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SHORT COMMUNICATION

Use of the random amplified polymorphic DNA (RAPD) assay for the detection of DNA damage and mutations: possible implications of confounding factors

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The aim of this study was to evaluate the potential of the random amplified polymorphic DNA (RAPD) assay to qualitatively detect the kinetics of benzo[*a*]pyrene (B[*a*]P)-induced DNA effects in the water flea *Daphnia magna* exposed to 25 and 50 $\mu g\,l^{-1}$ B[*a*]P for 7 and 6 days, respectively. Mortality was recorded on a daily basis in both experiments, and RAPD analysis was performed on samples collected every day following isolation of genomic DNA. The main changes occurring in RAPD profiles produced by the population of *Daphnia magna* exposed to 25 and 50 $\mu g\,l^{-1}$ B[*a*]P was a decrease and increase in band intensity, respectively. Most of the changes occurring in the RAPD patterns were likely to be the result of B[*a*]P-induced DNA damage (B[*a*]P DNA adducts, oxidized bases, DNA breakages) and/or mutations (point mutations and large rearrangements). In addition, reproducible changes also occurred in the profiles generated by control *Daphnia magna*. The results lead us to suggest that, in addition to B[*a*]P-induced DNA damage and mutations, factors such as variation in gene expression, steady levels of genetic alterations and changes in metabolic processes could induce some changes in RAPD patterns. Nevertheless, our data suggest that DNA damage and mutations appear to be the main factors influencing RAPD patterns. This study also emphasizes that unexpected variation in control profiles is not always associated with artefacts.

Keywords: RAPD, DNA damage, mutation, confounding factors, B[*a*]P, *Daphnia magna*.

Introduction

Despite growing concern over the presence of genotoxins in the aquatic environment, there is a lack of well-validated methods that can detect genotoxicity and mutagenicity of contaminants under *in vivo* conditions. Polymerase chain reaction (PCR) based methods such as the random amplified polymorphic DNA (RAPD) assay (Williams *et al*. 1990) and the arbitrarily primed PCR (AP-PCR) (Welsh and McClelland 1990) have been shown to have great potential in the detection of diverse genetic alterations. Recent studies include the detection of DNA damage and/or mutations induced by diverse chemical (Atienzar *et al*. 1999, 2001, Becerril *et al*. 1999, Lopez *et al*. 1999) and physical (Shimada and Shima 1998, Atienzar *et al*. 2000a) agents. Both the AP-PCR and RAPD methods are based on the selective amplification of genomic sequences that, by chance, are

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flanked by adequate matches to an arbitrarily chosen oligonucleotide primer sequence. These methodologies present numerous advantages. No prior knowledge of the genome (gene sequence or karyotype) under investigation is required, very little genomic DNA material is needed, and results are generated rapidly. Another remarkable advantage is the overall sensitivity of the techniques. And last but not least, RAPD and AP-PCR assays have the potential to detect a wide range of DNA damage (e.g. DNA adducts, DNA dimers, DNA breakage) as well as mutations (point mutations and large rearrangements). Despite these advantages, these PCR-based methods (but mainly the RAPD assay) have been criticized for their lack of reproducibility (Khandka *et al.* 1997) and the presence of non-parental bands (Ayliffe *et al.* 1994). Nevertheless, after rigorous optimization, the RAPD methodology has been reported as a robust assay with good reproducibility under defined conditions (Bielawski *et al.* 1995, Atienzar *et al.* 2000b). Finally, the cause of changes in profiles are often open to speculation unless specific analysis (e.g. cloning, sequencing or probing) is performed on the bands subjected to variation.

In an earlier study, we demonstrated the usefulness of the RAPD assay following *in vivo* exposure of the water flea *Daphnia magna* to benzo[*a*]pyrene (B[*a*]P) (Atienzar *et al.* 1999). However, RAPD profiles were generated at single point in time. In the present study, we aimed to evaluate the usefulness of the RAPD assay further; in particular we attempted to determine the kinetics of the DNA effects in *Daphnia magna* exposed to B[*a*]P and the reproducibility of the assay (as a function of time).

Materials and methods

Experimental design

Daphnia magna (*D. magna*, clone 5) were cultured as described in Atienzar *et al.* (1999). Freshly born neonates (less than 48 h post hatch) were exposed in groups of 20 animals to 25 and 50 $\mu g\,l^{-1}$ B[*a*]P for 7 (experiment 1) or 6 (experiment 2) days, respectively. It was decided to continue both experiments (which were run at different times) until the swimming behaviour of the *D. magna* was clearly affected. Test solutions were prepared from stock solutions of B[*a*]P at a concentration of 2 $g\,l^{-1}$ in dimethylformamide (DMF), then stored at 4°C prior to use. The B[*a*]P solutions were added to the *D. magna* culture medium using a volume of 100 µl of DMF in 1 l of medium, thereby ensuring a level below 0.05%, the maximum percentage of the solvent recommended by the American Society for Testing Materials (ASTM 1975). For each experiment, two control groups containing 20 animals were used. The controls consisted of a non-treated group and a group exposed to 100 µl DMF alone in 1 l of medium. A total of 180 (nine groups of 20) animals were used for each experiment, including the controls, solvent controls and the B[*a*]P-treated groups. Animals were fed during the experiments and the medium was changed three times a week as described earlier (Atienzar *et al.* 1999). The number of surviving animals was recorded, and samples (8–20 animals) were placed in 1.5 ml microcentrifuge tubes and stored at −80°C on a daily basis for 7 (experiment 1) or 6 (experiment 2) days. The whole population was sampled (i.e. maximum of 20 animals); when the number of survivors fell below eight, surviving *D. magna* were used from other beakers to get a minimal number of 8–10 animals per tube. Thus, DNA extraction was performed using between eight and 20 animals. This explains why the starting number of *D. magna* in both experiments was higher than 120 (6 × 20) for experiment 1 and 140 (7 × 20) for experiment 2 for the B[*a*]P-exposed groups.

Generation of D. magna *DNA profiles using RAPD*

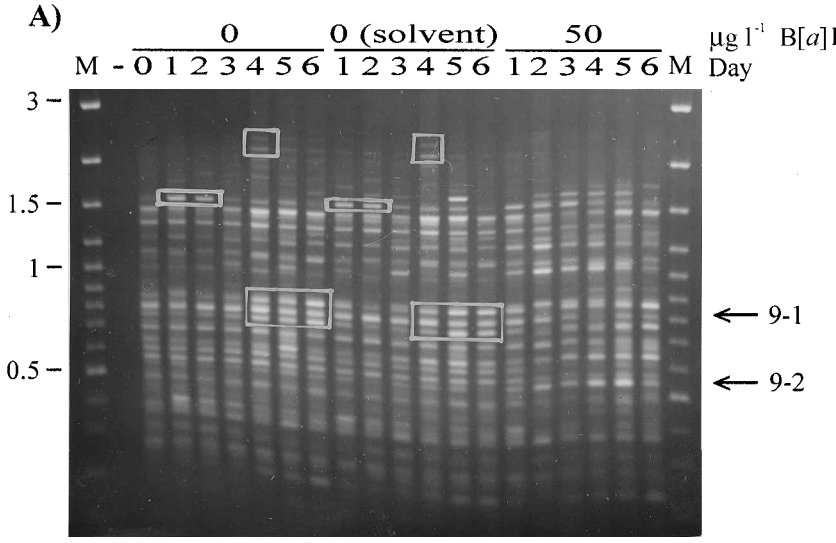
Total DNA from the *D. magna* was extracted and purified using a conventional phenol/chloroform method as described elsewhere in detail (Atienzar *et al.* 1999). The DNA concentration of each sample was determined by comparison with known concentrations of lambda phage DNA, as mentioned in Atienzar *et al.* (2001). DNA profiles of *D. magna* were generated in RAPD reactions performed under optimized conditions (Atienzar *et al.* 2000b). The 10-mer primers used (OPA9, OPB1, OPB5, OPB6, OPB7, OPB8, OPB10 and OPB17) were ordered from Operon Technologies (Southampton, UK); the

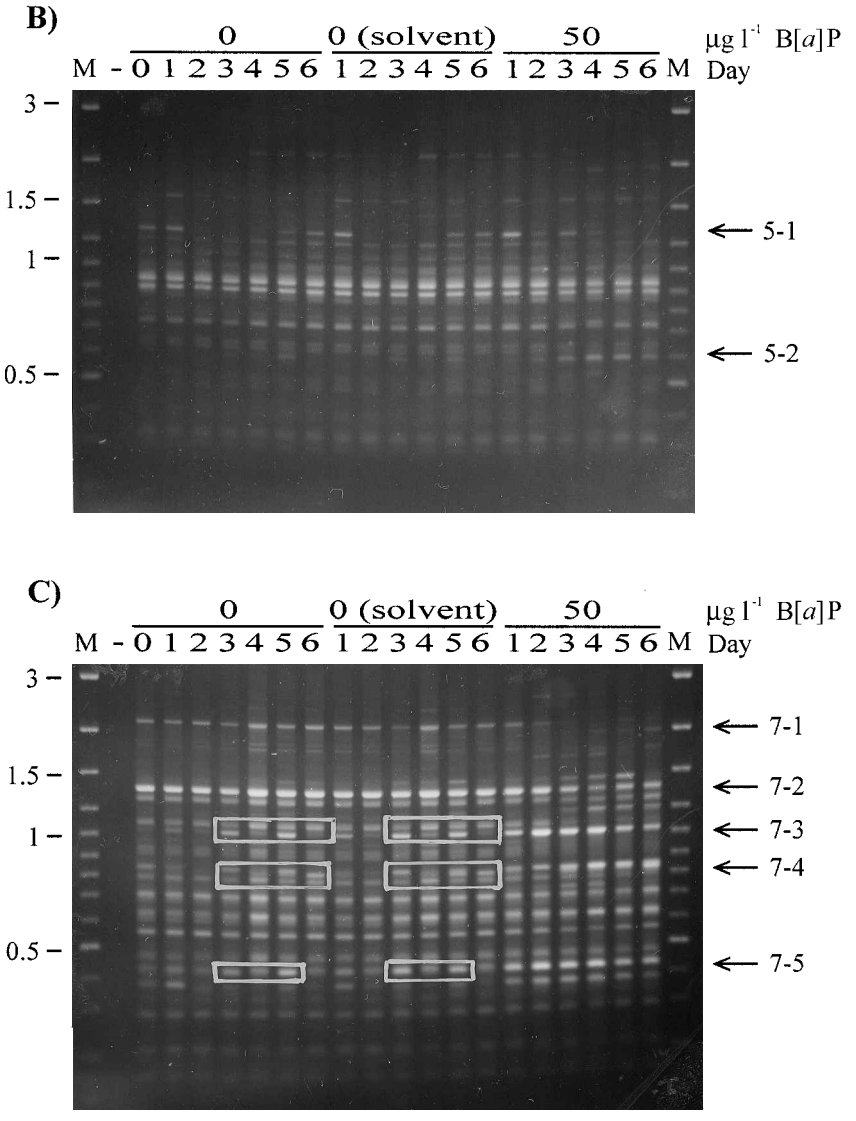
sequences of each primer can be obtained from http://www.operon.com. Electrophoresis and analysis of the RAPD profiles were performed as described in Atienzar *et al.* (1999). For comparison a DNA molecular size marker (GeneRuler^{TM} 100 bp DNA ladder plus; Immunogen International, Sunderland, UK) was used. The bands visualized, from top to bottom, were 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp.

Results

The survivorship results (data not presented) revealed that mortality was limited in the controls from both experiments, the highest proportion of dead animals being 10%, and that the control animals were healthy until the end of the experiments. In contrast, *D. magna* exposed to 25 $\mu g\,l^{-1}$ B[*a*]P were weak (unable to swim properly) from the fifth day of exposure, and only 15% of the animals survived to the end of the experiment. When the water flea was exposed to 50 $\mu g\,l^{-1}$ B[*a*]P, the animals were weakened from the second day until the end of the experiment. The number of surviving *D. magna* decreased gradually to a low level, and only 20% of this group survived to day 6.

The RAPD profiles obtained from the population exposed to 25 $\mu g\,l^{-1}$ B[*a*]P revealed that the intensity of some bands decreased mainly on the last 2 or 3 days of the experiment compared with the control profiles (data not shown). Furthermore, other primers revealed little or no obvious differences among profiles. The patterns that were obtained from the population exposed to 50 $\mu g\,l^{-1}$ B[*a*]P showed that the intensity of some bands decreased (e.g. 9-1, 7-2), while others increased (e.g. 9-2, 7-3, 7-4, 7-5) (figure 1). Moreover, band 5-2 appeared from day 2 until day 6 (figure 1B), whereas band 7-1 clearly disappeared after the second day of exposure (figure 1C). However, the major effect of exposure to 50 $\mu g\,l^{-1}$ B[*a*]P was that band intensity generally increased, whereas with 25 $\mu g\,l^{-1}$ B[*a*]P the main change in the profiles was a decrease in band intensity compared with control patterns. It is also noteworthy that the changes occurring in the RAPD profiles generated by the population exposed to 50 $\mu g\,l^{-1}$ B[*a*]P were more easily identifi-





able than those in the RAPD profiles produced by the population exposed to $25\,\mu g\,l^{-1}$ B[*a*]P.

The presence of some bands (e.g. 5-1, figure 1B) was not constant, even in control patterns, which did not facilitate the analysis. However, it was also clear that band 5-1 followed a specific pattern of variation in both non-exposed and solvent control groups which was perturbed when the *D. magna* were exposed to 50 but not to $25\,\mu g\,l^{-1}$ B[*a*]P (table 1). This phenomenon was also observed for a number of other bands (figure 1A and 1C; changes indicated by a frame), and the extent of variation seemed to depend on the choice of primer. For some primers, the variation in RAPD profile observed in both controls was identical, whereas for other primers little or no variation occurred between both profile controls (e.g. figure 1D).

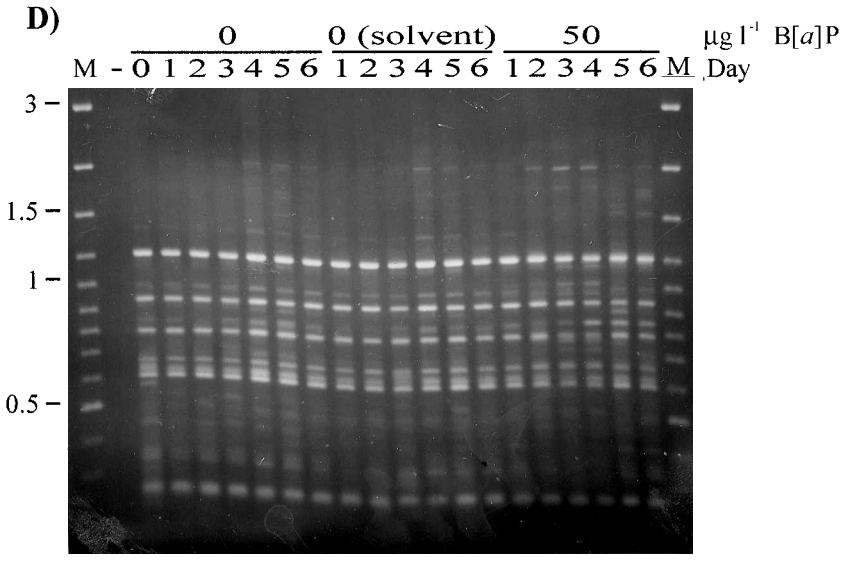


Figure 1. Kinetics of RAPD profiles of *D. magna* exposed to 50 µg l^{-1} B[*a*]P. The patterns were obtained using the 10-mer primers OPA9 (A), OPB5 (B), OPB7 (C) and OPB10 (D). M = GeneRuler^{TM} DNA ladder plus (Immunogen International). The molecular sizes (kb) of selected bands are shown on the left of each gel. – indicates no DNA control. Selected changes compared with control patterns are indicated by arrows. The frames indicate that the same variations occur in profiles generated by both controls.

Table 1. Presence (+) and absence (−) of band 5-1 in control, solvent control and B[*a*]P-exposed *D. magna* in both kinetic experiments.

	Control							Solvent control							B[*a*]P-exposed						
Days of exposure	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Band 5-1, first kinetic	+	−	−	−	+	+	+	+	−	−	−	+	+	+	+	−	−	−	+	+	+
Band 5-1, second kinetic	+	−	−	−	+	+	ND	+	−	−	−	+	+	ND	+	+	+	−	−	−	ND

ND = not determined.

Discussion

The exposure of *D. magna* to 25 µg l^{-1} B[*a*]P resulted in detectable DNA effects on the sixth and seventh day of exposure, whereas the animals exposed to 50 µg l^{-1} B[*a*]P showed evidence of DNA effects from the first day of exposure. The number of changes occurring in the RAPD profiles produced by the population of *D. magna* exposed to 50 µg l^{-1} B[*a*]P were greater and more easily detectable than those generated by the animals exposed to 25 µg l^{-1} B[*a*]P. In this study, the main change occurring in the RAPD profiles produced by the population of *D. magna* exposed to 25 and 50 µg l^{-1} B[*a*]P was a decrease and an increase in band intensity, respectively. This suggests that the DNA effects induced by both B[*a*]P concentrations were qualitatively and/or quantitatively different and may have been the results of DNA damage and/or mutations. These effects include changes in oligonucleotide priming sites due to mutations and/or DNA damage, as well as interactions of the *Thermus aquaticus* DNA polymerase with damaged DNA. The presence of diverse types of DNA lesions and mutations may also induce import-

ant structural changes that can significantly affect the binding of the primers (Bowditch *et al*. 1993, Atienzar 2000). In a set of experiments performed *in vitro*, we demonstrated that DNA damage such as B[*a*]P adducts, pyrimidine dimers and DNA breakage as well as point mutations in the sequence of the primer had a significant effect on RAPD profiles (Atienzar 2000).

Interestingly, band 5-1 generated by the controls and solvent controls followed a particular pattern (table 1) and some of the changes were very reproducible between both controls. This suggests that factors other than B[*a*]P-induced genetic alterations may have had some underlying effects on the RAPD profiles. The fact that *D. magna* can triple its body size within 6 days undoubtedly reflects substantial changes in gene expression. It is now well established that the methylation of specific cytosine residues in eukaryotic DNA controls the expression of some genes, leading to structural changes in the genomic DNA (Kohwi and Kohwi-Shigematsu 1991, Wolffe and Hayes 1999). It is worth mentioning that the patterns of methylation in the genomic DNA should remain unchanged during DNA extraction and PCR amplification. Thus, these structural changes induced in control genomic DNA of *D. magna* may have some observable effects on RAPD profiles. Although the first step of the PCR involves DNA denaturation at 95°C for 5 min, it is unlikely that the energy is sufficient to totally denature all the genomic DNA. However, changes in gene expression are probably not the only factors that can potentially affect the reproducibility of RAPD profiles. Other parameters such as genomic amplification and rearrangement, which are components of normal cellular development, may also effect RAPD profiles. There is also a possibility that DNA damage (e.g. 8-hydroxyguanine) in control animals could conceivably be induced by reactive oxygen species produced as a result of the normal metabolic processes that maintain steady-state levels of genetic damage (Hanawalt 1998) and may therefore contribute to differential RAPD profiles. Nevertheless, looking at the entirety of our results (Atienzar 2000), it seems that DNA damage and mutations (including point mutations and large rearrangements) have a very significant influence on RAPD patterns. Other factors such as variation in gene expression are likely to induce changes in these profiles, but it is proposed that these parameters have less impact than genotoxin-induced DNA damage and mutations, since structural constraint or distortion induced by DNA damage (e.g. DNA adducts, pyrimidine dimers) is stable (subject to repair), whereas most structural effects induced by changes in gene expression will be lost in the denaturation of the DNA during PCR. Indeed, changes in RAPD profiles did not always occur even when genomic DNA was extracted and amplified from actively growing *D. magna* (depending on the sequence of the primers), whereas DNA damage and mutations are expected to induce changes in the RAPD profile with most of the primers.

The changes in RAPD profiles that were generated by the control population of *D. magna* in the first kinetic experiment did not reveal as many changes (except band 5-1) as control patterns obtained from the second kinetic experiment (data not presented). This could be due to the fact that these experiments were run at two different times. During one of the kinetic experiments the temperature in the culture room was subjected to a relatively small but unusual variation (i.e. 20 ± 4°C instead of 20 ± 2°C), and it is possible that this external factor had an effect on metabolic processes and/or gene expression, and consequently on RAPD profiles.

Apart from this, it seems difficult to suggest any logical explanation for these differences.

The presence of variations in control RAPD profiles may lead some researchers to criticize the RAPD method because of the non-reproducibility of some bands generated by individuals of the same species. However, before concluding that false amplifications have occurred, it is necessary to test the PCR by using two DNA template concentrations, as recommended by Welsh *et al.* (1995). In this study, however, as the RAPD profiles generated from the non-exposed and the solvent controls were reproducible at precise times, there was no need to use two different DNA template concentrations. Because of this reproducibility, it seems likely that the changes in the RAPD profiles were not due to artefacts. This is why only repetitive bands (both absent or present) were considered for analyses, and we strongly recommend all users of this technology to do likewise.

In conclusion, this study shows that the RAPD method can be useful to qualitatively assess the kinetics of the DNA effects induced by B[*a*]P in populations of *D. magna*. The results also suggest that, while factors such as variation in gene expression, steady levels of genetic alterations and changes in metabolic processes may induce some alterations in RAPD profiles, DNA damage and mutations seem to be the main factors influencing variations in RAPD patterns. Finally, it is likely that most of the changes seen in the control RAPD profiles occur when the genomic DNA is extracted and amplified from actively growing organisms.

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