The Toxic Effects of Lindane On *Microcystis Aeruginosa* And The Influence of pH And DOM Dependent Effects of Lindane On *Microcystis Aeruginosa*

Deng Xiru

Xiamen Institute of Technology

Jiang Yingnan

xinxi cunchu xitong jiaoyubu zhongdian shiyanshi: Wuhan National Laboratory of Optoelectronics Data Storage and Application Lab

Xian Qiming (✉ xianqiming_edu@aliyun.com)

Nanjing University

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Abstract

The toxic effects of Lindane (γ -BHC) on *Microcystis aeruginosa* were studied under lab culture conditions. Total protein levels, as well as malondialdehyde (MDA) levels and superoxide dismutase (SOD) enzyme activity, in algal cells, were determined after exposure to different concentrations of Lindane. The bioaccumulation of Lindane, as well as the influence of pH and dissolved organic matter (DOM), on the toxic effects was also evaluated in algal cells. The growth of *M. aeruginosa* was inhibited by Lindane treatment (96 h), resulting in 50% of maximal effect (EC_{50}) concentration of 442 $\mu\text{g/L}$. In addition the lowest observed effect concentration (LOEC) was found to be 120 $\mu\text{g/L}$, the no observed effect concentration (NOEC) 60 $\mu\text{g/L}$, and the maximum acceptable toxicant concentration (MATC) 85 $\mu\text{g/L}$. With increasing concentrations of Lindane and exposure time, *M. aeruginosa* growth was significantly inhibited; in addition, total protein levels and SOD activity significantly decreased. MDA concentration, however, showed an insignificant increase after 96 h. Lindane has the potential for bioaccumulation in algal cells with a bioconcentration factor (BCF) of 340. Furthermore, the toxic effects of Lindane on *M. aeruginosa* were influenced by environmental factors, such as pH and DOM. The toxic effects decreased with increasing pH and humic acid concentrations. Ultrastructure cell images were used to depict Lindane induced apoptosis.

Introduction

Lindane (γ -BHC) is an organochlorine insecticide that is classified as a persistent organic pollutant (POP) and serious health problems can arise after exposure to it (Saez et al., 2012). It is a highly chlorinated compound that has been used worldwide, as a broad spectrum insecticide, on a variety of crops (Bidlan et al., 2004). The compound has also been used in human health applications to treat scabicide and pediculocide in the form of lotions and shampoos (Phillips et al., 2005). Lindane is the main insecticide produced in China. The highly stable nature of Lindane leads to its ready accumulation in the environment, and thus in organisms. Today, Lindane is no longer in production, however, it persists in the environment and has been found in water, sediment, soil, plant and animal samples (Carvalho et al., 2009; Cid et al., 2007; Concha-Grana et al., 2006; Vega et al., 2007).

Algae are primary producers in aquatic ecosystems and provide a source of food and energy for zooplankton, fish, and other aquatic organisms, the levels of algae are used to monitor and evaluate water environment quality (Kobraei and White, 1996). Blue-green algae (cyanobacteria) are a common, naturally occurring component in most recreational water environments. Excessive cyanobacteria populations in surface water are indicative of a eutrophic water environment. *Microcystis aeruginosa* is a single-celled alga which belongs to *Microcystis*, *Chroococcales*, *Cyanophyta*. *M. aeruginosa* is the dominant species in most eutrophic lakes in China and is found in eutrophic bodies of water all over the world (Saez et al., 2012). The aim of this study is to investigate the toxic effects of Lindane on *M. aeruginosa* as well as some environmental factors affecting its toxicity.

Materials And Methods

2.1 Reagents and materials

Lindane was purchased from J&K Scientific Ltd. (Beijing China, purity 99.3%) and humic acid (HA) was purchased from Sigma-Aldrich (Shanghai, China). All of the chemicals used in this study were analytical grade reagents. *Microcystis aeruginosa* (FACHB905) was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. It was cultured in BG11 medium (Olvera-Ramírez et al., 2000) at pH 7.1–7.5, 25 °C, and with a 12:12 light/dark cycle (2000 lx).

2.2 Algal Cell density measurements

A series of different concentrations of *M. aeruginosa* were prepared. Algae concentrations were measured by cell counting with a hemacytometer and determining their absorbances at 680 nm with a spectrophotometer. A significant linear correlation between cell number and absorbance was observed ($y = 0.0002x + 0.0116$, $R^2 = 0.9971$). The absorbance was then used as an index to measure the growth status of *M. aeruginosa* in the present study.

2.3 Acute and chronic toxicity test

M. aeruginosa was cultured in 250mL BG11 medium and different initial concentrations of Lindane (0, 50, 89, 158, 281, and 500 µg/L) were added. Experiments were performed in triplicate. The initial cell density was 1.7×10^4 cells/mL. The algal optical density at 680 nm (OD_{680}) was measured every 24 hrs by spectrophotometer and the growth inhibition rate (I_a) was calculated using the following equation:

$$I_a = (1 - N/N_0) \times 100\%$$

Where N represents the test group and N_0 represents the control group at OD_{680} . In accordance with the principle of the linear relationship between the natural logarithm of the toxicant concentration and the percentage of biological effect, we can calculate the concentration for 50% of maximal effect (EC_{50}) at the 96 hr time point through the probability method (Hoekstra, 1991). Lowest observed effect concentration (LOEC) is the lowest concentration which has a significant difference from the control and no observed effect concentration (NOEC) is the the highest concentration which has no significant difference from the control (Saker and Neilan, 2001). The maximum acceptable toxicant concentrations (MATC) were obtained using the following equation:

$$MATC = (LOEC \times NOEC)^{1/2}$$

2.4 Determination of the protein, SOD and MDA levels in algal cells

An algal solution (50 mL) was centrifuged for 10 minutes at 4000 rpm, the supernatant was discarded, and then the algal cells were washed using Mili-Q water (3x). Phosphate buffer solution (PBS) (0.05 M, pH = 7.8, 5 mL), a small amount of liquid nitrogen, and quartz sand were added to grind the cell material. The homogenate was then centrifuged for 10 minutes, at 4000 rpm; the resulting supernatant contained a

crude enzyme solution. Total protein levels in algal cells were determined using the Coomassie Brilliant Blue G-250 staining method (Bradford, 1976). The superoxide dismutase (SOD) enzymatic activity was determined according to the nitrogen blue tetrazolium photoreduction method (Beauchamp and Fridovich, 1971). Malondialdehyde (MDA) levels were measured according to the thiobarbituric acid method (Heath and Packer, 1968) with 10% trichloroacetic acid (TCA) substituting for PBS.

2.5 Determination of Lindane concentration in algal cells

An algal solution (70 mL) was centrifuged for 10 minutes, at 4000 rpm and the supernatant was discarded. The cell pellet was washed 3x using Mili-Q water. Acetic acid (100nL), a small amount of liquid nitrogen, and quartz sand were added to lyse algal cells. The homogenate was centrifuged for 10 minutes at 4000 rpm. The supernatant was filtered through a GF/C membrane (0.45 µm) and passed through a C18 column. The column was then washed with 10 mL methanol and 10 mL H₂O to clear away contaminants. The extract was eluted with 10 mL petroleum ether, and then concentrated to 1 mL using a vacuum rotary evaporator and gentle stream of pure nitrogen gas. Lindane concentration was determined using capillary gas chromatograph with electron capture detector (GC-ECD, Agilent 7890) fitted with HP-5 column (30m×0.25mm×0.25µm, Agilent, USA). The injector port temperature was 200 °C. The oven temperature started at 140 °C for 2 min, and increased to 260 °C at a rate of 10 °C min⁻¹. The temperature was then maintained at 260 °C for 10 min. The temperature of detector was set at 300 °C. A standard curve was prepared using Lindane solutions of various concentrations (5, 10, 20, 50, 100, 200, 500 µg/L). A linear regression equation ($y = 294.27x - 863.53$) ($R^2 = 0.9998$) for the standard curve was obtained for quantification of Lindane concentration in *M. aeruginosa* cells. The recovery efficiency and precision of the process were determined in three different concentrations of Lindane in these experiments we observed 84.6–91.9% recovery and 1.98–4.62% relative standard deviation (RSD). The bioconcentration factor of Lindane, in treated *M. aeruginosa* cells, was determined using the Kukkonen method (Kukkonen, 1991). The Kukkonen formula is below.

$$BCF = C_f / C_w$$

Here C_f represents the concentrations of Lindane (mg/kg) in the algal cells and C_w represents the concentration of Lindane in the water (mg/L).

2.6 The influence of pH and DOM

To determine the influence of pH and DOM, *M. aeruginosa* growth, after exposure to different concentrations of Lindane (5, 10, 20, 50, 100, 200, 500 µg/L) for 96 hrs, was assessed. In these experiments cells were grown in various pH conditions (pH 5, 7, 9) and various humic acid (HA) concentrations (0, 2.5, 5.0, 7.5, 10.0 mg/L). Cell growth was monitored every 24 hrs to evaluate the influence of pH and DOM.

2.7 The ultrastructural observations of *M. aeruginosa*

M. aeruginosa were grown in different concentrations of Lindane (5, 10, 20, 50, 100, 200, 500 µg/L) for 96 hrs and then samples were taken. Algal solutions were centrifuged for 10 minutes at 4000 rpm and the supernatant was discarded. Cells pellets were washed 3x, using 0.2M PBS (pH = 7.4). The algal cells were fixed with 2.5% glutaraldehyde (Sigma) and 2% paraformaldehyde, rinsed with 0.2 M PBS (pH = 7.4), and then suspended in 2% agar in PBS. Next, the small blocks of agar-suspended algae were post-fixed in 1% osmium tetroxide, dehydrated with ethanol, and embedded in Epon812 epoxy resin. Ultrathin sections were cut with a diamond knife and sequentially stained with 3% methanolic uranyl acetate followed by lead citrate (Pollio et al., 1993). The ultrastructure of *M. aeruginosa* was studied by transmission electron microscopy (TEM) using the JEM-200CX (JEOL, Japan).

2.8 Statistical analysis

The T-test was used to evaluate statistical differences among different test groups using the SPSS software (Version 16.0). All charts were generated with Origin 8.0 and Excel 2007.

Results And Discussion

3.1 Growth inhibition of *M. aeruginosa* by Lindane

Significant growth inhibition was observed in *M. aeruginosa* under increasing concentrations of Lindane and across various exposure times (Fig. 1). Through the Probit Regression function where $y = 0.6435x + 1.0806$ ($R^2=0.9964$), the toxicological data of Lindane on *M. aeruginosa* was obtained. The following toxicological values were determined using this method; EC_{50} 442 µg/L, LOEC 120 µg/L, NOEC 60 µg/L, and the MATC 85 µg/L. The LOEC value in this study is similar to the US EPA ECOTOX database reported value (300 µg/L) (US EPA, 2013).

3.2 Cellular effects of Lindane exposure in *M. aeruginosa*

The protein levels found in algal cells grown under different concentrations of Lindane, after 96 hrs of exposure, are shown in Fig. 2A. Protein levels decreased with increasing concentrations of Lindane. No reduction in protein levels were observed under low concentrations of Lindane (50–158 µg/L). On the contrary, significant reduction of protein level was observed for high concentrations of Lindane (281 and 500 µg/L). In these samples protein levels were 55.4% (281 µg/L) and 49.5% (500 µg/L) of the control sample ($p < 0.01$). These results indicated that Lindane exposure may disrupt protein synthesis in *M. aeruginosa*. Hans et al. (2001) reported that the protein content of *Anabaena* sp. (cyanobacteria) decreased significantly as Lindane dosage and exposure times increased.

The effect of Lindane exposure on SOD enzyme activity is shown in Fig. 2B. The SOD enzyme activity decreased with increasing concentrations of Lindane (50–500 µg/L for 96 hrs). In fact, marked enzymatic activity reduction occurred at all concentrations of 50 µg/L and higher ($p < 0.01$). SOD enzyme activity was only 12.8% of the control sample in 500 µg/L Lindane sample. The SOD enzyme catalyzes excess O_2^- to H_2O_2 and O_2 , in order to prevent free-radical toxicity and protect cells (Scandalios, 1993). In

this study, the activity of SOD decreased with increasing concentrations of Lindane. This decrease in SOD activity resulted in a decrease in the cell membrane's osmotic adjustment ability (Mittler, 2002; Zhu, 2002).

The cellular content of MDA increased gradually from 0.386 $\mu\text{mol/L}$ to 0.427 $\mu\text{mol/L}$ with increasing concentrations of Lindane (Fig. 2C.). The increasing MDA levels in the treatments varied from 4.0–10.6% compared to the control ($p < 0.05$). MDA is an oxidized product of membrane lipids. MDA has been shown to accumulate when plants are exposed to oxidative stresses. Cellular levels of MDA are considered to be an indicator of lipid peroxidation and cellular stress (Chaoui, 1997). In this experiment, MDA levels gradually increased in correlation to Lindane concentration levels. These results suggested Lindane promotes lipid peroxides in *M. aeruginosa*. Overall, we observed, Lindane exposure caused a reduction in total cellular protein content and SOD activity, as well as elevated MDA levels in *M. aeruginosa*. Moreover these results indicate high concentrations of Lindane may destroy the anti-oxidative system of *M. aeruginosa*.

3.3 Bioaccumulation of Lindane in *M. aeruginosa*

The concentration of Lindane in *M. aeruginosa* was determined using GC-ECD after exposure to different concentrations of Lindane (Supplementary material 1). As expected, the cellular concentration of Lindane increased with increasing initial concentrations of Lindane in the algal solution. The BCF of Lindane in *M. aeruginosa* was obtained when the initial concentration of Lindane was 400 $\mu\text{g/L}$ and algae cell population was 2.0×10^4 cells/mL (Supplementary material 2). The BCF value of 340 (log BCF, 2.53) indicated that Lindane has a relatively strong bioaccumulation potential in *M. aeruginosa*. Lindane has been previously shown to bioaccumulated in different aquatic organisms (Geyer et al., 1997; Thybaud and Le Bras, 1988; Vigano et al., 1992). Moreover, the BCF value in this study is similar to previously reported values (Arnot and Gobas, 2006). This review reported the BCF values of Lindane in over a 140 aquatic organism which ranged from 0.52–3.32 for logBCF and acceptable log BCF ranging from 2.16–3.32.

3.4 The influence of pH and DOM on Lindane toxicity

The influences of pH and DOM on Lindane toxicity in *M. aeruginosa* are represented in Table 1. There was little impact observed on the LOEC, NOEC and MATC of the samples. The EC_{50} did increase with an increase in pH; this result implies weak alkaline conditions could reduce Lindane toxicity in *M. aeruginosa*. Jin et al. (2004) reported the optimal pH range is 8.5–9.5 for the growth of *M. aeruginosa*. Lindane is hydrolyzed in alkaline conditions. Hydrolysis is an important mechanism in the abiotic transformation of Lindane (Ngabe et al., 1993). Previous studies revealed that Lindane hydrolysis could be catalyzed by hydroxide and hydrogen ions, especially in alkaline conditions (Ngabe et al., 1993; Saleh et al., 1982). The values of LOEC, NOEC and MATC at pH 5 and 9 were slightly higher than that at pH 7 (Table 1). Liu et al. (2011) reported that the hydrolysis of Lindane was not significant under certain growth conditions; specifically pH 5 or pH 7, at 25°C. This study reported the half-life of Lindane hydrolysis was approximately 2310 d (pH 5) and 1386 d (pH 7), however the half-life of this reaction was

28.1 d at pH 9. Thus pH has a significant effect on the rate at which Lindane is hydrolyzed in environment. The products of Lindane hydrolysis, including 1,2,4-trichlorobenzene, 1,2,3-trichlorobenzene 1,3,5-trichlorobenzene and pentachlorocyclohexene have been identified and their distributions shown to be depended on pH (Liu et al., 2002). Maybe these products are less toxic and persistent than Lindane in the environment. Therefore, support previous data in that, a weak alkaline condition degrades the Lindane toxicant and favors *M. aeruginosa* growth.

Table 1
The influence of pH and DOM on the toxic effects of Lindane on *M. aeruginosa* (96h)

pH	HA (mg/L)	The regression equation	R ²	EC ₅₀ (µg/L)	LOEC (µg/L)	NOEC (µg/L)	MATC (µg/L)
5	0	y = 0.6766x + 0.9149	0.9923	419	121	63	87
7		y = 0.6435x + 1.0806	0.9964	442	120	60	85
9		y = 0.6607x + 0.9533	0.9787	457	128	66	92
7	2.5	y = 0.6330x + 1.0018	0.9988	554	147	73	104
	5	y = 0.6330x + 0.8135	0.9966	745	198	99	140
	7.5	y = 0.6140x + 0.8358	0.9756	882	225	110	157
	10	y = 0.6505x + 0.5610	0.9813	920	238	129	175

The toxic effects of Lindane on *M. aeruginosa* decreased dramatically with increasing concentrations of HA (Table 1). The EC₅₀ value of Lindane increased from 554 µg/L to 920 µg/L when the concentration of HA increased from 2.5 mg/L to 10 mg/L. DOM is regarded as natural chelator (Boggs et al., 1985). Previous studies have shown the hydrophobic component of DOM has a high affinity to pesticides, thus DOM improves solubility in water and migration of hydrophobic organic contaminants (Edwards and Cole, 1996; Thevenot et al., 2009). DOM has been reported to decrease the bioconcentration of organic chemicals in aquatic animals. These decreases in bioconcentration have been attributed to DOM interactions with chemical compounds. Under these conditions aggregates are formed that are too large and/or too polar to be taken up by test organisms (Haitzer et al., 1998). In the present study, the increased EC₅₀ value maybe the result of a stable chelate formation involving HA and Lindane.

3.5 The ultrastructure of *M. aeruginosa*

The ultrastructure photos of *M. aeruginosa*, exposed to different concentrations of Lindane are shown in Fig. 3 (1-cell growth; 2-cell division). Here, the *M. aeruginosa* cells grown in BG-11 medium, without Lindane, demonstrate a dense cytoplasm an intact cell wall and membrane. The thylakoids were abundant and with a compact, neat arrangement in the cytoplasm. The central nuclei were obvious, ribosomes were evenly distributed throughout the cells, and there were many phosphate granules around the central nucleus (Fig. 3 – A1, A2). Under the stress of Lindane (50 µg/L) exposure the vast majority of *M. aeruginosa* cells began to degrade and thylakoids gradually blurred (Fig. 3 – B1, B2). The

disintegration of thylakoids became more obvious and the nucleus began to appear as an empty cavity in cells treated with 89 µg/L of Lindane (Fig. 3 – C1, C2). In samples where the concentration of Lindane was greater than 158 µg/L, the thylakoids were further broken and empty nuclei cavities became more abundant (Fig. 3 – D1, D2). The structure of algal cells became blurred, and the nuclei gradually disappeared (Fig. 3 – E1, E2). When the concentration of Lindane was increased to 500 µg/L, cells had all but disintegrated, however the cell walls remained intact (Fig. 3 – F1, F2).

The structural integrity of a cell's periphery plays an important role in protecting the internal structure, as well as maintaining normal viability and cellular activity. These TEM images of *M. aeruginosa* indicated that Lindane can destroy the structure of these cells. This results in the cells' inability to perform photosynthesis thus leading to death.

Conclusions

Here we report that *M. aeruginosa* growth is inhibited by Lindane. This study demonstrated Lindane exposure resulted in an EC₅₀ 442 µg/L, LOEC 120 µg/L, NOEC 60 µg/L, and MATC 85 µg/L in *M. aeruginosa* after 96 hrs of exposure. The observed decrease in protein levels, SOD activity, and the increase in MDA levels suggested the main mechanism of Lindane toxicity might result from oxidative damage of lipid and other biological macromolecules. Furthermore, the BCF value of 340 indicates Lindane has the potential to bioaccumulate in *M. aeruginosa*. Here we observed the toxic effects of Lindane decreased as pH increased and as concentrations of HA increased. Finally, the ultrastructure images of *M. aeruginosa* showed that increasing concentrations of Lindane destroyed cellular structures. This destruction resulted in the loss of photosynthesis, subsequently preventing cellular metabolism and reproduction eventually leading to cell death.

Declarations

Conflict of interest

None

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Ethical Approval

Not applicable

Consent to Participate

None

Consent to Publish

None

Authors Contributions

DX and JY performed the experiments, wrote the manuscript and analyzed the data. XQ supervised the whole study.

Availability of data and materials

All the data are included within the text.

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Figures

Figure 1

The growth inhibition of *Microcystis aeruginosa* by Lindane. Each value is the mean $\pm$ S.D.

Figure 2

Cellular Effects of Lindane on *Microcystis aeruginosa* (96 hrs). (A) Protein content, (B) SOD activities, (C) MDA content. Each value is the mean $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$

Figure 3

Effect of Lindane on the cellular ultrastructure of *M. aeruginosa* (7000×). A, B, C, D, E, F: ultrastructure of *M. aeruginosa* in 0, 50, 89, 158, 281 and 500 μg/L Lindane, respectively; 1-cell growth, 2-cell division

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