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48	Abstract	A decontamination technique based in cyclodextrin extraction has been developed to eliminate nonylphenol (NP) and 16 polycyclic aromatic hydrocarbons (PAHs; the US Environmental Protection Agency priority pollutants list) from sewage sludge. In a first step, PAHs and NP were characterised in six sludges to determine contamination levels according to limit values proposed by the European Union Sludge Directive draft. There were few variations in the total PAHs content with levels of 1.88 to 3.05 mg kg ⁻¹ . Three-ring PAHs predominated, but fluoranthene and pyrene were also present. None of the sludge exceeded the PAHs limit proposed by the European Union's draft Directive. On the contrary, NP content in four of the six sludges was over the recommended limits of 50 mg kg ⁻¹ for NP ethoxylates. With the aim of obtaining NP values below the concentration limits proposed to use the sewage sludge as agricultural amendments, a preliminary study using hydroxypropyl-β-cyclodextrin (HPBCD) extractions as a decontamination technique was carried out. About 90 % of NP content was removed with only one extraction with HPBCD, whereas after three sequential extractions using an aqueous solution without HPBCD, the NP extraction percentage was less	

than 1 %. Simultaneously, PAHs extraction percentages obtained with HPBCD were also much higher than when aqueous solution was used, especially in the case of two- and three-ring PAHs. Finally, the potential environmental hazard of HPBCD leachates to aquatic organisms (*Daphnia magna*) was tested. These results indicate that the treatment of sewage sludge with cyclodextrin could allow their safe use as fertiliser in agriculture.

49	Keywords separated by ' - '	Polycyclic aromatic hydrocarbons - Nonylphenol - Sewage sludge - Cyclodextrin - Toxicity
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Decontamination of polycyclic aromatic hydrocarbons and nonylphenol from sewage sludge using hydroxypropyl-β-cyclodextrin and evaluation of the toxicity of leachates

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Abstract A decontamination technique based in cyclodextrin extraction has been developed to eliminate nonylphenol (NP) and 16 polycyclic aromatic hydrocarbons (PAHs; the US Environmental Protection Agency priority pollutants list) from sewage sludge. In a first step, PAHs and NP were characterised in six sludges to determine contamination levels according to limit values proposed by the European Union Sludge Directive draft. There were few variations in the total PAHs content with levels of 1.88 to 3.05 mg kg⁻¹. Three-ring PAHs predominated, but fluoranthene and pyrene were also present. None of the sludge exceeded the PAHs limit proposed by the European Union's draft Directive. On the contrary, NP content in four of the six sludges was over the recommended limits of 50 mg kg⁻¹ for NP ethoxylates. With the aim of obtaining NP values below the concentration limits proposed to use the sewage sludge as agricultural amendments, a preliminary study using hydroxypropyl-β-cyclodextrin (HPBCD) extractions as a decontamination technique was carried out. About 90 % of NP content was removed with only one extraction with HPBCD, whereas after three sequential extractions using an aqueous solution without HPBCD, the NP extraction percentage was less than 1 %. Simultaneously, PAHs extraction percentages obtained with HPBCD were also much higher than when aqueous

solution was used, especially in the case of two- and three-ring PAHs. Finally, the potential environmental hazard of HPBCD leachates to aquatic organisms (*Daphnia magna*) was tested. These results indicate that the treatment of sewage sludge with cyclodextrin could allow their safe use as fertiliser in agriculture.

Keywords Polycyclic aromatic hydrocarbons · Nonylphenol · Sewage sludge · Cyclodextrin · Toxicity

Introduction

After the progressive implementation of the Directives 91/271/EEC and 98/15/EEC concerning urban wastewater treatment, many countries are increasing the number of wastewater treatment plants (WWTP) to avoid the spread of disease, remove organic matter and some pollutants and preserve the quality of surface waters. Within a WWTP, large amounts of sewage sludge are generated which require disposal. Final destination of sewage sludge used to be incineration or landfills (Pousada-Ferradas et al. 2012), but a useful and interesting option is the production of compost and the direct application of stabilised sludge to land (Laternus et al. 2007). Although there are potential hazards in this practice, such as spread of pathogens, odours, heavy metals and persistent toxic organics, sludge application to agricultural land have positive effects on soil, such as modification of soil structure and addition of organic matter and nutrients for plants, such as N, P and K (Morillo et al. 2002; Aparicio et al. 2009).

The use of sewage sludge in agriculture is regulated by the European Union's (1986) Council Directive 86/278/EEC.

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This Directive controls the quality of sludge, the amended soils, the loading rate and the crops that may be grown on treated land. This Directive requires that heavy metal contents (Cd, Cr, Cu, Ni, Pb and Zn) in both the sludge and soil comply with the established limits, but it does not yet establish maximum values for organic contaminants.

To limit the concentration for certain organic pollutants in both sewage sludge and sludge-amended soils, the European Union (2000) launched a working document on sludge that although not being a formal regulation, the third draft of this document limits the values for some organic compounds, including polycyclic aromatic hydrocarbons (PAHs; the sum of phenanthrene, fluorene, fluoranthene, pyrene, benzo(b) fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, and indeno(1,2,3-c,d)pyrene) and the sum of nonylphenol (NP) and nonylphenol mono (NP1EO) and diethoxylates (NP2EO; referred as NPE). Concentration limits fixed in this Directive draft for land application of sludge are 50 mg kg⁻¹ dry matter for NPE and 6 mg kg⁻¹ for the sum of these nine PAHs.

PAHs are formed and released into the environment as by-products of incomplete combustions through natural, e.g., by forest fires, and man-made sources (Morillo et al. 2008). Some of them are highly toxic, present high persistence in the environment, low biodegradability and high lipophilicity. PAHs enter to wastewater-treatment plants through the sewerage and are almost completely removed from wastewater (up to 90 %), being concentrated in sludge because of their poor solubility in water and high adsorption capacity on solid particles (Aparicio et al. 2009).

On the other hand, NP and NPE are used as nonionic surfactants in a large variety of industrial and domestic applications (Navarro et al. 2009). Although the use of NPEs has been banned under Council Directive 2003/53/EC (European Union 2003), their environmental presence and risk is still high because of its historical and pervasive widespread use. Industrial and urban wastewaters and agricultural run-off discharge to WWTPs where the biodegradation of NPEs leads to the decrease in the length of ethoxy chain and an increase of hydrophobicity (Ifelebuegu 2011). This is why NP is the main alkylphenol associated with sewage sludge (90 %; Soares et al. 2008). Considering its endocrine disrupting properties, NP in sludge may impair environmental effects (Fernández-Sanjuán et al. 2009). The application of sewage sludge as soil fertiliser may produce effects to soil organisms or contaminate groundwater. To determine the content of PAHs and NP in sewage sludge will therefore be the first step to consider before their application to agricultural soils, in order to know the need of using technological treatments in the sludge management to reduce the concentration of these organic pollutants to levels that allow land application of sewage sludge according to the European Directive.

To assess the real hazard of these toxic chemicals in sewage sludge intended for agricultural land, it is therefore important to determine the available fraction. Traditionally, extraction techniques were aimed to determine the concentration of the total organic contaminants in soils and sludge. The use of non-exhaustive extractions still gains importance to determine the bioavailable fraction of contaminants with the aim of assessing the real environmental risk (Alexander 2000). This fraction needs to be reduced in sewage sludge to allow its land application.

Compounds with low water solubility and a high Kow coefficient show a high adsorption capacity and low availability to be desorbed from soils, sediments or sludge, decreasing the fraction that can really present a toxicological risk. Different extracting agents have been used to increase the solubility of organic contaminants but extracting only the really available fraction. Mild extraction procedures, such as extraction techniques with n-butanol or solvent mixtures (ethanol/water, methanol/ water, etc.), and extraction with surfactant agents like Triton X-100, have been used, but those molecules can represent also a health and environment hazard (Ying 2006). Other biodegradable complexing agents, such as cyclodextrins (CDs), which encapsulate poorly water soluble contaminants, have been used to enhance the water solubility of hydrophobic compounds and their removal from polluted sites, while minimising environmental impact (Petitgirard et al. 2009). CDs are polycyclic glucose oligosaccharides resulting from enzymatic degradation of starch by bacteria. CD molecules have a hydrophobic and non-polar cavity which permit the solubilisation of non-polar and low-polarity organic molecules of appropriate size and shape through the formation of water soluble inclusion complexes (Ginés et al. 1996; Villaverde et al. 2005a).

CDs in general are considered non-toxic, biodegradable molecules (Fenyvesi et al. 2005; Kiss et al. 2007; Wacker Chemie 2013). Hydroxypropyl-β-cyclodextrin (HPBCD) has been reported to exhibit a low tendency to adsorb onto soil particles (Badr et al. 2004) and that it is well tolerated in humans (Gould and Scott 2005), being an alternative to the natural α-, β- and γ-cyclodextrins, with improved water solubility and even more toxicologically benign. The chemical properties of CDs combined with their non-toxic character to humans have led to their use in pharmaceuticals, as food additives, as well as in the environmental decontamination procedures of wastewater (Olah et al. 1998) and soil (Fenyvesi et al. 2009; Hajdu et al. 2011; Villaverde et al. 2012). Some CDs, in particular, have been shown to enhance the solubility of several contaminants, such as PAHs, pesticides or biphenyls (Villaverde et al. 2005b; Morillo et al. 2012). In the case of hydroxypropyl-β-cyclodextrin (HPBCD), many studies have also demonstrated that a non-exhaustive aqueous extraction from soils was able to

174	predict the bioaccessible fraction of different PAHs (Rhodes	at -2°C until their characterisation for PAHs and NP con-	220
175	et al. 2010) or pesticides (Hartnik et al. 2008).	centration and all other experiments.	221
176	In this study, three specific objectives were proposed: (1)	Analysis of PAHs content in sewage sludge	222
177	to analyse the content of 16 PAHs (included in the US	Sludge samples were spiked with the surrogate standards at a	223
178	Environmental Protection Agency (US EPA) priority pollut-	concentration of 2 mg kg^{-1} ; 0.5 g of sludge were inserted in a	224
179	ants list) and NP in six sewage sludge from WWTPs from	glass tube with 10 mL hexane/dichloromethane (1:1, v/v) and	225
180	NE Spain, (2) to carry out a non-exhaustive extraction of	placed in an ultrasonic bath for 10 min. The solution was	226
181	PAHs and NP from sewage sludge with HPBCD as a possi-	then centrifuged (10 min at $1,090\times g$; Beckman Coulter	227
182	ble method for sludge decontamination and (3) to study the	Avanti J-25I). The two last steps were repeated three times	228
183	toxicological risk of the leachates obtained after HPBCD	using fresh solvent. The sonicated extracts were evaporated	229
184	extractions.	in a Turbo Vap LV from Caliper Life Sciences to almost	230
		dryness (1 mL approximately) for further clean up.	231
185	Experimental	The extracts were purified using solid-phase extrac-	232
186	Chemicals and reagents	tion (SPE) cartridges of neutral alumina of 5 g. The alu-	233
187	Sixteen PAHs considered of primary environmental concern	mina was solvated and conditioned with 40 mL hexane/	234
188	according to the US EPA were analysed: naphthalene,	dichloromethane (1:1, v/v). Analyte elution was performed	235
189	acenaphthylene, acenaphthene, fluorene, phenanthrene, an-	with 40 mL hexane–dichloromethane (1:1, v/v). The extract	236
190	thracene, fluoranthene, pyrene, benzo[a]anthracene, chrys-	was concentrated to a volume of less than 1 mL by a Turbo	237
191	ene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]	Vap LV evaporator, transferred into an amber glass vial and	238
192	pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene and	reconstituted with hexane to a final volume of 1 mL. At this	239
193	benzo[ghi]perylene. They were purchased from Supelco as	stage, anthracene- d_{10} was added as an internal standard to give	240
194	mix solution of 200 mg L^{-1} in methanol. The surrogate	a concentration of $1\text{ }\mu\text{g mL}^{-1}$. Blanks were prepared at the	241
195	standard was a mixture containing naphthalene d-8,	same time under the same conditions. With these conditions, a	242
196	acenaphthene d-10, phenanthrene d-10, chrysene d-12 and	good recovery was obtained for PAHs, but NP was poorly	243
197	perylene d-12, purchased from Supelco as a solution of	recovered with alumina SPE. Therefore, a parallel extraction	244
198	200 mg L^{-1} in methanol. Anthracene d-10, from Supelco,	and clean-up was performed with florisil to enhance the	245
199	was also used as internal standard. 4-NP was supplied by Dr.	recovery of NP.	246
200	Ehrenstorfer (Cromlab, Spain). 2-HPBCD was supplied by	Analysis of NP content in sewage sludge	247
201	Cyclolab (Budapest, Hungary). Solvents were supplied by	NP analysis was carried out according to the method	248
202	Merck (Germany), alumina SPE cartridges of 5 g by the	proposed by Fernández-Sanjuán et al. (2009). Deuterated	249
203	International Sorbent Technology (UK), florisil SPE from	standard 4-n-NP-D8 was added to a sample aliquot of 0.1 g	250
204	Waters (USA) and nitrogen, for drying with 99.995 % of	of the sieved sludge at $0.5\text{ }\mu\text{g/g}$. Samples were homogenised	251
205	purity, by Air Liquid (Spain).	and kept at room temperature overnight and subsequently	252
206	Sample collection and preparation	extracted by sonication (10 min) with 5 mL of hexane/	253
207	Six sewage sludge samples were collected from different	dichloromethane (1:1, v/v) and repeated a second time. A third	254
208	WWTPs located in Northeast Spain and designated as A,	extraction was performed with 5 mL of dichloromethane/	255
209	B, C, D, E and F. Sludges A and B were from WWTPs	acetone (1:1, v/v). After each extraction step, samples were	256
210	located in little urban areas and sludge was not digested.	centrifuged for 10 min and the extracts were combined and	257
211	Sludges C–F were collected in WWTPs of industrial and	evaporated in a Turbo Vap LV to almost dryness. Extracts	258
212	highly urbanised areas and were anaerobic digested. One	were subsequently cleaned up by SPE cartridges with	259
213	kilogramme of sample was collected from the sludge tanks	5 g of florisil, conditioned with 20 mL of hexane/	260
214	using precleaned amber glass pots and were transported	dichloromethane (1:1, v/v) and 20 mL of dichloromethane/	261
215	refrigerated to the laboratory. Sludge samples were placed	acetone (1:1, v/v). The sample extract was eluted with 20 mL	262
216	in glass pots, frozen at -18°C and lyophilised during 48 h	of hexane/dichloromethane (1:1, v/v) and 20 mL of	263
217	(10^{-2} mbar vacuum) in a freeze dryer (Lioalfa, Telstar,	dichloromethane/acetone (1:1, v/v). The eluent was evaporat-	264
218	Spain). Afterwards, samples were sieved through $500\text{ }\mu\text{m}$	ed to almost dryness and reconstituted with ethyl acetate to a	265
219	mesh to obtain a homogeneous material. Samples were kept	final volume of 1 mL. NP was determined by gas chromatog-	266
		raphy–mass spectrometry (GC-MS) according to the method	267
		reported below.	268

269 Instrumental analysis

270 Samples were analysed by a gas chromatographer (GC;
 271 Agilent GC 6890N) coupled to a quadrupole mass spectrom-
 272 eter (MS; Agilent MD 5975B). The system was operated in
 273 electron ionisation (70 eV). The separation was achieved
 274 with a 30×0.25-mm I.D. DB-5 MS column (J&W
 275 Scientific, Agilent Technologies) coated with 5 % phenyl-
 276 methylpolysiloxane (film thickness, 0.25 µm). The oven
 277 temperature was programmed from 80 (holding time,
 278 1 min) to 175 °C at 6 °C/min (holding time, 4 min), to
 279 235 °C at 3 °C/min and finally to 320 °C at 8 °C/min,
 280 keeping the final temperature for 5 min. The same GC-MS
 281 program was used to determine PAHs and NP. Injection was
 282 performed in the splitless mode. Helium was the carrier gas
 283 (50 cm/s). Injector, transfer line and ion source temperatures
 284 were 280, 250 and 200 °C, respectively. For increased sen-
 285 sitivity and specificity, quantification was performed in time-
 286 scheduled selected ion monitoring using three ions per com-
 287 pound (Martínez et al. 2004). Internal standard quantifica-
 288 tion was performed using the deuterated surrogate standards
 289 present in each elution window. Peak detection and integra-
 290 tion were carried out using Agilent ChemStation software.

291 Non-exhaustive extraction of PAHs and NP from sewage 292 sludge using HPBCD solutions

293 Batch extraction experiments were carried out using the
 294 sludge E, and this same sludge spiked with the 16 EPA-
 295 PAHs (spiked E). Real and spiked sludge were treated with
 296 HPBCD solutions for its decontamination in comparison to
 297 an aqueous extraction in the absence of HPBCD. For sludge
 298 spiking, 0.2 g of sludge E were inserted in a glass tube and
 299 spiked with 0.2 mL of 5 mg L⁻¹ PAHs mix solution prepared
 300 in acetone. During 1 h, the tube was shaken and vortexed for
 301 10 s every 10 min. After complete acetone evaporation
 302 (24 h), the tube was closed and extracted. The content of
 303 each PAH was increased in 5 mg kg⁻¹.

304 *Batch extraction experiments* Of the real and spiked sludge
 305 E, 0.2 g was inserted in 25 mL glass centrifuge tubes (Corex)
 306 with 10 mL of an aqueous solution which contained 0.01 M
 307 Ca(NO₃)₂ to maintain the ionic strength and 200 mg L⁻¹
 308 HgCl₂ to prevent the bacterial growth (named aqueous solu-
 309 tion). In parallel, 0.2 g of sludge E (real and spiked) was
 310 inserted in the Corex tubes with 10 mL of a solution con-
 311 taining HPBCD (10 mM), Ca(NO₃)₂ (0.01 M) and HgCl₂
 312 (200 mg L⁻¹) (named HPBCD solution). All batch extraction
 313 experiments were conducted in triplicate. The Corex tubes
 314 were shaken on an orbital platform shaker at 200 rpm for 1 h
 315 at a constant temperature of 20 °C. Blanks were prepared at
 316 the same time under the same conditions. After centrifuga-
 317 tion at 3,000 rpm for 10 min, target compounds were

extracted from an aliquot of the aqueous supernatant with
 hexane, and 1 mL of this organic phase was transferred into
 amber glass vials. The rest of the supernatant was decanted
 and then 10 mL of fresh aqueous or HPBCD solution were
 added. The extractions were carried out three times in total,
 and each extract was analysed individually. One millilitre of
 the hexane extract was dosed in a vial, and at this stage,
 anthracene-d₁₀ was added to a concentration of 1 µg mL⁻¹
 and analysed by GC-MS as previously described.

Toxicity evaluation of leachates

In order to study the toxicity of the leachates obtained after the
 extraction of PAHs and NP from sewage sludge using aqueous
 and HPBCD solutions, parallel extraction experiments were
 performed but without Ca(NO₃)₂ and HgCl₂ since these com-
 pounds would affect the condition of *Daphnia*. The leachates
 used in these experiments were obtained as described: 0.5 g of
 sludge E were inserted in 100 mL amber glass bottles with
 25 mL of ASTM hard water (ASTM 1999). In parallel, 0.5 g
 of sludge E were inserted in the 100 mL amber glass bottles
 with 25 mL of 10 mM HPBCD solution prepared in hard
 water. The suspensions were shaken for 1 h at a constant
 temperature of 20 °C. After separation by decantation, the
 leachates obtained were kept at 4 °C.

Bioassays with the grazer *Daphnia magna* were
 conducted to evaluate sub-lethal feeding effects of filtered
 fractions of leachates. Feeding responses to sludge leachates
 diluted 1 and 10 % in ASTM hard water in 24 h toxicity tests
 were performed following Barata et al. (2008). Groups of
 five neonates were exposed to 20 mL of test concentrations
 in 30 mL borosilicate flasks in the presence of food. The alga
Chlorella vulgaris was added at a concentration of 5×10⁵
 cells/mL⁻¹ (equivalent to 1.5 µg C mL⁻¹). Treatments
 consisted of an ASTM hard water control (W), 10 mM
 HPBCD solution controls and the selected leachates with
 five replicates each. Each group of replicates consisted of
 five vessels with animals and one blank. Blanks were used to
 assure that initial algal concentrations did not increase sig-
 nificantly over the exposure period. Individual feeding rates
 (number of algal cells ingested per animal per hour) were
 determined as the change in cell density during 24 h
 according to the method described by Barata et al. (2008)
 and converted to proportional feeding rates relative to ASTM
 W. Cell density was estimated from absorbance measure-
 ments at λ=650 nm using standard calibration curves based
 on at least 20 data points (*r*²>0.98). Proportional responses
 were arcsine transformed to meet ANOVA assumptions of
 normality and variance homocedasticity (Zar 1996).
 Differences in bioassay responses among treatments were
 compared by one-way ANOVA following post hoc Tukey's
 test at a *p*<0.05 significance level.

369 Results and discussion

370 Content of PAHs and NP in sewage sludge

371 PAHs

372 Table 1 shows the concentration of PAHs and NP in studied
373 sludges. Sludge F had the highest concentrations of total
374 PAHs (3.05 mg kg⁻¹ dry weight (dw)) while sludge E had
375 the lowest (1.88 mg kg⁻¹dw). Among individual PAHs, the
376 highest contribution was that of phenanthrene (15–30 %) and
377 pyrene (10–24 %) followed by naphthalene (14–23 %).
378 Anthracene and fluoranthene were found in fewer amounts
379 (9–21 and 9–13 %, respectively). Normally, when phenan-
380 threne and pyrene are present at high levels in the sludges, it
381 indicates a domestic sewage water origin (Miégle et al. 2003;
382 Villar et al. 2006).
383 The limit concentration proposed for ΣPAHs in EC
384 Working Document on Sludge (European Union 2000) is
385 6 mg kg⁻¹dw indicating that none of the sludges exceeded
386 this maximum level. ΣPAHs was relatively low when com-
387 pared with other studies. Dai et al. (2007) found concentra-
388 tions from 2.5 to 26 mg kg⁻¹ of 16 PAHs in six sewage
389 sludge from a WWTP receiving steel and dyeing industry
390 wastewater from Beijing. Khadhar et al. (2010) found ΣPAHs
391 levels from 0.1 to 7.8 mg kg⁻¹ in sewage sludge samples
392 from nine Tunisian WWTPs. Oleszczku (2009) found a total

content from 2.83 to 9.95 mg kg⁻¹ with pyrene and fluoran- 393
thene being the most abundant PAHs in the four sewage 394
sludges studied. Blanchard et al. (2004) found that sludges 395
from high traffic areas displayed high levels of pyrene, 396
fluoranthene and phenanthrene, contrasting with those from 397
residential zones which showed a more homogeneous PAHs 398
distribution. 399
In Spain, Abad et al. (2005) analysed 139 samples from 400
Catalonia and found a median concentration of 1.4 mg kg⁻¹, 401
but less than 3 % of the samples had PAHs concentrations 402
higher than the European Union-proposed limit. Pérez et al. 403
(2001) determinate the PAH content in six sewage sludge 404
samples and found that phenanthrene and pyrene were the 405
most abundant PAHs. In 17 WWTP sludge from the 406
Valencian Community, Gómez-Rico et al. (2007) found that 407
only one sample exceeded the limit of 6 mg kg⁻¹ PAHs and 408
that phenanthrene was the major PAH followed by fluorene 409
and pyrene. 410
The different PAH distribution found among the sludge 411
studied indicated different sources. Table 2 shows that main- 412
ly three-ring PAHs predominated (from 30 % in sludge B to 413
54 % in sludge F). Four-ring PAHs were predominant (38 %) 414
only in sludge B. Shen et al. (2007) analysed 44 sludge 415
samples from WWTPs which received river waters, munic- 416
ipal sewage or mixtures of industrial and domestic sewage in 417
the Yangtze River delta area and observed that the content of 418
two- and three-ring PAHs was much higher than of four-, 419

t1.1 **Table 1** Recoveries of nonylphenol and PAHs obtained from six sewage sludges (in milligrammes per kilogramme) and percentages of individual
t1.2 PAHs related to ΣPAHs

t1.2	Sludge	A		B		C		D		E		F	
		mg kg ⁻¹	%	mg kg ⁻¹	%	mg kg ⁻¹	%	mg kg ⁻¹	%	mg kg ⁻¹	%	mg kg ⁻¹	%
t1.3													
t1.4	Nonylphenol	44	—	4.5	—	72	—	199	—	72	—	53	—
t1.5	Naphtalene	0.55	23.51	0.27	13.59	0.37	13.65	0.30	14.34	0.40	21.32	0.47	15.26
t1.6	Acenaphtylene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
t1.7	Acenaphtene	0.00	0.00	0.03	1.56	0.07	2.65	0.09	4.20	0.00	0.09	0.06	2.07
t1.8	Fluorene	0.00	0.00	0.08	3.74	0.18	6.55	0.22	10.28	0.00	0.20	0.11	3.48
t1.9	Phenanthrene	0.51	21.90	0.30	14.98	0.82	30.45	0.57	26.97	0.46	24.48	0.83	27.15
t1.10	Anthracene	0.36	15.28	0.20	9.90	0.25	9.19	0.23	11.15	0.24	12.87	0.65	21.19
t1.11	Fluoranthene	0.29	12.50	0.22	10.77	0.24	8.82	0.24	11.38	0.20	10.64	0.37	12.21
t1.12	Pyrene	0.57	24.39	0.32	15.92	0.44	16.36	0.42	19.85	0.37	19.79	0.30	9.71
t1.13	Benzo(a)anthracene	0.00	0.00	0.11	5.55	0.06	2.20	0.04	1.85	0.04	2.18	0.07	2.31
t1.14	Chrysene	0.00	0.00	0.11	5.34	0.00	0.00	0.00	0.00	0.00	0.00	0.04	1.27
t1.15	Benzo(b)fluoranthene	0.00	0.00	0.23	11.15	0.10	3.57	0.00	0.00	0.07	3.61	0.03	1.13
t1.16	Benzo(k)fluoranthene	0.00	0.00	0.05	2.49	0.18	6.56	0.00	0.00	0.10	5.12	0.13	4.23
t1.17	Benzo(a)pyrene	0.06	2.43	0.10	5.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
t1.18	Indeno(1,2,3-cd)pyrene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
t1.19	Dibenzo(a,h)anthracene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
t1.20	Benzo(g,h,i)perylene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
t1.21	ΣPAHs	2.35	100	2.02	100	2.68	100	2.10	100	1.88	100	3.05	100

Table 2 PAHs content (in per cent) in the sewage sludge samples studied

Sludge	A	B	C	D	E	F
2-rings PAHs	23.51	13.59	13.65	14.34	21.32	15.26
3-rings PAHs	37.17	30.18	48.84	52.59	37.35	53.89
4-rings PAHs	36.89	37.58	27.38	33.08	32.60	25.49
5-rings PAHs	2.43	18.65	10.13	0.00	8.73	5.35
6-rings PAHs	0.00	0.00	0.00	0.00	0.00	0.00

five-, and sing-ring PAHs. They concluded that the source of PAHs with ≥ 4 benzene rings is usually from combustion at very high temperature, whereas PAHs with two and three benzene rings originate from contamination of petroleum or petroleum products. Studies carried out by Oleszczko (2007, 2008) show that mainly four-ring PAHs predominated in five sewage sludges from Poland.

Nonylphenol

The concentration level of NP was highly variable in each WWTP, from 4.5 to 199 mg kg⁻¹ (Table 1). The highest concentrations of NP were found in sludge D followed by E and C. The differences in NP content can be explained by the high variability of influent wastewater composition and by the wide range of variables involved on each sludge treatment processes (González et al. 2010). Sludges C, D, E and F, which showed the highest NP content, are digested sludges, and it is known that during anaerobic digestion, the degradation of NPE to NP occurs, but NP persists (Minamiyama et al. 2006; Céspedes et al. 2008). The loss of weight achieved during the sludge dehydration process can also explain the high NP levels found in such sludge. The concentrations were lower in raw sludge (sludges A and B) than in digested, as observed also by Lian et al. (2009). Aparicio et al. (2009) found a higher NP level in anaerobically digested dehydrated sludge than in primary and secondary sludge and explained it by NP1EO and NP2EO degradation to NP.

The European Union (2000) limits concentration proposed in the third draft of the Working Document on Sludge for NP and NPE for land application of sewage sludge was 50 mg kg⁻¹. Table 1 shows that by only considering the NP content, four of the six sludges analysed exceed this maximum value, and, therefore, these sludges would not be apt to be used as fertilisers, and NP should be decreased for their further use in agriculture in order to preserve the environmental quality, considering the inherent toxicity of this contaminant.

Non-exhaustive PAHs and NP extraction using HPBCD solutions

A non-exhaustive extraction of PAHs and NP from sewage sludge with HPBCD solutions has been used as a possible

method for decontamination prior the sludge application to agricultural land.

PAHs

Figure 1 shows the percentages of individual PAHs extracted after three sequential extractions by the aqueous and HPBCD solutions from sludge E (Fig. 1a, b, respectively). The amount of PAHs removed by HPBCD extraction (Fig. 1b) was much higher than by aqueous extraction in its absence (Fig. 1a), although the percentages of PAHs removed varied depending on the individual PAH. When the aqueous solution was used, only naphthalene and phenanthrene were removed, their total extraction percentages were in general low (13 and 1.3 %, respectively), with little differences within the three extractions. Four- and five-ring PAHs were not extracted. HPBCD was more efficient as an extraction technique for PAHs (Fig. 1b). Not only were naphthalene and phenanthrene extracted in much higher percentages (33 and 15 %, respectively) but also anthracene (8 %) and some four-ring PAHs as well, such as fluoranthene and pyrene (although with extraction percentages lower than 2 %).

In order to confirm if only the type of PAH, and not their amounts present, or the aging process influences on their individual availability, the sludge E was spiked with 16 EPA-PAHs and the extraction with the aqueous and HPBCD solutions were also carried out on spiked E (Fig. 1c, d). When the aqueous solution was used (Fig. 1c), two- and three-ring PAHs (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene) were removed after three sequential extractions but at different percentages, (57, 10, 11, 5, 2 and 0.6 %, respectively). The results achieved were very different when HPBCD solution was applied (Fig. 1d). Naphthalene, acenaphthene and fluorene reached the highest extraction percentages (89, 39 and 48 %, respectively), but fluorene, phenanthrene and anthracene were the PAHs with the highest increase in extraction percentages in comparison to the aqueous solution: from 5, 2 and 0.6 % to 48, 32 and 36 % (that is 10, 16 and 60 times higher, respectively). Four-ring PAHs, such as fluoranthene, pyrene, benzo[a]anthracene and chrysene, which were not removed by the aqueous solution, showed total extraction percentages about 1–3 % with HPBCD solution. Five- and six-ring PAHs were not removed at all.

It is worth to emphasise the importance of HPBCD first extraction. The extraction percentages obtained for naphthalene, acenaphthylene and acenaphthene (47, 12 and 13 %, respectively) were similar or slightly higher than those obtained after the three sequential extractions using the aqueous solution (57, 10 and 11 %, respectively). In the case of fluorene, phenanthrene and anthracene, these differences were still higher: the extraction percentages obtained after the first extraction by HPBCD solution (19, 12 and 13 %,

Fig. 1 Percentage of individual PAHs extracted after three sequential extractions with the aqueous and HPBCD solutions from sludge E (a, b, respectively) and from spiked sludge E (c, d, respectively)

respectively) were much higher than the total extraction percentages obtained after three extractions with the aqueous solution (5.30, 1.62 and 0.56 %, respectively). In the case of four-ring PAHs (fluoranthene, pyrene, benzo[a]anthracene and chrysene), which were not removed by the aqueous solution, only with the first HPBCD extraction was it possible to remove 50 % of the total amount extracted after the three sequential extractions. This behaviour was also observed in the extraction of sludge E without spiking, commented previously. The first HPBCD extraction step importance has been observed by other authors (Villaverde et al. 2005b).

It is well known that PAHs present a low aqueous solubility. The higher the number of benzene rings, the higher their hydrophobicity and the lower their water solubility. For this reason, the use of water as an extractant agent in PAHs removal from contaminated sludge is relatively ineffective. The increase observed in PAHs extraction percentages after HPBCD in spiked and non-spiked sludge E is related to the fact that HPBCD molecules are able to form inclusion complexes enhancing the water solubility of organic compounds, such as PAHs, and therefore their extraction from polluted soils or sludge (Villaverde et al. 2005b; Gómez et al. 2010). The extraction percentages obtained were different for the PAHs studied depending on the appropriate size and shape of the PAH with respect to HPBCD hydrophobic cavity dimensions.

Although the extraction of naphthalene shows the highest percentage obtained (Fig. 1c, d), the increase with HPBCD in

relation to the aqueous solution was not very high (only 1.5 times higher). On the contrary, phenanthrene and anthracene were the PAHs which showed the highest increase in extraction percentages (from 2 to 33 % and from 0.6 to 36 %, respectively). This is related with the appropriate size and shape of the phenanthrene molecule (0.58 nm width and 0.78 nm length) with respect to HPBCD hydrophobic cavity dimensions (0.6 nm of diameter and 0.79 nm of depth). As a consequence, phenanthrene showed a best molecular accommodation in the hydrophobic cavity of HPBCD molecules than other PAHs. Anthracene also showed a high increase in extraction percentage with HPBCD solution. Its molecule presents a higher length and a lower width than phenanthrene (0.5 nm width and 0.92 nm length) that permits its inclusion in the cavity of HPBCD, but the distance between the molecule of anthracene and the active points of HPBCD is not so close as in the case of phenanthrene.

The extremely lower extraction efficiency observed in the case of 4-rings PAHs (fluoranthene, pyrene, benzo[a]anthracene and chrysene) may be attributable in part to a lower availability of the contaminants (which present a lower water solubility) for being transferred to the aqueous phase due to the high tendency to remain adsorbed on the hydrophobic surfaces of the sludge. Another influencing factor may be that they present a width higher than that of the HPBCD cavity; for this reason, only a small part of the molecules of these compounds are included into the HPBCD cavity when the inclusion complex is formed, and a great part remains in

contact with water, reducing their extraction percentage due to stearic restraints (Semple et al. 2007).

The sequence of the extraction percentages obtained for six of the PAHs extracted (acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene) is in agreement with the sequence of solubility efficiency (Se) and the apparent formation constants (Kc) obtained by Morillo et al. (2012) who evaluated the influence of the three different CDs (including HPBCD) on the solubility of these PAHs selected among those 16 proposed by EPA as the most frequently occurring and/or dangerous in environmental samples. It was found that phenanthrene showed the highest Se and Kc values when they formed inclusion complexes with HPBCD, followed in decreasing order by anthracene, fluorene, fluoranthene, acenaphthene and pyrene. They concluded that these results were related with the molecular volume of the PAH and the dimensions of CD cavity, and as consequence, the points of interaction between the PAHs and the CDs.

In spite of the different individual PAHs content of sludge E in comparison with spiked sludge E, the tendency in the extraction by HPBCD is the same: the two- and three-ring PAHs are much favoured than those of the four-ring compounds. Taking into account that sludge from WWTPs contain also fluoranthene and pyrene (four-ring PAHs), it would be necessary to use a mix of HPBCD with another cyclodextrin with a high cavity dimensions, such as γ -CD or their derivatives, in order to extract effectively such compounds. On the other hand, the ageing of sludge E in comparison with spiked sludge E is another important factor. The PAHs present in the aged sludge have become sequestered within the structure of the sludge, in particular in the organic matter fraction, and therefore, their extractability is expected to be lower. Many research studies have shown that when the soil-pollutant contact time increases the biological and chemical availability of contaminants decrease (Villaverde 2007; McAllister and Semple 2010). That is probably the reason of the lower percentages extracted by HPBCD from sludge E in comparison to spiked sludge E: 33, 15 and 8 % instead of 89, 32 and 36 %, for naphthalene, phenanthrene and anthracene, respectively. However, in the case of 4-rings PAHs the percentages obtained were very similar and very low (< 3 %), indicating that HPBCD has a lower tendency to extract such PAHs.

The bioavailable fraction of contaminants in soils sediments and sludge (and not their total content) is the main factor determining their fate in the environment and their influence on living organisms. For this reason, several chemical techniques capable of reliably predicting the bioavailability of organic contaminants to biota have been studied. Oleszczuk (2008, 2009) used three different extractants, Tenax-TA, *n*-butanol or HPBCD, to determine the content of the bioavailable fraction of PAHs in sewage sludge,

observing a significant positive relationship between Tenax-TA-extracted fraction and growth inhibition of *Heterocypris incongruens*, whereas when using *n*-butanol or HPBCD, the content of the extracted fraction was usually twice higher. That is, higher amounts of PAHs were extracted with HPBCD than those actually bioavailable. Then the extraction of PAHs reached in this paper using HPBCD was more than enough to obtain a decontaminated sludge since the rest of PAHs not extracted are not bioavailable and, therefore, not potentially toxic to the environment.

Nonylphenol

The total content of NP in sludge E was 72 mg kg⁻¹ (Table 1), higher than the limit value proposed for the sum of all the NP ethoxylates compounds by the Draft Document of the European Union which was 50 mg kg⁻¹. This means that before this sewage sludge can be used as fertiliser for agricultural land, NP content has to be reduced.

The extraction percentage of NP from sludge E by the aqueous solution after the three sequential extractions was lower than 1 % (Fig. 2a), with no differences between the amount of NP extracted after each of the three sequential extractions. However, when HPBCD solution was used as extraction technique, 105±14 % of the NP was extracted. It is important to emphasise that with only the first extraction, 89 % of NP was removed and the other two extractions increased until 100 %. Hence, only one extraction, using HPBCD solution, would be sufficient to reduce NP content in a highly contaminated sludge under the limit value proposed by UE.

Fig. 2 Percentage of NP extracted after three sequential extractions by the aqueous (a) and HPBCD (b) solutions from sludge E

The results obtained in the present study are in agreement with the research studies by Kawasaki et al. (2001) since they showed that NP solubility (aqueous solubility 5.43 mg L^{-1}) increased more than 6,000 times when HPBCD was used in a particular range of concentrations (0–60 mM). This increase is due to the fact that NP molecules, which have a smaller size than HPBCD cavity, can be included within such hydrophobic cavity and form inclusion complexes. As result, NP hydrophilicity and water solubility increase, and therefore its removal from contaminated sites occurs. This is the reason why the extraction of the sludge using HPBCD gives as such extremely high recovery result of NP in the leachates, and, therefore, a low final content of NP is reached in the treated sludge.

Toxicity of leachates

The removal of PAHs and NP from sludge by HPBCD could be a good alternative to existing sludge treatment methods which are basically aimed to reduce the water content. The decontamination method we proposed in this study removes the contamination load, especially for NP, and sludge may easily obey with the limit values proposed by the European Union. In this sense, the procedure could be of great importance for improving the sludge disposal alternatives in terms of environmental safety and economic costs. It is also important to determine whether the leachates containing the contaminants possess environmental hazard. Therefore, to further test the potential environmental hazards of CDs leachates to aquatic organisms, the toxicity of sludge HPBCD and water leachates were assessed using *D. magna* feeding responses (Fig. 3), a model organism to test aquatic toxicity (Barata et al. 2008).

In all tests, *Daphnia* mortality was absent. HPBCD diluted 1 and 10 % in ASTM water did not affect significantly ($P < 0.05$) proportional feeding rates relative to controls (Fig. 3). This result confirms that HPBCD does not impair any effect on *Daphnia*. Sludge HPBCD and water leachates diluted 1 and 10 % in ASTM hard water inhibited significantly ($P < 0.05$) *Daphnia* feeding rates relative to their respective controls (Fig. 3). It is important to emphasise that both, water and HPBCD sludge leachates had similar toxicity, despite the different amount of contaminants present in each of them. Water leachates diluted at 10 % had higher toxicity than at 1 %, as expected.

According to Figs. 1 and 2 and Table 1, water leachates diluted 1 and 10 % should contain $0.04\text{--}0.39 \text{ } \mu\text{g L}^{-1}$ NP, and those of HPBCD, 13 and $130 \text{ } \mu\text{g L}^{-1}$. Estimated levels of PAHs in both water and cyclodextrin leachates were low ($0.17, 0.10, 0.57$ and $0.13 \text{ } \mu\text{g L}^{-1}$ for 10 % HPBCD leachates of pyrene, fluoranthene, phenanthrene or naphthalene, respectively). The combined presence of these compounds in water and HPBCD leachates, however, caused similar effects

Fig. 3 Feeding responses of *D. magna* juveniles exposed to pure ASTM hard water (W) and HPBCD (HPBCD) and their sludge leachates (sludges W and HPBCD) diluted 1 and 10 % in ASTM hard water. Results are expressed as proportional responses (mean $\pm$ SE) relative to W controls. Different letters indicate significant ($P < 0.05$) differences across treatments after one-way ANOVA and post hoc Tukey's multiple comparison tests

on *Daphnia* feeding rates, as evidenced in Fig. 3. Previous studies indicate that effects of naphthalene in *Daphnia* occur only above a milligramme-per-litre levels, pyrene and fluoranthene above $10 \text{ } \mu\text{g L}^{-1}$ and phenanthrene above $40 \text{ } \mu\text{g L}^{-1}$ (Olmstead and LeBlanc 2005). For NP, however, sublethal detrimental effects on feeding and reproduction occur at concentrations greater than $80 \text{ } \mu\text{g L}^{-1}$ (Sun and Gu 2005; Campos et al. 2012), being lethal above $300\text{--}400 \text{ } \mu\text{g L}^{-1}$ (Comber et al. 1993). Accordingly, NP residues present in HPBCD sludge leachates should impair *Daphnia* feeding rates, whereas those measured in water sludge leachates did not. The fact that we found similar toxic effects for both water and HPBCD leachates means that NP residues present in HPBCD leachates were not bioavailable to *Daphnia*. Indeed, it is likely that HPBCD encapsulated NP making it less bio-available to *Daphnia*. Such ameliorated toxic effects of NP have been described for humic substances (Sun and Gu 2005; Burgess et al. 2005). Our results indicate further that observed toxicity was due to water soluble compounds not removed by HPBCD. However, although the leachates produced have been demonstrated not toxic due to the contaminants extracted by HPBCD, they should be further treated within the WWTP in order to avoid the dispersion of such contaminants in the environment. For this reason, HPBCD should be considered as a useful tool in sewage sludge decontamination treatments to reduce for example compounds such as NP and generate non toxic leachates. In this way, sludge highly contaminated with NP

or PAHs could be treated with HPBCD generating a contaminant-free sludge with added value, which may amplify its use in agronomical practices.

Conclusions

Given the more restricted concentration limit values for some organic compounds in sewage sludge included in the draft of the European Union regulations, a preliminary study using HPBCD extractions has been proposed as a method for the decontamination of sludge. Among other contaminants, PAHs and NP are toxic compounds that are present in high concentrations in sludge and are prioritised by the European Union since their presence can have serious environmental implications because of their application to agricultural soils at doses that may produce long-term contamination of soil and groundwater. In this study, it has been demonstrated that these compounds are able to form inclusion complexes with HPBCD and increase their solubility. It has been shown that the application of HPBCD solutions to sludge can enhance the contaminants desorption and decontamination, at expenses of generating a leachate containing the contaminants released. We propose the use of HPBCD as a very effective tool for the remediation of contaminated sewage sludge, increasing its safe usage for agricultural practices without posing environmental or health risks. We also suggest that the leachate, although demonstrated not toxic because of those contaminants extracted by HPBCD, such as NP or PAHs, should not be released to receiving waters but rather be treated in the WWTP to avoid their dispersion in the environment. Aspects related to the optimisation of HPBCD extractions for the sludge decontamination of multiclass contaminants present in the WWTPs and evaluation of leachates in terms of contamination loads, toxicity and possible decontamination treatments will be deeply investigated in further research.

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AUTHOR QUERIES

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