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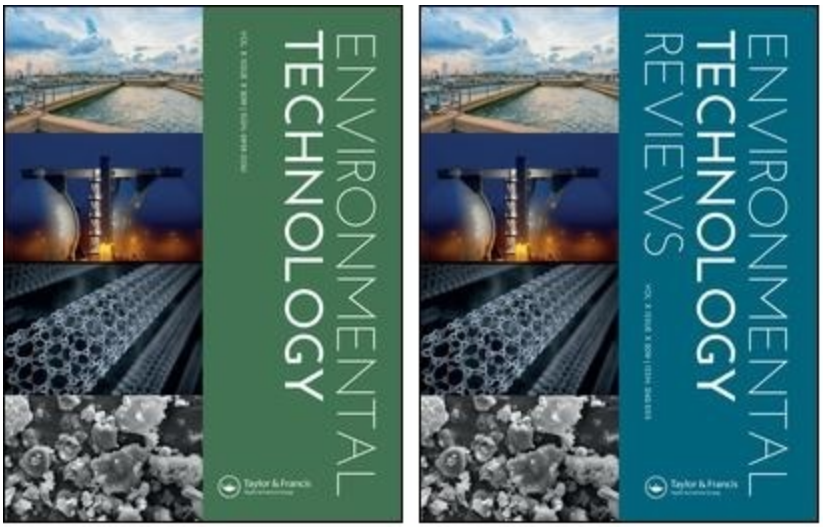


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# Developing the OECD 106 fate testing protocol for active pharmaceuticals in soil

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**Abstract**

The ability to determine accurately the fate of APIs in soil is essential for rigorous risk assessment associated with wastewater reuse or biosolid recycling to land, particularly in lower income countries where water and fertiliser is scarce. Four APIs (naproxen, ofloxacin, propranolol and nevirapine) with wide ranging functionality were used as examples in the development of the OECD 106 soil partitioning and/or degradation study, with naproxen used to illustrate applying the full methodology. The data showed key methodological criteria require careful consideration and testing to generate accurate and consistent results. Only glass fibre membranes were suitable for all APIs, without unduly adsorbing APIs to their surface, thus effectively restricting the minimum practical pore size to 0.7 µm. Polypropylene plastic centrifuge tubes were shown to be suitable, with careful determination of recoveries. Direct injection liquid chromatography-mass spectrometry could reliably resolve all 4 APIs down to less than µg $L^{-1}$ in soil solutions, although allowance for matrix effects via standard additions was required in some cases. Greatest analytical challenges were found for the highest molecular weight API with the greatest affinity for sorption to surfaces (ofloxacin). Key variables that can impact on partitioning such as solution pH and dissolved organic carbon concentrations were shown to vary within tests over time and should be accounted for.

**Key words:** soil; naproxen; method development; pharmaceuticals; liquid chromatography-mass spectrometry; OECD106

## 1 Introduction

There is substantial expansion of the use of active pharmaceutical ingredients (APIs) in low and lower-middle income countries (LLMIC) of Asia, Africa, Latin America and Australasia over the past two decades. In 2000, these countries accounted for 25 % of global pharmaceutical sales; in 2014 this figure was 32 %, with sales accounting for at least 25 % of the income of the largest pharmaceutical companies [1].

Many LLMICs are experiencing physical or economic water scarcity; to counter-act shortages many LLMICs use wastewater for irrigation. Globally, about 36 million ha of agricultural land is irrigated with wastewater [2]. The World Health Organisation has published guidelines on the safe use of wastewater in order to provide a management framework for safeguarding human health, while maximising the benefits of wastewater to agriculture [3-5]. Loadings of APIs in wastewater have been considered [2,3], but environmental risk assessments (ERA) for API inputs to soils and waters remain elusive in LLMICs [6-8]. However, even in high income countries it is only since 1998 and 2006, respectively, that the USA and European Union (EU) have required ERAs for all new marketing authorisation applications involving human medicinal products [9]. An assumption for ERAs in the EU is that wastewater is universally treated in wastewater treatment plants (WWTP) [10], an expectation that does not hold for LLMIC. Furthermore, the EU ERA is focussed only on exposure to APIs following application of sewage biosolids to soil [11], and does not include additional scenarios, such as irrigation of soils with wastewater and other contaminated water sources. In 2015, the UNEP International Conference on Chemicals Management highlighted the persistence and antimicrobial resistance of APIs as priority issues, and called for increased global knowledge of pharmaceuticals in all environmental compartments [12]. Understanding the mobility and fate of APIs in soil is therefore critical for any ERA in LLMICs.

The concentrations and distributions of APIs in soils are controlled by a number of processes, with sorption to soil solids [13], biodegradation and dilution/percolation through the soil profile particularly important [14,15]. The Organisation for Economic Co-operation and Development (OECD) 106 guideline was developed to assess the importance of the sorption (adsorption-desorption) of chemicals to soils using a batch-equilibrium method [16]. It is now a standard method, in both industrial and research contexts, for examining the sorption of APIs in soil. It has been endorsed for use by the EU [10, 17]. Notably, the method embraces the variety of soil types (with respect to organic carbon content, clay context, soil texture and pH) found in regions that rely on wastewater for irrigation [16,18].

It is important to note that OECD 106 is a guideline and not a Standard Operating Procedure. For this reason, Tier 1 Preliminary studies are outlined in OECD 106, to be undertaken prior to the Tier 2 Screening test and the Tier 3 Determination of Freundlich adsorption isotherms. Tier 1 studies include examining the adsorption of the test chemical on the surface of the test vessel, and the stability of the test substance. While not explicitly part of the Tier 1 assessment, it is made clear in the OECD 106 guideline that the development of an appropriate analytical method, with appropriate accuracy (bias, precision), reproducibility, recovery and detection limits, is also key to the successful performance of the test.

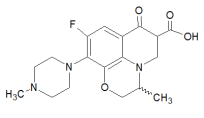
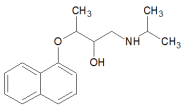
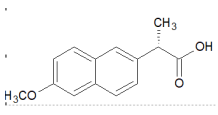
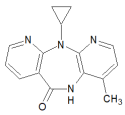
The study has developed a method, for testing APIs under the OECD 106 guideline, for examining the sorption of APIs with contrasting physico-chemical characteristics to two different soil types, taking account of all aspects of the process including laboratory ware, filter media and analytical optimisation. To demonstrate the effectiveness of the method development, 4 APIs of differing physico-chemical characteristics ofloxacin, propranolol, naproxen and nevirapine were used. Furthermore, the potential variation in pH and dissolved organic carbon (DOC), both of which could influence the sorption characteristic of the APIs of interest was monitored over the 120 hour test period.

## 2 Methods

### 2.1 API selection

The APIs studied represented different therapeutic classes and also had contrasting physico-chemical properties particularly the ionic charge at environmental pH (typically 5 – 9). Ofloxacin is a broad spectrum fluoroquinolone antibiotic, widely used in India and China [19], while propranolol is an extensively used beta-blocker, with reported poor removal in LLMIC WWTPs (Table 1) [20,21]. Naproxen is a non-steroidal anti-inflammatory drug routinely prescribed in Bangladesh, Pakistan, the Philippines and Thailand [22]. Finally, nevirapine (Table 1) is one of the most commonly prescribed antiretroviral drugs for preventing HIV-1 transmission in resource-poor countries [23].

**Table 1. Test API physico-chemical characteristics. Log $K_{oc}$ = log organic carbon:water partition coefficient. Log $K_{ow}$ = log octanol:water partition coefficient. * These values are not consistent with the low $K_{ow}$ and may reflect partitioning into other soil components (e.g. clay) in addition to, or instead of, organic carbon.**

| | Ofloxacin | Propranalol | Naproxen | Nevirapine |
|---|---|---|---|---|
| Structure of neutral ion |  |  |  |  |
| Ionic charge | Zwitterion | cation | anion | neutral |
| Formula | $C_{18}H_{20}FN_3O_4$ | $C_{16}H_{21}NO_2$ | $C_{14}H_{14}O_3$ | $C_{15}H_{14}N_4O$ |
| CAS No. | 82419-36-1 | 318-98-9 | 22204-53-1 | 129618-40-2 |
| MW (g $mol^{-1}$) | 361.4[1] | 260.2[1] | 231.1[1] | 267.1[1] |
| Water sol. (mg $L^{-1}$) | 25,000[1] | 6,000[1] | <3,000[1] | 0.7[11] |
| $pK_a$ | 5.97, 8.28[2] | 9.53[4] | 4.15[4] | 2.8[9] |
| Log $K_{oc}$ | 4.64-5.70*[2,3] | 3.21-4.69[2] | 1.98-2.72[5,6,7] | n/a |
| Log $K_{ow}$ | -0.39-0.65[4] | 2.58-3.48[4] | 2.99-3.18[4] | 1.75-2.5[8] |

[1][24-27]; [2][17]; [3][28]; [4][29]; [5][30]; [6][31]; [7][32]; [8][33]; [9][34].

## 2.2 Soil selection

Soils used for testing should be representative of the environments where the substances of interest will be applied, as well as being free from anthropogenic influences to reduce the risk of soils being contaminated with test substance. In the current study, two soils were chosen according to the OECD 106 guideline [16]. These soils were purchased from Lufa Speyer (Germany) and had a ≥ 5 year history of no pesticide, biocidal fertiliser, or organic manure application, resulting in a soil that should be as 'clean' from contaminants as possible. Sample handling by Lufa Speyer followed ISO 10381-6 (1993) and Good Laboratory Practice. Soil samples were provided air - dried and sieved to < 2 mm.

Table 2 shows the properties of the two soils, how they match with the OECD 106 Guideline and which LLMICs have similar soils. Both soils matched the requirements specified by OECD 106, except for the clay content of the loam soil, which was 0.8 % above the maximum.

**Table 2. Properties of the two soils used in this study in comparison with the OECD 106 guideline (mean values of different batch analyses ± s.d.)**

| | Sandy loam | Loam | OECD loamy sand | OECD silt loam |
|---|---|---|---|---|
| **pH (0.01 M $CaCl_2$)** | 5.7 ± 0.6 | 7.3 ± 0.1 | < 4.0 - 6.0 | 5.5 - 7.0 |
| **TOC (%)** | 0.7 ± 0.0 | 2.0 ± 0.2 | < 0.5 - 1.5 | 1.5 - 3.0 |
| **Clay content (%)** | 6.5 ± 1.6 | 25.8 ±1.8 | < 10 – 15 | 15 - 25 |
| **Cation exchange capacity (MEq/100 g)** | 7.5 ± 0.9 | 33.0 ± 4.5 | | |
| **LLMIC examples with similar soil properties** | SE China (Guangxi and Yunnan provinces).<br>NW India (Odisha and West Bengal).<br>NE Democratic Republic of Congo | Malaysia<br>El Salvador | | |

[1] [35]; [2] [16]; [3][36].

## 2.3 Laboratory ware

All re-usable plastic and glass apparatus was cleaned (2 % v/v Decon® for ≥ 24 h, rinsed with high purity water (HPW, 18.2 MΩ.cm resistivity), then soaked for ≥ 24 h in 10 % v/v HCl, and finally rinsed with HPW). Apparatus was dried in a Class 100 laminar flow hood, as a final step, and stored in resealable plastic bags. Sterile polypropylene centrifuge tubes (50 mL) were used, as were sterile syringes constructed only of polypropylene and polyethylene (i.e. no latex, rubber silicone, styrene or DEHP). Filter holders were also polypropylene and clear glass autosampler vials with silicon septa were used to minimise API losses (all purchased from Fisher Scientific, UK). Glass fibre filter membranes (GF/F, 0.7 µm nominal pore

diameter: Fisher Scientific, UK) were wrapped in aluminium foil and ashed at 450 °C for 6 h before use. Polycarbonate and cellulose nitrate filters were prepared by being washed with approximately 1 mL of sample before collection.

### 2.3.1 Laboratory ware selection

Polypropylene centrifuge tubes (50mL) were chosen as they could hold the correct amount of solution and soil with enough head space left for adequate mixing on the shaker, when placed horizontally. Although glass may be considered more appropriate for organics such as APIs, they would not be practical in this case. To assess sorption of the APIs to the walls of the centrifuge tubes, 3 x 30 mL 10 mM $CaCl_2$ (Fisher Scientific, UK) were shaken overnight in separate tubes, then spiked with a mixed API solution and then shaken for a further 4 h at room temperature.

Filter membranes were required that would remove suspended solids from the soil suspensions, but not at the same time sorb the test APIs. The pore size required was 0.7 µm or below for analysis by HPLC-HRAM-MS and fluorescence spectrophotometry. Comparisons were made between concentrations of APIs in a range of standards made up in HPW before and after filtration and recoveries calculated. Three filter types were tested for propranolol, naproxen and ofloxacin; glass fibre (0.7 µm nominal pore diameter), polycarbonate (0.2 µm) and cellulose nitrate (0.2 µm). Only glass fibre filters were assessed for nevirapine.

## 2.4 Sample storage

To ensure that the samples were stable during storage freshly made mixed stock solutions of the APIs were made in HPW and diluted into a calibration range (0.1, 1, 10, 50, 100 µg $L^{-1}$). These were analysed and compared against a calibration range that had been made from individual API standard stock solutions (in HPW) and stored for a minimum of 6 months at -20 °C.

## 2.5 Instrumentation - Liquid chromatography – mass spectrometry

A high performance liquid chromatography-high resolution accurate mass-mass spectrometer (HPLC-HRAM-MS) was used to detect and quantify APIs. This consisted of an Ultimate U3000 UHPLC liquid chromatography system and a Q Exactive Focus Orbitrap mass spectrometer (both Thermo Scientific, UK). Gaseous phase ions were generated from solution by electrospray ionisation before detection by mass spectrometry, allowing for the sensitive analysis of both ionised species and neutral compounds [37]. The HRAM detection simplified sample preparation, allowing for lower waste solvent volumes and shorter analysis times, as neither solid phase extraction nor pre-concentration were not required at experimental concentrations. Peak detection was achieved using Xcalibur software (Figure S1 of the electronic supporting information).

## 2.6 Summary of the OECD 106 soil – solution test procedure requirements

The OECD adsorption-desorption test procedure is described below, although API data are not presented here. The method was run without the addition of APIs but including the sediment and water in order to monitor any changes in pH and dissolved organic carbon concentrations in the test solutions over the 120 hour test period as these are critical parameters controlling the partitioning of organics.

### 2.2.1 Adsorption

The principle of this method is that a known concentration of a test substance is added to a known weight of soil in a 10 mM $CaCl_2$ solution. The soil suspension is shaken for a set period, and then centrifuged (and perhaps filtered) prior to analysis. It is an indirect method as only the aqueous phase is analysed and the difference between the amount of API added and recovered from the liquid phase is assumed to be the amount adsorbed to the soil.

A 6 g aliquot of soil are added to 50 mL polypropylene centrifuge tubes along with 30 mL of 10 mM $CaCl_2$ solution, in triplicate for each time point. These are put onto a reciprocal shaker (132 rpm), laid horizontally to allow the system to equilibrate overnight. Compounds of interest are added to the tubes, which were then returned to the shaker. Tubes were sacrificed at pre-selected times after API addition (0, 3, 6, 24, 48, 72, 96, 120 hours) centrifuged (4000 RPM, 15 minutes) then filtered using 0.7 µm glass fibre filters (Fisher Scientific, UK). Samples are stored at -20 °C until analysis. Experiment take place at room temperature (20 - 25 °C) and in the dark to ensure that photodegradation of the APIs did not occur.

### 2.2.2 Desorption

Desorption of APIs after sorption equilibrium has been reached provides information on how reversible the sorption of APIs is to the soils. This is important to know because it can provide ERAs with more data on the overall fate and mobility of an API in soils, especially those in wet climates or with repeated wastewater irrigation schemes. The OECD 106 method can take this into account (OECD, 2000). Soils are prepared as above and spiked with the same concentration of APIs after the system was shaken overnight. The tubes are then shaken in the dark at room temperature (18-22 °C) until adsorption equilibrium is reached and then all the tubes centrifuged (4000 RPM, 15 mins) and the supernatant removed, a subsample is taken and filtered to be analysed on HPLC-MS. The exact volume of 10 mM $CaCl_2$ was then replaced and the soil pellet is suspended again by 5 seconds on a minishaker. Samples are then sacrificed at 0, 3, 6, 24, 48, 72, 96 and 120 hours, centrifuged and filtered as before storing at -20 °C until analysis along with matching calibration solutions.

For this method development, to identify changes in the soil solutions after 10 mM $CaCl_2$ replacement, pH and DOC was measured from separate tubes handled at the same time but without APIs in them. pH and DOC were determined in the soil-water solutions at the pre-selected times using a Shimadzu TOC (total organic carbon) 5000A analyser for DOC and a

calibrated HANNA HI 9025 microcomputer pH meter fitted with a Camlab epoxy tough single junction combination pH electrode. Desorption is calculated as the percentage of the test substance which is desorbed related to the quantity of substance previously adsorbed at desorption equilibrium taking into account the incomplete removal of the supernatant after the sorption experiment (approximately 3 mL) [16]. The apparent desorption coefficient ($K_{des}$) is the ratio between the mass of the API on the soil and the mass concentration of the desorbed API in the aqueous solution once desorption equilibrium has been reached [16].

### 2.2.3 Soil solution ratio

The OECD 106 guideline sets out several parameters when selecting the soil : solution ratio including using at least 1 g of soil, achieving preferably >50 % sorption of the test substance to the soil and the ratio can be as high as 1:1 (low $K_d$) or as low as 1:100 (high $K_d$) depending on the estimated $K_d$ of the API using molecular modelling software and an available soil:solution ratio matrix (Figure S2) [16]. As an example a for the naproxen test a 1:5 ratio (6 g soil to 30 mL 10 mM $CaCl_2$) was chosen as the best balance between generating significant sorption (predicted to be somewhere between 20% and 80% sorption) but still ensuring the amount of naproxen left in solution was sufficiently high to accurately determine. The results showed that the losses from solution after shaking for four hours would give quantifiable results.

## 3 Results and Discussion

### 3.1 Liquid chromatography – mass spectrometry method development

A key aim was to develop an optimised LC-MS method for the simultaneous quantification of the four APIs used in the study. This included assessing HPLC columns,

mobile phase composition, gradient elution, ionisation mode (positive or negative) and scanning method.

Three C18 HPLC columns were examined, including a Kinetex 2.6 µm EVO C18 100 Å (2.1 x 100 mm), a Waters XBridge BEH C18 2.5 µm (2.1 x 50 mm) Column XP and a Waters XSelect CSH C18 2.5 µm (2.1 x 50 mm); all columns had a pre-column filter fitted (HiChrom 0.5 µm). The Waters columns were selected as they had lower back pressures and produced better peak shapes, relative to the Kinetex column. The aqueous mobile phases considered were 0.1 % (v/v) ammonia and 0.1 % (v/v) formic acid, both in HPW. The use of 0.1 % (v/v) ammonia improved analyte signal response but carry over was detected, particularly for ofloxacin and propranolol. As a result, 0.1 % (v/v) formic acid was chosen as the aqueous phase (eluent C). Methanol (100 %) used as the organic mobile phase (eluent D). The high sensitivity of the mass spectrometer facilitated an injection volume of 5 µL. This reduction in injection volume helped reduce carryover, as did an injector needle wash before and after each injection using 1:1 (v/v) HPW : methanol. The gradient elution programme ran from 95:5 to 0:100 C:D over 6 minutes, then held for 2 minutes and returned to the starting ratio for 2 minutes. The retention times showed that the four APIs were clearly resolved from each other initially by testing individually and then finally as a mixture (Figure 1) allowing the APIs to be spiked into soil incubations as a mixture, and reducing both sample processing and analysis time.

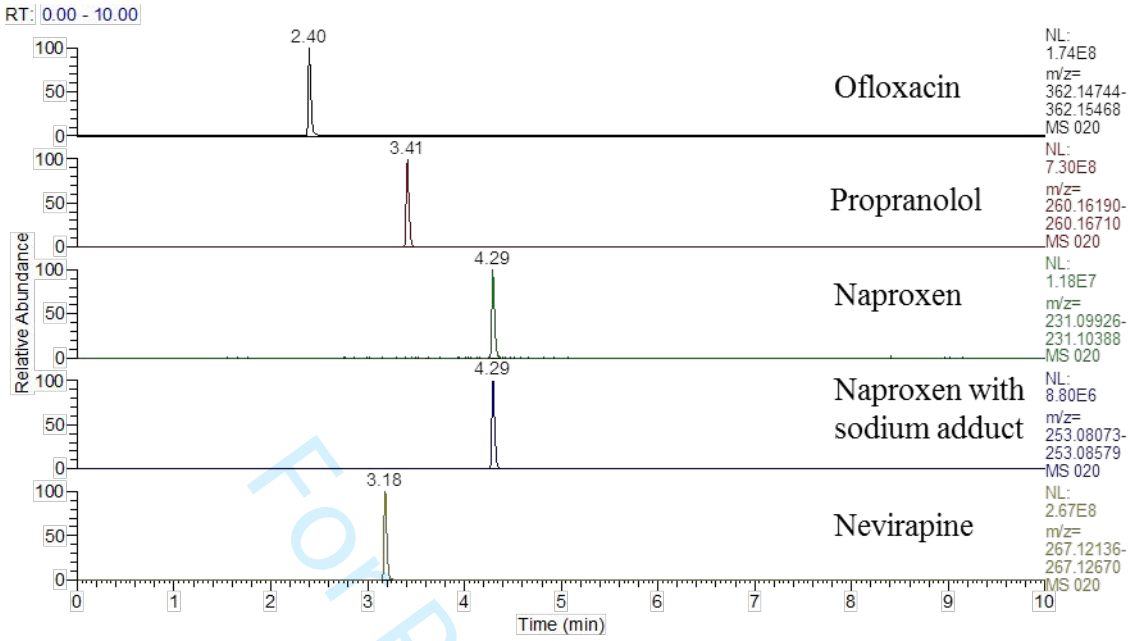



**Figure 1. Example total ion chromatogram of the APIs studied (100 µg $L^{-1}$ in HPW), using a Waters XBridge BEH C18 2.5 µm 2.1x50 mm Column XP.**

The eluent stream was diverted to waste from 0.1 – 1.5 minutes of the analytical run before entering the mass spectrometer in order to reduce contamination of the ionization source with salts from the sample matrix. All mass spectrometric analyses were performed in the positive ion mode. Parallel reaction monitoring (collision energy 30 eV) was used initially to target the specific ions and enable confident API identification with respect to background signals. Once it was apparent that the matrices of the soil filtrates did not interfere with the linearity of the calibration, full scan mode (*m*/*z* 100-1000) was used for all analyses.

Prior to all analysis, the MS response was assessed using a test mix (n-butylamine, caffeine, MRFA, and Ultramark 1621, Pierce LTQ Velos ESI, Thermo Fisher Scientific, UK). Methanol (HiPerSolv Chromanorm, VWR, UK) and HPW containing 0.1 % (v/v) formic acid (Optima Thermo Fisher Scientific, UK) were added and purged through the system before samples were analysed.

Due to problems with degradation or loss of ofloxacin to the glass autosampler vials, a batch of standards was made up in advance and frozen in 1 mL aliquots and defrosted alongside samples in extended periods of analysis longer than 48 hours.

### 3.1.1 Data analysis

All samples were analysed at least in duplicate. The raw data were analysed using Xcalibur software (Thermo Scientific, UK). Peaks were detected and smoothed using ICIS Peak Integration (Figure S1). The APIs were identified by retention time and *m*/*z* values measured to 5 decimal places. Sodium is a common contaminant in LC-MS, with main sources being glassware and mobile phases used. As naproxen forms a sodiated adduct, the peak areas of the parent ion and the sodiated adduct were combined. It is this level of scrutiny that is required to ensure the quality of data obtained via OECD tests on APIs.

Calibration standards included at least six separate concentrations and spanned at least two orders of magnitude (Table S1 in the electronic supporting informaton). Because of the wide range in the concentrations of the standards and with the loss of homoscedasticity, regression lines were weighted, using the Excalibur software, to adjust the best fit line by a factor related to an inverse 1/x function of the concentration [38]. This served to reduce the relative error (variance) of each standard calibration measurement and made the relationship more relevant (reduced bias) at the lower end of the concentration range [39]. All API calibrations were linear and positive, with $R^2$ values > 0.99 (Table S2).

### 3.1.2 Analytical figures of merit

**Precision**

The precision of the analytical measurements was calculated to quantify random error over the length of an analytical run. To achieve this, the relative standard deviation (RSD) was

calculated at three concentrations of API in HPW using at least 7 repeat measurements at each concentration (Table S3) [40].

With the exception of ofloxacin, the APIs showed good precision according to the EU decision 2002/657/EC as each RSD was < 20 % (Table S4) [40]. This decision specifies common criteria for the interpretation of analytical results to ensure that sample data are comparable between laboratories. Ofloxacin showed > 20 % RSD at the lower and middle concentrations. Ofloxacin is known to sorb to glass vials, and this is more pronounced at lower concentrations [41]. As the samples used for the precision experiment were in the glass vials of the autosampler for different lengths of time, loss of ofloxacin to glass vials would likely have varied.

**Limits of detection**

Instrument LOD was calculated using at least 5 low calibration concentrations to ensure that the data presented were both robust and analytically relevant (Table 3). LOD was derived using the standard deviation of the regression line intercept for each calibration, rather than the more usual standard deviation of replicate calibration blank measurements (Equation S1; [42].

**Table 3 LODs for APIs in the different matrices used in this study (µg $L^{-1}$)**

| | HPW | Loam | Sandy loam |
|---|---|---|---|
| **Ofloxacin** | 0.36 | 0.16 | 0.14 |
| **Propranolol** | 0.39 | 1.95 | 0.51 |
| **Naproxen** | 0.14 | 0.82 | 0.99 |
| **Nevirapine** | 0.15 | 0.28 | 0.25 |

As can be seen from Table 4, LODs below µg $L^{-1}$ were achieved, which is in agreement with reported values [43]. Instrument LODs for all APIs were adequate for this study.

### 3.1.3 Matrix effects

Initially, matrix effects on the measurement of APIs were quantified by spiking filtered soil suspensions in 10 mM $CaCl_2$ and comparing the results to HPW. The soil suspensions were prepared by shaking overnight, centrifuging at 4000 rpm for 15 min, followed by filtration through 0.7 µm GFF at different concentrations, and assessing the linearity and suppression of peak areas at each concentration compared to spiked HPW. A set of calibration solutions (in HPW) was prepared in parallel using the same API standard stock solutions, for comparison.

Matrix effects were observed but were not consistent for each API. Where an effect was identified, it was always quenching of the signal (Figure 2). Ofloxacin in sandy loam had the greatest matrix effect compared to HPW, with an 80 % decrease in the average peak area measured but linearity was not affected. These data indicate that calibration standard solutions should be matrix matched to the samples due to the signal quenching.

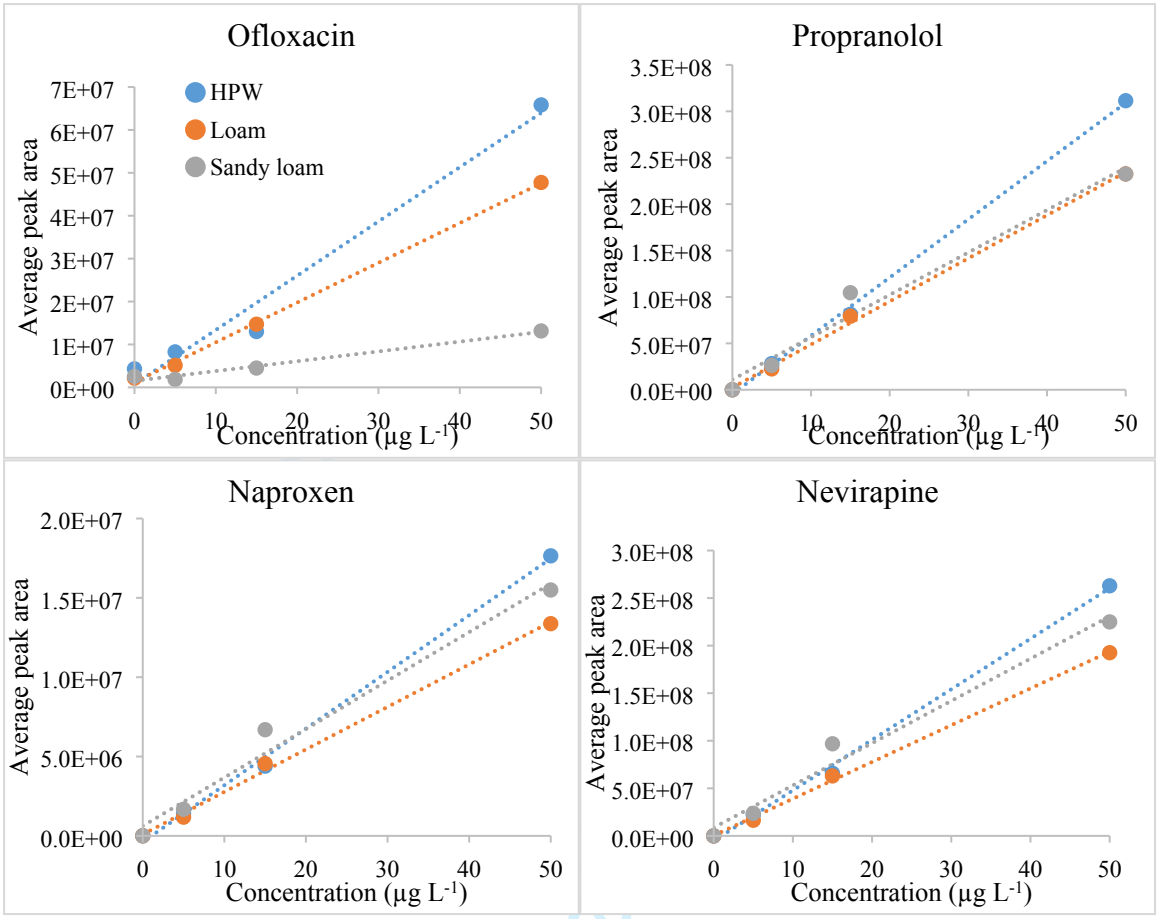



**Figure 2 Soil filtrate matrix effects. Legend is shown in ofloxacin graph and consistent throughout this set of graphs. Error bars are present but not visible ($\bar{x}$ ± s.d. n=3)**

Samples were incubated with shaking for 120 h to check that changes in the soil matrix, such as desorption of organic carbon or other organic material, disaggregation of colloids etc, which might occur over the 120 h sorption experiment, affected API measurements. Separate soil suspensions (6 g soil 30 mL 10 mM $CaCl_2$) without added APIs were shaken for 24 h and 120 h (to match the longest incubation experiment) and these incubations finished at the same time. The shaken soil suspensions were centrifuged and filtered as before and APIs spiked into the filtrates in the concentration range 0.1 – 100 µg $L^{-1}$. The data showed that any temporal changes in matrix did not affect analyte response in the instrument (Figure 3), with the largest difference < 20 % (ofloxacin in loam soil).

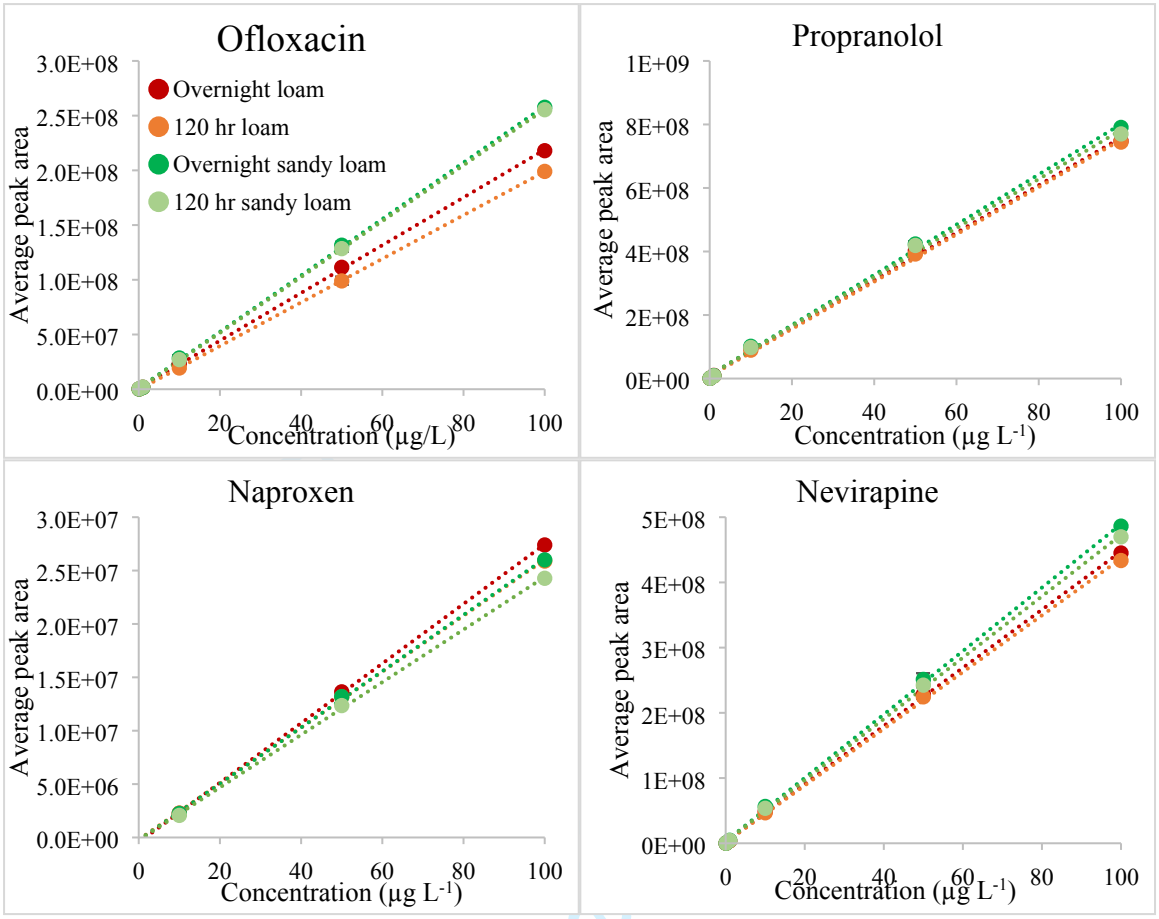



**Figure 3** **120 h soil matrix effects. Legend is shown in ofloxacin graph and is consistent throughout this set of graphs. Error bars are present but not visible ($\bar{x}$ ± s.d. n=3)**

For ease of analysis, all calibration standards were made up in soil filtrates representing the 24 h incubation. While there was some variation in specific APIs, the data were generally consistent across the time period and a 120 h calibration added little analytical value while increasing both workload and costs.

## 3.2 API loss to laboratory ware

### 3.2.1 Plastic ware

The results show that these APIs are unlikely to be lost to the centrifuge tubes as all recoveries were > 90 % (Table 4). As a result, all sorption experiments were undertaken in 50 mL polypropylene centrifuge tubes.

**Table 4 Spike concentrations of APIs and % recoveries of APIs from 50 mL polypropylene centrifuge tubes ($\bar{x}$ ± RSD n = 3)**

| | Spike concentration (µg $L^{-1}$) | Recovery (%) |
|---|---|---|
| **Ofloxacin** | 500 | 98.6 ± 4.2 |
| **Propranolol** | 240 | 101 ± 1 |
| **Naproxen** | 58 | 91.7 ± 17.1 |
| **Nevirapine** | 50 | 115 ± 4 |

### 3.2.2 Filter membranes

Glass fibre filters showed the lowest sorption of APIs and hence highest recovery for all three APIs, relative to the organic material-based membranes (Figure 4). Nevirapine also showed quantitative recovery and was only assessed for the glass fibre filters. The OCED 106 guideline requires filters with a pore size of 0.2 µm if centrifugation does not reach this level. These are not available in glass fibre membranes causing problems for compliance with the guideline but due to the better recoveries for these four APIs these filters were chosen for the experiments.

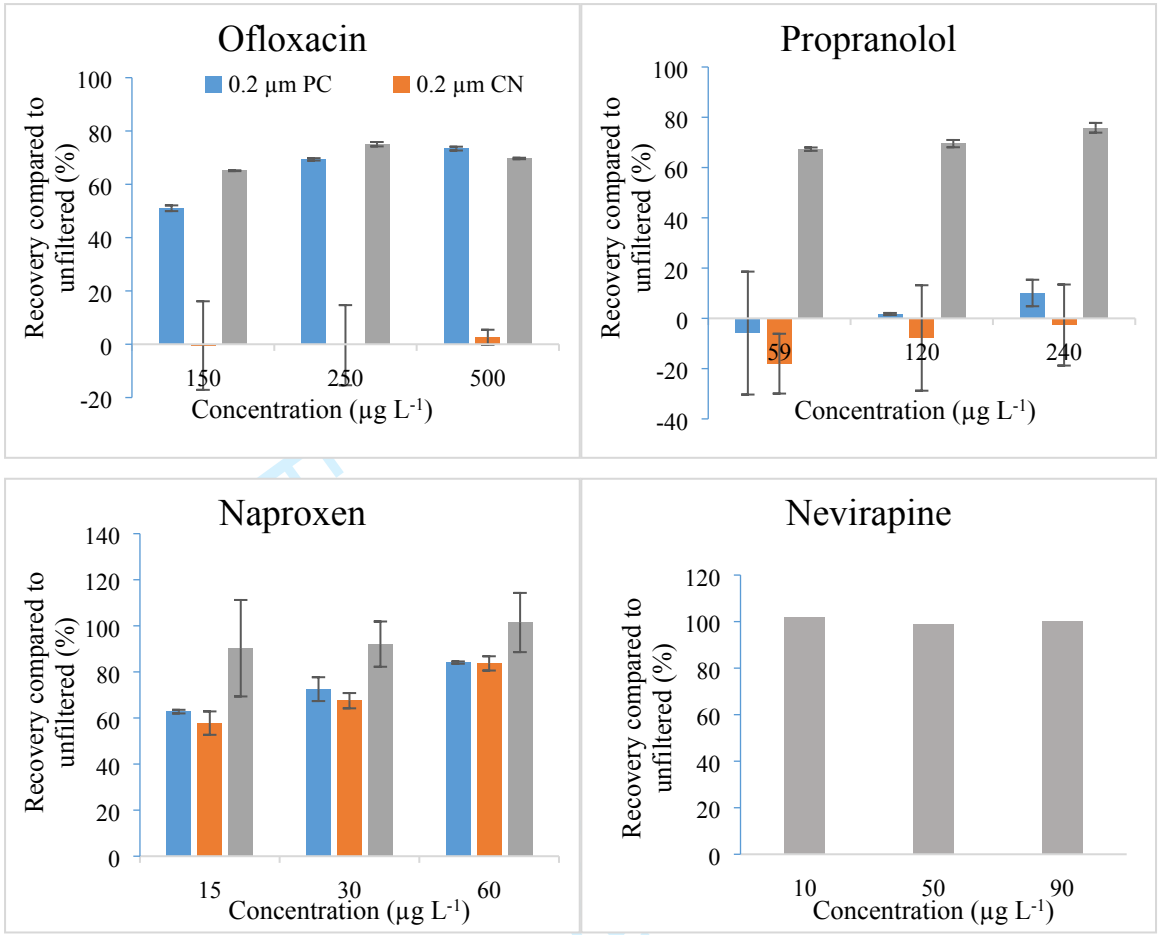



**Figure 4 Recovery of APIs following filtration (PC – polycarbonate, CN – cellulose nitrate, GF – glass fibre) ($\bar{x}$ ± RSD, n=3; except nevirapine, n=2 no RSD ). Legend is shown in ofloxacin graph.**

### 3.3 API and sample storage

The aim of this experiment was to test that the samples could be successfully stored at -20 °C without effecting the concentration of API in the samples. This was necessary as the HPLC-HRAM-MS was not always immediately available so adequate sample preservation was required until it could be used.

Concentrations of ofloxacin were significantly lower in the calibration solutions that had been stored frozen (ANOVA, $p \leq 0.05$). The range was 15-34 %, with the largest observed difference at 10 µg $L^{-1}$ and the lowest at 100 µg $L^{-1}$ (Figure S3). The other APIs were less influenced by this storage method. If samples were stored at -20 °C, a set of standard solutions was prepared alongside samples and stored in the same manner for consistency. Fresh API

calibration solutions were used to test the sensitivity and repeatability precision of the instrument.

### 3.4 Soil solution changes

The pH and DOC of the soil solutions are key variables in controlling the partitioning of organic chemicals and are rarely determined throughout the course of an equilibrium experiment. Data provided here shows that there was variation in the properties of the matrix after a period of shaking. pH of the loam soils started at 7.2 before reducing by 0.5 pH units to 6.7 over the 120 hours (Figure 5A). In contrast the sandy loam soil started at 6.1 then increased by 0.5 pH units to 6.6. The DOC results showed that the loam soil solution had a greater concentration of DOC throughout the experiments starting at 30 mg $L^{-1}$ before increasing over the shaking period to 44 mg $L^{-1}$ (Figure 5B). Sandy loam DOC started at 3.6 mg $L^{-1}$ before increasing to 9.4 mg $L^{-1}$. These are important considerations because such factors are rarely considered in soil testing experiments. Any increase in DOC in solution over the duration of the test is likely to lead to stabilisation of an API in solution, therefore reducing the calculated Koc and may also influence the attainment of equilibrium. The pH of soil suspensions influences the net charge on ionisable APIs and they will be fully ionised (> 99 %) when the pH is at ± 2 pH units from their $pK_a$ [18]. Variations in soil suspension pH over the length of the sorption experiment could therefore have an impact on the ionisation state of the APIs in question. Variation in pH could cause more significant changes to sorption behaviour in other compounds with a $pK_a$ around 6 under these conditions. Soil pH will also influence the pH-dependent charge on the organic matter, clay minerals and metal sesquioxide components of the soil, which may influence API sorption [44].

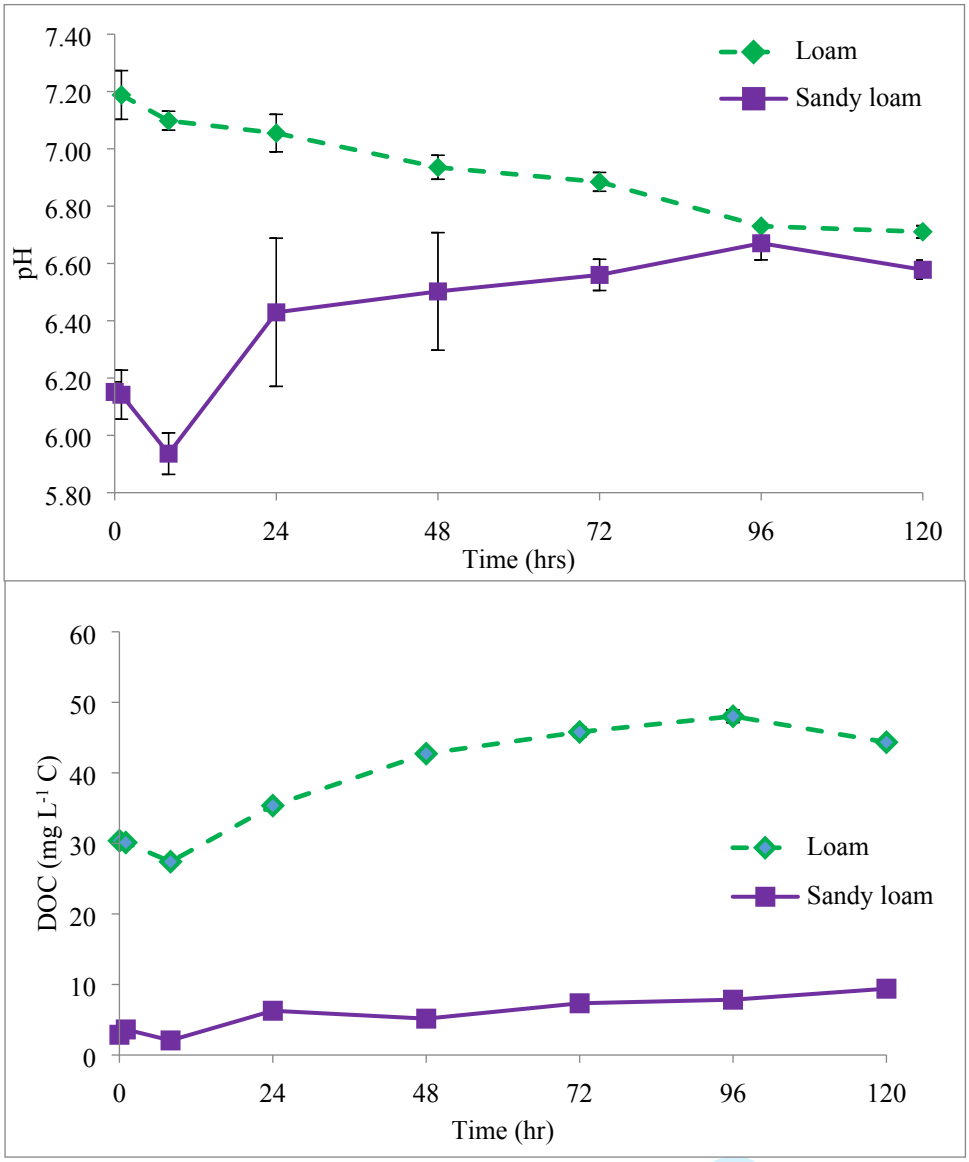



**Figure 5 A - pH of soil solutions over 120 hours shaking ($\bar{x}$ ± S.D. n = 3). B - DOC in soil solutions over 120 hours shaking ($\bar{x}$ ± S.D. n = 3)**

## 4. Conclusions

The ability to measure and understand the behaviour of APIs in soil is essential for any rigorous risk assessment associated with wastewater reuse or biosolid recycling to land, particularly in LLMIC where water and fertiliser availability are limited. An existing OECD testing methodology was fully evaluated for key variables which could impact on the quality of the data generated. Four APIs with wide ranging functionality, charge, molecular weight and sorption characteristics were used and data for naproxen provided as a case study to demonstrate the outcomes of applying such a technique to APIs. A full assessment of the sample preparation, sample stability, analytical method choice and performance and potential matrix interferences have been completed to enable practitioners and scientists to replicate the methodology for these and other APIs.

The data from the study shows that the choice of materials used for testing is crucial, with any filtration requiring glass fibre membranes, which restricts the minimum practical pore size to 0.7 µm. Adsorption experiments, such as the OECD 106 guideline, can be performed in polypropylene plastic centrifuge tubes with careful measurement of recoveries, which were 100% +/- 15% for the 4 APIs tested.

HPLC-HRAM-MS was shown to deliver LODs below 1 µg $L^{-1}$ for most matrices, with excellent precision and clear resolution of the eluting APIs. Ofloxacin was the most challenging API to measure, due to its high affinity for surface adsorption leading to instrument carry-over, and through loss to apparatus under certain conditions. This illustrated the importance of careful analytical quality control to ensure that useable data are obtained in the subsequent fate studies. Sample storage was shown to be viable and there were no long- term (5 day) impacts of matrix changes on analytical performance. Some matrix effects were observed for all 4 of the APIs tested, with quenching of the signal in all cases, particularly for ofloxacin,

consequently it is recommended that all analyses are undertaken alongside matrix-matched calibration.

Important aspects such as changes in solution pH and DOC over the course of the partitioning experiments should how important it is to monitor these critical variables which impact on the partitioning and fate of chemicals in the soil/water environment.

Overall, the data presented in this study shows the importance of systematic method development prior to undertaking soil risk assessment studies for APIs.

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## References


[1] Gautam, A. and X. Pan (2016). The changing model of big pharma: impact of key trends. *Drug Discovery Today* **21**(3): 379-384.

[2] Thebo, A. L., P. Drechsel, E. Lambin and K. Nelson (2017). A global, spatially-explicit assessment of irrigated croplands influenced by urban wastewater flows. *Environmental Research Letters* **12**(7): 074008.

[3] WHO (2006b). Guidelines for the safe use of wastewater, excreta and greywater. Volume 1 and 2. Geneva, WHO.

[4] WHO (2006c). Guidelines for the safe use of wastwater, excreta and greywater. Volume 3: Wastewater and excreta use in aquaculture. Geneva, WHO.

[5] WHO (2006d). Guidelines for the safe use of wastwater, excreta and greywater. Volume 4: Excreta and greywater use in agriculture. Geneva, WHO.

[6] Kookana, R. S., M. Williams, A. B. A. Boxall, D. G. J. Larsson, S. Gaw, K. Choi, H. Yamamoto, S. Thatikonda, Y.-G. Zhu and P. Carriquiriborde (2014). Potential ecological footprints of active pharmaceutical ingredients: an examination of risk factors in low-, middle- and high-income countries. *Philosophical Transactions of the Royal Society B: Biological Sciences* **369**(1656).

[7] Sorensen, J. P. R., D. J. Lapworth, D. C. W. Nkhuwa, M. E. Stuart, D. C. Gooddy, R. A. Bell, M. Chirwa, J. Kabika, M. Liemisa, M. Chibesa and S. Pedley (2015). Emerging contaminants in urban groundwater sources in Africa. *Water Research* **72**: 51-63.

[8]Tang Y., Guo L.L, Hong C.Y., Bing Y.X., Xu Z.C. (2017) Seasonal occurrence, removal and risk assessment of 10 pharmaceuticals in 2 sewage treatment plants of Guangdong, China. *Environmental Technology,* 40, 458-469.

[9] Huggett, D. (2017) Environmental risk assessment and new drug development. ADC Review DOI: DOI: 10.14229/jadc.2017.29.08.001.

[10] EMEA (2006). Guideline on the environmental risk assessment of medicinal products for human use. EMA/CHMP/SWP/447/00. London, European Medicines Agency.

[11] Reyes-Contreras C., Neumann P., Barriga F., Venegas M., Dominguez C., Bayona J.M. and Vidal G. (2018) Organic micropollutants in sewage sludge: influence of thermal and ultrasound hydrolysis processes prior to anaerobic stabilization. *Environmental Technology,* Published online: 22 Oct 2018.

[12] Nature (2015). Time to get clean. *Nature Editorial* **526**: 164.

[13] Septian A, Oh S. and Shin W.S. (2018) Sorption of antibiotics onto montmorillonite and kaolinite: competition modelling. *Environmental Technology,* 40, 2940-2953.

[14] Chefetz, B., T. Mualem and J. Ben-Ari (2008). Sorption and mobility of pharmaceutical compounds in soil irrigated with reclaimed wastewater. *Chemosphere* **73**(8): 1335-1343.

[15] Lin, K. and J. Gan (2011). Sorption and degradation of wastewater-associated non-steroidal anti-inflammatory drugs and antibiotics in soils. *Chemosphere* **83**(3): 240-246.

[16] OECD (2000). Test No. 106: Adsorption -- Desorption Using a Batch Equilibrium Method. Paris, OECD Publishing.

[17] Drillia, P., K. Stamatelatou and G. Lyberatos (2005). Fate and mobility of pharmaceuticals in solid matrices. *Chemosphere* **60**(8): 1034-1044.

[18] Lees, K., M. Fitzsimons, J. Snape, A. Tappin and S. Comber (2016). Pharmaceuticals in soils of lower income countries: Physico-chemical fate and risks from wastewater irrigation. *Environment International* **94**: 712-723.

[19] Zhang, Q.-Q., G.-G. Ying, C.-G. Pan, Y.-S. Liu and J.-L. Zhao (2015). Comprehensive Evaluation of Antibiotics Emission and Fate in the River Basins of China: Source Analysis, Multimedia Modeling, and Linkage to Bacterial Resistance. *Environmental Science & Technology* **49**(11): 6772-6782.

[20] Sun, Q., M. Lv, A. Hu, X. Yang and C.-P. Yu (2014). Seasonal variation in the occurrence and removal of pharmaceuticals and personal care products in a wastewater treatment plant in Xiamen, China. *Journal of Hazardous Materials* **277**: 69-75.

[21] Subedi, B., K. Balakrishna, R. K. Sinha, N. Yamashita, V. G. Balasubramanian and K. Kannan (2015). Mass loading and removal of pharmaceuticals and personal care products, including psychoactive and illicit drugs and artificial sweeteners, in five sewage treatment plants in India. *Journal of Environmental Chemical Engineering* **3**(4, Part A): 2882-2891.

[22] McGettigan, P. and D. Henry (2013). Use of non-steroidal anti-inflammatory drugs that elevate cardiovascular risk: an examination of sales and essential medicines lists in low-, middle-, and high-income countries. *PLoS Med* 10(2): e1001388.

[23] Wood, R. (2005). Nevirapine toxicity--implications for management of South African patients. *South African Medical Journal* **95**(4): 253-257.

[24] ChemSpider. (2015a). Naproxen. Retrieved 25 January, 2016, from http://www.chemspider.com/Chemical-Structure.137720.html?rid=b050566c-0ba9-4e0a-9f55-d63544f680d2

[25] ChemSpider. (2015b). Nevirapine. Retrieved 06 June, 2017, from http://www.chemspider.com/Chemical-Structure.4308.html?rid=d436f6df-a8b3-4eea-aacc-4a0db7f7f938

[26] ChemSpider. (2015c). Ofloxacin. Retrieved 01 September, 2015, from http://www.chemspider.com/Chemical-Structure.4422.html

[27] ChemSpider. (2015d). Propranolol hydrochloride. Retrieved 05 January, 2016, from http://www.chemspider.com/Chemical-Structure.56610.html?rid=8997f39b-5c2d-4d78-bc9d-a2a1b46e9256

[28] Nowara, A., J. Burhenne and M. Spiteller (1997). Binding of Fluoroquinolone Carboxylic Acid Derivatives to Clay Minerals. Journal of Agricultural and Food Chemistry 45(4): 1459-1463.

[29] DrugBank 4.3. (2013). Naproxen. Retrieved 25 January, 2016, from http://www.drugbank.ca/drugs/DB00788.

[30] Barron, L., J. Havel, M. Purcell, M. Szpak, B. Kelleher and B. Paull (2009). Predicting sorption of pharmaceuticals and personal care products onto soil and digested sludge using artificial neural networks. Analyst 134(4): 663-670.

[31] Xu, J., L. Wu and A. C. Chang (2009). Degradation and adsorption of selected pharmaceuticals and personal care products (PPCPs) in agricultural soils. Chemosphere 77(10): 1299-1305.

[32] Durán-Álvarez, J. C., B. Prado-Pano and B. Jiménez-Cisneros (2012). Sorption and desorption of carbamazepine, naproxen and triclosan in a soil irrigated with raw wastewater: Estimation of the sorption parameters by considering the initial mass of the compounds in the soil. Chemosphere 88(1): 84-90.

[33] Madikizela, L. M., N. T. Tavengwa and L. Chimuka (2017). Status of pharmaceuticals in African water bodies: Occurrence, removal and analytical methods. Journal of Environmental Management 193: 211-220.

[34] Kasim, N. A., M. Whitehouse, C. Ramachandran, M. Bermejo, H. Lennernäs, A. S. Hussain, H. E. Junginger, S. A. Stavchansky, K. K. Midha, V. P. Shah and G. L. Amidon (2004). Molecular Properties of WHO Essential Drugs and Provisional Biopharmaceutical Classification. Molecular Pharmaceutics 1(1): 85-96.

[35] Lufa Speyer. (2015). Analyses Data Sheet for Standard Soils according to GLP. Retrieved 19 February, 2016.

[36] FAO (2009) Food and Agriculture Organisation of the United Nations. Harmonized World Soil Database. Version 1.1, March 2009.

[37] Ho, C. S., C. W. K. Lam, M. H. M. Chan, R. C. K. Cheung, L. K. Law, L. C. W. Lit, K. F. Ng, M. W. M. Suen and H. L. Tai (2003). Electrospray Ionisation Mass Spectrometry: Principles and Clinical Applications. *The Clinical Biochemist Reviews* 24(1): 3-12.

[38] Kiser, M. and J. Dolan (2004) Selecting the Best Curve Fit. LCGC North America **22**, 112-117.

[39] Gu, H., G. Liu, J. Wang, A.-F. Aubry and M. E. Arnold (2014). Selecting the Correct Weighting Factors for Linear and Quadratic Calibration Curves with Least-Squares Regression Algorithm in Bioanalytical LC-MS/MS Assays and Impacts of Using Incorrect Weighting Factors on Curve Stability, Data Quality, and Assay Performance. *Analytical Chemistry* **86**(18): 8959-8966.

[41] Ciarlone, A. E., B. W. Fry and D. M. Ziemer (1990). Some observations on the adsorption of tetracyclines to glass and plastic labware. *Microchemical Journal* **42**(2): 250-255.

[40] European Commission (2002). Commission decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC). Brussels.

[44] Hyun, S. and L. S. Lee (2004). Hydrophilic and hydrophobic sorption of organic acids by variable-charge soils: effect of chemical acidity and acidic functional group. Environmental Science & Technology 38(20): 5413-5419.

[42] ICH Harmonized Tripartite Guideline (2005) Q2(R1) Validation of analytical procedures: text and methodology.

[43] Martínez-Hernández, V., R. Meffe, S. Herrera López and I. de Bustamante (2016). The role of sorption and biodegradation in the removal of acetaminophen, carbamazepine, caffeine, naproxen and sulfamethoxazole during soil contact: A kinetics study. *Science of The Total Environment* **559**: 232-241.

# Developing the OECD 106 fate testing protocol for active pharmaceuticals in soil

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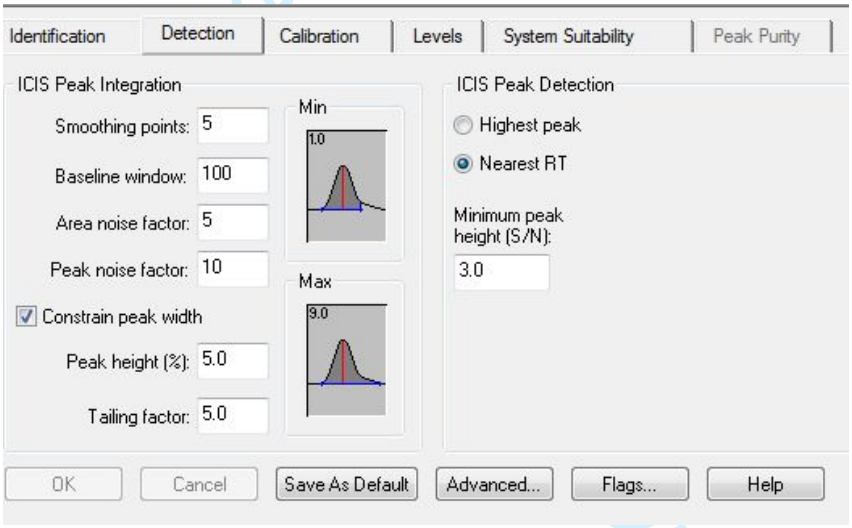


**Figure S1. Peak detection and processing method from Thermo Scientific Xcalibur software**

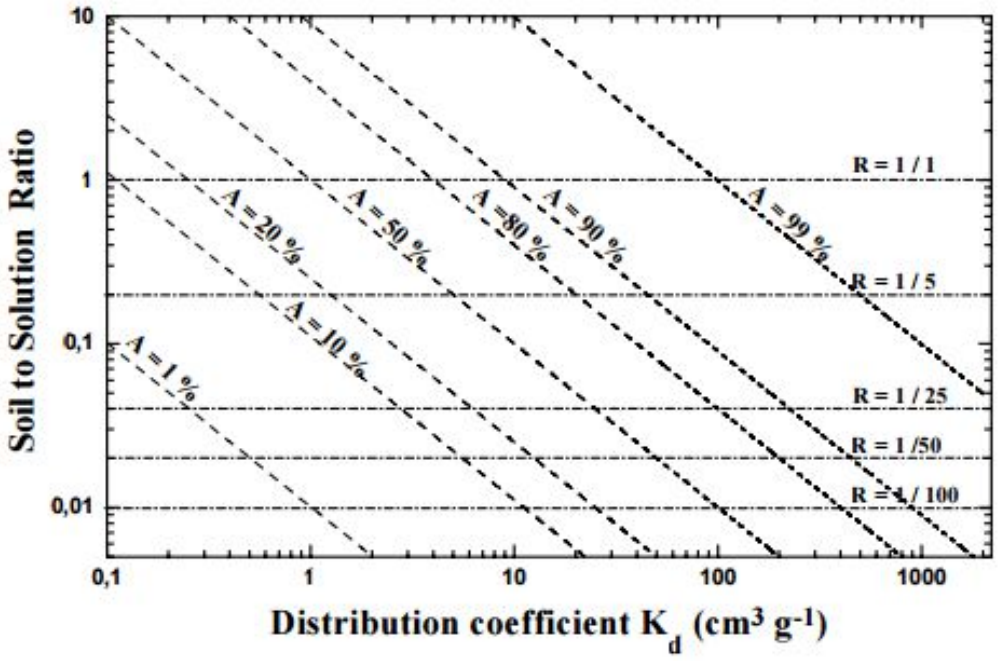


**Figure S2 Relationship between soil : solution ratios and $K_d$ at various percentages of adsorbed test substance; A = adsorption, R = soil : solution ratio [1].**

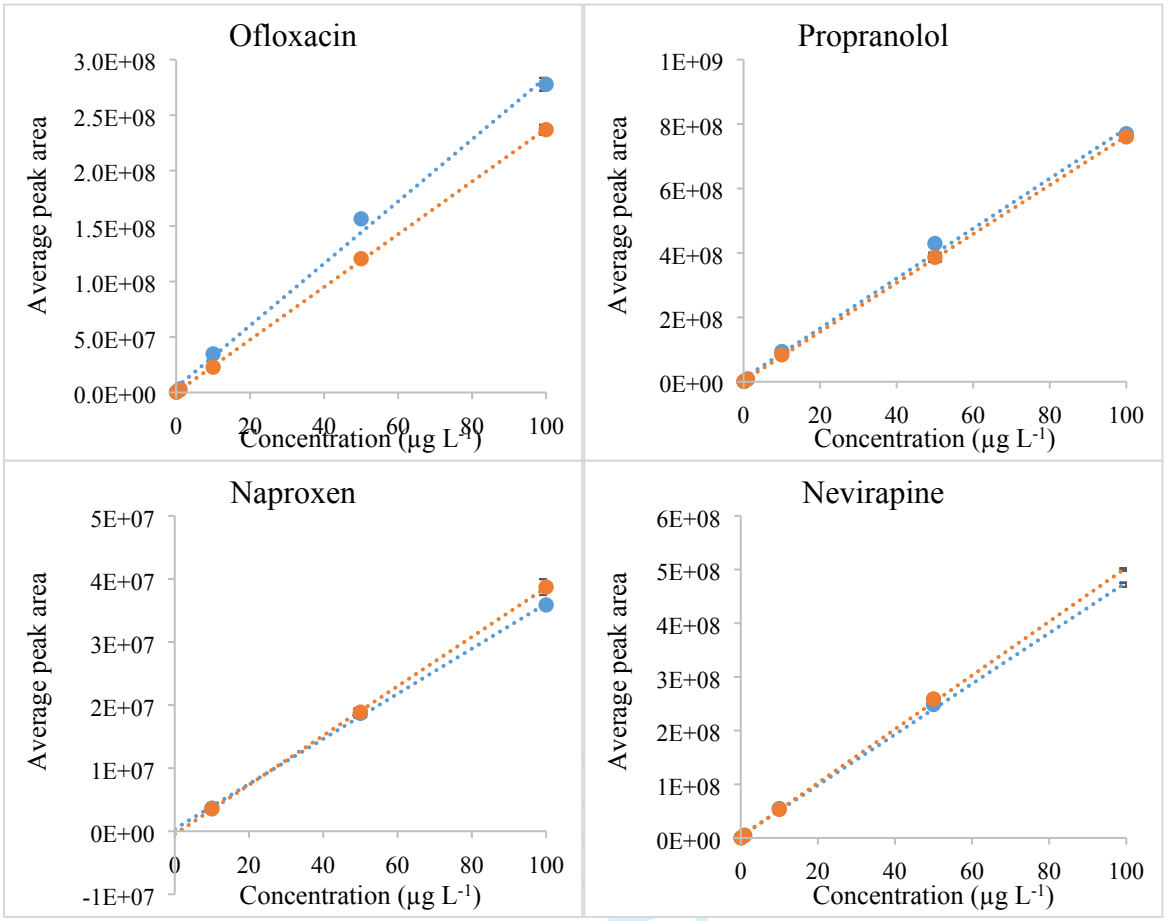


**Figure S3. Effects of storage on API calibrations. ● = freshly prepared, ● = frozen. Error bars were calculated but are not visible in most cases ($\bar{x} \pm$ s.d. n=3)**

**Table S1. Calibration concentrations for each API (µg $L^{-1}$)**

| Cal label | Ofloxacin | Propranolol | Naproxen | Nevirapine |
|---|---|---|---|---|
| C1 | 0.1 | 0.8 | 0.3 | 0.4 |
| C2 | 0.625 | 5 | 1.875 | 2.5 |
| C3 | 1.25 | 10 | 3.75 | 5 |
| C4 | 5 | 40 | 15 | 20 |
| C5 | 10 | 80 | 30 | 40 |
| C6 | 20 | 160 | 60 | 80 |
| C7 | 25 | 200 | 75 | 100 |

**Table S2. Calibration data in all matrices**

| API | Matrix | Equation | $R^2$ |
|---|---|---|---|
| **Ofloxacin** | HPW | $y = 2.48x10^6 \chi + 802007$ | 0.9945 |
| | Loam | $y = 2.21\ x10^6 \chi + 105035$ | 0.9925 |
| | Sandy loam | $y = 2.75\ x10^6 \chi + 18175$ | 0.9997 |
| | SWW | $y = 0.93\ x10^6 \chi + 930210$ | 0.9689 |
| | SWW + loam | $y = 0.76\ x10^6 \chi + 25681$ | 0.9917 |
| **Propranolol** | HPW | $y = 7.87x10^6 \chi + 3.66x10^6$ | 0.9925 |
| | Loam | $y = 7.51x10^6 \chi + 4.87x10^6$ | 0.9950 |
| | Sandy loam | $y = 7.51x10^6 \chi + 2.08x10^7$ | 0.9934 |
| | SWW | $y = 6.20x10^6 \chi - 5552\ \chi^2 + 1.27x10^6$ | 0.9994 |
| | SWW + loam | $y = 8.11x10^6 \chi - 7194\ \chi^2 + 2.37x10^6$ | 0.9981 |
| | SWW + sandy loam | $y = 6.34x10^6 \chi - 6409\ \chi^2 + 0.8\ x10^6$ | 0.9995 |
| **Naproxen** | HPW | $y = 0.29x10^6 \chi - 464824$ | 0.9981 |
| | Loam | $y = 0.27x10^6 \chi - 438441$ | 0.9986 |
| | Sandy loam | $y = 0.25x10^6 \chi - 453599$ | 0.9984 |
| | SWW | $y = 0.42x10^6 \chi - 74745$ | 0.9994 |
| | SWW + loam | $y = 0.46x10^6 \chi - 51935$ | 0.9992 |
| | SWW + sandy loam | $y = 0.41x10^6 \chi - 74212$ | 0.9997 |
| **Nevirapine** | HPW | $y = 5.13x10^6 \chi - 199410$ | 0.9993 |
| | Loam | $y = 0.26x10^6 \chi - 438441$ | 0.9986 |
| | Sandy loam | $y = 5.24x10^6 \chi + 445596$ | 0.9986 |
| | SWW | $y = 4.45x10^6 \chi + 69675$ | 0.9995 |
| | SWW + loam | $y = 6.82x10^6 \chi + 1.93x10^6$ | 0.9955 |
| | SWW + sandy loam | $y = 4.84x10^6 \chi + 345717$ | 0.9986 |

**Table S3 Concentration of APIs used to calculate precision**

| | Low (µg $L^{-1}$) | Middle (µg $L^{-1}$) | High (µg $L^{-1}$) |
|---|---|---|---|
| **Ofloxacin** | 1.25 | 10 | 25 |
| **Propranolol** | 10 | 80 | 200 |
| **Naproxen** | 7.5 | 60 | 150 |
| **Nevirapine** | 7.5 | 60 | 150 |

**Table S4. Relative standard deviation of HPLC-HRAM-MS method for APIs at low, middle and high concentrations**

| | Low (%) | Middle (%) | High (%) |
|---|---|---|---|
| **Ofloxacin** | 94 | 23 | 17 |
| **Propranolol** | 1 | 3 | 6 |
| **Naproxen** | 4 | 4 | 4 |
| **Nevirapine** | 3 | 4 | 4 |

**Equation S1 HPLC-HRAM-MS Limit of detection [2]**

$$LOD = \frac{(3.3\ \sigma)}{S}$$

Where σ = standard deviation of the y-intercepts of the regression line and S = slope of the calibration curve.

**References**

[1] OECD (2000). Test No. 106: Adsorption -- Desorption Using a Batch Equilibrium Method. Paris, OECD Publishing.

[2] ICH Harmonized Tripartite Guideline (2005) Q2(R1) Validation of analytical procedures: text and methodology.