

Anti-inflammatory and  
analgesic drugs in WWTP  
influent and effluent  
streams and the occurrence  
in the aquatic environment

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<b>Title and subtitle of the report</b> Anti-inflammatory drugs in WWTP influents and effluent and the occurrence in the aquatic environment.	
<b>Summary</b> IVL has performed a follow-up study on anti-inflammatory and analgesic drugs on commission from the Swedish EPA. In the first project was sewage water and sludge investigated. This investigation was focused on the occurrence of these drugs in surface water, sediment and fish in a watercourse influenced by the local WWTP. For this purpose was an analytical method developed capable to concentrate, separate and detect free and conjugated anti-inflammatory drugs.  Eight pharmaceutical drugs were analysed: paracetamol, acetyl salicylic acid, salicylic acid, ibuprofen, naproxen, ketoprofen, diclofenac and two metabolites of ibuprofen. Conjugated anti-inflammatory drugs were detected at low concentrations ( $\leq 1$ % of total) in influent and effluent. In the water and sediment samples were all the analysed drugs detected but at somewhat different pattern than in the sewage water. The anti-inflammatory drugs and paracetamol were not detected in fish collected in the vicinity of the WWTP discharge point.	
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## Sammanfattning

IVL Svenska Miljöinstitutet har på uppdrag av Naturvårdsverket genomfört en uppföljning av screeningundersökningen från år 2005 som behandlade förekomsten av läkemedel bl a. anti-inflammatoriska läkemedel i den svenska miljön (Andersson et al. 2006). Resultaten från denna undersökning visade att reningsverken i norra Sverige uppvisade högre halter av en del av anti-inflammatoriska läkemedel än de i söder samt att reningsverk i en del fall hade högre halter i utgående vatten av läkemedlen än i det inkommande vattnet.

I provtagningsprogrammet ingick inte tillräckligt med prover från berörda recipienter för att kunna bedöma hur belastningen ser ut i miljön kring reningsverken.

För att belysa dessa frågor bättre genomfördes provtagningar i Piteå älvs vattensystem uppströms och nedströms reningsverket vid Sandholmen, som var ett av de reningsverk som hade de högsta utgående halterna av anti-inflammatoriska läkemedel.

Syftet med föreliggande studie var:

- att undersöka om anti-inflammatoriska läkemedlen kommer till reningsverket i konjugerad form och om man på så vis kan förklara varför halterna i utgående vatten i en del fall är högre än inkommande,
- att undersöka om anti-inflammatoriska läkemedlen kan påvisas i vatten, sediment och fisk nedströms ett avloppsreningsverk.

Följande ämnen ingick i uppdraget: ibuprofen, ketoprofen, naproxen och diklofenak vilka även ingick i den tidigare undersökningen. Till dessa lades i denna undersökning även två andra viktiga läkemedel, paracetamol och acetylsalicylsyra, samt metaboliterna salicylsyra, hydroxy-ibuprofen och karboxy-ibuprofen.

För att kunna analysera den konjugerade formen av anti-inflammatoriska läkemedel utvecklades en analysmetod för ändamålet. Metoden bygger på fastfasextraktion av konjugaten följt av en separation av fria och konjugerade föreningar. De konjugerade anti-inflammatoriska läkemedlen hydrolyserades därefter med enzymet glukuronidas till den fria formen som kunde analyseras med den metod som tidigare har använts (Andersson et al. 2006).

Halten av anti-inflammatoriska läkemedel i utgående vatten var vid det första av två provtagningsstillfällena högre än i inkommande vatten. I det inkommande avloppsvatten var bidraget från konjugerade former mindre än 1 % av totalhalten. Diklofenak var ett undantag och upp till 7 % konjugerad diklofenak detekterades. Sammantaget visar undersökningen att förekomsten av konjugerade former av antiinflammatoriska läkemedel (NSAID:s) inte i sig kan förklara att halten i utgående vatten i en del fall är högre än inkommande vatten. Detta fenomen kan orsakas av provtagningen (fel i synkronisering av provtagningen) och till en mindre del av osäkerheten i analysen.

Konjugerade NSAID:s i influent och effluent (% av fria)

Datum	IB-OH	IB-COOH	Diklofenak
Influent 3/8	19	9	1
Effluent 3/8	21	8	7
Influent 8/8	8	4	0.2
Effluent 8/8	22	7	1

Förkortningar: Hydroxy-ibuprofen (IB-OH); Karboxy-ibuprofen (IB-COOH). Antiinflammatoriska läkemedel (NSAID:s).

Fördelningen av anti-inflammatoriska läkemedel i avloppsvattnen avspeglade den registrerade försäljningen väl; paracetamol uppvisade de högsta halterna i såväl avlopps- som ytvattenprover från Piteå älv följt av ibuprofen och naproxen. Diklofenak kunde inte påvisas i ytvatten vilket kan vara en effekt av fotokemisk nedbrytning av föreningen.

Salicylsyra avvek markant från mönstret och utgjorde en obetydlig fraktion i avloppet men var det anti-inflammatoriska läkemedel som återfanns i näst högst halt i ytvatten och sedimentprover. Förklaringen till detta är att salicylsyra förekommer i växter (växthormon) och därför återfinns i den växtlighet (träd och örter) som bl.a. finns naturligt ingår i Piteå älvs avrinningsområde.

I sedimenten återfanns också paracetamol i högst halter följt av salicylsyra, diklofenak, ibuprofen och naproxen. Orsaken till att de mer vattenlösliga föreningarna paracetamol och salicylsyra återfinns i sedimenten kan förklaras med att bindningen till sedimenten inte styrs av ren affinitetsfördelning utan av kemisk inbindning respektive katering.

Analys utfördes också på muskel, lever och galla från fisk insamlade i Piteå älv. Inga anti-inflammatoriska läkemedel kunde detekteras i fiskproverna vilket sannolikt beror på att föreningarna är protolyserade och därmed har ett lågt  $K_{ow}$  vid det aktuella pH-värdet (Weigel et al. 2004).

## Summary

As an assignment from the Swedish Environmental Protection Agency, IVL has performed a screening study of six non-steroidal anti-inflammatory drugs (NSAID:s) and one analgesic drug, paracetamol, in influent and effluent waters from a waste water treatment plant (WWTP), and in the aquatic environment in the vicinity of the WWTP. The following compounds were included: ibuprofen, ketoprofen, naproxen and diclofenac. The two high volume substances paracetamol and acetylsalicylic acid were also included as well as the metabolites salicylic acids, hydroxyl-ibuprofen and carboxy-ibuprofen. Thus, in total nine substances.

The aim of this project was to investigate the occurrence of conjugated forms of the mentioned drugs in influent and effluent waters in a WWTP and if this could explain the observation that effluent water samples sometimes contained higher concentrations than in the influent water. The occurrence of NSAID:s and paracetamol in the aquatic environment was also investigated. For this purpose, an analytical protocol developed capable of separating and determining both conjugated and free forms of the drugs. In the second part of the project samples of water, sediment and fish were collected upstream and downstream of a WWTP (Sandholmen) in the Piteå River system.

The composition of analysed drugs in the influents mirrored the annual sales figures quite well. The highest concentrations in effluent and surface water were detected for paracetamol followed by ibuprofen, naproxen, ketoprofen and diclofenac. Diclofenac was not detected in surface water, possibly due to photochemical degradation (Buser et al. 1998; Agüera et al. 2005).

The detected concentrations of conjugated NSAID:s in sewage influent and effluent water were generally low and their contribution to the total amount of NSAID:s was in this investigation below 1%. Diclofenac was an exception as 1-7 % was detected as conjugated in influent water samples. Therefore, the occurrence of conjugated NSAID:s in the influent can not solely explain the observation that the effluents occasionally have higher concentrations of NSAID:s than the influent.

Salicylic acid was a minor component in sewage water but was a dominating NSAID in surface water and sediment. Salicylic acid is also a phytohormone and is therefore ubiquitous in plants and this probably explains these results.

NSAID:s and paracetamol could not be detected in any of the fish samples. This may be explained by the fact that the NSAID:s are ionised at the ambient pH in Piteå River giving the compounds low log  $K_{ow}$ 's and bio-concentration potentials (Weigel et al. 2004).

# 1 Introduction

The results from the screening study 2005 (Andersson et al. 2006) showed large variations in the concentrations of the different pharmaceutical substances between different waste water treatment plants (WWTPs). The results also showed that several waste water treatment plants contained higher concentrations of these substances in the effluent than in the incoming water. This is rather contradictory but has been reported previously in scientific literature (Baronti et al. 2000; Nasu et al. 2001).

Non-steroidal anti-inflammatory drugs, abbreviated to NSAID:s are pharmaceuticals with, analgesic (reduce pain), antipyretic (reduce fever) and anti-inflammatory effects. The most important drugs of this group of drugs are aspirin (acetylsalicylic acid), ibuprofen, and naproxen, ketoprofen and diclofenac. Paracetamol has also antipyretic and analgesic properties, but has not anti-inflammatory properties and is therefore not classified as an NSAID.

The estimated sold of the different pharmaceuticals discussed in this report are summarised in Figure 1.

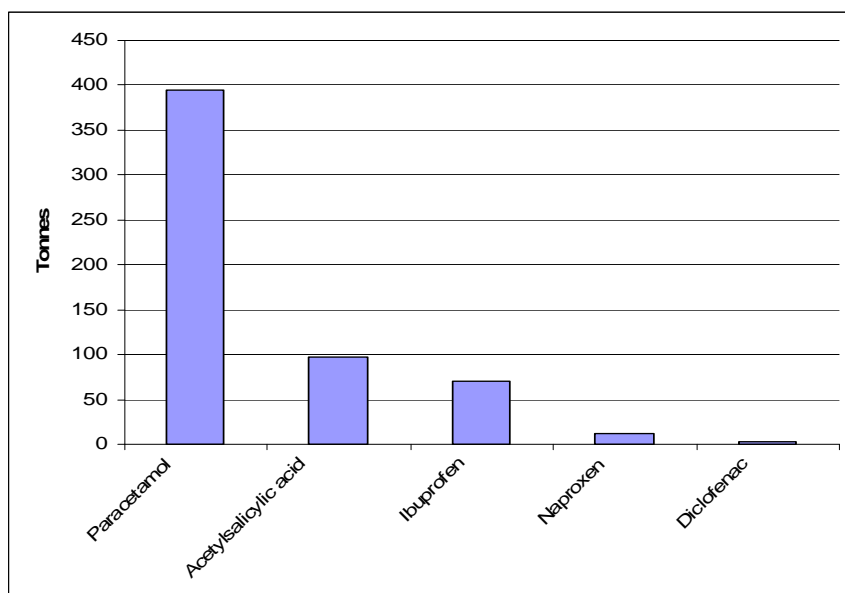


Figure 1. Approximately amount sold of different pharmaceuticals in Sweden 2005. The data is sales data from Apoteket AB for 2005. In cases of combination drugs, an estimate of the DDD has been used when no number was listed at the WHO website (<http://www.whocc.no/atcddd/>).

Upon excretion, non-steroidal anti-inflammatory drugs (NSAID:s) and paracetamol are conjugated in the liver and are excreted in urine as water-soluble glucuronides and sulfates. In the previously screening, the analytical method used disregarded the conjugated NSAID:s and only detected the free fraction of NSAID:s. Consequently, the total concentration in incoming water may have been underestimated.

In the WWTP extracellular  $\beta$ -glucuronidase, produced by fecal bacteria (*Escherichia coli*), will hydrolyse the conjugated NSAID:s back to the active parent compounds. This process seems to be

guided by rather fast kinetics. For example the glucuronid conjugates of steroids are mostly hydrolysed in the sewers before even reaching the WWTP (Baronti et al. 2000). However, the sulfate conjugates are more resistant and may bypass the biological treatment in WWTPs and may be released into the aquatic environment as conjugates (D' Ascenzo et al. 2003; Reddy et al. 2005). It has also been shown that glucuronides may be transformed to the corresponding sulphate in the activated sludge (Gomes et al. 2009). Thus, it was theoretically possible to explain the higher concentration in effluent water with a significant fraction of conjugated NSAID:s in incoming water.

In order to examine this theory a sampling program was elaborated at a particular WWTP that showed higher concentrations in effluent compared to influent in the previously performed screening study.

Furthermore, the screening program 2005 which mainly was focused on concentrations in water from WWTPs could not answer the question regarding the impact on the environment in the vicinity of WWTPs. The occurrence of NSAID:s in the aquatic environment was therefore more thoroughly investigated in this project.

The aim of this investigation was to: (a) develop an analytical method capable of separating and detecting free and conjugated NSAID:s, (b) develop an analytical method for determination of the total concentration paracetamol (free plus conjugated) in different environmental matrixes, (c) investigate if the elevated concentrations in the effluent can be explained by the occurrence of conjugated forms of the NSAID:s in the influent and (d) investigate if NSAID:s and paracetamol may occur in the environment (water, sediment and fish) receiving effluent water from the WWTP.

## 2 Methods

### 2.1 Sampling

The sampling was carried out in the north of Sweden, in Piteå at Sandholmen WWTP and in its recipient. The results from the screening 2005 showed that the effluent water from this plant contained the highest concentration of NSAID. The sampling programme in this investigation is summarized in Table 1. More details of the samples are given in Appendix 1.

Table 1. Sampling program

WWTP		Recipient			Total
Influent	Effluent	Surface water	Sediment	Fish	
2	2	4	4	3	15





Figure 2. Map over the sampling area: 1. Hemlunda (water-, sediment- and fish samples), 2. Sandholmen (water-, sediment- and fish samples), 3. South Sandholmen (Water and sediment), 4. Pitsund, (water-, sediment- and fish samples), ○ (WWTP-discharge point).

## 2.2 Sampling of WWTP water

The Sandholmen waste-water treatment plant in Piteå is dimensioned for 35000 person equivalents and is affiliated to 30500 person equivalents (Figure 2). The plant is constructed for mechanical, chemical and biological treatment of the waste-water.

Flow-proportional composite water samples of both the influent and effluent water streams were collected in cleaned plastic bottles and stored frozen until analysed. The samples were stored frozen since it is a mild preservation method. An alternative is to acidification the sample with a strong mineral acids (*e.g.* HCl) but was avoided since diclofenac quite readily is converted into a cyclic product under these conditions (Blum et al. 1996).

A bottle with ultra pure water (Milli-Q) was used as a field blank. The field blanks were exposed to the sampling environment during the sampling.

## 2.3 Sampling of environmental samples

Water and sediment samples were collected at four sampling stations; one located upstream, one at the WWTP discharge point and two down streams the local WWTP at Sandholmen. Fish were

collected at the same sampling stations as water and sediment except for the discharge point (Figure 2). The sampling of fish was permitted by Hemlunda FVO as water administrator and the northern Stockholm ethical board for animal research (N352/06).

Water samples were collected in cleaned plastic bottles and stored frozen until analysed. A bottle with ultra pure water (Milli-Q) was used as a field blank.

Sediment samples were collected by means of an Ekman dredge. The superficial sediment was (0-2 cm) transferred to clean plastic jars with a stainless steel spoon. The samples were stored in a freezer (-18°C) until analysed. A plastic jar filled with diatomaceous earth (10 % water) exposed to the surrounding environment during the sampling time was used as field blank.

Fish samples were collected by means of fishing nets. The fish were individually wrapped in cleaned aluminium foil and stored in a freezer (-18°C) until analysed.

## 2.4 Analysis

Analytical methods capable of separating conjugated and un-conjugated (free) NSAID:s in environmental samples were not available. Such methods exist only for steroids but these could not be directly applied to NSAID:s. Therefore, a method was developed capable of concentrating and measuring the free and the conjugated species of the NSAID:s. The results from the analytical development are summarized in Appendix 3.

### 2.4.1 Pharmaceutical and metabolites included in the analytical program

In addition to the four NSAID:s (ibuprofen, ketoprofen, naproxen, diclofenac) analysed in the previous investigation (Andersson et al. 2006), two high volume pharmaceuticals, paracetamol (CAS 103-90-2) and acetylsalicylic acid (CAS 50-78-2), was included in this study (Figure 3).

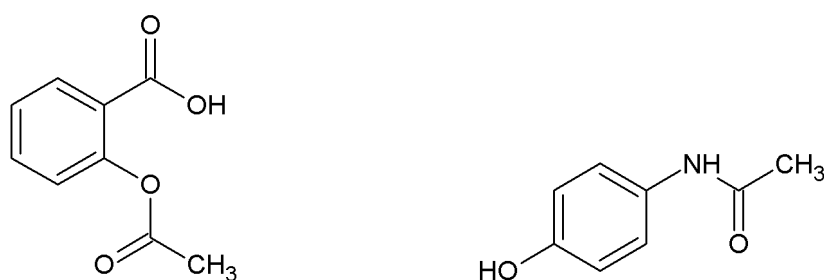


Figure 3. The chemical structure of acetylsalicylic acid and paracetamol

Furthermore, some known metabolites of the NSAID:s were also included. In humans, the NSAID:s are mainly metabolised in the liver by the phase I and phase II enzymatic system. The phase I pathway implies introduction of a hydroxy or a carboxyl group to the molecule rendering it more polar. In the phase II pathway the NSAID:s metabolites are conjugated to water soluble derivatives that facilitate excretion such as glucuronic acid.

Thus, ibuprofen is conjugated but also metabolised to hydroxyl-ibuprofen (2-(4-(3-hydroxy-2-methylpropyl)phenyl)propanoic acid) and carboxy-ibuprofen (2-[4-(1-hydroxy-1-oxopropan-2-yl)phenyl]propanoic acid) (Figure 4). These metabolites have been detected both in WWTP-effluents and surface water in concentrations higher than the mother compound and was therefore included in this investigation (Buser et al. 1999; Weigel et al. 2004). Since commercial standards were not available the identification of these compounds must be considered as tentative but the MS-spectrum obtained was in good agreement with previously published spectra (Buser et al. 1999; Weigel et al. 2004).

Salicylic acid was also included since it is the principal metabolite of acetylsalicylic acid.

Diclofenac is metabolized to a variety of hydroxylated (3-, 4-, 5-hydroxy), methoxylated (3-; 4-methoxy) and hydroxyl-methoxy- metabolites. However each metabolite occurs in quite low yields and was therefore not included in the analytical program (Blum et al. 1996; Kaphalia et al. 2006; Gröning et al. 2007).

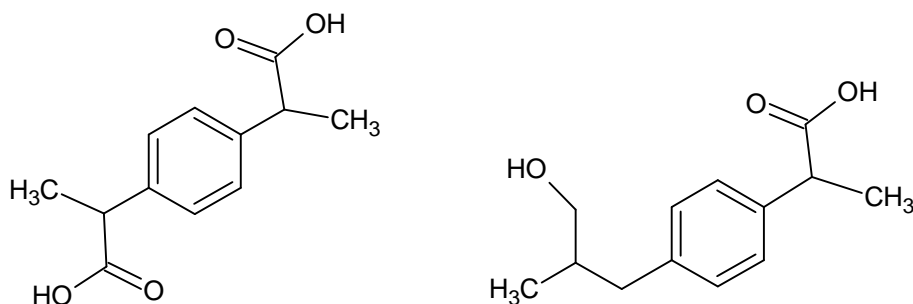


Figure 4. The chemical structure of carboxy-ibuprofen and hydroxy-ibuprofen.

Due to the lack of reference compounds of the NSAID:s metabolites, besides salicylic acid are the reported concentrations of the NSAID:s metabolites considered semi quantitative in the present report.

#### 2.4.2 Water analysis

Water samples (200-1000 ml) were analysed with regard to free and conjugated NSAID:s and the analgesic drug paracetamol. The samples were filtrated (pre-heated GF/C-filter) before solid phase extraction (SPE-extraction). The SPE-column (ENV+ 200 mg) was cleaned and activated prior use with hexane, ethyl acetate, methanol and water. The filtrated water sample was acidified with phosphoric acid (~ pH 2), and ascorbic acid (50 mg) was added to prevent sample oxidation (Vanderford et al. 2006). The sample was then spiked with surrogate standards and subsequently concentrated on the SPE-column (~15 ml/min). After the sample had passed through, the column was rinsed with a buffer (pH 2) and freeze-dried. The analytes were eluted using a mixture of methanol and acetone as mobile phase. The eluate was evaporated to dryness using nitrogen gas and re-dissolved in phosphate buffer (pH 2). Separation of free and conjugated NSAID:s were performed by first extracting the free fraction from the buffer with hexane:MTBE (3+1). This organic extract contained the un-conjugated NSAID:s.

In a second step the buffer, containing the conjugated NSAID:s and paracetamol, was hydrolysed with  $\beta$ -glucuronidase (se below). The released NSAID:s was extracted with hexane:MTBE (3+1).

The extract of the two fractions of free and conjugated NSAID:s were then subjected to derivatisation with methylchloroformate (se below), cleaned-up on a silica gel column and analysed with gas chromatography-mass spectrometry (GC-MS).

The polar compound paracetamol was left in the buffer solution after the extraction of the de-conjugated NSAID:s. Paracetamol was recovered from the buffer solution by three MTBE-extractions after addition of sodium chloride. This extract was dried over sodium sulphate and subsequently derivatised.

### **2.4.3 Sediment analysis**

The analytical method used was directed to only detect free analytes since sediment samples were not expected to contain conjugated species.

Freeze-dried sediment samples (~10 g of sediment fresh weight) were spiked with recovery standards and mixed well. After addition of ascorbic acid (50 mg) and acidification the sample was extracted with (a) acetone and (b) a mixture of acetone:methyl-*tert*-butylether (MTBE). The procedure was performed using first an ultrasonic bath (5 min) and then gentle agitation on a shaking board (30 min). The extracts were combined and mixed with water and subsequently extracted twice using hexane:MTBE (3+1) as solvent. The buffer phase was saved for paracetamol analysis and the extract was dried over sodium sulphate for 15 min. The dried organic phase was evaporated to complete dryness and re-dissolved in hexane. The hexane phase was then back-extracted, derivatised, and cleaned-up on a silica gel column prior to GC-MS analysis as described below.

Paracetamol was extracted from the phosphate buffer with MTBE after addition of sodium chloride. The combined extract was then dried over sodium sulphate (15 min) and the solvent was exchanged to hexan:MTBE (3+1). The extract was subsequently acetylated with acetic acid using pyridine as catalyst (se below).

### **2.4.4 Fish analysis**

An analytical protocols for both free and conjugated analytes in fish was developed since fish has the ability to conjugate pharmaceuticals (Clark et al. 1991).

#### *Tissue*

The fish was thawed immediately before analysis and subcutaneous tissue was dissected for analysis.

The fish sample (muscle, 10 g) was fortified with recovery standards, acidified and homogenized in acetone followed by a 30 minute-period of agitation. The extraction was repeated with a second aliquot of acetone. The organic solvent in the combined extracts was evaporated and the residue (tissue water) was diluted with buffer (pH 2) and extracted twice with hexane:MTBE (3+1). The organic extract was dried over sodium sulphate for 15 min and evaporated to complete dryness. The evaporated sample was re-dissolved in hexane and cleaned-up using the back-extraction technique (se below).

The buffer solution, containing the conjugated NSAID:s including paracetamol, was subjected to enzymatic hydrolyses (se below). The hydrolysate was extracted with hexane:MTBE (3+1) after acidification. The extract containing the hydrolysed conjugates of NSAID:s was combined with the

extract containing the free NSAID:s. Finally, this combined extract was subjected to derivatisation, cleaned-up on a silica gel column and analysed using a GC-MS.

Finally, paracetamol was extracted from the phosphate buffer with MTBE after addition of sodium chloride. The combined extract was then dried over sodium sulphate (15 min) and the solvent was exchanged to hexan:MTBE (3+1). The extract was subsequently acetylated with acetic acid using pyridine as catalyst (se below).

#### *Bile*

The content in the bile was used as a sensitive indicator of exposure (Oikari et al. 1984; Adolfsson-Erici et al. 2002).

The bile sample was chemically hydrolysed according to (Sawchuk et al. 1995). Briefly, the bile sample was mixed with ascorbic acid and the oxygen was evacuated with aid of a nitrogen gas flow. Subsequently was KOH added, still under N<sub>2</sub>-flow, to a final concentration of 1 M. The hydrolysis was performed at 70°C for 1 h. The hydrolysed sample was after acidification treated in the same way as tissue samples.

### **2.4.5 Enzymatic hydrolysis**

The evaporated extract was dissolved in an ammonium acetate buffer (pH 6.5). The enzyme was added and the sample was incubated for 1 hour at 37°C (for details, se Appendix 3).

### **2.4.6 Clean-Up and derivatisation**

#### *NSAID:s except paracetamol*

Each sample containing large amounts of matrix molecules was subjected to a developed **back-extraction** (liquid-liquid extraction) routine where the extract, dissolved in hexane, was vortexed with borax buffer (pH 8.3) and methanol (Andersson et al. 2006). The buffer phase, containing the NSAID:s, was then transferred to a new test tube, diluted with water, acidified and subjected to two rounds of hexane:MTBE (3+1) extraction. The extract was derivatised (methyl esterification) using methyl chloroformate (MCF) according to (Butz et al. 1993; Weigel et al. 2002) with minor modifications (Andersson et al. 2006). The solvent was evaporated under a stream of nitrogen before derivatisation. The dried extract was re-dissolved in reagent solvent mixture containing acetonitril/methanol/water/pyridine. A aliquot of MCF was added and the sample vortexed. Subsequently, another aliquot of MCF was added and the sample was again vortexed 10 s and left for 10 minutes to complete the reaction. The derivatization was stopped by the addition of water and finally the methyl esters of the NSAID:s were extracted with hexane: MTBE (9+1). The derivatised extract was then subjected to a silica gel chromatographic purification step on a silica gel column. Biphenyl was added prior to GC-MS analysis as volumetric standard.

#### *Paracetamol*

The MTBE-extracts of the water-, sediment- and fish samples containing paracetamol was dried over sodium sulphate and the solvent was exchanged to hexan:MTBE (3+1). The sample was derivatised with acetic acid anhydride and dry sodium acetate at 65°C for 1 hour. Biphenyl was added as volumetric standard analysis with GC-MS.

## 2.5 Instrumentation

The sample extracts were analysed on a 6890N gas chromatograph coupled to a 5973N mass selective detector (Agilent). The injection, 1  $\mu$ l, was done in splitless mode at 240°C. The fused silica capillary column (VF-5MS 30 m x 0.25 mm i.d. x 0.25  $\mu$ m film thickness, Varian) was held at 45°C for 1 min., ramped 15°C/min to 200°C, 5°C/min until 300°C and held at 300°C for 5 min. Helium was used as carrier gas. The detector was used in selected ion monitoring mode (SIM) with electron ionisation at energy of 70 eV. The analytes were identified by their characteristic retention time and one quantification ion (Q-ion) and one or two supporting ions (S-ion) used to increase specificity was recorded (Table 2a-b).

Quantification was based on comparison of peak abundance to the known response of the internal standard (2,4,5-TP). The reported analyte concentrations were corrected according to the determined surrogate standard losses. The acceptable recovery rates for the added recovery standards were 60% or above.

Table 2. Ions used in MS-analysis of NSAID:s as MCF-derivatives and paracetamol as acetate.

Substance	Rt (min)	Trg-ion	Q1-ion	Q2-ion
<i>Biphenyl</i>	9.8	154	-	-
Acetylsalicylic acid	9.91	120	152	-
Salicylic acid	10.75	135	133	166
Ibuprofen	11.00	161	177	220
<i>1-naphthoic acid</i>	10.90	155	127	186
Hydroxy-Ibuprofen	12.31	119	118	178
<i>2,4,5-TP</i>	12.90	198	282	223
Paracetamol	13.24	109	151	193
<i>3-chloro-4-hydroxyacetanilid</i>	13.24	143	185	-
Carboxy-Ibuprofen	13.53	205	264	-
Naproxen	15.93	185	244	-
Ketoprofen	17.43	209	268	-
Diclofenac	18.49	214	242	309

Abbreviations: Names in italics are the recovery standards and injection standard (biphenyl).  
Rt = retention time. Trg = Target ion. Q1 and Q2 = qualifier ions.

### 2.5.1 Quality control

The following quality criteria were used to ensure correct identification and quantification of the target compound: (a) the retention time should match those of the standard compounds within  $\pm$  0.05 min., (b) the intensity ratios of the selected ions (target- and qualifier-ions) are within  $\pm$  15% of expected / theoretical value (c) the signal-to-noise ratios are greater than 3:1 (Haglund et al. 1993). Generally, one "Target ion" was used as quantification ion (Trg.-ion) and one or two "Qualifier ions" (Q-ion) were recorded to increase specificity.

Field blanks were collected at each sampling station. A method blank was included for each sample batch analysed to assess background interferences and possible contamination of the samples. Concentrations below field blank levels are treated as not detected.

Possible background levels of analytes were subtracted from measured sample values (Keith 1991; Miller et al. 1993)

### 3 Results and discussion

The results from the measurements are given in Appendix 2.

#### 3.1 STP-influent and effluent

The anti-inflammatory drugs (NSAID) were determined as free (not conjugated) and conjugated compounds but paracetamol was determined as the sum of free and conjugated species due to analytical difficulties. Therefore, the paracetamol results are presented separately and not together with the NSAID:s.

All NSAID:s except acetylsalicylic acid and paracetamol were detected in all the influent and effluent samples. Paracetamol occurred in the highest concentrations in both influent and effluent water (24-59 µg/l; Figure 5) followed by ibuprofen (2.6-8.0 µg/l) and naproxen (2.3-7.3 µg/l) and mirrored the sales of drugs quite well (Figure 1).

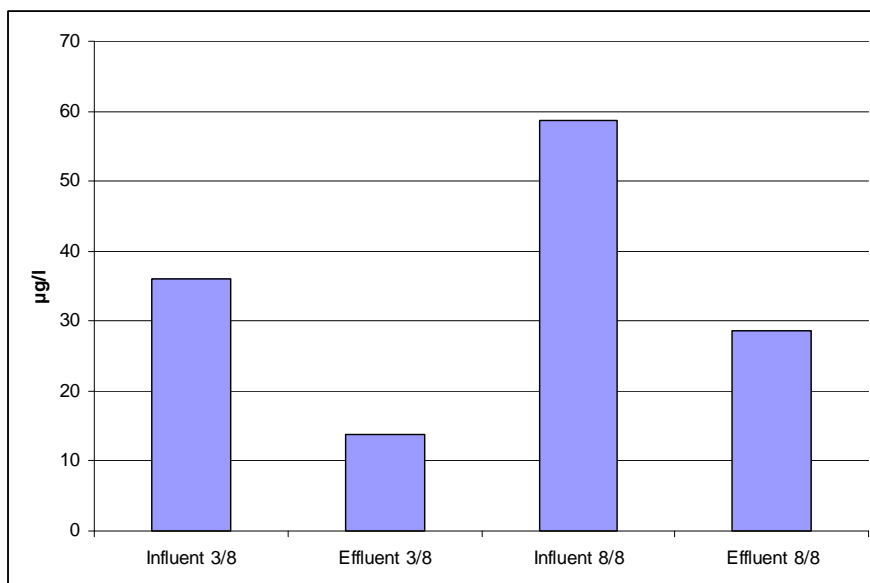


Figure 5. The sum concentrations of free plus conjugated paracetamol in influent and effluent (µg/L) at two sampling days in August 2006.

The measured concentrations of NSAIDs in the sewage water from Sandholmen WWTP are in agreement with the previous study (Andersson et al. 2006) with one exception, the concentrations of naproxen in this investigation were 3-10 times lower.

In a European perspective were the ibuprofen and the diclofenac concentrations in Hemlunda WWTP equal. For example Reematsma (2006), reported 0.1-1 µg/l of ibuprofen and 1-5 µg/l of diclofenac in European WWTP effluents compared to 2.6-3.5 µg/l of ibuprofen and 0.23 µg/l

diclofenac in Hemlunda WWTP effluent. Furthermore, influent water collected from Howdon WWTP (the UK) contained diclofenac (997-1036 ng/l), ibuprofen (7741-33764 ng/l) and paracetamol (5529-69570 ng/l). As the compounds pass through the works there was a reduction and in the final effluent the concentration of diclofenac ranged from 261 to 598 ng/l, ibuprofen 1979-4239 ng/l and the concentration of paracetamol were <20 ng/l (Roberts et al. 2006).

The concentrations of paracetamol in influents in a European WWTP were reported being 6-492 µg/l (Ternes 1998; Kasprzyk-Hordern et al. 2008). In effluent the concentrations were significantly lower due to rapid degradation (Löffler et al. 2005).

Metabolites of ibuprofen were consistently detected in all influent and effluent water samples (Figure 6). The concentrations of these metabolites (0.02-2.2 µg/l) were however lower than the corresponding mother compounds (2.3-8.0 µg/l). The hydroxyl-ibuprofen metabolite was detected in the highest concentrations (0.85-2.2 µg/l).

The concentrations of salicylic acid were low in both influent and effluent water. The origin of this compound is probably acetylsalicylic acid. But there are other derivatives of salicylic acids used in personal care products for example used as UV filters (e.g. homosalate, ethylhexyl salicylate) that may contribute to the salicylic acid detected in the STP water (Cuderman et al. 2007; Rodil et al. 2008).

The previous observation that the concentrations of NSAID:s were higher in the effluent water than in the influent water (Andersson et al. 2006) may be a result of hydrolysis of incoming conjugated NSAID:s in the WWTP. This theory was investigated here by sampling flow-proportional day samples of influent and effluent samples for two days.

The concentrations of NSAID:s in the influent and effluent waters collected at the first sampling occasion, day one, showed indeed higher concentrations of ibuprofen, hydroxy-ibuprofen and naproxen in the effluents compared to the influent while for the second sampling occasion day two the situation was the reversed (Figure 6).

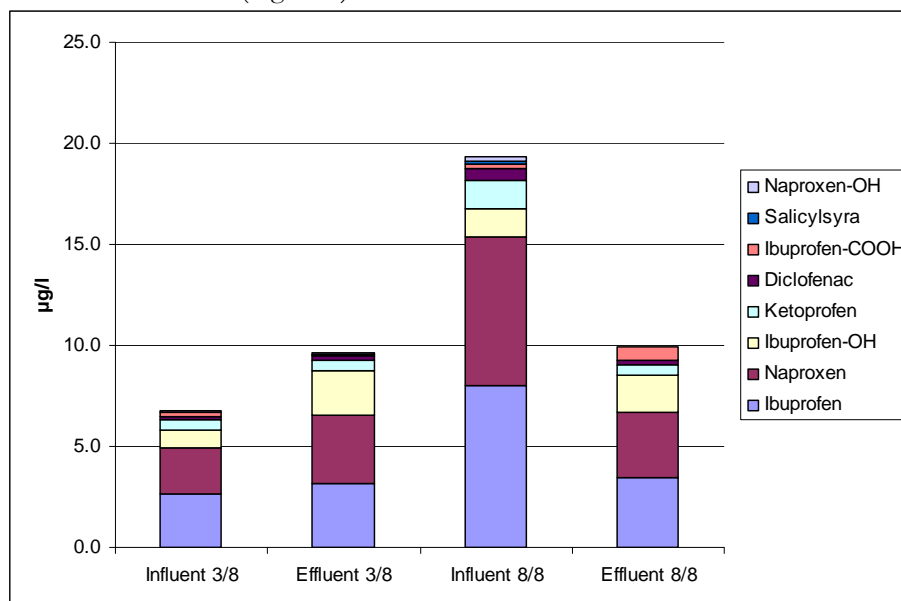


Figure 6. Free NSAID:s in influent and effluent water (µg/L) at two sampling days in August 2006. Ibuprofen-COOH and ibuprofen-OH are the carboxylated and hydroxylated metabolites of ibuprofen respectively.



Conjugated NSAID:s were present in both influent and effluent water samples but the concentrations were mostly less than 1 % of the de-conjugated (free) fraction (<0.2-35 ng/l) and thus too low to support the discussed theory that a significant amount of NSAID:s reach the WWTP as conjugate. These results are in agreement with results showing that conjugated compounds are de-conjugated before reaching municipal sewage treatment plants due to glucuronidases released from bacteria in human faeces *e.g.* E.coli (Baronti et al. 2000; Swartz et al. 2006).

The two conjugated metabolites of ibuprofen were also regularly detected in the sewage water. The highest concentrations were determined for the hydroxy-ibuprofen (110-460 ng/l) and the carboxy-ibuprofen metabolite showed a magnitude lower concentrations (1.4-42 ng/l).

The relative amount of the conjugates of ibuprofen and its metabolites to the free fraction are summarised in Table 3 and show that the ibuprofen metabolites are to a higher degree conjugated compared to the mother compound. This suggests that these metabolites are the more persistent sulfate conjugates and not the readily degradable glucuronides (D' Ascenzo et al. 2003; Reddy et al. 2005).

Table 3: Conjugated NSAID:s in influent and effluent (% of free)

Date	Ibuprofen	IB-OH	IB-COOH
Influent 3/8	0.14	19	9
Effluent 3/8	1.1	21	8
Influent 8/8	0.02	8	4
Effluent 8/8	0.08	22	7

Abbreviations: Hydroxy-ibuprofen (IB-OH); Carboxy-ibuprofen (IB-COOH).

The removal rate of the NSAID:s in the WWTP was low and in agreement with previous measurements (Andersson et al. 2006).

### 3.2 Surface water

All surface water samples contained detectable concentrations of not conjugated paracetamol (69-360 ng/l) and NSAID:s (<0.2-14 ng/l) while conjugated NSAID:s was not found in any of the samples. In the samples collected up-streams the WWTP, were paracetamol (170 ng/l), and salicylic acid (12 ng/l) clearly detected (Figure 7 and 8).

Comparable concentrations of paracetamol were determined in River Taff (the UK) and ranged from 216 to 1388 ng/l. In the Poland River Warta the concentrations ranged from 11 to 58 ng/l (Kasprzyk-Hordern et al. 2007).

Measurements in 139 streams across 30 USA states during 1999-2000 showed a median concentration of paracetamol of 0.11 µg/l with a maximum on 1.0 µg/l. The compound was detected in 23.8% of the streams. Ibuprofen was detected in 9% of the streams with an average concentration of 0.2 µg/l (maximum 1.0 µg/l). Concentrations on other NSAIDs were not reported (Kolpin et al. 2002).

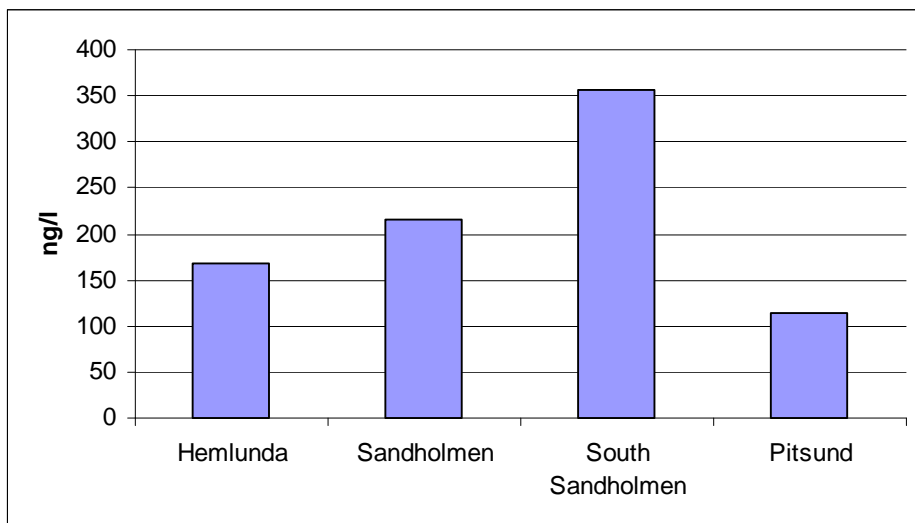


Figure 7. Concentrations of paracetamol in surface water in the Piteå River system.

Ibuprofen (0.48 ng/l) and naproxen (0.46 ng/l) were also detected but at trace level concentration (Figure 8). This indicates influence from the many societies upstream Hemlunda.

At the discharge point and at sampling point 3 (South Sandholmen) all substances but diclofenac were detected. The concentrations were in the low ng/l-range and correspond well with previously reported data in surface water (Weigel et al. 2004).

Diclofenac was not detected in any of the surface water sample. This compound is photochemically active and it is therefore possible that it has been degraded (Buser et al. 1998).

Water collected from River Vantaa Finland contained somewhat higher concentrations of NSAIDs compared to the present investigation. Ibuprofen ranged from 12 to 69 ng/l (median 32 ng/l), diclofenac 10-55 ng/l (median 25 ng/l), naproxen 16-32 ng/l (median 20 ng/l) and ketoprofen 8-28 (median 16 ng/l) (Vieno et al. 2007).

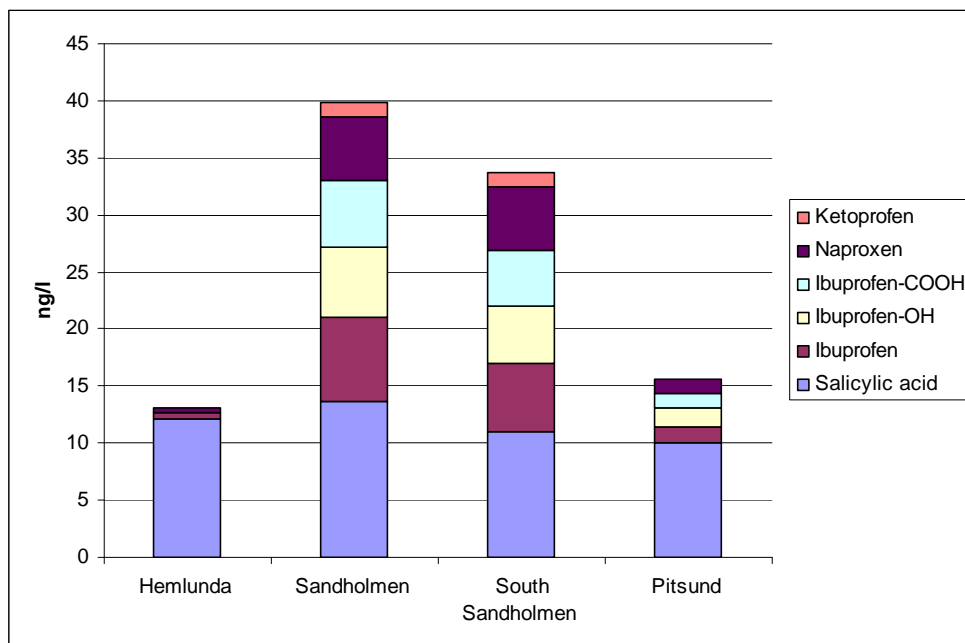


Figure 8. Total concentrations (free plus conjugated) of different NSAID:s in surface water in the Piteå River system. Ibuprofen-COOH and ibuprofen-OH are the carboxylated and hydroxylated metabolites of ibuprofen respectively.

Salicylic acid was, along with paracetamol, frequently found in the river system whereas the other NSAID:s showed low concentrations up-streams and lower concentrations downstream the WWTP, possibly due to dilution (Figure 8).

Salicylic acid is a phytohormone and is therefore ubiquitous in plants and fruits (Blacklock et al. 2001; Hayat et al. 2007). The occurrence of salicylic acid in some common plant species that exist within the Piteå River catchments area was therefore investigated. Chemical analysis showed that salicylic acid is a chemical constituent of grass, sallow (*Salix caprea*), birch (*Betula pendula*), aspen (*Populus tremula*) and in coniferous tree such as pine (*Pinus sylvestris*) and spruce (*Picea abies*). We therefore concluded that natural sources could be responsible for the observed distribution of salicylic acid in the river system.

### 3.3 Sediment

The concentrations of NSAID:s in the sediment samples were generally in the low  $\mu\text{g}/\text{kg}$  level ( $<0.2\text{--}6.0 \mu\text{g}/\text{kg dw}$ ; Figure 9) which may be a result of a low organic content in the sediments and the low  $\log K_{ow}$  for the NSAID:s at the actual pH (Weigel et al. 2004). Acetylsalicylic acid and the metabolites of ibuprofen were not detected in any of the sediment samples. The absence of these compounds in the Piteå River sediments was probably a result of a low affinity to the sediments and rapidly transformation (Löffler et al. 2005).

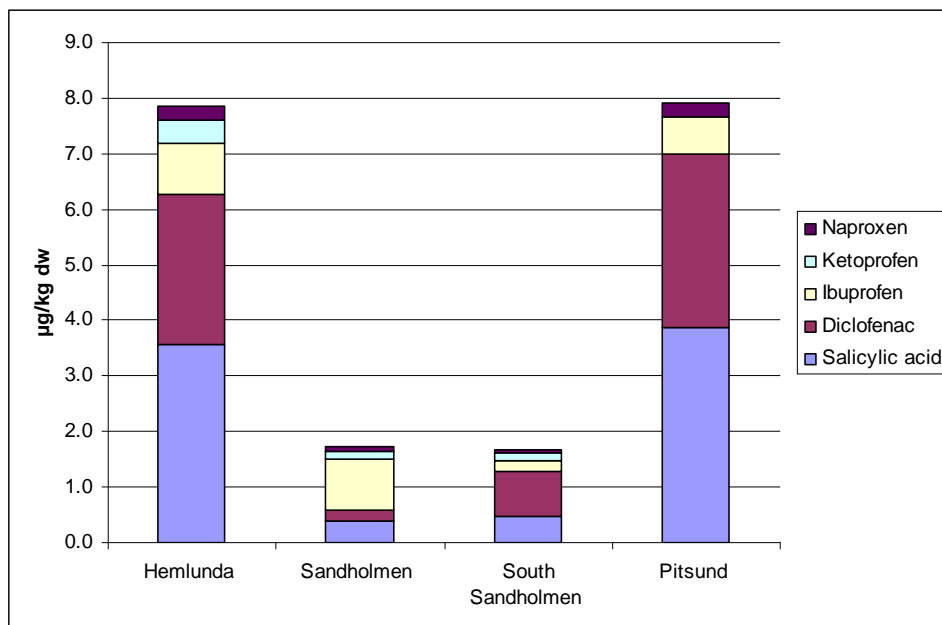


Figure 9. NSAID:s in sediment samples from the Piteå River system (µg/kg dw).

The highest concentrations of NSAID:s in sediment samples were detected at Hemlunda, upstream the WWTP and at Pitsund downstream the WWTP (Figure 9 and 10). The low concentrations at the discharge point Sandholmen was explained by the fact that the “sediment” consisted of sole sand with a low content organic carbon (0.3 %; Table 4) giving the sediment a low adsorption capacity for organic compounds such as pharmaceuticals. The low concentrations of NSAID:s found in the sediment samples from South Sandholmen (station 3 in Figure 1) can not be explained by low organic content (Table 4) but by strong currents in this area of the Piteå River bring about rapid re-suspension and erosion of the sediment top layer.

Table 4: Sediment characteristics

Sample	dw %	LOI %
Hemlunda	40	4.0
Sandholmen	75	0.3
South Sandholmen	51	2.6
Pitsund	25	8.2

Abbreviation: dw = dry weight 24 h at (105°C).  
LOI = loss of ignition (2 h at 550°C).

Paracetamol occurred in the highest concentrations in all the sediment samples (Figure 10) and again, salicylic acid was the second most important substance probably due to its natural sources.

The  $K_{ow}$  can not explain the enrichment of salicylic acid ( $\log K_{ow}$  2.4) and paracetamol ( $\log K_{ow}$  0.7) in the sediments. Salicylic acid have chelating properties (Merck Index) and may be enriched in the sediments by this mechanism like chlorocatechols (Remberger et al. 1993) and tetracyclines (Bogialli et al. 2006).

Diclofenac have a quite high  $K_{ow}$  ( $\log K_{ow}$  4.5; (Löffler et al. 2005; Kasprzyk-Hordern et al. 2008) explaining the relative high concentrations in the sediment samples down steams the WWTP.

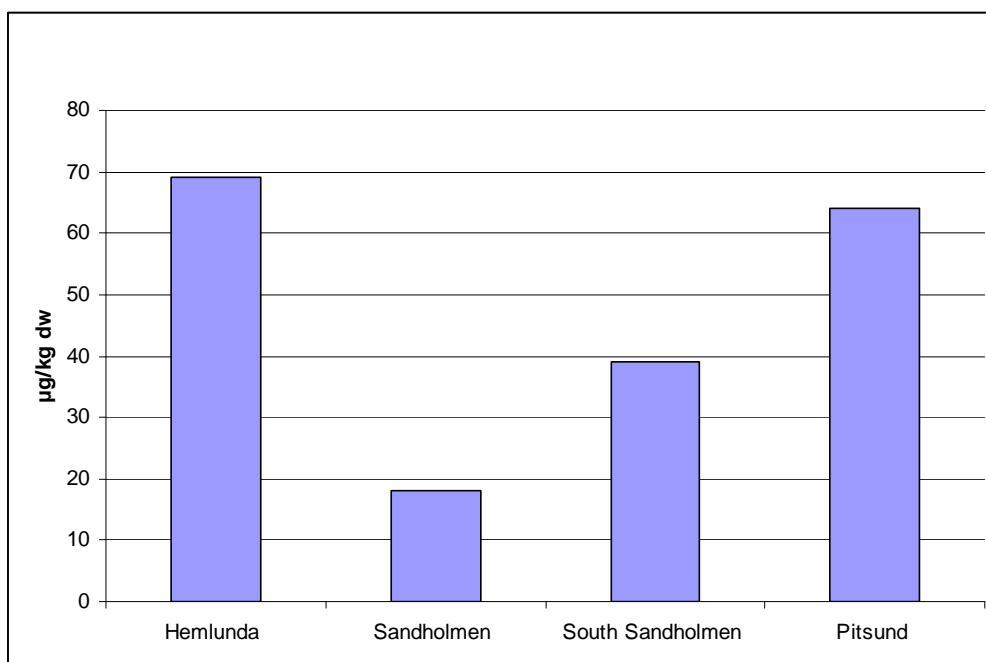


Figure 10. Paracetamol in sediment samples from the Piteå River system (µg/kg dw).

It is well documented that paracetamol released to surface water is rapidly transferred to the sediment despite the low  $K_{ow}$  (0.5) and high  $pK_a$  (9.5) and is therefore expected to be present in this part of the environment. Most of the sediment-bound paracetamol has been proved to be involved in a strong binding (*e.g.* covalent binding) and could not be extracted by simple solvent extraction (Löffler et al. 2005).

Spiked sediments were used to develop and validate the analytical method designed for paracetamol in sediment and sludge samples. The analyte (paracetamol) and recovery standard (3-chloroparacetamol) showed in these spiking experiments a low recovery rates. Furthermore, the analysis of the sediment samples collected in the Piteå River revealed that paracetamol could be detected in all sampling points but Sandholmen despite the relative high concentrations in the effluent water (Figure 5) and surface water (Figure 7).

Since oxidation was expected to be a part of the problem was EDTA or ascorbic acid added to the sample prior to extraction (Remberger et al. 1993). The addition of EDTA had no effect on the outcome but ascorbic acid significantly improved the recovery in all samples examined. This means that paracetamol appeared to be rapidly adsorbed and transformed but that the reaction to a certain extent was reversible (Table 10).

A possible mechanism that may explain this rapid reaction/adsorption is that paracetamol can be oxidised to the reactive metabolite N-acetyl-4-benzoquinone imin and further to 1,4-benzoquinone (Bedner et al. 2006). These metabolites may form covalent bounds with different structures of the humic fraction in the sediments by free radical coupling reactions via the semi-quinone imin or other radicals (LaKind et al. 1989). The oxidation of paracetamol to a phenoxy radical and a quinone, is probably necessary for this reaction (Bollag et al. 1985) and can be mediated chemically *e.g.* by manganese oxide (Chen et al. 1991; Ononye et al. 1994; Zhao et al. 2006), iron oxides

(LaKind et al. 1989; Li et al. 2003) or different enzymes from micro organisms (Simmons et al. 1989). Thus, the main part of the paracetamol in the sediments was presumably not detected by the analytical method used. An assessment of this can only be made after elucidation the nature of the binding.

### **3.4 Fish**

NSAID:s and paracetamol could not be detected in the fish muscle (<0.2-0.6 µg/kg f.w.) samples at any of the sampling stations. In addition, fish bile was analysed, after enzymatic hydrolysis, since it is considered as a more sensitive marker for exposure (Oikari et al. 1984; Adolfsson-Erici et al. 2002) but, NSAID:s and paracetamol could not be detected in the bile samples. These results seem to be logic as all the substances but paracetamol is ionised at the pH in the river (7.5) giving the compounds low octanol/water partition coefficient, log  $K_{ow}$  (Weigel et al. 2004). Paracetamol is not ionized at the river pH (pKa 9.5) but has a low log  $K_{ow}$  (0.5-09; (Kasprzyk-Hordern et al. 2008) and is thus not expected to bio-accumulate in fish.

## **4 Conclusions**

- The concentrations of conjugated NSAID:s were generally low in sewage water and can not solely explain the observation of higher concentrations in effluent compared to influent. The phenomenon of higher effluent concentrations compared to influent concentrations is more likely to be a sampling and / or analytical artefact.
- The distribution of the analysed NSAID:s in the WWTP influent and effluent mirrored the sales of the pharmaceuticals quite well.
- Two metabolites of ibuprofen were significant constituents in WWTP effluent water, surface water and sediments.
- NSAID:s discharged from the WWTP to the aquatic environment were detected in surface water and sediment at low ng/l and µg/kg dw concentrations respectively.
- Diclofenac was detected in sediment but not in river water probably due to rapid photolytic degradation.
- NSAID:s were not detected in the fish collected in the vicinity of the WWTP, implying low exposure to biota.
- Salicylic acid was a minor component in WWTP influent- and effluent water but significant in surface water and sediment due to natural sources.

## **5 Acknowledgements**

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## Appendix 1 – List of samples

MR-number	Type	Matrix	Site	Information	Coordinates
MR6001	Free and conjugated	Water – influent	Sandholmen ARV WWTP	2008-08-03	X: 7254361 Y: 1763998
MR6002	Free and conjugated	Water – effluent	Sandholmen ARV WWTP	2008-08-03	See above
MR6003	Free and conjugated	Water – influent	Sandholmen ARV WWTP	2008-08-08	See above
MR6004	Free and conjugated	Water – effluent	Sandholmen ARV WWTP	2008-08-08	See above
MR6007	Free and conjugated	Surface water	Hemlunda (#1 on map)	2008-08-09	X: 7258669 Y: 1760019
MR6010	Free and conjugated	Surface water	Sandholmen (#2 on map)	2008-08-09	X: 7253257 Y: 1764578
MR6013	Free and conjugated	Surface water	South Sandholmen (#3 on map)	2008-08-09	X: 7252160 Y: 1765154
MR6015	Free and conjugated	Surface water	Pitsund (#4 on map)	2008-08-09	X: 7248562 Y: 1766800
MR6005	Free	Sediment 0-2 cm	Hemlunda (#1 on map)	2008-08-09	See water sample
MR6008	Free	Sediment 0-2 cm	Sandholmen (#2 on map)	2008-08-09	See water sample
MR6011	Free	Sediment 0-2 cm	South Sandholmen (#3 on map)	2008-08-09	See water sample
MR6014	Free	Sediment 0-2 cm	Pitsund (#4 on map)	2008-08-09	See water sample
MR6006	Free and conjugated	Fish perch	Hemlunda (#1 on map)	2008-08-09	See water sample
MR6009	Free and conjugated	Fish perch	Sandholmen (#2 on map)	2008-08-09	See water sample
MR6012	Free and conjugated	Fish perch	South Sandholmen (#3 on map)	2008-08-09	See water sample

Explanations: Free = not conjugated NSAID:s; Conjugated = conjugated, e.g. glucuronides or sulphates, of NSAID:s.

## Appendix 2 – Results

Sample	Type	Matrix	Unit	Acetylsali- cylic Acid	Salicylic Acid	Ibuprofen	Ibuprofen- OH	Ibuprofen- COOH	Naproxen	Ketoprofen	Diclofenac	Paracetamol
MR6001	Free	WWTP Influent	µg/L	<0.05	<0.015	2.6	0.85	0.19	2.3	0.49	0.19	36
MR6002	Free	WWTP effluent	µg/L	<0.025	0.07	3.2	2.2	0.02	3.4	0.53	0.23	14
MR6003	Free	WWTP Influent	µg/L	<0.05	0.12	8.0	1.4	0.25	7.3	1.4	0.54	59
MR6004	Free	WWTP effluent	µg/L	<0.025	0.03	3.5	1.8	0.61	3.2	0.57	0.22	29
MR6007	Free	Surface water	ng/L	<25	12	0.48	<0.2	<0.2	0.46	<0.5	<0.2	170
MR6010	Free	Surface water	ng/L	<25	14	7.4	6.1	5.8	5.6	1.2	<0.2	220
MR6013	Free	Surface water	ng/L	<25	11	6.1	4.9	4.9	5.5	1.2	<0.2	360
MR6015	Free	Surface water	ng/L	<25	10	1.5	1.6	1.3	1.2	<0.5	<0.2	110
MR6005	Free	Sediment	µg/kg dw	<5	3.6	0.97	<1	<1	0.29	<0.5	3.5	69
MR6008	Free	Sediment	µg/kg dw	<5	0.9	6.0	<1	<1	<0.2	<0.5	0.19	18
MR6011	Free	Sediment	µg/kg dw	<5	0.5	0.21	<1	<1	<0.2	<0.5	0.85	42
MR6014	Free	Sediment	µg/kg dw	<5	3.9	0.74	<1	<1	0.31	<0.5	3.1	64
MR6006	Free	Fish	µg/kg fw	<10	<0.6	<0.6	<1	<1	<0.2	<0.5	<0.2	<5
MR6009	Free	Fish	µg/kg fw	<10	<0.6	<0.6	<1	<1	<0.2	<0.5	<0.2	<5
MR6012	Free	Fish	µg/kg fw	<10	<0.6	<0.6	<1	<1	<.02	<0.5	<0.2	<5
MR6001	Conj.	WWTP Influent	ng/L	<50	11	3.7	170	17	2.5	<0.2	2.7	nd
MR6002	Conj.	WWTP effluent	ng/L	<25	8.0	35	460	1.4	34	3.3	17	nd
MR6003	Conj.	WWTP Influent	ng/L	<50	11	1.3	110	10	2.0	<0.2	1.1	nd
MR6004	Conj.	WWTP effluent	ng/L	<25	18	2.7	390	42	3.5	<0.2	3.2	nd
MR6007	Conj.	Surface water	ng/L	<25	<0.5	0.30	<0.5	3.9	<0.2	<0.5	<0.2	nd
MR6010	Conj.	Surface water	ng/L	<25	<0.5	0.77	1.2	0.63	<0.2	<0.5	<0.2	nd
MR6013	Conj.	Surface water	ng/L	<25	<0.5	0.30	1.2	0.51	<0.2	<0.5	<0.2	nd
MR6015	Conj.	Surface water	ng/L	<25	<0.5	0.25	0.5	0.5	<0.2	<0.5	<0.2	nd
MR6006	Conj.	Fish	µg/kg fw	<10	<0.6	<0.6	<1	<1	<0.2	<0.5	<0.2	<5
MR6009	Conj.	Fish	µg/kg fw	<10	<0.6	<0.6	<1	<1	<0.2	<0.5	<0.2	<5
MR6012	Conj.	Fish	µg/kg fw	<10	<0.6	<0.6	<1	<1	<0.2	<0.5	<0.2	<5

Explanations: dw = dry weight; fw = fresh weight; nd = not determined.

## **Appendix 3 – Method development: analysis of free and conjugated non-steroid anti-inflammatory drugs (NSAID:s)**

### **Background**

The present method development is based on the analytical method used in the screening project 2005 (Andersson et al. 2006). In this investigation were ibuprofen, naproxen, ketoprofen and diclofenac analysed in different types of samples such as water, sediments and sludge. The procedure for derivatisation, clean-up and detection (GC-MS) was therefore already established but methods for concentration, isolation and hydrolysis of conjugated species of the NSAID:s was lacking and was the main objective in the present method development.

Some new compounds, expected to be important constituent in the WWTP water and possibly in environmental samples, were also added to the analytical protocol. The high volume pharmaceuticals acetylsalicylic acid and paracetamol (4-hydroxyacetanilid) were included together with the principal metabolites of ibuprofen (hydroxy- and carboxy-ibuprofen) and acetylsalicylic acid (salicylic acid).

Paracetamol could not be analysed under the same condition as the NSAID:s. Therefore, it was necessary to find a proper procedure for this compound as well.

Thus, the present development work was aimed at establishing an analytical method capable to (a) extract free and conjugated NSAID:s from water samples, (b) separate free and conjugated NSAID:s, (c) quantitatively hydrolyse the conjugated NSAID:s and (d) concentrate and quantify paracetamol.

### **Materials and methods**

The solvents methanol, hexane, acetone, methyl-*tert*-butylether (MTBE) used in the experiments were delivered from Rathburn, (Rathburn Chemical Ltd., Peeblesshire, Scotland).

Two commercial SPE-columns were studied for their, ability to concentrate the conjugated forms of the drugs discussed above: ENV+ (200 mg; Sorbent AB) and LiChrolut (200 mg; Merck, Darmstadt, Germany).

The NSAID:s ibuprofen, naproxen, ketoprofen and diclofenac as well as the derivatisation reagent methylchloroformat were delivered from Sigma-Aldrich Sweden AB.

Paracetamol was commercially available as glucuronid conjugate as well as the two model compounds for conjugated compounds, 4-methylumbelliferone glucuronide and 4-methylumbelliferone sulfat (Sigma-Aldrich).

The remaining analytes could not be obtained as conjugate commercially but were essential for the method development. This dilemma was solved by using a human urine sample containing all the target NSAID:s as conjugates. In this sample were about 92 % of the NSAID:s conjugated. The total concentrations of the NSAID:s (free plus conjugated) in the urine sample were determined after enzymatic hydrolysis. This result was used to compare the results obtained in the method development experiments including concentration, fractionation and hydrolysis.

The conjugated NSAID:s were hydrolysed with  $\beta$ -glucuronidase with sulphatase activity (1000 units/ml of H-3AF; *Helix pomatia*; Sigma, St. Louis, MO, U.S.A.). Supra pure water used in the experiments and was produced by a Milli-Q equipment (Millipore, Milford, MA).

Recovery standards used for all analytes but paracetamol were 2,4,5-trichlorophenoxy propionic acid and 1-naphtoic acid (Aldrich Chemie Steinheim, Germany).

A proper recovery standard for paracetamol analysis was needed. Theoretically, a possible candidate was 3-chloro-4-hydroxyacetanilid (3-chloroparacetamol) but was not commercially available.

3-chloroparacetamol was therefore synthesised and proved to be a good recovery standard. The synthesis of 3-chloroparacetamol was performed as follows. 2-chloro-4-hydroxyanilin (Sigma-Aldrich) was dissolved in water. Acetic acid anhydride (Fluca Buchs, Switzerland) was added and the mixture was heated on a heating block (50°C) for two hours and occasional shaken. The synthesis was chilled and water was carefully added. The synthesis product was extracted with organic solvent. Remaining traces of the aniline was removed by shaking the extract with HCl (1 M). The solvent was evaporated and the product was re-crystallized from hexane yielding a final product with a purity of 98 % (GC-MS).

## **Instrumentation**

### **GC-MS**

The sample extracts were analysed on a 6890N gas chromatograph coupled to a 5973N mass selective detector (Agilent). The injection, 1  $\mu$ l, was done in splitless mode at 240°C. The fused silica capillary column (VF-5MS 30 m x 0.25 mm i.d. x 0.25  $\mu$ m film thickness, Varian) was held at 45°C for 1 min., ramped 15°C/min to 200°C, 5°C/min until 300°C and held at 300°C for 5 min. Helium was used as carrier gas. The detector was used in selected ion monitoring mode (SIM) with electron ionisation at energy of 70 eV. The analytes were identified by their characteristic retention time and one quantification ion (Q-ion) and one or two supporting ions (S-ion) was recorded to increase the specificity (Table 2). Quantification was based on comparison of peak abundance to the known response of the internal standards. The analyte concentrations were corrected according to the determined surrogate standard losses.

Table 2. Ions used in MS-analysis of NSAID:s as MCF-derivatives and paracetamol as acetate.

Substance	Rt (min)	Trg-ion	Q1-ion	Q2-ion
<i>Biphenyl</i>	9.8	154	-	-
Acetylsalicylic acid	9.91	120	152	-
Salicylic acid	10.75	135	133	166
Ibuprofen	11.00	161	177	220
<i>1-naphtoic acid</i>	10.90	155	127	186
Hydroxy-Ibuprofen	12.31	119	118	178
<i>2,4,5-TP</i>	12.90	198	282	223
Paracetamol	13.24	109	151	193
<i>3-chloro-4-hydroxyacetanilid</i>	13.24	143	185	-
Carboxy-Ibuprofen	13.53	205	264	-
Naproxen	15.93	185	244	-
Ketoprofen	17.43	209	268	-
Diclofenac	18.49	214	242	309

Abbreviations: Names in italics are the recovery standards and injection standard (biphenyl). Rt = retention time. Trg = Target ion. Q1 and Q2 = qualifier ions.

## HPLC-UV

Analysis of paracetamol glucuronide, 4-methylumbelliferone glucuronide and 4-methylumbelliferone sulfate were performed on a HPLC instrument. The analytical system consisted of a pump (Costa Metric 4100, Thermo Separation Products), a UV-detector (LDC Milton Roy Consta Metric SM 4000) 254 nm) and a injector (Rheodyn model 7126) fitted with a 50  $\mu$ l loop. The analytical column was a reversed phase column (Licosphere 100, 5  $\mu$ , 205 x 4.6 mm; Hewlett Packard). The mobile phase was phosphate buffer (0.05M, pH 2) containing acetonitril (5 %) and the flow rate was 1.5 ml/min.

## Water sample preparation

The general analytical procedure used in the present investigation is described below. The clean-up procedure, when needed, was performed according to Andersson et al. (2006).

- The SPE-column (ENV+; 200 mg) was activated with methanol ethyl acetate (5 ml), methanol (5 ml) and phosphate buffer (5 ml; 0.05 M; pH 2).
- Before concentrated on the SPE-columns was the sample (200-1000 ml) pH-adjusted with phosphoric acid to pH 2. Prior to concentrate was surrogate standards added and the sample was mixed well. These surrogate standards were used for the quantification of the free fraction of NSAID:s and paracetamol.
- The flow rate during SPE-concentration was about 20 ml/min.
- The SPE-column was first dried on the vacuum manifold and then freeze-dried over night (-50°C; 7.5 x 10<sup>-2</sup> mbar).

- Unless otherwise stated, the analytes were successively eluted with first methanol (0.75 ml; allowed to soak into the column 10 min) and then a solvent mixture containing acetone and methanol (4:1; 5 ml).
- The combined eluate was evaporated to dryness using nitrogen gas, reconstituted in phosphate buffer (2 ml; 0.05 M; pH 2) and extracted three times with hexane:MTBE (2 ml; 3+1). This combined extracts contained the free fraction of NSAID:s.
- The remaining conjugated NSAID:s and paracetamol in the buffer were hydrolysed with enzyme (37°C; 1 hour) after adjusting the pH to 6.5 with ammonium acetate buffer.
- After acidification of the hydrolysate were the liberated NSAID:s recovered by three extractions with hexane:MTBE (2 ml; 3+1) after addition of surrogate standards. These surrogate standards were used for the quantification of the conjugated fraction of NSAID:s. The extract was dried over sodium sulphate and evaporated to dryness prior to derivatisation.
- Paracetamol (free plus conjugated) was recovered by extracting the hydrolysed sample twice with MTBE after addition of sodium chloride (about 200 mg).
- The NSAID:s were derivatised with methyl chloroformate prior to GC-MS analysis (see below). The phenolic pharmaceutical paracetamol was acetylated with acetic acid with pyridine as base (see below).

## Results

### *Choice of SPE-column*

Two solid phase columns (SPE) were compared for their ability to concentrate conjugated anti-inflammatory drugs and paracetamol: ENV+ (200 mg) and LiChrolut (200 mg). The columns were activated prior use with methanol and water.

In this experiment was known amounts of paracetamol-, 4-methylumbelliferone glucuronide and 4-methylumbelliferone sulfate used as test compound. These compounds were used in the initial experiment since they were expected to be good surrogates for the target analytes.

The concentration was performed at pH 2 and 6. After concentration, the SPE-columns were air dried on the vacuum manifold and subsequently eluted with methanol. The methanol eluates were analysed on a HPLC with UV-detector (254 nm) and the results from this initial experiment is summarised in Table 6.

Table 6. Recovery rate of conjugated compounds.

Compound	ENV+ (pH 2)	LiChr. EN (pH 2)	ENV+ (pH 6)	LiChr. EN(pH 6)
4-MU-G	95	78	<	<
4-MU-SO <sub>4</sub>	87	65	<	<
Paracetamol-G	95	78	<	<

Abbreviations: 4-MU-G = 4-methylumbelliferone glucuronide; 4-MU-SO<sub>4</sub> 4-methylumbelliferone sulfate; ENV+ (200 mg); LiChr = LiChrolut EN (200 mg).



The results from this experiment (Table 6) were in agreement with Weigel (2004), and showed that the ENV+ column was the column of choice and that acidification (pH 2) of the sample was necessary to obtain good recovery.

### ***SPE-extraction experiments***

In the recovery experiments were a synthetic sample prepared by dilute a urine sample (1 ml), containing all target analytes as conjugates in ultra pure water (200 ml; Milli-Q water). The synthetic sample was acidified (pH 2) and concentrated on the SPE-column (ENV+; 200 mg). The column was washed with buffer (pH 2) and dried for 15 min on the vacuum unit. Subsequently were the analytes eluted with methanol, hydrolyzed by enzyme and finally derivatised prior to GC-MS analysis. The undiluted urine sample was used as a reference and was treated as a sample besides that the SPE-treatment was omitted. The recovery of the SPE-concentrated conjugated pharmaceuticals was compared to the undiluted original urine sample. The results of these experiments are summarized in Table 7.

Table 7. Recovery of conjugate NSAID:s using ENV+ as SPE (ng/ml).

Compound	ENV+	Total	Recovery. %
Ibuprofen	18000	19000	92
Naproxen	4400	4600	96
Ketoprofen	3100	3200	98
Diclofenac	100	97	104

Explanations: Diluted urine sample (200 ml) concentrated on ENV+ (200 mg). Total = the content of the NSAID:s in urine sample that was hydrolyzed and extracted from the original urine sample.

The results in table 7 clearly showed that the ENV+ column concentrated all NSAID:s quantitatively: the ENV+-column extracted 92-104 % of the conjugated NSAID:s. Thus, was the ENV+-column used in the further experiments.

### ***Solvent extraction experiments***

Paracetamol was partly (69 %; Table 8) extracted with MTBE or ethyl-acetate but not with the solvent mixture hexane:MTBE (3+1) used to extract the NSAID:s. Quantitative extraction was obtained when sodium chloride was added (about 10 % w/w) prior to extraction with MTBE or ethyl-acetate (Table 8).

Table 8 Liquid extraction of paracetamol

Solvent	Recovery (%)
Hexane:MTBE (3+1)	0
Hexane:MTBE (1+1)	5
MTBE + NaCl	105
MTBE - NaCl	69
EA + NaCl	104
EA - NaCl	70

Sample (3 ml) was extracted at pH 2 with different solvents (a 1.5 ml) three times.

The great difference in extractability made it possible to completely isolate paracetamol from the NSAID:s and was accomplished by extracting the free NSAID:s with hexane:MTBE (3+1) from the sample leaving paracetamol left in the water phase. After addition of sodium chloride could paracetamol be extracted with MTBE or ethyl-acetate quantitatively.

### **Separation of free and conjugated NSAID:s.**

The ENV+ column was capable to extract both free and conjugated NSAID:s but the aim was to analyse the free and conjugated NSAID:s separately. Therefore, an attempt was made to separate free and conjugated NSAID:s by selective elution from the ENV+ column. Unfortunately, these experiments were not successful since the conjugated NSAID:s and a fraction (10-42 %) of the free diclofenac, naproxen and ketoprofen eluted simultaneously.

To overcome this problem was a procedure including first SPE and then solvent extraction procedure explored and these experiments showed that:

- free and conjugated NSAID and paracetamol were quantitatively recovered on the SPE-column,
- free NSAID:s were extracted with the solvent mixture hexane/MTBE (3+1) but paracetamol needed a stronger solvent *e.g.* MTBE.

Based on these results was a three step procedure constructed and validated. The first step implied sample concentrated on the ENV+ column. The column containing the concentrated analytes was freeze-dried over night prior to elution. All analytes (free and conjugated) were eluted first with methanol (0.75 ml) and then with acetone:methanol (4:1). The combined eluate was evaporated to dryness using nitrogen gas and the residue was reconstituted in phosphate buffer (2 ml; 0.05 M; pH 2). In step number two, was the separation of the free and conjugated compounds carried out. This was made by extracting the concentrated sample (SPE-concentrate) dissolved in the phosphate buffer, with a organic solvent mixture of hexane:MTBE. Two mixtures were tested: 10 % MTBE and 25 % MTBE in hexane. The obtained extracts were analysed before and after enzymatic hydrolyses and the results are summarized in Table 9.

Table: 9. Extraction of free NSAID:s with different organic solvents.

Compound	10 % no enz.	10 % enz.	25 % no enz.	25 % enz.
Ibuprofen	1200	1200	1100	1300
Naproxen	480	520	490	490
Ketoprofen	180	190	150	150
Diclofenac methyl ester	58	57	160	140

Explanation: 10 % and 25 % mean 10 and 25 % MTBE in hexane respectively.  
Enz. = enzymatic hydrolyses.

The results showed that free NSAID:s could be extracted quantitatively with 25 % MTBE in hexane leaving the conjugated substances and paracetamol left in the buffer solution. This conclusion was based on the facts that the amount of NSAID:s measured was independent of enzymatic hydrolysis implying that conjugated species were not extracted into the organic solvent used. In this extract was not even trace amounts of paracetamol detected.

Finally in the step 3, the conjugated NSAID:s and paracetamol in the buffer were hydrolysed and the liberated NSAID:s were extracted according to the method for free substances.

The performance of the whole procedure was investigated in an experiment using five parallel samples (200 ml) containing free and conjugated NSAID:s. Paracetamol was not incorporated in these experiments. The samples were concentrated on ENV+ columns and the columns were freeze-dried and subsequently eluted. Free and conjugated NSAID:s were separated by liquid-liquid extraction prior to enzymatic hydrolysis of the conjugated compounds. All samples were derivatized with methylchloroformate (MCF) and analysed by GC-MS. The results from this experiment are summarised in Table 10.

Table 10. Extraction efficiency and RSD of NSAID:s

Förening	RSD		Recovery
	Fri	Conj	%
Acetylsalicylic acid	4	77	31
Salicylic acid	4	14	112
Ibuprofen	5	2	103
Ibuprofen-OH	4	6	119
Ibuprofen-COOH	4	8	106
Naproxen	3	3	107
Ketoprofen	3	5	109
Diclofenac	4	14	114

Explanations: Ibuprofen-OH is the hydroxyl metabolite and Ibuprofen-COOH the carboxylate metabolite of ibuprofen. RSD = Relative Standard Deviation (%).

The results were satisfactory showing high recovery and low standard deviations (Table 9). Acetyl salicylic acid deviate from this picture. It was concluded that the used derivatization method gives semi quantitative results for this compound. Diclofenac and salicylic acid showed the highest standard deviation of the analytes by unknown reason.

### **Breakthrough volume**

The breakthrough volume of the SPE-column (ENV+; 200 mg) was investigated by concentrating sample volumes between 200-1000 ml spiked with equal amount of urine containing the conjugated NSAID:s.

Table 11. Recovery rate at different sample volume concentrated on SPE-column (ENV+; 200 mg).

Compound	250 ml	500 ml	750 ml	1000 ml
Acetyl salicylic acid	124	27	24	42
Salicylic acid	91	112	112	110
Ibuprofen	101	104	105	102
Hydroxy-ibuprofen	122	108	119	115
Carboxy-ibuprofen	105	105	104	103
Naproxen	111	106	108	107
Ketoprofen	116	106	106	113
Diclofenac	139	132	129	128

Explanations: Ibuprofen-OH is the hydroxyl metabolite and Ibuprofen-COOH the carboxylate metabolite of ibuprofen.

The results, summarised in Table 11, showed that no breakthrough was detected for any of the conjugated or free NSAID:s even when the column was loaded with 1000 ml sample (Figure 11).

## Hydrolysis of conjugate

The enzymatic hydrolyse was made according to (Blount et al. 2000). Briefly, the urine sample containing the conjugated NSAID:s was pH-adjusted to 6.5 by addition of ammonium acetat buffer  $\beta$ -glucuronidase enzyme with sulphatase activity was added and the sample was incubated at 37°C. The optimal incubation time was evaluated by incubate samples containing conjugated NSAID:s with the enzyme. Samples were periodically removed for analysis of the concentration of free NSAID:s. The duration of the experiment was 24 h. The yield of NSAID:s was constant within an incubations time of 1-24 hours. Thus, 1 hour incubation time was sufficient to quantitatively hydrolyse the NSAID-conjugates.

The enzymatic method was also compared to acid hydrolysis according to (Blum et al. 1996) and hydrolysis with sodium hydroxide according to (Sawchuk et al. 1995). The final concentration of HCl and KOH in the sample during the hydrolysis were 5 respective 1 M. The reaction was carried out at 70°C for 1 h. The results from this comparison are presented in Table 12.

Table 12. Comparison of chemical and enzymatic hydrolysis of conjugated NSAID:s.

Compound	Enzymatic	KOH	HCl
	$\mu\text{g/l}$	$\mu\text{g/l}$	$\mu\text{g/l}$
ASA	220	nd	10
Salicylic acid	3100	2100	2500
Ibuprofen	28000	29000	28000
Ibuprofen-OH	12000	11000	12000
Ibuprofen-COOH	26000	25000	23000
Naproxen	4800	5700	3200
Ketoprofen	4800	5500	5200
Diclofenac cyclic	9	31	520
Diclofenac	160	460	nd

Explanations: nd = not detected.

With a few exceptions gave the enzymatic and chemical hydrolysis comparable results. Acidic conditions gave lower naproxen results (Table 12) and transformed diclofenac quantitatively to its cyclic form (Figure 11). The quantitative results calculated for the cyclic form of diclofenac in Table 12 are considered semi quantitative.

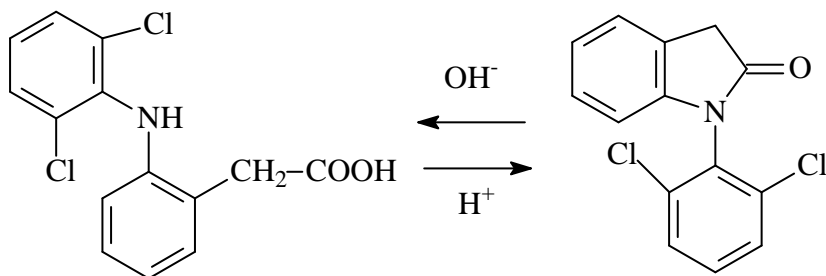


Figure 11. Production of the cyclic product 1-(2,6-dichlorophenyl)-2-indolinon (CAS 172371-96-9) of diclofenac under acidic conditions ( $\text{H}^+$ ) and the reverse reaction under alkaline conditions ( $\text{OH}^-$ ).

The reaction, producing the cyclic product of diclofenac, proved to be reversible and the mother compound could be recovered after treatment of the cyclic derivative with sodium or potassium hydroxide under the same conditions as used for chemical hydrolysis of conjugates. In a single hydrolyses experiment with the cyclic product of diclofenac, using KOH as base, attained a recovery of 85 %.

The diclofenac results exhibited great deviation between the studied hydrolyses methods (Table 12). The reason behind this is unclear but no attempt was made to investigate this discrepancy further.

## **Derivatisation**

### *NSAID:s*

The derivatisation of ibuprofen, naproxen, ketoprofen and diclofenac has been described elsewhere (Andersson et al. 2006). The method is based on Weigle et al. (2002, and Butz and Stan (1993), with minor modifications. Briefly, the dried extracts were dissolved in reagent solvent containing acetonitril/methanol/water/pyridine. A small aliquot of MCF was added and the sample vortexed (10 seconds). Subsequently, another aliquot of MCF was added the samples were vortexed for 10 seconds and left for 10 minutes to complete the reaction. The derivatisation was stopped by the addition of water. The samples were then extracted with hexane: MTBE (9+1; 2 ml), dried over sodium sulphate and concentrated. If needed was the derivatised extract subjected to clean-up on a silica column prior to GC-MS analysis (Anderson et al. 2006).

This methodology was also used for acetylsalicylic acid, salicylic acid and the metabolites of ibuprofen. The MCF-reagent was compared to the diazomethane reagent and the comparison revealed that all NSAID:s were reproducibly derivatised with MCF but acetylsalicylic acid. The measurements of this compound must therefore be considered as semi quantitative (se Table 11).

### *Paracetamol*

In order to attain good chromatographic properties and strong ions in MS-spectra was a number of derivatives of paracetamol explored: trimethylsilylether (TMS), di-*tert*-butyldimethylsilylether (DBDMS), and acetate. The silylated derivatives gave good chromatographic properties and strong ions in the mass spectrum.

Unfortunately, the silyl reagents produced two derivatives: mono- and di-silyl derivatives in varying proportions and the reaction were strongly influenced by the presents of sample matrixes.

The acetate derivative on the other hand gave one derivative and most important reproducible result. The draw back was a somewhat lower responses (15 %). The acetate derivative of paracetamol was therefore used for quantitative GC-MS analysis. Paracetamol and the surrogate standard 3-chloro-4-hydroxyacetanilid were acetylated in the solvent mixture hexane:MTBE (3+1) with the reagent acetic acid anhydride and pyridine as base. The reaction was accomplished at 65°C for 1 hour.

Paracetamol could be derivatised with the reagent methylchloroformate under the same conditions as used for NSAID:s but in this investigation was the acetate derivative preferred.

## **Conclusions**

We have developed an analytical method for the quantification of 7 common anti-inflammatory pharmaceuticals and the analgesic drug paracetamol. The method is capable to distinguish free and conjugated species of the anti-inflammatory compounds. The proposed method is accurate and precise and has a high sensitivity making it suitable for analysis of environmental samples.

The developed analytical method has successfully been applied on samples from WWTP and different environmental samples such as surface water, sediments and fish.