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A NOVEL SELECTIVE ENRICHMENT TECHNIQUE FOR USE IN BIODEGRADATION STUDIES

by

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ABSTRACT

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As more and more new organic compounds are being synthesized, the threat for environmental pollution caused by these chemicals increases accordingly. Consequently, it is necessary that we increase our knowledge of the various mechanisms by which these substances are degraded in the environment. Biodegradation is considered one of the convenient methods to assess the environmental hazards of a pollutant. To study the mechanism of biodegradation it is imperative to have an active bacterial culture which is normally acquired by the enrichment culture technique. The classical enrichment technique was designed on the principle that the solubility of the test substance in aqueous phase will never become a microbial growth-limiting factor. However, we all know that the fat soluble substance has a very low solubility in water and this renders the conventional enrichment technique less effective for selecting an active fat soluble substance degrading mciroorganism.

Investigation in our laboratory shows that the biodegradation of fat soluble substances mainly takes place at the fat-water interface and the extent of this interface will determine the availability of substrate to the degrading microorganisms and thus control the primary biodegradation rate. The isolation procedure involves finely emulsifying the test substance and stabilizing the emulsion with sodium ligninsulfonate. Microorganisms capable of degrading n-alkanes, aromatic hydrocarbons and polychlorinated biphenyls have been isolated by using this technique. This enrichment technique is useful in the study of the biodegradation of fat soluble substances and may contribute to our understanding regarding the persistence of such substances in the environment.

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Abstract

A selective enrichment technique has been developed for the rapid isolation of the lipophilic compound degrading microorganisms. This method is based on the fact that biodegradation of the lipophilic substance takes place mainly at the interface of the substance-water and the areal extent of this interface will determine the availability of substrate to the degrading microorganisms and thus control the primary biodegradation rate as well as the biomass of the microorganisms. The isolation procedure involves finely emulsifying the test substance and stabilizing the emulsion with sodium ligninsulfonate. Microorganisms capable of degrading n-alkanes, aromatic hydrocarbons, phenols and polychlorinated biphenyls have been isolated by using this technique.

INTRODUCTION

By definition, an enrichment technique implies the creation of an artificial environment which will either permit the desirable microorganisms to multiply at a faster rate than others or will suppress the growth of all but the desirable microorganisms (1). Enrichment technique have made an important contribution to the development of modern microbiology in the studies of microbial degradation and pathways. The literature on enrichment technique is vast and widely scattered, however, most of them deal with the isolation of microorganisms which will grow on hydrophilic compounds. Only limited information is available for isolating the lipophilic compound degrading microorganisms.

As more and more new organic compounds are being synthesized, the threat of environmental pollution caused by these new chemicals increases accordingly. Most are of lipophilic nature (hydrophobic) which are inherently more resistent to biodegradation than the hydrophilic ones. This is probably due to the fact that most microorganisms require an aqueous phase to carry out their metabolic activity and the hydrophobic nature of these new chemicals makes themselves less susceptible to microbial attack. Studies at these laboratories have been concerned with the biodegradation of persistent toxic organic substances and the experiments presented here are related to the isolation of lipophilic compound degradation microorganism by an enrichment technique.

MATERIALS AND METHODS

Source of inoculum: Activated sludge from the local municipal sewage plant was used as the major source of inoculum in the biodegradation study since the bacterial types (Alcaligenes, Acinetobacter, Flavobacterium, Pseudomonas and Escherichia) and their concentration ($7 \times 10^7 - 2 \times 10^8$ ml^{-1}) in the sludge were observed to remain relatively stable all year around. Occasionally, samples of activated sludge or soil from local industrial plants were also used as the bacterial seedings.

Growth medium: In order to control the variables normally encountered in a growth medium containing complex organic nutrients such as yeast extract and peptone, the following chemically-defined mineral medium was chosen as the basal medium with the following ingredients (g ℓ^{-1}): K_2HPO_4, 1.3; KH_2PO_4, 0.82; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.01; $CoCl_2 \cdot 6H_2O$, 0.001; $MnCl_2 \cdot 4H_2O$, 0.001; NaCl, 0.05. The final pH of the medium was 6.9 and was sterilized at 121°C for 15 min. Sodium ligninsulfonate and the test substances were sterilized separately before adding to the basal medium.

Preparation of emulsion: One hundred mg of the test substance (if the test substance was a solid at room temperature, it was dissolved in a minimal amount of n-hexane) was added to a sonic cup containing 10 ml of distilled water and 5 mg of sodium ligninsulfonate. The mixture was then subject to pulse ultra-sonification for approximately 1 min until it was emulsified. The emulsion was freshly prepared prior to the initiation of each experiment.

Preparation of emulsion-agar plate: Fifty mg of the test substance was made into 10 ml of a fine emulsion as described above under aseptical conditions and the emulsion then mixed with 500 ml of sterile mineral agar (approx. 50°C) for pour plate. Plates made in this manner were semi-translucent and colony development on these plates was slow (2 - 7 days).

Enrichment culture: Various amounts of the test substance in emulsion form were added to 125 ml Erlenmeyer flask containing 50 ml of mineral medium and 5 mg of sodium ligninsulfonate to give final concentrations of the test substance of 10, 20, 50, 100 and 300 mg ℓ^{-1}. Fresh activated sludge (0.1 ml) was added to each flask as the inoculum and the flasks were incubated at room temperature (21°C) on a gyrotary shaker for 1 - 4 weeks or longer. The samples were checked daily under a phase contrast microscope and the test substance was monitored for biodegradation using gas chromatography. If evidence of biodegradation was noticed, the culture broth was then used for the isolation of the degrading microorganism on emulsion agar plates.

Biodegradation measurement: In all experiments, only the primary biodegradation of the test substance was followed (the loss of parent compound). Five ml samples of the culture broth and 1 ml of n-hexane were mixed vigoroughly in a 15 ml conical glass centrifuge tube on a vertex mixer for 1 min. The emulsion was broken by centrifugation at 2,000 x g for 10 min and the clear hexane extract was used for g.c. analysis. Two gas chromatographs equipped with FID detectors were employed for the analyses. A Beckman GC-65 was fitted with dual 1.8 m x 6.3 mm o.d. stainless steel columns containing 2% OV-1 on Chromsorb

G-AW-DMCH. A Hewlett-Packard 5730A was fitted with dual 1.8 m x 6.3 mm o.d. glass columns containing 10% OV-1 on Chromsorb W-HP. The oven temperature was programmed from 100 - 250°C at 5-8°C min^{-1} and the temperatures at the injector port and detector were at 250 and 300°C, respectively.

Growth determination: Cultures were grown in 2 liters Bellco spinner flask at room temperature (21°C) and at various time intervals samples were withdrawn for dry weight determination (2).

Manometric technique: Oxygen consumption was measured at 20°C in a Gilson differential respirometer. Cells were harvested from the growth culture, washed and resuspended in cold 0.05 M phosphate buffer (pH 7.0). A typical reaction mixture contained 1 ml of cells, 1 ml of 0.05 M phosphate buffer, 0.9 ml of water, 0.1 ml of substrate and 0.15 ml of 20% KOH in the center well for CO_2 absorption. The final fluid volume was 3.2 ml.

RESULTS

The first successful application of this enrichment technique in this laboratory involved the isolation of a PCBs degrading Pseudomonas sp. from the activated sludge. In this case the development of this technique for the rapid isolation of lipophilic compound degrading microorganism was explored by using this bacterium and the commercial PCBs mixture (Aroclor 1221). Initially, an attempt was made to incorporate the PCBs into the growth medium by dissolving it in appropriate solvents such as acetone. As expected, the major portion of the added PCBs was found to separate out as soon as the acetone solution was added to the growth medium. No significant degradation of PCBs was noticed in the growth medium despite the growth of microorganisms. Further investigation revealed that most sewage microorganisms could utilize acetone as carbon and energy source for growth. Fig. 1 indicates that microorganisms in activated sludge were capable of rapid oxidizing acetone or acetate and this implies that it is inadvisable to use acetone as solvent in the enrichment technique as it may serve as an alternate carbon source.

However, when ultrasonically treated PCBs (as emulsion) was added to the mineral medium containing sodium ligninsulfonate as an emulsion stabilizer, significant degradation of PCBs (34%) was detected within 9 days. Further incubation to 11 and 14 days resulted in 71% and 99% of the added PCBs biodegradated, respectively (Fig. 2). From this mixture cultures, PCBs degrading Pseudomonas sp. 7509 was isolated which was capable of degrading PCBs from concentration of 300 mg ℓ^{-1} to a level of less than 1 mg ℓ^{-1} within 48 hours. It was noticed, however, that the

growth rate of P. sp. 7509 was greatly retarded if non-emulsified PCBs was employed with the growth medium. Examination by phase contrast microscopy of cultures grown with unemulsified PCBs revealed that the growth rate in terms of cell numbers was intimately associated with the dispersion of the PCBs droplets in the medium as shown in Fig. 3. It can be seen that most of the PCBs droplets were surrounded by the bacterial cells implying the possible involvement of bacterial action in the dispersion of PCBs droplets in the growth medium. Examination using an interference phase contrast microscopy showed that the cells actually attached themselves to the surface of PCB droplets, suggesting that microbial degradation of PCBs could be taking place at the PCB-water interface (Fig. 4). Similar observations were also noticed when this enrichment technique was applied to the isolation of other lipophilic compounds degrading microorganisms such as a p-cresol degrading bacterium (Fig. 5).

To further substantiate the usefulness of this enrichment technique for rapidly isolating and culturing the lipophile degrading microorganisms, P. sp 7509 was tested in the growth yield study. Since in the growth medium used, PCBs were the only carbon and energy source, the near-linear cell yield as a function of concentration of PCBs emulsion indicates that PCBs must be utilized by the cells for growth (Fig. 6).

The rate of PCB degradation by bacteria can also be followed by the rate of oxygen consumption (3). The results presented in Fig. 7 indicate a relationship between oxygen consumption and the concentration of PCBs in the flask, i.e., the amount of oxygen used by the cells is a direct function of PCBs concentration in the flask. It should be noted that with

all PCB concentrations employed in this experiment, there was no lag period for oxygen utilization, suggesting that the bacterial cells oxidized PCBs immediately, i.e., the cells grown by this enrichment method possess vitality sufficient to attack the lipophilic compound. UV-VIS spectrum of sodium ligninsulfonate recovered from the culture broth showed little difference from the control, indicating that sodium ligninsulfonate was not degraded in the process and hence did not provide an alternate carbon and energy source for the cells.

DISCUSSIONS

Investigators studying the biodegradation of lipophilic substances in the aquatic environment have always been confronted with the problem of finding a means to dissolve or disperse such substances in growth media. A number of enrichment methods have been used in the isolation of PCB degradation bacteria, including dissolving the PCBs in acetone (4,5), ethanol (6) and diethyl ether (7). All of these methods have their drawbacks. Acetone and ethanol are good substrates for microorganisms and provide alternate carbon sources for bacterial growth. Diethyl ether is immiscible with aqueous media. In addition, the fact that PCBs cannot be kept in the growth media at concentrations above their solubilities by the above techniques means that bacterial cells get little chance to contact the PCBs and consequently the likelihood of enhancing PCB degrading microorganisms is decreased. Finally, the use of organic carbon rich nutrients such as glucose and peptone in the enrichment medium (6) is of questionable value in that a great many different microorganisms can readily utilize such nutrients. This will affect which bacteria predominate in the growth medium and these need not necessarily be the lipophilic degrading ones.

The enrichment technique described here overcomes most of the problems encountered with methods used previously. The production of a fine emulsion of the lipophilic substance through ultrasonic means provides a much larger lipophile water interfacial area and so allows the microorganisms to overcome the substrate limiting problem which may determine subsequent bacterial growth rate. The use of sodium lignin-sulfonate rather than commercial emusifiers in the growth medium is due

to its stability to microbial action. While it provides a means to stabilize the emulsion, it is not utilized by the bacteria for growth. Therefore, most microorganisms isolated by this technique are very active in degrading the lipophilic compounds.

It should be pointed out that this enrichment technique is useful only when the test substance can be metabolized by the microorganism. Certain lipophilic substances, such as DDT, are only degraded by cometabolism (8). However, if necessary, a cometabolite can be applied in this enrichment technique. In conclusion, it would seem that the enrichment technique described here can be used reliably to obtain some lipophilic substanced degrading microorganisms from the environment.

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Fig. 1. Oxidation of Acetate and Acetone by activated sludge.

—●— Endogenous; —■— Acetate; —▲— Acetone.

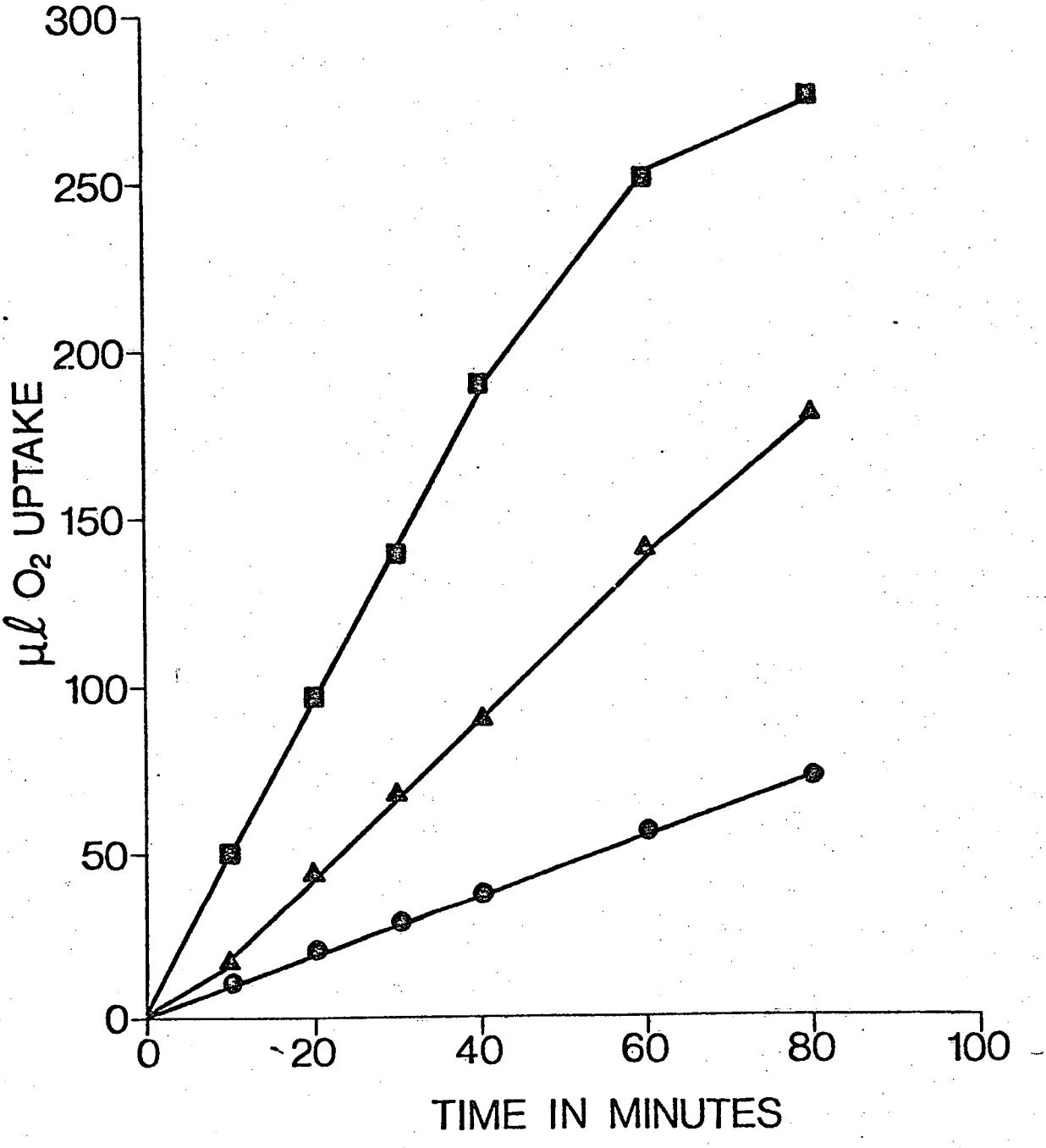

300
250
200
150
100
50
0
µl O2 UPTAKE
0
20
40
60
80
100
TIME IN MINUTES

Fig. 1

Fig. 2. FID gas chromatograms of n-hexane extracts of Aroclor 1221 at zero time, after 9 days, 11 days and 14 days incubation with activated sludge.

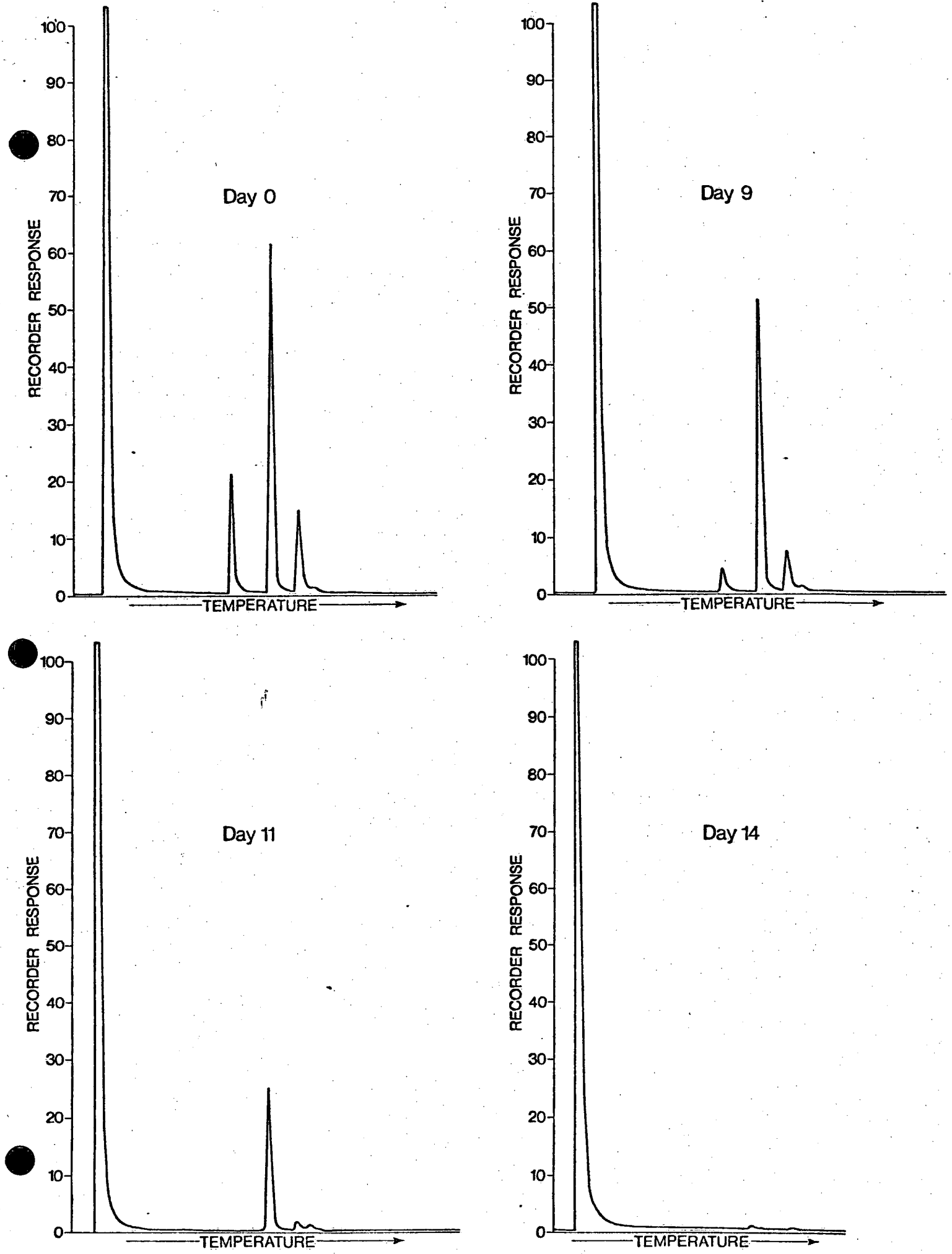
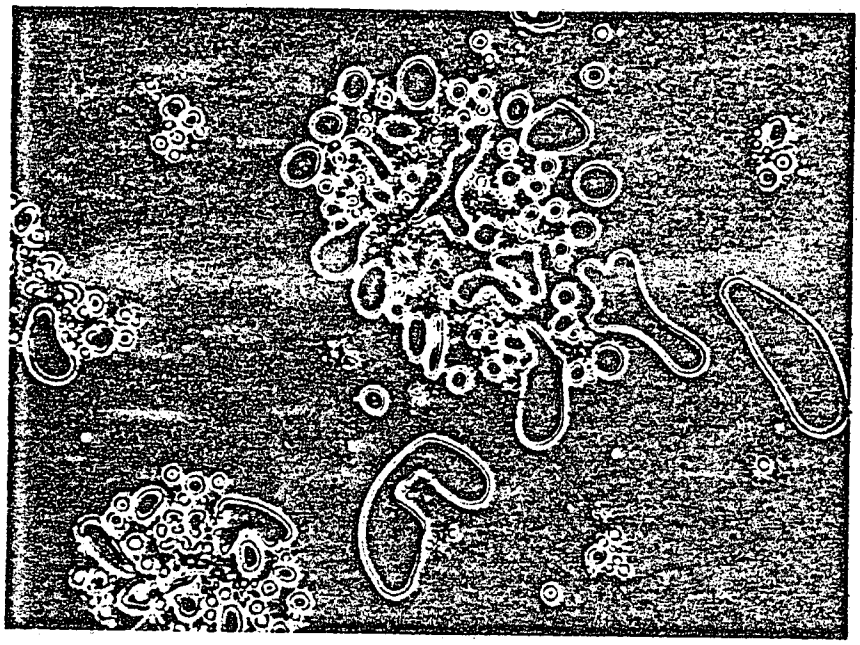
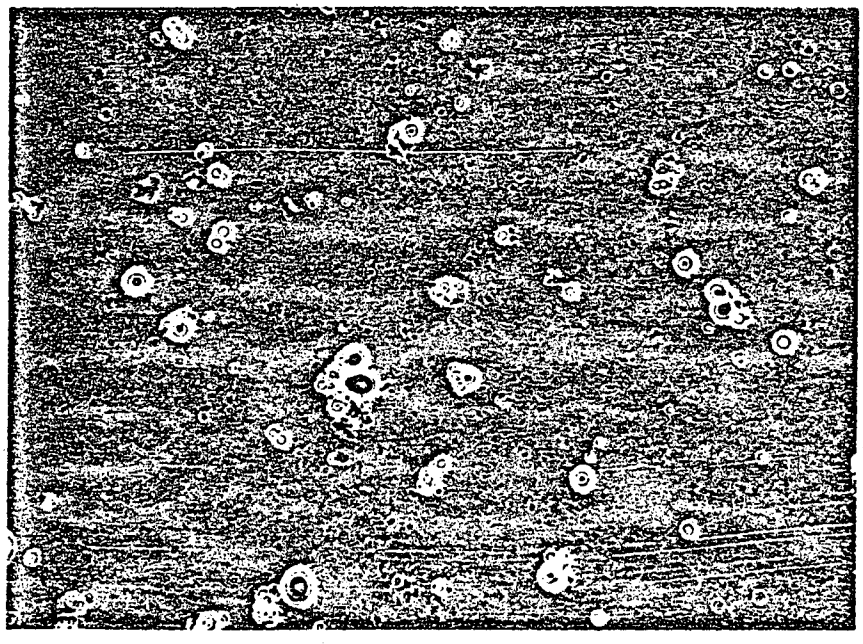

Day 0
Day 9
Day 11
Day 14
RECORDER RESPONSE
TEMPERATURE
0
10
20
30
40
50
60
70
80
90
100

Fig. 2

Fig. 3. Micrographs of the culture broth taken at different growth stages showing the dissociation of PCB droplets. 3a. early log phase; 3b. middle log phase. phase contract at 400X



a



b

Fig. 3

Fig. 4. Interference phase micrograph showing the bacterial growth at the PCBs-water interface. Interference phase contrast at 1000X.

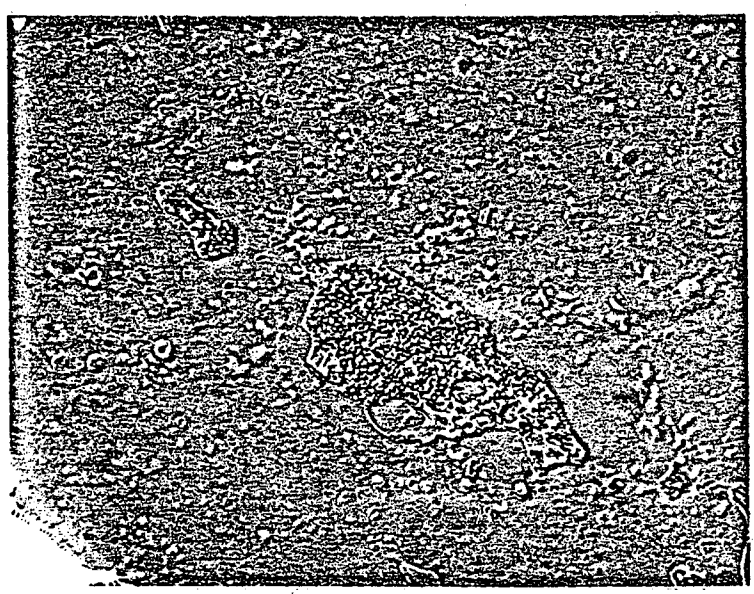


Fig. 4

Fig. 5. Interference phase micrograph showing the growth of a p-cresol degrading bacterium at the p-cresol-water interface (1000X).

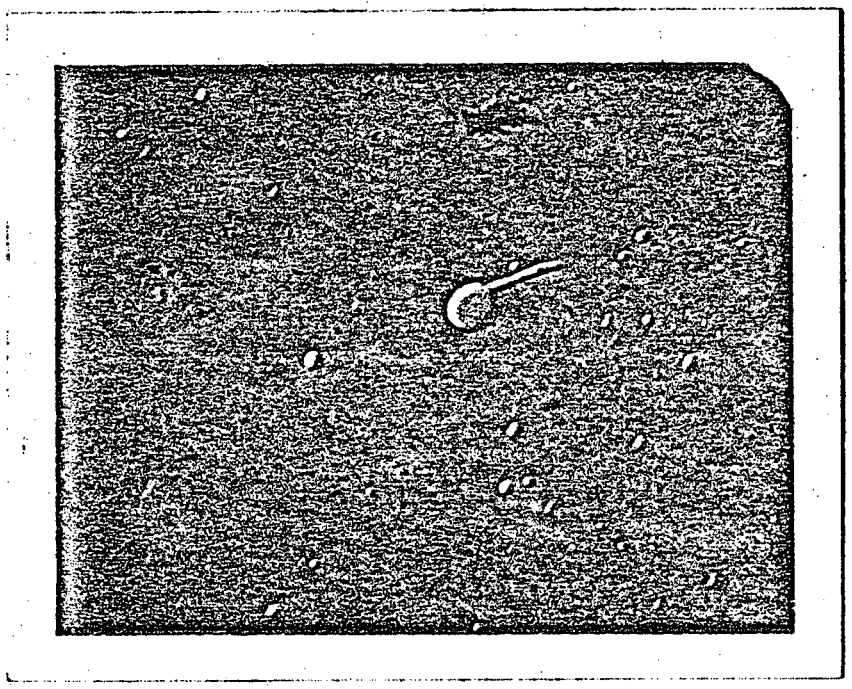


Fig. 5

Fig. 6. The growth yield of P. sp. 7509 as a function of Aroclor 1221 concentration in the growth medium.

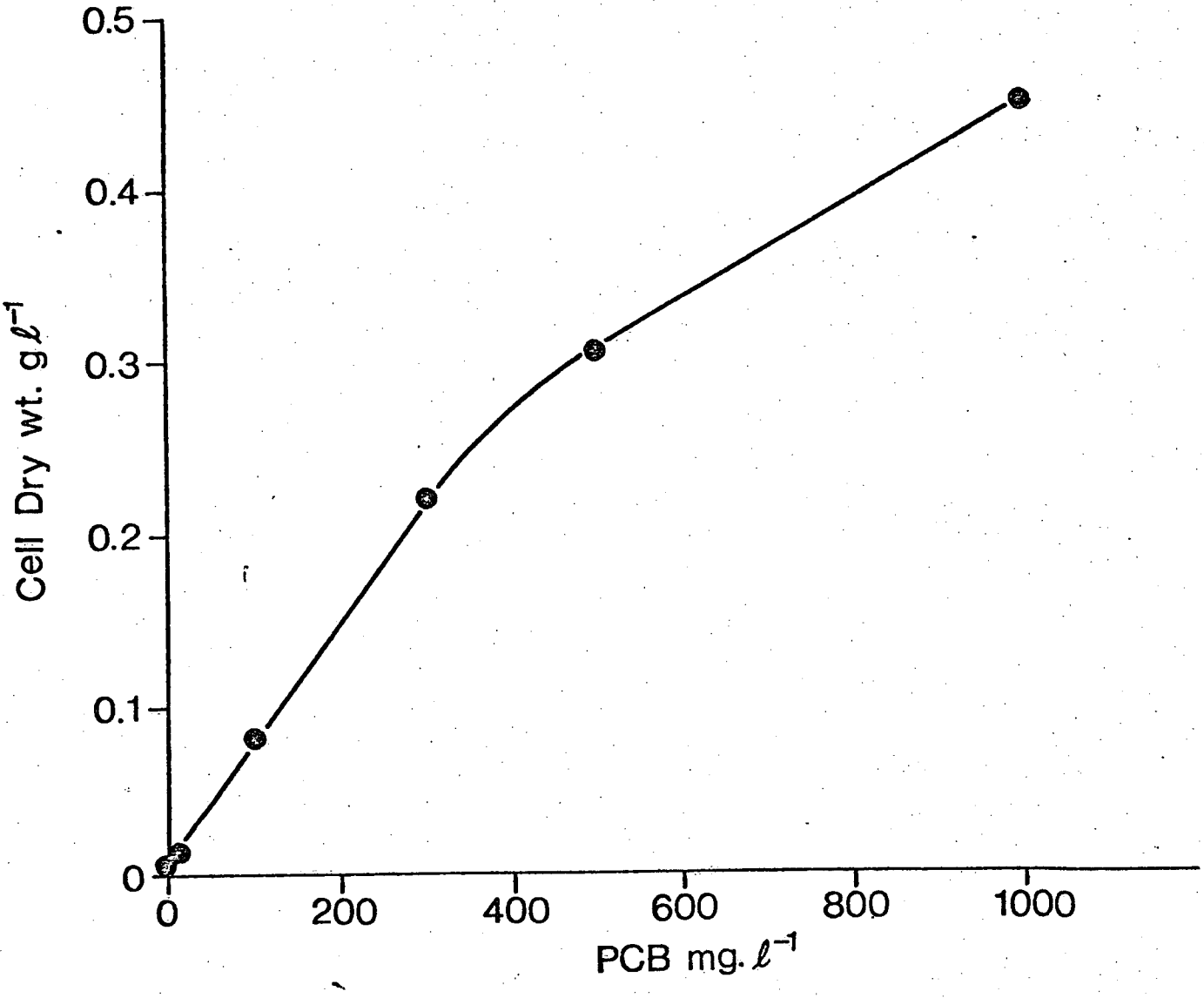

0.5
0.4
0.3
0.2
0.1
0
Cell Dry wt. g ℓ^{-1}
0
200
400
600
800
1000
PCB mg. ℓ^{-1}

Fig. 6

Fig. 7. Oxygen uptake as a function of the Aroclor 1221 concentrations in the flask.

Fig-7

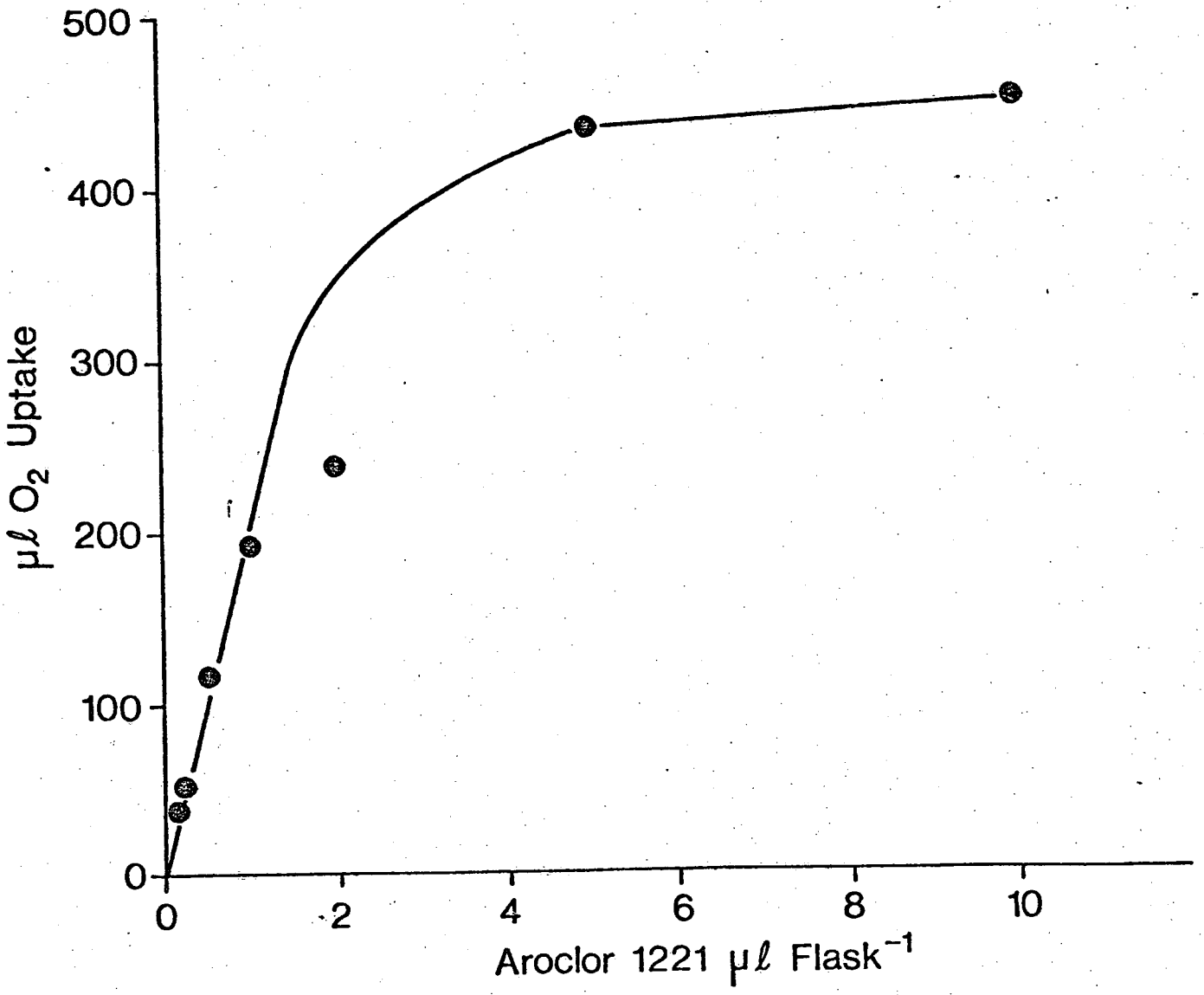

500
400
300
200
100
0
µℓ O_2 Uptake
0
2
4
6
8
10
Aroclor 1221 µℓ Flask^{-1}

Fig. 7

15893