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Biotransformation of an Organochlorine Insecticide, Endosulfan, by *Anabaena* Species

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This study assesses the role of the blue-green algal species present in the soil in the dissipation of endosulfan and its metabolites in the soil environment. Two *Anabaena* species, *Anabaena* sp. PCC 7120 and *Anabaena flos-aquae*, were used in this study. *Anabaena* sp. PCC 7120 produced three principal biotransformation compounds, chiefly endosulfan diol (endodiol), and minor amounts of endosulfan hydroxyether and endosulfan lactone. Trace amounts of endosulfan sulfate were detected. In comparison, the biotransformation of endosulfan by *Anabaena flos-aquae* yielded mainly endodiol with minor amounts of endosulfan sulfate. An unknown compound was produced up to 70% from endosulfan spiked in the medium inoculated by *A. flos-aquae* after 8 days of incubation. Therefore, the endosulfan fate was dependent on the species. Within 1 day of incubation, two *Anabaena* species produced low amounts of β-endosulfan after application of α-endosulfan. These results suggest the presence of isomerase in the *Anabaena* species. Further studies using a fermentor to control the medium pH at 7.2 to minimize chemical hydrolysis of endosulfan revealed a major production of endodiol with minor amounts of endosulfan sulfate and the unknown compound. These results showed that the production of the unknown compound might be dependent on the alkaline pH in the medium and that the production of endodiol by *A. flos-aquae* might be biologically controlled. This study showed that two algal species could contribute in the detoxification pathways of endosulfan in the soil environment.

KEYWORDS: Endosulfan; endosulfan diol; endosulfan sulfate; *Anabaena flos-aquae*; *Anabaena* sp. PCC 7120; fermentor

INTRODUCTION

Even though significant increases in agricultural productivity have resulted from the control of agricultural pests with synthetic chemical pesticides (*1*), there is widespread concern about the presence of pesticide residues in food and the environment. Endosulfan (1,2,5,6,7,7-hexachloro-5-norbornene-2,3-dimethanol cyclic sulfite) is an organochlorine insecticide mainly used to control *Helicoverpa* species in the upland soil in Korea. Fish are very susceptible to endosulfan toxicity at a level of 1−20 ng/L. Several intensive studies on the degradation of endosulfan in soil or water environments have been conducted (*2*−*10*). There are two principal mechanisms of endosulfan degradation due to the oxidation or hydrolysis caused by chemical or biological systems. Endosulfan sulfate and endosulfan diol (endodiol) are known to be produced by oxidation and hydrolysis, respectively. Endodiol is a nontoxic metabolite to fish and other organisms; thus, hydrolysis producing endodiol may be an important detoxification pathway of endosulfan. However, endosulfan sulfate has a similar toxicity compared to the parent compound endosulfan. In addition, endosulfan sulfate has a much longer tolerance in the soil environment in comparison to endosulfan (*11*). Therefore, the production of endosulfan sulfate seems to cause long persistence of endosulfan in soil. Endosulfan sulfate is produced by several microorganisms including *Phanerochaete chrysosporium* (*2*, *3*), *Mucor thermohyalospora* MTCC 1384 (*4*), and *Trichoderma harzianum* (*5*). However, the last two fungi produce endodiol as a major endosulfan metabolite in culture medium. Martens (*6*) reported the degradation of endosulfan to endodiol as a primary metabolite followed by endosulfan sulfate in flooded soil, when incubated with several fungi species. Guerin and Kennedy (*7*) and Guerin (*8*) detected the formation of endodiol when endosulfan was incubated with bacteria under anaerobic conditions. In addition, Miles and Moy (*9*) studied extensive

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degradation of endosulfan in an aqueous nutrient medium inoculated with mixed soil microorganisms and identified the degradation pathway of endosulfan including the formation of different metabolites such as endosulfan sulfate, endodiol, endosulfan ether, endosulfan hydroxyether, and endosulfan lactone. Recently, Sutherland et al. (*10*) have found a tentative metabolite endosulfan monoaldehyde as a hydrolysis metabolite and endosulfan sulfate in a strongly buffered culture medium (pH 6.6) to give a minimum chemical hydrolysis.

Cyanobacteria are free living, photoautotrophic microorganisms that have shown their capabilities to degrade both naturally occurring compounds and synthetic chemicals, especially pesticides (*12*−*14*). Therefore, cyanobacteria have been considered to be potent alternative organisms for chemical and physical treatments to transform environmentally persistent, toxic materials. For example, three blue-green algae, *Synechococcus elongates*, *Nostoc linckia*, and *Phormidium tenue*, strongly participate in the degradation of monocrotophos and quinalphos in soil (*14*). Here, we report the biotransformation of endosulfan by two blue-green algal species, *Anabaena* sp. PCC 7120 and *Anabaena flos-aquae*, in a culture medium and the metabolites. We also postulate the role of the two *Anabaena* species in the soil environment to dissipate endosulfan.

EXPERIMENTAL PROCEDURES

Microorganisms. *Anabaena* sp. PCC 7120 and *A. flos-aquae* were obtained from American Type Culture Collection (ATCC) and Korea Research Institute of Bioscience and Biotechnology, respectively. *Anabaena* species were grown in Allen's liquid medium without nitrate on a shaker at room temperature in a light intensity of ∼1700 lx by fluorescent lamps with a 12 h/12 h (light/dark) cycle. Their growth was monitored by measurement of chlorophyll *a* content (*15*). All operations were carried out under sterile conditions in order to avoid bacterial contamination.

Chemicals. α-Endosulfan, β-endosulfan, endosulfan sulfate, endodiol, endosulfan ether, and endosulfan hydroxyether were purchased from Chem Service Inc. (West Chester, PA). $NaNO_3$, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, ferric citrate, and ethylenediaminetetraacetate (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals used were of the highest grade commercially available.

Determination of Endosulfan Degradation and Metabolite Production. Experiments were carried out in batch cultures. One hundred milliliters of Allen's medium in 250 flasks stoppered with cotton plugs was inoculated with each *Anabaena* species. Four days later, α-endosulfan was supplemented to the inoculated medium to give a final concentration of 10 μg/mL. An uninoculated culture medium served as a control. All of the flasks were sealed and incubated on a shaker at room temperature in a light intensity of ∼1700 lx by fluorescent lamps with a 12 h/12 h (light/dark) cycle. Algal growth was monitored by measuring chlorophyll *a* content (*15*). Degradation was assessed by measurement of endosulfan in triplicate flasks. Sampling was done at various periods of incubation and analyzed by gas chromatography with an electron capture detector (GC-ECD). For a pH-controlled experiment, all procedures were conducted the same as above. However, 1 L of Allen's medium in a Bioneer fermentor (Bioneer Co., Seoul, Korea) was inoculated by *A. flos-aquae*. Sampling (10 mL) was done at various periods of incubation. The pH value of the fermentor was set to 7.2.

The remaining endosulfan and its metabolites were extracted from 5 mL of the crushed bacterial suspension by a glass homogenizer with an equal volume of nanograde hexane by vortexing for 30 s twice. The organic layer was collected in a vial and dried with nitrogen gas. Two milliliters of hexane was added to the dried sample, and a 2 μL volume of each hexane extract was subjected to GC-ECD analysis in a Varian Star 3400 CX with an electron capture detector on a DE-5 fused silica capillary column (30 m × 0.32 mm i.d. with 0.25-mm film coating) in a linear temperature gradient from 110 to 190 °C over 8.5 min. Chromatographic patterns were analyzed with Turbochem software (Seoul, Korea).

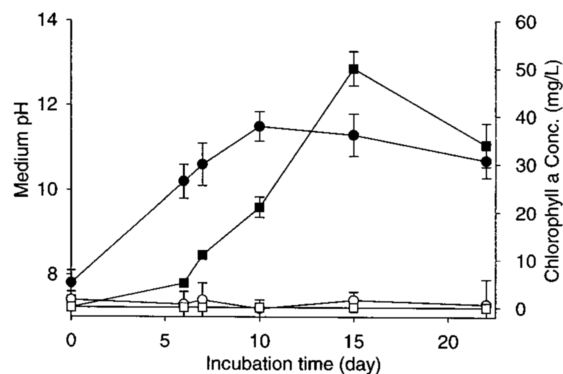


Figure 1. Growth of *A. flos-aquae* and change of the medium pH.

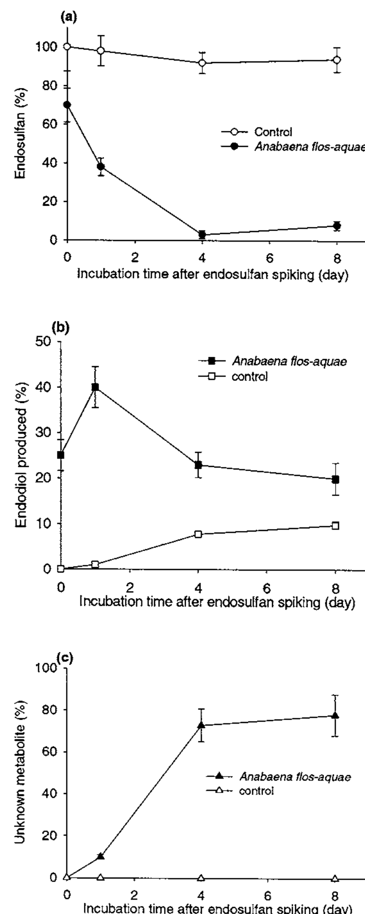


Figure 2. Endosulfan degradation after spiking in the medium inoculated with *A. flos-aquae*: (a) endosulfan remaining (%); (b) production of endodiol; (c) production of unknown compound.

RESULTS AND DISCUSSION

The growth of *A. flos-aquae* continued for up to 15 days after inoculation, and then the growth ceased (**Figure 1**). The increase of pH in the medium was found until 10 days after inoculation with *A. flos-aquae*. Chlorophyll *a* content and pH in the *A. flos-aquae* inoculated medium was up to about 50 mg/L and 12.5, respectively. Endosulfan spiked after 7 days of inoculation. However, there were no changes in either chlorophyll *a* content or pH in the control medium (**Figure 1**). Endosulfan concentration decreased immediately after spiking into the medium, declining after 4 days of incubation to <10%, as shown in **Figure 2a**. Endosulfan spiked in the control was not dissipated during this time. The major metabolite detected was endodiol (**Figure 2b**), and endosulfan sulfate and β-endosulfan were also

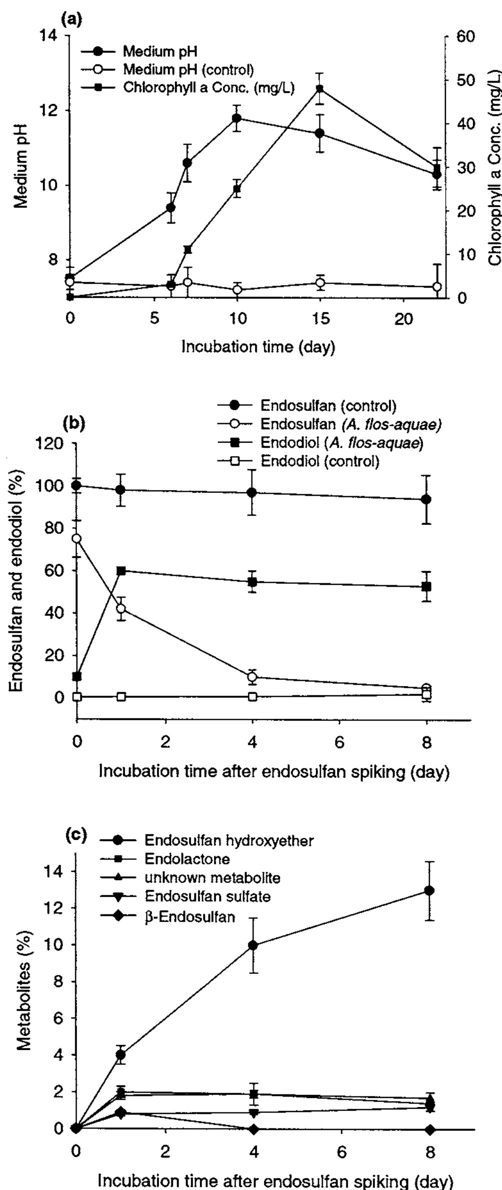


Figure 3. Endosulfan degradation after spiking in the medium inoculated with *Anabaena* sp. PCC 7120: (a) algal growth in the medium; (b) degradation of endosulfan spiked and the production of endodiol; (c) production of metabolites.

found in trace amounts. The endodiol produced disappeared gradually, with 20% of the initial endosulfan remaining as endodiol after 8 days from spiking. Interestingly, an unknown compound was detected that increased with incubation time as the basis of the detected peak area in GC chromatogram as shown in **Figure 3c**. However, there is no indication of what proportion of the total endosulfan became the unknown peak. On the other hand, *Anabaena* sp. PCC 7120 produced endodiol and endosulfan hydroxyether as the principal metabolites and endosulfan sulfate and β-endosulfan as minor metabolites (**Figure 3b**,**c**). *Anabaena* sp. PCC7120 also produced the unknown compound. However, the amount of the compound produced was insignificant.

Cyanobacteria can degrade both naturally occurring aromatic hydrocarbons and man-made xenobiotics. Cerniglia et al. (*16*) tested the *Oscillatoria* sp. strain JCM for naphthalene metabolism and showed formation of 1-naphthol from naphthalene. Four species of cyanobacteria, such as *N. linkia*, *N. muscorum*, *Oscillatoria animalis*, and *Phormidium foveolarum*, can degrade parathion-methyl to 4-nitrophenol in the medium. *Anabaena* sp. PCC 7120 possesses dechlorination activity of lindane (*17*, *18*), leading to the formation of 2,3,4,5,6-pentachloro-1-cyclohexene and 1,2,3- and 1,2,4-trichlorobenzene. *Anabaena* sp. can transform 2,4,6-trinitrotoluene (TNT) to azoxytetranitrotoluene and hydroxyaminodinitrotoluene (*19*). However, several studies have shown some impact of pesticides on the growth of cyanobacteria. Mohapatra and Mohanty (*20*) demonstrated that dimethoate and endosulfan inhibited growth and decreased survivability of *Anabaena doliolum*. Atrazine and hexazinone also inhibited the growth of *Anabaena flos-aquae* and *Selenastrum capricornutum* (*21*). Therefore, the impact of pesticides on the growth of *Anabaena* species can reduce the removal or dissipation of pesticides in soil.

Our results show evidence of two metabolic pathways of endosulfan by two different *Anabaena* species. Two *Anabaena* species produced endodiol as a primary product and a trace amount of endosulfan sulfate. Endodiol is a nontoxic compound; thus, we believe this is a hydrolysis pathway for a detoxification of endosulfan in the soil environment. However, the question arises how they produced endodiol. As we showed, because there is an increase of pH in the medium, chemical hydrolysis might influence the rate of endodiol production. None of the enzymes has been reported for endosulfan hydrolysis. One interesting study has been done investigating the biological hydrolysis of endosulfan in pure culture medium (*10*). The authors suggested the use of a strongly buffered culture medium (pH 6.6) to minimize chemical hydrolysis of endosulfan. In addition, the medium included the detergent Tween 80 for increasing the amount of endosulfan in contact with mixed bacteria. No bacterial growth was detected in the control cultures in the absence of endosulfan as a carbon source.

In the presence of endosulfan, growth of a mixed culture of bacteria occurred concomitantly with endosulfan decrease (*10*). Endosulfan was subjected to degradation by oxidation and hydrolysis. Conclusively, endosulfan sulfate formation was found to be favored as oxidative production, and a novel hydrolysis product tentatively identified as endosulfan monoaldehyde was found. From these findings, the two factors of pH and contact seem to be very important for enzymic endosulfan degradation by microorganisms. There was one more distinct factor remaining as oxygen because the biological oxidation reaction needs a supply of oxygen.

Therefore, we assume that the endodiol production in our study might result from chemical hydrolysis due to the increase of pH in the medium alone. However, we could not exclude the participation of biological hydrolysis because the production of endosulfan sulfate and endosulfan hydroxyether, as shown in **Figures 2** and **3**, could involve biological oxidation in both *Anabaena* species. To understand the mechanism of production of endodiol in the medium, we used a fermentor to prevent the increase of the medium pH and to supply oxygen in the medium constantly to protect against oxygen shortages. These conditions may enhance the biological oxidation of endosulfan in the medium.

A. flos-aquae in the pH-controlled experiment using a fermentor produced mainly endodiol (**Figure 4**). Chlorophyll *a* content was increased in the *A. flos-aquae* inoculated medium and was used to monitor growth, but no growth occurred in the control (**Figure 4a**). The spiked endosulfan dramatically declined after 2 days of incubation, with 20% of the endosulfan remaining after 7 days of incubation. After 4 days of incubation, the endosulfan spike gradually disappeared and reached 50% in control medium (**Figure 4b**). Endodiol was produced and

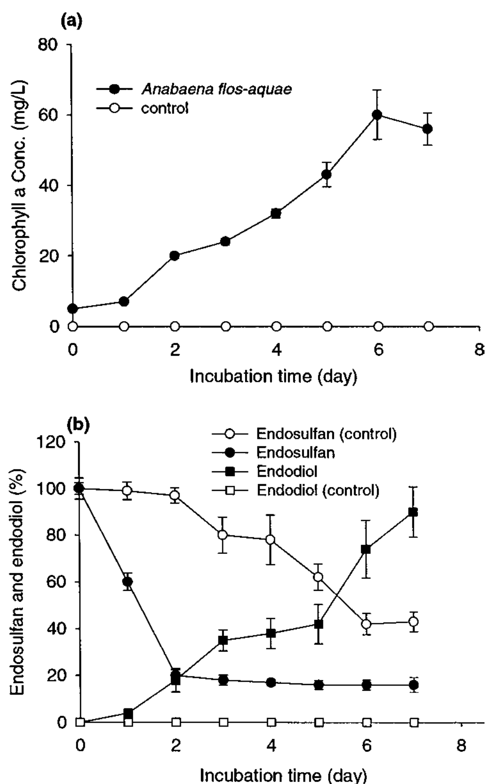


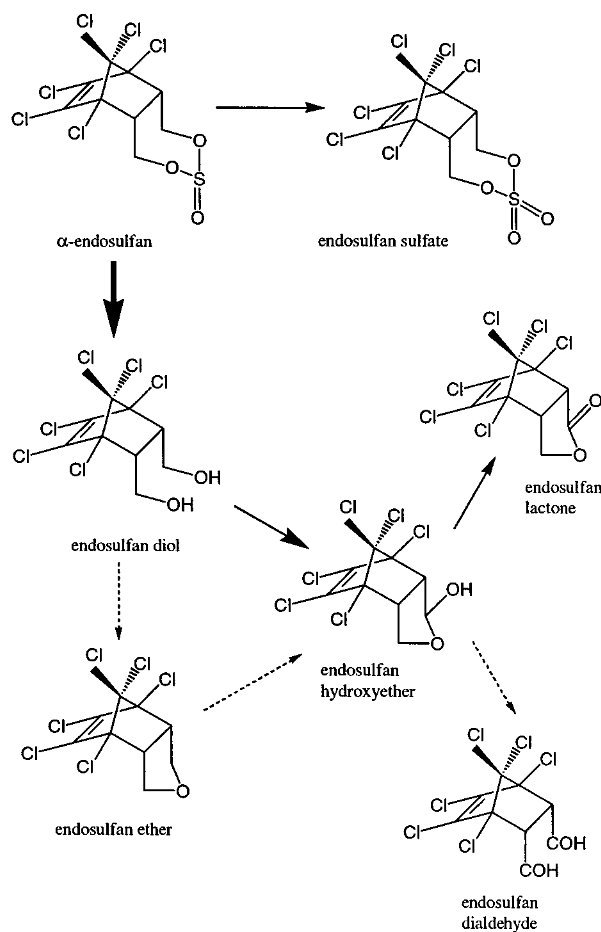
Figure 4. Endosulfan degradation after spiking in a medium inoculated with *A. flos-aquae* using a fermentor controlling the pH at 7.2: (a) algal growth in the medium; (b) endosulfan remaining (%) and the production of endodiol (%).

endosulfan sulfate was detected in trace amounts. In this experiment, only a small amount of the unknown compound was determined, <1%, as the basis of the peak area expressed in the GC chromatogram. Even when the pH in the medium was controlled, *A. flos-aquae* produced abundant endodiol. With these findings, there may be at least two mechanisms to produce endodiol.

There are several more considerable factors for microorganisms living in soil. Photolysis may be another factor. Therefore, endodiol production can be significantly influenced by pH and light. As many studies have suggested, cyanobacteria including *Anabaena* species are present in soil. In agricultural soils, high water potential is determined as the presence of salt ions. This strong water potential may contribute to or enhance biotransformation of pesticides by soil microorganisms. Han and New (*22*) suggested maximal removal of 2,4-dichlorophenoxyacetic acid (2,4-D) by soil organisms occurring at the highest water potential (ψ) of -0.1 MPa, and degradation decreased progressively down to $\psi = -5.5$ MPa with no breakdown at $\psi = -22$ MPa. Awasthi et al. (*23*) also identified moisture content as one of the most influential factors in endosulfan degradation. They demonstrated pH, concentration of endosulfan, and size of inoculum to be the principal factors in endosulfan degradation.

In conclusion, in two different experiments we have shown that endosulfan transformation by two *Anabaena* species occurs by oxidation and hydrolysis reactions. In addition, both *Anabaena* species participate in the detoxification process of endosulfan in soil to produce endodiol as a nontoxic metabolite. We have proposed a possible biotranformation pathway of endosulfan by *Anabaena* species, as shown in **Scheme 1**. Further studies are needed to evaluate the impact of water potential by salt ions present in agricultural soils on endosulfan biotransformation by *Anabaena* species.

Scheme 1 Proposed Endosulfan Biotransformation Pathway by *Anabaena* Species



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