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# Interpretation of Ultrastructural Changes in *Cladophora glomerata* Resulting from Hyamine Toxicity

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Electron microscopy has been seldom used in algal assays even though scientists have demonstrated that fine structural changes at the subcellular level are convincing evidence of the toxicity of chemicals (Dodge 1973).

There are a number of conventional algal assays used for toxicity studies, for instance, the $^{14}C$ technique and bottle tests (Miller et al. 1978), except that they are designed mostly for unicellular algal species as opposed to filamentous species.

Filamentous algae, and Cladophora in particular, are difficult to handle in assays because of their acropetal mode of branching. Not only is the log-growth phase in the pre-culture difficult to determine but it is difficult to obtain a uniform inoculum in terms of biomass, filament age, branch(let) maturity and cell sensitivity. Besides, the filaments self shade. Even in productivity studies, we experienced considerable variability between duplicate samples (Wong et al. 1979).

Cladophora glomerata grows profusely on rocky substrates, and is a nuisance in many of the Great Lakes' waterways (Wolfe and Sweeney 1982). It predominates over other attached algae and macrophytes in the growing season (Wong et al. 1978). Because of its low nutrient requirements, Cladophora flourishes at its maxiumum rate of growth even under conditions of relatively low nutrient supply (Wong and Clark 1976). For many years, much effort has been devoted to controlling excessive growth of Cladophora in the Great Lakes (International Joint Committee 1975).

Palmer (1956), and Maloney and Palmer (1956), in their study of chemical algicides, reported on the use of ammonium quaternary compounds to inhibit growth of

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Stigeoclonium (another filamentous green alga). The aims of this study are, first, to assess the possible use of electron microscopy as an assay technique for attached algae, and second, to determine the effectiveness of an ammonium quaternary compound (Hyamine) in inhibiting the growth of Cladophora glomerata.

## MATERIALS AND METHODS

The test filamentous species, Cladophora glomerata (L.) Kütz. (UTCC #13) was obtained from the University of Toronto Culture Collection (UTCC). The alga was originally isolated from the Thames River, Ontario. The algal culture was maintained in Bold's Basal Medium (BBM) (Hutchinson and Stokes 1975) supplemented with a vitamin mix ($B_{12}$, biotin and thiamine), and 5 mg/L Si as $Na_2SiO_3 \cdot 9H_2O$.

Chlorella fusca Shihira et Krauss, a unicellular alga, obtained from the laboratory at the Canada Centre for Inland Waters, Burlington, Ontario, was the other species used in the tests.

Hyamine 3500, was purchased from the Rohm and Haas Company. This algicide is an 80% concentrate in ethanol, containing the active ingredient n-alkyl (80% tetradecyl, 40% dodecyl and 10% hexadecyl) dimethyl benzyl ammonium chloride. The molecular weight of the chemical is 359.6. Stable and nonvolatile, it has been recommended by the company (Rohm and Haas 1978) as a disinfectant in swimming pools at low concentrations.

Algal assays were conducted in duplicate in 250-mL Erlenmeyer flasks contanining 100 mL BBM solution. The toxicity range of Hyamine for Cladophora was tested at the beginning of the study. In the final assays, the Hyamine concentrations were set at 0.2, 0.3, 0.4, 0.5, 0.8 and 1.0 mg/L. Zero Hyamine concentration was used as a control. About 0.5 g (wet weight) of Cladophora filament was used as the inoculum. The cultures were incubated for 4 d at 20° C in an environmental chamber where the light and dark cycle was set at 12:12 and the light intensity was 60 $\mu$Ein/$m^2$/sec.

On day 4, a small amount of plant material was removed from each concentration for morphological examination using a light microscope. One set of samples was put aside for electron microscopic preparation; the other set was incubated for another 3 wk for further morphological evaluations. These tests were repeated at the end of the experiment.

For electron microscope studies, Cladophora filaments were fixed in 4% (cacodylate-buffered) glutaraldehyde for

6 hr and then postfixed in 2% (cacodylate-buffered) osmium tetroxide for 1 hr. The dehydration ethanol series was 30%, 50%, 70%, 90%, 95% and 100%.

For infiltration, the Spurr's medium (Hayat 1989) to ethanol ratios were 1:3, 1:2, and 2:3 v/v. The Cladophora filaments were then embedded in 100% Spurr's medium in an aluminum dish. The samples were polymerized at 70° C overnight.

A dissecting microscope was used to locate the apical cells on the plastic. After they were marked, small columns of specimens were cut out with a jeweller's saw. Thin sections were prepared with a glass knife on a microtome. These sections were then stained with uranyl acetate and lead citrate. Observations were made using a Siemens (ELMISKOP 102, Montréal, Quebec) electron microscope.

To assess the effect of Hyamine on Chlorella cells, a conventional method based on growth measurements was used (Miller et al. 1978). A Chlorella pre-culture was prepared. The Hyamine concentrations used were the same as those employed in the Cladophora assays. On day 7, when the pre-culture reached its logarithmic growth phase, the Chlorella cells were ready for inoculation. The initial cell concentration in the test solutions was $40 \times 10^3$ cells/L. After inoculation, the culture flasks were mounted on a platform shaker and incubated for 14 d in a growth chamber. Cells numbers were enumerated at 4-d intervals and at the end of the experiment when the algal biomass reached its growth plateau.

## RESULTS AND DISCUSSION

Ammonium quaternary compounds have been shown to be more toxic to Stigeoclonium, a filamentous alga, than to Pimephales promelas (fathead minnow) (Maloney and Palmer 1956). In our study, we found that Hyamine at sublethal concentrations was more toxic to Cladophora, a filamentous alga, than to Chlorella, a unicellular green alga (Table 1).

Although the tests used two different approaches, the results were found to be consistent. With Chlorella cells, the biomass dropped at 0.60 mg/L Hyamine, and with Cladophora filaments, thylakoid membrane disruption in the apical cells was observed at 0.50 mg/L. The lethal dose of Hyamine for both test species was 0.80 mg/L.

Cladophora filaments propagate through apical cell division. The newly formed apical cells were much narrower and smaller than the branch cells. Measured at 350 μm long and 28 μm wide, they averaged about one-half

Table 1. Toxicities of Hyamine on Chlorella fusca (unicellular alga) estimated from the cell count technique, and on Cladophora (filementous alga) estimated by ultrastructural changes.

| Hyamine conc. (mg/L) | Chlorella fusca avg. yield ($10^5$ cells/mL) after 14 days | Cladophora glomerata visible changes after 4 days |
|---|---|---|
| control | 14.5 | --- |
| 0.15 | 13.6 | (no observation) |
| 0.20 | 15.3 | no visible changes |
| 0.30 | 13.9 | no visible changes |
| 0.40 | no data | loss of starch grains |
| 0.50 | 14.6 | no starch; thylakoid bands broken into small bundles; many pyrenoids appear in pairs |
| 0.60 | 10.8 | (no observation) |
| 0.80 | no growth | no starch; fused chloroplasts, many with vesicles in stroma; thylakoid membrane disintegrating; pyrenoids in clusters of 4; mitochondria small - some contain an empty matrix, others concentric cristae |
| 1.00 | no growth | decomposed cells |

the width and one-fifth the length of the mature cells in the main filaments (Fig. 1a).

In cross-section, the greater part of the cell lumen was occupied by a central vacuole (Fig. 1b). Surrounding this central vacuole was a thin layer of cytoplasm where large numbers of chloroplasts were found. In the middle of each mature chloroplast was a starch grain with bands of thylakoid membranes filling the stroma (Fig. 1c). Internal to the chloroplasts in the cytoplasm were the nuclei. Between chloroplasts, pyrenoids were found, and in between thylakoid membranes were the osmiophilic bodies (chloroplast lipids).

When Cladophora filaments were exposed to low Hyamine concentrations, the chloroplasts appeared to be the prime target (antiplastidial). At 0.50 mg/L, after a 4-d incubation, the thylakoid bands in many of the

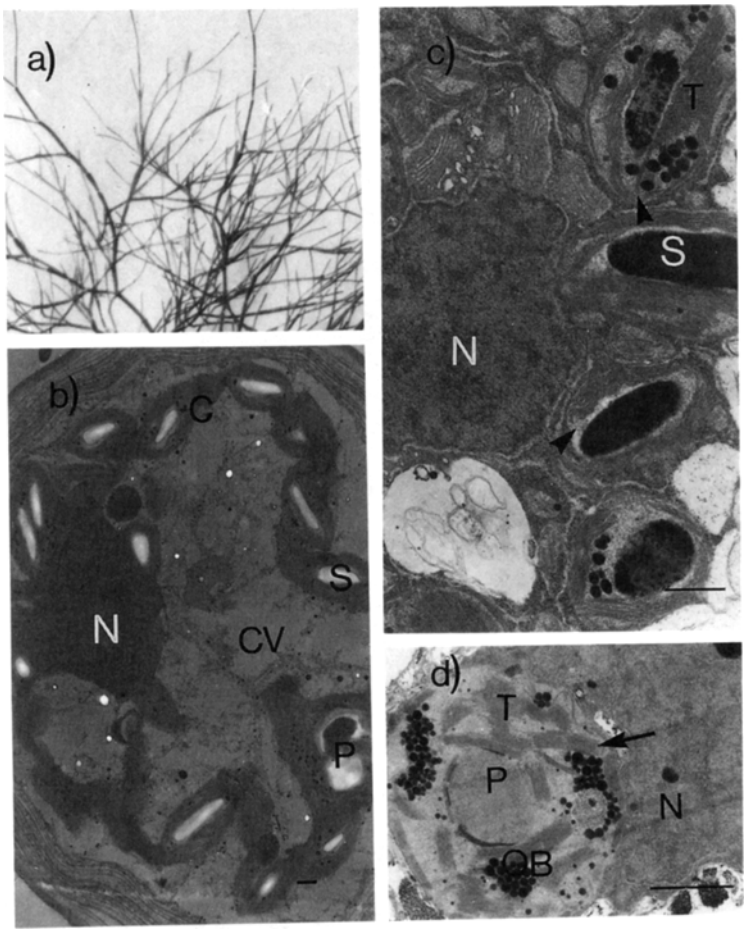


Figure 1a). Light micrograph of Cladophora glomerata.

Figure 1b). Cross-section of a control apical Cladophora cell. (C)= chloroplast; (N)= nucleus; (P)= pyrenoid; (S)= starch; (CV)= central vacuole. Bar= 1 $\mu$m.

Figure 1c). An enlarged portion of a control apical cell. Each chloroplast contains a large starch grain (S) with thylakoid membranes (T) terminating close to the chloroplast envelope (pointers). Bar=0.5$\mu$m.

Figure 1d). In exposure to 0.5 mg/L Hyamine, many chloroplasts in apical cells reveal broken bundles of thylakoid membranes (arrow). (OB)= osmiophilic bodies. Bar= 1 $\mu$m.

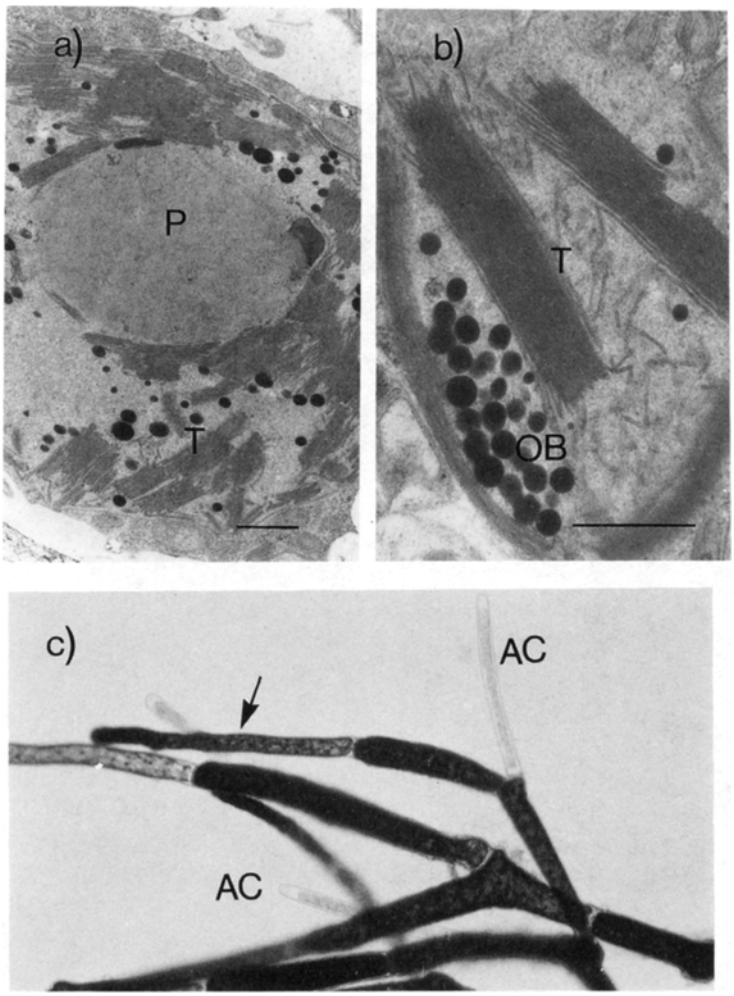


Figure 2a). The pyrenoid of a 0.5 mg/L Hyamine treated cell. Bar = 0.5 $\mu$m.
Figure 2b). Fragmentation of thylakoid membranes in a 0.5 mg/L Hyamine solution. Bar = 0.5 $\mu$m.
Figure 2c). Light micrograph of Cladophora glomerata after exposure to 0.5 mg/L Hyamine for 25 days. The discoloured cells are dead apical cells (AC) and the crooked one (arrow) is a surviving apical cell.

chloroplasts appeared wavy while some bands were broken into short bundles (Fig. 1d). Broken thylakoid bundles were also seen in the stroma surrounding the pyrenoids (Fig. 2a). At high magnifications, these detached membranes were observed as small fragments (Fig. 2b).

With so many non-functional chloroplasts, large numbers of apical cells became pale and lost the ability to divide. Thus, by halting apical cell division, Hyamine at low concentration generated an algistatic effect on Cladophora filaments (i.e. a temporary suppression of growth). Many apical cells recovered after 25 days of incubation (Fig. 2c).

Most herbicides act primarily on membrane systems such as the Golgi complex (Orr and Hess 1982). The Golgi complex is vital because it serves not only as the electron transport system but as the site where polysaccharides are synthesized. Hence, whenever there is a disruption in this system, there will be dilation of the endoplasmic reticulum, production of large number of vesicles and the development of abnormal cytoplasmic membranes (tonoplasts) in the central vacuole of the apical cells (unpublished data).

Unlike most other organic compounds, for example, algimycin or di- and tri-chloromethane, Hyamine is a cationic surfactant which induces no harmful effects to Cladophora cells below 0.50 mg/L. It is only when its concentration exceeds that of the critical micelle concentration that it enhances solubilization in cell membranes by forming protein-lipid-surfactant mixed micelles (Capaldi 1977).

One reason that Cladophora glomerata was able to survive low level Hyamine toxicities was because its reticulate chloroplast is composed of many individual chloroplasts - a winter form of C. glomerata has been shown to contain only one large chloroplast in the cells (Wong and Wainwright, in press). Apparently with this summer form Cladophora, some mature chloroplasts were more tolerant to low toxic stress.

In summary, electron microscopy is a much more reliable tool for assays with filamentous algae than more conventional methods such as cell harvesting, and Hyamine is an effective chemical for inhibiting apical cell division in Cladophora filaments at low concentrations. So, if the objective of algicide application is to halt Cladophora growth at its peak and allow less competitive algal species to thrive in the reconstruction of a balanced biotic community (Fitzgerald 1971), then the selection of Hyamine quaternary ammonium compounds may be worth considering.

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