PERFLUORINATED SURFACTANTS INHIBIT THE GROWTH OF CILIATE PROTOZOAN *TETRAHYMENA PYRIFORMIS*

M. A. Ud-Daula[1], Š. Zezulka[1,2] S. Fiedler[1] and K.-W. Schramm[1,3*]
[1]GSF-National Research Centre for Environment and Health, Institute of Ecological Chemistry, Ingolstädter Landstrasse 1, D-85764 Neuherberg, Germany
[2]Institute of Experimental Biology, Faculty of Science, Masaryk University, Kotlářská 2, CZ-61137 Brno, Czech Republic
[3]TUM-Technische Universität München, Department für Biowissenschaftliche Grundlagen, Weihenstephaner Steig 23, D-85350 Freising, Germany, *schramm@gsf.de, schramm@wzw.tum.de*

Abstract
The aim of this work was to evaluate the effect of two species of perfluorinated surfactants, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) on the growth of a ciliate *Tetrahymena pyriformis* and to determine their effective concentration (EC). The toxicity measurements were conducted using critical dilution assay in 96-well plates by monitoring the increment of cells as compared to an untreated control. Samples were exposed to the concentrations ranging from 0 to 100 mg/L of each compound and incubated at 28°C for 24 and 48 hours, respectively. Their optical density was then measured spectrophotometrically at 550 nm. Both PFOA and PFOS caused the inhibition of growth of the ciliate but the effect of PFOS was more significant as compared to PFOA. After 48 hours the EC_{50} value of PFOS was established to 85.8±11.6 mg/L (134 µM) and EC_{10} of PFOA 65.4±14.3 mg/L (145 µM). Both compounds affected the growth of *Tetrahymena* even in very low concentrations, but significant growth inhibition was recorded only in samples exposed to concentrations higher than 25 mg/L.
Keywords: Perfuorinated surfactants; Tetrahymena pyriformis; Growth; Toxicity; Effective concentration.

INTRODUCTION

Perfluorinated compounds (PFCs) have emerged as a new class of global environmental pollutants. Among PFCs, perfluorinated sulfonates and perfluorinated carboxylates have attracted much attention in recent years. These compounds in general, and perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in particular, comprise a class of environmentally persistent chemicals [1]. With their unique physicochemical properties, they have a broad spectrum of applications as surfactants, refrigerants and polymers, and also as components of pharmaceuticals, fire retardants, lubricants, adhesives, paints, cosmetics, agrochemicals and food packaging [2]. Perfluorochemicals have been detected not only in the physical environment, but also in humans and wildlife. These contaminants have been found in oceanic waters. Several studies have reported the presence of perfluorinated chemicals in a variety of wildlife species, including freshwater and marine mammals, fish, birds and shellfish [3].
Because of the high-energy carbon-fluorine bond, most of PFCs are persitent in the environment and resist hydrolysis, photolysis and biodegradation. They are mostly non-volatile, have high molecular weights, and can repel both water and oils [4].
Despite the widespread use of these compounds, relatively little is known about the fate and effects of PFOS and PFOA, particularly under semi-field conditions. In view of their widespread occurrence, extreme persistence, and the fact that they can bioaccumulate [5], the aim of this work was to evaluate the effect of PFOA and PFOS on the growth of a ciliate *Tetrahymena pyriformis* by means of growth inhibition test and to determine the effective concentration (EC) of both compounds.

MATERIALS AND METHODS

Chemicals

For experiments perfluorooctanoic acid (PFOA) from Fluorochem, UK, and perfluorooctane sulfonate (PFOS) in form of tetraethylammoium salt from Aldrich, Germany, were chosen. Both compounds were dissolved in

methanol-water system (1:4, v/v) using pure methanol (Aldrich, Germany) and Milli-Q water. The initial concentration of stock solutions was 1000 mg/L. Stock solutions were then diluted to the final concentrations by direct addition of cultivation medium or pre-diluted in 20% methanol solution. Final methanol concentration in the samples did not exceed 2% and its toxic effect was subsequently substracted from the data.

Protozoan cultivation

The ciliate protozoan *Tetrahymena pyriformis*, strain GL, was cultivated axenically without shaking in proteose peptone – yeast extract medium (PPY) [6] containing 0.35% proteose peptone, 0.07% yeast extract, 0.35% glucose (all from Merck, Germany) and trace elements. The pH-value of the medium was established to 7.2±0.05 and to avoid a pH-shift during the culture growth or due to the addition of test chemicals a biological buffer (MOPS, Aldrich, Germany) was added. This PPY medium was used for cultivation of experimental stock, pre-experimental cultures and as a nutrient supply during the growth tests.

Growth inhibition test

Toxicity assay was performed as a critical dilution assay in 96-well plates by monitoring the increment of *Tetrahymena* cell as compared to an untreated control. One day old pre-experimental logarithmically growing culture (approx. $1 – 2.10^5$ cells per mL) was added to the samples with PFOA and PFOS concentrations ranging from 0 to 100 mg/L and incubated at 28°C. The density of the cell population was measured spectrophotometrically at 550 nm using SLT Spectra microplate reader (Spectra III, SLT, Austria). The measurements were done after 24 (T1) and 48 (T2) hours of exposure. On the basis of optical densities of the samples a percentage of growth inhibition was calculated (the difference of OD of control and treated sample related to OD of control) and the value of effective concentration (EC_{50} for PFOS and EC_{10} for PFOA, respectively) was evaluated.

RESULTS AND DISCUSSION

Both PFOS and PFOA caused an affection of *Tetrahymena* growth. Selected concentration series (0 to 100 mg/L) represents possible environmental loading of surface waters, which are natural for many zooplanktonic species including protozoans. Studies focused on the concentration of PFCs in the environment referred about background concentrations ranging from 0 to tens ng/L, but in case of some ecological accident the concentrations could reach tens of mg/L. Similar studies made on human population stated similar findings in blood of normal population and workers from some factories, which produce PFCs [7].

Some studies with other organisms show stronger effect of PFOS in comparison with PFOA [8]. In our experiment we recorded similar response. Relatively high maximal concentration of PFOA (approx. 100 mg/L) was not sufficient to inhibit more than 20% of the growth rate in comparison to control sample (Fig.1). To the contrary of this, PFOS caused at the same concentration nearly 90% inhibition of growth (Fig. 2). The effect of low concentrations was not significant, data showed either only very weak inhibition or even stimulation (0 ± 5%). The least significant differences between samples and control were recorded in concentrations higher than 25 mg/L for both compounds (ANOVA, Fisher-LSD test, P=0.05; data not shown).

Effective concentrations of PFOA and PFOS at different levels were established after 48 hours of exposure (Table 1). The length of exposure period (48 hours) was chosen with respect to natural time border between exponential phase of growth and subsequent phase leading to the slower growth and a flashpoint of the culture.

Table 1. Effective concentrations of PFOA and PFOS evaluated from the growth response of *Tetrahymena pyriformis* after 48 hours of cultivation

Compound	Endpoint	Value (mg/L)
PFOA	EC10	65.4±14.3
PFOS	EC50	85.8±11.6

Concentration series of both compounds were prepared either by direct dilution of their stock solutions in PPY medium (decreasing concentration of methanol) or by pre-dilution once again in methanol/water solution and subsequent delivery to PPY (equal concentration of methanol).

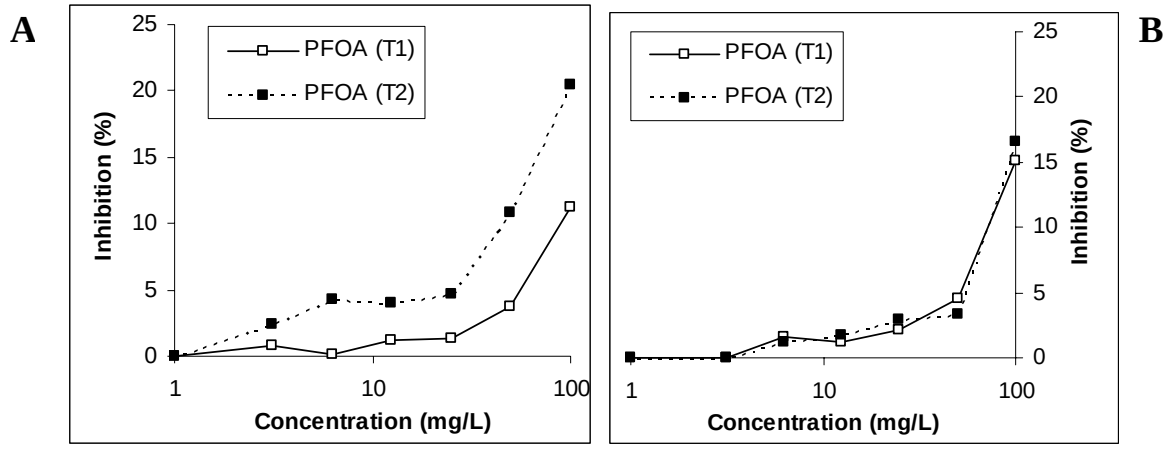

Figure 1. Inhibition of *Tetrahymena pyriformis* growth in the presence of perfluoroocanoic acid after 24 hours (T1) and 48 hours (T2) of exposure. PFOA solution was diluted directly in PPY medium (A) or pre-diluted in the same methanol/water solution and then delivered to PPY (B). PFOA concentrations (0 to 100 mg/L) are expressed by means of a logarithmic scale. Data points represent mean value of 6 replicates.

Samples exposed to decreasing methanol concentration (Figs 1A and 2A) did not show any significant difference in comparison with those exposed to equal methanol treatment (Figs 1B and 2B). Some authors [e.g. 9] describe effects of toxic compounds on *Tetrahymena* morphology, but in case of PFOA and PFOS we did not record any significant change, because even the cells in the control sample were of different size and shaped from standard oblong *Tetrahymena* cell to more or less rounded cells.

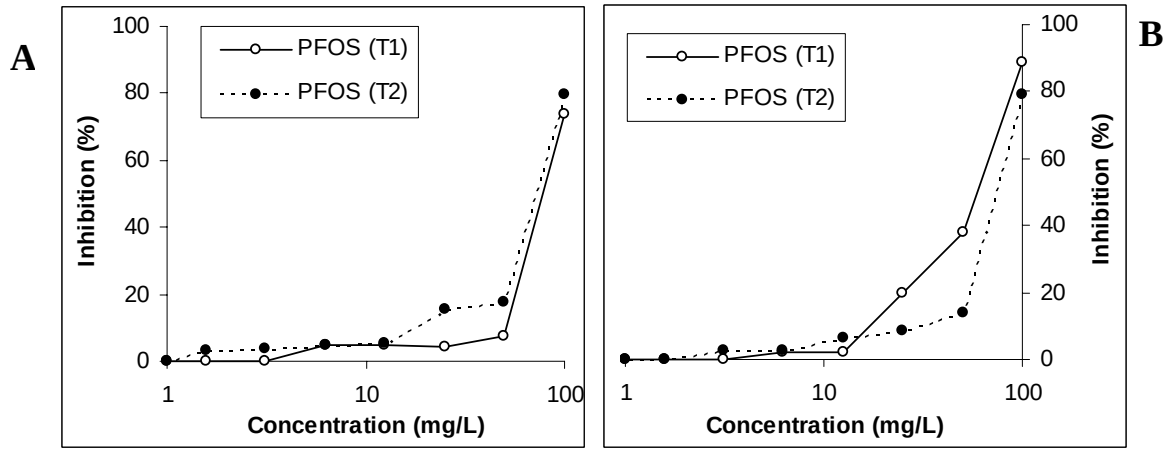

Figure 2. Inhibition of *Tetrahymena pyriformis* growth in the presence of perfluorooctane sulfonate after 24 hours (T1) and 48 hours (T2) of exposure. PFOS solution was diluted directly in PPY medium (A) or pre-diluted in the same methanol/water solution and then delivered to PPY (B). PFOS concentrations (0 to 100 mg/L) are expressed by means of a logarithmic scale. Data points represent mean value of 6 replicates.

One of the factors of PFCs availability for organisms could be the state of surfactant molecules in the solution. More hydrophobic species of perfluorinated surfactants, like PFOS and its tetraethylammonium salt (TEA-PFOS), are known for their ability to create micelles in aqueous solutions. The formation of micelles is

dependent mainly on the concentration of surfactant. By means of various methods it is possible to find so-called critic micelle concentration (CMC) which is typical for each compound and some physico-chemical conditions of the environment – the temperature, charge and other factors such as the presence of various ions e.g. from dissolved salts.

López-Fontán et al. [10,11] observed, that CMC value for TEA-PFOS at 28°C is approx. 1mM. Nevertheless the starting point of micelle formation could be lower, around 477 μM, when the first ion pairs of $PFOS^-$ and TEA^+ occur. A study with another protozoan *Paramecium caudatum* [12] showed, that a time period of stress-induced backward swimming of this protozoan is in good correlation with CMC value of the tested compound. Compounds with low CMC could be less toxic than those with high value of CMC like PFOS.

CONCLUSIONS

The growth inhibition test with *Tetrahymena pyriformis* showed, that this protozoan is sensitive to both tested perfluorinated compounds, PFOA and PFOS. Both these compounds exhibited acute toxic effect on protozoan growth, but the effect of perfluorooctane sulfonate was more significant than in case of PFOA. However, effective concentrations recorded are orders of magnitude higher than the usual environmental exposure, but possible risk connected to the bioaccumulation ability of these compounds in food chains should not be underestimated.

References

1. Yamashita, N., Kannan, K., Taniyasu, S., Horii, Y., Petrick, G., Gamo, T., 2005. A global survey of perfluorinated acids in oceans. *Marine Pollution Bulletin*, **51**, 658-668.
2. Key, B.D., Howell, R.D., Criddle, C.S., 1997. Fluorinated organics in the biosphere. *Environmental Science and Technology*, **31**, 2445-2454.
3. Mulkiewicz, E., Jastorff, B., Składanowski, A.C., Kleszczyński, K., Stepnowski, P., 2006. Evaluation of the acute toxicity of perfluorinated carboxylic acids using eukaryotic cell lines, bacteria and enzymatic assays. *Environmental Toxicology and Pharmacology*, ***in press***, doi:10.1016/j.etap.2006.11.002
4. Bossi, R., Riget, F.F., Dietz, R., Sonne, C., Fauser, P., Dam, M., Vorkamp, K., 2005. Preliminary screening of perfluorooctane sulfonate (PFOS) and other fluorochemicals in fish, birds and marine mammals from Greenland and the Faroe Islands. *Enviromental Pollution*, **136**, 323-329.
5. Giesy, J.P., Kannan, K., 2001. Global distribution of perfluorooctane sulfonate in wildlife. *Environmental Science and Technology*, **35**, 1339-1342.
6. Plessner P., Rasmussen L., Zeuthen E., 1964. Techniques used in the study of synchroneous Tetrahymena. In: *Synchrony in Cell Division and Growth*. (ed. E. Zeuthen), Intersciences, New York, pp. 534–565.
7. Olsen, G.W., Burris, J.M., Mandel, J.H., Zobel, L.R., 1998. Serum perfluorooctane sulfonate and hepatic and lipid clinical chemistry tests in fluorochemical production employees. *Journal of Occupational and Environmental Medicine*, **41**, 799–806.
8. Sanderson, H., Boudreau, T.M., Mabury, S.A., Solomon, K.R., 2004. Effects of perfluorooctane sulfonate and perfluorooctanoic acid on the zooplanktonic community. *Ecotoxicology and Environmental Safety*, **58**, 68-76.
9. Dias, N., Mortara, R.A., Lima, N., 2003. Morphological and physiological changes in Tetrahymena pyriformis for the in vitro cytotoxicity assessment of Triton X-100. *Toxicology in Vitro*, **17**, 357–366.
10. López-Fontán, J.L., González-Pérez, A., Costa, J., Ruso, J.M., Prieto, G., Schulz, P.C., Sarmiento, F., 2006. The critical micelle concentration of tetraethylammonium perfluorooctylsulfonate in water. *Journal of Colloid and Interface Science*, **294**, 458–465.
11. López-Fontán, J.L., González-Pérez, A., Costa, J., Ruso, J.M., Prieto, G., Schulz, P.C., Sarmiento, F., 2006. Thermodynamics of micellization of tetraethylammonium perfluorooctylsulfonate in water. *Journal of Colloid and Interface Science*, **297**, 10–21.
12. Matsubara, E., Harada, K., Inoue, K., Koizumi, A., 2006. Effects of perfluorinated amphiphiles on backward swimming in *Paramecium caudatum*. *Biochemical and Biophysical Research Communications*, **339 (2)**, 554-561.