

Photodynamic control of human pathogenic parasites in aquatic ecosystems using chlorophyllin and pheophorbid as photodynamic substances

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Abstract When used at low concentrations and added to the water body, water-soluble chlorophyllin (resulting from chlorophyll after removal of the phytol) and pheophorbid (produced from chlorophyllin by acidification) are able to kill mosquito larvae and other small animals within a few hours under exposure of solar radiation. Under laboratory conditions, the use of chlorophyllin/pheophorbid as photodynamic substances for pest control in water bodies promises to be not only effective and ecologically beneficial but also cheap. The LD₅₀ (50% of mortality in the tested organisms) value in *Culex* sp. larvae was about 6.88 mg/l, in *Chaoborus* sp. larvae about 24.18 mg/l, and in *Daphnia* 0.55 mg/l. The LD₅₀ values determined for pheophorbid were 8.44 mg/l in *Culex*, 1.05 mg/l in *Chaoborus*, and 0.45 mg/l in *Daphnia*, respectively. In some cases, chlorophyllin and pheophorbid were also found to be (less) active in darkness. The results presented in this paper show that chlorophyllin is about a factor of 100 more effective than methylene blue or hematoporphyrine, which were tested earlier for the same purpose. It is also much cheaper and, as a substance found in every green plant, it is 100% biodegradable.

Introduction

Parasitic diseases such as malaria and schistosomiasis are a plague for millions of people mainly in underdeveloped and developing countries and often prohibit the economic

development of the affected areas. Great efforts have been made in the past to extinct or at least control these diseases by means of medical treatment, pesticides, exhausting wetland, etc. but with limited success. An important problem of underdeveloped areas suffering from parasitic diseases is poverty, which cuts off the supply of material necessary for countermeasures.

More than 300 parasitic worm diseases and 70 protozoan-elicited diseases are described for humans (Cox 2002). Host finding in parasites is based on a well-adapted sensory system (Haeberlein and Haas 2008; Haas et al. 2002; Haberl et al. 1995; Brachs and Haas 2008). A number of human parasites live in aquatic ecosystems at least for some time of their developmental cycle. The global distribution of these organisms is not limited to subtropical and tropical countries, but some are found and are medically important in mid-latitude waters such as in Europe. One of the effects of global warming will probably be the invasion of malaria in Germany, which is only inhibited by the temperate climate (Goklany 2004).

One of the most important tropical infectious diseases in the world is malaria (Trampuz et al. 2003; Sachs and Malaney 2002; Greenwood et al. 2005). At least 300 million cases of malaria occur each year, which causes the death of more than 1 million people (WHO 2007). Most cases of malaria occur in Africa (about two thirds of the cases and about 90% of the mortality); also the tropical regions of Asia and Latin America are partially severely affected (Anonymous 2007).

Malaria is a protozoan parasitosis, which is caused by different *Plasmodium* species (phylum Apicomplexa). Most important are *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax*, and above all *Plasmodium falciparum*, the most severe representative. *P. falciparum* causes the greatest incidence of illness and death (about 80% of the

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malaria infections and 90% of deaths caused by malaria). *Plasmodium* shows alternation of generations, the human pathogenic species: *Anopheles* (final host) and man as intermediate host.

Also helminthosis caused by parasitic worms, such as nematodes, cestodes, and trematodes, cost many thousand lives every year. Bilharziosis (schistosomiasis) is a major problem in Egypt and other countries worldwide. It is a worm disease caused by the trematod *Schistosoma* (phylum Plathelminthes). *Schistosoma mansoni* (Africa and South America) and *Schistosoma japonicum* (Southeast Asia) cause gastrointestinal symptoms, while *Schistosoma haematobium* (Africa) causes mostly urogenital disorders. About 200 million people worldwide are infected, and between 200,000 to 1 million people die each year from the complications (Engels et al. 2002; WHO 2006). The adult worms live in the venous system of the liver and guts of man. A male and a female worm form a couple, where the female lives in a dorsal fold of the male and only separates from the male to lay eggs in the capillary system of the gut or the bladder (*S. haematobium*). The eggs elicit inflammation reactions and break through the vessels into the gut or bladder, respectively, and are set free with urine or feces. The alternate hosts of the sporocyst are snails (*Biomphalaria* for *S. mansoni*, *Bilinus* for *S. haematobium*, or *Tricula aperta* for *S. japonicum*).

The intention of this project was to develop methods for pest control, which are very inexpensive and non-toxic and, by this, also applicable for poor regions in the world. The methods are based on the photodynamic activity of certain colored organic substances, which, in the presence of light, produce singlet oxygen (1O_2) and other reactive oxidative species (ROS) that have the

potential to damage and kill specific developmental stages of pest organisms or their vectors, respectively (He and Häder 2002; Tominaga et al. 2004; Abdel-Kader et al., submitted for publication). Several organic substances induce photodynamic activity and produce reactive oxygen species such as the high reactive singlet oxygen and radicals, which have the potential to kill certain life stages of the parasites (cercaria) or the corresponding vectors (e.g., mosquito larvae). The most promising application of such substances is the suspension in parasite-contaminated water. The method strictly focuses on the expense factor. In contrast to previous attempts, inexpensive natural substances chlorophyll and its derivatives (chlorophyllin and phaeophorbid), which can easily be extracted from plants, were used. To make the hydrophobic substance water soluble, the phytol group responsible for hydrophobicity is removed by alkaline treatment (transfer into chlorophyllin). Slight acidification of the chlorophyll extracts results in a color change from green to olive-yellow (Richter 1996). This is an indication that pheophorbid (pheophytin without phytol) has been generated from chlorophyllin by hydrolysis. It is characterized by the lack of the central atom, magnesium, present in chlorophyll. The organic solvents ethanol and benzine can be recycled.

Materials and methods

Organisms

Culex sp. larvae were collected in a pond near the institute of biology in Erlangen and directly used for experiments.

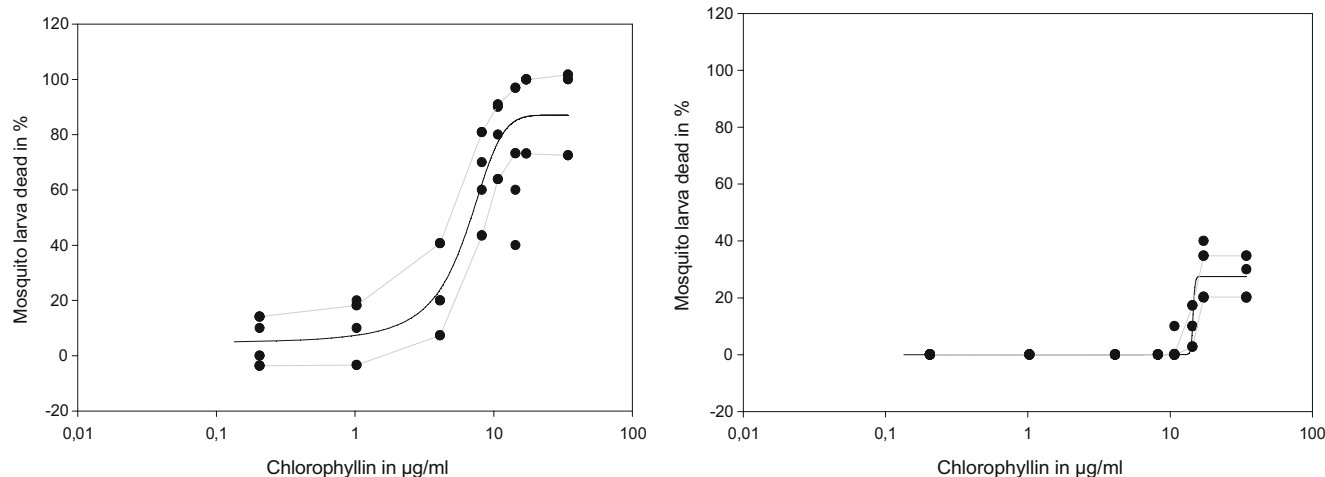


Fig. 1 Percentage of dead mosquito larvae (*Culex*) in dependence of the chlorophyllin concentration after 3 h of light exposure (*left*) or 3 h in darkness (*right*). Each sample (ten larvae) is represented by a *single*

dot. The *black line* is the sigma-shaped fit curve obtained by Sigma Plot, and the *gray ones* are the corresponding upper and lower borders of a 95% confidence level

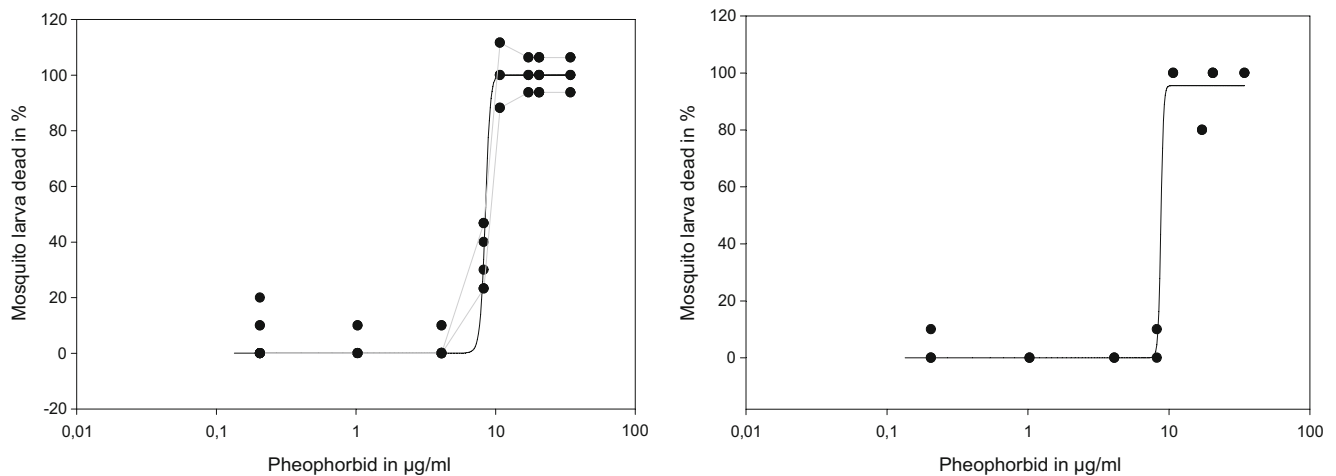


Fig. 2 Percentage of dead mosquito larvae (*Culex*) in dependence of the pheophorbid concentration after 3 h of light exposure (*left*) or 3 h in darkness (*right*). Each sample (ten larvae) is represented by a *single*

dot. The *black line* is the sigma-shaped fit curve obtained by Sigma Plot, and the *gray ones* are the corresponding upper and lower borders of a 95% confidence level

Chaoborus sp. larvae and *Daphnia* sp. were purchased from Zoo Baum (Nuremberg, Germany). Common carps (*Cyprinus carpio*) were provided from the Landesanstalt für Fischerei und Teichwirtschaft, Außenstelle Karpfenteichwirtschaft, Höchststadt Aisch, Germany.

Isolation of chlorophyll, modifications, storage, and material recycling

Different sources for isolation of chlorophyll were used, e.g., grass, stinging-nettle, dandelion, green cabbage, water hyacinth, spinach, etc. From all tested plants, deep-frozen spinach delivered the highest concentration of chlorophyll.

Chlorophyll was extracted in a waterbath at 55°C from the leaves, either with 96% ethanol or with 100% ethanol, after adding CaCO₃, which prevents formation of pheophytin. The extract was filtered and petroleum benzene was added. The upper lipophilic phase was taken and saponified with methanolic KOH. This treatment converts chlorophyll into water-soluble chlorophyllin. KOH precipitation yields high chlorophyllin concentrations. To calculate the concentration, this fallout was measured with a spectrophotometer. The extract was stored in a dark flask at 4°C. In some experiments, the effect of saponified pheophorbid (pheophytin without phytol) was determined. Pheophorbid was produced by acidification (HCl) of chlorophyllin. Before use, the stock

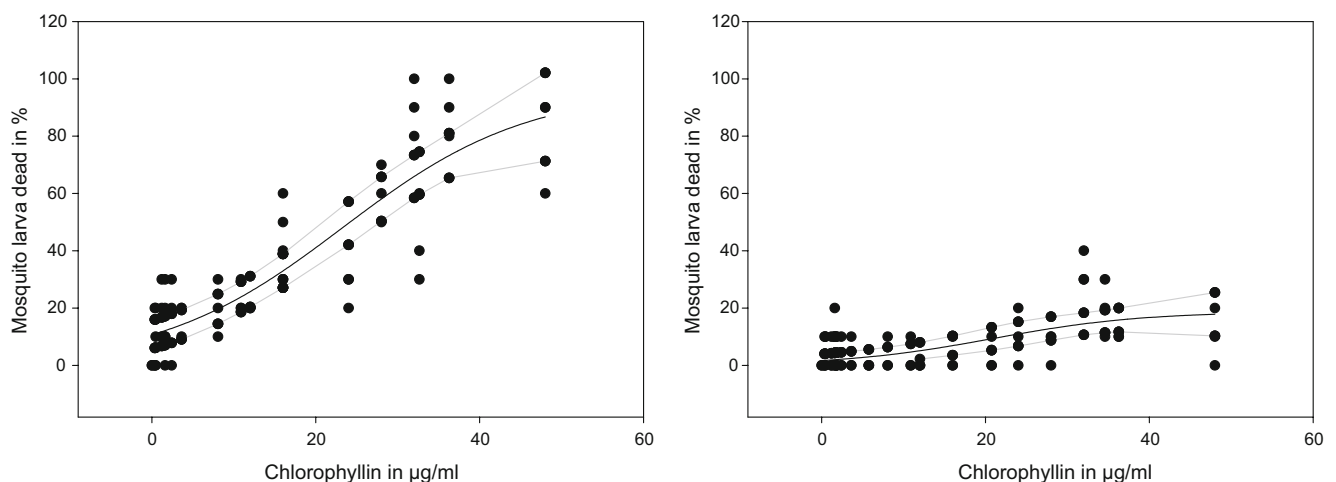


Fig. 3 Percentage of dead mosquito larvae (*Chaoborus*) in dependence of the chlorophyllin concentration after 3 h of light exposure (*left*) or 3 h in darkness (*right*). Each sample (ten larvae) is represented

by a *single dot*. The *black line* is the sigma-shaped fit curve obtained by Sigma Plot, and the *gray ones* are the corresponding upper and lower borders of a 95% confidence level

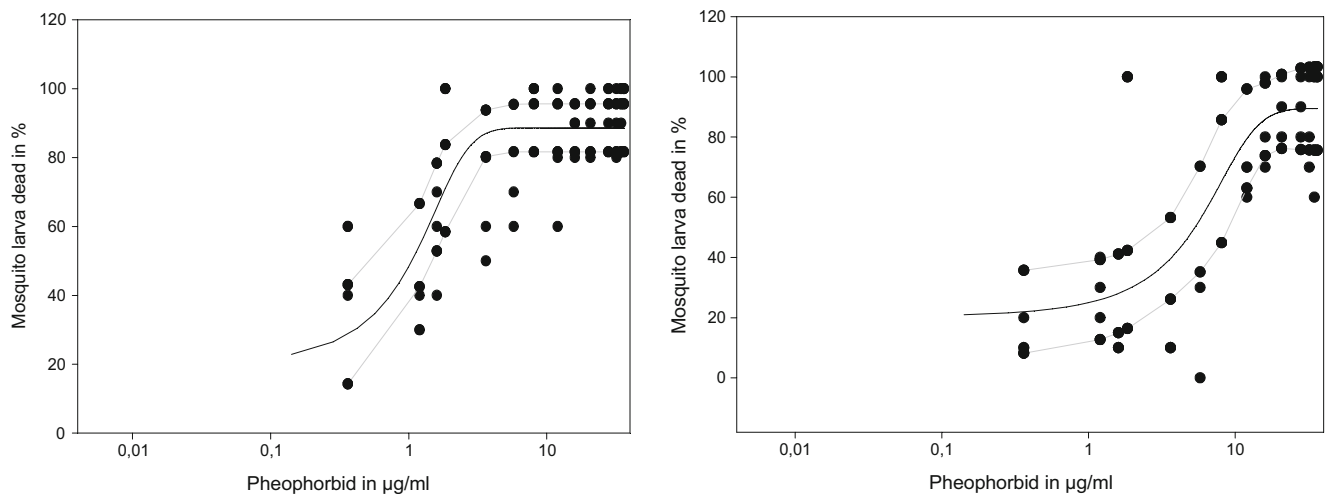


Fig. 4 Percentage of dead mosquito larvae (*Chaoborus*) in dependence of pheophorbid concentration after 3 h light exposure (*left*) or 3 h in darkness (*right*). Each sample (ten larvae) is represented by a

single dot. The *black line* is the sigma-shaped fit curve obtained by Sigma Plot, and the *gray ones* are the corresponding upper and lower borders of a 95% confidence level

solution was adjusted to pH 7.0. Petroleum benzine, which was used for isolation, could be reused several times. Nearly all ethanol could be recycled by distillation. The water content of the recycled ethanol was about 4%. The recycled EtOH was fully suitable to extract chlorophyll from plant material.

Sample treatment and determination of LD₅₀ values

Defined amounts of chlorophyllin dissolved in methanolic KOH were evaporated in darkness on a heating plate under air flow. Each concentration was applied in three repetitions

and three independent approaches. Water and KOH-treated (corresponding to highest chlorophyllin concentration) samples served as controls. Each chlorophyllin sample was dissolved in 30 ml of water, and subsequently, ten organisms were added. One-year-old carps were incubated in 10-l water basins with a chlorophyllin concentration of 15 mg/l. Carp larvae were incubated in chlorophyllin-containing water with 15 mg/l. All samples were kept in darkness overnight for incubation. After incubation, three samples were irradiated with simulated solar radiation (Sol 1200 W, mercury lamp 0383, Dr. Hönle, Martinsried, Germany) for 3–4 h. The lamp output was PAR:

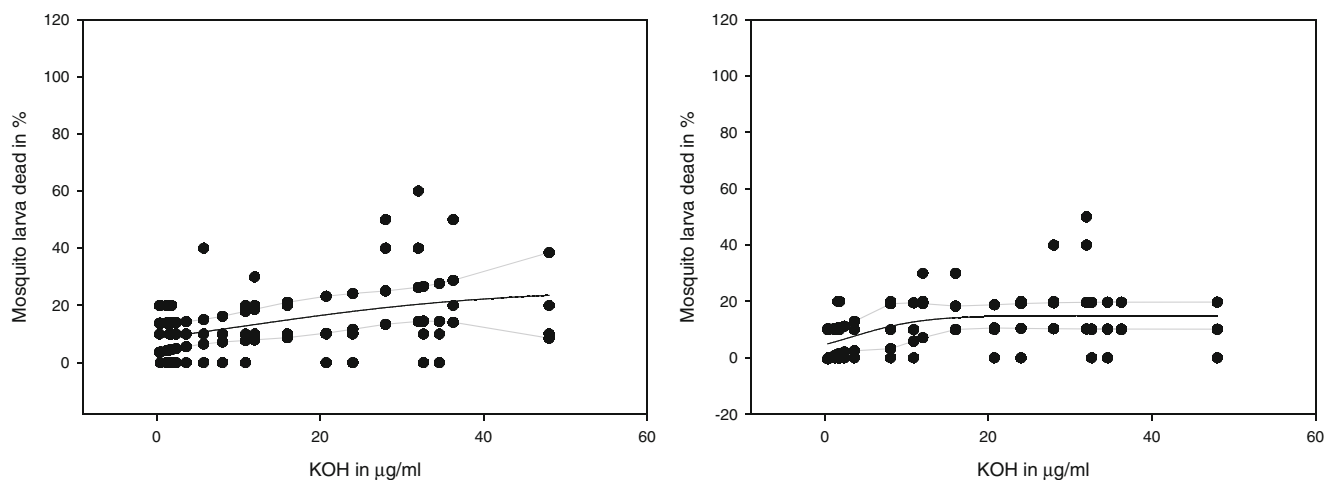


Fig. 5 LD₅₀ curve of *Chaoborus* larvae in presence of KOH after 4 h light exposure (*left*) or 3 h in darkness (*right*). Each sample (ten larvae) is represented by a *single dot*. The *black line* is the sigma-

shaped fit curve obtained by Sigma Plot, and the *gray ones* are the corresponding upper and lower borders of a 95% confidence level

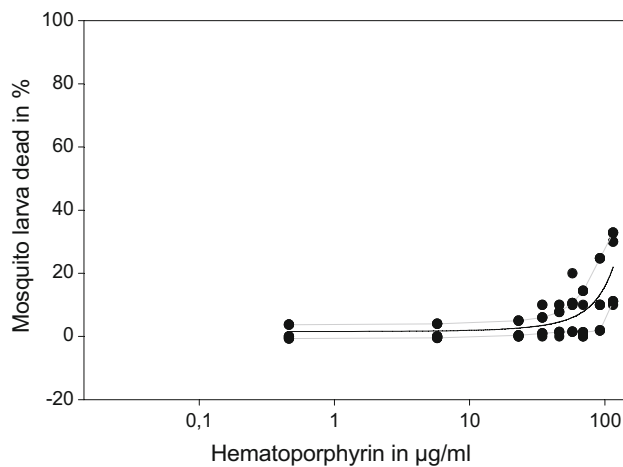
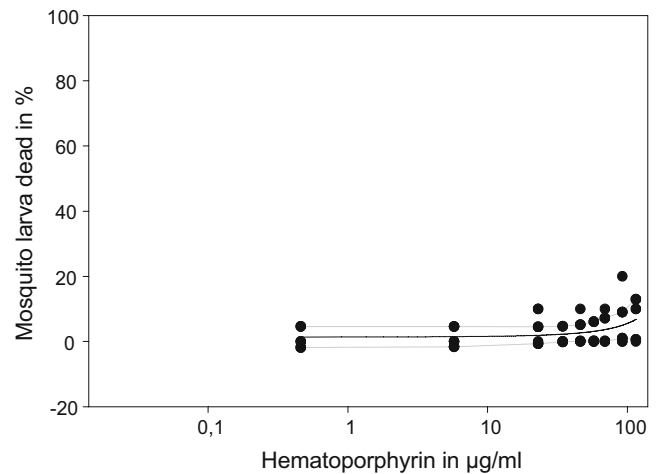


Fig. 6 Percentage of dead mosquito larvae (*Culex*) in dependence of hematoporphyrin concentration after 4 h of light exposure (*left*) or 4 h in darkness (*right*). Each sample (ten larvae) is represented by a *single*



dot. The *black line* is the sigma-shaped fit curve obtained by Sigma Plot, and the *gray ones* are the corresponding upper and lower borders of a 95% confidence level

149.66 W/m², UV-A 32.67 W/m², and UV-V 0.77 W/m² (determined with spectroradiometer OL 754, Optronics, USA). The emission spectrum of the lamp is shown elsewhere (Klisch et al. 2001). Three parallel samples were kept in darkness as dark controls. This scheme was performed for all concentrations. After irradiation, the vitality of the organisms was determined. Individuals showing no vital signs (movements, reflexes after tipping with a needle) were counted as dead, while not affected organisms and organisms with reduced viability (compared to untreated control) were counted as survivors. Lethal-concentration curves of mortality (LD curves) were calculated. They have a sigmoidal form described by the

following equation (Eq. 1; Tahedl and Häder 1999), described as in previous work (Tahedl and Häder 1999; Willemann 2002; Millán de Kuhn et al. 2006) to interpret the experimental data:

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

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

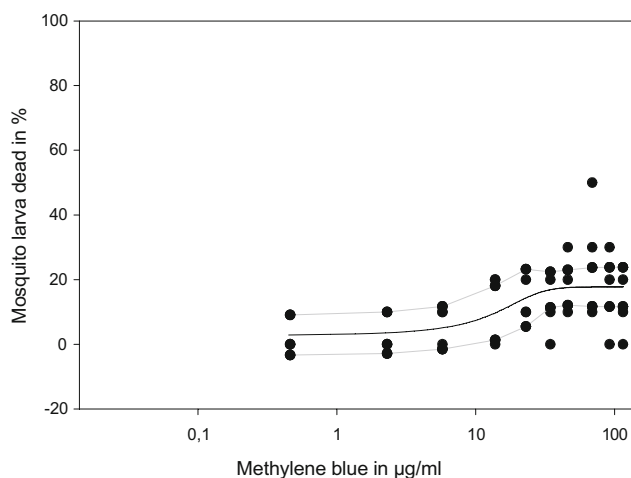
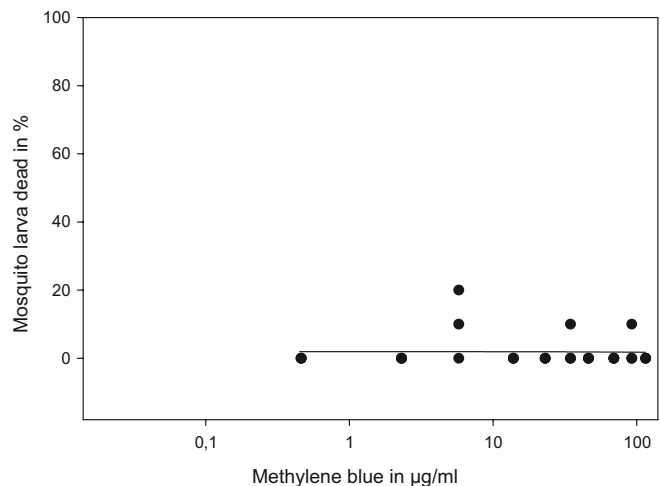


Fig. 7 Percentage of dead mosquito larvae (*Culex*) in dependence of methylene blue concentration after 3 h of light exposure (*left*) or 3 h in darkness (*right*). Each sample (ten larvae) is represented by a *single*



dot. The *black line* is the sigma-shaped fit curve obtained by Sigma Plot, and the *gray ones* are the corresponding upper and lower borders of a 95% confidence level

Table 1 Summary of LD₅₀ values

Experimental series	LD ₅₀ in μg/ml light	LD ₅₀ in μg/ml dark
<i>Daphnia</i> chlorophyllin 3 h	0.55	1.1
<i>Daphnia</i> pheophorbid 3 h	0.45	0.67
<i>Culex</i> chlorophyllin 3 h	6.88	No severe effect
<i>Culex</i> pheophorbid 3 h	8.44	8.8
<i>Chaoborus</i> hematoporphyrin 3 h	157.78	365.1
<i>Chaoborus</i> methylene blue 3 h	No severe effect	No severe effect
<i>Chaoborus</i> chlorophyllin 3 h	2418	No severe effect
<i>Chaoborus</i> pheophorbid 3 h	1.05	5.31
<i>Chaoborus</i> KOH 3 h	No severe effect	No severe effect

The data were processed using the software SigmaPlot for Windows 2000 by SPSS. This model corresponds with the equation proposed by Emmens (Tahedl and Häder 1999) to interpret effect–concentration relationships. The software fits the values with a nonlinear regression. The SigmaPlot curve fitter uses the Marquardt–Levenberg algorithm to determine the parameters of the independent variables that give the best fit between the equation and the data. This algorithm determines the values of the parameters iteratively so that the sum of the squared differences between the values of the observed and predicted values of the dependent variable is minimized.

$$SS = \sum_{i=1}^n (y_i - \hat{y}_i)^2 \quad (2)$$

where y_i is the observed and \hat{y}_i the predicted value. The parameters EC₅₀, b , and y_0 (see Eq. 1) are optimized in

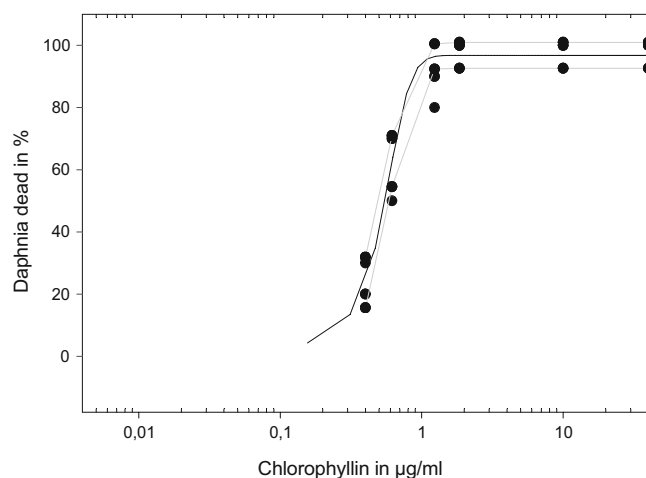


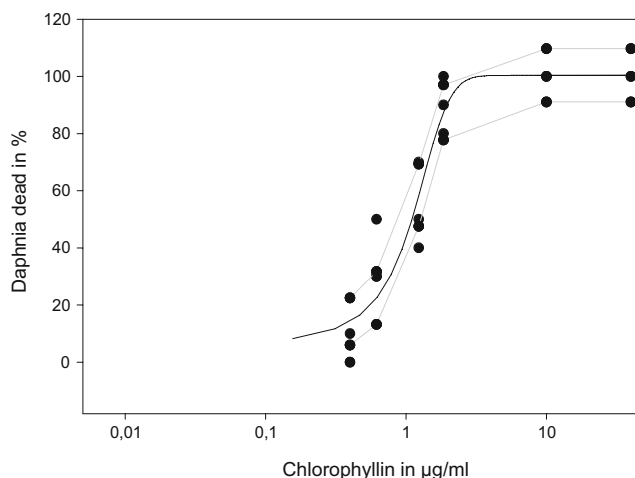
Fig. 8 Percentage of dead *Daphnia* in dependence of the chlorophyllin concentration after 3 h of light exposure (left) or 3 h in darkness (right). Each sample (ten larvae) is represented by a single

order to minimize SS. Confidence intervals of the optimized parameter set are calculated from the covariance matrix at a two-side error level of 5%.

Results

Initial experiments in which organisms were immediately irradiated after addition of chlorophyllin did not yield high mortality. This is most likely due to the fact that chlorophyllin was bleached before it was incorporated by the organisms. Several hours of dark incubation led to a significant accumulation of chlorophyllin inside mosquito larvae, which could be documented by means of fluorescence microscopy (data not shown). Chlorophyllin accumulation was also observed in cercaria of different trematodes and in yeast cells (data not shown).

After incubation, chlorophyllin was found to kill different organisms at remarkably low concentrations. The LD₅₀ value in *Culex* sp. larvae was about 6.88 mg/l, in *Chaoborus* sp. larvae about 24.18 mg/l, and in *Daphnia* 0.55 mg/l (Figs. 1, 2, 3, 5 and 8). For pheophorbid, the LD₅₀ values were 8.44 mg/l in *Culex*, 1.05 mg/l in *Chaoborus*, and 0.45 mg/l in *Daphnia* (Figs. 2 and 4, data for *Daphnia* not shown). In some cases, chlorophyllin and pheophorbid were also found to be active in darkness. Pheophorbid resulted in LD₅₀ values of 8.8 mg/l *Culex*, which is close to the determined value of the light-exposed samples. In *Chaoborus* LD₅₀ values were five times higher (5.31 mg/l) compared to those in light-exposed samples, and in *Daphnia*, 50% mortality was found to be in a range of 8.8 mg/l (Figs. 2, 4, and 8). It was found that during the puparium, mosquito larvae are relatively insensitive against chlorophyllin treatment. During metamorphosis, the chrys-



dot. The black line is the sigma-shaped fit curve obtained by Sigma Plot, and the gray ones are the corresponding upper and lower borders of a 95% confidence level

alis is encapsulated and totally stops food uptake. Most likely due to this reason, the accumulation of chlorophyllin inside the organism is limited and the photodynamic effect is reduced. For comparison, the efficiency of two other photodynamic substances, hematoporphyrin and methylene blue, was tested in *Chaoborus* larvae. The LD₅₀ for hematoporphyrin was 157.78 mg/l (darkness, 365.1 mg/l), while methylene blue was not effective at all concentrations tested (Figs. 6 and 7). All LD₅₀ values are summarized in Table 1.

One-year-old common carps (*C. carpio*) were not affected, while carp larvae did not survive the dark incubation with chlorophyllin.

Discussion

Abdul Kader and his research group of the National Institute of Laser Enhanced Science (NILES) performed very promising experiments using hematoporphyrin for pest control. In a recent work performed by Abdel-Kader et al. (2008a, b), it was demonstrated that larvae of *Culex* are sensitive against hematoporphyrin (0.07 μmol/ml). Also larvae of *Musa domestica* could be killed with this substance (10 μmol/ml). In the course of his PhD thesis, Allah El Tayeb (El Tayeb 2003) could demonstrate the effectiveness of hematoporphyrin against *Culex* and eggs of the snail *Lymnaea natalensis* (vector of the trematode *Fasciola hepatica*). In this work, the penetration of the photosensitizer inside the organism was shown by means of fluorescence microscopy and the pronounced effects on the ultrastructure of inner organs and muscles by means of TEM.

The intention of the chlorophyllin experiments was to find a cheap and practical method to control parasites or their vectors, respectively, for large scale application. For bulk experiments, we consider several natural photosensitizers derived from plants including chlorophyll derivatives. Chlorophyll can easily be extracted by organic solvents from local weeds, such as *Eichornia crassipes*, which grows fast in the water canals in Egypt. The extraction of chlorophyll from *Eichhornia* was tested and was easily possible (but with relatively low chlorophyll concentration per fresh weight) by means of the method described above (unpublished results). Alternative sources for chlorophylls are byproducts from food processing plants, which extract and discard chlorophyll. Other potential photosensitizers might include cytochromes, hemoglobin, and other cyclic and linear tetrapyrroles, such as phycobilins, which can easily be extracted from cyanobacteria. But the use of the later substances was not the scope of the research.

The data clearly show the potential of chlorophyllin and pheophorbid as a possible counter measure against pest

organisms. Prerequisite for successful application is the incorporation of the substance inside the organism, where chlorophyllin can exert its photodynamic property. The results show that dark incubation for several hours is necessary for accumulation inside the pest organism. This can be achieved by the application of the chlorophyllin during the night. After sunrise, the photodynamic action will start. Photodynamic effects of chlorophyll and its derivatives (pheophytin and conjugates) are also of interest, e.g., in photodynamic therapy research (Lee et al. 2004; Kessel and Smith 1989). By means of fluorescence techniques and biochemical methods, it was found that chlorophyllin induces apoptosis, necrosis, and expression of heat shock proteins in *Chaoborus* larvae (Wohllebe and Grimm, manuscript in preparation).

The experiments show that chlorophyll derivatives are much more effective to kill mosquito larvae than other photoactive substances like methylene blue or hematoporphyrin. From the curves and the LD₅₀ values, we obtained evidence that chlorophyllin is much more effective than the two other chemicals in the tested concentration range. Unfortunately, other small animals like *Daphnia* and fish larvae are also affected by chlorophyllin, which makes some precautions necessary before application. An advantage is the easy and cheap way of producing the chlorophyll derivatives and the ability to recycle all extraction substances needed. Preliminary experiments indicate that chlorophyll and its derivatives may also be an antiparasite measure in fish farming. Intensive fish cultivation with high density of animals is often the cause of massive appearance of fish parasites such as *Ichthyophthirius* or *Myxobolus* (Haas et al. 1998; Kallert et al. 2005, 2007). Unlike fish larvae, elder fish were unharmed and survived chlorophyllin treatment at concentrations, where, e.g., *Culex* larvae are severely affected. In the course of a future project, the applicability of chlorophyll derivatives in aqua culture is going to be investigated.

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