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ORIGINAL PAPER

# Unexpected removal of the most neutral cationic pharmaceutical in river waters

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**Abstract** Contamination of surface waters by pharmaceuticals is now widespread. There are few data on their environmental behaviour, particularly for those which are cationic at typical surface water pH. As the external surfaces of bacterio-plankton cells are hydrophilic with a net negative charge, it was anticipated that bacterio-plankton in surface-waters would preferentially remove the most extensively-ionised cation at a given pH. To test this hypothesis, the persistence of four, widely-used, cationic pharmaceuticals, chloroquine, quinine, fluphenazine and levamisole, was assessed in batch microcosms, comprising water and bacterio-plankton, to which pharmaceuticals were added and incubated for 21 days. Results show that levamisole concentrations decreased by 19 % in microcosms containing bacterio-plankton, and by 13 % in a parallel microcosm containing tripeptide as a priming agent. In contrast to levamisole, concentrations of quinine, chloroquine and fluphenazine were unchanged over 21 days in microcosms containing bacterio-plankton. At the river-water pH, levamisole is 28 % cationic, while quinine is 91–98 % cationic, chloroquine 99 % cationic and fluphenazine 72–86 % cationic. Thus, the most neutral compound, levamisole, showed greatest removal, contradicting the expected bacterio-plankton preference for ionised molecules. However, levamisole was the most hydrophilic molecule, based on its octanol–water solubility coefficient ($K_{ow}$). Overall, the pattern of pharmaceutical behaviour within the incubations did not reflect the relative hydrophilicity of the pharmaceuticals predicted by the octanol–water distribution coefficient, $D_{ow}$, suggesting that improved predictive power, with respect to modelling bioaccumulation, may be needed to develop robust environmental risk assessments for cationic pharmaceuticals.

**Keywords** Levamisole · Quinine · Chloroquine · Fluphenazine · Persistence · River

## Introduction

There are over 4000 pharmaceuticals available for human and veterinary use (Boxall et al. 2012). Following administration, unretained pharmaceuticals and any transformation products are excreted into surface waters, either directly or via treated and untreated wastewaters and biosolids. As a consequence, contamination of aquatic systems by pharmaceuticals is now extensive (Hughes et al. 2013; Cizmas et al. 2015). There remains, however, a marked lack of knowledge on the aquatic transport and fate of pharmaceuticals, and their biological effects (Boxall et al. 2012; Brooks et al. 2012; Kümmerer 2008; LaLone et al. 2014; Shore et al. 2014; Tijani et al. 2016). Effects of pharmaceuticals on terrestrial organisms have been reported, with one of the most publicised examples being the contribution of diclofenac residues to the almost total decline of the *Gyps* spp. vulture populations on the Indian subcontinent (Oaks et al. 2004). While such dramatic effects on aquatic organisms have not been reported to date, behavioural changes have been observed. For example, environmentally measured concentrations of the benzodiazepines oxazepam and diazepam were shown to markedly alter the social behaviours of the wild European

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perch (*Perca fluviatilis*) and adult zebrafish (*Danio rerio*), respectively (Brodin et al. 2013; Giacomini et al. 2016).

The prediction of adverse outcome pathways (OECD 2013) for a pharmaceutical contaminant is, *inter alia*, dependent on understanding its mode of action (MoA) giving rise to one or more molecular-initiating events within an organism, either through specific ligand-receptor interactions or through non-specific receptor interactions, which then leads to one or more adverse effects. Understanding the MoA in theory allows effects predictions to be extrapolated to other compounds with similar molecular characteristics, along with the ability to 'read across' to organisms from different taxonomic groups that share the same receptors (Hutchinson et al. 2013). Other important properties relevant to the manifestation of adverse effects include the persistence of, and hence exposure to, the pharmaceutical and the extent of accumulation within susceptible biota (Arnold et al. 2014).

For a given environmental loading, the degree of exposure of an organism to a pharmaceutical in surface waters is dependent on the extent of biological and physico-chemical mechanisms on in situ, measured concentrations of the parent pharmaceutical and any known transformation products. For example, in an assessment of the ability of riverine bacterio-plankton to transform the benzodiazepine, diazepam and its photo-degradation product, 2-amino-5-chlorobenzophenone (ACB), diazepam was much more persistent than ACB, which was mineralised to ammonium by the bacterio-plankton in all experiments (Tappin et al. 2014). This indicated that a removal of diazepam, and other benzodiazepines photo-degrading to ACB (West and Rowland 2012), could be effected *even* if the parent compound was not readily biotransformed. However, there is a lack of published studies on the role of microbes in the environmental cycling of pharmaceuticals, coupled with little discussion on biotic mechanisms of pharmaceutical removal.

In this study, the persistence of a range of commonly prescribed human and veterinary pharmaceuticals in incubations containing riverine bacterio-plankton was examined. Chloroquine and quinine are quinoline derivatives used extensively in Asia, Africa and South America for the treatment of malaria, although they also have a range of other medical applications (Achan et al. 2011; Price et al. 2014); the MoA of their antimalarial properties is not well understood (www.drugbank.ca). Chloroquine is excreted from humans either as unaltered or as N-desethyl chloroquine, and quinine unaltered or as 3-hydroxyquinine (Mirghani et al. 2003; Projean et al. 2003). Fluphenazine is a trifluorinated methyl phenothiazine derivative used to manage chronic psychoses including schizophrenia (Uchida et al. 2011); it is excreted in urine and faeces unchanged, or as 7-hydroxyfluphenazine, fluphenazine sulfoxide and metabolite conjugates (McEvoy et al. 2004). Thus, these compounds will enter surface waters via wastewater discharge and irrigation, and leaching from sewage solids applied to land. Concerns regarding the presence of neuroendocrine-active chemicals in surface waters, and their potential biological effects, have been raised, particularly those pharmaceuticals, including fluphenazine, that act on widely conserved dopamine receptors (Villeneuve et al. 2010a, b). Levamisole is an imidazothiazole derivative used as a human and veterinary anthelmintic (Lanusse et al. 2009). It is also used as a human immuno-modulator and as an adulterant in cocaine (Bertol et al. 2011). For veterinary use, the compound is generally applied topically, thus enhancing its transport to surface waters.

There are no published data on the transformations of these pharmaceuticals in surface waters, although it has been predicted that lower than 1 % of the quinine load entering a sewage treatment plant would be transformed (Jones et al. 2002). Furthermore, there are few data on their toxicity in the aquatic environment. Bioassays using the invertebrate *Daphnia magna* and alga *Chlorella vulgaris* suggested that chloroquine may be harmful to aquatic organisms (Zurita et al. 2005), while fluphenazine showed inhibitory effects on a range of bacterial species at concentrations of ca. 40–200 μmol $L^{-1}$ (Dastidar et al. 1995). Levamisole has been cited as a high-priority compound for detailed environmental risk assessment (Capleton et al. 2006; von der Ohe et al. 2011). These pharmaceuticals are all included in the World Health Organization's List of Essential Medicines (WHO 2015). Furthermore, they are ionisable molecules that can be protonated at surface water pH, and there is a paucity of data on the fate of pH-dependent cationic pharmaceuticals in these environments.

The potential problem of environmentally persistent pharmaceutical pollutants was recently recognised by the International Conference on Chemicals Management (ICCM 4, autumn 2015) as an Emerging Policy Issue of the Strategic Approach to International Chemicals Management (http://www.saicm.org/). Furthermore, three pharmaceuticals were included on the European Union (EU) watch list of March 2015 (EU 2015), with a view to proposing statutory Environmental Quality Standards within the EU Water Framework Directive, while the latest United States Environmental Protection Agency draft Contaminant Candidate List (CCL4) includes eight pharmaceuticals and two pharmaceutical precursors (https://www.epa.gov/ccl/chemical-contaminants-ccl-4), including quinoline. These regulatory concerns reflect the tension between the need for medicines to maintain and improve health and managing their potential environmental impact.

## Materials and methods

The full details of the design, testing and validation of the incubation procedure have been reported previously (Tappin et al. 2012, 2014).

### Media and chemicals

River water required for the microcosms and preparation of bacterio-plankton inocula were collected from Gunnislake, at the tidal limit of the River Tamar located in south-west England, UK. The river drains a rural, agriculture-dominated catchment, and water quality is generally good (Tappin et al. 2012, 2014), although the river and its catchment are impacted by historical metal mining activity. River turbidity is low (generally lower than 15 mg $L^{-1}$) and the oxygen saturation is high (generally >90 %) at the tidal limit, while the mean pH is 7.4 (Environment Agency, unpublished data). The pharmaceuticals used in the study were purchased as levamisole hydrochloride, quinine hemisulphate monohydrate, chloroquine diphosphate and fluphenazine hydrochloride (Sigma-Aldrich, AR grade). A tripeptide comprising the amino acids glycine, leucine and tyrosine (GLY; Sigma-Aldrich, ≥98 % purity), and representing labile organic matter, was also used to assess a priming effect on the bacterio-plankton population (Guenet et al. 2010) in the incubations involving levamisole only. Stock solutions of these compounds were made up in prepared incubation water and stored at 4 °C in the dark prior to use. Structural details of the compounds are given in Table 1.

### Microcosm experiments

*Preparation of incubation water*

River water was filtered (GF/F; 0.7 μm nominal pore diameter) to remove suspended particles, passed through a strong anion-exchange resin (Dowex® X-100, 200 mesh; water flow rate 80 mL $h^{-1}$) to remove nitrate, UV-irradiated (400-W medium-pressure Hg lamp, 6 h) to remove dissolved organic carbon (DOC) and then re-filtered through a 0.2-μm filter membrane (Whatman Anodisc 47, aluminium oxide) to remove any remaining particulate matter. These processes reduced nitrate and DOC to concentrations which ensured that alternative carbon and nitrogen substrates were, as far as possible, removed and that the river water matrix was compatible with direct analysis of samples by electrospray ionisation–mass spectrometry (ESI–MS). As a final step, the water was sterilised by autoclaving (115 °C, 15 min).

*Preparation of the inoculum*

The bacterial inoculum was prepared using river water collected within 24 h prior to introduction into the microcosm. This water was filtered through a 1.6-μm-pore-diameter membrane (combusted GF/A) to remove zooplankton and particle-bound bacterio-plankton (Ainsworth and Goulder 1998) and then re-filtered through a 0.2-μm-pore-diameter membrane filter (Whatman Anodisc 47). The bacterio-plankton retained on the membrane was resuspended in a small volume of the 0.2 μm filtered water to provide the inoculum, which was then added to the pre-treated river water to produce a final, representative bacterio-plankton concentration.

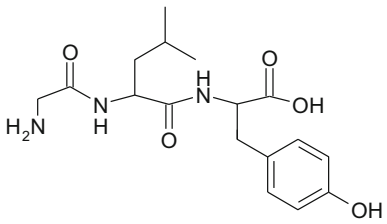
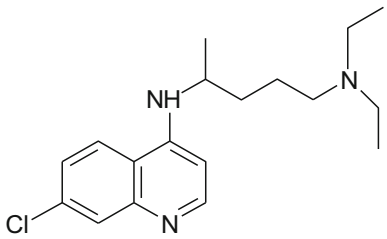
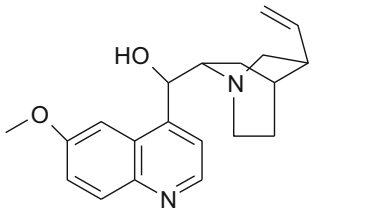
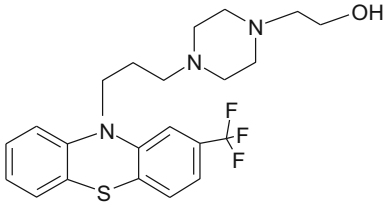
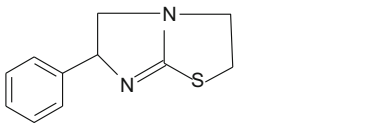
*Incubation experiments*

Incubation water (60 mL) was transferred to a 125-mL screw-capped amber glass bottle to which was added 30–60 μL of stock pharmaceutical solution and 1 mL of the bacterio-plankton inoculum. Starting concentrations of the compounds were ca. 15–30 μmol $L^{-1}$. Control microcosms of prepared river water containing bacterio-plankton inoculum only and the pharmaceuticals only were also set up to account for sorption effects and to investigate the effects of the pharmaceuticals on the bacterio-plankton communities. In a separate microcosm, 30 μmol $L^{-1}$ of GLY was added with levamisole. The 125-mL bottles were loosely capped, placed in a re-sealable plastic bag and transferred to an orbital shaker. Microcosms were prepared in duplicate at ambient temperature in the dark. An incubation time of 21 days was selected to reflect the river water transit time in the Tamar catchment. At day 0 and day 21, incubated samples were filtered using combusted GF/F filters and sub-samples collected for analyses.

### Chemical and microbiological analyses

The pharmaceuticals were analysed by ESI–MS using a Finnegan MAT LCQ MS, a quadrupole ion trap mass spectrometer with an external source atmospheric pressure interface capable of electro-spray ionisation. Solutions were introduced into the instrument by direct injection from a 250-μL Hamilton syringe at a rate of 3 μL $min^{-1}$. Prior to injection, each sample and standard was diluted 1:1 with methanol amended with 0.1 % (v/v) formic acid. The signal sensitivity for each pharmaceutical, in positive-ion mode, was optimised by adjustment of instrumental parameters using inbuilt tuning procedures. Ion counts were integrated for 2 min, and 5 × 2 min replicates were recorded per sample. Ion count stability was followed in real time via single ion monitoring. Quantitation of the analyte was achieved by generating an external standard

**Table 1** Structural information on the compounds studied

| Compound[a] | IUPAC name[a] | CAS no. | Structure[a] |
|---|---|---|---|
| Peptide ($C_{17}H_{27}O_5N_3$) | Glycyl-L-leucyl-L-tyrosine | 4306-24-5 |  |
| Chloroquine diphosphate ($C_{18}H_{26}ClN_3$) | *N*′-(7-chloroquinolin-4-yl)-*N*,*N*-diethyl-pentane-1,4-diamine | 50-63-5 |  |
| Quinine hemisulphate monohydrate ($C_{20}H_{24}N_2O_2$)[a] | (*R*)-(6-methoxyquinolin-4-yl)((2*S*,4*S*,8*R*)-8-vinylquinuclidin-2-yl)methanol | 303137-00-0 |  |
| Fluphenazine hydrochloride ($C_{22}H_{26}F_3N_3OS$) | 2-[4-[3-[2-(trifluoromethyl)-10*H*-phenothiazine-10-yl]propyl]-piperazine-1-yl]ethanol | 69-23-8 |  |
| Levamisole hydrochloride ($C_{11}H_{12}N_2S$)[a] | (6S)-6-phenyl-2,3,5,6-tetrahydroimidazo[2,1-b][1,3]thiazole | 16595-80-5 |  |

[a] Main molecule only

calibration curve on each analytical day and bracketing individual samples with a drift calibration standard to account for variations in instrumental sensitivity. Changes in sensitivity were accounted for using an algorithm developed for automated nutrient analysis (Skalar Analytical BV 2004); in this implementation, baseline drift was assumed unimportant (Eq. 1).

$$U''_{(n)} = \left(U'_{(n)} * (D1/((((D(b)-D(a))/(b-a)) * (n-a)) + D(a)))\right) \quad (1)$$

$U''_{(n)}$ = drift-corrected ion count for sample n, $U'_{(n)}$ = ion count for sample n, $D1$ = ion count of the first drift standard, $D(a)$ = ion count of the preceding drift standard, $D(b)$ = ion count of the following drift standard, $n$ = sample number, $a$ = sample number of preceding drift standard and $b$ = sample number of following drift standard. Standards were matrix-matched with the water used for the incubations. The identification of *m*/*z* values for potential biotransformation products was informed by the EAWAG Biocatalysis and Biodegradation Database (EAWAG-BDD; Ellis and Wackett 2012), now redesigned as enviPath (Wicker et al. 2016). Nitrate + nitrite and orthophosphate were determined by colorimetry with a precision of lower than 1 % (Skalar Analytical BV 2004) and ammonium by *o*-phthaldialdehyde fluorescence with a precision of 4 % (Holmes et al. 1999).

Viable counts of bacterio-plankton were determined using 100 µL aliquots from the incubations. These were diluted in phosphate-buffered saline solution and 100 µL of

each dilution spread on half strength Luria–Bertani agar (Merck, Germany) and incubated at 30 °C for two days. Colonies were enumerated as colony-forming units (cfu) $mL^{-1}$ of the original suspensions. The extraction of DNA and PCR amplification of 16S ribosomal RNA genes for bacterio-plankton community analysis was undertaken on cells retained on a 0.2-μm membrane filter. The retained cells were disrupted in a Hybaid Ribolyser™ Cell Disruptor, and the DNA was subsequently extracted into hexadecyltrimethylammonium bromide and phenol–chloroform–isoamyl alcohol, followed by resuspension in 50 μL nuclease-free water (Griffiths et al. 2000). Nested PCR amplifications were performed on these samples using Super Taq DNA polymerase (HT Biotech Ltd, UK) and G-Storm Thermal Cyclers (GRI, UK). DNA amplification was undertaken in a 50 μL sample using 1 μmol $L^{-1}$ of the universal primers for eubacterial 16S rRNA genes ($27_f$ and $1492_r$) (Lane 1991) with 1 unit Super Taq DNA polymerase. The amplified DNA fragments were re-amplified using forward primer 341 and reverse primer 907 (Muyzer et al. 1995). Denaturing gradient gel electrophoresis (DGGE) analysis (Muyzer et al. 1993) was performed on GC-clamped products of the second PCR amplification using the Bio-Rad Dcode system to separate DNA on a 8 % polyacrylamide gel in Tris acetate EDTA buffer (pH 8.0) with a 20–60 % denaturant gradient, in which 100 % denaturant was 7 molar urea amended with 40 % formamide (Muyzer et al. 1993). Electrophoresis was performed at 60 °C, run at 60 V (16 h), and the DNA banding visualised using Sybr Green I stain (Lonza Rockland, USA) with detection and image capture on a Storm 860 Molecular Imager (Molecular Dynamics, UK).

## Results and discussion

### Bacterio-plankton incubations

The abiotic control studies showed that none of the compounds were lost through sorption to the walls of the incubation bottles or through chemical degradation, during the experiments. The bacterio-plankton inocula were viable throughout the incubation period, as concentrations of colony-forming units increased by an order of magnitude in the microcosms containing pharmaceuticals (from lower than 10,000 cfu $mL^{-1}$ at day 0 to 82,000 cfu $mL^{-1}$ for levamisole and 220,000 cfu $mL^{-1}$ for fluphenazine at day 21). There was also a large increase in ammonium concentrations (from 0.7 to 68.0 μmol $NH_4^+$-N $L^{-1}$) after 21 days in the levamisole microcosms to which the GLY peptide had been added (Fig. 1), a further indication of bacterio-plankton mineralisation of dissolved organic matter. The $NH_4^+$ produced represented ca. 74 % of the peptide nitrogen, and its degradation was consistent with previous results for such incubations containing GLY only or GLY plus pharmaceutical (Tappin et al. 2012, 2014). In addition, our previous results, using clone libraries, showed that bacterio-plankton sampled from the Tamar water microcosms contained Gram-negative (α-proteobacteria, β-proteobacteria) and Gram-positive (Firmicutes) groups previously reported for freshwaters, which are capable of biotransforming xenobiotic contaminants (Tappin et al. 2014).

### Persistence of pharmaceuticals

Between days 0 and 21, the concentration of levamisole decreased from 33.2 to 26.9 μmol $L^{-1}$ (19 % removal) in the biotic incubation (Fig. 1; *t* test, $p < 0.001$, $n = 10$) and to 28.6 μmol $L^{-1}$ (13 % removal) in the biotic incubation with added peptide (Fig. 1a; *t* test, $p = 0.002$, $n = 10$). While solid-solution partition coefficients ($K_d$) are not available for levamisole, $K_d$s for ionisable pharmaceuticals are generally relatively low. The measured decreases in dissolved levamisole concentrations were almost certainly due to uptake into the bacterio-plankton, rather than simple sorption to cell surface components as the suspended solid concentration was lower than 1 mg $L^{-1}$ in the incubations. A DGGE fingerprinting of the amplified 16S rRNA marker genes of the bacterio-plankton communities incubated with and without levamisole showed that species composition (genotype) profiles were similar under all incubation conditions. In contrast to levamisole, the concentrations of quinine, chloroquine and fluphenazine remained constant in the microcosms over the 21-day period (Fig. 2), indicating that these pharmaceuticals were persistent.

Passive uptake of low molecular mass (lower than 500 Daltons) compounds into prokaryotic Gram-negative and -positive bacterio-plankton is more likely to occur with hydrophilic moieties, as the outer cell walls of these bacterio-planktons are hydrophilic (Hancock and Bell 1988; Nikaido and Vaara 1985). They also have a net negative charge which, in principle, should favour the electrostatic sorption of positively charged compounds. The octanol–water solubility coefficients (log $K_{ow}$) of the pharmaceuticals in this study indicate that levamisole was the most hydrophilic (Table 2). Their acid dissociation constants ($pK_a$), on the other hand, indicate that fluphenazine, chloroquine and quinine would be extensively positively ionised at pH 7.4, the mean pH of Tamar river water (Table 2), potentially promoting sorption to the cells by electrostatic attraction, while increasing their inherent water solubility (Gulde et al. 2014; Rendal et al. 2011a). These physico-chemical characteristics can be combined to yield a net hydrophilicity, the octanol–water distribution coefficient (log) $D_{ow}$, which may better explain any

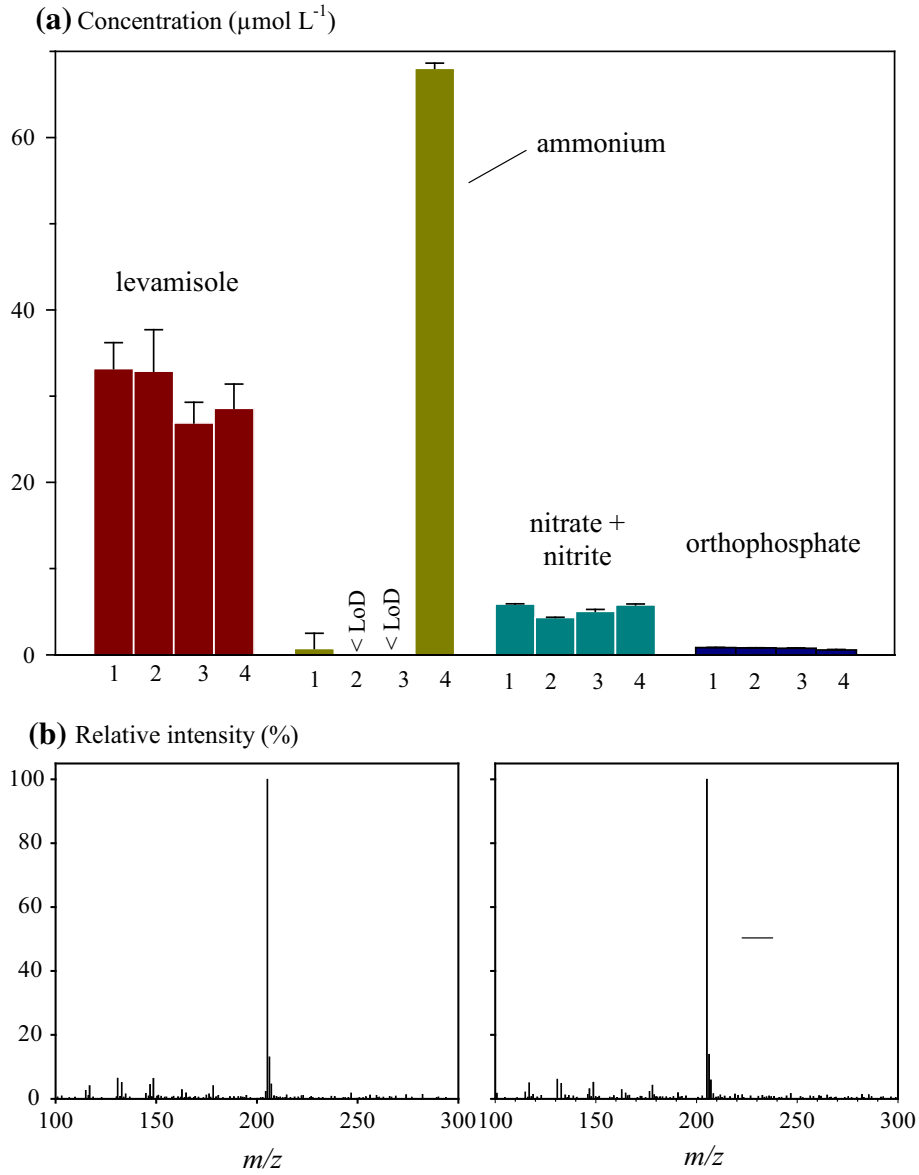



**Fig. 1** **a** Concentrations of levamisole, ammonium, nitrate + nitrite and orthophosphate in each microcosm at days 0 and 21. 1, $t$ = day 0; 2, $t$ = day 21 abiotic control; 3, $t$ = day 21 bacterio-plankton + levamisole; 4, $t$ = day 21 bacterio-plankton + levamisole + labile organic matter. *Error bars* represent ± 1σ of the results from duplicate incubations with each sample analysed 3–5 times ($n$ = 6–10). <LoD, lower than limit of detection. Concentrations of levamisole were reduced by 19 and 13 % in incubations 3 and 4, respectively. **b** Ion chromatograms (relative abundance of ion (%) vs. $m/z$) of levamisole in solution in a standard (*left panel*) and at day 21 (*right panel*). The *horizontal bar* shows the range in $m/z$ values of possible transformation products predicted by the EAWAG Biocatalysis and Biodegradation Database

observed loss of pharmaceutical from solution (Manallack et al. 2013; Warhurst et al. 2003). For a monoprotic base (e.g. levamisole), the net hydrophilicity can be calculated from Eq. 2:

$$\log D_{ow} = \log K_{ow} - \log\left[1 + 10^{(pK_a - pH)}\right] \quad (2)$$

For a diprotic, basic pharmaceutical (e.g. quinine, chloroquine and fluphenazine), Eq. 3 is used, where $pK_{a1} > pK_{a2}$:

$$\log D_{ow} = \log K_{ow} - \log\left[1 + 10^{(pK_{a1} - pH)} + 10^{(pK_{a1} + pK_{a2} - 2pH)}\right] \quad (3)$$

The calculated log $D_{ow}$ values at pH 7.4 are given in Table 2. In principle, the smaller the value, the more hydrophilic the compound and thus the easier for uptake into the cells of the bacterio-plankton present in the current study. The results of these calculations suggest that chloroquine is the most hydrophilic pharmaceutical, fluphenazine the least and levamisole and quinine intermediate, but this did not correspond with the measured loss of pharmaceutical from solution in this study. Similarly, poor correlations between estimated log $D_{ow}$ and the biotransformation of the basic pharmaceuticals atenolol, ifenprodil and propranolol in batch incubations with river water have been reported elsewhere (Yamamoto et al. 2009). The lack of predictive power using $D_{ow}$ may be attributed to poorly constrained log $K_{ow}$ and/or $pK_a$ values, particularly for basic pharmaceuticals (ECETOC 2013; Kah and Brown 2008; Manallack et al. 2013). Additionally, it may contribute to our lack of insight into the importance of

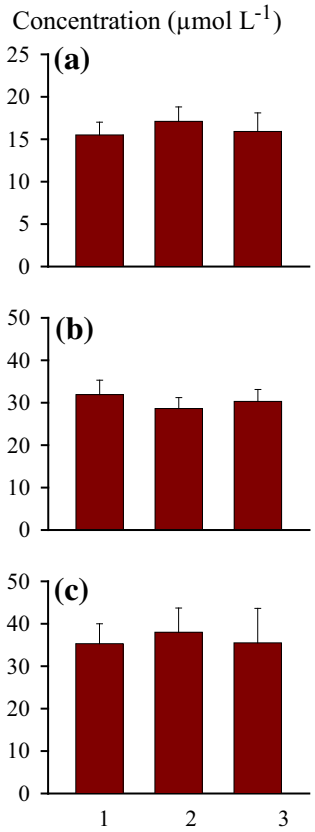



**Fig. 2** Concentrations of **a** fluphenazine, **b** quinine and **c** chloroquine in each microcosm at days 0 and 21. 1, $t$ = day 0; 2, $t$ = day 21 abiotic control; 3, $t$ = day 21 bacterio-plankton plus pharmaceutical. *Error bars* represent $\pm$ 1$\sigma$ of the results from duplicate incubations with each sample analysed five times ($n$ = 10). Concentrations of the pharmaceuticals were not significantly different after 21 days under the different incubation conditions

hydrophobic partitioning of ionisable pharmaceuticals relative to possible mechanisms of hydrophilic interaction (ECETOC 2013; Manallack et al. 2013). Whatever the explanation, an important observation to be drawn from the current study is that it was the most hydrophilic pharmaceutical, based on log $K_{ow}$ alone, and least ionised, based on p$K_a$ alone, that was removed from river water containing bacterio-plankton.

Studies of pharmaceutical uptake by eukaryotes (animals and plants) have reported that it is the non-ionised forms of the pharmaceutical that shows greatest partitioning into cells (Rendal et al. 2011b; Warhurst et al. 2003) while recently, the extent of bacterial transformation of pharmaceuticals incubated with sewage sludge was shown to be generally proportional to the fraction of unionised, neutral species present (Gulde et al. 2014). It may be concluded, therefore, that of the total pharmaceutical pool present, it is the non-specific biotransformation of the neutral or weakly ionised pharmaceutical fraction that is most likely to occur in surface waters, irrespective of cell type.

The loss of levamisole did not result in identifiable biotransformation products detected by ESI–MS (Fig. 1), even though these were predicted from the EAWAG-BBD, via initial de-alkylation of the tertiary amine to yield secondary and primary amines. Indeed, the dominance of the observed de-alkylation of pharmaceuticals containing a tertiary amine has been reported recently (Gulde et al. 2016). The $m/z$ values of the predicted transformation products are marked in Fig. 1 and did not correspond with detected peaks. This suggests that, if biotransformation molecules were produced, they were not released into solution but were further metabolised rapidly or were present at concentrations below the limit of detection under these conditions.

The three pharmaceuticals that were persistent all contain at least one tertiary amine, while chloroquine also contains a secondary amine group (Table 1). As noted above, the EAWAG-BBD predicts bacterio-plankton alteration of these functional groups via de-alkylation (Gulde et al. 2016). In contrast, the mono-N-substituted heterocyclic ring in the quinoline derivatives quinine and chloroquine is more difficult to decompose (Alexander 1999), though heterocyclic ring opening of quinoline by

**Table 2** Acid dissociation constant (p$K_a$)[a], octanol–water solubility coefficient (log $K_{ow}$)[a] and octanol–water distribution coefficient (log $D_{ow}$) data for chloroquine, quinine, levamisole and fluphenazine

| | p$K_{a1}$ | p$K_{a2}$ | log $K_{ow}$ | % Ionised[b] (at pH 7.4[c]) | log $D^{d}_{ow}$ (at pH 7.4[c]) |
|---|---|---|---|---|---|
| Chloroquine | 9.94–10.87 | 7.44–8.50 | 3.93–5.28 | >99 | −0.67 to 2.42 |
| Quinine | 8.4–9.05 | 4.13–4.32 | 2.51–3.44 | 91–98 | 0.85 to 2.40 |
| Levamisole | 6.98 | | 1.84–2.36 | 28 | 1.70 to 2.22 |
| Fluphenazine | 7.84–8.21 | 3.98 | 3.97–4.4 | 72–86 | 3.10 to 3.83 |

Calculated extent of pharmaceutical ionisation is also shown

[a] These data include both measured and predicted values and were obtained from www.drugbank.ca and references/links given within

[b] Calculated from: $\frac{1}{1+10^{-1(\mathrm{pH}-\mathrm{p}K_{a1})}}$ (ECETOC 2013)

[c] Environment Agency of England, unpublished data

[d] Calculated from Eqs. 2 and 3 in the text

bacteria has been reported (Griese et al. 2006). The EAWAG-BDD does not take into account the polarity of the molecule under consideration, however, and the persistence of these pharmaceuticals is thus probably attributable to their extensive (positive) ionisation under the incubation conditions and lack of cell uptake; the presumed electrostatic attraction appeared to have no measurable influence (Gulde et al. 2014; Rendal et al. 2011a, b). While quantitative estimates of the relative speeds of cell uptake by ionised and non-ionised basic pharmaceuticals are lacking, equivalent studies on acidic moieties show uptake speeds $10^3$–$10^4$ lower for the ionic forms relative to the neutral species (Rendal et al. 2011b).

## Investigation of a priming effect

The addition of labile organic matter (LOM) to accelerate bacterio-plankton biotransformation of more persistent organic matter is known as the priming effect, although there are few systematic studies of this phenomenon in aquatic systems (Gontikaki et al. 2013; Guenet et al. 2014). The presence of LOM, as wastewater effluent DOC added at relatively high concentrations, has been proposed to enhance pharmaceutical loss from river and estuarine coastal waters (Benotti and Brownawell 2009; Lim et al. 2008), but reports of decreased biotransformation of the target molecule, or no change, have also been reported and a range of mechanisms has been invoked to explain these varied outcomes (Bengtsson et al. 2014). In the current work, levamisole was incubated with the peptide as LOM equivalent to ca. 500 μmol C $L^{-1}$ and ca. 90 μmol N $L^{-1}$, concentrations which are typical of UK rural and semi-rural rivers, and of the same magnitude as the concentrations of DOC reported to enhance pharmaceutical loss in surface waters (Benotti and Brownawell 2009; Lim et al. 2008). While levamisole concentrations decreased in the presence of bacterio-plankton and LOM, the loss was not significantly different to that observed in parallel incubations without added LOM (*t* test, $p = 0.142$, $n = 11$). This outcome indicates that the biotransformation of levamisole occurs in surface waters, but that there would not necessarily be a priming effect, constraining transformation of the molecule to lower than 20 % of the total load.

## Environmental implications

The data from this study suggest that all of the molecules examined would be stable, to varying degrees, in surface waters, at least with respect to biotransformation by riverine bacterio-plankton. This finding, coupled to their probable continual loading to the environment, would suggest they may be persistent or, in the case of levamisole, pseudo-persistent in surface waters. While it is difficult to contextualise this outcome in relation to potential environmental impacts on aquatic organisms due to the absence of data on other ameliorating processes (e.g. photo-degradation or sorption to sediments), it may be hypothesised that, individually, these compounds will not inflict acute toxic effects at the concentrations anticipated. This assessment is supported by the viability of the bacterio-plankton communities shown in this work, coupled to the lack of community succession throughout the incubations for all the pharmaceuticals (indicated by the consistency of the DGGE fingerprint patterns across the experiments). Nevertheless, environmental implications arising from continual and chronic exposure of aquatic organisms to these pharmaceuticals, either as single entities or as mixtures, remain to be addressed.

Malaria is endemic in the low- and low–middle-income countries (LLMIC) of Asia, Africa and central and South America (http://www.cdc.gov/malaria/about/distribution.html), and it is in these regions that use of chloroquine and quinine to combat malaria is likely to be highest and contamination of wastewater with these pharmaceuticals most frequent. Many LLMIC make extensive use of wastewater for the irrigation of agricultural and horticultural crops, particularly in southern Asia and western South America (Lautze et al. 2014; Lees et al. 2016). The implication is that these quinolones will be continually added to surface waters, with possibly unforseen environmental consequences given their poorly understood modes of action.

Dopamine receptors occur in almost all vertebrate and invertebrate animals (Barron et al. 2010), while some bacteria and many plant species synthesise dopamine. Unlike in animals, the function(s) of, and receptor(s) for, dopamine in plants has yet to be elucidated (Kulma and Szopa 2007). The presence in surface waters of dopamine inhibitors like fluphenazine and other frequently prescribed behaviour-altering psychiatric and antidepressant pharmaceuticals is a cause for concern. It has been reported that the antipsychotic pharmaceutical haloperidol, a dopamine inhibitor, may affect the behaviour of some fish species (Villeneuve et al. 2010a, b), while benzodiazepines also elucidate behavioural changes in fish (Brodin et al. 2013; Giacomini et al. 2016). If these groups of neurotransmitters are persistent in the aquatic environment, then unanticipated and undesired impacts may emerge (Brodin et al. 2014).

The inability to accurately model the bioaccumulation of ionisable pharmaceuticals using established physico-chemical metrics, including the compounds examined in this study but also more widely, will prevent the development of a robust and comprehensive risk assessment of pharmaceutical fate and impact in aquatic systems. Given the projected increases in pharmaceutical use across the

globe (IMS 2015), it would appear prudent to significantly improve our predictive capability with respect to bioaccumulation.

## Conclusions

This study found that microbially mediated pharmaceutical loss from river water containing bacterio-plankton occurred, to an extent, but only for a pharmaceutical expected to be largely unionised under the conditions of the experiment. No losses occurred, under similar conditions, for the three pharmaceuticals expected to be ionised to a much greater degree. This observation is consistent with the paradigm that, in the absence of specific uptake mechanisms, small non-polar compounds are more easily sorbed by living cells, compared with small polar moieties. The contrasting net hydrophilic values calculated for the pharmaceuticals indicated that the explanation is less straightforward, although some of the data underpinning the calculations may not be well constrained. Thus, an improved predictive bacterio-plankton cell uptake capability for ionisable cationic pharmaceuticals in aquatic systems would appear to be imperative.

Although the study of pharmaceuticals in surface waters of high-income countries is now in its fourth decade, there remains little systematic understanding of pharmaceutical transport, fate and impact. This is all the more concerning for lower-income countries, where pharmaceutical use is forecast to increase significantly in the foreseeable future, with attendant increased losses of pharmaceuticals to the environment, and losses in many cases fuelled by the aspiration for the increased use of wastewater for the irrigation of agricultural and horticultural crops. This will only enhance the widespread diffuse contamination of aquatic systems by pharmaceuticals, with potential unforeseen consequences.

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