JGiesy@aol.com
02/12/2006 07:40 PM

To dlbacon@mmm.com
cc
bcc
Subject ES&T paper

I reviewed the attached paper (see review) for ES&T and rejected it. I have attached a confidential copy of the manuscript and supporting information as well as my review.

Sincerely,

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Review-2-12-06.doc

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supporting informatin.pdf

Exhibit 2081
State of Minnesota v. 3M Co.,
Court File No. 27-CV-10-28862

3MA01320043

General:

The concentrations studied in this study were very great and certainly not ecologically relevant. It could be argued that the selection of these concentrations was appropriate since the study was meant to determine the mechanisms of action of PFOS. However, in the case of perfluorinated compounds, such as PFOS, a critical aspect of the toxicity of the compounds is their binding to serum albumin and proteins in the liver. In rats, PFOS has been observed to have a relatively steep dose-response relationship. It has been speculated that this might be due to saturation or the plasma binding sites such that excess PFOS would be available to interact with other key biomolecules. Said, another way, if the circulating concentration of PFOS does not exceed the binding capacity of the blood, there would be no toxicity observed. For this reason, not only the magnitude of exposure (dose), but also the kinetics (dose rate) is important in studies of potential mechanisms. For this reason, it is the reviewer's opinion that the conclusions of the study are not valid. At best, it should be stated that the observed responses would be potential mechanisms of action if conditions of dose and dose rate are such that free PFOS would be available to interact with other biomolecules. The reviewer considers this a fatal flaw of the research that should preclude publication in ES&T. While this is a common mistake made by many researchers, especially in the area of perfluorinated compounds, ES&T should not encourage such publications.

The exposure duration was sufficient to accumulate measurable concentrations of PFOS, but as pointed out above, with this class of compounds it is not appropriate to substitute increased concentrations over shorter durations to achieve a target tissue concentrations. The reviewer would have preferred to have seen a longer exposure to lower concentrations to achieve the same levels of PFOS in tissues.

The exposure did not result in a range of tissue concentrations that would have been useful in determining the threshold for effects. Since fish exposed to 0.1 and 1.0 mg/L did not result in concentrations in the liver that were significantly greater than those in the control fish.

He relatively great background concentration in the control fish brings the results into question. In fact, the study did not have a "control", but rather another low dose that could not be distinguished from the other two lowest doses. In general, the study design was not acceptable.

The manuscript is well organized and generally well written. The English grammar and syntax is acceptable, but could be improved by a technical editor of English.

The methods applied, excluding dose issues were appropriate and well described.

The figures and tables are all necessary and succinct.

Paragraphs need to be indented.

Were the fish exposed to any prophylactic treatments with antibiotics? This could have a profound effect on the induction of certain detoxifying enzymes and **must** be discussed.

Specific:

Line 50: The use of the term "alarmingly" is judgmental and should be dropped without giving additional context.

Line 70: "allows to isolate" needs to be rewritten

Line 216 It is poor form to start a topic sentence with a citation to literature, a table or figure. Use a subject-verb format.

Line 216: The results observed on these biochemical responses is likely an artifact of the high concentrations (near acutely lethal) concentrations of PFOS used. Since these responses are considered to be fairly generalized responses to damage to the gill and or liver, they are not unique to PFOS and not surprising.

Line 246: The information presented indicates that the effects observed were not specific to PFOS and thus, no conclusions about the mechanism of action can be made from these results. Furthermore, due to the relatively high concentrations to which the fish were exposed, these results can not be used to predict what responses would be likely to occur during long-term, "chronic" exposures to environmentally relevant concentrations of PFOS. In fact, it is likely that the entire response pattern wold be different during more chronic exposures. For this reason, the information presented can not be used to determine the "critical" mode of toxicity.

The reviewer suggest that it would have been appropriate to have a positive control as well as a negative control. A more general stressor such as an osmotic stress, such as reduced salinity or even heavy metal insult would be likely to cause the same generalized responses observed in this study. The lack of such a positive control makes it impossible to make any inferences about mode of action and certainly not a "critical" or specific mode of action of PFOS. The results obtained, suggest that PFOS, which is a relatively strong surfactant was having direct effects on the surface of the gills. This conclusion is supported by the changes in blood ions. As pointed out by the authors, these effects, especially those on ALT was most likely due to direct and non-specific effects on membranes. The reviewer agrees, but then, a number of toxicants such as metals can also cause such effects. So this sheds little light on the mode of action, which was the started purpose of the study.

Line 273: The correlations between serum protein content that were observed in this study that differed from those of other researchers is likely due to the relatively great doses and short-term exposures. Again, the experimental design precludes being able to interpret the results accurately in an ecologically relevant context.

Line 277: Again, the result on osmoregulation suggested by changes in the blood ion balance, is likely due to the exposure to a surfactant and a non-specific membrane disruption.

Line 298: The large numbers of genes affected by the 100 mg/L exposure are indicative of general tissue/cell disruption from an acute insult and are not useful in determining the potential "critical" mechanism of action of PFOS.

Journal of Separation Science

QUANTITATIVE DETERMINATION OF PERFLUORINATED SURFACTANTS IN WATER BY LC-ESI-MS/MS

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QUANTITATIVE DETERMINATION OF PERFLUORINATED SURFACTANTS IN WATER BY LC-ESI-MS/MS

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Keywords

Polyfluorinated surfactants, HPLC-ESI-MS/MS,

1. Abstract

Perfluorooctanoate (PFOA), and perfluorooctane sulfonate (PFOS) and its derivatives are surfactants which have emerged as an important class of global, persistent environmental contaminants. In order to detect their main sources, to elucidate their environmental fate, and to discover potential sinks, methods for reliable quantitative determination at ppt-levels (ng/L) are needed.

The commonly employed method for water analysis involves preconcentration by solid phase extraction (SPE) followed by liquid chromatography coupled to electrospray-ionisation tandem mass spectrometry (HPLC-ESI-MS/MS). All steps must be carefully optimized in order to arrive at reliable quantitative-analytical data. Two major aspects must be considered: 1) during SPE, contaminations may arise from materials containing traces of PFOA/S; 2) electrospray ionisation yields are suppressed by matrix components and depend upon the analyte concentration in the extracts.

The method is employed to determine the levels of PFOA/S in the river Roter Main near Bayreuth.

2. Introduction

The perfluorinated surfactants (PFS) perfluorooctanoate (PFOA), perfluorooctane sulfonate (PFOS), and derivatives of the latter have been manufactured for over 50 years. At the end of the last decade, their annual production was about 4,650 metric tons [1]. Due to their unique molecular properties, i.e. being both water- and fat-repellent, they have been used for treatment of textiles or paper for rendering them water- and fat-repellent, in cosmetics, insecticide formulations, fire fighting foams, hydraulic fluids, in the photographic industry, for metal plating, and for production of semiconductors [2, 3]. PFS are fully anthropogenic, and they are stable at high temperatures and towards acids, bases, oxidants, and reductants. Therefore, they are fully persistent [2], are globally distributed and ubiquitous [4, 5] and are found in all compartments of the hydrosphere and biosphere [6-18].

Techniques sufficiently sensitive for environmental monitoring are gas chromatography-mass spectrometry (GC-MS) after derivatisation [19], and liquid chromatography coupled to electrospray-ionisation tandem mass spectrometry (LC-ESI-MS/MS) of the non-derivatised PFS [6-14]; the latter is the more commonly employed method.

Two major problems must be considered when PFS are determined: a) they are employed for the production of numerous plastic equipments used in laboratories such as vessels, cartridges, or tubings, and therefore care must be taken to keep blank levels at a minimum; b) when employing LC-ESI-MS/MS, matrix components and the actual concentration of analytes in the final extract may suppress electrospray ionisation yields [20].

3. Experimental

3.1 Chemicals and equipment

Perfluorooctanoic acid (95 %, Lancaster Eastgate, UK), perfluorooctane sulfonate potassium salt (98 %, Fluka, Buchs, Germany), ammonium acetate (99.0 %, Fluka, Buchs, Germany), acetic acid (Fluka, Buchs, Germany), methanol (picograde, Promochem, Wesel, Germany), and acetonitrile (picograde, Promochem, Wesel, Germany) are used as obtained.

All equipment is pre-cleaned by rinsing with bi-distilled water and methanol and washed in a laboratory glassware washing machine with alkaline detergent (Neodisher, Dr.Weigert, Hamburg, Germany). SPE connectors, valves, and adapters are sonicated in a beaker with water for 10 min, followed by methanol for another 10 min. This is done three times.

3.2 Sample Collection.

Water samples have been collected along the river Roter Main at Bayreuth in Northern Bavaria, Germany, in April and July 2005. The first location was 1 km upstream of the local waste water treatment plant (WWTP) near the city centre next to a street with low traffic volume; the second was 100 m upstream of the WWTP where the river is surrounded by meadows; the third was 100 m downstream of the outlet of the WWTP where the river is surrounded by meadows; and the fourth was about 1 km further downstream. Samples from the WWTP were also taken.

Samples were collected in 2-L screw-capped high-density polypropylene (PP) bottles (VWR, Darmstadt, Germany). At each location, two 2-L water samples were taken, immediately transported to the laboratory, and stored at 4°C in the darkness. Prior to preparation, the samples were allowed to reach room temperature.

3.3 Solid-Phase Extraction (SPE)

River and waste water samples are thoroughly mixed; when containing suspended particles they are transferred to 250-mL screw-cap PP bottles and centrifuged (High-Performance Centrifuge, Avanti J-25, Beckman, USA) at 12 000 rpm for 10 minutes. All samples are filtered using folded paper filters (597 1/2, Schleicher & Schuell, Dassel, Germany). For preconcentration, C18 cartridges are used (6 mL, 200 mg, Oasis HLB Waters Corp., Milford, USA), conditioned with 6 mL methanol followed by 10 mL deionised water (1 drop/s). A filtered water sample of 500 mL is passed through a cartridge (5 mL/min) under reduced pressure (0.4 Bar); the eluate is discarded. The cartridge is washed with 5 mL methanol/water (2:3, v/v), dried with air under reduced pressure (0.4 Bar) for 30 minutes, the analytes are eluted with 4 mL methanol (1 drop/s), and the eluate is collected in 5-mL PP tubes. The solvent is evaporated under a gentle nitrogen stream, and the dry residue is redissolved in 500

µL of a 1+1 mixture of aqueous ammonium acetate (10 mmol/L) and acetonitrile. The extract is filtered through a HPLC-membrane filter (0.45 µm, Roth, Karlsruhe, Germany) and transferred into a PP autosampler vial (0.75 mL, Supelco, Bellefonte, USA). When necessary, filtered extracts are diluted (1+1, 1+4, 1+9) by transferring defined volumes (250 µL, 100 µL, 50 µL) of the extract using Eppendorf pipettes into other PP-vials and adding a 1+1 mixture of aqueous ammonium acetate (10 mmol/L) and acetonitrile to a final volume of 500 µL.

3.4 Liquid Chromatography/Tandem Mass Spectrometry

A 10-µL aliquot of an SPE extract is injected onto a C18 column (ACE-EPS, 150 x 2 mm, 5 µm, 120 Å, Prontosil, Bischoff, Leonberg, Germany) and separated with a mobile phase consisting of 40 vol-% of aqueous ammonium acetate, 10 mmol/L, pH = 3.1, and 60 vol-% acetonitrile. Flow rate is 300 µL/min; column temperature is 40 ºC. Total run time is 6.5 min. Once a week, the HPLC-column is rinsed overnight with 50 % acetonitrile/water. This is done daily when highly concentrated (>20 ng/L) samples are analysed.

The column is hyphenated via an electrospray ionisation source to a tandem mass spectrometer (API 3000 LC/MS/MS; Applied Biosystems/MDS SCIEX, Foster City, USA). Mass spectra are acquired by negative electrospray ionisation. Cone voltage is – 4.3 kV, dwell time 0.2 s; the nebuliser, curtain, and collision gas flow rates are 8, 10, and 4 L/min, respectively; nebuliser temperature is 350 ºC. Argon is used as collision gas, and the collision energy is optimised for each compound. The multiple reaction mode (MRM) is employed for quantification, with the parent and daughter ions of m/z 413/369 for PFOA, and m/z 499/99 + 80 for PFOS. In the latter case the abundance of the ion at m/z 80 (SO_3^-) is higher but m/z 99 (FSO_3^-) is monitored for selectivity for fluorinated surfactants; for example, of the three peaks eluting between 3.9 and 5.8 min (Figure 1) the first one obviously is not PFOS. The two following peaks indicate branched and linear isomers [21]; under the assumption that the response factors for both isomer groups are equal, quantification is based on the integration of the two peaks between 4.3 and 5.7 min.

For calibration, a stock solution of PFOA is prepared by dissolving 53 mg of perfluorooctanoic acid (95 %) in 100 mL acetonitrile, resulting in a concentration of 500 mg/L. An equally concentrated stock solution of PFOS is prepared by dissolving

55 mg of perfluorooctane sulfonate potassium salt (98 %) in 100 mL acetonitrile. Then, 1 mL of each are combined in a PP-volumetric flask (100 mL) and diluted with the 1+1 mixture of aqueous ammonium acetate (10 mmol/L) and acetonitrile to obtain a mixture of PFOA + PFOS at concentrations of 5 mg/L each. One mL of this mixture is transferred with an Eppendorf pipette to a 5-mL PP-tube and 4 mL of the 1+1 mixture of aqueous ammonium acetate (10 mmol/L) and acetonitrile is added to obtain a solution of intermediate concentration (1 mg/L each). Finally, a low-concentrated standard mixture of 10 µg/L each is obtained by transferring 1 mL of the former solution into a 100-mL volumetric flask and adding 99 mL of the 1+1 mixture of aqueous ammonium acetate (10 mmol/L) and acetonitrile. All these solutions are stored in a refrigerator. The low-concentrated standard mixture is used for daily preparations of working solutions in the range from 1 to 10 µg/L for calibration, standard addition, and spiking experiments.

The following procedure is recommended for analysis of surface waters: first, daily a calibration curve with solutions of commercial standards in the 1+1 mixture of aqueous ammonium acetate (10 mmol/L) and acetonitrile is prepared in the range between 0.2 and 10 µg/L. Then a couple of dilutions are prepared of each extract, as many as necessary to arrive at a concentration of both analytes below 3 µg/L but above the instrumental LOQ (50 ng/L for PFOA and 100 ng/L for PFOS). The appropriate dilution (e.g. 1+4) is selected for standard addition; for this, six vials containing the same volume of the extract are used to prepare the most suitable dilution (100 µL, if the dilution 1+4 is found to be appropriate). Known but varying volumes (400, 300, 200, 100, 50 µL) of a working standard solution are added to the other five vials, and then each is filled to the same final volume (500 µL). Concentration and volume of the stock solution added is chosen to increase the concentration of the extract by about 30% in each successive vial. The highest concentration of the analytes in such solutions must still be in the linear range. A calibration curve obtained in this way is used to evaluate all samples collected on the same day of the same location and diluted in the same way.

3.5 Quality Control.

Spike and recovery experiments with tap water and river water samples (500 mL) are performed, to determine the accuracy of analysis. Three tap water samples

(500 mL each) are spiked to 3 ng/L of PFOA + PFOS by adding 500 µL of a working standard solution (3 µg/L) with an Eppendorf pipette, and three additional ones are spiked to 1.5 ng/L by adding 250 µL of the same standard mixture. River waters are spiked with 1, 2, 3 ng/L by adding various volumes of differently concentrated working solutions (250 µL of 2 µg/L, 500 µL of 2 µg/L and 500 µL of 3 µg/L). Extraction efficiency is determined by analysing sequentially diluted SPE extracts, because suppression of the ESI-yield is less pronounced at low concentrations. The MS-signal is evaluated versus an external calibration curve prepared with an aqueous solution of commercial standards.

4. Results and Discussion

The first step in quantitative determination of environmental trace chemicals must always address potential blank contaminants if the analyte is a widely employed chemical. When beginning this project, numerous problems arose from high blanks, as laboratory equipment made from plastic may contain traces of PFOA/S. They must be cleaned meticulously as described in the experimental part. Tubing, adapters and any other equipment made from Teflon can be a major source of high blank levels and should not be used. Traces of PFOA (≈2 ng/L) have been found in methanol, nothing in acetonitrile. Cartridges conditioned and eluted with methanol as described yield blanks equivalent to a concentration of 0.03 ng/L PFOA for a 500-mL sample.

The blank concentrations of PFOA in deionised, Millipore-filtered and tap water range from 0.18 and 0.22 ng/L; for unknown reason, bidistilled water is higher (0.35 ± 0.04 ng/L). As deionised water shows the least variability (0.22 ± 0.05 ng/L), it is chosen for conditioning of cartridges, for blank determinations, preparation of mobile phase, and as dilution solvent.

When all precautions are taken, blanks of ≈ 0.03 ng/L PFOA are achieved. This must be subtracted from the results when low-concentrated samples (< 1ng/L) are analysed. In all cases, PFOS is below detection limit (0.05 ng/L).

With an enrichment factor of 1000 by SPE, the limits of detection (LOD's, signal to noise (S/N) ratio 3) for surface water are 0.025 ng/L for PFOA and 0.05 ng/L for PFOS, limits of quantification (LOQ's, S/N ratio 6) are 0.05 and 0.1 ng/L, respectively.

SPE-recoveries from deionised water spiked at two different levels (1.5 and 3 ng/L) are 99-100% (± 7% rsd) for PFOA and 83-94% (± 7% rsd) for PFOS. River water samples spiked with both analytes at low level (1 ng/L) show recoveries of 97% (± 2%

rsd) for PFOA and 82% (± 2% rsd) for PFOS. Recoveries of river water samples spiked at 2 or 3 ng/L are 79-83% (± 3% rsd) for PFOA and 69-72% (± 3% rsd) for PFOS.

Ionisation yields – and thus the concentrations calculated for a given sample – depend on the actual concentration of analytes in the extracts (Fig. 2), especially of the weak acid PFOA. Upon co-elution of matrix components and at high analyte concentrations, electrospray ionisation efficiency is suppressed. This is due to the fact that only at low concentrations the surface charges on a primary spray droplet are sufficient to fully ionise all analyte and matrix molecules.

Therefore, the slopes of the calibration curves obtained by standard addition to extracts of river and waste water may be up to 2-fold lower (Fig. 3) compared to pure aqueous standard solutions. As suppression of ionisation is highly variable between sample batches, always an own standard addition sequence is performed because otherwise quantitative determination is unreliable.

Concentration of the measured extract (c_{LC}) is calculated according to equation:

$$c_{LC} = A / Slope \quad [ng/L] \qquad (1)$$

where A is the peak area of the MRM-chromatogram, and S is the slope of the calibration line obtained by standard addition. The concentration in the water sample (c_S) is calculated by:

$$c_S = (c_{LC} \times D_f) / (E_f \times R) \qquad (2)$$

with D_f being the dilution factor (1+0 = 1, 1+1 = 2, 1+4 = 5, etc.), E_f the enrichment factor (e.g. 1000 when a sample of 500 mL results in an extract of 500 µL), and R recovery ([%]/100).

The reproducibility determined by triplicate injections of river water extracts is within 5% for both analytes. The precision of analysing a river water sample by triplicate extraction is within 10%. Combined standard uncertainty (u) based in this analytical method is 9.2 %. The expanded relative uncertainty U (k=2) is 18.4 % for both compounds. The greater contribution of uncertainty arises from the variability of recovery and from the chromatographic quantification (regression line calculation, peak integration).

The established method was applied to the analysis of samples collected from the Roter Main River (Fig. 4). PFOA and PFOS were detected in all samples taken in July 2004. Their concentrations 1 km and 100 m upstream of the WWTP were 2.2 – 2.6 ng/L of PFOA and 3.2 – 3.4 ng/L of PFOS; 100 m downstream of the WWTP they

were significantly increased (14 ng/L PFOA, 26 ng/L PFOS), and about 1 km downstream they were still high (12 ng/L PFOA, 14.5 ng/L). This indicates that household wastewaters contribute significantly to the general background pollution of the hydrosphere. The dotted lines in Figure 4 indicate the values obtained when using an aqueous standard solution for calibration. Correspondingly, any budget calculations based on a insufficiently validated analytical method may be wrong by up to 50%.

5. Conclusions

When establishing a calibration for quantitative determination of PFS in various water samples (tap water, river water, waste water) by LC-ESI-MS/MS, potential sources of blank contamination must be carefully identified and minimised, as plastic materials used for sample preparation may contain the analytes.

The suppression of electrospray ionisation – and thus analyte signal – depends on its concentration and the presence of co-eluting compounds; this requires considerable efforts of optimisation to ensure reliable quantification. At present, standard addition is the only reliable way of PFOA/S quantification using LC-ESI-MS/MS. If stable–isotope labelled standards were available, mass spectrometric isotope dilution would be more convenient.

3MA01320056

Acknowledgments

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6. References

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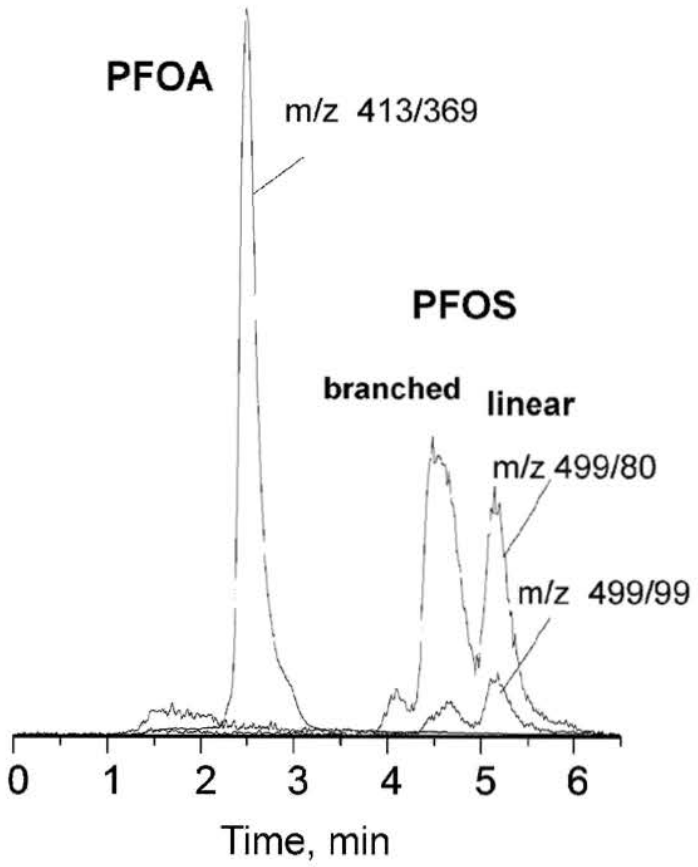


Figure 1. LC-ESI-MS/MS chromatogram of a typical SPE-extract of a river water sample (enrichment 1000-fold) with concentration of 41 ± 2 ng/L of PFOA and 18 ± 1.7 ng/L of PFOS.

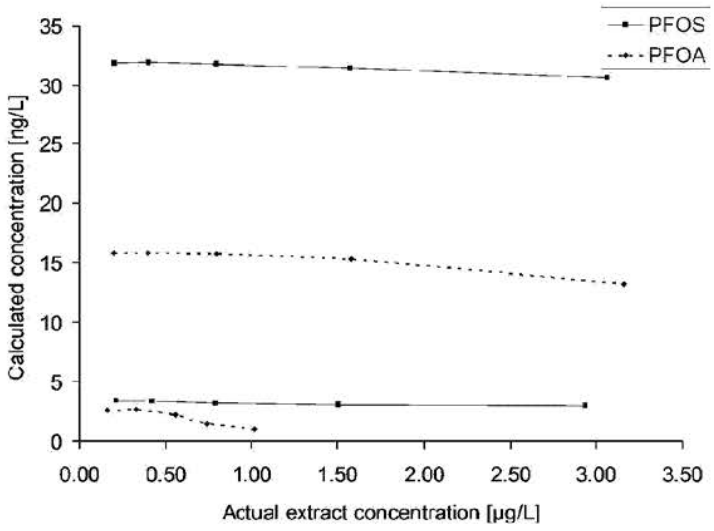
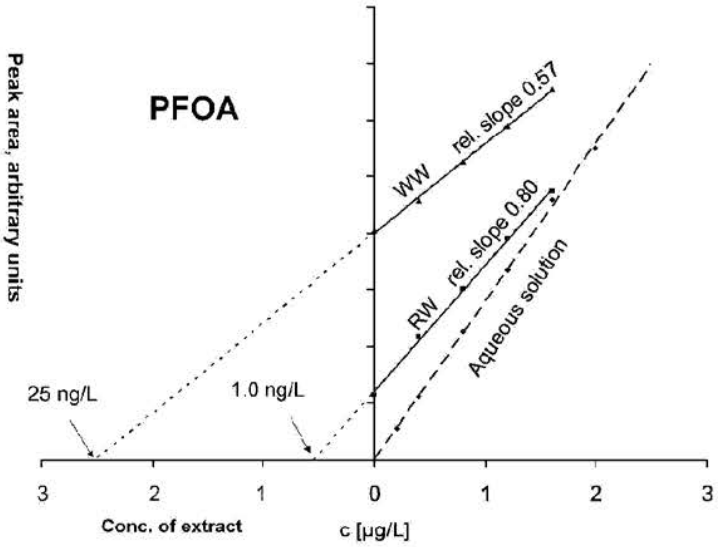


Figure 2. Dependence of the calculated concentrations of PFOA and PFOS in waste water and river water samples on the actual concentration in the extracts diluted to various extents.



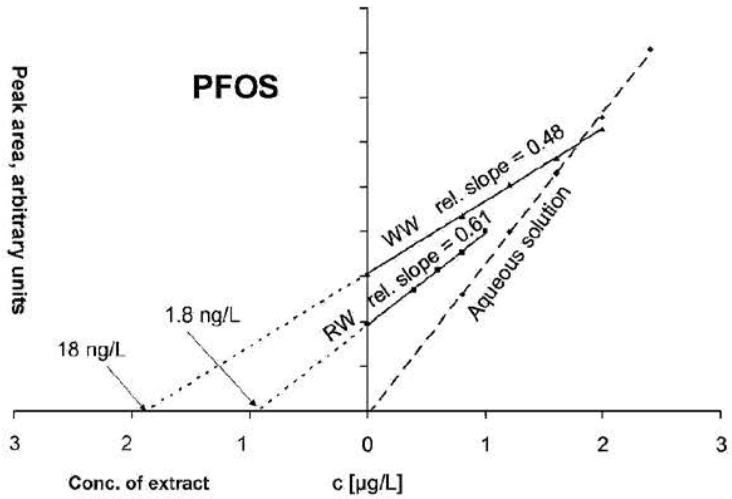


Figure 3. Comparison of calibration curves obtained with a solution of PFOA and PFOS: in 1+1 aqueous ammonium acetate (10 mmol/L)/ acetonitrile (---); by standard addition to a river water extract (RW) (1 µg/L PFOA, 1.8 µg/L PFOS), diluted (1+1), (¯); by standard addition to a waste water extract (WW) (25 µg/L PFOA, 18 µg/L PFOS), diluted (1+9), (¯). The slope of the calibration line obtained with the pure compounds is arbitrarily set to 1.0. The concentrations indicated at the intercept of extrapolated regression line and abscissa are calculated by taking SPE-enrichment factor and extract dilution into considerations and represent the final calculated values.

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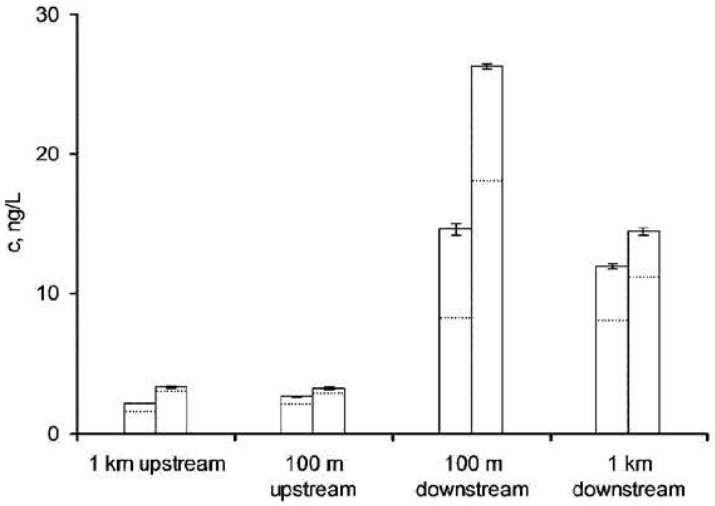

Figure 4. Typical concentration of PFOA (left columns) and PFOS (right columns) in river water before and after the inflow of treated waste water (July 2005). The dotted lines (···) indicate values obtained when using an aqueous standard solution for calibration.

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Table 1. Sequence of primers used in real-time PCR (RT-PCR) analysis.

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
reference gene	GGCTGCACCATTAGGAT	TGTGGAATAGGAGCACC
UDP-glycosyltransferase	GAGGCCATCTACCACG	CGTTGGTGACAGCATT
CYP 450 1A	CAGTTCAGAGTCCTGTG	CTCCTGTGCAGCGTAG
CYP 450 2G1	AGCGGACAGTTGTTCT	TGCGGTCTCTCAAGTG
CYP 450 olf1	CGGACAGTTGTTCTGG	TGTATCAGTGCGGTCTCT
MAP kinase 1 interacting protein	GGTACAGCATTAGCCGC	CCGGTTGAACTGCACA
ferritin heavy subunit	TACGCCTCCTACGTCT	ACTGGTTGACGCTCTT
14kDa apolipoprotein	CTTCTCAGCCTAGACTGG	GGTCTGTACGGACACT
hemopexin like protein	GGCTGACACCATCGAA	AGACGAATGCAGCATC
Glutathione-S-transferase	CCAATGTAACCATAGGCT	CCACAGTGGCTTGTTT
alpha-2-HS-glycoprotein	CAAATACCACGTACAGCC	ACCGAGGAATCAACAG