

Metadata of the article that will be visualized in OnlineFirst

1	Article Title	Decontamination of polycyclic aromatic hydrocarbons and nonylphenol from sewage sludge using hydroxypropyl-β-cyclodextrin and evaluation of the toxicity of leachates
2	Article Sub- Title	
3	Article Copyright - Year	Springer-Verlag Berlin Heidelberg 2013 (This will be the copyright line in the final PDF)
4	Journal Name	Environmental Science and Pollution Research
5		Family Name Morillo
6		Particle
7		Given Name Esmeralda
8		Suffix
9	Corresponding Author	Organization Institute of Natural Resources and Agrobiology of Seville (IRNAS-CSIC)
10		Division
11		Address Apdo. 1052, Seville 41080, Spain
12		e-mail morillo@imase.csic.es
13		Family Name Sánchez-Trujillo
14		Particle
15		Given Name Ma. Antonia
16		Suffix
17	Author	Organization Institute of Natural Resources and Agrobiology of Seville (IRNAS-CSIC)
18		Division
19		Address Apdo. 1052, Seville 41080, Spain
20		e-mail
21		Family Name Lacorte
22		Particle
23		Given Name Silvia
24		Suffix
25	Author	Organization Department of Environmental Chemistry, (IDAEA-CSIC)
26		Division
27		Address Jordi Girona, 18-26, Barcelona 08034, Spain

28		e-mail	
29		Family Name	Villaverde
30		Particle	
31		Given Name	Jaime
32		Suffix	
33	Author	Organization	Institute of Natural Resources and Agrobiology of Seville (IRNAS-CSIC)
34		Division	
35		Address	Apdo. 1052, Seville 41080, Spain
36		e-mail	
37		Family Name	Barata
38		Particle	
39		Given Name	Carlos
40		Suffix	
41	Author	Organization	Department of Environmental Chemistry, (IDAEA-CSIC)
42		Division	
43		Address	Jordi Girona, 18-26, Barcelona 08034, Spain
44		e-mail	
45		Received	23 February 2013
46	Schedule	Revised	
47		Accepted	11 June 2013
48	Abstract	<p>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</p>	

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
50	Foot note information	Responsible editor: Philippe Garrigues

Decontamination of polycyclic aromatic hydrocarbons and nonylphenol from sewage sludge using hydroxypropyl- β -cyclodextrin and evaluation of the toxicity of leachates

Ma. Antonia Sánchez-Trujillo · Silvia Lacorte · Jaime Villaverde · Carlos Barata · Esmeralda Morillo

Received: 23 February 2013 / Accepted: 11 June 2013
© Springer-Verlag Berlin Heidelberg 2013

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.

Responsible editor: Philippe Garrigues

M. A. Sánchez-Trujillo · J. Villaverde · E. Morillo (✉)
Institute of Natural Resources and Agrobiological of Seville (IRNAS-CSIC), Apdo. 1052, 41080 Seville, Spain
e-mail: morillo@imase.csic.es

S. Lacorte · C. Barata
Department of Environmental Chemistry, (IDAEA-CSIC),
Jordi Girona, 18-26, 08034 Barcelona, Spain

68 This Directive controls the quality of sludge, the amended
69 soils, the loading rate and the crops that may be grown on
70 treated land. This Directive requires that heavy metal con-
71 tents (Cd, Cr, Cu, Ni, Pb and Zn) in both the sludge and soil
72 comply with the established limits, but it does not yet estab-
73 lish maximum values for organic contaminants.

74 To limit the concentration for certain organic pollutants in
75 both sewage sludge and sludge-amended soils, the European
76 Union (2000) launched a working document on sludge that
77 although not being a formal regulation, the third draft of this
78 document limits the values for some organic compounds,
79 including polycyclic aromatic hydrocarbons (PAHs; the sum
80 of phenanthrene, fluorene, fluoranthene, pyrene, benzo(b)
81 fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo
82 (ghi)perylene, and indeno(1,2,3-c,d)pyrene) and the sum of
83 nonylphenol (NP) and nonylphenol mono (NP1EO) and di-
84 ethoxylates (NP2EO; referred as NPE). Concentration limits
85 fixed in this Directive draft for land application of sludge are
86 50 mg kg^{-1} dry matter for NPE and 6 mg kg^{-1} for the sum of
87 these nine PAHs.

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

98 On the other hand, NP and NPE are used as nonionic
99 surfactants in a large variety of industrial and domestic
100 applications (Navarro et al. 2009). Although the use of
101 NPEs has been banned under Council Directive 2003/53/
102 EC (European Union 2003), their environmental presence
103 and risk is still high because of its historical and pervasive
104 widespread use. Industrial and urban wastewaters and agri-
105 cultural run-off discharge to WWTPs where the biodegrada-
106 tion of NPEs leads to the decrease in the length of ethoxy
107 chain and an increase of hydrophobicity (Ifelebuegu 2011).
108 This is why NP is the main alkylphenol associated with
109 sewage sludge (90 %; Soares et al. 2008). Considering its
110 endocrine disrupting properties, NP in sludge may impair
111 environmental effects (Fernández-Sanjuán et al. 2009). The
112 application of sewage sludge as soil fertiliser may produce
113 effects to soil organisms or contaminate groundwater. To
114 determine the content of PAHs and NP in sewage sludge will
115 therefore be the first step to consider before their application
116 to agricultural soils, in order to know the need of using
117 technological treatments in the sludge management to reduce
118 the concentration of these organic pollutants to levels that
119 allow land application of sewage sludge according to the
120 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 concen-
tration 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 avail-
ability to be desorbed from soils, sediments or sludge, de-
creasing 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 en-
hance the water solubility of hydrophobic compounds and
their removal from polluted sites, while minimising envi-
ronmental impact (Petitgirard et al. 2009). CDs are
polycyclic glucose oligosaccharides resulting from enzy-
matic 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 chem-
ical properties of CDs combined with their non-toxic char-
acter to humans have led to their use in pharmaceuticals, as
food additives, as well as in the environmental decontami-
nation 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, pesti-
cides 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 et al. 2010) or pesticides (Hartnik et al. 2008).	220
175		221
176	In this study, three specific objectives were proposed: (1)	
177	to analyse the content of 16 PAHs (included in the US	
178	Environmental Protection Agency (US EPA) priority pollut-	
179	ants list) and NP in six sewage sludge from WWTPs from	
180	NE Spain, (2) to carry out a non-exhaustive extraction of	
181	PAHs and NP from sewage sludge with HPBCD as a possi-	
182	ble method for sludge decontamination and (3) to study the	
183	toxicological risk of the leachates obtained after HPBCD	
184	extractions.	
185	Experimental	
186	Chemicals and reagents	
187	Sixteen PAHs considered of primary environmental concern	
188	according to the US EPA were analysed: naphthalene,	
189	acenaphthylene, acenaphthene, fluorene, phenanthrene, an-	
190	thracene, fluoranthene, pyrene, benzo[a]anthracene, chrys-	
191	ene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]	
192	pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene and	
193	benzo[ghi]perylene. They were purchased from Supelco as	
194	mix solution of 200 mg L ⁻¹ in methanol. The surrogate	
195	standard was a mixture containing naphthalene d-8,	
196	acenaphthene d-10, phenanthrene d-10, chrysene d-12 and	
197	perylene d-12, purchased from Supelco as a solution of	
198	200 mg L ⁻¹ in methanol. Anthracene d-10, from Supelco,	
199	was also used as internal standard. 4-NP was supplied by Dr.	
200	Ehrenstorfer (Cromlab, Spain). 2-HPBCD was supplied by	
201	Cyclolab (Budapest, Hungary). Solvents were supplied by	
202	Merck (Germany), alumina SPE cartridges of 5 g by the	
203	International Sorbent Technology (UK), florisil SPE from	
204	Waters (USA) and nitrogen, for drying with 99.995 % of	
205	purity, by Air Liquid (Spain).	
206	Sample collection and preparation	
207	Six sewage sludge samples were collected from different	
208	WWTPs located in Northeast Spain and designated as A,	
209	B, C, D, E and F. Sludges A and B were from WWTPs	
210	located in little urban areas and sludge was not digested.	
211	Sludges C–F were collected in WWTPs of industrial and	
212	highly urbanised areas and were anaerobic digested. One	
213	kilogramme of sample was collected from the sludge tanks	
214	using precleaned amber glass pots and were transported	
215	refrigerated to the laboratory. Sludge samples were placed	
216	in glass pots, frozen at -18 °C and lyophilised during 48 h	
217	(10 ⁻² mbar vacuum) in a freeze dryer (Lioalfa, Telstar,	
218	Spain). Afterwards, samples were sieved through 500 µm	
219	mesh to obtain a homogeneous material. Samples were kept	
	at -2 °C until their characterisation for PAHs and NP con-	220
	centration and all other experiments.	221
	Analysis of PAHs content in sewage sludge	222
	Sludge samples were spiked with the surrogate standards at a	223
	concentration of 2 mg kg ⁻¹ ; 0.5 g of sludge were inserted in a	224
	glass tube with 10 mL hexane/dichloromethane (1:1, v/v) and	225
	placed in an ultrasonic bath for 10 min. The solution was	226
	then centrifuged (10 min at 1,090×g; Beckman Coulter	227
	Avanti J-25I). The two last steps were repeated three times	228
	using fresh solvent. The sonicated extracts were evaporated	229
	in a Turbo Vap LV from Caliper Life Sciences to almost	230
	dryness (1 mL approximately) for further clean up.	231
	The extracts were purified using solid-phase extrac-	232
	tion (SPE) cartridges of neutral alumina of 5 g. The alu-	233
	mina was solvated and conditioned with 40 mL hexane/	234
	dichloromethane (1:1, v/v). Analyte elution was performed	235
	with 40 mL hexane–dichloromethane (1:1, v/v). The extract	236
	was concentrated to a volume of less than 1 mL by a Turbo	237
	Vap LV evaporator, transferred into an amber glass vial and	238
	reconstituted with hexane to a final volume of 1 mL. At this	239
	stage, anthracene-d ₁₀ was added as an internal standard to give	240
	a concentration of 1 µg mL ⁻¹ . Blanks were prepared at the	241
	same time under the same conditions. With these conditions, a	242
	good recovery was obtained for PAHs, but NP was poorly	243
	recovered with alumina SPE. Therefore, a parallel extraction	244
	and clean-up was performed with florisil to enhance the	245
	recovery of NP.	246
	Analysis of NP content in sewage sludge	247
	NP analysis was carried out according to the method	248
	proposed by Fernández-Sanjuán et al. (2009). Deuterated	249
	standard 4-n-NP-D8 was added to a sample aliquot of 0.1 g	250
	of the sieved sludge at 0.5 µg/g. Samples were homogenised	251
	and kept at room temperature overnight and subsequently	252
	extracted by sonication (10 min) with 5 mL of hexane/	253
	dichloromethane (1:1, v/v) and repeated a second time. A third	254
	extraction was performed with 5 mL of dichloromethane/	255
	acetone (1:1, v/v). After each extraction step, samples were	256
	centrifuged for 10 min and the extracts were combined and	257
	evaporated in a Turbo Vap LV to almost dryness. Extracts	258
	were subsequently cleaned up by SPE cartridges with	259
	5 g of florisil, conditioned with 20 mL of hexane/	260
	dichloromethane (1:1, v/v) and 20 mL of dichloromethane/	261
	acetone (1:1, v/v). The sample extract was eluted with 20 mL	262
	of hexane/dichloromethane (1:1, v/v) and 20 mL of	263
	dichloromethane/acetone (1:1, v/v). The eluent was evaporat-	264
	ed to almost dryness and reconstituted with ethyl acetate to a	265
	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;	318
271	Agilent GC 6890N) coupled to a quadrupole mass spectrom-	319
272	eter (MS; Agilent MD 5975B). The system was operated in	320
273	electron ionisation (70 eV). The separation was achieved	321
274	with a 30×0.25-mm I.D. DB-5 MS column (J&W	322
275	Scientific, Agilent Technologies) coated with 5 % phenyl-	323
276	methylpolysiloxane (film thickness, 0.25 µm). The oven	324
277	temperature was programmed from 80 (holding time,	325
278	1 min) to 175 °C at 6 °C/min (holding time, 4 min), to	326
279	235 °C at 3 °C/min and finally to 320 °C at 8 °C/min,	327
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	<i>Batch extraction experiments</i> 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(NO ₃) ₂ to maintain the ionic strength and 200 mg L ⁻¹	
308	HgCl ₂ 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(NO ₃) ₂ (0.01 M) and HgCl ₂	
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	318
	hexane, and 1 mL of this organic phase was transferred into	319
	amber glass vials. The rest of the supernatant was decanted	320
	and then 10 mL of fresh aqueous or HPBCD solution were	321
	added. The extractions were carried out three times in total,	322
	and each extract was analysed individually. One millilitre of	323
	the hexane extract was dosed in a vial, and at this stage,	324
	anthracene-d ₁₀ was added to a concentration of 1 µg mL ⁻¹	325
	and analysed by GC-MS as previously described.	326
		327
	Toxicity evaluation of leachates	328
	In order to study the toxicity of the leachates obtained after the	329
	extraction of PAHs and NP from sewage sludge using aqueous	330
	and HPBCD solutions, parallel extraction experiments were	331
	performed but without Ca(NO ₃) ₂ and HgCl ₂ since these com-	332
	pounds would affect the condition of <i>Daphnia</i> . The leachates	333
	used in these experiments were obtained as described: 0.5 g of	334
	sludge E were inserted in 100 mL amber glass bottles with	335
	25 mL of ASTM hard water (ASTM 1999). In parallel, 0.5 g	336
	of sludge E were inserted in the 100 mL amber glass bottles	337
	with 25 mL of 10 mM HPBCD solution prepared in hard	338
	water. The suspensions were shaken for 1 h at a constant	339
	temperature of 20 °C. After separation by decantation, the	340
	leachates obtained were kept at 4 °C.	341
	Bioassays with the grazer <i>Daphnia magna</i> were	342
	conducted to evaluate sub-lethal feeding effects of filtered	343
	fractions of leachates. Feeding responses to sludge leachates	344
	diluted 1 and 10 % in ASTM hard water in 24 h toxicity tests	345
	were performed following Barata et al. (2008). Groups of	346
	five neonates were exposed to 20 mL of test concentrations	347
	in 30 mL borosilicate flasks in the presence of food. The alga	348
	<i>Chlorella vulgaris</i> was added at a concentration of 5×10 ⁵	349
	cells/mL ⁻¹ (equivalent to 1.5 µg C mL ⁻¹). Treatments	350
	consisted of an ASTM hard water control (W), 10 mM	351
	HPBCD solution controls and the selected leachates with	352
	five replicates each. Each group of replicates consisted of	353
	five vessels with animals and one blank. Blanks were used to	354
	assure that initial algal concentrations did not increase sig-	355
	nificantly over the exposure period. Individual feeding rates	356
	(number of algal cells ingested per animal per hour) were	357
	determined as the change in cell density during 24 h	358
	according to the method described by Barata et al. (2008)	359
	and converted to proportional feeding rates relative to ASTM	360
	W. Cell density was estimated from absorbance measure-	361
	ments at λ=650 nm using standard calibration curves based	362
	on at least 20 data points (r ² >0.98). Proportional responses	363
	were arcsine transformed to meet ANOVA assumptions of	364
	normality and variance homocedasticity (Zar 1996).	365
	Differences in bioassay responses among treatments were	366
	compared by one-way ANOVA following post hoc Tukey's	367
	test at a p<0.05 significance level.	368

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 Σ_{PAHs} 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. Σ_{PAHs} 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 Σ_{PAHs}
 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 PAHs related to Σ_{PAHs}

t1.2	Sludge	A		B		C		D		E		F	
t1.3		mg kg ⁻¹	%	mg kg ⁻¹	%	mg kg ⁻¹	%	mg kg ⁻¹	%	mg kg ⁻¹	%	mg kg ⁻¹	%
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	Σ _{PAHs}	2.35	100	2.02	100	2.68	100	2.10	100	1.88	100	3.05	100

t2.1 **Table 2** PAHs content (in per cent) in the sewage sludge samples studied

t2.2	Sludge	A	B	C	D	E	F
t2.3	2-rings PAHs	23.51	13.59	13.65	14.34	21.32	15.26
t2.4	3-rings PAHs	37.17	30.18	48.84	52.59	37.35	53.89
t2.5	4-rings PAHs	36.89	37.58	27.38	33.08	32.60	25.49
t2.6	5-rings PAHs	2.43	18.65	10.13	0.00	8.73	5.35
t2.7	6-rings PAHs	0.00	0.00	0.00	0.00	0.00	0.00

420 five-, and sing-ring PAHs. They concluded that the source of
 421 PAHs with ≥4 benzene rings is usually from combustion at
 422 very high temperature, whereas PAHs with two and three
 423 benzene rings originate from contamination of petroleum or
 424 petroleum products. Studies carried out by Oleszczku (2007,
 425 2008) show that mainly four-ring PAHs predominated in five
 426 sewage sludges from Poland.

427 *Nonylphenol*

428 The concentration level of NP was highly variable in each
 429 WWTP, from 4.5 to 199 mg kg⁻¹ (Table 1). The highest
 430 concentrations of NP were found in sludge D followed by E
 431 and C. The differences in NP content can be explained by the
 432 high variability of influent wastewater composition and by the
 433 wide range of variables involved on each sludge treatment
 434 processes (González et al. 2010). Sludges C, D, E and F,
 435 which showed the highest NP content, are digested sludges,
 436 and it is known that during anaerobic digestion, the degrada-
 437 tion of NPE to NP occurs, but NP persists (Minamiyama et al.
 438 2006; Céspedes et al. 2008). The loss of weight achieved
 439 during the sludge dehydration process can also explain the
 440 high NP levels found in such sludge. The concentrations were
 441 lower in raw sludge (sludges A and B) than in digested, as
 442 observed also by Lian et al. (2009). Aparicio et al. (2009)
 443 found a higher NP level in anaerobically digested dehydrated
 444 sludge than in primary and secondary sludge and explained it
 445 by NP1EO and NP2EO degradation to NP.

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

455 Non-exhaustive PAHs and NP extraction using HPBCD
 456 solutions

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

method for decontamination prior the sludge application to 459
 agricultural land. 460

PAHs 461

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

479 In order to confirm if only the type of PAH, and not their
 480 amounts present, or the aging process influences on their
 481 individual availability, the sludge E was spiked with 16 EPA-
 482 PAHs and the extraction with the aqueous and HPBCD
 483 solutions were also carried out on spiked E (Fig. 1c, d).
 484 When the aqueous solution was used (Fig. 1c), two- and
 485 three-ring PAHs (naphthalene, acenaphthylene, acenaphthene,
 486 fluorene, phenanthrene and anthracene) were removed after
 487 three sequential extractions but at different percentages, (57,
 488 10, 11, 5, 2 and 0.6 %, respectively). The results achieved
 489 were very different when HPBCD solution was applied
 490 (Fig. 1d). Naphthalene, acenaphthene and fluorene reached
 491 the highest extraction percentages (89, 39 and 48 %, respec-
 492 tively), but fluorene, phenanthrene and anthracene were the
 493 PAHs with the highest increase in extraction percentages in
 494 comparison to the aqueous solution: from 5, 2 and 0.6 % to
 495 48, 32 and 36 % (that is 10, 16 and 60 times higher, respec-
 496 tively). Four-ring PAHs, such as fluoranthene, pyrene,
 497 benzo[a]anthracene and chrysene, which were not removed
 498 by the aqueous solution, showed total extraction percentages
 499 about 1–3 % with HPBCD solution. Five- and six-ring PAHs
 500 were not removed at all.

501 It is worth to emphasise the importance of HPBCD first
 502 extraction. The extraction percentages obtained for naphtha-
 503 lene, acenaphthylene and acenaphthene (47, 12 and 13 %, 504
 505 respectively) were similar or slightly higher than those
 506 obtained after the three sequential extractions using the
 507 aqueous solution (57, 10 and 11 %, respectively). In the case
 508 of fluorene, phenanthrene and anthracene, these differences
 509 were still higher: the extraction percentages obtained after
 510 the first extraction by HPBCD solution (19, 12 and 13 %, 509

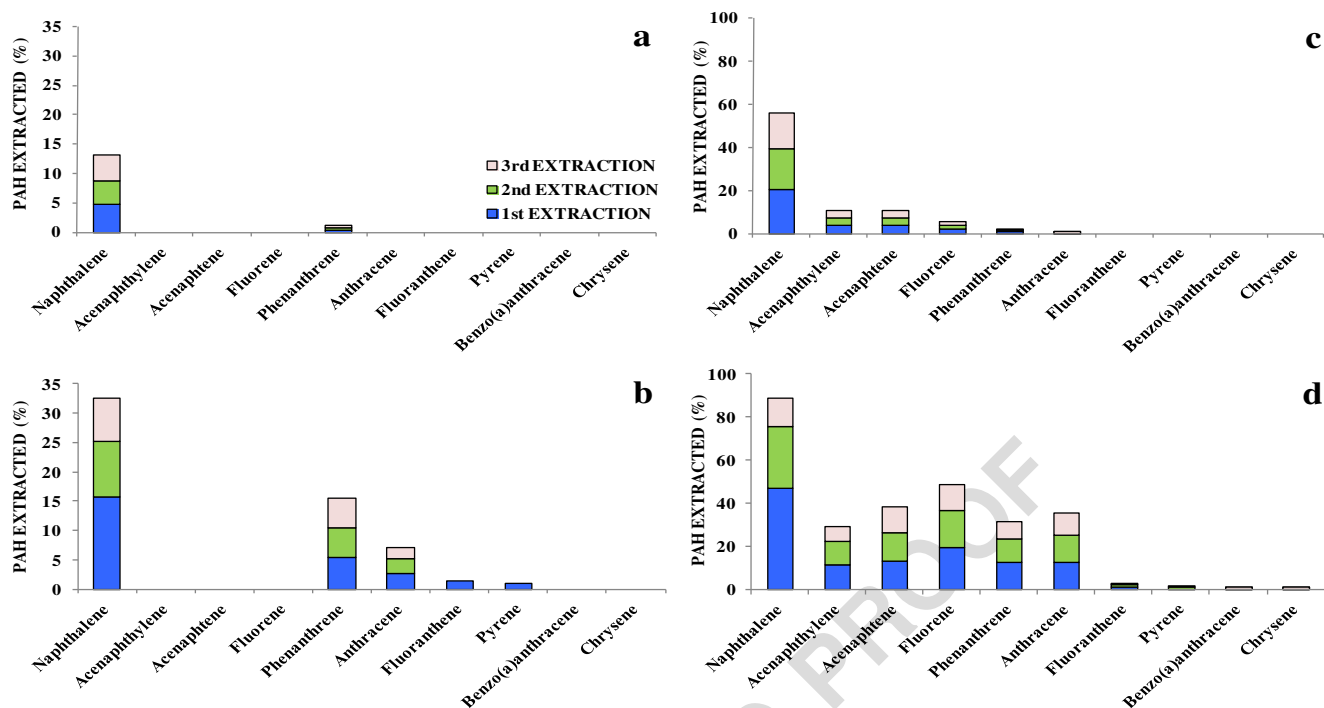


Fig. 1 Percentage of individual PAHs extracted after three sequential extractions with the aqueous and HPBCD solutions from sludge E (a, b, respectively) and from spiked sludge E (c, d, respectively)

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

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

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

relation to the aqueous solution was not very high (only 1.5
 538 times higher). On the contrary, phenanthrene and anthracene
 539 were the PAHs which showed the highest increase in extrac-
 540 tion percentages (from 2 to 33 % and from 0.6 to 36 %, re-
 541 spectively). This is related with the appropriate size and
 542 shape of the phenanthrene molecule (0.58 nm width and
 543 0.78 nm length) with respect to HPBCD hydrophobic cavity
 544 dimensions (0.6 nm of diameter and 0.79 nm of depth). As a
 545 consequence, phenanthrene showed a best molecular accom-
 546 modation in the hydrophobic cavity of HPBCD molecules
 547 than other PAHs. Anthracene also showed a high increase in
 548 extraction percentage with HPBCD solution. Its molecule
 549 presents a higher length and a lower width than phenanthrene
 550 (0.5 nm width and 0.92 nm length) that permits its inclusion
 551 in the cavity of HPBCD, but the distance between the mol-
 552 ecule of anthracene and the active points of HPBCD is not so
 553 close as in the case of phenanthrene.
 554

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

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

568 The sequence of the extraction percentages obtained for
569 six of the PAHs extracted (acenaphthene, fluorene, phenan-
570 threne, anthracene, fluoranthene and pyrene) is in agreement
571 with the sequence of solubility efficiency (Se) and the ap-
572 parent formation constants (Kc) obtained by Morillo et al.
573 (2012) who evaluated the influence of the three different
574 CDs (including HPBCD) on the solubility of these PAHs
575 selected among those 16 proposed by EPA as the most
576 frequently occurring and/or dangerous in environmental
577 samples. It was found that phenanthrene showed the highest
578 Se and Kc values when they formed inclusion complexes
579 with HPBCD, followed in decreasing order by anthracene,
580 fluorene, fluoranthene, acenaphthene and pyrene. They con-
581 cluded that these results were related with the molecular
582 volume of the PAH and the dimensions of CD cavity, and
583 as consequence, the points of interaction between the PAHs
584 and the CDs.

585 In spite of the different individual PAHs content of sludge
586 E in comparison with spiked sludge E, the tendency in the
587 extraction by HPBCD is the same: the two- and three-ring
588 PAHs are much favoured than those of the four-ring com-
589 pounds. Taking into account that sludge from WWTPs con-
590 tain also fluoranthene and pyrene (four-ring PAHs), it would
591 be necessary to use a mix of HPBCD with another cyclodex-
592 trin with a high cavity dimensions, such as γ -CD or their
593 derivatives, in order to extract effectively such compounds. On
594 the other hand, the ageing of sludge E in comparison with
595 spiked sludge E is another important factor. The PAHs pres-
596 ent in the aged sludge have become sequestered within the
597 structure of the sludge, in particular in the organic matter
598 fraction, and therefore, their extractability is expected to be
599 lower. Many research studies have shown that when the soil-
600 pollutant contact time increases the biological and chemical
601 availability of contaminants decrease (Villaverde 2007;
602 McAllister and Semple 2010). That is probably the reason
603 of the lower percentages extracted by HPBCD from sludge E
604 in comparison to spiked sludge E: 33, 15 and 8 % instead of
605 89, 32 and 36 %, for naphthalene, phenanthrene and anthra-
606 cene, respectively. However, in the case of 4-rings PAHs
607 the percentages obtained were very similar and very low
608 (< 3 %), indicating that HPBCD has a lower tendency to
609 extract such PAHs.

610 The bioavailable fraction of contaminants in soils sedi-
611 ments and sludge (and not their total content) is the main
612 factor determining their fate in the environment and their
613 influence on living organisms. For this reason, several chem-
614 ical techniques capable of reliably predicting the bioavail-
615 ability of organic contaminants to biota have been studied.
616 Oleszczuk (2008, 2009) used three different extractants,
617 Tenax-TA, *n*-butanol or HPBCD, to determine the content
618 of the bioavailable fraction of PAHs in sewage sludge,

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

Nonylphenol

629
630 The total content of NP in sludge E was 72 mg kg⁻¹
631 (Table 1), higher than the limit value proposed for the sum
632 of all the NP ethoxylates compounds by the Draft Document
633 of the European Union which was 50 mg kg⁻¹. This means
634 that before this sewage sludge can be used as fertiliser for
635 agricultural land, NP content has to be reduced.

636 The extraction percentage of NP from sludge E by the
637 aqueous solution after the three sequential extractions was
638 lower than 1 % (Fig. 2a), with no differences between the
639 amount of NP extracted after each of the three sequential
640 extractions. However, when HPBCD solution was used as
641 extraction technique, 105±14 % of the NP was extracted.
642 It is important to emphasise that with only the first extraction,
643 89 % of NP was removed and the other two extractions
644 increased until 100 %. Hence, only one extraction, using
645 HPBCD solution, would be sufficient to reduce NP content
646 in a highly contaminated sludge under the limit value pro-
647 posed by UE.

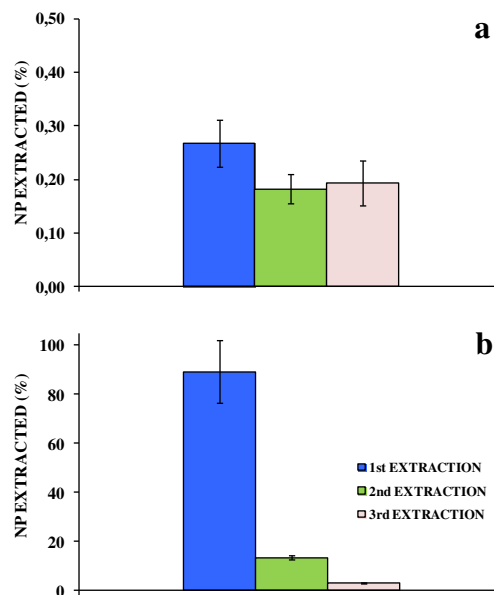


Fig. 2 Percentage of NP extracted after three sequential extractions by the aqueous (a) and HPBCD (b) solutions from sludge E

648 The results obtained in the present study are in agreement
 649 with the research studies by Kawasaki et al. (2001) since
 650 they showed that NP solubility (aqueous solubility
 651 5.43 mg L^{-1}) increased more than 6,000 times when
 652 HPBCD was used in a particular range of concentrations
 653 (0–60 mM). This increase is due to the fact that NP mole-
 654 cules, which have a smaller size than HPBCD cavity, can be
 655 included within such hydrophobic cavity and form inclusion
 656 complexes. As result, NP hydrophilicity and water solubility
 657 increase, and therefore its removal from contaminated sites
 658 occurs. This is the reason why the extraction of the sludge
 659 using HPBCD gives as such extremely high recovery result
 660 of NP in the leachates, and, therefore, a low final content of
 661 NP is reached in the treated sludge.

662 Toxicity of leachates

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

679 In all tests, *Daphnia* mortality was absent. HPBCD dilut-
 680 ed 1 and 10 % in ASTM water did not affect significantly
 681 ($P < 0.05$) proportional feeding rates relative to controls
 682 (Fig. 3). This result confirms that HPBCD does not impair
 683 any effect on *Daphnia*. Sludge HPBCD and water leachates
 684 diluted 1 and 10 % in ASTM hard water inhibited signifi-
 685 cantly ($P < 0.05$) *Daphnia* feeding rates relative to their re-
 686 spective controls (Fig. 3). It is important to emphasise that
 687 both, water and HPBCD sludge leachates had similar toxic-
 688 ity, despite the different amount of contaminants present in
 689 each of them. Water leachates diluted at 10 % had higher
 690 toxicity than at 1 %, as expected.

691 According to Figs. 1 and 2 and Table 1, water leachates
 692 diluted 1 and 10 % should contain $0.04\text{--}0.39 \text{ }\mu\text{g L}^{-1}$ NP, and
 693 those of HPBCD, 13 and $130 \text{ }\mu\text{g L}^{-1}$. Estimated levels of
 694 PAHs in both water and cyclodextrin leachates were low
 695 ($0.17, 0.10, 0.57$ and $0.13 \text{ }\mu\text{g L}^{-1}$ for 10 % HPBCD leachates
 696 of pyrene, fluoranthene, phenanthrene or naphthalene, re-
 697 spectively). The combined presence of these compounds in
 698 water and HPBCD leachates, however, caused similar effects

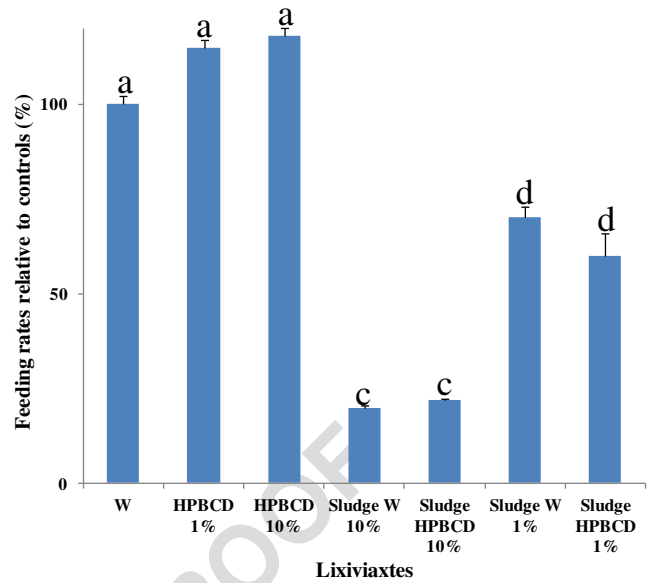


Fig. 3 Feeding responses of *D. magna* juveniles exposed to pure ASTM hard water (W) and HPBCD (HPBCD) and their sludge leachates (sludges W and HPBCD) diluted 1 and 10 % in ASTM hard water. Results are expressed as proportional responses (mean±SE) relative to W controls. Different letters indicate significant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

699 on *Daphnia* feeding rates, as evidenced in Fig. 3. Previous
 700 studies indicate that effects of naphthalene in *Daphnia* occur
 701 only above a milligramme-per-litre levels, pyrene and fluo-
 702 ranthene above $10 \text{ }\mu\text{g L}^{-1}$ and phenanthrene above
 703 $40 \text{ }\mu\text{g L}^{-1}$ (Olmstead and LeBlanc 2005). For NP, however,
 704 sublethal detrimental effects on feeding and reproduction
 705 occur at concentrations greater than $80 \text{ }\mu\text{g L}^{-1}$ (Sun and
 706 Gu 2005; Campos et al. 2012), being lethal above 300–
 707 $400 \text{ }\mu\text{g L}^{-1}$ (Comber et al. 1993). Accordingly, NP residues
 708 present in HPBCD sludge leachates should impair *Daphnia*
 709 feeding rates, whereas those measured in water sludge leach-
 710 ates did not. The fact that we found similar toxic effects for
 711 both water and HPBCD leachates means that NP residues
 712 present in HPBCD leachates were not bioavailable to
 713 *Daphnia*. Indeed, it is likely that HPBCD encapsulated NP
 714 making it less bio-available to *Daphnia*. Such ameliorated
 715 toxic effects of NP have been described for humic substances
 716 (Sun and Gu 2005; Burgess et al. 2005). Our results indicate
 717 further that observed toxicity was due to water soluble com-
 718 pounds not removed by HPBCD. However, although the
 719 leachates produced have been demonstrated not toxic due
 720 to the contaminants extracted by HPBCD, they should be
 721 further treated within the WWTP in order to avoid the
 722 dispersion of such contaminants in the environment. For this
 723 reason, HPBCD should be considered as a useful tool in
 724 sewage sludge decontamination treatments to reduce for
 725 example compounds such as NP and generate non toxic
 726 leachates. In this way, sludge highly contaminated with NP

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

730 **Conclusions**

731 Given the more restricted concentration limit values for some
 732 organic compounds in sewage sludge included in the draft of
 733 the European Union regulations, a preliminary study using
 734 HPBCD extractions has been proposed as a method for the
 735 decontamination of sludge. Among other contaminants,
 736 PAHs and NP are toxic compounds that are present in high
 737 concentrations in sludge and are prioritised by the European
 738 Union since their presence can have serious environmental
 739 implications because of their application to agricultural soils
 740 at doses that may produce long-term contamination of soil
 741 and groundwater. In this study, it has been demonstrated that
 742 these compounds are able to form inclusion complexes with
 743 HPBCD and increase their solubility. It has been shown that
 744 the application of HPBCD solutions to sludge can enhance
 745 the contaminants desorption and decontamination, at ex-
 746 penses of generating a leachate containing the contaminants
 747 released. We propose the use of HPBCD as a very effective
 748 tool for the remediation of contaminated sewage sludge,
 749 increasing its safe usage for agricultural practices without
 750 posing environmental or health risks. We also suggest that
 751 the leachate, although demonstrated not toxic because of
 752 those contaminants extracted by HPBCD, such as NP or
 753 PAHs, should not be released to receiving waters but rather
 754 be treated in the WWTP to avoid their dispersion in the
 755 environment. Aspects related to the optimisation of
 756 HPBCD extractions for the sludge decontamination of
 757 multiclass contaminants present in the WWTPs and evalua-
 758 tion of leachates in terms of contamination loads, toxicity
 759 and possible decontamination treatments will be deeply in-
 760 vestigated in further research.

761 **Acknowledgements** Support from projects CTM2006-04626 and
 762 CTM2009-07335, Spanish Ministry of Science and Innovation (co-
 763 funded by Fondo Europeo de Desarrollo Regional, FEDER), are greatly
 764 appreciated. M.A. Sánchez-Trujillo acknowledges a research contract
 765 from Consejo Superior de Investigaciones Científicas (CSIC, JAEPre
 766 0800763) cofinanced by Fondo Social Europeo (FSE).
 767

768 **References**

770 Abad E, Martínez K, Planas C, Palacios O, Caixach J, Rivera J (2005)
 771 Priority organic pollutant assessment of sludges for agricultural
 772 purposes. *Chemosphere* 51:1358–1369
 773 Alexander M (2000) Aging, bioavailability, and overestimation of risk
 774 from environmental pollutants. *Environ Sci Technol* 34:4259–
 775 4265

Aparicio I, Santos JL, Alonso E (2009) Limitation of the concentration
 of organic pollutants in sewage sludge for agricultural purposes: a
 case study in South Spain. *Waste Manag* 29:1747–1753
 ASTM (1999) Standard methods for measuring the toxicity of
 sediment-associated contaminants with freshwater invertebrates.
 E 1706-95b. In: Annual book of ASTM standards. ASTM, Phila-
 delphia, pp. 65–68
 Badr T, Hanna K, de Brauer C (2004) Enhanced solubilization and
 removal of naphthalene and phenanthrene by cyclodextrins from
 two contaminated soils. *J Hazard Mat B112*:215–223
 Barata C, Alañón P, Gutierrez-Alonso S, Riva MC, Fernández C, Tarazona
 JV (2008) A *Daphnia magna* feeding bioassay as a cost effective and
 ecological relevant sublethal toxicity test for environmental risk
 assessment of toxic effluents. *Sci Tot Environ* 405:78–86
 Blanchard M, Teil MJ, Ollivon L, Legenti L, Chevreuil M (2004)
 Polycyclic aromatic hydrocarbons and polychlorobiphenyls in
 wastewater and sewage sludges from the Paris area (France).
Environ Res 95:184–197
 Burgess RM, Pelletier MC, Gundersen JL, Perron MM, Ryba SA
 (2005) Effects of different forms of organic carbon on the
 partitioning and bioavailability of nonylphenol. *Environ Toxicol*
Chem 24:1609–1617
 Campos B, Piña B, Fernández-Sanjuán M, Lacorte S, Barata C (2012)
 Enhanced offspring production in *Daphnia magna* clones exposed
 to serotonin reuptake inhibitors and 4-nonylphenol. Stage- and
 food-dependent effects. *Aquat Toxicol* 109:100–110
 Céspedes R, Lacorte S, Ginebreda A, Barceló D (2008) Occurrence and
 fate of alkylphenols and alkylphenol ethoxylates in sewage treat-
 ment plants and impact on receiving waters along the Ter River
 (Catalonia, NE Spain). *Environ Pollut* 153:384–392
 Comber MHI, Williams TD, Stewart KM (1993) The effects of
 nonylphenol on *Daphnia magna*. *Water Res* 27:273–276
 Dai JY, Xu MQ, Chen JP, Yang XP, Ke ZS (2007) PCDD/F, PAH and
 heavy metals in the sewage sludge from six wastewater treatment
 plants in Beijing, China. *Chemosphere* 66:353–361
 European Union (1986) Council Directive 86/278/ECC: protection of
 the environment, and in particular of the soil, when sewage sludge
 is used in agriculture. *Official Journal L* 181:6–12
 European Union (2000) EC working document on sludge, 3rd draft: 27
 April 2000. ENV.E.3/LM, European Union, Brussels, Belgium
 European Union (2003) Council Directive 2003/53/EC of the European
 Parliament and the Council of 18 June 2003 amending for the 26th
 time Council Directive 76/769/EEC relating to restrictions on the
 marketing and use of certain dangerous substances and prepara-
 tions (nonylphenol, nonylphenol ethoxylate and cement). *Official*
Journal of the European Union 17.7.2003. L 178/24
 Fenyvesi E, Gruiz K, Verstichel S, De Wilde B, Leitgib L, Csabai K,
 Szaniszló N (2005) Biodegradation of cyclodextrins in soil.
Chemosphere 60:1001–1008
 Fenyvesi E, Molnár M, Letigib L, Gruiz K (2009) Cyclodextrin-
 enhanced soil-remediation technologies. *Land Contam Reclam*
 17:585–597
 Fernández-Sanjuán M, Rigol A, Sahuquillo A, Rodríguez-Cruz S,
 Lacorte S (2009) Determination of alkylphenols and alkylphenol
 ethoxylates in sewage sludge: effect of simple pre-treatment. *Anal*
Bioanal Chem 394:1525–1533
 Ginés JM, Pérez-Martínez JI, Arias MJ, Moyano JR, Morillo E, Ruiz-
 Conde A, Sánchez-Soto PJ (1996) Inclusión of the herbicide 2,4-
 dichlorophenoxyacetic acid (2,4-D) with beta-cyclodextrin by dif-
 ferent processing methods. *Chemosphere* 33:321–334
 Gómez J, Alcántara MT, Pazos M, Sanromán MA (2010) Soil washing
 using cyclodextrins and their recovery by application of electro-
 chemical technology. *Chem Eng J* 159:53–57
 Gómez-Rico MF, Font R, Aracil I, Fullana A (2007) Analysis of
 organic pollutants in sewage sludges from the Valencian Commu-
 nity (Spain). *Environ Contam Toxicol* 52:306–316

- 842 González MM, Martín J, Santos JL, Aparicio I, Alonso E (2010) Occurrence and risk assessment of nonylphenol and nonylphenol ethoxylates in sewage sludge from different conventional treatment processes. *Sci Total Environ* 408:563–570
- 843
- 844 Gould S, Scott R (2005) 2-Hydroxypropyl- β -cyclodextrin (HP- β -CD): a toxicology review. *J Food Chem Toxicol* 43:1451–1459
- 845
- 846 Hajdu C, Gruiz K, Fenyvesi E, Nagy ZM (2011) Application of cyclodextrins in environmental bioassays for soil. *J Incl Phenom Macrocycl Chem* 70:307–313
- 847
- 848 Hartnik T, Jensen J, Hermens JLM (2008) Nonexhaustive β -cyclodextrin extraction as a chemical tool to estimate bioavailability of hydrophobic pesticides for earthworms. *Environ Sci Technol* 42:8419–8425
- 849
- 850 Ifelebuegu AO (2011) The fate and behavior of selected endocrine disrupting chemicals in full scale wastewater and sludge treatment unit processes. *Int J Environ Sci Technol* 8:245–254
- 851
- 852 Kawasaki N, Araki M, Nakamura T, Tanada S (2001) Inclusion behavior of 4-nonylphenol into cyclodextrin derivatives. *J Colloid Interf Sci* 238:215–218
- 853
- 854 Khadhar S, Higashi T, Hamdi H, Matsuyama S, Charef A (2010) Distribution of 16 EPA-priority polycyclic aromatic hydrocarbons (PAHs) in sludges collected from nine Tunisian wastewater treatment plants. *J Hazard Mater* 183:98–102
- 855
- 856 Kiss T, Fenyvsi F, Pasztor N, Feher P, Varadi J, Kocsan R, Szente L, Fenyvesi E, Szabo G, Vecsernyes M, Bacskay I (2007) Cytotoxicity of different types of methylated β -cyclodextrins and ionic derivatives. *Pharmazie* 62:557–558
- 857
- 858 Laturnus F, Arnold K, Gron C (2007) Organic contaminants from sewage sludge applied to agricultural soils. *Environ Sci Pollut Res* 14:53–60
- 859
- 860 Lian J, Liu JX, Wei YS (2009) Fate of nonylphenol polyethoxylates and their metabolites in four Beijing wastewater treatment plants. *Sci Total Environ* 407:4261–4268
- 861
- 862 Martínez E, Gros M, Lacorte S, Barceló D (2004) Simplified procedures for the análisis of polycyclic aromatic hydrocarbons in water, sediments and mussels. *J Chromatogr A* 1047:181–188
- 863
- 864 McAllister L, Semple KT (2010) Measurement of bioaccessibility of organic pollutants in soil. In: S.P. Cummings (ed) *Bioremediation, methods in molecular biology*, vol. 599. Humana Press, New York, pp. 1–14
- 865
- 866 Miégle C, Dugay J, Hennion MC (2003) Optimization, validation and comparison of various extraction techniques for the trace determination of polycyclic aromatic hydrocarbons in sewage sludges by liquid chromatography coupled to diode-array and fluorescence detection. *J Chromatogr A* 995:87–97
- 867
- 868 Minamiyama M, Ochi S, Suzuki Y (2006) Fate of nonylphenol polyethoxylates and nonylphenoxy acetic acids in an anaerobic digestion process for sewage sludge treatment. *Water Sci Technol* 53:221–226
- 869
- 870 Morillo E, Maqueda C, Reinoso R, Undabeytia T (2002) Effect of two organic amendements on norflurazone retention and release by soils of different characteristics. *Environ Sci Technol* 36:4319–4325
- 871
- 872 Morillo E, Romero AS, Madrid L, Villaverde J, Maqueda C (2008) Characterization and sources of PAHs and potentially toxic metals in urban environments of Seville (South Spain). *Water Air Soil Pollut* 187:41–51
- 873
- 874 Morillo E, Sánchez-Trujillo MA, Moyano JR, Villaverde J, Gómez-Pantoja ME, Pérez-Martínez JI (2012) Enhanced solubilization of six PAHs by three synthetic cyclodextrins for remediation applications. Molecular modeling of the inclusion complexes. *Plos ONE*. doi:10.1371/journal.pone.0044137
- 875
- 876 Navarro A, Endo S, Gocht T, Barth JAC, Lacorte S, Barceló D, Grathwohl P (2009) Sorption of alkylphenols on Ebro River sediments: comparing isotherms with field observations in river water and sediments. *Environ Pollut* 157:698–703
- 877
- 878 Olah J, Cserhati T, Szejtli J (1998) β -cyclodextrin enhanced biological detoxification of industrial wastewaters. *Water Res* 22:1345–1351
- 879
- 880 Oleszczuk P (2008) Application of hydroxypropyl[β]cyclodextrin to evaluation of polycyclic aromatic hydrocarbon losses during sewage sludges composting. *J Environ Sci Health A* 43:10–17
- 881
- 882 Oleszczuk P (2007) Changes of polycyclic aromatic hydrocarbons during composting of sewage sludges with different properties and PAHs content. *Chemosphere* 67:582–591
- 883
- 884 Oleszczuk P (2009) Application of three methods used for the evaluation of polycyclic aromatic hydrocarbons (PAHs) bioaccessibility for sewage sludge composting. *Bioresource Technol* 100:413–420
- 885
- 886 Olmstead AW, LeBlanc GA (2005) Joint action of polycyclic aromatic hydrocarbons: predictive modeling of sublethal toxicity. *Aquat Toxicol* 75:253–262
- 887
- 888 Pérez S, Guillamón M, Barceló D (2001) Quantitative analysis of polycyclic aromatic hydrocarbons in sewage sludge from wastewater treatment plants. *J Chromatogr A* 938:57–65
- 889
- 890 Petitgirard A, Djehiche M, Persello J, Fievet P, Fatin-Roge N (2009) PAH contaminated soil remediation by reusing an aqueous solution of cyclodextrins. *Chemosphere* 75:714–718
- 891
- 892 Pousada-Ferradas Y, Seoane-Labandeira S, Mora-Gutierrez A, Nunez-Delgado (2012) A risk of water pollution due to ash-sludge mixtures: column trials. *Int J Environ Sci Technol* 9:21–29
- 893
- 894 Rhodes AH, McAllister LE, Semple KT (2010) Linking desorption kinetics to phenanthrene biodegradation in soil. *Environ Pollut* 158:1348–1353
- 895
- 896 Sánchez-Avila J, Tauler R, Lacorte S (2012) Organic micropollutants in coastal waters from NW Mediterranean Sea: sources distribution and potential risk. *Environ Internat* 46:50–62
- 897
- 898 Semple KT, Doick KJ, Wick LY, Harms H (2007) Microbial interactions with organic contaminants in soil: definitions, processes and measurement. *Environ Pollut* 150:166–176
- 899
- 900 Shen RY, Luo YM, Zhang GY, Teng Y, Li ZG, Wu LH (2007) Contamination of PAHs in sludge samples from the Yangtze River delta area. *Pedosphere* 17:373–382
- 901
- 902 Soares A, Guieysse B, Jefferson B, Cartmell E, Lester JN (2008) Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. *Environ Int* 34:1033–1049
- 903
- 904 Sun H, Gu X (2005) Comprehensive toxicity study of nonylphenol and short-chain nonylphenol polyethoxylates on *Daphnia magna*. *Bull Environ Contam Toxicol* 75:677–683
- 905
- 906 Villar P, Callejón M, Alonso E, Jiménez JC, Guiraúm A (2006) Temporal evolution of polycyclic aromatic hydrocarbons (PAHs) in sludge from wastewater treatment plants, comparison between PAHs and heavy metals. *Chemosphere* 64:535–541
- 907
- 908 Villaverde J (2007) Time-dependent sorption of norflurazon in four different soils: use of β -cyclodextrin solutions for remediation of pesticide-contaminated soils. *J Hazard Mater* 142:184–190
- 909
- 910 Villaverde J, Pérez-Martínez JI, Maqueda C, Ginés JM, Morillo E (2005a) Inclusion complexes of alpha- and gamma-cyclodextrins and the herbicide norflurazon: I. Preparation and characterization. II. Enhanced solubilisation and removal from soils. *Chemosphere* 60:656–664
- 911
- 912 Villaverde J, Maqueda C, Morillo E (2005b) Improvement of the desorption of the herbicide norflurazon from soils via complexation with β -cyclodextrin. *J Agric Food Chem* 53:5366–5372
- 913
- 914 Villaverde J, Posada-Baquero R, Rubio-Bellido M, Láiz L, Sáiz-Jimenez C, Sánchez-Trujillo MA, Morillo E (2012) Enhanced mineralisation of diuron using a cyclodextrin-based bioremediation technology. *J Agric Food Chem* 60:9941–9947
- 915
- 916 Wacker Chemie (2103) CawasoW7 HP Material Safety data Sheet. <http://www.wacker.com/cms/en/productsmarkets/products/product.jsp?product=8971>
- 917
- 918 Ying GG (2006) Fate, behavior and effects of surfactants and their degradation products in the environment. *Environ Int* 32:417–431
- 919
- 920 Zar JH (1996) *Bioestatistical analysis*. Bioestatistical analysis. Prentice-Hall, Upper Saddle River

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

- Q1. Kindly check if the names of authors are correctly captured and presented.
- Q2. Please check if data on authors' affiliations are correctly presented.
- Q3. Please check figure captions if correctly captured.
- Q4. References were renumbered because Refs. "Council Directive 2003/53/EC of the European Parliament and the council of 18 June 2003 amending for the 26th time Council Directive 76/769/EEC relating to restrictions on the marketing and use of certain dangerous substances and preparations (nonylphenol, nonylphenol ethoxylate and cement). Official Journal of the European Union 17.7.2003. L 178/24" and "Council Directive 86/278/ECC (1986) Protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. Official Journal L, 181, 04/07/1986, PP. 6–12" were modified to "European Union (2003) Council Directive 2003/53/EC of the European Parliament and the Council of 18 June 2003 amending for the 26th time Council Directive 76/769/EEC relating to restrictions on the marketing and use of certain dangerous substances and preparations (nonylphenol, nonylphenol ethoxylate and cement). Official Journal of the European Union 17.7.2003. L 178/24" and "European Union (1986) Council Directive 86/278/ECC: protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. Official Journal L 181:6–12", respectively. Please check if appropriate.
- Q5. Sánchez-Avila et al. (2012) was not cited anywhere in the text. Please provide a citation. Alternatively, delete the item from the list.