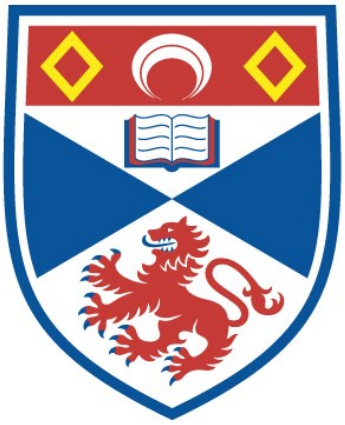
WASTEWATER NUTRIENT REMOVAL BY MARINE MICROALGAE

Rupert Justin Craggs

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Wastewater Nutrient Removal by Marine Microalgae (B)

by

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Submitted for the Degree of Doctor of Philosophy
at the University of St. Andrews

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February 1994

Declaration

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Abstract

Although, microalgal wastewater treatment systems represent an efficient and economic alternative to conventional processes, the use of marine microalgae to remove nutrients from wastewaters has not been extensively researched and few studies have been made in temperate and high latitude areas, where climate may limit treatment. In this study, 102 marine microalgal species, including 66 local endemic isolates from St Andrews Bay, Fife, Scotland, were screened under batch and continuous culture. Species were selected for their ability to remove high concentrations of ammonium and ortho-phosphate from primary treated sewage (diluted 1:1 with sterile seawater) while remaining dominant in culture. Abiotic removal of ammonium and ortho-phosphate at high pH was found to be low from saline media, indicating that much of the nutrient removal from the seawater:wastewater mixture was by algal uptake. Many of the best-treating species grew over a wide range of temperature (10-25 °C), and their growth was not inhibited by the low salinity of the 1:1 diluted wastewater.

Seven best-treating species continuously removed >80 % ammonium and >70 % ortho-phosphate when cultured in 20 litre mini-ponds (modelled on high-rate ponds) under ambient summer conditions over two weeks. These were all endemic isolates including six bacillariophyceaen isolates (of which three were strains of *Phaeodactylum tricornutum*), and a species of the cyanophyceaen *Oscillatoria*. Two isolates (*Oscillatoria* and an unidentified bacillariophyceaen SA91B33) with adherent properties, continuously removed 100 % of both ammonium and ortho-phosphate when tested in a corrugated raceway

designed to provide a large surface area for attachment. Preliminary experiments further showed the best-treating species to be capable of removing nutrients from eel aquaculture effluent. The abilities of marine microalgal species to remove high concentrations of nutrients, remain in unialgal culture and grow over a range of environmental conditions are indicative of their potential for use in wastewater treatment systems in temperate areas.

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This Ph.D. is dedicated to my parents Jane and David Craggs

and especially to Marls

for their love, support and encouragement.

"nihil viior alga" - "there is nothing worse than algae"

Horatius, Roman Poet 30 BC

"How about sewage?"

Craggs, English Algologist 1994 AD

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Abbreviations

The following abbreviations are used throughout this thesis:

B	Bacillariophyceaen isolate
BOD	Biochemical oxygen demand
cm	centimetre(s)
^{o}C	degrees Centigrade
C	Chlorophyceaen isolate
CY	Cyanophyceaen isolate
d.w.	distilled water
d	day
et al.	et alia (and others)
E	Einstein
EE	Eel effluent isolate
E-S	Erd-Schreiber
Fig.	figure
g	gram(s)
g	force of gravity (centrifugation)
h	hour(s)
l	litre(s)
mol	moles
M	molar
m	metre(s)
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mmol	millimoles
mm	millimetre(s)
M-Q	Milli-Q water
µg	microgram(s)
µl	microlitre(s)
nm	nanometers
N	Normal
$N\text{-}NO_3^-$	nitrate-nitrogen
$N\text{-}NO_2^-$	nitrite-nitrogen
$N\text{-}NH_4^+$	ammonium-nitrogen
N-Urea	urea-nitrogen
No.	number
OD_{570}	optical density at 570 nanometers
PAR	Photosynthetically active radiation
‰	parts per thousand
%	per cent
pers. comm.	personal communication
$P\text{-}PO_4$	ortho-phosphate-phosphorous
R	Rhodophyceae
SA	St Andrews
s	second
UV	ultraviolet light
w/v	weight per volume

Chemical symbols have their conventional meaning.

Chapter One

Introduction

1.1 General Introduction

Sewage has been discharged to coastal waters since Greek and Roman times but until recently there has been little regard for the environmental consequences or health hazards of this practice (Waldichuk, 1985). Marine disposal of sewage has been a traditional, cost-effective means of wastewater treatment for many coastal communities throughout the world (Cooper & Lack, 1987; Huntington *et al.*, 1992). It relies upon a number of natural processes including dilution, dispersion, assimilation, oxidation and bacterial killing (Paul & Midmer, 1989; Gay *et al.*, 1991; Huntington *et al.*, 1992).

Since many towns and cities are located in coastal areas (Myers, 1985), increasing urbanisation and industrialisation, have resulted in larger volumes of concentrated wastewaters being discharged at sea (Agg, 1973; Gloyna, 1971; Dix, 1981). The natural processes associated with marine disposal are no longer sufficient to treat wastewaters efficiently (Harlin & Darley, 1988) and the long-term effectiveness and acceptability of this practice is now of great concern.

In many parts of the world an increasing number of coastal areas are affected by nutrient enrichment and eutrophication (Price *et al.*, 1985; Harremoës, 1987; Vollenweider *et al.*, 1992). Algal blooms that have developed in the south eastern North Sea and parts of the Adriatic and Mediterranean Seas are thought to result from increased nutrient concentrations from the marine disposal of sewage (Gay *et al.*, 1991). Many UK coastal areas also show evidence of eutrophication (Raffaelli *et al.*, 1989; Rheinallt, 1989; Clark, 1992) which has been attributed to nutrient inputs through the discharge of domestic sewage and sewage sludge.

In this introduction I will review the current state of affairs regarding coastal sewage disposal and how marine microalgal treatment systems could provide a solution to the problems of coastal eutrophication. The environmental impact of coastal discharge, the current legislation for wastewater treatment and the ability of conventional treatment methods to meet these standards will be described. Alternative treatment processes will then be outlined and the advantages of a microalgal treatment system discussed. Microalgal treatment will be reviewed, and the use of marine microalgae in coastal treatment systems considered. Finally the aims of the research in this thesis will be stated.

1.2 Marine Disposal of Wastewaters

Wastewater inputs into coastal waters and estuaries are either by direct discharge through outfall pipes or by dumping of sewage sludge at sea. Approximately half of the sewage produced in Scotland and Wales and around one quarter of that produced in England is discharged through outfalls to the sea (Cooper & Lack, 1987). This amounts to over 3.5 million m^3 of wastewater discharged every day (S. Ratcliffe pers. comm., DOE, London, 1992). Much of the sewage discharged to UK coastal waters receives little or no treatment (Table 1.1) and may have serious consequences for the quality of the receiving waters in terms of environmental pollution from particulate material, nutrients and organic matter, risk to public health by microbial flora, organic compounds and heavy metals and reduced aesthetic appearance.

Table 1.1 UK coastal outfall data (>10,000 population) modified from Gay *et al.* (1991).

Type of Treatment	Number of Outfalls
None	49
Preliminary	54
Preliminary and primary	11
Secondary	3
Total	117

a) Particulate Material

Gerlach (1981) noted that due to their low salinity and high suspended solids, wastewaters discharged at sea flocculate and precipitate, and may physically smother sediments. The combination of the high biochemical oxygen demand (BOD) waste and reduced penetration of oxygenated seawater can result in anoxic conditions in the sediments (McIntyre, 1981). The suspended solids may also clog the gills of suspension feeders, leading to localised death around the outfall. The increased turbidity may also reduce phytoplankton and macroalgal photosynthesis by reducing light penetration.

b) Nutrients

Wastewaters contain high levels of the inorganic nutrients ammonium, nitrate and ortho-phosphate (Veber *et al.*, 1984; de la Noüe *et al.*, 1992). Direct discharge of wastewater through outfalls alone contributes 1.5 million tonnes of nitrogen and 1 million tonnes of phosphorous into the North Sea every year. This has been estimated to account for 12 % of nitrate and 25 % of phosphorus in UK estuaries and coastal waters (Gay *et al.*, 1991; Clark, 1992). Since nitrogen and phosphorous are the two main limiting nutrients for phytoplankton in the marine environment this may have serious ecological effects (Dix, 1981).

The increase in the level of inorganic nutrients greatly enhances the growth of phytoplankton and macrophytes which act as a stimulus to many food chains. This in itself, is not detrimental to the marine environment, but if the input of nutrients becomes too great eutrophication may cause an alteration of the ecological balance of organisms in the water (Clark, 1992). A large increase in the phytoplankton biomass may result in fish kills, either by clogging their gills, or through the release of toxic compounds. The phytoplankton 'bloom' may be so intense that the water is discoloured.

Settlement of dead plankton from these blooms on the sea bed, together with the high levels of organic matter from the wastewater severely reduces the oxygen concentration in bottom waters due to aerobic bacterial degradation (O'Sullivan, 1971). This can be sufficient to cause death or exclusion of many benthic organisms from the area (McIntyre, 1981; Topping, 1987; Clark, 1992).

c) Organic Enrichment and Biochemical Oxygen Demand

Sewage outfalls contribute over 1 million tonnes BOD to the North Sea every year (Clark, 1992). Heterotrophic breakdown or chemical oxidation of sewage-derived inorganic and organic material can lead to depletion of oxygen in sediments and the overlying water. An increase in sediment loadings of organic material can lead to changes in the natural flora and fauna (Clark, 1992; Gay *et al.*, 1991; Mason, 1991).

d) Microbial Flora

Sewage contains a wide variety of enteric bacteria and viruses. The presence of *E. coli* and faecal streptococci in a water body are used as indicators of faecal pollution, their occurrence signifies the presence of more harmful micro-organisms (Sebastian & Nair, 1984). There are two main health risks from sewage contaminated waters. First, those using the water for recreational activities (bathing, surfing, sailing etc.) can be at risk as some water is often ingested and open cuts are a pathway for transmission. Second, there is a risk to the hygienic quality of shellfish grown in polluted inshore waters.

The ingestion of contaminated sea food is probably the major health risk resulting from sewage discharge to the marine environment (Clark, 1992). Filter feeders, especially bivalve molluscs, flourish in areas surrounding sewage outfalls but also accumulate human pathogens on their gills. More serious problems occur if algal blooms of dinoflagellate species,

such as *Gonyaulax* sp. or *Gymnodinium* sp. develop as a result of nutrient enrichment (Mann, 1982). These species produce a neurotoxin which causes paralytic shellfish poisoning in mammals (O'Sullivan, 1971; Anderson, 1989). Although total numbers of human viruses in sewage are low, there is evidence that these viruses can survive for over a year in the sediments surrounding sewage outfalls (Cooper & Lack, 1987).

e) Organic Compounds

Many complex organic compounds such as phenols, dioxins, pesticide residues and polychlorinated biphenyls enter sewers via run off from roads or by direct and indirect discharges from industry. Natural processes are often unable to degrade these compounds or brake them down very slowly (Dix, 1981; Clark, 1992; de la Noüe *et al.*, 1992). Consequently, organic compounds may be accumulated by organisms which filter large volumes of water and by their predators higher up the food chain (Norton *et al.*, 1984; Law *et al.*, 1988). Toxic effects such as carcinogenesis and mutagenesis have been demonstrated for some aquatic organisms in response to these compounds (Neff, 1979).

f) Heavy Metals

At low concentrations many heavy metals are essential to life, but in high concentrations they can be toxic. Many marine organisms (e.g. algae and fish) are known to accumulate heavy metals which could reach toxic levels (Furr *et al.*, 1981; Clark, 1992; de la Noüe *et al.*, 1992).

g) Aesthetics

There are over 1000 outfalls used in the UK and many cause adverse environmental effects and aesthetically undesirable conditions. Some outfalls are short and cause discharge of untreated sewage above the low water mark, leaving gross fouling of recreational beaches with faecal solids and other "objectionable floatables". The improper siting of outfalls can

result in sewage plumes at the sea surface, assorted floating material and large numbers of scavenging gulls (Cooper & Lack, 1987; Paul & Midmer, 1989).

1.3 Coastal Water Quality Legislation: The European Community Urban Waste Water Treatment Directive (91/271/EEC)

For the reasons discussed above, concern over the environmental effects of wastewater discharge at sea has been mounting for some time, although, until recently, there was little legislation to control discharges. In May 1991, the Council of the European Communities adopted a Directive concerning urban wastewater treatment (Commission of European Communities, 1991). This Directive which must be implemented by 31 st December 1998, will have a considerable impact on the practise of wastewater treatment and disposal in the UK. In particular:

"The disposal of sludge to surface waters by dumping from ships, by discharge from pipelines or by other means must cease. "

Discharges to coastal waters *"must receive a minimum of primary treatment, with BOD and suspended solids of the incoming wastewater reduced by at least 20 % and 50 % respectively."*

Secondary treatment will be required for discharges to estuaries and in areas of high recreational or amenity value. In "sensitive areas", such as shallow estuaries, or enclosed bays, additional treatment for the removal of nutrients will be necessary to protect the receiving waters from possible development of eutrophic conditions (Huntington *et al.*, 1992). In these areas:

"...nutrient levels of discharges must be reduced to 10 mg N l^{-1} and 1 mg P l^{-1}, which is a minimum 70-80 % reduction total N and 80 % reduction of total P."

Although this Directive will require that treatment plants are built at all outfall sites, only a few of the plants will have nutrient removal facilities. The acceptable nutrient levels of these discharges, 10 mg N l^{-1} and 1 mg P l^{-1} (714 mmol N m^{-3} and 32 mmol P m^{-3}), may still cause eutrophication in coastal waters as nitrogen is the most frequently limiting nutrient in these areas (DeBoer & Ryther, 1977; Mann, 1982; Harremoës, 1987). The new treatment plants will add to the already increasing quantities of sewage sludge produced. Since the dumping of sludge at sea is to cease by the end of 1998, either alternative methods of sludge disposal or alternative treatment methods which do not produce sludge will have to be found.

1.4 Conventional Methods of Wastewater Treatment

Conventional sewage treatment systems do little to remove inorganic nutrients and are designed primarily to remove BOD and suspended solids and to meet bacterial and viral standards of receiving waters (Paul & Midmer, 1989; Gay *et al.*, 1991). This involves the separation of the solid fraction from the liquid phase which results in sludge production (WHO, 1987). There are various stages of treatment, each providing an effluent of higher quality (Table 1.2).

Primary treatment settles out much of the organic material comprising the BOD and suspended solids, but, the effluent still has high BOD and nutrient concentrations and high levels of bacterial contamination (Gay *et al.*, 1991). The BOD and bacterial contamination may be further

Table 1.2 Conventional methods of wastewater treatment. (Modified from Oswald, 1988a & c).

Treatment Stage	Methods	Treatment Efficiency
Preliminary	Screening to remove large solids. Comminution or maceration to reduce to slurry. Grit is extracted in a constant velocity channel or grit chamber.	Little reduction in sewage concentration.
Primary	Settlement of suspended organic matter.	Removes ≤ 33 % BOD and ≤ 66 % suspended solids. Removes 30-90 % of pathogens. Poor removal of nutrients (20 % total N and ≤ 15 % total P).
Secondary	Bacterial oxidation of the organic material using activated sludge or percolating filters.	BOD, suspended solids and pathogens Reduced by 90-99 %. Nutrient removal < 30 %.
Tertiary	Inorganic nutrient removal. Chemical processes (flocculation, selective ion exchange for NH_4^+, breakdown chlorination, high pH precipitation and anoxic reduction). Physical processes (NH_3 stripping, filtration, precipitation, thermal degradation, electrodialysis and reverse osmosis). Biological processes (nitrification, denitrification).	All processes have variable efficiency of removal.
Quaternary	Removal of refractory organics, organic toxicants, herbicides and pesticides. Activated carbon absorption.	Variable efficiency of removal.
Quinary	Removal of heavy metals, organic compounds and soluble minerals (Na^+, K^+, Mg^{2+}, Ca^{2+}).	Variable efficiency of removal.

reduced by secondary aerobic processes, which require considerable mechanical energy to supply oxygen and still leave an effluent with over 70% of the inflow concentrations of inorganic nutrients (Oswald 1988a; Gay *et al.*, 1991; de la Noüe *et al.*, 1992). Nutrients can be removed by physical and chemical tertiary processes, but these have variable efficiency, depending upon the nutrient to be removed. Since tertiary treatment requires extended residence times in expensive reactors and uses special chemicals, it is usually too costly to be implemented. Residues may also be left in the effluent which can lead to secondary pollution (Waldichuk, 1985; Oswald, 1988a; Robinson *et al.*, 1989; Metcalf & Eddy, 1991). Biological tertiary treatment (Table 1.2) is extensively employed (Randall, 1990), but, nitrification is a lengthy process, requiring neutral wastewater and has variable performance due to daily fluctuations in wastewater loading (Harremoës, 1987).

Each additional step following primary settlement doubles the relative cost of treatment. Complete tertiary processes aimed at removing ammonium, nitrate and phosphate are about four times more expensive than primary treatment, and quaternary and quinary treatments are about eight and sixteen times more expensive (Oswald, 1988b). Conventional treatment methods do not recycle nutrients which are lost to receiving waters, transformed to gas or precipitated (Oswald, 1970). A further problem is also created through the disposal of large quantities of the highly polluted sludge produced by these methods. Member states of the EC currently produce over 5.5 million dry tonnes of sewage sludge per year (Bowden, 1987). In the UK, annual sludge production is over 1 million tonnes dry solids of which 24 % is disposed of at sea, 53% is disposed of to land, 16% is disposed of to landfill and 7% is incinerated (Newman & Bowden, 1989). The costs of sludge treatment and disposal in the UK are greater than £250 million per year which accounts for approximately half

the total costs of wastewater treatment (Bruce & Davis, 1989; Lowe, 1990). The use of treatment methods which do not produce sludge would therefore be of considerable economic benefit.

Conventional treatment methods appear to be unable to remove nutrients from wastewaters efficiently and cost-effectively and create further economic and environmental problems through the production and disposal of sewage sludge (Bayes *et al.*, 1989; Blakey, 1989). Accordingly, if wastewaters are to be treated economically and discharged without adversely affecting the quality of the receiving waters alternative methods of treatment must be found.

1.5 Alternative Treatment Methods

Biological processes appear to be the most versatile solution to the problems of conventional wastewater treatment (Oron *et al.*, 1979). These are passive, low-energy systems, which may recycle nutrients in the wastewater into a commercially valuable product (de la Noüe *et al.*, 1986). The two main biological processes are wetlands and microalgal ponds.

1.5.1 Wetlands

Wetland systems offer an efficient way to remove up to 90% BOD and suspended solids (Wolstenholme & Bayes, 1990; Wood, 1990). High levels of pathogens and toxic compounds may also be removed (Arthur, 1986; Zhang *et al.*, 1990). Both natural wetland systems (Boyt *et al.*, 1977; Fisher, 1990; Wood, 1990) and constructed wetlands (Horne, 1994) have been used. These include monocultures of aquatic plants (Burgoon *et al.*, 1991) such as reed beds of *Phragmities australis* (Armstrong & Armstrong,

1990), *Typha* sp. (Wood, 1990) or water hyacinth (*Eichhornia crassipes*) (Soerjani, 1987; Tripathi & Shukla, 1991). Wetlands provide an economic alternative to conventional primary and secondary treatment but, they have low and variable nutrient removal and have to be emptied every 10-20 years (Arthur, 1986; Wolstenholme & Bayes, 1990). Moreover, they produce an effluent with high nutrient concentrations, which, if discharged into coastal waters, could lead to eutrophication.

1.5.2 Microalgal Systems

It is generally recognised that microalgae play an important role in the self purification of natural waters (Soeder, 1980). Microalgae may offer an inexpensive alternative for wastewater treatment because they use solar energy to supply the oxygen needed for aerobic degradation. They also recycle the nutrients responsible for eutrophication into potentially valuable biomass (Moraine *et al.*, 1979; Belsare *et al.*, 1987; de la Noüe & De Pauw, 1988). Microalgal treatment ponds involve anaerobic bacterial processes, aerobic bacterial processes and microalgal oxygenation and assimilation. Ponds operate continuously and produce very small quantities of sludge. The suitability of microalgae for use in treating wastewater is a result of their high productivity (up to 40 g m^{-2} d^{-1}) which reflects their simplicity of organisation (single cells of small size and simple morphology), efficient absorption of sunlight (up to 5 % of total solar energy conversion), and the ready availability of water, carbon dioxide and nutrients to all cells (Chapman & Chapman, 1983; Tam & Wong, 1983; Lobban *et al.*, 1985; Terry & Raymond, 1985; de la Noüe & De Pauw, 1988; Oswald, 1994). Many phytoplankton species are also capable of scavenging nutrients at low concentrations and can quickly reduce nutrients to very low levels (Mann, 1982). Furthermore, algae can be grown in continuous culture rather than

on a crop basis, maximising the use of available space (Terry & Raymond, 1985). Therefore microalgal treatment ponds may provide a suitable alternative to conventional treatment systems as they remove both organic and inorganic components of the wastewater and do not produce large quantities of sludge for further disposal.

1.6 Microalgal Wastewater Treatment

1.6.1 Historical Development

The use of organic wastes to encourage algal growth in fish culture ponds has been practised in Europe and Asia since ancient times (Hickling, 1971). Microalgal treatment of wastewaters has been studied for more than 90 years and evolved from lagooning domestic sewage in natural basins, pools and ditches to prevent intrusion into surface water supplies (Belsare & Belsare, 1987). In the early 1900's, experimental lagoons were constructed in California, North Dakota, Texas, and elsewhere in the USA as a means of treating wastewater (Caldwell, 1946). The success of these lagoons led to the acceptance of "waste stabilisation ponds" as a sewage treatment system by the United States Public Health Service Authorities (Meyers, 1948).

Research into the role of microalgae in sewage ponds by Oswald and his co-workers at Berkeley, California, USA, began in 1949. Oswald & Gotaas (1957) developed the high-rate pond, for combined wastewater treatment and mass production of algal biomass and recognised the economic advantage of such a process (Oswald, 1963). By 1962, there were 1,647 stabilisation ponds in the USA for the treatment of municipal wastes (Porges & Mackenthun, 1963) and possibly an equal number for the treatment of industrial or agricultural wastes (Porges, 1963)

During the mid-1960's Oswald and co-workers showed that algal systems could be used effectively in bioregenerative life support systems for spacecraft and submarines as photosynthetic gas exchangers and renewable food sources (Oswald *et al.*, 1965; Shelef *et al.*, 1970). At the same time, they also demonstrated that bioconversion of solar energy to chemical energy could be performed efficiently through the culture of algal biomass on wastewaters. The algae could be fermented to methane with conversion efficiencies of 50-70 % (Golueke & Oswald, 1959). High-rate pond design has been further improved by Shelef and co-workers in Israel (reviewed in Shelef & Azov, 1987). The "intensive algal water treatment system," is superior economically and ecologically to activated sludge plants and provides high quality effluent which may be reused for unrestricted irrigation or safely discharged into receiving waters (Shelef *et al.*, 1978b; Berend *et al.*, 1980).

Microalgal wastewater treatment systems have been successfully used all over the world (Table 1.3). By 1971, ponds were used in at least 39 countries from polar regions to the equator, and found to be both economical and practical for use in small villages to large cities (Gloyna, 1971). At least a dozen microalgal sewage processing systems based on the work at Berkeley are presently in place and functioning in several California municipalities, where the climate permits the year-round operation. Two high-rate ponds, one at St Helena, and the other at Holister, have been operating effectively for over 15 years (Green *et al.*, 1994).

1.6.2 Photosynthetic Oxygenation

Oswald coined the term "photosynthetic oxygenation" to describe the intensive process of wastewater treatment by microalgae and heterotrophic

Table 1.3 The application of microalgal wastewater treatment systems around the world.

Country	Pond System	Reference
Australia	Stabilisation	Parker *et al.* (1959)
	High-rate	Dodd & Anderson (1977)
Brazil	Stabilisation	Mara & Pearson (1986)
Canada	Pond	Pouliot & de la Noüe (1985)
Chile	Pond	Ayala & Bravo (1984)
Denmark	High-rate	De Pauw & Vaerenbergh (1983)
France	Stabilisation	Racault *et al.* (1994)
Germany	High-rate	Witt *et al.* (1981)
India	High-rate	Mahadevaswamy & Venkatamaran (1986)
Ireland	High-rate	Strain *et al.* (1986)
Israel	High-rate	Shelef *et al.* (1980)
Italy	High-rate	Balloni *et al.* (1983)
Malaysia	High-rate	Phang (1990)
Mexico	High-rate	Paniagua-Michel *et al.* (1987)
New Zealand	Stabilisation	Chapman & Chapman (1983)
Philippines	Ponds	Goldman (1979)
Scotland	High-rate	Fallowfield & Garrett (1985a & b)
South Africa	High-rate	Grobbelaar *et al.* (1988)
Sweden	Ponds	Guterstam (1990)
Thailand	High-rate	Goh (1985)
USA	High-rate	Ludwig & Oswald (1952) to Green *et al.* (1994)

bacteria (Oswald *et al.*, 1953) (Figure 1.1). In this process microalgal photosynthesis produces oxygen to drive heterotrophic decomposition of organic waste to inorganic nutrients (carbon dioxide, ammonium, and water). These are incorporated into algal biomass, which may then be separated from the effluent (Richmond & Grobbelaar, 1986; Oswald, 1988a, b & c; Birch & Bachofen, 1988; Etnier & Guterstam, 1991). Thus, oxidation of wastewaters with high BOD loading may be achieved inexpensively using solar energy, and nutrients may be recycled into algal biomass in the same pond (de la Noüe *et al.*, 1986).

During periods of intense photosynthesis, when all carbon sources including carbon dioxide have been exhausted, microalgae use bicarbonate as an inorganic carbon source for photosynthesis. Removal of bicarbonate raises the pH of the pond to 10 or more. High pH alters the physiochemical environment in the pond and may increase nutrient removal through precipitation of phosphate and ammonia volatilisation (Nurdogan & Oswald, 1994). Many studies have shown high levels of nutrient removal by microalgae from wastewaters rich in nitrogen and phosphorous compounds (Shelef *et al.*, 1978a; Przytocka-Jusiak *et al.*, 1984; Rodrigues & Oliveira, 1987; de la Noüe & Eidhin, 1988; Oswald, 1988; Tam & Wong, 1989).

Microalgal wastewater treatment systems are generally more efficient than conventional systems in removing enteric bacteria and viruses (Parhad & Rao, 1976; Shelef *et al.*, 1977). The environmental conditions which are favourable for algal growth such as sunlight (Chamberlain & Mitchell, 1978; Knorr & Torrella, 1994), and conditions within the pond including: oxygen saturation, high $pH_{,}$, high temperature and high concentrations of humic substances are all unfavourable for the survival of enteric bacteria and viruses (Sebastian & Nair, 1984; Richmond, 1986a; Paniagua *et al.*, 1987;

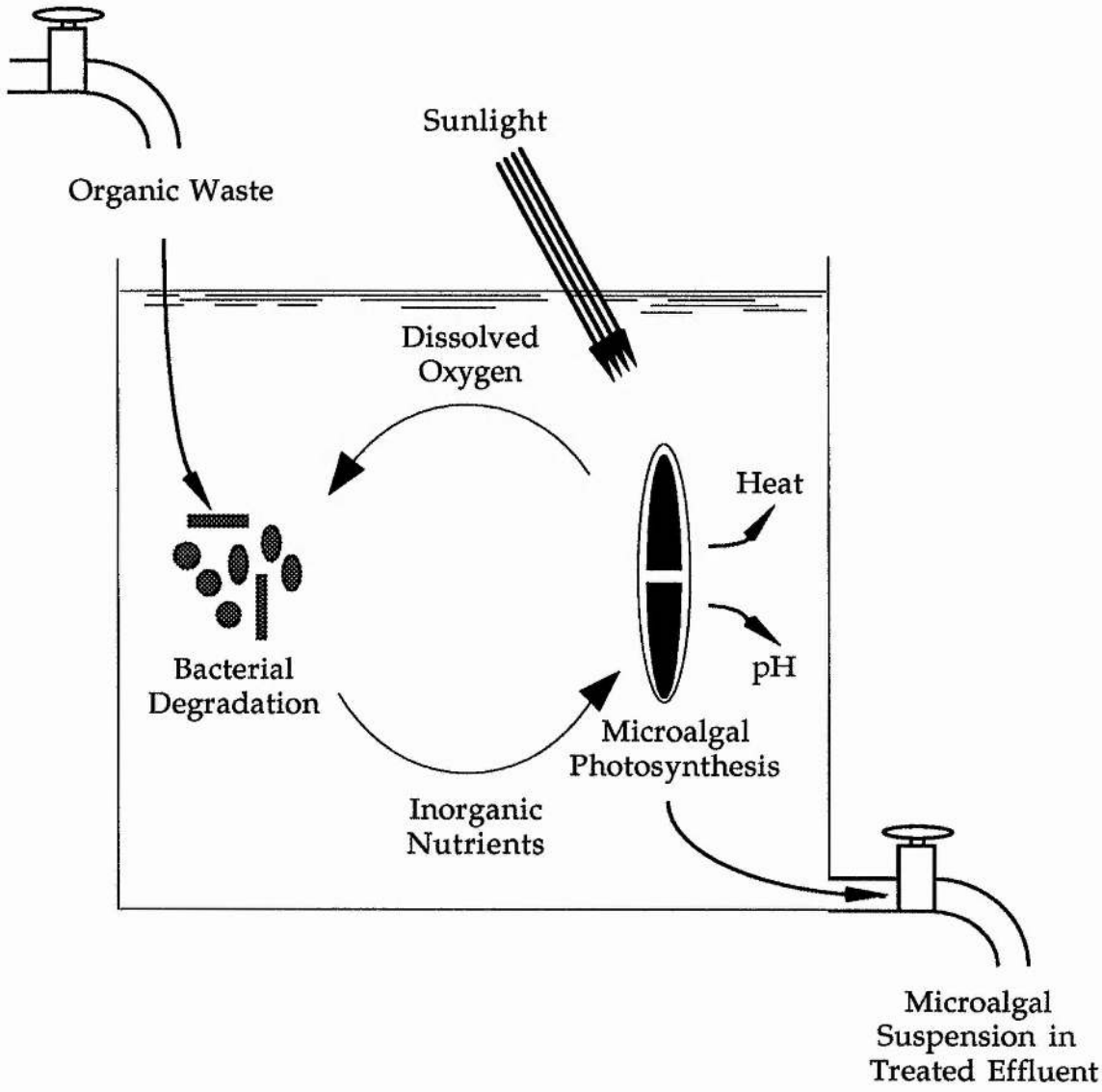

Figure 1.1 Photosynthetic oxygenation (adapted from Oswald, 1988a & c).

Pantastico, 1987; WHO, 1987; Oswald, 1988a; Tam & Wong, 1989; Curtis *et al.*, 1992). The microalgal treatment process is generally less sensitive to the presence of toxic substances (heavy metals and organic compounds) than conventional bacterial treatment processes (WHO, 1987). Moreover, microalgae have the capacity to remove these substances from the waste by accumulation (Tam & Wong, 1983; Oswald, 1988a; Wilkinson *et al.*, 1989; Gadd, 1990; Avery *et al.*, 1993a & b).

1.6.3 Microalgal Treatment Systems

Microalgal ponds are man-made, shallow bodies of water in which wastewater is allowed to stand for a time under the influence of the natural processes of purification by micro-organisms and natural conditions (sunshine, wind, temperature). Therefore, maximum treatment is achieved at minimal cost. Retention times depend on pond design, initial organic loading, temperature and the quality of effluent required for final disposal or reuse (Ellis, 1983; Abeliovich, 1986; WHO, 1987).

Non-toxic wastewaters of various origin and nature (municipal, industrial, agricultural or aquacultural) may be treated by microalgal systems, provided a suitable climate and low cost land are available (Gloyna, 1971; de la Noüe & De Pauw, 1988). Microalgal treatment systems have several other advantages over conventional wastewater treatment processes: they are more reliable; more economical to build and operate; easier to operate; and more tolerant of shock loads (Belsare & Belsare, 1987; Agunwamba, 1991; Green *et al.*, 1994). Properly designed and maintained microalgal ponds may also enhance the propagation of wildlife and contribute to the aesthetic appeal of an area (Oswald, 1972). The various

types of microalgal treatment systems are summarised in Table 1.4 (WHO, 1987). They reflect advances in design and efficiency of treatment.

1.6.4 Waste Stabilisation Ponds

Stabilisation ponds efficiently settle and degrade the organic material in sewage but do not remove nutrients; nutrient removal is not a requirement in many parts of the world since the effluent is reused in irrigation and therefore provides a valuable fertiliser. There are three types of stabilisation pond: anaerobic; facultative; and maturation. These may be used singly or connected either in parallel or series to suit requirements for the treated effluent (Table 1.4). However, there are various problems associated with the operation of each of these including objectionable odours, scum formation, weed growth, mosquitoes, greenhouse gas (methane) emission and the need for them to be desludged every 5-10 years (Pearson *et al.*, 1987; WHO, 1987; Oswald, 1988a).

1.6.5 High-Rate Ponds

The high-rate pond is an improvement in the design of the facultative stabilisation pond which enables more control of the wastewater treatment process by matching algal growth and oxygen production to the BOD of the waste (Table 1.4) (Oswald *et al.*, 1953; Shelef *et al.*, 1980; Grobbelaar *et al.*, 1988; Belsare & Belsare, 1987; Oswald, 1988a). Problems associated with facultative ponds include thermal stratification, high daytime pH and depletion of oxygen at night. These are alleviated by continuous mixing of the high-rate pond with a paddlewheel (Shelef *et al.*, 1978a; Grobbelaar *et al.*,

Table 1.4 Types of microalgal wastewater treatment ponds (Gloyna, 1971; WHO, 1987; Pearson *et al.*, 1987; Oswald, 1988a & c).

Pond type	Characteristics	Treatment	Uses
Anaerobic	Depth: 2.5-5.0 m, Retention time: 1-5 days Anaerobic throughout	Sedimentation of organic matter Anaerobic digestion and part mineralisation to CO_2, CH_4 and H_2S gases	Replace primary settling tanks, sludge thickeners, digesters and dryers Effluent has 40-60% suspended solids and BOD of inflow
Facultative	Depth: 1.5-2.0 m Retention time: 7-50 days Larger surface area Aerobic top layer due to algal oxygenation and anaerobic bottom sludge layer due to bacterial degradation separated by facultative layer	Dissolved, colloidal and suspended organic matter are metabolised by aerobic and facultative bacteria in upper and middle layers consuming O_2 and producing CO_2 which is transformed into algal biomass Facultative bacteria also consume combined oxygen from nitrates and sulphates when free oxygen is exhausted Remaining suspended solids settle to anaerobic layer	Replaces secondary treatment. Effluent contains high concentrations of dissolved oxygen. Algae, bacteria, rotifers and microcrustaceans are present but practically no suspended solids which will settle

Table 1.4 Types of microalgal wastewater treatment ponds (continued).

Pond type	Characteristics	Treatment	Uses
High-rate	Depth: 0.5m Retention time: 2 days Raceway Continuously mixed by gentle stirring using paddlewheel (as low as 5 cm sec^{-1} depending upon conditions) Aerobic throughout	Mixing promotes algal growth for concurrent nutrient removal and recycling into algal biomass Very little sludge accumulation	Replaces secondary and tertiary treatment removing more than 90% of the carbonaceous BOD, and up to 80% of the nitrogen and phosphate Virtual absence of pathogenic bacteria and viruses in the effluent, recovery of biomass as a valuable by-product and the absence of odour problems
Maturation	Depth: 1-1.5m Retention time: 3-10 days Aerobic	Removes a very high percentage of faecal bacteria, viruses, protozoa and other pathogens. It also removes some suspended matter and some nutrients, and further reduces the concentration of biodegradable organic matter.	Effluent has low BOD and suspended solids but nutrient levels depends on whether inflow from facultative pond or high rate pond

1988). Mixing also promotes nutrient removal and algal growth and, when stopped, induces flocculation and settlement of the algae (Oswald, 1988a).

1.6.6 Advanced Integrated Ponding Systems

The most sophisticated microalgal wastewater treatment system is the advanced integrated wastewater ponding system (Table 1.5). This has been developed over the last four decades by Oswald and co-workers at Berkeley, California (Oswald, 1991). It consists of a series of earthwork reactors which are designed to treat and reclaim the water, nutrients and usable energy from wastewater efficiently. The system comprises primary ponds (advanced facultative ponds) with internal anaerobic fermentation pits, high-rate ponds, tertiary settling ponds, and quaternary holding ponds (maturation ponds) (Green *et al.*, 1994).

Advanced facultative ponds provide primary treatment through settlement of organic solids in anaerobic fermentation pits. Organic compounds are completely converted to methane, nitrogen gas, carbon dioxide, stable residues and inorganic nutrients. Recirculation of some of the microalgal-rich effluent from the high-rate pond forms an aerobic, weakly alkaline layer on the surface of the facultative pond, which enables the methane to be "scrubbed," and the carbon dioxide and nutrients recycled in microalgal biomass. The methane may be collected and used for energy production, while the costs associated with conventional sewage sludge digestion, handling and disposal are eliminated (Green *et al.*, 1994).

The high-rate pond saves additional energy in secondary treatment through the use of solar energy to provide photosynthetic oxygenation for microbial oxidation of the organic residues. This is approximately one-tenth of the energy cost of the most efficient mechanical aeration. The

Table 1.5 Treatment in an advanced integrated wastewater ponding system (modified from Oswald, 1988a & c).

Treatment process	Criteria of effectiveness	How accomplished in integrated ponds
Preaeration	Odour removal	By recirculation of aerobic effluent from high rate pond.
Grit and screenables removal and sedimentation	Suspended solids removal	Wastes are inserted at the bottom of a fermentation pit where most solids tend to remain until their organic fraction is decomposed by anaerobic bacteria. The inert fraction remaining has a small volume and many years are required to fill the volume provided for inert accumulations.
Flotation	Grease and floatables removed	Inert floatables collect on specially designed and located scum ramps, which can be cleaned either manually or by mechanical equipment. Grease often becomes saponified at high pH and becomes biodegradable.
Methane fermentation	Combustible gas production	An anaerobic environment is created in the bottom of the ponds, permitting methane fermentation to occur. Special gas collection equipment can be installed for energy generation.
Biological oxidation	BOD removal	Oxygen in amounts equal or greater than the wastewater BOD is produced by photosynthetic micro-algae that are maintained in suspension by gentle flow mixing in a high rate pond. During algal growth the pH value in the pond increases to well above 9.0.
Removal of algae	% of suspended solids removed	Following gentle mixing in a high rate pond, algae tend to flocculate and settle in the settling ponds. Algae accumulated in the settling ponds can be removed for use or can remain in the pond for several months. Since two settling ponds are always provided, removal can be by simple decanting and drying.

Table 1.5 Treatment in an advanced integrated wastewater ponding system (continued).

Treatment process	Criteria of effectiveness	How accomplished in integrated ponds
Nutrient removal	N, P removed	Organic nitrogen is converted to ammonium or to N_2 gas. N_2 gas is produced through the heterotrophic denitrification in the anaerobic bottom of the primary pond and escapes to the atmosphere along with methane. Ammonium is taken up by the algae during growth. Surplus ammonium is converted to NH_3 which escapes to the air during gentle mixing at high pH. Phosphorous is taken up by algae or precipitated at high pH as calcium phosphate.
Disinfection	Removal of enteric bacteria.	Bacterial numbers are decreased by time, temperature and high pH. A pH greater than 9.2 for 24h is lethal to coliform and other enteric bacteria. pH values above 9 occur daily in high-rate ponds. The decline in Virus numbers results from long detention periods.
Heavy metal removal	% of removal	Algae have a high negative surface charge and therefore, an affinity for heavy metals which usually have a strong positive charge. By recirculation of high rate pond effluent containing algae, heavy metals adsorb to algae and settle out in the facultative pond. A high pH precipitates residual heavy metals in high-rate ponds.
Total dissolved solids removal	% of removal	Calcium phosphate and calcium and magnesium carbonate precipitate at high pH. Potassium and magnesium are incorporated into algal cells. Sodium is not removed to any significant extent by microalgae, and may increase slightly in long-detention ponds.
Refractory organics	% of removal	Long detention times permit time for organic refractory degrading micro-organisms to develop. High oxygen potentials in high rate ponds permit development of actinomycetes which degrade compounds refractory to facultative heterotrophs.

algal-bacterial concentrates are then removed from the oxidised waste in settling ponds and the treated wastewater may be held in maturation ponds for seasonal applications of irrigated water or for controlled discharge (Oswald, 1991).

The advanced integrated wastewater ponding system not only provides a solution to the problem of sludge disposal, but also offers the possibility of energy production from the methane gas. High rate ponds may be used to reduce nutrient levels to meet even the most stringent of EC standards and this together with the removal of enteric bacteria and viruses, heavy metals and refractory organics would enable unrestricted discharge of the effluent at sea. Microalgal ponds are also useful for secondary and tertiary treatment of effluents from conventional treatment processes (Veber *et al.*, 1984; Oswald, 1988a; Tam & Wong, 1989) and have been successfully used to treat a variety of wastewaters, including manures from all farmed animals and industrial effluents (Table 1.6).

Previously, research into microalgal wastewater treatment systems has tended to be in terms of pond engineering and process design (Gloyna, 1971; Green & Oswald, 1994; WHO, 1987; Oswald, 1988b; Pearson *et al.*, 1994). The microalgae in the ponds are referred to in terms of their contribution to the treatment process rather than by species. A study of wastewater treatment in terms of the abilities of different algal species to treat wastewaters and the response of these species to the conditions representative of treatment ponds would therefore be of great value to the understanding of the biology of these systems.

Table 1.6 Summary of other wastewaters treated by microalgal ponds.

Wastewater	Reference
Pig	De Pauw *et al.* (1980); Lincoln & Hill (1980); Fallowfield & Garrett (1985a, b); Strain *et al.* (1986); de la Noüe & Bassères (1989).
Cattle	Lincoln *et al.* (1983); Ayala & Bravo (1984); Paniagua-Michel *et al.* (1987).
Sheep	Ayala & Bravo (1984).
Goat	Phang (1990).
Chicken	Mahadevaswamy & Venkatamaran (1986); Paniagua-Michel *et al.* (1987); Pantastico (1987).
Duck	Phang (1990).
Palm oil	Phang (1990).
Rubber waste	Phang (1990).
Winery waste	Oswald (1994).
Cannery waste	Oswald (1994).
Tomato waste	Rodrigues & Oliveira (1987).

1.6.7 Biomass Production

Microalgae are among the most prolific producers of plant biomass (Goldman, 1979; Shelef & Soeder, 1980; Richmond, 1986b; Borowitzka & Borowitzka, 1988a). The algal biomass produced in microalgal treatment ponds may be discharged to natural waters in the treated effluent, where it may provide food for aquatic organisms or release nutrients slowly (Grobbelaar *et al.*, 1988). However the BOD of the algal biomass may be similar to that of the wastewater and therefore in many areas the algae would have to be removed to meet discharge standards.

Production of microalgal biomass with commercial value would not only offset the cost of removing the microalgal biomass from the treated effluent, but enable recycling of nutrients from the wastewater. The versatility of microalgal systems makes it possible to combine wastewater treatment and the production of a usable biomass (de la Noüe & De Pauw, 1988). Microalgal biomass has many potential uses including single cell protein for humans, livestock and aquaculture; as an energy source by fermentation to methane gas or alcohol or hydrogenation to hydrocarbons; and for the extraction of many fine chemicals such as pigments, oils, carbohydrates, pharmaceutical agents and vitamins. Many of these uses are reviewed by Aaronson & Dubinsky (1982), Metting and Pyne (1986) and de la Noüe & De Pauw (1988) and are summarised in Table 1.7.

1.7 Marine Microalgae

The use of marine microalgae in wastewater treatment ponds has not been widely studied. This is probably the result of historical factors rather than an intrinsic difference between freshwater and marine algae (Borowitzka, 1988a). In coastal areas, a marine microalgal based advanced

Table 1.7 Potential uses of microalgal biomass

Use	References
Organic fertiliser	Rodriguez-lopez (1983); Lembi & Waaland, (1988)
Single cell protein	Pantastico (1987); Becker (1988).
Animal feed	Shelef *et al.* (1978a); Lincoln & Hill (1980); Mahadevaswamy & Venkatamaran (1986); Becker (1988).
Poultry feed	Saxena *et al.* (1983); Mahadevaswamy & Venkatamaran (1986); Becker (1988).
Pig feed	Brune & Walz (1978); Lincoln *et al.* (1978).
Aquaculture Feed	Goldman (1979a); Paniagua-Michel *et al.* (1987); Pantastico (1987); Herrero *et al.* (1991); Benemann (1992).
Integrated aquaculture	Ryther *et al.* (1972); Proulx & de la Noüe (1985a & b); Pantastico (1987); Phang (1990).
Fuel: Fermentation	Benemann *et al.* (1977); Oswald (1988a).
Fuel: Hydrogenation	Borowitzka (1988b); Birch & Bachofen (1988); de la Noüe & de Pauw (1988); Hall & Rao (1989).
Fine Chemicals	Aaronson & Dubinsky (1989); Hall & Rao (1989).
Pigments	Borowitzka (1988c).
Oils	Aaronson & Dubinsky (1982); Borowitzka (1988b).
Carbohydrates	Aaronson & Dubinsky (1982); Borowitzka, (1988c); de la Noüe & De Pauw (1988).
Pharmaceutical agents	Aaronson & Dubinsky (1982); Burton & Ingold (1984).
Vitamins	Lem & Glick (1985); Richmond (1988); Borowitzka (1988c).

integrated ponding system would have several additional benefits to treating the wastewater.

Freshwater is a limiting resource in arid parts of world and costly to make potable in others (Myers, 1985). Therefore, the use of potable freshwater to wash away sewage can be regarded as wasting this valuable resource, especially if the treated effluent is discharged at sea. Seawater, however, is in plentiful supply so even the most concentrated effluents could be diluted down to a level at which marine microalgae could provide efficient treatment. The need for conservation of freshwater resources in the future may even require that a supply of seawater be used in large coastal cities for sanitary purposes (Russell-Hunter, 1970).

In terms of nutrient removal, high-rate ponds tend to function as nitrogen limited systems (Golueke *et al.*, 1967; Weissman *et al.*, 1978). Coastal waters are commonly nitrogen limited, so the discharge of effluents with a high phosphorus content into coastal waters would cause less eutrophication than if discharged into phosphorous limited freshwaters (McCarthy, 1980; Glibert, 1988). Carbon is normally also limiting in high-rate ponds (Oswald, 1970), and the high bicarbonate concentration (20 mmol m^{-3}) of seawater (Burris, 1977) offers a dual advantage by providing a carbon source and natural buffering capacity (Rebello, 1982). Seawater is a hostile environment for enteric bacteria and viruses, further reducing their survival in microalgal ponds (Fujioka *et al.*, 1981; Waldichuk, 1985).

Increasing salinity is a growing problem in rivers, streams and wetlands of many parts of the world and can limit agricultural production of traditional crops. The maximum salinity of water that can be used for crop irrigation is 3 ‰, water which is too saline for traditional crops may be used for both wastewater treatment and the production of marine or

brackish water microalgae, invertebrates and fish (Parker *et al.*, 1991). Microalgae may be easily harvested through aquatic food chains by filter-feeding or grazing fish or invertebrates for which they represent a natural food (Ryther, 1983; Wong & Tam, 1984; Proulx & de la Noüe, 1985a & b; De Pauw & Persoone, 1988; Etnier & Guterstam, 1991). In contrast to freshwater aquaculture, there are several species of marine organisms with high economic value which can be grown in wastewater enriched culture systems. Such groups are bivalve molluscs including oysters, clams, mussels and scallops or penaeid shrimps, all of which feed on unicellular algae (Ryther, 1983). Marine microalgae produce many commercial products and marine biotechnology has great potential for the production of sugars and polysaccharides, pharmaceuticals, dyes, bioflocculants, pigments, vitamins, lipids and oils, especially when nutrients are supplied by wastewaters (Curtin, 1985; de la Noüe & De Pauw, 1988).

Previous studies of the use of marine microalgae for wastewater treatment were mainly made by Ryther and co-workers at the Woods Hole Oceanographic Institute, Massachusetts, USA (Ryther *et al.*, 1972). However, the aim of their research was to investigate the use of secondarily treated wastewater as a nutrient source for the mass culture of marine microalgae. The microalgal biomass was then fed to bivalve molluscs. Continuous culture experiments ranged from small laboratory studies which measured the growth of a few marine species on wastewater:seawater mixtures (Goldman & Stanley, 1974), to small scale outdoor experiments in two 2000 l (4 m^2 surface area) circular ponds (Goldman *et al.*, 1974a & b) and on to large scale outdoor experiments in six 35000 l (150 m^2) ponds (D'Elia *et al.*, 1977). Marine microalgae have also been used to treat pig slurry (De Pauw & De Leenheer, 1979) and to remove nitrogenous compounds from mariculture systems (Alderson & Howell, 1973; Siddall, 1974).

Research into the production of microalgae as a source of biomass for energy began in 1978 by the Commission of the European Communities. Under the title "Mariculture on Land", this project involved researchers in Germany, France, Italy and Brazil, and mass culture facilities were constructed in the latter two countries. The project stressed the use of arid coastal lands and seawater for the culture of marine microalgae for fermentation to methane (Wagener, 1981). Marine microalgal treatment systems have also been employed in closed seawater recirculating systems (Honn & Chavin, 1975; Gerhardt, 1981). Attached assemblages of benthic microalgae have been developed for use in aquarium filtration systems (Adey, 1987; Adey & Goertemiller, 1987) and recently their application has been extended to wastewater treatment (Adey *et al.*, 1993). These previous studies have mainly concentrated on using wastewater:seawater mixtures to produce algal biomass for integrated aquaculture systems or fermentation to fuel. Few authors have made a detailed study of the ability of microalgal species to remove nutrients from sewage effluent and a comprehensive screening of marine species from all divisions has not been made.

1.7.1 Selection of Marine Microalgae

The following characteristics are important when selecting marine microalgal species which may have use in wastewater treatment: high and consistent nutrient removal capability; tolerance to a wide range of nutrient concentrations and salinities; the ability to grow heterotrophically; long term dominance in culture; high specific growth rate; resistance to predation and bacterial contamination; tolerance to low oxygen levels; ease of harvest; production of usable biomass or extracts (Marelie *et al.*, 1982; Abeliovich, 1986; Borowitzka, 1988c; Oswald, 1988a, b & c). Microalgal species with some of the characteristics outlined above are shown in Table 1.8.

Table 1.8 Selection of marine microalgal species for potential use in wastewater treatment

Species	Division	Characteristic	Reference
Chaetoceros calcitrans	Bacillariophyceae	Dominant in mass culture	Wohlgeschaffen *et al.* (1992)
		Aquaculture	Nelson *et al.* (1992);
Nitzschia longissima	Bacillariophyceae	Growth on wastewaters	Palmer (1969)
Nitzschia ovalis	Bacillariophyceae	Growth on wastewaters	Palmer (1969)
Phaeodactylum tricornutum	Bacillariophyceae	Growth and dominant on wastewaters	Ryther *et al.* (1972); Goldman & Stanley (1974); Goldman *et al.* (1974a); Goldman & Ryther (1976a)
		Dominant in mass culture	Wohlgeschaffen *et al.* (1992)
		Aquaculture	Herrero *et al.* (1991)
Skeletonema costatum	Bacillariophyceae	Growth on wastewaters	Goldman & Stanley (1974)
		Dominant in spring bloom Loch Striven	Marshall & Orr (1927)
		Aquaculture	Regan (1988)
Thalassiosira weissflogii	Bacillariophyceae	Dominant in mass culture	Wohlgeschaffen *et al.* (1992)
		Aquaculture	Regan (1988)
Botryococcus braunii	Chlorophyceae	Hydrocarbon fuel	Birch & Bachofen (1988)
Chlamydomonas reginae	Chlorophyceae	Aquaculture	Regan (1988)
Chlorella salina	Chlorophyceae	Aquaculture	Spectorova *et al.* (1982)
Chlorella stigmatophora	Chlorophyceae	Aquaculture	Fabregas & Herrero (1987a)
Dunaliella salina	Chlorophyceae	ß-Carotene, glycerol	Borowitzka & Borowitzka (1988b)
Dunaliella tertiolecta	Chlorophyceae	Dominant in mass culture	Wohlgeschaffen *et al.* (1992)
		Aquaculture	(Spectorova *et al.* (1982); Herrero *et al.* (1991)
		ß-Carotene, glycerol	Borowitzka & Borowitzka (1988b)
Nannochloropsis oculata	Chlorophyceae	Aquaculture	Witt *et al.* (1981); Regan (1988)

Table 1.8 Selection of marine microalgal species for potential use in wastewater treatment (continued).

Species	Division	Characteristic	Reference
Stichococcus bacillaris	Chlorophyceae	Aquaculture	Regan (1988)
Rhodomonas baltica	Chryptophyceae	Aquaculture	Regan (1988)
Rhodomonas marina	Chryptophyceae	Aquaculture	Regan (1988)
Rhodomonas sp.	Chryptophyceae	Aquaculture	Regan (1988)
Oscillatoria animalis	Cyanophyceae	Growth on wastewaters	Palmer (1969)
		Aquaculture	Pantastico (1987)
Spirulina platensis	Cyanophyceae	Aquaculture Harvestibility	Benemann (1992)
Amphidinium carterae	Dinophyceae	Aquaculture	Witt *et al.* (1981)
Oxyrrhis marina	Dinophyceae	Aquaculture	Regan (1988)
Micromonas pusilla	Prasinophyceae	Aquaculture	Regan (1988)
Tetraselmis rubens	Prasinophyceae	Aquaculture	Witt *et al.* (1981)
Tetraselmis sp.	Prasinophyceae	Growth on wastewaters	Goldman & Stanley (1974)
		Aquaculture	Spectorova *et al.* (1982); Regan (1988)
Tetraselmis suecica	Prasinophyceae	Dominant in mass culture	Wohlgeschaffen *et al.* (1992)
		Aquaculture	Fabregas *et al.* (1984, 1985b & 1987b); Herrero *et al.* (1991)
Tetraselmis tetrathele	Prasinophyceae	Dominance in mass culture	Materassi *et al.* (1984)
Tetraselmis verrucosa	Prasinophyceae	Aquaculture	Regan (1988)
Chrysochromulina chiton	Prymnesiophyceae	Aquaculture	Regan (1988)
Coccolithophora sp.	Prymnesiophyceae	Aquaculture	Regan (1988)
Coccolithus sp.	Prymnesiophyceae	Aquaculture	Regan (1988)
Isochrysis galbana	Prymnesiophyceae	Dominant in mass culture	Wohlgeschaffen *et al.* (1992)
		Aquaculture	Spectorova *et al.* (1982); Fabregas *et al.* (1985a); Herrero *et al.* (1991);
Pavlova lutheri	Prymnesiophyceae	Growth on wastewaters	Goldman & Stanley (1974)
		Dominant in mass culture	Wohlgeschaffen *et al.* (1992)
Phaeocystis poucheti	Prymnesiophyceae	Aquaculture	Regan (1988)
Prymnesium parvum	Prymnesiophyceae	Linoleic acid	Regan (1988)
Porphyridium purpureum	Rhodophyceae	Food product 'nori' Binding agents	Vonshak (1988)

One of the main problems with mass cultivation of microalgae is contamination by other microalgal species (Goldman, 1979; Shelef & Soeder, 1980; Materassi *et al.*, 1984; De Pauw *et al.*, 1984; Richmond, 1986c). Maintenance of a unialgal culture in open air systems usually requires strict management and manipulation of the environment including temperature, nutrient composition and dilution rate (Borowitzka, 1988c). Less than 50 of the 30,000 species of microalgae identified have been studied in some detail, with respect to their metabolism and chemical composition (Soeder, 1981; Borowitzka, 1988a; Richmond, 1986b). Furthermore, details of physiology, biochemistry, and potential for mass culture are known for only a few of those studied (Borowitzka, 1988c; Benemann, 1989; de la Noüe *et al.*, 1992). Therefore the physiology of microalgal species capable of treating wastewaters needs to be studied.

Although unialgal cultures of microalgal species can be obtained from culture collections, Borowitzka (1988c) stressed the importance of selecting microalgal strains that are suited to the climatic conditions under which the microalgal treatment will occur. Local endemic species should be most suited to the prevailing climate (Witt *et al.*, 1981; Oswald, 1988c) and they may be introduced to treatment ponds through the dilution of the sewage with seawater. Any comprehensive screening of the potential of marine microalgae to treat wastewaters ideally should include endemic species.

The traditional use of the sea for disposal of wastewaters and the relatively recent adverse effects of this practise on the environment has meant that little research into the use of wastewater treatment processes to prevent eutrophication has been made in the UK. Although the climate of the UK is not the most favourable for the use of a photosynthetic wastewater treatment process, outdoor semi-continuous mass cultures of

marine species including *Phaeodactylum tricornutum* have already been successfully grown using artificial nutrients in the UK (Ansell *et al.*, 1963a & b; 1964). Year-round treatment of wastewaters by freshwater microalgal cultures has also been demonstrated in the northern cold climate of Quebec, Canada, where *Scenedesmus obliquus* was cultivated on secondary effluent in greenhouses to maintain temperatures during the winter (Pouliot & de la Noüe, 1985). The productivity of freshwater mass algal culture systems, even in the temperate latitude of the UK has been shown to be comparable to that of traditional agricultural crops (Fallowfield & Garrett, 1985a & b).

Apart from the limitations to algal growth imposed by environmental conditions, there are several other problems associated with microalgal wastewater treatment systems. These include the large areas of land required for ponds, the lack of an efficient and economic method of harvesting the algal biomass and the difficulty of maintaining the algae in suspension (de la Noüe & De Pauw, 1988; Oswald, 1988a & c; Raven, 1988; Benemann, 1989). Therefore a screening of marine microalgal species for use in a wastewater treatment system should also address these drawbacks.

1.8 Aims

The preceding sections of this chapter have highlighted: the problems for marine disposal of wastewaters; the EC guidelines for the nutrient content of coastal discharge which have to be met by 1998; the shortfall of conventional treatment methods in meeting these guidelines; and how a marine microalgal wastewater treatment process may offer an efficient and economic alternative to conventional methods. Although extensive research has been made into microalgal treatment systems using freshwater species, the use of marine species has been little explored. Even less is known of the

potential of microalgal species from temperate climates for wastewater treatment. A comprehensive screening of microalgal species of every division has not been made, and few authors have included endemic species in their studies.

The specific aims of this study were as follows:

a) To screen a wide range of marine microalgal species for their potential to remove nutrients from wastewater and remain dominant in culture. In order to select marine microalgal species appropriate for a detailed study of wastewater treatment.

b) To determine the range of tolerance to environmental conditions of the best-treating species from the screening experiments and to evaluate their ability to remove nutrients under continuous culture.

c) To test the best-treating species selected from the continuous culture experiments for treatment under ambient temperate conditions using larger-scale apparatus modelled on a high-rate pond.

Chapter Two

Selection and Standardisation of Isolation Techniques, Culture Conditions and Analytical Methods

2.1 *Introduction*

This chapter describes the selection and standardisation of experimental methods for a protocol to simultaneously screen a large number of microalgal species for nutrient removal from wastewaters. The screening protocol was established by: 1) selection of culture conditions under which stock cultures would be maintained and screening experiments conducted; 2) selection and standardisation of analytical methods for determination of algal biomass and physical and chemical characteristics growth media; 3) selecting the optimal wastewater dilution for use in the screening experiments.

Analytical methods were chosen for their reliability, reproducibility and rapidity and were used throughout the research of this thesis to monitor the ability of microalgal species to treat seawater diluted wastewater. Since these experiments were to be made using wastewater and seawater sampled over three years, it was necessary to determine the chemical and physical characteristics of these waters and the variability of these characteristics with time.

2.2 *Materials and Methods*

2.2.1 *Microalgal Culture*

Algal growth is greatly affected by the conditions (temperature, light and mixing) under which microalgae are cultured and the physical and chemical characteristics of the culture medium (nutrient levels, salinity and pH) (Becker & Venkatamaran, 1982; Richmond, 1983; Fabregas *et al.*, 1987; Oswald, 1988b). Therefore, it was essential to standardise and control these factors so that comparisons could be made between screening experiments.

The optimum temperature for the culture of microalgae of temperate origin is between 10 and 15 °C (Starr, 1979), and one tenth of full daylight intensity (ca. 20 $\mu E\ m^{-2}\ s^{-1}$) is known to be optimal for culturing and maintenance of microalgal species (Starr, 1964; Guillard, 1979). Coastal phytoplankton grown under a 12 h:12 h photoperiod have optimal growth and nutrient removal rates (Brand & Guillard, 1981; Pryztocka-Jusiak *et al.*, 1984; Davidson *et al.*, 1991). Therefore these culture conditions (15 °C, 18-22 $\mu E\ m^{-2}\ s^{-1}$ PAR, and 12 h:12 h photoperiod) were used for the isolation of endemic species and in all experiments under laboratory conditions.

A Fisons series II growth cabinet (model 140G2/TC with Philips TLD 36W/35 fluorescent lamps) was used for the isolation of endemic microalgal species. A Conviron growth cabinet (model S10h, with Sylvania cool white fluorescent lamps) was used for maintenance of microalgal stock cultures, determining microalgal growth characteristics in batch culture, and for the batch culture screening experiments.

Seawater from St Andrews Bay, Fife, Scotland was used to make up Erd-Schreiber (E-S) culture medium (McLachlan, 1979) and to dilute the wastewater. Prior to experimental use, seawater was filtered through a sand filter and a 1 μm cartridge filter, and then heat sterilised by autoclaving (115 °C for 30 min.). E-S culture medium was used in all aspects of algal culture, including isolation of endemic species, maintenance of stock cultures, and determination of growth characteristics of the algae in batch culture. The E-S medium (2.36 mol m^{-3} $N\text{-}NO_3^-$ and 55.9 mmol m^{-3} $P\text{-}PO_4^{3-}$) was made up from:

> 1 ml mixed salt solution (20 g $NaNO_3$; 2 g $Na_2HPO_4.12H_2O$ dissolved in 100 ml distilled water (d.w.) and autoclaved at 121 °C for 15 min).

50 ml soil extract (1 kg finely sieved garden soil mixed with 2 l tap water, autoclaved at 107 °C for 1 h, settled for 1 d, the supernatant decanted and autoclaved at 121 °C for 35 min).

1 l 95 % seawater (diluted with d.w.).

Prior to use the E-S medium was equilibrated to the temperature of the inoculum culture (15 °C).

2.2.2 Isolation of Endemic Species

Endemic species were collected in plankton tows (55 µm net) during October 1990 and April 1991. Tows were conducted in the waters surrounding the primary sewage outfall in St Andrews Bay. Samples were stored in the Fisons culture cabinet for use in isolation experiments. Species were isolated by either pipette isolation or raw enrichment methods.

a) Pipette isolation (Hoshaw & Rosowski, 1979)

The tow sample was diluted serially with seawater to enable individual cells or colonies to be isolated. The individual cells were separated using Pasteur pipettes, observed through a dissecting microscope (Zeiss Stereomicroscope, SR D7082 Oberkochen) with a 50x objective. The isolated cells were inoculated into test-tubes containing 5 ml of sterile E-S culture medium and incubated for two weeks.

b) Raw enrichment (Guillard, 1979)

The tow sample was diluted 1:1 with wastewater and incubated for two weeks.

The dilution culture technique (Guillard, 1979) was used to achieve unialgal cultures of the isolate species. The average cell density of each

species was calculated from ten counts of isolate culture using a Neubauer haemocytometer, and serial dilutions using E-S medium were then made to obtain samples containing a single cell or colony. Replicate samples (10 ml) of each dilution were incubated in test-tubes for up to one month in the Fisons cabinet, and those which were found to be in unialgal culture were maintained as stock cultures. The purity of the microalgal isolates was checked and species identified using a compound microscope (Nikon Labophot) with x10 and x40 objectives). Dominant contaminants which grew up from the unialgal stock cultures from the culture collections were also isolated by this method.

Completely unialgal cultures are impossible to sustain out of doors in the real world (Borowitzka, 1988a, Oswald, 1988b). Detailed microscopic examination of the algal cultures used in this research inevitably found a few contaminants within each culture. However, in the cultures which were described as unialgal, contaminants remained at negligible levels throughout the duration of experiments.

2.2.3 *Culture Collection Species*

Microalgal species with established treatment capabilities or which had some of the qualities outlined in Chapter 1 (Section 1.7.2; Table 1.8) were obtained for use in this study from culture collections. Thirty-six species in unialgal culture (Table 2.1) were purchased or gifted from microalgal culture collections. Of the 29 species purchased, 20 were from the culture collection of the Marine Biological Association at Plymouth, England (which were already cultured on E-S medium), seven were from Biobred Ltd, Manchester, England, and two were from Culture Collection of Algae and Protozoa, Dunstaffnage Marine Research Laboratory, Oban,

Table 2.1 The 102 species of microalgae used in this experimental study.

Culture Collection Species	Strain	Algal Isolates	
Bacillariophyceae		**Bacillariophyceae**	**Chlorophycota**
[4]*Chaetoceros calcitrans*		SA90B1	SA90C1
[1]*Nitzschia longissima*	LA815	SA90B2	SA90C2
[3]*Nitzschia ovalis*		SA90B3	SA90C3
[5]*Phaeodactylum tricornutum*	100	SA90B3a	SA90C4
[5]*Skeletonema costatum*	106	SA90B4	SA90C5
[5]*Thalassiosira weissflogii*	541	SA90B5	SA91C6
		SA90B6	SA91C7
Chlorophyceae		SA90B7	SA91C8
[2]*Botryococcus braunii*	807/1	SA90B8	SA91C9
[5]*Chlamydomonas reginae*	399	SA90B9	SA91C10
[5]*Chlorella salina*	309	SA90B10	SA91C11
[5]*Chlorella stigmatophora*	85	SA90B9/10	SA91C12
[1]*Dunaliella salina*	LA40	SA90B11	SA91C13
[5]*Dunaliella tertiolecta*	83	SA91B12	SA91C14
[3]*Nannochloropsis oculata*		SA91B13	SA91C15
[5]*Stichococcus bacillaris*	82	SA91B14	
		SA91B15	**Cyanophyceae**
Chryptophyceae		SA91B16	SA91CY1
[1]*Rhodomonas baltica*	LP305	SA91B17	
[3]*Rhodomonas marina*		SA91B18	**Rhodophyceae**
[2]*Rhodomonas* sp.	995/2	SA91B19	SA90R1
		SA91B21	
Cyanophyceae		SA91B22	
[1]*Oscillatoria animalis*	LA945	SA91B23	
[1]*Spirulina platensis*	LA940	SA91B24	
		SA91B25	
Dinophyceae		SA91B26	
[5]*Amphidinium carterae*	450	SA91B27	
[5]*Oxyrrhis marina*	209b	SA91B27a	
		SA91B28	
Prasinophyceae		SA91B29	
[5]*Micromonas pusilla*	27	SA91B30	
[4]*Tetraselmis rubens*		SA91B31	
[1]*Tetraselmis* sp.	LA20	SA91B32	
[4]*Tetraselmis* sp.		SA91B33	
[5]*Tetraselmis suecica*	305	SA91B34	
[5]*Tetraselmis tetrathele*	272	SA91B35	
[5]*Tetraselmis verrucosa*	456	SA91B36	
		SA91B37	
Prymnesiophyceae		SA91B38	
[5]*Chrysochromulina chiton*	146	SA91B39	
[1]*Coccolithophora* sp.	LA635	SA91B40	
[4]*Coccolithus* sp.		SA91B41	
[5]*Isochrysis galbana*	I	SA91B42	
[5]*Pavlova lutheri*	75*	SA91B43	
[5]*Phaeocystis poucheti*	64	SA91B44	
[5]*Prymnesium parvum*	94	SA91B45	
		SA91B46	
Rhodophyceae		SA91B47	
[5]*Porphyridium purpureum*	539		

Culture collections: [1]Biobred Ltd, [2]CCAP, [3]Gatty Marine Laboratory, [4]Millport Marine Biological Station, [5]Plymouth Culture Collection.

Isolation codes designate endemic algal species.

Scotland. Four species were gifted by Dr Jane Lewis from the University Marine Biological Station, Millport, Scotland (also cultured on E-S medium) and the remaining three species were gifted by colleagues at the Gatty Marine Laboratory, University of St Andrews. Unialgal stock cultures of all microalgal species were maintained in the Conviron cabinet in 200 ml batch cultures on E-S medium.

2.2.4 *Selection of Biomass Determination Method*

The efficiency of four biomass determination methods (haemocytometer cell counts, optical density (OD), chlorophyll *a* and cellular protein) were compared using serial dilutions of cultures of four microalgal species (*Chlorella salina, Nannochloropsis oculata, Nitzschia ovalis* and *Phaeodactylum tricornutum*). The cultures were shaken to ensure the culture was homogeneous before sampling and all spectrophotometric analyses were made in 1 cm light path cuvettes, using a Pye Unicam SP6-450 UV/VIS spectrophotometer.

a) Haemocytometer Cell Counts (Stein, 1979)

Mean cell densities were calculated from ten replicate counts per culture sample.

b) Optical Density (OD) (Sorokin, 1979, Tam & Wong, 1989)

Measurements were made directly from 3 ml aliquots of each algal culture in disposable polystyrene cuvettes at 540 nm against a blank of E-S medium.

c) Chlorophyll *a* (Parsons *et al.*, 1984)

Ten millilitres of culture was centrifuged (1500 *g*, 10 min), the supernatant was removed and the pellet was mixed with 5 ml 90 % acetone

and few drops of $MgCO_3$ solution (to prevent acidity). The tube was covered with Nescofilm (Jensons Ltd, Leighton Buzzard, England) and left for 24 h in darkness at 4 °C for complete extraction. The extract was then shaken, centrifuged (1500 *g*, 10 min) to sediment the algal debris, and the OD of the supernatant was measured as described in Parsons *et al.* (1984) against a Milli-Q (M-Q) water reagent blank. The chlorophyll *a* concentration of the sample was calculated from:

$$\text{Chlorophyll } a \ (\mu g \ ml^{-1}) = C = 11.85 \ OD_{664} - 1.54 \ OD_{647} - 0.08 \ OD_{630}$$

Then:

$$\text{Chlorophyll } a \ (\mu g \ l^{-1}) = \frac{C \times v}{V}$$

Where:

v: the volume of extraction agent (ml)

V: the volume of sample centrifuged (l)

d) Determination of Protein (Sedmark & Grossberg, 1977)

The algal pellet (see chlorophyll *a* determination) was resuspended in 0.5 ml of 0.1 M NaOH and left for 12 h to extract the protein. After centrifugation (1500 *g*, 10 min), a 0.1 ml aliquot of the extract was mixed with 5 ml Coomassie Brilliant Blue reagent (G-250, Pierce, PO Box 117, Rockford, Illinois, USA, 61105). After 30 min, absorbance was read at 595 nm against a M-Q water reagent blank, and compared to a standard curve of bovine serum albumin (0.1-100 $\mu g \ ml^{-1}$; Sigma, fraction V).

2.2.5 *Selection of Optimal OD Wavelength*

Initially, the wavelength selected for use in the present study was OD_{540}, as preliminary measurements of OD at 438, 540 and 678 nm made on a dilution series of *Phaeodactylum tricornutum* showed that all three wavelengths were suitable for determination of biomass. The subsequent use of a microtitre plate spectrophotometer (Dynatech, MR5000, Billingshurst, England) enabled simultaneous measurement of the OD of all the algal species by using the 96-well microplate (Dynatech, M29A) format. Absorbance measurements of a dilution series of *Phaeodactylum tricornutum* with triplicate 300 µl aliquots in the wells of a microplate were compared at 405, 450, 490, 570 and 630 nm.

2.2.6 *Limits of Exponential Growth*

The size of the algal inoculum affects the growth rate of microalgal cultures and their ability to remove nutrients. Higher growth rates and nutrient removal are found with larger inocula (Tam & Wong, 1989) which should be at least 10 % of the total volume of the culture (Liao *et al.*, 1983). Growth characteristics of 83 algal species were measured by inoculating triplicate flasks of 75 ml of E-S medium with 10 ml of actively growing cell culture. The OD_{570} of three 300 µl aliquots from each flask were measured daily for two weeks using the microplate format against a blank of E-S medium. Prior to sampling, each culture was shaken vigorously and any algal settlement or attachment to the flask bottom was resuspended using a 'rubber policeman'. The duration of the exponential growth phase and exponential growth rate of each algal species was calculated from a semilog (base 2) plot of algal OD_{570} against time.

2.2.7 *Analytical Measurements*

The temperature of media was measured using a mercury in glass thermometer. The pH was determined using a Russell combination pH electrode (Type CWL) read from a Philips digital pH meter (Type PW9409) and calibrated with pH 7 and pH 10 buffers. Conductivity (siemens) was analysed using a Mullard conductivity cell (Type E7591/B) connected to a Mullard conductivity bridge (Type E756). Salinity (‰) was calculated from the chloride ion concentration (mmol m^{-3}):

$$\text{Salinity S (‰)} = \text{Chloride concentration (mmol } m^{-3}) \times 64.0$$

which was measured using a Corning (920) direct reading digital chloride meter.

Dissolved nutrients were measured against M-Q water reagent blanks by standard colorimetric methods selected for their reliability and convenience. Initially, tests were scaled down to a 10 ml sample volume and made in triplicate on samples which had been centrifuged at 1500 *g* for 10 min using a MSE Centaur 2 centrifuge to sediment any particulate matter. The absorbance of the reaction mixture was measured with 1 cm light path disposable polystyrene cuvettes using a Pye Unicam SP6-450 UV/VIS spectrophotometer. Nutrient concentrations (mmol m^{-3}) were calculated by comparing the average absorbance of triplicate samples to that of standards of known concentration which were calibrated against standard curves.

All methods, except the ammonium molybdate method for the determination of ortho-phosphate, were subsequently adapted to a 96-well microplate (Dynatech, M29A) format for use with a microtitre plate spectrophotometer (Dynatech, MR5000). Scaling down to microassays enabled rapid nutrient determination of a large number of microalgal

culture samples (300 µl) in triplicate. This was facilitated by using microplate centrifugation to spin down the algae (450 *g*, 10 min, Beckman CPR with microplate carrier for GH3.7 rotor), octapipette multichannel pipetting (Dynatech, S8/50) to transfer aliquots of the supernatants to clean microplates for nutrient analysis, and a multidispenser pipette (Eppendorf) to add reagents. Mixing of reagents between successive additions was effected by gently tapping the microplate or using a specially made mixerplate (a microplate with 200 µl pipette tips (with sealed ends) protruding from the base of drilled out wells) which enabled simultaneous mixing of all of the 96 wells of a microplate.

a) Nitrate-Nitrogen

$N\text{-}NO_3^-$ was determined using NAS Szechrome reagent (Diphenylamine Sulphonic Acid Chromogene) purchased from Park Scientific Ltd, Northampton, UK. The method was scaled down to the microplate format with a final volume of 275 µl. Samples were compared to 71.4 mmol N m^{-3} standards of KNO_3.

b) Nitrite-Nitrogen

$N\text{-}NO_2^-$ was analysed by the method described in Snell (1981) scaled down for use in the microplate format (final volume 300 µl). Samples were compared to 99.9 mmol N m^{-3} standards of $NaNO_2$.

c) Ammonium-Nitrogen

$N\text{-}NH_4^+$ was measured by the method described in Parsons *et al.* (1984). The microplate format was read at 630 nm using a 250 µl sample, 10 µl of both phenol and ammonium nitroprusside, and 30 µl of oxidising reagent (final volume 300 µl). Samples were diluted with M-Q water (1:4) prior to addition of reagents and were compared to a 1:4 diluted 293.8 mmol N m^{-3} standard of $(NH_4)_2\,SO_4$.

d) Urea-Nitrogen

N-$CO(NH_2)_2$ was broken down to ammonium by urease using a Sigma Diagnostics (P.O. Box 14508, St Louis, Mo. 63178, U.S.A.) urea nitrogen kit (procedure No. 640). Ammonium was then determined by the method described above.

e) Ortho-phosphate

Initially, P-PO_4^{3-} was analysed by the method of Parsons *et al.* (1984). However, the microtitre plate spectrophotometer could not measure absorbance at 885 nm, and disposable polystyrene cuvettes could not be used for the screening of multiple samples because they are known to interfere with absorbance measurements at this wavelength. An adaptation of the malachite green method, originally developed for the microplate determination of ATPase (Henkel *et al.*, 1988) was therefore used.

The OD_{630} of an ortho-phosphate standard curve was measured with time (min) after the addition of a 200 µl aliquot of malachite green reagent to 50 µl of each standard in triplicate. Malachite green reagent was prepared fresh from stock solutions of ammonium molybdate (5.72 % w/v in 6 N HCl), 0.0812 % (w/v) malachite green (technical grade, BDH Chemicals Ltd, Poole, UK), and M-Q water mixed at a ratio of 1:1:4, respectively. Polyvinyl alcohol, a protein stabilising agent used in the original method (Henkel *et al.*, 1988) was not required in the present analysis.

At all ortho-phosphate concentrations, the absorbance maximum was reached after five minutes and was stable for a further ten minutes, after which absorbance declined (Fig. 2.1). Absorbance declined more quickly for higher ortho-phosphate concentrations (Fig. 2.1) until, after two hours reaction time, precipitation of the reaction mixture caused a sudden increase. The standard curves for 5, 10 and 15 min reaction times are shown in Figure 2.2. All ortho-phosphate determinations were made using this

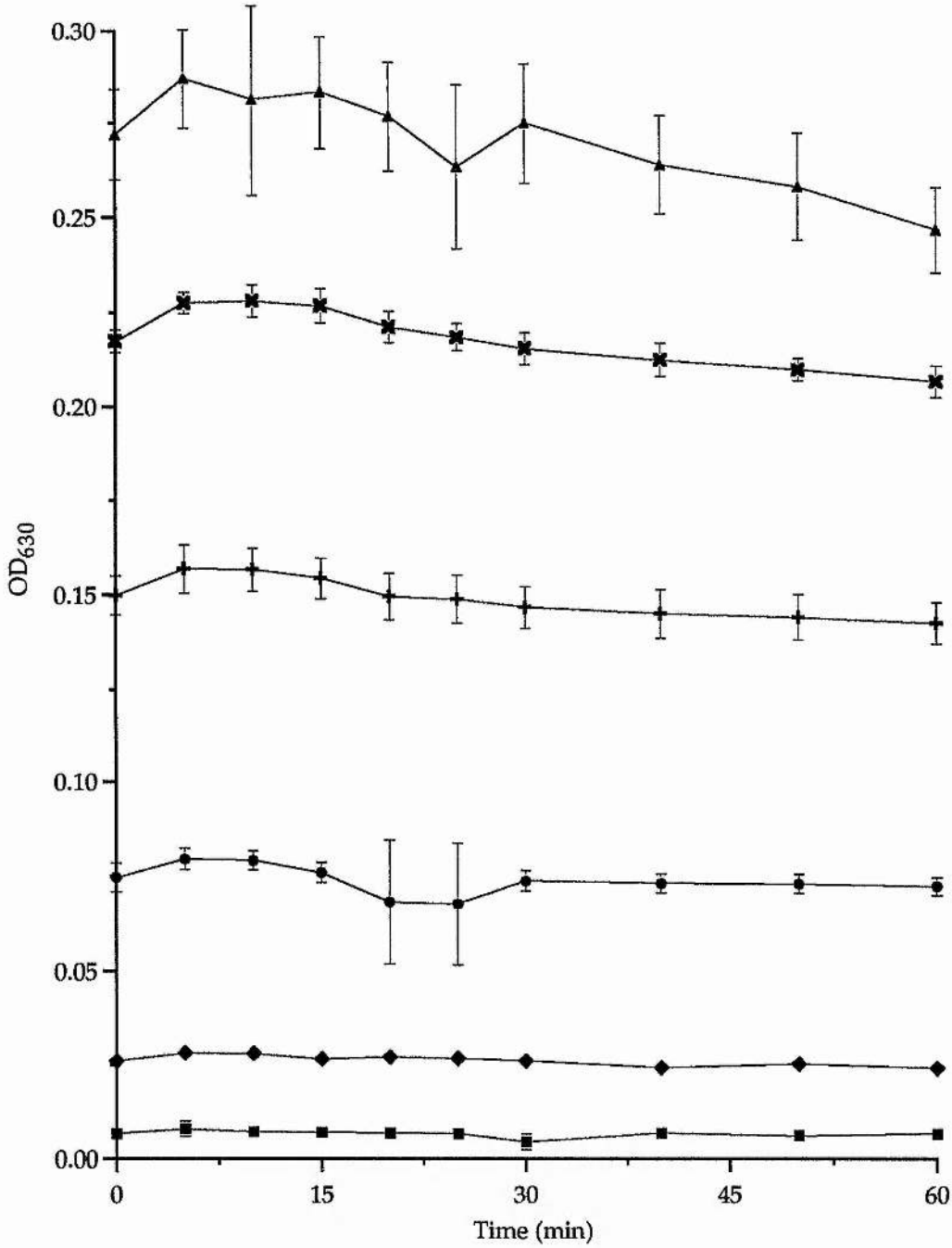

Figure 2.1 Reaction time (min) of the malachite green ortho-phosphate assay, measured at 630nm for a dilution series of 30.1 (▲-▲), 23.2 (✖-✖), 15.4 (✚-✚), 7.7 (●-●), 3.0 (◆-◆), and 0.8 (■-■) mmol P m^{-3}. Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

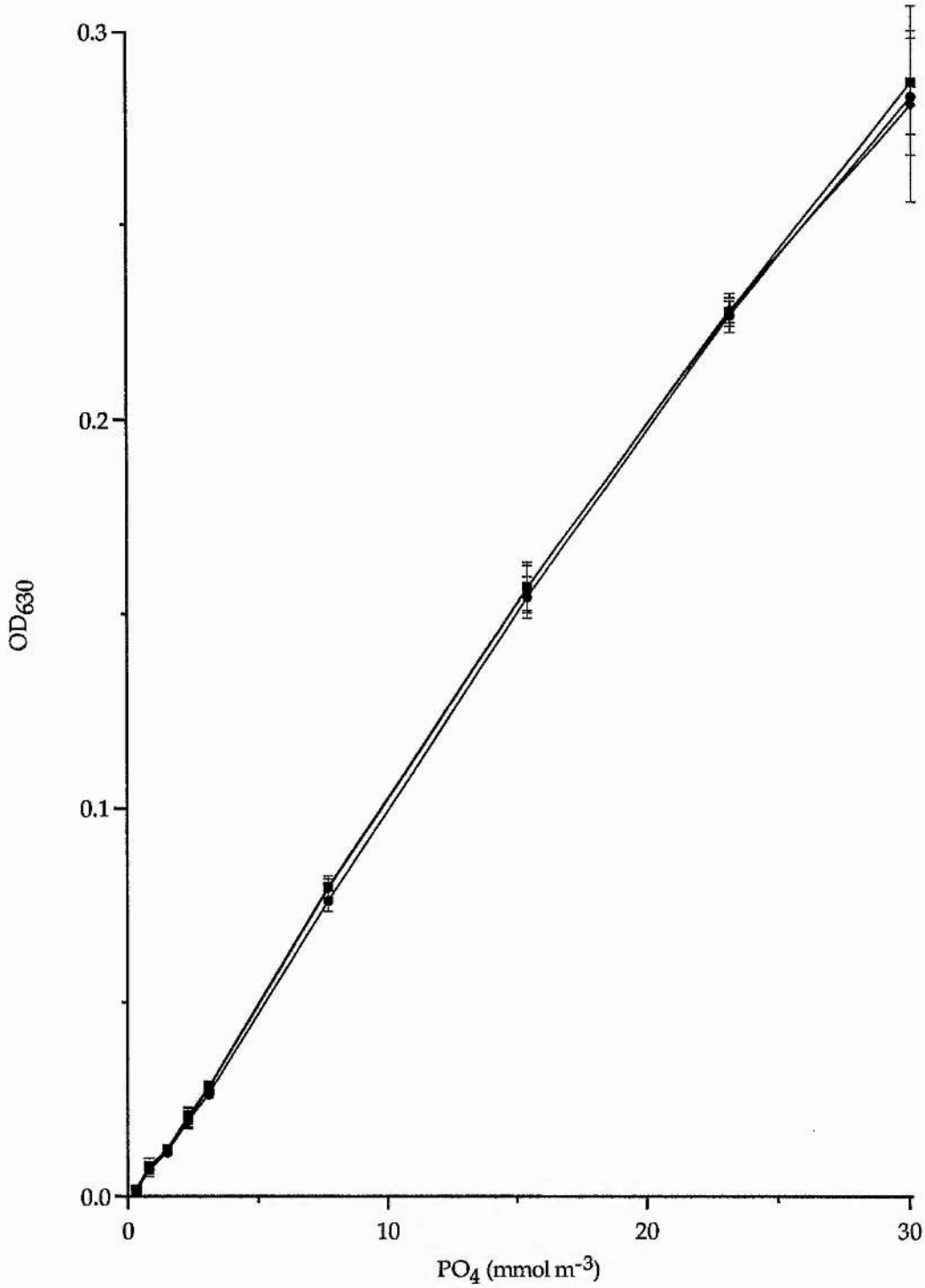

Figure 2.2 Standard curve for malachite green ortho-phosphate assay measured at 630 nm after 5 (■-■), 10 (◆-◆), and 15 (●-●) minutes reaction time. Values are means ± s.d. of triplicate samples of each concentration. The s.d. may be too small to be seen.

rapid, sensitive and reliable assay, measured 10 min after the addition of the malachite green reagent and compared to a 30.1 mmol P m^{-3} standard of K_2HPO_4.

2.2.8 *Source and Characteristics of Wastewater and Seawater*

Wastewater (primary sewage effluent) was collected from the treatment works at St Andrews, which receives on average 6.8 million litres (calculated from average flow rate) of wastewater per day from domestic sources. The wastewater is the supernatant left after preliminary screening and primary sedimentation processes. To establish the typical characteristics of this effluent, measurements of physical and chemical properties were made at monthly intervals from March 1991 to October 1993 and the mean and range values were calculated. Temperature, pH, conductivity, and salinity were measured directly. Dissolved nutrient concentrations ($N\text{-}NO_3^-$, $N\text{-}NO_2^-$, $N\text{-}NH_4^+$, $N\text{-}CO(NH_2)_2$ and $P\text{-}PO_4^{3-}$) were determined from triplicate samples. Prior dilution of the wastewater with M-Q water by 1:9 and 1:1 was necessary for measurement of $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ respectively.

The physical and chemical properties of seawater samples were also analysed at monthly intervals, and the mean and range values were calculated.

2.2.9 *Determination of Optimal Wastewater Dilution*

Dilutions of wastewaters of between 2 and 50% have been found suitable for algal growth and treatment (Ryther *et al.*, 1972; Goldman *et al.*, 1974a; Strain *et al.*, 1986; Paniagua-Michel *et al.*, 1987; Pantastico, 1987; Tam

& Wong, 1989), however the optimal dilution will vary with the particular characteristics of the wastewater used. Primary sewage effluent diluted with sterile seawater was used in this study to screen algal species for their ability to remove nutrients. No attempt was made to remove bacteria and naturally occurring plankton from the wastewater by filtration. The optimal dilution of the wastewater for growth of marine microalgal species was determined through preliminary experiments in which 16 algal species were grown in triplicate on a range of wastewater:seawater mixtures (1:0, 3:1, 1:1, 1:3, and 1:19). Salinities varied from 3 ‰ in the 1:0 dilution to 32 ‰ in the 0:1 dilution. A 10 ml inoculum of exponential phase algae was added to flasks containing 90 ml of diluted wastewater and the OD_{540} of triplicate samples was measured at weekly intervals for three weeks.

2.3 Results

2.3.1 Isolation and Identification of Endemic Microalgae

A total of 66 endemic isolates were obtained by pipette isolation and wastewater enrichment from the October 1990 and April 1991 plankton tows (Table 2.1). Microscopic analysis of the fresh tow samples revealed a variety of algal types, including green filamentous species and bacillariophyceaen species of all sizes. However, many of these species, especially the green filamentous species, died during the two week incubation period.

Those endemic isolates which have not yet been fully identified were defined (by class) as either Bacillariophyceae (B, unicellular or aggregated diatoms), Chlorophyceae (C, coccoid or flagellated green algae), Cyanophyceae (CY, filamentous chains) or Rhodophyceae (R, coccoid pink algae) and designated by an isolation code (Table 2.1). For example SA90B2

represents St Andrews, 1990, Bacillariophyceae Isolate No. 2. Unialgal cultures of all species were routinely maintained in exponential growth under laboratory conditions optimal for microalgal species from temperate climates.

2.3.2 *Selection of Biomass Determination Method*

For all the species tested, similar results were obtained using the four different methods of determining algal biomass. The results with *Nannochloropsis oculata* are shown in Figure 2.3. Measurements of biomass by all of the methods correlated well with culture dilution at low sample concentrations, but only cell counts and OD_{540} provided linear results over the entire range of dilutions tested (Fig. 2.3). Incomplete extraction of chl *a*, even after homogenisation, was a problem with both *Chlorella salina* and *Nitzschia ovalis*. Measurement of optical density (OD_{540}) was selected as the biomass determination method since measurements could also be made on samples taken directly from the culture, which enabled more species to be tested simultaneously.

The absorbance values measured for the five microtitre plate spectrophotometer filters, all correlated well with culture dilution (Fig. 2.4), and most linear results were obtained at 405, 490, and 570 nm. The wavelength chosen for the microplate format OD measurements was 570 nm since it was closest to the 540 nm wavelength originally used.

2.3.3 *Limits of Exponential Growth*

The OD_{570} of 83 microalgal species and isolates grown in batch culture on E-S medium using a 10 % inoculum was measured daily for two

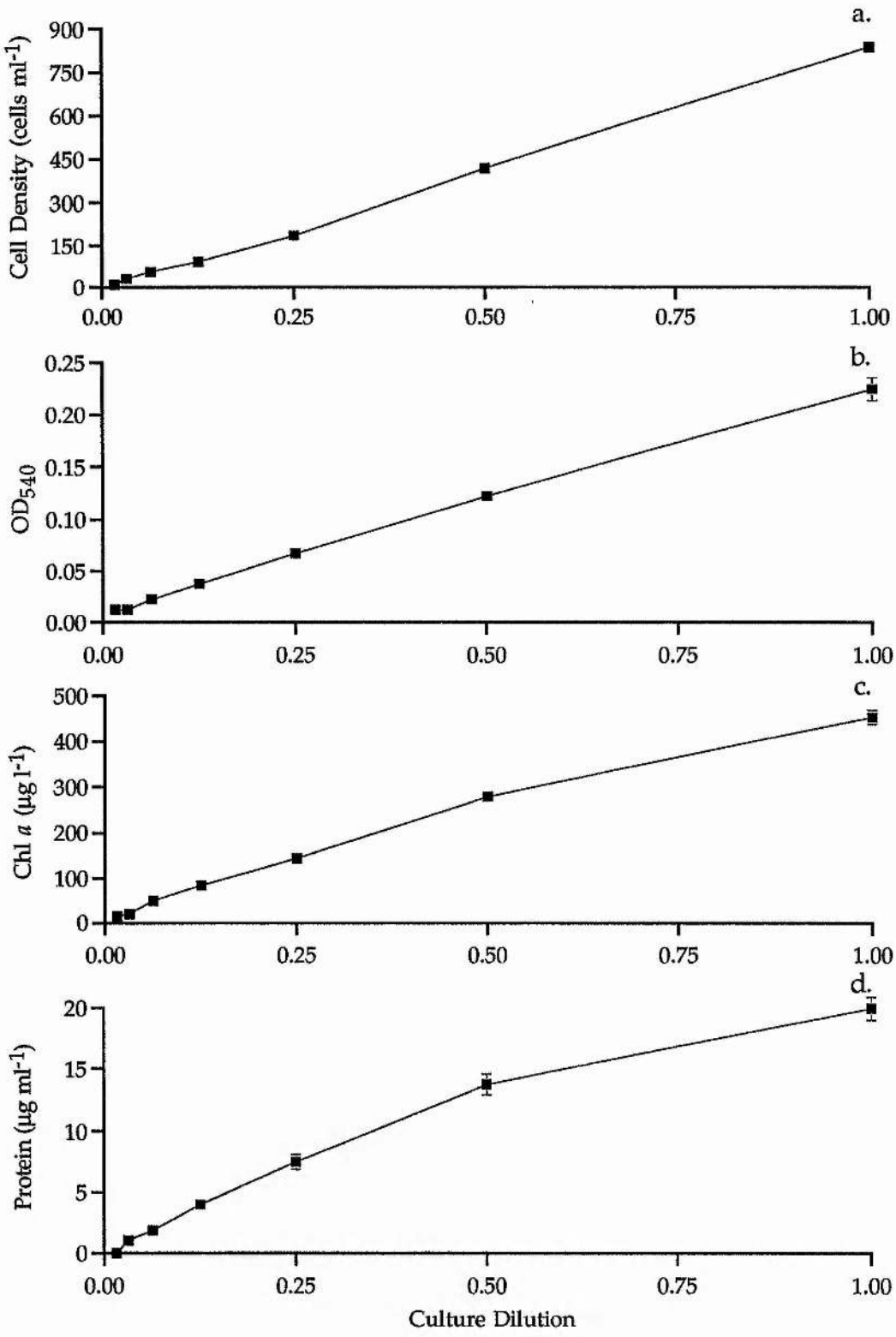

Figure 2.3 Comparison of four biomass determination techniques. a.) cell density (cells ml^{-1}), b.) OD_{540}, c.) chl *a* ($\mu g\ l^{-1}$), and d.) protein ($\mu g\ ml^{-1}$) for a dilution series of *Nannochloropsis oculata* (1.00 corresponds to undiluted culture). Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

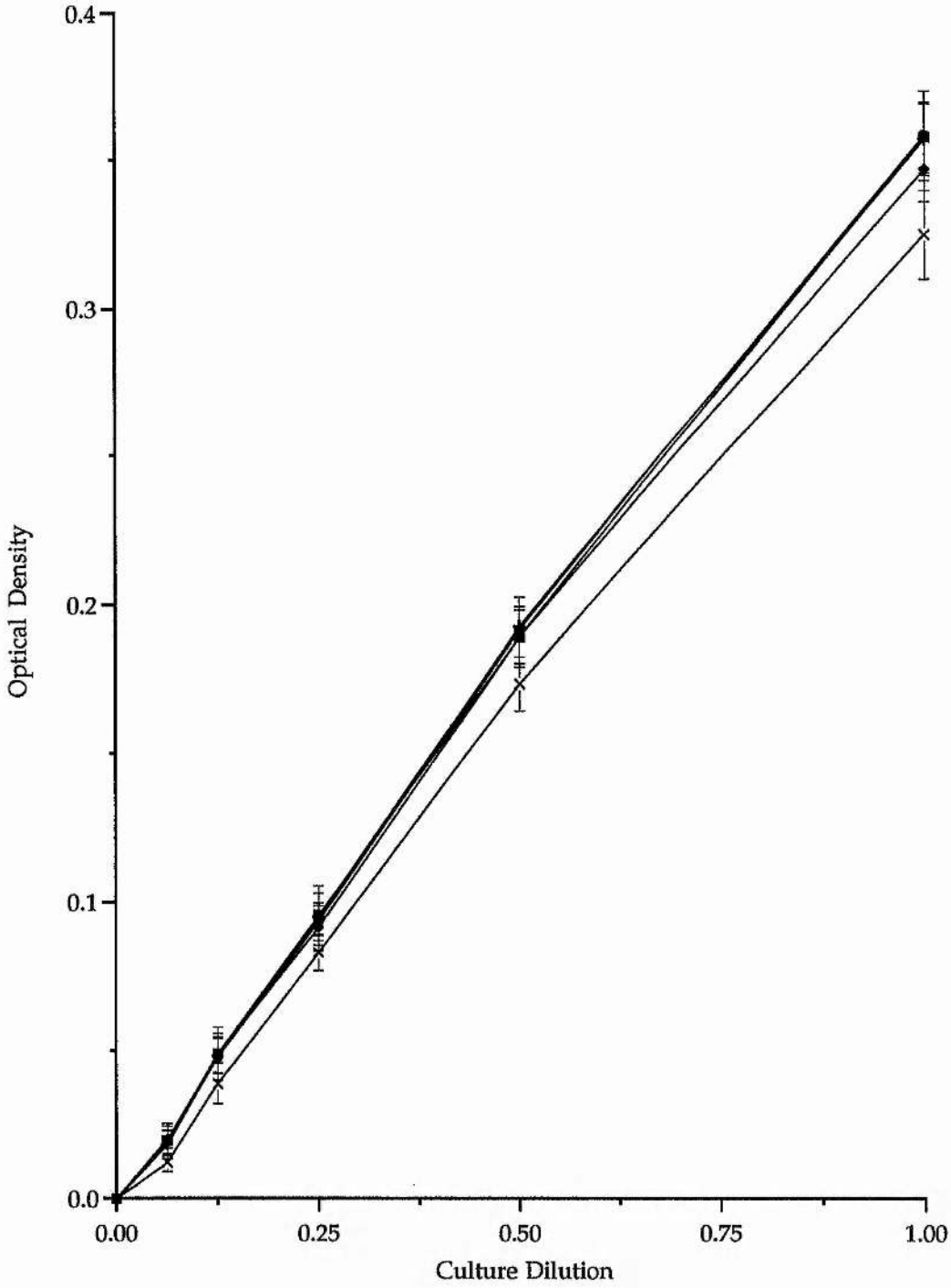

Figure 2.4 Comparison of absorbance values measured using filters of wavelengths (405 nm (■-■), 450 nm (◆-◆), 490 nm (●-●), 570 nm (✚-✚), and 630 nm (✖-✖)) in the microtitre plate spectrophotometer, for a dilution series of *Phaeodactylum tricornutum* (1.00 corresponds to undiluted culture). Values are means ± s.d. of triplicate samples for each dilution.

weeks. The aim of this was to determine the growth rate, doubling time and duration of exponential growth phase from the change in OD_{570} (Appendix 2; Table A 2.1). All but eight of the 47 species and isolates for which growth was able to be measured were found to be in exponential growth phase on day seven of the experiment. Aggregation of the algal cells affected the sampling of the remaining 36 of the species and distorted the OD_{570} measurements. However, in most cases, seven day old cultures provided exponential phase inocula for the screening experiments.

2.3.4 Wastewater and Seawater Characteristics

The mean and range values of the physical and chemical characteristics of wastewater and seawater are given in Table 2.2. Variations of these characteristics over the year between November 1992 and October 1993 are shown in Figures 2.5 and 2.6. The main nutrients in the wastewater were ortho-phosphate and ammonium (Table 2.2), while other nitrogen sources were present only in low concentrations. The concentration of both ortho-phosphate and ammonium, generally remained constant over the period of testing (Fig. 2.6; Table 2.2). Since the St Andrews sewerage and storm water drainage systems are connected, rainfall affected the nutrient concentrations in the primary effluent. On days with heavy rainfall ammonium and ortho-phosphate concentrations decreased, while nitrate and nitrite concentrations increased slightly. There was little variation in the nutrient concentrations of the seawater samples which were much lower than those in the wastewater, and thus made an insignificant contribution to the nutrient levels in the diluted wastewater (Table 2.2; Fig. 2.6). The physical characteristics of both the wastewater and seawater were relatively constant over the period of measurement, except temperature which changed seasonally (Figure 2.5).

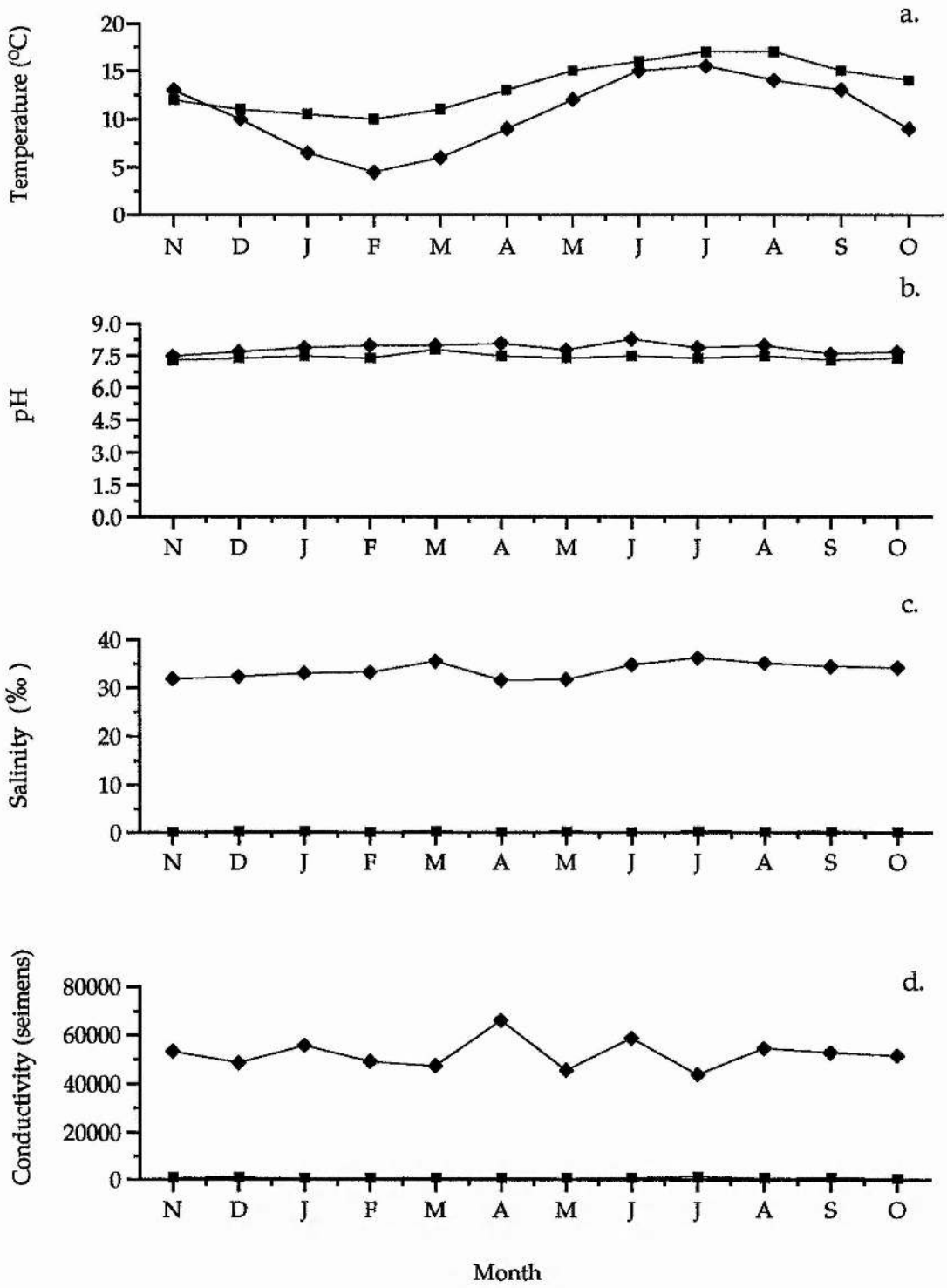

Figure 2.5 Variation in physical characteristics of primary sewage effluent (■-■) and St Andrews Bay seawater (◆-◆) measured over one year (11/92 to 10/93). a.) temperature (°C), b.) pH, c.) salinity (‰) and d.) conductivity (seimens). Values are single readings taken at monthly intervals.

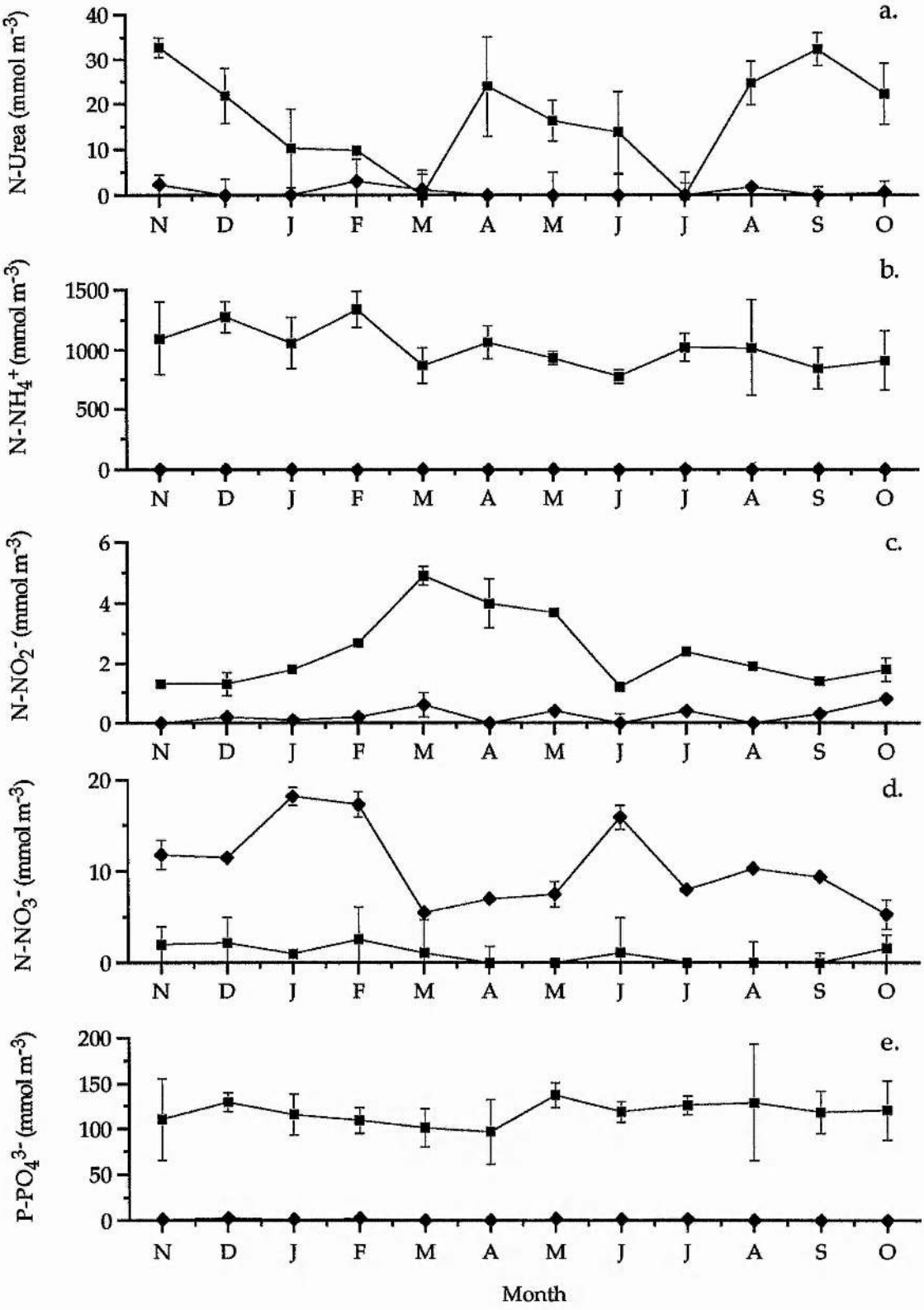

Figure 2.6 Variation in nutrient concentrations (mmol m^{-3}) of primary sewage effluent (■-■) and St Andrews Bay seawater (◆-◆) measured over one year (11/92 to 10/93): a.) urea, b.) ammonium, c.) nitrite, d.) nitrate, e.) ortho-phosphate. Values are means ± s.d. of triplicate samples taken at monthly intervals. Note variation in scales.

Table 2.2 Nutrient characteristics and physical properties of primary sewage effluent and seawater measured between March 1991 and October 1993.

Characteristic	Primary Effluent		Seawater	
	Mean ± s.d.	Range	Mean ± s.d.	Range
Temperature	13.9 ± 2.1	19.0 - 10.0	12.0 ± 2.6	15.5 - 6.0
pH	7.4 ± 0.2	7.8 - 6.9	7.8 ± 0.4	8.5 - 7.0
Salinity (‰)	0.2 ± 0.1	0.4 - 0.1	32.9 ± 3.2	36.3 - 22.0
Conductivity (seimens)	813.2 ± 166.7	3650.1 - 382.0	53574.5 ± 9243.0	67944.2- 26224.4
[1]BOD (mg l^{-1})	170.3 ± 48.8	291 - 82	n.d.	n.d.
[1]COD (mg l^{-1})	382.4 ± 111.6	704 - 190	n.d.	n.d.
[1]SS (mg l^{-1})	98.4 ± 22.5	160 - 40	n.d.	n.d.
Urea-N (mmol m^{-3})	21.3 ± 10.7	42.8 - 0.0	0.5 ± 2.3	4.1 - 0.0
N-NH_4^+ (mmol m^{-3})	920.1 ± 179.6	1854.8 - 323.7	1.8 ± 1.3	4.2 - 0.0
N-NO_2^- (mmol m^{-3})	2.8 ± 2.1	7.4 - 1.2	0.3 ± 0.4	1.1 - 0.0
N-NO_3^- (mmol m^{-3})	19.6 ± 13.2	37.4 - 0.0	9.4 ± 10.9	24.3 ± 0.0
P-PO_4^{3-} (mmol m^{-3})	117.9 ± 23.7	218.4 - 61.6	1.7 ± 1.1	3.4 - 0.0

[1]Data from Fife Regional Council

BOD: Biochemical oxygen demand
COD: Chemical oxygen demand
SS: Suspended solids

n.d. not done

2.3.5 *Determination of Optimal Wastewater Dilution*

Sixteen microalgal species and isolates were grown on a dilution series of wastewater with seawater (1:0, 3:1, 1:1, 1:3, and 1:19) to determine the dilution at which most grew best. Four of the 16 microalgae tested (SA90B4, SA90C1, *Phaeodactylum tricornutum,* and *Tetraselmis* sp.) grew on all wastewater dilutions including undiluted wastewater. Figure 2.7 shows the OD_{540} at each wastewater dilution for *Phaeodactylum tricornutum.* Cultures at all dilutions remained unialgal but highest growth was obtained with the 3:1 and 1:1 dilutions, and the 1:1 wastewater dilution had no lag phase. Therefore the 1:1 seawater dilution of wastewater was chosen as the medium on which to screen the microalgae.

2.4 *Discussion*

Experiments described here established both the optimal conditions for the simultaneous screening of a large number of microalgal isolates, and analytical techniques, based on microplate assays, for the measurement of algal biomass and nutrient concentrations.

Optical density was found to be the most reliable, reproducible and rapid method of determining microalgal growth and biomass; the other methods tested were too time consuming to be used for the simultaneous measurement of many species. Direct measurement of OD is generally accepted to correlate well with cellular density (Sorokin, 1979; Lyon & Woo, 1980; Fabregas *et al.,* 1984). All five filters (405-630 nm) of the microtitre plate spectrophotometer were suitable for measuring OD, and wavelengths between 438 and 678 nm are known to give accurate readings (Sorokin, 1979; Richmond, 1983). It was not practical to standardise the OD of the algal inoculum since algal species have very different pigment

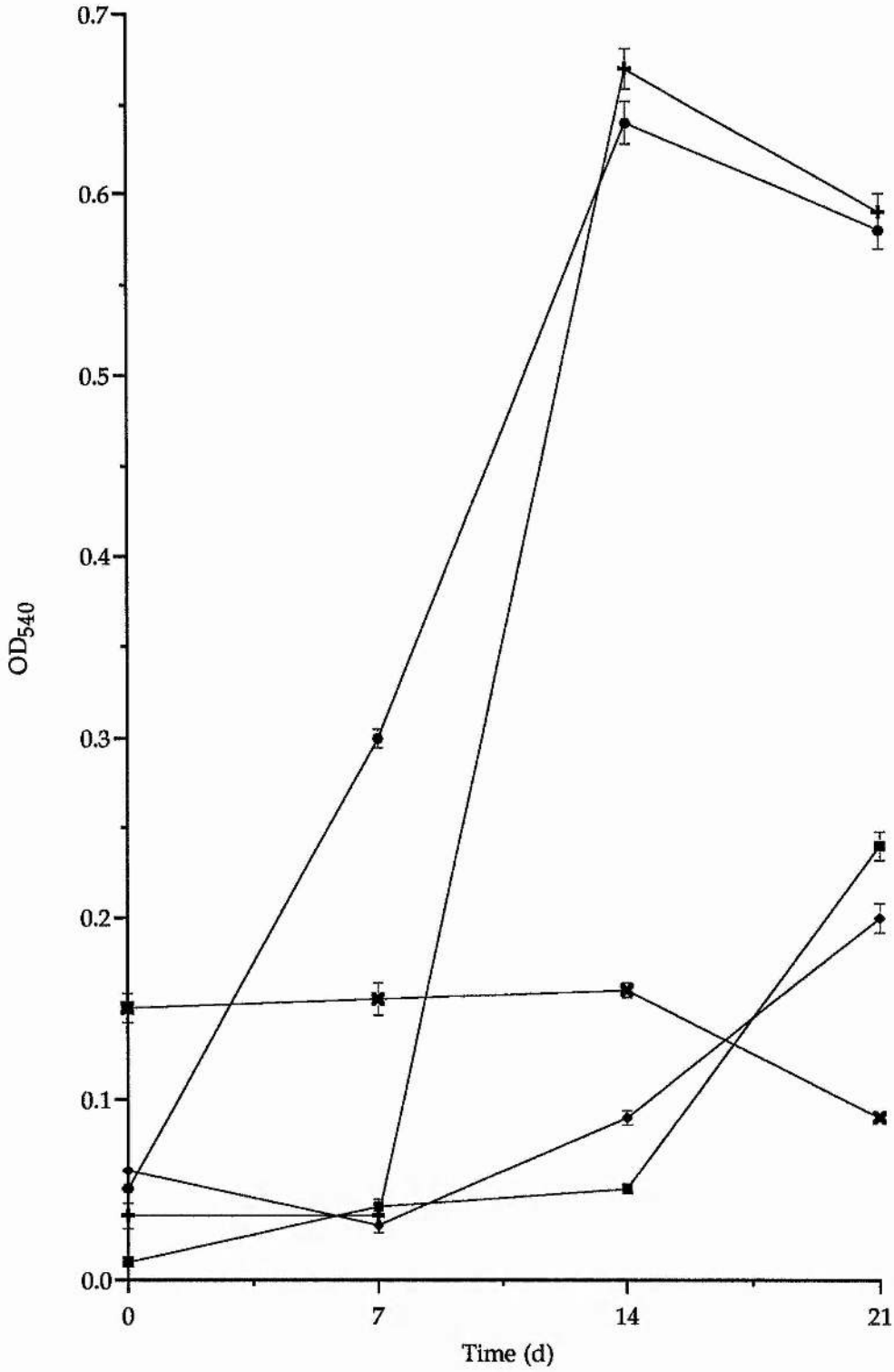

Figure 2.7 The effect of wastewater:seawater dilution on the relative growth (OD_{540}) of *Phaeodactylum tricornutum*. The following wastewater:seawater mixtures were used: 1:0 (✱-✱), 3:1 (✚-✚), 1:1 (●-●), 1:3 (◆-◆), and 1:19 (■-■). Values are means ± s.d. of triplicate samples from triplicate cultures. The s.d. may be too small to be seen.

compositions, and the time required to make cell counts of all cultures prior to experiments would have limited the number of microalgae that could have been screened. Growth characteristics indicated that seven day old cultures gave exponential phase algae at densities suitable for inocula.

Ammonium and ortho-phosphate were the main nutrients in the primary sewage effluent and had the most stable concentrations (Table 2.2; Fig. 2.6), which is typical of most wastewaters (Grobbelaar *et al.*, 1988; Oswald, 1988a). Therefore, only the changes in concentration of these two nutrients were measured in the screening experiments. A 1:1 dilution of the wastewater with seawater was found to be the best growth medium for the marine microalgal microalgae tested. Marine phytoplankton have been successfully grown on 1:1 seawater diluted secondary sewage effluent by several other authors (Ryther *et al.*, 1972; Goldman & Stanley, 1974; Goldman *et al.*, 1974a & b). Sebastian & Nair (1984) found high levels of treatment by *Scenedesmus obliquus*. on 1:1 freshwater diluted secondary effluent. In the present study, some marine microalgae were even capable of growing on undiluted wastewater.

Chapter Three

Batch Culture Screening of Marine Microalgal Nutrient Removal from Wastewater Diluted 1:1 with Seawater

3.1 *Introduction*

Although the true treatment capability of a microalga can only be determined through continuous culture, this was not practical for the simultaneous screening of 102 species and isolates. In contrast, batch cultures allow for the precise control of the growth conditions and easy assessment of the rate of growth. They are therefore a helpful tool for establishing, in part or completely, the conditions for optimal growth and treatment of the wastewater on which a continuous culture could then be based (Goldman, 1979; Fabregas *et al.*, 1984; Fabregas *et al.*, 1985a & b). Therefore a series of batch culture experiments was designed to screen simultaneously a wide variety of marine microalgae for their ability to remove inorganic nutrients (ammonium N-NH_4^+ and ortho-phosphate P-PO_4^{3-}) from wastewater diluted 1:1 with seawater and to remain dominant in culture. Initial screening experiments were performed under controlled environmental conditions, which are optimal for the growth of microalgal species from temperate regions (Section 2.2.1).

Under a given set of culture conditions, some algal species will be naturally more competitive than others (Birch & Bachofen, 1988). Therefore the screening experiments were repeated in an open greenhouse to determine whether those species selected under controlled conditions were capable of treating wastewater under ambient summer conditions. Microalgal high-rate ponds only receive light through the pond surface, have a large volume (e.g. 2,000-600,000 l), and are open cultures (Ryther *et al.*, 1972; Dodd, 1986; Oswald, 1988b). The effect of culture apparatus and volume on the ability of the best-treating microalgae to remove nutrients under ambient conditions was also investigated.

3.2 *Materials and Methods*

3.2.1 *Small-scale Batch Screening under Controlled Conditions*

One hundred and two algal species and isolates were screened for the ability to remove nutrients from wastewater diluted 1:1 with seawater (Section 2.2.9). Of these, 36 species were obtained from culture collections and 66 were endemic isolates (Sections 2.2.3, 2.3.1; Table 2.1).

Small-scale experimental batch cultures of 50 ml volume were made up in sterile 100 ml flasks (sealed with cotton wool plugs and aluminium foil caps) and grown in the Conviron controlled environmental incubator (Section 2.2.1). Cultures were mixed by swirling the flasks once a day. Wastewater diluted 1:1 with seawater had a salinity half that of E-S medium (31.4 ± 0.7 ‰), and contained ammonium, rather than nitrate, as the main nitrogen source. Consequently, algal cultures were pre-adapted to 1:1 diluted wastewater prior to the screening experiments. Three replicate cultures of each algal species or isolate were pre-adapted by mixing 25 ml of exponential phase stock algal culture (Section 2.3.3) with 25 ml of wastewater and incubating for one week. The screening experiments began by replacing half the culture volume (25 ml) of the pre-adapted algal culture in each flask with freshly made up 1:1 diluted wastewater. Nutrient ($N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$) concentrations remaining in each algal culture at the end of the treatment period were determined simultaneously using the microplate format (Sections 2.2.7). Algal culture purity was confirmed by microscopic examination (Section 2.2.2) at the time of nutrient measurement. The screening in each experiment was repeated in the form of a sequential batch culture by replacing half the culture volume with freshly made up 1:1 diluted wastewater.

The nutrient concentrations in control cultures of 1:1 diluted wastewater to which algae had not been added may have changed over the duration of the screening experiment as a result of the activity of bacteria present in the wastewater. Therefore the nutrient ($N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$) concentration removed by each algal species was calculated by subtracting the average nutrient concentration remaining in the triplicate algal cultures from that in control flasks without algae. The mean removal value for the replicate experiments was then calculated.

3.2.2 *Small-scale Batch Culture Screening under Ambient Conditions*

Small-scale experimental batch cultures described in Section 3.2.1 were also grown under conditions of ambient light and temperature in an open greenhouse. Since small cultures are much more susceptible to temperature fluctuations than cultures of a larger volume, the flasks were placed in a perspex water-bath which contained enough water to cover the culture medium inside the flasks. Daily temperature range was measured using a maximum-minimum thermometer immersed in the water-bath. The daily variation in light intensity was measured in this and subsequent experiments using a quantum photometer (Q101-4; Macam photometrics Ltd, Livingston, Scotland). The sequential batch cultures of the screening experiment and prior pre-adaptation to 1:1 diluted wastewater under ambient culture conditions were as described in Section 3.2.1. In addition, microscopic examination of the culture was made one week after the final nutrient analysis.

3.2.3 *Medium-Scale Open Batch Culture Screening under Ambient Conditions*

Medium-scale experimental batch cultures of 400 ml volume were made up in 500 ml beakers and grown in the open greenhouse. A black plastic sleeve covered the outside of each beaker up to the 400 ml mark so that light could only penetrate through the culture surface, simulating the conditions in an algal wastewater treatment pond. Algal cultures were placed on an orbital shaker (Lh Engineering Ltd, Mk V) which mixed the cultures by rotating a marble within each beaker. Sequential batch cultures of the screening experiment and prior pre-adaptation to both 1:1 diluted wastewater under ambient culture conditions were as described in Section 3.2.1 using scaled up volumes for the 400 ml cultures. Evaporation from the open cultures was compensated for by making up the volume with sterile distilled water at the time of sampling for nutrient measurement. Daily temperature range was measured using a maximum-minimum thermometer immersed in a sleeve covered culture beaker containing 400 ml water.

3.2.4 *Large-Scale Open Tub Batch Culture Screening under Ambient Conditions*

Large-scale experimental batch cultures of 6 l volume were made up in 10 l white polypropylene tubs and grown in the open greenhouse. A black plastic sleeve covered the outside of each tub up to the 6 l mark. Algal cultures were mixed by stirring with a glass rod once a day. The sequential batch cultures of the screening experiment and prior pre-adaptation to 1:1 diluted wastewater under ambient culture conditions were as described in Section 3.2.1, with scaled up volumes for the 6 l cultures. Evaporation from

the open cultures was compensated for as in Section 3.2.3. Daily temperature range was measured using a maximum-minimum thermometer immersed in a sleeve covered culture tub containing 6 l water. The temperature range, solar irradiance and photoperiod in this and all previous batch culture screening experiments are compared to the controlled culture conditions in Table 3.1.

3.3 Results

3.3.1 Small-scale Batch Screening under Controlled Conditions

Of the original 102 species and isolates screened, 84 survived pre-adaptation to 1:1 diluted wastewater. Of those that did not survive, six species (*Nitzschia ovalis, Oscillatoria animalis, Spirulina platensis, Oxyrrhis marina, Chrysochromulina chiton*, and *Coccolithophora* sp.) were from the culture collections and the other 12 were all endemic bacillariophyceaen isolates. The pre-adapted microalgae were screened twice for their ability to remove ammonium and ortho-phosphate over seven days treatment time. Thirty-five of these species and isolates remained in unialgal culture and displayed a range of abilities to remove nutrients (Fig. 3.1). Fifteen species and isolates showed little difference in their nutrient removal ability. All removed >98.1 % of the ammonium and >85.2 % of the ortho-phosphate compared to control concentrations (824.4 ± 58.5 mmol m^{-3} $N\text{-}NH_4^+$ and 49.4 ± 3.2 mmol m^{-3} $P\text{-}PO_4^{3-}$). Of the remainder, 14 species and isolates removed ammonium and ortho-phosphate at lower rates, including six species and isolates which, despite removing >83.8 % of the ammonium, removed <53.5 % of the ortho-phosphate compared to controls. The ortho-phosphate concentration increased in the cultures of the other six species (which included *Pavlova lutheri, Prymnesium parvum, Stichococcus bacillaris,*

Figure 3.1 Key

1 SA90B2
2 *Coccolithus* sp.
3 *Tetraselmis tetrathele*
4 SA91B12
5 SA91B43
6 SA90B4
7 *Tetraselmis* sp.
8 SA91C6
9 SA91B27
10 SA90C1
11 SA90C3
12 *Tetraselmis suecica*
13 *Phaeodactylum tricornutum*
14 SA91B39
15 SA90B7
16 SA91CY1
17 SA90R1
18 *Porphyridium purpureum*
19 SA91B32
20 SA91C13
21 *Chlorella stigmatophora*
22 SA90B3
23 SA91C10
24 *Nannochloropsis oculata*
25 *Tetraselmis rubens*
26 SA91B25
27 *Chlorella salina*
28 SA91B37
29 *Tetraselmis verrucosa*

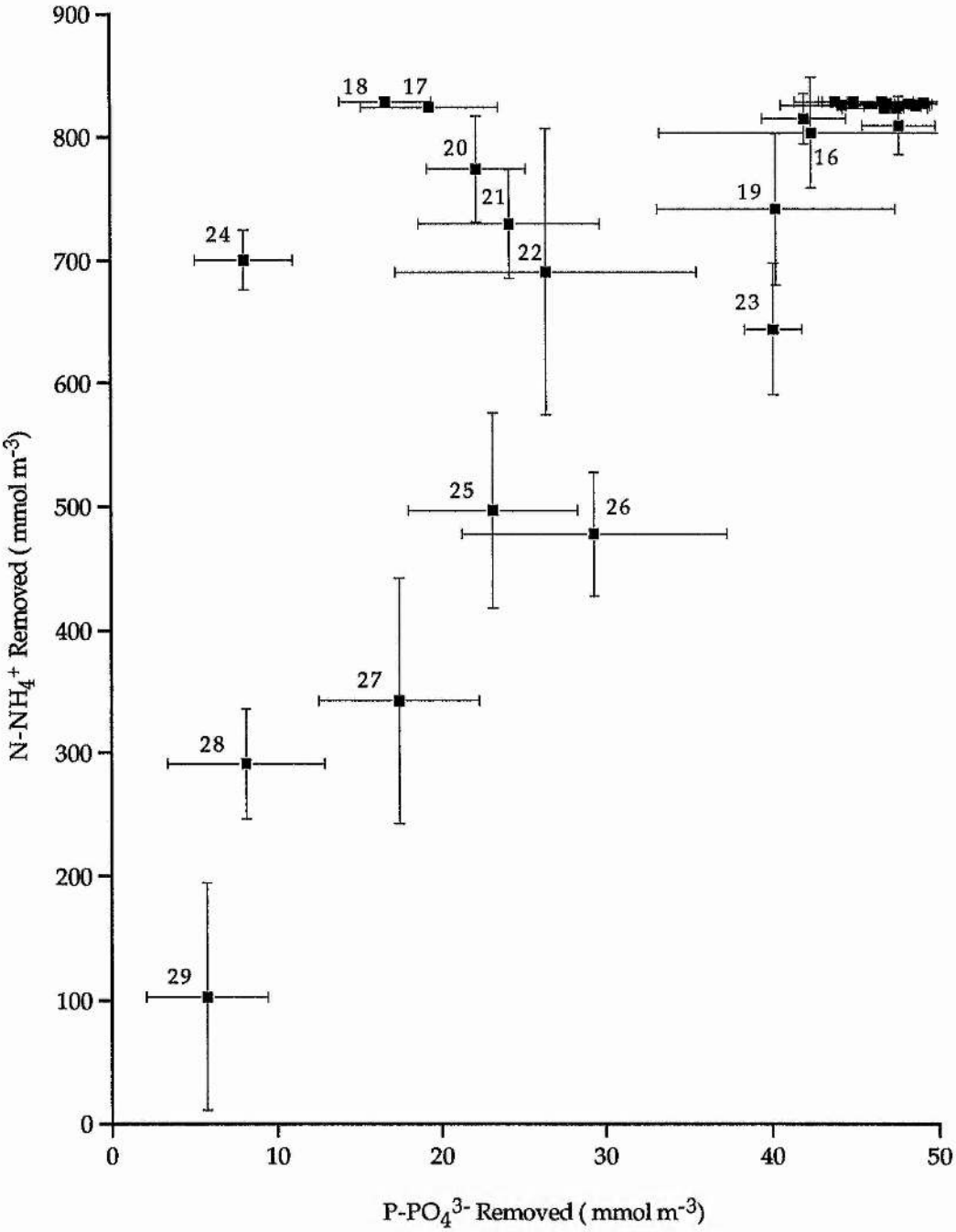

Figure 3.1. Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species cultured for seven days on wastewater diluted 1:1 with seawater in small-scale (50 ml) batch cultures under controlled conditions. Only the 29 species and isolates which remained unialgal and removed both $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ are shown. Values are means of means ± s.d. of duplicate experiments in which final nutrient concentrations in triplicate algal cultures were compared to final concentrations in controls without algae (824.4 ± 58.5 mmol m^{-3} $N\text{-}NH_4^+$, 49.4 ± 3.2 mmol m^{-3} $P\text{-}PO_4^{3-}$). The s.d. may be too small to be seen.

and all three species of *Rhodomonas*), although in some cases they removed as much as 60.7 % of the ammonium compared to controls (Appendix 3; Fig. A 3.1). A direct relationship between removal of the two nutrients was shown by the 35 species and isolates which remained in unialgal culture (determined by simple linear regression, $r=0.807$, $F_{1,32}=59.63$, $p<0.001$).

Daily nutrient analysis revealed that some species removed ammonium and ortho-phosphate in less than two days (Appendix 3; Fig. A 3.2). Accordingly, a second screening series was undertaken with a treatment time of two days. Sixty species were used, including the 35 species and isolates from the seven day screening which had remained in unialgal culture. The remaining 25 culture collection species were re-screened since many had been identified in the literature for their potential for use in a microalgal wastewater treatment process (Section 1.7.2; Table 1.8). All species survived pre-adaptation, and four repetitions of the sequential batch culture were made from which the mean removal values were calculated. Although the control nutrient levels (295.5 ± 50.0 mmol m^{-3} $N\text{-}NH_4^+$ and 35.2 ± 7.4 mmol m^{-3} $P\text{-}PO_4^{3-}$) of the wastewaters used in the two day treatment experiments were lower, results of nutrient removal were similar to those of the seven day experiments (Fig. 3.2). Twenty-four species remained in unialgal culture, of which 11 showed little difference in treatment ability with >91.4 % removal of ammonium and >90.4 % removal of ortho-phosphate compared to control flasks without algae (Fig. 3.3). Of the remainder, three species removed >76.2 % of the ammonium but <46.9 % of the ortho-phosphate, while for six species (*Chlorella stigmatophora*, SA90B5, SA91B27, *Porphyridium purpureum*, *Rhodomonas* sp., and SA90B3) the ortho-phosphate concentration compared to control flasks increased, despite removal of as much as 95.0 % of the ammonium (Appendix 3; Fig A 3.3). A direct relationship between removal of the two nutrients was also

Figure 3.2 Key

1 SA91B33
2 SA90C2
3 SA91B43
4 *Tetraselmis suecica*
5 SA90B2
6 SA90C3
7 *Tetraselmis tetrathele*
8 *Tetraselmis* sp.
9 *Phaeodactylum tricornutum*
10 SA91B12
11 SA90B4
12 SA91C10
13 *Chlorella salina*
14 *Dunaliella tertiolecta*
15 SA91C13
16 SA91B39
17 SA90C1
18 *Coccolithus* sp.

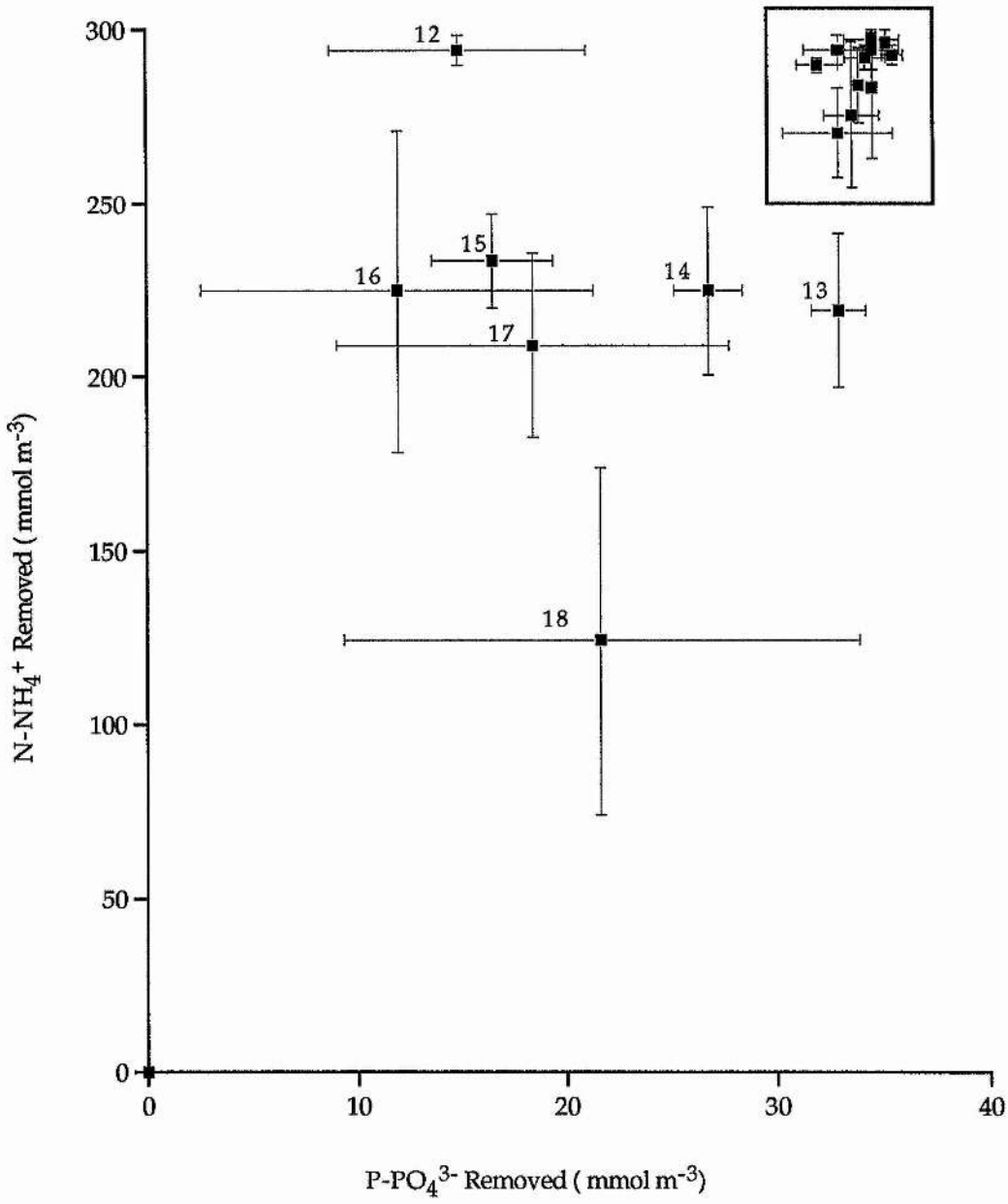

Figure 3.2. Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species cultured for two days on wastewater diluted 1:1 with seawater in small-scale (50 ml) batch culture under controlled conditions. Only the 18 species and isolates which remained unialgal and removed both $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ are shown. Values are means of means ± s.d. of four replicate experiments in which final nutrient concentrations in triplicate algal cultures were compared to final concentations in controls without algae (295.5 ± 50.0 mmol m^{-3} $N\text{-}NH_4^+$, 35.2 ± 7.4 mmol m^{-3} $P\text{-}PO_4^{3-}$). Species within the box are shown in detail in Fig. 3.3.

Figure 3.3 Key

1 SA91B33
2 SA90C2
3 SA91B43
4 *Tetraselmis suecica*
5 SA90B2
6 SA90C3
7 *Tetraselmis tetrathele*
8 *Tetraselmis* sp.
9 *Phaeodactylum tricornutum*
10 SA91B12
11 SA90B4

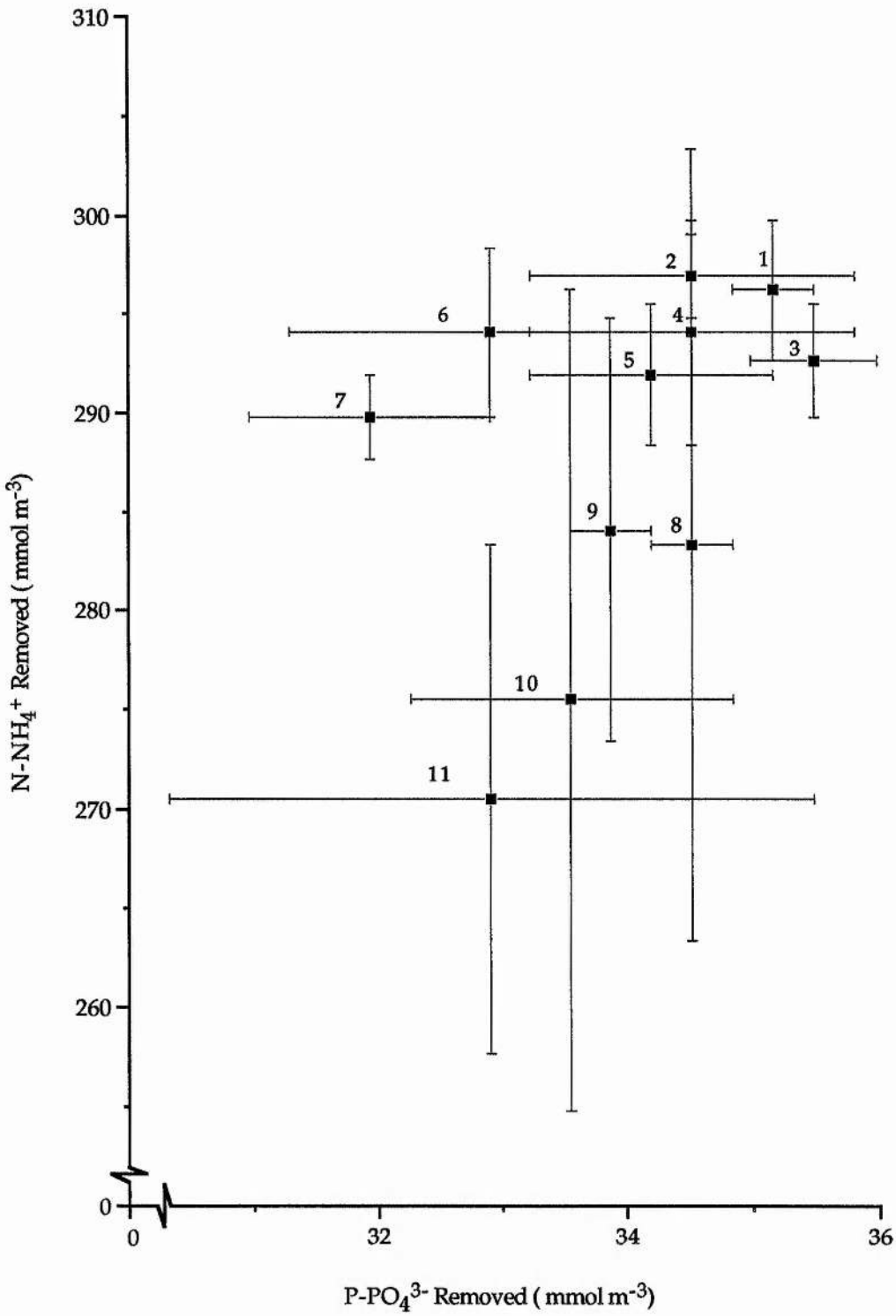

Figure 3.3 Detail of Figure 3.2. Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by best-treating algal species during two-day screening in small-scale (50 ml) batch cultures under controlled conditions. Values are means of means ± s.d.

found in this experiment (determined by simple linear regression, r=0.724, $F_{1,23}$=25.35, p<0.001).

3.3.2 *Small-scale Batch Culture Screening under Ambient Conditions*

The 60 species and isolates screened in the two day experiment under controlled conditions were re-screened under ambient conditions in an open greenhouse. Thirty-six species and isolates survived pre-adaptation to 1:1 diluted wastewater under ambient conditions. Of the 24 species which did not survive, sixteen species (*Chaetoceros calcitrans, Nitzschia longissima, Nitzschia ovalis, Thalassiosira weissflogii, Chlamydomonas reginae, Dunaliella salina, Oscillatoria animalis, Spirulina platensis, Amphidinium cartarae, Oxyrrhis marina, Micromonas, pusilla, Chrysochromulina chiton, Coccolithophora* sp., *Pavlova lutheri, Phaeocystis poucheti* and *Prymnesium parvum)* were from the culture collections and the other eight species were all endemic bacillariophyceaen isolates. Single cultures of the pre-adapted species were screened twice for their ability to remove ammonium and ortho-phosphate over a treatment time of two days.

The results of the two day nutrient removal under ambient conditions were similar to both the seven day and two day experiments under controlled conditions even though culture temperature and irradiance were not constant (Table 3.1). Thirty-four species and isolates remained in unialgal culture and displayed a similar range of abilities to remove nutrients (Fig. 3.4). Five species and isolates removed >91.5 % of both the ammonium and the ortho-phosphate, compared to control concentrations (458.2 ± 12.8 mmol m^{-3} $N\text{-}NH_4^+$ and 32.3 ± 0.3 mmol m^{-3} $P\text{-}PO_4^{3-}$). Of the remainder, 20 species and isolates removed ammonium and

Table 3.1 Summary of the controlled and ambient culture conditions measured during batch culture screening experiments.

Condition	Controlled	Ambient		
	Small-scale	Small-scale	Medium-Scale	Large-scale
Temperature (°C)	14-16	10-31	10-32	12-23
Light Intensity ($\mu E\ m^{-2}\ s^{-1}$)	18-22	500-2000	500-2000	500-2000
Photoperiod (l:d) (h)	12:12	15:9	15:9	18:6

Figure 3.4 Key

1 SA91C13
2 SA90B4
3 SA90B2
4 SA92B48
5 SA91B43
6 SA90C3
7 SA91CY1
8 SA92C17
9 *Dunaliella tertiolecta*
10 SA90C1
11 SA91B33
12 SA91C6
13 *Coccolithus* sp.
14 *Tetraselmis* sp.
15 SA91B25
16 SA91B12
17 *Tetraselmis suecica*
18 *Chlorella salina*
19 *Tetraselmis tetrathele*
20 *Porphyridium purpureum*
21 *Tetraselmis rubens*
22 SA91B47
23 SA91B39
24 SA90B5
25 SA92C16

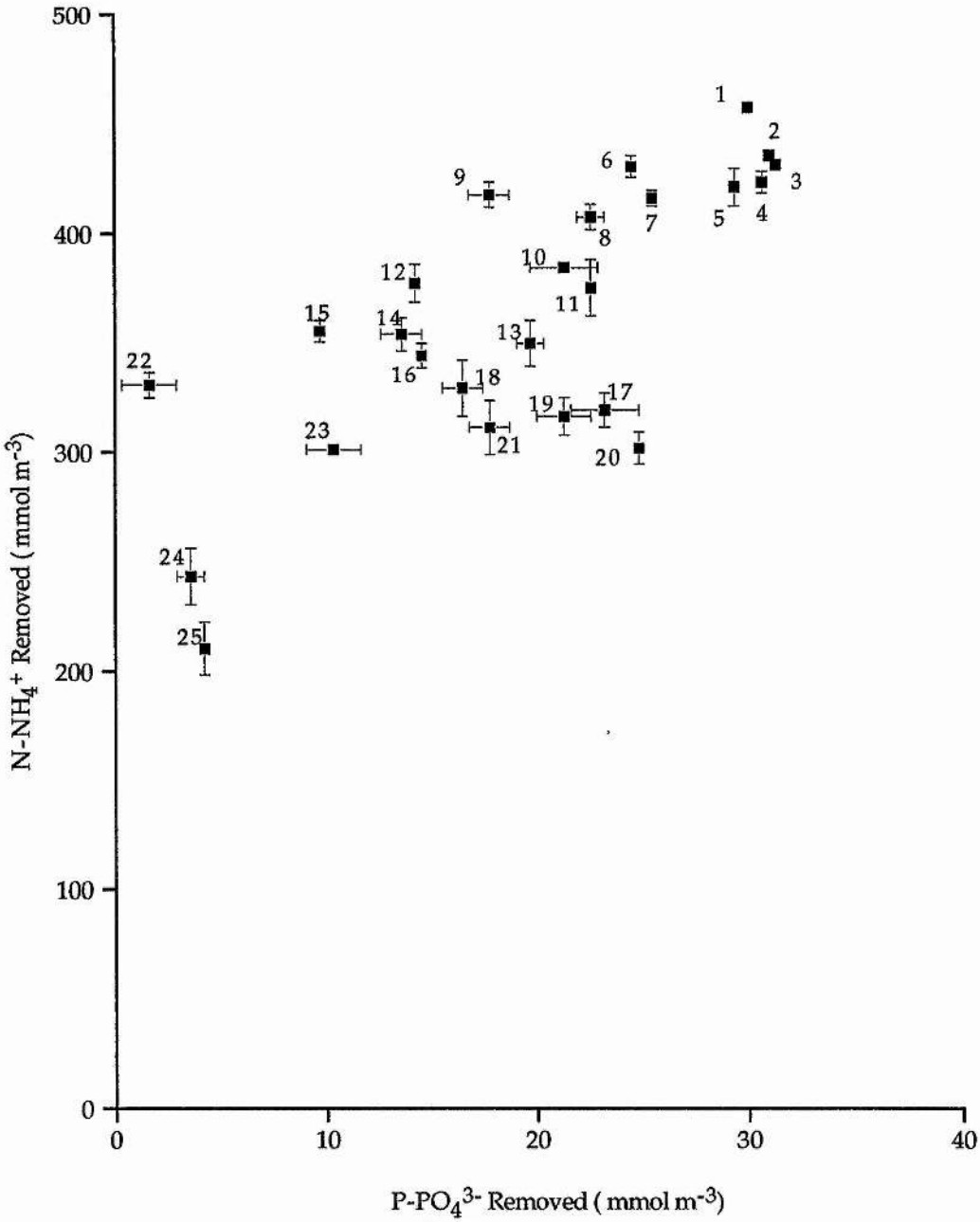

Figure 3.4 Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species cultured for two days on wastewater diluted 1:1 with seawater in small-scale (50 ml) batch cultures under ambient conditions. Only the 25 species and isolates which remained unialgal and removed both $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ are shown. Values are means of means ± s.d. of duplicate experiments in which final nutrient concentrations in algal cultures were compared to final concentrations in controls without algae (458.2 ± 12.8 mmol m^{-3} $N\text{-}NH_4^+$, 32.3 ± 0.3 mmol m^{-3} $P\text{-}PO_4^{3-}$). The s.d. may be too small to be seen.

ortho-phosphate at lower rates, while ammonium or ortho-phosphate concentrations increased for the other 11 species and isolates. Some species removed as much as 73 % of ammonium compared to controls, even though the ortho-phosphate concentration increased (Appendix 3; Fig A 3.4). A direct relationship between removal of the two nutrients was also shown for the 34 species and isolates which remained in unialgal culture (determined by simple linear regression, $r=0.778$, $F_{1,34}=52.07$, $p<0.001$).

Some of the species which demonstrated good nutrient removal in the screening experiments under controlled conditions did not survive screening under ambient conditions. *Phaeodactylum tricornutum* died out during the experiment, and *Dunaliella tertiolecta* and *Coccolithus* sp. died out during the week between the last nutrient measurement and final culture analysis, despite removing >76.3 % of the ammonium and >61.3 % ortho-phosphate.

3.3.3 *Medium-scale Open Batch Screening under Ambient Conditions*

The 25 best-treating species and isolates were selected from the three screening experiments under both controlled and ambient culture conditions for their ability to consistently remove a high percentage of nutrients compared to controls, and for remaining in unialgal culture (Table 3.2). Fourteen of these microalgae were endemic isolates from St. Andrews Bay.

These species were screened in single medium-scale open batch cultures which contained 400 ml of medium. A mixed culture of species (wastewater species) which had grown up in an open batch culture of 1:1 diluted wastewater was also tested. All species survived pre-adaptation to

Table 3.2 The 25 best-treating microalgal species and isolates remaining in unialgal culture ranked in order of combined nutrient removal ($N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$). Values are means of means ± s.d. % removal of three small-scale (50 ml) batch culture screening experiments, two under controlled culture conditions (7-day and 2-day) and one under ambient culture conditions (2-day).

Algal Species	Algal Class	Source	% $N\text{-}NH_4^+$ Removal	% $P\text{-}PO_4^3$ Removal
SA90B2	Bacillariophyceae	Endemic	97.7 ± 2.7	98.0 ± 1.2
SA91B43	Bacillariophyceae	Endemic	97.1 ± 3.7	95.5 ± 4.1
SA90B4	Bacillariophyceae	Endemic	95.6 ± 3.6	94.9 ± 1.0
SA92B48	Bacillariophyceae	Endemic	92.4 ± 0.0	95.0 ± 0.0
SA90C3	Chlorophyceae	Endemic	97.8 ± 2.7	88.2 ± 8.6
SA91CY1	Bacillariophyceae	Endemic	94.1 ± 3.3	82.5 ± 3.5
Tetraselmis suecica	Prasinophyceae	Plymouth	89.9 ± 14.3	86.4 ± 10.8
SA91B33	Bacillariophyceae	Endemic	91.1 ± 9.2	85.0 ± 15.0
Tetraselmis tetrathele	Prasinophyceae	Plymouth	89.1 ± 14.2	84.9 ± 13.7
Tetraselmis sp.	Prasinophyceae	Millport	91.1 ± 10.0	78.3 ± 25.7
SA91B12	Bacillariophyceae	Endemic	89.5 ± 10.5	79.7 ± 24.6
SA91C6	Chlorophyceae	Endemic	91.1 ± 8.7	70.2 ± 26.2
SA92C17	Chlorophyceae	Endemic	88.9 ± 0.0	70.0 ± 0.0
SA90C1	Chlorophyceae	Endemic	85.0 ± 12.1	69.8 ± 16.1
SA91C13	Chlorophyceae	Endemic	90.9 ± 8.8	61.6 ± 22.2
Dunaliella tertiolecta	Chlorophyceae	Plymouth	83.6 ± 7.5	65.6 ± 10.6
Coccolithus sp.	Prymnesiophyceae	Millport	72.8 ± 23.9	73.6 ± 17.5
Phaeodactylum tricornutum	Bacillariophyceae	Plymouth	64.9 ± 46.9	69.4 ± 33.6
SA91B39	Bacillariophyceae	Endemic	80.0 ± 13.5	54.2 ± 30.0
Chlorella salina	Chlorophyceae	Plymouth	62.5 ± 14.8	60.0 ± 24.6
Tetraselmis rubens	Prasinophyceae	Plymouth	64.1 ± 3.8	50.9 ± 4.1
Porphyridium purpureum	Rhodophyceae	Plymouth	80.1 ± 14.8	22.5 ± 49.7
SA90C2	Chlorophyceae	Endemic	49.7 ± 50.8	40.1 ± 58.1
Nannochloropsis oculata	Chlorophyceae	Gatty	78.9 ± 6.0	-7.9 ± 24.1
Chlorella stigmatophora	Chlorophyceae	Plymouth	59.4 ± 21.0	1.3 ± 37.7

1:1 diluted wastewater and ambient conditions and were screened for their ability to remove ammonium and ortho-phosphate over two days treatment time. Seventeen cultures remained unialgal, of which 14 removed >96.2 % of the ammonium and >98.5 % of the ortho-phosphate, compared to control concentrations of 296.9 ± 5.0 mmol m^{-3} $N\text{-}NH_4^+$ and 86.5 ± 0.3 mmol m^{-3} $P\text{-}PO_4^{3-}$ (Fig. 3.5). Although the other three cultures removed >96.4 % of the ammonium they removed less than 67.5 % of the ortho-phosphate. The mixed culture of wastewater species only removed 26.9 % of the ammonium and 13.81 % of the ortho-phosphate concentration compared to controls.

Four cultures (*Chlorella stigmatophora, Dunaliella tertiolecta, Nannochloropsis oculata,* and *Coccolithus* sp.), were contaminated by microalgal species occurring naturally in the wastewater (a coccoid chlorophyceaen, and a pennate bacillariophyceaen). Three species of *Tetraselmis* (*T. rubens, T. suecica* and *T. tetrathele*) were contaminated with the endemic isolates SA90B2 and SA90B4. The species *Phaeodactylum tricornutum* died out during the two day experiment.

3.3.4 Large-scale Open Tub Batch Culture Screening under Ambient Conditions

The 17 species and isolates which remained in unialgal culture and the mixed culture occurring naturally in the wastewater from the medium-scale open batch culture experiment were screened in single large-scale open batch cultures which contained 6 l of medium. All species and isolates survived pre-adaptation to 1:1 diluted wastewater and ambient conditions, and were screened for their ability to remove ammonium and ortho-phosphate over two days treatment time. Treatment by many of the

Figure 3.5 Key

1 *Tetraselmis* sp.
2 SA90B2
3 SA90C2
4 SA91B12
5 SA91B43
6 SA91B39
7 SA91B33
8 SA91CY1
9 SA91C13
10 *Chlorella salina*
11 SA90B4
12 SA90C3
13 SA90C1
14 SA92B48
15 SA91C6
16 SA92C17
17 *Porphyridium purpureum*
18 Mixed culture of wastewater species

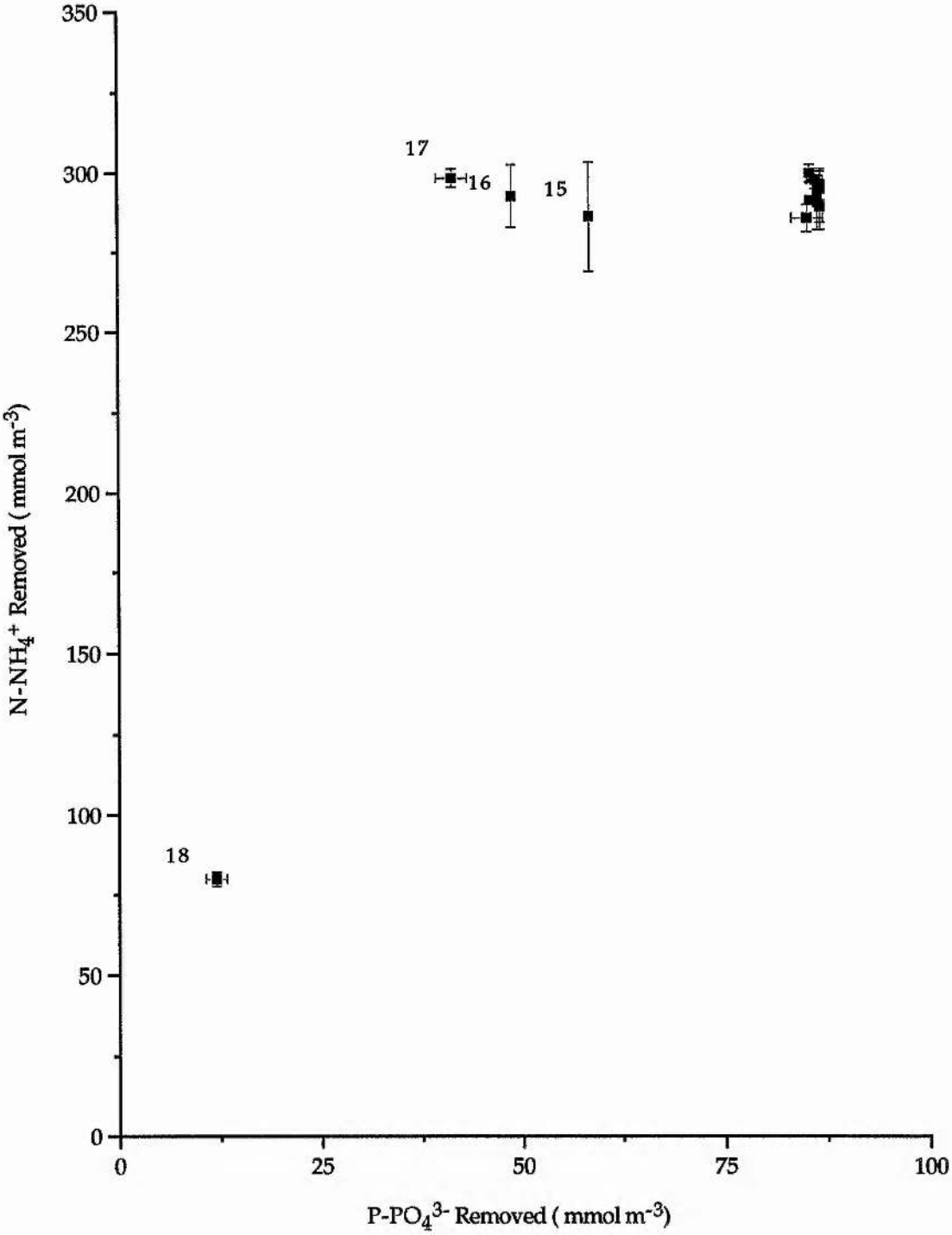

Figure 3.5 Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species cultured for two days on wastewater diluted 1:1 with seawater in medium-scale (400 ml) batch cultures under ambient conditions. Values are means of means ± s.d. of duplicate experiments in which final nutrient concentrations in algal cultures were compared to final concentrations in a control without algae (296.9 ± 5.0 mmol m^{-3} $N\text{-}NH_4^+$, 86.5 ± 0.32 mmol m^{-3} $P\text{-}PO_4^{3-}$). The s.d. may be too small to be seen.

species was unaffected by the increase in culture volume. Fourteen species and isolates remained in unialgal culture, of which 12 removed >99.4 % of the ammonium and >89.5 % of the ortho-phosphate compared to control concentrations (265.5 ± 5.7 mmol m^{-3} $N\text{-}NH_4^+$ and 44.2 ± 0.7 mmol m^{-3} $P\text{-}PO_4^{3-}$) (Fig. 3.6). Although the other two species removed >99.0 % of the ammonium, they removed less than 76.9 % of the ortho-phosphate. The mixed culture of wastewater species only removed 45.0 % of the ammonium and 49.2 % of the ortho-phosphate concentration compared to controls.

Three chlorophyceaen endemic isolates (SA90C3, SA91C13, SA92C17), were contaminated by algal species occurring naturally in the wastewater (Section 3.3.3).

3.4 Discussion

Batch culture screening experiments selected 14 species and isolates of marine microalgae, from more than 100, which demonstrated the ability to remove almost all ammonium and ortho-phosphate from 1:1 diluted wastewater with seawater. These microalgae also remained in unialgal culture during sequential batch culture, suggesting dominance over the mixed population of naturally occurring wastewater species which had a lower ability to remove nutrients. Many of the best-treating species and isolates identified under controlled conditions were also selected in the small-scale experiments under ambient environmental conditions, although three microalgae (*Phaeodactylum tricornutum* and two endemic isolates) were unable to tolerate or adapt to the natural range of conditions. Scaling up the culture volume from 50 ml in flasks to 6 l in open polypropylene tubs showed that many of the species selected at small-scale also treated well at

Figure 3.6 Key

1 SA90B4
2 SA90C2
3 *Chlorella salina*
4 *Porphyridium purpureum*
5 SA91B43
6 SA91CY1
7 SA92B48
8 SA91B12
9 SA90B2
10 *Tetraselmis* sp.
11 SA91B39
12 SA91B33
13 SA91C6
14 SA90C1
15 Mixed culture of wastewater species

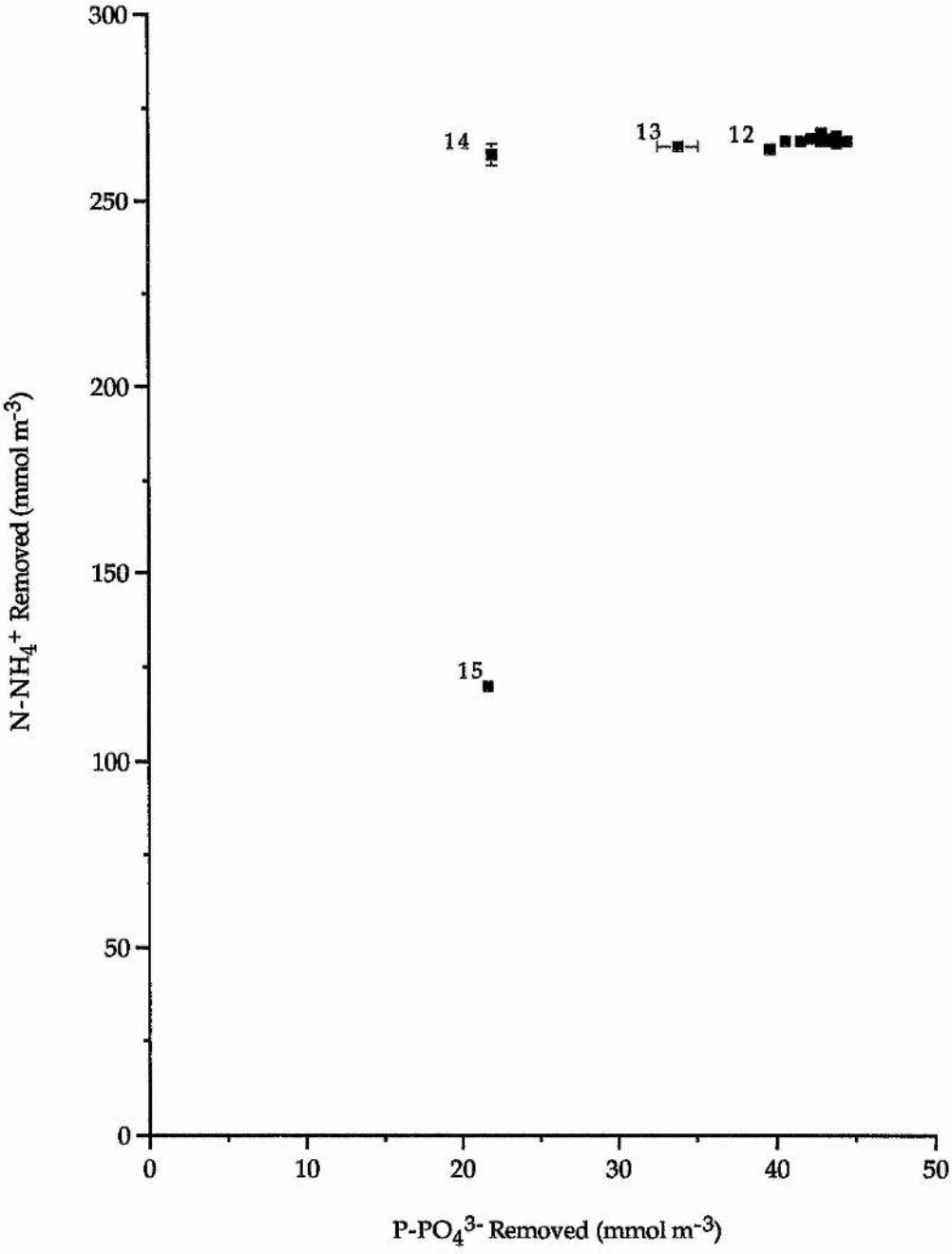

Figure 3.6 Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species cultured for two days on wastewater diluted 1:1 with seawater in large-scale (6 l) batch cultures under ambient conditions. Values are means of means ± s.d. of duplicate experiments in which final nutrient concentrations in algal cultures were compared to final concentrations in a control without algae (265.5 ± 5.7 mmol m^{-3} $N\text{-}NH_4^+$, 44.2 ± 0.7 mmol m^{-3} $P\text{-}PO_4^{3-}$). The s.d. may be too small to be seen.

higher volume. Culture contamination accounted for the poorer treatment of some of the 25 best-treating species and isolates in open culture.

Algal species from two different taxonomic divisions (Bacillariophyceae, and Chlorophyceae) are represented in the 14 best-treating algae. These species correspond to the algae which grow naturally on the surface of freshwater percolating filters (Curds & Hawkes, 1975) and the species found to dominate outside algal mass cultivation ponds of wastewater:seawater mixtures (Goldman *et al.*, 1974a & b; Fanuko, 1984). It is of note that of the 14 species selected, 11 had been isolated from the area directly surrounding the wastewater outfall in St. Andrews Bay. The other microalgal species tested here either did not survive, were unable to remain in unialgal culture, or showed a variety of nutrient removal capabilities, presumably due to differences in tolerance or ability to adapt to culture conditions or 1:1 diluted wastewater as a growth medium.

Three endemic isolates of *P. tricornutum* (SA90B2, SA90B4 and SA91B43) with different morphologies and the strain of *P. tricornutum* obtained from the Plymouth Culture Collection were found to be amongst the best-treating species in the screening experiments under controlled conditions. *P. tricornutum* has many characteristics which make it suitable for use in wastewater treatment. It can tolerate high pH (Goldman *et al.*, 1982b; Regan, 1988), stores ortho-phosphate as polyphosphate granules during luxury uptake (Kuenzler & Ketchum, 1962), and has a salinity range of 20-70 ‰. *P. tricornutum* is only dominant at temperatures below 19 °C in algal mass cultivation ponds utilising 1:1 diluted wastewaters (Goldman & Carpenter, 1974; Goldman & Ryther, 1976a). This may explain why, in the present study, the culture collection strain died during screening under ambient conditions in which the temperature varied from 10 to 35 °C. The

three endemic isolates appeared to be more tolerant or adaptable to the natural fluctuating conditions.

Species of *Tetraselmis* were also identified for their high treatment ability under controlled conditions. *Tetraselmis* also has many characteristics which make it suitable for use in wastewater treatment. It is tolerant of a wide range of pH, remains dominant over other naked flagellate microalgae and has a temperature range of 2-35 °C, (Regan, 1988). In the experiments reported here, *Tetraselmis* sp. had good nutrient removal under ambient conditions, but the other species of *Tetraselmis* had poorer treatment and became contaminated in open culture by the endemic strains of *P. tricornutum*. Perhaps these species were less suited to the ambient temperature conditions than the endemic isolates of *P. tricornutum*.

The higher treatment ability of *Porphyridium purpureum* under ambient conditions may be explained by its 25 °C temperature optimum (Vonshak *et al.*, 1985). In contrast, the temperature optimum of *Dunaliella tertiolecta* is above 30 °C (Goldman & Ryther, 1976a), which may have disadvantaged this species in the present study as culture temperature rarely exceeded this value during the experiments (Table 3.1). Goldman & Ryther (1976a) also found that at temperatures below 30 °C this species was out competed by algae such as the diatoms *P. tricornutum* and *Thalassiosira pseudonana*. Culture pH may also have contributed to the death of *Dunaliella tertiolecta* cultures. This alga is extremely sensitive to alkaline pH (Goldman *et al.*, 1982a), while cultures which had died were found to have pH 9.1.

Cross contamination of cultures increased in the open batch experiments when cultures were open to the air, although contamination was mainly by the algal species which occurred naturally in the wastewater. Nutrient removal by contaminated cultures, and by the culture of mixed wastewater species, was much lower than for the unialgal cultures of the

best-treating species. Competition between the species in a mixed algal culture may account for the reduced nutrient removing capability (D'Elia *et al.*, 1979).

The value of using controlled experiments for the selection of algal species capable of treating wastewaters was demonstrated by the observation that 13 of the 14 species finally selected in the ambient tub experiments were also selected under controlled conditions. The fourteenth species had not been isolated at the time of this study. The controlled culture conditions were, however, very different from ambient conditions, especially in terms of irradiance and the consequent heating effect on the culture (Table 3.1). These factors probably explain why 24 species, many of which were from the culture collections, died during the pre-adaptation to ambient conditions (Russell-Hunter, 1970; Goldman & Ryther, 1976a; Starr, 1979).

In the present study, rates of ammonium removal by the 14 best-treating algae (824.4 mmol N m^{-3} over seven days, and 265.5 to 458.2 mmol N m^{-3} over two days) were similar to those measured by other authors (Ganapati, 1975; Groenweg *et al.*, 1980; Shelef *et al.*, 1980; Chevalier & de la Noüe, 1985b; Lavoie & de la Noüe, 1987; de la Noüe & Bassères, 1989; Tam & Wong, 1989; Tadros & Philips, 1992), but ortho-phosphate removal rates (49.4 mmol P m^{-3} over seven days, and 35.2 to 86.5 mmol P m^{-3} over two days) were both more efficient and more rapid than previously measured (Groenweg *et al.*, 1980; Shelef *et al.*, 1980; Veber *et al.*, 1984; Lavoie & de la Noüe, 1987; de la Noüe & Bassères, 1989; Tam & Wong, 1989). It is of note that the nitrogen:phosphorus atomic ratios of the 1:1 diluted wastewater used in the initial seven day small-scale screening under controlled conditions and small-scale screening under ambient conditions (17:1 and 14:1 respectively), are close to the calculated optimum

Redfield ratio (15:1) for culturing phytoplankton species (Rhee, 1978). Those for the two day small-scale screening under controlled conditions and for the medium-scale and large-scale open culture experiments under ambient conditions were much lower (8:1, 3:1, and 6:1 respectively). These values reflect the natural variation in nutrient concentrations of the wastewater. Nevertheless, almost complete removal of both ammonium and phosphorous by the 14 best-treating algae was found in all experiments, suggesting that under batch culture the nutrient concentrations and N:P ratio of the diluted wastewater have little effect on the treatment capability of these species.

The direct relationship found for the removal of ammonium and ortho-phosphate by unialgal cultures of microalgal species in all screening experiments suggests that the ratio of ammonium to ortho-phosphate removal by microalgal species is independent of the total amounts removed. This is probably a reflection of the relative amounts of ammonium and ortho-phosphate required for algal metabolism.

The screening experiments were successful in identifying microalgae with the ability to remove nutrients efficiently from 1:1 diluted wastewater. Although caution must be used when extrapolating to a larger scale, scaling up of the batch culture volume in these experiments had little effect on the nutrient removing ability of many of the best-treating microalgal species. By screening species in small-scale batch cultures under ambient conditions, algal species which were able to adapt to, or were tolerant of natural environmental parameters (especially temperature) were selected. These characteristics may benefit the algal species used in wastewater treatment ponds under a temperate climate.

Chapter Four

Investigation of Culture Conditions Affecting Algal Growth and Nutrient Removal From 1:1 Diluted Wastewater

4.1 Introduction

The growth of a microalgal culture is governed by a complex interaction of conditions such as temperature, light level and quality, nutrient availability, salinity, pH, mixing and culture apparatus (Vonshak *et al.*, 1982; Richmond, 1983; de la Noüe *et al.*, 1986; Fabregas *et al.*, 1987a; Oswald, 1988b). Algae have optima for each of these conditions, although they may be tolerant of a wide range (Jiménez & Niell, 1991), or able to adapt to sub-optimal conditions if imposed gradually, as occurs in nature (Soeder & Hegewald, 1988).

In this study, batch cultures were used to identify the range of tolerance and optima of some of these physiological conditions for the best treating species from Chapter 3. Microalgal species which are tolerant of a wide range of environmental conditions or which are capable of adapting to environmental extremes, may enable a wastewater treatment process to operate throughout the year, even in a temperate climate.

The batch culture screening experiments described in Chapter 3 selected species which not only treated wastewater when culture conditions were controlled (15 ^{o}C, 18-22 μE m^{-2} s^{-1} PAR, 12 h:12 h) but also under ambient conditions which varied considerably over the light:dark cycle (10-31 ^{o}C, 0-2,000 μE m^{-2} s^{-1} PAR, 18 h:6 h). Since the growth of algal species in temperate regions is mostly temperature limited, especially in winter (Vonshak *et al.*, 1982; Bedell, 1985; de la Noüe *et al.*, 1986; Richmond, 1988), experiments were designed to investigate the growth of the best-treating algae over the temperature range experienced during the light:dark cycle.

Algae growing in coastal waters are often subjected to widely varying salinities due to changes in the seawater:freshwater ratio and have been shown to grow over a wide range of salinities (McLachlan, 1961, Yarish *et al.*,

1979). Since the salinity of a microalgal wastewater treatment pond can fluctuate widely as a result of heavy rainfall or high rates of evaporation, it was important to determine the effect of salinity on the growth of the best-treating microalgal species.

The batch culture screening experiments described in Chapter 3 were conducted without aeration using 1:1 diluted wastewater. Under these conditions, carbon dioxide may be limiting and the absorption of bicarbonate during photosynthesis may increase the culture pH to as high as 11 or more (Richmond, 1983; Fabregas *et al.*, 1984; Soeder & Hegewald, 1988). The pH of a culture affects many processes associated with algal growth and metabolism, as well as the availability and uptake of nutrient ions (Richmond, 1983; de la Noüe *et al.*, 1986; Borowitzka & Borowitzka, 1988b). At high pH and elevated temperature, ammonium may be transformed to ammonia gas and lost from the culture through 'stripping' (Witt *et al.*, 1981; De Pauw & Van Vaerenbergh, 1983; Chevalier & de la Noüe, 1985a & b), and ortho-phosphate may be removed through precipitation, especially in media, such as seawater, which are rich in calcium, aluminium and magnesium (Goldman *et al.*, 1982a; Belsare & Belsare, 1987). Abiotic factors may have contributed to the high rates of nutrient removal measured for the best-treating species in the batch culture screening experiments described in Chapter 3. Experiments were designed to investigate the increase in culture pH with photosynthesis and its effect on nutrient removal. The effect of pH and salinity on abiotic nutrient removal was also determined.

4.2 *Materials and Methods*

4.2.1 *Effect of Temperature on Algal Growth and Nutrient Removal*

The effect of temperature on algal growth and nutrient removal in 1:1 diluted wastewater was investigated using a thermo-gradient bar (Horrill, 1989), similar in design to that described by Grime *et al.* (1981). The thermo-gradient bar was constructed from a solid aluminium plate (91.4 x 45.7 x 5.8 cm thick) with two hollows, each 43 cm x 5 cm x 2.5 cm, cut into its underside, 5 cm from either end. Watertight compartments were formed from these hollows by sealing them with 45 cm x 7 cm lengths of aluminium sheet screwed down firmly onto rubber gaskets. A hole was drilled through both sides of the plate into these compartments and connectors fitted for attachment of inflow and outflow hoses. A thermogradient was set up and maintained along the length of the plate by passing heated water through one compartment and cooled water through the other. Heating and cooling were achieved using two Grant FH 15-A flow heaters and a Grant FC 25 flow cooler. Water flowed to and from the bar in a lagged hose pipe. Twelve parallel channels (83 cm long, 2.6 cm wide and 1.7 cm deep) were cut into the upper surface of the plate, into which growth tubes were placed. The thermogradient bar was supported within an angle iron frame and all exposed surfaces were insulated with 6 cm thick expanded polystyrene (Fig. 4.1). Lighting was supplied by six fluorescent tubes (40 watt Thorn "warm white" (25 $\mu E\ m^{-2}\ s^{-1}$) 12 h:12 h photoperiod) from (1.2 m) above the bar so that any heating effects were minimised.

The temperatures at either end of the bar were monitored using rod thermistors inserted into holes drilled into the plate. Calibration of the thermogradient along the bar was conducted with end temperatures of 4 and 36 ^{o}C. The thermogradient was monitored using seven disc shaped thermistors at distances along the length of the bar corresponding to the

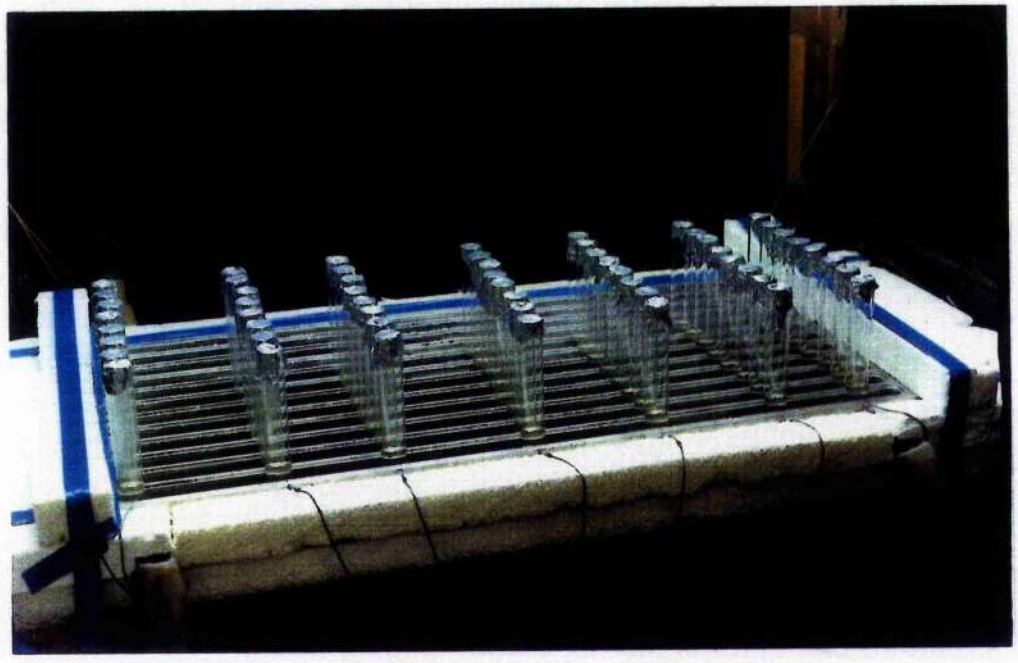

Figure 4.1 Thermogradient bar apparatus supported within an angle-iron frame and insulated with expanded polystyrene. Algal species were grown in tubes positioned along the temperature gradient at 5, 10, 15, 20, 25, 30 and 35 °C.

temperatures 5, 10, 15 ,20, 25, 30, and 35 °C. The thermistors were fastened to the front edge of the plate with "Blu-tac", which also provided thermal insulation. Temperatures were recorded once every four hours over one week using a 9 channel, intermittent Grant (D89-U) chart recorder and were found to be stable over both the light:dark cycle and over the seven days. Calibration was checked prior to, and daily, during each experiment.

Batch cultures were set up in flat bottomed culture tubes containing 5 ml fresh 1:1 diluted wastewater and covered with foil caps to minimise evaporation. The tubes were placed in the channels at each of the temperatures so that the wastewater temperature had equilibrated prior to the start of the experiment. Each of the tubes along one channel were inoculated with 5 ml algal culture of a single species. Ten of the best-treating species selected in Chapter 3 were tested (SA90B2, SA90B4, SA91B33, SA91B39, SA91B43, SA92B48, SA90C1, SA91C6, SA91CY1, and *Tetraselmis* sp.), along with a non-algal control for which 5 ml of 1:1 seawater:autoclaved distilled water was used instead of algal culture. Nutrient concentrations (Section 2.2.7) and relative algal growth (Section 2.3.2; OD_{570} against a blank of the diluted effluent without algae which was kept in the dark) were measured on days 0, 2, and 7. Tubes were swirled with a whirlimixer, and three 300 µl aliquots removed for analysis.

4.2.2 Algal Growth on Seawater Dilutions of Wastewater

Two parallel experiments were conducted to investigate the effect of salinity on algal growth in seawater diluted with wastewater. Dilutions of 1:0, 3:1, 1:1, 1:3, and 0:1 seawater:wastewater were made up to produce a gradient of wastewater concentration and a counter gradient of salinity. In

addition, a salinity gradient of the same dilutions was made up using M-Q water with added E-S nutrients.

Ten of the best-treating microalgal species (SA90B2, SA90B4, SA91B12, SA91B39, SA91B43, SA92B48, SA90C2, SA90C3, *Chlorella salina*, and *Tetraselmis* sp.) were tested using batch culture in microtitre plates. A 20 ml volume of each effluent dilution was inoculated with 2 ml of exponential phase algal culture (Section 2.3.3). After thorough mixing, 300 µl aliquots were pipetted in triplicate into the flat-bottomed wells of a 96-well microtitre plate (Dynatech M29A). The plates were incubated in the Conviron culture cabinet (Section 2.2.1) at 100% humidity to prevent evaporation. Relative algal growth was measured daily by determination of OD at 570 nm against a blank of the diluted effluent with no added algae (Section 2.3.2).

4.2.3 The Effect of pH on Abiotic Nutrient Removal

Two flasks containing 200 ml 1:1 distilled water:seawater and one with 200 ml 1:1 seawater:wastewater adapted algal culture were set up. A further 200 ml of 1:1 seawater:wastewater was added to each of the flasks which were placed in the Conviron environmental incubator (Section 2.2.1) and continually stirred by a magnetic bar. The pH of all three flasks was continuously monitored (Section 2.2.7) during the experiment. During the light period over 24 h, at intervals of one hour, the pH of one of the flasks without algae was raised to match that of the algal culture by addition of 1 M NaOH, while the remaining flask was the untreated control. Nutrient concentrations in all three flasks were then measured (Section 2.2.7).

4.2.4 *The Effect of Culture Salinity on Nutrient Removal at Elevated pH*

The ammonium and ortho-phosphate concentrations in a sample of 1:1 diluted wastewater were measured and three seawater solutions were made up with corresponding nutrient concentrations but different dilutions (1:0, 1:1, 0:1) with distilled water. Three 250 ml conical flasks were filled with 200 ml of each of these solutions and another flask with 200 ml 1:1 diluted wastewater. The flasks were continually stirred by a magnetic bar and continuous measurements of pH (Section 2.2.7) were made from each flask while 1 M NaOH was added. For every 0.1 rise in pH, up to pH 11.0, a sample of medium was taken and ammonium and ortho-phosphate concentrations determined (Section 2.2.7).

4.3 *Results*

4.3.1 *Effect of Temperature on Algal Growth and Nutrient Removal*

Algal cultures were preadapted to wastewater in the Conviron at 15 °C, and used to inoculate culture tubes containing temperature equilibrated 1:1 wastewater:seawater to test the effect of a rapid changes in temperature on their growth and nutrient removal capability. After two days culture, all the algae showed optimal growth and nutrient removal at 15 °C, except the isolate SA91CY1 which had a temperature optimum of 20 °C (Table 4.1, Figs. 4.2, 4.3, 4.4 & 4.5). Generally, the optimum temperature range of microalgae was between 10 and 20 °C, although three had wider ranges (Table 4.1, Figs. 4.2, 4.3, 4.4 & 4.5). Growth and nutrient removal by all microalgae declined above 25 °C, and all except isolates SA92B48 and SA91C6, died out at 35 °C. None of the species or isolates totally removed ammonium in the two days, although three isolates

Table 4.1 The temperature optima and ranges of unadapted and adapted cultures of 10 marine microalgal species and isolates cultured in 1:1 diluted wastewater on a thermogradient of 5 to 35 °C.

	Temperature unadapted culture		Temperature adapted culture	
Algal Species	**Optimum Temperature (°C)**	**Optimum Range (°C)**	**Optimum Temperature (°C)**	**Optimum Range (°C)**
SA90B2	15	10 - 25	20	5 - 25
SA90B4	15	10 - 20	20	10 - 25
SA91B33	15	10 - 20	20	5 - 25
SA91B39	15	10 - 20	15	10 - 25
SA91B43	15	10 - 20	20	10 - 25
SA92B48	15	5 - 25	20	5 - 25
SA90C1	15	10 - 20	20	15 - 25
SA91C6	15	10 - 20	10	5 - 15
SA91CY1	20	10 - 20	25	10 - 25
Tetraselmis sp.	15	5 - 20	20	10 - 25

(SA90B2, SA90B4 and SA92B48) and*Tetraselmis* sp., totally removed ortho-phosphate at some temperatures (Figs. 4.2, 4.3, 4.4 & 4.5). Nutrient concentrations in control tubes without algae did not change.

A second experiment tested the growth and nutrient removal by temperature acclimated algal cultures. Algal cultures which had been grown on diluted wastewater at each temperature of the thermogradient for one week, were used to inoculate culture tubes which had been equilibrated to the same temperature. Nutrient removal was measured after two days, and culture OD after one week. Most of the temperature-acclimated algae had optimal growth and nutrient removal at 20 °C, which was 5 °C higher than when all inocula were from stock cultures grown in the Conviron at 15 °C (Table 4.1, Figs. 4.2, 4.3, 4.4 & 4.5). Acclimation to the thermogradient extended the optimum temperature range of many of the species to between 5 and 25 °C (Table 4.1, Figs. 4.2, 4.3, 4.4 & 4.5). Growth and nutrient removal by all species declined above 25 °C. Four species (SA91B33, SA91B39, SA91B43, and SA92B48) died out at 30 °C and the remaining species, except isolates SA90B4 and SA91C6 and *Tetraselmis* sp., died out at 35 °C. Eight of the species (SA90B2, SA90B4, SA91B33, SA91B39, SA91B43, SA92B48, SA90C1 and *Tetraselmis* sp.) totally removed ammonium and ortho-phosphate at some but not all temperatures in the two days culture (Figs. 4.2, 4.3, 4.4 & 4.5). Nutrient concentrations in the control tubes without algae showed little difference over the temperature gradient.

4.3.2 Algal Growth on Seawater Dilutions of Wastewater

When seawater is diluted with wastewater, salinity is decreased but wastewater derived properties such as nutrient concentration, OD, and bacterial numbers are increased. To test the effect of hyposalinity on the

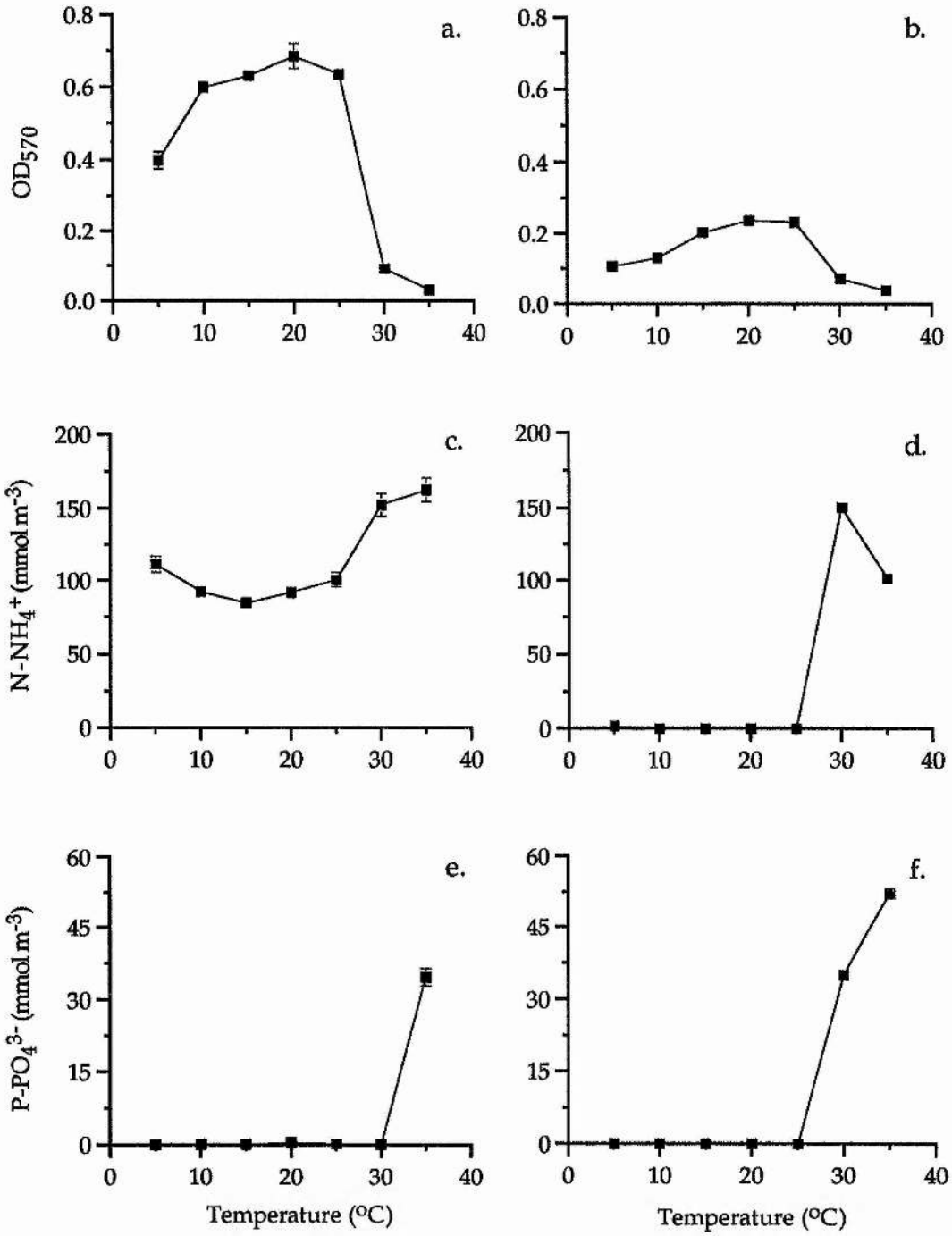

Figure 4.2 Culture OD and ammonium and ortho-phosphate concentrations of batch cultures of isolate SA90B2 grown on 1:1 diluted wastewater on a temperature gradient from 5 to 35 °C. Cultures were inoculated with either temperature unadapted algae (a., c. & e.) or with temperature adapted algae (b., d. & f.). Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

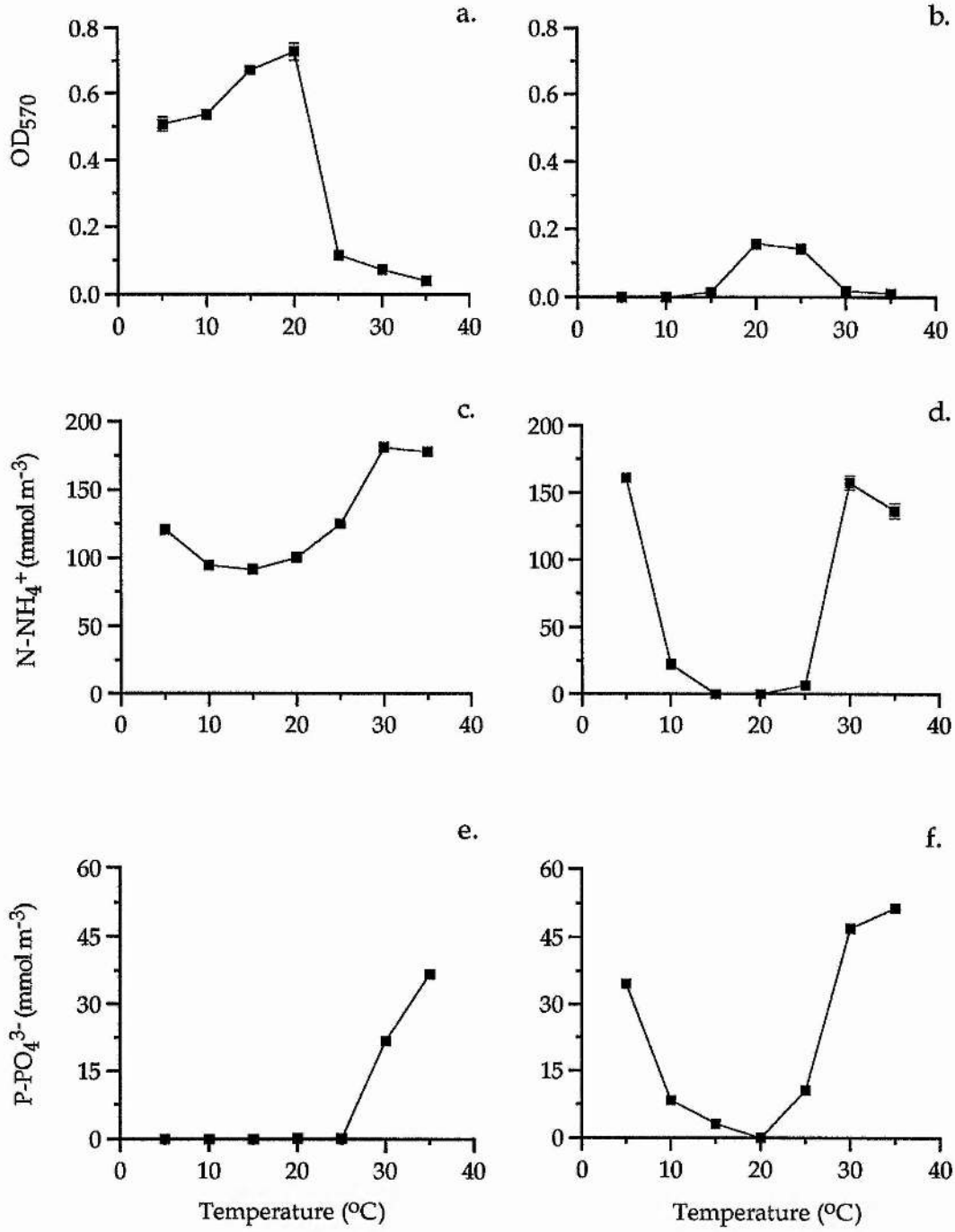

Figure 4.3 Culture OD and ammonium and ortho-phosphate concentrations of batch cultures of isolate SA90B4 grown on 1:1 diluted wastewater on a temperature gradient from 5 to 35 °C. Cultures were inoculated with either temperature unadapted algae (a., c. & e.) or with temperature adapted algae (b., d. & f.). Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

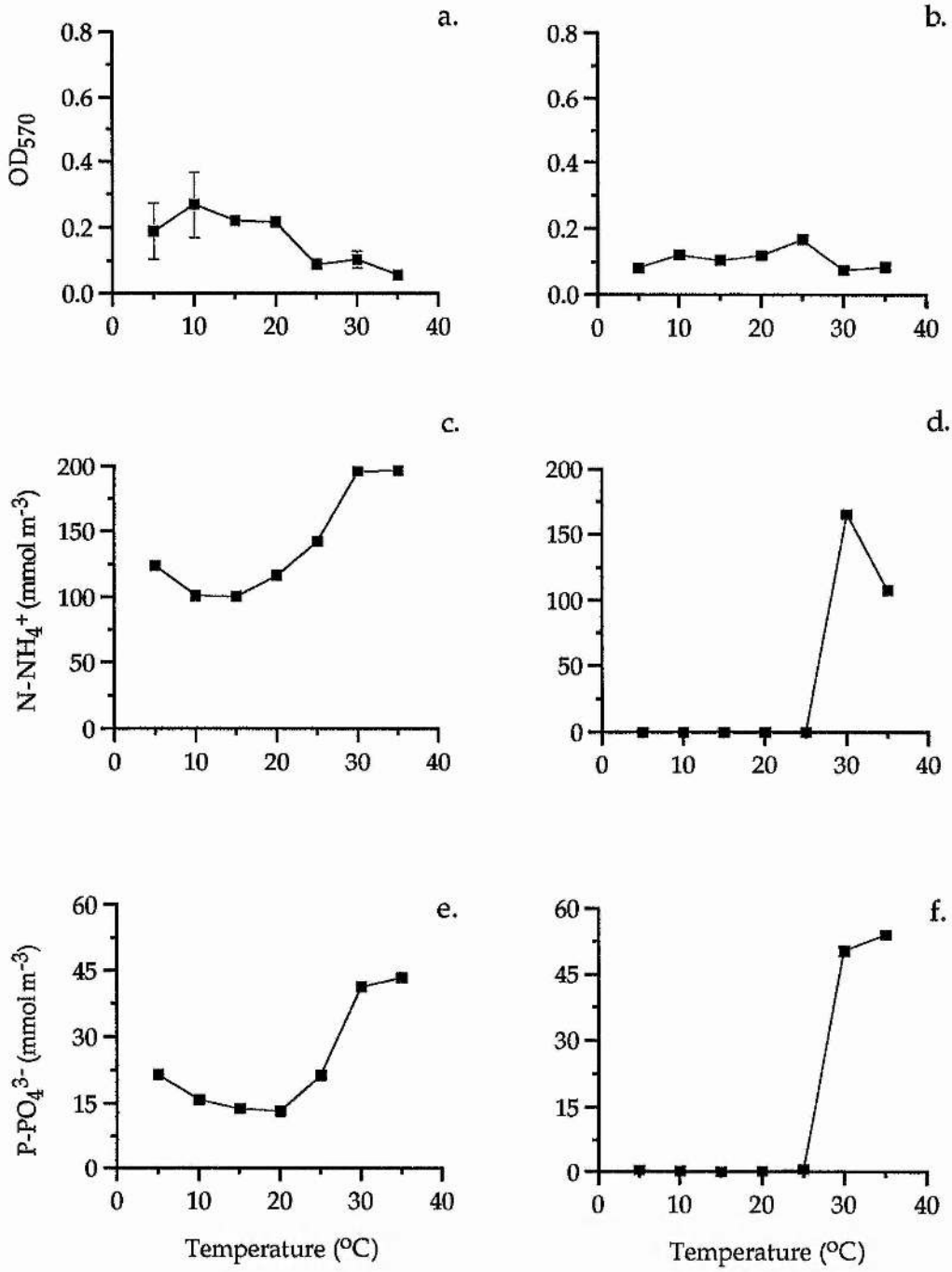

Figure 4.4 Culture OD and ammonium and ortho-phosphate concentrations of batch cultures of isolate SA91B33 grown on 1:1 diluted wastewater on a temperature gradient from 5 to 35 °C. Cultures were inoculated with either temperature unadapted algae (a., c. & e.) or with temperature adapted algae (b., d. & f.). Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

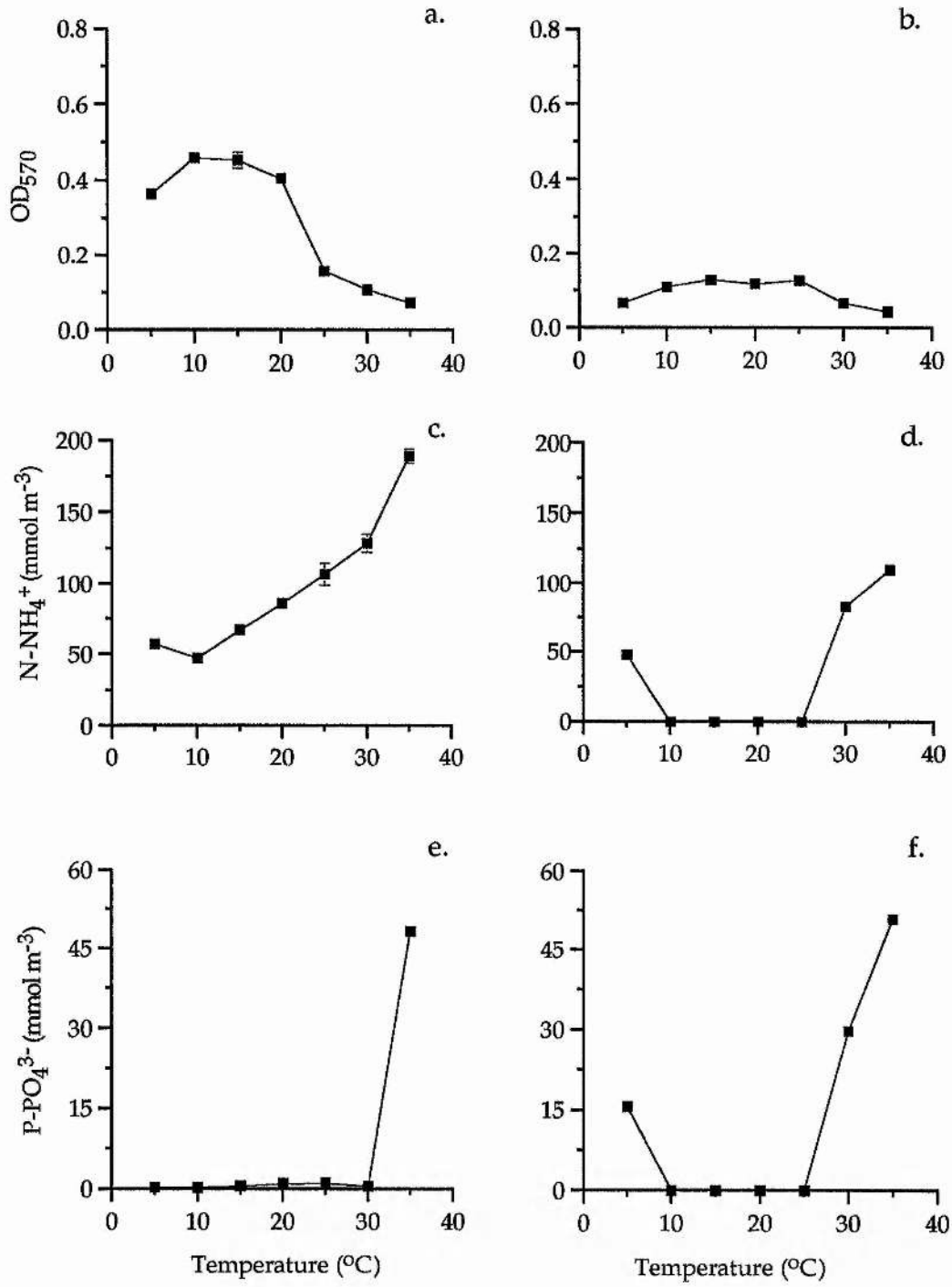

Figure 4.5 Culture OD and ammonium and ortho-phosphate concentrations of batch cultures of *Tetraselmis* sp. grown on 1:1 diluted wastewater on a temperature gradient from 5 to 35 °C. Cultures were inoculated with either temperature unadapted algae (a., c. & e.) or with temperature adapted algae (b., d. & f.). Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

growth of the best-treating species and isolates, ten were grown either in seawater diluted with wastewater, or in seawater diluted with M-Q water, giving a range of salinities of 0-32 ‰. Equal nutrient concentrations in the M-Q water dilutions were achieved by the addition of E-S nutrients (Section 2.2.1). Although the nitrogen content of the E-S media (2.36 mol m^{-3} N-NO_3^-, 55.9 mmol m^{-3} P-PO_4^{3-}) was similar to that of the undiluted wastewater (2.41 mol m^{-3} N-NH_4^+), growth of all the algae tested was higher at every dilution of the wastewater (Figs. 4.6a, 4.7a).

All microalgae had similar growth characteristics on the gradient of seawater:wastewater, with optimal growth at salinities between 8 and 24 ‰. Algae showed lower growth on undiluted wastewater and even less growth on undiluted seawater, which had comparatively low nutrient concentrations (0.2 mmol m^{-3} N-Urea, 1.5 mmol m^{-3} N-NH_4^+, 0.7 mmol m^{-3} N-NO_2^-, 10.1 mmol m^{-3} N-NO_3^- and 1.4 mmol m^{-3} P-PO_4^{3-}). However, in some cases, species responded differently if salinity was increased but nutrient concentration was kept constant. Typical results are given in Figures 4.6 & 4.7. Most microalgae (represented by SA90C3) had a similar growth response when seawater was diluted with either wastewater or M-Q water (Fig. 4.7), but the three endemic strains of *Phaeodactylum tricornutum* including isolate SA90B2 had a very different growth response to wastewater than to M-Q dilutions, and grew well over the whole salinity range, with higher growth at lower salinity (Fig. 4.6). Thus, for species such as isolate SA90B2, (SA90B4 and SA91B43) which were all endemic strains of *P. tricornutum*, the inhibitory effect observed when seawater is highly diluted with wastewater may not be due solely to hyposalinity, but to a property of the wastewater.

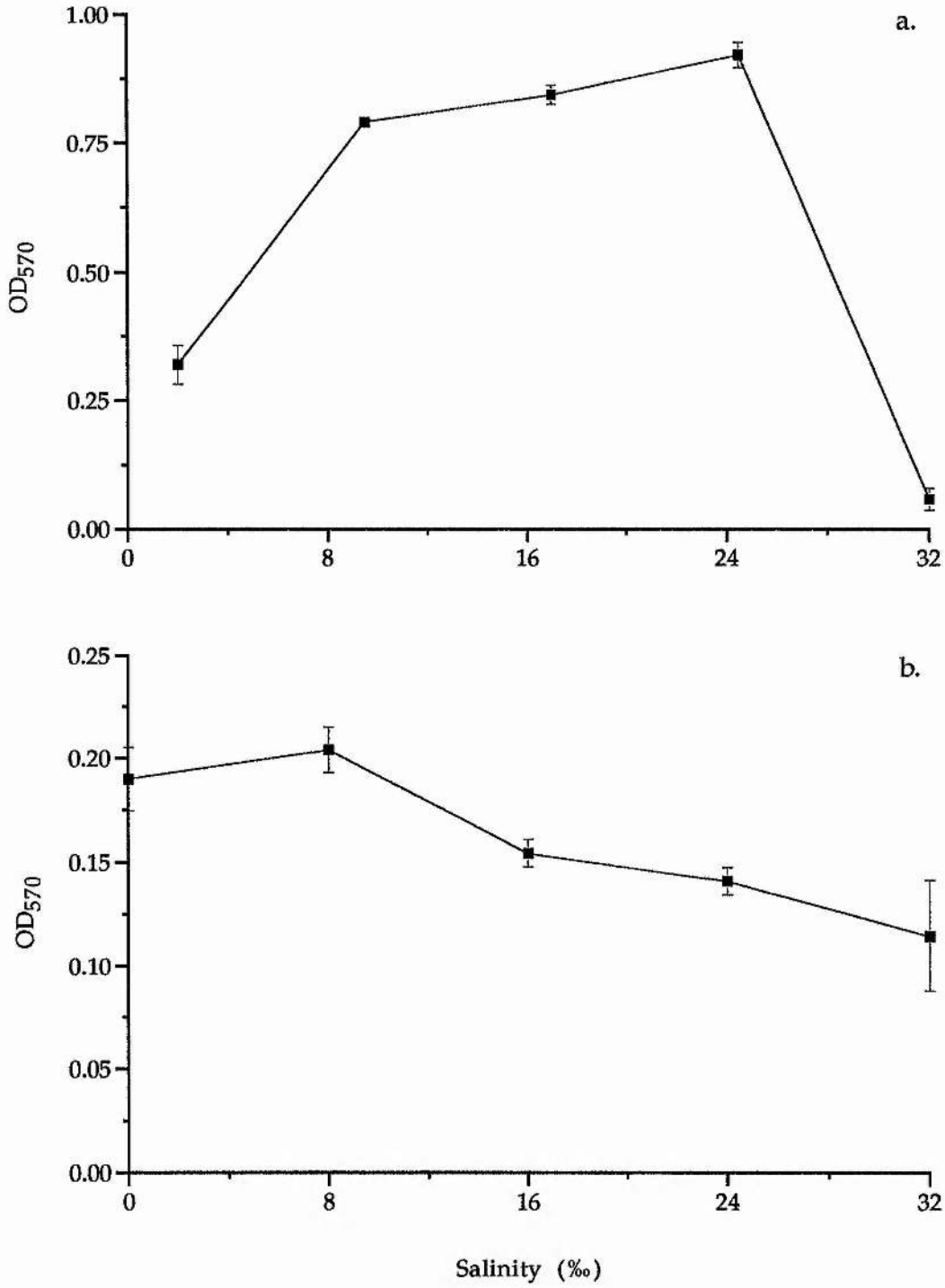

Figure. 4.6 Relative growth (OD_{570}) to early stationary phase for isolate SA90B2 on a gradient of seawater diluted with either wastewater (a.) or with M-Q water (b.). Values are means ± s.d. from three replicate microplate well cultures. The s.d. may be too small to be seen.

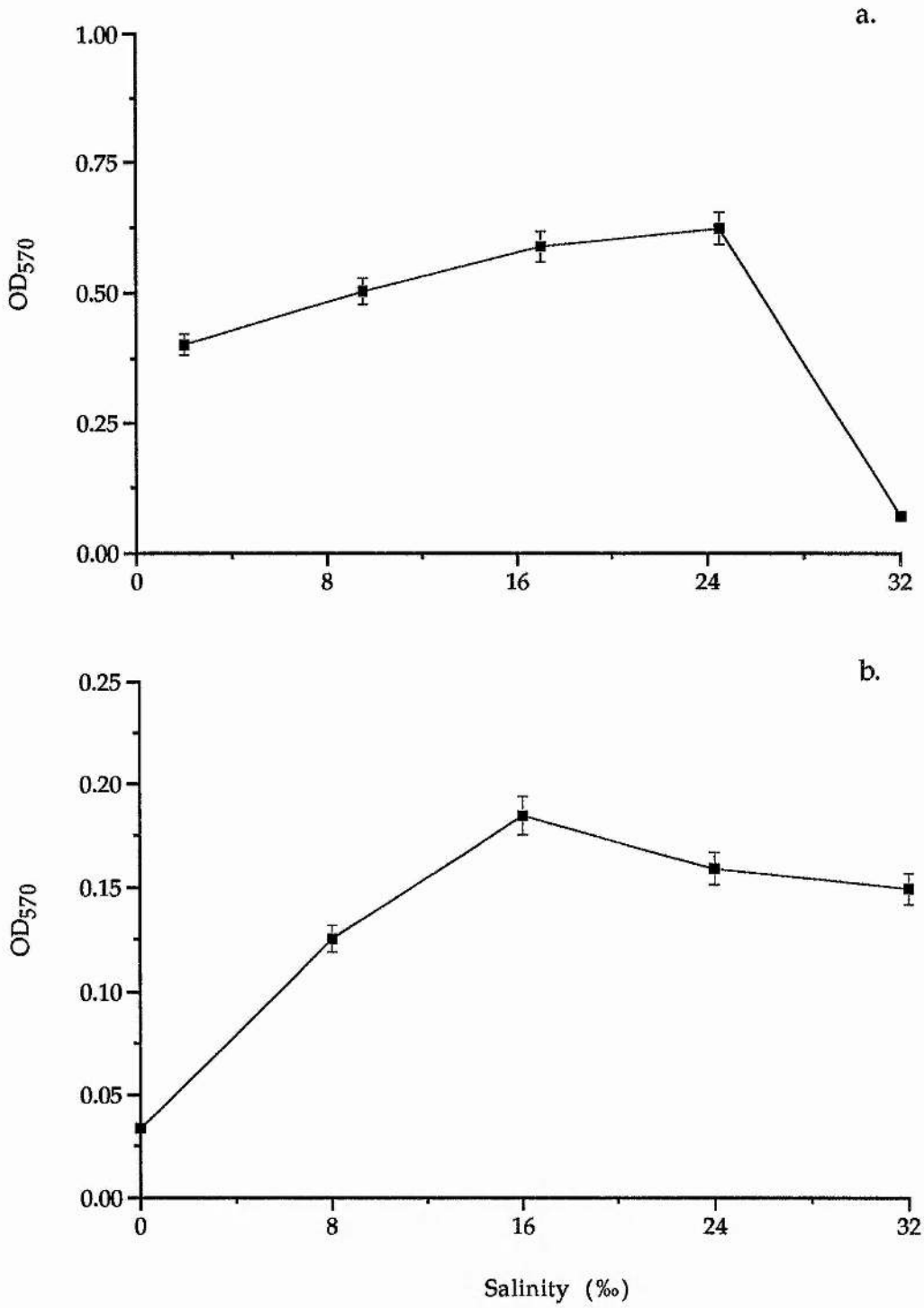

Figure 4.7 Relative growth (OD_{570}) to early stationary phase for isolate SA90C3 on a gradient of seawater diluted with either wastewater (a.) or with M-Q water (b.). Values are means ± s.d. from three replicate microplate well cultures. The s.d. may be too small to be seen.

4.3.3 *The Effect of pH on Abiotic Nutrient Removal*

The pH of an algal culture of isolate SA90B2 grown on 1:1 diluted wastewater was shown to rise and fall over the light:dark cycle (Fig. 4.8c). This experiment was designed to determine if pH mediated abiotic nutrient removal contributed to the nutrient removal measured for this species. The pH of 1:1 diluted wastewater without added algae was adjusted to that of the algal culture. Ammonium was totally removed from the algal culture in 26 h, whereas only 30 % was removed in the pH adjusted wastewater (Fig. 4.8a). Ortho-phosphate was completely removed by the algal culture in 11 h (Fig. 4.8b). Nearly 80 % of the ortho-phosphate was also removed from the pH adjusted wastewater during this period, but removal did not begin until the pH rose above pH 9.5. This pH was not reached until after 6 h in the algal culture (Fig. 4.8c), when 70 % of ortho-phosphate had already been removed (Fig. 4.8b). The control without algae and no pH adjustment showed little change in nutrient concentrations (Fig. 4.8a & b).

Abiotic removal did not occur overnight even though the pH was maintained above 10.0 (Fig. 4.8). Above pH 9.5, a white precipitate formed in the wastewater. To verify that it was ortho-phosphate that had been precipitated, a 1 ml sample of the pH adjusted medium was centrifuged, the centrifugate dissolved in a few drops of 1 M HCl and the volume made up to 1 ml with M-Q water and nutrient concentrations measured. The centrifugate contained ortho-phosphate but no ammonium (Fig. 4.9) and the concentration of ortho-phosphate closely followed the concentration removed from solution (Fig. 4.9b). The large change in ortho-phosphate concentration in the centrifugate overnight was probably due to sedimentation within the flask which the magnetic stirrer was unable to resuspend.

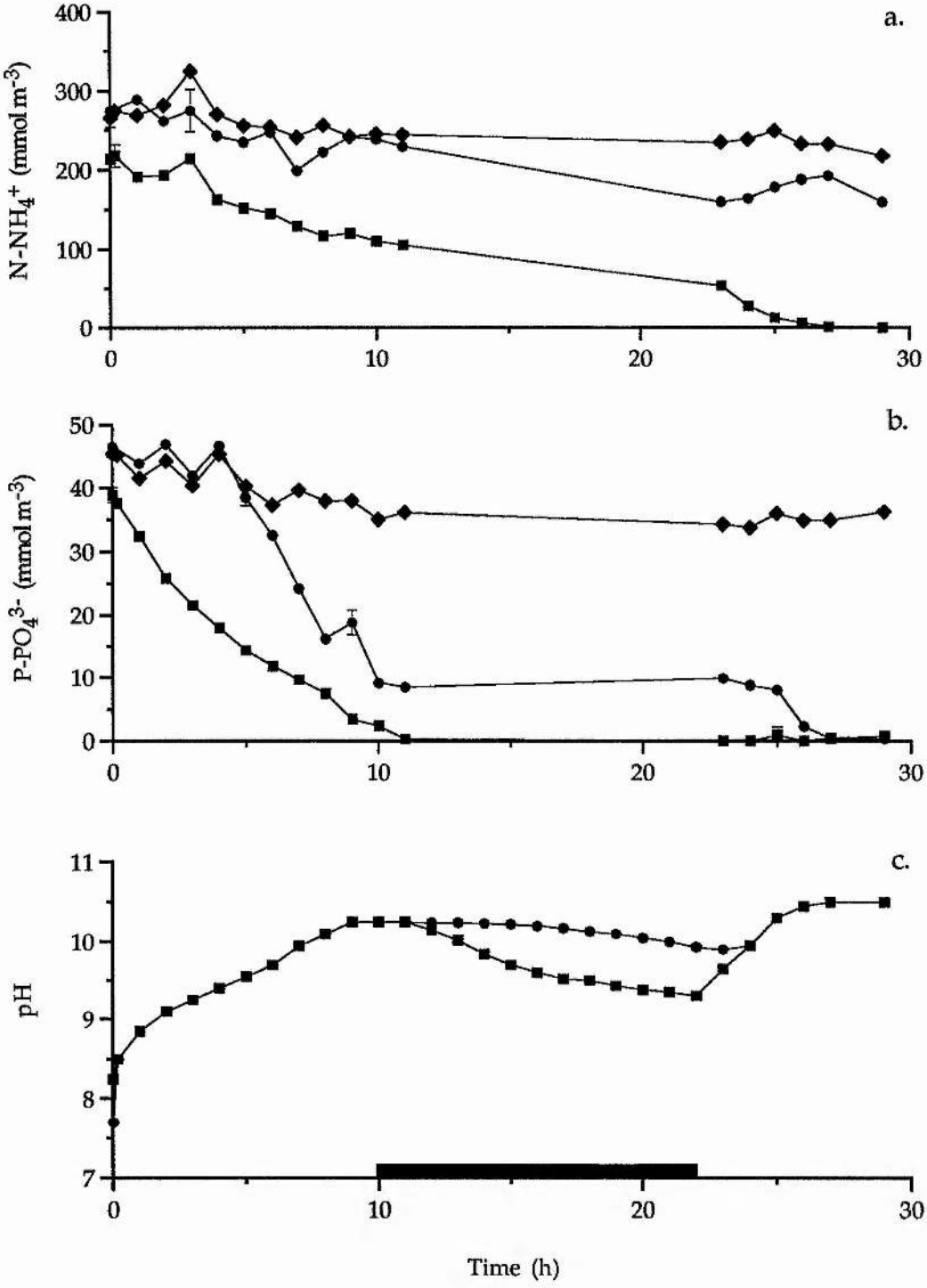

Figure 4.8 Ammonium (a.) and ortho-phosphate (b.) concentrations and pH (c.) in batch cultures of 1:1 diluted wastewater with algae (■-■), without algae but with pH adjusted to that of algal culture, except during the dark period (●-●) and without algae and with no pH adjustment (♦-♦) over 30 hours culture. Nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen. The bar marks the dark period.

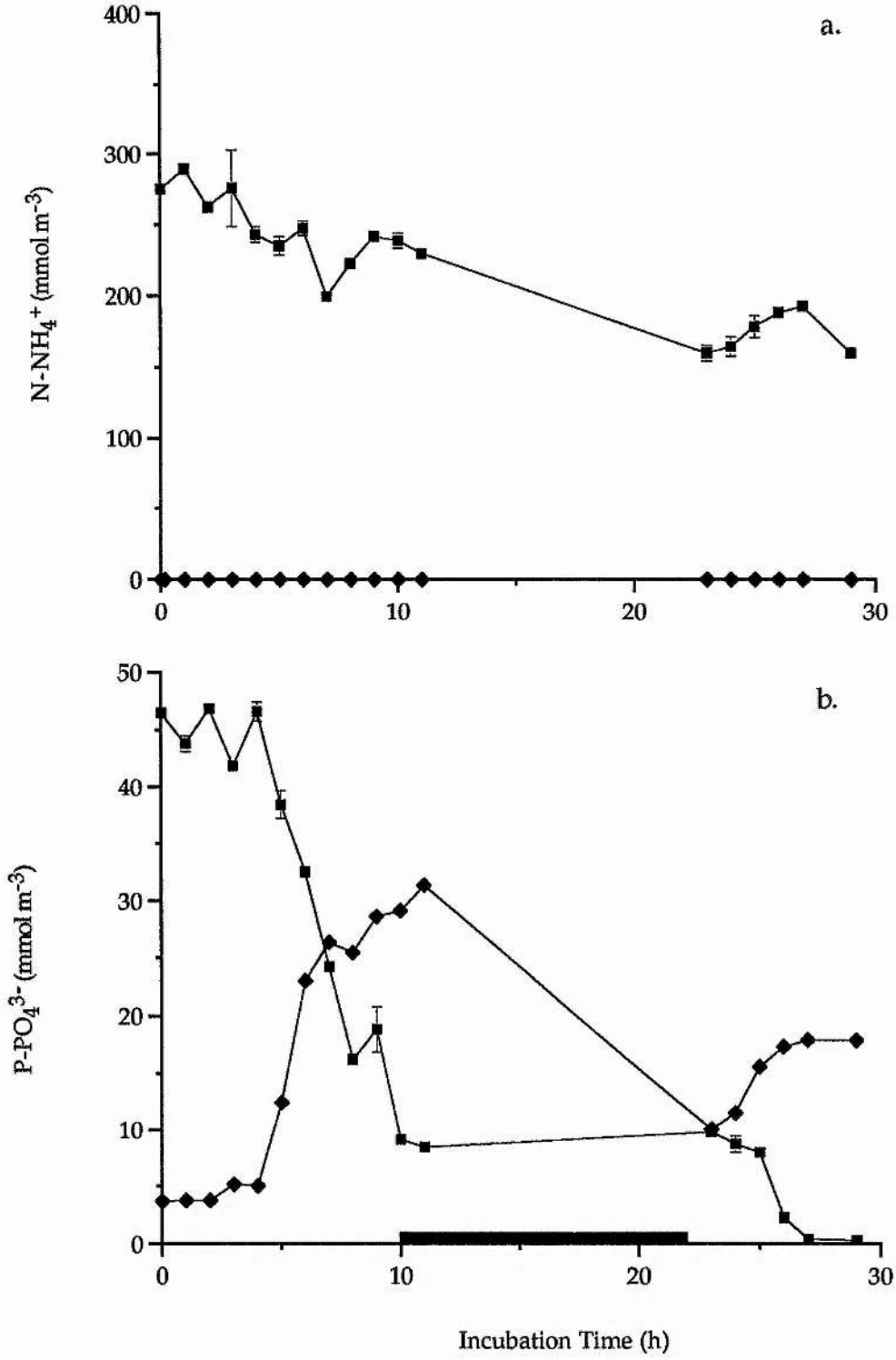

Figure 4.9 Detail of the ammonium (a.) and ortho-phosphate (b.) concentrations in solution (■-■) and in precipitate (◆-◆) of the batch culture of 1:1 diluted wastewater without algae but with pH adjustment over 30 hours culture. Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen. The bar marks to dark period.

4.3.4 The Effect of Culture Salinity on Nutrient Removal at Elevated pH.

The effect of salinity on pH mediated abiotic nutrient removal was determined. Nutrient removal occurred at elevated pH in all dilutions of seawater (1:0, 1:1 and 0:1) and in the 1:1 diluted wastewater (Fig. 4.10). Ammonium removal began at pH 8.5 and concentrations decreased linearly with increase in pH in all solutions. However, only 23.5 % of the original concentration had been removed at pH 11.2 (Fig. 4.10a). Ortho-phosphate was totally removed from all solutions, but the pH range over which this occurred depended upon the salinity of the medium. For distilled water the range was between pH 8.7 and pH 10.1 whereas in seawater and the 1:1 dilutions of seawater and wastewater the range was slightly higher, between 9.0 and 10.5 (Fig. 4.10b).

4.4 Discussion

4.4.1 Effect of Temperature on Algal Growth and Nutrient Removal

It was not unexpected that the algal species grown from inocula from the Conviron had temperature optima of 15 ^{o}C, since these species had been screened for nutrient removal and maintained in stock cultures at this temperature. Following temperature acclimation of cultures on the thermo-gradient, both the temperature optima and ranges of most of the species increased. The optimum of 20 ^{o}C is higher than was expected for these temperate species, since the maximum temperature of seawater from St Andrews Bay in the summer was only 15.5 ^{o}C (Chapter 2; Table 2.2). The optimum temperature of algal species in culture are often higher than those in which the species naturally live (Admiraal, 1977). Possibly, the growth of

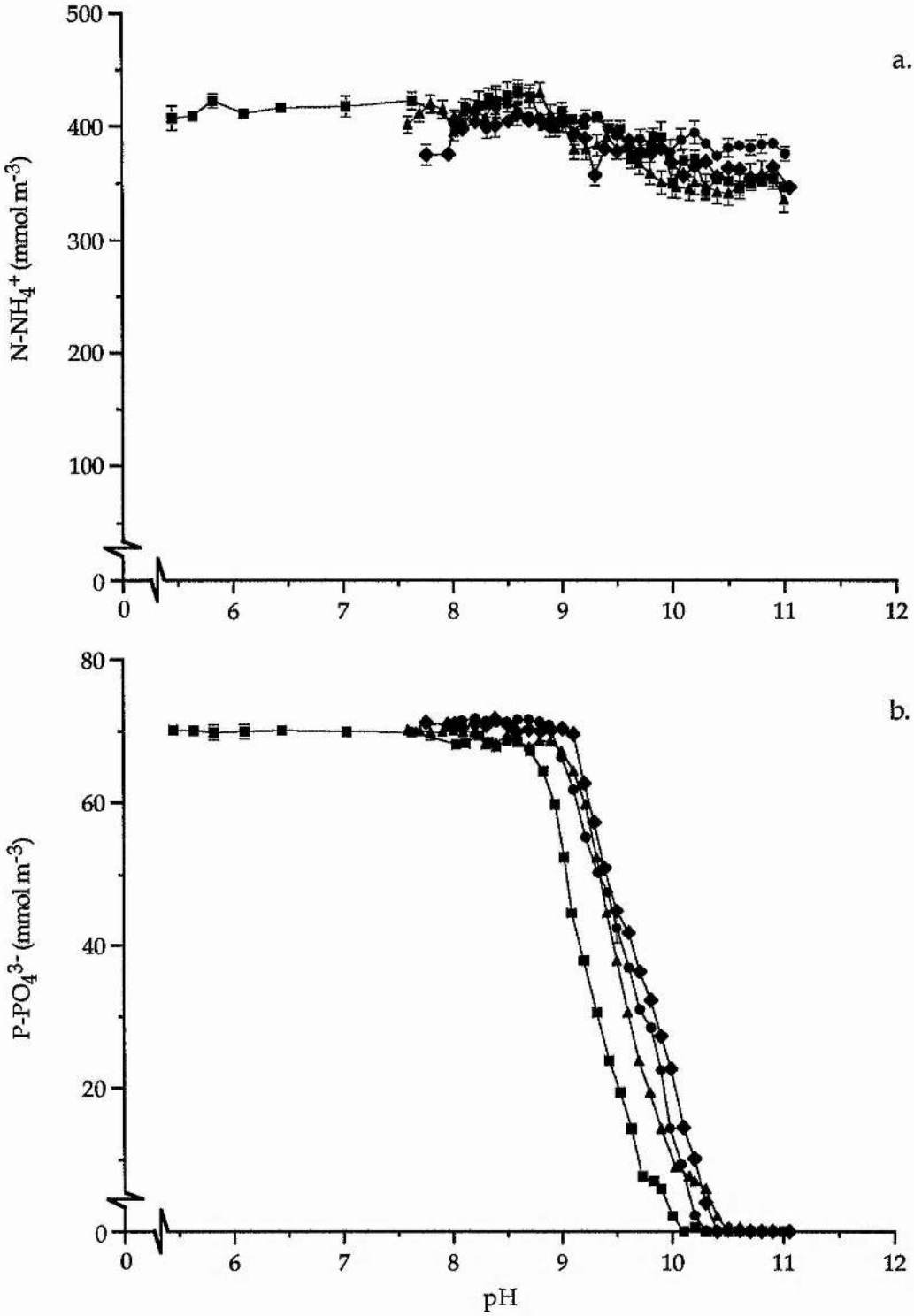

Figure 4.10 Ammonium (a.) and ortho-phosphate (b.) concentrations in distilled water (■-■), 1:1 seawater:distilled water (◆-◆), seawater (●-●) and 1:1 wastewater:seawater (▲-▲) immediately after pH adjustment with addition of 1 M NaOH. Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

many of these species in St Andrews Bay is temperature limited over much of the year.

Most species grew better at temperatures below their optima than above (Figs. 4.2, 4.3, 4.4 & 4.5). The inhibitory effect of higher than optimal temperature has been found for many microalgal species (Nelson *et al.*, 1992). During winter months, when seawater temperatures may be as low as 6 °C, temperature may limit the use of some of the best-treating microalgal species in wastewater treatment system. Temperature acclimated cultures of three algal species (SA90B2, SA91B33 and SA92B48) removed 100 % of both ammonium and ortho-phosphate during the two days culture at 5 °C (Figs. 4.2 & 4.4; Table 4.1), and therefore show promise for use in such a system during the winter.

4.4.2 *Algal Growth on Seawater Dilutions of Wastewater*

The different growth responses of 10 of the best-treating algae to a salinity gradient of seawater diluted with M-Q water indicated the variety of salinity tolerances of these marine algae. The similar growth responses of these species on seawater diluted with wastewater are not unexpected because they were selected for their nutrient removal ability from 1:1 seawater:wastewater. The growth of microalgae was probably higher on the wastewater than on the seawater dilutions with E-S nutrients since they use ammonium more readily as a nitrogen source than nitrate (Thompson *et al.*, 1989; Dortch, 1990; Cochlan & Harrison, 1991a, b & c; Raven *et al.*, 1992). The decline in the growth of the endemic strains of *Phaeodactylum tricornutum* such as SA90B2 at low dilutions of wastewater suggested that for these strains, other properties of the diluted wastewater had more effect on algal growth than salinity. The inhibitory effect of excessive ammonium

cannot be discounted as the ammonium content of undiluted wastewater used in this experiment was 2.4 mol m^{-3} $N\text{-}NH_4^+$. Algal photosynthesis is inhibited at ammonium concentrations above 2.0 mol m^{-3} $N\text{-}NH_3$, if the culture pH exceeds 8.0, when non-toxic NH_4^+ dissociates to toxic NH_3 (Abeliovich, 1980; Azov & Goldman, 1982). The pH in the batch culture of one of the best-treating algae (SA90B2) rose above pH 10.0 after 8 h culture (Fig. 4.8). However, this would not affect the microalgae in treatment ponds since 1:1 dilution of the wastewater with seawater would reduce ammonium concentrations below this inhibitory level.

Species SA90C3 is typical of many marine microalgae which have a salinity optimum in the range 16-32 ‰ (Laing & Utting, 1980; Fabregas *et al.*, 1984; Fabregas *et al.*, 1985b). Below the salinity optimum, the decline in growth is proportional to the decrease in salinity (Jiménez *et al.*, 1990). However, other microalgal species have been found to be capable of adapting to changes in salinity, ranging from freshwater 0 ‰ to oceanic seawater 35 ‰ (Fabregas *et al.*, 1987a).

4.4.3 The Effect of pH and Salinity on Abiotic Nutrient Removal

The batch culture experiments investigating pH changes during wastewater treatment by one of the best-treating algae have shown that culture pH varies with light:dark cycle (Fig. 4.8). This is a result of bicarbonate removal during high rates of microalgal photosynthesis (Richmond, 1983; Fabregas *et al.*, 1984; Soeder & Hegewald, 1988). In the present study, when the pH of 1:1 diluted wastewater was raised by addition of 1 M NaOH, ortho-phosphate began to precipitate at pH 9.5, and was totally removed at pH 10.5, although more than 70 % of ammonium still remained in solution at pH 11.4 (Fig. 4.8). However, in algal cultures,

over 50 % of ammonium and 70 % of ortho-phosphate were removed before the medium reached pH 9.5 (Fig. 4.8), indicating that nutrient removal in algal batch cultures was mainly due to algal uptake and not due to the abiotic effects of increased pH. Other authors have found ammonium removal from algal cultures to be mainly due to uptake and assimilation (Goldman and Stanley, 1974; Matusiak *et al.*, 1976). In this study ortho-phosphate precipitation was shown to occur at pH 9.5 or above, with concentrations in the dissolved precipitate corresponding to the concentration removed from the culture medium (Fig. 4.9). The salinity of the culture medium also affected the pH at which ortho-phosphate precipitation began. At a higher salinity the pH at which precipitation occurred was increased (Fig. 4.10) which may have been a result of the buffering capacity of seawater (Rebello & Moreira, 1982).

Since the effects of many of the environmental conditions (temperature, light and nutrients) which influence microalgal growth are interrelated (Vonshak *et al.*, 1982; Henry, 1988; de la Noüe & De Pauw, 1988), laboratory studies cannot provide a complete picture of the response of algae to natural conditions (Admiraal, 1977). Therefore, the conclusions reached about growth rate measurements in cultures must be applied with caution to algae growing in the field. Many microalgal species are able to adapt to the conditions under which they are cultured so that their range of tolerance to environmental conditions may be altered with time (Craig *et al.*, 1988). However the experiments described here have shown that salinity, temperature and pH are all conditions that affect the growth of marine algal species and that many of the best-treating microalgal species are capable of growing and removing nutrients over a wide range of temperatures and salinities.

Chapter Five

Batch Culture Screening for Nutrient Removal from Aquaculture Effluent

5.1 *Introduction*

Over-fishing, environmental change and/or pollution (Lee & Jones, 1991; Symes, 1991; Carmargo, 1992; Mann, 1993), have contributed to the decline in fish catches throughout the world (OECD, 1989; FAO, 1992). As a result, natural fisheries of food species (e.g. salmon, eels, trout and yellowtail) can no longer meet current and projected demands (OECD, 1989; Parker *et al.*, 1991). Aquaculture, particularly of high value species has therefore become an expanding and commercial industry (Neiland, 1990; Parker *et al.*, 1991; FAO, 1992; Fridley, 1993).

Conventional, extensive fish rearing methods are restricted to areas with sufficient quantities of high quality water (OECD, 1989; Arbiv & van Rijn, 1992), and many practices have caused pollution problems through the discharge of effluents rich in organic matter and inorganic nutrients (Mozes, 1992; Munday *et al.*, 1992; Rosenthal *et al.*, 1992). Advanced aquacultural systems with effluent treatment and recirculation and, consequently, reduced environmental impact, are beginning to be used (Rogers, 1984; Knösche, 1991). The low water demand and minimal effluent discharge of closed recirculating systems has permitted the siting of farms where traditional aquaculture practises would be prohibited (Arbiv & van Rijn, 1992). Further optimisation of conditions for fish culture such as temperature, pH and oxygen to achieve maximal food conversion and growth rates is also enabled by recirculation of effluent (Sadler, 1979).

Many recirculating aquaculture practices use advanced wastewater treatment systems which combine activated sludge and fixed film processes to remove organic waste and ammonium. Rotating biological contactors have been found to be one of the most efficient systems (Gabel, 1984; Rogers, 1984; Knösche, 1991). The organic load of the effluent is reduced

through heterotrophic decomposition and ammonium is converted to nitrate by nitrifying bacteria. The efficiency of the nitrification process is highly variable and may have serious consequences on fish survival if the concentration of the intermediary compound, nitrite, increases (Lan *et al.*, 1992; Weirich *et al.*, 1993). Due to poor denitrification, nitrate often accumulates in the recirculating effluent (Gabel, 1984; Bovendeur *et al.*, 1990; Knösche, 1991; Mozes, 1992), and although much less toxic than nitrite, high concentrations in the effluent may contribute to increased stress and decreased fish appetite (N. Hazon, pers. comm., University of St Andrews). A further drawback of these systems is that oxygen levels in the treated effluent are highly depleted by bacterial decomposition. Re-oxygenation of the effluent is therefore necessary before it is returned to the fish tanks, which considerably adds to the costs of aquaculture (Knösche, 1991). Effluent treatment methods for recirculating systems which efficiently and economically improve the water quality of the reused effluent (in terms of levels of nitrogenous compounds and oxygen) would be of great benefit to the aquaculture industry.

The incorporation of a microalgal component to the recirculating system may represent an alternative method. Ammonium could be directly removed from the effluent by algal biomass without the production of any toxic intermediaries (Groenweg *et al.*, 1980; Shelef *et al.*, 1980; de la Noüe & Bassères, 1989; Tam & Wong, 1989). Furthermore photosynthetic oxidation would drive bacterial decomposition and aerate the treated effluent (Grobbelaar *et al.*, 1988; Oswald, 1988a & c). The microalgae will also increase disinfection and produce biomass, which if harvested may also have potential for use as an aquaculture feed (Witt *et al.*, 1981; Pantastico, 1987; Fabregas & Herrero, 1986; de la Noüe & de Pauw, 1988; Villon *et al.*, 1989).

Although, the use of microalgae to remove nutrients from aquaculture effluents has been studied, there are few reports of the use of marine species (Alderson & Howell, 1973; Siddall, 1974; Honn & Chavin, 1975; Gerhardt, 1981). The aim of this chapter was therefore to examine the ability of marine microalgae to treat fish farm effluents. Experiments were conducted to screen a wide range of marine microalgae simultaneously for their ability to remove inorganic nutrients (ammonium N-NH_4^+, nitrate N-NO_3^- and ortho-phosphate P-PO_4^{3-}) from eel aquaculture effluent, to remain dominant, and grow well under culture conditions representative of an aquaculture system.

5.2 Materials and Methods

5.2.1 Microalgae

A total of 106 algae were screened for their ability to remove nutrients from eel aquaculture effluent. Of these, 36 species were obtained from culture collections, 66 were endemic isolates from St Andrews Bay (Sections 2.2.3, 2.3.1; Table 2.1) and 4 were isolated from eel (*Anguilla anguilla*) aquaculture effluent.

5.2.2 Aquaculture Effluent

Two types of eel aquaculture effluent were used in this study and their physical properties and nutrient composition are shown in Table 5.1. Activated effluent was sampled from the outflow of fish tanks of a small closed recirculating unit at Inverness, Scotland which is used in research to optimise fish growth and reduce stress effects in eel (*Anguilla anguilla*) culture (provided by Dr N. Hazon, Gatty Marine Laboratory, University of St Andrews). In this system, recirculating effluent is treated using a rotating

biological contactor aerator (Stahlermatic system, Germany) which consists of plastic discs to provide growth surfaces for fixed film bacteria. Rotation of the discs aerates and resuspends the activated sludge in a chamber through which the aquaculture effluent passes (Gabel, 1984). Eel fingerlings (Western Aquaculture, Bristol) were cultured in 40 % seawater at 23 °C (N. Hazon, pers comm). Eels were fed standard commercial diets (BP Nutrition, Invergordon) under normal aquaculture feeding regimes dependant upon fish size. Two samples of activated effluent were used. The second was taken two months later than the first when high concentrations of nitrate had accumulated as a result of poor denitrifying activity in the rotating biological contactor treatment system.

Untreated effluent was obtained directly from an eel tank which was operated under the same conditions (temperature, salinity, stocking density, feeding regime) as the pilot plant. Samples were taken from tank water which had not been changed for five days.

5.2.3 Screening Experiments

Algae were preadapted to untreated eel aquaculture effluent for one week prior to the screening experiments by inoculating triplicate test-tubes containing 10 ml of effluent with 2 ml of algal culture in exponential phase. The test-tubes were sealed with cotton wool plugs and placed in a Conviron controlled environmental incubator (Section 2.2.1) at 23 °C. Each screening began by adding 5 ml of effluent to three replicate test-tubes of each species containing 5 ml pre-adapted algal culture. The cultures were mixed by swirling once a day. Nutrient ($N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$) concentrations remaining in each algal culture were determined simultaneously at the end of the period of culture using the microplate

format (Section 2.2.7). Algal culture purity was confirmed through microscopic examination (Section 2.2.2) after seven days growth. Ammonium, nitrate and ortho-phosphate removal was calculated by subtracting the average nutrient concentration remaining in the triplicate algal cultures from that in control flasks of diluted effluent to which algae had not been added.

5.3 *Results*

Of 106 microalgae screened, 63 survived pre-adaptation to untreated eel aquaculture effluent. Of those that did not survive, eight species (*Nitzschia ovalis, Thalassiosira weissflogii, Oscillatoria animalis, Spirulina platensis, Amphidinium carterae, Micromonas pusilla, Tetraselmis* sp. (Biobred Ltd) and *Coccolithophora* sp.) were from culture collections and the other 35 were all endemic isolates of St Andrews Bay. Pre-adapted microalgae were screened twice for their ability to remove ammonium and ortho-phosphate from each of the three effluent samples (untreated, first activated and second activated) over two days treatment time and the mean removal values calculated.

5.3.1 *Nutrient Removal from Untreated Eel Aquaculture Effluent*

Thirty-nine species and isolates remained in unialgal culture and displayed a range of abilities to remove nutrients from untreated eel effluent (Fig. 5.1). Eighteen species and isolates removed high concentrations of nutrients. Of these, three removed >91.8 % of the ammonium and >99.4 % of the ortho-phosphate, compared to control concentrations (301.5 ± 7.3 mmol m^{-3} $N\text{-}NH_4^+$ and 26.6 ± 0.7 mmol m^{-3} $P\text{-}PO_4^{3-}$) (Fig. 5.1). A further four of these 18 species and isolates removed

Figure 5.1 Key

1 SA90C1
2 SA90B2
3 SA91B43
4 SA92B48
5 EE92C4
6 SA91C10
7 SA91B42
8 *Tetraselmis* sp.
9 *Tetraselmis suecica*
10 SA91B33
11 EE92C3
12 SA91B39
13 SA91C6
14 SA90B5
15 SA90B4
16 *Tetraselmis tetrathele*
17 SA90C2
18 *Tetraselmis rubens*
19 *Chlorella salina*
20 SA90B3
21 *Phaeodactylum tricornutum*
22 SA92C16
23 SA91B12
24 *Chaetoceros calcitrans*
25 SA91CY1
26 SA91B47
27 *Chrysochromulina chiton*
28 *Porphyridium purpureum*
29 *Tetraselmis verrucosa*
30 *Stichococcus bacillaris*

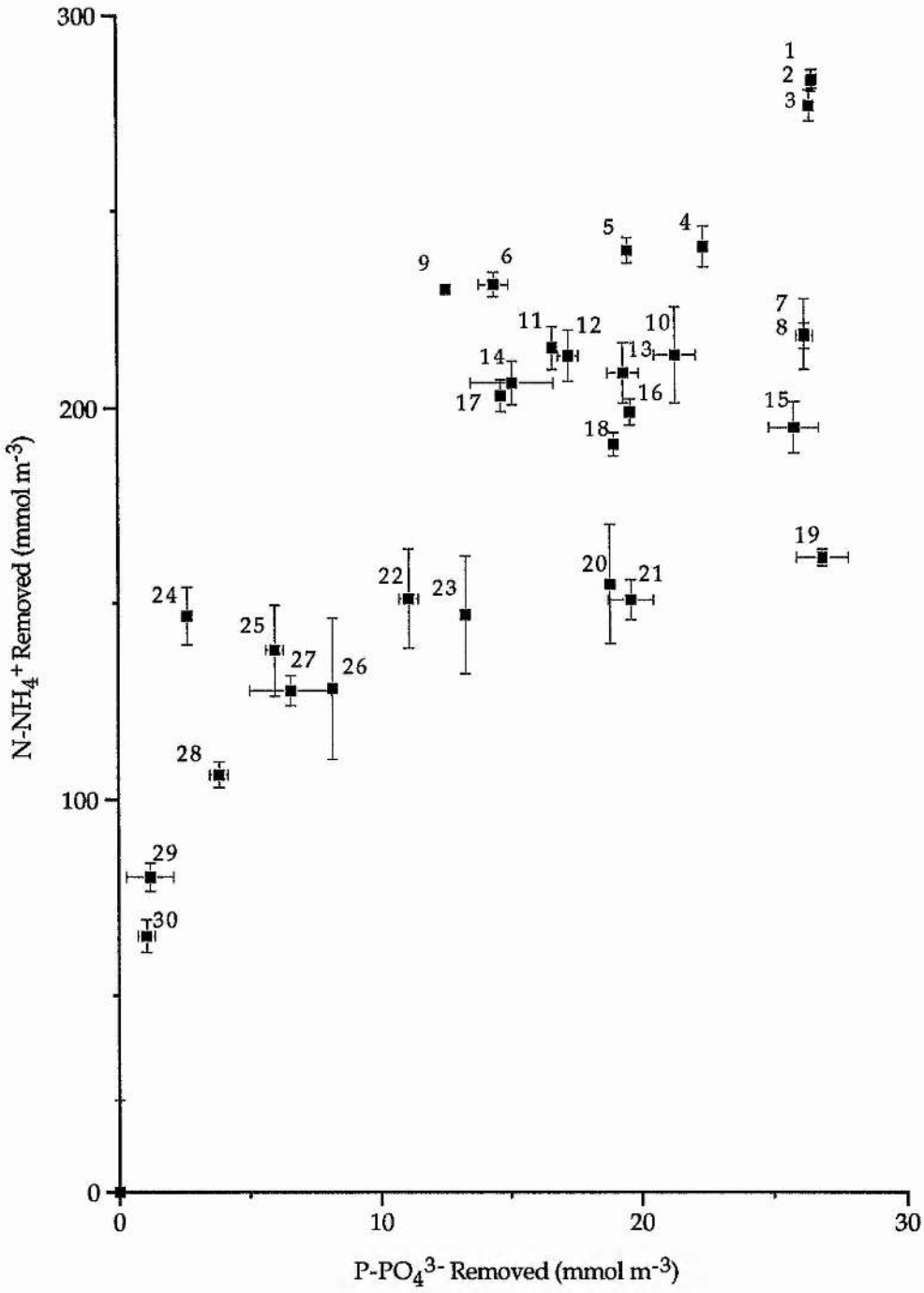

Fig. 5.1 Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species cultured for two days on untreated eel effluent in small-scale (10 ml) batch cultures under controlled conditions. Only the 30 species and isolates which remained unialgal and removed both $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ are shown. Values are means of means ± s.d. of duplicate experiments in which final nutrient concentrations in triplicate algal cultures were compared to final concentrations in controls without algae (301.5 ± 7.3 mmol m^{-3} $N\text{-}NH_4^+$, 26.6 ± 0.7 mmol m^{-3} $P\text{-}PO_4^{3-}$). The s.d. may be too small to be seen.

>97.1 % of the ortho-phosphate, but only removed between 72.6 % and 53.6 % of the ammonium compared to controls.

Of the remaining 21 species and isolates, 12 removed ammonium and ortho-phosphate at lower rates, while in the cultures of nine species the ortho-phosphate concentration increased. These included *Chlamydomonas reginae, Chlorella stigmatophora, Nannochloropsis oculata, Pavlova lutheri,* all three species of *Rhodomonas* and the eel aquaculture effluent isolates EE92C1 and EE92C2. However, some of these species removed as much as 65.8 % of the ammonium compared to controls (Appendix 5, Fig. A 5.1). A direct relationship between removal of the two nutrients was shown by the 39 species and isolates which remained in unialgal culture (determined by simple linear regression, $r=0.769$, $F_{1,37}=53.36$, $p<0.001$) (Figure 5.1).

5.3.2 Nutrient Removal from First Activated Eel Aquaculture Effluent Sample

Although nitrate was the main nitrogen source in the first activated eel effluent sample, and ortho-phosphate concentrations were much higher than in untreated effluent, nutrient removal by microalgae from both effluents was similar (Figs. 5.1, 5.2). Forty-three species and isolates remained in unialgal culture and displayed a range of abilities to remove nutrients from the activated effluent (Fig. 5.2). Only one species removed >90 % of all three nutrients compared to control concentrations (1771.4 ± 15.3 mmol m^{-3}, $N\text{-}NO_3^-$, 7.1 ± 0.9 mmol m^{-3} $N\text{-}NH_4^+$ and 415.2 ± 5.9 mmol m^{-3} $P\text{-}PO_4^{3-}$) (Fig. 5.2). Two other species removed >98.3 % ammonium and >93.7 % nitrate but <52.0 % ortho-phosphate compared to control flasks without algae (Fig. 5.2). Of the remainder, 17 species removed >90 % of the ammonium but between 78.4 % and 12.2 % of the nitrate, and

Figure 5.2 Key

1 SA90B2
2 SA91B33
3 SA90B4
4 SA91B39
5 SA90C1
6 SA91B43
7 SA90B3
8 SA90B5
9 SA91C6
10 SA90C3
11 SA91C10
12 SA91B42
13 SA91CY1
14 SA90C4
15 *Oxyrrhis marina*
16 SA91B47
17 *Tetraselmis tetrathele*
18 SA92B48
19 *Tetraselmis suecica*
20 *Phaeodactylum tricornutum*
21 *Rhodomonas marina*
22 EE92C1
23 SA90C3
24 EE92C2
25 SA92C16
26 *Prymnesium parvum*
27 *Rhodomonas baltica*
28 *Rhodomonas* sp.
29 *Stichococcus bacillaris*
30 *Chlorella salina*
31 *Chrysochromulina chiton*
32 *Tetraselmis* sp.
33 *Chlorella stigmatophora*
34 *Nannochloropsis oculata*
35 *Chaetoceros calcitrans*
36 SA90C2
37 *Skeletomema costatum*
38 *Tetraselmis verrucosa*
39 *Porphyridium purpureum*
40 *Nitzschia longissima*
41 *Chlamydomonas reginae*
42 *Tetraselmis rubens*

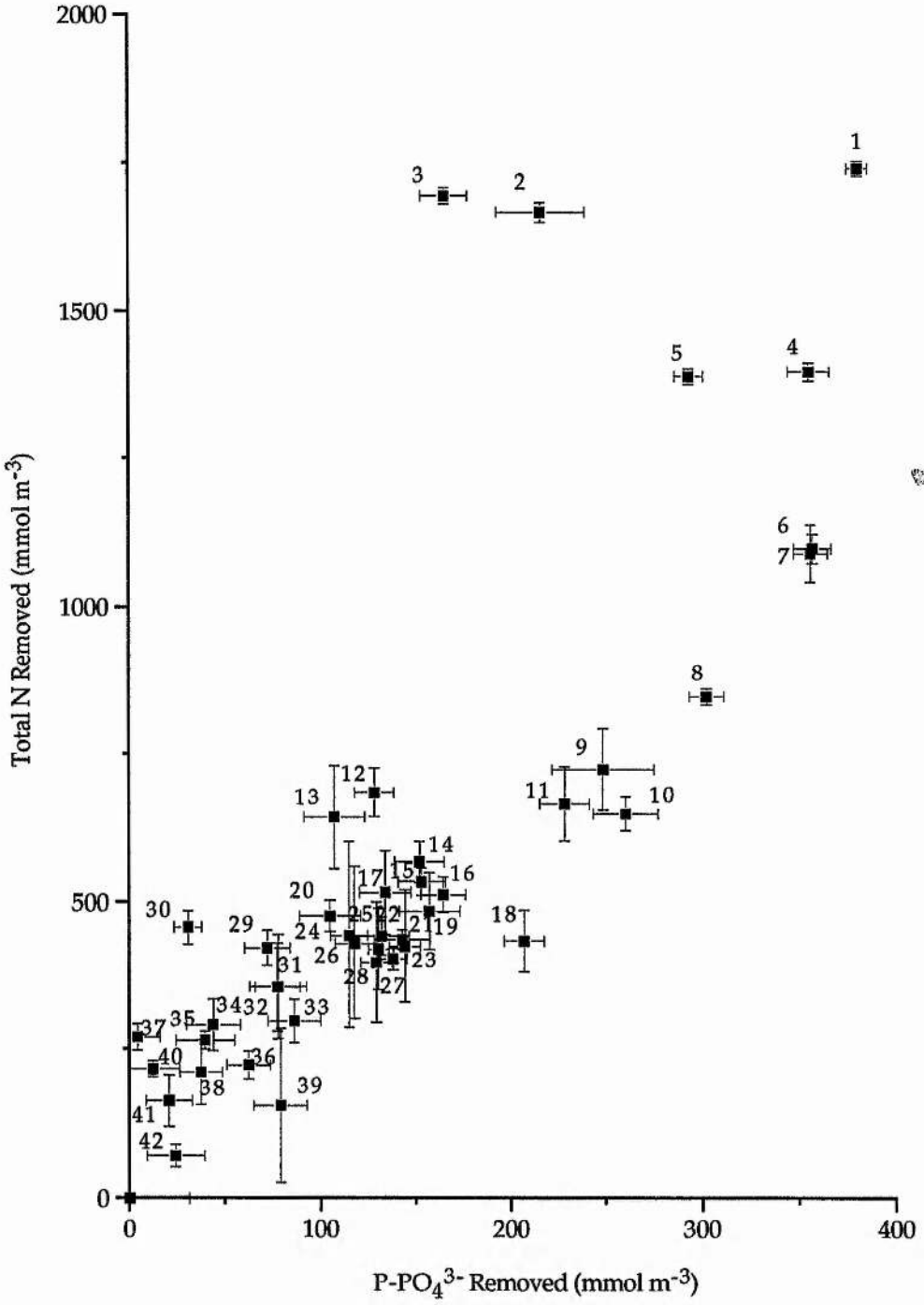

Fig. 5.2 Amounts of total nitrogen ($N\text{-}NH_4^+$ and $N\text{-}NO_3^-$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species and isolates cultured for two days on "activated" eel aquaculture effluent in small-scale (10 ml) batch cultures under controlled conditions. Only the 42 species and isolates which remained unialgal and removed both total-N and $P\text{-}PO_4^{3-}$ are shown. Values are means of means ± s.d. of duplicate experiments in which final nutrient concentrations in triplicate algal cultures were compared to final concentrations in controls without algae (1778.5 ± 16.2 mmol m^{-3} Total-N and 415.2 ± 5.9 mmol m^{-3} $P\text{-}PO_4^{3-}$).

between 86.0 % and 15.0 % of ortho-phosphate. The ammonium concentration increased in the cultures of two species (*Nannochloropsis oculata* and the eel isolate EE92C3) although these species removed 16.5 % and 37.1 % nitrate respectively. The ortho-phosphate concentration increased in the culture of one species (*Botryococcus braunii*), while this species removed 30.0 % ammonium and 14.5 % nitrate (Appendix 5; Fig. A 5.2). A direct relationship between removal of the two nutrients was also displayed (determined by simple linear regression, $r=0.782$, $F_{1,41}= 64.60$, $p< 0.001$).

Two species (*Dunaliella tertiolecta* and *Coccolithus* sp.) initially demonstrated good nutrient removal from both untreated and activated eel effluent but died during the five days between the nutrient measurement and culture analysis. These species also died after removing high levels of nutrients from 1:1 diluted wastewater.

5.3.3 Nutrient Removal from Second Activated Eel Aquaculture Effluent

Both nitrate and ammonium concentrations in the second sample of activated eel effluent were much higher than in the first (Table 5.1). Consequently only eight of the forty species and isolates remaining in unialgal culture removed nitrate, and removal was no more than 34.6 % of the control concentration (12.4 ± 0.4 mol m^{-3} $N\text{-}NO_3^-$). However the forty species and isolates remaining in unialgal culture displayed a range of abilities to remove ammonium and ortho-phosphate (Fig. 5.3), and sixteen species and isolates removed >90 % ammonium and >80 % ortho-phosphate compared to control concentrations (274.5 ± 6.1 mmol m^{-3} $N\text{-}NH_4^+$ and 581.2 ± 1.3 mmol m^{-3} $P\text{-}PO_4^{3-}$). Of the remaining species and isolates, 22

Table 5.1 Physical properties and nutrient composition of untreated and activated eel aquaculture effluents used in experiments. Values are means ± s.d. of triplicate samples.

Property	Untreated	Activated[1]	Activated[2]
OD_{570} ($\times 10^{-2}$)	1.8 ± 0.3	13.6 ± 0.2	4.5 ± 0.4
pH	7.6	6.8	6.5
Salinity (‰)	12.8	14.9	13.1
Conductivity (seimens)	18125	19950	17950
$N\text{-}NO_3^-$ (mmol m^{-3})	4.4 ± 0.1	1.8 ± 0.02[3]	12.4 ± 0.5[3]
$N\text{-}NO_2^-$ (mmol m^{-3})	2.0 ± 0.1	13.6 ± 0.3	38.8 ± 0.3
$N\text{-}NH_4^+$ (mmol m^{-3})	301.5 ± 23.6	7.1 ± 0.9	274.5 ± 6.1
N-Urea (mmol m^{-3})	3.8 ± 0.2	2.0 ± 0.1	15.2± 0.8
$P\text{-}PO_4^{3-}$ (mmol m^{-3})	26.6 ± 0.2	415.2 ± 31.1	581.2 ± 1.3

[1]First activated sample
[2]Second activated sample
[3]($\times 10^{-3}$)

Figure 5.3 Key

1 SA92B48
2 SA92C17
3 SA91B43
4 SA90B2
5 SA90B5
6 SA91B39
7 *Chlorella salina*
8 *Tetraselmis rubens*
9 EE92C3
10 SA90C3
11 SA90B4
12 SA90C1
13 SA91B33
14 SA90B3
15 SA92C16
16 *Nitzschia longissima*
17 SA90C2
18 SA91C10
19 SA91B42
20 SA91C6
21 *Porphyridium purpureum*
22 *Oxyrrhis marina*
23 SA91CY1
24 *Tetraselmis suecica*
25 *Dunaliella tertiolecta*
26 *Chlorella stigmatophora*
27 *Chrysochromulina chiton*
28 *Tetraselmis verrucosa*
29 *Rhodomonas* sp.
30 *Chlamydomonas reginae*
31 *Tetraselmis* sp.
32 *Tetraselmis tetrathele*
33 *Rhodomonas baltica*
34 *Botryococcus brauni*
35 *Prymnesium parvum*
36 *Chaetoceros calcitrans*
37 *Rhodomonas marina*
38 *Skeletomema costatum*

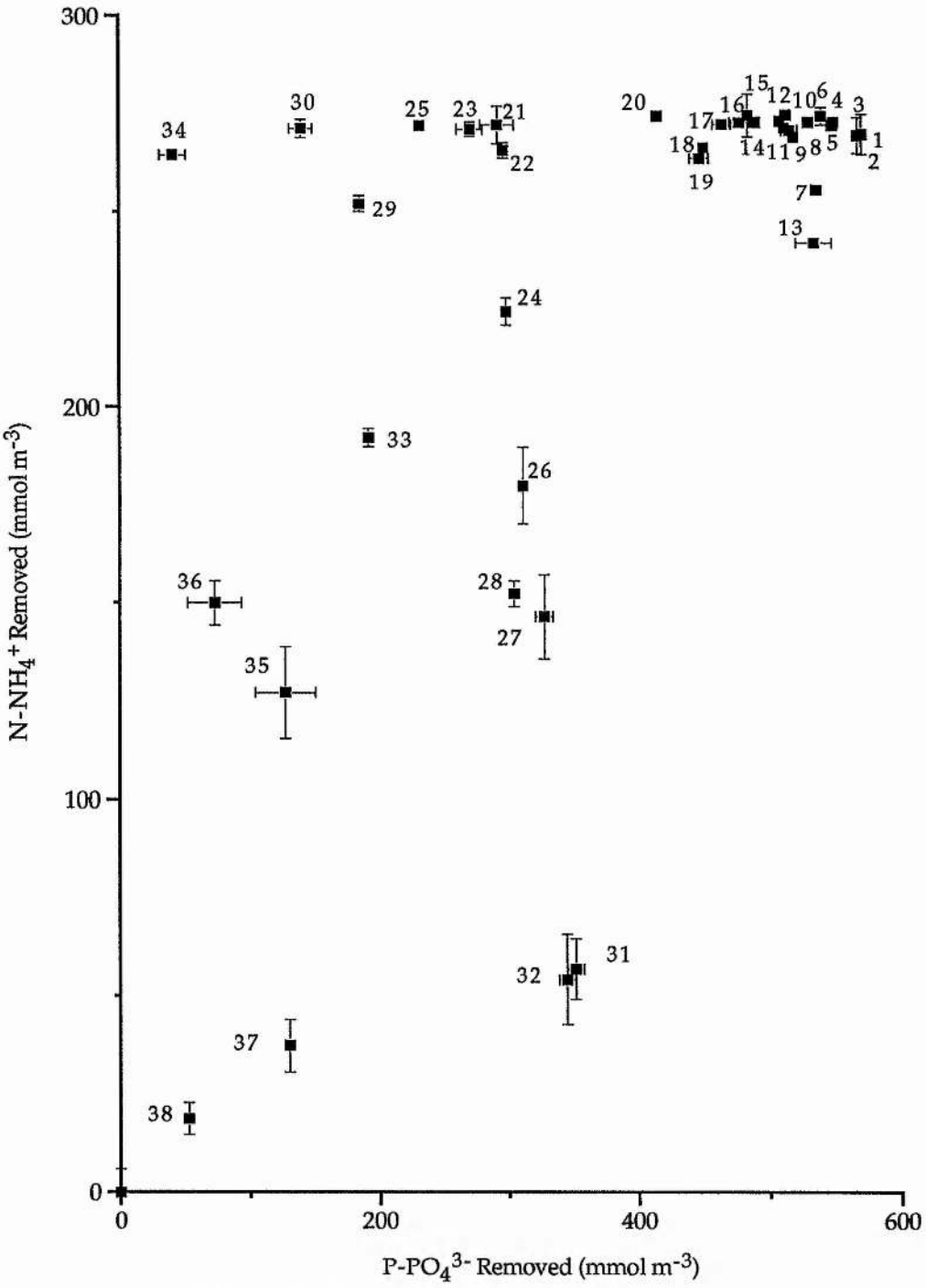

Fig. 5.3 Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species cultured for two days on "activated" eel effluent in small-scale (10 ml) batch cultures under controlled conditions. Only the 40 species and isolates which remained unialgal and removed both ammonium and ortho-phosphate are shown. Values are means of means ± s.d. of duplicate experiments in which final nutrient concentrations in triplicate algal cultures were compared to final concentrations in controls without algae (275.5 ± 6.1 mmol m^{-3} $N\text{-}NH_4^+$, 581.2 ± 1.3 mmol m^{-3} $P\text{-}PO_4^{3-}$). The s.d. may be too small to be seen.

removed ammonium and ortho-phosphate at lower rates. Eleven of these species and isolates removed high levels of ammonium (>91.8 %) but removed between 79.8 % and 7.1 % of ortho-phosphate compared to controls. The ortho-phosphate concentration increased in the cultures of the two remaining species (*Dunaliella salina* and *Pavlova lutheri*) although they removed 66.8 % and 34.8 % of ammonium respectively (Appendix 5; Fig. A 5.3).

5.4 Discussion

Batch culture screening of more than 100 species and isolates of marine microalgae selected 18 microalgae which demonstrated the ability to grow and remove high concentrations of ammonium and ortho-phosphate from untreated eel aquaculture effluent (Fig. 5.1). These species also remained in unialgal culture during sequential batch culture under optimum conditions required for eel growth (23 °C, 12.8 ‰ salinity) and low light intensities.

Many of these microalgae were also found to grow and treat activated eel effluent (Fig. 5.3), and to remove high concentrations of nitrate when ammonium concentrations were low. Fifteen of the best-treating species selected on untreated effluent were amongst the top 25 species identified when the results from the three screening experiments on untreated and activated effluent were combined (Table 5.2). The other microalgae tested either did not survive, were unable to remain in unialgal culture, or removed nutrients to a lesser extent, presumably due to differences in tolerance of culture conditions, or of eel aquaculture effluent as a growth medium.

Table 5.2 The 25 best-treating microalgal species and isolates remaining in unialgal culture ranked in order of combined mean nutrient removal. Mean ± s.d. % $N\text{-}NH_4^+$ and % $P\text{-}PO_4^{3-}$ removal were calculated from three batch culture screening experiments, one using untreated eel effluent and two using "activated" eel effluent.

Algal Species	Algal Class	Source	$\%N\text{-}NH_4^+$ Removal	$\%P\text{-}PO_4^{3-}$ Removal
SA92C17	Chlorophyceae	Endemic	98.2 ± 0.9	97.5 ± 0.1
[n]SA90B2	Bacillariophyceae	Endemic	96.6 ± 1.9	95.2 ± 0.3
[n]•SA91B43	Bacillariophyceae	Endemic	96.5 ± 4.2	93.3 ± 0.6
[n]SA90C1	Chlorophyceae	Endemic	97.9 ± 1.5	86.0 ± 0.3
[n]•SA91B39	Bacillariophyceae	Endemic	88.9 ± 3.2	80.5 ± 0.9
•SA92B48	Bacillariophyceae	Endemic	91.9 ± 1.8	77.4 ± 1.5
[n]SA90B4	Bacillariophyceae	Endemic	89.5 ± 2.7	74.9 ± 2.1
SA90B5	Bacillariophyceae	Endemic	89.2 ± 4.3	74.1 ± 3.5
[n]SA90B3	Bacillariophyceae	Endemic	83.0 ± 3.8	80.2 ± 1.3
SA90C3	Chlorophyceae	Endemic	101.3 ± 0.8	61.7 ± 0.7
[n]•SA91B33	Bacillariophyceae	Endemic	85.7 ± 3.6	74.7 ± 3.9
SA91C6	Chlorophyceae	Endemic	85.6 ± 2.8	68.0 ± 3.9
SA91C10	Chlorophyceae	Endemic	91.0 ± 1.9	62.2 ± 2.2
SA91B42	Bacillariophyceae	Endemic	76.8 ± 3.4	68.8 ± 0.7
[n]EE92C4	Chlorophyceae	Eel Effluent	88.6 ± 1.3	55.1 ± 0.4
Oxyrrhis marina	Dinophyceae	Plymouth	95.1 ± 0.8	43.9 ± 0.4
Dunaliella tertiolecta	Chlorophyceae	Plymouth	99.1 ± 0.2	39.8 ± 0.2
SA90C2	Chlorophyceae	Endemic	87.6 ± 2.3	50.0 ± 0.7
SA91C16	Chlorophyceae	Endemic	80.9 ± 3.0	52.1 ± 0.8
Nitzschia longissima	Bacillariophyceae	Biobred Ltd	86.6 ± 0.7	42.5 ± 0.9
Chlorella salina	Chlorophyceae	Plymouth	61.8 ± 2.2	66.9 ± 2.2
Tetraselmis suecica	Prasinophyceae	Plymouth	80.5 ± 5.5	45.5 ± 2.3
[n]*Tetraselmis tetrathele*	Prasinophyceae	Plymouth	57.5 ± 3.5	55.1 ± 0.9
•SA91CY1	Cyanophyceae	Endemic	74.3 ± 5.0	31.6 ± 1.5
EE92C3	Chlorophyceae	Eel Effluent	16.9 ± 7.2	71.2 ± 1.1

• Denotes species with adherent properties

[n] Denotes species capable of removing high concentrations of nitrate

The species selected for high nutrient removal and dominance on untreated eel aquaculture effluent were similar to those identified in the screening experiments using 1:1 diluted wastewater with seawater (Chapter 3). Algae were from two taxonomic divisions (Bacillariophyceae and Chlorophyceae), and of the 18 species selected, 14 were endemic isolates from the sewage outfall in St. Andrews Bay. Three endemic isolates of *Phaeodactylum tricornutum* (SA90B2, SA90B4 and SA91B43) were found to be amongst the best-treating species, while the strain of *P. tricornutum* obtained from the Plymouth Culture Collection showed poor treatment. The culture temperature (23 °C) was 4 °C above the optimum for this species (Goldman *et al.*, 1974; Goldman & Ryther, 1976), and may have contributed to the poor treatment by this strain. Four species of *Tetraselmis* were the only culture collection species amongst the best treating microalgae, and they were also identified in Chapter 3 for their ability to treat 1:1 diluted wastewater.

Rates of ammonium removal from the eel effluent were similar to those measured by other authors for removal from 1:1 diluted wastewater (Groenweg *et al.*, 1980; Shelef *et al.*, 1980; de la Noüe & Bassères, 1989; Tam & Wong, 1989) and those found in Chapter 3. More than 276.8 mmol N m^{-3} was removed from the untreated eel effluent by the three best-treating algae. Although ortho-phosphate concentrations in the untreated effluent were relatively low compared to other wastewaters (Groenweg *et al.*, 1980; Shelef *et al.*, 1980; de la Noüe & Bassères, 1989; Tam & Wong, 1989), the high removal rates (>302.2 mmol P m^{-3}) found for the best-treating species on activated effluent confirms the ability of these microalgal species to remove this nutrient.

The direct relationship found for the removal of ammonium and ortho-phosphate by the microalgae remaining in unialgal culture in the first two screening experiments suggests that the ratio of ammonium to

ortho-phosphate removal by microalgae is independent of the amounts removed. This is probably a reflection of the relative amounts of ammonium and ortho-phosphate required for algal metabolism (Reed, 1978). There was no relationship with the second activated sample possibly because of the excessive nitrate concentration.

The nitrogen:phosphorus ratios of the eel effluents used in the three screening experiments were 11:1 (untreated), 4:1 (first activated sample) and 22:1 (second activated sample). The low ortho-phosphate removal by the majority of algal species in effluents with N:P ratios less than the calculated optimum (Redfield ratio, 15:1) for culturing phytoplankton species (Rhee, 1978), shows the influence of N:P ratio on microalgal nutrient removal.

This study demonstrated the capability of marine microalgae to remove nutrients from recirculating eel aquaculture effluent. Although caution must be taken when extrapolating to a large scale, incorporation of a marine microalgal pond within existing recirculating systems may greatly reduce nutrient levels in the culture medium. This would have two benefits. First, as most microalgae remove ammonium in preference to nitrate (Dortch, 1990; Cochlan & Harrison, 1991a, b & c; Raven *et al.*, 1992) (Table 5.4), microbial nitrate production would cease and nitrate would no longer accumulate in the effluent. Second, costly aeration of the treated effluent before reuse would not be needed since this would adequately be achieved through algal photosynthesis. In addition, several of the best-treating species grow better when attached to surfaces than in suspension, and may adhere to the filter discs. Possibly, direct improvement of rotating biological contactor systems could be achieved through establishing an algal biomass on the biofilm. The use of these species could maintain a treated effluent which is algal free and enable easy

harvest of the algal biomass. Microalgal ponds incorporated into recirculating systems may have application for effluent treatment of aquaculture effluents of other species such as Sea Bass and Sea Bream which could also be cultured at sites away from the coast on seawater of reduced salinity.

Chapter Six

Wastewater Nutrient Removal by Marine Microalgae under Continuous Culture and Controlled Conditions

6.1 Introduction

In the previous chapters, batch culture was shown to provide a reliable and repeatable method for simultaneous screening of a large number of microalgae for the ability to remove nutrients from 1:1 diluted wastewater. However, batch culture is of limited use at large scale, since the growth rate of the algae tends towards zero, either because of an accumulation of a product which cannot be tolerated, or because of a lack of a particular nutrient or light (Richmond, 1983). Once the nutrients have been removed, part of the culture must be exchanged for untreated wastewater and the whole process restarted. Therefore, only small volumes of wastewater are treated over relatively long periods of time and the process is labour intensive. The problems of batch culture are overcome by using continuous culture, in which wastewater is added and algal culture removed at a controlled rate (Droop, 1966; Vonshak, 1986). The algal culture is held in the exponential phase of growth and culture conditions can be kept constant and optimal for efficient nutrient removal (Ukeles, 1979; Richmond, 1983; Fogg, 1987). Consequently, continuous cultures have higher production than batch cultures, and enable outdoor cultures of far greater volume to be maintained (De Pauw *et al.*, 1980; Witt *et al.*, 1981).

This chapter describes experiments to investigate the ability of the 14 best-treating microalgal species selected in Chapter 3 to treat wastewater in small-scale continuous cultures under controlled environmental conditions. The ability of these species to remove high levels of nutrients continuously from 1:1 diluted wastewater was measured and compared to treatment by a mixed culture of algae which occur naturally in the wastewater (wastewater species). The dominance of the best-treating species over the naturally occurring species was also determined.

6.2 *Materials and Methods*

6.2.1 *Microalgae*

The 14 best-treating microalgae selected in Chapter 3 were used in this study. All showed >90 % removal of ammonium and ortho-phosphate from 1:1 diluted wastewater and remained in unialgal culture. Eleven were isolates from the waters surrounding the wastewater outfall in St Andrews Bay including SA90B2, SA90B4, SA91B12, SA91B33, SA91B39, SA91B43, and SA92B48 (Bacillariophyceae), SA90C1, SA90C2, SA91C6 (Chlorophyceae) and SA91CY1 (Cyanophyceae). The remaining three species (*Chlorella salina* (Chlorophyceae), *Tetraselmis* sp. (Prasinophyceae) and *Porphyridium purpureum* (Rhodophyceae) were obtained in unialgal culture from culture collections (Table 2.1). Algal inocula were taken from unialgal stock cultures which were in exponential growth phase (Section 2.3.3).

6.2.2 *Apparatus*

Continuous culture vessels were designed and constructed from one litre round glass flasks with an outflow tube which extended from near the base of the flask (Fig. 6.1). The cultures were set up in a greenhouse, although temperature was maintained between 13-18 °C by an external circulating water bath using a dip cooler (Tecam Cambridge Ltd, UK) and a heater (Tecam, Tempette, model TE-7). Cultures were illuminated by ambient light supplemented by artificial light (150-170 $\mu E\ m^{-2}\ s^{-1}$, 12 h:12 h photoperiod) supplied by three mercury vapour lights (Trulite, 400W, MBF/U, GEC, London, UK). Diluted unfiltered primary sewage effluent was pumped peristaltically (Watson-Marlow Ltd, Falmouth, UK; model MHRE 200) to each culture vessel from a single 10 l glass storage carboy which was kept in the dark within a black plastic cover. Mixing was

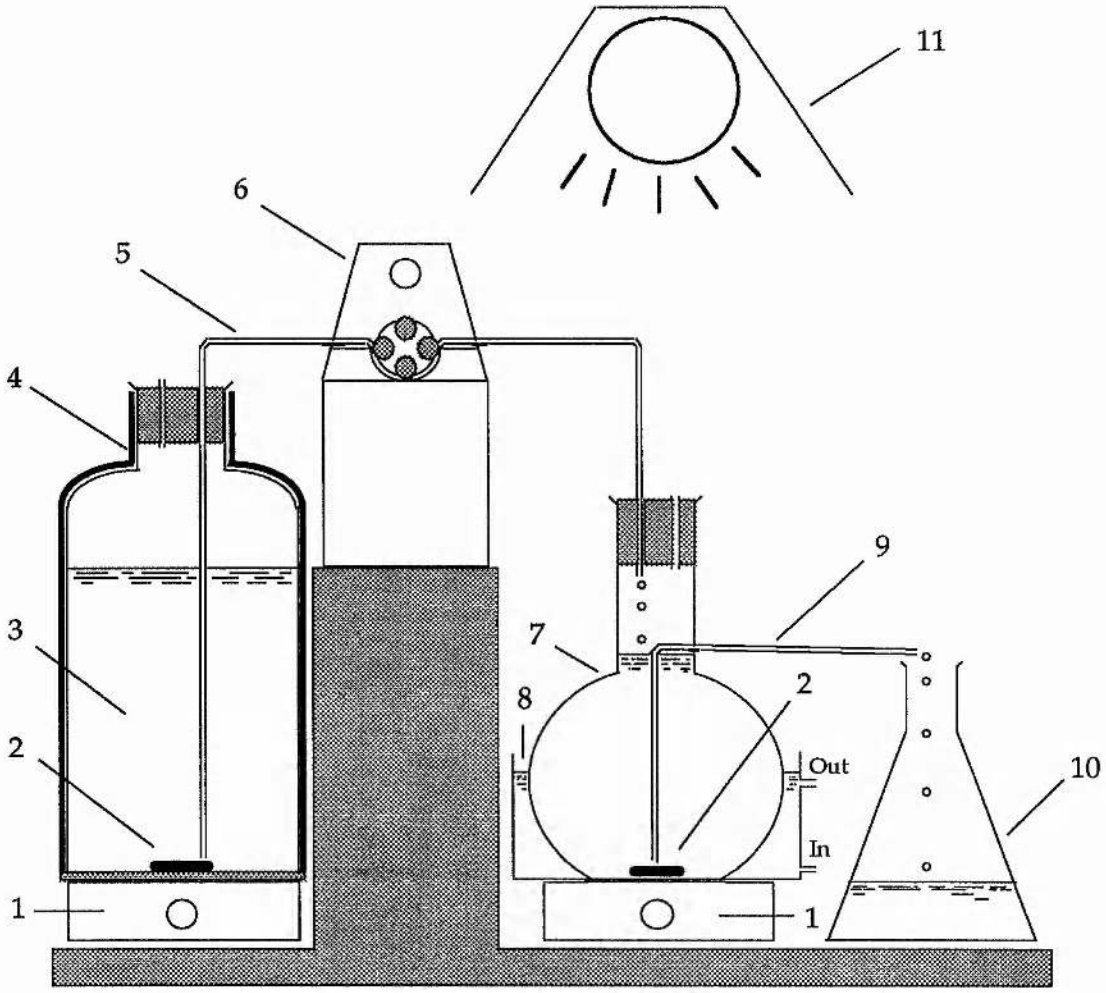

Figure 6.1 Schematic diagram of complete continuous culture unit. 1: magnetic stirrer; 2: plastic coated stirring bar; 3: 10 l nutrient supply bottle; 4: black plastic sleeve; 5: nutrient feed line; 6: peristaltic pump; 7: continuous culture vessel (1 l round glass flask); 8: cooling bath (13-18 °C); 9: overflow tube; 10: effluent collection flask; 11: mercury vapour light (150-170 $\mu E\ m^{-2}s^{-1}$, 12 h:12 h photoperiod).

accomplished with a plastic-coated magnetic stirring bar. Treated medium flowed out of the culture through the outflow tube, from which 10 ml samples were collected for analysis. When sampling was not in progress, the outflow dripped into a collection flask. A schematic diagram of a complete continuous culture unit is shown in Figure 6.1.

6.2.3 Operation

The continuous culture unit was set up using sterile equipment. Algal species were preadapted to 1:1 diluted wastewater and to conditions in a greenhouse by culturing 250 ml of exponential phase algal culture (grown on E-S media; Section 2.3.3) and 250 ml of wastewater in a flask for one week. A control culture to measure nutrient removal by wastewater species was also set up by adding 250 ml of sterile seawater to 250 ml of wastewater.

To maintain an algal culture of sufficient biomass to treat the wastewater, the dilution rate for continuous culture must be equal to the exponential growth rate of the algae. Algal growth rate varies with species and depends upon the conditions of culture including, climate, wastewater, culture apparatus (Oswald and Gotaas, 1955; De Pauw & Vaerenbergh, 1983; Strain *et al.*, 1986). Therefore, the dilution rate for the conditions in this study was determined by a preliminary batch culture experiment. A further 500 ml of 1:1 diluted wastewater was added to each flask and daily measurements of algal biomass (OD_{570}; Section 2.3.2) were made over one week. The continuous culture dilution rates (equal to the exponential growth rates), were calculated for the 14 species and found to be similar, approximating to 0.5 doublings day^{-1} (Appendix 6; Table A 6.1). Algae were maintained in continuous cultures at this dilution rate for 14 days. At daily

intervals, 10 ml samples of the inflow and outflow of each culture were taken for determination of ammonium and ortho-phosphate concentrations (Section 2.2.7) and microscopic examination (Section 2.2.2). The percentage removal of nutrients by each algal culture (% removal) was calculated from:

$$\% \text{ removal} = \frac{(I - O)}{I} \times 100$$

Where:

I: inflow nutrient concentration

O: outflow nutrient concentration

Nutrient removal by the algae was compared to that by the mixed culture of wastewater species which had grown up in the control culture (dominated by unidentified chlorophyceaen and bacillariophyceaen species).

6.3 Results

The nutrient concentrations in the inflow and outflow from one litre continuous cultures of 14 species grown under controlled conditions over 14 days, together with the mean percentage removal and culture state, are shown in Table 6.1. Eight of the microalgal species continuously removed >80 % of both ammonium and ortho-phosphate during the experiment (Table 6.1). A time course of the change in nutrient concentrations in the outflow from a culture of a representative species, isolate SA91CY1, is shown in Figure 6.2. Initially, high rates of nutrient removal were achieved by the other six species tested, but these declined over the treatment period, resulting in lower mean percentage removal (Table 6.1). A time course of

Table 6.1 Percentage removal of ammonium and ortho-phosphate by 14 marine microalgal species and a mixed culture of wastewater species grown in one litre continuous cultures on 1:1 diluted wastewater over 14 days continuous culture. The purity of the cultures on the final day of the experiment is also shown. Nutrient concentrations in the inflow were (315.2 ± 48.1 $N\text{-}NH_4^+$ mmol N m^{-3}; 56.6 ± 5.8 $P\text{-}PO_4^{3-}$ mmol P m^{-3}). Values are means ± s.d. of daily measurements over two weeks.

Algal Species	% $N\text{-}NH_4^+$ Removal	% $P\text{-}PO_4^{3-}$ Removal	Culture State
Tetraselmis sp.	83.9 ± 3.0	90.1 ± 1.4	Unialgal
SA90B2	89.1 ± 1.7	98.8 ± 0.9	Unialgal
SA90B4	88.3 ± 5.7	98.8 ± 1.4	Unialgal
SA91B33	84.0 ± 6.9	98.3 ± 1.6	Unialgal
SA91B39	83.9 ± 6.6	90.5 ± 3.4	Unialgal
SA91B43	84.1 ± 6.6	97.0 ± 1.9	Unialgal
SA92B48	82.5 ± 6.3	99.2 ± 1.1	Unialgal
SA91CY1	95.8 ± 1.6	95.9 ± 2.3	Unialgal
Chlorella salina	74.5 ± 12.3	76.2 ± 9.0	Contaminated
P. purpureum	71.3 ± 14.6	64.4 ± 17.3	Contaminated
SA91B12	73.2 ± 6.1	68.6 ± 1.8	Contaminated
SA90C1	70.5 ± 10.8	60.6 ± 16.8	Contaminated
SA90C2	58.1 ± 13.4	70.4 ± 17.5	Contaminated
SA91C6	74.4 ± 7.6	76.1 ± 8.0	Contaminated
Wastewater sp.	44.5 ± 6.5	50.3 ± 3.9	Mixed Culture

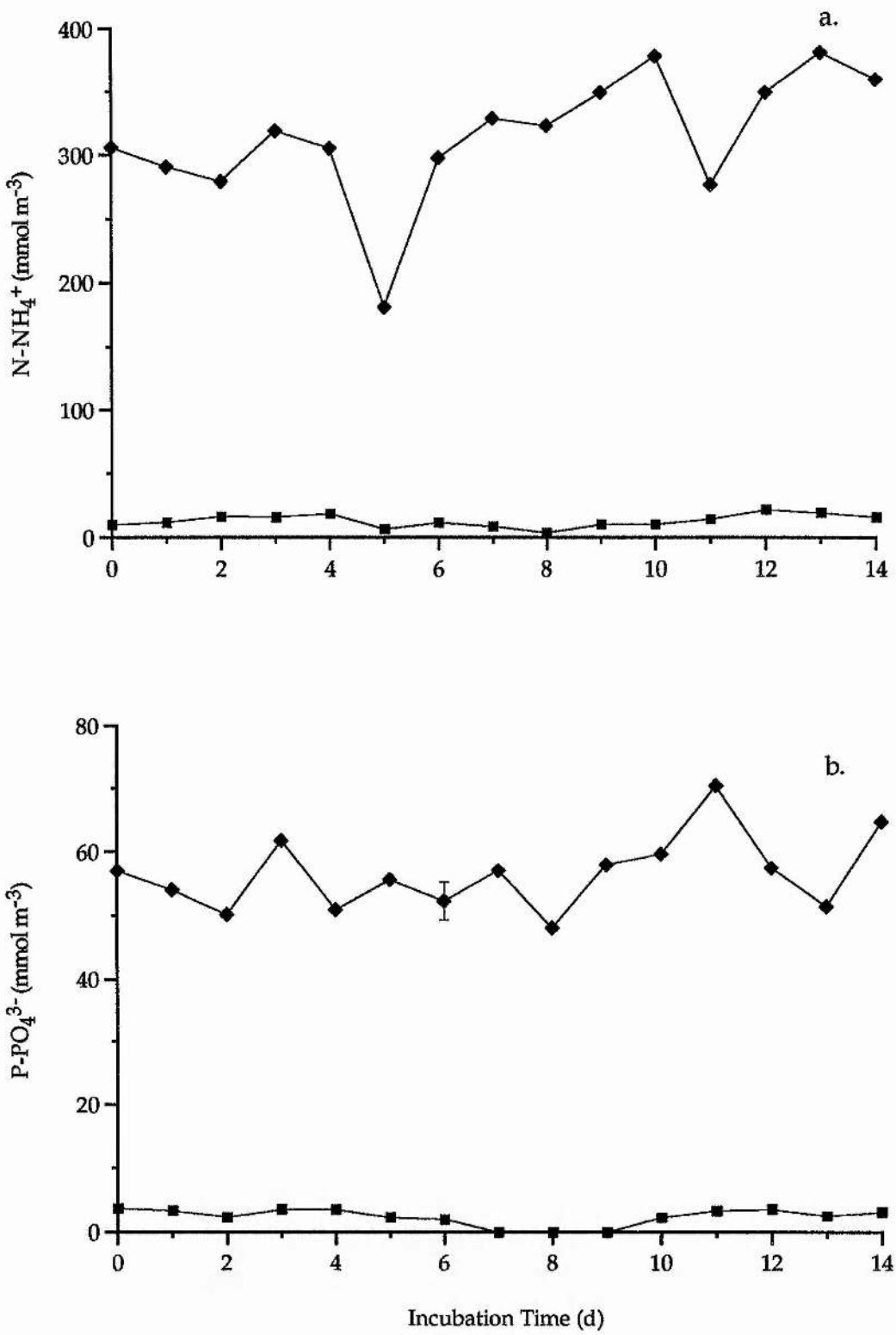

Figure 6.2 Concentrations of ammonium (a.) and ortho-phosphate (b.) in the inflow (◆-◆) and the outflow (■-■) of a continuous culture of isolate SA91CY1 over a 14 day period of treatment. Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

the change in nutrient concentrations in the outflow from a culture of a representative species, isolate SA90C2 is shown in Figure 6.3. Nutrient removal by the mixed culture of wastewater species was considerably lower than the marine species, at 44.5 % for ammonium and 50.3 % for ortho-phosphate, but was uniform over the culture period (Table 6.1; Fig. 6.4). The nutrient concentration of the diluted wastewater inflow (which was the same for all species) generally remained uniform throughout the experiment (315.2 ± 48.1 mmol m^{-3} $N\text{-}NH_4^+$ and 56.6 ± 5.8 mmol m^{-3} $P\text{-}PO_4^{3-}$), although the ammonium concentration did increase slightly over the culture period (Figs. 6.2, 3, 4).

The eight best-treating species which included six endemic isolates of the division Bacillariophyceae, a Cyanophyceaen and *Tetraselmis* sp. (Prasinophyceae), all remained in unialgal culture. The six remaining species became contaminated by wastewater species during the first few days of the experiment.

6.4 Discussion

Unialgal cultures of marine microalgal species were capable of removing high levels of nutrients from continuous culture on wastewater diluted 1:1 with seawater. Treatment was comparable to, or better than, that by freshwater species under similar conditions (De Pauw *et al.*, 1980; Lincoln & Hill, 1980; Shelef *et al.*, 1980; Martin *et al.*, 1985a & b; de la Noüe & Bassères, 1989; Megharaj *et al.*, 1992). Contamination of cultures by microalgal species which occurred naturally in the wastewater resulted in a decline in nutrient removal (Table 6.1; Fig. 6.3). This may have been due to a lower growth rate as a result of competition between the species (D'Elia *et*

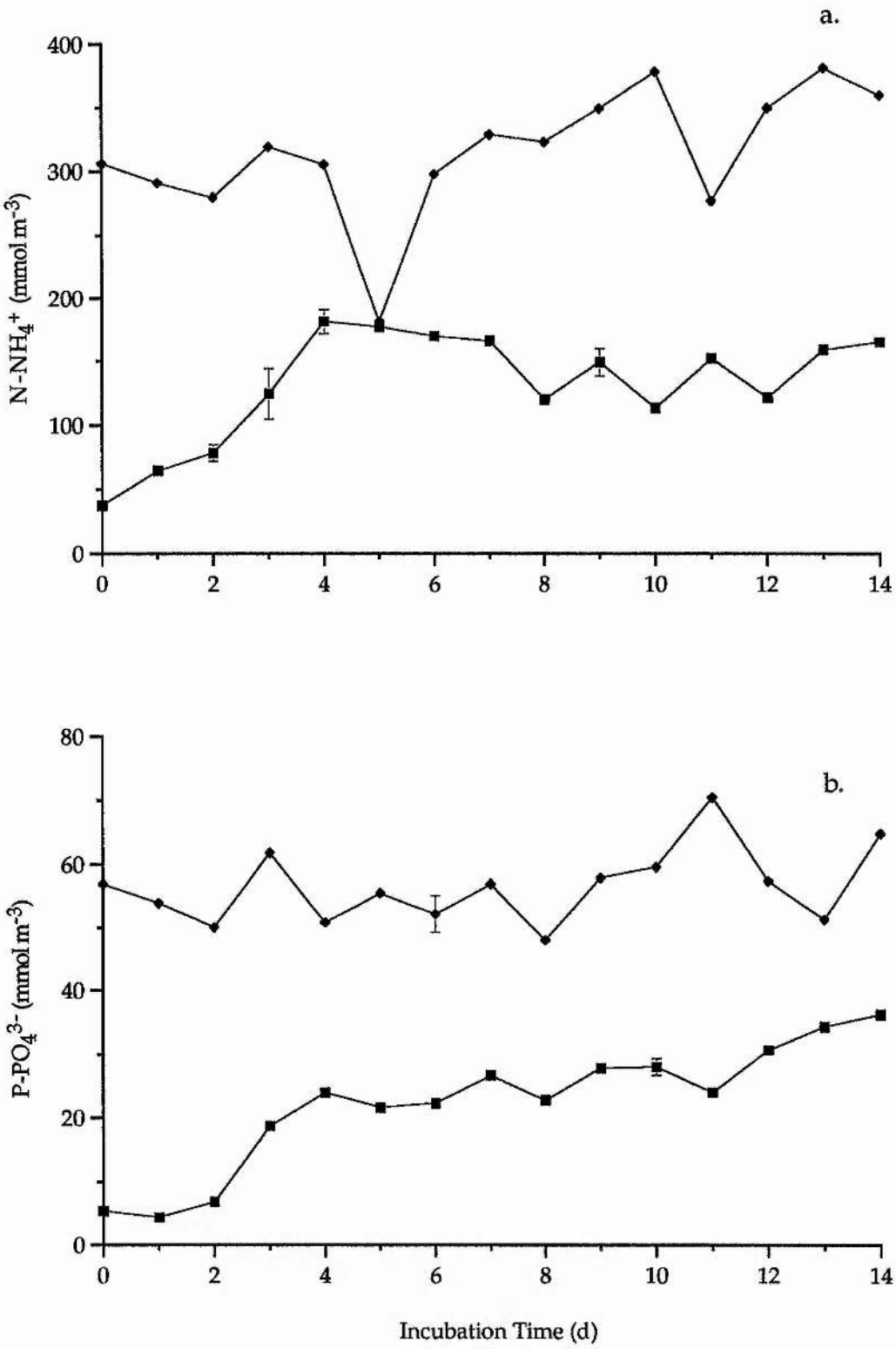

Figure 6.3 Concentrations of ammonium (a.) and ortho-phosphate (b.) in the inflow (◆-◆) and the outflow (■-■) of a continuous culture of isolate SA90C2 over a 14 day period of treatment. Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

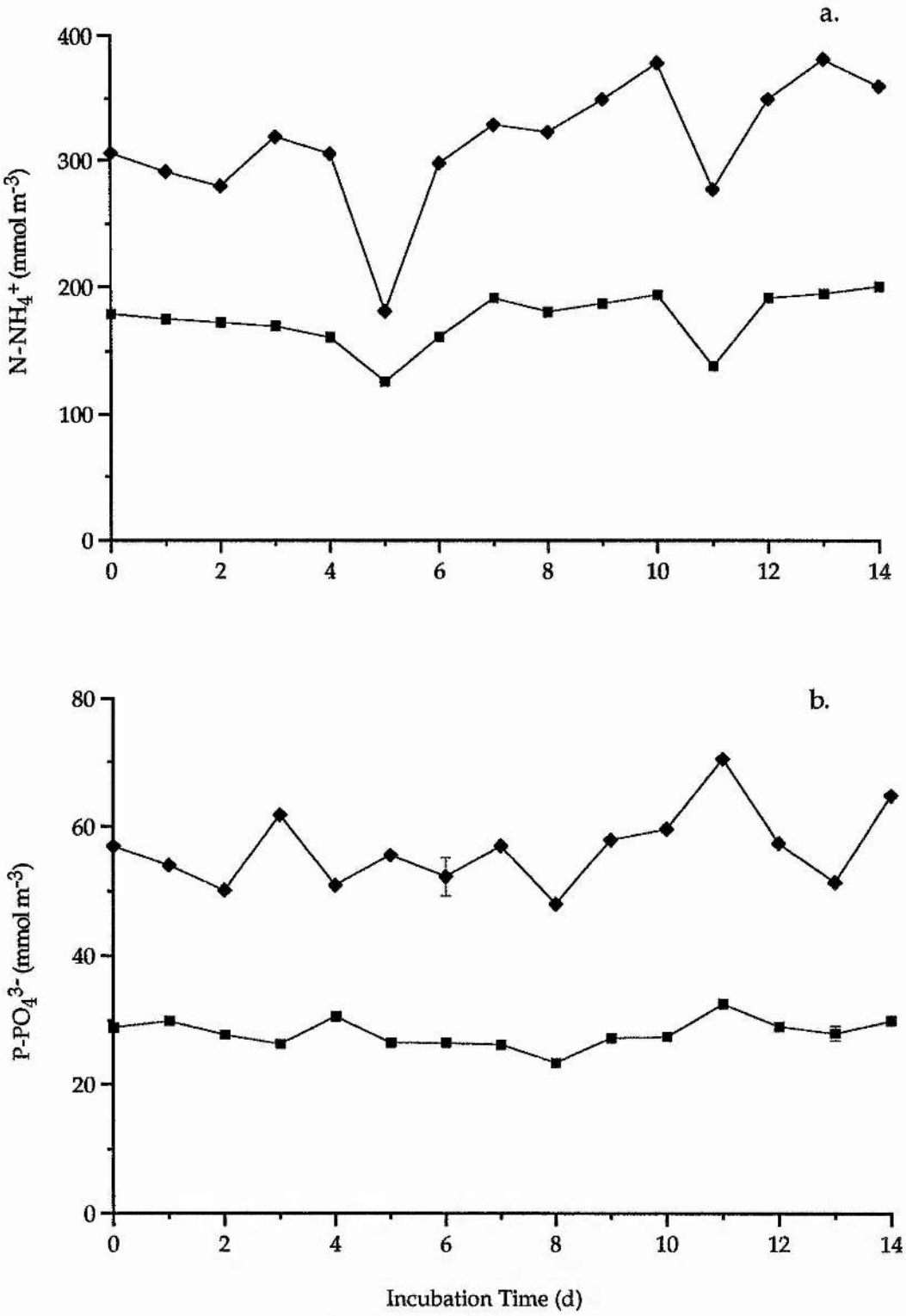

Figure 6.4 Concentrations of ammonium (a.) and ortho-phosphate (b.) in the inflow (◆-◆) and the outflow (■-■) of a continuous culture of algal species occurring naturally in the wastewater (wastewater sp.) over a 14 day period of treatment. Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

al., 1979), and may also explain the low treatment by the mixed culture of wastewater species.

Of the eight microalgae which remained in unialgal culture, one was a species of *Tetraselmis* and seven were endemic isolates, including six species of Bacillariophyceae and one of Cyanophyceae. Three of the bacillariophyceaen isolates have been identified as strains of *Phaeodactylum tricornutum*. Both this species and a species of *Tetraselmis* were found to be among the best-growing species in a previous study using a similar apparatus to that described here (Goldman & Stanley, 1974). Three other species, *Nitzschia* sp., *Skeletonema costatum* and *Pavlova lutheri*, were also found to grow well on 1:1 diluted secondary sewage effluent by these authors. In Chapter 3 of the present study, batch cultures of these species became contaminated by wastewater species. Goldman and Stanley (1974) used filtered wastewater which probably excluded any wastewater species from their experiments, and enabled the three species to grow in unialgal culture. Therefore species which grow well on 1:1 diluted wastewater are not necessarily dominant over other species.

P. tricornutum has been found to be dominant to many other species when grown on secondary sewage effluent (diluted 1:1 with filtered seawater) (Ryther *et al.*, 1972; Goldman & Stanley, 1974a & b; Goldman & Ryther, 1976). In this study, four other species of Bacillariophyceae, a Cyanophyceaen and a species of *Tetraselmis* with nutrient removal and dominance in culture comparable to that of *P. tricornutum* have been selected.

Chapter Seven

Wastewater Nutrient Removal By Marine Microalgae In Mini-Ponds Cultured Under Ambient Conditions

7.1 Introduction

The culture of microalgae under controlled environmental conditions in the laboratory is very different to that in large-scale outdoor systems which are subject to fluctuating natural conditions (de la Noüe *et al.*, 1992). The results from the experiments in the previous chapters showed that some marine microalgal species may have potential for use in a wastewater treatment process. However, to fully determine this potential, the ability of these species to treat wastewaters had to be tested in outside large-scale continuous cultures.

This chapter describes experiments run in ambient conditions during the summer, using mini-ponds which were modelled on high-rate microalgal treatment ponds. High-rate ponds are very large continuous cultures which are illuminated from their surface by sunlight and open to the atmosphere. The photosynthetic efficiency of the microalgal culture is increased by gentle mixing of the culture and maintaining the optimum depth for light penetration (0.3-0.5 m) (Terry & Raymond, 1985; Dodd, 1986; Richmond, 1986b; Oswald, 1988b).

The aims of these experiments were to determine: 1) whether a high degree of treatment was maintained under large-scale continuous culture and fluctuating ambient conditions; 2) if treatment was maintained during the dark period of the 24 h light:dark cycle; and 3) the relationship of dissolved oxygen (DO) and pH of the algal cultures to the efficiency of treatment.

7.2 *Materials and Methods*

7.2.1 *Algae*

The eight best-treating cultured microalgae selected in the continuous culture experiments described in Chapter 6 were used in this study. Of these seven were isolates from the waters surrounding the wastewater outfall in St Andrews Bay including SA90B2, SA90B4, SA91B33, SA91B39, SA91B43, and SA92B48 from the division Bacillariophyceae, and SA91CY1 from the division Cyanophyceae. The eighth species was *Tetraselmis* sp. (Prasinophyceae). Algal inocula were taken from unialgal stock cultures in exponential growth phase. (Section 2.3.3).

7.2.2 *Apparatus*

Continuous culture mini-ponds were constructed from 20 l white polypropylene vats covered with a black plastic sleeve up to the 20 l mark and placed in a waterbath. An outflow tube was added to each mini-pond by cutting a hole in the side of the vat and fitting a small diameter glass tube through the hole. The connection was sealed with a gasket made of rubber tubing, and the desired depth of culture (30 cm) was maintained by securing the external end of the outflow at an angle using a retort stand and clamp. Diluted, unfiltered primary sewage effluent was pumped peristaltically (Watson-Marlow Ltd, Falmouth, Cornwall, UK; model 502S) to each mini-pond from a 1000 l fibre-glass storage tank and flowed into each culture through a glass tube opening just above the bottom of the mini-pond. Mixing was accomplished with a plastic-coated stirring bar. Medium flowed out of the culture through the outflow tube, from which 20 ml samples were collected for analysis. When sampling was not in progress, medium dripped into a collection pipe. A schematic diagram of a complete mini-pond

continuous culture unit is shown in Figure 7.1a and two operating mini-ponds are shown in Figure 7.1b.

7.2.3 Operation

The continuous culture unit was set up using sterile equipment. Algae were preadapted to 1:1 diluted wastewater and ambient conditions by culturing 5 l of exponential phase algal culture (grown on E-S media; Section 2.3.3) and 5 l of wastewater in a mini-pond for one week. A control culture to measure nutrient removal by wastewater species was also set up by adding 5 l of sterile seawater to 5 l of wastewater.

The dilution rate for the culture conditions of these experiments was determined by a preliminary batch culture experiment. A further 10 l of 1:1 diluted wastewater was added to the mini-pond culture of the first species tested (SA90B4) and daily measurements of algal biomass (OD_{570}; Section 2.3.2) were made over ten days. The continuous culture dilution rate (equal to the exponential growth rate), was found to be 0.2 doublings day^{-1} (Appendix 7; Fig. A 7.1). A continuous mini-pond culture of each algal species was run for at least 14 days at this dilution rate, so that their treatment could be compared. At daily intervals, 20 ml samples of the inflow and outflow were taken for determination of nutrient concentrations (ammonium, nitrite, nitrate and ortho-phosphate; Sections 2.2.7), algal biomass (Section 2.3.2), and microscopic examination (Section 2.2.2). The percentage removal was calculated as described in Section 6.2.3.

Continuous measurements of DO and temperature were made from cultures of isolates SA90B2, SA90B4, SA91B39 and SA91CY1, while continuous measurements of pH (Section 2.2.7) were taken from all cultures. Dissolved oxygen and temperature were measured using an

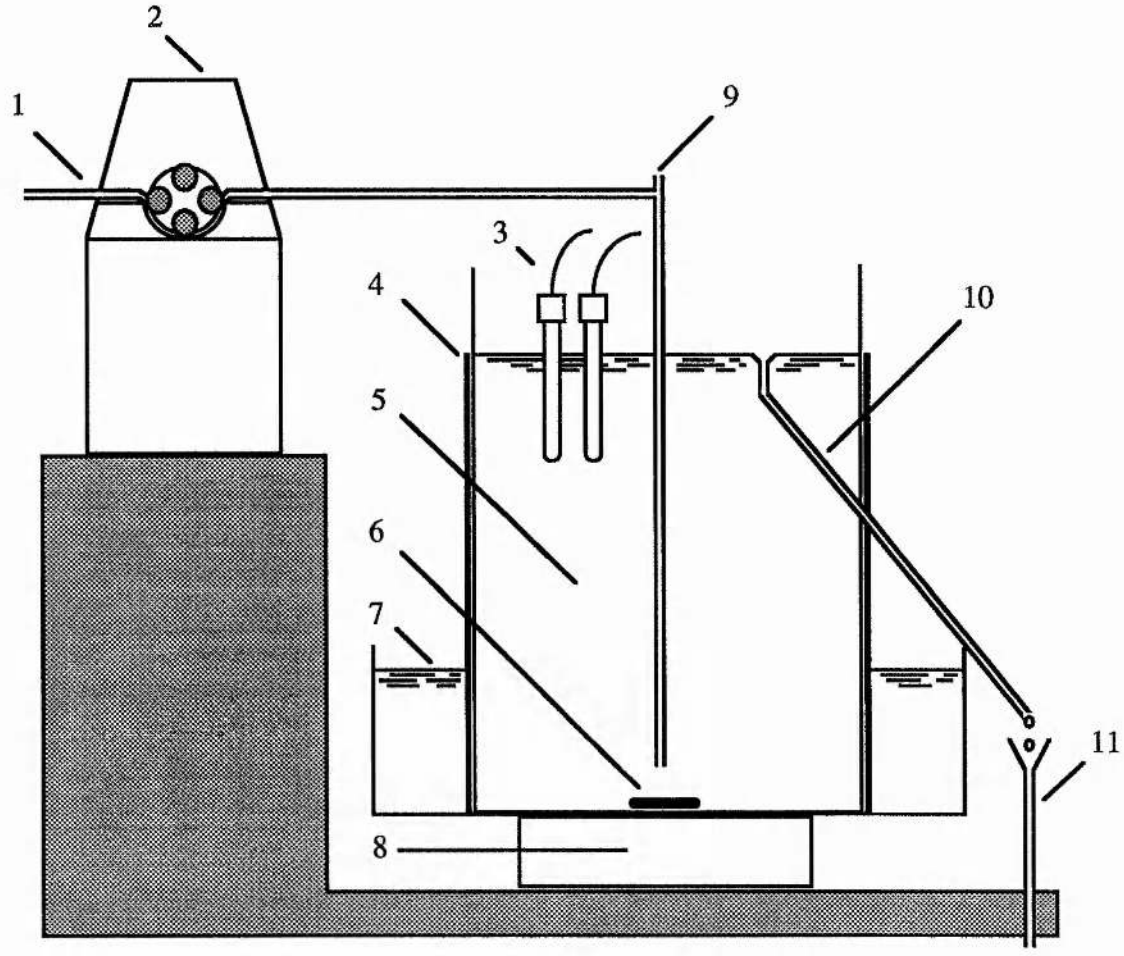

Figure 7.1a Schematic diagram of a complete continuous culture unit. 1: nutrient feed line from supply tank; 2: peristaltic pump; 3: pH/DO electrodes; 4: black plastic sleeve; 5: continuous culture vessel (20 l polypropylene mini-pond); 6: plastic coated stirring bar; 7: water bath; 8: magnetic stirrer; 9: inflow tube with air vent; 10: outflow tube; 11: waste pipe.

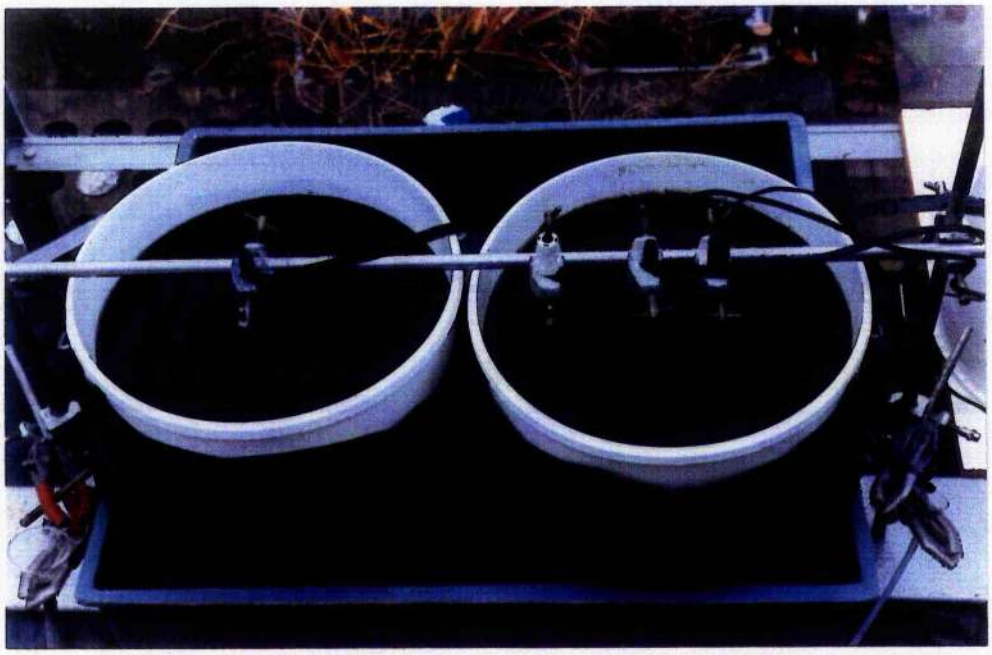

Figure 7.1b Two mini-pond continuous culture units in operation.

oxygen/temperature sensor (Kent Industrial Measurements Ltd, Chertsey, England; model 7131) and read by an oxygen meter (Kent, model 7130). Ambient light intensity was measured continuously over the period of investigation using quantum photometer (Section 3.2.2) with the PAR sensor placed just below the water surface within a mini-pond with no algal inocula.

The effect of the light:dark cycle on nutrient removal was also investigated for seven of the eight isolates (SA90B2, SA90B4, SA91B33, SA91B39, SA91B43, SA92B48, SA91CY1). Concentrations of ammonium, nitrite, nitrate and ortho-phosphate, algal biomass (OD_{570}) and physical parameters (temperature, DO and pH) of a mini-pond culture of each isolate were measured at 2 h intervals over a 24 h period for analysis against light intensity.

7.3 Results

The nutrient concentrations and algal biomass in the inflow and outflow of a 20 l mini-pond culture of isolate SA91B33, grown in ambient conditions for seven days preliminary batch culture and 31 days continuous culture, are shown in Fig. 7.2. During the batch culture, decrease in ammonium and ortho-phosphate concentrations in the outflow corresponded to the increase in algal biomass (Fig. 7.2). Algal biomass (OD_{570}) and nutrient concentrations of the outflow changed little over the following 31 days continuous culture (Fig. 7.2), indicating that the culture was in steady state. Mean nutrient removal was 89.4 % $N\text{-}NH_4^+$ and 85.4 % $P\text{-}PO_4^{3-}$ of the nutrient concentrations of the inflow (468.8 ± 54.6 mmol N m^{-3} and 75.6 ± 8.6 mmol P m^{-3}; Table 7.1).

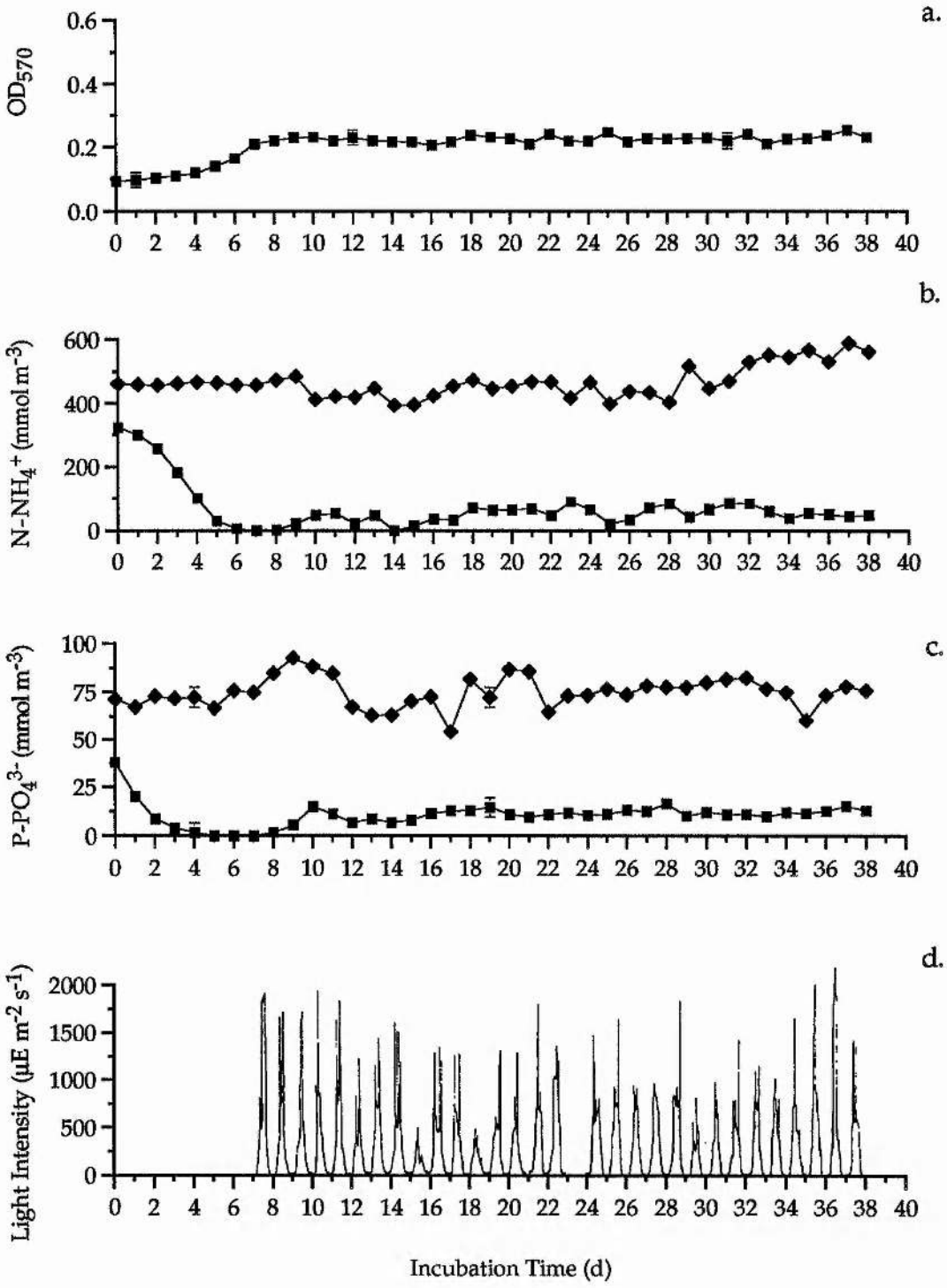

Figure 7.2 Culture biomass (a.), ammonium concentration (b.) and ortho-phosphate concentration (c.) in the 1:1 diluted wastewater inflow(◆-◆) and outflow (■-■) of a 20 litre mini-pond of isolate SA91B33 in relation to light intensity (d.) over seven days initial batch culture and 31 days continuous culture. Biomass and nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

Table 7.1 Percentage removal of ammonium and ortho-phosphate from 20 litre continuous mini-ponds of eight marine microalgal species and isolates on 1:1 diluted wastewater over 14 days. The purity of the cultures on the final day of the experiment is also shown. Values are means of means ± s.d. of daily measurements.

Algal species	% $N\text{-}NH_4^+$ Removed	% $P\text{-}PO_4^{3-}$ Removed	Culture State
SA90B2[1]	91.7 ± 4.3	97.8 ± 2.0	Unialgal
SA90B4[2]	88.7 ± 7.7	68.6 ± 19.4	Unialgal
SA91B33[3]	89.4 ± 4.9	85.4 ± 4.0	Unialgal
SA91B39[4]	86.5 ± 4.3	68.9 ± 7.6	Unialgal
SA91B43[4]	90.2 ± 3.8	72.4 ± 12.1	Unialgal
SA92B48[4]	91.1 ± 3.4	86.8 ± 4.3	Unialgal
Tetraselmis sp.[4]	77.4 ± 3.4	28.7 ± 2.6	Contaminated
SA91CY1[5]	95.9 ± 2.1	82.6 ± 1.7	Unialgal

[1]Inflow 400.4 ± 15.4 mmol N m^{-3}, 66.2 ± 2.9 mmol P m^{-3}

[2]Inflow 562.6 ± 18.0 mmol N m^{-3}, 60.2 ± 1.7 mmol P m^{-3}

[3]Inflow 468.8 ± 54.6 mmol N m^{-3}, 75.6 ± 8.6 mmol P m^{-3}

[4]Inflow 497.7 ± 60.7 mmol N m^{-3}, 76.2 ± 4.9 mmol P m^{-3}

[3]Inflow 443.4 ± 28.5 mmol N m^{-3}, 75.5 ± 11.2 mmol P m^{-3}

The inflow and outflow nutrient concentrations and algal biomass of mini-pond cultures of seven more microalgae, including six endemic isolates (SA90B2, SA90B4, SA91B39, SA91B43, SA92B48 and SA91CY1) and *Tetraselmis* sp. measured over 14 days continuous culture are shown in Figs. 7.3-7.9. All microalgae, except *Tetraselmis* sp. (which became contaminated by wastewater species during preadaptation), remained unialgal with little change in biomass (OD_{570}) during the culture period (Figs. 7.2a-7.9a), indicating that these cultures were also in steady state.

The large standard deviations for the biomass measurements of SA91CY1 (Fig. 7.8) were a result of cell aggregation, which affected the reproducibility of readings between replicates. Low values of algal biomass were obtained for some isolates (SA91B33, SA91B39, SA91B43, SA9248 and SA91CY1) compared to that of the two planktonic isolates, SA90B2 and SA90B4. This was due to the adherence of the non-planktonic isolates to the sides of the mini-pond. The control culture, which had no algal inoculum, became contaminated by the two planktonic isolates, SA90B2 and SA90B4, during the first week of continuous culture, a further indication of the dominance of these two isolates.

The nutrient concentrations of the diluted wastewater inflow remained uniform during all experiments (Table 7.1). All microalgae except *Tetraselmis* sp. which was contaminated continuously, removed >80 % of ammonium from the wastewater over the 14 day period (Table 7.1; Figs. 7.2b & c-7.9b & c). Four isolates (SA90B2, SA91B33, SA92B48 and SA91CY1) continuously removed >80 % of ortho-phosphate. Ortho-phosphate removal by two isolates (SA90B4 and SA91B43) also began at >80 %, but this declined over time, and by day 14, more than 50 % of the inflow concentration remained in the outflow (Table 7.1; Figs. 7.4c, 7.7c). Ortho-phosphate removal by isolate SA91B39 was constant at a lower level (68.9 %).

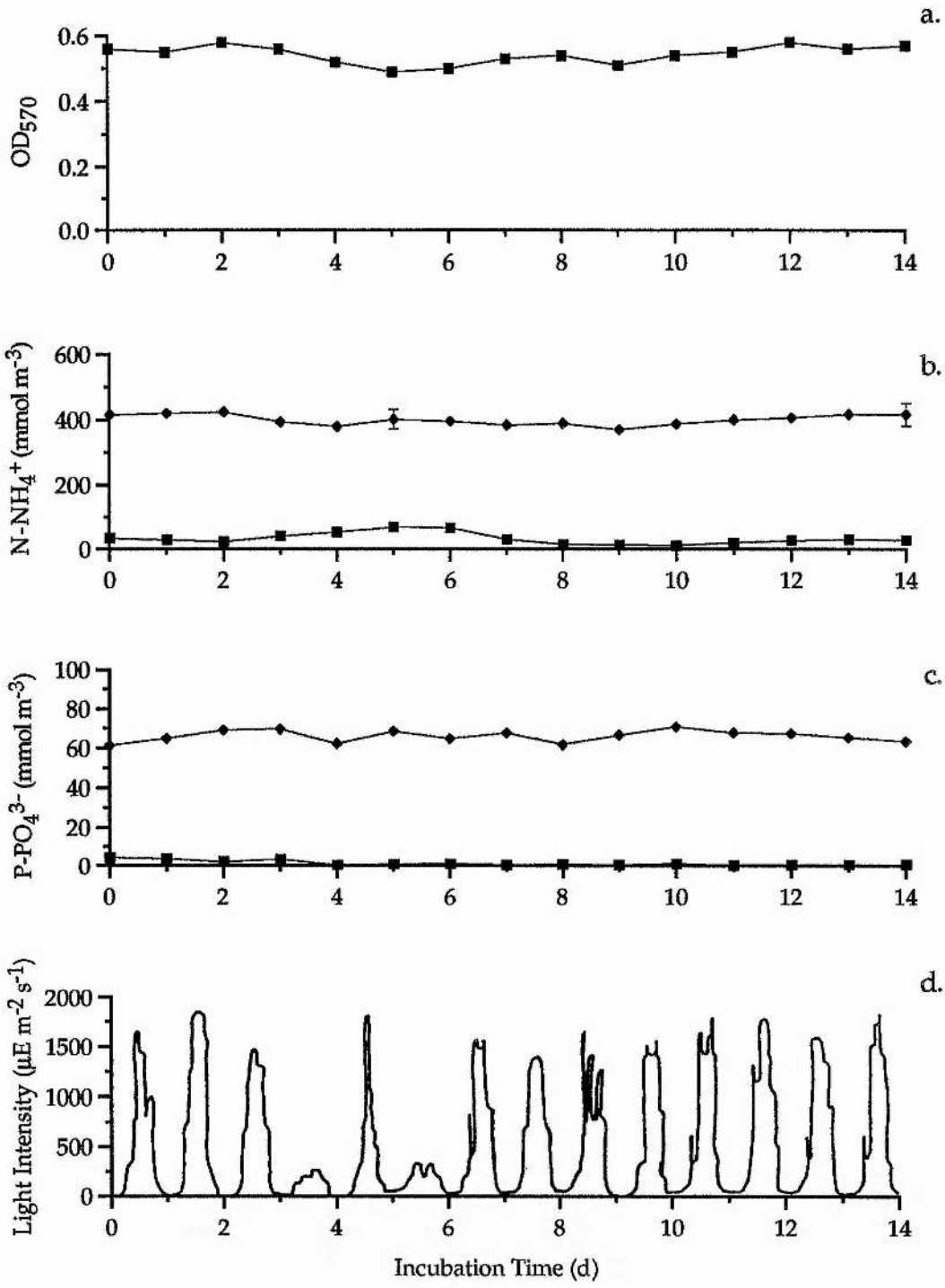

Figure 7.3 Culture biomass (a.), ammonium concentration (b.) and ortho-phosphate concentration (c.) in the 1:1 diluted wastewater inflow(◆-◆) and outflow (■-■) of a 20 litre mini-pond of isolate SA90B2 in relation to light intensity (d.) over 14 days culture. Biomass and nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

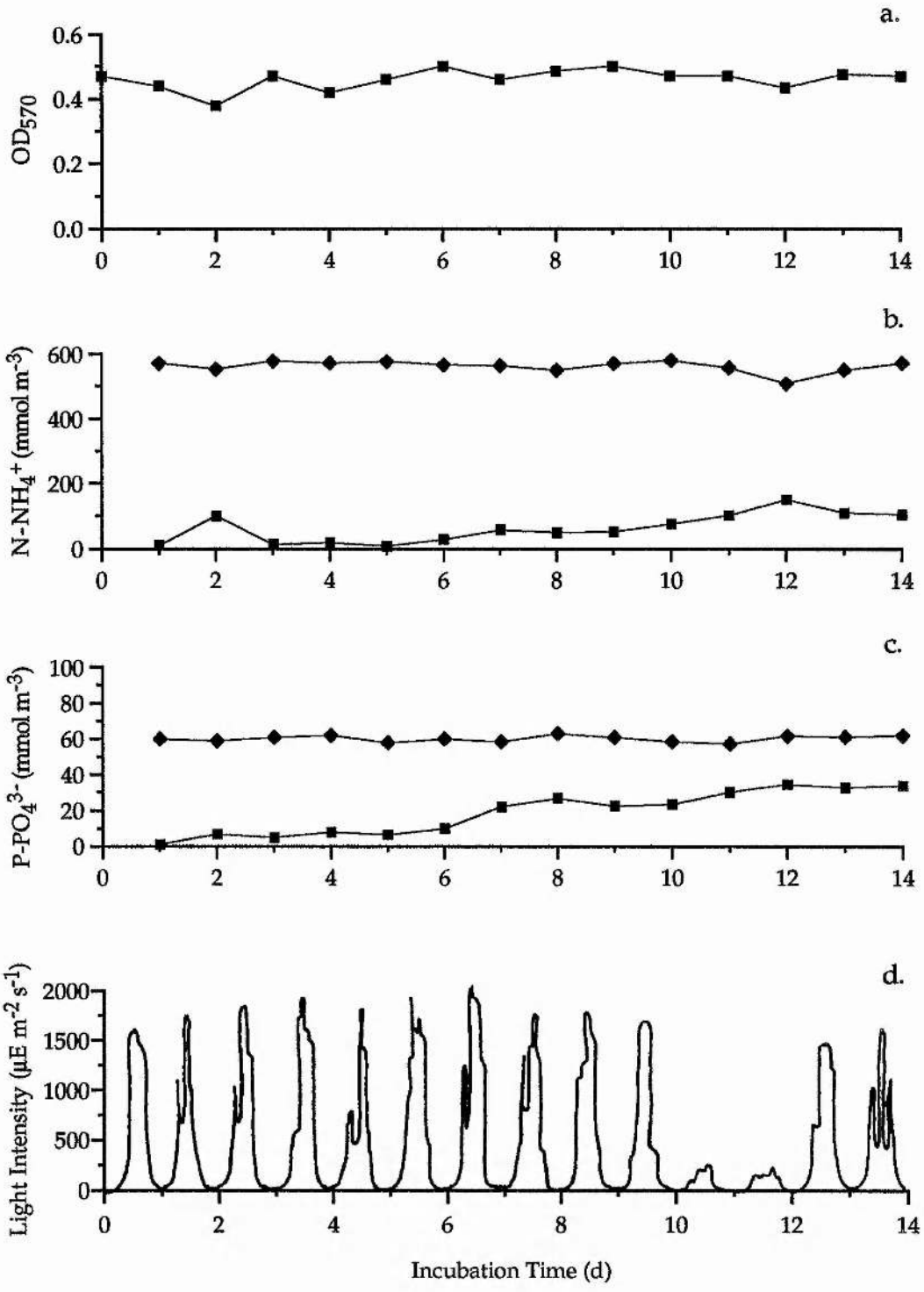

Figure 7.4 Culture biomass (a.), ammonium concentration (b.) and ortho-phosphate concentration (c.) in the 1:1 diluted wastewater inflow(◆-◆) and outflow (■-■) of a 20 litre mini-pond of isolate SA90B4 in relation to light intensity (d.) over 14 days culture. Biomass and nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

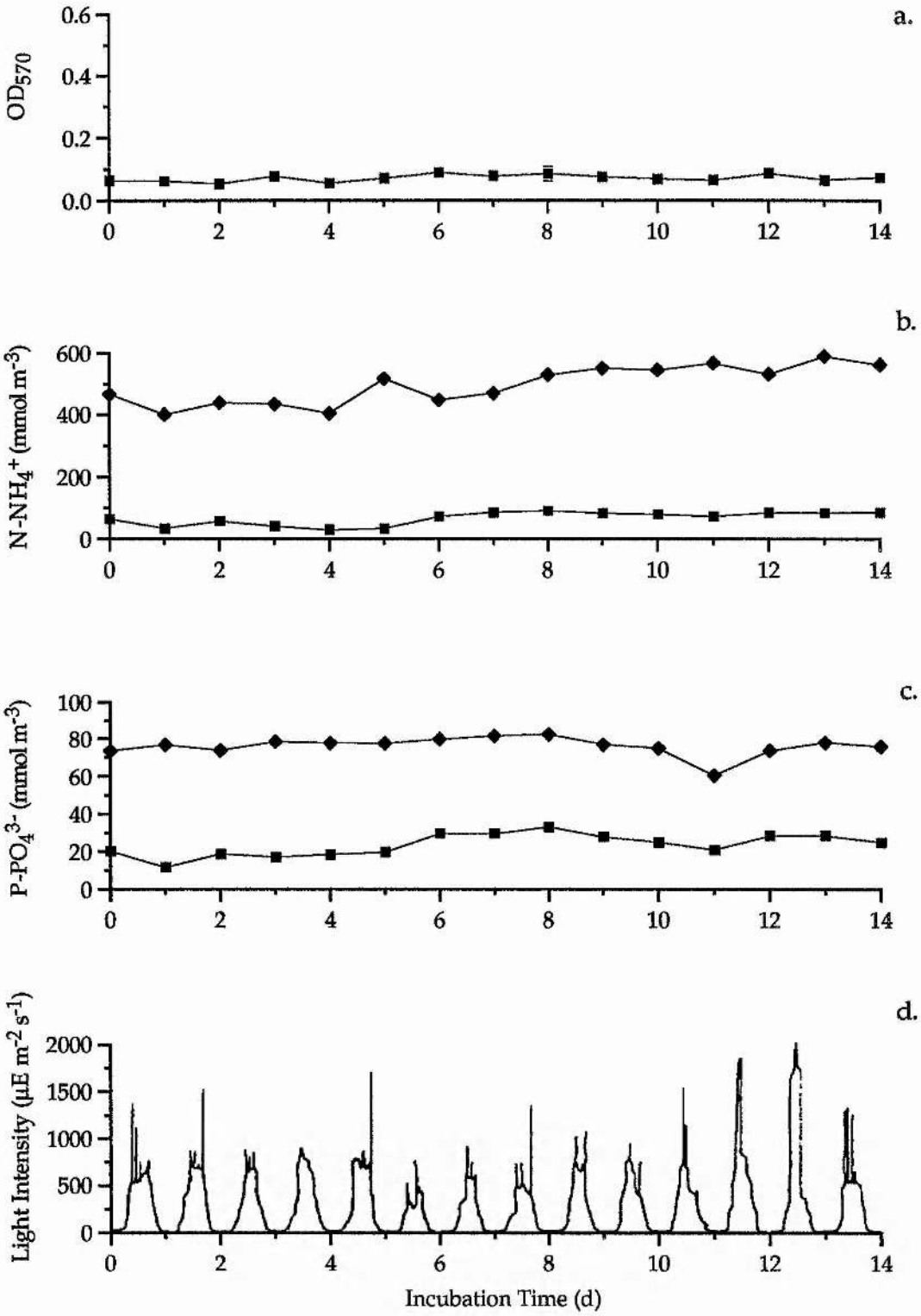

Figure 7.5 Culture biomass (a.), ammonium concentration (b.) and ortho-phosphate concentration (c.) in the 1:1 diluted wastewater inflow (♦-♦) and outflow (■-■) of a 20 litre mini-pond of isolate SA91B39 in relation to light intensity (d.) over 14 days culture. Biomass and nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

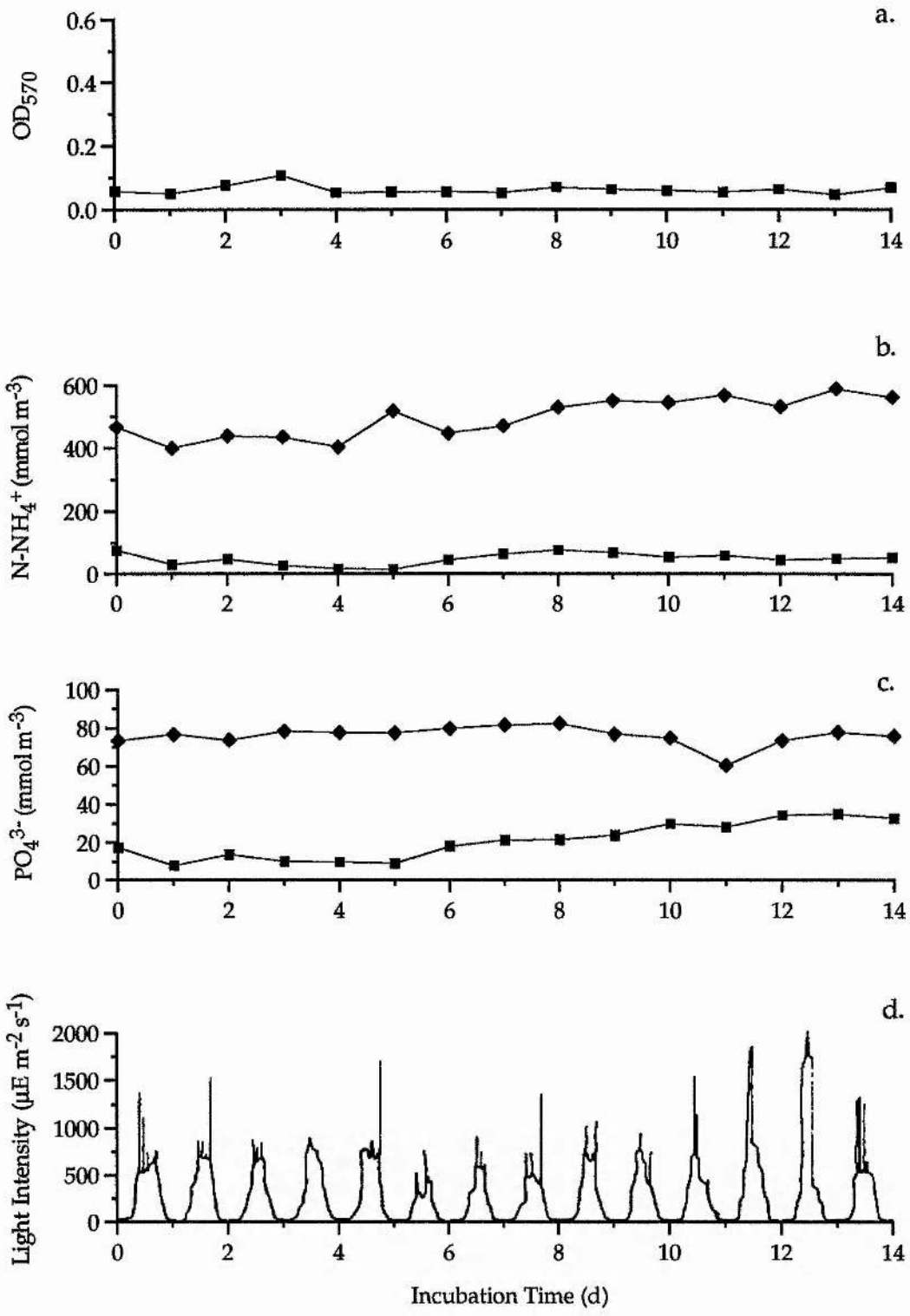

Figure 7.6 Culture biomass (a.), ammonium concentration (b.) and ortho-phosphate concentration (c.) in the 1:1 diluted wastewater inflow (◆-◆) and outflow (■-■) of a 20 litre mini-pond of isolate SA91B43 in relation to light intensity (d.) over 14 days culture. Biomass and nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

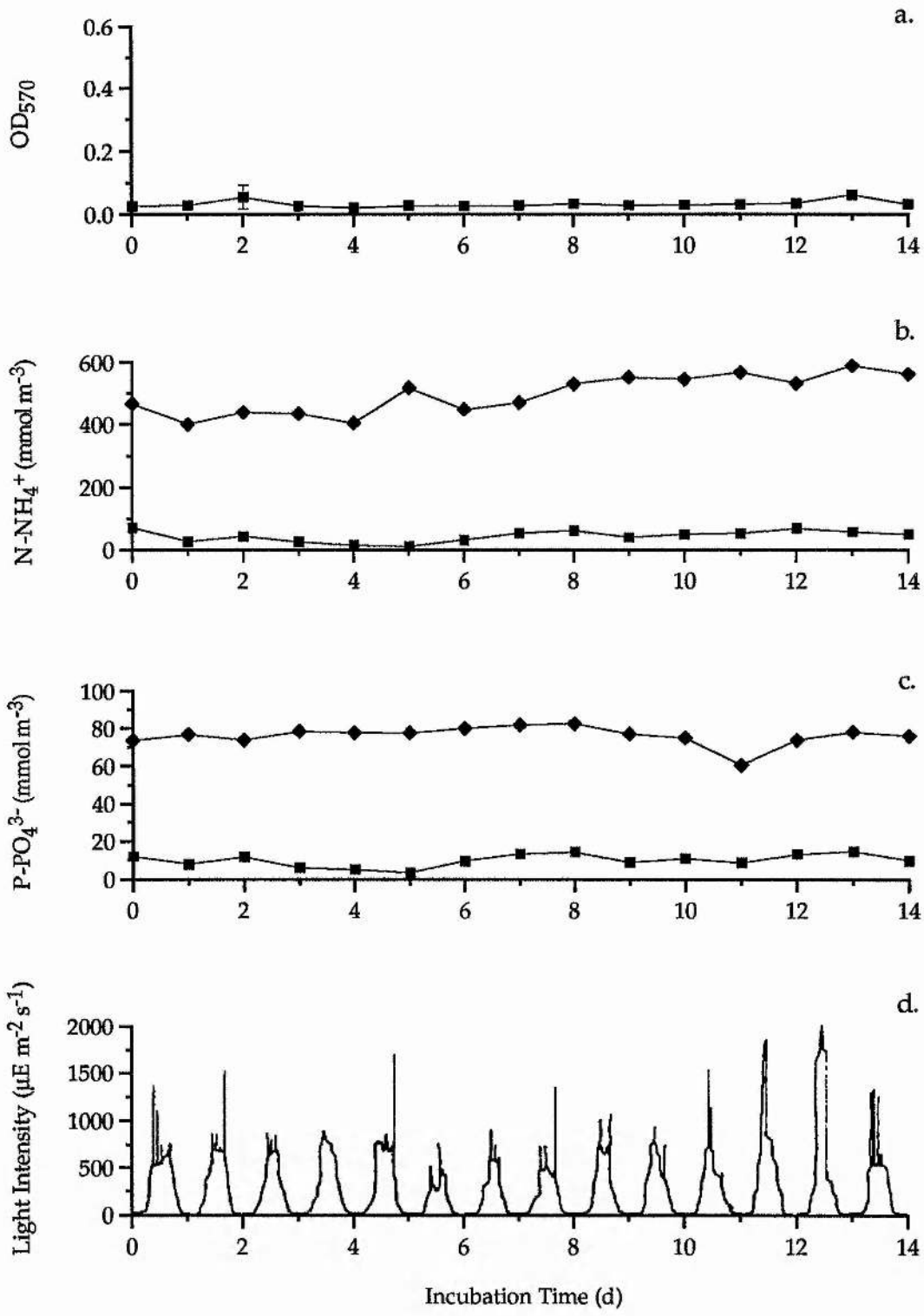

Figure 7.7 Culture biomass (a.), ammonium concentration (b.) and ortho-phosphate concentration (c.) in the 1:1 diluted wastewater inflow (◆-◆) and outflow (■-■) of a 20 litre mini-pond of isolate SA92B48 in relation to light intensity (d.) over 14 days culture. Biomass and nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

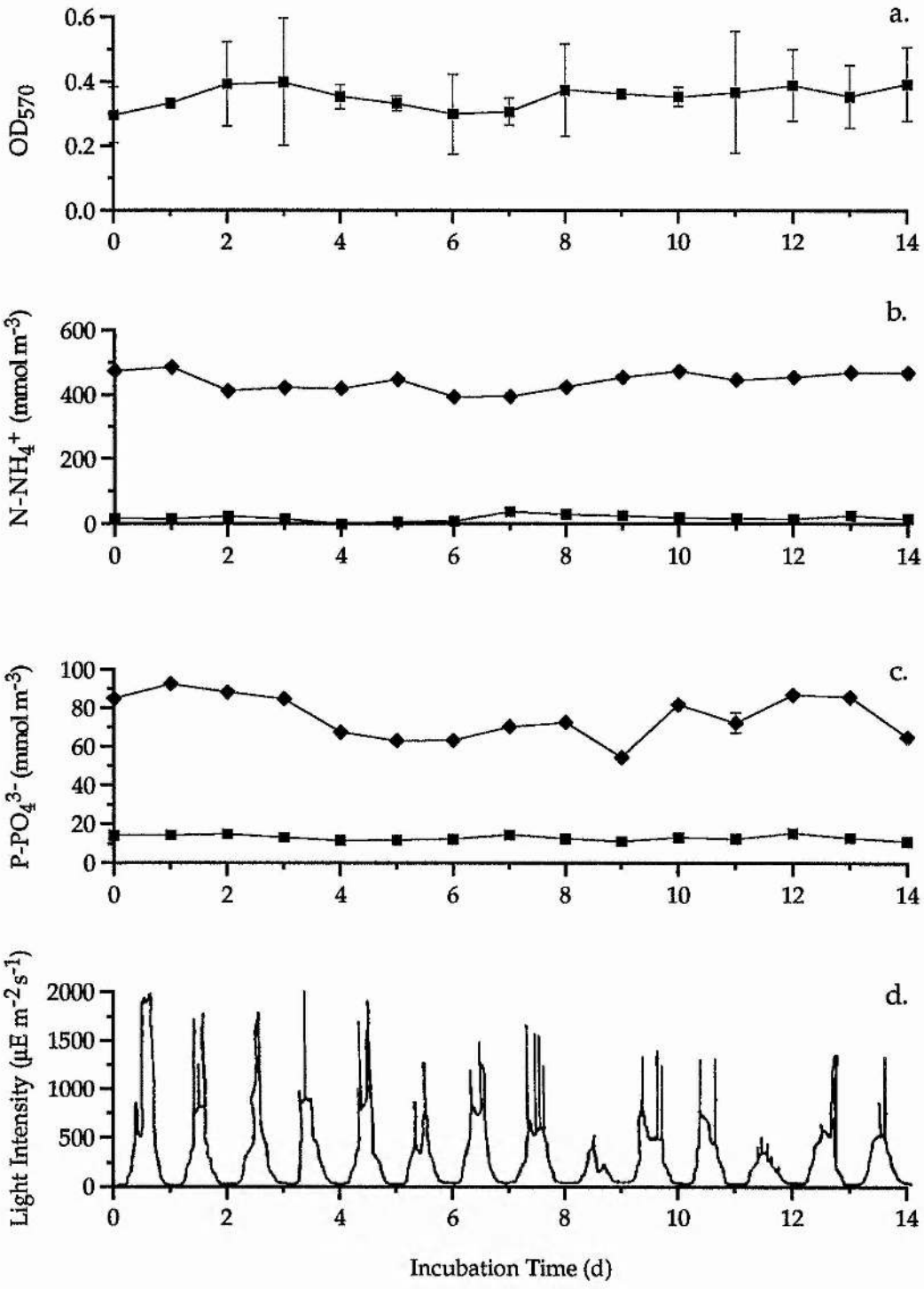

Figure 7.8 Culture biomass (a.), ammonium concentration (b.) and ortho-phosphate concentration (c.) in the 1:1 diluted wastewater inflow(◆-◆) and outflow (■-■) of a 20 litre mini-pond of isolate SA91CY1 in relation to light intensity (d.) over 14 days culture. Biomass and nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

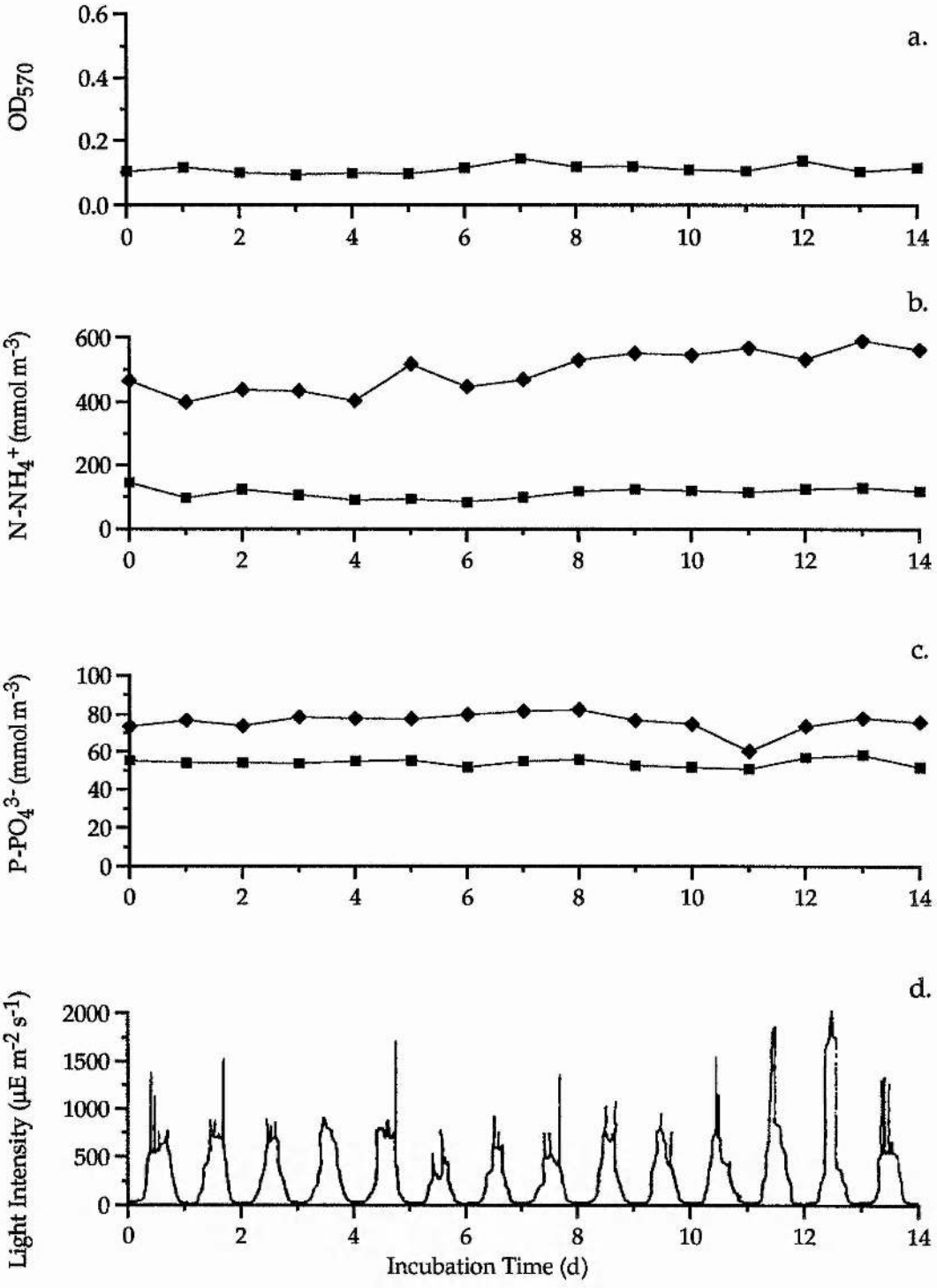

Figure 7.9 Culture biomass (a.), ammonium concentration (b.) and ortho-phosphate concentration (c.) in the 1:1 diluted wastewater inflow (◆-◆) and outflow (■-■) of a 20 litre mini-pond of *Tetraselmis* sp. in relation to light intensity (d.) over 14 days culture. Biomass and nutrient values are means ± s.d. of triplicate samples. The s.d. may be to small to be seen.

Contamination of the culture of *Tetraselmis* sp. resulted in much lower nutrient removal (77.4 % for $N\text{-}NH_4^+$ and 28.7 % for $P\text{-}PO_4^{3-}$) compared to the unialgal cultures.

Ambient light intensities were similar during all experiments, with a maximum incident light intensity of 2000 $\mu E\ m^{-2}\ s^{-1}$ (Figs. 7.2d-7.9d). Ammonium removal by all the bacillariophyceaen isolates decreased on days of low light intensity (days 3-5 for isolate SA90B2 (Fig. 7.3d), days 10-11 for isolate SA90B4 (Fig. 7.4d), and from day 5 for isolates SA91B39 (Fig. 7.5d), SA91B43 (Fig. 7.6d) and SA92B48 (Fig. 7.7d)), but remained >70 % for all isolates. When higher light intensities resumed, ammonium removal returned to >80 %. Ortho-phosphate removal by the three isolates SA91B39, SA91B43 and SA92B48 also decreased on days of low light intensity, whereas no apparent change was noticed in the culture of isolate SA90B2. A decrease in removal may have been offset by the decline in ortho-phosphate concentration of the diluted wastewater inflow at this time (Fig. 7.3c.). The decline in removal of ortho-phosphate by isolate SA90B4 (Fig. 7.4c) was unaffected by the low light intensities. Periods of low light intensity appeared to have little effect on nutrient removal by the isolate SA91CY1 and *Tetraselmis* sp. (Figs. 7.8 & 7.9).

Physical parameters of the mini-pond cultures of all species showed close relationships with changes in light intensity and hence with algal photosynthesis. Figure 7.10 shows the values for temperature, pH and dissolved oxygen in the culture of isolate SA90B2. Over the 14 day experimental period, the mean culture temperature was $20.5 \pm 3.3\ ^oC$ and ranged from 15.0 to 29.9 ^oC (Fig. 7.10a.). Generally, the pH was constant at around pH 9.1 ± 1.8; a maximum of pH 10.5 was reached during the day, although on days 3 and 5 when light intensities were low, pH decreased to 4.0 (Fig. 7.10b.). The DO concentration increased during daylight hours,

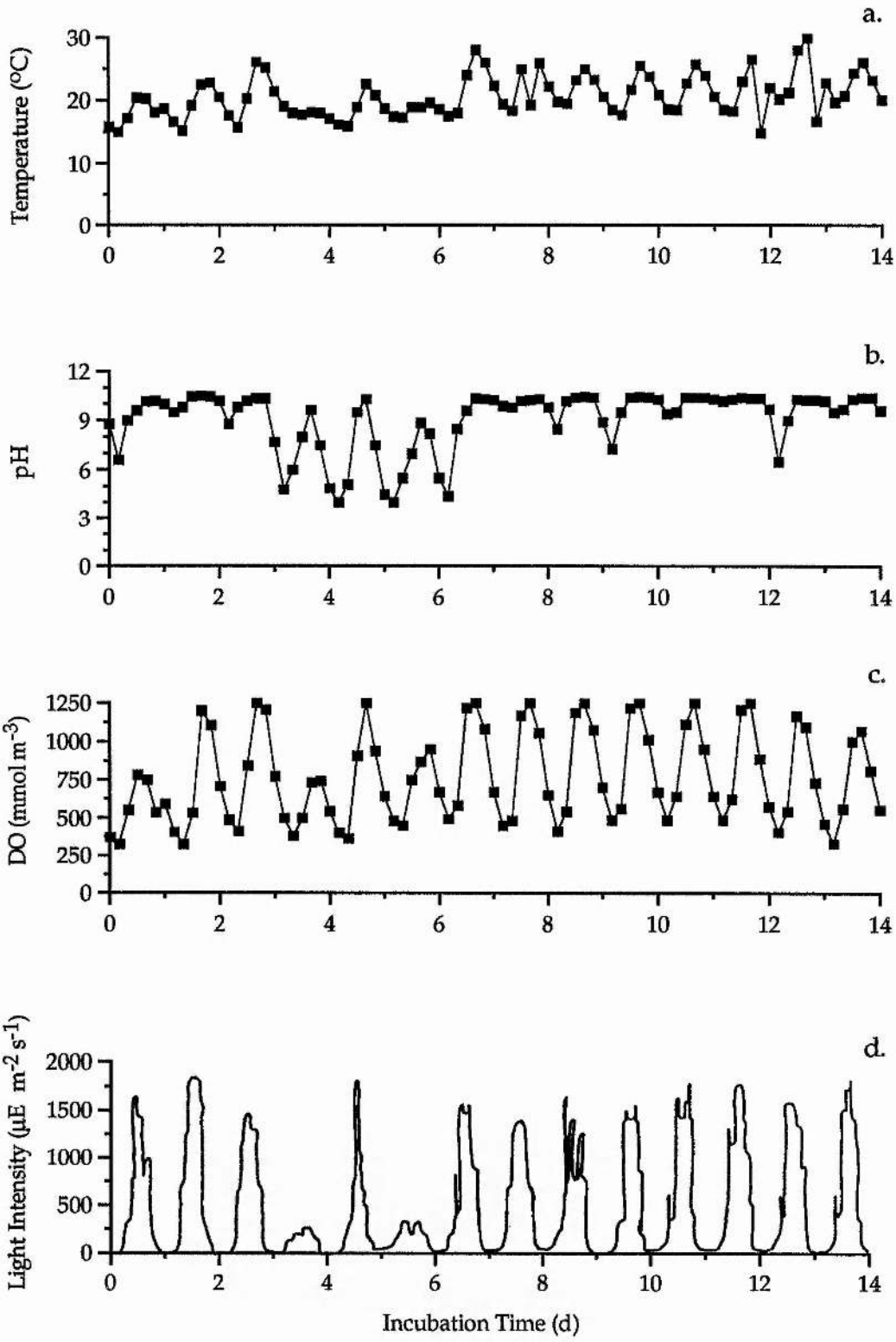

Figure 7.10 Temperature (°C) (a.), pH (b.), DO (mmolm^{-3}) (c.) of a 20 litre mini-pond of isolate SA90B2 in relation to light intensity (d.) over 14 days continuous culture.

Table 7.2 Mean ± s.d. and range values of physical characteristics (temperature, DO and pH) of the culture medium in 20 litre continuous mini-ponds of seven marine microalgal species grown on 1:1 diluted wastewater over 14 days.

Algal species	Temperature (°C)		DO (mmol m^{-3})		pH	
	Mean ± s.d.	Range	Mean ± s.d.	Range	Mean ± s.d.	Range
SA90B2	20.6 ± 3.3	28.0 - 14.9	762.4 ± 300.1	1250.0 - 325.0	9.1 ± 1.8	10.5 - 4.0
SA90B4	19.8 ± 2.8	27.3 - 15.3	633.8 ± 158.1	1250.0 - 325.0	9.9 ± 0.6	10.8 - 7.9
SA91B33	n.d.	n.d.	n.d.	n.d.	9.7 ± 0.3	10.4 - 9.3
SA91B39	19.7 ± 2.9	29.4 - 14.2	751.6 ± 316.3	1250.0 - 250.0	9.8 ± 0.3	10.6 - 9.3
SA91B43	n.d.	n.d.	n.d.	n.d.	9.6 ± 0.2	10.1 - 9.2
SA92B48	n.d.	n.d.	n.d.	n.d.	10.3 ± 0.3	10.9 - 9.8
SA91CY1	19.4 ± 2.5	25.7 - 14.0	554.9 ± 256.1	1250.0 - 112.5	9.2 ± 0.4	10.0 - 8.3

n.d. not done

presumably because of algal photosynthesis, but declined during the night because of both algal and bacterial respiration (Shelef *et al.*, 1980; Grobbelaar *et al.*, 1988) (Fig. 7.10c). DO exceeded 1250 mmol m^{-3} O_2 (200 % saturation, the limit of sensitivity of the electrode) in the late afternoon on most days. The values of all three parameters declined on days 3 and 5 when light intensity was low, but recovered to their original levels on day 6 when higher light intensities resumed. The values of temperature, DO and pH are summarised in Table 7.2.

To determine whether the nitrogen present as ammonium was actually removed from the culture and not converted to nitrite and nitrate by nitrifying bacteria (Oswald, 1988a), the concentrations of these nutrients were also measured during the experiments with six species (SA91B33, SA91B39, SA91B43, and SA92B48, *Tetraselmis* sp. and SA91CY1). The main nitrogen source in the diluted wastewater inflow was ammonium and both nitrate and nitrite contributed less than 1 % of the total nitrogen concentration (Fig. 7.11). Nitrite and nitrate concentrations were slightly higher in the mini-pond outflow than in the inflow (Fig. 7.11) but these concentrations were still less than 1 % (<5 mmol m^{-3}) of the total nitrogen and remained constant throughout the experiment.

Nutrient concentrations in both the diluted wastewater inflow and outflow from the mini-ponds of the seven endemic isolates were made at 2 h intervals over 24 h. The results for species SA90B2, which are representative of those for all species, are shown in Fig. 7.12. Both ammonium and ortho-phosphate removal decreased slightly overnight, but never fell below 70 %, while nutrient concentrations in the diluted wastewater inflow generally remained constant (Figs. 7.12b, c). Nitrite and nitrate concentrations were low (<5 mmol m^{-3}), and remained unchanged over the period of measurement (Fig, 7.13).

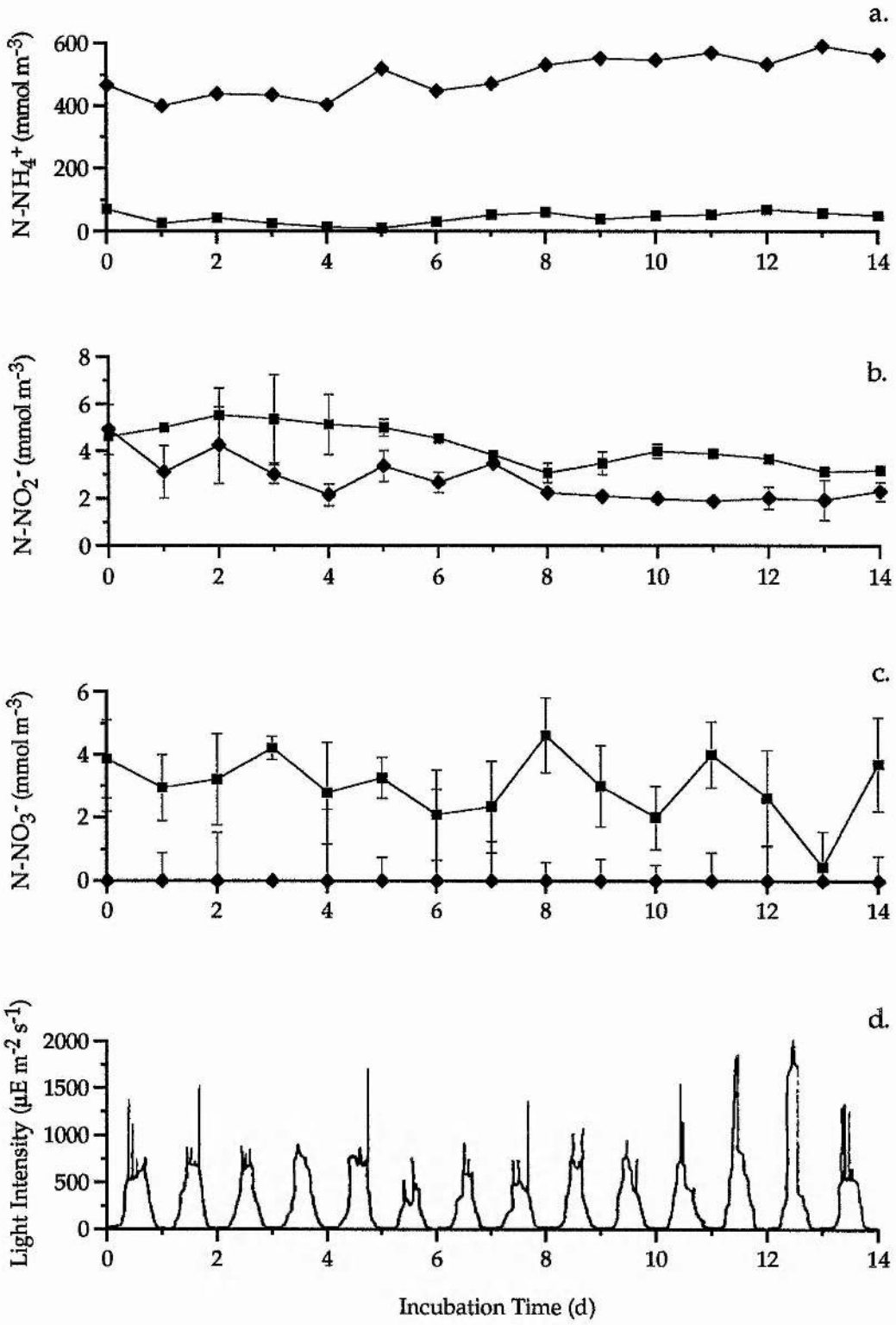

Figure 7.11 Ammonium (a.), nitrite (b.) and nitrate (c.) concentrations in the 1:1 diluted wastewater inflow (◆-◆) and outflow (■-■) of a 20 litre mini-pond of isolate SA92B48 in relation to light intensity over 14 days culture. Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

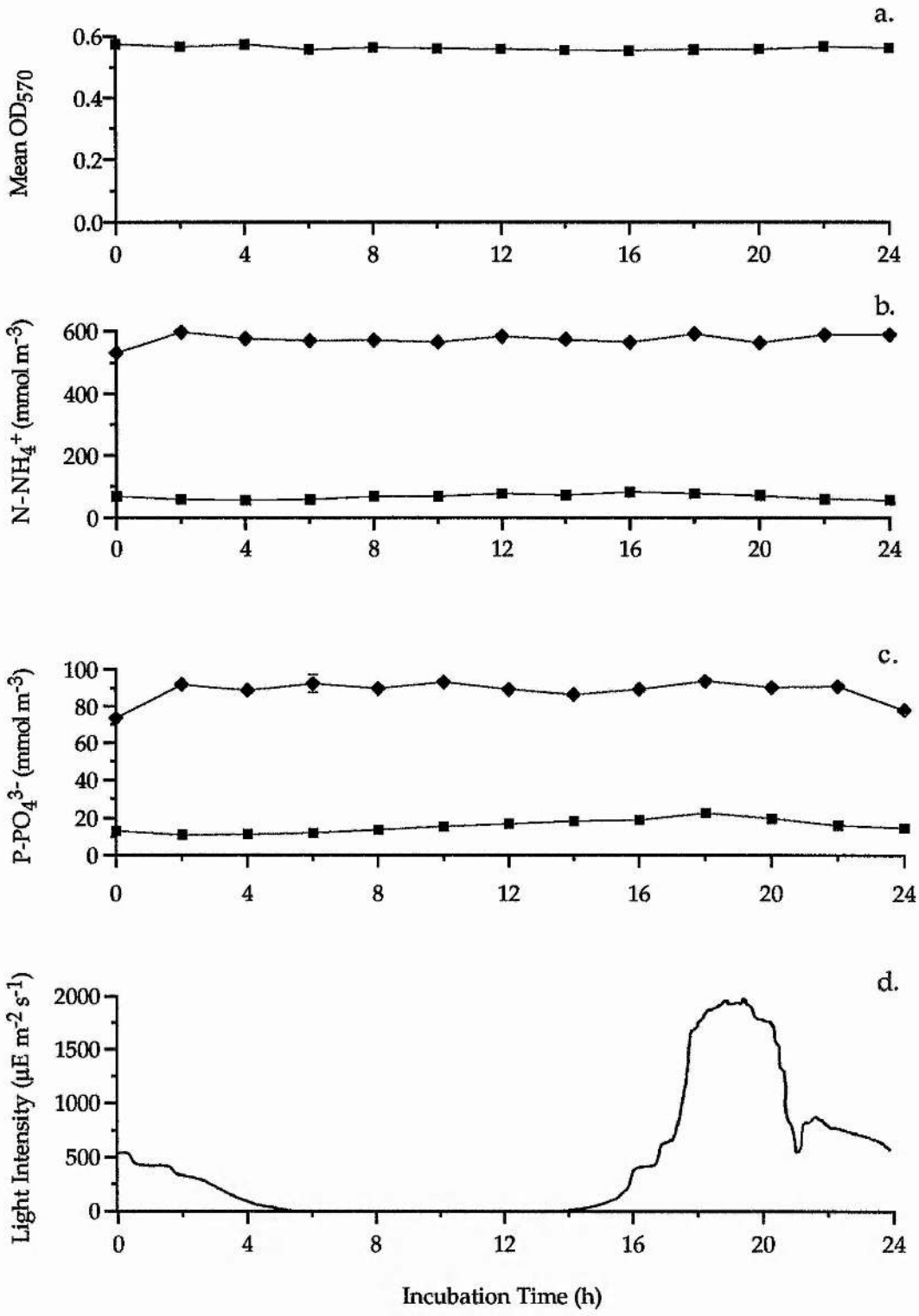

Figure 7.12 Culture biomass (a.), ammonium concentration (b.) and ortho-phosphate concentration (c.) in the 1:1 diluted wastewater inflow (♦-♦) and outflow (■-■) of a 20 litre mini-pond of isolate SA90B2 in relation to light intensity (d.) over 24 hours culture. Biomass and nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

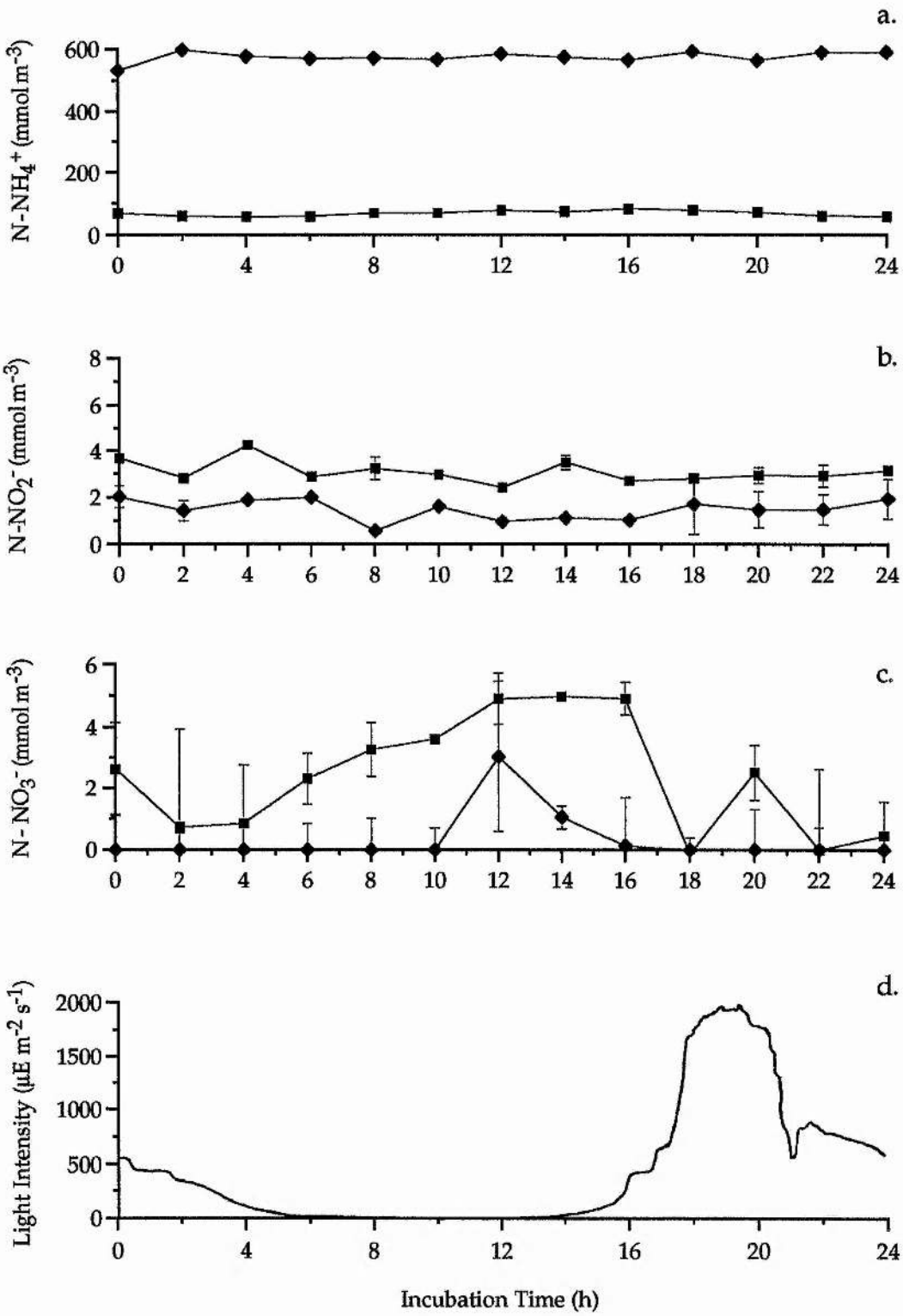

Figure 7.13 Ammonium (a.), nitrite (b.) and nitrate (c.) concentrations in the 1:1 diluted wastewater inflow (◆-◆) and outflow (■-■) of a 20 litre mini-pond of isolate SA90B2 in relation to light intensity (d.) over 24 hours culture. Values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

Continuous cultures of five of the isolates (SA91B33, SA91B39, SA91B43, SA92B48 and SA91CY1) were maintained for a further six weeks to determine whether the nutrient removal by these algae could be sustained over prolonged periods of culture. Ammonium removal by all isolates remained at >80 % (Table 7.3). Two isolates SA91B33 and SA91CY1 continued to remove >80 % ortho-phosphate, while removal by isolate SA92B48 declined to 63.4 %, and that of both SA91B39 and SA91B43 remained at approximately 50 % of the concentration of the inflow. All cultures remained unialgal.

7.4 Discussion

This study describes the abilities of eight marine microalgae, including seven endemic isolates and *Tetraselmis* sp., to remove nutrients from diluted wastewater in 20-l mini-pond cultures under ambient summer conditions. All microalgae were found to remain in steady state and to remove high levels of nutrients from wastewater, although there were slight differences in the abilities of species to treat the wastewater. The efficiency of nutrient removal (>80 %) shown by these algae is comparable to the efficiencies recorded for algal cultures by other workers (Dunstan & Menzel, 1971; Dunstan & Tenore, 1972; Megharaj *et al.*, 1992) even though the nutrient concentrations in the present study were over twice those used by these authors.

The steady state cultures of all microalgae examined in the present study were similar with respect to algal biomass and physical parameters, but exhibited differences in uptake of the two principle nutrients, ammonium and ortho-phosphate. Four isolates (SA90B2, SA91B33, SA92B48 and SA91CY1) continually achieved >80 % removal of both nutrients, while

Table 7.3 Percentage removal of ammonium and ortho-phosphate from 20 litre continuous mini-ponds of five marine microalgal isolates on 1:1 diluted wastewater after two months (n = 3). The purity of the cultures on the final day of the experiment is also shown. (Inflow nutrient concentrations were 489.1 ± 13.3 mmol m^{-3} $N\text{-}NH_4^+$ and 79.8 ± 1.3 mmol m^{-3} $P\text{-}PO_4^{3-}$).

Algal species	% $N\text{-}NH_4^+$ Removal	% $P\text{-}PO_4^{3-}$ Removal	Culture State
SA91B33	98.7 ± 3.9	97.3 ± 2.3	Unialgal
SA91B39	98.3 ± 4.1	55.3 ± 25.8	Unialgal
SA91B43	89.7 ± 4.8	44.2 ± 29.1	Unialgal
SA92B48	97.4 ± 3.2	63.4 ± 17.3	Unialgal
SA91CY1	98.8 ± 4.3	87.9 ± 1.5	Unialgal

ortho-phosphate removal by two isolates (SA90B4, SA91B43), despite initially being >80 %, declined to 50 % over the experiment, and isolate SA91B39 only continually removed 70 %. A reduction in the removal of ortho-phosphate by algal cultures is often indicative of nitrogen limitation (de la Noüe & Bassères, 1989) and has been noted in earlier studies of continuous cultures of marine microalgae grown on seawater diluted wastewater (Dunstan & Menzel, 1971; Dunstan & Tenore, 1972) and continuous cultures of freshwater microalgae grown on artificial medium (Robinson *et al.*, 1988; Megharaj *et al.*, 1992). However, it is unlikely that nitrogen was limiting in the present study as the N:P atomic ratio of the diluted wastewater was considerably higher (21:1 for B4 and 14:1 for B43) than the average for assimilation by marine microalgae (10:1; Ryther & Dunstan, 1971). These two isolates possibly have a reduced ability to remove ortho-phosphate since another isolate, SA90B2, had good ortho-phosphate removal, even though all three are endemic strains of *Phaeodactylum tricornutum*.

From the point of view of wastewater treatment, the presence of ortho-phosphate in the outflow of treatment ponds would be of little significance to marine eutrophication, since nitrogen and not phosphorous is frequently the limiting nutrient for phytoplankton growth in tropical and temperate coastal marine waters (McCarthy, 1980; Glibert, 1988). Although the ability to assimilate $N\text{-}NH_4^+$ in the dark has been shown to vary from species to species (Eppley *et al.*, 1971; Paasche, 1971), the continued high rates of ammonium and ortho-phosphate removal by all isolates during the dark period of the daily light:dark cycle is in agreement with more recent findings. At high cell densities, removal of NO_3 and NO_2 and PO_4^{3-} is unaffected by the light:dark cycle (Nalewajko & Lee, 1983; Marsot *et al.*, 1992).

The continuous mini-pond culture system is a very useful apparatus for the determination of the nutrient removal from diluted wastewater by microalgal species. Mini-ponds are inexpensive to set up, easy to operate and require little maintenance. Mini-pond systems were also used by Dunstan & Menzel, (1971) to treat wastewater although these had a volume of 15 l and were artificially illuminated and cooled. The wastewater used by these workers was chlorinated secondary effluent, stored frozen before use and diluted 1:4 with filtered seawater. The ponds described in the present study use untreated primary effluent, required no artificial illumination or temperature control and relied on a single microalgal species to reduce inorganic nutrient concentrations.

All seven endemic isolates were effective in removing ammonium, and therefore offer potential for the combined secondary and tertiary treatment of sewage before discharge into the sea. SA91B33 and SA91CY1 appear to be the most promising isolates, since they not only remove high concentrations of both ammonium and ortho-phosphate over long periods of culture, but have the added benefit of aggregating and adhering to the sides of the culture apparatus. One of the main limitations to the use of microalgae for the treatment of wastewaters is their subsequent removal from the treated effluent (Benemann *et al.*, 1980). Aggregation and adherence of the algal biomass to the wall of the culture vessel, providing a treated effluent which is virtually algal free, may provide one solution to this problem. The ability of some Cyanophyceaen species to aggregate when grown in suspension, allowing easy harvesting of biomass, has been studied previously (Talbot & de la Noüe, 1988). However, there have been no studies on aggregation of Bacillariophyceaen species.

Further attempts to identify these endemic isolates were only moderately successful. The cyanophyceaen was found to be a strain of

Oscillatoria sp. (pers. comm. G. Codd, University of Dundee). However, the four diatom species still remain to be identified, since these small (<2 μm) diatoms had few distinguishing markings on their frustules even when examined using scanning electron microscopy (pers. comm. D. Patterson, University of St Andrews).

Chapter Eight

A Corrugated Raceway For The Treatment of Wastewater by Marine Microalgae

8.1 Introduction

The design, construction and operation of microalgal wastewater treatment systems has been influenced by two major factors: the need for adequate mixing to maintain efficient treatment (Terry & Raymond, 1985; de la Noüe & De Pauw, 1988); and the problem of separating the microscopic algal biomass from the treated effluent economically, to complete the process (Richmond & Becker, 1986; Pantastico, 1987; Oswald, 1988b).

Thorough mixing of microalgal ponds ensures homogeneous conditions by avoiding sedimentation of algal cells and increasing the efficiency of light utilisation in the culture. Mixing also prevents thermal stratification and the occurrence of nutrient and pH gradients, supersaturation of oxygen and the depletion of carbon dioxide at the pond surface, and anaerobic conditions on the bottom (Persoone *et al.*, 1980; Terry & Raymond, 1985; Richmond, 1986c; Oswald, 1988a & b; Sukenik, 1991).

Various systems have been designed to enhance the mixing of algal mass cultures. Continuous flow mixing of shallow circulating raceways using paddlewheels has long been accepted (Moraine *et al.*, 1979; Oswald, 1988b). Other designs have employed air-water lifts (Soong, 1980; Vonshak *et al.*, 1982; Laws *et al.*, 1983), or mixing boards in rectangular ponds (Wagener, 1982; Materassi *et al.*, 1984). Some have used pumps to recirculate the culture in sloping culture units. Various types of sloping culture units have been used which include plates with transverse baffles (Setlik *et al.*, 1970; Heussler *et al.*, 1978), corrugated surfaces (Roubicek *et al.*, 1985) and a series of troughs (Goldman, 1979). More modern systems circulate the algal culture within closed polyethylene or plastic photobioreactors with a high surface area to volume ratio. These may be

vertical panels (Tredici *et al.*, 1991) or horizontal, vertical or coiled tubes (Laing & Jones, 1988; Borowitzka & Borowitzka, 1989; Lee & Low, 1992; Vonshak, 1992; Richmond *et al.*, 1993). Although high levels of treatment may be achieved using these types of apparatus, the additional costs in construction and operation severely restrict their economic use in microalgal wastewater treatment systems.

The BOD of the algal biomass in the effluent from microalgal treatment ponds is usually above the discharge standards for natural water bodies (Ryther, 1983). Therefore the algal biomass has to be removed before the effluent is discharged. By their very nature, microalgae are small (>20 μm). This, coupled with the facts that culture densities are relatively low, most species have a specific gravity slightly greater than that of the water, and many have a strongly negative charge on their surface which keeps them dispersed, makes harvesting difficult and costly (Moraine *et al.*, 1979; Oswald, 1988b).

The various harvesting methods available have been extensively reviewed (Lazer *et al.*, 1976; Benemann *et al.*, 1980; Mohn, 1980, 1988). These methods include flotation, sedimentation, precipitation, centrifugation, filtration, flocculation, autoflocculation and bioflocculation. The most successful techniques are centrifugation, filtration and flocculation (Mohn, 1988), but incompatibility between efficiency of the harvest methods and their cost-effectiveness restricts their application in microalgal wastewater treatment (Benemann *et al.*, 1980).

Immobilisation of algae in beads of carageenan or alginate may offer a novel and elegant way to recover the algal biomass, because the beads sediment in a few seconds and can be used repeatedly (Hall & Rao, 1989; Robinson *et al.*, 1989; Tyagi & Vembu, 1990; Garbisu *et al.*, 1991; de la Noüe

et al., 1992; Travieso *et al.*, 1992). However, the cost of the immobilisation may also be prohibitive for use in large-scale systems.

An alternative approach is to capitalise on natural characteristics of the microalgae in the design of treatment apparatus. In Chapter 7 two species of microalgae (SA91B33, SA91CY1), which continually removed high concentrations of ammonium and ortho-phosphate from 1:1 diluted wastewater, were noted for their ability to aggregate and adhere to the surface of the mini-pond. These properties are useful in two respects. First, there is no need to separate the algal biomass from the pond outflow, since the algae remain attached to the surface of the culture apparatus, leaving a treated effluent which is virtually algal free. Second, by inclining the surface of the apparatus, wastewater will trickle through the adhered algal biomass without the need for any additional mechanical mixing. An apparatus designed specifically for these adherent species would therefore have lower capital investment and lower operational costs.

The surface-adherent properties of species SA91B33 and SA91CY1 indicate that an apparatus with a high surface area to volume ratio would be most suitable for their use in wastewater treatment. This chapter describes the design construction and operation of such an apparatus for wastewater treatment using these species.

8.2 Materials and Methods

8.2.1 Algae

Algal inocula were taken from exponential phase cultures of the two endemic isolates (SA91B33, SA91CY1) and used to set up two continuous cultures which were the same as those described in Chapter 6. These

provided exponential growth phase, 1:1 diluted wastewater adapted cultures for seeding the raceways.

8.2.2 Apparatus

Corrugated raceways were constructed from four 20 cm wide strips of clear corrugated plastic sheeting. The strips were joined by overlapping two corrugations of the higher strip over two corrugations of the lower strip and sealing with silicone sealant (Vallance Ltd, Birmingham, England). (Preferably this should be "aquaseal," since the fungicide in the sealant used initially inhibited development of the algal mat on its surface.) Side walls of clear plastic sheet were attached and sealed with the sealant to produce a water tight continuous raceway 2.5 m long. The apparatus was enclosed in a wooden frame for support. The corrugation increased the surface area of the raceway and formed discrete micro-ponds. Upon inclination of the raceway at a slight angle, medium added at the top overflowed from one micro-pond to the next, this slowed the flow of wastewater down the raceway and reduced problems of desiccation by increasing the holding capacity. Each raceway contained 78 micro-ponds and had a total holding capacity of 2.1 l and surface area of 0.6 m^2. The raceway was protected from rain and falling debris by a glass cover, which was raised to facilitate ventilation. Inflow and outflow tubes passed through the end walls of the raceway. Unfiltered 1:1 diluted primary sewage effluent was pumped peristaltically (Watson-Marlow Ltd, Falmouth, England; model 502S) to each raceway from a 1000 l fibre-glass storage tank and dripped from the inflow tube into the first micro-pond. Medium trickled from one micro-pond to the next and finally out of the raceway into a collection vessel, through the outflow tube. A schematic diagram of a complete continuous corrugated raceway culture unit is shown in Figure 8.1a and a

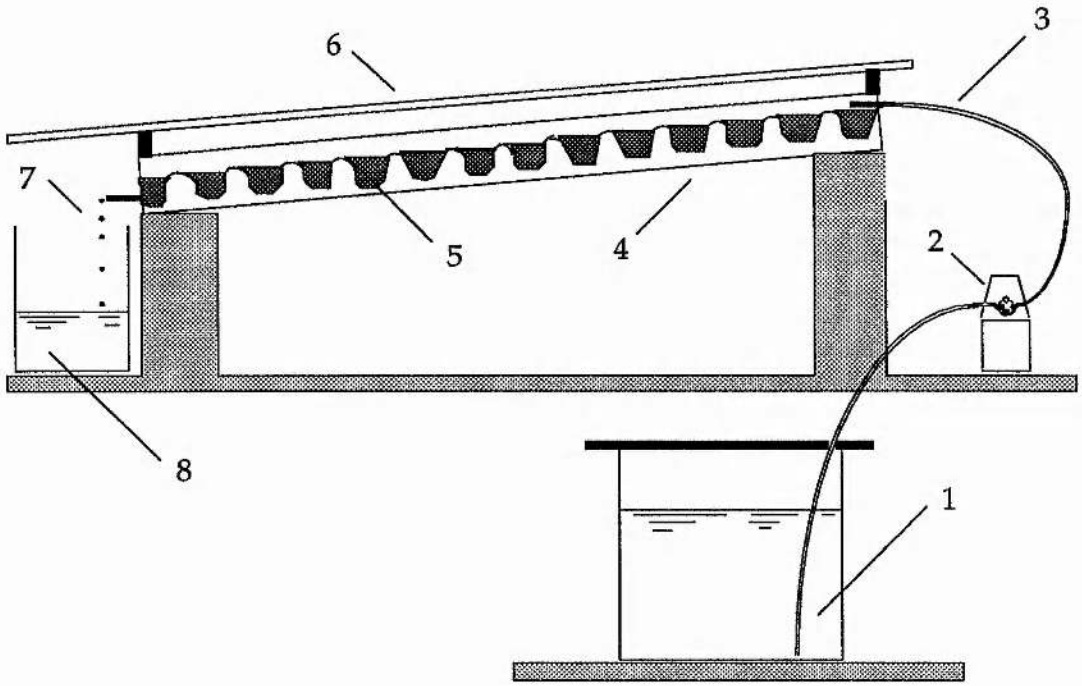

Figure 8.1a Schematic diagram of a complete continuous culture unit (a.): 1: diluted wastewater supply tank; 2: peristaltic pump; 3: wastewater inflow; 4: corrugated raceway; 5: algal culture within micro-pond; 6: glass rain cover; 7: outflow; 8: treated wastewater.

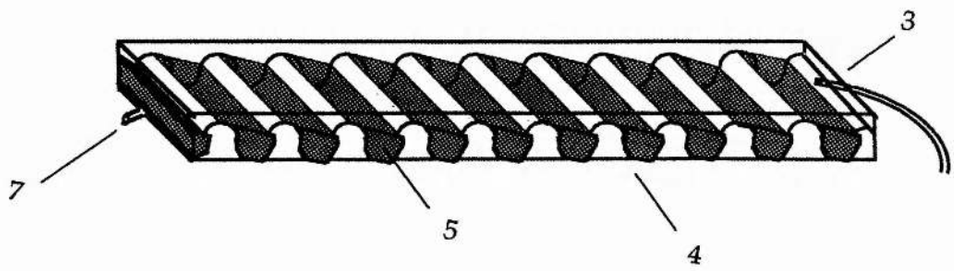

Figure 8.1b Detail of a corrugated raceway

Figure 8.1c Two corrugated raceway continuous culture units in operation.

Figure 8.1d Close-up of adhered algal culture on the surface of a corrugated raceway.

detail of a corrugated raceway is shown in Figure 8.1b. Two operating corrugated raceway units and a close-up of the adhered algal culture on the surface of a corrugated raceway are shown in Figures 8.1c & d respectively.

8.2.3 Operation

For one week, each raceway (one for each species) was seeded daily with 500 ml of algal culture. At the start of the treatment period (day 0) wastewater diluted 1:1 with seawater was added continuously at the same pumping rate used for the mini-ponds (4 l per day), so that treatment using the two types of apparatus could be compared directly. The raceways were run as continuous cultures for three months. Twenty millilitre samples of the 1:1 diluted wastewater inflow and corrugated raceway outflow were taken daily for measurement of nutrient concentrations (ammonium, nitrite, nitrate and ortho-phosphate; Sections 2.2.7), pH (Section 2.2.7), and for microscopic examination (Section 2.2.2).

The effect of diurnal variation in light intensity on nutrient removal was investigated for the two isolates. Concentrations of ammonium, nitrite, nitrate and ortho-phosphate were measured at 2 h intervals over a 24 h period. Parallel measurements of light intensity (PAR) were also made.

8.3 Results

The inflow and outflow nutrient concentrations of the cultures of isolates SA91CY1 and SA91B33 grown on corrugated raceways under ambient conditions are shown in Figs. 8.2 and 8.3. For both species, the reduction in nutrient concentrations to zero (Figs. 8.2 and 8.3) corresponded to the growth of a dense algal culture on the surface of the raceways

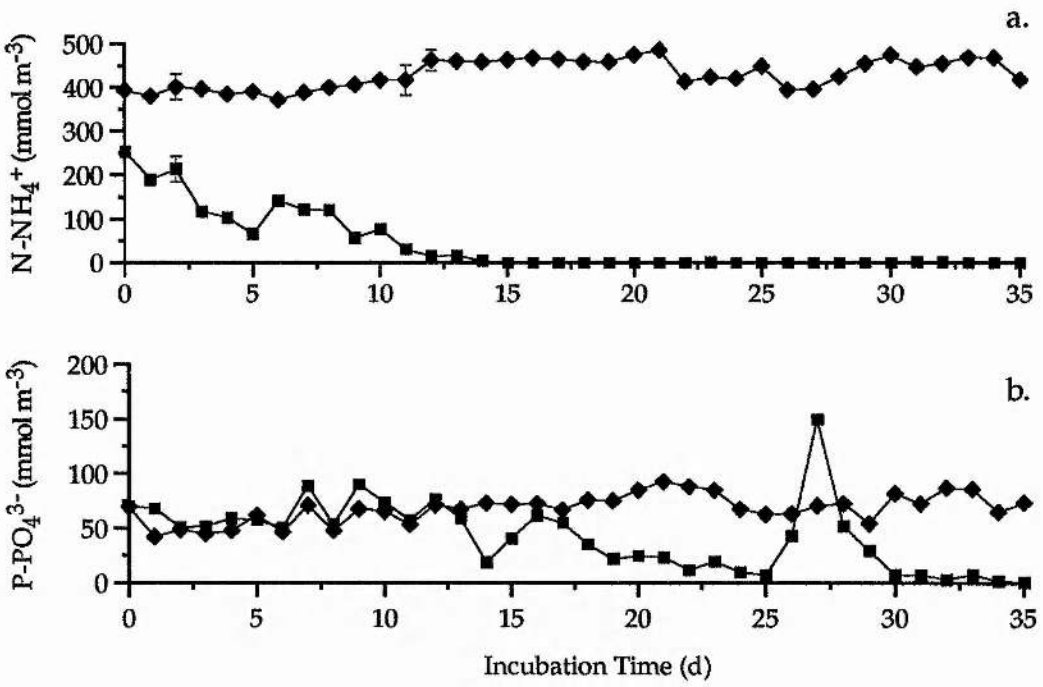

Figure 8.2 Ammonium (a.) and ortho-phosphate (b.) concentrations in the 1:1 diluted wastewater inflow (♦-♦) and outflow (■-■) of a corrugated raceway of isolate SA91CY1 over 35 days culture. Nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

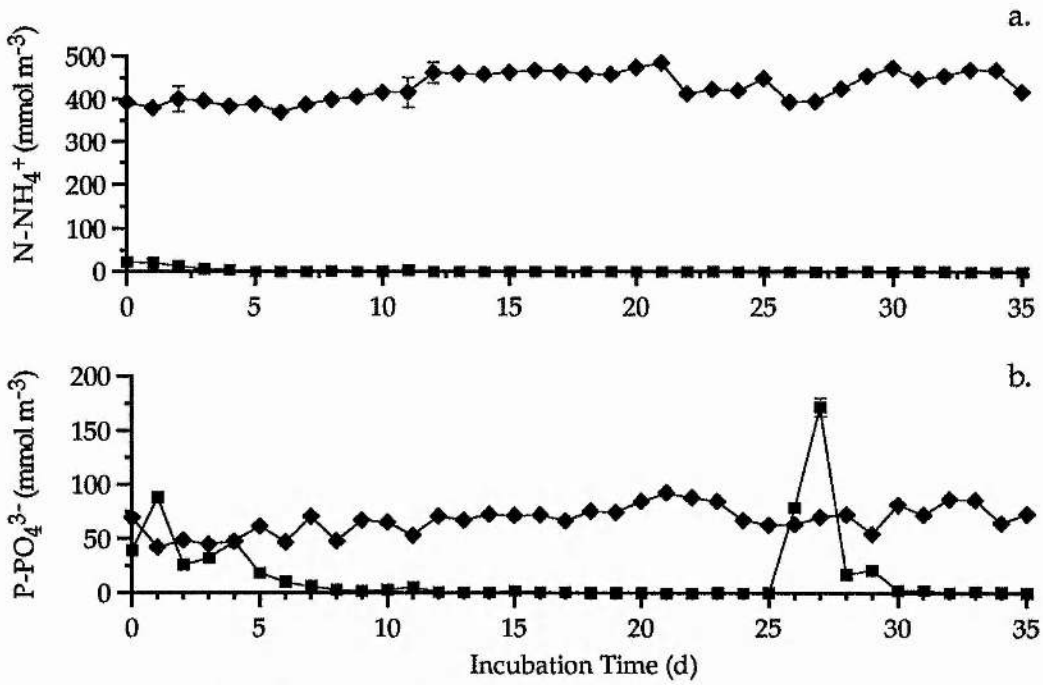

Figure 8.3 Ammonium (a.) and ortho-phosphate (b.) concentrations in the 1:1 diluted wastewater inflow (♦-♦) and outflow (■-■) of a corrugated raceway of isolate SA91B33 over 35 days culture. Nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

(Fig. 8.1d). The culture of isolate SA91CY1 took longer to establish than that of SA91B33, which probably accounts for the higher initial nutrient removal by the bacillariophyceaen species (SA91B33). The two species totally removed both nutrients, the ammonium being completely removed before the ortho-phosphate. The increase in ortho-phosphate concentration of the outflow between days 26 to 29 corresponded to the partial drying out of both raceways which was caused by a blockage in the inflow of diluted wastewater on culture days 25 to 26 (Figs. 8.2b and 8.3b). However the ammonium concentration of the outflows remained unchanged (Figs. 8.2a & 8.2b).

Figures 8.4 and 8.5 show detail of the nutrient removal by the two raceway cultures on days 36 to 50, for direct comparison to mini-pond cultures which were also operated over this 14 day period (Chapter 7). The raceway cultures of both species continued to remove 100 % of ammonium and ortho-phosphate (Table 8.1a), inflow nutrient concentrations remained uniform at 497.7 ± 60.7 mmol N m^{-3} and 76.2 ± 4.9 mmol P m^{-3}. The mean pH of the cultures were 9.8 ± 0.2 and 9.7 ± 0.1 for SA91CY1 and SA91B33 respectively over the 14 days, although that of the inflow was only 7.6 ± 0.1 (Table 8.1b).

To determine whether $N\text{-}NH_4^+$ was actually removed from the culture and not converted to nitrite and nitrate, the concentrations of these nutrients were also measured over the 14 day period. Nitrite concentration (2.8 ± 0.9 mmol N m^{-3}) in the inflow was less than 1 % of the $N\text{-}NH_4^+$ concentration (497.7 ± 60.7 mmol N m^{-3}), while nitrate was absent. Outflow concentrations of nitrite and showed little change from the inflow over the experimental period (Table 8.1a) and nitrate remained absent.

The nutrient concentrations in the diluted wastewater inflow and outflow from the two corrugated raceways, measured over a 24 h period are

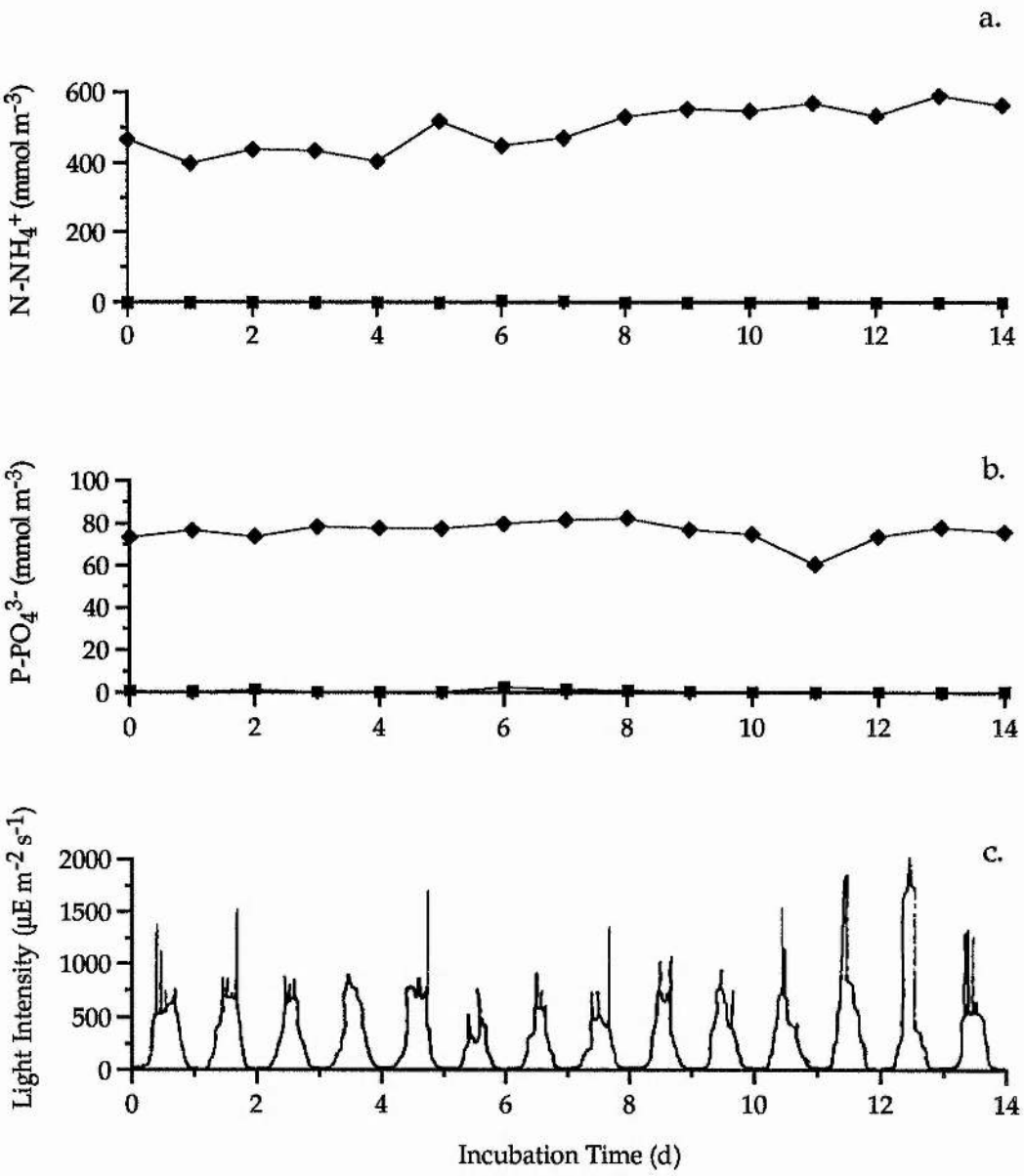

Figure 8.4 Ammonium (a.) and ortho-phosphate (b.) concentrations in the 1:1 diluted wastewater inflow (◆-◆) and outflow (■-■) of a corrugated raceway of isolate SA91CY1 in relation to light intensity (c.) over 14 days culture. Nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

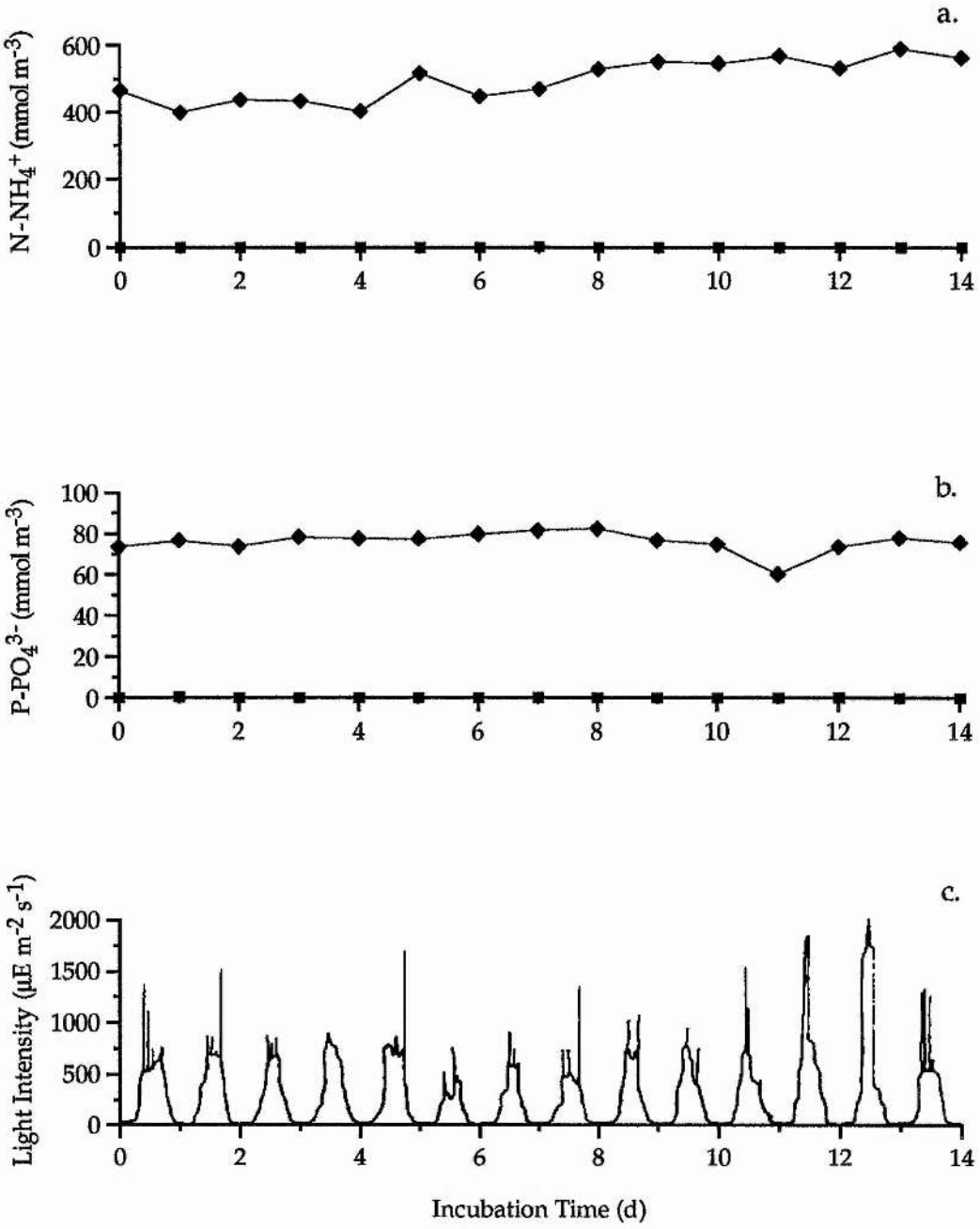

Figure 8.5 Ammonium (a.) and ortho-phosphate (b.) concentrations in the 1:1 diluted wastewater inflow (◆-◆) and outflow (■-■) of a corrugated raceway of isolate SA91B33 in relation to light intensity (c.) over 14 days culture. Nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

Table 8.1a Percentage removal of ammonium, nitrite and ortho-phosphate from corrugated raceways of two marine microalgal species on 1:1 diluted wastewater over 14 days (n = 14). Inflow concentrations were 497.7 ± 60.7 mmol m^{-3} $N\text{-}NH_4^+$, 2.8 ± 0.9 mmol m^{-3} $N\text{-}NO_2^-$, 76.2 ± 4.9 mmol m^{-3} $P\text{-}PO_4^{3-}$.

Algal Species	% $N\text{-}NH_4^+$ Removal	% $N\text{-}NO_2^-$ Removal	% $P\text{-}PO_4^{3-}$ Removal
SA91CY1	100.0 ± 0.4	51.7 ± 10.7	99.4 ± 0.8
SA91B33	100.0 ± 0.2	82.9 ± 7.1	100.0 ± 0.3

Table 8.1b Mean and range pH of the inflow and outflow from corrugated raceways of two marine microalgal species on 1:1 diluted wastewater over 14 days (n = 14).

Algal Species	pH	
	Mean ± s.d.	Range
SA91CY1	9.8 ± 0.2	10.0 - 9.4
SA91B33	9.7 ± 0.1	9.9 - 9.5
Inflow	7.6 ± 0.1	7.73 - 7.50

shown in Figures 8.6 and 8.7 and Table 8.2. Both ammonium and ortho-phosphate removal remained at 100 % over the light:dark cycle, (Figs. 8.6a & d, & 8.7a & d). The nitrite concentration showed little change over the period of measurement, with changes in the outflow reflecting those of the inflow (Table 8.2) and nitrate was absent in both.

Ammonium and ortho-phosphate removal remained at 100 % (Table 8.3) for both species, and the cultures remained unialgal, over a further two months culture. During this period, the concentration of nitrite relative to ammonium was less than 1 % and nitrate was absent.

8.4 Discussion

This chapter describes the abilities of two endemic isolates SA91CY1 and SA91B33 to remove nutrients from diluted wastewater in a specially designed corrugated raceway under ambient summer and autumn conditions. Both isolates were found to continuously remove all the ammonium and ortho-phosphate from wastewater over the three months of the experiment, which demonstrated the stability of the algal culture on the raceway surfaces.

Although a control raceway without an algal culture was not operated in this study, the reduction of the nutrient concentrations in the outflow from the raceways as the algal cultures were established (Figs. 8.2 & 8.3) indicated that nutrient removal was due to the microalgae. The increase in the ortho-phosphate concentration of the outflow when the two cultures partially dried out (Figs. 8.2 & 8.3) may have been due to re-solution of precipitated ortho-phosphate. Ortho-phosphate may have been precipitated within the algal biomass at high culture pH. The pH of both cultures was above 9.5 throughout the culture period. At this pH,

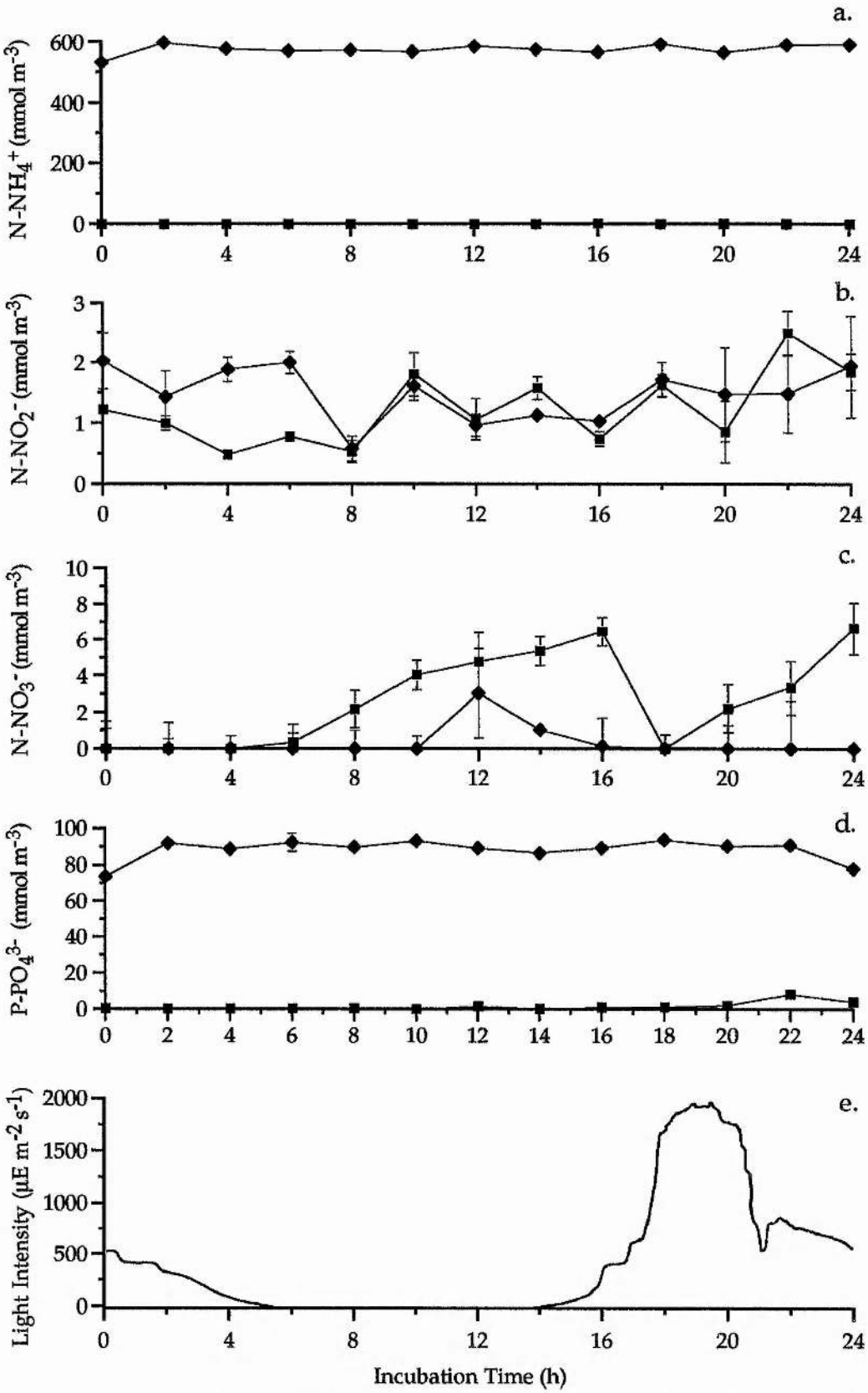

Figure 8.6 Ammonium (a.), nitrite (b.), nitrate (c.) and ortho-phosphate (d.) concentrations in the 1:1 diluted wastewater inflow (♦-♦) and outflow (■-■) of a corrugated raceway of isolate SA91CY1 in relation to light intensity (e.) over 24 hours culture. Nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

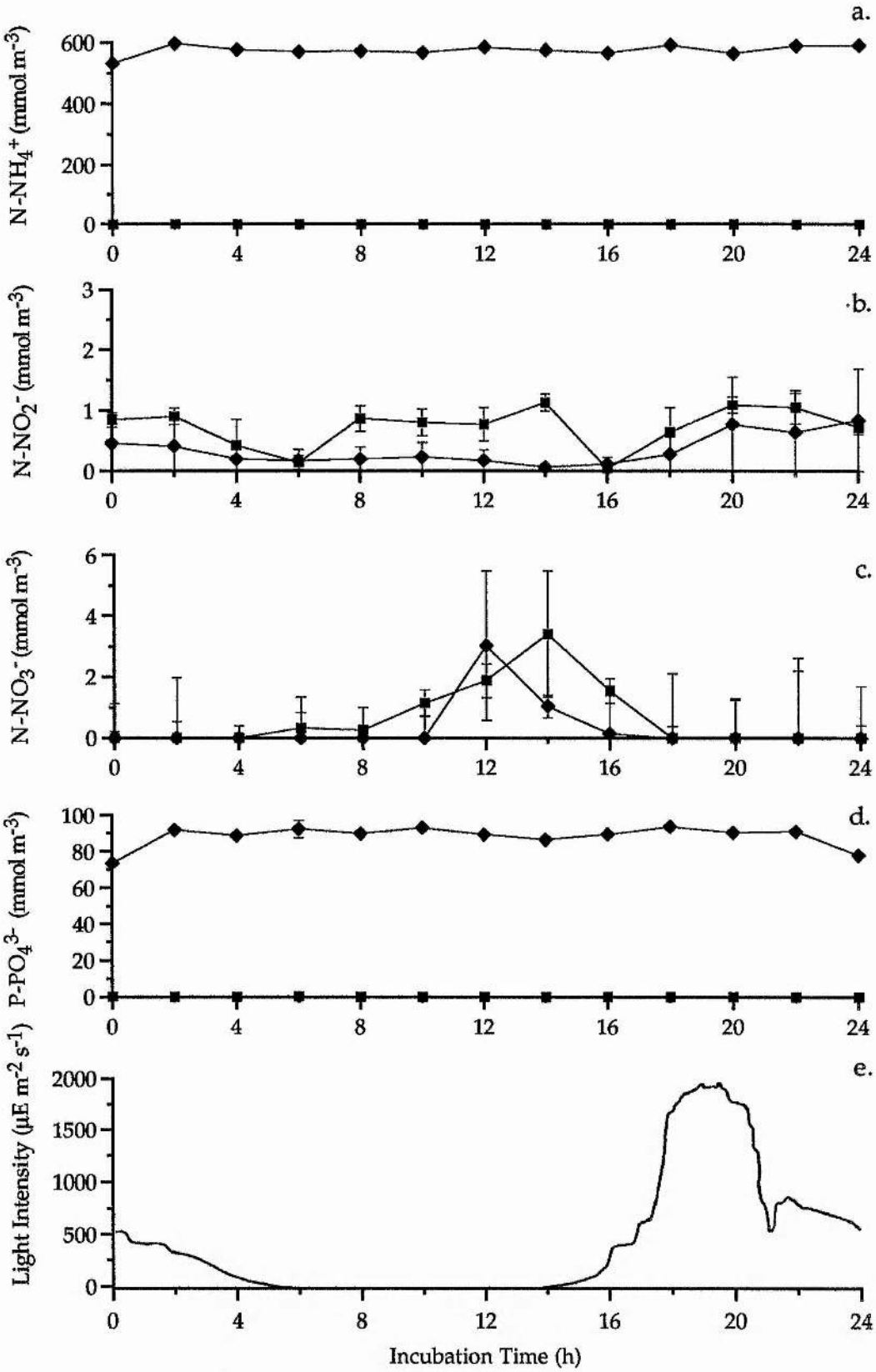

Figure 8.7 Ammonium (a.), nitrite (b.), nitrate (c.) and ortho-phosphate (d.) concentrations in the 1:1 diluted wastewater inflow (♦-♦) and outflow (■-■) of a corrugated raceway of isolate SA91B33 in relation to light intensity (e.) over 24 hours culture. Nutrient values are means ± s.d. of triplicate samples. The s.d. may be too small to be seen.

Table 8.2 Percentage removal of ammonium, nitrite and ortho-phosphate from corrugated raceways of two marine microalgal species on 1:1 diluted wastewater over 24 h (n = 12). The inflow concentrations were 575.1 ± 16.3 mmol m^{-3} $N\text{-}NH_4^+$, 1.5 ± 0.4 mmol m^{-3} $N\text{-}NO_2^-$, 88.6 ± 6.0 mmol m^{-3} $P\text{-}PO_4^{3-}$.

Algal species	% $N\text{-}NH_4^+$ Removal	% $N\text{-}NO_2^-$ Removal	% $P\text{-}PO_4^{3-}$ Removal
SA91CY1	100.0 ± 0.1	16.9 ± 40.0	98.3 ± 2.5
SA91B33	100.0 ± 0.1	50.8 ± 20.0	100.0 ± 0.2

Table 8.3 Percentage removal of ammonium, nitrite and ortho-phosphate from corrugated raceways of two marine microalgal species on 1:1 diluted wastewater after 3 months culture. Values are means ± s.d. of triplicate samples. Inflow concentrations were 553.9 ± 16.3 mmol m^{-3} $N\text{-}NH_4^+$, 3.3 ± 0.2 mmol m^{-3} $N\text{-}NO_2^-$, 79.2 ± 1.0 mmol m^{-3} $P\text{-}PO_4^{3-}$.

Algal species	% $N\text{-}NH_4^+$ Removal	% $N\text{-}NO_2^-$ Removal	% $P\text{-}PO_4^{3-}$ Removal
SA91CY1	100.0 ± 0.1	51.1 ± 6.1	100.0 ± 0.1
SA91B33	100.0 ± 0.1	90.6 ± 3.0	100.0 ± 0.3

ortho-phosphate precipitation is possible, but transformation of ammonium to ammonia gas would be very low (Section 4.4.3).

The mechanisms of nutrient removal in the raceways appear to be similar to those in the mini-ponds, with ammonium removed before the ortho-phosphate. Therefore, treatment in both raceways and mini-ponds was representative of high-rate ponds, which function as nitrogen limited systems (Golueke *et al.*, 1967; Weissman *et al.*, 1978)

The higher treatment by the algal isolates cultured on the raceways than in the mini-ponds (Tables 7.1 & 8.1) may due to a number of factors. Raceways have a larger surface area enabling a higher proportion of the culture to be exposed to direct light than in mini-ponds. There was also little wash-out of cells as the algae adhere to the raceway surface. Therefore they may have supported a higher algal biomass per unit volume. The raceways were operated so that treated and untreated wastewater never came into contact, whereas in the mini-pond, untreated effluent was continually mixed into the culture, inevitably increasing nutrient concentrations in the outflow. Furthermore, higher daily temperatures may have been attained in the raceways than in the larger volume mini-pond cultures. Torzillo *et al.* (1986) describe similar results for closed, low volume photobioreactors which had higher temperatures and higher productivity compared to larger volume open ponds.

This study has demonstrated the capability of two unialgal cultures of marine microalgae to treat wastewater efficiently during summer conditions in a temperate region. By exploiting the adherent properties of these species, the corrugated raceway microalgal treatment system does not require mixing and enables harvesting of the algal biomass by simply scraping it off, which is the most efficient and cost effective method of separating biomass from treated effluent (Reid and Assenzo, 1961).

Chapter Nine

Discussion

The experiments described in this thesis demonstrate the potential of marine microalgae for use in wastewater treatment systems in temperate areas. Marine microalgae were selected for the ability to remove >80 % of ammonium and ortho-phosphate which are the main nutrients in primary sewage effluent. A summary of the nutrient removal from batch culture (Chapter 3) and continuous culture (Chapters 6, 7 & 8) experiments under both controlled and ambient conditions is shown in Table 9.1. These rates of nutrient removal were typical of or greater than those which have been previously found for freshwater species under similar culture conditions (Lincoln & Hill, 1980; Martin *et al.*, 1985a & b; Lavoie & de la Noüe, 1987; de la Noüe & Bassères, 1989; Tam & Wong, 1989; Megharaj *et al.*, 1992). They are also comparable to the nutrient removal measured for marine microalgae cultured on wastewater (Dunstan & Menzel, 1971; Dunstan & Tenore, 1972; Goldman & Stanley, 1974; Goldman *et al.*, 1974a & b). However, caution must be taken when comparing results of this study to those of other authors since differences in experimental conditions also need to be considered.

The present study showed that the 102 marine microalgae tested had a variety of abilities to remove nutrients from wastewaters (Chapter 3 & 5). Decrease in the nutrient removal ability of a number of algal species was attributed to contamination by microalgal species occurring naturally in the wastewater (Chapter 6). Moreover, a mixed culture of wastewater species had lower nutrient removal than the unialgal cultures of the best-treating species selected in this study (Chapter 6). Possibly, the effects of competition between the species in the contaminated and mixed cultures contributed to their lower nutrient removal (Hulburt, 1970; D'Elia *et al.*, 1979).

Table 9.1 Summary of the nutrient removal by the best-treating marine microalgal species in the four different culture systems used in this study.

Culture System	% Ammonium Removed	% Ortho-phosphate Removed
Batch Culture	>99 %[1]	>90 %[1]
Continuous Culture (controlled conditions)	>80 %	>90 %
Continuous Mini-Ponds (ambient conditions)	>80 %	>80 %[2]
Continuous Raceways (ambient conditions)	100 %	100 %

[1]Two-day removal experiment.
[2]For some species ortho-phosphate removal declined with time.

Seven marine species were selected for their ability to remove high levels of nutrients and remained unialgal in open continuous culture in ambient conditions, suggesting dominance over the wastewater species. Indeed, two of the marine species were shown to outcompete a mixed culture of wastewater species under these conditions. One of the main problems for the mass culture of microalgae is contamination by unwanted species (Shelef & Soeder, 1980; Becker & Venkataraman, 1982; De Pauw *et al.*, 1984; Richmond, 1986c). Many conditions govern the competitive advantage of one species over another (Goldman & Ryther, 1976a; Nelson *et al.*, 1979; Goldman *et al.*, 1982b). Although the results of laboratory studies investigating competition between species are variable (Dortch *et al.*, 1982; Dortch, 1990) and there is some debate as to whether laboratory studies are applicable to the field (Grover, 1991), the present study has shown the seven best-treating species to be dominant in culture under all experimental conditions from small-scale batch cultures to large-scale open continuous cultures. The ability of these marine species to remain in unialgal culture may enable a microalgal wastewater treatment process to operate more efficiently by optimising conditions for the growth of a particular species.

The wide temperature range (10-25 °C) over which the best-treating species were shown to remove nutrients suggests that control of temperature may not be required to maintain a high rate of nutrient removal over winter (Chapter 4). In particular, three species were capable of totally removing nutrients from wastewater at 5 °C and low light intensities (25 $\mu E\ m^{-2}\ s^{-1}$), whereas the lowest temperature of 1:1 wastewater:seawater in winter would be 8 °C (calculated from Chapter 2; Table 2.2). Therefore, heat provided by the inflowing wastewater may be sufficient to maintain high treatment levels over winter (Witt *et al.*, 1981), and transparent polyethylene covers (Richmond, 1988) or greenhouses (Guterstam & Todd, 1990; de la Noüe *et al.*, 1992) to raise winter temperatures, may not be

necessary for the species selected in the present study. The salinity of the culture may also be permitted to vary as the best-treating microalgal species were found to grow well over a wide range (8-24‰) of wastewater:seawater dilutions (Chapter 4).

The versatility of the marine microalgal species in this study to treat other types of wastewaters was demonstrated by the ability of six of the seven best-treating species to remove >70% nutrients from eel aquaculture effluent (Chapter 5). In the absence of ammonium these species were capable of removing high concentrations of nitrate, suggesting that they may also be capable of treating other wastewaters which are rich in nitrate. Freshwater microalgae have been shown to treat many different types of wastewater (Chapter 1; Table 1.6), but there have been few investigations using marine microalgae (De Pauw & De Leenheer, 1979).

The research described in this thesis has several differences to that which has already been published on wastewater treatment by microalgae. There have been few studies of treatment by marine species, particularly in temperate areas. Most of the previous research with marine species has been by Goldman, Ryther and co-workers at the Woods Hole Oceanographic Institution, Massachusetts, USA. Although the ability of natural populations of marine microalgae to remove nutrients from wastewater has been studied (Goldman *et al.*, 1974a & b), the main aim of the work of this group was to use wastewater as a nutrient source to mass culture marine microalgae for the cultivation of bivalve molluscs in an integrated aquaculture (Dunstan & Menzel, 1971; Ryther *et al.*, 1972; Goldman & Ryther, 1976b; Goldman, 1979; Goldman, 1982a & b; Ryther, 1983). Other studies have also used wastewater for the production of marine microalgal biomass for fermentation to methane to produce energy (Wagener, 1981 & 1982; Balloni *et al.*, 1983). The present study has

specifically investigated the ability of marine microalgae to remove nutrients from primary sewage effluent. Previous studies (including research at the Woods Hole Oceanographic Institute; Sebastian & Nair, 1984; Chevalier & de la Noüe, 1985b; Lavoie & de la Noüe, 1987) have used secondary effluent. This has a lower BOD than primary effluent and has nitrate rather than ammonium as the main nitrogen source. The microalgae tested in the present study were shown to be capable of removing high levels of nitrate from the activated eel aquaculture effluent (Chapter 5). Moreover, many of these workers filtered the effluent to remove algal species occurring naturally in the wastewater. In the present study, unfiltered effluent was used so that only marine microalgae which were dominant to the wastewater species were selected. This study has shown that marine microalgae may be used for the combined secondary and tertiary treatment of wastewaters.

The screening protocol adopted in this study was novel since it involved the simultaneous screening of microalgae using different culture conditions ranging from laboratory batch cultures to outside continuous cultures. Earlier studies have tended to use either batch culture (Tam & Wong, 1989; Garbisu *et al.*, 1991; Tadros & Phillips, 1992) or mass culture (De Pauw & De Leenheer, 1979; Goldman, 1979; Balloni *et al.*, 1983). A comprehensive screening of the nutrient removal ability of over 100 species and isolates from eight taxonomic divisions has not been previously made; typically, only one or two species have been investigated (Tam & Wong, 1989; Garbisu *et al.*, 1991; Tadros & Phillips, 1992). Even fewer studies have used endemic microalgae, and these only used one or two isolates (Goldman & Stanley, 1974; Witt *et al.*, 1981). The seven best-treating microalgae in the present study were all endemic isolates which had been isolated from the waters around the sewage outfall in St Andrews Bay specifically for use in this study. Possibly, these microalgae were already

adapted to or tolerant of the ambient environmental conditions of light and temperature, or the high nutrient concentrations and reduced salinity associated with this outfall.

Six of the seven best-treating microalgae have been identified as Bacillariophyceae, (of which three were endemic strains of *Phaeodactylum tricornutum*), and the other as a species of *Oscillatoria.* The strains of *P. tricornutum* and *Oscillatoria animalis* from the culture collections either died out or were outcompeted in this study, which provides further justification for the need to include endemic isolates in a comprehensive screening of microalgae. Algal species which have been cultured for extended periods under controlled conditions may have different physiological, morphological and reproductive characteristics to species growing in ambient conditions (Craig *et al.*, 1988). This may have contributed to the relatively poor performance of the culture collection species.

Bacillariophyceaen species have also been found to dominate phytoplankton blooms in marine waters (Marshall & Orr, 1927; Hulburt, 1970) and seawater:wastewater mixtures in large-scale culture systems including: 15 l continuous cultures (Dunstan & Menzel, 1971), 400 l outdoor tanks (Dunstan & Tenore, 1972), 2,000 l outdoor mass cultures (Goldman *et al.*, 1974a, b; Goldman & Ryther, 1976b) and 35,000 l outdoor mass cultures (Goldman & Ryther 1976a & b; D'Elia *et al.*, 1977). One species in particular, *P. tricornutum,* has been reported to dominate seawater mixed with secondary sewage effluent in continuous cultures under laboratory conditions (Ryther *et al.*, 1972; Goldman & Stanley, 1974) and in large-scale outdoor pond cultures (Goldman & Ryther, 1976a & b; D'Elia *et al.*, 1977; Goldman & Mann, 1980). Three of the isolates selected in the present study were identified as strains of *P. tricornutum,* but three other marine

bacillariophyceaen isolates and a species of the cyanophyceaen, *Oscillatoria* (SA91CY1) which had nutrient removal abilities similar to or better than these strains were also selected. These species may never have been selected if the more ecologically based approach of previous studies had been used.

Five of the seven best-treating isolates in the present study, including the species of *Oscillatoria,* had adherent properties (Chapter 7). By designing a culture apparatus to enhance the growth of these microalgae greater nutrient removal rates were achieved than with cultures grown in mini-ponds (Table 9.1). This demonstrates the potential advantage of designing microalgal treatment systems to suit particular algal species, rather than relying on a mixed assemblage of microalgal species with variable nutrient removal rates to grow up which occurs with most systems in use today (Oswald, 1998a & b). Corrugated raceways have two further advantages in that they do not require mechanical mixing and enable simple and economic separation of the algal biomass from the treated effluent, both of which would reduce the operational cost of this system. Corrugated raceways have not previously been operated as non-recirculating systems using adherent microalgal species. However a similar approach to treating wastewaters using periphyton from coral reef communities attached to screens has been developed (Adey *et al.,* 1993). Study of the characteristics (physiology, morphology) of the species isolated by the screening techniques described here may enable optimisation of the treatment process for complete removal of nutrients (Table 9.1).

Further research, preferably over an entire year, is necessary to completely assess the potential of a marine microalgal treatment process in temperate areas, and to determine how treatment by different algal species is affected by low culture temperature and low ambient light intensity during winter. Investigations of wastewater treatment by marine

microalgae in larger culture systems also needs to be investigated to determine the effects of scaling up on species dominance and treatment ability. Microalgae are capable of removing BOD, heavy metals and enteric bacteria from wastewaters (Sebastian & Nair, 1984; Oswald, 1988a & c; Tam & Wong, 1989), and further experiments could measure the potential of the best-treating species from this study for removing these pollutants. Screening experiments should also be made with other wastewaters to determine the types of wastewater which marine species are capable of treating and whether the same species are always selected which was found for the two types of wastewaters used in the present study.

Adherence of microalgal species to surfaces may provide an economic solution to the problem of separating algal biomass from treated effluent. Isolation of benthic marine microalgal species for use in further screening experiments may identify other species with this beneficial property. To offset the costs of a microalgal treatment process and the costs of separating of the algal biomass from the treated effluent, the commercial use of the algal biomass should also be investigated (Table 1.7). The present study has demonstrated that marine microalgal wastewater treatment systems, may be of use for secondary and tertiary treatment of wastewaters in temperate areas. Efficient nutrient removal by marine microalgal systems could be used to reduce the concentrations of eutrophication causing nutrients discharged to coastal waters. Further research into the application of this potentially important process is necessary.

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Appendices

Table A 2.1 Growth characteristics of microalgal species in batch culture on Erd-Schreiber medium.

Algal Species	Specific Growth Rate, k ± s.d	Doubling Time, G (d) ± s.d.	Log Phase Beginning (d)	Log Phase End (d)
Bacillariophyceae				
[4]*Chaetoceros calcitrans*	0.31 ± 0.05	3.3 ± 0.5	4	11
[1]*Nitzschia longissima*	0.69 ± 0.07	1.5 ± 0.1	7	10
[3]*Nitzschia ovalis*	0.51 ± 0.03	2.0 ± 0.1	6	12
[5]*Phaeodactylum tricornutum*	0.60 ± 0.02	1.7 ± 0.1	1	7
[5]*Skeletonema costatum*	0.43 ± 0.06	2.4 ± 0.3	1	6
[5]*Thalassiosira weissflogii*	0.28 ± 0.04	3.7 ± 0.6	9	13
Chlorophyceae				
[2]*Botryococcus braunii*	No Growth			
[5]*Chlamydomonas reginae*	No Growth, Culture Settlement			
[5]*Chlorella salina*	0.16 ± 0.04	6.5 ± 1.7	9	13
[5]*Chlorella stigmatophora*	0.38 ± 0.02	2.7 ± 0.2	6	15
[1]*Dunaliella salina*	No Growth, Culture Settlement			
[5]*Dunaliella tertiolecta*	0.37 ± 0.04	2.8 ± 0.3	6	11
[3]*Nannochloropsis oculata*	0.39 ± 0.03	2.6 ± 0.2	5	13
[5]*Stichococcus bacillaris*	0.08 ± 0.00	13.4 ± 0.8	8	17
Chryptophyceae				
[1]*Rhodomonas baltica*	No Growth, Culture Settlement, Cell Aggregations			
[3]*Rhodomonas marina*	No Growth, Culture Settlement, Cell Aggregations			
[2]*Rhodomonas sp.*	0.41 ± 0.10	2.6 ± 0.5	2	5
Cyanophyceae				
[1]*Oscillatoria animalis*	Little Growth, Low Density, Cell Aggregations			
[1]*Spirulina platensis*	No Growth, Low Density, Cell Aggregations			
Dinophyceae				
[5]*Amphidinium carterae*	No Growth			
[5]*Oxyrrhis marina*	No Growth			
Prasinophyceae				
[5]*Micromonas pusilla*	0.31 ± 0.01	3.2 ± 0.1	5	12
[1]*Tetraselmis* sp.	0.24 ± 0.04	4.2 ± 0.8	5	12
[4]*Tetraselmis* sp.	0.37 ± 0.06	2.8 ± 0.4	6	11
[4]*Tetraselmis rubens*	0.43 ± 0.10	2.4 ± 0.5	3	8
[5]*Tetraselmis suecica*	0.48 ± 0.06	2.1 ± 0.3	6	11
[5]*Tetraselmis tetrathele*	0.40 ± 0.04	2.5 ± 0.2	6	12
[5]*Tetraselmis verrucosa*	0.32 ± 0.09	3.5 ± 1.1	11	16
Prymnesiophyceae				
[5]*Chrysochromulina chiton*	No Growth, Bacterial Contamination			
[1]*Coccolithophora* sp.	No Growth, Bacterial Contamination			
[4]*Coccolithus* sp.	0.52 ± 0.13	1.9 ± 0.2	9	11
[5]*Isochrysis galbana*	0.31 ± 0.04	3.3 ± 0.5	7	13
[5]*Pavlova lutheri*	0.38 ± 0.02	2.6 ± 0.1	6	13
[5]*Phaeocystis poucheti*	0.42 ± 0.08	2.5 ± 0.4	5	12
[5]*Prymnesium parvum*	Little Growth, Low Density			
Rhodophyceae				
[5]*Porphyridium purpureum*	No Growth, Culture Settlement			

Culture collections: [1]Biobred Ltd, [2]CCAP, [3]Gatty Marine Laboratory, [4]Millport Marine Biological Station, [5]Plymouth Culture Collection

Isolation codes designate endemic algal species.

Table A 2.1 Growth characteristics of microalgal isolates in batch culture on Erd-Schreiber medium (continued).

Algal Isolates	Specific Growth Rate, k ± s.d	Doubling Time, G (d) ± s.d.	Log Phase Beginning (d)	Log Phase End (d)
Bacillariophyceae				
SA90B1	0.40 ± 0.05	2.6 ± 0.3	3	9
SA90B2	0.38 ± 0.04	2.7 ± 0.3	4	11
SA90B3	0.28 ± 0.07	3.8 ± 1.1	5	14
SA90B4	0.23 ± 0.04	4.4 ± 0.6	5	13
SA90B5	0.33 ± 0.07	3.2 ± 0.8	2	16
SA90B6	0.36 ± 0.03	2.8 ± 0.2	3	10
SA90B7	No Growth, Cell Aggregations			
SA90B8	No Growth, Cell Aggregations, Culture Settlement			
SA90B9	0.15 ± 0.03	7.0 ± 1.2	4	16
SA90B10	No Growth Cell Aggregations, Culture Settlement			
SA90B11	1.0 ± 0.04	1.0 ± 0.0	1	8
SA91B12	No Growth, Cell Aggregations			
SA91B13	No Growth, Cell Aggregations, Mixed Culture			
SA91B14	No Growth, Cell, Aggregations, Mixed Culture			
SA91B15	No Growth, Cell Aggregations			
SA91B16	Little Growth, Cell Aggregations distort OD			
SA91B18	0.40 ± 0.04	2.5 ± 0.2	3	8
SA91B19	No Growth, Cell Aggregations, Mixed Culture			
SA91B21	0.20 ± 0.06	5.7 ± 2.0	3	11
SA91B22	0.21 ± 0.04	5.0± 1.1	4	8
SA91B23	0.37 ± 0.09	2.9 ± 0.7	4	8
SA91B24	No Growth			
SA91B25	0.33 ± 0.01	3.1 ± 0.1	0	10
SA91B26	No Growth, Cell Aggregations			
SA91B27	No Growth, Cell Aggregations distort OD measurement			
SA91B29	No Growth, Cell Aggregations			
SA91B30	No Growth, Cell Aggregations			
SA91B36	No Growth			
SA91B37	0.55 ± 0.03	1.8 ± 0.1	7	11
SA91B38	0.40 ± 0.05	2.5 ± 0.3	7	13
SA91B39	0.22 ± 0.04	4.7 ± 0.8	1	16
SA91B40	0.15 ± 0.03	6.9 ± 1.4	7	16
SA91B41	No Growth, Cell Aggregations			
SA91B42	No Growth			
SA91B43	0.21 ± 0.01	4.8 ± 0.2	5	12
SA91B44	No Growth, Culture Settlement			
SA91B45	0.63 ± 0.06	1.6± 0.2	6	10
Chlorophyceae				
SA90C1	No Growth			
SA90C2	No Growth			
SA90C3	0.51 ± 0.07	2.0 ± 0.3	9	15
SA91C6	0.08± 0.03	15.1 ± 6.0	4	13
SA91C9	0.29 ± 0.08	3.7 ± 1.0	0	11
SA91C10	0.46 ± 0.02	2.1± 0.1	5	13
SA91C11	No Growth			
SA91C13	No Growth			
Cyanophyceae				
SA91CY1	Filamentous aggregations distort OD measurement			
Rhodophyceae				
SA90R1	0.73 ± 0.03	1.4 ± 0.1	9	13

Figure A 3.1 Key

1 SA90B2
2 *Coccolithus* sp.
3 *Tetraselmis tetrathele*
4 SA91B12
5 SA91B43
6 SA90B4
7 *Tetraselmis* sp.
8 SA91C6
9 SA91B27
10 SA90C1
11 SA90C3
12 *Tetraselmis suecica*
13 *Phaeodactylum tricornutum*
14 SA91B39
15 SA90B7
16 SA91CY1
17 SA90R1
18 *Porphyridium purpureum*
19 SA91B32
20 SA91C13
21 *Chlorella stigmatophora*
22 SA90B3
23 SA91C10
24 *Nannochloropsis oculata*
25 *Tetraselmis rubens*
26 SA91B25
27 *Chlorella salina*
28 SA91B37
29 *Tetraselmis verrucosa*
30 *Rhodomonas marina*
31 *Rhodomonas baltica*
32 *Rhodomonas* sp.
33 *Pavlova lutheri*
34 *Stichococcus bacillaris*
35 *Prymnesium parvum*

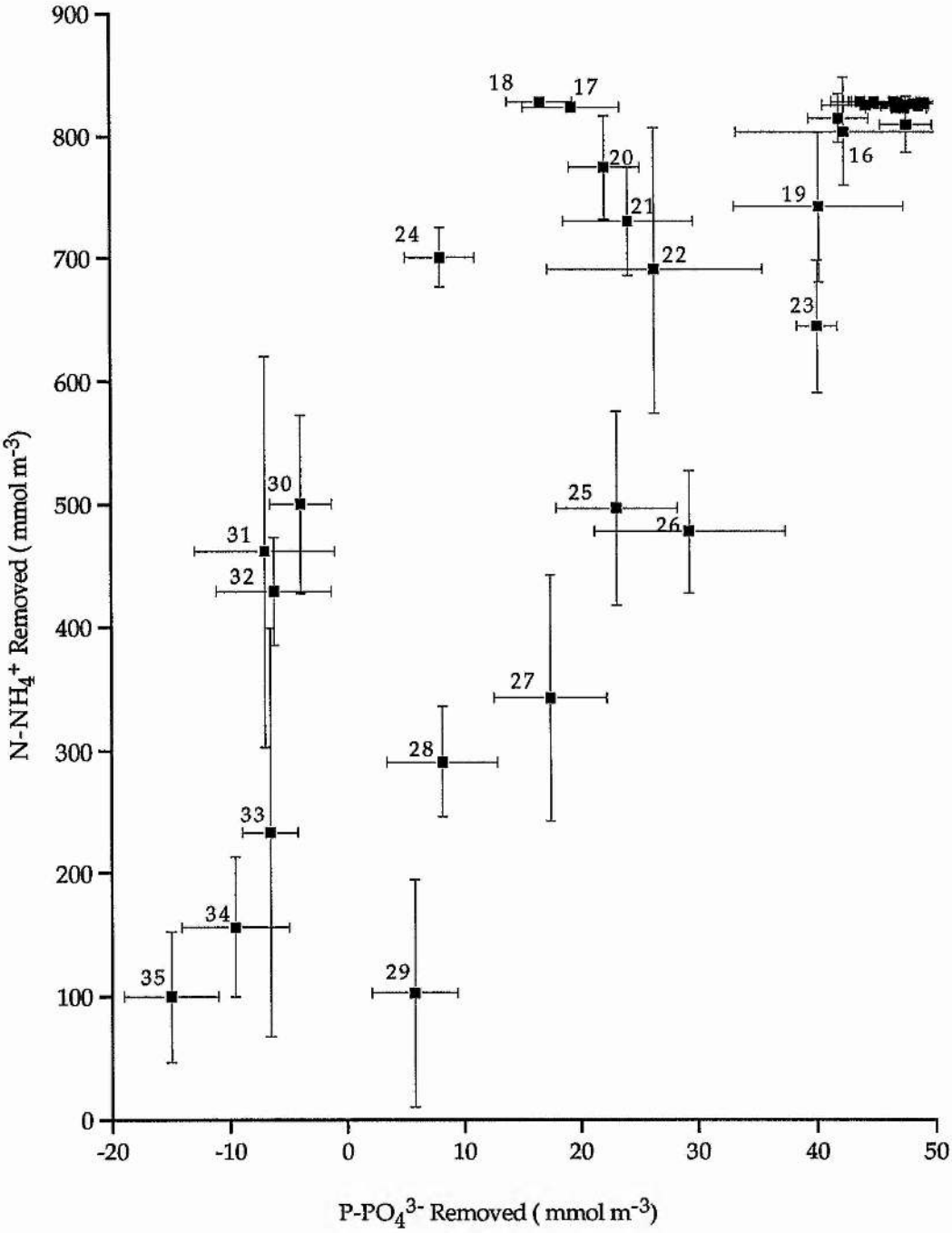

Figure A 3.1. Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species and isolates cultured for seven days on wastewater diluted 1:1 with seawater in small-scale (50 ml) batch cultures under controlled conditions. Values are means of means ± s.d. of duplicate experiments in which final nutrient concentrations in triplicate algal cultures were compared to final concentrations in controls without algae (824.4 ± 58.5 mmol m^{-3} $N\text{-}NH_4^+$, 49.4 ± 3.2 mmol m^{-3} $P\text{-}PO_4^{3-}$). The s.d. may be too small to be seen.

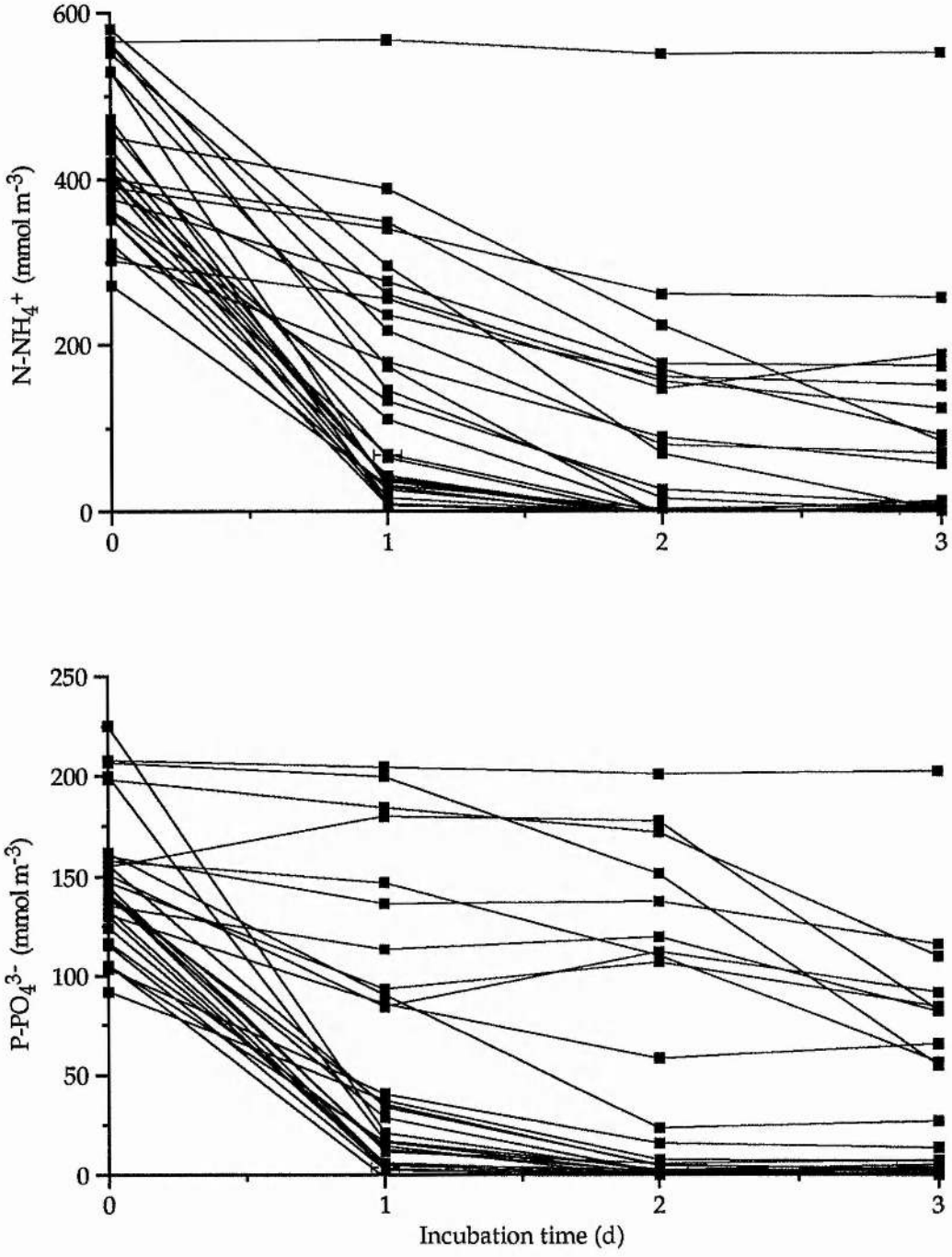

Figure A 3.2 Ammonium (a.) and ortho-phosphate (b.) concentrations measured daily in small-scale batch cultures of 29 microalgal species and isolates cultured on 1:1 diluted wastewater for four days. Values are means of triplicate cultures.

Figure A 3.3 Key

1 SA91B33
2 SA90C2
3 SA91B43
4 *Tetraselmis suecica*
5 SA90B2
6 SA90C3
7 *Tetraselmis tetrathele*
8 *Tetraselmis* sp.
9 *Phaeodactylum tricornutum*
10 SA91B12
11 SA90B4
12 SA91C10
13 *Chlorella salina*
14 *Dunaliella tertiolecta*
15 SA91C13
16 SA91B39
17 SA90C1
18 *Coccolithus* sp.
19 SA91B27
20 *Porphyridium purpureum*
21 SA90B3
22 *Chlorella stigmatophora*
23 *Rhodomonas* sp.
24 SA90B5

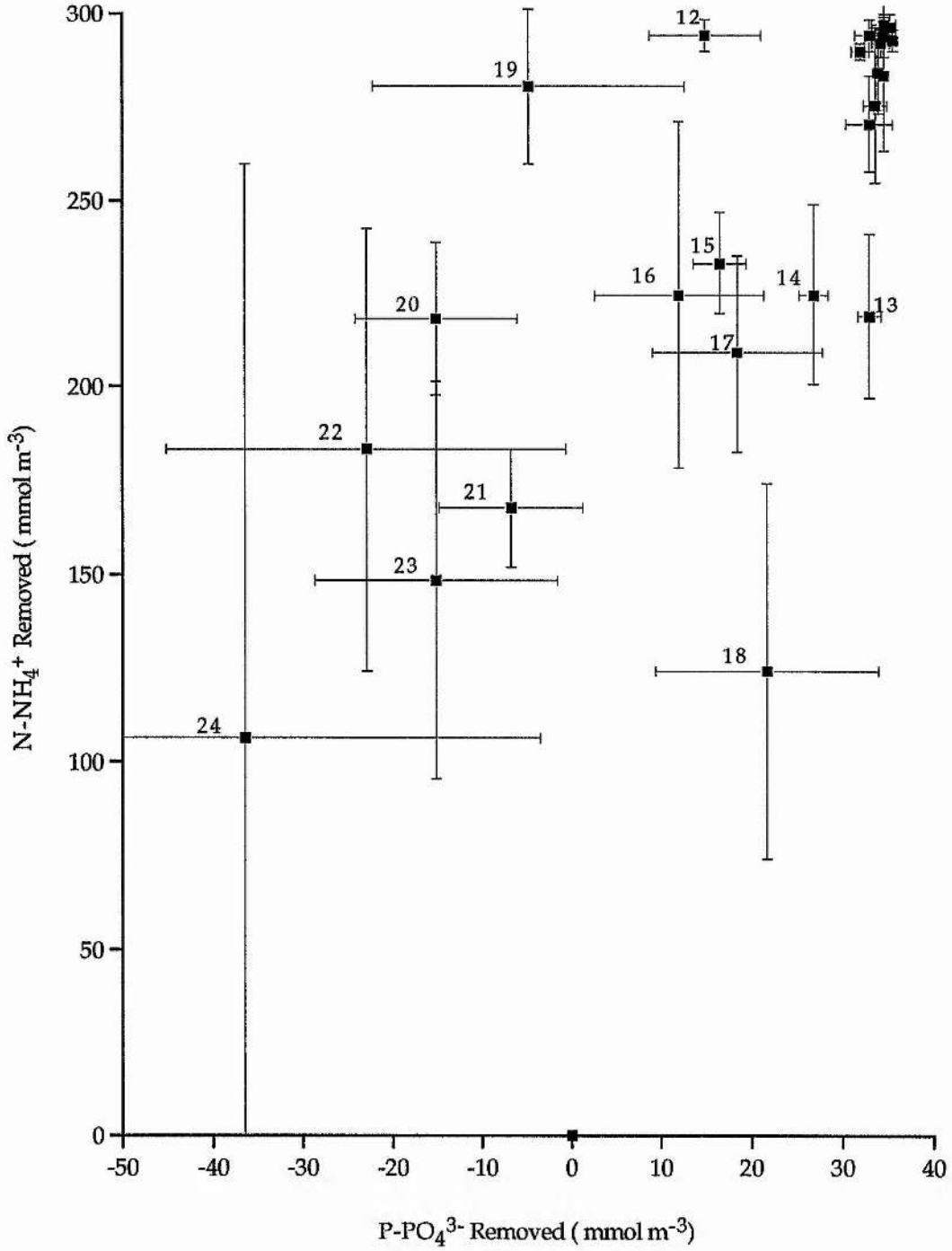

Figure A 3.3 Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species and isolates cultured for two days on wastewater diluted 1:1 with seawater in small-scale (50 ml) batch culture under controlled conditions. Values are means of means ± s.d. of four replicate experiments in which final nutrient concentrations in triplicate algal cultures were compared to final concentations in controls without algae (295.5 ± 50.0 mmol m^{-3} $N\text{-}NH_4^+$, 35.2 ± 7.4 mmol m^{-3} $P\text{-}PO_4^{3-}$).

Figure A 3.4 Key

1 SA91C13
2 SA90B4
3 SA90B2
4 SA92B48
5 SA91B43
6 SA90C3
7 SA91CY1
8 SA92C17
9 *Dunaliella tertiolecta*
10 SA90C1
11 SA91B33
12 SA91C6
13 *Coccolithus* sp.
14 *Tetraselmis* sp.
15 SA91B25
16 SA91B12
17 *Tetraselmis suecica*
18 *Chlorella salina*
19 *Tetraselmis tetrathele*
20 *Porphyridium purpureum*
21 *Tetraselmis rubens*
22 SA91B47
23 SA91B39
24 SA90B5
25 SA92C16
26 *Chlorella stigmatophora*
27 SA91B27
28 SA91C10
29 *Nannochloropsis oculata*
30 *Stichococcus bacillaris*
31 *Botryococcus braunii*

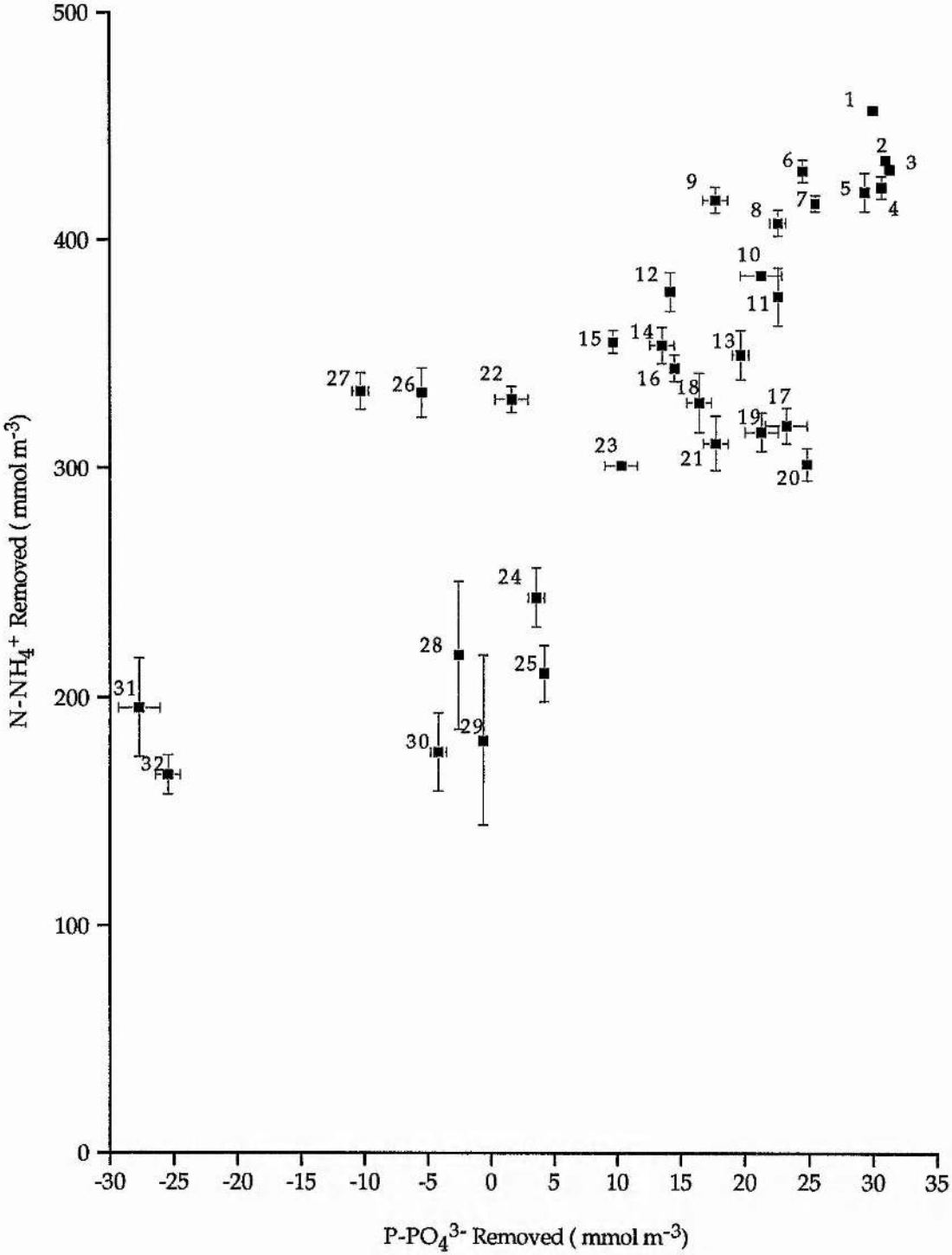

Figure A 3.4 Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species and isolates cultured for two days on wastewater diluted 1:1 with seawater in small-scale (50 ml) batch cultures under ambient conditions. Values are means of means ± s.d. of duplicate experiments in which final nutrient concentrations in algal cultures were compared to final concentrations in a control without algae (458.2 ± 12.8 mmol m^{-3} $N\text{-}NH_4^+$, 32.3 ± 0.3 mmol m^{-3} $P\text{-}PO_4^{3-}$). The s.d. may be too small to be seen.

Figure A 5.1 Key

1 SA90C1
2 SA90B2
3 SA91B43
4 SA92B48
5 EE92C4
6 SA91C10
7 SA91B42
8 *Tetraselmis* sp.
9 *Tetraselmis suecica*
10 SA91B33
11 EE92C3
12 SA91B39
13 SA91C6
14 SA90B5
15 SA90B4
16 *Tetraselmis tetrathele*
17 SA90C2
18 *Tetraselmis rubens*
19 *Chlorella salina*
20 SA90B3
21 *Phaeodactylum tricornutum*
22 SA92C16
23 SA91B12
24 *Chaetoceros calcitrans*
25 SA91CY1
26 SA91B47
27 *Chrysochromulina chiton*
28 *Porphyridium purpureum*
29 *Tetraselmis verrucosa*
30 *Stichococcus bacillaris*
31 SA90B3
32 *Chlorella stigmatophora*
33 *Rhodomonas* sp.
34 *Pavlova lutheri*
35 *Nannochloropsis oculata*
36 *Chlamydomonas reginae*
37 *Rhodomonas marina*
38 EE92C1
39 *Rhodomonas baltica*

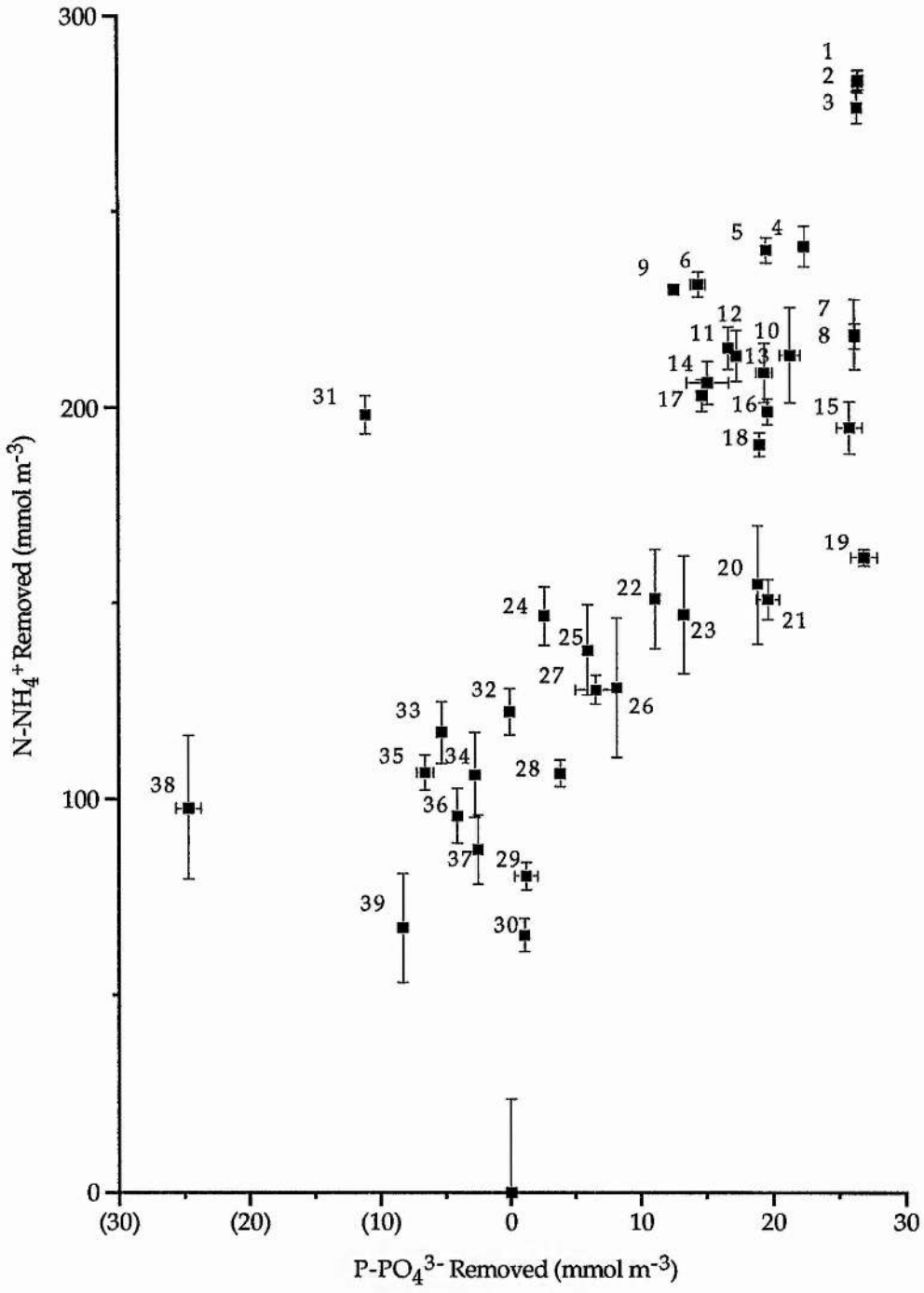

Fig. A 5.1 Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species and isolates cultured for two days on untreated eel effluent in small-scale (10 ml) batch cultures under controlled conditions. Values are means of means ± s.d. of duplicate experiments in which final nutrient concentrations in triplicate algal cultures were compared to final concentrations in controls without algae (301.5 ± 7.3 mmol m^{-3} $N\text{-}NH_4^+$, 26.6 ± 0.7 mmol m^{-3} $P\text{-}PO_4^{3-}$). The s.d. may be too small to be seen.

Figure A 5.2 Key

1 SA90B2
2 SA91B33
3 SA90B4
4 SA91B39
5 SA90C1
6 SA91B43
7 SA90B3
8 SA90B5
9 SA91C6
10 SA90C3
11 SA91C10
12 SA91B42
13 SA91CY1
14 SA90C4
15 *Oxyrrhis marina*
16 SA91B47
17 *Tetraselmis tetrathele*
18 SA92B48
19 *Tetraselmis suecica*
20 *Phaeodactylum tricornutum*
21 *Rhodomonas marina*
22 EE92C1
23 SA90C3
24 EE92C2
25 SA92C16
26 *Prymnesium parvum*
27 *Rhodomonas baltica*
28 *Rhodomonas* sp.
29 *Stichococcus bacillaris*
30 *Chlorella salina*
31 *Chrysochromulina chiton*
32 *Tetraselmis* sp.
33 *Chlorella stigmatophora*
34 *Nannochloropsis oculata*
35 *Chaetoceros calcitrans*
36 SA90C2
37 *Skeletomema costatum*
38 *Tetraselmis verrucosa*
39 *Porphyridium purpureum*
40 *Nitzschia longissima*
41 *Chlamydomonas reginae*
42 *Tetraselmis rubens*
43 *Botryococcus braunii*

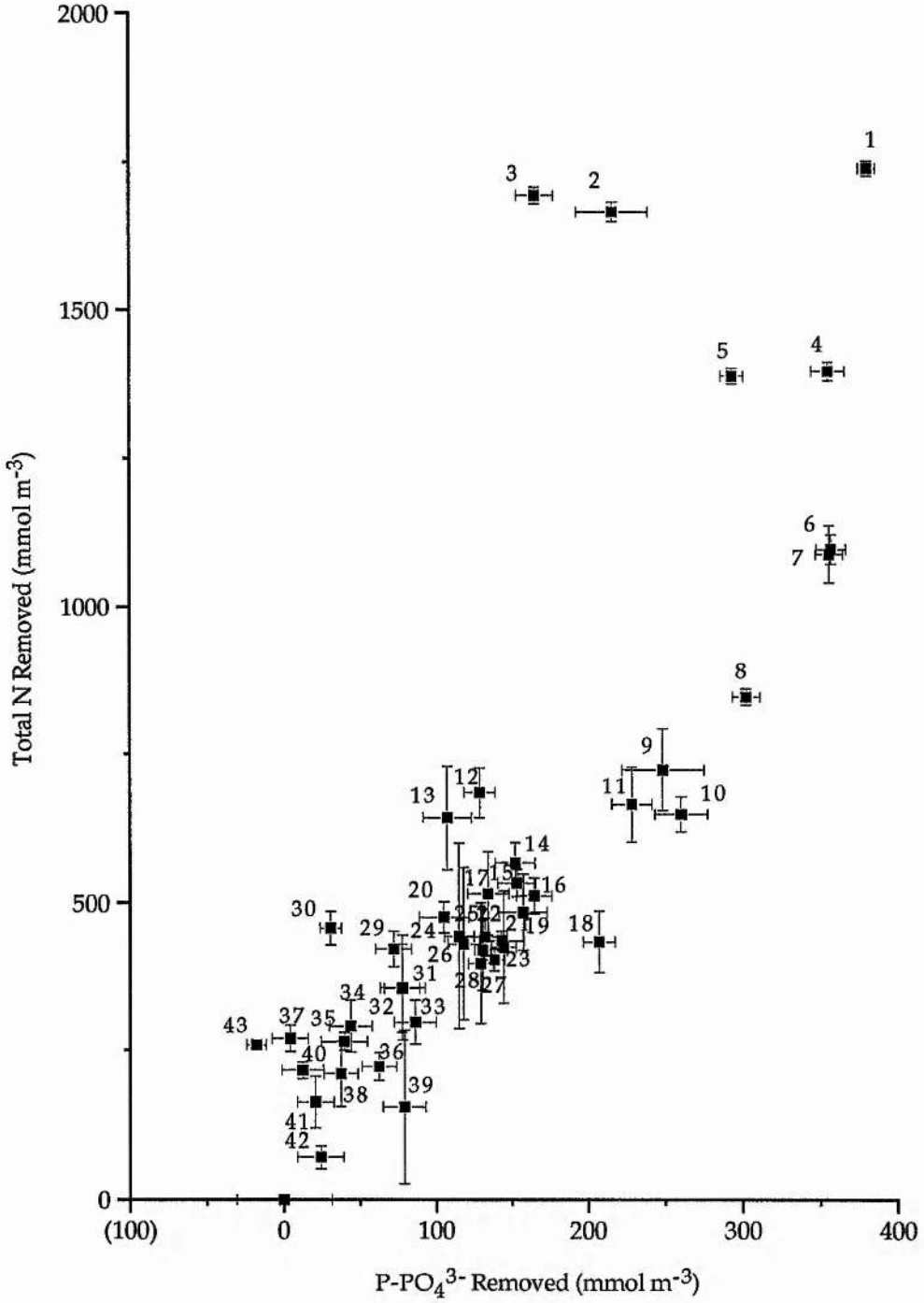

Fig. A 5.2 Amounts of total nitrogen ($N\text{-}NH_4^+$ and $N\text{-}NO_3^-$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species and isolates cultured for two days on "activated" eel aquaculture effluent in small-scale (10 ml) batch cultures under controlled conditions. Values are means of means ± s.d. of duplicate experiments in which final nutrient concentrations in triplicate algal cultures were compared to final concentrations in controls without algae (1771.4 ± 15.3 mmol m^{-3}, $N\text{-}NO_3^-$, 7.1 ± 0.9 mmol m^{-3} $N\text{-}NH_4^+$ and 415.2 ± 5.9 mmol m^{-3} $P\text{-}PO_4^{3-}$).

Figure A 5.3 Key

1 SA92B48
2 SA92C17
3 SA91B43
4 SA90B2
5 SA90B5
6 SA91B39
7 *Chlorella salina*
8 *Tetraselmis rubens*
9 EE92C3
10 SA90C3
11 SA90B4
12 SA90C1
13 SA91B33
14 SA90B3
15 SA92C16
16 *Nitzschia longissima*
17 SA90C2
18 SA91C10
19 SA91B42
20 SA91C6
21 *Porphyridium purpureum*
22 *Oxyrrhis marina*
23 SA91CY1
24 *Tetraselmis suecica*
25 *Dunaliella tertiolecta*
26 *Chlorella stigmatophora*
27 *Chrysochromulina chiton*
28 *Tetraselmis verrucosa*
29 *Rhodomonas* sp.
30 *Chlamydomonas reginae*
31 *Tetraselmis* sp.
32 *Tetraselmis tetrathele*
33 *Rhodomonas baltica*
34 *Botryococcus brauni*
35 *Prymnesium parvum*
36 *Chaetoceros calcitrans*
37 *Rhodomonas marina*
38 *Skeletomema costatum*
39 *Dunaliella salina*
40 *Pavlova lutheri*

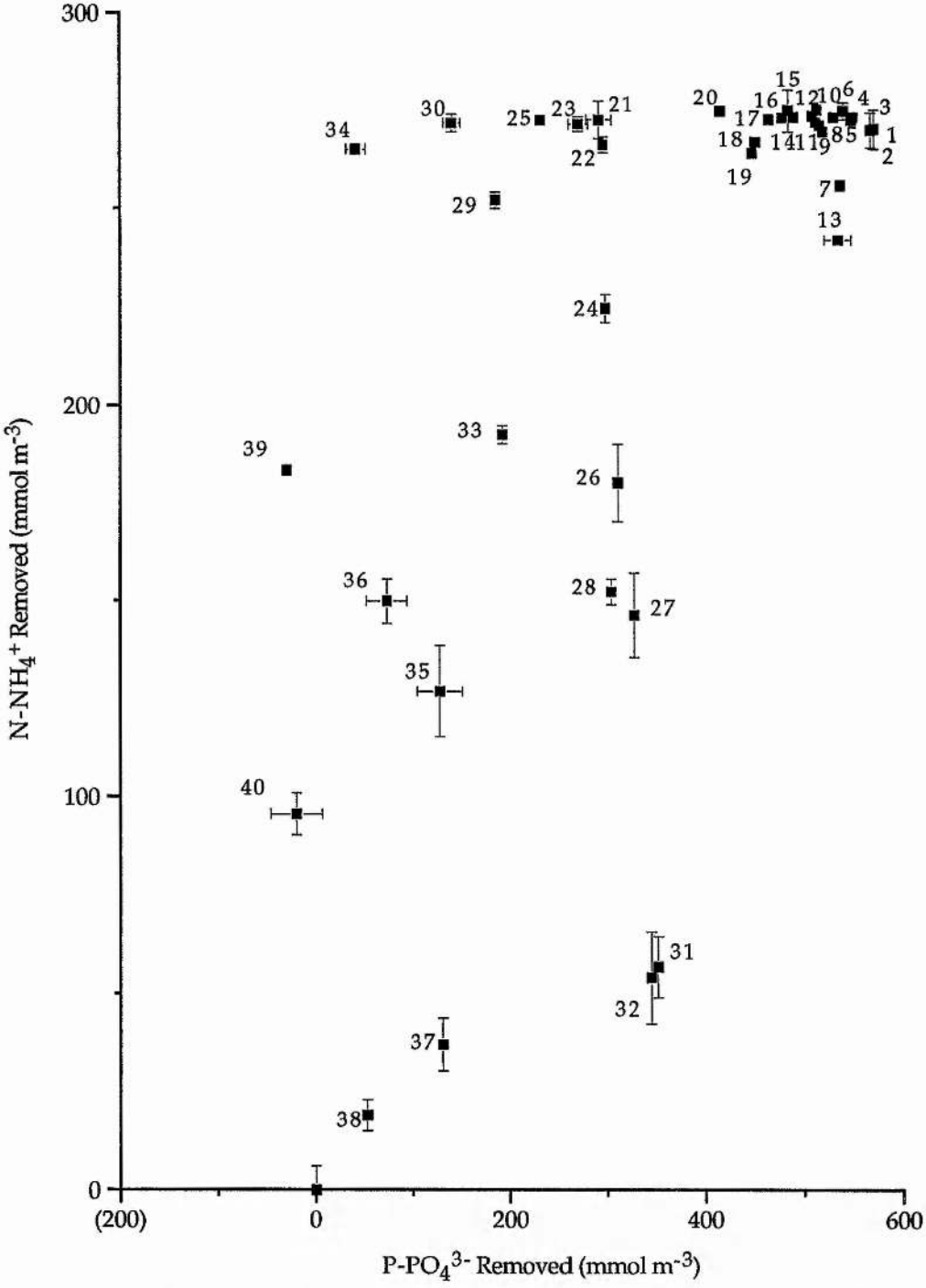

Fig. A 5.3 Amounts of ammonium ($N\text{-}NH_4^+$) and ortho-phosphate ($P\text{-}PO_4^{3-}$) removed by algal species and isolates cultured for two days on "activated" eel effluent in small-scale (10 ml) batch cultures under controlled conditions. Values are means of means ± s.d. of duplicate experiments in which final nutrient concentrations in triplicate algal cultures were compared to final concentrations in controls without algae (275.5 ± 6.1 mmol m^{-3} $N\text{-}NH_4^+$, 581.2 ± 1.3 mmol m^{-3} $P\text{-}PO_4^{3-}$). The s.d. may be too small to be seen.

Table A 6.1 Specific growth rates of 14 microalgal species and isolates grown in batch culture on 1:1 diluted wastewater for seven days.

Algal Species	Specific Growth Rate, k
SA90B2	0.59
SA90B4	0.58
SA91B12	0.47
SA91B33	0.52
SA91B39	0.54
SA91B43	0.56
SA92B48	0.45
SA90C1	0.57
SA90C2	0.45
SA91C6	0.39
SA91CY1	0.52
Chlorella salina	0.46
Tetraselmis sp.	0.58
Porphyridium purpureum	0.39
Mean	0.51

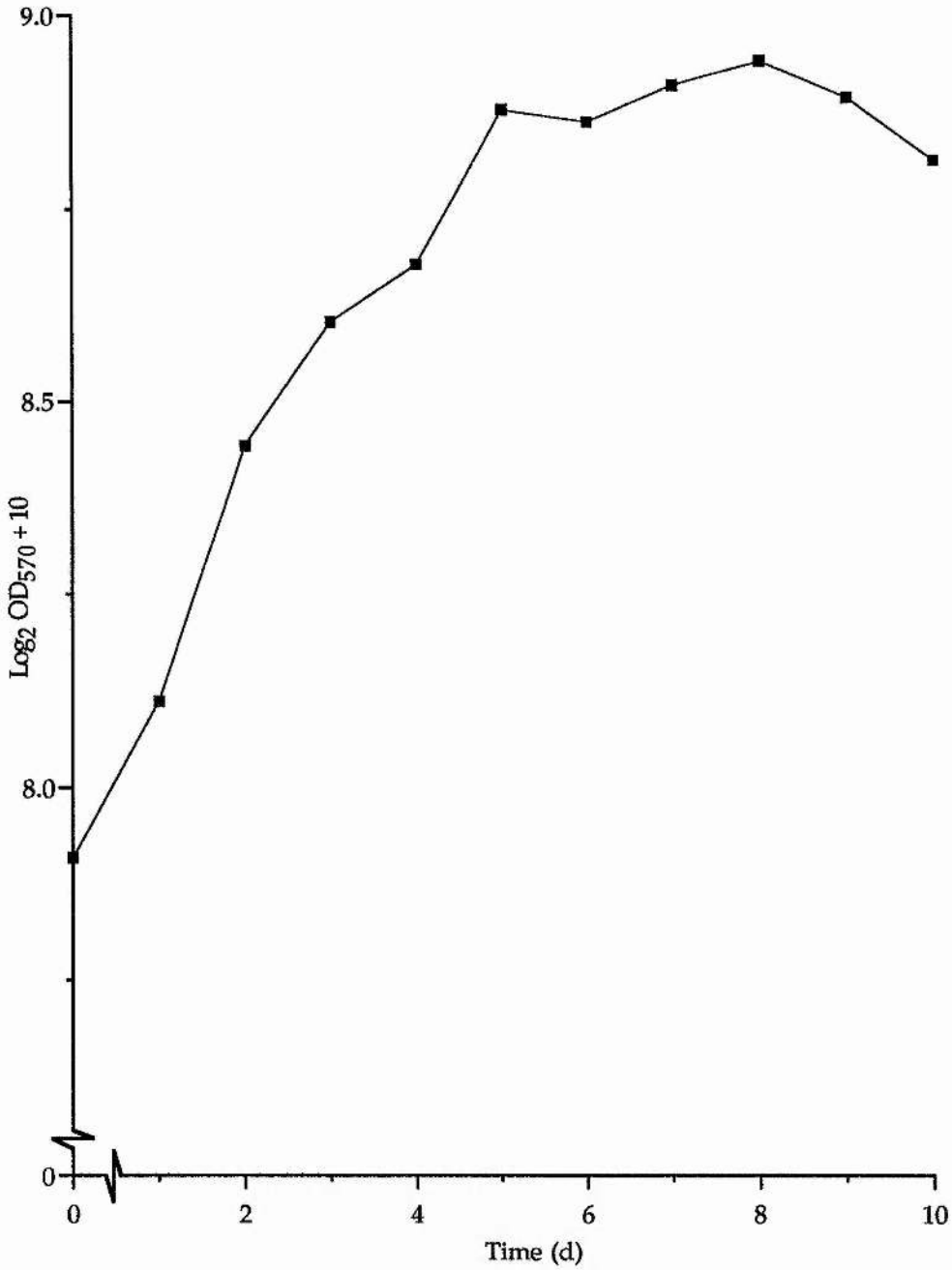

Figure A 7.1 Growth of isolate SA90B4 under batch culture in a mini-pond on 1:1 diluted wastewater over 10 days. The exponential growth rate was calculated from the gradient of the graph over the first 5 days and was found to be: $k = 0.19$.