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## Effect of land use on pollution status and risk of fish endocrine disruption in small farmland ponds

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1 **Effect of land use on pollution status and risk of fish endocrine disruption in small farmland ponds**

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25 **Abstract:** To study whether the intensity of agricultural activities affects pesticides loads in pond environment, a  
26 large number of Belgian farmland ponds were surveyed in spring 2004. Temporal distribution of pollutants was  
27 also investigated over restricted survey ponds sampled three times round year 2007. Sedentary pond  
28 Prussian carp juveniles were also captured to determine their brain aromatase activity (AA) and plasma  
29 vitellogenin (VTG) levels. Heavy metal distribution was also examined in various pond matrices. Among the  
30 pesticides analysed, only herbicides were detected. Contamination of pond water by atrazine was frequently  
31 observed during spring 2004, while isoproturon and glyphosate were detected round year 2007. Levels of  
32 herbicides were inversely related to the distance of ponds to crop field, and values peaked in April or October.  
33 Absence of endocrine disruptors in pond water was confirmed by lack of modulation in VTG and AA in male fish.  
34 Heavy metals were present in all the pond matrices, but overall contamination levels were low. The results  
35 demonstrated that Belgian ponds were mainly contaminated by herbicides, and that pond sedentary fish were not  
36 affected by endocrine disruptors. They also demonstrated a marked effect of land use intensity on herbicide  
37 pollution which can be mitigated by an adjustment of the buffer zones.

38

39 **Keywords:** Pollution, ponds, Prussian carp, endocrine disruption, land use intensity, herbicides, heavy metals

## 40 **Introduction**

41 Pools and small ponds have in addition to their aesthetic and agricultural values a high conservation value (Scheffer  
42 et al. 2006). Their biotopes are characterized by heterogeneous communities of aquatic organisms and they often  
43 contain rare or unique species (Wood et al., 2003; Williams et al., 2003; Nicolet et al., 2004). Several studies have  
44 reported the contribution of small ponds to regional diversity to be higher than that of other freshwater  
45 ecosystems such as lakes and rivers (Davies et al., 2008; Williams et al., 2008). In predominantly agricultural areas,  
46 small water bodies offer a special refuge to many aquatic biotas and thus contribute to mitigate the decline in  
47 overall biodiversity.

48 Because of the limited water exchange compared to large water bodies, various pollutants can accumulate in pond  
49 environments, which may impact the aquatic organisms inhabiting the ponds. The effects of land use intensity on  
50 pesticide accumulation in the pond environment, and its effects on pond biota, are, however, poorly documented.  
51 Despite the ban of atrazine in the EU, this herbicide is still used in other countries and, because of its rather

52 resistant nature, it is still often detected in sediments and surface waters (Wu et al., 2009; Sun et al., 2010).  
53 Herbicides, such as diuron, isoproturon and [glyphosate herbicides](#) are nowadays widely used worldwide and their  
54 accumulation has been reported in pond water (Greulich et al., 2002; Howe et al., 2004; Bonnet et al., 2007; Rohr  
55 et al., 2008). Besides their direct inhibitory actions on trophic resources, such as aquatic plants and insects,  
56 herbicide molecules have various deleterious effects on aquatic organisms. While data on the potential hormonal  
57 effects of atrazine are still controversial in fish (Spano et al., 2004, Nadzialek et al., 2008; Tillitt et al., 2010),  
58 numerous studies demonstrated that this pesticide, at environmentally relevant concentrations, has marked  
59 endocrine disrupting effects that affect the central nervous and immune system of frogs and salamanders (Sparling  
60 et al., 2001; Forson & Storfer, 2006; Kloas et al., 2009; Wang and Keller, 2009; Lasserre et al., 2009; Hayes et al.,  
61 2010a). High concentrations of [glyphosate herbicides](#) and the associated surfactants have also various detrimental  
62 effects on amphibian tadpoles, such as the disturbance in the metamorphosis process, tail damages, and gonad  
63 abnormalities (Howe et al., 2004; Barni et al., 2007; Brausch & Smith 2007; Dinehart et al., 2009). It has also been  
64 shown that combinations of chemicals, including atrazine, diuron, isoproturon and their breakdown products, at  
65 environmentally relevant concentrations are more toxic to fish and amphibians than the parent compounds in  
66 isolation (Spano et al., 2004; Fatima et al., 2007; Bonnet et al., 2007). Some pesticides that are already known to  
67 be resistant to degradation might be accumulated in small pond environments, as there is no dilution or flushing  
68 effect. Insecticides or industrial compounds such as aldrin or nonylphenol can induce sex-reversal in fish and  
69 amphibians by altering various neuroendocrine pathways including those controlling reproductive functions. They  
70 may induce an increase in the expression and activity of P450 aromatase, leading to an increase in oestrogen  
71 production that subsequently induces VTG accumulation in the liver and bloodstream (Folmar et al., 2001; Jobling  
72 et al., 2006; Hayes et al., 2006; Wingfield and Mukai, 2009; Nadzialek et al., 2011). Production of VTG induced by an  
73 increase in P450 AA in males has been used as a reliable biomarker to assess the occurrence of endocrine  
74 disruption (Folmar et al., 2001; Desforges et al., 2010; Nadzialek et al., 2011).

75 Presence of PAHs may also be suspected in the different pond matrices since they are ubiquitous in the  
76 environment due to mostly industrial activities but also due to other anthropogenic sources. PAHs are known to be  
77 strongly persistent with good dispersal properties (Guo et al., 2011). Apart from their known carcinogenic,  
78 mutagenic and endocrine disrupting properties (Brasseur et al., 2007; Guo et al., 2011, Rhind et al., 2011), PAHs  
79 have been shown to alter both the specific and non-specific immune system as well as the health status of different

80 fish and other animals living in aquatic environment (Lampi et al, 2006; Renaud & Deschaux, 2006; Gillardin et al.,  
81 2009; Milinkovitch et al., 2011; Danion et al., 2011).

82 Amongst the persistent pollutants, accumulation of heavy metals is frequently assessed for monitoring the health  
83 status of water bodies (Maas et al., 2010; Rimondi et al., 2012). Apart from geochemical processes, increase in  
84 heavy metals in pond environments may be caused by various sources, such as industrial and traffic emissions as  
85 well as runoff from habitations, especially in urbanised areas. Although it has been demonstrated that metal  
86 pollution loads are markedly higher in urbanized industrial landscapes than in agricultural areas (Imperato et al.,  
87 2003), agricultural operations such as sewage sludge or fertilizer use may increase metal contamination in soils,  
88 especially of cadmium (Cd), copper (Cu) and zinc (Zn) (Nicholson et al., 2003, Maas et al., 2010). Intensification of  
89 agricultural activities and other human activities may increase the runoff release of heavy metals in pond  
90 environments, but the effect of land use intensity on heavy metal pollution is not yet quantified. Accumulation of  
91 heavy metals in pond matrices may induce detrimental effects on aquatic organisms, for instance by altering their  
92 physiological and defence ability (Desouky, 2006; Reynders et al., 2008; Ng et al., 2011; Rimondi et al., 2012).

93 The objective of our study was to evaluate pesticide and heavy metal pollution status as well as risk of fish  
94 endocrine disruption in Belgian small farmland ponds in relation to land use level. To achieve this objective (1) we  
95 investigated the effect of land use level on herbicide loads in pond water as well as on heavy metal concentrations  
96 in various pond matrices during two large surveys covering the entire Belgian territory; (2) we verified whether  
97 there is seasonal variation in pesticide pollution in pond water, focussing on various problematic pollutants; and (3)  
98 we assessed a number of indicators of reproductive endocrine disruption in Prussian carp (*Carassius auratus*  
99 *gibelio*) juveniles resident in ponds located in intensive or extensive agricultural areas.

## 100 **Materials and methods**

### 101 *Seasonal changes in pesticide loads in pond water*

### 102 *Survey designs and analysed pesticides*

103 Two surveys were conducted in order to investigate whether changes in pesticide pollution in pond  
104 water vary with land use intensity. In the first survey conducted during May - June 2004, 126 ponds  
105 across almost the entire territory of Belgium (Fig. 1a) were sampled for water. For the selection of

106 the investigated ponds, the dominant land use type in the immediate neighbourhood of the ponds  
107 was the main criterion (see also Declerck et al., 2006). We tried as much as possible to uncouple land  
108 use and geographical position. To do so, we first selected forty two geographically spread clusters  
109 such that each cluster contained several ponds that were located within a circular area of  
110 approximately 20 km<sup>2</sup> (Declerck et al., 2006). Within each cluster, we then selected three ponds  
111 that correspond to a gradient in agricultural land use intensity, ranging from intensive agricultural  
112 activities (mostly cropland; “intensive ponds”), over intermediate agricultural land use (dominated by  
113 pastures; “extensive ponds”) to more pristine (“natural”, mostly located in protected nature areas).  
114 Crop presence was noticed around the majority of ponds: crop land was within a radius of 10 or 20 m  
115 for intensive or extensive ponds, while for natural ponds, no crop activities was present or crop land  
116 was observed within a radius > 100 m. Other details concerning the investigated sites have been previously  
117 described by Declerck et al. (2006). Pesticide analyses focussed mostly on herbicides (atrazine, simazine,  
118 propazine, terbuthylazin, triazine metabolites, diuron and isoproturon) but also on insecticides, namely  
119 endosulfan and lindane.

120 A second survey was carried out in 2007. This survey was more limited in number of ponds but involved three  
121 samples to cover temporal dynamics, involved a broader range of pesticides, and also included industrial  
122 pollutants. Fifteen small ponds were selected covering the three land use categories (Fig. 1b); these ponds were a  
123 subset of the ponds sampled in 2004. Water was sampled three times (April, July and October 2007) with an  
124 interval time long enough to cover the dissipation time of various pesticide molecules in water conditions (Mouvet  
125 et al., 1997; Sorensen et al., 2003; Tissier et al., 2005). The pollutants that were quantified were: (1) Herbicides:  
126 atrazine, simazine, propazine, terbuthylazin, triazine metabolites, diuron isoproturon, glyphosate and  
127 aminomethyl-phosphonic acid (AMPA); (2) Insecticides: aldrine, chlorfenvinphos,  $\alpha$ -endosulfan, dicofol; (3)  
128 Industrial compounds: octylphenol, nonylphenol, aldrine, and four polycyclic aromatic hydrocarbons (PAHs:  
129 benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene) and benzo(ghi)perylene). A summary of the number  
130 of investigated ponds and types of analysed pesticides is presented in table 1.

131

132 *Water sampling and pesticide analyses*

133 For analyses of the targeted pesticides, a pool of 2-L water was taken at eight randomly selected sampling  
134 stations in each pond (4 in the middle of the pond and 4 close to the edge). Just after sampling, water was  
135 filtered over a 20µm mesh screen in order to remove large organic particles, and then one litre of the water  
136 sample was kept at 4°C. In the laboratory, the water samples were stored at -20°C. Before solid phase extraction  
137 (SPE) of pesticides, the water samples were filtered over a 10 µm Whatman screen to eliminate remaining large  
138 organic particles, and then successively over 2.0 and 0.6 µm Millipore screens. C<sub>18</sub> cartridges (6 ml-Oasis  
139 column, Waters) were clean-conditioned by methanol and MEQ water before samples were loaded at a rate of  
140 6ml/min and eluted by acetonitrile. After SPE, the eluted samples were stored at -20°C until HPLC (only for  
141 triazines) or GC-MS analyses were applied as previously described in Nadzialek et al. (2010). Before injection, 1 ml  
142 of the eluted sample was filtered (0.2µm) and this filter was rinsed with 1 ml acetonitrile before solvent  
143 evaporation under N<sub>2</sub>. The dry residue was suspended in 90 µl of hexane and 10 µl of a solution (in hexane) of [<sup>2</sup>H<sub>12</sub>]  
144 Chrysene (200 pg injected), used as internal standard. The detection limit was 10 ppb for all pesticides. Glyphosate  
145 and its metabolite AMPA were quantified in water by a LC-MS method. After SPE clean-up as for other  
146 pesticides, samples were derivatized with 9-fluorenylmethyl chloroformate following the method described by  
147 Koeber et al. (2001) and Ibanez et al. (2006). Then, eluted concentrated samples were analyzed with an  
148 Alliance 2690 liquid chromatography system and a Micromass Quattro Ultima Platinum triple quadrupole mass  
149 spectrometer (Waters/Micromass, Manchester, U.K.). The quantification limit of both glyphosate and AMPA  
150 was 10 ppb.

151 *Reproductive disruption in fish*

152 The objective of this part of our research was to evaluate the risk of reproductive disruption in sedentary pond  
153 fish. Prussian carp (*Carassius auratus gibelio*, a goldfish subspecies) was selected as resident fish sentinel  
154 because it was one the most abundant fish species present in the ponds (results not shown). Six intensive or  
155 extensive ponds of the fifteen ponds already surveyed for pesticide loads in 2004 and 2007 were selected. Ten  
156 juveniles (5 males and 5 females) (35 – 65g) from each pond were captured in 2008 and 2009, weighed and blood  
157 sampled for plasma to determine plasma VTG levels. Then, fish were dissected for gonad and brain samples to  
158 estimate the gonado-somatic index (GSI, 100\*gonad weight/body weight) and the brain aromatase activity.

159 Blood samples were taken from the caudal vessel using heparinised syringes. The samples were centrifuged for 20  
160 minutes at 4500 rpm and the plasma was collected and stored at -80°C until assayed for VTG. Plasma  
161 concentrations of VTG were quantified by ELISA, using a carp commercial VTG ELISA kit (SPID BIO, France) with a  
162 detection limit of 10ng/ml and an intra- and inter- coefficient of variation of 4.85% and 5.85%, respectively. Brain  
163 aromatase activity was quantified by a radioimmunoassay enzymatic method described by Noakson et al. (2001)  
164 and Gonzales & Piferrer (2003) (see also Mandiki et al., 2005). Briefly, the brain homogenate sample was mixed  
165 with a generating solution (NAA) containing 1mM  $\beta$ NADPH (Sigma N-0505, Steinheim, Germany), 25nM  
166 androst-4-ene-3,17-dione-[1 $\beta$ -H<sup>3</sup>] (NEN, Boston, USA), and 275nM androst-4-ene-3,17-dione (Sigma,  
167 Steinheim, Germany). More specifically, 100 $\mu$ l of tissue homogenate, representing 10 mg of brain tissue, was  
168 mixed with 150 $\mu$ l of 300nM NAA and 50 $\mu$ l buffer solution and incubated for 60 min in a water bath at 30°C  
169 with gentle shaking. A control tissue homogenate mixed with a substrate without NADPH to prevent aromatase  
170 activity was co-incubated simultaneously. The enzymatic reaction was stopped by the addition of  
171 trichloroacetic acid (TCA, 10%), and then samples were centrifuged (8000 rpm, 10 min). The concentrations of  
172 proteins in the pellets were determined by the Lowry method (Lowry et al., 1951). The supernatant was  
173 extracted with chloroform for the aqueous phase, which was mixed with dextran-coated (5%) charcoal (0.5%)  
174 and quantified using a liquid scintillation counter. The intra-and inter coefficients of variation of the assays  
175 were 5.55 and 6.85%, respectively.

#### 176 *Heavy metal distribution*

177 During summer 2003 or spring 2004, we sampled different pond matrices (water, suspended solids, sediment, and  
178 snails) to analyse the following heavy metals: cadmium-Cd, tin-Sn, zinc-Zn, copper-Cu, lead-Pb and mercury-Hg  
179 (the latter only in water samples of 2004). The number of ponds and pond matrices investigated are  
180 summarized in table 2. For each pond, water was sampled at 8 locations as in pesticides design using a tube  
181 sample integrating the entire water column. The water taken at the different locations was pooled and filtered  
182 over 2mm to remove larger organic and other materials. Then, 2 litres of water of each pond was brought to  
183 the laboratory in a cooling box (4 °C). Upon arrival in the laboratory, the water sample was filtered through a  
184 125  $\mu$ m Teflon mesh screen to remove large organic particles, and then over a 1.2  $\mu$ m filter. These filters were  
185 used for analyzing heavy metal concentrations in suspended solids, whereas 10 ml of the filtrate was used to  
186 quantify heavy metal concentrations in the water. Filters were cleaned with HNO<sub>3</sub> 10% prior to use. For Hg



187 analyses in water samples, acid cleaned (1% HNO<sub>3</sub>) glass bottles were used and filtration was performed as  
188 soon as possible with appropriate filtration equipment pre-treated for trace metal analysis to avoid  
189 contamination. Sediment samples were taken from the upper sediment layer (0-3 cm) at eight sampling  
190 stations in each pond (4 in the middle of the pond and 4 close to the edge). They were placed in plastic bottles  
191 and dried at 60°C during 48 hours. Before analyses, they were calcinated at 650°C and then acidified (HClO<sub>4</sub>-  
192 HF-HNO<sub>3</sub>) at 110°C overnight before being evaporated and stored in a 5%HNO<sub>3</sub> solution. In addition to the  
193 abiotic pond matrices, bioaccumulation of heavy metals was tested in snails of the genus *Lymnea* that were  
194 collected in the ponds. Snails were present in 14% (18/99) and 27% (34/126) of the small ponds sampled during  
195 summer 2003 or spring 2004, respectively. A total of 48 ponds were investigated with 30-45 snails for each  
196 pond depending on population size of the snails. In the laboratory, snails were lyophilised and digested by  
197 acidified solution (H<sub>2</sub>O<sub>2</sub>-HNO<sub>3</sub>), and stored in nitric acid (5%HNO<sub>3</sub>). Then, concentrations of heavy metals  
198 were measured in the digested solutions using a high resolution quadruple-inductively coupled plasma-mass  
199 spectrophotometer (HR-ICP-MS). Multi-element standard solutions at different concentrations (0, 0.02, 1, 5,  
200 20, 100 and 200 ppb) were used for calibration. Total variation coefficients of four replicate measurements  
201 varied between 1.66-5.45%. Heavy metal levels in water were compared with the Belgian and international  
202 reference quality for surface waters: 1, 50, 50, 200-300 µgL<sup>-1</sup> and 770 ngL<sup>-1</sup> for Cd, Pb, Cu, Zn and Hg,  
203 respectively (Delbeuck, 2007, Reynders et al., 2008; USEPA, 2009, Rimondi et al., 2012), and those in the  
204 sediment were compared with the probable effect concentrations reported by MacDonald et al (2000): 4.98,  
205 149, 128, 459 µgL<sup>-1</sup> for Cd, Cu, Pb and Zn, respectively.

## 206 *Statistical analyses*

207 Values are mean ± SD, and data expressed in percentages were log-transformed before statistical analyses.  
208 Homogeneity of data was tested by Bartlett's test, and then ANOVA-II was used to test the main effect of the  
209 three types of ponds and season. Significance of differences between groups was estimated using the Scheffe's  
210 multiple range tests with a significance level at  $P < 0.05$ . Calculations were performed using Statistica software  
211 (STATSOFT, Tulsa, OK, USA). Regression analysis was used to test the relationships between atrazine (the more  
212 frequent herbicide) levels in water and distance to crop fields as well as between sediment concentrations and  
213 snail concentrations for each metal.

214 **Results**

215 ***Pesticide levels in pond water***

216 During the water sampling of 2004, atrazine was found in 42 ponds, at concentrations varying between 0.011-  
217 1.259 µg/L. Atrazine was detected in 33% of the 126 investigated ponds, with their number increasing with  
218 land use intensity: 6% of the ponds in nature reserves, 13% of the extensive land use ponds, and 23% of the  
219 intensive land use ponds (Fig. 2a). In those ponds where atrazine was detected, concentrations were  
220 significantly ( $P < 0.05$ ) higher in intensive land use ponds than in extensive land use ponds or ponds in natural  
221 areas (Fig. 2b). Overall, atrazine levels in the ponds were inversely related ( $R = -0.73$ ,  $P = 9.44 \times 10^{-8}$ ) to the  
222 distance from the nearest crop field. Presence of other herbicides molecules was generally low but showed  
223 also a trend of more contamination in intensive ponds than in extensive ponds. Indeed, while no triazine  
224 metabolites were detected, simazine was detected in 5 intensive land use and 3 extensive land use ponds  
225 (6.3% of the investigated ponds), at low concentrations (0.010 – 0.156 µg/L). Diuron was detected in (8.7% of  
226 the ponds, again predominantly located in agricultural areas (6 intensive land use ponds, 3 extensive land use  
227 ponds, and 2 ponds in nature reserves), at low concentrations varying between 0.010 – 0.161 µg/L. No other  
228 targeted pesticides (isoproturon, endosulfan and lindane) were detected during the 2004 sampling campaign.

229 During the 2007 sampling campaign, no triazines or other pesticides were detected except for isoproturon and  
230 glyphosate. Concentrations of these two herbicides were relatively low but showed important seasonal  
231 variations. Isoproturon levels were significantly ( $P < 0.05$ ) higher in October and April than in July (Fig. 3a). All  
232 intensive land use ponds and all extensive land use ponds were contaminated by isoproturon, and all but three  
233 extensive land use ponds had isoproturon irrespective of sampling season. All five ponds in natural areas were  
234 also contaminated by isoproturon in October but three samples of April and July did not reveal contamination.  
235 Similar to our observations for atrazine in 2004, isoproturon concentrations were higher ( $P < 0.05$ ) in intensive  
236 land use ponds (0.15-3.81 µg L<sup>-1</sup>) compared to extensive land use ponds (0.90-2.13 µg L<sup>-1</sup>) or ponds in natural  
237 areas (0.01-0.61 µg L<sup>-1</sup>) (Fig. 3b).

238 Overall, glyphosate concentrations were low, with overall higher concentrations ( $P < 0.05$ ) in October than in  
239 July and April (Fig. 4a), All five intensive land use ponds were contaminated by glyphosate herbicides whatever  
240 the sampling season, while this was observed in only three extensive land use ponds and ponds in natural

241 **areas**. In addition, in those ponds where **glyphosate herbicides** were detected concentrations were significantly  
242 higher ( $P < 0.05$ , Fig. 4b) in intensive land use ponds (0.541 – 2.075  $\mu\text{g/L}$ ) than in other pond types (extensive  
243 land use: 0.130 – 0.397  $\mu\text{g/L}$ , natural areas: 0.060 – 0.190 ponds).

244 AMPA levels showed the same profiles as for **glyphosate herbicides** (Figure 4a-b). Concentrations of  
245 insecticides (aldrine, chlorfenvinphos,  $\alpha$ -endosulfan, dicofol) and industrial compounds (octylphenol and 4-(para)-  
246 nonylphenol and 4 PAH molecules) were below the detection limits in all ponds.

### 247 ***Reproductive disruption features***

248 As expected, plasma VTG levels were markedly higher ( $P < 0.001$ ) in female (16-88  $\mu\text{gml}^{-1}$ ) than in male (0.00-  
249 1.47  $\mu\text{gml}^{-1}$ ). VTG values were lower than the detection limits (0.010  $\mu\text{gml}^{-1}$ ) for 50% of the tested males (Fig.  
250 5a). Levels of brain aromatase activity were markedly lower ( $P < 0.001$ ) in males (0.00-1.78  $\text{fmolmg}^{-1}\text{prot}^{-1}\text{min}^{-1}$ )  
251 than in females (6.55-17.94  $\text{fmolmg}^{-1}\text{prot}^{-1}\text{min}^{-1}$ ), and values did not differ between fish from intensive and  
252 extensive land use ponds (Fig. 5b). Only one intersex individual was observed in each type of ponds (2 out of  
253 120 fish screened = 1.7%). In these intersexual fish, a high level of aromatase activity (19-23  $\text{fmolmg}^{-1}\text{prot}^{-1}\text{min}^{-1}$ )  
254 and plasma VTG values (48-71  $\mu\text{gml}^{-1}$ ) comparable to values observed in females was observed. Both for  
255 females (1.8-3.4%) and males (0.65-1.25%), GSI values varied strongly between individuals, and no significant  
256 differences were observed between males from intensive land use (0.85 $\pm$ 0.1%) and extensive land use  
257 (0.94 $\pm$ 0.3%) ponds. The two intersex fish displayed higher GSI values (2.45%) than normal males and females.

### 258 ***Heavy metals***

259 **For all analyzed heavy metals, no effect of land use intensity was observed in any of the studied pond matrices**  
260 **(Fig. 6a-d). In the pond water, total concentrations ( $\text{ngL}^{-1}$ ) of Cd (range 0.00-0.44), Sn (range 0.00-0.14), Pb**  
261 **(range 0.08-2.66), Zn (range 0.85-5.82) and Hg (range 0.56-19.97) were near the detection limits (Fig. 6a).**  
262 **Concentrations of Zn were significantly ( $P < 0.05$ ) higher in 2007 compared to 2004, but concentrations were**  
263 **generally low ( $< 0.1 \mu\text{gL}^{-1}$ ) in most of the ponds.** In suspended materials (Fig. 6b), Zn levels were the highest  
264 (0.1-1.7  $\mu\text{gg}^{-1}$ ) of all metals, and concentrations were very low for the other metals, varying between 0-20  $\text{ngL}^{-1}$   
265 for Cd and Sn and 0.1-0.9  $\mu\text{gg}^{-1}$  for Pb and Cu. As expected, contents of heavy metals in sediment (Fig. 6c) were  
266 significantly higher than in water ( $P < 0.0001$ ) or suspended materials ( $P < 0.001$ ). The lowest values in

267 sediment were observed for Cd or Sn (0.05-1.72 or 1.4-4.56  $\mu\text{g g}^{-1}$ ) and the highest ones for Zn (61.98-505  $\mu\text{g g}^{-1}$ ). Levels for Pb and Cu varied between 9.17-93.80 and 3.73-84.40  $\mu\text{g g}^{-1}$ , respectively. For all the investigated  
268 heavy metals in sediment, values were lower than the minimum effect concentrations, except in 8.8% of the  
269 agricultural or natural ponds. Accumulation of heavy metals in snail homogenates (Fig. 6d) reflected tightly the  
270 levels found in the sediments **and not those in water or suspended materials**. Indeed, sediment concentrations  
271 were significantly correlated to snail concentrations (Table 3) for all metals **except for Cu and Sn**; specifically Pb  
272 (R = 0.99, p = 0.000) and Zn (R= 0.65, p = 0.001) sediment concentrations were well related to snail  
273 concentrations.

## 275 **Discussion**

### 276 ***Pesticide pollution***

277 **Regular follow-ups of pesticide pollution loads are often made for large lakes and rivers, but information is**  
278 **generally lacking for small water bodies such as ponds. Here we tested whether ponds suffer from pesticide**  
279 **contamination and whether this is related to current land use and land use intensity. In our survey of 126 ponds**  
280 **atrazine was detected in 33% of the investigated ponds, but the overall concentrations were low. Given the ban**  
281 **on this herbicide by the European Union (2004/248/EG), the fact that we still detected it in 1/3 of the ponds**  
282 **sampled in 2004 may indicate that atrazine molecule does not break down easily in pond water conditions. In**  
283 **other surface waters, the half-time for the dissipation of atrazine is reported ranging between 4 to 11 months**  
284 **depending to water physico-chemical conditions (Mouvet et al., 1997; Sorensen et al., 2003; Tissier at al., 2005).**  
285 **But apart from a possible long-term stability of atrazine in the pond water conditions, the current observation**  
286 **could also be an evidence of a continued use of atrazine since the sampling campaign was conducted not far**  
287 **from the ban regulation. The percentages of atrazine contamination increased markedly with the increase in**  
288 **land use intensity, with both the number of contaminated ponds and the average concentrations of atrazine in**  
289 **the contaminated ponds being higher in intensive land use compared to the other ponds and higher in the**  
290 **extensive land use ponds than in the ponds in natural areas. In our 2007 survey, no triazine herbicides,**  
291 **insecticides or industrial compounds were detected. In this survey, however, we detected the presence of the**  
292 **herbicides isoproturon and glyphosate (and its derivative AMPA) in the majority (glyphosate) or even in all ponds.**  
293 **Such results indicate historical changes in the use of pesticides in relation to the EU regulation concerning atrazine;**

294 which led to the use of other pesticide molecules. For both the herbicides detected, there was an increase in  
295 concentration from ponds in natural areas over extensive to intensive land use ponds. Overall, our analyses did not  
296 reveal very high concentrations but did show that herbicides are detected frequently, with a clear impact of land  
297 use on the probability of occurrence and observed concentrations. Declerck et al. (2006) showed that land use  
298 intensity in the immediate neighbourhood (100 to 200 m) of the ponds strongly impact water quality variables such  
299 as turbidity and nutrient concentrations. Here we show that cropland and agricultural activities in general in the  
300 neighbourhood of the ponds lead to increased concentrations of herbicides in the water. Irrespective of this clear  
301 impact of land use, a considerable fraction of ponds in nature reserves were also contaminated. Overall, our results  
302 do reveal lower concentrations of herbicides than measured in rivers and lakes (Delbeuck, 2005, 2008). Given the  
303 importance of distance to cropland and land use intensity in the immediate neighbourhood of the ponds, our  
304 results suggest that the creation of a sizeable buffer zone (>>50 m) where no crops may be cultured around ponds  
305 might help to mitigate spray-drift input in ponds.

306 Due to the current widespread use of phenyl urea in agricultural production and as a total herbicide in urban  
307 areas, isoproturon is among the contaminants most frequently encountered in rivers, streams, lakes, marine  
308 waters and groundwater in Belgium and other European countries (Thurman et al., 2000; Gerecke et al., 2001;  
309 Sorensen et al., 2003; Delbeuck, 2005, 2008, 2010). It has also been reported that heavy rainfall subsequent to  
310 herbicide applications in spring may lead to contamination of aquatic environments by run-off (Polard et al., 2011).  
311 Indeed, it is well known that herbicide contamination negatively affects the welfare of aquatic animals by altering  
312 the food availability and habitat or some interactions between biotas such as dominance of predators. All  
313 herbicides detected in the present study have been reported to act directly on various physiological pathways  
314 of aquatic animals. High levels of atrazine are still of concern for groundwater in Belgium (Delbeuck, 2010) as in the  
315 US and other countries (Gillion et al., 2006 in Tillitt et al., 2010). Atrazine has been reported to act as endocrine  
316 disruptor, but the data are still controversial in fish (Kazeto et al., 2004; Spano et al., 2004; Hinfrey et al., 2006;  
317 Nadzialek et al., 2008; Tillitt et al., 2010). Studies on amphibians converge to the conclusion that atrazine used at  
318 environmental concentrations has marked endocrine disrupting effects that affect the central nervous and immune  
319 system of frogs and salamanders (Sparling et al., 2001; Forson & Storfer, 2006; ; Kloas et al., 2009; Lasserre et al.,  
320 2009; Hayes et al., 2010a, b). Despite massive use of isoproturon for various purposes and its possible release in the  
321 environment, information is still scarce about the long term effects of exposure to chronic low levels of this

322 herbicide in animals. Greulich et al. (2002) reported that levels of 10 µgL<sup>-1</sup> of isoproturon in water can affect the  
323 detoxification enzymatic system in tadpoles of various amphibian species. Our study confirms earlier observations  
324 that isoproturon and glyphosate herbicides are nowadays among the most frequently encountered pesticides  
325 (Delbeuck, 2005, 2008). High concentrations of glyphosate herbicides have been found in pond environments in  
326 both Europe and the US, with deleterious effects on aquatic animals, including the disturbance in the  
327 metamorphosis process, tail damages, endocrine disruption, and gonad abnormalities (Howe et al., 2004,  
328 Brausch & Smith, 2007, Dinehart et al., 2009, 2010). Since glyphosate herbicides are now massively used  
329 everywhere, more studies are needed to clarify the long-term effects of low concentrations of these pesticides  
330 on aquatic organisms.

331 Simazine and diuron were detected at very low concentrations and in only a few ponds (survey 1). None of the  
332 tested insecticides (aldrine, chlorfenvinphos, α-endosulfan, dicofol) or industrial compounds (octylphenol,  
333 nonylphenol and 4 PAHs) were detected in survey 2. This may be due to the fact that the ponds are relatively  
334 isolated in the landscape, and thus less likely to become contaminated except for application in their immediate  
335 neighbourhood. Despite the fact that these insecticides have been banned in European countries, they are still  
336 found in rivers and streams (Delbeuck, 2007) at low concentrations due to their persistence in sediments and a  
337 regular solubilisation process through the water column. Many studies on rivers and streams do show  
338 contamination by phenolic compounds (Micic & Hofmann, 2009) and PAHs (Delbeuck, 2007, 2008, 2010; Wu et al.,  
339 2011; An et al., 2012 ).

#### 340 ***Endocrine disruption in resident fish***

341 To test whether the presence of some pesticides or other pollutants were missed in the pond water and to better  
342 understand their effects on aquatic pond animals, reproductive disruption biomarkers were examined in resident  
343 Prussian carp. This subspecies of goldfish is amongst the more abundant fish species found in farmland ponds in  
344 Belgium, and has already been used as a sensitive sentinel for detecting exposure to various endocrine disruptors  
345 such as 17-β estradiol and 4-nonylphenol (Soverchia et al., 2005; Popesku et al., 2008). In the present study,  
346 intersex incidence (1.7%) was comparable to that observed in other regions in reference rivers for some cyprinid  
347 fish species (0-41%) such as chub *Leuciscus cephalus*, roach *Rutilus rutilus*, and gudgeon *Gobio gobio* (Van Aerle et  
348 al., 2001; Jobling et al., 2002; Randak et al., 2009; Hinfray et al., 2010). Moreover, VTG concentrations in males

349 (0.00-1.47  $\mu\text{gml}^{-1}$ ) did not indicate a disturbance in endocrine production and were in the same order of magnitude  
350 as those measured in some male cyprinid fish such as roach (0.047-1.502  $\mu\text{gml}^{-1}$ ) from reference stream sites (Tyler  
351 et al., 2005). VTG values were lower than values reported for male cyprinid chub from contaminated stream sites,  
352 which can reach 49  $\mu\text{gml}^{-1}$  (Hinfray et al., 2010). In relation to VTG levels, low brain aromatase activity confirmed  
353 the lack in up-regulation of endocrine production. Values for aromatase activity were indeed comparable to those  
354 reported for some cyprinid fish from river systems (Noaksson et al., 2001; Hecker et al., 2007; Hinfray et al., 2010).  
355 Since the two latter investigated endocrine features have been reported as reliable biomarkers of pesticide  
356 exposure for various aquatic organisms (Folmar et al., 2001; Hayes et al., 2006; Wingfield & Mukai, 2009; Nadzialek  
357 et al., 2011; Desforges et al., 2010), the lack of their up-regulation in fish inhabiting the ponds reflects the absence  
358 (or presence at very low concentrations) of estrogenic pollutants in the pond water. **The results of the present  
359 study indicate that fish inhabiting the farmland ponds studied were not affected by endocrine disruptors.**

### 360 ***Heavy metals***

361 For the tested heavy metals (Cd, Sn, Pb, Cu, Zn, Hg), levels in water and suspended materials were about 100  
362 times lower than those usually measured in the same matrices from rivers and streams in Belgium (Bervoest &  
363 Blust, 2003; Delbeuck, 2007). Apart from Sn, the tested heavy metals are considered problematic in surface  
364 waters in Belgium and in many other countries, but in the present study none were found at concentrations  
365 higher than the European or US criteria of good quality surface water (Delbeuck, 2007; Reynders et al., 2008;  
366 USEPA, 2009; Rimondi et al., 2012). Zn concentrations increased markedly in 2004 compared to 2003, but  
367 values were still markedly lower ( $<0.1\mu\text{gL}^{-1}$ ) than those reported for some lakes in Wallonia (0.93  $\mu\text{gL}^{-1}$ ,  
368 Delbeuck, 2007) and contaminated sites in rivers in Flanders (109-2369  $\mu\text{gL}^{-1}$ , Reynders et al., 2008). This  
369 increase in Zn levels may be related to higher rainfall during the sampling campaign in 2004 compared to that  
370 in 2003, resulting in higher runoff of heavy metals in the pond water or in an increased in situ geochemical  
371 transfer of some metals from the sediment layer to the water column. As expected, concentrations of heavy  
372 metals were higher in sediment than in water and in suspended solids, but values were still lower than those  
373 found in other, larger water bodies (Delbeuck, 2007; Reynders et al., 2008). In some ponds, levels for Cu and Pb  
374 were higher than those typically reported for rivers in Flanders (Bervoest and Blust, 2003), but for all the tested  
375 heavy metals, values were lower than minimal effect concentrations as determined for aquatic organisms  
376 (MacDonald et al., 2000).

377 No significant effect of land use intensity on heavy metal concentrations was observed. It has been shown that  
378 the concentrations of some heavy metal, such as Cu, Cd and Zn, may be increased in agricultural areas due to  
379 sewage sludge and fertilizer use (Nicholson et al., 2003, Mass et al., 2010), but overall especially industrial  
380 activities are historically associated with heavy metal pollution (Loska et al., 2004; Reynders et al., 2008).  
381 Isolated ponds such as those tested in the current study may be relatively spared from pollution by heavy  
382 metals and industrial such as PAHs. Exceptions would be ponds located in the immediate vicinity of sources, or  
383 ponds that are regularly flooded by large water.

384 We established a significant relation between heavy metal concentrations in sediment and accumulation in the  
385 snails, indicating that snails are reliable sentinel species for heavy metal pollution in pond environment.  
386 Concentrations of heavy metals in snails were for some metals, such as Cd and Cu, comparable to those  
387 reported in other snail species (such in *Melanopsis praemorsa* and *Theodoxus jordani*) or higher than values  
388 reported for other species (such in *Perna perna*, *Dosinia sp* or *Donax rugosus*), even though the water bodies in  
389 these other studies were characterized by higher concentrations of heavy metals (Swailed et al., 1994;  
390 Sidoumou et al., 2006). In contrast, while Sn was present in all pond matrices, only trace levels were observed  
391 in the snails, indicating a low uptake or high elimination rate (Grosell et al., 2009; Moloukhia & Sleem, 2011) of  
392 this metal by the snails. It has been reported that snail species differ strongly in heavy metal accumulation and  
393 are more sensitive to metal pollution than other aquatic animals such as fish (Voet et al., 2006; Ng et al., 2011;  
394 Moloukhia & Sleem, 2011). In general, metal exposure is followed by a rapid activation of detoxification  
395 pathway defence, such as the increase of specific granules in the digestive gland cells for metal sequestration  
396 and the induction of metallothionein production by lysosomal granules in the endoplasmic reticulum for metal  
397 binding and excretion (Desouky, 2006, Voet et al., 2009; Ng et al., 2011). Despite the efficiency of the  
398 detoxification mechanism, long term exposure of aquatic animals to metal pollution is detrimental to their  
399 fitness because the energy requested for detoxification defence is re-allocated from normal physiological  
400 functions such as growth and reproduction (De Coen & Janssen, 2003; Ng et al., 2011). Some toxicological  
401 studies have also suggested a link between metal exposure and reproductive impairment in fish populations  
402 (Boyle et al., 2008); high levels of some heavy metals such as Cd could delay oocyte maturation and ovulation  
403 by disturbing the vitellogenesis process rather than the pituitary pathway control in Prussian carp (Szczerbik et  
404 al., 2006). A recent study demonstrated that Cu or Cd itself has no direct effect on VTG expression but may



405 increase the estrogenic-VTG induction level comparing to a single estrogenic exposure in goldfish (Chang et al.,  
406 2011), but no abnormal increase in the levels of plasma vitellogenin was observed both in males and females in  
407 the current study. In the current study, concentrations Cu or Cd were low in all the tested pond matrices and  
408 no alteration in brain aromatase activity or circulating vitellogenin level was observed in Prussian carp  
409 inhabiting representative pond environment of the Belgian intensive or extensive land use ponds.

#### 410 **Conclusions**

411 The overall results of our survey study demonstrated that small Belgian ponds were mainly contaminated by some  
412 herbicides and sporadic high discharges of heavy metals in sediment and snails, **and that pond sedentary fish were**  
413 **not affected by endocrine disruptors**. Our results also demonstrated a marked effect of agricultural land use  
414 intensity on herbicide pollution; **which would be mitigated by** an adjustment of the land use in the immediate  
415 neighbourhood of the pond or the use of sufficiently large buffer zones.

416

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420

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648

649 **Table captions:**

650 Table 1 Summary of pesticide surveys covering almost the entire Belgium territory (spring 2004) or the main  
651 pesticide pulverisations periods (April, July and October) over 2007

652 Table 2 Summary of the number of ponds and pond matrices tested for heavy metals (Cd, Sn, Zn, Cu, Pb and  
653 Hg) during summer 2003 and spring 2004

654 Table 3 Coefficients of correlations between values for heavy metal (Cd, Zn, Cu, Pb) concentrations in snails and  
655 in sediments sampled in ponds located in intensive and extensive agricultural areas or in natural reserves  
656 during spring 2004. Sediment concentrations were closely related to snail values, except for only Sn; Hg was  
657 not assayed in snails or sediment

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660 **Figure captions**

661

662 **Fig. 1a** Map of Belgium showing the locations of the 42 investigated clusters (circular symbols). Within a  
663 cluster, three small ponds were selected along a maximal gradient of surrounding agricultural land use  
664 intensity, ranging from relatively natural area (“ponds in natural areas”), pastures with low cattle density  
665 (“extensive land use ponds”) to areas with intensive agriculture, especially cropland (“intensive land use  
666 ponds”).

667 **Fig. 1b** Locations of the fifteen small ponds selected in the Northern (Flanders) and Southern (Wallonia) regions of  
668 Belgium in areas of intensive or extensive land use or in natural reserves. The ponds were selected among the 126  
669 ponds sampled during spring 2004 using the same gradient of land use intensity

670 **Fig. 2a-b: Atrazine occurrence (Fig. 2a) and concentrations (2b)** in water sampled during spring 2004 in ponds  
671 located in relatively natural areas, areas with extensive and areas with intensive agricultural land use. Atrazine  
672 contaminations were higher ( $P < 0.05$ ) in intensive land use ponds than in extensive land use ponds or ponds in  
673 natural ponds. n = number of ponds in which atrazine was detected.

674 **Fig. 3** Seasonal changes (3a, n = number of ponds) and variation in isoproturon levels (3b, n = number of  
675 samples) in water sampled three times in ponds located in intensive and extensive agricultural areas or in  
676 natural reserves during 2007. Isoproturon contamination was more frequent in October and April, and values  
677 were higher ( $P < 0.05$ ) in ponds located in agricultural areas than ponds in natural areas.

678 **Fig. 4** Seasonal changes (4a, n = number of ponds) and variation in glyphosate and AMPA levels (4b, n = number  
679 of samples) in water sampled three times in ponds located in intensive and extensive agricultural areas or in  
680 natural reserves during year 2007. Glyphosate and AMPA contamination was more frequent in October and  
681 April, and values were higher ( $P < 0.05$ ) in intensive land use ponds than in extensive land use ponds or in  
682 ponds in natural areas

683 **Fig. 5** Profiles of plasma vitellogenin levels (5a) and brain aromatase activity (5b) in sedentary Prussian carp  
684 juveniles sampled in ponds located in intensive and extensive agricultural areas in 2008 and 2009. N = 30  
685 juveniles. No modulation was observed in plasma VTG or brain aromatase activity in males, whatever the type  
686 of pond the fish were sampled from.

687 **Fig. 6** Variation in heavy metal concentrations in water (6a), suspended solids (6b), sediment (6c) or snail  
688 homogenates sampled in ponds in intensive and extensive agricultural areas or in ponds in natural reserves  
689 during summer 2003 or spring 2004. No significant effect of land use intensity on heavy metal concentrations  
690 was observed whatever the pond matrix.

691

692

693 **Table 1**

	Once sampling over Spring 2004	Three-month sampling over 2007
Number of ponds	126	15
Pesticide types :		
- Herbicides	- atrazine, - simazine, - propazine, - terbutylazin, - triazine metabolites, - diuron, - isoproturon	- atrazine, - simazine, - propazine, - terbutylazin, - triazine metabolites, - diuron, - isoproturon, - glyphosate, - aminomethyl-phosphonic acid (AMPA)
- Insecticides	- endosulfan, - lindane	- aldrine, - chlorfenvinphos, - endosulfan, - dicofol
- Industrial compounds		- octylphenol, - nonylphenol, - 4 polycyclic aromatic hydrocarbons (PAHs: benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene) and benzo(ghi)perylene)

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697 **Table 2**

	Summer 2003	Spring 2004
Water	99	126
Suspended solids	99	-
Sediment	-	68
Snails	14	34

698 - No sampling

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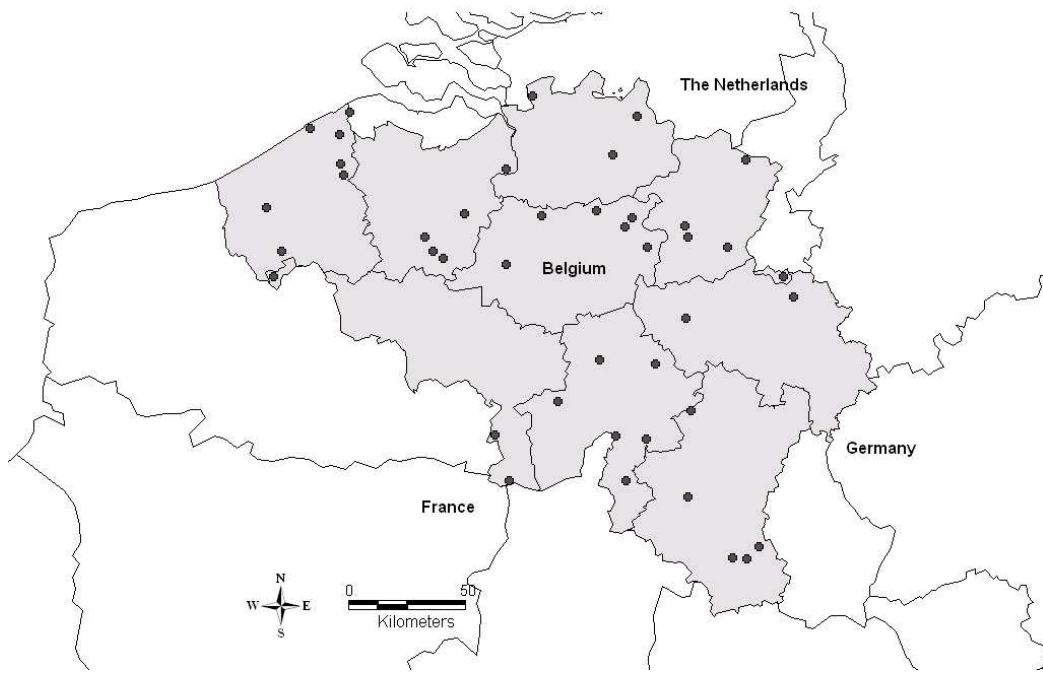
701 **Table 3**

	R	p
Cd	0.49	0.027
Pb	0.99	0.000
Cu	0.38	0.071
Zn	0.65	0.001

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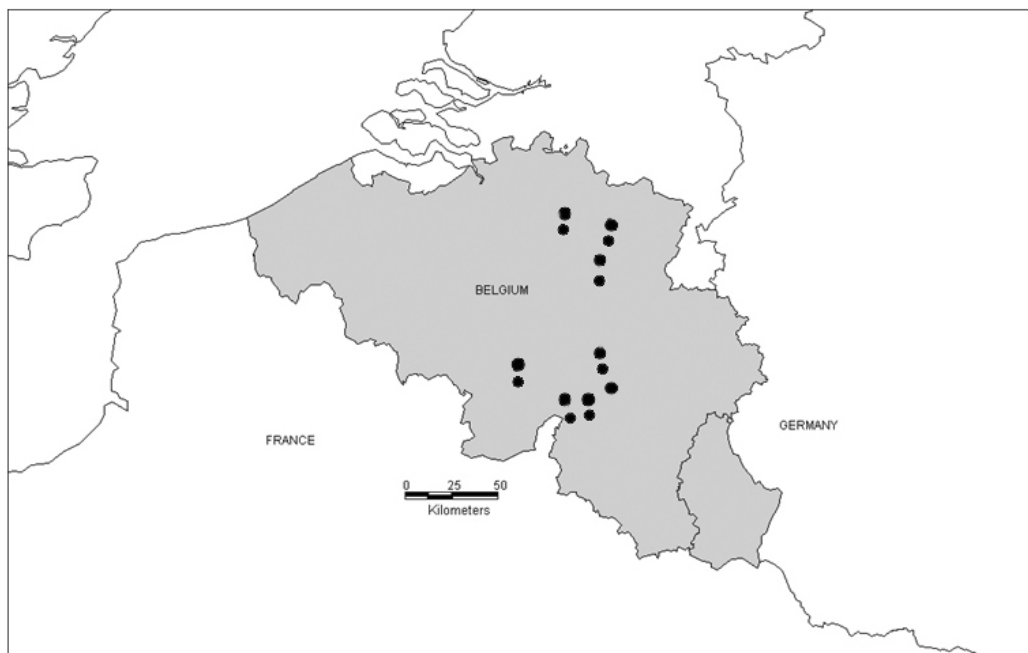
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705 **Fig. 1a**



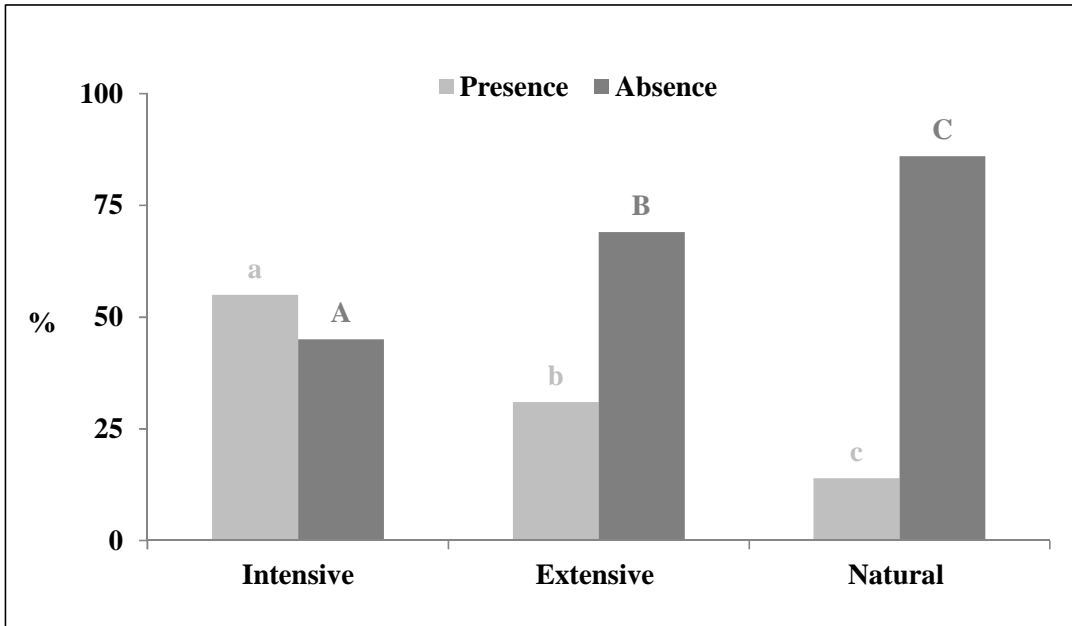
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707 **Fig. 1b**

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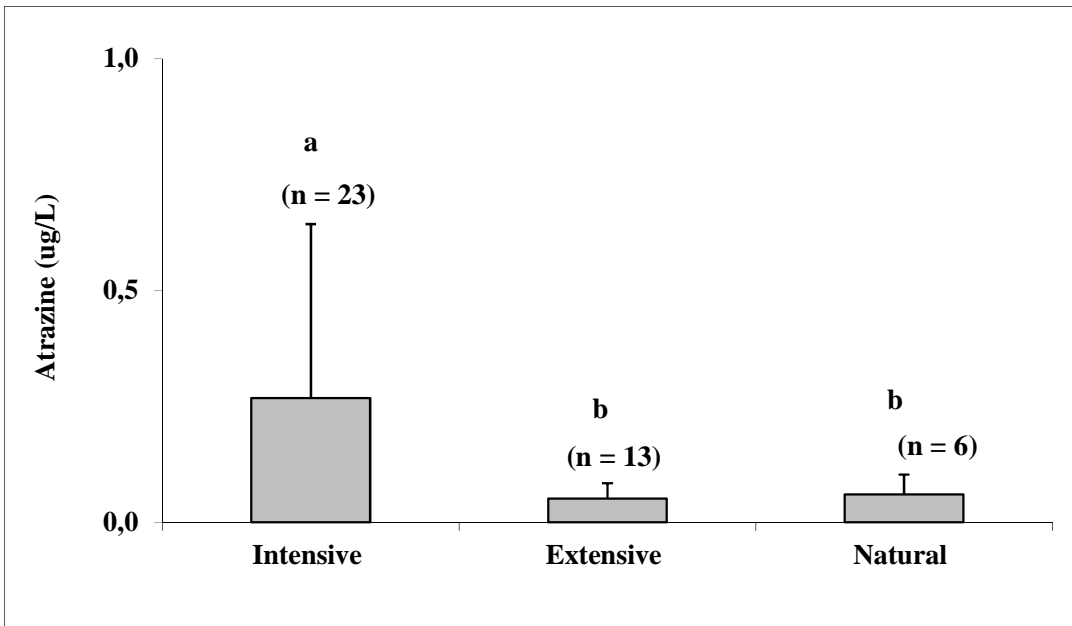
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712 Fig. 2a



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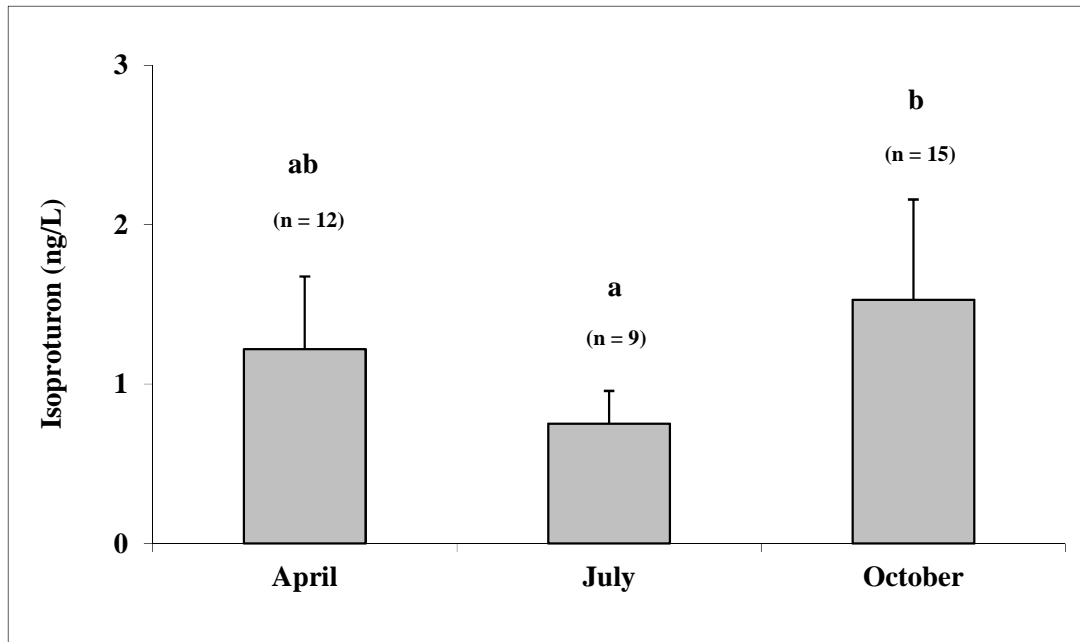
714 Fig. 2b

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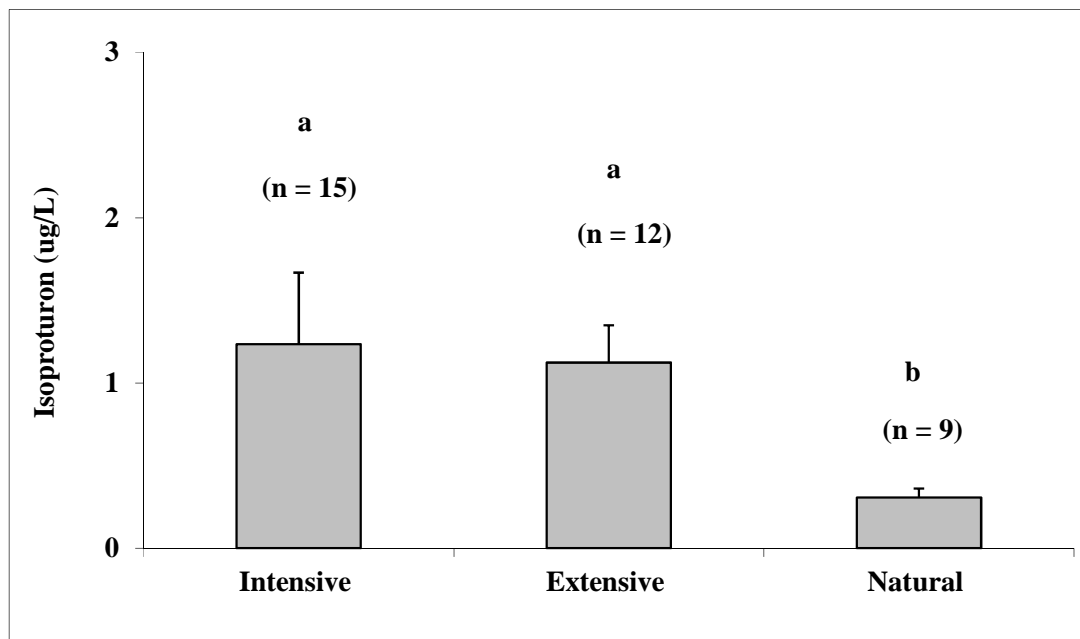
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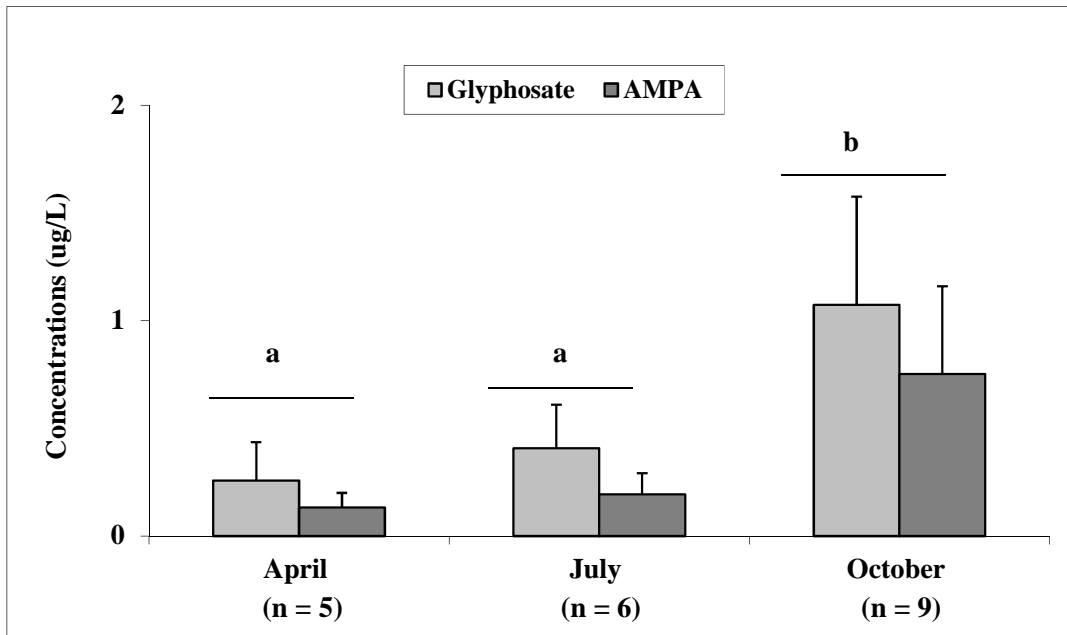
720 Fig. 3a



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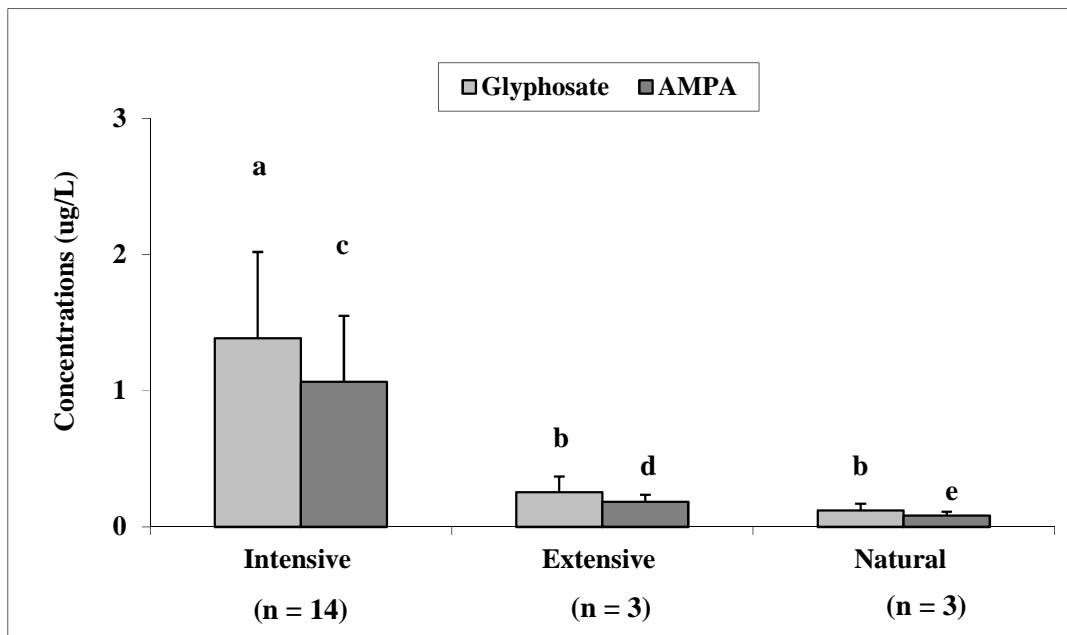
722 Fig. 3b

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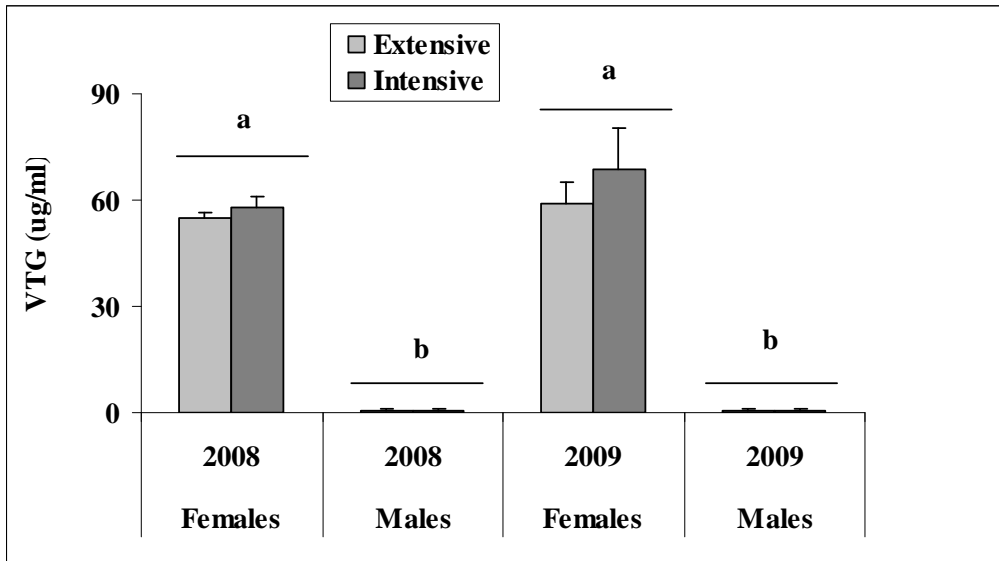
726 Fig. 4a



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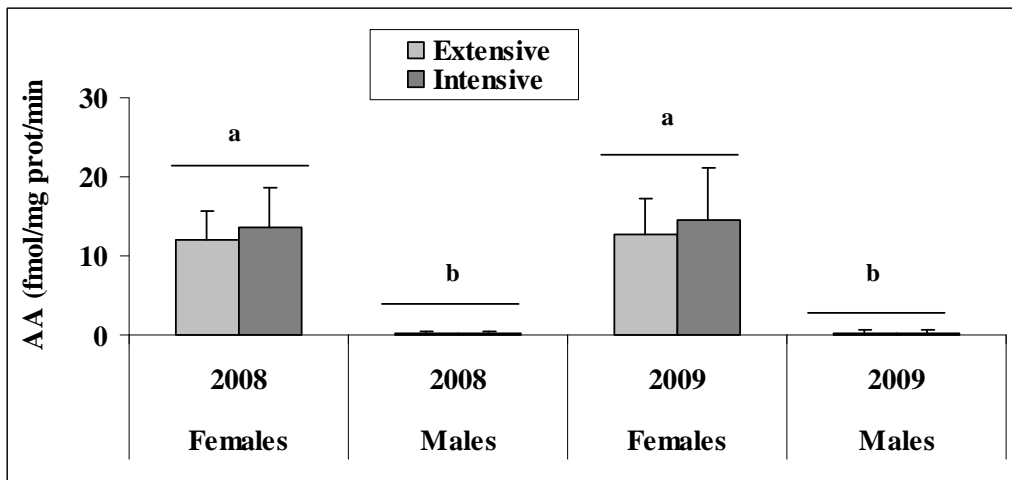
728 Fig. 4b

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731 Fig. 5a



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733 Fig. 5b

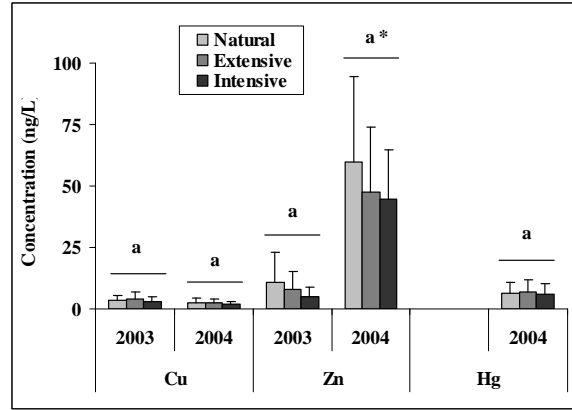
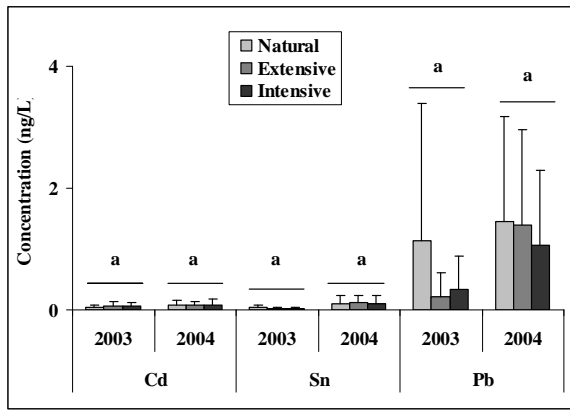
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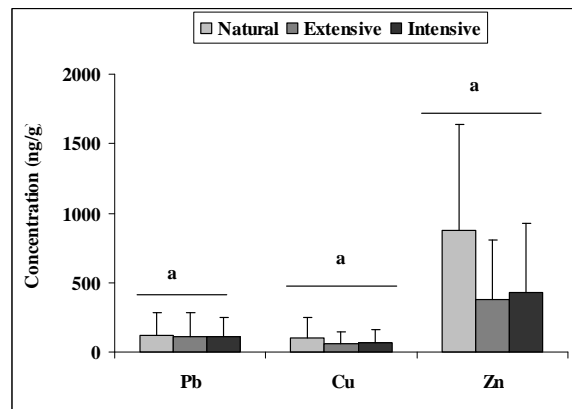
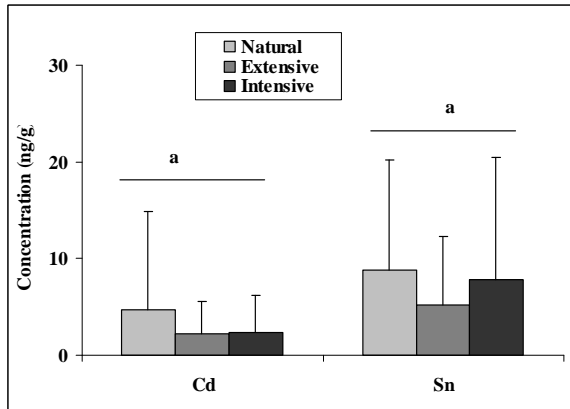
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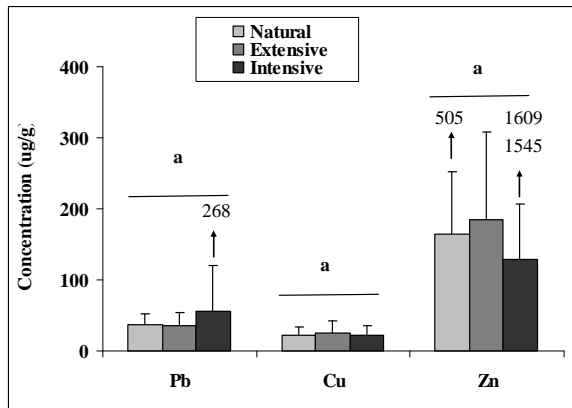
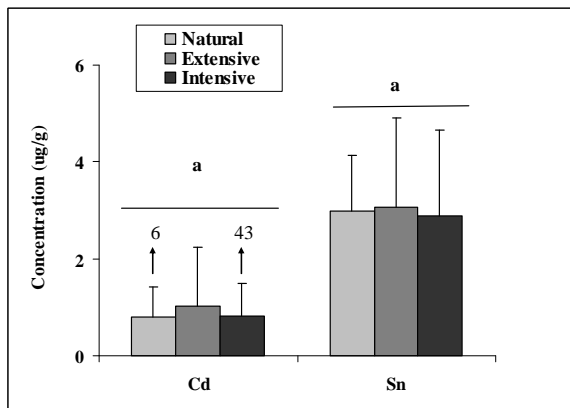


739 Fig. 6a



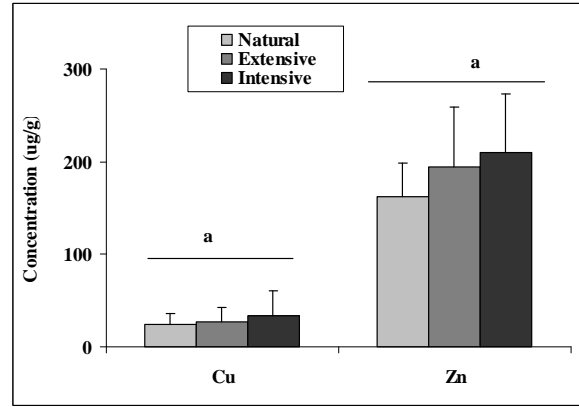
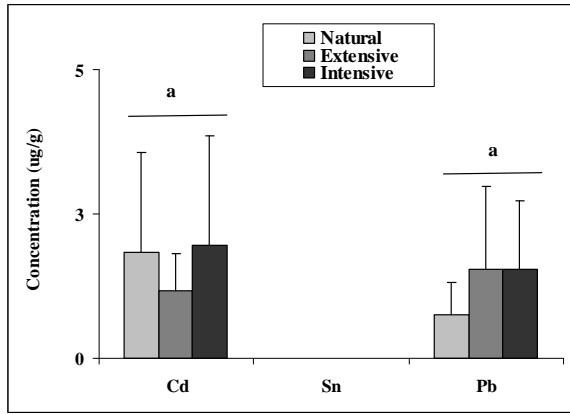
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741 Fig. 6b



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743 Fig. 6c



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745 Figure 6d

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