Folia biologica (Kraków), vol. **57** (2009), No 1-2
doi:10.3409/fb57_1-2.91-96

Effects of Norflurazon on Green and Brown Hydra

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Accepted September 15, 2008

KOVAČEVIĆ G., KALAFATIĆ M., LJUBEŠIĆ N. 2009. Effects of norflurazon on green and brown hydra. Folia biol. (Kraków) **57**: 91-96.

For the first time effects of norflurazon on green (*Hydra viridissima* Pallas, 1766) and brown hydra (*Hydra oligactis* Pallas, 1766) in high- and low-light conditions were investigated in order to establish the extent of damage that this substance inflicts, with special emphasis on the "bleaching effect" and the effect on hydra-algae symbiosis. Green hydra is a typical example of an endosymbiotic organism. The gastrodermal myoepithelial cells of green hydra contain endosymbiotic algae. Norflurazon is a selective translocational herbicide that induces a "bleaching effect" on newly developed chloroplasts, resulting in a decrease of photosynthetic activity and viability of the organism. In the experiment, morphological (binocular), cytological and histological (Bouin fixative, dehydration, paraplast embedding, Hämalaun-eosine staining, light microscope) and conventional transmission electron microscopy (cTEM) (glutaraldehyde, dehydration, raisin, uranyl-acetate, Pb-citrate) were used. Depending on the concentration and light conditions, norflurazon caused mortality, deformations, changes in behavior, locomotion and asexual reproduction, changes in the structure of all 3 layers in hydras, changes in the position and shape of endosymbiotic algae inside the hydra body as well as ultrastructural changes of treated hydras and endosymbiotic algae. Under low concentrations of norflurazon the effects on hydra were similar to the controls, while in the highest concentrations especially manifested were antichloroplastal and antimitochondrial effects. Norflurazon caused a great extent of damage and induced deleterious effects also upon other cellular components such as cellular membranes, ER, Golgi apparatus, ribosomes. Newly developed buds in symbiotic green hydras were not bleached. After a recovery period, the green hydra individuals that had survived re-established regular endosymbiosis with algae and recovered completely, whereas brown hydras recovered only partially.

Key words: Green and brown hydra, norflurazon, bleaching, endosymbiosis, antichloroplastal and antimitochondrial effect.

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Symbiotic associations are of wide significance in evolution, providing biological advantages and contributing to biological diversity. Endosymbiosis is a form of symbiosis in which within the same cytoplasm there exist at least two genomes of different evolutionary origin (EBRINEGER & KRAJČOVIČ 1994). Endosymbiosis research is one of the most important and the most interesting issues of modern evolutionary biology. Hydra has been used as a suitable experimental organism for more than 250 years, especially in evolutionary and ecotoxicological research (BEACH & PASCOE 1998; NIDARIĆ *et al*. 1995).

Hydras are aquatic cosmopolitan invertebrates that belong to the phylum Cnidaria (Hydrozoa, Hydroida) (HOLSTEIN & EMSCHERMANN 1995), and exhibit great regeneration ability and are usually found in unpolluted freshwater. The body of the hydra is comprised of 3 regions – a hypostome with tentacles, a body with the budding region and foot with a basal disc, as well as 3 layers. The ectoderm is the outer cellular layer built of myoepithelial, interstitial (I-cells) and nerve cells, cnidoblasts and cnides. The mesoglea is located between ectoderm and gastroderm and is built of fibrillar matter. The gastroderm is the inner cellular layer that surrounds the gastro-vascular cavity. It consists of gastrodermal myopeithelial and zymogene cells.

Green hydra (*Hydra viridissima* Pallas, 1766) is representative of a typical endosymbiotic organism. This species contains endosymbiotic algae in its gastrodermal myoepithelial cells, each alga in one symbiosome, surrounded by a perialgal space. Up to 20 individuals of green algae can be found in

one cell (HOLSTEIN & EMSCHERMANN 1995), i.e. in one hydra up to 200000 algae can exist. Algae occupy about 10 % of the cellular lumen. Within the gastrodermal myopeithelial cells, algae are regularly placed one above the other in the basal part of the cell. Green hydra is the only hydra species hosting "zoochlorellae", in which algae use the metabolic products of hydra and hydra uses the algal photsynthates. About 10 % of the fixed CO_2 from alga becomes incorporated into the protoplasm of hydra in the form of glycogen. Symbiotic hydras can survive periods of starvation longer and better than aposymbiotic hydras (DOUGLAS 1994; HABETHA *et al*. 2003). Brown hydra (*Hydra oligactis* Pallas, 1766) is an aposymbiotic hydra which does not form a symbiotic relationship.

Norflurazon (nf) is a selective translocational organic herbicide that causes a "bleaching effect" on newly developed chloroplasts (CHAMOVITZ *et al.* 1990; WRISCHER *et al.* 1998). Nf belongs to the piridazinone group of herbicides and is one of the most studied compounds with a bleaching effect in high - light conditions. It is the active compound of the herbicide SAN 9789. It causes the bleaching of cells with newly formed chloroplasts, i.e. during tylakoid development. Nf does not affect already formed chloroplasts (BÖGER 1996). The "bleaching effect" is achieved by inhibition of carothenoid biosynthesis in plants (CHAMOVITZ *et al*. 1990; SALOPEK & LJUBEŠIĆ 1994), resulting in chlorophyll depletion and inactivation of the photosynthetic apparatus. Nf blocks the phytoen-desaturase enzyme, which is localized on the chloroplast membrane. It is used in protection of the cultures of cotton, peanuts, lemon, apples, etc. The effects of nf has been investigated in cyanobacteria (CHAMOVITZ *et al*. 1990), *Euglena gracilis* (TSCHIERSH *et al*. 2002), algae (MERZLYAK *et al*. 1996), plants (ABROUS *et al*. 1998; SALOPEK & LJUBEŠIĆ 1994) and some animals such as planarians and birds.

Since the effect of nf on hydras has not been studied before, the aim of this work was to establish the effects of nf on morphological, cytological, histological and ultrastructural changes, asexual reproduction and overall viability of green and brown hydras, as well as the effect on hydra-algae symbiosis, with special emphasis on the "bleaching effect". We also established the extent to which the endosymbiotic relationship in green hydra is stable, resulting in higher viability of green hydra in an nf-intoxicated environment, therefore presenting an evolutionary advantage in comparison to aposymbiotic brown hydra.

Material and Methods

Hydras were obtained from Jarun and Maksimir lakes in Zagreb, from the Turopolje region near Zagreb, and from Whiteknights lake in England (by courtesy of Dr. E. A. ROBSON) from the surface of submerged plants. The collected organisms were maintained as a clone culture in the lab in glass dishes (11 cm in diameter and 5.5 cm height) in aerated aquarium water in daylight (photoperiod 10 hrs light and 14 hrs dark) at room temperature 21°C. They were fed once a week with the nauplia of *Artemia salina*. After each feeding they were transferred to clean aquarium water. Undamaged hydras, without buds, of the same size and developmental stage, were chosen for the experiment. During the experiment, hydras were not fed.

Green and brown hydras were treated with 5 concentrations of water solution of nf (SAN 9789, Sandoz Ltd., Basel, Switzerland; purity 80%): $2x10^{-4}$, $2x10^{-5}$, $2x10^{-6}$, $2x10^{-7}$ and $2x10^{-8}$ mol/L (ABROUS *et al*. 1998; SALOPEK & LJUBEŠIĆ 1994). A total of 30 hydras of each species was subjected to each concentration. Controls contained 30 hydras of each species in clean aquarium water. Experiments were performed in high – (70 μmol/m^{2}s) and low – (15 μmol/m^{2}s) light conditions and lasted for 21 days. Fresh herbicide solution was changed every 7 days. Hydras were transferred into clean aquarium water afterwards. The recovery period lasted for 7 days.

Morphological changes (mortality, deformations, changes in locomotion and behavior, asexual reproduction, the "bleaching effect") were documented in hydras in glass dishes of 6 cm diameter and 3.5 cm height and observed daily with a Zeiss Jena binocular. Cytological and histological changes (changes in the structure of all 3 layers of hydras and changes in the position and shape of endosymbiotic algae), were recorded from 3 hydras from each concentration and controls by immersion for 24 hrs in Bouin fixative and dehydrated through an ethanol series, embedded in paraplast and dyed with Hämalaun-eosine on the first 3 days of the experiment. Observations were performed under a Reichert light microscope. Micrographs were obtained using a Pentax-Z-70 camera, with Kodak Gold 100 film. Changes in ultrastructure (chloroplasts, mitochondria, cell membranes, cell walls, ER, Golgi apparatus) in green and brown hydra were observed by conventional TEM (cTEM). Hydras from each concentration and controls were fixed in 2 % glutaraldehyde on the 3rd day of the experiment. After post-fixation the material was dehydrated through an ethanol series, embedded into resin and contrasted with uranyl-acetate and Pb-citrate. Micrographs were obtained using the Zeiss EM10A and FEI Morgagni 269D electron microscopes, with Kodak and Imago films.

Results and Discussion

Norflurazon caused mortality, changes in morphology, behavior, locomotion, asexual reproduc-

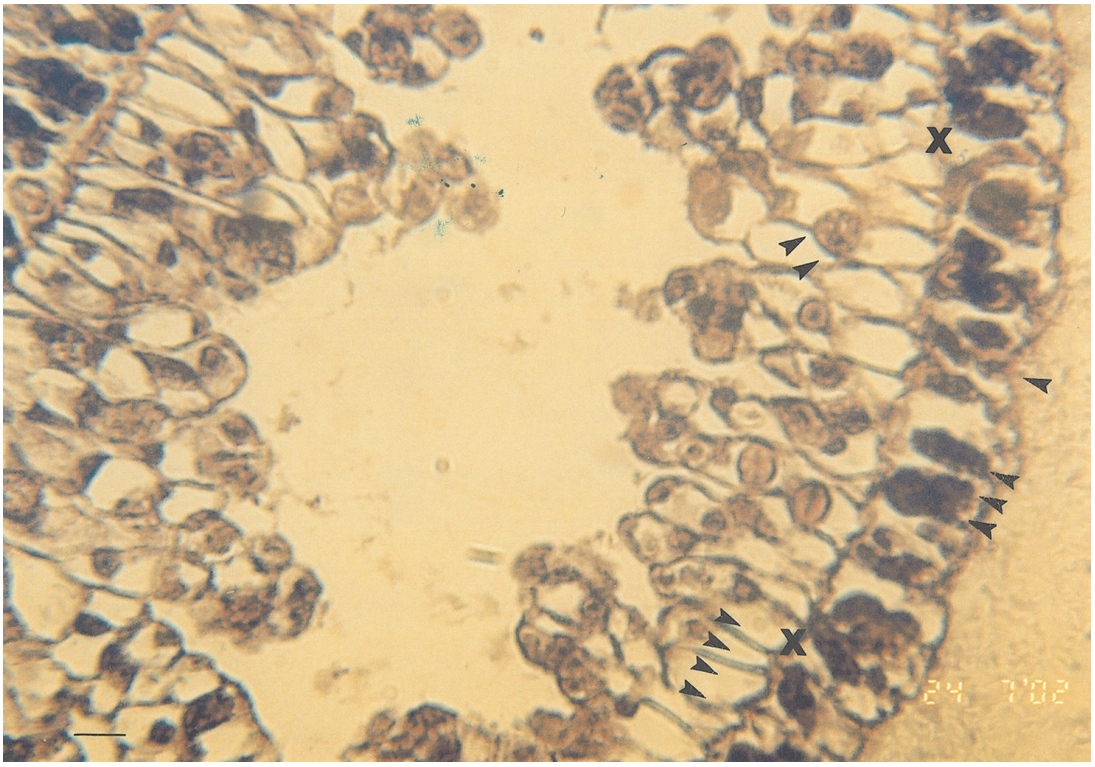

Fig. 1. *Hydra oligactis*. $2x10^{-6}$ mol/L of norflurazon in low-light conditions on the second day of the experiment. Damaged ectoderm (arrowhead) and mesoglea (x). Zymogene cell differentiation and migration towards the mesoglea (2 arrowheads). Individual I-cells in ectoderm (3 arrowheads). Gastrodermal myopeithelial cells vacuolized (4 arrowheads). Bar = 20 μm.

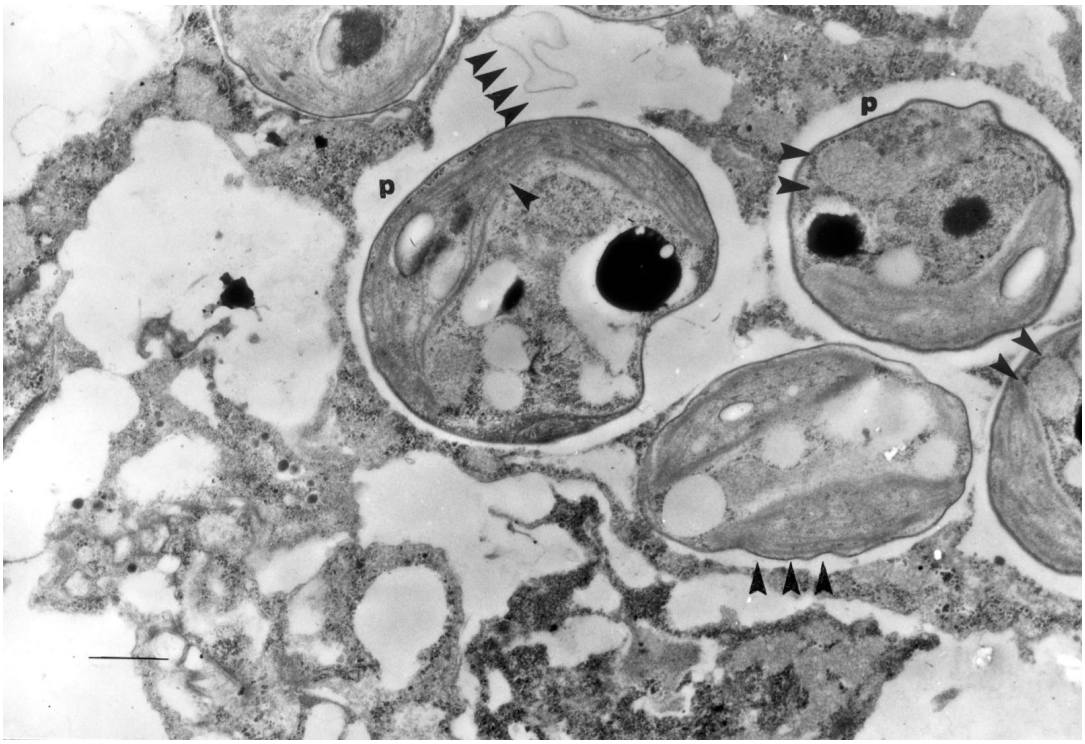

Fig. 2. TEM of gastrodermal layer of *Hydra viridissima* with endosymbiotic algae after the treatment in $2x10^{-5}$ mol/L of norflurazon in high-light conditions. Chloroplasts (arrowhead) and mitochondria (2 arrowheads) of algae with disturbed structure. Wrinkled cell wall (3 arrowheads). Osmyophylic structures in hydra cells (4 arrowheads). Perialgal space (p). Bar = 1 μm.

tion, damage to cellular and ultracellular structures and affected overall viability and fitness of green and brown hydra. The effects of nf on hydras were exacerbated by increasing concentration and in the high-light conditions (Figs 1-2; Tables 1-4), due to the more deleterious effect of nf in high-light con-

ditions (BÖGER 1996; CHAMOVITZ *et al.* 1990). In all the trials, aposymbiotic brown hydra revealed a greater extent of damage than symbiotic green hydra (Tables 1-4). In the two lowest nf concentrations, the treated animals were similar to the control.

Mortality is the basic parameter describing the toxicity of xenobiotics (KALAFATIĆ *et al*. 2003). Complete mortality of green hydra was noted in the 3 highest concentrations in the high-light conditions, whereas in the low-light conditions it was only partial. The LC_{50} for green hydra was noted between $2x10^{-6}$ and $2x10^{-7}$ mol/L of nf. Complete mortality of brown hydra in the high-light conditions was observed in all the concentrations, while in the low-light conditions the LC_{50} was noted between $2x10^{-6}$ and $2x10^{-7}$ mol/L of nf. Already from this parameter it could be concluded that nf in synergy with high-light conditions induced a harmful effect on the studied organisms and caused higher damage than in the low-light conditions (Table 1).

Sublethal effects of toxicants are of interest because they point to the possibility of real long-term damage caused by the particular substance to organisms. Morphological changes were first noted as contractions of the animals (Table 2), resulting from the reaction of the nervous system to chemical stimuli, which is a known defensive mechanism in unfavorable environment (NIDARIĆ *et al*. 1995). Some of the animals were round-shaped, hardly reacting to mechanical stimuli, lying at the bottom of the dish or exhibiting a twisting of the body. Some brown hydras remained constantly contracted and deformed, depending on the light intensity and nf concentration. The most noteworthy morphological damage was observed for the tentacles, a usual reaction of hydras to xenobiotics (KALAFATIĆ & KOPJAR 1994; KALAFATIĆ *et al*. 2001). Most of the tentacles were shortened and some were completely degraded. In green hydras tentacles recovered completely, while in brown hydras the deformations partially remained. Since the majority of the cellular material was spent on the processes of regeneration (KOVAČEVIĆ *et al.* 2001), reduced asexual reproduction was detected in treated hydras as compared to the controls (Table 3). The foot region was extremely degraded (Table 4), producing large quantities of mucous.

Parallel with morphological changes, cytological and histological changes occurred in all 3 layers of hydras. Severe damage to ectodermal

Table 1

Mortality (%) of green (g) and brown (b) hydra. Control animals (C). High-light (h) and low-light (l) conditions

mol/L nf	g-h	g-l	b-h	b-l
$2x10^{-4}$	100	0	100	100
$2x10^{-5}$	100	10	100	100
$2x10^{-6}$	100	10	100	100
$2x10^{-7}$	60	0	100	40
$2x10^{-8}$	0	0	100	20
C	0	0	0	0

Table 2

Contractions (%) of green (g) and brown (b) hydra in the experiment. Control animals (C). High-light (h) and low-light (l) conditions

mg/L nf	g-h	g-l	b-h	b-l
$2x10^{-4}$		100		
$2x10^{-5}$		30		
$2x10^{-6}$		0		
$2x10^{-7}$	0	0		40
$2x10^{-8}$	20	20		20
C	0	0	0	0

Table 3

Green (g) and brown (b) hydra budding (%). Control animals (C). High-light (h) and low-light (l) conditions

mg/L nf	g-h	g-l	b-h	b-l
$2x10^{-4}$		20		
$2x10^{-5}$		30		
$2x10^{-6}$		20		
$2x10^{-7}$	20	0		10
$2x10^{-8}$	20	10		20
C	40	70	0	20

Table 4

Foot damage (%) to green (g) and brown (b) hydra in the experiment. Control animals (C). High-light (h) and low-light (l) conditions

mg/L nf	g-h	g-l	b-h	b-l
$2x10^{-4}$		100		
$2x10^{-5}$		0		
$2x10^{-6}$		100		
$2x10^{-7}$	0	0		20
$2x10^{-8}$	20	20		20
C	0	0	0	0

epithelial cells was present, especially to the apical region that produces the extracellular mucous layer (Fig. 1), which protects hydras from mechanical stimuli and unfavorable environmental conditions. Ectoderm degradation could lead to easier diffusion of the toxicant to the organism and cause considerable damage. The mesoglea was partly missing, thinned or discontinued (Fig. 1). The gastrovascular lumen was reduced and filled with degraded cellular elements. Cellular membranes were only slightly contrasted. Gastrodermal myoepithelial cells in treated brown hydras were occasionally vacuolized (Fig. 1). I-cells and zymogene cells were reduced in comparison to the controls (KALAFATIĆ *et al.* 2001). I-cells act as a somatic reserve of embryonic elements, capable of transformation to other cell types. Zymogene cells have the capacity to differentiate and transform into gastrodermal I-cells and mucous cells that can migrate through mesoglea in the ectoderm, where they become ectodermal I-cells or mucous cells, capable of regenerating the damaged parts of the body. I-cells and zymogene cells were spent in the processes of regeneration of the damaged animals and were less abundant after the treatment (Fig. 1). The regenerative processes of I- and zymogene cells resulted in the hydras partial (brown hydra) or full (green hydra) regeneration (KALAFATIĆ & KOPJAR 1994). At sites lacking the mesoglea, migration of differentiated I- and zymogene cells was more rapid, providing more successful regeneration, especially in the contracted animals in which cellular layers were in close contact (KALAFATIĆ & KOPJAR 1995).

In green hydras, algae were irregularly distributed throughout the body and in places they do not usually appear (foot and tentacles) (KOVAČEVIĆ *et al.* 2001) as the result of cytoskeletal damage to gastrodermal myoepithellial cells (KALAFATIĆ & KOPJAR 1994). The "bleaching effect" during the experiment did not occur. Algae retained their green color throughout the experiment. The newly developed buds were not bleached (KOVAČEVIĆ *et al.* 2001), possibly due to the complex symbiotic relationship resulting from preadaptations blocking the bleaching of the chloroplasts and newly formed buds. Damage may be expressed in another way, i.e. through direct chloroplast degradation.

Transmission electron microscopy confirmed the described damage. Of special interest were antichloroplastal and antimitochondrial effects that resulted in low viability, degradation and mortality of the organisms. Chloroplasts were damaged, swollen, torn apart or degraded (Fig. 2). Plastoglobules appeared as the result of chloroplastal degradation (CZERPAK *et al.* 1992). The antimitochondrial effect was unexpected. Mitochondria were swollen, damaged, changed in shape or had a reduced matrix only in treated green hydras exposed to the high-light conditions (Fig. 2). This points to photoactivation in hydras exposed to the high-light conditions. Earlier studies confirmed the antichloroplastal and antimitochondrial effect of antibiotics in treated organisms and the evolutionary connection of these organelles with eubacteria (EBRINGER & KRAJČOVIČ 1987). Cellular membranes were destroyed. Fragments of rough ER and Golgi apparatus were present, which was also noticed in the treatment of hydras with insecticides (KALAFATIĆ *et al*. 1991). Ribosomes were reduced in number, as compared to the controls. Concerning the substantial amount of ultrastructural damage, osmyophylic inclusions appeared in the vacuolized gastrodermal cells of hydras (Fig. 2) (KOVAČEVIĆ *et al*. 2001). Cnidae were displaced as the result of tentacle damage and spread throughout the hydras' bodies. Algae were irregularly placed inside the gastrodermal host cells. Their cell walls were wrinkled as the result of degradation; algae changed their shape from round to oval (Fig. 2). In brown hydra the overall ultrastructural changes were more extensive.

After the 7-day recovery period, green hydras in the 3 lower concentrations recovered completely, with endosymbiotic algae regularly placed inside the host cells again. The brown hydras that survived retained permanent deformations. Regeneration processes were much more successful in low-light conditions. During the experiment aposymbiotic brown hydra were much more damaged than symbiotic green hydra in all the treatments. These results reveal that the symbiotic relationship in green hydra is stable and represents an evolutionary advantage compared to aposymbiotic hydras.

Acknowledgements

The presented results are a product of the scientific project "Molecular phylogeny, evolution and symbiosis of freshwater invertebrates" carried out with the support of the Ministry of Science, Education and Sport of the Republic of Croatia.

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