SCREENING TEST BATTERY FOR PHARMACEUTICALS IN URINE AND WASTEWATER

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(Received 22 April 2004; Accepted 18 August 2004)

Abstract—A test battery for identifying ecotoxicological hazards was applied to six pharmaceuticals (carbamazepine, diclofenac, ethinylestradiol, ibuprofen, propranolol, and sulfamethoxazole), to their mixtures, and to urine spiked with pharmaceuticals to test the suitability of biotests for screening urine and wastewater and for monitoring the efficiency of wastewater treatment. The test battery comprised the bioluminescence inhibition test with Vibrio fischeri, the yeast estrogen screen, and a photosynthesis inhibition assay in algae based on chlorophyll fluorescence measurements. Mixture and additional experiments with a cocktail of pharmaceuticals added to urine confirmed the applicability of the test systems as an integrated measure of the overall micropollutant burden. Because the concentration of pharmaceuticals in wastewater is low and the nutrients and salts may have a negative impact on the bioassays, urine and wastewater samples were cleaned and concentrated by solid-phase extraction (SPE). The compounds of interest ranged from polar to nonpolar and from positively charged to neutral and negatively charged. Consequently, the SPE method was optimized for universality rather than for specificity. Results of preliminary experiments with raw and treated urine and wastewater indicate the suitability of the proposed test battery for screening urine and wastewater.

Keywords—Concentration addition Independent action Toxicity mechanism Solid-phase extraction Whole-effluent toxicity testing

INTRODUCTION

Concern is growing over the increasing number of pharmaceuticals detected in the environment [1,2]. Only a small number of pharmaceuticals are produced in high volumes, and most of these have been found in wastewater and receiving waters only in low concentrations [3]. However, a large knowledge gap exists regarding the overall load of pharmaceuticals in the environment. Furthermore, pharmaceuticals usually are well metabolized in the human body, so not only the parent compounds but also their metabolites may pose an environmental hazard. Very little is known about the actual impacts of pharmaceuticals and their metabolites on aquatic biota. Whole-effluent toxicity testing [4,5] and test batteries of (micro-)bioassays [6,7] may help to assess the hazard and site-specific environmental risk of pharmaceuticals.

Biotests generally cannot distinguish between pharmaceuticals and other pollutants unless the source of pollution is known or the biotest targets the specific mode of toxic action by a given pharmaceutical. An example is a test battery of in vitro assays for endocrine disruption that was applied to the toxicological profiling of sediments [8]. An alternative approach is bioassay-directed fractionation [9] that aims to isolate and identify the causative toxic agent. This approach is a very helpful tool if a small number of compounds dominates the overall toxicity, but it is unsuitable and has never been applied to municipal wastewater, where we expect a high number of compounds at low concentrations.

Effect-based screening tools have the advantage of indicating the impact of all chemicals present in a given environmental sample. Unlike in chemical analysis, a compound cannot be overlooked in effect-based analysis, because even compounds at concentrations less than those causing a visible effect may contribute to mixture toxicity [10]. However, effect-based screening tools are prone to artifacts caused by a disturbance of the toxic response in the tests by natural components of the sample or incomplete extraction methods. The assumption of concentration addition of the components often implies that an ecotoxicological test system is an integrative measure of the overall toxic effect of the mixture. However, the assumption of concentration additivity needs to be tested before practical application of the test systems. We postulate that common toxic mechanisms dominate overall toxicity, because their effects are concentration additive. This hypothesis also implies that certain intricate, specific effects of single compounds will not greatly affect the overall toxicity of the mixture.

Here, we propose an ecotoxicological test battery for identifying pharmaceutical hazards. It contains a selective test system, the yeast estrogen screen (YES) for the detection of estrogenic compounds, and two more integrative tests, the bioluminescence inhibition test with Vibrio fischeri and the chlorophyll fluorescence test with the green algae Desmodesmus subspicatus. The chlorophyll fluorescence test allows identification of both specific photosynthesis inhibition and nonspecific effects.

The first aim of the present study was to evaluate the effects of six selected pharmaceuticals (carbamazepine, diclofenac, ethinylestradiol, ibuprofen, propranolol, and sulfamethoxazole), both alone and in a mixture. The mixture experiments were used to test the often-stated hypothesis that concentration addition is a realistic worst-case scenario for mixtures in the environment [11].

The second aim of the present study was to test a cocktail of these pharmaceuticals in the presence of urine or wastewater, both to evaluate the matrix effects and to develop an
appropriate sample preparation method based on solid-phase extraction (SPE). No cleanup procedure generally is used in whole-effluent toxicity testing when preparing the sample for the toxicity test [4,5]. However, the high concentrations of nutrients, salts, and colored molecules in urine and wastewater made it necessary to introduce such a step. The SPE method developed here also might serve as a cleanup or preconcentration step for more diluted samples, such as wastewater treatment plant effluents and receiving waters. Finally, we show some preliminary results regarding urine and wastewater before and after treatment in a bioreactor and a wastewater treatment plant, respectively, to discuss the suitability of the proposed test battery for use in site-specific risk assessment and for monitoring the efficiency of wastewater treatment.

Our overarching goal was to design and validate a screening test battery for application in the Novaquatix research project (http://www.novaquatix.eawag.ch), in which urine is separated at the source (i.e., in the toilet) [12,13]. The urine is then treated to recover nutrients for fertilizer production and to remove micropollutants and, thus, avoid releasing them into wastewater treatment plants. To monitor the treatment efficiency, we need a robust and reliable test battery. In addition to this specified application, this battery also should be applicable to other types of environmental samples, such as wastewater effluents or landfills.  

**MATERIALS AND METHODS**

**Chemicals**

The pharmaceuticals propranolol (Chemical Abstracts Service Registry Number [CAS RN] 525-66-6; purity, >98%), sulfamethoxazole (CAS RN 723-46-6, no purity reported), 17α-ethinylestradiol (CAS RN 57-63-6; purity, >98%), diclofenac (CAS RN 15307-856-5, no purity reported), ibuprofen (CAS RN 15687-27-1; purity, >98%), and carbamazepine (CAS RN 298-46-4, no purity reported) were obtained from Sigma (Buchs, Switzerland). For the chlorophyll fluorescence test, the reference compound was diuron 3-(3,4-dichlorophenyl)-1,1-dimethylurea (CAS RN 330-54-1; purity, 99.4%; Riedel-de Haen, Buchs, Switzerland), and for the YES, the reference compound was 17β-estradiol (CAS RN 50-28-2; purity, >97%; Fluka, Buchs, Switzerland). All solvents and salts were obtained from Fluka. Stock solutions were prepared in water and then acidified for the bases and made slightly alkaline for the acids to improve solubility. If dissolution in water was not possible, 0.1 M solutions in ethanol were prepared, and ethanol was either evaporated in the test vials (YES) or accounted for by adding the same amount of ethanol to the controls. Each toxicity experiment (single compounds and mixtures) was performed with at least three replicates and at least two independently prepared solutions. Concentrations are reported as nominal values. Concentrations in solutions were checked by chemical analysis as reported previously [14].

**Bioluminescence inhibition test**

The 30-min bioluminescence inhibition test with the marine bacterium *V. fischeri* was performed according to the protocol of the International Standard Organization (ISO 11348-3) [15]. This test often is referred to as the Microtox test (so named after one of the providers of the instrumentation). Freeze-dried bacteria obtained from Dr. Bruno Lange (Düsseldorf, Germany) were reconstituted in saline buffer containing 4 mM KCl, 10 mM MgCl₂, 10 mM MOPS (3-[[N-morpholino]propanesulfonic acid), and 346 mM NaCl with the pH adjusted to 7.0 ± 0.2 with HCl/NaOH. Exactly 500 μl of diluted bacterial suspension were mixed with 500 μl of sample in the saline buffer (plus a maximum 2.5% [v/v] of ethanol at the highest-exposure concentration) and incubated for 30 min at 288 ± 1 K. The luminescence output was then read with a LUMISTox 300 luminometer (Dr. Bruno Lange). Data evaluation was performed according to ISO 11348-3 (Eqn. 1), and the concentration–effect curves were analyzed as described below:

\[
\text{bioluminescence inhibition (%) } = \left(1 - \frac{\text{light intensity}_{\text{sample}}}{\text{light intensity}_{\text{control}}} \right) \cdot 100 \quad (1)
\]

**Chlorophyll fluorescence test**

The green unicellular algae, *D. subspicatus* (Chodat) SAG 86.81, obtained from the SAG Sammlung von Algenkulturen at the University of Göttingen (Göttingen, Germany), were grown in batch cultures in the medium of the Organisation for Economic Co-Operation and Development (OECD) Test Guideline 201 for the algal growth inhibition test [16]. The algae were transferred into a new medium every 3 to 4 d, and a new culture was started from an agar plate after a maximum of seven transfer cycles to maintain the growth permanently in the exponential phase. The growth rate in the exponential phase was 0.051 ± 0.006 h⁻¹.

The algae cultures were harvested in the exponential growth phase, centrifuged, and diluted to an optical density at 685 nm of 0.05. Then, 10 ml of the suspension were transferred to sterile, 20-ml glass vials with Teflon®-lined caps. Each test was performed with two controls and with 6 to 10 samples having different chemical concentrations. Each experiment was repeated two to five times. The glass vials were incubated for 2 h in a water bath at 278 K with continuous illumination of 200 μE m⁻² s⁻¹. The samples were then transferred to an illuminated shaker (100 rpm at 278 K, continuous illumination of 260 μE m⁻² s⁻¹; Multitron; Infors AG, Bottmingen, Switzerland).

After exposure, 1 ml of the cell suspension was transferred to the glass cuvette of a ToxY-PAM fluorometer (prototype manufactured by Gademann Instruments, Würzburg, Germany; series production by Heinz Walz, Effeltrich, Germany), and the effective quantum yield of energy conversion at photosystem II reaction centers, *Y*, was assessed using Equation 2, where *F* is the momentary fluorescence yield and *F*₉₅ is the maximum fluorescence yield induced by a saturation pulse [17]. The inhibition of photosystem II quantum yield was calculated using Equation 3 [18]:

\[
Y = \left( \frac{F_\text{M} - F}{F_\text{M}} \right) \quad (2)
\]

\[
\text{photosynthesis inhibition (％) } = \left(1 - \frac{Y_{\text{sample}}}{Y_{\text{control}}} \right) \cdot 100 \quad (3)
\]

**Yeast estrogen screen**

The recombinant YES was performed as described by Routledge and Sumpter [19], with minor changes and data evaluation as reported by Rutishauser et al. [20].

**Evaluation of concentration–effect curves**

The concentration–effect curves were fitted to a log–logistic function (Eqn. 4) using Prism 4.0 Software (GraphPad, San
Diego, CA, USA), which computed the best fit for experimental data with the least-squares method, with a fixed minimum at a 0% effect and a fixed maximum at a 100% effect. Adjustable parameters were the slope $m$ and the concentration causing a 50% effect ($EC_{50}$):

$$\text{effect (\%)} = \frac{100}{1 + 10^m (\log EC_{50} - \log \text{concentration})}$$  \hspace{1cm} (4)

The $EC_{50}$ values of the single compounds and mixtures are given in molar units (M), and the $EC_{50}$ values of environmental samples and urine are reported in dilution factor units (Eqn. 5):

$$\text{dilution factor} = \frac{\text{volume of initial sample}}{\text{volume of sample after dilution}}$$  \hspace{1cm} (5)

Note that for samples processed by SPE, the dilution factor actually may be greater than one. That is, the sample is more concentrated in the bioassay than in the original sample.

**Mixture experiments**

In the bioluminescence inhibition and chlorophyll fluorescence tests, mixture experiments were performed with a mixture of five pharmaceuticals, which were mixed in the ratio of their $EC_{50}$ values in the given test system. The fraction $p_i$ of any given mixture component $i$ is thus defined according to Equation 6:

$$p_i = \frac{EC_{50,i}}{\sum_{j=1}^{n} EC_{50,j}}$$  \hspace{1cm} (6)

The concentration–effect curves and $EC_{50,max}$ values were derived according to Equation 4, with a total concentration $c_{mix}$ on the concentration axis. The concentration $c_{mix}$ is the sum of the concentration of the $n$ components $i, c_i$ (Eqn. 7):

$$c_{mix} = \sum_{i=1}^{n} c_i$$  \hspace{1cm} (7)

For compounds that act in a concentration-additive manner, all toxic units (i.e., the ratios of $c_i$ to the effect concentration at any effect level $y$, or $EC_y$ [derived from Eqn. 4]), must add up to one (Eqn. 8) [21]:

$$\sum_{i=1}^{n} \frac{c_i}{EC_y} = 1$$  \hspace{1cm} (8)

For concentration addition, $EC_{y, mix}$ can, accordingly, be derived for each effect level $y$ from Equation 9:

$$EC_{y, mix} = \left( \sum_{i=1}^{n} \frac{p_i}{EC_y} \right)^{-1}$$  \hspace{1cm} (9)

The prediction of the alternative mixture concept of independent action [22] can be calculated with Equation 10:

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} \left[ 1 - E(c_i) \right]$$  \hspace{1cm} (10)

where $E(c_{mix})$ corresponds to the predicted effect of the mixture and $E(c_i)$ corresponds to the effect of mixture component $i$. The 95% confidence intervals of the mixture toxicity predictions were estimated by a resampling method (Monte Carlo simulation), assuming a log-normal distribution of errors of all input parameters and 1,000 random resampling steps of Equations 9 and 10 implemented in Mathematica (Ver. 5.0; Wolfram Research, Champaign, IL, USA).

**Urine**

Raw urine from healthy young male adults was used in the evaluation phase. It was used directly after sampling and without filtration. The pH was approximately 6.2. In actual experiments, we used male urine collected from public waterless urinals and a no-mix toilet at the Swiss Federal Institute for Environmental Science and Technology [12], diluted by a factor of 1.4 to 1.8, and stored over several weeks in a storage tank where the pH had changed to 9.0 [23]. The stored urine was filtered through glass-fiber filters (Whatman, Clifton, NJ, USA) before use.

**Solid-phase extraction**

Solid-phase extracts were prepared from 10 ml of urine (5–20 ml in the evaluation phase) or water, spiked with a total of 1.2 to 1.8 mM of a cocktail of pharmaceuticals of the following composition: cocktail (a): Ratio of the $EC_{50}$ values in the bioluminescence inhibition test (0.27 mM propranolol, 0.78 mM sulfamethoxazole, 0.13 mM diclofenac, 0.13 mM ibuprofen, and 0.49 mM carbamazepine) plus 1 µM ethinylestradiol or cocktail (b): 17% propranolol (0.2 mM), 0.2% ethinylestradiol (2.4 µM), 17% diclofenac (0.2 mM), 33% ibuprofen (0.4 µM), and 33% carbamazepine (0.4 mM). Before extraction, the samples were stabilized by adding 100 µl of methanol and diluted with 10 ml of water. The pH was adjusted to 3, 7, or 11 with 1 M HCl or 1 M NaOH. Polypropylene cartridges (volume, 6 ml) and polytetrafluoroethylene frits (Supelco, Bellefonte, PA, USA) were filled with the SPE material. Whenever available, cartridges filled by the provider were used. The following phases were evaluated: 100 mg of LiChrolut® EN (ethylvinylbenzene-divinylbenzene copolymer; Merck, VWR, Dietikon, Switzerland) plus 250 mg of LiChrolut RP-C18; 200 or 250 mg of Carbopack (ENVI-Carb 120/400 mesh; Supelco); 500 mg of Isolute C18 (Separtis, Grellingen, Switzerland); 200 mg of Isolute Env+ (polystyrene-divinylbenzene copolymer; Separtis); 200 mg of Oasis HLB (N-vinylpyrrolidone-divinylbenzene copolymer; Waters, Bergen op Zoom, The Netherlands); 200 mg of Chromabond EASY (Macherey Nagel, Oensingen, Switzerland); and Extrelute NT 3 for a 3-ml sample (Merck). Extrelute is a diatomic, earthy, and its extraction is similar to a conventional liquid–liquid extraction. Therefore, the experimental procedure was different than that for other types of SPE and was performed as recommended by the manufacturer.

The SPE columns were conditioned in a 12-port vacuum manifold (Visiprep; Supelco) by flushing three times with 2 ml of hexane, one time with 2 ml of acetone, three times with 2 ml of methanol, and three times with 2 ml of water (pH adjusted, plus 10% methanol) whenever acetone was the elution solvent. The columns were flushed with one time with 10 ml of dichloromethane/methanol (8:2 [v/v]), one time with 5 ml of methanol, and three times with 5 ml of water (pH adjusted) whenever dichloromethane/methanol was the elution solvent. After the sample was sucked slowly (one or two drops per second) through the column, it was washed with 1 ml of pH-adjusted water/methanol (9:1 [v/v]). The solid phase was then dried in a nitrogen stream for 1 h, and the analytes were eluted four times with 1 ml of acetone or four times with 1 ml of dichloromethane/methanol (9:1 [v/v]). After evaporating the eluent with $N_2$, the sample was redissolved in 500 µl of ethanol and aliquoted for the bioassays.

For the YES, the ethanololic solution was pipetted directly into the microtiter plates and evaporated. For the luminescent
inhibition test, the ethanol solution was diluted with salt water,
and for the photosynthesis inhibition test, it was evaporated
in the test vial and redissolved with the algal suspension. Con-
centration–effect curves were determined with Equation 4, us-
ing the dilution factor (Eqn. 5) as a concentration measure.
This factor refers to the relative dilution compared to the initial
10 ml of urine with a given total concentration of pharma-
ceuticals. The recovery of the SPE was defined by Equation
11:

\[
\text{recovery (\%) = } \frac{\text{EC50(cocktail without SPE)}}{\text{EC50(cocktail after SPE)}} \times 100 \quad (11)
\]

Another relevant criterion for the quality of the SPE method is
the matrix effect (Eqn. 12):

\[
\text{matrix effect } = \frac{\text{EC50(cocktail in urine after SPE)}}{\text{EC50(cocktail in water after SPE)}} \quad (12)
\]

If the matrix effect is less than one, interference is present
because of the intrinsic toxicity of urine. If the matrix effect
is greater than one, urine decreases the extraction efﬁciency
of the drug. The matrix effect should be close to one for
optimum performance.

Table 1. Descriptors of the concentration–effect curves (Eqn. 4) for
the various pharmaceuticals, the reference compound ethanol, and the
equipotent mixture (ratio of median effect concentration [EC50]) in
the bioluminescence assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log(1/EC50[M])a</th>
<th>Slope (m)²</th>
<th>n</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol</td>
<td>3.56 ± 0.02</td>
<td>1.65 ± 1.91</td>
<td>17</td>
<td>0.979</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>3.15 ± 0.02</td>
<td>1.92 ± 0.17</td>
<td>17</td>
<td>0.932</td>
</tr>
<tr>
<td>Ethinylestradiol</td>
<td>&lt;3.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td>3.89 ± 0.01</td>
<td>2.59 ± 0.12</td>
<td>17</td>
<td>0.994</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>3.87 ± 0.02</td>
<td>1.22 ± 0.08</td>
<td>17</td>
<td>0.958</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>3.31 ± 0.01</td>
<td>1.73 ± 0.10</td>
<td>17</td>
<td>0.979</td>
</tr>
<tr>
<td>Ethanol</td>
<td>−0.18 ± 0.02</td>
<td>3.65 ± 0.67</td>
<td>25</td>
<td>0.926</td>
</tr>
<tr>
<td>Mixture</td>
<td>3.43 ± 0.01</td>
<td>2.85 ± 0.17</td>
<td>29</td>
<td>0.982</td>
</tr>
</tbody>
</table>

- Values are presented as the mean ± standard error.

RESULTS AND DISCUSSION

**Bikluminescence inhibition**

The concentration–effect curves of the single pharmaceu-
ticals in the bioluminescence inhibition test were rather close
together and had similar slopes (Fig. 1). Ethinylestradiol did
not show any effect up to the solubility limit. The EC50 values
(Table 1) vary by a factor of six, from 130 to 700 µM.
The EC50 value of carbamazepine lay within a factor of 1.5 to a
value reported elsewhere [24].

**Inhibition of chlorophyll ﬂuorescence**

The EC50 values of the pharmaceuticals in the chlorophyll
fluorescence assay are listed in Table 2. They cover almost
two orders of magnitude of toxicity, and they do not correlate
directly with EC50 values of the bioluminescence inhibition
test or with hydrophobicity as expressed by the logarithm of
the octanol–water partition coefﬁcient. The EC50 values for
propranolol, ibuprofen, and diclofenac lie within a factor of
eight to previously reported, 96-h EC50 values for growth
inhibition of the same algae species as determined according
to the OECD Test Guideline [25].

Table 2. Descriptors of the concentration–effect curves (Eqn. 4) for
the various pharmaceuticals, ethanol, and the mixture in a ratio of the
educated effect concentration (EC50) values of the mixture components
in the chlorophyll fluorescence assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log(1/EC50[M])a</th>
<th>Slope (m)²</th>
<th>n</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol</td>
<td>5.61 ± 0.07</td>
<td>0.98 ± 0.14</td>
<td>17</td>
<td>0.708</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>3.08 ± 0.01</td>
<td>11.94 ± 2.33</td>
<td>11</td>
<td>0.874</td>
</tr>
<tr>
<td>Ethinylestradiol</td>
<td>4.38 ± 0.01</td>
<td>6.90 ± 0.01</td>
<td>11</td>
<td>0.639</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>3.30 ± 0.01</td>
<td>22.49 ± 16.19</td>
<td>16</td>
<td>0.750</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>3.35 ± 0.02</td>
<td>11.46 ± 5.26</td>
<td>15</td>
<td>0.384</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>&lt;3.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>−0.47 ± 0.06</td>
<td>0.82 ± 0.09</td>
<td>10</td>
<td>0.962</td>
</tr>
<tr>
<td>Mixture</td>
<td>3.39 ± 0.02</td>
<td>4.20 ± 0.84</td>
<td>39</td>
<td>0.542</td>
</tr>
</tbody>
</table>

- Values are presented as the mean ± standard error.
Concentration additivity in mixture experiments

The five pharmaceuticals were mixed in the ratio of their EC50 values. The experimental concentration–effect curve for the bioluminescence inhibition assay (Fig. 1) followed the prediction for independent action up to an effect level of 50%. At a higher effect level, the prediction for concentration addition represented a better description of the experimental concentration–effect curve. The difference between the two prediction models is small (only a factor of 1.7 at the EC50mix).

The experimental results in the chlorophyll fluorescence assay agreed well with the prediction of concentration addition (Fig. 2). In this experiment, a mixture of propranolol, sulfamethoxazole, ethinylestradiol, diclofenac, and ibuprofen was mixed in the ratios of the EC50 values in this test system. Only at effect levels less than 20% was the scatter of the experimental data so strong that no clear conclusion could be drawn.

Cleuvers [26] tested binary mixtures of pharmaceuticals in algae and daphnia. The effect of binary mixtures of ibuprofen and diclofenac in the 96-h algal growth inhibition test agreed well with the prediction of concentration addition, whereas clofibric acid mixed with carbamazepine showed independent action [25]. In daphnia, mixture effects were stronger than in algae, with a slightly synergistic effect of the ibuprofen/diclofenac mixture and concentration addition for clofibric acid mixed with carbamazepine [25].

The purpose of these screening experiments in the present study was not to perform a diagnostic mode-of-action analysis but to show that concentration addition can be used as a “realistic worst-case estimation” of mixture toxicity in these test systems and with these types of chemicals [11, 26]. Overall, our experimental mixture results in both the bioluminescence inhibition test and the chlorophyll fluorescence test were within a factor of three to the predicted concentration addition. Given other uncertainties in testing environmental samples, we conclude that these two test systems are suitable for detecting the cumulative effects of pharmaceuticals in environmental samples.

Yeast estrogen screen

As expected, propranolol, sulfamethoxazole, ibuprofen, and diclofenac did not show any estrogenic effect in the YES up to millimolar concentrations, at which they started to become cytotoxic (data not shown). The EC50 value for the estrogenic activity of ethinylestradiol (log(1/EC50(M)) = 9.47 ± 0.05) and the reference compounds 17β-estradiol (log(1/EC50(M)) = 9.54 ± 0.04) agreed well with earlier reported results [20]. In a mixture experiment with the pharma-cocktail (b), full overlap was seen between the concentration–effect curve of ethinylestradiol and of the mixture when the concentrations were expressed in terms of ethinylestradiol.

The YES is thus an example of a selective test system, because the effect of estrogenic compounds is not disturbed by other compounds that are present unless their concentration is so high that they are cytotoxic toward yeast cells. The literature reports that the effects of estrogenic compounds on the estrogen receptor and, in particular, in the YES are concentration additive [10, 27], and because the other drugs did not interfere with the results of ethinylestradiol, the YES is suitable for application as a selective test in the proposed test battery.

Matrix effects of urine

Because pharmaceuticals and their metabolites are excreted predominantly via urine and enter the aquatic environment mainly via municipal wastewater, we initially tested the toxicity of raw urine. Urine is a kind of worst-case background matrix for wastewater, because it typically is diluted by a factor of 100 on its way to the wastewater treatment plant. Our assumption is that if we have a suitable method for preparing samples of urine, this method also will work in wastewater.

We used raw urine from healthy adult male adults in the evaluation phase, because its quality changed little over the evaluation phase. By using urine instead of wastewater, we avoided possible time-dependent contamination from runoff or other sources or contamination peaks that might occur in municipal wastewater. Apart from inorganic salts and nutrients (nitrogen, phosphorus, and sulfur compounds), urine contains many organic compounds that are breakdown products of biomolecules and food, among them hydroxyindoles, ethyl mercaptan and other sulfides, phenols and cresols, as well as substituted benzoic and phenylacetic acids [28]. These organic constituents of urine share with each other and with the pharmaceuticals and their metabolites occurring in urine the characteristic that their log $K_{ow}$ typically is less than three, and they likely will add up to the cumulative burden of baseline toxicants. This does not mean that urine poses an environmental hazard per se, but it may disturb the bioassays of the test battery. In the photosynthesis inhibition assay, urine also had a stimulating effect on algal growth [29], because *D. subspicatus* were grown in the low-nutrient medium of the OECD Test Guideline [16]. In the presence of urine, the algae grew larger cells of a deeper green color. Whereas urine may be added to the controls, a control for monitoring the treatment efficiency of urine and wastewater treatment cannot be clearly defined. In the bioluminescence inhibition test, raw urine exhibited an EC50 value of 0.31 (expressed in dilution factor units) (Fig. 3).

The inorganic salts and compounds of very low hydropho-
bic平 can be readily removed by SPE. After SPE, the fertil-
izing effect of urine in the photosynthesis inhibition assay
disappeared (data not shown), and the chlorophyll fluorescence was
independent of the urine extract (data not shown), and the
toxicity of raw urine in the bioluminescence inhibition test
decreased by a factor of two to four (dependent on pH and
the data evaluation method) (Fig. 3). Sample pretreatment with
SPE is thus suitable for raw urine, because it removes
the negative side effects of the urine matrix on the test systems
without losing too much of the toxic effect of the organic
micropollutants in urine (for a detailed discussion of micro-
pollutant recovery, see the section below).

Evaluation of SPE method

The sample preparation for a test battery of bioassays must
be a general procedure that assures a compromise between
isolating a maximum yield of the pharmaceuticals and pol-
lutants and removing the interfering substances from the ma-
trix [30]. The effectiveness of a series of polymeric and re-
versed phases in extracting neutral and ionogenic compounds,
as well as more hydrophobic and more hydrophilic compounds
together, was thus evaluated.

We have not included ion-exchange resins in the evaluation,
although they often are used to selectively extract acidic or
basic drugs [31]. Such materials must be considered if the
study focuses on a specific group of compounds. In the present
study, we aim to catch all compounds that pose a hazard to
aquatic life. In a mixture composed of a large number of com-
pounds with highly differing modes of action, the bioacumu-
lation-driven, nonspecific effects in particular will exhibit
a cumulative effect [32]. We therefore focus on extraction
material with biomimetic properties, such as C18 and other
more hydrophobic and lipid-like material.

The starting point of the evaluation was the SPE method
with the LiChrolut EN/RP-C18 (Merck) previously used for
cleaning up and concentrating wastewater samples for steroid
analysis and for the YES [20,33]. The SPE extract of the raw
urine showed no estrogenic effect (Fig. 4). The recovery of
ethinylestradiol was 79% in water and 80% in urine (Fig. 4).
If the entire pharma-cocktail (a) was added to water and urine,
the recovery was 77 and 111%, respectively (data not shown).

These recoveries are satisfactory. When Carbopack was used
as an alternative SPE material, the recovery of ethinylestradiol
in urine decreased to 49%. The concentration–effect curves of
ethinylestradiol were not altered by the presence of other non-
estrogenic drugs, even in significantly higher concentrations.

Evaluation of solid phases. A more systematic evaluation
of the solid phases used for SPE was performed with the
bioluminescence inhibition assay, because the YES selectively
detects only the estrogenic compounds. At pH 3, recovery of
the pharma-cocktail was satisfactory with all phases except
Chromabond and Extrelute when using acetone as the elution
solvent (Table 3). Elution with dichloromethane/methanol pro-
duced lower recoveries (Table 3).

The maximum recoveries were significantly higher than
100%. These results were reproducible with both compositions
of the pharma-cocktail and in several repetitions of the ex-
pertiment. Note that recoveries significantly greater than 100%
were found only in the bioluminescence inhibition test, not in
the YES. Several explanations for this are possible. First, de-
spite conditioning, the solid phase still contained impurities
that are active in the bioluminescence inhibition test (but not
in the YES). Second, the extracts contain 2.5% ethanol at the
highest concentration and, possibly, some residues of the ac-
etone from the SPE elution. Third, the concentration–effect
curves do not all have the same slope; therefore, the calculated
recovery depends on the effect level. Here, we have used data
for the 50% effect level for assessing recovery (Eqn. 11). As
one example of an extraction with LiChrolut EN/RP-C18 at
pH 3 (Fig. 5) shows, none of these possibilities is alone re-
ponsible for the observed discrepancies, but all could con-
tribute to them.

Influence of pH. The pH of the sample usually is adjusted
to a value in which all analytes are neutral. This is not possible
in our case, because many anti-inflammatory drugs are acids,
most β-blockers are weak bases, and many drugs are even
zwitterionic. Our test set contains the acids diclofenac and
ibuprofen and the base propranolol. We therefore evaluated
the pH dependence of the extraction efficiency for LiChrolut
Table 3. Solid-phase extraction with different solid phases at pH 3 with recovery assessed using the bioluminescence inhibition assay

<table>
<thead>
<tr>
<th>Material</th>
<th>Elution with acetone</th>
<th>Elution with dichloromethane/methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>Matrix effect</td>
</tr>
<tr>
<td>LiChrolut EN/RP-C18</td>
<td>143 (128–160)/159 (148–172)</td>
<td>0.92 (0.78–1.09)/1.00 (0.83–1.20)</td>
</tr>
<tr>
<td>Carboxp</td>
<td>115 (91–146)</td>
<td>1.31 (0.73–2.35)</td>
</tr>
<tr>
<td>Isolute C18</td>
<td>99 (90–109)</td>
<td>0.63 (0.57–0.72)</td>
</tr>
<tr>
<td>Isolute Env+</td>
<td>148 (133–151)</td>
<td>0.46 (0.44–0.48)</td>
</tr>
<tr>
<td>Oasis HLB</td>
<td>121 (90–165)</td>
<td>0.82 (0.55–1.22)</td>
</tr>
<tr>
<td>Chromabond</td>
<td>84 (69–101)</td>
<td>0.72 (0.61–0.98)</td>
</tr>
<tr>
<td>Extrelute</td>
<td>75 (68–81)</td>
<td>0.58 (0.48–0.70)</td>
</tr>
</tbody>
</table>

Equation 11 (the 95% confidence intervals are shown in parentheses).
Equation 12 (the 95% confidence intervals are shown in parentheses).
Pharma-cocktail (a) composition: Ratio of the median effective concentration values in the bioluminescence inhibition test plus 1 μM ethinylestradiol.
Pharma-cocktail (b) composition: 17% propranolol, 0.2% ethinylestradiol, 17% diclofenac, 33% ibuprofen, and 33% carbamazepine.
ND = not determined.

EN/RP-C18, Oasis HLB, and Carboxp (Table 4). Clearly, the recovery was highest at pH 3. However, in an experiment with propranolol alone, which is positively charged at pH 3, only 40% were recovered after SPE with LiChrolut EN/RP-C18 at pH 3 (data not shown). The recovery was still only 57% at pH 7 and only 72% at pH 9, so we opted for extraction at pH 3 for the urine and wastewater samples.

Optimizing the washing step. After the sample has been sucked through the SPE cartridges, they are washed before the drying step. Washing with 1 ml of water/methanol (9:1 [v/v]) proved to be sufficient for removing salts and other matrix components. All the present results reported in the tables and figures were taken from experiments using this washing procedure. Washing with more water or stronger solvents (up to 20 ml of dichloromethane/methanol [1:9, v/v]), which often is recommended in sample preparation for chemical analysis, strongly decreased the recovery. The recovery (Eqn. 11) after Carboxp extraction, as determined with the bioluminescence inhibition assay, decreased from 84% without washing to 28% after washing with 10 ml of dichloromethane/methanol (1:9 [v/v]). In the YES, the recovery of ethinylestradiol in urine plus the pharma-cocktail was 121% with 1 ml of water/methanol (9:1 [v/v], LiChrolut EN/RP-C18). It decreased to 3% after washing with 5 ml of dichloromethane/methanol (1:9 [v/v], Carboxp).

Breakthrough. The maximum urine loading was determined for LiChrolut EN/RP-C18 and Carboxp. The extraction efficiency of LiChrolut EN/RP-C18 was hardly affected by 5 and 10 ml of urine (matrix effect of 0.84 and 0.98, respectively). With 20 ml of urine, the SPE was overloaded, and the matrix effect increased to 2.32. For Carboxp, matrix effects of 1.92, 2.16, and 6.09 were observed for 5, 10, and 20 ml of urine, respectively. Thus, with the amount of LiChrolut EN/RP-C18 chosen, no more than 10 ml of urine should be extracted to avoid overloading the column. The amount of Carboxp used was smaller than that of LiChrolut EN/RP-C18, so the results are not directly comparable between the two phases. Nevertheless, Carboxp appears to show a generally higher matrix effect, presumably because of the higher extraction efficiency of the urine components.

In conclusion, LiChrolut EN/RP-C18 proved to be the appropriate phase for extracting pharmaceuticals from urine as a sample preparation for the bioassays in our test battery. We found no perfect pH range if both acids and bases are present in the mixture, but extraction at pH 3 gave satisfactory results. Because of the high content of organic compounds in urine, great care must be taken not to overload the SPE columns. We recommend a maximum of 10 ml of urine per 200 mg of LiChrolut EN/100 mg of LiChrolut RP-C18. Washing should be done with 1 ml of water/methanol (9:1 [v/v]) adjusted to pH 3 and elution done four times with 1 ml of acetone.

Application of test battery to monitor treatment efficiency of urine and wastewater

Preliminary experiments have been performed to evaluate whether the method proposed and examined here is suitable for monitoring the efficiency of urine and wastewater treatment with respect to the removal of micropollutants. Figure 6 shows the toxicity in the bioluminescence inhibition assay of urine collected in a public no-mix toilet at Swiss Federal Institute for Environmental Science and Technology [12]. The toxicity of the collected urine is slightly lower than that of raw urine because of the dilution in this model of a no-mix toilet. After
treatment for 2 d in a sequencing batch reactor with a solid retention time of greater than 30 d, the toxicity was reduced by 70% [29].

Wastewater samples were taken from the influent of the biological stage (after the primary clarifier) of a technical pilot plant treating municipal wastewater. After concentration by a factor of 10, the sample exhibited a 70% effect in the chlorophyll fluorescence assay and a 97% effect in the bioluminescence inhibition assay, whereas the toxicity in the effluent of the wastewater treatment plant was reduced to 10% and to 20 to 30%, respectively. With the same preconcentration factor, 60% induction was observed with the YES in the influent, but no effect was detectable in the effluent. These results indicate that the SPE is a necessary pretreatment procedure for testing wastewater. It should be noted that these preliminary data are not meant to provide quantitative results of the treatment efficiency, only to demonstrate the applicability of the approach.

CONCLUSION

The proposed screening test battery is suitable for assessing the cumulative impact of pharmaceuticals and other micropollutants in urine, wastewater, and environmental samples after a short cleanup and preconcentration step with SPE. Although whole-effluent toxicity testing usually avoids sample preparation, we showed that SPE was necessary for investigating difficult matrices, such as urine and raw wastewater, and for switching to an appropriate matrix. The latter point is important, because each test system requires a different technique and medium for sample addition. The samples also had to be preconcentrated for the treated urine and wastewater. After careful evaluation, we recommend using LiChrolut EN/RP-C18 as the solid-phase material and performing the extraction at pH 3.

We have already applied this test battery in the Novaquatis project [12,13] to monitor the efficiency of urine treatment methods with respect to the removal of micropollutants. The first preliminary results are promising: The proposed test battery was successfully used to survey the degradation of selected pharmaceuticals added to urine during treatment with a urine batch bioreactor [14,29]. In the latter study [29], a direct comparison with the results of chemical analysis was helpful in interpreting the results and as additional validation for the applicability of the test battery.

In the future, we will run further application studies to evaluate the strength and limitations of the proposed test battery for screening of treatment efficiency and compare its performance, advantages, and disadvantages directly with chemical analytical methods.

Acknowledgement—The present study was partially financed by Novaquatis. We thank Barbara Rutishauser, Judit Lienert, Nathalie Chèvre, and Maja Lüssi.

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