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Some Aspects of the Effects of Heavy Metal Pollution on Microbal Sediment Populations

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SOME ASPECTS OF THE EFFECTS OF HEAVY METAL POLLUTION ON MICROBIAL SEDIMENT POPULATIONS

BY

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ABSTRACT

Sediment populations were tested for metal resistance by the spread plate method on Trypticase Soy Agar (TSA) amended with 50 ug/ml of Hg, Cd, Pb, Sn, or Zn. Those metals that were found in high concentration in Pawtuxet River sediment (i.e., Pb, Sn, and Zn) were less inhibitory to the sediment population than either Hg or Cd, present only in low amounts. Metal content of the Pawtuxet River sediment was determined by atomic absorbtion spectrophotometry. Several sediment isolates were selected and resistance patterns to the five metals were determined by the replicate method. A wide range of resistance was found to exist amoung the isolates. Resistance levels of these isolates were approximated by a gradient plate study, the metal concentration ranging from 0-200 ug/g. These tolerance levels were further pinpointed using metal amended Trypticase Soy Broth (TSB) to determine the minimal inhibitory concentration (MIC). The isolates were more resistant to those metals (i.e., Pb, Sn, Zn) that were present in the sediment from which the organisms were isolated.

Unpolluted sediment was subjected to pollution level amounts of a metal mixture containing Hg, Cd, Pb, Sn, and Zn, and Pb alone to determine the response of the microbial flora to the metals. Population levels in the test reactors initially decreased as compared to the nonpolluted control, but recovered later to levels equivalent to or greater than

the control, and resulted in the selection of metal resistant predominant colony types. A cross tolerance study of predominant colony types demonstrated that Pb alone was not inhibitory, but that the metal mixture was toxic. Colony types selected by the metal mixture or Pb were generally homogeneous.

Plasmid screening of selected sediment isolates Flavobacterium PWX2 and Aeromonas PWX7 was accomplished via agarose gel electrophoresis to determine the relationship of plasmids to metal and/or multiple metal resistance. Flavobacterium PWX2, resistant to each metal (Hg, Cd, Pb, Sn, Zn) contained at least five plasmids; the plasmid pattern did not vary when the isolate was grown in each metal. Aeromonas PWX7 contained two or more plasmids, but yielded two distinct plasmid profiles: two plasmids were present when grown in Hg, while only one (the larger of the two found with Hg) was present when the isolate was grown in the presence of Pb or Sn.

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INTRODUCTION

Heavy metal pollution of Narragansett Bay and surrounding waters is a major pollution concern since the Bay receives extensive use as a recreation source and as a primary source for the shellfish industry. The Bay area is densely populated, with more than 90% of the state population living within 10 miles of its boundry. There also are many industrieswhich contribute to metal pollution. Primary industrial metal pollution sources include the electroplating and jewelery industries, sewage disposal, and spent lubricating oil (8). It is estimated that approximately 226 metric tons of spent lube oil enters the Bay from Providence each year via storm drains and sewage treatment effluent. It has been calculated that 19% of this total (44 metric tons) is attributable to dumping spent oil on roads and in sewers (15).

Toxicity of metals that are found in the estuary depends on several environmental and chemical factors. Although at low concentrations many of these metals are necessary for growth, they become toxic at high concentrations. Toxicity is mainly due to denaturation of proteins within the organisms. Toxicity is also dependent on the availability of metal ions to the microorganisms. Availability is determined by how much of the ions are bound to particulate matter or found as insoluble precipitate (12). When metals are bound,

either wholly or in part, a decrease or disappearance of toxicity may result. Humic acids, clays and other organic compounds in sediments strongly bind to metals. In polluted areas, petroleum can complex to metals such as mercury. Mercury levels in the hydrocarbon layer have been shown to be 4000 times more than in sediment, and 300,000 times higher than found in the water column (12). Other metals including zinc, lead, copper, and nickel have been reported concentrated in hydrocarbons found in aquatic systems (6). Organic material found in activated sludge also bind high levels of metals, and is thought to reduce toxicity of heavy metals.

The effects of pH are also important. In general, under acidic conditions metals are found as free ionic cations (12) while at an alkaline pH, ions precipitate as insoluble hydroxides or oxides. Thus low pH increases availability, whereas high pH removes metals from the system. For example, metal polluted mine streams have acidic conditions and metals present are in ionic forms.

The pH also influences the binding of metals by clay, humic acids, and other organic compounds (14). Adsorbtion of metals by humic acids under acidic conditions increases with increased pH, and binding is greater than that of clay. The situation becomes reversed at the critical pH above which the humate-metal complexes begin to become solubilized. The pH range at which maximum adsorbtion occurs depends on the precipitated humic acid and the metal ion involved.

When the pH rises above 6, metal ions become almost totally sorbed and/or precipitated, especially in the absence of bacterial conversion of metals to soluble complexes. Clays play the major role under these conditions, binding strongly to hydroxyl metal species. Thus binding outcompetes solubilization, and the extent of binding again depends on pH and the types of clays and organic compounds involved (14).

Ionic interactions may alter toxicity as well. Other cations can reduce the inhibitory effects of metals; for example, calcium and magnesium are reported to diminish toxicity of zinc, cadmium, and other metals such as nickel, cobalt, manganese, iron, and copper to several bacteria. Anions reduce harmful effects by forming precipitates, which reduces availability of metal ions to bacteria. Hydroxyl, thiosulfate, carbonate and bicarbonate ions participate in this process. Sulfides are also important in forming insoluble precipitates.

Several mechanisms of resistance are known. As previously mentioned, sulfides form insoluble complexes with metals. Many organisms are capable of forming H_2S, and these organisms are very tolerant to metals. Production of sulfides competes for the binding of heavy metals with sulfhydryl groups (R-SH) found in proteins. Thus, as the concentration of sulfides increases, sulfhydryl groups are competitively inhibited from binding with metal ions (Me^{2+}) which may cause denaturation of proteins.

Low sulfide concentration

$$R\text{-}SH + Me^{2+} + H_2S = R\text{-}S\text{-}Me^{+}$$

Denaturation

High sulfide concentration

$$R\text{-}SH + Me^{2+} + H_2S = R\text{-}SH + H_2S\text{-}Me^{+}$$

No denaturation

As they protect themselves, they also protect associated organisms not able to to produce H_2S (12).

Production of organic compounds, which chelate metal ions, reduce metal availability and toxicity. Such extracellular chelates include citric acid, oxalic acid, methionine, and glutathione. Sulfhydryl compounds are produced intracellularly and serve the same function.

Uptake and accumulation can also confer metal tolerance. Nonspecific binding can occur to various portions of the cell surface. Cadmium, zinc, and other metals adhere to polygalacturonic acid, a major component of the cell's outer layer. Slime layers act as an impermeable barrier in which metals are absorbed and precipitated.

Metabolic dependent transport has also been reported. The amount of nonspecific metal binding is not significant in these cases. At high metal concentrations, intracellullar precipitates can occur; detoxification is then able to take place if the ions are compartmentalized and transformed. Transformations of metals will be discussed separately for each metal. Decreased uptake or impermeability also acts to

decrease toxicity (12).

I. Mercury

More data has been obtained on mercury as a toxic pollutant than any other of the heavy metals. This interest stemmed from the mercury poisonings in Japan due to seafood consumption from mercury polluted waters (33). Mercury is widely distributed in the environment at low concentrations, but higher in areas where contamination has occurred (17). Concentrations of mercury up to 0.86 ug/g of sediment are reported from several stations in the Chesapeake Bay (6), but values for mercury levels range up to 14.2 ug/g of sediment (Lonergan, pers. comm.) for the Pawtuxet River outfall into Narragansett Bay. Mercury found in these and other aquatic environments are due to industrial outfall, waste disposal, ship maintenance and antifouling agents, and agricultural runoff. In areas of high contamination, mercury levels increase with depth; the reverse is true in nonpolluted areas. Mercury tends to bind strongly to all suspended materials, especially clays and organic complexes (e.g., humates) (6).

Mercury occurs in many forms, both as inorganic and organic species. Inorganic mercury exists in three valence states; metallic or elemental mercury (Hg^{o}), which is volatile and insoluble in water; mercuric ions (Hg^{2+}) that

form complexes such as $HgCl_4$; and mercuric ore, most common in the form of HgS (cinnabar). Organic forms tend to be more toxic, up to 10 to 100 times more toxic than inorganic species. This is especially true of methylmercury, which shows a long retention time in biological tissues. The metal may enter the food chain at the various trophic levels, where it may ultimately accumulate in fish.

The methylation process can occur both aerobically and anaerobically. It can also occur biotically and abiotically. Three pathways have been proposed for the methylation process. (1) Sediment and sewage inhibitants secrete methylcobalamin (Vitamin B_{12}), which serves as a methyl group donor. (2) Normal microbial flora on the gills and guts of fish are capable of methylation. (3) Totally abiotic process without microbe or microbial byproduct involvement. Many organisms have been shown to methylate inorganic mercury compounds to form methylmercury (24) and methylcobalamin was found to stimulate methylation in some species (17). In Clostridium cochlearium, methylation appears to be a form of detoxification. This organism normally produces methylcobalamin, and when cured of this ability, it becomes more sensitive to inorganic mercury whereas sensitivity to methylmercury is the same for both the parent and mutant strains. The same is true concerning the uptake of mercury compounds. Since no strain of Clostridium cochlearium can form elemental mercury (considered another from of

detoxification), methylmercury formation appears to be the detoxification mechanism (26). Only a small amount of volatile mercury appears to exist in the methylated form, approximately 0.05% (6). Presence in fish of methylmercury has been attributed to among other sources intestinal bacteria present which have the capability of forming methylmercury (32).

Abiotic transformation of mercury to methylmercury is dependant on many variables. Important factors include the presence of ligands, stepwise transformations of several metals, and photochemical reactions. Trimethyltin can methylate several metals including Pb, Au, and Pt. Trimethyllead in turn methylates mercury more readily than does its tin equivalent (6). Transmethylation of metals is also effected by environmental factors such as salinity, or more specifically the chloride ion content, which determines the rate of mercury methylation (4). Photochemically, acetate reacts with mercury in the presence of light to produce methylmercury (19).

Three possible mechanisms of resistance to mercury were considered during early research: synthesis of mercury binding thiols, alteration of permeability, and elimination of the toxic substance by formation of volatile compounds. Mercury volatilization was found to be the detoxification mechanism in all cases studied. In the case of organomercurials, Hg^{2+} is split from the compound by mercuric

lyase, and subsequently converted to the elemental form by mercuric reductase. Recent evidence suggests that mercury methylation is an alternate form of detoxification, previously discussed.

Elemental mercury can be formed from either organic or inorganic mercurial compounds. Elemental mercury (Hg^o) is a volatile form due to the fact that it has a lower vapor pressure than inorganic forms (e.g., $HgCl_2$) and some organic species (e.g., phenylmercury). Many different bacteria have been shown to volatilize mercury at a concentration of 10 ug/ml from growth medium. This can be accomplished both aerobically and anaerobically. Volatilization is more efficient under aerobic conditions, removing 21.5-87.2% of the metal within 24 hours. Under anaerobic conditions, 12.7-78.1% of the mercury is lost from the culture system. The enzyme responsible for this reaction is mercuric reductase, which has been found associated with plasmids. Curing of isolates containing these plasmids resulted in a loss of volatilization and growth. Thus formation of elemental mercury is considered a mechanism of detoxification. The extent of transformation of mercury to Hg^o is not related to growth rate or increase in cell number. Pseudomonas species are reported to be the predominant organism of the mercury resistant isolates of Chesapeake Bay, and play a significant role in the volatilization process. Due to the efficiency of volatiliztion, distribution and

numbers of Pseudomonas and other species, these isolates appear to play a major role in the cycling of inorganic mercury in the environment (24).

Resistance to mercury is plasmid mediated, and has been well documented (23), (24), (32). Resistance has been reported to be conferred chromosomally in one species of Pseudomonas (24). Resistance is always inducible, with no constitutive resistance known to exist (32). Plasmids belong to two general classes depending on the range of resistance for which it codes. Narrow-spectrum plasmids confer resistance to Hg^{2+}, merobromin, and fluorescein mercuric acetate while broad-spectrum plasmids encode for resistance against phenylmercuric acetate, thimerosal, methyl- and ethylmercuric compounds, as well as those substances covered by narrow-spectrum plasmids (32).

Resistance is encoded for by the mer operon, which codes for two enzymes, mercuric reductase and mercuric lyase (32). Moreover, the gene exists as a transposon, which can be transferred as an independent unit. It as been shown that the transposon can be transferred from the chromosome to plasmids containing broad antibiotic resistance. This plasmid can then be integrated onto a chromosome of a second organism of the same or different species, and transferred further to other plasmids. One such element has been found in Escherichia coli. These events all occur independently of host recombination (10).

Mercury resistant isolates were found to transform inorganic and organic species to elemental mercury. These isolates can also methylate inorganic forms (23). Mercury volatilization (i.e., elemental mercury formation) is catalyzed by mercuric reductase, encoded for by the mer operon. This occurs in Pseudomonas, Escherichia coli, and Staphylococcus aureus. This enzyme is found intracellularly in the soluble protein fraction, and is not associated with membranes. The functional moiety is a bound FAD group, which in order to be active requires a thiol reagent.

There is evidence for a second set of enzymes encoded for by the mer gene. Two separate organomercurial lyases have been found in resistant isolates, but are not well understood. Each differs slightly in its properties and substrate preferences. To be active both require a reduced thiol reagent, preferably L-cysteine.

Methylation appears to be mediated by a separate genetic system (23), (24) because although there has been extensive research concerning methylation, there have been no reports to date that the reaction is plasmid mediated.

II. Cadmium

The toxicity of cadmium was first observed as the endemic disease, Itai-Itai, seen in metal mining and smelter areas of Japan. The disease causes pain within the bone

which explains the Japanese name of the disease meaning Ouch-Ouch. It was noted in 1935; the first outbreak caused 100 fatalities, while in a second three deaths occurred. It was not until 1961 that the disease was attributed to cadmium (30). Cadmium is normally relatively rare and is found naturally at low concentrations in the environment (17). Levels in Narragansett Bay and Providence River range up to 2.1 ug/g (34). Most common sources of cadmium include metal processing (especially refining of zinc), mine wastes, electroplating, fertilizers, and pesticides (17).

The nature and extent of biological interactions depend on the species in which cadmium is present (17). Methylated species are more toxic than the cationic (Cd^{2+}) form, but are less stable than methylmercury in the aqueous environment. These species may be of questionable significance as toxic pollutants in aquatic environments, due to their volatility.

Cadmium in the presence of cobalamin was converted to a volatile form, and when this volatile form reacted with Hg^{2+}, methylmercury was produced, which suggests that the volatile compound was methylcadmium. The volatile compound has not been positively identified.

A variety of organisms have exhibited resistance to cadmium. Yeasts, Coryneforms (including the genera Corynebacterium, Brevibacterium, Arthrobacter, and Nocardia) and Pseudomonas, in decreasing order are resistant (7). In a conflicting report, Gram negative organisms are reported to

be more resistant than Gram positive organisms (4).

Plasmid mediated resistance to cadmium has been demonstrated in Staphylococcus aureus to be a stable, inheritable phenotype (32). This phenomenon has not been found in any other Gram negative or positive organisms (27), and appears to be due to an alteration in cadmium uptake, rather than a transformation process. Those strains containing plasmids do not take up as much cadmium as the sensitive plasmidless types.

Resistance is due to two separate genes, cadA and cadB. Transduction of plasmids containing either cadA or cadB into sensitive Staphylococcus aureus resulted in 100 and 10 fold increases in resistance. The cadA gene causes reduced cadmium accumulation due to a cadmium efflux, a constitutive characteristic (27). The efflux is due to an alteration of the energy dependant Mn^{2+} transport system, and the presence of the cadB gene did not alter accumulation. The cadA gene is carried on a penicillinase plasmid, and is located between the mer and penicillinase loci (21). Resistance does not depend on prior growth on cadmium. The biological mechanism for this process is not known.

The cadB gene produces a substance that appears to bind cadmium in energy inhibited cells (27). Little else is known about this gene as it is always masked by cadA characteristics.

III. Lead

Lead has been found to occur in Narragansett Bay sediments in levels up to 44 ug/g (8), and to 192 ug/g in the Providence River sediment (34). Major contributors include leaded gasolines, spent lube oils, lead paint and pipes. This metal tends to accumulate in sediment and sewage sludge. It also becomes concentrated when absorbed into tissue, more so than either mercury or cadmium (17).

Lead appears as both organic and inorganic species. Some of the more common lead forms are methylated lead compounds. The methylated forms are more toxic than the inorganic compounds. Like methylcadmium, these methylated forms are less stable in the aqueous phase than methylmercury (32). Tetramethyllead is the most common methylated form. This compound can be formed in sediment by the addition of trimethyllead acetate in all cases, and in some cases by trimethyllead nitrate (17). Lead oxides have been reported to demethylate methylcobalamin, which is an important methyl source for many metals. This reaction proceeds more rapidly under dilute acidic conditions than in water (33).

Inorganic lead salts react very slowly with methylcobalamin to produce several compounds including tri- and tetramethyllead.

Trimethyllead is a capable methylator, transforming several nontoxic metals and mercury to their methylated

forms. This compound methylates more readily than trimethyltin. Strong anions including CN^- and SCN^- retard trimethyllead transformation of mercury.

Toxicity of lead has been shown to be a function of speciation (11). The availability of lead is determined by pH, and phosphate and lead concentrations. At a pH of 6 and above little lead is in its free ionic form at high phosphate concentration, regardless of the lead levels. As phosphate levels decrease, there is a corresponding increase in the ionic lead concentration. Ionic lead is not expected or seen at pH 8 at any phosphate concentration. Increased levels of phosphate exhibit a detoxifying effect.

Several types of bacteria have been shown to transform lead. Pseudomonas, Acinetobacter, Flavobacter, and Aeromonas have been shown to transform trimethyllead acetate to tetramethyllead (17), (32). Tetramethyllead was not formed from inorganic compounds on defined media by these groups (17). Lead binding by the cell envelope in resistant Aeromonas has been reported, but the mechanism of methylation is not known. It has been proposed that lead is likely to undergo methylation in vivo.

In lead resistant strains, it has been suggested that lead ions are precipitated on the cell surface in the form of phosphate (1). Plasmid mediated resistance to inorganic lead compounds have been found in Staphylococcus aureus. The plasmid confers resistance to penicillin as well as to a

variety of other metals. These resistance markers appear to form a cluster on the plasmid, with no other markers within it (22). No evidence exists that the resistance involves the transformation of lead (32).

IV. Tin

Tin concentrations range up to 97 ug/g for Narragansett Bay and the Providence River sediments (34). Primary sources include tin plated containers, solder, and organotin compounds found in fungicides and antifouling agents (17).

Tin is found as several different compounds, organic forms being more toxic than inorganic or elemental forms. The toxicity of tin is enhanced in combination with organic groups, the toxic effects varying according to the type and number of organic constituents. Alkyl groups are more toxic than aryls, and triorganotins are more harmful than di- or tetraorgano forms.

Several tin compounds have been reported to be biomethylated. A Pseudomonas strain capable of methylating cadmium appears able to produce methyltin, a volatile form (17), (32). Methyltin is more toxic than inorganic forms, and like other metals it is less stable in water than methylmercury. The mechanism of methylation is unknown.

Tin compounds also participate in transformation reactions. Butyl and phenyltin compounds are transformed to

pure trimethyltin, and no other organotins are produced. Biomethylated tin can abiotically methylate mercury to form methylmercury (17).

Toxicity of inorganic tin has been observed to increase with the use of agar as a gelling agent (13). Gelatin and silica gel added to the medium as solidifying agent decreased toxicity. No similar effect have has been noted with any other metals.

There is no evidence to date of any plasmid mediated tin resistance.

V. Zinc

Concentrations up to 277 ug/g have been reported for zinc in the Narragansett Bay and Providence River sediments (34). Little data has been reported on the metal concerning methylation, toxicity, or resistance mechanisms. A possible relationship does exist concerning gene products in cadmium resistant isolates causing an increase in zinc tolerance (27). No further work has been done in this area.

A topic of major concern relating to heavy metal tolerant organisms is the association of antibiotic resistance. Both single and multiple resistances have been demonstrated for a variety of antibiotics (2).

Reports of resistant organisms include clinical

isolates, domestic sewage inhibitants, as well as estuary strains. Staphylococcus aureus is reported resistant to the antibiotics penicillin, erythromycin, and tetracycline, while at the same time tolerant to mercury, lead, cadmium, and zinc (7). Pseudomonas aeruginosa and some enteric strains show similar resistance patterns (32).

Estuary isolates of Bacillus and Mycobacterium species, as well as Pseudomonads and Coryneforms were found to be resistant to metals including mercury, lead, cadmium, and to several nonheavy metals such as molybdenum and arsenic. Resistance to many commonly used antibiotics were also reported. Resistance to ampicillin and chloramphenicol were most frequent, while an intermediate level of resistance was observed toward streptomycin. Isolates were least resistant to kanomycin, gentamycin, and tetracycline. Different taxa showed varied resistance patterns that differed according to metal tolerance.

It has been shown that there is a high correlation between metal and antibiotic resistance (2), (7) and that these resistances have in some case been found on the same plasmid (22). A major health hazard potentially exists regardless if metal and antibiotic tolerances occur on the same or more than one plasmid. A second health hazard that has occurred is the accumulation of heavy metals throughout

food chains (bioamplification). Concentrations may reach lethal levels in higher trophic levels. This is best illustrated by consumption of tainted seafood from mercury polluted waters and sediments in Japan. Minamata disease is a severe neurological disorder caused by prolonged methylmercury exposure. This form of mercury was found to concentrated in fish and shellfish, and reached high enough concentrations to prove fatal. Between 1953 and 1970, many outbreaks occurred, resulting in 43 deaths. A second incident later claimed six lives.

The accumulation in fish is not well understood. Proposed mechanisms (32) include (1) direct adsorbtion of methylmercury (possibly microbially produced) from the water column and subsequent concentration due to long retention; (2) direct methylation of inorganic compound by fish tissue; (3) formation of methylated compounds by normal fish flora; and (4) ingestion of preformed methylmercury from the food supply. Bacteria are considered to be at the bottom of the chain in this last mechanism and therefore important (17).

Cadmium is also concentrated at various trophic levels. Bacteria accumulate cadmium in the cell wall and membrane, which may lead to further concentration in its predators. Shellfish concentrate cadmium primarily in the liver, gills, and kidneys. Bioamplification is not evident on the whole organism level (30).

Although there are no other reports of other heavy metal

accumulation within food chains, the possibility does exist. Metals show a variety of similar characteristics, and metal pollutant accumulation should not be ignored as a possible health threat. More research is required to determine if this is a legitimate problem.

MATERIALS AND METHODS

I. STUDY OF INHIBITORY EFFECTS OF HEAVY METALS ON EXPRESSED MICROBIAL SEDIMENT POPULATIONS

a. Sample collection

Samples of Pawtuxet River sediment were obtained from the shoreline of Pawtuxet Cove 200 meters south of the river mouth, and placed in sterile plastic bags (Nasco Whirl-Pac, Cole-Parmer Instrument Co., Chicago, Ill.). The temperature of the sediment, as well as the pH and salinity of the water were recorded.

b. Sample preparation and CFU number determination

One gram wet weight of sediment was weighed and placed into a 9 ml sterile distilled water blank. From this initial 1:10 dilution A series of 1:10 dilutions were then made, from which 0.1 ml of the proper dilutions were spread plated onto Trypticase Soy Agar (TSA, BBL, Cockeysville, MD.), and to TSA amended with 50 ug/ml of Hg, Cd, Pb, Sn, and Zn. Each metal was kept as a stock solution of 10,000 ug/ml at a pH of 2. All metals except Pb were in an inorganic salt form: $HgCl_2$,

$CdCl_2x2.5H_2O$ (Mallinckrodt Chemical Works, St. Louis, Mo.), $SnCl_2x2H_2O$ (Matheson, Coleman, and Bell, Norwood, OH), and $ZnCl_2$ (Fisher Scientific Co., Fairlawn, NJ). Pb was in the form of an organic salt, $Pb(C_2H_3O_2)_2x3H_2O$ (Mallinckrodt). NH_4NO_3 (Fisher) was added to the medium as an inorganic nitrogen source at a concentration of 0.25% w/v.

Plates were then incubated under anoxic conditions at the ambient temperature of the sample at the time of collection in a National Appliance Company (Portland, Oregon) anaerobic incubator flushed three times with N_2 (final oxygen content <0.02% by volume). The plates were then counted for total CFU number, with the TSA plates acting as controls (equivalent of 100%). The percentage of organisms that grew on TSA plus each metal indicated the effect of the metals on growth of the expressed population.

II. STUDY OF HEAVY METAL RESISTANCE PHENOMENON IN SELECTED ISOLATES

a. Determination of resistance patterns

The replicate plate method was used to select sediment isolates resistant to various combinations of Hg, Cd, Pb, Sn, and Zn. The spread plate method was employed to obtain countable CFUs on TSA, to be used as a master plate. From this plate replicates were made to TSA plus Hg, Cd, Pb, Sn, and Zn at 50 ug/ml of each metal ion. As a check for replication, a final replicate was made to TSA. Isolates

were then selected yielding a variety of resistance patterns to the five metals and demonstrating the phenomenon of multiple metal resistance.

b. Approximate tolerance levels to heavy metals

The gradient plate method was employed to determine the selected isolates' tolerance to each metal. The gradient ranged from 0-200 ug/ml of Hg, Cd, Pb, Sn, and Zn. TSA was used as a base, and was poured into an angled petri dish. After the base had solidified it was overlaid with TSA amended with 200 ug/ml of each metal to obtain the gradient. A single streak inoculum was streaked across the gradient, and the point at which growth ceased indicated the approximate level of resistance to that metal.

c. Minimal inhibitory concentration (MIC)

Using approximate tolerance values, a narrow range of metal concentrations were tested to more accurately determine the isolates' tolerance levels. A series of five metal amounts were added to 10 ml of Trypticase Soy Broth (TSB, BBL) in acid washed glassware, and plain TSB served as a control. Growth was read as positive or negative; the lowest concentration of metal in which no growth occurred was the minimal inhibitory concentration (MIC).

III. DETERMINATION OF METAL CONCENTRATION IN SEDIMENTS

In order to determine the amount of metals found in sediment, the metals must be eluted from the sediment by an

acid hydrolysis treatment. Only at a low pH will the metals become solubilized and not adsorb to sediment particles or the glass container.

a. Sample preparation

1. Dry an aliquot of sediment for 48 hours at room temperature, being sure that there will be at least 1.0 g of sediment dry weight.

2. Weigh out 1.0 g of dried sediment, and place into 150 ml beaker. Add 50 ml distilled water and 3.0 ml concentrated HNO_3 (Allied Chemical, Morristown, NJ).

3. Take to dryness on hot plate without boiling (approximately two hours).

4. Add 5.0 ml concentrated HNO_3, cover with watch glass, and reflux gently until residue becomes lighter in color (approximately two hours).

5. Cool, and add 5.0 ml 1:1 HCl (Allied Chemical) and H_2O and return to hot plate to dissolve residue.

6. Wash down beaker and watch glass with distilled water, and filter through acid washed (0.1N HCl, Allied Chemical) filter paper. If there is less than 25 ml filtrate, bring to volume in volumetric flask.

b. Atomic Absorbtion Spectrophotometry

Metal determinations were performed on a Perkin-Elmer Atomic Absorbtion Spectrophotometer (Model 5000, Analytical Instruments, Norwalk, Ct.). The levels of the metals Cd, Pb, Sn, and Zn were determined by flame ionization detection

(Analytical Methods for Atomic Absorption Spectrophotometry, Perkin-Elmer). Hg was be detected by the flameless hydride reduction method (MHS-10 Mercury/Hydride System, Perkin-Elmer) using sodium borohydride (Fisher). For each method, five readings of a sample were taken, and an average value calculated.

IV. PRESENCE OF PLASMIDS IN SELECTED ISOLATES

a. Culture conditions and storage

Selected isolates were carried as frozen permanent stocks in TSB to which 1.5% glycerol (Mallinckrodt) was added. The glycerol lowers the freezing point of the culture broth, preventing cell destruction during the freezing process. TSB and metal amended (50 ug/ml) TSB were inoculated and incubated overnight to log phase. Sterile glycerol was added and the cultures were stored at -18°C. Working stocks were carried on TSA slants stored at 4°C, and transfered each month.

b. Inoculum and preparation for plasmid screening

Ten ml of TSB was inoculated from working cultures and incubated overnight at 22°C. An 0.5 ml aliquot of the overnight culture was used to inoculate 50 ml of TSB and 50 ug/ml metal amended TSB in acid washed 250 ml Erlenmeyer flasks. Cultures were incubated at 22°C and shaken at 200 rpm in a PsychroTherm Incubator (New Brunswick Scientific Co., Inc., New Brunswick, NJ) overnight to provide cells in

log phase growth. Chloramphenicol (Sigma Chemical Co., St. Louis, Mo.), (34 mg/ml in ethyl alcohol) was filter sterilized and 0.25 ml was added to each flask to amplify the number of plasmid copies per cell. The chloramphenicol stock was stored at $4^{o}C$. After addition of chloramphenicol the flasks were incubated an additional 12-16 hours at $22^{o}C$ at 200 rpm.

c. Nucleic acid extraction

The procedure for plasmid extraction was obtained from Birnboim and Doly (4) as modified by Ish-Horowicz and Burke (16).

1. Ten ml of amplified culture was centrifuged (International Clinical Centrifuge, Model CL, Needham, Ma.) for 5 minutes at approximately 3000 rpm and the supernatant discarded.

2. Resuspend cell pellet in 0.2 ml SOLUTION I, to which is added 20 mg/ml of freshly prepared lysozyme (Sigma Chemical Co.).

SOLUTION I can be stored at $4^{o}C$ after autoclaving.

Composition of SOLUTION I:

50 mM Glucose (J.T. Baker Chemical Co., Phillipsburg, NJ)

25 mM Tris-HCl (Sigma Chemical Co.)

10 mM Na_2EDTA (Sigma)

3. Transfer the suspended cells to a sterile 1.5 ml Eppendorf tube and let stand for 5 minutes at room

temperature.

4. Add 0.4 ml of freshly prepared SOLUTION II, invert gently to mix, let stand on ice for 10 minutes.

Composition SOLUTION II:

0.2N NaOH (Fisher)

1% Sodium dodecyl sulfate (SDS, Sigma)

Make SOLUTION II fresh from stocks of 10N NaOH and 20% SDS for each experiment.

5. Add 0.3 ml of SOLUTION III, invert sharply to mix, and let stand on ice for 10 minutes.

Composition SOLUTION III:

60 ml 5M Potassium Acetate (Mallinckrodt)

11.5 ml glacial acetic acid (Allied Chemical, Morristown, NJ)

28.5 ml sterile distilled water

6. Centrifuge (Sorvall RC-5B, DuPont Instruments, Norwalk, Ct., SS-34 rotor) for 20 minutes at 12,000xg. A tight pellet should form.

7. Transfer 180 ul of the supernatant to a sterile 1.5 ml Eppendorf tube.

8. Add 120 ul of ice cold isopropanol to each tube, mix well and precipitate DNA for 15-60 minutes at -18°C.

9. Centrifuge (Fisher Micro-Centrifuge, Model 235A) for 2 minutes at room temperature at 15,000xg.

10. Discard the supernatant, and dry the nucleic acid

pellet with a gentle flow of gas (N_2, or CO_2).

11. Dissolve the pellet in 100 ul of TE 7.5 buffer.

Composition TE 7.5 buffer:

10 mM Tris (Sigma)

1 mM Na_2EDTA

pH 7.5

12. Resuspended pellet is stored at 4^oC until analyzed.

d. Agarose gel electrophoresis

Preparation of the gel

To 200 ml of Tris-borate buffer add 1.6 g of agarose M (LKB 2206-101, LKB-Produkter AB, Bromma, Sweden) to yield a 0.8% gel concentration. The solution is brought to boiling for 5 minutes on a magnetic stirrer. Add 10 ul of 0.5 mg/ml stock of ethidium bromide (EtBr, Sigma) per 200 ml of gel solution and Tris-borate buffer. The gel is now ready to be poured.

Composition of Tris-borate buffer

10.7 g Tris (Sigma)

5.5 g Boric Acid (Sigma)

0.84 g Na_2EDTA (Sigma)

1 liter distilled H_2O

pH 8.3

Samples of extracted nucleic acid are prepared as follows for electrophoresis:

1. Add 4 ul of a heat treated 1 mg/ml RNAse A

(Sigma) stock to a sterile 1.5 ml Eppendorf tubes and place on ice. The RNAse stock is stored at -18°C between use. The RNAse was boiled for 5 minutes in a water bath to inactivate any DNAse present.

2. To each tube add 6 ul of 0.1% v/v bromphenol blue (BPB, Sigma) in 40% glycerol, mix, and place on ice. The BPB stock solution was stored at 4°C.

3. Add 20 ul of each extract to the proper tubes and mix thoroughly.

d.1 Running nucleic acid through the gel

Once the gel was solidified, 20 ul of each prepared sample was loaded per well. Voltage from the power unit (Buchler 3-1500, Buchler Instruments, Inc., Fort Lee, NJ) was set at a maximum of 5 volts/cm (i.e., 100v for 200 cm gel). The gel was run at room temperature, but should not exceed 30°C.

d.2 Viewing and photographing the gel

The gel is examined on a UV box (Transilluminator Model TM-36, Ultra-Violet Products, Inc., San Gabriel, Ca.). Photographs were taken with a Polaroid MP-3 Land Camera, using Polaroid 4x5 Land Film, Type 57 (Cambridge, Ma.). A 75 cm x 75 cm Wratten Gelatin Filter No. 9 (Eastman Kodak Co., Rochester, NY) was employed to filter most of the UV light.

d.3 Determination of plasmid molecular weights

Molecular weights of extracted plasmids were determined by comparison to markers of known molecular weights. A Hind-III digest of Lambda DNA (New England BioLabs, Beverly, Ma.) has seven fragments of known molecular weights. The size of these fragments are:

Kilobase (kb)	Molecular weight (daltons)
23.3	15.1
9.5	6.2
6.4	4.2
4.2	2.7
2.2	1.4
1.8	1.2
0.53	0.34

There is an inverse relationship between the migration of DNA of the covalently closed circular form (CCC) and the molecular weight (Meyers et al). Molecular weights of the environmental isolates' plasmids were calculated by plotting the logarithm of relative migration of the known marker bands versus the logarithm of the known molecular weights (Hind III digest), obtaining a linear standard curve. From this standard curve the weight of unknown plasmids could be determined.

V. POPULATION DYNAMICS

To determine the role of heavy metal pollution in population dynamics, organisms from the unpolluted sediments

and water of the Succotash Salt Marsh were subjected to pollution levels of the heavy metals found at a polluted site. Metal levels in the unpolluted sediment were determined by atomic absorbtion spectrophotometry and were found only in trace quantities (Lonergan, pers. comm.). The following protocol was used:

1. Line three 10 gallon aquaria with plastic bags, and fill with 3-4" of salt marsh sediment. To each tank add 9 liters of salt marsh water, and add:

(1) 1 liter H_2O to the first tank to serve as a control.

(2) Add each metal at concentrations found at the Pawtuxet River site to the second tank as follows:

Metal	Concentration (ug/ml)	Amount 10,000 ug/ml Stock Solution
Hg, Cd	100*	100 ml
Pb	320**	320 ml
Sn	197	197 ml
Zn	135	135 ml

Bring total volume to 10 liters.

*Hg, and Cd levels are much higher than those found in Pawtuxet River sediments.

**Lead acetate used in series 1, lead oxide in series 2.

The concentration of lead was lower than the level found at Pawtuxet due to low solubility.

(3) To the third tank add 320 ml of 10,000 ug/ml Pb stock.

Bring total volume to 10 liters.

2. Provide aeration for each tank, and cover with plastic to prevent evaporation.

3. Sediment samples are taken aseptically, and one gram wet weight of sediment was placed into 9 ml sterile distilled H_2O. From this intitial 1:10 dilution, a series of 1:10 dilutions were made, from which 0.1 ml of the proper dilutions spread plated on appropriate media: control sediment to TSA, metals polluted sediment to TSA + metal mixture, and lead polluted sediment to TSA + Pb. Metal concentrations for the media were the at same levels as found in the tanks. This routine was repeated daily intil a proper dilution series was developed.

4. Plates were incubated at room temperature, and CFU numbers as well as colony types and frequencies were recorded.

VI. CROSS TOLERANCE STUDY

The predominant colony types from each sediment (control, all metals, and lead) were tested to determine if the organisms could tolerate the environments afforded by each of the other two sediments. The procedure used is as follows:

1. The predominant colony types were transferred by sterile toothpicks from the dynamics study spread plates to TSB containing nothing (control), a metal mixture, and lead.

2. Look for growth (positive) or lack of growth (negative) in each tube, and report as + or -.

This test determined if the isolates could tolerate conditions other than in which they were grown.

VII. ORGANISM IDENTIFICATION

Identification of selected isolates was determined using morphological, cultural, and biochemical characteristics. Each isolate was identified to genus using two primary sources: Bergey's Manual of Determinative Bacteriology (Buchannan and Gibbons, 1974); and A Guide to the Identification of the Genera of Bacteria (Skerman, 1959). Isolate PWX2 has been keyed to the genus Flavobacterium, and PWX7 was keyed to the genus Aeromonas. The results of the biochemical tests may be found in appendix A.

RESULTS AND DISCUSSION

A majority of the environmental parameters of the Pawtuxet River sediments (Table 1) remained constant throughout the sampling period. The temperature ranged from 1^{o} to $8^{o}C$, well within the defined psychrophilic temperature

Table 1. Environmental parameters of Pawtuxet River sediment and water.

DATE	TIME	TIDE	TEMPERATURE (°C)	% SALINITY	pH	Eh (mV)
2-9-82	12:00 N	Low	3°	0.4	6.57	NR*
2-12-82	12:50 PM	Low	1°	0.4	6.05	NR
2-19-82	10:00 AM	Low	1°	0.4	6.25	+175
3-5-82	10:40 AM	Low	4°	0.05	6.30	+250
3-26-82	10:45 AM	High	8°	0.4	6.20	-255

*NR-not recorded

range. On each test date, the pH was between 6.0 and 6.5, and with one exception salinity remained at 0.4%. The tide conditions seemed only to effect Eh readings: for the values recorded, the Eh was positive at low tide indicating aerobic conditions, while negative values were observed at high tide indicating anaerobiosis. Thus the sample site represents a uniform environment with the exception of the 3-26-82 sample where the Eh was drastically different from the other samples.

Sediment populations from the metal polluted Pawtuxet River were tested for metal resistance on TSA containing 50 ug/ml of Hg, Cd, Pb, Sn, or Zn (Figure 1, Table 2). Samples were incubated at both room and ambient (temperature of the sample at time of collection) temperature. Hg and Cd both exhibited significant inhibition of colony expression (CE); the mean CE reached only a maximum of 10.5% under room or ambient temperature incubation for Hg, while Cd allowed the maximum average of 20.4% growth under either incubation condition. The other three metals Pb, Sn, and Zn showed little to moderate inhibitory effects on mean CE values. At room temperature, Pb allowed 49.1% CE, while under ambient incubation 95.0% CE took place. Sn was less inhibitory yielding 61.4% CE at room temperature, while under ambient conditions inhibition was depressed as the mean CE reached 96.3%. The CE values for Zn were 87.9% and 43.9% under room

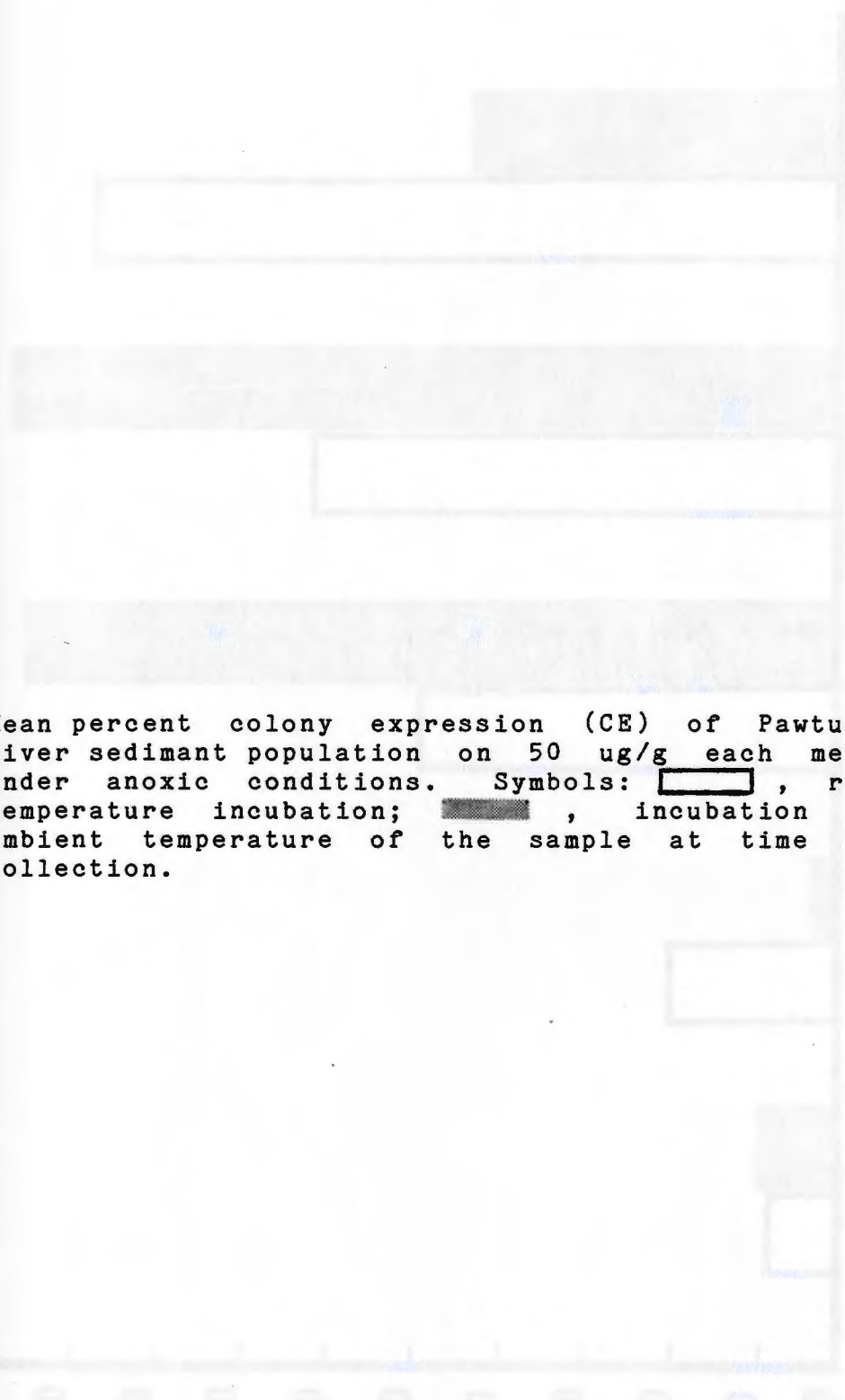


Fig. 1. Mean percent colony expression (CE) of Pawtuxet River sedimant population on 50 ug/g each metal under anoxic conditions. Symbols: ☐, room temperature incubation; ▒, incubation at ambient temperature of the sample at time of collection.

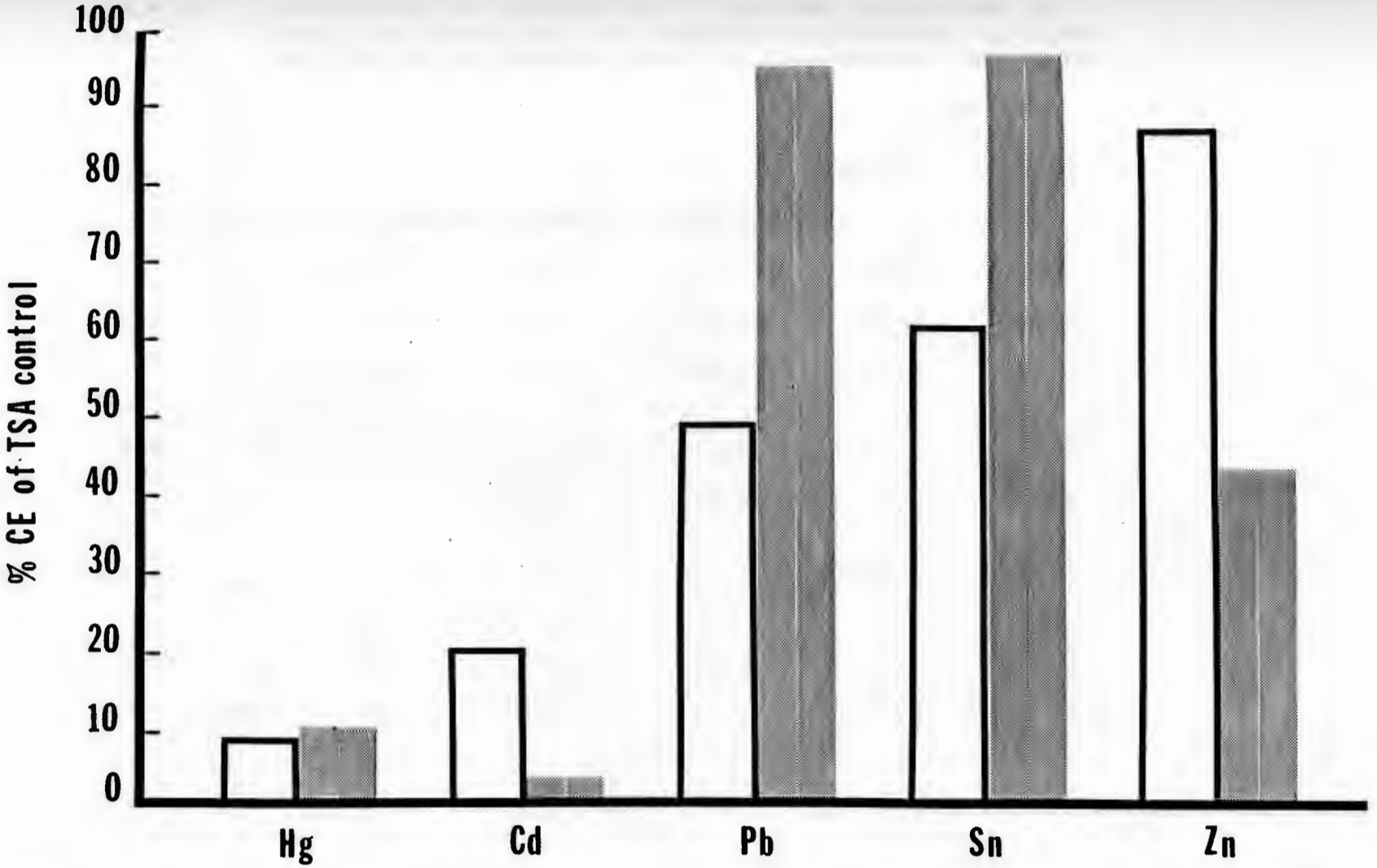
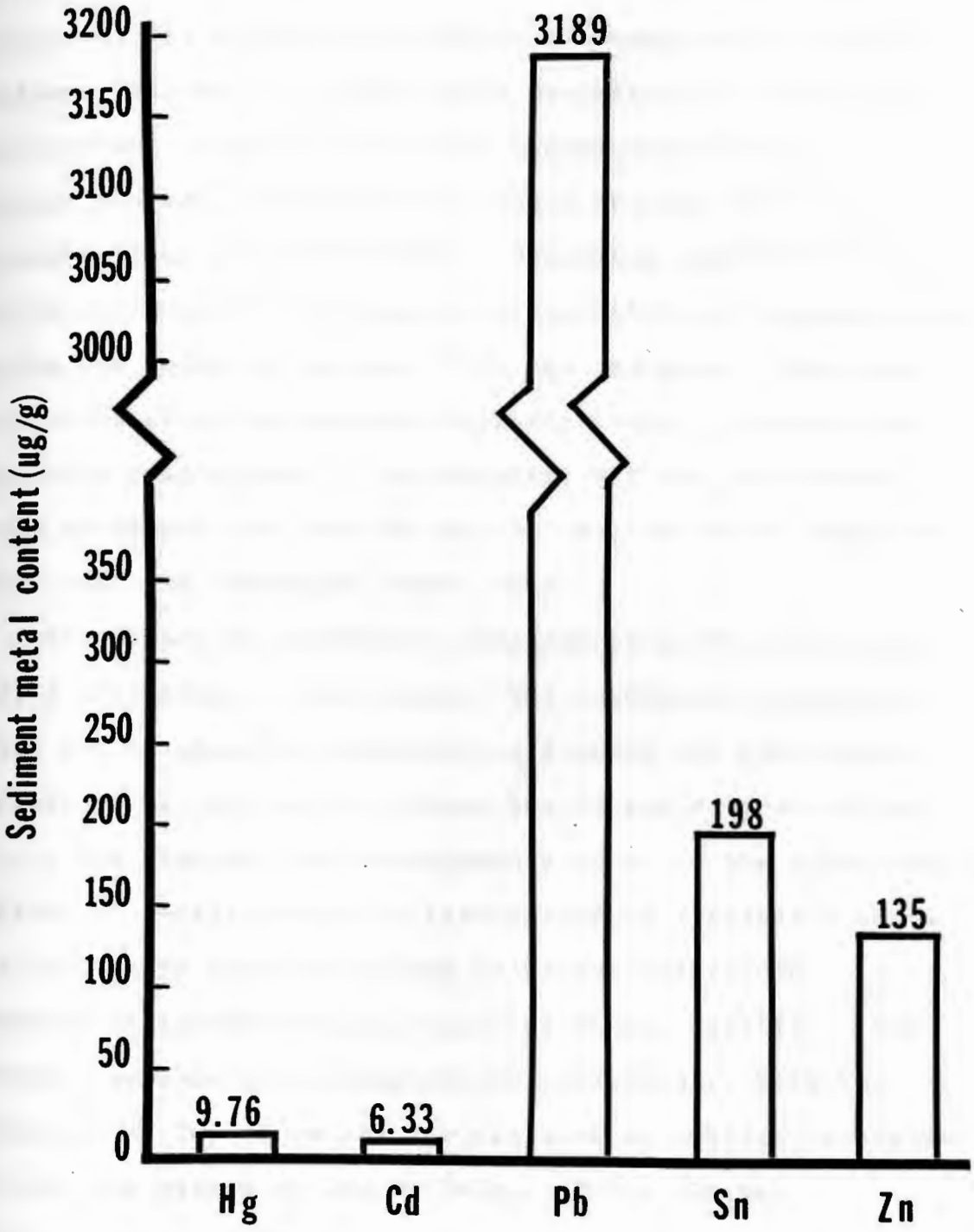

% CE of TSA control
100
90
80
70
60
50
40
30
20
10
0
Hg
Cd
Pb
Sn
Zn

Table 2. Expression of Pawtuxet River sediment population on metal isolation media at ambient (temperature of sample at time of collection) and room temperature incubation.

Temperature of Isolation — RT-Room temperature; AMT-Ambient tempurature

Date	Isolation	CE Values on TSA + 50 ug/g of: Hg^{+2}	Cd^{+2}	Pb^{+2}	Sn^{+2}	Zn^{+2}
2-9	RT	11.8	54.6	74.0	88.3	101.7
	AMT	0.0	0.0	49.2	54.2	10.5
2-12	RT	2.7	0.0	13.9	81.7	67.3
	AMT	NC	NC	45.6	0.0	0.0
2-19	AMT	18.9	0.0	208.9	202.7	154.0
3-26	RT	12.6	6.5	59.3	14.1	94.9
	AMT	12.7	12.5	76.3	128.2	10.9

Fig. 2. Heavy metal content of Pawtuxet River sediment determined by atomic absorbtion spectrophotometry (n=3).


Sediment metal content (ug/g)
3200
3150
3100
3050
3000
350
300
250
200
150
100
50
0
3189
198
135
9.76
6.33
Hg
Cd
Pb
Sn
Zn

temperature and ambient incubation conditions respectively.

The moderate to high percentage of resistances in the sediment populations to Pb, Sn, and Zn were anticipated because of the high concentrations of these metals in the sediment (Figure 2). Since metal resistance is induced by the presence of metals one would expect selection for populations resistant to these metals present at high concentrations in the sediment. Organisms resistant to Hg and Cd would not be expected to be selected as frequently due to the low levels of Hg and Cd in the sediment. This data is consistant with the previous investigations of aerobic and anaerobic populations of Narragansett Bay and Providence River sediments (35) and the aerobic population of Pawtuxet River sediment (Lonergan, pers. comm.).

The effect of incubation temperature on CE inhibition varied according to each metal. The incubation temperature could not be shown to stimulate or depress the inhibitory effects of Hg, Cd, or Pb. Since the Eh was determined for only a few samples, the relationship of Eh to the inhibitory effects of metals should be interpreted as a possible trend. The inhibitory value of Sn was increased under room temperature incubation only when the Eh was positive. The reverse was true under negative Eh conditions. With the exception of Zn, CE values are elevated at ambient incubation for all the metals at low Eh (e.g., -255). Zn was consistantly more inhibitory at room temperature regardless

of the Eh value.

Metal levels of Pawtuxet River sediment were determined by atomic absorbtion spectrophotometry (Figure 2). An average of three sediment samples yielded the following values (ug/g): the Pb level was 3189, Sn was 198, and Zn at 135. With the exception of Zn, each of these values were much higher than reported for other Narragansett study sites (Eisler, et al, 1977) and several magnitudes higher than reported control sites such as the Philadelphia oceans control site. Hg (9.76 ug/g) and Cd (6.3 ug/g) were present in relatively low levels, but were still above values found in the Providence River.

Resistance patterns for selected isolates toward 50 ug/g of each metal were determined by the velvetine replicate plate method (Table 3). This technique poses a problem due to lack of consistancy of replication of a given colony throughout a series of plating media. Therefore, the resistance patterns of colonies to metals determined by this method may not always be accurate (e.g., lack of replication of a colony on a particular metal amended medium might incorrectly be interpreted as sensitivity to that metal). Several isolates were chosen yielding variable resistance patterns ranging from complete sensitivity to total resistance to all metals. Several different colony types

Table 3. Resistance patterns of selected sediment isolates to TSA amended with 50 ug/g of Hg, Cd, Pb, Sn, or Zn as determined by the replicate plate method.

Isolate	Hg	Cd	Pb	Sn	Zn
PWX1	S	S	S	S	S
PWX2	R	S	R	R	S
PWX3	S	R	S	R	R
PWX4	S	S	S	R	R
PWX5	S	R	R	R	R
PWX6	R	R	R	R	R
PWX7	R	S	R	R	S

S-sensitive to $\leq$50 ug/ml of metal

R-resistant

Isolate	Colony morphology
PWX1	Large, opaque, round, yellow center, entire.
PWX2	Small, opaque, round, convex, entire.
PWX3	Large, fried egg (white with yellow center), round, convex, entire.
PWX4	Small, yellow-white, round, opaque, convex.
PWX5	Large, opaque, round, yellow center, entire.
PWX6	Large, clear with brown center, convex, entire.
PWX7	Large, clear with brown center, convex, entire.

were observed, and in two cases the same colony types (isolates PWX1 and 5, and isolate PWX6 and Aeromonas PWX7) had different resistance patterns. Each of the seven resistant isolates exhibited resistance to two or more of the metals tested thus suggesting that isolates from a site polluted with a variety of metals most often demonstrate multiple metal resistance. Resistance patterns of the selected isolates were widely varied, ranging from complete sensitivity of PWX1 to 50 ug/g of each metal to total resistance by PWX6 to the same metals. Sensitivity to Hg and Cd was observed in four of the seven isolates, while only isolate PWX1 expressed sensitivity to 50 ug/g of Sn.

Each of the above isolates were tested by the gradient plate method to determine the approximate level of metal resistance over a range of 0 to 200 ug/g of metal (Table 4). With the exception of isolate PWX6, which showed low resistance levels to all metals, tolerance to Pb, Sn, and Zn were significantly higher than toward Hg, and Cd. Resistance to Pb, Sn, and Zn ranged between 90 to $\geq$200 ug/g, while Hg and Cd tolerance ranged from sensitivity of $\leq$50 ug/g to 90 ug/g. These data indicate that organisms are more tolerant to the metals that are present in the polluted sediments from which they were isolated.

Using the values obtained from the gradient plate experiment, a more accurate resistance value was determined

Table 4. Approximate metal tolerance levels of selected sediment isolates when exposed to 0-200 ug/g of metal amended TSA gradient plate.

Isolate	Hg	Cd	Pb	Sn	Zn
PWX1	S	S	110	90	190
PWX2	90	75	≥200	≥200	≥200
PWX3	15	25	≥200	≥200	110
PWX5	30	10	190	125	95
PWX6	S	20	S	30	15
PWX7	35	40	≥200	≥200	≥200

by growing the isolates in a series of metal concentrations in TSB (Table 5). The lowest value inhibiting growth was the minimal inhibitory concentration (MIC). Generally, the isolates were more tolerant to each metal in the liquid medium system than on the gradient plates. Hg and Cd were found more inhibitory than the other metals. Sensitivity to Hg ranged from between 25-50 ug/g for isolate PWX3 to >500 ug/g in Flavobacterium PWX2 and Aeromonas PWX7. Cd was less inhibitory as each isolate was resistant to >500 ug/g, with the exception of PWX3 that could tolerate only 25-50 ug/g. Tolerance to metal levels exceding 500 ug/ml, the highest concentration tested, were observed in isolates PWX1, 3, 5, Flavobacterium PWX2 and Aeromonas PWX7 with respect to Pb, Sn, and Zn.

Resistance values were greater than the gradient plate values when the organisms were grown in metal amended TSB. This phenomenon has been previously reported for Sn (13), but has not bqen observed for any other metal to date. In the case of Sn the enhanced toxicity in the presence of agar-agar was accredited to the formation of organotin compounds with greater toxicity than cation forms of the metal.

POPULATION DYNAMICS

The data from the polluted sediments demonstrated the high incidence of metal resistant bacteria in these sediments It was desired, therefore, to examine the population dynamics

Table 5. Minimal Inhibitory Concentrations (MIC) of Hg, Cd, Pb, Sn, and Zn on selected sediment isolates up to 500 ug/g.

Isolate	Hg	Cd	Pb	Sn	Zn
PWX1	75-100	>500	>500	>500	>500
PWX2	≥500	>500	>500	>500	>500
PWX3	25-50	>500	>500	>500	>500
PWX5	75-100	>500	>500	>500	>500
PWX7	>500	>500	>500	>500	>500

in a freshly polluted sediment to determine the nature of acquired resistance. Colonies were typed by morphology (Table 7) to monitor the changes in predominant colonies within the population. Sediment from the Succotash Salt Marsh was used for this series of experiments as it represents a non-metal polluted source. Three tanks were used in each series; a control, a Pb polluted tank, and a tank receiving a mixture of metals representative of the Pawtuxet River Site.

In the first experiment (series 1) lead was added to tanks 2 and 3 as lead acetate which added a potential nutrient supplement of 0.094 g/l of acetate. The sediments were not mixed by stirring subsequent to the addition of Pb or the metal mixture. The total of colony forming units (CFU) in the control tank were initially higher than in the metal mixture or lead polluted sediments (Figure 3, Table 6). The total control CFU peaked on day 6 at 2.45×10^4 organisms/ml, with a secondary peak occurring on day 17 of 6.8×10^3 organisms/ml. The significance of these peaks may be questionable, since there was a general decline in CFU values throughout the study period. There was a general decline in expressible CFU in the control tank throughout the sampling period. Predominant colony types fluctuated, with type A most frequently predominating throughout the test period (Figures 4a-k, Table 8).

Addition of the metal mixture to the sediment depressed

initial CFU levels 1-1.6 logs lower than the control, which continued until day 5, when the expressible population began its recovery. After 13 days the CFU reached a maximum value of 8.26×10^4 organisms/ml, approximately the initial value of the control sediment. A significant and rapid decline occurred for the remainder of the sampling period. Predominant colony types (Figures 4a-k, Table 8) in the polluted reactor showed less variation than the control, with types A and H being the most common isolates.

The initial number of CFU wase also depressed in lead polluted sediments, but to a lesser degree than with the metal mixture. The total CFU quickly increased so that by day 2 control values are surpassed by 1.27 logs to a peak value of 2.27×10^7 organisms/ml after 10 days. CFU levels began to decline after 10 days at approximately the same rate as that of the control. After three days, the predominant colony types consisted of types A and H, as was found in the metal mixture polluted sediment. The succession of predominant colony types are represented in Figures 4a-k,

The elevated CFU numbers observed in the lead amended sediment might be attributable to the fact that Pb was added in the form of the salt lead acetate. The presence of acetate may have acted as an additional nutrient source, and stimulated the growth of organisms able to metabolize acetate.

This environmental simulation, while not an accurate

duplication of natural events, does indicate an initial decrease in the number of viable organisms in the sediments polluted with metals followed by a return to recoverable organisms at levels present in the control. Thus it would appear that adaptation-selection for resistance rapidly occurs in a freshly metal polluted sediment. The unfortunate choice of lead acetate as the pollutant form of lead may indicate that external nutrient (i.e., acetate) accelerate the process of developed resistance observed in this population. The gradual die-off over time in this system is typical of laboratory ecosystems and should not be confused as a direct effect of the metal pollutants.

In the second experiment lead oxide (PbO) was substituted for lead acetate to eliminate any of the effects of acetate, and all additives were thoroughly mixed with the sediment by stirring prior to the addition of the water overlay.

During series 2, initial CFU control levels (Figure 5, Table 9) were approximately one log less than the control in series 1. After a slight decline, a maximum of 1.51×10^4 organisms/ml was reached on day 12. Several different colony types became predominant (Figure 6a-k, Table 10). Type A was observed frequently during the first several days, and type H was predominant on days 12, 15 and reappeared after a 10 day absence on day 25.

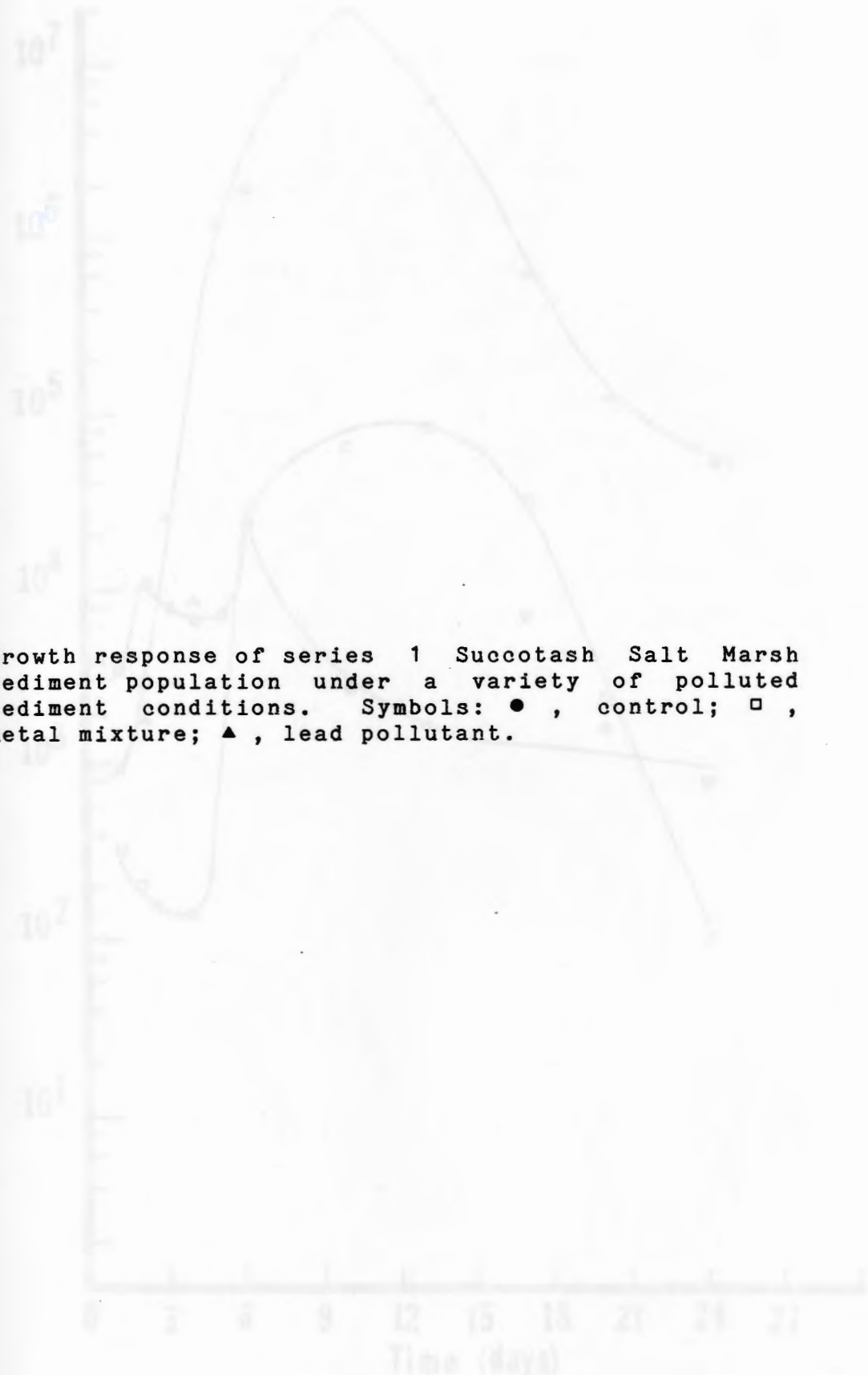


Fig. 3. Growth response of series 1 Succotash Salt Marsh sediment population under a variety of polluted sediment conditions. Symbols: ● , control; □ , metal mixture; ▲ , lead pollutant.

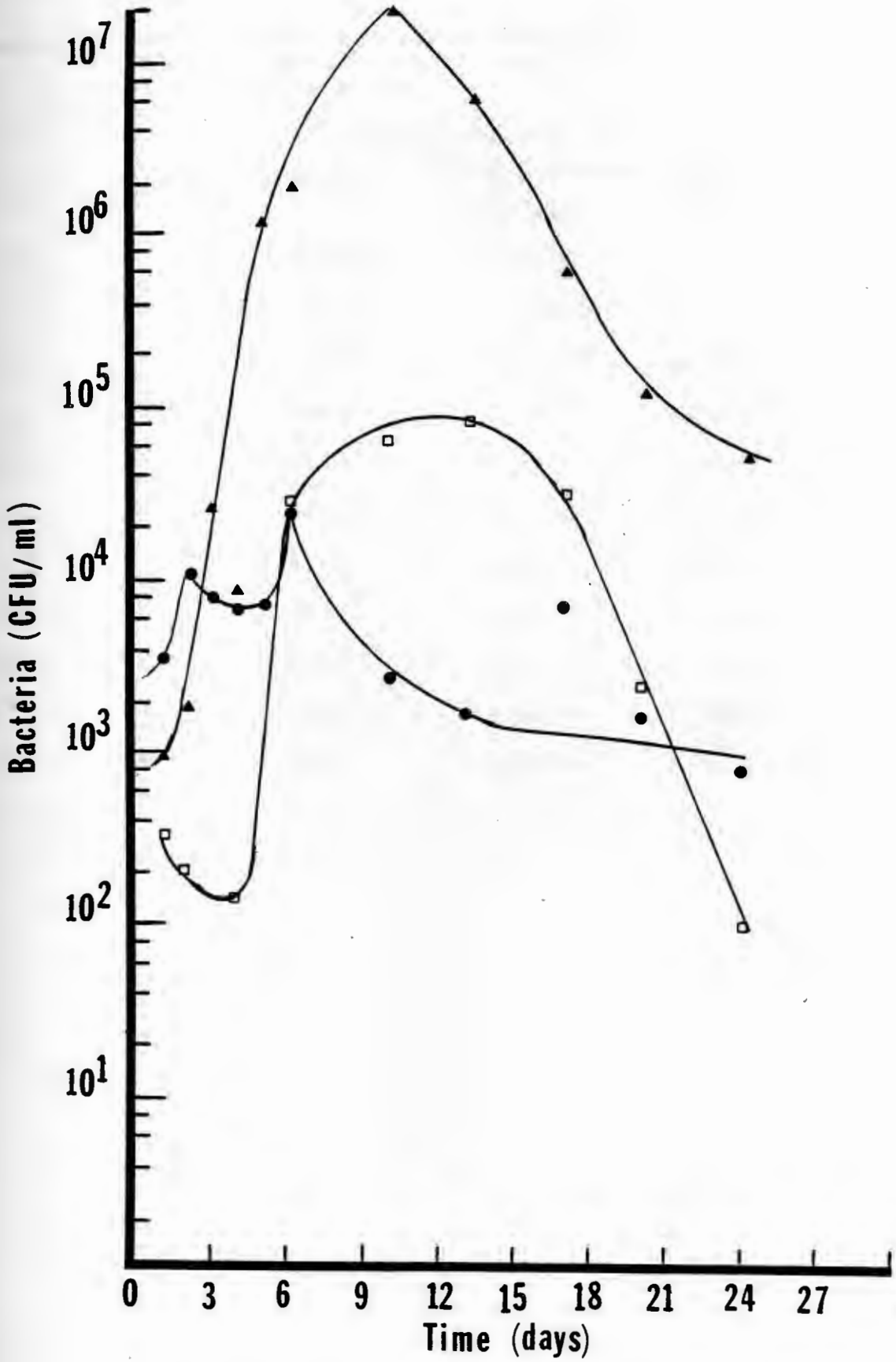

Bacteria (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
3
6
9
12
15
18
21
24
27
Time (days)

Table 6. Bacteria concentrations (CFU/ml) found in the control, metal mixture, and lead polluted sediments, series 1.

DATE	DAY	CONCENTRATION (CFU/ml) CONTROL	METAL MIXTURE	LEAD
2-16	1	3.95×10^3	3.5×10^2	9.5×10^2
2-17	2	1.14×10^4	2.0×10^2	1.95×10^3
2-18	3	7.9×10^3	$<1.0 \times 10^2$	2.6×10^5
2-19	4	6.7×10^3	1.5×10^2	8.0×10^3
2-20	5	7.2×10^3	$<1.0 \times 10^2$	1.14×10^6
2-21	6	2.45×10^4	2.7×10^4	1.95×10^6
2-25	10	3.1×10^3	6.5×10^4	2.27×10^7
2-28	13	1.8×10^3	8.25×10^4	1.65×10^7
3-4	17	6.8×10^3	3.36×10^4	6.05×10^6
3-7	20	1.6×10^3	2.4×10^3	1.28×10^6
3-11	24	7.5×10^2	1.0×10^2	5.1×10^5

Table 7. Morphology of colony types found in population dynamics test sediment during both series 1 and 2.

DESIGNATION	COLONY MORPHOLOGY
A	White, smooth, convex, entire
B	Yellow w/white edge (eggyolk), smooth, undulate
C	Yellow, smooth, convex, entire
D	Pink, smooth, convex, entire
E	Yellow, flat, entire
F	Yellow-white, rough, flat, undulate
G	White, smooth, flat, slightly undulate
H	White, punctiliform, smooth, convex, entire
I	Brown, smooth, convex, entire
J	Translucent, smooth, convex, entire
K	White w/clear edge, smooth, convex, entire
L	Brown w/tan center, raised, entire
M	White, rough, entire
N	Tan w/white edge, smooth, convex, entire
O	White, rough, flat, irregular
P	Opaque, raised
Q	Opaque, punctiliform, entire
R	Yellow-white, smooth convex, entire
S	White (large), smooth, convex, wavy
T	White, flat, "star center", irregular
U	Yellow-white, smooth, convex, irregular
V	White w/granular edge, irregular
W	Yellow-brown, smooth, convex, entire
X	Opaque, smooth, convex, entire
Y	Large orange w/white edge, flat, irregular
Z	Tan, opaque, flat, undulate
AA	Red, smooth, convex, entire
BB	Orange, smooth, convex, entire
CC	Brown w/opaque edge, punctiliform, smooth, convex, entire
DD	Yellow w/clear edge, smooth, convex, entire
EE	Brown w/yellow edge, smooth, convex, entire
FF	Orange, smooth, convex, undulate
GG	White, smooth, raised, entire

Table 8. Number and type of predominant colonies found in each test sediment.

DATE	DAY	CONCENTRATION BACTERIA (Types, organisms/ml) CONTROL	METAL MIXTURE	LEAD
2-16	1	A 3.15×10^{3}	A 3.5×10^{2}	H 3.0×10^{2} A 2.5×10^{2}
2-17	2	A 5.0×10^{3} B 2.5×10^{3}	O 1.5×10^{2} A 5.0×10^{1}	B 1.25×10^{3} A 6.5×10^{2}
2-18	3	N 3.4×10^{3} A 1.3×10^{3}	-	B 1.35×10^{5} N 9.0×10^{4}
2-19	4	NT*	A 1.5×10^{2}	H 8.0×10^{4}
2-20	5	NT	-	NT
2-21	6	R 2.35×10^{3}	R 2.7×10^{3}	A 1.95×10^{6}
2-25	10	I 1.5×10^{3} H 8.0×10^{2}	H 6.5×10^{4}	H 2.27×10^{7}
2-28	13	H 7.0×10^{2} A 6.0×10^{2}	H 8.25×10^{4}	A 1.65×10^{6}
3-4	17	T 4.5×10^{3} J 1.05×10^{3}	H 3.05×10^{4}	H 6.05×10^{6}
3-7	20	H 8.0×10^{2} A 4.0×10^{2}	A 2.4×10^{3}	A 1.10×10^{6} H 1.65×10^{5}
3-11	24	A 5.0×10^{2} W 2.5×10^{2}	A 1.0×10^{2}	A 5.1×10^{5}

*NT-Not typed

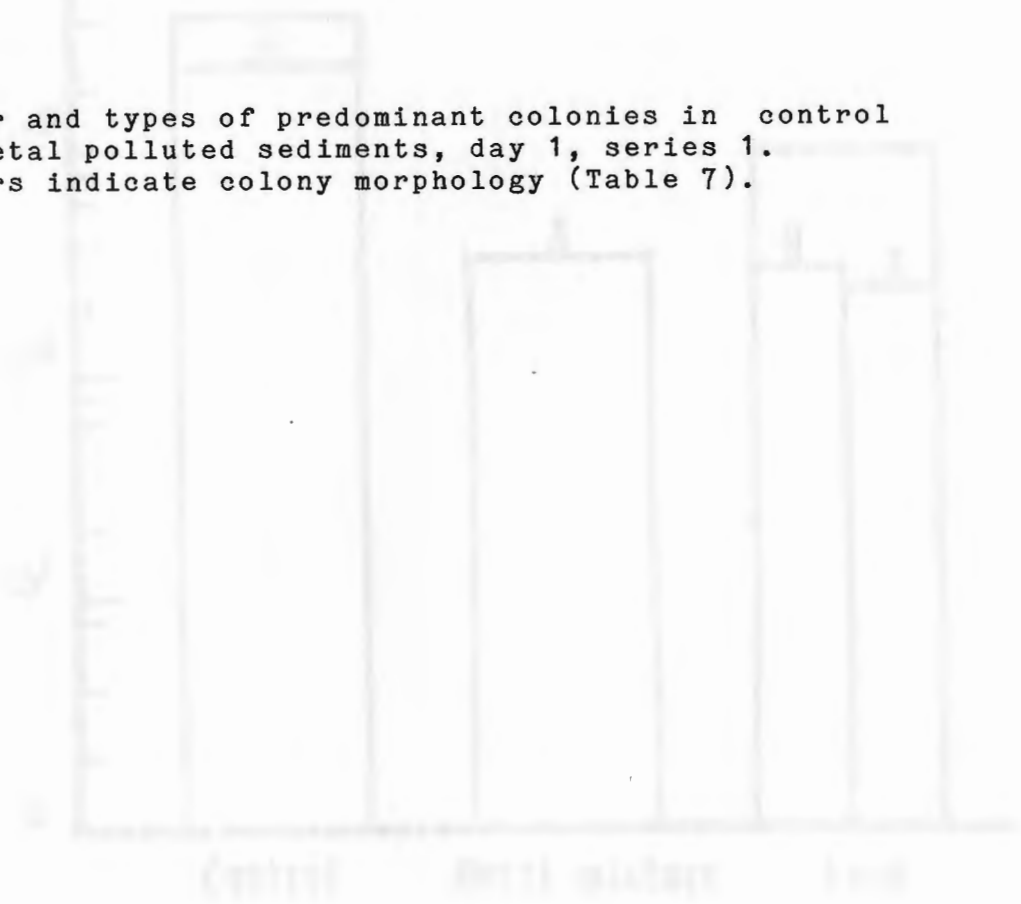
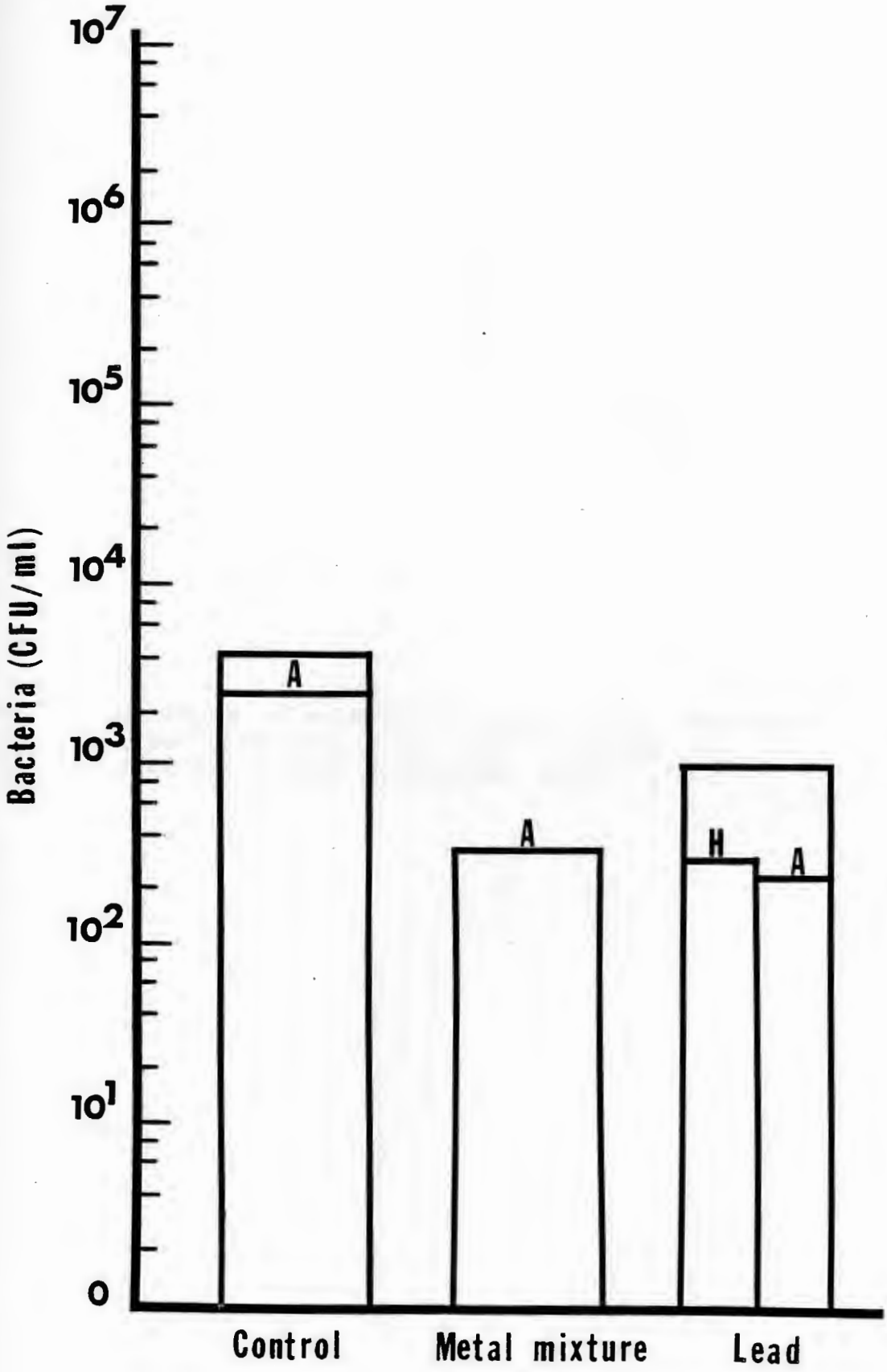


Fig. 4a. Number and types of predominant colonies in control and metal polluted sediments, day 1, series 1. Letters indicate colony morphology (Table 7).


Bacteria (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
A
A
H
A
Control
Metal mixture
Lead

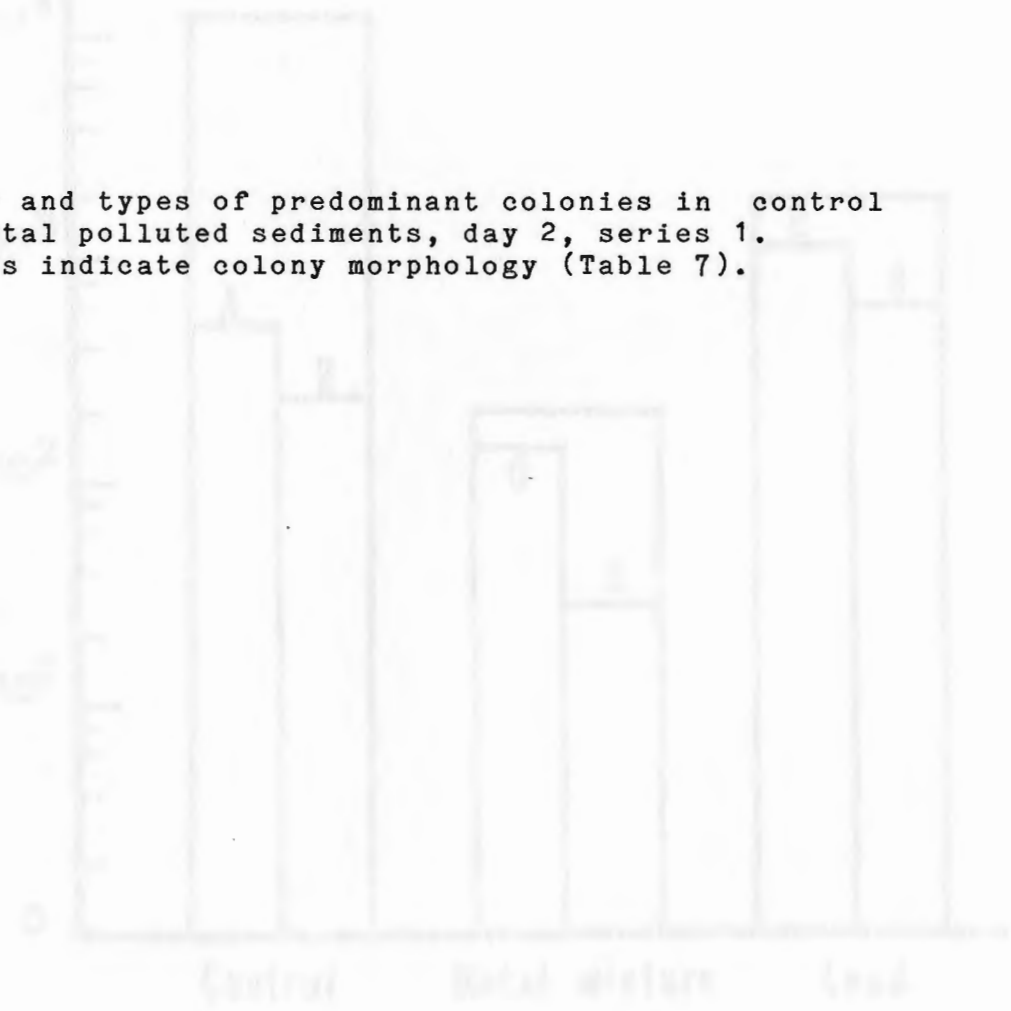
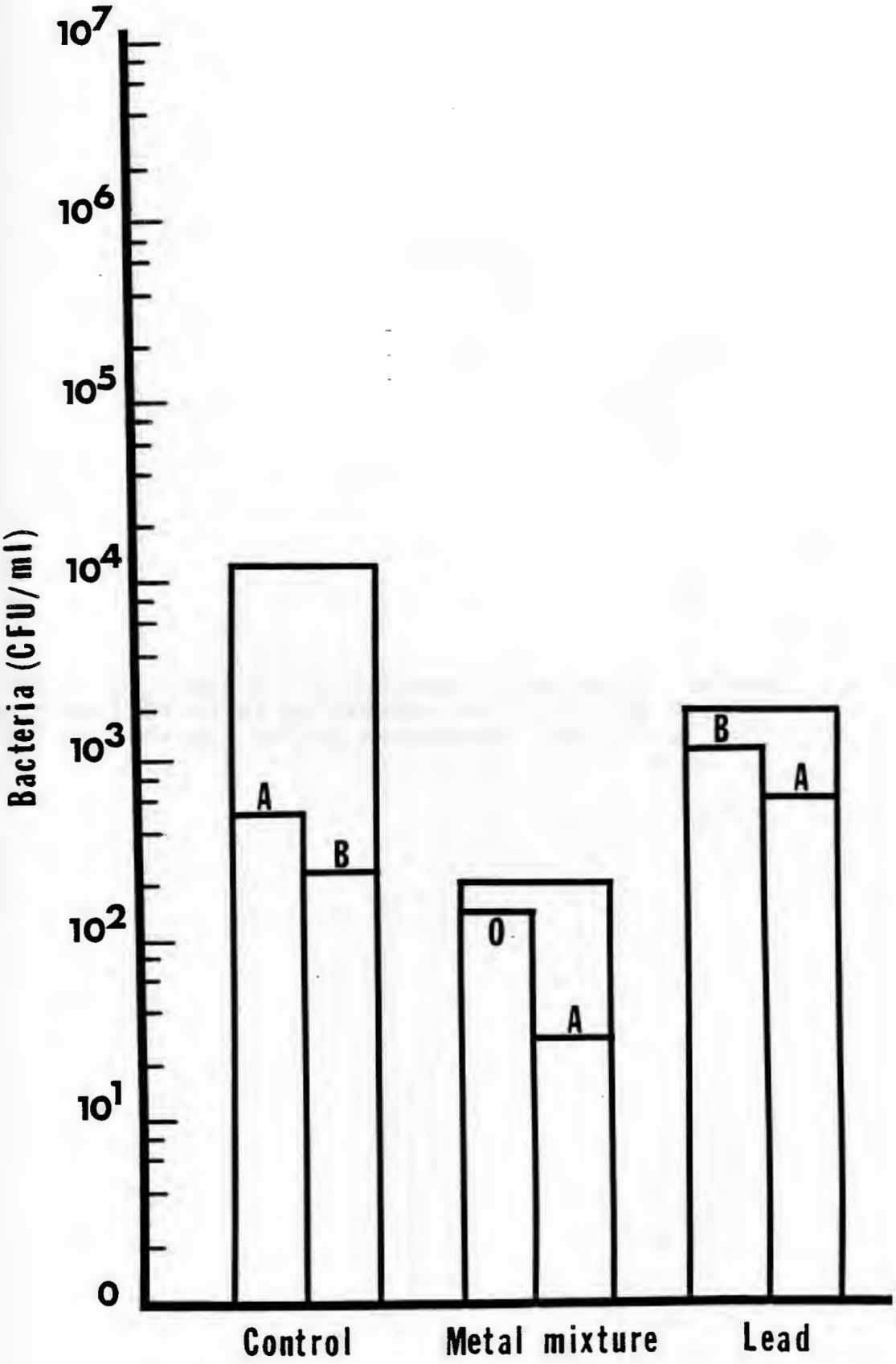


Fig. 4b. Number and types of predominant colonies in control and metal polluted sediments, day 2, series 1. Letters indicate colony morphology (Table 7).


10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
Bacteria (CFU/ml)
A
B
0
A
B
A
Control
Metal mixture
Lead

ig. 4c. Number and types of predominant colonies in control and metal polluted sediments, day 3, series 1. Letters indicate colony morphology (Table 7).

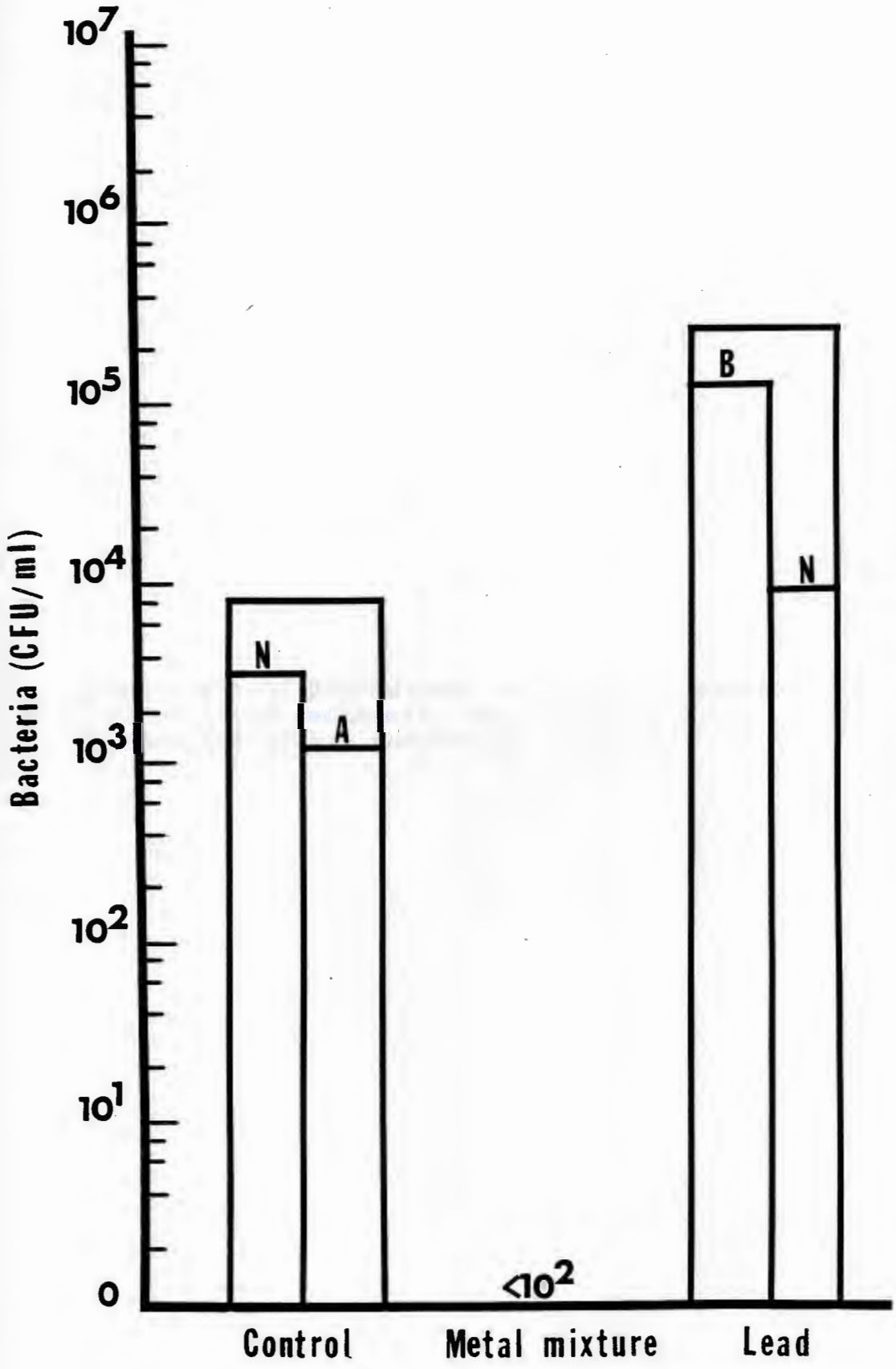
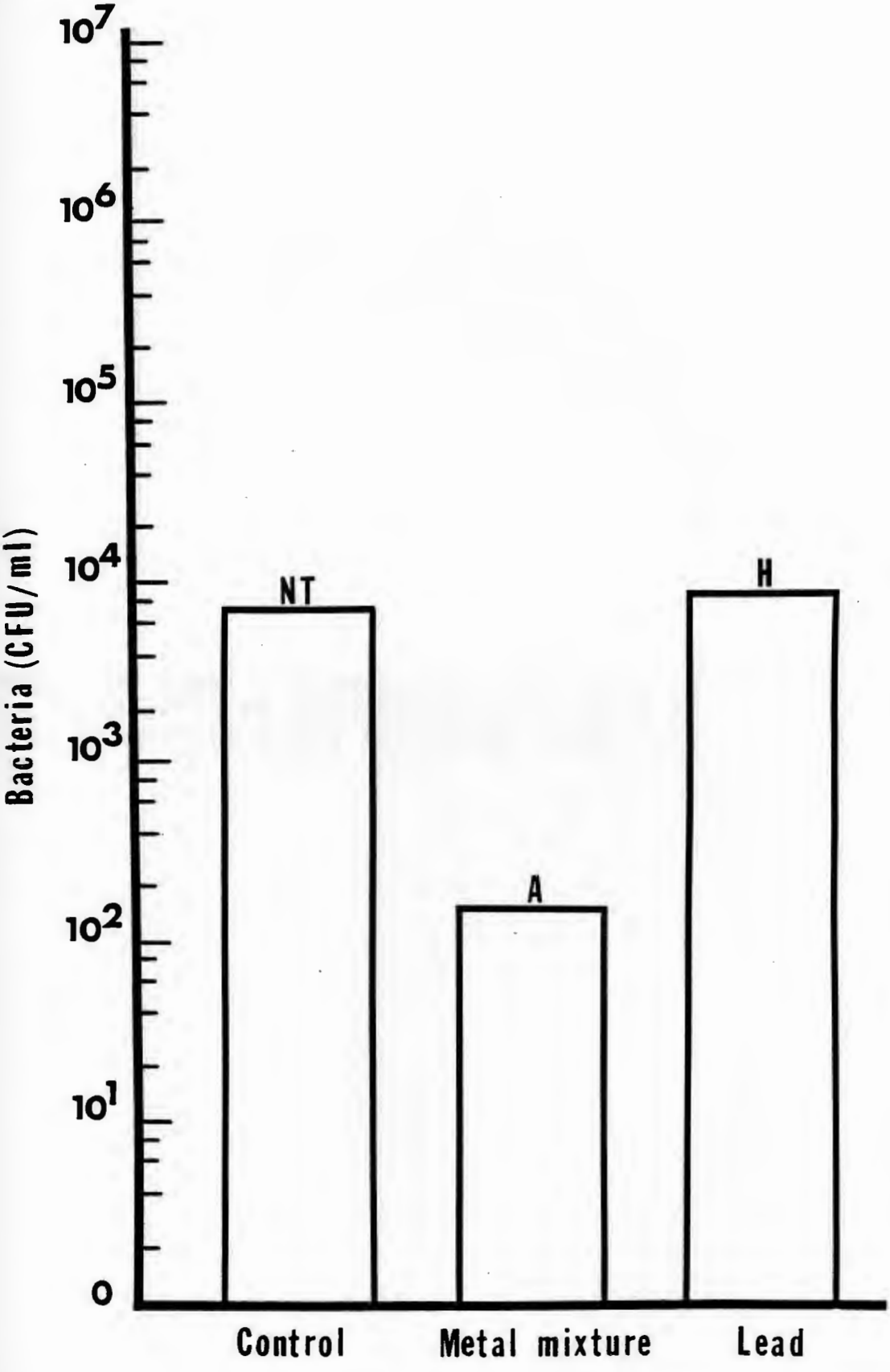
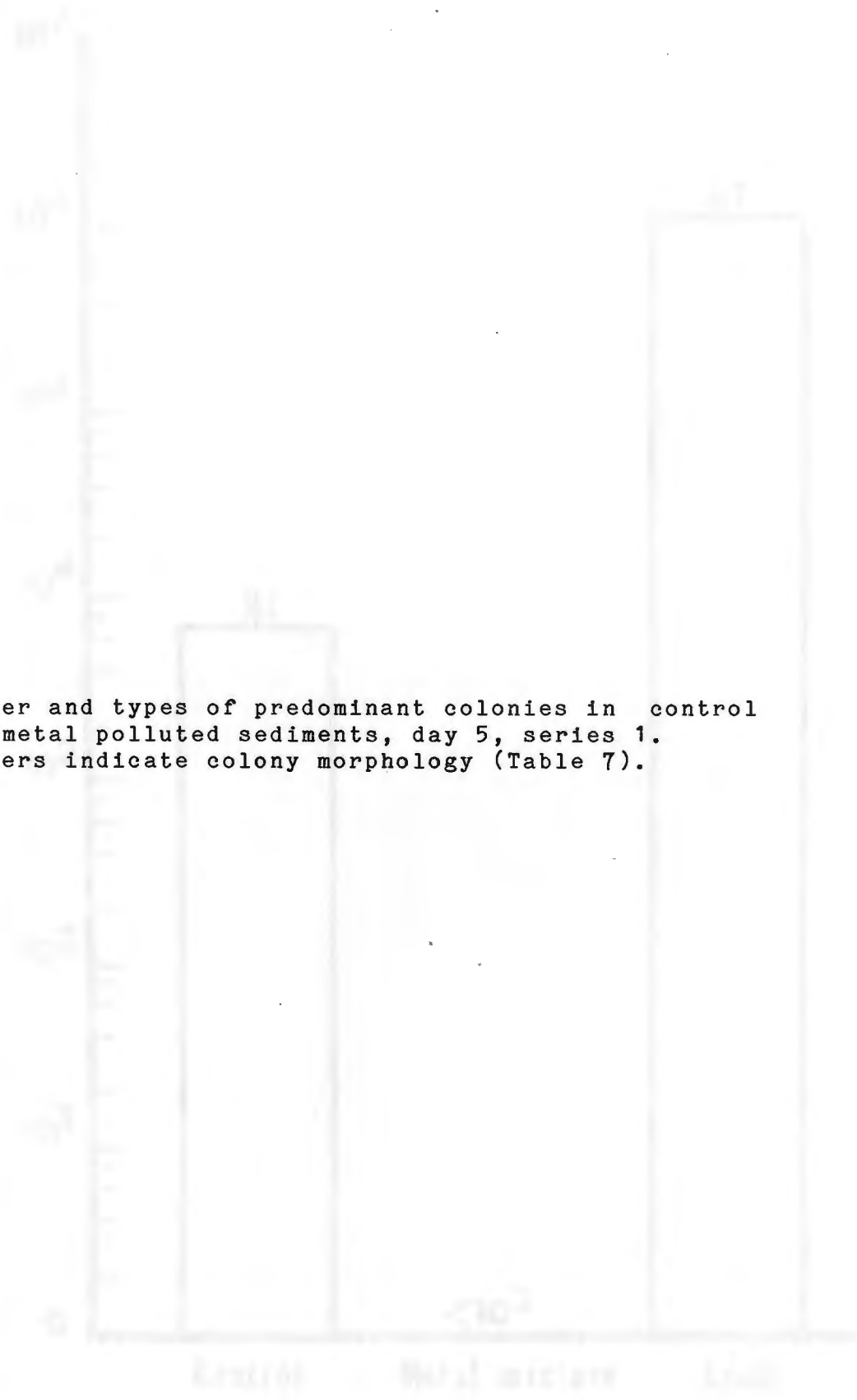
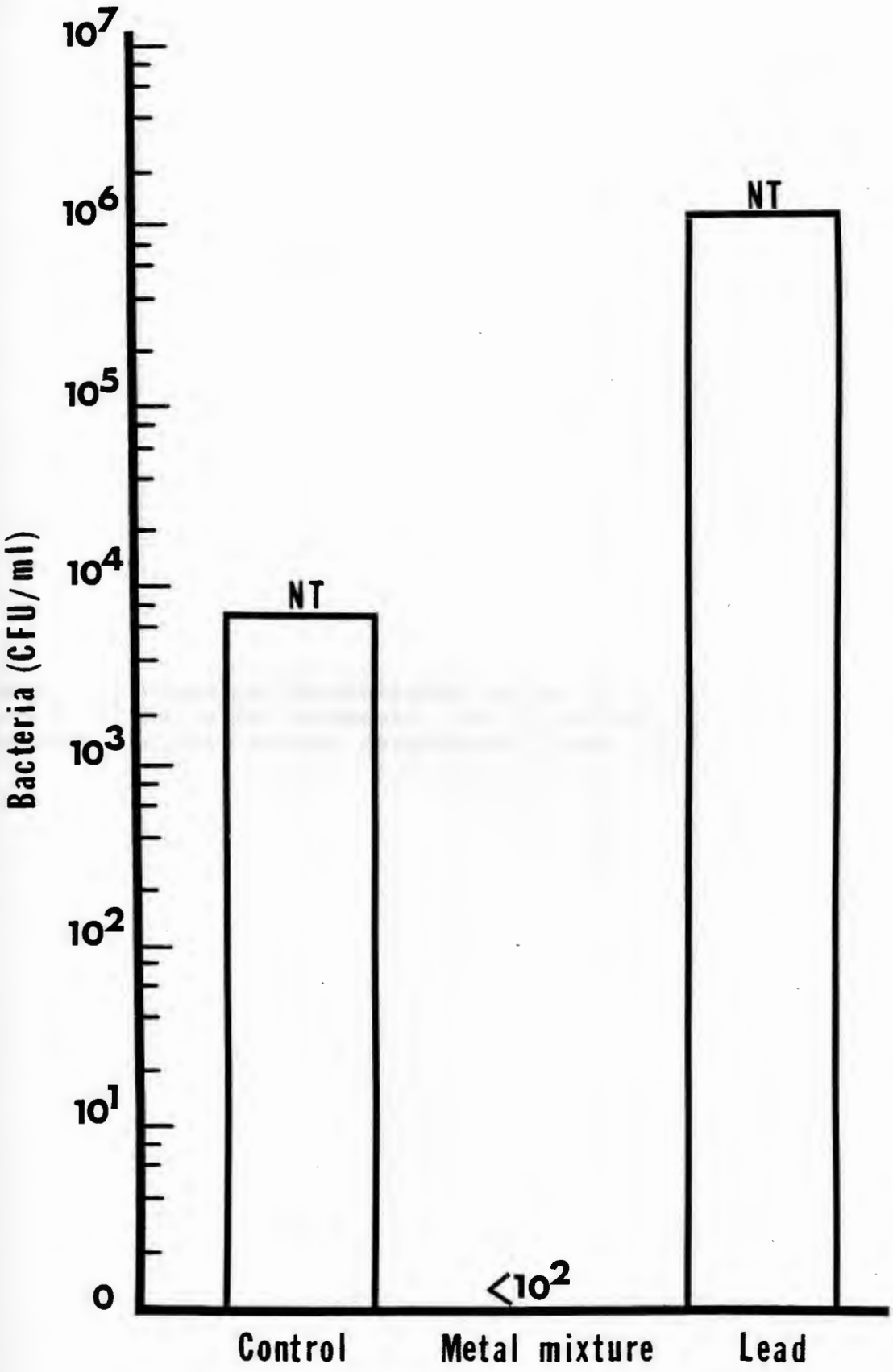

10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
Bacteria (CFU/ml)
N
A
B
N
<10^2
Control
Metal mixture
Lead

Fig. 4d. Number and types of predominant colonies in control and metal polluted sediments, day 4, series 1. Letters indicate colony morphology (Table 7).


10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
Bacteria (CFU/ml)
NT
A
H
Control
Metal mixture
Lead



ig. 4e. Number and types of predominant colonies in control and metal polluted sediments, day 5, series 1. Letters indicate colony morphology (Table 7).


Bacteria (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
NT
NT
<10^2
Control
Metal mixture
Lead

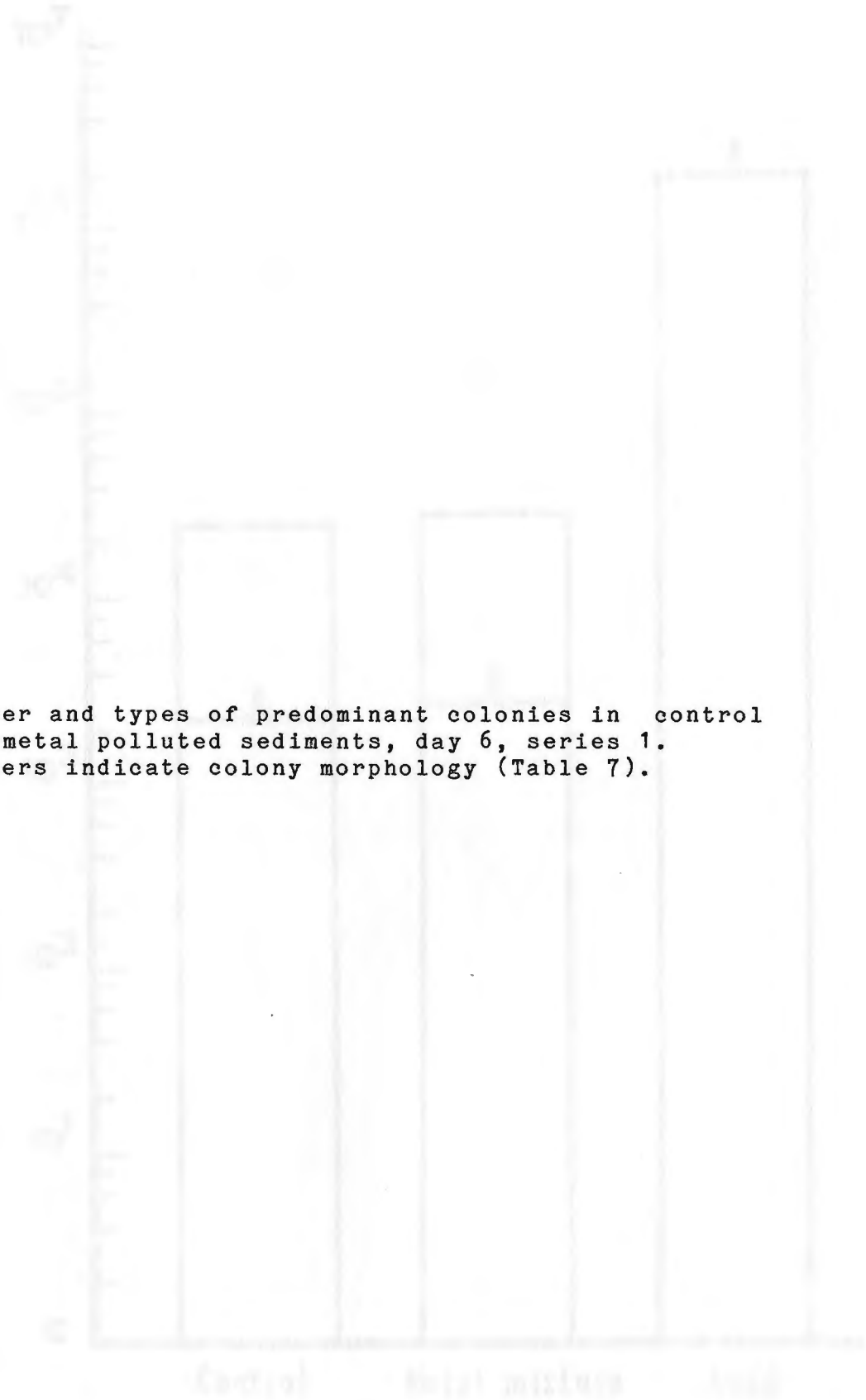
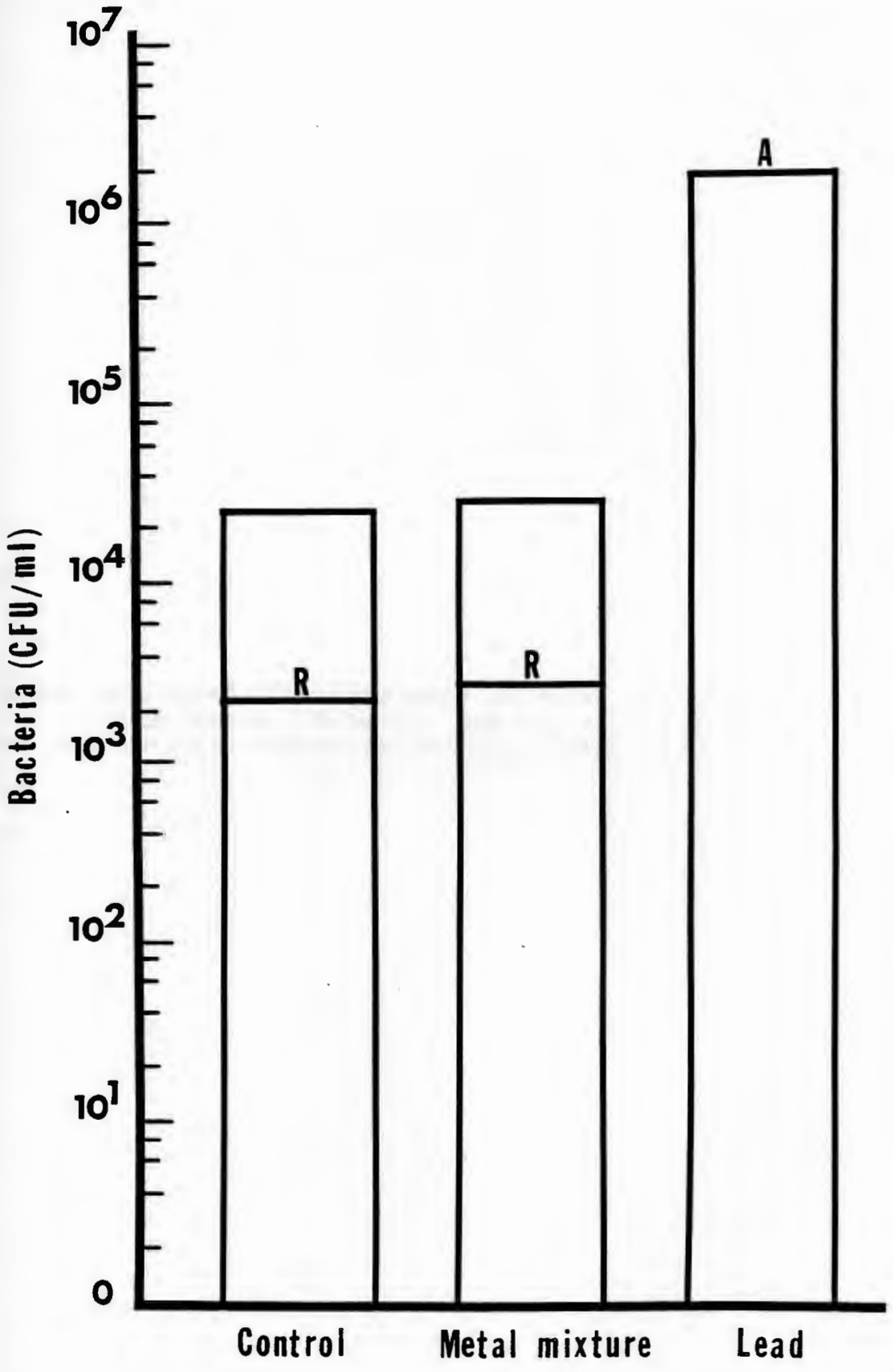
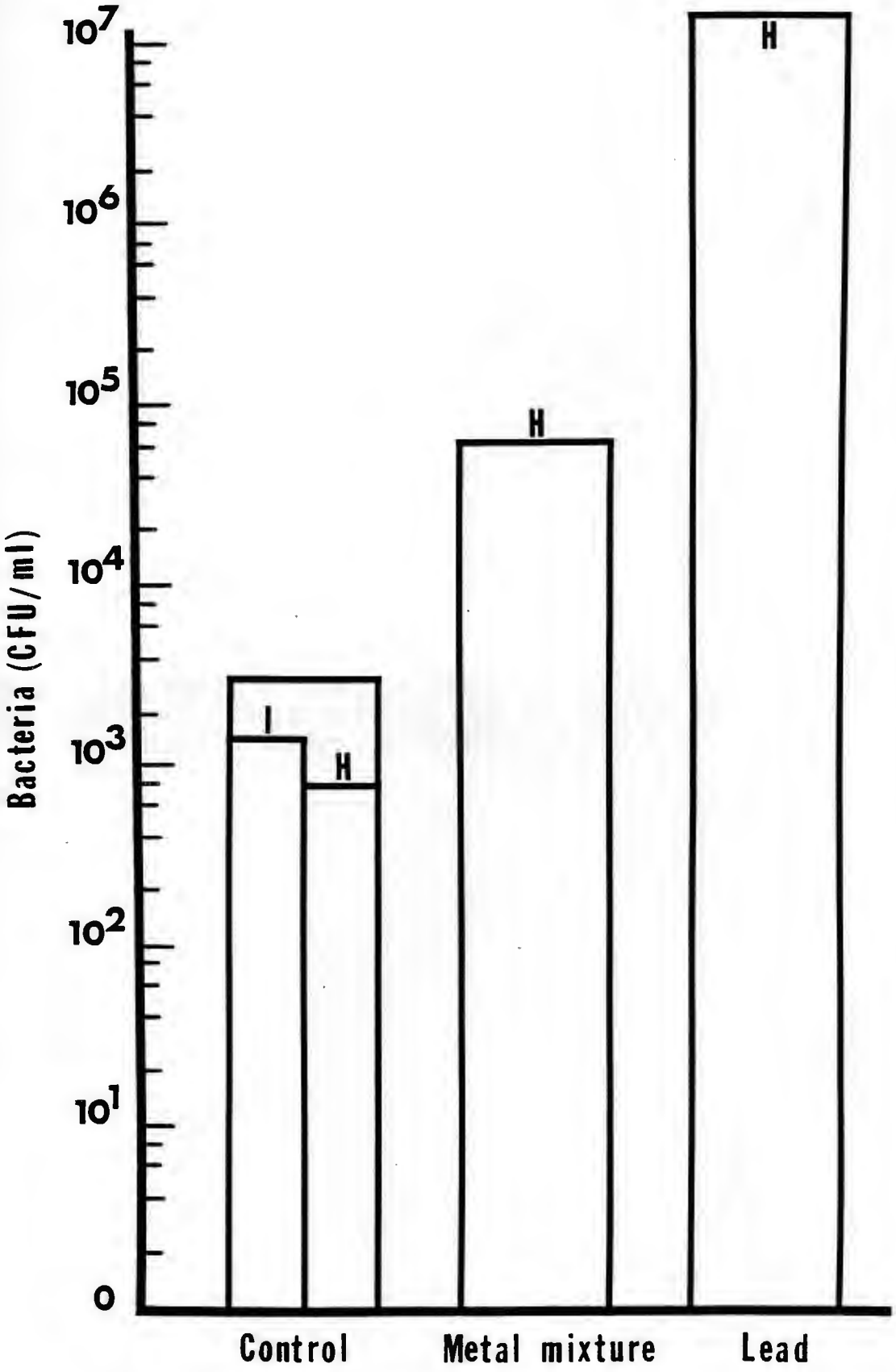
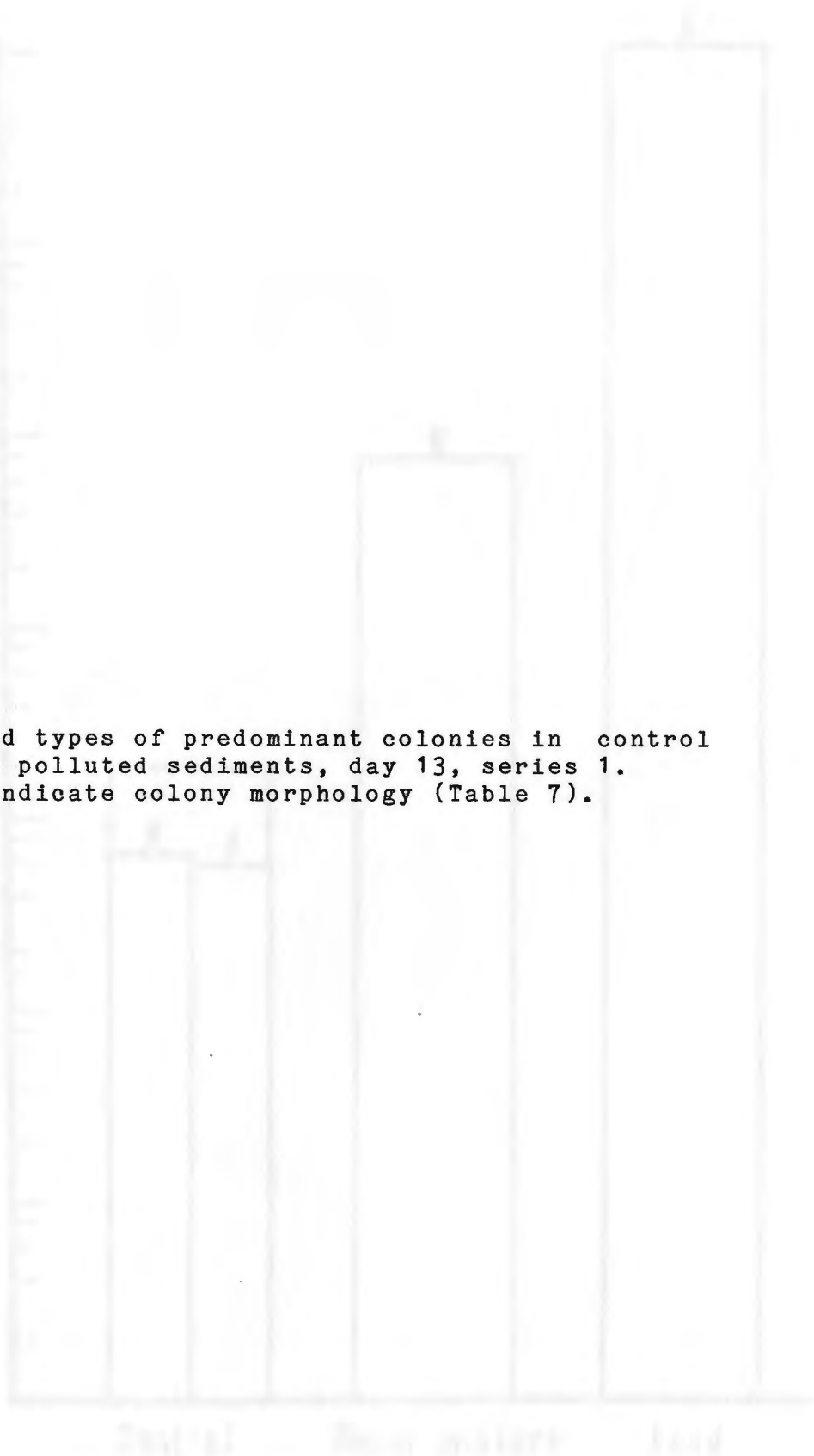


Fig. 4f. Number and types of predominant colonies in control and metal polluted sediments, day 6, series 1. Letters indicate colony morphology (Table 7).

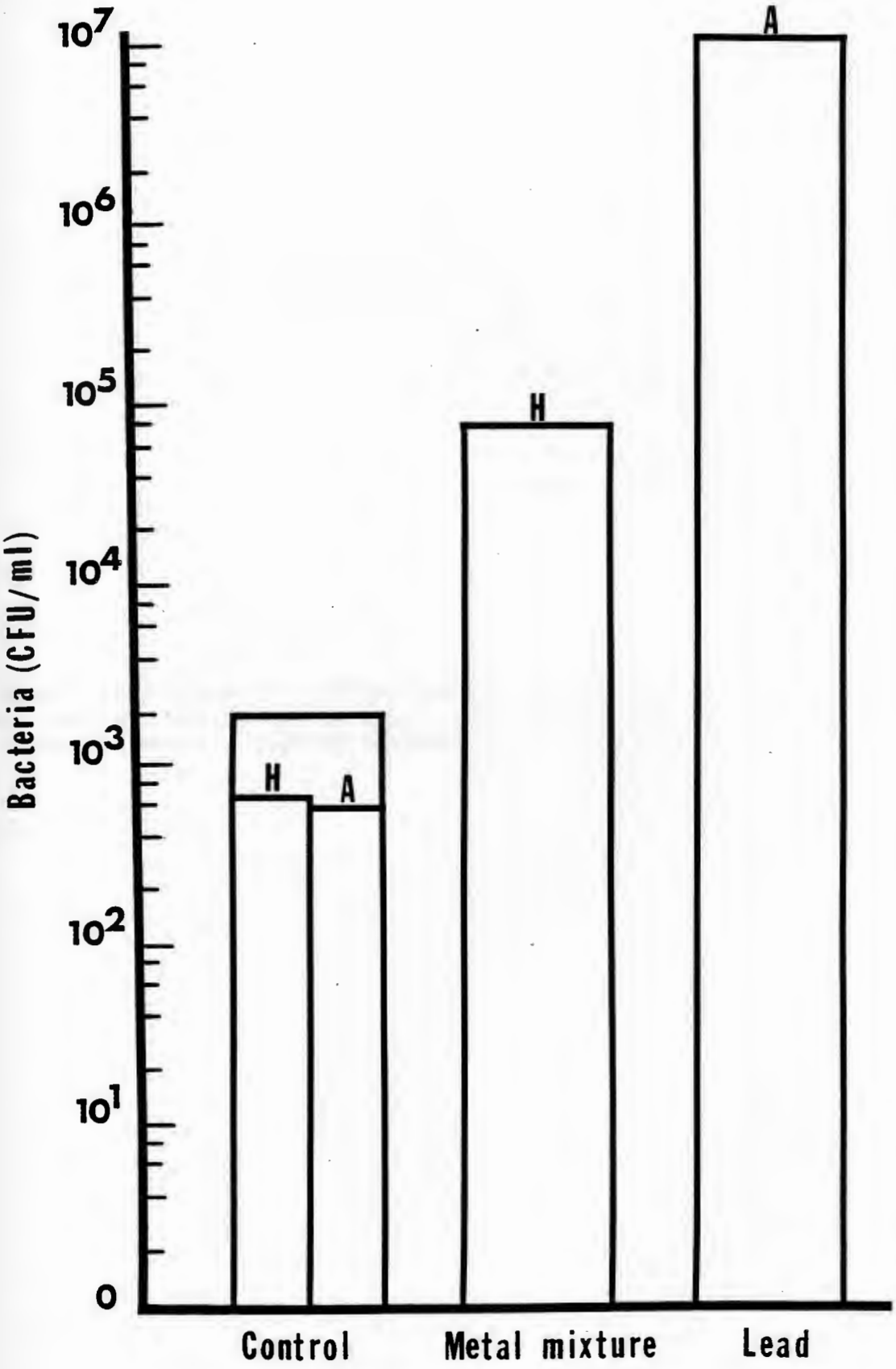

10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
Bacteria (CFU/ml)
A
R
R
Control
Metal mixture
Lead

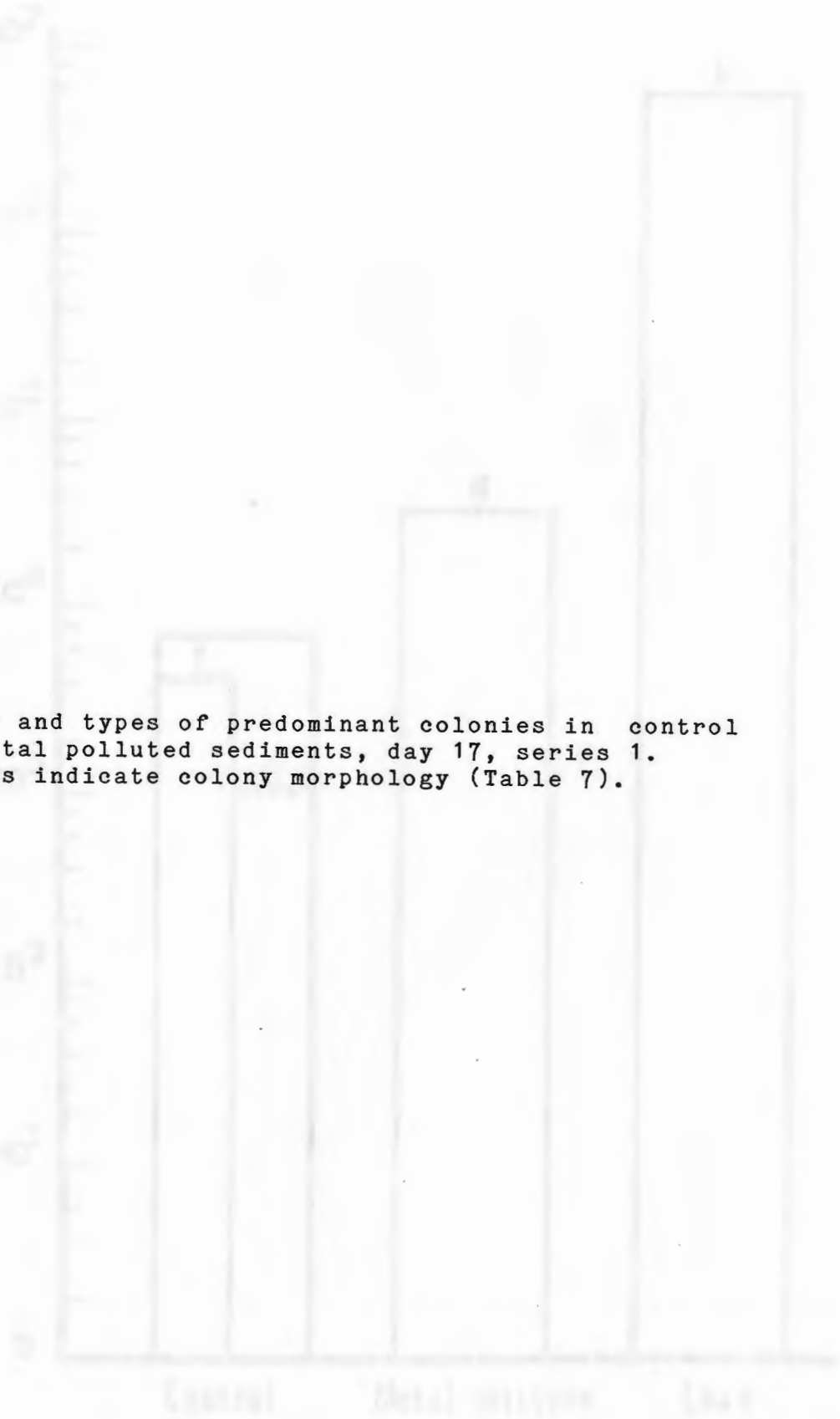
g. 4g. Number and types of predominant colonies in control and metal polluted sediments, day 10, series 1. Letters indicate colony morphology (Table 7).


Bacteria (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
I
H
H
H
Control
Metal mixture
Lead

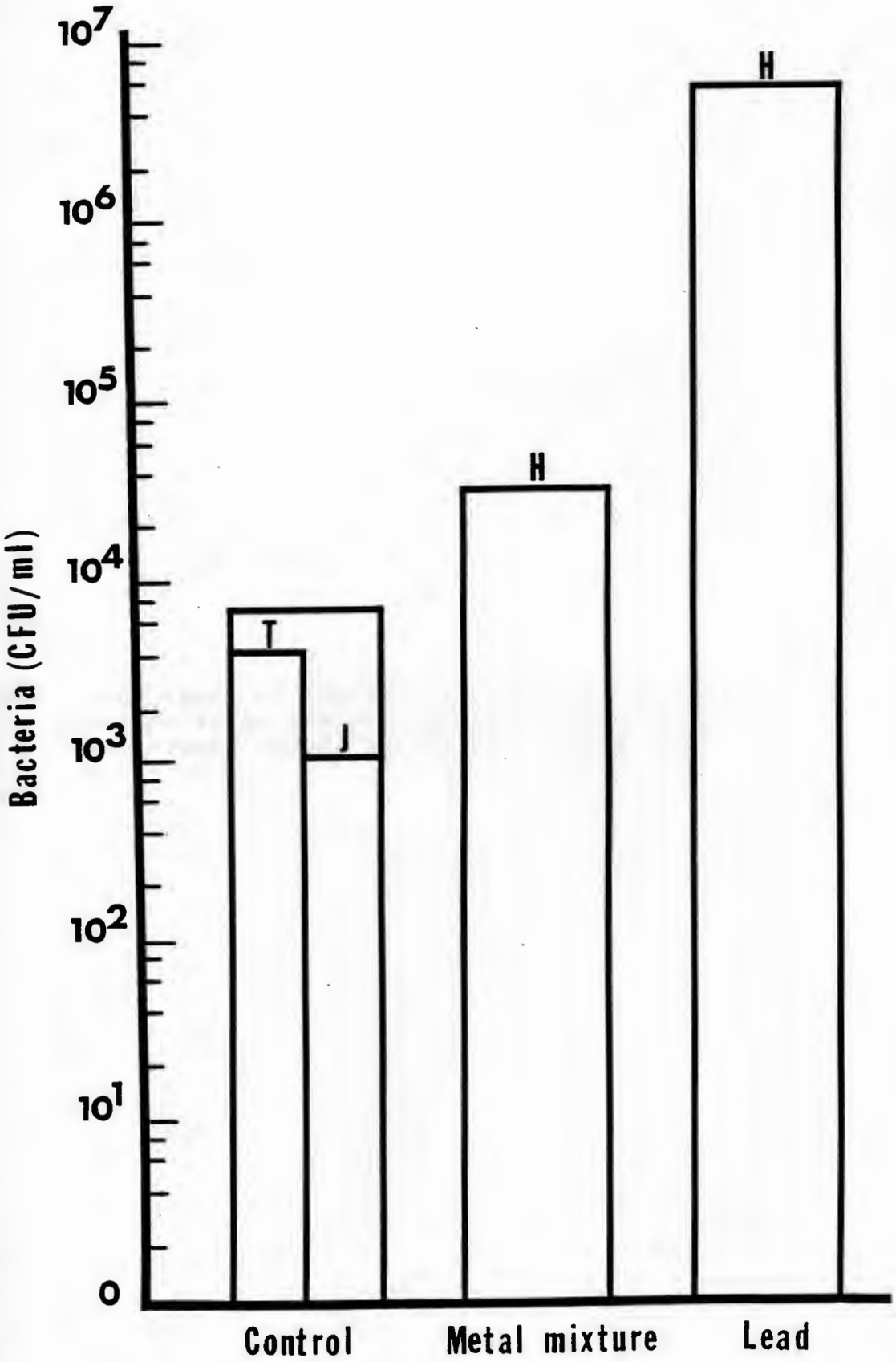


ig. 4h. Number and types of predominant colonies in control and metal polluted sediments, day 13, series 1. Letters indicate colony morphology (Table 7).

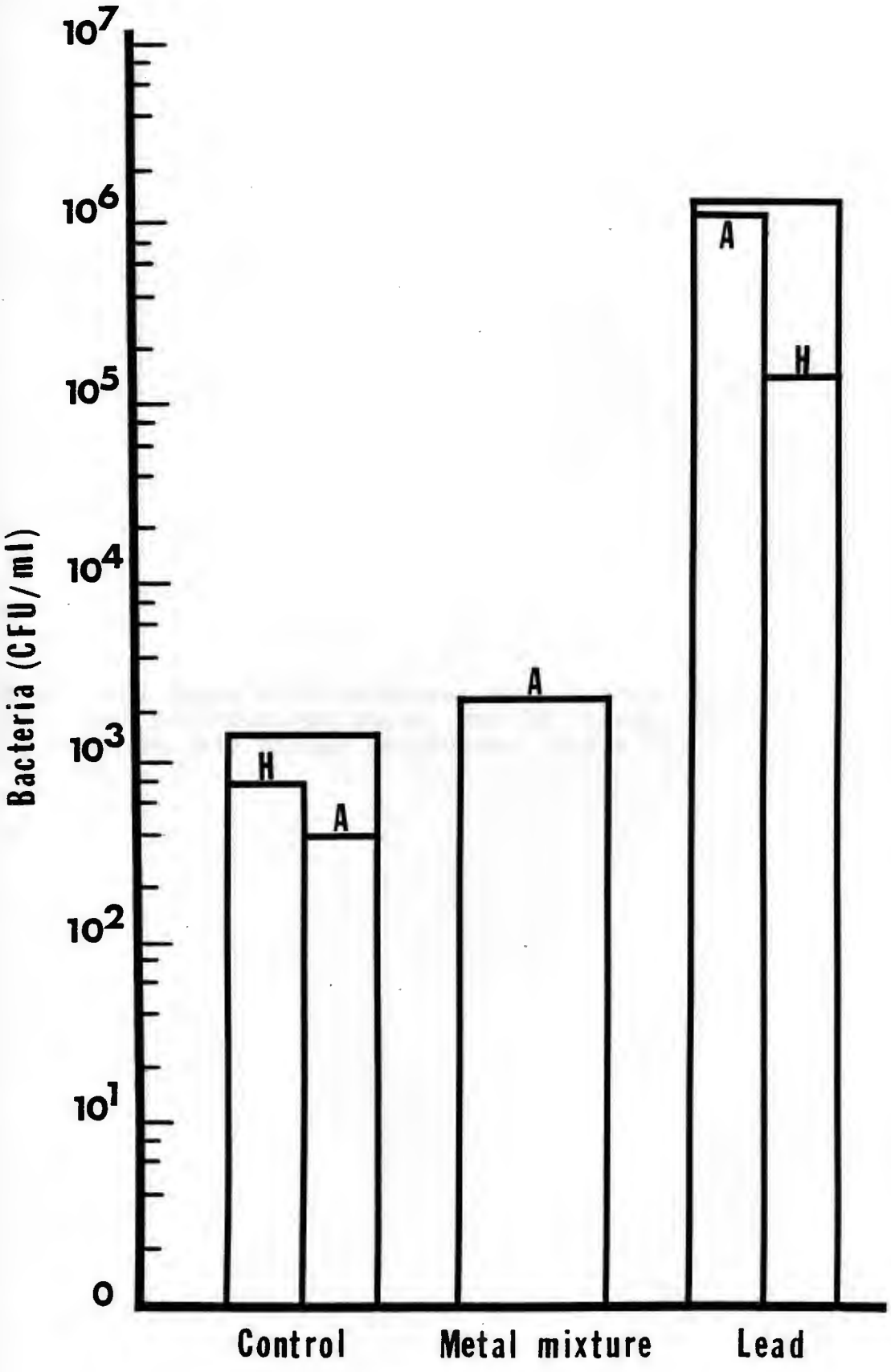

Bacteria (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
H
A
H
A
Control
Metal mixture
Lead

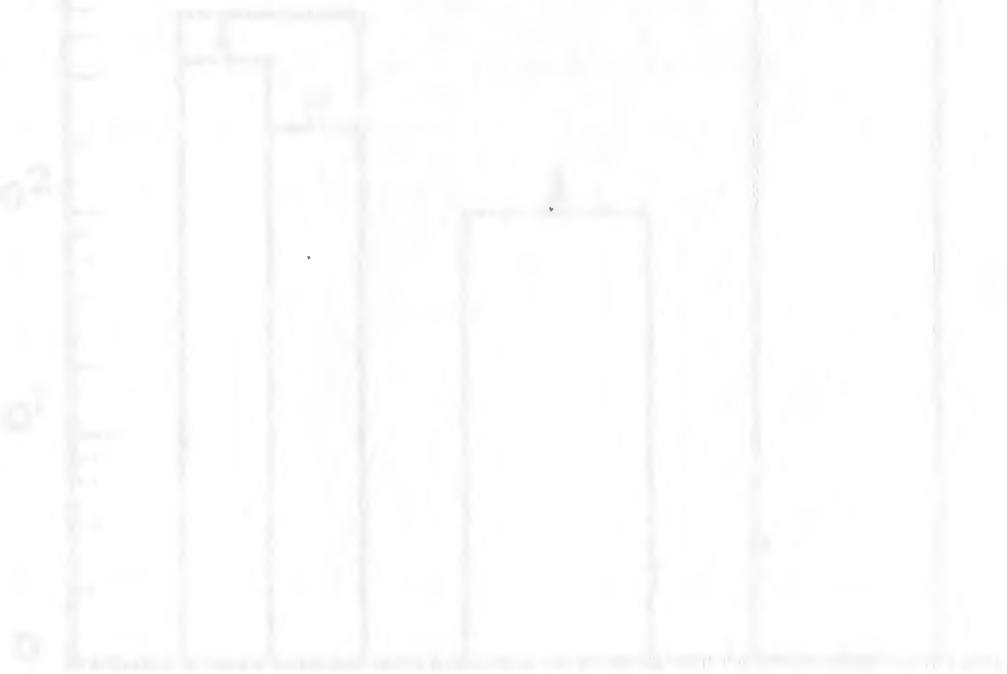


g. 4i. Number and types of predominant colonies in control and metal polluted sediments, day 17, series 1. Letters indicate colony morphology (Table 7).

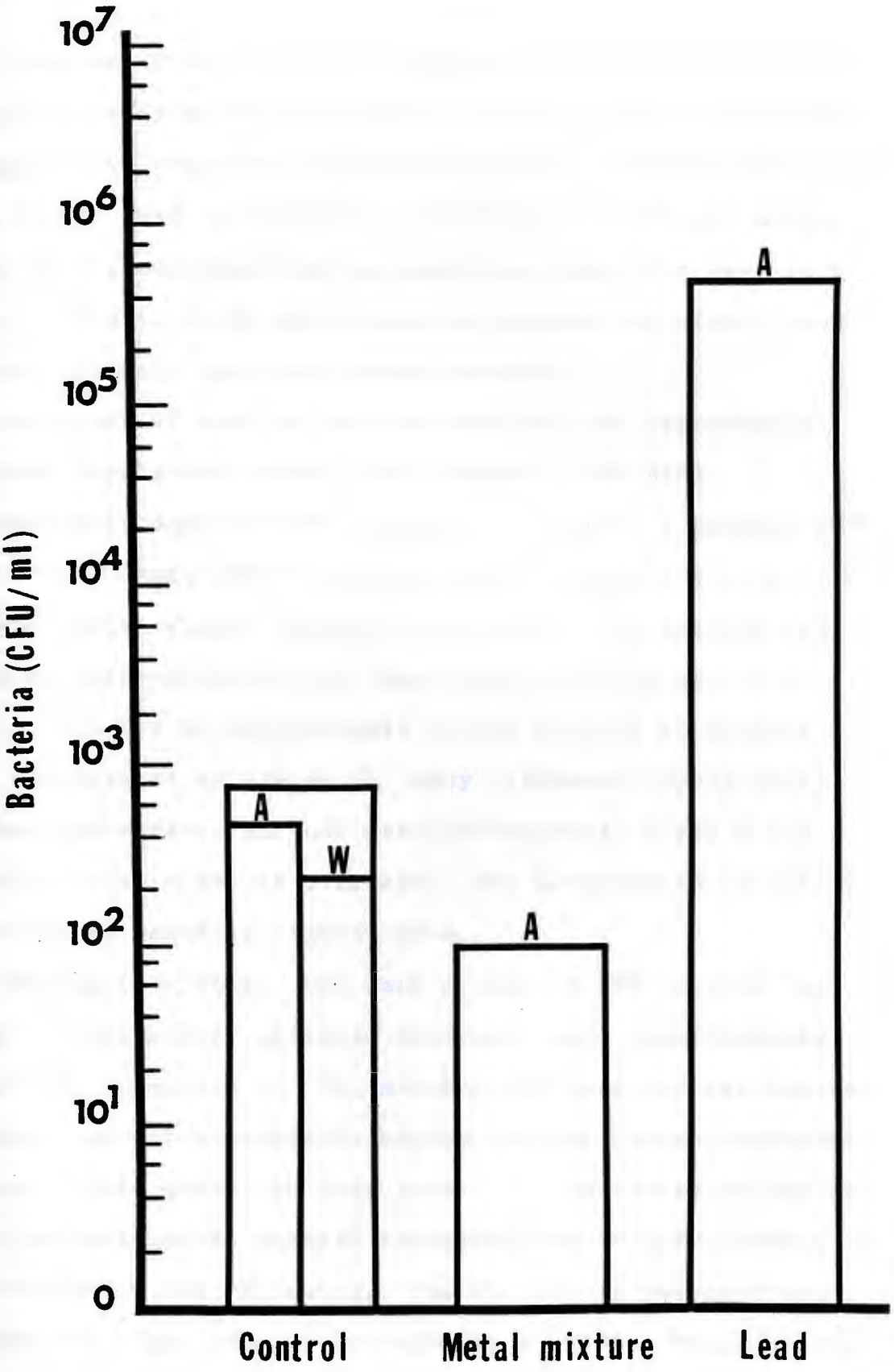

Bacteria (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
T
J
H
H
Control
Metal mixture
Lead

g. 4j. Number and types of predominant colonies in control and metal polluted sediments, day 20, series 1. Letters indicate colony morphology (Table 7).


Bacteria (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
H
A
A
A
H
Control
Metal mixture
Lead



g. 4k. Number and types of predominant colonies in control and metal polluted sediments, day 24, series 1. Letters indicate colony morphology (Table 7).


10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
Bacteria (CFU/ml)
A
W
A
A
Control
Metal mixture
Lead

Depression of initial CFU numbers occurred immediately after the addition of all metals to the sediment. Organism concentration peaked at 18 days at 6.0×10^5, approximately 1.5 logs higher than in series 1. A number of different colony types were predominant but no more than for a few days at a time. Types A, D, H, and I were all present at significant levels, but none were completely dominant.

Addition of lead to the sediment did not appreciably decrease population levels from series 1, and were approximately equal to the control. On day 7, a maximum CFU of 3.32×10^5 organisms/ml occurred, and a secondary peak of 1.36×10^4 organisms/ml appeared on day 18. The maximum CFU value was approximately two logs lower than the series 1 peak. This may be attributable to the absence of acetate that was present in series 1. Many different colony types became predominant, and not until 18 days did types N and A establish themselves as dominant. The progression of colony types can be found in Figures 6a-k.

During this study, the peak number of CFU in both the metal mixture and Pb polluted sediments were significantly lower than in series 1. The maximum CFU level of the control sediment was not appreciably higher in this series, although initial levels were 0.88 logs less. The lower CFU values in metal mixture and Pb amended sediments may be attributable to one or both of the following. The Pb salt in series 2 was changed from lead acetate to lead oxide (PbO). This results

in the removal of acetate as a nutritional supplement from the metal mixture and lead polluted sediments. The solubility of lead oxide (0.0068 g/100 ml) is much less than lead acetate (44.3 g/100 ml), and results in a greater amount of Pb in the form of PbO present in the sediment. The metals and PbO were also mixed into the sediment by stirring, and caused increased contact between the metal ions and sediment organisms.

The metal mixture and Pb amended sediments showed selectivity for certain colony types in a fashion similar to series 1, but to a lesser degree. The difference in the degree of selectivity may be attributed to the removal of acetate as a potential selective agent. In the metal mixture tank, four predominant colonies were present, while only three were observed in the Pb tank. Some overlap in the predominant types occurred between the two tanks (types A and H). The control tank showed virtually no selection at all.

In each population dynamics series, a large segment of the sediment population subjected to heavy metal pollution acquired resistance. This can be observed by the fact that after an initial decline in the metal mixture and lead polluted sediment populations, the subsequent recovery was equivalent to or greater than control population levels. The environment does not be become sterile, but in fact selects for a different portion of the population.

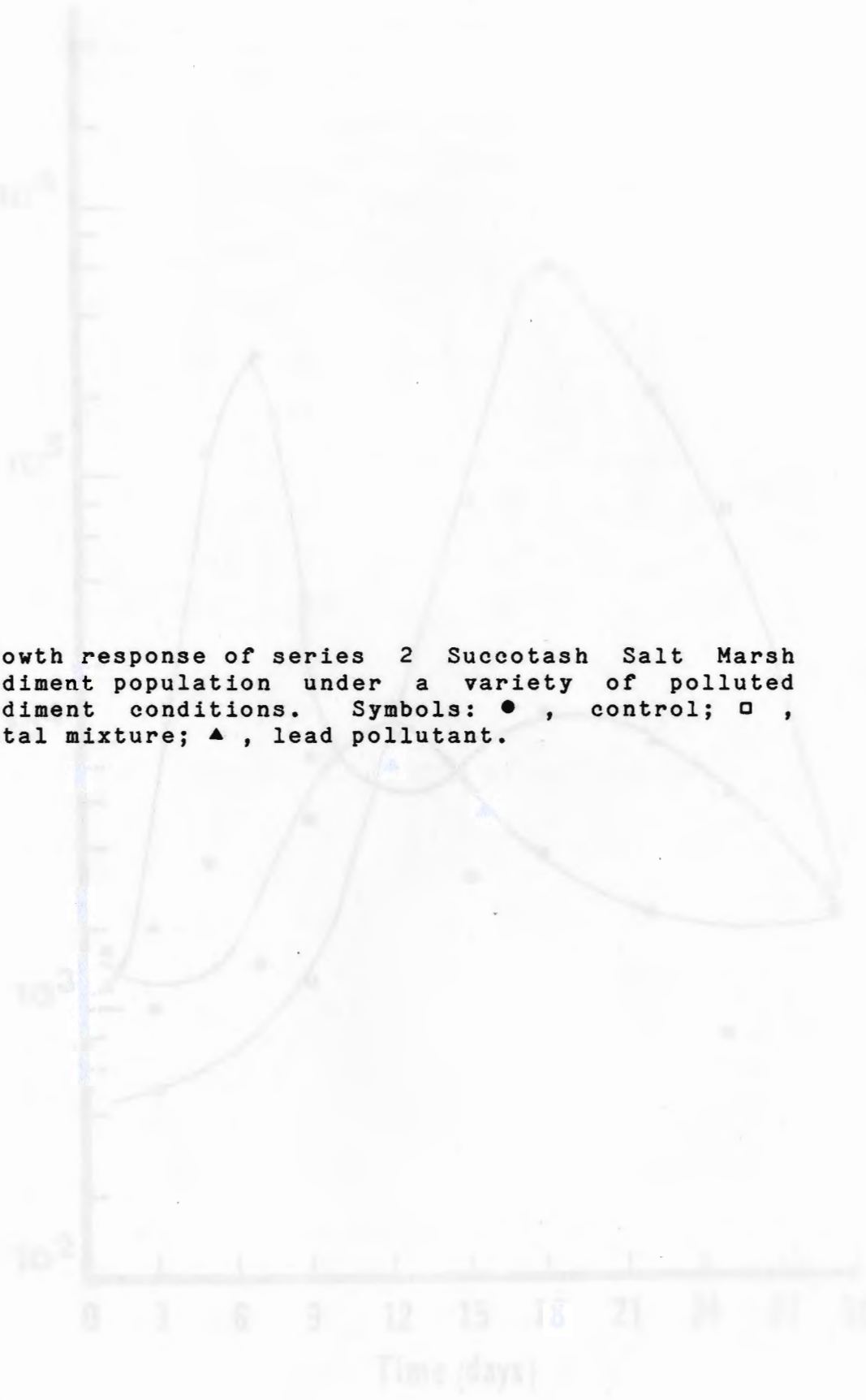


Fig. 5. Growth response of series 2 Succotash Salt Marsh sediment population under a variety of polluted sediment conditions. Symbols: ● , control; □ , metal mixture; ▲ , lead pollutant.

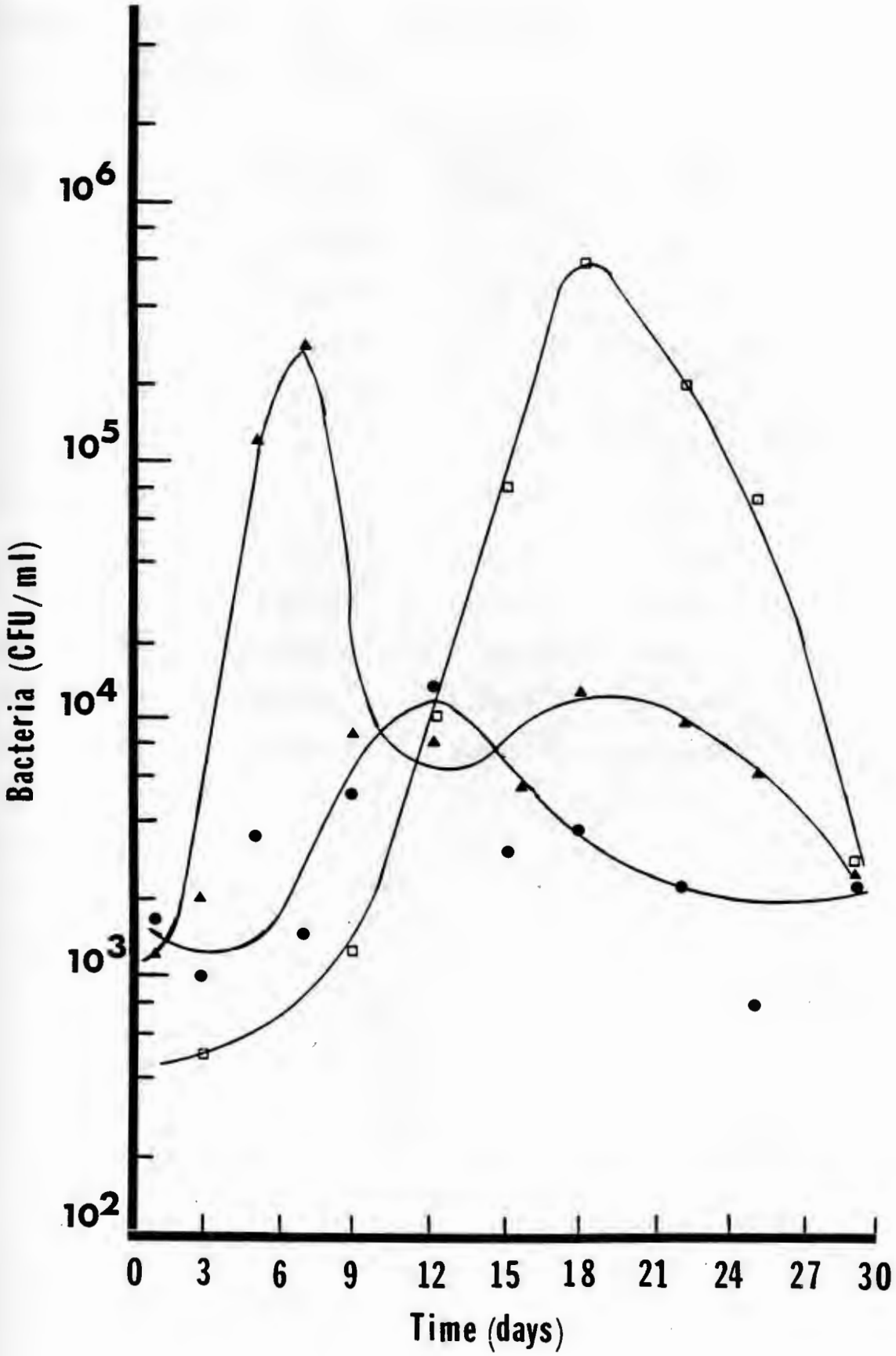

Bacteria (CFU/ml)
10^6
10^5
10^4
10^3
10^2
0
3
6
9
12
15
18
21
24
27
30
Time (days)

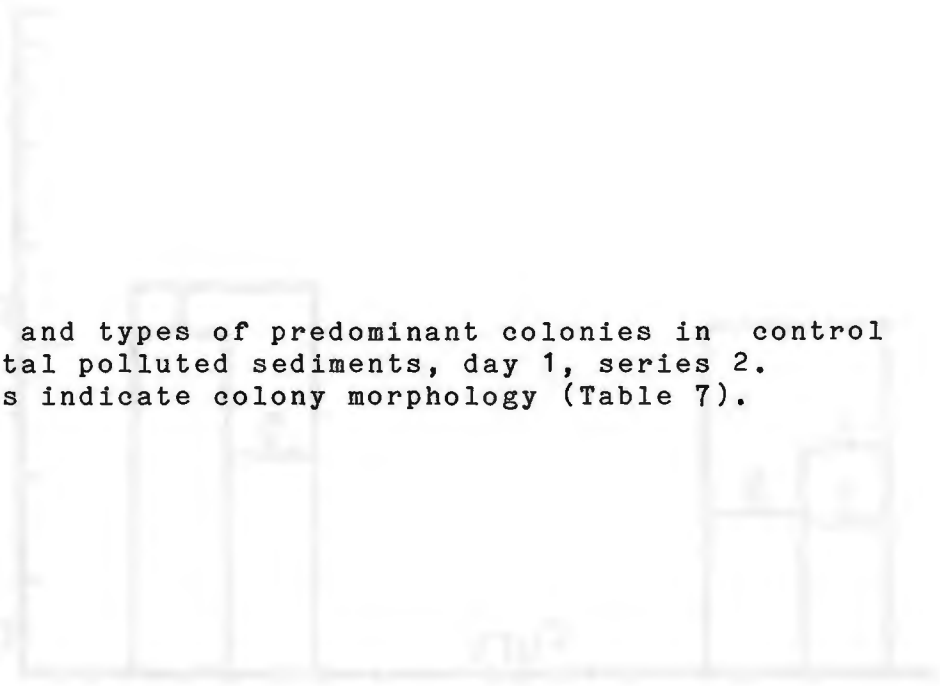
Table 9. Bacteria concentrations (CFU/ml) found in the control, metal mixture, and lead polluted sediments, series 2.

DATE	DAY	CONCENTRATION (CFU/ml) CONTROL	METAL MIXTURE	LEAD
4-4	1	1.7×10^3	$<1.0 \times 10^2$	1.25×10^3
4-6	3	1.0×10^3	5.0×10^2	2.0×10^3
4-8	5	3.55×10^3	$<1.0 \times 10^2$	1.12×10^5
4-10	7	1.55×10^3	$<1.0 \times 10^2$	3.32×10^5
4-12	9	4.65×10^3	1.2×10^3	9.0×10^3
4-15	12	1.51×10^4	1.03×10^4	8.55×10^3
4-18	15	3.15×10^3	7.95×10^4	5.4×10^3
4-21	18	1.85×10^3	6.0×10^5	1.36×10^4
4-25	22	2.35×10^3	2.20×10^5	9.95×10^3
4-28	25	8.5×10^2	7.8×10^4	6.55×10^3
5-2	29	2.25×10^3	3.0×10^3	2.8×10^3

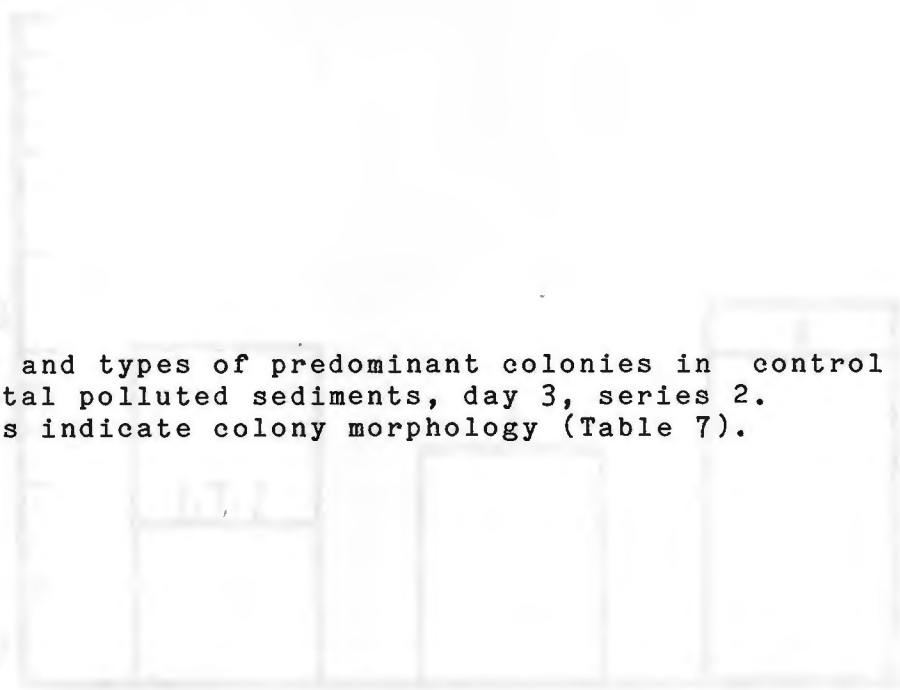
Table 10. Number and type of predominant colonies found in each test sediment, series 2.

DATE	DAY	CONCENTRATION BACTERIA (Types, organisms/ml) CONTROL	METAL MIXTURE	LEAD
4-4	1	X 1.2×10^2 C 5.0×10^2	-	K 3.5×10^2 X 3.0×10^2 A 2.0×10^2
4-6	3	I,T,Z 3.0×10^2	A 5.0×10^2	A 1.0×10^3
4-8	5	A 2.6×10^2	-	N 7.6×10^4 I 3.5×10^4
4-10	7	NT (SP*)	-	N 2.73×10^5 I 5.75×10^4
4-12	9	J 3.8×10^3 A 6.5×10^2	I 1.05×10^3 H 1.5×10^2	H 9.0×10^3
4-15	12	H 1.21×10^4 A 1.75×10^3	I 9.5×10^3 A 6.0×10^2	CC 3.9×10^3 J 3.05×10^3
4-18	15	H 1.4×10^3 A 4.5×10^2	H 6.5×10^4 D 1.3×10^4	H 4.0×10^3 B 8.5×10^2
4-21	18	I 1.0×10^3 N 2.5×10^2	I 5.45×10^5 D 5.5×10^4	N 6.25×10^3 H 1.55×10^2 A 1.3×10^2
4-25	22	O 1.2×10^3 J 5.0×10^2	A 2.19×10^5	N 3.95×10^3 A 2.3×10^3
4-28	25	H 3.0×10^2 C,BB 2.0×10^2	H 7.35×10^4	N 4.95×10^3 A 8.0×10^2
5-2	29	H 7.5×10^2 C,GG 3.5×10^2	A 2.05×10^3 D 9.5×10^2	N 2.1×10^3 X 3.5×10^2

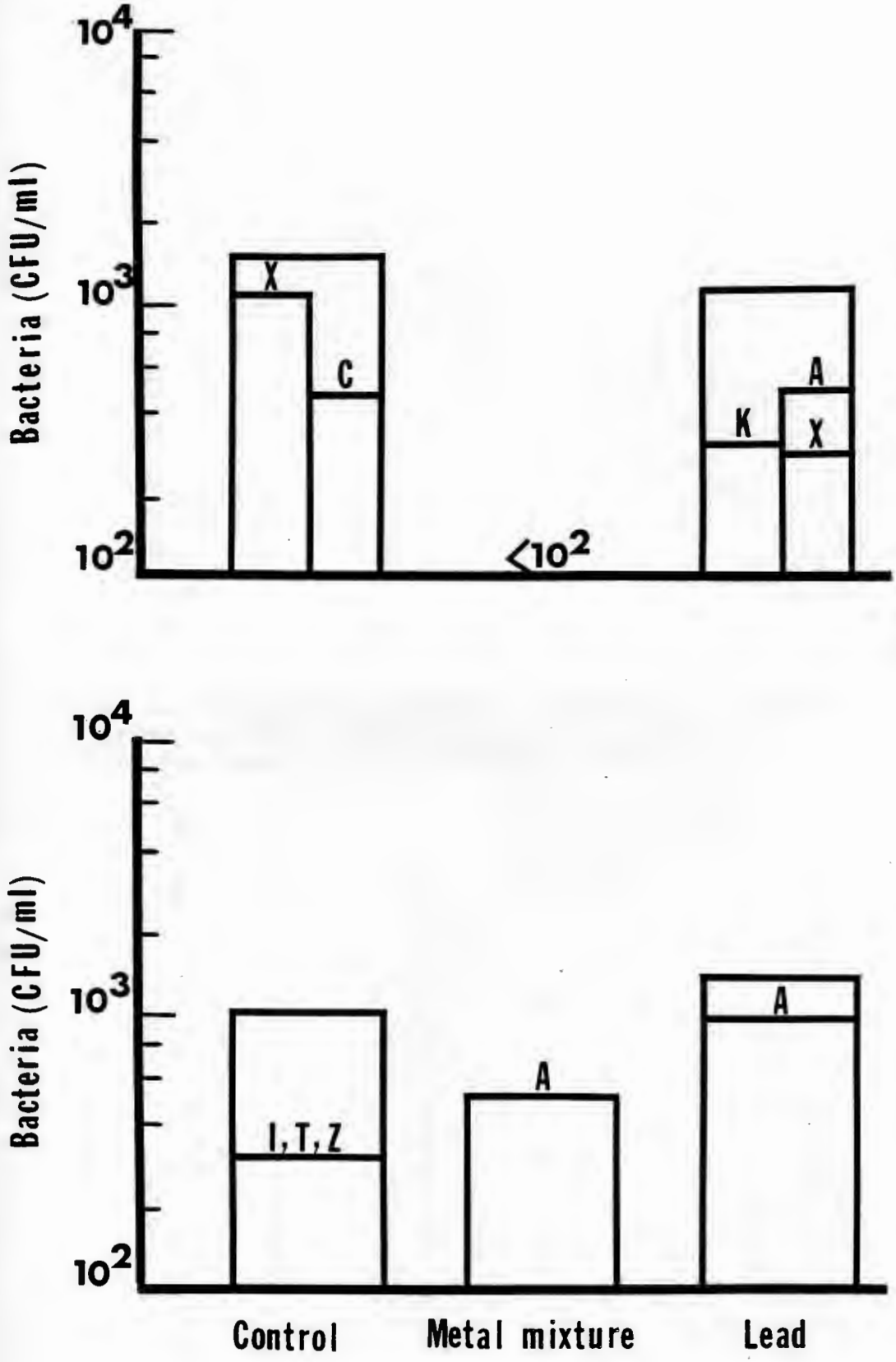
*NT-Not typed
SP-spreader



ig. 6a. Number and types of predominant colonies in control and metal polluted sediments, day 1, series 2. Letters indicate colony morphology (Table 7).



ig. 6b. Number and types of predominant colonies in control and metal polluted sediments, day 3, series 2. Letters indicate colony morphology (Table 7).


Bacteria (CFU/ml)
10^4
10^3
10^2
X
C
<10^2
K
A
X
Bacteria (CFU/ml)
10^4
10^3
10^2
I, T, Z
A
A
Control
Metal mixture
Lead

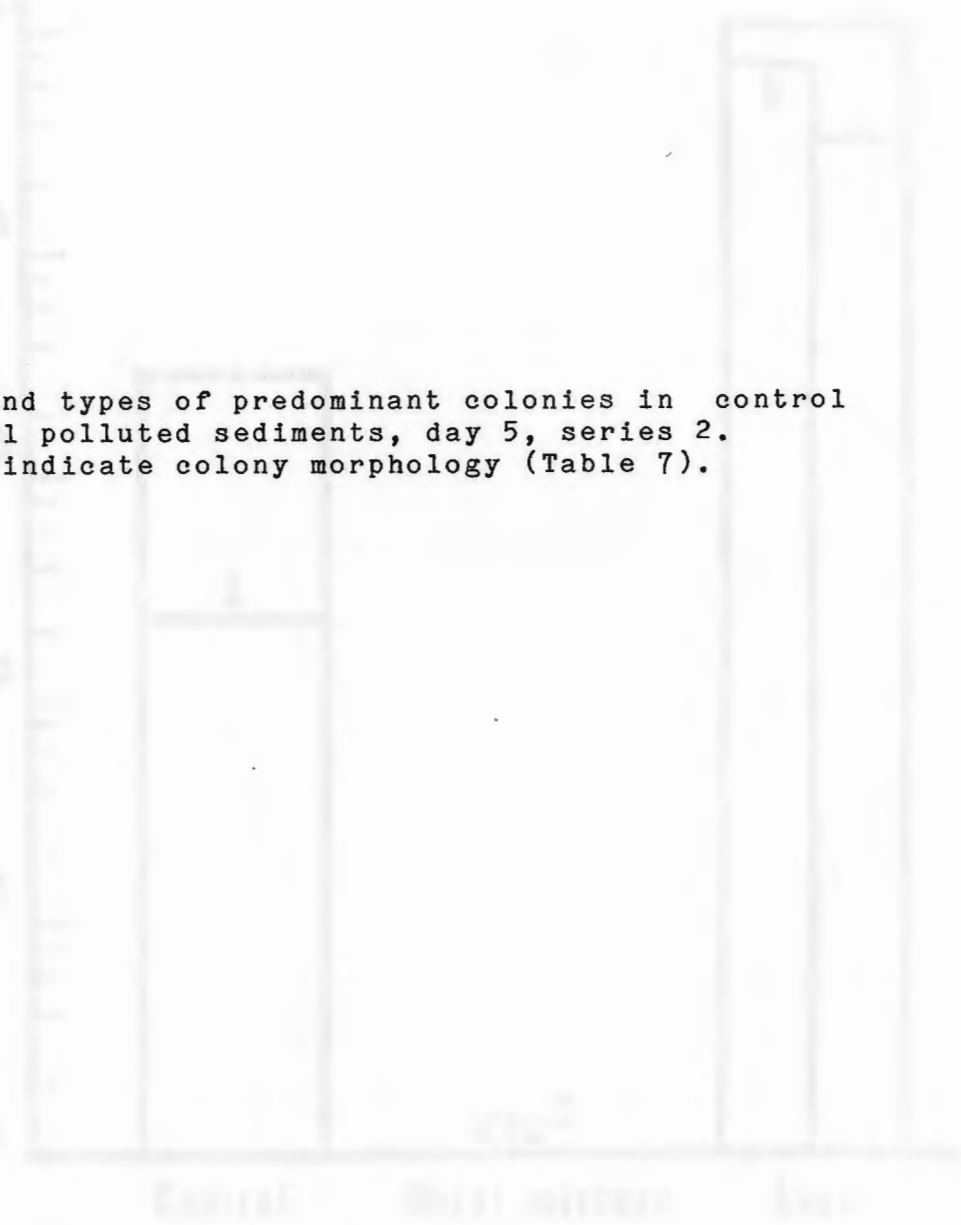
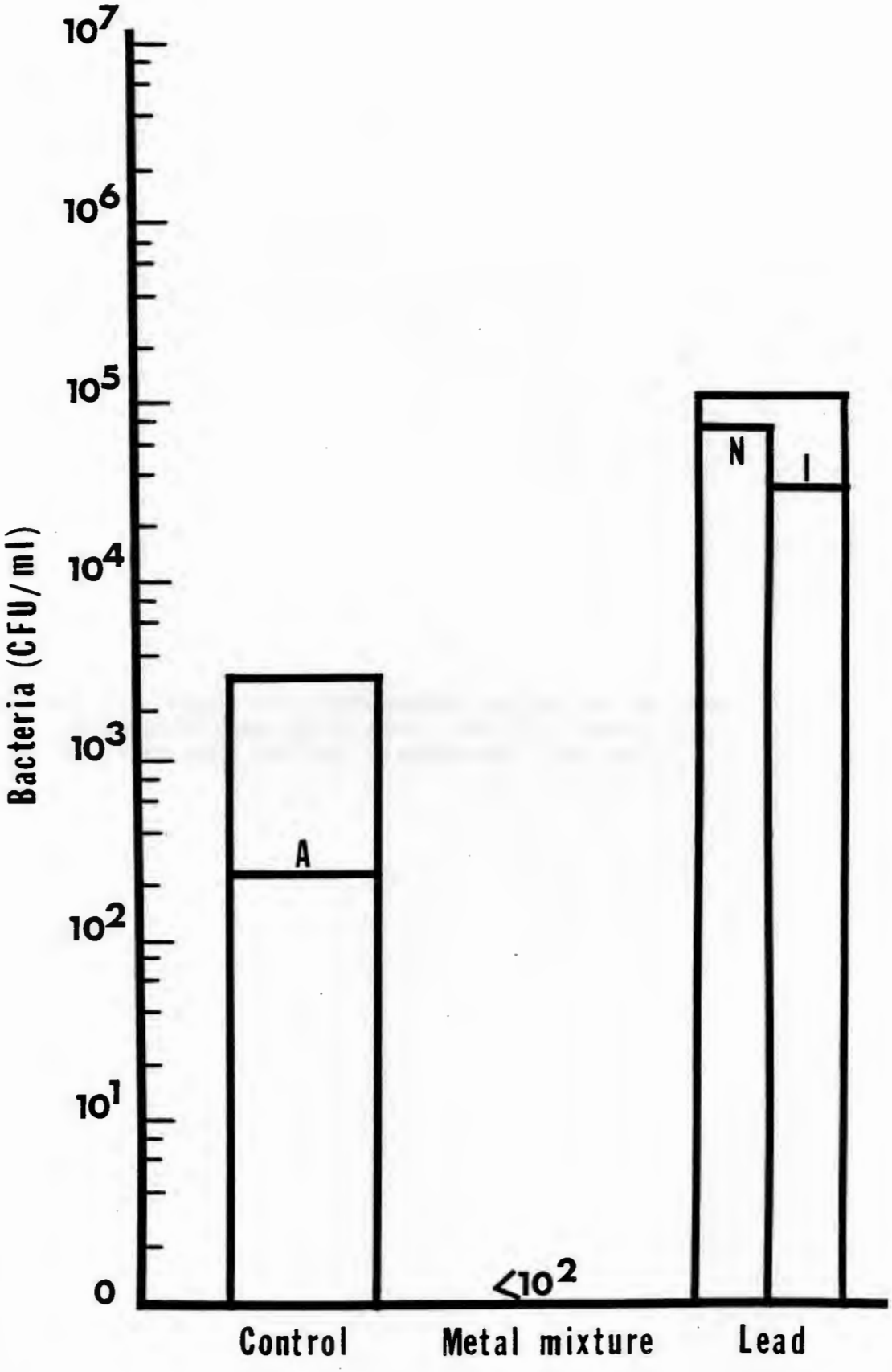
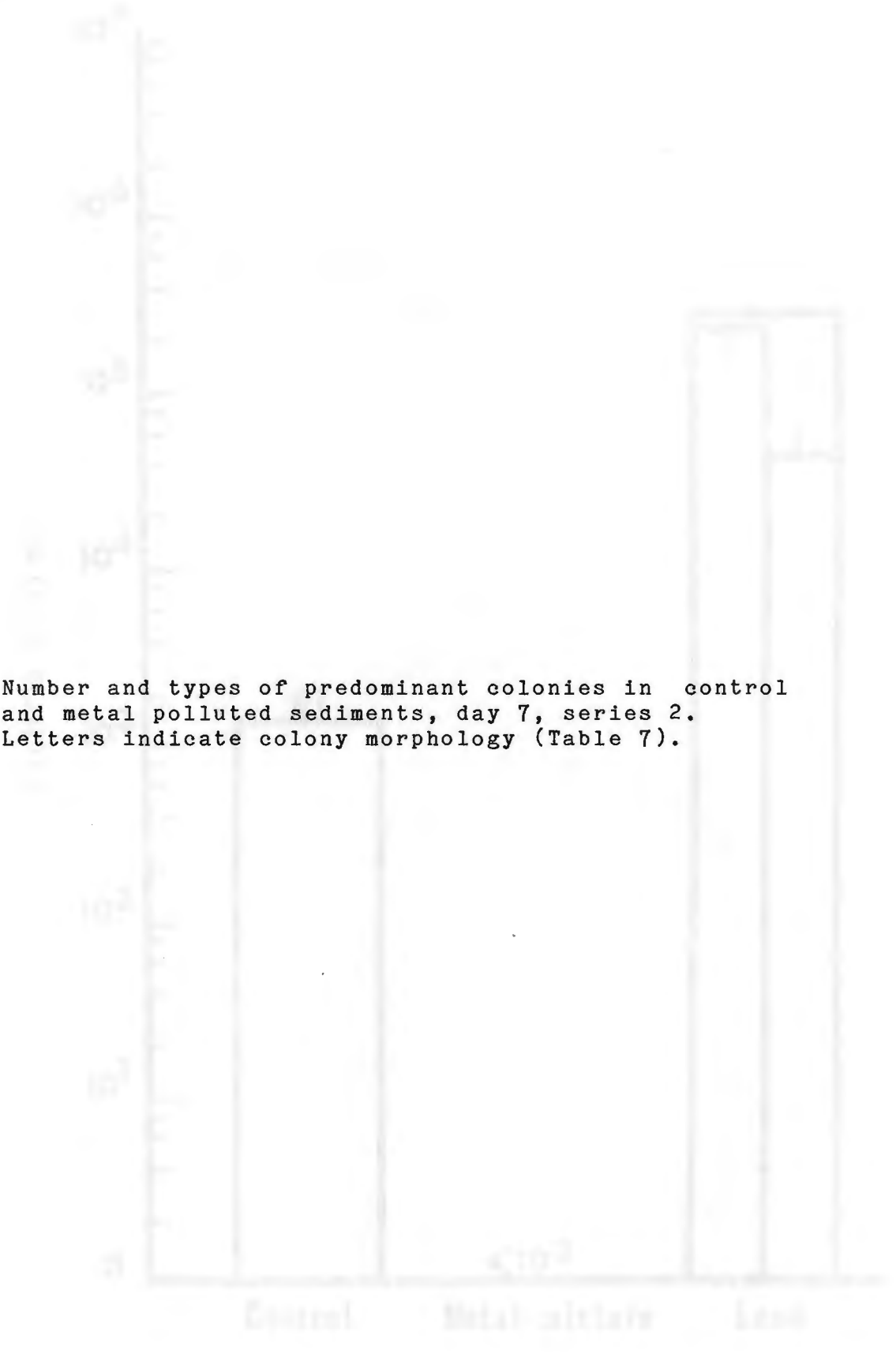
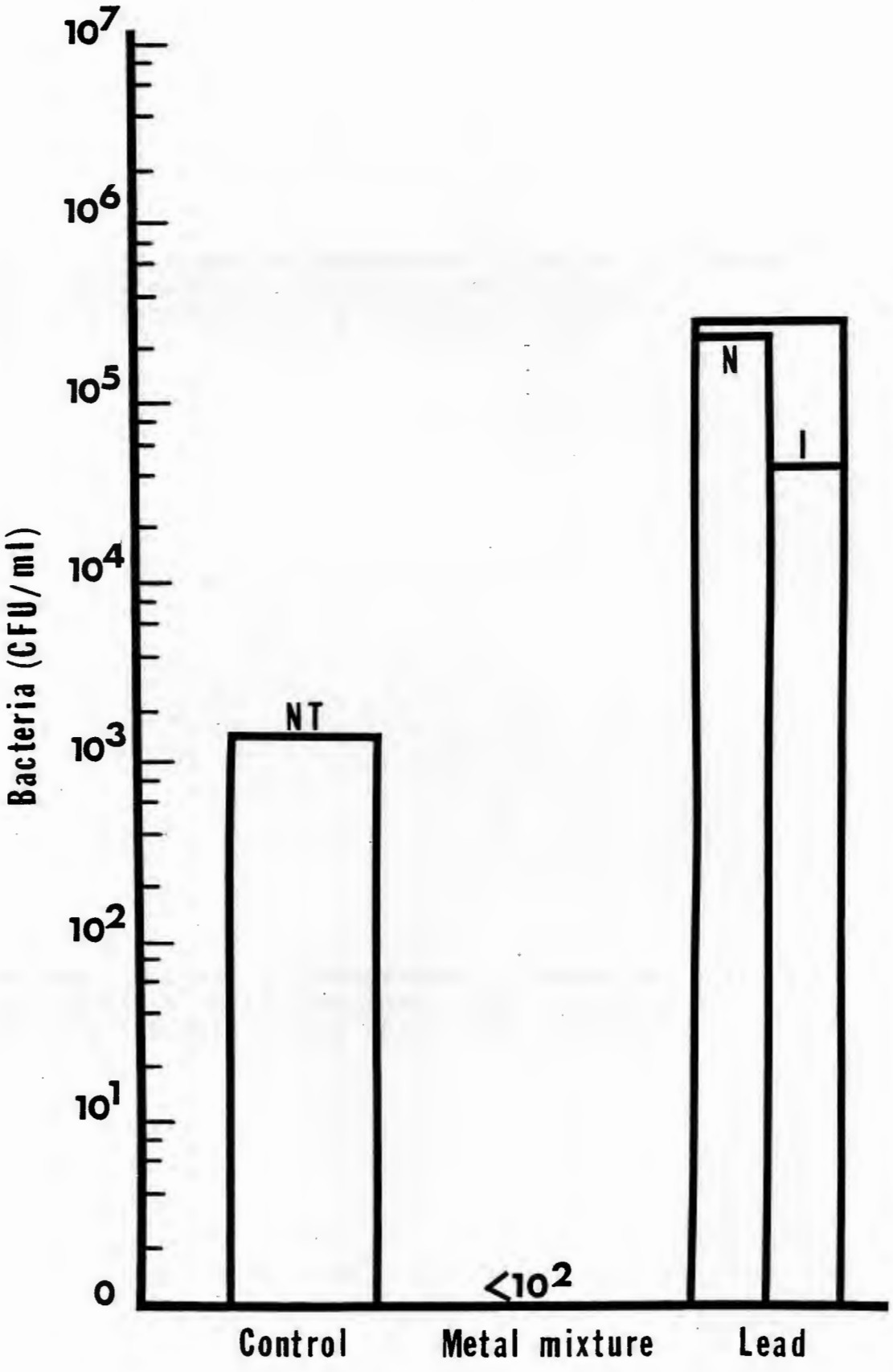


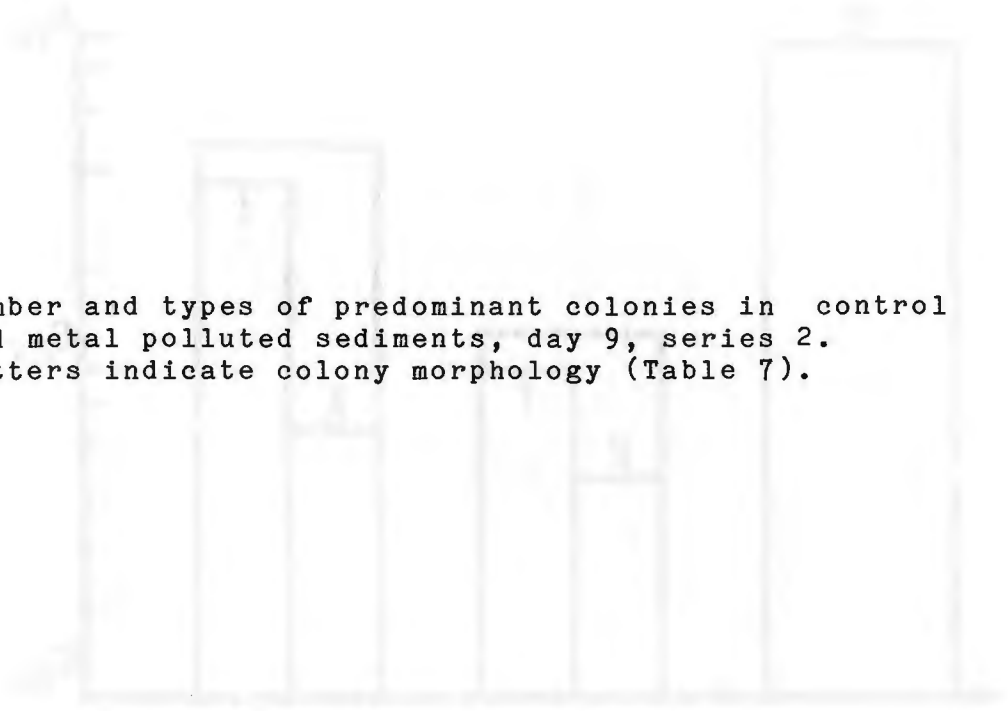
Fig. 6c. Number and types of predominant colonies in control and metal polluted sediments, day 5, series 2. Letters indicate colony morphology (Table 7).


10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
Bacteria (CFU/ml)
A
N
I
$<10^2$
Control
Metal mixture
Lead

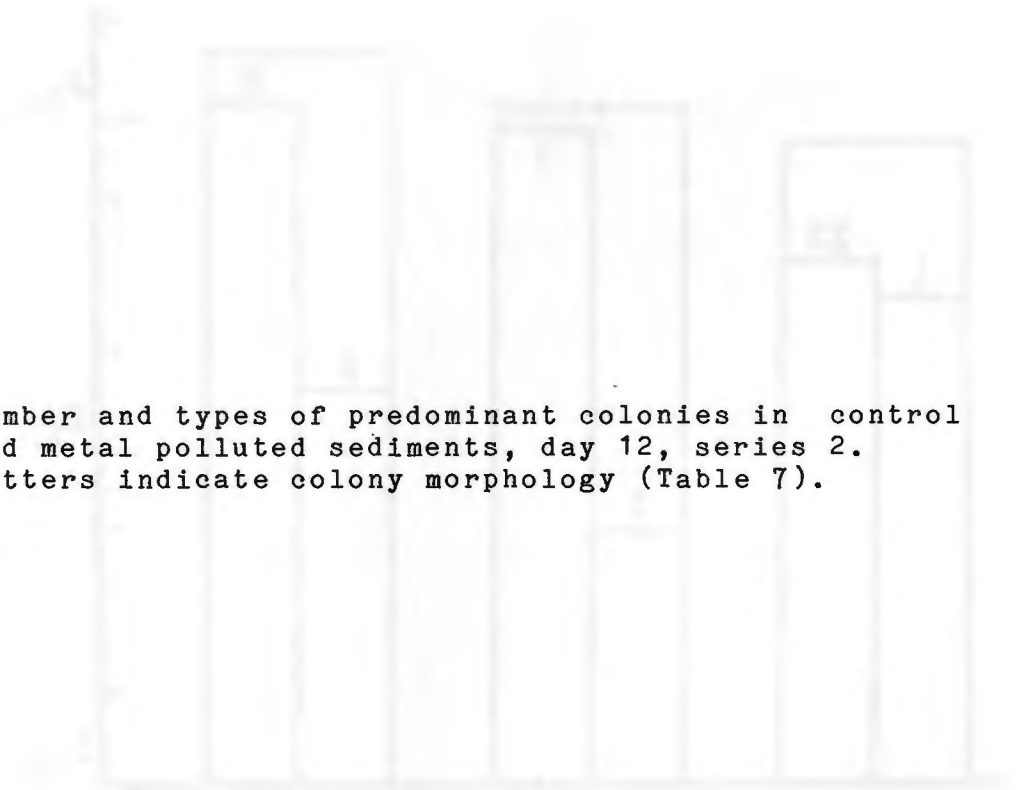


ig. 6d. Number and types of predominant colonies in control and metal polluted sediments, day 7, series 2. Letters indicate colony morphology (Table 7).

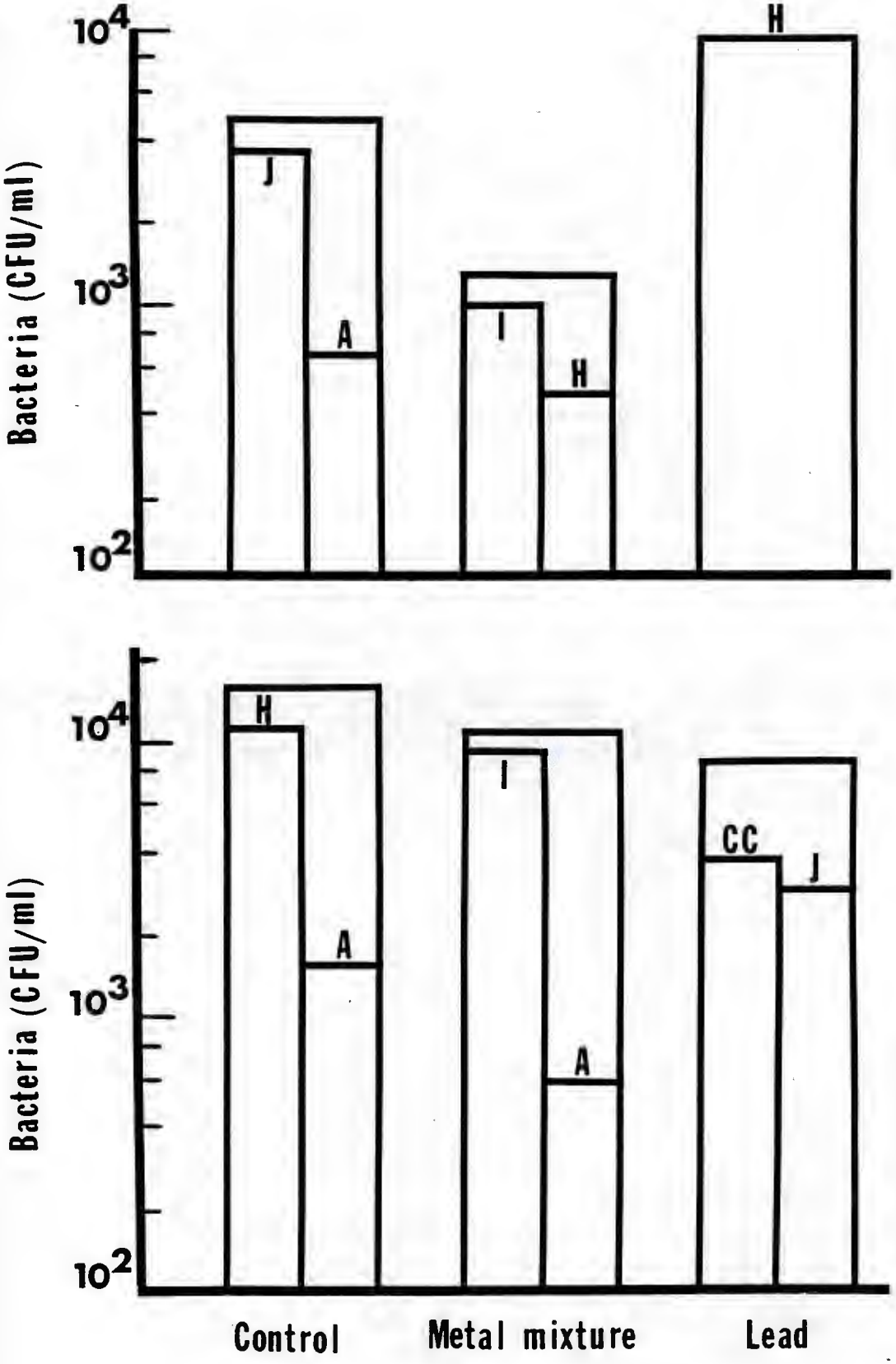

Bacteria (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
NT
N
I
<10^2
Control
Metal mixture
Lead



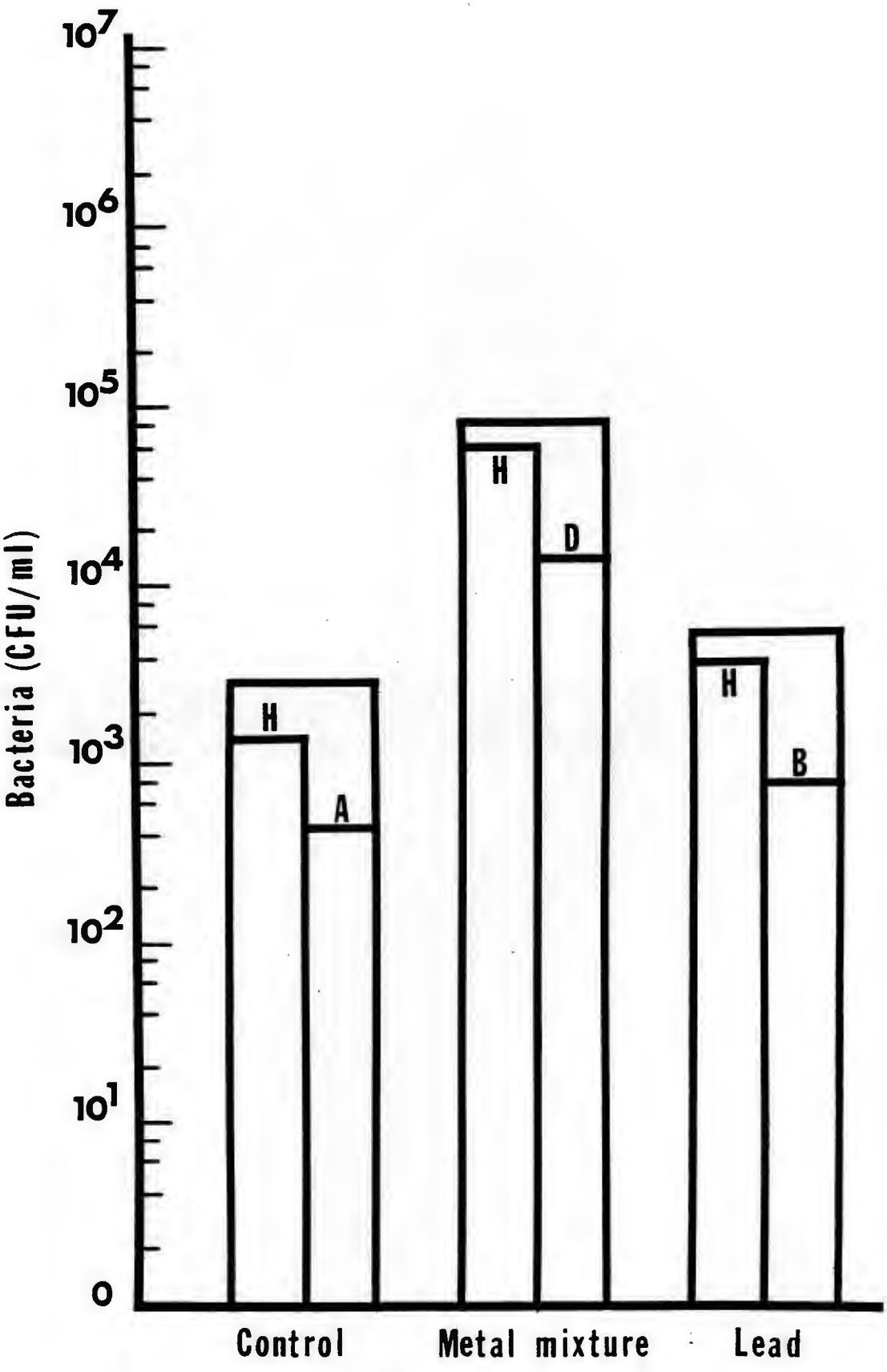
ig. 6e. Number and types of predominant colonies in control and metal polluted sediments, day 9, series 2. Letters indicate colony morphology (Table 7).



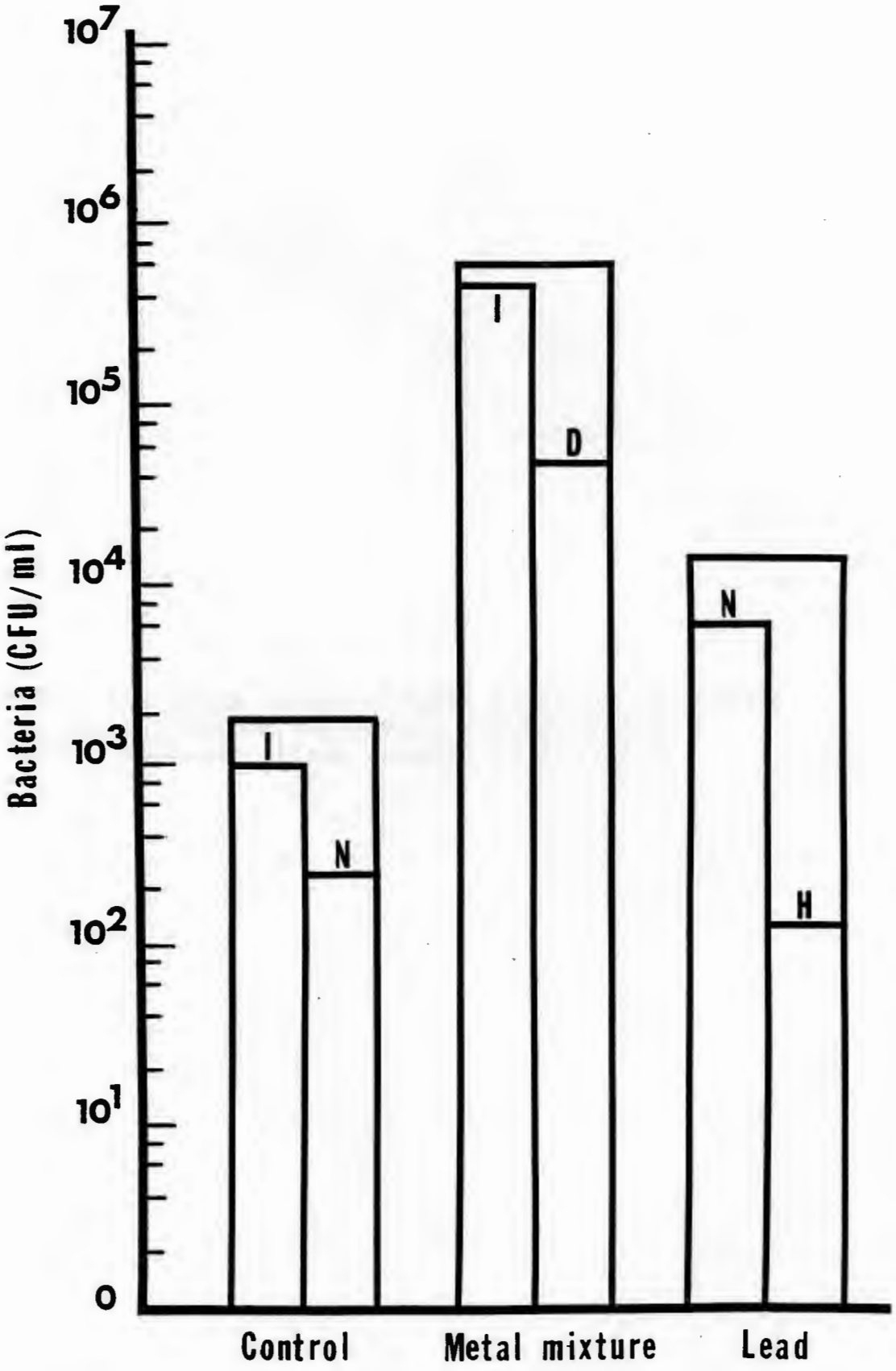
ig. 6f. Number and types of predominant colonies in control and metal polluted sediments, day 12, series 2. Letters indicate colony morphology (Table 7).


Bacteria (CFU/ml)
10^4
10^3
10^2
J
A
I
H
H
Bacteria (CFU/ml)
10^4
10^3
10^2
H
A
I
A
CC
J
Control
Metal mixture
Lead

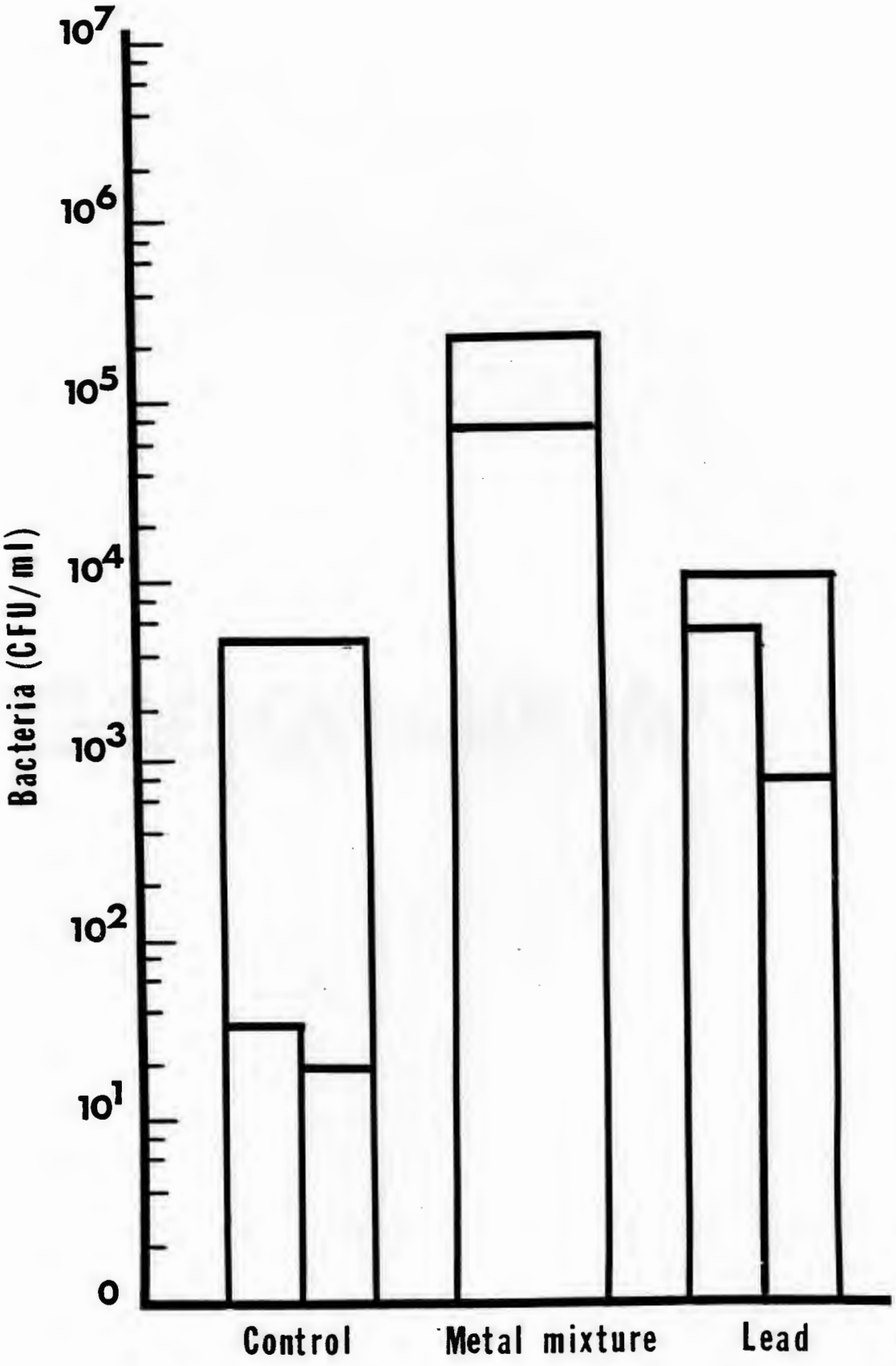
ig. 6g. Number and types of predominant colonies in control and metal polluted sediments, day 15, series 2. Letters indicate colony morphology (Table 7).


Bacteria (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
H
A
H
D
H
B
Control
Metal mixture
Lead

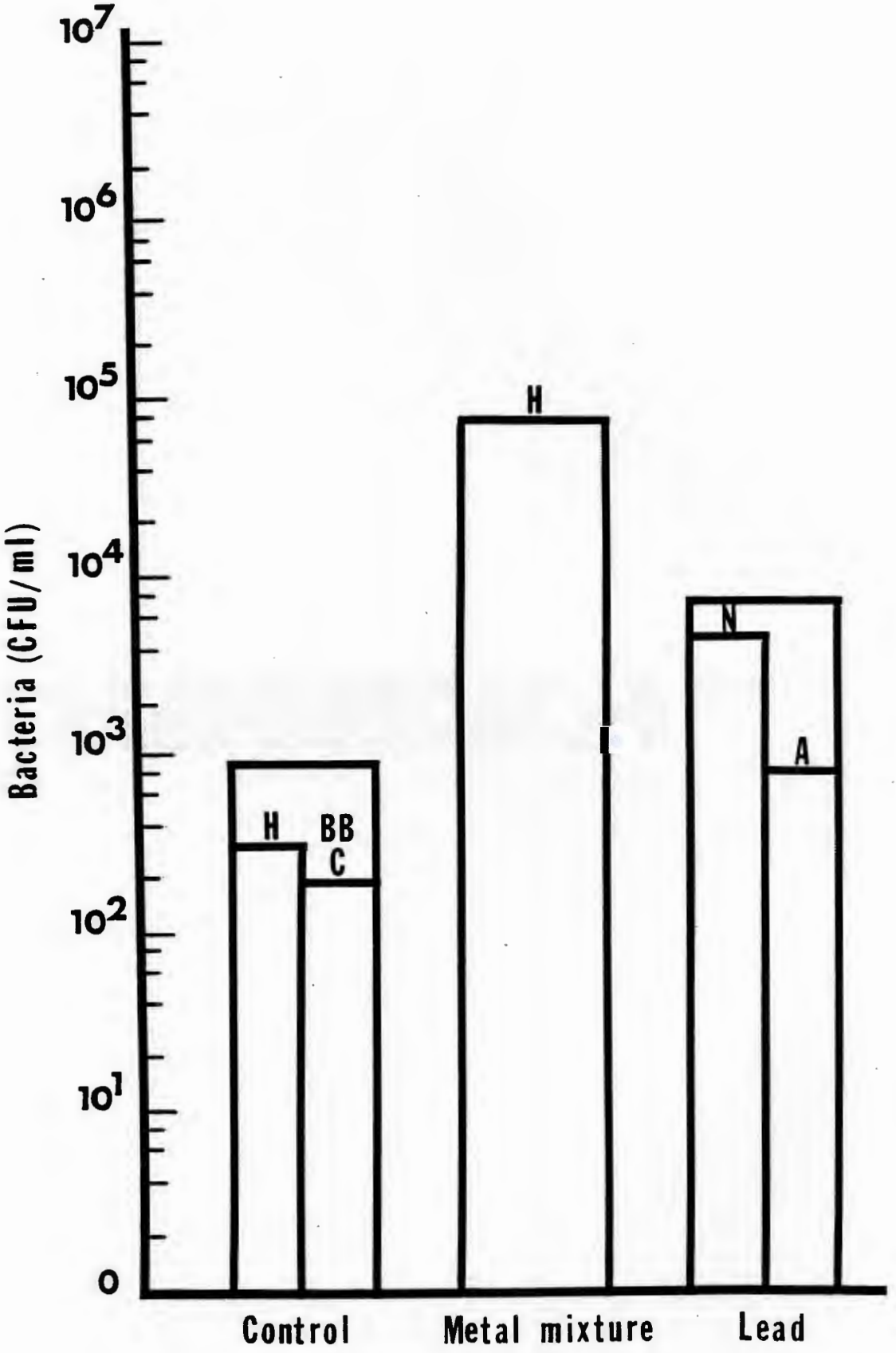
g. 6h. Number and types of predominant colonies in control and metal polluted sediments, day 18, series 2. Letters indicate colony morphology (Table 7).


Bacteria (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
I
N
I
D
N
H
Control
Metal mixture
Lead

ig. 6i. Number and types of predominant colonies in control and metal polluted sediments, day 22, series 2. Letters indicate colony morphology (Table 7).


10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
Bacteria (CFU/ml)
Control
Metal mixture
Lead

ig. 6j. Number and types of predominant colonies in control and metal polluted sediments, day 25, series 2. Letters indicate colony morphology (Table 7).


Bacteria (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
H
BB
C
H
N
A
Control
Metal mixture
Lead

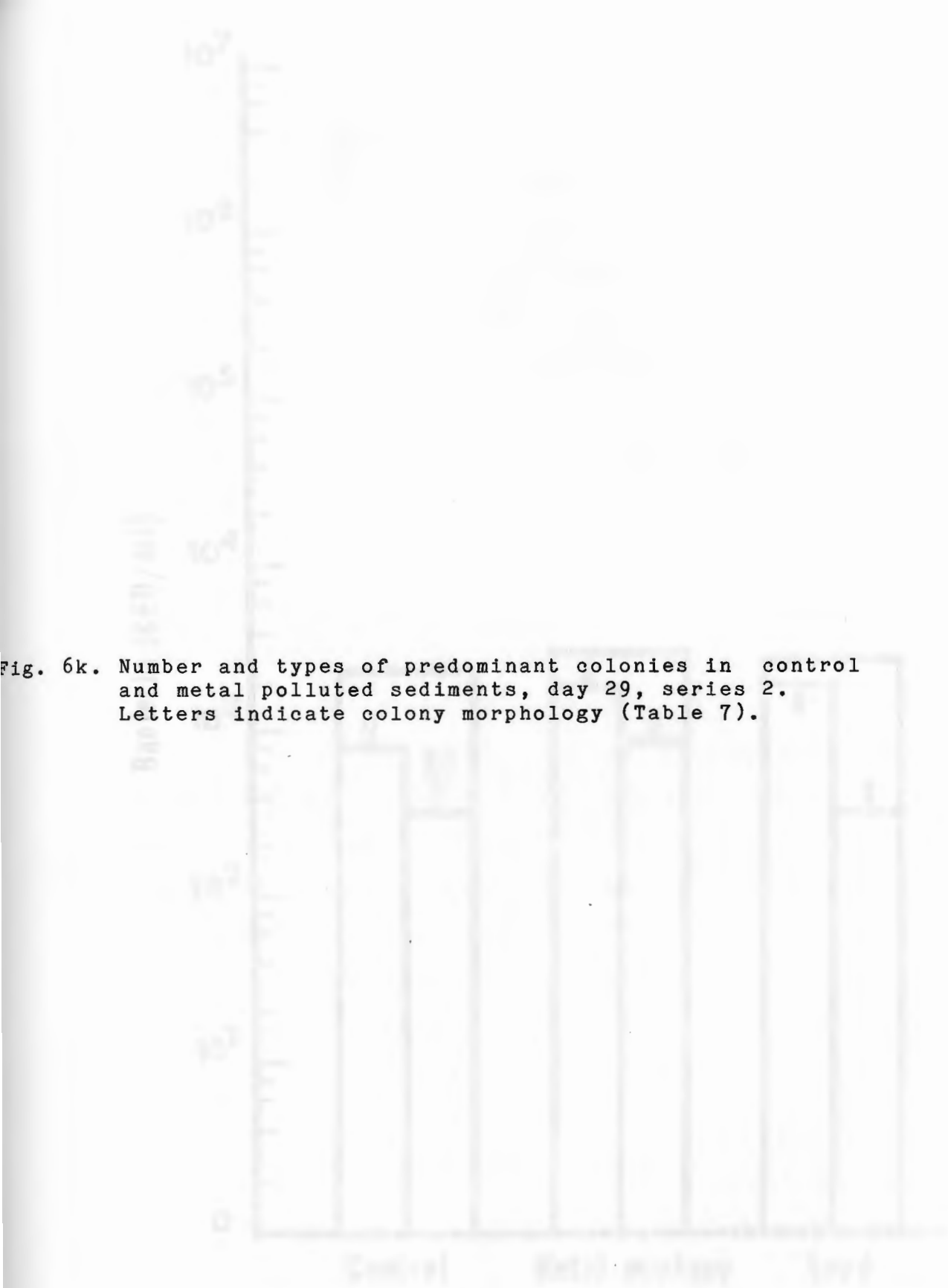
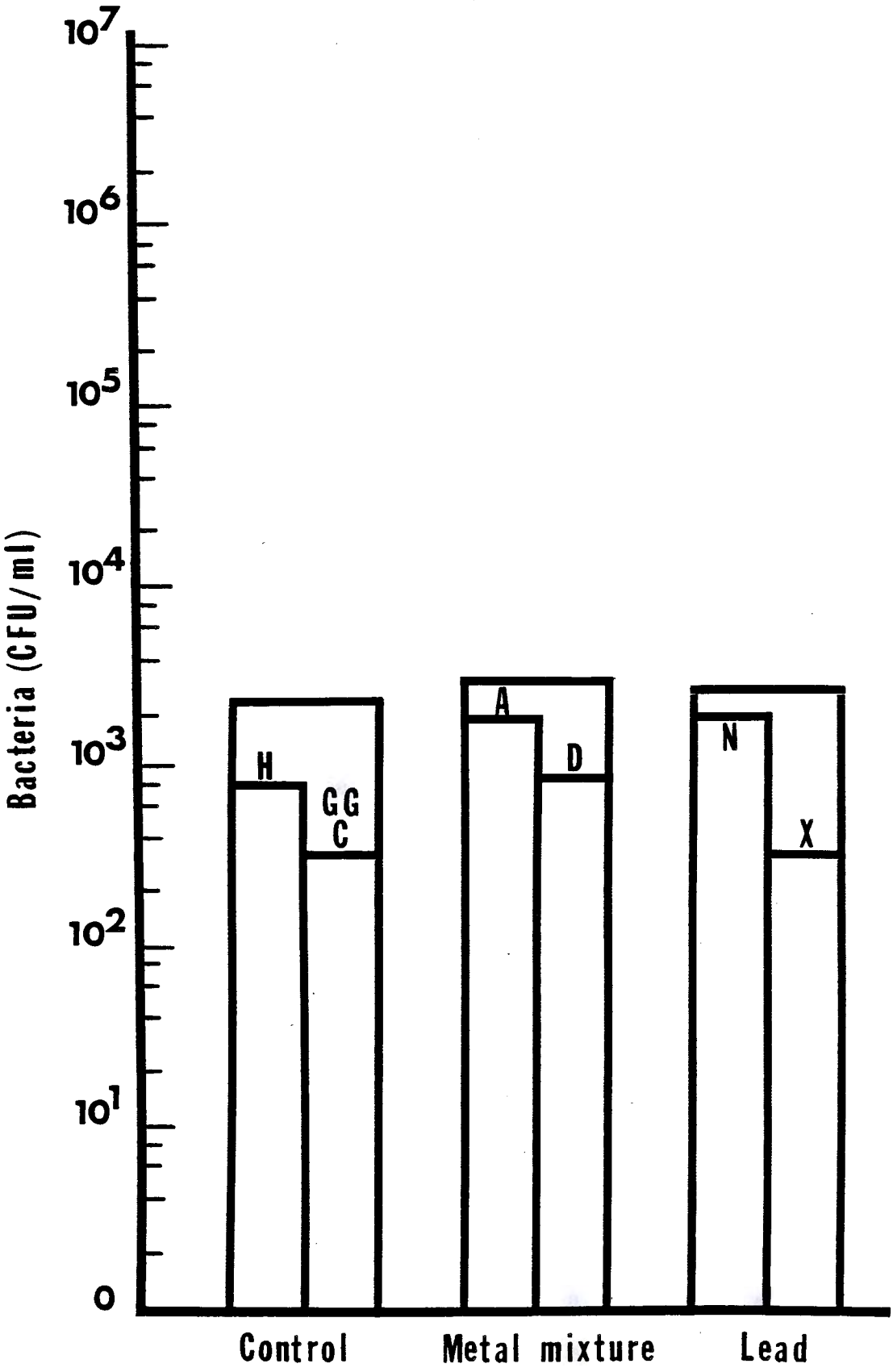


Fig. 6k. Number and types of predominant colonies in control and metal polluted sediments, day 29, series 2. Letters indicate colony morphology (Table 7).


Bacteria (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
H
GG
C
A
D
N
X
Control
Metal mixture
Lead

A cross tolerance test was used to determine if the predominant colony types from each sediment (i.e., control, metal mixture, and lead) could tolerate conditions found in each of the other two sediments. The sample consisted of five colonies of each predominant type, inoculated into TSB, TSB + metal mixture, and TSB + Pb. The experiment was run twice, and the results were similar for both series 1 (Table 11) and series 2 (Table 12).

Colony type H was representative of both control samples, and showed growth in in TSB (control) and slow growth in lead amended TSB. No growth was observed in the metal mixture amended media.

From the mixed metal polluted sediment, type H from series 1 and type A from series 2, growth was positive in the control, metal, and lead media. Type H showed delayed growth in the lead medium as was observed in the type H control isolate. In each series, the growth patterns were consistant for the respective colony types, indicating that each type consisted of a homogeneous population.

Colony type N was predominant in both lead sediment series, and showed positive growth in both the control and lead tests. No growth was observed in mixed metal amended medium. In series 2, colony type N appears to be composed of the same organism. However, type N of series 1 is not equal throughout. Isolate number 4 from the lead amended sediment exhibits the ability to grow in TSB plus a metal mixture,

Table 11. Growth response of the predominant colony type when exposed to a variety of pollution conditions to determine if growth can occur in an unfamiliar environment, series 1.

4-28-83

From control sediment to:

	TSB	TSB + METAL MIXTURE	TSB + Pb*
1	+	-	+(d)
2	+	-	+(d)
3	-	-	+(d)
4	+	-	+(d)
5	+	-	+(d)

Predominant colony type: H

From metal mixture polluted sediment to:

	TSB	TSB + METAL MIXTURE	TSB + Pb
1	+	+	+(d)
2	+	+	+(d)
3	+	+	+(d)
4	+	+	+(d)
5	+	+	+(d)

Predominant colony type: H

From lead polluted sediment to:

	TSB	TSB + METAL MIXTURE	TSB + Pb
1	+	-	+
2	+	-	+
3	+	-	+
4	+	+	+
5	+	-	+

Predominant colony type: N

*(d)-delayed growth

Table 12. Growth response of predominant colony types when exposed to a variety of pollution conditions to determine if growth can occur in an unfamiliar environment.

From control sediment to:

	TSB	TSB + METAL MIXTURE	TSB + Pb
1	+	-	+
2	+	-	+
3	+	-	+
4	+	-	+
5	+	-	+

Predominant colony type: H

From metal mixture polluted sediment to:

	TSB	TSB + METAL MIXTURE	TSB + Pb
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+

Predominant colony type: A

From lead polluted sediment to:

	TSB	TSB + METAL MIXTURE	TSB + Pb
1	+	-	+
2	+	-	+
3	+	-	+
4	+	-	+
5	+	-	+

Predominant colony type: N

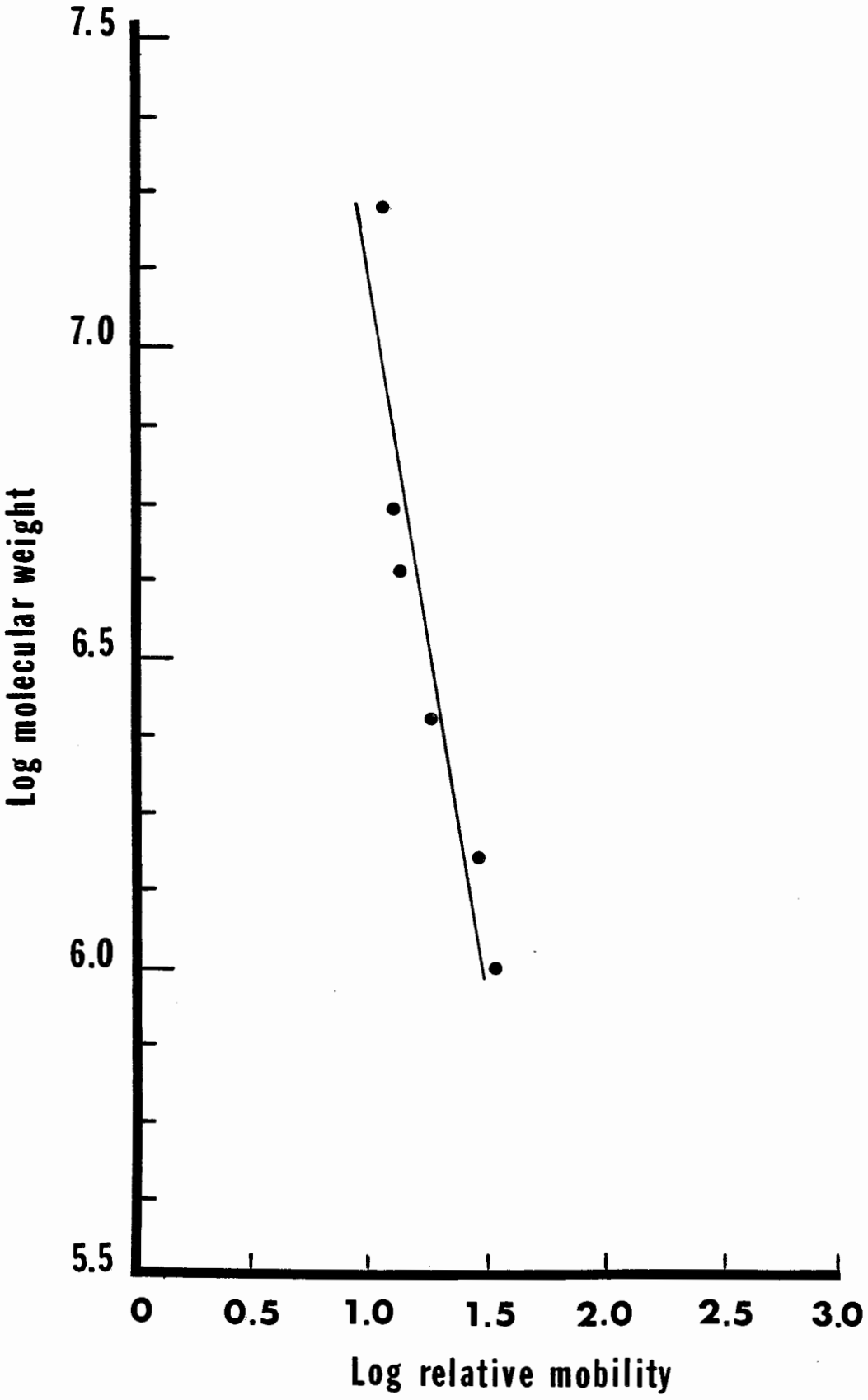
whereas none of the other isolates show this capability. With the exception of isolate 4, the presence of lead alone does not appear to select for resistance to all the metals present in the mixture.

PRESENCE OF PLASMIDS IN PAWTUXET RIVER SELECTED ISOLATES

Plasmids have been shown to confer resistance to several different heavy metals. Hg and Cd tolerance are known to be encoded by plasmids in environmental and clinical isolates, while plasmid mediated lead resistance has been demonstrated in certain clinical isolates. No plasmid resistance mechanisms have been shown for Sn or Zn, although a mechanism has been suggested linking increased Zn tolerance to gene products found in Cd resistant isolates. Environmental isolates were screened for plasmids to investigate if resistance to Pb, Sn, and/or Zn is possibly plasmid mediated. The role of plasmids in multiple metal resistance is also important and was investigated.

Agarose gel electrophoresis was used to screen Flavobacterium PWX2 and Aeromonas PWX7 for the presence of plasmids, and the molecular weights were determined by using the fragments of lambda DNA-Hind III digest which are of known molecular weights (Figure 7). Flavobacterium PWX2 is resistant to the metals Hg, Cd, Pb, Sn, and Zn, and contains five or more plasmids ranging in molecular weight from

Fig. 7. Log molecular weight versus log relative mobility of λ-DNA Hind III digest. Mobility of fragment one arbitrarily set equal to 10.


7.5
7.0
6.5
6.0
5.5
Log molecular weight
0
0.5
1.0
1.5
2.0
2.5
3.0
Log relative mobility

8.71×10^6 to 43.65×10^6 daltons (Figure 8, Table 12). One or more of these plasmids may act to confer resistance to one or more metals in the mixture. There appeared to be no difference in the plasmid patterns when the organism was grown in TSB + each metal at 50 ug/ml, and TSB + metal mixture.

Aeromonas PWX7 ultimately lost its resistance to Cd and Zn at 50 ug/ml, but remained resistant to 50 ug/ml each of Hg, Pb, and Sn. After the loss of Cd and Zn resistance this isolate contained two different plasmid patterns depending on which metal it was grown (Figure 9, Table 12). When grown in TSB + 50 ug/ml Hg, two plasmids were present at molecular weights of 12.58×10^6 and 19.49×10^6 daltons. When the organism was grown in TSB amended with 50 ug/ml of Pb or Sn, only the larger plasmid of 19.49×10^6 molecular weight was present. This data suggests that the large plasmid may indeed encode for multiple metal resistance.

Table 13. Size and molecular weight of plasmid DNA isolated from *Flavobacterium* PWX2 and *Aeromonas* PWX7.

	Molecular weight (daltons)	Kilobase (kb)
Flavobacterium PWX2		
1	43.65×10^6	29.10
2	31.62×10^6	21.08
3	12.58×10^6	8.39
4	9.55×10^6	6.37
5	8.71×10^6	5.80

All plasmids are present when the organism is grown in TSB amended with Hg, Cd, Pb, Sn, and/or Zn is present.

Aeromnonas PWX7.		
1	19.49×10^6	12.99
2	12.58×10^6	8.39

Plasmids 1 and 2 are present in Pb or Sn amended TSB.
Plasmid 2 only present in Hg amended TSB.

Fig. 8. Agarose gel electophoresis of plasmid DNA from Flavobacterium PWX2. The organism was grown in TSB amended with 50 ug/g of: (A) Hg, (B) Cd, (C) Pb, (D) Sn, and (E) Zn. Lane (F) is purified λ-DNA Hind III digest standard.

A B C D E F

A B C D E F

'ig. 9. Agarose gel electophoresis of plasmid DNA from Aeromonas PWX7. The organism was grown in TSB amended with 50 ug/g of: (A) Hg, (B) Cd, (C) Pb, (D) Sn, and (E) Zn. Lane (F) is purified λ-DNA Hind III digest standard.

A B C D E F

A B C D E F

CONCLUSIONS

1. Sediment populations are more likely to be resistant to metals present in high concentrations in the sediment from which they were isolated than to metals absent or present only in low amounts. Pb, Sn, and Zn were found at high concentrations in Pawtuxet River sediment and were found only at best moderately inhibitory. Hg and Cd, present at low levels in the sediment, were found highly toxic.

2. The temperature of incubation only affected the inhibitory effects of Sn and Zn. Zn was more inhibitory at ambient temperature incubation regardless of Eh values, while Sn showed increased inhibition at ambient temperature only under positive Eh values.

3. Selected sediment isolates were more tolerant to Pb, Sn, and Zn than Hg or Cd when grown on a solid medium (TSA). Resistance levels were further elevated for each metal when the isolates were grown in a liquid medium (TSB).

4. Exposure of an unpolluted sediment population to Pb or a metal mixture results in an initial depression of population levels. Recovery becomes equivalent to or greater than control levels indicating that a large segment of the population acquires resistances to Pb and the mixture of metals respectively. The metal mixture and Pb leads to selection of particular colony types, which become predominant. Selection is more rapid and defined than in the

control.

5. Selection appears to be generally homogeneous. Pb alone as a selective agent is not in itself toxic, as isolates from control sediments exhibited growth in a lead amended medium. Exposure of an isolate to lead, with one exception, does not confer resistance to the metal mixture.

6. Isolate Flavobacterium PWX2 is resistant to the metal mixture, and contains at least five plasmids, one or more of which may encode for metal resistance. Aeromonas PWX7 has two or more plasmids, and exhibits different plasmid patterns depending in which metal it has been grown. Both plasmids are present when the isolate is grown in Hg, while only one is present when grown in either Sn or Pb.

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Appendix A: Biochemical test reults used in identifying *Flavobacterium* PWX2 and *Aeromonas* PWX7.

	ORGANISM IDENTIFICATION	
ISOLATE	*Flavobacterium* PWX2	*Aeromonas* PWX7
BIOCHEMICAL TESTS		
Fermentaion of:		
Glucose	+	+* (d)
Fructose	+*	+*
Sucrose	+* (w,d)	-* (d)
Mannose	-	-
Lactose	-	-
Rhamnose	-	-
Dulcitol	-*	-
Mannitol	+* (w)	-
Xylose	-	-
Inulin	-	-
Inositol	-	-
Maltose	+	+*
Cellabiose	-*	+* (w,d)
Catalase	+* (w)	+
Oxidase	+	+
Citrate (24 hr.)	-	-
(48 hr.)	+	-
MR	-	-
VP	-	-
TSI (24 hr.)	K/K/-	K/K/-
(48 hr.)	K/K/-	A/A/-
Motility	-	+
Mucate	+	-
Urease	-	-
Amylase	-	-
Gelatinase	-	+
BTB	facultative	facultative
Arginine dehydrolase	+* (d)	+
Ornithine decarboxylase	-	+
Lysine decarboxylase	+	+
Nitrate reduction	+(gas)	-
Casein hydrolysis	-	+*
Bile Esculin	+	+* (d)
NaCl		
3%	+	+
6.5%	+	+
15%	-	-
20%	-	-

pH		
3	+	+
5	+	+
7	+	+*
9	+	+* (w)
Phenol		
0.1%	-	-
0.3%	-	-
Pigment production		
15°C	-	-
20°C	-	-

*(d)-delayed growth
(w)-weak growth

MORPHOLOGY

PWX2 Gram negative rod; (approximately 2.28x3.0 u)
PWX7 Gram negative rod, (approximately 1.5x2.28 u)