Fall 1996

Ecophysiology of metal-reducing Aeromonas and Shewanella strains from Great Bay Estuary

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ECOPHYSIOLOGY OF METAL-REDUCING *AEROMONAS* AND *SHEWANELLA* STRAINS FROM GREAT BAY ESTUARY.

BY

VICTORIA KNIGHT

B.S., University of Rhode Island, 1992

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy

in

Microbiology

September, 1996

This dissertation has been examined and approved.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Blakemore for taking a chance and giving me the opportunity to work in his laboratory. During the past few years he has taught me a great deal, however, the greatest lesson he taught me was the ability to think like a scientist. I would like to thank the members of my dissertation committee Dr. Jones, Dr. Lesser, Dr. Margolin, and Dr. Tisa for their continued guidance and support. I thank Dr. N. Kinner for the use of the ion chromatograph for Se (VI) measurements. I thank Robert Mooney for expert technical assistance, encouragement, and friendship. I thank Nancy Blakemore for her continued help especially, with the preparation of each of my posters. I thank the graduate school for a Graduate Research Enhancement Award, and a Teaching Assistant Summer Fellowship. I would like to thank the center for marine biology for a CMB fellowship which allowed financial support for Se (VI) measurements.

Throughout my dissertation work several undergraduates have worked on various aspects of this research. I would like to thank Steve Wudyka, Hannah Vostrofsky, Katie Stepeneck, Felica Vinci, Christine Matson, and Christopher Rovaldi their effort and input greatly facilitated completion of this work.

My friends, especially Dan Strong, Kelly Pendergast, Mary Smith and Vasu Maganti have been a great influence upon me: their encouragement through the bad times; their support through the good times; and their ability to make me realize that there is more to life than finishing one more experiment! I would like to thank my parents for their continued support and encouragement. This dissertation is dedicated to them as a symbol of my appreciation.

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ABSTRACT:

ECOPHYSIOLOGY OF METAL-REDUCING *AEROMONAS* AND *SHEWANELLA* STRAINS FROM GREAT BAY ESTUARY.

By

Victoria Knight

University of New Hampshire, September, 1996,

The seasonal abundance and distribution of mesophilic aeromonads at seven sites in Great Bay Estuary, NH were monitored over a 24 month period. Total heterotrophic bacteria, total and fecal coliforms, salinity, temperature, pH, total suspended solids, and chlorophyll were also measured in the samples. Aeromonads were isolated at each site throughout the year with the highest incidence and population density in the late summer to early fall. Aeromonad abundance correlated positively with the incidence of coliforms, temperature, and salinity.

Aeromonas hydrophila ATCC 7966 grew anaerobically by using NO_3^-, fumarate, Fe (III), Co (III), or Se (VI) as terminal electron acceptors. Of thirty isolates from Great Bay, 100% were capable of reducing NO_3^-, Fe (III), and Co (III), and 43% carried out dissimilatory Se (VI) reduction. Final cell yields of *A. hydrophila* ATCC 7966 increased in direct proportion to the amount of oxidant provided (over the range 1.25-10 mM). Cells of *A. hydrophila* ATCC 7966 contained *c* -type cytochromes (420, 522, 553 nm). Hydrogen-reduced cytochromes were oxidized by Fe (III) or NO_3^-. Extracts of cells

grown anaerobically with Fe (III), reduced it at a rate of 116 nmol·min^{-1}·mg protein^{-1}. Electron transport was inhibited by μm concentrations of : HOQNO (76 %), quinacrine (88 %), dicumarol (58 %) oxygen (96%), CCCP (83 %) and sodium azide (36%) and to a lesser extent by rotenone (18%). The results were consistent with the involvement of FAD dehydrogenase, quinones, cytochromes and an iron reductase in the respiratory chain.

Co-culturing *Aeromonas veronii* and a dissimilatory iron-reducer *Shewanella alga* resulted in enhanced growth, citrate degradation and iron reduction as compared to the extent observed with either strain grown axenically. By 48 hours, the co-culture consumed twice as much citrate and produced half as much formate and twice as much acetate. The synergistic link between these two organisms was apparently formate: produced by *A. veronii,* it was, in turn, used as a substrate for iron reduction by *S. alga.*

Ecophysiology of Metal-Reducing *Aeromonas* and *Shewanella* Strains from Great Bay Estuary.

INTRODUCTION

Members of the genus *Aeromonas* are gram negative, oxidase positive, facultatively-anaerobic rods belonging to the *Vibrionaceace* family. The role of *Aeromonas* as opportunistic pathogens of both animals and humans has been well documented but, little is known of their ecology and physiology. Prior to this study aeromonads had only been reported in New Hampshire waters on one previous occasion (as part of a nationwide study). This study was initiated to develop an understanding of the incidence, and seasonal distribution of mesophilic aeromonads in Great Bay Estuary, and the factors that regulate their survival.

CHAPTER ONE

SEASONAL DISTRIBUTION OF MESOPHILIC AEROMONADS IN GREAT BAY ESTUARY NEW HAMPSHIRE.

ABSTRACT:

The seasonal abundance and distribution of mesophilic aeromonads was determined for a seven site transect from freshwater seaward through Great Bay Estuary, New Hampshire and in its tributaries. Samples were collected monthly for two years for several biotic (mesophilic aeromonads, total heterotrophic bacteria, total coliforms, and fecal coliforms) and physicochemical parameters (pH, temperature, salinity, total suspended solids, and chlorophyll) were measured monthly over a two year interval. Mesophilic aeromonads were isolated from each site throughout the year, with an increase in abundance from 0 to 60 $CFU \cdot ml^{-1}$ with the highest density during the summer and early fall. Heterotrophic bacteria ranged from 50 to $5 \cdot 10^3$ $CFU \cdot ml^{-1}$ with the highest density during the early fall. Total and fecal coliforms were isolated throughout the year at all sites with the incidence ranging from 0 to and 63 $CFU \cdot ml^{-1}$, respectively. The salinity ranged from 0 % at the freshwater site to approximately 3 % at Hilton Park and Adam's Point. The remaining sites were intermediate to these values and fluctuated between 0.1 and 1.5 % depending upon seasonal effects due to changes in precipitation and run off. The temperature ranged from 0-22 °C. Total suspended solids varied from approximately 5 x

10^{-3} to 3 x 10^{-1} ppm and chlorophyll a content ranged between 0.1 to 11 mg^{1}. Multiple regression analysis revealed significant correlation between the density of aeromonads an both total and fecal coliforms ($p = 0.0002$, and $p = 0.0075$, respectively). Moreover, populations of all these groups of heterotrophic organisms varied predictability with temperature. Prior to this study, the incidence and seasonal distribution of aeromonads had not been reported in New Hampshire waters.

INTRODUCTION:

Klyver and Van Niel (1936) first proposed the genus *Aeromonas* to accommodate gram-negative enteric-like polarly flagellated bacteria that are autochthonous to aquatic habitats (13). Unlike enteric bacteria, however, these organisms are oxidase positive. Aeromonads currently reside within the *Vibrionaceae* family. On the basis of DNA-DNA hybridization studies, a new family, the *Aeromonadaceae* (15), has been proposed. The genus contains 8 phenospecies included within two major groups: the mesophilic (and motile) aeromonads; such as A. *hydrophila*, *A. caviae*, and *A. sobria*, and the psychrophilic, non-motile fish pathogens grouped as *A. salmonicida* (13). Results of conventional biochemical tests are consistent with grouping these bacteria into 8 phenospecies (13). However, DNA-DNA hybridization revealed the presence of 14 hybridization groups (12, 49). Therefore, identification beyond the phenospecies level requires the use of molecular tools. Characterization methods have included Restriction Fragment Length Polymorphism analysis (RFLP, 45), Randomly Amplified DNA polymorphism (RAPD, 58), comparison of PCR-amplified 16S rDNA sequences (16) and DNA-DNA hybridization (49). A rapid multiwell DNA hybridization plate for differentiation of aeromonads to the species level has been developed (58). Huys (1996) reported the use of Amplification Length Fragment Polymorphism analysis (AFLP) which incorporates the use of a preliminary restriction digest of genomic DNA followed by a highly stringent DNA amplification of the generated fragments using primers specific for the restriction site (25). This method has allowed discrimination among members of each of the 14 DNA hybridization groups (25).

Aeromonads have long been recognized as opportunistic pathogens of a wide range

of poikilothermic and homeothermic animals including humans (1, 13, 34, 47, 59). Recent local extinctions of amphibians have brought *A. hydrophila* into the spotlight as the causative agent of "red leg" in frogs: a septicemic infection which causes hemorrhagic ulcerations and ultimately death (11). Between 1974-1982, 11 populations of Boreal toads (*Bufo boreas boreas*) in the West Elk Mountains of Colorado disappeared with *A. hydrophila* implicated as the causative agent (11). Although *Aeromonas* is part of the indigenous microbiota of amphibians, environmental stress potentiates disease by this opportunistic pathogen (11). *A. hydrophila* (*Bacillus hydrophilus*)was first isolated from frogs in 1891 and upon reinoculation produced septicemia in both warm- and cold-blooded animals (26). Aeromonads have also caused extensive losses in the fishing industry as the etiological agent of "red sore disease". In one documented case, 37, 000 fish were killed in a 13 day period in one North Carolina lake (39). In addition, during the Fall of 1976, 95% of the white perch (*Roccus americanus*) were killed and 50% of the total catch was discarded due to unsightly red sores (22). The incidence of fish infected with red sores is directly related to the density of aeromonads in the water supply (21, 22, 24). Aeromonads are also responsible for three types of infections in snakes and amphibians: an acute septicemia characterized by lethargy, weakness and convulsions; pneumonia spread by the snake mite (*Optionysus natricis*); and ulcerative stomatis or "mouth rot" in snakes which is characterized by frothy exudate around the mouth and their inability to eat (31). Additionally, aeromonads have also produced sepsis in dogs, pneumonia in dolphins, abortion in cattle, blackrot in hen eggs, and diarrhea in piglets (31).

Aeromonads were originally thought to be pathogenic only for immunocompromised humans (1, 31). The role of aeromonads as pathogens of imunocomptent humans however, emerged in 1968 when Von Graeventiz and Menson published a report of 27 *Aeromonas*-associated opportunistic infections (64). Aeromonads produce a wide range of localized and systemic infections (1, 31) with 50-60 % presenting

as an acute and self-limiting gastroenteritis (1). Infection usually follows ingestion of contaminated water (8, 18, 28). There is a marked peak of *Aeromonas*-associated gastroenteritis throughout the summer months (32). The second most prevalent form of disease occurs as wound infections ranging from a mild cellulitis to fulminant myonecrosis, normally occurring following a traumatic incident in contaminated water (29). A microbiological study resulted when a diver wounded in the Anacostia River, VA became infected (56). The concentrations of total bacteria and aeromonads on the skin of divers were found to increase to be comparable to concentrations in the water after divers had been swimming for 30 minutes in the Anacostia River (14). Wounds caused by fin cuts, or puncture wounds due to fish bones or fish hooks commonly lead to *Aeromonas* infections, particually in immunocompromised anglers (29, 60). The incidence of infections increases with abundance of aeromonads in the water (14, 29). Post-operative infections have also been reported and their incidence can be correlated with the use of medicinal leeches which harbor aeromonads within their intestines (1).

Aeromonads produce several known virulence factors: an extracellular enterotoxin, a cytotoxic endotoxin and a beta hemolytic hemolysin, often referred to as aerolysin (1, 31). Aeromonads also produce an alpha hemolytic hemolysin which is thought to play a lesser role in pathogenicity. Aeromonads produce two siderophores (amonobactin and an enterobactin-like molecule). These are used *in vivo* to sequester ferric iron (38, 64). Growth of aeromonads in heat-inactivated serum requires the presence of amonobactin (27). Siderophore production (and ability to sequester iron) is directly related to virulence; perhaps due to an increased ability to resist the lytic activity host complement (38, 64). Pathogenicity by this opportunist is caused by the extracellular enterotoxin. Its production can be correlated with aerolysin production and with a positive Voges-Proskauer test (1, 12, 31). *Aeromonas sobria* and *A. hydrophila* account for the majority of human infections and both actively produce aerolysin (7, 30, 32). It is interesting to note that *A. caviae*

accounts for very few known *Aeromonas*-associated human infections and 95 % of *A. caviae* isolates screened also do not produce aerolysin (42, 44). Environmental isolates screened for hemolysin production and pathogenic potential exhibit patterns similar to those of clinical isolates (7, 10, 30, 33). Therefore, environmental isolates do present a public health concern for humans (7, 30, 33) in the context of recreation, in aquaculture, or as contaminants of drinking water.

Aeromonads are ubiquitous in water and have been isolated from all but the most extreme environments ranging from bottled drinking water (62), pristine alpine lakes (Grand Tetons, WY; 24) Louisiana bayous (24), the aphotic zone of the Atlantic ocean (24) and sewage sludge (48) . In a study encompassing the entire United States and part of Puerto Rico, aeromonads were isolated from 92 % of 147 water sources sampled (24). Aeromonads are ubiquitous in freshwater and can also be isolated from brackish and marine environments (24). Unlike one species of the closely related genus, *Vibrio* they do not have a sodium requirement and grow equally well in medium with, and without the addition of seawater. In fact, aeromonads can become the most prevalent organism in marine, or estuarine habitats having high concentration of organics due to input of anthropogenic waste (7, 22). Use of genetically marked *A. hydrophila* in microcosms revealed that this organism survived best in fresh water but also in salt water, after an initial die off during the first nine days. The *Aeromonas* population in salt water recovered by day twelve and slowly dropped off again by day 28 (35). Aeromonads can be isolated throughout the year with a sharp increase in incidence during the summer to early fall (17, 20, 23, 24). *Aeromonas* incidence can be positively correlated to temperature (17, 23). Aeromonads are sensitive to low temperatures, in fact at 4 °C they do not conserve enough energy to divide and it is thought that they enter a viable but non culturable state (53). The relationship between *Aeromonas* and several physical and chemical water quality parameters has varied over a broad range of environmental locations examined (51). The

incidence of aeromonads has been correlated to temperature (17, 21, 23), chlorophyll a (20, 52, 54), total nitrogen (20, 52, 54), total phosphorous (52, 54), fecal coliforms (4, 10, 20), or heterotrophs (21). Rippey and Cabelli proposed the use of aeromonads as indicators of water trophic status (52, 54). Their incidence has been correlated with indicators of productivity: chlorophyll a; phosphorous; and Secchi depth (48, 52, 54). They are also sensitive to subtle changes in water quality (53). The doubling time of aeromonads decreased by 4 hours in oligotrophic waters amended with enough eutrophic water to increase the phosphorous concentration by as little as 2 $\mu g \cdot l^{-1}$ (53). Addition of glucose (5 mg $C \cdot l^{-1}$) and phosphorous (10 $\mu g \cdot l^{-1}$) increased their growth rate in oligotrophic waters by 70 % (55). Aeromonads also respond to the input of anthropogenic (specifically sewage) waste and can rapidly become the predominant organisms (7, 22). Growth of indigenous aeromonads increased at two sites of industrial waste (pulp mill and rum refinery) due to the discharge of a nutrients which increased the concentrations of nitrogen, phosphorous and total organic carbon (7, 22). The effluents from both sites rapidly became anoxic due to high productivity which was lethal for the indigenous aquaculture species (7, 22). Although aeromonads are indigenous to aquatic habitats, at polluted sites they can rapidly become the dominant species (7).

Aeromonads often occur as false positives in the currently employed rapid identification systems for coliforms such as Colilert™ and Colisure™. In fact, the closing of 12 New Hampshire beaches during the summer of 1995 could have resulted from populations of indigenous aeromonads which may have contributed to false positive coliform tests based upon the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide which like *E. coli* aeromonads can cleave (41). Recent research suggests that a selective medium incorporating the antimicrobial cefsulodin (2) might provide better discrimination between coliforms and aeromonads. *Aeromonas* and *Flavobacterium* were both inhibited by this antibiotic (2). Aeromonads as false positives in coliform identification analysis are

problematic. Although the incidence of *Aeromonas* increases with higher levels of pollution, they are normally not of human fecal origin but are indigenous to most waters (6). Their relative abundance is higher with an increase in the concentration of total organic matter (6). In eutrophic waters coliform incidence correlates directly with numbers of aeromonads (4, 51) whereas in hypereutrophic or oligotrophic waters *Aeromonas* persists longer than do coliforms (4, 5, 9, 62). In addition, the two most common pathogenic *Aeromonas* species, *A. hydrophila*, and *A. sobria*, persist longer in oligotrophic waters (5). Thus, coliforms are not consistently useful indicators of the incidence of this opportunistic pathogen: this underscores the point made by others, that coliforms alone may not be a useful predictor of the true microbiological quality of the water (5, 51, 62).

Great Bay Estuary is located on the border of New Hampshire and Maine. Great Bay Estuary is one of the largest estuaries on the eastern coast of the United States and is comprised of the Piscataqua River, Great Bay and Little Bay (28). The Estuary is a tidally dominated system and is the confluence of seven major rivers (28). While the Estuary is not normally heavily polluted (although, ironically, at the time of this writing a major oil spill of > 1000 gallons from a tanker adrift in the Piscataqua River has seriously polluted the sediments of the lower river as far upstream as Adam's Point), shellfish closures, eelgrass die off, and increasing development all suggest that this ecosystem is showing signs of stress. The incidence and seasonal distribution of this opportunistic pathogen have not been previously studied. A study was undertaken to determine the incidence and seasonal distribution of aeromonads in Great Bay Estuary and its tributaries. In an effort to understand factors controlling *Aeromonas* populations, water quality parameters were also measured in an effort to determine any possible correlations between the incidence of aeromonads and several biotic and physical water quality characteristics.

MATERIALS AND METHODS:

Sample collection: Water samples were collected monthly from August 1994 - June 1996 from seven sites in Great Bay Estuary and its tributaries (Fig. 1). Samples were collected at 0.5 to 1 m at low tide in sterile sample bottles and transported back to lab on ice, where they were processed within 2 hours.

Medium and growth: Mesophilic aeromonads, total heterotrophic bacteria, and both total and fecal coliforms were enumerated by using membrane filtration (3). Water samples were filtered through 0.45 μm grided GN-6 filters (Gelman, AnnArbor MI), and placed on appropriate culture media. Mesophilic aeromonads were enumerated on Rimler-Shotts *Aeromonas* medium (57) which consisted of the following ($g \cdot l^{-1}$) constituents: Sodium thiosulfate, 6.8; sodium deoxycholate, 1.0; ferric ammonium citrate, 0.8; yeast extract (Difco, Detroit, MI), 3.0; NaCl, 5.0; L-lysine-HCl, 5.0; L-ornithine-HCl, 6.5; maltose, 3.5; cysteine, 0.3; thymol blue (0.4%), 3 ml. The medium was adjusted to pH 7 with NaOH. After autoclaving, novobiocin (50 mg)was added from sterile stocks. Total heterotrophic bacteria were enumerated on salt water complete (SWC) medium (19) which consisted of the following ($g \cdot l^{-1}$) constituents:peptone, 5.0; yeast extract, 1.0; glycerol, 3.0 ml; 75% modified artificial sea water, 1000 ml. Artificial sea water (37) was modified to contain the following ($g \cdot l^{-1}$) constituents: NaCl, 23.48; $MgCl_2 \cdot 6H_2O$, 10.65; Na_2SO_4, 3.92; $CaCl_2 \cdot 2H_2O$, 1.46; KCl , 0.66; $NaHCO_3$, 0.19 without pH adjustment. Samples collected from freshwater sites were placed on SWC lacking artificial seawater. Total and fecal coliforms were enumerated on m-ENDO agar (Difco, Detroit MI) at 37 °C and 44.5

°C, respectively. All incubations were at 37 °C for 24 hours unless otherwise noted. Incubation at 37 °C allowed further selection for aeromonads by decreasing background of hetertrophs. Incidence of aeromonads were unaffected.

Physical parameters: At each site, the following physical parameters were measured: temperature, salinity, pH, total suspended solids (TSS), and chlorophyll a. concentration. Temperature was measured by using a mercury thermometer (range - 35-50 °C). Salinity was determined by using a refractometer (Atago, Japan). The pH was measured by using an Orion model pH meter (Orion Scientific, Cambridge MA).

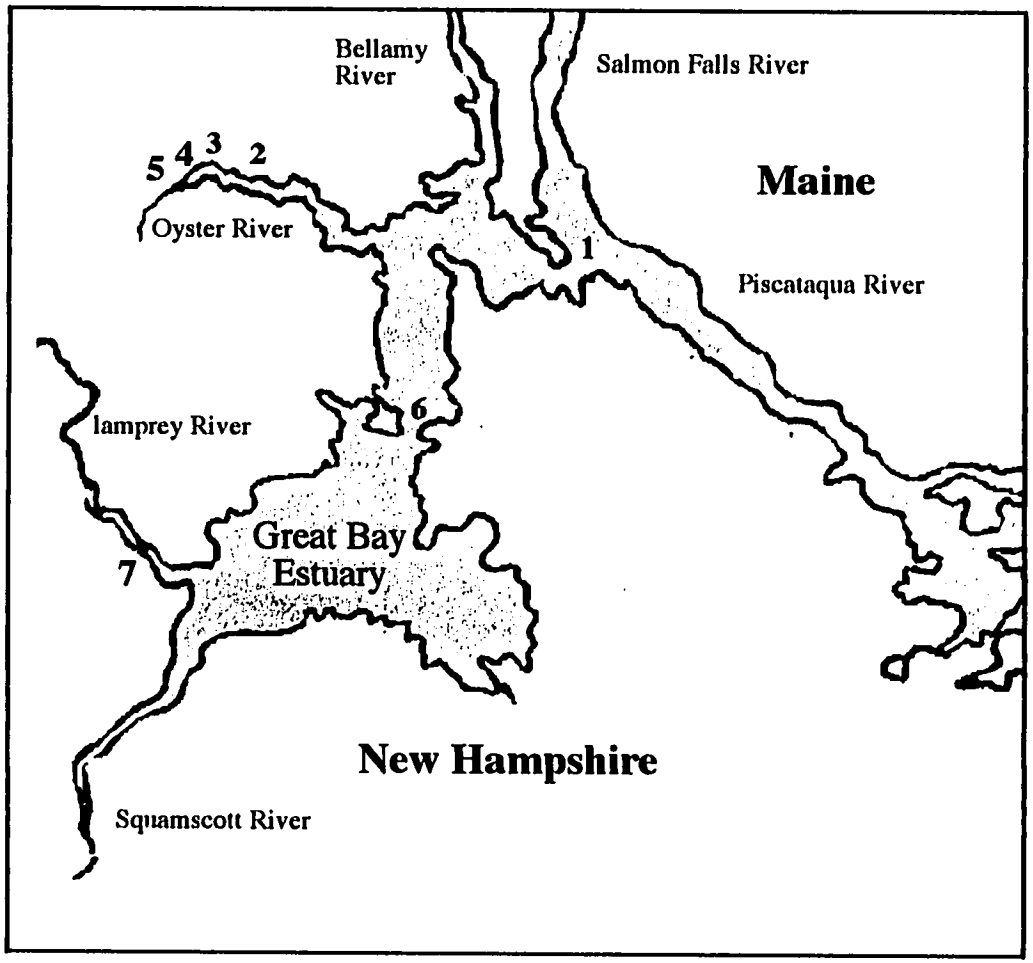
Total suspended solids: Water samples were filtered in duplicate through pre-dried, pre-weighed P/N glass fiber filters (Gelman, Ann Arbor MI). The filters were dried at 60°C to a constant weight and were then reweighed (3).

Chlorophyll a: Water samples were filtered in duplicate through P/N glass fiber filters (Gelman, AnnArbor MI). Chlorophyll was extracted with 5 ml acetone (90%) overnight at 4°C in the dark. Samples were centrifuged at 5, 000 x g for 5 min to remove debris and the chlorophyll a concentration was measured by using the trichromatic method (3). The concentration of chlorophyll a was calculated as follows chlorophyll a = 11.85 E_{664} - 1.54 E_{647} - 0.08 E_{630} and mg·chlorophyll $\cdot l^{-1}$ = (chl .a x vol)/(vol.water filtered x 10).

Identification of isolates: Presumptive aeromonads were cloned three times on Rimler-Shotts *Aeromonas* medium and were maintained on SWC. Standard biochemical tests (See Table 1) were used to confirm aeromonad identification (12, 50)

Statisistical analysis: Enumeration data were analyzed by using the StatView statistics

program (Abacus Concepts Co., Berkeley CA). Data were subjected to a spearman rank correlation analysis. This type of analysis is used for non-normally distributed data. A p of < 0.05 was considered significant. Each parameter was analyzed individually with incidence of aeromonads. In order to compare total multiple parameters a stepwise multiple regression analysis was used. This analysis scanned data for parameters to add or remove. $P < 0.05$ resulted in addition of parameter and $p > 0.1$ resulted in removal of parameter.



Site	Location
1	Hilton Park
2	Bunker Creek
3	Jackson's Landing
4	Mill Pond Dam
5	Durham Mill Pond
6	Adam's Point
7	Joyce's Kitchen

Figure 1 Location of sampling sites in the Great Bay estuarine complex.

Test	*Aeromonas*	*Pseudomonas*	*Vibrio*
Oxidase	+	+	+
Growth 0 % NaCl	+	+	-
Growth 1% NaCl	+	+	+
Arginine dihydrolase	+	+	-
Oxidative	+	+	+
Fermentative	+	-	+
Acid in mannitol	+	-	+
Acid in inositol	-	-	-

Table 1 Identification of *Aeromonas* to the genus level .

RESULTS:

Hilton Park: Heterotrophs ranged from approximately 40 CFU·ml^{-1} in the winter months to approximately 3·10^{3} CFU·ml^{-1} during the early fall (Fig. 2 A and 3 A). *Aeromonas* densities ranged from 0-64 CFU·ml^{-1} with the greatest abundance detected during the early fall of each year (Fig. 2 A-3 A). Total and fecal coliforms ranged from 0 to 6 and 3 respectively, with the highest abundance in the early fall (Fig. 2 A-3 A). The salinity at this site ranged from 11 -34 ppt being lowest in the winter months due to increased precipitation (Fig. 2 B and 3 B) and runoff. The concentration of total suspended solids ranged from 9.3 x 10^{-3} to 2.9 x 10^{-1} ppm. Chlorophyll a concentrations ranged from 0.36-3.17 mgl^{-1} with the highest concentration in September, 1995.

Bunker Creek: Heterotrophs ranged from approximately 60 CFU·ml^{-1} in the winter months to approximately 2·10^{3} CFU·ml^{-1} during the early fall (Fig. 4 A and 5 A). *Aeromonas* densities ranged from 0-85 CFU·ml^{-1} with the greatest abundance detected during the early fall of each year (Fig. 4 A and 5 A). Total and fecal coliforms ranged from 0 to 2 and 5 CFU·ml^{-1} respectively, with the highest abundance during the early fall (Fig. 4 A and 5 A). The salinity ranged from 3- 33.5 ppt with the low in the winter months due to increased precipitation (Fig. 4 B and 5 B). The concentration of total suspended solids ranged from 8.88 x 10^{-3} to 6.64 x 10^{-2} ppm. The chlorophyll a ranged from 0.21-5.52 mg·l^{-1} with the highest concentration in September, 1995.

Jackson's Landing: Heterotrophs ranged from approximately 70 CFU·ml^{-1} in the winter months to approximately 5·10^{3} CFU·ml^{-1} during the summer and early fall (Fig. 4 A

and 5 A). A speciously high concentration of heterotrophs occurred in November, 1994 ($5 \cdot 10^3$ CFU·ml^{-1}). *Aeromonas* densities ranged from 0 - 69 CFU·ml^{-1} with the greatest abundance detected during the early fall of each year (Fig. 4 A and 5 A). Total and fecal coliforms ranged from 0 to 4 and 2 CFU·ml^{-1} respectively, with the highest abundance during the early fall (Fig. 4 A and 5 A). The salinity at this site ranged from dramatically from 8-31.5 ppt depending upon the season (Fig. 2 B and 3 B) with the low in the winter months due to increased precipitation and run off. The concentration of total suspended solids ranged from 9×10^{-3} to 4×10^{-2} ppm. The chlorophyll a ranged from 0.5 - 11 mg·l^{-1} with the highest concentration in September, 1995.

Mill Pond Dam: Heterotrophs ranged from approximately 45 CFU·ml^{-1} in the winter months to approximately $2 \cdot 10^3$ CFU·ml^{-1} during the early fall (Fig. 8 A and 9 A). *Aeromonas* densities ranged from 0 - 41 CFU·ml^{-1} with the greatest abundance during the early fall of each year (Fig. 8 A and 9 A). Total and fecal coliforms ranged from 0 to 4, and 2 respectively, with the highest abundance in the early fall (Fig. 8 A and 9 A). The salinity at this site ranged from 0 1 ppt with the low in the winter months due to increased precipitation (Fig. 8 B and 9 B). The concentration of total suspended solids ranged from 1.2×10^{-3} - 2.9×10^{-2} ppm (Fig. 8 B and 9 B). The concentration of chlorophyll a ranged from 0.1 - 1.7 mg·l^{-1} with the highest concentration in the fall of 1995.

Durham Mill Pond: Heterotrophs ranged from approximately 60 CFU·ml^{-1} in the winter months to approximately $3.3 \cdot 10^3$ CFU·ml^{-1} during the early fall (Fig. 10 A and 11 A). *Aeromonas* densities ranged from 0 - 18 CFU·ml^{-1} with the greatest abundance detected during the early fall of each year (Fig. 10 A and 11 A). Total and fecal coliforms ranged from 0 to 7 and 3 CFU·ml^{-1}, respectively, with the highest abundance during the early fall (Fig. 10 A and 11 A). The salinity at this site was always 0 ppt (Fig. 10 B and 11

B). The concentration of total suspended solids ranged from 9.2 x 10^{-3} to 8.42 x 10^{-2} ppm. The chlorophyll a ranged from 0.45-3.14 mg·l^{-1} with the highest concentration in September, 1995 (Fig. 10 B and 11 B).

Adam's Point: Heterotrophs ranged from approximately 50 CFU·ml^{-1} in the winter months to approximately 2·10^3 CFU·ml^{-1} during the early fall (Fig. 12 A and 13 A).*Aeromonas* densities ranged from 0 - 5 CFU·ml^{-1} with the greatest abundance detected during the early fall of each year (Fig. 12 A and 13 A). Total and fecal coliforms ranged from 0 to 2 and 3 CFU·ml^{-1}, respectively, with the highest abundance during the early fall (Fig. 12 A and 13 A). The salinity at this site ranged from 5 - 33 ppt with the low in the spring due to increased precipitation and run off (Fig. 12 B and 13 B). The concentration of total suspended solids ranged from 12.5 x 10^{-2} to 3 x 10^{-1} ppm. The chlorophyll a ranged from 0.46-5.83 mg·l^{-1} with the highest concentration in September, 1995.

Lamprey River: Heterotrophs ranged from approximately 2·10^2 CFU·ml^{-1} in the winter months to approximately 1.9·10^3 CFU·ml^{-1} during the early fall (Fig. 14 A and 15 A). *Aeromonas* densities ranged from 0.1-63 CFU·ml^{-1} with the greatest abundance detected during the early fall of each year (Fig. 14 A and 15 A). Total coliforms ranged from 0.1 to 95 CFU·ml^{-1} and fecal coliforms ranged from 1.2-63 CFU·ml^{-1} with the highest abundance of each detected during the early fall of each year (Fig. 14 A and 15 A). The salinity ranged from 0-14 ppt depending upon the season (Fig. 14 B and 15 B). The concentration of total suspended solids ranged from 5 x 10^{-3} to 1.1 x 10^{-1} ppm. The chlorophyll a concentration ranged from 0.34 - 4.13 mg·l^{-1} with the highest concentration in September, 1995 (Fig. 14 B and 15 B).

Statistical analysis: Spearman rank correlation test (Table 2) demonstrated correlation

($p = < 0.0001$) of aeromonad density with: pH, density of heterotrophs, total coliforms and fecal coliforms. Correlations were detected with salinity ($p = 0.0389$), temperature ($p = 0.0019$), or total suspended solid ($p = 0.0014$) and no correlation with the concentration of chlorophyll a ($p = 0.5763$), or rainfall ($p = 0.1305$). Multiple regression analysis revealed a correlation between aeromonad density and the incidence of both total ($p = 0.0002$) and fecal ($p = 0.0075$) coliforms (Table 3).

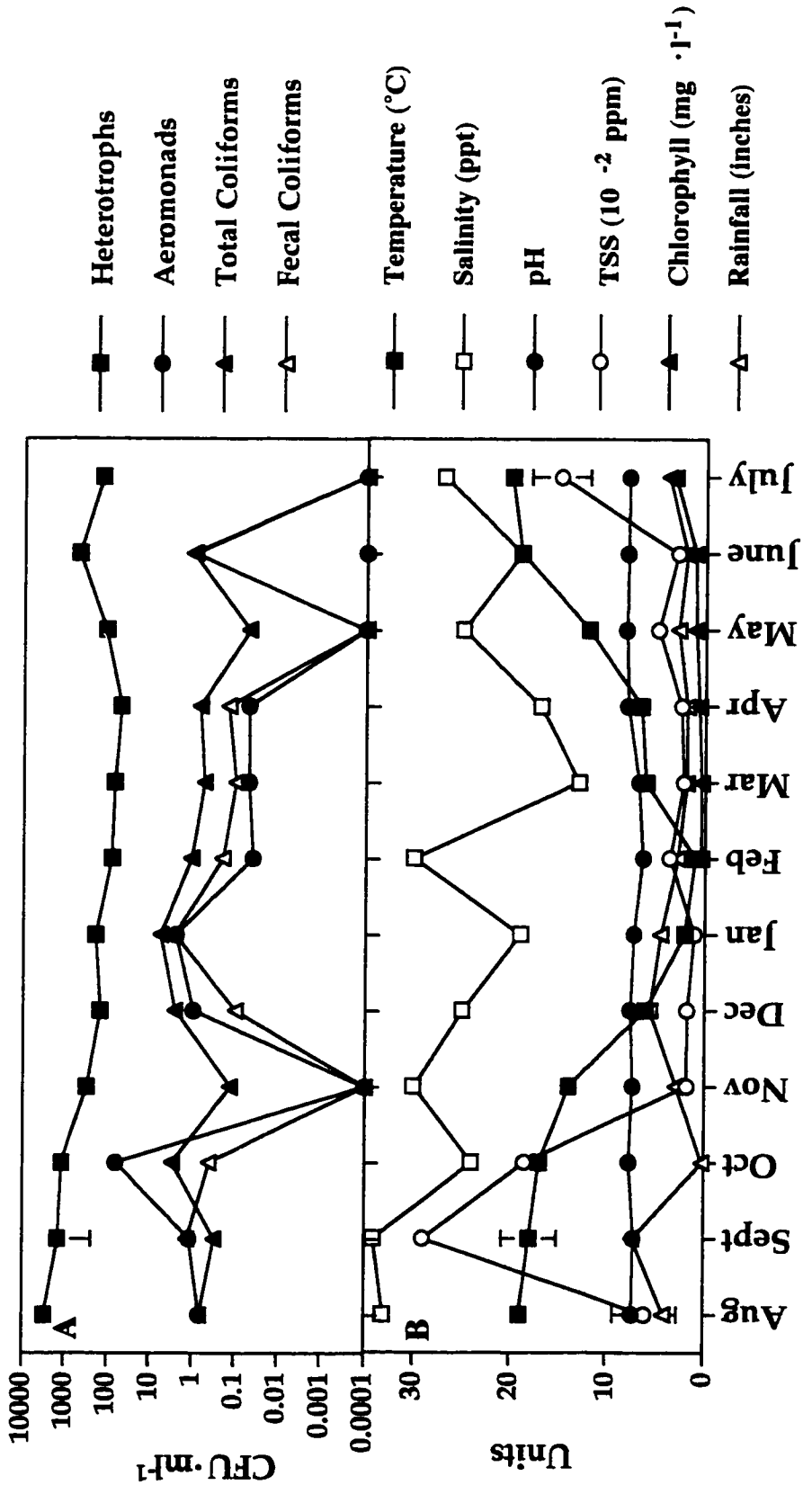


Figure 2 Biotic (A) and physicochemical (B) parameters at Hilton Park (Fig. 1, site 1). Samples were collected at low tide from August 1994 - July 1995.

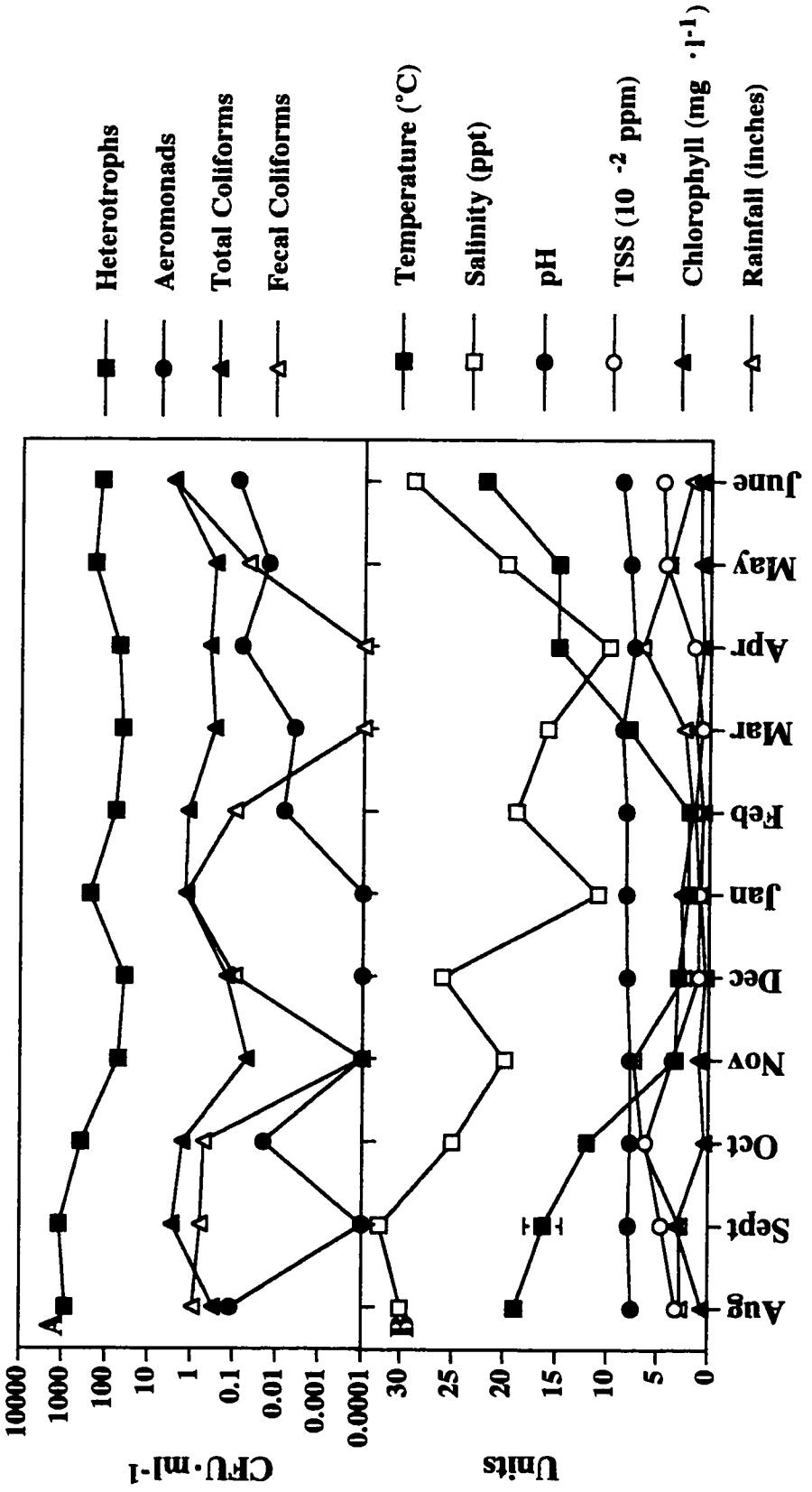


Figure 3 Biotic (A) and physicochemical (B) parameters at Hilton Park (Fig. 1, site 1) Samples were collected at low tide from August 1995-June 1996.

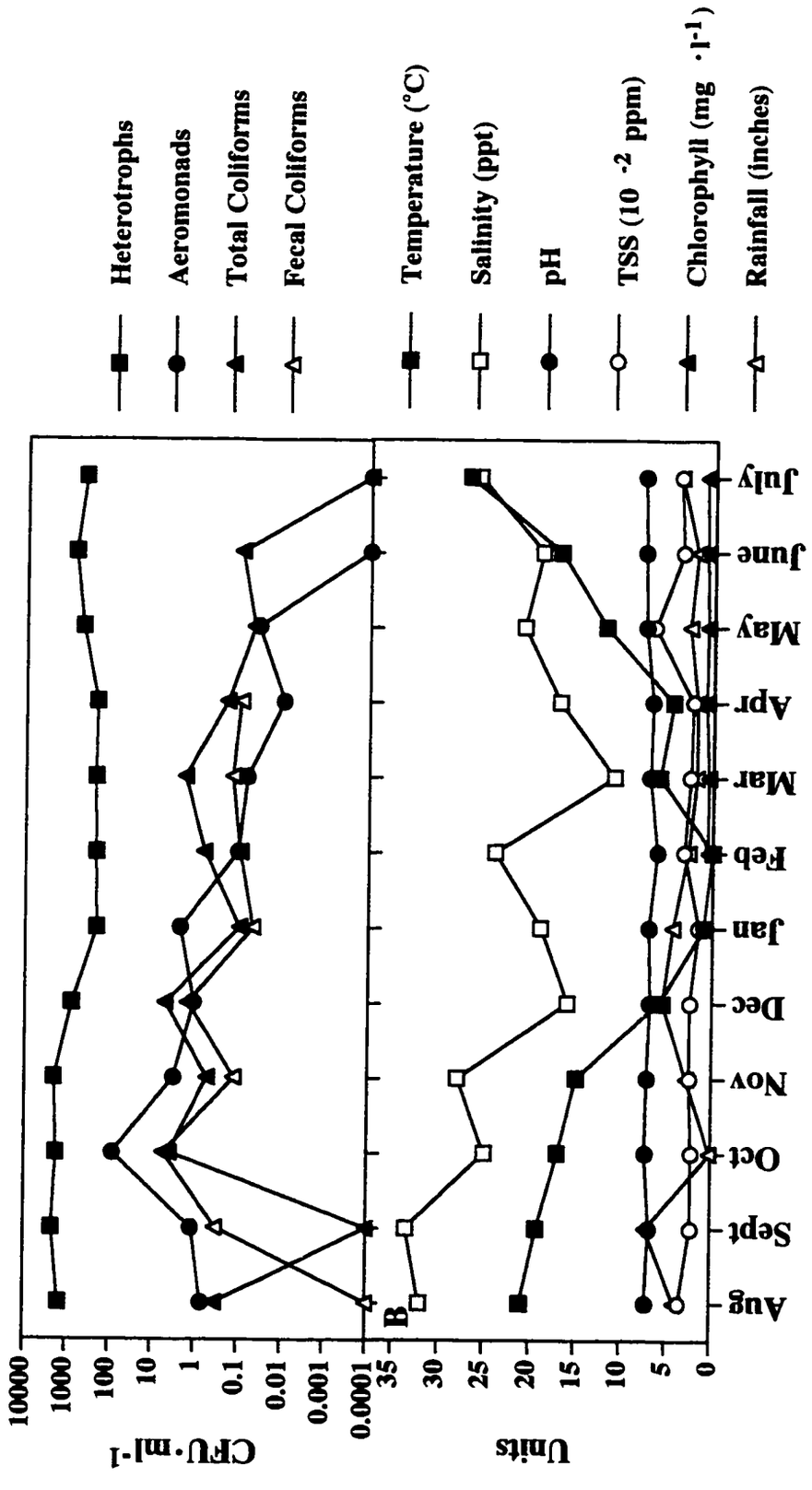


Figure 4 Biotic (A) and physicochemical (B) parameters at Bunker Creek (Fig. 1, Site 2). Samples were collected at low tide from August 1994-July 1995

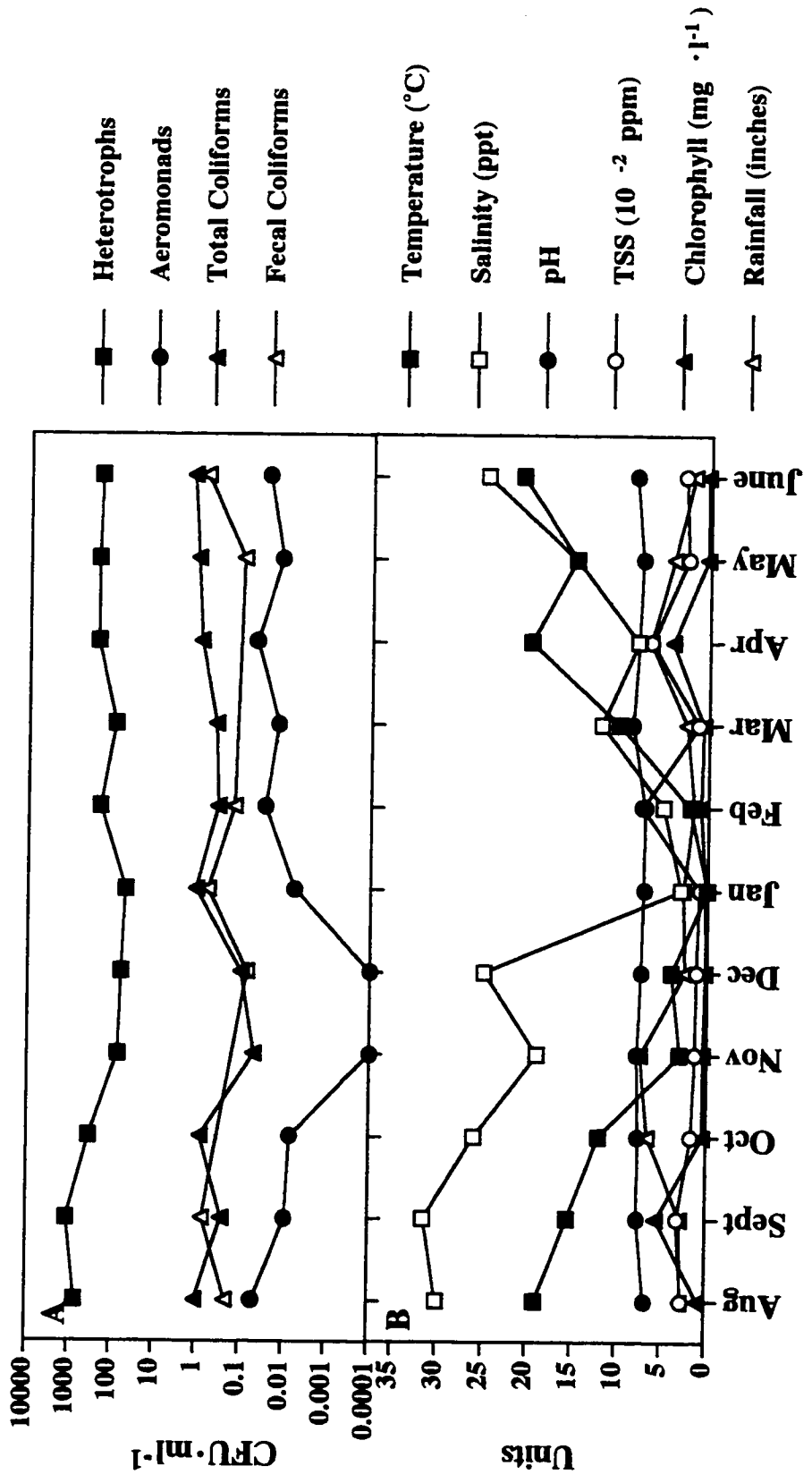


Figure 5 Biotic (A) and physicochemical (B) parameters at Bunker Creek (Fig. 1, site 2). Samples were collected at low tide from August 1995-June 1996.

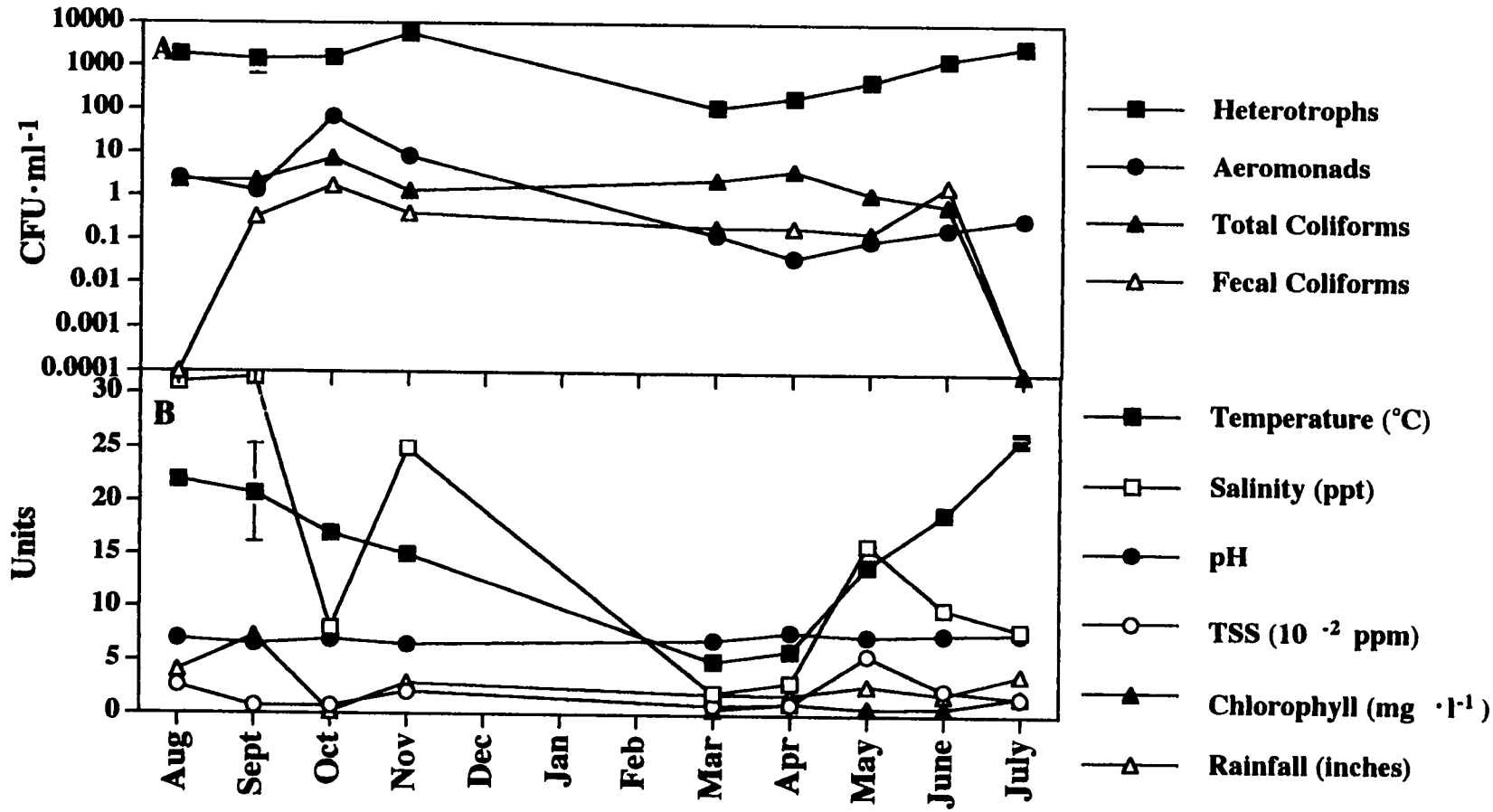


Figure 6 Biotic (A) and physicochemical (B) parameters at Jackson's landing (Fig. 1, site 3). Samples were collected at low tide from August 1994- July 1995. Samples were not collected from December-Febuary.

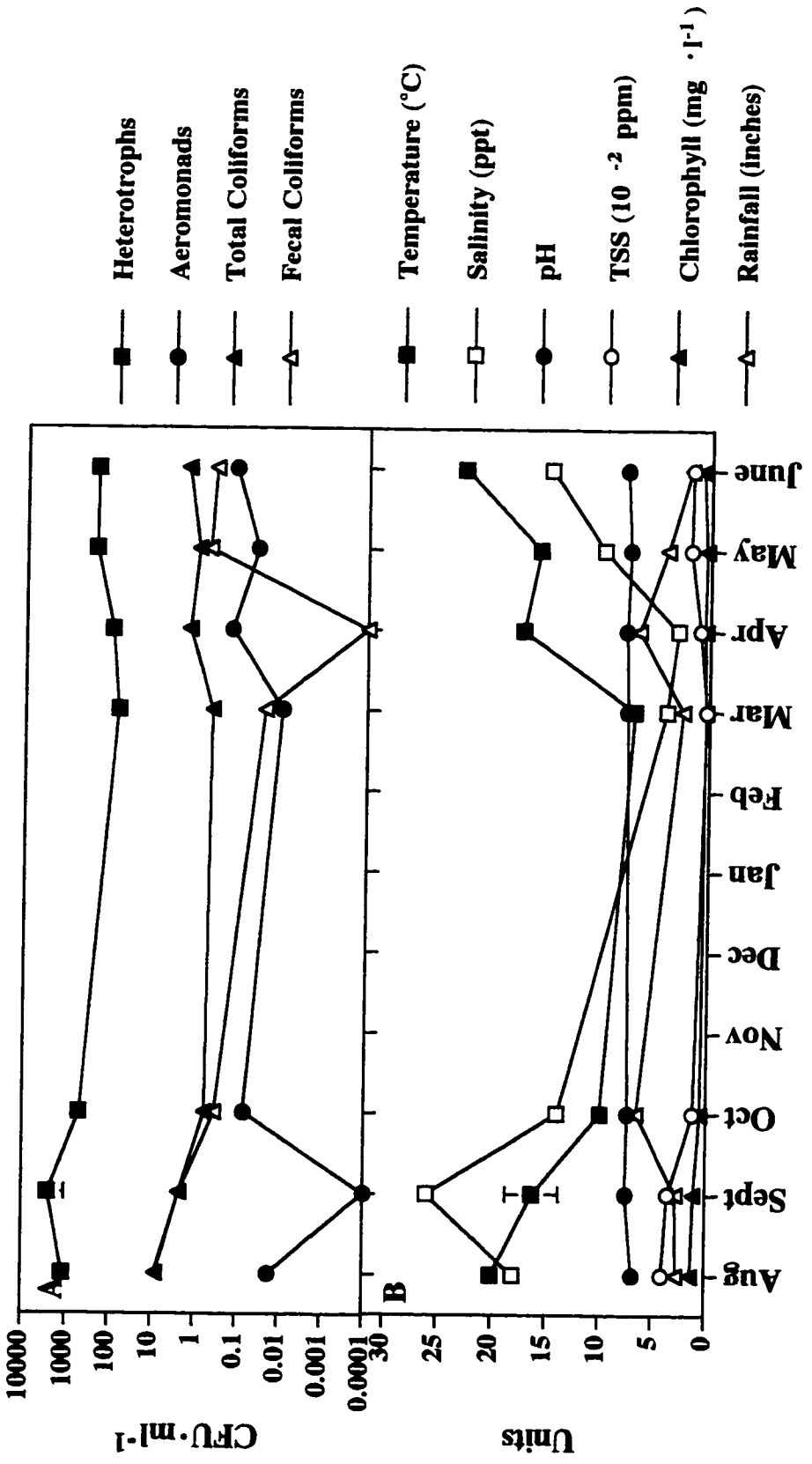


Figure 7 Biotic (A) and physicochemical (B) parameters at Jackson's Landing (Fig. 1, site 3). Samples were collected at low tide from August 1995-June 1996. Samples were not collected from November to Febuary.

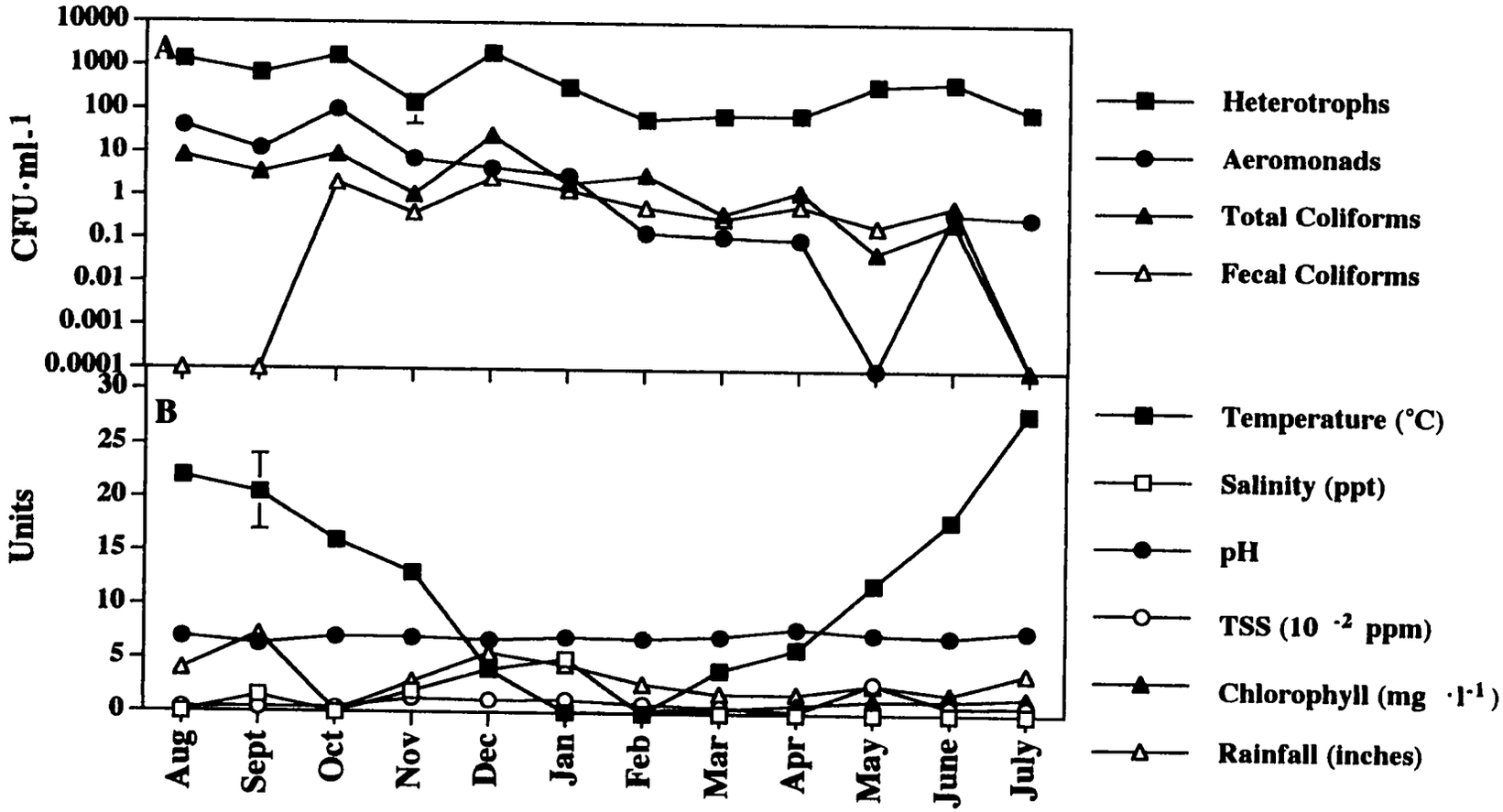


Figure 8 Biotic (A) and physicochemical (B) parameters at Mill Pond Dam (Fig. 1, site 4). Samples were collected at low tide from August 1994-July 1995

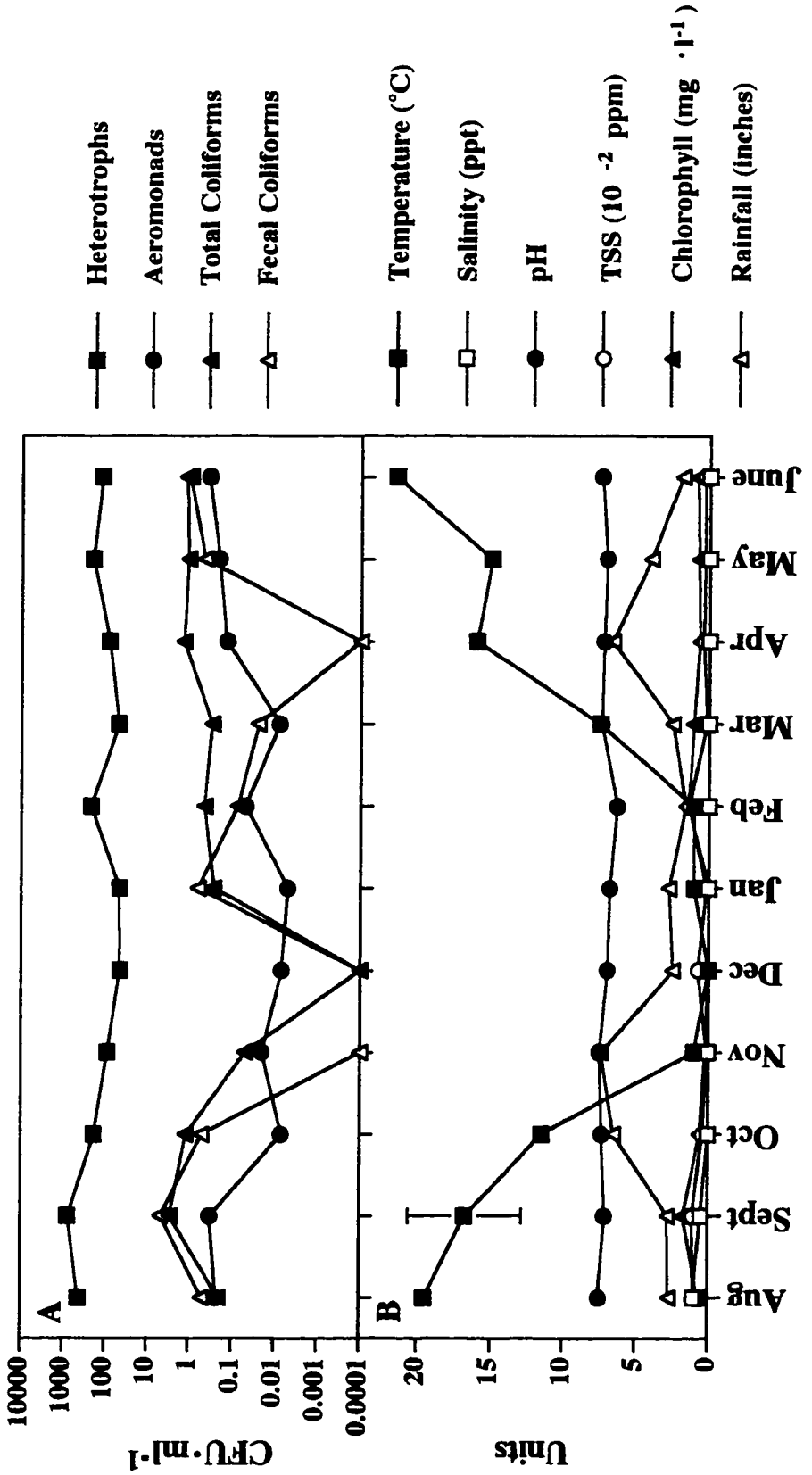


Figure 9 Biotic (A) and physicochemical (B) parameters at Mill Pond Dam (Fig. 1, site 4). Samples were collected at low tide from August 1995-June 1996.

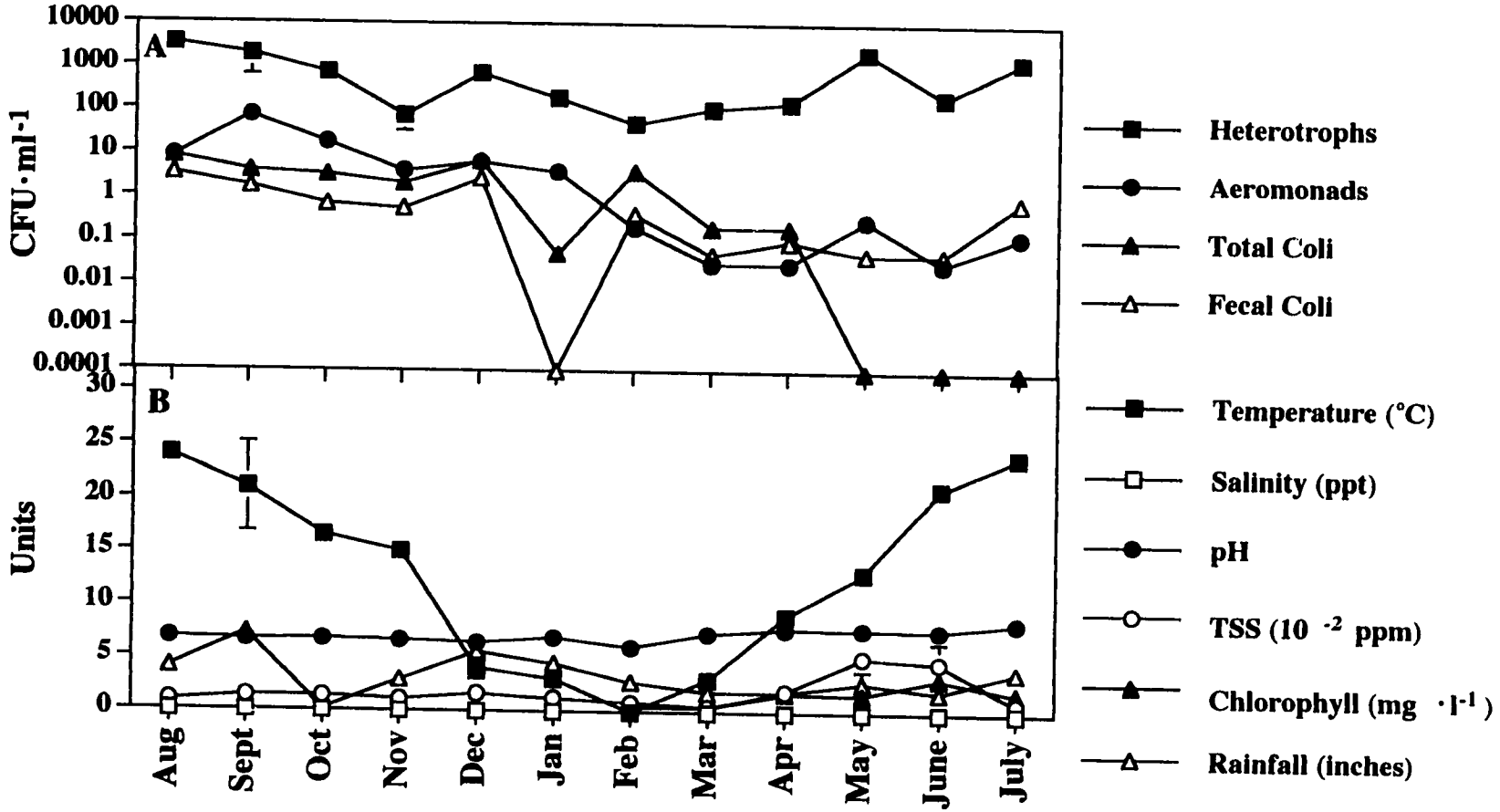


Figure 10 Biotic (A) and physicochemical parameters at Durham Mill Pond (Fig. 1, site 5). Samples were collected at low tide from August 1994-July 1995

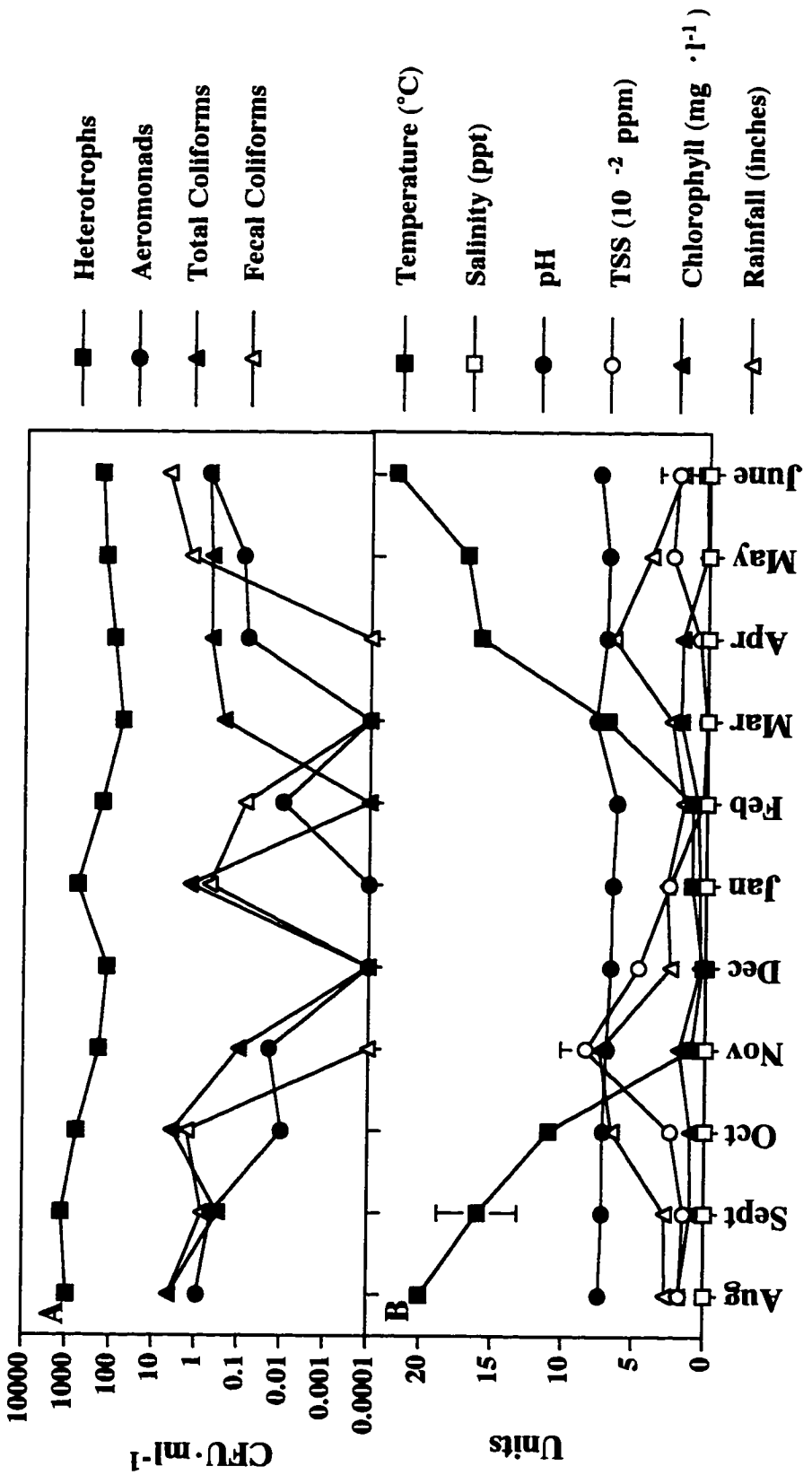


Figure 11 Biotic (A) and physicochemical (B) parameters at Durham Mill Pond (Fig. 1, site 5). Samples were collected at low tide from August 1995-June 1996.

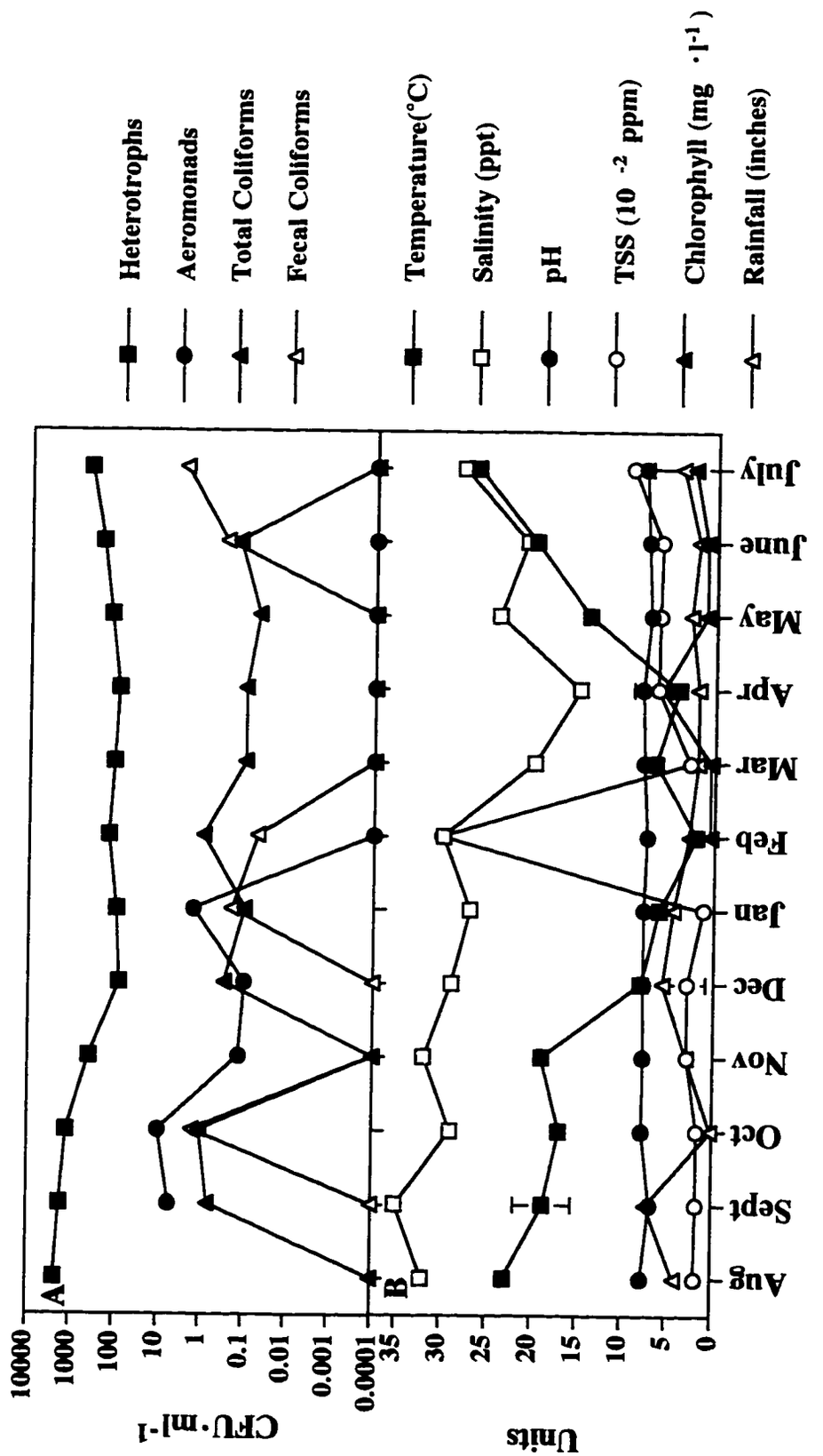


Figure 12 Biotic (A) and physicochemical (B) parameters at Adam's Point (Fig. 1, site 6). Samples were collected at low tide from August 1994-July 1995.

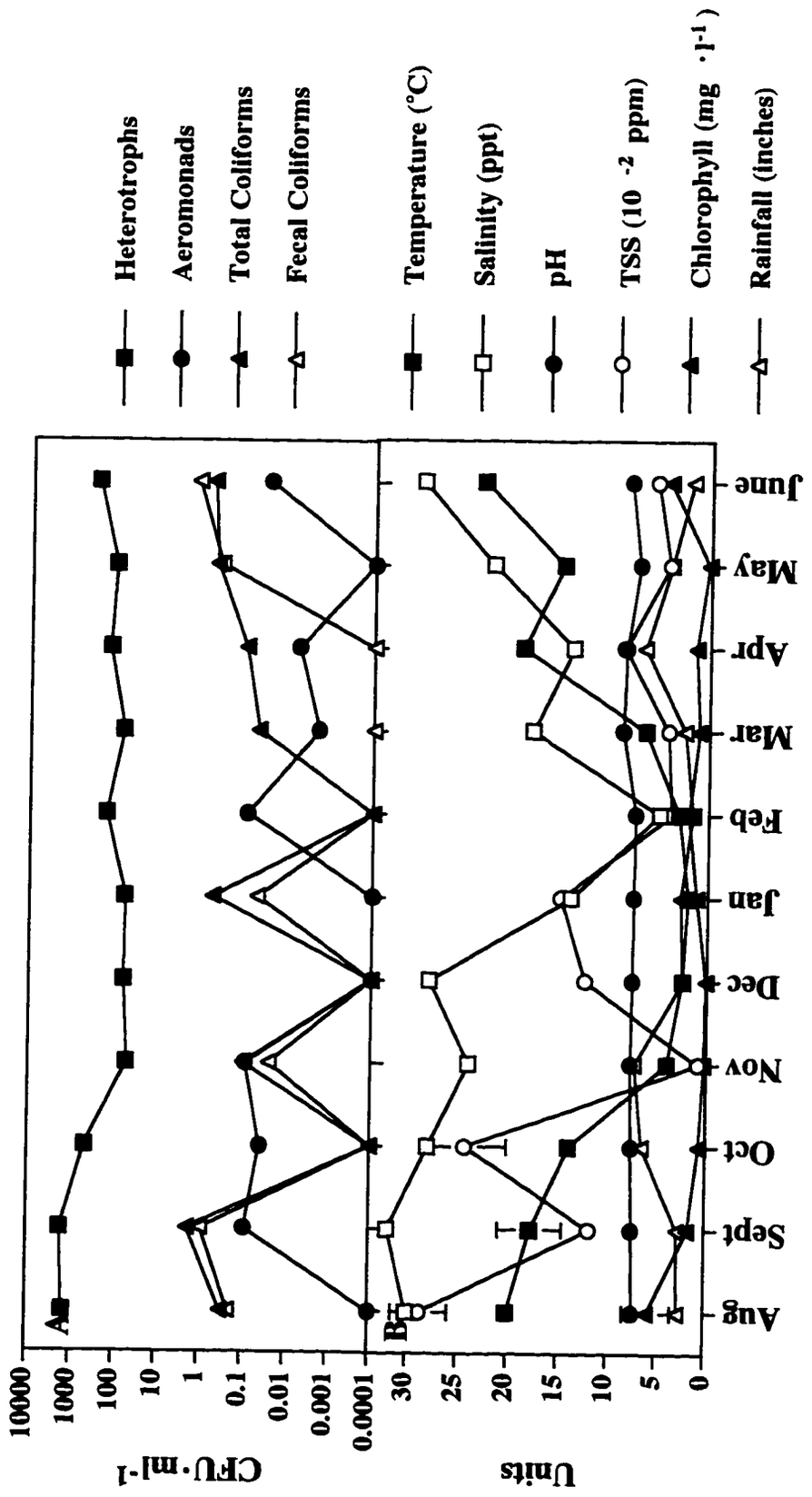


Figure 13 Biotic (A) and physicochemical (B) parameters at Adam's Point (Fig. 1, site 6). Samples were collected at low tide from August 1995-June 1996.

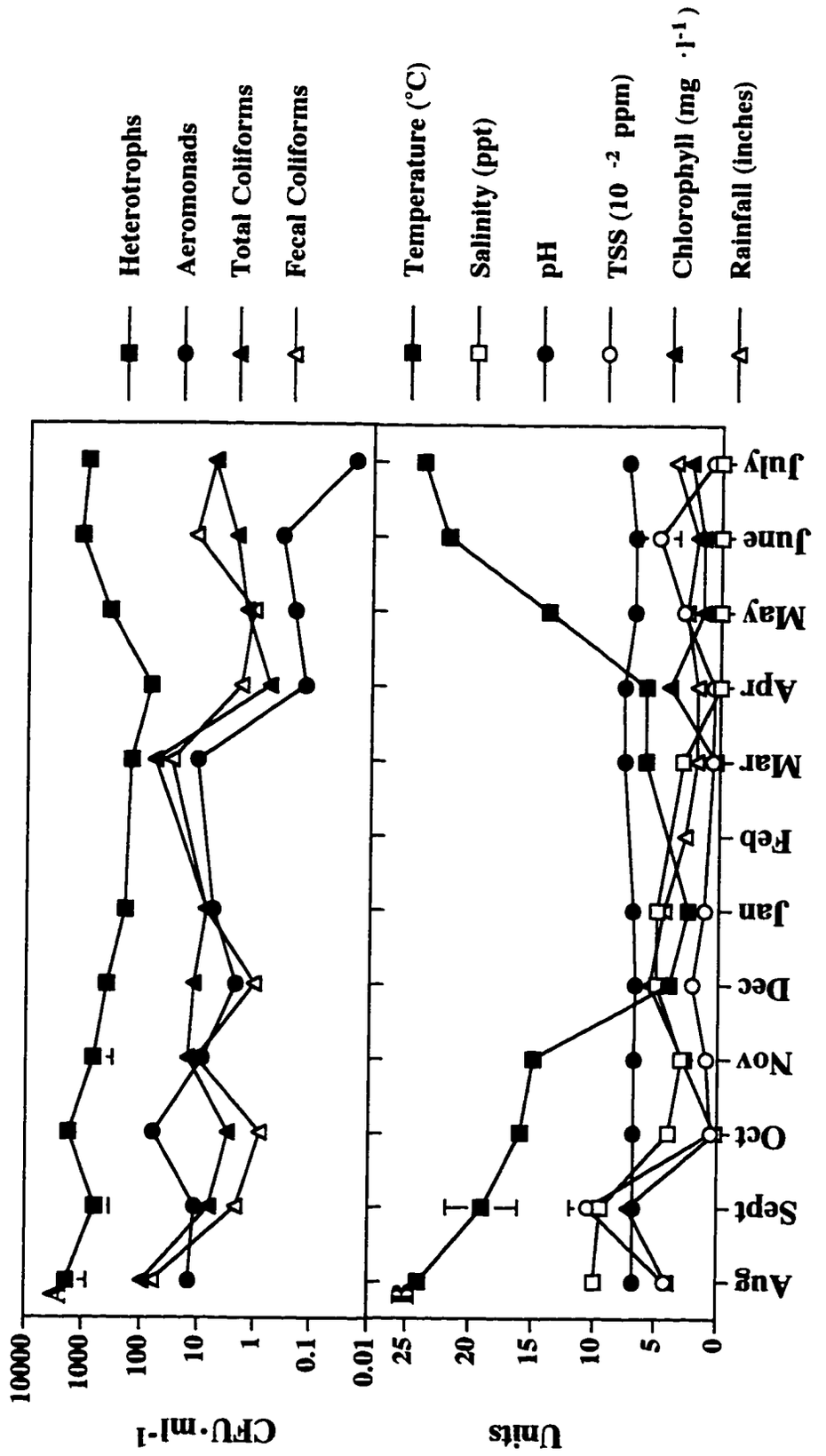


Figure 14 Biotic (A) and physicochemical (B) parameters at Joyce's Kitchen (Lamprey River, Fig. 1, site 7). Samples were collected at low tide from August 1994-July 1995.

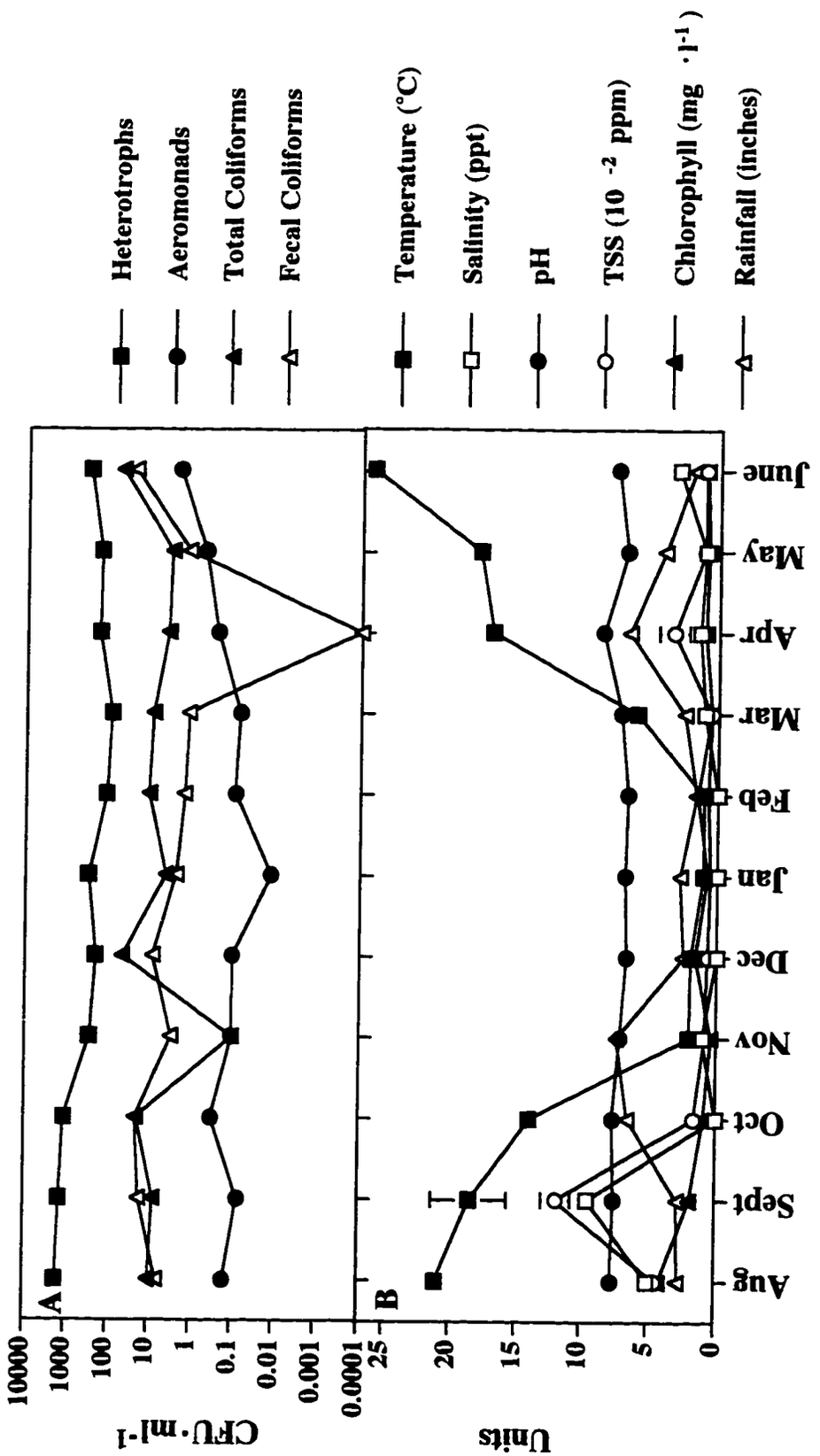


Figure 15 Biotic (A) and physicochemical parameters at Joyce's Kitchen (Lamprey River, Fig. 1, site 7). Samples were collected at low tide from August 1995-June 1996.

Parameter	p value
Temperature	0.0019
Salinity	.0389
pH	< 0.0001
Total suspended solids	0.0014
Chlorophyll a	0.5763
Heterotrophic plate count	< 0.0001
Total coliforms	< 0.0001
Fecal coliforms	< 0.0001
Rainfall	0.1305

Table 2 Spearman rank correlation for *Aeromonas* abundance with biotic and physicochemical parameters measured in this study.

Parameter	Standard coefficient	t-value	P-value
Total coliforms	0.461	3.916	0.0002
Fecal coliforms	0.321	2.723	0.0075

Table 3 Multiple regression for *Aeromonas* abundance with total and fecal coliforms. $n = 113$, $r^2 = 0.566$.

DISCUSSION:

Aeromonas has been reported in NH on only one occasion (in 1978, as part of a broad U. S. Survey Involving only one coastal site; 24). Therefore, a monitoring program was undertaken to determine the abundance and seasonal distribution of mesophilic aeromonads in a seven-site transect of Great Bay Estuary, New Hampshire and in its tributaries (encompassing both freshwater and tidally influenced sites). Two of the sites were truly estuarine (Site 1, and site 6). The Mill Pond (site 5) was freshwater. The other sites were under tidal influence and the salinity fluctuated between approximately 1-15 ppt. Samples for several biotic and physicochemical parameters were collected monthly for two years.

Aeromonads were isolated throughout the year from all sites with an increase in abundance during the summer and early fall. The high incidence during the summer months could be due to both temperature effects and nutrient loading. Mesophilic aeromonads are very sensitive to low temperatures. At 4 °C, aeromonads do not conserve enough energy to divide, and it is thought that they enter a viable but non-culturable (VBNC) state (53). However, we isolated aeromonads at all but the Adam's Point site, in low abundance (0.1-0.4 $CFU \cdot ml^{-1}$) during the winter months with water temperatures as low as 1 °C. Others have shown that *Vibrio vulnificus* was not culturable from water samples or shellfish from Great Bay Estuary between October and June (46). The incubation temperature we used for quantification of *Aeromonas* was 37 °C which we determined to be optimal for growth. This is an unusually high optimal temperature for the isolation of indigenous aquatic organisms. However, we were interested in the mesophilic, and not the psychrophilic aeromonads; and more importantly this temperature provided

further selection i.e. the background of non-aeromonads was reduced. Controls in which the number of aeromonads were quantitated at both 30 °C and 37 °C revealed minimal differences between the incubation temperatures (data not shown). In fact, at 37 °C the number of aeromonads was slightly higher due to lower background of non-aeromonads.

A stepwise multiple regression analysis correlated aeromonad densities with the abundance of both total ($p = 0.0002$) and fecal ($p = 0.0075$) coliforms. This supports results of other studies in which the incidence and abundance of aeromonads (enumerated at 37 °C) correlated with coliform densities in both freshwater and estuarine environments (17, 21, 23, 51, 54). In eutrophic waters, coliforms can be used to accurately predict the concentration of mesophilic aeromonads (9, 29, 54, 62). Great Bay Estuary, although largely undeveloped, receives large amounts of anthropogenic input and is therefore likely to fall within this category. Seven major rivers flow into the Great Bay Estuary and of these most are receiving treated waters from municipal wastewater (28). The effluent discharge drains at most of these local wastewater treatment facilities are used for both effluent discharge and storm overflow (28). Therefore, after heavy rain untreated effluent may be discharged directly into the receiving water (28). Effluent from individual sanitary sewers, agricultural run off and non-point source pollution all combine to release a high organic load into the estuary (28). It has been estimated that 14.6×10^4 kg·yr^{-1} and 3.9×10^2 kg·yr^{-1} phosphorous, respectively is discharged into Great Bay Estuary as a result of point and non-point source pollution (28). As a result the productivity in the estuary is expected to be high. Aeromonads would be expected to thrive in this environment. Rippey and Cabelli (1980) proposed the use of mesophilic aeromonads as indicators of water trophic status (52, 54). Aeromonad densities can be correlated with indicators of productivity such as elevated phosphorous, and total nitrogen and low secchi depth. In addition they respond subtle changes in the water quality (55). The doubling time of aeromonads decreased by 4 hours in oligotrophic waters amended with enough eutrophic

waters to increase the phosphorous concentration by as little as 2 $\mu g \cdot l^{-1}$ (53).

In this study, Spearman rank correlations revealed that *Aeromonas* density was strongly correlated ($p = <0.0001$) with total coliforms, fecal coilforms, pH and temperature. In all water sources studied the incidence of aeromonads increased with temperature (20, 21, 52). In several thermally altered lakes the incidence of *Aeromonas* was directly related to the ambient temperature (17, 23). In our study, little correlation occurred between aeromonad densities and salinity ($p = 0.0389$), this can be explained by seasonal variation due to precipitation and run off. Little correlation occurred with either total suspended solids ($p = 0.0014$) or chlorophyll a ($p = 0.0019$). This can be explained by the high sediment loading at both estuarine sites providing the highest total suspended solids, and chlorophyll at the sites where aeromonads were commonly least abundant. Differences between the correlations are expected to be the result of the statistical analysis used. Spearman rank correlation analysis compares each parameter individually with the incidence of aeromonas (dependant variable). This analysis is non-parametric and often used for non-normally distributed data (as in the case of the data collected here which contained lots of zeros). The drawback however, is that each parameter is individually analyzed therefore, not taking into account tof other parameters. Therefore, a stepwise multiple regression was also used. This analysis compares all parameters collectivly with the incidence of aeromonads. Each parameter is added or removed based upon it's P value ($p < 0.05$ to add, and $p > 0.1$ to remove).

At the Lamprey River Site the abundance of both *Aeromonas*, and total and fecal coliforms were consistently high. In fact, at this site the densities of coliforms exceeded the allowable concentration for coliforms in New Hampshire recreational waters (88 $CFU \cdot ml^{-1}$ /100 ml, 43) for 91 % (21/23 months) of the sampling months. Although this site is within tidally influenced areas of the Newmarket wastewater discharge site, the input from waterfowl which are ever present may be more likely responsible for these high

numbers. Aeromonads are associated with the intestinal tracts of birds and are often found at densities of 1.2×10^7 cell·g^{-1} feces (35). In fact, aeromonads have been isolated in numbers two orders of magnitude higher than those of *Salmonella* (35).

Our results are consistent with those obtained by others for water sources on the eastern coast of the United States. Such studies have consistently revealed the highest densities of aeromonads during the summer and early fall. The incidence of aeromonads in each case increases with temperature. In Florida waters, aeromonad density ranged from 11-2400 CFU·ml^{-1} with the highest densities detected during the summer months (63). The temperature ranged from 11-30 °C (63). In a thermally altered lake in North Carolina, aeromonad density ranged from 20-300 CFU·ml^{-1} and the temperature from 18-28 °C (20). Further north, in Virginia, aeromonads were seasonally distributed with densities ranging from 3 -110 CFU·ml^{-1} and temperature from 6-28°C (51). In Rhode Island waters, aeromonads were found at densities of 0-100 CFU·ml^{-1} with temperatures ranging from 7-25 °C (54). We found aeromonad densities in Great Bay to range between 0 and 65 CFU·ml^{-1} with the highest abundance in the summer and early fall. The temperature ranged from 0-22°C. These data suggest that factors which regulate aeromonad densities, may be different in the NE and SE United States. However, it could be as simple as the ability of aeromonads to survive at low temperature. In the northern states, once the temperature drops below approximately 4 °C Aeromonas may enter a VBNC state (53). Therefore, unlike in southern waters, in the spring once the temperature increases, they may need to reestablish themselves within the estuary. Thereby resulting in cyclic population densities with deeper oscillation than in southern waters.

Great Bay Estuary, New Hampshire is a shallow temperate estuary. We isolated mesophilic aeromonads were readily isolated from its waters at diverse sites throughout the entire year. Ours is the first report of *Aeromonas* in New Hampshire waters. By examining population densities seasonally we have obtained evidence consistent with a role

of temperature, in regulating the abundance of this opportunistic pathogen in Great Bay Estuary. In addition in Great Bay Estuary as in many other eutrophic waters, total and fecal coliforms can be used as indicators of aeromonad abundance.

This is of considerable significance with respect to current standard methods of water analysis. The aeromonad counts over a two year period were frequently on the order of 0.1 % of the heterotrophic plate count, 50 % of the total coliforms, and > 100% of the fecal coliform counts. Since mesophilic aeromonads comprise one source of false positives in rapid coliform tests (by "quick" methods involving MUG hydrolysis), they may contribute to statistics which result in implementation of protective public health practices (such as closing of New hampshire recreational waters when the coliform count exceeds 88 CFU·100 ml^{-1}). On the other hand, the fact that *A. hydrophila* is an opportunistic pathogen and is indigenous to natural waters suggests that this water quality standard should not be amended.

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CHAPTER TWO

DISSIMILATORY METAL REDUCTION BY MESOPHILIC AEROMONADS ISOLATED FROM GREAT BAY ESTUARY, NEW HAMPSHIRE.

ABSTRACT:

Aeromonas hydrophila ATCC 7966 grew anaerobically on the non-fermentable carbon and energy source glycerol with nitrate, fumarate, Fe (III), Co (III)-EDTA, or Se (VI) as sole terminal oxidant. No growth occurred with sulfate, sulfite, thiosulfate, TMAO, Mn (IV) or Cr (VI). Concomitant growth and reduction occurred with nitrate, Fe (III), Co (III), and Se (VI). Final cell yield and the extent of reduction by *A. hydrophila* ATCC 7966 were in direct proportion to the amount of terminal oxidant provided. Thirty mesophilic aeromonads were isolated from Great Bay Estuary, New Hampshire and its tributaries by using a method which did not select either for metal-reducing microorganisms or anaerobic respirers. Of these, 100 % were capable of nitrate reduction, 100 % were capable of dissimilatory Fe (III) reduction, and 100 % were capable of cobalt (III) reduction, and 43 % were capable of Se (VI) reduction. Fe (III) reduction supported anaerobic growth of *A. hydrophila* ATCC 7966 with glycerol, succinate, and to a lesser extent, with lactate as energy sources. Iron reduction was enzymatic as evident from several observations: the quantity of iron reduced increased with temperature with a sharp

decline (Q_{10} between 37 and 45 °C = 139) at 45 °C; no reduction occurred when ferric oxyhydroxide (unchelated) was separated from the cells by dialysis tubing, and the pH of the culture medium throughout growth remained circumneutral (pH 6.74).

Dissimilatory metal and metalloid reduction by members of the genus *Aeromonas* has not previously been reported. These findings add an important genus of aquatic bacteria to the limited number of genera known to be capable of dissimilatory metabolism with metals and suggest that anaerobic respiration may be an important means of survival and growth of this important bacterium in anaerobic aquatic habitats.

INTRODUCTION:

The role of mesophilic aeromonads as opportunistic pathogens of both cold- and warm-blooded animals including humans has been well documented (2, 22). In addition, most studies have focused upon the incidence of aeromonads in the natural environment with regard to their role in public health (18). Less is known of their physiology and ecology. Aeromonads are facultative anaerobes which grow aerobically, or anaerobically by fermentation (18, 22). We undertook a study to determine if mesophilic aeromonads were capable of anaerobic respiration.

In the absence of oxygen, diverse bacteria can conserve energy for growth by coupling the oxidation of H_2, organic acids, alcohols, or aromatic compounds to the reduction of nitrate, nitrite, sulfate, sulfite, fumarate, trimethylamine-N-oxide (TMAO), thiosulfate, or metals as terminal electron acceptors (27, 39). For an electron acceptor to be of value for electron disposal in respiration it must have a proper redox potential; one that is low enough to be non-toxic for the bacterium but high enough to be energetically useful when coupled to organic carbon oxidation (39). Microbial transformation of metals and complex organic compounds is an important means of biogeochemical cycling, and potentially of bioremediation (15, 27, 39). Metal-reducing organisms are important in biogeochemical cycling within aquatic sediments by mineralizing carbon compounds and by mobilizing or immobilizing diverse metals (15, 18, 27).

The iron concentration in sediments often exceeds that of other potential electron acceptors such as O_2, NO_3^-, and SO_4^{2-} providing the potential for significant nutrient release through organic matter mineralization (15, 27, 39, 48). Iron in sediments often exists as a poorly crystalline iron oxide (3, 11). Use of this form of iron by metal reducers

depends upon their close association with the oxide precipitates and may depend upon their ability to chelate iron (3, 11). Dissimilatory iron reduction was first described by Balashova and Zavarzin in 1980 (4). They described dissimilatory reduction coupled to hydrogen oxidation by a *Pseudomonas* isolate (4) recently characterized as *Shewanella putrefaciens* (39). Obuekwe and Westlake (1982) isolated several dissimilatory metal reducers from oil fields and mine tailings. These were originally identified as *Pseudomonas ferrireducens* and *Pseudomonas sp. 200* (40, 41). These organisms have each been re-assigned (to *Shewanella putrefaciens*) on the basis of 16S rRNA sequence analysis (39). Myers and Nealson (1988) reported a metal-reducing organism strain MR-1(36) recently characterized as *Shewanella putrefaciens* (39) capable of dissimilatory reduction with both Mn (IV) and ferric iron (38). It appears that members of this genus are ubiquitous, many of the original Fe (III)-reducing organisms have recently been re-characterized on the basis of 16S rRNA sequence analysis as *Shewanella putrefaciens* (39). In addition, *Shewanella* isolates have been isolated from environments as diverse as the Baltic sea, Green Bay (WI), and the Black Sea (39). A second species within this genus, *Shewanella alga,* was isolated from Great Bay Estuary, N. H. (6, 47). This organism coupled iron reduction with H_2 (6), lactate (6), or formate oxidation (Chapter 4). *Shewanella alga* also grew by using Co (III), Ur (VI), thiosulfate, (but, interestingly, not sulfate), nitrate, nitrite, or fumarate as terminal electron acceptors (6, 10, 16, 37, 38, 40).

A second major group of iron reducers includes members of the genus *Geobacter* (27, 39). *Geobacter metallireducens* strain GS-15, an obligate anaerobe, was isolated from iron-rich sediments of the Potomac river (28, 29). Studies with this organism set the stage for rewarding explorations of anaerobic degradation of complex organic molecules with iron as the terminal oxidant (28). In fact, *G. metallireducens* strain GS-15 was the first organism reported to completely mineralize an aromatic compound (toluene) to CO_2 anaerobically in axenic culture (28); a process formerly considered to be possible only with

mixed microbial communities. *Geobacter metallireducens* was also capable of dissimilatory metabolism via reduction of Mn (IV), Ur (VI), or nitrate (18, 29). A second member of this genus isolated from a hydrocarbon-contaminated ditch was recently characterized as *Geobacter sulfurreducens* (7). *G. sulfurreducens* grows on acetate with the reduction of Co (III), S^o, fumarate, or malate (7) . A close relative, *Desulfuromonas acetoxidans*, is the first organism known to couple the complete oxidation of acetate to CO_2 with Fe (III) reduction (46). *D. acetoxidans* is also capable of dissimilatory metabolism with reduction of Mn (IV), sulfur or malate as terminal oxidants (46).

It appeared that sulfate reducers such as *Desulfovibrio* in natural sediments are active in iron reduction (27, 39). Lipid analysis of sediments indicated that sulfate-reducing bacteria were an appreciable percentage of the bacterial population in iron reduction zones (39). Pure cultures of *Desulfovibrio* have also been shown to be capable of dissimilatory Fe (III) reduction (8, 2 27, 39). Reduction required actual contact between Fe (III) and the cells and did not occur as a result of chemical reduction by H_2S, a product of sulfate reduction (8, 27).

Several fermentative bacteria, including several different *Bacillus* strains, have been isolated and characterized as metal reducers (27, 39); for one strain, iron reduction has been shown to support growth (27, 39). *Peleobacter carbinolicus* (32), a bacterium formerly thought to grow strictly by fermentation, was recently shown to grow by anaerobic respiration Fe (III) or S^0 reduction.

Selenium is one of the least plentiful (0.001 ppm) but most toxic elements in the earth's crust (12). Selenium is biologically interesting because small differences exist between concentrations which are essential (0.05 - 0.1 ppm) and those which are toxic (4 ppm). The discovery that toxic concentrations of selenium were adversely affecting the bird population at Kesterson wildlife refuge in California emphasized the importance of the biogeochemistry of this element (33). Selenium is a metalloid which behaves chemically

like sulfur (12). In the environment it is found predominantly as selenate (VI) and selenite (IV). Many soil organisms reduce Se (VI) to elemental selenium aerobically as a detoxification strategy, but few have been shown to couple Se (VI) reduction with anaerobic growth (5, 11, 26). *Wolinella succinogenes* reduced Se (VI) anaerobically with fumarate as the terminal electron acceptor. However, reduction occurred only when growth had reached stationary phase (52). Reduction did not occur with Se (VI) as the sole electron acceptor (52). Macy (1989) reported dissimilatory reduction of Se (VI) to Se (IV) by *Pseudomonas* strain AX (34). When grown in co-culture with a selenite oxidizer, *Pseudomonas* Strain E, Se (VI) was reduced to elemental selenium (34). To date, only two organisms are knownto be capable of dissimilatory Se (VI) reduction to elemental Se (33, 44). *Thauera selenatis* (33) grows by reducing Se (VI) as sole oxidant to elemental selenium. It is interesting to note that this organism is also capable of nitrate reduction, and although the nitrate and selenate reductases have proven to be two distinct enzymes, reduction beyond Se (IV) to Se^0 required concomitant denitrification (33). Reduction of selenite by this organism is mediated by the nitrite reductase, or a component of the reductase complex (33). A second organism, SES-3 isolated from highly selenous water in Mono Lake, CA grows with selenate as sole terminal electron acceptor (44). Strain SES-3 reduced Se (VI) to elemental selenium (44). Like many metal reducers, this organism can grow with reduction of As (VI), Fe (III), thiosulfate, S^0, Mn (IV), TMAO, or fumarate (25, 51).

Selenium reduction in nature is often overlooked because unlike the more prevalent potential electron acceptors such as Fe (III), or Mn (IV), which are often found in mM quantities, Se (VI) naturally occurs only in μM concentrations (12, 43, 50). However, reduction of this toxic potential electron acceptor results in the formation of the non-toxic, and insoluble elemental selenium (33). This provides real possibilities for the use of microbial reduction in the bioremediation strategies of this toxic metalloid (12, 43). The

concentration of selenium in most soils is 0.1-2 ppm. However, in soils derived from Cretaceous rock found on the western coast of the United States, concentrations of Se (VI) are often as high as 100 ppm (12). The high selenium concentrations coupled with the intense irrigation of this arid region provides Se (VI) concentrations as high as 0.3 ppm in the ground water (12, 33). Irrigation waters are often collected in evaporation ponds and it is here that selenium becomes concentrated causing mortality of the indigenous wildlife. In highly contaminated sites microbial reduction may be the primary mechanism for selenium ion transformation to less toxic and insoluble elemental selenium. Indigenous microbes might provide a means for bioremediation of this compound (12, 33, 43). Macy et al. (1989) used a mixed culture bioreactor system to convert soluble selenate to precipitated non-toxic elemental selenium (33).

Cobalt is often deposited in the environment in high concentrations as a byproduct in the production of radionuclides (15). Synthetic chelating agents such as ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentacetic acid (DTPA), and nitrilotriacetic acid (NTA) form strong water-soluble complexes with metal ions and radionuclides and have therefore, been used to remove metal oxides on the walls of nuclear reactors (15). However, co-disposal of these water-soluble cobalt-chelator complexes have resulted in increased migration of this hazardous waste (15). Co (II), the product of Co (III) reduction, forms a weak complex with chelators such as EDTA and as a result often dissociates from the chelator as is deposited in the soil. This allows containment and possible remediation of the site (15). To date only two metal-reducers have been reported to grow by dissimilatory Co (III) reduction: *S. alga* (6, 16) isolated from Great Bay Estuary, and *Geobacter sulfurreducens* (7).

Metal mobilization in Great Bay Estuary, New Hampshire is directly influenced by microbial activity and seasonal effects (19). Fe (II) production is maximal in the spring and summer months; consistent with the period of maximal microbial activity and bioturbation

(19). The iron cycle in this estuary also involves Fe (II) oxidation (iron sulfide dissolution) which occurs in surfical sediments maximally during the winter months (19). The Fe (III) concentration in Great Bay Estuary sediments often exceeds that of other electron acceptors such as O_2, NO_3^-, and SO_4^{2-} (19) providing the potential for significant nutrient release through organic matter mineralization coupled to iron reduction (19, 53). Tugel et al. (1986) observed iron reduction in enrichment cultures made with surficial sediments from Great Bay Estuary, New Hampshire, but did not isolate bacteria capable of dissimilatory iron reduction (53).

Interest in the iron cycle of Great Bay Estuary was stimulated by an early finding that members of the genus *Aeromonas* isolated from this environment carry out dissimilatory metal reduction of Fe (III) in axenic culture (23) or, in a least one case, could be shown to indirectly support iron cycling through syntrophic associations with other organisms (Chapter 4). To better understand the ecophysiology of members of the genus *Aeromonas*, a study was undertaken to determine the range of electron acceptors used in anaerobic respiration by mesophilic aeromonads. This study resulted in the isolation of 30 mesophilic aeromonads from Great Bay Estuary and its environs. The isolations were based upon established selective and differential plating techniques; not the ability to reduce metals. These isolates were then examined for their ability to grow anaerobically in a dissimilatory manner using the alternate electron acceptors mentioned above, especially Fe (III).

MATERIALS AND METHODS:

Bacterial Strains: *Aeromonas hydrophila* ATCC 7966 was obtained from the American Type Culture Collection (Rockville, Maryland). Mesophilic aeromonad isolates were obtained from water samples, frogs and shellfish from Great Bay Estuary, NH or its freshwater tributaries (Chapter 1). Aeromonad isolates were cloned and identified to the species level by using standard biochemical tests (Table 1, chapter 1).

Media and cultivation: Mesophilic aeromonads were isolated on Rimler-Shotts *Aeromonas* medium (49) by using membrane filtration (1). All incubations were at 37°C unless otherwise noted. This temperature, which provided selection at initial stages of isolation, was optimal for growth of all isolates as well as for the ATCC strain. For aerobic growth, cells were grown with shaking in Tripticase Soy Broth (TSB, Difco, Detroit MI). For anaerobic growth, cells were grown in basal salts medium (BSM) which contained the following ($g \cdot l^{-1}$): $NaHCO_3$, 2.5; NH_4Cl, 1.5; NaH_2PO_4, 0.6; KCl, 0.1; yeast extract (Difco, Detroit MI), 0.1; Wolfe's vitamins (55), 10 ml; and Wolfe's minerals 10 ml (55). This medium was supplemented with 30 mM glycerol and 20 mM Fe (III) as Fe (III) citrate for *A. hydrophila* ATCC 7966 or Fe (III) oxyhydroxide chelated with equimolar NTA for the aeromonad isolates. Growth with alternate electron acceptors was in BSM supplemented with 30 mM glycerol and alternate electron acceptor as follows (mM): $NaSO_4$, 20; Na_2SO_3, 20; MnO_2, 20; thiosulfate, 20; TMAO, 20; $NaNO_3$, 20; fumarate, 20; Na_2SeO_4,10, Co(III)-EDTA, 1; or $CrCl_3 \cdot 6H_20$, 1; unless otherwise noted. Differences between acceptor concentrations were due to toxcity of metal or metalloid. Amorphous Fe (III) oxyhydroxide was synthesized from $FeCl_2 \cdot 6H_20$ as previously

described (30). Co(III)-EDTA was synthesized from $CoCl_2 \cdot 6H_20$ by peroxide oxidation as previously described (17). Standard anaerobic technique was used throughout (35). Culture medium was boiled and cooled under a steady stream of N_2:CO_2 (80:20). Traces of oxygen were removed by passing these gasses through hot copper filings. Triplicate cultures were initiated at an approximate concentration of 2 x 10^6 cells·ml^{-1} with cells from aerobically grown cultures. For reductant studies, cells were inoculated at an initial concentration of 2 x 10^7 cells·ml^{-1} into BSM with reductant (reductants and concentrations listed in Table 1) but lacking electron donor and incubated for 24 hours. These were then transferred to BSM with appropriate oxidants and reductants. After 72 hours growth, triplicate samples were analyzed for cell counts and for Fe (II) production. Fe (II) concentrations were normalized to cell numbers to allow comparison between substrates. Incubation periods were predetermined by preliminary data to encompass time required for growth on the respective acceptor.

Analytical Techniques: Fe (II) production was assessed by using ferrozine after treatment of the sample with 0.5 N HCl (31). Ferrous ethylenediamine-HCl was used as a standard. Growth was determined by using acridine orange direct counts (AODC) after samples were treated with oxalate to solubilize iron (to relaese bacteria bound to particuate iron). A modification of the epifluorescence technique (20) was used as previously described (31). Nitrate was measured by using sulfanilic acid and α-naphthol after zinc reduction to nitrite (13). Cobalt was detected as Co (III) by measuring absorbance at 535 nm as previously described (7). Selenium was measured by using ion chromatography with an AS4A (Dionex, Sunnyvale, CA) column using an eluent of 1.8 mM Na_2CO_3: 1.7 mM $NaHCO_3$ with a flow rate of 2.0 ml·min^{-1} as previously described (42). Chromium was measured with a colorimetric assay by using *s*-diphenylcarbazide (54).

RESULTS:

Growth: *Aeromonas hydrophila* ATCC 7966 grew by anaerobic respiration using nitrate and fumarate as terminal electron acceptors (Fig. 1). No growth above that of the control lacking added electron acceptor occurred with sulfate, sulfite, thiosulfate, Mn (IV) or TMAO (Fig. 1). *Aeromonas hydrophila* grew to a final cell density of 6×10^8 cells·ml^{-1} within 36 hours anaerobically in BSM supplemented with 30 mM glycerol and 20 mM nitrate (Fig. 2 A). The organism reached a final cell density of 4×10^8 cells·ml^{-1} (Fig. 2 A) within 60 hours when grown anaerobically in BSM supplemented with 30 mM glycerol and 20 mM ferric citrate. It reached a final cell density of 1×10^8 cells·ml^{-1} (Fig. 2 A) within 24 hours when grown anaerobically in BSM supplemented with 30 mM glycerol and 2 mM Co (III)-EDTA. Anaerobic growth in basal salts medium supplemented with 30 mM glycerol and 10 mM Se (VI) yielded 2×10^7 cells·ml^{-1} (Fig. 3 A) within 96 hours. No growth above that of the control (1×10^7 cells·ml^{-1}, Fig 2 A, 3 A) lacking terminal electron acceptor occurred within 120 hours with 1 mM Cr (VI) (Fig. 3 A) . Final cell yields of *A. hydrophila* ATCC 7966 after 48 hours were directly proportional ($n = 15$, $p < 0.001$, $r^2 = 0.894$) to the amount of nitrate provided over the range 1.25-5 mM (Fig. 4 A). Final cell yields after 60 hours were directly proportional ($n = 15$, $p < 0.001$, $r^2 = 0.968$) to the amount of Fe (III) citrate provided over the range 1.25-10 mM (Fig. 5 A). The final cell yield of *A. hydrophila* ATCC 7966 after 24 hours was directly proportional ($n = 15$, $p < 0.001$, $r^2 = 0.971$) to the amount of Co (III)-EDTA provided over the range 0.5-1.5 mM (Fig. 6 A). Final cell yields after 120 hours growth were directly proportional ($n = 15$, $p < 0.001$, $r^2 = 0.981$) to the amount of Se (VI) provided over the range 1.25-10 mM (Fig. 7 A). Growth of *A. hydrophila* ATCC 7966 in BSM supplemented with 30 mM glycerol did

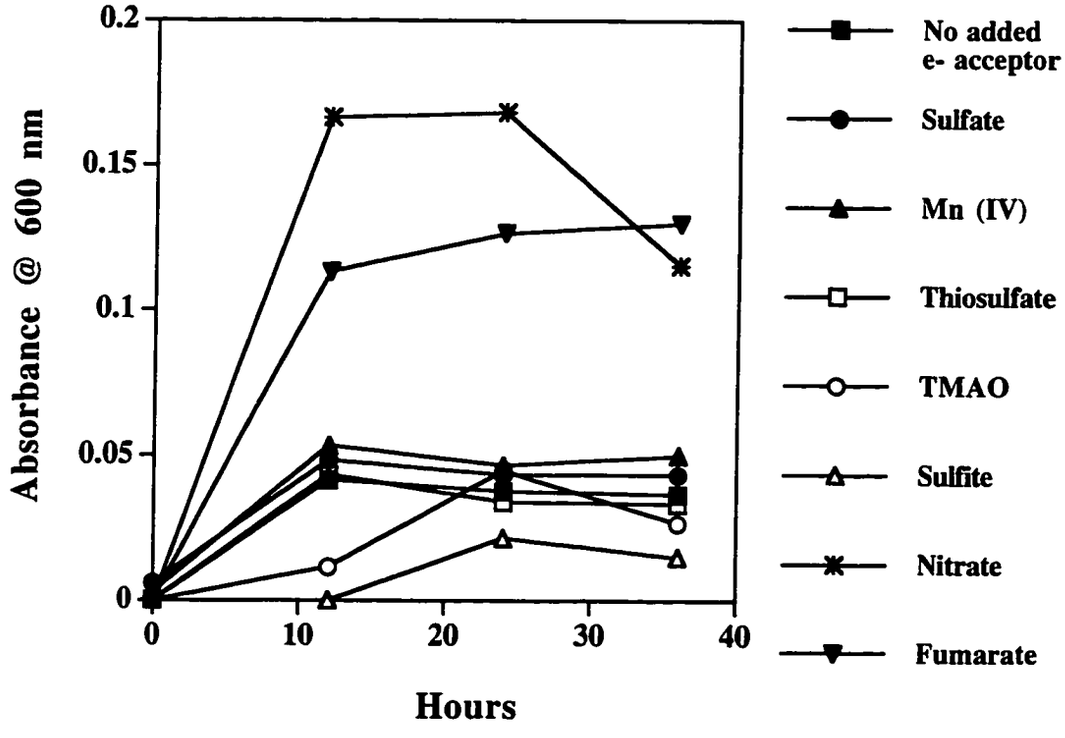

Figure 1 Anaerobic growth of *A. hydrophila* ATCC 7966 in BSM supplemented with 30 mM glycerol and alternate electron acceptors (20 mM).

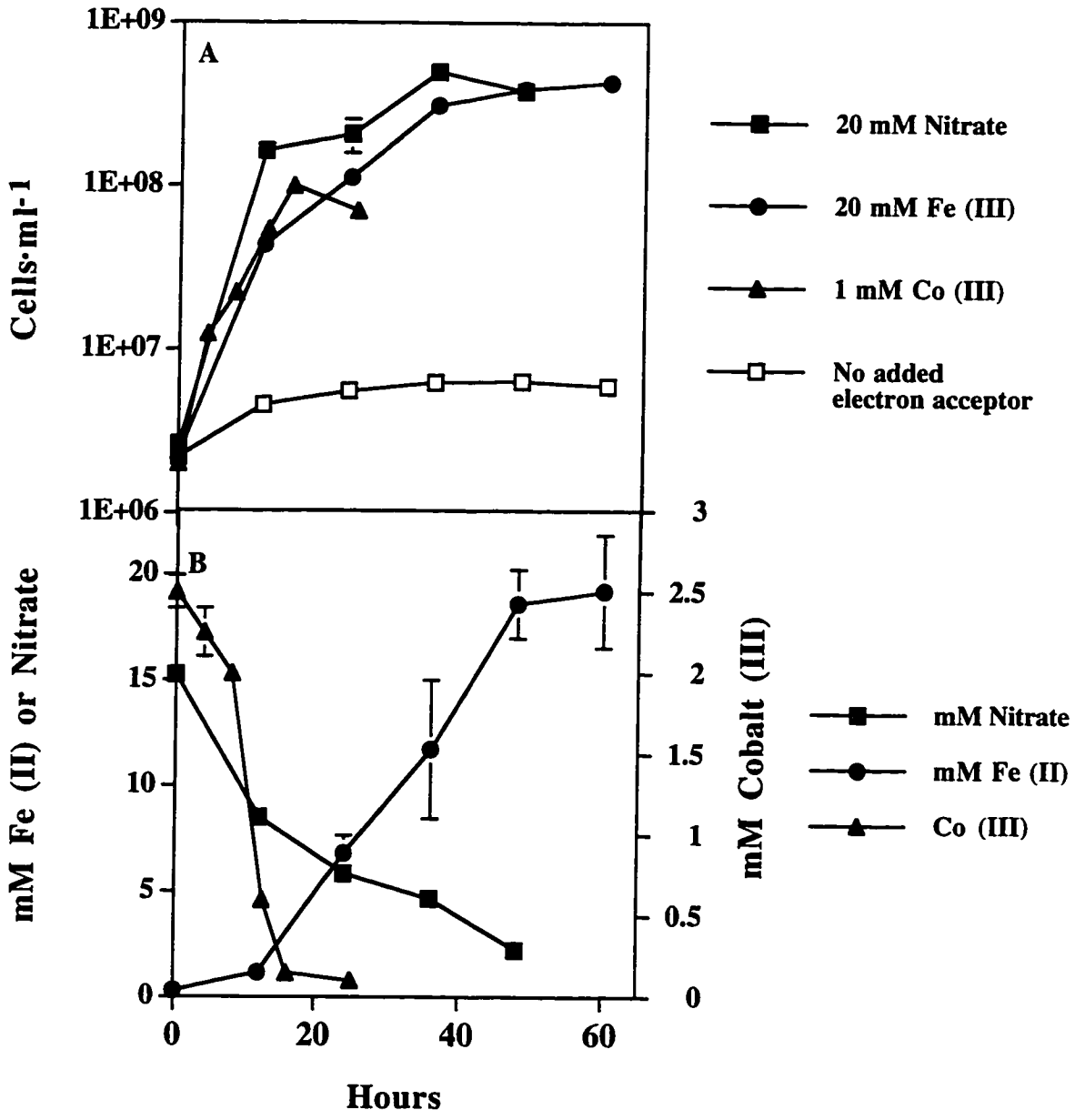

Figure 2 Anaerobic growth (A) and reduction (B) by *A. hydrophila* ATCC 7966 in basal salts medium supplemented with 30 mM glycerol and alternate electron accepetors. Electron acceptors were provided as follows: 20 mM Fe (III) citrate, 20 mM $NaNO_3$ or 2 mM Co (III)-EDTA

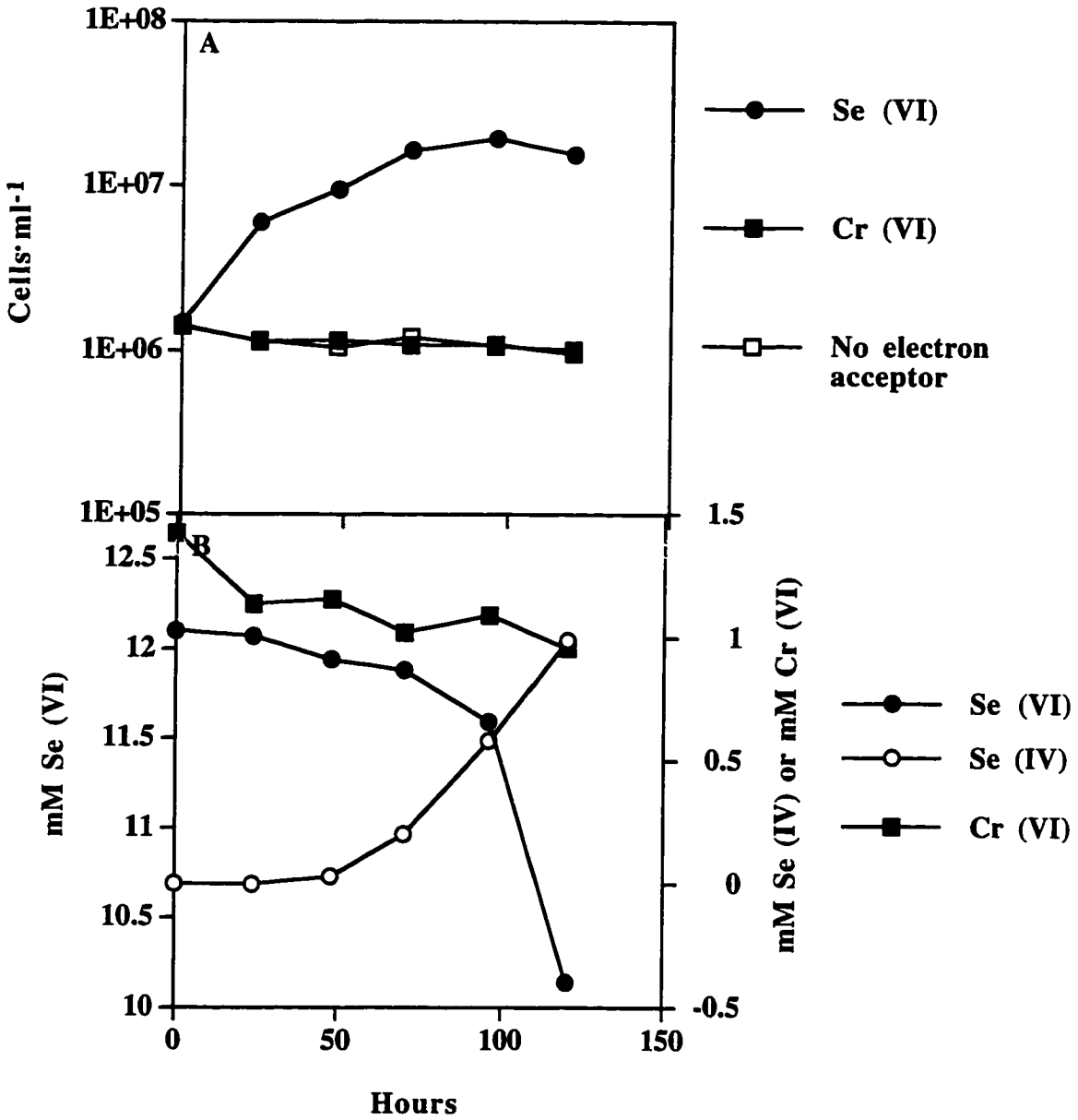

Figure 3 Anaerobic growth (A) and reduction (B) by *A. hydrophila* ATCC 7966 in BSM supplemented with 30 mM glycerol and alternate electron acceptors. Electron acceptors were provided as follows: 10 mM Se (VI) and 1 mM Cr (VI).

not occur when 20 mM unchelated Fe (III) oxyhydroxide was separated from the cells by dialysis tubing (Fig 10). Growth of the control (unchelated Fe (III) oxyhydroxide mixed with the cells) reached a final cell density of 1.2×10^8 cell· ml^{-1} after 48 hours (Fig. 10).

Extent of nitrate reduction: *A. hydrophila* ATCC 7966 reduced 12 mM nitrate within 48 hours when grown anaerobically in BSM supplemented with 30 mM glycerol and 20 mM nitrate. Nitrate reduction occurred concomitantly with growth (Fig. 2 B). The extent of nitrate reduction was directly proportional ($n = 15$, $p < 0.001$, $r^2 = 0.868$) to the amount of nitrate provided over the range 1.25-5 mM (Fig. 4 B).

Extent of iron reduction: *A. hydrophila* ATCC 7966 reduced 20 mM Fe (III) within 48 hours (Fig. 2 B) when grown anaerobically in BSM supplemented with 30 mM glycerol and 20 mM ferric citrate. The extent of Fe (II) reduced was directly proportional ($n = 15$, $p < 0.001$, $r^2 = 0.970$) to the amount of Fe (III) provided (Fig. 5 B) over the range 1.25 - 10 mM. Fe (III) reduction in BSM supplemented with 30 mM glycerol did not occur when 20 mM unchelated Fe (III) oxyhydroxide was separated from the cells by dialysis tubing (Fig 10). Cells reduced 6.8 mM Fe (III) provided as unchelated Fe (III) oxyhydroxide after 48 hours of growth (Fig. 10).

Extent of cobalt reduction: *A. hydrophila* ATCC 7966 reduced 1.2 mM Co (III) within 18 hours when grown anaerobically in BSM supplemented with 30 mM glycerol and 1 mM Co (III)-EDTA. Co (III) reduction occurred concomitantly with growth (Fig. 2 B). The amount of Co (III) reduced was directly proportional ($n = 15$, $p < 0.001$, $r^2 = 0.976$) to the amount of Co (III) provided over the range 0.5-1.5 mM (Fig. 6 B).

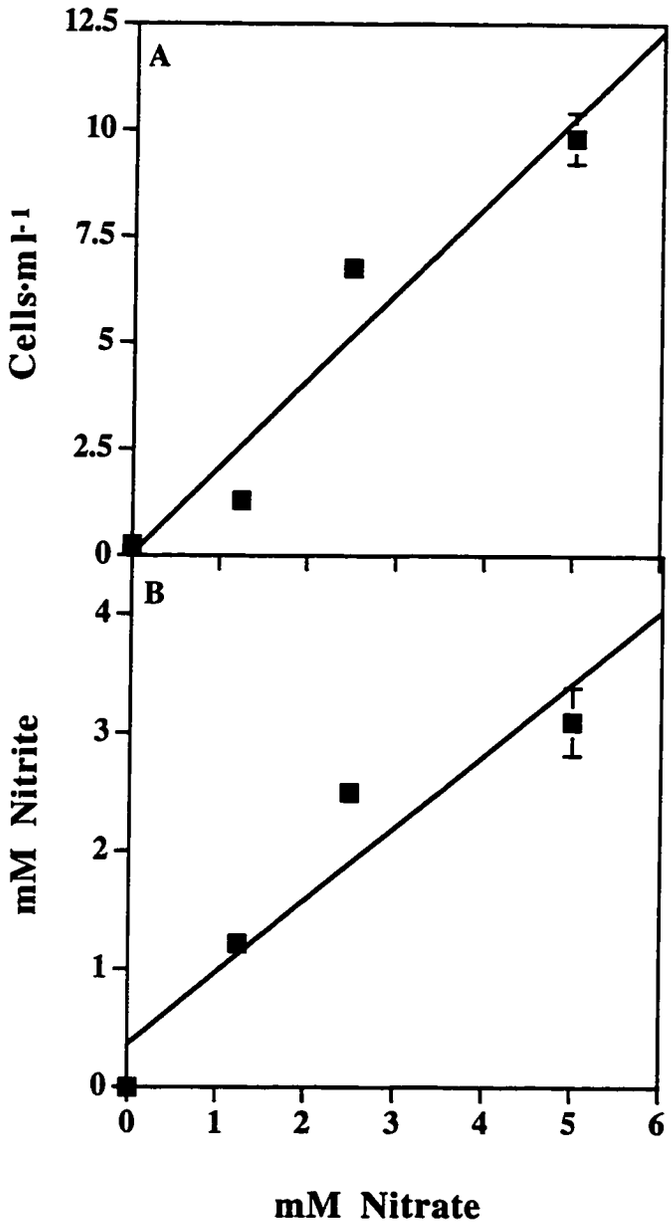

Figure 4 Relationship between final cell yield (A), nitrate reduced (B) and nitrate provided to *A. hydrophila* ATCC 7966 grown anaerobically in BSM supplemented with 30 mM glycerol.

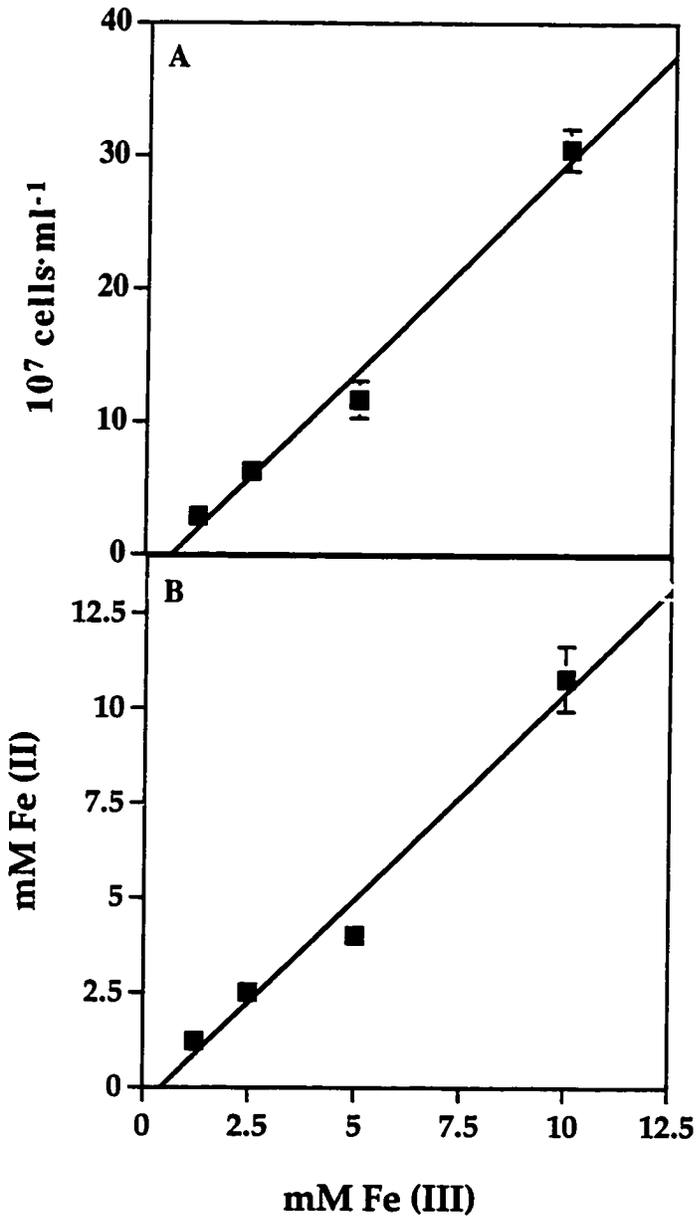

Figure 5 Relationship between final cell yield (A), Fe (III) reduced (B), and mM Fe (III) provided to *A. hydrophila* ATCC 7966 grown anaerobically in BSM supplemented with 30 mM glycerol.

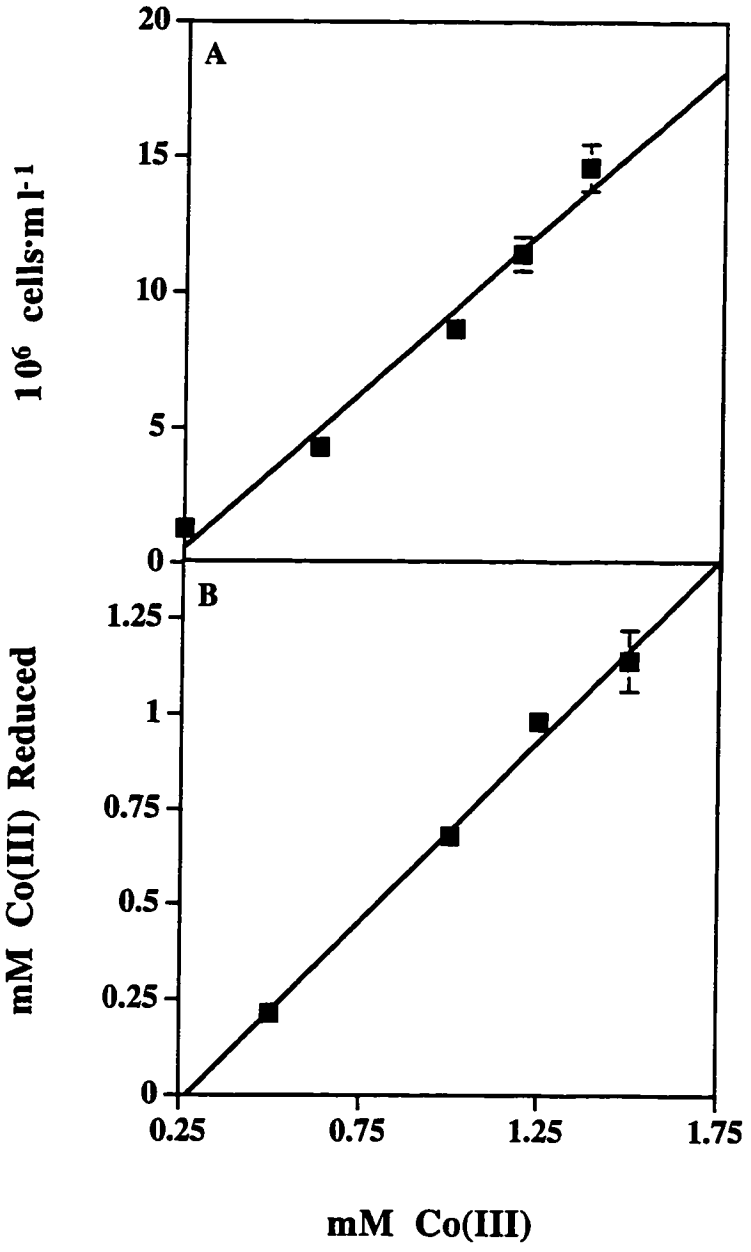

Figure 6 Relationship between final cell yield (A), Co (III) reduced (B), and mM Co (III) provided to *A. hydrophila* ATCC 7966 grown anaerobically in BSM supplemented with 30 mM glycerol.

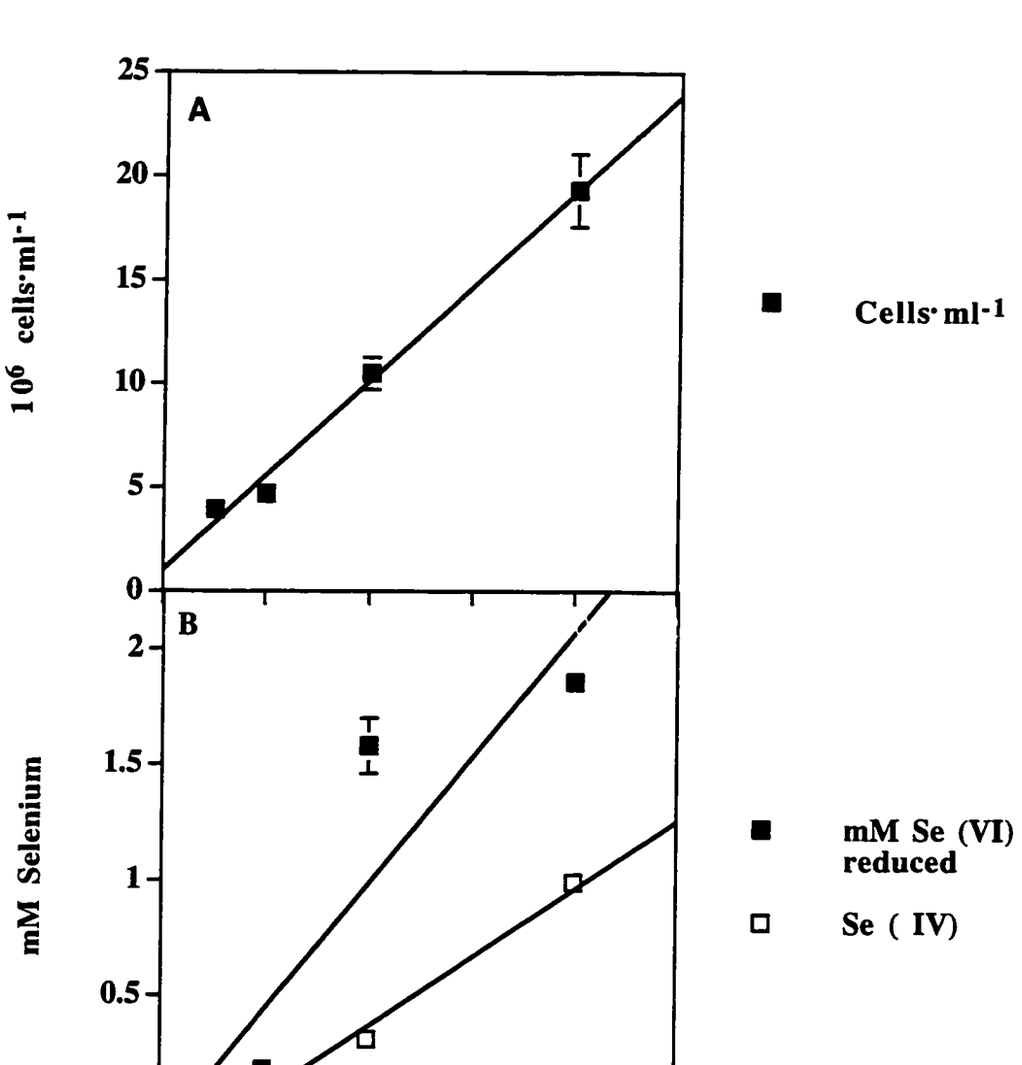

Figure 6 Relationship between final cell yield (A), Se (IV) reduced (B), Se (IV) produced (B) and mM Se (VI provided to *A. hydrophila* ATCC 7966 grown anaerobically in BSM supplemented with 30 mM glycerol.

Extent of selenium reduction: *A. hydrophila* ATCC 7966 reduced 2 mM Se (VI), and produced 1 mM Se (IV) within 120 hours (Fig. 3 B) when grown anaerobically in BSM supplemented with 30 mM glycerol and 10 mM Se (VI). The difference between these two values is elemental selenium (as evidenced by red precipitate) which is removed by filtration prior to analysis. The amount of Se (VI) reduced ($n = 8$, p 0.0042, $r^2 = 0.769$) and Se (IV) produced ($n = 8$, $p < 0.001$, $r^2 = 0.986$) were directly proportional to the amount of Se (VI) provided (Fig. 7 B) over the range 1.25 - 10 mM.

Extent of chromium reduction: *A. hydrophila* ATCC 7966 reduced 0.5 mM Cr (VI) within 120 hours (Fig. 3 B) when grown anaerobically in BSM supplemented with 30 mM glycerol and 1 mM Cr (VI).

Survey of isolates: Thirty isolates collected from Great Bay Estuary and its tributaries were examined for their ability to reduce metals. Characterization of these isolates to the species level by using conventional phenotypic biochemical characteristics revealed that 18 were *A. hydrophila*, 9 were A. *sobria* and were 3 *Aeromonas* spp. Of the 30 isolates tested 100 % were capable of nitrate reduction (97 % reduced greater 12.5 mM, 61 % reduced greater than 15 mM, and 19 % greater than 17.5 mM after 48 hours [Fig. 8 A] when grown in BSM supplemented with 30 mM glycerol and 20 mM nitrate). One hundred percent were capable of Fe (III) reduction; 26 (84%) reduced greater than 5 mM, 25 (83%) reduced greater than 10 mM and 16 % reduced greater than 15 mM Fe (III) after 48 hours (Fig. 8 B) when grown in BSM supplemented with 30 mM glycerol and 20 mM NTA-chelated ferric oxyhydroxide. All isolates reduced Co (III); 23 (74 %) reduced greater than 0.25 mM Co (III), 14 (45 %) reduced greater than 0.5 mM Co (III), and 44 % reduced greater than 0.75 mM Co (III) within 24 hours (Fig. 8 C). Forty three percent of isolates Se (VI); 8 (26%) reduced greater than 0.5 mM, 5 (16%) reduced greater than 1 mM, and 1 (MP31*)

reduced greater than 2 mM Se (VI) within 120 hours.

Electron donors utilized: *Aeromonas hydrophila* 7966 reduced Fe (III) citrate with glycerol, succinate, and lactate, producing 11.1, 3.13, and 1.02 mM Fe (II) respectively, after 72 hours (Table 1). A small amount of reduction occurred with H_2 (0.51 mM). Concentrations of reductants used and the amounts of Fe (II) produced are shown in Table 1. The extent of Fe (II) reduction was normalized (to cells·ml^{-1}) to allow comparison between substrates. Reduction occurred with pyruvate (3.41 mM). However, this is presumably due to acid production (the pH of spent culture medium was 4.8) by fermentation because significant growth occurred in BSM without the addition of ferric iron (data not shown).

Temperature effects: Final cell yield and the extent of Fe (III) reduction by *A. hydrophila* ATCC 7966 grown in BSM supplemented with 30 mM glycerol and 20 mM Fe (III) citrate increased in direct proportion to temperature over the range 15-37 °C with a sharp decrease at 45 °C. The Q_{10} (effect of temperature) was calculated for the amount (mM) of Fe (III) reduced per hour between 15 and 25 °C, 25 and 37 °C, and 37 and 45 °C and were 3.1, 2.17, and 139, respectively.

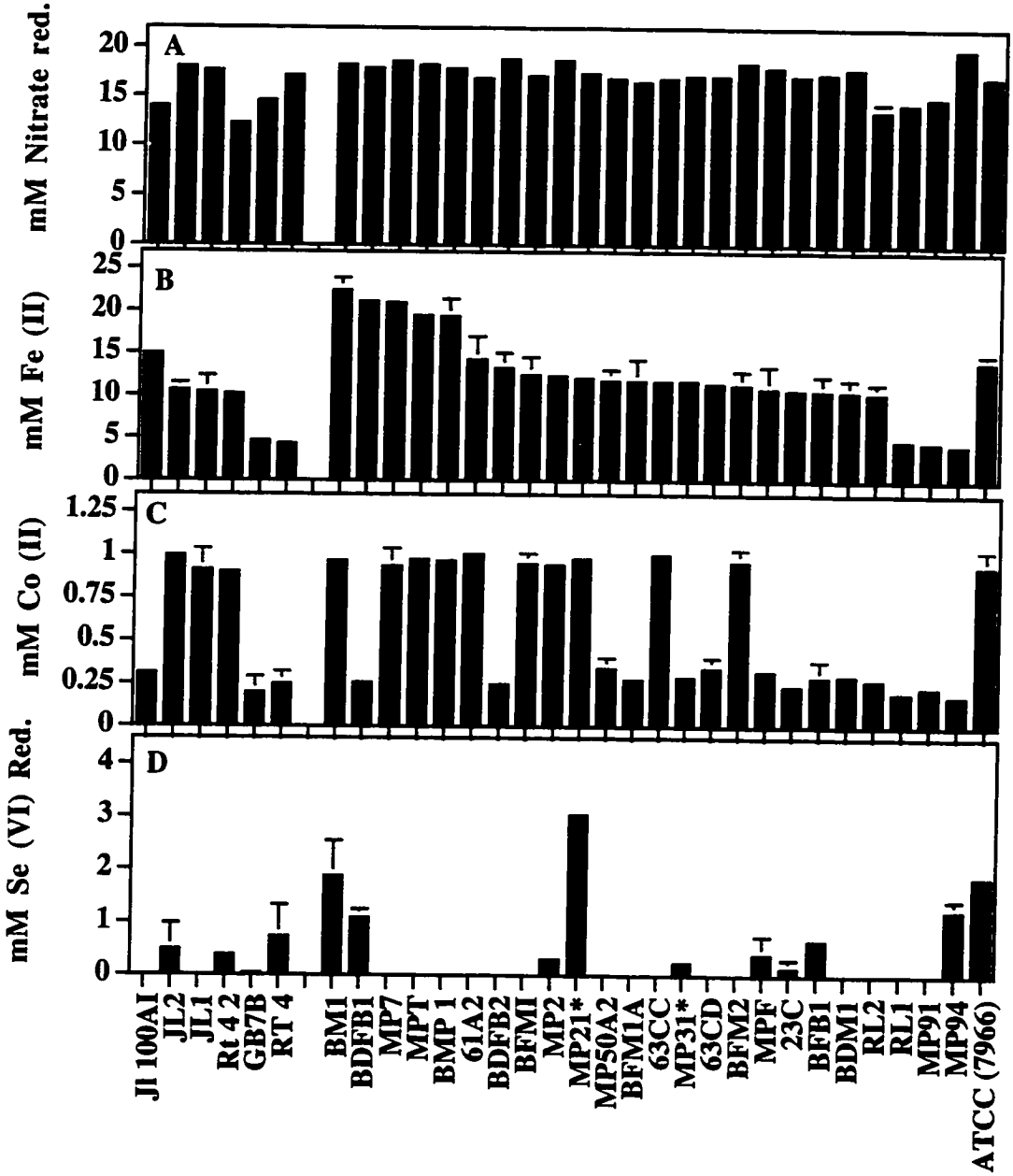

Figure 8 Metal reduction by aeromonad isolates grown anaerobically in BSM supplemented with 30 glycerol and alternate electron acceptors as follows: (A) 20 mM nitrate; (B) 20 mM Fe (III)-NTA; (C) 2 mM Co (III)-EDTA; and (D) 10 mM Se (VI). Results are the extent of reduction after incubation times as follows: nitrate, 48 hours; Fe (III), 60 hours; Co (III), 24 hours; and Se (VI), 120 hours.

Oxidant	Amount provided (mM)	Fe (II) produced (mM)
Acetate	15	0
Caproate	5	0
Ethanol	15	0
Formate	15	0
Glycerol	15	11.1 ± 0.6
Hydrogen	5 cc	0.5 ± 0.1
JP-8[a]	6.25×10^{-4} ml·ml^{-1} (v/v)	0
Lactate	15	1.0 ± 0.1
Malate	5	0
Methanol	5	0
Phenol	0.5	0
Pyruvate	15	3.4 ± 0.7
Succinate	15	3.1 ± 0.5
Toluene	0.5	0

[a] Jet fuel

Table 1 Range of oxidants used as substrates for Fe (III) reduction by *A. hydrophila* ATCC 7966. Values are the extent of Fe (III) reduction after 72 hours growth. Each sample was adjusted for both cell numbers and effect of blank to allow comparison.

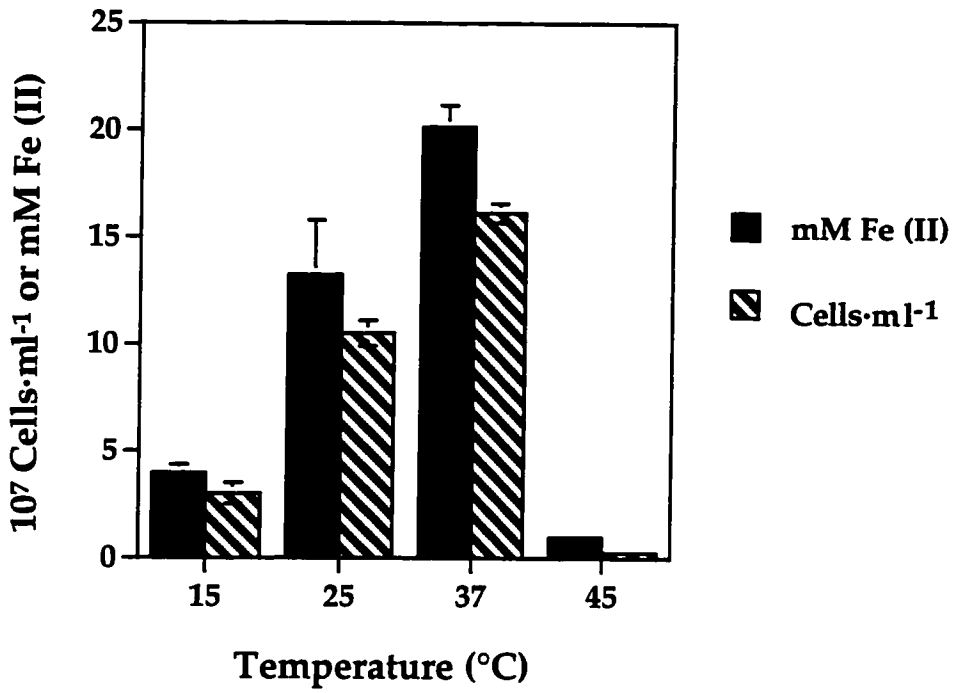

Figure 9 Relationship between incubation temperature, final cell yield and Fe (III) reduction by *A. hydrophila* ATCC 7966 grown anaerobically in BSM supplemented with 30 mM glycerol and 20 mM Fe (III) citrate.

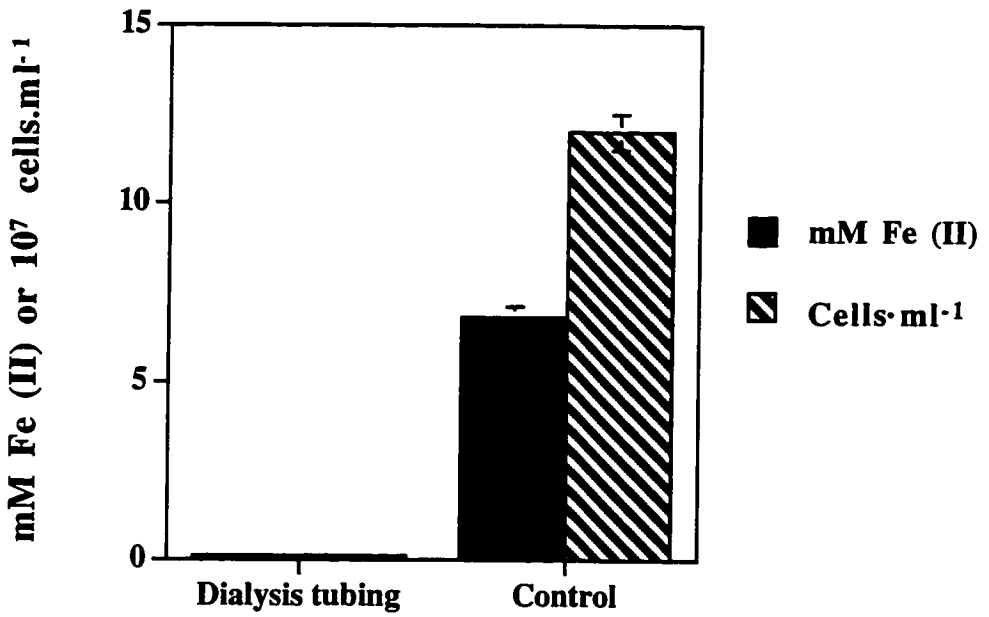

Figure 10 Final cell yield and Fe (III) reduction after 48 hours by *A. hydrophila* ATCC 7966 grown in BSM supplemented with 30 mM glycerol and 20 mM Fe (III) oxyhydroxide (unchelated) separated from the cells by dialysis tubing. The control Fe (III) oxyhydroxide mixed with the cells.

DISCUSSION:

Mesophilic aeromonads are facultative anaerobes that are ubiquitous in aquatic habitats. They have previously been known to grow aerobically or anaerobically by fermentation (18). We now report that they carry out anaerobic respiration with nitrate, Fe (III), Co (III), Se (VI), or fumarate. Thirty mesophilic aeromonads were isolated from Great Bay Estuary and its tributaries (Chapter 1). These isolates were collected by using established selective and differential plating techniques for mesophilic aeromonads (Chapter 1): not by enrichment for metal reducers or anaerobic respirers.

Fe (III) reduction was observed by all thirty isolates with 83% reducing greater than 10 mM Fe (III) within 48 hours. To avoid problems associated with Fe (III) reduction due to pH or Eh effects, we used the non-fermentable energy source glycerol. Initial studies with amorphous Fe (III) oxyhydroxide gave poor growth and reduction. By chelating the iron with citrate, EDTA, or NTA increases in both final cell yields and the extent of reduction were obtained (data not shown). Although iron in sediments often exists as poorly crystalline iron oxides (3, 11), use of this form of iron by metal reducers often depends upon their close association with the oxide precipitates and may depend upon their ability to chelate iron (3, 11). For studies with *A. hydrophila* ATCC 7966, Fe (III) oxyhydroxide was replaced by Fe (III) citrate. It was determined that this isolate did not grow by fermentation of citrate. Growth and iron reduction by aeromonad isolates was tested in BSM supplemented with ferric oxyhydroxide chelated with equimolar NTA. Fe (III) reduction by *Aeromonas* is dissimilatory in nature: growth and Fe (III) reduction occurred concomitantly, and no growth occurred without the addition of Fe (III). In addition, the extent of iron reduction and final cell yields were directly proportional to the

quantity of Fe (III) supplied. Iron reduction was enzymatic as evident from several observations: the quantity of iron reduced increased with temperature with a sharp decline at 45 °C (Q_{10} from 37 to 45 °C was calculated to be 139); no reduction occurred when ferric oxyhydroxide (unchelated) was separated from the cells by dialysis tubing, and the pH throughout growth remained circumneutral at 6.74. For reductant experiments *A. hydrophila* 7966 was inoculated into BSM with reductant, but lacking electron acceptor. This allowed the cells to adapt to the energy source prior to initiating each test with approximately the same cell density. At the end of 72 hours of growth the amount of Fe (II) produced was standardized to cell number to allow comparison between tests. Although this organism appeared to use diverse terminal oxidants, the range of reductants used was minimal. Of the substrates tested, reduction occurred with glycerol, and succinate and to a lesser extent with lactate. The Fe (III) reduction observed with pyruvate was due to fermentation of this substrate as evidenced by significant growth in controls lacking Fe (III).

Reduction of Fe (III) provides many possibilities for anaerobic bioremediation of complex organic molecules. Most waste in need of bioremediation is degraded under anoxic conditions. Current bioremediation strategies require pumping oxygen through the water or soil which stimulates aerobic decomposition but is costly. Anaerobic degradation of complex organic molecules coupled to Fe (III) reduction has been reported by several Fe (III) reducers (21, 28). In addition, the end product of Fe (III) reduction, unlike those of other potential electron acceptors, are innocuous and environmentally benign minerals such as siderite, vivianite and magnetite (27, 39). More importantly, Fe (III) is ubiquitous in nature and often found at mM concentrations as required to be of use as a terminal oxidant (27, 39, 48). Iron reduction in estuarine habitats is directly related to microbial activities and bioturbation (19). Microbial reduction has been previously documented in Great Bay sediments and appears to play a significant role in biogeochemical cycling of this metal (19,

53).

These data also show that mesophilic aeromonads can grow by means of dissimilatory selenate reduction. In one previous report, aeromonads were isolated as part of a large group of organisms from a selenite contaminated site (5). However, in that study only the resistance of these organisms to selenite and not growth rates were examined (5). Dissimilatory reduction of selenium is a trait not shared by many known metal reducers. At this time, only two organisms are known to grow in this manner (33, 44). *Shewanella alga* (Chapter 4) is able to grow in a dissimilatory manner with Co (III), Ur (VI), Mn (IV), thiosulfate, nitrate, TMAO or fumarate (6), however, it cannot grow using Se (VI) as a terminal oxidant (16). Selenium is often overlooked as a potential electron acceptor because the concentrations found in the natural environment are often minimal (μM). However, in areas of selanous rock such as the western coast of the United States (12) selenium concentrations in the ground water can often reach toxic concentrations (300 $\mu g \cdot ml^{-1}$). The San Joaquin valley, CA is a unique environment, high selenate concentrations coupled with intense irrigation of this important agricultural region often yield toxic selenate concentrations in ground water (12, 44). Excess irrigation waters are often collected in large irrigation ponds for evaporation which allows concentration of the indigenous minerals. As in the case of the Kesterson reservoir, selenate concentrations had a profound effect on the wildlife which resulted in high mortality of the indigenous birds. Chemical reduction requires the addition of strong oxidants which themselves are equally hazardous to the environment (12). Microbial transformation of selenium appears to be a novel solution. Dissimilatory selenate reduction results in precipitation of non-toxic elemental selenium. This allows for selenium collection and can be exploited as a means of bioremediating contaminated sites (12). Macy (1993) designed a bioreactor using the selenate respirer *Thauera selenatis* (33). The nature of this organism which requires concomitant denitrification in order to reduce Se (VI) beyond Se (IV) essentially solved two

problems. The irrigation waters also contained high concentrations (50 ppm) of nitrate (33) due to anthropogenic input. This reactor decreased both the toxic Se (VI), and nitrate concentrations in the H_2O (33).

Cobalt (III) is found in low concentrations in most environments. However, at the sites of many nuclear reactors in which ^{60}Co (III) is formed as a byproduct in the production of radionuclides this toxic metal is a very real problem (15). Periodically the inside of the reactors are cleaned with chelators such as EDTA is problematic because the Co (III) forms a strong, waters soluble complex with the EDTA. This promotes spreading od this toxic metal and hinders containment. The product of reduction, Co (II) however, forms a weak complex with the chelator and therefore is no longer water-soluble. Although this does not detoxify the metal it does allow containment. The use of anaerobic respiration by microbes with Co (III) has only recently been noted and previous to this study only two metal reducers *D. sulfurreducens* (7) and *Shewanella alga* (which was isolated previously in our laboratory from Great Bay Estuary; 6, 16), were known to grow with Co (III) as the sole terminal acceptor. Co (III) reduction by aeromonas adds to the limited number of cobalt reducers and extends the range of alternate electron acceptors used by this genus.

Chromium contamination of the environments is extensive as a result of mining operations (27). Reduction of soluble Cr (VI) to insoluble Cr (IV) is likely to be useful in remediating contaminated waters and soils (27). Chromium reduction has been previously reported by a novel Cr (VI)-reducing aeromonad *A. dechromatica.* (8) However, the reduction of small quantities and low growth rates observed are suggestive of developing resistance to the toxicity of this metal. We obtained similar results with *A. hydrophila* ATCC 7966. Aerobic Cr (VI) reduction has been reported by many organisms (27) including *A. hydrophila* (9) however, in no case has this been coupled to anaerobic growth (27).

Molar growth yields were calculated to be $2.6 \cdot 10^4$, $2.9 \cdot 10^4$, $1.4 \cdot 10^4$, and $1.1 \cdot 10^4$ cells·mole acceptor reduced^{-1} for Fe (III), nitrate, Co (III), and Se (VI), respectively. Although these results were calculated with cell numbers and not dry weights they do provide information about growth yields on these various oxidants. Growth on Se (VI) would be expected to yield a greater cell yield per mole because each mole of selenate reduced results in a six 6 electron transfer (compared to the 1 electron transferred for the other acceptors used). The growth yield on this substrate however, was the lowest of all acceptors tested. Evaluation of the redox potentials of each oxidant provide an explanation for this observed growth yield. The initial reaction from Se (VI) to Se (IV) conserves energy for growth however, the second reaction from Se (IV) to elemental selenium requires energy. It is thought that the second reaction occurs as a detoxification strategy and without removal of the toxic end product Se(IV) growth could not continue.

Thirty mesophilic aeromonads isolated from Great Bay Estuary were evaluated for their ability to reduce metals and metalloids. Of these two isolates (RT 4, and BDFB1) exhibited significant reduction of each acceptor tested. Aeromonad isolates were collected by selective and differential plating techniques, and not by enrichment cultures for metal reducers. This suggests that metal reduction is a trait shared by many members of this genus. However, it does appear that several isolates were consistently "better" reducers. Differences between these isolates and their less active counterparts warrants further investigation and may shed light on the physiology of this interesting genus.

Dissimilatory metal reduction by mesophilic aeromonads has not previously been reported. Their role as facultative pathogens of fish, amphibians, and humans have been well documented (2, 22), but little is known with regard to their ecological niche. Mesophilic aeromonads may be quite active in iron reduction in the changing conditions within the estuary because they are found ubiquitously in both freshwater and brackish habitats. A separate study of the seasonal distribution of aeromonads in Great Bay Estuary

(Chapter 1) showed that these bacteria can be isolated throughout the year with highest numbers occurring in the late summer months. Our findings add to the limited number of genera known to be capable of dissimilatory metal metabolism and suggest a means for enhancing the survival of this important aquatic bacterium in anaerobic aquatic habitats.

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CHAPTER THREE

CELL MECHANISM OF DISSIMILATORY FE (III) REDUCTION BY *A. HYDROPHILA* ATCC 7966.

ABSTRACT:

Aeromonas hydrophila ATCC 7966 was grown in batch culture aerobically (O_2 as terminal electron acceptor), and anaerobically with Fe (III) citrate, or nitrate as the terminal acceptor. Extracts of cells grown anaerobically with Fe (III), reduced ferric iron, at a rate of 116 nmol·min^{-1}·mg protein^{-1}. Extracts of cells grown aerobically (O_2 as terminal acceptor) with Fe (III), or anaerobically with nitrate, reduced Fe (III) at 95% and 63.5% respectively, of this rate. Low background or endogenous rates (approximately 60 nMol·min^{-1}·mg protein^{-1}) of reduction occurred with heat killed cell extracts. Membrane and cytosol fractions of *A. hydrophila* ATCC 7966 reduced Fe (III) at 108 nmol·min^{-1}·mg protein^{-1} and 24 nmol·min^{-1}·mg protein^{-1}, respectively. This is consistent with a membrane associated ferric reductase. Dithionite-reduced minus air-oxidized absorption spectra obtained from washed, whole cells cultured aerobically or anaerobically (in medium containing 20 mM ferric iron), or anaerobically with 20 mM nitrate exhibited maxima at wavelengths (420, 522, and 553 nm) characteristic of *c*-type hemoproteins. Hydrogen-reduced cytochromes from cells grown aerobically or anaerobically with Fe (III) were oxidized by Fe (III) or nitrate. Hydrogen-reduced cytochromes from cells grown

anaerobically with nitrate were oxidized by nitrate, but not by Fe (III). Azide (1 mM) completely blocked oxidation of cytochromes by Fe (III) or nitrate in cells grownaerobically with Fe (III). This was also true of cells cultured anaerobically with nitrate. In contrast, azide had no detectable effect on cells grown anaerobically with Fe (III). Fe (III) reduction by cells cultured under all three growth conditions was inhibited by as much as 80% of the control (no inhibitor) by μM concentrations of: quinacrine, dicumarol, HOQNO, or CCCP, and to a lesser extent by azide. Rotenone was without effect. These results are consistent with electron transport-mediated Fe (III) reduction in *A. hydrophila* cells, in which reducing equivalents entering the respiratory chain at FAD dehydrogenase subsequently pass from quinones via cytochromes to a single iron reductase.

INTRODUCTION:

The chemisomotic theory of Mitchell (1961) proposes that reducing equivalents (NADH, FADH) enter the respiratory chain via a (pyridine nucleotide or flavins) dehydrogenase and are subsequently passed among carriers vectorially arranged in the respiratory membrane. These electron transport components include non-heme iron sulfur proteins, quinones, cytochromes, and finally a terminal oxido-reductase (1, 14). Electron transport supports an electrochemical proton motive force which is coupled to energy conservation as ATP via a membrane-bound ATPase (14). In the absence of oxygen, diverse bacteria may use a variety of alternate terminal electron acceptors including nitrate, nitrite, sulfate, sulfite, thiosulfate, fumarate, trimethyl amine N-oxide (TMAO) or metal ions (8, 18). Components of the associated respiratory chains have been elucidated by the use of respiratory inhibitors (6). Inhibitor studies with the dissimilatory Fe (III) reducer *Pseudomonas ferrireducens* strain 200 revealed two distinct dissimilatory ferric reductases. A constitutive reductase was produced during both aerobic and anaerobic growth and appeared to be involved in electron transport with Fe (III) as the terminal electron acceptor. A second reductase, induced in cells only under low oxygen growth, appeared to act primarily as an electron sink (1). Fe (III) reduction by *Escherichia coli* K12, an organism which is not a dissimilatory iron reducer, does not involve an electron transport chain (26). Pleiotropic mutants of *E. coli* K12 which lacked cytochromes or quinones expressed Fe (III) reduction rates similar to those of the parent strains (26). In addition, cyanide, an inhibitor of the terminal cytochrome oxidase, and ATP, each had stimulatory rather than inhibitory effects on Fe (III) reduction. Cell extracts of *E. coli* K12 grown anaerobically with nitrate reduced Fe (III) at 25% (56 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$) of the rate of extracts of

the well known dissimilatory Fe (III) reducer *Geobacter metallireducens* (see below) strain GS-15 (210 nmol·min^{-1}·mg protein^{-1}, 5). Moreover, the Fe (III) reductase of *E. coli* K12, unlike those of dissimilatory Fe (III) reducers, is a soluble enzyme and is more likely to be involved in the release of Fe (III) from siderophores (26).

Experimental approaches such as the use of respiratory inhibitors and generation of pleiotropic respiratory mutants have provided strong evidence for the role of cytochromes in electron transport to Fe (III) as the terminal electron acceptor in diverse bacteria (1, 18, 25). All known dissimilatory Fe (III) reducers with one exception (9), contain *C*-type cytochromes (8, 9, 15, 20, 24). Mutants lacking *c*-type cytochromes are not capable of Fe (III) reduction (25). The use of 2-heptyl-4-hydroxy quinoline N-oxide (HOQNO), which blocks the transfer of electrons from cytochromes of type *b* to those of type *c* (6), almost completely inhibited Fe (III) reduction in *P. ferrireducens* strain 200 (1). Total cytochrome content (*b*- and *c*-type) was directly correlated to the amount of ferric reductase activity (20) and cells starved for Fe (III) lost both their pigmentation and their ability to reduce iron (20). Quinones also appeared to be important in electron transport with Fe (III). The addition of menaquinone to fumarate-, nitrate- or ferric-reductase mutants of *P. ferrireducens* strain 200 restored their ability to use these electron acceptors (15).

The effect of nitrate on Fe (III) reduction has been the subject of numerous studies (4, 19, 21) since Ottow (1970) demonstrated the inhibitory effect of nitrate on Fe (III) reduction by a number of organisms (22). In an attempt to determine the mechanism of this reduction, Ottow isolated nitrate reductase-less mutants (23). The extent of Fe (III) reduction by cultures of these mutants was less than that of the parent strains. However, in the presence of nitrate, Fe (III) reduction by the mutants did not decrease, suggesting that nitrate and Fe (III) were reduced by the same enzyme. Obuekwe and Westlake (1981) reported nitrate inhibition of Fe (III) reduction in cultures incubated for long periods of time (21); during short term incubations, rates of Fe (III) reduction were stimulated by nitrate

(21). It was proposed that the long term net decrease in Fe (III) reduction was, in fact, due to chemical oxidation of Fe (II) by nitrite produced from nitrate. *S. putrefaciens* MR-1 grown aerobically or microaerobically, reduced nitrate, nitrite, or Fe (III) by using three distinct reductases (4). Fe (III) reduction by a novel ferric reductase identified in *S. putrefaciens* MR-1 was unaffected by the addition of nitrate, nitrite, fumarate or TMAO (17). The nitrate reductase of the obligate anaerobe *G. metallireducens* strain GS-15 was produced only when cells were grown on nitrate (5); not on Fe (III). In addition, the rate of Fe (III) reduction by cell extracts of GS-15 grown anaerobically with nitrate was 50 % of that of cells cultured anaerobically with Fe (III) (5). These findings suggest a high degree of variability among bacteria in the manner in which they may use Fe (III) as a terminal oxidant as well as variation in the regulation of this process.

We previously reported dissimilatory Fe (III), Se (IV), and Co (III) reduction by mesophilic aeromonads (7, Chapter 3). Members of the genus *Aeromonas* are gram negative, facultatively anaerobic, oxidase-positive rods, that are indigenous to freshwater and marine habitats. Little is known concerning the ecology of this opportunistic pathogen. Anaerobic respiration with metals could be important in survival of *Aeromonas* in anoxic water and sediments. To better understand the role of metals in the biology of *A. hydrophila* , we have investigated the mechanism for dissimilatory Fe (III) reduction by cultured cells and cell extracts. Although thirty mesophilic aeromonads (*A. hydrophila, and A. sobria)* were recently isolated (Chapter 1) from habitats proximal to Durham were dissimilatory Fe (III) reducers, we have focussed our energy metabolism studies on *A. hydrophila* ATCC strain 7966.

MATERIALS AND METHODS:

Growth: *A. hydrophila* ATCC 7966 was grown in Basal Salts Medium (BSM) which consisted of the following ($g \cdot l^{-1}$): $NaHCO_3$, 2.5; NaH_2PO_4, 0.6; NH_4Cl, 1.5; KCl, 0.1; yeast extract, 0.1; Wolfe's Vitamins (27), 10 ml; Wolfe's Minerals (27), 10 ml. The medium was supplemented with 20 mM Fe (III) citrate and 30 mM glycerol unless otherwise noted. Nitrate medium was BSM lacking ferric citrate and supplemented with 30 mM glycerol plus 20 mM $NaNO_3$. For anaerobically grown cells, BSM was prepared by using standard anaerobic technique (13). The medium was boiled and cooled under a stream of N_2:CO_2 (80:20) which had been passed over hot copper to remove traces of oxygen. Cells were also grown aerobically with shaking at 200 rpm. All cultures were incubated at the optimal temperature of 37 °C unless otherwise noted.

Terminal electron acceptor competition studies: *A. hydrophila* ATCC 7966 was grown in BSM aerobically with Fe (III), or nitrate, or anaerobically with nitrate (Fig. 1 A). Cells were collected by centrifugation at 10,000 x G for 10 min, washed and resuspended in 10 mM HEPES (N-2-Hydroxyethyl piperazine-N-2-ethane sulfonic acid) buffer (pH 7). Cells were sparged with N_2 for 10 min and added (to a final cell density of 1.35×10^{10} $cells \cdot ml^{-1}$) to 10 ml 30 mM bicarbonate buffer (pH 7), supplemented with 5 mM Fe (III) and 5 mM glycerol (Fig. 1 A). After 12 hours, Fe (II) was measured by using ferrozine. To determine the effect of nitrate or nitrite on Fe (III) reduction, terminal electron acceptor competition studies were performed. Cells were grown anaerobically with Fe (III) citrate, collected by centrifugation at 10, 000 x g for 10 min, washed twice and resuspended with in 10 mM HEPES buffer (pH 7). The washed cells were added (to a final cell density of

1.35 x 10^{10} cells·ml^{-1}) to 30 mM bicarbonate buffer containing 5 mM Fe (III) and 5 mM glycerol (electron donor) to which 5 mM nitrate, or 1 mM nitrite were added (Fig. 2 A).

Fe (III) reductase: *A. hydrophila* ATCC 7966 was grown anaerobically in BSM supplemented with 30 mM glycerol and either 20 mM Fe (III) citrate or 20 mM $NaNO_3$ for 16 hours at 37°C. Cells were collected by centrifugation at 10,000 x g for 10 min, washed twice and resuspended in 10 mM HEPES buffer (pH 7). Cells were broken by using a French pressure cell (3 passages at 15,000 psi). For the separation of membrane and cytosol fraction, cell extracts were centrifuged for 10 min at 10, 000 x g to remove unbroken cell. Cell extracts were then centrifuged at 200,000 x g to collect the membrane fraction. Fe (III) reductase activity was measured as the accumulation of Fe (II) by a modification of the assay of Dailey and Lascelles (3) as previously described (16). The assay was performed in anaerobic cuvettes (1 cm path length; TCS-Medical products Co., Huntington Valley PA). The assay mix (2 ml total volume) consisted of the following: glycerol, 10 mM; Fe (III) citrate, 0.12 mM; ferrozine, 0.4 mM; KPO_4 buffer (pH 7.5), 28 mM; and approximately 0.5 mg cell extract protein. Accumulation of Fe (III) was measured by following the absorbance at 562 nm by using a Beckman DU-640 spectrophotometer (Beckman Fullerton, CA). A molar extinction coefficient of 28,000 was used (3).

Cytochrome spectra: *A. hydrophila* ATCC 7966 was grown for 16 hours in BSM aerobically or anaerobically with 20 mM Fe (III) citrate or anaerobically with 20 mM $NaNO_3$. Cells were collected by centrifugation at 10,000 x g for 10 min, washed twice and resuspended in 10 mM HEPES buffer (pH 7) to a final cell density of $5.4.10^{11}$ cells·ml^{-1} (1.25 mg $protein^{-1}$·ml^{-1}). Dithionite-reduced minus air-oxidized absorption spectra were collected as previously described by others (2). To determine the reoxidation

of cytochromes by Fe (III) or nitrate, cell cytochromes were reduced with hydrogen prior to adding Fe (III) or nitrate. Cells placed in a Thunburg cuvette with 500 µl of 5 mM Fe (III) citrate or 5 mM nitrate in the side arm were sparged with a steady stream of hydrogen for 5 min. The contents of the side arm were mixed with the cells immediately before measuring spectra in a Beckman DU-640 spectrophotometer (Beckman, Fullerton, CA). The control contained 500µl 10 mM HEPES buffer in the side arm. Azide was added from concentrated stock to a final concentration of 1×10^{-3} M.

Inhibitor Studies: *A. hydrophila* ATCC 7966 was grown in BSM containing 30 mM glycerol and 20 mM Fe(III) citrate, either aerobically or anaerobically for 16 hours at 37°C. For nitrate grown cells, the BSM contained 20 mM $NaNO_3$ in lieu of ferric citrate. Cells were collected by centrifugation at 10,000 x g for 10 min, washed twice in, and resuspended in 10 mM HEPES buffer (pH 7). Cell suspensions were sparged with N_2:CO_2 (80:20) for 10 min and dispensed in triplicate into 10 ml 30 mM bicarbonate buffer (pH 7) to a final concentration of 1.35×10^{10} cells·ml^{-1} . These were each supplemented with 5 mM glycerol and respiratory inhibitors were added as shown in Table 1. Controls were used to determine the effect of solvents. Cell suspensions were incubated for 10 min with the appropriate respiratory inhibitor prior to the addition of 5 mM Fe (III) citrate to start the reaction. After incubation for 12 hours at 37°C, the concentration of Fe (II) was determined. Percent inhibition over that of cells without respiratory inhibitor was determined after correcting for controls (reagent blank and solvent control lacking inhibitor).

Analytical techniques: HCl-extractable Fe (II) was determined by using ferrozine as previously described (11). Ferrous ethylene diamine sulfate was used as a standard. Cell numbers in cultures grown with Fe (III) were determined by epifluorescence

microscopically following acridine orange staining as previously described (12). Protein concentration was determined by using the BCA assay (Pierce Chemical Co., Rockford IL); a colormetric modification of the biuret reaction.

Inhibitor	Inhibition Site	Concentration (M)	Solvent[a]
Quinacrine	(Flavins) dehydrogenase	1×10^{-4}	H_2O
Rotenone	NADH dehydrogenase	1×10^{-4}	Acetone
Dicumarol	Quinones	1×10^{-5}	0.05 N NaOH
HOQNO[b]	Cytochrome b	1×10^{-5}	Ethanol
Azide	Terminal oxidase	1×10^{-3}	H_2O
CCCP[c]	Protonophore	1×10^{-5}	Ethanol

[a] Inhibitors were dissolved in minimal solvent and inhibition due to solvent was subtracted from samples.
[b] 2-heptyl-4-hydroxyquinoline *N*-oxide.
[c] Carbonyl-cyanide-*m*-chlorophenylhydrazine. Disrupts the proton motive force.

Table 2 Concentration and sites of inhibition of respiratory inhibitors.

RESULTS:

Culture history and iron reduction by washed cells: Culture history affected the extent of cell Fe (III) reduction. Suspensions of washed cells previously grown aerobically with 20 mM Fe(III), reduced 80 % of the amount of Fe (III) reduced by cells cultured anaerobically with iron (Fig. 1 B). Cells previously grown anaerobically with 20 mM nitrate reduced 18 % of the Fe (III) reduced by cells cultured anaerobically with iron (Fig. 1 B). Washed cells previously grown aerobically with 20 mM nitrate reduced 24% of the amount of Fe (III) reduced by cells cultured anaerobically with iron (Fig. 1 B). The amount of Fe (III) reduced by washed cell suspensions provided with nitrate or nitrite was 24% and 12% respectively, (Fig. 2 B) of that of cells without the addition of alternate electron acceptor. Only 5% of the amount of Fe (III) reduced by cells cultured anaerobically with Fe (III) was reduced if the electron donor was omitted (Fig. 2 B).

Fe (III) reductase activity: Extracts of cells grown anaerobically with Fe (III) reduced Fe (III) at a rate of 116 nmol·min^{-1}·mg protein $^{-1}$ (Table 1). Extracts of cells grown aerobically (O_2 as terminal acceptor) with Fe (III) reduced Fe (III) at 98% (114 nmol·min^{-1}·mg protein $^{-1}$) of this rate (Table 1). Extracts of cells grown anaerobically with nitrate reduced Fe (III) at 41% (48 nmol·min^{-1}·mg protein $^{-1}$) of the rate of cells cultured anaerobically with Fe (III). Addition of NADH as sole electron donor resulted in 41%, 11%, and 82% respectively of the rate observed with cell extracts provided with glycerol as electron donor for cells previously cultured anaerobically with Fe (III), or nitrate, or aerobically with iron (Table 1). The extent of Fe (III) reduction by heat killed (100 °C, 10 min) cells cultured anaerobically with Fe (III), or nitrate, or aerobically with Fe (III) were

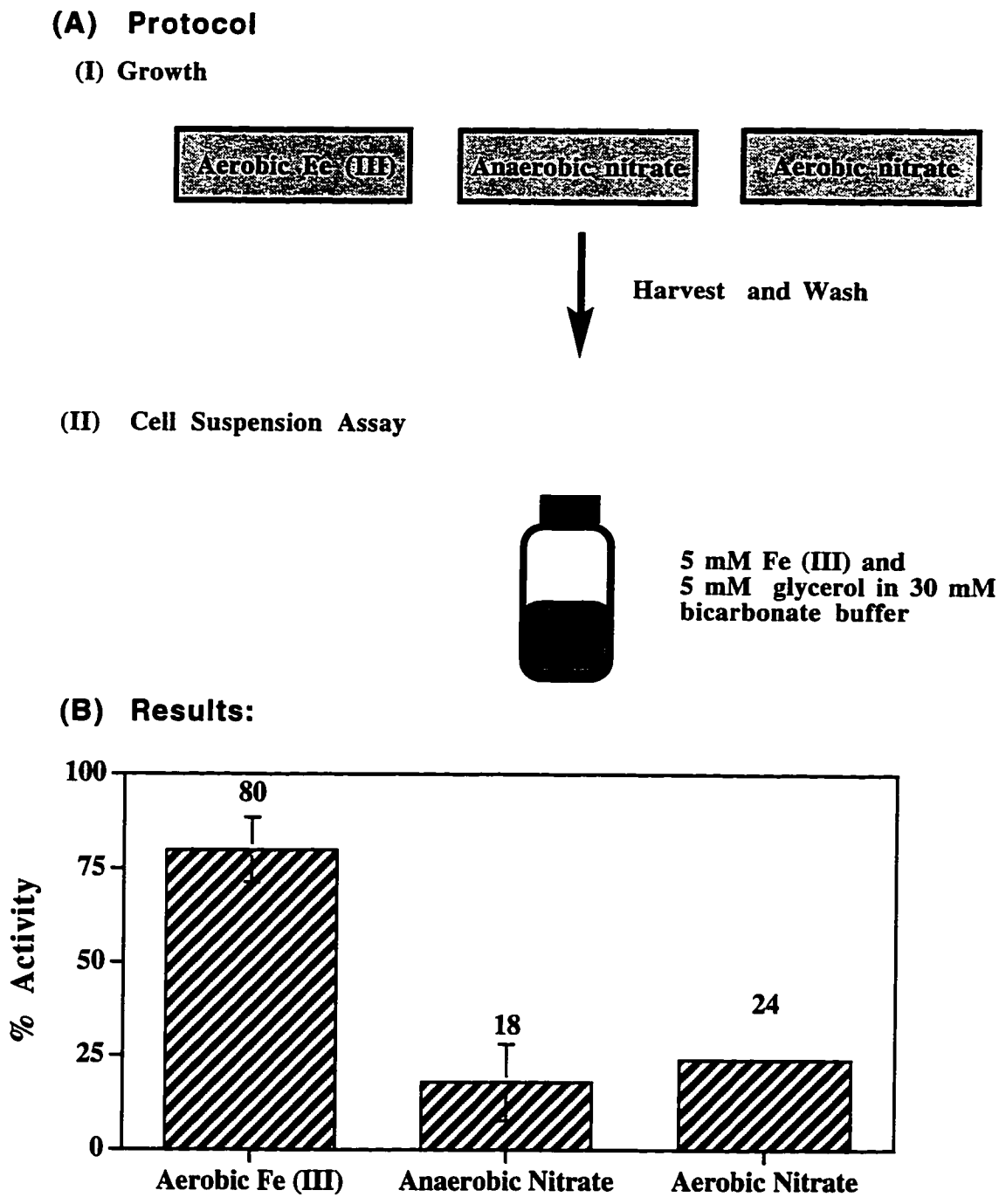

Figure 1 Effect of cell culture history on Fe (III) reduction in suspensions of washed cells grown aerobically with Fe (III), anaerobically with nitrate, or aerobically with nitrate. Experimental design (A) and percent activity of Fe (IIII) reduction (B) from that of cells grown anaerobically with Fe (III).

(A) Protocol:

(I) Growth

Harvest, wash, resuspend cells

(II) Cell Suspension Assay

5 mM Fe (III) citrate and 5 mM glycerol in 30 mM bicarbonate buffer. Incubations were at 37°C.

No electron donor | 5 mM nitrate | 1 mM nitrite

(B) Results:

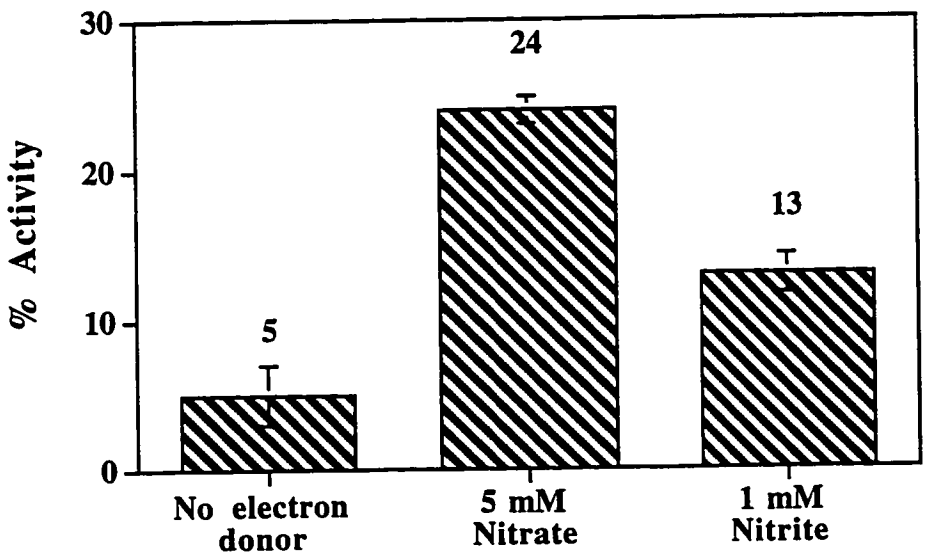

Figure 2 Effect of potential acceptors on Fe (III) reduction by suspensions of washed cells grown anaerobically with Fe (III). Experimental design (A) and percent activity of Fe (III) reduction (B) from that of cells without the addition of electron acceptor

11, 10, and 0 nmol·min^{-1}·mg protein $^{-1}$ respectively, (Table 1).

In order to locate reductase activity, cell extracts of cells previously grown anaerobically with Fe (III) were separated by ultracentrifugation to membrane and cytosol fractions. Fe (III) reduction rates for the membrane and cytosol fractions were 93% and 20% respectively, of the rate of un-fractionated cell extracts (Table 1). Heat killed (100 °C, 10 min) cell fractions did not reduce Fe (III).

Cytochromes: Dithionite-reduced minus air-oxidized absorption spectra obtained with cells grown aerobically or anaerobically with Fe (III), or anaerobically with nitrate (Figs. 3-5) contained peaks characteristic of *c*-type cytochromes (absorption maxima at 420, 522, and 553 nm). Hydrogen-reduced cytochromes of cells grown aerobically (Fig. 3) and anaerobically (Fig. 4) with Fe (III), but not those of cells grown anaerobically with nitrate (Fig. 5) were oxidized by the addition of Fe (III). Hydrogen-reduced cytochromes of cells grown under any of the growth conditions (Figs. 3-5) were oxidized by the addition of nitrate. Oxidation of H_2-reduced hemoproteins in cells grown anaerobically with Fe (III) was unaffected by azide (Fig. 4). In contrast, oxidation of H_2-reduced hemoproteins in cells grown aerobically with Ferric iron (Fig. 3), or anaerobically with nitrate (Fig. 5), was blocked by azide.

Inhibitor studies: The extent of Fe (III) reduction by suspensions of washed cells grown anaerobically with Fe (III) was inhibited (see Fig. 6) over that of the control cells (without inhibitor) by the addition of quinacrine (11% activity), dicumarol (37% activity), HOQNO (39% activity), CCCP (18% activity), to a lesser extent by azide (64% activity) but not by rotenone (100% activity). Fe (III) reduction by suspensions of washed cells grown aerobically with Fe (III) was inhibited (see Fig. 6) by the addition of quinacrine (10% activity), dicumarol (46% activity), HOQNO (75 % activity), CCCP (75% activity),

azide (20% activity) and to a lesser extent by rotenone (34% activity). Fe (III) reduction by cells grown anaerobically with nitrate was inhibited (see Fig. 6) by the addition of quinacrine (19% activity), CCCP (28% activity), azide (13 % activity) and to a lesser extent dicumarol (88% activity), and HOQNO (52% activity) but not rotenone (100% activity). After boiling for 10 minutes, cells grown under any of the three conditions failed to reduce iron (> 92 % inhibition of the control).

Culture History	nmol·min^{-1}·mg protein $^{-1}$		
	10 mM glycerol	10 mM glycerol Heat Killed	No glycerol 0.5 mM NADH
Aerobic, Fe (III)	114 ± 19	0 ± 11	95 ± 0
Anaerobic, NO_3^-	48 ± 25	10 ± 0	5 ± 6
Anaerobic, Fe (III)	116 ± 28	11 ± 19	48 ± 0
Cytosol	24 ± 22	0 ± 8	ND[a]
Membrane	108 ± 23	0 ± 23	ND

[a] Not done

Table 2 Fe (III) reductase activity in cell extracts of *A. hydrophila* ATCC 7966 grown in BSM supplemented with various electron acceptors.

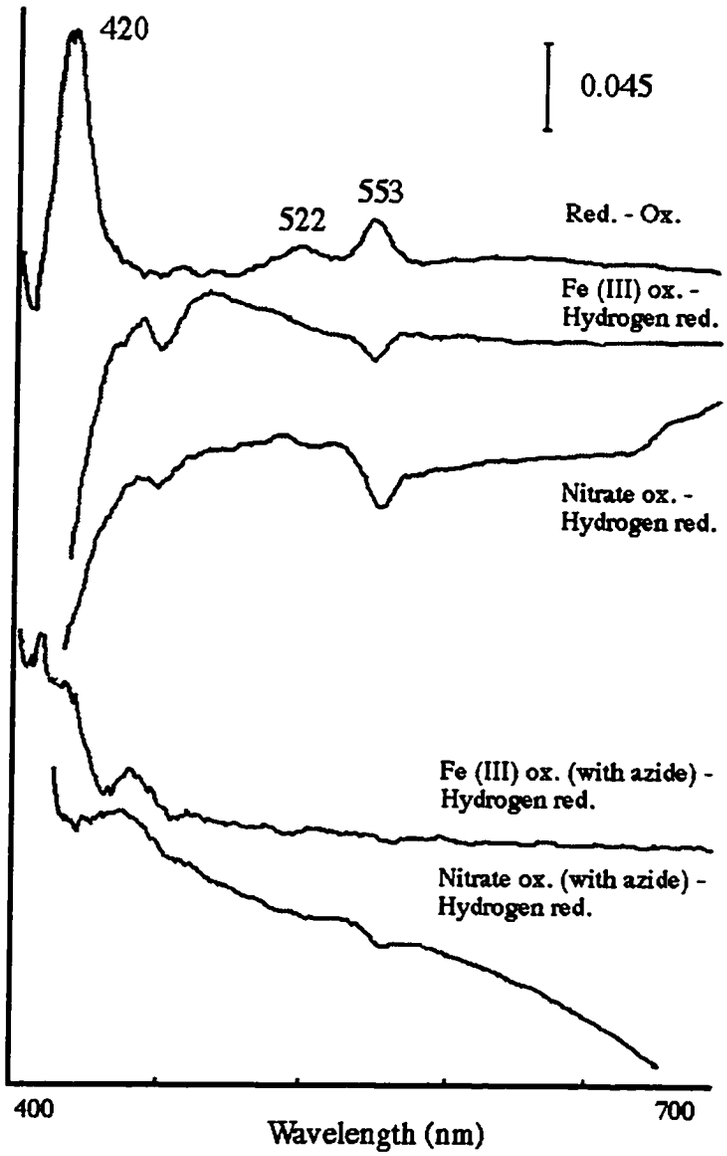

Figure 3 Differential cytochrome spectra of intact, washed cells of *A. hydrophila* ATCC 7966 grown aerobically in BSM supplemented with 30 mM glycerol and 20 mM Fe (III) citrate. Red.= reduced and Ox. = oxidized.

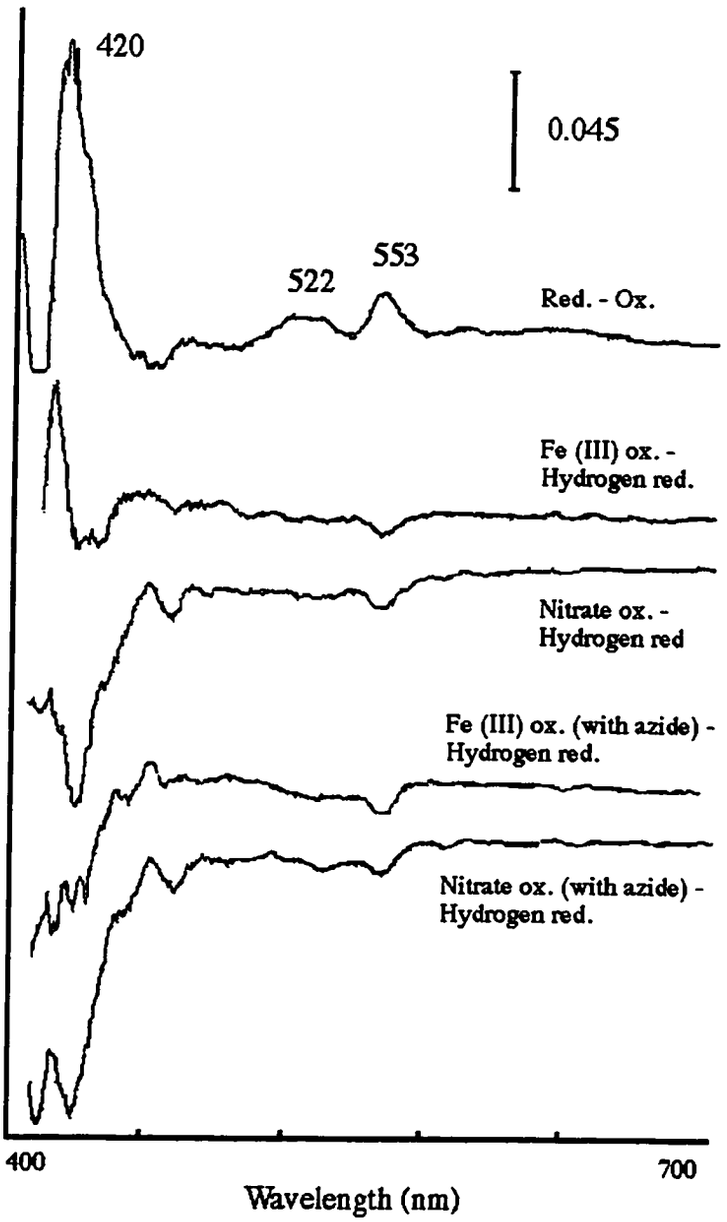

Figure 4 Differential cytochrome spectra of intact, washed cells of *A. hydrophila* ATCC 7966 grown anaerobically in BSM supplemented with 30 mM glycerol and 20 mM Fe (III) citrate. Red.= reduced and Ox. = oxidized.

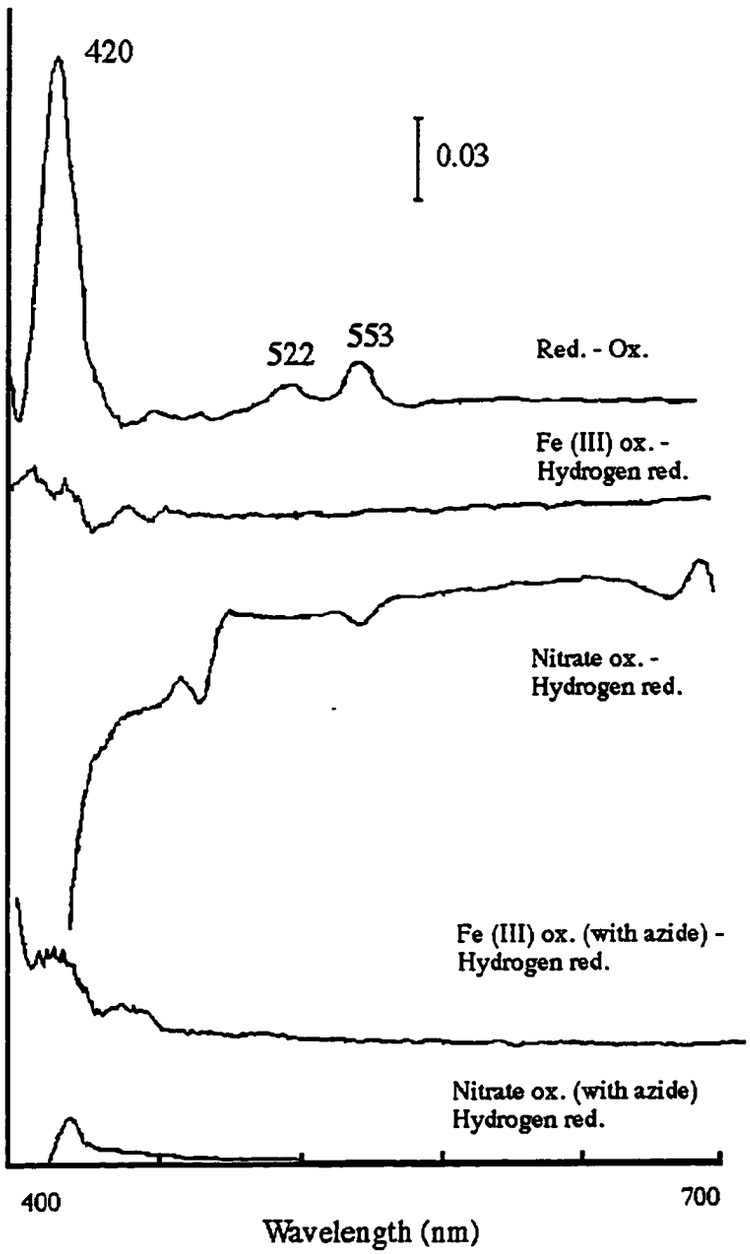

Figure 5 Differential cytochrome spectra of intact, washed cells of *A. hydrophila* ATCC 7966 grown anaerobically in BSM supplemented with 30 mM glycerol and 20 mM $NaNO_3$. Red.= reduced and Ox. = oxidized.

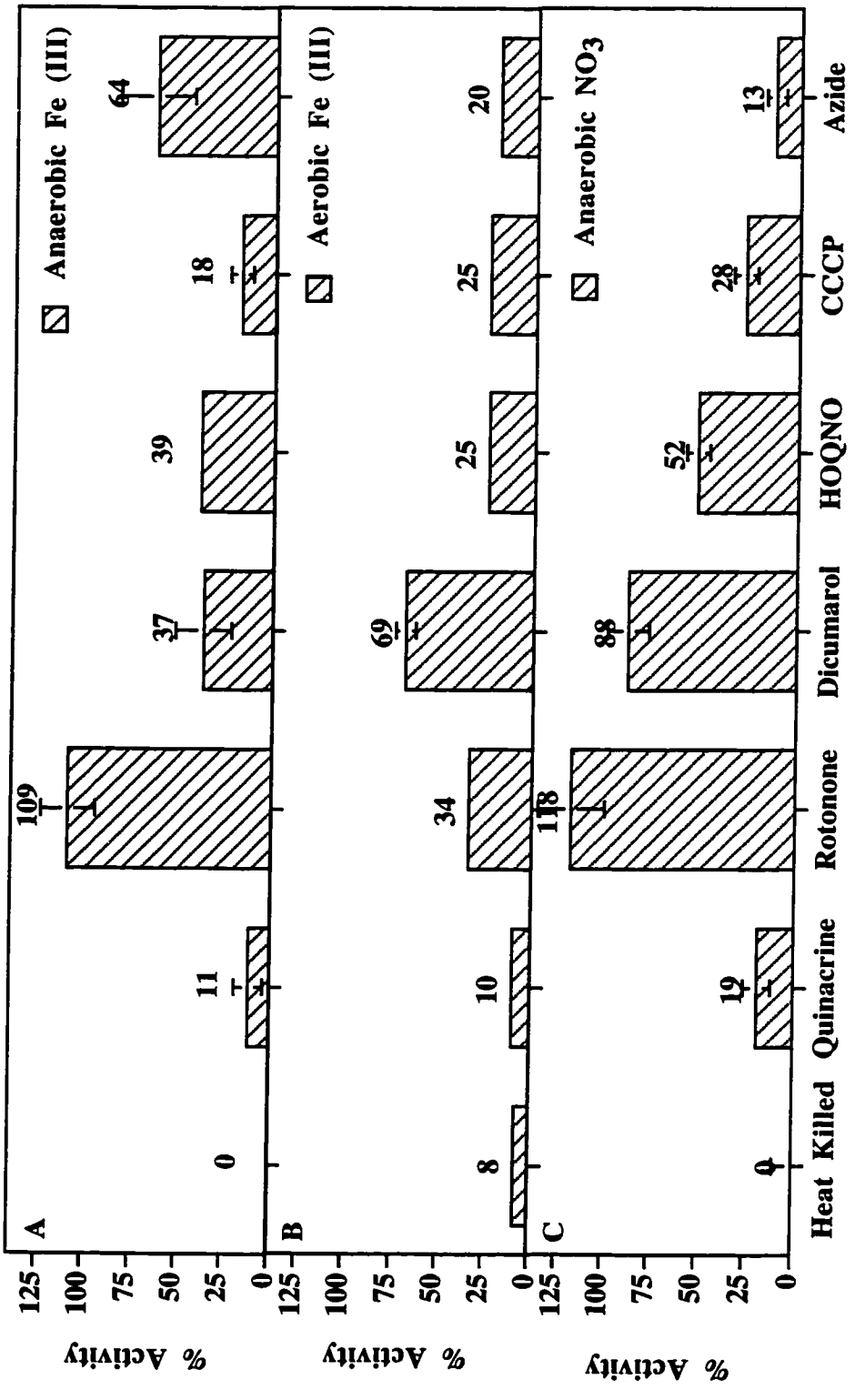

Figure 6 Percent activity of Fe (III) reduction by washed cell suspensions of *A. hydrophila* ATCC 7966 previously grown anaerobically (A) with Fe (III), aerobically (B) with Fe (III), or anaerobically with nitrate (C). Percent activity from that of cell suspensions without the addition of inhibitor. Concentrations of inhibitors shown in table 1.

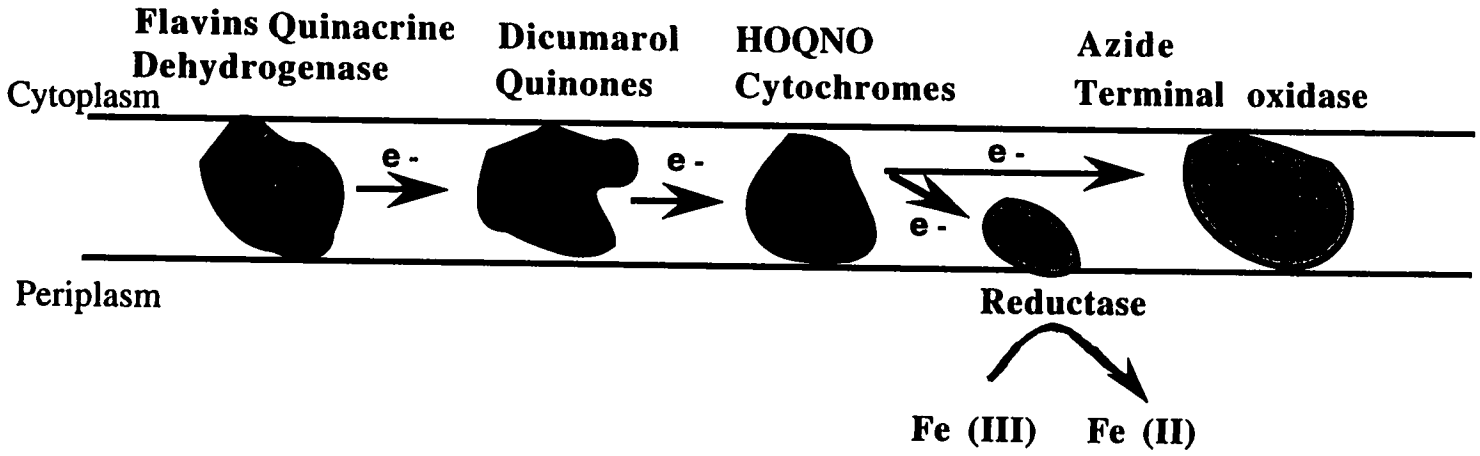

Figure 7 Proposed electron transport chain for *A. hydrophila* ATCC 7966 coupled to Fe (III) reduction.

DISCUSSION:

Aeromonas hydrophila ATCC 7966 grew with any of several terminal electron acceptors including O_2, nitrate, Fe (III), fumarate, Se (VI), or Co (III). This provided an opportunity to evaluate the role of culture history on Fe (III) reduction and to examine the mechanism of dissimilatory Fe (III) reduction by *A. hydrophila* ATCC 7966. Addition of nitrate or nitrite to suspensions of washed cells previously grown anaerobically with Fe (III) inhibited Fe (III) reduction 76% and 88 %, respectively. This implicated nitrate reductase in Fe (III) reduction by *Aeromonas* or, perhaps, competitive diversion of electrons from Fe (III) to NO_3^-. Fe (III) reduction was inhibited 82 % over that of cells grown anaerobically with Fe (III) by cells grown under nitrate-reducing conditions. Cells grown anaerobically with nitrate reduced Fe (III) at 60% of the rate of cells grown anaerobically with Fe (III). This is consistent with the involvement of a ferric reductase that is inhibited by nitrate. These results were similar to those obtained by others (5) with the dissimilatory iron-reducer *Geobacter metallireducens* strain GS-15 (5). Nitrate-grown *G. metallireducens* cells reduced Fe (III) at 50 % the rate of Fe (III)-grown cells (5). Fe (III) reduction by cell extracts of *A. hydrophila* ATCC 7966 previously grown aerobically with Fe (III) was 95 % of the rate of cell extracts of cells previously grown anaerobically with Fe (III). This suggests that ferric reductase in *A. hydrophila* is constitutively produced, with slight increase to full activity in cells grown under Fe (III)-reducing conditions. *Pseudomonas ferrireducens* sp 200 produces two distinct reductases (1). A constitutive reductase which is involved with electron transport to Fe (III) is produced under aerobic and anaerobic conditions (1). The second reductase is induced under microaerobic conditions and acts predominantly as an electron sink (1), not being

associated with energy conservation.

Room temperature dithionite-reduced minus air-oxidized absorbance spectra confirmed the presence of *c*-type hemoproteins in cells grown under all three growth conditions employed in this study. Cytochromes (*C* -type) have been detected in nearly all known dissimilatory Fe (III) reducers (9, 18). Hydrogen-reduced hemoproteins from cells grown aerobically and anaerobically with Fe (III), but not those grown anaerobically with nitrate were oxidized by t Fe (III). Hydrogen-reduced cytochromes of cells grown under all three conditions were oxidized by nitrate. It is interesting to note that hydrogen-reduced cytochromes from cells grown anaerobically with nitrate were not oxidized by the addition of Fe (III) and that the rate of Fe (III) reduction by such cells was also inhibited by 37 %. Reoxidation of hydrogen-reduced cytochromes by Fe (III) or nitrate, was completely blocked by the addition of azide in cells grown aerobically with Fe (III), or anaerobically with nitrate. Azide had no detectable effect on cells grown anaerobically with Fe (III).

Rotenone inhibited Fe (III) reduction in suspensions of washed cells previously grown aerobically with Fe (III). Washed suspensions of cells grown anaerobically with Fe (III) or with nitrate were not affected by the addition of rotenone. This suggests that NADH dehydrogenase of *A. hydrophila*, unlike that of many other Fe (III) reducers (1, 5, 18), is repressed or inactive under anaerobic conditions. Quinacrine inhibited Fe (III) reduction by 80% or more of the control rate in suspensions of washed cells grown under any of the culture conditions. This suggests that FAD dehydrogenase is important in channelling reducing equivalents into the electron transport chain. The addition of HOQNO inhibited Fe (III) reduction by cells grown under all three of the culture conditions employed in this study. This is consistent with the use of cytochromes in electron transport to Fe (III). Cytochromes (*C*-type) have been detected in all dissimilatory iron reducers (9, 15, 20, 24) with one exception (10). In addition, pleiotropic mutants of *Shewanella putrefaciens* which lack cytochromes are not capable of Fe (III) reduction (25). Mutants of

E. coli K 12 which lack quinones and cytochromes, reduced Fe (III) at rates comparable to those of the parent strain. However, this reductase, unlike that of dissimilatory Fe (III) reducers, is a soluble enzyme and is not involved in dissimilatory metal reduction (26). In our study, dicumarol inhibited Fe (III) reduction in cells grown aerobically and anaerobically with Fe (III). When the effect of the solvent was subtracted, the effect of dicumarol with nitrate grown cells was minimal. Quinones are expected to play a vital role in Fe (III) reduction. Addition of menaquinone to pleiotropic mutants which lack quinones restored their ability to reduce Fe (III) (17). It was curious that the extent of Fe (III) reduction by cells grown aerobically with Fe (III), or anaerobically with nitrate was inhibited 81, and 86 %, respectively, by the addition of azide. This suggests that a second terminal oxidase may function under these conditions. The ferric reductase of *A. hydrophila* ATCC 7966 appears to be membrane associated. 93 % (104 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$) of the Fe (III) reductase activity of whole cell extracts was associated with the membrane fraction (Table 1). Endogenous rates 24 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$) were associated with the cytosol fraction (Table 2). This is consistent with a dissimilatory ferric reductase (16, 26). Assimilatory ferric reductases such as those associated with siderophores are soluble enzymes (26). Our data indicate that dissimilatory Fe (III) reduction by *A. hydrophila* involves the use of a respiratory chain. Reducing equivalents appear to enter the chain via a FAD dehydrogenase, and are subsequently passed via quinones to cytochromes and a membrane associated Fe (III) reductase (Fig. 7).

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CHAPTER FOUR

SYNERGISTIC IRON REDUCTION AND CITRATE DISSIMILATION BY *SHEWANELLA ALGA* AND *AEROMONAS VERONII.*

ABSTRACT:

Two bacterial isolates from Great Bay Estuary, New Hampshire, when co–culturered carried out anaerobic dissimilation of citric acid with Fe(III) as the terminal electron acceptor. Neither isolate oxidized citrate with Fe(III) anaerobically in axenic culture. The Fe(III) reducer, *Shewanella alga* strain BrY, did not grow anaerobically with citrate as an energy source. The citrate-utilizer, *Aeromonas veronii*, did not reduce iron axenically with a variety of electron donors including citrate. The onset of iron reduction by the co-culture occurred with the maximum rate of citrate dissimilation and just prior to initiation of growth by either organism (as detected by viable plate counts). Anaerobic growth rates and final cell densities of each bacterial strain were greater in co–culture than in axenic cultures. After 48 h of growth, the co-culture had consumed 27 mM citrate as compared with 12 mM dissimilated by the axenic culture of *A. veronii.* Aditionally, after 48 h the co-culture produced half as much formate (6 mM) and twice as much acetate (40 mM) as did *A. veronii* grown axenically (12 mM and 20 mM respectively). Formate produced from citrate by *A. veronii* appears to have supported growth and Fe (III) reduction by *S. alga.* Although not obligatory, nutrient coupling between these two

organisms illustrates that fermentative (*A.veronii* type) organisms can convert organic compounds such as citrate to those used as substrates by dissimilatory Fe(III) reducers,including *S. alga.* This synergism broadens the range of substrates available for iron reduction, stimulates the extent and rate of organic electron donor degradation, as well as that of iron reduction, and enhances growth of each participant.

INTRODUCTION:

Dissimilatory iron reduction is an important means by which some bacteria participate in anaerobic carbon cycling (8, 13, 28). Microbial Fe(III) reduction may be a significant global process in that dissimilatory iron reduction can, under some conditions, outcompete sulfate reduction as a means of anaerobic organic matter mineralization (16, 29, 32). The organisms participating in dissimilatory iron reduction have not been completly described most studies to date have been carried out with pure cultures of rather few species such as *Shewanella putrefaciens* or *Geobacter metallireducens*. Studies have shown a highly diverse repertoire of terminal oxidants used by *S. putrefaciens* (27), the outer membrane location of a hemoprotein which may participate in dissimilatory iron reduction in this organism (25), the ability of *G. metallireducens* to completely oxidize aromatic compounds to CO_2 using Fe(III) as the terminal electron acceptor (18, 15) and the ability of the latter organism to produce copious extracellular fine-grain magnetite during dissimilatory iron reduction (16). Recently, *Pelobacter carbinolicus*, a close relative of both *Geobacter* and known sulfate-reducing bacteria, and formerly thought to be strictly fermentative, was shown to be capable of anaerobic respiration with Fe(III) (20). The majority of known bacteria capable of dissimilatory iron reduction use fermentation products as substrates. For instance, *S. putrefaciens* couples lactate oxidation with Fe(III) reduction (26) and *Shewanella alga* strain BrY (3) oxidizes H_2 or lactate with Fe(III). Degradation of aromatic compounds including mono-chlorinated substrates has been coupled to dissimilatory iron reduction in enrichment cultures (12, 22).

Because most organic waste in need of bioremediation reposes under anoxic conditions, dissimilatory iron reduction by microbes could be useful in anaerobic

bioremediation technologies. It is likely that successful *in situ* anaerobic bioremediation strategies would exploit the metabolic potential of mixed assemblages of autochthonous microorganisms rather than axenic cultures. To date, few investigators have employed mixed cultures to study dissimilatory iron reduction. By using sediments amended with iron and various carbon sources, Lovley and Phillips (16) showed that iron-reducing organisms in enrichment cultures can inhibit sulfate reduction and methane production. Tugel et al. (33) observed iron reduction in enrichment cultures made with surficial sediments from Great Bay Estuary, New Hampshire, but did not isolate bacteria capable of dissimilatory iron reduction. Lovley and Phillips (18) showed that glucose oxidation in sediments in which iron reduction was the terminal electron accepting process, required a mixed (undefined) microbial consortium. Bell et al. (2) observed the formation of magnetite during anaerobic iron reduction when several uncharacterized riverine sediment isolates from a enrichment culture were co-cultured, but not when any of them were grown axenically. As part of an investigation to evaluate the possibility of chemical (non-enzymatic) Fe(III) reduction by cultured microorganisms, Lovley et al., (19) observed synergistic iron reduction by co-cultures of *Escherichia coli* and *G. metallireducens* provided with glucose as the carbon and energy source but not by either organism cultured alone.

To extend the work of Tugel et al. (33) bacteria were previously isolated from sediments and water of Great Bay Estuary were capable of dissimilatory iron reduction (3, unpublished results). I decided to investigate whether a defined two-organism co-culture might show enhanced growth or rates of dissimilatory iron reduction and/or a more extensive capacity for carbon mineralization as compared to those of the component bacteria grown axenically. We chose citrate as a model organic acid. We expected citrate to be readily degraded as it is by lactic acid bacteria (via citrate lyase) to acetate, oxaloacetate, pyruvate and lactate (9). These products are substrates used by known iron-reducing

bacteria (3, 4, 5, 30). Citric acid is widely distributed in plants, animals and prokaryotes and is therefore likely to be a substrate encountered in natural habitats. Although we used it as a convenient and relevant organic electron donor, a more complete understanding of the environmental fate of this molecule should be important in the light of its value as a "natural" compound having widespread commercial use.

MATERIALS AND METHODS:

Source of organisms: The two organisms used in this study, *Shewanella alga* strain BrY and *Aeromonas veronii* strain BrA, were isolated from tidally influenced sediments at the confluence of Bunker Creek and the Oyster River (which, in turn, empties into Great Bay Estuary), New Hampshire. Enrichment culture, serial dilution to extinction and conventional anaerobic culture techniques (3) were used. *Shewanella putrfaciens* MR-4 was provided by Dr. K. Nealson (Center for Great Lakes Studies, University of Wisconsin).

Cell growth: Growth of these bacteria axenically and in co-culture was evaluated in a basal salts medium containing the following constituents ($g \cdot l^{-1}$): $NaHCO_3$, 2.5; KCl, 0.1; NH_4Cl, 1.5; $NaH_2PO_4 \cdot H_2O$, 0.6; Wolfe's vitamins, 10 ml (34); and trace mineral solution, 10 ml (34). To these were added ferric citrate (50 mM) and sodium formate (30 mM; when appropriate). Standard anaerobic technique was used throughout (1). Unless specified, the gas phase was N_2:CO_2; 80:20. Trace O_2 was removed by passing the gas through hot copper filings. Cultures were grown in triplicate in 250 ml serum vials each containing 50 ml culture medium (24). Replicate broth cultures were initiated with a 10% (v/v) inoculum of exponentially growing culture (approximate initial concentration 10^5 $CFU \cdot ml^{-1}$). Viable counts were obtained by using plate counts of samples removed periodically and plated in an anaerobic glovebox on seawater complete (SWC) medium consisting of ($g \cdot l^{-1}$): peptone, 3.0; yeast extract, 1.0; agar, 15; glycerol, 3 ml; in 3/4-strength artificial seawater (ASW) consisting of ($g \cdot l^{-1}$): NaCl, 23.48; $MgCl_2 \cdot 6H_2O$, 10.65; Na_2SO_4, 3.92; $CaCl_2 \cdot 2H_2O$, 1.46; KCl , 0.66; $NaHCO_3$, 0.19 (23) without pH adjustment. All cultures were incubated at

30°C. Acridine orange direct counts were carried out as previously described (16) to determine plating efficiencies. For culture identification, Hugh and Leifson's (7) oxidation/fermentation broth (Difco, Detroit, MI) consisting of the following constituents ($g \cdot l^{-1}$) was used: Bacto peptone, 2.0; Bacto proteose peptone, 5.0; NaCl, 5.0; K_2HPO_4, 0.3; bromophenol blue, 0.03; agar, 2.0. Triple sugar iron agar (Difco, Detroit, MI) consisting of ($g \cdot l^{-1}$) was also used: Bacto beef extract, 3.0; Bacto yeast extract, 3.0; Bacto peptone, 2.0; glucose, 1.0; lactose, 10.0; sucrose, 10.0, ferrous sulfate, 0.2; NaCl, 5.0; sodium thiosulfate, 0.3; phenol red, 0.024; agar, 13.0.

HPLC analysis: Samples (20 µl) of centrifuged and filtered (0.45 µm) culture supernatant fluids were analyzed for organic acids by using a Beckman Model 110A chromatograph equipped with BioRad HPX-87H cation-exchange analytical and guard columns and a Beckman Model 160 detector equipped with a zinc lamp and 210 nm filter for organic acid detection. The solvent was 0.005 M H_2SO_4. Column temperature was maintained at 65°C by means of a water jacket connected to a circulating water bath. Compounds were identified by using authentic compounds as internal standards.

Iron analysis: Fe(II) was quantitated colormetrically with ferrozine (18) at 562 nm with ferrous ethylene diamine sulfate as a standard. Samples were acidified for 15 min with 0.5 N HCl prior to iron analysis (17). The detection limit of this assay is in the nM range.

Formate dehydrogenase: Cultures were grown for 48 h in 50 mM ferric citrate broth supplemented with 30 mM formate. Cells were collected by centrifugation at 10,000 x g for 10 min at 4°C. Concentrated cells were washed once in phosphate-buffered saline (PBS), containing the following constituents ($g \cdot l^{-1}$): Na_2HPO_4, 1.236; $NaH_2PO4.H_2O$, 0.18; NaCl, 8.5. The cells were then centrifuged and resuspended in PBS. Cells were disrupted with a

French pressure cell (2 passages, each at $1.4 \cdot 10^8$ Pa). Protein concentration was quantitated by using the BCA protein assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a standard. Formate dehydrogenase activity was measured as a decrease in absorbance at 600 nm by using a Hitachi model U-2000 spectrophotometer. Formate was the electron donor. Phenazine methosulfate and dichlorophenolindophenol (DCPIP) were the intermediate and final electron acceptors, respectively (6). A molar extinction coefficient of 21 $mM^{-1} \cdot cm^{-1}$ for DCPIP (6) was used to calculate the specific activity. The specific activity was defined as units$\cdot mg^{-1}$ protein where a unit (U) refers to 1 µmole formate decomposed per minute.

RESULTS:

Organisms and growth: The two strains used in this study were gram-negative, facultatively anaerobic, non-spore forming, motile, polarly flagellated rods. Strain BrY was a strain of *Shewanella* as determined from phenotypic characters (oxidase +; oxidative but not fermentative [7]; use of thiosulfate as an electron acceptor) and analysis of the 16S rRNA sequence analysis (K. Nealson and D. Saffarini, personal communication). Rossello-Mora et al. (31) have identified strain BrY as *Shewanella alga.* The other organism used was isolated from sediments at the same location as was strain BrY. Based upon phenotypic characters (oxidase +; oxidative and fermentative; acid and gas in TSI medium and other traits) it is a strain of *Aeromonas veronii* (10). Cells of these two organisms were microscopically indistinguishable from one another. However, when cultured aerobically on the same plate, *S. alga* produced small, pink colonies easily distinguishable from the larger, white ones characteristic of the *A. veronii* isolate. Therefore, we used this latter feature to assess the relative growth responses of these two organisms in mixed culture.

Unlike *S. alga, A. veronii* grew axenically with citrate as the sole carbon and energy source. Maximum densities of axenically grown cells ($2 \cdot 10^8$ CFU·ml $^{-1}$) occurred by 30 h (Fig. 1 A). Co-culturing these bacteria consistently had a pronounced effect on growth of both strains. The lag interval preceding maximum growth *of A. veronii* decreased by 10 h. The maximum viable count of *A. veronii* was increased 4-fold (to $8 \cdot 10^8$ CFU·ml^{-1}), and the culture doubling time increased 7-fold from 12 to 1.75 h (Fig. 1 A). Growth of *S. alga* was also significant ($1\text{-}2 \cdot 10^9$ CFU·ml^{-1}) by 30 h in co-culture (Fig. 1 A) whereas this organism did not grow axenically in this medium. Maximum growth of *S.*

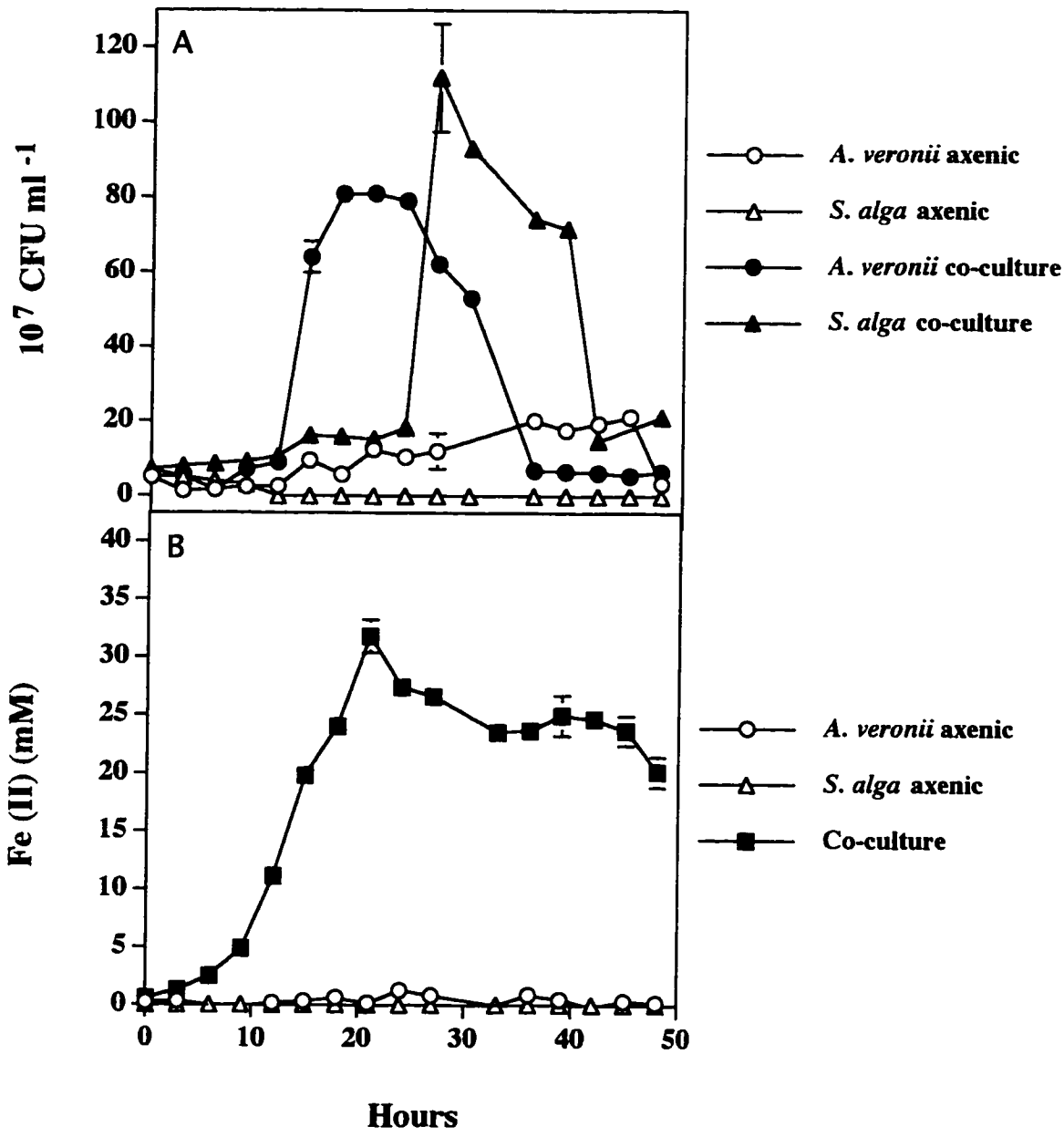

Figure 1 Anaerobic growth (A) and Fe (III) reduction (B) by *A. veronii* and *S. alga* axenically and in co-culture in basal salts medium containing 50 mM ferric citrate.

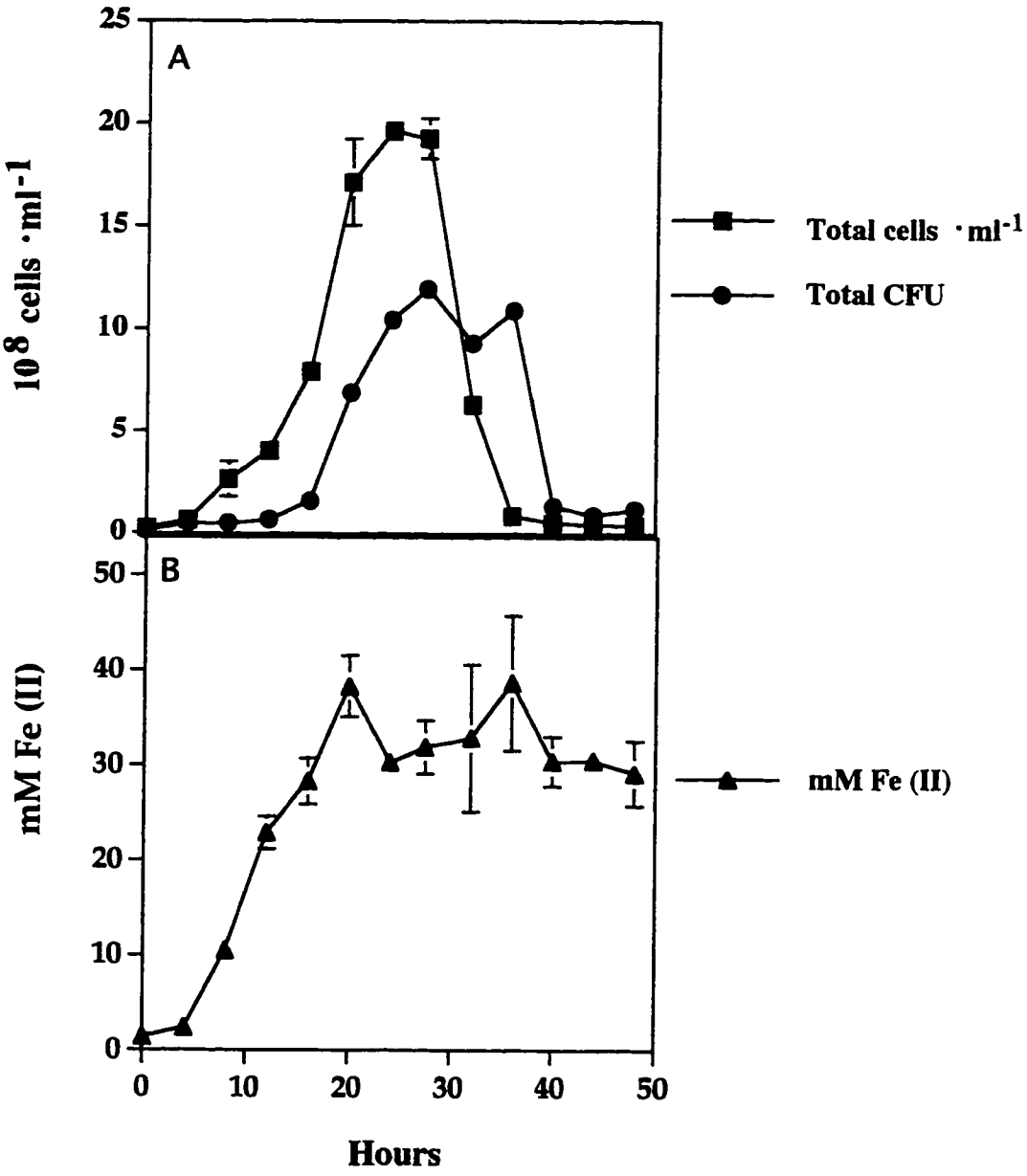

Figure 2 Growth (A) measured by total cell counts or total CFU and Fe (III) reduction (B) by co-culture of *S. alga* and *A.veronii* in basal salts medium supplemented with 50 mM ferric citrate.

S. alga lagged behind that of *A. veronii* by about 10 h in mixed culture. Both organisms in mixed culture showed a rapid decrease in viable counts after reaching maximum cell density (Fig 1 A). This appeared to be due to cell lysis as evidenced by a rapid decrease in acridine orange total counts and viable plate counts of the same culture (Fig. 2). The plating efficiencies of the organisms in co-culture were determined by comparing combined plate counts with total acridine orange direct counts. Cell numbers as determined from plate counts were 50 % of those determined by acridine orange direct counts.

Iron reduction: *S. alga* strain BrY reduced Fe(III) in axenic culture with pyruvate, lactate, H_2 (3) or formate (see below). The fact that the pH of the medium remained circumneutral and uninoculated controls showed no iron reduction, are consistent with biological rather than chemical reduction of iron. Moreover, previous results (3) indicated that Fe (III) reduction by *S. alga* required cell contact with the iron. The quantity of Fe(III) reduced by either strain grown axenically on iron citrate was barely detectable (Fig. 1 B) by the ferrozine assay which detected nM ferrous iron. By 20 h however, co-cultures (Fig. 1 B) had produced 32 mM Fe(II). The decrease observed thereafter was undoubtedly due to precipitation of ferrous iron as a mineral phase having the appearance of the white mineral ferrous carbonate (siderite).

Citrate utilization: Over the culture growth period the mixed culture utilized citrate at roughly twice the rate (0.52 mmol·hr^{-1} of that of axenic *A. veronii.* By the end (48 h) of axenic growth, *A. veronii* consumed 10 mM citrate as compared to 20 mM dissimilated by the co-culture (Fig. 3 A). (The apparent increase within five hours is presumably due to sampling error). By the end of culture growth, we detected roughly 40 mM acetate (Fig. 3 B) and 6 mM formate (Fig. 3 C) in the co-culture as compared to 20 mM and 12 mM, respectively, in the axenic culture of *A. veronii.* Lactate was detected as a product in spent

culture fluids of *A. veronii.* However, its concentration (3.5 mM) was the same as that detected in the co-culture (3.1 mM). Small amounts of ethanol were produced by *A. veronii* grown axenically and by the co-culture but ethanol was not a substrate for Fe(III) reduction.

Growth with formate: In this study, *S. alga* grew axenically with 30 mM formate (Fig. 4) but not without the addition of Fe(III). Iron reduction and culture growth occurred concomitantly as determined from direct counts made with acridine orange (Fig. 2). Culture growth as measured by using viable plate counts (Fig.4) appeared to lag behind iron reduction. The final viable cell densities (measured by standard plate counts) of *S. alga* cultured axenically (Fig. 5 A), as well as the quantity of Fe(III) which the organism reduced by 48 h (Fig. 5 B) each increased in direct proportion to the formate concentration over the range 10-20 mM. Because formate was not previously recognized as being a sole carbon and energy source for *S. alga* (3), we verified formate utilization by re-examining our frozen laboratory stocks of the original isolate, as well as a culture maintained over the interim elsewhere by Frank Caccavo (University of Montana, Bozeman). Axenic growth of *A. veronii* in basal salts medium was not inhibited by formate concentrations over the range 5-20 mM (Fig. 6).

Formate dehydrogenase: Formate dehydrogenase (FDH) activity (Table 1) was detected in cell extracts of *S. alga* (specific activity, 0.27 $U \cdot mg^{-1}$ cell protein) and in the control (*S. putrefaciens* strain MR-4; specific activity 0.84 $U \cdot mg^{-1}$ cell protein). FDH specific activity of strain MR-4 was 3 times that of *S. alga* and 23 times that detected in *A. veronii* (specific activity, $3.6 \cdot 10^{-2}$ $U \cdot mg^{-1}$ cell protein).

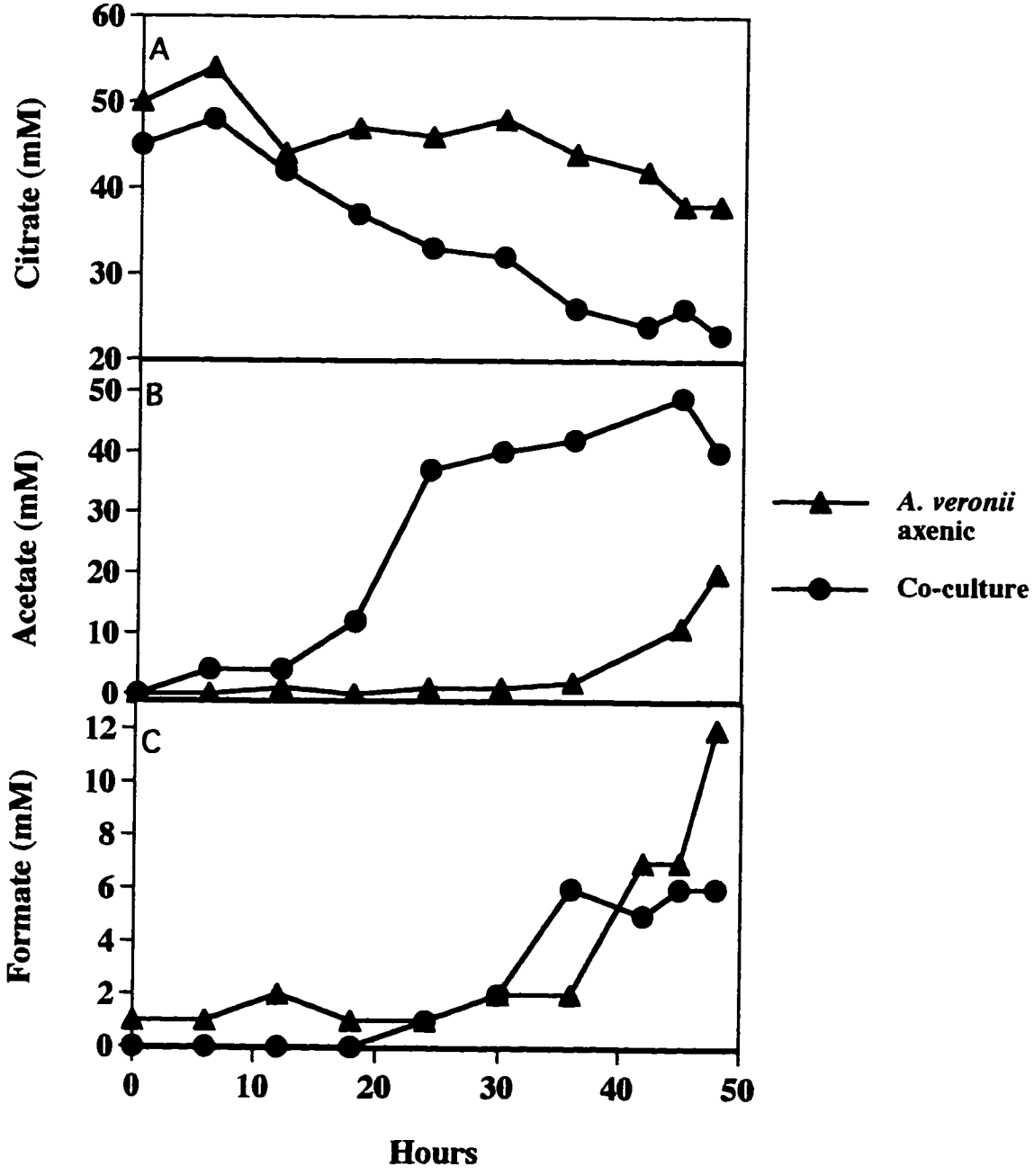

Figure 3 Citrate dissimilation and end product formation by *A. veronii* axenically and in co-culture with *S. alga*. Cultures were grown in basal salts medium containing 50 mM ferric citrate. (A) Citrate dissimilation. (B) Acetate production. (C) Formate production.

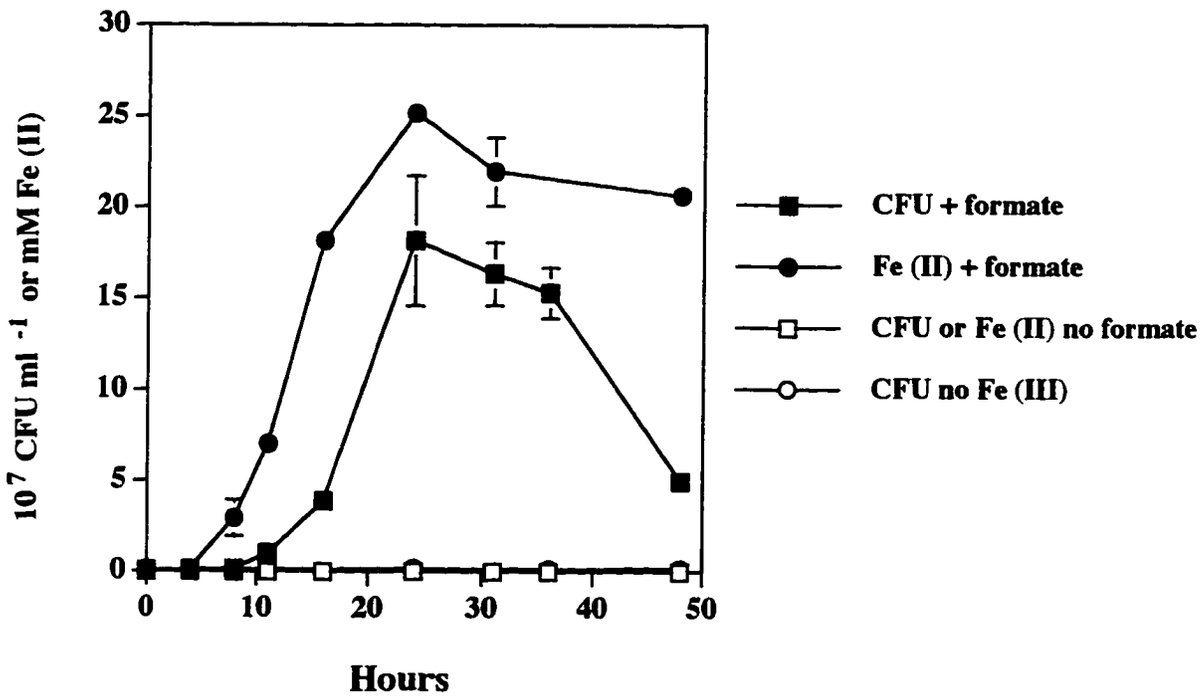

Figure 4 Anaerobic growth and Fe (III) reduction by *S. alga* grown axenically in basal salts medium containing 50 mM ferric citrate with, and without, 30 mM formate. Results shown are means and standard deviations of triplicate cultures.

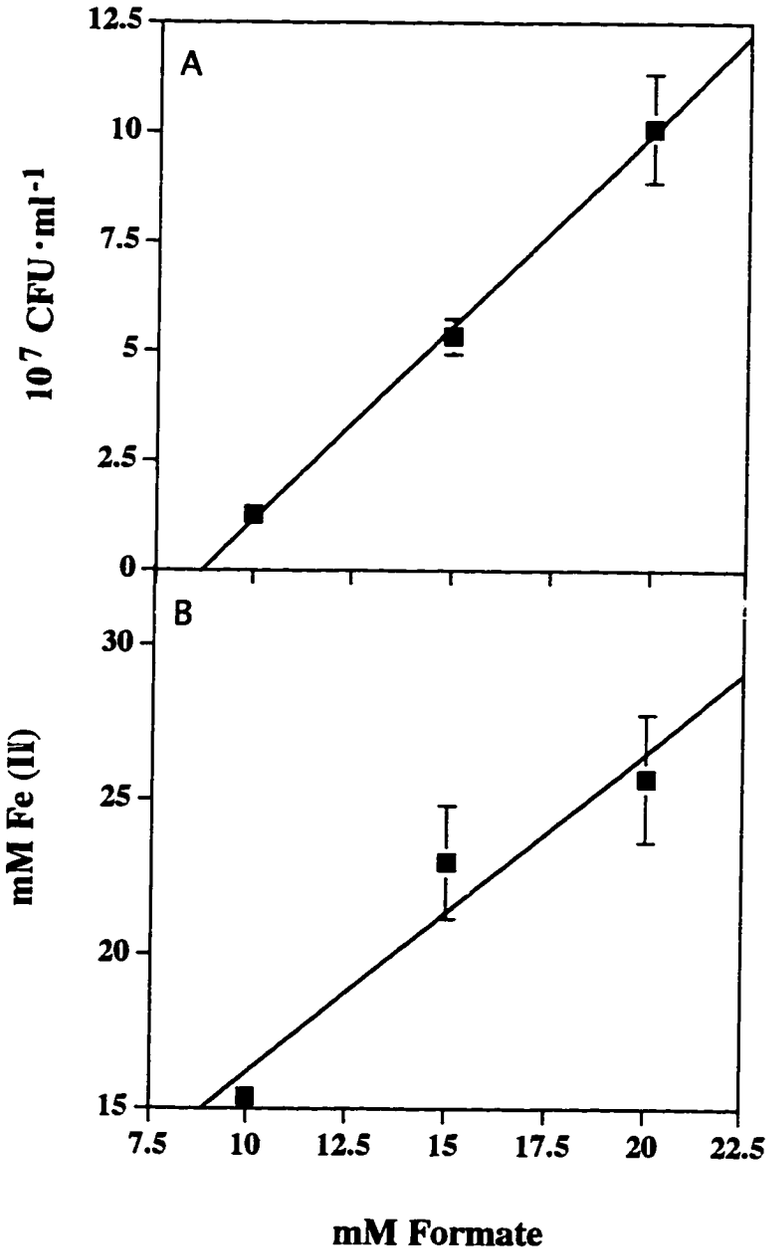

Figure 5 Effect of formate on axenic *S. alga* growth (A) and Fe (III) reduction (B). *S. alga* was axenically grown anaerobically in basal salts medium with 50 mM ferric citrate with different formate concentrations. Results shown are means and standard deviations of triplicate cultures after 48 hours growth.

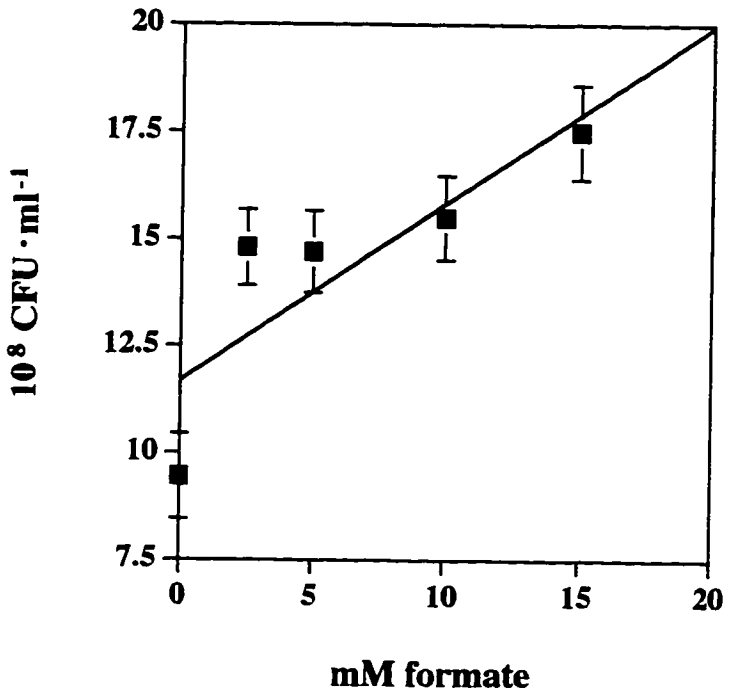

Figure 6 Growth of *A. veronii* axenically in basal salts medium supplemented with 50 mM ferric citrate and 2.5-15 mM formate

	Specific Activity (μM formate decomposed·min^{-1}·mg protein^{-1})	
Isolate	**Live**	**Heat Killed**
S. alga	2.7×10^{-1}	5.1×10^{-3}
A. veronii	3.6×10^{-2}	7.0×10^{-2}
S. putrefaciens strain *MR4*	8.4×10^{-1}	6.9×10^{-3}

Table 1 Formate dehydrogenase specific activity. 1 unit represents 1 μM formate decomposed·min^{-1}·mg cell protein^{-1}

DISCUSSION:

In situ bioremediation efforts can be expected to benefit from the metabolic diversity found in the autochthonous microbiota of most natural environments. Microbial food chains and synergistic relationships known to exist in nature remain inadequately studied. There is increasing interest in the combined catabolic potential of mixed assemblages of microbes and the manner in which they might collectively metabolize compounds which are degraded only slowly or incompletely by axenic cultures. Mixed culture studies of organic matter mineralization involving Fe(III) reduction are few (2, 18) and, except for one reported as part of a more general study (19), have not involved defined mixtures of characterized organisms. My results indicate that Fe(III) reduction by *S. alga* was synergistically coupled to fermentation of citric acid by *A. veronii*, a compound which *S. alga* by itself did not anaerobically degrade. Growth of both organisms was stimulated in mixed culture and the extent of citrate degradation and iron reduction were enhanced. The onset of iron reduction in the mixed culture coincided with the period of citrate dissimilation and formate production and these preceded the linear growth response of either organism. The direct cell count data (Fig. 2) indicate that iron reduction is more tightly coupled to cell growth early in the experiment than appears from viable plate counts. This discrepancy is presumably due to binding of the bacteria to particulates and presence of indistictable cells. Cell binding to small aggregates which later disappeared would explain the lag seen between the onset of iron reduction and the increase in growth as measured by viable (plate) counts.

S. alga strain BrY has been reported to use a variety of electron acceptors, although rather few donors (H_2, lactate, ethanol, pyruvate or formate) are used anaerobically (3, this

study). Because axenic *A. veronii* did not produce from citrate, lactate or pyruvate, it appears that the synergistic link between the two bacteria in this study was formate and perhaps H_2 produced from formate by *A. veronii*; these are used by *S. alga* to support extensive Fe(III) reduction and growth (3). The significant formate dehydrogenase activity in *S. alga* (and low or no activity in *A. veronii*) is consistent with enzymatic formate removal by *S. alga.* We observed concomitant growth and Fe(III) reduction by *S. alga* axenically with formate as the sole electron donor. End product removal does not appear to be responsible for the increase in cell densities of *A. veronii* because we could detect no inhibitory effects on the growth of axenic *A. veronii* with formate concentrations as high as 20 mM. Final cell yields and the extent of Fe(III) reduction for axenically grown *S. alga* were in direct proportion to the amount of formate provided over the range 5-20 mM. Ethanol was also produced in small quantities which may account for the loss of some of the reducing equivalents produced from citrate. We also observed enhanced growth of both organisms (reduction in lag; seven-fold stimulation of doubling time; four-fold increase in final cell densities) and significantly enhanced iron reduction in co-culture.

The organisms used in this study were isolated from the same estuarine sediments in which Fe(III) reduction has been shown to be an important biogeochemical process (11, 33). Isolation and axenic growth of *S. alga* strain BrY from this environment provided an opportunity to examine the coupling of Fe(III) reduction to H_2 and fatty acid oxidation by a member of this sediment community (3). Our work illustrates that the seasonally robust Fe(III) reduction observed in Great Bay Estuary (11, 33) can be driven by natural and anthropogenic sources of parent compounds which the dissimilatory Fe(III) reducers, themselves, may not have the capacity to degrade. My results obtained with this defined two member co-culture provide experimental evidence consistent with a two-step model of anaerobic organic carbon cycling (17) involving fermentation by one group of microbes which is coupled to iron respiration by another. Use of catabolic end products of

associated microbes can be expected to vastly expand the repertoire of parent compounds available for anaerobic organic carbon cycling by Fe(III) reducers, as well as the extent of carbon mineralization and growth of the microbial participants, even if they have more limited anaerobic dissimilative capacity coupled to iron respiration in pure culture.

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SUMMARY

Members of the genus *Aeromonas* are gram negative, oxidase positive, facultatively anaerobic rods. Within this genus there are two major groups, the psychrophilic non-motile fish pathogens (*A. salmonicida*) and diverse species of mesophilic and motile organisms which can be differentiated by standard biochemical tests to 3 species: *A. hydrophila*; *A. sobria*; and *A. caviae*. Mesophilic aeromonads are indigenous to freshwater, brackish and marine environments. Their role as opportunistic pathogens of both warm- and cold-blooded animals (especially amphibians, reptiles, and fish), is well documented. As suggested by recent increases in literature citations in which it has been associated with human illnesses, *Aeromonas* has been described as an "emerging" pathogen. Review of the literature (see, for instance, Chapter 1) indicates, however, that relatively little is known concerning the organism's ecology or ecophysiology. Factors which promote its survival in environments differing in physicochemical characteristics such as temperature, salinity, dissolved oxygen, and nutrient loading are not well known. To learn more concerning the ecology of this organism, a study was undertaken to determine the incidence and seasonal distribution of *Aeromonas* in seven sites throughout Great Bay Estuary and its tributaries. With the exception of the Durham Mill Pond site each of the study sites was tidally influenced. Each site was sampled monthly over a two year period. Aeromonads were isolated from each site throughout the entire year with an increase in incidence during the late summer to early fall. The incidence of aeromonads were highest at sites proximinal to freshwater. Of several biotic and abiotic parameters measured at each site, statistical analysis revealed a correlation between the incidence of aeromonads and of total and fecal coliforms. These results are consistent with those in

published reports indicating that counts in eutrophic waters of coliforms and fecal coliforms to accurately predict the incidence of aeromonads.

Members of the genus *Aeromonas* are metabolically quite diverse bacteria. They grow aerobically, as well as anaerobically by fermentation. Although *Aeromonas* is described as being capable of reducing nitrate, no study of which we are aware has shown that this activity is coupled to energy conservation or to growth. Likewise, products of nitrate reduction have not been reported. We isolated 30 strains of *Aeromonas* from New Hampshire waters local to Durham varying in these and other parameters. The isolates were obtained by direct plating to a selective and differential medium (usually following membrane filtration of water samples). One hundred percent of our isolates were capable of reducing nitrate under anaerobic conditions with the non-fermentable electron donor glycerol. Growth yields and the extent of nitrate consumed by cells of *A. hydrophila* ATCC 7966 were directly proportional to the quantity of nitrate provided over the range 1.25-5 mM. Neither gas formation nor nitrous oxide were detected as products from cells cultured anaerobically with nitrate by using acetylene blockage (Data not shown). Therefore, these nitrate reducers were not capable of denitrification.

Of the thirty isolates tested in addition to *A. hydrophila* ATCC 7966 all were capable of dissimilatory Fe (III) reduction. Growth yields and the extent of ferric iron reduced by cells of *A. hydrophila* ATCC 7966 were directly proportional to the quantity of ferric iron provided over the range 1.25-10 mM Fe (III). All strains were capable of Co (III) reduction. The final cell yields and extent of reduction were proportional to the amount of oxidant provided over the range 0.5-1.5 mM Co (III). Forty three percent of strains were capable of dissimilatory Se (VI) reduction. Final cell yields and extent of reduction increased in direct proportion to amount of oxidant provided over the range 1.25-10 mM Se (VI). Reduction of selenate to elemental selenium is energetically unfavorable. The redox potential of the selenite/elemental selenium couple suggests that this is an energy requiring

reaction. Conservation of energy from the selenate/selenite-coupled reaction is expected to drive this unfavorable reaction.

We did not detect reduction of sulfate, thiosulfate, sulfur, TMAO or DMSO by *Aeromonas*. Moreover, at least one aeromonad used in this work *A. veronii* strain BrA was incapable of dissimilatory Fe (III) reduction (see Chapter 4). Thus, members of this genus appear to be less versatile in the choice of terminal electron acceptor than are members of the genus *Shewanella* which are notable in this regard.

Anaerobic respiration with metals provides a means whereby these organism can survive and grow under anaerobic conditions in the absence of fermentable carbon and energy sources. Moreover, the growth yields associated anaerobic respiration are consistently higher than those from fermentation provided abundant oxidant is available. It is noteworthy that virtually all strains in this study isolated from diverse habitats and representing seasonal and salinity differences as well as free-living and host (amphibian)-associated isolates, were all capable of dissimilatory iron reduction. It should be noted that the isolation procedures used did not select for iron-, nor nitrate-reducing (nor other alternate terminal electron accepting) ability. Thus, it would seem that anaerobic respiration with metals, with nitrate and, perhaps the metalloid selenium, is common to the group.

This work expands by one the number of microbial taxonomic groups capable of dissimilatory metal reduction. Like the metal reducers of the genus *Shewanella*, aeromonads are facultatively anaerobic bacteria indigenous to aquatic habitats. Anaerobic respiration can be expected to be of ecological importance to aeromonads by extending their anaerobic capabilities to growth on non-fermentable substrates and providing an alternative to fermentation as a means of survival under anoxic conditions. Moreover, dissimilatory metal reduction is energetically more favorable than is fermentation. In the presence of abundant acceptor, Fe (III) should be nearly the energetic equivalent of O_2. Reeburgh reported that in the Skan Bay, Alaska, Fe (III) concentrations in the sediments can reach as

high as 50 mmol.l-1 sediment. The products of Fe (III) reduction (unlike H_2S produced by sulfate reducers) are relatively innocuous minerals such as siderite, vivianite, or magnetite. Dissimilatory metal reduction also promises several advantages in anaerobic bioremediation strategies. Most organic waste in need of bioremediation is found under anaerobic conditions. Current bioremediation strategies require pumping oxygen through the site which is costly. Anaerobic bioremediation by bacteria using nitrate and sulfate as terminal electron acceptors has been well documented. Bioremediation of complex organic molecules under Fe(III)-reducing conditions is under investigation. Although the Fe (III) reduction rates observed in this study were equivalent to those of the obligate anaerobe *Geobacter metallireducens*, *Aeromonas* is not likely to be a candidate for *in situ* anaerobic bioremediation unless strains can be genetically engineered to delete virulence characteristics or their release into the environment can be minimized or prevented.

Selenium is found in the environment as selenate and selenite (Se (VI), and Se (IV), respectively). Selenate is usually found in low concentrations (0.1-2 ppm) in the natural environment. However, both selenate, and selenite are toxic to biota at relatively low concentrations (4 ppm). Reduction of either of these forms results in the formation of insoluble, elemental selenium. This allows for detoxification, or sequestration of this toxic metalloid. Bioreactors have been used to remove selenium (as insoluble elemental selenium) from contaminated water and therefore demonstrate the ability of bioremediation of this toxic metalloid.

The organisms used in this study were isolated from the same estuarine sediments in which Fe(III) reduction has been shown to be an important biogeochemical process. Isolation and axenic growth of *S. alga* strain BrY from this environment provided an opportunity to examine the coupling of Fe(III) reduction to H_2 and fatty acid oxidation by a member of this sediment community. Our work illustrates that the seasonally robust Fe(III) reduction observed in Great Bay Estuary can be driven by natural and

anthropogenic sources of parent compounds which the dissimilatory Fe(III) reducers, themselves, may not have the capacity to degrade. Results obtained with a defined two member co-culture provide experimental evidence consistent with a two-step model of anaerobic organic carbon cycling involving fermentation by one group of microbes which is coupled to iron respiration by another. Use of catabolic end products of associated microbes can be expected to vastly expand the repertoire of parent compounds available for anaerobic organic carbon cycling by Fe(III) reducers, as well as the extent of carbon mineralization and growth of the microbial participants, even if they have more limited anaerobic dissimilative capacity coupled to iron respiration in pure culture.

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