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Guidelines for alkylphenols estimation as alkylphenol polyethoxylates pollution indicator in wastewater treatment plant effluents

Y. Moliner-Martínez,[a] J. M. Pastor-Carbonell,[b] A. Bouzas,[b] A. Seco,[b] M. R. Abargues*[b] and P. Campíns-Falcó[a]

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A solid-phase microextraction coupled with a gas chromatography-mass spectrometry method has been developed for the estimation of technical nonylphenol in the presence of other endocrine disruptors, belonging to different chemical families. The profile of the technical nonylphenol found in real samples was tested, and, given that it was similar to that provided for the standard used, reliable results were obtainable. Endocrine disruptors such as 4-*n*-nonylphenol, bisphenol A and 4-*tert*-octylphenol were simultaneously analysed. The best conditions achieved enabled the analysis of all analytes using a sample volume of 15 mL or even only 300 μL. Using such a low sample volume reduced the filtration process, which, in turn, significantly reduced the analysis time. The limits of detection achieved ranged between 0.006 and 0.5 μg L^{-1}. Wastewater samples collected in three sampling campaigns (2006, 2007 and 2008) revealed an important presence of 4-*tert*-octylphenol and technical nonylphenol in several sampling points especially during the summer season. This finding is indicative of alkylphenol polyethoxylates pollution.

Introduction

The wide use of manufactured products has led to the appearance of several micropollutants, such as surfactants, pesticides, plastic reinforcements and hormone disruptors, in natural environments. Some of these chemicals are known as endocrine disruptor chemicals (EDCs), which are able to disrupt the endocrine system. The European scientific and regulatory community has agreed on the following definition of EDC: "an endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function".[1]

The consequences of exposure of wildlife to these substances, especially aquatic wildlife[2–7] or babies,[8] are harmful, given that these micropollutants are persistent and toxic and could accumulate in aquatic organisms.[9] European and international environmental authorities and organizations have included EDCs within the priority substances to be controlled, so as to protect wildlife health,[10–13] despite no concentration limit being set for most EDCs.

Wastewater treatment plants (WWTPs) can remove EDCs from the environment,[14,15] although the removal could be incomplete in that EDCs could reach continental waters and cause harmful effects on aquatic wildlife. EDCs are a global concern due to their widespread occurrence, persistence, bioaccumulation and potential adverse effects on ecosystem functioning and human health.[9] Different kinds of substances can actually be included within the term 'endocrine disruptors'.[11,16–21] Some of them, such as technical nonylphenol (t-NP), 4-*tert*-octylphenol (OP) or bisphenol A (BPA), have an anthropogenic origin and come from industrial activities.

Alkylphenol polyethoxylates (APEOs) are a group of non-ionic surfactants found in detergents, paints, herbicides, pesticides and plastic surfactants and have important industrial and domestic applications. Nonylphenol polyethoxylates (NPEOs) are about 80% of APEOs, and octylphenol polyethoxylates (OPEOs) are the remaining 20%. This represents 500 thousand tons of APEOs annually produced worldwide.[22] APEOs can be degraded during the wastewater treatment process to alkylphenols: OP and t-NP. These alkylphenols are more toxic and lipophilic than APEOs. The estrogenic activity observed for alkylphenols appeared to be confined to *para*-substituted compounds.[23] The mentioned estrogenic activity becomes stronger with the increase in the number of the alkyl carbons. This activity is maximized with a nonyl-chain, whereas phenol shows weak estrogenic activity.[24] t-NP (branched 4-nonylphenol) is a mixture of different branched nonylphenols,[25,26] and together with OP they are degradation products of APEOs, NPEOs and OPEOs respectively.[27,28] However

[a]*Departament de Química Analítica, Facultat de Química, Universitat de València, C/ Dr Moliner 50, 46100 Burjassot, Valencia, Spain*

[b]*Departament d'Enginyeria Química, Escola Tècnica Superior d'Enginyeria, Universitat de València, Avinguda de la Universitat s/n., 46100 Burjassot, Valencia, Spain. E-mail: Miguel.Abargues@uv.es; Fax: +34 96 3544898; Tel: +34 96 3543927*

4-*n*-nonylphenol (4-NP) is not a metabolite of APEOs, thus its occurrence in the environment is infrequent.[29,30] Other authors identified the t-NP toxicity in 1984.[31]

Solid-phase microextraction (SPME), which was initially developed in 1989 by Pawliszyn,[32,33] is a simple, low cost and solvent free technique. Gas chromatography-mass spectrometry (GC/MS) was used as the analysis technique in most of these studies,[34–36] showing low limits of detection (LODs) required to detect the usual low EDC concentrations in water. Despite some studies on SPME of EDCs that have been published so far,[20,21,37–43] none of them seem to have dealt with the nature of the source of EDCs as previously described.

The aim of the Directive 2000/60/EC[44] is to achieve and ensure a good quality for surface, groundwater, transitional and coastal waters. Moreover, Directive 2008/105/EC[13] lays down environmental quality standards (EQS). 4-NP and OP are legislated as priority substances in Annex II of the European Directive 2008/105/EC, which delves on APEO pollution. t-NP (CAS number 84852-15-3) and 4-NP (CAS number 104-40-5) are both considered to be priority compounds, although only EQS for 4-NP were included in the above mentioned Directive. t-NP was added to the list of priority substances in the Directive 2011/0429,[45] which also established EQS for this substance.

The study area is focused on the eastern Spanish coast, in particular in the region of Comunitat Valenciana, in which a wastewater treatment plant network, for a total of 28 sample points, was selected in three sampling campaigns held in 2006, 2007 and 2008. For the purpose of this investigation, a screening procedure was developed in order to test the presence of t-NP and OP as well as to measure the occurrence of APEOs, 4-NP, and BPA, which present oestrogenic activity due to *para*-substituted compounds.[23] Table 1 details parameters such as chemical structures, octanol–water partition coefficients (log K_{ow}), ion monitoring and identification and retention time (t_r).

The characteristics of t-NP peaks, such as height, width and number of peaks, in real water samples were also studied in order to test the usefulness of the standard used.

Research

Chemicals and reagents

Analytical reagent grade chemicals were used in this study. Sulphuric acid (≥95%) was purchased from VWR International (Fountenay sous Bois, France). Sodium hydroxide was purchased from Panreac (Barcelona, Spain) and methanol was purchased from Merck (Darmstadt, Germany).

The micropollutants technical nonylphenol (t-NP) (Pestanal, purity grade 94%) and 4-*n*-nonylphenol (4-NP) (>99.9%) studied were supplied by Riedel-de Haën (Seelze, Germany); bisphenol A (BPA) (>97%) and 4-*tert*-octylphenol (OP) (>97%) were purchased from Sigma-Aldrich (Steinheim, Germany). Pure water with a resistance of 18.0 MΩ was obtained by means of a Milli-Q water purification system (Millipore, Bedford, MA, USA). Helium (>99.9992%) was purchased from Carburos Metálicos (Barcelona, Spain).

Stock standard solutions were prepared in methanol with a concentration up to 1000 mg L^{-1}. More dilute solutions were prepared from stock solutions directly in pure water with a maximum concentration of 1 mg L^{-1} and were stored in darkness at 4 °C until use. The conservation period of solutions was six months.

Apparatus

The measurements were performed using a gas chromatography-mass spectrometry system (GC/MS) using 6890N GC with 5973 inert MS Detector (Agilent, Palo Alto, CA, USA) fitted with a split/splitless injection port and operated by MSD Chemstation Software (Agilent, Palo Alto, CA, USA). The capillary column was a fused-silica HP-5 MS (30.0 m × 250 μm I.D., 0.25 μm film thickness; Agilent Technologies, San José, USA.). The liner was a splitless inlet, single-taper, glass-wool, deactivated liner (Agilent Technologies, San José, USA).

The GC/MS was operated in splitless mode and the injection port temperature was held isothermally at 280 °C. The transfer line and the ion source were held at 280 °C and 250 °C, respectively. The MS worked in Selected-Ion-Monitoring (SIM) mode and the electron impact energy was set to 69.9 eV. Helium was used as carrier gas at a constant flow rate of 1.0 mL min^{-1}. The GC column temperature program involved an initial oven temperature set at 50 °C, held for 1 min; 30 °C min^{-1} to 140 °C, held for 1 min; 20 °C min^{-1} to 280 °C, held for 4 min, for a total run time of 16 min.

In order to establish the target ions and their relative abundance for micropollutants, the GC/MS was carried out in full scan mode (scan range from 100 to 300 *m*/*z*); the quantification and characteristic ions are shown in Table 1. Sample analyses were conducted in selected ion monitoring and identification mode. The analytical signals used were the peak area of monitoring ion for each EDC. Moreover, the mixture of t-NP, which is composed of 22 *para*-substituted isomers, does not produce only one peak. This phenomenon was studied and it was concluded that all peaks between 9.17 and 9.55 min correspond with the t-NP mixture, so these peaks were used as analytical signals.[46,47]

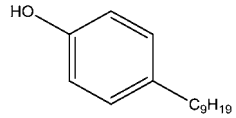
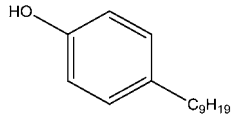
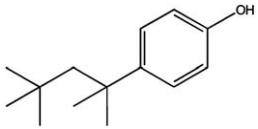
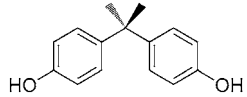
Sampling

The sampling points studied were WWTP effluents located along the coastal region of Comunitat Valenciana (located at the eastern Mediterranean Spanish coast[9]). The sampling campaigns were carried out in 2006 (March, July and November), 2007 (June and September) and 2008 (March, July and November). The studied WWTPs treated urban, industrial and mixed wastewaters. The wastewater samples were collected in 250 mL brown glass bottles (labbox labware S.L., Barcelona, Spain), which were submerged and completely filled, with no bubbles. In order to avoid cross-contamination in the sampling, step bottles were cleaned using a nitric acid bath (10% (v/v)) for 48 h and finally rinsed with ultrapure water after use. The samples were filtered and stored at 4 °C and protected from light until their analysis.

SPME-GC/MS analysis

A SPME assembly with replaceable extraction fibres (Supelco, Bellefonte, PA, USA) was used. Polydimethylsiloxane–divinylbenzene of 65 μm (PDMS–DVB) and polyacrylate (PA) of 85 μm fibres were purchased (Supelco, Bellefonte, PA, USA).

Table 1 Chemical structure, trivial and IUPAC names, quantification and identification ions of analysed compounds and retention times. The obtained standard deviations for retention times were lower than 0.02 min. The octanol–water partition coefficient (log K_{ow}) values for all compounds as predicted from the ALOGPS 2.1 computer program provided by Virtual Computational Chemistry Laboratory[48]

Chemical structure	Trivial name	IUPAC name	Abbreviation	log K_{ow}	CAS number	Molecular weight (g mol^{-1})	Monitoring ions (m/z)	Identification ions (m/z)	t_r (min)
 C_9H_{19} is a branched chain	Technical nonylphenol	4-Nonylphenol (branched)	t-NP	4.5	84852-15-3	220.35	135	107, 135, 136	9.17–9.55
 C_9H_{19} is a linear chain	4-*n*-Nonylphenol	4-Nonylphenol	4-NP	5.76	104-40-5	220.35	107	121, 135, 149	10.14
	4-*tert*-Octylphenol	4-(1,1,3,3-Tetramethylbutyl) phenol	OP	4.1	140-66-9	206.32	135	107, 135, 136	8.60
	Bisphenol A	4,4′-(Propane-2,2-diyl) diphenol	BPA	3.32	80-05-7	228.29	213	119, 165, 198, 213, 228	11.80

Prior to their first use, PDMS–DVB fibres were conditioned at 250 °C for 0.5 h and PA fibres were conditioned at 280 °C for 1 h using the injection port of the GC system, as indicated by the manufacturer.

All experiments were performed using direct immersion SPME (*i.e.*, the fibre was completely immersed in the solution). Once the extraction time was established, the fibre was removed and placed in the injection port of the GC for analyte desorption. Several experimental variables were studied, namely fibre coating (PA and PDMS–DVB fibres), ageing of the fibre, sample volume (15 mL and 300 μL), extraction time (10 to 30 min), desorption time (1–3 min) and injection temperature (250 °C and 280 °C). All the experiments were carried out at room temperature.

Results and discussion

Optimization of the analytical procedure

SPME procedure. PA and PDMS–DVB fibres were tested in order to achieve the best sensitivity and signal-to-noise ratios. PA fibre was selected because PDMS–DVB was significantly contaminated with BPA (data not shown) and consequently the LOD achieved was worse. The literature indicates that relative high blanks are obtained for BPA with PDMS–DVS fibres due to the BPA contained in the epoxy resin in order to connect the fibre needle to the fibre holder.[27]

SPME extraction time. Extraction times between 5 and 60 min were studied. Although, the increase of the extraction time improved the analytical responses, 30 min was selected as the achieved sensitivity was suitable for the trace analysis of ECDs.[12,13]

Fibre aging. The ageing of the fibre was studied by measuring the area of the chromatographic peak of a real sample containing t-NP below LOD and spiked with t-NP (0.2 μg L^{-1}) after the extraction with three different fibres, in particular a new fibre and two fibres used 40 times and 80 times, respectively. Three replicates were made for each fibre. The evaluation of the results was carried out by applying the one-tailed *t*-statistical test for 2 degrees of freedom at a significance level of $\alpha = 0.05$, comparing the results obtained for each old fibre with those achieved with the new fibre. Similar signals were found, for the studied fibres, at a 95% confidence level. Thus, each fibre may be used for at least 80 extractions without causing loss in the extraction efficiency.

Sample volume. Wastewater samples with high content of particulate matter require a filtration step prior to the analysis. In some cases, this step can be tedious and time-consuming as high volumes of samples must be filtered. In an attempt to overcome this problem, 15 mL and 300 μL of sample volume

Table 2 Analytical parameters obtained for the target analytes with DI-SPME-GC-MS processing $V_1 = 15$ mL and $V_2 = 300$ μL of sample

	Analytical parameters									
	V_1					V_2				
	Linear working range (μg L^{-1})	R^2	RSD% ($n = 5$) (%)	LOD (μg L^{-1})	LOQ (μg L^{-1})	Linear working range up to (μg L^{-1})	R^2	RSD% ($n = 5$) (%)	LOD (μg L^{-1})	LOQ (μg L^{-1})
Technical nonylphenol	0.05–100	0.95	13	0.05	0.15	0.05–100	0.95	15	0.05	0.15
4-*n*-Nonylphenol	0.01–100	0.98	20	0.01	0.03	0.01–100	0.98	19	0.01	0.03
4-*tert*-Octylphenol	0.006–100	0.98	16	0.006	0.020	0.006–100	0.98	18	0.006	0.020
Bisphenol A	0.5–300	0.96	19	0.5	1.5	0.5–300	0.95	20	0.5	1.5

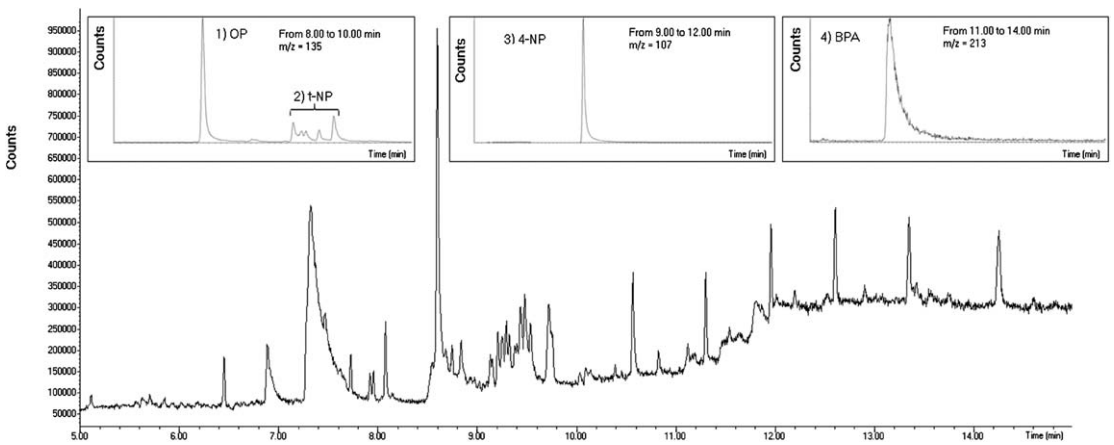


Fig. 1 TIC (scan range from 100 to 300 *m*/*z*) and SIM chromatograms (see Table 1 for more explanation) for a water sample providing target compounds at levels below LODs, spiked with a mixture of (1): OP (0.1 μg L^{-1}), (2): t-NP (0.4 μg L^{-1}), (3): 4-NP (0.2 μg L^{-1}) and (4): BPA (2 μg L^{-1}). Retention times were 8.60, 9.17–9.55, 10.14 and 11.80 min for OP, t-NP, 4-NP and BPA, respectively.

were studied. A disposable syringe filter-unit was used if the analysis was carried out with 300 μL. Table 2 shows the results obtained by processing a 15 mL and a 300 μL water sample. Satisfactory results were obtained for all EDCs analysed when using 300 μL instead of 15 mL of sample volume without loss in the following quality assurance parameters. The limits of detection (LODs) were calculated as the minimum concentration of a compound present in a sample that produces a signal-to-noise ratio of 3. The limits of quantification (LOQs) were determined in the same way for a factor of 10 times the signal-to-noise ratio. The repeatability of the method was calculated as the relative standard deviation (RSD%) for 5 independent analyses of 5 independent portions of the same sample.

GC/MS system. Desorption times in the GC injection port between 1 and 3 min were tested for all the analytes. The results revealed that 1 min was enough for the completion of analyte desorption. Two different injection port temperatures were evaluated, 250 °C and 280 °C. Better responses were obtained with 280 °C injection port temperature, and, hence, this temperature was selected for further analysis.

Fig. 1 shows the total ion chromatogram (TIC) (see Table 1 for the ions used) for t-NP (0.4 μg L^{-1}), OP (0.1 μg L^{-1}), 4-NP (0.2 μg L^{-1}) and BPA (2.0 μg L^{-1}). Fig. 1 also includes the SIM chromatograms corresponding to each analyte. Given that different peaks were obtained, the SIM chromatogram for t-NP (see Fig. 1) proves that this compound is a mixture of different branched nonylphenols. Thus, the identification of this

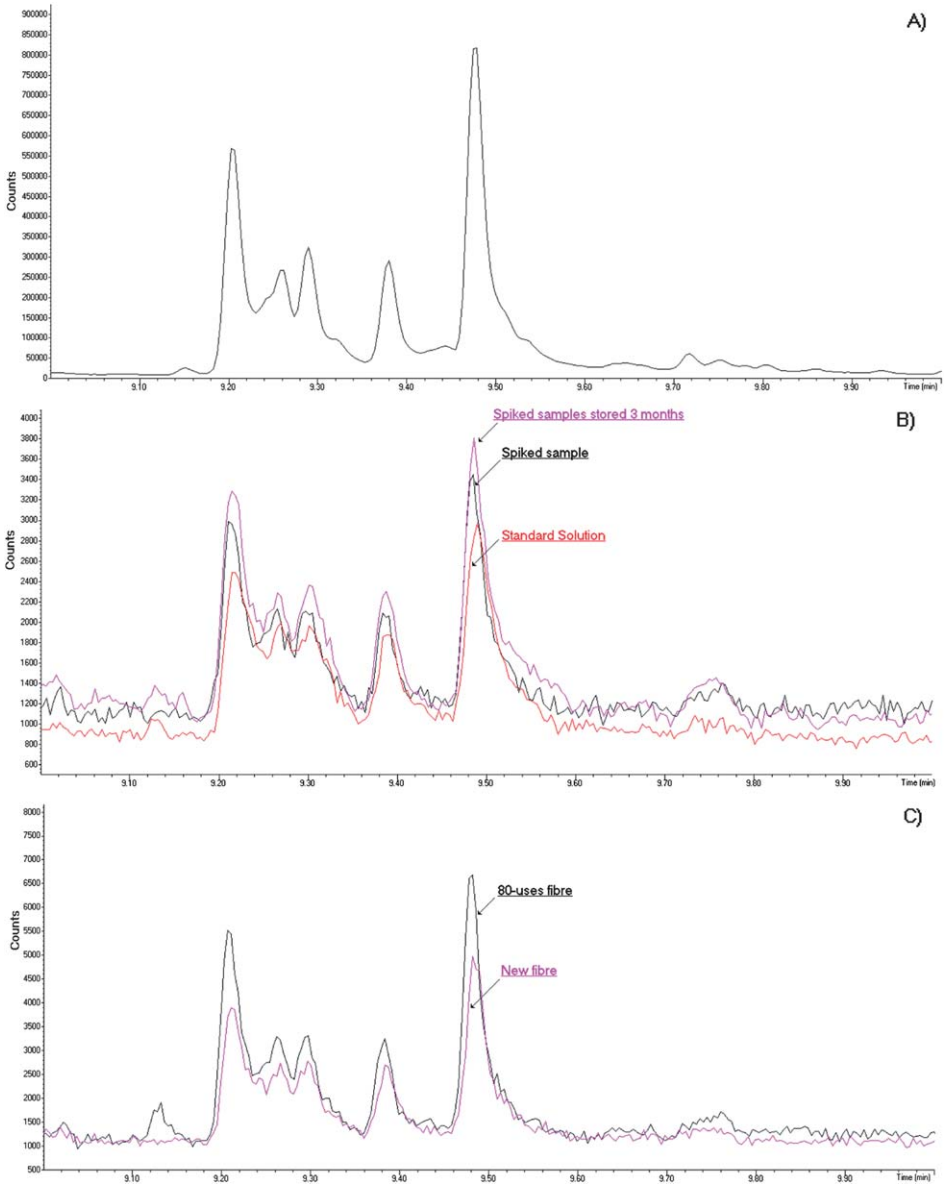


Fig. 2 (A) Chromatographic peaks of t-NP (25 μg L^{-1}), (B) comparison of chromatographic peaks of t-NP as a function of the sample matrix: standard solution, spiked WWTP effluent sample with t-NP and spiked water samples with t-NP stored for 3 months (1.25 μg L^{-1}) and (C) comparison of the chromatographic peaks of t-NP as a function of fibre ageing: new fibre and fibre after 80 extractions (1.25 μg L^{-1}). In all cases, the monitoring ions were 135 (*m*/*z*).

compound in water samples could be complicated if the characteristics of these peaks change depending on the water sample, extraction time, fibre and other experimental factors. In an attempt to evaluate this effect, the dependence of the peak characteristics on the sample (a standard or real sample) and on the fibre properties was studied. The SIM chromatogram obtained for t-NP is provided in Fig. 2A. Fig. 2B compares the chromatograms obtained for t-NP in a standard solution with those from a spiked WWTP effluent sample with t-NP (1.25 μg L^{-1}) and those from a spiked WWTP effluent sample with t-NP (1.25 μg L^{-1}) stored for 3 months. As can be seen, no differences could be found when comparing these chromatograms. Fig. 2C shows the two SIM chromatograms obtained for a spiked WWTP effluent sample with t-NP (1.25 μg L^{-1}), the first one with a new fibre and the second one with a fibre used for 80 extractions. As stated above, the SIM chromatogram did not present any significant differences. The results showed that the sensitivity and peak characteristics of these spiked WWTP effluent samples were independent of the sample matrix, the storage time or the extraction phase. Moreover, no 4-NP was found, which indicates that t-NP does not contain the linear isomer as mentioned in the introduction section.[29,30]

Analytical parameters. Table 2 details the analytical parameters obtained for the EDCs studied in this research under the best conditions and using the monitoring ions given in Table 1. The application of the suggested procedure to the target analytes analysis showed linear working ranges up to 100 μg L^{-1} for t-NP, 4-NP and OP. A linear working range up to 300 μg L^{-1} was established for BPA. Satisfactory regression coefficients were achieved, ranging between 0.95 and 0.98 as shown in Table 2.

Table 2 also shows %RSD obtained for the target analytes (1.0, 1.0, 0.1 and 0.2 μg L^{-1} for t-NP, 4-NP, OP and BPA respectively). Satisfactory values, near to 20%, were obtained in all cases. LODs and limits of quantification (LOQs) are also listed in Table 2. LOD was experimentally obtained from the concentration corresponding to 3 times the signal-to-noise ratio and LOQ was determined as 10/3 times the LOD. The achieved LODs match with real samples and were found to be between

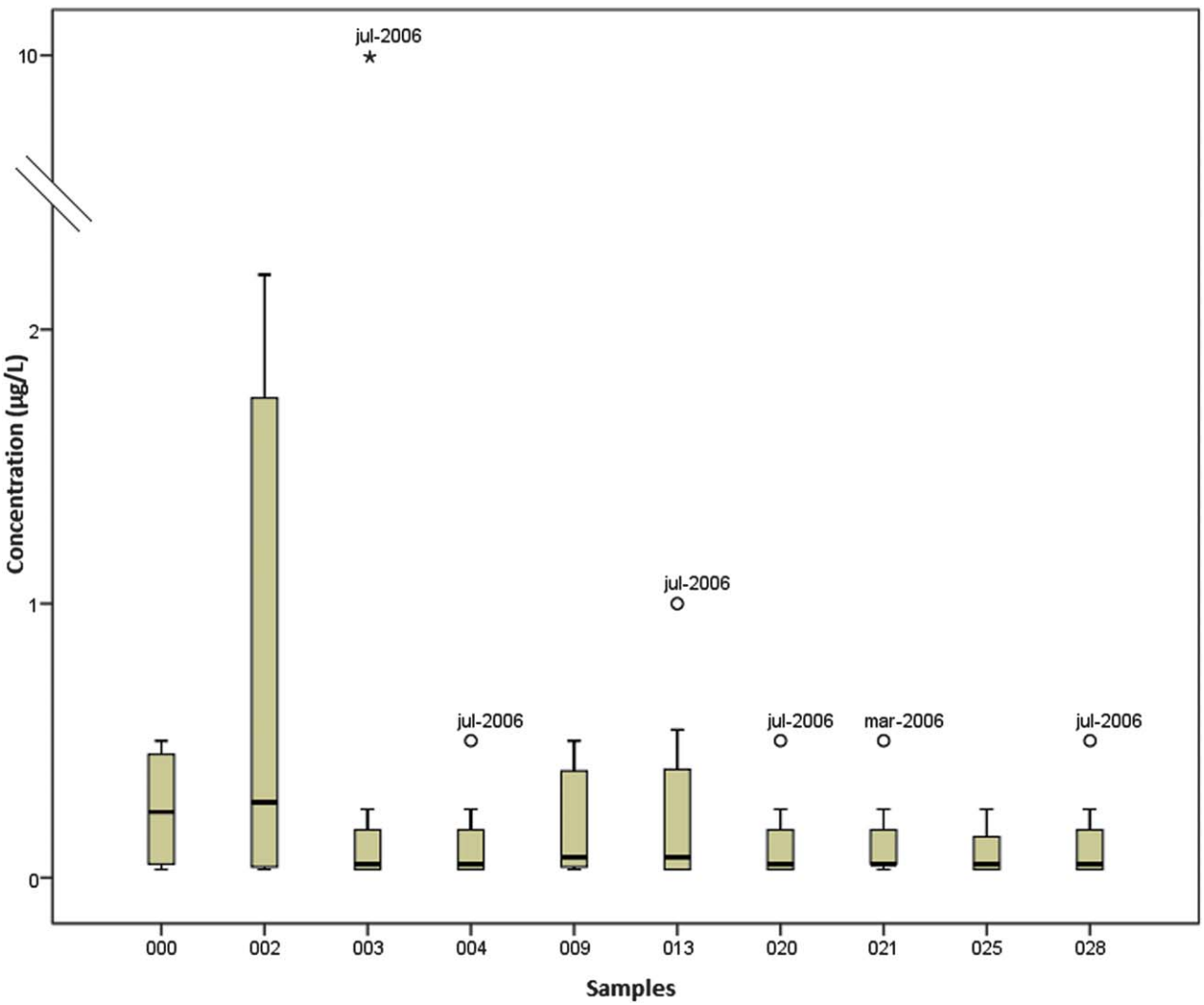


Fig. 3 Box plot of t-NP concentrations depending on the seasonal period (concentration found in μg L^{-1} *vs.* WWTP effluent). The horizontal line represents the median value, boxes represent the interquartile ranges and whiskers, the 95% confidence intervals. The circles represent statistical outliers (1.5 times the interquartile range) and the asterisks represent extreme values (greater than 3 times the interquartile range).

0.006 and 0.5 μg L^{-1}. The LODs were assessed according to the standards by reducing the concentration successively. Thus, the same above described procedure for determination of the LOD and LOQ was used in real samples, which were spiked with concentrations below the LOD. The LODs obtained were suitable for trace analysis of ECDs in water samples.[12,13,27]

Wastewater sample analysis. The region of Comunitat Valenciana is a highly populated area (about 4 million people), and under the influence of a high industrial and touristic pressure in the coastal areas, especially in the summer time (July–September). The results obtained revealed the presence of t-NP in all sampling campaigns in several WWTPs. 4-NP was kept below the LOD in 2006 and 2007 campaigns. Nevertheless, the concentrations in four WWTP effluents in 2008 were between the LOD and LOQ. t-NP was found in 18 sampling points in 2006 at concentrations between below the LOD and 10 μg L^{-1}. Besides, in 2007 the t-NP was found in 19 sampling points, although in 15 of these sampling points, the concentration was below the LOD. In the other sampling points, the concentrations varied between 0.1 and 2.2 μg L^{-1}. Finally in 2008, the number of sampling points in which t-NP was found was 10, with concentration ranging between 0.05 and 0.79 μg L^{-1}. Statistical analyses were performed using IBM SPSS Statistics for Windows Version 19.0 (SPSS Inc, Chicago, Illinois, USA). Fig. 3 shows the box-plot for t-NP. As expected, the higher concentrations of these compounds were found during the summer period, probably due to the increase in the touristic pressure in these coastal areas.

The results obtained for OP reveal that it was found in 8 and 10 sampling points in the 2006 and 2007 sampling campaigns, respectively. The higher concentrations (between 0.12 and 0.19 μg L^{-1}) were identified in the same WWTP effluent. In the 2008 sampling campaign, the sampling points in which OP was found increased to 15, 13 of which had concentration levels ranging between 0.04 and 2.4 μg L^{-1}. The box-plot in Fig. 4 summarizes the above data for OP.

As stated in the introduction section, only micropollutants 4-NP and OP are mentioned in the Annex I of the Directive 2008/105/EC. Environmental quality standards (EQS) for inland surface waters, both annual average (AA-EQS) and maximum

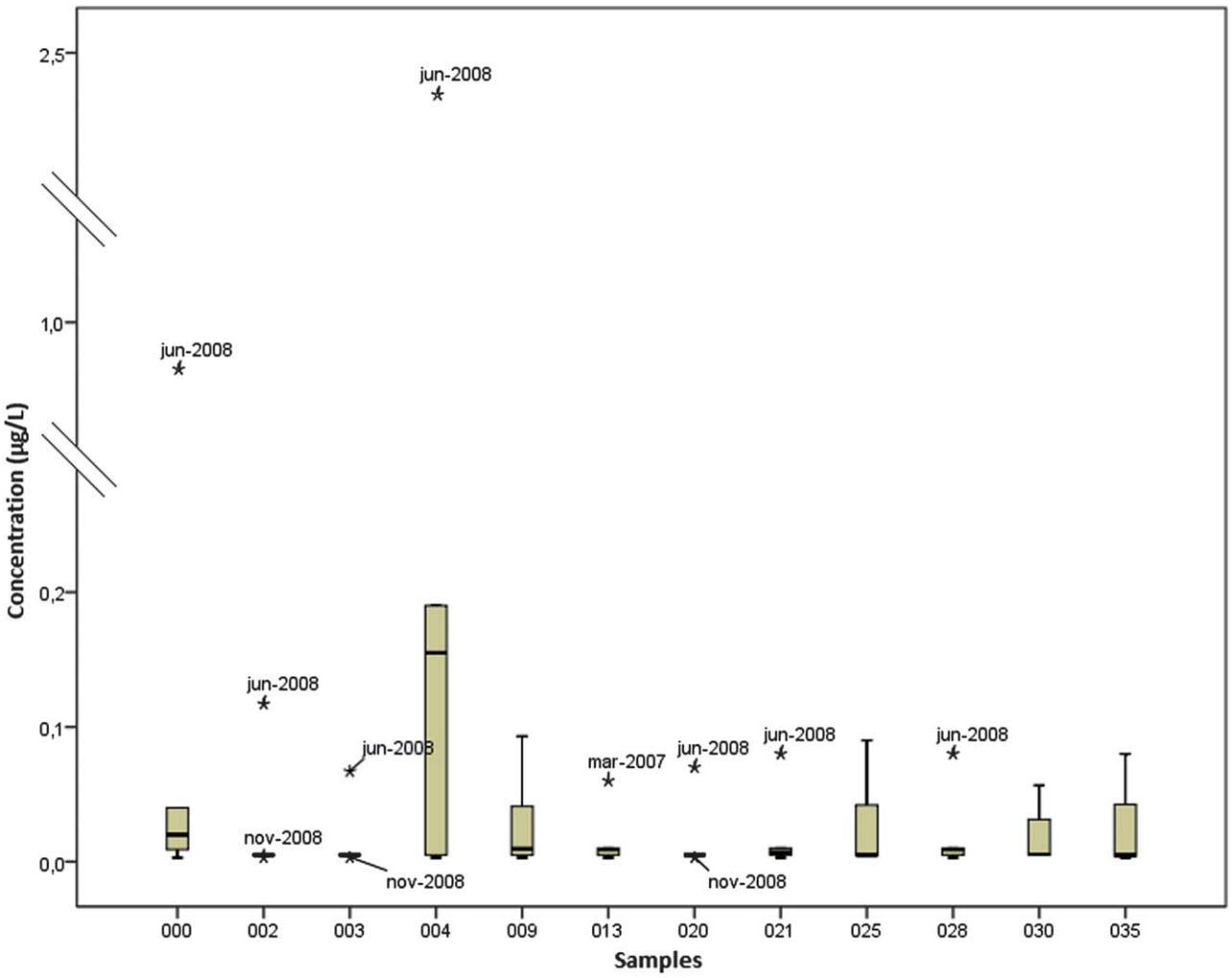


Fig. 4 Box plot of OP concentrations at different seasonal periods (concentration found in μg L^{-1} *vs.* WWTP effluent). The horizontal line represents the median value, boxes represent the interquartile ranges and whiskers, the 95% confidence intervals. The circles represent statistical outliers (1.5 times the interquartile range) and the asterisks represent extreme values (greater than 3 times the interquartile range).

allowable concentration (MAC-EQS), are 0.3 and 2.0 μg L^{-1} for 4-NP, respectively. In the case of OP, only AA-EQS have been regulated in the Directive 2008/105/EC, being 0.1 μg L^{-1}. AA-EQS and MAC-EQS for 4-NP were higher than the results achieved. Thus, coastal waters in the research were unaffected by WWTP effluents, since the effluent concentrations were lower than the EQS established for inland surface waters.[13,45] Despite 11% of the WWTP samples exceeding the OP EQS, WWTP effluents did not contribute to an increase of OP concentration in surface waters above the EQS established, as the dilution factor lowered the particular concentrations with respect to EQS.

Nevertheless, the contribution of BPA to coastal waters was reduced. The concentrations detected were mainly lower than LOD levels, and, in fact, only one WWTP effluent sample in the 2006 sampling campaign was between the LOD and LOQ. Hence, like OP and t-NP, the studied WWTPs did not contribute to an increase in the BPA concentration in the coastal area.

Conclusions

This study suggested a SPME-GC-MS method for the analysis of t-NP, 4-NP, BPA and OP, all of which are considered as endocrine disruptor compounds. There are two main advantages of the procedure put forward. The first one is the simultaneous analysis of different endocrine disruptor compounds from different sources while the second one involves using filtered samples directly for analysis by using a low sample volume of 15 mL or even 300 μL for analyte extraction, in order to save time and reduce the amount of samples used in the filtration process.

This procedure was put into practice in three sampling campaigns of WWTP effluents along the region of Comunitat Valenciana held in 2006, 2007 and 2008. t-NP was found in several sampling points, while 4-NP was below LOD in 98% of sampling points studied and between LOD and LOQ in 2% of the sampling points. These results owed to the fact that t-NP is a degradation metabolite of NPEOs (the largest source of these compounds), whereas 4-NP does not originate from NPEO. OP was also found in several sampling points, especially in 2008 and also in the summer season. This was probably due to the increase in the touristic pressure in this coastal area, although at lower levels than those found for t-NP. These findings are consistent with the composition of APEOs (80% nonylphenol ethoxylates and 20% octylphenol ethoxylates). Moreover, BPA were generally found below LODs, only 1% of the studied samples ranged between LOD and LOQ.

The WWTP discharge points under study generally presented concentrations lower than the EQS established for OP and 4-NP in the Directive 2008/105/EC. Although, 11% of these WWTP discharge points presented higher concentrations of OP than the established EQS, the effect of WWTPs under study on coastal waters was inexistent, as deduced from the reduced identified concentrations and the dilution factor used.

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