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Morphological features and comet assay of green and brown hydra treated with aluminium

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Abstract
Hydras are simple aquatic organisms, members of the phylum Cnidaria. Green hydra (*Hydra viridissima* Pallas) is endosymbiotic and brown hydra (*Hydra oligactis* Pallas) is a non-symbiotic species. Aluminium is one of the most abundant elements in the Earth's crust, but its effects upon living organisms have not been much studied until recently. The aim of this research was to explore the potential environmental effects of aluminium ions by studying aluminium-induced changes in two closely related hydra species, and to trace the extent damage caused. For the first time, a modified alkaline comet assay was used to study primary DNA damage in green and brown hydra cells. Aluminium toxicity triggered mortality, morphological, behavioral and DNA changes. DNA tail length and intensity changes were greater in brown than in green hydras, but behavioral responses to the presence of aluminium ions were observed more rapidly in green hydra. The toxicity also affected reproduction. Brown hydra was more susceptible to aluminium than green hydra, confirming the evolutionary advantage provided by symbiosis. Biomonitoring protocols using hydra and the comet assay could be developed to provide a valuable and rapid method for determining the quality of freshwater.

Keywords: Aluminium, brown hydra, comet assay, DNA damage, green hydra, morphological changes, symbiosis

1. Introduction

Endosymbiosis is one of the most important and most interesting subjects in biology. Symbiotic associations are of wide significance in evolution, provide biological advantages and contribute to biodiversity (Margulis and Sagan, 2002; Seckbach, 2001).

Hydra is a simple cosmopolitan freshwater invertebrate organism, a member of the phylum Cnidaria, class Hydrozoa, order Hydroida, suborder Hydrina, family Hydrydae (Holstein and Emschermann, 1995). It is usually found in unpolluted freshwater on leaves of submerged plants but has recently been found in some polluted habitats (Kalafatić et al., 2003). Hydra has a simple, cylindrical body with a budding region, an adhesive foot at one end and a mouth surrounded by six to eight tentacles at the other (Burnett, 1973). The body is comprised of two cellular layers separated by mesoglea and hydras have a tremendous rate of regeneration (Kalafatić et al., 2001). Green hydra forms a symbiotic relationship with algae of *Chlorella* genus (Douglas, 1994).

Hydra is a suitable test organism in ecotoxicological and evolutionary research (Burnett, 1973; Douglas, 1994; Kalafatić and Kopjar, 1994). As a metazoan organism, with a simple body plan, changes can be observed following exposure to xenobiotics like pesticides, heavy metals and antibiotics (Fradkin et al., 1978; Kalafatic and Tomaškovic, 1999; Kovačević et al., 2005, 2007). Hydra is a useful experimental object for establishing of lethal and sub-lethal doses of toxicants as well as in understanding the effect of pollutants on freshwater ecosystems (Beach and Pascoe, 1997).

The comet assay enables the evaluation of genetic damage at the single cell level (Singh et al., 1988). The method has been modified several times (e.g. Collins, 2004), and one for hydra is described in this paper. A multi-species approach towards ecotoxicological testing is fundamental for improving environmental management and

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ecological risk assessment. In this context, there is a need to find aquatic species that can be used as biomonitors for water quality assessment (Van Straalen, 2002).

The toxicity of aluminium ions has not been much studied until recently (Exley, 2003; Hermann, 2001). Aluminium is almost insoluble in water, a property that limits its biological availability (Macdonald and Martin, 1988). However, with industrial developments, toxic gas emissions and acid rain, aluminium toxicity has become an environmental problem because it becomes more soluble in acid water in its mobility (Goenaga and Williams, 1988). Aluminium, eluted from soils by acid rain, drains to surface and subterranean waters, thereby presenting a potential environmental danger for freshwater organisms. The aim of the present research was to explore the potential environmental toxicity of high levels of aluminium ions on two closely related hydra species, green symbiotic and brown non-symbiotic hydra.

2. Materials and Methods

Morphological changes

A static toxicity test was performed using individuals of green (*Hydra viridissima* Pallas, 1766; strain S1J-J1) and brown (*Hydra oligactis* Pallas, 1766; strain S1M-K1) hydra. Animals were collected from the Zagreb lakes of Jarun and Maksimir. Collected animals were grown in culture under stable laboratory conditions in 350 ml of aerated aquarium water in glass dishes, 11 cm in diameter, 5.5 cm in height (photoperiod 10 hrs light/15 μmol/m^2s, 14 hrs dark).

Five animals of each species were treated under laboratory conditions (21.5°C) with 50 ml of one of the following 12 concentrations of aluminium-sulphate (Al) $Al_2(SO_4)_3$.$18H_2O$ (25, 50, 80, 100, 150, 200, 250, 350, 475, 480, 490 and 500 mg/l) in aerated aquarium water in glass dishes, 6 cm in diameter, 3.5 cm in height (photoperiod 10 hrs light/15 μmol/m^2s, 14 hrs dark) (see Table 1 for mineral composition of the water). The aluminium-sulphate was purchased from Kemika, Croatia. Each batch of animals were given subacute exposure for three days and compared with the control groups. After the exposure, the animals were put in 50 ml of clean aerated aquarium water and left to recover for 20 days. The aquarium water, during the recovery period, was not changed and aeration was kept at the same level as during the experimental exposure. The pH of the aluminium-supplemented water, for each concentration of Al, is shown in the Table 2. For a positive control, individuals of both hydra species were put into aerated aquarium water, acidified with 2% H_2SO_4 to the pH values given in the Table 2. This was done in order to compare possible changes induced by Al with those induced by increased acidity (Rundgren and Nilsson, 1997). The experiments were repeated three times.

A binocular light microscope Carl Zeiss, Jena, was used for morphological studies. Observed parameters were: mortality, asexual and sexual reproduction, tentacle reduction, reaction to mechanical stimuli with a preparation needle, deformations, mucous secretion and migration to the water surface.

Table 1. Chemical composition and characteristics of the aquarium water used in the experiments.

Parameter	Value
pH	8.06
Electroconductivity	442 μS/cm
Oxidativity (O_2)	1.4 mg/l
Cl^-	23.86 mg/l
NO_2^-	<0.03 mg/l
NO_3^-	20.36 mg/l
NH_4^+	<0.02 mg/l
PO_4^{3-}	<20 μg/l P
F^-	78.0 μg/l
SO_4^{2-}	27.13 mg/l
K^+	2.63 mg/l
Na^+	12.06 mg/l
Ca^{2+}	43.54 mg/l
Mg^{2+}	24.68 mg/l
Al	10.70 μg/l
Fe	<20 μg/l
Cu	<5 μg/l
Coliform bacteria	0
Escherichia coli	0
Colonies 37°C/48h	0
Colonies 22°C/72h	0
Color	Absent

Table 2. The pH of the aluminium solutions ($Al_2SO_4.18H_2O$) in the experiments.

$Al_2SO_4.18H_2O$ (mg/l)	pH
Control	8.06
25	7.25
50	6.93
80	6.72
100	6.36
150	6.29
200	6.14
250	5.71
350	5.35
425	4.38
450	4.35
475	4.31
480	3.93
490	3.91
500	3.85

Comet assay

After the treatment period, animals from each of the concentrations of Al were transferred into a micro-test-tube containing 10 µl of chilled distilled water. Hydras were homogenized with a 100 µl micropipette by sucking the animals in and out several times. During the entire homogenization the tube was kept on ice. The comet assay was carried out under alkaline conditions, as described by Singh et al. (1988) with the specific modifications described below.

Agarose gels were prepared on fully frosted slides coated with 1% and 0.6% normal melting point (NMP) agarose. Homogenized hydras (10 µl) were mixed with 0.5% low melting point (LMP) agarose, placed on the slides and covered with a layer of 0.5% LMP agarose. The slides were immersed for 1.5 hrs in freshly prepared ice-cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Na-sarcosinate, pH 10) with 1% Triton X-100 and 10% dimethyl sulfoxide (Kemika, Zagreb, Croatia). Alkaline denaturation and electrophoresis were carried out at 4°C under dim light in freshly prepared electrophoretic buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0). After 15 min of denaturation, slides were randomly placed side by side in a horizontal gel-electrophoresis tank, facing the anode. Electrophoresis lasted another 10 min at 0.8 V/cm. After electrophoresis, slides were gently washed with a neutralization buffer (0.4 M Tris-HCl, pH 7.5) three times at five-minute intervals. Slides were stained with ethidium bromide (20 µg/ml) and examined using a 400× magnification fluorescence microscope (Zeiss) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. A total of 100 comets were scored per slide. Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, "hedgehogs" and superimposed comets. Using a black and white camera, the microscope image was transferred to a computer-based image analysis system (Comet Assay II, Perceptive Instruments Ltd.). Tail length (µm) and tail intensity (% of DNA in tail) were used as a measure of DNA damage. Entire DNA damage evaluation was performed in duplicate.

Statistics

Mean ± standard deviation (s.d.) was presented for the morphological changes and mean ± standard error (s.e.) for the comet assay. Calculations were performed using Statistica 5.0 (StatSoft, USA) to test the difference of the tail length and intensity values between treated and control animals with the Student's *t*-test. The dependence of the comet assay endpoints to the Al concentration was evaluated by calculating the Spearman correlation coefficient. Multiple regression and coefficient were calculated for endpoint predicting. *P* values of less than 0.05 were considered significant.

3. Results

Morphological changes

Aluminium toxicity triggered mortality, numerous morphological and behavioral changes, showing a deleterious effect upon both hydra species (Figs. 1–6). Higher concentrations triggered more prominent changes: tentacle reduction (Figs. 1, 2, 5), incoherent reactions to mechanical stimuli (Fig. 6), migration to the water surface, contractions (Figs. 1, 2) and apocrine mucous secretion from the ectodermal layer. Cytotoxic effects were observed at the apical region as a degradation of ectodermal myoepithelial cells. Brown hydra showed higher aluminium susceptibility in most of the assessed parameters (Figs. 3–6). Control animals did not show any changes, except for budding (Table 4). Positive controls, at lowered pH, did not show any changes.

In 475 mg/l Al, none of green hydras died while 86% (±18%) of brown hydras died. In 480 mg/l 73% (±18%) green hydras and 86% (±9%) brown hydras died. In 490 mg/l 80% (±16%) green hydras and all brown hydras died. In 500 mg/l Al all specimens of both hydra species died (Fig. 3).

Table 3. Comet assay parameters in the cells of green and brown hydra treated with different concentrations of Al for three days.

Hydra	Al concentration (mg/l)	Mean tail length (±s.e.) (µm)	Mean tail intensity (±s.e.) (% of DNA)
Brown	0	10.8±0.18	0.82±0.22
	25	11.8±0.18[1]	1.10±0.27
	250	12.8±0.39[1]	2.32±0.60[1]
	475	12.0±0.73	8.29±1.29[1,2]
Green	0	6.1±0.08	0.46±0.12
	25	6.4±0.14	0.24±0.10
	250	6.0±0.13	0.30±0.13
	475	8.7±0.52[1,2]	4.28±1.01[1,2]

[1]Significant compared to the control; [2]Significant compared to the lower concentration.

Table 4. Correlation and predictability of the tail length and tail intensity values for green and brown hydra with respect to exposure to different concentrations of Al for three days.

Statistical parameter	Hydra	Tail length	Tail intensity
Spearman correlation coefficient R	Brown	0.80	0.90
	Green	0.40	0.40
Multiple regression coefficient R^2	Brown	0.31	0.89
	Green	0.66	0.73

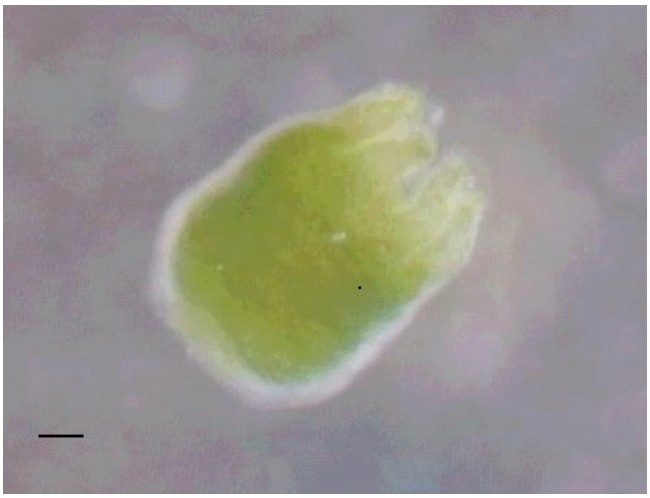

Figure 1. Green hydra contraction and tentacle reduction during the experiment. Bar 500 µm.

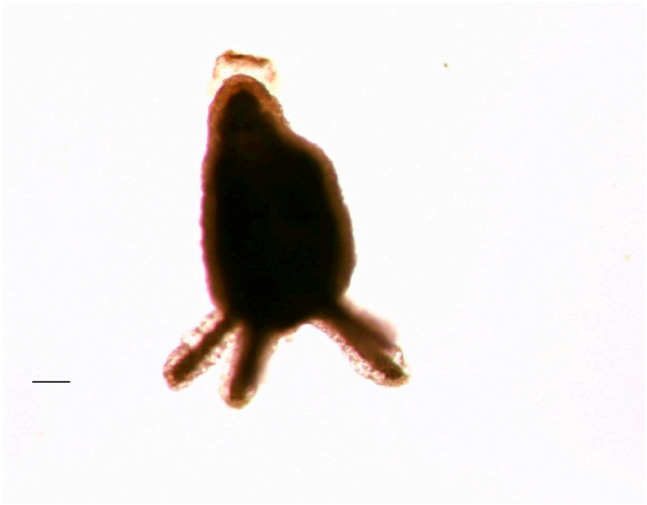

Figure 2. Brown hydra contraction and tentacle reduction during the experiment. Bar 500 µm.

Both asexual (budding) and sexual reproduction were affected. Budding was noted in 26% (±18%) of the animals in 25 mg/l Al, 93% (±9%) of the animals in 50–80 mg/l Al and 86% (±9%) in 100 mg/l Al in green hydras compared to 21% (±16%) of the animals in the control group. None of the brown hydras budded in any of the concentrations compared to the 6% (±9%) of the animals in the control group (Fig. 4). Sexual reproduction was noted only once in both animal species. A green hydra exhibited sexual reproduction developing an egg after the first day of the experiment in 250 mg/l of Al. A brown hydra began to produce an egg on the second day of the recovery.

Tentacle reduction was present in 33 (±33%) of green and 20% (±16%) of brown hydras in 25–100 mg/l Al, in 26% (±37%) of green and 13% (±18%) of brown hydras in

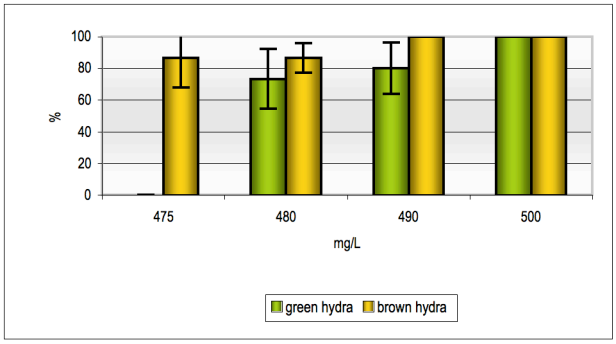

Figure 3. Mortality of green and brown hydra in the experiment (mean±s.d).

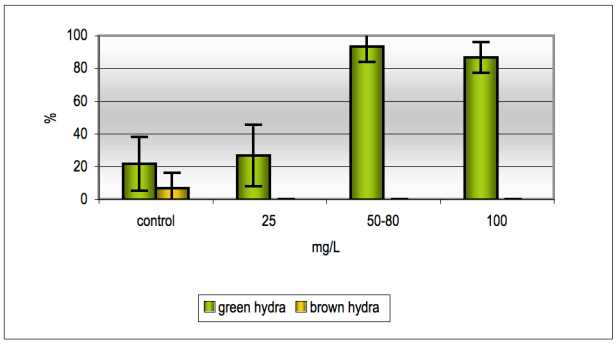

Figure 4. Budding of green and brown hydra in the experiment (mean±s.d).

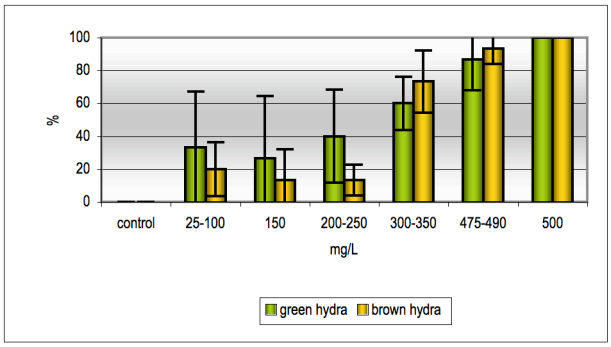

Figure 5. Tentacle reduction of green and brown hydra in the experiment (mean±s.d).

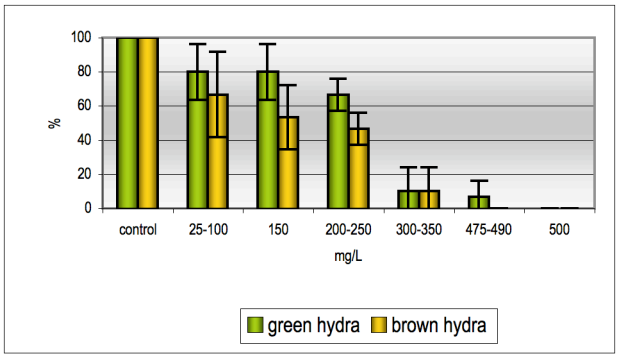

Figure 6. Reaction to mechanical stimuli of green and brown hydra in the experiment (mean±s.d).

150 mg/l Al, in 40% (±28%) of green and 13% (±9%) of brown hydras in 200–250 mg/l Al, in 60% (±16%) of green and 73% (±18%) of brown hydras in 300–350 mg/l Al and in 86% (±18%) of green and 93% (±9%) of brown hydras in 475–490 mg/l Al (Fig. 5).

Eighty percent (±16%) of green and 66% (±24%) of brown hydras reacted by contraction normally like the control (100%) to mechanical stimuli in 25–100 mg/l Al, 80% (±16%) of green and 53% (±18%) of brown hydras reacted normally in 150 mg/l Al, 66% (±9%) of green and 46% (±9%) of brown hydras reacted normally in 200–250 mg/l Al, 10% (±14%) of green and 10% (±14%) of brown hydras reacted normally in 300–350 mg/l Al, 6% (±9%) of green hydras reacted normally in 475–490 mg/l Al (Fig 6).

Migration to the water surface was noted early at the lowest aluminium dose. Green hydras migrated on the second and brown hydras on the first day of the treatment. Migration outside the experimental dish is reported for the first time for hydras exposed to toxic metal solutions. Two green hydras migrated out of the 475 mg/l of Al and two individuals of brown hydras migrated out of the 425 mg/l Al solution.

Comet assay

In green hydra, a significant increase in comet endpoint values was only observed in the highest concentration of Al. Mean value of tail length was 8.7±0.52, and of tail intensity 4.28±1.01 compared to the control where values were 6.1±0.08 and 0.46±0.12, respectively. Brown hydra tale length values for animals treated with 25 and 250 mg/l (11.8±0.18 and 12.8±0.39, respectively) were significantly higher compared with the control (10.8±0.18). Tail intensity values were significantly elevated at concentrations of 250 mg/L and 475 mg/l Al (2.32±0.60 and 8.29±1.29, respectively). In 25 mg/l Al, although increased (1.10±0.27), the tail intensity did not significantly differ from the control (0.82±0.22) (Table 3).

As shown in Table 4, for brown hydra, the Spearman correlation coefficient indicated an excellent correlation between Al concentration and both comet assay parameters (R=0.80 for the tail length and R=0.9 for the tail intensity). In contrast, a poor correlation was found (R=0.40) in green hydra for both comet assay parameters. Multiple regression coefficient (Table 4) in brown hydra indicated poor predictability of the tail length values (R^2=0.31) but excellent for the tail intensity values (R^2=0.89). In green hydra, the predictability of the tail length was higher than in the brown one (for R^2=0.66). However, the predictability for tail intensity was lower (R^2=0.73).

4. Discussion

Mortality is an important parameter in detection of toxicity (Zupan and Kalafatić, 2003). Brown hydra exhibited greater susceptibility to aluminium ions than green hydra. Values at high concentrations (475 mg/l, 480 mg/l, 490 mg/l, 500 mg/l) of aluminium were used to determine the 72 hr LC_{50} and LC_{100}. The LC_{50} was established for green hydra in the range 475–480 mg/l Al. The LC_{100} was less than 500 mg/l Al as no hydras survived this exposure.

Of special interest for ecotoxicological research are the sublethal effects, which can have long-term consequences on ecosystems, populations and species (Calervo et al., 1998; Zupan and Kalafatić, 2003). A dose 25 mg/l of Al for 72 hours, stimulated green hydra growth and caused no detected damage to the animals. It is known that low doses of toxicant may induce organism growth, which is termed hormesis (Stebbing, 1982; Žnidarić et al., 1987). Hormesis was more pronounced in aluminium concentrations of 50, 80 and 100 mg/l Al, but these levels induced the minor changes in animal morphology. In all the treated brown hydras, budding was inhibited, because the majority of the cell reserves were spent on regenerating damaged body parts (Kovačević et al., 2001). Green hydra at low Al concentrations, exhibited its fitness by sexual reproduction while brown hydra did develop sex organs in aquarium water enriched with Al.

Tentacle reduction in the range of 25–250 mg/l Al and mucous secretion of the foot were more pronounced in green hydra. This could be explained by the fact that the tentacles are a very sensitive part of the hydra body. Green hydra tentacles are smaller that those of brown hydra. Tentacles are in direct contact with the toxicant, consist of small cells of simple squamous epithelium, and exhibit the greatest morphological changes (Kalafatić and Kopjar, 1994). Mucous secretion represents a defensive mechanism (Burnett, 1973) that helps in protection and detoxication. Again, this was more pronounced in green hydra reflecting the evolutionary advantage of symbiosis.

Mechanical stimuli, touching the animals with a needle, caused control animals to react by immediate body contractions. With the increase of the aluminium toxicity, hydras showed slower contractions with almost no contractions in the highest concentrations of Al. Contractions are visible immediately after treatment as the animals react to the external stimulus (Burnett, 1973). As a result, the surface area of the body decreases and there is also closer contact of cellular layers (Žnidarić et al., 1995). Between the 250 and 300 mg/l Al, there was a threshold of hydras' susceptibility to Al, in terms of tentacle reduction and reactions to stimuli. Cytotoxic effects were also observed at the apical region; the ectodermal myoepithelial layer became degraded in both hydra species, mostly at higher Al concentrations.

Brown hydras were more affected, at a given Al concentration, than green hydras. However, green hydras started exhibiting the negative effects of Al earlier. This

may be due to difference in relative body surface to volume ratio. Brown hydras have a smaller ratio, so toxins may be absorbed by diffusion more slowly. However, the overall effect seems to be more harmful for brown hydra, once the toxin has been absorbed into the animal's system. Migration of hydras to the water surface enables the animals to evade unfavorable environmental conditions (Burnett, 1973). Brown hydras, exposed to Al, migrated earlier than green hydras, showing that conditions were more unfavorable for the non-symbiotic species, compared to the symbiotic species.

The toxic form of aluminium Al^{3+} can replace other, biofunctional cations such as Mg^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} (Cowburn et al., 1990; Macdonald and Martin, 1988) and interfere with metabolic processes. Studies on fish (Havas and Rosseland, 1995) and other freshwater organisms (Gensemer and Playle, 1999; Michailov et al., 2003) have revealed that Al exposure can cause mortality and changes in growth, morphology, behavior and reproduction. Such studies have been carried out on planarians (Calervo et al., 1998; Kalafatic and Tomaškovic, 1999), hydras (Kovačević et al., 2006), and algae (Kinross et al., 2000).

Chemicals including aluminium are released into the atmosphere (WHO, 1997) and most eventually reach, and contaminate, the ground or surface waters. Water pollution therefore presents a serious problem to aquatic ecosystems (Takeshi et al., 2004). So far, the comet assay has been used in polychaete species (*Capitella* spp.) (Bach et al., 2005), mullet (*Mugil* sp.) and catfish (*Netuma* sp.) (De Andrade et al., 2004), mussel (*Dreissena* sp.) (Klobučar et al., 2003) and (*Mytilus* sp.) (Rocher et al., 2006). In the present study, using the comet assay, only the highest concentration of Al (475 mg/l) failed to show a significant increase in hydra tail length value in brown hydra compared with the control. This may be due to the high toxicity of the treatment since more than 85% of the isolated brown hydra cells appeared as "ghost cells". Such ghosts, seen under the microscope, indicate cell death. Since the comet assay detects DNA lesions at a single cell level (Collins, 2004; Tice et al., 2000), it enables differences in the damage level, caused by the cellular toxicant level, to be determined.

Due to the cytotoxicity of the highest concentration of Al used, multiple regression analysis showed poor predictability of tail length values. However, if the comet assay results for 475 mg/l Al are omitted from the calculations, the regression coefficient of the tail length value rises from R^2=0.31 to R^2=0.82, which is even higher than for green hydras. High coefficient indicates excellent predictability of the tail length values for Al treatment. However, the tail intensity values were not much affected. By omitting the results for the highest concentration, the coefficient changed from 0.89 to 0.99 (range 0–1). For green hydras only the treatment with 475 mg/l Al showed significant increase in both measured comet assay parameters, but unlike brown hydra, the green hydra cells did not appear as "ghost cells".

The above results indicate a much lower genetic susceptibility of green hydras to Al compared with brown hydra. The observed lower susceptibility of green hydras to DNA damage by aluminium could be mediated by the endosymbiotic *Chlorella*. This alga produces canthaxanthin and other carotenoids that are potential antioxidants (Bin-Hua et al., 2006; Inbaraj et al., 2006). If released from the alga into the hydra's cell, these compounds could help protect hydra's genome from damage.

In the comet assay, the tail intensity values, in both brown and green hydra, showed a higher sensitivity and a stronger correlation with the concentration of Al, and were thus better predictors of damage than tail length values. This could be explained by the theory of comet tail formation. During electrophoresis, DNA does not migrate as the fragments do. The tail of the comet is the result of the relaxed DNA loops migrating as they are pulled by the electric field to a limited distance from the core. The length of those loops determines the length of the comet tail. Since tail intensity indicates the number of DNA breaks, it is assumed that beyond some critical amount of damage, the tail intensity is increased rather than tail length (Collins et al., 1997). Due to its higher genetic susceptibility, brown hydra appears to be a better biomonitor for aquatic toxicology studies compared with green hydra. The comet assay was also shown to be sensitive enough to detect the effects of aluminium, a contaminant of freshwater. Due to its higher correlation and predictivity, the tail intensity was shown to be a more relevant comet assay parameter for DNA damage than tail length. Introducing hydras and the comet assay for biomonitoring freshwater could provide one criterion for assessing the environmental quality of fresh water (Van Straalen, 2002).

The Al levels used in the present short term study are not likely to be found in nature, but exposure to lower Al levels for a much longer period could cause damage to sensitive freshwater organisms, including hydra populations in their natural habitat. Industrial waste disposal may induce the sorts of acute symptoms observed in the present study. It is interesting that reduced pH, by itself, in aquarium water, did not cause the toxic effects observed in the presence of Al, that resulted in a comparable pH. Therefore, we conclude that it is the toxicity of aluminium, and not acidity that damaged the green and brown hydras.

Green hydra was more resistant to unfavorable environmental conditions than brown hydra. Finally, after exposure to deleterious levels of Al ions, the surviving green hydras recovered and regenerated completely after 72 hrs when placed in aquarium water without added aluminium. Brown hydras that survived Al exposure, took twice as long to regenerate, confirming the advantage that symbiosis confers on the green species.

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