Pharmaceuticals in the Terrestrial Environment:
Resistance to Bacterial Degradation and Environmental Implications

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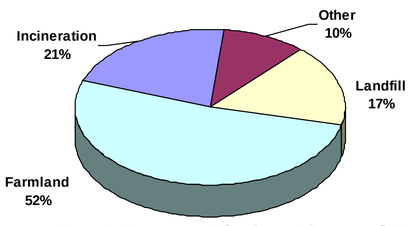
Background

Pharmaceutical Selection

An extensive literature search was first performed to identify pharmaceuticals previously found in the environment. Selection criteria were then developed to aid in the choice of pharmaceuticals suitable for these studies. The criteria included, availability of toxicity data, information on human metabolites, UK usage (t a^{-1}), environmental occurrence, information on biodegradation and photodegradation and removal rates in sewage treatment works.

From this process the pharmaceuticals Prozac® (fluoxetine hydrochloride), Valium® (diazepam) and their human metabolites were selected for further investigation (Figure 1).

Disposal of Sewage Sludge by Application to Agricultural Land



Since the EU banned sea disposal of sewage sludge in 1998 the application rate of sewage sludge to land has risen significantly (Figure 2) and is set to rise further. Land application of sewage sludge may thus be an important transport route for sewage sludge associated organic chemicals, such as pharmaceuticals, into the environment.

Figure 2. UK Sewage Sludge Disposal 99/00
Sources: OFWATT, Scottish Executive & DOE Northern Ireland

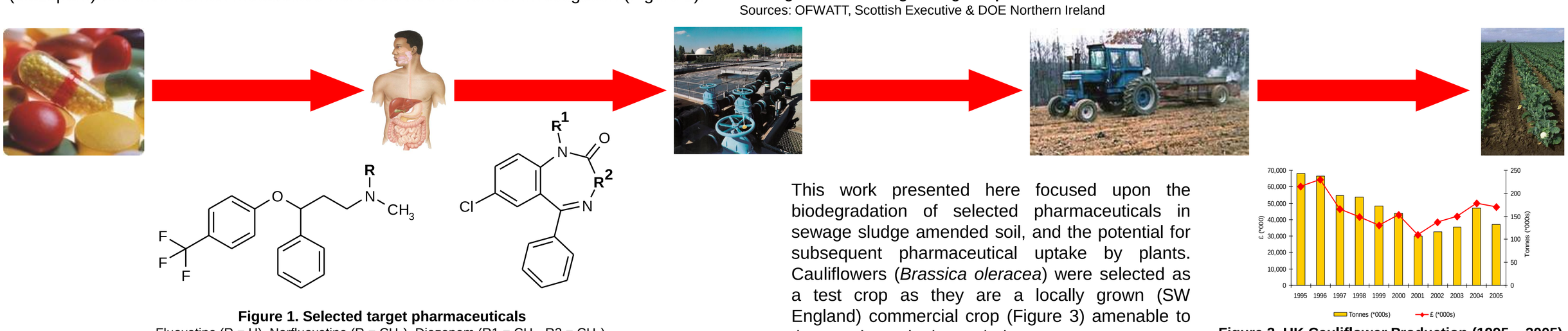


Figure 1. Selected target pharmaceuticals
Fluoxetine (R = H), Norfluoxetine (R = CH_3), Diazepam (R1 = CH_3, R2 = CH_2), Temazepam (R1 = CH_3, R2 = OH) and Oxazepam (R1 = H, R2 = OH).

This work presented here focused upon the biodegradation of selected pharmaceuticals in sewage sludge amended soil, and the potential for subsequent pharmaceutical uptake by plants. Cauliflowers (*Brassica oleracea*) were selected as a test crop as they are a locally grown (SW England) commercial crop (Figure 3) amenable to tissue culture cloning techniques.

Figure 3. UK Cauliflower Production (1995 – 2005)
Source: DEFRA

Biodegradation in Liquid Cultures with Acclimatised Environmentally Relevant Bacterial Populations

- Sewage-sludge amended soil used to create enrichment cultures for use as inoculum.
- 5μg target compound (Fluoxetine HCl, Norfluoxetine HCl, Diazepam, Temazepam and Oxazepam) added (0.2μg mL^{-1}) to each exposure.
- Developed methods (ultrasonic solvent extraction and tandem SPE) used for extraction and clean-up of samples from days 0, 35, 45 & 60.
- HPLC-ESI(+)-MS^n used for analysis
- Use of deuterated internal standards (d_5-Fluoxetine HCl & d_5-Oxazepam) allowed the generation of quantitative data *via* ratio calibration.

Statistics (F-test, t-test) @ 95% confidence interval (n = 3) found no significant differences in concentration after 60 days when compared with abiotic controls for Fluoxetine, Norfluoxetine, Diazepam and Temazepam, i.e. no evidence of biodegradation (Figure 3).

Significant losses (~80%) of Oxazepam were seen after 60 days due to both abiotic and biotic factors.

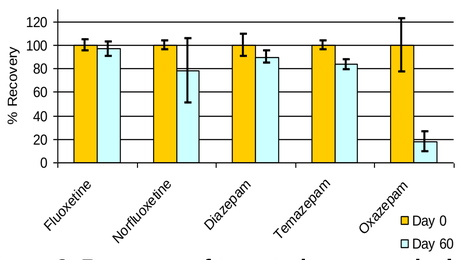


Figure 3. Recovery of target pharmaceuticals from liquid cultures after 60 days

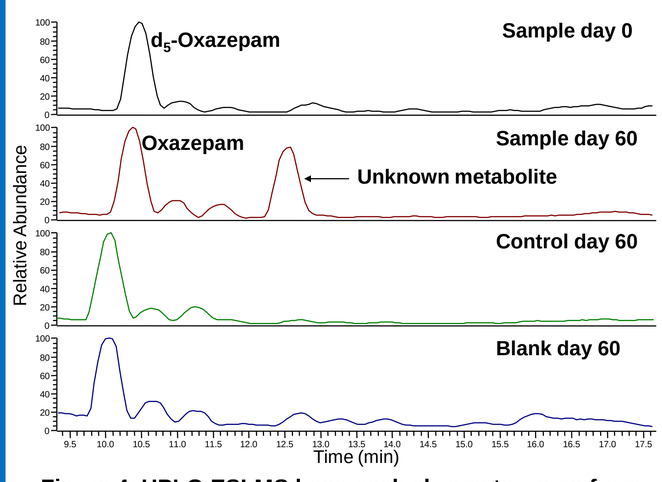


Figure 4. HPLC-ESI-MS base peak chromatograms from the liquid culture biodegradation of Oxazepam

An unknown metabolite was found to be present in all 3 replicates from day 60 (Figure 4).

The full mass spectrum of the unknown metabolite was identical to that of keto-Nordiazepam, however the Rt was ~ 3 minutes later. It was therefore suspected that the unknown metabolite may be enol-Nordiazepam.

Tautomerism experiments with basified Nordiazepam in methanol and CD_3OH to generate deuterated analogs were unable to produce enough enol-Nordiazepam for MS^2 fragmentation.

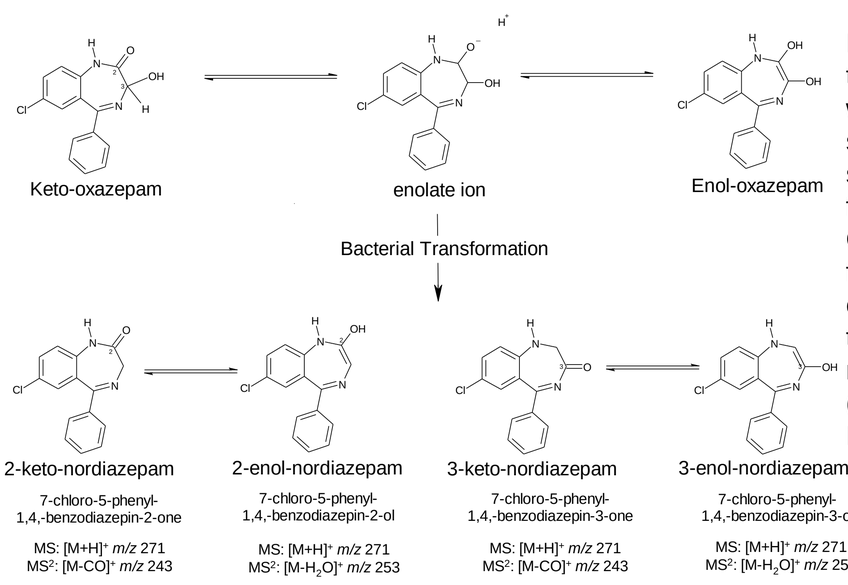


Figure 5. Biotransformation of Oxazepam to Nordiazepam

However equivalent tautomerism experiments with Oxazepam as the starting material were successful in the formation of enol-Oxazepam, whose MS^2 fragmentation provided evidence to support the theory that the unknown metabolite formed (Figure 5) was enol-Nordiazepam.

Biodegradation in Sewage Sludge Amended-Soil

- Sewage-sludge amended soil used as inoculum.
- 1.5μg Fluoxetine HCl added 15 g sewage sludge amended soil.
- Developed methods (ultrasonic solvent extraction and tandem SPE) used for extraction and clean-up of samples over 270 days.
- Use of d_5-Fluoxetine HCl allowed the generation of quantitative data *via* ratio calibration.
- HPLC-ESI(+)-MS^n used for analysis

Statistics (F-test, t-test) @ 95% confidence interval (n = 3) found no significant differences in concentration after 270 days when compared with abiotic controls i.e. no evidence of biodegradation (Figure 6).

Fungal and bacterial colony growth monitored.

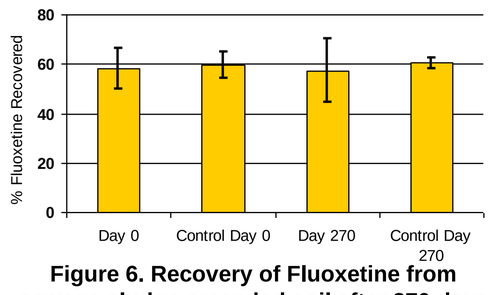


Figure 6. Recovery of Fluoxetine from sewage sludge amended soil after 270 days

Uptake of Pharmaceuticals by Plants

- 10 μg Fluoxetine HCl per 35 mL pot of tissue culture media (NB: not soil; equiv ~ 250 ng g^{-1})
- Cauliflower explants grown under controlled conditions for 8 weeks.
- Curd, leaves, stem, roots and media were sampled separately.
- Developed method (ultrasonic solvent extraction and tandem SPE) used for extraction and clean-up.
- Use of d_5-Fluoxetine HCl (internal standard; 2 μg pot^{-1}) allowed the generation of quantitative data *via* ratio calibration.
- HPLC-ESI(+)-MS^n, operated in SIM and SRM modes was used for analysis

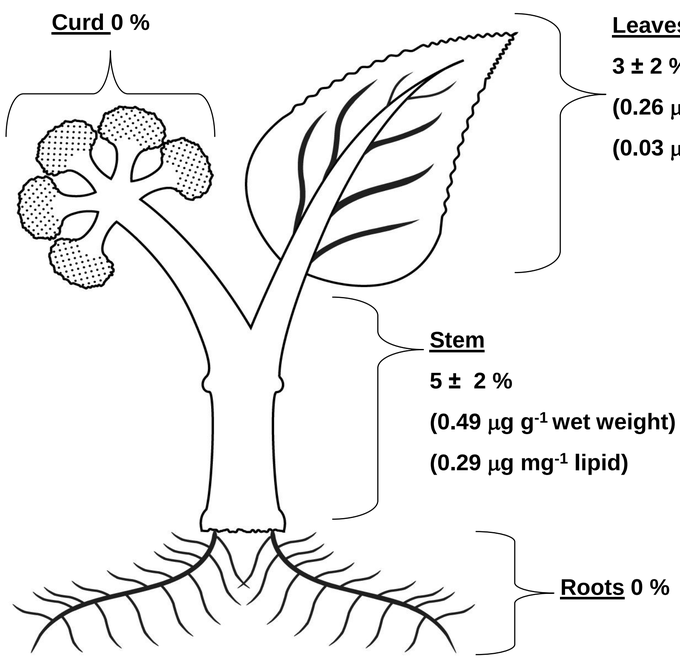


Figure 8. Summary of recovered translocated Fluoxetine within cauliflower tissue cultures

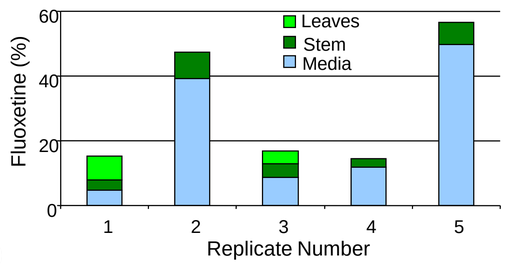


Figure 7. Recovery of Fluoxetine from individual cauliflower tissue culture plant replicates

Evidence for the translocation of Fluoxetine from media to stems was found in all samples, and further transport to leaves was seen in 2 of 4 samples (Figures 7 & 8). These results indicate the uptake of pharmaceuticals into crops may be a viable transport route within the environment, however further research is required.

Conclusions

Oxazepam was the only target analyte to undergo significant degradation under environmentally relevant conditions, forming one major biotransformation product. This product is proposed to be the enol tautomer of Nordiazepam, which is another bioactive 1,4-Benzodiazepine. These results are of concern as it indicates the behaviour of these pharmaceuticals in the environment is likely to be of a persistent nature. With persistent compounds there is a risk of accumulation within an environment e.g. field soil. When compounds undergo accumulation the risk of contamination of other environmental components becomes more likely e.g. exposure and potential bioaccumulation in terrestrial organisms and plants.

The finding that Fluoxetine underwent uptake into cauliflower stem and leaves is a significant finding as regards current knowledge about the transport and fate of pharmaceuticals in the environment. This study is believed to be the first research into pharmaceutical uptake by a crop plant and provides evidence that entrance into food chains may be a viable transport route in the environment. Further plant uptake research using more environmentally sound conditions (i.e. soil) must be completed before any major conclusions can be drawn, but results highlight the need for further research. Should plant uptake of pharmaceuticals prove to be a common phenomenon further contamination risks arising from this should be assessed. For example heavily contaminated crops could pose a risk to terrestrial organisms including livestock, any birds that may feed upon crop grains or berries, and the human food chain.