INTRODUCTION

The wide use of pesticides has become a pervasive threat to many natural aquatic ecosystems. These anthropogenic contaminants originate from a variety of sources (municipal, agriculture, silviculture, industries, etc.) as a result of human activities. They are used to protect human beings from the insect vectors of disease-causing pathogens, to protect crop plants from competition with abundant but unwanted plants species, and to protect crop plants and livestock from diseases and depredations by fungi, insects, mites, and rodents.¹

Some pesticides have been found to be harmful to certain algal species but not to others. They affect biological processes at the cellular, population, community, and ecosystem levels of organisms.¹ Disruption of photosynthesis or energy metabolism is one of the major physiological processes affected by pesticides.¹ Their degree of toxicity depends on their physico-chemical properties (solubility in water, residence time, form in which they are employed, half-life of products, breakdown of products, etc.) and the hydrological characteristics of the receiving water bodies.²

The effects of toxic chemicals have been measured on a variety of algal species by using different cultural methods and a number of different biological responses. The use of microalgae as test organisms is popular because of their structural simplicity, abundance in nature, and the ease of obtaining commercially available algal cultures for laboratory testing.³ Algae commonly tested range

Original Article

Growth and photosynthesis inhibition by agricultural pesticides in three freshwater microalgae

Nicolas G Guanzon Jr*a AND Hiroyuki Nakahara

Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-01, Japan

ABSTRACT: Growth rate and photosynthesis of Microcystis aeruginosa, Scenedesmus quadricauda and Aulacoseira granulata exposed to different concentrations of the agricultural pesticides CNP (p-nitrophenyl 2,4,6-trichlorophenyl ether), MEP [O,O-dimethyl O-(3-methyl-4-nitrophenyl) thio phosphate], ISP [isoprothiolane (C₁₂H₁₈O₄S₂)], and TBT (tri-n-butyltin chloride) were determined. The effective concentration (EC₅₀) for growth and photosynthesis in each species of microalgae was then calculated. Inhibition of growth and photosynthesis in the three microalgae was greatest when exposed to CNP and TBT. Microcystis aeruginosa and A. granulata showed a higher tolerance, whereas S. quadricauda showed a higher sensitivity. Except for MEP, the EC₅₀ values for growth obtained in the three microalgae were higher than those for photosynthesis. The growth–photosynthesis response relationship showed that, for CNP and TBT, growth of the three organisms tested were less inhibited than their photosynthesis at a lower exposure (0.001–0.05 µg/L). At a higher exposure (0.10–1.0 µg/L), the response between relative growth rates and relative photosynthesis was proportional. For MEP and ISP, a proportional response existed between relative growth rates and relative photosynthesis in all test organisms. These results suggest that the inhibition of growth and photosynthesis by agricultural pesticides differs for the three microalgae. The differences can be explained in terms of the physico-chemical properties of the four pesticides and the physiological and morphological properties of the three microalgae.

KEY WORDS: agricultural pesticides, EC₅₀, freshwater microalgae, growth, photosynthesis.
from common, to rare and exotic freshwater, and marine species of various division. In the present study, the inhibitory effects of the following agricultural pesticides: herbicide CNP (p-nitrophenyl 2,4,6-trichlorophenyl ether), insecticide MEP [(O,O-dimethyl O-(3-methyl-4-nitrophenyl) thiophosphate], fungicide ISP (isoprothiolane), and algicide TBT (tri-n-butyltin chloride), on growth and photosynthesis of the three dominant microalgae (Microcystis aeruginosa, Scenedesmus quadricauda, and Aulacoseira granulata) in Lake Biwa were evaluated. Effective concentration (EC50) for growth and photosynthesis in each species of microalga was also determined. These four kinds of agricultural pesticides were chosen for this study so as to compare their inhibitory effects on microalgae. Furthermore, there is no information available on their effects on the three experimental organisms tested.

MATERIALS AND METHODS

Test pesticides

The test pesticides CNP, MEP, ISP, and TBT were agricultural pesticide standards obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Test microalgae

Axenic clone cultures of microalgae used in the present study included Microcystis aeruginosa, Scenedesmus quadricauda, and Aulacoseira granulata) in Lake Biwa were evaluated. Effective concentration (EC50) for growth and photosynthesis in each species of microalga was also determined. These four kinds of agricultural pesticides were chosen for this study so as to compare their inhibitory effects on microalgae. Furthermore, there is no information available on their effects on the three experimental organisms tested.

Culture conditions and treatment of pesticides

Clonal cultures of M. aeruginosa and S. quadricauda were grown axenically in 10 mL batches of Conway-thiamine (CT) medium at 25°C, and A. granulata was grown in Conway-silicate (CSI) medium at 20°C. Light was provided at an irradiance [photosynthetically active radiation (PAR)] of approximately 250 μE/m² per s using cool-white fluorescent lamps set to a 14 h photoperiod. The various concentrations of test pesticides were introduced into the cultures during the exponential growth phase. All the microalgae were exposed to final concentrations of 0.001 μg/L, 0.01 μg/L, 0.05 μg/L, 0.10 μg/L, 0.50 μg/L, and 1.0 μg/L. These concentrations were within the range found in contaminated natural environments. Analytical grade acetone was used as the carrier solvent. Stock solutions of these pesticides were prepared fresh for experiments in sterilized culture medium. Prior to exposure to the different concentrations of pesticide, 1.0 mL of cultured microalgal cells was pipetted from each 10 mL of experimental culture and fixed with 0.05 mL of formalin to be used as an initial sample for growth rate calculations. Then, 1.0 mL of each different pesticide concentration dissolved in the growth medium was axenically added simultaneously to each culture. The various concentrations of added pesticides were measured to give the desired final exposure levels used in the present study. Control cultures, which received no pesticide, were also included. In addition, 1 mL of cultured cell sample was pipetted from each treatment for growth rate calculations prior to the addition of new culture medium. The experiment was performed twice with four replicates for each exposure.

Growth rate measurement

Algal cells were counted using a Burker Turk hemacytometer for M. aeruginosa and S. quadricauda, and a Sedgwick–Rafter counting chamber for A. granulata under an Olympus light microscope with ×400 magnification (Olympus Co., Tokyo, Japan). Relative growth rates were then calculated and expressed as cell divisions per day following the formula of Fogg and Thake.

Photosynthesis measurement

Photosynthesis measurements were carried out in a dark room using an oxygen meter (YSI Model 53; YSI Inc., Ohio, USA) equipped with a compensation vessel, which was kept in a water bath and fitted with a motor to shake the vessel, and linked to a recorder (FBR-253A; TOA Electronics Ltd, Kobe, Japan). Light was provided by an incandescent lamp (110 V, 300 W). The PAR (400–700 nm) of the light source was measured using an underwater quantum sensor (LI-1000 Datalogger; LI-COR, Nebraska, USA). Water temperature in the water bath was controlled using a Komatsu Yamato Coolnics Circulator (CTE-220; Komatsu Co., Osaka, Japan). Photosynthesis in M. aeruginosa and S. quadricauda was measured at 25°C, and at 20°C.
for *A. granulata*. Because their photosynthesis reaches saturation at approximately 350 μE/m² per s, light-saturated photosynthesis was measured at approximately 1500 μE/m² per s in the present investigation.

Net photosynthesis was measured immediately after the introduction of the pesticides, and then 24 h later, using duplicate 10 mL samples for each pesticide dose. Upon completion of the measurements, the samples were placed immediately in 10 mL sampling bottles and fixed with 0.05 mL formalin to be used for the final growth rate calculations. Mean relative photosynthesis was calculated for each different pesticide dose and expressed as mg O₂/h per 10⁶ cells. Effective concentrations (EC₅₀); that is, the concentration resulting in a 50% reduction of growth and photosynthesis, of the three microalgae were then computed.

**RESULTS**

**Growth and cell morphology**

Figure 1 and Table 1 show the relative growth rates and EC₅₀ values of the three microalgae upon exposure to different concentrations of pesticide. The relative growth rates of the three microalgae were lowest upon exposure to different CNP concentrations, decreasing exponentially with the level of pesticide concentration in the medium (Fig. 1a). As a consequence, their EC₅₀ values were attained at a lower exposure level. Chlorotic *S. quadricauda* cells and fragmented *A. granulata* filaments were observed after exposure to concentrations of 0.50 mg/L and 1.0 mg/L. At these concentrations, moribund and bleached cells were noticed in all three species of microalgae. Mortalities in the three experimental organisms were highest upon exposure to a concentration of 1.0 mg/L. With exposure to different TBT concentrations, the relative growth rates of the three microalgae were also low (Fig. 1d). As for CNP, these also decreased exponentially with concentration in the medium. Chlorotic *S. quadricauda* cells and fragmented *A. granulata* filaments were observed at the highest exposure (1.0 μg/L). Moribund and bleached *M. aeruginosa*, *S. quadricauda* cells, and *A. granulata* filaments were also abundant at this concentration but less so compared to those observed in CNP. In MEP, growth rates of the three microalgae also decreased exponentially with pesticide concentration in the medium (Fig. 1b). At the highest exposure (1.0 μg/L), few chlorotic *S. quadricauda* cells and fragmented *A. granulata* filaments were observed, and moribund and bleached *M. aeruginosa* and *S. quadricauda* cells, and *A. granulata* filaments were...
few. An abundance of healthy cells were observed even at a higher exposure. The relative growth rates of the three microalgae were highest upon exposure to different ISP concentrations (Fig. 1c). However, they showed the same pattern for the other three pesticides, whereby growth decreased exponentially with the level of pesticide in the medium. Even at the highest exposure concentration, chlorotic *S. quadricauda* and fragmented *A. granulata* filaments were very scant. Cells of the three microalgae were mostly healthy at lower exposures (0.001–0.10 μg/L). Low mortalities were observed in the three microalgae upon exposure to all concentrations of ISP.

The blue-green alga *M. aeruginosa* showed the highest relative growth rates in CNP and MEP (Fig. 1a,b), whereas the diatom *A. granulata* showed the highest values in ISP and TBT (Fig. 1c,d). The green alga *S. quadricauda* showed the lowest relative growth rates in all pesticides (Fig. 1).

**Photosynthesis**

Figure 2 and Table 1 show the relative photosynthesis and EC<sub>50</sub> values of the three microalgae upon exposure to different concentrations of pesticide. Photosynthesis of the three experimental microalgae decreased exponentially with increasing pesticide concentration in the medium. The lowest photosynthetic rates and EC<sub>50</sub> values were obtained upon exposure to different CNP concentrations (Fig. 2a; Table 1), followed closely by TBT (Fig. 2d), then MEP (Fig. 2b) and, finally, by ISP (Fig. 2c). Among the three microalgae tested, *M. aeruginosa* showed the highest EC<sub>50</sub> values for photosynthesis in all pesticides (Table 1). *Aulacoseira granulata* showed the lowest EC<sub>50</sub> value for photosynthesis in ISP, whereas *S. quadricauda* showed the lowest value in TBT.

**Growth–photosynthesis response relationship**

Figures 3–6 show the growth–photosynthesis response relationship in the three microalgae exposed to different concentrations of pesticide. Values were computed based on 100% of untreated samples and expressed as a percentage of those untreated.

The growth–photosynthesis response relationship showed that, except for the insecticide MEP, the relative growth rates of the three organisms were greater than their relative photosynthesis at all exposure levels (Figs 3–6). This is very evident in the pesticides CNP and TBT at lower exposure concentrations.

<table>
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<th>CNP</th>
<th>MEP</th>
<th>ISP</th>
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levels of 0.001–0.10 μg/L (Figs 3,6). At higher concentration levels of 0.50 μg/L and 1.0 μg/L, a proportional response existed. In MEP, a proportional response existed between relative growth rates and relative photosynthesis (Fig. 4). Both growth and photosynthetic processes were almost equally inhibited at all exposure levels.

DISCUSSION

The growth and photosynthetic rates of the three microalgae were inhibited most after exposure to the herbicide CNP (Figs 1a,2a) and, as a result, their EC50 values were obtained at a lower concentration (Table 1).

Pesticides are known to inhibit the growth and photosynthesis of aquatic macro- and microphytes.9 Among the pesticides, herbicides were found to have an acute toxicity to algae. They can penetrate into the cell immediately, especially when light is present, and disrupt photosynthesis or energy metabolism. Furthermore, they inhibit the uptake of inorganic nutrients, especially in the form of nitrogen and phosphorus, which are critical to the growth and reproduction of aquatic algae.2 Various kinds of herbicides have been shown to reduce lipid biosynthesis in algal cultures.11 These lipids are important structural elements in the cell membrane and in the membranes of various cell organelles, and also control the movement of substances into the cell, as well as the movement and metabolism of materials within the cell.2 Phytotoxic effects of an herbicide include blocking of electron transport in the mitochondria, interfering with respiration, and inhibiting transpiration.12,13

The same observations have been reported by other investigators on the effects of some herbicides on other species of microalgae. Under natural conditions, tests have shown that the herbicides atrazine and diuron are highly toxic to some algae at concentrations below 0.1 p.p.m.9 Atrazine reduces algal productivity and biomass in standing water at a concentration level as low as 10 μg/L.14 Natural phytoplankton communities are inhibited by herbicides at a concentration level of 20 μg/L;15 however, at higher levels, the inhibition of algal biomass is great in artificial streams.16

Growth and photosynthetic rates of the three microalgae were also reduced upon exposure to the algicide TBT, the compounds of which are toxic to plants.9 It has been found to inhibit the productivity of both freshwater and marine planktonic algae,17 and to reduce photosynthetic activity in aquatic algae by inhibiting the Hill reaction in Photosystem II.9 Being an algicide, TBT has been
Effects of pesticides in microalgae

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Fig. 3 Growth rate-photosynthesis response relationship in: (a) *Microcystis aeruginosa*; (b) *Scenedesmus quadricauda*; and (c) *Aulacoseira granulata* exposed to various *p*-nitrophenyl 2,4,6-trichlorophenyl ether concentrations. (●) Relative growth rate; (x) relative photosynthesis.

Fig. 4 Growth rate-photosynthesis response relationship in: (a) *Microcystis aeruginosa*; (b) *Scenedesmus quadricauda*; and (c) *Aulacoseira granulata* exposed to various O,O-dimethyl O-(3-methyl-4-nitrophenyl) thiophosphate concentrations. (●) Relative growth rate; (x) relative photosynthesis.

Fig. 5 Growth rate-photosynthesis response relationship in: (a) *Microcystis aeruginosa*; (b) *Scenedesmus quadricauda*; and (c) *Aulacoseira granulata* exposed to various isoprothiolane concentrations. (●) Relative growth rate; (x) relative photosynthesis.
zineb is lethal to some strains of *Anabaena* and *Nostoc*, even at very low concentrations. 27

Results of the present study showed that, except for the pesticide MEP, the EC50 values for growth in the three microalgae were higher than those for photosynthesis (Table 1). In CNP, the observed EC50 value for growth in *M. aeruginosa* was more than 50 times greater than that for photosynthesis. But in *S. quadricauda* and *A. granulata*, their EC50 values for growth was approximately 30 and 35 times larger than those for photosynthesis, respectively. For TBT, the EC50 value for growth in *M. aeruginosa* was 80 times higher than that for photosynthesis, while in *S. quadricauda* and *A. granulata*, their EC50 values for growth was more than 100 and 150 times greater than those for photosynthesis, respectively. In MEP, the EC50 values for growth in the three microalgae were lower than those for photosynthesis, whereas in ISP, the EC50 value for growth in *M. aeruginosa* and *A. granulata* were higher than those for photosynthesis, respectively. As for *S. quadricauda*, its observed EC50 value for growth was almost the same as that for photosynthesis.

The growth–photosynthesis response relationship for MEP showed that a proportional response is observed between the relative growth rates and relative photosynthesis. Maybe, at first, MEP caused an alteration to cell division18 or damaged the cell membrane and then, finally, destroyed the cells;22 hence, photosynthetic rates of the three microalgae were reduced. In ISP, a proportional response also existed between relative growth rates and relative photosynthesis for the three microalgae (Fig. 5).

Among the four pesticides tested, the fungicide ISP showed fewer effects. ISP is a less absorptive pesticide in which its concentrations decrease with dilution and diffusion in a reservoir.23 Some fungicides are known to inhibit growth and photosynthesis, and are even lethal to many species of algae.8 A study on the preliminary screening for potential algicides has shown that 2 mg/L of dichlone selectively killed the cyanobacteria *Cylindrospermum licheniforme* and *M. aeruginosa* in ponds.24 At a concentration of 10 μg/L, it prevented the growth of *Anabaena* sp.,25 while the growth of *Anacystis nidulans* was prevented using a concentration of 140 μg/L of dichlone.26 The fungicide designed specifically to kill algae. Algicides are frequently used as a component of antifouling paints for ships and bouys.9

The organophosphorus insecticide MEP was found to be the third most harmful pesticide. Although insecticides are designed specifically to kill insects, they have also been found to be toxic to algae.18,19 They cause alterations of the cell cycle, increases cell volume, and irregular cell division.18 They also act as inhibitors of photosynthesis in aquatic algae.20,21 In cultures of the green alga *Chlamydomonas reinhardii*, the organophosphorus insecticide, phosalone, damaged the cell membrane, caused the leakage of cell proteins and, finally, caused the destruction of the cell.22 Inhibition of nitrogen fixation by trichlorfon leads to a reduction in photosynthetic pigments and this, in turn, decreases the rate of photosynthesis.18

In the present investigation, the observed photosynthetic rates of the three microalgae decreased exponentially with the level of MEP concentration in the medium (Fig. 2b). Probably, the reduction of their photosynthetic rates resulted in the retardation of their growth rates.

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three experimental organisms, the blue-green alga *M. aeruginosa* and the diatom *A. granulata* showed a higher tolerance to the four pesticides, whereas the green alga *S. quadricauda* showed a lower resistance. Blue-green algae reproduce very fast and possess mucilaginous sheaths that can block the penetration of pesticide molecules into the cell. As for diatoms, their cell walls are predominantly made of silicon, which can prevent adherence of pesticide intracellularly. The green algae have a higher sensitivity to pesticide because of their exposed cell membrane. Differences in growth and photosynthesis inhibition depend on the physico-chemical properties of the four pesticides (formulation and mode of action) tested. Individual effects of active substances are often caused by small parts of large molecules, whereby such a minor chemical difference can cause large differences in the effects of substances on specific organisms. Vulnerability of a particular species to a potentially toxic contaminant such as a pesticide is very dependent on cultural methods, which is very dependent on cultural methods, which

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