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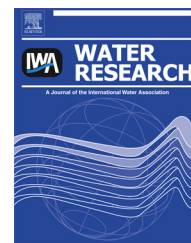
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# Stereoselective biodegradation of amphetamine and methamphetamine in river microcosms

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## ABSTRACT

Here presented for the first time is the enantioselective biodegradation of amphetamine and methamphetamine in river microcosm bioreactors. The aim of this investigation was to test the hypothesis that mechanisms governing the fate of amphetamine and methamphetamine in the environment are mostly stereoselective and biological in nature. Several bioreactors were studied over the duration of 15 days (i) in both biotic and abiotic conditions, (ii) in the dark or exposed to light and (iii) in the presence or absence of suspended particulate matter. Bioreactor samples were analysed using SPE-chiral-LC-(QTOF) MS methodology. This investigation has elucidated the fundamental mechanism for degradation of amphetamine and methamphetamine as being predominantly biological in origin. Furthermore, stereoselectivity and changes in enantiomeric fraction (EF) were only observed under biotic conditions. Neither amphetamine nor methamphetamine appeared to demonstrate adsorption to suspended particulate matter. Our experiments also demonstrated that amphetamine and methamphetamine were photo-stable. Illicit drugs are present in the environment at low concentrations but due to their pseudo-persistence and non-racemic behaviour, with two enantiomers revealing significantly different potency (and potentially different toxicity towards aquatic organisms) the risk posed by illicit drugs in the environment should not be under- or over-estimated. The above results demonstrate the need for re-evaluation of the procedures utilised in environmental risk assessment, which currently do not recognise the importance of the phenomenon of chirality in pharmacologically active compounds.

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## 1. Introduction

It is well known that the incomplete removal of pharmaceuticals and illicit drugs during sewage treatment results in their sustained emission to the aquatic environment (Castiglioni et al., 2011). Less well understood is that during sewage treatment and in the aquatic environment, chiral drugs can undergo stereoselective mechanisms controlling their fate. It has been reported that chiral pharmacologically active

compounds such as anti-inflammatory drugs, beta-blockers and antidepressants were observed in varying non-racemic proportions before and after sewage treatment indicating their stereoselective fate (Kasprzyk-Hordern and Baker, 2012; Gasser et al., 2012; MacLeod et al., 2007; MacLeod and Wong, 2010; Nikolai et al., 2006; Fono and Sedlak, 2005; Fono et al., 2006; Matamoros et al., 2009; Buser et al., 1999; Kasprzyk-Hordern et al., 2010). Following wastewater treatment, differences in enantiomeric fractions in the wider aquatic

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environment were also observed suggesting enantioselective processes (Kasprzyk-Hordern and Baker, 2012; Fono et al., 2006; Buser et al., 1999; Kasprzyk-Hordern et al., 2010; Bagnall et al., 2012; Winkler et al., 2001).

This evidence of stereo-selectivity in the aquatic environment represents a new challenge for the aquatic scientist. More than half of pharmaceuticals in use are chiral and many of these are marketed as racemates, which are drugs consisting of an equimolar mixture of two enantiomers (Kasprzyk-Hordern, 2010). Enantiomers of the same drug have identical physicochemical properties except optical activity but may differ in their biological properties. Distribution, metabolism or excretion from the body usually favour one enantiomer over the other. This results from the fact that enantiomers stereoselectively react in biological systems for example with enzymes. Furthermore, stereoselective biological transformation of drugs causes the enantiomeric composition of chiral compounds may be changed. Metabolites of achiral compounds can also be chiral (e.g. achiral albendazole or risperidone are transformed into chiral metabolites) (Kasprzyk-Hordern, 2010). Considering this, current ecotoxicological data regarding racemic formulations needs to be reassessed as this data is based on the assumption that environmental concentrations of chiral drugs are racemic. Neither is this assumption correct nor are enantiomers equal in terms of their ecotoxicity. For example in a study undertaken by MacLeod et al. (2007) an enrichment of fluoxetine with S(+)-fluoxetine was observed as a result of wastewater treatment. Such a phenomenon is of potentially significant ecotoxicological consequence as toxic effects of fluoxetine enantiomers are species dependent with S(+)-fluoxetine being more toxic than R(-)-fluoxetine in *Pimephales promelas* (Stanley et al., 2007). Propranolol was also found to be enriched with S(-)-propranolol (MacLeod et al., 2007) as a result of wastewater treatment. This is concerning as S(-)-propranolol is known to have higher toxicity towards *P. promelas* than its antipode (Stanley et al., 2006).

Illicit drugs are a group of highly biologically active chiral chemicals which have recently been identified as emerging environmental micropollutants (Kasprzyk-Hordern, 2010; Castiglioni et al., 2011). Kasprzyk-Hordern and Baker (2012) published the first report on enantiomeric profiling of chiral amphetamine-like illicit drugs during wastewater treatment and in the environment. In a study of 7 WWTPs utilising activated sludge and trickling filters over the period of 9 months, the enantioselective fate of amphetamine-like compounds was observed. MDMA was found in raw wastewater to be enriched with the R(-)-enantiomer due to preferential metabolism of S(+)-MDMA in humans. Mean enantiomeric fractions (EF) values for raw and treated wastewater were found to be 0.68 and 0.78 respectively indicating further enrichment of MDMA with the R(-)-enantiomer as a result of wastewater treatment, probably due to enantioselective microbial processes occurring. The highest stereoselectivity was observed in the case of activated sludge treatment, which indicates that different consortia of microorganisms might be responsible for degradation in different treatment processes. Amphetamine was also found to be enriched with the R(-)-enantiomer. Degradation of amphetamine was observed to be stereoselective with the S(+)-enantiomer being preferentially

degraded leading to further enrichment of amphetamine with the R(-)-enantiomer. Out of the two enantiomers of ephedrine (1S,2R(+)- and 1R,2S(-)-) only natural 1R,2S(-)-enantiomer was frequently detected in raw wastewater. However, synthetic 1S,2R(+)-ephedrine was detected in treated wastewater indicating stereoselective processes occurring, possibly chiral inversion, although no direct evidence was found. Hashim et al. (2011) also highlighted the possibility of chiral inversion of S(+)-naproxen leading to increased concentration of R(-)-naproxen during laboratory scale membrane bioreactor simulated wastewater treatment.

Stereoselective fate of amphetamine-like compounds was also observed in receiving waters. The extent of stereoselectivity was dependent on the type of chiral compound, proximity to wastewater treatment plants (and utilised technology) and season. Higher stereoselectivity was observed in the aqueous environment over the spring/summer time possibly due to higher microbial activity. MDMA was quantified in receiving waters at low ppt levels and it was found to be enriched with R(-)-enantiomer. Change in EFs (further enrichment with the R(-)-enantiomer) was observed with the course of the river, which might be due to microbial processes occurring or as a result of a discharge of non-racemic MDMA with treated wastewater. Similarly, amphetamine was found in receiving waters enriched with the R(-)-enantiomer. As a result of enrichment of ephedrine with 1S,2R(+)-enantiomer during wastewater treatment, this enantiomer was also detected in receiving waters, despite the fact that this enantiomer is not marketed (Kasprzyk-Hordern and Baker, 2012).

The results presented by Kasprzyk-Hordern and Baker (2012) are of high environmental significance as two enantiomers of each amphetamine-like compound reveal different potency and potentially also ecotoxicity. For example the pharmacological actions of both the MDMA and MDA enantiomers differ both quantitatively and qualitatively. S(+)-Enantiomers are thought to be more amphetamine-like stimulants, and R(-)-enantiomers are more hallucinogenic (Fantegrossi, 2008). S(+)-amphetamine has twice as high stimulant activity than R(-)-amphetamine (Kasprzyk-Hordern et al., 2010). As for amphetamine, the psychostimulant effects of methamphetamine are enantioselective, and the S(+)-enantiomer is much more active than the R(-)-enantiomer (Kasprzyk-Hordern and Baker, 2012).

There is a clear lack of information describing the stereoselective mechanisms in the aquatic environment: (1) Research as to the stereoselective fate of chiral pharmaceuticals and illicit drugs in the aquatic environment is necessary; (2) A greater understanding of the stereoselective ecotoxicity of each enantiomer is necessary. This research deals only with the first point but realises the necessity of the second. Evidence suggests that stereoselectivity is biological in nature; however, this hypothesis remains untested.

This is to the authors' knowledge the first report studying degradation of amphetamine and methamphetamine in river microcosms including effects of microbial degradation, photolysis and sorption, and by-products formation. The research reported in the manuscript tests the hypothesis that degradation of chiral illicit drugs (in this case amphetamine and methamphetamine) is stereoselective and biological in nature. To do so, several river water bioreactors were studied

over the duration of 15 days in both biotic and abiotic conditions, and in the dark or exposed to sunlight.

## 2. Materials and methods

### 2.1. Chemicals and reagents

The reference standards *S/R*(±)-amphetamine and *S/R*(±)-methamphetamine and internal standards (ISTD): *S/R*(±)-amphetamine-*d*11, *S/R*(±)-methamphetamine-*d*14 were purchased from LGC Standards (Teddington, UK). All internal standards were added to river water samples prior to SPE extraction and were used for analyte quantification. All reference materials, including ISTD, had purity of >98%.

Stock solutions (1 mg/L) of the drugs were prepared in methanol and were stored in the dark at  $-5\text{ }^{\circ}\text{C}$ . Working solutions were prepared by diluting stock solutions in methanol or mobile phase and were stored at  $4\text{ }^{\circ}\text{C}$ . HPLC grade methanol and water were obtained from Fisher Scientific UK (Loughborough, UK). Ammonium acetate was obtained from Sigma–Aldrich (Gillingham, UK).

### 2.2. Microcosm bioreactors

#### 2.2.1. Mixed compound initial scoping exercise

##### 2.2.1.1. Study of mixed compound bioreactors in river water microcosms: influence of biotic (microbial degradation) and abiotic processes (photochemical processes) – experiment 1.

River water collected from the River Avon at Saltford (West of Bath, UK, collected during June and July 2011) was used for microcosm bioreactor experiments. Initial biodegradation studies were conducted to investigate the fate of chiral drugs at enantiomeric level. The following processes were investigated: biodegradation, photodegradation and other abiotic processes. With this initial study a racemic standard containing a mixture of target illicit drugs: *S/R*(±)-amphetamine and *S/R*(±)-methamphetamine and caffeine was used. Caffeine is a chemical with proven high biodegradability in the aqueous environment. It was added to microcosms to verify the occurrence of biological processes throughout the duration of experiments. As can be observed in Fig S1 much

higher degradation of caffeine took place in biotic reactors when compared to abiotic reactors.

Degradation experiments were conducted in the light and dark (to study photochemical processes), with or without sodium azide (as an inhibitor to biotic processes). Eight conical flasks (made of borosilicate 3.3 glass with no visible light absorption and UV light cut-off at  $<275\text{ nm}$ ) used as bioreactors in microcosm experiments were autoclaved prior to use. All were subsequently filled with river water filtered through  $0.7\text{ }\mu\text{m}$  glass fibre filter (2 L each). Four bioreactors were spiked with sodium azide to a concentration of 1 g/L to inhibit biotic processes (Abiotic Reactors). Four bioreactors remained un-spiked in order to allow biotic processes to occur (Biotic Reactors). Each conical flask was placed onto a magnetic stirrer either in the light (two replicate microcosms with and two without sodium azide; Light Reactors) or the dark (two replicate microcosms with and two without sodium azide; Dark Reactors) as presented in Fig. 1. Daylight conditions were simulated using an Osram 400 W HQI BT daylight lamp, which was switched on for 8 h each day to mimic average sunlight conditions in the UK. The bioreactors were ventilated to prevent temperature rise. There was 1.5 m distance between the bottle base and the light source. Average photon flux measured at the level of the bottle base was  $388\text{ }\mu\text{mol/m}^2/\text{s}$ .

Each microcosm bioreactor was investigated in duplicate. Samples were taken three to five times over a fifteen-day sampling period and analysed with SPE-LC-QTOF-MS. Other parameters analysed during the sampling period included dissolved oxygen (DO), pH and temperature (Table 1).

##### 2.2.1.2. Study of mixed compound bioreactors in river water/sediment microcosms: influence of suspended particulate matter – experiment 2.

To determine the influence of suspended particulate matter and potential adsorption mechanisms on stereo-selective degradation, experiments were conducted (as described above) (i) in the light and dark, (ii) with and without sodium azide and (iii) with and without fortification of samples with 1 g/L of river sediment. River water was collected from the River Avon. Sediment was collected from the bed of the River Avon. Eight 2 L conical borosilicate 3.3 glass flasks (four containing 2 g of river sediment) were autoclaved prior to experimentation and were

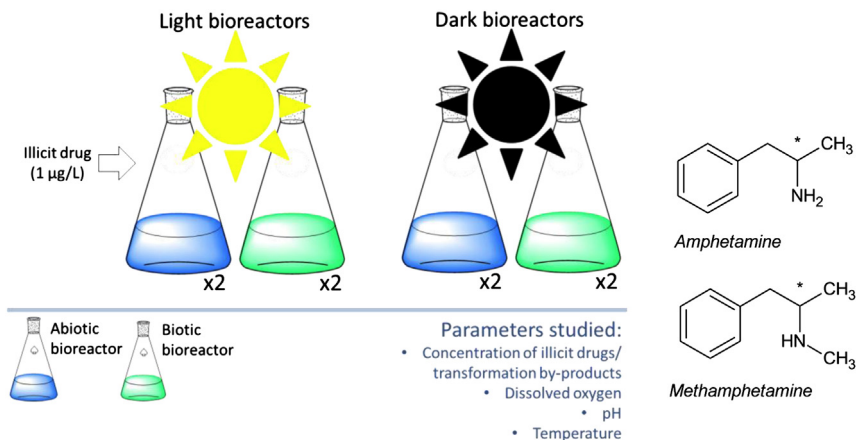


Fig. 1 – Schematic for the bioreactors.

**Table 1 – Temperature, dissolved oxygen (DO) and pH in microcosm bioreactors (DAR- Dark Abiotic Reactor, DBR- Dark Biotic Reactor, LAR- Light Abiotic Reactor, LBR- Light Biotic Reactor, DASR- Dark Abiotic Reactor with Sediment, DBSR- Dark Biotic Reactor with Sediment, LASR- Light Abiotic Reactor with Sediment and LBSR- Light Biotic Reactor with Sediment).**

Experiment 1			
	pH (mean ± SD)	Temp [°C] (mean ± SD)	DO [mg/L] (mean ± SD)
DAR	8.6 ± 0.3	22.6 ± 6.4	11.1 ± 6.9
DBR	8.7 ± 0.7	22.9 ± 6.6	8.9 ± 1.5
LAR	8.5 ± 0.4	23.2 ± 6.8	10.7 ± 6.7
LBR	8.9 ± 1.0	26.1 ± 1.0	9.5 ± 3.0
Experiment 2			
DAR	8.4 ± 0.6	25.8 ± 2.6	8.9 ± 0.8
DBR	8.7 ± 0.6	25.3 ± 2.4	9.3 ± 1.6
DASR	8.5 ± 0.5	25.4 ± 2.4	8.8 ± 1.0
DBSR	8.8 ± 0.9	25.8 ± 2.3	9.5 ± 1.8
LAR	8.6 ± 0.1	25.8 ± 2.8	8.7 ± 0.5
LBR	8.9 ± 1.1	26.3 ± 2.6	9.3 ± 1.2
LASR	8.6 ± 0.1	25.5 ± 2.8	8.5 ± 0.3
LBSR	9.0 ± 0.6	25.9 ± 2.7	9.1 ± 1.3
Experiment 3			
Amphetamine			
DBR	8.4 ± 0.5	28.5 ± 1.4	7.1 ± 1.6
LBR	9.3 ± 1.2	29.0 ± 0.7	9.0 ± 2.0
Methamphetamine			
DBR	8.4 ± 0.5	29.5 ± 1.7	7.0 ± 1.5
LBR	9.3 ± 1.1	29.7 ± 1.5	8.2 ± 3.0

used during this investigation. All were subsequently filled (2 L each) with river water. All microcosms were spiked with the mixed racemic standard of chiral drugs and caffeine. Four bioreactors (two containing sediment and two without) were additionally spiked with sodium azide to a concentration of 1 g/L. Each conical flask was placed onto a magnetic stirrer either in the light or the dark as per Fig. 1. Each microcosm bioreactor was investigated in duplicate. Samples were taken three to five times over a fifteen-day sampling period and analysed with SPE-LC-QTOF-MS. Other parameters analysed during the sampling period included DO, pH and temperature (Table 1).

### 2.2.2. Single compound amphetamine and methamphetamine bioreactors (experiment 3)

Following initial scoping, further bioreactor experimentation was conducted using river water (collected from Saltford in September 2011). Eight autoclaved 2 L conical flasks were filled with 2 L of unfiltered river water. Four 2 L borosilicate 3.3 glass flasks were spiked with racemic amphetamine and racemic amphetamine-d11. Of these amphetamine bioreactors, two were placed in the dark and two were in the light. Four additional 2 L flasks were spiked with racemic methamphetamine and racemic methamphetamine-d14. Of these methamphetamine bioreactors, two were placed in the dark and two were in the light. Deuterated counterparts to amphetamine and methamphetamine were used for the purpose of breakdown product identification. Samples were taken twice a week over a 15 day sampling period and analysed with SPE-LC-QTOF-MS. Other parameters analysed during the sampling period included DO, pH and temperature (Table 1).

### 2.3. Sampling and analysis using SPE-LC-QTOF-MS

Samples were analysed using methodology described by the authors elsewhere (Bagnall et al., 2012). Each 100 mL sample was filtered through Whatman GF/F 0.7 µm glass fibre filter (Whatman, UK) and spiked with internal standard (deuterated analogue of chiral drug) to a concentration of 200 ng/L (note that in the single compound amphetamine and methamphetamine bioreactors S/R(±)-MDMA-d5 was used as internal standard as deuterated analogues of these compounds were also a subject of investigation in the microcosms). Following this, samples were concentrated using SPE (solid-phase extraction). HLB cartridges were loaded at a flow rate of <6 mL/min and eluted with 4 mL of methanol at a rate of <1 mL/min. Extracts were then evaporated to dryness with a TurboVap evaporator (Caliper, UK, 40 °C, N<sub>2</sub>, <5 psi) and reconstituted in 0.5 mL of mobile phase. All samples were filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13 mm) and transferred to polypropylene 0.3 mL capacity vials (Waters, UK).

The samples were then analysed by SPE-chiral-LC-QTOF MS using an Acquity UPLC (Waters, UK) and a microTOFQ MS (Bruker Daltonik GmbH, Germany). Each extract was injected into the LC-QTOF in duplicate. A Chirobiotic V column, 250 × 2.1 mm, I.D. 5 µm (Sigma–Aldrich, UK) and 20 × 1.0 mm, I.D. 5 µm guard column (Sigma–Aldrich, UK) was used for chiral resolution. The chromatographic conditions for this column were: methanol containing 4 mM ammonium acetate and 0.005% formic acid at a flow rate of 0.1 mL/min. The column was maintained at 25 °C and the autosampler temperature was 4 °C. The chromatographic run time was 66 min and the injection volume was 20 µL.

The method provided very good sensitivity of measurements with method limits of quantification in river water of 4.8 and 5.0 ng/L for S(+)- and R(-)-amphetamine and 18.3 and 18.5 ng/L for S(+)- and R(-)-methamphetamine. Full baseline resolution of enantiomers (*R<sub>s</sub>* = 1.2) of amphetamine and methamphetamine allowed for reliable quantification of each enantiomer. Inter-day accuracy and precision were <10%. For detailed discussion on method development and validation please see Table S1 and the paper by Bagnall et al. (2012).

Enantiomeric fractions (EF) of studied chiral drugs were calculated using the following equation:

$$EF = \frac{E_{1rel}}{E_{1rel} + E_{2rel}} \text{ and } E_{1rel} = \frac{E_1}{E_{1IS}}, E_{2rel} = \frac{E_2}{E_{2IS}}$$

where E1 and E2 represent peak areas of the (+) and (-) enantiomers respectively and E<sub>1IS</sub>, E<sub>2IS</sub> represent corresponding peak areas of internal standards.

## 3. Results and discussion

### 3.1. The influence of abiotic mechanisms upon degradation and stereo-selectivity of chiral drugs

#### 3.1.1. Study of mixed compound bioreactors in river water microcosms: influence of biotic (microbial degradation) and abiotic processes (photochemical processes)

From the initial scoping exercise (experiment 1), it was established that biological activity was responsible for stereo-



selectivity witnessed with amphetamine and methamphetamine. Abiotic processes in both the light and the dark were not observed during this investigation and appeared not to contribute to degradation (change in absolute concentration) or changes in enantiomeric fraction over the fifteen day sampling period (see Fig. 2, Dark Abiotic Reactor and Light Abiotic Reactor). Neither the concentration of amphetamine nor methamphetamine in both light and dark abiotic bioreactors deviated by more than  $\pm 15\%$  from initial concentration.

Similarly with EF, no significant deviation beyond that of the analytical method was observed. No stereoselectivity was observed in any abiotic reactor in both dark and light conditions.

Under biotic conditions, both reduction from initial amphetamine and methamphetamine concentration and significant change in EF was witnessed. The most pronounced changes occurred in the light bioreactors with amphetamine, where total amphetamine concentration reduced from 693 ng/L to 135 ng/L within eight days and was not detected

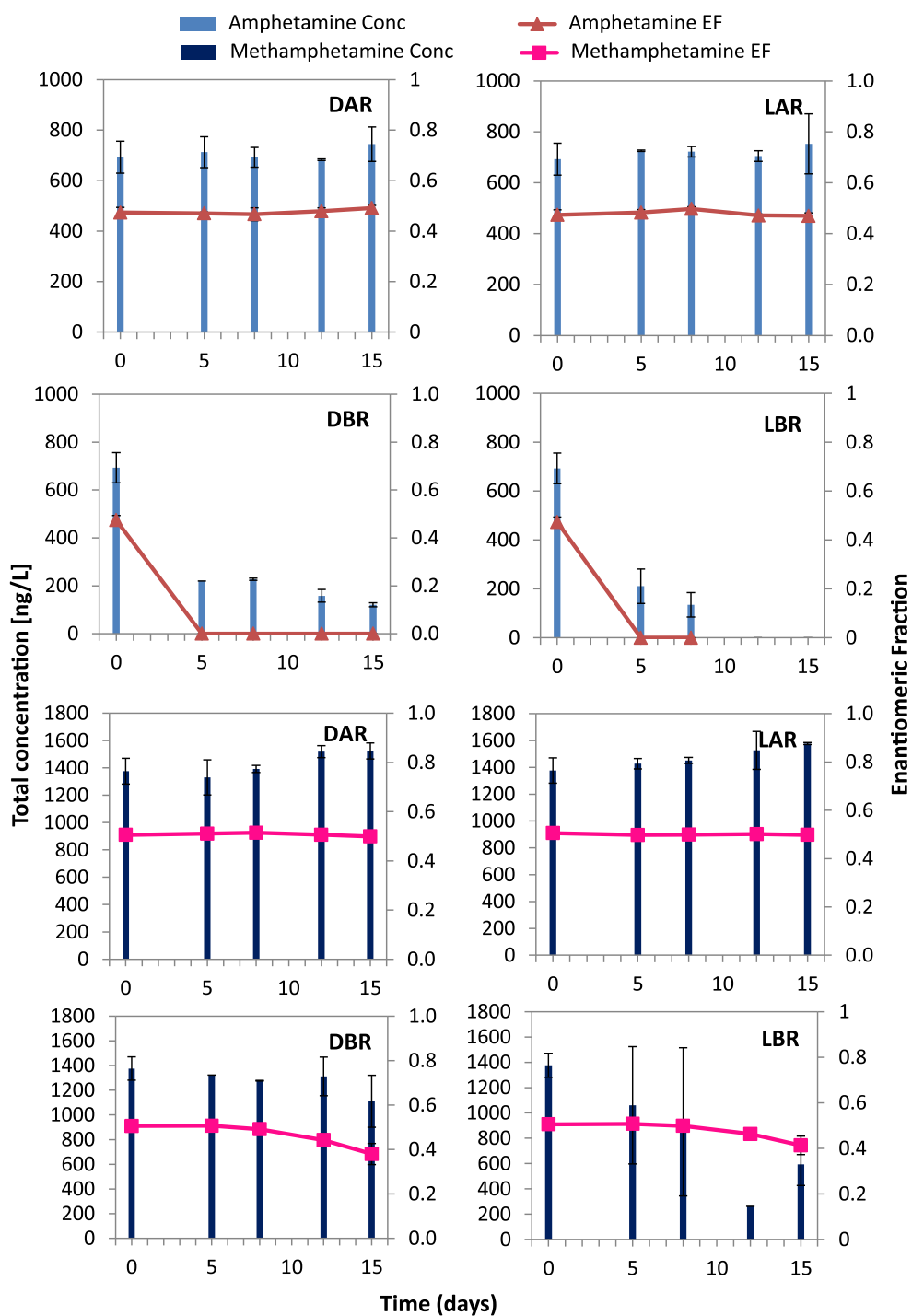


Fig. 2 – The stereoselective behaviour of amphetamine and methamphetamine in light and dark, biotic and abiotic conditions (DAR- Dark Abiotic Reactor, DBR- Dark Biotic Reactor, LAR- Light Abiotic Reactor, LBR- Light Biotic Reactor).

subsequently. Moreover, the EF of this compound changed from 0.47 to  $<0.02$  within five days indicating only the R(-)-enantiomer persisted from then on. Elimination of the S(+)-enantiomer was also witnessed with amphetamine in the dark, where EF changed from 0.47 to  $<0.02$  within five days. However, degradation of this compound was slower in the dark; the total concentration of amphetamine remaining on

day 15 was 121 ng/L. With methamphetamine, the initial EF of 0.51 reduced to 0.38 and 0.41 in the light and dark biotic bio-reactors respectively. Faster degradation of this compound was also witnessed in the light. The total concentration of methamphetamine on day 15 was 594 ng/L in the light (Light Biotic Reactor) as opposed to 1110 ng/L in the dark (Dark Biotic Reactor).

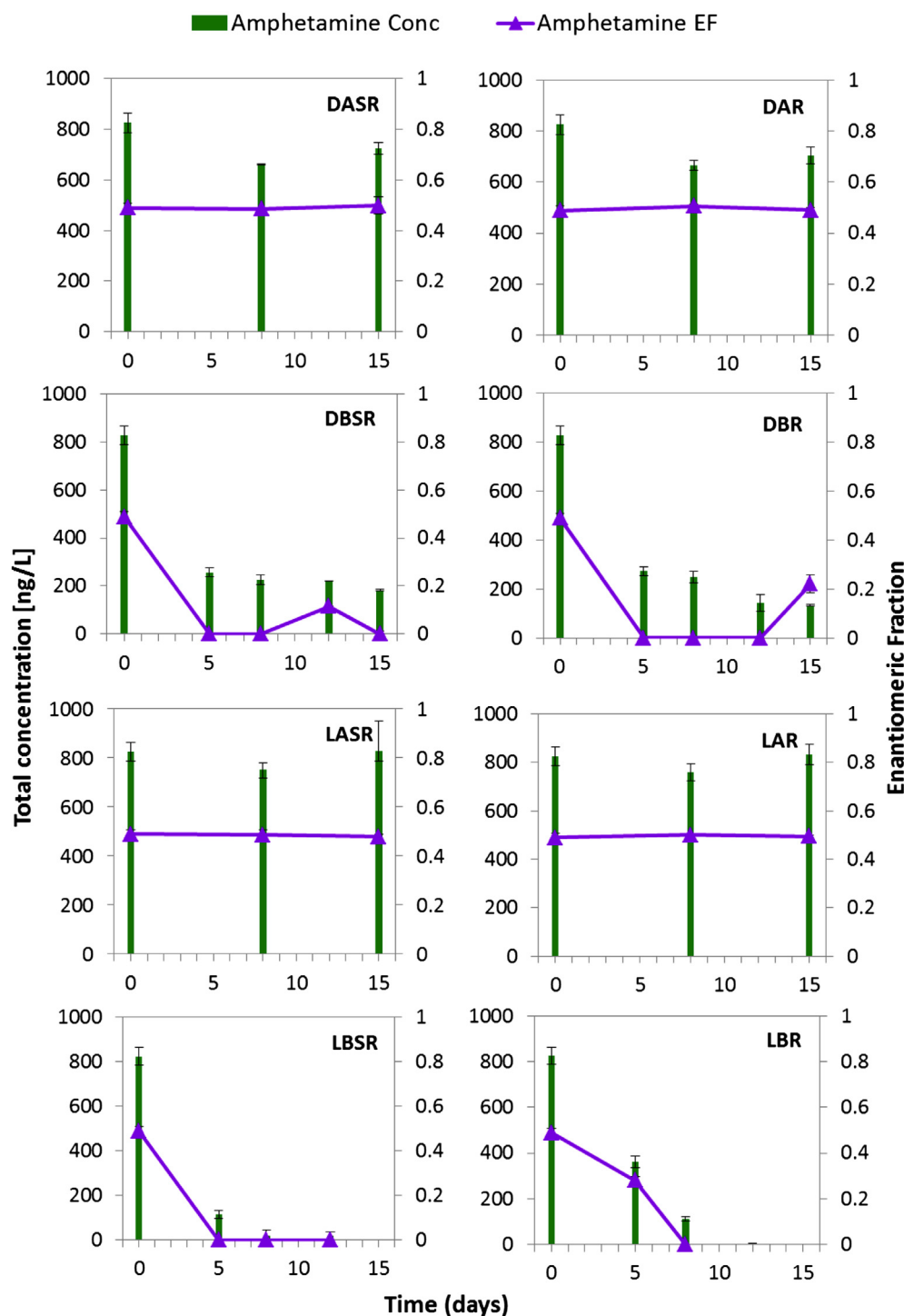


Fig. 3 – The stereoselective behaviour of amphetamine in the light and dark, under biotic and abiotic condition with and without sediment additions (DARS- Dark Abiotic Reactor with Sediment, DARS- Dark Abiotic Reactor, DBSR- Dark Biotic Reactor with Sediment, DBR- Dark Biotic Reactor, LASR- Light Abiotic Reactor with Sediment, LAR- Light Abiotic Reactor, LBSR- Light Biotic Reactor with Sediment, LBR- Light Biotic Reactor).

3.1.2. Study of mixed compound bioreactors in river water/sediment microcosms: influence of suspended particulate matter  
The second round of scoping investigations (experiment 2), in addition to confirming previous findings, established that adsorption appeared, as expected, to have limited impact

upon observed stereo-selectivity of transformation of amphetamine and methamphetamine (Figs. 3 and 4). There appeared to be no significant difference ( $t$ -test  $P > 0.05$ ) between concentration or EF over the 15 day sampling period between abiotic bioreactors with or without the addition of

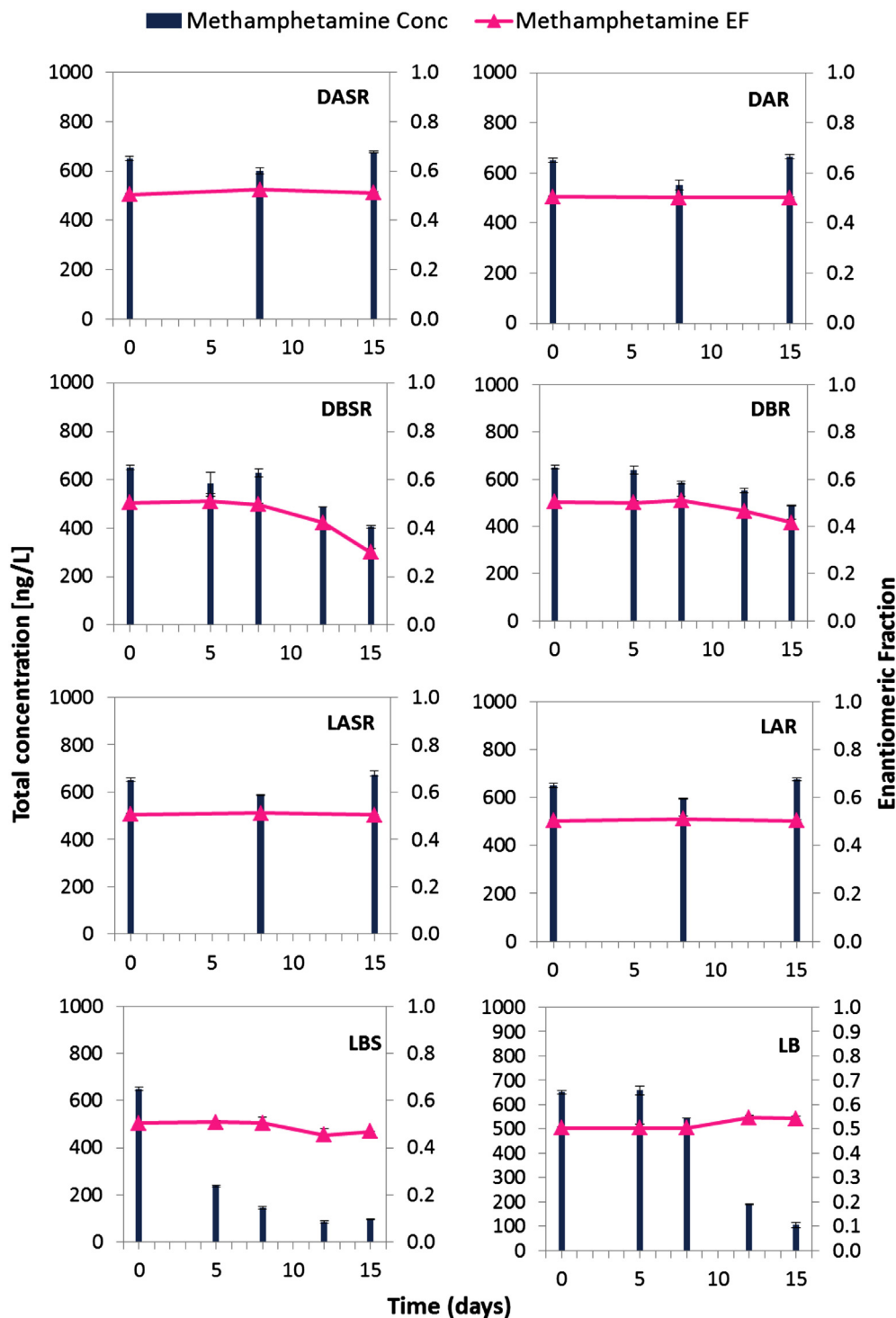


Fig. 4 – The stereoselective behaviour of methamphetamine in the light and dark, under biotic and abiotic condition with and without sediment additions (DAR- Dark Abiotic Reactor, DBR- Dark Biotic Reactor, DARS- Dark Abiotic Reactor with Sediment, DBSR- Dark Biotic Reactor with Sediment, LAR- Light Abiotic Reactor, LBR- Light Biotic Reactor, LASR- Light Abiotic Reactor with Sediment, LBSR- Light Biotic Reactor with Sediment).



sediment for either amphetamine (Fig. 3) or methamphetamine (Fig. 4) in the dark. However, under light conditions, this was not the case. Whilst dark biotic bioreactors with and without the addition of sediment behaved similarly for both amphetamine and methamphetamine, in the light it appeared that the addition of sediment increased the rate of degradation. For example, in the case of amphetamine, the light bioreactor with sediment addition degraded initial total amphetamine concentrations to 115 ng/L within eight days (Fig. 3). This was an 86% reduction in comparison to 56% observed by the light bioreactors without sediment addition. With methamphetamine, faster degradation was also observed in the light bioreactor containing sediment in comparison to the bioreactor without sediment. By day eight, the light bioreactor with sediment addition degraded initial total methamphetamine concentrations to 143 ng/L. This was a 78% reduction in comparison to 16% observed by the light bioreactors without sediment addition.

This investigation has elucidated the fundamental mechanism for degradation as being biological in origin. Furthermore, stereo-selectivity and changes in EF were only observed under biotic conditions. Neither amphetamine nor methamphetamine appeared to demonstrate adsorption to suspended particulate matter. Under dark and light abiotic conditions no changes in concentration or EF of amphetamine or methamphetamine could be established between bioreactors with or without the addition of sediment. This strongly suggests that adsorption had little impact upon the analyte concentration. This could therefore mean that adsorption has a minimal role in the degradation of amphetamine and methamphetamine in the aquatic environment. Interestingly, a difference between bioreactors with or without the addition of sediment could be established for amphetamine and methamphetamine under biotic conditions. With amphetamine, after five-

days, concentrations (and EF) decreased to 115 ng/L (<0.02) and 362 ng/L (0.28) respectively from the bioreactors with and without sediment addition. With methamphetamine after fifteen-days, concentrations (and EF) decreased to 94 ng/L (0.47) and 105 ng/L (0.54) respectively from the bioreactors with and without sediment addition. These differences in activity between the biotic bioreactors with and without sediment addition were exclusive to light conditions only. However, it cannot be assumed that in light conditions, amphetamine and methamphetamine are more likely to be adsorbed to suspended particulate matter. It is more likely that the addition of sediment provides more favourable environmental conditions for the biota to thrive under light conditions.

Evident in each set of bioreactor experiments is the apparent increase in degradation kinetics between bioreactors in the light and the dark. Consistently, bioreactors in the light degraded amphetamine and methamphetamine quicker than their dark equivalents. This was not however due to the combination of biodegradation and photo-degradation. In fact, these experiments demonstrated that amphetamine and methamphetamine were photo-stable under light abiotic conditions, as no reduction in concentration or change in EF could be established. It could therefore be concluded that the faster rate of degradation of amphetamine and methamphetamine encountered in the light was biological in nature and due to some advantage associated with light conditions. Such advantages could have been due to the presence of algae and the establishment of a diverse micro-community. The fixing of carbon and the subsequent compounds produced by algae could have resulted in greater bacterial abundance and diversity which could have been the advantage in comparison to the dark bioreactors. However, further work is needed to confirm this hypothesis.

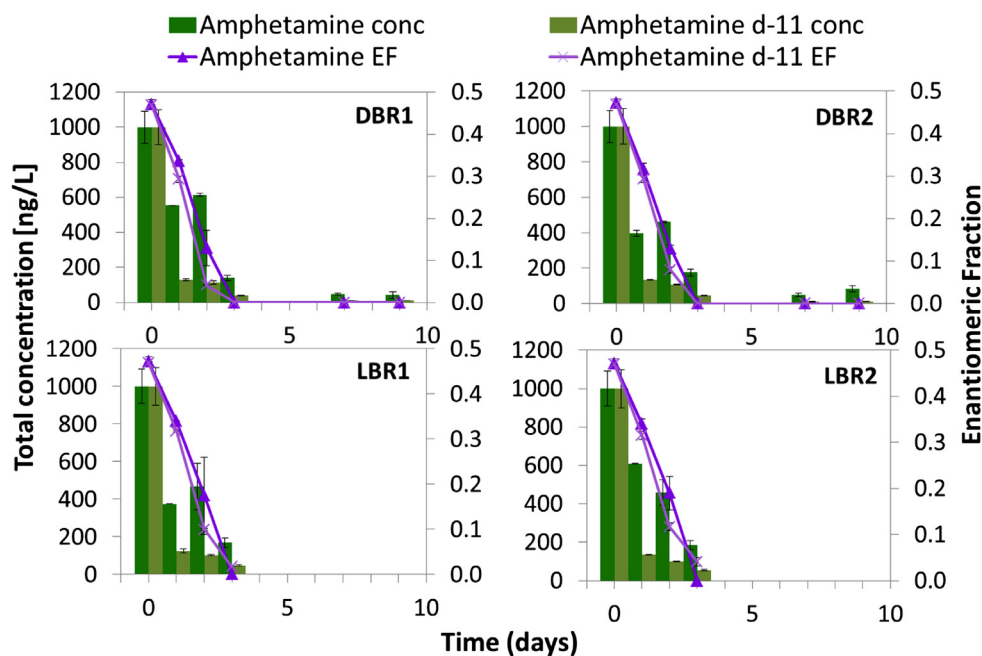


Fig. 5 – The stereoselective biodegradation of amphetamine in light and dark bioreactors (DBR- Dark Biotic Reactor, LBR- Light Biotic Reactor).

### 3.2. The stereoselective biodegradation of amphetamine and methamphetamine in river water microcosms

Following initial investigations, biodegradation as the predominant removal mechanism in the river water bioreactors was evident. Furthermore stereo-selectivity was witnessed for both amphetamine and methamphetamine. To confirm these findings, single compound bioreactors containing amphetamine or methamphetamine and their deuterated analogues were conducted.

#### 3.2.1. Amphetamine bioreactors

It was noticed that during the initial scoping study, much degradation of amphetamine occurred in the first five days. Therefore, increased sampling during the first week was

conducted. In fact, in the case of amphetamine (Fig. 5), >90% degradation was witnessed within the first three days, regardless of the light conditions. With additional sampling, it could also be seen that EF decreased from circa 0.5 to <0.02 within three days. The speed of the elimination of the S(+)-enantiomer could not be fully appreciated in the initial scoping exercise. Although amphetamine d-11 was also present, no breakdown products could be identified by chiral LC/QTOF-MS.

#### 3.2.2. Methamphetamine bioreactors

In the methamphetamine bioreactors (spiked with methamphetamine and its deuterated analogue, methamphetamine-d14) neither amphetamine nor amphetamine-d11 were added in the initial spike. This was confirmed by the lack of detection

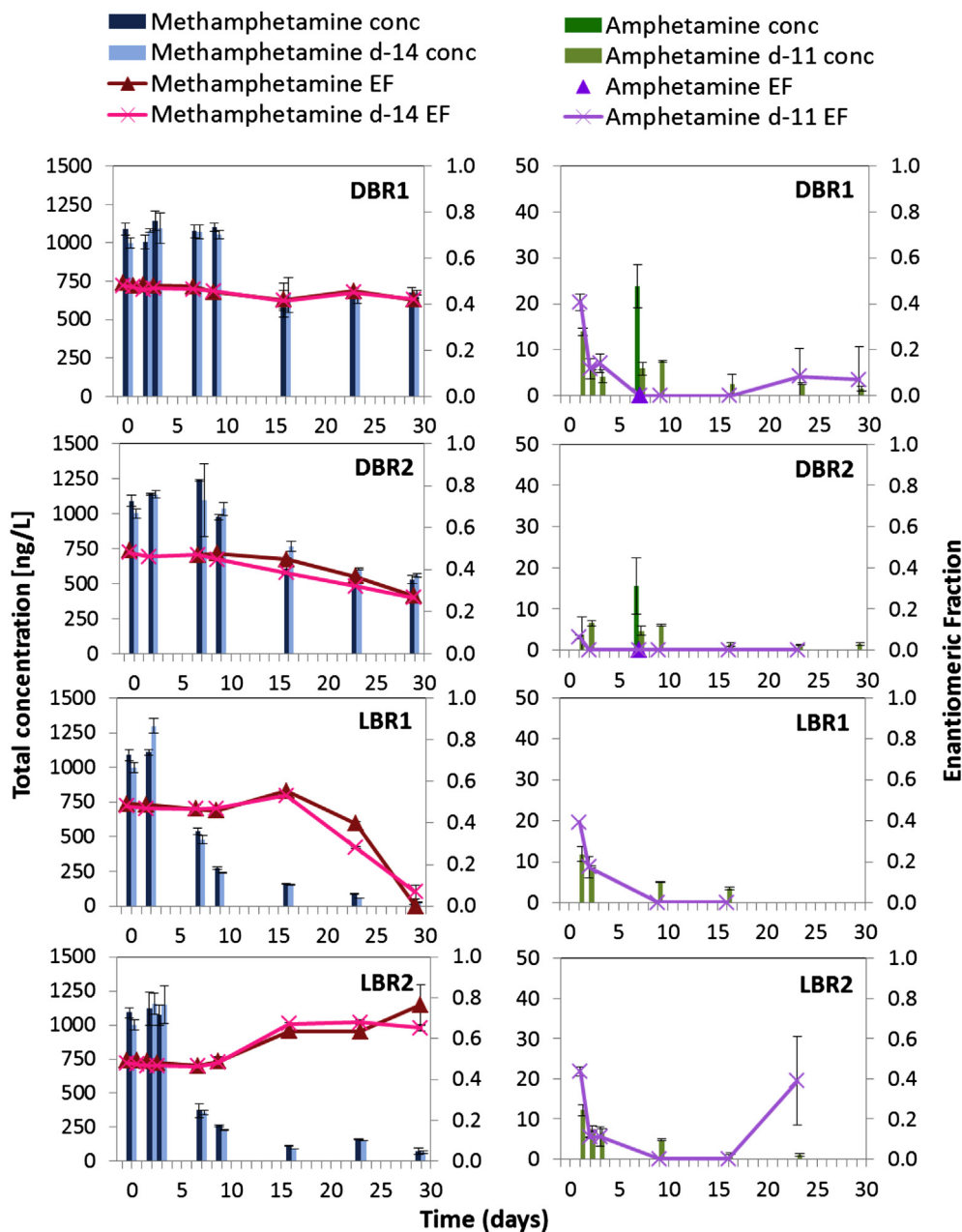


Fig. 6 – The stereoselective biodegradation of methamphetamine in light and dark bioreactors (DBR- Dark Biotic Reactor, LBR- Light Biotic Reactor).

on day zero of amphetamine or amphetamine-d11. However, after just one day, amphetamine-d11 was detected in both light and dark bioreactors (Fig. 6). The detection of amphetamine and amphetamine-d11 in methamphetamine microcosms is evidence that these compounds are breakdown products of methamphetamine and methamphetamine-d14 respectively. Further evidence lies in the enantiomeric fractions of the formed amphetamine. Fig. 6 shows that stereoselective biodegradation of methamphetamine, with preferential removal of S(+)-enantiomer led to the formation of amphetamine enriched with the S(+)-enantiomer.

In dark conditions, methamphetamine was more persistent, having decreased in total concentration from 1090 to 675 and 529 ng/L in each replicate bioreactor. Reduction in methamphetamine-d14 also occurred at a similar level; reduction in concentration was from 1000 to 675 and 560 ng/L in each replicate bioreactor. The dark methamphetamine bioreactors demonstrated reduction in concentration of methamphetamine and slight shifts in EF from racemic proportions to enrichment of the R(–)-enantiomer after twenty-nine days. With dark biotic reactors (DBR1), initial methamphetamine and methamphetamine-d14 EF were near racemic (0.48 and 0.49 respectively). This changed to 0.42 for both methamphetamine and methamphetamine-d14 after 29 days. However, in the light bioreactors, methamphetamine behaved differently and EF altered differently for each replicate despite similar reduction in total concentrations. After 29 days methamphetamine EF was 0.07 and 0.65 for light bioreactors 1 and 2 respectively indicating an enrichment with R(–)-methamphetamine (and formation of amphetamine enriched with S(+)-amphetamine) or with S(+)-methamphetamine (and formation of amphetamine enriched with R(–)-amphetamine) in LBR1 and LBR2 respectively. This unexpected outcome indicates the complexity of stereo-selective mechanisms under light conditions. Further experiments will need to be undertaken to test this phenomenon but it is evident that different microbial communities developed in two light bioreactors.

#### 4. Conclusions

The research outlined here revealed, for the first time, stereoselective degradation of amphetamines and formation of non-racemic by-products due to stereoselective biological processes occurring in river microcosms. Further experiments will be undertaken in the natural aqueous environment to confirm this phenomenon. Additional research will also be undertaken in order to verify if there is a possibility of enantioselective accumulation of the chiral drugs by the bioculture. Illicit drugs are present in the environment at low concentrations but due to their pseudo-persistence and non-racemic behaviour, with two enantiomers revealing significantly different potency in humans (and potentially different toxicity towards aquatic organisms), the risk posed by illicit drugs in the environment should not be under- or over-estimated. The above results demonstrate the need for re-evaluation of the procedures utilised in environmental risk assessment, which currently do not recognize the importance of the phenomenon of chirality in pharmacologically active compounds.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2013.06.057>.

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