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Fitness Parameters and DNA Effects Are Sensitive Indicators of Copper-Induced Toxicity in *Daphnia magna*

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This study compared the effects occurring at molecular and population levels in *Daphnia magna* exposed to copper concentrations in the range of 15–120 μg/l. The qualitative and quantitative modifications arising in random amplified polymorphic DNA (RAPD) profiles as a measure of DNA effects were compared with a number of key ecological fitness parameters, namely, the age-specific survival, age-specific fecundity, net reproductive rate, and intrinsic rate of population increase. Results suggested that growth, reproduction, and most of the fitness parameters as well as genomic template stability (a qualitative measure reflecting changes in RAPD profiles) were significantly affected at copper concentrations of 90 and 120 μg/l. Among the fitness parameters, the age-specific fecundity and net reproductive rate were the most sensitive parameters of toxicity. Changes in RAPD patterns generally occurred at copper concentrations of 90 and 120 μg/l, but with one primer, changes significantly arose at all copper concentrations. Overall, molecular and population parameters compared well and represented a sensitive means to measure toxicity induced by copper in *Daphnia magna*. In conclusion, the measurement of parameters at both molecular and population levels is valuable for investigating the specific effects of agents interacting with DNA. Ultimately, this methodology may allow the ecotoxicological examination of the link between molecular alterations and measurable adverse effects at higher levels of biological organization.

Key Words: **copper; RAPD; DNA effects; genomic template stability; fitness parameters; *Daphnia magna*.**

Although the detection of DNA damage has been widely studied using a number of laboratory methods, to the best of our knowledge, the study of DNA lesions and their consequences at higher levels of biological organization has seldom been attempted (Anderson and Wild, 1994; Jha, 1998). Not only can genotoxins shorten the life expectancy of organisms, but they can also result in alterations in population dynamics. Thus, they can have an effect at both intra- and inter-species biodiversity (Anderson and Wild, 1994; Depledge, 1998; Würgler and Kramers, 1992). Such changes may initiate direct and adverse ecological consequences. In this context, research is needed to improve our understanding of the consequences of DNA damage and mutations at the population level. While neoplasia in invertebrate species is considered to be low in comparison with tumor incidences reported in fish (Couch and Harshbarger, 1985), this is not to say that genotoxic agents are without effects. Kurelec (1993) suggested that, in invertebrates, genetic damage is manifested as a suite of pathological changes, the so-called "genotoxic disease syndrome." In this context, the effect of genotoxins on growth, fecundity, and mortality are particularly relevant.

In this study, the effects of copper were evaluated at the population and molecular levels in an invertebrate species. Copper is essential to living systems, in part through its fundamental role in electron transport, respiration, growth, and development (Linder, 1991). This essential element is associated with key enzymes and/or proteins, such as superoxide dismutase (Atienzar *et al*., 1998; Fridovich, 1995), metallothioneins (Hamer, 1986), and cytochrome *c* oxidase (Steffens *et al*., 1987) which are well represented in invertebrates (Linder, 1991). In vertebrate cells, copper has been implicated in the stabilization of chromosomes and it may be involved in transcription and/or specific transcription-stimulatory events affected by hormones (Linder, 1991). However, numerous studies have also reported that copper induces toxicity (Conradi and Depledge, 1998; Koivisto and Ketola, 1995) including genotoxicity (Eichhorn and Shin, 1968; Lloyd and Phillips, 1999). For instance, the binding of copper to DNA bases unwinds the double-helix (Eichhorn and Shin, 1968) and DNA damage can be generated. Lloyd and Phillips (1999) reported that the binding of copper to DNA is necessary for the generation of double-strand breaks, 8-hydroxydeoxyguanosine, and putative intrastrand cross-links in Fenton reactions.

The generation of genomic DNA profiles, using randomly primed polymerase chain reaction (PCR) protocols such as the arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990) and random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990) has proved valuable in many areas of biomedical research. RAPD and AP-PCR are powerful tools for gene mapping, population, pedigree analysis, phylogenetic studies, and strain identification (Grayson *et al*., 2000; Liu *et al*., 1999; Tinker *et al*., 1993). In addition, their use in survey-

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ing genomic DNA for evidence of various types of damage and mutation suggest that they may potentially form the basis of novel genotoxicological assays for the detection of DNA damage and mutations (Atienzar, 2000; Atienzar *et al*., 1999, 2000a; Becerill *et al*., 1999; Ionov *et al*., 1993; Kohno *et al*., 1994; Kubota *et al*., 1992, 1995; Lopez *et al*., 1999; Peinado *et al*., 1992; Savva, 1998; Shimada and Shima, 1998). Despite earlier criticism of the RAPD assay (Ellsworth *et al*., 1993; Khandka *et al*., 1997), subsequent reports have demonstrated that after rigorous optimization of the PCR parameters, the assay performs well, in terms of number of amplified bands, product yield, and clarity of the profiles, with a wide range of organisms including bacteria, plants, as well as invertebrate and vertebrate animals (Atienzar *et al*., 2000b).

In the context of the information provided above, the objectives of this study have been to determine whether the RAPD assay could detect copper-induced DNA effects in the fresh water flea, *Daphnia magna*, under *in vivo* conditions, to measure fitness and Darwinian parameters in this key species for ecotoxicological studies, and to assess how these endpoints (i.e. changes in RAPD profile and fitness parameters) compare in terms of detection of toxicity.

MATERIALS AND METHODS

***Culture of* Daphnia magna.** *Daphnia magna* (*D. magna*, clone 5) were maintained in Elendt's medium (Elendt and Bias, 1990), at a temperature of 20 ± 2°C with a photoperiod of 16 h light (1000 lux):8 h dark. Animals were fed with the alga *Chlorella vulgaris* ($1.2–2.4 \times 10^7$ cells per daphnid per day) and a booster solution of Frippack microencapsulated food (Salt Lake Brine Shrimp, Grantsville, UT) as described in Atienzar *et al*. (1999).

Preparation of test solutions. Test solutions for acute and chronic exposures were prepared from stock solutions of copper (in the form of $CuCl_2$, Sigma, Poole, UK) at a concentration of 3.90 and 0.24 mg/ml in distilled water, respectively, and were subjected to the same dilution (100 μl of the different solutions in 1 liter of M7 medium).

Toxicity tests. The acute toxicity of copper was assessed by determining the LC_{50} of the chemical for *D. magna* over a period of 48 h. Freshly born neonates (less than 48 h) were exposed in replicate groups of 20, to concentrations of copper equivalent to 10, 25, 62.5, 156.25, or 390.62 μg/l. Animals were fed (see culture of the organism) during the test, and surviving animals were counted to determine the 48-h LC_{50} and its 95% confidence limit.

The chronic toxicity of copper to *D. magna* was assessed under the same experimental conditions as for the acute toxicity tests. Animals were exposed to copper concentrations of 15, 30, 60, 90, or 120 μg/l for 15 days. Surviving animals were counted on days 1, 3, 5, 8, 10, 12, and 15. Moribund, non-swimming animals were removed from the culture at regular intervals on and between counting days. Animals were stored at −80°C prior to DNA extraction and RAPD profiling. As M7 medium contains 2 μg/l copper, all concentrations previously mentioned in acute and chronic toxicity tests can be corrected by adding 2 to each of the values. However, as this is negligible, non-rectified values have been used throughout the paper.

Growth and reproductive measurements. The length of every *D. magna* (apex to base) (except at the start of the experiment [day 1] where 20 *D. magna* were measured) surviving at days 1, 3, 5, 8, 10, 12, and 15 was measured by video capture and image analysis using a Quantimet 570 image analyzer (Cambridge Instruments, Cambridge, UK). Neonates were counted and recovered at daily intervals. The length of newborn neonates (generally 10 animals per replicate) released at first generation was also determined.

Calculation of fitness parameters. The intrinsic rate of natural increase of the *D. magna* population, r_m, was calculated using Lotka's equation: $\Sigma l_x m_x e^{-r_m x} = 1$ (Krebs, 1978; Lotka, 1925). For a cohort of animals observed from birth through to death at regular intervals, x is the age in days, l_x is the age-specific survival (number of living females on day x/number of females at start of life table); m_x is the age-specific fecundity (number of newborn individuals produced on day x/number of living females on day x). The net reproductive rate (R_o) can be calculated using the following formula: $R_o = \Sigma l_x m_x = \Sigma U_x$ with U_x being the realized fecundity. Minimum generation time (T_{min}) and inter-brood time (Bt) represent the time between birth and the deposition of the first batch of offspring, and the time between clusters or broods, respectively.

Generation of* D. magna *DNA profiles using RAPD. Total DNA from *D. magna* was extracted and purified using a conventional phenol/chloroform method as described in Atienzar *et al*. (1999). The extracted DNA was electrophoresed on 1.2% (w/v) agarose gels using a Tris-Borate-EDTA (TBE) buffer system (1X TBE = 90 mM Tris-base, 90 mM boric acid, and 2 mM EDTA) at 100 V for 2 h. The DNA concentration of each sample was determined by comparison with known concentrations of Lambda phage DNA (Sigma, Poole, UK). DNA profiles of *D. magna* were generated in RAPD reactions performed in a reaction volume of 25 μl containing 2 μM 10-mer primer (OPA9, OPB1, OPB5, OPB6, OPB7, OPB8, OPB10, OPB11, OPB12, OPB14, or OPB17, Operon Technologies, Southampton, UK; sequences for each primer can be obtained from the website at http://www.operon.com) 0.33 mM dNTP, 5.11 mM $MgCl_2$, 20 ng DNA, 2.8 units of *Thermus aquaticus (Taq*) DNA polymerase and 1X reaction buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.08% Nonidet P40), and 2.5 μg bovine serum albumin. These primers were used because they allowed satisfactory RAPD profiles to be obtained with different species belonging to the bacterial, vegetal, and animal kingdoms and in particular with *D. magna* (Atienzar *et al*., 2000b). Thermal cycling parameters consisted of a 5-min denaturation (95°C) followed by 40 cycles of 1-min denaturation (95°C), 1-min annealing at 50°C, and a 1-min extension (74°C) (with the final extension period adjusted to 10 min). All PCR chemicals were from Immunogen International (Sunderland, UK) except when otherwise mentioned.

Amplified DNA was mixed with one-fifth volume of gel loading buffer (analytical grade water containing 25% ficoll, 0.25% bromophenol blue, and 0.25% xylene cyanol, Sigma, Poole, UK) and 12–15 μl of this solution was loaded onto the agarose gel. PCR products were electrophoresed on 1.2% agarose gel using a TBE system buffer (see above) at 90 V for 6 h, stained with ethidium bromide (0.015% [v/v]), visualized under UV light, and photographed using a Polaroid CU5 camera system (Eastman Kodak, New York). The image of the gel was saved electronically for further analysis when necessary. The intensity of selected bands was determined using the Kodak Digital Science™ 1 D (Eastman Kodak, New York). For comparison, DNA molecular size marker (GeneRuler™ 100bp DNA ladder plus, Immunogen International, Sunderland, UK) was used. Bands visualized were from top to bottom: 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp.

Estimation of genomic template stability and transformation of the data. Each obvious change observed in RAPD patterns (disappearance of bands, appearance of new bands, and variation in band intensities) was given the arbitrary score of +1, and the average calculated for each experimental group of animals with 5 primers (OPA9, OPB7, OPB8, OPB10, and OPB17, representing approximately 50% of the primer used) which showed clear changes in RAPD. Primers which did not produce changes in RAPD profiles or which were too difficult to score were not used to calculate the genomic template stability (GTS). GTS (%) was calculated by the formula $100 - (100a/n)$, where n is the number of bands detected in control DNA profiles and a the average number of changes in DNA profiles. To compare the sensitivity of each parameter (GTS, l_x, m_x, r_m and R_o), changes in these values were calculated as a percentage of their control value (set to 100%).

Statistical analyses. The 48-h LC_{50} value and the 95% confidence limit were calculated using the Logit method and the package software SPSS 6.1 for

TABLE 1
Demographic Trends of *Daphnia magna* Populations following Exposure to Different Copper Concentrations

Copper concentrations (μg/l)	Longevity (days)	Days to become ovigerous	Maximum body size (mm)	Maximum eggs/female	No. of broods	Inter-brood period (days)	Total neonates	Age-specific survival (l_x)	Age-specific fecundity (m_x)	Net productive rate (R_o)	Intrinsic rate of natural increase (r_m)
0	15 ± 0	7 ± 0	4.6 ± 0.02	35.0 ± 4.2	3 ± 0	3 ± 0	1471.0 ± 20.4	6.7 ± 0.5	80.5 ± 0.4	55.5 ± 4.4	0.21 ± 0.02
15	15 ± 0	7 ± 0	4.9 ± 0.04	38.0 ± 2.8	3 ± 0	3 ± 0	1424.5 ± 33.2	6.0 ± 0.07	97.2 ± 1.9*	53.8 ± 0.1	0.18 ± 0.06
30	15 ± 0	7 ± 0	4.7 ± 0.08	29.5 ± 0.7	3 ± 0	3 ± 0	1409.0 ± 19.8	6.5 ± 0.3	83.5 ± 12.7	49.1 ± 3.8	0.22 ± 0.01
60	15 ± 0	7 ± 0	4.5 ± 0.01	26.5 ± 0.7*	3 ± 0	3 ± 0	1269.5 ± 61.5	6.2 ± 0.4	80.2 ± 6.9	46.1 ± 0.2*	0.12 ± 0.07
90	15 ± 0	7 ± 0	3.7 ± 0.07**	20.0 ± 5.7**	3 ± 0	3 ± 0	618.0 ± 14**	6.1 ± 0.3	40.3 ± 6.0**	19.9 ± 7.2**	0.08 ± 0.07*
120	10 ± 0**	8 ± 0**	2.5 ± 0.35**	5.0 ± 1.4**	0.5 ± 0.7**	—	2.5 ± 3.5**	4.2 ± 0.2**	0.3 ± 0.4**	0.1 ± 0.2**	−0.06**

Note. Values represent mean ± SD and were calculated using both replicates (i.e., 40 *daphnia* per concentration). All parameters were determined throughout the experiment until *Daphnia* were unable to swim properly; * and ** indicate a significant difference from control ($p < 0.05$ and $p < 0.01$, respectively).

Windows. Differences among growth rates were calculated using multiple regression analysis (as in Conradi and Depledge, 1998). Briefly, the data were $\log_{10}$-transformed. As the number of surviving animals decreased with the increase in the toxic effect, the regressions were weighted with $n^{1/2}$ (where n is the number of the surviving animals). Analysis of variance (ANOVA) was performed using the computer software package Statgraphics (Statgraphics plus for Windows version 3.1, Statistical Graphics, USA). Changes in GTS, key fitness parameters (l_x, m_x, R_o, and r_m) and other parameters (longevity, maximum body size, maximum number of eggs/female, etc) were also statistically tested using ANOVA. The LSD (least significant differences) test was used to reveal statistical differences.

RESULTS

Acute Toxicity

The 48-h LC_{50} of copper for *D. magna* (clone 5) was 165.1 μg/l copper. The upper and lower values of the 95% confidence limit were 147.7 and 192.2 μg/l copper, respectively ($p < 0.05$).

Changes in Growth, Reproduction and Mortality

Demographic trends for the populations of *D. magna* exposed to differing concentrations of copper are shown in Table 1. In the range 15–120 μg/l copper, consistent reductions occurred in the maximum number of eggs per female and maximum body size. In contrast, the longevity and the number of broods were only affected at 120 μg/l of copper. *D. magna* exposed to the highest copper concentration could not survive for more than 10 days whereas animals from the other groups survived throughout the experiment (Table 1, Fig. 1A). The mean period for *D. magna* to become ovigerous was identical among groups, and animals exposed to gradual increases in copper concentrations displayed the same time between broods except for the animals exposed to 120 μg/l copper (Table 1). The populations growing at 120 and 90 μg/l copper had a smaller body size than controls ($p < 0.001$), and there were no significant differences between control and other copper-exposed groups (Fig. 1B). In addition, the sizes of sub-24-h juveniles at days 8 and 10 were similar among all groups ($p < 0.01$, data not shown). Finally, the total number of offspring was significantly reduced at 90 and 120 μg/l copper ($p < 0.01$; Fig. 1C, Table 1) compared to the control.

Changes in Fitness Parameters

Alterations to key fitness parameters, age-specific survival (l_x), age-specific fecundity (m_x), net reproductive rate (R_o), and intrinsic rate of population increase (r_m) are presented in Table 1 and Figure 4, before and after transformation, respectively. *D. magna* exposed to 120 μg/l copper exhibited a significant reduction in l_x compared to the controls ($p < 0.01$). In contrast, the remaining fitness parameters (age-specific fecundity, net reproductive rate, and intrinsic rate of population increase) were more sensitive indicators of toxic effects than age-specific survival, as they were significantly different for at least the 2 highest copper concentrations (90 and 120 μg/l copper) ($p < 0.05$). R_o and m_x appeared to be the most sensitive fitness parameters for the detection of toxic effects and were also significantly different from control at 60 and 15 μg/l copper, respectively.

RAPD Profiling

DNA amplified was extracted from healthy organisms aged 15 days from all groups except for *D. magna* exposed to 120 μg/l copper. Genetic material obtained from those latter animals was carried out on weak but living animals aged 10 days. In total, 11 oligonucleotide primers were used in the analysis and the presence of changes in the RAPD profiles obtained from the exposed population depended on the primer used. DNA profiles presented in Figure 2 were generated using 4 primers and a mixture of 4 individuals from each replicate. Profiles generated by these primers revealed differences between control and exposed individuals, with visible changes in the number and size of amplified DNA fragments, and both increases and decreases of DNA band intensities. Arrows on the right of each gel (Fig. 2) show some of the obvious modifications. Although some changes in RAPD profiles arose

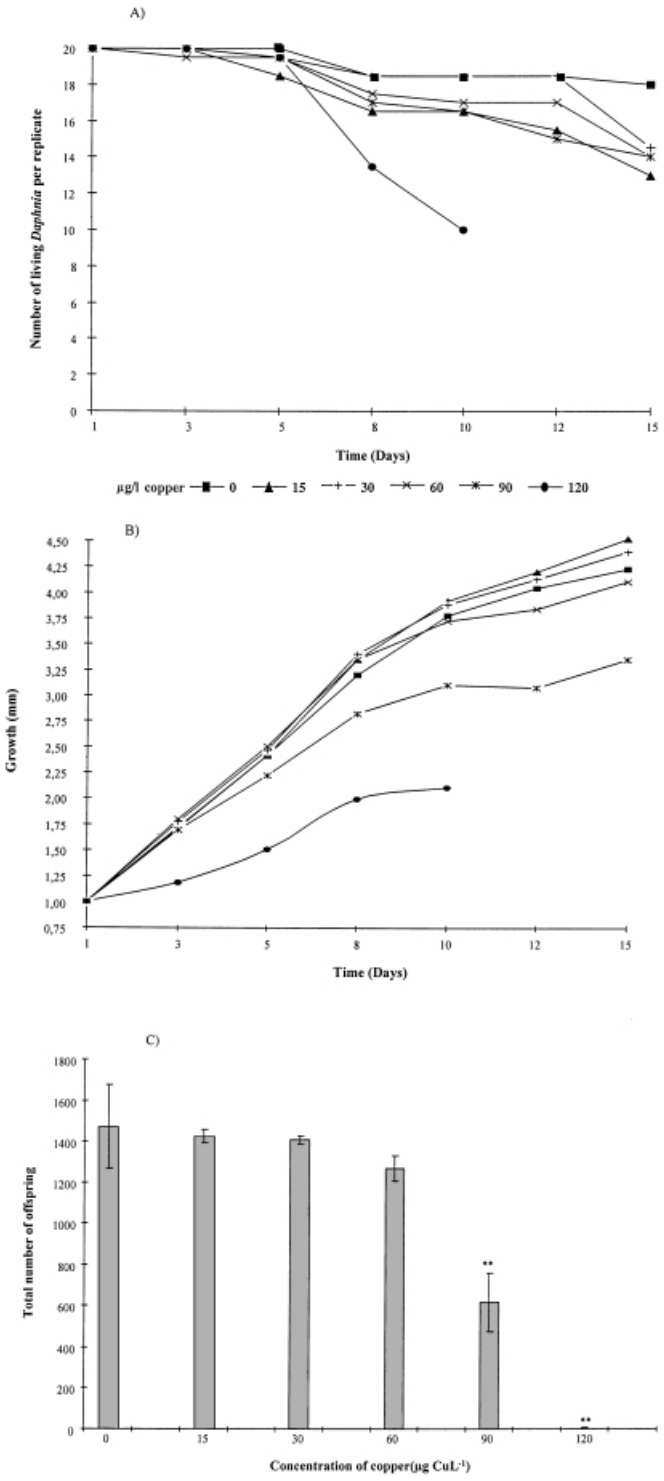

FIG. 1. Variation in number of living *Daphnia magna* per replicate (A), growth (B), and total number of offspring (C) in populations of *Daphnia magna* exposed to copper. **Indicates a significant difference from control ($p < 0.01$).

at 15 and 30 μg/l copper (e.g. bands 9-2, 7-3 [increase in band intensity], 9-4 [disappearance of bands]), most of the modifications occurred in patterns of *D. magna* exposed to 60, 90, and 120 μg/l copper. Extra bands generally appeared for some or all of the 3 highest concentrations (e.g. bands 10-1, 10-2, 14-3) whereas band 9-4 was only present in the control profiles. In addition, an increase in band intensity was the major event arising in the patterns generated by the animals exposed to the 2 lowest copper concentrations. In contrast, every type of modification was well represented in the patterns produced by *D. magna* exposed to the 3 highest copper concentrations. Figure 3 shows that band intensity (selected from 28 bands) followed 2 different tendencies. Intensity of 60% of the bands (17 out of 28) increased and decreased in the ranges 15–60 and 90–120 μg/l copper, respectively, compared to control intensities (curve b). In contrast, the average intensity of the remaining bands (40%) increased at all copper concentrations (curve a). Finally, some of the modifications (e.g. bands 14-1, 14-2, and 14-3) generated by a mixture of *D. magna* (Fig. 2D) generally followed the same tendency than those obtained from single individuals in both replicates (data not shown). For instance, the intensity of band 14-1 was generally brighter at 120 μg/l copper, whereas the intensity of band 14-2 progressively decreased in the range 60–120 μg/l copper. Moreover, band 14-3 appeared in profiles generated by the *D. magna* exposed to 90 and 120 μg/l copper.

Comparison of Fitness Parameters with Genomic Template Stability

To compare the sensitivity of the fitness parameters presented in Table 1, changes in each factor were calculated as a percentage of their control value (set to 100%). All the parameters presented in Table 1 were measured throughout the experiment except for the population of *D. magna* exposed to 120 μg/l copper, which were carried out for 10 days. Changes in RAPD profiles were expressed as reductions in GTS (a qualitative measure reflecting the obvious changes to the number and intensity of DNA bands in DNA patterns generated by toxicant-exposed *D. magna*) in relation to profiles obtained from control animals. GTS calculated with 5 primers (OPA9, OPB7, OPB8, OPB10, and OPB17) is presented in Table 2. As already mentioned, primers that did not produce any changes in RAPD profiles or that were too difficult to score were not used to calculate the GTS. A comparison between GTS and fitness parameters is presented in Figure 4. Results reveal that all parameters measured were significantly different at 90 and 120 μg/l copper ($p < 0.05$) except l_x, which was only significantly different at the highest copper concentration. The general tendency of all measured parameters was a progressive decrease with rising copper concentration, except for the age-specific fecundity which was significantly higher than control at 15 μg/l copper ($p < 0.05$). Overall among fitness parameters, m_x and R_o were the most sensitive parameters. Although GTS calculated with 5 primers was only significantly different at 90 and 120 μg/l copper, GTS calculated from single 10-mer primer OPA9 was significantly different from control at all copper concentrations ($p < 0.05$).

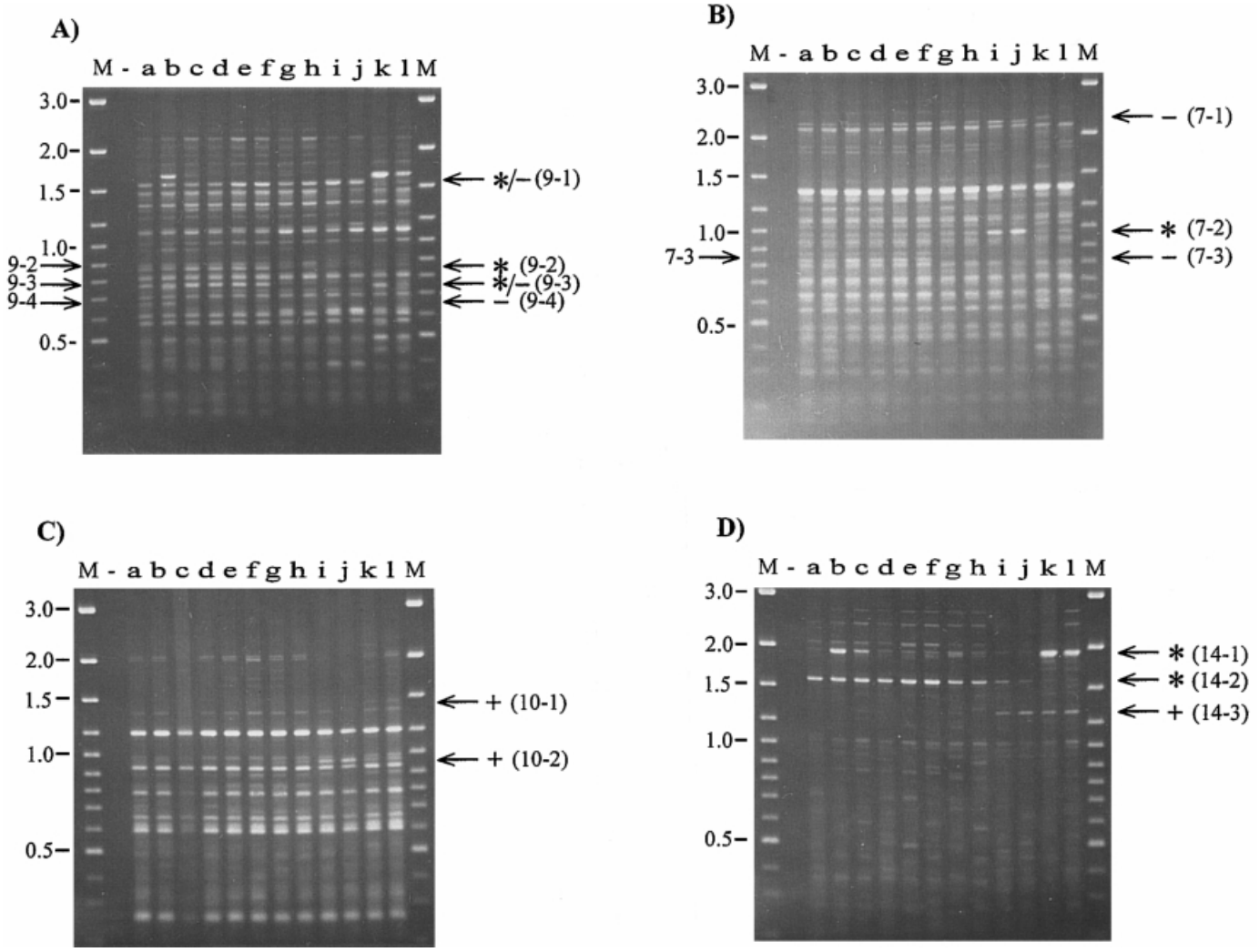

FIG. 2. RAPD profiles of *Daphnia magna* exposed to copper. M, DNA molecular size marker (GeneRuler™ 100 bp DNA ladder plus, Immunogen International; the molecular sizes, in kilobases, of selected bands are indicated on the left); - indicates no DNA control. RAPD profiles were generated using 10-mer primer OPA9 (gel A), OPB7 (gel B), OPB 10 (gel C), and OPB14 (gel D) and a mixture of 4 *D. magna* for each concentration and replicate. *D. magna* were exposed to 0 (a-b), 15 (c-d), 30 (e-f), 60 (g-h), 90 (i-j), and 120 (k-l) μg/l copper; a, c, e, g, i, k, and other letters refer to replicate 1 and 2, respectively. Selected changes are indicated by arrows in comparison to control patterns. *Variation in band intensities; +, appearance of a new band; –, disappearance of a band.

DISCUSSION

Despite growing concern over the presence of genotoxins in the aquatic environment, there is a lack of knowledge pertaining to the potential consequences of DNA damage and mutations at population level in the aquatic biota. In this study, changes in RAPD profiles that reflect DNA effects were compared to fitness parameters in *D. magna* exposed to copper concentrations in the range 15–120 μg/l. DNA effects include DNA damage as well as mutations and possibly other effects (see below) at the DNA level, which can be induced by chemical or physical agents that directly and/or indirectly interact with genomic DNA. Parameters measured at molecular (GTS) and population (growth, reproduction, fitness parameters) levels were mainly affected at 90 and 120 μg/l copper. Among the fitness parameters, the age-specific fecundity and net reproductive rate were the most sensitive parameters of toxicity as they were also significantly different at 15 and 60 μg/l copper. In contrast, age-specific survival was only significantly different at the highest copper concentration, suggesting that mortality is not a sensitive parameter. Although GTS calculated with 5 primers was only significantly affected at 90 and 120 μg/l copper, it is important to mention that one primer significantly detected DNA effects at all copper concentrations tested. Thus, the RAPD assay can be particularly sensitive. Overall, molecular and population parameters represented a sensitive means to measure toxicity induced by copper in *D. magna*. Similarly, Sadinski *et al*. (1995) reported that DNA adducts and micronuclei can be sensitive measures of sublethal DNA damage, as well as possible short-term indicators of

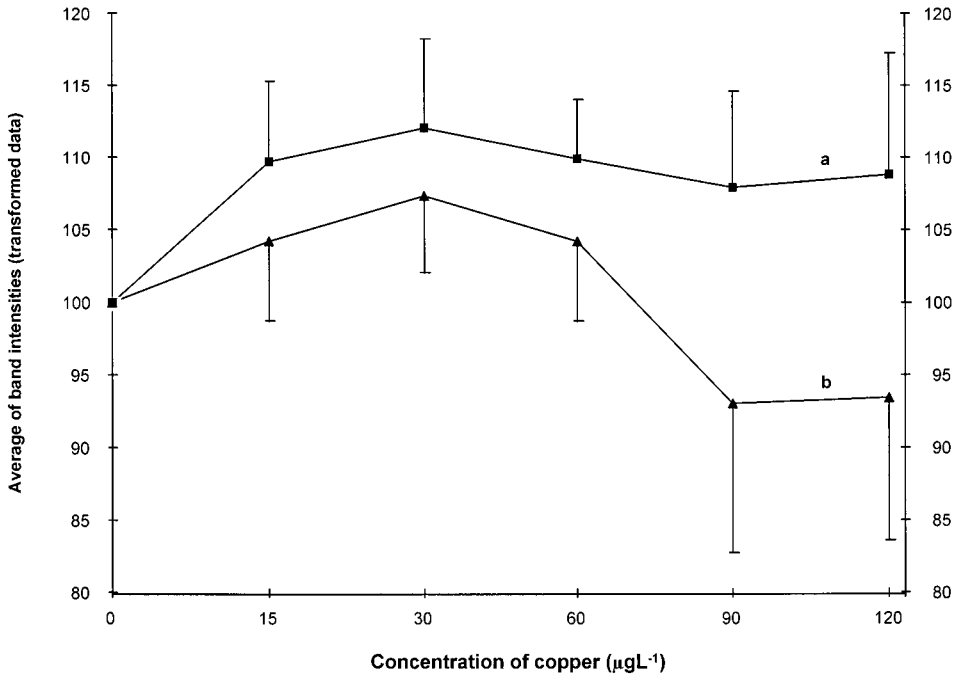

FIG. 3. Variation in band intensities selected from RAPD profiles of *Daphnia magna* exposed to copper. A total of 28 relatively intense bands (generated by 5 primers) appearing across the width of the gels were arbitrarily selected. The band intensities were calculated as a percentage of their own control value (set to 100%). As band intensity followed 2 different patterns, average and standard deviation were calculated with 11 (squares, curve a) and 17 (triangle, curve b) bands.

indirect effects on fitness in amphibians exposed to benzo-(a)pyrene [B(a)P]. Atienzar *et al*. (1999) reported that B(a)P-induced DNA effects also compared favorably with the traditional indices of fitness in *Daphnia magna*. Studies have also shown that cytogenetic damage correlates with development and survival of early life stages of marine invertebrates, which could have detrimental consequences at the higher levels of biological organization (Jha *et al*., 2000).

The measure of molecular and population parameters present several advantages. First of all, in ecotoxicology, it is fundamental to accumulate data at different levels of biological organization in order to fully understand the effect of a toxicant on organisms. Secondly, the measure of some parameters at the population level facilitate the interpretation of the data at the molecular level. For instance, a significant reduction in growth correlates with a significant inhibition in DNA replication, suggesting that the extent of DNA damage may be important in the majority of the cells. In the present study, it seems that for the 2 highest copper concentrations, DNA replication was significantly reduced due to a higher level of DNA damage. On the other hand, as growth and reproduction displayed similar values compared to the control in the range 15–60 μg/l copper, it could be assumed that DNA lesions were efficiently repaired and that DNA replication was not significantly inhibited.

TABLE 2
Genomic Template Stability Calculated with Five 10-mer Primers

[Cu] (μg/l)	Primer					Mean	SD
	OPA9	OPB7	OPB8	OPB10	OPB17		
0	95.3	100.0	100.0	100.0	100.0	99.1	2.1
15	86.0	100.0	100.0	100.0	100.0	97.2	6.2
30	86.0	94.7	87.5	100.0	100.0	93.7	6.7
60	74.4	89.5	91.7	90.0	100.0	89.1	9.2
90	67.4	84.2	83.3	90.0	95.4	84.1	10.5
120	60.5	78.9	75.0	80.0	90.9	77.1	11.0

With respect to copper toxicity, it has been reported that lethal effects on *Daphnia* occurred at 10 μg/l copper (Baird *et al*., 1991). In another study, Winner and Farell (1976) revealed that copper toxicity was increased at concentrations beyond 40 μg/l. However, the great variability among different studies makes direct comparisons difficult, owing to differences in medium quality, amounts of food, and the use of different clones of the same species (Koivisto and Ketola, 1995; Meador, 1991; Soares, 1992). In the present study, 15 μg/l copper stimulated reproduction, since age-specific fecundity was significantly higher than control, as previously reported in other studies (Dave, 1984). However, the total number of neonates generated by the population of *D. magna* exposed to 0 and 15 μg/l copper were statistically identical, because the number of surviving animals decreased at 15 μg/l copper in comparison to the control and other groups (except for animals exposed to 120 μg/l copper). Thus, despite the significant increase in reproduction (m_x) at 15 μg/l copper in comparison to the control, overall there was no beneficial effect in *D. magna* exposed to the lower copper concentration. The stimulation in reproduction can be explained by hormesis, an unspecific stimulation by a chemical or physical agent (Luckey, 1975). The

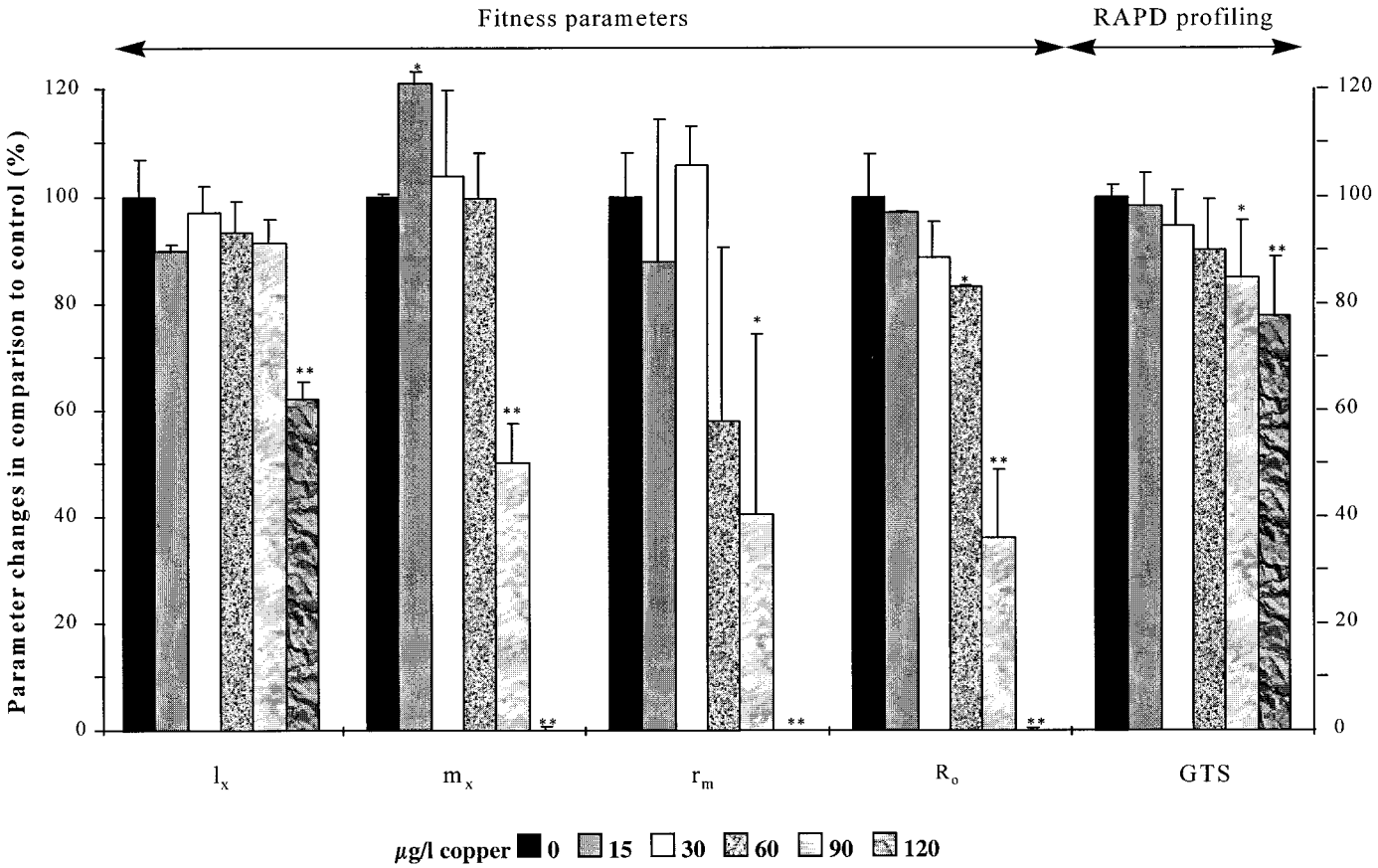

FIG. 4. Comparison between key fitness parameters and genomic template stability in populations of *Daphnia magna* exposed to copper. l_x: age-specific survival; m_x: age-specific fecundity; R_o: net reproductive rate; r_m: intrinsic rate of natural increase; GTS: genomic template stability; * and ** indicate a significant difference from control ($p < 0.05$ and $p < 0.01$, respectively). Error bars represent standard deviation.

decrease in growth following sublethal copper exposure has also been demonstrated in various species of crustaceans (e.g. Conradi and Depledge, 1998). By attaining reproductive maturity at a smaller body size, *D. magna* are able to buffer the impact of lower body growth rate on the age at the first reproduction (Lynch, 1985). In addition, the effect on growth was not due to a limitation in food levels as the size of the neonates at first reproduction was not affected (Enserink *et al*., 1993) but was due to a direct toxic effect of copper.

The second objective of the study was to evaluate the potential of the RAPD method to detect copper-mediated DNA effects in *D. magna*. Recently, the RAPD technique has been successfully used to detect DNA effects induced by benzo-(a)pyrene (Atienzar *et al*., 1999), mitomycin C (Becerril *et al*; 1999), ultraviolet radiation (Atienzar *et al*., 2000a), and 17-β-estradiol (estrogen)/4-n-nonylphenol (xenoestrogen) (Atienzar, 2000; manuscript submitted) in aquatic species under *in vitro* and *in vivo* conditions. In this study, *D. magna* were exposed to copper because it is known to induce a range of DNA damage such as single- and double-strand breaks, modified bases, abasic sites, DNA-protein cross-links, and even bulky adducts representing intrastrand dimerization of adjacent purine bases (dimers) (Carmichael *et al*., 1992; Lloyd and Phillips, 1999). Copper, as with other transition metals, catalyses the Fenton type reduction of hydrogen peroxide to form hydroxyl radical, one of the most reactive radical oxygen species (Drouin *et al*., 1996).

RAPD profiles detect alterations to genomic DNA through the use of arbitrarily primed PCR reactions. These effects include changes in oligonucleotide priming sites and variations in the activity of the *Taq* DNA polymerase. In the present study, variation in band intensity, disappearance of bands, and appearance of new PCR products occurred in profiles generated from the exposed organisms. Copper-induced DNA damage, e.g., Cu(I)-DNA complex, bulky adducts, oxidized bases, may significantly interfere with the PCR events. It has been reported that in naked double-stranded DNA, the transition from weak complex DNA-Cu(II) to strong complex DNA-Cu(I) (George *et al*., 1987) could induce conformational B-to-Z conversion in certain DNA fragments (Prütz *et al*., 1990). In addition, dimers can alter the structure of the DNA (Hanawalt, 1998; Wang *et al*., 1993). If so, such structural changes are likely to have a significant effect on the kinetics of PCR events. New PCR products can be amplified because

some sites become accessible to the primers after structural change or because the same mutations have occurred in the genome. A loss of an amplicon can only be the outcome if the same structural changes occur in 75–90% of the cells or if the same mutations arise in the same percentage of cells (Atienzar, 2000). However, it is very unlikely that mutations occur in a large portion of cells, because most of the DNA damage (which can lead to mutations during DNA replication) will be efficiently repaired. DNA lesions such as bulky adducts are expected to have detrimental effects on RAPD profiles. Not only can they induce structural changes, but they can also reduce the polymerization of the DNA and/or block the *Taq* DNA polymerase (Nelson *et al*., 1996), which will result in a decrease in band intensity, or alternatively, in a disappearance of amplified products in the case of extensive DNA damage (Atienzar, 2000). As already mentioned, new PCR products may reveal a change in the DNA sequence due to point mutations and/or large rearrangements. A single point mutation within the primer site can generate significant changes in RAPD patterns (Williams *et al*., 1990; Atienzar, 2000). Nevertheless, the RAPD assay also has the potential to detect point mutations outside the priming site, as they can induce structural changes (Bowditch *et al*., 1993). It is also well known that DNA repair and replication of damaged DNA can lead to point mutations (Livneh *et al*., 1993). Due to the rapid growth of *D. magna* (Fig. 1B) and the presence of DNA damage, it is very likely that mutations occurred after replication. Our preliminary results suggest that 10–25% of the cells need to be affected by mutation and/or DNA damage to allow a visible change in the RAPD profile (Atienzar, 2000). As "hot spot" interactions between DNA and copper have been reported in the literature (Prütz *et al*., 1990; Rodriguez *et al*., 1995), it is likely that hot spot mutations are generated following DNA replication.

Although DNA damage and mutations induced by genotoxins are likely to be the main factors affecting RAPD profiles, our preliminary results suggest that other factors may also contribute to changes in patterns but probably to a lesser extent (manuscript submitted; Atienzar, 2000). These factors could be responsible for some of the changes occurring in control RAPD patterns (see, for instance, Figs. 2A and 2D). Numerous studies have shown that gene expression correlates with a change in DNA structure (Kohwi and Kohwi-Shigematsu, 1991; Wolffe and Hayes, 1999), which in turn may induce changes in RAPD profiles. Other parameters such as genomic amplification and rearrangement, which are components of normal cellular development, may also influence RAPD profiles. There is also a possibility that DNA damage (e.g., 8-hydroxyguanine) in control animals could conceivably be induced 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. In addition, cell physiology, which is known to have a direct influence on DNA (Oshimura and Barrett, 1986), may also have an effect on RAPD profiles. Therefore, the changes that occur in control RAPD patterns could be explained by a change in one or a combination of the factors previously mentioned between both replicates.

The RAPD technique clearly shows promise in the detection of pollutant-induced DNA effects. The main advantages of the RAPD method lie in its rapidity, applicability to any organism (since no information on the nucleotide sequence, cell cycle, or chromosome complement is required), and sensitivity to detect a wide range of DNA damage and mutations. However, RAPD is a qualitative method and the nature and amount of DNA effects can only be speculated. Thus, in the context of the evaluation of pollutant-induced DNA effects, a powerful approach would be to perform the RAPD method to obtain qualitative data that would give an overview of the DNA effects, and then to use more specific methods such as the ^{32}P-postlabeling, DNA strand break measurements (comet assay), or cytogenetic assays to generate quantitative data.

In conclusion, fitness parameters and DNA effects are sensitive markers to detect copper-induced toxicity in *D. magna*. Thus, the RAPD assay to detect DNA effects in conjunction with other biomarkers from higher levels of biological organization would prove a powerful ecotoxicological tool.

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