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Toxicity Identification Evaluation of Organic Pollutants Based on Solid-Phase Micro-Extraction and Gas Chromatography/Mass Spectrometry

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Since 1992, Toxicity Identification Evaluation (TIE) protocols have been used (Norberg-King et al. 1992; Durhan et al. 1993; Mount and King 1993) by the U.S. Environmental Protection Agency (EPA) to identify toxic pollutants in complex effluents. The characteristic and benefits of TIE techniques, which include Gas Chromatography-Mass Spectrometry (GC-MS) analysis, have also been described (Burkhard et al. 1991). The experimental method included an extraction step, followed by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) fractionation and chemical analysis by GC-MS of the toxic fractions (Reineke et al. 2002).

Since 1989, Solid Phase Micro-Extraction (SPME), introduced by Belardi and Pawliszyn in 1989, is being applied to organic characterization of all kinds of environmental samples, generally linked to gas chromatography (Eisert and Levsen 1996); it has also been used to assess aquatic toxicity in complex mixtures (Parkerton et al. 2000; Alonso et al. 2001a).

SPME has been applied successfully in sewage characterization (Alonso et al. 2001b) and could be applied in TIE protocols representing a cost/effective tool in order to avoid the time-consuming RP-HPLC fractionation step. This paper presents its application.

MATERIALS AND METHODS

Water samples (400 ml, n=16) were taken from a sewage system prior to treatment at a sewage treatment plant that receives municipal and industrial sewage. The sampling was performed by a 900 Max Portable Sampler (American SIGMA, USA) on two days (working and weekend) for 24 hours/day, at 1-hr intervals. Water quality parameters (pH, conductivity, temperature and dissolved oxygen) were measured *in situ* at 10 minutes interval and samples were stored at 4-5ºC until analysis.

Extraction of organic compounds from water samples was performed with Solid Phase Micro-Extraction (SPME) Fiber (Assembly 70 µm Carbowax/DVB Stable flex, Supelco 7336-U) by immersion in a vial containing 3.5 ml of sample with

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intense constant magnetic stirring at 22 ºC for 3 hours. This solid-phase and conditions were chosen as per a previous study with wastewater samples (Alonso et al. 2001b).

Desorption of organic compounds from the fiber was performed into the helium stream of the heated injector port (250ºC splitless 5 min) of a HP 6890N Gas Chromatograph equipped with a HP 5973N mass selective detector (Agilent Technologies). The column (HP-5MS, 30 m, 0.25 mm x 30 m x 0.25 μm, Agilent 19091S-433) was initially set to 50ºC, held for 5 minutes, increased to 250ºC at 10ºC/min and held for 10 min. Detection was performed in the EI-SCAN mode. Quadrupole and source were set at 150 ºC and 230 ºC, respectively.

After chromatogram recording, the mass spectrum of each peak was compared with Wiley (Enhanced Chemstation G1701DA, Agilent Technologies) and Nist98 (NIST/EPA/NIH Mass Spectra library, Agilent Technologies) libraries in order to identify organic compounds. When the quality of a match was higher than 90%, the corresponding standard was injected in the chromatographic system in order to confirm the identity of the compound detected. Standards samples were prepared by spiking a known quantity of the corresponding compound in MilliQ water and were processed in the same way as samples, in order to minimize errors related to recovery efficiency. External standard procedure was used to quantify the identified compounds.

Three toxicity tests were used in this study: *Daphnia* sp. acute immobilisation test (OECD 1993), the algae growth inhibition test modified to 96 well micro-titre plates (Ramos et al. 1996) and cytotoxicity tests on the RTG-2 fish cell line (Babín et al. 2001). Briefly, the *Daphnia* sp. acute immobilization test studies toxic effects on swimming capability of not more than 24 hours old daphnids exposed to a range of sample dilutions. In the alga growth inhibition test, effects of a range of sample dilutions, are studied on the growth of a unicellular green algal species. *Daphnia magna* and *Chlorella vulgaris* were exposed to 10 %, 1% and 0.1% dilutions of each sample in reconstituted water with hardness of 250 mg $CaCO_3$/L and pH 7.9 ± 0.3 (ISO 6341, 1982) in duplicate. After 48 h and 72 h, respectively, immobilized daphnids and the number of algae were recorded.

The RTG-2 fish cell line (ATCC, CCL N.55) was exposed to a 75% dilution of each sample, using three replicate wells per concentration, in Minimum Essential Eagle Medium with Earle's salts (EMEM) for 24 hours, test controls (medium without additions) were run in parallel. They were taken as reference in order to determine the percentage of inhibition in each sample. Four end-points were used in order to measure the cytotoxicity, EROD, ß-galactosidase, neutral red stain and protein content. EROD activity was measured directly in the wells without removing the exposure medium (Bols et al. 1999). Briefly, 50 μl/well of a 0.825 μM solution of 7-ethoxyresorufin in EMEM medium was added, measuring the fluorescence (TECAN-spectrafluor fluorescence spectrofluoremeter, excitation wavelength of 530 nm and emission wavelength of 590 nm) at 60 min. The fluorescence plate reader was also used to quantify ß-galactosidase activity

(Pablos et al. 1998); fluorescence was measured (360 nm excitation, 465 nm emission) at 60 min after addition of 20 µl/well (0.18mM) of substrate (MFU-galactoside) to the medium. Neutral Red staining of the lysosomes in order to evaluate cell viability was performed as described by Borenfreund and Puerner (1985) but adapted to use 96-well plates (Castaño et al. 1996) reading the absorbance at 550 nm. Then, the protein content was measured on the same plate, to evaluate the total cellular mass, using the Kenacid Blue Protein assay described by Castaño et al. (1996).

Results were initially tested for normality and homogeneity of variance and data analysis was performed using one-way ANOVA, followed by a Duncan´s test for the multiple comparison procedures. When data failed to be normally distributed, the Wilcoxon´s rank test was applied.

RESULTS AND DISCUSSION

Previous toxicological and chemical analyses suggested the toxicity to be associated with the organic fraction. Solid-Phase MicroExtraction in combination with Gas Chromatography and Mass Spectrometry detection (GC-MS) was selected as a time/cost effective and powerful technique to detect organic compounds in complex mixtures. The analysis confirmed this hypothesis as shown in Table 1, up to 32 organic compounds were detected in the collected samples.

Diazinon and quinalphos (organothiophosphonic pesticides) are unusual compounds in this type of sample and were detected in a few samples collected on the working day. The identification of these pesticides was confirmed with the corresponding analytical standards.

Diazinon and quinalphos were quantitatively determined using the SPME/GC-MS SIM (ions 179, 306 for Diazinon and 146, 298 for Quinalphos) technique and external standard procedure (recovery > 90%, detection limits 1 and 0.5 ng/ml, respectively). Figure 1 shows the concentrations of pesticides found in the samples collected at 60 minutes interval; as can be noted both compounds appear at the same, short period of time, corresponding to a discrete spill during the working day. Maximum concentrations of diazinon and quinalphos rose to 450 ng/mL and 125 ng/mL, respectively.

A very good relationship between toxicity and pesticide concentrations was observed appearing as the principal cause of *Daphnia magna* mortality. As can be seen in Figure 1, the same samples show toxicity to *Daphnia magna* in the acute immobilisation test. Mortality reached 100% with a 10% dilution of the sample in the medium test; this sample has about 30 equitox/m^3, where equitox/m^3 is the inverse of the percentage of effluent able to produce 50 % of *Daphnia* mortality. On the other hand, samples collected during the weekend did not show toxicity to *Daphnia magna.*

Table 1. Organic compounds detected in the collected samples

CAS Number	Compound Name	CAS Number	Compound name
584-84-9	2,4-Toluene diisocyanate	80-05-7	Bisphenol A
98-00-0	2 Furanmethanol	90-11-9	Naphthalene, 1-bromo
128-37-0	Butylated Hydroxytoluene	13593-03-8	Quinalphos
771-51-7	1H-Indole-3-acetonitrile	57-10-3	Palmitic acid
5466-77-3	2-Ethylhexyl 4-methoxycinnamate	112-80-1	Oleic Acid
333-41-5	Diazinon	98-86-2	Acetophenone
3380-34-5	Quinalphos	1000149-85-9	Isolimonene
120-72-9	Indole	470-67-7	1,4 Cineole
95-20-5	1H-Indole, 3-methyl	17422-33-2	6-Chloroindole
89-78-1	Menthol	57-11-4	Stearic acid
1009-61-6	Ethanone, 1,1′-(1,4-phenylene)bis	140-29-4	Benzyl Cyanide
23676-09-7	Benzoic acid, 4-ethoxy-, ethyl ester	584-84-9	Benzene, 2,4-diisocyanato-1-methyl
84-66-2	Diethyl Phthalate	142-91-6	Isopropyl palmitate
84-74-2	Dibutyl Phtalate	1000222-86-6	Oxime,- methoxy-phenyl
117-81-7	bis(2-Etilhexil)ftalato	120-51-4	Benzyl Benzoate
5400-75-9	2H-Benzimidazol-2-one, 1,3-dihydro-5-methyl	1222-05-5	Cyclopenta[g]-2-benzopyran,1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl

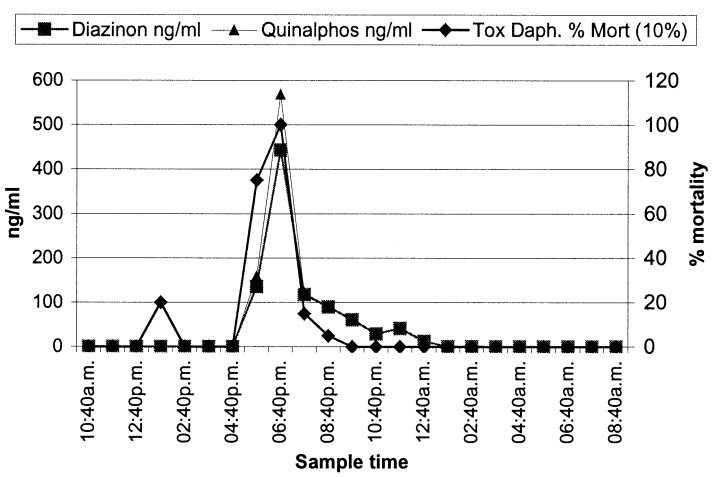

Figure 1. Diazinon and quinalphos concentrations relationship with *D. magna* toxicity in working day samples.

According to bibliographic data (Burkepile et al. 2000; Fernandez-Casalderrey at al. 1995; EEDB 2000), diazinon and quinalphos have a LC_{50} (48 h) to *Daphnia magna* between 0.6 ng/ml and 3.4 ng/ml, and 0.18 ng/ml and 0.56 ng/ml, respectively. Therefore, *Daphnia magna* mortality in the samples appears to be directly related with quinalphos and diazinon concentrations.

Table 2. Percentages of inhibition (vs. control tests) obtained in each sample for both sampling days

Working day						Weekend					
	Algae	Fish cell toxicity (% inh)					Alg	Fish cell toxicity (% inh)			
N	% Inh	B-Gal	EROD	Prot.	R.N.	Nº	ae	B-Gal	EROD	Protei	R.N.
1	-168	-13	-1	-8	-2	**24**	Lost Sample				
2	-24	-80	-6	-3	1	**25**	-1	-27	3	2	-1
3	-302	-63	6	-2	10	**26**	-95	-6	5	-6	-5
4	-359	-112 (*)	**49 (*)**	**25 (*)**	**51**	**27**	-29	-7	5	-6	2
5	-392	-126 (*)	8	4	7 (*)	**28**	-188	-6	9	-1	-6
6	-136	-166 (*)	6	-9	9	**29**	-201	-42	20 (*)	0	-4
7	-281	-105	24 (*)	3	31	**30**	-158	-92	12	5	-11
8	-283	-50	1	-23	-14	**31**	-180	-51	10	10	0
9	-350	-17	-1	-4	-3	**32**	-230	-26	13	5	0
10	-472	-64	3	-11	-9	**33**	-178	-12	15	-5	-2
11	**30**	8	10	-10	-8	**34**	-403	-14	12	-8	-1
12	**36**	7	7	-5	-4	**35**	-552	-31	7	-17	-3
13	-27	-14	10	-4	2	**36**	**53**	-39	22 (*)	-6	2
14	-17	-31	6	-11	-3	**37**	-504	-15	10	2	6
15	-21	-24	11	-5	-4	**38**	-269	-45	10	16 (*)	2
16	-593	-32	9	-15	-2	**39**	-244	-34	17	12	1
17	-467	-37	7	-11 (*)	-1	**40**	-333	-25	20 (*)	11 (*)	3
18	-369	-22	5	-19	0	**41**	-201	-11	16	11	2
19	-513	-35	0	4	-4	**42**	-158	-40	15	16 (*)	0
20	-273	-28	1	6	-2	**43**	-4	-8	17	7	-1
21	-383	-101	-7	11	0	**44**	-193	-22	5	1	0
22	-5	-61	3	10	20	**45**	-181	-202 (*)	-7	6	-3
23	-195	-111 (*)	-16	2	3	**46**	-52	-167 (*)	9	11	3 (*)

(*) Significant difference (LSD´s multiple range test, $p < 0.05$) between sample and control.

Table 2 shows the percentages of inhibition (compared with control tests) obtained in each sample for both sampling days. As can be seen in Table 2, most samples present negative percentages, with the exception of three samples (number 11, 12 and 36), that show toxicity to *Chlorella vulgaris*, and only one (number 4) that presents cytotoxicity with a percentage of inhibition higher than 25 % for the three endpoints used. These values could reveal the presence of toxic compounds not detected in the SPME-GC/MS analysis, because this assay covers only organic and chromatographically stable compounds.

The negative values for algae inhibition could be explained by the algal growth induction associated with the presence of nutrients in the effluent (Sanchez 2002).

Regarding RTG-2 results, only sample 4 showed cell toxicity, while induction of β-galactosidase was observed for several samples. β-galactosidase induction has been described elsewhere (Babin et al. 2001). The induction of this enzymatic cytotoxicity is recognized as a sublethal dysfunction appearing at concentrations below those producing toxicological effects (Babin et al. 2001).

This induction was not observed for other parameters which ranged around the mean and only occasionally showed difference higher than 10 %.

Chemical analysis did not detect potential contaminants for the observed algae and fish cell toxicity (samples 11, 12 and 4, respectively), and the measured toxicity was not high enough to allow the application of further TIE fractionation procedures.

No relevant toxic organic compound was detected in samples presenting algae or fish cell toxicity; this fact reveals the necessity of using a battery of bioassays in order to cover all types of toxic substances.

In summary the employed SPME-GC/MS technique, when joined with a battery of ecotoxicological tests and a high sampling frequency permits detection of a discrete spill of toxicants in complex effluents. This procedure serves as a good tool to assess these type of effluents.

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REFERENCES

Alonso C, Fernández C, García P, Tarazona JV, Carbonell G (2001a) SPME/GC-MS for the determination of Linear Alkyl Benzenes (LABs) in environmental samples. In: Gelpi (ed) Advances in Mass Spectrometry,15: Wiley&Sons, p 853

Alonso C, Fernández C, Tarazona JV (2001b) Screening of organic contaminants in industrial effluents by Solid-Phase Microextraction/Gas Chromatography-Mass Spectrometry. Proc 11th SETAC Europe Annual Meeting 21-25 May 2000, p 175

Babin M, García P, Fernández C, Alonso C, Carbonell G, Tarazona JV (2001) Toxicological characterisation of sludge from sewage treatment plants using toxicity identification evaluation protocols based on *in vitro* toxicity tests. Toxicol in Vitro 15:519-524

Belardi RP, Pawliszyn J (1989) The application of chemically modified fused silica fibers in the extraction of organics from water mix samples and their rapid transfer to capillary columns. Wat Pollut Res J Canada 24: 179-191

Bols NC, Schirmer K, Joyce ME, Dixon DG, Greenberg BM, Whyte JJ (1999) Ability of polycyclic aromatic hydrocarbons to induce 7-ethoxyresorufin-o-deethylase activity in a trout liver cell line. Ecotoxicol Environ Saf 44:118-128

Borenfreund E, Puerner JA (1984) A simple quantitative procedure using monolayer cultures for toxicity assys (HTD/NR 90). J Tissue Culture Meth 9:7-9

Burkepile DE, Moore MT, Holland MM (2000) Susceptibility of five nontarget organisms to aqueous diazinon exposure. Bull Environ Contam Toxicol 64: 114-121

Burkhard LP, Durhan EJ, Lukasewycz MT (1991) Identification of nonpolar toxicants in effluents using toxicity-based fractionation with gas chromatography/mass spectrometry. Anal Chem 63: 277-283

Castaño A, Cantarino MJ, Castillo P, Tarazona JV (1996) Correlations between the RTG-2 cytotoxicity test EC50 and *in vivo* LC50 rainbow trout bioassay. Chemosphere 32:2141-2157

Durhan EJ, Norberg-King TJ, Burkhard LP (1993) Methods for aquatic toxicity identification evaluations: Phase II. Toxicity identification procedures for samples exhibiting acute and chronic toxicity. EPA 600/R-92-080. US Environmental Protection Agency, Duluth, MN

Eisert R, Levsen K (1996) Solid-Phase microextraction couple to gas chromatography: a new method for the analysis of organics in water. J Chrom A 733: 143-157

Environmental Effects Database (EEDB) (2000) Office of Pesticide Programs. Pesticide Ecotoxicity Database, Ref. Number 344. Environmental Fate and Effects Division, US Environmental Protection Agency, Washington, DC

Fernández-Casalderrey A, Ferrando MD, Andreu-Moliner E (1995) Chronic toxicity of diazinon to *Daphnia magna*: Effects on survival, reproduction and growth. Toxicol Environ Chem 49: 25-32

ISO 6341 (1982) Water quality: Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) (15 March 1982)

Mount DI, Norberg-King TJ (1993) Methods for aquatic toxicity identification evaluations: Phase III. Toxicity confirmation procedures for samples exhibiting acute and chronic toxicity. EPA 600/R-92-081. US Environmental Protection Agency, Duluth, MN

Norberg-King TJ, Mount DI, Amato JR, Jensen DA, Thompson JA (1992) Toxicity identification evaluations: Characterization of chronically toxic effluents, Phase I. EPA 600/6-91-005F. U.S. Environmental Protection Agency, Duluth, MN

OECD (1993) Guidelines for Testing of Chemicals 202, Organization for Economic Co-operation and Development Publications Service 75775 Paris Cedex 16, France

Pablos MV, Boleas S, Tarazona JV (1998) Use of Mfu-Galactoside enzymatic activity as ecotoxicological end-point on rainbow trout red blood cells. Bull Environ Contam Toxicol 61: 786-792

Parkerton TF, Stone MA, Letinski DJ (2000) Assessing the aquatic toxicity of complex hydrocarbon mixtures using solid phase microextraction. Toxicol

Lett 112-113: 273-282
Ramos C, de la Torre AI, Tarazona JV, Muñoz MJ (1996) Desarrollo de un ensayo de inhibición de *Chlorella vulgaris* utilizando un test en microplacas. Rev Toxicol 13: 97-100
Reineke N, Bester K, Huhnerfuss H, Jastorff B, Weigel S (2002) Bioassay-directed chemical analysis of river Elbe surface water including large volume extractions and high performance fractionation. Chemosphere 47: 717-723
Sanchez P (2002) Valoración ecotoxicológica de la contaminación de origen agrario. Incorporación de bioensayos en los protocolos de evaluación del riesgo ambiental. PhD thesis. Complutense University, Madrid, Spain