THE EFFECTS OF SUBLETHAL CONCENTRATIONS OF MERCURIC CHLORIDE

ON AMMONIUM-LIMITED SKELETONEMA COSTATUM (GREV.) CLEVE.

by

LOUISE CLOUTIER-MANTHA

B.Sc., McGill University, 1976

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Department of Botany)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August, 1978

© Louise Cloutier-Mantha, 1978

In presenting this thesis in partial fulfillment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted to the Head of my Department or his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Botany

The University of British Columbia
2075 Wesbrook Place
Vancouver, B.C.
Canada, V6T 1W5

Dated this ___Nov 7/78_____

ABSTRACT

The effects of sublethal additions of mercuric chloride
were studied in the marine diatom Skeletonema costatum (Grev.)
Cleve grown in ammonium-limited chemostats and batch cultures.

In the short-term Hg exposure (up to 5 hours), unexposed
chemostat effluents were simultaneously perturbed with
5 µM NH_4Cl and Hg concentrations ranging from 0.04 to 5.53
nM $HgCl_2$. In the long-term Hg exposure (679.5 hours), ammo-
nium-starved effluents were only perturbed with 5 µM NH_4Cl.

In the short-term Hg exposure, when the effluent from the
chemostat culture was starved for 1.5 hours, Hg decreased the
affinity for the substrate (increased Ks value) and the rate
of ammonium assimilation or the internally controlled uptake
rate, Vi_{max}. When the effluent was starved for 30 hours, only
Vi_{max} was reduced. These effects occurred between 1.84 and
3.68, and at 0.18 nM $HgCl_2$ in effluents starved for 1.5 and 30
hours, respectively. The maximum rate of uptake, Vs, was not
depressed.

In the long-term Hg exposure, at least 0.37 nM $HgCl_2$
decreased the specific growth rate and the maximum cell
density, while the chlorophyll a per cell increased. A period
of population decline was followed by resumption of growth.
Morphological alterations were observed before and after the
recovery.

In the long-term experiment, six days of continual

exposure to 0.37 nM $HgCl_2$ gradually increased the Ks value without affecting Vs and Vi_{max}. The results from exposure to 3.68 nM $HgCl_2$ were similar to the short-term Hg exposure, since both the substrate affinity (Ks value) and the assimilatory rate (Vi_{max}) were impaired. In addition, the maximal uptake rate, Vs, was also reduced after exposure to 3.68 nM $HgCl_2$ for six days in the long-term experiment. After resumption of growth in the Hg-treated cultures, when a new steady-state was established, the affinity for the substrate and assimilatory rates increased in phase D (day 23) compared to phase A (day 6).

The recovery of growth and nutrient uptake rates in phase D, may have been partially mediated by the acquisition of Hg tolerance and the appearance of cells of a different stage of the sexual life cycle, as suggested by differences in cell size and chemical composition.

An attempt was made to determine whether a short-term physiological response (Hg induction of metallothionein synthesis) could be responsible for the recovery. The 250 nm absorbance profile, of nutrient-saturated cultures exposed for 90 to 116 hours to sublethal concentrations of mercury, showed no large absorbance peak in the medium molecular weight pool, corresponding to metallothionein, as it occurs in animals exposed to heavy metals.

The intracellular distribution and levels of Cu, Zn, and Hg in S. costatum, grown in nutrient-saturated batch cultures, were affected by 0.37 nM $HgCl_2$. A concentration equal to or

greater than 1.84 nM $HgCl_2$ reduced the growth rate and cell density, possibly due to the accumulation of Hg in the high m.w. pool. Exposure to 1.84 nM $HgCl_2$ prior to a second addition of 5.53 nM reduced Hg levels in the high m.w. pool. Upon Hg exposure, Zn levels decreased in the high and low m.w. fractions but gradually increased in the medium m.w. pool. Copper slightly increased in the high m.w. pool but remained constant in the medium and low m.w. pools, in relation to total intracellular levels. High levels of Cu and Zn in the low m.w. pool suggests that a substance of a lower m.w., than usually reported for metallothionein, may be involved in the storage and detoxification of heavy metals in S. costatum.

Thesis Supervisor

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

CHAPTER I

INTRODUCTION

Section 1. Environmental Impacts, Distribution and Concentration of Mercury

During the 1950's and 1960's, several dramatic declines in wildlife populations occurred due to the ingestion of mercurials. For example, sead-eating and piscivorous birds declined in Sweden. In Yugoslavia, Iraq, Pakistan and Guatemala community poisoning outbreaks were caused by wheat-dressed alkyl mercurial fungicides (Krenkel, 1973). Disasters at Minimata (1953) and Niigata (1965) are amongst the best documented cases of human poisonings.

In Canada (1968-1969), high levels of Hg were found in pheasants and partridges in the Prairies (Femreite, 1970). The axial muscles of pike from the Saskatoon River contained up to 10 mg·kg^{-1}Hg (Wobeser et al., 1970). Consequently, sport and commercial fisheries were closed in the Saskatoon River network and parts of the Great Lakes, as well as hunting in the Prairies. More than one million pounds of fish were incinerated ($\geq$ 0.5 mg·kg^{-1}Hg in muscle tissue, Dept. Fish. and Forestry, 1971).

Usually the major offenders of Hg pollution are: 1) the chlor-alkali industry where inorganic Hg is used in the electrolytic preparation of chlorine and caustic soda, and

2) metal recovery from Pb-Zn deposits. Mercury is also used in; 1) the fabrication of electrical and control instruments, 2) antiseptics and preservatives in pharmaceutical products and paints, and 3) fungicides and slimicides in agriculture and the pulp industry.

The disclosure about mercury toxicity has led to a general decline in the utilization and commercial production of mercurials and a strengthening of legislation on by-product disposal and discharge. In spite of a reduced input into the environment, there are still important and inadequately quantified losses from anthropogenic sources, such as mining and smelting of Hg ores, combustion of fossil fuels and oils, and improper waste disposal of sludges.

In the ocean, the primary sources of Hg are; degassing of the earth's crust, industrial pollution by atmospheric jet streams, precipitation, volcanism, and upwelling of deep water (Gardner, 1975). Weathering, leaching of Hg-containing soils (Nelson et al., 1977), river transport to oceans and environmental fluxes influenced by man are less or as important as the natural atmospheric translocation of Hg from continents to oceans (Rice et al., 1973; Windom et al., 1975).

Due to differences in sampling, analytical techniques and the short residence time of Hg in the mixed layer of the ocean, it is difficult to generalize on Hg levels in different water bodies (Andren and Harriss, 1975; Gardner, 1975; Kuiper, 1976). The lowest Hg levels reported ranged from 11.2 $ng \cdot l^{-1}$ in the southern hemisphere to 33.5 $ng \cdot l^{-1}$ in the northern

hemisphere; the north eastern Atlantic coastal zone was an anomaly having only 14.7 ng·l^{-1} (Gardner, 1975). Unusually high values of 364 µg·l^{-1} occurred in certain specific areas (Rice et al., 1973; Williams et al., 1974). Freshwater streams ranged from 17 to 125 ng·l^{-1} Hg (Keckes and Miettenen, 1972; Krenkel, 1973). In North Atlantic plankton, particulate Hg values ranged from 0.2 to 0.4 mg·l^{-1} (dry weight) in unpolluted areas, to 5.3 mg·l^{-1} in coastal polluted waters. Variations in Hg levels were not correlated to species composition but to the distance from the pollution sources (Windom et al., 1973).

Even though background levels are low and may be of little concern, health hazards are associated with the ingestion of Hg-contaminated foods, through magnified accumulation in food chains (Cook, 1977). The toxicity of Hg is due to its affinity for thiols, selenols, phosphates, amino and carboxyl terminal groups of amino acids and various cell components (Seyferth, 1978).

Bioassays of single element toxicity performed in situ on natural phytoplankton populations indicated that toxicity thresholds were as follows: Hg <10 nM; Cu∼ 100 nM; Pb, Cd, and As(V)∼ 300 nM; Zn ∼1µM; Ni, Co, Sb, Se, and As(III) > 1 µM (Hollibaugh et al., unpublished manuscript). In Skeletonema costatum, comparable reduction of cell division was obtained with 5-10 µg·l^{-1} Hg, 25-100 µg·l^{-1} Cd and 50-200 µg·l^{-1} Cu (Berland et al., 1977). In Chlorella pyranoidosa, Hg was more inhibitory than Cu (Kamp-Nielsen, 1971) and Cu was more toxic

than Zn in <u>Amphidinium</u> <u>carterae</u>, <u>Thalassiosira</u> <u>pseudonana</u>, <u>S</u>. <u>costatum</u>, and <u>Phaeodactylum</u> <u>tricornutum</u> (Bræk <u>et</u> <u>al</u>., 1976).

Section 2. <u>Factors Influencing Mercury Toxicity</u>

2.1 <u>Chemical Composition of Medium</u>

Heavy metal toxicity varies widely according to experimental variables and conditions. The influence of various factors affecting heavy metal toxicity on algae (Kamp-Nielsen, 1971; Hannan and Patouillet, 1972; Rice <u>et al</u>., 1973), and on microorganisms and invertebrates have been studied (Pyefinch and Mott, 1948; Corner and Sparrow, 1956; Fitzgerald and Faust, 1963; Wisely and Blick, 1966; Lewis <u>et al</u>., 1973; Whitfield and Lewis, 1976; Gibson <u>et al</u>., 1975).

The sensitivity of a test organism in a pollution assay is affected by: 1) physical factors such as light (Steemann Nielsen and Wium-Andersen, 1971; Kamp-Nielsen, 1971; Overnell, 1976), temperature (De Filippis and Pallaghy, 1976a; Blinn <u>et al</u>., 1977; Knowles and Zingmark, 1978), salinity (Hunter, 1949), pH (Wisely and Blick, 1966), time and concentration of exposure (Erickson, 1972; Zingmark and Miller, 1973); 2) chemical factors such as the degree of chelation, and nutrient and dissolved oxygen concentrations (Corner and Sparrow, 1956; Fitzgerald and Faust, 1963; McBrien and Hassal, 1967; Erickson

et al., 1970; Lewis et al., 1973; Nelson and Colwell, 1975;
Overnell, 1975a); and 3) biological factors such as size of
the inoculum (Steemann Nielsen et al., 1970; Shieh and Barber,
1972; Ben-Bassat and Mayer, 1975; Overnell, 1976), and the
physiological state of cells (Gibson, 1972; Zingmark and
Miller, 1973).

Of all the potential factors capable of influencing metal
toxicity, the chemical composition of the growth medium can be
the most difficult factor to define and control. The choice
of an appropriate medium relies both on the specific requi-
rements of the test organisms and the type of study being
conducted. The inhibitory levels determined in chelator-rich
media cannot be compared to those found in chelator-free media
(Jensen et al., 1976; Kayser, 1976; Overnell, 1976).

It is well established that the addition of natural and
synthetic chelating agents can complex trace metals (Gardiner,
1976) and influence their uptake (Cossa, 1976; Schulz-Baldes
and Lewin, 1976; George and Coombs, 1977). Natural seawater
contains undefined complexing agents such as humic and fulvic
acids (Singer, 1973) in different amounts. Lewis et al.,
(1971; 1973) found that EDTA-enriched natural seawater varied
in its ability to support growth of young stages of a copepod,
presumably because of the presence of natural complexing
agents. In cultures of Fragilaria and Asterionella with soil
extract additions, mercurials were significantly less toxic
than in completely defined medium (Tompkins and Blinn, 1976).
The presence of decomposed natural plankton and detritus

increased Cu tolerance in T. pseudonana (Erickson, 1972). The absolute growth rates of marine phytoplankton were lower in mid-winter seawater in spite of higher nutrient levels (Jensen et al., 1974). Chelated iron counteracted the effects of Hg on C. pyranoidosa (Kamp-Nielsen, 1971) and on three coastal diatoms (Jensen et al., 1976). The addition of EDTA increased the tolerance of P. tricornutum, when the molar ratio of EDTA : Cu >1 (Bentley-Mowat and Reid, 1977), and the growth rates of T. pseudonana which was previously inhibited by Cu (Erickson, 1972). In Chlorella vulgaris and Oocystis, EDTA had both stimulatory and adverse effects (Fitzgerald and Faust, 1963). Although alkali ions could alleviate Cu toxicity (Overnell, 1975a), they failed to neutralize Hg inhibition (Steemann Nielsen and Wium-Andersen, 1971).

Heavy metal toxicity was intimately connected with nutritional stress in algae (Hannan and Patouillet, 1972; Hannan et al., 1973) and in Daphnia (Winner et al., 1977). In nutrient poor waters, even $3.0 \ \mu g \cdot l^{-1} Hg$ arrested the growth of Chlorella (Stokes et al., 1973). A toxic Hg threshold (60 $\mu g \cdot l^{-1}$) was demonstrated for a summer phytoplankton assemblage but not for the spring population (Blinn et al., 1977), possibly due to the combined effects of lower nutrient levels and higher temperatures during the summer. Low nutrient levels decreased the normal excretion rate of dissolved organics (Betz, 1977), thus resulting in an increase in Hg toxicity. These compounds may be involved in Hg volatilization (Ben-Bassat and Mayer, 1977; 1978).

2.2 Speciation of Mercury

In phytoplankton, organomercurials are more toxic than inorganic and elemental Hg. The ^{14}C uptake of Nitzschia delicatissima was reduced by 50% with: 1 $\mu g \cdot l^{-1}$ phenyl mercuric acetate (PMA); 0.5 $\mu g \cdot l^{-1}$ methyl mercuric dicyanidiamide and MEMMI[1]; and 10 $\mu g \cdot l^{-1}$ diphenyl mercury (Harriss et al., 1970; White, 1970). The potassium content and O_2 evolution of D. tertiolecta (Overnell, 1976) and the lipid synthesis in freshwater algae (Matson et al., 1972) were more depressed by methylmercury than $HgCl_2$. Even though PMA was more toxic than $HgCl_2$, the accumulation of Hg in the presence of PMA was less than with $HgCl_2$ in Chlorella (De Filippis and Pallaghy, 1976a), implying that toxicity is not always due to the lipoid solubility of organomercurials. Cultures of aerobic heterotrophic bacteria were more resistant to PMA than $HgCl_2$ (Nelson and Colwell, 1975). Hannan and Patouillet (1972) reported that $HgCl_2$ was more toxic to various algae than dimethylmercury. The primary productivity of Fragilaria crotonensis and Asterionella formosa was more affected by $Hg(NO_3)_2$ than $HgCl_2$ (Blinn et al., 1977).

----

[1] N-methylmercuric-1,2,3,6-tetrahydro-3,6-methano-3,4,5,6,7,7-hexachlorophthaliamide.

Section 3. <u>Accumulation of Metals</u>

Some aspects of the exchange kinetics between metals and
algae have been elucidated (Glooschenko, 1969; Davies, 1974;
1976; Fujita and Hashizume, 1975). Metal accumulation is
principally influenced by the species of the metal, the alga
and its physiological state. The uptake varies as a function
of the time and concentration of exposure (Shieh and Barber,
1972; Davies, 1974; Cossa, 1976). Mercury accumulation was
greater in dividing than non-dividing cells (Burkett, 1975;
Richardson <u>et al</u>., 1975) and also greater in dead and moribund
cells than live cells probably due to the cessation of active
excretion of mercury (Fujita and Hashizume, 1975; Bentley-
Mowat and Reid, 1977).

Metal accumulation is usually accomplished in two phases.
The first one consists of a Freundlich adsorption isotherm
which is immediate, rapid and passive. The metal adsorbs onto
cell surfaces until a definite number of sites are saturated
(Davies, 1974; 1976; Dolar <u>et al</u>., 1971). This adsorption
phase is followed by an active but slower uptake, during which
the metal is translocated across the cell membrane (Shieh and
Barber, 1972; Schulz-Baldes and Lewin, 1976). This phase of
uptake was temperature dependent in <u>Chlorella</u> (De Filippis and
Pallaghy, 1976c). During accumulation, a simultaneous
exchange of metals occurs between algae and the medium (Ben-
Bassat and Mayer, 1977; 1978; Betz, 1977). Excreted Hg can
either be inorganic (Betz, 1977) or organic, since Hg may be

released as a compound complexed with a natural metabolite, capable of reducing Hg^{+2} to Hg^0 (Ben-Bassat and Mayer, 1978).

The incorporation of metal into the cell is rapid. In _Synedra ulna_, Hg was deposited on thylakoid surfaces and then transferred into the cytoplasm (Fujita _et al._, 1977). In _Anacystis nidulans_, Hg was associated with a phycocyanin-rich fraction and with 60,000, 180,000, and 230,000 m.w. fractions derived from peripheral and intrinsic lamellar components (Hammans _et al._, 1976). In ^{203}Hg-labelled plant tissues, Hg granules were almost exclusively sequestered within nucleoli and between spaces occupied by chromatin (De Filippis and Pallaghy 1975).

Section 4. _Effects of Mercury_

The inhibitory levels of mercurials on algal growth (Ben-Bassat _et al._, 1972; Hannan and Patouillet, 1972; Tompkins and Blinn, 1976), photosynthesis and respiration (Zingmark and Miller, 1973; De Filippis and Pallaghy, 1976a) have been determined for a number of defined conditions.

The effects of various mercurials on phytoplankton are summarized in Table I. In general, diatoms prove to be more sensitive to heavy metals than dinoflagellates (Kayser, 1977). Out of seven species, _S. costatum_ was especially sensitive to Hg (Overnell, 1976). _Gymnodinium splendens_ was the most sensitive to $Hg(CH_3COO)_2$ followed by _Scrippsiella faeroense_ and _Prorocentrum micans_ (Kayser, 1976). Freshwater

TABLE I. Literature summary of the effects of mercurials on phytoplankton.

TEST ORGANISM	CELLULAR PROCESSES	EFFECTS	MERCURIALS	CONCEN-TRATIONS (μM)	REMARKS	REFERENCE
DIATOMS:						
Asterionella formosa	g.r. "	p. inh. f. "	HgCl$_2$ "	0.921 1.842	batch "	Tompkins and Blinn, 1976.
Fragilaria crotonensis	" "	stim. f. inh.	" "	0.184 0.368	" "	
Chaetoceros sp. *Cyclotella sp.* *Phaeodactylum tricornutum*	g.r. " "	inh. " "	HgCl$_2$ " "	0.368 " "	batch " "	Hannan and Patouillet, 1972.
Skeletonema costatum	g.r.	inh.	HgCl$_2$	0.002	chemostat	Rice et al., 1973.
	g.r. & P/S C:N ratio	inh. unchanged	HgCl$_2$ "	0.018-0.037 "	batch "	Berland et al., 1977.
	P/S	50% inh.	HgCl$_2$	2.500	batch	Overnell, 1976.
Nitzschia delicatissima	P/S "	50% inh "	MEMMI[1] methyl dicyanidiamide PMA diphenyl Hg	0.5 $\mu g \cdot l^{-1}$ 0.5 " 1.0 " 10.0 "	batch 24 h treatment	Harriss et al., 1970.
DINOFLAGELLATES:						
Amphidinium carterae *Gymnodinium splendens*	g.r. g.r. & P/S " " "	p. inh. f. inh. p. " f. " f. "	HgCl$_2$ Hg(CH$_3$OO)$_2$ " " "	0.004 0.184-1.842 1.842 0.368 0.368	turbidostat (added once) " (daily addition) " batch	Zingmark and Miller, 1973. Kayser, 1976.
Prorocentrum micans	g.r. & P/S " " " "	p. inh. f. " p. " f. " p. "	Hg(CH$_3$OO)$_2$ " " " "	0.368 1.842-3.683 0.184-0.368 0.368 0.037	turbidostat (added once) (daily addition) " batch "	
Scripsiella faeroense	g.r. & P/S " " "	f. inh p. " f. " p. "	Hg(CH$_3$OO)$_2$ " " "	3.683 1.842 3.683 0.037-0.184	turbidostat (added once) " batch "	
NATURAL POPULATIONS						
Marine phytoplankton	primary productivity	p. inh.	HgCl$_2$	1.842		Knock and Mason, 1971.
	P/S	50% inh.	HgCl$_2$	0.004		Zingmark and Miller, 1973.
	P/S "	inh. "	HgCl$_2$ methyl Hg	0.018 0.5 $\mu g \cdot l^{-1}$		Knauer and Martin, 1972.
North Sea coastal plankton	g.r.	p. inh.	HgCl$_2$	0.006-0.018	*in situ* enclosure	Kuiper, 1976.
Lake phytoplankton	P/S " "	40% inh. 85% " stim.	HgCl$_2$ " "	0.221 3.683 0.048-0.107	*in situ* enclosure " "	Blinn et al., 1977.

GREEN ALGAE:						
Scenedesmus sp.	g.r.	inh.	$HgCl_2$	0.110	batch	Bringham and Kuhn, 1959.
S. dimorphus	g.r.	unchanged f. inh.	$HgCl_2$ methyl Hg	0.037 10 ug l^{-1}		Maltida *et al.*, 1971.
Chlamydomonas reinhardtii	g.r.	f. inh.	$HgCl_2$	7.366	batch, highly chelated medium	Ben-Bassat *et al.*, 1972.
Chlorella pyrenoidosa	g.r. K$^+$ efflux	p. inh. stim.	$HgCl_2$ "	0.368-1.050	batch	Kamp-Nielsen, 1971.
	dark respiration "	stim. inh.	$HgCl_2$ "	30.000-81.030 500.000	batch	Shieh and Barber, 1972.
Chlorella (Emerson strain)	g r.	unchanged	$HgCl_2$	1.000	batch	De Filippis and Pallaghy, 1976a.
	biomass	stim.	PMA	0.1 μM	"	
	PAR P/S respiration DNA, RNA, protein "	stim. p. inh. " stim. "	$HgCl_2$ " " " " PMA	1.000 " " " " 0.1 μM	" " " " "	
Dunaliella tertiolecta	P/S " K$^+$ efflux "	inh. " stim. "	$HgCl_2$ $(CH_3)_2Hg$ " $HgCl_2$	0.037-0.368 0.1-1.0 0.368-3.683	semi-continuous cultures, 10-15 min exposure	Overnell, 1975a.
HAPTOPHYTE	g.r.	84% inh.	$HgCl_2$	10.000	batch	Davies, 1974; 1976.
Isochrysis galbana	g. " "	p. inh. f. "	" "	7.500 10.500	" "	

g.r.= growth rate; P/S= photosynthesis; inh.= inhibition; f.= full; p.= partial; stim.= stimulation
1: this chemical is defined on page 7

phytoplankters are usually more tolerant than their marine counterparts and nanoplankters are frequently the most resistant. When Cu was added to a large in situ enclosure, large centric diatoms (e.g., Chaetoceros) and dinoflagellates disappeared, whereas, microflagellates and pennate diatoms (e.g., N. delicatissima) survived and were responsible for the recovery of productivity (Thomas and Seibert, 1977).

Teratological studies suggest that developing or metabolically active stages are more susceptible to metal intoxication (Pyefinch and Mott, 1948; Wisely and Blick, 1966). In A. nidulans, stationary phase cells were less vulnerable to Hg poisoning than logarithmically growing cells (Hammans et al.,1976), probably due to the presence of metabolites in the growth medium. During the lag phase, the growth medium is conditioned by excretion from live cells and dissolved organics leaching from dead and moribund cells. These products may either complex with toxic metallic ions or stimulate volatilization (Zingmark and Miller, 1973; Davies, 1974; Kayser, 1976; Ben-Bassat and Mayer, 1977;1978; Betz, 1977). Mercury was less toxic when added during the log phase than concurrently with the inoculation of cells (Zingmark and Miller, 1973).

The effects of mercurials on photosynthesis are species specific. Mercuric chloride inhibited plastocyanin activity in isolated chloroplasts (Kiminura and Katok, 1972) and $Hg(CH_3COO)_2$ degraded phycocyanin in A. nidulans (Hammans et al., 1976). In Chlamydamonas reinhardtii, Hg interfered

with PS I and PS II reactions (Overnell, 1975b). Although $\underline{S}$. $\underline{costatum}$ was the most sensitive alga tested, 2.5 µM $HgCl_2$ reduced photosynthesis by only 50% (Overnell, 1976).

An investigation on $\underline{C}$. $\underline{pyranoidosa}$ pointed to excretion of potassium ions as a primary effect of Hg poisoning (Kamp-Neilsen, 1971). Sublethal levels of heavy metals caused potassium leakage at concentrations similar to or greater than those required for the inhibition of photosynthesis in $\underline{D}$. $\underline{tertiolecta}$ and $\underline{P}$. $\underline{tricornutum}$ (Overnell, 1975a; Davies, 1976). In $\underline{Ankistrodesmus}$ $\underline{braunii}$ and $\underline{Euglena}$ $\underline{gracilis}$, Hg inhibited galactosyl transferase activity, galactolipid and chlorophyll syntheses (Matson $\underline{et}$ $\underline{al}$., 1972). The same inhibition appeared to occur in $\underline{Fragilaria}$ and $\underline{Asterionella}$ species (Tompkins and Blinn, 1976). Galactolipids are one of the major chloroplast lipids. In $\underline{Chlorella}$, $HgCl_2$ stimulated DNA and RNA syntheses while PMA initially decreased DNA, RNA and protein syntheses. Both mercurials had a similar effect on biovolume and excretion (De Filippis and Pallaghy, 1976a). In $\underline{S}$. $\underline{costatum}$, (Berland $\underline{et}$ $\underline{al}$., 1977) and natural populations (Thomas $\underline{et}$ $\underline{al}$., 1977), the C:N ratio remained constant despite a reduction in growth and photosynthesis.

In metal-treated algae, morphological alterations have occurred in $\underline{P}$. $\underline{tricornutum}$ and $\underline{Chlorella}$ (Nuzzi, 1972), and in $\underline{Thalassiosira}$ isolated from Hg-treated $\underline{in}$ $\underline{situ}$ enclosures (D. Seibert, personnal communication). $\underline{Ditylum}$ $\underline{brightwellii}$ underwent osmotic disturbances resulting in considerable swelling (Bentley-Mowat and Reid, 1977). After 12-14 days of

exposure to 50-100 $\mu g \cdot l^{-1} HgCl_2$, colonies of A. formosa deviated from their normal stellate configuration to form large cylindrical stacks of up to 30 colonies, agglomerated by mucilagenous secretions (Tompkins and Blinn, 1976). Cell volumes of I. galbana almost doubled at 10.5 μM $HgCl_2$ probably due to the impairment of methionine production, which is involved in cell division (Davies, 1974). The dinoflagellate, S. faeroense, responded to $Hg(CH_3COO)_2$ by the bursting of thecae, releasing of naked, motile cells, and formation of vegetative resting stages (Kayser, 1976).

Section 5. Mercury Resistance

The tolerance of certain algae to elevated metal levels has been assessed in comparative studies (e.g., Bentley-Mowat and Reid, 1977). Tolerance may be acquired by the development of an exclusion mechanism (Davies, 1976), by volatilization of Hg from the medium (Ben-Bassat and Mayer, 1975), or by an acclimation response (Stokes et al., 1973; Stockner and Antia, 1976; Say et al., 1977). Tolerance may also be innate, as shown by Pediastrum boryanum which could survive and reproduce at 1 $mg \cdot l^{-1}$ $HgCl_2$ in spite of a cellular concentration factor of 1.77 x 10^{14} (Richardson et al., 1975). Differences in threshold levels for F. crotonensis and A. formosa correlated with surface to volume ratios (Blinn et al., 1977).

Tolerance to heavy metals can be acquired following recovery from initial metal inhibition (Kamp-Nielsen, 1971;

Ben-Bassat et al., 1972; Fujita and Hashizume, 1975; Davies, 1976; De Filippis and Pallaghy, 1976a; 1976b; Berland et al., 1977).

Resistance due to volatilization of Hg is common in microorganisms. In Staphylococcus aureus (Weiss et al., 1977), Pseudomonas aeruginosa and P. puteda (Clark et al., 1977), recovery from the initial inhibition of PMA was mediated by the phenylmercury hydrolysis of PMA to benzene and Hg^{+2} and the reduction of Hg^{+2} to Hg^0 by mercuric reductase followed by a rapid volatilization of Hg^0 from the medium. The same process may have occurred in Hg-resistant marine bacteria (Nelson et al., 1973). Volatilization was genetically controlled in E. coli and Aerobacter aerogenes (Komura and Kaziro, 1971; Summers and Lewis, 1973). Cells of Chlorella, which became Hg-resistant, reestablished normal growth rates and were more efficient in Hg^0 volatilization than Hg-sensitive cells (De Filippis and Pallaghy, 1976c). The cell extract from Chlorella contained a natural, low molecular weight, non-enzymatic, light-induced, reducing compound capable of diffusing Hg^0 from the spent medium (Ben-Bassat and Mayer, 1977; 1978). Pretreatment of Chlorella with 10-20 μM $HgCl_2$ prevented DCMU inhibition of light-induced Hg^0 volatilization due to competition between $HgCl_2$ and DCMU, while an uncoupler of PS I, methylamine, transiently stimulated O_2 evolution and volatilization (Ben-Bassat and Mayer, 1978).

Section 6. Assessment of Experimental Design

Two basic experimental approaches have been used in pollution studies to determine the effects of pollutants. Bioassay organisms have been exposed to acute (lethal) and chronic (sublethal) levels over short to long time periods.

Batch cultures have been intensively used and only a few studies were conducted in natural aquatic systems in an attempt to determine, the effects of pollutants on natural populations (Kuiper, 1976; Blinn et al., 1977) and their ecological repercussions (Dunstan and Menzel, 1971; Gibson et al., 1975; Grice et al., 1977).

With batch cultures, all essential growth-promoting elements and toxic substances are added at once and the cultures grow logarithmically until the exhaustion of nutrients occurs. Since the duration of the bioassay is usually short, batch culture bioassays may overestimate toxicity. The observed toxic levels may be misleadingly high because the density and physiological state of the population vary with the utilization of the most limiting nutrient. For example, tolerance to Cu varied by a factor of 30 in Nitzschia palea grown in batch cultures (Steemann Nielsen and Wium-Andersen, 1971).

However, batch cultures are useful in estimating toxic ranges, screening for different mercurials (Harriss et al., 1970; Nuzzi, 1972) or suitable test organisms (Overnell, 1976), and determining the action of a toxicant on various

metabolic processes.

A few studies have been done using continuous cultures such as a turbidostat (Kayser, 1976), or a chemostat (Rice et al., 1973). In the chemostat, algal growth rate is controlled by the rate of medium inflow or dilution rate (Harrison et al., 1976). Culture volume, cell density and cellular chemical composition are relatively constant for a specific dilution rate. Experimental time is theoretically unlimited and variables, which make the assay more sensitive, are easily controllable (e.g., nutrients and mode of metal exposure). This technique is suitable for the maintenance of nutrient-limited populations and may be more realistic than batch cultures because phytoplankton of the upper photic zone are often nutrient-stressed, especially during the summer months. In the future, this mode of culturing may prove more useful in assessing and understanding the effects of lower levels of metals.

Section 7. Purpose of this Study

In general, there is a lack of research documenting the effects of environmentally encountered mercury levels. Moreover, since most investigations have used concentrations of 3 to 6 orders of magnitude higher than the concentrations used in this study, the short-term terminal rather than the long-term subtle effects of mercury have been examined (Table I).

In the past, a myriad of studies have determined the

effects of mercurials on different cellular processes and/or the effects of some factors influencing toxicity. A few studies reported the effects of heavy metals on biomass parameters in continuous cultures (Rice et al., 1973; Kayser, 1976; Bentley-Mowat and Reid, 1977) and some briefly related tolerance to nutrients but under saturated conditions (Hannan et al., 1973; Morel et al., 1978).

Nutrient limitation can limit primary production and under these conditions of nutrient deficiency, chances of survival will depend on the nutrient uptake kinetic responses of the nutrient-stressed populations. The ability of a species to increase the uptake affinity for the limiting nutrient will favour the chances of successful interspecific competition. However, the imposition of a secondary stress (pollution) on a population that is already nutrient-stressed may impair this competitive ability.

Recovery in algae previously inhibited by heavy metals, may be attributed to several factors. One possible reason for the recovery from inhibition may be the production of complexing agents such as metallothionein. This was investigated with different levels of mercury exposure.

Thus, it appears that this work is the first study which documents the effects of long-term mercury exposures, at nearly ecological levels, on nutrient-limited diatoms, grown in continuous cultures (chemostats).

CHAPTER II

EFFECTS OF SHORT AND LONG-TERM EXPOSURES TO SUBLETHAL
LEVELS OF Hg ON NUTRIENT KINETICS

Section 1. Introduction

Nutrient uptake kinetics in phytoplankton can be des-
cribed in terms of the following equation which is similar to
the Michaelis-Menten equation for enzyme kinetics:

$$(1) \quad V = V_{max} \cdot S \ (K_s + S)^{-1}$$

where V = rate of nutrient uptake (hr^{-1}); V_{max} = maximum rate
of uptake (hr^{-1}); S = concentration of the limiting nutrient
(μM), and K_s = half-saturation constant (μM), the value of S
when $V = V_{max}/2$. Values of K_s are species specific and a low
K_s value indicates that a species has a high capacity or
affinity to take up a limiting substrate across the cell
membrane. Newer terminology associated with the disappearance
and the rate of uptake of the limiting nutrient has been
described by Conway et al. (1976) and it is presented in the
following paragraphs.

In nutrient-limited cultures, uptake and assimilation of
the limiting nutrient usually occurs in 3 phases (Fig. 1).
The first phase consists of a rapid rate or surge uptake, V_s,
which appears to represent the transport of the nutrient
across the cell membrane and into an internal pool. It occurs
over the time period T_s. During the second phase, the rate of
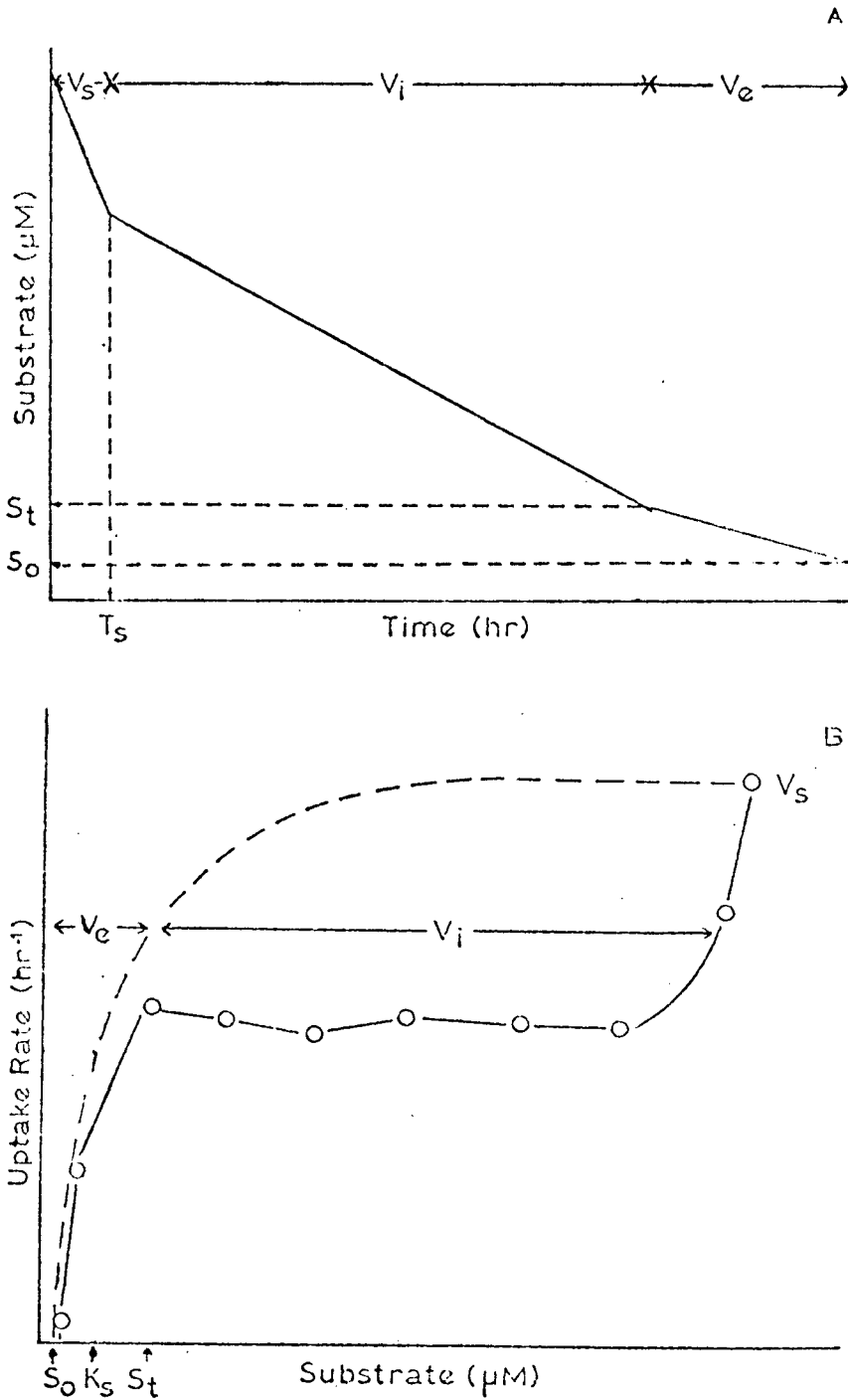
FIGURE 1. Nutrient uptake kinetic terminology. (A) The disappearance of the limiting nutrient with time. (B) The rate of uptake of the limiting nutrient as a function of the substrate. (From Conway et al., 1976). Symbols are defined in Section 2.1

uptake ,V_i, decreases as the internal pool becomes full. At this point, the rate of assimilation of the limiting nutrient, V_i, (the mobilization from the internal pool into larger molecules via assimilatory enzymes) becomes the rate limiting step controlling the uptake rate. During the third phase, the external substrate concentration is low. The internal pool, containing the limiting nutrient, becomes gradually depleted as the rate of assimilation exceeds the uptake rate. In this phase, the uptake rate, V_e, is under external control since it is limited by the low external concentration of the limiting nutrient in the medium. Thus, the three phases involved in the translocation of the substrate across the cell membrane are V_s, V_i and V_e.

The substrate concentration at the juncture between the two phases of uptake, V_i and V_e, is defined as S_t and indicates the beginning of the next phase, V_e. During the third phase of uptake, V_e may cease when the concentration of the limiting nutrient is completely or partially depleted. In the latter case, the residual concentration of the limiting nutrient still remaining is defined as S_o. The actual half-saturation constant (actual K_s) is determined by adding the apparent half-saturation constant (apparent K_s) and the S_o value. The apparent K_s is calculated by assuming that $S_o = 0$.

In this study, V_{max} was estimated using V_s. The value of V_s, which represents only one datum point on the uptake hyperbola (see dashed line in Fig. 1B), probably under-estimates the true value of V_{max}. The maximum assimilation

rate, Vi_{max}, was estimated by fitting an hyperbola to the Ve and Vi data.

Section 2. Materials and Methods

2.1 Inoculum

Chemostat cultures were inoculated with an unialgal culture of Skeletonema costatum (Grev.) Cleve (Northwest Pacific Culture Collection, #18, University of British Columbia), isolated from Patricia Bay, British Columbia, Canada.

2.2 Chemostat Cultures

All glassware was autoclaved and culture medium was filtered through a 0.22 μm Millipore filter in an attempt to keep bacteria at negligible levels. S. costatum was grown in ammonium-limited chemostats in artificial seawater (Appendix A) made from reagent grade salts and enriched with constituents of 'f' medium (Appendix A). Concentrations (μM) of the major nutrients in the inflow medium were: NH_4Cl, 10.0 (f/100); Na_2SiO_3, 35.3 (f/3); KH_2PO_4, 3.68 (f/20). Concentrations (nM) of trace metals (f/20) and vitamin (f/20) were: $CuSO_4$= 3.93; $ZnSO_4$= 7.65; $CoCl_2 \cdot 6H_2O$= 4.39; $MnCl_2 \cdot 4H_2O$= 0.91; $Na_2MoO_4 \cdot 2H_2O$= 2.61 and vitamin B_{12} = 0.05 μg·l⁻¹. Iron and EDTA were added as ferric sequestrene (1.17 μM).

The chemostat system was previously described (Davis

et al., 1973). Continuous cultures were grown in borosilicate, 2 l and 6 l flat bottom boiling flasks (Pyrex) maintained at 17 ± 0.5 °C. Flasks were not coated with silicone (Siliclad, Clay Adams) since preliminary results indicated that adsorption of Hg onto walls was not decreased and no silicate leached from the inner walls of the culture flasks. The cultures were continuously stirred with teflon covered magnetic stirring bars at 120 rpm and were maintained without aeration to avoid volatilization. They were also under continuous illumination (50 $\mu E \cdot m^{-2} \cdot s^{-1}$). Spectral distribution from daylight fluorescent lamps (Sylvannia Powertube, VHO and Duro-Test, UHO) was corrected by using a 0.3 cm thick Plexiglas sheet (# 2069 Rohm and Hass), in an attempt to simulate 5 m underwater light under sunny conditions for the Jerlov type 3 coastal water (Holmes, 1957).

2.3 Analyses

Growth was monitored by following changes of in vivo fluorescence using a Turner Model 111 fluorometer and of cell density using an inverted light microscope. Length and width of cells were measured using an ocular micrometer. Nutrient analyses were performed using a Technicon AutoAnalyzer®. The ammonium method was basically that of Koroleff (1970) as automated by Slawyk and MacIssac (1972). The methods for silicate and phosphate followed the procedures of Armstrong et al., (1967) and Murphy and Riley (1962), respectively. Both

methods were automated by Hager et al., (1969).

The ^{14}C technique was used for measuring the rate of photosynthesis (Strickland and Parsons, 1972). Five μCi of NaH[14]CO_3 (New England Nuclear, Boston) were added to 100 ml of chemostat effluent, mixed, separated into two, 50 ml bottles and incubated for 2 hours under original conditions. No dark incubation was conducted. Cells were collected onto a 25 mm diameter 0.45 μm Millipore filter. Filters were dissolved in scintillation cocktail (Scintiverse, Fisher Scientific Co. Ltd.) and counted for radioactivity with a Unilux III Nuclear Liquid Scintillation System, Nuclear Chicago.

Chlorophyll a was determined by the trichromatic method (Strickland and Parsons, 1972). Two hundred and fifty ml of chemostat effluent were filtered onto a glass fiber filter covered with 1 ml of a saturated MgCO_3 solution. Pigments were extracted for 20 hours in cold, dark conditions in 90% acetone. Cells were homogenized, centrifuged and the absorbance spectrum (750, 665, 663, 645, 630 and 430 nm) of the supernatants for chlorophylls, carotenoids and phaeophytins were determined using a Perkin-Elmer Model 124D double beam spectrophotometer. Phaeophytin a was measured by adding 3 drops of 10% HCl to the above supernatants and reading the absorbances at the same wavelenghts.

For the Hg analysis, 200 ml of effluent were filtered onto a 47 mm diameter 0.45 μm Millipore filter. Filters and filtrates were analyzed for particulate and soluble Hg, respectively. These Hg fractions were measured by a cold

vapor method (Hatch and Ott, 1968) using a flameless atomic absorption spectrophotometer (Pharmacia U.V. Control and Optical Units, Model 100). Mercurials were oxidized via a $H_2SO_4/KMnO_4/K_2S_2O_8$ digestion and reduced by the reagent $(NH_4OH)_2 \cdot H_2SO_4 + NaCl$. This was followed by the addition of $SnSO_4$ resulting in the release of elemental Hg vapour. This analytical technique does not differentiate between the various species of inorganic and organic Hg. The detection limits of the method were 0.18 to 5.52 nM Hg (0.05-1.50 $\mu g \cdot l^{-1}$). All reported Hg concentrations are a mean of duplicate samples.

The concentration of $HgCl_2$ during any time interval (equation 2) and the average concentration during the entire experiment (equation 3) were computed as follows:

$$(2) \qquad C_{n+1} = C_n \; e^{-D \, (t_{n+1} - t_n)} + C_0$$

$$(3) \qquad \bar{C} = \sum (C_{n+1} - C_n / D) \; (T^{-1})$$

where n = number of time intervals; C_{n+1} = concentration at t_{n+1} (nM); C_n = concentration at t_n (nM); C_0 = added concentration (nM); D = dilution rate (hr^{-1}); $t_{n+1} - t_n$ = time intervals between two additions (hr); $\bar{C}$ = average concentration (nM) during the entire experiment and T = time of the entire experiment (679.5 hr). Since in these calculations losses of Hg are only due to the dilution rate, these calculated Hg concentrations are referred to as the 'expected' Hg concentrations.

The specific growth rate (μ) was computed using the following equation (Davis et al., 1973):

$$(4) \quad \mu = D + (1/t) \ln (x_{n+1}/x_n)$$

where μ = specific growth rate (hr^{-1}); D = mean dilution rate over the time interval (hr^{-1}); x_{n+1} and x_n = cell densities at times t_{n+1} and t_n (10^7 cells·l^{-1})

Variations in the analyses used in these experiments are presented in Table II. Subsamples of chemostat culture were used to determine the standard deviation, except for the Hg analyses.

2.4 Experimental Design

Batch cultures were used to determine the range of sublethal concentrations of HgCl$_2$ capable of inhibiting the growth rates of nutrient-saturated cultures. Prior to inoculation, cells were centrifuged and resuspended in artificial seawater to avoid a transfer of complexing agents into the fresh medium. The medium was enriched with 'f/25' and the source of nitrogen was nitrate.

In the short-term Hg-exposure, a 6 l chemostat culture was grown under ammonium limitation with a dilution rate of 0.04 hr^{-1}. The dilution rate was determined using the following equation:

$$(5) \quad D = F \cdot V^{-1}$$

where F = flow rate (ml·hr^{-1}) and V = volume of the culture (ml). Chemostat samples were analyzed daily for fluorescence, cell density and nutrient concentrations. A steady-state was reached when no trend in these parameters was observed for

TABLE II. Standard deviation for analyses used in the short and long-term mercury exposure experiments.

Parameter measured	Mean s.d.	Units	%	n
Cell numbers[1]	1.37 ± 0.33	10^7 cells·1^{-1}	24	10
Fluorescence[1*]	45.59± 1.56	relative units	3	22
Chlorophyll a	22.19 ± 2.85	μg chl a·10^7 cells^{-1}	13	5
"			10*	18
	5.09 ± 0.30	μg C·10^7 cells^{-1}·hr^{-1}	6	10
			7*	30
Ammonium	2.0 ± 0.15	μM	8	10
Hg analysis[2] particulate Hg	20.0 ±1.20	ng l^{-1}	6	6
soluble	3.68 ± 0.22	nM Hg	6	6

% - standard deviations expressed as a percentage of the mean
[1]: also see standard deviations in Appendix B and Figure 3.
[2]: used n/2 sets of standards done on different days. The standard deviations were calculated and expressed as a percentage of the mean. The later percentage was converted into the units used for each type of analysis. The highest standard deviation (6%) was used in this table.
* :the standard deviation is expressed as a mean of the standard deviation of n/2 sets of duplicate measurements.

several days. Then 500 ml of chemostat effluent was collected and simultaneously perturbed with 5 µM NH_4Cl and one of the following concentrations of $HgCl_2$: 0.37, 1.84, 3.68, or 5.53 nM. In an attempt to increase the toxic effect of $HgCl_2$ on nutrient uptake, 1 l of effluent was collected for 12 hours and allowed to starve for 24 hours such that the average starvation period was 30 hours. Then it was perturbed as described above, with 5 µM NH_4Cl and one of the following concentrations of $HgCl_2$, 0.18, 0.37, 1.84 or 3.68 nM.

Details of the batch mode perturbation technique have been described (Caperon and Meyer, 1972). Basically, the technique consisted of quickly injecting $HgCl_2$ and NH_4Cl simultaneously into the effluent and mixing thoroughly. Ammonium determinations were made as frequently as every 6 minutes in order to closely follow the disappearance of ammonium from the medium. Phosphate and silicate were not added along with ammonium since their concentrations in the chemostat effluent were sufficient to ensure no limitation during the perturbation experiment. From the experiments using short-term Hg exposure, it appeared that semi-continuous additions of 3.68 nM $HgCl_2$ over a long period of time could affect ammonium-limited cells. In designing the long-term Hg exposure experiment, a lower concentration was also used in case the effects of 3.68 nM $HgCl_2$ were too severe.

In the long-term experiment, a 6 l chemostat culture (D= 0.039 ± 0.005 hr^{-1}) was divided into three, 2 l chemostat cultures operating at the following nearly identical dilution

rates: I= 0.041 ± 0.002 hr^{-1}; II= 0.040 ± 0.002 hr^{-1}; III= 0.039 ± 0.001 hr^{-1}. These dilution rates were chosen, such that small variations in the dilution rate would not change the concentration of ammonium in the culture medium. The original steady-state was maintained for another 2 days to ensure that the cultures were not disturbed by the splitting of the 6 l chemostat culture, and then Hg additions were started. For 30 days, cultures I and III were semi-continuously exposed to 0.37 and 3.68 nM HgCl$_2$, respectively. A HgCl$_2$ reference solution (3.68 mM, or 1000 mg·l^{-1}, Fisher Scientific Co. Ltd.) was diluted to form substock solutions of 0.37 and 3.68 μM which were made fresh every fourth day. A total of two ml of each HgCl$_2$ substock was injected through the inflow port of each chemostat cork. Generally, the additions were made every 8 hours during the first 10 days and every 12 hours for the rest of the experiment and exceptions are noted on Fig. 6 in section 2.3. The control, culture II, received an equivalent volume of distilled deionized water.

Chemostat effluent was collected for 12 hours, (i.e., starved for an average period of 6 hours), and used for ^{14}C uptake experiments, cell counting and Hg analysis. The remaining effluent was used for a perturbation experiment conducted 5 hours later, resulting in an average cell starvation period of 11 hours. Five hundred ml of effluent were collected for chlorophyll a determination.

Three perturbation experiments (chemostat effluents I, II, and III) were performed simultaneously and nutrient con-

centrations were determined every 6 minutes during the initial 18-30 minutes, and then every 18 minutes for the rest of the perturbation experiment.

The uptake kinetic parameters, apparent Ks and Vi_{max} were computed using the equations outlined by Conway et al., (1976) and statistically evaluated using the methodology of Cleland (1967).

Section 3. Results

3.1 Results from some Preliminary Studies

The effects of Hg on S. costatum were first investigated using nutrient-saturated batch culture assays to determine a range of sublethal concentrations (Fig. 2). Additions of 0.37 to 7.37 nM $HgCl_2$ gradually decreased the in vivo fluorescence while only 3.68 and 7.37 nM decreased the maximum growth rate without inducing a lag period. After 88 hours of exposure to $HgCl_2$, final cell densities were gradually reduced from 2.50 to $0.30 \cdot 10^7$ cells$\cdot l^{-1}$ over a sublethal concentration range of 0.37 to 7.37 nM $HgCl_2$, respectively.

3.2 Growth Phases during the Long-term Mercury Exposure

The original data including dilution rate, in vivo fluorescence, cell numbers, specific growth rate, NH_4^+ effluent concentration, chlorophyll a, carotenoid:chlorophyll
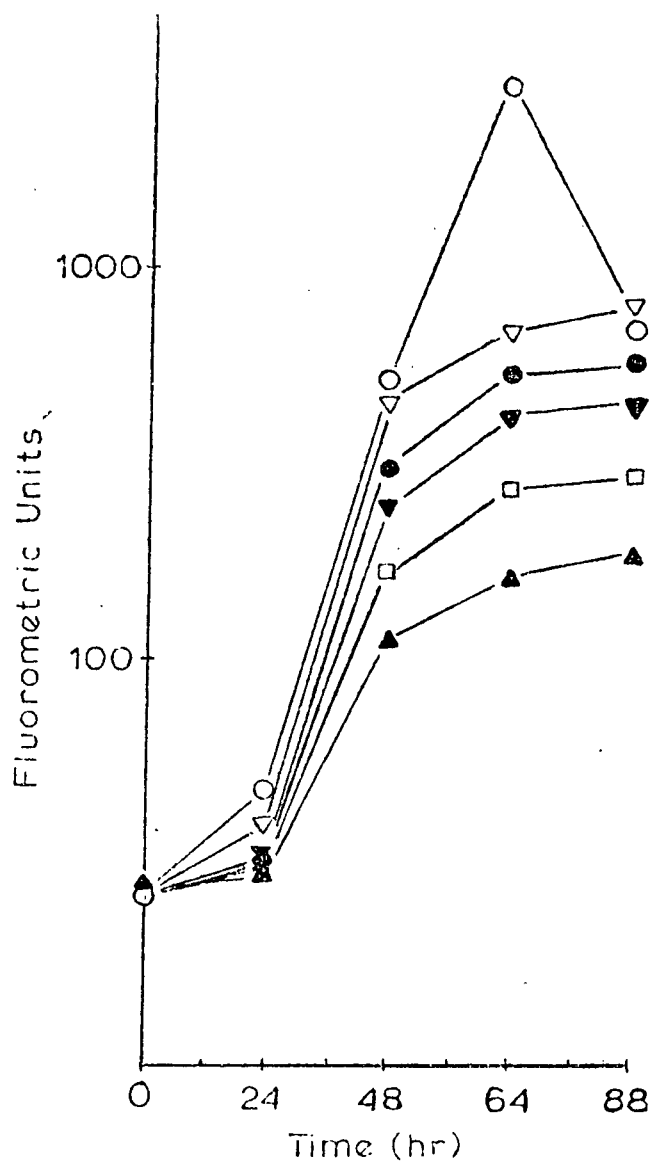
FIGURE 2. Changes in in vivo fluorescence in nutrient-saturated batch cultures exposed to the following additions of $HgCl_2$ (nM): (○) 0.00; (▽) 0.37; (●) 0.93; (▼) 1.84; (□) 3.67 and (▲) 7.37.

a, photosynthetic rate, and cell dimensions are tabulated in Appendix B.

Changes in biomass (in vivo fluorescence and cell numbers) of the 3 chemostat cultures are shown in Fig. 3. Although both measurements display the same trend, in vivo fluorescence was less variable than cell numbers.

The growth of ammonium-limited S. costatum during the long-term exposure to sublethal doses of $HgCl_2$ was divided into four phases (Fig. 4). The duration of phases A and B varied, whereas, phases C and D were similar in the three chemostats. During phase A, the control chemostat culture (II) remained at the steady-state previously held by the parent chemostat, except for a few days in phase A (Fig. 3). The semi-continuous additions of $HgCl_2$ impaired the ability of chemostat culture I and III cell populations to return to the original steady-state. In phase B, Hg-treated culture populations drastically declined while the control suffered a milder population loss due to changes in the life cycle; the life cycle will be described latter. Cell densities of cultures II, I, and III were reduced to 17.0, 3.0 and 0.09%, respectively, of their original densities. During the recovery period (phase C), cell densities rapidly increased. In phase D, a new steady-state was established in all cultures (Fig. 3A).
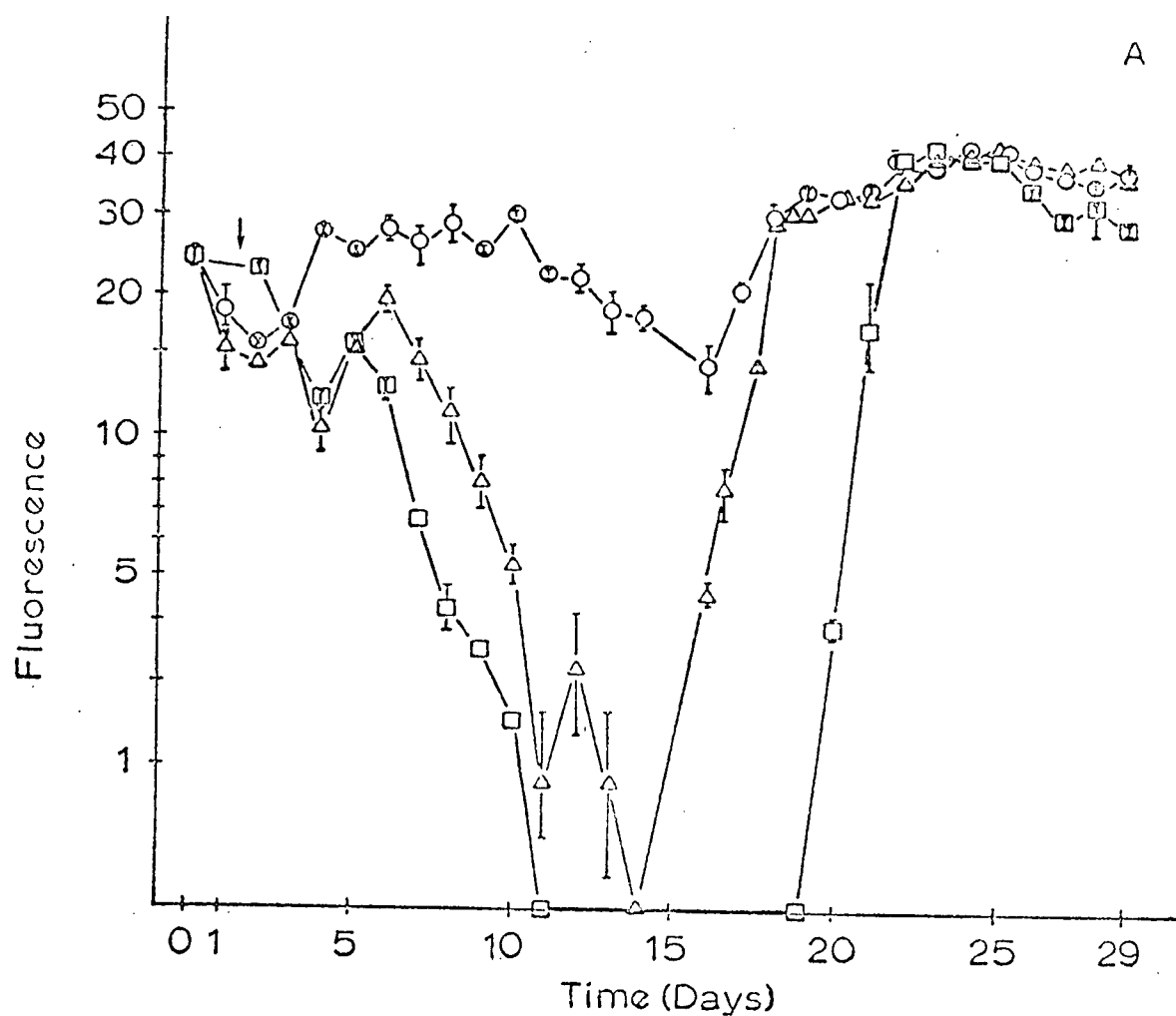
FIGURE 3. Changes in (A) in vivo fluorescence and (B) cell numbers of ammonium-limited cells during the long-term mercury exposures (nM HgCl$_2$) to; (○) 0.00, (∧) 0.37 and (□) 3.68 . The arrow represents the time at which the first addition of mercury was made. The bars show the standard deviations of each measurement.

B
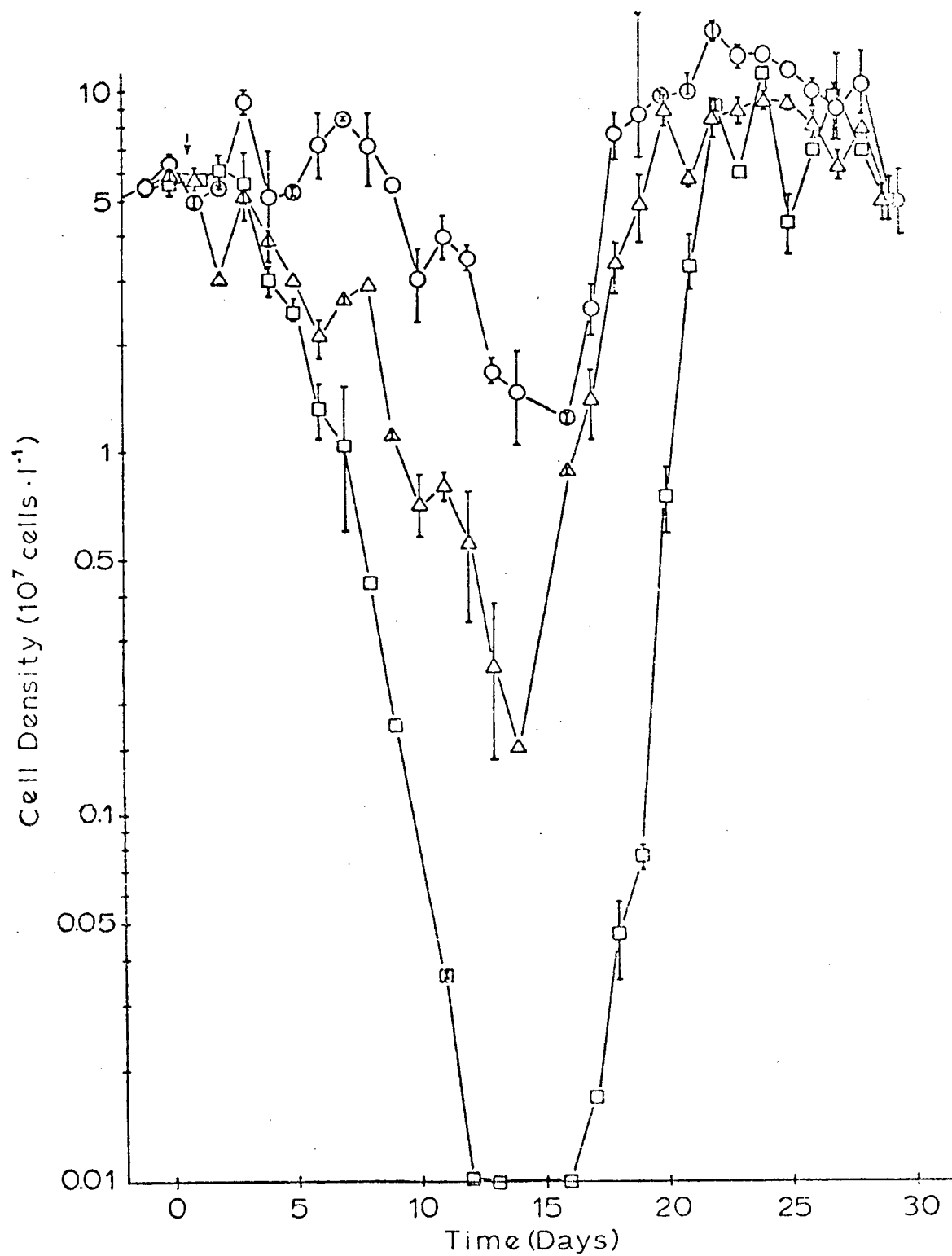
FIGURE 3B.
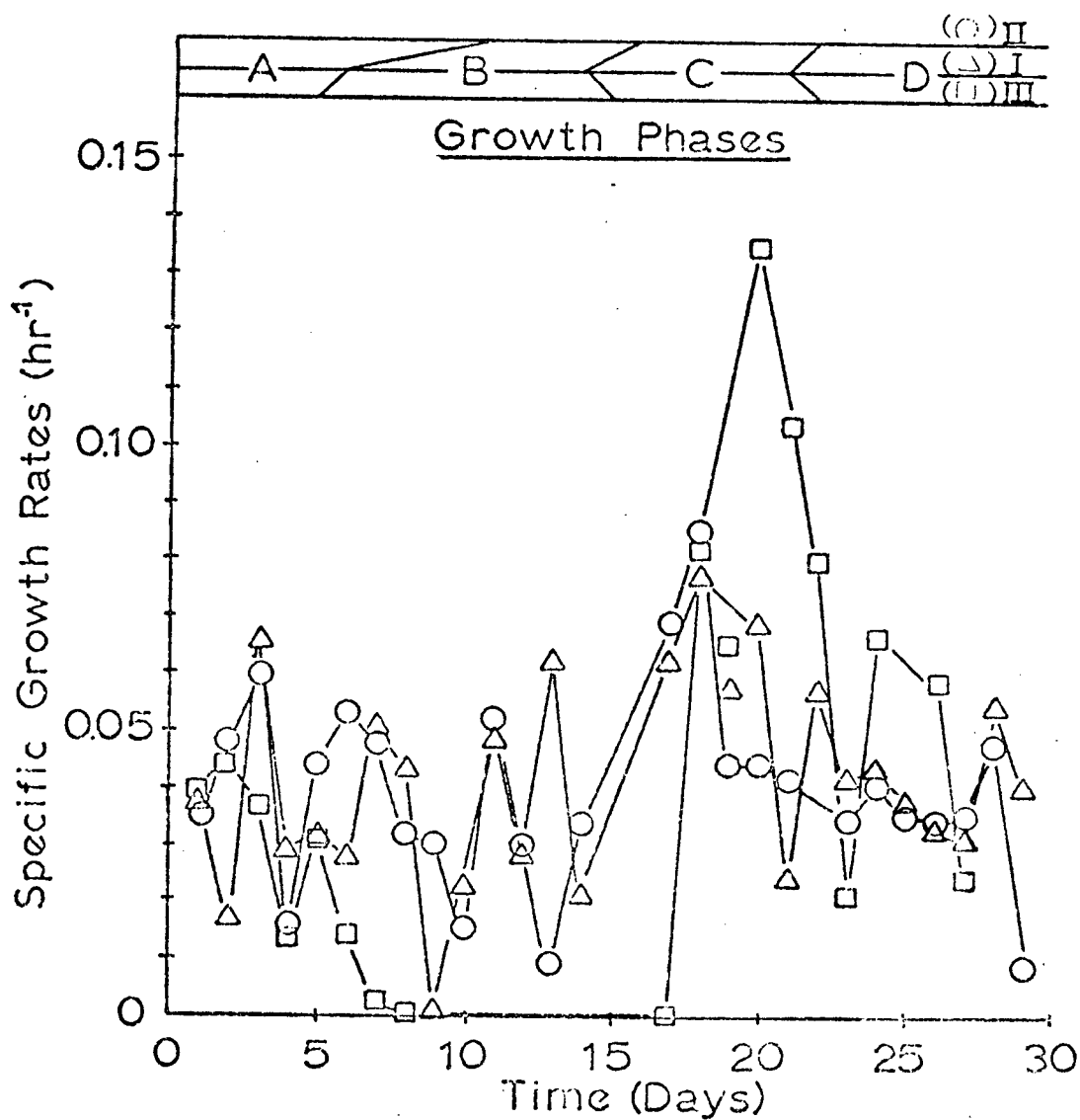
FIGURE 4. Changes in the specific growth rates of ammonium-limited cells during the long-term mercury exposures (nM HgCl$_2$) to: (O) 0.00, ($\wedge$) 0.37 and ($\square$) 3.68. The four growth phases are: phase A: maintenance of the steady-state; phase B: decline in specific growth rates; phase C: increase in specific growth rates and ; phase D: return to a new steady-state.

3.3 Specific Growth Rates and Nitrogen Quotas

The effect of $HgCl_2$ on specific growth rates is shown in Fig. 4. On day 1, specific growth rates varied between $0.035 - 0.040$ hr^{-1} in all cultures but by day 6, a definite trend of declining specific growth rate was set in all cultures. In chemostat III, a decrement in cell division began as early as day 3 and specific growth rates remained undetectable from days 12 to 16. Cultures II and I sustained a low specific growth rate during phase B. In phase C, cultures II, I, and III successively recovered with specific growth rates as high as 0.085, 0.077 and 0.135 hr^{-1}, respectively, and division rates up to 3 times per day. Although these maxima occurred only one day, relatively high specific growth rates were maintained for 5, 7 and 11 days at exposures of 0.00, 0.37 and 3.68 nM $HgCl_2$, respectively. New steady-state growth rates resumed after approximately 20 days.

The amount of nitrogen per cell (μM N·10^7 cells^{-1}) followed the opposite trend to cell density. As cell densities and ammonium assimilation decreased, the nitrogen per cell increased from about 1.7 in phase A, to 6.38, 25.03, and 15.87 in cultures II, I, and III, respectively, at the end of phase B. During phase C, specific growth rates sharply increased, and the nitrogen per cell concomitantly decreased as the cultures became nitrogen-limited again. In phase D, the nitrogen per cell for the control culture was 50% lower than in phase A probably due to differences in cell volume and

the amount of nitrogen per cell.

3.4 Effects of Mercury on Photosynthesis

Changes in chlorophyll a and ^{14}C uptake during phase A are presented in Fig.5. Chlorophyll a per cell increased with time. The largest amounts were observed on day 8 (Fig. 5A). During phase A, the average carotenoid : chlorophyll a ratios were 2.39, 2.17 and 2.04 for cultures II, I and III, respectively (Appendix A). A pigment ratio of 2.39 was also reported for ammonium-starved S. costatum (Harrison et. al., 1977). Since direct chlorophyll a determinations were not conducted after day 8, the chlorophyll a : fluorescence ratio was determined from values obtained in phase A and was used to convert fluorescence readings to chlorophyll a. This ratio was 0.33, 0.43 and 0.45 for chemostats II, I and III, respectively. After 22-29 days, chlorophyll a quotas in chemostats II, I, and III were 1.41 ± 0.53, 2.17 ± 0.54, and 2.33 ± 0.90 μg chl a·10^7 cells^{-1}, respectively (Appendix B). Values for phaeophytin a were too variable to be used with confidence.

The ^{14}C uptake gradually decreased with time until day 5 (Fig. 5B). Between days 1 and 4, a 50% reduction in photosynthesis occurred in chemostats II and III, while no obvious trend was observed in chemostat I. On day 6, chemostats II, I, and III achieved photosynthetic rates of 2.52, 5.39, and 4.54 μg C · 10^7 cells^{-1}·hr^{-1}, respectively. The
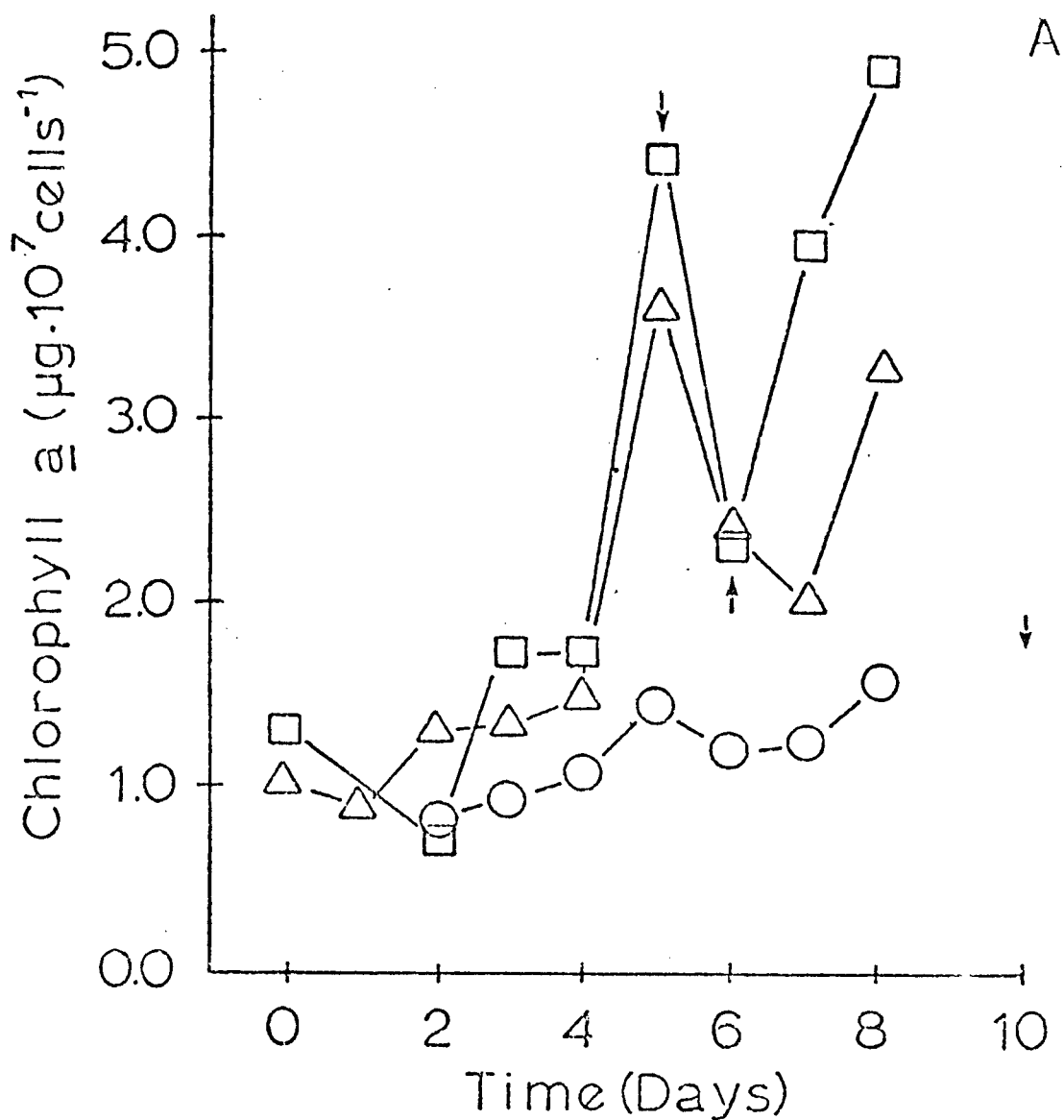
FIGURE 5. Changes in (A) chlorophyll a, and (B) photosynthetic rate and (C) photosynthetic assimilation rate during phase A of the long-term mercury exposures (nM $HgCl_2$) at ($\bigcirc$) 0.00; ($\triangle$) 0.37 and ($\square$) 3.68 . The arrow indicates the end of phase A for each culture.
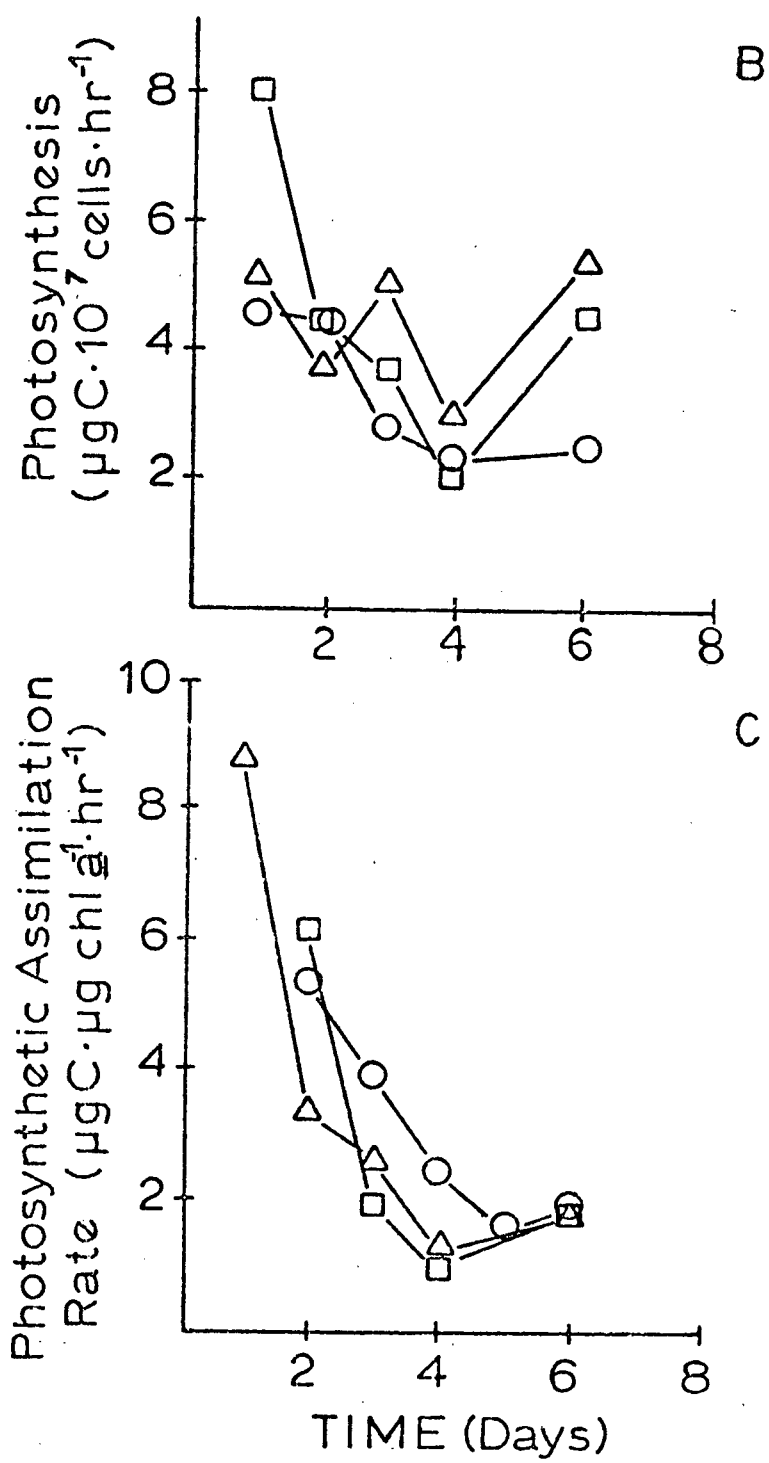
FIGURE 5B and 5C.

photosynthetic assimilation rate was reduced to about the same value on day 6 for all treatments (Fig. 5C).

3.5 Morphological Observations

During phases A and B, chains of 1 to 2, elongated, curved and highly vacuolated cells, connected by short silica rods, and cell clusters around lysed cells, were more frequently observed in Hg-treated chemostats than in chemostat culture II.

Statistical analyses, including the analysis of variance and three a posteriori range tests (Duncan's multiple range test, Newman-Keul's test and Tukey's test) were used in an attempt to determine whether the effects of the three treatments on cell dimensions were significantly different from each other (0.00, 0.37 and 3.68 nM $HgCl_2$). Up to day 5, the cell dimensions in the three treatments were not significantly different. During the later stages of phase A, cells exposed to 3.68 nM $HgCl_2$ were significantly longer than the two other chemostat populations. In phases C and D, cells were significantly shorter than in phase B in all chemostats. Chemostat III cells were longer than cells of chemostats I and II cells, the latter being statistically indistinguishable.

Scanning electron micrographs revealed most of the characteristics already observed with the inverted microscope, (e.g., short silica rods). Only in the Hg-treated cultures, at the end of phase C, populations consisted of

post-auxospore cells,(1-3%) and chains of 4 to 6 cells(1-8%) connected with longer silica rods. Consequently, these populations were more heterogeneous than the control. Although no gametes and relatively few post-auxospore cells were observed, synchronized sexual reproduction may have occurred, due to the increased frequency of shorter cells toward the end of phase C in all cultures. The wide cells which were formed during phase C, quickly became narrower under ammonium-limited conditions in phase D. This may explain the absence of these wide cells in chemostat culture II since the cells were always under ammonium limitation.

3.6 Mercury Analyses

Variations in the expected total Hg concentrations in culture I are shown in Fig. 6. Since the same pattern of Hg fluctuations with time was observed for culture III, it was not included in Fig. 6. Expected total concentrations (calculated using equation 2) represent maximum Hg levels. The accumulation or volatilization of Hg by the cells, the adsorption onto frustules and inner walls of culture flasks are not considered in these calculations. Expected total concentrations take into account only the changes in concentrations as a result of the semi-continuous additions and losses due to the dilution rate of the culture. The average expected total concentrations (calculated using equation 3), in cultures I and III, were 0.33 ± 0.07 and
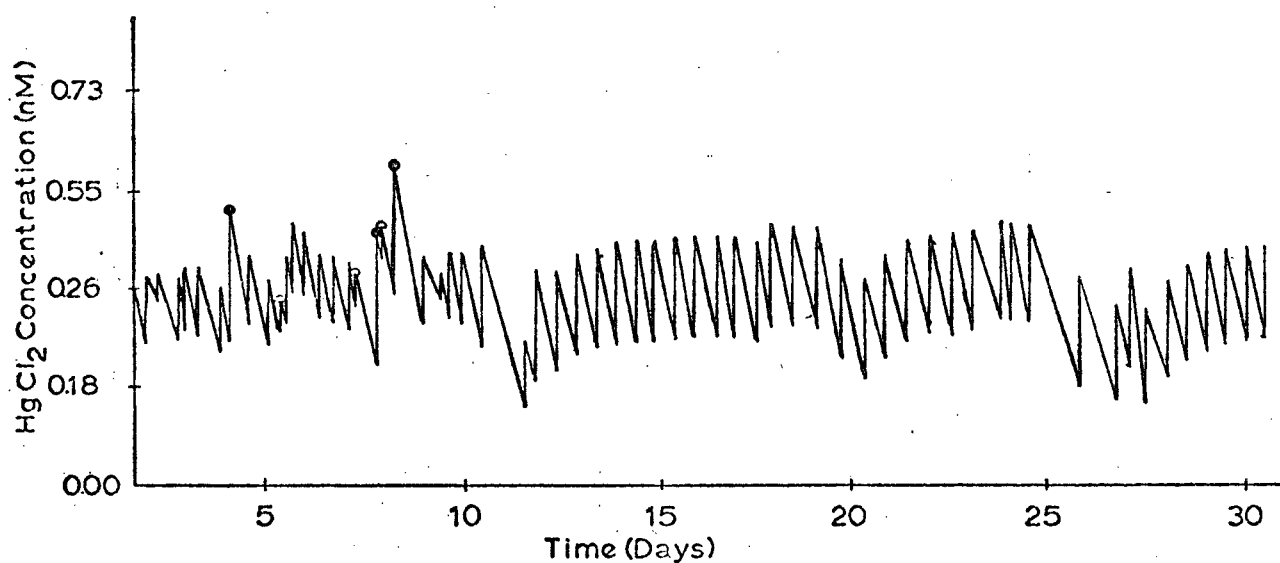
FIGURE 6. Variations in the expected total mercury concentrations in chemostat I during the long-term mercury exposure to 0.37 nM $HgCl_2$. The average expected total concentration, which only takes into account the mercury losses due to the dilution rate of the chemostat, was 0.33±0.07 nM $HgCl_2$. During the first ten days, the concentration of the addition was 0.12 nM $HgCl_2$ except when (◌) 0.06 and (●) 0.24 were added. The expected total concentrations of mercury between each addition and the average expected concentration over the entire experiment were calculated using equations 2 and 3, respectively.

3.46 ± 0.78 nM $HgCl_2$, respectively for the entire experiment. Based on studies on Cu toxicity, (Sunda and Guillard, 1976), it is possible that Hg toxicity may be related to mercuric ion activity. However, the present work deals with total Hg concentrations, because there is no technique which directly measures mercuric ion activity.

Table III shows that the concentration of particulate Hg increased with concentration and time of Hg exposure. Particulate Hg values in cultures exposed to 0.37 and 3.68 nM $HgCl_2$, accounted for 22-58% and 15-41%, respectively, of the total expected concentrations. In cultures I and III, most of the soluble Hg could not be recovered, possibly due to the analytical technique used. The small amounts of Hg in the control cells were probably due to contamination from reagent grade salts used in the preparation of the culture medium (Table III).

3.7 Short-term Nutrient Kinetics

The nutrient uptake kinetic responses of cultures exposed to sublethal doses of $HgCl_2$, for 5 minutes to 5 hours, are shown in Fig. 7. The values of the nutrient uptake kinetic parameters are presented in Table IV.

The effects of Hg on the uptake kinetics of NH_4^+-limited S. costatum starved for 1.5 hours, occurred at a threshold concentration between 1.84 and 3.68 nM $HgCl_2$ (Table IV). The latter addition reduced Vi_{max} by 37% and increased Ks from

TABLE III. Concentrations of mercury in the long-term experiment. Filters and filtrates from 200 ml of chemostat effluent were analyzed for particulate and soluble mercury, respectively. Each value is the mean of duplicates. 'Measured' refers to values obtained from the mercury analysis. 'Expected total' refers to values obtained using equations 2 and 3, and 'expected soluble' refers to the difference between the total expected and measured particulate value. The percentage loss (% Loss) is the difference between the 'expected' and 'measured' soluble Hg, divided by the 'expected' soluble Hg, and multiplied by 100 . Atg = attog = 10^{-18}g.

	CONTROL			EXPOSED TO 0.37 nM			EXPOSED TO 3.68 nM		
DAY	2	4	6	2	4	6	2	4	6
MEASURED:									
Particulate Hg:									
(atg·cell^{-1})	----	0.91	1.61	2.45	5.16	7.56	8.32	33.46	207.93
(nM)	----	0.07	0.11	0.07	0.18	0.11	0.52	1.36	1.18
Soluble Hg: (nM)	N.D.	N.D.	0.18	0.33	N.D.	N.D.	0.41	N.D.	0.55
EXPECTED:									
Total Hg : (nM)	0.00	0.00	0.00	0.33	0.32	0.40	3.46	3.32	3.24
Soluble Hg :(nM)	0.00	0.00	0.00	0.25	0.13	0.29	2.95	1.95	2.06
% LOSS:	----	----	----	----	100%	100%	86%	100%	73%
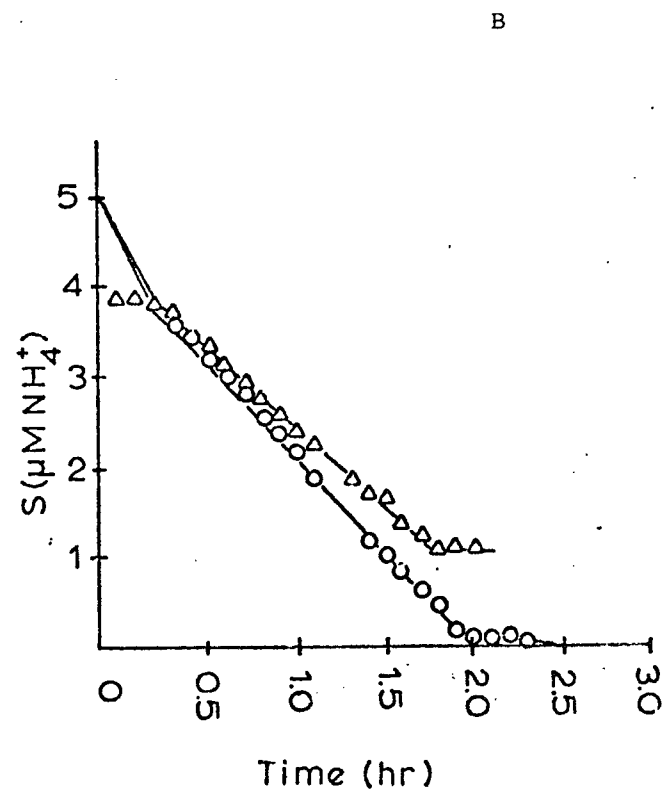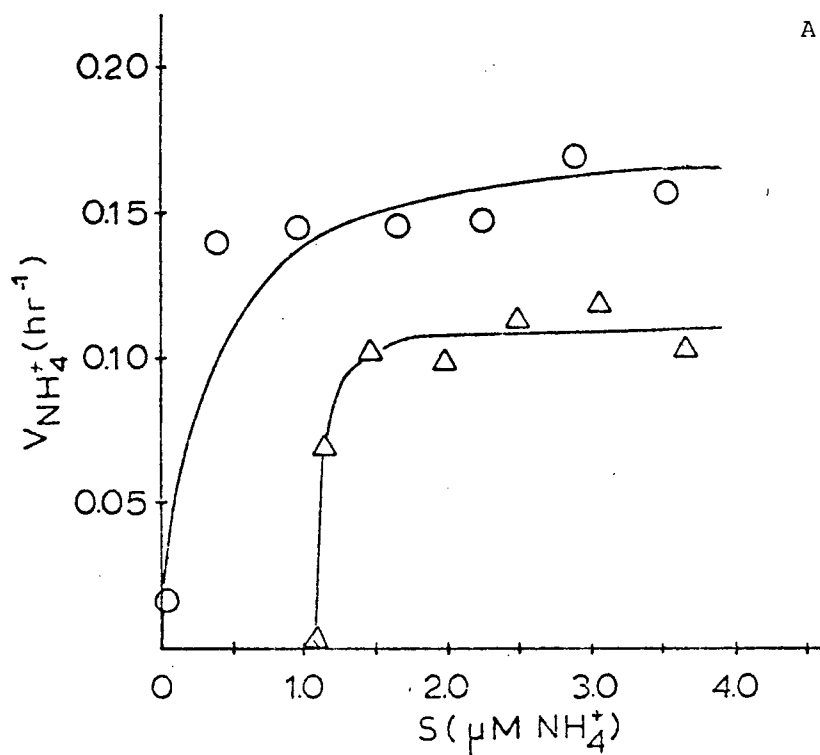
FIGURE 7. Ammonium uptake rates as a function of the substrate, for ammonium-limited cells starved for (A) 1.50 hours and (C) 30.0 hours, during the short-term (up to 5 hours) mercury-exposure (nM $HgCl_2$) to 0.00 (O) and 3.68 (△). Figures (B) and (D) show the disappearance of ammonium with time.

C

D

46
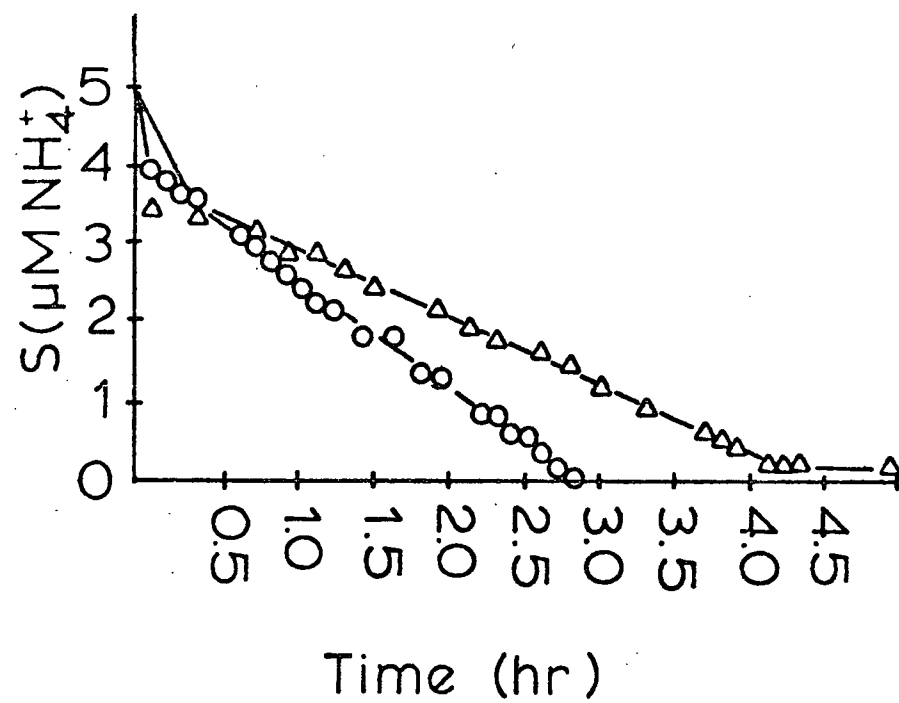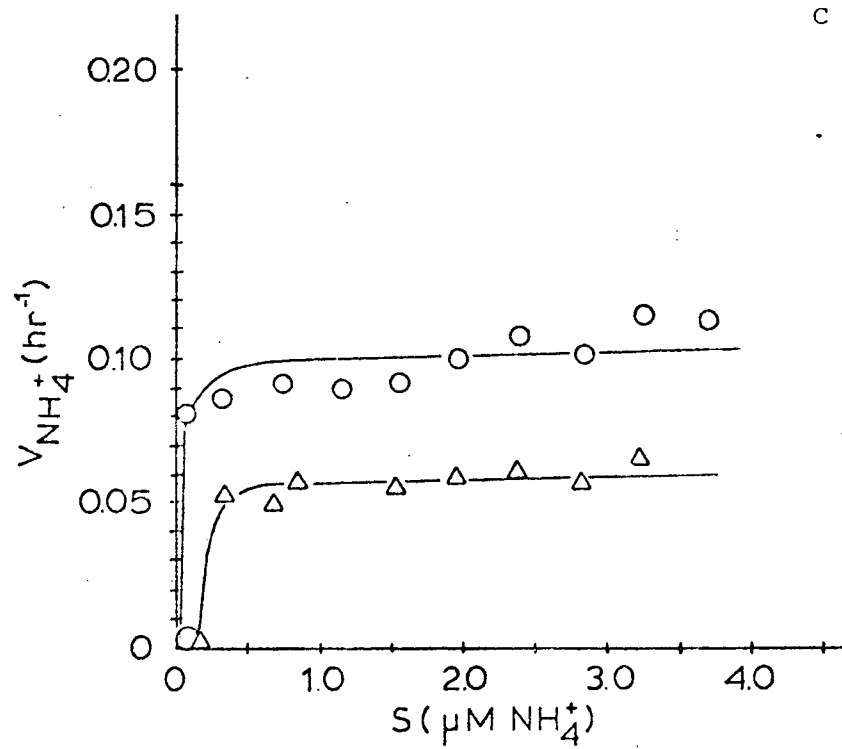
TABLE IV. Nutrient kinetic response to short-term mercury exposure. The nutrient kinetic parameters are defined in section 1 of this chapter. The Ks values are the actual Ks = apparent Ks + So. Values of the standard errors are shown for Vi_{max} and Ks; the Vs values represent the average uptake rate during the time period, Ts, over which the surge uptake occurs. N.D. = not detectable.

1.5 HOURS OF STARVATION

CONCENTRATION (nM $HgCl_2$)	Vs (hr^{-1})	Ts (hr)	Vi_{max} (hr^{-1})	Ks (μM NH_4^+)	So (μM NH_4^+)
0.00	0.44	0.26	0.178 ± 0.017	0.27 ± 0.13	N.D.
0.37	0.50	0.26	0.148 ± 0.006	0.21 ± 0.05	N.D.
1.84	0.93	0.16	0.167 ± 0.011	0.01 ± 0.02	N.D.
3.68	0.45	0.26	0.112 ± 0.004	1.11 ± 0.07	1.08
5.53	1.89	0.06	0.117 ± 0.005	0.85 ± 0.07	0.83

30.0 HOURS OF STARVATION

CONCENTRATION (nM $HgCl_2$)	Vs (hr^{-1})	Ts (hr)	Vi_{max} (hr^{-1})	Ks (μM NH_4^+)	So ($\mu M \cdot NH_4^+$)
0.00	1.74	0.06	0.012 ± 0.003	0.02 ± 0.00	N.D.
0.18	0.46	0.26	0.070 ± 0.010	0.51 ± 0.29	0.32
0.37	2.18	0.06	0.060 ± 0.000	0.54 ± 0.11	0.50
1.84	0.55	0.26	0.050 ± 0.000	0.00 ± 0.03	N.D.
3.68	0.50	0.26	0.060 ± 0.000	0.20 ± 0.08	0.16

0.28 to 1.11 µM NH_4^+. The increase in Ks was primarily due to the cessation of uptake when 1.08 µM NH_4Cl still remained in the medium (Fig. 7A). The Vs values were similar when all values were calculated over the same time interval of 0.26 hour. In NH_4^+-limited effluents starved for 30 hours, Vi_{max} was significantly reduced by exposure to a concentration as low as 0.18 nM $HgCl_2$. Increased time of starvation of NH_4^+-limited chemostat effluents (from 1.5 to 30 hours) resulted in a decrease in the Ks value of the control cultures from 0.27 to 0.02 µM NH_4Cl (Table IV). Since this latter value of Ks for the control culture was near the limits of detection, this made it difficult to determine the effects of Hg exposure on Ks.

/Short-term Hg exposure (1.50 hours of starvation) did not appear to affect Vs (Table IV), since the mean uptake rate over the concentration range from 0.00 to 5.53 nM $HgCl_2$ was 0.51 ± 0.06 hr^{-1} when the uptake was calculated at a time of 0.26 hr. Variations in the Vs values reflect the difficulty in making this measurement. However, both NH_4^+ starvation and Hg treatment resulted in a very sharp and reduced Ve region of the uptake curve (Fig. 7A and C). The So values (Table IV) represent the amount of NH_4^+ remaining at the end of the perturbation experiment when Ve = 0 for 18 to 60 minutes. However, on some occasions the uptake slowly resumed after a few hours.

Of secondary interest, is the effect of duration of starvation of effluent from the chemostat on the nutrient

uptake response (Table V). When effluents were starved for 11 hours, Ks and St values decreased while Vi_{max} remained constant. This indicated that ammonium-limited populations responded to 11 hours of nitrogen deficiency by increasing their affinity for the substrate. Between 11 to 30 hours, the rate of mobilization of nitrogen from the internal nutrient pool into the assimilatory system was decreased. Between 30 and 72 hours, ammonium deficiency had adverse effects on all the phases of the nutrient uptake rates.

3.8 Long-term Nutrient Kinetics

Nutrient uptake kinetic parameters measured during phases A and D of the long-term Hg exposure are presented in Table VI. In culture III, the nutrient kinetics deviated considerably from the regular pattern as early as day 4, whereas culture I showed significant deviations only by day 8 (Fig. 8). Despite the presence of 1.3 to 1.5 μM NH_4Cl in culture I effluent, the spike addition of NH_4^+ resulted in a normal value for Vs of 1.21 hr^{-1}, after which no significant uptake took place for 7.1 hr (Fig. 8B, day 8). However, in culture III (day 6), the maximum recorded Vs was 0.70 hr^{-1}, in contrast to culture II (day 5) where a Vs of 1.19 hr^{-1} was measured. These comparisons were made at a standard time interval of 0.10 hour.

In general, during phase A in the Hg-treated cultures, the Ve region of the uptake curve gradually became gentler

TABLE V. The effects of duration of starvation of chemostat effluent on the nutrient uptake kinetics. The uptake kinetic parameters are defined in section 1 of this chapter. The Ks values are the actual Ks, where actual Ks = apparent Ks + So; n. d. = not detectable.

STARVATION TIME (hr)	V_s (hr^{-1})	T_s (hr)	Vi_{max} (hr^{-1})	Ks ($\mu M\ NH_4^+$)	So ($\mu M\ NH_4^+$)	Source
0.00	0.27	2.60	0.124	0.50	0.20	Conway and Harrison, 1977
1.50	0.44	0.26	0.178	0.27	n. d.	Table IV
11.00	0.37	0.30	0.129	0.59	n. d.	Tavle VI
30.00	1.74	0.06	0.102	0.02	n. d.	Table IV
72.00	0.17	0.70	0.088	0.10	n. d.	Conway and Harrison, 1977

TABLE VI. Nutrient uptake kinetic response to the long-term mercury exposure. The nutrient uptake kinetic parameters are defined in section 1 of this chapter. The Ks values are the actual Ks where actual Ks = apparent Ks + So. Values of standard errors are shown for Vi ($Vi = Vi_{max}$) and Ks. N.D. = not detectable.

The units of the nutrient uptake kinetic parameters are; $Vs = hr^{-1}$, $Ts = hr$, Vi ($Vi = Vi_{max}$) $= hr^{-1}$, $Ks = \mu M\ NH_4^+$, $So = \mu M\ NH_4^+$.

CHEMOSTAT CULTURE II (CONTROL)

DAYS	2	4	5	6	19	26
Vs	0.37	0.62	1.19	----	0.94	0.38
Ts	0.30	0.20	0.10	----	0.10	0.30
Vi	0.129±.006	0.080±.003	0.145±.009	0.129±.011	0.145±.006	0.129±.011
Ks	0.59 ±.14	0.25 ±.09	0.44 ±.11	0.49 ±.16	0.06 ±.02	0.12 ±.08
So	0.26	0.20	N.D.	N.D.	N.D.	N.D.

CHEMOSTAT CULTURE I (EXPOSURE TO 0.37 nM $HgCl_2$)

DAYS	2	4	5	6	19	23
Vs	0.28	0.31	0.28	0.99	1.62	0.48
Ts	0.50	0.30	0.60	0.10	0.10	0.20
Vi	0.104±.005	0.110±.017	0.186±.078	0.152±.024	0.122±.004	0.183±.009
Ks	0.18 ±.10	1.22 ±.57	0.03 ±1.67	1.34 ±.71	0.01 ±.02	0.01 ±.02
So	0.04	0.25	N.D.	N.D.	N.D.	N.D.

CHEMOSTAT CULTURE III (EXPOSURE TO 3.68 nM $HgCl_2$)

DAYS	2	4	5	6	23	26
Vs	0.37	0.54	0.63	0.70	0.58	0.30
Ts	0.40	0.20	0.20	0.10	0.20	0.20
Vi	0.134±.007	0.123±.013	0.121±.020	0.061±.008	0.170±.007	0.105±.003
Ks	0.35 ±.07	1.36 ±.36	1.80 ±.52	2.83 ±.23	0.15 ±.03	0.05 ±.02
So	0.04	0.63	0.85	2.43	N.D.	N.D.
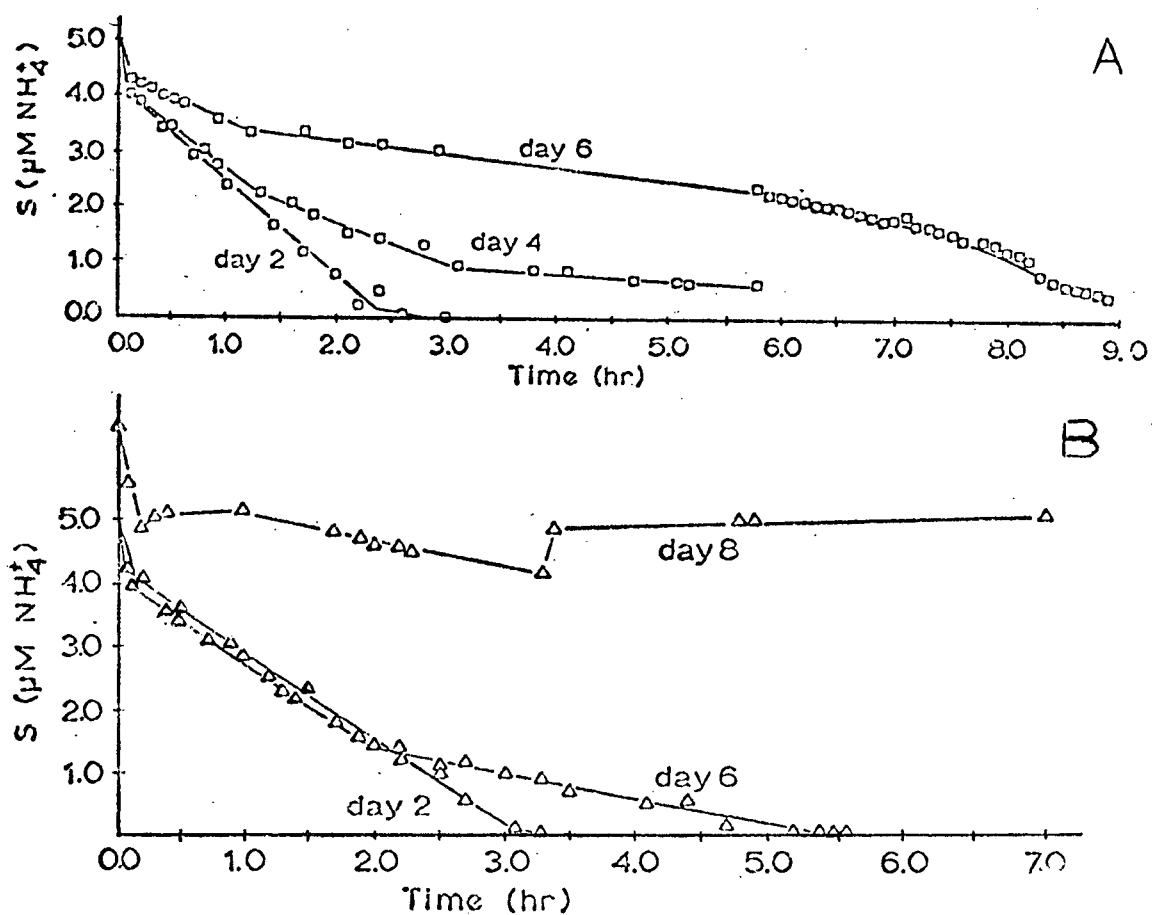
FIGURE 8. The disappearance of the substrate with time during a perturbation experiment conducted on different days during phase A of the long-term mercury exposure (nM $HgCl_2$) to (A) 3.68 ($\square$), and (B) 0.37 ($\triangle$).
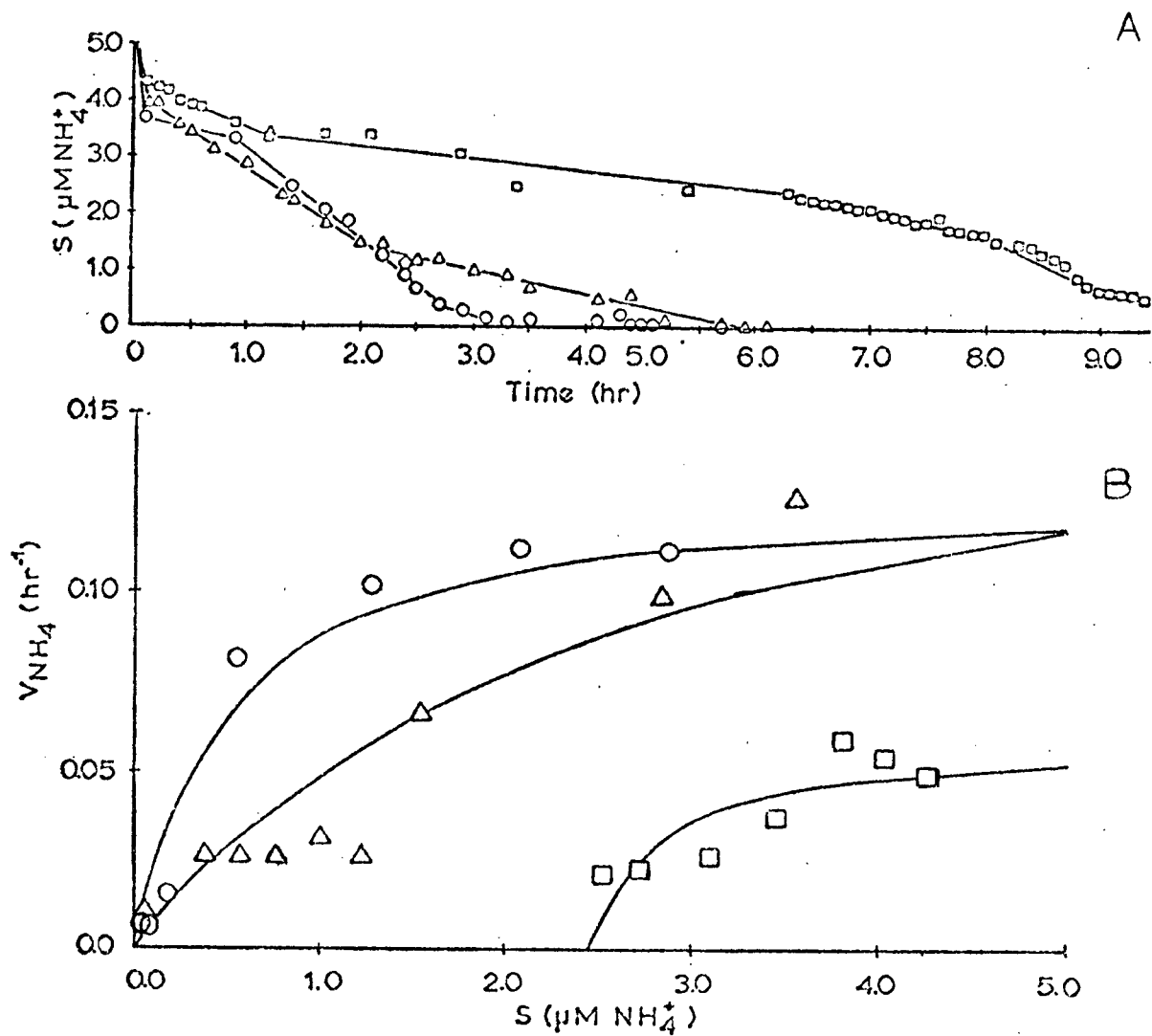
FIGURE 9. (A) Disappearance of ammonium with time, after the addition of 5 μM NH$_4$ · on day 6 (phase A) of the long-term mercury exposure (nM HgCl$_2$) to (○) 0.00; (△) 0.37 and (□) 3.68 . These data were used to calcul - ate the ammonium uptake rates as a function of the substrate in figure (B).

with duration (Fig. 8) and concentration (Fig. 9) of Hg exposure. This change was reflected by the increase in Ks values or a loss of affinity for the substrate in culture III (Table VI). It appeared that Vs was also reduced in chemostat culture III compared to the control culture II.

In phase D, Hg-treated cultures had a higher Vi_{max} and lower Ks (Fig. 10) than in phase A (Fig. 9). In the control, Vi_{max} values were similar to values obtained in phase A but Ks values were reduced in phase D (Table VI).

Section 4. Discussion

4.1 Effects of Mercury on Photosynthesis

Under ammonium limitation (e.g., in the control culture II), the reduction in photosynthetic assimilation rate was due to a decrease in photosynthesis and a slight increase in chlorophyll a per cell. Transient reductions in photosynthesis have also been observed in nutrient-limited (Thomas, 1975a; 1975b) and nutritionally perturbed natural phytoplankton communities (Falkowski and Stone, 1975). The simultaneous effects of mercury and ammonium limitation (days 1 - 5 in phase A and in phase D) resulted in a greater enhancement of chlorophyll a synthesis than under ammonium limitation alone. The small increase in photosynthetic assimilation rate in mercury-treated cultures (day 6) was
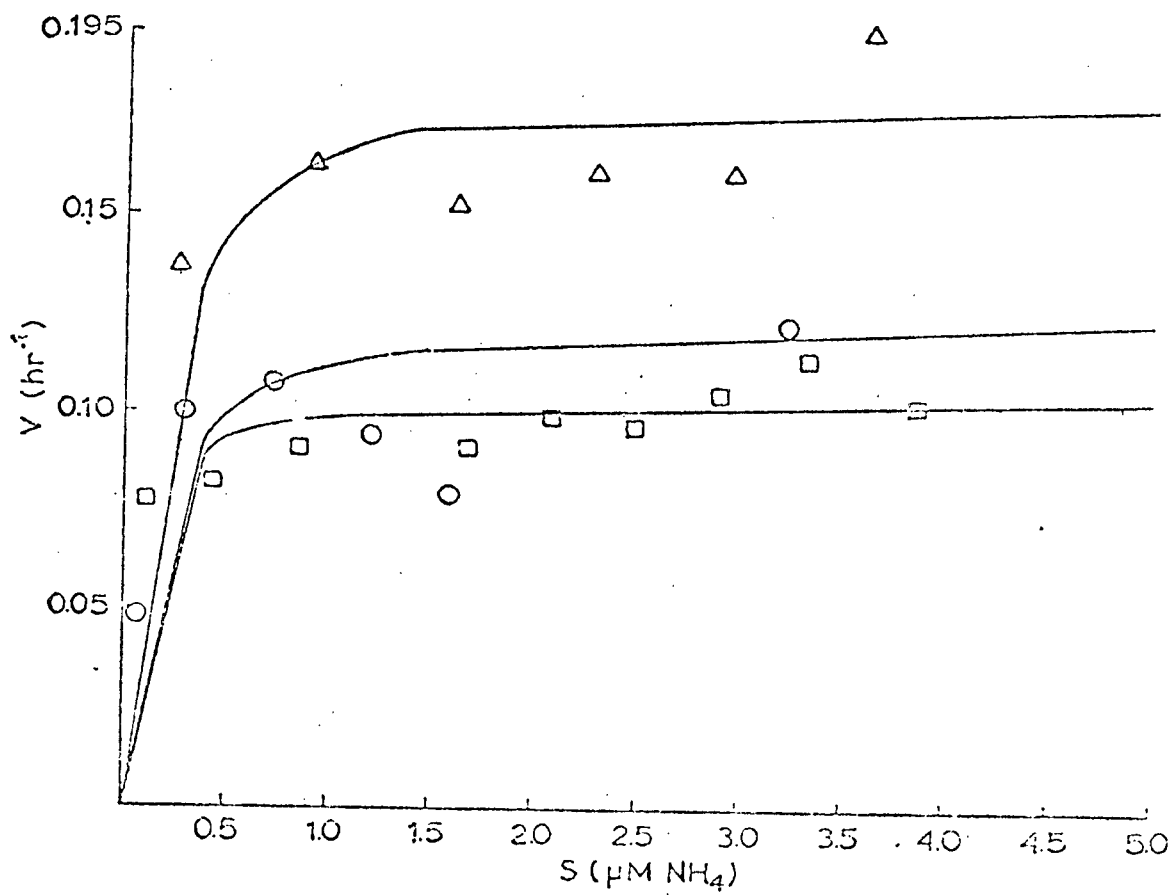
FIGURE 10. Ammonium uptake rates as a function of the substrate after the recovery (phase D) from the long-term mercury exposure (ηM $HgCl_2$) to (O) 0.00; ($\triangle$) 0.37 and ($\square$) 3.68 .

probably due to the cessation of ammonium limitation, since the amount of nitrogen and chlorophyll a per cell increased in spite of an increase in particulate mercury up to 207.93 atg·cell^{-1} (attog = 10^{-18} g).

In phase D, _in vivo_ fluorescence was comparable in all treatments. In the control and mercury-treated cultures, cell numbers doubled and returned to their original levels while cell volume still increased upon mercury exposure. If the nitrogen and chlorophyll a per cell were expressed per unit of cell volume, the values would appear to be similar among all cultures.

The increase in chlorophyll a content may be due to a stimulation of synthesis, or to a duplication of organelles without the formation of a new frustule. This would produce elongated cells of greater cellular content (e.g., DNA, RNA and proteins). This has been observed in silicate-starved _Navicula pelliculosa_ (Coombs et al., 1967). Thus, mercury might arrest cell division by interfering with the regulation of the silicate metabolism.

4.2 Effects of Mercury on Growth Parameters

The effect of mercury on biomass and nutrient kinetics were proportional to the concentration of the additions (e.g., culture III was the first culture to be disturbed from its steady-state). In culture III, high particulate mercury values were reached sooner than in culture I (Table III).

Periods of cell loss in nutrient-limited cultures have been reported (Davis et al., 1973). As cells became narower in all nutrient-limited chemostat cultures, there was an increased likelihood for the recurrence of sexual reproduction. For a silicate-limited cultures of S. costatum a decrease in cell density, an increase in the amount of the limiting nutrient per cell and the occurrence of wide, short cells indicated that synchronized sexual reproduction occurred (Davis et al., 1973). Similar observations were made for the Hg-treated cultures, and to a very limited extent, in the control culture. Additions of mercury, which resulted in the earlier decline in cultures I and III populations (phase A), did not appear to interfere with presumed sexual reproduction processes (phase C) since resumption of growth occurred simultaneously in all cultures.

4.3 Effects of Mercury on Nutrient Uptake Kinetics

In the short-term mercury exposure and in culture I, the maximum rate of uptake, Vs, was not altered. In cultures I and III (days 2 to 4), the actual Ks increased, indicating a loss in the affinity for the substrate, while the maximum assimilatory rate, Vi_{max}, remained unchanged. Although, the actual Ks (apparent Ks + So) increased in culture I, the apparent Ks values were reduced. In culture III, the apparent Ks values were also reduced as well as the maximum uptake rate, Vs. The enzymatic inhibition by heavy metals is usually

classified as noncompetitive (Lehninger, 1975). Even though uncompetitive and noncompetitive inhibition can result in a decrease in Vi_{max} or Vs, the additional decrease in the apparent Ks values indicated that mercury uncompetitively inhibited the uptake in ammonium-limited cultures. Under long-term, low level (0.37 nM $HgCl_2$) exposure and under short-term higher level (>1.84 nM $HgCl_2$) exposure, the uptake rate at low levels (estimated by Ks) appeared to be more sensitive than the initial uptake (Vs). Much higher levels of mercury were required to decrease Vs or Vi_{max}. The reasons for the greater sensitivity of the rate of uptake at low concentrations are not clear. The reduction in Vs or Vi_{max} in the long-term high level (3.68 nM $HgCl_2$) exposure could be due to the high affinity of mercurials for sulfhydryl groups, resulting in the binding of some mercuric ions onto cell membrane enzymes (either enzymes involved in the hydrolysis of ATP or carrier enzymes). Changes in nutrient kinetics from the short-term experiment (1.5 hours of starvation), after the recovery (phase D) in the long-term experiment, and changes in cell morphology in culture III, suggest that the threshold concentration for recovery from mercury inhibition , occurred between 1.84 and 3.68 nM $HgCl_2$. The threshold concentration where the first effect of mercury inhibition was observed on nutrient uptake kinetics was reduced to 0.18 nM $HgCl_2$ by an increased period (30 hours) of ammonium starvation.

4.4 Recovery from Initial Mercury Inhibition

Recovery from mercury inhibition in continuous (Fujita and Hashizume, 1975; Kayser, 1976) and batch cultures (De Filippis and Pallaghy, 1976c; Berland et al., 1977) are known. They have been attributed to mercury losses from the medium (Davies, 1974) either by volatilization when cell densities are high or a decrease in particulate mercury through an uptake exclusion mechanism(Ben-Bassat et al., 1972; Ben-Bassat and Mayer, 1975; 1977; De Filippis and Pallaghy, 1976b).

In phase C of the long-term mercury exposure, growth resumed when cell densities were minimum and possibly when mercury particulate values were highest. Greatest metal accumulation occurred in mercury-resistant Chlorella (De Filippis and Pallaghy, 1976c), in Isochrysis galbana and Dunaliella tertiolecta, after the initiation of a few cell divisions (Davies, 1974). The increase in specific growth rates in mercury-treated cultures was probably due to the appearance of cells of a new life cycle stage with a different cellular chemical composition (as seen from the nitrogen and chlorophyll a content). In nutrient-limited diatoms, during vegetative reproduction, changes in the cellular chemical composition, reduction in width and elongation of cells possibly produced populations of different physiological stages. Werner (1971) also found that during cell diminution in Coscinodiscus asteromphalus, changes in chemical composition

occurred and the sensitivity to metabolic inhibitors varied with different life cycle stages or cell widths.

After recovery, improvement of nutrient uptake (increase in the affinity for the substrate and the rates of uptake) occurred in the mercury-treated cultures. The acquisition of mercury-tolerance may be due to several factors. It could have been acquired from genetic recombination, as a consequence of sexual reproduction. Alternatively, some or all cells in phase C may also have developed a physiological resistance upon mercury exposure. The selective pressure operating on phase C cells may have resulted in the survival of a few cells capable of coping with high external or internal concentrations of Hg, and therefore growth during the recovery phase would be initiated by these cells. Berland et al., (1977) suggested that recovery may be due to a return to the initial physiological state of the cells but no suggestion of mechanism was given. The results from the long-term mercury exposure in this study suggest that a change in cell type may be as important as a pure physiological adaptation mediated by a biochemical change in cell metabolism.

4.5 Mercury Losses

Losses were independent of the initial doses. These losses from the used medium were probably not due to volatilization within the cultures. In continuous cultures, the regulation of a constant volume inside the culture flasks

depends on a constant air pressure in the dead air space. Consequently, the partial pressure of mercury above the surface of the medium, and the amount of Hg adsorbed onto the walls and in the solution were probably at equilibrium. The actual low recovery of soluble mercury may be due to the presence of a heat-stable photosynthetic metabolite in the used medium (Ben-Bassat and Mayer, 1977), responsible for the reduction of Hg^{+2} to Hg^0. Thus, the major loss may come from the analytical procedures for mercury determinations, which require heating of aqueous samples at $95°C$ during 2 hours. Therefore, the heat-stable photosynthetic metabolite would enhance the rate of volatilization during the heating process.

4.6 Applications to the Natural Environment

In natural waters, mercuric ion activity would probably be less than in the artificial seawater due to the presence of unknown quantities of natural complexing materials such as detritus, dissolved organics. Therefore, total mercury concentrations equivalent to those used in cultures may not produce the same effects in natural seawater.

During periods of seasonal nutrient limitation, mercury pollution may seriously interfere with the competitive ability of a diatom. Under nutrient-limited conditions, the ability to take up the limiting nutrient is severely reduced (Ks value is increased) by exposure to a secondary stress such as mercury. This may result in a change in the dominant species

or replacement by different algal groups such as flagellates
(Thomas and Seibert, 1977).

4.7 Evaluation of Chemostat Studies

In this study, the use of chemostats in determining the
effects of nearly ecological total concentrations of mercury
on an unialgal population, already under a primary stress
(nutrient limitation), has allowed a closer imitation of an
ecological situation than if batch cultures had been used.
Long-term, low level effects rather than short-term, high
level effects may simulate more closely the dispersal mode of
industrial pollutants into the natural environment. This
simulation is best achieved using continuous cultures.

The threshold concentration where the effects occur, are
almost an order of magnitude lower in the long-term compared
to the short-term experiments. It is for this reason that the
use of continuous cultures to determine threshold effects is
strongly urged. The noticeable changes in cell morphology and
physiology, during vegetative and especially during sexual
reproduction, tend to make diatoms unsuitable test organisms
for bioassays. From these experiments, the use of chemostat
cultures for pollution bioassay is considered as a refined
technique. Since conditions which induce certain life cycle
stages in algae are ill-defined, the suitability of the
chemostat in pollution studies could be improved by using
species which are less variable during their life cycle.

CHAPTER III

THE EFFECT OF Hg EXPOSURE ON INTRACELLULAR DISTRIBUTION OF Hg, Cu, AND Zn

Section 1. Introduction

Repeated exposure of phytoplankton to trace elements may increase tolerance (De Filippis and Pallaghy, 1976c), but it is not known whether a complexing agent such as metallothionein plays a role in this process. Metallothionein is a low molecular weight (m.w.) protein (10,000) whose synthesis is stimulated by exposure to heavy metals. This protein can bind and detoxify heavy metals such as Ag, Cd and Hg. It can also store Cu and Zn when they occur in excess of the levels required for metalloenzymes (Bremmer and Davies, 1975 ; Brown and Chatel,1978). Deleterious effects may occur when the rate of heavy metal accumulation exceeds the rate of metallothionein synthesis or its binding capacity (Winge et al., 1973; Brown and Parsons, 1978). Hence, heavy metals such as Cd and Hg may accumulate in the high m.w. pool and exert toxic effects by: 1) substituting for Cu and Zn in metalloenzymes (Brown, 1977; Brown and Parsons, 1978), 2) altering the quarternary or tertiary enzyme structure, or 3) binding to active or other sites leading to conformational changes.

Metallothionein has been ubiquitously found in land and

marine animals (Piscator, 1964; Buhler and Kagi, 1974; Howard and Nickless, 1977a; 1977b), but its presence in phytoplankton remains to be clarified. McLean et al., (1972) may have found a metallothionein-like fraction binding Cd and Zn in blue-green algae, exposed to radioactive Cd.

The initiation of logarithmic growth in algae previously inhibited by heavy metals may be attributed to several factors. One possible reason for the recovery from inhibition may be the production of complexing agents such as metallothionein.

The aim of this study was to investigate the possibility of a short-term biochemical adaptation in response to heavy metal exposure in S. costatum and to determine whether metallothionein would be responsible for sequestering heavy metals and the subsequent recovery. The effects of $HgCl_2$ on the intracellular levels and distribution of Cu and Zn are also examined.

Section 2. Materials and Methods

2.1 Batch Cultures

Cultures of Skeletonema costatum were grown in artificial seawater (Davis et al., 1973) enriched with modified 'f/2' medium (Appendix A). Batch cultures were grown in borosilicate, 6 l flat bottom boiling flasks continuously

stirred at 120 rpm. Growth was monitored by changes in in vivo fluorescence with a Turner Model 111 fluorometer and in cell density using an inverted microscope.

2.2 Experimental Conditions

During the first 70 hours of the experiment, two unialgal log phase cultures were grown with no pre-exposure to Hg (cultures A and B) while two other cultures (C and D) were exposed to 1.84 nM $HgCl_2$; after 70 hours, one culture of each subset (cultures B and D) was perturbed with 5.53 nM $HgCl_2$ (Table VII). Culture E was exposed to 0.37 nM $HgCl_2$ over a period of 116 hours (Table VII).

2.3 Analyses

Early stationary phase cells were harvested by centrifugation for 6 minutes at 650·g at 6°C in 50 ml polycarbonate test tubes. The cells from the control culture were harvested after 70 hours while the cells from the Hg-exposed cultures were harvested between 90 and 116 hours. The cells remaining in the supernatants were collected onto a 0.45 μM Millipore filter, resuspended in artificial seawater and recentrifuged as above.

One gram of cells (wet weight) was homogenized in 3ml of 0.9% NaCl for 5 minutes using a TRI-R STIR-R Model 563C variable speed lab homogenizer at a speed setting of 4.5. The homogenate was centrifuged at 27,000·g for 10 minutes using a

TABLE VII. Exposure to different concentrations of $HgCl_2$ at different times during a batch culture experiment.

TREATMENT	CONCENTRATION (nM $HgCl_2$) ADDED AT	
	0.00 hr	70.00 hr
Culture A	0.00	0.00
Culture B	0.00	5.53
Culture C	1.84	0.00
Culture D	1.84	5.53
Culture E	0.37	0.00

Sorval Superspeed RC2-B automatic refrigerated centrifuge. The supernatant was fractionated on a Sephadex G-75 (Pharmacia) column (9 X 60 cm) with 0.01 M NH_4HCO_3 elution buffer. Fractions of 2 ml were collected.

Ultraviolet absorbance was determined with a Perkin-Elmer Model 124D double beam spectrophotometer. Absorbances at 250 and 280 nm determined the relative amount of metal-bound substances and aromatic amino acids, respectively. Peaks were identified as being in the position of the high (enzyme-containing) and low m.w. intracellular pool by comparing their positions in relation to the medium m.w. fractions obtained from naturally occurring duck liver metallothionein. The identification of metallothionein was done by comparing its elution profile with those of Piscator (1964) and Leber (1974) as described in Brown and Chatel (1978). Copper and Zn levels were determined in each fraction using direct aspiration with a Perkin-Elmer Model 306 flame atomic absorption spectrophotometer with deuterium background correction. Total mercury was measured by a cold vapor method on the combined fractions of each peak, utilizing a 30 cm cell (Pharmacia UV Control and Optical Units Model 100).

Section 3. Results

3.1 Growth

At the beginning of the experiment, all cultures grew
exponentially (Fig.11). Growth rate was unaffected by 0.37 nM
$HgCl_2$ while concentrations equal to or greater than 1.84 nM
$HgCl_2$ reduced it. After 70 hours, control cultures (A and B)
had a growth rate of 1.92 ± 0.37 divisions per day and the Hg-
exposed cultures (C and D) had a reduced growth rate of
1.31 ± 0.27 divisions per day. The cell densities in the pre-
exposed cultures (C and D) were reduced by 25% after 24 hours,
and by 55% after 46 hours of pre-exposure to 1.84 nM $HgCl_2$.

3.2 Distribution of Hg, Cu and Zn

The 250 nm absorbance profile for all cultures (Fig.12)
shows the relative abundance of metal-bound substances. It
consisted of two major absorbance peaks, one in the high
m.w. pool (fractions 2 to 7) and a major peak in the low
m.w. pool (fractions 12 to 20). In contrast to the absorbance
profiles of cytoplasmic fractions derived from animals exposed
to heavy metals, there was no large absorbance peak in the
medium m.w. pool.

The typical gel elution profile of a nutrient-saturated
culture exposed to 1.84 nM $HgCl_2$ (culture C) is shown in
Fig. 13. This profile was characterized by: 1) high levels of
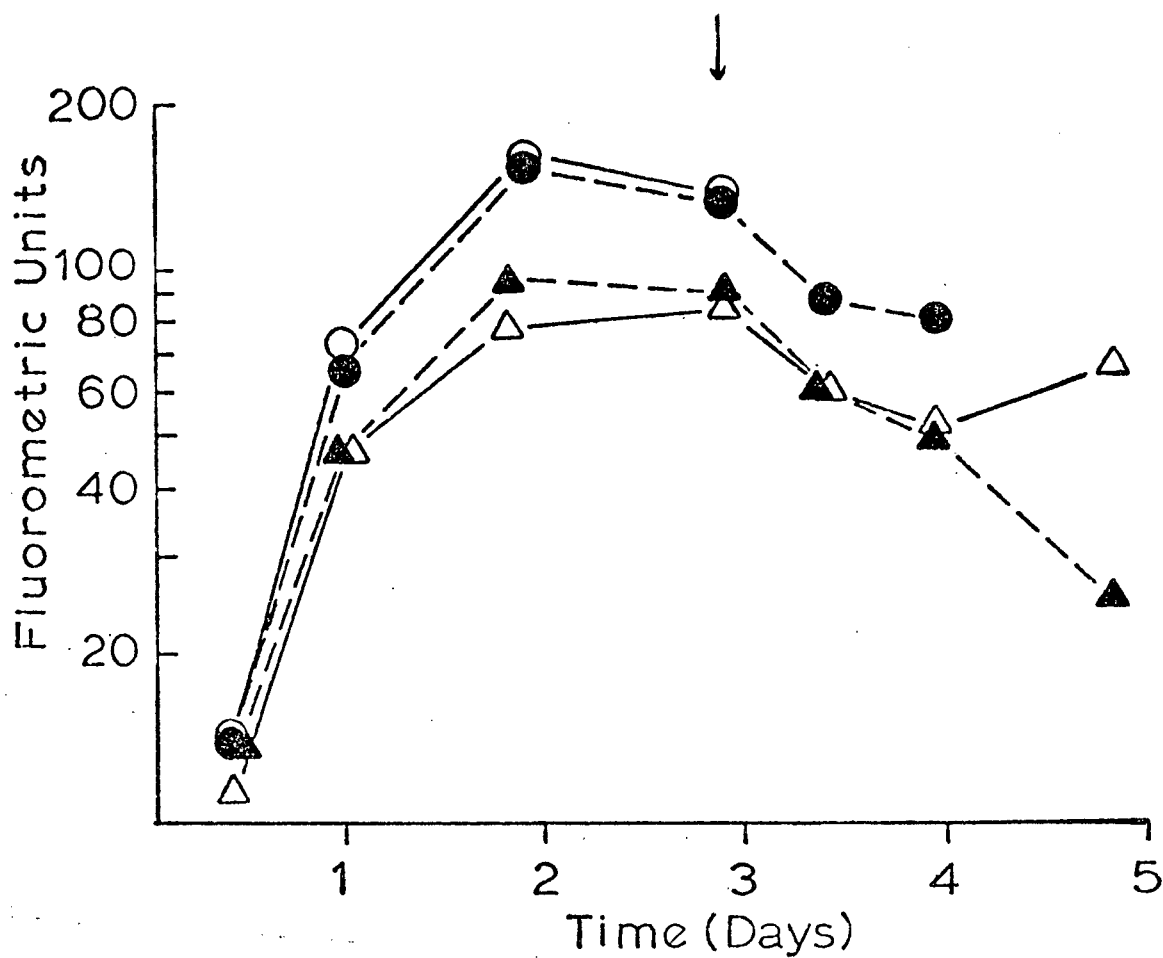
FIGURE 11. Changes in the in vivo fluorescence in the following batch cultures: (◯) culture A; (●) culture B; (△) culture C and (▲) culture D. The time and concentration of mercury exposure are given in Table VII. The arrow indicates the time (70 hours) at which the addition of 5.53 nM $HgCl_2$ was made to cultures B and D.
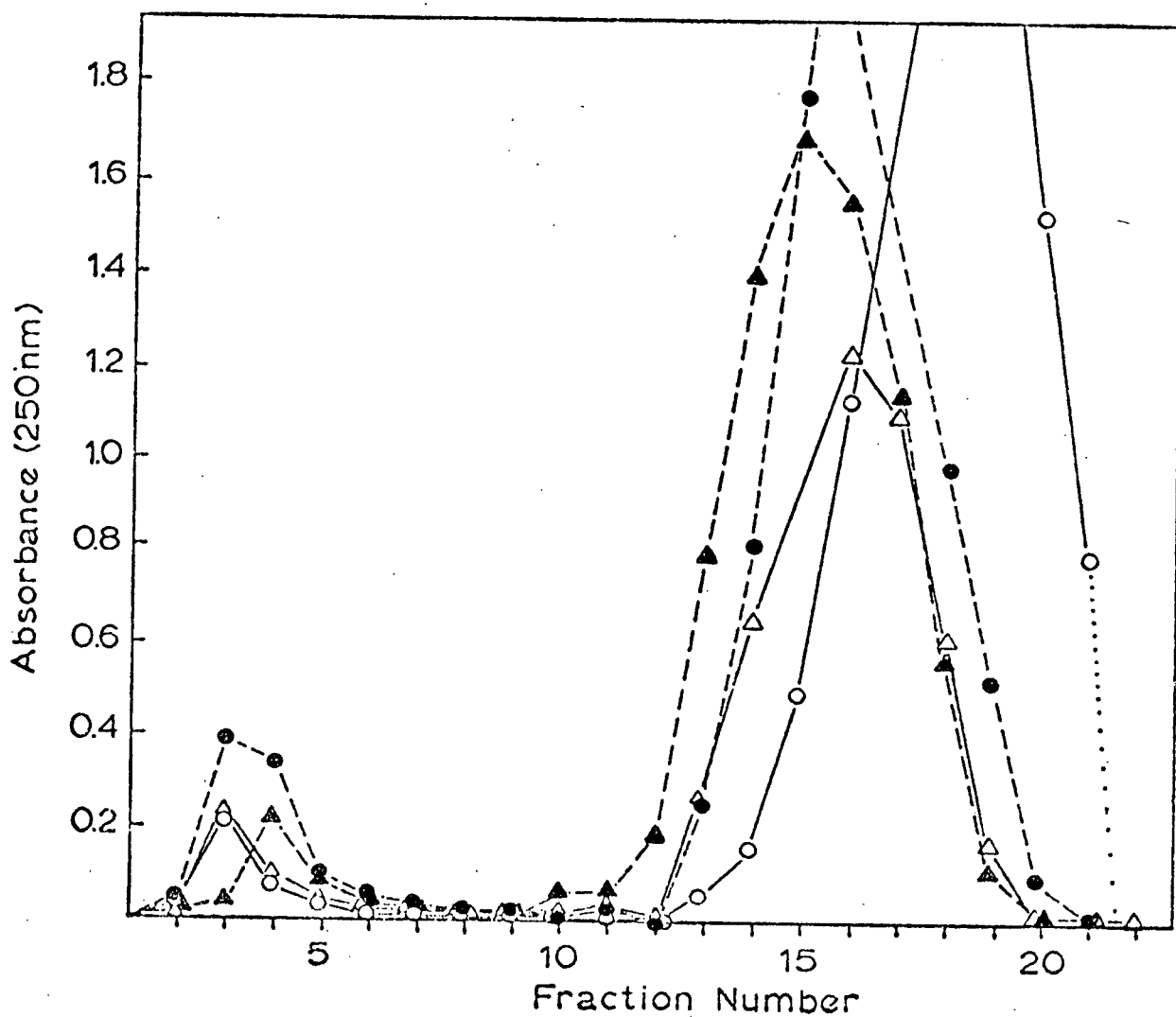
FIGURE 12. The 250 nm absorbance profile of fractions collected from
the followoing cultures: (◯) culture A; (⬤) culture B; (△) culture
C and (▲) culture D. The time and concentration of mercury exposure
are given in Table VII.
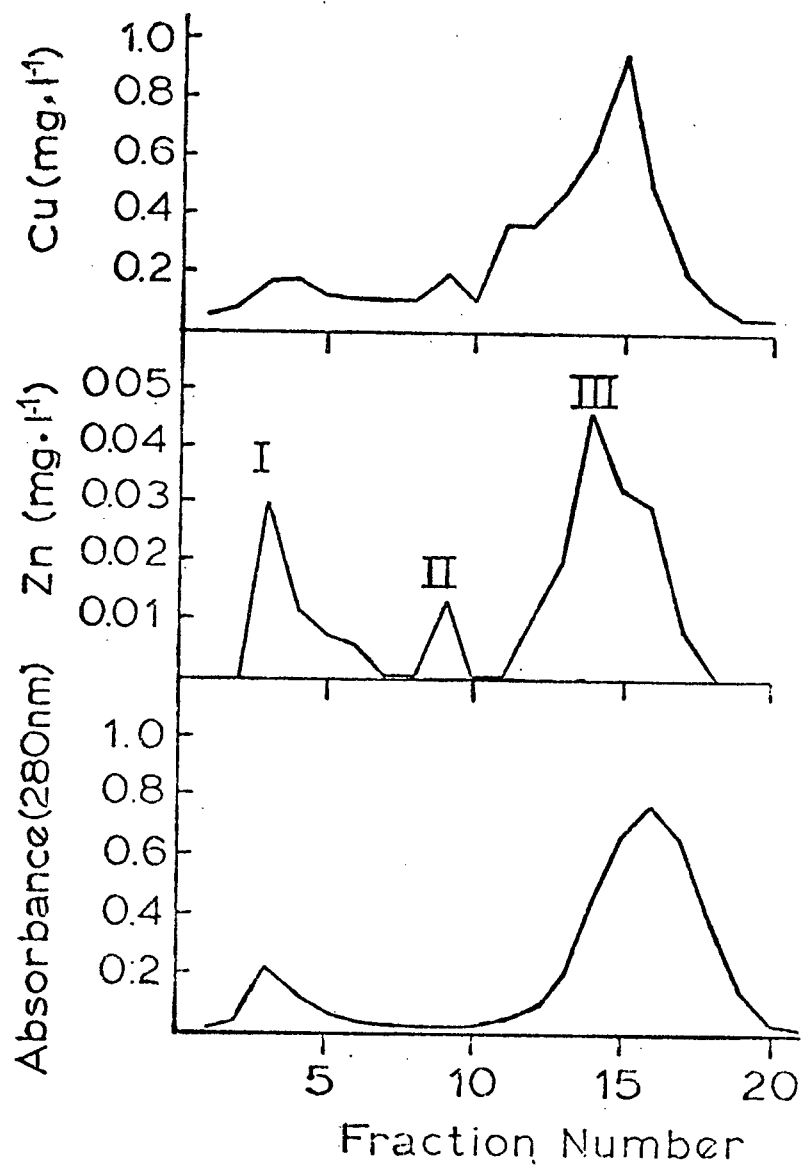
FIGURE 13. The gel elution profile of the 280 nm absorbance and of the amounts of Cu and Zn in intracellular pools of batch culture C, initially exposed to 1.84 nM $HgCl_2$. I = high molecular weight pool; II = medium molecular weight pool, and III = low molecular weight pool.

Cu and Zn in the low m.w. pool (III), 2) large amounts of Zn and lesser amounts of Cu in the high m.w. pool (I), and 3) smaller amounts of Cu and Zn in those fractions which may correspond to the position of metallothionein (II) as determined in previous studies (Bouquegneau et al., 1975). Gel elution profiles from other Hg exposures were similar in shape but the level of metals in each peak varied (Table VIII).

In all cultures, total intracellular Zn and Cu levels decreased and increased, respectively, as a function of Hg concentrations (Table VIII). Total intracellular Zn decreased by 38 to 50% due to Hg exposure. A decrease in Zn occurred in the high and low m.w. fractions and a gradual increase of Zn occurred in the medium m.w. fractions. Total Cu increased by 20% in cultures B and D which were perturbed with a highest additions of $HgCl_2$ (5.53 nM). Copper increased in the high m.w. fractions, whereas Cu levels in the medium m.w. fractions remained constant (ca. 12.5% of total intracellular Cu).

Total intracellular Hg was only detectable in cultures B and D which were perturbed by the highest additions of $HgCl_2$ (5.53 nM). In culture B, 85% of the Hg appeared in the high m.w. pool. Pre-exposure of S. costatum to 1.84 nM $HgCl_2$ (culture D) reduced total Hg levels by 75% when compared to culture B which had no pre-exposure. In culture D, all the accumulated Hg was found over the high m.w. pool. Although Hg values for cultures A, C and E were lower than the Hg

TABLE VIII. Distribution of total Zn, Cu and Hg in the intracellular pools of nutrient-saturated cells with (cultures C, D and E) and without (cultures A and B) previous exposure to $HgCl_2$. Time and concentration of $HgCl_2$ exposure for the different cultures are given in Table VII. Data are the compilation of metal levels ($\mu mole \cdot g \cdot cells^{-1}$ (wet weight)) from profiles such as in Fig. 13. ND = not detectable.

TREATMENT	Zn (µmole·g cells⁻¹)				Cu (µmole·g cells⁻¹)				Hg (µmole·g cells⁻¹)			
	TOTAL	HIGH MW POOL	MEDIUM MW POOL	LOW MW POOL	TOTAL	HIGH MW POOL	MEDIUM MW POOL	LOW MW POOL	TOTAL	HIGH MW POOL	MEDIUM MW POOL	LOW MW POOL
CULTURE A	.0231	.0083	ND	.0148	.270	.032	.035	.203	ND	ND	ND	ND
CULTURE E	.0136	.0065	ND	.0071	.275	.044	.034	.197	ND	ND	ND	ND
CULTURE C	.0115	.0035	.0009	.0071	.253	.041	.032	.180	ND	ND	ND	ND
CULTURE B	.0144	.0046	.0011	.0087	.328	.042	.040	.246	.0138	.0117	.0021	ND
CULTURE D	.0130	.0035	.0017	.0078	.326	.062	.040	.223	.0034	.0034	ND	ND

LEAF 74 OMITTED IN PAGE NUMBERING.

standards, a qualitative examination of the Hg analysis showed that total intracellular Hg increased with Hg concentrations, and the highest levels of Hg were found in the high m.w. pool followed by the medium m.w. fractions.

Section 4. Discussion

Even though a concentration as low as 0.37 nM $HgCl_2$ failed to reduce growth rates, it was sufficient to change the levels and distribution of intracellular Cu and Zn in S. costatum. The displacement of Zn by Hg on metallothionein has also been observed by Kagi and Vallée (1960). Displacement of Zn and Cu by other heavy metals appears to be a general characteristic of metal-induced binding proteins.

In S. costatum, more Cu than Zn occurred in the medium m.w. fractions, which is not typical of metallothioneins. This indicates that this protein may not play a major role in the detoxification of heavy metals. In ducks, when the high m.w. (enzyme-containing) pool was apparently Zn saturated, excess Cu and Zn occurred in metallothionein (Brown and Chatel, 1978). In Cd and Hg-induced thioneins in rat livers, Zn appeared in approximately half the binding sites (Winge et al., 1975), or in residual amounts with smaller amounts of Cu in animals (Kagi and Vallée, 1960).

In S. costatum, the decrease of total intracellular Zn at any Hg concentration and of Hg upon Hg-preexposure may be regulated by an exclusion mechanism. When the freshwater

green alga <u>Chlorella</u> was exposed to Zn, a temperature sensitive component of the Zn uptake was inhibited and the number of Zn exchange sites on the cell wall was reduced (De Filippis and Pallaghy, 1976c).

At exposures equal to or greater than 1.84 nM $HgCl_2$, reduction of growth rates coincided with the detection of Hg in the high m.w. pool. A similar pattern was also found in chum salmon and a summer 'zooplankton' assemblage (Brown and Parsons, 1978) when deleterious effects were observed. In animals, failure to detoxify heavy metals occurs as the rate of bioaccumulation of these heavy metals exceeds the rate of metallothionein synthesis or its binding capacity. The excess of heavy metals then appears in the high m.w. pool with simultaneously occurring pathological effects (Bouquegneau <u>et al</u>., 1975; Yoshikawa, 1976; Brown and Parsons, 1978). Deleterious effects can be caused by the displacement of Cu and Zn from metalloproteins by heavy metals rendering the proteins non-functional (Friedberg, 1974; Bremmer and Davies, 1975). Since elements of subgroups IIb (eg. Zn, Cd and Hg) have a high binding affinity for sulfhydryl groups, denaturation of protein at the tertiary and quarternary structural levels could occur.

The decrease of Zn in the high and low molecular weight pools, accompanied by a simultaneous increase of Zn in the medium m.w. pool was found in <u>S. costatum</u> in this study. This observation has also been reported for the summer 'zooplankton'. The medium m.w. pool in <u>S. costatum</u> does not

appear to be the main storage site for Cu and Zn. In $\underline{S}$. $\underline{costatum}$ approximately 70% and 60% of total Cu and Zn, respectively were found in the low m.w. pool like in 'zooplankton' and unlike higher organisms. This pool may act as a reservoir of Zn and possibly of Cu, for the enzymes in the high m.w. pool. It may play a dominant role in the metabolism and detoxification of trace metals when they are present in excessive amounts. The low m.w. pool contains organics such as amino acids, nucleic acids, etc., which are known as metal complexing agents. Zinc-taurine, Cu-taurine and Cu-betaine homarine complexes were separated from heavy metal exposed oysters, $\underline{Ostrea}$ $\underline{edulis}$ and $\underline{Crassostrea}$ $\underline{gigos}$, in which no evidence of animal-like metallothionein was found (Howard and Nickless, 1977a; 1977b).

In summary, three observations indicate that metallothionein in $\underline{S}$. $\underline{costatum}$ may not play as major a role in the detoxification of Hg as in vertebrates; 1) the failure to detect a major absorbance peak in the medium m.w. pool, 2) the greater amount of Cu than Zn (about three fold) in the medium m.w. fractions and, 3) the increase of Hg in the high m.w. pool. Despite higher levels of Hg occurring in the high m.w. pool, it is possible that the amino acids of the low m.w. pool may be involved in the detoxification of lower Hg levels or the acquisition of tolerance.

SUMMARY

The aim of this study was to examine the growth and
nutrient uptake kinetic responses of an ammonium-limited
diatom during short or long-term exposures to sublethal
concentrations of $HgCl_2$. The cosmopolitan neritic centric
diatom Skeletonema costatum, which is a predominant species
during the vernal bloom, was used in these experiments.

To achieve this goal, two major steps were followed. At
first, a gross range of sublethal concentrations was
determined using batch cultures enriched with 'f/25' medium.
Secondly, the effects of short-term mercury exposures on
ammonium-limited cells grown in chemostat cultures were
determined by simultaneously adding ammonium and one of the
sublethal concentrations of $HgCl_2$. Only concentrations equal
to or greater than 1.84 nM $HgCl_2$ decreased the assimilation
rate (Vi_{max}) and the affinity for the substrate. In an
attempt to increase the sensitivity to a secondary stress
(Hg), effluents from the chemostats were starved for an
average period of 30 hours. These experiments indicated than
ammomium starvation lowered the threshold effect of $HgCl_2$ to
0.18 nM.

The results of these first two steps provided useful
information in the designing of the long-term mercury exposure
experiment. In the latter experiment, two ammonium-limited
chemostat cultures were exposed to 0.37 and 3.68 nM $HgCl_2$.

Mercury was semi-continuously added at regular time intervals during a period of 30 days. The lowest concentration used in this experiment did not decrease the maximum growth rates of nutrient-saturated cultures (cultures grown in 'f/2' and 'f/25') nor did it affect the nutrient uptake kinetics of ammonium-limited cultures in the short-term mercury exposure.

In this experiment, the imposition of long-term mercury exposure on top of a primary stress (nutrient limitation), affected the different phases of uptake and the growth of exposed populations. Decimation of the populations in the mercury-treated cultures resulted in conditions of nutrient saturation, during which minimum cell densities occurred. A minor growth rate decline was observed in the control culture but conditions of nutrient limitation were maintained. Growth rates simultaneously resumed in all cultures implying that mercury additions did not interfere with the occurrence of sexual reproduction in mercury-treated cultures. This period of growth decline was followed by a return to a new steady-state.

In the long-term exposure to 0.37 nM $HgCl_2$, a decrease in the affinity for the substrate (increase in Ks) occurred. The maximum (Vs) and internally controlled (Vi_{max}) rates of uptake were not affected in the long-term exposures to 0.37 nM $HgCl_2$ and in the short-term exposures up to 1.84 nM $HgCl_2$. Long-term exposure to 3.68 nM $HgCl_2$ decreased; 1) the substrate affinity (increased Ks), 2) the initial rapid transport of the substrate across the cell membrane at high (5 μM NH_4Cl)

nutrient levels, Vs, and 3) the internally controlled assimilatory rate, Vi_{max}.

In general, mercury inhibition on the nutrient kinetics of ammonium-limited cells appeared to be uncompetitive.

During the new steady-state in Hg-treated cultures, the affinity for the substrate (Ks), and the assimilatory rate (Vi_{max}) of uptake were increased in phase D (day 23) compared to phase A (day 6). Recovery appeared to be partial in the chemostat exposed to 3.68 nM $HgCl_2$. Improvement in nutrient kinetics and changes in cell morphology in all cultures probably reflected the acquisition of mercury tolerance. These changes were partially related to the appearance of cells of a different life cycle stage.

An attempt was made to determine whether a short-term physiological adaptation (mercury induced synthesis of metallothionein) could be responsible for the recovery through the sequestration of heavy metals by intracellular complexing agents. The failure to detect an ultra-violet absorbance peak in the medium molecular weight pool, where metallothionein usually occurs in animals exposed to heavy metal and high levels of Hg in the high m.w. pool, suggested that this protein may not play a major role in the metabolism and detoxification in short-term Hg exposure. In the long-term mercury exposure, the absorbance and the metal profiles of cells which recovered from mercury inhibition were not measured and therefore the role of metallothionein as a detoxification protein could not be ascertained.

Concomitantly, the effects of sublethal concentrations of $HgCl_2$ (0.37 to 5.53 nM $HgCl_2$) on the intracellular levels and distribution of Hg, Cu and Zn was also examined using batch cultures. Increasing mercury concentrations resulted in the decrease and increase of intracellular levels of Zn and Cu, respectively, as well as the increase of intracellular Hg. Mercury concentrations equal to or higher than 1.84 nM resulted in the accumulation of Hg in the high molecular weight fractions which could be responsible for the decrease in growth rates. In cells pre-exposed to a lower mercury concentration prior to the addition of a second and higher concentration, the intracellular Hg levels decreased. This was possibly due to the development of a physiological adaptation, e.g., an exclusion mechanism.

REFERENCES

Andren, A.W. and R.C. Harriss: Observations on the association between mercury and organic matter dissolved in natural waters. Geochem. Cosmochem. Acta 39, 1253-1257 (1975)

Armstrong, F.A.J., C.R. Stearns and J.D.H. Strickland: The measurements of upwelling and subsequent biological processes by means of the Technicon AutoAnalyser® and associated equipment. Deep-Sea Res. 14, 381-389 (1967)

Ben-Bassat, D., D.G. Skelef and G. Gruner and H.I. Shuval: Growth of _Chlamydamonas_ in a medium containing mercury. Nature (London) 240, 43-44 (1972)

-- and A.M. Mayer: Volatilization of mercury by algae. Physiol. Plant. 33, 128-132 (1975)

-- and --: Reduction of mercuric chloride by _Chlorella_: Evidence for a reducing factor. Physiol. Plant. 40, 157-162 (1977)

-- and --: Light-induced Hg volatilization and O_2 evolution in _Chlorella_ and the effect of DCMU and methylamine. Physiol. Plant. 42, 33-38 (1978)

Bentley-Mowat, J.A. and S.M. Reid: Survival of marine phytoplankton in high concentrations of heavy metals, and uptake of copper. J. Exp. Mar. Biol. Ecol. 26, 249-264 (1977)

Berland, B.R., D.J. Bonin, O.J. Guérin-Ancey, U.I. Kapkov and D.P. Arlhac: Action des métaux lourds à des doses sublétales sur les caractéristiques de la croissance chez la diatomée _Skeletonema costatum_. Mar. Biol. 42, 17-30 (1977)

Betz, M.: Investigations on the simultaneous uptake and release of mercury by _Dunaliella tertiolecta_. Mar. Biol. 41, 89-97 (1977)

Blinn, D.W., T. Tompkins and L. Zaleski: Mercury inhibition on primary productivity using large volume plastic chambers _in situ_. J. Phycol. 13, 58-61 (1977)

Bouguegneau, J.M., C. Gerday and A. Disteche: Fish mercury-binding thionein related to adaptation mechanisms. FEBS. Lett. 55, 173-177 (1975)

Bræk, G.S., A. Jense and A. Mohus: Heavy metal tolerance of marine phytoplankton. III. Combined effects of copper and zinc ions on cultures of four common species. J. Exp. Mar. Biol. Ecol. 25, 37-50 (1976)

Bremmer, I. and N.T. Davies: The induction of metallothio-nein in rat liver by Zn injection as restriction of food intake. Biochem. J. 149, 733-738 (1975)

Bringhmann, G. and R. Kuhn: The toxicity effects of waste water on aquatic bacteria, algae, and small crustaceans. Gesund. Ing. 80, 115 (1959)

Brown, D.A.: Increase of Cd and Cd:Zn ratio in the molecular weight pool from apparently normal liver of tumor bearing flounders (Parophrys vetulus). Mar. Biol. 44, 203-209 (1977)

-- and K.W. Chatel: Interactions between cadmium and zinc in cytoplasm of duck liver and kidney. Chem-Biol. Interact. (in press) (1978)

-- and T.R. Parsons: Relationships between cytoplasmic distribution of mercury and toxic effects to zooplankton and chum salmon (Onchorhycus keta) exposed to Hg in a controlled ecosystem. J. Fish. Res. Bd. Can. 35, 880-884 (1978)

Buhler, R.H.O. and J.H.R. Kagi: Human hepatic metallothio-nein. FEBS. Lett. 39, 229-234 (1974)

Burkett, R.D.: Uptake and release of methyl-mercury-203 by Cladophora glomerata. J. Phycol. 11, 55-59 (1975)

Caperon, J. and J. Meyer: Nitrogen limited growth of marine phytoplankton. Part II. Uptake kinetics and their role in nutrient limited growth of phytoplankton. Deep-Sea Res. 19, 619-632 (1972)

Clark, D.L., A.A. Weiss and S. Silver: Mercury and organomercurial resistances determined by plasmids in Pseudomonas. J. Bacteriol. 132, 186-196 (1977)

Cleland, W.: The statistical analyses of enzyme kinetic data. Adv. Enzymol. 29, 1-32 (1967)

Conway, H.L., P.J. Harrison and C.O. Davis: Marine diatoms grown in chemostats under silicate and ammonium limitation. II. Transient response of _Skeletonema costatum_ to a single addition of the limiting nutrient. Mar. Biol. 35, 187-199 (1976)

-- and --: Marine diatoms grown in chemostats under silicate or ammonium limitation. IV. Transient response of _Chaetoceros debilis_, _Skeletonema costatum_ and _Thalassiosira gravida_ to a single addition of the limiting nutrient. Mar. Biol. 43, 33-44 (1977)

Cook, J.: Environmental pollution by heavy metals. Intern. J. Environmental Studies 9, 253-266 (1977)

Coombs, J., W.M. Darley, O. Holm-Hansen and B.E. Volcani: Studies of the biochemistry and fine structure of silica shell formation in diatoms. Chemical composition of _Navicula pelliculosa_ during silicon-starvation synchrony. Pl. Physiol., Lancaster 42, 1601-1606 (1967)

Corner, E.D.S. and B.W. Sparrow: The modes of action of toxic agents. I. Observations on the poisoning of certain crustaceans by copper and mercury. J. Mar. Biol. Assn. U.K. 35, 531-548 (1956)

Cossa, D.: Sorption du cadmium par une population de la diatomée _Phaeodactylum tricornutum_ en culture. Mar. Biol. 34, 163-167 (1976)

--: The growth kinetics of _Isochrysis galbana_ in cultures containing sublethal concentrations of mercuric chloride. J. Mar. Biol. Assn. U.K. 54, 157-169 (1974)

--: An assessment of the bases of mercury tolerance in _Dunaliella tertiolecta_. J. Mar. Biol. Assn. U.K. 56, 31-37 (1976)

Davis, C.O., P.J. Harrison and R.C. Dugdale: Continuous culture of marine diatoms under silicate limitation. I. Synchronized life cycle of _Skeletonema costatum_. J. Phycol. 9, 175-180 (1973)

De Fillipis, L.F. and C.K. Pallaghy: Localization of zinc and mercury in plant cells. Micron. 6, 111-120 (1975b)

-- and --: The effects of sub-lethal concentrations of mercury and zinc on _Chlorella_ I. Growth characteristics and uptake of metals. Z. Pflanzenphysiol. Bd. 78, 197-207 (1976a)

-- and --: II. Photosynthesis and pigment compositon. Z. Pflanzenphysiol. 78, 197-206 (1976b)

-- and --: III. Development and possible mechanisms of resistance to metals. Z. Pflanzenphysiol. 79, 323-335 (1976c)

Department of Fisheries and Forestry: Pollution and the fisheries. Ottawa (1971)

Dolar, S.G., D.K. Keeney and G. Chesters: Mercury accumulation by Mariophyllum spicatum L. (watermilfoil). Env. Lett. 1, 191-198 (1971)

Dunstan W.M. and D.W. Menzel: Continuous cultures of natural populations of phytoplankton in dilute, treated sewage effluent. Limnol. Oceanogr. 16, 623-632 (1971)

Erickson, S.E., N. Lackie and T.E. Maloney: A screening technique for estimating copper toxicity to estuarine phytoplankton. J. Wat. Poll. Control. Fed. 48, R-270-278 (1970)

--: Toxicity of copper to Thalassiosira pseudonana in unenriched inshore seawater. J. Phycol. 8, 318-323 (1972)

Falkowski, P.G. and D.P. Stone: Nitrate uptake in marine phytoplankton: Energy sources and the interactions with carbon fixation. Mar. Biol. 32, 74-84 (1975)

Femreite, N.: Mercury hazards in Canada and their possible hazards as a source of mercury contamination. Env. Poll. 1, 119-131 (1970)

Fitzgerald, G.P. and S.R. Faust: Factors affecting the algicidal and algistatic properties of copper. Appl. Microbiol. 11, 345-351 (1963)

Friedberg, F.: Effects of heavy metal binding on protein structure. Quart. Rev. Biophys. 7, 1-33 (1974)

Fujita, M. and K. Hashizume: Status of mercury in the freshwater diatom, Synedra ulna. Wat. Res. 9, 889-894 (1975)

--, K. Iwasaki and E. Takabatake: Intracellular distribution of mercury in freshwater diatom, Synedra cells. Env. Res. 14, 1-13 (1977)

Gardiner, J.: Complexation of trace metals by ethylenediaminetetraacetic acid (EDTA) in natural waters. Wat. Res. 10, 507-514 (1976)

Gardner, D.: Observations on the distribution of dissolved mercury in the ocean. Mar. Poll. Bull. 6, 43-46 (1975)

George, S.G. and T.L. Coombs: The effects of chelating agents on the uptake and accumulation of cadmium by Mytilus edulis. Mar. Biol. 39, 261-268 (1977)

Gibson, V.R., T.J. Lawson and G.D. Grice: Cepex: The effects of copper on zooplankton during the June 1974 experiment. CEPEX, Tech. Inform. Release #2, (August 1975)

Gibson, C.E.: The algicidal properties of copper on a green and blue green alga and some ecological implications. J. Appl. Ecology 9, 513-518 (1972)

Glooschenko, W.A.: Accumulation of ^{203}Hg by the marine diatom Chaetoceros. J. Phycol. 5, 224-226 (1969)

Grice, C.D., M.R. Reeve, P. Koeller and D.W. Menzel: The use of large volume, transparent, enclosed sea-surface water columns in the study of stress on plankton ecosystem. Helgolander wiss Meeresunters 30, 118-133 (1977)

Guillard, R.R.L. and J.H. Ryther: Studies on marine planktonic diatoms. I Cyclotella nana (Hustedt) and Detonula confervacea (Cleve). Can. J. Microbiol. 8, 229-239 (1962)

Hager, S.W., L.I. Gordon and P.K. Park: A practical manual for the use of the Technicon AutoAnalyzer® in seawater nutrient analyses, 31 pp. Tech. Rep. 68-33, Department of Oceanography, Oregon State University, Corvallis (1968)

Hammans, J.W.K., C.P.L.M. Rabou and H.Q. Pietersen: Participation of pigment complexes in uptake and incorporation of mercury ions by Anacystis. Photosynth. 10, 440-446 (1976)

Hannan, P.J. and C. Patouillet: Effect of mercury on algal growth rates. Biotech. and Bioeng. 14, 93-101 (1972)

--, P.E. Wilkness, C. Patouillet and R.A. Carr: Measurements of mercury sorption by algae. Naval Research Laboratory, Washington D.C. 20375, NRL Report 7628 (1973)

Harrison, P.J., H.L. Conway and R.C. Dugdale: Marine diatoms grown under silicate and ammonium limitation. I. Cellular chemical composition and steady-state growth kinetics of Skeletonema costatum. Mar. Biol. 35, 177-186 (1976)

--, --, R.W. Holmes and C.O. Davis: Marine diatoms grown in chemostats under silicate limitation. III. Cellular chemical composition of Chaetoceros debilis, Skeletonema costatum and Thalassiosira gravida. Mar. Biol. 43, 19-31

(1977)

Harriss, R.C., D.B. White and L.B. MacFarlane: Mercury com-
pounds reduce photosynthesis by phytoplankton. Science
736-737 1970 (1970)

Hatch W.R. and W.L. Ott: Determination of sub-microgram
quantities of Hg by atomic absorption spectrophotometry.
Analyt. Chem. 40, 2085-2087 (1968)

Hollibaugh, J.T., D.L.R. Seibert and W.H. Thomas: A
comparison of the acute toxicities of ions of ten heavy
metals to phytoplankton from Saanich Inlet, Canada,
unpublished manuscript

Holmes, R.W.: Solar radiation, submarine daylight and photo-
synthesis, 109-128. In: Treatise on marine ecology and
paleoecology, Ecology 1, New York: Geological Society of
America (1957)

Howard, A.G. and G. Nickless: Heavy metal complexation in
polluted molluscs II. Oysters (Ostrea edulis and
Crassostrea gigos), Chem.-Biol. Interact. 17, 257-263
(1977a)

-- and --: Heavy metal complexation in polluted molluscs. I.
Limpets (Patella vulgata and Patella intermedia). Chem.-
Biol. Interact. 16, 107-114 (1977b)

Hunter, R.W.: The poisoning of Marinogammus marinus by
cupric sulfate and mercuric chloride, J. Exp. Biol. 26,
113 (1949)

Jensen, A., B. Rystad and S. Melsom: Heavy metal tolerance
in marine phytoplankton. I. The tolerance of three algal
species to zinc in coastal seawater. J. Exp. Mar. Biol.
Ecol. 15, 145-157 (1974)

--, -- and --: Heavy metal tolerance of marine
phytoplankton. II. Copper tolerance of the species in
dialysis and batch cultures. J. Exp. Mar. Biol. Ecol. 22,
249-256 (1976)

Kagi, J.H.R. and B.L. Vallée: Metallothionein: a cadmium and
zinc containing protein from equine and renal cortex, J.
Biol. Chem. 239, 3460-3465 (1960)

Kamp-Nielsen, L.: The effects of deleterious concentrations
of mercury on the photosynthesis and growth of Chlorella
pyrenoidosa. Physiol. Plant. 24, 256-261 (1971)

Kayser, H.: Waste water assay with continuous agal cultures: The effects of mercuric acetate on the growth of some marine dinoflagellates. Mar. Biol. 36, 61-72 (1976)

--: Effect of zinc sulphate on the growth of mono and multispecies cultures of some marine plankton algae. Helgolander wiss Meeresunters 30, 682-696 (1977)

Keckes, S. and J.K. Miettenen: Mercury as a marine pollutant, 276-289. In: Marine pollution and sea life. Fishing News (Books) Ltd, London, (1972)

Kester, D.R., I.W. Duedall, D.H. Connors and R.M. Pytkowicz: Preparation of artificial seawater. Limnol. Oceanogr. 12, 176-178 (1967)

Kiminura, M. and S. Katok: Studies on electron transport associated with photosystem II. Functional site of plastocyanin; inhibitory effect of $HgCl_2$ on electron transport and plastocyanin in chloroplasts. Biochem. Biophys. Acta. 283, 268-278 (1972)

Knauer, G.A. and J.H. Martin: Mercury in a marine pelagic food chain. Limnol. Oceanogr. 17, 868-876 (1972)

Knowles, S.C. and G. Zingmark: Mercury and temperature interactions on the growth rates of three species of fresh water phytoplankton. J. Phycol. 14, 104-109 (1978)

Komura, I. and I. Kaziro: Mechanisms of $HgCl_2$ resistance in microorganisms. I. Vaporization of a Hg compound from $HgCl_2$ by multiple drug resistant strains of E. coli. J. Biochem. (Japan) 70, 885-893 (1971)

Koroleff, F.: Information, technique and methods for sea-water analyses. Interlab. Rep. 3, 19-22 (1970)

Krenkel, P.A.: Mercury: environmental considerations. Part I. C.R.C. Environ. Control 3, 303-404 (1973)

Krock, H.J. and D.T. Mason: Bioassay of lower trophic levels. Vol. VI . Eng. Res. Lab., University of California, Berkeley Rept. 71-8, 123 pp. (1971)

Kuiper, J. :Development of North Sea coastal plankton communities in separate plastic bags under identical conditions. Mar. Biol. 44, 97-109 (1976)

Leber, A.P.: A mechanism for cadmium and zinc induced tolerance to cadmium toxicity: involvement of metallothionein. 72 pp. Purdue University, Ph.D. Dissertation, University Microfilms, Ann Arbor Michigan, (1974)

Lehninger, A.L.: Biochemistry. Worth Publ., New York, 1041 pp. (1975)

Lewis, A.G., A. Ramnarine and M.S. Evans: Natural chelators - an indication of activity with a calanoid copepod Euchaeta japonica. Mar. Biol 11, 1-4 (1971)

--, P. Whitfield and A. Ramnarine: The reduction of copper toxicity in a marine copepod by sediment extract. Limnol. Oceanogr. 18, 324-326 (1973)

Matida, Y., H. Kumoda, S. Kimura, U. Siaqa, T. Nose, M. Yokote and H. Kawatsu: Toxicity of mercury compounds to aquatic organisms by accumulation of the compounds by the organisms. Bull. Freshwater Fish. Res. Lab., Tokyo 21, 197-227 (1971)

Matson, R.S., G.E. Mustoe and S.B. Chang: Mercury inhibition on lipid biosynthesis in freshwater algae. Env. Sci. Tech. 61, 158-160 (1972)

McBrien D.C.H. and K.A. Hassak: The effects of toxic doses of copper on Chlorella vulgaris. Physiol. Plant. 20, 113-117 (1967)

McLachlan, J.: Growth media-marine, 25-51. In: Handbook of phycological cultures, methods and growth measurements, Ed. J.R. Stein, Cambridge University Press, Cambridge, (1973)

McLean, F.I., O.J. Lucis, Z.A. Shakh and E.E.R. Jansz: The uptake and subcellular distribution of Cd and Zn in microorganisms. Fedn. Proc. Am. Socs. Exp. Biol. 31, 699 (1972)

Morel, N.M.L., J.G. Rueter and F.M.M. Morel: Copper toxicity to Skeletonema costatum (Bacillariophycae). J. Phycol. 14, 43-48 (1978)

Murphy, J. and Riley, J.P.: A modified single solution method for the determination of phosphate in natural waters. Anal. Chem. Acta. 27, 31-36 (1962)

Nelson, H., W. Blair, F.E. Brinckman, R.R. Colwell and W.P. Iverson: Biodegradation of phenylmercuric acetate by mercury resistant bacteria. Appl. Microbiol. 26, 321-326 (1973)

--, J.D. and R.R. Colwell,: The ecology of mercury-resistant bacteria in Chesapeake Bay. Microbiol. Ecol. 1, 191-218 (1975) --, B.R. Larsen, E.A. Jenne and D.H. Sorq: Mercury dispersal from lode sources in the Kuskokwina River Drainage, Alaska. Science 198, 820-824 (1977)

Nuzzi, R. :Toxicity of mercury to phytoplankton. Nature 237, 38-40 (1972)

Overnell, J: The effects of heavy metals on photosynthesis and cell potassium in two species of marine algae, Dunaliella tertiolecta and Phaeodactylum tricornutum. Mar. Biol. 29, 99-103 (1975a)

--: The effect of some metal ions on photosynthesis in a freshwater alga. Pestic. Biochem. Physiol. 5, 19-26 (1975b)

--: Inhibition of marine algal photosynthesis by heavy metals. Mar. Biol. 38, 335-342 (1976)

Peterson, G.R. H.V. Warren, R.E. Delavault and R. Fletcher: Heavy metal content of some freshwater fishes in British Columbia. B.C. Fish and Wildlife Branch, Tech. Circular, (1970)

Piscator, M.: On cadmium in normal human kidney with a report on the isolation of metallothionein from livers of cadmium exposed rabbits. N. Hyg. Tidskr. 45, 76-82 (1964)

Pyefinch, K.A. and J.C. Mott: The sensitivity of barnacles and their larvae to copper and mercury. J. Exp. Biol. 25, 276-298 (1948)

Rice, H.V., D.A. Leighty and G.C. McLeod: The effects of some trace metals an marine phytoplankton. C.R.C. Microbiol. 3, 27-44 (1973)

Richardson, T.R., W.F. Millington and H.M. Miles: Mercury accumulation in Pediastrum boryanum (Chlorophyceae). J. Phycol. 11, 320-323 (1975)

Say, P.J., B.M. Diaz and B.A. Whitton: Influences of zinc on lotic plants. I. Tolerance of Hormidium species to zinc. Freshwater Biol. 7, 357-376 (1977)

Schulz-Baldes, M. and R.A. Lewin: Lead uptake in two marine phytoplanktonic organisms. Biol. Bull. 150, 118-127 (1976)

Seyferth, D.: An infamous element. Science 200, 679-680 (1978)

Shieh, Y.J. and J. Barber: Uptake of mercury by Chlorella and its effects on potassium regulation. Planta (Berl.) 109, 43-60 (1972)

Singer, P.C.:Trace metals and metal-organic interactions in natural waters. Ann Arbor Sci. Publ. Inc., Ann Arbor. 380 pp. (1973)

Slawyk, G. and J.J. MacIsaac: The comparison of two automated ammonium methods in a region of coastal upwelling. Deep-Sea Res. 19, 521-524 (1972)

Steemann Nielsen, E., L. Kamp-Nielsen and S. Wium-Andersen: Influence of deleterious concentrations of copper of the growth of Chlorella pyrenoidosa. Physiol. Plant.23, 828-840 (1970).

-- and S. Wium-Andersen: The influence of Cu on photosynthesis and growth in diatoms. Physiol. Plant. 24, 480-484 (1971)

Stockner, J.G. and N.J. Antia: Phytoplankton adaptation to environmental stresses from toxicants, nutrients and pollutants - a warning. J. Fish. Res. Bd. Can. 33, 2089-2096 (1976)

Stokes, P.M., T.C. Hutchinson and K. Krauter: Heavy metal tolerance in algae isolated from contaminated lakes near Sudbury, Ontario. Can. J. Bot. 51, 2155-2168 (1973)

Strickland, J.D.H. and T.R. Parsons: A practical handbook of seawater analysis. Fish. Res. Bd. Can., Ottawa, Bull. 167, 267-279 (1972)

Summers, A.O. and E. Lewis: Volatilization of $HgCl_2$ by mercury resistant plasmid bearing strains of E. coli, Staphylococcus aureus and Pseudomonas aeruginosa. J. Bacteriol. 113, 1070-1072 (1973)

Sunda W. and R.R.L. Guillard: The relationship between cupric ion activity and the toxicity of copper to phytoplankton. J. Mar. Res. 34, 511-529 (1976)

Thomas, W.H.: On nitrogen deficiency in tropical Pacific oceanic phytoplankton: Photosynthetic parameters in poor and rich water. Limnol. Oceanogr. 15, 380-385 (1975a)

--: Effect of ammonium and nitrate concentration on chlorophyll increases in natural tropical Pacific phytoplankton populations. Limnol. Oceanogr. 15, 386-394 (1975b)

-- and D.L.R. Seibert : Effects of copper on the dominance and the diversity of algae: Controlled ecosystem pollution experiment. Bull. Mar. Sci. 27, 23-33 (1977)

--, O. Holm-Hansen, D.L.R. Seibert, F. Azam, R. Hodson and M. Takahashi: Effects of copper on phytoplankton standing crop and productivity. Bull. Mar. Sci. 27, 34-43 (1977)

Tompkins, T. and D.W. Blinn :The effects of mercury on the growth rate of *Fragilaria crotonensis* Kitton and *Asterio nella formosa* Hass. Hydrobiol. 49, 111-116 (1976)

Weiss, A.A., S.D. Murphy and S. Silver: Mercury and organo-mercurial resistance determined by plasmids in *Staphylococcus aureus*. J. Bacteriol. 132, 197-208 (1977)

Werner, D.: Der entwicklungscyclus mit sexualphase bei der marinen Diatomee *Coscinodiscus asteromphalus*. II. Oberflächenabhängige differenzierung während der vegetativen zellverkleinerung. Arch. Mikrobiol. 80,115-133 (1971)

White, D.B.: The effects of some mercurials on photosyn-thesis of naturally occuring phytoplankton populations and on the marine diatom, *Nitzschia delicatissima* Cleve. M.Sc. thesis, 53 pp., Department of Oceanography, Florida State University and College of Arts and Sciences, (1970)

Whitfield, P. and A.G. Lewis: Control of the biological availability of trace metals to a calanoid copepod in coastal fjord. Est. Coast. Mar. Sci. 4, 255-266 (1976)

Williams, P.M., E.J. Robertson, K. Chew and H.V. Weiss: Mercury in the South polar seas and in the Northeast Pacific ocean. Mar. Chem. 2, 287-299 (1974)

Windom, H.L., F.E. Taylor and R. Stickney: Mercury in North Atlantic plankton. J. Cons. perm. int. Explor. Mer. 35, 18-21 (1973)

--, -- and E.M. Waiters: Possible influence of atmospheric transport on the total mercury content of south eastern Atlantic continental shelf surface waters. Deep-Sea Res. 22, 629-633 (1975)

Winge, D.R., J. Krasno and A.V. Colucci: Cadmium accumulation in rat liver: correlation between bound metal and pathology, 300-301. In: Trace element metabolism in animals , 2. Ed. W.G. Hoeskstra, J.W. Suttie, H.E. Ganther and W. Mertz, University Park Press, Baltimore (1973)

-- , R. Premakumar and K.V. Rajagolapan: Metal-induced formation of metallothionein in rat liver. Arch. Biochem. Biophys. 170, 242-252 (1975)

Winner, R.W., T. Keeling, R. Yeager and M.P. Farrell: Effect of food type on the acute and chronic toxicity of copper to Daphnia magna. Freshwater Biol. 7, 343-349 (1977)

Wisely, B. and R.A.P. Blick: Mortality of marine inverte-brates larvae in mercury and zinc solutions. Aust. J. Mar. Freshwater Res. 18, 63-72 (1966)

Wobeser, G., N.O. Nielsen and R.M. Dunlop: Mercury concen-tration in tissues of fish from the Saskatchewan River. J. Fish. Res. Bd. Can. 27, 830-834 (1970)

Yoshikawa, H.: Comparison of cadmium treatment and phenobar-bitol treatment for the mitigation of acute cadmium toxicity. Ind. Health 14, 45-46 (1976)

Zingmark, R.G. and T.G. Miller: The effects of mercury on the photosynthesis and growth of estuarine and oceanic phytoplankton, 45-57. In: Physiological ecology of estuarine organisms, Ed. F.T. Vernberg, University of South Carolina Press, Columbia, S.C., (1973)

APPENDIX A. Artificial Seawater Recipe

APPENDIX B. Data derived from the long-term mercury exposure of ammonium-limited $\underline{S}.$ $\underline{costatum}$ to 0.00, 0.37 and 3.68 nM $HgCl_2$. D = dilution rate ($10^{-2} \cdot hr^{-1}$); Fluor = $\underline{in\ vivo}$ fluorescence, using door 3; Cell no = cell numbers ($10^7 cells \cdot 1^{-1}$); Cell lenght (μm); μ = specific growth rate (hr^{-1}); Effl. = effluent concentration of ammonium (μM); Q = the amount of nitrogen per cell ($\mu M\ N \cdot 10^7$ cells); Chl $\underline{a}$ = the amount of chlorophyll $\underline{a}$ per cell (μg chl $\underline{a} \cdot 10^7$ cells^{-1}); D_{430} : D_{665} = absorbance ratio of carotenoid : chlorophyll $\underline{a}$; Chla $\underline{a}$* = estimated chlorophyll $\underline{a}$ using the chlorophyll $\underline{a}$: fluoresence ratio obtained during phase A; P./S.= photosynthetic rate (μg $C \cdot 10^7$ cells$^{-1} \cdot hr^{-1}$).

^{2} fluorescence values obtained using door 30.
^{3} "   "   "   "   " 10.

ARTIFICIAL SEAWATER RECIPE

SALTS	$g \cdot 94.4 \ 1^{-1}$	$g \cdot 1^{-1}$	m.w.	CONCENTRATION (M)
NaF	0.25	.00265	41.99	6.30×10^{-5}
H_3BO_3	2.20	.23305	61.83	3.77×10^{-3}
KBr	8.20	.08686	119.01	7.30×10^{-4}
$NaHCO_3$	16.40	.17373	84.01	2.07×10^{-3}
KCl	56.70	.60063	74.56	8.06×10^{-3}
Na_2SO_4	335.60	3.555	142.04	2.50×10^{-2}
NaCl	2003.0	21.218	58.44	3.63×10^{-1}
$SrCl_2 \cdot 6H_2O$	2.00	.02118	266.62	7.95×10^{-4}
$CaCl_2 \cdot 2H_2O$	127.20	1.347	147.02	9.17×10^{-3}
$MgCl_2 \cdot 6H_2O$	906.90	9.607	203.31	4.73×10^{-2}

Ref: Kester et al., 1967

COMPOSITION OF 'f' MEDIUM

SALTS	STOCK SOLUTIONS $(g \cdot 1^{-1})$	T.M. MIX $(mg \cdot 1^{-1})$	F MIX $(mg \cdot 1^{-1})$	F MEDIUM (M)
$NaNO_3$	150.0		150.0	1.77×10^{-3}
Na_2SiO_3	30.0		30.0	1.06×10^{-4}
KH_2PO_4	10.0		10.0	7.35×10^{-5}
$CuSO_4$	19.6	19.6	0.0196	7.85×10^{-8}
$ZnSO_4$	44.0	44.0	0.044	1.53×10^{-7}
$CoCl_2 \cdot 6H_2O$	20.9	20.9	0.029	8.78×10^{-8}
$MnCl_2 \cdot 4H_2O$	3.6	3.6	0.0036	1.82×10^{-8}
$Na_2MoO_4 \cdot 2H_2O$	12.6	12.6	0.012	5.2×10^{-8}
Ferric Sequestrene[2]	10.0	10,000	10.0	
B_{12}	1.0	1.0	.001	
Fe[2]	1.30	1,300	1.3	2.33×10^{-5}
EDTA[2]	8.72	8,720	8.72	2.34×10^{-5}

Ref: Guillard and Ryther, 1962; 2: McLachlan, 1973

Chemostat Culture II (Control)

DAY	D	FLUOR.	CELL NO.	CELL LENGTH	μ	EFFL.	Q	CHL. a	D430:D665	CHL. a*	P./S.
0	3.60	23.9±0.9	6.37±0.42	15.90±9.63							
1	4.08	18.3±2.4	5.90±0.43	16.28±7.74	.035					1.24	4.58
2	4.09	15.5±0.1	5.88±0.10	16.74±5.24	.048	0.00	1.70	0.83	3.18	1.02	3.37
3	4.00	17.8±0.3	9.32±0.63	21.45±6.30	.060	0.05	1.07	0.918	2.14	0.55	2.77
4	4.00	27.0±0.7	5.20±0.59	19.80±8.46	.016	0.16	1.89	1.095	2.56	1.71	2.26
5	4.10	24.3±0.4	5.61±0.27	23.38±9.39	.044	0.00	1.78	1.459	2.25	1.43	
6	4.36	27.5±1.4	7.22±0.45	18.40±6.94	.053	0.00	1.39	1.214	2.47	1.26	2.52
7	4.00	35.3±2.5	8.39±0.04	19.99±8.58	.048			1.249	1.98	1.39	
8	3.90	28.1±2.5	7.07±1.63	17.13±8.97	.032	0.22	1.38	1.600	2.14	1.31	
9	4.10	24.6±2.4	5.50±0.01		.030					1.48	
10	3.97	29.7±0.6	3.00±0.69		.015					3.27	
11		21.8±0.3	3.98±0.54		.052					1.81	
12	3.96	21.8±1.5	3.14±0.22		.030					2.29	
13	3.96	18.5±1.9	1.69±0.15		.014					3.61	
14	3.80	17.0±0.7	1.48±0.43		.034	0.53	6.38			3.79	
15											
16		14.1±1.7	1.26±0.03							3.69	
17	3.96	20.7±0.8	2.51±0.40	16.37±5.51	.069	0.74	3.69			2.72	
18	3.88	28.8±1.3	7.61±1.05	16.71±7.00	.085	0.13	1.30			1.25	
19	3.92	32.2±0.8	8.62±2.01		.044					1.23	
20	3.92	32.5	9.58±0.30		.044	0.09	1.03			1.12	
21	3.83	33.2±0.8	9.99±0.11	12.97±4.78	.041	0.17	0.98			1.10	
22	4.05	38.9±1.4	14.44±0.63		.055	0.06	0.69			0.89	
23		38.0±0.5	12.36±0.87		.034	0.00	0.61			1.01	
24		40.3±1.3	12.49±0.00		.040					1.06	
25	3.70	40.0±1.3	6.53±1.33	14.81±6.67	.035	0.00	0.87			2.02	
26		39.6±0.5	10.0±0.86		.034	0.43	1.00			1.31	
27	3.94	37.0±1.8	8.93±1.66		.035					1.37	
28	3.90	39.0	10.71±2.05		.047					1.20	
29		37.3±1.0	5.16±0.97		.009					2.39	

Chemostat Culture I (Exposure to 0.37 nM HgCl$_2$)

DAY	D	FLUOR.	CELL NO.	CELL LENGTH	μ	EFFL.	Q	CHL. a	D430:D665	CHL. a*	P./S.
0	3.70	23.8±0.4	5.90±0.36	15.90±9.63					2.51		
1	4.06	15.5±1.0	5.80±0.41	16.28±7.74	.038			0.94	2.41	1.75	5.17
2	4.38	14.0	2.99±0.08	18.53±8.71	.017	0.00	1.72	1.29	2.55	1.16	4.19
3	4.20	16.3±0.7	5.26±0.30	20.3±5.66	.066	0.05	3.33	1.34	1.55	2.03	5.09
4	4.10	10.1±0.9	3.90±0.35	25.38±10.70	.029	0.16	1.87	1.49	2.10	1.81	2.99
5	4.40	15.8±0.5	3.03±0.00	18.7±8.51	.032			3.60	1.53	1.45	
6	4.09	19.4±0.9	2.08±0.24	26.07±11.96	.027	0.13	4.74	2.38	2.32	3.30	5.39
7	4.15	14.5±1.5	2.65±0.05	25.04±12.60	.051			2.03	2.53	3.18	
8	3.90	11.2±1.6	2.86	32.85±13.84	.043	0.93	3.17	3.34	2.04	3.20	
9	4.20	8.1±1.1	1.13±0.02		.001					4.30	
10	4.29	5.3±0.6	0.71±0.13		.023					4.95	
11	4.30	1.8±0.8	0.81±0.07		.049					2.84	
12	4.30	3.3±1.0	0.56±0.22		.028					1.40	
13	4.30	1.9±0.7	0.25±0.14		.062					5.73	
14	4.20	18.3 1.5[2]	0.15		.021	6.27	25.03			5.50	
15											
16		42.7±6.8[2]	0.84±0.02	11.37±6.77							
17	4.00	10.1±2.5	1.4±0.21	13.38±5.08	.062	2.26	5.53			3.13	
18	4.18	28.0±0.4	3.29±0.51	18.92±9.65	.077	0.15	3.00			3.69	
19	4.15	29.8±0.8	4.80±1.09		.057					2.69	
20	4.01	32.5±1.8	8.46±0.46		.064	0.10	1.17			1.67	
21	4.04	32.1±3.0	5.67±0.19		.024	0.15	1.74			2.46	
22	3.90	34.8±2.1	8.55±1.03		.057	0.06	1.16			1.77	
23		39.1±1.6	8.87±0.67		.041	0.00	1.13			1.91	
24		41.2±2.2	9.43±0.52		.043					1.90	
25	3.60	40.1±1.1	9.28±0.46	16.47±6.31	.038	0.00	0.72			1.88	
26		37.7±1.6	8.16±0.79		.033	0.09	1.21			2.01	
27	4.13	36.5±2.1	6.08±0.34		.026					2.61	
28	4.10	34.8±0.3	7.79±0.18		.054					1.94	
29		38.5±0.5	4.99±0.59		.040					3.35	

Chemostat Culture III (Exposure to 3.68 nM $HgCl_2$)

DAY	D	FLUOR.	CELL NO.	CELL LENGTH	μ	EFFL.	Q	CHL. a	D430:D665	CHL. a*	P./S.
0	3.90	23.3	5.56±0.44	15.90±9.63							
1	3.95		5.68	16.28±7.74	.040				1.87		8.10
2	4.04	22.5±0.3	6.08±0.6	16.80±7.41	.043	0.00	1.76	0.71	3.20		4.51
3	4.00	15.7±0.5	5.57±1.19	21.33±6.60	.037	0.06	1.64	1.75	2.31	1.65	3.67
4	3.94	11.9±0.3	2.97±0.27	34.76±10.54	.014	0.16	1.77	1.72	2.49	1.26	2.02
5	3.90	15.8±10.3	2.45±0.12	29.51±11.22	.031			4.43	1.85	1.79	
6	4.09	12.7±0.5	1.32±0.23	28.23±9.70	.014	0.04	7.54	2.35	2.26	2.88	4.54
7	4.00	6.6±0.5	1.06±0.46	35.37±22.79	.031			3.96	1.88	4.29	
8	3.80	4.3±0.5	0.433	23.08±5.60	.003	3.13	15.87	4.92	1.70	2.78	
9	3.90	3.5	0.18	8.05±6.12	.001				0.63	4.43	
10	4.06	2.5								8.67	
11	3.90	15.5±0.4[2]	0.0362								
12	3.96	3.3±2.1[2]	0.00252								
13	3.94	17.2±4.7[2]									
14	3.88	7.7±0.3[2]				7.17					
15											
16		5.7±1.5[2]									
17	3.80	2.8±1.5[2]	0.0167								
18	3.90	5.0±1.4[2]	0.046±0.011		.081	9.46	11.84				
19	3.92	11.3±1.2[2]	0.076±0.005		.060						
20	4.02	11.8±0.5[3]	0.75±0.16		.135	4.99	6.70				
21	3.81	53.3±0.4[3]	3.45±0.59	9.16±4.63	.103	1.42	3.49				
22	3.90	39.5±1.4	9.23±0.34		.080	0.06	1.68			1.91	
23		41.5±1.0	6.00±0.26		.021	0.08	1.65			3.08	
24		39.6±1.4	11.39±0.37		.066					1.55	
25	3.60	39.6±0.5	4.30±0.83	22.0±9.28	.000	0.00	2.33			4.11	
26		33.4±1.0	6.96±0.25		.058	0.06	1.43			2.14	
27	3.81	29.5±0.6	9.77±2.89		.024					1.35	
28	3.90	30.2±2.5	6.81±0.21							1.98	
29		28.3±0.6	5.07±0.67							2.49	