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# Fractionation and toxicity evaluation of waste waters

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**Abstract**

Several toxicity-based procedures have been proposed for waste water risk assessment but the toxic agents could never be identified in these very complex mixtures. A procedure was adopted using disposable solid-phase extraction cartridges to extract organic chemicals and preparative HPLC to fractionate them in relation of their hydrophobicity. Acute toxicity of whole samples and their fractions was measured on *Daphnia magna*, using a commercially available biokit. The procedure was applied to leachate from an industrial landfill and a textile effluent. In both cases the toxic effects due to xenobiotics were highest in the most hydrophobic HPLC fraction. The compounds responsible for the observed toxicity were identified and quantified by GC–MS. Reconstructed mixtures were analysed to assess their fitting with GC profiles and tested for toxicity to compare the responses of individual chemicals and mixtures. 

*Keywords:* Water analysis; Environmental analysis; Hydrophobicity; Toxicological analysis; Phthalates

## 1. Introduction

Chromatographic techniques are widely employed to determine molecular descriptors used in ecotoxicology for modelling the environmental fate of chemicals. For instance, reversed-phase liquid chromatography (RPLC) was used to determine the octanol–water partition coefficient ($K_{ow}$) [1,2], an important physicochemical parameter for defining the hydrophobicity of organic chemicals that influences their toxicity and bioaccumulation in aquatic organisms. Solute retention in RPLC is governed by partitioning of the eluate between the mobile and the stationary phase, the former (usually a mixture of water and methanol) similar to water and the latter (octylated or octadecylated silica) similar to *n*-octanol.

However, gas–liquid chromatography (GLC) and headspace coupled to GLC can be employed to estimate vapour pressure [3] and Henry's constants [4], respectively.

These are the most important physical properties governing the rate of volatilisation from surface water to the atmosphere [5] and the equilibrium partitioning of a chemical between air and water.

Global distillation and cold trap condensation is the theory proposed to explain long-range transport of persistent organic pollutants (POPs) and their trapping in cold [6] and high-altitude [7] zones. Risebrough [8], in a far-sighted paper, proposed comparing the global distribution of non-polar organic chemicals to a *global gas chromatographic system*.

Chromatographic techniques can, therefore, be considered very useful not only for extracting and separating hundreds of pollutants occurring in wastes and surface waters but also for predicting their environmental risk, simply on the basis of the

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retention times of the peaks even before they can be definitively identified. However, the prediction of toxicity to aquatic organisms based solely on hydrophobicity is unreliable in the case of polar and reactive molecules with a specific mode of action, so toxicity testing is the ultimate answer for assessing the risk associated with a chemical or a mixture of chemicals.

For these reasons coupling preparative RPLC with biotests is a very powerful screening procedure for assessing the risk of complex mixtures of chemicals dissolved in a water medium, such as waste waters and polluted surface waters [9–11]. This approach, used in the project Protocol for the Evaluation of Residues in Industrial Contaminated Effluents (PERICLES), funded by the European Commission (ENV4-CT950021), 1996–1999, could also provide indications for the most appropriate analytical technique for identifying and quantifying toxic pollutants: high-performance liquid chromatography–mass spectrometry (HPLC–MS) or GLC–MS were used, depending on the range of hydrophobicity of the fractions obtained by RPLC shown be the most toxic to the organisms selected for the biotests.

We applied this protocol to two types of waste waters, a landfill leachate and a textile effluent, and were able in both cases to identify the main chemicals responsible for toxicity.

## 2. Experimental

### 2.1. Sample extraction

Waste water samples were filtered on 0.45-μm Millipore membrane cellulose acetate filters and two 50-ml samples were passed in parallel through two LiChrolut EN (3 ml, Merck, Germany) cartridges. One of them was eluted with 5 ml of ethyl acetate and the other with methanol. A 1-ml volume of the ethyl acetate extract was stored for GC–MS analyses and the remaining 4 ml was mixed with 40 ml of *Daphnia* medium. This aqueous solution was used to prepare dilutions for ecotoxicological assays after solvent evaporation by nitrogen stripping. The methanol extract was concentrated to 1 ml and used for HPLC fractionation.

### 2.2. HPLC fractionation

Two-hundred μl samples of the concentrated methanol extract containing the organic pollutants recovered by the LiChrolut EN phase were injected into a preparative column (LiChrospher 100 Merck, RP-18, 10 μm, 244 mm×10 mm I.D.) using a Jasco liquid chromatograph Model LC900 equipped with a UV 975 variable-wavelength detector under the following conditions: $\lambda$=220 nm from 0 to 27 min, $\lambda$=245 nm from 20 to 60 min.

Methanol–water was used as eluent in a gradient from 60:40 to 70:30 in 20 min, hold for 5 min, from 70:30 to 80:20 in 5 min, hold for 22 min. The hold-up time ($t_0$) was determined as the retention time of dimethylformamide. Methanol was then evaporated from the HPLC fractions and their volume was adjusted to 50 ml with the *Daphnia* medium.

A calibration curve was calculated using reference compounds with known *n*-octanol–water partition coefficients (Table 1):

$$k' = 0.4028 + K_{ow} - 0.0212\,(\log K_{ow})^2 - 0.3222, \quad r^2 = 0.9988 \qquad (1)$$

where $k'$ is the capacity factor defined as: $k' = (t_R - t_0)/t_0$ with $t_R$ and $t_0$ the retention times of the reference compounds and dimethylformamide, respectively.

Cumulative fractions were collected from consecutive injections at the following times: fraction 1 from 4 to 6 min, fraction 2 from 5 to 7 min, fraction 3 from 7 to 12 min, fraction 4 from 12 to 21 min and fraction 5 from 21 to 52 min. The corresponding log $K_{ow}$ ranges (Table 1) can be calculated from the calibration curve equation reported above.

### 2.3. Ecotoxicological assays

A microbiotest using dormant eggs (ephippia) of the freshwater cladoceran *Daphnia magna* purchased from Creasel, Belgium, was employed to measure sample toxicity. Ephippia were treated as described in the manufacturer's procedure to induce hatching and the toxicity test was carried out on less than 24-h-old daphnids, diluting effluent fractions or pure chemicals in a medium which was a mixture of

Table 1
Capacity factors ($k'$) and *n*-octanol–water coefficient partitions of chemicals used as reference compounds (Eq. (1))

| Reference compound | Log $k'$ | Log $K_{ow}$ | Ref. |
|---|---|---|---|
| Simazine | 0.23 | 1.51 | [2] |
| 1,2,3,4-Tetrachlorobenzene | 1.05 | 4.46 | [1] |
| 2-Hydroxydesethylatrazine | −0.60 | −0.53 | Calculated from simazine according to Rekker and de Kort [12] |
| Desisopropylatrazine | −0.25 | 0.19 | Calculated from simazine according to Rekker and de Kort [12] |
| Terbutylazine | 0.65 | 2.83 | Calculated from simazine according to Rekker and de Kort [12] |

non-chlorinated tap water–mineral water (1:1) with a final hardness of 170 mg $l^{-1}$ (expressed as $CaCO_3$). Dilutions were prepared from the aqueous extract prepared as described in Sections 2.1 and 2.2. Each dilution was tested in duplicate putting 10 animals in glass beakers containing 40 ml of the test solution. Two control beakers were prepared for each series to measure spontaneous mortality.

The 24 and 48 h $IC_{50}$ (median immobilisation concentration) for *Daphnia magna* were also determined for the chemicals identified in the toxic fractions for which no reliable toxicity data were found in the literature. Exposure concentrations were analytically determined in the case of low-solubility chemicals, whose starting solutions were prepared by saturating the *Daphnia* medium with the chemical to be tested.

At least five concentrations were tested for each series of definitive acute toxicity tests. The percentage of immobilisation was recorded at 24 and 48 h using probit analysis to calculate the $IC_{50}$.

### 2.4. Analyses

LiChrolut EN organic extracts, prepared as described in Section 2.1, and aqueous HPLC fractions, extracted again with LiChrolut EN cartridges, were analysed by GC–MS with a HP 5890/HP 5971 apparatus. The chromatographic column was a Meridian MDN-5S 30 m×0.25 mm I.D. column of 0.25 μm film thickness (Supelco, Bellefonte, PA, USA). The injector was set at 280°C, used in the splitless mode, head pressure 40 kPa, with the oven programmed for a temperature gradient from 50 to 289°C. The mass spectrometer source was 160°C and the quadrupole was programmed for acquisition of masses from 40 to 500 (scan mode) after a solvent delay of 4 min.

Quantitative analyses were done using calibration curves with specific ions for the pollutants.

Exposure concentrations in the ecotoxicological assays were determined at the end of the tests.

3,5-Dichloroaniline and bis(2-ethylhexyl) phthalate were analysed by GC with electron-capture detection (ECD) after extraction with *n*-hexane. 4-Nonylphenol was analysed by reversed-phase HPLC analysis on a $C_{18}$ column (250 mm×5 mm I.D.) using fluorescence detection ($\lambda_{ex}$=230 nm, $\lambda_{em}$=290 nm), directly injecting the aqueous medium used for the test (20 μl).

## 3. Results and discussion

This work focused on the risk associated with organic micropollutants in waste waters; a previous paper considered the contribution of inorganic species and of organic macroconstituents which are polar enough to dissolve largely in the aqueous medium to a great extent [13]. However, the role of the organic micropollutants in the two effluents considered here is important: in terms of toxicity units (T.U.=the reciprocal of the 48 h $IC_{50}$ on *Daphnia magna*), the LiChrolut EN extracts contributed 58% and 62% of the initial effluent toxicity of the landfill leachate and the textile industry effluent, respectively [13]. Since the toxicity of the two types of waste water itself was high in comparison to other industrial wastes [14], we can argue that the risk associated with organic micropollutants in these wastes is considerable.

Extractable organic micropollutants are usually the

Table 2
Percentage of *Daphnia* immobilisation caused by the HPLC fractions (% of the original sample volume in parentheses)

| | Fraction | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Log $K_{ow}$ range | −0.59 to −0.28 | −0.28 to 0.52 | 0.52 to 1.7 | 1.7 to 2.81 | 2.81 to 5.16 |
| *Landfill leachate (16.7%)* | | | | | |
| 24 h immobilisation | 0 | 0 | 0 | 0 | 80 |
| 48 h immobilisation | 10 | 0 | 10 | 10 | 100 |
| *Textile effluent (20%)* | | | | | |
| 24 h immobilisation | 0 | 0 | 0 | 20 | 100 |
| 48 h immobilisation | 0 | 10 | 10 | 30 | 100 |

most persistent and toxic portion of the waste water organic component while the components not retained by solid-phase extraction (SPE) phases correspond to very polar chemicals, which are readily biodegradable and show low toxicity for aquatic organisms, unless they are member of very dangerous classes of compounds such as pesticides or other biocides, purposely synthesised to kill unwanted organisms. For these reasons the environmental risk of the organic micropollutants in an aquatic environment usually increases with hydrophobicity, which can be roughly estimated by the $K_{ow}$ value of the chemical [15,16].

The LiChrolut EN phase can recover chemicals with a wide range of $K_{ow}$ values and the further HPLC fractionation splits the components in relation to their $K_{ow}$ range. The first HPLC fractions (1, 2 and 3), with low $K_{ow}$ ranges, are amenable to HPLC–MS analysis while the more hydrophobic fractions (4 and 5) should be more suitable for GC–MS.

As the aim of ecotoxicological screening is to assign priority to the toxic components of the effluents, a knowledge of the polarity range of the chemicals to be analysed helps in selecting the most appropriate technique. In view of the limited amount of sample that could be obtained by HPLC fractionation only a few dilutions can be tested on *Daphnia magna* and the $IC_{50}$ values cannot be determined. Nevertheless, the toxicity of the five fractions obtained by HPLC can be compared, considering the highest dilution causing 100% *Daphnia* immobilisation at 48 h (Table 2).

The highest toxicity was recovered in fraction 5 for both the landfill leachate and the textile effluent including organic micropollutants with a log $K_{ow}$ ranging from 2.81 to 5.16. The upper cut-off was fixed at 5.16 because it was considered useless to prolong analytical times to collect extremely hydrophobic chemicals which are not soluble enough in water to cause acute toxicity to *Daphnia* [17].

Table 3
Landfill leachate: quantitative analysis of fraction 5

| Compound | Concentration in the original volume of sample ($\mu g\ l^{-1}$) |
|---|---|
| 2-Phenoxyethanol | 3 |
| Triphenyl phosphate | 15.2 |
| 2-Butoxyethanol acetate | 1.3 |
| 2-Methylthiobenzothiazole | 0.6 |
| Dibenzyl phthalate | 35.8 |
| Dibutyl phthalate | 204 |
| Diethyl phthalate | 1197 |
| Dimethyl phthalate | 8.6 |
| Bis(2-ethylhexyl) phthalate | 6808 |
| Benzylbutyl phthalate | 3805 |
| 3,5-Dichoroaniline | 20.72 |

Fraction 5 of the two waste waters was extracted by LiChrolut EN and the extract was analysed by GC–MS and GC–ECD. The compounds identified and quantified by these techniques are listed in Tables 3 and 4.

Then, we divided the pollutant concentrations in Tables 3 and 4 by a factor of 6 for the landfill leachate and 5 for the textile effluent to take account of the dilution at which the fractions were tested and we compared them with their 48 h $IC_{50}$ (Table 5). In this way it was possible to assess how much each chemical quantified in the fractions contributed to the

Table 4
Textile effluent: quantitative analysis of fraction 5

| Compound | Concentration in the original volume of sample ($\mu g\ l^{-1}$) |
|---|---|
| Dibutyl phthalate | 45.3 |
| Diethyl phthalate | 20.7 |
| Bis(2-ethylhexyl) phthalate | 254.1 |
| 4-Nonylphenol | 432.0 |

toxic effect on *Daphnia*. Toxicity data are taken from the literature, when available, and were determined in our laboratory if not available or reliable. Most of the toxicity data reported in the literature refers to experiments carried out without direct measurement of the exposure concentration which is however crucial in the case of very hydrophobic chemicals. These compounds are in fact difficult to dissolve in water and tend to adsorb onto glassware; the nominal concentration is, therefore, much higher than the bioavailable concentration, sometime exceeding the solubility value, and the toxicity data are usually underestimated.

Furthermore, experimental measures of the standards taken in exactly the same conditions as real samples make for greater accuracy than using only the literature values.

As the analytical concentration of one component, bis(2-ethylhexyl) phthalate in the landfill leachate and 4-nonylphenol in the textile effluent, exceeded our $IC_{50}$, these two compounds seem to be the main ones responsible for mortality. The highest tested concentrations of bis(2-ethylhexyl) phthalate (0.8 mg $l^{-1}$) and 4-nonylphenol at 0.065 mg $l^{-1}$ gave 90% and 80% mortality, respectively. It is reasonable, therefore, to suppose that 1.1 mg $l^{-1}$ of bis(2-ethylhexyl) phthalate and 0.1 mg $l^{-1}$ of 4-nonylphenol could cause 100% mortality.

The compounds identified and quantified in fraction 5 were mixed in the same proportion occurring in the initial waste water and tested for toxicity at three different dilutions (Table 6).

The reconstructed fractions are a slightly less toxic than the true fraction 5, the difference falling within the variability of this kind of ecotoxicological assay. The responses of mixtures seem to indicate, however, that the chemicals found in the two fractions have no synergistic or more than additive effects.

## 4. Conclusions

Chromatography can support ecotoxicological re-

Table 5
Exposure concentrations giving 100% immobilisation and $IC_{50}$ value (mg $l^{-1}$)

| Compound | Exposure concentration | | 48 h $IC_{50}$ | |
|---|---|---|---|---|
| | Leachate | Textile | Literature | This work |
| 3,5-Dichloroaniline | 0.0034 | | | 0.31 |
| 2-Methylbenzotiazole | 0.00008 | | | 5.2 |
| Dibutyl phthalate | 0.034 | 0.009 | 5.2 [18] | 3.9 |
| 4-Nonylphenol | | 0.086 | | 0.05 |
| Bis(2-ethylhexyl) phthalate | 1.134 | 0.051 | | 0.24 |

Table 6
Toxicity of reconstructed fractions

| Original volume (%) | Landfill leachate | | Textile effluent | |
|---|---|---|---|---|
| | Immobilised at 24 h (%) | Immobilised at 48 h (%) | Immobilised at 24 h (%) | Immobilised at 48 h (%) |
| 5 | 0 | 35 | 5 | 5 |
| 10 | 20 | 55 | 0 | 10 |
| 20 | 50 | 100 | 5 | 90 |

search and environmental sciences not only providing very useful and powerful machines and analytical techniques to identify pollutants but also applying partitioning theory developed for chromatographic processes to environmental compartment.

Toxicity identification evaluation (TIE) has long been recognised as a rapid technique for the hazard assessment of waste waters [9–11]. However, the chemicals responsible for toxicity have never been characterised, probably because the two methods have never been employed in an interactive way. This study shows that it is possible to relate ecotoxicological assays with chemical analyses, and it is not necessary to identify all the chemicals in the sample. The use of SPE and HPLC fractionation make it possible to identify a limited number of compounds likely to be responsible for the toxic effect. For organic compounds the HPLC fractionation we propose gives information on the toxicity and bioaccumulation potential of pollutants, relating them to $K_{ow}$. The use of experimental measurements of toxicity is recommended for uncertain literature data. A final proof of the identification of the pollutants responsible for toxicity comes from experiments with a reconstructed mixture corresponding to the real sample.

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