

**A NEW SYSTEM TO BIOASSAY PESTICIDES PRESENT IN THE
SURFACE MICROLAYER USING FLOATING PROPAGULES OF AN
AERO-AQUATIC HYPHOMYCETOUS FUNGUS
*PSEUDOAEGERITA MATSUSHIMAE***

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Abstract. The surface microlayer often contains pesticides at levels which may be more than a hundred thousand times those sampled in the remainder of the water column. Standard bioassay systems cannot assess these pesticides since no system yet devised has made use of organisms inhabiting the surface microlayer. Using floating propagules of *Pseudoaegerita matsushimae*, an aerobic aquatic hyphomycetous fungus, a bioassay system was devised in which the percentage germination of such propagules after exposure to various levels of pesticides were plotted to produce a dose-response germination scale onto which subsequent data could be fitted. The following pesticides and pesticide mixtures were used: PCP; DDT; Methoxychlor; Bis(tributyl)tin Oxide; Malathion; Captan; 1 part PCP/1 part DDT; 2 parts PCP/1 part DDT; 3 parts PCP/1 part DDT; 2 parts DDT/1 part PCP; 3 parts DDT/1 part PCP; 1 part PCP/1 part Methoxychlor; 2 parts PCP/1 part Methoxychlor; 3 parts PCP/1 part Methoxychlor; 2 parts Methoxychlor/1 part PCP; 3 parts Methoxychlor/1 part PCP. The bioassay system revealed increased sensitivity to pesticides and pesticide mixtures which affect respiratory metabolism. Some pesticide mixtures were more effective in inhibiting germination than their individual components while others appeared much less toxic to the propagules than their unassisted components.

1. Introduction

The surface microlayer, the top 60–150 μm of the water surface (Maguire *et al.* 1982; Duce *et al.*, 1972), often contains pesticides at levels which may exceed one hundred thousand times those sampled in the remainder of the water column. This phenomenon is explained by the extremely high concentrations of naturally derived and anthropogenic lipophilic materials which may be present or may have been introduced to this zone (Sodergren, 1979), and which act as effective pesticide concentrators (Eisenreich *et al.*, 1978). Incidentally, a non-anthropogenic surface layer would account for pesticides recorded at the air-water interface in the apparent absence of anthropogenic additives, e.g. oil (Maguire *et al.*, 1982). The potential for pesticide accumulation is obviously much greater when petroleum derivatives are present as thin films or slicks (Seba and Corcoran, 1969).

There are no permanent inhabitants of the air-water interface and thus conventional systems cannot be applied to assess the impact of pesticides present in the

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zone. The challenge therefore, was to design and develop a system to bio pesticides present in the surface microlayer. Initially, several common insect species were considered. Corixidae (Water boatmen), Notonectidae (Back swimmers), Gerridae (Water striders) and Hydrometridae (Marsh treaders), can be found at the air-water interface. However, these insects are air breathers and do not maintain contact with the water in order to respire. Mosquito larvae were ruled out as potential candidates. Pesticides reaching the surface microlayer often dissolved in mineral oils or other petroleum distillates. These distillates are lethal to mosquito larvae, killing them by clogging their spiracles and preventing respiration, and are themselves often employed as control agents (Spencer, 1982).

Aero-aquatic fungi are specialized hyphomycetes found primarily in shallow lentic systems which receive large inputs of energy in the form of fallen leaves. These fungi are unique in that they colonize submerged detritus but reproduce by extending hyphae up through the water into the air where their asexual reproductive structures are produced. The propagules are unique, buoyant structures which float because they entrap air during development (Michaelides, 1982; Michaelides and Kendrick, 1982). When mature, the propagules dehisce and float upon the surface of the water until they encounter a floating leaf which they can colonize. They are ideally situated to be the first aquatic fungi, and perhaps even the first aquatic organisms, to colonize autumn-shed leaves as they enter the pond.

Floating propagules are also, from our point of view, ideally situated to maintain intimate contact with any pesticides formulations present in the surface microlayer. One species, *Pseudoaegerita matsushimae* (Matsushima) Webster, produces abundant, large ($\sim 80 \mu\text{m}$ diameter) easily manipulated propagules. These are readily harvested from submerged decaying *Acer saccharum* and *Fagus grandifolia* leaves removed from shallow lentic systems and maintained in culture (Premdas and Kendrick, 1991).

Earlier studies revealed that $\sim 97\%$ of *P. matsushimae* propagules would germinate if placed on damp sterile pieces of filter paper and incubated at 25°C for a minimum of 6 h. Furthermore, the frequency of germination was found to be unaffected by the presence of common petroleum distillates and petroleum-based pesticide formulations (Premdas and Kendrick, 1991). An earlier study indicated that propagule production in aquatic hyphomycetes were affected by varying levels of PCP (Michaelis and Locher and Premdas, 1988). We postulated that if the subsequent germination of aero-aquatic propagules was also affected by exposure to pesticides, this research could be used in bioassaying such pesticides. Studies were initiated to investigate the potential of aero-aquatic fungi for use in a bioassay system.

2. Materials and Methods

2.1. PROCUREMENT OF FUNGAL PROPAGULES

Field work was carried out at a small, relatively unpolluted woodland pond at the Laurel Creek Conservation Area (Waterloo, Ontario, Canada). This site

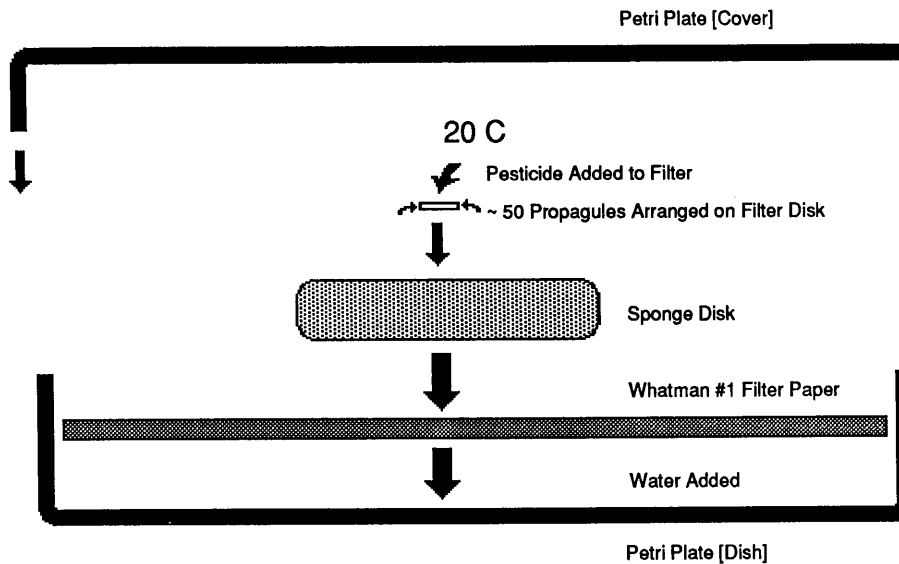


Fig. 1. Schematic of damp propagule germination chamber.

selected because the aero-aquatic fungi present, and many aspects of this pond ecology have been extensively studied (Michaelides, 1982). Submerged, decaying maple leaves (*Acer saccharum* Marsh) were sampled, treated and incubated according to Fisher (1979). Following this process, numerous aero-aquatic propagules could be observed sporulating from the leaf surface. Propagules of *P. matsushii* could then be identified and aseptically removed for testing using a sterile needle.

2.2. THE BIOASSAY SYSTEM

Bioassay tests were conducted in a damp chamber (Figure 1). A petri plate was lined with 9.0 cm (diameter) Whatman #1 filter paper. A sponge disk 3.5 cm (diameter) \times 0.5 cm (thick) was placed in the plate. Distilled water was added to completely saturate the disk and the entire apparatus left in place for at least 1 hour to fully saturate the sponge. After this period, excess water was drained off, leaving at least 1–2 mm at the bottom of the plate.

Propagules of *P. matsushimae* were quickly transferred from their leaf substrate to sterile, moist, 0.5 cm diameter Whatman #1 filters using sterile needles. Approximately 50 propagules were placed on each filter disk. Propagules were manipulated using fine needles to ensure that they were spread evenly over the filter disk, and that no overlapping or clumping occurred.

Loaded filters were then carefully transferred onto saturated sponge disks using the edge of a clean scalpel blade. Filters were placed so that the surface on which

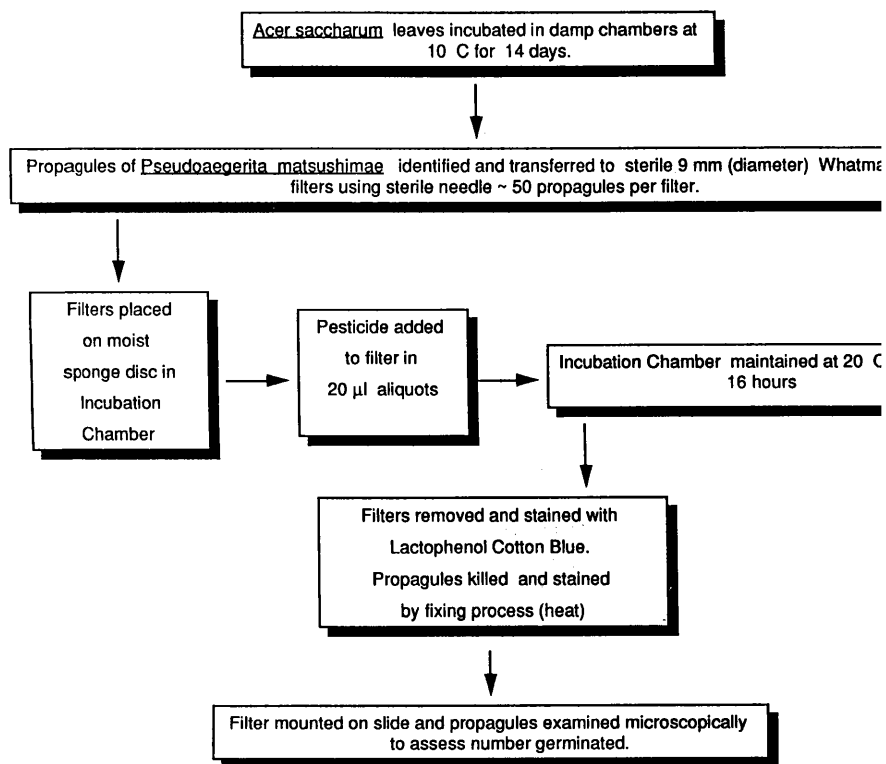


Fig. 2. Flow-chart of bioassay method.

propagules rested faced upwards. Filters soon became fully saturated with water. Water levels in the plate were kept constant, to ensure that propagules were moist and in contact with their filter disks throughout the experiment. Aliquot (0.1 ml) of pesticide solutions were then added to filters containing propagules. The quantity was sufficient to completely cover a 0.5 cm diameter filter disk without dislodging any propagules.

The plates were then covered, thus effectively becoming damp chambers and were placed in an incubator overnight (16 h) at 20°C. Subsequently, filters were mounted on slides, stained using Lactophenol Cotton-blue and heated to kill the propagules. Preparations were then examined microscopically to assess the number of propagules germinated (Figure 2). The presence of germ tubes was used as the criterion for establishing germination (Figures 3 and 4). Bioassays were carried out in triplicate of five times at each pesticide or pesticide mixture concentration tested.

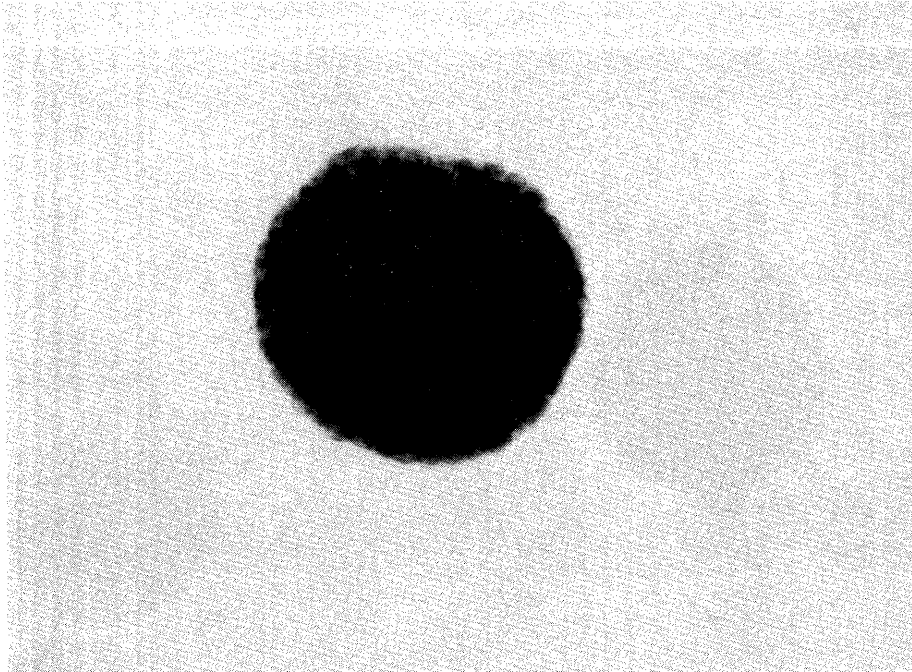


Fig. 3. Ungerminated propagule of *Pseudoaegerita matsushimae*. Magnification $\times 40$. Propagule measures approximately 40×40 micrometers.

2.3. PESTICIDES AND CARRIER FORMULATIONS

With the exception of Captan, pesticides were bioassayed in solution in petroleum based carriers. The effects of these carriers on the germination of *P. matsushimae* propagules was reviewed in an earlier publication (Premdas and Kendrick, 1964). The following carriers were employed: analytical-grade kerosene (K), pharmaceutical-grade light mineral oil (LM), commercial-grade light mineral oil (CL), 10% Tween-80 surfactant (T-80), emulsifiable oil concentrate (EC), distilled water (DW).

Commercial grade pesticides often contain highly toxic impurities which may synergize or antagonize the net toxicity of the primary compound. To minimize these effects, stock solutions were made up using analytical grade pesticides. The following pesticides and combinations of pesticides were bioassayed: PCP, DDT; Methoxychlor; Bis(tributyl)tin Oxide; Malathion; Captan; 1 part PCP/1 part DDT; 2 parts PCP/1 part DDT; 3 parts PCP/1 part DDT; 2 parts DDT/1 part PCP; 3 parts DDT/1 part PCP; 1 part PCP/1 part Methoxychlor; 2 parts PCP/1 part Methoxychlor; 3 parts PCP/1 part Methoxychlor; 2 parts Methoxychlor/1 part PCP and 3 parts Methoxychlor/1 part PCP. With the exception of Captan, pesticides were dissolved or suspended in carrier formulations likely to be used.

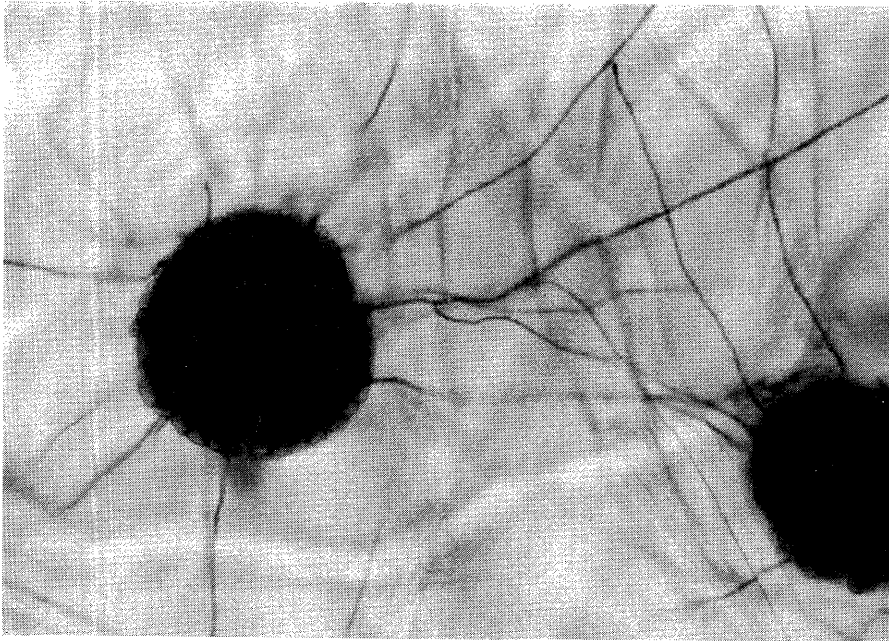


Fig. 4. Germinated propagule of *Pseudoaegerita matsushimae*, note presence of germ tubes indicating that successful germination has taken place. Magnification $\times 35$. Propagule measures approximately 40×40 micrometers.

in field application situations and which would tend to accumulate in the surface microlayer as anthropogenic lipophiles (Table I).

Pesticides were added to the appropriate carrier in a dark foil-wrapped bottle and stirred continuously for 24 h in the dark at room temperature (21°C) to ensure solution. Subsequent concentrations from 100 g/L to 0.1 mg/L were made up by serial dilution of the stock. All solutions were stored in the dark at 10°C between experiments to prevent photolysis and to ensure chemical stability. Experiments were begun within one day after a stock solution was made up and germination completed within 1 week.

2.4. STATISTICAL TREATMENT OF DATA

Pesticides and pesticide mixtures were bioassayed 5 times at each concentration. Data were pooled and analysed by the Probit method using a Cricket StatgraphTM integrated statistical graphics package. IC_{50} s were obtained directly from computer-generated graphs as described by Sokal and Rohlf (1981). Examples, Captan and Pentachlorophenol, are shown in Figures 5 and 6, respectively.

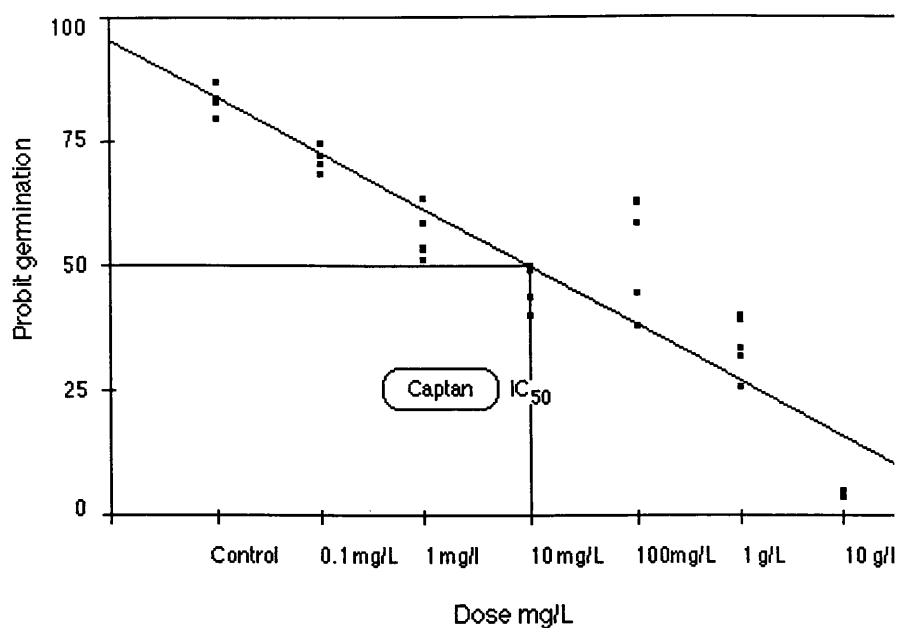
TABLE I
 IC₅₀s of pesticides bioassayed using *Pseudoaegerita matsushimae*
 propagule bioassay.

| Pesticide | Carrier | IC ₅₀ |
|---------------------------------|---------|------------------|
| PCP | K | 10 mg/l |
| | LM | 16 mg/l |
| | CLM | 10 mg/l |
| DDT | LM | 10 mg/l |
| | K | 31 mg/l |
| Methoxychlor | LM | 13 mg/l |
| | K | 20 mg/l |
| Bis(tributyl)tin Oxide | T-80 | 8.0 mg/l |
| | LM | 2.5 mg/l |
| Malathion | EC | 18 mg/l |
| 1 part PCP/1 part DDT | K | 16 mg/l |
| 2 parts PCP/1 part DDT | K | 9.5 mg/l |
| 3 parts PCP/1 part DDT | K | 10 mg/l |
| 2 parts DDT/1 part PCP | K | 24 mg/l |
| 3 parts DDT/1 part PCP | K | 40 mg/l |
| 1 part PCP/1 part Methoxychlor | K | 18 mg/l |
| 2 parts PCP/1 part Methoxychlor | K | 18 mg/l |
| 3 parts PCP/1 part Methoxychlor | K | 28 mg/l |
| 2 parts Methoxychlor/1 part PCP | K | 60 mg/l |
| 3 parts Methoxychlor/1 part PCP | K | 126 mg/l |
| Captan | DW | 10 mg/l |

3. Results and Discussion

3.1. PESTICIDE TOXICITY

The results of the bioassay are shown in Table I. Our studies suggest that pesticide toxicity can be carrier mediated and confirm the findings of other authors in this field. (Bis)tributyltin oxide was more efficient at inhibiting germination when dissolved in light mineral oil than in Tween-80 surfactant. PCP was more toxic to *P. matsushimae* when dissolved in either kerosene or commercial lubricating oil rather than in light mineral oil. The toxicity of DDT was enhanced when dissolved in light mineral oil as compared to its toxicity in kerosene. The same was true for Methoxychlor, the DDT analogue tested during this study. The effectiveness of Sportak (40% Prochloraz) for treating *Pyrenophora teres* was significantly improved when LC477 surfactant was added to the application mixture (Khardar and Stevens, 1986). Similarly, fungitoxicity of Dithane M45 (1.4 kg a.i./ha) against *Puccinia sorghi* infecting corn was increased by Triton XR surfactant (2.5% v/v).



| Source | Sum of Squares | Deg. of Freedom | Mean Squares | F-Ratio | Prob>F |
|--------------|----------------|-----------------|--------------|---------|--------|
| Model | 28.947 | 1 | 28.947 | 189.009 | 0.000 |
| Error | 5.054 | 33 | 0.153 | | |
| Total | 34.001 | 34 | | | |

Coefficient of Determination (R²) 0.851
 Adjusted Coefficient (R²) 0.847
 Coefficient of Correlation (R) 0.923
 Standard Error of Estimate 0.391

Fig. 5. Sample graph (probit technique) of pesticide bioassay using Captan. Each point represents a single test involving ~50 propagules.

(Pitblado, 1986).

Mixtures of 1, 2 and 3 parts PCP/1 part DDT, and 1 and 2 parts PCP/Methoxychlor were more effective in inhibiting *P. matsushimae* propagule germination than either DDT or Methoxychlor. However, any addition of DDT or Methoxychlor to PCP decreases the toxicity of the latter. Mixtures which employed 2 and 3 parts DDT or Methoxychlor to 1 part PCP were many times more toxic to this species than either unassisted DDT, Methoxychlor or PCP.

Insecticide-fungicide combinations are frequently used to control inse

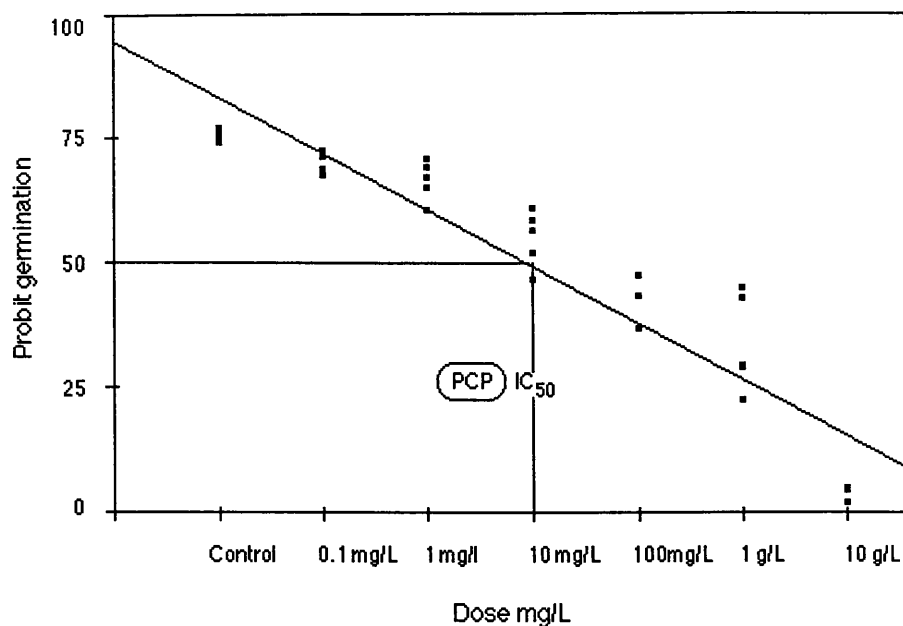
fungal pathogens. Mergamma (262 g/L Maneb, 132 g/L Lindane) was reported to provide excellent control of *Limonius agonus* (Wireworm) infecting barley (Smith, 1986). Agrox DL Plus (15% diazinon, 25% Lindane, 15% Captan) provided effective control of *L. agonus* on corn (Smith, 1986). TF 3508 (512 g/L Lindane, 60 g/L Thiram, 60 g/L Carbendazim) applied to canola seeds to treat *Leptosphaeria maculans*, eradicated infection and improved subsequent emergence and growth of seedlings (Smith, 1986). A Captan-Diniconazole mixture was more effective than Captan in controlling apple scab (*Venturia inaequalis* (Cooke) Winter) and *Gymnosporangium clavipes* (guinea rust) on apple (Warner and Herbert, 1986). Combinations of Captan and TF 3621 (25% Captan, 15% Benalaxyl) (0.5 : 0.1 a.i./kg seed) provided significantly greater control of *Pythium spp.* on sweet corn than Captan alone (0.66 a.i./kg seed) (Letebdre, 1986). Foliar applications of Captan and Easout 70 (thiophonate-methyl) (75 : 77 g a.i./100L) were more effective than unassisted Captan (75 g a.i./100L) against *Colletotrichum gloeosporioides* (Pers. Sacc. infecting blueberry (Hildebrand and Newbery, 1986).

3.2. ECOLOGICAL IMPLICATIONS

Aero-aquatic fungi colonizing submerged leaves are periodically forced to survive under microaerobic conditions. In shallow woodland ponds, these conditions may occur during summer when high temperatures reduce the oxygen saturation potential and exchange is exceeded by demand. In temperate zones, low oxygen tensions also occur during winter, when gaseous exchange is impossible as a result of heavy ice cover. Fisher and Webster (1979) report continued growth of aero-aquatic fungi under microaerobic conditions and suggest possible survival even after 6 months at zero oxygen tension. Also, woodland ponds are often high in ionic and polyphenolic leaf exudates, e.g. cations, tannins, phenols and lignin metabolites, which may be inhibitory to some organisms. Levels are probably greater during Autumn when increased numbers of leaves enter the pond. Michaelides (1982) found high levels of SO_4 and very low amounts of NO_2 and NO_3 ions during his study of the same pond investigated here.

P. matsushimae displayed increased sensitivity to respiratory inhibitors. This fungus was more sensitive to PCP and (Bis)tributyltin oxide, inhibitors of oxidative phosphorylation, and to Captan, a thiol complexing agent than to other biocides. In many cases, the fungi were more resistant to insecticides and insecticide/pesticide mixtures than other organisms reported in the literature. However, initial studies showed *P. matsushimae* to be as sensitive to Captan as other fungi. It is apparent that high mammalian or teleost toxicities do not always guarantee high fungitoxicity. Future studies will concentrate on increasing the sensitivity of this system in addition to screening a larger number of aero-aquatic species. It is tempting to imply that increased pesticide resistance is linked to environmental hardiness. However, further study is probably necessary before any real conclusions can be drawn.

Lentic ecosystems are at greatest risk to pesticides applied during fall :



| Source | Sum of Squares | Deg. of Freedom | Mean Squares | F-Ratio | Prob>F |
|--------|----------------|-----------------|--------------|---------|--------|
| Model | 28.432 | 1 | 28.432 | 240.858 | 0.000 |
| Error | 3.895 | 33 | 0.118 | | |
| Total | 32.327 | 34 | | | |

| | |
|--|-------|
| Coefficient of Determination (R ²) | 0.879 |
| Adjusted Coefficient (R ²) | 0.876 |
| Coefficient of Correlation (R) | 0.938 |
| Standard Error of Estimate | 0.344 |

Fig. 6. Sample graph (probit technique) of pesticide bioassay using pentachlorophenol. Each represents a single test involving ~ 50 propagules.

spring when propagule densities on water surfaces are at their peaks (Premdas and Kendrick, 1991). Even if the residence of pesticides in the surface microlayer is of short duration, critical damage to the pond ecosystem might occur. Relatively short exposures, > 3 h, to some formulations bioassayed were sufficient to kill propagules or permanently inhibit germination (Premdas and Kendrick, 1991).

In an actual situation, lowered germination potential would result in decreased colonization of new leaves entering the system. These leaves constitute an important but initially unusable energy source for most of the lentic fauna. Aero-a-

fungi which colonize these leaves act as the energy intermediates, degrading leaves and converting them partly into mycelial structures which other aquatic organisms consume. It is probably insufficient to estimate potential environmental damage to such systems solely on the basis of known, or extrapolated, toxicity of the predominant contaminant. Deliberate or inadvertent mixtures of commercial biocides (pesticides and fungicides) may present a far greater risk to the aquatic environment.

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