Do pharmaceuticals affect freshwater invertebrates?
A study with the cnidarian *Hydra vulgaris*

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Abstract

Pharmaceuticals enter natural waters through sewage effluent and landfill leachates and present an unknown risk to aquatic species including freshwater invertebrates. In this study the acute and chronic toxicity of 10 drugs, commonly prescribed in the UK i.e. ibuprofen, paracetamol, acetylsalicylic acid, amoxicillin, bendroflumethiazide, furosemide, atenolol, diazepam, digoxin, amlodipine were assessed using the cnidarian *Hydra vulgaris*. In a 7 day exposure period there were no effects on survival at concentrations up to 1.0 mg l\(^{-1}\) and after 17 days neither feeding nor bud formation were adversely affected. However the ability of dissected polyps to regenerate a hypostome, tentacles and foot was inhibited by diazepam, digoxin and amlodipine at 10 µg l\(^{-1}\). It is suggested that other drugs targeted at mammalian receptor systems may also affect aquatic invertebrates although it is unlikely, at their low environmental concentrations, that those examined in this study actually present a risk.

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1. Introduction

Pharmaceuticals are designed to interact with biological systems in order to bring about beneficial effects principally in man but also in domestic and farm animals. In the UK there are currently 19,835 Marketing Authorisations for medicinal products covering 5,091 active ingredients (Medicines Control Agency, pers. commun.). These target about 500 distinct biochemical receptors (Daughton and Ternes, 1999) and are available as either over the counter or prescription only medicines. Although they have been designed largely for specific effects in man, drugs could potentially bring about changes in other animals which may be exposed. Environmental problems may arise when excreted drugs (and those disposed of unused) enter water as the parent compound or a metabolite, via sewage effluent from domestic dwellings and hospitals or from landfill leachates. There are increasing demands from both the scientific communities and the popular press (Raloff, 1998; Pearce, 1999) for information on the potential toxicity of pharmaceuticals in water, yet there is an almost complete lack of data concerning their effects on aquatic fauna, particularly the invertebrates which play a major role in the structure and functioning of freshwater ecosystems. Hence it is important that we understand the extent to which invertebrates may be affected by the presence of active pharmaceutical agents. New drugs are added annually to the British National Formulary (BMA and RPS, 2001) but for most there are no available data on environmental fate, transformation or effects and there is still no approved environmental risk assessment (ERA) procedure for pharmaceuticals used in human therapy in Europe although several draft documents have been discussed. To determine a realistic
ERA, chronic toxicity data for key species are ideally required (Girling et al., 2000). It is this information which is currently lacking and which can only be obtained through chronic, sub-lethal toxicity studies.

Many pharmaceuticals have already been detected (generally at ng l⁻¹ or low μg l⁻¹ concentrations) in sewage effluent and other environmental samples including surface, ground and even drinking water (Halling-Sørensen et al., 1998; Ternes, 1998). The available toxicity data for pharmaceuticals, much of which generally relate to acute lethal responses determined in the water flea *Daphnia* sp. (Lilius et al., 1995; Henschel et al., 1997; Wollenberger et al., 2000) indicate that high, environmentally unrealistic concentrations are needed to cause acute toxicity. However, to date, the chronic, sub-lethal effects of only ethinylestradiol (EE2) on aquatic species have been investigated. EE2, which is prescribed as a female contraceptive, has been identified as a potent endocrine disrupter which causes an increase in plasma vitellogenin in fish in the field (Purdom et al., 1994) and inhibits testicular growth in laboratory fish maintained at concentrations as low as 2 ng l⁻¹ (Jobling et al., 1996). We have recently demonstrated (Watts et al., 2002) that concentrations as low as 100 ng l⁻¹ EE2 caused increased juvenile recruitment in populations of the amphipod *Gammarus pulex* and a change in sex ratio from approximately 1:1 to 2:1 (female:male). In addition studies with the insect *Chironomus riparius* revealed deformities in the mouthparts following chronic exposure to EE2 at concentrations as low as 10 ng l⁻¹ (Watts et al., in press).

These results suggest that invertebrates as well as fish are at risk from long-term, low level EE2 exposure, despite low acute toxicity e.g. the 96 h LC50 for *G. pulex* is 1.7 mg l⁻¹ (Watts et al., 2001) and for *C. riparius* 9.5 mg l⁻¹ (Segner et al., in press). This consequently raises the possibility that other pharmaceuticals could exhibit similar effects, i.e. lethal only at very high environmentally unrealistic concentrations but damaging at very low concentrations over long exposure times. The lack of data clearly indicates that the ecotoxicity of pharmaceuticals at environmentally relevant concentrations is a major unaddressed area (Daughton and Ternes, 1999).

The purpose of this study was to determine if common pharmaceuticals entering freshwater adversely affect the survival and life history characteristics of freshwater invertebrates to the extent that population structure could be modified. The investigation was carried out with the sedentary cnidarian *Hydra* which occurs in many freshwaters (Campbell and Bode, 1983) and is widely used for studying freshwater pollutants (Benson and Boush, 1983; Hyne et al., 1993; Beach and Pascoe, 1998; Pollino and Holdway, 1999; Karntanut and Pascoe, 2000) and in screening tests to assess the teratogenic potential of drugs (Wilby and Tesh, 1990). The pharmaceuticals were selected from lists of the most widely prescribed drugs, compiled in recent studies in the UK, including Wales (personal communication Dr. D. John, Welsh School of Pharmacy), Germany (Ternes, 1998) and Denmark (Stuer-Lauridsen et al., 2000) and taking into account the amounts produced and where available, their actual concentrations in sewage effluent/surface water as well as information relating to fate, biodegradability and persistence. Although it is unlikely that freshwater invertebrates would ever be exposed to such concentrations causing acute lethal toxicity it was considered important for comparative purposes to assess acute effects before progressing to chronic studies at environmentally relevant concentrations.

2. Methodology

2.1. Culture of test organism

*Hydra vulgaris* were cultured in *Hydra* medium (Lenhoff, 1983) at 20 ± 1 °C with a 16:8 h light: dark regime using separate glass aquaria (33 × 24 × 21 cm) and following the procedure described by Beach and Pascoe (1998).

2.2. Test chemicals

The following 10 pharmaceuticals were examined: ibuprofen (non-steroidal anti-inflammatory, antipyretic and analgesic); paracetamol (acetamidophenol an analgesic–antipyretic); aspirin (acetylsalicylic acid, a non-steroidal anti-inflammatory, antipyretic and analgesic); amoxicillin (broad spectrum penicillin); bendroflumethiazide (thiazide diuretic for hypertension); furosemide (diuretic); atenolol (β-adrenoceptor blocker); diazepam (benzodiazepine anxiolytic); digoxin (cardiac glycoside); amiodipine (calcium channel blocker). All the pharmaceuticals used were obtained as the active pharmaceutical ingredient (API) to avoid complications from other components present in commercial preparations. Amiodipine besylate was kindly supplied by Pfizer Limited, Global Research and Development, Sandwich, Kent, UK and the other chemicals were obtained from Sigma-Aldrich Company Limited, Poole, Dorset, UK.

2.2.1. Preparation and Analysis of drug solutions

One hundred mg l⁻¹ stock solutions of each drug were prepared by dissolving the API in water (ibuprofen, paracetamol, acetylsalicylic acid and amoxicillin), 10% ethanol (bendroflumethiazide, furosemide and atenolol) or 100% ethanol (diazepam, digoxin and amiodipine) with ultrasonication used where necessary to help with dissolution. For each drug, nominal concentrations of 10, 100 μg l⁻¹, 1.0 and 10 mg l⁻¹ were prepared by dilution in *Hydra* medium with ethanol (0.01–10.0%) as indicated in Table 3. Control solutions used throughout
the study were: *Hydra* medium and 0.01%, 0.1%, 1.0% and 10.0% ethanol solvent controls. As an additional control the toxicity of the heavy metal cadmium was assessed for comparison with results determined in previous studies. The concentrations of test solutions of individual pharmaceuticals in *Hydra* medium were examined by recording UV absorbance and UV spectra. Calibration curves were derived from absorbance readings. The same set of data was obtained from serial dilutions of each individual compound in ethanol, in which all compounds readily dissolved. Reproducibility of spectra, linearity of calibration and the actual absorbance readings at the wavelength of maximum absorbance were compared between both sets and used to assess actual concentrations. Measurements were carried out on a CE6600 UV spectrometer (Cecil Instruments Ltd.) in 1 cm quartz cuvettes. Spectra were recorded from 190 to 300 nm. Analyses were carried out of the 1 and 10 mg l\(^{-1}\) concentrations of each drug from which all other concentrations were obtained by direct dilution.

### 2.3. Acute toxicity—effects on polyp structure

Toxicity tests were carried out by transferring a single non-budding *Hydra* polyp from the stock aquarium to 3 ml of fresh test solution in a glass vial (2 × 2.5 cm) which had been previously equilibrated with test solution. Five replicate animals were used for each test concentration (10, 100 \(\mu\)g l\(^{-1}\), 1.0, 10 mg l\(^{-1}\)) and for the *Hydra* medium and solvent controls. The same procedure was repeated for all 10 pharmaceuticals and their appropriate controls. Toxicity was assessed by microscopically recording the morphological status of each polyp each day and assigning a score (Table 1) from 10 (normal) to 0 (disintegrated) as devised by Wilby (1988) and used by Karntanut and Pascoe (2000). The experiment was carried out in a temperature controlled room under the same conditions as used for the *Hydra* culture and was continued for 7 days.

Test solutions were renewed daily. Water quality (hardness, conductivity, dissolved oxygen and pH) of the *Hydra* medium was measured throughout the study. The drug concentrations were analysed as described above. Statistical analysis of toxicity scores was performed using non-parametric Kruskal–Wallis tests and comparison between treatments by the non-parametric Mann–Whitney test.

### 2.4. Chronic toxicity—effects on feeding and bud production

All animals survived the acute toxicity study and the exposure was continued under the same experimental conditions to determine if their ability to feed and to produce buds had been impaired. Five newly hatched *Artemia* nauplii (the food also provided to *Hydra* in culture) were presented to each polyp on alternate days and the number ingested within 1 h was recorded. This experiment continued for 10 days with test solutions renewed after each feeding period. The number of new buds produced was also recorded daily.

The mean number of *Artemia* ingested was compared with oneway ANOVA or non-parametric Kruskal–Wallis followed by Tukey multiple pair wise or Mann–Whitney tests to identify specific differences. ANOVA was also used to compare bud production.

### 2.5. Chronic toxicity—effects on polyp regeneration

(a) Following the 7 day initial exposure and the 10 day feeding/budding study, digestive region tubes were dissected from all polyps in the 10 \(\mu\)g l\(^{-1}\) concentration of each drug and transferred to a glass vial containing

<table>
<thead>
<tr>
<th>Score</th>
<th>Morphology of polyp</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Extended tentacles and body reactive</td>
</tr>
<tr>
<td>9</td>
<td>Partially contracted, slow reactions</td>
</tr>
<tr>
<td>8</td>
<td>Clubbed tentacles, body slightly contracted</td>
</tr>
<tr>
<td>7</td>
<td>Shortened tentacles, body slightly contracted</td>
</tr>
<tr>
<td>6</td>
<td>Tentacles and body shortened</td>
</tr>
<tr>
<td>5</td>
<td>Totally contracted, tentacles visible</td>
</tr>
<tr>
<td>4</td>
<td>Totally contracted, no visible tentacles</td>
</tr>
<tr>
<td>3</td>
<td>Expanded, tentacles visible</td>
</tr>
<tr>
<td>2</td>
<td>Expanded, no visible tentacles</td>
</tr>
<tr>
<td>1</td>
<td>Dead but intact</td>
</tr>
<tr>
<td>0</td>
<td>Disintegrated</td>
</tr>
</tbody>
</table>

From initial healing of the wound (score 3) regeneration to a normal polyp (score 10) occurs (from Wilby (1988)).
3 ml of test solution. Each digestive region was then observed microscopically at 24, 48 and 72 h and the degree of regeneration assessed using Wilby’s (1988) graded scoring system (Table 2) in which 10 indicates complete regeneration to a polyp with mouth, 4-6 tentacles and a peduncle while 0 indicates disintegration of the preparation.

(b) Regeneration was also examined in excised polyp digestive regions which had not previously been exposed to any drug. These were prepared from Hydra taken directly from culture and then exposed to 10 μg l−1 of each drug. As before five replicate preparations were employed.

3. Results

3.1. Water quality and drug concentration

Water quality parameters recorded for the Hydra medium during the study were hardness (207.6 mg l−1 as CaCO₃), conductivity (445 μS cm−1), pH (7.6) and DO (7.2 mg l−1). Concentrations of all drug solutions were found to be within 10% of nominal and so the nominal concentrations were used in all data presentations and analyses. The toxicity data obtained with cadmium controls were in line with those seen in earlier experiments providing confidence in the validity of the results for the drug tests.

3.2. Acute toxicity—effects on polyp structure

All Hydra survived 7 days in the test solutions and controls except for those in the 10% ethanol control and in the highest concentration (10 mg l−1) of diazepam, digoxin and amlodipine, which had been prepared using 10% ethanol. Median toxicity scores indicating the effects of each drug to Hydra after 7 day exposure are shown in Table 3 and range from 7 (shortened tentacles and body slightly contracted) to 9 (polyp partially contracted and with slow reactions). Control animals showed scores of 9 or 10 (normal polyp with extended tentacles and reactive body). Although some polyps exposed to the drugs had a significantly lower score than seen in the control the effects were generally unlikely to be environmentally relevant, although for amlodipine a

<table>
<thead>
<tr>
<th>Pharmaceuticals</th>
<th>Median score (range in parentheses) at different concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 μg l−1</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>9 (8–9) a</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>9 (9–10)</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>9 (9–10)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>9 (8–10)</td>
</tr>
<tr>
<td>Bendroflumethiazide</td>
<td>9 (9)</td>
</tr>
<tr>
<td>Furosemide</td>
<td>9 (8–10)</td>
</tr>
<tr>
<td>Atenolol</td>
<td>9 (9–10)</td>
</tr>
<tr>
<td>Diazepam</td>
<td>8 (7–9) b</td>
</tr>
<tr>
<td>Digoxin</td>
<td>9 (8–9)</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>9 (8–10)</td>
</tr>
<tr>
<td>Cont. 0.01% ethanol</td>
<td>9 (9) b</td>
</tr>
<tr>
<td>Cont. 0.1% ethanol</td>
<td>–</td>
</tr>
<tr>
<td>Cont. 1% ethanol</td>
<td>–</td>
</tr>
<tr>
<td>Cont. 10% ethanol</td>
<td>–</td>
</tr>
<tr>
<td>Cont. Hydra medium</td>
<td>10 (9–10) a</td>
</tr>
</tbody>
</table>

a,b in the same column indicates significant difference p ≤ 0.05 from control with the same letter (non-parametric Kruskal–Wallis followed by Mann–Whitney test to identify specific difference).

D indicates all polyps dead.

Hydra medium was used as the control at all concentrations of ibuprofen, paracetamol, acetylsalicylic acid and amoxicillin.

0.01% ethanol was the solvent control for the remaining drugs at 10 μg l−1 and for bendroflumethiazide, furosemide and atenolol at 100 μg l−1.

0.1% ethanol was the solvent control for diazepam, digoxin and amlodipine at 100 μg l−1 and bendroflumethiazide, furosemide and atenolol at 1.0 mg l−1.

1.0% ethanol was the solvent control for diazepam, digoxin and amlodipine at 1 mg l−1 and bendroflumethiazide, furosemide and atenolol at 10 mg l−1.

10% ethanol was the solvent control for diazepam, digoxin and amlodipine at 10 mg l−1 respectively.
significant $p < 0.05$) concentration-related reduction in score was noted.

### 3.3. Chronic toxicity—effects on feeding and bud production

The mean number of *Artemia* ingested by *Hydra* previously exposed for 7 days to each drug is shown in Table 4 and it can be seen that for ibuprofen and acetylsalicylic acid at the lower test concentrations, feeding is significantly elevated ($p < 0.05$) compared to that for animals in control *Hydra* medium. Typically feeding decreases with increasing drug concentration and for the water soluble ibuprofen and acetylsalicylic acid this decrease is statistically significant ($p < 0.05$, oneway ANOVA/non-parametric Kruskal–Wallis). However for some of the drugs prepared in alcohol, the reduced feeding is mirrored by a similar reduction in the solvent controls.

The mean number of buds produced during the 10 day feeding period ranged from 0.2 to 2.0 but there was no statistical difference ($p \geq 0.05$, oneway ANOVA) in bud production between the different drugs or between different concentrations of the same drug.

### 3.4. Chronic toxicity—effects on polyp regeneration

(a) The median regeneration scores for isolated digestive regions following 7 day exposure of the adult polyp to 10 $\mu$g l$^{-1}$ of drug and 10 days feeding are shown in Table 5. By 72 h, digestive tubes in control *Hydra* medium achieved a score of 10 (fully formed polyp with a mouth, 4–6 tentacles and a peduncle) and those in the 0.01% ethanol control reached score 8. In most drug solutions regeneration reached score 7–10 however, for digestive regions from polyps which had been exposed to diazepam, digoxin or amlodipine most failed to regenerate at all with median scores of 0, indicating major inhibition by these drugs at 10 $\mu$g l$^{-1}$.

(b) The regeneration scores for digestive regions from polyps which had not previously been exposed are shown in Table 6. Although the effect is not so clear as in the previous study, inhibition of regeneration was also apparent with diazepam, digoxin and amlodipine.

### 4. Discussion

Pharmaceuticals differ from other pollutants entering water in two key respects; (i) they are actually designed...
to have effects upon biological systems by modification of physiological/biochemical function, and (ii) unlike many other pollutants, e.g. agrochemicals, which are discharged or released sporadically, pharmaceuticals are continuously introduced into surface waters causing life cycle exposures of the biota.

It is unlikely that freshwater invertebrates would ever be exposed to drug concentrations causing acute lethal toxicity and studies which have been carried out to date, largely with Daphnia sp. (Lilius et al., 1995; Henschel et al., 1997; Wollenberger et al., 2000) have reported LC50 values at the mg l\(^{-1}\) level. In a study with the synthetic estrogen 17\(\alpha\)-ethinylestradiol (Pascoe et al., 2002) a 96 h LC50 of 3.8 mg l\(^{-1}\) was recorded for H. vulgaris. Nevertheless, it is important to confirm with other drugs, that acute toxic effects do not occur before progressing to chronic studies. This investigation has demonstrated with 10 widely prescribed pharmaceuticals that H. vulgaris polyps survive 7 days exposure at concentrations of 10, 100 \(\mu\)g l\(^{-1}\) and 1.0 mg l\(^{-1}\). At 10 mg l\(^{-1}\) animals in diazepam, digoxin and amlodipine did not survive because of the high ethanol concentration required to maintain solubility at this concentration. Therefore these pharmaceuticals do not present a risk of acute lethal toxicity to Hydra and furthermore, the sublethal response criteria examined (bud formation and feeding) were not consistently modified in a way which would suggest adverse effects. However, regeneration of isolated digestive regions to fully functional polyps with a hypostome, tentacles and a foot was inhibited by three of the drugs examined i.e. diazepam, digoxin and amlodipine, at 10 \(\mu\)g l\(^{-1}\). Inhibition was greater in digestive regions from polyps which had previously been exposed for 17 days to the drugs (Table 5) compared with those which had not been previously exposed (Table 6).

In human clinical use digoxin inhibits Na\(^+\)/K\(^+\) AT-Pase in the cell membrane so preventing the exchange of extracellular K\(^+\) for intracellular Na\(^+\) and resulting in a secondary increase in intracellular Ca\(^{2+}\). Amlodipine is a calcium antagonist which blocks voltage sensitive Ca\(^{2+}\) channels, preventing the entry of Ca\(^{2+}\) into the cell while diazepam enhances Cl\(^-\) conductance in the neurones by potentiating the effect of the neurotransmitter gamma-aminobutyric acid (GABA). Since all three drugs function clinically in different ways, it is not clear by what mechanism(s) these substances inhibited the regeneration process. Regeneration was not inhibited in the solvent control preparations but, as with all toxicity studies involving solvents, it must be considered a possibility that an interaction between the solvent and drug caused the inhibition of regeneration. However it is interesting to note that Concas et al. (1998) have recently demonstrated the presence of GABA receptors in H. vulgaris and believe them to be involved in neuroregulation of the feeding response. Furthermore, in vitro assays revealed that the effects of GABA were potentiated by 100 \(\mu\)M diazepam. The authors concluded from this and other studies with neurosteroids, that in Hydra, an animal with probably the most primitive nervous system to have evolved, modulation of GABA receptors closely resembles that seen in the mammalian brain and in other vertebrates and suggest that these receptors have been highly conserved throughout evolution. It is possible therefore that other receptor systems and biochemical pathways occurring in Hydra and higher invertebrates may also be subject to the actions of
pharmaceuticals which enter and pollute natural waters in sewage effluent, although effects may not be seen at the low concentrations to be expected in the environment. For example Pascoe et al. (2002) reported that the synthetic estrogen 17α-ethinylestradiol inhibited regeneration and sexual reproduction in Hydra but only at concentrations of 320 and 500 µg l⁻¹ respectively.

5. Conclusion

Acute and chronic exposures of the cnidarian H. vulgaris to 10 commonly prescribed pharmaceuticals indicate that these substances do not present an acute lethal risk or adversely affect feeding or bud formation at concentrations up to 1.0 mg l⁻¹. However, three of the drugs examined i.e. diazepam, digoxin and amlodipine did inhibit polyp regeneration at 10 µg l⁻¹.

Acknowledgements

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