Environmental ChemOinformatics

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ECO – Deliverable : 6

Report about the results of STR fellowships

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PU	Public	X
PP	Restricted to other program participants	
CO	Confidential, only for members of the consortium (including the Commission Services	

Recruitment of STR

Due to the difficulties with the recruitment of the STRs, the submission of Deliverable No. 6 was postponed by approximately 7 months. Despite intense and repeated announcements of positions in Euraxess and at several other job lists (e.g., Nature jobs, CCL list), we found that it was extremely difficult to find suitable and qualified candidates who could fill-in the announced short-time fellowships.

Below, we provide an overview of recruitment process for each laboratory of the ECO partners.

At the beginning of 2011, two candidates applied for a position at the Hochschule Fresenius. Both candidates were eligible. Miss Marta Llorca was immediately recruited for a three-months fellowship, while the other candidate, Miss Eliana Da Silva, will start her fellowship in January 2012.

For the STR positions at Leiden University there were five applicants. Two of them were not eligible due to the EU eligibility requirement on the maximum duration of R&D experience (they had more than 4 years of R&D after their MSc degrees). One candidate, Mr. Tobias Frömel was recruited. He has completed a 3-months fellowship, which he started on February, 15th 2011. Another candidate, Mr. Christian Eckstein has been also recruited and will start a one-year fellowship on 15 August 2011.

For the STR positions at INIA, group of Dr. Jose Navas, there have been 6 applicants. As the project leader had the chance to recruit a very good candidate (Mona Connolly), it was decided by the recruitment committee - in agreement with the project officer - to pool all STR person months of INIA to this position.

For Italian partner, Prof. R. Todeschini, there were 12 applications at the beginning of this year. Five candidates were not eligible. The following fellows were recruited:
Miss Monika Gajewska started her fellowship (10 months) on 01 May 2011. She will present her first results at the midterm review meeting. Miss Evanthia Giagloglou will

start her fellowship on 01 September 2011. Miss Tine Ringsted will join the ECO on 01 October 2011.

For the Swedish group, Prof.Tomas Öberg, 4 candidates have applied so far. One of them, Mr. Stefan Brandmaier, has been recruited and will start a 3-months fellowship on September 15th. The recruitment process is currently running for the remaining 9 person-months.

For HMGU 20 candidates applied. 8 candidates were not eligible. Two candidates were recruited by other ECO partners. One candidates could not join ECO because of restrictions of the formal employment procedure in France (Miss Fiorella Ruggiu is currently employed as a PhD student at the University of Strasbourg and she cannot stop/postpone her PhD contract to participate in ECO STF) while other candidates were primary interested in long-term PhD positions or/and had other offers. Several candidates were employed as indicated below: Mr. Michal Switnicki has started his fellowship in Dr. Igor Tetko's group on 15 June 2011. Miss Elisa D'Onofrio will start her fellowship on 01 September 2011 and Mr. Matteo Cassotti will start his fellowship on 01 January 2012. The recruitment process for remaining fellow-months is ongoing. There are several other candidates that are evaluated for possible employment and will be likely soon offered positions during the nearest time.

During the reporting period two STRs, namely Tobias Frömel and Marta Llorca finished their STR at the laboratories of Prof. Dr. Willie Peijnenburg (Leiden University, LU, The Netherlands) and Prof. Dr. Thomas Knepper (Hochschule Fresenius, HSF, Germany), respectively. The detailed reports of both STRs are attached as Annex I.

Training of STR

The primary objective of the training of both STRs was to contribute to their education in environmental sciences, such as modern analytical methods as well as ecotoxicology.

Both students learned different techniques and methodologies, which will be required to provide compound specific risk assessment. Both ECO students were attached to ongoing projects of the respective host institutes. The themes of their researches extended the scopes of their PhD thesis and were within the scope of ECO.

Additionally both students got training within different experimental methods being on-top to their routine work. During their stay in their host labs, they were able to actively participate in crosstalk and collaboration, not only between students and their mentors in the respective host Institutes but also with the other ECO participants.

Additionally Miss Marta Llorca as well as Mr. Tobias Frömel took part at the Winter School 2011 in Idstein (21-25. February). Mr. Tobias Frömel also participated the Summer School 2010 as external visitor. Both of them also actively prepared and implemented an important part of the training in analytical methods of their specific research areas for the other ECO fellows. Both students have worked according to their individual Career Development Plans (CDPs). Also, two other ECO fellows, Mr. Stefan Brandmaier and Miss Monika Gajewska took part at the Winter School 2011 in Idstein as the external visitors. Moreover, Mr. Stefan Brandmaier prepared and implemented a presentation for the advanced training in QSAR during both the Summer School 2010 in Munich and the Winter School 2011 in Idstein.

The data collected by the STRs will be made publicly available at the web site of ECO and additionally will be published in international peer-reviewed scientific journals. This will bring both students forward towards their PhDs and will provide an easy mechanism to measure the progress and success of the ECO-ITN.

Evaluation of the scientific results

The achieved scientific results of Miss Marta Llorca during her stay at HSF were tremendous. She developed an analytical method for a new class of environmental relevant perfluorinated compounds as well as performed degradation and monitoring studies.

Mr. Tobias Frömel increased his scientific background in the field of ecotox studies at the University of Leiden, the Netherlands. He was capable to conduct the envisaged

studies during the time frame given and obtained new, very valuable results for the short chain perfluorinted acids. These results will be presented during the upcoming ECO school.

Both STRs demonstrated very fast integration in the host labs and also participated in several socially events of the respective laboratories.

Conclusions and outlook

In summary, the both ECO short-term fellowships were a great success for both the selected fellows and their host institutes. The ECO-ITN partners are looking forwards towards the results of the new STR fellowships, which have started recently.

Marie Curie Initial Training Network

Environmental Chemoinformatics (ECO)

Final project report /2011

8 July 2011

Physicochemical assessment, biodegradation and environmental analysis of perfluoroalkyl phosphonic compounds

Duration of Short Term fellowship:

3.5 months

Early stage researcher:

Marta Llorca

Project supervisor:

Prof. Dr. Thomas Knepper (IFAR)

Dra. Marinella Farré (IDAEA-CSIC)

Prof. Damià Barceló (IDAEA-CSIC)

Research Institution:

Hochschule Fresenius-Institute for Analytical Research (IFAR)

Introduction

Perfluorinated compounds (PFCs) are a class of organofluorine which are used as an inert material due their physical, chemical and biological stability. Due the strong carbonfluorine (C-F) bond associated with PFCs, some of these compounds have high stability and environmental persistence and can be bioaccumulated and biomagnified assessing the presence of these ones in environmental samples , biological samples and food . Because of their persistence and their potential to accumulate they are of toxicological concern . The study of possible metabolites and degradation products should be done in order to establish a direct impact into environment. One group of these compounds are the perfluoroalkyl phosphonic acids (PFAPs) which have been detected in water from Canada and the effect of these compounds has been assessed in rats concluding that the mono-PFPAs and di-PFPAs may also have significant lifetimes in the human body and it was demonstrated that the mono-PFPAs may bind to blood cells underestimating their concentration in plasma and sera samples. The biological fate of the mono-PFPAs and di-PFPAs determined in the study suggested that there was a potential exposure for human and that if exposure does occur, they may be long-lived in the body.

Under the frame of "Environmental ChemOinformatic Marie Curie Initial Training Network", it was proposed the study of: i) to establish different physicochemical properties of of three different PAPS (perfluorohexyl phosphonic acid, perfluorooctyl phosphonic acid, and perfluorodecyl phosphonic acid), ii) to assess the possible biodegradation and released products in effluent water, iii) adapt and validate an analytical method for the analysis of PFPAs and iv) to assess the occurrence of PFPAs in different water samples from Germany. This project was developed in laboratory facilities of Hochschule Fresenius-Institute for Analytical Research (IFAR), in the group of Prof. Dr. Thomas Knepper and in IDAEA-CSIC laboratories.

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order to establish a direct impact into environment. One group of these compounds are the perfluoroalkyl phosphonic acids (PFAPs) which have been detected in water (10) from Canada and the effect of these compounds has been assessed in rats (11) concluding that the mono-PFPAs and di-PFPAs may also have significant lifetimes in the human body and it was demonstrated that the mono-PFPAs may bind to blood cells underestimating their concentration in plasma and sera samples. The biological fate of the mono-PFPAs and di-PFPAs determined in the study suggested that there was a potential exposure for human and that if exposure does occur, they may be long-lived in the body.

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Experimental section

Chemicals and reagents

Perfluoroalkyl phosphonic acids included in this study were: perfluorohexylphosphonic acid (PFHxPA), perlfuorooctylphosphonic acid (PFOPA) and perfluorodecylphosphonic acid (PFDPA) [chemical purity > 98 %; methanol] supplied for Wellington Laboratories Inc. (Canada).

6-Chloroperfluorohexylphosphonic acid (ClPFHxPA) [chemical purity > 98 %; methanol] was used as a surrogate and was purchased from Wellington Laboratories Inc. (Canada).

Ultra pure Milli-Q water was prepared by a Millipore-Q-system (Millipore, Milford, MA, USA). Methanol (MeOH) suprasolv, ammonium acetate salt ($AcNH_4$: MW, 77.08; $\geq$98 %), ammonia, n-hexane and acetonitrile (ACN) were obtained from Merck (Darmstadt, Germany).

Physicochemical properties

Partition coefficient water-hexane. In order to assess the fate of PFPAs compounds in environment, the partition coefficient between water and hexane was calculated according OECD guideline 123 (12). 10 ml of a mixture n-hexane: miliQwater (1:1) was prepared in a PP tube with PFPAs at 100 µg/L. The mixture was vortexed 1 min and shaked in an orbital digestor. In order to establish the equilibrium between water-hexane, an aliquot of 0.5 ml of hexane and an aliquot of 0.5 ml of water were taken at different times: 0, 1, 2, 24, 48 and 96 hours.

0.175 ml of water aliquot was diluted with 0.075 ml of MeOH (7:3) and then 2.5 µl of surrogate-internal standard was added in order to obtain a final concentration of 10 µg/L.

0.175 ml of hexane aliquot was dried under N_2 (g), reconstituted in a 0.250 ml of Water:MeOH (7:3) and 2.5 µl of surrogate-internal standard was added in order to obtain a final concentration of 10 µg/L.

Adsorption experiments in sludge. PFPAs Adsorption - Desorption experiments were carried out according to the procedure described by the OECD guideline 106 (13), using the indirect method. These experiments were performed using two types of sewage sludge from Beuerbach (Hesse, Germany): activated sludge and final sludge. Very briefly, the procedure

was as follows: the samples were dried under ambient conditions and homogenized. Approximately 2 g of dried sample were introduced into a PP tube and 10 ml of MilliQ-water with $CaCl_2$ (0.01 M), according to ISO 10390-1 (14), in order to minimize the cation exchange. This first part of the experiment was carried out in quadruplicates and two procedural blanks were included consisting in MilliQ-water $CaCl_2$ (0.01 M). The mixture was vortexed 1 min and stirred overnight (~12 h) in an orbital digester. The first aliquot of water (200 µl) was taken as a blank before start the spiked experiments. Then, three of the PP tubes were spiked with PFPAs at optimum level in order to achieve 100 µg/L. The other PP tube with sludge was used as a blank. The prepared tubes were vortexed and stirred in an orbital digester along all experimental process. During the sampling process 200 µl aliquot of water were taken after the following times: 0, 2.5, 4, 7.5, 24 and 48 hours.

Water aliquots were introduced into a PP eppendorf and centrifuged at 4000 rpm, at room temperature for 20 min. 0.175 ml of the supernatant was filtered by 0.45 µm Nylon filter and filled up with 0.075 ml of MeOH. 2.5 µl of surrogate-internal standard was added to obtain a final concentration of 10 µg/L.

Aerobic degradation experiments. Degradation experiments were carried out in order to test if any transformation process can be done by aerobic organisms of WWTPs. The experiments were performed according to OECD guideline 309: Aerobic Mineralization in Surface Water – Simulation Biodegradation Test (15). For experiment purposes, wastewater effluent was taken from Beuerbach WWTP (Hesse, Germany). The wastewater effluent was distributed in seven 250 mL amber glass bottles. 5 bottles were spiked with a mixture of PFPAs to obtain a final concentration of 500 µg/L. Three of the bottles were used to follow the biotransformation processes and the other two ones were spiked with NaN_3 in order to stop the biological activity and to assess other possible physicochemical processes as hydrolysis or glass adsorption. In addition, two non spiked wastewater effluents were used as a blank.

During 3 month the bottles were stirred 24 h/day in an orbital digester and the pH was controlled and maintained constant. Dark conditions were used in order to minimize the algae growth, and aerobic conditions were maintained under aeration.

During first week every two days an aliquot of 0.5 ml was taken from each bottle and once a week during the rest of the experiment. These samples were filtered through 0.45 µm

cellulose filter. Then, 0.35 ml of each one was transferred into a PP vial and filled up with 0.15 ml of MeOH using ClPFHxPA as internal standard.

In order to adapt the organisms to phosphonic degradation one more experiment was carried out in parallel. The effluent blank water was spiked with 10 ng/ml of triphenylphosphinoxid (TPPO). This experiment was performed based on an earlier published work by Knepper et al. (16) were the effluent WWTP water organisms were able to degrade TPPO in 21 days in a biologically active fixed-bed bioreactor (FBBR) experiments simulating a WWTP system. The effluent water was spiked with TPPO and aerobic conditions were maintained shaking experimental bottles in an orbital digester 24 h/day with aeration of the water 20 min, once a day. After 21 days the water was spiked with PFPAs at the same concentration that the previous experiments carried out without TPPO. The same conditions and sampling times were maintained.

Algae adsorption experiments. *Desmodesmus subspicatus* (pond scum, green weed) was used in algae adsorption experiments. In this case, the experiments were carried out adding 6.75, 12.5, 25, 50 and 100 ng/mL of corresponding PFPA into transparent glass bottles. The bottles were exposed to natural light and stirred in an orbital digester along the experiment time. Aliquots of 250 µl were taken after 0, 0.5, 1, 5, 24, 144 and 288 hours of exposure and diluted 1:1 with MeOH in order to stop any biological process. A blank experiment containing the algae medium and algae medium spiked with PFPAs at 100 ng/ml were carried out in order to avoid any possible cross contamination during experiments, analysis process or adsorption of the analytes in the glass. The algae medium was constituted with different nutrients ($NaHCO_3$, $CaCl_2$, NH_4Cl, $MgSO_4$, $MgCl_2$ and KH_2PO_4 at mg/L levels) and trace elements (Na_2EDTA, $FeCl_2$, $MnCl_2$, H_3BO_3, Na_2MoO_4, $ZnCl_2$, $CoCl_2$, $CuCl_2$) at pH of 8. The pH of the samples was continuously controlled.

A sample of 0.5 ml was taken from each bottle at experimental time. These samples were filtered through 0.45 µm cellulose filter. Then, 0.35 ml of each were transferred into a PP vial and filled up with 0.15 ml of MeOH using ClPFHxPA as internal standard.

PFPAs in water samples

To assess the presence of PFPAs in different types of water, an analytical method was developed based on solid phase extraction (SPE) followed by LC-MS/MS. SPE procedure was based on the adaption of a previous method published by Llorca et al. (2). In this case, the method was modified in order to improve the efficiency of PFPAs during SPE. The optimized conditions were: i) conditioning: 2 x 2 ml of MeOH, 2 x 2 ml of miliQ water; ii) loading: 150 ml of water at 1 ml/min and dried under N_2; iii) elution: 2 x 2 ml of MeOH (1% NH_3).

The quality assurance include: method limits of detection and quantification (MLOD and MLOQ), the efficiency of the method evaluated by recoveries at three different spiked levels (15, 30 and 60 ng/L) of MiliQ water and effluent water, and the reproducibility intraday of the method at the same spiked levels.

In order to assess the presence of these compounds in water different samples were analyzed, including mineral bottled water, tap water, river surface water and effluent water from Beuerbach WWTP (Hesse, Germany). A total number of 34 samples were analyzed. A blank of miliQ water and all the solvents used during extraction and clean-up procedure were extracted in parallel in order to discriminate possible cross contamination.

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Instrumental analysis

Degradation and algae experiments were followed by analysis using liquid chromatography coupled to a hybrid quadrupole-linear ion trap analyzer (LC-QqLIT). The separation of the analytes was carried out in a Synergi 4u Fusion-RP 80A (50 x 2.0 mm) column in a Perkin Elmer Series 200 HPLC chromatograph (Norwalk, CT, USA). The chromatograph was coupled to a QqLIT mass spectrometer Q Trap 3200 (Applied Biosystems, Foster City, CA, USA) using a Turbo Ionspray interface in electrospray negative mode. The acquisition of the samples was done by selected reaction monitoring mode (SRM). The possible metabolites formation was assessed using the Scan mode in the first quadrupole working under negative and positive ionization conditions. The injection volume was set at 5 µl for the SRM mode, and at 30 µl using the Scan mode.

The analysis of adsorption experiments in sludge, algae experiments and water samples were carried out by liquid chromatography coupled to a triple quadrupole analyzer (LC-QqQ). The separation of the analytes was carried out in a MZ-Aqua Perfect C18 (5µm, 50 x 2.1 mm) column in a HP HPLC chromatograph (Norwalk, CT, USA). The chromatograph was coupled to a QqQ mass spectrometer API 2000 (Applied Biosystems, Foster City, CA, USA) using a Turbo Ionspray interface in electrospray negative mode. The acquisition of the samples was done by selected reaction monitoring mode (SRM) with an injection volume of 5 µl.

In both cases mobile phases were consisting in (A) water:MeOH (95:5) 5 mM $AcNH_4$ and (B) water:MeOH (10:90) 5 mM $AcNH_4$ with the gradient as follows: starting at 70% of A and at 0.5 min decreased to 20% at 2 min. From 2 to 6 min the %A decreased until 10% and was maintained 0.5 min. After this time the %A returned to 70% in 1.5 min and was maintained for 6 min. The total run time was 12 min at flow rate of 400 µl/min.

Main transitions used for quantification and identification, as well as, retention time for the 3 analytes are summarized in table 1.

Table 1: Main transitions of PFPAs

	Retention time (min)	**Transitions**	**Ratio**
PFHxPA	3.9	399 > 399 399 > 79*	1.5
PFOPA	5.1	499 > 499 499 > 79*	1.2
PFDPA	5.8	599 > 599 599 > 79*	1.7

* Quantification transition

Results and discussion

Partition coefficient water-hexane

Table 2 shows the obtained results in the water-hexane partition experiments.

Table 2: partition coefficient (D) hexane-water

	Concentration (µg/L)								
	PFHxPA			PFOPA			PFDPA		
	Hexane	Water	D	Hexane	Water	D	Hexane	Water	D
24h	< 0	94.1		3.12	73.7	0.042	1.61	22.8	0.071
48h	< 0	106		0.691	89.5	0.008	1.24	24.8	0.050
96h	1.29	112	0.012	0.97	85.1	0.011	3.39	18.6	0.182

D = [Concentration hexane / Concentration water]

As it can be seen, these compounds were more hydrophilic due the phosphonic group than hydrophobic due the perfluoroalkyl chain.

This experiment concluded that these compounds can be found in environmental waters.

Adsorption experiments in sludge

Figure 1 summarizes adsorption/desorption experiments. As can be seen, long chain compounds were adsorbed to sludge, whereas for the shortest one the compound can be found in both phases. With these experiments were established the percentages distributed in both phases after the equilibrium achieved after 10 h of contact. In the case of PFDPA this percentage of adsorption in sludge was around 80% of exposure and 70% for the activated sludge. In the case of PFOPA the percentage of adsorption was similar to PFDPA adsorption in sludge but in activated sludge the percentage of adsorption was lower (45%).

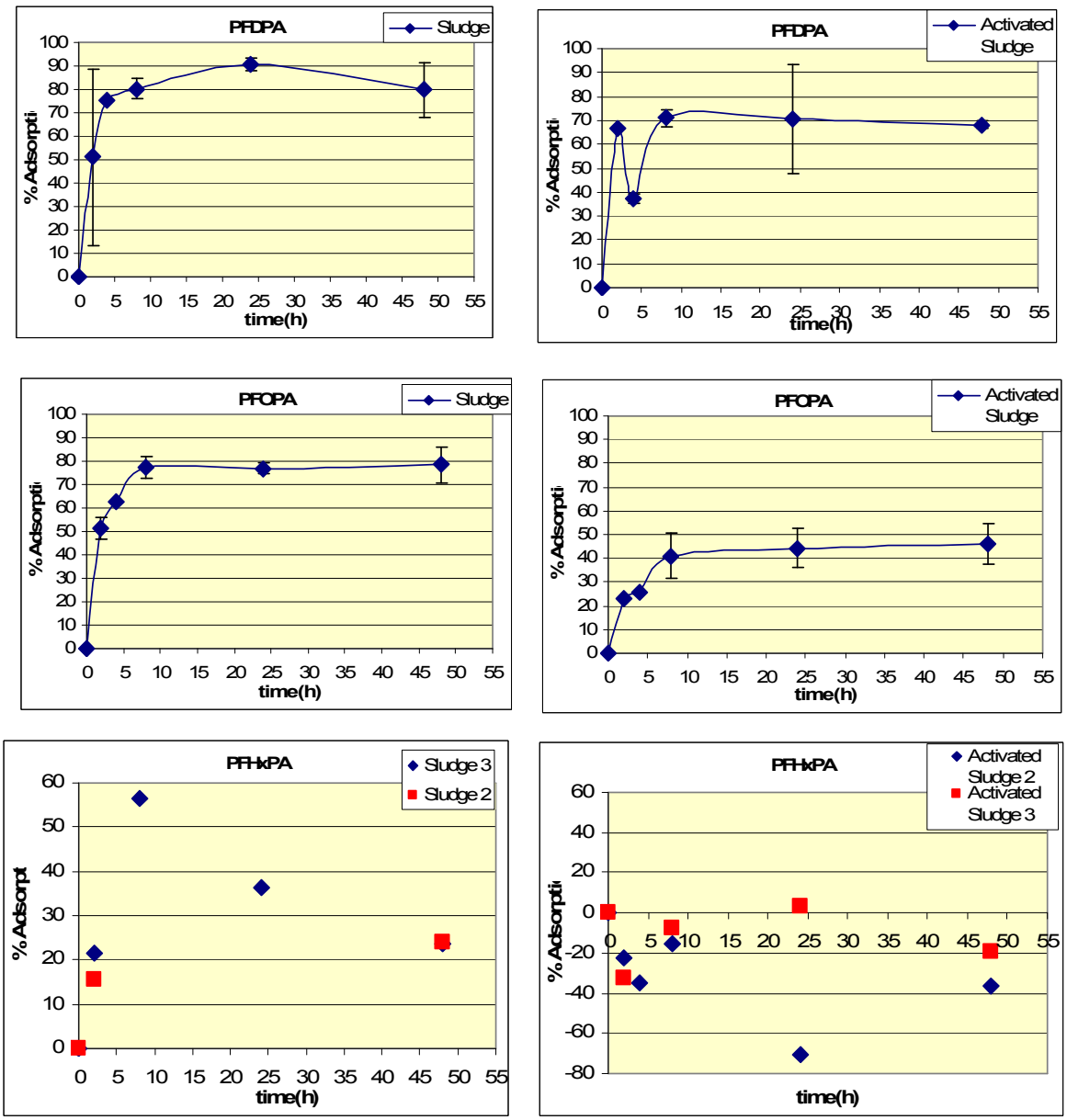

Figure 1: percentage of adsorption vs time of exposure.

The distribution coefficient (K_d) based on equation of figure 2 was calculated, where A_{eq} is the percentage of adsorption at equilibrium (%), V_0 is the initial volume of aqueous phase and m_{soil} is the mass of the soil used in the experiment.

$$K_d = \frac{A_{eq}}{100 - A_{eq}} \cdot \frac{V_0}{m_{soil}} (cm^3 g^{-1})$$

Figure 2: distribution coefficient equation according OECD guideline 106 (13).

The K_d calculated for sludge were: [11.9 ± 1.7] cm^3/g for PFOPA and [14.7 ± 5.6] cm^3/g for PFDPA. For activated sludge the results of the K_d were: [12.4 ± 5.2] cm^3/g for PFOPA and [42.7 ± 5.9] cm^3/g for PFDPA. As can be seen in both cases, the K_d of PFDPA was higher than PFOPA values. This result was expected since PFDPA has a higher C chain than PFOPA and, consequently, the molecule is likely to be more lipophilic.

In the case of PFHxPA experiments the equilibrium was not established.

Aerobic degradation experiments

Aerobic degradation experiments performed for PFHxPA and PFOPA concluded that no degradation processes occurred along all the experiment exposure.

Figure 3 shows the results obtained during the analysis of effluent water experiments for PFDPA.

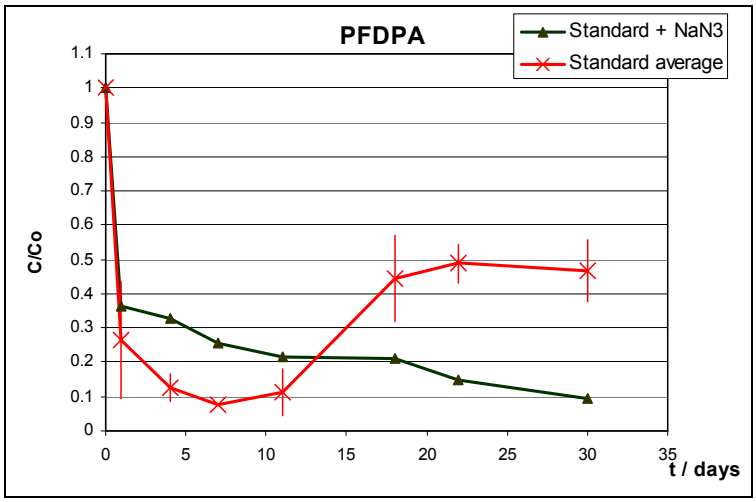

Figure 3: relative level of PFDPA in effluent water vs. time of exposure. Error bars of triplicates.

As it can be seen, between the 2nd and the 10th day of experiment, the levels of PFDPA (red colour line) were lower than the level of this compound adsorbed in the glass (experiment with standards and NaN_3, green line). It could indicate that this compound was degraded, metabolized or adsorbed by the organisms. The possible degradation or metabolization was ruled out because there was not found any degradation product during the Scan analysis.

Along this experiment it was observed the generation of a membrane or biofilm inside the water experiment, which began the 2nd experimental day and the filtration of the aliquot by 0.45 µm cellulose filter was difficult. It could be that the decrease of the PFDPA was related to the generation of this membrane.

In order to discriminate the fate of PFDPA, four aliquots of 0.5 ml were extracted without filtration using dilution [1:1] with the follow solvents: A) miliQ water, B) acetonitrile, C) n-hexane and D) sodium dodecyl sulphate (SDS) at 1% in miliQ water. All the mixtures were vortexed 1 min, mixed in an ultrasonic bath 20 min and centrifuged at 10000 rpm, 20 min. 0.35 ml of the supernatant from the experiments A and D were transferred into a PP vial. 0.35 ml of the supernatant of the experiments B and C were transferred into a PP vial, dried under a gentle stream of N_2 and reconstituted with 0.35 ml of miliQ water. All the extracts were filled up with 0.15 ml of MeOH and ClPFHxPA was introduced as internal standard. The samples were analyzed by SRM and Scan mode (in positive and negative mode) by LC-QqLit instrument. Some of the obtained chromatograms in SRM mode can be seen in Figure 4. The results obtained with the different extraction experiments show how the level of PFDPA (in intensity terms) increased following the order: miliQ water ~ n-hexane < ACN < SDS. Using ACN as extracting agent we obtained the precipitation of the external proteins retained in the membrane or biofilm generated. The PFDPA came from the destruction of this membrane. The use of SDS as a disrupting cellular agent, the higher level of PFDPA came from extracellular and intracellular source. These results could indicate that part of the compound was adsorbed by the organisms using this one as a source of phosphate or due the similarity between PFDPA and one of the phospholipids used in the extracellular membrane. This hypothesis is in agreement with previous review by Pearsons et al. (17) which suggested that bacteria will adapt to utilize this ones as source of energy. Another possibility is that this compound could be included in cells and excreted material could generate a membrane complex arising from the outer membrane of the cell envelope as in

previous published work by Rothfiel et al. (18) in the study of growing cultures of *Escherichia coli* and *Salmonella typhimurium* and the excretion process of lipopolysaccharides, phopholipids and proteins into the culture fluid. As it can be seen in the Figure 3, the level of PFDPA increased between 15th and 30th day of experiment. These could indicate that the cells started to excrete PFDPA or the death of the responsible organism.

In order to identify the organisms responsible of PFDPA adsorption, microscope analysis was carried out. In addition, algae organisms were discarded because the experiments were carried out in the dark. However, due to the complexity of the matrix, the identification of the organisms was not feasible.

Results obtained using TPPO as an agent to adapt the organisms presents in effluent water from WWTP to phosphonic degradation showed the same generated membrane but in this case the levels of PFDPA didn't follow a coherent adsorption being randomly distributed between the glass and the adsorption in the organisms or membrane.

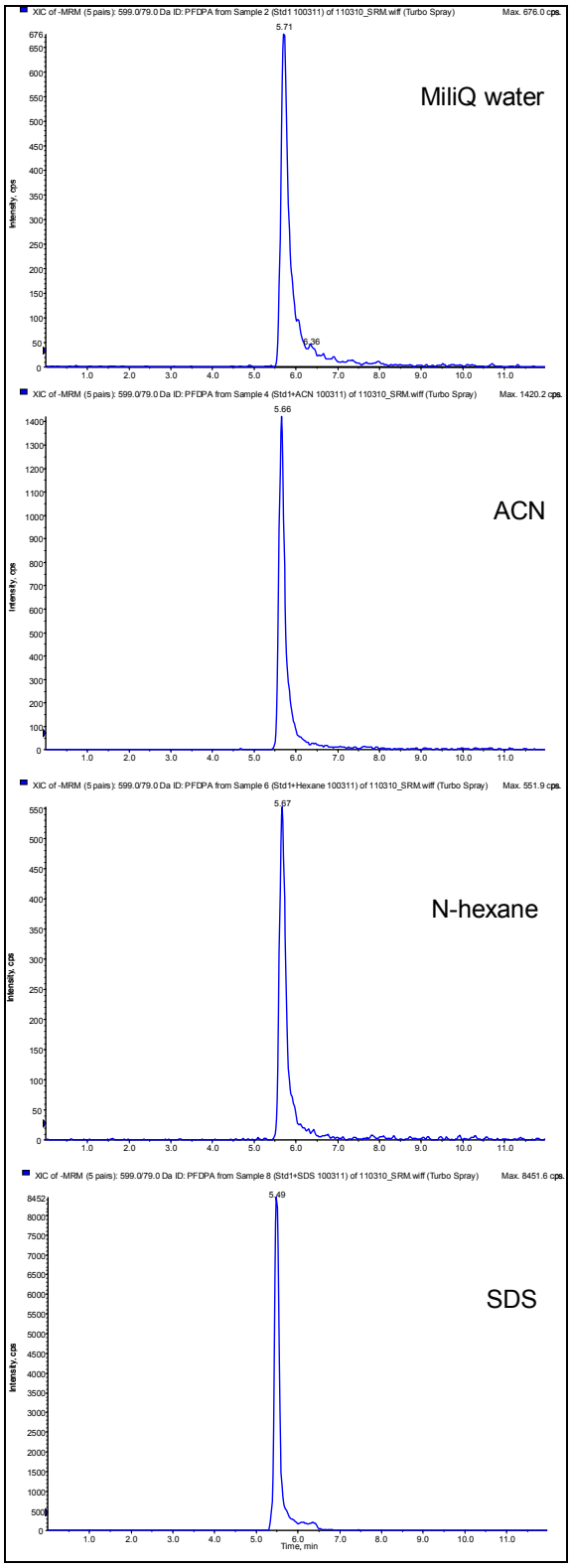

Figure 4: Chromatograms in SRM mode of experiments using miliQ water, acetonitrile, n-hexane and SDS.

Algae adsorption experiments

The algae adsorption experiments didn't conclude any type of adsorption although disappearance of algae green colour in bottle experiments with PFDPA was observed.

Due the disappearance of green colour it was proposed to measure the absorbance of the most concentrated experiment bottles for every analyte. The results showed that the absorbance of PFDPA was four times lower than the absorbance of the other two PFPAs. These preliminary results could indicate that the presence of PFDPA inhibits algae growth or maybe it only inhibits the chlorophyll generation.

More studies are required in order to establish the process of the inhibition of the green colour in PFDPA experiments.

Validation parameters of PFPAs water extraction

Different quality parameters were studied in order to assess the applicability of the method in miliQ water and WWTP effluent water including: method limits of detection and quantification (MLOD and MLOQ, respectively), the percentage of recovery at three different spiking levels (15, 30 and 60 ng/L) and the reproducibility intraday at every spiked level expressed as a percentage of relative standard deviation (%RSD). Results are presented in tables 3 and 4.

Table 3: quality parameters calculated in miliQ water

	MiliQ water							
	MLOD (ng/L)	MLOQ (ng/L)	15 ng/L		30 ng/L		60 ng/L	
			% Rec	% RSD	% Rec	% RSD	% Rec	% RSD
PFHxPA	0.64	2.1	82	3	63	8	81	20
PFOPA	0.35	1.2	76	12	80	6	88	23
PFDPA	0.50	1.7	124	4	58	1	100	17

Table 4: quality parameters calculated in WWTP effluent water

	WWTP effluent water							
	MLOD (ng/L)	MLOQ (ng/L)	15 ng/L		30 ng/L		60 ng/L	
			% Rec	% RSD	% Rec	% RSD	% Rec	% RSD
PFHxPA	0.46	1.5	107	13	110	3	82	13
PFOPA	0.44	1.5	76	22	65	11	61	28
PFDPA	0.83	2.8	107	12	76	5	56	10

%Rec: percentage of recovery (n=3)
%RSD: reproducibility intraday (n=3)
MLOD: method limit of detection
MLOQ: method limit of quantification

Good quality parameters were obtained for the analysis of PFPAs in clean waters (miliQ water optimization) and dirty ones (effluent WWTP water optimization). The selected compounds were detected below 1 ng/L in all waters and percentages of recoveries in the range of spiked levels were between 58 – 124% in miliQ water and between 56 – 107% in WWTP effluent water. The reproducibility intraday was below 30% in all the cases.

PFPAs in water samples

The analysis of miliQ water as a blank of all analytical procedure and all the solvents used during extraction and clean-up procedure extracted in parallel in order to discriminate possible cross contamination concluded that no PFPAs were present in the solvents or included during analytical procedure.

Table 5: results of water monitoring

	PFHxPA			PFOPA		
Sample Name	**Average (ng/L)**	**SD**	**%RSD**	**Average (ng/L)**	**SD**	**%RSD**
Mineral Water 1	< MLOD			< MLOD		
Mineral Water 2	< MLOD			< MLOD		
Frankfurt Tap Water 1	<MLOD			< MLOQ		
Frankfurt Tap Water 2	< MLOD			< MLOD		
Frankfurt Tap Water 3	< MLOD			< MLOD		
Idstein Tap Water 1	< MLOD			< MLOD		
Potable water 1	27	0.01	11	< MLOD		
Water after charcoal treatment	46	0.01	13	< MLOD		
Rhine Sand Trap water	53	0.01	16	< MLOD		
Nidda river 1	23	1.8	8	< MLOD		
Nidda river 2	< MLOD			9.4	0.6	6
Nidda river 3	51	4.1	8	< MLOD		
Nidda river 4	< MLOD			6.6	0.3	5
Nidda river 5	63	13	21	< MLOD		
Pfuhlgraben, Wehrda	< MLOQ			< MLOD		
Usa, Friedberg, Unterhal	< MLOQ			< MLOD		
Erlenbach, Bad Vilbel	< MLOQ			< MLOD		
Landgraben, Trebur, Brue	2.8	0.7	25	< MLOD		
Horlof, Niederflorstadt	< MLOQ			< MLOD		
Wetter, Assenheim	2.6	0.1	5	< MLOD		
Solz, Sorga	< MLOQ			< MLOD		
Rosbach, Nieder-Woellsta	< MLOQ			< MLOD		
Wehre, Niederhone	< MLOQ			< MLOD		
Schwarzbach, Astheim, Me	3.0	0.5	16	< MLOD		
Beinesgraben, Bauschheim	2.5	0.5	20	< MLOD		
Zellersbach, Roehrigshof	2.6	0.8	30	< MLOD		
Bach aus Pferdsdorf, Wil	< MLOQ			< MLOD		
Wickerbach, Floer Sheim	< MLOQ			< MLOD		
Weihe, Untersuhl	< MLOD			< MLOD		
Hauptgraben, Astheim	< MLOD			< MLOD		
Eitra (Fischbach), Bodes	< MLOD			< MLOD		
Solz, Weiterode	< MLOQ			< MLOD		
WWTP effluent 1	< MLOD			3.9	0.3	7
WWTP effluent 2	< MLOQ			< MLOQ		

MLOD, MLOQ: method limit of detection and quantification, respectively

As can be seen in table 5, three over thirty-four analyzed samples showed positive levels for PFOPA in the range of 3.9 – 9.4 ng/L. The most contaminated samples were from Nidda River and from WWTP effluent.

In the case of PFHxPA the levels were established between 2.5 – 63 ng/L. These compounds have been reported before by Eon et al. (10) at levels between 0.88 and 3.4 ng/L of PFOPA in Canadian Surface water and between 0.76 and 2.5 in effluent water. These levels are comparables to levels reported in this work although these last ones presented higher range and included positive results in the analysis of PFHxPA.

The main difference between the concentration obtained for PFHxPA and PFOPA could be explained using the physicochemical parameters calculated previously in this work. As can be seen in the section of "Adsorption experiments in sludge", the percentage of adsorption established at equilibrium for PFOPA was between 45% in activated sludge and 80% in sludge. This indicated that the major part of PFOPA introduced into the WWTP system would be found in sludge or activated sludge of WWTP at higher levels than in water. In the case of PFHxPA no equilibrium between water and sludge (or activated sludge) was observed. Our results indicted that this compound could be randomly distributed between water and soil fractions and this can support our water results.

PFDPA was analyzed in all water samples but the results were below MLOQ in all cases.

Conclusions

This work reports some physicochemical parameters of a new class of perfluorinated acids detected before in Canada rivers and WWTP effluents. However, higher concentrations of PFHxPA and PFOPA were found in the present study of a German river and in a WWTP effluent.

The degradation experiments concluded that PFPAs were not biodegraded, although accumulation or adsorption of PFDPA was assessed. More studies are required in order to elucidate the fate and environmental impact of this compound. Also complementary study has to be performed in order to assess the type or possible type of organisms that can adsorb this compound and the mechanism of adsorption.

The identification of these related products could be a tool to help the registration, evaluate, authorize and restrict, if it would be necessary, these chemical substances although further studies are necessary.

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Marie Curie Initial Training Network

Environmental Chemoinformatics (ECO)

Final project report

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ECOTOXICITY OF HIGHLY FLUORINATED COMPOUNDS

Duration of Short Term fellowship:

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Early stage researcher:

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Introduction

The ubiquitous detection of several perfluorinated surfactants, such as perfluorooctane sulfonates (PFOS) and perfluorooctanoic acid (PFOA), and their adverse environmental and toxicological effects have rendered them one of the most investigated compounds during the last years. The abnormal properties and activities imparted by the presence of the fluorine atoms have been shown to impede prediction of various properties.

However, prediction of environmentally significant properties and activities of highly fluorinated compounds is crucial to understand their fate and their effects on biota. Thus, a comprehensive model covering a wide range of perfluoroalkyl chain lengths and different functional groups should be established. In order to achieve this, ecotoxicological measurements of certain compounds that have been preselected by means of statistical experimental design, must be performed.

Within this scope, the ecotoxicity of several compounds on different species had already been investigated. However, results of perfluorocarboxylic acids have not been as assumed and should therefore be thoroughly reinvestigated. Especially toxicity of perfluorobutanoic acid (PFBA) to the green algae *Pseudokirchneriella subcapitata* and the cladoceran *Daphnia magna* had been much higher than assumed. Taking into account that novel fluorosurfactants which will be released might incorporate shorter perfluoroalkyl chain lengths, it is crucial to understand their toxicological and ecotoxicological potential.

Materials and Methods

Perfluorinated compounds were purchased from different chemical suppliers and had purities of > 95%.

Acute toxicity to algae was carried out with the "PAM test" (pulsed amplitude modulation) developed by the Rijksinstituut voor Volksgezondheid en Milieu (RIVM, National institute for Public Health and the Environment) and the University of Amsterdam [1]. Briefly, photosynthetic activity of the green algae *Pseudokirchneriella subcapitata* is measured by a pulsed-amperometric fluorometer under the influence of the selected chemical at different concentrations. The end-point of this measurement is the fluorescence yield compared with a blank after 4.5 h.

Acute toxicity to *Chydorus sphaericus* was assessed by the help of a test developed at RIVM [1]. Briefly, a minimum number of 20 juvenile (age < 24 h) animals were exposed to a series of at least six concentrations of the test chemical and a blank. The animals were divided into at

least four groups and the test was carried out in 2 mL HPLC vials with a test volume of 250 µL. Immobilization was assessed after 24 h and 48 h under a microscope after gently shaking the HPLC vial. EC_{50} and EC_{10} values were calculated with the Probit software provided by the US Environmental Protection Agency.

Concentrations of the compounds in assays with PFBA (with pH adjustment), PFOA (with pH adjustment), perfluorononanoic acid (PFNA) and 1H,1H,8H,8H-perfluorooctane-1,8-diol (THPFOdiol) were confirmed by means of Liquid Chromatography – Electrospray Ionization - Tandem Mass Spectrometry (HPLC-ESI-MS/MS). Samples were drawn right after the test and immediately diluted 1:10 (V:V) with methanol to prevent adsorption. For further measurement, these samples were subsequently diluted with water/methanol (1:1; V:V) to fit into the calibration curve. For measurement of PFOA, PFNA and THPFOdiol, internal standard $^{13}C_2$-PFOA was added to the samples and used for quantification.

Results and Discussion

Acute toxicity to green algae Pseudokirchneriella subcapitata

It was found out that problems associated with previous measurements were related to the acidity of the perfluorinated carboxylic acids. Even though problems in assessing the pKa values of perfluorinated acids exist [2], they are likely to be very low due to the electron-withdrawing effects of the perfluoroalkyl chain.

The ecotoxicity test to algae is carried out in so-called 'Dutch standard water' (DSW), which is milli-Q water containing 100 mg/L $NaHCO_3$, 20 mg/L $KHCO_3$ and 180 mg/L $MgSO_4*7H_2O$. Considering no buffer activity of MgSO4, only the carbonate species have buffering effect, resulting in a buffer capacity of ca. 1.4 mM.

Therefore, concentrations of any perfluorinated acid above 1.4 mM will cause a major change in pH, which in turn will lead to a toxic effect on the algae. To circumvent this problem, the stock solutions used for the assay were adjusted to a pH above 8, just like the DSW, with hydrochloric acid and sodium hydroxide solution.

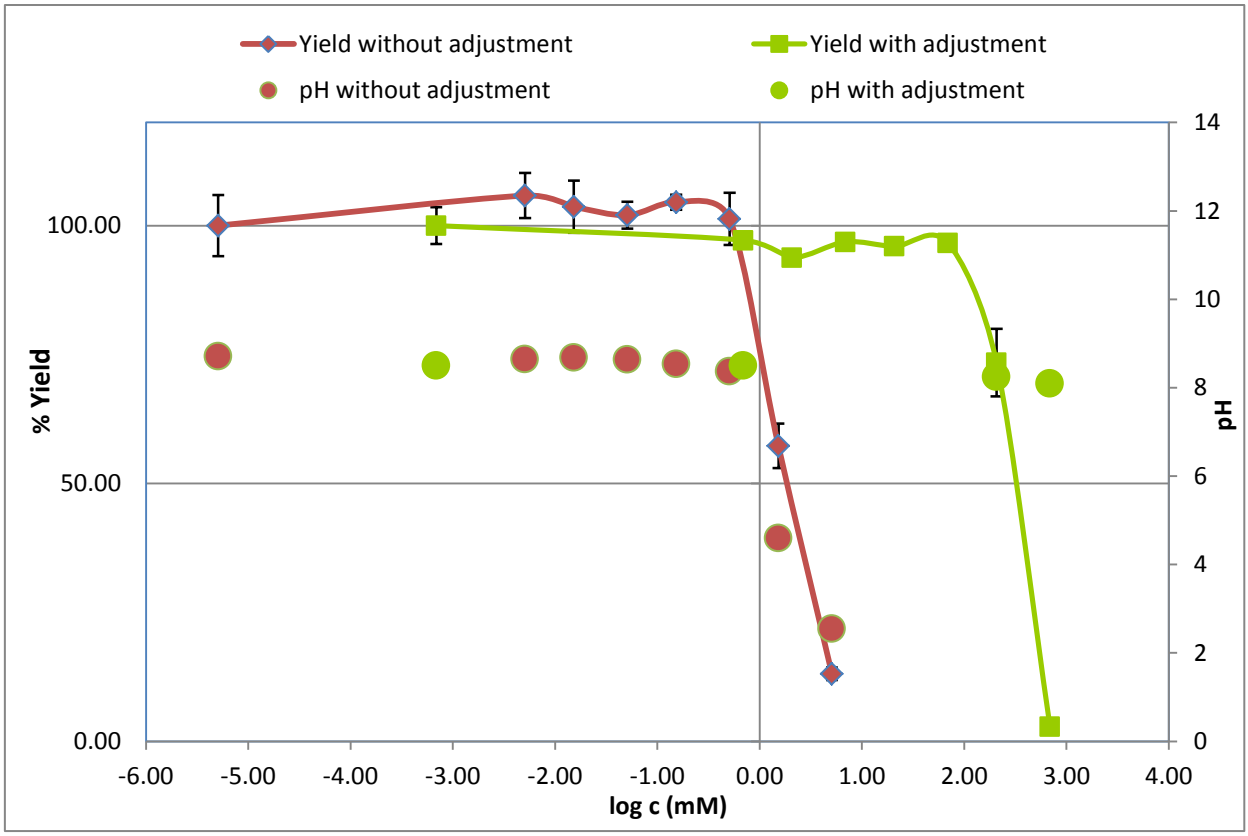


Figure 1: Logarithmic dose-effect curve for PFBA with and without pH adjustment. The dots represent the pH in the test vials at the respective PFBA concentrations. Red: without pH adjustment; green: with pH adjustment

Figure 1 shows the logarithmic dose-effect graph for PFBA and contrasts the curves with and without pH adjustment. It is obvious that the pH drop at log c = 0.2 affects an increased toxicity, which is not the case when pH is adjusted. Without pH adjustment, the toxicity is more than two orders of magnitude lower than with non-adjusted stock solution (EC_{50} = 1.76 mM vs. 279 mM). Since perfluorinated acids are supposed to occur in their dissociated form in the environment, the adjustment of pH is actually reasonable.

It could be shown that the previously measured values for other perfluorinated acids were equally biased by a pH effect. Figure 2 demonstrates that also for PFOA, pH adjustment is necessary. Here again, PFOA was measured with and without adjustment. A third assay was performed using the commercially available ammonium perfluorooctanoate (APFO). The dose-response curves for pH-adjusted PFOA and APFO were very similar, which is also expressed by very similar EC_{50} values.

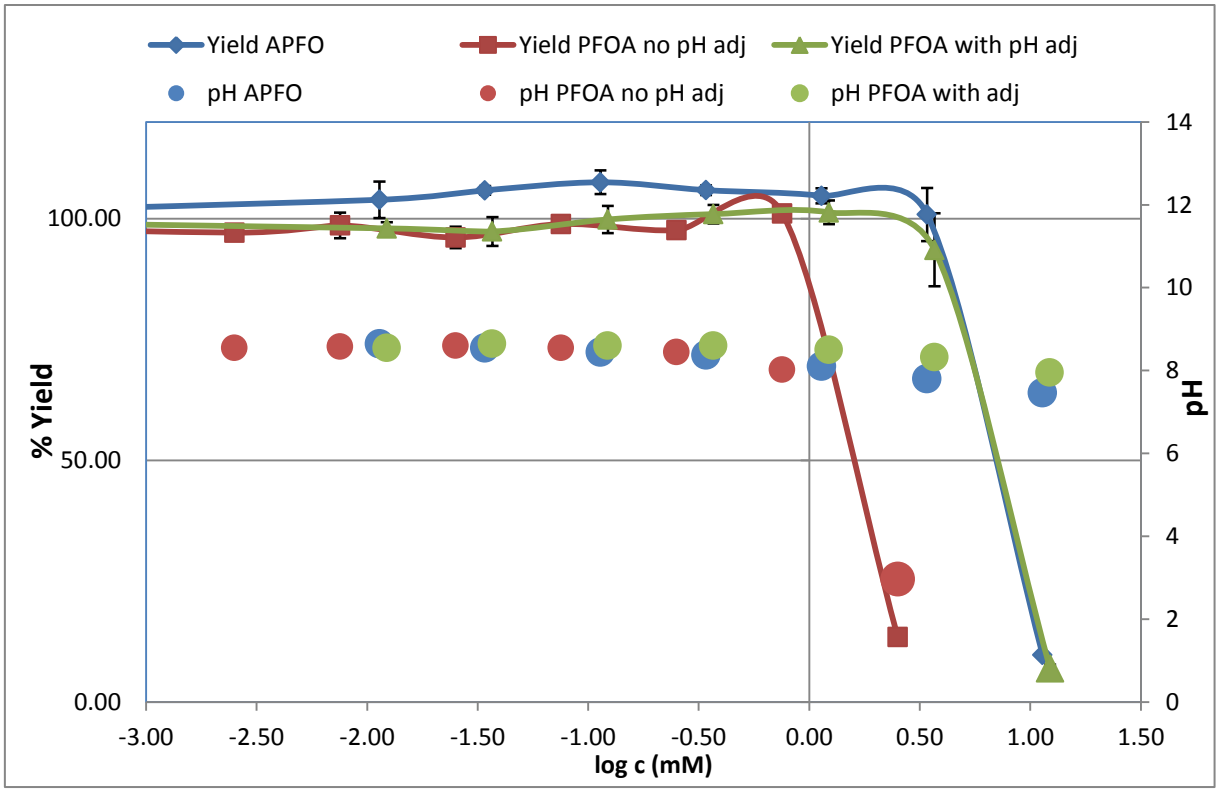
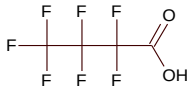
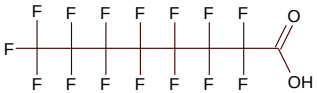
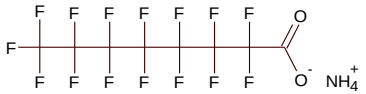
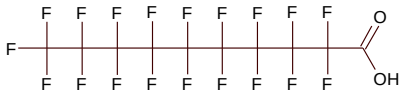
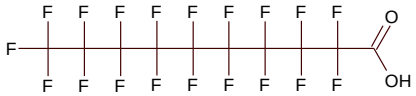
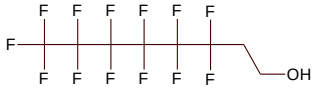
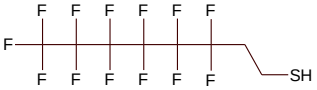


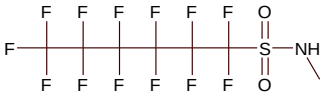
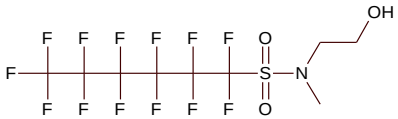
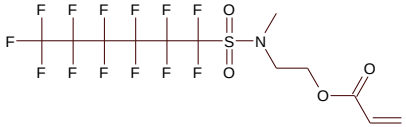
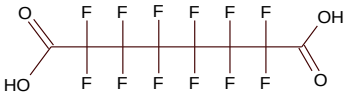
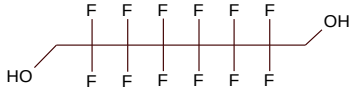
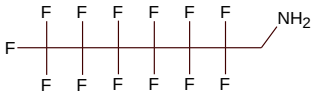
Figure 2: Dose-response curves for APFO, PFOA without pH adjustment and PFOA with pH adjustment. Dots indicate the pH measured in the vials after the test. Please note that nominal concentrations are given for better comparison of the data between PFOA without pH adjustment and APFO.

Various other perfluorinated compounds were tested in for acute toxicity on algae. The results are shown in Table 1. For a number of compounds, low water solubility prevented measurements of EC_{50} values, because even at the highest concentrations, no effect was observed.

The comparison of THPFOdiol and PFSubA clearly indicates that toxicity of a compound is not only related to the perfluoroalkyl chain length. In this case, the EC_{50} values will have a difference of at least two orders of magnitude, although both contain six difluoromethylene groups. The toxic effect of the two alcohol groups is much more pronounced than that of the two carboxylic acid functions. However, this may be different for other species, because the PAM test is very specific since an effect will only be observed if the photosynthetic system is affected.

Table 1: Overview of results for acute toxicity of fluorinated compounds to green algae

Structure	Chemical name	Acronym	Concentrations verified	EC_{50} PAM test[a] [mM]	EC_{10} PAM test[a] [mM]
	Perfluorobutanoic acid[b]	PFBA	yes	257 (236 – 279)	156 (131 – 185)
	Perfluorooctanoic acid[b]	PFOA	yes	5.96 (5.43 – 6.53)	3.68 (3.27 – 4.15)
	Ammonium perfluorooctanoate	APFO	no	6.94 (6.07 – 7.94)	4.31 (3.60 – 5.16)
	Perfluorononanoic acid[b]	PFNA	yes	0.732 (0.599 – 0.894)	0.232 (0.169 – 0.519)
	Perfluorodecanoic acid[b]	PFDA	no	> S_w > 0.438 mM	
	1H,1H,2H,2H-Perfluorooctanol	6:2-FTOH	no	> S_w > 0.04	
	1H,1H,2H,2H-Perfluorooctanethiol	6:2-FTSH	no	> S_w > 0.038	

Structure	Chemical name	Acronym	Concentrations verified	EC_{50} PAM test[a] [mM]	EC_{10} PAM test[a] [mM]
	N-Methyl perfluorohexanesulfonamide	N-MePFHxSA	no	> S_w > 0.02	
	N-Methyl,N-(2-hydroxyethyl)-perfluorohexanesulfonamide	N-MePFHxSAEt	no	> S_w > 0.02	
	N-Methyl-perfluorohexane sulfonamidoethyl acrylate	N-MePFHxSAEtAc	no	> S_w > 0.02	
	Perfluorooctanedioic acid	PFSubA	no	> 130	
	1H,1H,8H,8H-Perfluorooctane-1,8-diol	THPFOdiol	yes	0.659 (0.592 – 0.735)	0.182 (0.143 – 0.231)
	1H,1H-Perfluoroheptylamine	DHPFHpAm	no	> 0.151	

> S_w : higher than water solubility

[a] 95% confidence interval in brackets **[b] stock solutions neutralized with sodium hydroxide, therefore toxicity of the anion is assessed**

Acute toxicity to Chydorus sphaericus

The acute toxicity of PFBA was also measured to the cladoceran *Chydorus sphaericus*, which is a common species in Central European waters. Previous measurements yielded an EC_{50} value of 2.51 mM.

However, also in this assay, a medium is used which contains low buffer concentration, in this case 0.77 mM sodium bicarbonate. Therefore, when the PFBA concentration exceeds the bicarbonate concentration, a major pH reduction will occur, although this could not be proven in the test because of the very small volume used.

Table 2: Results for acute toxicity of PFBA to *Chydorus sphaericus*

	24 h		48 h	
	[mM]	**95% CI [mM]**	**[mM]**	**95% CI [mM]**
EC_{50}	30.6	25.4 - 35.2	22.3	16.8 - 26.6
EC_{10}	16.6	11.0 - 20.9	13.1	7.5 - 17.2

95% CI: 95% confidence interval

The test was carried out with a stock solution with pH adjustment. The resulting values are much higher than previous ones with an EC_{50} value of 22.3 mM after 48 h (Table 2). This value is comparatively high and shows that toxic effects of PFBA are not likely to occur in the environment, where concentrations are usually in the pM to nM range.

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