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First evidence of fish genotoxicity induced by heavy metals from landfill leachates: The advantage of using the RAPD-PCR technique

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ABSTRACT

Municipal leachates are loaded with heavy metals that can contaminate surface water before discharge into a receiving body of water. The aim of this study is to evaluate the genotoxic effects of heavy metals generated by domestic waste on the common roach *Rutilus rutilus* in the last of the four interconnected ponds at the Etueffont landfill. We used random amplified polymorphic DNA (RAPD) since it has been shown to be a powerful means of detecting a broad range of DNA damage due to environmental contaminants. Our results show the ability of RAPD analysis to detect significant genetic alterations in roach DNA, after contamination with a set of metals contained in the landfill leachates in comparison to a roach from a non-polluted reference pond. Analysis of electrophoresis profiles indicates apparent changes such as the appearance of new bands or disappearance of bands as compared to the control. In fact, mixed smearing and laddering of DNA fragments in muscle samples support the genotoxic effects of metal deposits in the roach. This study is the first evidence found via the RAPD-PCR technique in the detection of pollutant impacts on fish exposed to landfill leachates.

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1. Introduction

Landfill leachate generated by biodegradation of solid wastes may pollute both ground and surface waters (Khatabi and Aleya, 2007; Öman and Junestedt, 2008) as it filters out of a landfill site. Widely used in Europe and North America, landfilling is the most attractive option for waste disposal, though it may be a source of large quantities of organic and inorganic matter and heavy metals (Bulc, 2006). Organisms living in constructed wetlands such as landfill leachate treatment systems are subject to chemical pressure and must tolerate high levels of heavy metals and high organic and nutrient loads (Schwarzbauer et al., 2002). Such is the case for the domestic landfill treatment station located in Etueffont (Territoire de Belfort, France) which consists of four interconnected natural lagooning ponds that collect leachates downstream via a draining system prior to water discharge into a small stream. We have indeed shown in these lagoons that, despite environmental stress, biological communities including phytoplankton and zooplankton (Khatabi et al., 2006), macro-invertebrates (Khatabi and Aleya, 2007) and bacteria (Grisey et al., 2010) develop well. In addition, it has been demonstrated that

macrophytes – the cattail (*Typha latifolia* L.) and the reed (*Phragmites australis* L.) – have a capacity for heavy metal storage in the phytomass without effects on their growth (Grisey et al., 2012). In 2007, three thousands roach *Rutilus rutilus* were introduced into the last pond in an effort to add the missing trophic compartment (fish) to the classical food web, i.e. phytoplankton–zooplankton–fish and to later examine the potential hazards of heavy metals for the fish.

As has been previously demonstrated for the Etueffont landfill (Khatabi and Aleya, 2007) and other sites (Al-Yaqout and Hamoda, 2003; Ziyang et al., 2009), landfill leachate is one of the major sources of metals discharged into surrounding environments, yet metals are important environmental hazards due to biomagnification (Masters, 1997), long-term accumulation in living organisms, in water and sediments (Linnik and Zubenko, 2000; Che et al., 2006; Palaniappan and Karthikeyan, 2009) and toxicity (Szefer et al., 1990). Moreover, many studies of fish genotoxicity demonstrate the role of metals in triggering DNA damage (Vargas et al., 2001; Valko et al., 2006; Barbosa et al., 2010). Metal toxicity can be influenced by various factors such as pH, alkalinity, temperature (Adhikari et al., 2006), oxygen and hardness (Ghillebaert et al., 1995). Heavy metals in mixture are more toxic to fish than separate ones (Alabaster and Lloyd, 1982), which could adversely affect human health (Gerhard et al., 1998; Nowak and Kozłowski, 1998; Lee et al., 2006). Accordingly, the toxicity of

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landfill leachate has been assessed by several researchers using a number of different living organisms, including luminescent bacteria *Vibrio fischeri* (Silva et al., 2004), aquatic invertebrates (Zaltauskaite and Cypaite, 2008; Khattabi and Aleya, 2007) and aquatic vertebrates such as fish (Alkassasbeh et al., 2009). Fish are ecophysiologicaly affected and their distribution prone to changes as a consequence of degradation in water quality (Aleya et al., 1994; Hued and Bistoni, 2005). In addition, they play a key role in community dynamics due to the place they occupy in the trophic chain. Among potential fish bioindicator species for environmental stress, the zebrafish *Danio rerio* is the most extensively studied experimental model. However, the roach *R. rutilus* (Linnaeus) could perhaps satisfy the main requirements advanced for bioassay testing because it is a native viviparous and one of the most common and widely distributed species in Europe (Kubecka, 1993). It is found in all kinds of water bodies (lakes, rivers, channels, brackish pools, estuaries, etc.).

To assess these potential effects, several classic assays have been used to examine the genotoxic impacts of heavy metals on fish, mainly the micronucleus assay and the comet assay (Mitchelmore and Chipman, 1998; Cambier et al., 2010; Omar et al., 2012). However, the random amplified polymorphic DNA (RAPD) analysis is more robust as it is a powerful PCR-based technique that involves the amplification of random segments of genomic DNA (De Wolf et al., 2004). In addition, the assay does not need prior knowledge of the genome under investigation, and arbitrarily chosen short primers (generally a 10 bp sequence) are used at low stringency to amplify multiple segments from genomic DNA. RAPD assay permits the detection of genetic alterations, after contamination with pollutants in aquatic organisms, as well as detection of intrapopulational polymorphism. The appeal of the RAPD technique is its requirements of only small quantities of DNA. For example, it has been evaluated as a potential tool to detect the genotoxicity of nitrofurazone in the marine ciliate *Euplotes vannus* (Zhou et al., 2011), ultraviolet radiation in the marine macroalgae *Palmaria palmata* (Atienzar et al., 2000), copper toxicity in the cladoceran *Daphnia magna* (Atienzar et al., 2001), 4-n-Nonylphenol and 17-beta estradiol in the crustacean barnacles *Elminius modestus* (Atienzar et al., 2002) and tritiated water in the bivalve mollusc *Mytilus edulis* (Hagger et al., 2005). More recently, the RAPD methodology has been applied in detecting the genotoxic potential of some chemicals and metals in fish (Castaño and Becerril, 2004; Zhiyi and Haowen, 2004; Mohanty et al., 2009; Rocco et al., 2010; Orioux et al., 2011; Osman et al., 2012). However, as far as we know, there is no information regarding the potential polymetallic genotoxic effects on the roach (i) in situ and (ii) especially the potential polymetallic genotoxic effects from leachates originating in domestic wastes. Since fish is a healthy and valuable food, investigating toxic metals in fish muscle becomes priority because of the potential risk to humans who consume them (Amundsen et al., 1997; Sandor et al., 2001; Burger and Gochfeld, 2005; Yilmaz and Doğan, 2008; Yildirim et al., 2009; Fallah et al., 2011). Moreover, fish muscle is a reliable source of DNA (Chakraborty et al., 2006; Weber et al., 2003) used with success in several populations and taxonomical studies.

The aim of this study was thus to investigate the potential genotoxic effect of polymetallic pollution in the muscles of roach with RAPD-PCR in the last of the four interconnected ponds of the Etueffont landfill (Belfort, France).

2. Materials and methods

2.1. Study site

The Etueffont municipal solid waste landfill was opened in 1974. It is located north-east of Belfort (France) (Fig. 1a, Appendix 1) and extends over a total surface area of 2.2 ha underlain by impermeable schistous layers. The width, length and

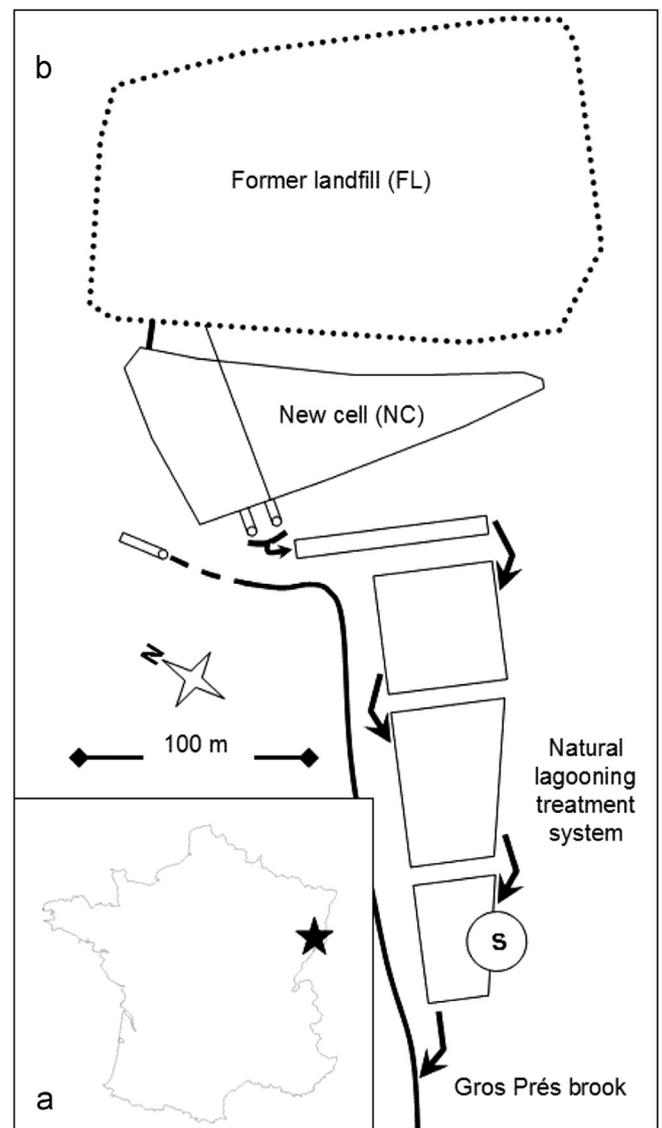


Fig. 1. Study site. (a) Etueffont landfill location and (b) schematic map of the site: former landfill (FL), new cell (NC), S: fourth sampling pond of the natural lagooning treatment system.

depth are respectively 110, 200 and 5 m. The landfill, operating in the open air, contains 200,000 t of household refuse ground prior to discharge. The leachates are collected downstream via a draining system and treated in a four-pond lagooning system. Two sand filters with perforated drains, were placed upstream from the first pond (Aleya et al., 2007). These geotextiles consisted of synthetic fibres (polymeric materials) made of flexible porous fabrics by standard weaving machinery (woven geotextile). From 1976 to 1998 domestic wastes were deposited directly onto the soil surface. This dump zone is referred to as the former landfill (FL). After 1998, the waste was dumped into a new watertight cell (NC). Waste disposal at the site stopped in 2002 in accordance with French legislation (Grisey et al., 2010). Part of the leachates, originating in the FL and NC, were collected and then treated by a natural lagooning system composed of four ponds (Khattabi and Aleya, 2007). As previously said, in 2007 three thousand roach *R. rutilus* were introduced into the last pond (Fig. 1b).

2.2. Roach capture

In summer 2010 roach were caught in the fourth pond (fish size: 128.01 mm \pm 3.15) using a fishing rod and also in an unpolluted reference lagoon at Bailly Francheville (LFVl) (fish size: 142.75 mm \pm 1.707). Fish were initially introduced at the same time in control and exposed ponds. The fish were first wrapped in polyethylene plastic, put into an isolated container, and brought to the laboratory, where they were immediately frozen and stored at -20°C until dissection. The water chemistry samples were collected in quantities of 150 ml, also from the fourth pond and from the reference lagoon LFVl in summer 2010.

2.3. Heavy metal analysis

After collection of water samples, the bottles were stored at 4 °C for preservation before preparation and analysis. The samples were filtered by means of a 0.45- μm membrane. Twenty-five millilitres of each sample was prepared with 6 ml HNO_3 before analysis. Concentration of heavy metals in water samples was determined with the inductively coupled plasma optical emission spectrometer (ICP, OES) (Varians, 720-ES). International certified reference materials for the water (SRM 1643e) were analysed at the beginning and end of each batch of samples to assess accuracy and precision. The emission lines that were employed were As (I) 188.980, Cd (II) 214.439, Cu (I) 327.395, Mn (II) 257.610, Ni (II) 231.604, Pb (II) 220.353 and Zn (I) 213.857.

2.4. Genomic DNA extraction

To minimise contamination, all the materials used in the experiments were previously washed in 15 per cent sodium hypochlorite solution and rinsed in ultra pure water; a sterilised stainless steel knife was used to cut the tissues.

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the supplier's instructions. For each sample, approximately 20 mg of muscle was frozen in liquid nitrogen and homogenised in 180 μl tissue lysis buffer ATL with 20 μl proteinase K (12 h at 56 °C). The DNA solution was treated with 100 $\mu\text{g ml}^{-1}$ RNase A (15 min at 37 °C) and the pellet discarded after centrifugation (14,000 rpm for 3 min). Buffering conditions were adjusted and DNA was loaded onto the DNeasy Mini spin column. Total genomic DNA was eluted with 120 μl buffer AE, and stored at -20 °C until used. The concentration and purity of DNA were estimated using both NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) and 3 per cent agarose gel electrophoresis.

2.5. RAPD analysis

RAPD reactions were performed using PuRe Taq Ready-To-Go™ PCR beads (GE Healthcare Biosciences, Pittsburgh, Pennsylvania, USA). Information on the primer set used is given in Appendix 2. RAPD-PCR was performed on two pools of genomic DNAs extracted from ten individuals of both control (LFVl) and test (B4) fishes. The genomic DNAs from ten individuals were blended to suppress the intra-population genetic polymorphism potentially revealed by RAPD. Each RAPD reaction contains thermostable polymerases (AmpliTag™ DNA polymerase and Stoffel fragment), BSA (2.5 μg), dNTPs (0.4 mM each dNTP), buffer [3 mM MgCl_2 , 30 mM KCl and 10 mM Tris, (pH 8.3)], genomic DNA, 25 pmol of random primer and ultra-pure water, for a final volume of 25 μl . Fifty nanograms of genomic DNA were subjected to amplification in a Mastercycler thermal cycler (Eppendorf, Le Pecq, France) for 45 cycles (1 min at 94 °C to denature DNA, 2 min at 36 °C for annealing, then 2 min at 72 °C for the extension step), followed by a final 10 min extension at 72 °C. The reaction mixtures were stored at -20 °C prior to use. The PCR products were resolved by electrophoresis on 3 per cent agarose gel at 35 V for 3 h. Gels were stained with ethidium bromide and the bands visualised with a UV lamp and documented using an Image system.

3. Results and discussion

3.1. Heavy metals

General water quality characteristics of the two sampling sites are summarised in Tables 1 and 2. These results show that the fourth pond contains very high metal concentrations, exceeding international water quality standards (Canadian Council of Ministers of the Environment, 1999; US Environmental Protection Agency, 1999; Flemish Government, 2000), compared to the reference Francheville

Table 1

Physico-chemical variables the fourth pond of the lagooning system (B4) and Bailly Francheville (LFVl) lagoon.

	B4	LFVl
NH_4^+ ($\mu\text{g l}^{-1}$)	1420 \pm 1850	1500 \pm 860
NO_2^- ($\mu\text{g l}^{-1}$)	80 \pm 50	bdl
NO_3^- ($\mu\text{g l}^{-1}$)	1070 \pm 1000	1300 \pm 170
PO_4^{3-} ($\mu\text{g l}^{-1}$)	180 \pm 210	bdl
T (°C)	19.9 \pm 0.3	20.03 \pm 0.25
pH	7.6 \pm 0.1	7.1 \pm 0.14
DO (mg l^{-1})	6.9 \pm 1.01	8.6 \pm 0.25
Conductivity ($\mu\text{S cm}^{-1}$)	770.0 \pm 3.78	672.67 \pm 10.01

Table 2

Concentration of heavy metals at the sampling sites.

	B4	LFVl	CQC	USEPA	FQC
As	2278.3 \pm 647	0.0021 \pm 0.00	–	–	–
Cd	90 \pm 35.8	0.0011 \pm 0.00	0.02	2.2	1.0
Cu	7525.1 \pm 5568	0.0021 \pm 0.00	24	9.0	50
Mn	549.64 \pm 636	0.0213 \pm 0.0041	–	–	–
Ni	8606 \pm 3890	0.0021 \pm 0.00	25	52	30
Pb	90 \pm 35.8	0.0021 \pm 0.00	17	2.5	50
Zn	7405.9 \pm 4010	0.0136 \pm 0.0032	30	120	200

$n=6$ for B4 and $n=3$ for LFVl.

CQC, Canadian quality criteria for aquatic freshwater life.

USEPA, US Environmental Protection Agency.

Flemish quality criteria.

Lagoon which exhibited good water quality characteristics. We indeed recorded high to very high levels of Cd, Cu, Zn and Ni: 90 \pm 35.8; 7525.1 \pm 5568; 7405.9 \pm 4010 and 8606 \pm 3890 $\mu\text{g l}^{-1}$ respectively. Significant differences were found between the two sites (ANOVA, $F=6.68$; $P<0.01$). The high level of heavy metals, mainly Cd, Cu, Zn and Ni, at B4 may be attributed to the leachates contained in the 200,000 t of refuse landfilled in 1999, 4 per cent of which are trace metals (Khattabi et al., 2006). Furthermore, we have recently reported that the high values of all the heavy metals recorded in the water inflow did not affect the growth of the two predominant plant species (*T. latifolia* and *P. australis*), thus the most urgent step was to further investigate the impact of a high metal concentration on fish.

3.2. Analysis of DNA fragmentation

The purity and integrity of the DNA template are crucial for good RAPD analysis (Zhou et al., 1997). The 260 nm/280 nm absorbance ratio obtained from the roach muscle extraction is in the range of 1.85–1.9. This indicates a high DNA purity grade of extraction. The genomic DNA purified from the roach *R. rutilus* muscle from the LFVl Lagoon reference, showed a very low stained smear pattern upon electrophoresis, with no evidence of DNA-ladder (see Fig. 2, lane 1). In contrast, the genomic DNA purified from the Etueffont B4 pond revealed internucleosomal fragmentation (ladder pattern) mixed with a smear-like pattern, generally regarded as a molecular hallmark of apoptosis and necrosis (Fig. 2, lane 2). Indeed, a regulatory role of iron, copper and zinc in endonuclease activity and apoptosis has been suggested by several authors (McCabe et al., 1993; Shiokawa et al., 1994; Burkitt et al., 1996).

3.3. RAPD fingerprinting pattern

Comparing the amplified product using different DNA template concentrations, the product derived from the roach DNA of 50 ng is richer than the sample of 5 ng DNA template and clearer than the one obtained from the 500 ng DNA template (Fig. 3). Thus, in our experimental conditions, the 50 ng genomic DNA template is the optimal concentration for this RAPD reaction, though the amount of genomic DNA used in this PCR reaction can vary from as little as 5 ng to as much as 500 ng (Muralidharan and Wakeland, 1993; Zhou et al., 1997). The same concentration was also used to amplify the DNA product of zebrafish (Zhiyi and Haowen, 2004).

3.4. Effect of heavy metals on RAPD profile

To see the genetic effect of heavy metal contamination, RAPD analysis was performed on DNA extracted from muscle of *R. rutilus*

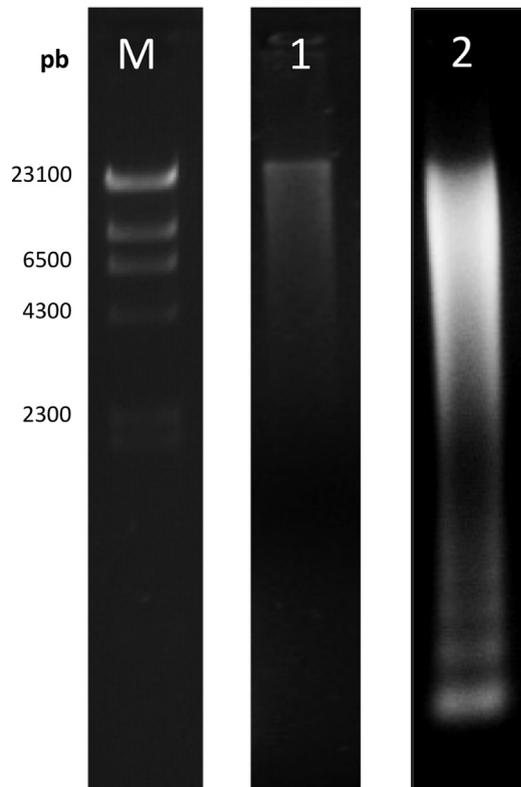


Fig. 2. Agarose gel electrophoresis of purified genomic DNA from *Rutilus rutilus*. Purified genomic DNAs extracted from ten *Rutilus rutilus* fish collected from the unpolluted reference lagoon at Bailly Francheville (lane 1) and from the fourth pond of the natural lagooning system (lane 2). DNAs were separated by electrophoresis on 3.0 per cent agarose gels in TBE buffer, and visualised after staining with ethidium bromide. Total DNA isolated from control fish was of high molecular weight (greater than 23 kb) and unfragmented (lane 1). M, Lambda DNA *Hind*III molecular weight marker.

caught in B4 and LFvl. Six different random primers were tested, each giving a specific amplification profile. All RAPD pattern modifications were presented in Fig. 4. The number of total fragments obtained with the six different primers tested on DNA isolated from control fish was nineteen (Fig. 4, fragments indicated by white stars on the gels). RAPD profiles evidenced substantial differences between control and exposed fish. Our results corroborate those of Omar et al. (2012) and Osman et al. (2012) who reported that the genetic diversity in fish populations living in polluted sites is altered in comparison to non-polluted areas. Our results are also supported by several studies evidencing the genotoxic effect of heavy metal pollution on fish health (Shinn et al., 2009; Svecevićius, 2010; Orieux et al., 2011; Svecevićius et al., 2012). However, as no data are available from heavy metals originating from landfill leachates, we cannot compare our findings to those of the literature.

Apparent changes were observed, such as appearances of some new bands or the disappearance of bands as compared to the control. A maximum of one band disappeared among the exposed fish with primers 2 and 4, while in primer 3 a maximum of two new bands appeared in the fish exposed to heavy metal pollution (Fig. 4, P2 to P6, see arrow on the gels).

According to Atienzar and Jha (2006) and Noel and Rath (2006), interaction of genotoxic agents with DNA can induce alterations to the structure and function of DNA, including DNA adducts and breakage. Mutations and changes were translated with appearance and/or disappearance of bands as well as variation in band intensities. Admitting these findings, we can assume that the differences between the profiles of RAPD-PCR product of exposed

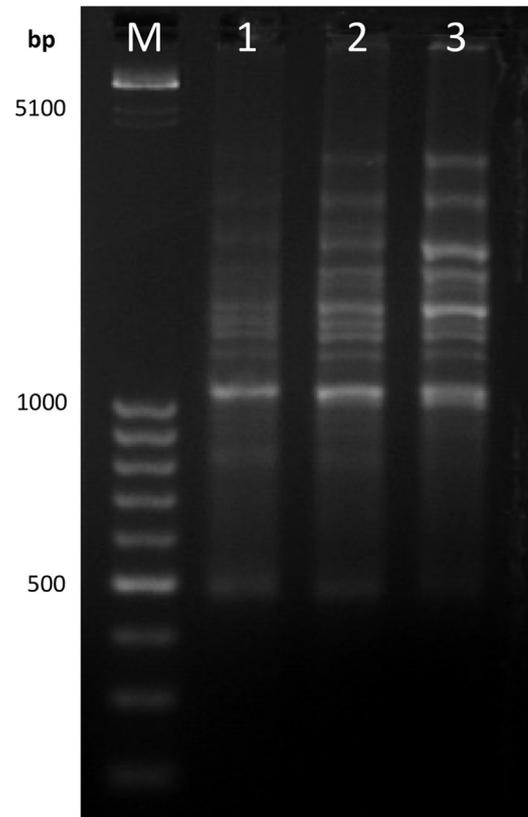


Fig. 3. RAPD patterns obtained from different amounts of genomic DNA with primer 1. The quantities of DNA template tested varied as follows: lane 1, 5 ng; lane 2, 50 ng; and lane 3, 500 ng. The amplified PCR products were resolved by electrophoresis through a 3 per cent agarose gel. RAPD reaction with 50 ng genomic DNA template produced distinct bands (lane 2). Lanes indicated by M contains Lambda DNA double digested with *Hind*III and *Eco*RI as molecular marker.

and non-exposed roach are the result of metal toxicity. In fact, RAPD assay does not provide information on the nature and extent of these genotoxic-induced DNA alterations, but is often used as a quantitative tool (Atienzar and Jha, 2006).

RAPD banding patterns from pooled genomic DNA were similar in both the control and exposed roach with primer 1 (see Fig. 4, P1) confirming that DNA from the pooling of ten roach were sufficient to remove the intra-population variability due to genetic polymorphism, which is a similar outcome to the results of Cambier et al. (2010). Since not all the regions of the genome are equally sensitive to the genotoxic impact of toxicants, inactive chromatin regions are protected and less prone to modifications than active ones (Cambier et al., 2010). Therefore, one should not expect that all RAPD primers can reveal the genotoxic effect of metal. Yet, tested primers 2–6 could reveal different RAPD banding patterns between the controls and exposed genomic DNA from the pooled roach species (Fig. 4, P2–P6), suggesting differences in sensitivity depending on primer sequence. The appearance of new bands, or the disappearance of bands in the five primers, indicate a clear ability of metals such as Cd, Zn, Cu and Ni to induce DNA damage in fish. It has been shown that Cd is capable of inducing DNA damage and mutations such as point mutations, small insert and deletions, rearrangements, ploidy changes, single- and double-strand breaks, base substitution, oxidised bases, and even bulky adducts at specific loci of DNA in fish cells (Castaño and Becerri, 2004). Moreover, Cu induced a significantly higher level of oxidative DNA damage, suggesting that DNA damage in fish can serve as a sensitive biomarker for changes in water quality as well as the presence of genotoxic chemicals (Bertin and Averbeck, 2006; Mustafa et al., 2012). Nickel is carcinogenic and bioaccumulative that may alter cell

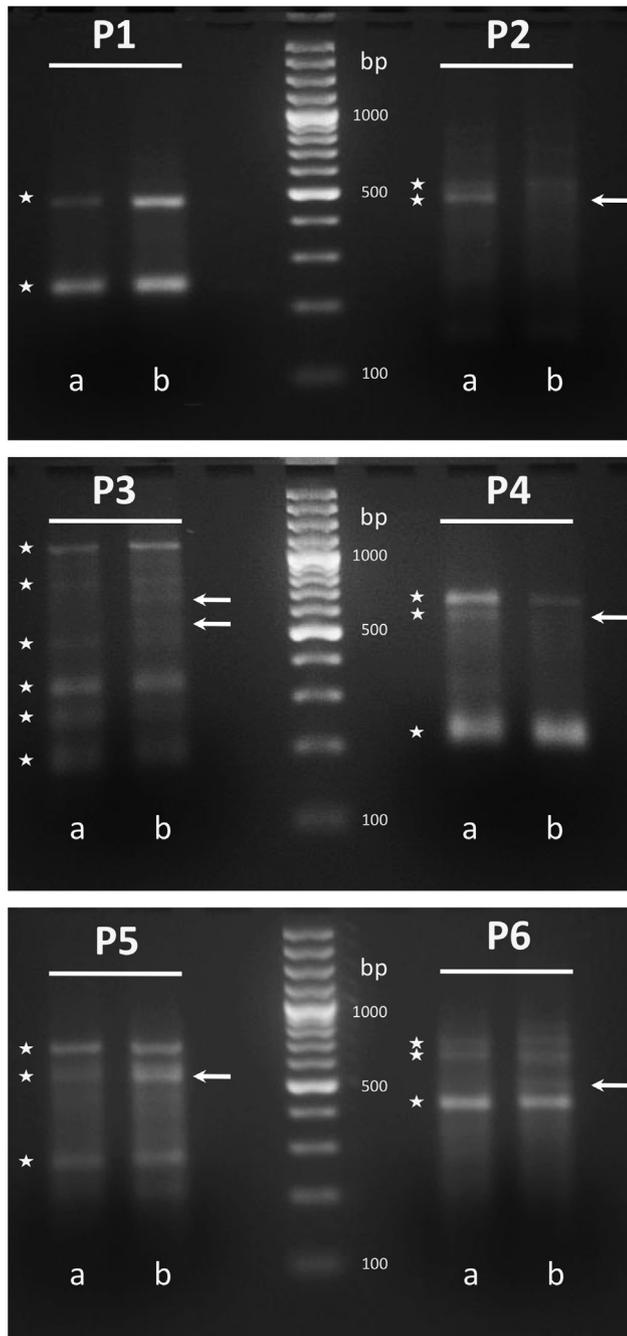


Fig. 4. RAPD-PCR patterns from genomic DNA of ten *Rutilus rutilus* fish from the (a) lagoon Bailly Francheville (LFV1) and (b) the fourth pond of the lagooning system (B4). RAPD profiles were generated using primers P1, P2, P3, P4, P5 and P6. For information one the six arbitrary primers used for RAPD analysis see Appendix 2. Amplification products were fractionated in a 3 per cent agarose gel. The gels were stained with ethidium bromide and visualised under UV light. The appearance and disappearance of bands and intensity variations were indicated by white arrows. The nineteen DNA fragments corresponding to the number of total fragments obtained with the six different primers tested on genomic DNA isolated from control fishes were indicated by white stars. The DNA standard was Thermo Scientific GeneRuler 100 bp Plus DNA Ladder (Fisher Scientific, France).

proliferation, genetic expression and calcium-dependent enzyme activity, and interfere in DNA methylation and repair (Buschini et al., 2009; Zhou et al., 2009). Eisler (1998) summarised data on the toxic mode of action of nickel in fish and concluded that toxic and carcinogenic effects of nickel compounds are associated with nickel-mediated oxidative damage to DNA and proteins and to

inhibition of cellular antioxidant defenses. As regards Zn, Bagdonas and Vosyliene (2006) and Obiakor et al. (2010) indicated that even a short-term exposure of fish to Zn concentrations higher than those prescribed as maximum acceptable concentrations could produce genotoxic effects on erythrocytes. Then, as indicated by Alabaster and Lloyd (1982) and Enserink et al. (1991), when mixed, metals may have additional chronic toxicity compared with their individual effect. These results may explain why DNA damage occurred in fish subjected to polymetallic pollution in the fourth Etueffont pond.

4. Conclusion

This study demonstrated that the polymetallic pollution reaching the last pond of the Etueffont landfill is genotoxic to roach *R. rutilus* as evidenced by differences in RAPD fingerprinting patterns between control and exposed roach. Our results give the first evidence from the RAPD-PCR technique in detecting the impact of pollutants on fish exposed to landfill leachates. Further studies are needed to better elucidate the relation cause-effect between heavy metals and genotoxicity on *R. rutilus* so as to deepen the cause of the damages observed.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ecoenv.2013.12.014>.

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