Acute and chronic toxicity of short chained perfluoroalkyl substances to *Daphnia magna*

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**Abstract**

The aim of this study was to evaluate the aquatic toxicity of a C4–C6 chemistry based fluoroalkylated polymer and the perfluoroalkyl carboxylic acids, PFBA, PFHxA and PFOA to *Daphnia magna*. The acute toxicity decreased with decreasing carbon chain length, but the polymer did not show a dose related effect. In a chronic toxicity test performed with PFHxA, mortality was observed at similar concentrations as in the acute toxicity test, indicating that toxicity did not increase with increasing exposure time. Effects on mortality, reproduction and population growth rate occurred at similar concentrations, indicating no specific effect of PFHxA on sublethal endpoints. C4–C6 chemistry is thus less hazardous to daphnids than C7–C8 chemistry. Yet, these compounds are persistent, hard to remove from the environment and production volumes are increasing.

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**1. Introduction**

Because of their water, oil and stain repelling qualities (Prevedouros et al., 2006), poly- and perfluoroalkyl substances (PFASs) are applied in a wide range of materials, including surface tension lowering products, such as metal cleaners, and water-repellent coatings for leather, paper and textiles (Prevedouros et al., 2006; DuPont, 2010). Especially the perfluoroalkyl acids (PFAAs), having a fully fluorinated carbon tail of varying lengths and a sulfonic, phosphonic or carboxylic head group, are used in the production of larger fluoropolymers. The production of these fluoropolymers can cause the release of PFAAs into the environment as residues (DuPont, 2010) or possibly as a result of biodegradation of their precursors (Prevedouros et al., 2006; Dinglasan-Panlilio & Mabury, 2006; Russell et al., 2008; Liu and Mejia Avendano, 2013). Once released into the environment, fluorinated compounds are not readily degraded, because of the strong polar bond between carbon and fluorine, and are therefore persistent (Parsons et al., 2008). This has raised concerns about the environmental presence of PFAAs and these compounds have indeed been shown to be present in most environmental compartments, but are most abundant in aquatic environments (Kwadijk et al., 2010; Möller et al., 2010; Ahrens, 2011; Houde et al., 2011). Therefore, most attention should be attributed to the fate and effects of PFAAs in aquatic ecosystems.

Recently, a program has been proposed in the United States to reduce the release of the currently abundant perfluorooctanoic acid (PFOA) into the environment by 95% by 2015 (U.S. EPA, 2006). Efforts made by participating manufacturers included switching from the so-called C7–C8 chemistry to C4–C6 chemistry (DuPont, 2010), thereby replacing toxic substances with putatively less toxic ones, since it was demonstrated that accumulation and in vitro toxicity decrease with decreasing carbon chain length (Buhrke et al., 2013; Dai et al., 2013). Although PFAAs with shorter carbon chains (C4–C6) are present in much lower concentrations in biota (Houde et al., 2011), these shorter-chained PFAAs are also persistent and difficult to remove from wastewaters (Guo et al., 2010; Thompson et al., 2011; Eschauzier et al., 2012) and possible solutions for this problem are expensive. This may eventually lead to fairly high concentrations of short chained PFAAs in the environment (Guo et al., 2010; Wilhelm et al., 2010; Thompson et al., 2011; Eschauzier et al., 2012), raising concerns about their aquatic toxicity. However, toxicity data is dominated by human and in vitro toxicity and, in contrast to C12–C8 compounds (Ding et al., 2012; Dai et al., 2013), few studies have been published on the aquatic...
ecotoxicity of short chained PFAAs, while chronic aquatic toxicity tests on these compounds are virtually lacking. Toxicity tests on fluoro polymers are even scarcer. The aim of the present study was therefore to assess the acute aquatic toxicity of two short chained PFAAs and a fluoroalkylated polymer and to compare the acute toxicity of these C4–C6 chemistry based compounds to the toxicity of the previously widely applied PFOA. The six carbon chained PFAA was selected for chronic toxicity testing as we expected a higher toxicity of this compound compared to the four carbon chained PFAA. To this end, daphnids (Daphnia magna) were exposed to the selected perfluoroalkyl substances in acute and chronic toxicity experiments. The test species D. magna was selected because it is easy to culture and reproduces parthenogenetically. Furthermore, D. magna is globally present in most standing freshwater habitats and a major food source for higher trophic levels. Due to these benefits, it has been widely applied in ecotoxicity assessments and several validated OECD guidelines are available (OECD, 2004, 2008).

2. Materials and methods

2.1. Test organism

Daphnid neonates (younger than 24 h; clone 4) used in this study were obtained from Grontmij, Amsterdam, and were cultured in Elendt M4 medium according to OECD guideline 211 (OECD, 2008). For an extensive description of the specific culture conditions, see Waaijers et al. (2013a).

2.2. Test compounds

Acid forms of the PFAAs perfluorobutanoic acid (PFBA, >98%), perfluorohexanoic acid (PFHxA, >97%) and perfluorooctanoic acid (PFOA, >96%) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The fluorotelomere-based polymer Capstone® P-620HS (24.5–25.5%) was obtained from DuPont. Their CAS-numbers, chemical properties and structures are summarized in Table 1. Preparation of stock solutions was performed in autoclaved polypropylene vessels, which have limited sorption of PFASs to the vessel surface compared to glass (Ji et al., 2008). Stock preparation was performed by dilution in demineralized water, and mixing by magnetic stirring. The solubility of PFOA in water is high, namely

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**Table 1**

CAS-numbers and chemical properties of PFBA, PFHxA, PFOA and Capstone® P-620HS (n.a. = not available).

<table>
<thead>
<tr>
<th>Compound</th>
<th>CAS number</th>
<th>Molecular structure</th>
<th>Molecular formula</th>
<th>Acidity (pKa)</th>
<th>Predicted solubility (sw; mg/L)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFBA (C4)</td>
<td>375-22-4</td>
<td><img src="image" alt="PFBA Structural Formula" /></td>
<td>C₄F₇O₂</td>
<td>0–1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.47×10&lt;sup&gt;2&lt;/sup&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>* Sigma–Aldrich, 2013a,b</td>
</tr>
<tr>
<td>PFHxA (C6)</td>
<td>307-24-4</td>
<td><img src="image" alt="PFHxA Structural Formula" /></td>
<td>C₆F₁₁O₂</td>
<td>2–3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.95×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>b DuPont, 2010</td>
</tr>
<tr>
<td>PFOA (C8)</td>
<td>335-67-1</td>
<td><img src="image" alt="PFOA Structural Formula" /></td>
<td>C₈F₁₅O₂</td>
<td>2–3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.4×10&lt;sup&gt;3&lt;/sup&gt;&lt;sup&gt;f&lt;/sup&gt;</td>
<td>* DuPont, 2012</td>
</tr>
<tr>
<td>Capstone® P-620HS</td>
<td>n.a.</td>
<td><img src="image" alt="Capstone® P-620HS Structural Formula" /></td>
<td>n.a.</td>
<td>2–3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>n.a.</td>
<td>* Simplified example of fluoropolymer; Russell et al., 2008.</td>
</tr>
</tbody>
</table>

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**Abbreviations**

CAS no. chemical abstracts service number
C4–C6 chemistry produces molecules with a carbon chain length of four to six
C7–C8 chemistry produces molecules with a carbon chain length of seven to eight
EC<sub>50</sub> concentration that causes 50% effect compared to the corresponding control
ISO international organization for standardization
LC<sub>50</sub> concentration that causes 50% mortality of the test population
LC-MS/MS high performance liquid chromatography (coupled to) tandem mass spectrometry
OECD organization for economic co-operation and development
PFAS poly- and perfluoroalkylated substance
PFAA perfluoroalkyl acid
PFBA Perfluobutanoic acid (C4)
PFHxA Perfluorohexanoic acid (C6)
PFOA Perfluorooctanoic acid (C8)
PFDA Perfluorodecanoic acid (C10)
PFDoA Perfluorodecanoic acid (C12)
REACH Registration, Evaluation, Authorization and Restriction of Chemicals

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3.4 g/L (U.S. EPA, 2002; Bhbatrai and Gramatica, 2010; Ding and Peijnenburg, 2013; de Voogt, 2014, Table 1), and the predicted solubility of shorter carbon-chained PFAAs is even higher, probably due to their higher polarity. The solubility of Capstone® P-620HS, however, was only confirmed visually, and it is thus possible that the compound existed in a suspension rather than a dissolved form in the stock solution. The PFAAs were supplied in acid forms, decreasing the pH below the boundary conditions of the test. Therefore dissolved sodium hydroxide ($Na^+ (aq) + OH^− (aq)$) was added to all PFAA concentrations until the prescribed pH values between 6 and 8 were reached.

### 2.3. Quality assurance and quality control

To test the sensitivity of the daphnid culture maintained in our laboratory, acute toxicity tests were performed at regular intervals (about every three months), with the reference toxicant $K_2Cr_2O_7$. These tests showed that the sensitivity of the daphnid culture was within the limits set by the guideline (EC50, 24 h = 0.6–2.1 mg/L) (OECD, 2004; Vaaajers et al., 2013a).

To validate the acute toxicity test, the water quality parameters pH and $O_2$-saturation were measured in the controls and the lowest and highest concentrations of each compound at the end of the experiment, as recommended by the guideline (n = 2 per concentration per compound) (OECD, 2004). The water quality parameters pH and $O_2$-saturation (range 7.00–7.82 and 8.54–9.42 mg/L respectively) remained well within the requirements as described in OECD guideline 202 (OECD, 2004). To validate the chronic toxicity test, in addition to pH and $O_2$-saturation, hardness of water was also tested, as described by the guideline (OECD, 2008). The water quality parameters of the chronic experiment were measured before and after medium refreshments (n = 2 per concentration). The water quality parameters pH and $O_2$-saturation (range 7.34–8.69 and 8.36–10.95 mg/L respectively) remained well within the limits indicated by OECD guideline 202 (OECD, 2008), but the hardness of the water exceeded the recommended value of <250 mg/L in a few samples. Additionally, water temperature was kept constant at 20°Celsius throughout all tests as described by the guidelines (OECD, 2004; 2008).

To determine if daphnid immobility could be caused by the sodium buffer used to adjust the pH, the toxicity of sodium hydroxide was tested. NaOH was added to all the PFAA solutions, however, with increasing PFBA concentrations most NaOH was consumed, probably through the reaction of PFBA with sodium hydroxide rather than dissolved in the solution. Therefore dissolved sodium hydroxide ($Na^+ (aq) + OH^− (aq)$) was added to all PFAA concentrations until the prescribed pH values between 6 and 8 were reached.

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### 2.4. Acute toxicity tests

Acute immobility caused by the test compounds after 48 h of exposure was tested according to OECD guideline 202 (2004). Daphnid neonates were exposed to the four compounds at five to eight concentrations (including control) in ISO medium (Supporting Information, Table S1). Four to six replicates per concentration were tested, each replicate consisting of a 50 mL polypropylene Greiner tube with the appropriate amount of the stock solution dissolved in 20 mL of ISO medium, containing five daphnid neonates (younger than 24 h). The uncovered vessels were randomly distributed in a climate controlled fume hood ($20 ± 1°C$) with a 16:8 h light:dark photoperiod. The daphnids were randomly distributed over the test vessels and not fed during the experiments. After 24 and 48 h of incubation, the daphnids were checked for immobilization. Daphnids were considered immobile when they were not able to swim after 15s of gentle stirring, according to the guideline (OECD, 2004).

### 2.5. Chronic toxicity test

The chronic toxicity of the C6 compound perfluorohexanoic acid (PFHxA) was determined in a 21d daphnid reproduction test, following OECD guideline 211 (2008), except where noted. Per test concentration (Supporting information, Table S2) 15 replicates were prepared, each consisting of one parthenogenetic reproducing female daphnid kept in 40 mL PFHxA containing Elendt M4 medium in a 50 mL Greiner tube. The tubes were randomly distributed in a climate controlled fume hood (20 ± 1°C), with a light–dark regime of 16:8 h. The experiment was started by introducing one neonate (younger than 24 h) into each tube using a disposable transfer pipette. Each day the number of animals not responding to gentle stimulation by tapping on the tube was scored. Juveniles and ephippia (winter eggs) were also counted and removed daily. The daphnids were fed daily with a suspension of the algae Scenedesmus subspicatus, with a concentration of $3.0 \times 10^5$ cells/mL, supplied by Gronntjø (Amsterdam). The volume of algal suspension fed to the daphnids was 450 μL at day 0–2, 700 μL at day 3–5, and 900 μL from day 6 until the end of the experiment. The daphnids were transferred to new exposure tubes containing renewed test concentrations three times a week.

### 2.6. Chemical analysis

To determine the actual concentrations of the test compounds in the acute and chronic toxicity tests, water samples were taken from two replicates of the controls and the lowest and highest concentrations of the PFAAs and stored in 50 mL polypropylene Greiner tubes at –7°C. Additionally, for the chronic experiment, the three highest concentrations were sampled. All concentrations between the lowest and highest concentrations were interpolated. Water samples were collected at the beginning and end of the acute tests and at weekly time intervals of the chronic experiment, according to OECD guideline 202 and 211 (2004; 2008). The water samples were diluted to fit within the linear range of the calibration curve of the HPLC system. See Table S1 and S2 in the supporting information of Eschauzier et al. (2013) for the internal standards used for the quantification of the analytes. After dilution, samples were mixed 1:1 with MeOH:H2O and filtered through an Acrodisc LC 13 GHPPall in insert PPvial and stored at 4°C until analysis.

PFAAs were analysed by injecting 20 μL from the extract into a High Performance Liquid Chromatograph (HPLC, Shimadzu, Kyoto, Japan) connected to a tandem mass spectrometer (4000 Q Trap, Applied Biosystems, Toronto, Canada) which was operated in the negative ionization mode with scheduled MRM. A Kinetex XB-C18 (ID 2.1 mm; length 75 mm, particle size 2.6 μm, Phenomenex, Torrance, USA) gradient elution with a flow of 0.2 mL/min was applied with the following solvent composition: A: 40:60 MeOH:H2O and B, 95:5 MeOH:H2O (both with 2 mM ammonium acetate). After an equilibration time of 3.5 min, the solvent composition decreased from 60% at the start of the analysis to 10% eluant B at 2 min, and further decreased to 0% B at 2.9 min. The solvent composition increased after 5 min to 60% B again at 5.8 min.
2.7. Data analysis

Using the acute survival data, concentration–response relationships were plotted and the corresponding 48 h EC50 values were calculated according to Haanstra et al. (1985) by fitting a logistic curve (Eq. (1)) through the data of the studied toxicity endpoints, that is percentage mobility, cumulative reproductive output per female and population growth rate, against the actual PFAA concentration in the water.

\[
y(x) = \frac{c}{1 + e^{b(\log_{10} x - \log_{10} a)}}
\]

In this equation \(y(x)\) is the response of the endpoint at concentration \(x\) (%), \(a\) is the EC50 (mg/L), \(b\) is the slope of the curve, \(c\) is \(y(0)\), which equals the average mobility of the control and \(x\) is the actual concentration of PFAS in the water (mg/L) (Waaijers et al., 2013b). EC50 values for the different compounds were compared using generalized likelihood ratio tests according to Van Gestel and Hensbergen (1997).

During the chronic test survival and number of neonates was recorded daily. Using the PFHxA survival data a concentration–response relationship was plotted, but because of the sharp transition from 93.3% survival at 770 mg/L to 0% survival at 1777 mg/L, it was not possible to calculate a 21d LC50 value. Using the acute survival data, concentration \(x\) (%), a is the EC50 (mg/L), \(b\) is the slope of the curve, \(c\) is \(y(0)\), which equals the average mobility of the control and \(x\) is the actual concentration of PFAS in the water (mg/L) (Waaijers et al., 2013b). EC50 values for the different compounds were compared using generalized likelihood ratio tests according to Van Gestel and Hensbergen (1997).

Data analyses were performed with SPSS software (V21.0.0), except for r and its standard error and for survival, which was performed with R (version 3.0.2).

3. Results

3.1. Acute toxicity of perfluorinated compounds

During the acute toxicity tests 100% control survival was recorded. Clear concentration–response relationships were obtained for PFBA, PFHxA and PFBA (Fig. 1) from which EC50 values for mobility were derived. The 48 h EC50 values for PFBA, PFHxA and PFOA were 5251 mg/L (95% CI: 3889–6614), 1048 mg/L (95% CI: 802–1294) and 239 mg/L (95% CI: 190–287) respectively (see Supporting information Table S3 for EC5 and EC10). PFBA and PFHxA were significantly \((p < 0.001)\) less toxic than PFOA, while PFBA and PFHxA were significantly \((p < 0.001)\) less toxic than PFBA. From the toxicity test with NaOH, it appeared that a concentration of 410 mg/L was not toxic, while a concentration of 1639 mg/L was toxic as low mortality was observed at this concentration. This implies that in the PFBA test NaOH may have contributed to the observed toxicity as the highest concentration NaOH used in this test was 1639 mg/L. NaOH concentrations in the other tests were below...
410 mg/L and therefore NaOH did not contribute to the observed toxicity of the other compounds. For Capstone© P-620HS there was no relationship between the concentration and the effect, hence an EC50 value could not be calculated (Supporting information Fig. S1).

Fig. 2. Average (±SE) survival (%, % of initial animals) of *Daphnia magna* (n = 10–15 and n = 27 for control) after 21d of exposure to PFHxA concentrations (±SE) in Elendt M4 medium (mg/L). For visualization, control concentrations (0.03 mg/L) are set at 1 mg/L.

Fig. 3. Average cumulative reproductive output (number of neonates per female) of *Daphnia magna* per concentration (n = 8–15 and n = 27 for control) over time (3A) and after 21d of exposure (±SE) to PFHxA concentrations (±SE) in Elendt medium (mg/L). Letters in Fig. 3A indicate significant differences between concentrations (p ≤ 0.001). Standard errors in Fig. 3A are not shown for clarification of the figure. The EC50 in Fig. 3B is plotted as (776 mg/L) with 95% confidence interval, and the logistic curve represents the fitted concentration–response relationship. For visualization, control concentrations (0.03 mg/L) are set at 1 mg/L in Fig. 3B.
3.2. Chronic toxicity of PFHxA

Control survival was 96%, well above the limits set by the guideline (>80%) (Supporting Information Fig. S2). During the experiment, five daphnids died because of improper handling and were excluded from the analysis. The PFHxA survival data after 21 days of exposure only showed a dose—response relationship at the highest test concentration (Fig. 2). Because of the sharp transition from 93.3% survival at 770 mg/L to 0% survival at 1777 mg/L, the non-linear dose—response model was unable to calculate a LC50 value.

Daphnids that had not reproduced at the end of the experiment appeared to be males and were excluded from reproduction and population growth analysis. There was no significant (p > 0.05) difference in age at first reproduction between the PFHxA concentrations and the control. The average number of neonates per control female after 21 days was 54.5 neonates per daphnid (Fig. 3A). The reproductive output of daphnids exposed to 3 and 83 mg/L PFHxA was significantly (p < 0.0001) higher than that of the controls and the reproductive output of daphnids exposed to 770 mg/L and 1777 mg/L PFHxA was significantly (p = 0.001 and p < 0.0001 respectively) lower than that of the controls (Fig. 3A). From the reproduction data obtained at the end of the 21-day exposure period, a clear concentration—response relationship was obtained (Fig. 3B) from which an EC50 value of 776 mg/L (95% CI: 735–816) was derived.

The daily observations on survival and reproduction were integrated into the population growth rate. For this parameter also a clear concentration—response relationship was obtained (Fig. 4). The EC50 value for the chronic effects of PFHxA on per capita population growth rate was 853 mg/L (95% CI: 978–917). No significant (p > 0.05) differences were observed between the EC50 values for reproduction and population growth rate. See Supporting Information Table S4 for all EC50 and EC105.

4. Discussion

The aim of this study was to compare the toxicity of the new C4–C6 chemistry based compounds to the toxicity of the previously widely used C7–C8 chemistry based compounds by performing acute toxicity tests on PFBA, PFHxA, PFOA and a fluorotelomer-based polymer called Capstone© P-620HS. It was observed that acute toxicity of the tested perfluoroalkyl acids decreased with decreasing carbon chain length. The acute EC50 value for PFOA (239 mg/L) was in agreement with values obtained by Ding et al. (2012; 220 mg/L), but were lower than the values reported by Ji et al. (2008; 477 mg/L). However, Ji et al. (2008) reported nominal concentrations and made use of glass beakers, which might have caused sorption of the compound to the glass surface, leading to lower actual exposure concentrations. Also the acute EC50 value of PFBA (5251 mg/L) agreed with values obtained by Ding et al. (2012; EC50 > 4280). Since NaOH may have contributed to the observed toxicity in the PFBA experiment, PFBA may actually be even less toxic. For PFHxA, Hoke et al. (2012) reported an EC50 value of >96.5 mg/L from the literature (Loveless et al., 2009), which was, however, wrongly cited since the latter study was performed with rats rather than with daphnids. Therefore, no toxicity data for PFHxA to D. magna was available until now, and hence, the present study is the first one to provide these. The toxicity of this C6 compound (EC50 value 1048 mg/L) is between that of the C8 and the C4 compounds. Thus as previously observed for in vitro toxicity (Buhire et al., 2013) and bioaccumulation in daphnids of C12–C8 compounds (Dai et al., 2013), the present study showed that the acute toxicity to daphnids decreased from C8 to C4 compounds.

PFHxA was expected to show a higher toxicity in the chronic toxicity test since toxicity generally tends to increase with increasing exposure time (Marinković et al., 2011). Yet, the comparison between the acute EC50 for mobility (1048 mg/L) and the range of concentrations causing chronic mortality (770–1777 mg/L) reveals that the toxicity of PFHxA did not increase with increasing exposure time. Hence, for this compound the acute EC50 for mobility is a good estimate for its chronic effects on mortality.

The data of the per capita number of offspring showed an increase up to 145 mg/L of PFHxA (significantly for 3 mg/L and 83 mg/L), indicating that the compound enhanced reproduction at sublethal concentrations. At higher concentrations, the per capita number of offspring did decline, resulting in an EC50 of 776 mg/L. A chronic reproduction test with PFOA has been performed earlier by Ji et al. (2008), however this study used too low concentrations to provide substantial toxicity data and reported, as mentioned earlier, nominal concentrations. Effects on mortality, reproduction and population growth rate occurred at similar concentrations, indicating no specific effect of PFHxA on sublethal endpoints.
In riverine samples, McLachlan et al. (2007) reported maximum concentrations of PFHxA and PFOA of 18 and 200 ng/L respectively, while a concentration of 116 ng/L for PFOA was reported by Möller et al. (2010). Comparing these field data with the presently obtained effect concentrations reveals that toxic concentrations are at least a factor of 1,000,000 higher than the field concentrations and even when incidental spills are considered the factor remains very high (Eschauzier et al., 2012). Moreover, the acute EC50 values are far above 10 mg/L for all compounds, so according to the REACH classification the compounds have a very low toxicity, a classification which also holds for the chronic toxicity of PFHxA. This would imply that both aquatic hazard and risk of these compounds are low; however, only a single aquatic species was tested so far. Furthermore, the C4–C6 compounds are persistent (U.S. Environmental Protection Agency, 2002), difficult to remove during drinking water preparation (Eschauzier et al., 2012), and increased production of short chained PFASs may be foreseen.

5. Conclusions

It is concluded that the tested C4–C6 chemistry based compounds are less toxic to daphnids than the C7–C8 chemistry based compound PFOA. Moreover, the mode of action of the C6-compound PFHxA is probably non-specific and its toxicity does not increase with increasing exposure time. C4–C6 chemistry is thus less hazardous to daphnids than C7–C8 chemistry. Yet, these compounds are persistent, hard to remove from the environment and production volumes are increasing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2014.12.025.

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