Uptake and disposition of pharmaceuticals by Bluegill exposed at constant concentrations in a flow-through aquatic exposure system

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Pharmaceuticals and personal care products (PPCPs) have been identified as ubiquitous contaminants in WWTP influents, effluents and surface water (Ternes, 1999; Kolpin et al., 2002; Fatta-Kassinos et al., 2011).

Fish exposed to effluent-dominated water may accumulate PPCPs
- USEPA pilot study of 36 PPCPs in fish tissues (Ramirez et al., 2009, ET&C, 28, 2587–2597).
- Antidepressant pharmaceuticals were found in wild fish tissues (Schultz et al., 2010, ES&T, 44, 1918-1925; Brooks et al., 2005, ET&C, 24, 464–469).

To better understand the potential for effects, we instigated a laboratory ADME (absorption, distribution, metabolism, and excretion) study for fish exposed to typical PPCPs.
Experimental Design

Bluegill (*Lepomis macrochirus*) or Pumpkinseed (*Lepomis gibbosus*)

**Chemicals:**
- Temazepam
- Methocarbamol
- Sulfamethoxazole
- Rosuvastatin
- Diclofenac
- EtoH (vehicle and control)

**Flow-through system**

- *Day 0, 10, 20, 30*
  - Water sample
  - Fish tissues: muscle, liver, brain, bile, blood

**Chemicals**:

- *Waste*
  - Tempo
  - Methocarbamol
  - Sulfamethoxazole
  - Rosuvastatin
  - Diclofenac
  - EtoH (vehicle and control)

**Experimental Design**

- Water sample
  - Internal standards
  - QuEChERS kit
  - Pfree cartridge
  - LC-MS/MS
  - uptake

- Solid tissues
  - QuEChERS kit
  - Pfree cartridge
  - LC-QTOF

- Liquid tissues
  - LC-QTOF
  - Metabolism
QuEChERS method:

- QuEChERS: Quick, Easy, Cheap, Effective, Rugged and Safe
- Multiresidue method commonly used for analysis of pesticides in food.
- Recently, applied to analyze emerging contaminants, such as estrogens (Jakimska et al., 2013) in whole fish and pharmaceuticals (Bueno et al., 2013) in whole mussels.

Three steps:
- Aqueous salt extraction, acetonitrile back extraction, and dispersive solid phase extraction (d-SPE)

References:
Two salt extraction kits tested:

- Non-buffer kit (Agilent 5982-5850: 4 g MgSO\(_4\); 0.5 g NaCl)
- Buffer kit (Agilent 5982-5650CH: 4 g MgSO\(_4\); 1 g NaCl; 1 g NaCitrate; 0.5 g disodium citrate sesquihydrate)

- For most chemicals, no obvious difference demonstrated in recoveries between non-buffer and buffer kits.
- However, diclofenac had a smaller SD for buffer kit recoveries.
- For polar ion chemicals, buffered extraction chosen over unbuffered extraction for smaller pH fluctuation.

References:
Five dispersive solid phase extraction (d-SPE) kits tested:

- Recoveries for the d-SPE kits were uniform, except for kit with highest PSA content.
- Sulfamethoxazole, methocarbamol showed slight increase with 50, 150 mg amounts of PSA, but response of diclofenac decreased dramatically for 400 mg PSA.
- GCB is good for most of the PPCPs, but exhibited strong absorbance for diclofenac.
- Supelco Z-Sep/C18 kit showed best overall responses for most PPCPs.

References:
Optimized QuEChERS Extraction Procedure

1. Muscle (2 g) or liver (0.5 g)
2. Add 5mL water and 2 CHs
3. Spiking 20 ng internal standards
4. Homogenization (vortex 1 min)
5. Add 10 mL ACN (1% acetic acid)
6. Vortex 1 min
7. Add buffer extraction kit *
8. Optional Enzyme Hydrolysis
9. Optional
10. Centrifugation (if necessary)
11. LC-MS/MS
12. Manual shaking 1 min
13. Centrifuge 5 min
14. Transfer 6 mL ACN to d-SPE **
15. Vortex 1 min; Centrifuge 5 min
16. Transfer 4 mL ACN, dry by N₂
17. 0.4 mL 8:2 water:ACN

CHs: ceramic homogenizer; ACN: acetonitrile

* Agilent 5982-5650 kit; ** Supelco Z-Sep/C18 Kit
• Agilent 1200 LC system coupled with 6410 Triple Quadrupole MS with ESI source
• Positive mode for sulfamethoxazole, methocarbamol, rosvastatin, temazepam
• Negative mode for diclofenac
• Injection volume: 10 µL
• Mobile phase
  – Positive: 0.1 M formic acetate buffer (A) and methanol (B)
  – Negative: 1% acetic acid (v/v) (A) and methanol (B)
• Flow: 0.4 mL/min
First exposure experiment

Chemicals:
- Sulfamethoxazole; methocarbamol; rosvastatin; temazepam; diclofenac
- EtOH as blank control

- Single exposure for each compound.
- The nominal concentration is 1,000 ng/L for each.
- Most of the recoveries in water phase are close to 100%.

![Chemical structures](image)

Sulfamethoxazole $\log K_{ow}=0.48$
Methocarbamol $\log K_{ow}=0.48$
Rosuvastatin $\log K_{ow}=2.05$
Temazepam $\log K_{ow}=2.15$
Diclofenac $\log K_{ow}=0.7$
Temazepam in fish tissues:

- Temazepam showed strongest uptake in fish tissues among the five chemicals tested. The concentration ranges are <LOQ (0.84)-1.97 ng/g ww in muscle, 11.15-32.59 ng/g (ww) in liver, 5.85-14.0 ng/g in brain, and 2,350-4,160 ng/mL in bile (preliminary estimates).
- No obvious difference was found between non-enzyme hydrolysis and enzyme hydrolysis samples.
- Slight increase of concentration from day-10 to day-20 in muscle and bile; but decrease in liver and brain.
- Bioaccumulation factors (BCFs) were 0.72-1.99 L/kg in muscle, 10.4-32.9 L/kg in liver, 5.63-14.1 L/kg in brain; and 2,370-3,780 in bile (preliminary estimates).
Methocarbamol and rosuvastatin in fish tissues:

- Concentrations of methocarbamol in muscle were at range of 0-0.22 ng/g, below the LOQ (0.26 ng/g). Rosuvastatin was not measured in muscle.
- Methocarbamol and rosuvastatin were sporadic measured in liver and brain, but below or just a little higher than LOQ.
- Methocarbamol and rosuvastatin were not measured in bile.
Uptake and Depuration Experiment

Expected aqueous concentrations of SMX, TEM and DCF were 2,000 ng/L, and MET is 4,000 ng/L.

1: Single exposure;
2: Tem/Met;
3: Mix-all
Uptake and Depuration Kinetics

- Temazepam and methocarbamol in fish muscle

1: Single exposure;
2: Tem/Met;
3: Mix-all

Note: provisional data, not for citation
Uptake and Depuration Kinetics

- Temazepam and methocarbamol in fish liver

1: Single exposure;
2: Tem/Met;
3: Mix-all

Note: provisional data, not for citation
Uptake and Depuration Kinetics

- Temazepam and methocarbamol in fish brain

1: Single exposure;
2: Tem/Met;
3: Mix-all

Note: provisional data, not for citation
- Temazepam and methocarbamol in fish bile

1: Single exposure; 2: Tem/Met; 3: Mix-all

Note: bile samples cleaned up with a phospholipid-removing SPE procedure

Note: provisional data, not for citation
Bioconcentration Factors and Partitioning Coefficient

Note: bioconcentration factors (BCF) are in log form (log BCFs); pH adjusted octanol–water partition coefficient also in log form (log Dow).
Uptake and Elimination Rate Constants

Bluegill exposed to temazepam and methocarbamol

**Ku—uptake rate coefficients**

![Graph A](image)

**Ke—elimination rate coefficients**

![Graph B](image)

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- single—exposed to temazepam or methocarbamol;
- binary—mixture of temazepam and methocarbamol;
- mix all—mixture of all four pharmaceuticals.
Conclusions

• QuEChERS and dispersive-SPE method with isotope labelled internal standards obtained satisfied recoveries and method detection limits.

• Temazepam showed the strongest uptake in fish tissues. Methocarbamol sulfamethoxazol and diclofenac displayed lower uptake in some tissues. Rosuvastatin was least likely to be taken up in fish tissue.

• Uptake and depuration results suggest that tissue concentrations are closely linked to ambient concentrations.

• Identification of various metabolites is necessary to evaluate the potential of ADME processing.

Note: provisional data, not for citation
Thank you!

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Diclofenac in fish tissues:

- Diclofenac was measured at the concentration of <LOQ (0.24)-2.38 ng/g ww in muscle; and in one liver sample (22.28 ng/g ww); and in two bile samples (0.78 and 1.53 ng/mL).
- Diclofenac was not detected in fish brain tissue.
- The BCFs are 0.12-2.92 L/kg in muscle, 28.36 L/kg in the one liver sample, 0-1.99 in bile.
Sulfamethoxazole in fish tissues:

- Concentration of sulfamethoxazole was low in muscle. Most were below LOQ (0.38 ng/g ww), except one enzyme hydrolysis sample (0.39 ng/g ww).
- Sulfamethoxazole was measured at the range of <LOQ (0.58 ng/g ww)-2.02 ng/g ww in liver, and 0.32-3.60 ng/g ww in brain, 0.15-2.34 mg/mL in bile.
- The BCFs are 0.41 L/kg in the one muscle sample, 0.33-2.96 L/kg in liver, 0-3.04 L/kg in brain, 0-2.44 in bile.
The nominal concentrations of sulfamethoxazole, temazepam and diclofenac are doubled to 2,000 ng/L.

The nominal concentrations of methocarbamol and rosuvastatin are quadrupled to 4,000 ng/L.

The recoveries of most compounds in water phase are near 100%.

**Chemicals:**
- Sulfamethoxazole; methocarbamol; rosuvastatin; temazepam; diclofenac
- EtOH as blank control
Multiresidue analysis of PPCPs in tissues

- Ultrasonic extraction + SPE (solid phase extraction) purification
  - USEPA method for PPCPs and some metabolites
  - Ramirez et al., 2007, Analytical Chemistry, 79(8): 3155-3163
  - Ramirez et al., 2009, ETC, 28, 2587–2597

- Pressurized liquid extraction + silica gel or GPC purification
  - Antibacterials, carbamazepine, musks, alkylphenol, UV filters
  - Kim et al., 2011, JCA, 1218, 3511–3520
  - Subedi et al., 2011, JCA, 1218, 6278–6284

- SPME with isotope corrected matrix method
  - Antibacterials, UV filters in fish muscle, brain, blood
  - Zhang et al., 2011, Analytical Chemistry, 83: 6532-6538

Costly, time consuming, and solvent consuming!
β-Glucuronidase: Type HP-2S, Sigma (G7770), from Helix pomatia
Buffer: pH 5.0 0.2M sodium acetate buffer
Enzyme solution: 2% (v/v) β-Glucuronidase in sodium acetate buffer
CHs: ceramic homogenizer

Muscle (2g)  
Other tissue (0.5g)  
Spiking 20 ng internal standards  
0.4 mL enzyme solution  
4.6 mL buffer, 2CHs  
Homogenization (vortex 1 min)  
Enzyme hydrolysis 37°C, 4h  
As non-enzyme method

Spiking of DCF-GLUC in 2.0g muscle, and after enzyme hydrolysis, the recoveries based on the calculation of DCF are 87±3%.

Recoveries, LODs and LOQs

<table>
<thead>
<tr>
<th></th>
<th>Recovery (%)</th>
<th>Reporting limit b</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 ng/g ww spiking (n=5)</td>
<td>1 ng/g ww spiking (n=7)</td>
<td>LOD (ng/g)</td>
<td>LOQ (ng/g)</td>
</tr>
<tr>
<td><strong>Muscle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2 g basis)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>100±2</td>
<td>50±12 a</td>
<td>0.11</td>
<td>0.38</td>
</tr>
<tr>
<td>Methocarbamol</td>
<td>115±4</td>
<td>88±4 a</td>
<td>0.08</td>
<td>0.26</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>108±8</td>
<td>130±21</td>
<td>0.25</td>
<td>0.85</td>
</tr>
<tr>
<td>Temazepam</td>
<td>99±6</td>
<td>109±5</td>
<td>0.25</td>
<td>0.84</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>104±2</td>
<td>109±5</td>
<td>0.07</td>
<td>0.24</td>
</tr>
</tbody>
</table>

a Background concentrations for sulfamethoxazole and methocarbamol are found at 1.39 ng/g and 0.10 ng/g wet weight (ww); b Signal to noise method.

<table>
<thead>
<tr>
<th></th>
<th>Recovery (%)</th>
<th>Reporting limit a</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 ng/g ww spiking (n=3)</td>
<td>2 ng/g ww spiking (n=3)</td>
<td>LOD (ng/g)</td>
<td>LOQ (ng/g)</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.5g basis)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>116±2</td>
<td>115±3</td>
<td>0.17</td>
<td>0.58</td>
</tr>
<tr>
<td>Methocarbamol</td>
<td>90±6</td>
<td>86±5</td>
<td>0.17</td>
<td>0.57</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>106±4</td>
<td>105±13</td>
<td>0.20</td>
<td>0.67</td>
</tr>
<tr>
<td>Temazepam</td>
<td>107±5</td>
<td>106±4</td>
<td>0.39</td>
<td>1.30</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>109±2</td>
<td>134±11</td>
<td>0.18</td>
<td>0.59</td>
</tr>
</tbody>
</table>

a Signal to noise method.
uptake in fish muscle:

- For the higher exposure concentration batch, temazepam was measured the high concentration in fish muscle up to 7.65 ng/g ww.
- Methocarbamol was detected in fish muscle at the higher exposure batch.
- Diclofenac and sulfamethoxazole were measured in some fish muscle with low concentration. Rosuvastatin was not measured in any fish.
- Further metabolite identifications are needed.

### Table 1. The second exposure

<table>
<thead>
<tr>
<th>Compound</th>
<th>Detection</th>
<th>Concentration ng/g</th>
<th>BCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temazepam</td>
<td>10/16</td>
<td>0.91-7.65</td>
<td>0.59-6.13 L/kg</td>
</tr>
<tr>
<td>Methocarbamol</td>
<td>10/15</td>
<td>0.52-1.20</td>
<td>0.11-0.43 L/kg</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>3/12</td>
<td>ND – 1.23</td>
<td>0-0.67 L/kg</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>10/12</td>
<td>ND - &lt;LOQ</td>
<td>/</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>ND</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

Spk= spiking 1 ng/g ww
Melissa M. Schultz, 37, associate professor of chemistry at the College of Wooster, passed away this spring following a car accident. Melissa was an outstanding environmental chemist and dedicated her scientific career to mentor undergraduate students to follow in her footsteps.

To honor her memory, we are establishing a permanent SETAC undergraduate travel fund in her name to continue providing undergraduate research students with the opportunity to attend our annual SETAC meetings.

Given the many lives Melissa has touched, we are hoping to reach this goal at this years’ SETAC meeting. If you would like to donate to this fund, just access the endowment page of the SETAC website: http://www.setac.org/?page=SNAEndowmentCom and click on “Donate online”. Simply add “In memory of Melissa Schultz” under “Donor Comments” and SETAC will keep track of the donation for the purpose of the Melissa Schultz student travel award.
How It All Started

Boulder Creek, CO

Fourmile Creek, IA