THE ROLE OF COLLOIDS IN PROVIDING A SOURCE OF IRON TO PHYTOPLANKTON

by

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We accept this thesis as conforming to the
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ABSTRACT

Iron-rich colloidal material was found to be associated with diatoms in the natural environment. To determine if this association could be important to the organism, the supply of iron from colloidal forms to phytoplankton was investigated. Laboratory bioassays with the marine diatom Thalassiosira pseudonana demonstrated that freshly precipitated colloidal iron could readily support diatom growth. However, when these colloids were aged or subjected to short periods of heating, the iron availability was drastically reduced. The iron availabilty was not increased with addition of the complexing agent EDTA. The reduction in availability appears to be linked to increased thermodynamic stability of the colloidal hydrous ferric oxides. The probable mechanism of this reduction is decreased colloidal dissolution rates. The supply of iron from colloids to phytoplankton appears to be determined by the chemistry of the colloidal iron material rather than by the physical association of colloidal iron and cell walls.

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INTRODUCTION

Iron is an essential micronutrient for virtually all forms of life. Phytoplankton require more iron than any other trace metal (Anderson and Morel, 1980) and iron deficiencies in phytoplankton have been found to cause decreased rates of both photosynthesis and assimilation (Glover 1977). In higher plants, Spiller and Terry (1980) have shown that iron deficiency decreased chlorophyll A, chlorophyll B, carotene and xanthophyll concentrations as well as the photosynthetic electron transport capacity. Their study showed that with progressive iron deficiency, the number of grana per chloroplast and the number of thylakoids per grana both decreased. Similar responses to iron deficiency are expected in phytoplankton. The effects of iron deficiency, however, are reversible when iron is made available to the cell. With phytoplankton, this occurs through chemical interactions with various sources of iron in the surrounding environment. The present study deals with the supply of iron to phytoplankton from colloidal hydrous iron oxides which, for the sake of convenience, will henceforth simply be termed colloidal iron.

Iron exists in sea water as a soluble hydrated ion, as dissolved complexes formed with organic and inorganic ligands, and also as a component of suspended particulate or colloidal organic and inorganic material. Since iron forms highly insoluble hydrous ferric oxides in oxygenated sea water, and the levels of organic ligands that might increase its solubility are usually relatively low, it has been suggested that most iron is present as colloidal ferric hydroxide (Lewin and Chen, 1973). However, the oxidation rate of ferrous iron is considerably slower than previously thought (Yu-Jean and Kester, 1978) and, since ferrous iron is more soluble than ferric iron, the calculated dissolved iron concentration may underestimate the true concentration if substantial iron reduction was occurring (e.g. photooxidation of organic complexes or ferric ion complexation by reducing ligands).

Although the biologically available forms of iron have not been satisfactorily determined, it is generally accepted that they are composed mainly of the mononuclear species formed with various inorganic or organic ligands. Chelating agents such as ethyelenediaminetetraacetic acid (EDTA), which is a strong synthetic chelator of ferric iron, are known to enhance phytoplankton growth (Lewin and Chen, 1971), indicating that the chelation of iron directly or indirectly affects it's availability. Since EDTA does not appear to be transported across the cell membrane (Huntsman and Barber, in press) it suggests that EDTA acts only to increase the pool of soluble iron. Furthermore, there is evidence that some phytoplankton, under iron deficient conditions, have a physiological response which results in the production and release of organic compounds including some termed siderophores that are highly specific for ferric iron (Anderson and Trick, personal communication). It is possible that siderophores increase iron availability by either increasing the pool of soluble iron or by acting as carrier compounds which chelate ferric iron and transport it across the cell wall. However, Anderson and Morel (1980) have suggested that the ferric iron species must first be reduced before uptake. They have suggested this reduction could occur either by photo-oxidation of ferric-organic complexes, by chelation by reducing siderophores, or by reduction at the cell membrane.

The dissolved species of iron believed to be biologically available should exist in equilibrium with hydrous colloidal oxides that presumably are the predominant form of iron in seawater. Thus as the concentration of the available soluble species is reduced, replacement should occur through dissolution of the colloid. Colloidal hydrous oxides are therefore the ultimate source of iron for phytoplankton.

The distinction between colloidal and soluble iron species cannot be rigorously defined. In general, a colloidal dispersion is considered one in which the discontinuous phase (in this case iron) is subdivided into units that are large compared with simple molecules, but small enough that interfacial forces as well as inertial forces are significant in governing the properties of the system (Sennett and Olivier, 1965). Particles with diameters less than 1.0 μm are usually considered to have these charateristices. Because of these charateristices colloidal material may remain suspended indefinitely, in contrast to particulate matter. In the past, the term 'dissolved' has been used for metals in aqueous solutions that pass through a .45 μm pore size filter. Although this is a practical working definition, it is not representitive of metals in true solution (i.e. mononuclear species) since some colloidal metals will not be removed by the filtration process. For the purposes of this investigation, the following classification scheme was chosen: iron existing as free hydrated ions or as complexed species was considered dissolved while aggregates up to 1.0 μm in diameter were termed colloids. Solids larger than 1.0 μm in diameter were considered particulates.

There has been some suggestion that phytoplankton are able to directly utilize iron-rich colloidal matter adsorbed on their surfaces. Harvey (1937) observed an association between colloidal iron and marine diatoms in laboratory cultures and after estimating the iron requirements of the cell, the dissolved iron concentrations, and the iron diffusion rate, he concluded that for phytoplankton to escape iron limitation this adsorption was necessary. This conclusion was later supported by Goldberg (1952). More recent estimates of true dissolved iron concentrations, however, are considerably higher than those used by Harvey (Bryne and Kester, 1976) which casts some doubt on his conclusions.

At present, there is still little known about the ability of colloidal iron (i.e. colloidal hydrated ferric oxides) to supply iron to phytoplankton. The purpose of this study was to determine whether colloidal iron occurred associated with diatoms in the natural environment, and to investigate how its manner of preparation affects its ability to supply iron to phytoplankton. Diatoms collected from different environmental locations were thus examined by scanning electron microscopy (S.E.M.) for the presence of surface associated colloidal material. Where this material was found, the elemental composition was estimated with energy dispersive X-ray analysis (EDAX). In addition, laboratory studies were made using a bioassay organism to test the ability of iron colloids prepared in different ways to provide a source of biologically available iron. The colloidal forms tested were: freshly precipitated ferric hydroxide, freshly precipitated ferric hydroxide that was treated by heating and ageing (yet remained X-ray amorphous), and crystalline goethite and hematite.

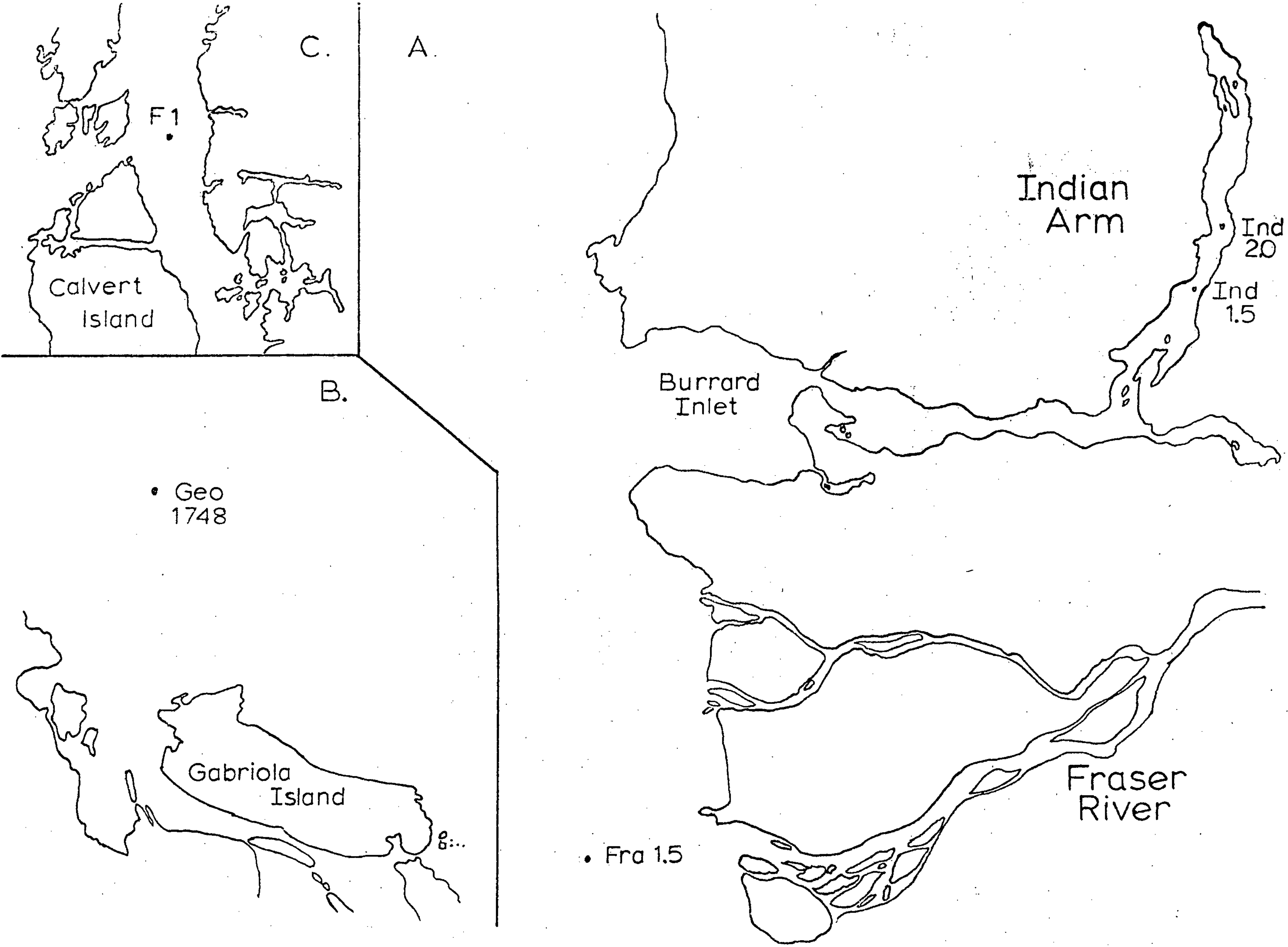
MATERIALS AND METHODS

Field Study

Water samples were collected at a number of depths from stations in Indian Arm, the Strait of Georgia, and Fitzhugh Sound as well as within the Fraser River salt wedge and plume (Fig. 1). The water was collected in National Institute of Oceanography polypropylene samplers and then transferred to acid-cleaned 250 ml polypropylene bottles. Aliquots of the samples (50 ml) were immediately drawn into polypropylene syringes (see Appendix 1) and filtered through 0.1 μm Nuclepore filters (13 mm diameter). Five milliters of prefiltered (0.1 μm), glass-distilled water was then passed through the sample filter to remove salt which would interfere with the S.E.M. examination of the sample. The filters were then transferred to sterile petri dishes, dried at room temperature, and frozen.

For S.E.M. examination, the dried filters were mounted directly on a metal stub with spectrographic graphite paint, sputtered with gold (ca. 100 angstrom coating) and examined with an Ortec scanning electron microscope. Where diatoms were found and particulate or colloidal material was present on the frustules, the iron content of the material was estimated with energy dispersive X-ray analysis (Ortec). To limit the electron beam exposure to the area of interest, the magnification was increased to maximum or until the colloid (or particle) filled the field of view. The data are plotted as a spectrum of X-ray energies, and the peak height of a given energy level is

Figure 1. Field sampling locations. In Indian arm, filter samples were taken from water collected from 5, 20, 50 and 200m at Ind. 2.0 and the depths of 5, 20, 50 and 175m from Ind. 1.5. In the Strait of Georgia (Geo. 1748), water from 5, 20, 50 and 375m was sampled. At Fra. 1.5, samples were taken from 1 m while further upstream, water was sampled from the apex of the salt wedge. (The location of sampling varied due to movement of the wedge during the tidal cycle.)


C.
F1
Calvert Island
A.
Indian Arm
Ind 2.0
Ind 1.5
Burrard Inlet
B.
Geo 1748
Gabriola Island
Fraser River
Fra 1.5

proportional to the amount of that element in the sample (Fig. 2). Difficulties arise which prevent the quantitative measurement of the elements present. These difficulties include irradiation of the surrounding area by electron beam scatter, X-ray fluorescense, differing detection capabilities depending on the atomic numbers, and absorption of the released X-rays by atoms within the object. In addition, both the thickness and orientation of the object with respect to the detector affects the X-ray collection efficiency. Due to these problems the technique is best used only to estimate the elemental composition of objects. In view of the limitations of the method, the following criteria were used to identify iron-rich colloidal material: spectra of colloids of interest were compared with the spectra of the adjacent areas of the filter or frustules and when the FeKα peak heights of the colloids were substantially higher than that of the adjacent areas, the colloids were considered to be iron-rich (Fig. 3). On two separate occasions where iron-rich colloids were found associated with frustules, transects across the frustules and colloids were analyzed for iron content.

In order to test if the association of colloidal matter with diatom frustules was a result only of the chemistry of the siliceous frustule (i.e. not controlled by the organism), borosilicate glass plates were used to model the frustule surface. These glass plates have a similar surface chemistry to that of the frustule and present a smooth surface where colloidal material can not be physically entrapped. Water from

Figure 2. X-ray energy spectrum of a colloidal particle collected in a field sample. The horizontal axis represents the energy levels and the vertical axis the number of X-rays measured. Each peak represents a single element but in some cases masking can occur by overlapping peaks. The presence of two iron peaks, representing the FeKα and Kβ X-ray energies, indicate a high iron content.

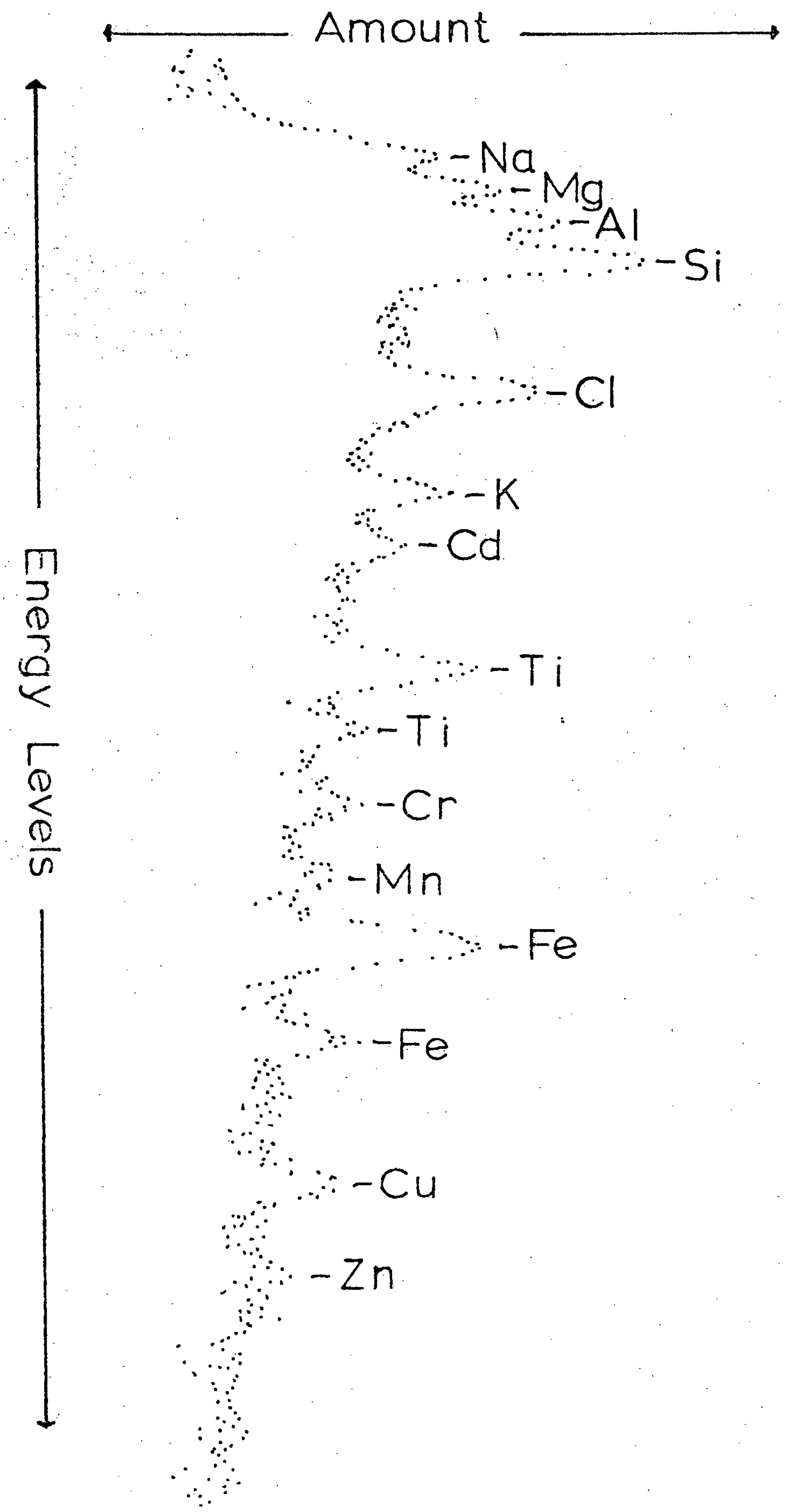
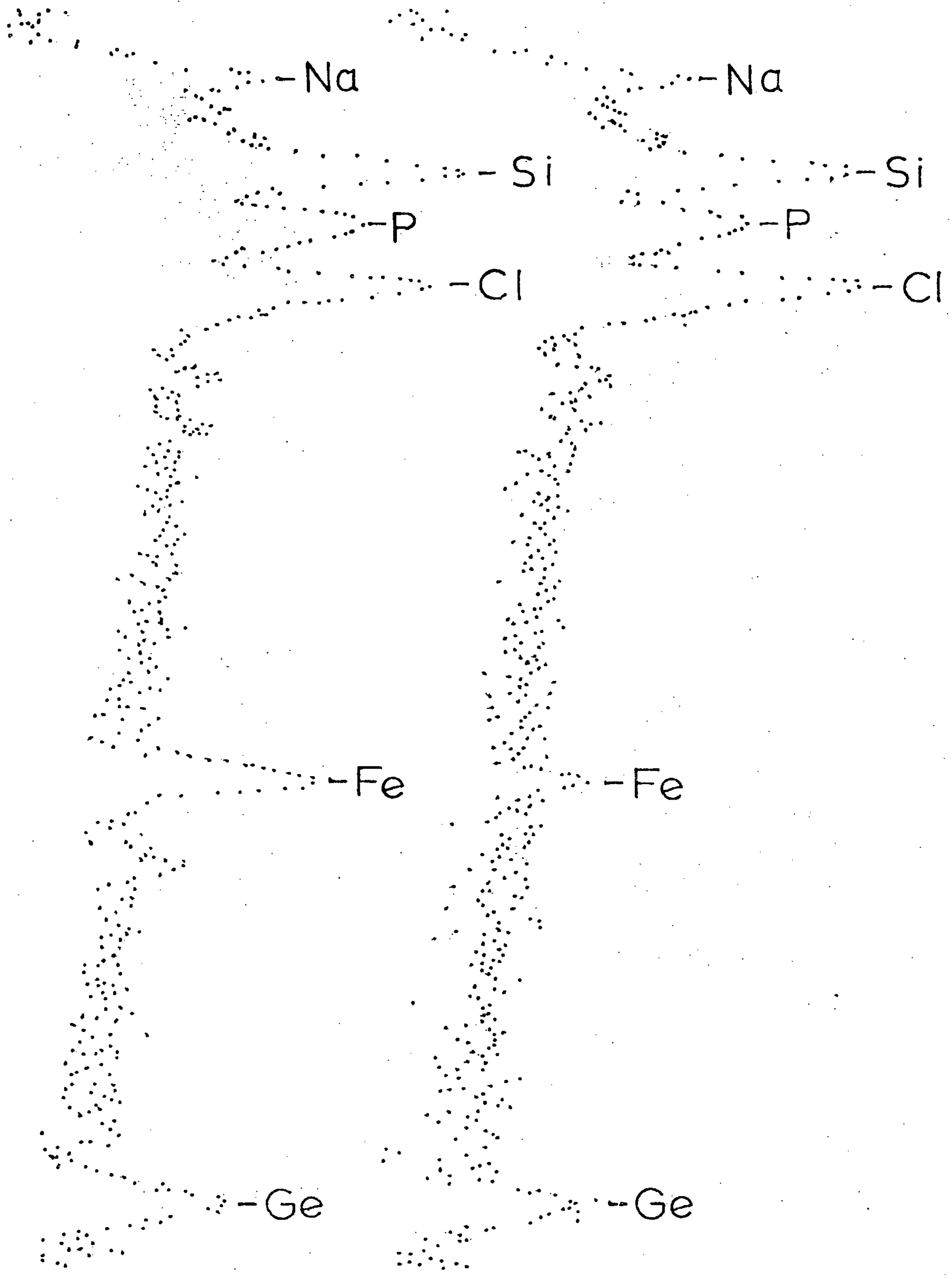

Amount
Energy Levels
Na
Mg
Al
Si
Cl
K
Cd
Ti
Ti
Cr
Mn
Fe
Fe
Cu
Zn

Figure 3. Determination of iron-rich colloids was done by comparing spectra of the colloid (lower) and adjacent areas of the filter or frustule (upper). Colloids having differences in iron peak heights such as shown were considered to be iron-rich.


Na
Si
P
Cl
Fe
Ge
Na
Si
P
Cl
Fe
Ge

the Fraser River salt wedge, which has abundant suspended solids (50 - 500 mg l^{-1}), was pumped through a polypropylene tube with a peristaltic pump and passed continuously through a chamber containing vertically mounted glass plates. (The plates were mounted vertically to prevent particle settling.) Both the chamber and the mounting stage were constructed of methyl methacrylate. After a period of exposure (about 2 hours) the glass plates were removed, dip-rinsed in filtered (0.1 μm) distilled water (to remove salt), placed in sterile dust-free petri dishes, and allowed to dry. The plates were then analyzed in the same manner as the filtered samples.

Laboratory Study

(i) Bioassays

The bioassays were conducted to test the ability of different colloidal iron stocks to support organism growth. The marine diatom Thalassiosira pseudonana (Hustedt), Hasle and Heimdal (WHOI clone 3-H), from the N.E. Pacific culture collection (the University of British Columbia) was used in all bioassays. Both the stock cultures and bioassays were conducted in batch using the artificial sea water medium 'AQUIL' (Morel *et al.*, 1979). All cultures were grown at 15°C and illuminated under Cool White twisted fluorescent lights on a 16:8 hour light dark cycle at a radiation level of 95 μEin m^{-2} s^{-1}. Cell inocula from iron-limited, EDTA-free, stock cultures were added to the bioassay flasks to give initial cell concentrations of 1000-2000 cells ml^{-1}. Cell concentrations were monitored daily during the bioassay with a Coulter Counter (model Zf). The length of the bioassays varied between 5 and 28 days depending on the nature of the experiment. Differences in populations were considered significant if they differed by two standard deviations.

(ii) Medium Preparation

The standard ocean water (SOW) used to prepare the bioassay medium was prepared in 20 liter quantities by the addition of salts to glass distilled water (see Appendix 2). The SOW, without added nutrients or metals, had undetectable concentrations of Fe, Cu, Cd, Mn, V, Ni, and Zn (measured by direct injection graphite furnace atomic absorption spectrophotometry). Four liters of medium were normally prepared at one time for each bioassay to ensure comparable medium chemistry throughout the flasks. The AQUIL nutrient solutions (NO_3^-, PO_4^{3-} and $Si(OH)_4$ passed through a column containing Chelex-100) and the metal mix (Cu, Mn, Zn, Co, Mo but excluding Fe and EDTA) were first added to the SOW. This mixture was then bubbled with filtered (0.4 μm), acid-cleaned (6N H_2SO_4) carbon dioxide to attain a pH of 5.5 (to prevent salt precipitation during autoclaving). The medium was then autoclaved (121°C, 30 mins) in a 4-liter glass aspirator bottle. Upon cooling, the pH was measured and, if necessary, the medium was bubbled with clean air (as above) to pH 8.0. Aliquots of the medium (250 ml) were then transferred to previously autoclaved 500 ml polycarbonate Erlynmeyer flasks and the cell inoculations were added.

Additions of the prepared iron stocks (see below) were made either before or after autoclaving of the medium depending on the experiment. In all experiments, iron was added to give a total concentration of 4.5×10^{-7}M. All additions and inoculations were made in a class 100 laminar flow hood that had all possible metal parts replaced with polypropylene. The bioassay apparatus was soaked in 6N HCl and rinsed three times with glass distilled water prior to use. Three replicates of each test were run and every bioassay series included three flasks in which no iron was added. The pH of the individual cultures was measured initially (7.9 - 8.1) and at the end of the bioassays (8.7 - 9.5). (The pH increase was due to biological action.) Bacterial contamination was tested with tryptic soy broth and only low levels were infrequently found, presumably from the non-autoclaved iron stocks.

(iii) Iron Stock Preparations and Treatments

Fresh colloidal ferric hydroxide stocks were prepared at a concentration of 4.5×10^{-4}M by the addition of $FeCl_3$ to glass distilled water. No adjustments for pH, which fell to 2.5, were made. The rapid formation of polynuclear ferric colloids in such ferric salt solutions is well documented (Murphy, et al (1976), van der Giessen (1968), Atkinson, et al (1977)). Colloid size was not determined but Murphy et al (1976) have estimated the size to be 2-16 nm after 24 hrs of ageing. The colloids were added to the culture media either directly after stock preparation or after various treatments of the stock including: a) autoclaving (121°C, 15 psi) for 15 mins., 30 mins. and 1, 4, 12, and 24 hour periods, b) heating to 100°C for 5, 10 and 15 minute intervals, c) heating at 50°C, 70°C, and 90°C for 5 minutes and d) ageing at room temperature for one week and three months. A Fe-SOW colloidal stock was prepared as above, adjusted to the pH of the colloid stock in distilled water and added to cultures either directly or after 15 minutes of autoclaving.

Fe-EDTA stock solutions were prepared by the addition of ferric chloride (4.5×10^{-4}M) and EDTA (5.0×10^{-3}M) to glass distilled water. The presence of the chelator EDTA in the iron stock prevents polynuclear colloid formation by the chelation of the free iron (Lewin and Chen, 1973). The Fe-EDTA stocks were added to cultures either 1-2 days after preparation (to allow equilibration) or after the treatments of autoclaving (15 mins) or ageing (18 months at 10°C).

A colloidal goethite (αFeOOH) stock was prepared following the method of Forbes et al. (1974). An acidified ferric nitrate solution (pH 1.9) was aged for 48 hours at room temperature and then heated at 60°C (pH 11.7) for three days. The precipitate thus formed was confirmed to be goethite by X-ray diffraction. The average colloidal diameter in the polydispersed sol was determined with S.E.M. and found to be about 0.8 μm. The colloidal goethite aggregates were rinsed with distilled water and stored at 10°C in a polyethylene bottle until use. (Iron loss to the container walls was negligible.) To determine the volume of the iron stock to be added to cultures, the iron concentration of an acidified (pH 1.0), heated (70°C for 2 days) aliquot of the goethite stock was measured with flame atomic absorption (Techtron AA-4).

A colloidal hematite (αFe_2O_3) stock was prepared by the method of Matijevic and Scheiner (1978). An acidic ferric nitrate solution was heated for three days at 100°C to create a monodispersed colloidal sol. X-ray diffraction confirmed that the precipitate produced was hematite. The size of the monodispersed sol was determined to be 0.1 μm with S.E.M.. The aggregates were rinsed with glass-distilled water and stored at 10°C in a polyethylene bottle until use. The iron concentration was determined in the same fashion as with the goethite stock. Both the goethite and hematite stocks were disaggregated in an ultrasonic bath before addition to culture media.

(iv) Physical and Chemical Analysis

The physical and chemical properties of the fresh and autoclaved colloidal iron stocks were compared by a number of methods. These included X-ray diffraction, thermal gravimetry, Mossbauer spectroscopy, gel filtration, and by measuring the relative dissolution rates. For these comparisons the period of autoclaving was 15 minutes unless otherwise noted.

For the X-ray diffraction analysis, the autoclaved and non-autoclaved solutions were adjusted to pH 7.5 and rapidly mixed to produce aggregates by colloidal coagulation. (This isoelectric pH was determined experimentally.) After the aggregates had settled they were extracted by pipet, deposited on an X-ray slide and dried at room temperature. The X-ray diffraction patterns of these dried samples was scanned between 10 and 50 degrees 2θ using Mn filtered FeKα radiation. Fresh and autoclaved (15 min, 30 min, 1 hr, 4 hr, 12 hr, and 24 hr) colloidal iron stocks were also scanned by camera techniques using 6 hour exposure periods.

Thermal gravimetric analysis was performed on both fresh and autoclaved colloidal iron stocks using subsamples of the batches previously prepared for X-ray diffraction analyses. The samples were held at 115°C for 4 mins to remove moisture followed by a 40°C min^{-1} temperature increase to 700°C. The percent weight change of the sample was recorded.

For Mossbauer spectroscopy, the autoclaved and non-autoclaved colloidal iron stocks were adjusted to their isoelectric pH (ca. 7.5) and rapidly mixed to produce aggregates of iron that were allowed to settle. The aggregates were repeatedly rinsed by centrifugation with distilled water to remove the chloride ion. The supernatant was discarded and the pellets transferred to Mossbauer cells. These cells were then sealed with epoxy and quick frozen in liquid nitrogen. The spectrum of each sample was then measured for a 24 hour period at liquid nitrogen temperatures (see appendix 4).

To determine the gel filtration rates, a polycarbonate column (60 cm) was filled with Sephadex G-10. Because this gel readily adsorbs iron from solution, it was first treated with a 1 M zirconium solution to block the available adsorption sites of the gel and thus permit the passage of iron (Murphy et al, 1975). A 1.0 ml aliquot of the iron stock was introduced at the top of the gel and eluted with a 1 M zirconium solution at a flow rate of 6.0 ml hr^{-1}. Three milliter fractions were collected and, after acidification (1% HCl), the iron concentrations were determined by direct injection graphite furnace atomic absorption spectrophotometry.

To measure the colloidal dissolution rates, the dissolved ferric iron was measured over time with the colorimetric chelating reagent sulphosalicylic acid by the method of Langford et al (1977). As free ferric ions are chelated by this reagent, the equilibrium between dissolved and colloidal iron is disrupted, causing the solid to dissolve. The rate of this solution under a given set of conditions is in part related to the thermodynamic stability of the colloids and is seen to decrease with increasing stability. Thus, the rates of colour development in the autoclaved and non-autoclaved iron stocks indicate the relative stabilities of the colloidal matter. In this experiment, the pH of the sulphosalicylic acid solution was first adjusted to that of the iron stocks (2.5) and then the solution was mixed (1:1) with the sample in a 10 cm spectrophotometer cell. The cell was immediately placed in a spectrophotometer (Bausch and Lomb 2000) and the absorbance at 500 nm measured for 30 mins.

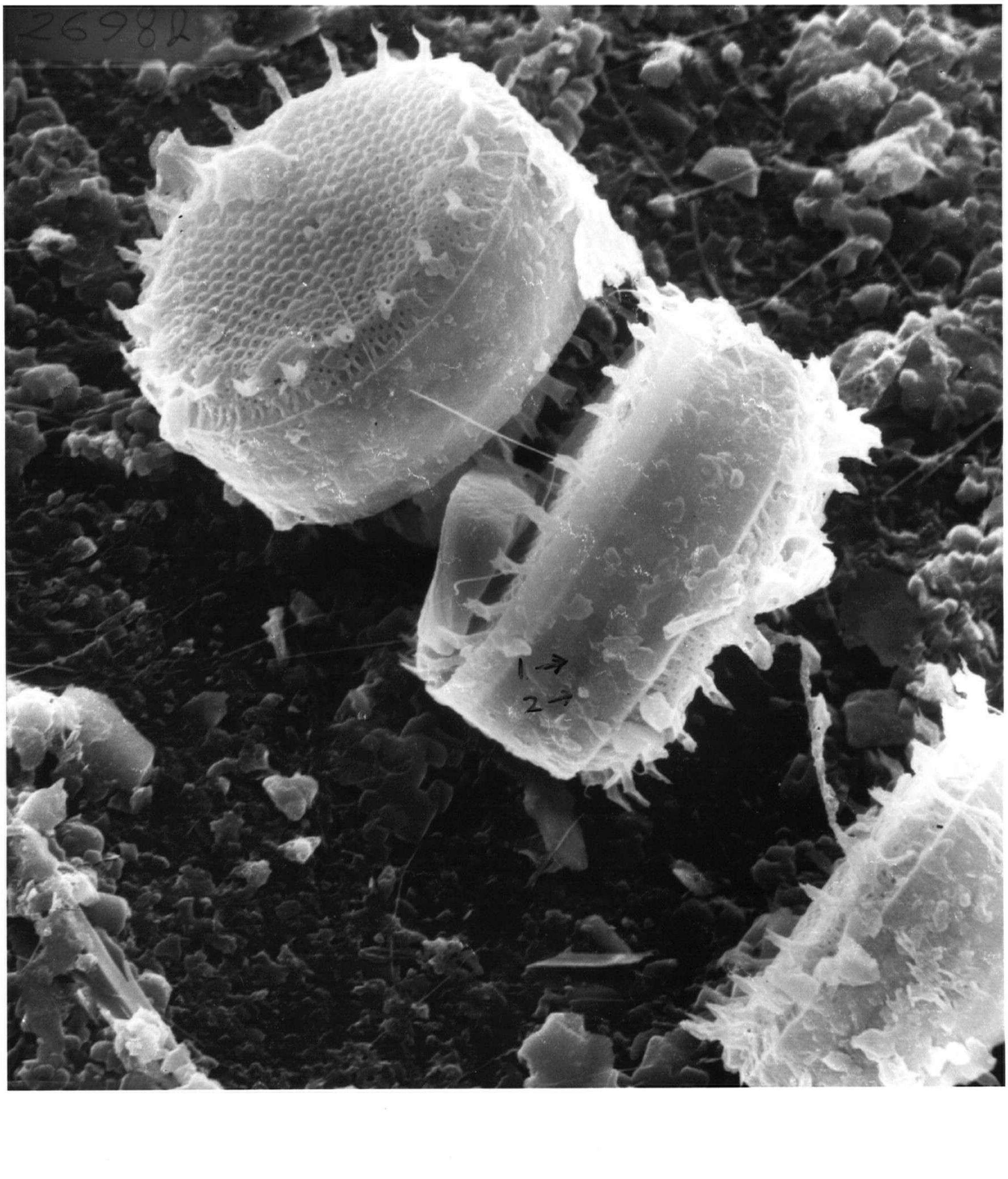
RESULTS

Field Study

Colloidal material was found at all of the sampling locations (Fig. 1). At those stations where samples were taken from a series of depths (Ind. 0.0, Ind. 1.5, Ind. 2.0 and Geo 1748), the amount of colloidal material retained on the 0.1 μm pore diameter filter was found to increase with increasing depth. One exception to this was Fra. 1.5 where the maximum was at the surface within the water of the Fraser River plume. Extremely high levels of colloidal and particulate material were found in the Fraser River salt wedge. At each station some of the colloidal material was found to be iron-rich. For example, about 26% of the colloidal material examined in the bottom waters of Indian Arm (at Ind. 2.0) had substantial amounts of iron.

Diatoms were found in the upper part of the water column at every station and in the bottom waters of Indian Arm and the Fraser River (in the salt wedge). Some diatom frustules within the Fraser River plume, the Fraser river salt wedge and in the deep waters of Indian Arm had colloidal matter associated with their surfaces. In many cases this colloidal matter contained substantial amounts of iron. Figures 4-7 show some examples of iron-rich colloids associated with diatoms. Quantitative estimates of this association were not made. The iron content along a transect crossing a diatom frustule towards an iron-rich colloid is shown in Fig. 8. The euphotic zones within Indian

Figure 4. Iron-rich colloid:diatom association was found on a number of occasions. These spectra were taken from the areas shown in the photograph.


26982
1→
2→

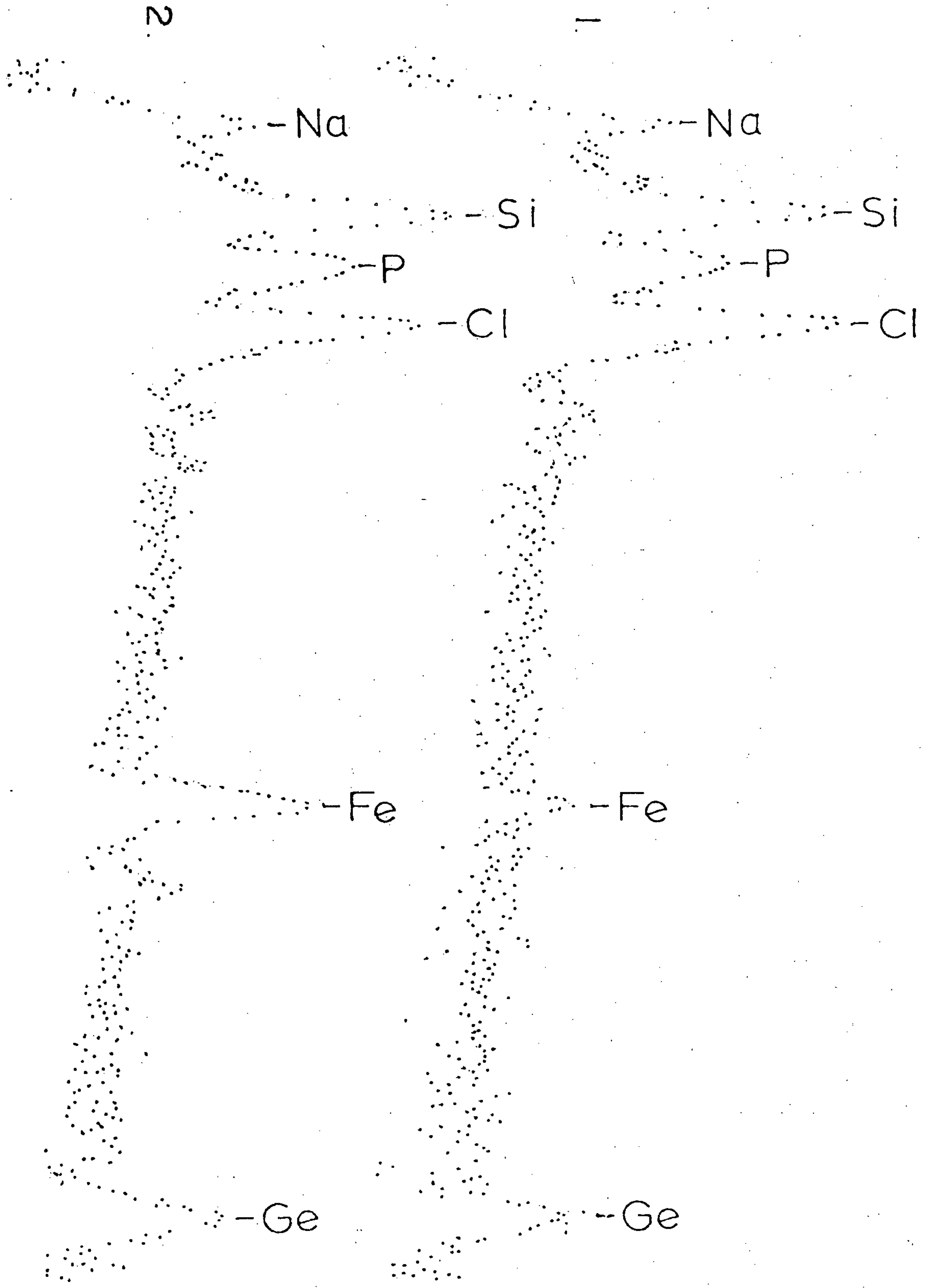
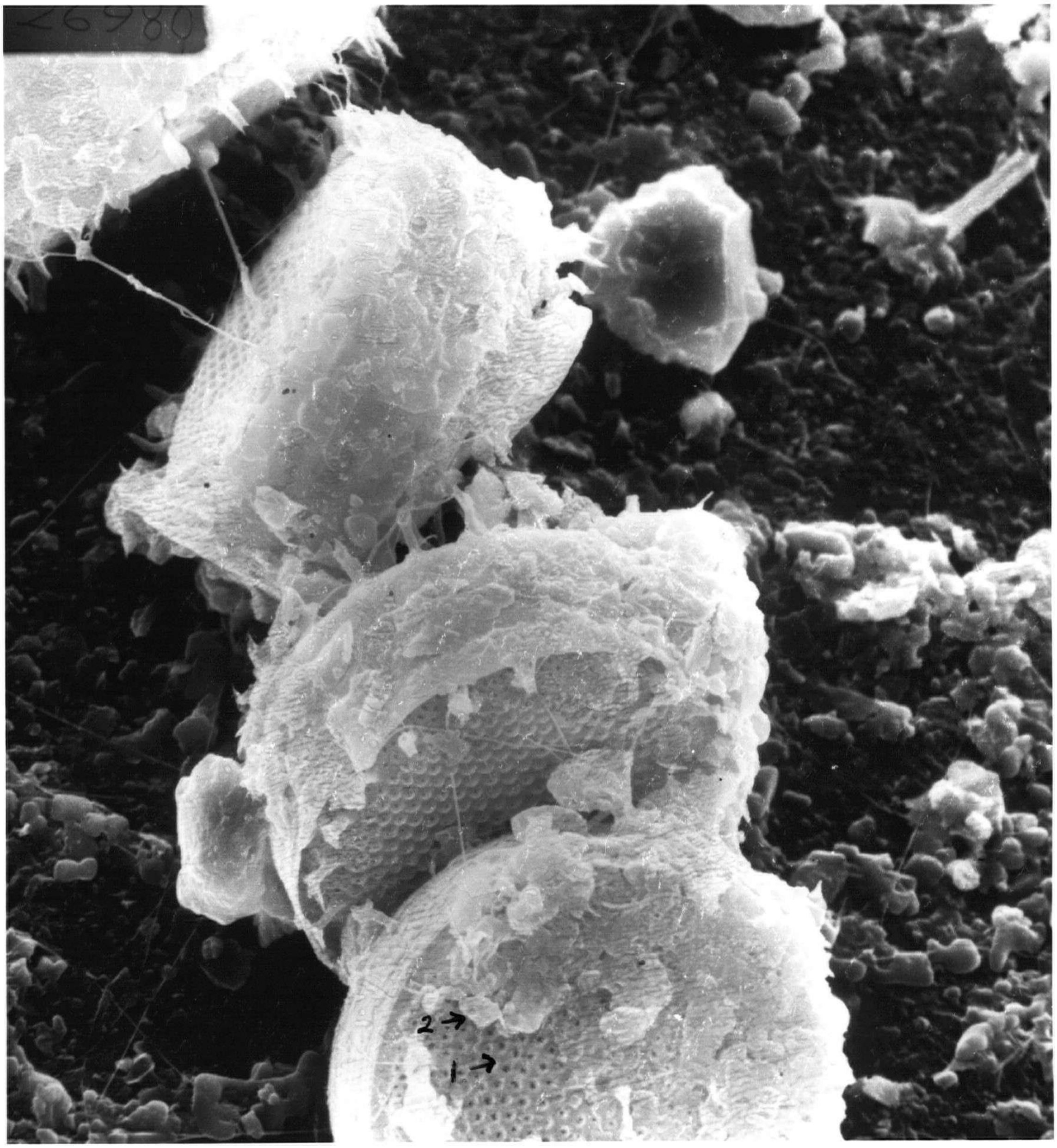

2
1
-Na
-Na
-Si
-Si
-P
-P
-Cl
-Cl
-Fe
-Fe
-Ge
-Ge

Figure 5. Another example of iron-rich colloid:diatom association. These spectra were taken from the areas shown in the photograph.


26980
2
1

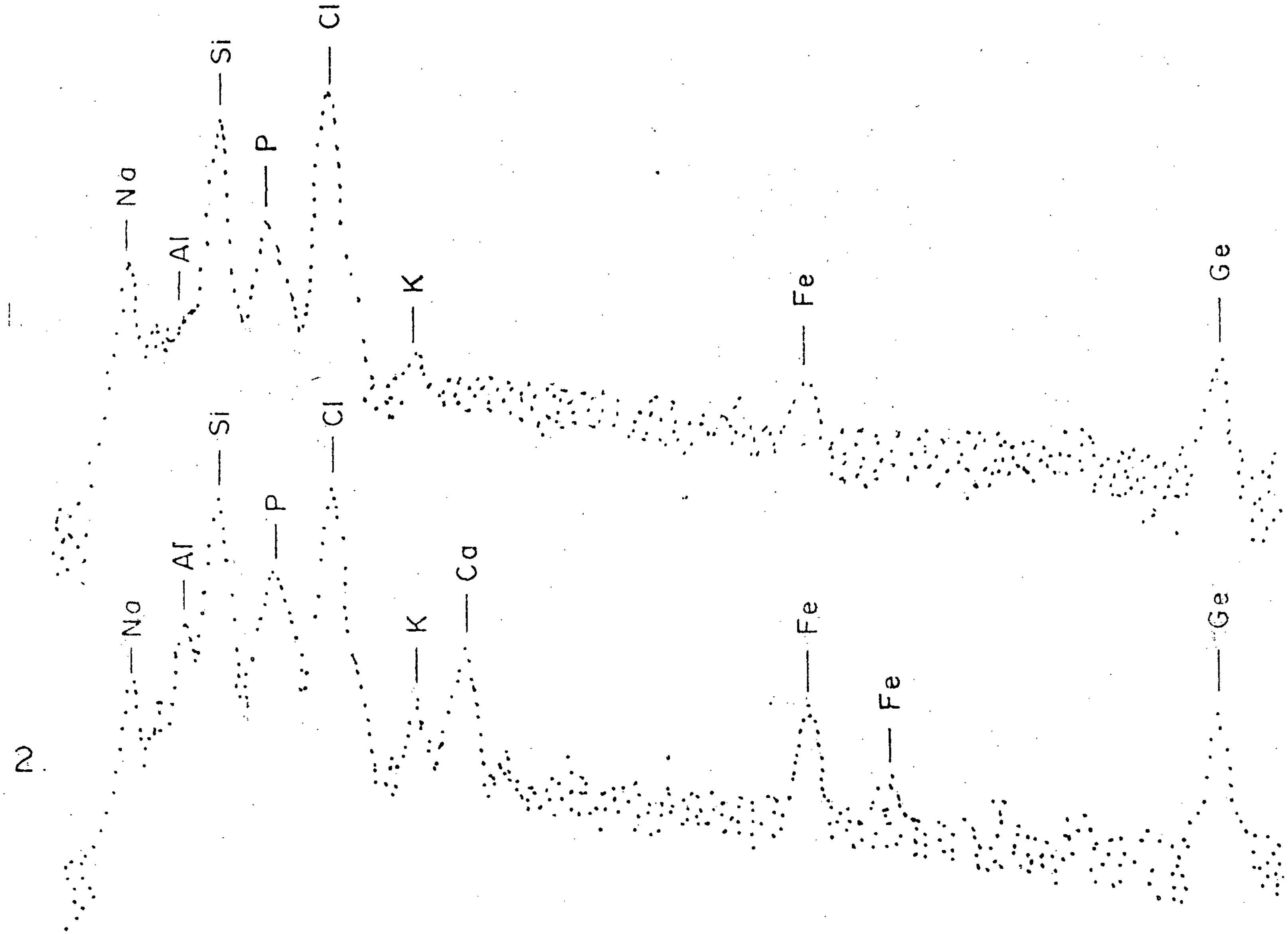
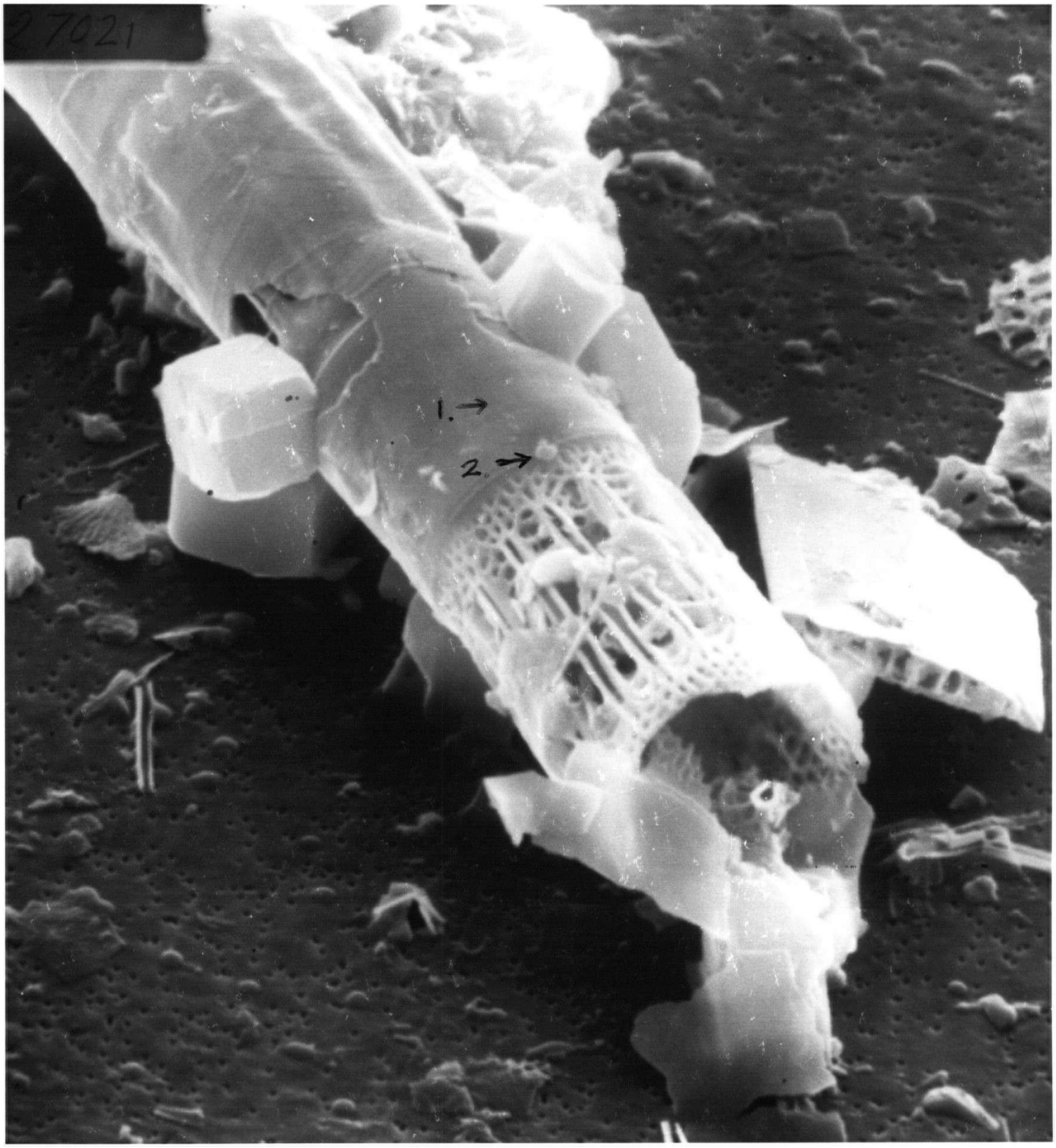

1
Na
Al
Si
P
Cl
K
Fe
Ge
2
Na
Al
Si
P
Cl
K
Ca
Fe
Fe
Ge

Figure 6. Another example of iron-rich colloid:diatom association. These spectra were taken from the areas shown in the photograph.


27021
1.
2.

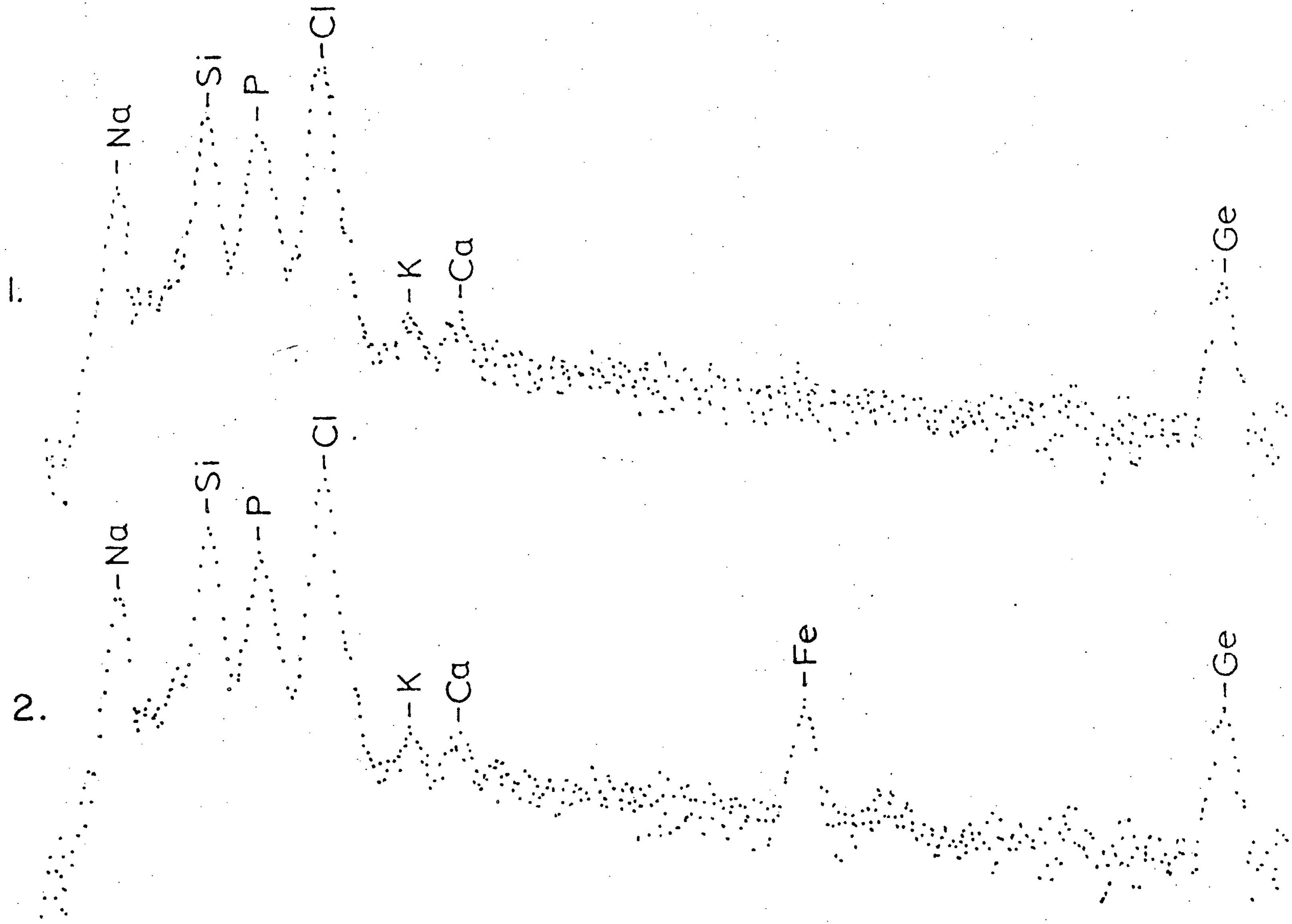
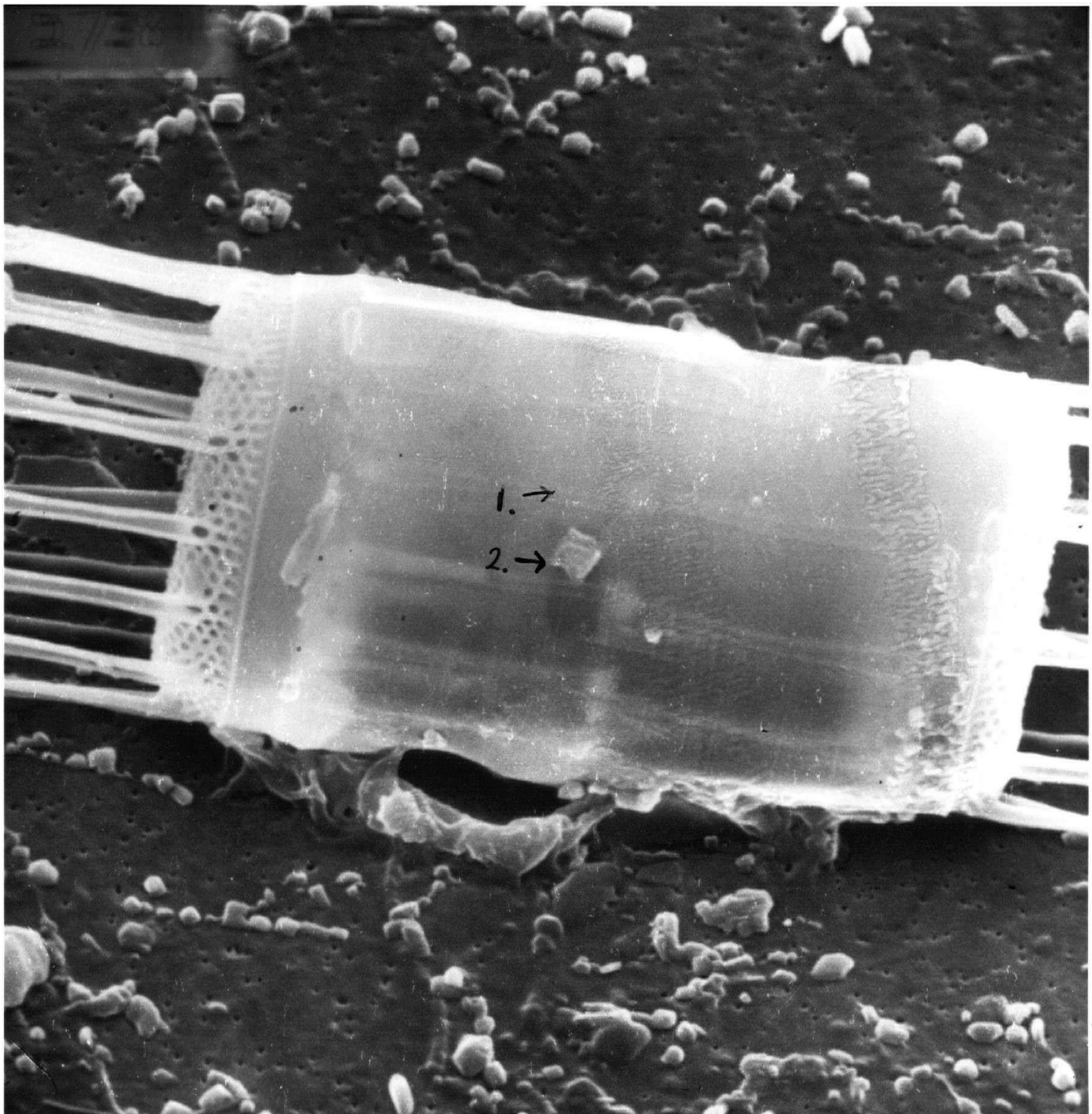

1.
-Na
-Si
-P
-Cl
-K
-Ca
-Ge
2.
-Na
-Si
-P
-Cl
-K
-Ca
-Fe
-Ge

Figure 7. Another example of iron-rich colloid:diatom association. These spectra were taken form the areas shown in the photograph.


1. →
2. →

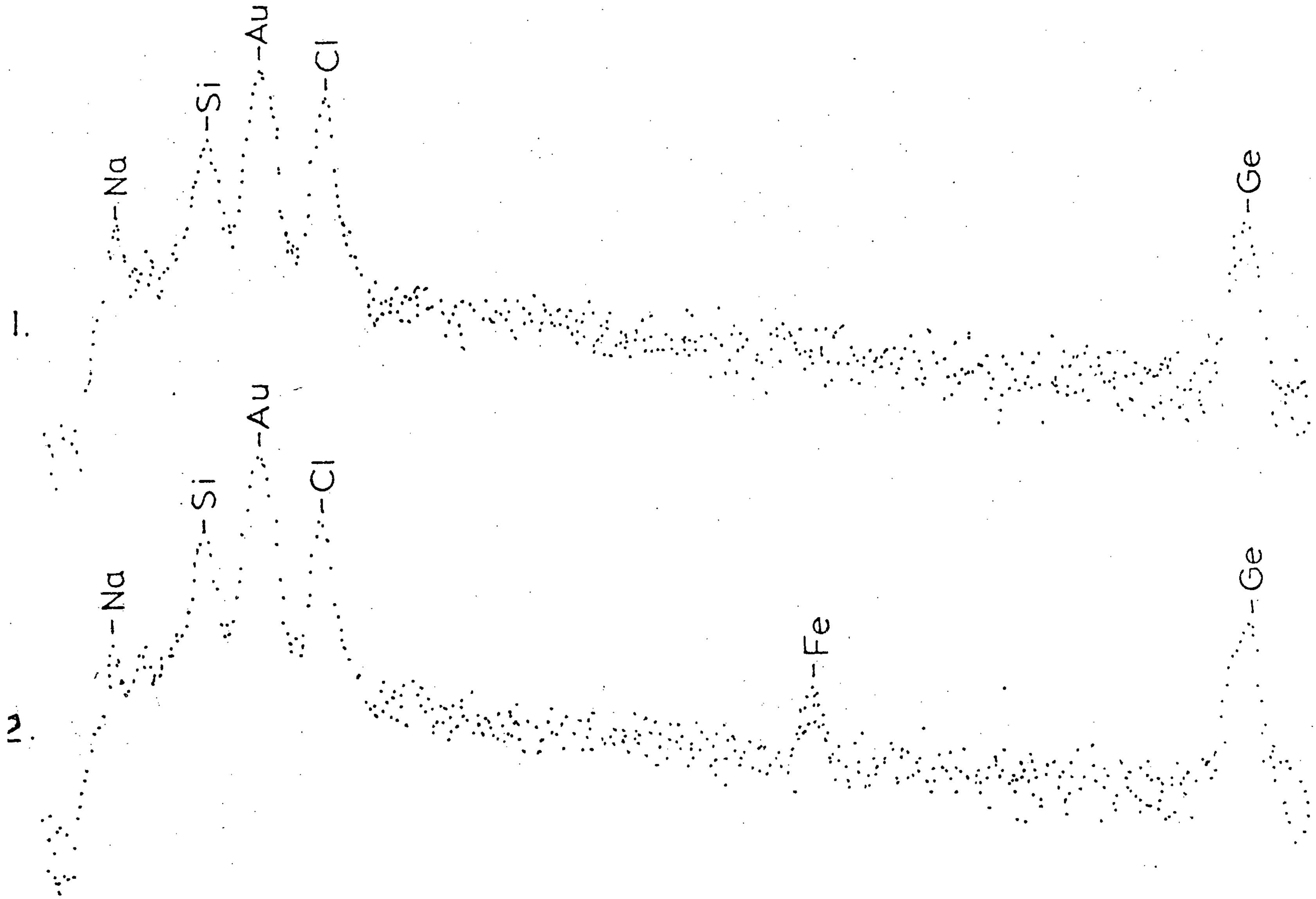
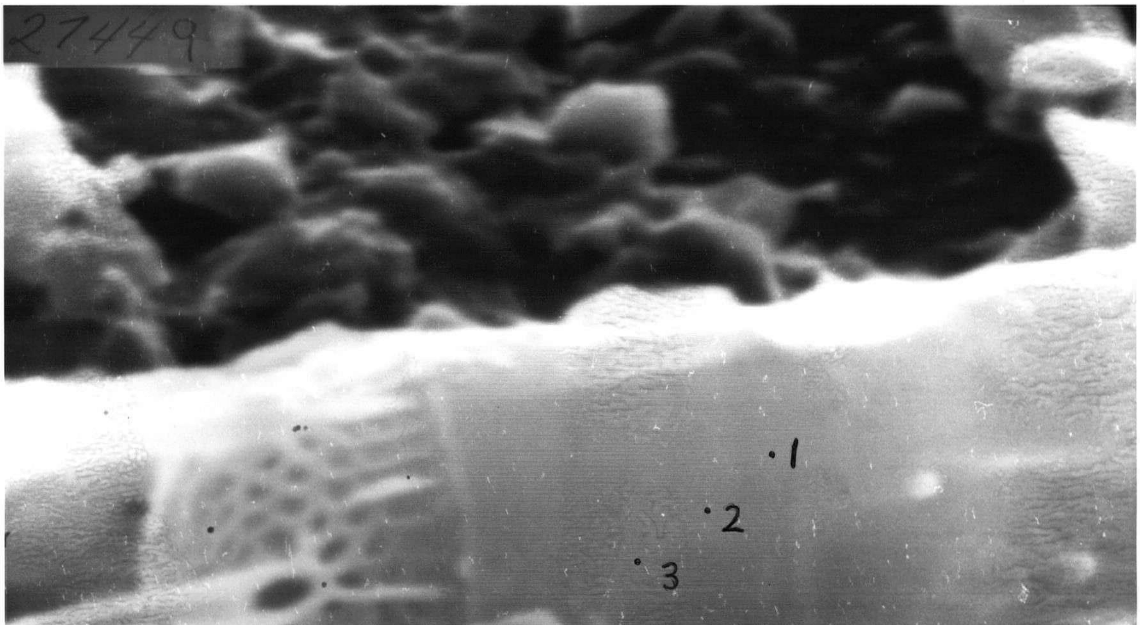
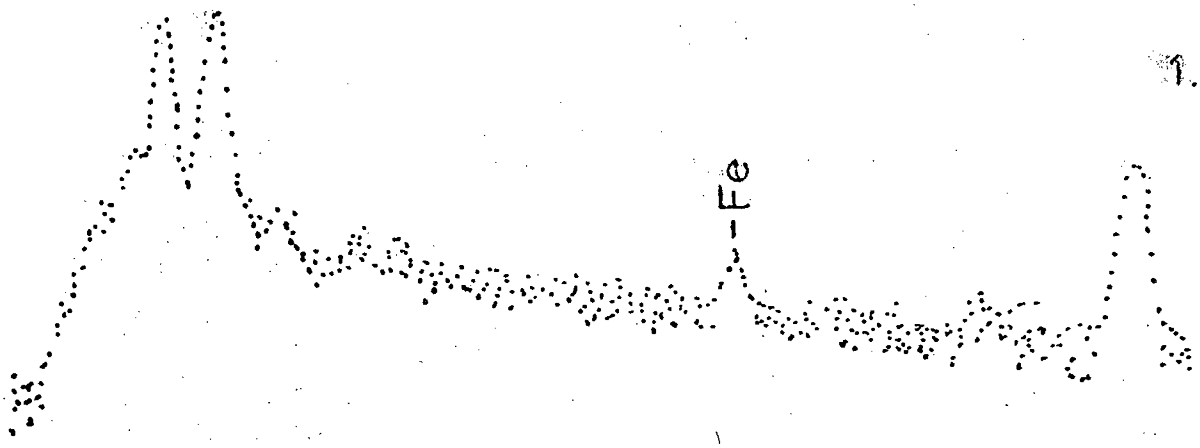
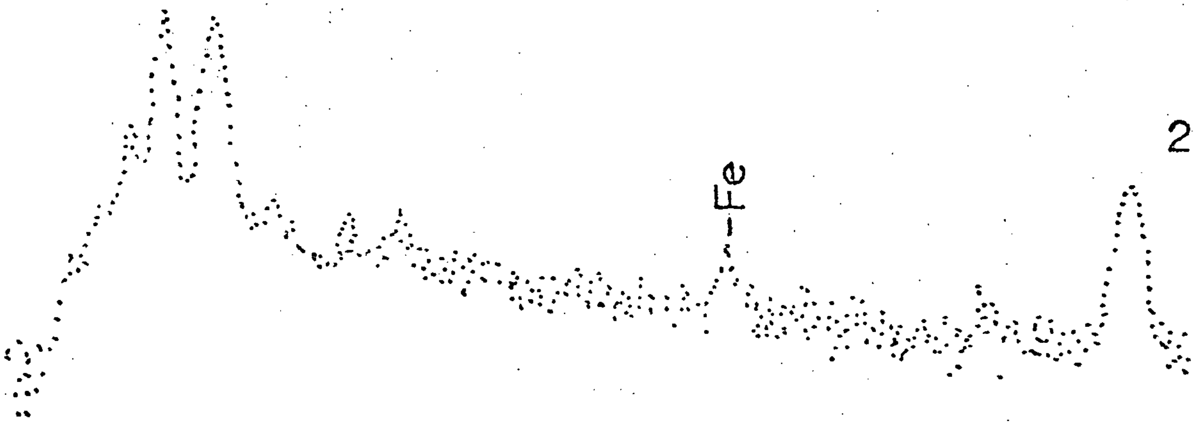

1.
Na
Si
Au
Cl
Ge
2.
Na
Si
Au
Cl
Fe
Ge

Figure 8. Iron content along a transect of a colloid associated frustule was measured to attempt to determine whether colloidal dissolution was occurring. The spectra were measured from 1 to 3 towards the colloid. There appears to be a slight increase in the iron content towards the colloid, however, the differences are probably only due to electron beam scatter during measurement.

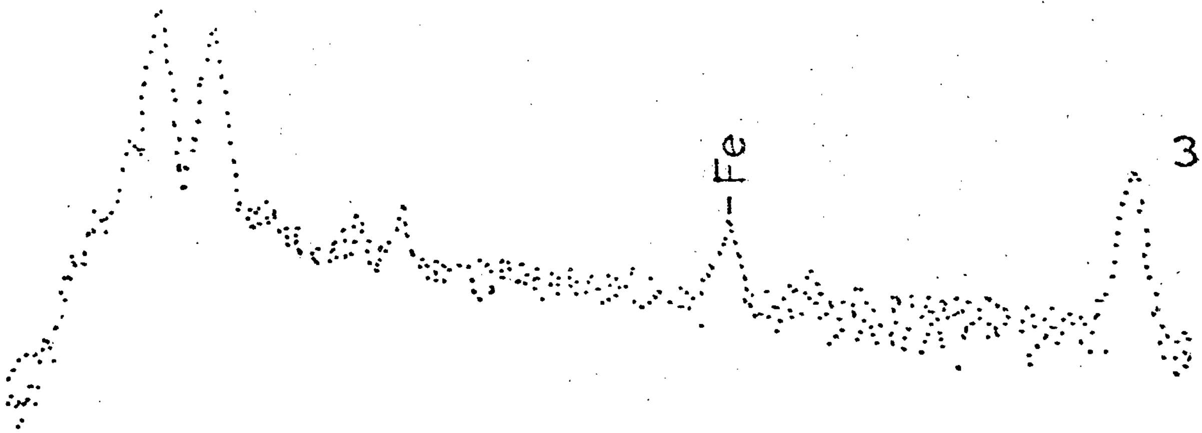

27449
1
2
3
c



1.



2.



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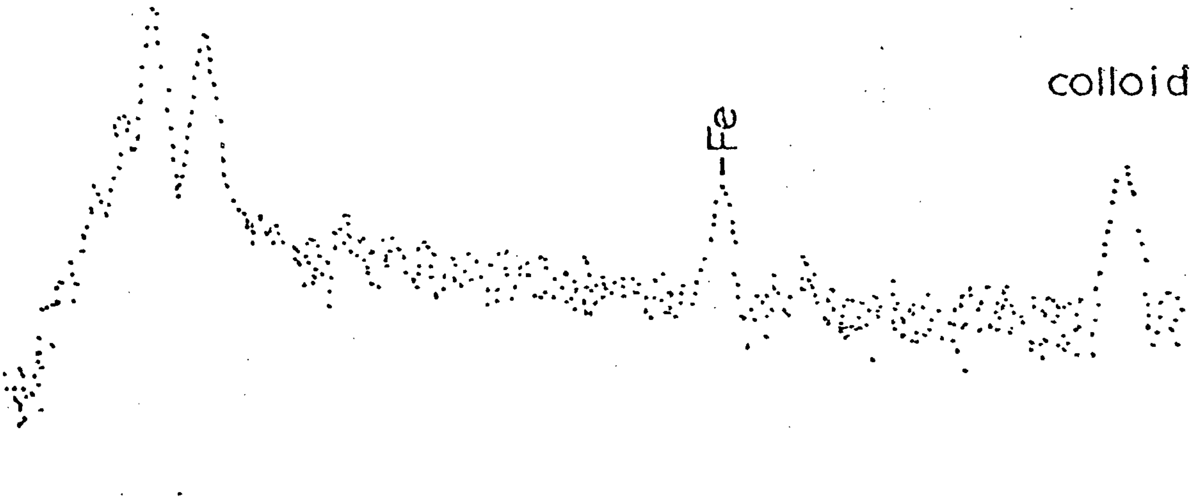


colloid

Arm, the Strait of Georgia, and Fitzhugh Sound contained many diatoms; however, no colloidal association was observed. Additionally, little colloidal matter was present on the filters from these areas.

As mentioned in the materials and methods section, borosilicate glass plates were exposed to sample water and then examined by S.E.M. to determine the degree of control the siliceous frustule might exert over the apparent colloidal association. Colloidal material was found to be associated with the plates and in some cases these colloids were found to be iron-rich.

Laboratory Study

Bioassays

The growth in cultures supplied with fresh colloidal iron and iron-EDTA stocks was found not to be significantly different demonstrating that EDTA did not enhance the supply of iron from freshly precipitated colloidal hydroxide to *T. psuedonna* (Fig. 9). The same series of experiments was run using glutamic acid with similar results. To determine if the iron remained available with no chelating agent in the medium during autoclaving, fresh colloidal iron was added to the medium before autoclaving. Growth in these cultures was found to be no different than in cultures with fresh colloidal iron added after autoclaving (Fig. 10). Since EDTA was shown not to increase the iron availability, cultures having fresh colloidal iron added

Figure 9. Cell growth with fresh colloidal (□) and iron-EDTA stocks (△) added after medium autoclaving is shown. The X-axis represents days of the bioassay and the Y axis the log of the cell number ml^{-1}. One S.D. was less than the symbol size. Growth in the two tests was not significantly different.

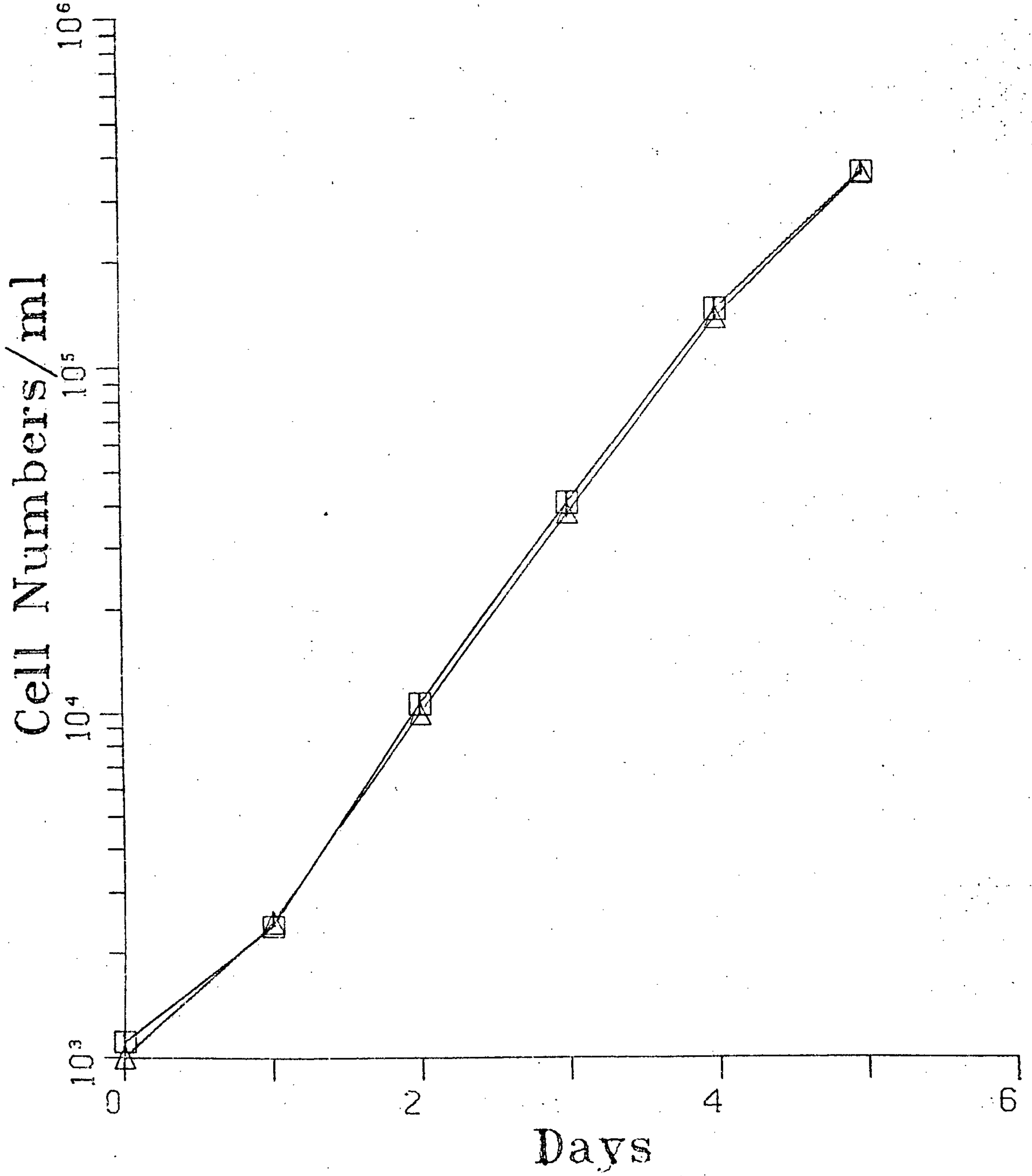
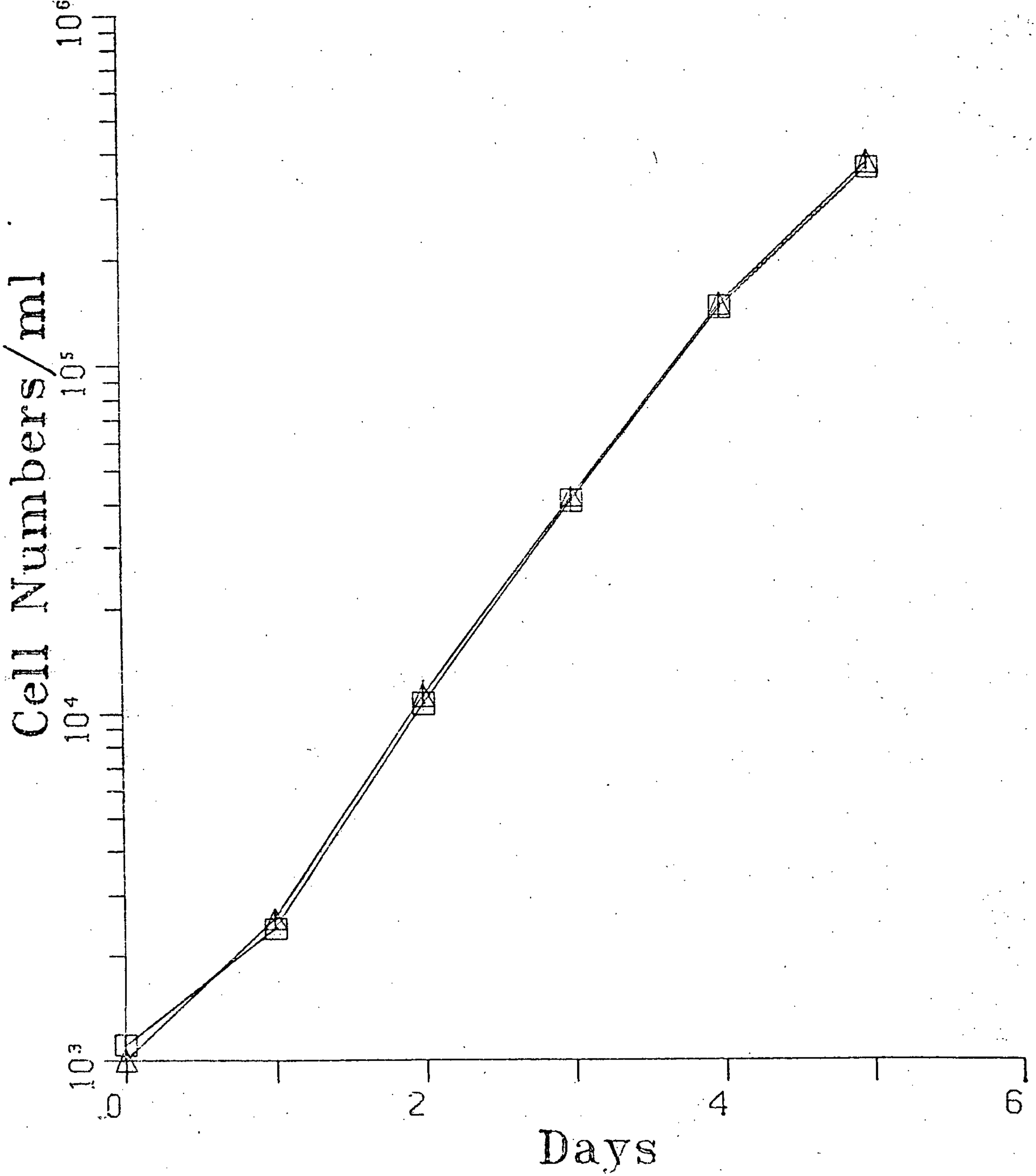

Cell Numbers/ml
10^6
10^5
10^4
10^3
0
2
4
6
Days

Figure 10. Cell growth with fresh colloidal stock addition before medium autoclaving (□) was found not to be significantly different from additions after medium autoclaving (△). One S.D. was less than the symbol size.


Cell Numbers/ml
10^6
10^5
10^4
10^3
0
2
4
6
Days

after medium autoclaving were chosen as the control for subsequent experiments.

The fresh colloidal iron stock was then autoclaved, heated, and aged to determine what treatments of the stock might reduce the availability of the iron. Autoclaving of the fresh colloidal iron stock before addition to the cultures caused a reduction in the rate of organism growth (Fig. 11). This growth reduction did not vary significantly with different periods of autoclaving (Fig. 12). The growth limiting factor, as determined by the addition of both nutrients (NO_3^- , PO_4^{3-} , and $Si(OH)_4$) and metals (Cu, Mn, Zn, Co, Mo, and Fe), was found to be iron.

To determine if the increased pressure during autoclaving (15 psi), contributed to this change in the iron stock, aliquots of a fresh colloidal iron stock were heated to 100°C (at atmospheric pressure) for 5, 10 and 15 minute intervals before addition to cultures. The growth in these experiments did not differ significantly and was similar to that of cultures supplied with autoclaved iron stock (Fig. 13). Fresh colloidal iron was again the only nutrient addition that increased organism growth. Cell numbers in cultures supplied with the iron stocks heated for 5 and 10 minutes were found to increase very slowly over a period of 21 days after growth had ceased in cultures without added iron and in the control.

To provide a better indication of the degree of heating required to cause this change in iron availability, aliquots of a fresh colloidal iron stock, prepared at room temperature (ca. 21°C), were heated for 5 minute intervals at 50°C, 70°C and 90°C

Figure 11. Growth in cultures supplied with autoclaved colloidal stock (□) was significantly less than that of the non-autoclaved fresh colloidal iron (△). This reduction in growth was found to be due to iron limitation (by nutrient addition). One S.D. was less than the symbol size.

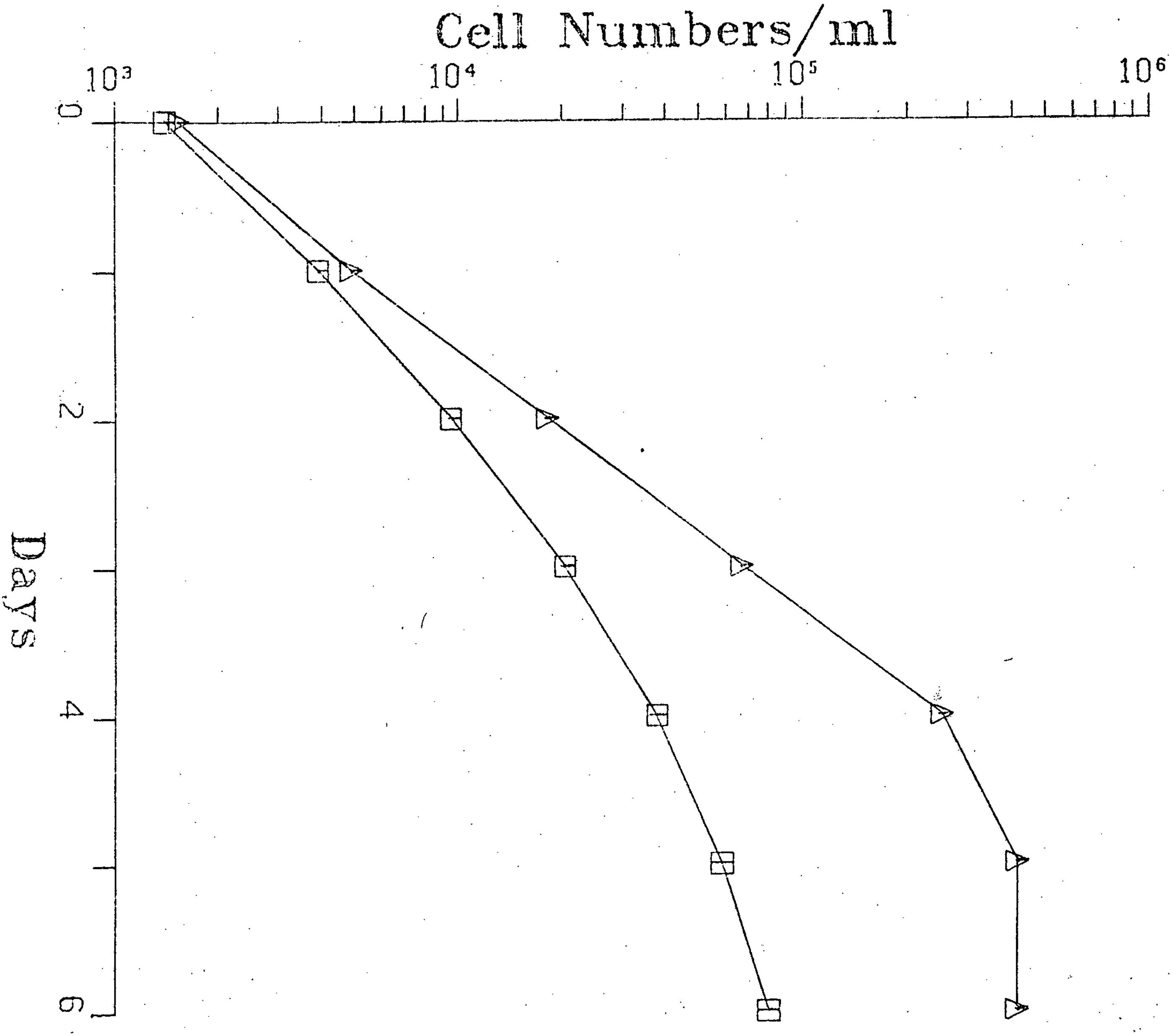

Cell Numbers/ml
10^3
10^4
10^5
10^6
Days
0
2
4
6

Figure 12. Fresh colloidal stocks were autoclaved for 15 minutes (Δ), 4 hours (◇), and 24 hours (¤) periods before addition to cultures. Growth in these cultures was significantly below the control (□) but not substantially different from one another. Bar represents one S.D. where greater than symbol size.

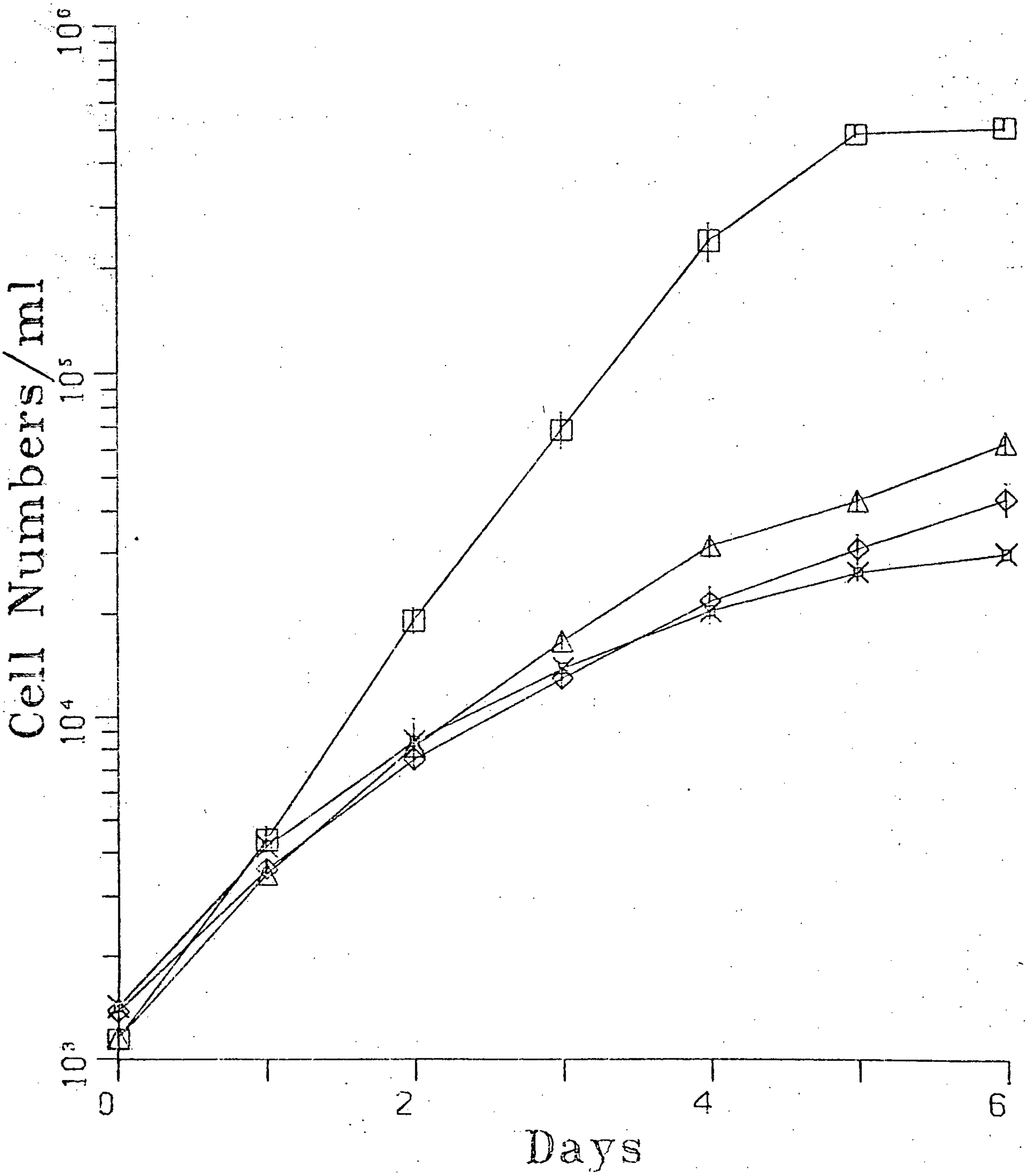
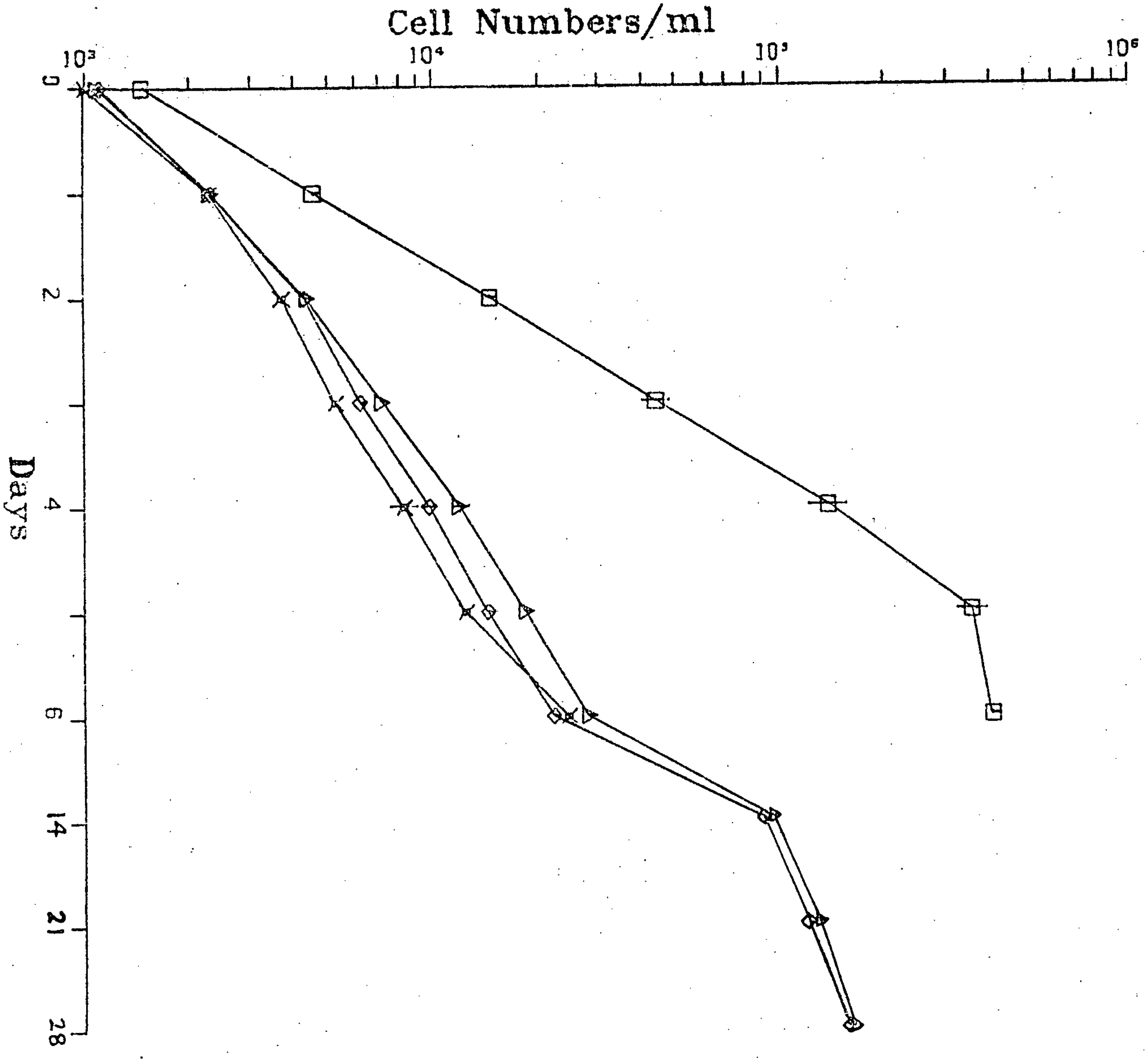

Cell Numbers/ml
10⁶
10⁵
10⁴
10³
0
2
4
6
Days

Figure 13. Growth in cultures supplied with iron stocks heated (100°C) for 5 mins. (△), 10 mins. (◇), and 15 mins. (⌘) was similar and significantly less than the control (□). Growth in these cultures, however, continued during a monitoring period of 28 days. Bar represents one S.D. where greater than symbol size.


Cell Numbers/ml
10³
10⁴
10⁵
10⁶
Days
0
2
4
6
14
21
28

before being added to the cultures. Cultures supplied with the aliquot heated to 50°C had less growth than the control, but significantly better growth than cultures supplied with the aliquots heated to 70°C and 90°C (Fig. 14). There was no significant difference in growth between the experiments at the two higher temperatures.

Since reduced organism growth was not found when the iron was autoclaved in the culture medium (Fig. 10), the effect of ionic strength on the availability of the colloidal iron was investigated. A Fe-SOW colloidal stock was autoclaved (15 min) before being added to cultures. Growth in these cultures was found not to vary significantly from the control and was substantially greater than the autoclaved Fe-distilled water stock (Fig. 15).

To determine the effect of ageing on the availability of iron, fresh colloidal iron stocks (in distilled water at room temperature and pH 2.5) were aged for one week and three months before being added to cultures. Three months of ageing resulted in a significant growth reduction while one week of ageing did not affect growth (Fig. 16). The growth pattern of the cultures supplied with three month old iron stock was similar to those of cultures supplied with an autoclaved iron stock (Fig. 17). Additions of EDTA to the cultures did not enhance growth. Trace metal and nutrient additions demonstrated that the limiting factor was iron.

To determine how the presence of a chelating agent in the iron stock affected the change in iron availability, fresh iron-

Figure 14. Growth in cultures supplied with an iron stock heated to 50°C for 5 minutes (Δ) was significantly below that of the control (□) but better than those cultures supplied with iron stocks heated to 70°C (◇) and 90°C (⌑). Bar represents one S.D. where greater than symbol size.

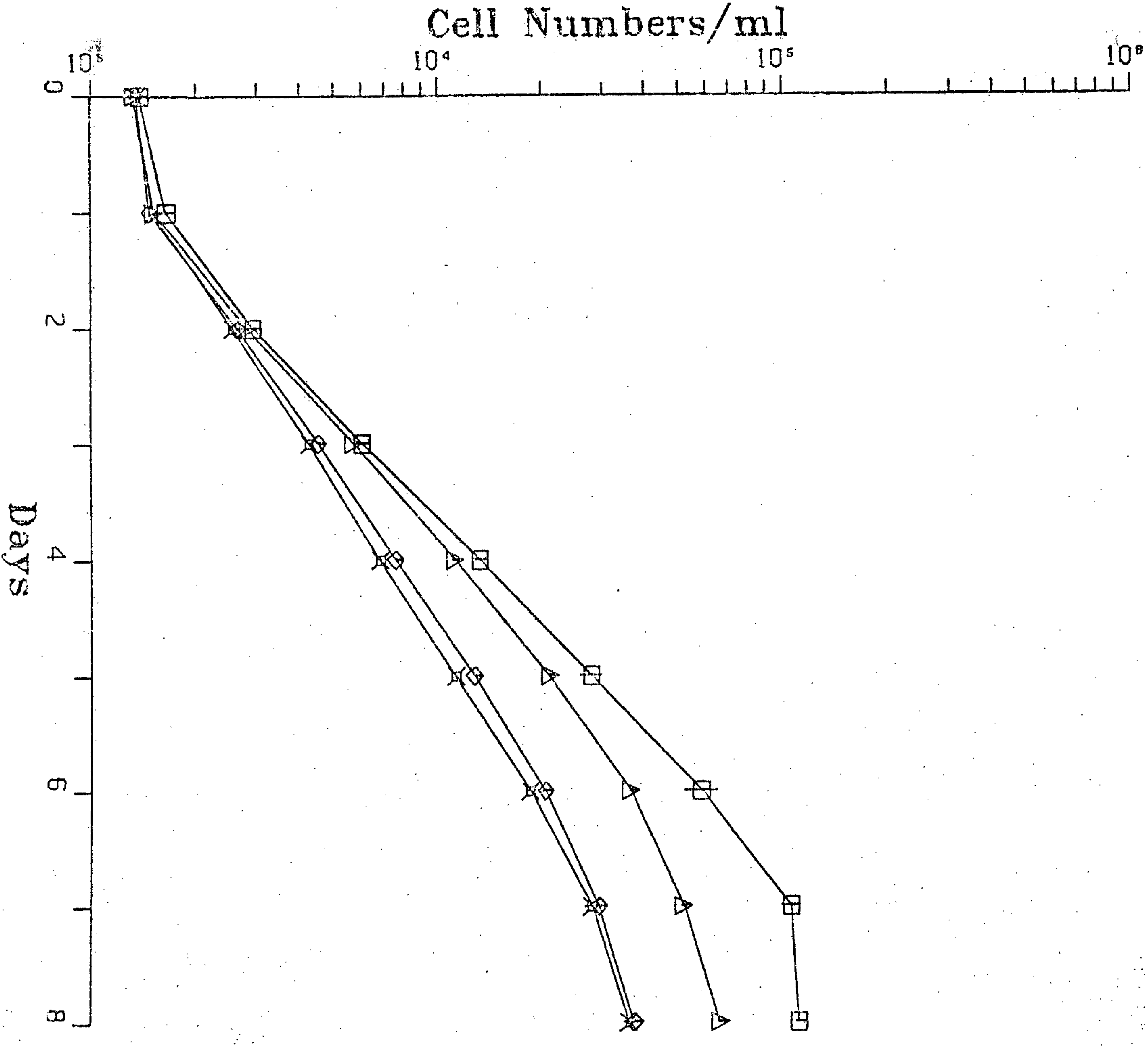

Cell Numbers/ml
10³
10⁴
10⁵
10⁶
Days
0
2
4
6
8

Figure 15. Growth in cultures supplied with distilled water autoclaved iron stock (□) and autoclaved Fe-SOW stock (△) were compared to the control (◇). The increased ionic strength of the Fe-SOW stock appeared to prevent the change in availability. Bar represents one S.D. where greater than symbol size.

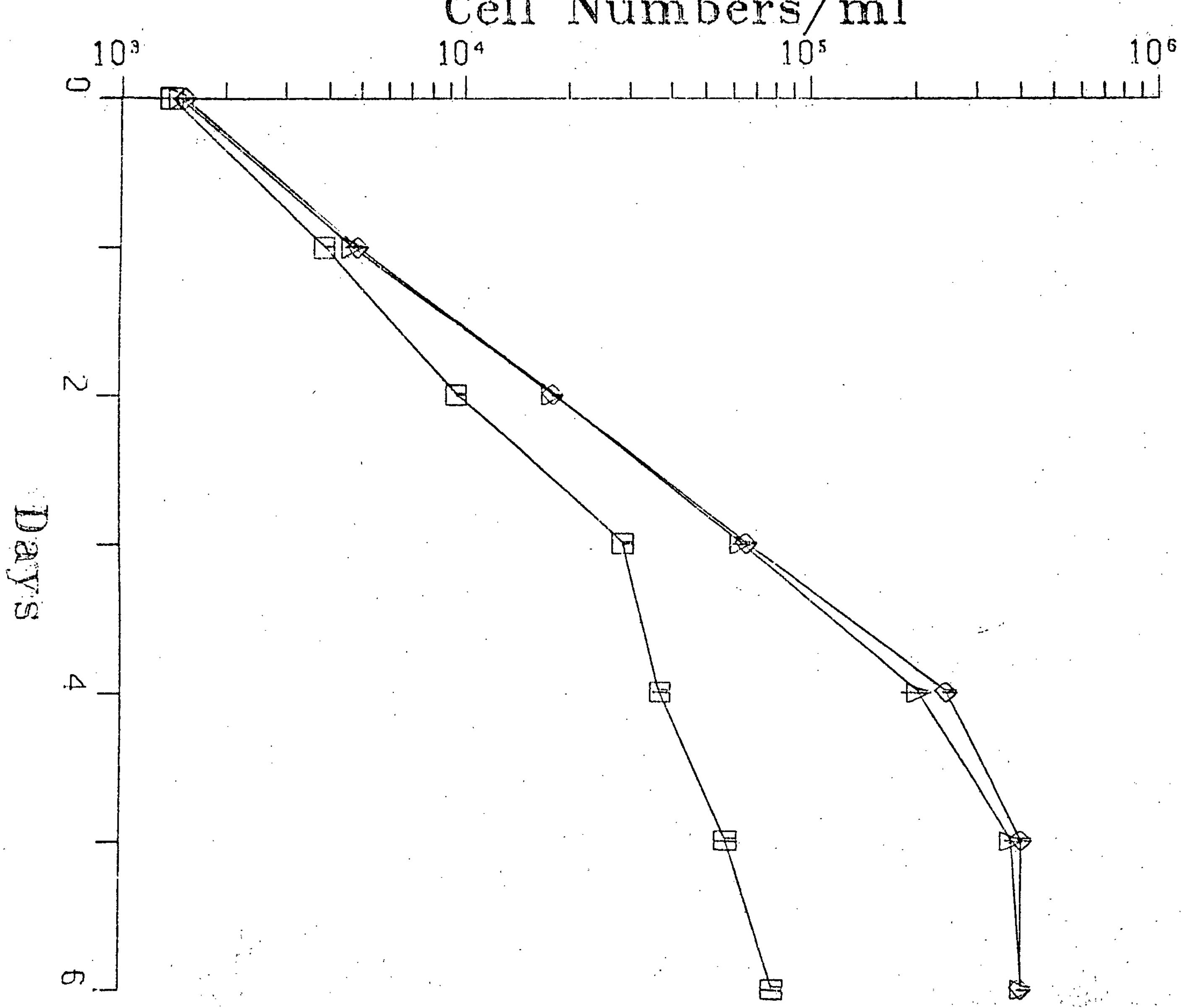

Cell Numbers/ml
10³
10⁴
10⁵
10⁶
Days
0
2
4
6

Figure 16. The effect of iron stock ageing on cell growth was found to vary with the period of ageing. One week of ageing at 21°C (△) was not significantly different from the control (□) while 3 months of ageing (◇) produced a much lower rate of growth. The reduced growth was again found to be due to iron limitation. One S.D. was less than the symbol size.

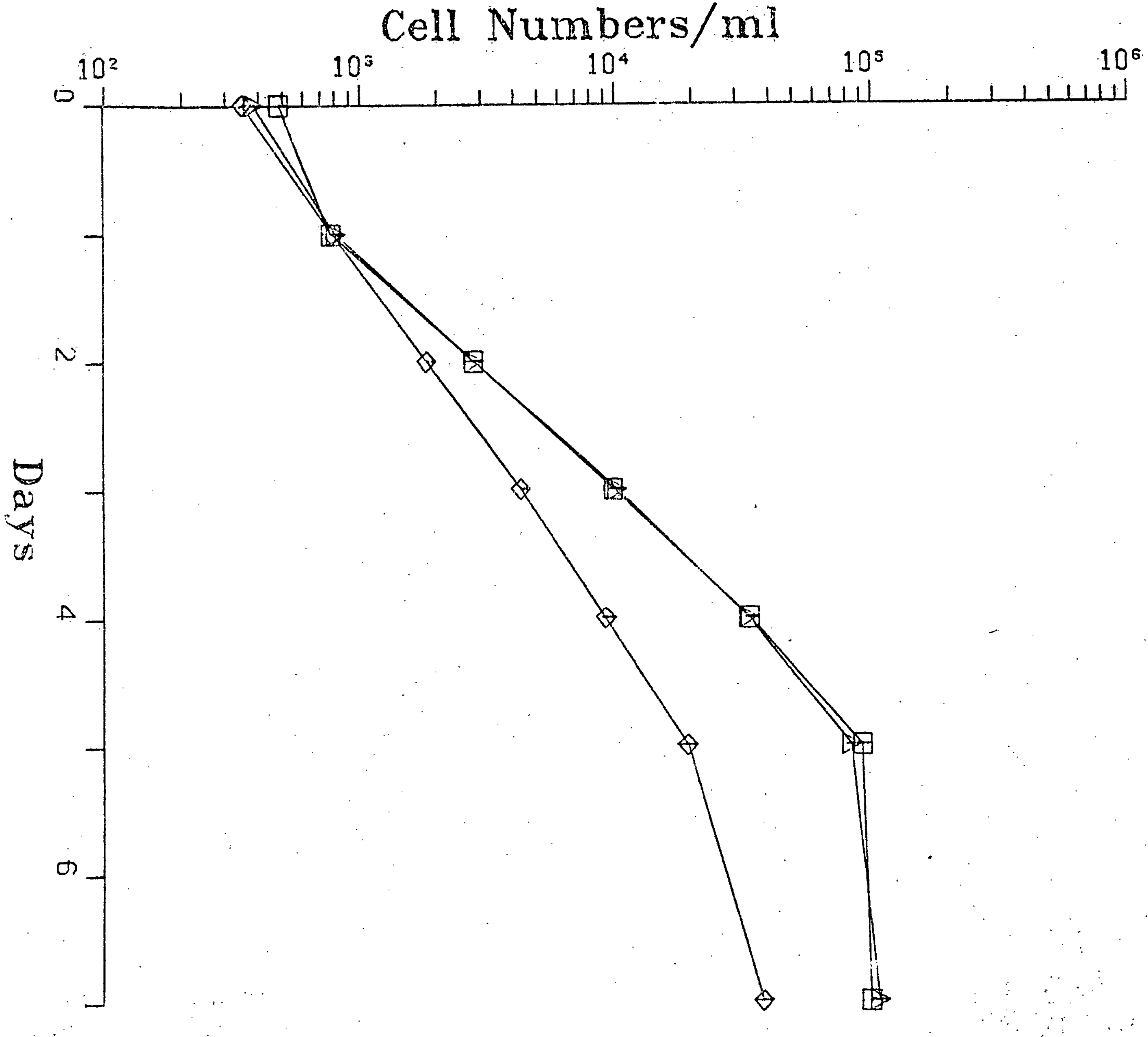
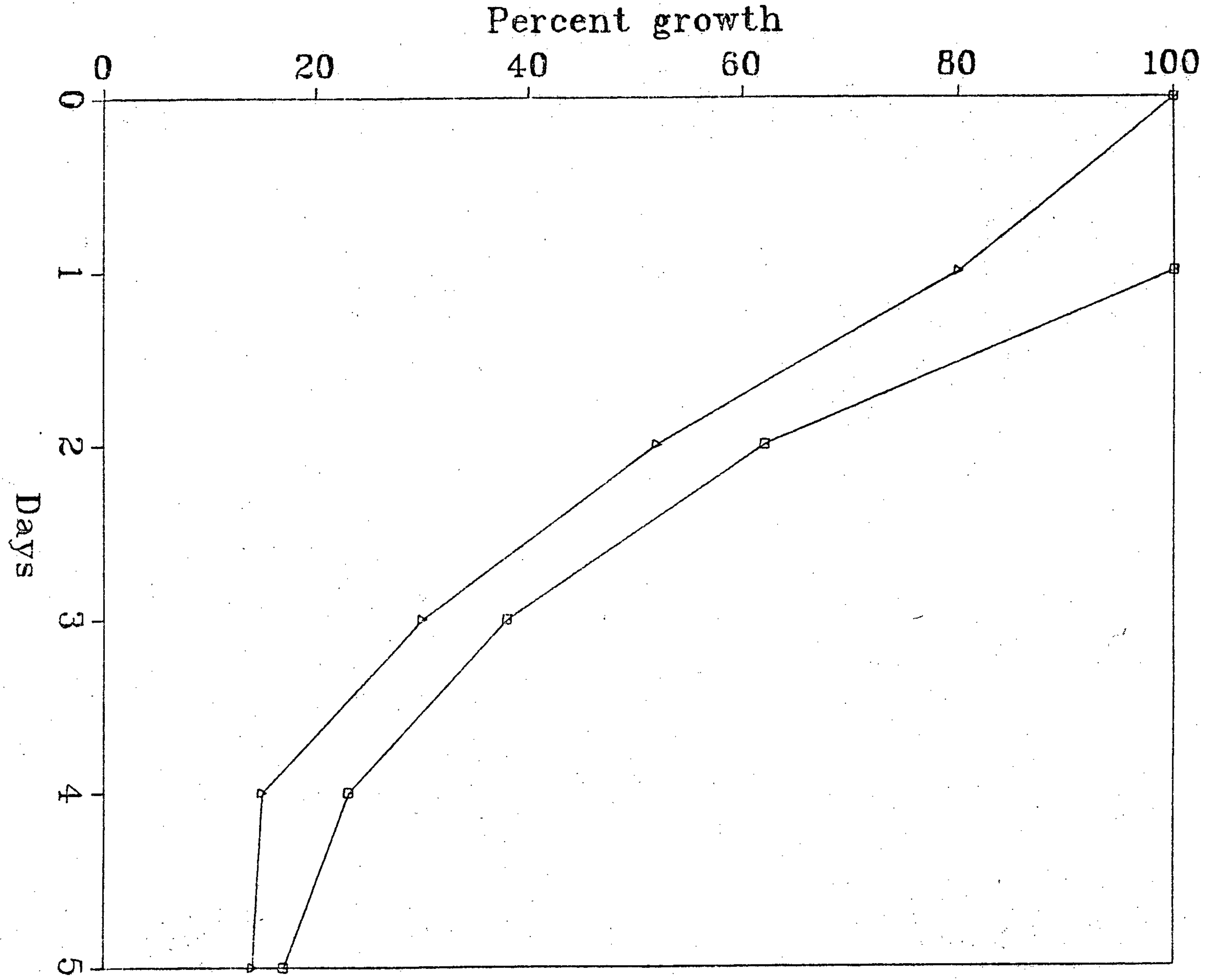

Cell Numbers/ml
10²
10³
10⁴
10⁵
10⁶
0
2
4
6
Days

Figure 17. The growth patterns of cultures supplied with iron stocks heated at 100°C for 5 minutes (△) and aged at 21°C for 3 months (□), were found to be similar. The growth in these tests are expressed as a percent of the control growth over time.


Percent growth
0
20
40
60
80
100
0
1
2
3
4
5
Days

EDTA stocks (Fe: 4.5×10^{-4}M, EDTA: 5.0×10^{-3}M) were autoclaved (15 mins.) or aged (14 months at 10°C) before addition to the cultures. (Because of a report by Lockhart and Blakely (1975) on the degradation of EDTA, the percent loss of EDTA during the autoclaving and the bioassays was estimated (appendix 3) and found not to be significant.) Growth in cultures supplied with these iron stocks was not significantly different from each other or from that of cultures supplied with fresh colloidal iron (Fig. 18).

Colloidal goethite and hematite were tested to determine their ability to supply iron to the organism. The growth in cultures supplied with these two forms of iron was similar but significantly below that of cultures supplied with freshly prepared colloidal iron (Fig. 19). The growth patterns were also similar to those of cultures with no added iron. Additions of EDTA to the cultures did not enhance growth. Growth limitation was again found to be due to iron limitation. Using a light microscope, both colloidal goethite and hematite were observed to be attached to the living diatom cells in the test cultures.

In each bioassay series there was always substantial growth for the first 2-3 days in cultures that had no added iron. However, cultures supplied with autoclaved, heated and three month old iron stocks had significantly lower growth when compared with those cultures without added iron (Fig. 20). It therefore appears that the addition of these stocks actually reduced the available concentration of iron.

Figure 18. When cultures were supplied with Fe-EDTA stocks that were autoclaved (△) or aged for 18 months at 21°C (◇), the growth was not significantly different from the control (□). One S.D. was less than the symbol size.

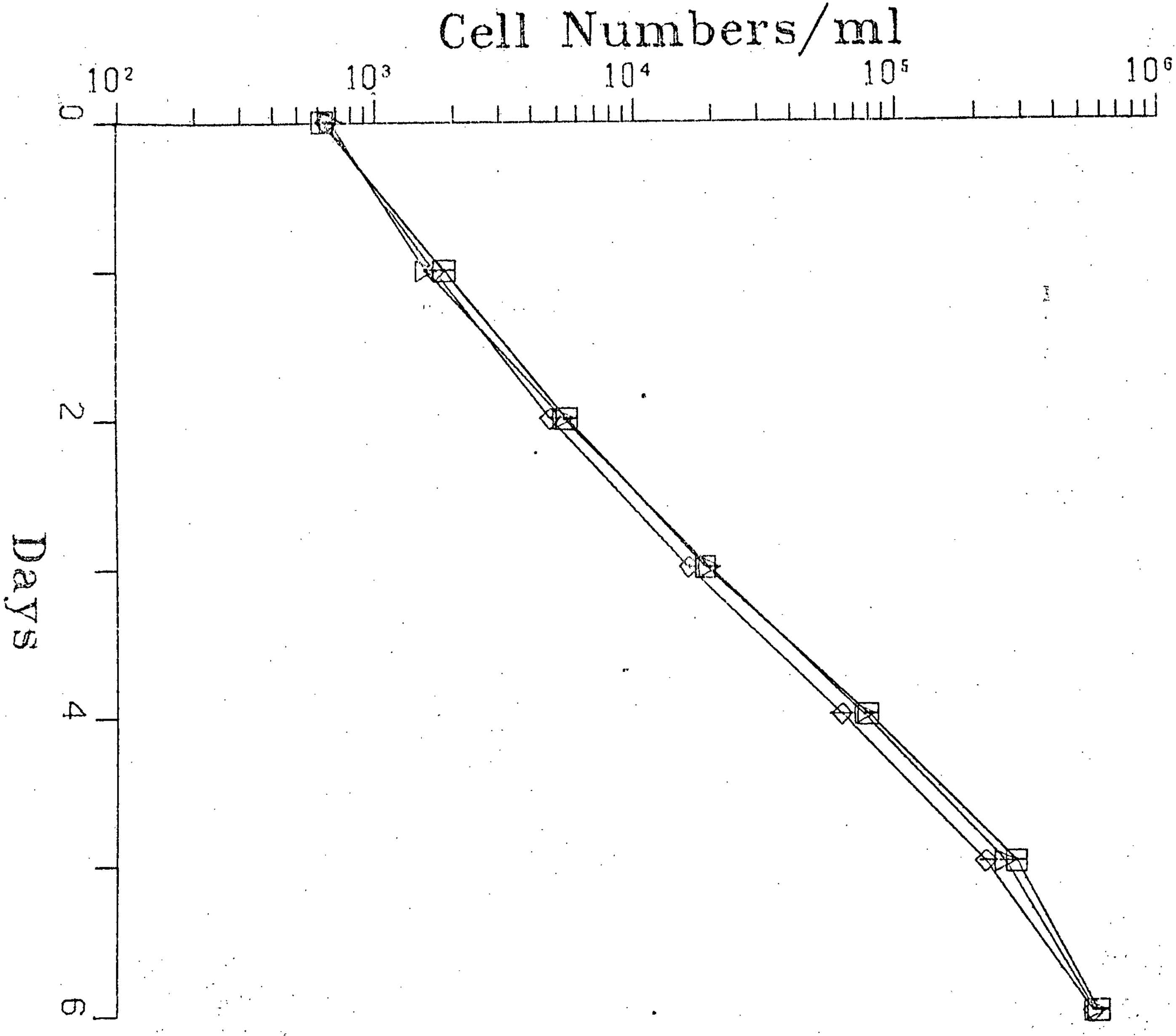

Cell Numbers/ml
10^2
10^3
10^4
10^5
10^6
Days
0
2
4
6

Figure 19. Growth in cultures supplied with colloidal goethite (◇) and hematite (△) was found to be significantly below that of the control (□). Reduced growth was found to be due to iron limitation. Bar represents one S.D. where greater than symbol size.

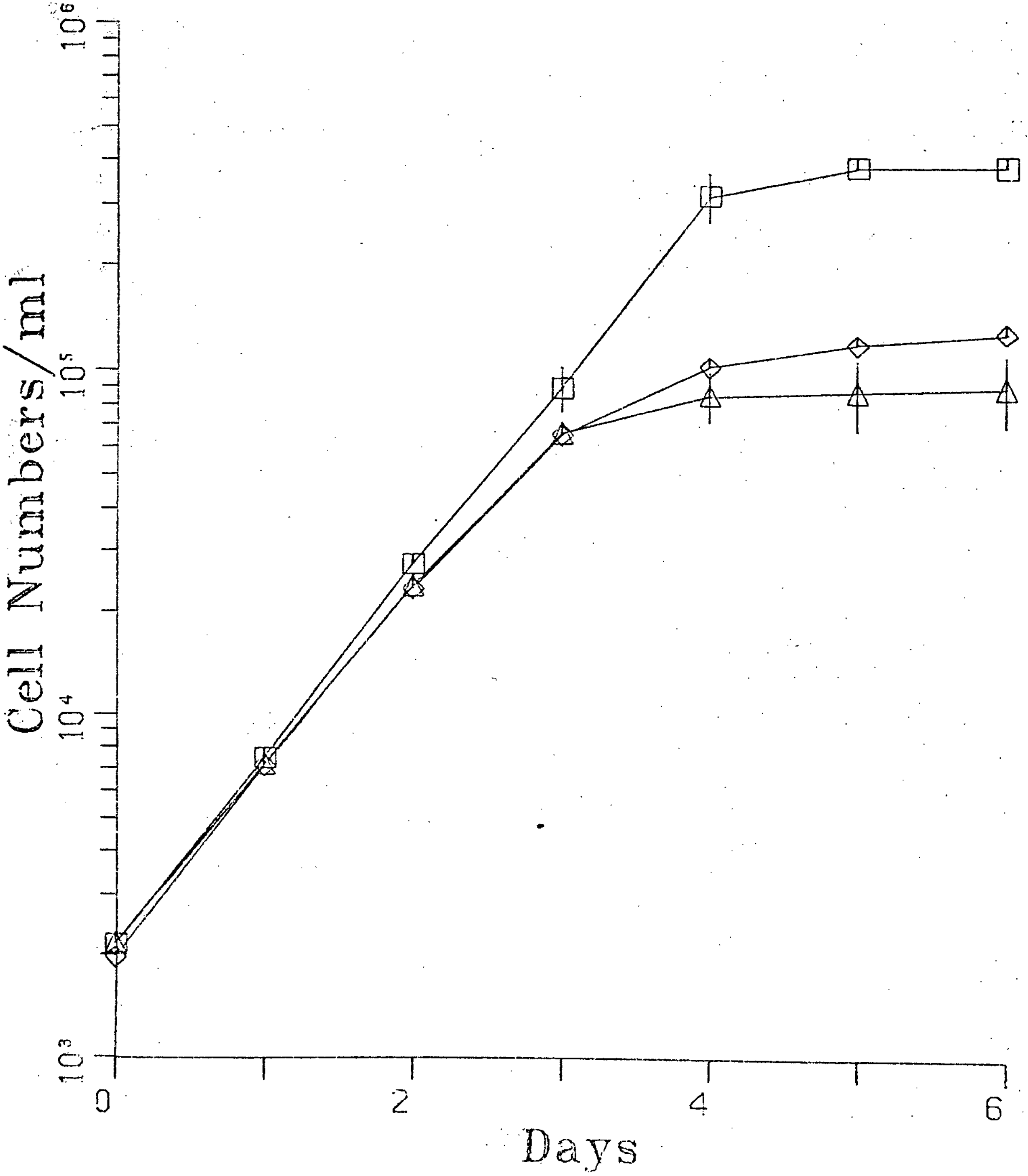
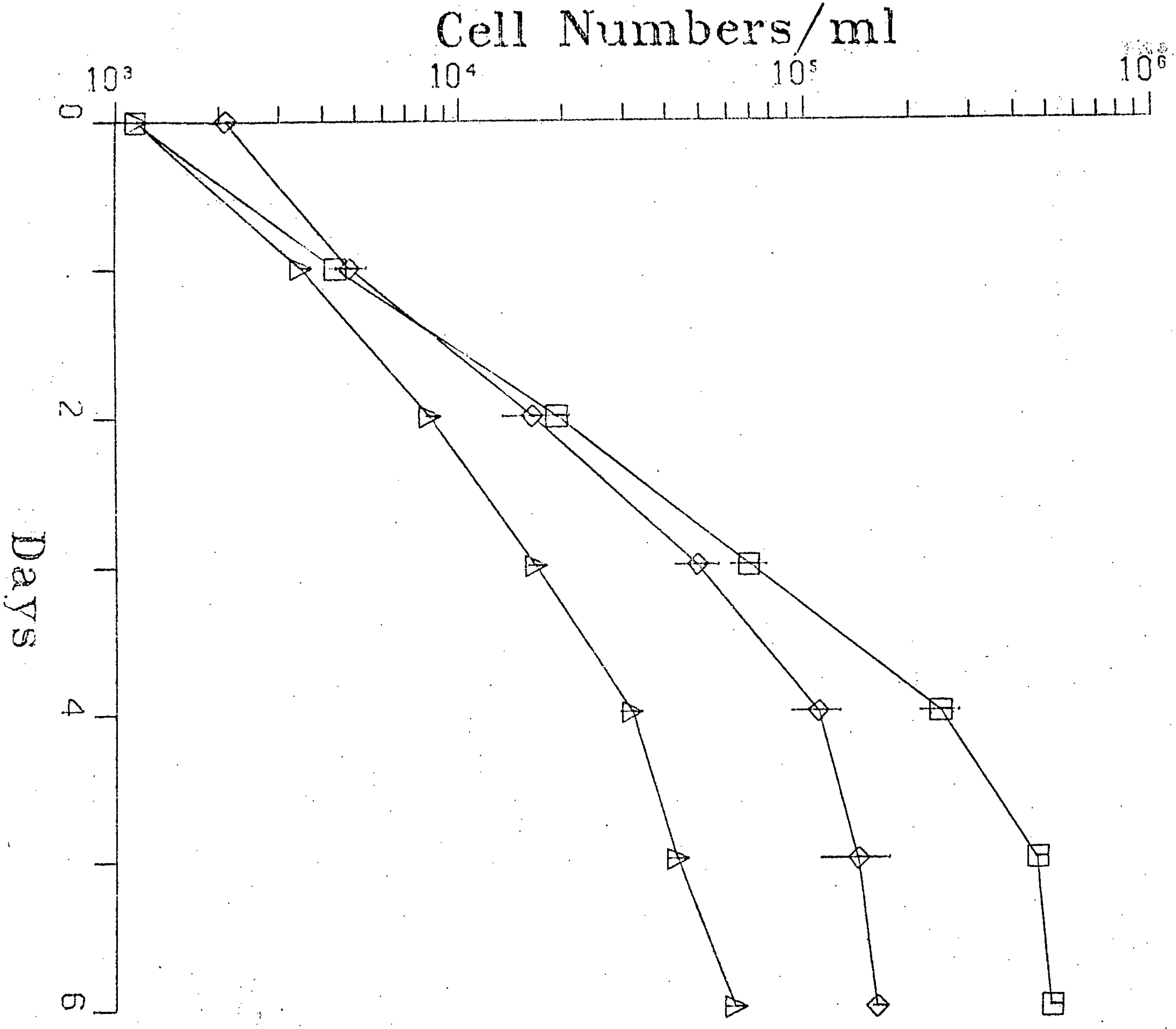

Cell Numbers/ml
10⁶
10⁵
10⁴
10³
0
2
4
6
Days

Figure 20. Growth in cultures without iron addition (◇) occurred for the first 2-3 days of the bioassays but was significantly less than the control (□). Cultures supplied with autoclaved colloidal stock (△) had significantly lower growth than the cultures without added iron. Bar represents one S.D. where greater than symbol size.


Cell Numbers/ml
10³
10⁴
10⁵
10⁶
0
2
4
6
Days

Analysis of the Autoclaved Iron Stock

To determine how autoclaving changed the colloidal iron stock, the autoclaved and fresh colloidal iron stocks were examined using X-ray diffraction, Mossbauer spectroscopy, thermal gravimetry, gel filtration and dissolution rate measurements. No analysis was performed on the three month old iron stock because of insufficient sample quantities.

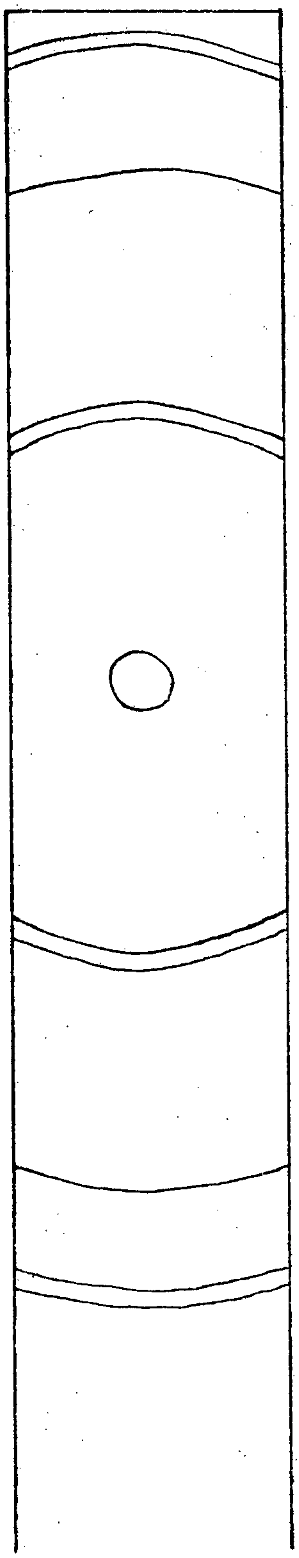
(i) X-ray diffraction

No crystallinity was detectable in the freshly prepared or autoclaved (30 mins.) iron stocks. Microcrystallites, however, were observed in the autoclaved stock using a petrographic microscope with crossed Nichols. Although crystallinity was not detected by X-ray diffraction, the iron in the sample appeared to be birefringent. (Microcrystallites could remain undetected with the X-ray techniques used.) When iron stocks were autoclaved for different lengths of time (15 min, 30 min, 1 hr, 4 hr, 12 hr, and 24 hr), a diffraction pattern was detected using a camera technique in the iron stock autoclaved for 24 hr (Fig. 21).

(ii) Mossbauer spectroscopy

Mossbauer spectroscopic analysis did not demonstrate any significant difference between the autoclaved and non-autoclaved stocks. The hyperfine splitting and the chemical isomer shift of the two stocks were identical within the error of the technique

Figure 21. The X-ray diffraction pattern of the iron stock after a 24 hour period of autoclaving. The bands on the film represent the diffraction pattern of a crystal structure. No bands were detected after shorter periods of autoclaving.



(table 1). (For a brief description of Mossbauer spectroscopy see appendix 4.) There was, however, a slight increase in the peak width (Γ) of the autoclaved stock.

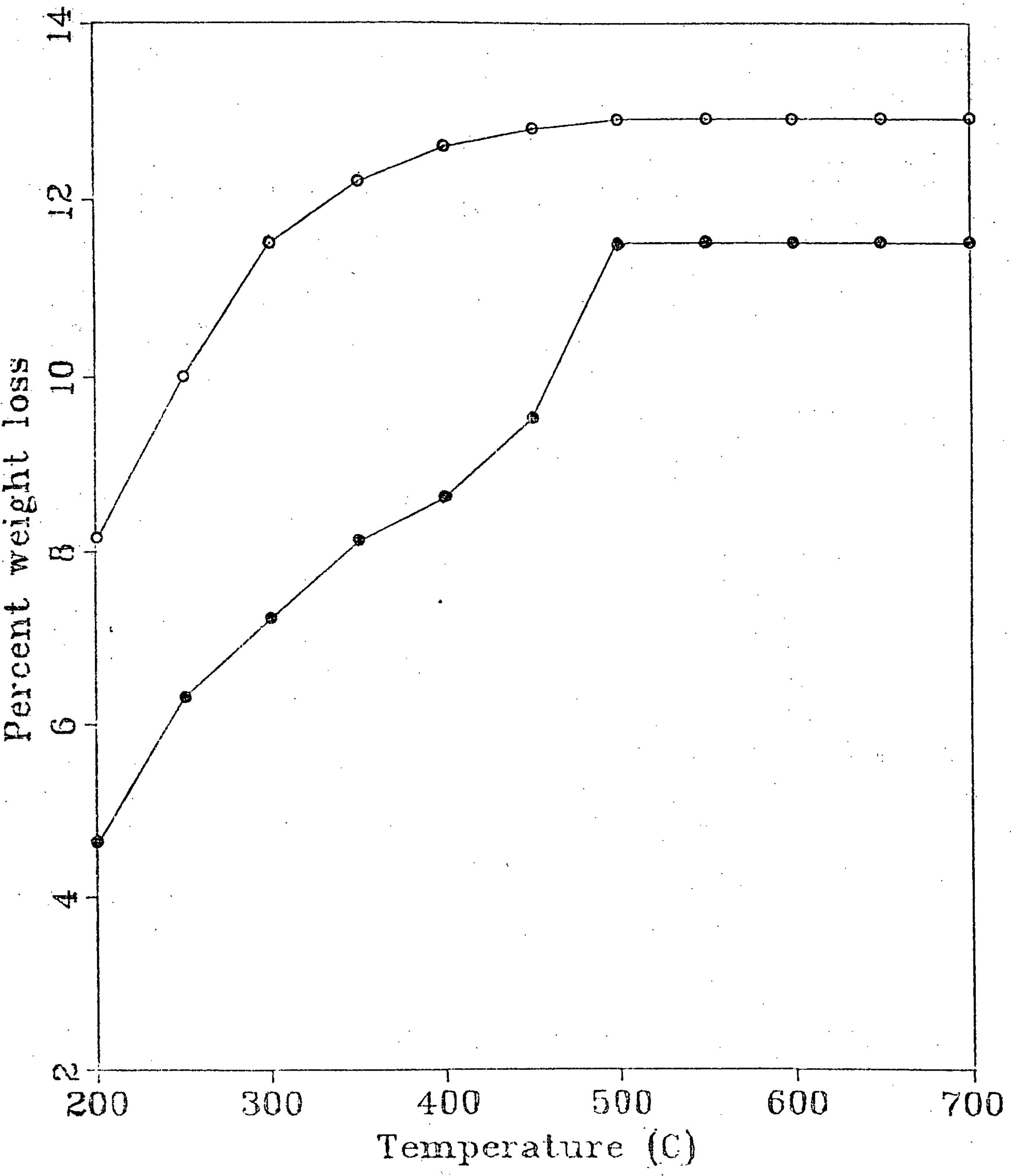
Table 1. Mossbauer analysis of the autoclaved and fresh iron stock. The error of the analysis is about .02.

Iron Type	Splitting (σ)	Shift (Δ)	Peak Width (Γ)	
			(Γ_1)	(Γ_2)
Fresh colloidal iron	.665	.428	.634	.525
Autoclaved colloidal iron	.673	.441	.775	.648

(iii) Thermal gravimetic analysis

The percent weight change of the autoclaved and non-autoclaved colloidal iron stocks upon heating is shown in Fig. 22. The non-autoclaved colloidal iron was found to have a higher percent weight loss than the autoclaved material. The non-autoclaved sample had a steadily decreasing percent weight loss with increasing temperature, while the autoclaved sample had a rapid weight loss between 450°C and 500°C. Both samples had no further weight change above 500°C.

Figure 22. Thermal gravimetric analysis . The percent weight loss, through the temperature range tested, is shown for the fresh (o) and autoclaved (•) iron stocks. The results show that the autoclaved stock had a lower degree of percent weight loss which suggests a lower degree of hydration. In addition, the autoclaved iron stock had a rapid weight loss between 450°C and 500°C.


Percent weight loss
2
4
6
8
10
12
14
200
300
400
500
600
700
Temperature (C)

(iv) Gel filtration

The results of this analysis show that, with autoclaving, the colloidal size (or molecular weight) of the particles was increased (Fig. 23). This increase could be due to either coagulation of the existing polycationic colloids or a dissolution-reprecipitation process.

(v) Dissolution rates

The dissolution rates of the colloids in the two stocks were compared by colorimetric measurement of the dissolved ferric iron concentrations over time. The absorbance change of the two samples over a 30 minute interval is shown in Fig. 24. The non-autoclaved iron stock had a higher initial absorbance and a greater rate of increase than that in the autoclaved stock. Absolute concentrations were not calculated since the experiment was used only to illustrate a difference between the iron stocks.

Figure 23. Comparison of the gel filtration rates . The gel filtration rate of the fresh colloidal stock (△) was measured and compared to that of the autoclaved colloid stock (□). The results are plotted as iron concentration vs volume eluted from the column. The gel bed volume was determined to be 15 milliters. The autoclaved stock was found to pass directly through the column indicating that the colloids were too large to enter the gel beads. However, the non-autoclaved stock passed slower through the column presumably because the colloids passed through the gel beads. These results suggest that the colloidal size of the fresh iron stock increased upon autoclaving.

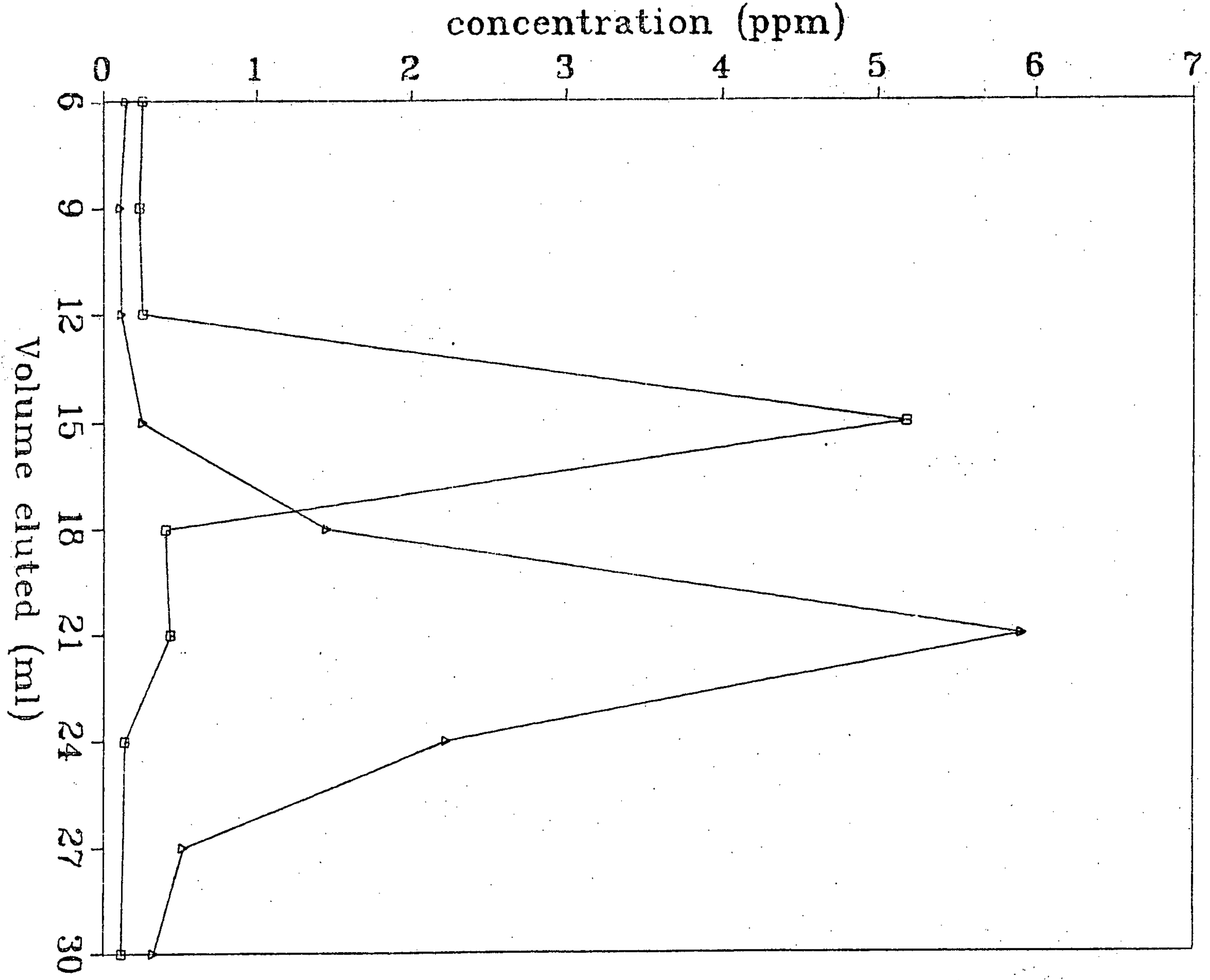
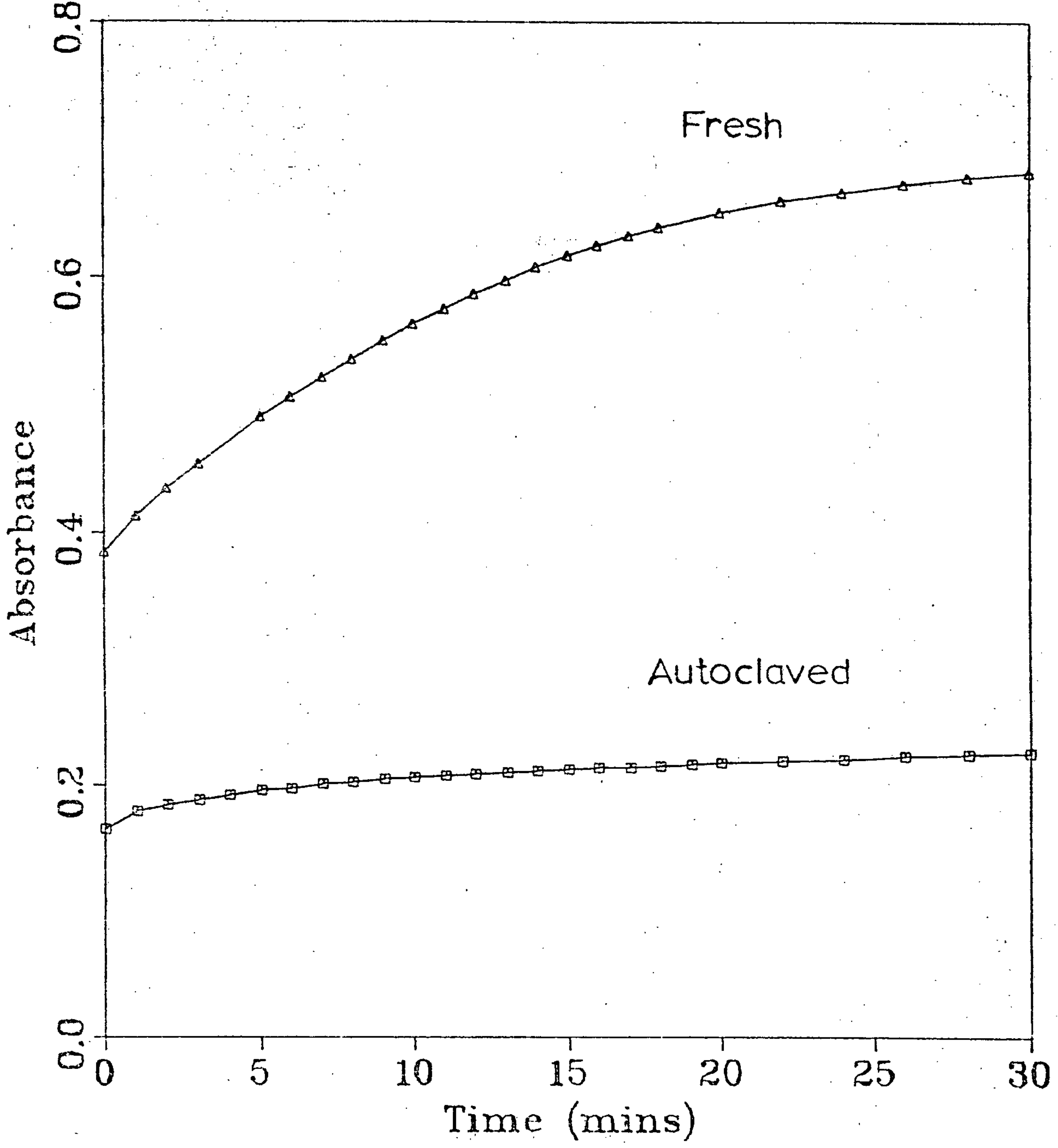

concentration (ppm)
0
1
2
3
4
5
6
7
6
9
12
15
18
21
24
27
30
Volume eluted (ml)

Figure 24. Comparison of the dissolution rates. The dissolution rates of autoclaved (□) and fresh colloidal stocks (△) were measured colorimetrically over 30 mins and are plotted as absorbance over time. The results show that the dissolution rate of the fresh colloidal stock is much more rapid than that of the autoclaved stock.


Fresh
Autoclaved
Absorbance
0.0
0.2
0.4
0.6
0.8
Time (mins)
0
5
10
15
20
25
30

DISCUSSION

Field Study

Colloids were found to be associated with diatom frustules in three localities sampled: the bottom waters of Indian Arm (Ind 2.0), the surface water in the Fraser River plume (Fra 1.5) and the Fraser River salt wedge. The water in these localities was similar in that each contained abundant colloidal material. In some cases the associated colloids were iron-rich. Although this association was not found to be ubiquitous among the localities sampled, the results of the glass plate experiments suggest that it is possible for any frustule surface to become coated by colloidal material. Factors that could have contributed to the absence of observed colloidal association with diatom frustules in the surface waters of Indian Arm, the Strait of Georgia and Fitzhugh Sound include the lower colloid:diatom ratios in these areas and the limited resolution of the S.E.M.. (Although the S.E.M. is capable of very high resolution, it is greatly decreased with the lower beam current used in this case to reduce the damage to the biological material.)

It is possible that the observed colloid-diatom association, in areas with abundant colloidal material, may have been an artifact of the filtration process (by settling of colloidal material onto the frustules). In these localities, however, colloidal material was found associated with areas of diatom frustules not exposed to particle settling from above (i.e. underneath frustule overhangs). In addition, exposed surfaces of frustules were in some cases found to be 'material free', yet the surrounding area of the filter contained large amounts of colloidal material. These observations suggest that at least some of the associated colloids are not artifacts and that colloidal material is in fact associated with diatoms in the natural environment. This is supported by observations of surface associated colloidal material in laboratory cultures (Harvey 1937, Wells, Honours thesis and present study).

Results from the glass plate experiments suggest that the association of colloidal material with diatoms need not be controlled by the diatom and may be the result of a passive process. However, organisms without siliceous frustules may behave differently. Massalski and Leppard (1979) observed the association of clay particles with freshwater algae and bacteria and found that fibrillar colloids, produced within the cell, acted to bridge the clays to the cell surface. Such an association between the colloid and the cell would appear to be actively controlled by the cell. At present, the role of the colloidal association with organisms is unknown but it has been suggested that it is of nutritional value (Harvey 1937, Goldberg 1952). However, it remains to be shown that metals within colloids are available to organisms. The bulk of the laboratory work in this study was directed towards answering this question.

If the iron-rich colloidal material associated with diatoms was being utilized, it is prerequisite that dissolution must occur before uptake. Thus, a re-distribution of iron across the frustule surface from the solubilization of the colloid might be expected. In fact, there does appear to be a slight gradational increase in iron content across the frustule towards the colloid (Fig 8), however the difference is not great and could be an artifact of the electron beam scatter. It is apparent that EDAX does not have the resolution necessary to define these small differences if they exist.

Bioassays

Previously published reports have suggested that metal chelating agents such as EDTA are required in phytoplankton cultures to achieve optimal growth (Provasoli, 1963, Canterford, 1979). Chelating agents are believed to enhance growth by preventing precipitation and maintaining the iron in a soluble state. As a result, it was desirable to exclude chelators when testing the ability of colloids to supply iron to the organism. Thus the purpose of the initial experiments was to develop a bioassay technique that achieved optimum growth of the organism without the addition of a chelating agent. The growth in cultures supplied with fresh colloidal iron was not significantly different from those cultures supplied with iron complexed by EDTA (Fig. 9). From these results it is apparent that optimal growth of *T. psuedonana* in Aquil can be obtained without a chelating agent if the iron is supplied in the form of a freshly prepared ferric hydroxide colloidal suspension.

Experiments were then conducted to determine what treatments of the colloidal iron stock, prior to its addition to cultures, would reduce the iron availability. These treatments included autoclaving (121°C, 15 psi), heating, and ageing. In all cases the treated iron stocks were added to previously autoclaved culture media. Growth in cultures supplied with treated iron stocks was compared to that of the control (fresh untreated colloidal iron stocks added after autoclaving of medium) to determine if these treatments were responsible for a change in iron availability.

The results showed that autoclaving (15 mins, 15 psi, 121°C) of the iron stock caused a reduction in organism growth (Fig. 11) which was found to be due to iron limitation. Longer periods of autoclaving did not cause further substantial growth reduction (Fig. 12). Growth reduction also occurred when iron stocks were heated (100°C, 1 atm pressure), suggesting that pressure variations (during autoclaving) did not contribute to the change in iron availability. Although growth in cultures supplied with heated iron stocks was reduced it continued for a period of 21 days of monitoring after growth in the control had ceased (Fig 13). Even after 28 days, growth was continuing indicating the iron was still being made available to the organism but at a very slow rate. It is possible that cell numbers could have eventually reached the final level seen in the control cultures.

The decreased growth rate in cultures supplied with the iron stocks heated to 50°C and 70°C (5 mins.) (Fig. 14) demonstrates that while the decrease in availability was gradual, it occurred within the narrow temperature range between

21°C and 70°C. Again, the growth in the cultures supplied with the heated iron stock had not ceased at the end of the experiment. The iron stock ageing experiments showed that while one week of ageing did not reduce growth, patterns of reduced growth did occur after three months of iron stock ageing (at 21°C).

EDTA additions to the cultures supplied with heated or aged iron stocks was found not to enhance growth. However, addition of EDTA to the iron stocks before autoclaving or extended ageing prevented the reduction in iron availability (Fig. 19). Since EDTA prevents the formation of polynuclear ferric colloids, by chelation of the aquo-ions, these results suggest that the formation of iron colloids is a necessary precursor to the process that reduces the iron availability. Thus the chemical and/or physical changes occurring in the colloidal iron as a result of heating and extended ageing would appear to cause the reduction in iron availability. These changes are well documented and will now be briefly discussed.

Processes Occurring in Ferric Salt Solutions

The transformations that occur in colloidal iron stocks during heating and ageing are presumably similar and comparible to those reported to occur during hydrolysis of ferric salts (Murphy et al 1976, van der Giessen 1968, Dousma and de Bruyn 1976). After addition to distilled water, ferric chloride will dissociate and the Fe^{+3} ions liberated are rapidly hydrated, the coordination sphere containing six H_2O molecules forming an octahedron. This complex then rapidly releases a hydrogen ion to form $[Fe(H_2O)_5(OH)]^{2+}$ (van der Giessen 1968). The electrostatic repulsion of the hydrated ions can be overcome through a combination of van der Waals and hydrogen bonding, which causes the hydrated ions to associate with one another resulting in the formation of colloidal sized polynuclear complexes. The rate of this aggregation is largely controlled by the pH, temperature, the concentration of the aquo-ions, and the type of anions present. In the colloidal iron stocks prepared for these experiments, aggregation occurred within minutes, as was shown by a change in color from a translucent yellow to a deep amber. Murphy et al (1976) found that these colloids were spheres 1.5 - 3.0 nm in diameter which later linked to form larger rod-like structures. Adjacent hydrated ions within the polynuclear complex may undergo olation to form an OH (or ol) bridge between the ions resulting in the loss of water (van der Giessen, 1968, Dousma and deBruyen, 1976). A proton may then be split off from the ol bridge to form an oxo bridge. This latter process is called oxolation. The olation and oxolation processes are illustrated in Fig. 25. While the initial loss of protons from the aquo-ions occurs very rapidly, the olation and oxolation processes proceed slowly at room temperature and may take hundreds of hours for completion (van der Giessen, 1968). The rate of these processes, however, greatly increases at higher temperatures.

While the forces causing aggregation of the aquo-ions and ordering within the polynuclear complexes are chemical in nature, they are similar to those interfacial forces controlling ion adsorption to surfaces. Since interfacial forces are important in the supply of iron from the colloid to the organism, I have chosen to illustrate the factors controlling the aggregation and subsequent ordering processes with a general description of the energies of adsorption. While perhaps not strictly valid in a chemical sense, it will provide a basic understanding of interfacial forces that will be necessary to assess later conclusions.

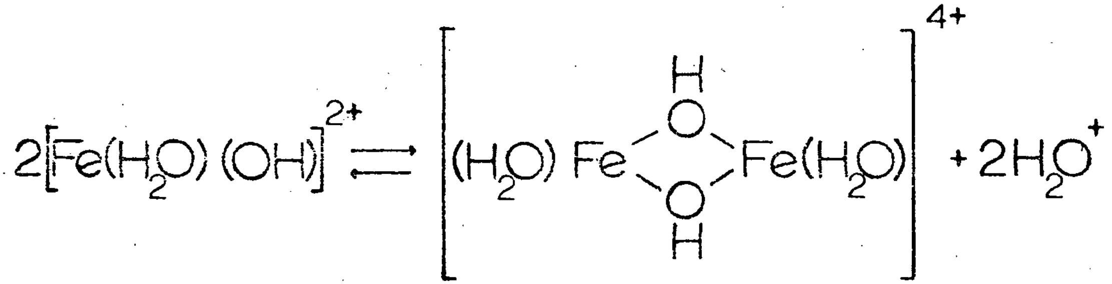
The relationship between energies of adsorption and distance from a surface are best described using the Lennard-Jones graph (Fig. 26). Both the energy input and release are represented by the vertical axis while the horizontal axis depicts distance from the surface. With adsorption, an approaching ion will slip within a defined distance (A) from the surface to form a non electron sharing bond (eg. electrostatic or van der Waals), thereby releasing the adsorption energy (Ea). Both the distance (A) and the Ea will vary not only with different ions and adsorbates, but also with different locations

Figure 25. The hydrolysis, olation and oxolation processes iron atoms undergo upon addition to an aqueous solution are shown. Initially, the hydrated ferric ion rapidly looses a proton. The resultant hydrated ion may then undergo olation (3) and subsequently oxolation (4). These bridging reactions proceed very slowly at room temperature. With time, however, randomly orientated ol and oxo bridges will form within the colloid.

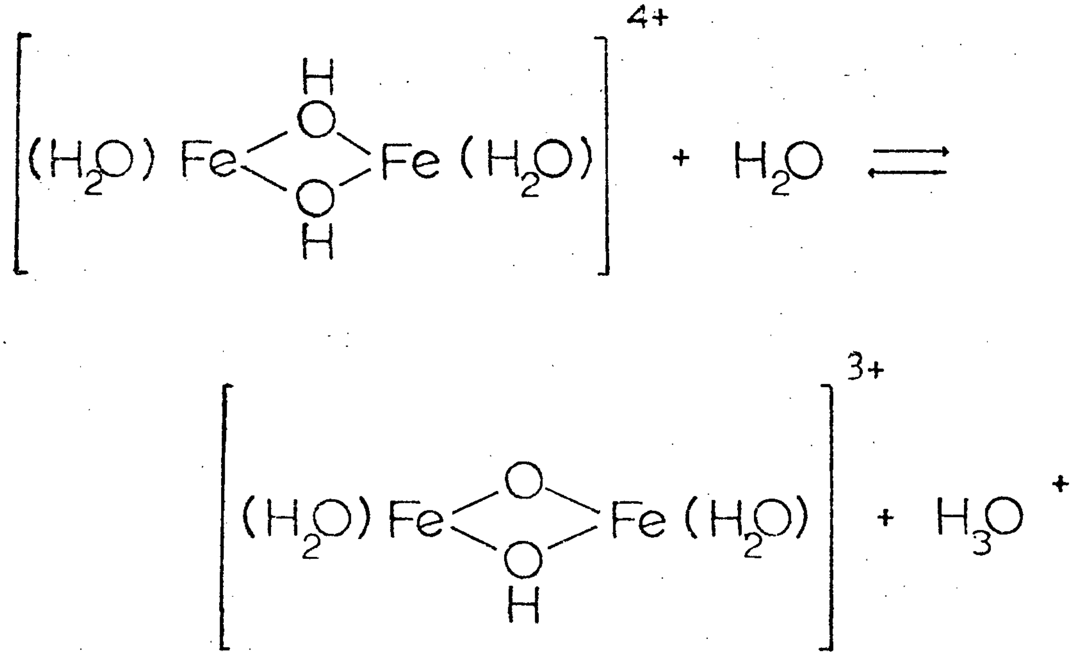
1. $Fe^{3+} + 6H_2O \rightleftharpoons Fe(H_2O)_6^{3+}$

2. $Fe(H_2O)_6^{3+} + H_2O \rightleftharpoons [Fe(H_2O)_5(OH)]^{2+} + H_3O^+$

3.



4.

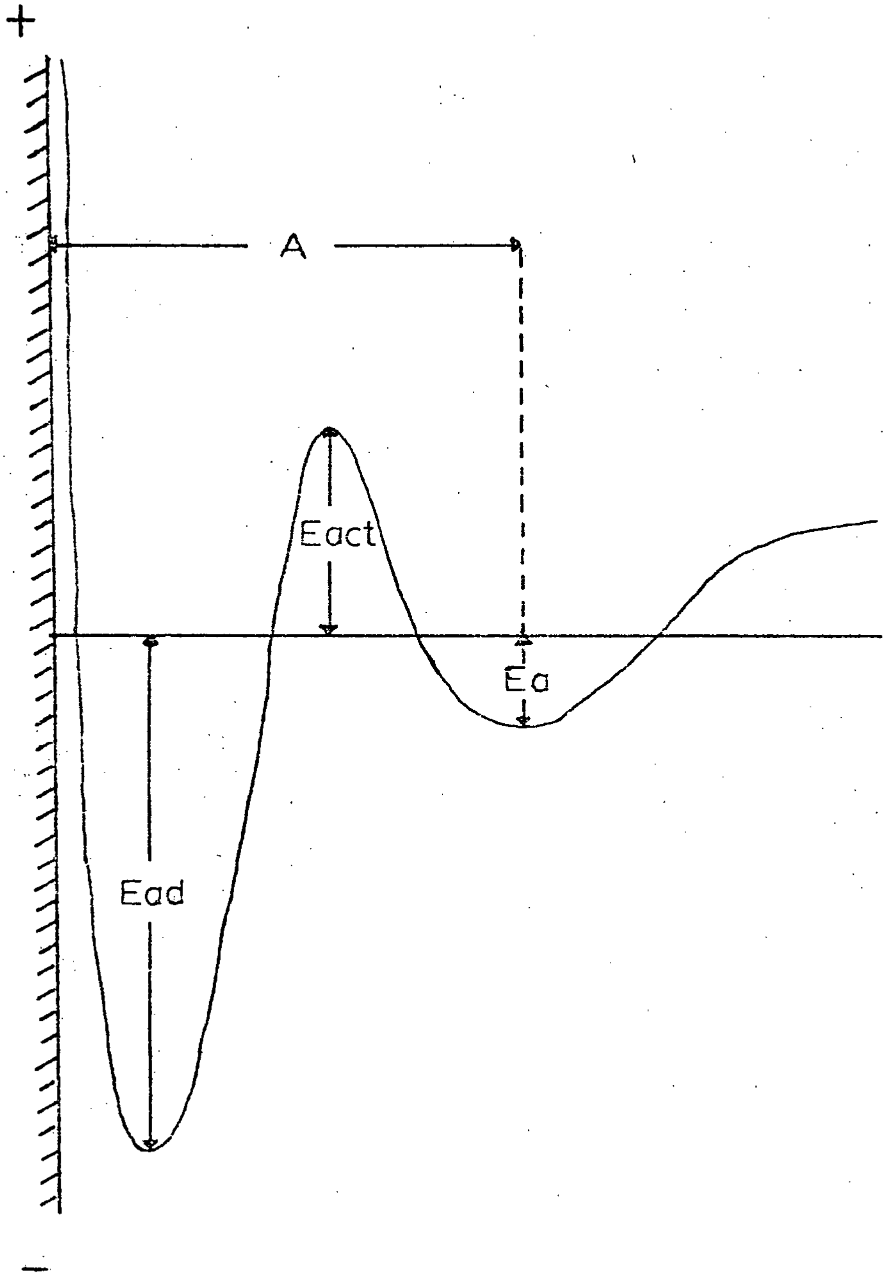


on heterogenous surfaces. Desorption (or ion release) may occur with an energy input equal to Ea. Because this is usually small the adsorption is relatively weak; this form of adsorption is termed physical adsorption.

Since energy is required to move the adsorbed ion closer to the surface, the distance (A) will fluctuate with the energy imparted to the ion by molecular collisions. There exists a critical distance from the surface within which there will be a spontaneous sharing of electrons between the ion and the surface, forming a more stable bond and reducing the adsorption distance (A). This reaction is much more exothermic (Ead) than physical adsorption and is termed chemical adsorption (or chemisorption). The energy input necessary to cause this change is known as the energy of activation (Eact). It will vary with different ions and locations on the heterogenous surface. Since release of the chemisorbed ion requires an input equal to both the Eact and Ead, chemisorption is less readily reversed. As a result, the chemisorbed ion is incorporated into the structure of the solid and desorption is then controlled mainly by the bond stability.

If we now consider the change that occurs in the colloidal iron stocks used in this study, the same principals apply. The hydrated ferric ions in the initial colloidal sized polynuclear complexes are linked by relatively weak bonding (van der Waals and hydrogen) such as in physical adsorption. If the Eact is supplied, adjacent hydrated ions will move to within the critical distance and part of the water-hydroxide sheath

Figure 26. Lennard-Jones graph . The energy of ions approaching a surface is depicted in this graph. Ea is the energy released upon physical adsorption and Eact is the activation energy required for the ion to shift to chemosorption; a much more exothermic reaction (Ead). The energies shown will vary with different ions and surfaces.


+
A
Eact
Ea
Ead
−

surrounding each iron atom will be lost as H_2O (Fig. 25), establishing ol bridges between the ferric ions (much the same as the change from physical to chemical adsorption). This reaction results in condensation (i.e. dehydration) of the polynuclear complexes. Once the initial olation occurs, further condensation is catalyzed by the presence of the ol bridges and over time a network of bridges within the polynuclear complex would be formed. (Catalysis occurs by a reduction in the activation energy.) Thus because of the increase in the strength of bonding between the ferric ions of the colloid, the thermodynamic stability of the oxide phase is increased. The overall rate of this change in the colloidal iron stock would be determined by both the rate of hydrated ion aggregation and by the rate of the reaction which subsequently transforms the disorganized, amorphous solid that initially forms into one having a more ordered structure and greater thermodynamic stability.

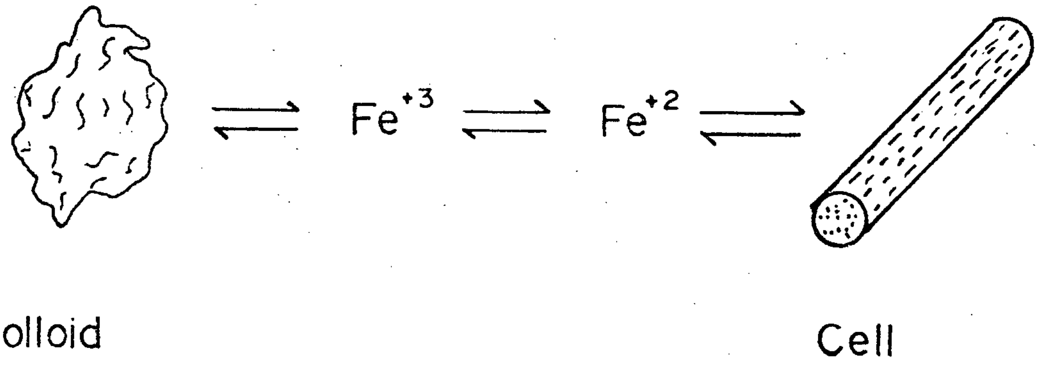
Although the processes of aggregation and subsequent dehydration have been described separately, one must understand that both may occur simultaneously. As polynuclear complexes form and grow by aggregation of aquo-ions from solution, olation and oxolation will occur within the colloids. The extent to which these reactions occur during aggregation is dependent on the temperature of the solution. With increasing temperatures, the energy of the interacting molecular units increases permitting a greater rate of reaction. Thus the relationship between aggregation, olation and oxolation will change with different conditions.

While the initial bridging of the iron atoms will be randomly orientated, over time the structure will change to a crystalline one. Charateristically, the structure within the hydrous ferric oxides will undergo constant alterations over time progressing through two or more crystalline forms before the most thermodynamically stable structure is achieved (Langmuir and Whittlemore, 1971). (The most stable structure varies under different conditions.) Thus one must not consider hydrous ferric oxides as static polynuclear complexes but as constantly changing units.

To determine whether crystallization of the colloidal iron would reduce the growth of the organism, the ability of colloidal goethite and hematite to supply iron to the organism was tested. Goethite and hematite are probably the most thermodynamically stable ferric oxyhydroxide and oxide under earth surface conditions (Langmuir and Whittlemore, 1971). The growth in cultures supplied with these forms of iron was significantly below that of cultures supplied with fresh colloidal iron (Fig. 20). This reduction was found to be due to iron limitation. Although association of colloidal goethite and hematite with the living diatoms of the cultures was observed, the growth patterns were similar to those of cultures with no added iron indicating that there was little or no iron supplied to the organism. These results suggest that the availability of colloidal iron changes in parallel with the thermodynamic stability of the structure within the colloid. It is also apparent that iron availability is not directly related to the proximity of the colloid to the organism as suggested by Harvey (1937) and Goldberg (1952).

If variation in the thermodynamic stability of the structure within the colloid does affect the availability of iron, it must be related to its affect on the mononuclear iron species, one or more of which are believed to be available. For the moment, let us assume that one or a number of dissolved ferric species are available to the organism. Upon uptake of the dissolved ferric species by the organism, their replacement in the medium would occur through a re-equilibration of the solution with the colloidal iron. In a given solution, the rate of such replacement from a colloid is controlled by both the rate of monomer removal from the colloid, which is dependent on the active surface area, and the magnitude of the activation energy of the dissociation reaction. This energy of activation increases with increasing stability of the ferric ion bonding configuration. Thus, the thermodynamic stability of the polynuclear complexes may be related to iron availability through changes in the colloidal dissolution rate.

However, another effect of increasing thermodynamic stability is a reduction in the mononuclear iron species concentration. While these reduced concentrations may not be of importance if the iron species released from the colloid is in a biologically available form, a more significant effect might occur if reduction or conversion to some other specific species is necessary before uptake is possible. If, for the moment, it is assummed that reduction of the ferric ion is necessary before uptake by the organism can occur (Anderson and Morel, 1980), then a possible pathway of iron supply to the organism might be as shown below. A decrease in the dissolved ferric iron concentration may decrease the rate of reduction (step 2) and thus the iron supply to the organism. If the rate of reduction (or any other ferric ion conversion) is the rate determining step, addition of EDTA to the medium containing heated or aged iron would increase this rate, and thus organism growth, by increasing the concentration of the mononuclear iron species. However, addition of EDTA to cultures supplied with heated or



A Possible Pathway of Iron Supply

aged iron stocks did not enhance growth suggesting that low mononuclear iron concentrations was not the major cause of growth reduction but rather that the reduced dissolution rate of the colloids controlled organism growth.

Growth in cultures supplied with heated, autoclaved, and three month old iron stocks was not only less than the control, but was significantly lower than that of cultures with no added iron. A possible explanation for this phenomenon is the adsorption of residual available iron (present by contamination) to the added colloids of the stock. The metal scavenging abilities of colloidal hydrous oxides are well documented and adsorption of the residual available iron in the cultures may occur within hours (Kim and Zeitlin, 1971). The initial adsorption may be physical, however, if the stability of the colloidal oxide of these stocks was higher, the structuring present would catalyze chemisorption of the iron (Leja, personal comm.). The ions would then be incorporated into the colloid structure and release of these adsorbed ions would then be determined by the stability of the colloid. Although the residual iron would also be scavenged by colloids in the control cultures (fresh colloidal iron addition), the growth demonstrates that these colloids provide a good supply of iron to the organism.

While autoclaving of the fresh colloidal iron stock before addition to the cultures was shown to reduce organism growth, the initial laboratory experiments showed that autoclaving of the Aquil medium with iron (Fe: 4.5×10^{-7}M) had no deleterious effect on growth (Fig. 10). It was found that the higher ionic strength of the solution was, in part, responsible for the lack of reduction in iron availability. This salt effect might result because of the occlusion of foreign ions by the precipitate which then interferes with the formation of oxide bonds between the iron atoms (Langmuir and Whittlemore, 1971). Thus, a hydrous oxide phase of lower thermodynamic stability might be expected in the higher ionic strength solution after the same period of heating suggesting more rapid dissolution rates and higher mononuclear iron concentrations. This in turn would be expected to increase the iron availability. In addition to higher ionic strength, the lower iron concentration in the medium during autoclaving (Fe: 4.5×10^{-7}M) would also decrease the rate of hydrated ion aggregation and thus the rate of restructuring.

In summary, it is suggested that autoclaving, heating and extended ageing all cause the colloidal phase to undergo similar structural changes that presumably increase their thermodynamic stability. These structural modifications, moreover, appear to decrease the biological availability of the colloidal iron by reducing the colloid dissolution rate. To investigate this hypothesis further, the autoclaved and fresh colloidal iron stocks were analytically compared.

Analysis of the Autoclaved and Fresh Iron Stocks

To determine whether the structure of the colloidal iron preparations was measurably altered by short periods of autoclaving, various properties of the autoclaved and fresh colloidal iron stocks (both in distilled water) were compared. The thermodynamic stability of the colloidal phase is determined by its surface area and structure, the latter being in turn determined by factors such as mineralogy, degree of ordering degree of hydration and by the presence of impurities. The effect of impurities can be disregarded in the present case because of the method of stock preparation. Thus the relative thermodynamic stabilities of the colloidal iron stocks should increase as the degree of ordering increases and the degree of hydration and the surface area decrease. The techniques used to estimate these characteristics included X-ray diffraction, Mossbauer spectroscopy, thermal gravimetric analysis, and gel filtration.

No ordering was detected in the autoclaved (15 min) or fresh iron stock by X-ray diffraction using either wavelength scanning or camera techniques. However, after 24 hrs of autoclaving, evidence of crystallization was detected by the camera technique. This suggests that early stages of ordering might be present after 15 minutes of autoclaving. The inability to detect ordering in the latter stock is likely due to the limitations of the diffraction technique.

Mossbauer spectroscopy, which was also used to compare the degree of ordering in the two iron stocks showed a slight increase in peak width of the autoclaved colloidal stock that could possibly be due to alteration in the electron spin orientations of some iron atoms (i.e. altered bond formation). These observations, however, could be equally well explained by differences in sample thickness or by alterations in the orientation of the atomic ferromagnetic field during freezing of the sample. The inability of Mossbauer spectroscopy to measure a significant difference between the stocks demonstrates that reduction in iron availability requires very little change in the measurable chemical environment of the atom. However, the ferromagnetic properties of iron interfere to some extent in the analysis and it may be possible to achieve a better comparison of the stocks with more extensive Mossbauer studies.

The degree of hydration of the autoclaved and fresh colloidal stocks was compared with thermal gravimetric analysis. The samples were first heated to 115°C for 4 minutes (to drive off the water molecules associated with pores and surfaces) and then to 700°C. Because the initial heating (4 mins, 115°C) would also cause some loss of chemically bound water (through olation), the technique was used only to compare the stocks rather than to measure their actual degrees of hydration. The autoclaved stock was found to have a lower degree of hydration than that of the fresh stock (Fig. 22) suggesting that olation or oxolation between iron atoms had occurred in the autoclaved colloids. Additionaly, the autoclaved stock had a rapid water loss between 450°C and 500°C. One possible explanation for this phenomenon may be the release of trapped or chemically bound water molecules. During the ordering process some water molecules can be incorporated within the structure and at higher temperatures sufficient energy may be provided to release this water. The temperature at which this release occurs will vary depending on the strength and configuration of the bonds incorporating the water molecules into the structure. The narrow temperature range of release in this case would suggest that these molecules must have had similar bonding configurations. Another possibility for this sudden water release might be a simultaneous change in atomic bonding configurations (i.e. structure) within the solid that resulted in the loss of hydroxide ions (through further olation) as water. In either case, the sudden weight change between 450°C and 500°C further suggests that ordering occurred within the colloids during autoclaving.

In an attempt to compare the surface areas of the colloidal iron stocks, their gel filtration rates were measured. The results showed that the colloidal size increased upon autoclaving of the iron stock (Fig. 23). Although the size of well crystallized colloids can be used to estimate their surface area, the surface areas of amorphous ferric hydroxides cannot be estimated in this fashion as they have a porous, gel-like structure through which ions can migrate. Thus, measurement of the exterior surface area greatly underestimates the actual surface area. If the colloid size increase in the autoclaved iron stock was due only to coagulation of existing polynuclear complexes, it would not appreciably reduce the actual surface area of the colloidal stock (although exchange between the internal surface and exterior solution would be slowed). However, Murphy _et al_ (1976) suggested that during heating of such polymeric colloidal suspensions, a dissolution-reprecipitation reaction occurs that results in increased structural order. If this did occur in the heated iron stocks, the porosity of the colloids would be reduced (and thus the surface area), which in turn would reduce the number of sites available to react with the solution. However, this reprecipitation process was seen by Murphy _et al_ after a period of weeks in solutions that were four orders of magnitude more concentrated than the stocks used in this study and did not result in an appreciable change in colloidal size (although aggregates were later seen to form). Thus the increase in colloid size is probably due to just coagulation of existing colloids, a phenomenon that should not appreciably alter the thermodynamic stability of the hydrous oxide phase. This suggests that the increased stability was mainly due to increases in the degree of condensation and, hence, of ordering.

Since the rate of dissolution is expected to covary with changes in thermodynamic stability, the dissolution rates of the colloidal phase in the two stocks were compared. The results indicate that the dissolution rate of the fresh colloidal iron stock was more rapid than that of the autoclaved iron stock (Fig. 24). This provides evidence supporting the hypothesis that decreased growth in cultures supplied with the heated iron stocks was due to reduced rates of colloidal dissolution. The very low rate of dissolution in this stock could be the reason for the continued low rate of growth seen in cultures supplied with heated (100°C) iron stocks.

While no single technique provided unequivocal evidence of a change in the thermodynamic stability of the colloidal iron, the combined evidence suggests that the stability increased during autoclaving. Thus while the changes which made the colloidal iron non-available could not be precisly documented, it is evident that the decrease in availability occurred following only minor changes in the thermodynamic stability.

CONCLUSIONS

Colloidal material was found associated with diatom frustules in three of the field localities sampled: the bottom waters of Indian Arm (Ind 2.0), the Fraser River plume (Fra 1.5) and within the Fraser River salt wedge. In many cases the associated colloidal matter was iron-rich. It does not appear that this association was an artifact of the sampling process. Factors that could have contributed to its absence in the other areas sampled are the large number of cells found relative to the small amount of colloidal material and the limited resolution of the scanning electron microscope. Results from the glass plate experiments suggest that the association of colloidal material with diatoms may be due to the surface properties of the siliceous frustule and need not be biologically controlled by the diatom itself.

The bioassay results showed that while freshly precipitated ferric hydroxide was readily available, autoclaving, heating, or ageing of that same stock reduced the availability of the iron. The reduction in iron availability was shown to be a gradual process occurring over time that was greatly accelerated at higher temperatures. Although the rate of growth was reduced in cultures supplied with heated colloidal iron stocks, cell numbers in these cultures continued increasing for at least 21 days after growth had ceased in the control. This indicates that iron was being made available but that the rate of supply was very low.

Although addition of EDTA to the cultures supplied with autoclaved and aged colloidal iron stocks did not increase iron availability, growth was not reduced when EDTA was added to the iron stock before these treatments. Since the presence of EDTA would prevent the aggregation of iron in such stocks (by chelation of the ferric ions), these results indicate that the formation of colloidal ferric hydroxide is a necessary precursor to the process reducing the availability of the iron stock. This in turn suggests that the process is related to changes in colloidal iron.

Changes that occur in ferric salt solutions, such as those in iron stocks used in these bioassays, are well docummented. Initially, polynuclear colloids form by aggregation of the hydrated ferric ions and over time these colloids become more stable through internal rearrangement and condensation reactions. Higher temperatures accelerate this ordering process. Such ordering changes must, moreover, result in an increase in the thermodynamic stability of the colloidal iron. To test whether the stability affected the supply of iron to the organism, goethite and hematite were tested as sources of iron. These well-crystallized minerals are probably the most stable ferric oxyhydroxide and oxide under earth surface conditions. Both were found to be poor sources of iron to T. psuedonana even though association of the colloids with living diatoms was observed. These results support the hypothesis that organism growth decreases as the thermodynamic stability of the colloidal iron increases.

To determine whether reduced iron availablity in the treated colloidal iron stocks can be related to measurable changes in their chemical and physical properties, the autoclaved and fresh iron stocks were compared using techniques that would provide information with respect to their degree of polymerization, hydration and crystallinity. These techniques were X-ray diffraction, Mossbauer spectroscopy, gel filtration and thermal gravimetric analysis.

No evidence for a regular crystal structure was detected in the autoclaved and fresh colloidal stocks by either X-ray diffraction or Mossbauer spectroscopy. A longer period of autoclaving (24 hrs), however, produced detectable crystallinity, indicating that early stages of ordering could be present in the colloidal stocks autoclaved for 15 minutes. Thermal gravimetric analysis demonstrated that the overall degree of hydration was lower in the autoclaved stock. Further, the rapid loss of water between 450°C and 500°C indicates that some form of structure was present in the autoclaved colloidal iron. These results suggest that the thermodynamic stability of the colloids increased during the process of autoclaving. Although colloidal size was found to increase with autoclaving, this was most likely due to colloidal coagulation which in itself would not alter appreciably the thermodynamic stability of the colloids. The results of the analytical comparison were unable fully to characterize the non-available form of colloidal iron.

The change in iron availability of the colloidal stocks appears to parallel increases in thermodynamic stability and could be due to either reduced dissolution rates or reduced mononuclear iron species concentrations. Dissolution rates of the autoclaved and fresh colloidal stocks were compared and the rate in the autoclaved stock was found to be substantially lower. Reduced dissolved iron concentrations could affect organism growth if conversion of the iron form released by colloid dissolution is necessary before uptake can occur. However, since addition of EDTA to iron stocks that were aged or autoclaved did not enhance growth, it appears that colloid dissolution rates may be the controlling factor determining the iron supply to the organism. This is a very important consideration when examining the effect of natural chelating agents (e.g. siderophores and humic materials) on organism growth in estuarine and oceanic environments.

If changes in the thermodynamic stability of the iron is related to the reduced growth seen in the experiments, the shift between available and non-available iron must be viewed as a continuing process since the ordering of ferric (oxy)hydroxides continues over time. The point at which the iron within colloids becomes 'non-available' will depend on the degree and form of ordering and the iron demand of the population. As the experiments have shown, the true biological availability of a colloidal metal cannot be determined with short term bioassays. Even in the case of colloidal goethite and hematite, an equilibrium exists between the colloidal and mononuclear species of iron such that replacement will occur as a mononuclear species is removed biologically. The iron within the colloids is then actually 'available' although the rate of supply is very slow. Thus, when I use the term 'non-available', it is with respect to the iron demand that allows optimal growth of the population.

The results of this investigation suggest that the method of iron stock preparation and the treatment of that iron stock before addition to cultures directly controls the biological availability of the iron. Attempts to fully characterize the change in the colloidal stocks that had reduced the iron availability were unsuccessful. The change, however, appears to be related to increases in the degree of polymerization and decreases in the degree of hydration that necessarily increases the thermodynamic stability of the colloidal hydrous oxide. While techniques such as scanning electron microscopy and energy dispersive X-ray analysis show the association of colloidal metals to organisms both in the laboratory and in the natural environment, the availability of this metal is probably determined by the thermodynamic stability of the colloid rather than the proximity of the colloid to the cell.

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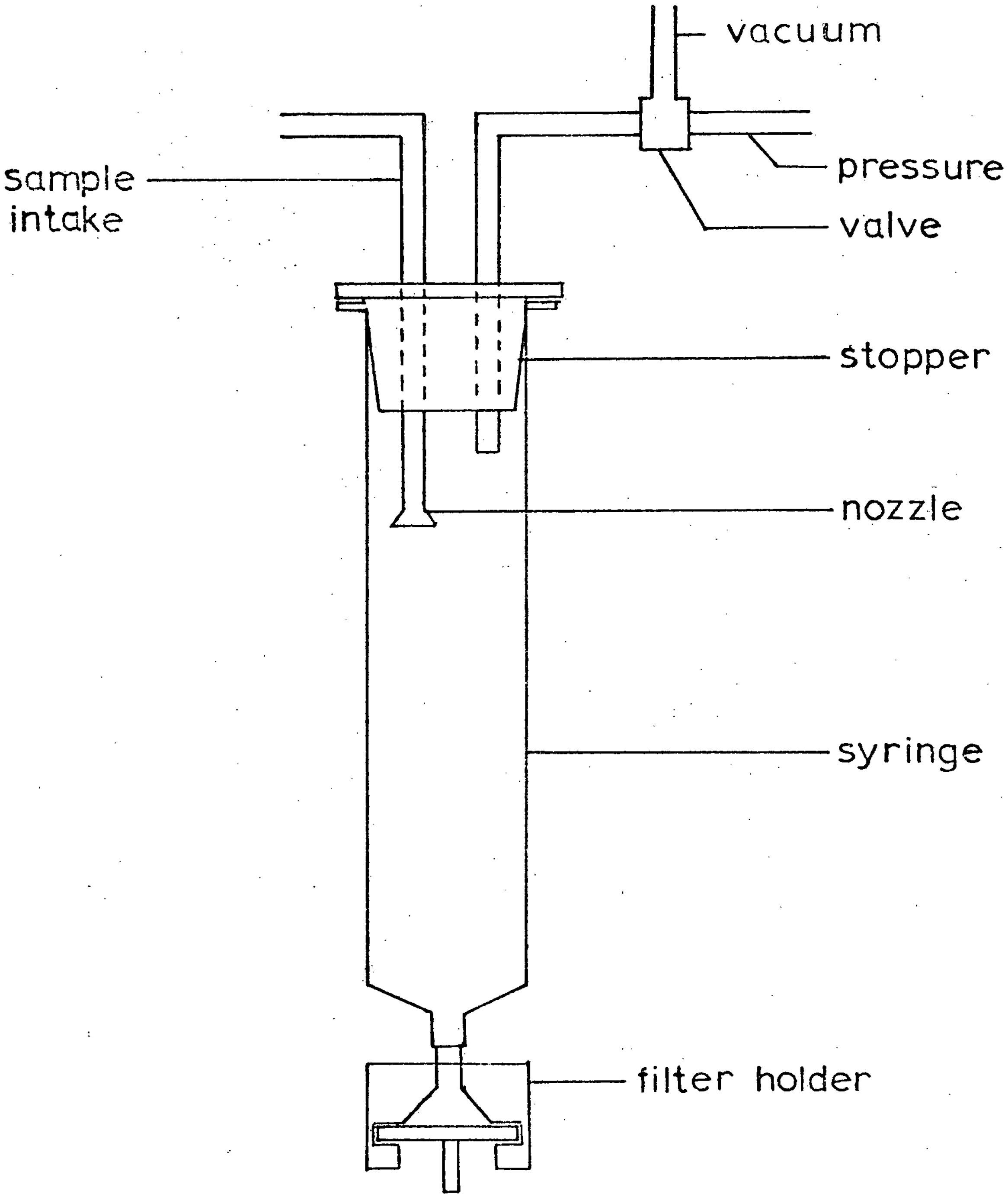
APPENDIX 1

The filtration apparatus used for the collection of the field samples was designed to reduce sample contamination (Fig. 27). Polypropylene syringes were capped with silicone rubber stoppers that were fitted with two polypropylene nozzles. One nozzle was connected, through a two way valve, to either vacumm or nitrogen pressure while the other nozzle was connected to the sample intake tube. All tubing was constructed of polyvinyl chloride and the syringe stand of methyl methacrylate.

Before sample filtration, 1.0 N HCl was drawn through the sample intake tube into the syringe (by vacumm) to clean the apparatus. The acid was then expelled from the syringe by nitrogen pressure with adjustment of the two way valve. The syringe and intake tube were then rinsed three times with distilled water by the same method. The syringe was fitted with a nuclepore 'pop-top' 13 mm diameter filter holder (containing a 0.1 μm polycarbonate filter) and the sample was drawn into the syringe. Nitrogen pressure was applied and the desired volume of sample filtered. The pressure was then released, the filter holder removed and the remaining sample discarded. The filter holder was reattached to the syringe and 5.0 milliters of distilled water was drawn into the syringe. Pressure was applied until all of the rinse water had passed through the filter.

Figure 27. Filtration sampling apparatus .


vacuum
sample intake
pressure
valve
stopper
nozzle
syringe
filter holder

APPENDIX 2

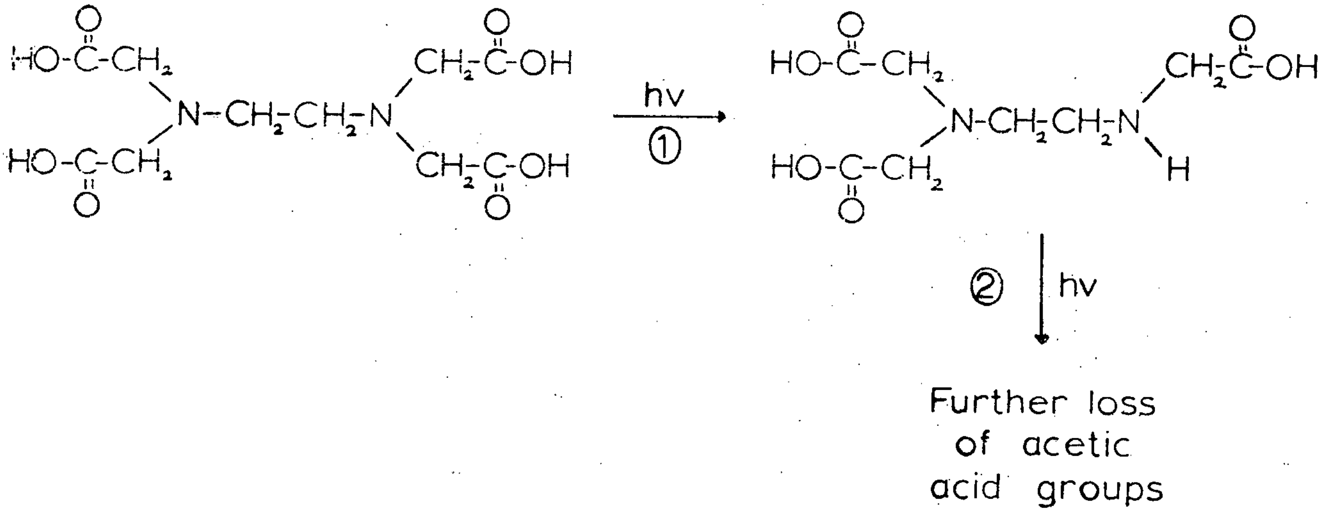
For the bioassay study, standard ocean water (SOW) was prepared in 20 liter quantities by the method of Morel _et_ _al_ (1979). The following salts were added to 18 liters of distilled water in the indicated quantities:

NaCl	490.6 g
$NaSO_4$	81.9 g
$CaCl_2$ $2H_2O$	30.8 g
KCl	14.0 g
$NaHCO_3$	4.0 g
KBr	2.0 g
H_3BO_3	0.6 g
$SrCl_2$ $6H_2O$	0.34 g
NaF	0.06 g

The salt solution was then bubbled with acid cleaned (6N H_2SO_4), filtered (0.4 μm) air overnight to bring the pH into equilibrium. $MgCl_3$ $6H_2O$ was dried (at 70°C) and 222.0 g was added to the equilibrated salt solution. The volume was made up to 20 liters and the solution bubbled (as above) for 6 hours for re-equilibration. The SOW was then passed through a ion exchange resin (Chelex, Na form, 100 mesh) to remove transistion metal contamination and stored in acid-cleaned 10 L glass flasks until use.

APPENDIX 3

Because of a recent report suggesting that photodegradation of EDTA occurs under high light intensities (Lockhart and Blakely, 1975), the effect of iron stock preparation techniques and coldroom bioassay conditions on EDTA was investigated. Iron, EDTA, and iron-EDTA solutions were placed under both light (95 μEin M^{-1} S^{-1}) and dark conditions in the coldroom (15°C) for the period of one week. Lockhart and Blakely (1975) suggested that under high light intensities the following reaction occurred



and suggested that the rate of '1' was much more rapid than '2'. Because increased temperatures may also cause this degradation, the effects of extended autoclaving on these solution was also tested. If these conditions did cause EDTA breakdown, then CO_2 production would be expected to occur. The CO_2 produced by the treatments of one week of coldroom light and dark conditions and

4 hours of autoclaving was measured (Fig. 28). The results show that the Fe-EDTA solution under coldroom light and autoclaved conditions produced more CO_2 than both the Fe and EDTA solutions combined. Similar results were seen when a Cu-EDTA solution was autoclaved. Increasing salinity was also found to increase the production of CO_2 in the experiments (Fig. 29). The results demonstrate that both high light intensities and heating catalyze the breakdown of EDTA and that the rate of breakdown increases when the EDTA is in the chelated form.

It was then necessary to determine if this EDTA breakdown was important with respect to loss of the chelation ability of the solution. If EDTA breakdown occurs as Lockhart and Blakely suggested, then the loss of one acetic acid group would greatly reduce the stability of the chelate-metal complex. Assumming that only reaction '1' occurred during the treatments, the upper limit of the percent EDTA 'lost' can be estimated. In reaction '1', each EDTA molecule would supply 2 carbon atoms or:

$$2 \times 12.01 \text{ g mole}^{-1} = 24.02 \text{ gC mole}^{-1}$$

Since the EDTA concentration was 5.0×10^{-2} M, then:

$$24.02 \text{ gC mole}^{-1} \times 5.0 \times 10^{-2} \text{ moles l}^{-1} = 1200 \text{ mgC l}^{-1}$$

The highest CO_2 level measured in the tests was the autoclaved (4 hrs) iron-EDTA solution at 41.12 mgC l^{-1}. The upper limit of EDTA 'lost' with this treatment can be calculated as:

Figure 28. The CO_2 produced from Fe, EDTA, and Fe-EDTA stocks with autoclaving (4 hrs) and under coldroom light or dark conditions (for 7 days) was measured. The results are shown as the peak area of the CO_2 curve (in intergrater units). The Fe-EDTA stock, with autoclaving and under coldroom light conditions, had significantly greater CO_2 production then either the Fe or EDTA stocks.

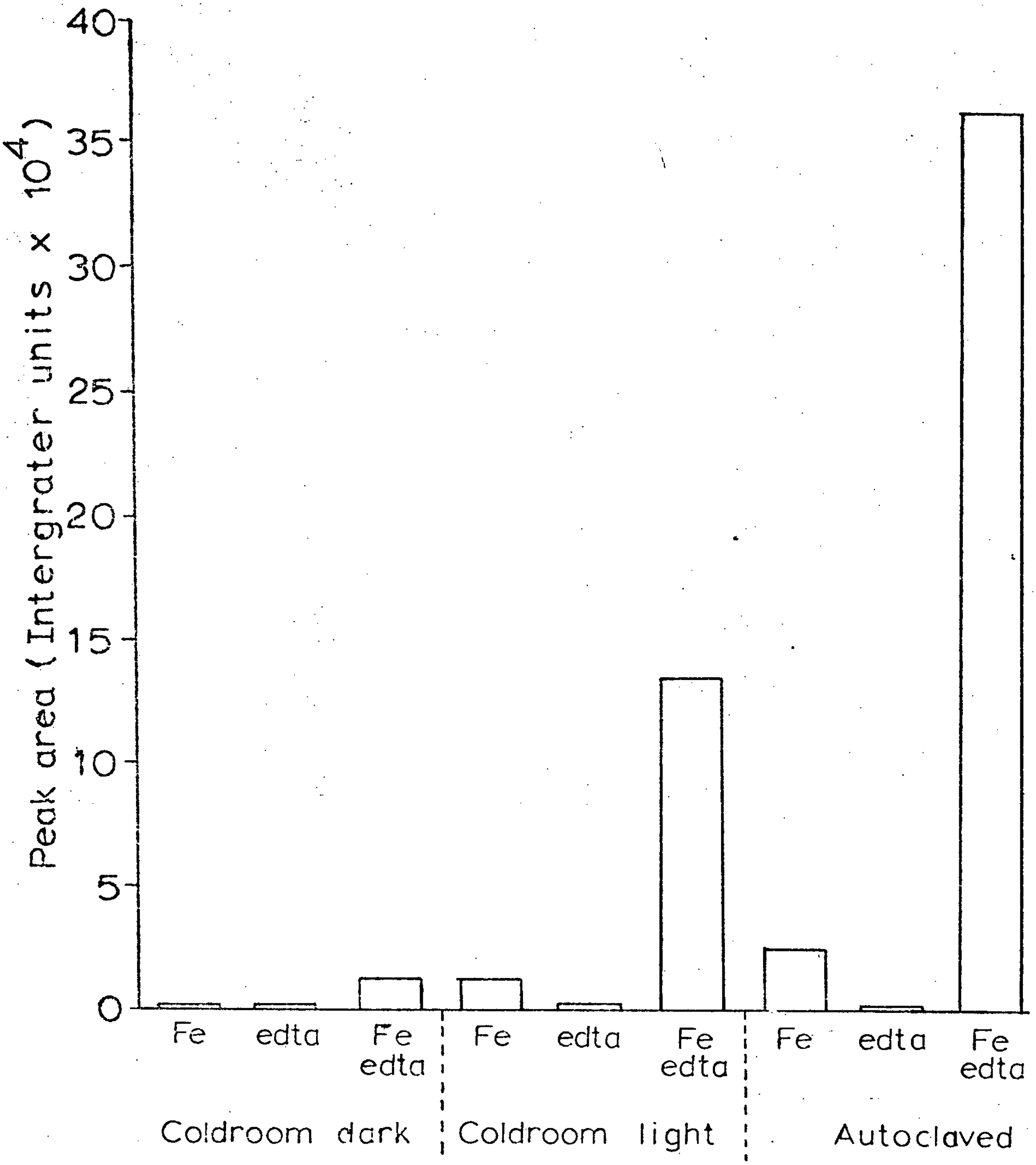
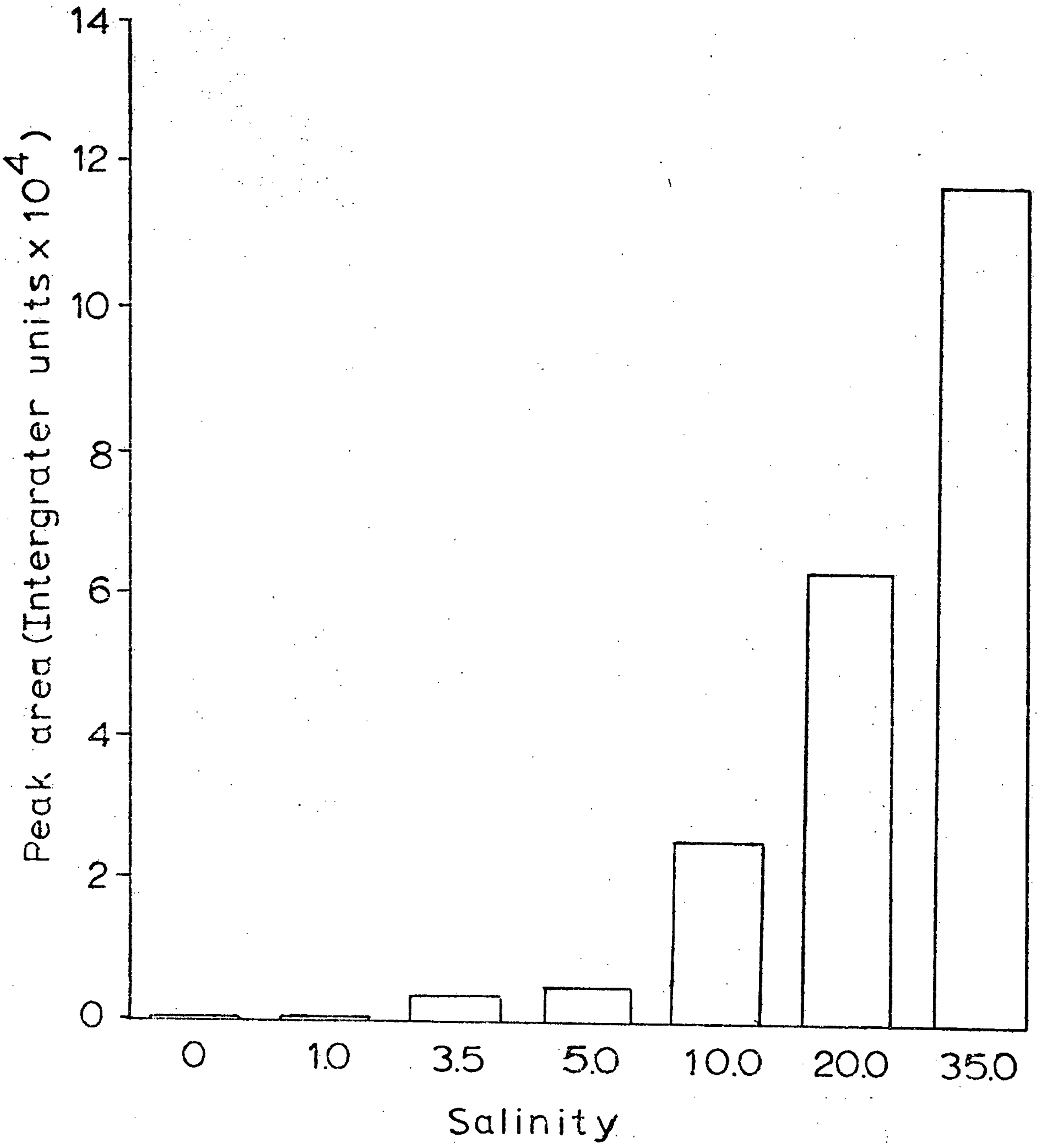

Peak area (Intergrater units x 10^4)
0
5
10
15
20
25
30
35
40
Fe
edta
Fe edta
Fe
edta
Fe edta
Fe
edta
Fe edta
Coldroom dark
Coldroom light
Autoclaved

Figure 29. An increase in salinity was found to increase the production of CO_2 in the Fe-EDTA stock. (see figure 28 for label details.)


Peak area (Intergrater units x 10^4)
14
12
10
8
6
4
2
0
0
1.0
3.5
5.0
10.0
20.0
35.0
Salinity

$$\frac{41.12 \text{ mgC } l^{-1}}{1200 \text{ mgC } l^{-1}} = 3.4\%$$

By this method, the upper estimate of the percent EDTA lost under coldroom light conditions is 1.3%. If reaction '1' and '2' occurred during EDTA breakdown these estimates would decrease. Because only 5.9 % of the total iron is complexed by EDTA in Aquil (from the speciation program 'Minequil'), these estimates suggest that the EDTA loss in the treatments used in this study was not sufficient to affect the bioassays.

APPENDIX 4

Mossbauer spectroscopy is the measurement of resonnant gamma-rays transmitted through a sample as a function of their Doppler velocity with respect to the source. The Doppler velocity is directly correlated with the energy of the gamma-ray so the spectrum is actually a record of transmission (or absorbance) as a function of energy of the incident radiation. The major difference from other forms of transmission spectroscopy is that long periods of counting (hours rather than minutes) are required because of the very small energy band scanned and the low photon flux densities necessary. The underlying principle of the Mossbauer effect is the absorbance of a unique energy (i.e., Doppler velocity) gamma-ray by the electron field of an atom resulting in its transformation into the excited state. Atoms in the excited state may then release the absorbed energy (emission). The key to the Mossbauer effect is the absorbance and emmision of the low energy gamma-rays without energy loss by either recoil or thermal broadening. (Thus the absorbance and emission energies are identical.) The recoilless absorbance and emission is optimized at low temperatures. Because of this the autoclaved and non-autoclaved colloidal samples were analysed at liquid nitrogen temperatures (78°K).

To interperet Mossbauer spectra an understanding of what affects the absorbance of gamma-rays is required. There are three principal hyperfine interactions to consider; chemical isomer shift, magnetic dipoles, and electric quadrupoles. The chemical isomer shift is a change in the electric monopole or Coulombic interaction between the electric and nuclear charge caused by a difference in the size of the nucleus. It is seen as a shift of the absorbance line with respect to that of the emission energy of the source without changing the shape of the spectra. Both the magnetic dipole and quadrupole interactions, on the other hand, generate multiple line spectra and can consequently give a great deal of information. Magnetic hyperfine interaction will occur if the atom is subjected to a magnetic field. The magnetic field will split the energy level of the nucleus into non-degenerate equal-spaced sublevels. This may be illustrated in the spectrum as a splitting of the peak. (For a more detailed explanation of this phenomenon see Gibb (1976).) Since iron is highly ferromagnetic, this hyperfine interaction is expected to affect the spectra in some way. The electric quadrupole interaction is directly related to the electron spin of the nucleus. Any nucleus with sufficiently high spin quantum number (I = 0.5) has a non-spherical charge distribution. The quadrupole splitting is sensitive to the symmetry of the environment of the atom. The absence of quadrupole splitting is indicative of cubic or near-cubic site symmetry. In contrast, the presence of quadrupole splitting indicates significant distortion.

While the chemical isomer shift merely causes a uniform shift of the absorbance lines (without altering their separation), both the magnetic and quadrupole interactions are directional dependant effects. As a result, when both are present the general interpretation of the spectrum can be quite complex. The Mossbauer analysis in this study, however, was used only to compare spectra of the autoclaved and non-autoclaved colloidal stocks and to interpret possible differences.