled BMAA
HPLC	high performance liquid chromatography
S/N	signal-to-noise (ratio)
ECH-SF	extremely high concentration – standard flow rate
VHC-HF	very high concentration – high flow run
HC-VHF	high concentration – very high flow run
SC-EHF	standard concentration – extremely high flow run

1 INTRODUCTION AND OBJECTIVES

To evaluate the effectivity of xenobiotic removal by submerged and/or floating aquatic macrophytes, it is first necessary to have a robust analytical method or set of methods capable of detecting the xenobiotics at low concentrations and accurately quantifying the xenobiotics – both in raw water samples and in plant material. A variety of potentially harmful common surface water contaminants ranging in size, origin, hydrophobicity and function, including a natural toxin, an antiviral agent, an analgesic and an antibiotic, were selected for this work. Consideration was given to the requirement for a single, or at most two, analytical methods to limit the time and expense of evaluating macrophytes and macrophyte function, because of the large experimental matrices that could result from three plants and four xenobiotics in various combinations at various concentrations. Thus, the ability to quantify all xenobiotics in one or two analytical methods was deemed essential.

The major advantage, other than efficiency, of high throughput sequential phytoremediation systems (HTSPSs) over natural or mixed vegetation artificial wetlands is that each macrophyte is characterised in terms of xenobiotic tolerance, uptake kinetics, bioaccumulation and biotransformation, in advance of system design. Thus, plant performance can be used to preselect plant sequence based on these characteristics. As such, once all macrophyte options have been evaluated in terms of tolerance, uptake, biotranformation/depuration kinetics, nutrient and flow requirements, and biomass production, the actual system is relatively simple to design and does not require extensive empirical testing for sustainability.

To construct an HTSPS to evaluate the concept, it was first necessary to evaluate selected aquatic macrophytes for their tolerance to the selected xenobiotics, their potential for uptake of the xenobiotics, the kinetics of such uptake, depuration, and the potential biotransformation of these xenobiotics by the macrophytes. Given the "proof of concept" nature of this project, only five macrophytes were selected for evaluation based on local availability to yield a final three or four plants to apply the "proof of concept" model HTSPS. Consideration was given to whether the available plants were:

- Indigenous (most desirable).
- Widespread and a common non-indigenous plant (acceptable).
- Widespread and a common plant considered a potential problem (not ideal but should be evaluated for possible use).
- Invasive weed (undesirable).

1.1 Common Surface Water Contaminants

Although there are several toxins of natural origin in fresh water, cyanotoxins are the most common and most abundant of natural toxins. Microcystin is the most studied toxin. It is produced by many bloom-forming and high density benthic mat cyanobacteria such as *Microcystis* and *Oscillatoria* (Hisbergues et al., 2003) that are common contaminants of both large dam reservoirs and smaller concrete reservoirs respectively. Blooms or high benthic densities of benthic species are typical in eutrophic water bodies (Downing & Van Ginkel, 2004). However, the most common cyanotoxin, β-N-methylamino-L-alanine (BMAA), is produced by 95% of tested free-living cyanobacterial strains (Cox et al., 2005; Esterhuizen & Downing, 2008). It therefore constitutes the most likely toxin to be found irrespective of the dominant bloom species. BMAA is also environmentally modulated (Downing et al., 2011; Scott et al., 2014) like the next most common cyanotoxin, microcystin (Downing et al., 2005a, 2005b). However, its ubiquity alone makes BMAA an ideal candidate for using in this work, particularly since similar work on microcystin has been published.

Emerging organic contaminants include herbicides, pesticides, phthalates, polychlorinated biphenyls and pharmaceuticals. Our intended application of green liver technology is treating inadequately treated wastewater to remove common toxins of household origin in conjunction with the inevitable cyanotoxins that are produced in polishing ponds where there are high levels of nutrients. Given this, the most suitable class of potential water contaminants for this work was pharmaceuticals. There is abundant literature on the level of pharmaceutical contamination of surface waters (Agunbiade & Moodley, 2014; Fick et al., 2007; Prasse et al., 2010; and many more) and the potential negative effects thereof on plants and animals (Abedini et al., 1998; An et al., 2009 Brandão et al., 2014; Contardo-Jara et al., 2014; Esterhuizen-Londt et al., 2011b, Fick et al., 2010; Ziková et al., 2010). Pharmaceutical contaminants of the Umgeni river system were reviewed, and specifically those that would survive potentially stressed conventional wastewater treatment (Brandt et al., 2013).

Agunbiade and Moodley (2014) reported the occurrence and concentrations of a variety of pharmaceutical contaminants in the Umgeni river system. This data served as a reference for selecting pharmaceuticals. The range of frequently isolated antibiotics is depicted in Figure 1 as published by Agunbiade and Moodley (2014). Ampicillin was found most consistently within a relatively narrow concentration range. The highest observed concentration was for the quinolone antibiotic nalidixic acid. Although tylosin was observed more frequently at relatively higher concentrations, it was not considered because of its primary use in agriculture. Ampicillin and nalidixic acid were selected given their significant differences in physicochemical properties, their abundance, and the frequency of abundance as reported.
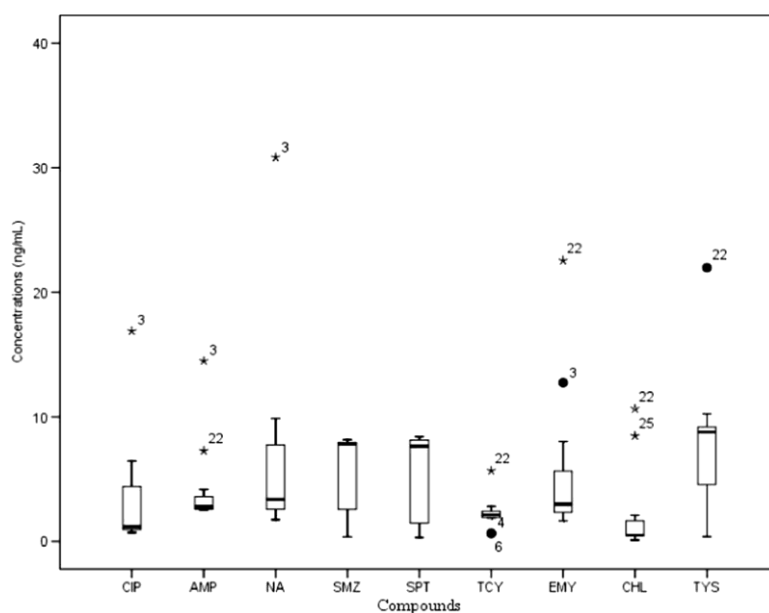
Figure 1: Box and whisker plots of antibiotics quantified in surface waters of the Umgeni river

(CIP) Ciprofloxacin, (AMP) ampicillin, (NA) nalidixic acid, (SMZ) sulfamethoxazole, (SPT) streptomycin, (TCY) tetracycline, (EMY) erythromycin, (CHL) chloramphenicol, and (TYS) tylosin

Figure 2 shows the abundance, and the frequency of abundance for analgesic and antipyretics as reported by Agunbiade and Moodley (2014). Acetaminophen is clearly the most abundant of analgesics and antipyretics in the surface waters of the Umgeni river system.
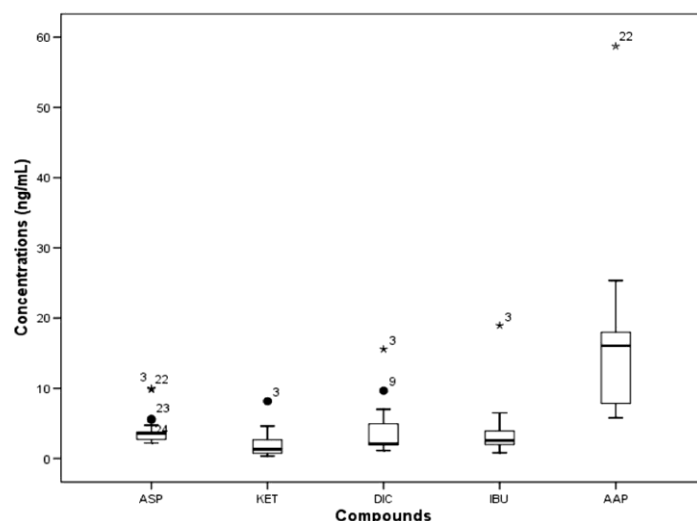
Figure 2: Analgesic/antipyretic drug concentrations in the Umgeni river system

(ASP) aspirin, (KET) ketoprofen, (DIC) diclofenac, (IBU) ibuprofen, (AAP) acetaminophen

In the search for a suitable antiretroviral drug, our selection was informed by current policy, namely, The South African Antiretroviral Treatment Guidelines (Department of Health, 2013). This source suggested nevirapine based on its use where either efavirenz or tenofovir is contraindicated in first-line treatment of all new patients. Typical doses and pharmacokinetics also suggest that this drug may be present in wastewater at detectable concentrations although there are no reports from South African waters. However, reports by Fick at al. (2010, K'oreje et al. (2012) and Prasse et al. (2010), amongst others, suggest a concentration range of between a few nanograms per litre to several hundred nanograms per litre. The selection of this drug therefore also posed an analytical challenge given the potentially low lower limit of quantification (LLOQ) and lower limit of detection (LLOD) that would be required. The xenobiotics listed in Table 1 were selected. The table also shows the diversity of chemical and biological characteristics.

Table 1: Selected xenobiotics with relevant chemical and biological characteristics and indication of occurrence in surface waters

Compound	Mass (Da)	logP	pK$_a$	Biological	Reported in South African waters
BMAA	118.134	N/A	N/A	Neurotoxin	Downing (2011) Esterhuizen et al. (2011)
Ampicillin	349.410	−0.81	2.7, 7.3*	Antibiotic	Agunbiade and Moodley (2014)
Acetaminophen	151.163	0.48	9.51	Analgesic	Agunbiade and Moodley (2014)
Nalidixic acid	232.235	1.50	1.0, 6.0*	Antibiotic	Agunbiade and Moodley (2014)
Nevirapine	266.888	2.50	2.8	Antiviral	No reports

* amine
N/A: not applicable due to derivatisation
logP: in octanol at 25°C

1.2 Published Analytical Methods

There are several methods for analysing BMAA from both water and biological tissues. Our extensive experience with this toxin, and comparison of available methods (Banack et al., 2010; Banack et al., 2011; Esterhuizen-Londt et al., 2011a) led us to adopt propyl chloroformate derivatisation using an amino acid derivatisation and analysis kit called EZ:faast™, which is produced by Phenomenex (USA). For full details see the WRC report entitled "The development of an analytical system for β-N-methylamino-*L*-alanine and investigation of distribution of producing organisms and extent of freshwater contamination" (Downing et al., 2011).

There are several options for detecting pharmaceutical contaminants in surface water (reviewed in Rossi & Sinz, 2002). The decision to use liquid chromatography mass spectrometry (LC-MS) was based on equipment availability and the high degree of sensitivity and selectivity obtainable in complex matrices.

1.3 Xenobiotic Uptake by Plants

Although the uptake and accumulation of antibiotics by crop plants is well documented (reviewed by Kümmerer, 2009) as is the uptake in hydroponically grown plants (Herklotz et al., 2010), there is limited literature on the tolerances to, or uptake of, pharmaceuticals by submerged or floating macrophytes, and even less so for those endemic to Southern Africa. Reports on the uptake of veterinary antibiotics by Italian rye grasses (Xian et al., 2010), the ability of *Myriophyllum aquaticum* and *Pistia stratiotes* to modify the antibiotic growth promoters, tetracycline, and oxytetracycline in aqueous wastewater systems (Gujarathi et al., 2005) amongst several others, suggest a widespread ability for plants to take up pharmaceuticals across a relatively wide size and solubility range. This has yet to be demonstrated for all plants suited to a high throughput submerged or floating-macrophyte xenobiotic removal system.

BMAA uptake has only been well documented for *Ceratophyllum demersum* although there are reports on BMAA in various plants. Work on *C. demersum* (Esterhuizen-Londt et al. 2011b, Esterhuizen et al., 2011) showed substantial and rapid uptake of BMAA with an appropriate plant response to the xenobiotic. It was not determined whether this response was adequate to protect the plant at environmentally relevant BMAA concentrations. However, should *C. demersum* be able to detoxify BMAA adequately and be able to maintain the toxin without subsequent depuration and without damage to primary metabolism at typical environmental levels, this plant may prove sufficient for BMAA removal.

2 EXPERIMENTAL PROCEDURES

The following section deals with the experimental procedures for developing simple rapid methods that require minimum sample preparation and can detect multiple analytes representing a wide chemical and functional range. The procedures include only one or two run chromatographic methods linked to mass spectrometry; the evaluation of locally available macrophytes for their tolerance to, uptake potential and kinetics and depuration of, and potential for biotransformation of the selected xenobiotics; and the construction and efficacy determination of a laboratory scale HTSPS by implementation of the analytical methods and kinetic data obtained in this work.

2.1 Xenobiotics

BMAA was purchased from Sigma. Standard solutions were prepared in 20 mM HCl and stored at −80°C. All other xenobiotics were donated by Adcock Ingram, Johannesburg, and stored in dry form at 4°C. All stock solutions were made in deionised water or water:acetonitrile (80:20) and stored at 4°C prior to dilution in deionised water to the required concentrations.

2.2 Sample Preparation

Stock solutions (1 M) of all pharmaceuticals were made in sterile deionised water and kept at −80°C. A stock solution of BMAA was made in 20 mM HCL. Serial dilutions were made in deionised water of all analytes except BMAA, which was diluted in 20 mM HCL to facilitate derivatisation. Ampicillin, acetaminophen, nalidixic acid and nevirapine were serially diluted with halving dilutions from 5 mg·L^{-1} down to 312 µg·L^{-1}. The concentration for BMAA standards ranged from 2.38 mg·L^{-1} to 149 µg·L^{-1}.

As described by Esterhuizen-Londt & Downing (2011c), Strata-X-C (33 µm particle size, 85 A pore size, 800 m^2·g^{-1} surface area) mixed-mode polymeric strong cation sorbent Giga^{TM} tubes (Phenomenex) were conditioned with 20 ml of pure methanol (liquid chromatography grade, Merck) at a flow rate of 20 ml·min^{-1} and equilibrated with 20 ml of formate buffer (pH 4) at a flow rate of 20 ml·min^{-1}.

BMAA (2.38 µg and 0.2382 µg respectively) was added to 1 L of acidified (1 ml of 2 N HCl) sterile reverse osmosis (Consolidated Water Conditioning CWC Series 7T) purified water in triplicate and concentrated using a solid-phase extraction Giga^{TM} tubes column (Phenomenex). A flow rate of 10 ml·min^{-1} was used. The column was washed with 20 ml each of 2% formic acid and 100% methanol (20 ml·min^{-1}) before elution in 5% ammonium hydroxide in a 1:1 ratio of methanol:acetonitrile (10 ml·min^{-1}). The sample was subsequently acidified and dried under a low nitrogen gas stream to remove hydroxide ions that could have interfered with derivatisation. The sample was resuspended in 1 ml of sterile reverse osmosis water, and 500 µl of this was derivatised and analysed on LC-MS as described below. Relevant solid-phase extraction eluent fractions were collected and analysed for BMAA.

The testing was scaled up to investigate recovery in a larger volume. BMAA (2.38 µg) was added to 20 L of reverse osmosis water and concentrated as described. The collected eluent was dried under a stream of nitrogen gas. It was resuspended in 1 ml reverse osmosis water and the entire volume was derivatised.

This process was repeated on water samples taken from a 30 L *C. demersum* culture tank with approximately 8 g·L^{-1} plant material (wet weight). Not only did the solution contain the supplied nutrients (50 mg·L^{-1} Nutrifeed™ hydroponic solution, Starke Ayres), but it also contained any other organic matter originating from the *C. demersum*, as well as any other organisms and their exudates growing in this non-axenic tank. These samples were sterilised by autoclaving prior to spiking with BMAA as many organisms are known to take up BMAA. Typically, spiked live pond water loses any free BMAA rapidly (Esterhuizen & Downing, 2011). Flow-through and wash fractions were collected and analysed for BMAA content as described above.

Ampicillin, acetaminophen, nevirapine and nalidixic acid were added at a concentration of 1 µg·L^{-1} each to deionised water. These xenobiotics were recovered from the water samples by gravity-feeding the water samples through 100% acetonitrile conditioned and deionised water equilibrated C18 solid-phase extraction columns (Phenomenex Strata-X/XL, 3 ml capacity or 20 ml capacity depending on volume of sample) and eluting with a predetermined stepwise gradient to limit contaminants while recovering as much of the analyte as possible. The stepwise gradient was designed by eluting with increasing percentages of acetonitrile and analysing all fractions collected.

This process was repeated on raw *C. demersum* culture tank water samples of 50 ml, 20 ml and 2 ml, which were spiked with ampicillin, acetaminophen, nevirapine and nalidixic acid at concentrations of 50 ng·L^{-1}, 250 ng·L^{-1}, 500 ng·L^{-1}, 1000 ng·L^{-1} and 2500 ng·L^{-1}. The relevant solid-phase extraction eluent fractions were collected, pooled and lyophilised overnight before resuspension in water for analysis as described below.

To optimise the extraction of the selected contaminants from the plant material, the *C. demersum* culture (as described earlier) was harvested, rinsed thoroughly in deionised water and snap-frozen in liquid nitrogen before grinding in liquid nitrogen to a fine powder. An initial spike of 1 g each of the four pharmaceutical xenobiotics was added to 200 mg of the lyophilised plant powder to evaluate extraction protocols. The following extractions were done on replicates of the spiked matrix material:

- 65°C aqueous extraction with agitation for two hours.
- 25°C 20% acetonitrile extraction with agitation for two hours.
- 25°C 40% acetonitrile extraction with agitation for two hours.

Higher organic concentrations were not used because of the partition coefficient of the most hydrophobic of the xenobiotics, indicating that complete extraction would occur at 40% or less.

The extracts were lyophilised, resuspended in 1 ml water and fractionated by solid-phase extraction. The two fractions previously determined to contain all analytes, were combined, lyophilised, resuspended in 1 ml water, and analysed as described below.

Matrix effects on analysis were determined by spiking 100 mg freeze-dried plant material with a range of concentrations corresponding to total raw water concentrations at a macrophyte-carrying capacity of 50 g *C. demersum* per litre. This typically yields between 1.1 g and 1.3 g dry weight. Since typical xenobiotic concentrations to be tested (based on published values) are between 500 ng·L^{-1} and 10 000 ng·L^{-1}, this corresponds to 380 pg·mg^{-1} (dry weight) at the lowest concentration assuming complete accumulation of the xenobiotic by the plant, up to 909 ng·mg^{-1}. Thereafter, 100 mg freeze-dried samples were spiked in replicates of five with 50 ng, 500 ng and 5000 ng each of the four pharmaceutical xenobiotics, extracted and fractioned as described above. The combined lyophilised and resuspended samples were analysed using LC-MS as described below.

2.3   Sample Analysis

BMAA was analysed using a Shimadzu 2010AB liquid chromatography coupled to a Shimadzu mass spectrometer (2010 EV) after derivatisation using the liquid chromatography form of the EZ:faast™ amino acids analysis kit (Phenomenex). BMAA was separated from other amino acids by liquid chromatography on a commercial column (Phenomenex AAA mass spectrometer 250 × 2.0 mm amino acid analysis column).

A solvent gradient was used with A: 10 mM ammonium formate in water and B: 10 mM ammonium formate in methanol (0.0 min = 68% B, 13.00 min = 83% B, 13.01 min = 68% B, 17.00 min = 68% B) at a flow rate of 0.25 ml·min^{-1} and 1 µl sample injection volume. The column temperature was kept constant at 35°C. The mass spectrometer electrospray ionisation source (positive ion mode) temperature was set at 250°C. The ion scan range was between 20 m/z and 600 m/z. The detector voltage was set at 1.5 kV unless otherwise stated. The interface voltage was set at 4.5 kV and the curved desolvation line voltage at −20 V with the heating block at 200°C. The data was analysed using LC-MS solutions Ver. 3 software.

The single quadrupole detector (Shimadzu 2010EV) with a Shimadzu LC-20AB liquid chromatography system was validated using a propyl chloroformate precolumn derivatised BMAA standard (Sigma). The resulting molecule was quantified against three internal standards: homoarginine, methionine-D3 and homophenylalanine. Multiple user, delayed derivatisation and delayed analysis assessments were conducted to verify the system and to determine how robust the method was. BMAA standards were derivatised in varying concentrations on one day and injected in triplicate to assess equipment accuracy and reproducibility. Three individuals undertook derivatisation of standards in triplicate on three consecutive days to determine derivatisation reproducibility. A calibration curve was constructed for quantification based on the ratio of peak areas of the representative molecular ion for BMAA (m/z = 333)

to that of methionine-D3 (m/z = 281). The LLOD and LLOQ values were determined experimentally by dilution to invisibility [signal-to-noise (S/N) ratio < 3 in a matrix] and reproducibility (standard deviation ±5% in a matrix) respectively.

Accuracy and precision were calculated from the five calibration standards of BMAA against the internal standard to validate the method. Precision represents a percentage relative standard deviation (%RSD) of the analyte to internal standard peak areas obtained from replicates ($n = 5$). Accuracy was calculated as a percentage variation relative to the nominal concentration of each point.

The chromatographic separation and detection of the pharmaceutical analytes were optimised in terms of solvents, solvent modifiers, solvent ratio gradient and run time. Detection parameters were optimised based on a general instrument tune rather than an analyte specific tune because of the desire for a single run for all xenobiotics. Manual tuning to resolve what appeared to be ion suppression was done by altering interface and curved desolvation line voltages. Detector voltage was optimised manually for sensitivity in matrices. The optimum analytical conditions are described below.

All pharmaceuticals were analysed on a Shimadzu 2010AB liquid chromatography device coupled to a Shimadzu mass spectrometer (2010 EV). Separation was achieved by reversed phase chromatography on a commercial column (Phenomenex Gemini-C18). A solvent gradient was used with A: 1 mM ammonium formate in water and B: acetonitrile (0.0 min = 5% B, 2.00 min = 20% B, 6.0 min = 95% B curve 3, 6.01 min = 5% B, 10 min = 5% B) at a flow rate of 0.35 ml·min^{-1} and 5 µl sample injection volume. The column temperature was kept constant at 35°C. The mass spectrometer electrospray ionisation source (positive ion mode) temperature was set at 250°C. The ion scan range was between 100 m/z and 400 m/z. The detector voltage was set at 1.5 kV. The interface voltage was set at 4.5 kV and the curved desolvation line voltage at −20 V with the heating block at 200°C. The data was analysed using LC-MS solutions Ver. 3 software.

Accuracy and precision were calculated from the five calibration points. Precision represents a %RSD of the analyte relative to the analyte peak areas ($n = 5$). Accuracy was calculated as a percentage variation relative to the nominal concentration of each point.

The biomass production rate was established by wet weight determination prior to exposure and seven days after the start of each experiment, except for the extremely high flow rate experiment, where this was deemed unnecessary

Nitrates were measured by reduction using copper cadmium and colorimetric analysis using Griess–Illosvay reagent. All measurements were made in triplicate and quantified of a standard curve ($r^2 = 0.995$).

2.4   Macrophyte Selection, Collection and Maintenance

Macrophytes were collected from nearby water sources based on availability while taking cognisance of the desirability of the plants and the need for diversity in terms of the nature of the plant. To this end, both floating and submerged plants, and both flowering plants and an alga were selected. *Spirodela polyrhiza, Egeria densa* and *Stuckenia pectinata* were collected from the Baviaans river and the Kouga river, Eastern Cape, South Africa. *C. demersum* was purchased from a local aquatic plant supplier. *Chara vulgaris* was sourced from agricultural animal drinking troughs near Alexandria in the Eastern Cape, where it is commonly used to remove nutrients and prevent the need for frequent water changes. In all cases, the macrophytes were collected in 25 L sampling bags in sufficient source water to submerge the plants, or, in the case of the floating plant *S. polyrhiza*, in 500 ml bottles. Macrophytes were identified based on morphological characteristics alone.

Only healthy growing plants were selected. These plants were thoroughly rinsed in water and all visible fauna and epiphytic growth removed. Where any indication of fungal growth was noted, plants were submerged overnight in 0.0005% malachite green. Macrophyte species were cultured in individual 30 L glass tanks in □ strength Nutrifeed™ hydroponic solution (Starke Ayres) for all plants except *C. demersum*, which was grown in ¼ strength Nutrifeed™. Approximate in-tank flow rates of 500 ml·min⁻¹ for *C. demersum and E. densa*, and 1 L·min⁻¹ for *S. pectinatus* were maintained. No internal flow was used for *C. vulgaris* and *S. polyrhiza*. The medium in each tank was changed weekly.

2.5 Exposure of Macrophytes

The tolerance for each macrophyte was determined by exposing the plant to each of the xenobiotics in growth medium for a defined period. Tolerance to a xenobiotic was defined as the absence of a negative impact on oxygen evolution after 24-hour exposure to the xenobiotic at the exposure concentration. The initial exposure concentration was 30 mg·L⁻¹, which is 1000-fold higher than reported maximum environmental levels in the Umgeni river system). Where negative effects were observed, the exposure concentration was reduced sequentially until no significant negative effects were observed.

To characterise the individual uptake kinetics of each plant for each xenobiotic, each plant was exposed in triplicate to all pharmaceuticals combined at equal concentrations ranging from 20 ng·ml⁻¹ to 3 µg·ml⁻¹ over a period of three days. This was done to reflect a real-world situation. Sampling was performed at 0 minutes, 30 minutes, 60 minutes, 90 minutes, 120 minutes, 1440 minutes, 2880 minutes and 4320 minutes. Plants were exposed in maintenance medium at a constant temperature of 22°C and an illumination of 25 µmol E⁻² s⁻¹. The amount of xenobiotic missing from the exposure vessel, relative to a plant-free control, was deemed to have been taken up by the plant. The rates of uptake were calculated per gram (wet weight) of plant material. Uptake rates were plotted against exposure concentration to determine whether the maximum uptake rate was below the expected maximum exposure concentration.

BMAA exposure has previously been demonstrated in *C. demersum* (Esterhuizen et al., 2011) as described above, but the fate of the BMAA once taken up has not been reported. The metabolism of the isotopically labelled BMAA and the potential for depuration of the toxin were therefore investigated. Stable isotope-labelled BMAA (L-BMAA-4,4,4-d₃,¹⁵N₂) was purchased from the Institute of Ethnomedicine (Jackson, Wyoming). *C. demersum* was purchased from Ultimate Aquatics (Port Elizabeth, South Africa). To allow acclimatisation, *C. demersum* was cultivated for four weeks prior to commencing the experiment in synthetic medium (Privasoli) at a constant temperature and illumination of 22°C and 25 µmol E⁻² s⁻¹, respectively. Following 24-hour exposure, all specimens were individually removed from the exposure beakers and washed with sterile distilled deionised water followed by methanol.

Replicate *C. demersum* sections of 2 cm long and consisting of stem and leaves were statically exposed in sterile water, each in an individual beaker, to 1 µM L-BMAA-4,4,4-d₃,¹⁵N₂ for 24 hours under illumination and temperature conditions as described above. Following 24-hour exposure, all specimens were individually removed from the exposure beakers and washed with sterile distilled deionised water followed by methanol.

To determine whether depuration of xenobiotics occurred, plants exposed to the highest concentration of antibiotics/analgesics for three days were thoroughly rinsed in fresh running water and transferred to a xenobiotic-free medium. Medium concentrations of all xenobiotics were measured after 24 hours to determine whether plants excreted any of the drugs. The depuration medium was treated as depicted in Figure 3 for pharmaceutical tests.

For BMAA biotransformation and retention, each post-exposure wash in both water and methanol was repeated in triplicate. Thereafter, the washed specimens were placed in individual flasks containing only sterile distilled deionised water for depuration. Sections remained in BMAA-free medium (water) for 72

hours or 120 hours, after which they were harvested by snap-freezing in liquid nitrogen and stored at −80°C until further processing.

In the absence of isotopically labelled pharmaceuticals, biotransformation was determined as follows: Plants exposed to the highest concentration of pharmaceuticals were rinsed thoroughly, patted dry on towelling paper, and the wet weight determined. Plants were snap-frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Pharmaceuticals were extracted and partially purified by solid-phase extraction as described below.

All plant sections were ground to a fine powder in liquid nitrogen and lyophilised overnight using a VirTis freeze dryer (−42.6°C, 170 mTorr vacuum). To extract free cellular amino acids from BMAA-exposed *C. demersum*, lyophilised plant matter was resuspended in 0.1 M trichloroacetic acid by vortexing. Thereafter, samples were sonicated in a water bath at 4°C for 10 minutes and vortexed again. Sonication and vortexing were repeated once more, after which samples were incubated at room temperature for 30 minutes. Following incubation, samples were centrifuged (Eppendorf MiniSpin) at 10 000 × g for 10 minutes at room temperature. The resultant protein pellet was washed in 0.1 M trichloroacetic acid by resuspension via vortexing. Following centrifugation as described above, the supernatant was pooled with that of the first extraction step. Protein-associated amino acids were extracted from the remaining protein pellet by acid hydrolyses with 6 N HCl as described by Esterhuizen et al. (2011). Following hydrolysis, hydrolysates were filtered using Ultrafree-MC 0.22 mm centrifugal filtration units, the filtrate dried down using a Savant SpeedVac® and the dried residue resuspended in 20 mM HCl.
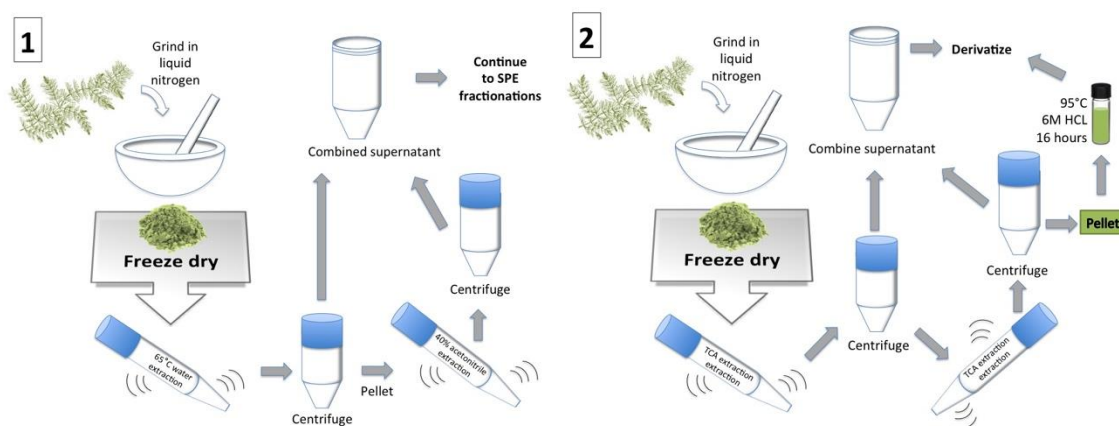

Figure 3: Extraction of pharmaceuticals (1) and BMAA (2) from plant material

(SPE) solid-phase extraction
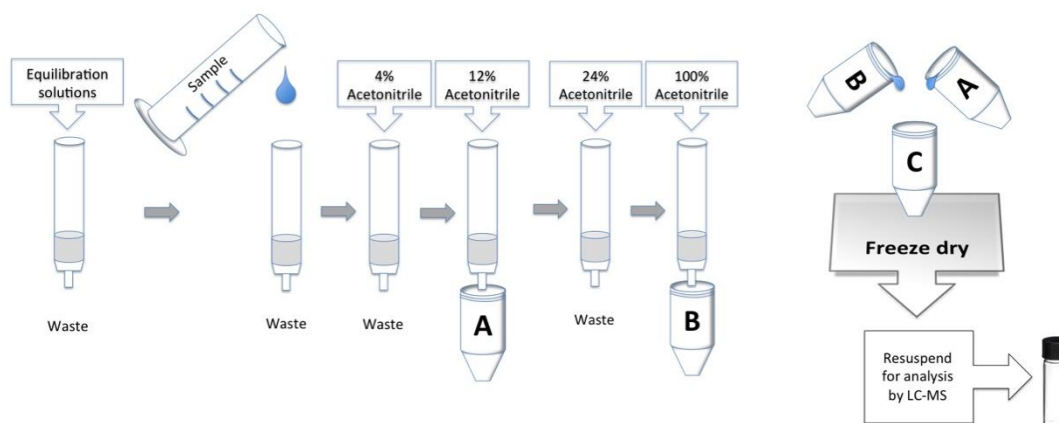*(TCA) trichloroacetic acid*

Figure 4: Extraction and sample preparation of xenobiotics from depuration medium and plant extracts

Xenobiotics absent from the initial exposure medium, which were not lost from the plant during the depuration experiment but not observed within the plant, were considered to have undergone biotransformation.

Free and protein-associated amino acid fractions were, following derivatisation with a propyl chloroformate derivative, separated using liquid chromatography and analysed using single quadrupole mass spectrometry as described by Downing et al. (2011). Detection and analysis of isotopically labelled amino acids were based on high-performance liquid chromatography (HPLC) retention times and mass spectrometric detection of amino acid isotopologue parent ions as described by Downing et al. (2014). Analysis of label distribution from BMAA to proteinogenic and non-proteinogenic amino acids was based on the relative abundance of amino acid isotopologues extracted from BMAA-exposed cultures, normalised against control cultures.

2.6 Evaluation of Potential Toxicity of Xenobiotics to Macrophytes

All exposed plants were monitored for adverse effects by determining their photosynthetic rates as measured by oxygen evolution after the stipulated exposure times and concentrations. An oxygraph containing a Hansatech oxygen electrode (Hansatech, King's Lynn, Norfolk, UK) was used to measure the rates of oxygen evolution or uptake. The electrode was calibrated with air-saturated distilled H_2O at 20°C as per the manufacturer's instructions. Replicate plant sections were briefly patted dry with towelling paper, weighed, and placed in 30 ml growth medium in 50 ml Falcon tubes containing each of the xenobiotics individually at the selected concentration for the relevant time period. Samples were dark-adapted for 15 minutes. The reaction chamber was illuminated with photosynthetic fluorescent tubes (Triton Dayglo©) under continuous illumination of 100 μmol of photons $m^{-2} \cdot s^{-1}$. The rate of oxygen evolution was measured over 10 minutes. Oxygen evolution was expressed per wet weight of plant material, as $nmol \cdot mg^{-1} \cdot min^{-1}$.

2.7 Xenobiotic Analysis

BMAA was analysed using a Shimadzu 2010AB liquid chromatography system coupled to a Shimadzu mass spectrometer (2010 EV) after derivatisation using the liquid chromatography form of the EZ:faast™ amino acids analysis kit (Phenomenex). BMAA was separated from other amino acids by liquid chromatography on a commercial column (Phenomenex AAA mass spectrometer 250 × 2.0 mm amino acid analysis column). A solvent gradient was used with A: 10 mM ammonium formate in water and B: 10 mM ammonium formate in methanol (0.0 min = 68% B, 13.00 min = 83% B, 13.01 min = 68% B, 17.00 min = 68% B) at a flow rate of 0.25 ml·min⁻¹ and 1 μl sample injection volume. The column temperature was kept constant at 35°C. The mass spectrometer electrospray

ionisation source (positive ion mode) temperature was set at 250°C. The ion scan range was between 20 m/z and 600 m/z. The detector voltage was set at 1.5 kV unless stated otherwise. The interface voltage was set at 4.5 kV and the curved desolvation line voltage at −20 V with the heating block at 200°C. The data was analysed using LC-MS solutions Ver. 3 software and BMAA quantified as described in Deliverable 1 of this project.

The chromatographic separation and detection of the analytes was optimised in terms of solvents, solvent modifiers, solvent ratio gradient and run time. Detection parameters were optimised based on a general instrument tune rather than an analyte specific tune because of the desire for a single run for all xenobiotics. Manual tuning to resolve what appeared to be ion suppression was done by altering interface and curved desolvation line voltages. Detector voltage was optimised manually for sensitivity in matrices. The optimum analytical conditions are described below.

All pharmaceuticals were analysed on a Shimadzu 2010AB liquid chromatography system coupled to a Shimadzu mass spectrometer (2010 EV). Separation was achieved by reverse-phase chromatography on a commercial column (Phenomenex Gemini-C18). A solvent gradient was used with A: 1 mM ammonium formate in water and B: acetonitrile (0.0 min = 5% B, 2.00 min = 20% B, 6.0 min = 95% B curve 3, 6.01 min = 5% B, 10 min = 5% B) at a flow rate of 0.35 ml·min^{-1} and 5 µl sample injection volume. The column temperature was kept constant at 35°C. The mass spectrometer electrospray ionisation source (positive ion mode) temperature was set at 250°C. The ion scan range was between 100 m/z and 400 m/z. The detector voltage was set at 1.5 kV. The interface voltage was set at 4.5 kV and the curved desolvation line voltage at −20 V with the heating block at 200°C. The data was analysed using LC-MS solutions Ver. 3 software.

2.8 Model System Design and Construction

A glass tank measuring 150 × 50 × 30 cm was constructed to allow variable partition types to generate up to ten sub-sections. Internal partitions are removable and can be configured in several ways to yield different compartment sizes. Sufficient partition slots exist for baffles to ensure adequate mixing in each compartment as illustrated in Figure 5. The tank had a workable total volume of between 75 L and 225 L. A 260 L reservoir and a 260 L waste collection tank were installed. The feed to the HTSPS was a gravity-feed from a pump-fed constant pressure head unit. Feeding of the high tank for gravity-feeding the system was achieved using a simple submersible constant flow pump. The secondary reservoir was filled by a constant flow submersible tank with overflow back into the reservoir tank. Overflow to the waste tank was achieved via a level-adjustable gravity siphon. Figure 5 shows this design. Light was provided by 324 W of fluorescent plant growth tubes (Pro-Flora®). No filters, traps or ion exchange resins were used in the system. All xenobiotic removal was due to the plants and/or photolysis.
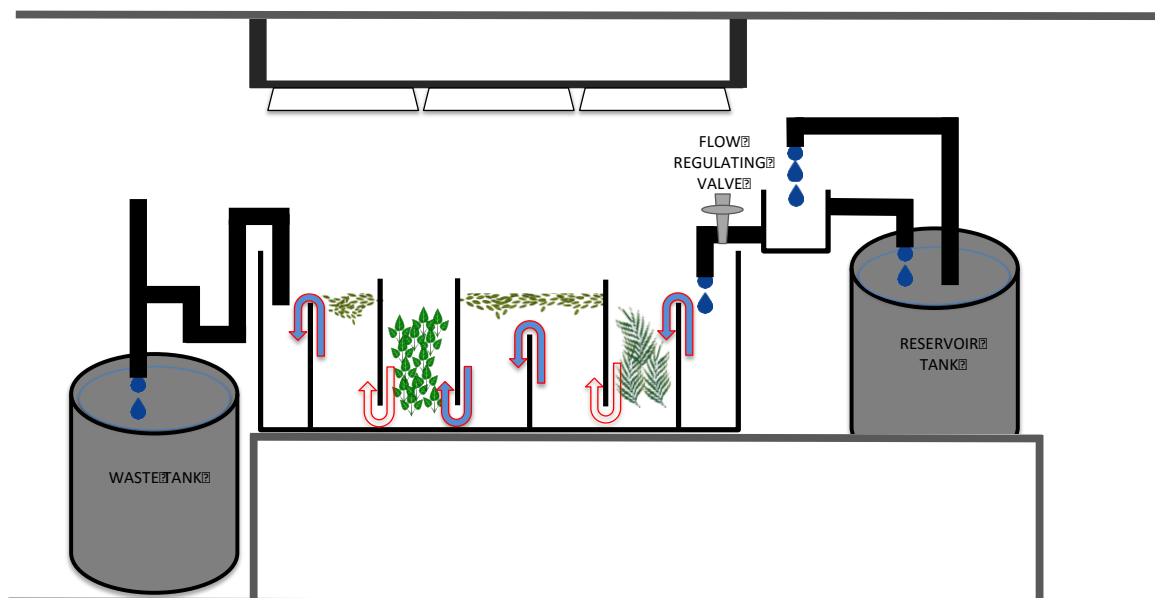
Figure 5: HTSPS model system design

To select a sequence for the macrophytes, tolerances of all macrophytes for all xenobiotics had to be considered in conjunction with the uptake kinetics, and biotransformation and depuration data. Tolerances were well above the concentrations used in HTSPS efficacy evaluation. *S. polyrhiza* was, however, somewhat sensitive to nevirapine and to a lesser extent to nalidixic acid. Plant sequence was therefore based primarily on the kinetics of xenobiotic uptake, nutrient requirements and biomass production at those nutrient levels. Thus, based on a biomass doubling-rate of just over five days under excess nutrients, *S. polyrhiza* was placed in the final compartment to balance biomass production with the much slower *C. vulgaris* and the other plants. Additionally, the relatively higher biotransformation rate of nalidixic acid by *S. polyrhiza* reduced the accumulation of this pharmaceutical in its native form, and allowed re-uptake of depurated nalidixic acid from upstream plants. Based on these considerations, only two plant sequences were tested:

- *E. densa* ➔ *C. vulgaris* ➔ *C. demersum* ➔ *S. polyrhiza.*
- *C. vulgaris* ➔ *E. densa* ➔ *C. demersum* ➔ *S. polyrhiza.*

2.9   Model System Evaluation

Nutrifeed™ hydroponic solution (Starke Ayres) was used at full strength to simulate highly eutrophied water as is typical of xenobiotic-rich source water. Similarly, xenobiotic concentrations were greater than 10 000 times typically reported surface water levels to represent highly contaminated source water prior to release into river flow or impounds. Thus, $0.1 \, mg \cdot L^{-1}$ or $0.05 \, mg \cdot L^{-1}$ of each of acetaminophen, nevirapine, nalidixic acid, ampicillin and BMAA were used as the "highly contaminated source water", and either $50 \, ng \cdot L^{-1}$ or $100 \, ng \cdot L^{-1}$ of each at the much higher flow rates, representing a system suitable for purification of typical antibiotic contaminated surface waters as widely reported.

Flow rates were selected to highlight the efficiency of the high throughput system relative to classical polishing ponds. As such, the slowest flow rate ($0.02 \, hour^{-1}$) used yielded a retention time of 48 hours. Subsequent flow rates of $0.04 \, hour^{-1}$, $0.08 \, hour^{-1}$ and $0.16 \, hour^{-1}$ were used at more appropriate xenobiotic concentrations of 500 times, 10 times and 5 times higher than typically very high reported surface water contamination concentrations for the selected xenobiotics (see Table 2). For ease of conceptualisation, the highest flow rate through a high throughput system such as this, but at the scale of an Olympic swimming pool, would allow a flow rate more than 100 L per minute.

Samples were collected at the downstream end of each compartment of the system, and at the inflow and outflow every six hours during exposure and depuration, and every three hours for flow rates of 0.08 hour⁻¹ and 0.16 hour⁻¹. Samples were collected in sterile 50 ml Falcon tubes, snap-frozen, and stored at −80°C until analysis.

Table 2: Table of experimental design

Name	Xenobiotic concentration ($\mu g \cdot L^{-1}$)	Flow rate (hour⁻¹)	Retention time (hours)	Exposure (hours)	Depuration (hours)
EHC-SF	100.00	0.021	48	96	48
VHC-HF	50.00	0.042	24	48	24
HC-VHF	0.10	0.084	12	24	24
SC-EHF	0.05	0.168	6	12	24

EHC: extremely high concentration   *EHF: extremely high flow*
VHC: very high concentration   *VHF: very high flow*
HC: high concentration   *HF: high flow*
SC: standard concentration   *SF: standard flow*

3   RESULTS, TREATMENT OF RESULTS AND DISCUSSION

3.1   Optimised Analysis

Figure 6 shows a typical unprocessed chromatogram of propyl chloroformate derivatised BMAA obtained by HPLC mass spectrometer analysis of m/z 333.
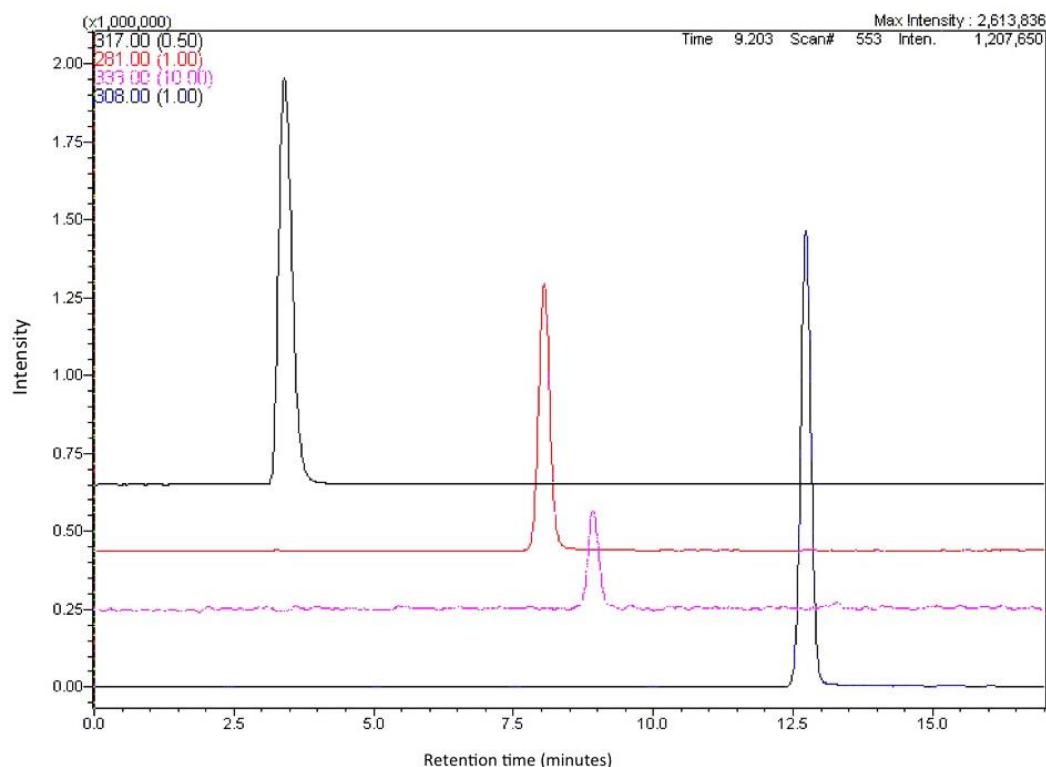

Figure 6: A typical chromatogram obtained from a standard solution

Figure 7 shows standard curve of BMAA based on the ratio of the peak areas of the internal standard methionine-D3 (m/z 281) and BMAA (m/z 333) R^2 = 0997 (n = 5).
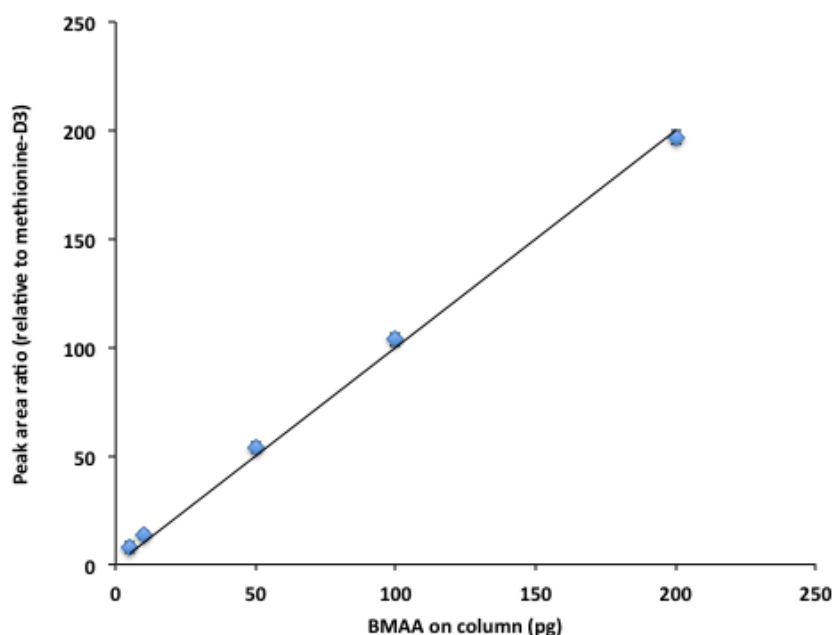
Figure 7: Standard curve of BMAA concentrations against the BMAA quasi-molecular ion peak area relative to internal standard methionine-D3

Table 3 lists the precision and accuracy as determined for the optimised LC-MS BMAA analytical method. Precision and accuracy are both acceptable for the intended use.

Table 3: Precision and accuracy of calibration standards for BMAA

Concentration	%RSD	% Accuracy
5 pg	25.56	65.70
10 pg	7.50	87.40
50 pg	9.34	95.09
100 pg	8.26	95.98
200 pg	9.64	93.13

A final optimised ammonium formate concentration of 1 mM was used to accommodate the wide range of hydrophobicity and pK_a values while retaining excellent separation of analytes and adequate ionisation levels. At higher ammonium formate concentrations, the relatively low pK_a of the nalidixic amine group resulted in co-elution with ampicillin. The observable increase in sensitivity at high concentrations of analytes, particularly nevirapine because of its high ionisation potential, did not warrant further optimisation to separate the ampicillin and nalidixic acid at lower pH since the limits of detection for nevirapine were already extremely low in water and acetonitrile with no solvent modifiers. Figure 8 shows a typical chromatogram of acetaminophen, ampicillin, nalidixic acid and nevirapine standard solutions at a concentration of 5 ng on column separated using the developed LC-MS method.
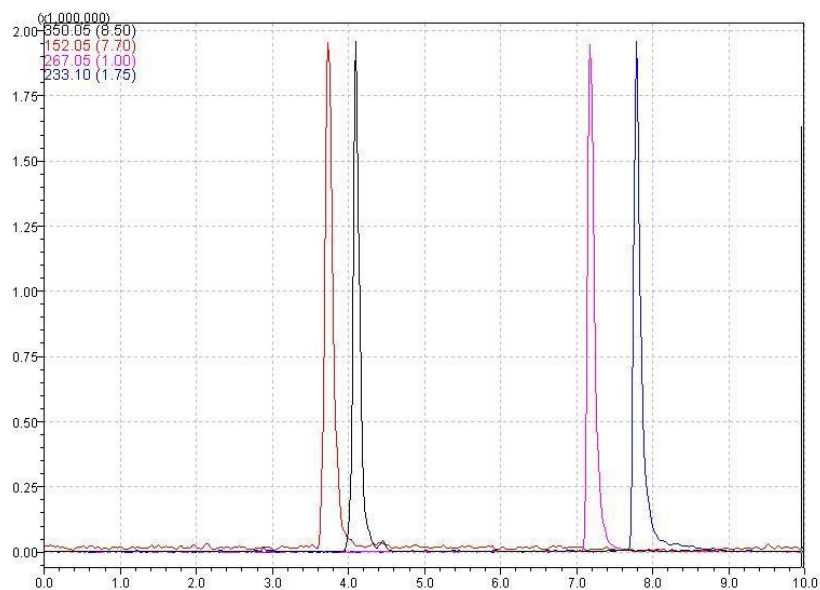
Figure 8: Typical chromatogram of standard solutions

Figure 9 shows the standard curves obtained for all pharmaceutical xenobiotics:

- A: acetaminophen [$y = 7 \times 10^{-12}x^2 + 3 \times 10^{-6}x$, $R^2 = 0999$].
- B: ampicillin [$y = 10^{-11}x^2 + 5 \times 10^{-6}x$, $R^2 = 0999$].
- C: nalidixic acid [$y = 4 \times 10^{-13}x^2 + 2 \times 10^{-6}x$, $R^2 = 0999$].
- D: nevirapine [$y = 2 \times 10^{-13}x^2 + 2 \times 10^{-7}x$, $R^2 = 0996$].

Error bars denote standard deviation ($n = 5$).
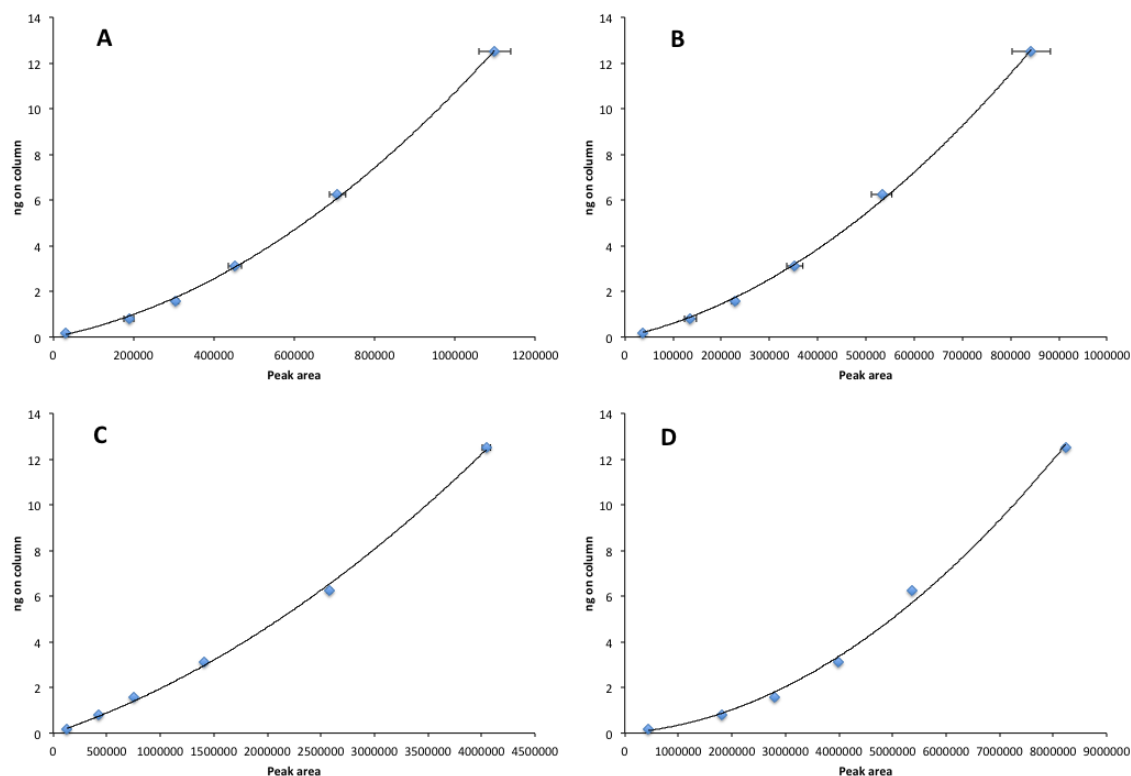
Figure 9: Standard curves for all pharmaceutical xenobiotics

The quadratic fit for standard curves was necessitated because of a pH to prevent co-elution of nalidixic acid and ampicillin. Although not necessarily typical, quadratic fits to standard curves occur frequently with electron spray ionisation-generated chromatograms (Rossi & Sinz, 2002) and can be substantially linearised using a stable isotopologue internal standards (Shi, 2003). It is worth noting that for nevirapine, dilution to LLOD did not alter the quadratic fit of data irrespective of source or curved desolvation line voltage or the use of solvent modifiers.

Table 4 lists the precision and accuracy of the calibration point analysis. Although it was possible within the usable levels of precision and accuracy to produce an additional two sequentially reduced concentration data points below the lowest point shown while retaining a S/N ratio of 10 or greater, the absence of a requirement for accurate or precise quantification did not warrant this. LLOD was considered the more important parameter since removal to limits of detection is the desired result.

Table 4: Precision and accuracy of calibration standards for pharmaceuticals

Compound	Concentration (mg/L)	%RSD	% Accuracy
Acetaminophen	0.156	16.24	66.20
	0.781	6.74	95.39
	1.563	2.19	99.80
	3.125	3.64	89.54
	6.250	2.93	89.94
	12.500	3.62	93.96
Ampicillin	0.156	23.11	70.93
	0.781	7.46	89.50
	1.563	8.06	94.02
	3.125	3.44	96.22
	6.250	6.01	88.10
	12.500	4.06	90.53
Nevirapine	0.156	4.35	66.47
	0.781	2.89	68.68
	1.563	2.28	64.53
	3.125	3.31	72.76
	6.250	8.09	90.69
	12.500	2.24	78.42
Nalidixic acid	0.156	25.56	28.78
	0.781	7.50	82.40
	1.563	9.34	88.91
	3.125	8.26	84.24
	6.250	9.64	75.02
	12.500	18.23	82.94

3.2 Analysis in Water and Plant Growth Medium and Plant Tissue

Raw water samples were taken from a 30 L *C. demersum* culture tank with approximately 8 g·L^{-1} plant material (wet weight). The samples contained not only supplied nutrients but also any other organic matter originating from the *C. demersum* as well as any other organisms and their exudates growing in this non-axenic tank. This adequately reflected the conditions that existed during the evaluation of the model green liver system.

Typical recovery rates from BMAA-spiked deionised water and spiked live pond water ranged from 82% to 103%, and 43% respectively. However, sterilisation of the live pond water raised the percentage recovery to 78% (±7%). The latter was comparable to apparent recovery rates in a complex amino acid matrix and probably reflects the difference between the method detection limit as a function of the instrument detection limit, and the practical detection limit. However, the difference between live and sterile pond water is a clear indication of the well-documented rapid uptake of BMAA by phytoplankton. No such difference was observed with the pharmaceuticals. Although of academic interest, this is of little interest from an evaluation perspective of xenobiotic uptake and transformation. It is simply the LLOD and LLOQ that are of any interest as these reflect the ability to determine the presence of active compounds. As such, any evaluation of uptake and bioaccumulation or biotransformation, and potential for depuration must be based on these limits in matrix. To that end, these are shown in Table 5.

Table 5: LLODs and LLOQs for all xenobiotics

	Matrix	LLOD (S/N>3)	LLOQ (S/N>10)
BMAA	Deionised water (on column)	3 pg	10 pg
	Pond water (per litre)	10 ng·L^{-1}	30 ng·L^{-1}
	Plant matrix (per gram dry weight)	1 pg·g^{-1}	10 pg·g^{-1}
Acetaminophen	Deionised water (on column)	78 pg	156 pg
	Pond water (per litre)	156 ng·L^{-1}	312 ng·L^{-1}
	Plant matrix (per gram dry weight)	125 ng·g^{-1}	500 ng·g^{-1}
Ampicillin	Deionised water (on column)	150 pg	312 pg
	Pond water (per litre)	312 ng·L^{-1}	500 ng·L^{-1}
	Plant matrix (per gram dry weight)	125 ng·g^{-1}	250 ng·g^{-1}
Nevirapine	Deionised water (on column)	5 pg	*30 pg
	Pond water (per litre)	3 ng·L^{-1}	# 100 ng·L^{-1}
	Plant matrix (per gram dry weight)	2 ng·g^{-1}	# 15.6 ng·g^{-1}
Nalidixic acid	Deionised water (on column)	20 pg	*50 pg
	Pond water (per litre)	6 ng·L^{-1}	# 100 ng·L^{-1}
	Plant matrix (per gram dry weight)	4 ng·g^{-1}	# 15.6 ng·g^{-1}

Estimated below calibration curve with (n<5), quantification will occur above 156 pg on column
 # Limited by calibration curve and sample loop volume
S/N: signal-to-noise ratio of method chromatogram

Figure 10 shows an example of quantification of acetaminophen in water with the LLOQ at 156 pg on column with a S/N ratio of 10.58.
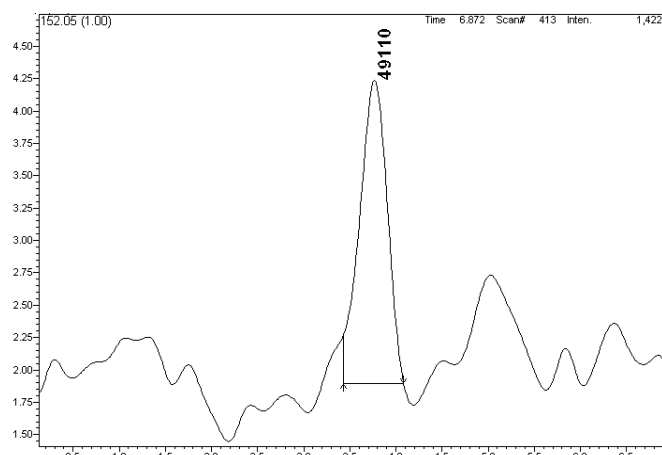

Figure 10: Example chromatogram of acetaminophen in water

Figure 11 shows examples of unprocessed, raw chromatograms of BMAA (m/z 333) [left] and nevirapine (m/z = 267) [right] analysed from plant matrix at a concentration of 10 ng·g and 250 ng·g (dry weight)$^{-1}$ respectively (approximately 400 g wet weight plant material, or approximately 9 L pond volume equivalent for this plant).
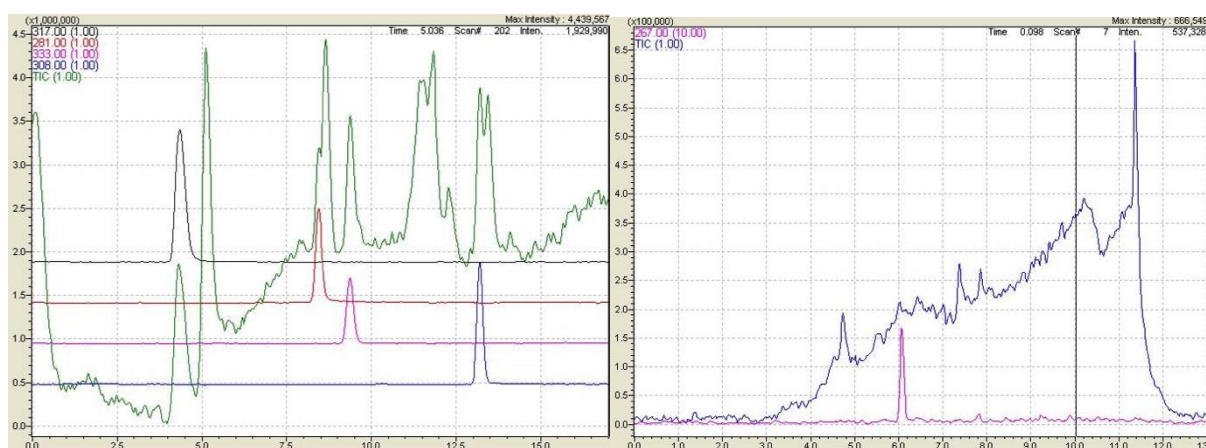
Figure 11: Typical chromatograms obtained for BMAA and nevirapine in a complex plant matrix at concentrations well below the expected accumulation concentrations of xenobiotics

3.3   Evaluation of Selected Macrophytes

Five plant species were collected and tested. The plants were tentatively identified as *Chara vulgaris*, *Ceratophyllum demersum*, *Stuckenia pectinata*, *Egeria densa* and *Spirodela polyrhiza*. All plants were maintained in 40 L glass tanks with media and flow rates as described above. None of the tested xenobiotics had a negative effect on *C. demersum, S. pectinata* or *E. densa* at the maximum tested concentration (see Figure 12, Figure 13 and Figure 14). The error bars in the figures denote standard deviation ($n = 3$).

Figure 12: Oxygen evolution by *C. demersum* after 24-hour exposure to various xenobiotics

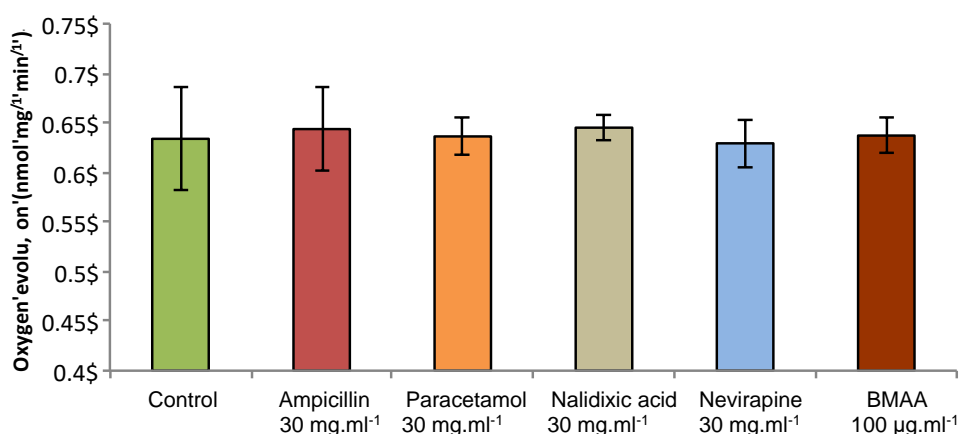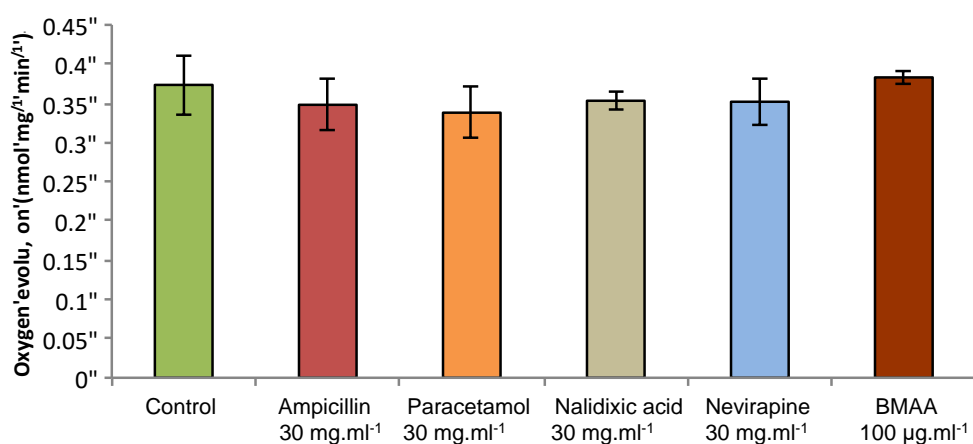**Figure 13: Oxygen evolution by *E. densa* after 24-hour exposure to various xenobiotics**
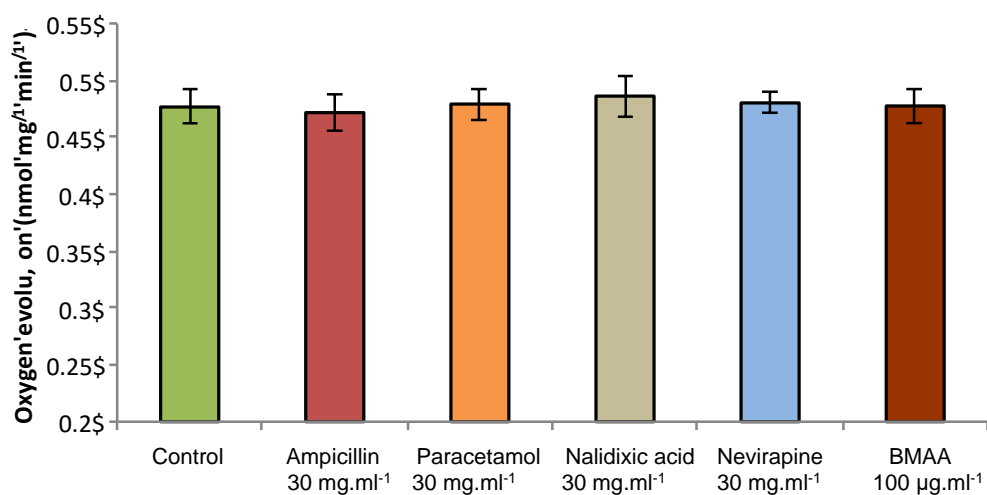

Figure 14: Oxygen evolution by *S. pectinata* after 24-hour exposure to various xenobiotics

Oxygen evolution was reduced on exposure to nevirapine in the case of *C. vulgaris* (Figure 15,) and for both nevirapine and nalidixic acid in the case of *S. polyrhiza* (see Figure 16). Reduction in the negatively impacting xenobiotic reduced the effect; where the effect was not significantly different from control values, the plant was deemed tolerant of that xenobiotic at that concentration. Statistically significant ($p > 005$) differences from the control are indicated by an asterisk in Figure 15 and Figure 16. Error bars denote standard deviation ($n = 3$). Although not significant, the reduction in oxygen evolution by *S. polyrhiza* by nevirapine at 1 mg·L^{-1} was considered a negative impact. *S. polyrhiza* should only be used where concentrations of nevirapine are well below this value.
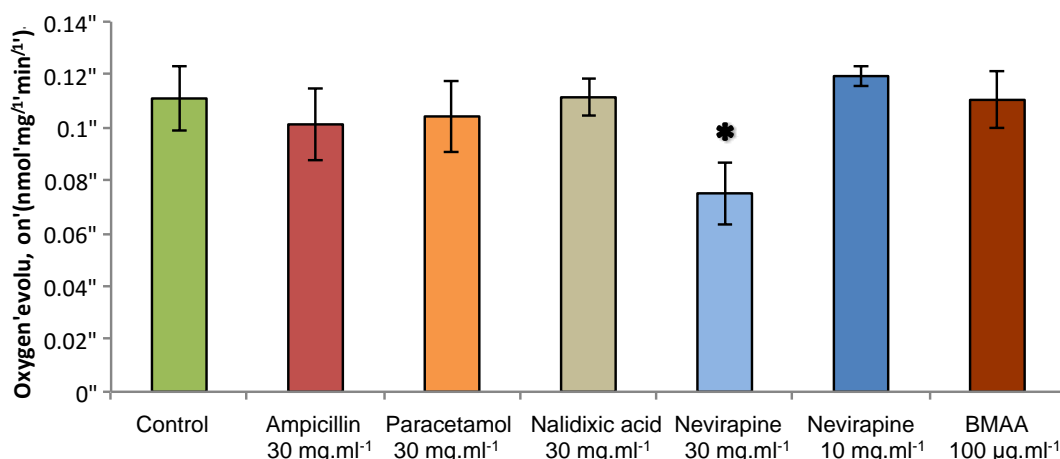
Figure 15: Oxygen evolution by *C. vulgaris* after 24-hour exposure to various xenobiotics
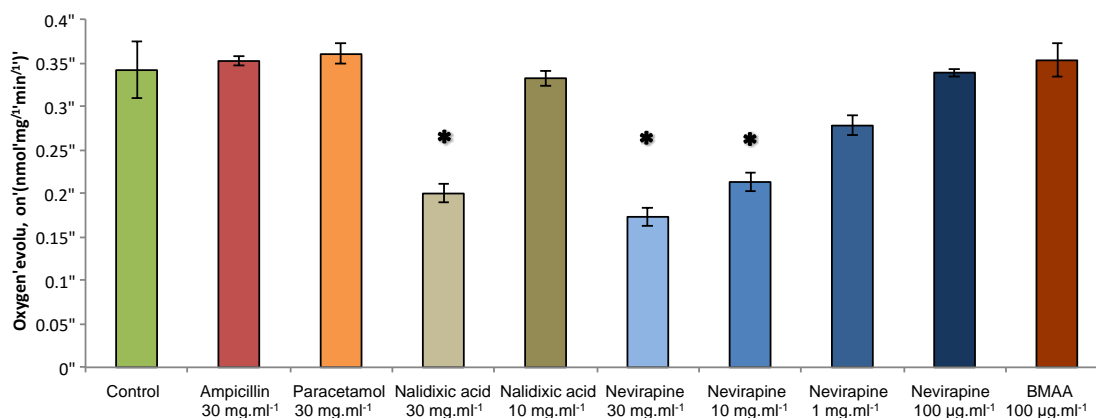
Figure 16: Oxygen evolution by *S. polyrhiza* after 24-hour exposure to various xenobiotics

3.4 Uptake Kinetics

The uptake of BMAA is well described for *C. demersum* (Esterhuizen-Londt et al., 2011a). The uptake is rapid and at an exposure concentration of 100 µg·L^{-1}. Within 24 hours, 90% removal is achieved with the toxin appearing in both the free and protein-associated plant cell fractions (Esterhuizen-Londt et al., 2011b). The following results therefore pertain to the pharmaceuticals specifically.

Spirodela polyrhiza

Figure 17 shows the uptake of pharmaceuticals at different concentrations over three days by *S. polyrhiza*. The uptake was initially rapid at all concentrations, with between 60% and 80% of the available pharmaceutical removed within 24 hours at a concentration of 20 µg·L^{-1} (Figure 17A). Further

change was insignificant except for ampicillin, which was almost completely removed within three days. At 50 µg·L⁻¹, uptake was also substantial with between 50% and 75% removal in the first 24 hours. There was a total removal of approximately 80% for all pharmaceuticals in the three-day exposure period (Figure 17B).

Similar uptake was observed at 100 µg·L⁻¹ for the more hydrophobic nevirapine and nalidixic acid. However, the uptake of ampicillin and paracetamol appeared not to increase from the 50 µg·L⁻¹ exposure uptake values. This may suggest saturation of uptake. Though, at later time points, uptake continued to about 70% after three days. This may also indicate induction of a biotransformation process allowing continued uptake at these elevated concentrations. This would seem to be the case at an exposure concentration of 200 µg·L⁻¹ where uptake was rapid, with approximately 50% of all pharmaceuticals removed in 24 hours and between 65% and 80% after three days (Figure 17D). At this highest concentration, ampicillin and paracetamol were taken up to a lesser extent as well.

Figure 17: Uptake of pharmaceuticals at different concentrations over three days by *S. polyrhiza*

Uptake of ampicillin (■), paracetamol (■), nevirapine (■) and nalidixic acid (■) at 20 µg·L⁻¹ (A), 50 µg·L⁻¹ (B), 100 µg·L⁻¹ (C) and 200 µg·L⁻¹ (D) over three days by S. polyrhiza. Error bars denote standard deviation (n = 3) but were removed from the 0-hour time point where they represent method rather than uptake variability.

Figure 18 shows the uptake over two hours at concentrations ranging from 0.2 mg·L⁻¹ to 3 mg·L⁻¹. A far higher variability was observed over the shorter period, with less pronounced tapering of the uptake rate after the initial rapid uptake during the first 30 minutes. Presumably this is due to much higher concentrations being used. It prevented equilibrium being attained between internalised and medium xenobiotics. The continued uptake may reflect the ability of the plant to biotransform drugs.
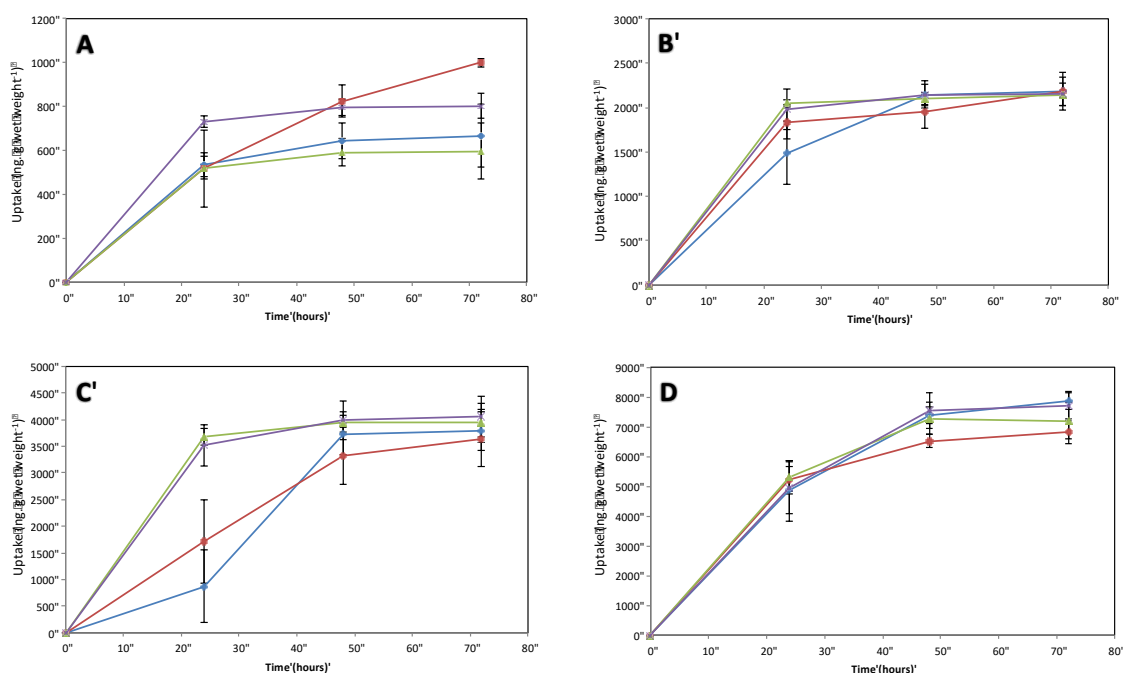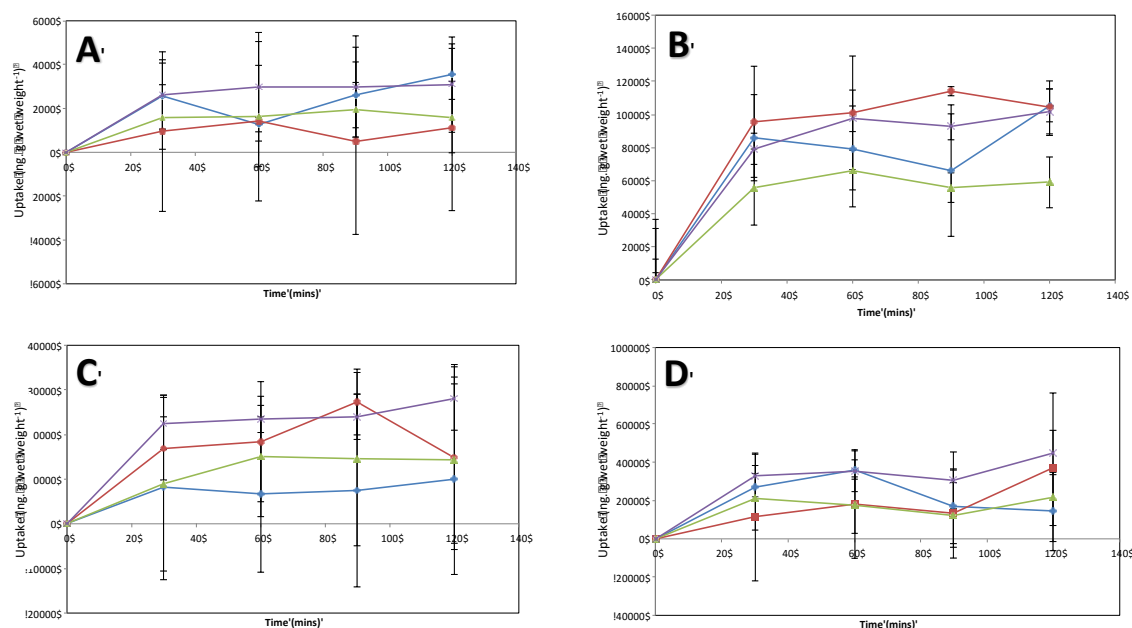
Figure 18: Uptake of pharmaceuticals at different concentrations over two hours by *S. polyrhiza*

Uptake of ampicillin (■), paracetamol (■), nevirapine (■) and nalidixic acid (■) at 200 µg·L⁻¹ (A), 600 µg·L⁻¹ (B), 1500 µg·L⁻¹ (C) and 3000 µg·L⁻¹ (D) over two hours by S. polyrhiza. Error bars denote standard deviation (n = 3) but were removed from the 0-hour time point where they represent method variability rather than uptake variability.

Chara vulgaris

Figure 19 shows the uptake of pharmaceuticals at different concentrations over three days by *C. vulgaris.* The uptake was initially rapid at all concentrations, but with significant differences between pharmaceuticals. Initial uptake of between 30% and 65% of the available pharmaceuticals within 24 hours was observed at a concentration of 20 µg·L⁻¹ (Figure 19A). The lowest uptake at 24 hours was for nalidixic acid. This did not increase over the following two days. The same pattern of limited uptake, decreasing or ceasing after 24 hours, was observed for all concentrations of nalidixic acid. This suggests a very low equilibrium ratio and possibly no biotransformation of this xenobiotic by *C. vulgaris.*

At 50 µg·ml⁻¹, uptake was also substantial with between 40% and 85% removal in the first 24 hours. There were insignificant changes thereafter. As for the lower concentrations, nalidixic acid was taken up to a lesser extent while the more hydrophilic pharmaceuticals were almost completely removed (Figure 19B). A similar pattern of uptake was observed at 100 µg·L⁻¹ (Figure 19C) and for an exposure concentration of 200 µg·L⁻¹ (Figure 19D). However, for the highest concentration (Figure 19D), nevirapine appeared to be taken up to a lesser extent as well, thus suggesting possible saturation and potentially an inability of *C. vulgaris* to biotransform the xenobiotic.

Figure 19: Uptake of pharmaceuticals at different concentrations over three days by *C. vulgaris*

Uptake of ampicillin (■), paracetamol (■), nevirapine (■) and nalidixic acid (■) at 20 µg·L⁻¹ (A), 50 µg·L⁻¹ (B), 100 µg·L⁻¹ (C) and 200 µg·L⁻¹ (D) over three days by C. vulgaris. Error bars denote standard deviation (n = 3) but were removed from the 0-hour time point where they represent method variability rather than uptake variability.

Figure 20 shows the uptake over two hours at concentrations ranging from 0.2 mg·L⁻¹ to 3 mg·L⁻¹. As with most of the other plants, uptake continues throughout the exposure period but is particularly delayed for nalidixic acid. At 3 mg·L⁻¹, the uptake appears to cease after 24 hours, thus suggesting saturation at this concentration within a very short time.

Figure 20: Uptake of pharmaceuticals at different concentrations over two hours by *C. vulgaris*

Ceratophyllum demersum

Figure 21 shows the uptake of pharmaceuticals at different concentrations over three days by *C. demersum.* The uptake was initially rapid at all concentrations, but significantly slower for ampicillin. This difference was lost after 48 hours and 72 hours. More than 80% of all xenobiotics were removed from exposure media at a concentration of 20 µg·L⁻¹ after three days (Figure 21A). At 50 µg·L⁻¹, ampicillin uptake was also slightly but not significantly slower. Rapid uptake in the first 24 hours resulted in residual xenobiotic levels of between 13% and 1% (Figure 21B). Similarly, high initial rates of uptake were observed at 100 µg·L⁻¹ (Figure 21C) and for an exposure concentration of 200 µg·ml⁻¹ (Figure 21D). However, for the higher concentrations, the amount of the different drugs taken up was more variable.
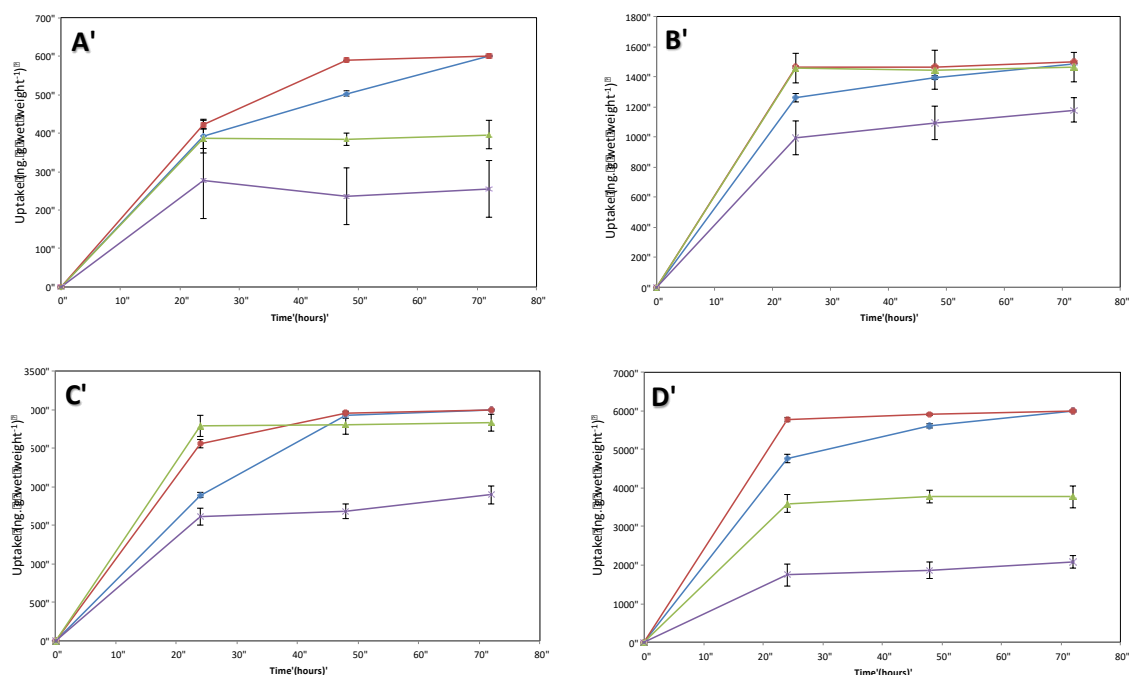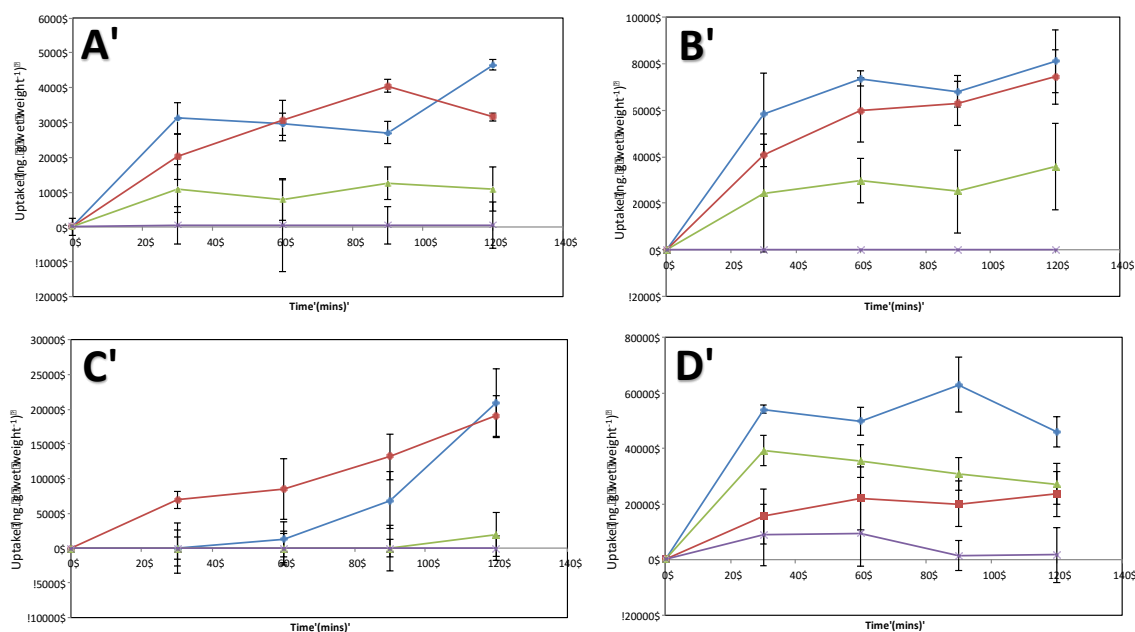

Figure 21: Uptake of pharmaceuticals at different concentrations over three days by *C. demersum*

Uptake of ampicillin (■), paracetamol (■), nevirapine (■) and nalidixic acid (■) at 20 µg·L⁻¹ (A), 50 µg·L⁻¹ (B), 100 µg·L⁻¹ (C) and 200 µg·L⁻¹ (D) over three days by C. demersum. Error bars denote standard deviation (n = 3) but were removed from the 0-hour time point where they represent method variability rather than uptake variability.

Figure 22 shows the uptake over two hours at concentrations ranging from 0.2 mg·L⁻¹ to 3 mg·L⁻¹. A far higher variability was observed over the shorter period, presumably as a function of variability of individual plant response to the xenobiotics. Over the longer exposure period, such initial variability was not evident. However, Figure 22 shows the ability of *C. demersum* to take up all the tested xenobiotics at a concentration unlikely to be observed in the environment, which was also shown to be non-toxic to the plant over a short period for any tested pharmaceuticals. At the highest concentration, uptake of the hydrophobic drugs appeared to saturate within 30 minutes. Ampicillin and paracetamol were taken up over the entire period suggesting an inability to biotransform the hydrophobic drugs rapidly for this short time frame, or the requirement for expression of a detoxification system.
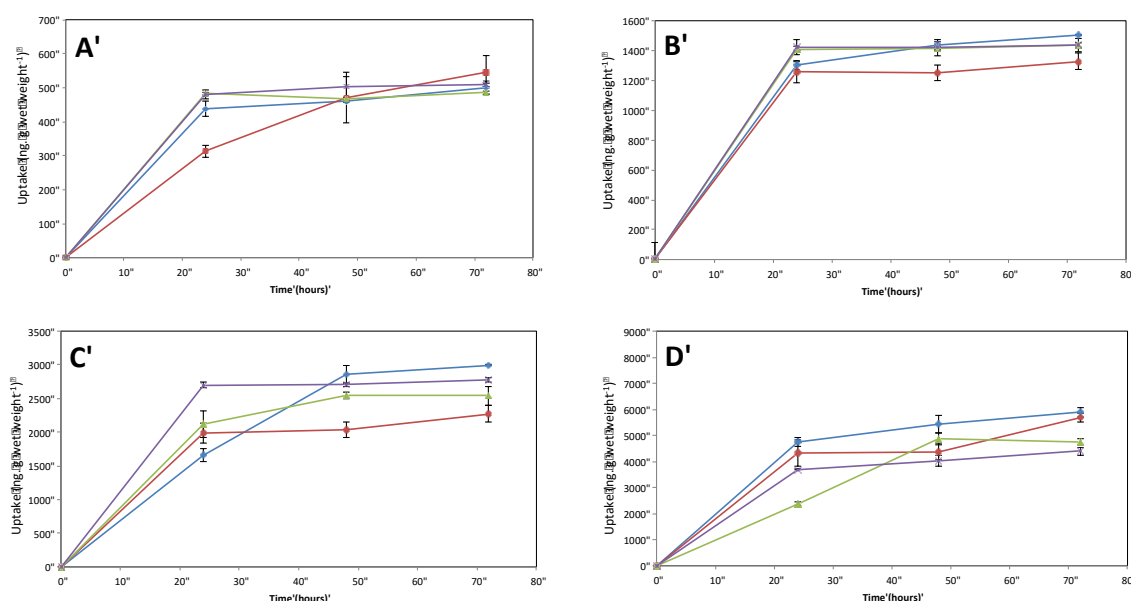
Figure 22: Uptake of pharmaceuticals at different concentrations over two hours by _C. demersum_

Uptake of ampicillin (■), paracetamol (■), nevirapine (■) and nalidixic acid (■) at 200 µg·L⁻¹ (A), 600 µg·L⁻¹ (B), 1500 µg·L⁻¹ (C) and 3000 µg·L⁻¹ (D) over two hours. Error bars denote standard deviation (n = 3) but were removed from the 0-hour time point where they represent method variability rather than uptake variability.

Stuckenia pectinata

Figure 23 shows the uptake of pharmaceuticals at different concentrations over three days by _S. pectinata._ The uptake was initially rapid at all concentrations. As with other plants tested, this rapid uptake over 24 hours at all concentrations suggests non-saturated uptake at environmentally relevant concentrations. There was some variation in rates for ampicillin and nevirapine at 20 µg·L⁻¹ (Figure 23A). At 50 µg·L⁻¹ and at 100 µg·L⁻¹, ampicillin uptake was also slightly but not significantly slower (Figure 23B and Figure 23C).

Figure 23: Uptake of pharmaceuticals at different concentrations over three days by _S. pectinata_

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Figure 24 shows the uptake over two hours at concentrations ranging from 0.2 mg·L⁻¹ to 3 mg·L⁻¹. Uptake over this short period is far more variable, suggesting variability in individual plants to respond to xenobiotics. The reduced uptake rate for ampicillin at concentrations higher than 0.2 mg·L⁻¹ is particularly interesting and suggests interference by other xenobiotics at higher concentrations. However, the higher concentrations are not environmentally relevant and no such interference should occur during model testing.
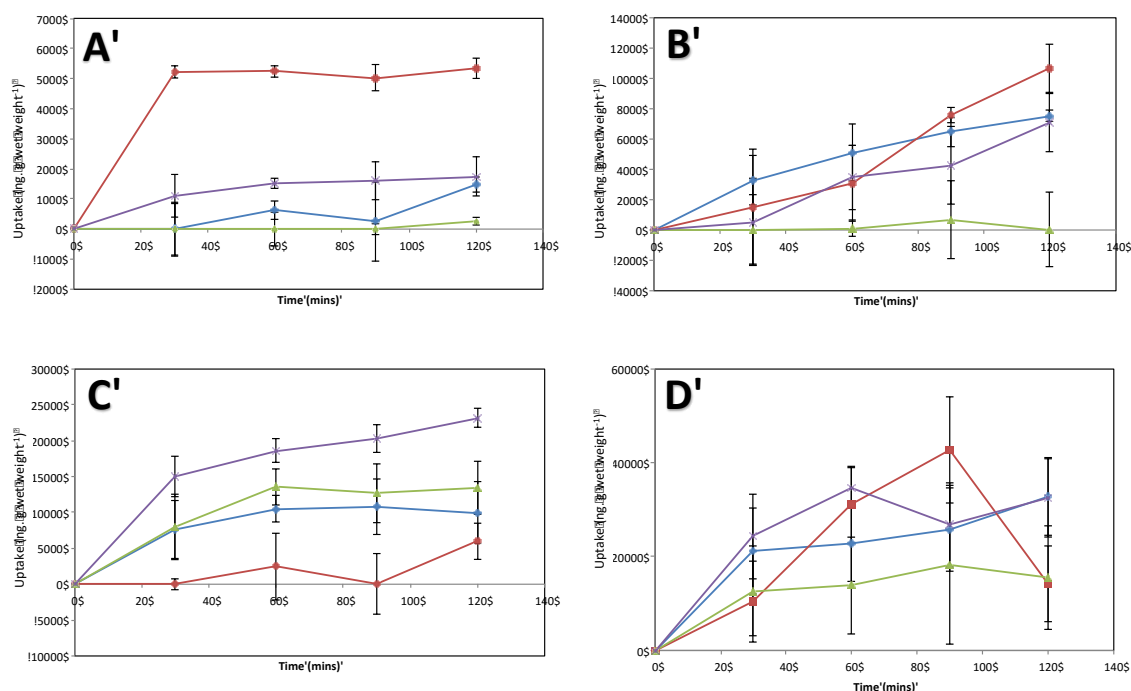
Figure 24: Uptake of pharmaceuticals at different concentrations over two hours by *S. pectinata*

Egeria densa

E. densa takes up acetaminophen and nalidixic acid at a higher rate than ampicillin in particular, but also for nevirapine at low concentrations (Figure 25). In all cases, the initial uptake rate is lower than other plants for all pharmaceuticals tested. This is fully discussed below. However, despite the relatively slower uptake rate, the plant is extremely robust making it a potentially suitable candidate. Furthermore, it has a relatively slow growth rate compared to other plants, negating the need for continuous biomass removal.
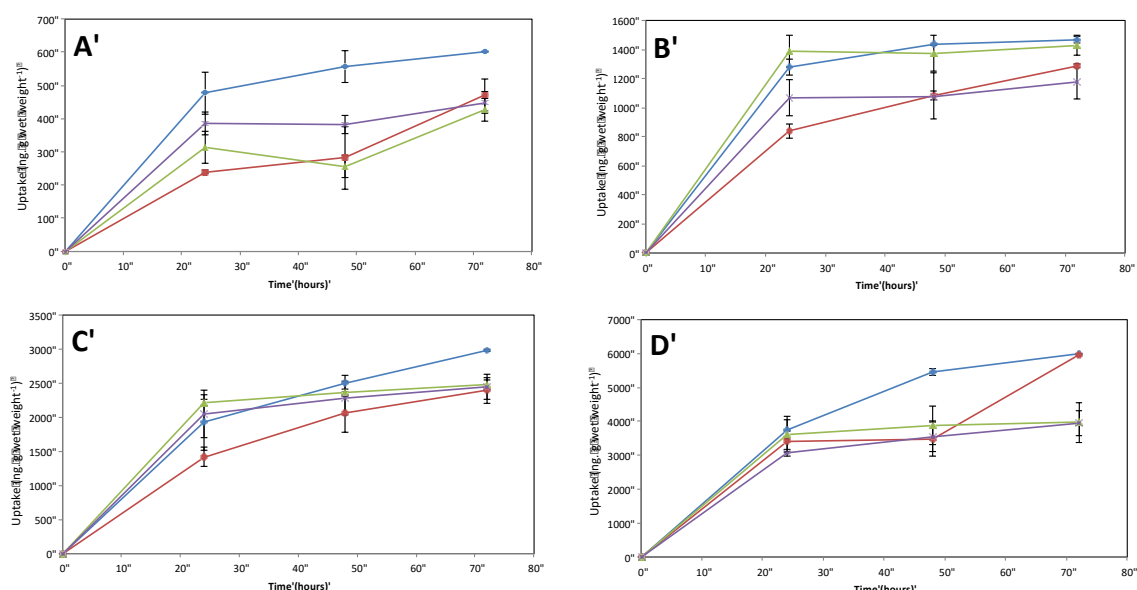
Figure 25: Uptake of pharmaceuticals at different concentrations over three days by *E. densa*

Uptake of ampicillin (■), paracetamol (■), nevirapine (■) and nalidixic acid (■) at 2 µg·L⁻¹ (A), 5 µg·L⁻¹ (B), 100 µg·L⁻¹ (C) and 200 µg·L⁻¹ (D) over three days by E. densa. Error bars denote standard deviation (n = 3) but were removed from the 0-hour time point where they represent method variability rather than uptake variability.

Figure 26 shows the uptake over two hours at concentrations ranging from 0.2 mg·L⁻¹ to 3 mg·L⁻¹. A noticeable variability in uptake of the various drugs is observed at higher concentrations. The pattern of uptake over this shorter period at higher concentrations is similar to that of the other plants tested, suggesting a common requirement for removal or compartmentalisation of internalised xenobiotics to maintain an equilibrium ratio favourable for continued uptake.
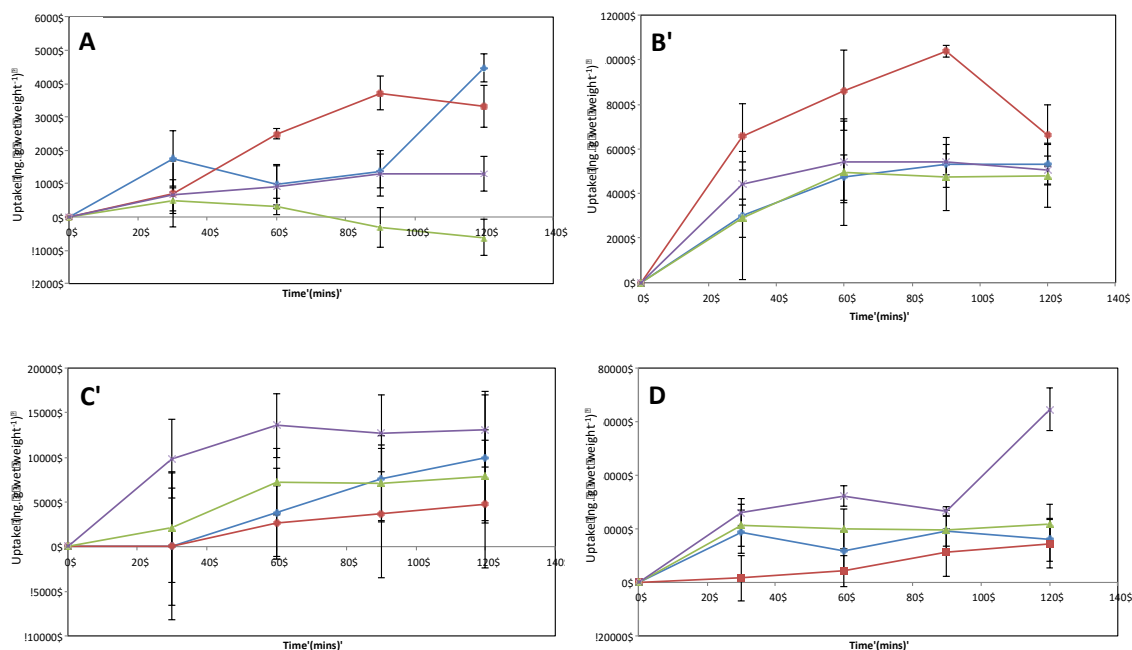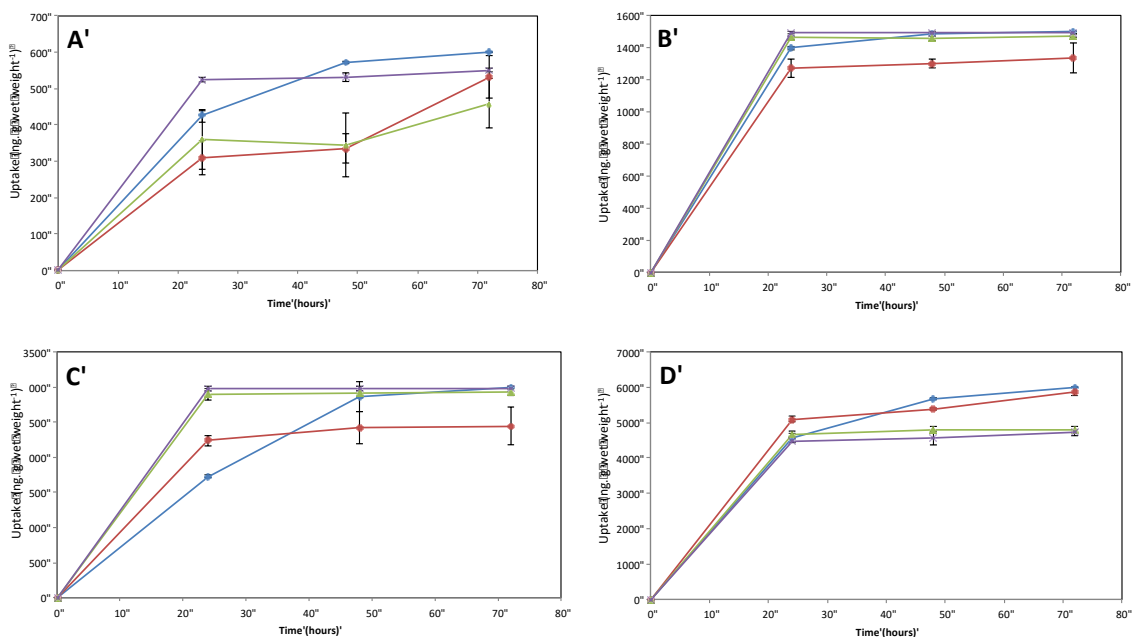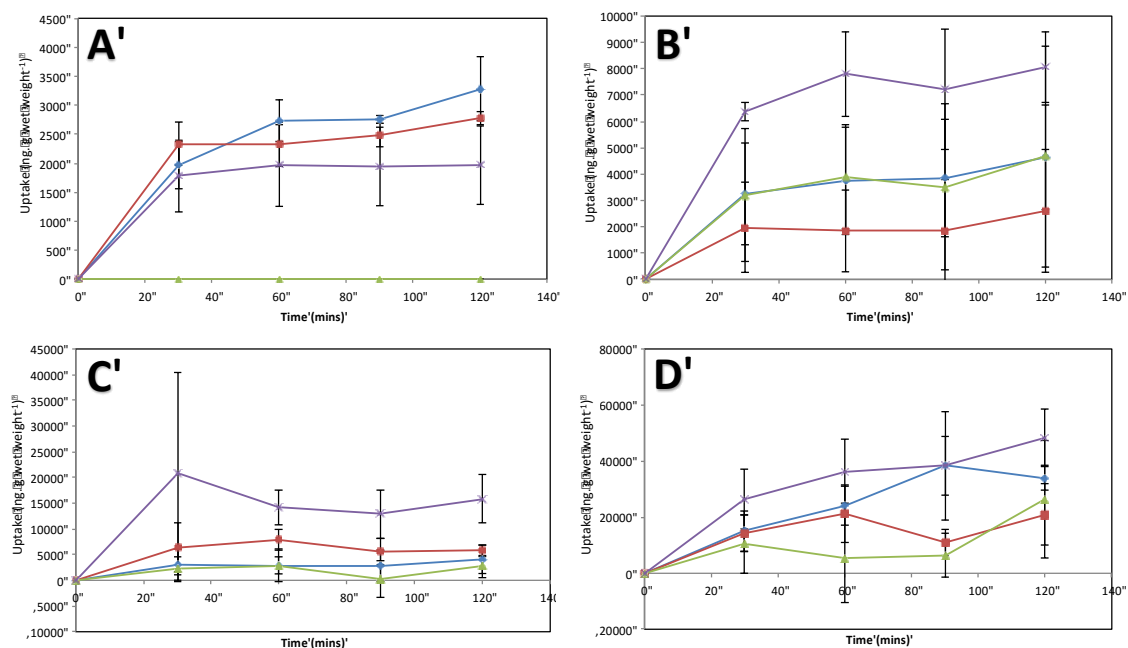

Figure 26: Uptake of pharmaceuticals at different concentrations over two hours by *E. densa*

Uptake of ampicillin (■), paracetamol (■), nevirapine (■) and nalidixic acid (■) at 200 µg·L⁻¹ (A), 600 µg·L⁻¹ (B), 1500 µg·L⁻¹ (C) and 3000 µg·L⁻¹ (D) over two hours by E. densa. Error bars denote standard deviation (n = 3) but were removed from the 0-hour time point where they represent method variability rather than uptake variability.

Saturation curves for all plants and pharmaceuticals

Figure 27 shows the smoothed saturation curves for all pharmaceuticals for all plants. Although some reduction in uptake rate does occur for the highest concentration, most notably for *C. vulgaris* with the hydrophobic drugs, saturation was not achieved and maximum uptake rate is therefore not reported. Uptake is proportional to xenobiotic concentration, and linear for most xenobiotics across environmentally relevant concentrations of all xenobiotics. Uptake saturation will therefore not occur with any of these plants for any of the tested xenobiotics at the concentrations that will be used to run the model green liver system.
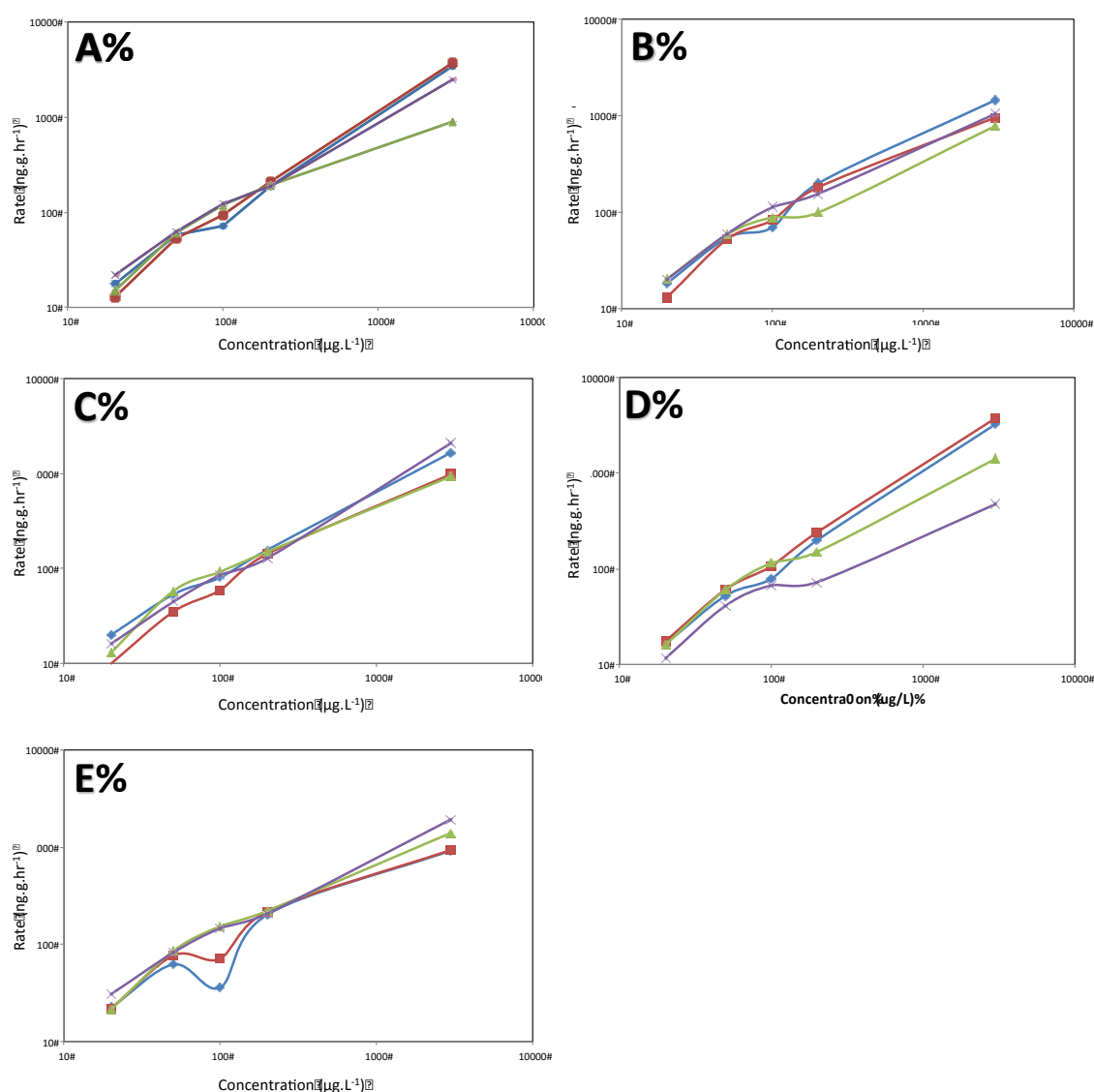
Figure 27: Saturation curves for all pharmaceuticals

Uptake rate as a function of substrate concentration for ampicillin (■), paracetamol (■), nevirapine (■) and nalidixic acid (■) in S. pectinata (A), C. demersum (B), E. densa (C), C. vulgaris (D) and S. polyrhiza (E).

3.5 Depuration

Spirodela polyrhiza

S. polyrhiza contained no detectable ampicillin prior to depuration. The plant lost 0.03% of the ampicillin, which was removed from the exposure medium in 72 hours in the depuration medium. None of the remaining 99.97% was retained in the plant in a detectable form.

S. polyrhiza contained a total of 2.89 ng·g^{-1} of acetaminophen prior to depuration. The absence of acetaminophen in the depuration medium after 72 hours suggests a 99.9% biotransformation efficiency.

S. polyrhiza contained a total of 0.62 ng·g^{-1} of nevirapine prior to depuration, despite a loss from exposure medium of 33 655 ng. The plant lost 0.39% of the nevirapine, which was removed from the exposure medium in 72 hours. The remaining 99.61% was either retained in the plant (3.7%) or biotransformed.

S. polyrhiza contained a total of 1528.79 ng·g^{-1} of nalidixic acid prior to depuration. The plant lost 1.8% of the nalidixic acid, which was removed from the exposure medium in 72 hours. The remaining 98.2% was retained in the plant. The relatively high depuration rate and plant retention rate make *S. polyrhiza* less suitable for nalidixic acid removal at high concentrations.

Chara vulgaris

C. vulgaris contained no detectable ampicillin prior to depuration, and 6.17 ng·g^{-1} of acetaminophen prior to depuration. The ampicillin and acetaminophen released from the plant during depuration was insignificant over 72 hours. No significant amount of ampicillin or acetaminophen was detectable in the plant during this period.

C. vulgaris contained a total of 0.38 ng·g^{-1} of nevirapine prior to depuration. The plant lost 0.18% of the nevirapine, which was removed from the exposure medium over 72 hours. Retention within the plant was insignificant.

C. vulgaris contained a total of 11367 ng·g^{-1} of nalidixic acid prior to depuration. The plant loss to depuration medium was 7.2% of the nalidixic acid, and approximately 0.13% was retained in the plant. *C. vulgaris* also has a poor uptake rate for nalidixic acid and is not the best candidate for removing this drug.

Ceratophyllum demersum

C. demersum contained no detectable ampicillin prior to depuration. The plant lost only 0.5% of the ampicillin, which was removed from the exposure medium during the depuration period of 72 hours. None of the remaining 99.5% was detected in the plant.

C. demersum contained 34 706 ng·g^{-1} of acetaminophen prior to depuration. The plant lost 1.25% of the acetaminophen, which was removed from the exposure medium in the first 24 hours in a xenobiotic-free medium, but this was taken up again over the remaining 48 hours of depuration. The plant retained 0.01% of the acetaminophen.

C. demersum contained a total of 0.12 ng·g^{-1} of nevirapine prior to depuration. The plant lost 1.8% of the nevirapine, which was removed from the exposure medium in the 72 hours in depuration medium. The remaining 98.1% was either retained in the plant or biotransformed.

C. demersum contained a total of 543 ng·g^{-1} of nalidixic acid prior to depuration. The plant lost 9.4% of the nalidixic acid, which was removed from the exposure medium in the first 24 hours in xenobiotic-free medium, but 0.3% was taken up again by 72 hours. The remaining 90.4% was either retained in the plant or biotransformed. However, for nalidixic acid the amount retained in the plant was relatively high at 0.97%.

Stuckenia pectinata

S. pectinata contained no ampicillin prior to depuration and no ampicillin was detectable in either the depuration medium or the plant at any point during the depuration experiment. The plant removed 88 120 ng·g^{-1} from the exposure medium and all of this was unaccounted for at the end of depuration.

S. pectinata contained a total of 63.4 ng·g^{-1} of acetaminophen prior to depuration. The acetaminophen released during 72 hours of depuration was insignificant as was the amount retained in the plant. Approximately 99.99% of the acetaminophen was biotransformed by *S. pectinata*.

S. pectinata contained a total of 0.44 ng·g^{-1} of nevirapine prior to depuration. The plant lost 0.33% of the nevirapine, which was removed from the exposure medium in 72 hours. The remaining 99.67% was either retained in the plant or biotransformed.

S. pectinata contained a total of 722.31 ng·g^{-1} of nalidixic acid prior to depuration. The plant lost 1.6% of the nalidixic acid, which was removed from the exposure medium during depuration for three days. The remaining 98.4% was either retained in the plant or biotransformed.

Egeria densa

E. densa contained no ampicillin prior to depuration. The plant lost 0.02% of the ampicillin, which was removed from the exposure medium in 72 hours in xenobiotic-free medium. The remaining 99.98% was either retained in the plant (0.53%) or biotransformed.

E. densa contained a total of 426.27 ng·g^{-1} of acetaminophen prior to depuration. The plant retained no acetaminophen after depuration, but lost only 0.25% of the acetaminophen, which was removed from the exposure medium during depuration in xenobiotic-free medium. The remaining 99.75% was biotransformed.

E. densa contained negligible nevirapine prior to depuration. The plant lost 0.36% of the nevirapine, which was removed from the exposure medium during three days of depuration. The remaining nevirapine was considered to have been biotransformed since an insignificant amount was detected in the plant after depuration.

E. densa contained a total of 295.75 ng·g^{-1} of nalidixic acid prior to depuration. The plant lost 13.2% of the nalidixic acid removed from the exposure medium during depuration and retained 0.1%. The remaining 86.7%% was biotransformed.

Depuration of all plants and pharmaceuticals

The high depuration rate observed for most pharmaceuticals in most plants at the very high exposure concentration used must be considered in conjunction with the retention amount of each drug in each plant and the calculated biotransformed percentage. The 3 mg·L^{-1} exposure concentration used to evaluate retention, biotransformation and depuration is 100-fold higher than reported environmental concentrations. It is highly probable that the retention, particularly of hydrophobic xenobiotics, is concentration dependent and the irreversible removal of the xenobiotics is biotransformation dependent. The depuration of these drugs, and particularly nevirapine and nalidixic acid, is therefore to be expected after removal from such a high exposure concentration. These experiments will be repeated at environmentally relevant concentrations as part of the next deliverable, where individual plants are tested in a model green liver system. This will provide real-world depuration rates to compare to biomass production rates for evaluating the full system.

3.6   Biotransformation Potential

Due to the substantially reduced budget, biotransformation could not be evaluated using stable isotope-labelled xenobiotics. However, isotope-labelled BMAA remaining from a previous study allowed for a

complete analysis of that toxin. In all other cases, absence from exposure media and plant tissue, together with lack of depuration was considered to represent biotransformation.

Spirodela polyrhiza

The amount of xenobiotics relative to control experiments that remained unaccounted for by retention in *S. polyrhiza*, or by depuration after removal to fresh xenobiotic-free medium, was considered to have been metabolically modified by the plant. At an exposure concentration of 3 mg·L^{-1}, this amount was:

- 35 293 ng·g^{-1} wet weight of plant material for ampicillin.
- 34 729 ng·g^{-1} wet weight of plant material for acetaminophen.
- 52 752 ng·g^{-1} wet weight of plant material for nevirapine.
- 72 722 ng·g^{-1} wet weight of plant material for nalidixic acid.

Of these amounts, 99.97% of the ampicillin, 99.99% of the acetaminophen, 99.3% of the nevirapine and 93.2% of the nalidixic acid were unaccounted for and deemed to have undergone biotransformation. *S. polyrhiza* appears to be most useful for the biotransformation of acetaminophen but has the lowest uptake rate for this xenobiotic.

Chara vulgaris

The amount of xenobiotics relative to control experiments that remained unaccounted for by retention in *C. vulgaris*, or by depuration after removal to fresh xenobiotic-free medium, was considered to have been metabolically modified by the plant. At an exposure concentration of 3 mg·L^{-1}, both ampicillin and acetaminophen removed from the exposure medium by *C. vulgaris* appeared to be transformed at 99.5% and 99.99% respectively. Unaccounted for nevirapine after depuration constituted 99.82% of that taken up during exposure, while only 92.6% of nalidixic acid could not be accounted for in *C. vulgaris.*

Ceratophyllum demersum

The amount of xenobiotics relative to control experiments that remained unaccounted for by retention in *C. demersum*, or by depuration after removal to fresh xenobiotic-free medium, was considered to have been metabolically modified by the plant. At an exposure concentration of 3 mg·L^{-1}, the apparent biotransformation for ampicillin was 99.5%, acetaminophen 98.74%, nevirapine 98.1%, and nalidixic acid 90.4%.

Labelled BMAA (BMAA-4,4,4-d$_3$,^{15}N$_2$) was taken up from the culture medium by *C. demersum,* with uptake data corresponding to previous studies (Esterhuizen-Londt et al., 2011b) that showed concentration dependent uptake of exogenous BMAA by *C. demersum*, which followed Michaelis–Menton kinetics. Figure 28 shows the change in the free and protein-associated BMAA content of *C. demersum* over a 144-hour period during which the plants were exposed to 1 µM BMAA-4,4,4-d$_3$,^{15}N$_2$ for the initial 24 hours. Thereafter, it was transferred to BMAA-free sterile water for a 120-hour depuration period.
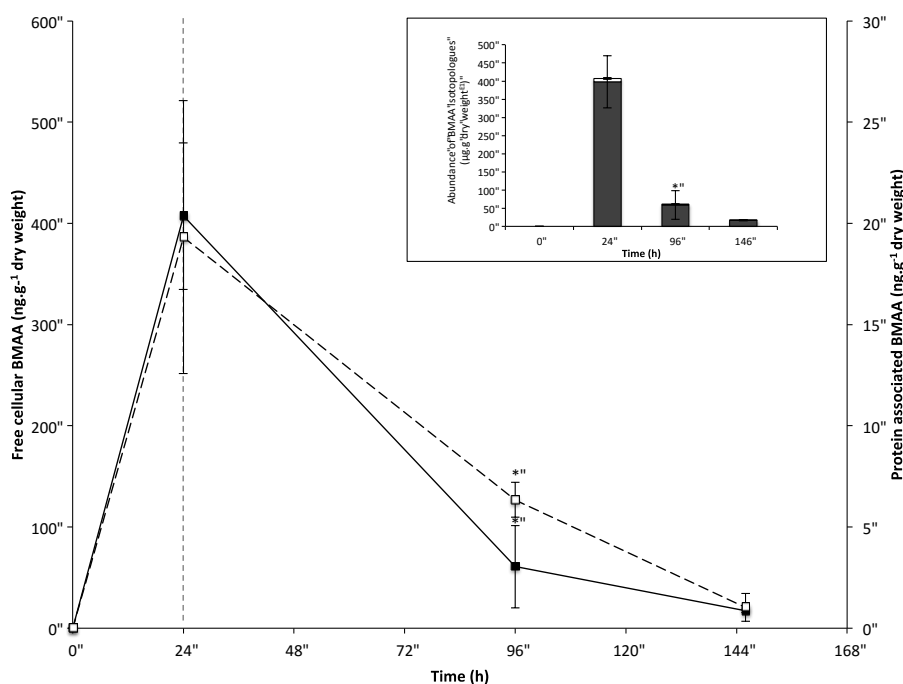
Figure 28: Change in the free and protein-associated BMAA content of *C. demersum* over a 144-hour period

Free (solid line) and protein-associated (broken line) cellular BMAA in C. demersum over a total of 144 hours. Plant sections were exposed to 100 µM L-BMAA-4,4,4-d3,^{15}N2 for 24 hours, after which plants were washed and transferred (indicated by a dotted line) to BMAA-free water for depuration (see insert). The abundance of BMAA isotopologues, where labelled BMAA is depicted by dark grey bars, and BMAA missing a single isotope labelled by open bars. Error bars depict standard deviations where n = 3. Significant differences (p<005) from previous time points are indicated by an asterisk (*).

During the depuration period, there was a significant (*p*<0.05) reduction in intracellular BMAA, with nearly complete removal of free and protein-associated BMAA from the plants within five days. No BMAA was detected in the media during depuration. The decrease in cellular BMAA could therefore not be attributed to excretion, but was either due to complete catabolism or covalent modification of the amino acid. In other studies on BMAA uptake in macrophytes, a marked decrease in cellular BMAA was also observed (Esterhuizen et al., 2011) – even in plants continuously exposed to exogenous BMAA (Contardo-Jara et al., 2013).

Detoxification mechanisms of xenobiotics in plants routinely involve covalent chemical modifications to the parent compound, yielding a conjugate or modified compound that is more hydrophilic and less toxic. These modified compounds are not excreted but compartmentalised within the cell (Coleman et al., 1997). Therefore, it is not particularly surprising that BMAA was not excreted during depuration, but as data suggest, rather sequestered inside the cell in a modified form. There was very limited transfer of BMAA labels to other cellular amino acids, which supports the fact that BMAA was covalently modified in *C. demersum*.

Figure 29 shows the changes in the abundance of amino acid isotopologues. The only significant increases in isotopologue abundance of any of the amino acids analysed were observed in arginine, which showed a 42% ± 24% increase in labelled arginine relative to native arginine following 24 hours of labelled BMAA exposure, and in asparagine and aspartate, which showed a 35% ± 13% and 28% ± 17% increase, respectively, in the double-labelled isotopologue relative to the single labelled isotopologue, following 120 hours of depuration.
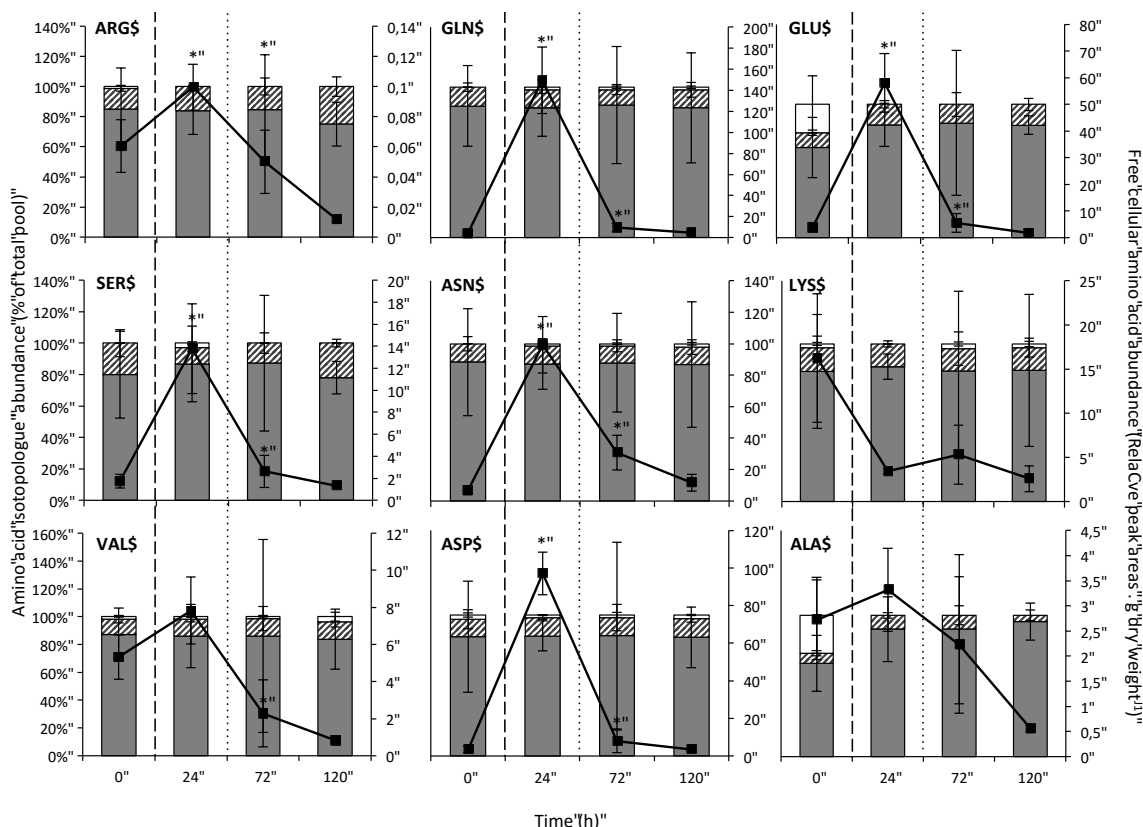
Figure 29: Changes in the abundance of amino acid isotopologues in *C. demersum*

The change in amino acid isotopologue abundance in C. demersum exposed to 1 µM LBMAA4,4,4-d3,15N2 for 24 hours (dashed line indicates transfer of plants to BMAA medium), and during a 120-hour depuration period (dotted line indicates transfer of plants to BMAA-free depuration medium). Bars show the abundance of amino acid isotopologues as a percentage of the total respective amino acid pool, where amino acid 0+ isotopologues are indicated by grey bars, 1+ isotopologues are indicated by diagonally striped bars and 2+ isotopologues are depicted by open bars. The total free cellular amino acid pool of respective amino acids is indicated by a solid line (secondary axis). All graphs depict liquid chromatography and/or mass spectrometry peak areas normalised against internal standard, methionine-D3. Error bars depict standard deviations where n = 3. Significant differences (p<005) from previous time points are indicated by (**) for isotopologue ratios and by (*) for total free cellular amino acid pools. (Arg) arginine; (Asn) asparagine; (Asp) aspartate.

Although these changes in the abundance of isotopes of these three amino acids are significant, the redistribution of labels is minimal, and it does not in any way account for the substantial observed decrease of free cellular BMAA. The redistribution of BMAA isotope labels most likely occurred during BMAA detoxification reactions, which may have resulted in the dissemination of chemical groups, free to be used in other anabolic processes such as amino acids biosynthesis. Therefore, based on the marked decrease of cellular BMAA over time in *C. demersum* and the negligible redistribution of BMAA labels, data show that BMAA is neither catabolised, nor does it undergo any significant atomic exchanges such as de-methylation/methylation or de-amination/amination reactions, but that the neurotoxin is covalently modified and sequestered in the cells of *C. demersum*.

Various studies have confirmed the phytotoxicity of BMAA, with data showing increases in oxidative stress, enzyme inhibition (Contardo-Jara et al., 2013; Esterhuizen-Londt et al., 2011a and plant development defects (Brenner et al., 2000). Data from this study suggest that *C. demersum* responds to BMAA as a phytotoxin and not as a nutrient. Contardo-Jara et al. (2013) suggest that due to its hydrophilic nature, BMAA would not be chemically transformed via cellular biotransformation systems that have been shown to biotransform the common cyanotoxin, microcystin, a system that involves the formation of a glutathione-microcystin conjugate catalysed by glutathione *S*-transferase. However,

chemical transformation of BMAA in plants via this or a similar biotransformation system should not be excluded based on the hydrophilic nature of BMAA. At physiological pH, due to the low pK_a value of the two-amino group BMAA is essentially uncharged and consequently very reactive (Nunn & Ponnusamy, 2009). During the biotransformation of toxic compounds, given that a phytotoxin already contains a reactive group that can react with compounds during phase II, phase I of the biotransformation process can be bypassed (Coleman et al., 1997). Therefore, considering the reactivity of BMAA under physiological conditions, it is very likely as data suggests that BMAA is covalently bound to some cellular molecule, resulting in the formation of a non-toxic BMAA-conjugate. This conjugate is sequestered within the plant, or perhaps, atypically for plant detoxification, modified and excreted in a modified form that was not detectable in the depuration medium.

Stuckenia pectinata

The amount of xenobiotics relative to control experiments that remained unaccounted for by retention in *S. pectinata* or by depuration after removal to fresh xenobiotic-free medium, was considered to have been metabolically modified by the plant. At an exposure concentration of 3 mg·L^{-1}, the apparent biotransformation efficiencies were 100% for ampicillin, 99.99% for acetaminophen, 99.63% for nevirapine and 98.4% for nalidixic acid.

Egeria densa

The amount of xenobiotics relative to control experiments that remained unaccounted for by retention in *E. densa*, or by depuration after removal to fresh xenobiotic-free medium, was considered to have been metabolically modified by the plant. At an exposure concentration of 3 mg·L^{-1}, the apparent biotransformation efficiencies for ampicillin was 99.54%, acetaminophen 99.75%, nevirapine 99.64%, and nalidixic acid 86.7%. *E. densa* has the highest biotransformation rate for nevirapine but not the highest uptake rate. The very high depuration rate for nalidixic acid makes this plant unsuitable for nalidixic acid removal.

Biotransformation rates for all plants and pharmaceuticals

As expected, the biotransformation rates were highest for hydrophilic xenobiotics and lowest for the most hydrophobic drugs. This is typical of three-phase detoxification (Sandermann, 1994). However, the biotransformation rates should not be considered the primary determinant of plant selection or plant position in a sequential system. The uptake rate is a primary determinant in selecting plants to optimise placement for maximum flow rate and minimum biomass requirements. However, the generally high biotransformation rates observed at this high exposure concentration do suggest that the selected plants might all be useful in a HTSPS.

In summary, Table 6, Table 7, Table 8 and Table 9 list all tolerances, uptake rates, and depuration and biotransformation rates for all plants and all pharmaceutical xenobiotics under the tested conditions.

Ampicillin

Table 6: Ampicillin tolerances, uptake rates, and depuration and biotransformation rates

	Tolerance (mg·ml^{-1})	Uptake (ng·g^{-1}·hour^{-1})	*Total removed (ng)	*Plant content (ng·g^{-1} wet weight)	Depuration (ng in medium)		Retention (ng·g^{-1} wet weight)		Estimated biotransformation (µg·g^{-1}·day^{-1})
					1 day	3 day	1 day	3 day	
S. polyrhiza	>30	938	22 517 (28 239)[#]	ND	1.56	5.55	ND	ND	35.3
C. demersum	>30	955	22 924 (8 941)[#]	ND	103.46	116.97	ND	ND	25.4
E. densa	>30	981	23 556 (11 518)[#]	ND	ND	4.50	ND	125.09	36.9
C. vulgaris	>30	3 750	>90 000	ND	4.92	1.67	ND	ND	>111.3
S. pectinata	>30	3 671	88 120 (3 255)[#]	ND	ND	ND	ND	ND	83.4

* After 24-hour exposure (3 mg·L^{-1})
ND: Not detected
[#] Standard deviation

The increase in in plant ampicillin in *E. densa* after three days' depuration was attributed to the uptake of lost ampicillin to the medium after reversing metabolic modification, with subsequent re-uptake. *C. vulgaris* did not take up ampicillin. *S. pectinata* was most effective at removing ampicillin and appeared to completely biotransform the antibiotic.

Acetaminophen

Table 7: Acetaminophen tolerances, uptake rates, and depuration and biotransformation rates

	Tolerance (mg·ml^{-1})	Uptake (ng·g^{-1}·hour^{-1})	*Total removed (ng)	*Plant content (ng·g^{-1} wet weight)	Depuration (ng in medium)		Retention (ng·g^{-1} wet weight)		Estimated Biotransformation (µg·g^{-1}·day^{-1})
					1 day	3 day	1 day	3 day	
S. polyrhiza	>30	923	22 156 (9 387)[#]	2.89	ND	ND	ND	ND	34.7
C. demersum	>30	1 446	34 706 (6 990)[#]	4.36	435.95	ND	1.85	4.34	38.4
E. densa	>30	1 652	39 640 (6 595)[#]	426.27	564.1	100.98	1.85	ND	62.0
C. vulgaris	>30	3 246	77 895 (1 761)[#]	ND	6.17	5.56	0.46	0.06	96.4
S. pectinata	>30	3 446	82 703 (1 303)[#]	63.44	ND	4.05	1.27	3.07	78.2

* After 24-hour exposure (3 mg·L^{-1})
ND: Not detected
[#] Standard deviation

C. vulgaris and *S. pectinata* were the most effective at removing acetaminophen and had the highest biotransformation rates.

Nevirapine

Table 8: Nevirapine tolerances, uptake rates, and depuration and biotransformation rates

	Tolerance (mg·ml⁻¹)	Uptake (ng·g⁻¹·hour⁻¹)	*Total removed (ng)	*Plant content (ng·g⁻¹ wet weight)	Depuration (ng in medium)		Retention (ng·g⁻¹ wet weight)		Biotransformation (ng·g⁻¹·day⁻¹)
					1 day	3 day	1 day	3 day	
S. polyrhiza	<1	1 402	33655 (22 183)#	0.62	81.76	131.73	0.19	0.04	52.6
C. demersum	>30	782	18 756 (8 863)#	0.124	381.74	354.13	0.173	1.92	20.4
E. densa	>30	929	22 296 (7 406)#	0.14	211.0	119.1	0.02	17.12	34.9
C. vulgaris	<10	1 411	33 858 (6 611)#	0.38	65.84	61.91	0.46	0.41	41.8
S. pectinata	<10	900	21 586 (10 141)#	0.44	10.92	71.23	0.22	0.20	20.3

After 24-hour exposure (3 mg·L⁻¹)
ND: Not detected
 # Standard deviation

The more hydrophobic nevirapine was removed less efficiently by all plants. *S. polyrhiza* was the most effective at biotransformation and had the highest uptake rate.

Nalidixic acid

Table 9: Nalidixic acid tolerances, uptake rates, and depuration and biotransformation rates

	Tolerance (mg·ml⁻¹)	Uptake (ng·g⁻¹·hour⁻¹)	*Total removed (ng)	*Plant content (ng·g⁻¹ wet weight)	Depuration (ng in medium)		Retention (ng·g⁻¹ wet weight)		Biotrans-formation (ng·g⁻¹·day⁻¹)
					1 day	3 day	1 day	3 day	
S. polyrhiza	<10	1 933	46 396 (15 644)#	1528.79	872.31	834.69	353.70	1 098.13	72.7
C. demersum	>30	1 043	25 028 (7 162)#	543.80	2 316.14	2 242.64	381.21	146.76	25.3
E. densa	>30	2 086	50 069 (8 870)#	295.75	3 572.21	6 612.92	128.04	59.32	72.1
C. vulgaris	>30	474	11 367 (8 360)#	85.10	1 289.41	822.12	16.30	14.19	13.1
S. pectinata	>30	2 484	59 603 (8 180)#	722.31	184.11	944.54	648.01	896.70	52.2

After 24-hour exposure (3 mg·L⁻¹)
ND: Not detected
 # Standard deviation

BMAA

BMAA was also rapidly and completely taken up by *C. demersum* at environmentally relevant concentrations. The tolerance of *C. demersum* for all other tested xenobiotics makes this useful for removal of BMAA irrespective of the tolerances of the other plants for BMAA.

3.7 Evaluation of the HTSPS

Figure 30 shows the HTSPS shortly after biomass harvesting of *C. demersum*, and while running at a high flow rate with very high concentration of xenobiotics. Direction of flow is from right to left.

Figure 30: Model HTSPS containing four macrophytes

Extremely high concentration – standard flow rate (EHC-SF) system

The data was collected from a 150 L system running a medium containing 0.1 mg·L^{-1} of each xenobiotic and a flow rate of 0.021 hour^{-1}. The plant sequence was:

C. vulgaris ➔ *E. densa* ➔ *C. demersum* ➔ *S. polyrhiza*

Figure 31 shows the xenobiotic concentrations over time at defined points along the system. Sampling points were at the inflow and at the outflow for each compartment. The time for establishing the system is included in the figures to show the absence of depuration at the start of each experiment since the same plants were used repeatedly for each flow rate/concentration combination (although the lowest concentrations were run first). The sequential reduction can clearly be seen in Figure 31. Furthermore, the relatively slower removal of nalidixic acid in Compartment A and subsequent relatively more rapid removal of nalidixic acid are in accordance with the kinetic data. The hydrophobic xenobiotics were removed significantly slower than the hydrophilic xenobiotics but with a lower depuration rate, particularly in the case of BMAA. Although this data gives an indication of xenobiotic removal, a more concise conceptualisation is achieved if the average concentration at each sampling point is plotted during the continuous exposure phase of the experiment. This is shown in Figure 32.

Figure 31: Xenobiotic concentrations over time at defined points along the EHC-SF system

Xenobiotic concentrations in each compartment over establishment, running and depuration phases for the EHC-SF model system. A is the input, B was sampled at the C. vulgaris compartment outlet, B at the E. densa compartment outlet, C at the C. demersum partition outlet, D at the centre of the S. polyrhiza compartment, and E in the centre of the drainage sump. F is the outlet. Nalidixic acid (solid black line), nevirapine (dashed black line), acetaminophen (solid grey line), ampicillin (dotted black line), BMAA (dashed grey line). Error bars denote standard deviation (n = 3). A 0 (zero) represents concentrations below detection limit using solid-phase extraction as previously described.
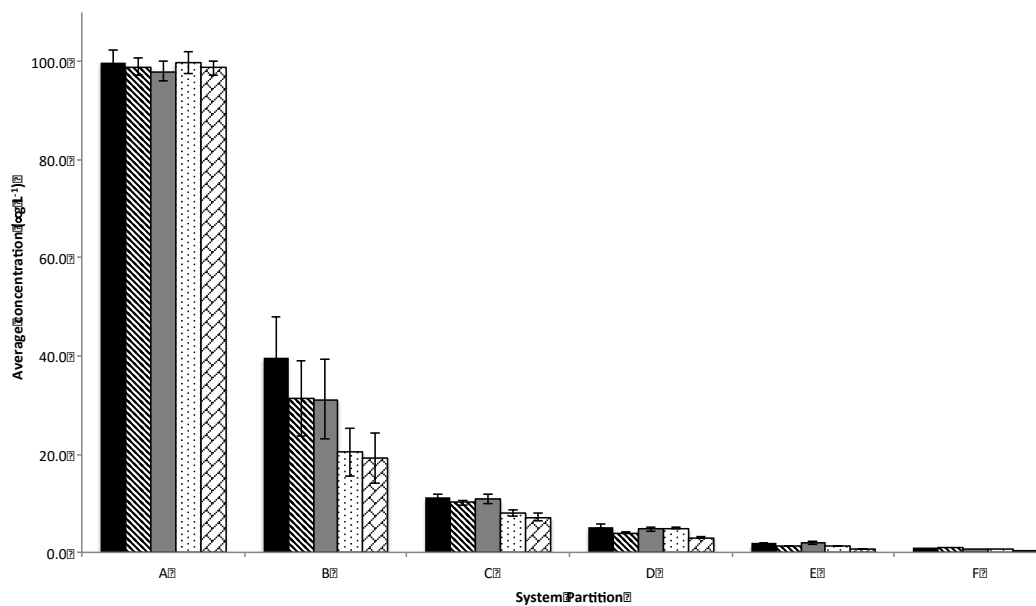
Figure 32: Sequential removal of xenobiotics by the EHC-SF system

A is the input, B was sampled at the C. vulgaris compartment outlet, B at the E. densa compartment outlet, C at the C. demersum compartment outlet, D at the base of the S. polyrhiza partition, and E in the centre of the drainage sump. F is the outlet. Nalidixic acid (solid black bar), nevirapine (diagonally hatched bar), acetaminophen (grey bar), ampicillin (speckled bar), BMAA (diagonal brick pattern). Error bars denote standard deviation (n = 8).

There was loss from the system by depuration. The total amount of each antibiotic lost from the system during the depuration phase was as follows:

- Nalidixic acid – 0.026 mg.
- Nevirapine – 3.282 mg.
- Ampicillin – 0.669 mg.
- Acetaminophen – 1.644 mg.
- BMAA – none detected.

It is considered that these unwanted releases, although they constitute a very small proportion of the total exposure amounts, will be further ameliorated by the removal of biomass as shown in Table 6.

Very high concentration – high flow rate (VHC-HF)

This data was collected from a 150 L system running a medium containing 0.05 mg·L^{-1} a flow rate of 0.042 hour^{-1}. The plant sequence was:

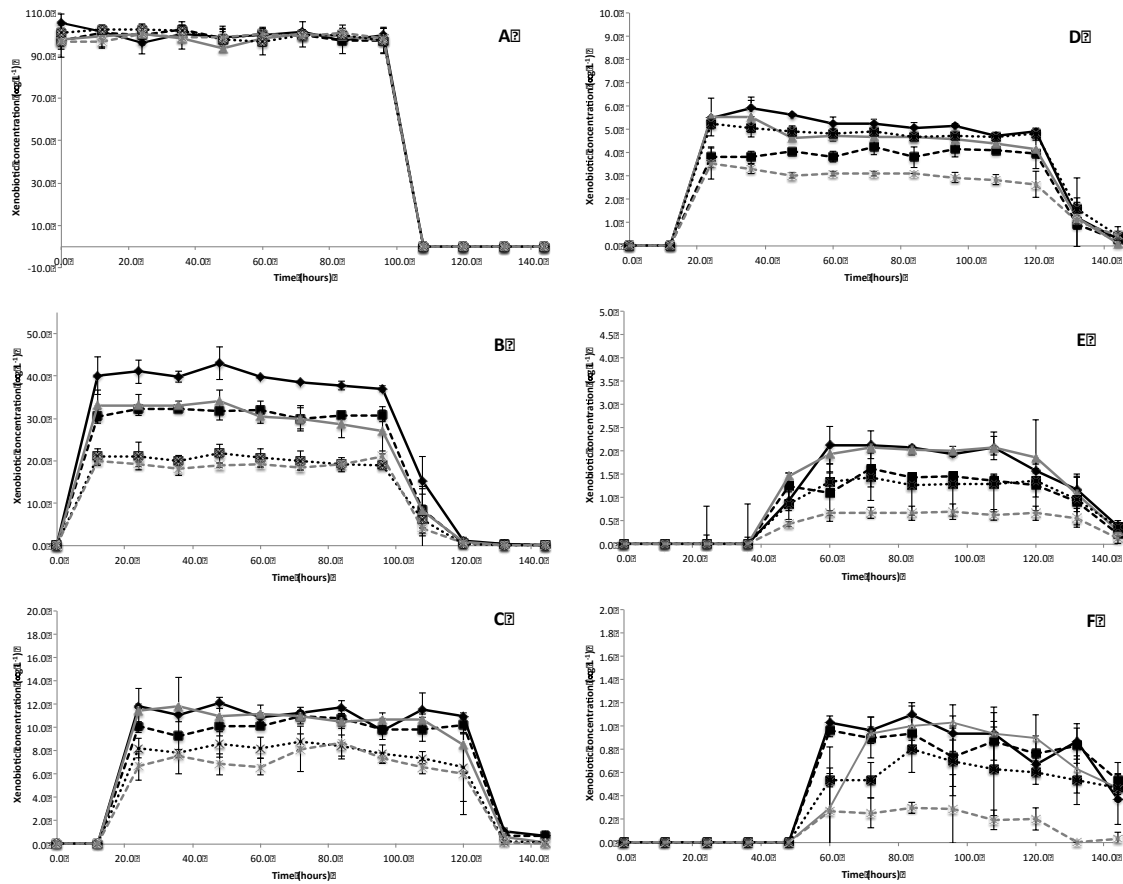*C. vulgaris ➔ E. densa ➔ C. demersum ➔ S. polyrhiza*
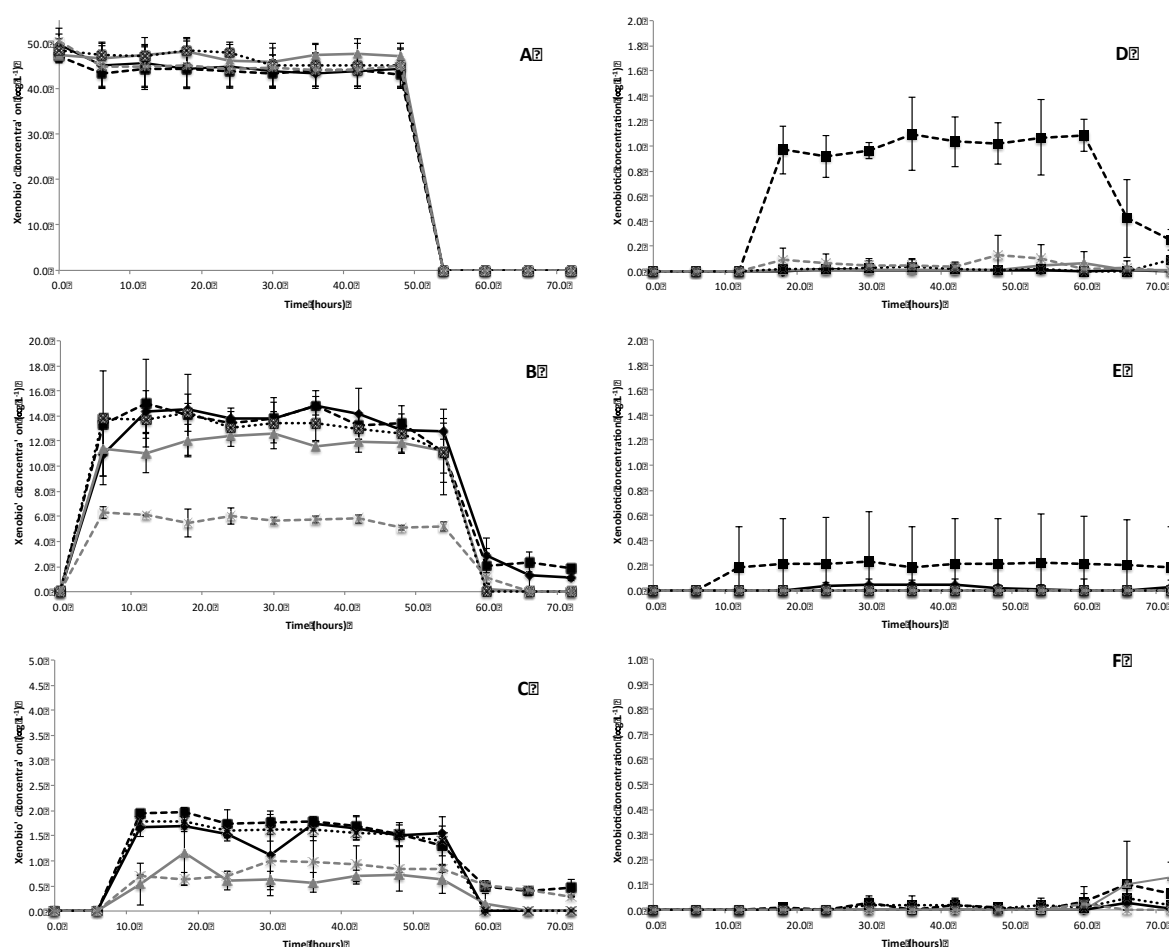
Figure 33: Xenobiotic concentrations over time at defined points along the VHC-HF system

Xenobiotic concentrations in each compartment over establishment, running and depuration phases for the VHC-HF model system. A is the input, B was sampled at the C. vulgaris compartment outlet, B at the E. densa compartment outlet, C at the C. demersum compartment outlet, D at the centre of the S. polyrhiza compartment, and E in the centre of the drainage sump. F is the outlet. Nalidixic acid (solid black line), nevirapine (dashed black line), acetaminophen (solid grey line), ampicillin (dotted black line), BMAA (dashed grey line). Error bars denote standard deviation (n = 3). 0 (zero) represents concentrations below detection limit using solid-phase extraction as previously described.

Despite the higher flow rate, a higher percentage removal was achieved in this run despite similar biomass loading. This suggests that the higher concentration may be excessive for this system configuration. As shown in Figure 33, almost complete removal was achieved and depuration was proportionately reduced. Figure 34 shows the average removal for each partition during the exposure phase of the experiment.
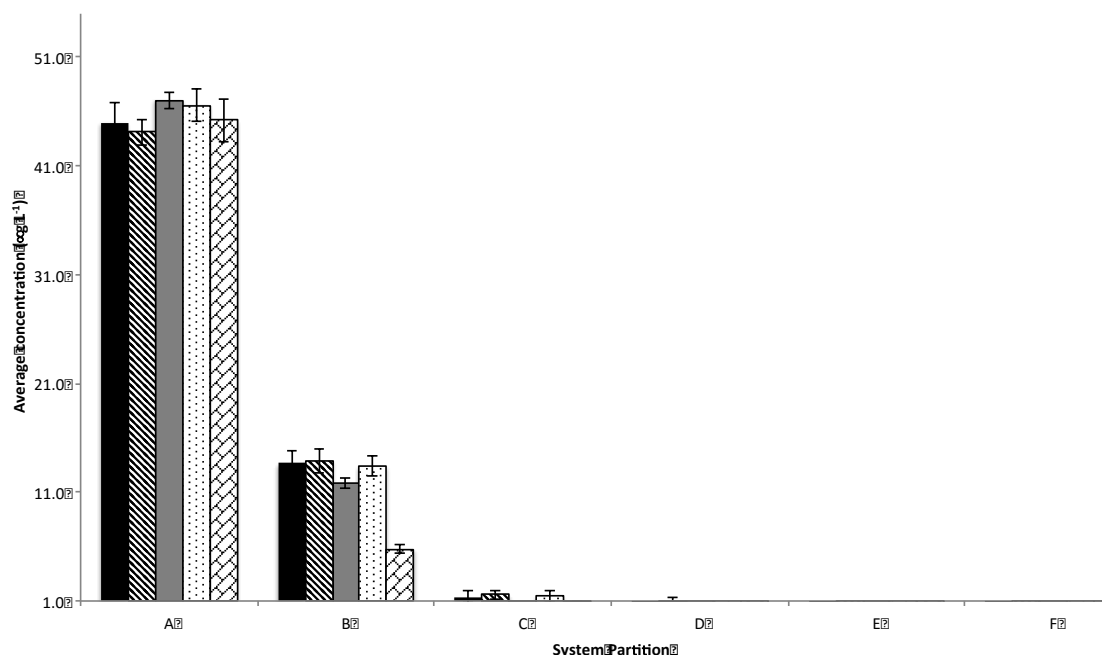
Figure 34: Sequential removal of xenobiotics by the VHC-HF system

A is the input, B was sampled at the C. vulgaris compartment outlet, B at the E. densa compartment outlet, C at the C. demersum compartment outlet, D at the base of the S. polyrhiza partition, and E in the centre of the drainage sump. F is the outlet. Nalidixic acid (solid black bar), nevirapine (diagonally hatched bar), acetaminophen (grey bar), ampicillin (speckled bar), BMAA (diagonal brick pattern). Error bars denote standard deviation (n = 8).

E. densa and *C. demersum* had improved biomass production and appeared healthier when running at this flow rate. Given the almost complete removal of nitrate at higher flow rates, this could indicate a requirement for placement earlier in the sequence. However, placing *E. densa* before *C. vulgaris* did not alter the xenobiotic removal dynamics. It did, however, reduce epiphytic algal growth on *C. vulgaris*. This was considered undesirable due to the potential for pipe and pump blockages. For this reason, and because of the relatively poor biomass production, *C. vulgaris* is not recommended for future HTSPS systems.

High concentration – very high flow rate (HC-VHF)

Data was collected from a 150 L system running a medium containing 100 ng·L^{-1} a flow rate of 0.084 hour^{-1}. The plant sequences were:

C. vulgaris ➔ E. densa ➔ C. demersum ➔ S. polyrhiza

E. densa ➔ C. vulgaris ➔ C. demersum ➔ S. polyrhiza

In both cases, all xenobiotics were below the limits of detection from Partition C onwards (and Partition D for nevirapine), even when using twice the optimised volume for solid-phase extraction as outlined above. No antibiotics were detected in the depuration medium during the depuration period constituting five system volumes.

Standard concentration – extremely high flow rate (SC-EHF)

Data was collected from a 150 L system running a medium containing 50 ng·L^{-1} a flow rate of 0.168 hour^{-1}. The plant sequence was:

C. vulgaris ➜ *E. densa* ➜ *C. demersum* ➜ *S. polyrhiza*

As for the HC-VHF system, xenobiotic concentrations were below the LLOD for all xenobiotics except for nevirapine by Partition C. No antibiotics were detected in depuration at the stated flow rate for five system volumes. This indicates that the system may be particularly useful in removing persistent low levels of xenobiotics from surface waters where nutrient removal can be balanced with the capacity for xenobiotic removal.

Biomass production

Biomass production was not significantly different for any particular set of conditions where the flow rate was equal to or greater than 0.02 hour^{-1} and nutrients were provided at stated concentrations. Table 10 lists the biomass production for each plant as a percentage yield per week and the actual yield forms the model system expressed as biomass per surface area per week and biomass per volume treated water per week.

Table 10: Biomass production in HTSPS model system running at 0.042 hour^{-1} ($n = 6$)

	C. demersum	*C. vulgaris*	*E. densa*	*S. polyrhiza*
Percentage yield per week	22.0 (± 0.5)	1.1 (± 0.8)	11.5 (± 0.3)	49.2 (± 2)
Grams biomass (wet) per surface area per week	293.3 g·m^{-2}	18.0 g·m^{-2}	166.7 g·m^{-2}	426.7 g·m^{-2}
Grams biomass (wet) per total water treated per week	41.9 g·m^{-3}	2.6 g·m^{-3}	23.8 g·m^{-3}	60.9 g·m^{-3}

The total biomass production from this small system for the purpose of maintenance was 135 g. Extrapolated to a large (50 × 20 m) system, this would result in approximately 180 kg usable biomass per week. Table 11 lists the potential xenobiotic transformation and total xenobiotic removal per tested xenobiotic for the model system based on biomass production, and system and kinetic data.

Table 11: Potential maximum complete removal of selected xenobiotics (mg·week^{-1}) per plant by biomass removal from the model HTSPS system* loaded with 100 µg·L^{-1} with a flow rate of 0.042 hour^{-1} of each xenobiotic

	C. demersum	*C. vulgaris*	*E. densa*	*S. polyrhiza*	**Total**
Acetaminophen	10.71	1.84	10.85	16.02	**39.41**
Ampicillin	10.69	2.10	6.46	15.81	**35.06**
Nevirapine	6.28	0.79	6.11	23.56	**36.74**
Nalidixic acid	7.79	0.25	12.62	32.57	**53.00**
BMAA	15.23	1.03	9.59	15.43	**41.28**

Typical contamination levels are below those used here, and actual removal rates will depend on exposure concentrations.

Given that the remaining biomass continues biotransformation as previously determined, this data represents the sustainability of xenobiotic removal at the stated rate, irrespective of potential reduced uptake as a function of limited capacity for storage of biotransformed molecules. However, no such limitation has been observed for these xenobiotics using these plants. Nonetheless, biomass removal in a system using high nutrient levels is not only essential for maintaining the system but would constitute a significant mechanism of xenobiotic removal, prevent depuration, and potentially be a source of biomass for value-adding processes.

The ease of use and simplicity of harvesting, combined with the relatively high biotransformation and xenobiotic uptake rates, make *S. polyrhiza* an excellent phytoremediation macrophyte. Where future implementation is indicated, a larger surface area/volume partition should be used to maximise its effectiveness where space allows. However, upstream hydrophobic antibiotic-tolerant plants such as *C. demersum* are required to protect the *S. polyrhiza,* which appears to have a somewhat lower tolerance for hydrophobic antibiotics.

The comparison of the theoretical data based on biotransformation potential and measured removal from the HTSPS system under those conditions shows that the system works between two and three times more efficiently than predicted by short-term kinetic data. This in turn suggests an adaptation to the xenobiotic with enhanced uptake and possibly biotransformation.

Nutrient removal

Figure 35 shows nitrate removal for all flow rates. As expected, the percentage removed depended on flow rate; a higher percentage was removed at lower flow rates. Nitrate removal correlated well with biomass production. For example, based on the Redfield ratio, the nitrate removed by *S. polyrhiza* should have yielded approximately 42 g of biomass in one week. The actual figure was 65 g. This discrepancy was attributed to ammonium in the feed that has not been quantified. Similar corresponding values for all plants were observed. On that basis, a phosphate removal in excess of 1 mg per m3 water treated would be achievable in this system.
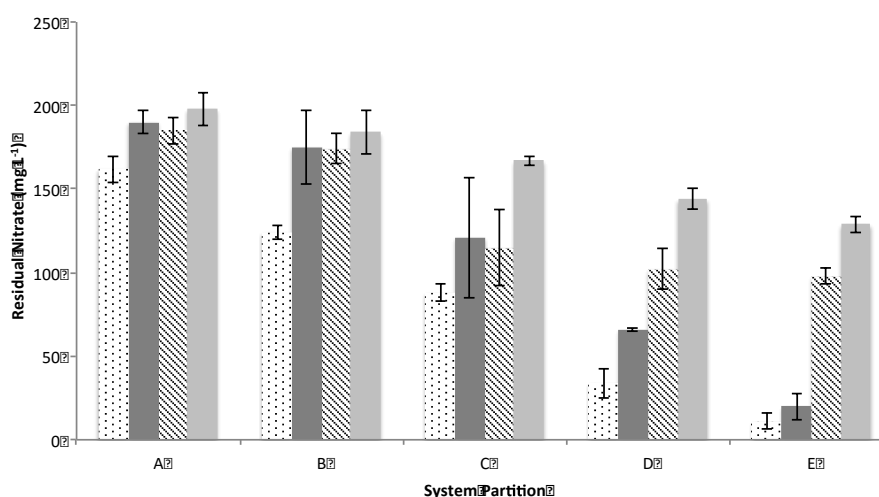

Figure 35: Nitrate removal at all flow rates

Nitrate removal by HTSPS system loaded with 200 mg·L^{-1} nitrate. The speckled bar represents a flow rate of 0.021 hour^{-1}, the dark grey bar 0.042 hour^{-1}, the diagonally patterned bar represents a flow rate of 0.084 hour^{-1} and the light grey bar 0.16 hour^{-1}. Error bars indicate standard deviation (n = 3).

The HTSPS system is thus also very efficient at removing nutrients. The source water nutrient composition in an implemented system will determine efficacy, but proportional concentrations of nitrogen and phosphorous will be required for balanced removal. Clearly a retention time of 24 hours or more was required for adequate nitrate removal from this system. This flow rate also allowed significant removal of the selected xenobiotics at a concentration of 0.050 mg·L^{-1}, a concentration approximately 1000 times greater than typical surface water antibiotic levels from polluted waters. Clearly, with the reduced footprint, the high throughput rate and the efficacy of xenobiotic removal, the system warrants a trial implementation in a 'real-world' situation.

4   CONCLUSIONS

Suitable methods were developed for detecting selected xenobiotics with appropriate sensitivity and accuracy in both raw water and in plant matrix. This allowed a range of available and appropriate submerged or floating macrophytes to be evaluated for (a) sensitivity to the selected xenobiotics, (b) uptake kinetics of the selected xenobiotics by the various plants, and (c) biotransformation and/or bioaccumulation and possible depuration of the xenobiotics by each plant. This data, in turn, informed the construction of the model HTSPS to determine the suitability of the concept for a future implementation at pilot scale.

The purpose of this project was to establish proof of concept and the feasibility of HTSPSs to remove selected xenobiotics at a flow rate higher than traditional maturation or polishing ponds. The data presented here clearly shows that the system is feasible. Xenobiotic removal exceeded expectations.

5   RECOMMENDATIONS

We recommend that the HTSPS concept be tested in a small facility with known water quality issues. The system is scalable, but we suggest a small-scale implementation where there is a constant flow of contaminated water, where there is space to construct the system, and where there is appropriate community involvement to manage the biomass production. We suggest that biomass processing to add value (in the form of fuel pellet production) be investigated prior to construction to ensure that excess biomass does not in itself become a waste problem.

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