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# Study of the uptake of non-steroid anti-inflammatory drugs in wheat and soybean after application of sewage sludge as a fertilizer

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## HIGHLIGHTS

- ► Wheat and soybeans were grown in soil fertilized with sewage sludge.
- ► Sludge as well as crops were analyzed for four anti-inflammatory drugs.
- ► Only two of the compounds (ibuprofen and diclofenac) were detected in the sludge.
- ► No uptake of any of the drugs could be shown in neither wheat nor soybean.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Non-steroid anti-inflammatory drugs (NSAIDs) are frequently occurring in sludge and waters from sewage treatment plants (STPs). Sludge obtained from sewage treatment is often applied as a fertilizer in agriculture and not many studies about the uptake of pharmaceuticals into crops can be found. In this paper, we present a greenhouse experiment to study the presence of four NSAIDs (naproxen, ketoprofen, diclofenac and ibuprofen) in two different crops (soybean and wheat) after application of sludge as a fertilizer. Two different amounts of sludge were added to the soil; the recommended amount (with respect to phosphorus content) and the double recommended amount. One treatment without sludge was also included as a blank. The crops were harvested after 60 as well as 110 days. Only diclofenac and ibuprofen were detected in the sludge in concentrations of 22 and 217 ng $g^{-1}$ dry weight, respectively. None of the NSAIDs were detected in the crops from any of the treatments. Compared to the amounts applied to the soil, detection limits correspond to an uptake of less than 2% for diclofenac and 0.8% for ibuprofen.

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## 1. Introduction

Fertilization of farmland with sewage sludge today is a heavily debated issue worldwide due to the concerns related to potential harm on the environment as well as human health from contaminants present in the sludge (Bengtsson and Tillman, 2004). Human pharmaceuticals raise a special interest due to their biological activity and the fact that a number of studies have shown their presence in several compartments of the environment (Halling-Sörensen et al., 1998; Belfroid et al., 1999; Heberer et al., 2002; Spongberg and Witter, 2008; Brausch et al., 2012; Dong et al., 2013) as well as adverse effects

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on aquatic species (Triebskorn et al., 2004; Gravel and Vijayan, 2007; Quinn et al., 2008; Quinn et al., 2009; Mehinto et al., 2010; Gonzalez-Rey and Bebianno, 2012).

NSAIDs (Non steroid anti-inflammatory drugs), including among others naproxen, ketoprofen, ibuprofen and diclofenac, belong to the pharmaceuticals most frequently detected in aquatic environments due to their extensive consumption (Gentili, 2007; Nieto et al., 2007; Sebök et al., 2008; Maijo et al., 2012; Migowska et al., 2012). Only in Sweden, over 200 t of these four substances were altogether sold during 2010 (Apotekens Service, 2011).

During 2008, 26% of the sewage sludge produced in Sweden was applied as a fertilizer onto farmland while 27% was used as a filling material in construction of parks, recreation grounds etc. and 20% for covering of old landfills (Formas, 2012).

Several studies have shown the presence of NSAIDs in incoming water to STPs (González-Barreiro et al., 2003; Radjenović et al., 2007; Jelić et al., 2011; Falås et al., 2012) as well as in digested sludge with detected amounts in sludge usually in the range of 10–100 ng $g^{-1}$ dry weight (Jelić et al., 2009; Sagristà et al., 2010; Saleh et al., 2011). There is a concern that if such sludge is used for the fertilization of food crops, NSAID residues might be taken up into the plants and thereby contaminate human as well as livestock food. Wu et al. showed the uptake of several pharmaceuticals and personal care products in soybeans grown in soils fertilized with biosolids and irrigated with contaminated water (Wu et al., 2010). Another study showed the uptake of 17α-ethynylestradiol and triclosan in pinto beans (Karnjanapiboonwong et al., 2011), but the sand and soil used in the experiment were spiked repeated times to maintain a concentration of 1 μg/g of the test substances which is well above actual environmental concentrations. In the same way, Eggen et al. showed the uptake of metformin, ciprofloxacin and narasin in forage and crop plants, but also here the soil was spiked to an initial concentration of 10 mg/kg of all compounds at the beginning of the experiment (Eggen et al., 2011). Winker et al. investigated the uptake of carbamazepine and ibuprofen in ryegrass fertilized with urine (Winker et al., 2010). Carbamazepine was shown to persist in soil as well as taken up into plants while no ibuprofen was detected in neither soil nor plants after three months. These studies show that uptake of certain pharmaceuticals into plants takes place. Nevertheless in all the cases, large amounts of the drug compounds were applied, not mimicking the real conditions where non-spiked sludge is applied onto farmland.

Several different methodologies have been applied for the extraction of pharmaceuticals from plant material, such as microwave assisted extraction, ultrasonic solvent extraction (Chitescu et al., 2012) and pressurized liquid extraction (Wu et al., 2010). In many cases, solid phase extraction (SPE) is also necessary as a subsequent clean-up and preconcentration step.

In our group we recently developed an alternative technique for the extraction of NSAIDs in sludge by applying hollow fiber liquid phase microextraction (HF-LPME), as a clean-up step after pressurized hot water extraction (PHWE), avoiding the consumption of high amounts of organic solvents employed in other methodologies (Saleh et al., 2011). Liquid chromatography–mass spectrometry analysis with electrospray-ionization interface (LC-ESI-MS) was employed to analyze the extracts obtained after the HF-LPME.

The aim of this study was to apply the PHWE + HF-LPME method for the determination of NSAIDs in plant material and to study the possible presence of these compounds in crops fertilized by sewage sludge in the amounts recommended for such application.

## 2. Experimental

### 2.1. Chemicals and solutions

Ketoprofen, naproxen, ibuprofen and diclofenac sodium salt were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Di-*n*-hexylether (DHE), ammonium carbonate (containing 30–33% $NH_3$) and sulfuric acid (95–97% pure) were supplied by Fluka (Buchs, Switzerland). Methanol, analytical reagent grade, used for preparation of standard solutions as well as mobile phase in LC separations, was obtained from Honeywell B&J brand (Sleeze, Germany). Glacial acetic acid, used for adjusting pH in the mobile phase, and ammonium acetate were purchased from Merck (Darmstadt, Germany). Reagent sodium hydroxide was obtained from Scharlau Chemie S.A. (Barcelona, Spain). Reagent water was obtained from a MilliQ water purification system (Millipore, Billerica, MA, USA).

Individual stock standard solutions containing 100 mg $L^{-1}$ of ketoprofen, naproxen, diclofenac and ibuprofen were prepared in methanol. Working solutions of a mixture of all four NSAIDs were prepared by appropriate dilution. The standards for calibration curves were prepared by dilution in the acceptor phase used for HF-LPME. All the solutions were kept at 4 °C in darkness. Acceptor buffer solution (0.1 M ammonium carbonate) was prepared in water by dissolving an appropriate amount of the salt.

### 2.2. Experimental setup

Samples of digested sludge were collected from Källby STP (Lund, Sweden) in October 2010 after storage outdoor for six months. This plant treats the sewage of a population of 86,000 persons (VASyd, 2011). The sewage water undergoes primary sedimentation, biological treatment with activated sludge and finally chemical precipitation of phosphate. The sludge from the biological and chemical treatment steps is returned to incoming water and sludge is only removed from the system during primary sedimentation. After dewatering, the sludge is anaerobically digested under mesophilic conditions (37 °C) for 20–30 days where after it undergoes further dewatering. The sludge had a pH of 8.0, dry substance content of 26% (weight), ignition loss of 61% of the dry substance and tot-N of 46 g/kg. Källby STP produces approximately 5000 t of sludge each year. After leaving the plant, the sludge undergoes an additional storage for six months outdoors prior to application onto farmland. The amount applied during fertilization is based on the phosphorous (P) content in each sludge stock, to result in a P supply of 16.4 kg P/ha (= 10,000 $m^2$)/year during a five-year period. For this specific sludge stock this figure corresponded to 14.8 t sludge/ha/year. Transformed into small scale conditions this is equal to 3 g/$dm^2$/year. All of the sludge used in the experiment originated from the same stock, and two different amounts were used, the recommended amount (10 g per plant pot) and double recommended amount (20 g per pot), each pot containing 1300 g of potting soil.

Soybean (*Glycine max*) and wheat (*Triticum aestivum*) were used for the uptake study. These species were chosen since wheat is one of the most common crops fertilized with sludge in Sweden and soybeans have shown uptake of other pharmaceuticals in a previous study (Wu et al., 2010). The seeds were obtained from Nelson Garden AB (Tingsryd, Sweden). For each sludge supply (10 and 20 g per 1300 g soil) 10 replicates were prepared for wheat and soybean respectively. Also five pots for each crop without any sludge addition were included as blanks resulting in altogether 50 pots. Plastic pots with the volume of 4 L were used for the experiment. Peat-based potting soil (Kronmull ®) was obtained from Weibulls Horto (Hammenhög, Sweden). The dry substance of the peat fraction corresponded to 80 kg/$m^3$ with an organic fraction of min. 95% and the humification degree H3–H5 (von Post). The soil contained 4% clay, 3% sand and 180, 90 and 195 g/$m^3$ of N, P and K respectively. The pH was 5.5–6.5.

The soil was added in small portions to the pots and mixed with the sludge by hand. Six seeds were planted in each pot, and at least four seeds germinated in each pot. All the pots were kept in a glass greenhouse under artificial light (16 h/day) and watered daily.

### 2.3. Sample preparation

A grab sample of the sludge used for fertilization was analyzed for NSAIDs according to a previous work from our group with slight modifications (Saleh et al., 2011). After 60 as well as 110 days, the plant parts above the soil (i.e. not the roots) were harvested and subjected to chemical analysis. During the duration of the experiment no seeds or beans were formed on neither wheat nor soybean plants, i.e. the analysis was performed on the leaves of each plant type. Since this study was aimed at assessing the risk of presence of pharmaceuticals mainly in the edible part of the crops, the roots were not harvested and analyzed. Also, it is difficult to separate the roots from the soil which could lead to false positives if sludge residues are still present on the roots. All samples were dried at 40 °C in an oven. The sludge was crushed by a mortar, and the plants were crushed by a blender, and sieved (0.5 mm) to obtain the same particle size.

#### 2.3.1. Extraction

PHWE was used for the extraction of NSAIDs. The same parameters as in the previous study for sewage sludge were applied (Saleh et al., 2011). A Dionex ASE-300 (Sunnyvale, CA, USA) Accelerated Solvent Extractor with stainless steel extraction cells (33 $cm^3$ volume) was used. Summarizing, 0.5 g of dried sample (sludge or plant material) was transferred into the cell, and mixed with 20 g of sea sand (previously washed with acetone and water and dried to 200 °C). The extraction conditions were: extraction solvent: 0.01 M NaOH in reagent water, extraction temperature: 120 °C, extraction pressure: 100 bar, preheating time: 6 min, static extraction time: 5 min, number of cycles: 5, flush volume: 90% of the cell volume and nitrogen purge time: 60 s. The final extraction volume was about 90 mL after 5 successive cycles and the extract was transferred into a 100 mL volumetric flask and adjusted to pH 1.5 with sulfuric acid before HF-LPME extraction.

#### 2.3.2. Clean-up and pre-concentration

Hollow fiber liquid-phase microextraction was applied as the clean-up and preconcentration step. Accurel Q3/2 polypropylene hollow fibers with a wall thickness of 200 μm (0.2 μm pore size) and an internal diameter of 600 μm (Membrana GmbH, Wuppertal, Germany) were used. The fibers were cut into 10 cm pieces and one of the ends connected to a 100 μL syringe (Hamilton, Bonaduz, Switzerland) holding the acceptor phase (ammonium carbonate 0.1 M). The syringe was used to introduce the acceptor phase into the membrane and to hold the membrane during the extraction. After the fiber was filled with acceptor phase, the end was sealed by a piece of aluminum foil. Then, the fiber was immersed in DHE during 15 s to fill the pores with organic solvent and in water during another 15 s to remove the excess of organic solvent.

All the experiments were carried out using a magnetic stirrer (IKA-Werke, Staufen, Germany) at 600 rpm.

When the extraction was completed (4 h), the fiber was removed from the donor solution, the closed end was opened and the extract (acceptor phase) was aspirated into the syringe and transferred into an autosampler vial for analysis by LC-MS.

In previous works in our group the parameters for HF-LPME were optimized (Saleh et al., 2011). Here, we only optimized the extraction time since it could differ between sludge and plant matrices. The performance was evaluated based on the enrichment factor (*Ee*), defined as in Eq. (1)

$$Ee = \frac{C_{Ae}}{C_{Di}} \quad (1)$$

where $C_{Ae}$ is the concentration of a compound in the acceptor solution at equilibrium and $C_{Di}$ is the concentration in the sample solution at the beginning of the extraction.

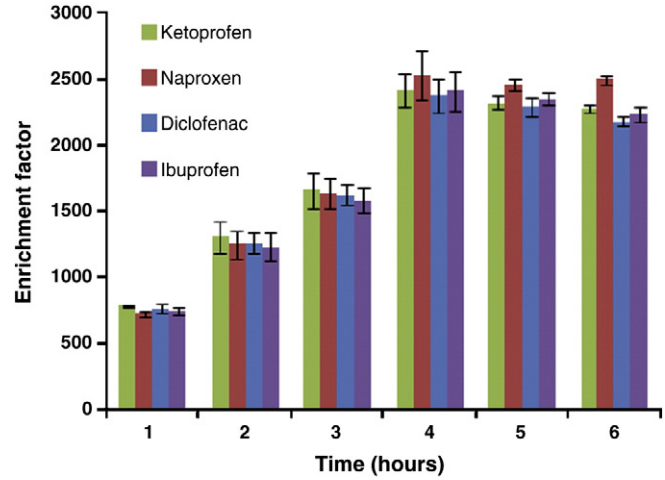


**Fig. 1.** Effect of extraction time for the NSAIDs in water when HF-LPME is used. Conditions used: Ammonium carbonate as acceptor phase, 600 rpm stirring rate and water adjusted at pH 1.5. Standard deviations (n = 3) are marked.

Fig. 1 shows the results obtained. As can be seen the enrichment factor quickly increases from one to four hours, while from four to six hours it slightly decreases, so four hours was chosen as the optimum extraction time. Relative standard deviations for extracted samples ranged between 1.6 and 18.3%, the values obtained for the extraction time chosen being between 10.8 and 14.3%.

### 2.4. LC-ESI-MS analysis

The LC system was composed by two Waters 515 pumps (Waters, Milford, MA, USA), a vacuum degasser, a Triathlon autosampler (Spark-Holland, Emmen, Netherlands) equipped with a 250 μL syringe, 100 μL sample loop, 15 μL needle tubing volume and 500 μL buffer tubing. The injection was made in μL pick-up mode with an injection volume of 10 μL. An ODS-2 Hypersil (5 μm, 100 × 2.1 mm, (Thermo Scientific, Waltham, MA, USA)) column, a $C_8$ precolumn (Phenomenex, Torrance, CA, USA) and a single quadrupole mass spectrometer (Waters Micromass ZMD) with electro-spray ionization (ESI) interface were used. Chromatographic separation was carried out using a gradient of mobile phases A (10 mM ammonium acetate adjusted to pH 4 with acetic acid) and B (methanol). The gradient started with linear increase of mobile phase B from 50% to 65% in 10 min and kept at 65% during 5 min, after that an increase to 85% in 1 min and kept during 5 min to clean the column. After this cleaning, initial conditions were fixed with an equilibration time of 5 min before the next analysis. The flow rate was 0.25 mL $min^{-1}$.

Data acquisition was performed in negative ion mode and optimized MS parameters for the analysis were the following: capillary voltage was 3.5 kV, cone voltage was 8 V for ketoprofen and naproxen; and 10 V for diclofenac and ibuprofen, ESI source block temperature was 150 °C, desolvation temperature was 350 °C, and desolvation gas flow was 540 L $h^{-1}$. Selective ion monitoring was used to detect ions with m/z ratios of 253 (ketoprofen), 229 (naproxen), 294 (diclofenac) and 205 (ibuprofen). Calibration curves for each compound from 0.05 to 10 mg $L^{-1}$ were prepared, and the correlation coefficients were higher than 0.998 in all cases.

Repeatability (n = 5) and reproducibility (n = 4) for the LC-analysis were also evaluated, by injections of samples with the concentration 0.1 mg $L^{-1}$ with relative standard deviation values in the range of 2.5–4.7% for intra-day and 5.1–17.3% for inter-day analyses.

## 3. Results and discussion

### 3.1. Determination of NSAIDs in sludge

Measured PHWE recoveries were used to calculate the concentration of the pharmaceuticals in sludge as described earlier (Saleh et al., 2011).

Surprisingly, only two drugs (diclofenac and ibuprofen) were detected in the stored sludge (Table 1). Previous studies (Sagristà et al., 2010; Saleh et al., 2011) have shown the presence of all four targeted NSAIDs in sludge, although in those cases the sludge analyzed was obtained directly from the STP without outdoor storage. This suggests that degradation took place during the storage. The recoveries obtained for diclofenac and ibuprofen were 89 and 49% respectively, in agreement with the earlier study (Saleh et al., 2011). Recoveries for ketoprofen and naproxen were not calculated due to the absence of these analytes in the sludge.

Standard addition after PHWE was used for the quantification of the analytes in PHWE extracts. Eq. (3) was used for the determination of the concentration of diclofenac and ibuprofen in the sludge. (Sagristà et al., 2010).

$$C_{\mathrm{Ae}} = \frac{m_{Di}}{\mathrm{A}} + \frac{m_{\mathrm{Si}}}{\mathrm{A}} \tag{3}$$

where $m_{\mathrm{Si}}$ is the initial amount of the compound in the sample, $m_{Di}$ is the initial amount in the donor phase, $C_{\mathrm{AE}}$ is the concentration in acceptor phase and A is a constant. If samples are spiked with different concentrations and analyzed and $C_{\mathrm{AE}}$ is plotted versus $m_{Di}$, the initial amount of each analyte in the sludge or plant material can be calculated as the intercept divided by the slope (1/A) of a linear regression line adjusted to the data points. The concentrations obtained were 22 ng $g^{-1}$ for diclofenac and 217 ng $g^{-1}$ for ibuprofen.

As can be seen in Table 1, these values are in the same range as in other works, however, all the NSAIDs analyzed were found in these previous works.

It is difficult to draw conclusions too far since this specific sludge stock was never analyzed for NSAIDs before storage and the potential actual decrease in analyte concentrations thereby cannot be deduced. However, since two independent studies have previously shown the presence of all four NSAIDs in non-stored sludge from the same STP at several different occasions (Sagristà et al., 2010; Saleh et al., 2011), these results indicate that degradation seems to take place during storage and that ketoprofen and naproxen seem more susceptible to degradation than diclofenac and ibuprofen. Highest amounts after storage are still found of ibuprofen, which probably is related to the fact that it is by far the most consumed of the four NSAIDs.

### 3.2. Determination of NSAIDs in crops

As mentioned in the experimental section, two different plant species were studied, soybean and wheat. Two different treatments were employed as well as a blank as control and both crop types were harvested after 60 as well as after 110 days.

The analysis method was the same as for the sludge. No presence of any NSAID was detected in the crops from any of the treatments. The mean weight of the plant material analyzed was 7.5 g dry weight per plant. Assuming that the detection limit for the two analytes are same as in reference (Saleh et al., 2011), which is reasonable as the extraction and analysis method is the same (0.4 ng/g for diclofenac and 1.4 ng/g for ibuprofen), the amount of drugs in each plant is less than 3 ng and 10.5 ng, respectively. To each pot, maximum of 20 g of sludge was added, containing 132 ng and 1300 ng, respectively (considering a dry weight of 30%). Thus, an amount of less than 2% of the diclofenac and less than 0.8% of the ibuprofen present in the sludge was taken up into the plants.

In the majority of previously published plant uptake studies spiking has been performed, either of the sludge or the soil. This means firstly that plants are exposed to higher concentrations than what actually occurs in the sludge and secondly a risk that compounds added directly to the soil in the beginning of the experiment are not as strongly sorbed as those native in the sludge and thereby more accessible for the plants which could lead to a possible overestimation of the uptake. When designing this study, care was taken to ensure realistic conditions, mimicking the situation on actual farmland. Sludge which had been subjected to the required storage (6 months) was used. Also, the same amount of sludge per g soil as should have been applied onto the field was used, calculated based on the P content of this specific stock and no spiking of neither sludge, nor soil was performed. The analysis of the sludge showed that two of the investigated analytes, ibuprofen and diclofenac, were present in the sludge in detectable and realistic concentrations and thereby available for the possible uptake by grown crops.

During the duration of the experiment (110 days in total), no seeds, nor beans were formed on neither wheat nor soybean plants which means that the specific parts used for food production could not be analyzed. Instead, the whole part of the plant above ground was harvested and analyzed. This means that potential concentrations found in plant samples would not necessarily have to reflect the concentration in the edible part of the plant. However, it is unlikely that detectable amounts of the analytes would accumulate in the seeds if not present in other parts of the plant tissue. The fact that no detectable amounts were found in plants from neither the treatment with the recommended sludge supply nor the one with the double recommended supply indicates that if an uptake of NSAIDs into wheat and soybean takes place it is very small and the potential risk to consumers is minor.

**Table 1**
Comparison of the concentrations obtained in different works.

| Concentration ng $g^{-1}$ (dry weight) | | | | |
|---|---|---|---|---|
| Ketoprofen | Naproxen | Diclofenac | Ibuprofen | Reference |
| 51.3–89.6 | 7.7–14.1 | 13.7–22.9 | 304–588 | Saleh et al. (2011)[a] |
| 29 | 138 | 39 | 122 | Sagristà et al. (2010) |
| 30.3–211.2 | n.a | 143.2–209.1 | 299.3–547.9 | Radjenović et al. (2007)[b] |
| n.d | n.d | 22 | 217 | This work[c] |

n.a.: not analyzed.
n.d: not detected.
[a] Sludge obtained during different months.
[b] Sludge obtained in different points in the sewage treatment plant.
[c] Average of three replicates.

## 4. Conclusions

This study shows that no detectable uptake of the non steroid anti-inflammatory drugs ketoprofen, naproxen, diclofenac and ibuprofen into wheat or soybean takes place during sewage sludge fertilization, even when a sludge amount twice as high as recommended is applied. Thus the risk of exposure to these compounds via crops grown in sludge-fertilized soil is considered very small. It also suggests that degradation takes place during outdoor storage of the sludge, thus resulting in partial or complete removal of these compounds.

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