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Assessment of the impact of chlorophyll derivatives to control parasites in aquatic ecosystems

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Abstract Several research groups have studied new biopesticides which are less toxic to the environment and capable of controlling the vectors of parasitic diseases, especially in aquatic ecosystems. Pest control by photodynamic substances is an alternative to chemical or other measures, with chlorophyll and its derivatives as the most studied substances supported by their easy availability and low production costs. The impact of chlorophyll derivatives on four different species, a small crustacean (*Daphnia similis*), a unicellular alga (*Euglena gracilis*) and two species of fish (*Astyanax bimaculatus* and *Cyprynus carpio*) were tested under short-term conditions. In addition, the effects of long-term exposure were evaluated in *D. similis* and *E. gracilis*. In short-term tests, mortality of *D. similis* (EC_{50} = 7.75 mg/L) was most strongly affected by chlorophyllin, followed by *E. gracilis* (EC_{50} = 12.73 mg/L). The fish species showed a greater resistance documented by their EC_{50} values of 17.58 and 29.96 mg/L in *C. carpio* and *A. bimaculatus*, respectively. A risk quotient is calculated by dividing an estimate of exposure by an estimate of effect. It indicated that chlorophyll derivatives can be applied in nature to control the vectors of parasitic diseases under short-term conditions, but long-term exposure requires new formulations.

Keywords Chlorophyll derivatives · Environmental impact · *Daphnia magna* · *Euglena gracilis* · Fish

Introduction

Vector-borne diseases are a global burden and are estimated to cause several million deaths and countless cases

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of diseases each year. More than 70 parasitic diseases caused by protozoa have been described in humans. The search for new insecticides capable of controlling different parasites combined with a low toxicity to the environment has been conducted by several research groups (Erzinger 2011).

Chlorophyll derivatives, such as chlorophyllin and pheophorbide can be used in photodynamic reactions that destroy parasites found in aquatic ecosystems. These substances can be produced from chlorophyll extracted from green plant material. By simple chemical modifications the hydrophobic chlorophyll can be made water-soluble in the form of chlorophyllin, which, in turn, can be modified to pheophorbide by acidification (Erzinger 2011).

Experiments with photodynamic substances yielded very promising results. The study by Abdel-Kader et al. (1999) demonstrated that *Culex* larvae are sensitive to a concentration of 0.07 mM chlorophyllin and *Musca domestica* larvae could be killed with this substance at a concentration of 10 µM. El-Tayeb (2003) demonstrated the effectiveness of hematoporphyrin against *Culex* eggs and the snail *Lymnea natalensis* (vector of the trematode *Fasciola hepatica*). The uptake of the photosensitizer into the organism has been shown by fluorescence microscopy as well as a pronounced effect on the ultrastructure of internal organs and muscles (El-Tayeb 2003, Abdel-Kader and El-Tayeb 2009).

Wohllebe et al. (2009) using chlorophyllin/pheophorbide under laboratory conditions as photodynamic substances for pest control showed that this treatment is not only effective and environmentally beneficial but also cost effective. The EC_{50} value (50 % mortality in test organisms) in *Culex* sp. larvae was about 6.88 mg/L and in *Chaoborus* sp. larvae approximately 24.18 mg/L. The EC_{50} values determined for pheophorbide were 8.44 mg/L in *Culex* and 1.05 mg/L in *Chaoborus*. The results presented by Wohllebe et al. (2009) show that chlorophyllin is more effective than hematoporphyrin or methylene blue by a factor of 100, which were previously tested for the same purpose.

Erzinger et al. (2011) showed that in *Chaoborus* sp. a dark incubation period of about 3 h is sufficient to induce mortality of about 90 % and ≥6 h resulted in nearly 100 % mortality when the organisms were exposed to light after the dark incubation. The temperature did not significantly influence the mortality of larvae in a treatment of 6 h in darkness and subsequent incubation for 3 h in light. At 10, 20C or 30 °C between 80 and 100 % of the treated larvae were killed when the intensity of light from a solar simulator was higher than 30 W/m^2. Lower irradiances were less effective.

Several authors have reported that chlorophyll derivatives have the ability to control different parasites in aquatic systems. Wohllebe et al. (2012) found that the protozoan parasite *Ichthyophthirius mulftifiliis* (Fouquet), which causes the white spot disease in many freshwater fish species, was killed (EC_{50} = 0.67 mg/L). Mahmoud et al. (2013) reported the molluscicidal activity (EC_{50} = 30 mg/L) for the snails *Lymnaea stagnalis*, *Biomphalaria* spp. and *Physa marmorata*.

The possibility of developing new products to control aquatic pests resulted in intellectual property registration of two new products based on chlorophyll derivatives. Abdel-Kader et al. (2005a, b, 2006, 2008a, b, 2009) obtained several patents using chlorophyll derivatives to control different parasites. El-Tayeb and Abdel-Kader (2009) registered the intellectual property called "Field application for malaria vector control using sunlight active formulated extract which is based on using plant extracts containing chlorophyll derivatives. In 2010 Erzinger and Häder developed a new biopesticide, which is nationally and internationally patented (Erzinger and Häder 2009, Häder and Erzinger 2011). The product is capable to eliminate multiple vectors of parasites that have an aquatic life stage. Its mechanism of activity is based on the release of singlet oxygen ($^1O_2$) through the interaction with light, which in turn results in the activation of the caspase cascade (Wohllebe et al. 2011), producing signaling proteins, which finally induce apoptosis of cells in the intestine endothelium, resulting in the death of the parasites. This was elucidated in mosquito larvae which had been exposed to low concentrations of chlorophyllin. After several hours of uptake in darkness the chlorophyllin could be located in the intestine by fluorescence microscopy. The use of acridine orange staining demonstrated that some cells of the intestine endothelium had undergone necrosis induced by $^1O_2$. Subsequent staining with propidium iodide and Hoechst 33342 showed that the cell nuclei of the intestine endothelium had entered apoptosis (Wohllebe et al. 2011).

In 2014, Kashiyama and Tamiaki (2014) published a review, which described various strategies by which organisms avoid phototoxicity of chlorophylls, emphasizing the costs of adaptive mechanisms against this risk of these biological processes. The objective of the present study was to evaluate the potential risk of environmental toxicity caused by the application of chlorophyll to control pest organisms in aquatic systems.

The objectives of the present research are to assess the ecotoxicological consequences of the application of chlorophyll derivatives to control vectors of human and animal parasites in aquatic ecosystems. Since these substances have been found to effectively kill mosquito larvae and fish parasites at low concentrations it is of importance to evaluate if they affect other organisms in aquatic ecosystems.

Materials and methods

Preparations of chlorophyllin

Chlorophyllin was prepared from deep frozen spinach (*Spinacia oleracea* L). Chlorophyll was extracted from the leaves in a water bath at 65 °C using 96 % ethanol, after adding 0.1 M $CaCO_3$, which prevents the formation of pheophytin (Rahmani and Csallany 1991). The extract was filtered and petroleum ether (50–100 °C) was added to the liquid fraction at room temperature. After shaking and separation, the upper lipophilic phase was removed and saponified with methanolic 1 M KOH. This treatment converts chlorophyll into the water-soluble chlorophyllin (Schertz 1928). The chlorophyllin concentration was determined using a spectrophotometer (Shimadzu UV-2501) as described by Porra et al. (1989). The extract was stored in a dark flask at 4 °C under N_2 atmosphere (Erzinger et al. 2011).

In the short-term experiments stock solutions of chlorophyllin were used at concentrations of 100 mg/L dissolved in methanolic KOH. The methanol was evaporated in darkness on a heating plate (80 °C) under airflow.

Organisms

Cell culture and growth conditions of Euglena gracilis

All experiments were performed using an axenic culture of the freshwater flagellate *E. gracilis* Klebs (strain Z) obtained from the culture collection in Göttingen (Germany). The cells were grown in a mixture of a complex and mineral medium described previously (Checcucci et al. 1976). New cultures were inoculated with 25 % from an exponential culture and grown at 22 °C under 20 W/m^2 continuous white light from fluorescence tubes in stationary 100-mL Erlenmeyer flasks.

Daphnia similis

D. similis cultures were grown in 1 L-glass beakers containing 800 mL of culture medium and 20 *Daphnia*. The culture medium was renewed and the offspring produced was removed twice a week. Brood *Daphnia* were removed after 4 weeks in culture and replaced with neonatal organisms. Cultures were maintained at 21 ± 0.5 °C under 12 h light/12 h dark. Cultured *Daphnia* were fed with a suspension of the unicellular green alga *Raphidocelis subcapitata* (formerly called *Selenastrum capricornutum*) twice daily. A peristaltic pump controlled by a timer delivered the food. The feeding rate was about 2×10^5 cells/ml per days.

Fish species

For the tests two fish species, *Astyanax bimaculatus* and *Cyprinus carpio,* were selected. *A. bimaculatus* is a genus native to the Amazon basin; it has a geographical distribution in tropical and subtropical America and represents a significant food source for fish holding higher positions in the food chain. Ordinarily, many small *Characiformes* that inhabit rivers, reservoirs and lakes are called minnows in Brazil. *A. bimaculatus*, popularly known as red-tailed tetra, reaches an average length of 10 cm and is caught by small business companies. It is widely distributed in South and Central America, and it is abundant in several watersheds in Brazil. It has a broad food spectrum, so it can adapt to whatever food is available. In addition, it produces small and numerous eggs that develop rapidly (Dias et al. 2005), warranting their success in colonizing dammed environments (Silva et al. 2010). Among the native species, the yellow-tailed tetra (*A. bimaculatus*) has a potential for aquaculture. It is an omnivorous species that feeds mainly on insects (Reis et al. 2003, da Silva 2009). It is well accepted as a fish snack and used as bait for sport fishing (Hayashi et al. 2004).

C. carpio, although not native to Brazil, was selected because it is used for cultivation in aquaculture especially in the southern states of the country. Both fish species were obtained from aquaculture in Municipal Foundation for Rural Development July 25, Joinville, Brazil. All fish stored in individual glass tanks for a period of 3 days before the experiment with optimal cultivation conditions in order to adapt the fish to the new environment and reduce stress induced by catching and transferring them to deionized water with the appropriate concentration of chlorophyllin. The water was buffered to maintain of pH of 7.2–7.5, an alkalinity of 30–35 mg/L, and a hardness of 40–50 mg/L as $CaCO_3$. The water was mixed thoroughly and aerated before transfer into the test chambers. Fish are acclimated to the test water by gradually changing the water in acclimation tanks from 100 % well water to 100 % reconstituted water over a 4- to 6-h period at 25 ± 1 °C as required by the test procedures (ABNT 2007, ABNT 2011). Toxicity tests were conducted under static conditions without aeration and the organisms were not fed during the test.

Judging from the behavior of the fish this acclimation period was sufficient. For both species juvenile specimens which showed good viability of about 6 cm length were selected.

Short- and long-term tests of toxicity in *Daphnia similis*

The methodology for the short-term tests with *D. magna* is described in the standard NBR 12713 (ABNT 2003).

Neonates of *D. similis*, 2–26 h old, were exposed to diluted samples of chlorophyllin for a period of 48 h (Flohr et al. 2005).

Dilution water was reconstituted with a total hardness of 40–48 mg/L of $CaCO_3$, pH 7.2 at 7.6 and conductivity of approximately 160 µS/cm. The quality control of each batch was carried out by a feasibility test, where a population of test organisms was exposed to the dilution water under the test conditions. The batch dilution water is considered acceptable for use if the immobility rate and/or mortality did not exceed 10 % over a period of 48 h.

Neonate *D. similis* were taken from the cultures and placed in new bottles with the same dilution water plus the appropriate concentration of chlorophyllin. Six neonates were used per sample with an age of 24 h which is considered a very sensitive stage in this species. The sensitivity of the test organisms was evaluated by determining the EC_{50} after 48 h. In parallel the response to potassium dichromate ($K_2Cr_2O_7$) was tested, which is reference substance commonly used for this species. The acceptable range of $K_2Cr_2O_7$ for *D. similis* was 0.04–0.17 mg/L, according to the methodology described in Araujo (2005).

In the long-term toxicity tests of (21 days) chlorophyllin concentrations were 0 (control), 1, 2, 2.5, 3, 3.5 and 4 mg/L. The fertility of three generations (the end of 21 days) was calculated from the number of offsprings. Each concentration sample contained one female *Daphnia* in 50 mL and there were 10 replicates for each concentration ISO 10706 (ISO 2000).

Short- and long-term tests of toxicity in *Euglena gracilis*

For the short-term tests the cells were exposed to chlorophyllin at the desired concentration for 24 h (one light/dark cycle). The first test was done 30 min after transferring the cells to the observation chamber where they were kept in darkness. The second test was performed after 24 h during which the cells again were kept in darkness. The endpoints of these tests were precision of gravitactic orientation, alignment in the water column, percentage of motile cells, mean velocity and form factor (compactness), all of which were determined using the automatic bioassay NGTOX (Erzinger et al. 2010), which is the second generation of the biomonitoring instrument ECOTOX (Tahedl and Häder 1999, 2001). The device consists of a miniaturized microscope, a Firewire camera (DMK 21F04, Imaging Source, Bremen, Germany), a stainless steel cuvette with glass windows, an infrared monitoring light source (infrared LED) and three stepper motor pumps to transfer water, samples and cultures to the observation cuvette. The internal functions are controlled by a built-in microprocessor linked to an external host computer to ensure a fully automatic performance.

In the test with *E. gracilis*, evaluating the motility and orientation parameters described above, the effect was considered significant if the inhibition values of all three replicates exceeded the threshold value of the NG-TOX for a given parameter (11.4 % for motility, 6.8 % for velocity, 3.4 % for compactness, 12.3 % for r-value and 3.1 % for upward swimming) (Azizullah et al. 2011). The highest concentration of a chemical with no significant effect is described as NOEC (no observed effect concentration). The highest concentration, at which no significant effect was observed for a given parameter, was considered as the G-value for that parameter (Azizullah et al. 2011).

The biotest NGTOX is programmed to work in several modi. The single toxin mode is used to produce a series of an automatic dilution (1:2, 1:4, 1:8, 1:16 and 1:32) of the toxin stock solution. Control measurements were done using cell cultures mixed with water instead of toxins. In addition, monitoring of a fixed toxin concentration over longer incubation times can be done using the control mode. In the current experiments measurements were performed immediately after exposure to the toxins and subsequently daily for up to 1 week. All measurements were performed in darkness to avoid interaction of gravitaxis and photoorientation.

For the long-term toxicity tests cultures of *E. gracilis* were grown in media with different concentrations of chlorophyllin (0, 1, 3, 7, 9, 13 and 16 mg/L) added, and the effect on the various movement parameters was observed after 7 days of growth. Cultures were grown in three independent replicates in 100-mL flasks with 50 ml of total volume in each flask. All cultures were inoculated with 10 mL at an initial cell density of about 80,000 cells per mL. The other growth conditions were as described above. There is no standardized method for quality control for the motility and orientation in *Euglena*. However, the minimum requirement was that >80 % of the cells were motile (Ahmed and Häder 2010).

Effects of chlorophyllin in fish in vivo

Three replicates with 10 young fish each were prepared for each concentration of chlorophyllin in 1000 ml of water at 20 °C and each experiment was repeated independently three times. KOH-treated samples (corresponding to the highest chlorophyllin concentration) served as controls and were compared with samples in water. All samples were incubated in darkness for 3 h. After incubation three samples were irradiated with simulated solar radiation (Sol 1200 W, mercury lamp 0383; Dr. Hönle, Martinsried, Germany) for another 3 h. The lamp output was PAR 149.66 W/m^2, UV-A 32.67 W/m^2, and UV-B 0.77 W/m^2 (determined with a spectroradiometer OL 754; Optronics, USA). The emission spectrum of the lamp has been

published elsewhere (Klisch et al. 2001). Three parallel samples were kept in darkness as dark controls. This scheme was performed for all chlorophyllin concentrations. After irradiation the vitality of the fish was determined. Abiotic parameters such as temperature, pH and dissolved oxygen were measured before and after of each analysis using a Hanna Multiprobe.

The endpoint for the fish tests was mortality. Individuals showing no vital signs (movements, reflexes after tipping with a needle) were counted as dead, while unaffected organisms and organisms with reduced viability (compared to the untreated controls) were counted as survivors (ABNT 2011).

Data analysis

Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Statistical analyses were also performed using one-way ANOVA. When the ANOVA showed significant significance, a Tukey test was to determine which concentrations were significantly different from the control group and the other treatments. This procedure allowed the determination of the standard NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) values. To detect any significant differences in individual growth, the nonparametric Mann–Whitney test (MW) was used. A value of $p \leq 0.05$ was considered to indicated significance in all tests.

The median effective concentration (EC_{50}) were estimated using Eq. (1) fitted to the dataset through the least squares method (Tahedl and Häder 1999):

$$y = \frac{y_0}{1 + \left(\frac{c}{EC_{50}}\right)^b} \quad (1)$$

where y is the response variable (percentage of dead organism), c the chlorophyll concentration, y_0 is the response when the concentration tends to infinity and b is a scale factor.

Confidence intervals were calculated from the covariance matrix at a two-side error level of 5 %. All data are expressed as mean ± standard deviation (SD).

Results

Initial experiments evaluated the toxicity in short-term experiments for the micro crustacean *D. similis*, the alga *E. gracilis* and two fish species. In all experiments the control samples (treated with KOH or water) showed no physiological modification or mortality in the studied species. The investigated risk factor was the hydrophilic chlorophyll derivative chlorophyllin. In long term studies no breakdown product of this substance has been found to have adverse effects (Wohllebe 2010). The other potential toxic substances, such as ethanol, methanol and petroleum ether used for extraction and chemical modification, were removed from the chlorophyllin by evaporation before application of the photosensitizer. The other substance to be considered is KOH; this was also tested and found not to induce any toxicity at the highest concentration used in the dilution series or any measureable change in the water pH. These evidences rule out the assessment of other risk factors, and the concentrations indicated below refer to chlorophyllin.

Evaluation of short-term toxicity

The effects of chlorophyllin in *E. gracilis* on motility, cell velocity, gravitaxis and cell compactness (=cell shape) were studied immediately after incubation, and EC_{50} values were calculated for all parameters and summarized Fig. 1.

In the short-term exposure experiments with chlorophyllin, all parameters gave a dose-dependent response but varied in their sensitivity giving different EC_{50} values. Alignment was the least sensitive parameter with an EC_{50} value of 16.33 mg/L (Fig. 1c) followed by the precision of gravitational orientation (r-value) with an EC_{50} of 14.70 mg/L (Fig. 1b) and compactness with an EC_{50} value of 14.00 mg/L (Fig. 1d). The velocity showed a low EC_{50} value (9.81 mg/L) (Fig. 1e). Motility was the most sensitive parameter with an EC_{50} value of 8.1 mg/L (Fig. 1a). The mean EC_{50} value obtained for the algae and derived from the five physiological parameters showed a sensitivity of 12.73 mg/L. The value obtained for *D. similis* was 7.77 mg/L (Fig. 2).

When exited with blue light, chlorophyllin emits a red fluorescence. Because the carapace of *Daphnia* is transparent, incorporated chlorophyllin showed an intensive fluorescence of the intestine which could be visualized using epifluorescence microscopy (NOVA 606, Nova Optical Systems) with no camera attached.

The effects of different concentrations of chlorophyllin in short-term toxicity tests in fish are summarized in Fig. 3. *A. bimaculatus* showed greater resistance to chlorophyllin ($EC_{50} = 29.96$ mg/L) which was higher than that of *C. carpio* ($EC_{50} = 17.58$ mg/L).

Evaluation of long-term toxicity

In order to assess the toxicity in long-term tests, we used the invertebrate *D. similis* and the alga *E. gracilis*. The precision of gravitactic orientation of *E. gracilis*, as quantified by the r-value (Häder et al. 1995), was less sensitive than the other parameters with a NOEC of

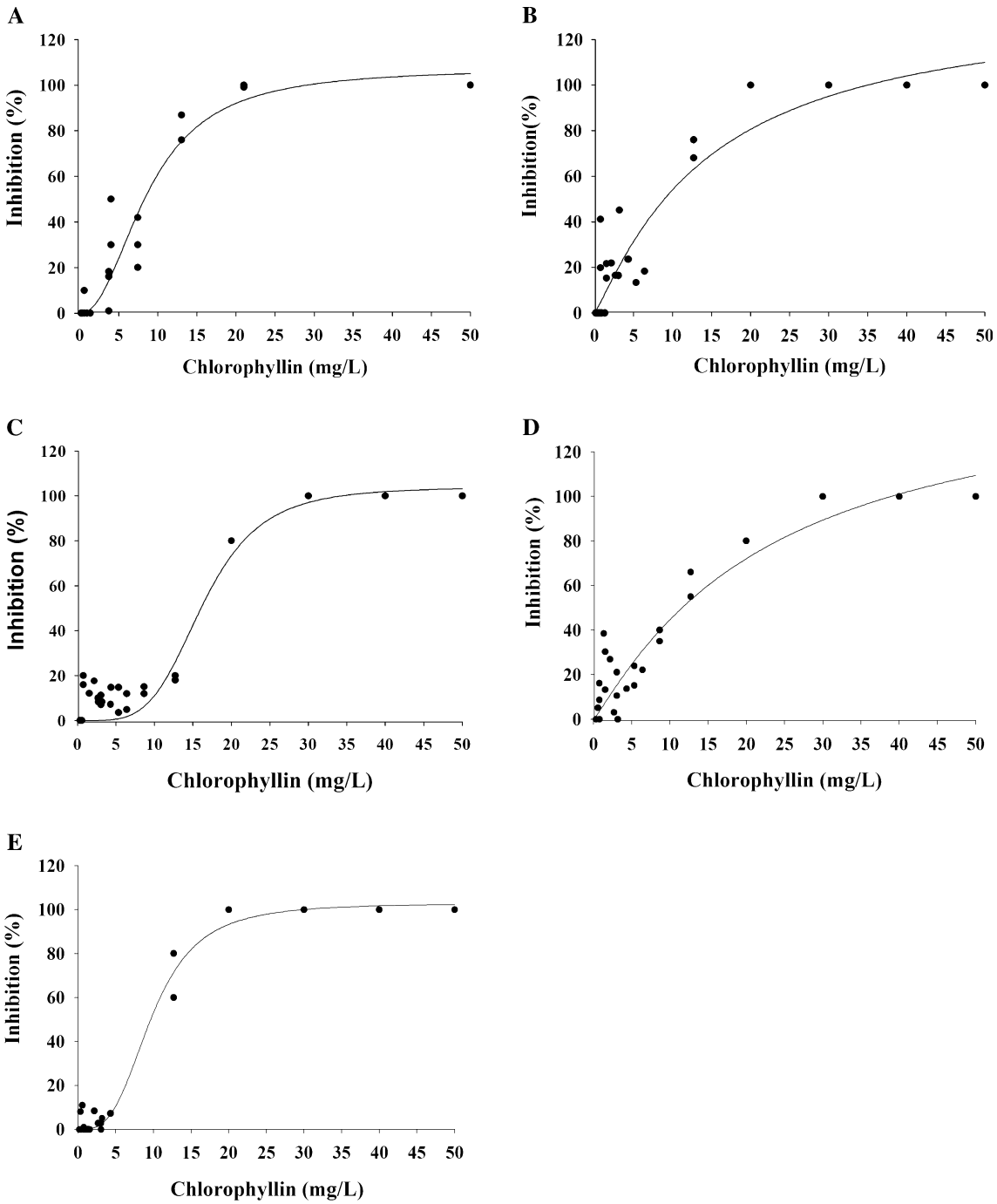

Fig. 1 Inhibition by chlorophyllin of **a** motility (EC_{50} = 8.81 mg/L), **b** precision of gravitactic orientation (EC_{50} = 14.70 mg/L, **c** alignment (EC_{50} = 16.33 mg/L), **d** compactness (EC_{50} = 14.00 mg/L and **e** velocity (EC_{50} = 9.81 mg/L) in *E. gracilis* after short-term exposure

8.7 mg/L, followed by velocity with a NOEC of 7.0 mg/L (Table 1). The mean NOEC value obtained for the long-term tests of algae of the five physiological parameters showed a sensitivity of 4.7 mg/L.

The test acceptability criteria according to the standard NBR 12713 (2003) were met for the chronic *Daphnia* toxicity test. A chronic test of *D. similis* was also established as a benchmark to compare the lethal concentration (EC_{50}) observed in the acute tests. Figure 4 shows the total number of neonates per adult in the long-term bioassay of *D. similis* exposed to different concentrations of chlorophyllin.

After 21 days exposure, no immobility was observed in the control group, validating the test. Data distribution was normal and homogeneous (ANOVA and Tukey test). The fecundity of *D. similis* exposed to chlorophyllin

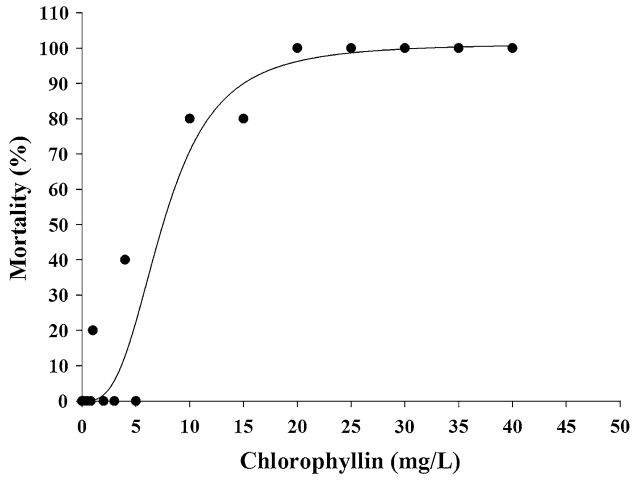

Fig. 2 Percentage of mortality of *D. similis* exposed to chlorophyllin in short-time tests for 48 h in light. EC_{50} = 7.75 mg/L. Each point corresponds to 20 *D. similis*

concentrations >3.0 mg/L was significantly different from that in the control significant ($p = 0.0361$). At concentrations above 4 mg/L there was no yield of offspring due to the toxicity of the medium. In the long-term tests NOEC for fecundity was 2.05 mg/L and LOEC was 3.81 mg/L. The Maximum Acceptable Toxicant Concentration (MATC) (Eq. 2) was calculated and reported from the results of a number of standard procedures designed by the United States Environmental Protection Agency (US EPA) and other organizations to maintain high accuracy and precision among all toxicity tests for regulatory purposes. The MATC is the geometric mean between these two values, such that:

$$MATC = \sqrt{(NOEC)(LOEC)} \quad (2)$$

In this study, the MATC obtained for the chlorophyllin was 2.79 mg/L.

Discussion

According to Wohllebe et al. (2009) upon irradiation solubilized chlorophyll transfers its excitation energy to oxygen resulting in the formation of singlet oxygen ($^1O_2$) and other reactive oxygen species (ROS) that have the potential to damage and kill specific developmental stages of pest organisms or their vectors. The application of very low concentrations of chlorophyllin to water inhabited by parasites reliably kills these larvae via the photodynamic activity of the substance in the presence of light. This same phenomenon was observed in acute and chronic tests for both *D. similis* and *E. gracilis*. Since the organisms were exposed to light during or after the incubation to chlorophyllin, the observed effects can be due to a photodynamic effect, i.e. formation of ROS. Future research will clarify if

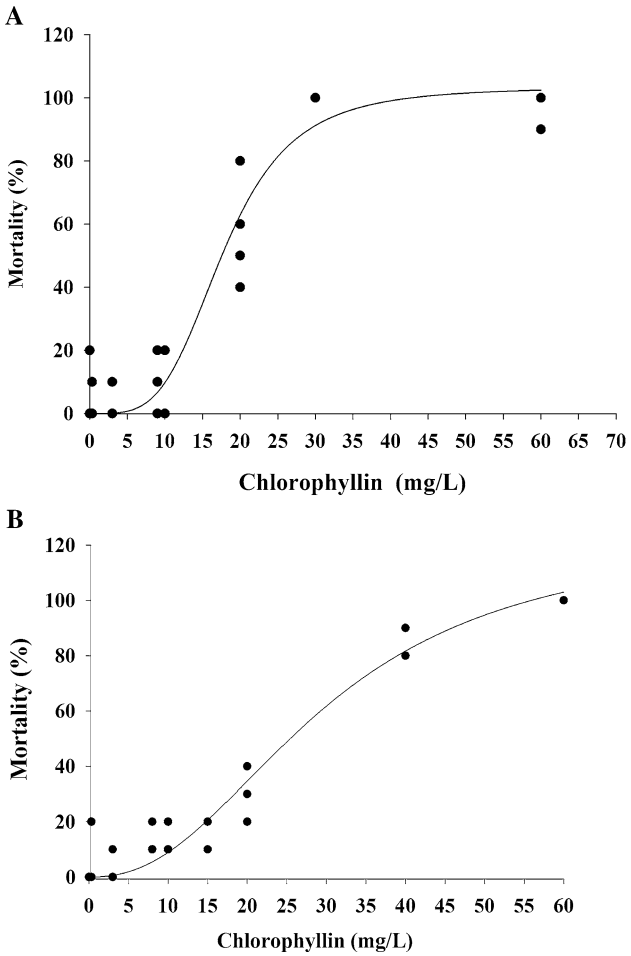

Fig. 3 Percent mortality of *C. carpio* **a** (EC_{50} = 17.58 mg/L) and *A. bimaculatus* **b** (EC_{50} = 29.96 mg/L) exposed to chlorophyllin, incubated in the dark for 3 h and subsequently for 3 h in simulated sunlight. Each point corresponds to ten fishes. The values shown are mean ± SD of the three replicates. One-way ANOVA was calculated for a 95 % confidence level. For all tests $p < 0.001$ was calculated

this is true for the test organisms in this study or whether another mechanism is involved.

Fundel et al. (1998) conducted studies on the effect of chlorophyll in *Daphnia*. At concentrations between 1.2 and 16.3 mg/L, 50 % of the chlorophyllin was observed to change into pheophytin in the absence of light; in the presence of light the conversion was significantly lower. This mechanism occurs during ingestion of chlorophyllin by phytoplankton cells (Strom 1993).

According to U.S. EPA (2004, 2007) the environmental toxicity can be evaluated by defining a risk quotient (RQ) which is calculated by dividing a tabulated value (provided by the agency for various test organisms) by the measured value of the effect.

$$Q = EEC/EC_{50} \quad (3)$$

where EEC is the expected environmental concentration (mg/L). EC_{50} is the estimated mean lethal concentration

Table 1 Percent inhibition of motility, swimming velocity, cell compactness (cell shape), alignment and gravitactic orientation (r-value) in *E. gracilis* after 7 days of growth at different concentrations of chlorophyllin

Chlorophyllin (mg/L)	Inhibition (%)				
	Motility	Precision of gravitactic orientation	Alignment	Compactness	Velocity
1.0	0.0 ± 0.23	2.7 ± 0.32	1.5 ± 0.15	1.8 ± 0.22	0.0 ± 0.00
3.0	26.2 ± 1.23*	1.1 ± 2.55	4.5 ± 1.45	15.7 ± 0.23*	2.9 ± 0.55
5.0	43.5 ± 0.67	7.1 ± 1.45*	5.1 ± 1.77	30.2 ± 2.44	15.0 ± 0.77*
7.0	66.0 ± 0.45	43.1 ± 3.55	20.7 ± 2.00	45.0 ± 1.56	40.1 ± 2.66
9.0	99.8 ± 5.66	70.0 ± 4.22	50.3 ± 1.34	55.6 ± 3.22	99.5 ± 1.36
13.0	100.0 ± 0.23	100.0 ± 0.44	70.0 ± 1.00	90.0 ± 1.34	100.0 ± 0.55
16.0	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00

Values given are mean ± standard deviation of three independent replicates

* Significant differences as compared to the control ($p < 0.05$)

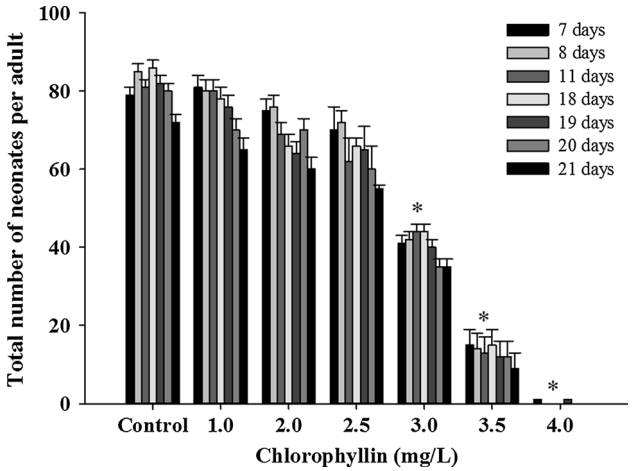

Fig. 4 Total number of neonates per adult in the long-term bioassay of *D. similis* exposed to different concentrations of chlorophyllin. Values given are mean ± standard deviation of three independent replicates. *indicates values significantly different ($p < 0.05$) from the controls as calculated by one-way ANOVA and Tukey test

(mg/L). The quotient RQ identifies situations of high or low risk. According to Goktepe et al. (2004), RQ values below <0.05 suggest that the chlorophyllin used in the study is of no concern for *Euglena*. RQ values between 0.05 and 0.5 indicate that the compounds could present some hazard that may be mitigated by restricted use. *Daphnia* gave a value of 0.08 in the short-term tests. Finally, RQ values above >0.5 require that further testing be conducted before taking regulatory action for the use of pesticides or compounds. According to the National Council for the Environment Resolution 357 (CONAMA 2005) the maximum chlorophyll concentration established for waters intended to supply drinking water for humans or life stock or used for recreation after conventional or advanced treatment is 60 μg/L.

In the short-term tests with *Daphnia* RQ values >0.05 were found (*D. similis* RQ = 0.077) while the tests with *E. gracilis* (RQ = 0.047), *C. carpio* (RQ = 0.002) and *A. bimaculatus* (RQ = 0.003) were lower. The finding that *A. bimaculatus* showed a greater resistance to chlorophyllin than *C. carpio* could be due to the fact that the former is a species capable to survive in adverse conditions (Paiva et al. 2006).

In order to determine the coefficient of environmental risk in long-term tests we also used the methodology described by U.S. EPA (2004, 2007), which requires to divide the 21 days average concentration in the water by the long-term NOEC of the aquatic invertebrates. The RQ for *D. similis* was 0.975. For algae, the RQ level of screening was routinely based on the lowest EC_{50} relative to NOEC. The RQ value for *E. gracilis* was 0.369.

In contrast to the micro-crustacean *D. similis* and the alga *E. gracilis,* where photodynamic activity was the main cause of mortality, the tested fish are not transparent so that light does not penetrate into their bodies. The possible cause of mortality could be a toxic effect of chlorophyllin on the gills of the fish. Gomes et al. (2012) conducted studies on *A. bimaculatus* in which they monitored anomalies of fish gills to assess fresh water quality. One of the stressors studied was the presence of chlorophyll derivatives. According to Shimada Borges et al. (2013), in studies with Nile tilapia (*Oreochromis niloticus*), the gills of teleost fish are potentially useful to assess eutrophication, because these organs are in direct contact with the water and act as a selective interface between the organisms and the environment. Besides their respiratory function the gills of fish are also responsible for the acid–base balance, osmoregulation, excretion and reception of stimuli (Sayer and Davenport 1987, Laurent and Perry 1990, Randal and Wright 1990, Bailly et al. 1992, Partearroyo et al. 1992). However, it was found that the gill covers in young fish can transmit a considerable fraction of the

impinging light (Wohllebe 2010) which is sufficient to activate the photosensitizer chlorophyllin. Therefore the chlorophyllin effect on young fish in this study could well be due to a photodynamic reaction on the gills.

Stringfellow et al. (2009) described that chlorophyll in the water can reduce dissolved oxygen, however, the effective concentrations being used for the control of pest organisms are so minute that they could not measurably reduce the oxygen concentration of the water body.

Concentrations for chlorophyllin have been determined to eliminate mosquito larvae as vectors for human illnesses such as malaria, dengue, yellow fewer and others (Wohllebe et al. 2009, Erzinger et al. 2011, Wohllebe et al. 2011). Likewise, chlorophyllin concentrations have been measured to kill economically important fish parasites (Wohllebe et al. 2012). Comparing those concentrations with those found in this study to affect ecologically important vertebrate and invertebrate test organisms indicates that the use of chlorophyllin in short-term applications does not pose an ecological risk for aquatic ecosystems. In contrast, long-term exposure of these test organisms to chlorophyllin requires some caution. One way of solving this problem could be the application in a specific formulation, which is taken up preferentially by the target organisms, while others are not affected. This is facilitated by the fact that many organisms in the food web feed by particle size (Fenchel 1980).

Conclusions

The potential of chlorophyll and its derivatives to control parasites and pest organisms in aquatic ecosystems is an interesting alternative to chemical or other forms of remedification. But using these substances requires some caution and requires an ecological risk assessment with a proper quality control. Judging from the RQ values and adopting the U.S. EPA recommendations, chlorophyll derivatives (chlorophyllin) can apparently be used to aquatic ecosystems in short-term applications without risk for the environment as indicated by invertebrate and vertebrate test organisms. However, for long-term applications some caution is warranted. Further research of specific formulations is necessary to reduce the potential risk of the environment and increase the stability of the biocide and to prevent its rapid degradation in the presence of light.

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Conflict of interest The authors declare that they have no conflict of interest.

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