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THE EFFECT OF ACEPHATE (ORTHENE®) ON THE GROWTH AND NUTRIENT UPTAKE OF TWO AQUATIC BACTERIA

by

Gary L. Williams

B.E.S. The University of Waterloo 1975

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in the Department
of
Biological Sciences

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SIMON FRASER UNIVERSITY

1982

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Approval

Name: Gary L. Williams

Degree: Master of Science

Title of Thesis: The effect of acephate (Orthene®) on growth and nutrient uptake of two aquatic bacteria

Examining Committee:

Chairman: Dr. Robert C. Brooke

Dr. L. J. Albright and Dr. P.C. Oloffs, Co-Senior Supervisors

Dr. Glen H. Geen

Dr. James E. Rahe, Public Examiner

Date approved April 5, 1982

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The effect of acephate (Orthene®) on growth and nutrient uptake of two aquatic bacteria

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ABSTRACT

The effects of high (1000 ppm) and low (1 ppm) concentrations of acephate on the rate of growth and nutrient uptake by 2 bacterial lake isolates grown in batch culture were examined. One of the isolates, identified as *Pseudomonas fluorescens*, was not affected by acephate at either concentration. The other, *Chromobacterium lividum*, showed an increase in doubling time, decreased maximum cell yield and reduced cell size when grown in the presence of 1000 ppm acephate. The observed effects of 1000 ppm acephate on *C. lividum* growth were reversible if the cells were reinoculated into fresh culture medium, and adaptation did not occur with repeated acephate treatments of 1000 ppm. The rate of total [^{14}C]glucose and [^{14}C]amino acid uptake by *C. lividum* was reduced by the high acephate concentration, primarily due to less ^{14}C-label being incorporated by the cells. Transport studies with [^{14}C]cycloleucine, an amino acid analogue that is not metabolized, indicated that the high acephate concentration did not affect membrane transport. It appears from these experiments that the higher concentration of acephate may affect biosynthetic processes of *C. lividum*.

DEDICATION

To Bonny,

with Affection and Gratitude

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A. INTRODUCTION

The widespread useage of pesticides has led to study of the effects of these compounds on aquatic microbial communities (Ware and Roan 1970; Baughman and Paris 1980). Acephate (O,S-dimethyl acetylphosphoramidothiolate) is an organophosphorus insecticide of particular interest in British Columbia because of its potential use to control spruce budworm. It is an acetylcholinesterase inhibitor but differs from many insecticides in having low mammalian toxicity and high water solubilty (Eto 1974; Martin 1974).

Heterotrophic bacteria are important in the utilization of both dissolved and particulate organic compounds in aquatic ecosystems because they concentrate nutrients in a form which can be used by other organisms (heterotrophic production) and mineralize organic substances to inorganic molecules for use by autotrophs. Heterotrophic production includes direct consumption of bacteria by zooplankton and other aquatic animals (Monakov 1972; Gophen _et al_. 1974; Peterson _et al_. 1978), or indirect consumption through a detritus based food chain (Mann 1972; Saunders 1977; Rich and Wetzel 1978; Cummins 1973; Berrie 1976).

The importance of bacteria in heterotrophic production is due to the fact that bacteria possess efficient transport mechanisms which function at very low substrate concentrations (Wright and Hobbie 1965,1966). These active transport systems are associated with the cytoplasmic membrane and at least three

distinct classes of nutrient transport systems have been described (Wilson and Smith 1978). In one of these classes of systems chemical alteration of the substrate occurs during transport, a process termed group translocation. This class does not operate in most obligate aerobes (Romano 1970; Dills *et al.* 1980). In the other two classes molecules are transported across the cytoplasmic membrane without chemical modification. One uses membrane bound carriers while the other possesses soluble binding proteins (Neu and Heppel 1965). Costerton *et al.* (1974) speculate that aquatic bacteria, which are predominantly Gram negative, have evolved efficient binding protein/intracellular degradative enzyme systems which function well at low substrate concentrations.

The objective of this research was to study bacterial nutrient membrane transport as influenced by acephate under laboratory conditions. Very little is known about the effects of acephate on bacteria. Focht and Joseph (1974) did not find any marked effect from repeated applications of acephate (20 ppm) on soil microbial numbers, ammonification, nitrification, sulfur oxidation or respiration. Bacteria isolated from sewage and soil were able to utilize acephate as their sole phosphorus source, although it was unsuitable as a carbon source (Rosenberg and Alexander 1979). They also found that cell-free extracts of two soil *Pseudomonas* species catalysed the disappearance of acephate, but ionic alkyl phosphorus compounds were not used because they were unable to penetrate into the cells. The only

published study showing the effect of aquatic microbiota on acephate was by Szeto *et al*. (1979) who found that autoclaving water samples prior to acephate addition greatly decreased the rate of acephate break-down, strongly suggesting microbial degradation.

The results reported herein describe the effects of acephate on the growth and nutrient uptake of two lake isolates tentatively identified as *Chromobacterium lividum* and *Pseudomonas fluorescens*.

B. MATERIALS and METHODS

Collection and Identification of Bacterial Isolates

Lei Lake (49°35'N,123°51'W), a coastal dystrophic lake situated at an elevation of 701 m with a surface area of 2.1 ha and a maximum depth of 11.5 m was selected for sampling. On July 10, 1979, water samples were taken from a depth of 0.5 m using 100-mL glass bottles. The samples were returned to the laboratory within several hours of sampling and 0.1-mL portions were spread on nutrient agar (Difco) plates and incubated at 15°C (approximate ambient temperature) for 1 week. Colonies were randomly selected and streaked until pure. Isolates were identified using morphological, cultural, physiological and biochemical characteristics following the diagnostic keys of Shewan *et al*. (1960) and Cowan and Steel (1970).

Morphological and cultural characteristics were evaluated as follows. Cultural characteristics were observed on nutrient agar plates. Motility was determined by observing growth in semi-solid (0.4%) agar and the results interpreted according to Tittsler and Sandholzer (1936). Cell morphology was observed under oil immersion using a Zeiss standard WL research light microscope and, the Gram stain used was the Hucker modification (Society of American Bacteriologists 1957).

Physiological parameters were studied using the following

techniques. Aerobic growth was evaluated after one week incubation at 4 and 37°C on nutrient agar plates, while anaerobic growth potential was determined by streaking nutrient agar plates with each culture and incubating them in a GasPak 100™ Anaerobic System (Becton, Dickinson and Company, Mississauga, Ontario) for 1-2 weeks. The media of King et al. (1954) were used to demonstrate the production of fluorescent pigments (pyocyanin and fluorescin) which were detected using UV light.

Biochemical tests were conducted using the following methods. Oxidative and fermentative utilization of glucose was studied using the method of Hugh and Leifson (1953) and acid production from glucose was tested by incubating cultures at 15°C in glucose basal salts media with phenol red indicator. Gelatin liquifaction was demonstrated by stabbing gelatin butts and incubating at 15°C for 1-2 weeks. The oxidase test of Kovacs (1956) was used, except that 0.1% ascorbic acid was added to the reagent to control autoxidation (Steel 1962). The catalase and litmus milk tests followed standard techniques (Gagnon et al. 1959; Cowan and Steel 1970).

Growth Experiments

Two hundred-mL cultures of *Chromobacterium lividum* isolate[1] and *Pseudomonas fluorescens* isolate were grown in 500-mL Erlenmeyer flasks at 15°C and at a shake rate of 100

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[1] "Isolate" following a species name refers to a Lei Lake study organism, differentiating it from the type species.

strokes/min in a reciprocating incubator. The glucose-nitrogen (GN) minimal culture medium contained glucose, 2.89 mM; NH_4Cl, 2.73 mM; Na_2HPO_4, 2.94 mM; $MgSO_4(7H_2O)$, 0.34 mM; nitrilotriacetic acid, 0.18 mM; KCl, 2.91 mM; and trace elements dissolved in distilled water (Bell 1976).

Culture medium was inoculated to 1% of its volume with a 46 h culture and concentrations of 1 or 1000 ppm technical acephate (98% active ingredient) were added to the flasks 15-30 min after inoculation. To obtain concentrations of 1000 ppm, 200.2 mg of acephate was added directly to culture flasks; while 0.2 mL of an aqueous acephate solution containing 1.0 mg/mL acephate were added to obtain concentrations of 1 ppm. Acephate (1000 ppm) was also added to sterile GN medium to test for microbial contamination of the insecticide. Each flask was prepared in triplicate except for _P. fluorescens_ isolate growth experiments which were in duplicate.

Growth rates were determined from optical density (OD) measurements taken at 540 nm using a Carl Zeiss PMQII spectrophotometer. One 2.5-mL aliquot from each of 3 flasks containing _C. lividum_ isolate culture, and 2 aliquots from each of 2 flasks containing _P. fluorescens_ isolate culture were measured per concentration.

Cell counts of _C. lividum_ isolate cultures were made using the acridine orange direct cell count (AODC) technique of Hobbie _et al._ (1977), which is briefly described here. Nuclepore membrane filters (0.2-μm pore size and 25-mm diameter) were

pre-stained in a 0.2% solution of irgalan black in 2% (v/v) acetic acid for 6-12 h, and rinsed in distilled water prior to filtration. A 2-mL sample was treated with 0.2 mL of a 0.1% acridine orange aqueous solution for 2 min and then filtered by vacuum (125 mm Hg). The filter was placed on a microscope slide, a drop of Cargille type B immersion oil placed on it and a cover slip placed on the filter. High bacterial numbers in the samples made dilutions of up to 1000x with GN medium neccessary to facilitate counting.

Counts were made using a Zeiss standard WL research microscope fitted with a IV FL epifluorescence condenser, a 100 W halogen lamp, a 450-490 band-pass filter, a FT 510 beam splitter, and a LP 520 barrier filter. At least 200 bacterial cells were counted from each of a minimum of 10 randomly chosen microscope fields, and the total calculated by extrapolation.

Bacterial cell sizes of the *C. lividum* isolate were monitored during exponential growth. Photomicrographs of two fields per filter, as prepared for the AODC, were taken after 32 and 51.5 h of the growth (Fig. 2) using Kodak Tri-X pan black and white film (400 ASA) with an exposure of 25-30 sec. The graphical statistical method of Cassell (1965) indicated that sizing 50 randomly chosen cells from each photomicrograph would provide a representative sample. The length and width of 300 cells were measured using a micrometer for each concentration at both sampling times.

The pH of 1000 ppm acephate-treated and untreated cultures

of C. lividum isolate was monitored to observe possible acephate-pH effects.

Uptake of ^{14}C-nutrients

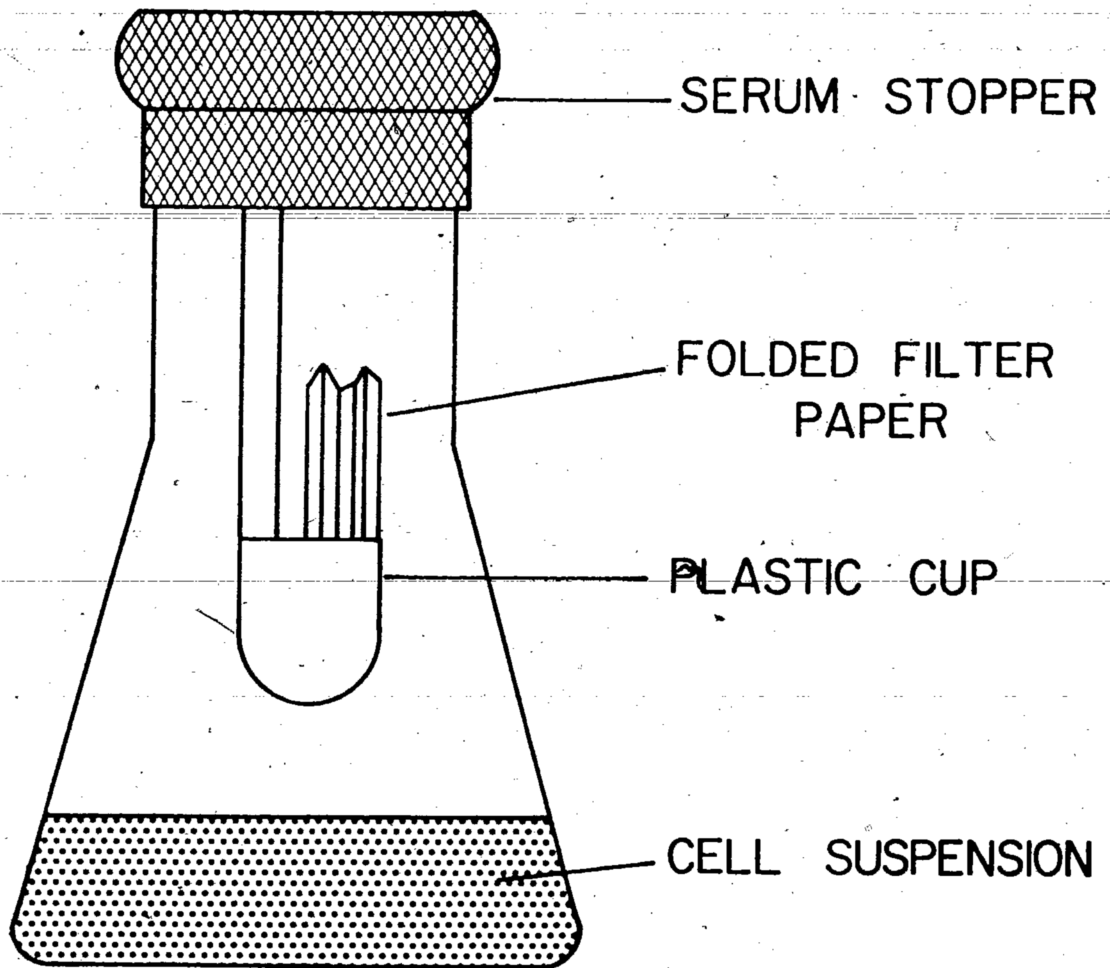
Cultures grown to an OD 540 nm of 0.3 were harvested by centrifugation at 10,400 g for 10 min at 4°C in a Sorvall RC2-B refrigerated centrifuge. The resulting pellet was resuspended in glucose-free GN medium (GFN) and recentrifuged. The washed cells were then resuspended in 150 mL of GFN broth and left for 1 h at 15°C prior to radioactive substrate addition. Acephate was added as descibed for the growth experiments or, it was added with the radioactive substrates to observe any immediate effects of the insecticide.

The radioactive carbon substrates were prepared as follows. D-[^{14}C(U)]glucose, diluted to a specific activity of 0.9 uCi/mM, was added to each 5-mL suspension of C. lividum isolate to yield a final concentration of 2.8 x 10^{-7} mM. The specific activty and final concentration of [^{14}C]glucose used in the P. fluorescens isolate uptake experiments were 1.8 uCi/mM and 5.4 x 10^{-7} mM, respectively. Uptake of a L-[^{14}C(U)]amino acid mixture consisting of ala, arg, asp, glu, his, ile, leu, lys, phe, pro, ser, thr, tyr, val, and gly (New England Nuclear) was also tested. The original mixture was diluted to a specific activty of 10 uCi/mg C and added to 5-mL samples to yield a final concentration of 1.96 x 10^{-2} mg C.

The rates of uptake were determined according to Hobbie and

Crawford (1969). This involved addition of 5 mL of cell suspension to each sterile 25-mL Erlenmeyer flask which was shaken at 100 strokes/min in a Dubnoff shaking water bath at 15°C. Killed controls were prepared by adding 0.4 mL of 36% formalin to the suspension. Each flask was capped with a serum stopper fitted with a plastic cup (Kontes Co.) containing a 25-mm x 51-mm piece of folded Whatman No. 1 filter paper (Fig. 1). The ^{14}C-nutrients, with or without acephate, were injected through the serum stoppers using 1-mL plastic syringes and 21 gauge hypodermic needles. At the end of 3- or 5-min incubation periods, 0.2 mL of 2 N H_2SO_4 were injected into each flask to stop uptake and remove $^{14}CO_2$ from solution. At the same time 0.2 mL of phenethylamine were injected into the plastic cup saturating the filter paper to absorb the $^{14}CO_2$. The flasks were then shaken for 1 h to collect all $^{14}CO_2$, after which the filter paper was added to a vial containing 10 mL of PCS II scintillation cocktail (Amersham Corporation). The remaining culture was filtered through a 47-mm diameter 0.22-μm pore size Millipore filter, washed with 10 mL of GFN, and the filter placed in a glass scintillation vial containing 10 mL of PCS II cocktail. The vials were left overnight to dissolve the filters and allow chemiluminescence to subside before being counted on a Beckman LSC-8000 scintillation counter. Quenching was corrected for by the external standards ratio method.

Fig.1. Apparatus used to recover respired $^{14}CO_2$ onto filter paper moistened with phenethylamine.


SERUM STOPPER
FOLDED FILTER PAPER
PLASTIC CUP
CELL SUSPENSION

Transport of [^{14}C]cycloleucine

Cycloleucine was chosen to investigate transport because past studies in this laboratory have shown that it is not metabolized. To ensure that cycloleucine was not utilized as as a substrate for growth, cultures of C. lividum isolate were prepared and grown as described above, except that cycloleucine (1-aminocyclopentane-1-carboxylic acid) replaced glucose as the carbon substrate in the GN medium. Microbial degradation of [^{14}C]cycloleucine (Amersham Corporation) was tested by measuring the mineralization of the substrate using the technique of Hobbie and Crawford (1969), as described previously. Absence of growth or respired $^{14}CO_2$ by cultures grown with cycloleucine as the substrate provided evidence that this compound was not metabolized and could be used to study transport independent of metabolism.

The rate of cycloleucine uptake was determined as follows. Acephate (1000 ppm) treated and untreated cells from 46 h cultures were each centrifuged and washed in GFN medium and then left for 1 h at 15°C. Two 75-mL aliquots, one of which was killed with 6 mL of 36% formalin, were placed in separate sterile flasks. To each flask 4 mL of [^{14}C]cycloleucine diluted to specific actvity 1 mCi/mM was added (10^{-5} M final concentration). At 3 min intervals 5-mL aliquots were removed and vacuum filtered (125 mm Hg) through 0.22-μm pore size 47-mm diameter Millepore filters. The filters were immediately washed with 10 mL of GFN medium, placed in scintillation vials

containing PCS II scintillation cocktail, and left to dissolve overnight before being counted in the scintillation counter.

The influx-efflux (transport in and out of cells) or chase experiments followed the method of Masuda and Albright (1978). Cultures were washed three times with GFN medium and suspended in 10 mL of the medium in an ice bath until needed. The suspension was diluted to an OD 540 nm of 0.2 with modified GN transport medium (GN medium with 400-μg chloramphenicol/mL). Each of three 50-mL Erlenmeyer flasks received 25 mL of the above suspension and was placed in a shaker water bath at 15°C. The contents of one of the flasks were killed by the addition of 0.4 mL of 36% formalin and served as the killed control. To each of the flasks 1 mL of radioactive cycloleucine solution was added (final concentration 10^{-5} M), with the contents of one of the flasks being diluted at 43 min with cold cycloleucine (0.01 M final concentration). All flasks were sampled at specific intervals by removing 1-mL aliquots which were filtered, rinsed and placed in PCS II scintillation cocktail prior to counting.

Statistical Analysis

Statistical analysis of the data was performed by computer using the MTS ANCOVA/ANCOVA program adapted by Greig and Osterlin (1978). This program uses Duncan's Multiple Range Test to distinguish significance following the initial ANOVA/ANCOVA when there are more than 2 means. When the ANCOVA indicated that regression lines were not parallel, or when Duncan's Multiple

Range Test was not sensitive enough, Scheffe's Simultaneous Confidence Intervals were determined using the Midas statistical package (Statistical Research Laboratory 1976). All figures in the text, with the exception of Figure 1, were drawn using computer generated regression lines.

C. RESULTS

Identification of Bacterial Isolates

The results of the morphological, cultural, physiological and biochemical tests of the Lei Lake isolates and the type species characteristics are shown in Table 1. The *C. lividum* isolate exhibited similar morphological, cultural and physiological characteristics with the type species, although biochemical test results were often ambiguous. The glucose oxidative and frementative and glucose acid tests gave ambiguous or variable reactions. Kovacs oxidase test proved difficult to interprete with the *C. lividum* isolate because the purple bacterial pigment interferred with reading a positive purple color change. The *P. fluorescens* isolate gave more definitive test results; only the litmus milk test was inconclusive.

Growth Experiments

The growth of *C. lividum* isolate in GN broth containing 0 (control), 1 and 1000 ppm acephate is shown in Fig.2. No change from the control was observed in the doubling time (the time required for a change in OD from 0.1 to 0.2), maximum cellullar yield or morphology in cultures exposed to 1 ppm acephate, but exposure to 1000 ppm acephate did cause differences. Maximum cellular yields were greater in the control than in the 1000 ppm

Table 1. Morphological, cultural, biochemical and physiological characteristics of Lei Lake isolates compared to type species (Buchanan and Gibbons 1974).

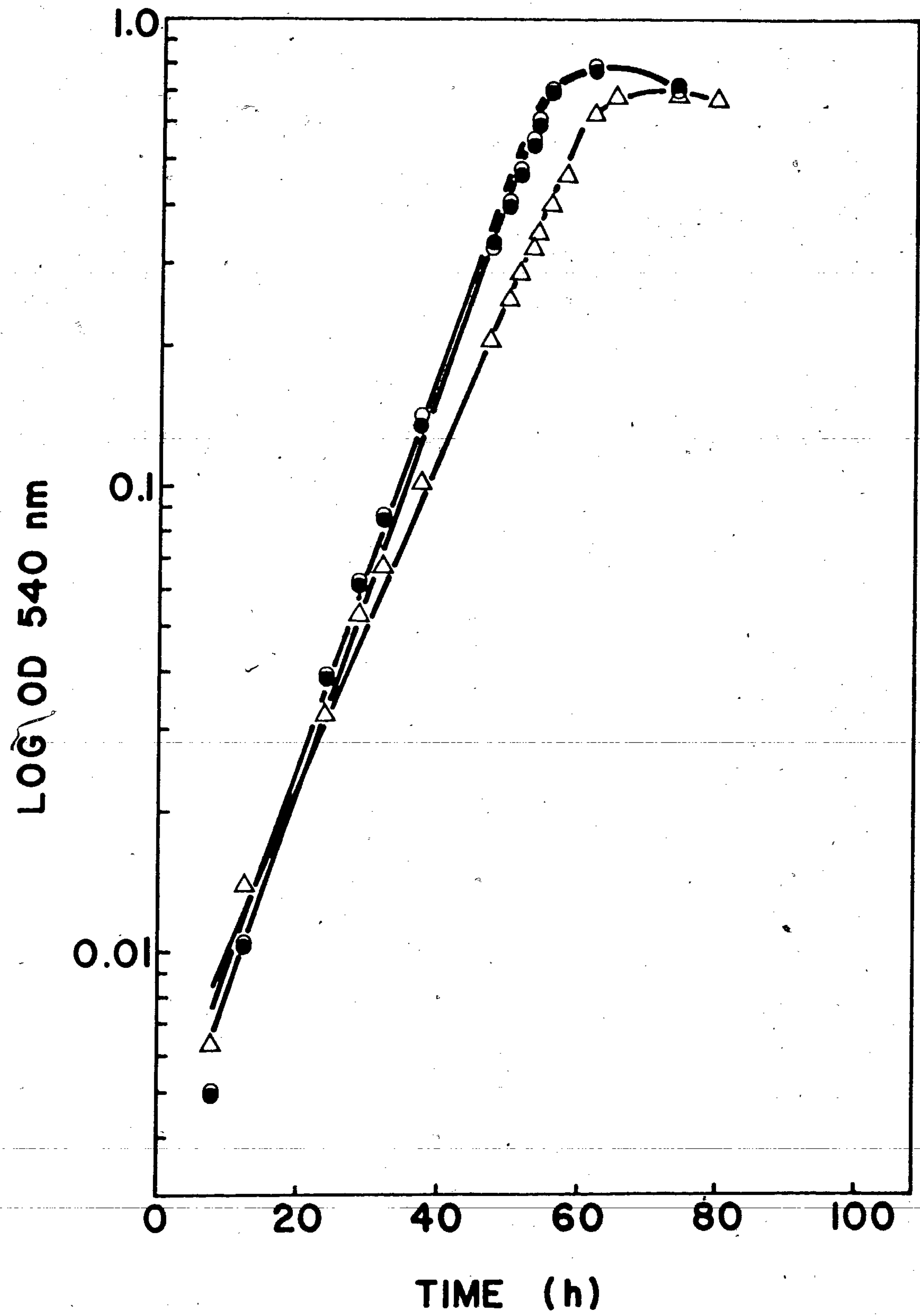
Character	*C. lividum*		*P. fluorescens*	
	type	isolate	type	isolate
<u>Morphological and Cultural</u>:				
size (μm)	0.8 x 2.5-6	0.7 x 2.3	0.7 x 2.5	0.7 x 2.3
shape	rod	rod	rod	rod
motility	motile	motile	motile	motile
colony appearance	round, purple	round, purple	round	irregular
Gram reaction	−	−	−	−
<u>Physiological</u>:				
growth at 4°C	+	+	+	+
growth at 37°C	−	−	−	−
obligate aerobe	+	+	+	+
Fluorescin pigment	−	−	+	+
<u>Biochemical</u>:				
glucose (oxidative)	+	±	+	+
glucose (fermentative)	−	±	−	−
glucose acid	+	±	+	+
Kovacs oxidase	±	±	+	+
catalase	+	+	+	+
gelatin liquefaction	−	−	+	+
litmus milk peptonized	−	−	+	±

\+ = positive

− = negative

± = unclear reaction

Fig.2. Effect of 0 (○), 1 (●) and 1000 (△) ppm acephate on the growth rate of *C. lividum* isolate (see Appendix 1). The equations of the lines used to calculate doubling times are:
control, logOD=0.0429(time)-2.5088;
1 ppm, logOD=0.0428(time)-2.5148;
1000 ppm, logOD=0.0355(time) -2.357


1.0
0.1
0.01
LOG OD 540 nm
0
20
40
60
80
100
TIME (h)

treatment; OD of 0.79 to 0.69, respectively. The medium pH dropped from 7.5 to 6.3 during growth in GN broth with or without acephate (Apps.2 and 3).

The linear portion of the growth curves from 8 to 54 h (Fig.2) were analyzed by a 1-way ANCOVA and Scheffe's test. There was a significant difference ($P<.05$) between the 1000 ppm treatment and both the control and 1 ppm treatments, but not between the control and 1 ppm treatment ($P>.05$). Regression lines for the linear or exponential growth period were used to calculate doubling times. The doubling time of the control culture was 7.02 h compared to 7.04 and 8.48 h for cultures exposed to concentrations of 1 and 1000 ppm acephate respectively.

Length and width measurements for *C lividum* isolate were analyzed by a 1-way nested ANOVA and Duncan's Multiple Range Test (Table 3). Width measurements were not significantly different ($P>.05$) for any of treatments. Cultures exposed to 1000 ppm acephate showed a significant ($P<.05$) decrease in cell length (compared to the control and 1 ppm treatment) but no significant diference ($P>.05$) was indicated between the control cultures and those exposed to 1 ppm acephate.

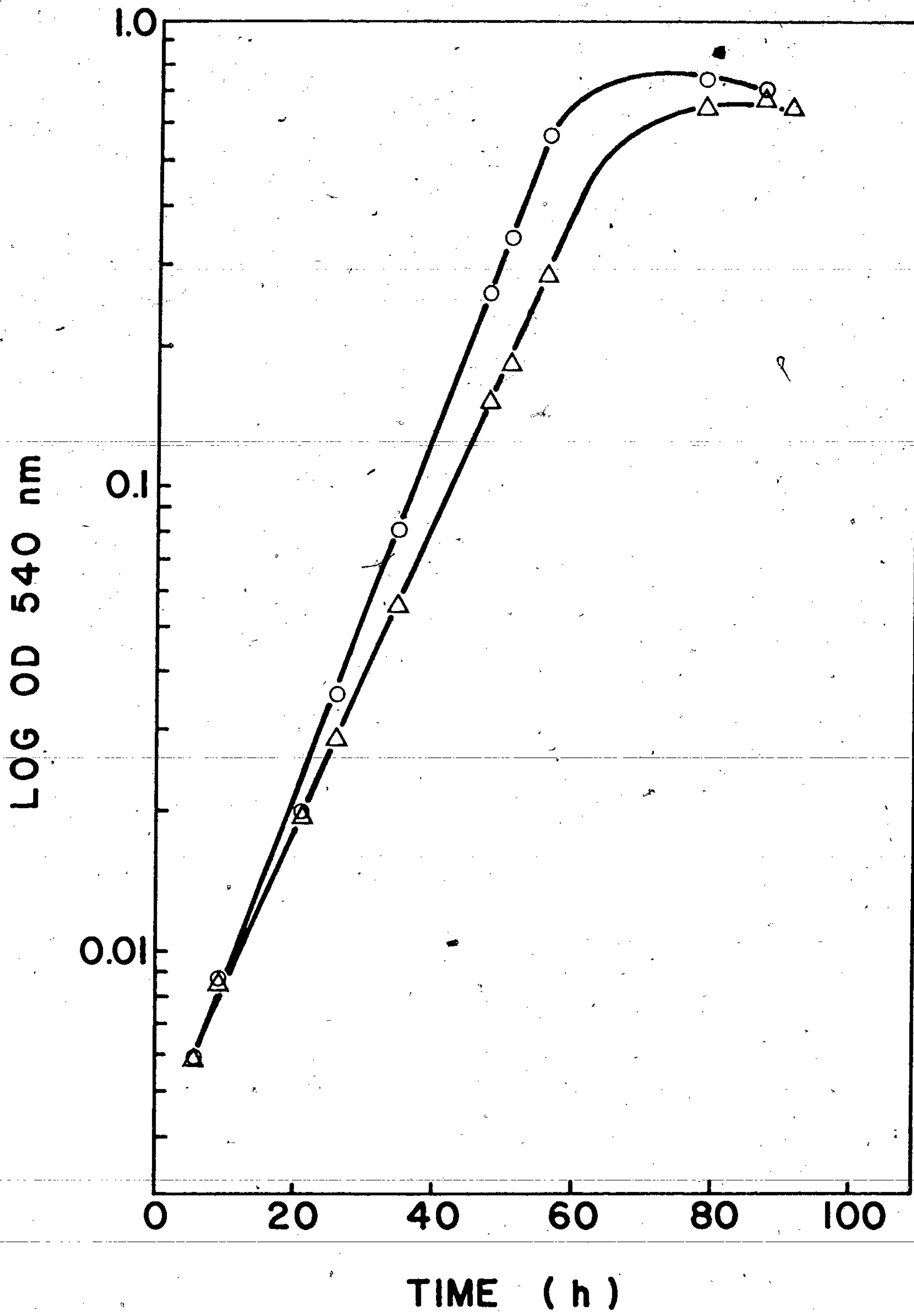
Results of growing *C. lividum* isolate, previously grown in GN medium containing acephate (Fig.2:1000 ppm), in acephate-free medium or in medium containing 1000 ppm acephate are shown in Fig.3. Analysis of the linear portion of the growth curves from 5 to 50 h by ANCOVA revealed a significant difference ($P<.05$)

Table 2. Effect of 1 and 1000 ppm acephate on the length and width (± 1SD) of C. lividum isolate cells (n = 300) after 32.0 and 51.5 h of growth.

Time	Treatment	Length (μm)	Width (μm)
32.0	control	1.98 ± 0.34	0.53 ± 0.09
	1 ppm	1.96 ± 0.36	0.54 ± 0.08
	1000 ppm	1.76 ± 0.31*	0.48 ± 0.07
51.5	control	1.93 ± 0.32	0.39 ± 0.05
	1 ppm	1.84 ± 0.33	0.40 ± 0.05
	1000 ppm	1.63 ± 0.26*	0.39 ± 0.04

*P < .05 (compared to control and 1 ppm)

Fig.3. Growth rate of C. lividum isolate, previously grown in 1000 ppm acephate, in the presence of 0 (○) and 1000 (△) ppm acephate (see Appendix 4). The equations of the lines used to calculate doubling times are:
control, logOD=0.0389(time)-2.442;
1000 ppm, logOD=0.0328(time)-2.3875

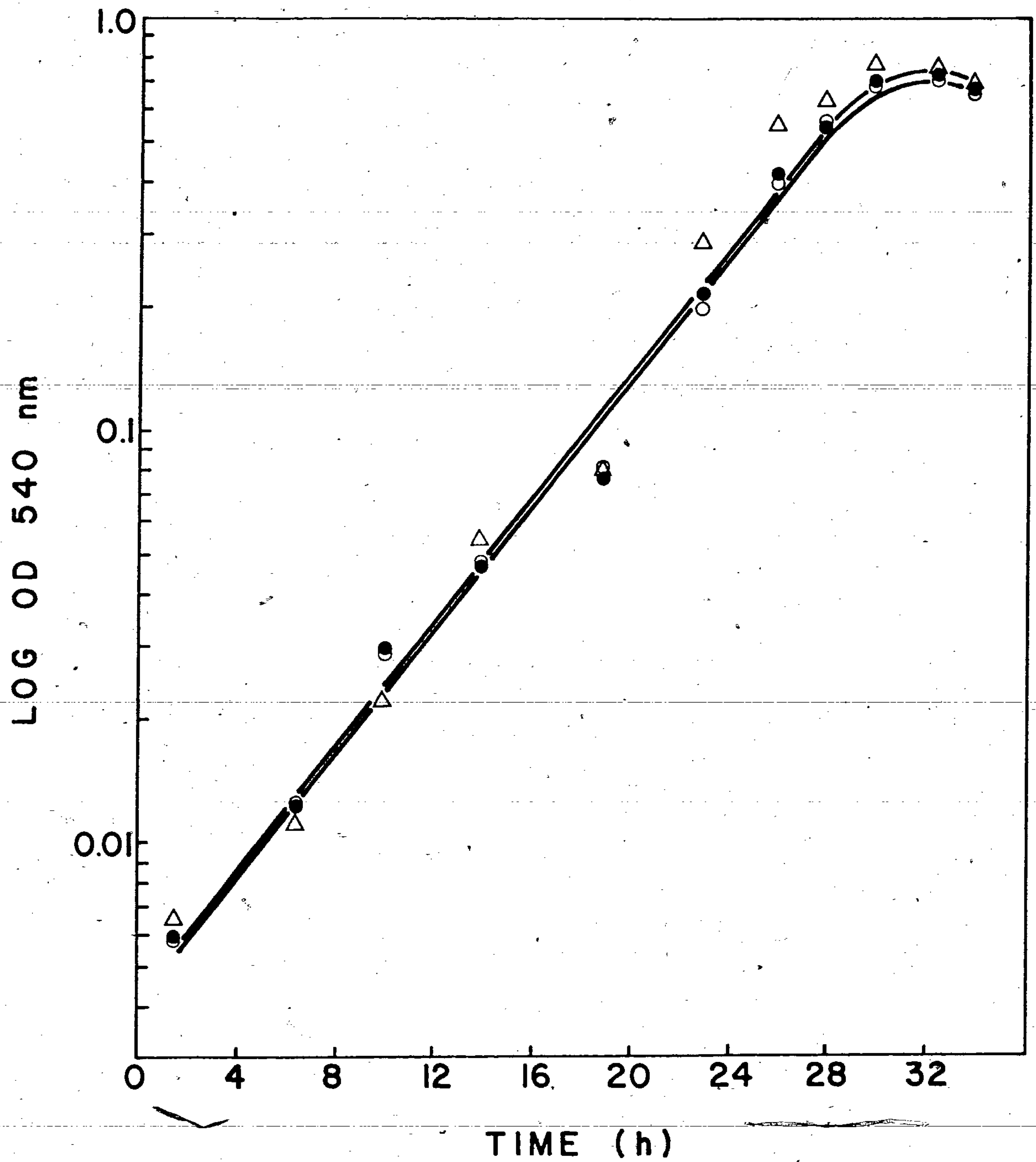

1.0
0.1
0.01
LOG OD 540 nm
0
20
40
60
80
100
TIME (h)

between treatments. Cultures grown in medium without acephate had a doubling time of 7.74 h compared to 9.18 h for those exposed to 1000 ppm acephate. These doubling times are within 10% of those calculated from cultures shown in Fig.2, and the difference is probably caused by experimental conditions rather than acephate.

C. lividum isolate cell numbers, measured using AODC, varied considerably between replicate counts, and made detection of inter-treatment differences difficult (Apps.5 to 7). It was necessary to dilute cultures up to 1000 times for counting which, combined with cell clumping may have contributed to the variability.

P. fluorescens isolate growth was not affected by acephate (Fig.4). The data were more variable than for _C. lividum_ isolate (Fig.2) probably because this species had more tendency to clump in the GN medium. For these reasons AODC enumeration was not attempted. Comparison of the linear portion of the growth curves (1.5 to 26 h) using 1-way nested ANCOVA and Duncan's Multiple Range Test showed no significant difference among treatments (P>.05). During this period of exponential growth the doubling time was about 4 h for cultures in all treatments.

Fig.4. Effect of 0 (○), 1 (●) and 1000 (△) ppm acephate on the growth rate of P. fluorescens isolate (see Appendix 8). The equations of the lines used to calculate doubling times are:
control, logOD=0.075(time)-2.392;
1 ppm, logOD=0.075(time)-2.393;
1000 ppm, logOD=0.075(time)-2.364


1.0
0.1
0.01
LOG OD 540 nm
0
4
8
12
16
20
24
28
32
TIME (h)

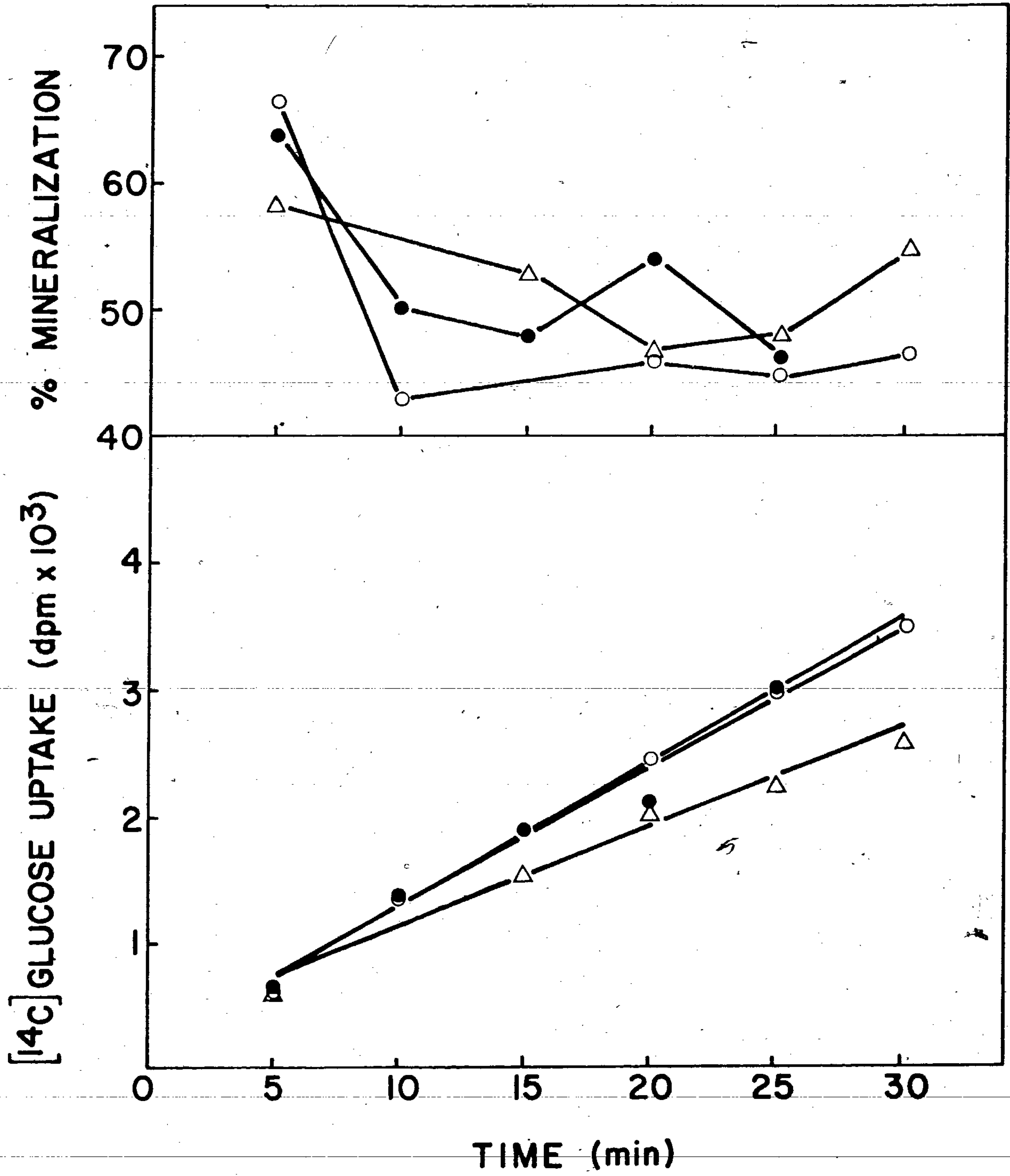
Uptake of ^{14}C-nutrients

C. lividum isolate exhibited linear aerobic uptake (including respired CO_2) of [^{14}C]glucose over the 30-min incubation period (Figs.5 and 6), compared with 18 min for [^{14}C]amino acids mixture (Figs.7 and 8). *P. fluorescens* isolate had rapid [^{14}C]glucose uptake rates so an 18-min incubation was used (Figs.9 and 10), but amino acid uptake was not assayed.

Per cent mineralization (that portion of total uptake respired as CO_2) was measured as an indicator of stress since environmental factors, such as pesticides may stress bacteria and increase CO_2 evolution. The data (Figs.5 to 10) do not clearly indicate higher levels of CO_2 evolution in the 1000 ppm acephate treated cells. The high rate of mineralization before 10 min, which was typical of most uptake experiments, probably resulted from cell suspension preparation (e.g. washing, starving, etc.).

C. lividum isolate cultures grown in the presence of 1000 ppm acephate showed lower rates of [^{14}C]glucose total uptake than either the control or cultures exposed to 1 ppm acephate (Fig.5). Analysis of the data by ANCOVA and Scheffe's test showed that there was a significant difference ($P<.05$) between the 1000 pppm treatment and both the control and 1 ppm treatments. No difference was indicated between the control and 1 ppm treatment ($P>.05$). In contrast, ANCOVA of the total uptake data for cultures exposed to acephate at the time of substrate addition (Fig.6) did not reveal a significant difference ($P>.05$)

Fig.5. Rate of total [^{14}C]glucose uptake and % mineralization for C. *lividum* isolate cell suspensions prepared from cultures grown in the presence of 0 (○), 1 (●) and 1000 (△) ppm acephate (see Appendices 9; 17 to 20).


70
60
50
40
% MINERALIZATION
4
3
2
1
[14C] GLUCOSE UPTAKE (dpm x 10³)
0
5
10
15
20
25
30
TIME (min)

between the control and either treatments. Concentrations of 1000 ppm acephate affected total uptake by reducing the amount of cell associated radioactivity without appreciably altering the % mineralization.

The uptake of amino acids by C. lividum isolate is shown in Figure 7 for cultures grown in acephate, and Figure 8 for cultures treated with acephate at the time of substrate addition. The data have been transformed by graphing uptake versus log time to simplify statistical calculations. Analysis of the total uptake data (Fig.7) by ANCOVA and Duncan's Multiple Range Test revealed that there was a significant difference ($P<.05$) between intercepts among treatments, but not slopes, between the 1000 ppm treated cells and both the control and 1 ppm treatment, but no significant difference ($P>.05$) between the control and 1 ppm treatment. The % mineralization was much greater in the 1000 ppm treatment than in the control or 1 ppm treatments. For cultures treated with acephate at the time of substrate addition (Fig.8) an ANCOVA and Duncan's Multiple Range Test indicated that there was no significant difference ($P>.05$) between slopes or intercepts between the treatments.

In contrast to C. lividum isolate total glucose uptake by P. fluorescens isolate was not affected when cells were grown in the presence of acephate (Fig.9), or treated with the insecticide at [^{14}C]glucose addition (Fig.10). ANCOVA of the total uptake data for both experiments indicated that there was no significant difference ($P>.05$) between treatments.

Fig.6. Rate of total [^{14}C]glucose uptake and % mineralization for *C. lividum* isolate cell suspensions prepared from cultures grown without acephate additions which were treated with 0 (○), 1 (●) and 1000 (△) ppm acephate at the time of glucose addition (see Appendix 10).

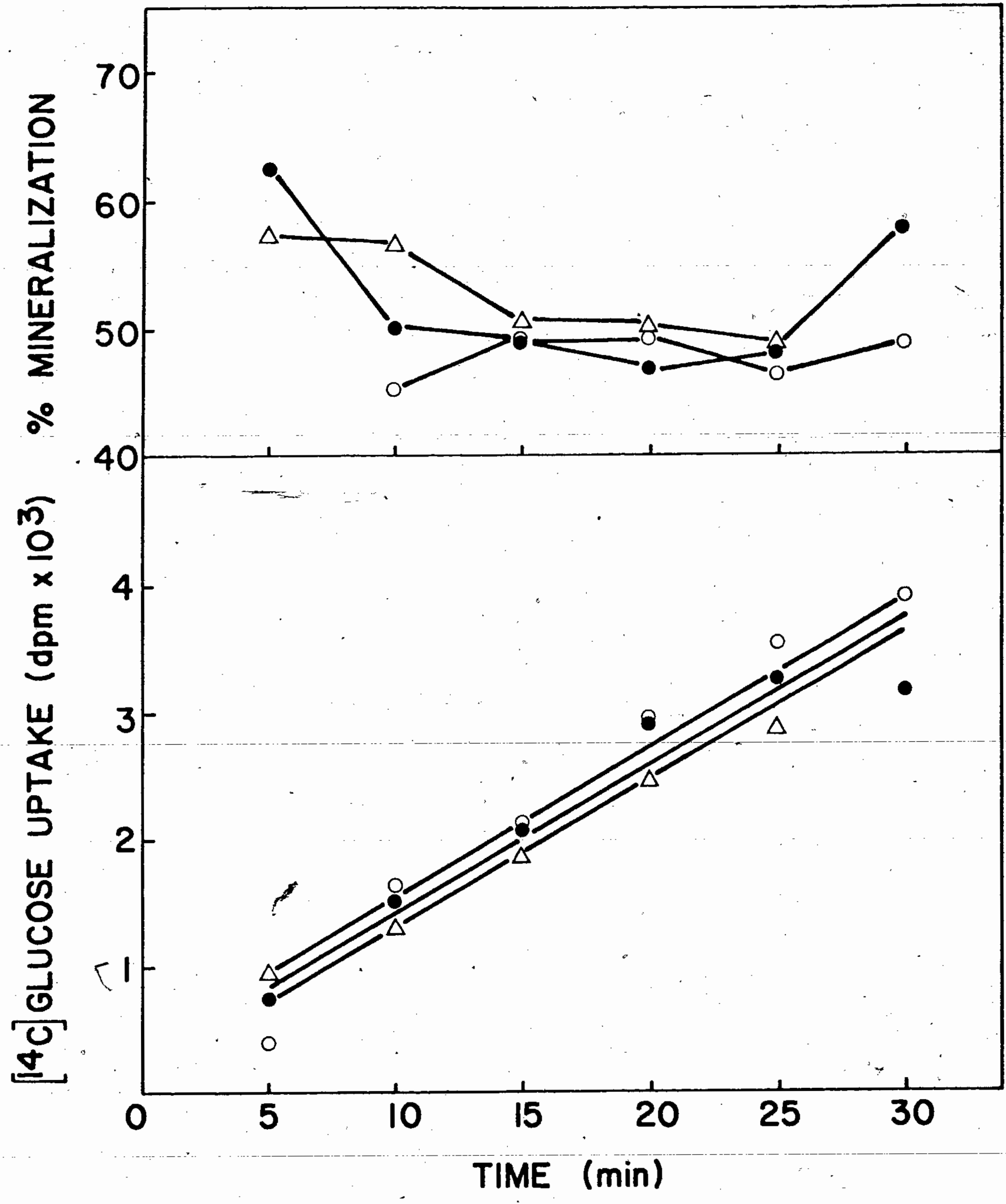

% MINERALIZATION
70
60
50
40
[14C] GLUCOSE UPTAKE (dpm x 10³)
4
3
2
1
0
5
10
15
20
25
30
TIME (min)

Fig.7. Rate of total [^{14}C]amino acids mixture (A.A.) uptake and % mineralization for *C. lividum* isolate cell suspensions prepared from cultures grown in the presence of 0 (○), 1 (●) and 1000 (△) ppm acephate (see Appendices 11, 21 and 22).

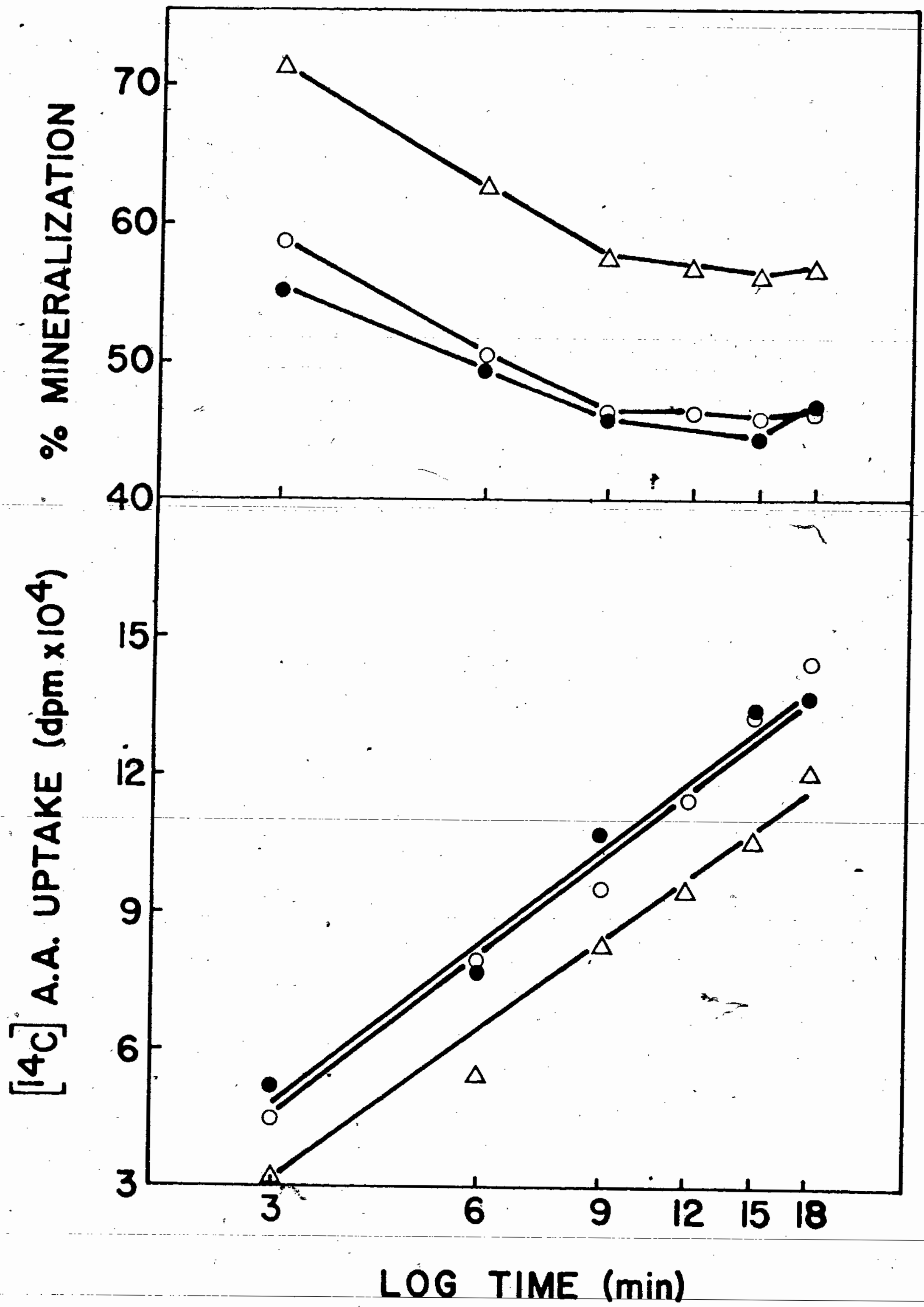

% MINERALIZATION
70
60
50
40
[14C] A.A. UPTAKE (dpm x 10^4)
15
12
9
6
3
3
6
9
12
15
18
LOG TIME (min)

Fig. 8. Rate of total [^{14}C]amino acids mixture (A.A.) uptake and % mineralization for *C. lividum* isolate cell suspensions prepared from cultures grown without acephate additions which were treated with 0 (○), 1 (●) and 1000 (△) ppm acephate at the time of amino acid addition (see Appendix 12).

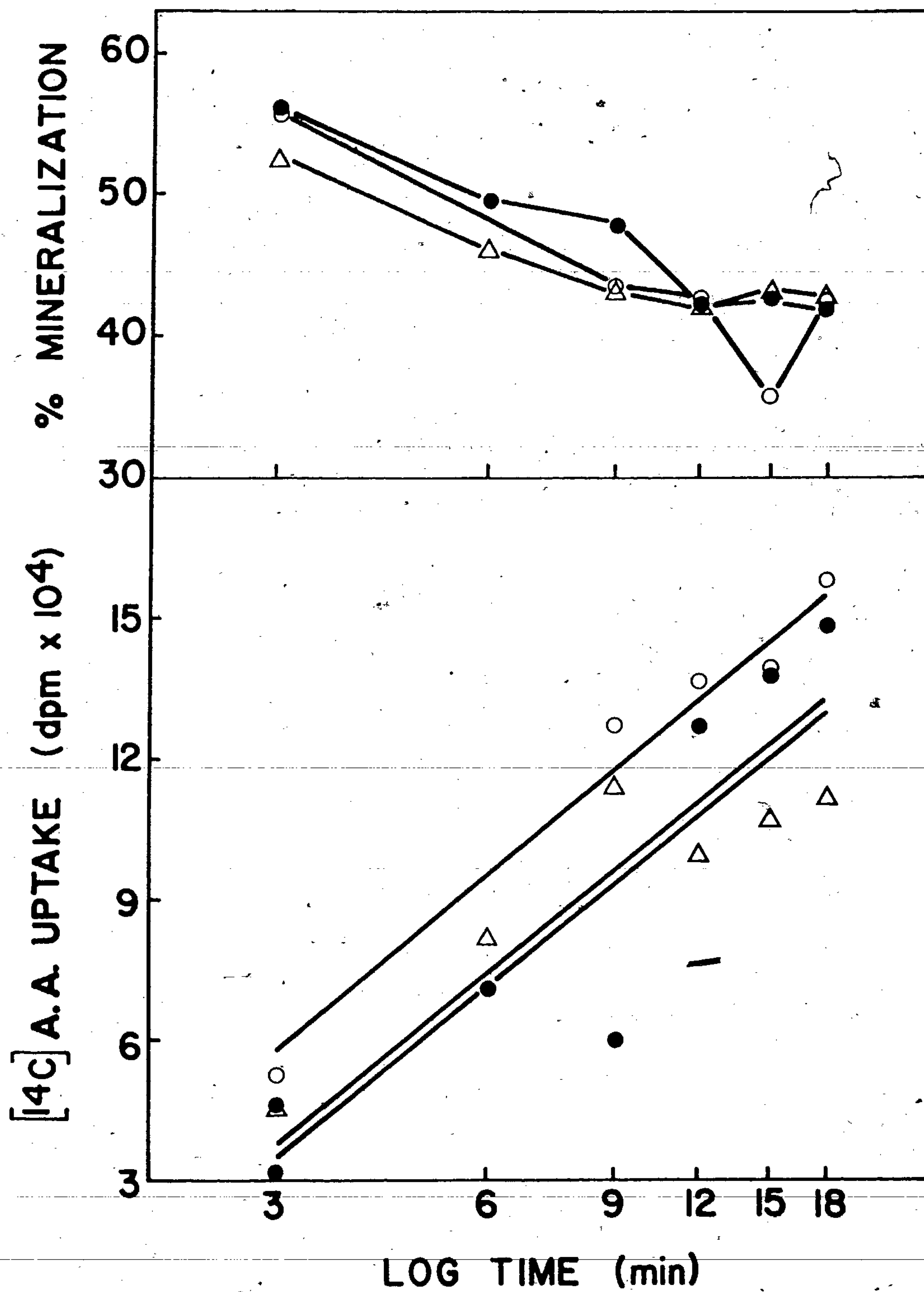

% MINERALIZATION
60
50
40
30
[14C] A. A. UPTAKE (dpm x 10^4)
15
12
9
6
3
3
6
9
12
15
18
LOG TIME (min)

Fig.9. Rate of total [^{14}C]glucose uptake and mineralization for *P. fluorescens* isolate cell suspensions prepared from cultures grown in the presence of 0 (○), 1 (●) and 1000 (△) ppm acephate (see Appendices 13, 23 and 24).

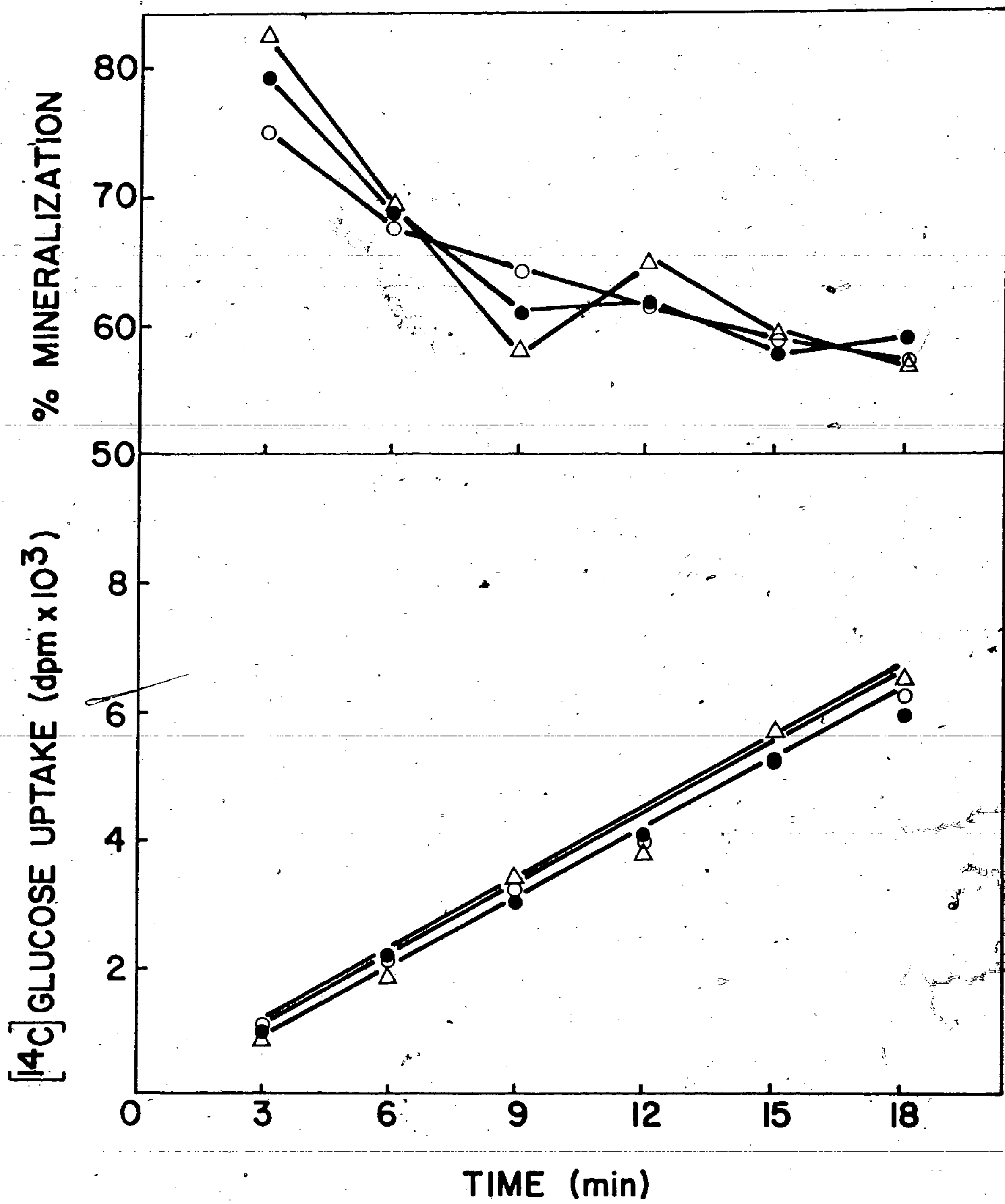
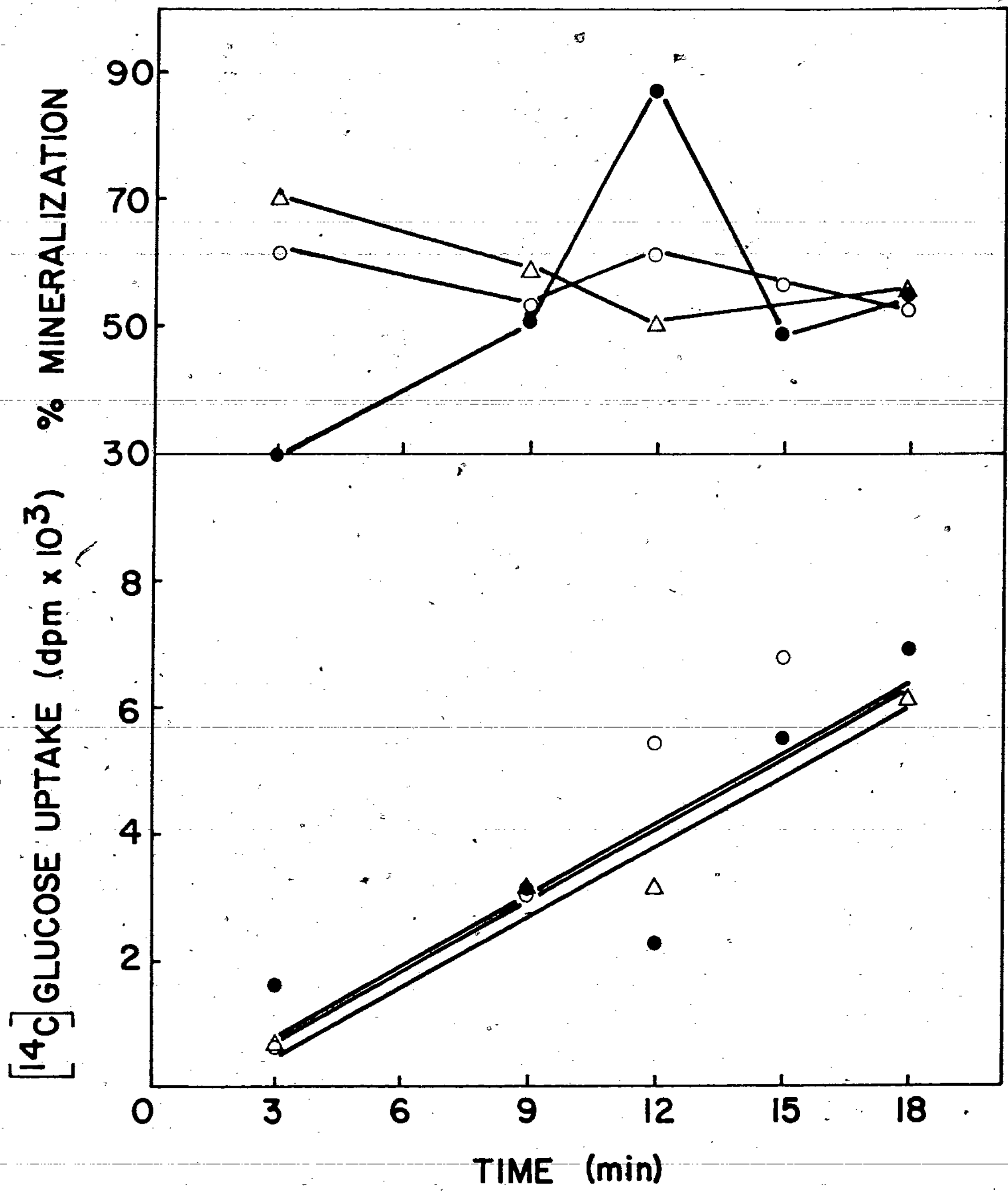

% MINERALIZATION
80
70
60
50
[14C] GLUCOSE UPTAKE (dpm x 10³)
8
6
4
2
0
3
6
9
12
15
18
TIME (min)

Fig.10. Rate of total [^{14}C]glucose uptake and % mineralization for *P. fluorescens* isolate cell suspensions prepared from cultures grown without acephate additions which were treated with 0 (○), 1 (●) and 1000 (△) ppm acephate at the time of glucose addition (see Appendices 14, 25 and 26).

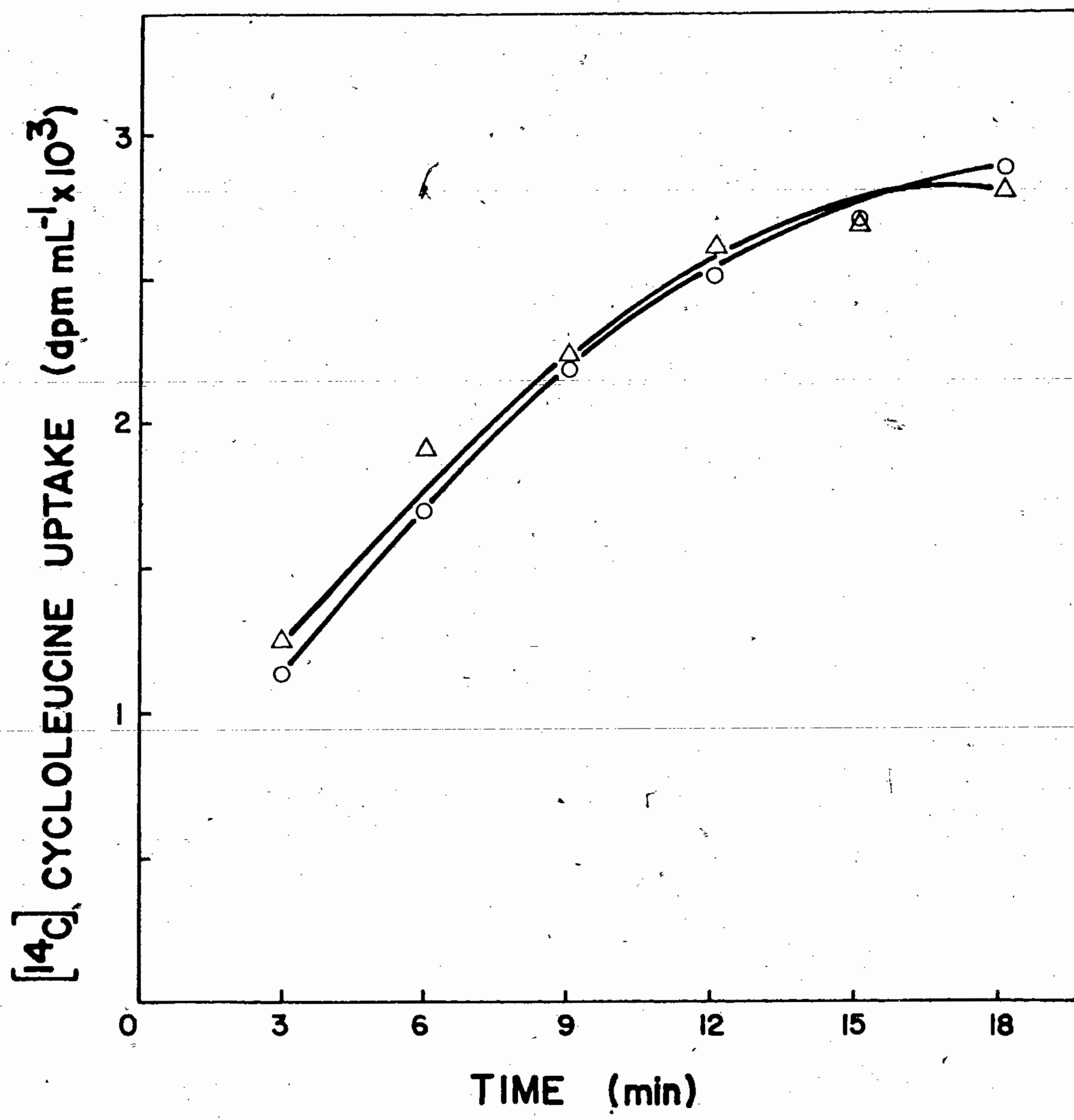

90
70
50
30
% MINERALIZATION
8
6
4
2
[14C] GLUCOSE UPTAKE (dpm x 10³)
0
3
6
9
12
15
18
TIME (min)

Transport of [^{14}C]cycloleucine

Cycloleucine was not able to support growth of C. lividum isolate when it was the sole carbon source nor was any $^{14}CO_2$ recovered during respiration experiments when [^{14}C]cycloleucine was used as the substrate. While these results indicate that cycloleucine was not metabolized, it was taken up by the cells. Aerobic uptake of [^{14}C]cycloleucine at 15°C reached saturation at about 15 min and was not affected by the presence of 1000 ppm acephate (Fig.11).

For influx-efflux experiments the cells were suspended in GN medium with chloramphenicol which prevented protein synthesis but maintained viable cells for use in the study of transport systems independent of growth. Concentrations of [^{14}C]cycloleucine in both the control and acephate-treated cultures reached a maximum after approximately 10 min, decreased slightly thereafter, and remained relatively constant between 35 and 50 min (Fig.12). At 43 min, the medium concentration of cycloleucine was increased to 0.01 M by the addition of unlabeled cycloleucine. The increase of unlabeled substrate caused a significant efflux of intra-cellular label similar to the initial uptake, and by 60 min the amount of label dropped to approximately the acid-killed control concentration. The loss of intra-cellular label suggests that [^{14}C]cycloleucine was being held in an intracellular pool rather than being incorporated into protein.

Fig.11. [^{14}C]cycloleucine uptake of *C. lividum* isolate cell suspensions treated with 0 (O) and 1000 (Δ) ppm acephate (see Appendix 15).


[14C] CYCLOLEUCINE UPTAKE (dpm mL-1 x 10^3)
3
2
1
0
3
6
9
12
15
18
TIME (min)

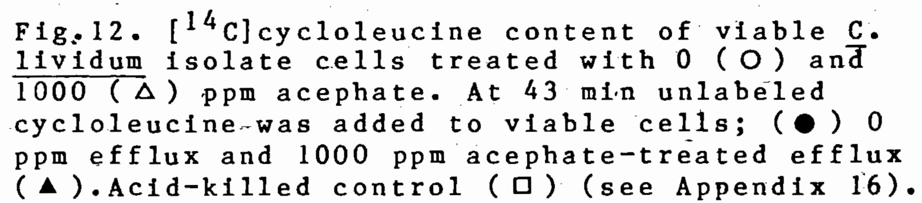
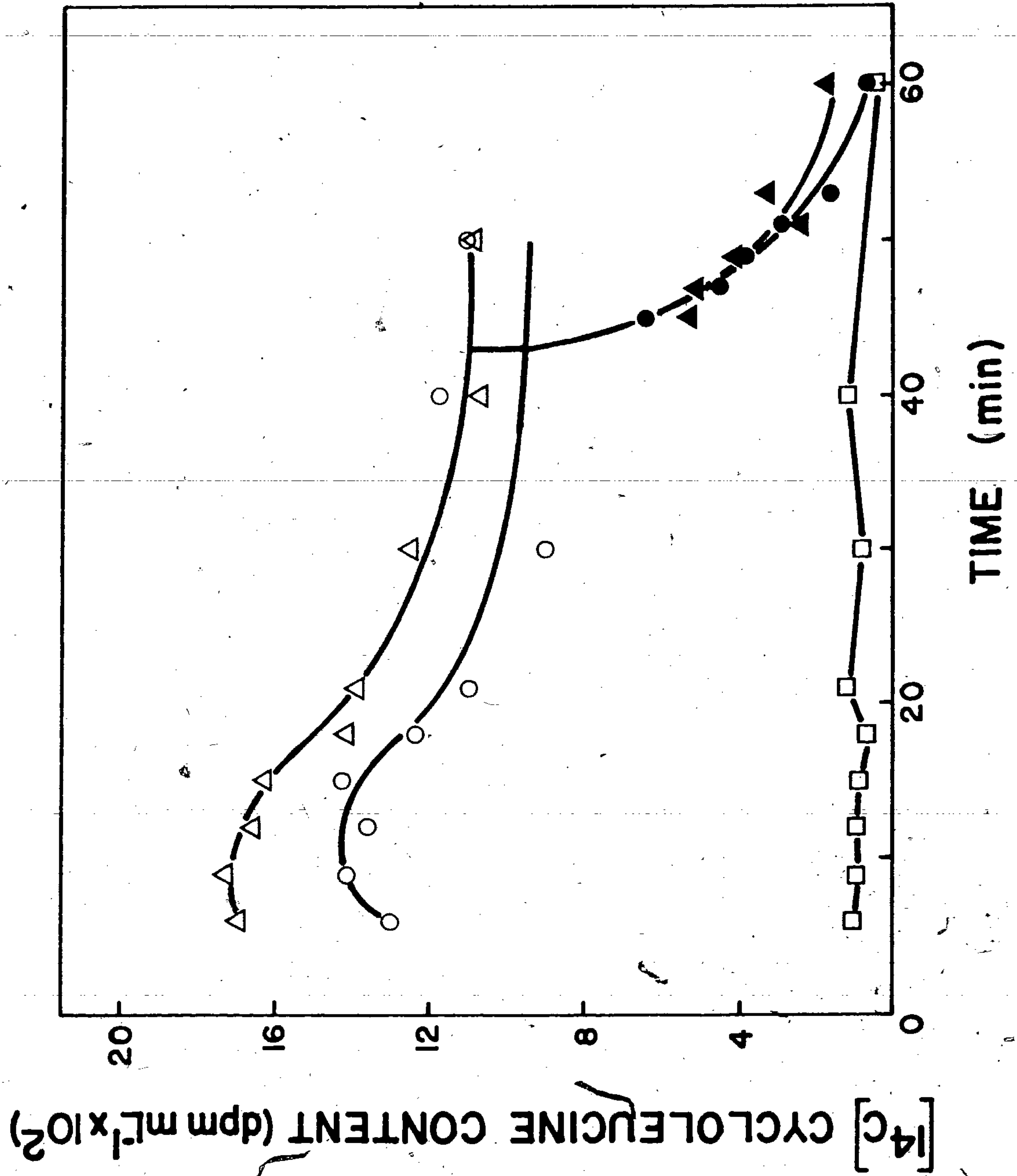


Fig. 12. [^{14}C]cycloleucine content of viable *C. lividum* isolate cells treated with 0 (○) and 1000 (△) ppm acephate. At 43 min unlabeled cycloleucine was added to viable cells; (●) 0 ppm efflux and 1000 ppm acephate-treated efflux (▲). Acid-killed control (□) (see Appendix 16).



[14C] CYCLOLEUCINE CONTENT (dpm ml-1 x 102)
0
4
8
12
16
20
TIME (min)
20
40
60

D. DISCUSSION

The characteristics determined for the Lei Lake isolates agreed well with those reported for the type species (Table.1) except for the biochemical test results for the C. lividum isolate. However, the distinctive cultural and physiological characteristics were strong diagnostic indicators for C. lividum and at least one other study has based Chromobacterium sp. identification mainly on these parameters (Bolter 1977).

The genus Chromobacterium is currently placed in the family Rhizobiaceae (Buchanan and Gibbons 1974) but there is strong evidence that the taxonomic position of the genus should be changed (Moffett and Colwell 1968; Heberlein et al. 1967; Sneath 1974), and possibly placed in the family Pseudomonadaceae.

P. fluorescens and C. lividum are common bacteria in soil and water (Doudoroff and Palleroni 1974; Sneath 1974). Pseudomonas sp. are typical inhabitants of lake water (van Niel and Stanier 1959; Kuznetsov 1970; Rheinheimer 1974), and may be the dominant bacterial genus present in aquatic environments (Lapteva 1977; Bell et. al. 1980). C. lividum appears to be less abundant in aquatic ecosystems. Bell et al. (1980) isolated two species of Chromobacterium from oligotrophic rivers in New Brunswick but C. lividum accounted from less than 1 to 3 % of the total population sampled. In Siberian lakes about 4 % of the species isolated were identified as belonging to Chromobacterium (Kuznetsov 1970).

The data presented for C. *lividum* isolate show that growth in medium containing 1000 ppm acephate medium decreases the rate of growth and uptake of glucose and amino acids, but not the rate of cycloleucine uptake. Thus it appears that acephate may not inhibit active transport directly, in C. *lividum* isolate.

To give perspective to 1 and 1000 ppm concentrations, it is useful to compare the toxicity of acephate with more familiar pesticides, and to know the amount of acephate which would be required to give these concentations in Lei Lake. Acephate has a 24-h LC_{50} of 900 to 2890 ppm for rainbow trout (*Salmo gairdneri* (Duangsawasdi and Klaverkamp 1979; Geen *et al.* 1980). Holden lists the 24-h LC_{50} of several pesticides for rainbow trout as: DDT, 4.2 ppb; malathion, 100 to 160 ppb; methyl parathion, 2.75 ppm (96 h); carbaryl, 3.5 to 4 ppm; 2,4-D(PGBEE),1.2 ppm; 2,4-DB(acid), 13.5 ppm. In order to obtain concentrations of 1 and 1000 ppm acephate in Lei Lake about 122 kg and 1.2×10^5 kg respectively would have to be homogeneously dispersed in the water column (based on a surface area of 2.1 ha and mean depth of 5.8 m).

Optical density was used to measure growth because it is proportional to the number or mass of cells in a bacterial suspension (Herbert 1961; Spaun 1962; Meynell and Meynell 1970). When light is transmitted through a suspension almost all of the apparent absorbance is attributed to light scattering by individual cells, and only a small extent to true absorption (Spaun 1962; Freifelder 1976). Less light scattering by C.

lividum isolate cultures exposed to 1000 ppm acephate probably caused lower OD measurements.

Although calibrations for cell number and dry weight with OD were not made, the cell numbers for *C. lividum* isolate cultures exposed to 1000 ppm acephate and those for the control were comparable at an OD of 0.3 (Appendix 5). But cell size and the maximum cellular yield of 1000 ppm acephate-treated *C. lividum* isolate cultures were reduced. Powell (1963) and Meynell and Meynell (1970) state that smaller cells scatter less light than larger ones at a given wavelength, so that reduced light scattering at a given OD by the acephate-treated cultures could be a result of lower biomass. Therefore treatment of *C. lividum* isolate cultures with 1000 ppm acephate may reduce biomass by affecting biosynthetic mechanisms.

Blakemore and Carey (1978) found that concentrations of polychlorinated biphenyls (PCBs) as low as 10 ppb increased generation times, lowered maximum cell yields and caused morphological changes in a marine pseudomonad, and attributed these effects to inhibition of biosynthetic mechanisms. In a followup study Blakemore (1978) showed that the synthetic rate and total amount of nucleic acid per cell were significantly reduced in 10 ppb PCB treated-cells and adenine accumulation was 30% of controls. These results suggested membrane alterations or inhibition of enzymes involved in nucleic acid synthesis.

The effects of 1000 ppm acephate on *C. lividum* isolate were reversible when the cells were inoculated into fresh culture

medium (Fig.3). Reversible effects indicate that this concentration is bacteriostatic for the bacterium, and may not be permanently bound to the cells. Blakemore and Carey (1978) found that a PCB concentration of 200 ppb was also bacteriostatic for some bacteria. C. lividum isolate exposed to repeated 1000 ppm concentrations of acephate did not show adaptation to the insecticide. Reduced doubling times would be expected if this organism was adapting to acephate.

Uptake data for C. lividum isolate exposed to 1000 ppm acephate during growth indicated that the amount of radioactive glucose and amino acids retained by the cells was significantly reduced compared to the control. However, the portions of $^{14}CO_2$ were similar. Uptake rates for the substrates tested depend on the rate of translocation into the cell, catabolic and anabolic pathways and respiration as CO_2. Yarbrough et al. (1980) considered the initial rate (that occurring within the first 15 sec) of glucose and proline transport to be representative of the true transport rate, and subsequent uptake to reflect other processes such as catabolism or incorporation into cellular material. Baross et al. (1975) found that the radioactivity retained by filtration after acid fixation with sulfuric acid was identical to the amount precipitated with trichloroacetic acid (proteins and nucleic acids). Fixation caused release of the substrate pools and low weight metabolites but did not affect intracellular macomolecular components. Interpretation of uptake data was discussed by Griffiths et al. (1981) in their

study of the effects of 50 ppt crude oil on ^{14}C-labeled glucose and glutamic acid uptake by marine sediment microorganisms. They reasoned that the effects of crude oil on transport into the cells could be differentiated from biosynthetic effects by comparing changes in the portions of cell associated radioactivity with that respired as $^{14}CO_2$. Changes primarily restricted to cell associated radioactivity reflected changes in biosynthetic mechanisms, while equal changes in cell associated and respired radioactivity indicated alteration of substrate transport into the cell. Since cell associated radioactivity was more strongly affected in uptake experiments with C. lividum isolate using cell suspensions prepared from cultures grown in 1000 ppm acephate (Figs.5 and 7), biosynthetic pathways may be affected either by reduced macromolecular synthesis or by a shift to synthesis of low weight metabolites. Upon fixation these small molecules would have been released from the cell.

Acephate (1000 ppm) did not affect the uptake or efflux of cycloleucine by C. lividum isolate. Cycloleucine was chosen as the substrate to investigate transport because it was not metabolized by C. lividum isolate and has been recommended for study of transport independent of metabolism (Akedo and Christensen 1962; Christensen and Jones 1962). As an analogue of L-leucine or L-valine it is transported by the leucine-isoleucine-valine transport system, which is a well known multiple transport mechanism in bacteria utilizing binding proteins (Ankaru 1978). Christensen (1975) states that

cycloleucine is a useful model substrate because it is reactive with 2 or more important transport systems.

For acephate to affect biosynthesis it would have to penetrate into the cell. No studies have been published describing membrane permeability or transport in _C. lividum_ but it appears that binding proteins located within the periplasmic space between the outer and cytoplasmic membrane are important in the transport of glucose and amino acids by Gram negative bacteria generally (Costerton _et al._ 1974; Meadow 1975; Wilson and Smith 1978; Romano _et al._ 1980). The use of acephate as a sole phosphorus source by soil pseudomonad enrichment cultures may indicate that acephate is able to penetrate into cells (Rosenberg and Alexander 1979), either with or without the the assistance of a transport system. However, acephate may be degraded extracellularly and the phosphorus moiety taken up separately.

Membrane structure is also an important factor in the permeability of cells. Replacement of saturated fatty acid with unsaturated fatty acid in membrane lipids increases the nonelectrolyte permeability of cells (McElhaney _et al._ 1973). Unsaturated fatty acid substitution decreases the orderly arrangement or rigidity of the membrane and increases the fluidity. Bacteria grown at a low temperature have membranes containing greater proportions of unsaturated fatty acid than those grown at a higher temperature (Esfahani _et al._ 1969; Fox 1972a,b). The maintenance of a fluid membrane is important in

the normal functioning of membrane activities, including transport.

Under the conditions of *C. lividum* isolate culture (15°C and pH 7.5 to 6.3) acephate would be expected to be ionized (Klaverkamp and Hobden 1980), but may penetrate the membrane directly or be transported. Duangsawasdi and Klaverkamp (1979) estimated Q_{10} values from rates of mortality for rainbow trout to be 1.3, which according to Shifrer *et al*. (1974, cited in Duangsawasdi and Klaverkamp 1979) may indicate that physical uptake processes (e.g. diffusion) are involved. Since acephate effects were only observed at a high concentration (1000 ppm) its concentration gradient may have assisted penetration into the cell.

If acephate affects *C. lividum* isolate why does it not appear to affect *P. fluorescens* isolate growth or glucose uptake when both organisms catabolize glucose using the Entner-Duodoroff pathway (Brock 1970)? Two possible explanations are given below.

Firstly, *C. lividum* isolate has a 7 h doubling time compared with 4 h for *P. fluorescens* isolate, and it is possible that the shorter doubling time is a result of more rapid metabolism. Khasawinah *et al*. (1978) studied the toxicity of methamidophos, an acephate activation product, on housefly cholinesterase and concluded that the relative stability and low *in vivo* degradation permitted adequate concentrations for a sufficiently long period of time to cause cholinesterase inhibition. Acephate

is known to be a poor *in vitro* inhibitor of housefly cholinesterase (Rojakovick and March 1972) and may behave similarly to methamidophos *in vivo*. Although bacteria do not possess cholinesterase, a high internal acephate concentration may be required to affect biosynthetic enzyme(s).

Secondly, *P. fluorescens* isolate may not be affected by acephate if it is able to utilize alternate glucose transport systems or biosynthetic pathways. There are at least 3 routes by which glucose may enter the Entner-Duodoroff pathway (Lynch and Franklin 1978) or be degraded (Wood 1955) in *P. fluorescens*. This organism is also able to use from 60 to more than 80 carbon sources for growth (Stanier *et al*. 1966) reflecting its enzymatic versatility. Acephate is a poor *in vivo* inhibitor of housefly acetylcholinesterase (Rojakovich and March 1972) and does not react with detoxifying enzymes such as microsomal oxidases and GSH-dependent transferases in resistant insects (Suksayretrup and Plapp 1977). Therefore acephate may affect a specific biosynthetic enzyme in *C. lividum* isolate but *P. fluorescens* isolate would avoid acephate effects by utilizing alternate pathways.

The results of the study presented here indicate that only a high concentration of acephate (1000 ppm) affected *C. lividum* isolate and it is unlikely that aquatic organisms would be exposed to concentrations of this magnitude in the natural environment. Neither test microorganism was affected when exposed to 1 ppm acephate, the upper limit of a theoretical

environmental dosage, indicating that the toxicity of acephate to bacteria is depends on high concentrations and the type of metabolism. Rabeni and Stanley (1979) and Geen *et al*. (1981) have shown that only minor and localized impacts on the biota were observed in streams treated with low (<1 ppm) concentrations of acephate.

E. CONCLUSION

The results of this study indicate that acephate present at a concentration of 1000 ppm in culture medium affects the rate of growth and glucose and amino acid uptake by C. lividum isolate, but does not affect the rate of cycloleucine uptake or efflux. The inhibition of cell associated uptake by acephate appears to be caused by interference with biosynthetic mechanisms. No effects of acephate at a theoretical environmental concentration (1 ppm) were observed in any of the experiments with C. lividum isolate. Acephate did not affect the rate of growth or uptake of glucose by P. fluorescens isolate when present at concentrations of 1 or 1000 ppm in culture medium.

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G. APPENDICES

Appendix 1. OD_{540} measurements (n = 3) for <u>C</u>. <u>lividum</u> isolate cultures during growth in the presence of 0, 1 and 1000 ppm acephate (Fig. 2).

Time (h)	Control			1 ppm			1000 ppm		
	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD
8	0.005 0.005 0.005	0.005	0.000	0.005 0.005 0.005	0.005	0.000	0.006 0.006 0.007	0.006	0.000
12.5	0.011 0.010 0.011	0.011	0.001	0.011 0.010 0.010	0.010	0.001	0.011 0.016 0.015	0.014	0.003
24	0.039 0.040 0.038	0.390	0.001	0.040 0.039 0.038	0.039	0.001	0.030 0.033 0.034	0.032	0.002
28.5	0.064 0.062 0.062	0.063	0.001	0.062 0.062 0.060	0.061	0.001	0.051 0.053 0.054	0.053	0.002
32	0.086 0.088 0.086	0.087	0.001	0.089 0.084 0.075	0.084	0.008	0.066 0.066 0.068	0.067	0.001
37.5	0.143 0.147 0.137	0.142	0.005	0.137 0.144 0.122	0.134	0.011	0.100 0.104 0.100	0.101	0.002
47.5	0.330 0.332 0.320	0.327	0.006	0.345 0.345 0.287	0.326	0.034	0.210 0.211 0.200	0.207	0.006

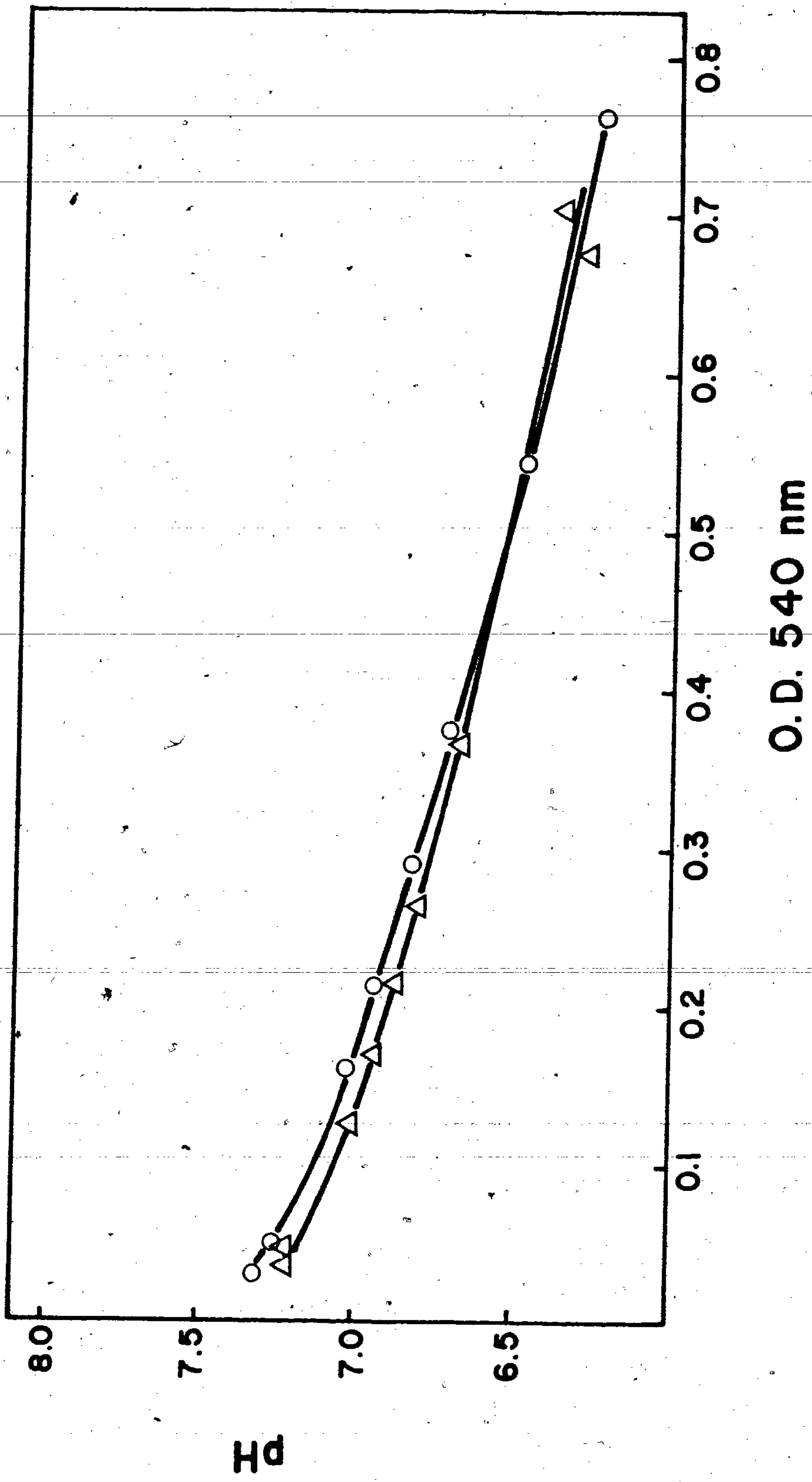
Appendix 1 (continued).

Time (h)	Control			1 ppm			1000 ppm		
	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD
50	0.415 0.425 0.380	0.407	0.024	0.435 0.415 0.340	0.397	0.050	0.254 0.254 0.243	0.250	0.006
51.5	0.490 0.490 0.440	0.473	0.029	0.500 0.510 0.400	0.470	0.061	0.286 0.290 0.273	0.283	0.009
53.25	0.580 0.580 0.510	0.557	0.040	0.580 0.585 0.465	0.543	0.068	0.326 0.330 0.310	0.322	0.011
54	0.640 0.622 0.550	0.604	0.048	0.640 0.620 0.505	0.588	0.073	0.350 0.355 0.330	0.345	0.013
56	0.750 0.755 0.630	0.712	0.071	0.750 0.745 0.590	0.695	0.091	0.400 0.410 0.383	0.398	0.014
58	-	-	-	-	-	-	0.470 0.470 0.446	0.462	0.014
62	0.780 0.820 0.770	0.790	0.027	0.780 0.820 0.770	0.790	0.027	0.640 0.640 0.590	0.623	0.029
63.5	0.780 0.790 0.770	0.780	0.010	0.780 0.800 0.780	0.787	0.012	0.670 0.660 0.660	0.663	0.006

Appendix 1 (continued).

Time (h)	Control			1 ppm			1000 ppm		
	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD
65	-	-	-	-	-	-	0.680 0.680 0.670	0.677	0.006
74	0.705 0.700 0.705	0.703	0.003	0.710 0.735 0.710	0.718	0.014	0.680 0.700 0.700	0.693	0.012
79.5	-	-	-	-	-	-	0.665 0.685 0.680	0.677	0.010

Appendix 2. The influence of C. *lividum* isolate upon medium pH when grown in the presence of 0 (○) and 1000 (△) ppm acephate.


pH
8.0
7.5
7.0
6.5
O.D. 540 nm
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

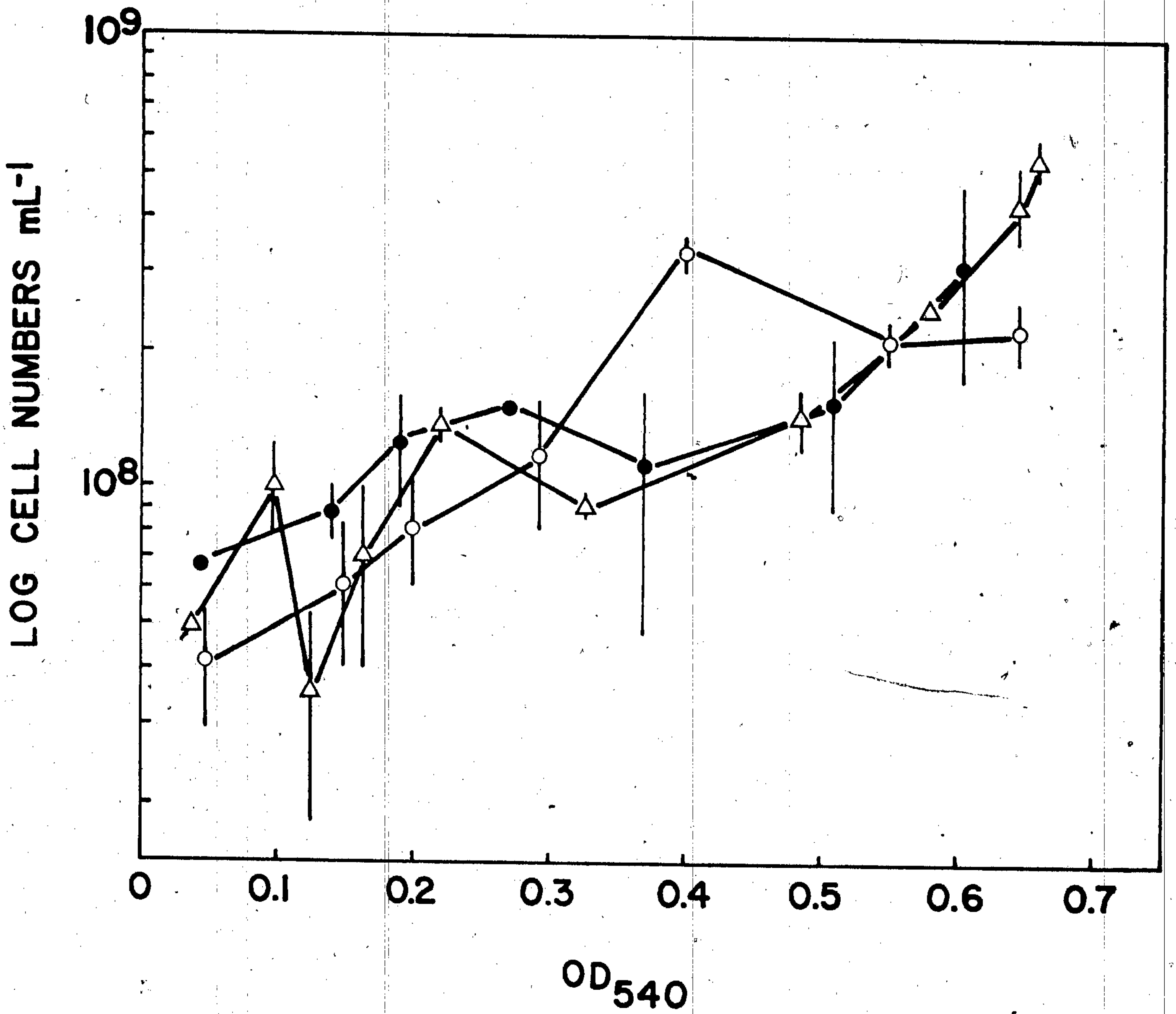
Appendix 3. Data for the influence of C. lividum isolate upon medium pH when grown in the presence of 0 and 1000 ppm acephate (Appendix 2).

Time (h)	Control						1000 ppm					
	OD_{540}			pH			OD_{540}			pH		
	OD	$\bar{x}$	± 1SD	pH	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD	pH	$\bar{x}$	± 1SD
22.25	0.028 0.037	0.033	0.010	7.27 7.36	7.32	0.06	0.040 0.034	0.037	0.004	7.21 7.24	7.23	0.02
29.5	0.048 0.052	0.050	0.003	7.30 7.23	7.27	0.05	0.050 0.046	0.048	0.003	7.20 7.24	7.22	0.03
42.75	0.143 0.182	0.163	0.030	7.12 6.95	7.04	0.12	0.145 0.108	0.127	0.030	6.95 7.09	7.02	0.10
45.5	0.190 0.240	0.215	0.040	7.03 6.85	6.94	0.13	0.180 0.160	0.170	0.010	6.88 7.00	6.94	0.08
48.0	0.277 0.305	0.291	0.020	6.92 6.73	6.83	0.13	0.225 0.205	0.215	0.010	6.79 6.92	6.86	0.09
51.0	0.356 0.400	0.378	0.030	6.81 6.60	6.71	0.15	0.275 0.252	0.264	0.020	6.73 6.87	6.80	0.10
55.0	0.510 0.575	0.543	0.050	6.58 6.37	6.48	0.15	0.380 0.355	0.368	0.020	6.61 6.76	6.69	0.11
66.0	0.780 0.750	0.765	0.020	6.22 6.27	6.25	0.04	0.680 0.675	0.678	0.040	6.26 6.32	6.29	0.04
69.0	0.750 0.730	0.740	0.010	6.31 6.33	6.32	0.01	0.710 0.700	0.705	0.010	6.34 6.39	6.37	0.04
75.5	0.720 0.720	0.720	0.000	6.27 6.33	6.30	0.04	0.680 0.670	0.675	0.010	6.36 6.42	6.39	0.04

Appendix 4. OD_{540} measurements (n = 3) for C. lividum isolate cultures, previously grown in 1000 ppm acephate, during growth in the presence of 0 and 1000 ppm acephate (Fig. 3).

Time (h)	Control			1000 ppm		
	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD
5.0	0.006 0.006 0.006	0.006	0.000	0.006 0.006 0.006	0.006	0.000
9.0	0.009 0.008 0.009	0.009	0.001	0.009 0.010 0.007	0.009	0.002
21.0	0.021 0.021 0.019	0.020	0.001	0.019 0.019 0.018	0.019	0.001
26.0	0.036 0.038 0.033	0.036	0.003	0.028 0.028 0.030	0.029	0.001
34.5	0.083 0.080 0.080	0.081	0.002	0.056 0.051 0.059	0.056	0.004
47.5	0.270 0.255 0.257	0.261	0.008	0.145 0.152 0.165	0.154	0.010
50.5	0.350 0.343 0.348	0.350	0.004	0.176 0.184 0.200	0.187	0.012
56.0	0.590 0.565 0.560	0.572	0.016	0.272 0.280 0.310	0.287	0.020
78.5	0.745 0.760 0.745	0.750	0.009	0.630 0.645 0.700	0.658	0.037
87.0	0.710 0.720 0.720	0.717	0.006	0.665 0.680 0.700	0.682	0.018
91.0	-	-	-	0.650 0.655 0.670	0.658	0.010

Appendix 5. Acridine orange direct counts of *C. lividum* isolate for 0 (○), 1 (●) and 1000 (Δ) ppm acephate-treated cells during growth.



LOG CELL NUMBERS mL-1
10⁹
10⁸
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
OD540

Appendix 6. OD_{540} measurements (n = 4) for duplicate flasks of _C. lividum_ isolate cultures during growth in the presence of 0, 1 and 1000 ppm acephate (Appendix 5).

Time (h)	Control			1 ppm			1000 ppm		
	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD
8.0	0.008 0.008 0.008 0.008	0.008	0.000	0.010 0.008 0.007 0.007	0.008	0.001	0.008 0.008 0.008 0.008	0.008	0.000
13.0	0.010 0.010 0.010 0.010	0.010	0.000	0.011 0.010 0.010 0.011	0.011	0.001	0.010 0.010 0.010 0.011	0.010	0.001
19.0	0.020 0.020 0.020 0.020	0.020	0.000	0.019 0.020 0.019 0.020	0.020	0.001	0.018 0.019 0.018 0.019	0.019	0.001
29.5	0.050 0.048 0.046 0.045	0.047	0.002	0.044 0.044 0.044 0.044	0.044	0.000	0.036 0.038 0.038 0.037	0.037	0.001
42.0	0.155 0.155 0.140 0.143	0.148	0.008	0.143 0.142 0.140 0.140	0.141	0.002	0.098 0.097 0.096 0.098	0.097	0.001
45.5	0.207 0.205 0.192 0.192	0.199	0.008	0.190 0.190 0.190 0.187	0.189	0.002	0.125 0.123 0.127 0.125	0.125	0.002

Appendix 6 (continued).

Time (h)	Control			1 ppm			1000 ppm		
	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD
	0.305 0.305 0.279 0.279	0.292	0.015	0.275 0.280 0.270 0.265	0.273	0.006	0.165 0.167 0.165 0.166	0.166	0.001
53.0	0.413 0.415 0.390 0.385	0.401	0.015	0.380 0.375 0.370 0.373	0.375	0.004	0.220 0.220 0.220 0.220	0.220	0.000
55.0	0.495 0.495 0.460 0.455	0.476	0.022	0.450 0.450 0.440 0.440	0.445	0.006	0.255 0.253 0.260 0.255	0.256	0.003
57.0	0.570 0.570 0.530 0.530	0.550	0.023	0.510 0.510 0.510 0.510	0.510	0.000	0.285 0.285 0.287 0.290	0.287	0.002
59.0	0.670 0.670 0.620 0.620	0.645	0.029	0.600 0.605 0.600 0.605	0.603	0.003	0.330 0.327 0.330 0.327	0.329	0.002
64.0	-	-	-	-	-	-	0.480 0.490 0.490 0.480	0.485	0.006

Appendix 6 (continued).

Time (h)	Control			1 ppm			1000 ppm		
	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD
66.6	0.750 0.740 0.770 0.770	0.758	0.015	0.760 0.760 0.760 0.760	0.760	0.000	0.580 0.580 0.585 0.590	0.584	0.005
68.5	-	-	-	-	-	-	0.650 0.645 0.640 0.640	0.644	0.005
72.0	0.760 0.740 0.730 0.730	0.740	0.014	0.740 0.730 0.710 0.710	0.723	0.015	0.660 0.660 0.640 0.670	0.658	0.013
80.0	-	-	-	-	-	-	0.680 0.670 0.660 0.670	0.670	0.008
81.0	-	-	-	-	-	-	0.670 0.670 0.660 0.660	0.665	0.006

Appendix 7. Acridine orange direct count data for *C. lividum* isolate cultures grown in the presence of 0, 1 and 1000 ppm acephate (Appendix 5).

Time (h)	Control			1 ppm			1000 ppm		
	Counts	$\bar{x}$	±1SD	Counts	$\bar{x}$	±1SD	Counts	$\bar{x}$	±1SD
1.0	2.67 E+6	-	-	2.79 E+6	-	-	2.55 E+6	-	-
29.5	3.92 E+7	4.17 E+7	1.22	6.57 E+7	6.74 E+7	0.23	4.75 E+7	4.94 E+7	0.27
	3.26 E+7			6.90 E+7			5.13 E+7		
	3.56 E+7								
	5.95 E+7								
42.0	9.22 E+7	6.12 E+7	2.08	7.14 E+7	8.80 E+7	1.19	8.69 E+7	1.01 E+8	0.23
	4.91 E+7			8.93 E+7			1.31 E+8		
	5.33 E+7			9.21 E+7			1.05 E+8		
	5.01 E+7			9.93 E+7			8.00 E+7		
45.5	6.74 E+7	8.24 E+7	2.11	7.84 E+7	1.25 E+8	0.35	1.61 E+7	3.56 E+7	1.75
	9.73 E+7			1.62 E+8			2.57 E+7		
				1.21 E+8			4.89 E+7		
				1.37 E+8			5.17 E+7		
49.5	9.76 E+7	1.19 E+8	0.38	1.52 E+8	1.50 E+8	0.04	8.85 E+7	7.01 E+7	3.01
	1.10 E+8			1.47 E+8			1.02 E+8		
	9.36 E+7						3.81 E+7		
	1.75 E+8						5.17 E+7		
53.0	3.83 E+8	3.39 E+8	0.38	1.86 E+8	1.12 E+8	0.64	1.29 E+8	1.38 E+8	0.12
	4.04 E+8			7.62 E+7			1.46 E+8		
	3.08 E+8			7.44 E+7					
	2.61 E+8								

Appendix 7 (continued).

Time (h)	Control			1 ppm			1000 ppm		
	Counts	$\bar{x}$	±1SD	Counts	$\bar{x}$	±1SD	Counts	$\bar{x}$	±1SD
57.0	2.31 E+8 1.97 E+8	2.14 E+8	0.24	1.99 E+8 1.09 E+8	1.54 E+8	0.64	-	-	-
59.0	2.51 E+8 2.07 E+8	2.26 E+8	0.35	1.57 E+8 3.68 E+8 4.12 E+8	3.12 E+8	1.36	8.77 E+7 9.57 E+7	9.17 E+7	0.57
64.0	-	-	-	-	-	-	1.60 E+8 1.28 E+8	1.44 E+8	0.23

Appendix 8. OD_{540} measurements (n = 4) for P. fluorescens isolate cultures during growth in the presence of 0, 1 and 1000 ppm acephate (Fig. 4).

Time (h)	Control			1 ppm			1000 ppm		
	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD
1.5	0.007 0.006 0.005 0.005	0.006	0.001	0.006 0.005 0.004 0.007	0.006	0.001	0.006 0.006 0.008 0.006	0.007	0.001
6.5	0.013 0.012 0.012 0.013	0.013	0.001	0.012 0.012 0.012 0.012	0.012	0.000	0.011 0.011 0.010 0.009	0.010	0.001
10.0	0.030 0.028 0.028 0.027	0.028	0.001	0.030 0.037 0.027 0.026	0.030	0.005	0.028 0.028 0.014 0.018	0.022	0.007
14.0	0.042 0.045 0.050 0.054	0.048	0.005	0.048 0.049 0.049 0.042	0.047	0.003	0.052 0.056 0.049 0.060	0.054	0.005
19.0	0.086 0.083 0.078 0.079	0.082	0.004	0.082 0.079 0.067 0.068	0.074	0.008	0.065 0.072 0.096 0.090	0.081	0.015
23.0	0.225 0.226 0.160 0.175	0.197	0.034	0.224 0.221 0.200 0.207	0.213	0.011	0.288 0.280 0.291 0.286	0.286	0.005

Appendix 8 (continued).

Time (h)	Control			1 ppm			1000 ppm		
	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD	OD	$\bar{x}$	± 1SD
26.0	0.450	0.396	0.055	0.455	0.418	0.035	0.560	0.548	0.010
	0.435			0.440			0.550		
	0.354			0.384			0.540		
	0.343			0.391			0.540		
28.0	0.570	0.554	0.024	0.560	0.541	0.022	0.640	0.623	0.020
	0.555			0.560			0.640		
	0.570			0.520			0.605		
	0.520			0.525			0.605		
30.0	0.700	0.661	0.057	0.685	0.663	0.021	0.750	0.766	0.022
	0.720			0.675			0.745		
	0.615			0.640			0.790		
	0.610			0.650			0.780		
32.5	0.680	0.685	0.019	0.685	0.694	0.008	0.720	0.724	0.005
	0.660			0.690			0.720		
	0.700			0.700			0.730		
	0.700			0.700			0.725		
34.0	0.650	0.658	0.012	0.660	0.668	0.009	0.690	0.699	0.010
	0.645			0.660			0.690		
	0.665			0.675			0.710		
	0.670			0.675			0.705		

Appendix 9. Data for rate of total [^{14}C]glucose uptake and % mineralization for C. *lividum* isolate grown in the presence of 0, 1 and 1000 ppm acephate (Fig. 5).

Time (min)	Control			1 ppm			1000 ppm		
	Uptake (dpm)			Uptake (dpm)			Uptake (dpm)		
	Total	Respired	% Min.*	Total	Respired	% Min.*	Total	Respired	% Min.*
5	633	422	66.6	684	436	63.7	653	381	58.4
10	1375	593	43.1	1379	693	50.3	-	-	-
15	-	-	-	1911	922	48.3	1546	818	52.9
20	2492	1151	46.2	2148	1164	54.2	2035	978	48.1
25	3000	1356	45.2	3012	1400	46.5	2281	1100	48.2
30	3530	1658	47.0	-	-	-	2619	1445	55.2

*% Min. = % mineralization, calculated as (respired uptake/total uptake) x 100

Appendix 10. Data for rate of total [^{14}C]glucose uptake and % mineralization for C. lividum isolate treated with 0, 1 and 1000 acephate at the time of glucose addition (Fig. 6).

Time (min)	Control			1 ppm			1000 ppm		
	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*
	Total	Respired		Total	Respired		Total	Respired	
5	405	-	-	743	466	62.7	950	543	57.2
10	1652	748	45.3	1507	756	50.2	1313	746	56.8
15	2142	1055	49.3	2100	1032	49.1	1877	950	50.6
20	2967	1460	49.2	2791	1310	46.9	2450	1231	50.2
25	3558	1656	46.5	3249	1560	48.0	2875	1402	48.8
30	3902	1907	48.9	3177	1838	57.9	-	-	-

*% Min. = % mineralization, calculated as(respired uptake/total uptake) x 100

Appendix 11. Data for rate of total [^{14}C]amino acids mixture uptake and mineralization for <u>C</u>. <u>lividum</u> isolate grown in the presence of 0, 1 and 1000 ppm acephate (Fig. 7).

Time (min)	Control			1 ppm			1000 ppm		
	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*
	Total	Respired		Total	Respired		Total	Respired	
3	44 694	26 243	58.7	52 811	29 078	55.0	32 038	22 786	71.1
6	79 887	40 421	50.6	76.558	37 963	49.6	54 329	34 012	62.6
9	95 386	44 277	46.4	107 235	48 903	45.6	86 427	49 464	57.2
12	114 060	52 932	46.4	-	-	-	94 399	53 560	56.7
15	132 396	60 661	45.8	133 080	58 904	44.3	105 073	58 864	56.0
18	144 194	66 764	46.3	136.601	63 014	46.1	119 981	67 869	56.6

*% Min. = % mineralization calculated as (respired uptake/total uptake) x 100.

Appendix 12. Data for rate of total [^{14}C]amino acids mixture uptake and % mineralization for _C. lividum_ isolate treated with 0, 1 and 1000 ppm acephate at the time of amino acid addition (Fig. 8).

Time (min)	Control			1 ppm			1000 ppm		
	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*
	Total	Respired		Total	Respired		Total	Respired	
3	52 811	29 376	55.6	31 129	17 450	56.1	45 970	24 078	52.4
6	-	-	-	71 164	35 234	49.5	82 095	37 675	45.9
9	127 086	55 231	43.5	59 512	28 529	47.9	114 172	49 087	43.0
12	136 406	58 043	42.6	127 412	53 598	42.1	99 876	41 904	42.0
15	138 995	49 412	35.6	138 663	58 784	42.4	107 811	46 186	42.8
18	157 923	67 422	42.7	148 332	61 969	41.8	111 412	47 554	42.7

*% Min. = % mineralization, calculated as (respired uptake/total uptake) x 100.

Appendix 13. Data for rate of total [^{14}C]glucose uptake and % mineralization for <u>P. fluorescens</u> isolate grown in the presence of 0, 1 and 1000 ppm acephate (Fig. 9).

Time (min)	Control			1 ppm			10[illegible] ppm		
	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*
	Total	Respired		Total	Respired		Total	Respired	
3	1144	859	75.1	1092	865	79.2	873	722	82.7
6	2153	1461	67.9	2175	1497	68.8	1884	1312	69.6
9	3262	2109	64.7	3141	1928	61.4	3479	2028	58.3
12	4007	2469	61.6	4189	2599	62.0	3845	2500	65.0
15	5258	3064	58.3	5282	3079	58.3	5779	3443	59.6
18	6304	3650	57.9	5995	3579	59.7	6551	3756	57.3

*% Min. = % mineralization, calculated as (respired uptake/total uptake) x 100.

Appendix 14. Data for rate of total [^{14}C]glucose uptake and % mineralization for P. *fluorescens* isolate treated with 0, 1 and 1000 ppm acephate at the time of glucose addition (Fig. 10).

Time (min)	Control			1 ppm			1000 ppm		
	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*
	Total	Respired		Total	Respired		Total	Respired	
3	838	608	72.6	528	153	97.2	1104	689	62.4
6	1902	1118	58.8	1554	1049	67.5	1722	1202	69.8
9	2818	1689	59.9	-	-	-	2886	1682	58.3
12	4294	2539	59.1	4159	2489	59.9	4494	2557	56.9
15	4806	2957	61.5	5502	3128	56.9	5305	3457	65.2
18	6087	3338	54.8	5797	3122	53.9	5015	3087	61.6

*% Min. = % mineralization, calculated as (respired uptake/total uptake) x 100.

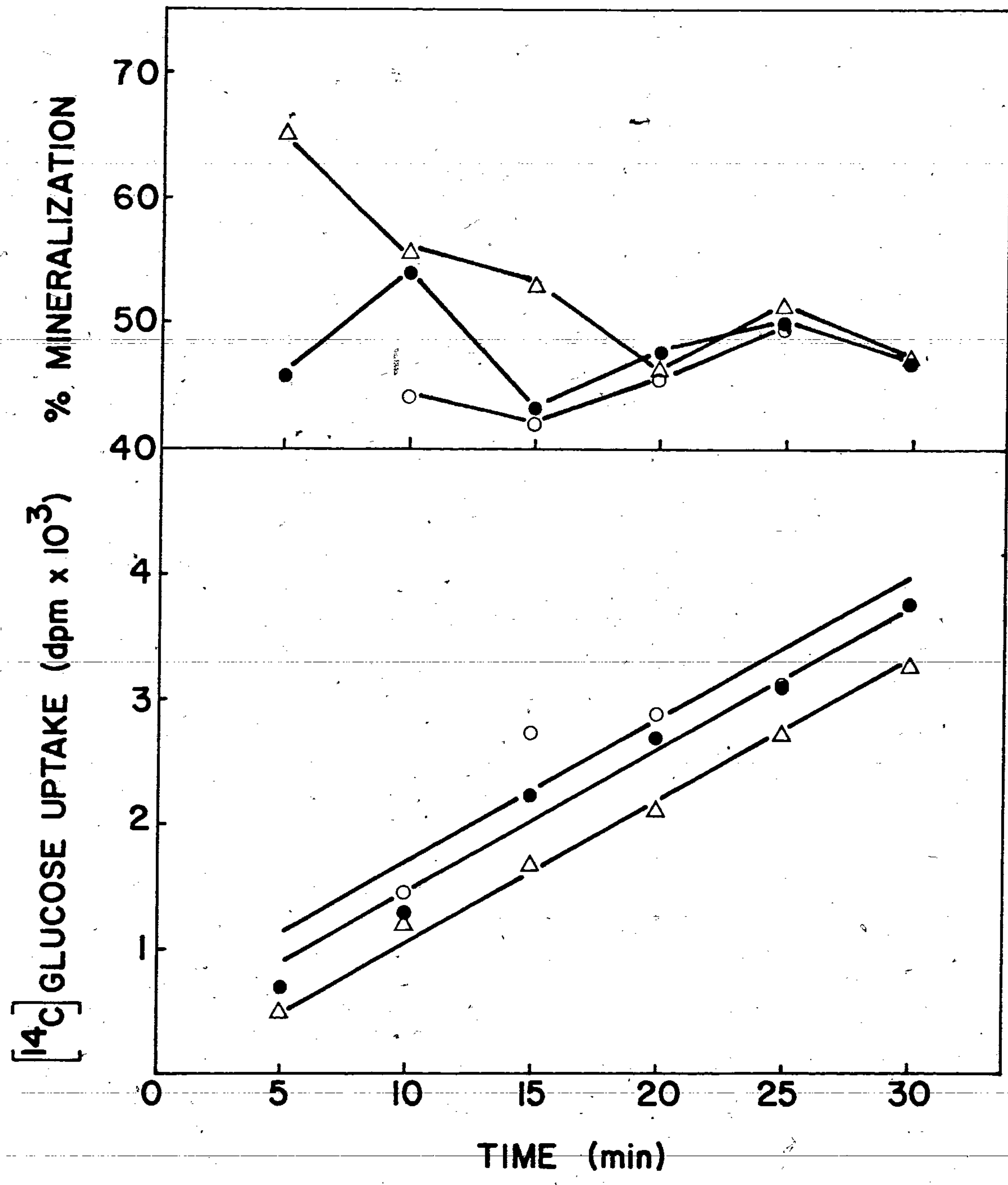
Appendix 15. Data for the rate of [^{14}C]cycloleucine uptake of viable *C. lividum* isolate cells treated with 0 and 1000 ppm acephate (Fig. 11). All data corrected for acid-killed controls.

Time (min)	Control (dpm)	1000 ppm (dpm)
3	1144	1242
6	1703	1915
9	2194	2248
12	2523	2623
15	2726	2701
18	2899	2819

Appendix 16. Data for the [^{14}C]cycloleucine content of viable C. *lividum* isolate cells treated with 0 and 1000 ppm acephate and acid-killed control (Fig. 12). At 43 min unlabled cycloleucine was added to viable cells.

Time (min)	Control			Acid-killed Control			1000 ppm		
	dpm	$\bar{x}$	± 1SD	dpm	$\bar{x}$	± 1SD	dpm	$\bar{x}$	± 1SD
6	1285 1329	1307	31	132 81	107	36	1765 1635	1700	92
9	1390 1435	1413	32	165 34	100	93	1782 1675	1729	76
12	1410 1315	1363	67	89 109	99	14	1606 1722	1664	82
15	1524 1326	1425	140	82 103	93	15	1578 1682	1630	74
18	1251 1226	1239	18	67 90	79	16	1467 1373	1420	67
21	1137 1068	1103	49	95 165	130	50	1412 1366	1329	33
30	851 944	898	66	69 101	85	23	1290 1205	1248	60
40	1400 960	1180	311	114 128	121	10	1176 971	1074	145
50	1111	-	-	-	-	-	1098	-	-
60	611	-	-	46 90	68	31	789	-	-

Appendix 17. Trial experiment of [^{14}C]glucose uptake and % mineralization for *C. lividum* isolate grown in the presence of 0 (○), 1 (●) and 1000 (△) ppm acephate.



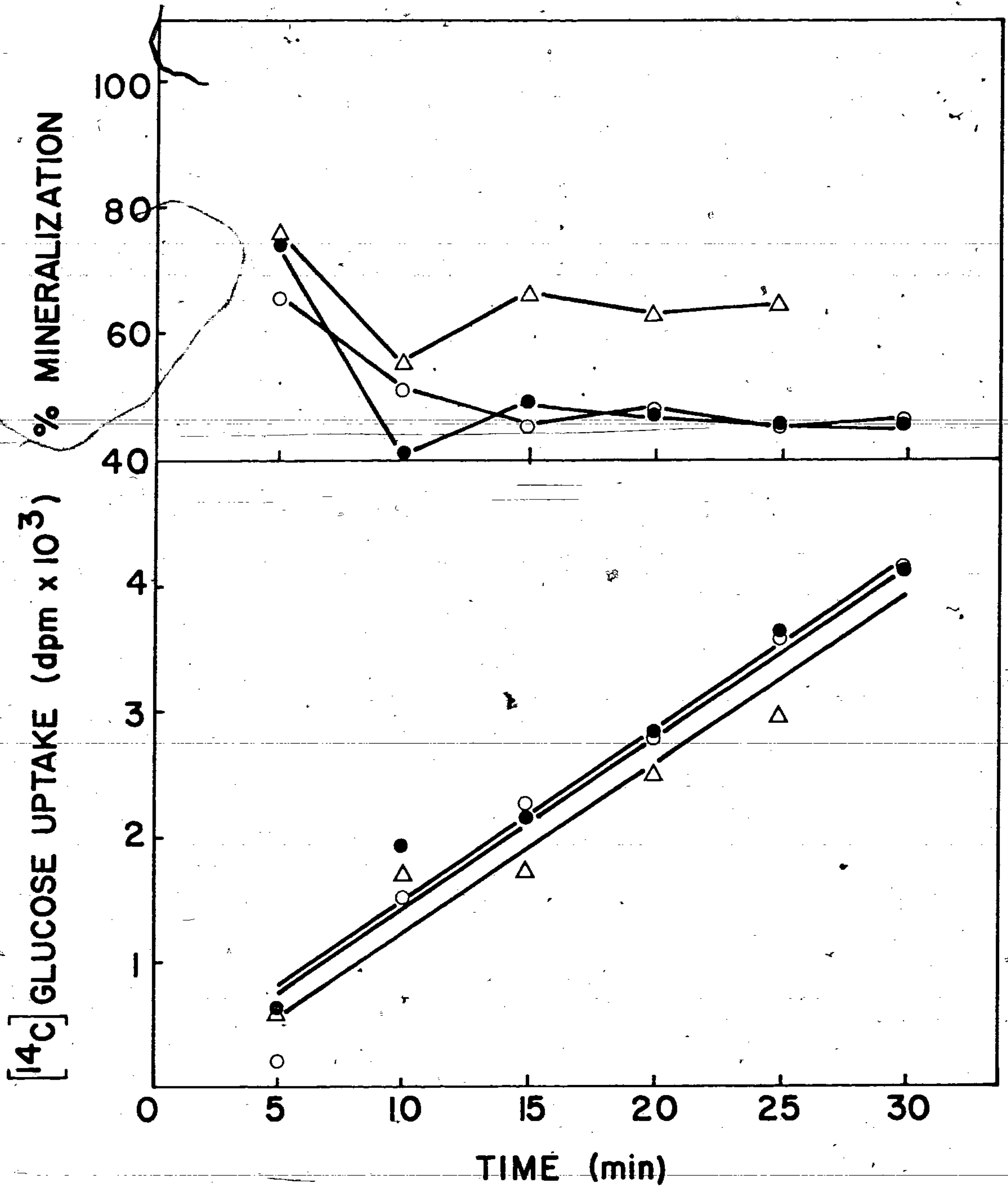
% MINERALIZATION
70
60
50
40
[14C] GLUCOSE UPTAKE (dpm x 10^3)
4
3
2
1
0
5
10
15
20
25
30
TIME (min)

Appendix 18. Data for rate of total [^{14}C]glucose uptake and % mineralization for _C. lividum_ isolate grown in the presence of 0, 1 and 1000 ppm acephate (Appendix 17).

Time (min)	Control			1 ppm			1000 ppm		
	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*
	Total	Respired		Total	Respired		Total	Respired	
5	-	-	-	693	317	45.7	489	317	64.8
10	1465	656	44.0	1284	691	53.8	1181	657	55.6
15	2739	1145	41.8	2228	958	43.0	1664	895	53.8
20	2871	1300	45.3	2684	1285	47.9	2082	955	45.9
25	3149	1553	49.3	3110	1553	49.9	2701	1381	51.1
30	-	-	-	3748	1755	46.8	3265	1534	47.0

*% Min. = % mineralization, calculated as (respired uptake/total uptake) x 100.

Appendix 19. Trial experiment of [^{14}C]glucose uptake and % mineralization for *C. lividum* isolate grown in the presence of 0 (○), 1 (●) and 1000 (△) ppm acephate.

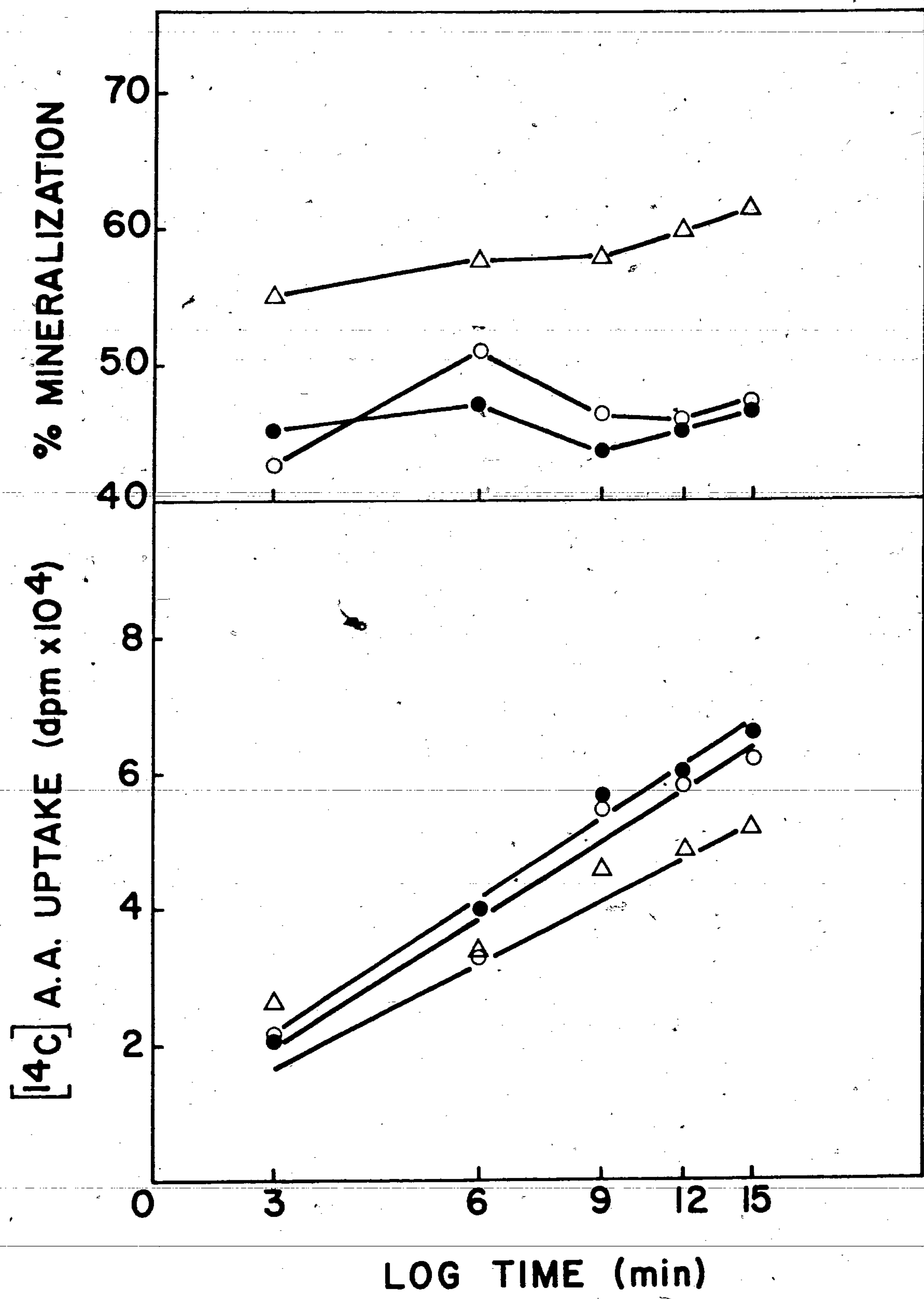

% MINERALIZATION
100
80
60
40
[14C] GLUCOSE UPTAKE (dpm x 10³)
4
3
2
1
0
5
10
15
20
25
30
TIME (min)

Appendix 20. Data for rate of total [^{14}C]glucose uptake and % mineralization for _C. lividum_ isolate grown in the presence of 0, 1 and 1000 ppm acephate (Appendix 19).

Time (min)	Control			1 ppm			1000 ppm		
	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*
	Total	Respired		Total	Respired		Total	Respired	
5	201	132	65.7	626	457	73.0	593	450	75.9
10	1528	784	51.3	1937	795	41.0	1712	941	55.0
15	2287	1038	45.4	2145	1058	49.3	1745	1158	66.4
20	2783	1345	48.3	2831	1339	47.3	2485	1554	62.5
25	3587	1618	45.1	3648	1664	45.6	2968	1895	63.8
30	4143	1908	46.1	4100	1875	45.7	-	-	-

*% Min. = % mineralization, calculated as (respired uptake/total uptake) x 100.

Appendix 21. Trial experiment of [^{14}C]amino acids mixture (A.A.) uptake and % mineralization for C. lividum isolate grown in the presence of 0 (○), 1 (●) and 1000 (△) ppm acephate.



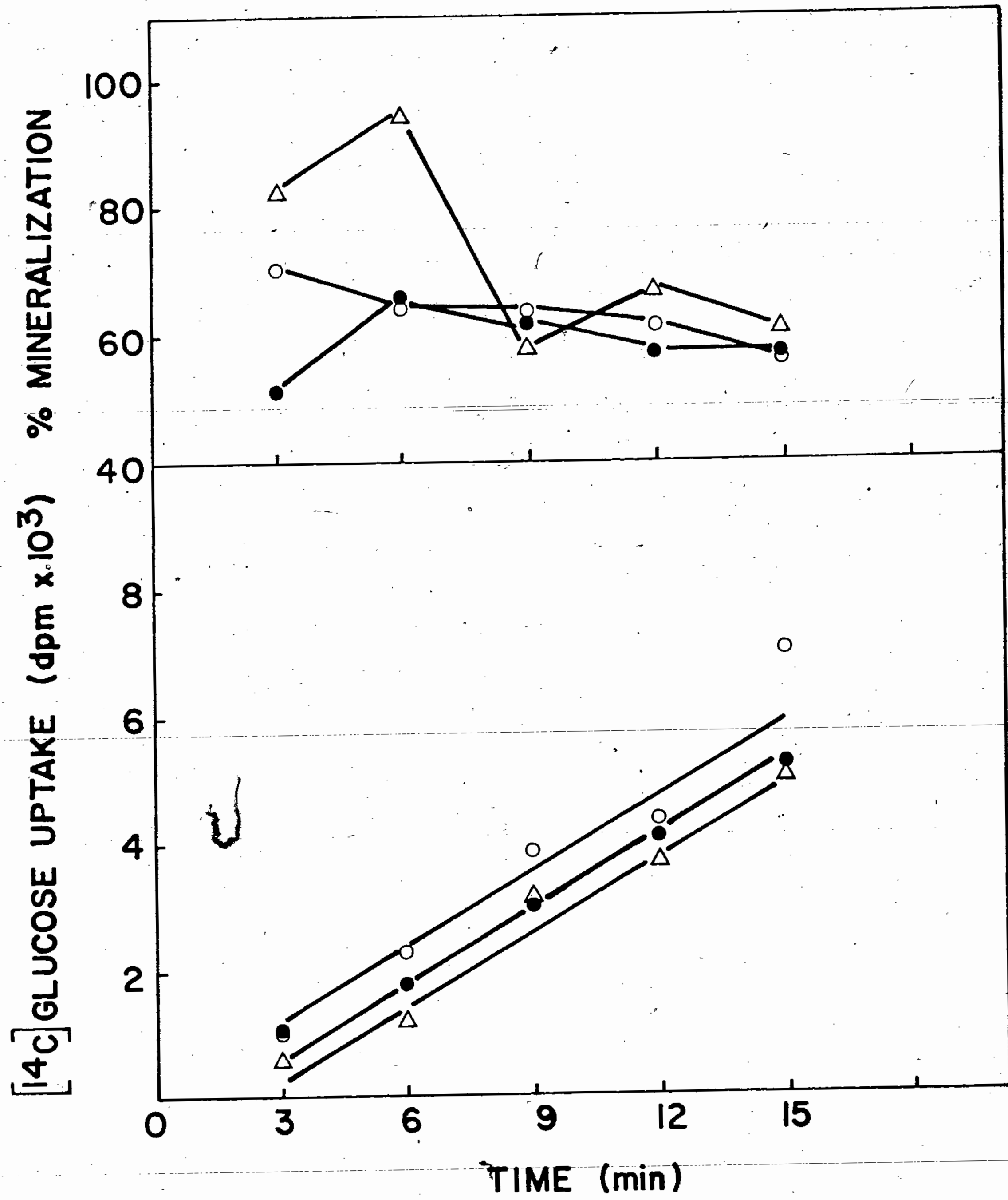
% MINERALIZATION
70
60
50
40
[14C] A. A. UPTAKE (dpm x 10^4)
8
6
4
2
0
3
6
9
12
15
LOG TIME (min)

Appendix 22, Data for total [^{14}C]amino acids mixture uptake and % mineralization for _C. lividum_ isolate grown in the presence of 0, 1 and 1000 ppm acephate (Appendix 21).

Time (min)	Control			1 ppm			1000 ppm		
	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*
	Total	Respired		Total	Respired		Total	Respired	
3	21 193	9 098	42.9	20 957	9 456	45.1	26 798	14 798	55.2
6	32 524	16 584	51.0	40 306	19 276	47.8	34 310	19 826	57.8
9	54 703	25 343	46.3	56 913	24 940	43.8	45 992	26 613	57.9
12	58.082	26.679	45.9	60 107	27 128	45.1	48 928	29 312	59.9
15	62 016	29 349	47.3	66 053	30 772	46.6	52 359	32 152	61.4

*% Min. = % mineralization, calculated as (respired uptake/total uptake) x 100.

Appendix 23. Trial experiment of [^{14}C]glucose uptake and % mineralization for *P. fluorescens* isolate grown in the presence of 0 (○), 1 (●) and 1000 (△) ppm acephate.



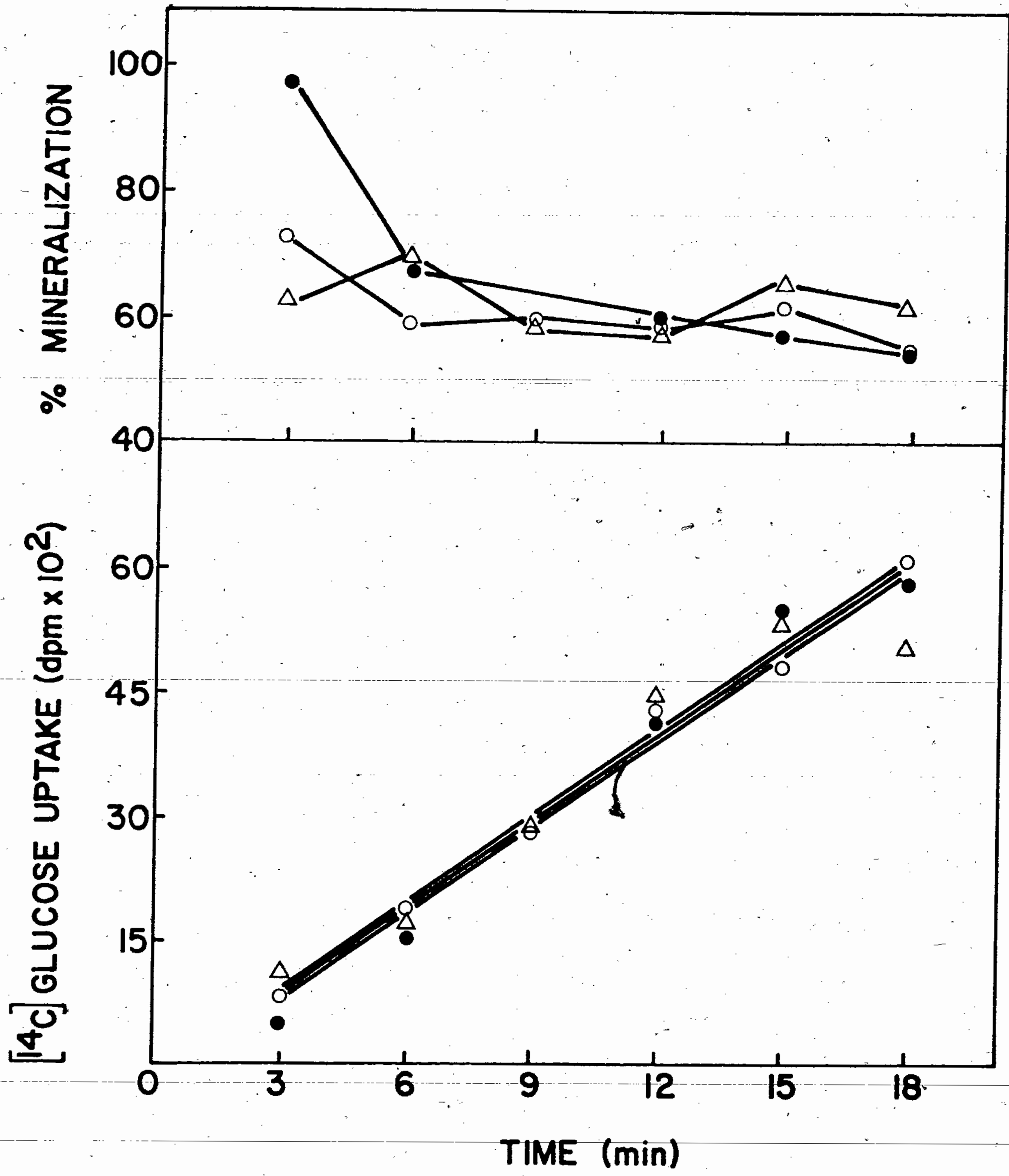
100
80
60
40
% MINERALIZATION
8
6
4
2
[14C] GLUCOSE UPTAKE (dpm x 10³)
0
3
6
9
12
15
TIME (min)

Appendix 24. Data for rate of total [^{14}C]glucose uptake and % mineralization for _P. fluorescens_ isolate grown in the presence of 0, 1 and 1000 ppm acephate (Appendix 23).

Time (min)	Control			1 ppm			1000 ppm		
	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*
	Total	Respired		Total	Respired		Total	Respired	
3	976	685	70.2	1065	548	51.5	539	445	82.6
6	2311	1487	64.3	1743	1141	65.5	1150	1081	94.0
9	3848	2432	63.2	2983	1846	61.9	3129	1874	59.9
12	4362	2678	61.4	4078	2307	56.6	3655	2427	66.4
15	7069	3964	56.1	5253	3027	57.6	4985	3038	60.9

*% Min. = % mineralization, calculated as (respired uptake/total uptake) x 100.

Appendix 25. Trial experiment of [^{14}C]glucose uptake and % mineralization for *C. lividum* isolate treated with 0 (○), 1 (●) and 1000 (△) ppm acephate at the time of glucose addition.


% MINERALIZATION
100
80
60
40
[14C] GLUCOSE UPTAKE (dpm x 10²)
60
45
30
15
0
3
6
9
12
15
18
TIME (min)

Appendix 26. Data for rate of total [^{14}C]glucose uptake and % mineralization for *P. fluorescens* isolate treated with 0, 1 and 1000 ppm acephate at the time of glucose addition (Appendix 25).

Time (min)	Control			1 ppm			1000 ppm		
	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*	Uptake (dpm)		% Min.*
	Total	Respired		Total	Respired		Total	Respired	
3	628	386	61.5	1609	474	29.5	656	455	69.4
9	3089	1644	53.2	3170	1619	51.1	3145	1838	58.4
12	3942	2419	61.4	2478	2153	86.9	3125	1566	50.1
15	5394	3041	56.4	5532	2675	48.4	-	-	-
18	6972	3626	52.0	6894	3761	54.6	6111	3362	55.0

*% Min. = % mineralization, calculated as (respired uptake/total uptake) x 100.