

Uptake of Diquat by Four Aero-Aquatic Hyphomycetes

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ABSTRACT

Cultures of four pond-dwelling fungi were challenged with various levels of the aquatic herbicide, diquat dibromide. Three of the fungi absorbed diquat and their growth was significantly inhibited. The fourth did not absorb diquat and was not inhibited by its presence in concentrations of up to 10 $\mu\text{g ml}^{-1}$.

INTRODUCTION

Most studies of the effects of xenobiotics on aquatic systems have been concerned with fish, macrophytes or plankton. The dependence of the higher trophic levels in shaded ponds and streams on the fungi colonising leaf-litter is now well known. Yet, as far as we are aware, no-one has studied the effects of xenobiotics on the fungi concerned, or the consequences for organisms which eat them. This paper documents such a study.

In recent years it has been established that there is a close relationship between the activities of conidial fungi colonising autumn-shed leaves in shaded streams and ponds and the feeding habits of detritivorous benthic aquatic invertebrates (Kaushik & Hynes, 1971; Baerlocher & Kendrick, 1973, 1981; Michaelides & Kendrick, 1982).

Those studies were concerned only with the components of natural systems and they revealed that, although the leaf material is the major energy source in the system, it is largely inaccessible to detritivores until it has been colonised and 'conditioned', mostly by fungi. Several

invertebrates feed preferentially on specialised hyphomycetous fungi which grow on, and in, submerged decomposing leaves. Since the leaves and their mycoflora represent the lowest trophic level, pollutants which are absorbed by either will probably affect organisms at the higher trophic levels.

This study tested the effects of the aquatic herbicide, diquat dibromide, on four aero-aquatic hyphomycetes from a pond in the Laurel Creek Conservation Area, Regional Municipality of Waterloo, Ontario, Canada. Subsequent effects on non-target organisms consuming these fungi were also investigated.

The pond is primarily a heterotrophic autumnal pool surrounded almost exclusively by deciduous trees. The maximum area of the basin at its highest water level is 578 m². Due to its heavily shaded location and consequent absence of much primary productivity, the pond receives its major supply of energy in the form of leaf-litter. The species of trees around the pond are: *Acer saccharum* (85% of the total), *Fagus grandifolia*, *Fraxinus americana* and *Tsuga canadensis*. *Acer saccharum* contributes most of the leaf litter (approximately 74%, according to Michaelides, 1982).

MATERIALS AND METHODS

The four fungi used belong to the aero-aquatic hyphomycetes, a specialised pond-dwelling ecological group. These fungi produce what are called 'bubble-trap propagules' which entrap air and float. The unusual development and ecological significance of these propagules are discussed by Michaelides & Kendrick (1982). The four species chosen were *Helicoon elegans* (Corda) Arnaud, *Pseudoaegerita matsushimae* (Matsushima) Webster, *Hormiactis ontariensis* Matsushima and *Beverwykella pulmonaria* (van Beverwijk) Tubaki.

Experiment 1: Effects of diquat dibromide on the mycelial growth of four aero-aquatic hyphomycetes

The fungi were grown on Potato Dextrose Agar (PDA) plates for 14 days. Four millimetre plugs were then removed from the periphery of colonies and used as inocula for 1-litre flasks containing 100 ml of pond water, 100 mg litre⁻¹ chloramphenicol (to suppress bacterial growth), and diquat

at concentrations of 0 (control), 0.2, 0.5, 1.0, 5.0 and 10.0 $\mu\text{g ml}^{-1}$. Four replicates were used in each treatment. The fungal cultures were incubated for 20 days in darkness, at 18°C, then the mycelia were harvested and dried at 45°C for 24 h.

Experiment 2: Extraction of diquat from fungi

The fungi were grown on PDA plates for 14 days. Four millimetre plugs were removed from the periphery of colonies and used as inocula for 1-litre flasks containing 100 ml of Modified Yeast Extract Glucose Broth (MYEGB), 100 mg litre⁻¹ chloramphenicol and diquat at concentrations of 0 (control), 0.2, 0.5, 1.0 and 10.0 $\mu\text{g ml}^{-1}$. Four replicates were used in each treatment. The fungi were incubated in the medium for 20 days in darkness at 18°C. Then the mycelium in each flask was recovered on a Whatman No. 1 filter paper and rinsed with distilled water to remove any unbound surface herbicide. It was then placed in a 50 ml cylinder with 1 ml of distilled water, macerated in a sonicator for 3.5 min to ensure that almost all cells were broken and then made up to 25 ml with distilled water.

A hot plate with a metal block (31 cm × 31 cm × 15 cm) containing 100 orifices, into which large test-tubes were fitted, served as a refluxing apparatus (Fig. 1). The emulsion was placed in a test-tube and 2.5 ml 18N sulphuric acid and 0.1 ml 2-octanol were added. The test-tube was placed in one of the holes in the metal block and a small glass funnel with a glass bubble inside was inserted in the top of the test-tube. The mixture was refluxed for 6 h at 150°C. The refluxed material was filtered through celite 545 and neutralised with 10N NaOH, transferred to a separating funnel and allowed to percolate slowly through a cation exchange column. The elution was done according to the method of Calderbank & Yuen (1966). The eluate was reduced with a mixture of sodium dithionite and sodium metabisulphite in 0.3N NaOH. The optical density was measured at 379 nm on a SP8-100 spectrophotometer at a wavelength scanning speed of 1 nm s⁻¹ and a chart speed of 5 s cm⁻¹.

Extractions from fungal tissue 'spiked' with diquat were performed in order to establish whether or not it was possible to extract the herbicide from this type of tissue. We are aware of the fact that the pesticide was added to prepared material and was not taken up naturally by living material.

However, 'spiking' is an accepted method for obtaining this type of

information. Standards were made and their absorbance was read at the same wavelength. The decay of the diquat cation reduced by sodium dithionite and sodium metabisulphite was observed for 15 min: no significant change was noted in the peak. A standard curve was produced showing the optical densities of the standards, with known diquat concentrations in $\mu\text{g ml}^{-1}$.

For each treatment, the same number of replicates were prepared for an experiment designed to establish the amount of diquat that adsorbs to

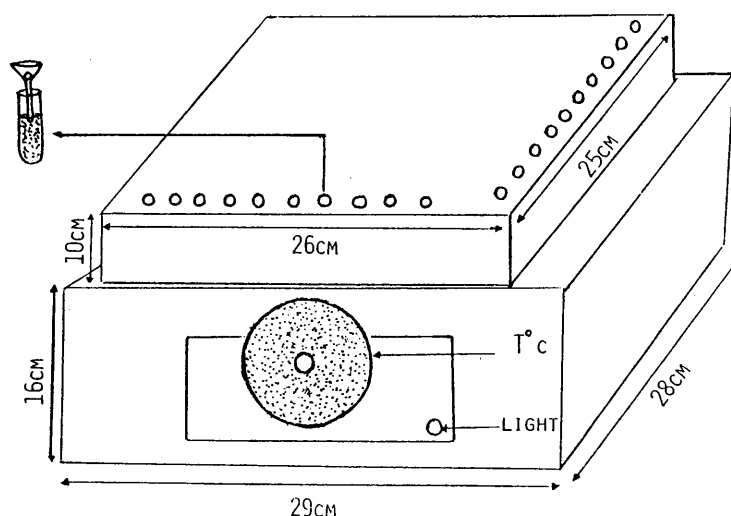


Fig. 1. Apparatus used in the hydrolysis step: consisting of a hot plate with a metal top containing 100 orifices, into which test-tubes are placed. The contents of the test-tubes are refluxed for 6 h at 150 °C.

the glassware used in the experiments. Yeast extract glucose broth amended with diquat was placed in 1-litre glass flasks and kept in the dark for 20 days at 15 °C. After 20 days, the diquat was extracted from the medium by the method of Calderbank & Yuen (1966). The optical density of each extract was read at 379 nm and the per cent loss of diquat to the glass walls of the containers was calculated. The experiment was repeated in order to find out whether or not shaking the flasks prior to the extraction had any effect on the amounts adsorbed onto the glass. The loss of diquat to the glass of the container was taken into account in each subsequent experiment.

RESULTS

Experiment 1: Effects of diquat dibromide on the mycelial growth of four aero-aquatic hyphomycetes

Helicoon elegans produced the most biomass in the control flasks and *Pseudoaegerita matsushimae* the least. All four aero-aquatic hyphomycetes were affected negatively by most additions of diquat to their growth media. At a diquat concentration of $0.2 \mu\text{g ml}^{-1}$, the growth of *P. matsushimae* was not significantly affected, but the other three fungi showed some inhibition, *Hormiactis ontariensis* being most severely affected. *H. elegans* and *Beverwykella pulmonaria* showed increasing inhibition at the higher concentrations of diquat. *P. matsushimae*,

TABLE 1
Student-Newman Keuls Table of Mycelial Growth in Pond Water
Containing Different Diquat Concentrations

<i>Fungus</i>	<i>Mean weight (g)</i>
<i>Pseudoaegerita matsushimae</i>	0.101 14 ^{a*}
<i>Beverwykella pulmonaria</i>	0.081 33 ^{a,b}
<i>Helicoon elegans</i>	0.059 74 ^b
<i>Hormiactis ontariensis</i>	0.019 69 ^c

* Values with different superscripts are significantly different.

however, was much less severely affected by higher concentrations of diquat than the other three fungi studied (Fig. 2).

Statistical analysis showed that, in all cases but one, there was a negative correlation between concentration and growth, that increasing diquat concentration had a strong inhibitory effect on fungal growth and that there was a correlation between the fungus used and growth at certain concentrations. Significant differences in growth were observed between the four aero-aquatic fungi (Table 1).

An equation which describes the growth patterns of the four fungi in the presence of the herbicides is as follows:

$$\log Y = A + B \log X$$

Log Y represents the logarithmic values of mycelial weight, log X represents the logarithm of the concentrations, A is the slope of the line and B is the value of the Y intercept.

The growths of *B. pulmonaria* (Fig. 3(a)) and *H. elegans* (Fig. 3(b)) are well described by an equation having a slope of -0.519 and a Y intercept

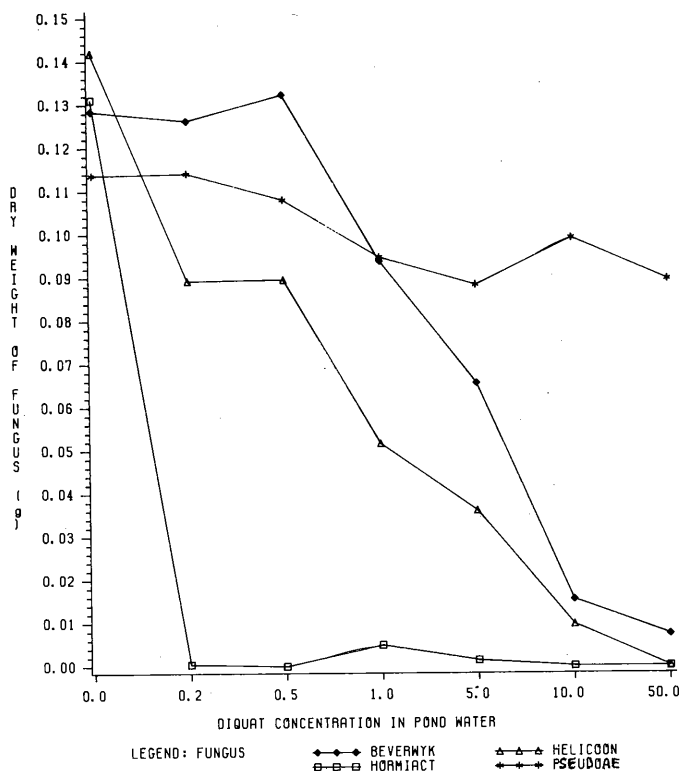


Fig. 2. Effects of diquat on mycelial growth. The diquat concentrations are expressed in $\mu\text{g ml}^{-1}$. *Pseudoaegerita matsushimae* is least inhibited by diquat, while *Hormiactis ontariensis* is inhibited most.

of -0.044 . The p value is 0.0001, meaning that there is a significant relationship between the log of the concentration and the dry weight of the fungus. *H. ontariensis* (Fig. 3(c)) was severely inhibited at all concentrations of diquat. *P. matsushimae* (Fig. 3(d)) was not strongly inhibited by diquat, so there was no significant relationship between the concentration and the dry weight of the fungus.

Experiment 2: Detection and extraction of diquat

A standard curve was produced showing the optical densities of the standards containing known amounts of diquat. All concentrations throughout the experiment were calculated by using the regression equation of the standard curve.

$$X = 7.2738 - 0.1293Y$$

The X values represent the diquat concentrations and the Y values represent the absorbance of the standards at 379 nm.

The adsorption of diquat onto the walls of the glassware used in the experiments was determined by comparing the amounts recovered from control flasks, immediately after addition of the pesticide, with the amounts recovered after 20 days.

Immediately after the addition of the pesticide, recovery ranged from 80% to 92%. After 20 days, recovery ranged from 62% to 79%. These sets of values are significantly different, indicating that there was a significant loss of diquat by adsorption to the glass.

When 20-day flasks were shaken for 5 min prior to the extraction of diquat from the media (Fig. 4), recovery ranged from 65% to 78%. These values are not significantly different from those obtained when the flasks were not shaken before extraction (Fig. 4(a)) and indicate that little or no diquat is released from the glass by shaking.

Extractions from fungal mycelia spiked with diquat gave recovery rates ranging from 80% to 89% (Fig. 4(b)). Diquat was also recovered from the mycelia of three of the four fungi grown in modified yeast extract glucose broth (MYEGB) containing the herbicide. For *B. pulmonaria* recovery was 42% to 75%; for *H. elegans*, 44% to 75%; for *H. ontariensis*, 34% to 81%, depending on the concentration of diquat present in the medium. No diquat was recovered from mycelia of *P. matsushimae* grown in four of the five concentrations and only 2% was recovered when the fungus was grown in media containing $5.0 \mu\text{g ml}^{-1}$ diquat (Fig. 4(c)).

Extractions from the media in which the fungi had grown showed that the amounts of diquat remaining in flasks in which *P. matsushimae* had grown were by far the highest, recovery ranging from 66% to 80% of the original amount (Fig. 4(d)). These values are significantly different from those for the media in which the other three fungi had grown. The media in which *B. pulmonaria* had grown contained only 20–30% of the original levels of diquat; those with *H. elegans*, 19–34%; and those with *H. ontariensis*, 17–34% of the original amounts of diquat.

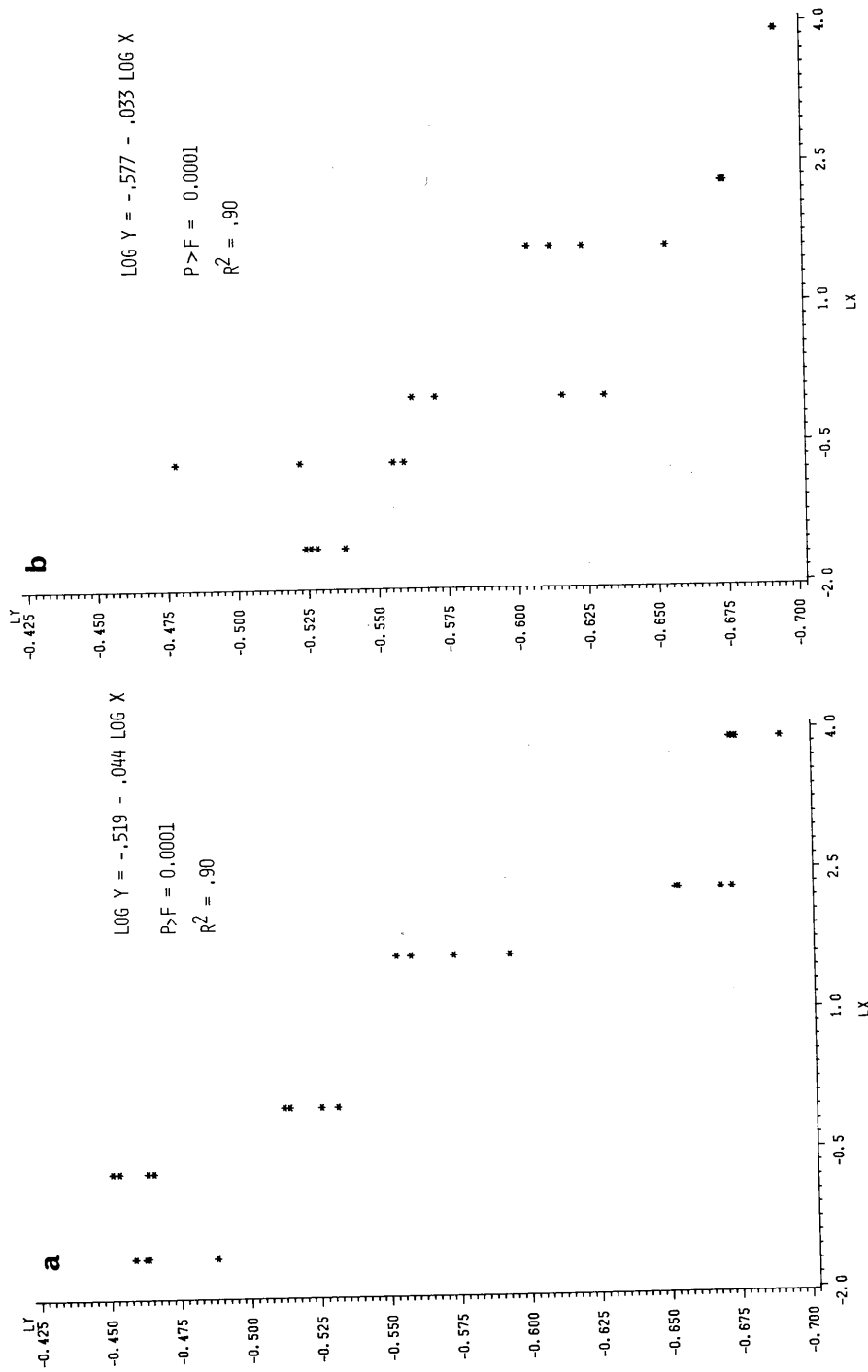


Fig. 3. The points are obtained from the equation: $\log Y = A + B \log X$. (a) The points describe the growth of *Breviortyella pulmonaria*. Slope = -0.519 , $R^2 = 0.90$. (b) The points describe the growth of *Helicoverpa pulmonaria*. Slope = -0.577 , $R^2 = 0.90$.

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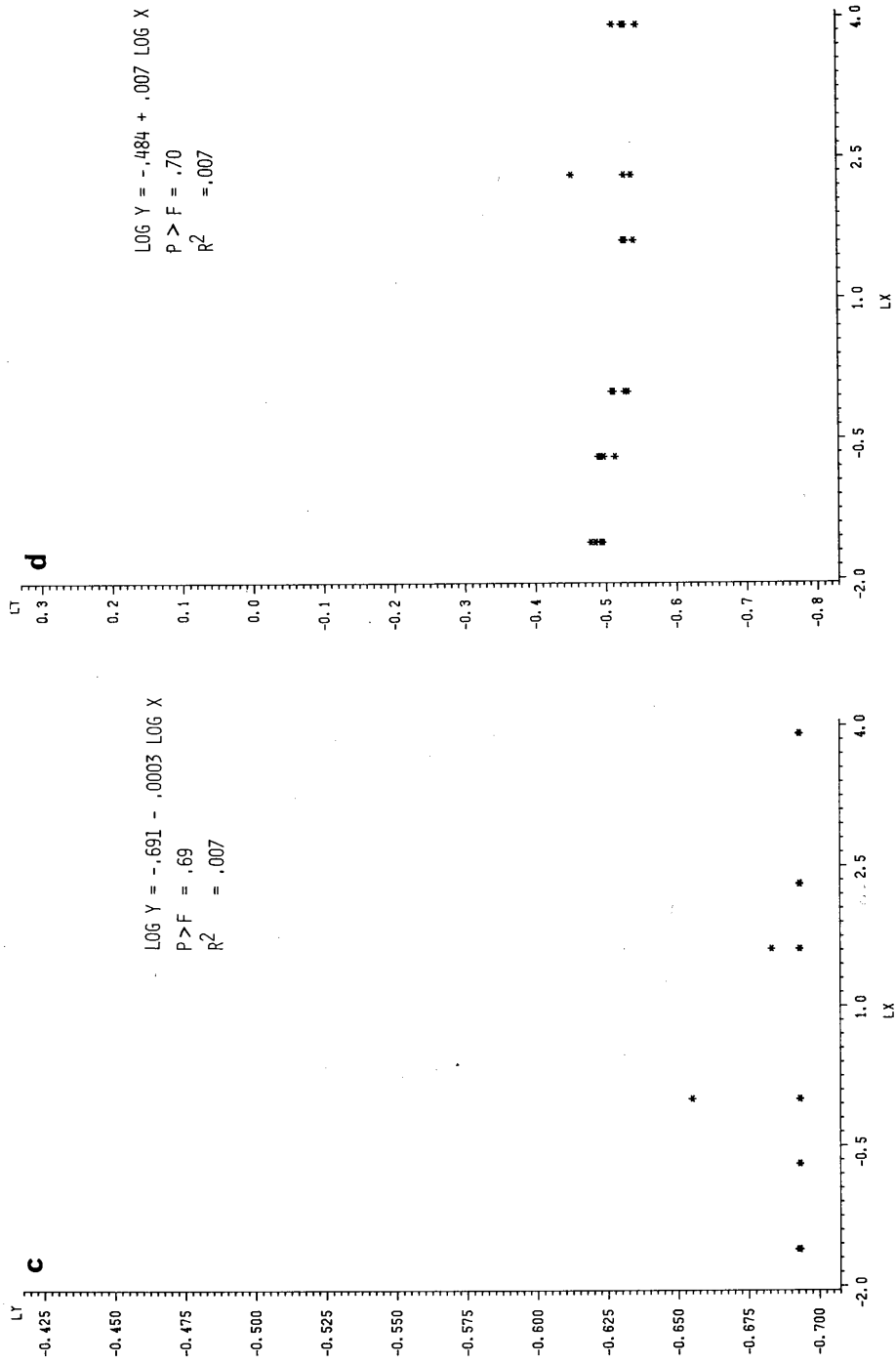


Fig. 3—contd. The points describe the growth of *Hormiactis ontariensis*. Slope = -0.691, $R^2 = 0.007$. (d) The points describe the growth of *Pseudoaegerita matsushimae*. Slope = -0.484, $R^2 = 0.007$.

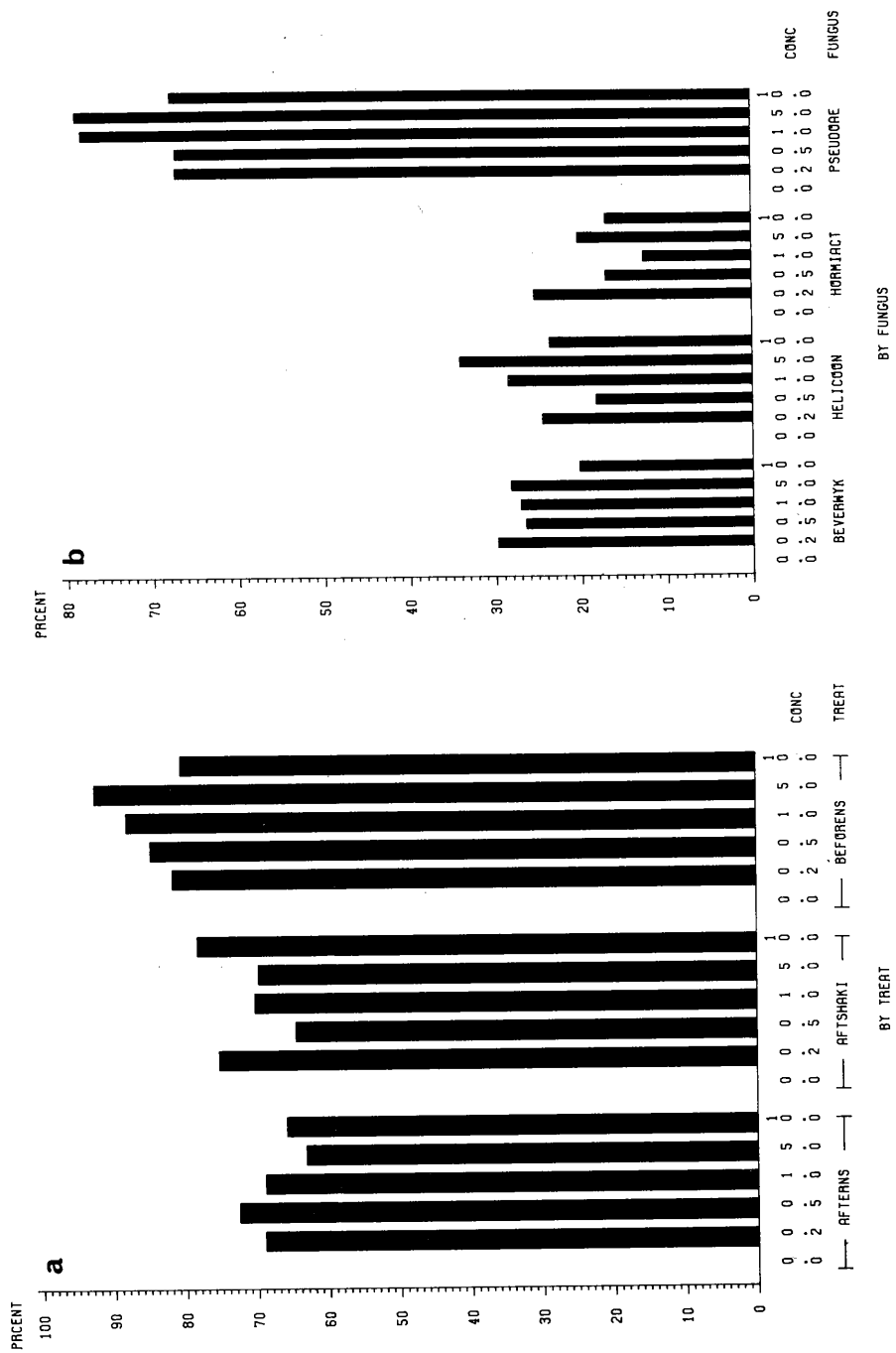


Fig. 4. (a) Per cent diquat recovered from the media in the adsorption to glass experiment. (Beforens) before the incubation, flasks shaken; (Aftshaki) after the incubation, flasks not shaken; (Aftshaki) after the incubation, flasks shaken. Concentrations are expressed in $\mu\text{g ml}^{-1}$. (b) Per cent recovery of diquat from media in which the fungi were grown. Concentrations are expressed in $\mu\text{g ml}^{-1}$.

Fig. 4. (a) Per cent diquat recovered from the media in the absorption to glass experimental apparatus before the incubation; (b) Per cent diquat recovered from the media in the absorption to glass experimental apparatus after the incubation, flasks not shaken; (c) Per cent diquat recovered from the media in the absorption to glass experimental apparatus after the incubation, flasks shaken. Concentrations are expressed in $\mu\text{g ml}^{-1}$. (d) Per cent recovery of diquat from media in which the fungi were grown. Concentrations are expressed in $\mu\text{g ml}^{-1}$.

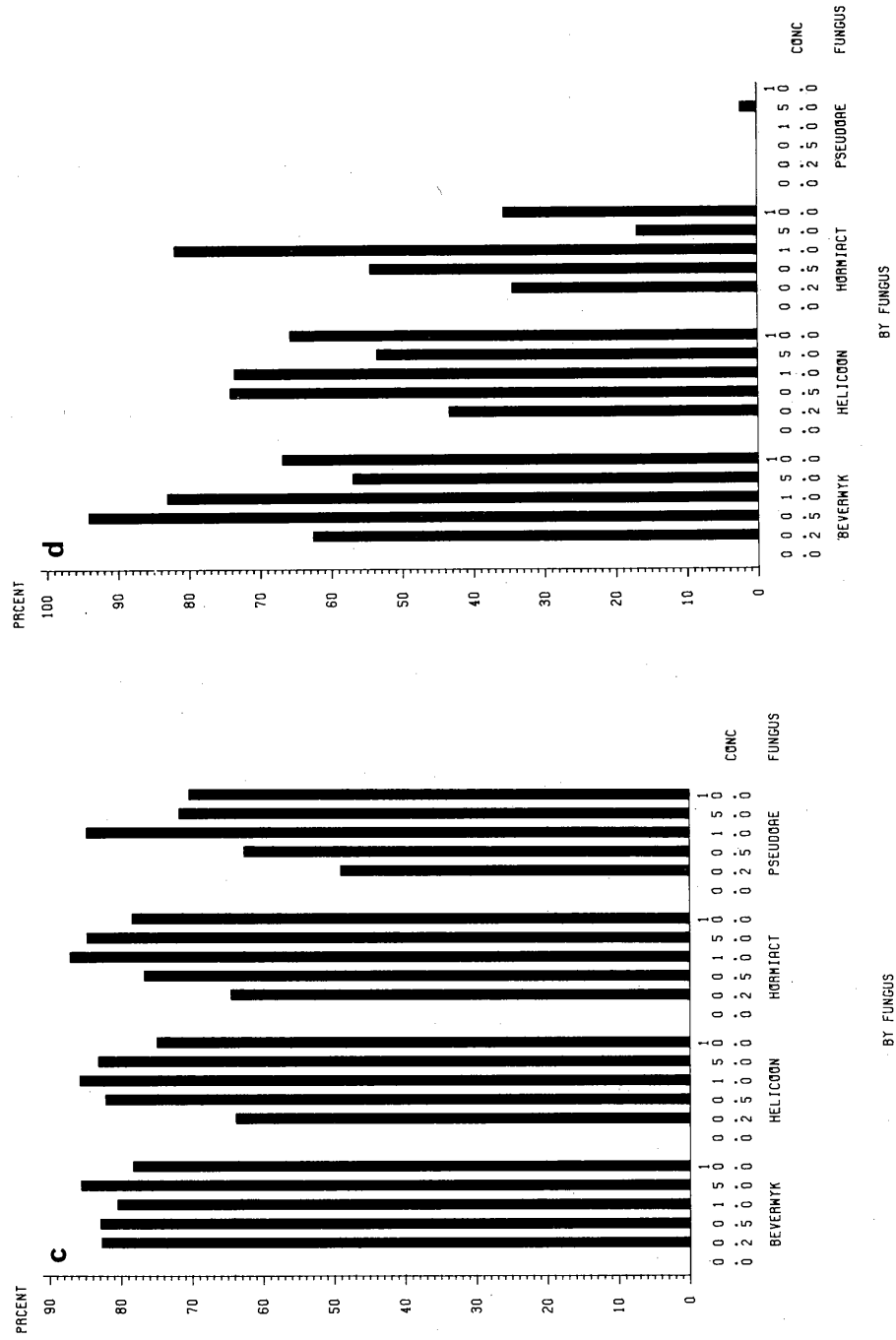


Fig. 4—contd. Per cent recovery of diquat from spiked fungal material. Concentrations are expressed in $\mu\text{g ml}^{-1}$. (d) Per cent recovery of diquat from the fungi grown in media containing the herbicide. Concentrations are expressed in $\mu\text{g ml}^{-1}$.

DISCUSSION

Diquat dibromide (1,1-ethylene-2,2-bipyridilium dibromide monohydrate) Reglone® (Chipman Chemical Co. Ltd) commercial formulation is an aquatic herbicide widely used to control unwanted aquatic macrophytes. The bipyridilium herbicides, when applied to plants as cations, become free radicals upon reduction by electrons from Photosystem I (Calderbank, 1968). In contact with oxygen, the free radicals become oxidised, and in contact with water, hydrogen peroxide, which is highly toxic to plants (Calderbank & Slade, 1976), is produced.

The effects of the bipyridilium herbicides on fish and aquatic invertebrates have been studied. Gilderhaus (1967) noted that 1–3 $\mu\text{g ml}^{-1}$ diquat did not affect bluegills *Lepomis microchirus* Rafinesque but was quite toxic to Cladocera.

Newman & Way (1965) noted small changes in arthropod populations of an aquatic ecosystem following exposure to diquat. Tatum & Blackburn (1962) recorded an increase in oligochaete and *Chaoborus* populations following applications of diquat.

Pierce *et al.* (1965) claimed that diquat used at concentrations ranging from 0.25 to 10 $\mu\text{g ml}^{-1}$ had no adverse effect on fish, invertebrates or plankton, but Blackburn & Weldon (1965) reported that 0.5 $\mu\text{g ml}^{-1}$ diquat in a subtropical pond affected the phytoplankton for a short period. Birmingham & Colman (1977) reported that Reglone® inhibited the growth of the blue-green (prokaryotic) algae, *Anabaena flos-aquae* and *Anacystis nidulans*, at concentrations greater than 0.03 $\mu\text{g ml}^{-1}$. Among the eukaryotic algae, the diatom *Navicula pelliculosa* was inhibited at concentrations greater than 0.3 $\mu\text{g ml}^{-1}$ but *Chlorella vulgaris* was unaffected at 3 $\mu\text{g ml}^{-1}$.

Cullimore (1975) noted that while the growth of a *Chlorella* sp. was unaffected by 10 $\mu\text{g ml}^{-1}$ diquat, the growth of *Chlorella vulgaris* and *Chlamydomonas terricola* exposed to 0.1, 0.5 and 10.0 $\mu\text{g ml}^{-1}$ diquat was reduced by 50%. Diquat was found to be effective against bacterial parasites of fish (Whipp, 1967).

Reglone® shows some toxicity toward soil fungi. Wilkinson (1969) reported that it had inhibitory effects on *Trichoderma viride* at a concentration of 10 $\mu\text{g ml}^{-1}$, and on *Rhizopus stolonifer* at a concentration of 100 $\mu\text{g ml}^{-1}$. *Fusarium culmorum* and *Aspergillus niger*, however, were little affected. Smith *et al.* (1976) suggested that *Aspergillus niger* degrades diquat. Wallnoeffer (1968) in Summers (1980) observed

that diquat is more inhibitory to fungi than paraquat, another bipyridilium herbicide. He found that *Rhizopus japonicus* and *Aspergillus niger* were completely inhibited by $20 \mu\text{g ml}^{-1}$ and $1200 \mu\text{g ml}^{-1}$ diquat, respectively. Funderburk (1969) claimed that the yeast *Lipomyces starkeyi* degrades diquat.

Some experiments with mammals have suggested that diquat is not very toxic to them (Comning *et al.*, 1969; Gage, 1968). Yet Vanholder *et al.* (1981), in reviewing eleven cases of diquat poisoning in man, reported that six were fatal. Most studies on the pollution of ponds by herbicides have ignored the fungal aspects of pond ecology. Research in this laboratory has recently emphasised the rôle of fungi in the decomposition of submerged leaves and the fact that the fungi involved, as well as the leaves, represent an important part of the diet of many benthic pond invertebrates.

If pesticides entering ponds inhibit the growth of fungi which act as important intermediaries in the food chain, much of the food normally available to the invertebrates would become inaccessible. Equally important, accumulation of pesticides in the fungal mycelium could result in the transfer of the pollutant to the higher levels of the food chain, and possibly in biological accumulation.

This study showed that some of the fungi grazed upon by pond invertebrates (*H. ontariensis*, *B. pulmonaria*, *H. elegans*) are strongly inhibited by the aquatic herbicide, diquat dibromide, while another, *P. matsushimae*, is little affected.

Extractions of diquat from the fungal tissue were performed in order to establish whether or not the fungal cells are permeable to the herbicide and to investigate the possibility of diquat accumulation. Results indicated that only three of the four fungi under study were permeable to diquat, extraction from *P. matsushimae* being unsuccessful.

The question arose as to whether or not diquat was metabolised by this fungus and converted into compounds that did not absorb in the 379 nm wavelength range. This did not appear to be the case, as most of the pesticide remained in solution at the end of the incubation period.

Birmingham & Colman (1977) studied the effects of diquat on algae. They suggest that diquat is mainly adsorbed through an ion exchange mechanism to organic binding sites on the surface of the algae, since 40% to 70% was desorbed by 5M ammonium chloride.

Although we cannot provide an explanation for our inability to extract diquat from *P. matsushimae*, we may suggest that electrical charges on the

cell membrane could prevent the strongly cationic diquat molecule from entering the cell, or that differences in the cell wall itself may have the same effect.

CONCLUSIONS

We have demonstrated that diquat has an inhibitory effect on several fungal elements of the pond system. Three of the four aero-aquatic hyphomycetes tested were relatively sensitive to diquat. These three fungi were apparently permeable to the herbicide, which could subsequently be extracted from them. Future experiments will attempt to discover to which fraction of the fungal cells the diquat is bound. The fourth fungus did not appear to adsorb or metabolise diquat, since the herbicide did not seriously affect its growth and could not be recovered from the mycelium. We can thus hypothesise that this fungus will have a competitive advantage in ponds treated with diquat: it should be possible to test this hypothesis experimentally.

Some preliminary feeding studies carried out in our laboratory suggest that small invertebrates such as snails (*Lymnaea* sp.), when provided with a choice of mycelia of the four fungal species grown in media containing diquat, prefer to graze on *P. matsushimae*, rather than on any of the other three fungi investigated. Fungi such as *P. matsushimae*, which do not accumulate the pollutants inside their cells, and thus represent uncontaminated food sources for invertebrates, might be regarded as elements favourable to the reestablishment of homeostasis in the pond system following its contamination. We will examine this aspect further in future publications.

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