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Identification of P-Glycoprotein Inhibitors in Contaminated Freshwater Sediments

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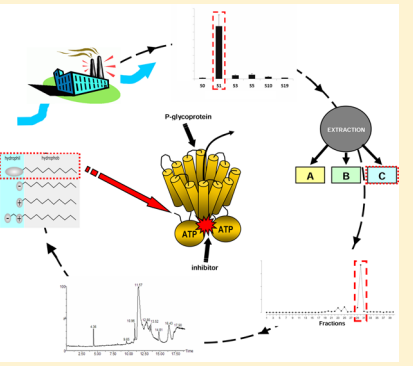
Supporting Information

ABSTRACT: P-glycoprotein (P-gp, ABCB1) is an important part of the multixenobiotic resistance (MXR) defense system in aquatic organisms. The main goal of this study was identification of P-gp inhibitors in contaminated sediments using the effect-directed analysis (EDA) approach. The samples were collected from the Gorjak creek (Zagreb, Croatia), a recipient of wastewater effluents from the pharmaceutical industry. Sediment samples were extracted and fractionated using a two-tiered approach. Resulting nonpolar, medium polar, and polar fractions were tested on the inhibition of P-gp activity using P-gp overexpressing PLHC-1/dox cells and calcein-AM as model substrate. The obtained EC50 values (up to 757 μg/g, expressed in toxicity equivalents of model P-gp inhibitor cyclosporine A) revealed high inhibitory potential of polar fractions of investigated sediments and clearly reflected the impact of pharmaceutical wastewater. P-gp specific ATPase assay and the cytotoxicity modulation experiments with colchicine indicated that most of the observed P-gp inhibition was due to the presence of noncompetitive inhibitors. A detailed chemical analysis by ultrahigh-performance liquid chromatography-quadrupole/time-of-flight mass spectrometry (UPLC-QTOFMS) revealed nonionic surfactants, including alcohol polyethoxylates (LAEOs) and polypropylene glycols (PPGs), as the major components of the most active subfractions. Testing of several LAEO and PPG commercial mixtures confirmed their potential to inhibit the fish P-glycoprotein and modulate toxicity of other xenobiotics present in complex environmental samples.



INTRODUCTION

The outer cell membrane represents the first line of nonspecific defense against xenobiotics. Efflux transport proteins within the cell membrane provide this defense by actively extruding xenobiotics out of the cells, preventing their accumulation inside the cell and consequently reducing their toxic potential. Such transporters were first described in human tumor cell lines and related tissues as major factors in development of the multidrug resistance (MDR) phenotype. Different mammalian models indicate that several members from the large ABC (ATP-binding cassette) family of proteins are major mediators involved in efflux of various drugs and/or their metabolites. Through binding and hydrolyzation of ATP, ABC proteins obtain energy for active transport of their substrates across cell membranes. In mammals, 49 members of the ABC protein superfamily are subdivided into seven families designated A through G (http://nutrigene.4t.com/humanabc.htm). The ABCB1 (MDR, P-glycoprotein), ABCG2 (breast cancer resistance protein, BCRP), and several members from the ABCC (multidrug resistance associated proteins, MRPs) family are directly involved in the efflux of xenobiotics, representing toxicologically most relevant ABC transport proteins.[1]

Because of its overexpression in various tumor tissues and cell lines, the P-glycoprotein (P-gp; ABCB1) became the first and best characterized ABC transporter. The initial observation that many populations of aquatic organisms can survive in a highly polluted environment triggered the first identification of the P-gp-like efflux activity in aquatic organisms.[2] The related phenomenon was soon termed the multixenobiotic resistance mechanism (MXR), and the presence and function of P-gp (ABCB1) has been identified in numerous aquatic organisms investigated so far.[3−5] Similarly to its role in the MDR in mammals, P-gp pumps many structurally different xenobiotics out of the cells of aquatic organisms, reducing their cytotoxicity.[6] Furthermore, the induction of P-gp-like genes, proteins, and/or transport activity has been shown in different tissues of fish and mussels living in polluted environments.[7−9] Finally, it has been demonstrated that specific classes of environmental compounds, both of natural and anthropogenic origin, can lead to inhibition of P-gp-like transport activity.[10] These P-gp inhibitors are called chemosensitizers, because they can cause an increase in sensitivity to other toxic compounds inside cells through competitive or noncompetitive blockage of P-gp mediated efflux.[11] Consequently, development of a methodological framework for the detection and identification of potent P-gp inhibitors in complex environmental samples is of ultimate ecotoxicological importance.

Aquatic sediments are a major sink for a number of anthropogenic pollutants. They contain highly complex mixtures

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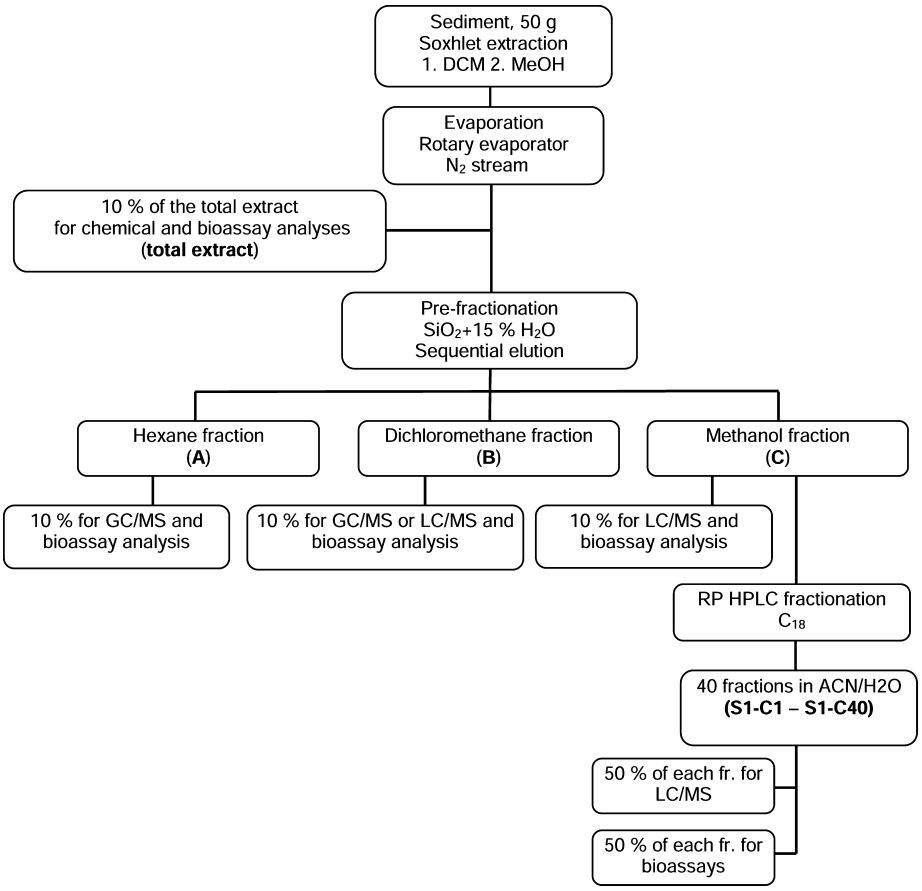

Figure 1. Schematic presentation of the extraction procedure used in the study.

of diverse pollutants, and their ecotoxicological prioritization remains a challenging task, requiring a complex analytical approach. Such procedures typically include combination of a detailed sample fractionation and advanced chemical analytical methods driven by biological effects. This, so-called effect-directed analysis (EDA) approach, has been successfully used in identification and prioritization of pollutants, e.g., for the identification of aryl hydrocarbon receptor (AhR) agonists, estrogenic and androgenic compounds, and mutagenic compounds as well as the pollutants responsible for chronic toxicity in various environmental matrices.[12−14]

However, despite the potential ecotoxicological importance of P-gp inhibitors, so far no EDA study has focused on the detection and identification of this type of chemical in complex environmental samples. One of the reasons is the lack of appropriate in vitro models. Although the cell lines that overexpress P-gp and other human xenobiotic transporters are readily available, environmentally more relevant in vitro tools, expressing transporters from aquatic organisms, have not been developed. Our group previously described the expression of a series of (eco)toxicologically relevant ABC transporters in the PLHC-1 hepatoma cell line derived from topminnow (*Poeciliopsis lucida*), one of the most frequently used in vitro models in aquatic toxicology. Following that work, we selected and characterized the doxorubicin resistant PLHC-1 subclone (PLHC-1/dox), which exhibits high overexpression of fish P-gp.[15] Finally, together with the previously established calcein-AM (Ca-AM) assay as a high throughput method for initial detection of P-gp inhibitors, we recently optimized the P-gp specific ATPase assay using the membrane vesicles isolated from PLHC-1/dox cells, offering the first specific method that can be used to reveal the type of interaction with fish P-gp.[16] Therefore, the main goal of this study was an EDA-based detection and identification of P-gp inhibitors in contaminated freshwater sediments impacted by the pharmaceutical industry.

EXPERIMENTAL SECTION

Study Site. The sediment samples were collected in February 2008 at several locations along the Gorjak Creek, a small watercourse in the area of the city of Zagreb, Croatia, highly influenced by wastewater discharges from the nearby pharmaceutical and baker's yeast production facilities.[17] The sampling design included a reference location, situated 500 m upstream from the wastewater discharge point, and 5 locations situated 100−4000 m downstream from the discharge point (Figure S1 in Supporting Information). Surface sediment samples (0−10 cm) were collected using a plastic coring device and transferred to the laboratory in Zagreb within 5 h. The wet sediment samples were homogenized, and an aliquot of about 1 kg per sample was air-dried at room temperature in a clean fume hood and then pulverized using a mechanical mill. Ground sediment fractions, having a particle size less than 63 μm, were isolated by dry sieving and stored at 4 °C until extraction.

Sediment Extraction and Fractionation. A complex scheme including sample extraction and two levels of extract fractionation was employed for the analysis of the collected sediment samples (Figure 1). Briefly, subsamples of 50 g of dry sediment were extracted by Soxhlet extraction in two separate cycles of 8 h each using methylene chloride (200 mL) and methanol (200 mL) as extracting solvents. The resulting extracts

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were combined and reduced to a small volume using rotary evaporation followed by evaporation under nitrogen stream using a TurboVap system (Caliper Life Sciences, Hopkinton, MA, USA). The residue was transferred into 4 mL screw-cap vials. Before further separation, 10% of the total (raw) extract was taken from each sample and divided into two identical aliquots for chemical and biological analyses. The remaining extracts were subjected to an additional separation step using silica gel deactivated with 15% water.[18] The total extract was applied to the top of the silica gel column (5 mL) and subsequently eluted with 25 mL of *n*-hexane, 30 mL of dichloromethane, and 30 mL of methanol to yield nonpolar (A), medium-polar (B), and polar fractions (C), respectively. Each of the fractions was reduced in volume by evaporation under the N_2 stream and transferred into 1.8 mL vials fitted with Teflon-lined screw caps. A 10% aliquot was taken from each of the collected three silica gel fractions in order to determine the relative distribution of P-gp inhibitors among the fractions.

The remaining extract (80%) was subjected to a detailed fractionation by preparative high-performance liquid chromatography (HPLC), using a Varian ProStar instrument, equipped with an autosampler (Model 410), photodiode array detector (Model 330), and fraction collector (Model 704). The polar fraction (C) was separated by preparative reverse-phase chromatography using an octadecyl silica (C18) column (250 × 10 mm) (Phenomenex, Torrance, USA), applying a binary gradient at a mobile-phase flow of 5 mL/min. The mobile phase A was a mixture of water and acetonitrile (8/2), and the mobile phase B was pure acetonitrile. The following gradient elution was applied: 0−5 min, 100% A; 5−10 min, the percentage of B linearly increased from 0 to 25%; 10−25 min, the percentage of B linearly increased from 25 to 100%; 25−40 min, isocratic hold at 100% B. The separate fractions were collected in uniform time intervals of 1 min. The fractions, obtained after HPLC fractionation, were divided into 2 identical aliquots for further chemical and bioassay analyses. All aliquots for biological analyses were evaporated to dryness, dissolved in dimethlysulphoxide (DMSO), and stored at 4 °C until further analyses.

Chemical Analysis. The applied analytical protocol was designed to provide a comprehensive characterization of the prepared extracts, covering a wide range of possible nontarget and target contaminants as described in our previous publications.[18,19] The most polar silica gel fraction and subfractions from the C_{18}-HPLC fractionation were analyzed by liquid chromatography/time-of-flight mass spectrometry (LC/QTOF MS). The details on LC/QTOF MS technique, applied in the study, can be found in the Supporting Information. The identifications of nontarget compounds by ultrahigh-performance liquid chromatography (UPLC)/QTOF were performed on the basis of accurate mass determination and chromatographic information as described earlier.[20] In addition, the extracts were checked for the presence of a number of suspected target contaminants.

Growth and Treatment of PLHC-1/wt and PLHC-1/dox Cells. PLHC-1 (*Poeciliopsis lucida* hepatocellular carcinoma) cells were obtained from the American Type Culture Collection (ATCC; LGC Promochem, Teddington, UK). PLHC-1/dox cell line was previously isolated and characterized in our laboratory.[15] The cells were grown at 30 °C in DMEM/F12 medium containing L-glutamine and 5% FBS.

Treatment of PLHC-1/dox Cells: Calcein-AM Assay, Membrane Vesicles Isolation, and the ATPase Assay. Transport activity of P-gp was measured using the Ca-AM assay as previously described,[21] with slight modifications (described in more detail in Supporting Information). Calcein fluorescence was finally measured using a microplate reader (Infinite M200, Tecan, Salzburg, Austria) at 485 nm excitation and 530 nm emission wavelengths. For the purpose of membrane vesicles preparation, PLHC-1/wt and dox cells were cultured in 175 cm^2 culture flasks till confluence and then scraped and centrifuged. The resulting pellet was frozen at −80 °C until further use. Plasma membrane vesicles were then isolated as described by Cornwell et al.[22] The pellet containing plasma membrane vesicles was resuspended in 0.5 mL of sample buffer, and total proteins were determined by the Lowry method.[23] ATPase activity was measured using the malachite green method with modifications.[16,24] The amount of the released inorganic phosphate was finally spectrophotometrically measured using a microplate reader at 625 nm. The release of inorganic phosphate was also measured in the presence of 1.2 mM orthovanadate to determine the vanadate sensitive fraction of the ATPase reaction.

Cytotoxicity Determinations and Cytotoxicity Modulation Experiments. Cytotoxicity was determined using PLHC-1/dox cells by the MTT reduction assay adapted according to the Mosmann's procedure.[25] The formazan salts were finally dissolved in isopropanol, and the plates were read using a microplate reader at 570 nm using 750 nm as a reference wavelength. Additional details are described in the Supporting Information.

Data Analysis. In the case of the calcein-AM and MTT assay, EC50 (MTT) or IC50 (Ca-AM) values were calculated using a classical sigmoidal four parameters dose−response model:

$$y = b + (a - b)/(1 + 10^{((\log \mathrm{EC50} - x) * h)})$$

where y is the response, b represents the minimum of response, a represents the maximum of response, h is the shape parameter, and x is the logarithm of inhibitor concentration. IC50 values denote concentrations that resulted in 50% of maximal increase in Ca-AM accumulation over the control cells treated with Ca-AM only, obtained upon addition of model P-gp inhibitor cyclosporine A (7.5 μM). EC50 value is the concentration of substance or sample that corresponds to 50% of maximal cytotoxic effect. The potency of samples to reverse the colchicine resistance of PLHC-1/dox cells was expressed as the modulation factor (MF) and calculated according to the equation:

$$\mathrm{MF}\ (\%) = (\mathrm{EC50}_{\mathrm{PLHC\text{-}1/dox}} - \mathrm{EC50\text{-}IN}_{\mathrm{PLHC\text{-}1/dox}}) / (\mathrm{EC50}_{\mathrm{PLHC\text{-}1/dox}} - \mathrm{EC50}_{\mathrm{PLHC\text{-}1/wt}}) \cdot 100$$

where $\mathrm{EC50}_{\mathrm{PLHC\text{-}1/dox}}$ and $\mathrm{EC50}_{\mathrm{PLHC\text{-}1/wt}}$ are EC50 values of specific drugs obtained for PLHC-1/dox and PLHC-1/wt cells, respectively. $\mathrm{EC50\text{-}IN}_{\mathrm{PLHC\text{-}1/dox}}$ is the EC50 of drug obtained with PLHC-1/dox cells in the presence of specific transport inhibitor (cytotoxicity modulator).

Cyclosporine A toxicity equivalents (CYC-FEL TEQ) were calculated as proposed by Brack et al.[26] using the fixed effect level approach. Ca-AM accumulation caused by sediments was related to IC20 values of cyclosporine A. All experiments were independently repeated 3−5 times. All calculations were preformed using R version 2.15.0 and Gnumeric Spreadsheet for Ubuntu-Linux 10.04.

RESULTS AND DISCUSSION

P-gp Inhibitory Potential of Contaminated Sediments. Using the Ca-AM efflux assay as a fast and reliable screening

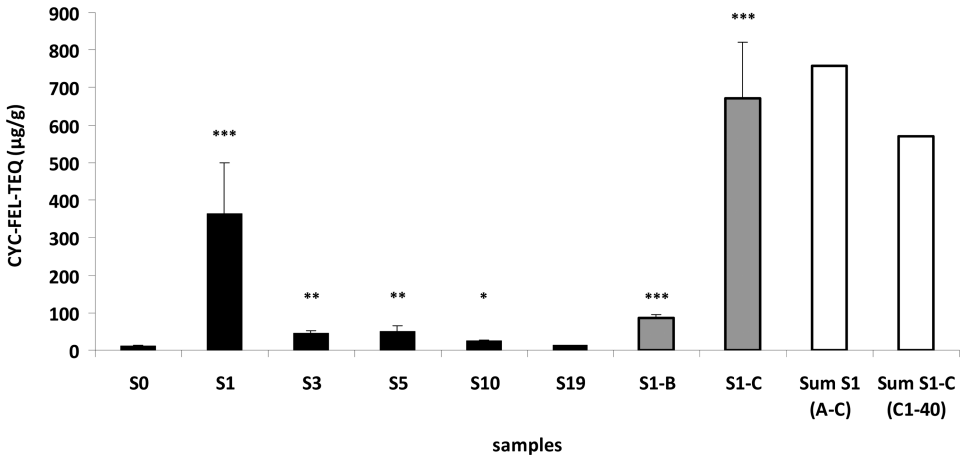

Figure 2. P-gp inhibitory potential of the Gorjak creek sediment samples as determined by the calcein-AM assay with PLHC-1/dox cells. Presented are results of the initial screening of nonfractioned sediment samples (black bars), inhibitory potential of major S1 fractions (gray bars; B-medium polar and C-polar fraction), along with the summed effect of the major S1 fractions (A + B + C) and summed effect of the S1−C polar subfractions (S1-C1 to C-40) (open bars). P-gp inhibitory effect of the nonpolar fraction A was below detection limit of the method. Asterisks denote statistically significant differences in comparison to the P-gp inhibitory effect of the sediment sample collected at the location S0 before the production facilities, at $P < 0.05$ (∗), 0.01 (∗∗), or 0.001 (∗∗∗). Results are expressed in toxic equivalents of model P-gp inhibitor cyclosporine A (CYC-FEL TEQ, μg/g of dry sediment) and are means ± SDs ($n = 3$).

Table 1. List of the Major Contaminant Classes Identified in the Polar (S1-C) Fraction of the Contaminated Sediment from the Gorjak Creek

compound name	abbrev	type of compound/origin	estimated concentration (μg/g)
polypropylene glycols	PPG	solvent, nonionic surfactant	120
alcohol polyethoxylates	$A_{13}EO_n$	nonionic surfactants	3
alkyldimethylbenzylammonium (chloride)	ADBAC	cationic surfactants	39
linear alkylbenzene sulfonates	LAS	anionic surfactants	65
azythromycin	AZI	macrolide	8.5[a]
erythromycin	ERY	macrolide	0.8[a]
desmethylazithromycin	DAZI	macrolide intermediate	8.8[a]
dehydroerythromycin	ERY-H_2O	ERY transformation product	3.3[a]
zolpidem	ZOL	psychiatric drug	5.0[a]
torsemide	TOR	diuretic	8.2[a]
terbinafine	TER	antifungal drug	2.8[a]
chlortalidone	CHL	diuretic	20.1[a]
warfarin	WAR	anticoagulant	1.4

[a]Data from the ref 19.

method, a high potential of all analyzed sediment samples to interact with the fish P-gp transport activity was observed (Figure 2; Table S1 in Supporting Information). Sediment sample collected at the reference location S0 situated 500 m upstream from the major source of pollution also showed the presence of P-gp inhibitors, but the inhibitory potential was almost 20 times lower than in the S1 sample collected immediately downstream from the pharmaceutical facility. Locations further downstream from the S1 site exhibited a gradual decrease in concentrations of P-gp inhibitors, in accordance with the expected pollution gradient. The concentration of P-gp inhibitors, expressed in CYC-FEL TEQ, at the most downstream location (S19) was 13.6 μg/g, which was very close to the value obtained for the reference location S0.

Initial fractionation of the reference sediment extract S0 and the extracts that showed the highest inhibitory potential (S1 and S5) on a silica gel column resulted in three subfractions having different polarities. In all fractionated samples, P-gp inhibitors were almost exclusively associated with the most polar fraction C (Figure 2; Table S1 in Supporting Information). The medium polar fraction (S1-B) showed a measurable inhibitory potential only in the most polluted S1 sample. For the majority of the samples, cumulative effects, calculated as the sum of the individual inhibitory effects of the three major subfractions, were almost identical to the effect of composite samples. The only exception was the most polluted sample S1, for which the summed subfractions S1-C and S1-B resulted in 757 μg/g of CYC-FEL TEQ, while the nonfractionated S1 sample contained only 364 μg/g of toxic equivalents (Figure 2). This discrepancy could be explained by the presence of some unknown toxicants in the composite sample S1, which were in the next step separated by the silica gel fractionation, resulting in higher P-gp inhibitory potential of the S1-C sample. Namely, the applied Ca-AM assay is based on the cleavage of Ca-AM by nonspecific esterases inside the cell, resulting in the release of fluorescent calcein. However, esterases were shown to be highly sensitive to inhibition by toxic compounds, masking the potential effects on P-gp transport activity.[27] In order to test this hypothesis, we performed additional tests: rhodamine 123 (Rh123) assay and the esterases inhibition assay using the cytosolic preparation of PLHC-1 cells.

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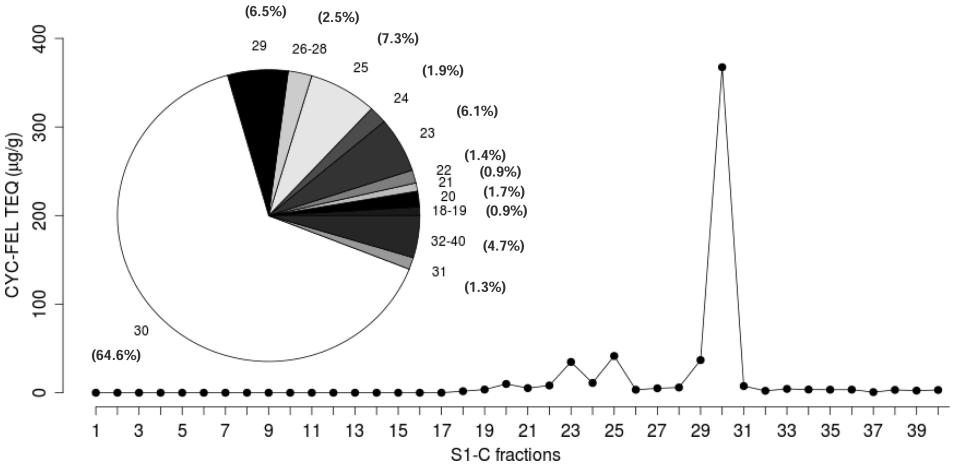

Figure 3. P-gp inhibitory potential of the S-1C individual subfractions and their relative contribution to the overall inhibitory potency of the S1-C fraction. Inhibitory potential of subfractions was determined by the calcein-AM assay with PLHC-1/dox cells and expressed in μg/g CYC-FEL TEQ. Relative contributions of subfractions were calculated and expressed with respect to the toxicity of the major S-1C fraction set at 100%.

Although Rh123 is less responsive as a P-gp probe in comparison to Ca-AM, it is not sensitive to esterases inhibition. Indeed, data using Rh123 as a model P-gp supstrate showed the highest inhibitory effect of nonfractionated S1 sample in comparison to the effect of major A−B−C fractions, and our results confirmed much higher esterases inhibitory effect of the composite S1 sample in comparison with the S1-C fraction (Figure S4 and Table S5 in Supporting Information). Despite this potential drawback, however, the Ca-AM efflux assay still represents the method of choice due to its higher response, simplicity, and high throughput potential. Nevertheless, in order to avoid underestimation of inhibitory potential, it is highly recommended to test the sample for its ability to inhibit esterases, especially when testing complex environmental samples of unknown chemical composition.

In further attempt to identify chemicals causing the observed P-gp inhibition, the polar fraction of the S1 sample was characterized in depth using the UPLC/QTOF technique.[20] The main classes of compounds found in the sample are listed in Table 1. As can be seen, the sample contained significant concentrations of different contaminant classes, including pharmaceutical compounds as well as complex assemblage of various anionic, nonionic, and cationic surfactants. Pharmaceuticals included macrolide antibiotics and their intermediates, diuretics torsemide and chlorthalidone, anticoagulant warfarin, antifungal drug terbinafine, and psychiatric drug zolpidem. The estimated concentrations of these pharmaceutical compounds at the most polluted location were in the range from 1 to 20 mg/kg. On the other hand, the concentration levels of surfactants were estimated to be even higher (up to 100 mg/kg), in particular linear alkylbenzene sulfonates (LAS), polypropylene glycols (PPG), and alkyldimetylbenzylammonium cationic surfactants (ADBACs). It should be noted that the enhanced presence of polypropylene glycols (PPGs) and ADBACs indicates predominately pharmaceutical origin of surfactants. PPGs are widely used in the pharmaceutical industry as inactive solvents of active drug ingredients, while ADBACs find their application as disinfectants in various formulations.

On the basis of the results achieved using simple silica gel fractionation, we focused our further research on a more detailed analysis of the C subfraction of the S1 sample, with an attempt to identify the most potent P-gp inhibitors in the sediment extract. The fraction S1-C was HPLC-fractionated into 40 subfractions (S1-C1 to S1-C40), and the fractions were subsequently tested for their inhibitory potential. The sum of CYC-FEL TEQ values for all individual subfractions (S1-C1 to C40) was 569 μg/g, corresponding to 85% of the total activity found in the composite S1-C fraction (672 μg/g of CYC-FEL TEQ; Figure 2 and Table S1 in Supporting Information). This indicated that some of the active substances, presumably belonging to the highly lipophilic compounds in terms of C_{18} HPLC elution, were lost during the fractionation step. It should be pointed out that the first 18 S1-C subfractions did not show any detectable effect on P-gp transport activity (Figure 3). The majority of other fractions showed measurable inhibitory potential, but most of the P-gp inhibition was associated with the fraction S1-C30. This fraction contained 367 μg/g CYC-FEL TEQ, which explained 65% of the total inhibitory effect observed in the S1-C fraction. Consequently, in the next step of our study, we performed a detailed chemical and biological analysis of the S1-C30 subfraction.

Chemical Identification of Contaminants in S1-C30 Fraction. The most potent fraction S1-C30 was characterized in detail by the UPLC/QTOF technique in order to get closer to the chemical identity of the substances causing the observed P-gp inhibitory effect. The UPLC chromatogram of this fraction is shown in Figure 4A. It contained several resolved peaks, and we were able to identify most of them, as shown in Table 2. Most of the peaks belonged to either nonionic or cationic surfactants, including dialkyldimethylammonim and alkyldimetylbenzylammonium cationic surfactants, as well as tridecanol polyethoxylate and PPG nonionic surfactants. The largest peak at 11.4 min was the PPG oligomer with 18 PEO units (PPG18). The mass spectrum of this compound, presented in Figure 4B, shows characteristic adducts of PPG18 with the sodium and ammonium ions, leading to the dominant singly charged ion $[M + Na]^+$ at m/z 1085.7607 and doubly charged ion $[M + 2H]^{2+}$ at m/z 532.3829. Other minor ions, present in the chromatogram, were formed by a subsequent loss of propoxy unit (C_3H_6O) from the protonated PPG18 molecule.

Another dominant component of this fraction (RT 12.8 min) was nonionic surfactant tridecanol polyethoxylate ($C_{13}EO_n$) with an average number of EO units of about 6. Furthermore, all ethoxymers of this commercial mixture were eluted under the same broad peak, which can be seen in the mass spectrum, displaying a characteristic pattern of $[M + H]^+$ ions of individual ethoxymers with a mass difference of 44 Da (Figure 4C). Apart

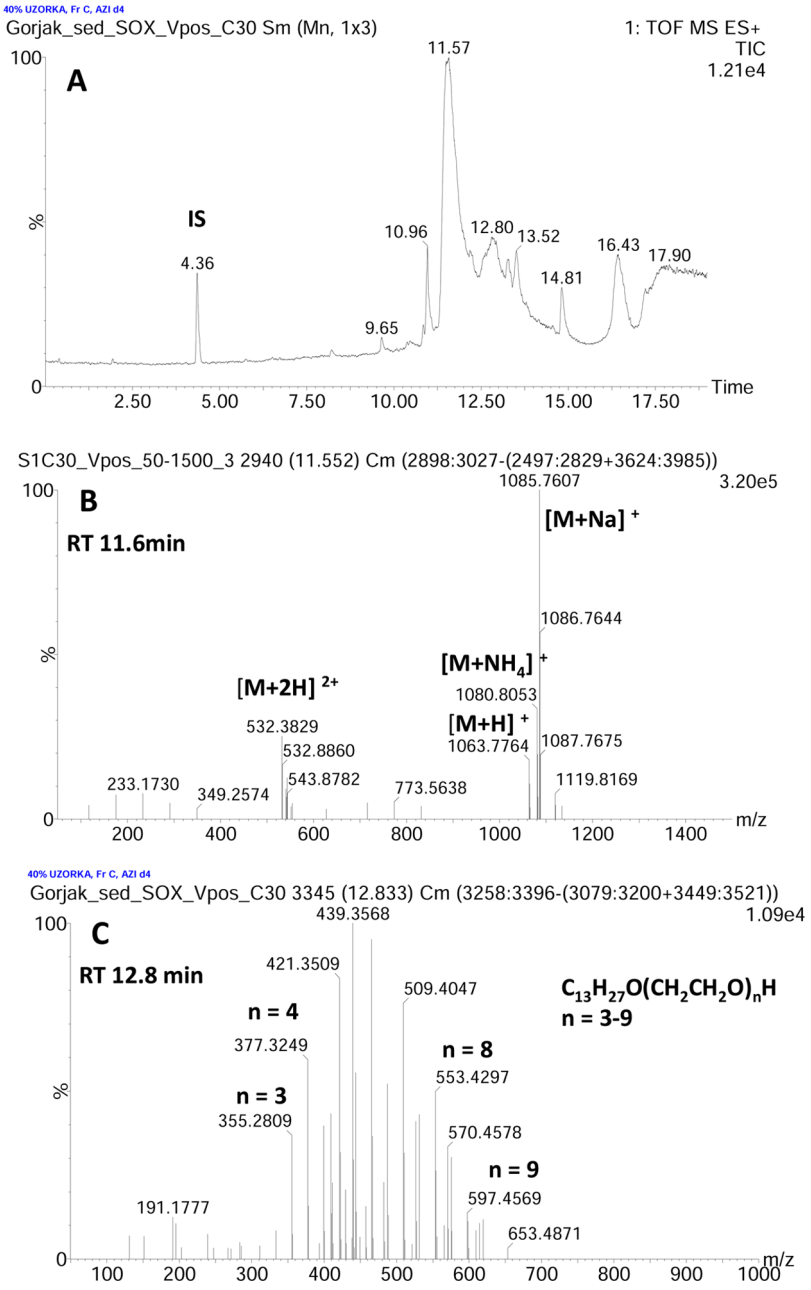

Figure 4. Analytical chemical identification of the major constituents of the fraction S1-C30. (A) UPLC/TOF MS chromatogram of the fraction S1-C30, (B) accurate mass spectrum of the peak at 11.6 min, which was identified as polypropyleneglycol with 18 PO units (PPG 18), and (C) accurate mass spectrum of the peak at 12.8 min, which was identified as tridecanol polyethoxylate with an average number of EO groups of 6.

Table 2. Major Contaminants Identified in the Most Active Fraction S1-C30 of the Sediment Extract from the Gorjak Creek

RT/ min	*m/z*	elemental composition	mass accuracy/ Δ mDa	compound name; origin
9.65	242.2825	$C_{16}H_{36}N$ $[M]^+$	−2.3	tetrabutylammonium; cationic surfactant
10.96	270.3138	$C_{18}H_{40}$ $[M]^+$	−2.3	dioctyldimethylammonium; cationic surfactant
11.41	1063.7760	$C_{54}H_{111}O_{19}$ $[M + H]^+$	+4.0	polypropyleneglycol (PPG), nPO = 18; nonionic surfactant; solvent
12.80	465.3773	$C_{25}H_{53}O_7$ $[M + H]^+$	−1.8	tridecanol polyethoxylate; $C_{13}EO_{3-8}$ (the *m/z* value is given for the most abundant oligomer $C_{13}EO_6$); nonionic surfactant
13.30	360.3612	$C_{25}H_{46}N$ $[M]^+$	−1.8	hexadecyldimethylbenzylammonium; cationic surfactant; antiseptic
13.51	312.3607	$C_{21}H_{46}N$ $[M]^+$	−2.3	octadecyltrimethylammonium; cationic surfactant
14.81	388.3919	$C_{27}H_{50}N$ $[M]^+$	−2.4	octadecyldimethylbenzylammonium; cationic surfactant; antiseptic

from nonionic surfactants, several cationic surfactants were also identified in the S1-C30 fraction. These included dioctyldimethylammonium (RT 10.96 min) and octadecyldimethylbenzylammonium (RT 14.8 min) quarternary ions. Mass spectra of the identified compounds are given in Supporting Information (Figure S2). None of the prominent pharmaceutical compounds,

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identified in the sediment extract and listed in Table 1, was found in the P-gp active fractions. The analysis of the S1-C30 fraction indicated possible importance of surfactants as potent P-gp inhibitors, and in the final step of our study, we performed an extensive testing of a number of commercial mixtures of nonionic and cationic surfactants.

P-gp Inhibition Potency of Surfactants. We tested a series of cationic and nonionic surfactants (Table S4 in Supporting Information), structurally similar to those found in the sediment samples, for their potential to inhibit P-gp activity. As can be seen in Figure 5, none of the cationic surfactants tested showed any P-gp inhibitory potential, in accordance with previously reported results for the mammalian P-gp.[28,29] On the contrary, most of the nonionic surfactants showed a highly significant P-gp inhibitory potential, ranging from 30% to 70% of the maximal inhibition observed with cyclosporine A as a model P-gp inhibitor (Figure 5). Among nonionic surfactants, we tested structurally different alcohol polyethoxylates, which showed a significant variability in their specific inhibitory effects, depending on the structure of their alkyl and polyethoxylate moiety, respectively. Higher responses were obtained for commercial mixtures having average alkyl chain length of about 12 (Genapol L series), while mixtures with larger chain lengths (C16−C18; Genapol T series) gave lower responses. IC50 values for the most active Genapols LA-030 and LA-070 were 2.1 and 1.2 μM, respectively (Table S4 in Supporting Information).

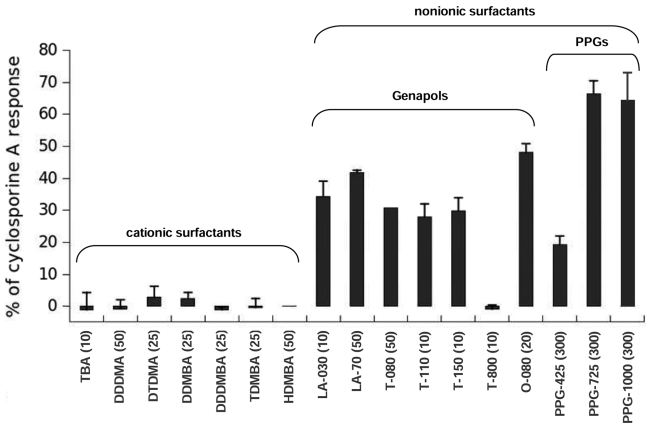

Figure 5. Determination of the P-gp inhibitory potential of commercially available mixtures of cationic and nonionic surfactants structurally similar to those found in the S-1C subfraction #30. Inhibitory potential of surfactants (nominal concentrations (μM) given in parentheses, abbreviations according to the list presented in Table S4 in Supporting Information) was determined by the calcein-AM assay with PLHC-1/dox cells and expressed in % of the maximal effect observed with model P-gp inhibitor cyclosporine A (7.5 μM) set at 100%. Data are means ± SDs (n = 3).

These observations are in agreement with the study published by Seelig and Gerebtzoff,[30] which showed that polyethoxylate surfactants interact with human P-gp in the concentration range that does not disorder cell membrane. Furthermore, it has been demonstrated that the degree of interaction with human P-gp depends on both the length of the alkyl chain and the number of ethoxy groups in the molecule.[31] Alkyl chain represents the hydrophobic part of the molecule that enables its partition in the lipid membrane, the first and prerequisite step for interaction with P-gp. In the second step, the polyethoxylate part of the molecule enters the transporter active site through the lateral cleft in the P-gp molecule oriented toward the membrane bilayer.[32] Once inside the P-gp cavity, polyethoxylate groups form the multiple hydrogen bonds within the active site of the transporter. The fine balance between the number of ethoxy groups and length of alkyl chain is of crucial importance for degree and type of interaction with P-gp, and it has been shown that the oligomer having 12 carbon atoms in the alkyl chain and 8 ethoxy groups showed the strongest interaction with the human P-gp transporter.[33] The predominant type of alcohol polyethoxylates in the S1-C30 fraction identified by UPLC/QTOF analysis was $C_{13}EO_6$, which has a very similar hydrophilic−lipophilic balance like $C_{12}EO_8$.

Apart from alcohol polyethoxylate type of nonionic surfactants, the UPLC/QTOF analysis revealed high concentration of the PPG oligomer with 18 PEO units. Therefore, we tested three PPGs with varying average number of PEO units of 7 (PPG 425), 12 (PPG 725), and 17 (PPG 1000). High inhibitory

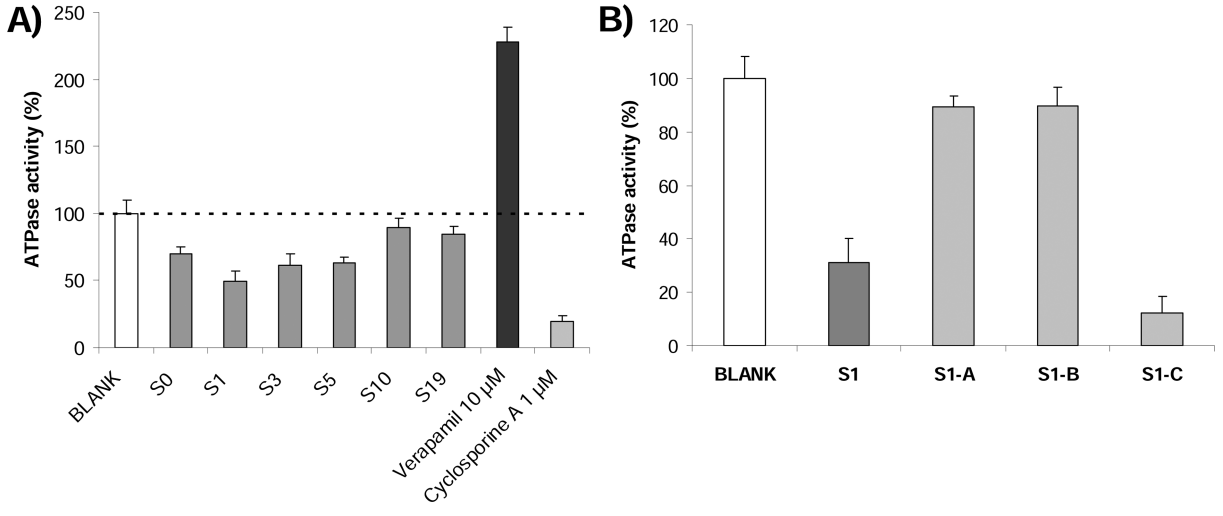

Figure 6. Identification of the type of P-gp inhibitory potential of tested Gorjak Creek sediment extracts using the fish P-gp ATPase assay. (A) Effects of the raw sediment extracts, verapamil (model P-gp ATPase competitive inhibitor) or cyclosporine A (model noncompetitive inhibitor); (B) effects of the extract of the S1 sediment sample and corresponding nonpolar (S1-A), medium polar (S1-B), and polar (S1-C) fractions. Blank denotes procedural control. Results are expressed as percentage of the baseline P-gp ATPase activity set to 100%. The values represent means ± SDs (n = 3).

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potential, reaching almost 70% of the maximal inhibition observed with cyclosporine A (7.5 μM), was determined for PPG 725 and PPG 1000 (Figure 5), while the inhibitory potential of PPG 425 was significantly lower. However, it should be noted that, despite the high maximal inhibition observed for PPGs, their relative affinities values were significantly lower (higher IC50 values) than in the case of Genapols (Table S4 in Supporting Information).

Type of the Identified P-gp Inhibitors. Although the Ca-AM assay offers a sensitive and high throughput tool for the detection of the P-gp inhibitory potential, it does not provide any information on the type of P-gp inhibitors present in analyzed samples; i.e., it is not possible to discern between the chemical inhibitors that block the transport activity by competition for substrate binding site(s) and substances that noncompetitively inhibit the P-gp transport, e.g., by blocking the ATP hydrolysis or by some other nonspecific mechanisms. To overcome this drawback, we used the fish P-gp ATPase assay recently optimized by our group.[16] None of the analyzed samples showed any activation of the ATPase activity in the wide range of tested concentrations (data not shown). However, all of the initial (nonfractionated) samples exhibited inhibition of the P-gp ATPase activity, with S1 being the most potent sample (Figure 6A), in agreement with data obtained using the Ca-AM assay (Figure 2). In addition, the results also indicated the predominant presence of noncompetitive inhibitors in the analyzed samples. Furthermore, inhibition of ATPase activity was almost completely associated with the polar fraction S1-C (Figure 6B), which is again in accordance with the results obtained by the Ca-AM assay. The same pattern, showing predominant association of P-gp inhibitors with the polar fraction, was observed in all other samples (not shown). In addition, using the MTT modulation assay, we confirmed that the predominant type of inhibitors present in S1-C sample are noncompetitive inhibitors. Addition of the S1-C sample modulated the cytotoxicity of the model P-gp substrate colchicine in a concentration-dependent manner, while no cytotoxicity modulation of the S1-C sample was observed in the presence of the model MRP (ABCC) inhibitor MK571 (Table S3 and Figure S3 in Supporting Information). Recent studies on human P-gp using the ATPase assay showed that nonionic surfactants can behave either like substrates or noncompetitive inhibitors of the transport activity, depending predominantly on the character of the hydrophobic part of the molecule, i.e., on the type and length of the alkyl chain.[33] It was shown that the increase in the alkyl chain length from 6 to 14 resulted in a general tendency of decreasing stimulating effect and increasing inhibitory effect on P-gp related ATPase activity. Our findings that tested alcohol polyethoxylates (C12−C16 alkyl chain), as well the as the alcohol polyethoxylates present in the S1-C30 fraction (C13 alkyl chains), behaving as noncompetitive inhibitors, are in accordance with the described findings for human P-gp.

Ecotoxicological Relevance of P-gp Inhibition by Nonionic Surfactants. Surfactants are high production volume chemicals,[34] which enter the aquatic environment in significant quantities despite the fact that most of the common surfactant types are readily biodegradable during the conventional wastewater treatment.[35] Approximately 50% of the total surfactant production belongs to the nonionic surfactants.[34] The first observations, suggesting that nonionic surfactants can modulate the activity of human P-gp, were reported back in the 1990s, but only recent studies showed that these compounds can act as specific substrates or inhibitors of P-gp at the concentrations much lower than those needed for disordering of the cell membrane. Our study is the first report, which identified nonionic surfactants of the alcohol polyethoxylate type and polypropylene glycols as potent inhibitors of the fish P-gp activity. Moreover, we found that these nonionic surfactants were major constituents of a contaminated sediment extract, exhibiting strong fish P-gp inhibitory potency. Consequently, although surfactants are generally considered only weakly to moderately toxic at the concentrations typically found the aquatic environment,[35,36] the results from this study reveal their potential to modulate the toxicity of other xenobiotics by inhibiting efflux activity of the fish P-glycoprotein, a critical component of the cellular defense machinery in aquatic organisms. Therefore, our results indicate the need for a careful reassessment of the ecotoxicological relevance of these ubiquitous environmental contaminants.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information, including data on chemicals used in the study, methodological details on LC/MS and biological analyses, map of the study site, data on calcein-AM and Rh123 assays, modulation of toxicity assays for tested sediment extracts and commercial mixtures of nonionic and cationic surfactants, and esterases inhibition data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Leslie, E. M.; Deeley, R. G.; Cole, S. P. C. Multidrug resistance proteins: Role of Pglycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol. Appl. Pharmacol.* **2005**, *204*, 216−237.

(2) Kurelec, B.; Pivcevic, B. Evidence for a multixenobiotic resistance mechanism in the mussel *Mytilus galloprovincialis*. *Aquat. Toxicol.* **1991**, *19*, 291−301.

(3) Kurelec, B. The multixenobiotic resistance mechanisms in aquatic organisms. *Crit. Rev. Toxicol.* **1992**, *22*, 23−43.

(4) Bard, S. M. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. *Aquat. Toxicol.* **2000**, *48*, 357−389.

(5) Sturm, A.; Segner, H. P-glycoproteins and xenobiotic efflux transport in fish. In *Biochemistry and Molecular Biology of Fishes Vol. VI. Environmental Toxicology*; Mommsen, T. P., Moon, T. W., Eds; Elsevier: Amsterdam, 2005; pp 496−533.

(6) Epel, D.; Luckenbach, T.; Stevenson, C. N.; Macmanus-Spencer, L. A.; Hamdoun, A.; Smital, T. Efflux transporters: Newly appreciated roles in protection against pollutants. *Environ. Sci. Technol.* **2008**, *42*, 3914−20.

(7) Smital, T.; Sauerbor, R.; Hackenberger, B. K. Inducibility of the P-glycoprotein transport activity in the marine mussel Mytilus

galloprovincialis and the freshwater mussel Dreissena polymorpha. *Aquat. Toxicol.* **2003**, *65*, 443−465.
(8) Tutundjian, R.; Cachot, J.; Leboulenger, F.; Minier, C. Genetic and immunological characterisation of multixenobiotic resistance system in the turbot (Scophthalamus maximus). *Comp. Biochem. Physiol., Part B* **2002**, *132*, 463−471.
(9) Williams, T. D.; Gensberg, K.; Minchin, S. D.; Chipman, J. K. A DNA expression array to detect toxic stress response in European flounder (*Platichthys flesus*). *Aquat. Toxicol.* **2003**, *65*, 141−157.
(10) Smital, T.; Luckenbach, T.; Sauerborn, R.; Hamdoun, A. M.; Vega, R. L.; Epel, D. Emerging contaminants - Pesticides, PPCPs, microbial degradation products and natural substances as inhibitors of multixenobiotic defense in aquatic organisms. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* **2004**, *552*, 101−117.
(11) Smital, T.; Kurelec, B. The chemosensitizers of multixenobiotic resistance mechanism in aquatic invertebrates: A new class of pollutants. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* **1998**, *399*, 43−53.
(12) Keiter, S.; Grund, S.; van Bavel, B.; Hagberg, J.; Engwall, M.; Kammann, U.; Klempt, M.; Manz, W.; Olsman, H.; Braunbeck, T.; Hollert, H. Activities and identification of aryl hydrocarbon receptor agonists in sediments from the Danube river. *Anal. Bioanal. Chem.* **2008**, *390*, 2009−2019.
(13) Thomas, K. V.; Langford, K.; Petersen, K.; Smith, A. J.; Tollefsen, K. E. Effect-directed identification of naphthenic acids as important in vitro xeno-estrogens and anti-androgens in North Sea offshore produced water discharges. *Environ. Sci. Technol.* **2008**, *43*, 8066−8071.
(14) Brack, W. Effect-directed analysis: A promising tool for the identification of organic toxicants in complex mixtures? *Anal. Bioanal. Chem.* **2003**, *377*, 397−407.
(15) Zaja, R.; Caminada, D.; Loncar, J.; Fent, K.; Smital, T. Development and characterization of P-glycoprotein 1 (P-gp1, ABCB1)-mediated doxorubicin-resistant PLHC-1 hepatoma fish cell line. *Toxicol. Appl. Pharmacol.* **2008**, *227*, 207−18.
(16) Zaja, R.; Loncar, J.; Popovic, M.; Smital, T. First characterization of fish P-glycoprotein (abcb1) substrate specificity using determinations of its ATPase activity and calcein-AM assay with PLHC-1/dox cell line. *Aquat. Toxicol.* **2011**, *103*, 52−63.
(17) Terzic, S.; Mikac, I.; Senta, I.; Ahel, M. Comprehensive molecular characterization of organic contaminants in freshwater sediments by gas chromatography/mass spectrometry and liquid chromatography/time-of-flight mass spectrometry. In *Progress in Environmental Science and Technology*; Li, S., Wang, Y., Cao, F., Huang, P., Zhang, Y., Eds.; Science Press: Beijing, China, 2009; Vol. *II*, Part A, pp 397−405.
(18) Smital, T.; Terzic, S.; Zaja, R.; Senta, I.; Pivcevic, B.; Popovic, M.; Mikac, I.; Tollefsen, K. E.; Thomas, K. V.; Ahel, M. Assessment of toxicological profiles of the municipal wastewater effluents using chemical analyses and bioassays. *Ecotoxicol. Environ. Saf.* **2011**, *74*, 844−851.
(19) Smital, T.; Terzić, S.; Lončar, J.; Senta, I.; Žaja, R.; Popović, M.; Mikac, I.; Tollefsen, K. E.; Thomas, K. V.; Ahel, M. Prioritisation of organic contaminants in a river basin using chemical analyses and bioassays. *Environ. Sci. Pollut. Res.* **2012**, DOI: 10.1007/s11356-012-1059-x.
(20) Terzic, S.; Ahel, M. Nontarget analysis of polar contaminants in freshwater sediments influenced by pharmaceutical industry using ultra-high-pressure liquid chromatography-quadrupole time-of-flight mass spectrometry. *Environ. Pollut.* **2011**, *159*, 557−566.
(21) Zaja, R.; Klobucar, R. S.; Smital, T. Detection and functional characterization of Pgp1 (ABCB1) and MRP3 (ABCC3) efflux transporters in the PLHC-1 fish hepatoma cell line. *Aquat. Toxicol.* **2007**, *81*, 365−76.
(22) Cornwell, M. M.; Safa, A. R.; Felsted, R. L.; Gottesman, M. M.; Pastan, I. Membrane vesicles from multidrug-resistant human cancer cells contain a specific 150- to 170-kDa protein detected by photoaffinity labeling. *Proc. Natl. Acad. Sci. U.S.A.* **1956**, *83*, 3847−3850.
(23) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(24) Henkel, R. D.; VandeBerg, J. L.; Walsh, R. A. A microassay for ATPase. *Anal. Biochem.* **1998**, *169*, 312−318.
(25) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.
(26) Brack, W.; Segner, H.; Moder, M.; Schuurmann, G. Fixed-effect-level toxicity equivalents - A suitable parameter for assessing ethoxyresorufin-O-deethylase induction potency in complex environmental samples. *Environ. Toxicol. Chem.* **2000**, *19*, 2493−2501.
(27) Hollo, Z.; Homolya, L.; Davis, C. W.; Sarkadi, B. Calcein accumulation as a fluorometric functional assay of the multidrug transporter. *Biochim. Biophys. Acta, Biomembr.* **1994**, *1191*, 384−388.
(28) Batrakova, E. V.; Li, S.; Alakhov, V. Y.; Miller, D. W.; Kabanov, A. V. Optimal structure requirements for pluronic block copolymers in modifying P-glycoprotein drug efflux transporter activity in bovine brain microvessel endothelial cells. *J. Pharmacol. Exp. Ther.* **2003**, *304*, 845−854.
(29) Shono, Y.; Nishihara, H.; Matsuda, Y.; Furukawa, S.; Okada, N.; Fujita, T.; Yamamoto, A. Modulation of intestinal P-glycoprotein function by cremophor EL and other surfactants by an in vitro diffusion chamber method using the isolated rat intestinal membranes. *J. Pharm. Sci.* **2004**, *93*, 877−885.
(30) Seelig, A.; Gerebtzoff, G. Enhancement of drug absorption by noncharged detergents through membrane and P-glycoprotein binding. *Expert Opin. Drug Metab.* **2006**, *2*, 733−752.
(31) Li-Blatter, X.; Seelig, A. Exploring the P-glycoprotein binding cavity with polyoxyethylene alkyl ethers. *Biophys. J.* **2010**, *99*, 3589−3598.
(32) Aller, S. G.; Yu, J.; Ward, A.; Weng, Y.; Chittaboina, S.; Zhuo, R.; Harrell, P. M.; Trinh, Y. T.; Zhang, Q.; Urbatsch, I. L.; Chang, G. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* **2009**, *323*, 1718−1722.
(33) Li-Blatter, X.; Seelig, A. P-glycoprotein-ATPase modulation: The molecular mechanisms. *Biophys. J.* **2012**, *102*, 1383−1393.
(34) Edser, C. Latest market analysis. *Focus Surfactants* **2006**, *5*, 1−2.
(35) Ying, G.-G. Fate, behavior and effects of surfactants and their degradation products in the environment. *Environ. Pollut.* **2005**, *32*, 417−431.
(36) Krogh, K. A.; Halling-Sørensen, B.; Mogensen, B. B.; Vejrup, K. V. Environmental properties and effects of nonionic surfactant adjuvants in pesticides: A review. *Chemosphere* **2003**, *50*, 871−901.