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SOME STUDIES OF SOIL BOUND RESIDUES

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Summary The binding of pesticide residues by humic fractions is briefly reviewed, and some consideration is given to the mechanisms involved and to procedures for studying these. Data are presented for the incorporation of labelled residues of ethirimol [5-n-butyl-2-(ethylamino)-6-methyl-4(3)-pyrimidone] in the clay-sized components of three English soils.

Résumé On fait un résumé de la fixation des residus pesticides par les composants humiques; on considire ensuite les mécanismes, et les méthodes qu'on emploie à les étudier. On présente les données de l'incorporation des residues d'ethirimol marques dans les composants grandeur argile de trois sols de l'Angleterre.

INTRODUCTION

When pesticides enter the soil they can interact especially with its colloidal constituents, and become involved in the soil biological processes from which humic substances are formed. The soil colloids are primarily composed of clays, oxides and hydrous oxides, particularly those of silicon, iron and aluminium, and of humic materials. Each of these components can exist independently in the soil, or they can be associated to provide complex inhomogeneous surfaces for interaction with ions, or with pesticides or other organic chemicals in their environments.

According to the American Institute of Biological Sciences Environmental Task Group a bound residue is "that unextractable or chemically unidentifiable pesticide residue in fulvic acid, humic acid and humin fractions after exhaustive sequential extraction with nonpolar organic and polar solvents". This definition, adopted by the EPA (1975), focuses attention entirely on the soil organic matter contribution to the binding process. To a large extent this is supported by the available data in the literature, although certain pesticides, notably paraquat (Knight and Tomlinson, 1967), and possibly under certain circumstances prometone and prometryne (Talbert and Fletchall, 1965), have a preference for the soil clay minerals. However, it must be remembered that initially, at least, the pesticide will interact with the first reactive surface with which it comes into contact. The reversibility of the binding process will determine whether or not it can later be transferred to another surface. Many studies of soil colloid-pesticide interactions have used commercial clay preparations and humic materials isolated from soils. In this communication we propose to review briefly some of the information which illustrates the importance of soil organic materials for the binding of pesticides and their residues, to briefly review the binding mechanisms which are thought to be involved and techniques for their study, and to present data from experiments carried out by us which investigated the fate of ethirimol [5-n-butyl-2-(ethylamino)-6-methyl-4(3)-pyrimidone] after incubation in three English soils.

BINDING BY SOIL ORGANIC COLLOIDS

Hayes and Swift (1978) have discussed the information which is available up to the present time with regard to the composition and shapes of the most important colloidal organic components of soil, and Stevenson (1976) has reviewed some of the possible chemical and physical interactions which can take place between pesticides and humic substances.

Microbial activity in soils is usually related to the soil organic matter content, and the extent to which bound residues are present in soil can also be related to the microbial activity. Metabolism of organic amendments give rise to relatively stable humic materials which are broadly subdivided into humic acids (precipitated by acid from solution in aqueous base), fulvic acids (soluble in aqueous base and acid), and humin (insoluble in aqueous base and acid) fractions. These fractions have been shown to be active in the binding of pesticides, and to incorporate their metabolites or altered products into their polymeric structures. Although polysaccharides, either associated with the humic materials or free in the soil environment, are important components of soil organic colloids it would appear that they are relatively unimportant in so far as the binding of pesticides is concerned.

In addition to their solubility properties, humic acids, fulvic acids, and humin materials vary in their elemental compositions, acidic group and free radical contents, and in their molecular weight distribution values. There is support for the view that these components are related generically; fulvic acids are often regarded as initial transformation products and it is often suggested that these can subsequently be transformed in sequence to humic acids and humin materials. If so it could be expected that, initially, bound residues would be concentrated in the fulvic acid fraction. Meikle et al (1976) have suggested that the greater solubility of fulvic acids might coincide with their more rapid formation and degradation and provide a higher probability for the incorporation of pesticide moieties in their structures. Their results for ^{14}C-labelled ditalimfos were consistent with this view since the specific activity of the fulvic fraction was greater than that of the humic acids, and the activity of these in turn was considerably greater than that of the humin materials. However, because of the relative proportions of fulvic (FA) to humic (HA) acids in chelating resin extracts (FA:HA ratio = 1:1.8) the total amount of label in the humic acids was greater, although the order was reversed when sodium hydroxide was used as the extractant when the FA to HA ratio was 1:0.6. This focuses attention on the fact that the extraction procedure used can be very important in considerations of the distributions of pesticide residues between the different components of humic materials. The conditions used during incubation are also important.

A study by Helling (1976), involving the use of several dinitroaniline herbicides, showed that an average of 50% of the bound residues were contained in the fulvic acid fraction compared with 15-20% in the humic acids, and 25-30% and sometimes as much as 40% was contained in the humin. This work also illustrated the importance of taking into account the extraction procedures. When they used the method of Gascho-Stevenson (1968) (which involved pretreatment with HF, followed by dialysis against $Na_4P_2O_7$ and NaOH solutions) very little fulvic acid was obtained, and the bound butralin residues were concentrated in the humic acid and humin materials. Bound residues of pirimicarb were almost completely extracted in NaOH solution and contained in the fulvic acid materials (Hill, 1976).

There are also data which imply that fulvic acids are the least important of the humic components for holding bound residues. For instance Richey et al (1977) found that the distribution of aldicarb residues was humin > HA > FA when the materials were extracted and fractionated by the procedures of Kononova (1966). Also Smith (1977) found that little bound residues of dichlorofos-methyl could be extracted at room temperature in one normal NaOH, but that the residues were released in the humic acids after refluxing with aqueous triethanolamine.

The work of Ambrosi et al (1977a and b) emphasizes how incubation conditions can affect the distribution of bound residues in the humic fractions. When the insecticide phosalone and the herbicide oxadiazon were separately incubated in moist soils the distribution of bound residues in the humic fractions were in the order FA > HA > humin, but a different distribution pattern was obtained when the compounds were incubated in flooded soils. These trends can be related to the active microbial populations. A good example of the influence of microorganisms can be found in the work of Katan et al (1976) and of Katan and Lichtenstein (1977). The earlier study reported less binding of parathion in sterile than in non-sterile soils, and the later work showed that the binding of five of the potential pesticide metabolites, containing the amino group instead of the nitro group, was 14 to 26 times greater than the original compound. Amino groups could be expected to form covalently linked Schiff base products by condensing with carbonyl functional groups in the humic materials.

BINDING MECHANISMS AND METHODS FOR THEIR STUDY

Paraquat and diquat are known to be adsorbed through ion-exchange processes on clays and humic substances (Burns and Hayes, 1974; Hayes et al, 1975). In the case of binding by montmorillonite clay the reaction is highly energetic (Hayes et al, 1975) and charge transfer processes between the electron clouds of the adsorbent and adsorbates are thought to enhance the binding processes. The extent of binding by the humic substances is governed by the shape of the humic polymers (Hayes, 1976) and this in turn is governed by the resident inorganic cations (Hayes and Swift, 1978). These considerations can be rationalised by the fact that the adsorbate molecules must diffuse to the adsorption sites, and diffusion processes are retarded in structures which have narrow pores or highly cross-linked structures (Burns and Hayes, 1974; Hayes et al, 1975).

Ion-exchange reactions which are not supplemented by other energetic adsorption mechanisms are reversible. Thus these processes should not in theory give rise to bound residues. Many pesticides are ionisable, and the extent to which they are ionised will depend on their pK_a values and on the pH of the media. Triazine herbicides, for example, are held by clays when the solution pH is less than their pK_a values (Weber, 1970), but under highly acid conditions hydroxonium ions can compete with the triazine cations for sites on the clay surfaces. Therefore treatments with acid or base would be expected to desorb such ionisable materials. Other relevant mechanisms for adsorption by clays include van der Waals forces, ligand exchange reactions, hydrogen bonding, and water bridging mechanisms, and all of these processes are reversible. Mortland (1970) has presented an appropriate review.

Analysis of isotherms for the adsorption and desorption of pesticides provide most information with regard to the reversibility of the binding processes. From analysis of isotherms obtained at different temperatures enthalpy values can be obtained, but more accurate data are given by microcalorimetry measurements (Hayes et al, 1975). X-ray diffraction procedures are used to determine the conformations of species which are held in the interlamellar spaces of expanding lattice clays, and neutron diffraction techniques are now being introduced for the same purpose. Spectroscopy techniques, in particular analysis with infrared (Mortland, 1970) are particularly useful when determining the contributions of van der Waals forces, ligand exchange and water bridging, and hydrogen bonding to the adsorption processes. Ultraviolet spectroscopy complements the infrared technique in studies of charge transfer reactions. More recently electron spin resonance and Mossbauer spectroscopy have helped to indicate the contributions to binding of coordinates of polar organic molecules with transition metals, and Raman spectroscopy is expected to provide a valuable technique for studies of molecules adsorbed on clay surfaces.

Thermal analysis techniques, such as differential thermal analysis, differential thermogravimetric analysis, and differential scanning calorimetry provide good

instrumental methods for studying the desorption of organic molecules from clays and oxide surfaces. Indications of the adsorption mechanisms can be obtained from an analysis of the thermograms obtained. However, the procedures are complicated by the presence of indigenous organic molecules, such as humic materials associated with the inorganic adsorbents.

The mechanisms outlined for the adsorption of pesticides by clays and oxides can operate for the interactions with humic substances. Of the techniques mentioned for investigations of interaction mechanisms, the analysis of adsorption and desorption isotherms, calorimetry, and spectroscopy procedures are most useful for studies with humic substances. However, chemisorption is the adsorption process most likely to give rise to bound residues. This process is highly energetic and involves the formation of a covalent linkage between the adsorbent and adsorbate. Examples of reactions which would give rise to chemisorbed species include the formation of Schiff bases, esters, hemiacetals and acetals, and carbamates. Such reactions could readily take place under appropriate reaction conditions between the carbonyl, carboxyl, phenolic-, enolic-, and alcohol-hydroxyl groups present in humic materials and appropriate functional groups in the adsorbate or in its biologically transformed products.

Because of the highly exothermic nature of chemical reactions, calorimetry provides a useful technique for observing chemisorption reactions. Also such reactions are implied when the rates and extents of adsorption are increased as the temperature is raised. Where the functional group linking the adsorbate-adsorbent species is labile it is possible to release the adsorbate by hydrolysis in acid or base or by enzymolysis. Richey _et al_ (1977), for instance, showed that soil bound residues of alicarb were extractable in organic solvents after treatment with β-glucosidase and cellulase enzymes in pH 5 buffer. This would infer that the alicarb was bound to a cellulose-type [β-(1→4) linked glucose units] polysaccharide structure, and a solvent soluble conjugate was released by the enzymolysis process.

Radiolabelling provides the most useful technique for studying the fate of organic chemicals in soil. By labelling the amendments in different positions it is possible, for example, to deduce the order in which different parts of the molecules are attached or metabolised by the soil microorganisms. Also if, in separate incubation experiments, the radioactivity of the bound residues of the same pesticide, initially labelled in different positions, should be different it can be inferred that it is a metabolite and not the parent compound which is bound (Richey _et al_, 1977). The isotope dilution procedure can provide confirmatory evidence for the existence of bound residues. Should radiolabelled compounds be released by addition of an unlabelled solution of the same material it would be highly likely that the released material was not held as a bound residue. Paraquat cannot be desorbed from montmorillonite by treatment with the usual reagents used to study the existence of bound residues, but it has been shown to exchange with itself by use of the isotope dilution procedure.

Binding of metabolites is implied when the rates of formation of bound residues can be related to the rates of degradation of the pesticides. It must be remembered that where radioactive chemicals are used the presence of radioactivity in the extracted organic components might arise from compounds which bear little resemblance to the pesticide. This is especially true where scrambling of the label takes place, and this is likely in instances where the pesticide acts as a substrate for the synthesis of humic substances by soil microorganisms.

BINDING BY THE SOIL CLAY-SIZE FRACTION

The clay-size fraction of soil, or the material < 2.0 μm e.s.d. has colloidal properties, and it is composed for the most part of quartz, carbonates, oxides and hydrous oxides, clays, and organic polymers. Where the oxides, clays and humic

materials can be associated a complex surface is presented for adsorption processes. Paraquat, for instance, might initially react with the organic component of the complex and be later transferred to the clay component with which it interacts most strongly. Most other pesticides would tend to remain in the humic constituents.

An outline of some of the results obtained when ethirimol [5-n-butyl-2-(ethylamino)-6-methyl-4(3)-pyrimidone] was reacted with the clay-size fractions of Peartree, Frensham, and Gore soils is presented here. Some data (supplied for the < 3 mm soil-particle dimensions by I.C.I. Plant Protection Ltd.) for the composition and properties of these soils are presented in Table 1. The clay-size fractions,

Table 1

Composition and properties of Peartree, Frensham, and Gore soils

Soil	Pear Tree	Frensham	Gore
Soil type	Sandy clay loam	Coarse sand	Calcereous sandy loam
Clay (%)	23.3	8.8	27.8
Organic Matter (%)	6.2	1.9	10.9
pH	6.95	6.70	7.35
CEC (meq/100 g)	18.2	5.7	31.1

isolated by sedimentation and centrifugation procedures, were further subdivided into the 2.0 - 0.2 μm- and < 0.2 μm-size ranges and the total carbon, organic carbon, and the cation-exchange capacity (CEC) was determined for each fraction. These data are presented in Table 2 where n.d. indicates values which were not determined.

Table 2

Carbon contents and CEC data for clay-size extracts of soils

	Size	Pear Tree	Frensham	Gore
Clay-size extracts (as % of soil)	2.0-0.2 μm	8.8	8.5	4.5
	< 0.2 μm	2.3	0.2	1.0
Total carbon (%)	< 0.18 mm	6.2	1.4	8.6
	2.0-0.2 μm	9.1	7.9	11.8
Organic carbon (%)	< 0.18 mm	4.3	1.0	4.8
	2.0-0.2 μm	6.1	n.d.	n.d.
CEC (meq/100 g)	< 0.18 mm	29.9	8.1	34.8
	2.0-0.2 μm	52.3	40.9	34.7

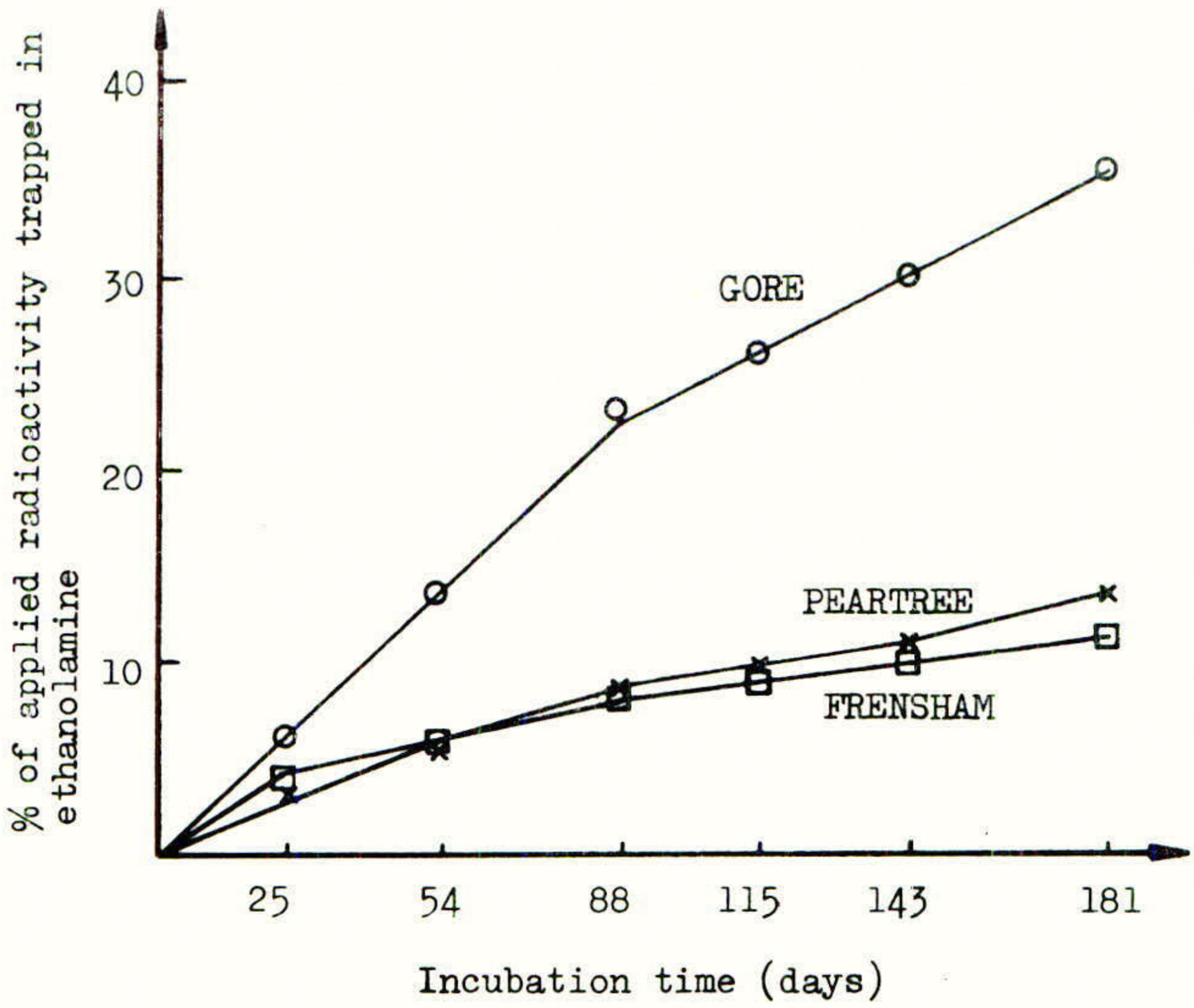
Adsorption of ^{14}C-labelled ethirimol by the soils and by their clay-size fractions was measured by the slurry technique, and scintillation counting was used to measure the radioactivity in the equilibrium solution. Linear (partition-type) isotherms were obtained for adsorption by the soils; for equilibrium solution concentrations of 90 μg of ethirimol g^{-1} of solution 0.9 mg, 0.45 mg, and 0.25 mg of adsorbate was bound by the Peartree, Frensham, and Gore soils, respectively. A similar shape of isotherm was obtained for the adsorption by the clay-size fraction in the case of the Gore soil, but the isotherms for adsorption by these fractions of the Peartree and Frensham soils were Langmurian. For equilibrium solution concentrations of 90 μg g^{-1} solution adsorption amounted to 6.5, 3.5, and 0.5 mg g^{-1} of adsorbent in the cases of the Peartree, Frensham and Gore clay-size fractions. When these fractions were washed with acid (to remove carbonates and exchangeable cations), treated with sodium hypochlorite (to oxidise the associated organic matter) and with citrate-dithionite buffer (to remove oxides and hydrous oxides) adsorption

was significantly increased to the same value (11 mg g^{-1} of residual clay-size materials) for all three samples at equilibrium solution concentrations of 90 $\mu g\ g^{-1}$ of solution. This indicates that the clay components in all three soils were capable of adsorbing to the same extents.

It would therefore appear that the contribution to the adsorption of ethirimol by the clays in the three soils was affected by the presence of coating materials. Although the clay and organic matter contents of the Gore were the greatest of the three soils, it also had the highest pH (Table 1) and contents of carbonates (from Table 2). It is clear also (Table 2) that considerable amounts of organic matter were associated with the clay-size fraction. Calcium was almost certainly the predominating exchangeable cation in the case of the Gore soil. This would result in a compact "ball of wool" type conformation (Hayes and Swift, 1978) of the humic substances which might cover much of the clay surfaces and impede the access of the ethirimol to the clays. Also this type of conformation would impede diffusion of adsorbate to the internal adsorbing sites in the humic polymers and allow adsorption to take place mainly at its external surfaces. This thesis could be investigated by removing the carbonates and investigating adsorption by fractions exchanged with H^+ and Na^+ ions.

In order to investigate the fate of ethirimol in the soil ^{14}C-labelled samples (10 ppm) were added to the Peartree, Frensham, and Gore soils and then incubated at 19-20°C under constant aeration conditions for periods of up to six months. After passing over the incubated soils air was passed through 0.1N H_2SO_4, and then through 2-methoxyethanol, and ethanolamine in order to trap volatile organic products and carbon dioxide. An insignificant amount of radioactivity was detected in the first two traps, but ^{14}C-activity, believed to be attributable to $^{14}CO_2$ was detected in the ethanolamine. The evolution with time of this radioactivity for the three soils is shown in Fig. 1. Mineralisation to $^{14}CO_2$ was greatest for the Gore soil and the trapped radioactivity amounted after six months to nearly 40% of that applied. After the same time interval the radioactivity trapped in the Peartree and Frensham soils totalled only about 12% of the applied chemical.

Fig. 1 - Radioactivity trapped in ethanolamine after incubation of ^{14}C-ethirimol in three soils



After incubation the soil samples were extracted sequentially with acetone, methanol, and distilled water. Fig. 2 presents the amount of residual radioactivity which remained in the Peartree, Gore, and Frensham soils after these exhaustive solvent extraction procedures. It can be seen that the amounts of bound residues remaining in the soils did not correspond to the adsorption behaviour of these soils. Adsorption decreased in the order Peartree > Frensham > Gore, and the bound residue contents of the soils decreased in the order Peartree > Gore > Fensham.

Fig. 2 - Applied radioactivity bound in the soils during incubation for 25-181 days

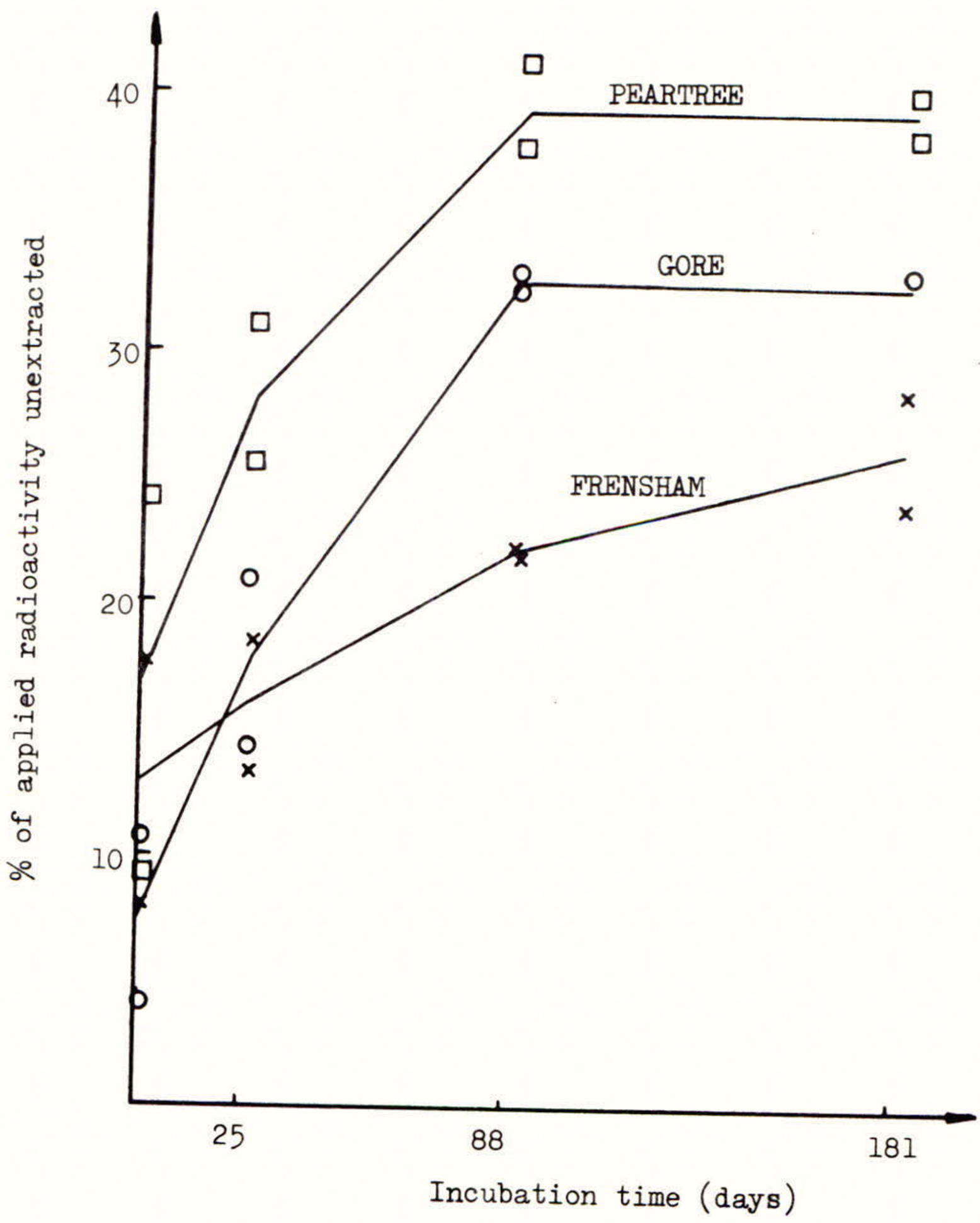


Table 3 presents data for the amounts of carbon-14 in the three soils, and in the clay-size fractions of those at the end of the incubation period. A Packard Sample Oxidiser, Model 306 was used and values were obtained for the total carbon contents (%) and the cation-exchange capacities of the samples. These data show that there was little difference between the concentration of bound residues in the two clay-size fractions. However, the concentration in the clay minerals was always greater than that in the wholesoil. It is not possible to relate the amounts of bound residue directly with the CEC or with the carbon contents of the samples.

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Table 3

Bound residues in the soils and clay components

		Pear Tree	Frensham	Gore
Bound Residues (ppm)	> 2.0 μm	3.47	1.44	2.25
	2.0–0.2 μm	6.73	11.99	5.06
	< 0.2 m	5.73	12.04	6.16
(%)	2.0–0.2 μm	5.53	4.45	6.80
	< 0.2 μm	3.72	3.34	2.07
Total carbon (%)	2.0–0.2 μm	10.4	7.3	12.5
	< 0.2 μm	6.7	6.6	9.0
CEC (meq/100 g)	2.0–0.2 μm	54.8	48.5	33.5
	<0.2 μm	57.4	73.3	44.9

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MEASUREMENT OF HERBICIDE EFFECTS ON NITROGEN FIXATION BY LEGUMES

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Summary The results of four experiments to assess the side-effects of alloxydim-sodium on growth, nodulation and nitrogenase activity of peas are presented. They are used to illustrate the difficulties of designing experiments to assess such side-effects of herbicides on legumes and the problems of assessing the significance of observed effects.

Résumé Les résultats de quatre expériences pour évaluer les effets secondaires de l'herbicide alloxydim-sodium sur la croissance, la production nodulaire et l'activité nitrogénasique du pois servent à illustrer les difficultés rencontrees par ceux que creent de telles expériences ainsi que les problèmes associés à l'évaluation des observations faites.

INTRODUCTION

The requirements of authorities responsible for the registration of pesticides are becoming increasingly stringent and far-reaching. The American Environmental Protection Agency (EPA) Guidelines to Manufacturers (1978) now includes the requirement for data on side-effects on soil micro-organisms. Nitrogen fixation is one of the microbial functions covered and the suggested acceptable protocols include measurements of nitrogenase activity by the acetylene reduction method.

During an evaluation of the selectivity and activity of the herbicide alloxydim-sodium (Richardson and Parker, 1978), it was noted that the herbicide had reduced root weight and nodule numbers of peas. This effect has now been examined in more detail and the results are presented here principally as an illustration of the problems associated with assessment of effects on nitrogen fixation.

METHOD AND MATERIALS

Peas (Pisum sativum var. Dark-Skinned Perfection) were grown as described by Richardson and Parker (1978) for post-emergence selectivity tests, except that Osmacote (15:12:15) at 3.5 g/kg of soil was used instead of John Innes base fertilizer. To try to ensure optimum nodulation and nitrogen fixation efficiency in these growth conditions, each seed was sown onto approximately 1g of peat-based inoculum of Rhizobium leguminosarum (RCR 1045). The inoculum was prepared as described by Vincent (1970). Five seeds were sown in each 12.5 cm pot and seedlings were thinned to leave two uniform plants per pot, with three replicate pots per treatment. Plants were grown in the greenhouse under sodium lights with a day-length of 14-16 h and minimum temperature of 15°C.

The herbicide was applied at the 3-leaf stage (6-leaf in experiment 4) using a laboratory pot sprayer operating at a pressure of 2.1 kg/cm^2 and moving at a constant speed 45cm above the plants. Herbicide application rates were 2 and 4 kg a.i./ha at spray volume of 345 l/ha. The rates suggested by the manufacturer

for experimental purposes range from 1 to 2.5 kg a.i./ha, depending on the target weed.

The effects of the herbicide were assessed 7 to 8 weeks after treatment. Root and shoot dry weights and nodule numbers were recorded. Nodules were excised from the roots using fine forceps, dried and weighed. Nitrogenase activity was assayed (Greaves et al., 1978) on nodulated primary lateral roots which were selected as being representative of the whole root system on the basis of nodule number and appearance.

Four experiments were done during a six month period (February to July, 1978). Experiments 1 and 2 were set up simultaneously but kept in different parts of the greenhouse. Experiments 3 and 4 were also done at the same time, except that seeds in the latter were sown 2 weeks before those in experiment 3.

RESULTS

At the time of spraying a number of plants were harvested in order to determine the variation present. Even though plants were thinned out from five to two per pot before treatment, in an attempt to leave uniform plants, considerable variation was still apparent. Thus the shoot fresh weights of replicate plants varied by a factor of about two in all experiments. Leaf areas were not recorded but a similar degree of variation would be present. No nodules could be detected by eye at this stage of growth.

The results of the four experiments are summarised in Table 1.

Table 1

Effects of alloxydim-sodium on growth, nodulation and nitrogenase activity in peas

	Application rate kg a.i./ha	Experiments 1	2	3	4
Shoot dry wt g/plant	0	1.57	1.82	2.7	5.9
	2	1.34	1.12	2.8	5.8
	4	1.37	1.42	2.9	5.2
	SE^{+}_{-}	0.08	0.13	0.2	0.3
Root dry wt g/plant	0	0.26	0.23	0.55	1.01
	2	0.20	0.16	0.67	1.27
	4	0.17	0.18	0.60	0.93
	SE^{+}_{-}	0.02	0.01	0.04	0.13
Nodule dry wt mg/plant	0	6.6	7.1	83.5	217.1
	2	5.4	4.9	74.8	182.0
	4	2.9	4.3	50.9	259.4
	SE^{+}_{-}	0.7	0.9	7.0	27.0
Nodule number /plant	0	41	38	301	317
	2	33	24	282	443
	4	24	36	233	429
	SE^{+}_{-}	4	7	45	42
Nitrogenase activity nM $N_2/h^{-1}/g^{-1}$ root	0	185	6607	4053	2256
	2	143	5482	1688	4484
	4	116	6950	2576	2502
	SE^{+}_{-}	18	557	223	504

All figures are the means of three replicate pots, six replicate plants.

Shoot Growth Statistically significant reductions of shoot growth ocurred at both dose rates in experiments 1 and 2. Plants in all experiments were green and healthy and showed no symptoms of herbicide damage.

Root Growth In the first two experiments significant reductions in root dry weight were recorded. In contrast, an increase in dry weight ocurred with the low application rate in experiment 3. A similar trend was observed in the last experiment but this was not statistically significant.

Nodules Reductions in nodule dry weight were recorded in the first three experiments especially at the high application rate. The results of experiment 4 did not show this effect. Despite these reductions in dry weight, nodule numbers were significantly reduced only in experiment 1 at the high dose rate. In experiment 4 nodule numbers were significantly increased at both dose rates.

Nitrogenase Activity As with other parameters, statistically significant reductions ocurred at the high rate of herbicide application in experiment 1 and a similar effect was noted, at both rates, in experiment 3. In contrast, in the last experminent the low application rate was associated with a significant increase in acetylene reduction. It is noticeable that, in experiment 1, the nitrogenase activity measured is considerably lower than that in subsequent experiments. These low figures were obtained from plants harvested in dull light conditions. The activities in experiment 2 were measured on the same day but on plants harvested following a short (2 h) period of bright sunshine. Similarly experiments 3 and 4 were analysed on a sunny day with high light intensity.

DISCUSSION

Recently Johnen et al (1978) have highlighted the problems of assaying side-effects of pesticides on legumes. They showed that large standard errors were associated with the results of the acetylene reduction method and that relatively large coefficients of variation were obtained with measurements of nodule numbers and weights. Thus these methods would only detect major differences between treatments. Shoot, root and total plant weights were much less variable and could, therefore, detect smaller differences. Recent work at the Weed Research Organization (Richardson, unpublished) has shown that some of the herbicides recommended for use in peas may affect plant growth. Reductions in shoot dry weight of up to 40 per cent were recorded. There is, however, no evidence from field trials or commercial practice that these recommended herbicides cause any detrimental effects on final yield. Effects on plant growth (or on nodulation and nitrogenase activity) obtained in laboratory experiments, therefore, may not be reflected in the field and the results should not be given undue emphasis.

While the results in this paper show a similar degree of variation to that reported by Johnen et al (1978) they illustrate an additional problem frequently encountered in studies of side-effects of chemicals on soil micro-organisms. That is the lack of reproduceability of repeat experiments. Thus in the first three experiments, the response to the chemical was noticeably different. Indeed, different results were obtained in experiments 1 and 2 which were done simultaneously. In the last experiment (4), the increase in nodulation and nitrogenase activity following treatment is, perhaps, not surprising. The plants in this experiment were twice as large when sprayed as in the other experiments. Leaf area was, therefore, greater and hence so was herbicide retention and, possibly, uptake. Further, the nodulation process would be more advanced, even though nodules were not visible to the naked eye.

Interpretation of such inconsistent results is extremely difficult. The frequency of reductions in growth and nodulation might suggest a potential harmful effect of the chemical. On the other hand, the results showing lack of effect or indeed increased nodulation and nitrogenase activity must be considered also.

The present experiments raise many questions about the practicality of the different means of measuring the side-effects of herbicides on legumes. The labour input to simultaneous nodule counting, nitrogenase assays and weight measurements, for example, is extremely high and this severely limits the numbers of replicates which can be handled. This in turn ffects the precision of the results obtained. In these experiments, the analyses required the services of 4 to 6 members of staff. Even so, the nodule counting,for example, often took four to five days. Obviously, operator error becomes a major factor in these circumstances.

The measurement of nitrogenase activity presents several problems. As activity increases with increasing light intensity,then the time of year at which plants are grown,and the time of day at which they are harvested, will affect the results. One answer is to use growth chambers with controlled light intensity and day length. However, suitable chambers are not always available at centres where the tests are performed. Even where such chambers are available, very large numbers of replicate plants would be required at each sample date to reduce standard errors sufficiently to allow detection of small effects.

Soil conditions may also affect design and interpretation of experiments. It is known that inorganic nitrogen inhibits nodulation and nitrogen fixation (Buckman and Brady,1969). In our experiments fertilizer was used, as it is generally in commercial practice in the U.K., and peas were thus not dependent on nitrogen fixation. Indeed, in other experiments (Greaves and Richardson, unpublished), it was noted that, whereas peas receiving no fertilizer had about 100 nodules per plant at the time of spraying those which were fertilized had none. It could be argued then, that effects of herbicides on nodulation may be of little importance where the crop is grown with nitrogen fertilizer. This is especially so if they occur early in the plant's growth and are corrected at a later stage when nitrogen-fixation assumes more importance to the plant.

It is recognised that the stage of growth at which herbicides are applied may markedly affect the plant's response. On the other hand, frequently it is forgotten that it is extremely difficult to obtain a uniform stand of plants, even in highly controlled experimental conditions. Thus our data show a two-fold variation in shoot fresh weight at spray time with-in each experiment, despite careful thinning of seedlings to achieve uniformity of the test plants. Such variability of the plants will obviously affect spray retention and thus response to the chemical. This will in turn increase the variability of the results and the difficulties of interpretation.

Johnen et al (1978) concluded from their experiments that, of the measurements made, nitrogenase activity, nodule weight, plant weight and total numbers of nodules appeared to be the most reliable indicators of pesticide side-effects. In the present work all these factors did, at some time, indicate side-effects of alloxydim-sodium which were potentially detrimental or beneficial. However, the lack of reproduceability shown in the four experiments casts doubt on the reliability of such methods. Further, the variability of the results, particularly of nodule estimations and nitrogenase activity, severely limits their use without massive replication within experiments. It would seem, therefore, that the most reliable indicator, with the greatest ease of application in practice, is plant weight or yield.

Despite the difficulties, it is clear that the evaluation of side-effects on nodulation and nitrogen-fixation plays an important part in ensuring the safe and efficient use of herbicides in legume crops. Research is continuing at the Weed Research Organization to determine more reliable means of measuring side-effects, with particular reference to effects of time of spraying and of nitrogen fertilization.

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NOTES

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ATP ASSAY FOR DETERMINING PESTICIDE EFFECTS ON MICROORGANISMS

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Summary In conjunction with conventional counting methods, ATP was evaluated as an indicator of relative microbial contents of thirteen soils and six freshwaters. In soils, ATP content compared well with the content and turnover of organic matter, measured by CO_2 evolution during incubation, and in waters with degree of eutrophication.
ATP in soils was also sensitive to changes resulting from natural variation and pesticide-induced stress. Studies on the herbicide 'Ivosit' (at 2.5, 25 and 125kg a.i./ha) and the alcohol-wetter mixture 'Offshoot T'(at 220 and 2,200 l a.i./ha), showed that changes in ATP were explained more fully if population counts were also considered.
The data suggest that ATP assay is useful for determining pesticide effects on microorganisms in natural environments.

Résumé Conjointement avec les méthodes conventionelles de comptage, l'ATP fut évalué comme indicateur des nombres rélatifs de microbes dans treize sols et six eaux douces. La concentration d'ATP dans le sol soutenait bien la comparaison avec la quantité de matière organique et son utilisation mesuré par l'évolution de CO_2 pendant incubation. Dans les eaux douces l'on pouvait faire comparaison avec le niveau d'eutrophication.
La concentration d'ATP dans les sols était sensible aux changements provenant de variation naturelle et de l'application des pesticides. Des études avec l'herbicide "Ivosit" (à 2.5, 25 et 125 kg m.a/ha) et le mélange d'alcool et de dispersant "Offshoot T" (à 220 et 2,200 l m.a/ha) montraient que les changements de quantité d'ATP s'expliquaient mieux si l'on considéraient aussi les comptes de populations.
Les données indiquent que l'essai d'ATP peut être utile pour déterminer les effets des pesticides sur les microorganismes dans les milieux naturels.

INTRODUCTION

When determining the effects of a pesticide on non-target microorganisms the experimental conditions should be as close to the natural environment as possible. Thus a comprehensive series of tests has been developed by the Microbial Ecology Unit at Jealotts Hill (Anderson, 1973, Johnen & Drew, 1977) This paper describes the use of the adenosine triphosphate (ATP) assay as one of these tests.

ATP is found only in living cells (Doxtader,1969) and can be determined using the bioluminescence reaction (McElroy, 1947, Strehler, 1968). Its concentration per cell depends on cell size,mass,and hence taxonomic type (Ausmus,1973) and on physiological state (Lee et al.,1971a and b). Therefore, changes in the ATP concentration of a pure microbial population may be used, in conjunction with total and viable counts, to indicate changes in activity and growth phase. Extraction of ATP from the complex microbial communities in soils and water presents, however, considerable problems of methodology and interpretation of results. In these environments ATP

Table 1

Properties of soils used

Soil	classification	O.M. %	CO_2 (1) from O.M.	pH (in H_2O)	sand (%)	silt (%)	clay (%)
1. Feltwell Fen	peat	65.0	63	6.4	71	19	6
2. Evesham	loam	8.0	45	7.6	51	25	24
3. Tarlton	"	7.5	26	8.0	45	31	24
4. Brooms Barn	sandy loam	4.1	34	9.2	61	19	20
5. Pear Tree	" "	6.4	12	5.9	64	17	19
6. Whiskers	" "	6.1	19	6.1	65	14	21
7. Broadricks	" "	3.1	17	6.5	72	12	16
8. Wisborough Green	silty clay loam	2.6	15	6.6	18	53	29
9. Heath Knap	coarse sand	4.3	nd	6.5	84	3	13
10. Speyer	loamy coarse sand	4.8	nd	7.6	80	7	13
11. Frensham	loamy sand	1.6	14	6.4	82	10	8
12. Lower Sand Field	coarse sand	2.4	5	7.5	91	4	5
13. Lilyfield	" "	1.8	2	5.6	95	3	2

Table 2

Microbial characteristics of soils after 7 days laboratory equilibration

Soil	ATP (µg/g dry soil)	propagules ($\times 10^{-8}$/g dry soil)	ATP/cell (2) (µg $\times 10^{-10}$)	Bacteria ($\times 10^{-6}$/g dry soil)	Actinomycetes ($\times 10^{-5}$/g dry soil)	Fungi ($\times 10^{-4}$/g dry soil)
1. Feltwell Fen	2.8	nd	-	37	55	22
2. Evesham	3.6	57	6	39	7	42
3. Tarlton	2.6	46	6	32	3	50
4. Brooms Barn	2.8	nd	-	32	11	38
5. Pear Tree	1.9	30	6	60	30	20
6. Whiskers	1.5	18	8	nd	12	nd
7. Broadricks	1.1	14	8	19	14	33
8. Wisborough Green	1.3	10	13	nd	12	nd
9. Heath Knap	1.2	11	11	13	15	35
10. Speyer	0.6	5	12	10	28	66
11. Frensham	0.3	2	15	13	12	42
12. Lower Sand Field	0.1	4	25	8	10	60
13. Lilyfield	0.1	1	10	20	5	20

(1) Evolution (mg C) from 100g dry soil during 7 days incubation.

(2) calculated from total propagule numbers.

nd = not determined

concentration does not indicate directly either biomass or activity (Greaves et al., 1973, Kaczmarek et al.,1976) but a combination of both.

Several methods of differing efficiency for extracting ATP from terrestrial and aquatic environments have been reported (Holm Hansen & Booth,1966, Doxtader,1969, Macleod et al., 1969, Lee et al.,1971a,b). Our methods have an extraction efficiency of 70% to 100%. (Davies, unpublished results) which combined with the low half life of ATP in soil (Conklin & Macgregor,1972) and water, makes it a sensitive indicator of changes affecting the microbial community.

Despite problems in the interpretation of results from the ATP assay, it has definite advantages of time and sensitivity when compared with other techniques which rely solely on enumeration to determine pesticide induced changes in mixed microbial communities. Combining ATP assay with enumeration methods enhances the value of each method and enables clearer understanding of pesticidal effects.

In this paper we have presented data which show how ATP concentrations, preferably coupled with enumeration of the main groups of microorganisms, can be used to characterise test systems and to monitor changes within them.

MATERIALS AND METHODS

ATP assay and population counts - The ATP assay was done using a Du Pont Luminescence Biometer and Du Pont reagents (Allen III,1972). Five replicate assays, each of duplicate biometer injections, were done at each sampling time. ATP was measured in soils after filtration of suspensions through a 20 μm mesh and extraction by the modified butanol-octanol method of Anderson & Davies (1973). Water was first filtered through a 250 μm mesh. After collection on 0.45 μm pore size filters ATP was extracted by boiling tris-HCl buffer (Holm Hansen, 1966).

Other biomass measurements were done on the same replicate samples used for ATP assay. The total number of propagules were counted in 1 cm^2 smears of suitably diluted soils or waters. The smears were observed microscopically after staining with the differential fluorescent stain described by Anderson & Slinger (1975) which indicates the numbers of living (not necessarily viable) and recently dead individuals in the community.

Counts of aerobic heterotrophic bacteria,fungi and actinomycetes were made using the dilution plate method with 1.0% w/v peptone as diluent; the selective media for isolation were soil extract (Pramer & Schmidt, 1964) and nutrient agar (Oxoid CM3) for soil and water bacteria respectively, peptone dextrose (Johnson,1957) for fungi, and dextrose casein (Jensen,1930) for actinomycetes. Three dilution series were made from each of the five replicates and plates were incubated for 7 to 14 days at 15° or 20°C in darkness. For algal counts samples were concentrated where necessary by centrifugation and the resultant pellets resuspended and counted in 0.1mm deep haemocytometers by phase contrast microscopy.

Soil Organic Matter Turnover - CO_2 evolution during turnover of soil organic matter was determined as described by Johnen & Drew, 1977.

Soils and Water - Soils (Table 1) from 12 sites were sampled to 25cm. One additional soil a "standard soil" for pesticide evaluation came from W.Germany (1). This had been stored for four years before use. All soils were sieved (2mm), moistened (to 40% of moisture holding capacity), and then stabilized (normally 7 days) at 20°C in darkness (Johnen & Drew, 1977) before testing or pesticide treating.

Water samples were obtained from the top 30cm of 3 ponds and 3 rivers. All measurements were performed on waters within 2 hours of sampling.

Pesticides and Pesticide Treatment - The rates used were: 'Ivosit' 2.5, 25 and 125 kg a.i./ha on Pear Tree and Lilyfield Soils (i.e. 1 x, 10 x and 50 x field rates) 'Gramoxone W' 90 kg a.i./ha on Frensham soil (80 x field rate) and 190 kg a.i./ha on Broadricks soil (170 x field rate). The alcohol-wetter mixture 'Offshoot T' was applied as aqueous solution at 220 and 2,200 l a.i./ha to Evesham soil (1 x and 10 x field rates). In the laboratory, 'Ivosit' and 'Offshoot T' were uniformly dispersed by hand mixing in equilibrated soil samples. In the field, 'Gramoxone W' was applied by watering can.

(1) Provided by LUFA Agricultural Investigation & Research Institute Speyer,W.Germany

RESULTS AND DISCUSSION

Microbial Characterisation of Soils and Water - ATP content and numbers of microorganisms counted in soils and waters are given in Tables 2 and 3. Most values are means of samples taken over several years, so are representative for each site disregarding season. They show that combining ATP assay with population counts indicates the microbial character of a system better than any single method.

Different soils contained different amounts of ATP (Table 2), these amounts being related to the organic matter contents. The loamy soils contained 1.3 to 3.6 µg ATP/g soil while sandy soils contained 0.1 to 1.2 µg ATP/g soil. Soil 10 did not fit this pattern as long storage (4 years) had resulted in lower microbial activity than expected for a soil of relatively high organic matter content. The lower ATP values in the sandier soils were also associated with lower numbers of propagules and hence with higher ATP content per cell. This suggests that either their microbial activity is higher or that their microbial composition is different.

When predicting the possible behaviour of systems, particularly soils, for pesticide studies, ATP content combined with enumeration may be useful. Turnover of organic matter in 11 of the soils (Table 2) shows that although there is not a linear relationship, ATP contents and total propagule counts are fairly good indicators of the soils' relative behaviour. Hence ATP contents of soils may give a quick indication of possible activity and could be used to plan sampling times and predict duration of pesticide tests on functional processes (Johnen and Drew, 1977).

Table 3

Microbial populations in fresh water from ponds and rivers

		ATP/ ml	propagules/ ml	algae/ ml	viable bacteria/ ml
Pond 1	Witley	0.12 ug	1.6×10^8	0.3×10^8	2.7×10^6
2	Popes Manor	0.03 ug	3.4×10^8	$<0.1 \times 10^2$	3.7×10^6
3	Billingbear	2.10 ug	20.0×10^8	7.8×10^8	nd
River 1	Thames	0.14 ng	7.0×10^6	7.0×10^3	1.0×10^6
2	Loddon	0.31 ng	6.0×10^6	$<0.1 \times 10^2$	1.0×10^6
3	Blackwater	0.41 ng	3.0×10^6	$<0.1 \times 10^2$	1.0×10^6

nd = not determined

Pond waters (Table 3) had higher ATP contents than river waters, the most eutrophic systems, Pond 3, having highest values for ATP, total propagules and algae. Correlations between ATP content and microbial numbers are, however, not evident, particularly for river waters.

Changes in ATP Contents of Soils - In addition to characterising different systems, ATP assay combined with enumeration methods show changes within a system. Greaves et al. (1973) and Kaczmarek et al. (1976) reported fluctuations in both ATP content and propagule counts in soils with no clear correlations. Such findings were confirmed by us for 2 laboratory incubated soils (Table 4).

In Lilyfield soil the differences in ATP and in numbers suggest that ATP concentrations might indicate activity changes rather than population changes as usually, the population composition on dilution plates remained relatively constant. The activity and composition of the microbial population of Pear Tree soil was more stable. ATP contents were higher with much less variation during 28 days incubation. Total numbers of propagules and proportions of bacteria, fungi and actinomycetes varied slightly and showed no clear correlations with ATP. The

amount of ATP extracted during this experiment was unusual for this soil, as was the large number of actinomycetes.

Table 4

Microbial content of 2 laboratory incubated soils

Lilyfield Soil	ATP ng/g dry soil	Propagules x 10^7/g dry soil	Bacteria x 10^6/g dry soil	Actinomycetes x 10^5/g dry soil	Fungi x 10^4/g dry soil
Time 0d	13	8	13	28	8
1d	130	18	13	27	6
2d	149	10	13	23	4
4d	127	19	10	19	5
8d	144	14	10	24	7
14d	249	24	12	18	7
24d	149	11	11	20	4
Pear Tree Soil	ug	x 10^8	x 10^6	x 10^6	x 10^4
Time 0d	2.3	54	26	31	13
1d	2.4	40	26	30	11
2d	2.5	36	24	26	10
4d	2.7	42	34	25	13
9d	2.9	23	44	31	13
15d	2.8	16	17	25	12
28d	2.7	21	21	18	12

These results suggest ATP is a sensitive indicator of change in the microflora. The exact nature of the changes can be confirmed only by population counts. If ATP content changes but not numbers, activity has probably altered; if numbers change but not ATP content, then the population composition has altered. A combination of methods therefore enables some changes to be interpreted in a way that is not possible for each method alone.

Effects of Pesticides on ATP content of Soils - As ATP concentration is a sensitive indicator of change in unstressed systems under uniform environmental conditions,in situations of stress,such as may be induced by pesticide treatment, greater changes may be detected and used to predict impact on a system.

In studies during several years, changes in ATP content of soil have been detected following pesticide application. Usually, however, the changes have been transient and of no practical importance. Two chemicals, 'Ivosit' and 'Offshoot T' did show clear effects on the ATP content and microbial populations of soils (Table 5). 'Gramoxone W' is included in Table 5 as this had no effects at abnormally high application rates at any time after treatment.

All statistically significant reductions in both ATP concentrations and in fungal numbers correlated strongly with increasing concentrations of chemical. Such a clear-cut trend is not apparent with total propagule number or numbers of bacteria or actinomycetes. Thus if direct counts had been used to interpret treatment effects, despite the indication of slight reductions in total numbers, no significant differences would have been apparent.

The ATP assay is therefore the best way of quantifying the result of stress in a system. The qualitative results of the stress may be partly elucidated by enumeration methods. Thus,here the reduction in numbers of fungi is an important factor. The detection of differences due to chemical treatment indicates the usefulness of the ATP assay in combination with population counts for monitoring pesticide effects. It must be stressed however, that extreme differences were caused by excessive rates of 'Ivosit' and 'Offshoot T' and that they are not necessarily indicative of results after normal field use.

Table 5

Effect of laboratory applications of 'Ivosit' and 'Offshoot T' and field application of 'Gramoxone W' on microbial populations of soils

Pesticide	Soil	rate (x FR)(1)	time	ATP µg	Total propagules x 10^{-8}	bacteria x 10^{-6}	actinomycetes x 10^{-5}	fungi x 10^{-4}
'Ivosit'	Peartree	x 50	1 month	0.14*	35	40*	184	3*
		x 10		0.80*	34	29	140	8*
		x 1		2.28	42	19	173	13
		0		2.65	42	21	179	12
	Lilyfield	x 50	3 months	0.02*	0.8	3	3	1*
		x 10		0.03*	1.0	8*	103*	2*
		x 1		0.04*	1.2	1	11	4*
		0		0.11	1.6	3	23	6
'Offshoot T'	Evesham	x 10	14 days	0.37*	66	550*	15*	0*
		x 1		0.61*	81	151*	12*	1*
		0		1.42	85	39	37	9
'Gramoxone W'	Frensham	x 80	7 years	0.47	6	10	45	21
		0		0.52	6	9	39	20
	Broadricks	x170	7 years	0.79	11	23	50	24
		0		0.80	11	25	46	10

(1) FR = recommended field rate

* significantly different from untreated soil at P = 5%

CONCLUSION

Characterisation of microbial content of 13 soils and of 6 freshwaters with a range of properties, showed that ATP assay, in combination with other methods of measuring the population, can provide useful information on the relative natures of test systems. ATP concentration in the soils compared well with their content and turnover of organic matter and might thus be used as a guide to soil behaviour when planning pesticide studies. Also ATP was a sensitive indicator of changes occurring within a system. Observed changes in ATP concentrations with time agreed with the findings of others (Kaczmarek et al.,1976; Greaves et al., 1973). In three soils, reduced ATP concentrations were observed following application of 'Ivosit' and 'Offshoot T'. The results indicate that ATP assay may be used as an indicator of changes in a test system and so for measuring potentially harmful effects on microorganisms caused by pesticides. Its inclusion in test programmes (Anderson,1973; Johnen & Drew, 1977 and Atlas et al., 1978) for determining side effects is thus advantageous.

The ATP assay is complex and expensive. Thus the nature of the test system and the information required should be carefully considered before adopting it as a routine test.

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THE EFFECT OF PARAQUAT INDUCED DEATH OF AQUATIC PLANTS ON THE HETEROTROPHIC ACTIVITY OF FRESHWATER BACTERIA

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Summary Bacterial heterotrophic activity was estimated before and after treatment of a weedy pond with paraquat. Activity increased during the period of plant decompostition and again during a subsequent algal bloom. It then returned to predicted levels but one year after treatment there was some indication that activity was greater than predicted.

Résumé L'activité hétérotrophique des bactéries a été estimée avant et après avoir traité, avec paraquat, un étang, couvert de mauvaises herbes. L'activité s'est augmentée pendant que les plantes se décomposaient et encore, plus tard, lorsque les algues fleurissaient. Elle est revenue alors aux niveaux attendus mais une année après le traitement il y avait quelque indication que l'activité était plus grande qu'on avait envisagée.

INTRODUCTION

Many studies have examined the effects of paraquat and other herbicides on the chemistry and the eucaryotic organisms of freshwaters. However few studies of changes to bacteria in similar field experiments have been made. These studies have shown that herbicide-induced plant death increased populations of heterotrophic bacteria in water, sediment (Fry et al, 1973) and on plant surfaces (Ramsay and Fry, 1976). One study of epiphytic bacteria (Fry and Ramsay, 1977) has shown that population increases were accompanied by an increase in bacterial heterotrophic activity. The aim of the work presented here was to examine and compare the changes of planktonic and epiphytic bacterial heterotrophic activity after paraquat was used to kill a natural population of aquatic macrophytes.

METHOD AND MATERIALS

The site used was a pond (surface area, 0.19ha; mean depth, 1.08m) on the Coedarhydyglyn estate near Cardiff (Grid reference ST 10387537). It contained a dense stand of aquatic macrophytes dominated by Apium inundatum (L.) Rchb. f., Cladophora glomerata (L.) Kütz, Elodea canadensis Michx., Lemna minor L. and Potamogeton natans L.

All samples were taken at 0900h between 12.3.74 and 27.7.76 and were used within 3h of sampling. Bulked surface water samples, composed of six 700ml aliquots, were taken aseptically at weekly intervals for most analyses. Heterotrophic activities for glycollate and acetate were analysed approximately fortnightly before herbicide treatment. Representative samples of plant material were taken at approximately monthly intervals. Samples for all variables were taken more frequently close to herbicide application between 10.6.75. and 29.7.75. Paraquat was applied as Gramoxone S on 17.7.75 to reach a final concentration of 1.04 mg l^{-1}.

Water samples were shaken 20x by hand and filtered through 380µm pore size bolting nylon before use. Epiphytes were removed from 60g fresh weight of plant material by treating for five minutes in a Colworth Stomacher - 400 (A.J. Seward & Co. Ltd., London) with 300ml of sterile distilled water. Plant debris was removed from this suspension with 380µm bolting nylon, washed with 300ml of sterile distilled water and the 10,000g pellet from this suspension, which contained the epiphytes, was washed three times to remove excess dissolved organic material.

Bacterial heterotrophic activity was determined from measurements of $^{14}CO_2$ mineralised from ^{14}C-organic substrates by methods similar to those of Fry and Ramsay (1977) except that between 5 and 10ml of water or epiphyte suspension was used instead of plant parts. The substrates were (U - ^{14}C) glucose, (1 - ^{14}C) glycollic acid and (U - ^{14}C) acetic acid (Radiochemical Centre, Amersham) at concentrations between 6 and 500 µg l^{-1}. Occasionally 1000 µg l^{-1} was used for very active samples. Maximum potential mineralisation rates (Vmax) and turnover times (Tt) were estimated by regression analysis of standard Michaelis-Menten plots.

Algal biomass was estimated from cold methanol extracted chlorophyll a (Talling and Driver, 1963). Plant biomass was assessed by cropping from three representative 0.5 m^2 quadrats. Dissolved oxygen was measured by the Winkler titration (Golterman and Clymo, 1969) and dissolved carbohydrate by the method of Gerchakov and Hatcher (1972) using 1l of 0.2 µm membrane filtered water concentrated to about 20ml under vacuum at 90°C. Total bacteria were counted by epifluorescense microscopy (Ramsay and Fry, 1976) using 0.45 µm black Sartorius membrane filters.

Predicted values and confidence intervals before and after paraquat treatment were estimated by time series analysis (Humphrey et al., 1977). All means were compared by analysis of variance; the calculations for these comparisons and for the multiple regression analysis were done with the Statistical Package for the Social Sciences (Nie et al, 1975). All data, except that of temperature, pH and dissolved oxygen, were transformed by $\log_{10}$ for all computations except for the mean values. The notation +xd has been used in the text to define the number (x) of days after paraquat treatment.

RESULTS

Paraquat treatment killed all the aquatic plants and by +25d most of the plant biomass in the pond had decomposed. Only 5.6 g dry weight m^{-2}, i.e. 1.8% of the original standing crop, remained. Plants were not found after this time until the next year when the maximum seasonal biomass, 29 g dry weight m^{-2}, was only 6.5% of the average maximum seasonal biomass for the previous two years. As the plants decomposed an algal bloom, mainly Euglena sp., developed and lasted from +17 until +68d. The algal biomass averaged 86.7 µg chlorophyll a l^{-1} during this period and reached a maximum value at +43d of 153.8 µg chlorophyll a l^{-1}. This was over 16 times the expected value.

There were increases in the heterotrophic activity of planktonic and epiphytic bacteria after herbicide treatment for all substrates (Table 1). Typical results presented (Fig. 1) show that increases in heterotrophic activity of planktonic bacteria, as indicated by increase in Vmax and decrease in Tt (Fig. 1a,c), occurred rapidly reaching an initial peak at +12d on average. Subsequently minima occurred at about +29d before increasing again to a second maximum at about +45d. The Vmax of epiphytic bacteria also increased rapidly (Fig 1b) to a maximum at +21d which was close to the end of the period of plant decomposition after which estimates could not be made. Usually the maximum activities after treatment were significantly greater than the values predicted from the previous year's data (Table 1), the degree of increase varying between x 7.5 and x 126. The times of occurrence of the first values significantly greater than the predicted Vmax suggest that the activity of

Table 1

Changes in heterotrophic activities of planktonic and epiphytic bacteria after paraquat treatment

Variable	Days after treatment on which occurred the first value significantly greater than the predicted value	the first peak value	the second peak value
Planktonic Vmax,			
glucose	3	15	43
glycollate	6	15	47
acetate	6	8	47ns
Planktonic Tt,			
glucose	2	11	36
glycollate	6	15	40ns
acetate	4	8	54
Epiphytic Vmax,			
glucose*	2	21	-
glycollate	6	21	-
acetate	5	21	-

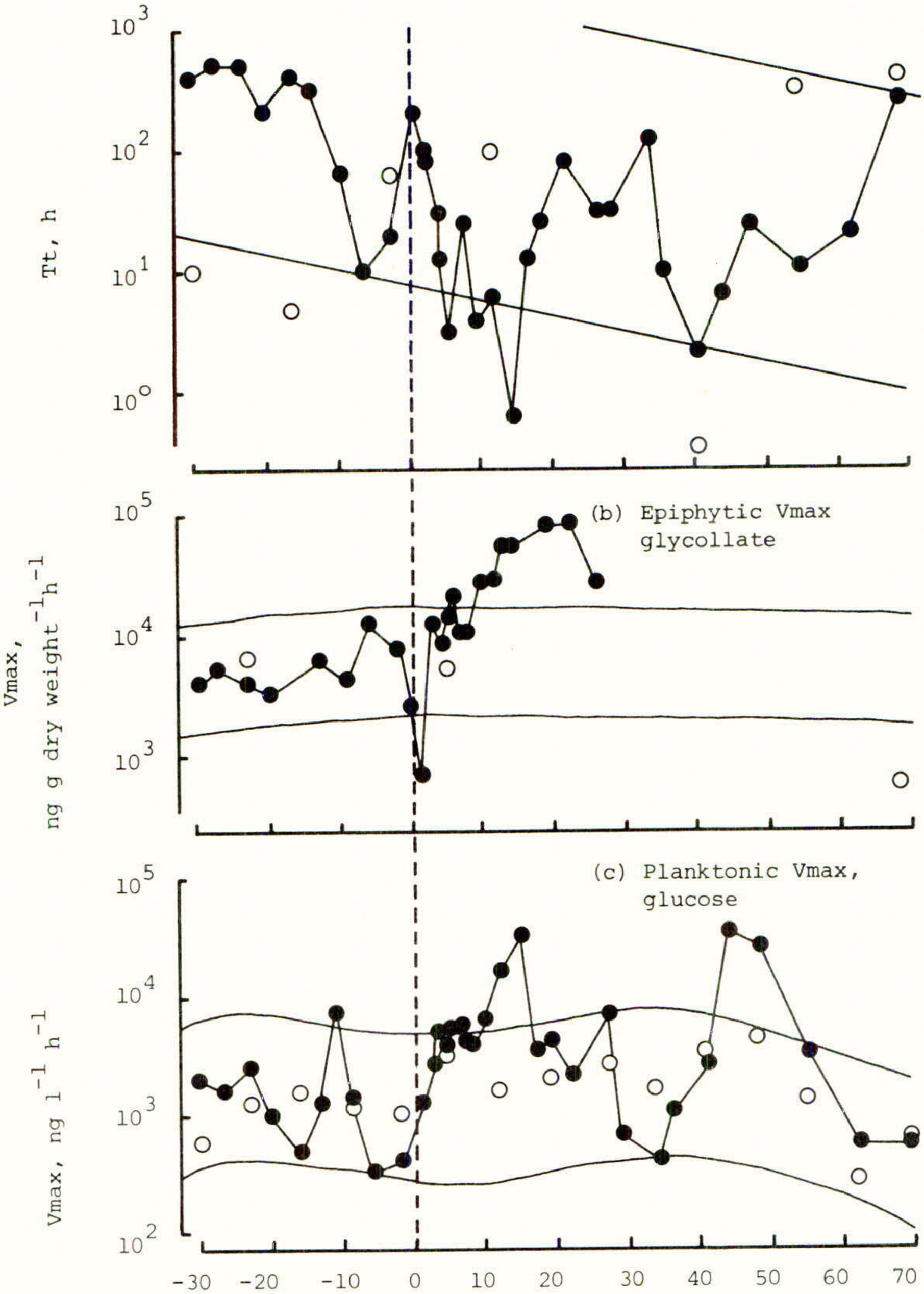
Predicted values and their 95% confidence intervals were estimated by time series analysis. All peak values significantly greater ($P < 0.05$) than predicted unless stated (ns); minimum values are used for turnover times; * = time series analysis showed no significant cyclic events so predicted values are the mean of the $\log_{10}$ of the pre-treatment results and 95% confidence intervals are calculated from $t_{(0.05)}$ x standard deviation of the results.

epiphytic bacteria increased faster than the planktonic organisms (Table 1). Minimum values for dissolved oxygen, 0.1 mg l^{-1} on +7d and +47d, both caused coarse fish deaths and were seperated by a maximum value of 14.1 mg l^{-1} on +22d.

The activity of planktonic bacteria always returned to within the 95% confidence intervals for predicted levels by +70d (Fig 1) and usually remained within these intervals until the following February (Fig 2) when measurements temporarily stopped. However many of the Tt values for glycollate and acetate were outside these levels. In the early summer of the year after herbicide treatment the planktonic and epiphytic bacterial heterotrophic activity in the pond was greater than expected as shown for the Vmax of acetate for planktonic bacteria (Fig. 2). Calculations for all variables indicate that 94% of the observations at this time were above predicted values and 69% were above the upper 95% confidence interval for the predictions. Also for all but one variable the mean value for this time was greater than the mean value obtained from similar dates before treatment, however of these only three of the possible nine differences were significant ($P < 0.05$).

Figure 1

Changes in heterotrophic activity, with upper and lower 95% confidence intervals of planktonic and epiphytic bacteria close to paraquat treatment in 1975 (●) and for the corresponding dates in the previous year (O)



The values of Vmax/bacterium (Table 2), calculated from the heterotrophic activity estimates and total bacterial counts, show that this variable increases for most substrates when estimates for the period of rapid plant decay, between +3 and +25d, are compared with the three week period immediately prior to herbicide application and all similar dates before treatment. As 75% of these comparisons showed significant differences the observed increases in Vmax/ bacterium are undoubtably real. There was little increase in this variable after the plants had decomposed.

Data obtained before paraquat treatment were used to calculate empirical multiple regression models of the activities of planktonic bacteria. Water temperature, pH, concentrations of dissolved oxygen and dissolved carbohydrate and total bacterial counts were used as regressor variables as these were likely to influence directly bacterial activity. With all the activity estimates, except the Tt for glucose, between 69% and 77% of the variance in the data used to construct the model was accounted for by the multiple regression equations (Table 3). In the Vmax prediction equations temperature accounted for about 86% of the total variance explained whilst the analogous figure for total bacterial count was 0.3%. These were the only important common features in the equations. These equations were then

Figure 2

Changes of Vmax for acetate mineralisation by planktonic bacteria before and after paraquat treatment

Solid horizontal lines are values predicted by time series analysis and their 95% confidence intervals. The date of herbicide application is indicated by the vertical broken line.

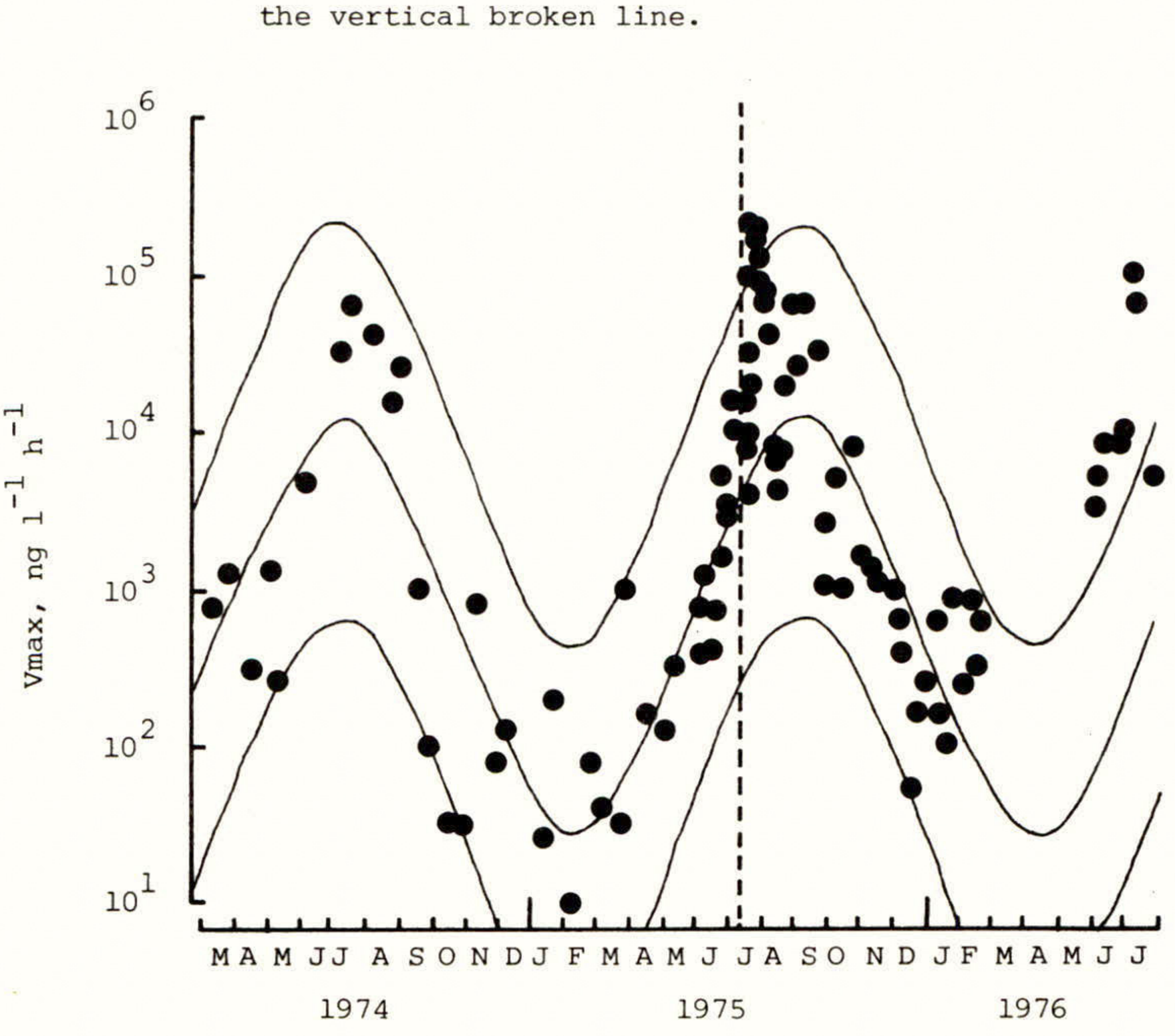


Table 2

Potential mineralisation rates (Vmax) per bacterium for substrates before and after paraquat treatment.

Variable	Means of Vmax/bacterium x 10^{-10}, µg bacterium^{-1} h^{-1}, for the period of rapid plant decay after treatment	for all similar dates before treatment	the three weeks immediately before treatment
Planktonic			
glucose	0.95	0.41	0.38*
glycollate	3.68	1.25	1.01**
acetate	10.52	3.57*	1.69***
Epiphytic			
glucose	0.92	0.32***	0.36
glycollate	0.83	0.85*	0.99
acetate	1.09	0.40***	0.44**

Mean value before treatment significantly different from the mean value during the period of rapid plant decay, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Table 3

Percentages of variance (R^2, %) in heterotrophic activities of planktonic bacteria explained by multiple regression models calculated from before treatment data

Dependent variable		R^2 (%) before treatment	for 10 weeks after treatment	for 54 weeks after treatment
Vmax,	glucose	70.2	33.2	70.1
	glycollate	69.4	10.5	75.5
	acetate	77.2	12.1	65.8
Tt,	glucose	30.5	0.0	0.0
	glycollate	73.8	1.6	26.9
	acetate	73.9	30.0	59.3

used to predict the activities for two periods after herbicide treatment and the percentage of the variance in the actual post-treatment data that was explained by the equations was calculated. The Vmax predictions for all substrates showed better agreement (Table 3) between the real and predicted values for the 54 week period after treatment than the 10 week period. This trend was also followed with the Tt predictions but these equations were generally not as successful in predicting either set of post-treatment values.

DISCUSSION

The results show a rapid rise in planktonic and epiphytic bacterial activity by +6d, peak values for these activities averaging at +12d and +21d respectively and planktonic activity minima following at +29d. As these events correspond with the period of plant decomposition it is likely that the heterotrophic activity of bacteria caused this decomposition and was probably responsible for the associated reduction in dissolved oxygen. These conclusions accord with those of work referred to in the introduction. The increase in Vmax/bacterium shows that activity increases more than total numbers during plant decay indicating a real increase in the relative activity of bacteria associated with plant decomposition which did not occur during the algal bloom.

The second burst of planktonic bacterial activity appeared after +29d, it peaked at about +45d and had ended by +70d. This time sequence agreed well with the occurrence of the algal bloom which was maximal at +43d and terminated by +68d. It was probably this bloom which, in the absence of much bacterial activity, caused the dissolved oxygen peak at +22d. This rise in bacterial activity was probably stimulated by release of organic substrates by the algae. Although similar relationships between phytoplankton and bacterial activity have been noted before (Gocke, 1977) the resulting drastic decreases in oxygen concentration have not.

Probably the activity values that were outside the confidence intervals calculated by time series analysis long after treatment deviated because the estimated periods of the seasonal cycles varied markedly from the 52 week period which would logically be expected. This argument is supported by the small number of significant differences observed with comparisons between the mean activities during early summer the year after treatment and similar dates before treatment. This possibility can only be resolved by calculation of time series models over many years which might better describe typical seasonal cycles.

The multiple regression analysis showed objectively that the factors which influenced planktonic bacterial activity during plant decomposition and the algal bloom were different from those which influenced activity throughout the rest of the work. This was because the equations explained more of the variance before treatment, and in the 54 weeks after treatment, than during the 10 week period immediately after paraquat application. The analysis also suggested that temperature was likely to be a major factor controlling planktonic Vmax in all but the immediate post-treatment period; this agrees with work from other aquatic habitats (Gocke, 1977). Total bacterial numbers apparently had little effect on Vmax. There was no evidence that the herbicide had any direct effect on the heterotrophic activity of the bacterial populations in this environment.

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