SOLUBILIZATION OF INORGANIC PHOSPHATES BY BACTERIA IN LAKE SEDIMENTS

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I declare that this thesis has been composed by myself, and that the results described are my own, except for the sediment phosphate analysis in Experiment I, Chapter 3 and the keto acid analysis Experiment 7, Chapter 4.

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ABSTRACT

Phosphate availability is a major factor controlling algal productivity in Loch Leven, a shallow eutrophic freshwater lake. Experimental work was designed to investigate four main aspects of the influence of microorganisms on the phosphate cycle in the loch.

1. Phosphate Flux Between Sediments and Water The following parameters were investigated using Jenkins cores as models. The effect of:

(a) Sediment type on anaerobic phosphate release and the rate of change in redox potential.

(b) Poising the Eh with nitrate, sulphate or thiosulphate on phosphate release.

(c) Aerating sediments on phosphate movement.

(d) Temperature on phosphate release and redox potential changes.

(e) Sediment gas production on phosphate release.

(f) Algal phosphate uptake on phosphate movement between the sediment and water phases.

2. Role of Microorganisms in Phosphate Release from Sediments Jenkins cores were treated with HCHO to determine the contribution that biological activity made to phosphate release from the sediments.

3. Inorganic Phosphate Solubilization by Bacteria Under Laboratory Conditions

(a) An alternative method of enumerating phosphate solubilizing bacteria was devised.

(b) The effect of sediment type on the distribution of phosphate solubilizing bacteria was investigated.

(c) The bacteria solubilizing inorganic phosphate were identified.

(d) The ability of the bacteria to solubilize various inorganic phosphates under laboratory conditions was investigated.

(e) The effect of various alternative energy sources on phosphate solubilization was determined.

(f) Acidity and chelation effects on phosphate solubilization were separated.

(g) An attempt was made to identify the metabolic byproduct bringing about solubilization.

4. The Importance of These Processes in the Loch Assuming the data obtained in the laboratory were related to the loch situation, calculations were made to assess the movement of phosphate from the sediment in to the water phase.

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CHAPTER 1 INTRODUCTION

A lake is a large body of water totally surrounded by land. Lakes appear to be permanent features of the landscape but they are geologically transitory and, given time, will eventually fill in and disappear (Hutchinson, 1957). For man the water stored in these lakes is an essential commodity for life as we know it today. Not only is water necessary for life processes but it is also essential for industrial and sanitary purposes and is extensively used for recreation. It is easy to see, therefore, that the preservation of our water supplies and the maintenance of their quality is of the utmost importance.

At one time maintaining the good quality of water was not a problem because the world population was small and scattered. However deterioration in water quality started early in man's history. As soon as man started to clear the native forests, till the land and keep flocks of grazing animals, nutrients were leached, from the denuded land into the rivers and lakes. The coming of the industrial revolution intensified the problem. Towns sprang up with the inherent problems of sewage disposal and factories had to find some method by which they could dispose of their industrial wastes. As a result raw sewage and industrial effluents were discharged directly into the waterways with disastrous consequences. These wastes were often toxic e.g. the wastes from coke ovens (Stafford, 1970) and resulted in stretches of rivers becoming free from plants and animals. When these wastes were algal nutrients enormous algal blooms became common place. Griffiths (1939) found written evidence of blooms as early as 1804, although at this time they were uncommon. However by the beginning of the twentieth century algal blooms were being recorded quite frequently and biological indices had been devised by which the amount of pollution in a water body can be measured (Campbell, 1939; Butcher, 1946; Cairns & Kaesler, 1965). Many lakes have a bloom at some time of the year except in uninhabited upland areas. Subsequently the quality of inland waters fell. Lake water became unsuitable for use as a domestic supply and was frequently too dirty for industrial purposes as many industries e.g. steel, textile, paper, distilling need a clean water source. Numerous laws have now been passed which limit the amount of waste material which can be discharged into rivers and lakes. In many countries, especially in Europe and the United States there is very strict legislation which sets out the concentrations of chemicals which may be discharged into a waterway. However many of these measures were taken too late and the characteristics of inland water bodies have been changed irrevocably unless there is an enormous financial investment provided to clean them up.

The terms eutrophic, mesotrophic and oligotrophic were introduced into the literature by Weber in 1907 to describe the general nutrient status of the soil solution in German bogs (Hutchinson, 1969). The terms are now used to describe the nutrient status, especially phosphate and nitrate, of lake waters. Oligotrophic waters may be classified as systems with a low primary productivity and eutrophic waters as those with a high primary productivity (Hudson & Marson, 1970). Both types of water may have low nutrient concentrations at any given time but eutrophic waters have the capacity of becoming nutrient rich if the primary productivity falls. In undisturbed lakes, eutrophication is a natural 'ageing process' which eventually terminates in the disappearance of the lake (Likens, 1972) due to the deposition of detritus on the lake sediments and the encroachment of land plants e.g. willow, alder, which help the lake to 'dry out'. The process of eutrophication is frequently accelerated by mans activities but allowance has to be made for the nature of the lake basin when eutrophication is discussed. Eutrophication must be distinguished from pollution as the latter refers to damage to the water body by poisonous substances whereas the former is only nutrient enrichment although this also damages the system.

Lake Productivity

Any given ecosystem contains two biological groupings, primary and secondary producers. Plants and chemoautotrophic bacteria obtain their nutrients from inorganic sources and produce organic matter whereas secondary producers (heterotrophic organisms) utilise an organic nutrient source. Thus, the primary productivity of a system controls the secondary productivity. The primary productivity is, in turn, controlled by the inorganic nutrient supply and also, frequently, the amount of available light.

Nitrogen, in the form of nitrate, and phosphorus, in the form of orthophosphate, are both essential nutrients. An algal cell contains carbon, nitrogen and phosphorus in the ratios 106 : 16 : 1 and if any of these elements are limiting the cells are unable to grow and divide and the size of the population is naturally controlled. Silica is important in controlling the productivity of diatoms as this element is an essential major constituent of the diatom frustule. Many other minor elements e.g. Mg, Mn, Co, S may help to control algal growth (Goldman, 1972) but they are rarely thought to be the growth limiting nutrients.

In eutrophic lakes a balanced algal population will be maintained consisting of many different types. When phosphates and/or nitrates are in excess vast blooms of one species may occur and the natural balance of the lake population will be disturbed. Blue green algae (Cyanophyceae) are the most common cause of an algal bloom although diatoms (Bacillariophyceae) and green algae (Chlorophyceae) are also frequent offenders. Blooms are undesirable for numerous reasons. The water becomes aesthetically displeasing, appearing like 'pea soup' and often has an unpleasant odour. Macrophytes in deeper water may be affected because the light is reduced by the dense surface growth (Mackenthum, 1973). The water may become 'anoxic' at night due to there being algal respiration but no photosynthesis and when a bloom 'crashes' the water may become deoxygenated due to bacterial activity which affects fish and other animal populations. Filters to water works and other industrial premises may become clogged by algae with serious and, often, expensive consequences. Blue green algae also have the added disadvantage that they sometimes produce toxins which can result in massive fish kills. For these reasons it is desirable that the primary productivity in a water body should be controlled.

Excessive quantities of nitrogen or phosphorus compounds are the most common cause of eutrophication (Deevey, 1972). These chemicals enter the system from many different sources including sewage and industrial effluents (Vollenweider, 1968), soil erosion, both natural and originating from mans activities (Golterman, 1973) and run off from agricultural land after ploughing and fertiliser application or discharge from farm wastes (Cooke, 1972). Any effluent which contains detergents will be particularly high in phosphates, this includes sewage and effluents from the textile industries. The sediments also contribute towards the nutrient status of the lake due to decomposition and recycling of nutrients or by steady leaching of chemicals to maintain an equilibrium with the water.

As well as affecting the primary productivity in lakes, high phosphate and nitrate concentrations have other direct or indirect effects. The bacterial population increases because this tends to be directly correlated with the size of the algal population (Overbeck, 1972) with many of the bacteria producing growth factors which are required by the algae (Thomas, 1973). The increase in the algal population also causes the grazing invertebrates and other animals to increase in numbers and overall there is a greater biological oxygen demand in the water. As a result many deep lakes become both oxygen and temperature stratified in the summer. In oligotrophic lakes where the biological oxygen demand is low the hypolimnion will not become depleted in oxygen but where the oxygen demand is high, as in eutrophic lakes, the hypolimnion will be anaerobic causing many chemical reactions to take place in the reduced sediment and considerable leaching of nutrients, especially phosphate, from the mud into the water.

Phosphorus has been found to limit primary productivity in fresh water more frequently than nitrogen (Vollenweider, 1968) and it is also easier to control phosphate concentrations in a lake because vast quantities of atmospheric nitrogen could be biologically fixed annually in the water, mainly by blue green algae, which is an uncontrollable aspect of the nitrogen cycle. A close study of the role that phosphorus plays in eutrophication and how the availability of this nutrient could be controlled may help to alleviate the problem of algal blooms.

Role of Phosphorus in Living Systems

Phosphorus is the twelfth most abundant element in the earths crust. The most usual valence number of phosphorus is 3 or 4 but it can have co-ordination numbers ranging from 1 to 6 (Mahan, 1969). In all its compounds except the alkali and alkaline earth metal phosphides, phosphorus is covalently bonded to its neighbouring atoms. Phosphorus is spontaneously oxidised by oxygen and is usually found in the form of its oxides (phosphates) in the natural environment.

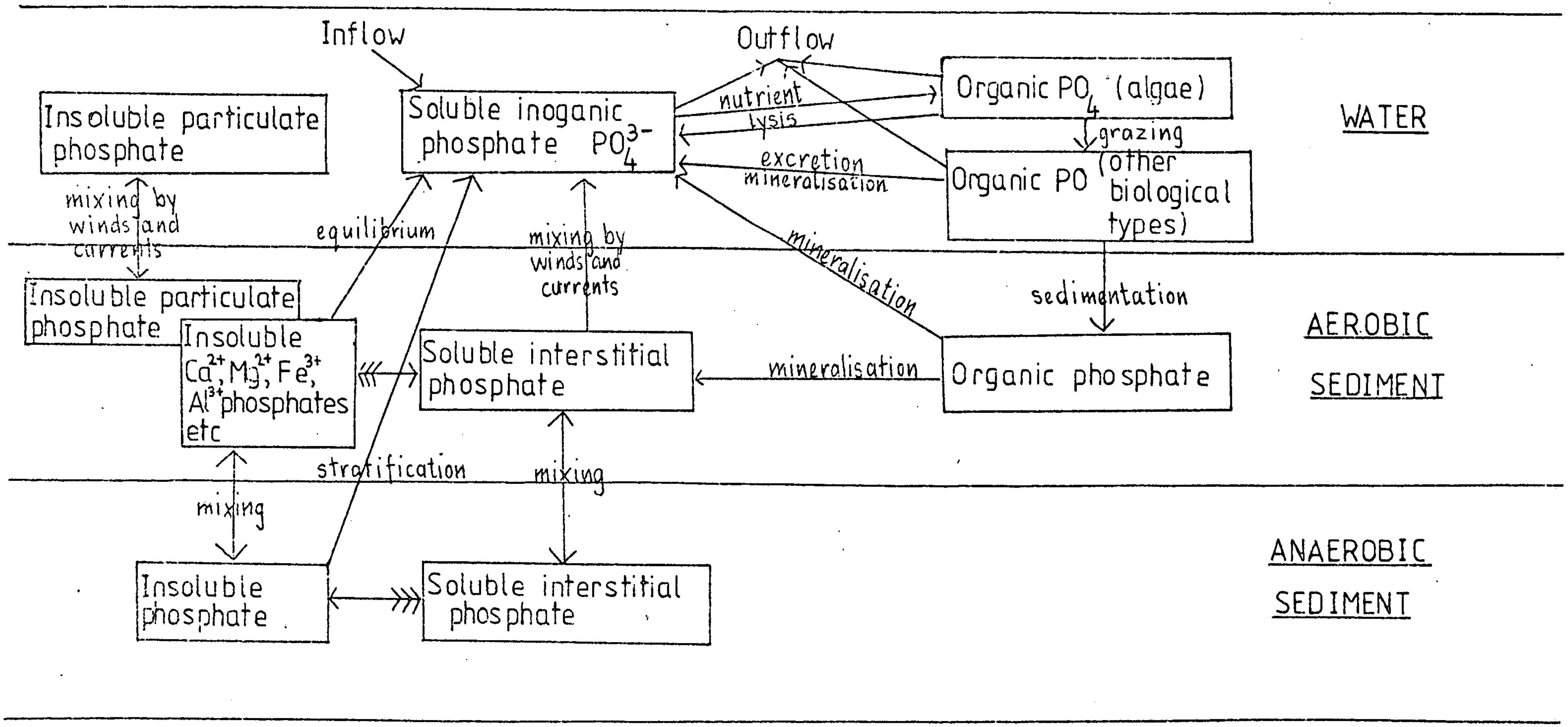
The variable valency of phosphorus makes it invaluable in living systems where it frequently acts as a high energy carrier. Adenosine triphosphate is the main high energy carrier. This can be converted to the diphosphate or monophosphate by losing the phosphate groups and the energy released by these reactions is used to bring about other cellular processes such as gluconeogenesis, muscle contractions and many other reactions (Leninger, 1970). Phosphorus is also found in many other compounds in living organisms such as nucleotides, lipids and phytin and as such is a fairly major constituent of a living system.

Phosphorus in Lakes

Sediments In lake sediments, the most abundant source of phosphorus is inorganic insoluble phosphates. The actual form in which the phosphorus is found depends upon such parameters as the pH, Eh and salt content of the environment. In acid sediments it is typically combined with iron and aluminium and in alkaline sediments calcium phosphate is dominant (Hesse, 1973). Typical phosphates found in sediments are apatite (e.g. $Ca_{10}(PO_4)_6F_2$), chlorapatite ($Ca_5(PO_4)_3Cl$), phosphoferrite ($FeMn_3(PO_4)_2\,3H_2O$), strengite ($Fe_3PO_4\,2H_2O$) and many others. Phosphorus is also found in the form of organophosphorus compounds such as phytin, nucleic acids and glycerophosphates. The organic phosphates originate from plant and animal remains and from microbial synthesis of inorganic phosphorus compounds. The interstitial water in the sediment has a high orthophosphate (PO_4^{3-}) concentration. This is because all the insoluble phosphates are in equilibrium with the water and phosphate is constantly dissolving or precipitating to maintain this equilibrium. Also microbial decomposition of organic compounds and excretion by animals in the sediment contribute to the high phosphate concentration in the interstitial water.

<u>Water</u> Lake water contains soluble and insoluble inorganic and organic phosphates. Soluble inorganic orthophosphate is the form of phosphorus which is available to algae as a nutrient. Polyphosphates, which originate from algae, are also inorganic in nature and can be utilised as a nutrient. Insoluble inorganic or

Fig 1. Phosphorus cycling in freshwater lakes



'particulate' phosphate originates mainly from disturbance of the sediments by winds and currents. The organic phosphates are found in living organisms i.e. fish, phytoplankton, zooplankton and bacteria. In living organisms these 'bound-up' organic phosphates may be termed as insoluble as they are unavailable as nutrients. However upon the death and lysis of algae, for example, they may become soluble and can be utilised by bacteria and zooplankton. Fish, birds and zooplankton excrete large quantities of organic and inorganic phosphates into the water (Scharpf, 1973).

Phosphorus Cycling in Lakes

(a) Main Pathways of Movement Many detailed studies of the phosphorus cycle in lakes have been published (e.g. Rigler, 1956; O'Melia, 1974; Porter, 1975). Rigler, (1975) gave a dynamic view of the phosphorus cycle in a freshwater ecosystem. The phosphorous cycle is complex but less so than the carbon and nitrogen cycles because these elements can be lost from the system in the form of gases. It is possible to construct a box diagram (Fig. 1) for phosphorus which shows the location of the major types of phosphorus in a lake and how they interact with each other. A complete picture of the phosphorus cycle in any given lake could be obtained by determining all the relevant values in the diagram. However many of these interactions are difficult to measure. For example, there are no satisfactory techniques for measuring the sedimentation of algae (Smith, 1974) which could add enormous quantities of organophosphorus to the sediments annually. Estimating the effects of winds and currents is difficult yet these may help phosphate release from the sediments by mixing the interstitial or particulate phosphate with the main body of the lake. There are many non-biological interactions taking place in the phosphorus cycle, especially in the sediments. Insoluble inorganic phosphates may be reduced to form soluble phosphates and thus released into the water phase or the opposite reaction could take place in oxidising conditions thus rendering the phosphate unavailable as a nutrient. A typical reaction would be

$$Mn^{3+}\ PO_4\ \text{(insoluble)}\ \xrightarrow[\text{oxidised}]{\text{reduced}}\ Mn^{2+}\ PO_4\ \text{(soluble)}$$

Constant leaching from the sediments due to equilibrium constants probably contributes vast quantities of phosphate to the water column annually (Stumm & Morgan, 1970) but if the phosphate concentrations in the water were high the opposite process would take place.

(b) <u>Role of Organisms</u> The organisms found in the sediments and waters of lakes can affect the phosphorus in many different ways.

(1) <u>Algae</u> If other conditions are not limiting algal growth is directly proportional to the supply of orthophosphate in the water, up to a certain value,

depending on the species. If there is excess phosphate available the algae can accumulate and store it in the form of polyphosphates which are entirely inorganic. Scenedesmus sp. and Chorella sp. can accumulate as much as 10% phosphorus by dry weight and this is known as luxury uptake (O'Kelley 1973). These polyphosphates can be utilised if the water becomes depleted in phosphate. When the algae die they either lyse, thus adding organic phosphates directly to the water phase, or they sediment out, thus contributing organic phosphates to the sediments.

(2) Invertebrates The zooplankton in a lake graze upon the phytoplankton and are frequently major contributors to the recycling of organic phosphorus in the water phase. All the invertebrates excrete both inorganic and soluble organic phosphates and invertebrates in the sediments probably play a major role in the decomposition of complex organophosphorus compounds. Nematodes and insect larvae in the sediments may help in the release of orthophosphate from the interstitial water to the main water column by disturbing the sediment surface when emerging. Invertebrate respiration helps to establish reducing conditions at the onset of stratification thus contributing to the subsequent release of reduced phosphates.

(3) Bacteria Microorganisms affect phosphorus in many different ways and play a major role in the phosphorus cycle.

(i) Breakdown of Organic Phosphates Organic phosphates in natural waters may be present in soluble, colloidal and suspended forms. Many bacteria have heterotrophic nutrition e.g. *Bacillus sp.*, *Pseudomonas sp.* etc., and are capable of degrading these organic phosphates, thus recycling the phosphorus. The bacteria bringing about the mineralisation of organic phosphates have been well characterised. The sorption and hydrolysis of organic phosphates in lake sediments is partly due to chemical interactions and partly due to the action of microorganisms. Sorption depends upon chemical interactions and hydrolysis is brought about by sediment microorganisms (Rodel & Armstrong, 1977). The hydrolysis is brought about by the action of phosphatase enzymes. These are a group of enzymes which catalyze the hydrolysis of both esters and anhydrides of phosphoric acid (Schmidt & Laskowski, 1961). These phosphatases cleave the bond between the phosphate group and the carbon chain thus releasing orthophosphate into the environment (Feder, 1973). The concentration of free phosphatase enzymes in lake water has been found to correlate with the amounts of phytoplankton and bacteria (Reichardt et al, 1967). Golterman, (1960) gives a detailed account of phosphorus mineralization in fresh water using two algal types, *Scenedesmus quadricauda* and *Stephanodiscus hantzchii*, as examples. However, approximately 30% of the phosphorus in algae is fairly resistant to degradation and forms refractory material in the sediments. Scharpf, (1973) has concluded that this material eventually can be degraded and utilised, but at rates of no more than a few percent per year.

(ii) <u>Production of Metabolic By-products</u> Many bacteria under certain favourable nutritional conditions are capable of producing metabolic by products which accumulate in the environment surrounding the cell. Some of these products are capable of affecting the form in which the phosphate is present in the sediment. The solubility of many inorganic phosphorus compounds is governed by the pH of the environment (Stumm & Morgan, 1970) and most phosphates are more soluble under acid conditions. Many bacteria produce acids especially by fermentation, and this would have the effect of lowering the pH of the microenvironment surrounding the cells, thus phosphates such as calcium or aluminium would become more soluble and therefore more available to algae as a nutrient. Also certain bacteria are capable of producing keto acids on sugar containing substrates (Webley, 1959). The keto acids can chelate the cation of an insoluble phosphate such $MgHPO_4$ thus rendering the phosphate anion soluble. The production of keto acids could therefore have the double effect of lowering the pH and chelating the cation.

By ?

(iii) <u>Creation of Reducing Conditions</u> Many phosphates are more soluble when in a reduced than in an oxidised state. Bacteria can help to establish suitable reducing conditions for the reduction of phosphates by first of all assisting invertebrates to use up oxygen and then by utilising other chemicals e.g. NO_3^-, SO_4^{2-} and thus lowering the redox potential of the sediments even further.

(c) Phosphorus Availability In many lakes the primary productivity is too high to be accounted for by the phosphorus entering from short term external sources. If the production of algae is equated with the input and output of phosphorus to the lake there is frequently a deficit, that is the input of phosphorus is insufficient to maintain the observed primary productivity of the lake (e.g. Holden & Caines, 1974). The sediments can act as a reserve of phosphorus which can contribute phosphate to the water phase. Alternatively sediments can absorb excess phosphate from the water (Harter, 1968; Li et al, 1972). Because the sediments are capable of contributing towards the nutrient loading of a lake it is important to know the exact conditions under which release or adsorption of phosphate (or other nutrients) will take place and the factors controlling these conditions. There is limited value in controlling the nutrient input from external sources if the internal input is adequate for production, but once this unknown contribution is quantified the control of the primary productivity by altering the nutrient loading to the lake may be feasible.

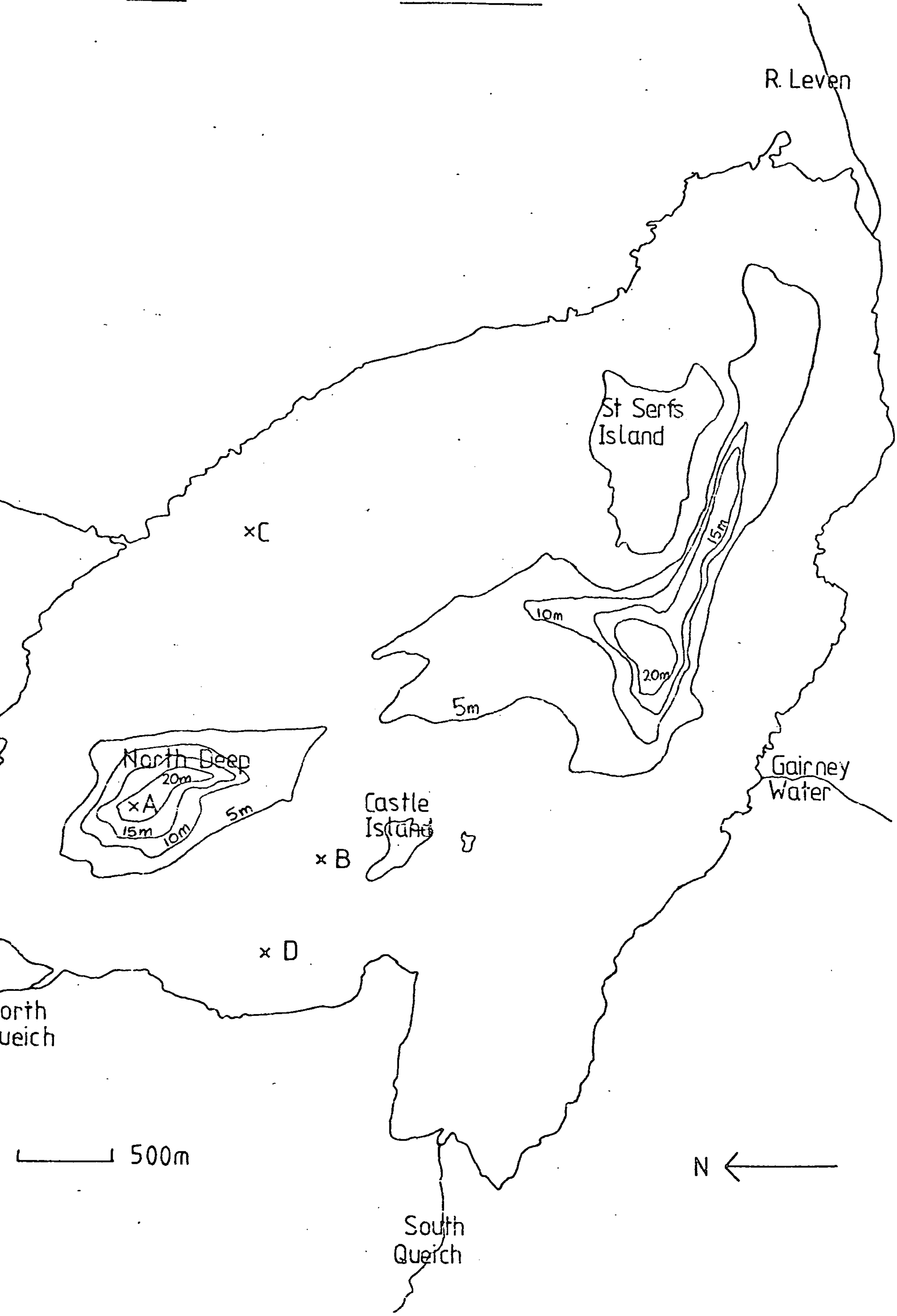
The General Physical and Biological Characteristics of Loch Leven

Loch Leven has long been a famous fishery, dating back to the fourteenth century. Scientific records of the plant and animal populations are available for the nineteenth and twentieth centuries. These records show that there have been considerable changes in the fish and plant populations of the loch in the past 100 years. The diversity of fish species has declined with brown trout (Salmo trutta) now being dominant. There are fewer macrophytes in the loch and although dense concentrations of blue green algae were occasionally recorded before the turn of the century there has been a bloom every year since 1963 (I.B.P. report, 1974).

The loch is a national nature reserve and is the most important water body in Scotland for numbers of migratory, breeding and overwintering water foul. A number of studies have been carried out on Loch Leven since the end of the nineteenth century and since 1963 regular observations have been made, after the initiation of chemical studies to explain the algal blooms. In 1964 it was proposed that an interdisciplinary investigation should take place as part of the International Biological Programme. When the I.B.P. study ended work continued on the Loch and is still being undertaken. There is, therefore, much chemical and biological information about the loch which is only 35 miles from Edinburgh.

Loch Leven was formed at the end of the last glaciation from kettle holes left in the glacial drift by retreating ice. The mean water level is 10.7m above sea level and the loch lies on a fertile plain where the soil is derived

Fig.2. Loch Leven



from old red sandstone and carboniferous rocks. The mean depth of the loch is 3.9m and the maximum depth is 25.5m. There are large areas of shallow water (Fig.2) and two deep holes, the North and South deeps, of over 20m (Smith, 1974). The sediments of Loch Leven are rich in iron and vary from coarse sand (grain size$>$2mm) along the shoreline to a fine silt (grain size$<$0.004mm) in the deep holes. The sandy areas are poor in organic carbon (0.05%) but the percentage gradually increases until in the deep holes it is about 8% (Calvert, 1974). There are three main inflows to the loch, the North and SouthQueich and Cairney Water and only one outflow; the River Leven. The prevailing winds are south westerly and as the loch is very exposed it is well mixed for most of the year.

The waters of Loch Leven are eutrophic as is shown by the high primary productivity, the chorophyll level rarely being below 40 ugl^{-1}(Bailey Watts, 1977). The surrounding land has long been used for intensive agriculture so there will be leaching of nutrients into the loch from this source especially from fertiliser application. The human population is congregated in two main centres, Kinross and Milnathort, each of which has a sewage treatment plant which discharges into the loch. A woollen mill situated on the South Queich discharges its effluent directly into the river. The mill used to contribute a large part of the total phosphorus entering the loch but that entering from other sources in now significantly greater (Holden, 1974; Caines, 1976). The total amount of phosphorus retained in the loch has decreased but vast algal blooms still occur regularly (Bailey-Watts, 1977).

Objectives of Present Investigation

Much of the work carried out on the release or adsorption of phosphate by sediments has concentrated on the physical and chemical aspects of the problem, for example, the effect of sediment type and pH. Living organisms also play an important role in the movement of phosphate in and out of the sediment. Bacteria, in particular, affect the phosphate flux in many different ways.

The work described in this thesis attempts to quantify the role that microorganisms play in the release of orthophosphate from lake sediments. The contribution that bacteria make to the creation of reducing conditions has been studied. Investigations have been made to ascertain the exact redox potential necessary for phosphate release. Attempts have been made to sterilise or partially sterilise sediments, with an assessment of their success, to separate biological and non biological factors leading to anaerobic phosphate release. The effect that sediment type, temperature, aeration and light have on phosphate release have been investigated. The properties and activity of phosphate solubilizing bacteria in Loch Leven were investigated because these bacteria have been studied in soils on numerous occasions but very little information is available about their distribution in lake sediments. The pH and chelation effects of the by-products produced by these bacteria were assessed. An assessment of the contribution that all these different mechanisms of phosphate release from the sediment were making to the water phase of Loch Leven was made in order to ascertain the source of the additional phosphates that sustain the enormous algal blooms.

CHAPTER 2 MATERIALS AND METHODS

(A) Sampling Four sampling sites were chosen so that sediments from a variety of depths with differing inorganic, chemical and organic carbon contents representative of the loch could be studied.

The sites were as follows (Fig 2):-

(1) The North deeps (A), a silty area 20m deep with sediments containing 8% organic carbon.

(2) An area just off Castle Island (B), 4m deep with 4% organic carbon. This site had a clayey type of sediment and was representative of a large area of the loch.

(3) Two sandy sites (C & D) near the shore, 1m deep with less than 0.5% organic carbon. Site C supported a macrophytic population consisting mainly of Potomogeten sp. but there were no macrophytes present at the other sampling sites (A, B & D).

Buoys were placed at all the sites so that samples were collected from the same location on all occasions.

Samples from the silty and clayey areas (A & B) were collected using a Jenkins corer (Mortimer, 1941) which gives a sample consisting of approximately 1 litre of sediment and 1 litre of water. There was usually more sediment in the cores taken from the deep areas where the mud is softer. The samples from the sandy sites were collected using a corer specifically designed for sampling shallow sandy areas in that it is pushed into the sand manually (Maitland, 1969). Both the Jenkins corer and sand corer gave a relatively undisturbed sample and care was taken not to disturb the sediment/water interface. The metal covers on the top and bottom of the cores were replaced with rubber bungs soon after sampling. The cores were immediately transported to the laboratory (35 miles) where various treatments were carried out on the day of sampling.

Water samples for chemical and bacterial analyses were collected in sterile glass jars. The jars were only ¾ filled to prevent deoxygenation of the samples.

(B) Treatments of Jenkins Cores

(1) Aerobic When the cores were left standing, exposed to the atmosphere, the rate of diffusion of oxygen was not great enough to prevent reducing conditions from being established. Consequently it was necessary to aerate the water phase. Glass spargers, placed in the water column as near to the sediment surface as possible without causing visible disturbance, were attached to a small pump. The aeration rate was adjusted by placing clips on the rubber tubing which led from the pump to the spargers. To prevent undue evaporation of the water petri dish lids were placed over the cores but even with these precautions the water level had to be adjusted daily using distilled water. Water samples, which were taken using a syringe, were placed in polythene bottles and immediately frozen.

(2) Anaerobic Sealing The bottom lid of the core was replaced

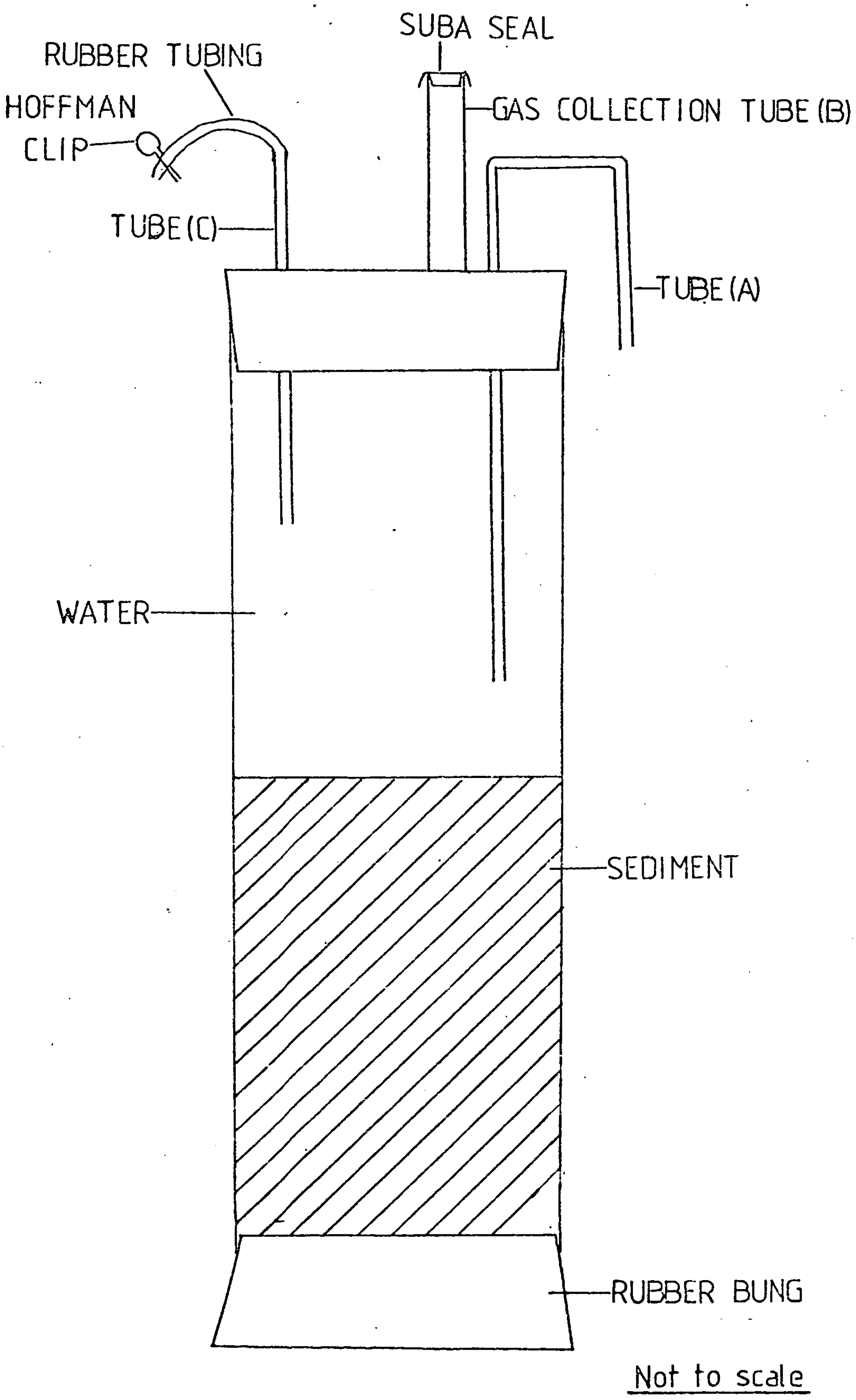


Fig 3 Anaerobic sealing of a Jenkins core.

with a tightly fitting rubber bung. The metal lid at the top of the core was removed and replaced with a rubber bung which had two narrow glass tubes and one wider tube inserted (Fig.3). All the gas bubbles were removed by placing tube A in distilled water and drawing the air out through B using a syringe. This was repeated with tube C. The system was sealed using a silicone based aquarium sealer.

Water samples were taken from the anaerobic cores by placing tube A in distilled water and drawing core water out through C using a syringe. The water was placed in a polythene bottle, then frozen. Gas which had collected in B was removed by once again immersing tube A in distilled water and drawing the gas out through the suba seal using a syringe with a fine needle. Care was taken at all times to prevent air entering the cores after they had been sealed.

(3) Segmentation of Cores Horizontal segments 3cm thick were cut from the sediment collected in the Jenkins cores. The core was clamped to a securely fixed retort stand, after the water had been siphoned off. The metal lid at the bottom of the core was replaced by a straight sided bung which just fitted inside the tube. The sediment and bung were pushed up the core using a narrow metal tube. When the mud emerged from the top of the core, 3cm slices were cut using a thin sheet of aluminium which had been sterilised by dipping it in methylated spirits and then

flaming. The slices were placed in sterile polythene bags. A certain amount of mixing between slices took place because as the sediment was pushed up the core the more liquid surface layers tended to remain on the side of the tube and mix with the deeper sediment. However this amount of cross contamination appeared to be unavoidable.

(4) Mini cores The standard size of Jenkins core has a volume of 2 litres and a smaller core would have been more convenient to use for certain experiments. A core 4.5cm diameter and 30cm long was chosen which had a volume of 500ml. The water from the large cores was siphoned into a clean container and the mud was then placed in the mini cores so that they were half filled (250ml). They were topped up with water and allowed to stand overnight at 4°C. A bung, similar to that on the anaerobic cores (Fig.3) was placed on the mini cores. Tube B was plugged with cotton wool and air was pumped in through tube C, the air flow being controlled by metal clips so that the sediment surface was undisturbed. The cores were aerated for 4 weeks at 10°C, this being the time required for the orthophosphate concentration in the water and the total number of viable bacteria on the sediment surface to reach a constant value. The cores were then ready for experimental use. Similar methods to those used with the standard cores were used to seal and sample the mini cores.

TABLE 1 Colony counts on casein peptone starch medium at $25^{\circ}C$ for 21 days to find the best method of dispersing bacteria from sediment and the most suitable diluent

Dispersant	Diluent	
	½ strength Ringers solution	0.5% peptone
glass beads	4.0×10^7	1.4×10^6
whirlimixer	6.0×10^5	8.0×10^4
1% calgon	2.1×10^4	7.6×10^3

Counts are the number of bacteria per ml wet sediment and are an average of three samples.

(C) Microbiological Methods

(1) Sampling and dilution The samples were collected in jars and cores as described previously. For the sediment analyses the water from the cores was siphoned off and discarded. 10ml of the sediment surface was sampled using wide mouthed pipettes and placed in soil dilution bottles. 90ml $\frac{1}{4}$ strength Ringers solution containing glass beads were added to the sediment sample and shaken for two minutes. The resultant suspension was diluted to give a final dilution of 10^{-5} using $\frac{1}{4}$ strength Ringers solution for 10 fold dilutions. Water samples were treated similarly. As the sand was difficult to pipette 20g was used instead of 10ml and the volume occupied by this amount was determined.

Shaking with glass beads appeared to be the most successful method for dispersing the bacteria from sediment or algae (Table 1). Whirlimixing or the use of Calgon (a detergent) gave lower counts than glass beads. Similarly the use of 0.5% peptone as diluent gave lower counts than $\frac{1}{4}$ strength Ringers solution (Table 1).

(2) Total viable counts Total viable counts of bacteria can be carried out using plate counts or fluorescence microscopy techniques (Jones & Simon, 1975). However the latter technique is less reliable than plate counts, which in themselves are not entirely accurate, and small change in methodology can give widely varying results. Thus throughout this work plate counts were used for viable counts of

TABLE 2 Media Used for Counting Bacteria

Casein, peptone, starch	Phosphate solubilising bacteria
casein 0.5g	glucose 0.5%
peptone 0.5g	NH_4SO_4 0.2%
starch 0.5g	$MgSO_4$ 0.05%
$MgSO_4$ $7H_2O$ 0.05g	KCl 0.01%
Fe EDTA 0.05g	yeast extract 0.05%
K_2HPO_4 0.2g	agar 2%
agar 15g	
glycerol 1.0ml	
distilled H_2O 1000ml	

TABLE 3 Numbers of Phosphate Solubilising Bacteria Isolated on Various Media ($x10^3$)

Glucose concentration % / Nitrogen source		0.1	0.3	0.5	0.7	0.9	1.0
Asparagine	0.2%	0	0.07	0.96	11.0	15.0	3.4
Nitrate	0.2%	0	0.68	21.0	20.0	33.0	14.0
NH_4 SO_4	0.2%	0	0.4	52.0	41.0	44.0	26.0

bacteria. Several media were assessed to obtain the higher count, these were nutrient agar, soil extract agar, and a casein, peptone starch medium (Collins, et al 1973). The casein, peptone starch medium gave the highest counts in the order of 10^7 organisms per ml, compared to $10^5 - 10^6$ for the other media investigated. This medium is very dilute and the lake bacteria were, perhaps, adapted to a nutrient poor environment. Thus for total viable counts 0.1ml of the dilutions were spread on to a casein, peptone starch medium (Table 2), incubated for 21 days at 25°C and the colonies were then counted. The plates were incubated in the dark to prevent algal growth. 25% of the colonies growing on plates incubated in the light were algae.

(3) Phosphate Solubilizing Bacteria Phosphate solubilizing bacteria were counted using a modification of the medium of Katznelson and Bose, 1959 (Table 2). The original medium was designed for soil bacteria and preliminary investigation showed that the medium was too rich for lake bacteria. By varying the glucose concentration and nitrogen source the most suitable combination was found (Table 3) to be a medium containing 0.5% glucose and 0.2% ammonium sulphate. The highest number of phosphate solubilising bacteria were isolated on this medium.

To estimate the numbers of these bacteria 10ml sterile medium was dispensed into sterile $\frac{5}{8}$" test tubes with metal caps and allowed to solidify. 0.1ml of the sediment in a series of 10 fold dilutions were inoculated on to the surface

Plate 1 Clearing in a precipitate of phosphate in agar medium by bacteria isolated from Loch Leven.

of the medium. The tubes were incubated for 4 days at 25°C and any tubes which showed clearing in the precipitate were scored as positive.

The bacteria were isolated by spreading diluted sediment on to plates containing the phosphate solubilizing medium. These were incubated at 25°C for 4 days and any colonies which gave a clearing in the precipitate (Plate 1) were subcultured on to nutrient agar.

(D) Chemical and Physical Methods

(1) Oxygen Analysis of Core Water The rate of respiration in the sealed Jenkins cores was assessed by measuring the rate at which oxygen disappeared from the core water. Initially oxygen electrodes were inserted into the cores but these proved unsuccessful therefore the Winkler method of oxygen analysis was utilised.

Reagents

(a) Manganese sulphate solution, dissolve 500g $MnSO_4\ 4H_2O$ in 1 litre distilled water.

(b) Alkaline iodide solution, dissolve 150g KI in small quantity of distilled water and add the supernatant of a solution containing 500g Na OH in 500ml water and dilute to 1 litre.

(c) Sodium thiosulphate solution - 0.0125 M (1ml = 0.1mgO_2).

(d) Starch indicator, dissolve 2g of soluble starch in 300ml of boiling water.

(e) Concentrated sulphuric acid.

Method A 70ml glass stoppered bottle was completely filled with a fresh sample of water from a core and a check was made that no air bubbles were trapped beneath the stopper. 0.24ml $MnSO_4$ solution and 0.24ml alkaline KI solution were added to the sample. The bottle was restoppered rapidly and mixed. The precipitate formed was allowed to settle and the mixing procedure was repeated twice more to ensure complete adsorption of oxygen. 0.28ml concentrated H_2SO_4 were then added and the sample was mixed thoroughly. A 25ml aliquot was titrated with 0.0125 M thiosulphate solution using starch as indicator.

$$\text{Dissolved oxygen (mg/l)} = \text{ml } Na_2 S_2 O_3 \times \frac{10^2}{25}$$

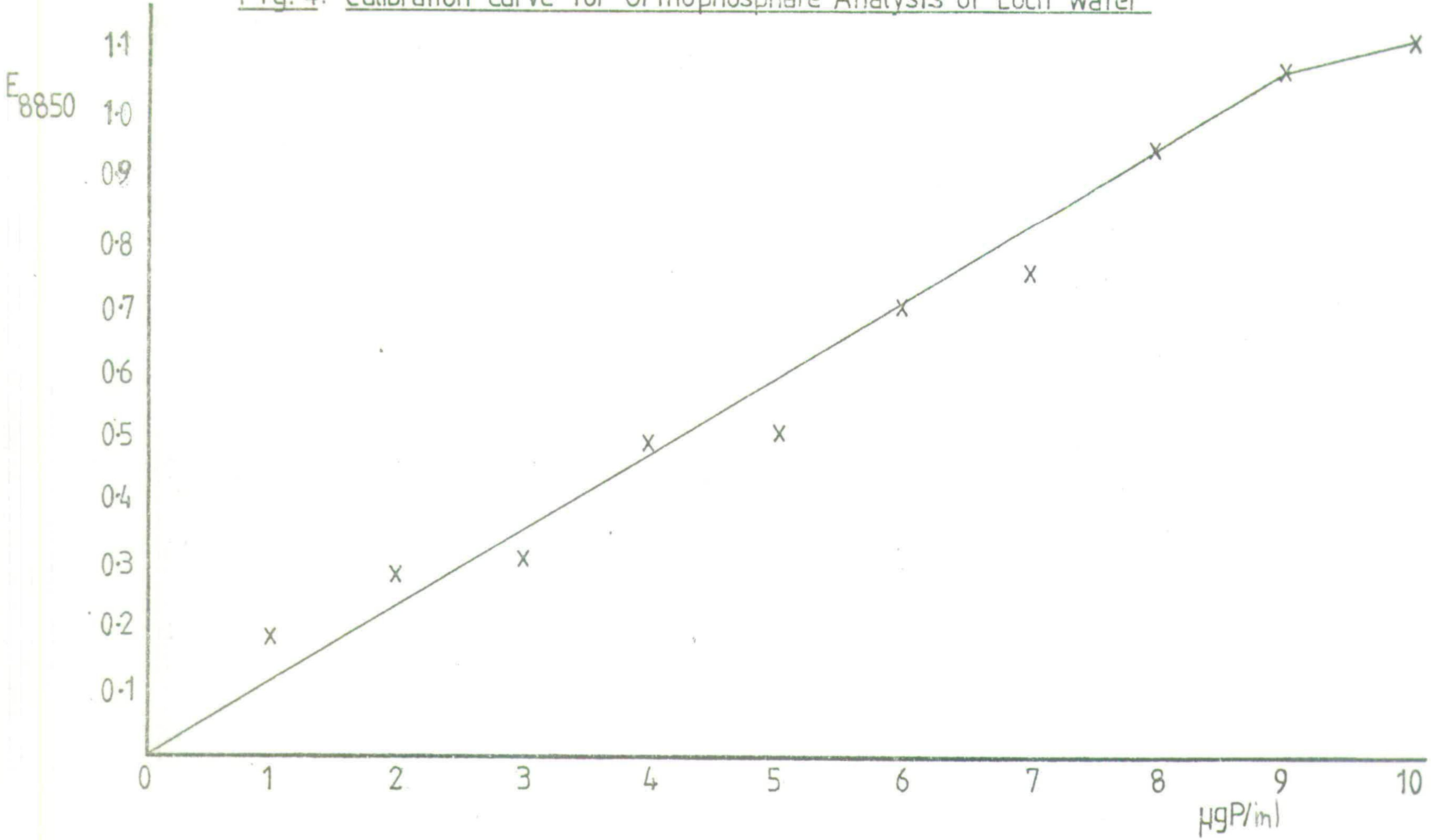
Thiosulphate diluted x10 at low O_2 concentrations

(2) Orthophosphate Analysis The orthophosphate concentration in a water sample may be ascertained by a number of different methods. The levels of orthophosphate in Loch Leven are very low and several methods were tried to find the most satisfactory (Fogg & Wilkinson, 1958; Gales et al, 1966; Strickland and Parsons, 1968; Holden & Caines, 1974). The method of Strickland and Parsons, (1968) gave the most reproducible results. Some of the methods were not sensitive enough to detect the low levels of orthophosphate in the water and the technique of Strickland and Parsons is more satisfactory in that it uses ascorbic acid as reducing agent rather than stannous chloride which is unstable.

Reagents

(a) Ammonium molybdate solution, 15g $(NH_4)_6 Mo_7 4H_2O$ was

Fig. 4. Calibration Curve for Orthophosphate Analysis of Loch Water



dissolved in 500ml distilled water.

(b) Sulphuric acid solution, 140ml conc H_2SO_4 was added to 900ml distilled water.

(c) Ascorbic acid solution, 27g ascorbic acid was dissolved in 500ml distilled water, this solution was stored frozen in 10ml aliquots as it is unstable at room temperatures for more than a few days.

(d) Potassium antinomyl tatrate solution, 0.34g of this reagent was dissolved in 250ml distilled water.

(e) Mixed reagent, 10ml molybdate solution, 25ml H_2SO_4 solution, 10ml ascorbic acid solution and 5ml tatrate solution were added together and mixed thoroughly. This solution was stable for up to 6 hours.

(f) Standard phosphate solution, 0.816g KH_2PO_4 was dissolved in 1000ml distilled water. This stock solution was diluted 10 fold before use to give a solution containing 6×10^{-1} µgP/ml.

Method The samples to be analysed were centrifuged at 6000 rpm for 10 minutes and 10ml of the supernatant were pipetted into a test tube. 1ml of mixed reagent was added to the 10ml of sample and shaken. After 5 minutes and within 2 - 3 hours the resultant blue colour was measured at 8850nm using 1 cm pathlength cuvettes.

A calibration curve was obtained by measuring the adsorbance of the standard phosphate solution at various dilutions. A straight line was obtained throughout the range measured. (Fig.4).

(3) Gas Analysis The gas collected from the sealed Jenkins cores was analysed for methane and nitrogen using a Pye GC 104 series gas chromatogram. A column containing molecular sieve 5A was used with an oven temperature of 50°C. The flow rate of hydrogen through the column was 15ml/min and 0.5ml samples were used.

(4) Redox Potential Measurements The redox potential of the water in the sealed Jenkins cores was measured using platinum/KCl combination electrodes. A sealed glass bottle containing an electrode was flushed out with oxygen free nitrogen and connected to tube A on the sealed core (Fig.3). Water was inserted through tube C thus forcing core water in to the blood bottle. This water did not come into contact with the atmosphere at all during the procedure and the Eh was read directly using a Pye unicam pH meter. This method was considered to be more reliable than leaving the electrodes permanently in the cores as they tended to be "poisoned" by sulphide produced under anaerobic conditions.

(5) Chromatography The substances responsible for the solubilization of inorganic phosphates were detected using chromatography.

Reagents

(a) Solvent, pyridine (60ml), butanol (30ml), distilled water (10ml).

(b) Reagent 1, 20ml acetone, 0.1ml saturated $AgNO_3$ and

0.05ml distilled water were mixed. The precipitate was allowed to settle and the supernatant was used.

(c) Reagent 2, 10gNaOH were dissolved in 7.5ml distilled water and diluted to 500ml with CH_3OH.

(d) Reagent 3, 5g$Na_2S_2O_3$ were dissolved in 100ml distilled water.

(e) Keto acid standards, 0.1g of the desired keto acid was dissolved in 10ml distilled water.

(f) Standard sugar solutions, a 1% solution of the relevant sugar was prepared.

Method The liquid cultures to be assayed were centrifuged at 6000 rpm for 30 minutes. 0.05 of the resultant supernatent was placed on to Whatman no. 1 chromatography paper. 0.005ml of the standard sugar solution and 0.01ml of the keto acid solution were also spotted on to the paper. The chromatogram was placed in the solvent, using descending chromatography, for 36 hours. After drying, the paper was developed by immersion in reagent 1, followed by reagent 2 and finally by immersion in reagent 3. The paper was dried between the treatments and after the final treatment. The sugars and keto acids showed up as brown spots on the paper.

CHAPTER 3 PHOSPHATE RELEASE AND ADSORPTION BY THE SEDIMENTS OF LOCH LEVEN

3-1 Introduction

The sediments of many eutrophic lakes contain large quantities of inorganic phosphate which, under certain conditions, may be released into the overlying water, this means that unavailable phosphate becomes available for biological assimilation (Li, etal 1972). Alternatively soluble phosphate can be chemically absorbed from the water phase by sediments and during the absorption process insoluble salts are formed, such as calcium or aluminium phosphates, thus rendering the phosphate unavailable as an algal nutrient.

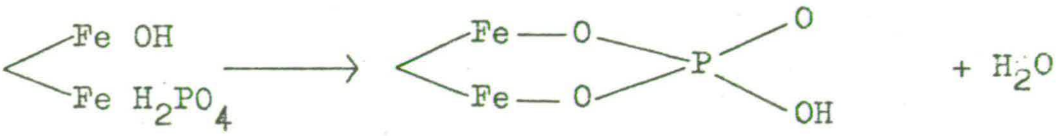
Role of Sediments in Phosphate Movement Many studies have shown that phosphate is adsorbed by the sediments under aerobic conditions (Jitts, 1959; Shukla, etal 1971; Syers, etal 1973). By this process sediments are therefore capable of accumulating a large proportion of the phosphate which enters the lake with the possibility of release to the water phase if reducing conditions develop.

Importance of Iron, Aluminium and Organic Matter on Phosphate Movement Most of the data available on this aspect is concerned with soil, therefore the following discussion relates more to the movement of phosphate in soils than in sediments. Work carried out on 40 acidic soil profiles has shown that extractable aluminium and free iron oxide were well correlated with phosphate adsorption and the concentration of free iron oxide was more important than aluminium in freely drained soils but not in waterlogged ones, (Lopez-Hernandez & Burnham, 1974). Alternatively, in studies of soils with a broad range of characteristics, results indicated that aluminium was primarily responsible for phosphorus adsorption with iron playing a minor role in local regions where the aluminium concentration was relatively constant (Vijayachandran & Harter, 1975). Both the above workers proposed that organic matter plays a major role in the adsorption process. Harter, (1969) has suggested that the phosphate is initially bonded to the anion exchange sites on organic matter and subsequently transformed into less soluble iron and aluminium phosphates. Other theories, for example, Hsu, (1965) maintain that the adsorption of phosphate involves purely inorganic reactions, but once again the importance of iron and aluminium complexes are emphasised. The amounts of surface reactive amorphous hydroxides of aluminium and iron dominate the process rather than the concentrations of Al^{3+} and Fe^{3+} in solution (Hsu, 1965). In studies of inorganic phosphate sorption by soils it has been shown that there is an initial rapid decrease in soluble phosphorus concentration followed by a slower decrease. This change involved a shift from a physically sorbed form of phosphorus to a chemisorbed form (Ryden, et al 1977a). Ryden, et al (1977b), proposes three stages of adsorption.

(i) chemisorption at protonated surface sites

$$\left<\begin{matrix}Fe - OH_2\\Fe - OH_2\end{matrix}\right.^{+} + H_2PO_4^- \longrightarrow \left<\begin{matrix}Fe - H_2PO_4\\Fe - OH_2\end{matrix}\right. + H_2O$$

(ii) chemisorption by replacement of surface hydroxyls



$$\left<\begin{matrix}Fe\ OH\\Fe\ H_2PO_4\end{matrix}\right. \longrightarrow \left<\begin{matrix}Fe - O\\Fe - O\end{matrix}\right> P \begin{matrix}O\\OH\end{matrix} + H_2O$$

(iii) a more physical sorption of phosphate as a potential determining ion.

All the adsorption mechanisms can be described by a modification of the Langmuir equation which states

$$\frac{1}{x} = \frac{1}{kbc} + \frac{1}{b}$$

x = amount of PO_4^{3-} sorbed

c = solution PO_4^{3-} concentration

b = sorption max.

k = a constant related to the sorption energy

<u>Effect of Acidity</u> Phosphate adsorption also depends upon the pH of the environment (McPherson, 1958; Mokwunye, 1975; White, & Taylor 1977). Mokwunye found that an increase of the pH of soils to which phosphate had been added increased the phosphate adsorbed by the soils. The amount of phosphate sorbed depends on the concentration of phosphate in the water. At low concentrations (1-100µM) the affinity of the adsorption was greatest at ph5.2 - 5.5, over a range of pH4.2 - 6.8, but at higher concentrations (100-1000µM)

the affinity was least at pH5.2 - 5.5 (White & Taylor, 1977). These results were interpreted in terms of the formation of basic aluminium and calcium phosphates. McPherson found that little phosphate was released at pH5.5 - 6.5 but further acidity caused a slight increase in phosphate in the water. Under acid conditions the salts tend to be ionised so that the phosphate ion is free to bind with hydrogen ions, thus counteracting the effect of the acid.

$$\underset{\text{ALKALI}}{HPO_4^{2-}} + H^+ \rightleftharpoons \underset{\text{ACID}}{H_2PO_4}$$

Calcium Concentration The concentration of calcium in the sediments affects the sorption of inorganic phosphate in that non calcerous sediments have been found to sorb more phosphate than calcerous sediments. Shukla etal, (1971) found that phosphate sorption was inversley related to $CaCO_3$ concentration. However in soils it has been shown that phosphate sorption depended upon the ionic strength and cation species of the matrix solution, the amount of phosphate being adsorbed increasing with greater ionic strength and being more with calcium ions than sodium ions (Ryden etal, 1977a). There is no explanation about the difference between soil and sediment and it would be expected that more phosphate would be sorbed at high calcium concentrations as calcium phosphate is highly insoluble (Weast, 1975).

Effect of Aerobic Conditions Until recently aerobic conditions were thought to be necessary for phosphate sorption (Hayes et al, 1952; Fitzgerald, 1970; Syers et al, 1973). Khalid et al, (1977) observed that if high levels of phosphate were added to flooded soils it could be adsorbed under reducing as well as aerobic conditions. However these soils were incubated in a $0.1M CaCl_2$ solution and this affects the phosphate adsorption (above) and possibly the excess calcium allowed more phosphate to be sorbed than usual. Shallow eutrophic lakes are rarely stratified yet they sustain large algal blooms which cannot be accounted for by the amount of phosphate entering from external sources (Holden & Caines, 1974). However all systems are in equilibrium or tend towards an equilibrium, if conditions remain stable, and it is possible that there is a minute but continual leaching of phosphate from the sediments. The rate of this leaching could be large enough to maintain the phosphate concentration at a level favourable for the continual production of plant populations (Pomeroy et al, 1975). As with adsorption of phosphate and anaerobic release the rate of liberation will depend on the pH of the system (Anderson, 1975) and the amount of organic matter present in the sediment (Wildung et al, 1974). Lerman, (1974) states that mixing a thin layer of the sediment surface with the water increases the phosphate concentration in the lake water by amounts comparable in magnitude with the concentration continually diffusing from the sediment. The wind may also aid phosphate release by disturbing the sediment surface (Anderson, 1974).

Factors Affecting Movement Across Surfaces Most of the movement of phosphate through the mud surface is a purely physical process (Hynes, & Greib 1970) depending upon the concentration gradient across the mud surface (Kamp-Nielson, 1974). The diffusion rate in sediments is generally about ½ - 1/20 of that of the ions in free solution (Manheim, 1970) probably because the movement of ions is hindered by the mud particles. There are also variable amounts of gas produced in sediments including N_2, CH_4 and H_2S (Martens, 1974; Zeikus, 1976; Duncan, pers. comm.) and the release of this gas from the sediment surface in the form of bubbles may aid the movement of phosphate into the water. Using experimental results Kamp-Nielson, (1975) constructed an empirical equation for the temperature dependent sediment - water exchange of phosphate in Lake Esrom, describing the exchange as the sum of desorption, diffusion and biological degradation processes. This equation was

$F = 0.563e^{0.203t} - 0.60 \ln Ps - 2.27$ F is the phosphate released $mgPm^{-2}day^{-1}$

Ps = interstitial phosphate conc. $mgPl^{-1}$

The actual movement of the phosphorus from the interstitial water into the lake water can also be described by a diffusion expression (Jorgensen, & Kamp Nielson 1975)

$PRS = 1.21 (Pi - Ps) - 1.70$ PRS = phosphate released from sediment

Pi = conc. P in interstitial water

Ps = conc. soluble in water phase

Neither of the above equations include any biological data which verifies the suggestion of Hynes & Greib, (1970) that phosphate movement through mud is a purely physical process. ?

Effect of Reducing Conditions Mortimer, (1941), in his classical work on the exchange of dissolved substances between mud and water in lakes found that phosphate was released from the sediments into the lake water under reducing conditions. This has been verified by later workers who have found that phosphate is released from the sediments rather than sorbed into them under anoxic conditions (Shippel et al, 1973). When these conditions prevail the water is frequently thermally stratified and the phosphate remains in the hypolimnium, except for a small amount diffusing into the epilimnion, until overturn when it becomes available to algae as a nutrient. In lakes which frequently stratify this anaerobic release is considered to be a very important process with a major portion of exchangeable sediment phosphate (45-87%) participating in a rapid exchange reaction at the onset of reducing conditions (Li et al, 1972). Once the oxygen concentration of the sediment falls below 2 mgl^{-1} the redox potential of the sediment is low enough to result in the mobilization of iron phosphates (Mortimer, 1971) although the pH can still prevent phosphate release if it is too high (Ponnamperona, 1972). In Lake Kinneret the exchangeable phosphate appears to be the iron bound fraction, its concentration in the pore water increasing simultaneously with the reduction of sulphates and precipitation of FeS (Serruya et al, 1974). The solubilization of the phosphate is probably due to reactions such as

$$\mathrm{Fe^{3+}\ PO_4\ (insol)} \longrightarrow \mathrm{Fe^{2+}\ PO_4\ (soluble)}$$

which are brought about with the onset of reducing conditions (Patrick & Mahapatra, 1968). Once the phosphate is in a soluble form, it is free to diffuse from the sediments into the water provided the mud surface is in a reduced state. If the mud surface was oxidised the phosphate would probably be reprecipitated due to the oxidation of the ferrous ions to ferric ions.

Role of Sediment Biological Processes The organisms in the sediment contribute towards this anaerobic release of phosphate by creating the necessary reducing conditions. At first, when there is oxygen present, both invertebrates and microorganisms will be active in removing the oxygen. Fungi, protozoa, actinomycetes, aerobic and facultatively anaerobic bacteria will all participate in utilising the oxygen. Under these conditions the normal aerobic respiratory metabolic pathways will be taking place. Once the oxygen has disappeared the invertebrate, fungal, protozoan, actinomycete and aerobic bacterial populations will cease to function, facultative and strictly anaerobic bacteria will become important. These organisms do not use oxygen as the terminal electron acceptor but use alternative inorganic (SO_4^{2-}, NO_3^{-}) or organic compounds. Many strict anaerobes are extremely sensitive to oxygen

TABLE 4 Suggested Effect of Eh on Microbial Metabolism in Lake Sediments (after Keeney, 1971)

Sequence of Events	Process	Eh (mv)	Bacterial Metabolism
1	normal oxidations	+600	Aerobic
2	NO_3^- reduction	+500	Aerobic *
	Mn^{4+} reduction	+400	Aerobic
	Fe^{3+} reduction	+300	Aerobic
3	SO_4^{2-} reduction	0	
4	H_2 production	-150	obligately anaerobic
5	CH_4 production	-220	

* Using anaerobic respiratory processes in presence of NO_3

and will die if exposed to it for any length of time (Hawker, & Linton 1972). There is a definite sequence in which the alternative electron acceptors are utilised and as each substance is used up the redox potential falls (Table 4).

The first alternative electron acceptor is NO_3-N. A few bacteria which are normally aerobic can use nitrate as a terminal electron acceptor by reducing either to nitrite only or further to molecular nitrogen, nitrous oxide etc. Gas formation by this process is referred to as denitrification. These reactions take place at Eh values below 300-350mV (Meek , 1969) and any available gaseous oxygen will be utilised in preference to nitrate. The presence of nitrate in a system maintains a redox potential of 100-150 mV, which will not fall below this value until the nitrate has been utilised (Bell, 1969).

A second alternative electron acceptor is sulphate which is reduced to S^{2-}. Very few types of bacteria can utilise sulphate as an electron acceptor, the property being confined to *Desulfovibrio sp.* and a few anaerobic sporeforming rods, *Desulfotomaculum sp.* Each molecule of sulphate can accept eight electrons, $SO_4^{2-} + 8e^- + 8H^+ \longrightarrow S^{2-} + 4H_2O$, and it is interesting to note that no sulphide appears in the environment until all NO_3-N has disappeared (Keeney, et al 1971). These processes take place at about -150mV.

Between -100 to -200mV the anaerobic fermentation of a range of simple and complex organic substrates takes place leading to the formation of organic acids. A few types of anaerobic bacteria use carbonate as an electron acceptor with methane gas as the end product. This process takes place in the Eh range -250 to -300mV. The only substrates utilised by these methane producing bacteria appear to be H_2, CO_2 and formate. These processes only take place in strictly anaerobic conditions as the bacteria are extremely sensitive to oxygen (Stanier, et al 1970).

Thus it can be seen that bacteria play a major role in the creation of reducing conditions in sediments. Without these facultative and strictly anaerobic bacteria the redox potential would probably remain at too high a level for phosphate release to take place. The ability of some of these bacteria to produce gas (N_2 and CH_4) probably aids the passage of phosphate from the sediment by disturbing the surface.

Experimental Programme on Phosphate Release and Adsorption in Jenkins Cores

Loch Leven is a highly eutrophic lake which sustains extensive algal blooms annually. The size of these blooms cannot be accounted for by the phosphate entering the loch from external sources (Holden, & Caines 1974). therefore the excess phosphate may be attributed to sediment release. It was therefore necessary to assess the role of certain parameters in various Loch Leven sediments to both understand where the excess phosphate was coming from and also to gain knowledge that may be applied to other ecosystems. Loch Leven is a shallow exposed loch which was believed to stratify rarely so redox potential measurements were taken throughout the year on sediments to confirm previous observations. Investigations were also carried out to determine the Eh value at which phosphate release could take place, because the higher this value, the greater the likelihood that suitable conditions would be present under field conditions. The effect of temperature on the rate of fall in redox potential and subsequent phosphate release was investigated, because Loch Leven is covered in ice (inverse stratification) for several weeks during the winter. Sterile and non sterile mud were compared to ascertain whether the fall in redox potential and subsequent phosphate release was a purely biological or chemical phenomenon or a mixture of both. As a large portion of Loch Leven was found to be very rarely stratified the aerobic release of phosphate was studied and compared with the anaerobic release. In this way it was possible to make an assessment of the contribution that the sediments of Loch Leven were making to the water and also to gain an understanding of some of the parameters controlling phosphate interactions in the sediments.

3-2 Chemical and Physical Characteristics of Loch Leven Sediments

The importance of various chemical and physical parameters of sediments in influencing the interchange of phosphate between sediments and the water phase was emphasised in the last section (3-1). This section discusses these parameters in the sediments from the North deep and a typical shallow area of Loch Leven using data obtained in the laboratory and by other workers.

The following examinations were carried out on fresh samples of cores or waters.

(a) Oven dry weight of 3cm sections Samples obtained in May 1976 were dried at 80°C overnight.

(b) X-ray fluorescence analysis Analysis for major elements was carried out on dried sediments obtained in April 1976 by Geology Department, University of Edinburgh.

(c) Phosphate analysis of sediments and interstitial water 3cm sections of sediment were analysed by the Freshwater Fisheries Laboratory, Pitlochry for different phosphate fractions using the procedure of Chang and Jackson, (1958). Orthophosphate concentrations in the interstitial water were measured by Dr. J. Drake, University of Vermont under an atmosphere of nitrogen to prevent undue oxidation of the iron taking place. The samples were collected during May 1978.

TABLE 5 X-ray Fluorescence Analysis of Sediment from Loch Leven (Geology Department, University of Edinburgh, 1976)

% Element in dry material

	Ca	K	Fe	Si	Al	P	Mg	% moisture
Deep area	1.29	0.11	4.56	29.96	7.25	0.06	1.00	83-91
Shallow area	1.17	0.14	3.97	26.31	5.01	0.09	0.75	78-85

TABLE 6 Phosphate Fractions (mgP/100g dried sediment) in Loch Leven Sediment Obtained using the Procedure of Chang & Jackson (1958) (R. Harriman, Freshwaters Fisheries, Pitlochry, 1978)

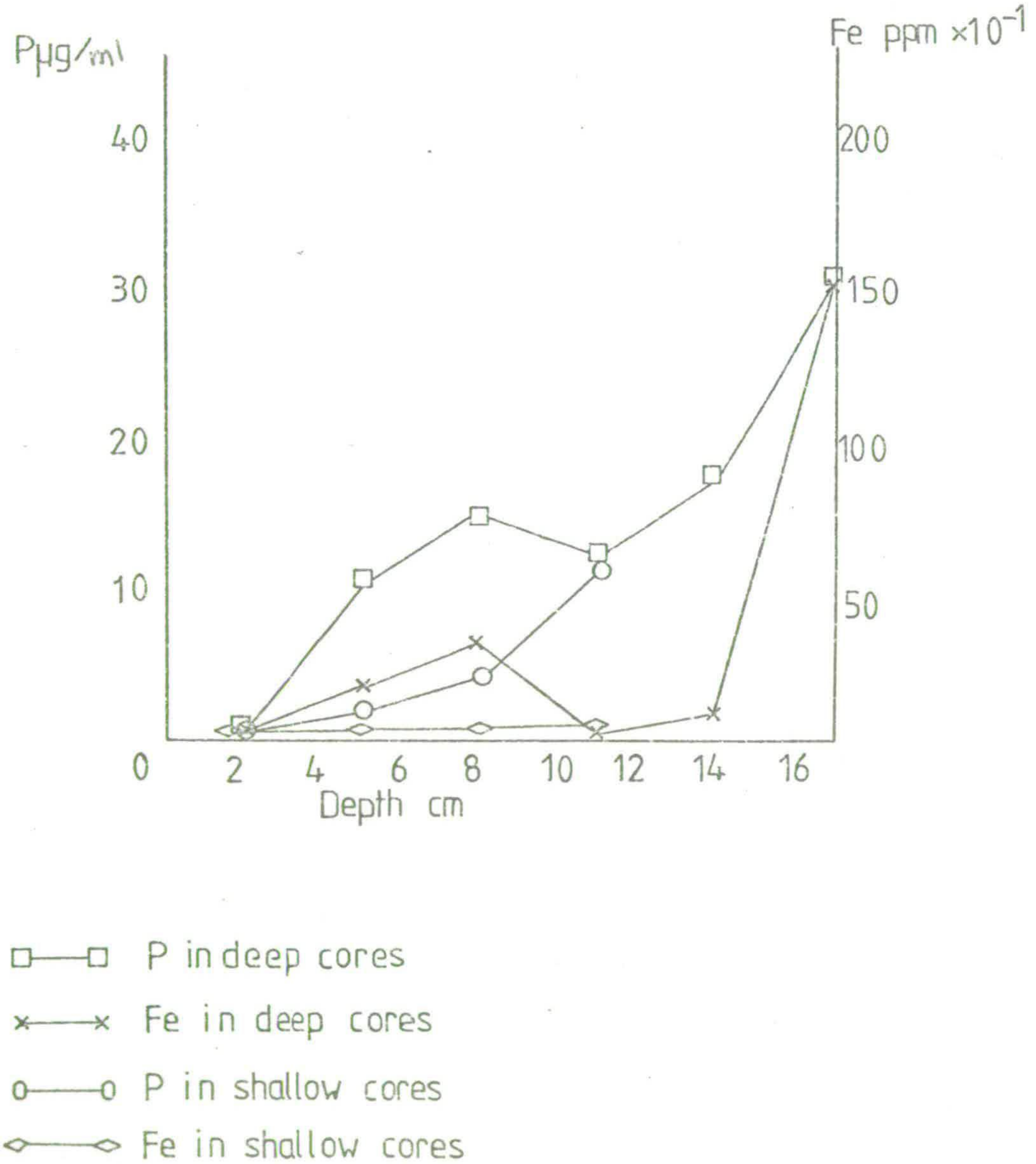
Depth cms	DEEP				SHALLOW			
	Al-P	Fe-P	Ca-P	TotalP	Al-P	Fe-P	Ca-P	TotalP
1-2	24.8	81.0	50.6	271	25.7	48.6	51.5	241
2-4	21.3	85.2	50.1	267	17.9	34.0	43.2	177
4-6	22.1	72.0	47.5	259	17.1	28.1	42.3	154
6-8	25.6	77.2	45.4	256	14.0	22.6	41.5	129
8-10	19.3	48.9	39.7	204	12.0	20.4	40.3	120
10-12	22.0	57.6	39.0	192	13.0	21.0	40.2	124
12-14	19.8	57.6	38.8	192	13.0	22.7	39.0	115
14-16	10.7	24.9	35.4	139	11.0	20.5	38.8	108
16-18	8.5	23.9	35.6	131	10.7	20.7	40.6	102
18-20	16.3	47.4	36.5	159				
20-22	17.1	54.8	34.7	167				

(d) Eh measurements At approximately monthly intervals during 1977 cores were used to measure the Eh of the sediment surface and of 1cm intervals down the core. These data were obtained by placing a platinum electrode on the end of a glass rod and clamping the rod to a metal stand which was calibrated in centimetres. A reference electrode containing saturated KCl was placed in the core water. The platinum electrode was lowered gently so that it just touched the sediment surface and the Eh was read directly from a Pye Unicam pH meter. The electrode was then pushed into the sediment and the Eh read every centimetre.

KCl ?

Results The X-R-F data (Table 5) show that the two sediment types are very similar in the top 20cm in the total element content per gm dry weight and similar per ml when taking into account the % moisture. However the data on the different forms of phosphate (Table 6) showed that the two sediment types differed from each other in the actual form in which the phosphate was present. The amounts of calcium and aluminium phosphate were similar but there was more iron in the deep area. This could be important when studying the anaerobic release of phosphate from the sediments because iron phosphate is the type most likely to be affected by the onset of reducing conditions due to the variable valency exhibited by iron. In both sediment types the amount of all types of phosphate decreased with increasing depth.

Fig. 5. Phosphate and Iron in the Interstitial Water of Jenkins cores taken from Loch Leven Deep and Shallow Areas.



It would appear that the phosphate moves up through the sediment, possibly it is solubilised in the reduced lower zones and reprecipitated as it reaches the more aerobic upper zones. The Chang and Jackson procedure is not altogether reliable because there is some overlap between each extracted fraction. There has been much critisism of the technique (Williams etal, 1976) but the same techniques were followed for each sample and the data appear to be suitable for comparative purposes. The interstitial water data (Fig.5) showed that the orthophosphate concentration increased with increasing depth. Comparing Table 4 with Fig. 5, the results show that where the phosphate concentration is lowest there is less iron phosphate present, that is deeper down the sediment there is less iron phosphate than near the top, whereas aluminium and calcium phosphate do not change appreciably. Thus the high phosphate concentrations can ? be correlated with high concentrations of iron phosphate in the sediment. Also in the interstitial water a high ? iron concentration is correlated with a high phosphate concentration. This observation once again indicated that phosphate release may be due to the reduction of ferric to ferrous phosphate.

Eh Measurements In spite of the inherent difficulties of measuring and interpreting Eh in a complex ecological situation containing many oxidation/reduction systems the data in Fig. 6 a and b clearly demonstrate the differences

Fig. 6a. Changes in the redox potential of the surface of Loch Leven deep and shallow sediment with season.

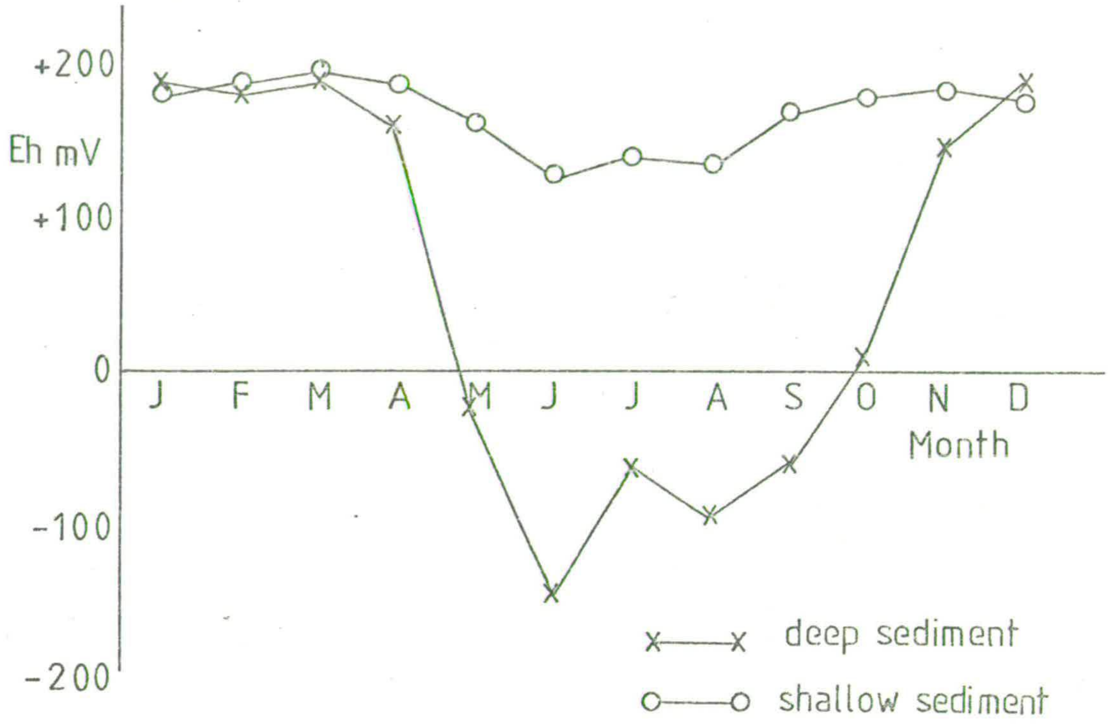
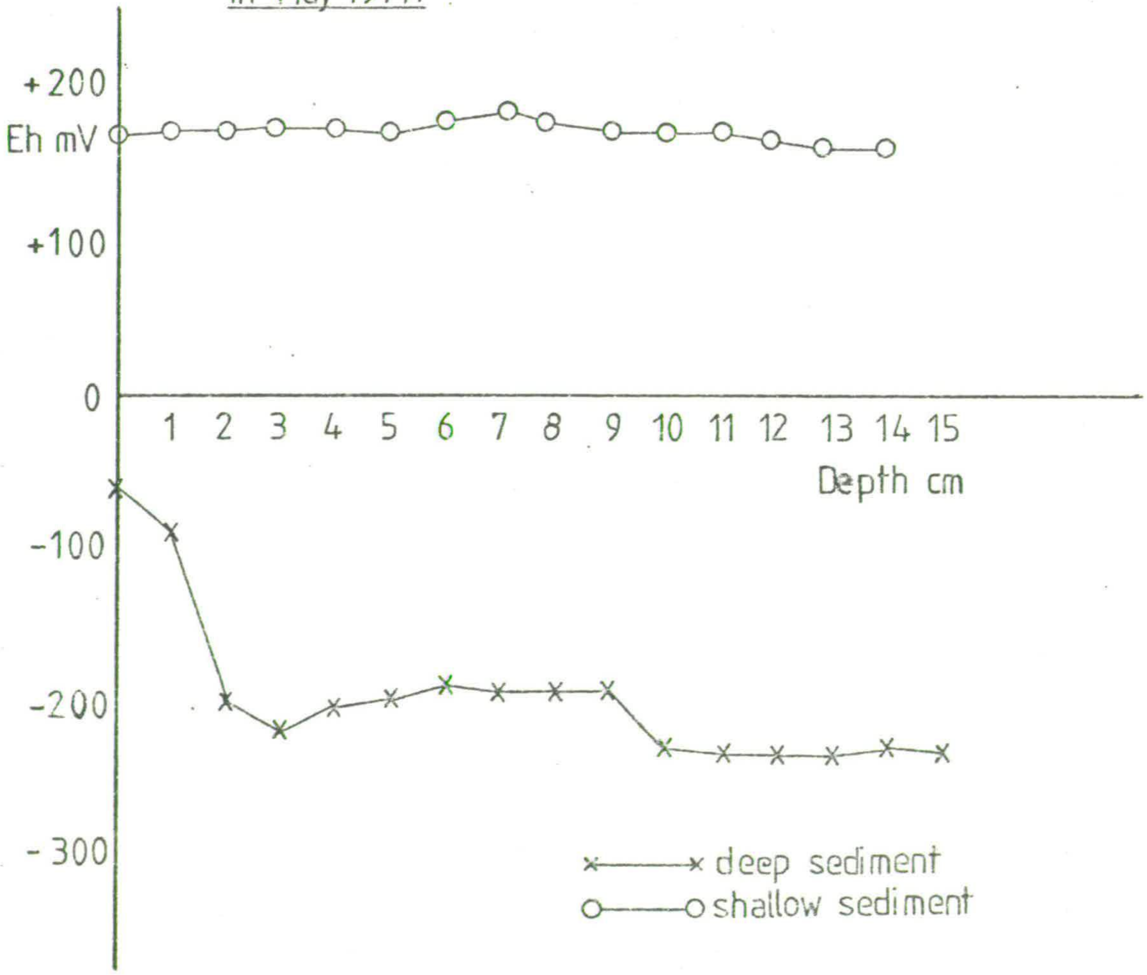


Fig. 6 b. The Eh profile of deep and shallow sediment sampled in May 1977.



between the two sediment types. Fig. 6 a showed that there was very little seasonal change in the redox potential of the shallow sediment and that the North deep was in an extremely reduced state during the summer. Other workers at Loch Leven have assumed that the loch stratified infrequently, even in the deep areas, because of the shallow and exposed nature of the loch. This pattern appears to take place in the shallow areas, the slight depression in redox potential in the summer could be due to greater biological activity and oxygen uptake with the higher temperature. However the loch water above the shallow areas is usually well enough mixed to be thoroughly oxygenated and this oxygen diffuses into the sediment, preventing it from becoming too reduced. However the deep sediment had a very low redox potential during the summer. The reduced state of the deep sediment was also apparent from the black colouration and the smell of the sediment as opposed to the light brown colour of the shallow area. These results indicated that the water directly over the sediment was anoxic for long periods at a time. If the water became oxygenated by strong winds causing mixing in the deeper areas, any oxygen which entered the sediment would be rapidly utilised by both chemical and biological mechanisms in that oxygen starved environment. In the autumn the Eh started to rise and the sediment remained more oxygenated throughout the winter. In the winter there are frequently strong winds (force 5 or 6), the water temperature is low so the rate of oxygen utilisation in the sediment is reduced and the mud can gradually become oxygenated. The redox potential of the shallow sediment remained reasonably constant with depth (Fig. 6b) indicating the area was oxygenated to a considerable depth throughout the year. The deep cores were usually more reduced below the surface except during January and February when they were aerobic throughout (Appendix 1). The upper areas of the deep sediment will occasionally receive some oxygen, especially during the winter and at overturn of stratification, but this oxygen does not diffuse much below the surface except during the winter. If phosphate release is brought about by the onset of reducing conditions these redox potential measurements could indicate that ideal conditions would exist in the North deep during the summer for reduction of phosphates to take place but very little would be released in the shallow areas.

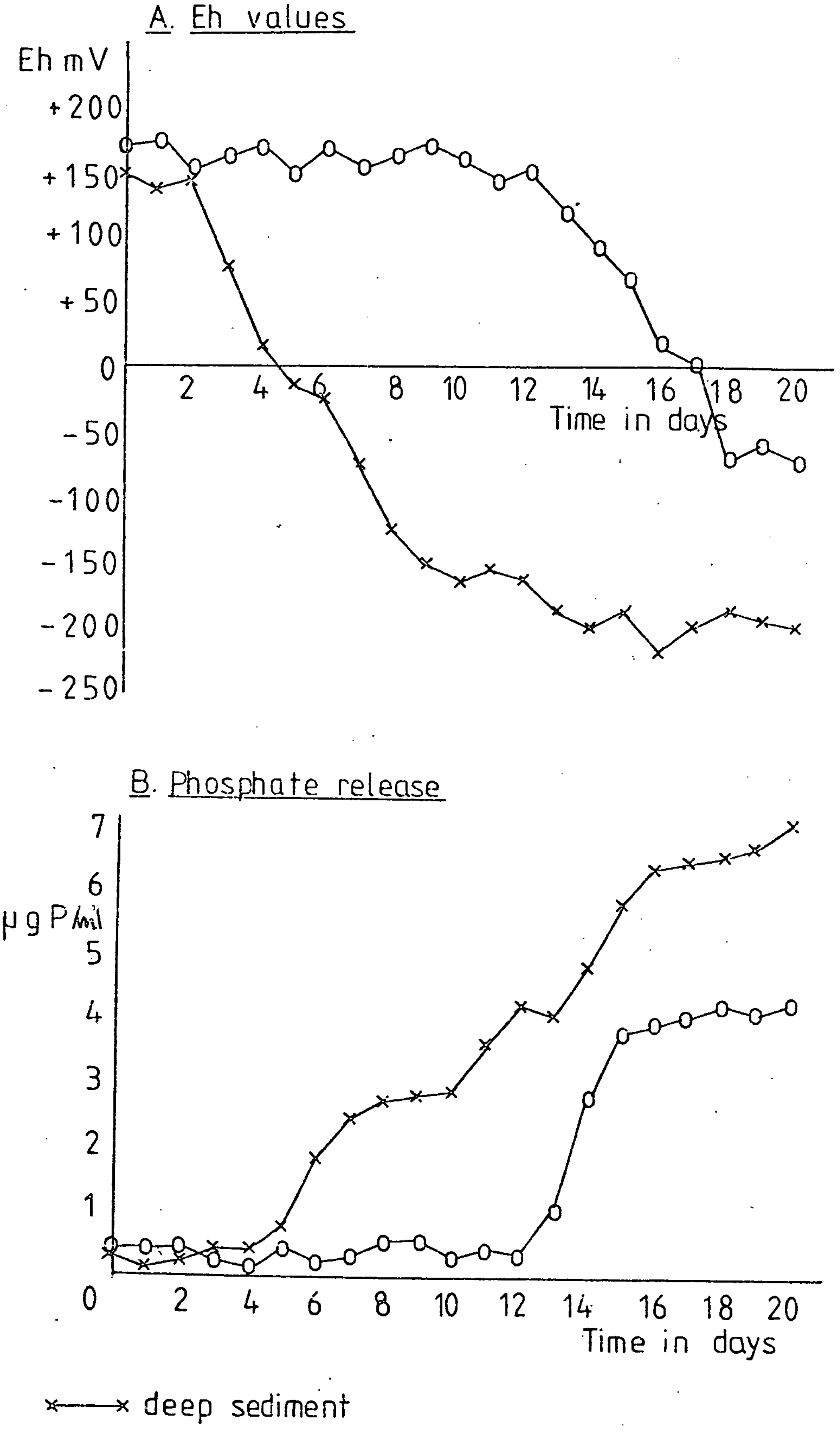
3-3 The Effect of Temperature and Sediment Type on the Anaerobic Release of Phosphate

In order to assess the importance of phosphate release from the sediments, experiments using Jenkins cores were undertaken using samples from both deep and shallow sites. The sediments from the two sites have a different organic matter and iron content and it seemed particularly relevant to investigate whether any relationship existed between these characteristics and phosphate release. Since seasonal temperature fluctuations may also affect the processes of phosphate release, this factor was also investigated.

Methods Two experiments were undertaken. In the first, designed to compare the two sediments under anaerobic conditions, three Jenkins cores were collected from both the North deep and shallow area on March 9th 1976. These cores were closed to the atmosphere and incubated for 20 days at 16°C in the dark. Water samples were taken daily and analysed for their orthophosphate content. The redox potential of the water phase was also measured daily.

In the second experiment, devised to show the effect of temperature on phosphate release, eighteen Jenkins cores were collected on July 21st 1977, nine from the North deep and nine from the shallow area. The cores were closed to the atmosphere and incubated at 25°C,

Fig.7. Comparison of fall in Eh and phosphate release in deep and shallow cores held anaerobically at 16°C



×——× deep sediment

o——o shallow sediment

Data mean of 3 cores

10°C and 4°C for 36 days in the dark, there being three of each type of core at every temperature. Water samples were taken about every 3 days and the orthophosphate concentration measured. The Eh of the water phase was measured each time a water sample was taken.

Results and Discussion The initial experiment showed that phosphate was released from Loch Leven sediments into the water phase under anaerobic conditions (Fig. 7). The data presented in Fig. 7a show that as the redox potential started to fall orthophosphate began to accumulate in the water phase. The results for the two sediment types were quite dissimilar to each other. Initially the redox potential of the water phase was similar in all the cores but that of the deep samples started to fall rapidly after 3 days whereas the shallow Eh remained stable for about 8 days and only then started to fall slowly. A lower Eh value was eventually reached in the deep samples then in the shallow ones. There was a difference in the rate at which orthophosphate accumulated in the water phase, far more entering the deep samples than the shallow and at a greater rate (Fig.7b). The concentration of orthophosphate reached a maximum of 4 μgP/ml after 17 days and did not rise thereafter whereas that of the deep cores was still rising after 20 days, having already reached a value of 7 μgP/ml.

The deep sediment has a higher organic carbon content

(8%) and also probably has more freshly sedimented material than the shallow area (4%) which would mean a greater availability of organic nutrients capable of inducing higher bacterial activity. The activities of different groups of bacteria in each sediment type are unknown but it seems clear that in the deep sediment, where reducing conditions are more prevelant, there will be a greater number of anaerobic bacteria than in the shallow more aerobic sediment. These bacteria would lead to the creation of reducing conditions by anaerobic respirations using NO_3^-, SO_4^{2-} and various fermentative processes (Keeney et al, 1971). The higher orthophosphate concentration in the deep core water could have been predicted from the greater amount of phosphate shown to be present in the deep areas (Table 6). The presence of this additional phosphate as iron phosphate would also help phosphate release since the insoluble ferric phosphate would be mobilised (Mortimer, 1971) to form soluble ferrous phosphate (Patrick & Mahapatra, 1968). Alternatively if hydrogen sulphide were present ferrous sulphide would be formed leaving the phosphate free to diffuse from the sediment (Serruya et al, 1974).

The second experiment showed that temperature was important in controlling phosphate release in that reducing conditions were established more slowly at low temperatures (Fig. 8). At 20°C there was a fairly rapid fall in the redox potential of both types of core but at 4°C the Eh took considerably longer to fall, never reaching very low values. This rapid fall in the Eh of the shallow sediment at 20°C differed from the results of the previous experiment (Fig. 7). A possible reason for this could be that in the cores taken during March all biological activity would be very low initially due to the temperature of the loch and would take a while to become fully realised whereas in July all the organisms would already be active. In both sediment types there was a high concentration of phosphate released into the water at 20°C although more was released in the deep cores and at a greater rate. At 4°C very little phosphate accumulated in the water of either sample type. The results for 10°C were, as expected, intermediate to those of 20°C and 4°C. The experiment was run for 36 days but after 18 days the redox potential and orthophosphate concentration at all temperatures remained reasonably stable and they are therefore not shown in the figure.

The effect of temperature on the establishment of reducing conditions and on phosphate release was expected. In living organisms all the reactions taking place are of a chemical nature and are governed by the Arrhenius equation which states that there is a linear ? relationship between temperature and rate of reaction (Stanier, etal 1970), thus at low temperatures the rate of enzymic reactions controlling both aerobic and anaerobic respiration will be considerably reduced. This means that the rate at which the Eh falls will decrease with decreasing temperature as has been shown. Even after suitable reducing conditions have been established the rate at which the phosphate is solubilised and diffuses through the sediment would be very slow as these processes are also governed by the Arrhenius equation. Thus temperature affected both phosphate release and fall in Eh.

Other seasonal changes in the loch such as the greater mixing of the water during the spring, autumn and winter by wind may also affect phosphate release by oxygenating the sediment to a greater extent. Also during the summer when more algae are present and sedimenting out, there may be more bacterial activity due to the supply of fresh nutrients thus the Eh could fall quite rapidly. However temperature is probably the overiding factor because if it is too low there will be very little biological activity even if there is an adequate supply of nutrients.

3-4 Separation of Biological and Chemical Factors Affecting Phosphate Release

In lake sediments there are many diferent types of organism contributing to the total biological activity and metabolising the oxygen in the sediment by respiratory processes. These organisms include bacteria, nematodes, insect larvae and benthic algae. The algae in the photic zone may, however, release more oxygen during daylight due to photosynthesis than they utilise for respiratory purposes (Keeney, et al 1971). There are many chemical oxidation reactions e.g. $Fe^{2+} \rightarrow Fe^{3+}$, $Mn^{2+} \rightarrow Mn^{3+}$ which take place in the sediments and these will use up some of the oxygen which has diffused from the water into the mud. Apart from helping to use up oxygen the invertebrate population will also probably aid phosphate release by moving through the sediments and disturbing the surface. By totally sterilising cores or adding selective substances such as antibiotics or nematocides which only kill a portion of the biological population it should be possible to separate the various chemical and biological parameters which are creating reducing conditions and contributing to phosphate release from the sediments.

Methods Fifteen cores were collected from the North deep on November 14th 1975 and treated as follows:-

Date	Treatment	No. of cores	Type of analysis
14.11.75	Cores closed to the atmosphere and HCHO was added to the water phase to give a final concentration of 5% Incubated 30 days, 16°C	3	Eh of water phase measured daily. HPO_4^{2-} of water samples done daily Total viable counts on the sediment surface were done after 30 days
14.11.75	Cores closed to the atmosphere and incubated for 30 days, 16°C	3	"
14.11.75	Cores closed to the atmosphere and HCHO added as above. Incubated 30 days 16°C. After 30 days H_2S was bubbled through the cores for 1 hour	3	Eh and HPO_4^{2-} analysis as above. HPO_4^{2-} analysis was carried out on the water after treatment with H_2S

Cont. ....

Date	Treatment	No. of cores	Type of analysis
14.11.75	None	3	Total viable counts of bacteria on the sediment surface carried out on day of sampling
14.11.75	Immersed in water at 80°C on 3 consecutive days	3	Daily analysis of the water phase for HPO_4^{2-}. Total viable counts of bacteria on the sediment surface carried out on day 3

Eight cores were collected from the North deep in March 1976. These had antibiotics supplied by Wellcome Laboratories Ltd., added (see table below). The combination of antibiotics used was designed to kill all bacteria, affecting both gram positive and gram negative types. The cores were treated as follows:-

Date	Treatment	No. of cores	Type of analysis
20.3.76	Cores closed to the atmosphere Antibiotics added:- Trimethoprim 1 µg/ml Sulphadiazine 20 µg/ml Incubated 30 days, 16°C	3	Water samples taken every 2 days and analysed for HPO_4^{2-} Eh of water measured every 2 days. Bacteria on the sediment surface counted after 30 days
20.3.76	Cores closed to the atmosphere. Incubated 30 days, 16°C	3	"
20.3.76	None	2	Total viable count of bacteria on the sediment surface on day of sampling

Twelve cores were taken from the North deep during May 1978. A nematocide and insecticide were added in various combinations (see table below) to find the effect the invertebrate population had on Eh and phosphate release. The nematocide and insecticide had been tested for their effect on lake bacteria by incorporating them into nutrient agar plates and innoculating them with lake sediment. They were found to have no effect on the bacterial population. The cores were treated as follows:-

Date	Treatment	No. of cores	Type of analysis
10.5.78	Cores closed to atmosphere. The nematocide (thibenzole) was added to give a final concentration of 318 ppm. Incubated 30 days, 16°C	3	Eh measurements done every 2 days Water samples analysed for HPO_4^{2-} every 2 days
10.5.78	Cores closed to atmosphere. The insecticide (temik) was added to give a final concentration of 10 ppm. Incubated 30 days, 16°C	3	"

Cont. ....

TABLE 7 The Effect of Sterilizing Agents on the Aerobic Bacterial Populations on the Sediment Surface of Jenkins Cores

	Heat	HCHO (5%)	Antibiotics	No treatment
Before treatment	2.0×10^6	4.0×10^7	6.1×10^7	5×10^7
After treatment	1.2×10^4	not detected	1.1×10^5	3×10^5

Heat, the cores were heated to 80°C for 1 hour on 3 consecutive days.

Antibiotics added to the water phase were trimethoprim (1 ug/ml) and sulphadiazine (20 ug/ml).

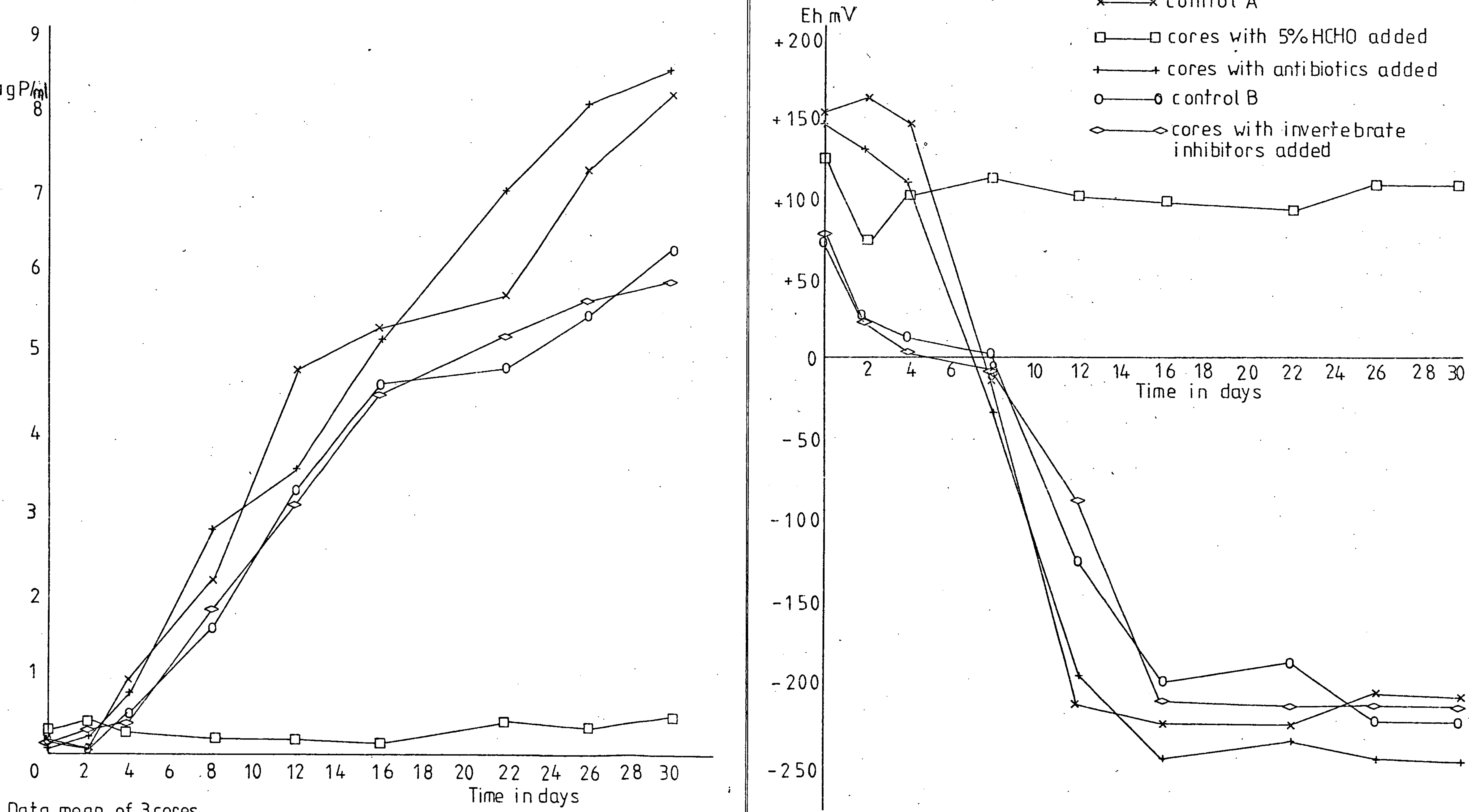
Counts per ml wet sediment on casein peptone starch medium after incubation at 25°C for 21 days.

Date	Treatment	No. of cores	Type of analysis
10.5.78	Cores closed to atmosphere. Insecticide and nematocide were added. Incubate 30 days, 16°C	3	Eh measurements done every 2 days Water samples analysed for HPO_4^{2-} every 2 days
10.5.78	Cores closed to atmosphere. Incubated 30 days, 16°C	3	"

Results and Discussion Of the two sterilizing agents used (HCHO and heat) formaldehyde was the only one which proved to be totally successful (Table 7). The heat treatment should have been sufficient to kill a high proportion of the bacteria. The first treatment should have killed all the vegetative cells present in the cores and the heat induced any spores to germinate which would then have been killed on day 2, the third day ensuring almost complete sterilization. However, although the population was reduced by two orders of magnitude there was still a sizeable bacterial population present in the core after treatment. Those species isolated after heating were mainly Bacillus sp. which indicated that the heat was adequate to kill any vegetative cells but that perhaps the spores were slow to germinate and were thus unaffected by the treatment. Repeating the heating process every other day may prove more successful in sterilizing the cores. The heating process also appeared to affect the phosphate concentration in the core water. After the cores had been heated 3 ugP/ml could be detected in the core water which indicated that the heat induced chemical changes in the sediment. As previously stated chemical reactions take place at a greater rate at higher temperatures which could explain the changes taking place. Therefore, in view of the chemical changes and lack of sterility heat was not thought to be a useful means of treating the Jenkins cores. There were no bacteria detected in the sediment after treatment with formaldehyde (Table 7) and it did not appear to affect phosphate concentrations in the core water so this method was used to give apparent sterilization of the cores. The antibiotics used did not affect the bacterial population to any great extent (Table 7) even though higher (1000x) concentrations of antibiotic were used then those recommended by the manufacturers. A probable explanation is that the organic matter present in the sediment had absorbed the antibiotics and therefore inactivated them. As long as there is a suitable receptor site the antibiotic will be adsorbed whether this is on dead organic matter or living bacteria. When the antibiotics were incorporated into a casein,

?

Fig.9. Separation of biological and chemical effects on redox potential changes and phosphate release in Jenkins cores from the North deep incubated anaerobically at 16°C.



A. Phosphate concentration in the water phase.

B. Changes in redox potential.

Data mean of 3cores

Control A Control cores for HCHO and antibiotic treatments
Control B Control cores for combined insecticide and nematocide treatment

Antibiotics, trimethoprim 1ug/ml and sulphadiazine 20ug/ml

Insecticide, thibenzole 318ppm
Nematocide, temik 10ppm

peptone, starch agar medium, no colonies developed, proving their effectiveness in controlling bacterial growth under these conditions. There was also a noticeable reduction in the numbers of bacteria in the control cores after incubation (Table 7) but the adverse reducing conditions, particularly the hydrogen sulphide present, may have caused many aerobic bacteria to die. After 30 days there may have been fewer available nutrients in the cores, once again tending to reduce the bacterial population. It must be noted that all the above data and comments refer to aerobic or facultative aerobic bacteria, no investigations were made on anaerobic bacteria. There have been reports of the inhibition of sediment bacteria by the addition of antibiotics (Kamp-Nielson, 1974), but no reliable assessment of the effects of the antibiotics was made although 100% kill was assumed to have occurred.

The effect of the various sterilizing agents on the rate of fall of the redox potential and on phosphate release is shown in Fig. 9. When formaldehyde was used, the redox potential did not change throughout the period of the experiment and no phosphate was released from the sediment. However, when hydrogen sulphide was bubbled through the cores for 1 hour, to induce non biological reducing conditions, phosphate was released to give a value of 8 µgP/ml in the water phase. These results indicated that the reducing conditions were a result of biological usage of the oxygen, without any significant chemical oxidations participating in the process. They also showed that the phosphate release was a chemical and physical phenomenon which took place once the correct redox potential had been reached. This purely chemical and physical release of phosphate under reducing conditions agrees with the results of other workers. Both Hynes & Greib, (1970) and Kamp-Nielson, (1974) concluded from their experiments on the movement of phosphate through mud that it depended upon physical and chemical processes once suitable conditions for phosphate release have been established. In the cores which had antibiotics added there was little difference between these and the controls. These results would be expected because the antibiotics did not affect the bacterial numbers and presumably activity to any great extent.

The addition of the nematocide and insecticide did not unduly affect the redox potential changes or the amount of phosphate released from the sediment. There are two possible explanations. The first, and most probable, judging from the antibiotic results, is that the additives were adsorbed by the sediment before affecting the relevant organisms. This was further verified by the fact that no increase in the number of dead larvae or worms was observed in the core water. The other explanation could be that the invertebrate population has a small influence on the rate of oxygen uptake and that the reducing conditions result mainly from bacterial respiration. The results also suggest that the invertebrates do not influence phosphate release by mixing the sediments. The high number of invertebrates present (East, & Morris 1977; Maitland, & Morris 1977) were likely to be having an effect because even if there were only a few animals present in each core they would use enough oxygen to affect the rate at which reducing conditions were established. More work requires to be carried out on this aspect using different nematocides and insecticides or the same ones at greater concentrations before any conclusions can be drawn.

3-5 Poising of Eh in Jenkins Cores and Subsequent Phosphate Release

Data discussed earlier in this section showed that release of phosphate from Loch Leven sediments is a chemical and physical process brought about by the onset of reducing conditions in the system. These investigations did not indicate the redox potential necessary before phosphate could be released. If the release was due to the reduction of ferric to ferrous then an Eh level between the nitrate and sulphate oxidation/reduction potential would be suitable. However if the release was due to the formation of FeS, the sulphate would have to be reduced with the formation of H_2S. If a high concentration of a certain chemical is added, the oxidation state of the system remains stable until the chemical is used up (either biologically or non biologically). Therefore by adding various poising agents to a series of cores the critical Eh value for phosphate release could be determined. The less negative this value, the sooner the phosphate will be released after the onset of reducing conditions in the sediments.

Methods The initial experiment (A) was designed to find the Eh values between which phosphate could be released from the sediments of Loch Leven.

Twelve Jenkins cores were collected from the North deep on October 15th 1976. They were sealed anaerobically and treated as follows:-

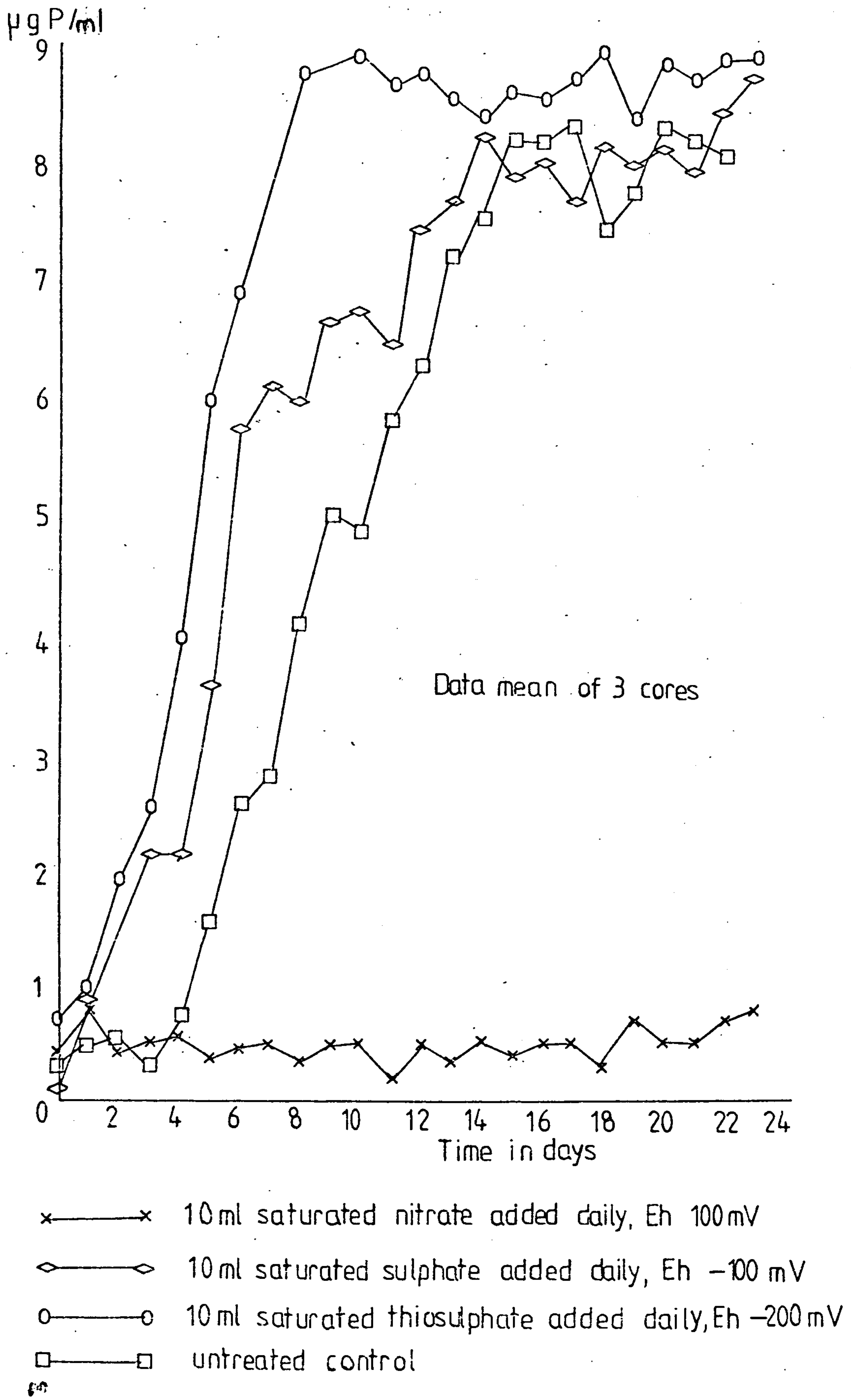
No. of cores	Treatment
3	10 mls saturated sodium thiosulphate solution added daily
3	10 mls saturated KNO_3 added daily
3	10 mls saturated Na_2SO_4 added daily
3	untreated control

The cores were incubated at 16°C in the dark for 23 days. Water samples were taken daily for orthophosphate analysis. The above chemicals were added to give a variety of Eh values. The reduction of iron may be important in phosphate release so nitrate (Eh value 100mV) and sulphate (Eh value -100mV) were added as iron is reduced between these values. Thiosulphate, which has a low Eh value of -200mV was added to ascertain if further phosphate was released at lower values. The redox potential of thiosulphate is not as low as that of H_2S but the latter is toxic which is why thiosulphate was used instead. The amounts of chemicals added were arbitrary and designed to give adequate poising of the Eh. In later experiments, different concentrations of certain chemicals were added to determine the effect on phosphate release.

For the second experiment (B) which was set up to show the effect of nitrate concentration in the water phase on phosphate release from the sediment, fifteen core samples were taken from the North deep on June 30th 1977. The water was siphoned off and the sediment dispensed into 30 mini cores. The cores were allowed to equilibrate, with aeration, for 4 weeks. Potassium nitrate was then added to the cores to give a final concentration in the water phase of 0, 1, 2, 3, 7 and 14 ppm NO_3 -N (5 cores at each concentration) and the cores were closed to the atmosphere. They were incubated at 20°C for 40 days in the dark and water samples were taken regularly for orthophosphate analysis.

In the third experiment (C) to examine the rate of oxygen utilization and gas release in cores containing different types of sediment, twelve Jenkins cores were collected from each of the deep and shallow areas on May 19th 1978. The cores were aerated overnight and then closed to the atmosphere. Potassium nitrate was added to give a final concentration in the water phase of 0, 1, 3 and 14 ppm NO_3 -N. Cores of both sediment types were set up in triplicate. The cores were incubated for 30 days at 16°C in the dark. The oxygen concentration of the water phase was measured every 2 days until the oxygen had been completely utilised. Water samples were taken every 2 or 3 days for orthophosphate analysis. Any gas which collected in the cores was sampled, the volume noted and analysed for nitrogen and methane. The amount of gas released from

Fig.10. The effect of poising the redox potential on phosphate release in cores from the North deep held anaerobically at 16°C in the dark.



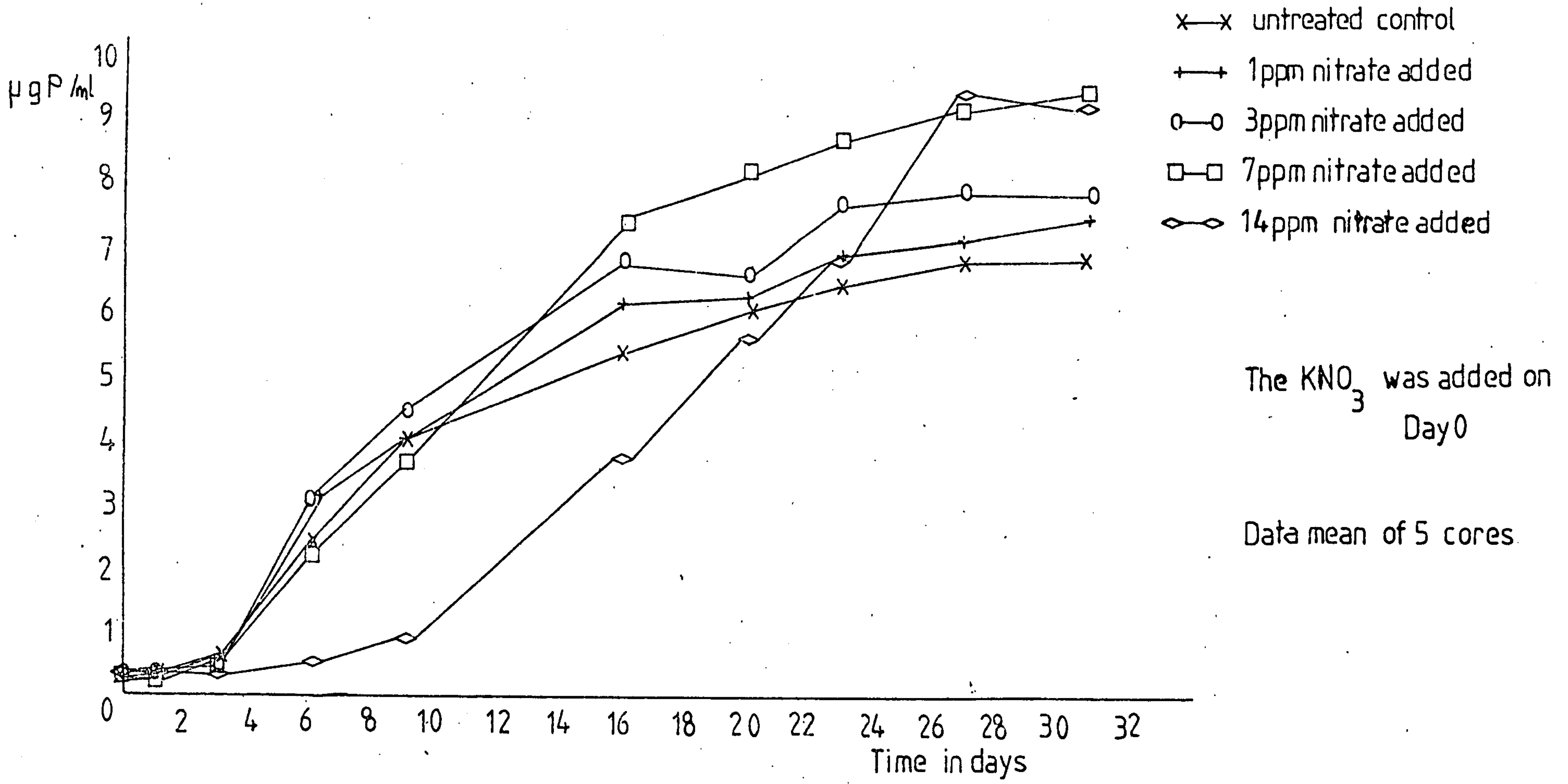
the sediments was felt to be important at the resultant disturbance of the mud may aid phosphate release.

<u>Results and Discussion</u> <u>Experiment A</u> In the initial experiment the addition of chemicals to the cores was shown to have an effect on phosphate release from the sediments (Fig. 10). After the addition of nitrate little phosphate was released but when sulphate and thiosulphate were added there was an immediate release of phosphate. This release was more rapid than in the control cores. The results showed that the phosphate release took place at an Eh value of between +100mV and -100mV. These observations indicated that the reaction bringing about the release of phosphate could be

$$Fe^{3+}PO_4 \text{ (insoluble)} \longrightarrow Fe^{2+}PO_4 \text{ (more soluble)}$$

The reduction of ferric phosphate to ferrous phosphate takes place between the afore mentioned levels and although other phosphates e.g. $MnPO_4$ could be released within this range the iron phosphate is the most likely as this is the dominant form in the sediment. It is unlikely that FeS plays a major role in phosphate release (Serruya, et al 1974) because when sulphate was added there was an immediate release of phosphate in the water, before hydrogen sulphide could have been formed. The more immediate release of phosphate in the sulphate and thiosulphate treated cores than in the controls appeared to be related to the rapid fall in Eh compared with the several days required in the control cores.

Fig.11. The effect of adding varying concentrations of nitrate to phosphate release in mini cores containing deep sediment.



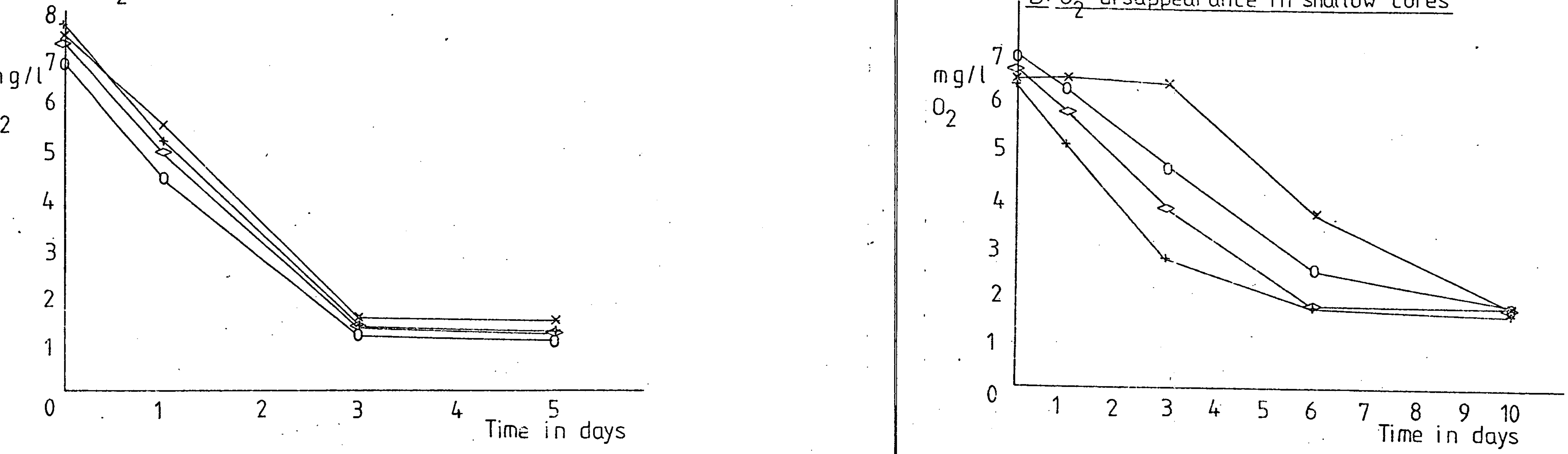
Experiment B The above results indicated that the nitrate concentration in the loch water may be an important factor in controlling phosphate release. At high nitrate concentrations the redox potential was too high for phosphate release to take place. Thus by adding a range of concentrations of nitrate to cores it should be possible to estimate the importance of nitrate in controlling certain aspects of the phosphate cycle. The results of the second experiment, shown in Fig. 11, indicated that the high nitrate concentrations could, indeed, play a significant role in controlling phosphate release. The control cores and the treatment with 3 and 7 ppm NO_3 -N added were initially very similar but there was a slight enhancement in the rate of phosphate release with 1 ppm NO_3 -N. The phosphate concentration remained at a low level for several days after 14 ppm NO_3 -N had been added. The addition of nitrate at all the concentrations tended to increase the amount of phosphate released after 24 days. There was no additional phosphate release after 30 days and the concentration in the water remained stable until the experiment was terminated after 40 days.

The initial rapid release of phosphate at 1 ppm NO_3 -N indicated that nitrate was a limiting nutrient suggesting that the addition of extra nitrate enhanced bacterial activity. Thus the sediments would be reduced more rapidy and the phosphate released at a greater rate. This explanation was investigated in experiment

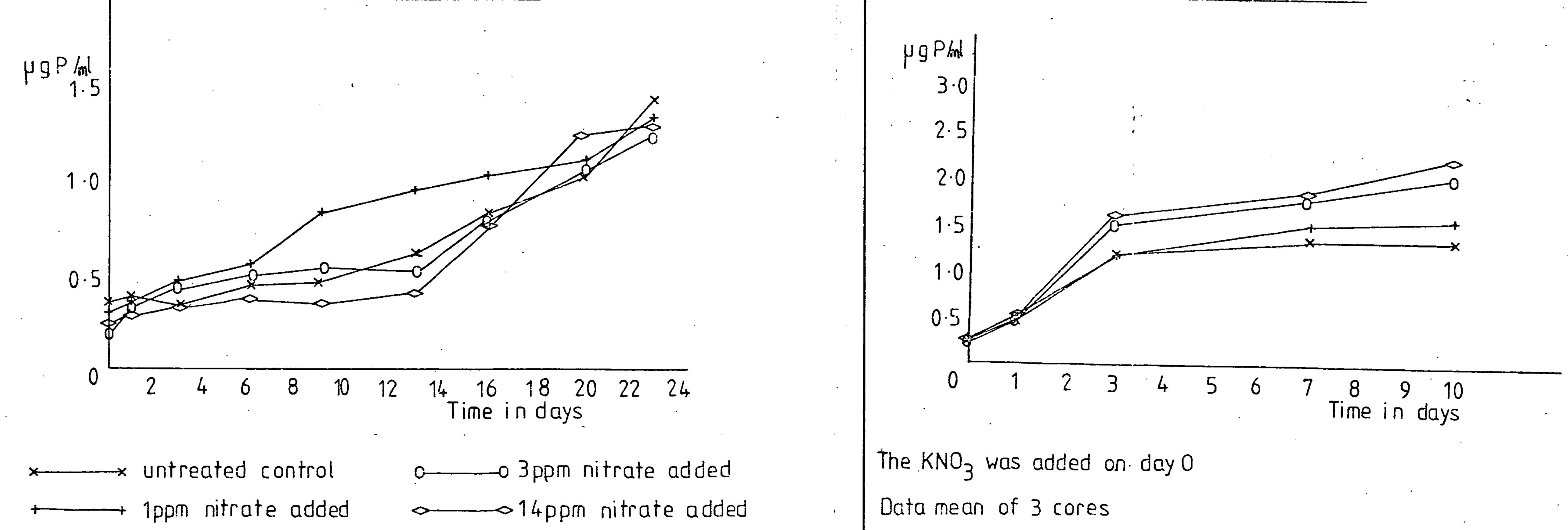
C by measuring sediment respiration and was shown to be true. However at the higher nitrate concentrations the salt would have the effect of poising the Eh and preventing phosphate release despite the higher rate of respiration. This trend could be seen most clearly at 14 ppm NO_3 -N. The high final concentrations of phosphate in the cores which had had nitrate added were unexpected. All the cores receiving the nitrate released large volumes of gas (not quantified in mini cores) which could be seen bubbling out of the sediment and disturbing the surface. This disturbance of the sediment which would tend to aid the release of any phosphate from the interstitial water into the core water may also be a factor accounting for the higher concentrations of phosphate which could be detected in the cores which had had nitrate added.

Experiment C The results of experiment B suggested that the addition of nitrate to cores may make a difference to the rate of sediment respiration if nitrate was limiting. However in that experiment the mini cores had been incubated for 4 weeks before the nitrate had been added, therefore in experiment C freshly collected cores were used for oxygen measurements. Although the water phase of the deep cores was oxygenated at the start of the experiment the sediment was in an extremely reduced state and much of the oxygen would be used up chemically as well as biologically.

Fig.12. Oxygen disappearance and phosphate release after the addition of nitrate-nitrogen to Jenkins cores taken from Loch Leven deep and shallow areas


A. O_2 disappearance in deep cores

B. O_2 disappearance in shallow cores


C. Phosphate release in shallow cores

D. Phosphate release in deep cores

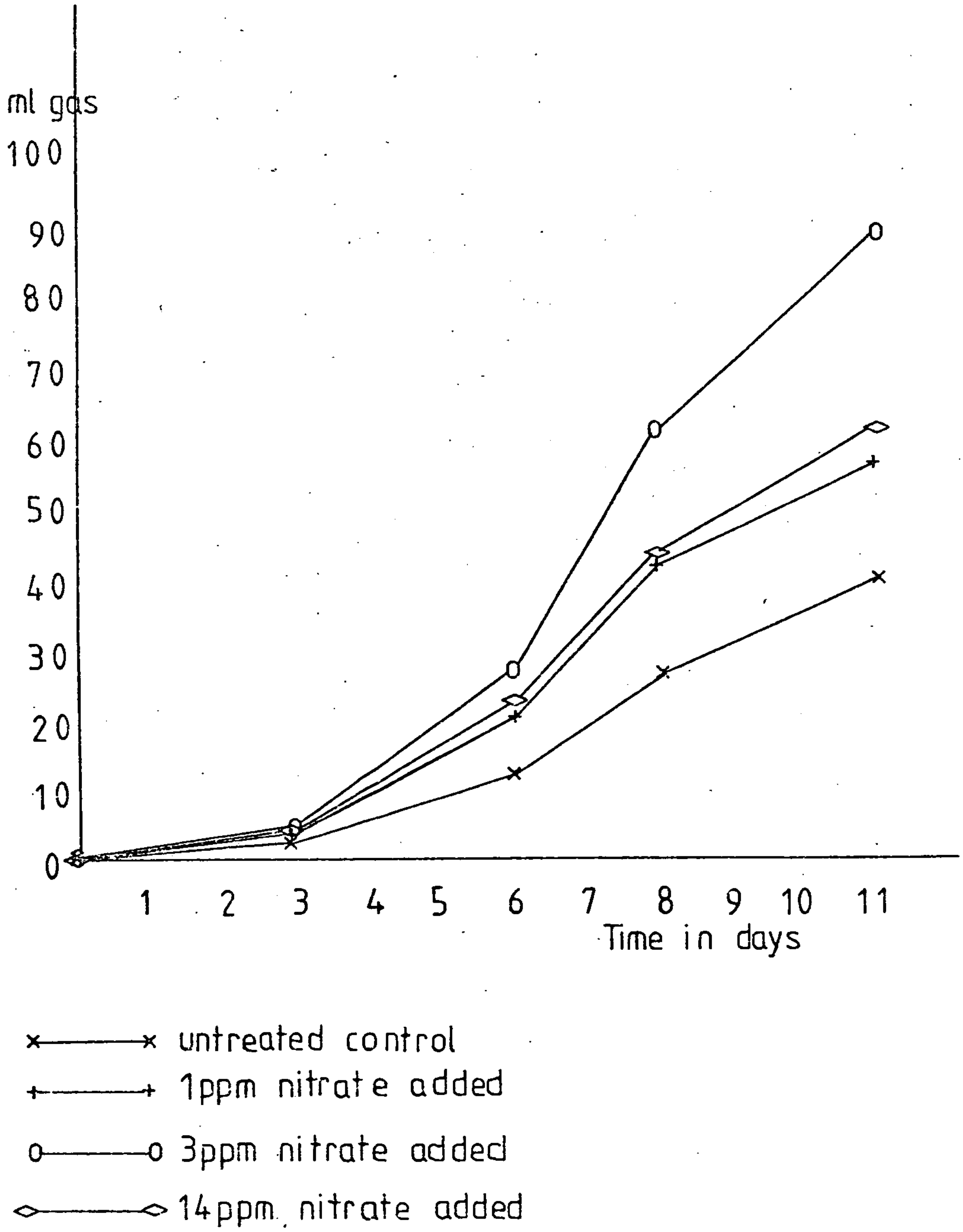
The KNO_3 was added on day 0

Data mean of 3 cores

However it was possible to see a small difference between the cores which had nitrate added and the control cores (Fig. 12A). In the nitrate cores there was a slightly higher rate of oxygen uptake than in the controls indicating that, perhaps, nitrate did affect the respiration rate. However in the shallow cores it was possible to see a great difference in the rates of oxygen uptake of the various treatments (Fig. 12b). There was a greater oxygen uptake rate at 1 ppm NO_3 -N than in the other treatments but there was still enhanced oxygen uptake at 3 and 14 ppm NO_3 -N. These results indicated that nitrate may have been limiting sediment respiration in the loch. There was an algal bloom at the time of sampling and the algae had utilised much of the available nitrate leaving very low concentrations (0.1 ppm NO_3N) in the water phase. An oxygen concentration of below about 1-5 mg/l was not recorded. The sampling from the cores required water to be introduced that may have contained a small amount of oxygen.

The phosphate release in the shallow cores, as shown in Fig. 12c was more rapid with 1 and 3 ppm NO_3 -N added than in the control cores but with 14 ppm NO_3 -N several days elapsed before the phosphate started to appear in the water phase. However in Fig. 12b 14 ppm NO_3 -N promoted a higher rate of respiration than in the control cores. Therefore these results indicate that nitrate poised the Eh value at a high

Fig 13 Gas release after the addition of nitrate-nitrogen to Jenkins cores taken from Loch Leven north deep



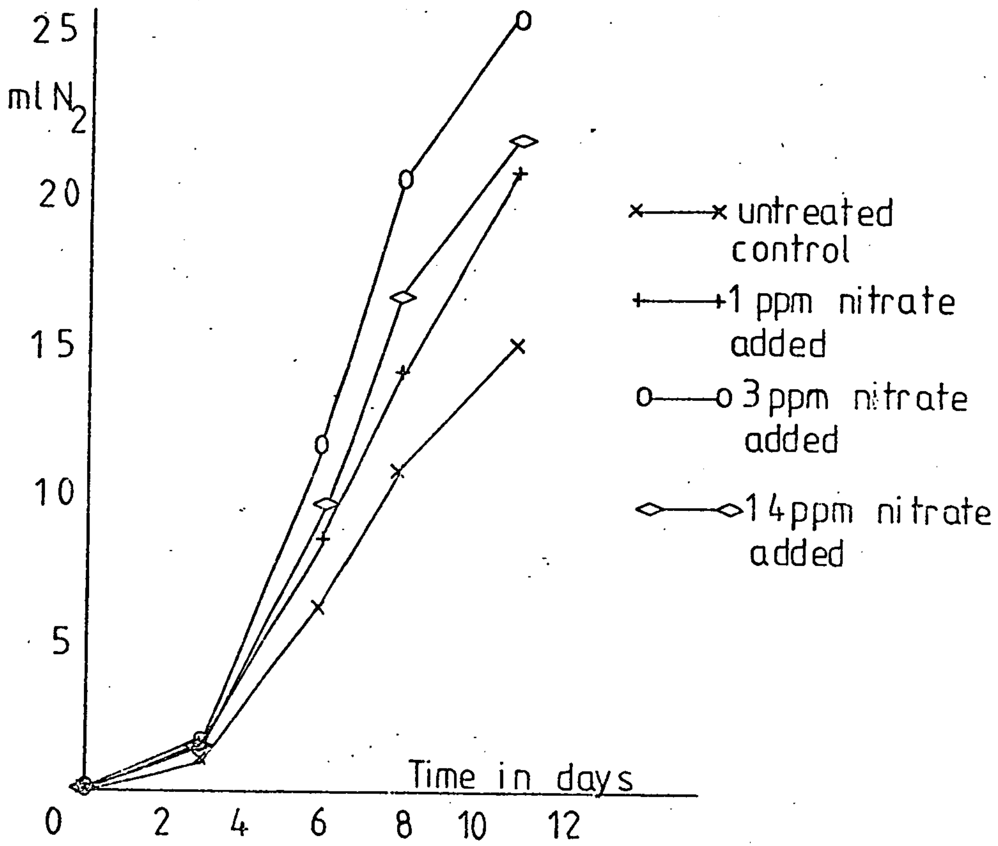
The KNO_3 was added on day 0

Data mean of 3 cores

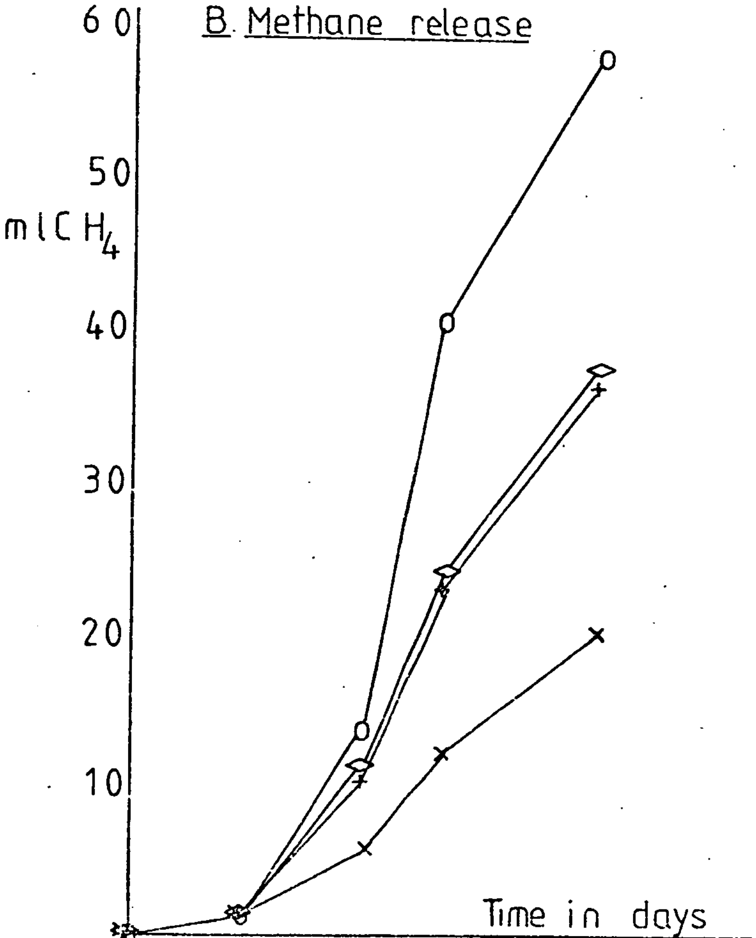
enough value to prevent phosphate release despite higher respiration rates. However enough nitrate must be present so that the salt is not used up too rapidly and can poise the Eh otherwise a faster rate of phosphate release is seen (1 and 3 ppm NO_3 -N). In the deep cores there was a different pattern of phosphate release (Fig. 12d) with 3 and 14 ppm giving the most rapid release and 1 ppm NO_3 -N being very similar to the controls. The higher respiration rates in the nitrate treated cores could account for the faster phosphate release at 3 and 14 ppm NO_3 -N but this would not be comparable with the poising effects found in the shallow cores or in previous experiments. An explanation for the greater amounts of phosphate at 3 and 14 ppm NO_3 -N can be seen in the data provided in Fig. 13. This figure shows that more gas was released in the cores which had been treated with nitrate and these released more phosphate. Large volumes of both nitrogen and methane were produced in the deep cores (Fig. 14 a, b) although more of both types of gas were produced at 3 ppm than with 14 ppm NO_3 -N. More nitrogen would have been expected in the 14 ppm NO_3 -N cores because of the additional nitrate being denitrified. There was no gas detected in the shallow cores which was unexpected, because denitrifying bacteria have been found in this sediment in similar numbers to the deep sediment (Duncan, pers. comm.). However the cores were in an extremely reduced state after 30 days since a black

Fig 14. Nitrogen gas and methane release after the addition of NO_3–N to Jenkins cores taken from Loch Leven North deep.

A. Nitrogen release



B. Methane release



Data mean of 3 cores

colouration of the water due to sulphide production was observed. When no gas was produced the results of phosphate release were as anticipated (Fig. 12c) with 1 ppm NO_3 -N enhancing the rate of phosphate release and higher concentrations of nitrate decreasing the rate of release by poising the redox potential.

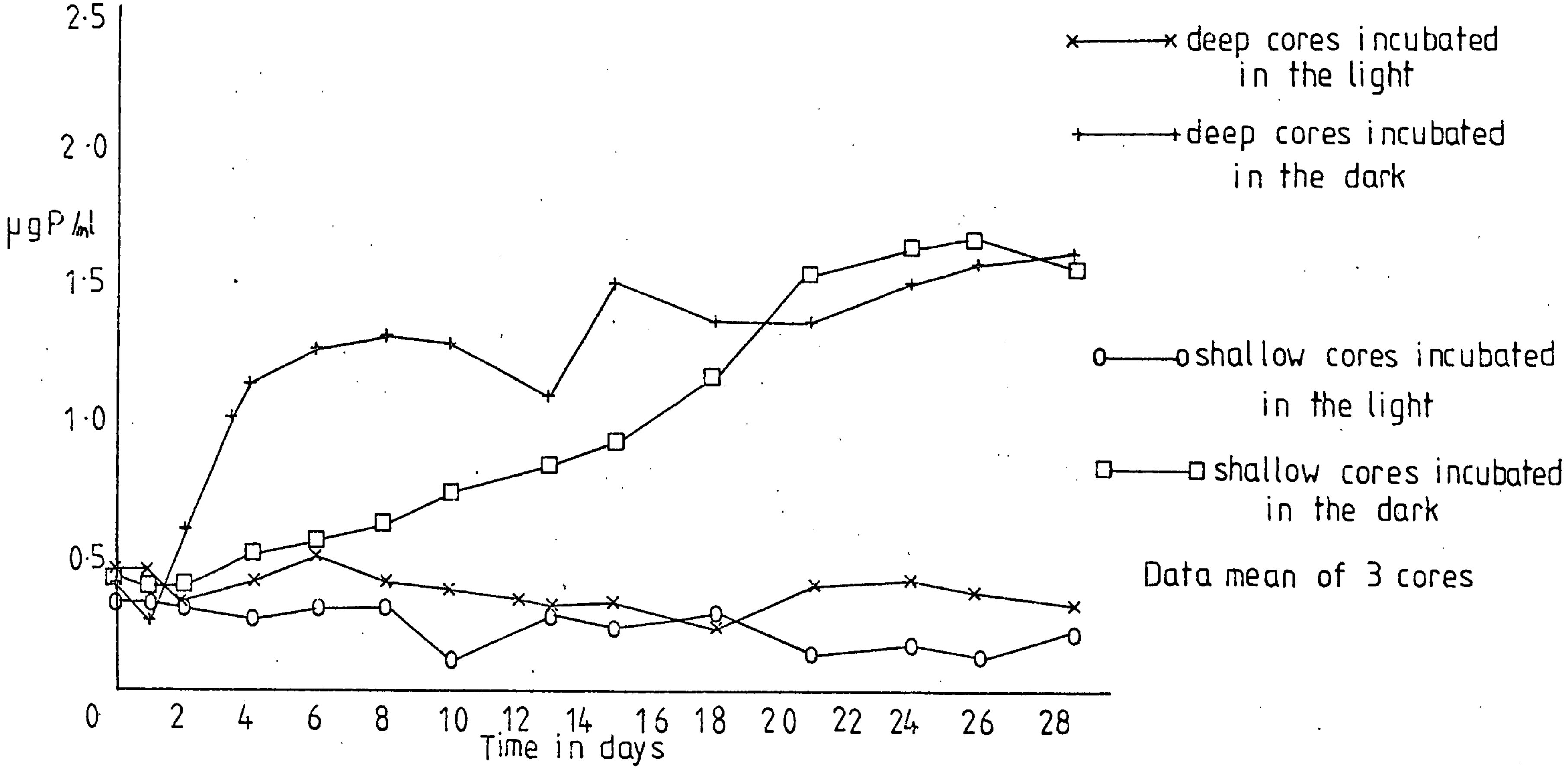
Thus from these three experiments three main conclusions can be drawn about the effects of nitrate on phosphate release. Firstly, if nitrate is limiting in a system and there is a sudden influx of nitrate, the respiration rate in the sediments will be increased which will tend to reduce the sediments rapidly thus releasing phosphate. Secondly, if the nitrate concentration is high enough this salt will poise the Eh preventing phosphate release despite the higher respiration rate. Finally, the additional gas produced from nitrate tends to disturb the sediments which allows phosphate to escape more rapidly from the interstitial water into the lake water. This latter factor of gas production, enhancing phosphate release, appears to be more important than the poising of the Eh by nitrate.

3-6 The Effect of Aeration on Phosphate Release in Jenkins Cores

The previous sections have dealt with phosphate release from the sediments under anaerobic conditions. However Loch Leven is a shallow exposed lake and results from measurements of redox potential in the sediment (Fig. 6) have shown that it is rarely stratified except in the deep areas. It seems likely that the deep areas are the only places where anaerobic phosphate release is likely to take place. Holden, & Caynes (1974,) calculated that not enough phosphate enters the loch from external sources to account for the algal blooms which occur annually and the results described previously suggest that only a small proportion of the phosphate required by the algae will be released under reducing conditions. The extra phosphate may, therefore, be leaching from the sediments under aerated conditions. By experimenting with aerated Jenkins cores an attempt was made to measure this leaching effect.

Methods 6 deep cores and 6 shallow cores were collected on May 3rd 1978. The cores were all aerated for 30 days at 20°C, three of each type in daylight by an east facing window and the remaining six cores in the dark. Water samples for orthophosphate analysis were taken regularly. A comparison with dark and light enabled initial experiments on the effect of algal growth on phosphate concen-

Fig.15. The effect of light and dark on phosphate release in aerated cores taken from Loch Leven deep and shallow areas.



tration in the water phase to be undertaken.

Results and Discussion The results (Fig. 15) showed that it was possible to have phosphate release under dark aerobic conditions from both sediment types and eventually an equilibrium was reached where the phosphate concentration in the water remained constant at around 1.6 μg P/ml. There was an accumulation of phosphate in the water in the dark but under light conditions no change in the phosphate concentration could be detected. In the light there was visible algal growth in the water and on the sides of the tubes. Golterman, et al (1969) found that *Scenedesmus sp.* could utilise any phosphate exuding from the mud of Dutch lakes and presumably in the light cores the algae were using any phosphate which entered the water. Initially the phosphate was released more rapidly from the deep sediment than the shallow but the equilibrium concentrations in the water of each type of sample were very similar. All the cores remained oxic throughout the experiment due to the aerating procedure. This state could be confirmed by observing the light brown colour of the sediment.

The processes taking place in the light would be loosely comparable to the loch situation. In the natural situation a proportion of the phosphate leaching from the sediments would immediately be utilised by the algae in the water therefore the concentration of orthophosphate would remain low although the organic phosphorus content may rise. The similarity between the equilibrium value of the two sediment types can be explained by the fact that they contain similar quantities of all the major types of phosphate except for iron phosphate (Table 6). There is more iron phosphate in the deep areas but this is highly insoluble under aerobic conditions and would not, therefore, contribute greatly to the equilibrium. The phosphates which will contribute towards the equilibrium are $MgHPO_4$ and $CaHPO_4$ which are present in equal concentrations in both sediment types.

This aerobic release of phosphate could be brought about by several factors. For any salt in contact with a solvent, such as water, there is always an equilibrium which must be maintained where a certain amount of the salt dissolves to form ions. This eqilibrium value can be calculated for any salt (Mahan, 1968)

e.g. $CaHPO_4\,(s) \longrightarrow Ca^{2+}(aq) + HPO_4^{2-}(aq)$

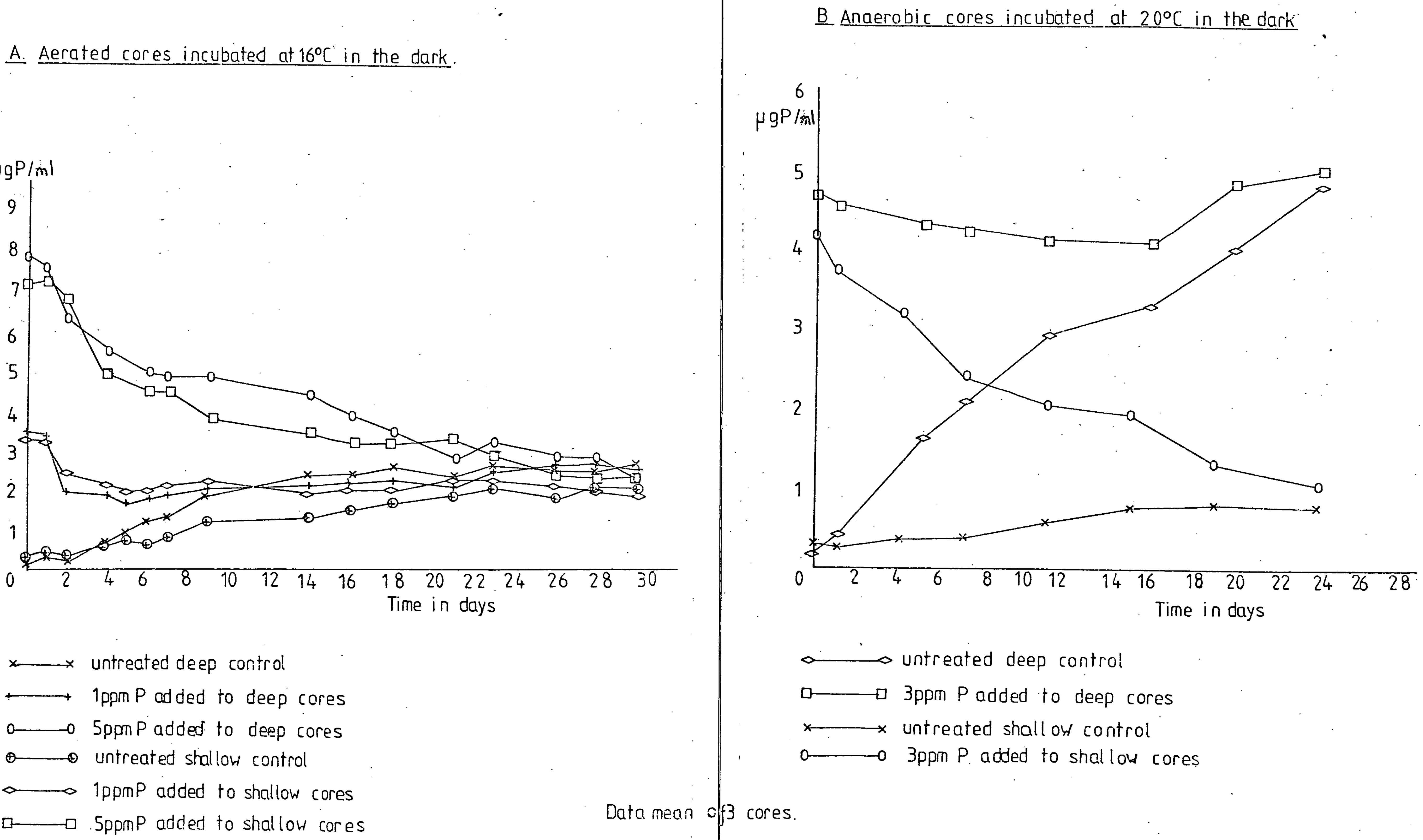
$$K = \frac{(Ca^{2+})\,(HPO_4^{2-})}{(Ca\,HPO_4)}$$

Thus it can be seen that the higher the concentration of the salt the more ions there will be present in the water to maintain the equilibrium. The situation is not static and, as well as dissolving, the salt is constantly reforming. Once the equilibrium is established, as in the dark cores, there will be equal amounts of the salts dissolving and reprecipitating. However in the light cores any phosphate dissolving is immediately utilised by algae and therefore there will be a constant movement of phosphate into the water from the sediment. Invertebrates will also play a role in releasing the phosphate from the sediment. They will be constantly moving about below the surface where the interstitial water is rich in phosphate and then disturbing the sediment surface thus allowing the phosphate to escape into the water. In the loch the wind will also tend to disturb the sediment surface thus aiding the release of phosphate although this was not the case in the cores as the spargers were carefully placed so that there was no disturbance of the sediment.

3-7 Phosphate Adsorption by the Sediments under Aerobic and Anaerobic Conditions

In the natural environment under normal conditions there is a continual inflow of phosphate into a lake from external sources, either natural leaching from the soil or man induced inflow. Frequently more phosphate enters the system than leaves through outflowing waters. This is the case in many Dutch lakes and also in Loch Leven. In the past much phosphate has entered Loch Leven from agricultural land and also from the woollen mill (Holden & Caines, 1974) and during this period as much as 189 kgP excess phosphorus remained in the loch per week. The situation is now improved and there is only a small amount of excess phosphorus remaining in the ecosystem; about 16kgP/wk (Caines & Harriman, 1976). The phosphate concentrations in the waters of many of these Dutch lakes and Loch Leven is not generally very high (usually in the range 1-10 µgP/ml) and although a lower concentration than anticipated could be accounted for by algal uptake during the summer, in winter the concentration of phosphate would be expected to rise. Normally this winter rise is not observed. It is widely recognised (Syers et al, 1973) that this additional phosphate is, in part, being adsorbed by the sediment which, over the years, will gradually become rich in phosphorus. Fitzgerald, (1970,) has shown that lake sediments are capable of adsorbing large quantities of phosphorus

Fig.16. Adsorption by Loch Leven sediments of phosphate added to the water phase.



A. Aerated cores incubated at 16°C in the dark.

B. Anaerobic cores incubated at 20°C in the dark.

Data mean of 3 cores.

annually. By adding phosphate to Jenkins cores it should be possible to estimate the rate at which Loch Leven sediments can adsorb phosphate.

Methods . In an initial experiment, nine Jenkins cores were collected from each of the North deep and shallow areas on October 21st 1977. KH_2PO_4 was added to six of each type of core to give a final concentration of 1 ?? and 5 ppm PO_4 -P (three of each type at each concentration) and the remaining cores were left untreated. All the cores were aerated in the dark at 16°C for 30 days. Water samples were taken every other day and analysed for orthophosphate.

For the second experiment 6 deep and 6 shallow cores were collected on June 22nd 1978. The cores were anaerobically sealed and three of each type had KH_2PO_4 added to give a final concentration of 3 ppm PO_4 -P in the water phase. The cores were incubated at 20°C for 28 days. Water samples were taken every 2 days for orthophosphate analysis.

Results and Discussion Under aerobic conditions the phosphate added disappeared rapidly from the water phase until a value of approximately 1.5 ug P/ml remained (Fig. 16a) which has been shown previously (P.71) to be the equilibrium value of phosphate in the water under aerated conditions in the dark. A similar value was reached in the control cores due to phosphate leaching from the sediment. These results show the enormous potential for adsorbing phosphate under the aerobic conditions prevailing in the loch throughout most of the year. The sediments therefore act as a 'sink', as proposed by Shukla, et al (1971) and Syers, et al (1973.) There was little difference between the rate of adsorption in the deep and shallow sediments which is perhaps surprising as the amount of organic matter is thought, by many workers (Harter, 1969; Lopez-Hernandez, & Burnham 1974; Vijayachandran, & Harter 1975) to play a major role in phosphate adsorption.

Phosphate that was added to cores which were anaerobically sealed, was slowly absorbed by the sediment (Fig. 16b). It seems possible that the initial absorption took place because the cores were still aerobic. However the deep cores were extremely reduced when they were collected and phosphate would have been expected to be released. The quantity of phosphate being released soon exceeded the amount that was being adsorbed by the sediments but it did demonstrate the principal that phosphate is absorbed under reducing conditions if the value in the water phase is high enough. The result was interesting because it has always been postulated previously that phosphate is released under reducing conditions but not adsorbed although these observations did not agree with the finding of Khalid, et al (1977). The shallow samples did not demonstrate the effect because no phosphate was released in the control cores as usually happens. The buoy had moved due to high winds and the cores did not resemble those normally collected from the shallow area and were of a more sandy nature. The sandy areas have a low content of iron phosphate so little phosphate is likely to be released under anaerobic conditions but the added phosphate could still be adsorbed. As has already been stated phosphate release under anaerobic conditions is probably due to the reduction of ferric to ferrous salts. However there are many phosphates present in the sediments which are not affected by changes in redox potential, such as calcium, magnesium or aluminium phosphates and the phosphate absorbed under reducing conditions could be forming these salts rather than iron or manganese salts.

There is probably a continual two way process taking place in the loch with phosphate continuously being absorbed by the sediments and released from them. Under oxidised conditions more will be absorbed than released but the opposite will happen under reducing conditions with the resultant effect that the phosphate concentration in the water would gradually increase under these anoxic conditions which agrees with the findings of Schippel, et al (1973) The sediments act as a phosphate buffer in regulating dissolved inorganic phosphate with the relative proportions of inorganic phosphorus sorbed into the solid phase and in solution thus determining whether inorganic phosphorus is sorbed or desorbed as proposed by numerous other workers.

3-8 Summary of Conclusions

The results discussed in this chapter indicated that many factors interact with each other to control the phosphate concentration in the loch. The release of phosphate from Loch Leven sediments under anaerobic conditions was confirmed. However the results showed that the rate at which the phosphate was released was controlled by temperature, biological respiration and also by the addition of chemicals to the water phase which were capable of poising the Eh. Addition of low concentrations of nitrate to the water phase was found to increase the biological respiration rate in the cores and thus reducing conditions were quickly established, which enhanced the rate of phosphate release. However high concentrations of nitrate prevented phosphate release despite increased oxygen uptake rates, by poising the Eh at too high a level for release to take place. The results indicated that gas release from the sediments enhanced phosphate release by disturbing the sediment surface and thus allowing the phosphate to escape into the water phase. The release and adsorption of phosphate under aerobic conditions was contrary to many theories (P. 74). Conclusions drawn from the results indicated that there was a dynamic situation in which phosphate was continually being adsorbed by and desorbed from the sediments. The direction of flow appeared to depend upon the phosphate concentration in the water phase. If this concentration was high then phosphate was adsorbed by the sediment, even under anaerobic conditions, but if low then phosphate was released from the sediments. The system was always tending towards an equilibrium of 1.5 ugP/ml in the water phase. The release of phosphate under anaerobic conditions was found to be due to the reduction of ferric phosphate to ferrous phosphate but it was deduced that calcium, magnesium and aluminium phosphates played the dominant role in the aerobic release of phosphate.

Sediment type was found to be important in controlling the anaerobic release of phosphate. The deep sediment which was rich in iron phosphate and also had a higher organic carbon content allowed the establishment of reducing conditions more rapidly than the shallow sediment and also more phosphate was released from the deep areas. Thus it was concluded that the concentration of iron in sediments may be important in controlling phosphate release.

Biological activity affected the phosphate interactions in the loch by affecting the redox potential. The lowering of the Eh was found to be a purely biological phenomenon although the resultant phosphate release was entirely due to chemical and physical reactions. Invertebrates also probably affected phosphate release by disturbing the sediment surface. Algae influenced the phosphate concentration in two ways. When growing, the algae utilise phosphate entering the water phase thus phosphate will continually leach from the sediment to maintain the equilibrium in the water (approximately 1-5 ug/ml). However the concentration is usually below this level in the summer when there are algal blooms. Also dead algae sedimenting to the bottom of the loch will provide additional nutrients in the sediment thus increasing biological activity which would mean a reduction of the redox potential in the sediment.

Annual Effects in the Loch

Temperature was found to be very important in controlling the rate at which the Eh fell and thus the rate at which phosphate was released. During the summer when loch temperatures are high (17 - 20°C) the Eh of the sediments could fall rapidly upon stratification thus allowing phosphate to be released. However during the winter when temperatures are low (4°C or less) the loch could be covered in ice for several weeks without there being any appreciable fall in Eh or release of phosphate.

The number of algae in the loch also affects the phosphate concentration on a seasonal basis. As mentioned above there will be a continual leaching of phosphate from the sediments during the summer when algal populations are high. During the winter when algal populations are low there is still an inflow of phosphate to the loch from external sources and once the equilibrium value of about 1.5 µgP/ml is established this phosphate will be adsorbed by the sediments. Thus during the summer there is a leaching of phosphate from the sediment but the opposite process takes place during the winter depending on the numbers of algal cells in the water.

The *redox potential* of the sediments was found to be relatively stable throughout most of the loch during the year. However the deep areas were found to be extremely reduced during the summer and as these are also the areas rich in iron phosphate there will be a release of phosphate during the summer.

CHAPTER 4 PHOSPHATE SOLUBILIZING BACTERIA

4-1 Introduction

Bacteria capable of solubilizing insoluble inorganic phosphates have been isolated from a number of different soils and lake sediments (Sperber, 1958; Louw & Webley, 1958; Harrison et al, 1972; Barea et al, 1976). In this process the bacteria do not act directly on the phosphate by producing phosphate solubilizing enzymes but the solubilization is brought about by the action of metabolic byproducts which are released into the environment surrounding the cell. There are many theories about the actual mechanism by which the phosphate is rendered soluble.

Sperber (1957, 1958) concluded from her work on soil organisms that the action was brought about by substances such as lactic or citric acids which were found to leach from the bacterial cells. Most phosphates are more soluble in acid conditions and the acids released from bacterial cells would lower the pH thus creating favourable conditions for solubilization to take place. Phosphate solubilizing bacteria isolated from lake sediments have been found to produce a wide range of acids such as citric and lactic acids. These acids were shown to be capable of clearing a precipitate of phosphate in agar media and were detected in lake sediments and living algal cells using paper chromatography (Harrison et al, 1972). However Harrison proposed that the solubilization was due to the formation of 5 or 6 membered ring compounds involving the cation from the phosphate and the acids rather than a pH effect. Other workers (Duff et al, 1963; Webley & Duff, 1965; Louw, 1970) suggested that the bacteria released keto acids which chelate the metal cation of the insoluble phosphate thus freeing the phosphate anion and rendering it soluble. These keto acids and chelates have been detected in supernatants of bacterial cultures using paper chromatography (Rabson & Tolbert, 1958). A widely held view is that carbohydrate metabolism is essential for phosphate solubilization and many carbohydrates have been detected in algal cells and lake sediments (Harrison et al, 1970; 1972). However other studies have found that bacteria can more actively solubilize phosphate when grown on alanine containing media than when glucose (Katznelson & Bose, 1959). In lake sediments higher numbers of phosphate solubilizing bacteria are found near areas where there is a high phosphatase activity, probably due to the higher concentration of nutrients in those areas (Ayyakkannu & Chandramohan, 1971). The phosphate solubilizing phenomenon has only been observed under aerobic conditions but whether this is because anaerobic conditions have been tested and proved negative or have not been tried is not entirely clear from the literature. However anaerobic bacteria should be capable of clearing a precipitate of phosphate as most anaerobic fermentations produce acid byproducts.

Many different types of organism possess this phosphate solubilizing property including fungi, actinomycetes and bacteria (Sperber, 1958) although no identification of the species of fungi and actinomycetes was made. The organisms isolated vary in their efficiency of solubilization to quite a large extent (Duff et al, 1963; Ayyakkannu & Chandramohan, 1970). The latter worker found <u>Bacillus</u> <u>sp</u>. to be the most active whereas Duff found that gram negative rods were the most efficient. However, although similar conditions were used for the tests, Ayyakkannu isolated his bacteria from marine sediments while Duff isolated his from soil.

Phosphate solubilizing bacteria are capable of acting on a wide range of different phosphates including simple phosphates such as $MgHPO_4$, $FePO_4$, $Ca_3(PO_4)_2$ or $AlPO_4$ or more complex naturally occurring phosphates such as gafsa and hydroxyapatite. Not all the phosphates are solubilized with equal efficiency and the amount of solubilization does not appear to depend upon the solubility coefficient of the phosphate (Louw & Webley, 1959; Harrison et al, 1972).

The distribution of these bacteria in the natural environment appears to be dependent on many factors. In soils it has been shown that the incidence of these bacteria is higher in the rhizosphere region of roots rather than barren ground (Louw, 1970) with even higher numbers on the roots themselves (Katznelson & Bose, 1959). Also the type of vegetation affects the numbers of these bacteria with more being found on the roots of lupins than wheat (Louw, 1970) and a very low incidence on flax and clover (Katznelson et al, 1962). Many of the bacteria have been found to require yeast extract and soil extract when grown in pure culture and as all plants exude growth factors required by bacteria this would account for the higher numbers near the roots. Plants also release carbohydrates from their roots and the phosphate solubilizing bacteria would be able to utilise these. The highest numbers of these bacteria have been isolated from soils rich in organic matter (Webley & Duff, 1965) and in sediments more phosphate solubilizing bacteria have been found in clayey rather than sandy areas. (Ayyakkannu & Chandramohan, 1971).

<u>Mechanisms of Solubilization</u> Two main mechanisms by which phosphate solubilization takes place have been referred to above, that is the effect of acid and a chelation mechanism, and microbial physiological mechanisms which bring about these processes will be described below.

<u>(a) Acidity</u> The salt of a weak acid is often more soluble in a strong acid than in water. This is because the weak acid has a higher disociation constant than the stronger one. The reaction involving phosphate and hydrochloric acid could be

$$MgHPO_4 + 2HCl \longrightarrow MgCl_2 + H_3PO_4$$

This is known as the 'turning out' of a weak acid by a strong one. In water magnesium phosphate dissolves according to the expression

$$(Mg^{2+})\ (HPO_4^{=}) \quad = \quad \text{SOLUBILITY CONSTANT}$$

If H^+ ions are added the concentration of phosphate is reduced due to the formation of phosphoric acid

$$HPO_4^{=} + 2H^+ \longrightarrow H_3PO_4$$

The formation of the phosphoric acid effectively reduces the hydrogen ion concentration and at the same time the phosphate ion concentration, therefore more magnesium ions can go into solution. This means that the phosphate is more soluble in a strong acid.

Thus if phosphate solubilizing bacteria released a stronger acid than phosphoric acid this would be a means by which phosphate could be solubilized. Many anaerobic bacteria release acids from the cells during a wide range of fermentations (Stanier et al, 1970) but very few aerobic organisms possess the characteristic. It has been suggested (above) that keto acids are released by phosphate solubilizing bacteria which means they are not using the usual glycolytic pathway but a more unusual pathway e.g.

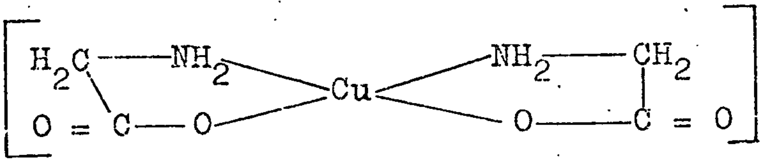
$$\text{glucose} \xrightarrow{\text{glucose dehydrogenase}} \text{gluconate} \xrightarrow{\text{glucose dehydrogenas}}$$

$$\text{2 keto -D- gluconate} \longrightarrow \text{2 -keto-6-gluconate} \longrightarrow$$

$$\text{- - - - -} \quad \text{TCA cycle} \longleftarrow \text{pyruvate} \longleftarrow$$

This pathway does mean that aerobic bacteria are capable of releasing acids and if these keto acids are stronger than phosphoric acid the phosphate will be solubilised.

(b) Chelation The word chelate is derived from the Greek word meaning claw which adequately describes the structure of a chelated molecule. Other terms which are used in a description of chelation are Ligand, which is a negative ion or polar molecule bound to a metal atom and the bond is a metal ligand band. Some ligands are attached to the metal atom by more than one donor atom in such a manner as to form a heterocyclic ring e.g. the ring formed by the glycinate ion:



This type of ring is called a chelate ring and the molecule or ion from which it is formed is known as a chelating agent. Transition metals are most commonly found in chelate rings but the alkaline metals are also capable of forming chelates. The chelating agent must fulfil two major criteria

(a) It must possess at least two appropriate functional groups, the donor atoms of which are capable of combining with a metal atom by donating a pair of electrons, that is basic co-ordinating groups or acidic groups which have lost a proton.

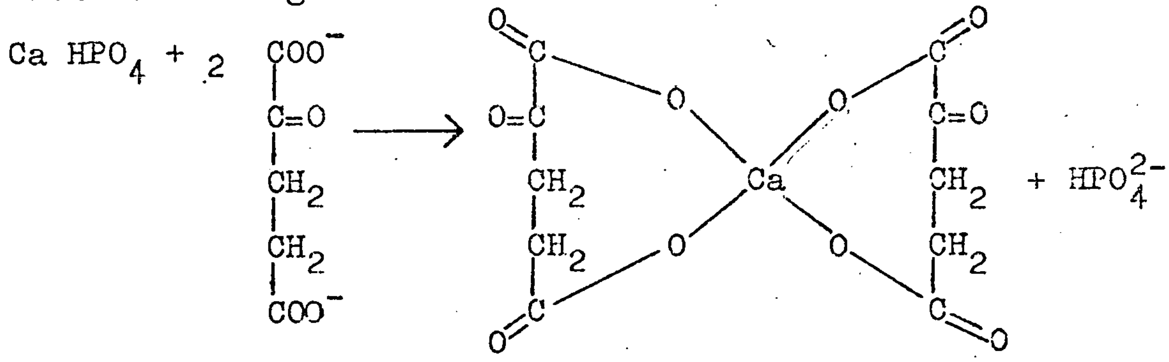
e.g.

BASIC CO-ORDINATING GROUPS	ACIDIC GROUPS
$=O$	$-COOH$
$-NH_2$	$-SO_3H$
$-O-R$	$-OH$ (enolic or phenolic)

(b) The functional groups should be so situated in the molecule that they permit the formation of a ring with a metal atom as the closing member.

The stability of the bond formed depends upon the nature of the metal and the ligand. The size and electronegativity of the metal influence the strength of the bond. The ligand molecule may have other atoms attached to it and the number, nature and position of these influence the stability of the metal ligand band.

Keto-acids as Chelating Agents As mentioned above the substances responsible for phosphate solubilization may be keto acids which are produced by a small number of bacteria which use an unusual metabolic pathway. Such acids as ketogluconic or ketoxylonic (produced during xylose metabolism) acids. These acids possess an acidic group (-COOH) and a basic co-ordinating group (=O) therefore they should be able to form a heterocyclic ring. A possible chelating reaction could be



Both of these mechanisms, pH and chelation, leave the phosphate in a soluble form. This phosphate could then either diffuse into the water phase or be readsorbed by metal ions present in the sediment (Chapter 3). The chelating mechanism is irreversible but the solubilization due to acid conditions is reversible, depending upon the pH of the environment. In lakes which are rarely stratified, which means there is no anaerobic release of phosphate, these phosphate solubilizing bacteria could play an important role in releasing phosphate from the sediment either by lowering the pH or producing chelating molecules.

Although there is a considerable amount of data available about numbers and activity of phosphate solubilizing bacteria in soil, there is very little information for lake sediments, and no investigations have been carried out on Loch Leven. The work about to be described had several objectives. Firstly, the enumeration procedures used by previous workers to count phosphate solubilizing bacteria (Sperber, 1958; Louw & Webley, 1958; Webley & Duff, 1965) are either inaccurate or cumbersome. When counting these bacteria on plates very few colonies can be counted before the clearing in a precipitate of phosphate covers the whole plate which makes the method statistically invalid. Webley, (1965) assessed the numbers of these bacteria using a chromatography method which detects the presence of keto acids thus indicating the presence of phosphate solubilizing organisms but this is not an easy method for routine work. A simpler, more accurate method was therefore devised. Secondly, the potential for phosphate solubilizing activity in sediments was assessed by determining the phosphate solubilizing ability and byproducts of the organisms on various naturally occurring sugars and phosphates. Thirdly an attempt was made to differentiate between the effect of pH and chelation in the solubilization process.

4-2 The Occurrence of Phosphate Solubilizing Bacteria in Loch Leven

The following experiments describe the distribution, population size and the identification of the phosphate solubilizing bacteria occurring in the water and sediments of Loch Leven.

Method Duplicate core samples were collected from the North deep, the shallow area, the sandy site which contained macrophyte growth and the additional sandy site which did not support a macrophyte population. Samples were collected on March 16th 1976, September 20th 1976, March 15th 1977 and April 29th 1977. Water samples were also collected on these dates.

The total viable bacterial population and the numbers of phosphate solubilizing bacteria in each sample were counted, triplicate counts were done for each site. The phosphate solubilizing bacteria were isolated by spreading 0·1 ml of the diluted samples on to the surface of plates containing the phosphate solubilizing medium. Any colonies which gave a clearing in the precipitate were subcultured.

The phosphate solubilizing bacteria which were isolated were maintained on nutrient agar slopes and later identified using the biochemical methods of Holding, (1971), in conjunction with Bergey's manual (1974).

Results and Discussion

TABLE 8 Counts of Bacteria in Loch Leven Sediments (no./ml wet sediment)

sediment type	Deep	Shallow	sand with macrophytes	Sand
total viable count	6.35×10^7	5×10^7	3.18×10^6	5.6×10^6
$PO_4^{\equiv}$ solubilisers	5.2×10^4	2.8×10^4	3.3×10^4	3.8×10^3
% $PO_4^{\equiv}$ solubilisers	0.082	0.056	1.03	0.063
% organic matter	8.0	4	0.5	0.5

Colony count on casein peptone starch agar at 25°C for 21 days

MPN counts on glucose phosphate agar at 25°C for 4 days

Total viable bacterial population The deep area was found to contain the highest number of bacteria (Table 8) with the shallow area having a slightly lower population. Both the sandy areas had a total bacterial count one order of magnitude lower than the shallow area. These differences could probably be accounted for by the differing organic carbon contents of the sites. The deep area, which had the highest count, also had the greatest organic carbon content (8%) which means there would be more nutrients available in this area than in the sandy areas which had a very low organic carbon content (0.5%). The shallow area was intermediate to these with an organic carbon content of 4%. The bacterial count in the loch water was about three orders of magnitude lower than in the sediment which would be because there are fewer nutrients in the water.

The bacteria in the different sediments and the water were similar types. There were many pigmented organisms (red, orange, yellow, purple) which appeared to be the dominant population although there were also numerous non pigmented bacteria. Most of the organisms on the casein peptone starch medium were gram negative rods showing that these were the dominant organisms in the loch. The bacteria present included *Caulobacter sp.*, *Pseudomonas sp.*, *Bacillus sp.*, *Cytophaga sp.* and *Chromobacterium sp.*, among others (Duncan, Patrick pers. comm.)

Phosphate Solubilizing Bacteria Several zones of clearing were observed on the 10^{-3} dilution plates (Plate 1) which indicated that phosphate solubilizing bacteria were present in Loch Leven sediment. An accurate count could not be obtained from the plates because at the 10^{-3} dilution, there were only about 10 organisms per plate which, if counted, do not give a statistically accurate answer but at lower dilutions other loch bacteria were too numerous and hid any solubilization effects. Thus the most probable number method (Chapter 2) for counting the phosphate solubilizing bacteria proved most useful, giving a count within 95% confidence limits. This method was easy to use and clearing in the tubes was readily visible.

Similar numbers of bacteria were isolated from each sediment type except the non macrophytic sand which had lower numbers (Table 8) and similar results were obtained each time a sample was taken. The sand had a low organic carbon content (0.5%) which, according to Webley, & Duff (1965) and Ayyakannu, & Chandramohan (1971) is a factor affecting the numbers of these bacteria although a rise from 4% organic matter to 8% did not affect the counts. The macrophytic sandy area had a similar count to the deep and shallow areas despite the low organic content suggesting that the plant population affects the numbers of phosphate solubilizers as shown by Louw (1970). A higher proportion of the bacteria were phosphate solubilizers in this area than at the other sites although they were still only a small percentage of the total population. The presence of

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macrophytes seems to have a greater influence than total organic carbon content in determining the population of these bacteria in Loch Leven. Similar results were obtained each time samples were taken.

The phosphate solubilizing bacteria differed from the main sediment population in that they were all non pigmented whilst the dominant bacteria in the loch are yellow or orange. Most of the bacteria appeared to belong to two different types, a gram positive rod which sporulated very occasionally and a gram negative, very motile rod. To verify that most of the bacteria isolated belonged to one of these groups they were identified. Biochemical tests (Holding & Collee, 1971) showed these bacteria to be *Bacillus carotarum* and *Pseudomonas putida* respectively. The two types were present in the loch in approximately equal numbers and most of the phosphate solubilizing organisms isolated belonged to one or other of these groups.

4-3 To Investigate the Effects of Sugar Source and Concentration on the Solubilization of Phosphate by Phosphate Solubilizing Bacteria

The many different types of sugar occurring naturally in plant and animal cells become available for decomposition after the death of the cells. The range of sugars which phosphate solubilizing bacteria could utilise to produce the solubilization effect was investigated in the following experiments. The more sugars which could lead to the solubilization the greater the possibility of the process occurring in the ecosystem. Also the effect of sugar concentration on the process was investigated as sugar concentrations in the sediment may be quite low and this may affect the amount of solubilization which takes place.

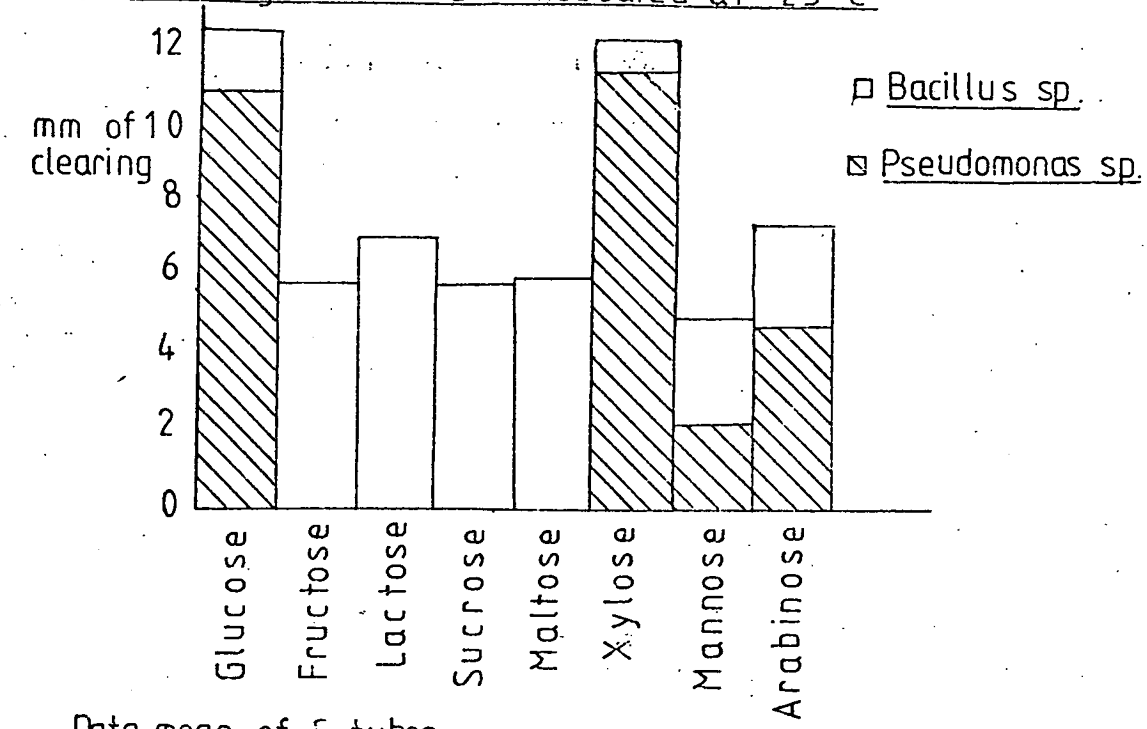
Method Three experiments were undertaken. The objective of the first was to determine the effect of sugar source on phosphate solubilization. Phosphate solubilizing medium was prepared using lactose, sucrose, maltose, mannose, xylose, glucose, arabinose and fructose as sugar sources at a concentration of 0.5%. A precipitate of $CaHPO_4$ was induced and the sterile medium was dispensed into sterile $\frac{5}{8}$" test tubes. An overnight shake culture of phosphate solubilizing bacteria was grown in nutrient broth at 37°C. 0.1 ml of this culture was innoculated on to the surface of the tubes which were then incubated for 4 days at 25°C. The clearing down the tubes was measured to determine the extent of solubilization.

The second experiment determined quantitatively the effect that sugar source had on solubilization. Liquid phosphate solubilizing medium containing the same sugars was prepared, but without a source of phosphate. This medium was dispensed into 250 ml conical flasks and sterilised. 1.0g sterile $MgHPO_4$ was added to each flask. Sterile medium in the flasks was shaken at 37°C for 2 weeks to allow equilibration to take place. 20 ml of medium was then removed from each flask, centrifuged at 6000 rpm for 20 minutes and the orthophosphate concentration in the supernatant measured. The flasks were inoculated with phosphate solubilizing bacteria and incubated for 2 days in a shaker. The number of bacteria per ml in the culture after 2 days was estimated using a haemocytometer slide. The culture was centrifuged at 6000 rpm for 20 minutes and the orthophosphate concentration of the supernatant was again measured and a chromatogram was prepared to detect any keto acids which were present. Control flasks containing a soluble phosphate were also measured for their orthophosphate content before and after the organisms had grown.

In the third experiment to determine the effect of sugar concentration on phosphate solubilization tubes were made up as in the first experiment. However instead of using different types of sugar, the glucose concentration was varied to give final concentrations of 0.1, 0.3, 0.5, 0.7, 0.9 and 1.0% glucose. The tubes were then

Fig.17. Effect of sugar source on phosphate solubilization by phosphate solubilizing bacteria.

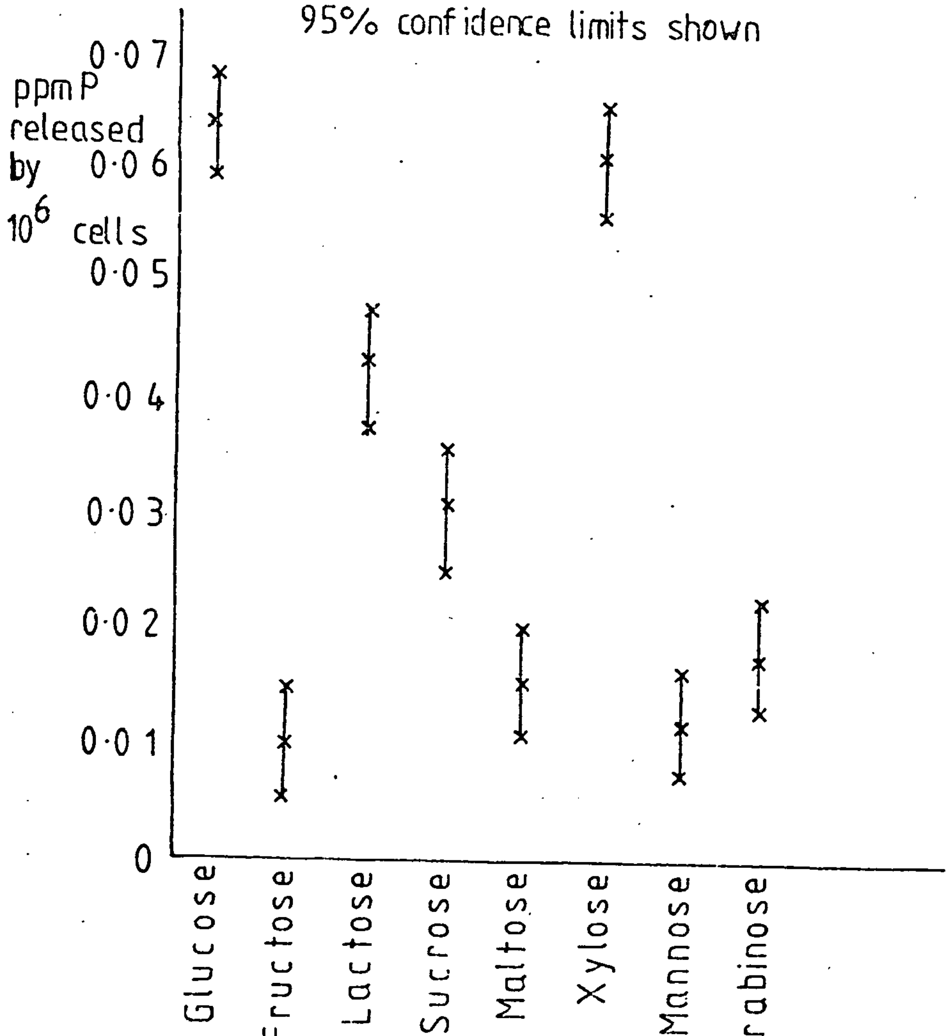
A. Clearing down tubes incubated at 25°C



Data mean of 5 tubes

B. Phosphate release in liquid medium by the Bacillus sp. incubated at 37°C for 2 days.

95% confidence limits shown



treated as in the first experiment.

Results and Discussion

The Effect of Sugar Type on Phosphate Solubilization

The results (Fig. 17) showed that the bacteria were capable of solubilizing the phosphate when grown on a wide range of sugars. Comparable results were obtained using the tubes and liquid cultures with positive observations obtained in both media for all the sugars. The amount of solubilization obtained did not seem to depend upon whether the sugars had a five or six carbon ring. However the best solubilization occurred when the organisms were grown on some mono rather than disaccharides. Some sugars require more energy for breakdown than others and the amount of energy required depends upon the stereochemistry of the sugar and the nature and position of any attached sub groups. Glucose and xylose are easily assimilable in their original form but other sugars, such as mannose, have to be converted to other sugars, such as glucose, before they can be metabolised which means that an additional energy requiring step is necessary. Similarly the bond in a disaccharide must be broken before the constituent monosaccharides can be metabolised. This could explain why glucose and xylose gave the best solubilization. Less energy was required for their breakdown and therefore it is possible that more byproduct was produced and released. These results agreed with those of Harrison, et al (1972) who showed that

TABLE 9 ....Cont.

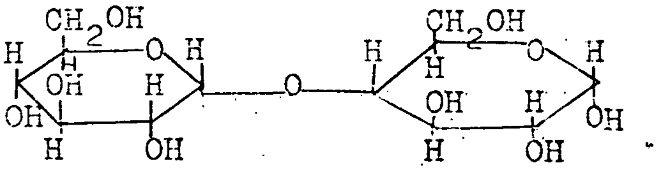
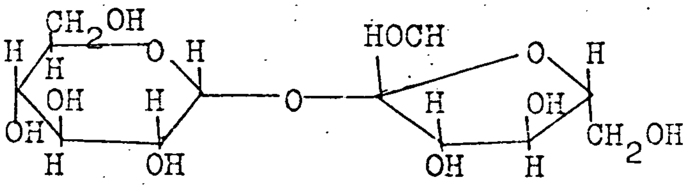
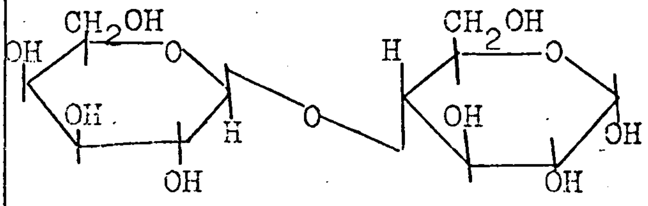
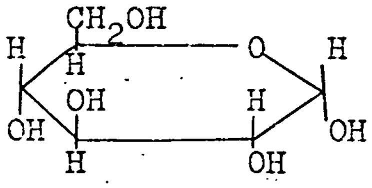
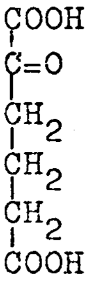
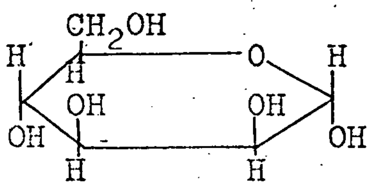
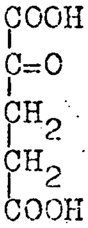
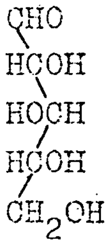
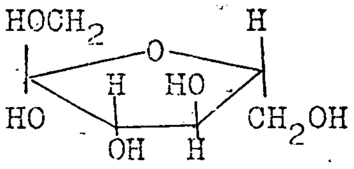
Sugar	Structure	Byproduct
Maltose $C_{12}O_{11}H_{22}$		2 x 2 keto-gluconic acid
Sucrose $C_{12}H_{22}O_{11}$		2 x 2 keto-gluconic acid
Lactose $C_{12}H_{22}O_{11}$		2 x 2 keto-gluconic acid

TABLE 9 The Sugars which gave the Phosphate Solubilising Effect and their Possible Byproducts

Sugar	Structure	Byproduct
Glucose $C_6H_{12}O_6$		2 ketogluconic acid  $\mathrm{COOH-C{=}O-CH_2-CH_2-CH_2-COOH}$
Mannose $C_6O_6H_{12}$		2 ketogluconic acid
Arabinose $C_5H_{10}O_5$		2 ketoglutaric acid  $\mathrm{COOH-C{=}O-CH_2-CH_2-COOH}$
Xylose $C_5H_{10}O_5$		2 ketoxylonic acid
Fructose $C_5H_{12}O_6$		2 ketogluconic acid

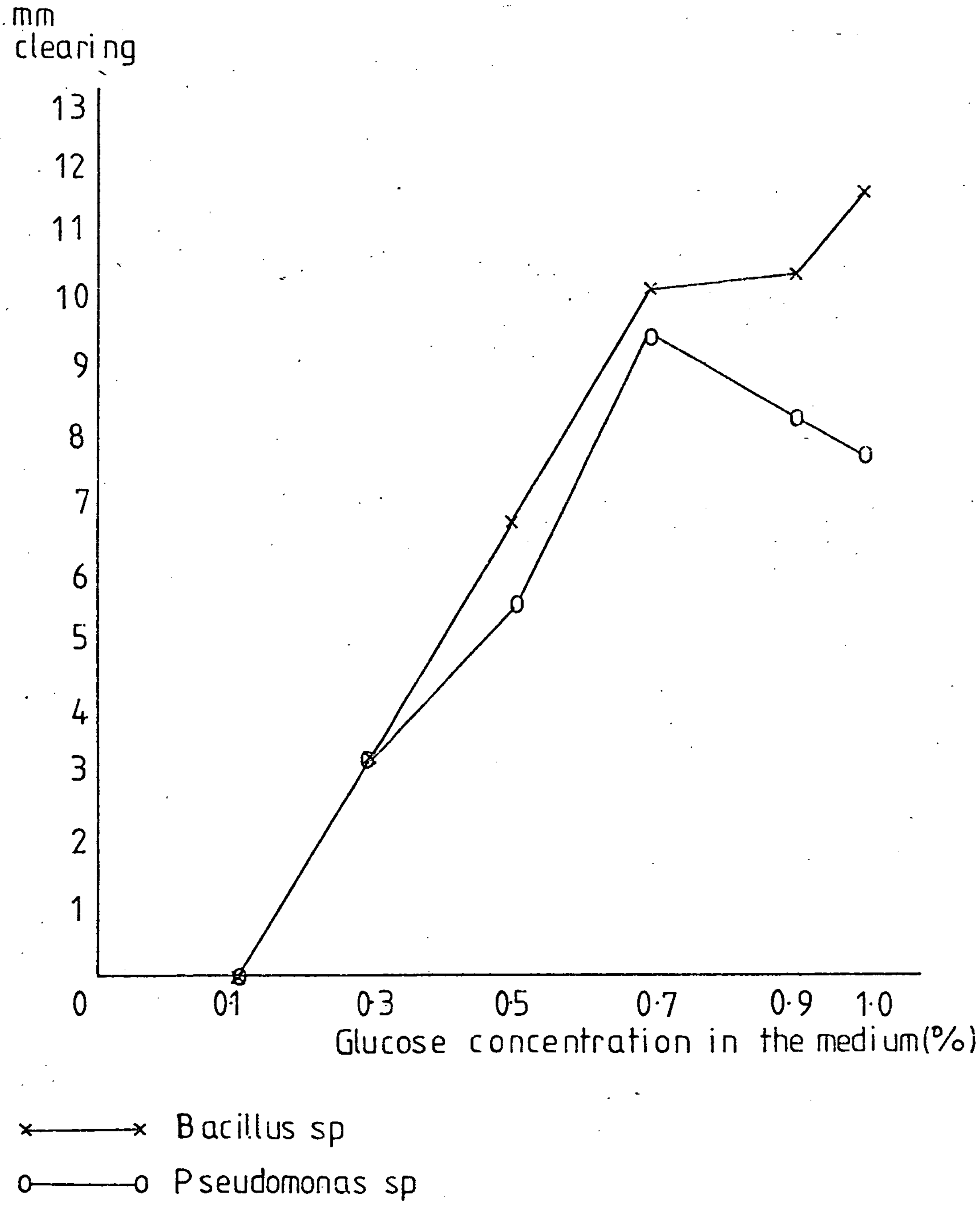
Cont. ....

organisms grown on xylose and glucose solubilised the phosphate more readily than when grown on other sugar sources. The amount of phosphate taken up by bacterial cells during growth was not detectable in the control flasks therefore no correction to the results was necessary.

The chromatograms of the culture supernatants showed up substances with similar r.f. values to ketogluconic and ketoglutaric acids. However the r.f. values of the various keto acids are all very similar and it is difficult to distinguish between them by this method. Further tests were needed to give a positive identification of the active substances although previous workers had taken r.f. values as being accurate (Duff, et al 1963; Webley, & Duff 1965). Thus tentative conclusions could be drawn about the possible byproducts which bring about solubilization. The possible byproducts from the various sugars are shown in Table 9.

The Bacillus sp. appeared to give solubilization on a wider range of sugars than the Pseudomonas sp. when grown in the tubes although the latter grew on all the sugar sources (it did not grow on the medium if no sugar was added). The results from the liquid Pseudomonas culture showed that a certain amount of solubilization was taking place with all the sugars but the amount was approximately two orders of magnitude less than the Bacillus sp. (Appendix 2). Ayyakkannu, & Chandramohan (1970,) found that Bacillus sp. were the most active organisms in solubilizing phosphate which agreed with the above results but Duff et al (1963,) showed that gram negative rods were the most active.

Fig.18. Effect of glucose concentration on the solubilization of phosphate as measured by clearing down tubes.



95% confidence limits ± 0·8

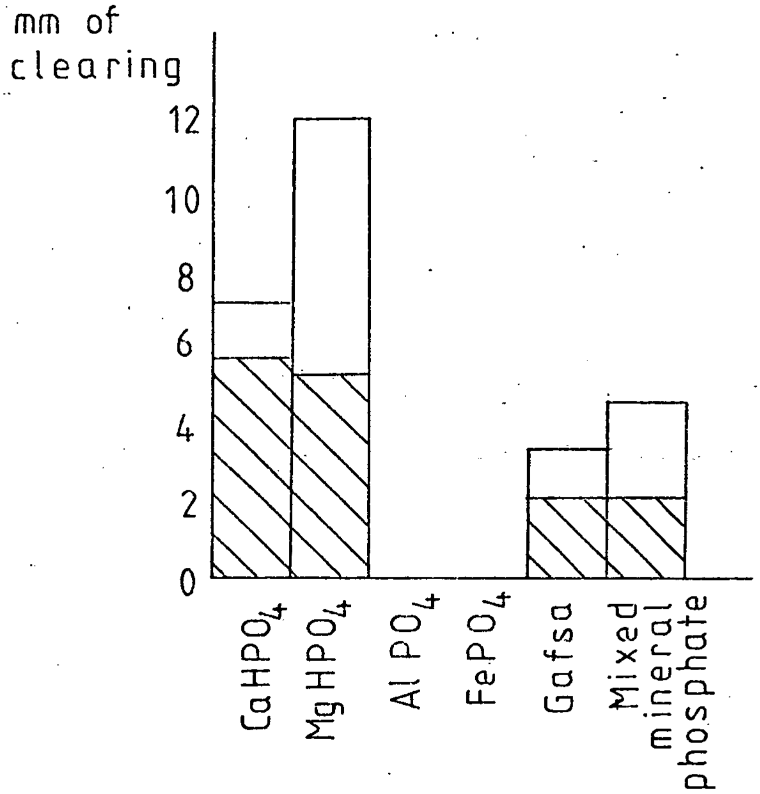
The activity will depend upon the actual species of organism isolated, not just the genus or type, therefore Duff may have isolated less active Bacillus sp. than the Bacillus caratarum isolated from Loch Leven.

The above results indicated the wide range of sugars which these bacteria could utilise and produce the solubilization effect. Many of these sugars are found in the lake environment, being the constituent sugars of algal cells (Hecky, et al 1973) and would therefore be available to the bacteria. This means that there is likely to be some sugar which the bacteria can utilise and give the phosphate solubilization effect in the lake sediment.

The Effect of Sugar Concentration on Phosphate Solubilization

Fig.18 shows that the concentration of glucose in the medium affected the efficiency of phosphate solubilization. Neither organism gave any clearing at 0.1% glucose showing that there was a minimum concentration of glucose required below which solubilization could not take place. The amount of clearing produced by the Pseudomonas sp. reached a peak at 0.7% glucose and then declined whereas the amount produced by the Bacillus sp. was still increasing at 1% glucose. A possible explanation for the lack of clearing at 0.1% glucose is that all the glucose metabolised at this low concentration is completely oxidised and none of the metabolic intermediates are produced in great enough quantity to be released. This minimum requirement of glucose for phosphate solubilization to take place could be important in the loch situation as sugar concentrations in the sediment could possibly be very low and thus no solubilization would take place. The fact that the Bacillus sp. produced more clearing at higher glucose concentrations than the Pseudomonas sp. could indicate that it was more tolerant of higher nutrient concentrations than the latter.

Fig.19. Solubilization of naturally occurring phosphates as measured by clearing down tubes.



95% confidence limits ± 1·23

0·1g phosphate/10mls medium

Tubes incubated at 25°C for 4 days

☐ Bacillus sp.

▧ Pseudomonas sp.

4-4 To Investigate the Effect of Phosphate Solubilizing Bacteria on Naturally Occurring Phosphates

In lake sediments there are many different types of inorganic phosphates, some being more soluble than others. The effect of phosphate solubilizing bacteria on a number of these phosphates was measured to determine the range of natural phosphates solubilized and to compare the relative amounts by which these phosphates were solubilized.

Method $\frac{5}{8}$" test tubes containing 0.1g of an insoluble inorganic phosphate were sterilised. 10 ml of sterile phosphate solubilizing medium containing no phosphate were added to the tubes which were mixed thoroughly and allowed to solidify. An overnight culture of phosphate solubilizing bacteria in nutrient broth was prepared and the surface of the tubes was innoculated with 0.1 ml of this culture. The tubes were incubated at 25°C for 14 days and the clearing in the precipitate down the tubes was then measured.

Results and Discussion Fig. 19 shows that the amount of clearing varied, as expected, with the type of phosphate used. The best clearing was obtained on $MgHPO_4$ followed by $CaHPO_4$. The results for two naturally occurring complex forms of phosphate, gafsa and a mixed mineral phosphate, were well below those obtained for magnesium and calcium phosphates and no clearing was obtained with $FePO_4$ or $AlPO_4$. The Bacillus sp. gave the best clearing in all cases.

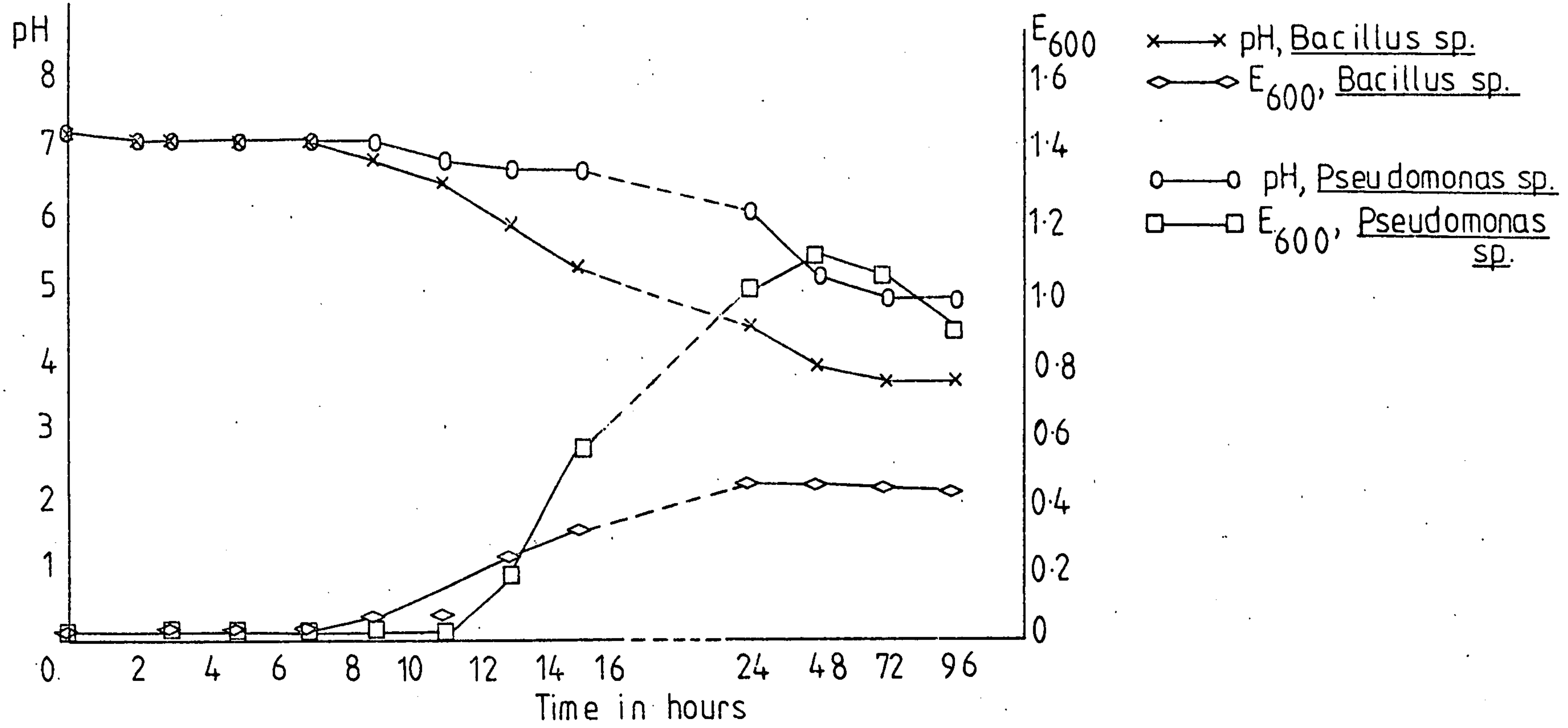
The results were as expected from the solubility products of the various phosphates but differed from those of Harrison, et al (1972) who found that the bacteria solubilized $CaHPO_4$ better than $MgHPO_4$. Weast, (1975) classes both $MgHPO_4$ and $CaHPO_4$ as slightly soluble in water but soluble in acid and from their respective positions in the periodic table the former should be slightly more soluble. Although appreciably lower results were obtained using gafsa and the mixed mineral phosphate the fact that some solubilization was observed could be significant ecologically. These complex forms of phosphate will be the forms in which the majority of the phosphate in the sediment is present so that if the bacteria had not affected them it is unlikely that they would have been very active in bringing about solubilization in the natural environment. Both $AlPO_4$ and $FePO_4$ which the bacteria were unable to solubilize, form trivalent ions and both are highly insoluble, although Harrison, et al (1972) and Louw, & Webley (1959) found that bacteria could solubilize these salts. A possible explanation is that the substance bringing about solubilization is unable to form a chelate with trivalent ions or these phosphates may be so insoluble that the chelating agent is unable to separate the cation and anion and thus form a chelate. This was an unexpected result because chelates are usually formed more easily by ions which can exhibit a variable valency such as aluminium or iron, rather than alkaline metals, such as magnesium or calcium.

4-5 Changes in pH and Production of Keto acids in Liquid Cultures of Phosphate Solubilizing Bacteria

Most phosphates are more soluble under acid rather than alkaline conditions. Therefore under acidic conditions it seemed important to attempt to separate the solubilization due to acidity alone from the additional effect of chelation. The change of pH in liquid cultures was measured and the production of keto acids with time was also investigated.

Method 1 litre of liquid phosphate solubilizing medium containing 0.5% glucose and a soluble source of phosphate (1g K_2HPO_4, 1g KH_2PO_4) instead of an insoluble phosphate was placed in 2 litre flasks and sterilised. The flasks were innoculated with phosphate solubilizing bacteria and incubated at 37°C in a shaker. The initial pH of the medium was measured and again after 3 hours using a Pye unicam pH meter. The absorbance of the culture at 600nm was also noted to estimate the stage in the growth cycle which the culture had reached. A small amount of the culture was centrifuged and 0.1ml of the supernatant was analysed for keto acids using chromatography. The pH and E600 were measured every 2 hours thereafter for 16 hours and the chromatography was carried out at 5, 7, 8 and 25 hours after the start of the experiment. Daily measurements of all parameters were made for several days to follow the survival of the organisms and to discover whether any keto acids remained in the culture medium or

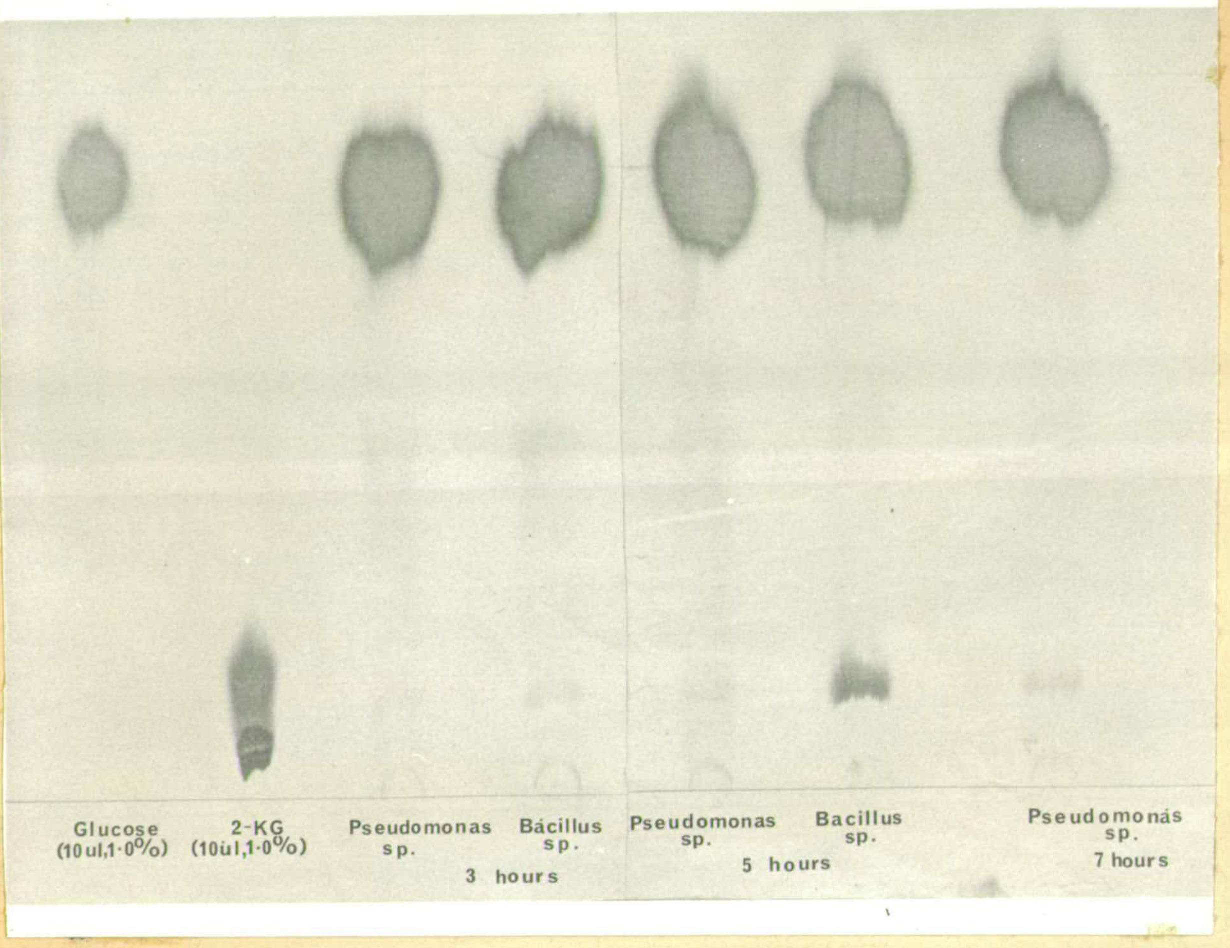
Fig. 20. Change in pH and E_{600} with time in liquid cultures of phosphate solubilizing bacteria incubated in a shaker at 37°C.



Data mean of 3 flasks

were remetabolised.

Results and Discussion The pH of the cultures remained stable until the organisms entered the log phase of the growth cycle when it started to fall rapidly, ultimately reaching the low values of pH 3.7 (Bacillus sp.) and pH 4.9 (Pseudomonas sp.) (Fig. 20). The pH of the Bacillus culture fell more rapidly than that of the Pseudomonas, although, initially, they had similar growth rates. As the Bacillus sp. entered log phase it started to produce ketogluconic acid (Plate 2) which accumulated to reach a fairly high concentration after 25 hours. The spot on the chromatogram was assumed to be ketogluconic acid and no further tests were carried out at this stage. Qualitative concentration estimates of the keto acids were measured by the size of the spot. The keto acid was still detectable in the culture medium at the end of the experiment (4 days) without any apparent change in concentration although all the glucose had disappeared by this stage. No ketogluconic acid could be detected in the Pseudomonas culture until 48 hours after the start of the experiment although by this time the bacteria had passed through the log phase and were beginning to lyse (E_{600} was starting to fall). An explanation is that the Pseudomonas sp. does not secrete the ketogluconic acid from the cell in large quantities, as does the Bacillus sp. but it is released into the culture when the cells start to die and lyse. The two species show completely different types of


Glucose (10ul,1·0%)
2-KG (10ul,1·0%)
Pseudomonas sp.
Bacillus sp.
3 hours
Pseudomonas sp.
Bacillus sp.
5 hours
Pseudomonas sp.
7 hours

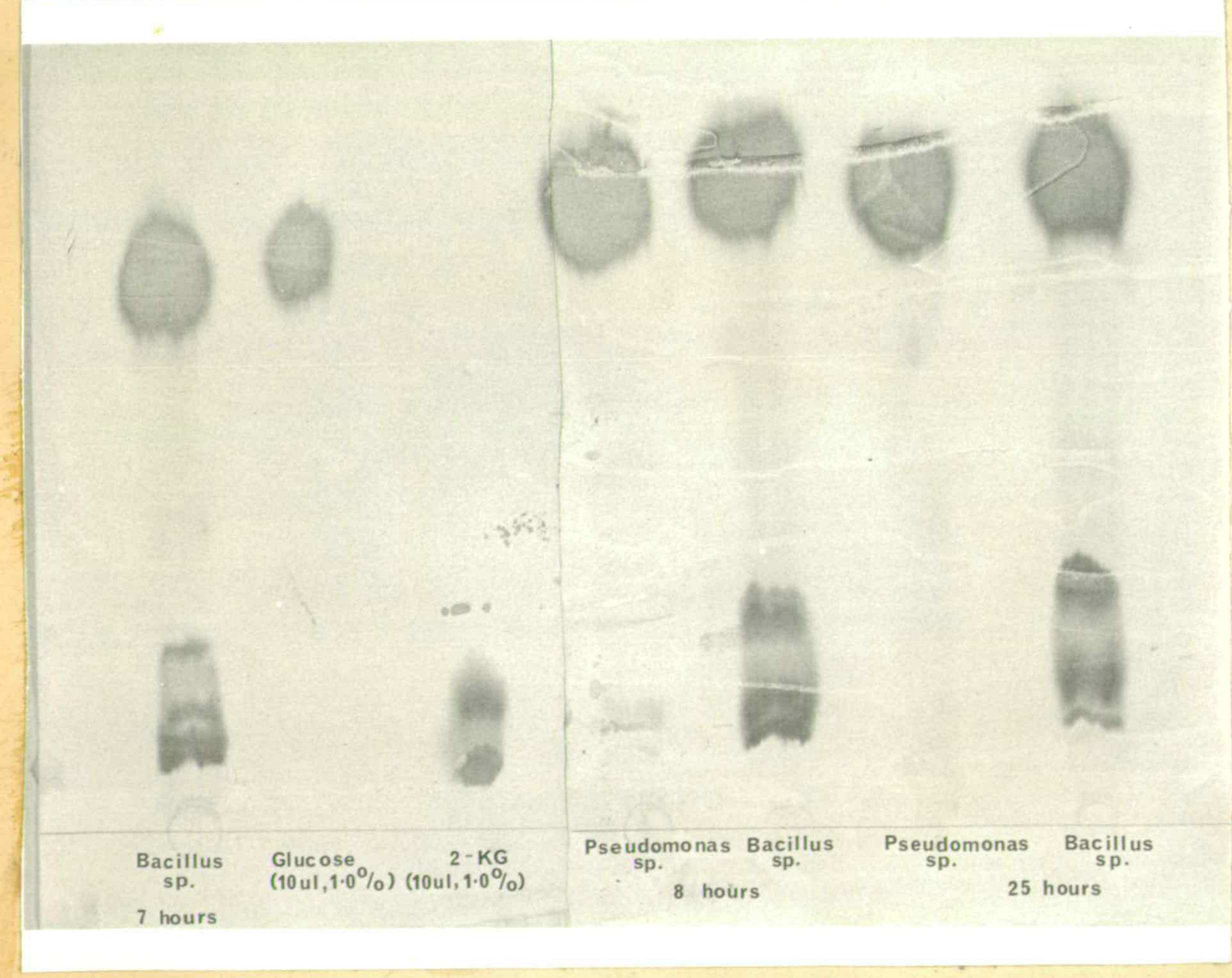

Bacillus sp.
7 hours
Glucose (10ul,1·0%)
2-KG (10ul,1·0%)
Pseudomonas sp.
Bacillus sp.
8 hours
Pseudomonas sp.
Bacillus sp.
25 hours

Plate 2 Production of 2 keto gluconic acid with time by the phosphate solubilizing bacteria grown in liquid phosphate solubilizing medium at 37°C.

growth curve. The growth rate was initially the same but the Bacillus stopped increasing in numbers after 24 hours and thereafter the numbers remained constant. The Pseudomonas culture produced many more bacteria, reached a peak after 48 hours and then the numbers started to decline.

These results explain why the Bacillus sp. solubilize phosphate more efficiently than the Pseudomonas sp. irrespective of whether the effect is due to acidity or chelation.

Both the bacteria are capable of growing on 2 keto gluconic acid as the sole source of energy and carbon. This was shown by ~~i~~noculating the bacteria on to plates containing a mineral salts medium with keto gluconic acid as the only carbon and energy source. A surprising result, therefore, was that the keto acid remained in the culture medium and was not remetabolised when the sugar source became depleted. However if the keto acid had formed a chelate with some of the metal ions in the culture medium, the complex may have been unable to pass through the bacterial cell wall and would therefore be unavailable as a nutrient.

4-6 Separation of Chelation and pH Effects in Phosphate Solubilization

From the previous experiments it was noted that the phosphate solubilization effect may be due to either acid or chelating mechanisms. The following experiments were designed to separate the two effects and ascertain which was the most important in bringing about phosphate solubilization.

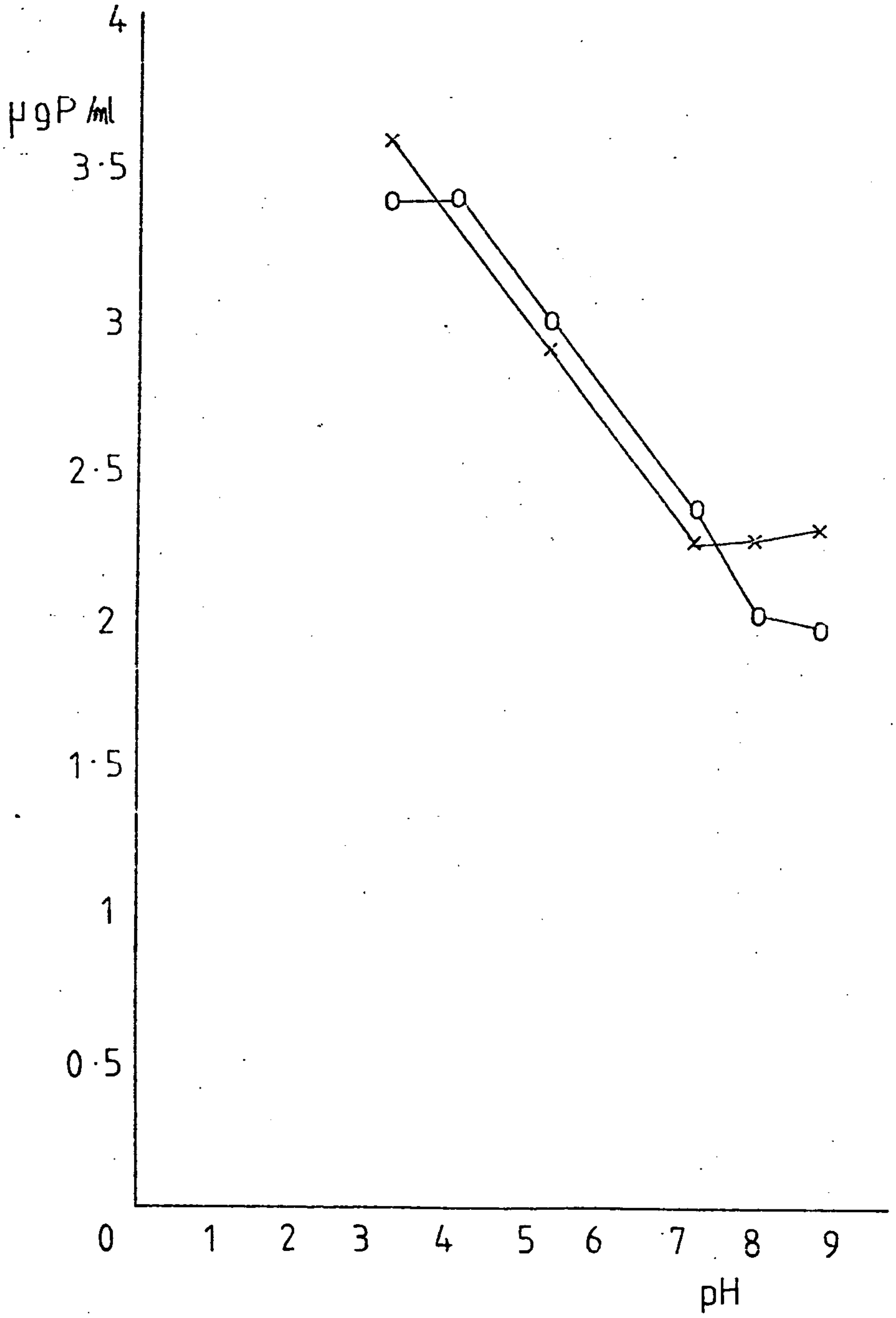
Method The objective of the initial experiment was to obtain a solubility curve for $MgHPO_4$ at various pH values and to determine whether the addition of 2 ketogluconic acid affected this curve. 0.1g $MgHPO_4$ and 10ml citrate buffer at pH 3, 4, 5, 6 or of tris buffer 7, 8, 9 (Cruickshank, 1970) were added to test tubes. The tubes were shaken. To another set of tubes containing 0.1g $MgHPO_4$ and 9.9ml of the buffers 0.1ml 10% 2 ketogluconic acid was added and the tubes were shaken. All the tubes were allowed to stand for 2 hours, being shaken every ½ hour. The clear supernatant was centrifuged at 6000 rpm for 15 minutes to remove any finely divided suspended material. The final supernatant was diluted 1000 fold with distilled H_2O and analysed for its orthophosphate concentration.

In the second experiment an attempt was made to eliminate the acid effect in cultures of phosphate solubilizing bacteria using buffered medium. 150ml sterile phosphate solubilizing medium containing 0.5%

glucose and 1.0g $MgHPO_4$ was dispensed into sterile 250ml flasks. A similar batch of medium made up using tris buffer instead of distilled water was also dispensed into 250ml flasks. These flasks were shaken for 2 weeks at 37°C., They were then innoculated with phosphate solubilizing bacteria (Bacillus sp.) and the initial pH and orthophosphate concentration of the cultures were measured. The cultures were incubated, with shaking at 37°C for 2 days. After incubation the final pH and the number of cells per ml were determined. The bacteria were then centrifuged out and the supernatant was analysed for orthophosphate and the presence of keto acids.

The final experiment was set up to find the effect of various substances on a phosphate precipitate in an agar medium. 2% water agar was prepared and a precipitate of $CaHPO_4$ was induced in the agar. This medium was dispensed in 15 ml amounts into petri dishes and allowed to solidify. 0.1ml of buffers pH 3.1, 4.0, 5.3, 6.1, 7.3 and 8.1, sulphuric acid, 2 ketogluconic acid, 2 ketoglutaric acid and sodium hydroxide were spotted on to the plates. 0.1ml of the supernatant of a culture of the Bacillus sp. was also placed on the agar. 10ml of the supernatant was freeze dried and then reconstituted with 1.0ml distilled water (10 fold concentration). 0.1ml of this concentrate was tested on the water agar. Any clearing in the precipitate induced by the above substances was noted. All the plates were then flooded with M NaOH and any disappearance of the clear zones was observed.

Fig.21. The effect of adding 2ketogluconic acid on the solubility of MgHPO at different pH values in liquid media.



o——o 2 ketogluconic acid added (0·1ml 10% 2KG/0·1gMgHPO_4)

×——× untreated controls

95% confidence limits ±0·09

TABLE 10 The Effect of Buffering Cultures of Phosphate Solubilising Bacteria on Phosphate Release

	Non Buffered	Buffered
Original pH	7.1	7.1
Final pH	5.6	5.9
PO_4 release (ppm P)	$(6.85 \pm 0.24) \times 10^{-7}$	$(2.71 \pm 0.24) \times 10^{-7}$

Culture media containing $MgHPO_4$ buffered using tris buffer, incubated 37°C for 2 days.

<u>Results and Discussion</u> In the first experiment, as anticipated, the phosphate was found to be more soluble under acid conditions (Fig. 21). The addition of ketogluconic acid appeared to make a difference to the amount of phosphate solubilised but the effect varied according to the pH value. At high and low pH values more phosphate was released in the tubes without the keto acid but at physiological pH values more phosphate was detected in the supernatant of the tubes with ketogluconic acid. The differences were very small but statistically significant.

In the second experiment so much acid was produced that, despite the buffer, the pH fell sharply (Table 10). However, the final pH of the buffered culture was marginally higher than the non buffered system and there was a difference in the amount of phosphate released in each of the cultures. Equal quantities of ketogluconic acid (as measured by dot size) were produced in each case. dot?

The above results indicated, as expected, that acidity was probably the major factor contributing to phosphate solubilization by the bacteria but that the keto acid could enhance the effect at physiological pH values. Although $MgHPO_4$ is only sparingly soluble in water it is more soluble under acid conditions. Thus the acid environment could initially solubilise the phosphate, freeing the cation which could then bond with the keto acid to form a chelate. The formation of the chelate would effectively remove the cation from solution and thus more phosphate would dissolve to maintain the equilibrium.

TABLE 11 The Effect of Various Substances on a Phosphate Precipitate in an Agar Medium

Substance (0.1ml)	pH	Clearing	Disappearance of clearing on addition of alkali
H_2SO_4	0.6	+	+
NaOH	12.0	-	N/A
Buffer	3.1	+	+
"	4.0	+	+
"	5.3	(∓)	N/A
"	6.1	-	N/A
"	7.3	-	N/A
"	8.1	-	N/A
"	9.0	-	N/A
2 ketogluconic acid	2.6	+	-
2 ketoglutaric acid	2.1	+	-
culture supernatant	3.7	(∓)	-
concentrated supernatant	3.5	+	-

N/A not applicable

At very low pH values the keto acid would not be ionised and thus would not chelate the cation so less PO_4^{3-} would be released.

Table 11 shows how, in the third experiment, various substances affect the solubility of phosphate. The phosphate was soluble under acid conditions, as shown by the clearing in the precipitate, but insoluble at higher pH values. The keto acids had a low enough pH value to solubilize the phosphate without the presence of any other acid. With the sulphuric acid and buffers the clearing disappeared upon the addition of alkali indicating that the reaction was reversible, depending upon the acidity:

$$\underset{\text{(alkali, insoluble)}}{H_2O + CaHPO_4} \longrightarrow \underset{\text{(acid, soluble)}}{Ca^{++} + H^{+} = HPO_4^{=} + OH^{-}}$$

The addition of acid or alkali change the equilibrium of the solution thus rendering the phosphate more or less soluble. However the clearing brought about by the keto acids was a non reversible reaction, that is it did not disappear upon the addition of a strong alkali. This indicated that the cation (Ca^{++}) has associated with the keto acid to form a chelate. The calcium would therefore be unavailable to reassociate with the phosphate which would remain in a soluble ionised form even after the addition of an alkali.

The culture supernatant obtained by direct centrifugation gave a small amount of clearing; the precipitate became slightly less opaque but not clear. However after concentrating the supernatant by freeze drying a clear zone was observed. This clearing did not disappear upon the addition of alkali. These results indicated that the phosphate solubilization effect was not entirely due to the low pH of the culture supernatant and also that the substance which brought about the solubilization was in the supernatant. The results confirmed that the phosphate was being solubilized by some product which had diffused from the bacterial cell. A probable mechanism is that the phosphate was initially solubilized by the low pH of the culture and then the cation was chelated by the keto acids secreted by the cells. Thus previous theories of either a pH effect or a chelation effect (e.g. Sperber, 1958; Harrison et al, 1972) were both partially correct but previous workers did not recognise the significance of both mechanisms

TABLE 12 Confirmatory tests for the identification of the byproduct released during glucose metabolism by the phosphate solubilizing bacteria

Test	Mobility relative to glucose			
	2 keto deoxygluconic acid	Byproduct from glucose metabolism	5 keto deoxygluconic acid	5 keto deoxygluconic acid
	(Ca salt)		(K salt)	(Ca salt)
Electrophoresis pyridine /acetic acid/H_2O (10 : 4 : 86) pH 5.3	1.07	1.07	-	-
Chromatography Butanol/acetic acid/H_2O (4 : 1 : 5)	0.3	0.3	0.67	1.30
Butanol/pyridine/H_2O (6 : 4 : 3)	0.15	0.15	0.20	0.11
Ethylacetate/acetic acid/formic acid/H_2O (18 : 3 : 1 : 4)	1.04	1.04	1.47	1.47

Test	2 keto deoxygluconic acid	Byproduct from glucose metabolism
Dubois phenol sulphuric acid test	negative	negative
anthrone test	negative	negative

4-7 Confirmatory Tests for the Identification of the Byproduct Released During Glucose Metabolism by the Phosphate Solubilizing Bacteria (from Dr. I.W. Sutherland, Microbiology Department Edinburgh)

The results obtained previously (4-5) indicated that the byproduct responsible for phosphate solubilization was a keto acid, probably 2 ketogluconic acid. This was deduced from the results of chromatograms produced using one solvent only and confirmatory tests were needed to draw definite conclusions.

Methods The byproduct released by the phosphate solubilizing bacteria during glucose metabolism was isolated and purified using electrophoresis. Various tests (Table 12) were then carried out to find the properties of the byproduct relative to those of 2 ketodeoxygluconic acid and 5 ketodeoxygluconic acid.

Results and Conclusions The results shown in Table 12 indicated that the byproduct from glucose metabolism behaved in a similar manner to 2 ketodeoxygluconic acid in all tests. 5 ketodeoxygluconic acid give entirely different results. Thus it was concluded that the byproduct released by the phosphate solubilizing bacteria during glucose metabolism was very probably 2 keto deoxygluconic acid.

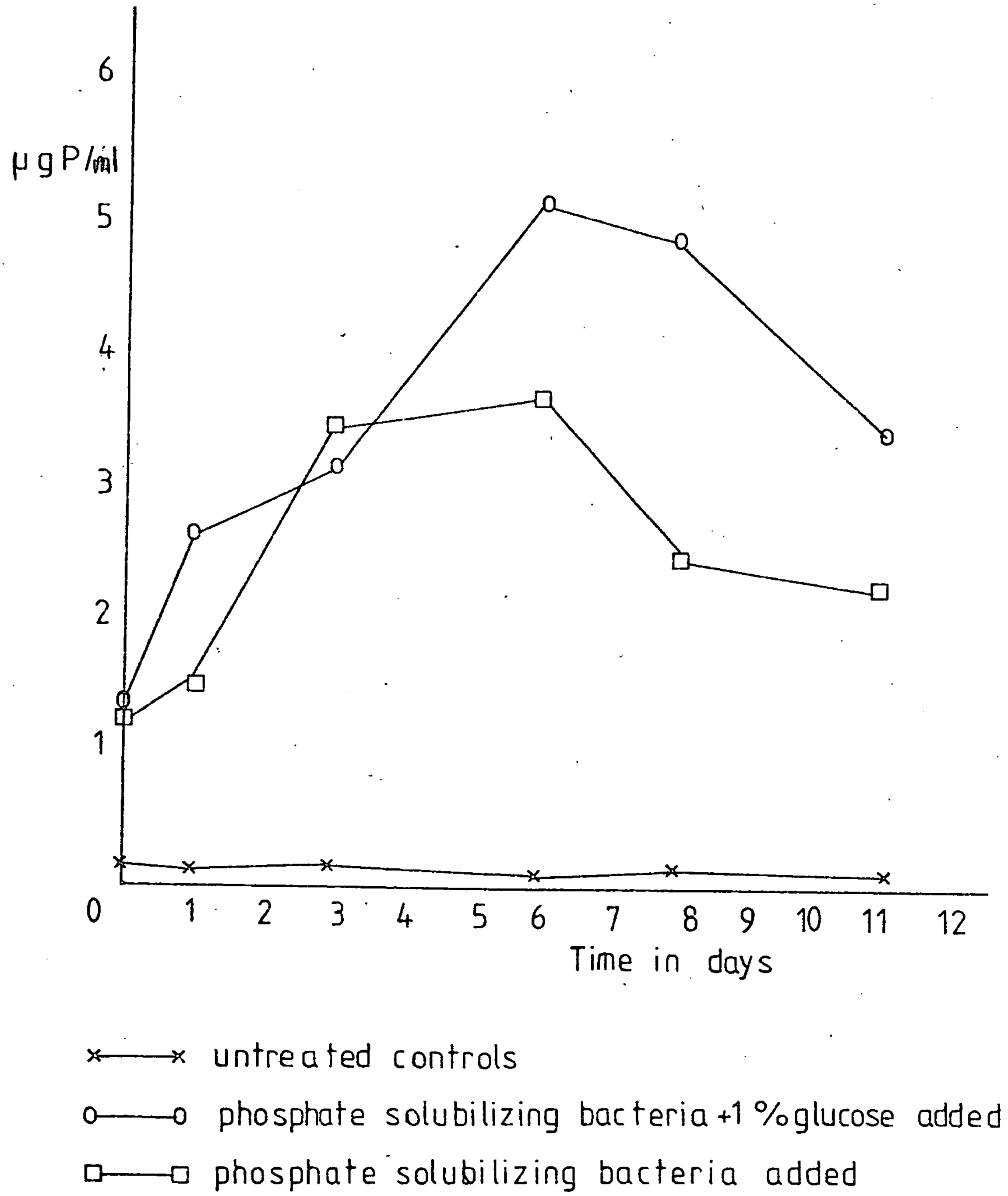
4-8 Addition of Phosphate Solubilizing Bacteria to Mini Cores

The work described previously in this chapter has dealt with the characteristics of phosphate solubilizing bacteria and some of the underlying mechanisms of phosphate solubilization. However, these organisms were originally isolated from lake sediment and their possible importance in the natural environment required to be assessed. In lakes which are rarely stratified, such as Loch Leven, these organisms may be important in releasing phosphates into the water from the sediment. Field estimates of their activity would be extremely difficult. A major problem would be separating phosphate solubilizing processes from other mechanisms of phosphate release. A highly artificial system was therefore selected and an attempt was made to relate the results to the natural situation. By adding the bacteria to small sediment/water cores a measure of their potential for phosphate release may be obtained.

Method 12 Jenkins cores were collected from the North deep on October 19th 1977. These were dispensed into mini cores as previously described and allowed to equilibrate for 4 weeks at 20°C.

A culture of phosphate solubilisers was obtained by innoculating nutrient broth with the Bacillus sp. and incubating at 37°C for 2 days in a shaker. The cultures were then centrifuged at 6000 rpm for 30 minutes, the

Fig. 22. Phosphate release in mini cores after the addition of phosphate solubilizing bacteria.



Data mean of 3 cores

The cores were incubated at 20°C in the dark supernatant was discarded and replaced with tris buffer. This washing process was repeated three times. The bacteria were finally suspended in a small quantity of tris to give a thick suspension of bacteria.

10 ml of the bacterial suspension were added to 5 cores. A further 5 cores had 10 ml of bacterial suspension added and glucose was also added to give a final concentration of 1% in the water phase. The cores were allowed to stand for three hours to allow the suspension to settle to a certain extent. A water sample was then taken from each core and frozen. Counts of phosphate solubilizing bacteria and total viable counts were carried out on the sediment surface of 2 cores from each treatment. The remaining cores were aerated in the dark at 20°C for 12 days. Water samples were taken every other day, frozen and later analysed for orthophosphate. After 12 days incubation the bacteria on the sediment surface were again counted.

<u>Results and Discussion</u> The results (Fig. 22) showed that the addition of phosphate solubilizing bacteria to the cores made a considerable difference to the amount of phosphate in the water phase. The concentration of phosphate in the control cores remained constant throughout the experiment. The orthophosphate concentration in the water of the treated cores initially rose and then after 6 days started to fall again. The results suggest that the high rate of solubilization in the first few days declines to a point at which absorbtion is greater than

TABLE 13 Counts of Bacteria in Mini Cores (no./ml wet sediment) and Amount of Phosphate Released per Organism per Day

	Control	PO_4^{3-} solubilisers added	PO_4^{3-} solubilisers + glucose added
START			
PO_4^{3-} solubilisers	3.2×10^4	2.8×10^5	2.8×10^5
total count	2.0×10^7	3.8×10^7	5.6×10^7
FINISH			
PO_4^{3-} solubilisers	4.0×10^4	7.0×10^5	2.9×10^5
total count	5.0×10^7	1.0×10^8	9.2×10^7

From the results (Fig. 22) P released per day

	µgP/1
Cores with PO_4^{3-} solubilisers added	0.4
Cores with PO_4^{3-} solubilisers + glucose added	0.6

Area of surface of core = 15.9 cm^2

Number of PO_4^{3-} solubilising bacteria in top centimetre

Cores with PO_4^{3-} solubilisers added	4.45×10^6 bacteria per core
Cores with PO_4^{3-} solubilisers + glucose added	4.6×10^6 bacteria per core

Amount of phosphate released per organism per day

Cores with PO_4^{3-} solubilisers added $\frac{0.4}{4.45 \times 10^6}$

$= 9.0 \times 10^8$ ugP/1/day

Cores with PO_4^{3-} solubilisers + glucose added $\frac{0.6}{4.6 \times 10^6}$

$= 1.3 \times 10^7$ ugP/1/day

solubilization. As has already been shown (Chapter 3) phosphate is usually adsorbed by the sediment under aerobic conditions until an equilibrium value of 1.5 µgP/ml is reached so that after the bacteria had stopped releasing excess phosphate a reduction to the equilibrium level would be expected. The amount of phosphate released by the original population would already be in equilibrium; a fact that explains why no phosphate release was detected in the control cores. However the additional bacteria did contribute significant quantities of phosphate to the water. Calculations based on the number of bacteria present in the cores at the end of the experiment and the amount of phosphate released in the cores per day show that the bacteria could play a major role in releasing phosphate from the sediments (Table 13). The cores remained aerobic throughout the experiment as indicated by the light brown colour of the sediment so the release of phosphate did not appear to be due to the establishment of reducing conditions.

The addition of glucose to the core water enhanced the release of phosphate over and above that released by the addition of bacteria without an extra energy source. These results agreed with Harrison, et al (1972) who showed in sediment supplementation experiments that the addition of glucose to sediments containing phosphate solubilizing bacteria enhanced the release of soluble phosphate. The original sediment probably only contained small quantities of easily assimilable sugars and the addition of glucose meant that the conditions in the cores were more similar to those of the pure cultures in which the bacteria had been grown.

The bacteria had a high survival rate when added to the cores and actually increased in number during the 12 days of the experiment. This means that the environmental conditions in the core were suitable for growth and survival over the period of the experiment.

4-9 General Discussion

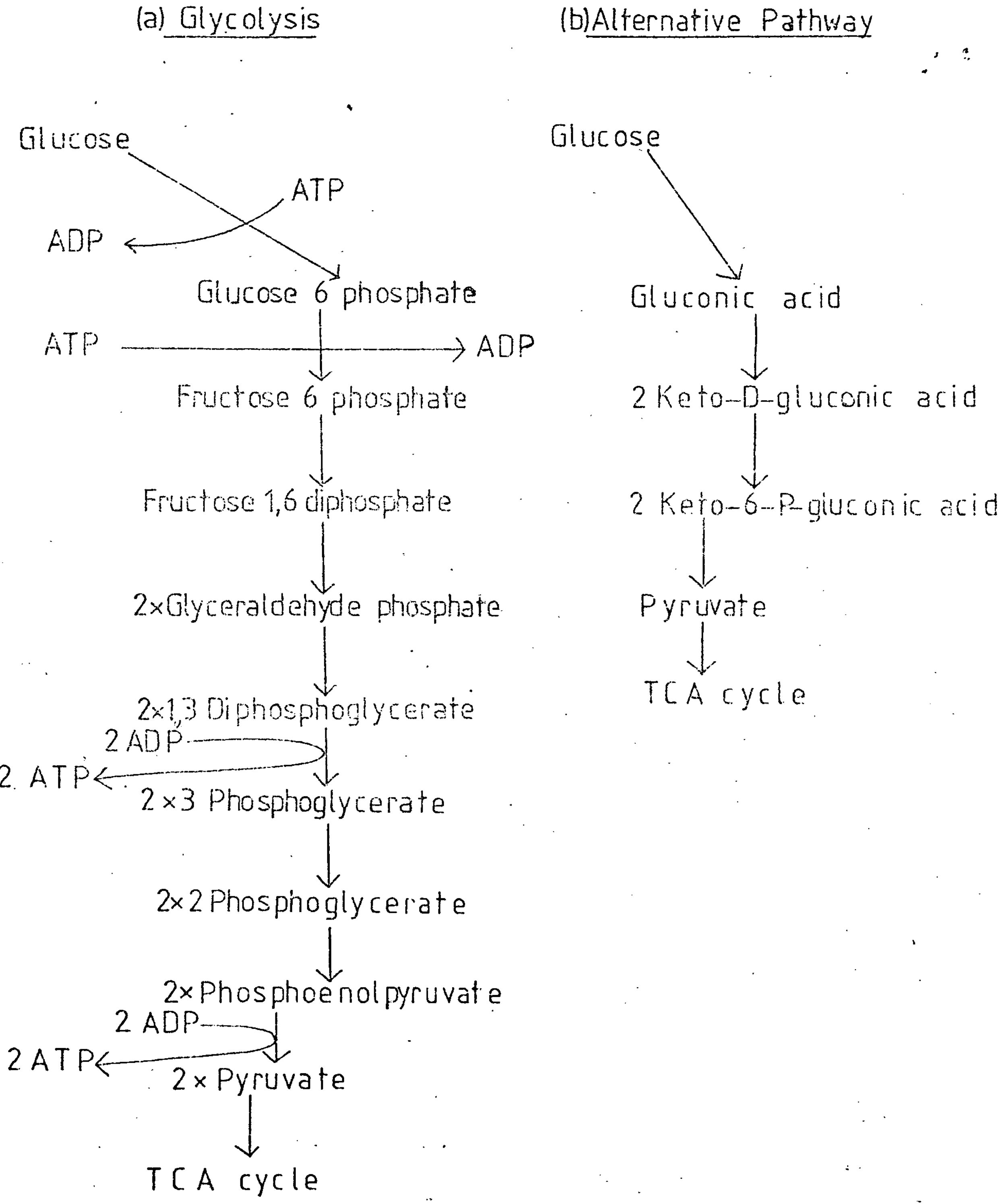
Bacteria capable of solubilizing insoluble inorganic phosphates were isolated from Loch Leven and were found to be a small but significant proportion of the total population. The use of plates containing a precipitate of phosphate was found to be useful and reliable for isolating the organisms but a more accurate method of ennumerating these bacteria using a most probable number technique looking at clearing down tubes was devised. This most probable number method was easy to use and gave reproduceable results, unlike plate counts, and is extremely useful for counting phosphate solubilizing bacteria. The principal species isolated were *Pseudomonas putida* and *Bacillus carotarum*, the latter being the most active in solubilizing phosphate. The restricted number of species in Loch Leven which exhibited the phosphate solubilizing property was unexpected because other workers, both in soil (Sperber, 1958) and in aquatic environments (Harrison et al, 1972), have found a wide range of bacteria with this property. The numbers of these bacteria were similar in all the sediment types, except the non macrophytic sand which has a lower count, despite the differing organic carbon content of each area. The high count in the macrophytic sandy area was thought to be due to a rhizosphere effect.

A certain minimum concentration of glucose was necessary for solubilization to take place. Below this concentration, although the organisms were still capable of growing, it is probable that insufficient keto acid was produced to leach from the cells thus no phosphate solubilization could be detected. However, although the natural environment as a whole may have a low sugar concentration, in the microenvironments surrounding the cells there may be a high enough sugar concentration for solubilization to proceed. A study of the sugar concentration in the sediments of Loch Leven would need to be carried out to gain an insight into the potential activity of these bacteria 'in situ'. However the organisms could utilise a wide range of naturally occurring sugars to give solubilization although the best effects were observed with mono rather than disaccharides. Both the species were nutritionally versatile growing on all the sugars tested as well as on other substances such as starch, peptone and 2 ketogluconic acid. Their ability to solubilise phosphate on substances other than carbohydrates was not tested. A study of this could prove interesting as the wider the range of substances on which they exhibit the property the more likelihood there is that they will be active in the loch. Nutritional adaptability is a good survival mechanism in the natural environment where the supply of nutrients could be extremely variable.

A number of naturally occurring inorganic phosphates were solubilized by the bacteria including complex mineral phosphates. The effect upon these complex forms of phosphate which can occur in the loch was deemed to be important. The degree of solubilization depended upon the solubility constant of the phosphate at a given acidity level. This result was explained by the fact that the solubilization was brought about by a combination of acidity and chelation. The initial solubilization took place due to a lowering of the pH which meant that the most soluble phosphates would be affected to the greatest extent. The cation was then chelated which left the phosphate anion in a permanently dissolved state. This work did not ascertain whether the bacteria released two separate acids, one which lowered the pH as well as the keto acid or whether the keto acid fulfilled both roles, that is initial solubiliser and chelating agent.

The results of adding phosphate solubilizing bacteria to the mini cores were interesting because they indicated that the bacteria had the potential of releasing large quantities of phosphate to the loch water. This could be most important in shallow lakes, like Loch Leven, where little phosphate is released under anaerobic conditions and therefore the major contribution of phosphate to the water from the sediments would have to be under aerobic conditions. Free sugars were probably quite scarce in the sediment although no data is available for Loch Leven. However the effect of the additional glucose indicated that free sugars probably were scarce in the loch but even without an extra energy source significant quantities of phosphate were released. There would probably be more sugar in the loch than in the cores because these had been incubated for 4 weeks before use. In the natural situation there would be a continual influx of sugars to the sediment from 'crashed' algal blooms and decaying terrestrial

Fig.23. Comparison of the Glycolytic Pathway and the Pathway Utilised by the Phosphate Solubilizing Bacteria for Glucose Metabolism.



(a) Glycolysis

(b) Alternative Pathway

vegetation as well as exudates from macrophytes and algae. Therefore a constant supply of sugars will be available for a small amount of solubilization to take place. The results from the cores indicated that the bacteria were capable of solubilizing phosphates under what would be nutrient poor conditions and this is of importance in the loch situation. The byproduct produced by the phosphate solubilizing bacteria during glucose metabolism was found to be 2 keto deoxygluconic acid. This substance was thought to be responsible for the solubilization of the inorganic phosphates. If these keto acids are being released from the bacterial cells it means that the organisms are using a very unusual pathway for sugar metabolism which could explain why the phosphate solubilizing property was restricted to a few types of bacteria. A typical pathway for glucose oxidation is the glycolytic pathway (Fig. 23a) which ultimately leads to the Krebb's cycle via pyruvate and acetyl co A. This is, energetically speaking, an efficient pathway which produces two molecules of ATP before it reaches the tricarboxylic acid cycle. The pathway which produces 2 ketogluconic acid (Fig. 23b) is an unusual one and is less energetically efficient than glycolysis. The bacteria may utilise the more unusual pathway because they lack some enzyme on the glycolytic pathway. However no data is available on this aspect. An alternative explanation could be that the secretion of keto acids with the resultant phosphate solubilization is a survival mechanism helping the bacteria to compete successfully with other organisms under adverse conditions but there appears to be no relationship between chelate production and phosphate deficiency. If the bacteria were found mainly in the water phase this could be possible as phosphate concentrations are frequently extremely low and if they were adhered to a piece of particulate phosphate the phosphate solubilizing bacteria would have an advantage over non phosphate solubilizing bacteria. However the densest populations of phosphate solubilisers are found in the sediment, where phosphate is rarely limiting and as each bacterial cell only requires 3 x 10^{-15}g of phosphorus (Luria, 1960) it would not be necessary to solubilize additional phosphate. A biochemical study of the bacteria would be necessary to clarify the situation as to why the organisms utilise this unusual pathway.

These organisms are unusual in that large quantities of acid are produced under aerobic conditions. Many anaerobic bacteria release acids from the cells during a wide range of fermentations (Stanier et al, 1970) but very few aerobic organisms possess this characteristic. The property has been intensively investigated in the acetic acid bacteria. The phosphate solubilizing bacteria can perhaps be compared with the acetic acid bacteria in that they are strict aerobes, can continue to grow under acid conditions and secrete large quantities of acid from the bacterial cell. Some of the acetic acid bacteria e.g. Gluconobacter sp. lack a functional tricarboxylic acid cycle whereas it is possible that the phosphate solubilisers lack an enzyme on the glycolytic pathway.

In fact studies on the metabolism of L - sorbose to 2 keto - L - gulonic acid have shown *Gluconobacter sp.* (an acetic acid bacterium) and *Pseudomonas putida* (the phosphate solubilizing organism) to be very similar in their activities (Kitamura & Perlman, 1975; Makover et al, 1975; Martin & Perlman, 1976). An examination of the ability of acetic acid bacteria to solubilise phosphate when grown on a carbohydrate source would be of interest.

TABLE 3.4 Estimate of Phosphate Release from Loch Leven Sediments

	Loch	Deep area	Shallow area	Core
Area in m^2	5.2×10^7	2.45×10^5	4.7×10^7	3.84×10^{-3}
Stratified days	–	60	0	–

Phosphate Release under Oxidised Conditions

	Amount per day in core ugP	Amount per day in Loch ugP	Amount per year in loch KgP
Shallow area	0.0125	1.52×10^8	59.0
Deep area	0.107	6.82×10^6	2.28

Phosphate Release under Anaerobic Conditions

	Amount per day in core ugP	Amount per day in Loch ugP	Amount per year in loch KgP
Shallow area	–	–	–
Deep area	0.35	2.19×10^7	1.31

Release by Phosphate Solubilising Bacteria

Volume of sediment to be considered = 5.2×10^5 m^3

There are 3×10^6 phosphate solubilisers per m^3 fresh sediment. Therefore in the loch there are 1.56×10^{12} phosphate solubilisers.

Each organism is capable of releasing 5.7×10^6 mgP day $^{-1}$

Therefore total release over 8 months = 2.16×10^3 kgP

Total annual contribution of phosphorus by sediment to the water = $59.0 + 2.28 + 1.31 + 2.16 \times 10^3$ kg

= 2.22×10^3 kgP

CHAPTER 5 GENERAL DISCUSSION

5-1 Quantitative Aspects of Phosphate Release in Loch Leven

Calculations were made to assess approximately the quantity of phosphate which might be released into the water phase from the sediments. These calculations must be treated with caution because the core results were obtained under highly artificial conditions.

Table 14 shows the criteria considered when the calculations for phosphate release from the sediment were carried out. The total area of the loch and the areas of the deep and shallow sediment were calculated (Calvert, 1973). The shallow area was assumed to stratify only rarely and the deep areas for approximately two months every year. These assumptions were based on the results obtained on the redox potential of the sediments. The rate of phosphate release in the dark was used in the calculation for aerobic release of phosphate. The amount of phosphate that each sediment type could release under aerobic and anaerobic conditions was then calculated. Calculations as to the amount of phosphate which phosphate solubilizing bacteria in the loch could release per annum were made. These latter calculations were based on the data obtained from adding these bacteria to mini cores. The calculation only gives an extremely approximate value because it is assuming that the bacteria are as active in the loch as in the cores and only takes into account the top centimetre of the sediment. However the top centimetre is probably the layer of greatest activity (Henrici & McCoy, 1938). Activity was assumed over 8 months of the year, the temperature being 4°C or below during the 4 winter months.

The results (Table 14) show that the total amount of phosphate being released from the deep sediments under both reducing and oxidising conditions is negligable when compared with the amount released from the large area of sediments beneath shallow water under oxidised conditions. The phosphate solubilising bacteria had an enormous potential for phosphate release, 2.16 x 10^3 kgP/yr. The effect of the phosphate solubilising bacteria could only be measured by adding additional organisms because the phosphate leached from the sediments of untreated samples would include that solubilized by the indigenous population. The amount of phosphate released under aerobic conditions from the deep and shallow sediments was probably an underestimate. In the loch the phosphate would be constantly removed from the sediment surface by currents and winds whereas in the cores it accumulates, slowing down the rate of diffusion of further phosphate from the sediment. Conversely the phosphate solubilizing bacterial activity in the loch is probably less than in the cores but even if this were taken into consideration the organisms could still release considerable quantities of phosphate into the loch annually.

Annual Budgets The total net annual input to the loch during 1976 was 832 kg P (Harriman, 1976). Taking into account the total release from the sediments, this input from external sources is only about 33% of the input from internal sources. When the input of phosphate from external sources has been related to the phosphate content of algal blooms there has frequently been a deficit which has been estimated to be as much as 2000 kg P/yr (Holden & Caines, 1974). The apparent deficit can be explained by the figures obtained in Table 14, a greater amount of phosphate than 2000 kg P/yr could, potentially, be released from the sediments. The release was originally thought to be due to the sediments becoming reduced (Holden & Caines, 1974) but the results have shown that the release of phosphate is due to the solubilization of sediment inorganic phosphates under oxidised conditions.

5-2 In Site Sampling and Interdisciplinary Studies

Although the Jenkins core is ideal for experimenting with small volumes of sediment and water it has many disadvantages. The samples collected were assumed to be relatively undisturbed but frequently the sediment surface is disturbed during sampling. This is verified by the fact that the phosphate concentrations in the core water immediately after sampling are usually higher than in the loch water (0.3 µgP/ml c.f. 0.1 µgP/ml). These values fall after standing overnight. There are also other disadvantages in using Jenkins cores. The polypropylene wall of the core can adsorb chemicals preventing their detection in the water. Bacteria will probably multiply on the core walls (Zobell effect) and in the small volume of the core this factor may enhance their activity compared with the larger water column in the loch. The water in the core is static and as substances diffuse from the sediments the concentration rises in the water with the result that an equilibrium situation develops and the leaching rate may be reduced. Thus a smaller amount may be released from the sediment of the cores than in the natural situation where water movement would continually remove phosphate from the close proximity of the sediment thus preventing an equilibrium situation arising. Results obtained in the cores in the first few days after sampling are therefore potentially more important than longer term results.

Better methods for studies of this type would be the use of larger volumes of sediment and water such as in large tanks. The ideal situation would be to use 'in situ' measurements. The use of enclosures has proved to be most successful in the Lake District in Esthwaite Water (Lund, 1973) and also in Loch Leven. In this method large volumes of the lake are enclosed in plastic tubes and the changes taking place in various parameters (algal populations, chemical concentrations etc.) can be monitored. Field treatments, for example, the addition of chemicals or the exclusion of sediment effects etc., have proved very useful in these tubes giving a more reliable assessment of the actual field situation than small cores. However there will still be significant effects from the sides of the tubes but the method is more suitable than removing samples from the lake because the experiments take place in a relatively undisturbed system.

The value of interdisciplinary studies has been demonstrated in this investigation. A large quantity of information was readily available about the chemical, physical and biological changes taking place in the loch, all of which helped to answer some of the questions which arose during the work on the biological processes associated with phosphate release.

5-3 Applications of this Work in Relation to Agriculture and Eutrophication

As phosphorus is an essential nutrient for plants, including algae, work involving the study of phosphorus mobility in lakes should have implications for agriculture and water eutrophication.

The phosphate solubilizing bacteria have been found to be most successful in releasing insoluble phosphate, even when cultured organisms were added to cores and therefore approaching the natural situation. The question could therefore be asked whether the addition of these bacteria to soils, possibly via seed incocculation, would help in the release of phosphate to crops, rather than adding phosphate fertilisers. This method has been tried in Russia although the success of the trials was questionable (Cooper, 1959). However other workers have shown that the type of vegetation (Katznelson & Bose, 1959; Louw, 1970) affects the number of these bacteria in soils and in future it might be possible to increase the root exudates so that these organisms can be more effective in phosphate solubilization.

Phosphate is widely known to be one of the major causes of eutrophication in lakes. Many attempts have been made to control algal production by reducing the phosphate input to the water from external sources (Shapiro, 1969; Hudson & Marson, 1970). The removal of phosphorus from sewage, using a simple method involving precipitation, has given reasonable results in the Lake of Zürich. In this lake there is now a higher oxygen concentration, improved light penetration and decreased phosphate concentrations in the water (Thomas, 1975). Sewage has been diverted from Lake Södra Bergundasjon in Sweden and it is hoped that the lake will become less eutrophic although the sediments still contribute large quantities of phosphorus to the water every year (Bengtsson, 1975). However the work described in this thesis shows that the major input to Loch Leven is from the sediments, therefore controlling external sources only goes a small way to alleviating the problem.

There have been numerous suggestions for controlling phosphate release from the sediments. Some of these methods have been tried with success such as the dredging out of sediment which would otherwise continue releasing nutrients after the external sources have been eliminated (Vollenweider, 1968) or by aeration of the anaerobic hypolimnion (Bengtsson, 1975b) although this latter method would not be applicable in Loch Leven as most of the release takes place under aerobic conditions. In small lakes polythene sheeting has been laid over the sediment (Hynes, [illegible] 1970) to prevent phosphate mobility but this method would not be feasible for larger areas.

A preferable longer term method, avoiding undue disturbance to the lake or excessive expenditure, is to allow the lake to purify itself. All major external point inputs of phosphate should be controlled which is a feasible proposition. This would mean that any algae growing in the lake would be utilising the phosphate which has been shown to leach continuously from the sediment. Many of these algae would leave the lake in the outflow, thus removing the phosphorus from the system. The sediments would gradually become depleted of phosphate which had accumulated over the years. This would take many years but eventually the lake should become less eutrophic with little phosphate entering from external sources or leaching from the sediments.

SUMMARY

Phosphate availability is a major factor controlling algal productivity in Loch Leven, a shallow eutrophic freshwater lake. Experimental work was designed to investigate four main aspects of the influence of micro-organisms on the phosphate cycle in the loch.

1. Phosphate Flux Between the Sediments and Water Phase

The following experiments were carried out on sediment-water interface samples in Jenkins cores.

(a) Sediments from the deep areas (8% organic carbon) released 9 times more phosphate (2.8 μgP/ml) after 10 days than shallow area sediments (4% organic carbon) when held anaerobically at 16°C.

(b) Redox potential measurements showed that the Eh of the deep sediment fell more rapidly (to -175 mV) than that of the shallow sediment (-60 mV). Poising of the Eh with nitrate, sulphate or thiosulphate produced evidence that phosphate was released between the nitrate (100 mV) and sulphate (-100 mV) levels. Nitrate concentration in the water phase influenced phosphate release from the sediments. These data indicated that the release of phosphate was due to the reduction of ferric to ferrous ions.

(c) Under aerated conditions cores from both sediment types reached an equilibrium of 1.5 μgP/ml after 24 days at 20°C, excess phosphate being assimilated into the sediment.

(d) Temperature studies showed that at 20°C, 4.3 μgP/ml was released after 10 days from the deep sediment whilst

at 4°C, 4 times less was released.

(e) Gas production, both N_2 and CH_4, in the sediments was found to enhance phosphate release.

(f) The only experiment in the light showed that algal uptake of phosphate was an important factor in keeping the phosphate concentration in the water below the equilibrium value of 1.5 μgP/ml. In the dark this level was maintained by the release and adsorption of phosphate by the sediments.

2. Role of Microorganisms in Bringing about Phosphate Release Treatment of cores with formaldehyde (5%) completely inhibited any fall in redox potential which indicated that biological activity and not chemical processes was the major factor in reducing the Eh. Release of phosphate was a purely chemical and physical phenomenon, dependent on the Eh of the system.

3. Inorganic Phosphate Solubilization by Bacteria Under Laboratory Conditions

(a) In addition to the solubilization of insoluble phosphate on agar plates an alternative method of counting phosphate solubilizing bacteria was developed using a most probable number technique involving clearing down test tubes containing agar media.

(b) The phosphate solubilizing bacteria occurred in all the sediments at between 1.0 and 0.06% of the total colony forming population. Numbers varied between 5×10^4 organisms/ml wet sediment (8% organic carbon area) and 3×10^3 organisms/ml wet sediment (0.05% organic carbon). In sediments with similar organic carbon contents, greater numbers were found near Potomogeton (pond weed) growth.

(c) The most numerous bacteria solubilizing inorganic phosphate were classified as Bacillus carotarum and Pseudomonas putida.

(d) The Bacillus sp. were more effective solubilizing organisms than the Pseudomonas sp.

(e) The bacteria could solubilize various phosphates to differing extents with Mg > Ca > gafsa and a mixed mineral phosphate. Iron and aluminium phosphates were unaffected by the bacteria.

(f) Energy source was found to affect the amount of solubilization obtained. Glucose and xylose > lactose > maltose, mannose, fructose and sucrose.

(g) The solubilization was due to the release of acidic substances which initially dissolved the phosphate by a chelating process.

(h) The chelating substance was most probably 2 keto deoxygluconic acid when the organisms were grown on glucose.

4. The Importance of these Processes in the Loch If the results from core experiments are related to the overall situation in the loch then 3.59 kg phosphorus will be released from the deep areas and 59.0 kg phosphorus from the shallow areas per annum. Phosphate solubilizing bacteria will contribute 2.16 x 10^3 kg phosphorus per annum. Thus the average annual input of phosphorus from external sources during the years 1974-78 was only 33% of that released from the sediments.

APPENDIX 2

Phosphate released in liquid medium by the Pseudomonas sp. incubated at 37°C for 2 days

Sugar	ppm P released by 10^6 cells
glucose	2.0×10^{-3}
fructose	3.2×10^{-4}
lactose	4.1×10^{-4}
sucrose	8.6×10^{-4}
maltose	1.7×10^{-5}
xylose	9.0×10^{-4}
mannose	9.2×10^{-5}
arabinose	3.1×10^{-4}

Data mean of 5 flasks

APPENDIX 1

Monthly depth profiles for the redox potential of cores taken from Loch Leven North Deep during 1977

Depth cms	J	F	M	A	M	J	Jl	A	S	O	N	D
0	+190	+180	+180	+100	-70	-145	-60	-90	-10	+60	+120	+185
1	+190	+180	+170	+120	-90	-180	-160	-170	-40	0	+120	+190
2	+180	+180	+180	+150	-200	-190	-165	-165	-90	-10	+100	+180
3	+190	+190	+175	+140	-210	-207	-190	-180	-95	0	+80	+185
4	+190	+190	+185	+130	-200	-205	-195	-215	-145	-5	+75	+185
5	+180	+180	+180	+130	-190	-210	-205	-200	-140	+5	+75	+185
6	+180	+180	+170	+135	-185	-217	-210	-210	-160	+10	+80	+185
7	+180	+180	+170	+140	-190	-220	-210	-210	-180	0	+90	+185
8	+175	+180	+170	+130	-190	-225	-210	-220	-185	-10	+90	+180
9	+180	+170	+175	+120	-190	-225	-210	-225	-180	-25	+95	+170
10	+180	+170	+170	+90	-210	-230	-210	-225	-180	-20	+85	+170
11	+180	+180	+170	+95	-210	-235	-210	-230	-175	-30	+90	+170
12	+175	+180	+175	+85	-210	-237	-210	-235	-180	-35	+90	+175
13	+170	+180	+170	+80	-210	-235	-210	-235	-185	-35	+90	+170
14	+170	+180	+170	+85	-200	-235	-215	-235	-190	-35	+90	+170
15	+170	+180	+170	+85	-210	-237	-215	-235	-200	-35	+90	+170

Data mean of 2 cores.

Eh measured in mV.

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