Identification of surfactant degradation products as toxic organic compounds present in sewage sludge

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A cost-effective strategy combining chemical analysis and bioassays for the identification of polar toxic compounds in sewage sludge is reported. ToxAlert® 100 bioluminescence inhibition assay was used in combination with chemical analysis involving extraction, clean-up, chromatographic separation and mass spectrometry detection. This methodology was applied to real samples of sludge from three wastewater treatment plants (WWTP) located in Catalonia (Spain) during a 3 month period. In the first step, sewage sludge was lyophilized, treated by sonication with a mixture of methanol and chloroform and finally cleaned up using a sequential solid phase extraction (SSPE) with an octadecylsilica cartridge (C_{18}) in series with a polymeric Lichrolut EN cartridge (Lic EN). In the second step, the toxicity of each fraction of the sludge sample was investigated using the ToxAlert® 100. The unequivocal identification and quantification of polar organic cytotoxic substances present in the fractionated extracts were determined by liquid chromatography-mass spectrometry (LC-MS). Major toxic compounds identified were: non-ionic polyethoxylated surfactants (nonylphenol polyethoxylates, alcohol polyethoxylates), their intermediates (polyethylene glycol polyethoxylated, nonylphenol carboxylates and polyethoxylated alcohol carboxylates), linear alkylbenzenesulfonates and heavy metals. The toxic response (in terms of bioluminescence inhibition using ToxAlert® 100), defined by the 50% effective concentration (EC_{50}), and the toxicity units (TU) for every standard non-ionic surfactant were calculated. The results provided the identification of polar cytotoxic compounds as well as the evaluation of their contribution to the total toxicity observed in sewage sludge.

Introduction

A relevant step in wastewater treatment processes is sewage sludge production. The amount of sludge produced in Europe is dependent on the number of wastewater treatment plants (WWTP). Due to the increasing number of WWTP being constructed all over Europe, it is envisaged that in 6 years' time the 6500 million tonnes of sludge currently produced will be doubled.

The sewage sludge from WWTP that receive effluents from the leather production industry is often heavily loaded with inorganic (*e.g.*, chromium derivatives) and organic constituents. A significant fraction of this group is made up of surfactants, such as non-ionic surfactants [nonylphenol ethoxylates (NPEO) and alcohol ethoxylates (AEO)], which are currently used to remove skin grease in the tannery industry.

Industrial and urban wastewaters contain a complex mixture of organic pollutants, mainly alcohol and alkylphenol ethoxylates and linear alkylbenzenesulfonate (LAS).[1] Most of these analytes, such as LAS, are efficiently removed in WWTP.[1] However, NPEO are degraded during water treatment, and their degradation products, such as nonylphenol carboxylic acids (NPEC) and nonylphenol (NP), are formed. NP is primarily used in the production of this type of non-ionic surfactant and also in the production of lubricants, resins and antioxidants. Because of the common usage and the degradation of NPEO to NP, this compound often enters the aquatic environment *via* industrial and municipal wastewater effluents.

The use of NPEO is being restricted in some countries due to the toxicity of its biodegradation products,[2] such as NP. In addition, NP, NPEC and other related compounds, such as octylphenol (OP), have been related to endocrine-disrupting effects.[3,4]

Although chemical analysis allows the identification and quantification of several chemicals, it does not provide sufficient information to assess the ecological risk caused by the reuse of sewage sludge. In this respect, the use of bioassays to evaluate the toxicity of wastes is strongly recommended.[5–8] However, few studies have examined the relationship between chemical analysis and toxicity, and most of these case studies have been concerned with inorganic substances, such as metals,[9,10] and non-polar organic compounds.[8,10,11] To our knowledge, this is the first time that a combined methodology has been applied to sewage sludge samples for the unequivocal identification and toxicity evaluation of polar organic toxic compounds. This work follows a previous paper from our group using a similar combined approach for wastewaters.[12]

Various biological assays may be used for this purpose[13] but, due to the ease of operation, reproducibility, sensitivity,[14,15] cost and standardization,[16] the toxicity study carried out in the present work was based on the bioluminescence inhibition of *Vibrio fischeri*, using the new ToxAlert® 100 developed by Merck. This system is based on the well-known Microtox®, which was the first commercial toxicity device based on the bioluminescence inhibition of *Photobacterium phosphoreus*. The sewage sludge samples evaluated for toxicity in the present study were analysed by sequential solid phase extraction-liquid chromatography-mass spectrometry (SSPE-LC-MS) in previous work by our group.[17]

The objectives of the present work were: (i) to assess the potential toxicity of sewage sludge from three different WWTP located in Catalonia (north-east of Spain) receiving domestic and industrial wastewater; and (ii) to combine chemical analysis by LC-MS with the ToxAlert® 100 to identify and quantify the toxicity caused by surfactants and their degradation products in sewage sludge.

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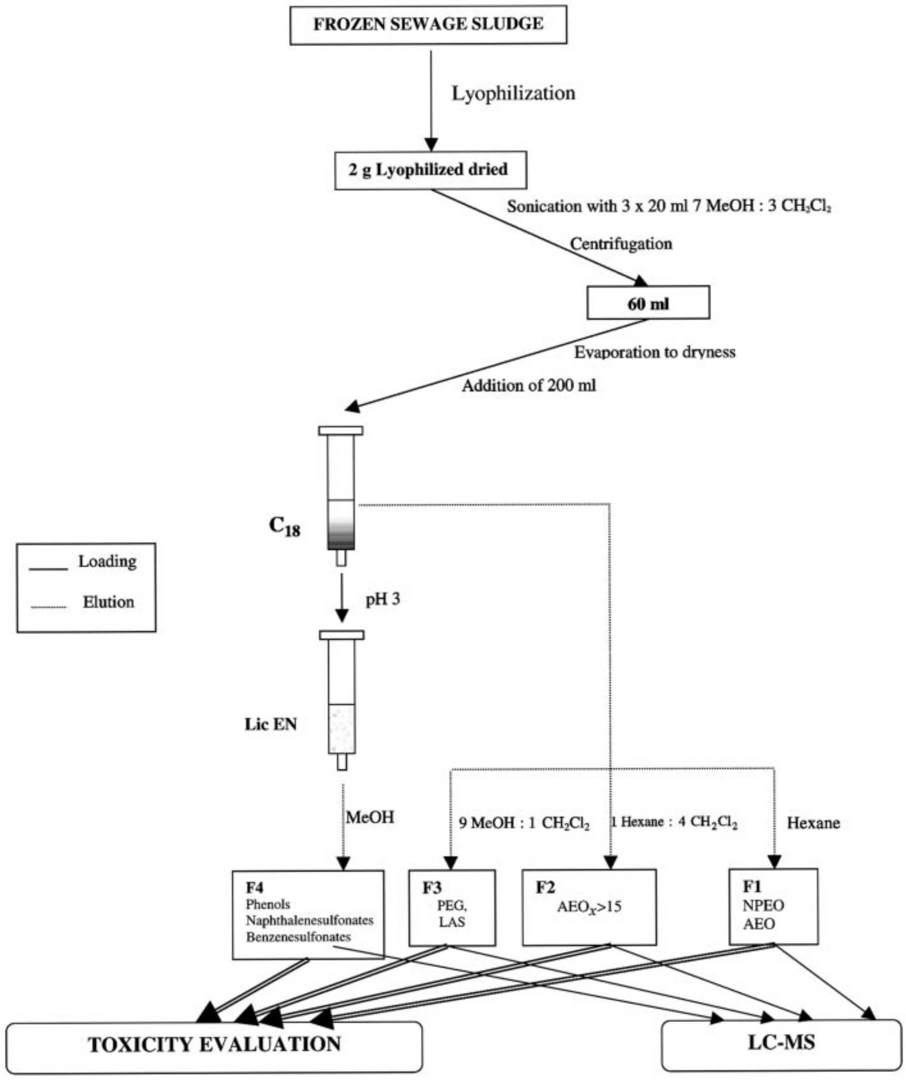


Fig. 1 General scheme of the combined analytical–toxicological procedure used in this work.

The present study aims to identify toxic compounds using an integrated analysis combining different procedures: fractionation steps, chemical analysis and toxicity evaluation.

Materials and methods

Chemicals and reagents

Liquid-dried photobacteria reagents *Vibrio fischeri* NRRL B-111 77 were purchased from Merck (Darmstadt, Germany). NaCl was of analytical reagent grade and was from Panreac (Barcelona, Spain). LC solvents were obtained from Merck, and acetic and sulfuric acid pro-analysis grade were from Panreac and Merck, respectively. The lack of standard test substances for some of the retrieved compounds should be pointed out. All the individual polyethoxylated surfactants [polyethylene glycols (PEG), nonylphenol (NP), nonylphenol polyethoxylates ($NPEO_x$) and alkyl alcohol polyethoxylates (C_nEO_x)], corresponding to a mixture with an average number of ethoxy units (x), were from Kao Corporation (Barcelona, Spain). Phenols were purchased from Merck.

Solid phase extraction (SPE) sorbents were octadecylsilica (C_{18}) obtained from Merck and polymeric Isolute ENV+ from IST (Cambridge, UK). Commercial linear alkylbenzenesulfonates (LAS) were supplied by Petroquímica Española S.A. (Cadiz, Spain) in a single mixture standard (not single standard solutions of each LAS). The proportional composition of the different homologues in the standard mixture is: C_{10} (3.9%), C_{11} (37.4%), C_{12} (35.4%), C_{13} (23.1%), C_{14} (0.2%).

Sewage treatment plants and sample collection

The sludge samples were collected from three WWTP located in Catalonia (Spain). One of these, Igualada-WWTP, receives influents with 20% of the total flow from the tannery industry and the rest urban waste. Manresa-WWTP receives only urban wastewater. These two plants consisted of a primary settlement process, followed by biological sewage treatment. The third plant, Montornes-WWTP (receiving 60% industrial effluent from different types of industry and the rest urban wastewater), consisted of a primary settlement process involving only physicochemical treatment.

The sampling was carried out during a 3 month pilot survey undertaken between May 1998 and July 1998 with monthly sampling.

Sludge was collected and immediately frozen and lyophilized for preservation.

Extraction and fractionation procedure

The method used for the extraction and clean-up of non-ionic surfactants and their degradation products was presented in a previous paper by Castillo *et al.*[17] Briefly, it involved the extraction (three times) of target compounds from 2 g of lyophilized sludge by sonication for 20 min with a mixture of methanol and CH_2Cl_2 (7 : 3) (3 × 20 ml). The extracts were

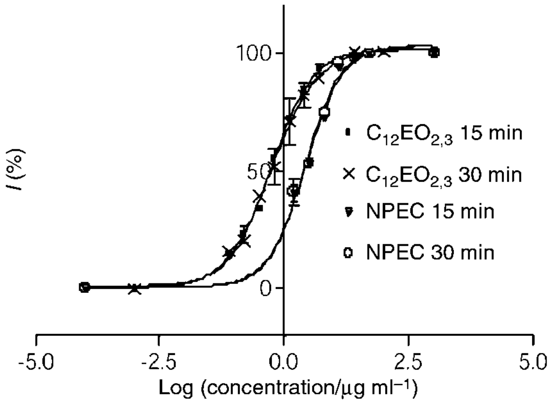


Fig. 2 Bioluminescence inhibition curves for two standard substances with exposure times of 15 min and 30 min. The curves show the percentage inhibition, *I*(%), *vs.* the logarithm of the concentration (µg ml^{-1}).

separated from the sediment by means of a centrifugation step. The three extracts were combined, evaporated to dryness and redissolved with 200 ml of HPLC water. The clean-up was performed by applying an SSPE procedure[18] based on the use of an octadecylsilica (C_{18}) sorbent from Merck in series with a polymeric Isolute ENV+ from IST. Medium polarity organic compounds were preconcentrated in the C_{18} cartridge. Differential elution was applied to this cartridge in order to obtain three fractionated extracts.

The eluent systems utilized for the stepwise desorption were as follows:[17] 2×5 ml of hexane for fraction F1 ($NPEO_x$, C_nEO); 2×5 ml of dichloromethane–hexane (4 : 1, v/v) for fraction F2 ($AEO_x < 15$); and 2×5 ml of methanol–dichloromethane (9 : 1, v/v) for fraction F3 (PEG, LAS, pentachlorophenol). The most polar phenols, benzenesulfonates (BS) and naphthalenesulfonates (NPS), in the acidified water residue were trapped by polymeric sorbent Isolute ENV+ and eluted with methanol to obtain fraction F4. Finally, total evaporation of the extracts was carried out with a gentle stream of nitrogen with reconstitution of the extracts to 1 ml with methanol. The general procedure used in the present work is shown in Fig. 1.

Cytotoxicity methodologies

Some micro-organisms belonging to the *Photobacterium* genus or *Vibrio* genus naturally emit light.[19] Bacteria with this property are known as luminescent bacteria and, in general, are marine species, such as *Vibrio fischeri*. The enzyme involved is bacterial luciferase which catalyses the following reaction:

$$FMNH_2 + O_2 + R - CO - H$$
$$\rightarrow FMN + R - COOH + H_2O + \text{light}$$

Bioluminescence is directly proportional to the metabolic status of the cell. Toxic substances cause changes to the cellular state and these changes are rapidly reflected in a decrease in bioluminescence.

In the present work, the determination of toxicity was performed by means of a ToxAlert®100 system from Merck, which uses *Vibrio fischeri*.

All standards and sample solutions were adjusted to 2% NaCl for optimal reagent performance. The reconstitution time for the dried bacterial reagent was 15 min. Briefly, the following protocol was used. After a pre-incubation time of 15 min, the different diluted extracts and standards and the control (pure sodium chloride solution) were added to the corresponding vials. Immediately after this addition, the bioluminescence of each vial was measured. The vials were incubated at 15 °C for a fixed time. After this period of incubation, the bioluminescence of each vial was measured again. Finally, thanks to the software incorporated into the device, the percentage of inhibition for each vial was obtained.

In all cases, the percentage inhibition [*I* (%)] was determined by comparing the response given by a saline control solution with that corresponding to the diluted extract or diluted standard. Therefore, the bioluminescence inhibition is:

$$I(\%) = [1 - (\text{dilution light}/\text{control light})] \times 100$$

The inhibition data obtained over a sufficiently wide range of concentration for a standard substance or a sample can be fitted to a sigmoidal inhibition curve, and the 50% effective concentration (EC_{50}) can be calculated for a standard

Table 1 Bioluminescence inhibition values (50% effective concentration, EC_{50}, toxicity unit, TU_s, and goodness of fit of the adjusted inhibition curve, R^2) for standard substances using ToxAlert®100

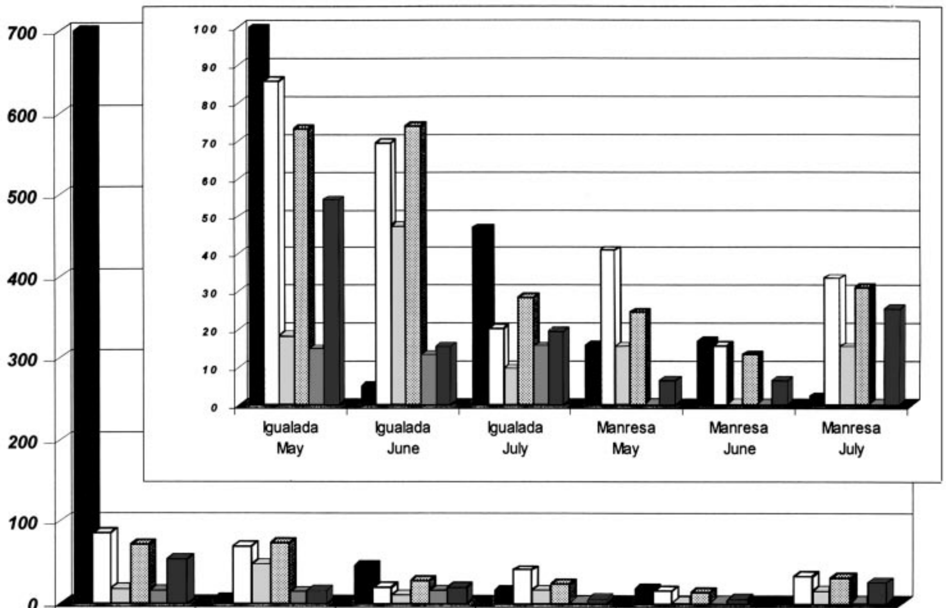
		ToxAlert®100	
Substance	EC_{50}/µg ml^{-1}	TU_s	R^2
$C_{10}EO_6$	3.25	0.307	0.997
$C_{12}EO_2$	0.55	1.82	0.991
PEG4	127.40	0.0078	0.995
Nonylphenol	0.36[a]	[a]	[a]
NPEO	2.70	0.370	0.982
NPEC	2.64	0.379	0.999
LAS	144	0.0069	0.997
Phenol	7.99	0.125	0.999
4-Chlorophenol	21.21	0.0471	0.996
2-Chlorophenol	34.82	0.0287	0.987
2,4-Dichlorophenol	2.85	0.3509	0.993
Pentachlorophenol	1.07	0.935	0.988

[a]Regression did not converge.

Table 2 Bioluminescence inhibition values (50% effective concentration, EC_{50}, toxicity impact index, TII_{50}, and goodness of fit of the curve, R^2) for the four fractions of each sludge sample

WWTP		Fraction	EC_{50}	TII_{50}	R^2
Igualada	May	F1	18.36	5.4466	0.998
		F2	804	0.1244	0.993
		F3	17707	0.0056	0.995
		F4	1356	0.0737	0.999
	June	F1	64.81	1.5430	0.999
		F2	1365	0.0733	0.995
		F3	8877	0.0113	0.998
		F4	20093	0.0005	0.998
	July	F1	50.85	1.9666	0.999
		F2	10472	0.0095	0.996
		F3	17044	0.0059	0.999
		F4	10617	0.0094	0.986
Manresa	May	F1	156	0.6410	0.990
		F2	Nq[a]	Nq	Nq
		F3	88037	0.0011	0.990
		F4	5925	0.0169	0.952
	June	F1	155	0.6452	0.993
		F2	359	0.2786	0.998
		F3	658	0.1520	0.991
		F4	3061	0.0327	0.998
	July	F1	39	2.5641	0.998
		F2	10433	0.0096	0.984
		F3	21511	0.0046	0.999
		F4	2778	0.0360	0.999
Montornes	May	F1	30	3.3333	0.998
		F2	9899	0.0101	0.986
		F3	Nq	Nq	Nq
		F4	Nq	Nq	Nq
	June	F1	50	2.0000	0.999
		F2	13426	0.0074	0.998
		F3	Nq	Nq	Nq
		F4	26402	0.0038	0.997

[a]Nq, not quantified.



	Igualada May	Igualada June	Igualada July	Manresa May	Manresa June	Manresa July
■ Total LAS / mg kg^{-1}	705	5	47	16	17	2.3
□ NPEO/mg kg^{-1}	86.1	69.5	20.4	41	15.8	33.6
□ NPEC / mg kg^{-1}	18.6	47.7	9.5	15.5	0	15.1
▨ NP / mg kg^{-1}	73.3	74.2	28.5	24.6	13.5	31.3
▨ Total Cr sludge content/ g Cr(V) kg^{-1}	14.9	13.2	15.7	0.49	0.39	0
■ TII50 F1 x 10	54	15.43	20	6.41	6	26

Fig. 3 Results of the chemical analysis, performed by SSPE-LC-APCI-MS, of the linear alkylbenzenesulfonates (LAS), nonylphenol polyethoxylates (NPEO), nonylphenol carboxylates (NPEC) and nonylphenol (NP). Total LAS is the sum of the different homologues, C_nLAS, for $n=10$–13, where n is the number of atoms of carbon in the linear chain. The toxicity impact index (TII_{50}) evaluated for the organic extract F1 and the total chromium content in each sludge are also indicated.

substance or a sample. The EC_{50} is the amount of a substance or a sample that produces 50% bioluminescence inhibition.

Together with the EC_{50} values, in the present work, the toxicity unit (TU) values were calculated for a more convenient graphical expression and interpretation of the toxicity data; the values of EC_{50} were converted to TU according to the formula of Sprague and Ramsay:[20]

$$TU=(EC_{50})^{-1}\times 100$$

This expression is the dilution factor, which must be applied to obtain a 50% effect, and is directly proportional to the toxicity.

For more toxic substances, the larger the bioluminescence inhibition and the larger the TU.

According to the previous formula, the toxicity unit for a standard substance, TU_s, is defined as:

$$TU_s=(EC_{50})^{-1}\times 100$$

The toxicity unit (TU_i) due to one substance (whose concentration is known in a sample) is calculated using:

$$TU_i=(\text{concentration of a substance in the sample}/EC_{50}\text{ of this standard substance})\times TU_s$$

From the EC_{50} of a sample, a new parameter, defined as the toxicity impact index (TII_{50}), can be calculated:

$$TII_{50}=(EC_{50})^{-1}\times 100$$

TII_{50} allows a comparison of the toxic impact of different sewage sludges or wastewaters without previous knowledge of their chemical analysis components.

Although TII_{50} and TU are calculated in a similar way, they are different parameters and cannot be mixed up, because TU is related to an amount of a known substance and TII_{50} is related to an amount of a complex mixture whose composition is unknown.

In the present work, the most commonly found polar organic substances detected by our group in different wastewaters and sludges were studied.[12,17,21] For these substances (working in duplicate), the inhibition values were determined at different concentrations over an adequate concentration range to fit an inhibition curve, and the EC_{50} and TU_s values were calculated. The bioluminescence inhibition values were measured after two incubation times, 15 and 30 min, but no significant differences were obtained (see Fig. 2). The results presented in this work were measured after an incubation time of 30 min. The EC_{50} and TU_s values are expressed in μg ml^{-1} and ml μg$^{-1}\times 100$, respectively. These values and the goodness of fit, expressed as R^2, are shown in Table 1.

The toxicity assessment of the sludge samples was performed over the four extracts obtained for each sample according to the extraction procedure described above. Each extract was studied over a sufficient range of concentration to fit an inhibition curve (working in duplicate); the EC_{50} and TII_{50} were calculated. The EC_{50}, TII_{50} and R^2 values are shown in Table 2.

LC-MS analysis

A VG Platform from Micromass (Manchester, UK), equipped with a standard atmospheric pressure ionization (API) source, which could be configured for atmospheric pressure chemical ionization (APCI) or pneumatically assisted electrospray ionization (ESI), was used for LC-MS analysis. The LC

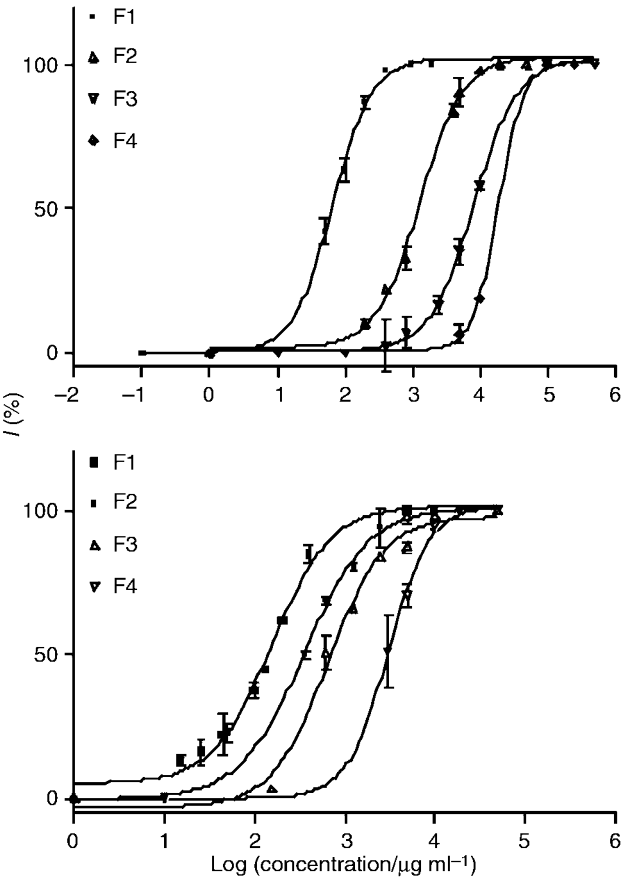


Fig. 4 Bioluminescence inhibition curves for the four organic extracts obtained for one of the most toxic samples, Igualada-June (top), and for the least toxic sample, Manresa-June (bottom). The curves show the percentage inhibition, I(%), *vs.* the logarithm of the concentration (µg ml^{-1}).

solvent was delivered by a Waters 616 gradient pump system controlled by a Waters 600 S controller from Waters-Millipore (USA). For APCI experiments, source and probe temperatures were set at 150 and 400 °C, respectively, the corona discharge voltage was maintained at 3 kV and the cone voltage was set at 30 V. The high voltage lens voltage was set at 0.20 kV. For ESI experiments, a voltage of 3.7 kV was applied to the needle tip. Nitrogen was used as nebulizing and drying gas at flow rates of 10 and 300 l h^{-1}, respectively. In full scan mode, the *m*/*z* range was from 70 to 350 in negative ion (NI) mode and from 100 to 1000 in positive ion (PI) mode.

Anionic compounds were separated by means of a Hypersil BDS C_{18} analytical column from Shandon (Cheshire, UK) of 250 mm length and 2 mm internal diameter, with a particle size of 5 µm, using water (B) and 80% acetonitrile–20% water (A) as mobile phases, both containing 5 mM triethylamine and 5 mM acetic acid. The elution gradient for the separation of LAS started with 100% B and was linearly decreased to 0% B over 20 min. The flow rate of the mobile phase was 0.3 ml min^{-1}. In the case of BS and NPS, the mobile phase composition was 100% B during the first 5 min, linearly decreased to 95% B over 3 min and kept isocratic for 10 min, decreased to 75% B over 17 min and continuously decreased to 60%, 30% and 0% B over 45, 50 and 55 min, respectively. The flow rate of the mobile phase was 0.8 ml min^{-1} and a post-column addition of 0.2 ml min^{-1} of methanol was necessary.

The most relevant results of the chemical analysis performed by SSPE-LC-APCI-MS are shown in Fig. 3.

Results and discussion

ToxAlert®100, based on the bioluminescence inhibition of *V. fischeri*, has been used to establish the EC_{50} and TU_s values of commonly detected compounds in industrial wastes. From the results shown in Table 1, it can be seen that the most toxic compounds studied are $C_{12}EO_2$, NP and pentachlorophenol. Nevertheless, NP formed turbid solutions at concentrations above 0.45 µg ml^{-1} and, for this reason, the I (%) values of the upward portion of the fitted sigmoidal curve are overestimated. The same problem was observed for LAS, although the solution measured up to inhibition values of around 80% was clear.

In general, the positive signal obtained using bioassays is an advantage, because it could be used as an alarm to detect the presence of these compounds at lower concentration levels during screening.

Table 2 shows the inhibition values obtained for each extract of every sample. The EC_{50} values are expressed in µg of sludge (ml of saline solution)$^{-1}$. From each EC_{50}, the TII_{50} values were calculated. For all cases, F1 was the most toxic fraction. This extract corresponds to NPEO and its degradation products NPEC and NP. Fraction F2 corresponds to the second more toxic extract and the toxicity in this case can be attributed to the presence of AEO compounds. Fraction F3 contains PEG, LAS and the less polar phenols. The acute toxicity of PEG and LAS is low according to the results in Table 1, and therefore the maximum contribution to the toxicity in this fraction can be assigned to phenols such as pentachlorophenol. Fraction F4 corresponds to the extract containing more polar phenols, BS and NPS. Since the acute toxicity for sulfonic acids is low,[22] the greater part of the toxicity of this fraction is attributed to phenols.

In general, a comparison of the toxicities produced by non-ionic surfactants in different WWTP shows that samples from plants that receive industrial influents, such as Igualada, are more toxic than samples from plants that receive only urban wastewater, such as Manresa. However, fraction F1 for the sample Manresa-July showed a high toxicity.

The most toxic sample analysed was Igualada-May. The Igualada-WWTP receives wastes from the tannery industry, which uses NPEO for removing skin grease. The least toxic sample was Manresa-June. The inhibition curves for the four extracts of Igualada-June (one of the most toxic samples) and of Manresa-June (the least toxic sample) are shown in Fig. 4.

The toxicity values reported in Table 2 refer to a mixture of substances and, in spite of the clean-up, the tested extracts have a high content of unknown substances. Part of the measured effect is due to synergistic and antagonistic influences[12] between the different substances present in the organic extracts. Therefore, unidentified compounds in complex mixtures may have certain toxic effects that cannot be explained. The percentage of total organic carbon (TOC) identified is less than 10%, and therefore more than 90% of TOC corresponds to unidentified chemicals.

From the data depicted in Table 2 and Fig. 3, it can be observed that the most toxic extracts were those corresponding to the less polar fractions for all samples, such as NPEO and its degradation products NPEC and NP. Another important analyte present at high concentration is LAS. Although these chemical species are easily degraded, their use is being increased and their presence is relevant in sewage sludge. During the SSPE, LAS is concentrated in F3, which is less toxic compared with F1 (50–1000 times less toxic).

Fig. 3 shows the chromium content for sludges from Igualada and Manresa. Sludges from Igualada had a much higher chromium content than those from Manresa. The EC_{50} values (expressed in µg ml^{-1}) obtained for chromium are 13 and 0.280 and the TU values are 0.077 and 3.534 using the test organisms *Photobacterium phosphoreum* and *Daphnia magna*, respectively.[23] In general, in industrial processes, salts of Cr(VI) are used as oxidizers; therefore after leather treatment and in the presence of a large amount of organic material, the major chromium content in the sludge will be Cr(III). The pH of the

Igualada sewage sludge was around 7.4; therefore most of the chromium would be $Cr(OH)_3 \cdot nH_2O\downarrow$, according to the Pourbaix diagram.[24] Thus the majority of chromium contained in the sludge is removed during the clean-up step and cannot substantially contribute to the final toxicity of the organic extracts.

Conclusions

A combined procedure consisting of chemical and biological methods was used for the isolation, identification and quantification of polar organic toxic substances in sewage sludge. The chemical methodology employed in the present work is based on the use of SSPE followed by LC-MS. For each organic extract, toxicity evaluation was carried out using the bioluminescence inhibition of *Vibrio fischeri* with Tox-Alert®100. This system is based on a standardized organism, is rapid and can be performed with a large number of dilutions at the same time.

For the studied samples, NPEO, AEO and their degradation products were identified as the major polar organic toxic substances present. The new protocol presented here should be of help for the implementation of the new Draft European Union Directive on the use of sewage sludge for soil amendment. In the Draft Directive, the levels of NPEO and LAS should be monitored before the sludge can be used for soil amendment.

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References

1 E. Matthijs, M. S. Holt, A. Kiewiet and G. B. J. Rijs, *Environ. Toxicol. Chem.*, 1999, **18**, 2634.
2 K. B. Sherrard, P. J. Marriott and M. J. McCormick, *Anal. Chem.*, 1994, **66**, 3394.
3 C. M. Lie, C. L. J. Frid, M. E. Gill, D. W. Cooper and D. M. Jones, *Environ. Sci. Technol.*, 1999, **33**, 1009.
4 J. E. Harries, S. Jobling, J. P. Sumpter, C. R. Tyler, A. Janbakhsh and P. Matthiessen, *Environ. Toxicol. Chem.*, 1999, **18**, 932.
5 R. Van Coillie, N. Bermingham, C. Blaise and R. Vezeau, *Aquatic Toxicology and Water Quality Management*, Wiley, New York, 1989, pp. 161–191.
6 B. Ferrari, C. M. Radetski, A.-M. Veber and J.-F. Ferard, *Environ. Toxicol. Chem.*, 1999, **18**, 1195.
7 P. G.-J. De Maagd, *Environ. Toxicol. Chem.*, 2000, **1**, 25.
8 G. D. Sayles, C. H. Acheson, M. J. Kupperle, Y. Shan, Q. Shou, J. R. Meier, L. Chang and R. C. Brenner, *Environ. Sci. Technol.*, 1999, **33**, 4310.
9 S. P. McGrath, B. Knight, K. Killham, S. Preston and G. I. Ipaton, *Environ. Toxicol. Chem.*, 1999, **18**, 659.
10 M. Lewis, D. Weber, R. Stanley and B. Albrecht, *Environ. Toxicol. Chem.*, 2000, **19**, 192.
11 N. Klinkow, J. Oleksy-Frenzel and M. Jekel, *Water Res.*, 1998, **32**, 2583.
12 M. Castillo and D. Barceló, *Anal. Chem.*, 1999, **71**, 3769.
13 I. E. Tothill and A. Turner, *Trends Anal. Chem.*, 1996, **15**, 178.
14 J. O. Lambertson, T. H. Dewitt and C. Swartz, in *Sediment Toxicity Assessment*, ed. G. A. Burton, Lewis, Boca Raton, FL, 1992, pp. 183–211.
15 M. Salizzato, V. Bertato, B. Pavoni, A. Volpi Ghirardini and P.-F. Ghetti, *Environ. Toxicol. Chem.*, 1998, **17**, 655.
16 M. W. Toussaint, T. R. Shedd, W. H. Van der Schailie and G. R. Leather, *Environ. Toxicol. Chem.*, 1995, **14**, 907.
17 M. Castillo, E. Martínez, A. Ginebreda, Ll. Tirapu and D. Barceló, *Analyst*, 2000, **125**, 1733.
18 M. Castillo, F. Ventura and D. Barceló, *Waste Manage.*, 1999, **19**, 101.
19 T. Brock, *Biology of Microorganisms*, Prentice-Hall, London, 8th edn., 1997, pp. 705–707.
20 J. B. Sprague and A. Ramsay, *J. Fish. Res. Board Can.*, 1965, **22**, 475.
21 M. Castillo, M. C. Alonso, J. Riu and D. Barceló, *Environ. Sci. Technol.*, 1999, **33**, 1300.
22 H. Greim, J. Ahlers, R. Bias, B. Broecker, H. Hollander, H.-P. Gelbke, H.-J. Klimisch, I. Mangelsdorf, A. Paetz, N. Schön, G. Stropp, R. Vogel, C. Weber, K. Ziegler-Skylakakis and E. Bayer, *Chemosphere*, 1994, **28**, 2203.
23 I. Johnson and A. Boxall, 2000, personal communication.
24 M. Pourbaix, *Atlas of Electrochemical Equilibrium in Aqueous Solutions*, Pergamon Press, Oxford, 1966.