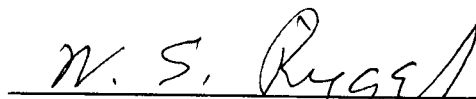


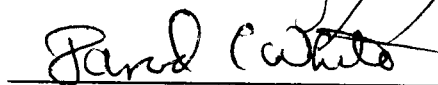
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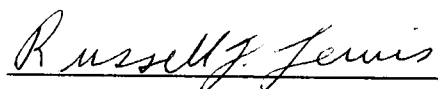
I am submitting herewith a dissertation written by Andrew Vaughn Ogram entitled "The Extraction and Purification of Microbial DNA from Sediment." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

  
\_\_\_\_\_  
Gary Saylor, Major Professor

We have read this dissertation  
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Accepted for the Council:

  
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Vice Provost  
and Dean of the Graduate School

THE EXTRACTION AND PURIFICATION OF  
MICROBIAL DNA FROM SEDIMENTS

A Dissertation  
Presented for the  
Doctor of Philosophy  
Degree  
The University of Tennessee, Knoxville

Andrew Vaughn Ogram

December 1988

To my parents  
with love and gratitude

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## ABSTRACT

A procedure for the extraction and purification of microbial DNA from sediments has been developed. This technique is based on the lysis of cells in the presence of the sediment and the subsequent extraction and purification of the DNA. The DNA recovered is of high quality as judged by ultraviolet spectrophotometry and is susceptible to cleavage by common restriction endonucleases, which renders it amenable for use in advanced techniques for the study of environmental community structure. Susceptibility to attack by restriction endonucleases is not a characteristic of all DNA obtained by this method, however. This procedure has the advantage over existing methods in that it allows a more representative sample of DNA to be recovered.

DNA adsorption isotherms were analyzed for a broad range of soil and sediment types, which allowed the major factors controlling DNA adsorption to be identified. Mineralogy and pH were the primary factors, with phosphate ion concentration content being less important. This knowledge led to the development of a procedure for the quantitative extraction of free DNA from sediments, an important phase in the total purification procedure.

This procedure also allowed the discrimination between extracellular and intracellular DNA to be made, which resulted in the first isolation and molecular analysis of sediment extracellular DNA to be made. A method for the rapid and accurate measurement of extracellular DNA concentrations was developed which utilizes high performance liquid chromatography and a fluorescence detection system.

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## CHAPTER 1

### INTRODUCTION

The analysis of the structure and function of natural microbial communities has traditionally relied upon cultural, physiological and biochemical techniques that are frequently time consuming and sometimes imprecise (50). By incorporating the techniques of molecular biology into microbial ecology, it is becoming possible to gain a great deal of information concerning the nature of microbial communities in a relatively short period of time and with a high degree of precision. The most commonly applied molecular techniques have involved the use of hybridization probes for the identification and enumeration of specific genetic sequences in a natural community, and the cloning of community DNA for population genetics studies is currently being developed. The primary requirement that any of these techniques have is that a representative sample, either as viable cells or as DNA, must be obtained, and be of sufficient purity for the desired application. Through nucleic acid hybridization technology and the use of specific gene probes, the identification and quantitation of specific genetic sequences within a microbial community is possible (79). Examples of environmentally relevant gene probes are listed in Table 1. These nucleic acid probes generally fall into two categories; species-specific probes (25,37,78) and function-specific probes (80). Species-specific probes are constructed from genetic sequences, usually from genes encoding ribosomal RNA, that are highly specific to one taxonomic group.

Table 1. Examples of Nucleic Acid Probes Used in Environmental Analysis

| Target                                  | Probe                                                    | Application                            | Ref.                |
|-----------------------------------------|----------------------------------------------------------|----------------------------------------|---------------------|
| 4-CB-degrading strains                  | pSS50                                                    | identification, enumeration, isolation | 72,76,77            |
| Naphthalene-mineralizing strains        | NAH-7                                                    | enumeration                            | 77                  |
| Gram-negative Mercury-resistant strains | <u>mer</u> operon                                        | identification, enumeration            | 7,8                 |
| <u>Bacillus subtilis</u>                | cloned fragment of 23 S rRNA                             | identification                         | 47                  |
| <u>Rhizobium</u>                        | <u>R. trifolii</u> chromosome                            | identification                         | 36                  |
| Nitrogen-fixing strain                  | <u>nif K,D,H</u> genes from <u>Klebsiella pneumoniae</u> | enumeration                            | Sayler, unpublished |

Depending upon the nature of the probe, it may be specific to a number of closely related species within the same genus, or to a specific strain within a species (23). Function specific probes, such as those that identify genes for nitrogen fixation or pollutant degradation, are not specific to one taxonomic group, but rather measure the potential of the community as a whole to perform a given function. It should be noted that function specific probes are usually qualitatively different from the more traditional measures of a given function, in that gene probes will provide a measure of the potential a community has for that function, but not the actual activity of the function. It would be necessary to use the more standard techniques if, for example, the total amount of nitrogen being fixed by a system must be known. This approach may also, however, be limited to potential activity if the analysis is conducted on samples taken from the environment.

Two recently developed gene probe techniques for use in molecular microbial ecology studies are DNA:DNA colony hybridization (8,80) and DNA:DNA hybridization of direct DNA extracts (38,79). Each of these techniques has its weaknesses and strengths which may be exploited depending upon the type of information desired. For most applications in community structure and function analysis, the total extraction and probing of community DNA may be preferable to colony hybridization. Colony hybridization has the requirement that cells be cultured before analysis may begin. Since many of the species in nature are not readily culturable (perhaps fewer than 10% of environmental isolates may be cultivated by standard laboratory media and growth conditions)

(54), most of the organisms in a typical sample may be excluded from analysis. Direct extraction and purification of DNA from an environmental sample may be more tedious than colony hybridization, but may yield a more truly representative sample. The benefits and drawbacks of both of these techniques and their contribution to efficient and specific analysis of microbial communities will be discussed in the following sections.

The colony hybridization technique for the detection of specific genetic sequences has been in use for over 12 years and was originally developed to facilitate the isolation of specific recombinant DNA clones in E. coli (35), but has only recently been applied to environmental analysis. The general procedure consists of growing cells on an appropriate solid medium, lysing the colonies and transferring the DNA to a DNA binding membrane, followed by hybridizing a  $^{32}\text{P}$ -labelled probe to specific sequences of the bound DNA. The number of colonies containing the target sequences are then enumerated, generally by autoradiography (35).

As mentioned previously, one of the major limitations of this procedure is the requirement that cells be cultured in the laboratory. There is no single growth medium available that will allow the cultivation of the majority of the hundred or more strains that may be present in a typical environmental sample (54). Strict anaerobes and species with unusual growth requirements, such as the iron and sulphur bacteria, are automatically excluded from analysis unless specific

care is taken to grow these organisms. These factors are frequently limitations in the traditional methods as well.

Another requirement implicit in colony hybridization is that all cells must be dislodged from the particulates (such as clay and silt) that are ubiquitous in environmental samples. This is absolutely essential in soil and sediment samples, and is frequently necessary in aquatic samples as well. A number of procedures have been developed for the separation of cells from particulates, most of which are based on blending in the presence of surfactants and the subsequent fractionation of cells from the particulates (24,62,91) but none of these procedures are completely successful (6,62). Since most environmental isolates produce an extracellular polysaccharide that facilitates the irreversible adhesion of the cell to surfaces, bacteria may become permanently bound to the organic matrix of particulates (29). This would make it difficult, if not impossible, to separate all viable cells from the environmental matrix. A sampling bias may then result from only the easily dislodged species being available for colony hybridization.

An alternative strategy to the colony hybridization technique is the direct extraction and isolation of DNA from the environment (38,67,91). By isolating microbial DNA directly from environmental samples, some of the limitations of the colony hybridization technique may be by-passed. Direct isolation and purification of DNA obviates the need for cultivation of organisms, thereby eliminating the major source of sampling error inherent in the colony hybridization technique. Direct extraction, like colony hybridization, has the

requirement that all cells be efficiently lysed so that the recovered DNA will not be biased in favor of those species that are easily lysed. Direct extraction can also be faster than colony hybridization. Isolated colonies from environmental samples are usually allowed two weeks to develop before gene probing commences, while only three days or less may be required to directly isolate DNA suitable for probing.

This technique will allow a more accurate analysis of many parameters than standard techniques would allow because no "bottle effect" is observed, i.e. no ecological changes due to increased surface area provided by incubation in containers are encountered. Analyses are performed directly on environmental samples with no incubation time in the laboratory required. When samples are removed from the environment to be used as microcosms for determining the potential for biodegradation of a given pollutant, for example, the microcosm cannot be considered truly representative of conditions in the field. DNA extracted from a sample would give a more accurate assessment of the biodegradation potential in the field at the moment of sample collection. Direct extraction of DNA also has the advantage over other techniques in that DNA is very easy to store. A single extraction may yield enough DNA for analysis with several probes. This probing may be performed at any time after extraction, with no loss of validity if it has been stored properly.

The major obstacle to direct extraction of DNA is frequently a logistic one: large samples must occasionally be dealt with, particularly when aquatic samples are to be analyzed. Since

cultivation of organisms is by-passed in this technique, enough sample must be collected so that sufficient DNA may be isolated to perform the experiment. This is usually not a problem when dealing with sediments and soils, where the biomass is usually high enough that samples of less than 100 grams contain more than enough DNA for analysis. Over 50  $\mu$ g of DNA have been recovered from a gram of fresh water sediment, yielding enough DNA for at least five hybridization analyses (Sayler et al. unpublished data). Aquatic samples typically have lower cell counts than sediments and soils, and volumes in excess of 1000 liters may be required in order to isolate enough DNA for analysis.

The first environmental DNA isolated from the environment was purified from soil samples in 1980 by Torsvik (91). Whole cells were first separated from the soil particles by a series of blendings and differential centrifugations, and the cells subsequently lysed by standard enzymatic lysis procedures. The DNA was contaminated by organic compounds that had carried through in the fractionation of the cells from the soil, and had to be removed by ion exchange/size exclusion chromatography and by hydroxyapatite chromatography. There have been at least two modifications of this techniques, both of which are currently in use in various laboratories. This technique has the limitation that cells must be efficiently separated from the soil matrix, with no bias as to species. As discussed in regard to the colony hybridization technique, this requirement is not easily met.

It should not be assumed that total DNA extraction is superior to colony hybridization in all circumstances. If the isolation of a

strain possessing a certain genotype is desired, it would not be possible to use direct DNA isolation techniques. It is also conceivable that in certain circumstances, colony hybridization may be more sensitive than direct isolation. If, for example, the numbers of a specific genotype are below the limits of detection of a direct isolation and probing procedure, but the strain is efficiently removed from its environmental matrix and is culturable, colony hybridization may be more likely to detect its presence. The limits of detection possible using labeled probes are continually decreasing; as little as 0.1 fg viral DNA have been detected in environmental samples (C. Gerba, personal communication).

#### Environmental Analysis Using Gene Probes

Among the earliest reported application of colony hybridization in the analysis of environmental samples was in the detection and enumeration of pathogenic *E. coli* in water samples collected from homes in rural Thailand (23). By screening colonies with a cloned gene coding for a specific enterotoxin, the need for a live mouse assay was bypassed, and up to a 10,000 fold increase in sensitivity over conventional methods was achieved.

Gene probes have been used extensively in environmental microbiology and biodegradation research. Both colony hybridization and the hybridization of directly isolated DNA have been used in the determination of cells capable of degrading naphthalene in activated sludge (79), which may then be used for the calculation of second

order degradation rate coefficients. The potential for the degradation in sediments of environmental pollutants as diverse as simple aromatic hydrocarbons, such as toluene (80), and polychlorinated biphenyls (74) have also been calculated with the use of specific gene probes. Conventional techniques for these types of measurements would require the cultivation of the environmentally isolated bacteria with the pollutant incorporated into the growth medium. Such studies are time consuming, and the results may be ambiguous due to the possibility of cross feeding or the appearance of false positives due to the growth of colonies on medium contaminants or micronutrients (80).

One of the most powerful environmental applications of the colony hybridization technique has been in the isolation of specific catabolic genotypes from environmental samples. Classical techniques require a time consuming series of successive enrichment cultures to isolate cultures of organisms capable of degrading specific pollutants. By using colony hybridization, Pettigrew and Sayler (74) have isolated strains (both singly and in a catabolic consortium) capable of degrading 4-chlorobiphenyl (4-CB) in approximately one-fourth the time required by the enrichment technique. The probe used for this study was pSS 50, a 53.2 kb plasmid coding for the catabolism of 4-CB. Even though this is a rather large plasmid and undoubtedly codes for more genes than the 4-CB pathway, in this study it appeared to be specific to the 4-CB catabolic phenotype.

Gene probes may also be used in conjunction with more conventional techniques for the isolation and characterization of

novel environmentally important genotypes. A highly specific probe for mercury resistance in Gram negative cells has been developed (7) and used to study the adaptation of microbial communities to mercury contamination (8). In addition to yielding valuable information on the response of natural communities to pollution, this study isolated Gram-negative colonies that were resistant to mercury, but did not share homology with the probe. This may represent a previously unknown mechanism for mercury resistance among Gram negative bacteria. One of the areas of intense research in environmental microbiology concerns the efficacy and fate of genetically engineered microorganisms (GEM's) in the environment (88). Gene probes are proving to be an indispensable method for evaluating both the fate of GEM's and the effects that they may have on the indigenous community (40,81). Jain et al. (41) have recently shown the applicability of the colony hybridization technique in measuring the maintenance and stability of introduced catabolic plasmids in ground water microcosms, while Holben et al. (38) have probed direct DNA extracts to track the fate of genetically modified Bradyrhizobium japonicum in soil microcosms.

Evaluation of the effects of GEM's on the structure and function of the microbial communities they invade is providing the impetus for development of function specific probes directed toward ecological parameters such as nitrogen fixation and photosynthesis. Until recently, the use of environmental nucleic acid probes has largely been restricted to applied concerns, such as biodegradation and heavy

metal resistance. Research on the effects of introduced bacterial species on ecological parameters in microcosms is possible, however, using isolated DNA and probes with DNA sequences specific for genes coding for nitrogen fixation, and photosynthesis.

The preceding discussion has almost exclusively concerned function specific probes; a number of species specific probes have also been developed for environmental analysis. Due to the highly conserved nature of ribosomal RNA's, most species specific probes are constructed from fragments coding for either 16s rRNA or for 23s rRNA. Examples of this type of probe have been constructed for Bacillus subtilis (48) and the fluorescent group of Pseudomonas (25). Another probe, specific to an Arthrobacter sp. isolated from a contaminated groundwater aquifer (78), has not been characterized as to its genetic origin, although it seems likely that this, too, is related to sequences coding for rRNA.

As the data base for rRNA sequences expands, more species and genus specific probes will become available. At present, kingdom specific probes for identification of Archaeobacteria, Eukaryotes and Eubacteria may be constructed from signature sequences derived from comparisons of 16s rRNA (D. Stahl, personal communication). These synthetic oligonucleotides may be used to define the structure of natural communities relative to the representation of each of the three kingdoms. Synthetic oligonucleotides specific to such ecologically important groups as sulfate reducing bacteria and methanogens are available through research on ribosomal RNA sequence analysis. Probing community nucleic acid extracts with probes

directed toward ribosomal RNAs might be particularly useful due to the increased sensitivity that can be attained. Ribosomes are present at levels up to 10,000 copies per cell, greatly increasing the number of targets for the probe (68).

#### Future Prospects for Gene Probes in Environmental Analysis

The possibilities of community structure and function analysis through the use of gene probes are almost unbounded. The major limitation of the technique, in its current state, is the lack of useful probes. As the molecular biology of naturally occurring bacteria becomes better understood, more probes will become available. New applications for molecular genetics in environmental microbiology are continually being found, and as the study of molecular genetics progresses, environmental microbiology will undoubtedly benefit.

As stated before, one of the primary requirements for ecosystem analysis using gene probes or molecular cloning is the need for a sample that is truly representative of the community. The purpose of this dissertation is to develop a quantitative procedure for the direct extraction of microbial DNA from sediments. This procedure is based on the mechanical lysis of microbial cells in the presence of the sediment, followed by the extraction and purification of the released DNA. The final product should be representative of the community as well as being of sufficient purity for most molecular biological techniques.

## CHAPTER 2

### LITERATURE REVIEW

#### Isolation of DNA from Pure Culture

Techniques for the isolation of DNA have been in an almost continual state of flux for over one hundred years, with hundreds of techniques being described in the literature. As analytical chemistry and the understanding of the structure of DNA increased over the years, isolation procedures were developed in order to yield DNA of higher purity and physical condition. The major obstacle in obtaining pure DNA has traditionally been protein contamination (61). Bacterial chromosomes are tightly associated with a group of highly basic proteins that serve to protect the DNA and lend structure to the nucleoid, much like the histones found in eucaryotes. When a bacterial nucleoid is isolated without the separation of the associated protein, about 20% of the mass of the complex is due to protein (89).

These proteins are bound to DNA primarily through ionic interactions, and may be displaced by increasing the ionic strength of the medium with certain salts (45-47). The most efficient anions for this treatment are those that bind with metals, indicating that metals may form bridges between DNA and proteins. Examples of anions which separate proteins from DNA are p-aminosalicylate, benzoate, and fluoride (45). High concentrations (4 M) of guanidinium chloride have also been used in the purification of both DNA and RNA. Proteins are

denatured by, and soluble in, guanidinium chloride and are dissociated from DNA (15).

Sodium acetate has been used extensively since 1898 to dissociate proteins from DNA (75,55). At acidic pH's, proteins form ionic complexes with DNA. This complexation may be avoided by adjustment of the pH with acetate so that the pH is between the isoelectric point of the protein and the point where the protein and the DNA precipitate out. The proteins are then removed by several rounds of ethanol precipitation (61), or by extraction with an organic solvent (47). Ammonium acetate has also been used for this purpose, and may be preferred because fewer salts coprecipitate with DNA in the presence of ethanol than when potassium acetate is used (17).

Detergents such as sodium dodecyl sulfate (SDS) and lithium dodecyl sulfate have also been used to dissociate nucleic acids from proteins (66). The mechanism of action seems to be through hydrophobic interactions between the protein and the hydrocarbon chain of the detergent, and the formation of micelles due to the hydrophilic group of the detergent. These chemicals also denature nucleases, thereby preventing their degradation of nucleic acids.

Early purification techniques were generally based on repeated rounds of sodium acetate treatment followed by ethanol precipitation of the DNA (75). The first use of organic solvents in the separation of proteins from nucleic acids was described by Sevag et al. in 1938 (83). They isolated nucleic acids from cultures of streptococci by first lysing the cells by sonication, followed by sedimentation of the cellular debris by centrifugation. The nucleoprotein complexes

remaining in solution were precipitated by acidification with hydrochloric acid, redissolved in water at a pH of 7, and an equal volume of ethanol was added to precipitate out some impurities. The nucleoprotein remaining in solution was then precipitated by acidification, washed with ethanol, and then redissolved. Proteins were then extracted out by the addition chloroform and a small amount of amyl alcohol for the prevention of foaming. Protein formed a "skin-like precipitate" at the interface between the aqueous and the organic layers, and these chloroform extractions were repeated until no precipitate was seen at the interface. The authors concluded that the presence of this precipitate is a sensitive test for the presence of protein, and that the absence of the precipitate is indicative of the absence of protein.

Phenol was also found to be a good solvent for proteins, provided that the protein had been separated from the DNA by the addition of salts prior to extraction with phenol. Phenol extractions were originally used in the purification of RNA; when salts are not added, the DNA is extracted along with the protein by the phenol, leaving RNA behind in the aqueous phase (46).

In a comparison of the phenol and chloroform extraction procedures, Saito and Miura (77) showed that phenol is a much more efficient extractant for the isolation of transforming DNA than chloroform is, yielding a recovery of around 46%. Phenol has the disadvantage of forming oxides when exposed to air which may damage DNA. Phenol is also quite toxic to cells, and must be removed from

the DNA by extraction with a volatile solvent, such as chloroform or ether, before any biological applications are attempted. The standard technique currently in use is to extract with a combination of buffer-saturated phenol and chloroform:isoamyl alcohol (24:1) in a 1:1 ratio, followed by extraction of the phenol in the aqueous phase by chloroform. Protease may be added prior to extraction in order to break up the protein and prevent DNA from being trapped in the organic phase by protein. Proteins have also been extracted from nucleic acid preparations by a combination of phenol and m-cresol (47), but the use of m-cresol has been abandoned.

RNA co-purifies with DNA in all of these techniques, and must be removed if one desires pure DNA. This is usually accomplished by digestion of the RNA with ribonuclease (RNase), followed by extraction of the RNase with a phenol:chloroform mixture. A combination of two types of RNase (T1 and A) is generally preferable to digestion with one alone. Ribonuclease A (isolated from bovine pancreas) is an endoribonuclease that cleaves RNA at the pyrimidines at the 3' phosphate linkage and the 5' phosphate linkages of the adjacent nucleotide. Ribonuclease T1 (isolated from Aspergillus oryzae) cleaves the 5' phosphate linkage of guanosines and the 5' phosphate linkage of the adjacent nucleotide. Once the RNase has been extracted, DNA is usually separated from nucleotides and digested RNA by precipitation in approximately 66% ethanol (55).

Another method of separating dissociated proteins and other cellular constituents from nucleic acids has been by centrifugation, utilizing the formation of either sucrose (66) or cesium chloride (55)

gradients. In sucrose gradient centrifugation, sucrose of decreasing concentration (and decreasing densities) is layered into a centrifuge tube. The sample density is adjusted so that it is less than the topmost (and lightest) sucrose layer, and applied to the top of the tube. The tube is centrifuged in a swinging bucket, during which time the sample components sediment to a zone of their own density. Both linear and convex gradients have been applied to nucleic acid purification (66).

Cesium chloride ( $\text{CsCl}$ ) gradients are established by mixing the sample with a  $\text{CsCl}$  solution and centrifuging at ultra high speeds (frequently in excess of 40,000 rpm). The high centrifugal force developed by the high speed centrifugation pushes the heavier cesium ions toward the bottom of the tube, setting up a linear density gradient. As with sucrose gradients, sample components are then separated on the basis of density, with proteins at the top, DNA in the middle, and RNA pelleted at the bottom of the tube (55). Care must be taken to avoid using chemicals that are insoluble in  $\text{CsCl}$ , such as SDS. If proteins are to be dissociated by a detergent, the  $\text{CsCl}$ -soluble detergent sodium dodecyl sarcosinate should be used. This method is not useful with samples high in protein content, as the protein will not layer on top of the gradient but will mix throughout the tube (66).

Since the late 1800's, a common feature of most purification techniques has been the precipitation of DNA away from some contaminants. Ethanol has been used most frequently, but others,

including isopropyl alcohol (57), 2-methoxyethanol (47), and polyethylene glycol (65), have been used. The general effect is that these solvents decrease the polarity of the medium enough that DNA is no longer soluble, and precipitates out. Ethanol is generally added at a rate of two volumes of ethanol to one volume of DNA solution (55), while isopropyl alcohol is added at a rate of 0.54 volumes (57). Ethanol has the advantage of being easily evaporated once the precipitated DNA is collected, while isopropyl alcohol has the advantage that it precipitates RNA and oligonucleotides less efficiently.

The efficiency with which ethanol precipitates DNA is primarily a function of the concentration of the DNA and the length of time allowed for the precipitation. DNA at a concentration of 0.005  $\mu\text{g/ml}$  was precipitated in two volumes of ethanol and 2.5 M ammonium acetate at an efficiency of 85% when precipitated overnight at room temperature. This efficiency decreased to 40% when no time was allowed for precipitation. One hundred per cent recovery was achieved for a concentration of 5  $\mu\text{g/ml}$  at room temperature overnight. Temperature does not appear to have any effect on the actual precipitation of the DNA, but may interfere considerably with its recovery by centrifugation. Only 33% of 0.005  $\mu\text{g/ml}$  DNA was recovered in an overnight precipitation at  $-70^{\circ}\text{C}$ , while 85% was recovered at room temperature. Ethanol solutions become quite viscous at low temperatures, which decreases the efficiency of recovery by centrifugation (17).

DNA may also be precipitated through the formation of an

insoluble salt with cetyltrimethylammonium ions (9). Many high molecular weight impurities, such as proteins, are also precipitated by ethanol and isopropanol, but not by cetyltrimethylammonium bromide (CTAB). CTA-nucleic acid salts are soluble in ethanol, while proteins are not. Nucleic acids may be purified of proteins by CTAB precipitation, dissolving in ethanol and reprecipitation by the addition of NaCl to the ethanol. Phenol extractions are usually used to remove proteins from these solutions, however. This procedure has the advantage over solvent based precipitations in that it does not significantly increase sample volume.

#### Chromatographic Techinques

One of the earliest chromatographic techniques developed for the purification of nucleic acids, and by far the most successful, utilizes the mineral hydroxyapatite. Nucleic acids are purified by adsorption to the hydroxyapatite with the selective elution of proteins and other contaminants. Purified DNA is then eluted from the hydroxyapatite in an active and unaltered form. Hydroxyapatite  $[\text{Ca}_{10}(\text{PO}_4)_6 \text{OH}_2]$  is a common mineral (most notably found in teeth) that was first synthetically produced by boiling the mineral brushite in NaOH, followed by successive washes in sodium phosphate buffer (Mitsui Toatsu, anonymous publication).

The first application of hydroxyapatite chromatography was introduced by Tiselius and colleagues in 1956 for the purification of proteins. Its use in the purification of nucleic acids was introduced

by the same laboratory in the following year, with important modifications being introduced by Britten et al. in 1969 (10,56) and by Markov and Ivanov in 1974 (56). In the Britten procedure, cells are lysed in the presence of 8 M urea and 0.24 M sodium phosphate buffer (NaP), pH 6.8. This lysate is mixed with hydroxyapatite at room temperature, and then poured into a suitable column. The aqueous phase is eluted, followed by elution with 8 M urea, 0.24 M NaP to remove contaminants that are bound through non-specific mechanisms (90). Urea is then eluted by 0.024 M NaP, and the DNA eluted by 0.48 M NaP. A certain amount of DNA would not be bound to the column, but could be recovered by passing the lysate through a second hydroxyapatite column. The recovered DNA was pure as judged by ultraviolet spectroscopy, with a negligible amount of RNA contaminating the final samples. Markov and Ivanov found that protein contamination was significant, but could be easily removed by phenol:chloroform extraction prior to loading onto the hydroxyapatite. These authors reported an efficiency of 80% recovery from cell lysates.

The mechanism of nucleic acid adsorption to, and hence its purification by, hydroxyapatite is not completely understood at this time. There appear to be multiple modes of adsorption of nucleic acids to hydroxyapatite, with the ionic attraction between the calcium and the phosphates in the nucleic acid being the primary mode of adsorption. Phosphates within the hydroxyapatite crystal lattice are exchanged with phosphates in the nucleic acid and physical adsorption of the nucleic acid to the mineral results. The nucleic acid may be eluted from the hydroxyapatite by increasing the molarity of phosphate

ions in the mobile phase of the system. This free phosphate displaces the phosphate groups of the nucleic acid, and the molecule desorbs. The more strongly the molecule is adsorbed, the higher is the molarity of phosphate ions required to elute the nucleic acid (10,12).

Single stranded DNA, single stranded and double stranded RNA, and RNA:DNA hybrids are all adsorbed less strongly to hydroxyapatite than is double stranded DNA. The decrease in strength of adsorption seen with double stranded RNA and RNA:DNA hybrids is thought to be due to the phosphate groups in these molecules being less exposed than they are in double stranded DNA. This results in fewer phosphate groups being available for exchange with the phosphates in the hydroxyapatite. As a result of this lower affinity for hydroxyapatite, double stranded RNA and RNA:DNA hybrids may be eluted at a lower phosphate molarity than double stranded DNA, and they may be separated from each other (59).

Single stranded DNA also has a lower affinity for hydroxyapatite than does double stranded DNA, but the explanation for this is not as straightforward as with the RNA cases. Since single stranded DNA is physically less constrained than double stranded DNA, it has more phosphates that are available for adsorption to hydroxyapatite than double stranded DNA, and one might assume that single stranded DNA would be bound more strongly than double stranded DNA. This is not the case, however, as single stranded DNA is eluted at a lower phosphate molarity than is double stranded DNA. Martinson has found that the degree of base stacking as well as the degree of base pairing

within single stranded DNA increases the strength of adsorption, indicating that the change in conformational entropy upon adsorption plays an important role in the relative strengths of adsorption. Single stranded DNA undergoes a much larger decrease in conformational entropy upon adsorption than the already highly ordered double helix of double stranded DNA (59,60).

Nucleic acids may also be eluted from hydroxyapatite by increasing the temperature rather than the phosphate ion concentration. The hydroxyapatite column is equilibrated in 0.12 M sodium phosphate buffer and typically heated to 60°C, a temperature and phosphate ion concentration at which single stranded DNA and RNA elutes and double stranded DNA remains bound. Double stranded DNA is eluted by raising the temperature to the melting point of the DNA, and the DNA elutes as single stranded DNA (10).

Hydroxyapatite chromatography has traditionally taken the form of open column chromatography rather than high performance liquid chromatography (HPLC). Open column chromatography has a number of disadvantages, among them being low flow rates resulting in long times required for purification and few theoretical plates which may lead to poor resolution and unsatisfactory purification. These difficulties are currently being overcome with the development of hydroxyapatite HPLC columns. The development of these HPLC columns has been slow due to the need for a small, yet pressure stable, hydroxyapatite particle. The first commercially available hydroxyapatite HPLC column was marketed by Bio-Rad (Richmond, CA), but was handicapped by having a short life due to pressure instability of

the packing. A second HPLC column has been developed by a Japanese firm, Mitsui-Toatsu, and marketed in the United States by Rainin Instrument Company, Emeryville, California. The Mitsui-Toatsu column relies on a novel method for the production of relatively uniform hydroxyapatite crystals, which yields a much more pressure stable packing than the Bio-Rad column. Columns are available from analytical scale up to a preparative scale column that has a total capacity of over 100 mg of DNA plus contaminants. The successful application of these preparative scale columns to DNA purification should greatly increase the ease and speed of large scale DNA isolations.

Chromatographic stationary phases other than hydroxyapatite have been used successfully in separating DNA and RNA molecules of different sizes and classes. Success in the actual purification of DNA has been more limited, with most products being primarily aimed at desalting, rather than extensive purification, of DNA. An example of this type is the Elutip-d disposable column produced by Schleicher and Schuell (Keene, NH). While the composition of the resin has not been made available to the public, it appears to work on an ion-exchange principle. DNA is bound to the column under low (20 mM) NaCl molarities and is eluted with a high molarity (1.0 M) NaCl buffer. Detergents, proteins and organic solvents interfere with the efficiency of the column, and should be removed from the DNA prior to loading, i.e. the DNA should be clean before being placed on the

column. The NACS Prepak column (marketed by Bethesda Research Laboratories), is similar to Elutip columns.

DuPont (Boston, MA) is currently marketing small disposable columns made specifically for the purification of small amounts (20  $\mu$ g to 1 mg) of DNA. These Nensorb columns, like the Schleicher and Schuell Elutip-d columns, are small open columns. The composition of the packing is not available but the purification seems to be based on a paired ion chromatography principle. DNA is loaded onto the column in the presence of triethylamine, which forms an ion pair with the nucleic acid. Contaminants are eluted with excess triethylamine, and the nucleic acid-triethylamine ion pair is eluted with a reverse phase solvent such as 50% methanol. Nensorb columns are not efficient purifiers of nucleic acids, and nucleic acids must have been through at least some preliminary purification procedures before loading onto the column.

These last columns were not designed for the rapid purification of DNA from cell lysates, but rather for the separation of DNA from salts, restriction enzymes, and unincorporated nucleotides left over from nick translation reactions. Hydroxyapatite remains the chromatographic stationary phase of choice for DNA purification.

### Cell Lysis

The first step in any DNA isolation procedure is the lysis of the cells from which the DNA is to be extracted. Due to the variety of cell types and degrees of susceptibility to lysis, a number of lysis procedures have been developed. These procedures may be divided into

two broad classes: enzymatic or chemical lysis; and mechanical disruption. Each technique has its advantages and disadvantages, and no single technique may be completely satisfactory for a given application. Enzymatic lysis is gentle and cellular components of interest may not be damaged by the lysis, but not all cells are lysed efficiently by enzymes. Mechanical cell disruption may be more general in its lysis of microorganisms, but it is harsh and may damage cellular components, such as shearing DNA (82).

Bacteriolytic enzymes generally have one of three modes of action: glycosidases which cleave polysaccharides; muramidases which split the bonds joining polysaccharides and peptides; and endopeptidases which attack polypeptides (5). The most commonly used procedures call for treatment of the cells with the muramidase lysozyme (isolated from egg whites). Lysozyme disrupts the cell walls of some bacteria by attacking peptidoglycan at the N-acetylglucosamine-N-acetylmuramic bond (18). Not all Gram positive species are susceptible to lysozyme action, and an enzyme with different substrate specificity may be used. An example of this is lysostaphin, an endopeptidase isolated from Staphylococcus staphylolyticus which is specific for the cross-linking peptides in staphylococcus (42). Most Gram negative bacteria are sensitive to lysozyme activity, but their outer membranes must first be destroyed by pre-treatment with either EDTA, by freeze-thawing (82), or by pretreatment with a detergent (5). The detergent sodium dodecyl sulfate may be added after treatment with lysozyme to aid in lysis (47). Examples of other enzymes that are

active against Gram negative cells are the lytic proteases isolated from Micromonospora and Bacillus subtilis (5).

Many Gram negative bacteria may be lysed in SDS alone (57), which serves to solublize components of the cell wall. Fuhrman et al. (28) recently found that freeze-thawing followed by heating a cell suspension with 1% SDS in a boiling water bath for 1-2 minutes quantitatively lysed microbes isolated from a marine sample.

Disruption using ultrasonic probes has been used successfully in some cases. Ultrasonic probes are lowered into the cell suspension and caused to vibrate rapidly. Bubbles formed at the tip of the probe vibrate rapidly, producing shear forces that break open the cells. A disadvantage of this method is that it may be difficult to obtain reproducible results since the degree of disruption is dependent upon several factors, including the viscosity of the suspension, the time of sonication, and the material that the container is made from (82).

One of the most frequently used mechanical lysis devices is the French pressure cell press and a number of similar devices, such as the Chailikoff press or the Ribi cell. The cell suspension is broken open by shear forces that develop as the cells pass through a small hole at very high pressures (up to 20,000 pounds per square inch). The French press is efficient at lysing Gram negative cells and Gram positive bacilli, but is not efficient at lysing Gram positive cocci and endospores (82). The Hughes press and the X press are similar to the French press, but use high pressures (10-80,000 psi) to force frozen cells through a small orifice (39).

Cells may also be broken open by grinding mills, which use

abrasive grinding in the presence of small balls or powders. Cells may either be dry (as lyophilized cells) or in suspension for use in most mills, with dry cells being crushed and wet cells being lysed through pressure shearing (39). Neither of these devices are particularly efficient at lysing cells, and in an extensive review on the subject, Hughes et al. (39) suggest that they are best used for the isolation of organelles.

Ballistic disintegration is effective at lysing most cell types. A cell suspension is placed in a homogenizer with glass or plastic beads and agitated vigorously. Cells are lysed by shear forces that develop as air passes between the glass beads. Examples of this type of device are the Bead Beater and the Braun disintegrator (39).

#### Measurement of DNA Concentration

The most common method of measuring DNA concentrations is by ultraviolet spectroscopy. DNA typically has absorbance maxima at 208 nm and approximately 260 nm, with the concentration being proportionate to the absorbance at 260 nm ( $A_{260}$ ), provided the sample is free of contaminants that absorb in this region. The relationship between the concentration of double stranded DNA and the absorbance at 260 nm with a 1 cm pathlength is:

$$\text{DNA (mg/ml)} = A_{260}/20$$

Single stranded DNA and RNA absorb somewhat more strongly per unit mass than double stranded DNA, and their concentration is expressed

as:

$$\text{single stranded DNA (mg/ml)} = A_{260}/23$$

This method is not particularly useful at measuring concentrations below 5  $\mu\text{g/ml}$ , but at higher concentrations yields an excellent evaluation of the purity of the sample. Protein absorbs strongly at 280 nm, and protein contamination may be evaluated by the ratio of absorbances at 260 nm and 280 nm. Protein free preparations should have an  $A_{260}/280$  ratio of 1.8 to 2.0, while contaminated preps are significantly lower. Nucleic acids also have a minimum at around 234 nm, with the absorbance at 230 nm being approximately equal to the absorbance at 280 nm (42). The ratio of absorbances at 260 nm and 230 nm is particularly sensitive to environmental contaminants, such as humic substances.

A number of fluorescent dyes, including ethidium bromide, mithramycin, DABA.2HCl, DAPI, diphenylamine and Hoescht 33258 have been used to measure DNA concentrations in cell extracts. Of these, Hoescht 33258 (73), DABA.2HCl (92), diphenylamine (13,73,51), and DAPI (73) have been used in environmental samples.

DABA.2HCl (m-diaminobenzoic acid dihydrochloride) has been used to measure DNA concentrations in soils (92) and as a measure of DNase activity in sea water (53). This method is based on the hydrolysis of DNA in perchloric acid and the specific detection of free deoxyribose by the addition of the dye (94). Torsvik and Goksoyr used a variation on this procedure whereby the samples are filtered onto glass fiber filter paper, yielding found a linear response between 1 and 20  $\mu\text{g}$  DNA

per sample. These authors warned that DABA.2HCl fluorescence is quenched by humic substances, and that there is a lack of specificity due to reaction of the dye with primary aldehydes. The diphenylamine technique, used in the analysis of seawater (73) and sediments (51) is similar to the DABA.2HCl technique in that it is also specific for deoxyribose, and is based on reaction of the DNA with the dye in perchloric acid.

DAPI (4',6'-diamidino-2-phenylindole) binds to DNA, thereby increasing its fluorescence over 20 times relative to non-bound DAPI (14). This fluorescence is constant in pHs between 5 and 10, but is quenched by the presence of high concentrations of cations as well as by low ionic strengths which might limit its utility in marine samples. Paul and Myers (73) have noted quenching of DAPI in the presence of magnesium ions, and have found it to have high blanks in sea water.

The fluorescent dye most commonly used in environmental samples today is Hoescht 33258 (bisbenzimidazole) (49), and has been applied to marine samples (73,20,21) and sediments (85). Hoescht 33258 binds specifically to A-T rich regions of DNA, (89), and fluorescence is highly dependent upon the base composition of the DNA studied, with fluorescence from Clostridium perfringens DNA (68% AT) being almost five times as great as fluorescence from Micrococcus luteus DNA (27% AT). Paul and Myers speculate that environmental DNA may have a base composition of approximately 50%, but this may not be universally true as activated sludge may have a considerably lower A-T content (Ogram, unpublished data). Considerable error could be introduced if standard

curves are made from calf thymus DNA, which has an A-T content of approximately 50%. Hoescht 33258 is not effected by the presence of magnesium, however, and may be more suitable for use in natural samples than DAPI is.

### Measurement of Environmental DNA

The search for an efficient procedure to isolate pure DNA from sediments and soils has been a long and arduous one. Most studies of nucleic acids found in the environment have involved destructive procedures which were directed at the measurement, rather than the purification, of the molecules due to the extreme difficulty in obtaining pure nucleic acids.

The largest obstacle in the purification of nucleic acids from environmental samples is the presence of humic substances; large, complex molecules that are ubiquitous in environmental samples. Humic substances are defined as organic chemicals that are naturally occurring, high molecular weight (ranging from several hundred to over 300,000), and difficult to degrade (2). The origins of humic substances are not clear, but current theory holds that they are the result of a multi-stage process. Plant components such as lignin are believed to be broken down into monomeric units by microorganisms, followed by eventual polymerization of the monomers. The major components of humic substances are humic and fulvic acids, which are large and poorly defined organic acids. Humic acids are operationally defined as being those soil or sediment organic matter components that

are alkaline extractable and acid precipitable, while fulvic acids are smaller molecules that are alkaline extractable and acid soluble. Both humic and fulvic acids are primarily composed of phenolic groups, with their major reactive groups being phenolics and carboxyls (86). A representation of a recently proposed generalization of the structure of humic acid is presented in Figure 1.

The earliest report on nucleic acids associated with soil organic matter was published in 1913 (4). In this study, as in most subsequent studies for the next 70 years, purified nucleic acids were not obtained, but were studied indirectly by isolating the various components of nucleic acids. Shorey obtained contaminated nucleic acids from a solution of soil fulvic acid by adding ethanol in the presence of sodium acetate. When the resulting precipitate was hydrolyzed by acid treatment, phosphoric acid, hypoxanthine, cytosine, and adenine were released.

Anderson used a similar approach (3,4), but with more sophisticated techniques, to isolate bases derived from soil associated nucleic acids. He used a standard alkaline extraction, acid precipitation technique for the extraction of humic acids from soils. The samples were then hydrolyzed by boiling in 72% (w/w) perchloric acid. The pH of the hydrolysates was adjusted to 4, which precipitated potassium perchlorate, sesquioxides, and some organic matter. The resulting supernatants were adjusted to pH 10 and passed through a Dowex-1 anion exchange column. The hydrolyzed bases were then selectively eluted from the Dowex by ammonium hydroxide-formic acid buffers at different pH's. The bases were further purified and

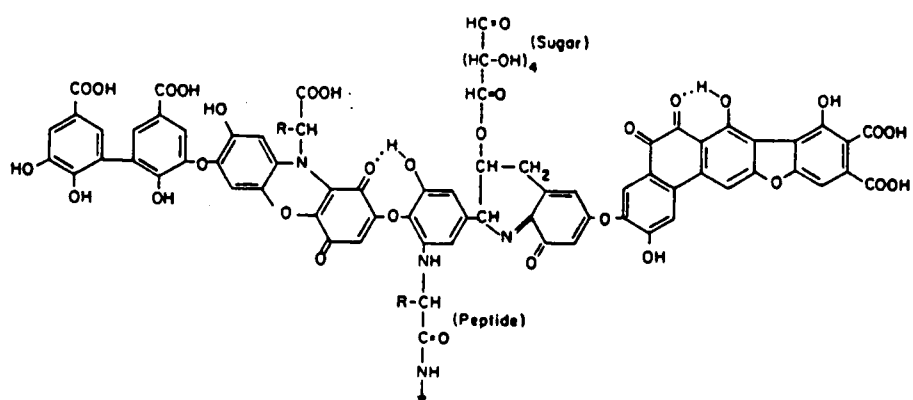


Figure 1. Structure of Humic Acid as Proposed by Stevenson (86).

their identities confirmed by paper chromatography. Through the relative concentrations of the bases, Anderson estimated that the GC content of DNA in the soil samples studied ranged from 59 to 74%.

A similar destructive technique for separating radiolabelled sediment microbial DNA and RNA has been developed for use in the radio-labelled precursor uptake technique for microbial activity measurements (44). Tritiated thymidine was added to freshly sampled sediments and incubated for various lengths of time, during which time active cells incorporated these labelled precursors into various cellular components. The sediment was dissolved by the addition of 2N HCl, indicating that this sediment was composed primarily of carbonates although no characterization of the sediment was reported. The dissolved sediment was filtered through a glass fiber filter, binding DNA and RNA to the filter. The filters were extracted with trichloroacetic acid, washed, and then extracted with NaOH. Low molecular weight compounds, but not DNA and RNA, are soluble in trichloroacetic acid, while the nucleic acids are soluble in NaOH and are removed from the filters at this point. The solution was then acidified, which resulted in the separation of RNA from DNA. RNA was hydrolyzed by the NaOH in the previous step, and will not precipitate upon acidification. The label in the supernatant was taken to be due to RNA, and that in the precipitate was due to DNA. This procedure is not one for purification, only for the separation of radio-labelled nucleic acids, and it is unlikely that pure compounds were isolated.

Nannipieri et al. (65) reported a different fractionation scheme for measuring non-labelled nucleic acids in soils. Their procedure

involves successive rounds of  $\text{HClO}_4$  extractions coupled with neutralization and extraction with sodium pyrophosphate. The supernatant from these extraction contain both RNA and DNA, which are distinguished by colorimetric assays specific to the different sugars in RNA and DNA.

The concentrations of extracellular, or dissolved, DNA have been measured in aquatic environments by a number of different methods. The persistence and fate of extracellular DNA in the environment is of great interest due to its importance as a nutrient and its potential in genetic exchange. Free DNA in natural systems is utilized primarily as a nutrient by bacteria, with nucleotides entering salvage pathways for the production of nucleic acids (71).

A variety of procedures have been used over the past 15 years for measuring dissolved DNA concentrations. Minear (63) concentrated filtered water collected from lakes by lyophilization, and then measured the amount of phosphorus in the samples by spectrophotometry. High molecular weight compounds were separated by passage through a gel permeation column, Sephadex K 50/100, and DNA determined by diaminobenzoic acid (DABA) fluorescence (63).

Paul and colleagues (20,21,69,73) have extensively used a technique that involves the filtration of seawater under low vacuums (less than or equal to 20 kPa) to avoid cell lysis. These filtered samples are then concentrated by precipitation with ethanol and partially purified by passage through an Elutip column (Schleicher and Schuell, Keene, NH). The concentration of DNA in the samples is then

determined by fluorescence with Hoescht 33258. This method may not be particularly accurate, as Deflaun and Paul (21) have reported that loss of fluorescence upon DNase digestion may be as little as 25%, indicating that Hoescht 33258 may not be specific for DNA in these environments.

### Purification of Environmental DNA

A landmark series in the annals of environmental DNA research was a trilogy spearheaded by Vigdis Lid Torsvik (24,91,92) which described the isolation of whole bacteria and fungi from soils, the measurement of the DNA contained in these soil microbes, and finally, the purification of soil DNA. All succeeding techniques developed for DNA purification from soils and sediments (except for the one described in this dissertation) were derived from these papers (38,85). The procedure involves the separation of bacteria from fungi and soil particles by successive blendings and low speed centrifugations that result in the sedimentation of soil particles and fungi, leaving the bacterial fraction in the supernatant (24). The bacterial fraction is then lysed by a standard detergent and lysozyme procedure, followed by the purification of the DNA. Torsvik found the bacterial fraction to be contaminated by humic acids, and extensive purification was required (91).

Torsvik tried a number of purification techniques, including phenol:chloroform extractions, ethanol precipitation, and two different chromatography techniques before arriving at the most efficient method. She found that the cellular fractions were still

significantly contaminated with humic acids, and a combination of anion exchange-size exclusion chromatography and hydroxyapatite chromatography was necessary to purify most samples. The lysate was mixed with 0.7 M KCl and 8 M urea, kept on ice overnight to precipitate out insoluble contaminants, and then passed through a DEAE-Sepharose CL-6B anion exchange resin. DEAE-Sepharose CL-6B is a weak anion exchanger and gel filtration resin with a molecular weight fractionation range of 10,000 to 4,000,000. Torsvik found that most humic acids remained bound to the top of the column, while a small proportion moved through the column ahead of the DNA front. The DNA co-eluted with some contaminants that absorbed strongly at 260 nm in ultraviolet spectroscopy. These contaminants were successfully separated from the DNA by the hydroxyapatite procedure developed by Markov and Ivanov. Torsvik suggested that the ion exchange column may be by-passed in samples not containing significantly high amounts of organic contamination (91).

A fundamental assumption in this approach is that a representative sample of bacteria is extracted from the soil. In view of the variety of mechanisms by which cells may become attached to surfaces, sometimes irreversibly, this is not a trivial assumption. In a quantitative study on the optimization of the separation of bacteria from soils, Bakken (6) reported that a slight modification of the homogenization and centrifugation method of Faegri et al. (24) released approximately 20% of the adsorbed bacteria for every round of homogenization and centrifugation. This efficiency was dependent upon

the soil type, with only about 10% of the cells present in a clay loam (with 23% clay) being released. These reported efficiencies are somewhat suspect because they are based on the very thing that they are trying to evaluate: the separation of bacterial cells from soil particles. These efficiencies are taken from initial acridine orange direct counts (AODC), which requires that cells be separated from the soil before they are counted. Cells that are not easily dislodged would be completely ignored in this measurement.

A modification of the Torsvik technique was presented by Steffan et al. (85) which was specifically designed for use with sediments containing moderately high (23%) clay contents. In this procedure, a homogenization buffer of 0.1% sodium dodecyl sulfate and 0.1 M phosphate buffer (pH 4.5) was used to decrease the adsorption of both cells and free DNA to the clay. They reported efficiencies of extraction of 50% after four rounds of homogenization and centrifugation. Once again, these efficiencies were based on AODC measurements or plate counts.

The next modification of this technique came in 1988 from Holben et al. (38). These researchers also used an approach similar to that proposed by Torsvik, with some important modifications made in the purification steps. Cells were separated from particulates by homogenization in a solution containing polyvinylpolypyrrolidone, a polymer that adsorbs humic acids. This step significantly improved the quality of the sample, and made subsequent purification easier. After separation of the cells from the particulates, cells were lysed

and DNA purified by cesium chloride-ethidium bromide gradient centrifugation.

A novel approach to isolating small amounts of DNA from soil has recently been proposed by B. H. Olson's (27) laboratory at the University of California, Irvine. This technique involves the lysis of cells prior to separating them from particulates, followed by the separation of the DNA from particulates and contaminants by agarose gel electrophoresis. This technique is currently limited by small sample size.

#### Adsorption of DNA to Sediments and Soils

An understanding of the behavior of DNA in sediments is essential if a procedure for the direct extraction of DNA is to be developed. The most important physical factor facing released DNA is its adsorption to the sediment.

The extent of adsorption of a chemical to a soil or sediment is usually evaluated by adsorption isotherms. In this type of analysis, the amount of adsorption at equilibrium and at constant temperature is measured for varying concentrations of the solute (33). These data are then analyzed by one of several mathematical models appropriate to the system being studied. Data gathered in this manner usually fit one of four types of generalized curves, and may then be analyzed through the application of an appropriate mathematical model. The type of curve resulting from this analysis may sometimes be used to infer generalizations concerning the mechanism of adsorption. The four basic types of isotherms, shown in Figure 2 (30), are:

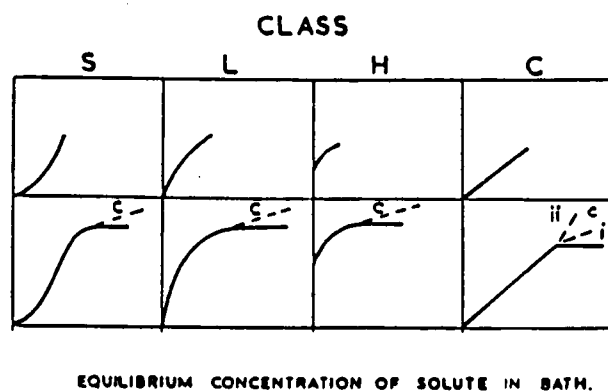


Figure 2. Classification of Sorption Isotherms, from Giles et al. (30).

1) The "S," or sigmoidal, type which results from multilayer adsorption and is described by the "BET" model (named after the initials of the developers of the model);

2) The "L," or Langmuir type, which is indicative of a system that may be saturated by the solute. This type is often applied to ion exchange mechanisms;

3) The "H," or high affinity type; and

4) The "C," or constant partition type, which indicates a porous sorbent into which the solute diffuses.

The adsorption of most solutes takes the form of the "L" type curve, and is described by either the Freundlich or the Langmuir model. The Freundlich model, an empirical relationship first proposed in 1909, is frequently applied to describe the adsorption of neutral organic molecules to soils and sediment. The equation is usually written as:

$$S = KC^n$$

where S is the amount adsorbed ( $\mu\text{g/g}$ ), K is the adsorption coefficient, C is the concentration in solution ( $\mu\text{g/ml}$ ), and n is a constant that is typically less than one. The higher the values of K and n, the more the adsorption (76).

Ionic exchange reactions are usually best fit to the Langmuir equation, developed in 1918 to describe the adsorption of gases to surfaces. This equation may take several different forms, but it is usually applied to sediments and soils as:

$$S = S_{\text{max}}KC/[C(1 + KC)]$$

where  $S$  is the amount adsorbed ( $\mu\text{g/g}$ ),  $C$  is the concentration in solution,  $S_{\text{max}}$  is the maximum adsorption capacity ( $\mu\text{g/g}$ ) and  $K$  is the Langmuir affinity coefficient ( $\text{ml}/\mu\text{g}$ ). Care must be taken in the interpretation of this data, since the precipitation of solutes from solution may yield data similar to Langmuir type adsorption (76). This problem has been noted in the analysis of phosphate adsorption to soils (93).

The extent to which a chemical is adsorbed is dependent upon the chemical structure of the solute, the composition of the liquid phase, and the nature of the sorbent. Non-polar, hydrophobic organic molecules, such as polychlorinated biphenyls, are sorbed through hydrophobic bonding and their adsorption coefficients are closely correlated with the organic carbon content of the sediment. Hydrophobic bonding is less a true bonding than it is an association; it is more energetically favorable for a hydrophobic molecule to be partitioned from the ordered and highly polar aqueous phase into the less polar organic phase of a sediment particle. The organic matter fraction behaves somewhat like a gel into which the molecule dissolves. Neutral polar organics may be sorbed to sediment organic matter through a variety of mechanisms, such as the short range van der Waals attractions, which include dipole-dipole and dispersive interactions, and hydrogen bonding (43).

The adsorption of ionic organic molecules is generally controlled by the clay mineral fraction of a soil or a sediment. The most important clay in the adsorption of most ionic organics, including DNA, are the smectites. Smectites, which includes montmorillonite,

are alumina silicate clays that have very large surface areas available for adsorption. These clays are typically charged negative as a result of isomorphous substitution of cations of a lower valence for one of a higher valence, i.e.  $\text{Mg}^{2+}$  for  $\text{Al}^{3+}$ . They are composed of layered sheets of silica tetrahedrons with an alumina octahedron in between (Figure 3). Two of these basic components may be separated by a spacing of 9.6 Å when completely collapsed, to over 30 Å when polar molecules are intercalated (26).

Most studies on organic adsorption to clays have been conducted on "cleaned" clays, i.e. clays which have been prepared in the laboratory so as to have no other clays or organic matter associated with them. Adsorption data taken from studies of this sort are not directly applicable to natural sediments since mineral surfaces are "blocked" to varying degrees by the presence of organic matter. This would decrease the surface area available for adsorption, thereby lowering the amount of adsorption by the mineral fraction. Karickhoff (42) has proposed a relationship to predict the adsorption of a given chemical to a sediment that takes the contributions of both the organic fraction and the clay mineral fraction into account:

$$K = K_m C_m f^m + K_{OC} OC f^{OC}$$

where  $K_m$  is the adsorption coefficient for the mineral fraction of the soil,  $C_m$  and  $OC$  are the fractional masses of clay minerals and organic carbon, and  $f^m$  and  $f^{OC}$  are the available fractions of these masses. Any attempt at extrapolation of adsorption data gained from cleaned

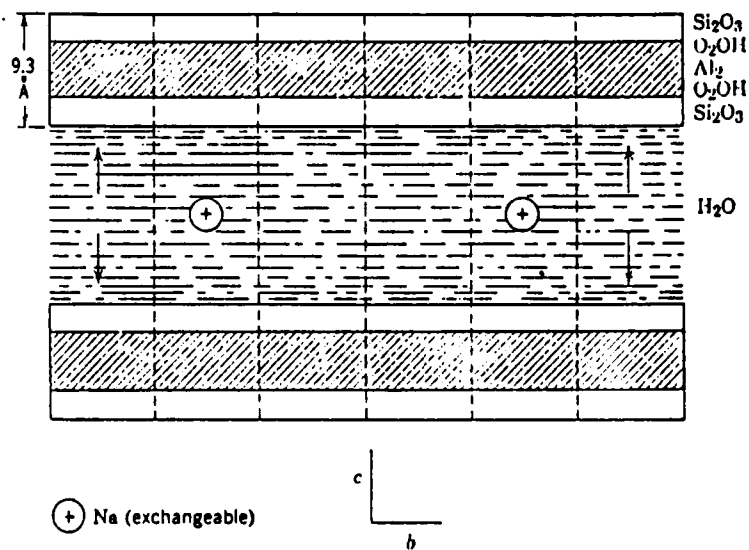


Figure 3. Sideview of Montmorillonite (58).

clays to natural sediments must account for the extent of coverage of the mineral by organic matter.

The adsorption of DNA to soils and sediments is primarily mediated by the mineral fraction, with montmorillonite having been shown to be a powerful sorbent of DNA. In their seminal work on the adsorption of DNA to cleaned montmorillonite, Greaves and Wilson (31) showed that DNA adsorption is highly pH dependent. Above pH 5, very little DNA is adsorbed and adsorption is confined to the outer surfaces of the clay. Below pH 5, large amounts of DNA move into the interlayer region of the clay, and there is a linear expansion of up to 19 Å, after which expansion tails off. Concomitant with this linear expansion is a linear increase in adsorption, up to at least 16 mg DNA being adsorbed per mg montmorillonite. It was proposed that as the pH drops below the pK<sub>a</sub> of the DNA (approximately pH 5), some bases are protonated as well as the phosphate groups. This leads to an overall positive charge on the molecule which increases its adsorption within the clay. As the pH increases, these protons are stripped off and the molecule becomes anionic. The anionic form of the molecule is excluded from the interior of the clay, and hence most of the surface area of the clay is unavailable for adsorption. The DNA may be adsorbed through cation bridging to the surface of the clay.

Adsorption of DNA to cleaned quartz sands has been shown to be primarily dependent upon cation concentration, with more adsorption being seen in the presence of divalent cations than monovalent cations. When a chelator of divalent cations, such as ethylene diamine tetraacetic acid, is introduced into the system, adsorbed DNA

is released. This indicates that the primary mechanism of adsorption is by cation bridging when in the presence of divalent cations. When monovalent cations are present in the absence of divalent cations, it was suggested that a double electrical layer was established and adsorption takes place through van der Waals forces (1,52).

Thus far, the only studies concerning the adsorption of DNA to sediments have been done on cleaned systems. As mentioned before, results gleaned from these types of experiments may be difficult to apply to natural sediments. While these studies are quite valuable and are necessary preliminary experiments, further work needs to be done to characterize DNA adsorption to natural soils and sediments.

## CHAPTER 3

### OBJECTIVES

The goal of this investigation is to expand the fundamental knowledge base on the factors effecting DNA recovery from environmental samples. This knowledge will be used to develop efficient and reproducible methods that will allow recovery in abundance of purified DNA representative of the microbial community; such DNA being required for advanced molecular approaches in microbial eology. Specific objectives will be:

I. To develop a lysis procedure that will be suitable for use in the presence of sediment particles and to evaluate the efficiency of lysis;

II. To analyze the characteristics of adsorption of DNA to a representative range of important types of soils and sediments, and to develop a procedure for the efficient extraction of DNA from sediment;

III. To develop a procedure for the purification of the extracted DNA;

IV. To distinguish between isolated intracellular and extracellular DNA, and to develop a rapid procedure for the measurement of extracellular DNA concentrations in sediments.

V. To evaluate the usefulness of the isolated DNA.

## CHAPTER 4

### MATERIALS AND METHODS

#### DNA Adsorption to Soils and Sediments

Five different soils, a lake sediment, and an acid-washed sand were used in this study, and the pertinent properties of these materials are listed in Table 2. All materials were ground to pass through an 80-mesh sieve (nominal diameter, 177  $\mu$ m).

Commercially obtained herring sperm DNA (Sigma Biochemicals, St. Louis) was used in all experiments. For the high molecular weight DNA studies, the DNA was dissolved in standard saline citrate (SSC) (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) after several passes through a 21-gauge needle. DNA for the low molecular weight DNA sorption isotherms was sheared by four passes through a French pressure cell press (Aminco Inc.) at 20,000 psi. The fragment size of this DNA was found to average 500 base pairs by agarose gel electrophoresis with lambda phage DNA restricted by Hind III as a size marker (data not shown).

The extent of DNA sorption to these soils was determined by a batch slurry method similar to that described by Green et al. All glassware was siliconized by treatment with dimethyl dichlorosilane to prevent DNA adsorption to glass. Two grams of soil was weighed into siliconized glass tubes, SSC added to soil to 1% on the basis of mass of soil, and autoclaved for 30 minutes to inactivate heat-labile nucleases. Four milliliters of DNA solution (concentration ranging

Table 2. Selected Properties of Soils and Sediments Used in Adsorption Studies

| Soil                | Texture   | Minerals                                                | Clay | Organic Carbon, % | CEC, <sup>a</sup> mequiv/100 g |
|---------------------|-----------|---------------------------------------------------------|------|-------------------|--------------------------------|
| Maury               | silt loam | vermiculite, montmorillonite, kaolinite, illite, quartz | 24.0 | 0.90              | 17.50                          |
| Iberia              | silt clay | montmorillonite, kaolinite                              | 49.3 | 2.16              | 40.80                          |
| Memphis             | silt      | montmorillonite, illite                                 | 10.0 | 0.76              | 6.50                           |
| Lily                | loam      | vermiculite, illite                                     | 14.0 | 1.98              | 4.85                           |
| Eustis              | fine sand | quartz, montmorillonite                                 | 3.2  | 0.60              | 5.20                           |
| acid-washed sand    | sand      | quartz                                                  | 0    | 0                 | ND <sup>b</sup>                |
| Ft. Loudon sediment | silt      | interstratified material, kaolinite, montmorillonite    | 26.0 | 3                 | ND <sup>b</sup>                |

<sup>a</sup>CEC = cation-exchange capacity.

<sup>b</sup>ND = not determined.

from 0 to 100  $\mu\text{g/ml}$ ) was added to the soils and three replicates at each concentration, mixed well, and shaken on a tumbling shaker overnight. These tubes were centrifuged at a low speed until the supernatant was clear, and the supernatant was analyzed for DNA concentration as described below. The amount of DNA removed from solution was calculated as DNA sorbed by the soil or sediment and the sorption data were analyzed by the Freundlich or Langmuir equations (76). pH was determined by using the appropriate buffer in a 2:1 solution:soil mixture after being stirred for 1 hour.

DNA concentrations were analyzed in one of two ways. Concentrations of high molecular weight DNA were determined as described by Paul and Myers (73). Two milliliters of supernatant was mixed with 1 ml of  $1.5 \times 10^{-5}$  M Hoescht 33258 fluorescent dye (Sigma Biochemicals) and allowed to sit in the dark for 10 minutes, and the concentrations were analyzed by a Perkin-Elmer Model MPF-44A fluorescent spectrophotometer set an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Standard curves were prepared with a range of DNA concentration in the appropriate soil extract. The DNA concentrations for the molecular weight studies were determined by using radio-labeled DNA. DNA solutions were spiked to 20,000 cpm/ml with  $^{32}\text{P}$ -nick translation labeled sheared herring sperm DNA before addition to soil, and the equilibrium concentrations were related to radioactivity remaining in solution, determined by the addition of 0.5 ml of supernatant to 10 ml of commercial scintillation cocktail (Beckman Ready-Solv HP/b), and liquid scintillation spectrometry (Tracor Analytic Model 6892). Since those soils and

sediments containing montmorillonite absorb water, the removal of solution from the supernatant by absorption was taken into account when concentrations were calculated.

#### Evaluation of Extraction Efficiency of DNA from Soils and Sediments

Two grams of air-dried soil were placed in plastic 15 ml tubes and mixed with 4 ml 100 µg/ml herring sperm DNA that had been spiked with  $^{32}\text{P}$ -DNA to yield 20,000 cpm/ml. Tubes were shaken overnight to load the sediments, centrifuged, and the supernatant removed and sampled to determine the concentration of  $^{32}\text{P}$ -DNA remaining in the sorbent.

The appropriate extraction buffer, either 0.12 M sodium phosphate (NaP), pH 8, or sodium carbonate buffer, pH 10 (0.025 M  $\text{NaHCO}_3$  + 0.025 M  $\text{Na}_2\text{CO}_3$ ) was added to the soil or sediment, mixed well, and centrifuged. The supernatant was sampled and measured for radioactivity. This process was repeated until all radioactivity was removed from the sorbent.

#### Isolation of DNA from Activated Sludge

Activated sludge was collected from the City of Knoxville waste treatment plant. The sludge was centrifuged at 5000 rpm for 30 min in a Sorvall GSA rotor. The clear supernatant was discarded, and the pellet washed three times with 200 ml 0.1 M sodium pyrophosphate, pH 8.8, and centrifuged as before. All supernatants were discarded.

Cells were lysed by a freeze-thaw cycle followed by digestion with lysozyme and homogenization in a Bead-Beater (Bio-spec Inst., Bartlesville, OK) with an operation time of 5 minutes. Forty ml 0.12 M NaP, pH 6.8 was added to the thawed cells, mixed, and 10 mg/ml lysozyme added to the sample and incubated at 37°C for 1 hour. This digestion was followed by digestion with 0.25 mg/ml Proteinase K. Lysis was completed by homogenization in a Bead-Beater.

The sample was centrifuged and the pellet extracted twice with 80 ml 0.12 M NaP, pH 8. The supernatants were combined and brought up to 0.5 M potassium acetate and stored on ice for 5 minutes in order to precipitate protein. Extracted 3 times with a one volume of a 1:1 mixture of phenol (saturated with 0.12 M NaP, pH 6.8) and chloroform:isoamyl alcohol (24:1). This was followed by 2 extractions with chloroform:isoamyl alcohol. DNA was precipitated by the addition of two volumes of ice cold ethanol to the aqueous phase, and left at -20°C overnight. DNA was pelleted by centrifugation, resuspended in 0.12 M NaP, pH 6.8, and dialyzed against this buffer overnight with four changes of the dialysis bath.

#### DNA Purification from Sediments

##### Sediments

Two sediment samples, one from a salt marsh (Range Point) and the other from brackish water (Bayou Texar), were collected near Pensacola, Florida, and a third sediment sample was collected from the Singleton Terminal area of Fort Loudon Reservoir, Knoxville, TN. The pertinent properties of these sediments are listed in Table 3.

Table 3. Selected Properties of Sediments Used in Development of Purification Techniques

| Sediment    | Source                               | pH  | %<br>Total<br>Carbon | %<br>Silt Sand Clay |      |      |
|-------------|--------------------------------------|-----|----------------------|---------------------|------|------|
|             |                                      |     |                      | Silt                | Sand | Clay |
| Fort Loudon | Fresh Water Reservoir, Knoxville, TN | 7.0 | 3                    | 66                  | 8    | 26   |
| Bayou Texar | Brackish water, Pensacola, FL        | 6.1 | 4                    | 15                  | 66   | 19   |
| Range Point | Salt Marsh, Pensacola Beach, FL      | 5.4 | 16.5                 | 55                  | 1    | 44   |

Total carbon was determined by a LECO carbon analyzer, and pH by mixing 10 grams of sediment with 10 ml of CO<sub>2</sub>-free water and taking the pH at equilibrium (8). Particle size distributions (clay, silt and sand contents) were determined by the hydrometer method (19).

#### Direct Lysis of Sediment Samples

Two hundred ml of 0.12 M sodium phosphate buffer (NaP), pH 8, was mixed with 100 grams of sediment (wet weight) and 2.5 grams sodium dodecyl sulfate and held at 70 C for one hour. Lysis was completed in a Bead-Beater with a 1:1 ratio of sediment to glass beads, and an operation time of 5 minutes. Efficiency of lysis was checked by acridine orange direct counts (36) and was found to be greater than 90%.

#### DNA Extraction from Sediment Samples

The sediment/glass bead mixture was centrifuged at 6084 x g in a Sorvall GS-3 rotor for 30 minutes and the supernatant recovered. In order to further recover the DNA, two successive extractions of the sediment were made with 0.12 M NaP, pH 8, by adding 400 ml buffer to the sediment, shaking, and then centrifuging as above. All supernatants were pooled. These extracts were then centrifuged at 16,000 x g in a Sorvall GSA rotor for 30 minutes to remove cell debris and other particulates.

#### Sample Concentration

Sample extracts were concentrated either by ethanol precipitation or by precipitation with polyethylene glycol (PEG). In the case of

ethanol precipitation, either no salts were added, or the sample was brought up to 0.3 M sodium acetate or 2.5 M ammonium acetate. Two volumes of ethanol were then added and mixture was kept at either -20°C or room temperature overnight. The sample was centrifuged and the pellets resuspended in 0.12 M NaP, pH 6.8, by several passages through a 60 ml syringe with an 18 gauge needle.

PEG precipitation was performed by adding NaCl to a final concentration 0.5 M, and 0.5 volumes of 50% PEG. This mixture was kept at 4°C overnight and centrifuged at 650 x g for 2 minutes. The supernatant was then aspirated off and discarded. The loose pellet was resuspended in approximately 50 ml of TE buffer (0.010 M Tris, 0.001 M EDTA, pH 8.0). PEG was removed by extraction with phenol (TE saturated). One half volume of phenol was added, stirred for 5 minutes, and centrifuged at 4,000 x g for 20 minutes. The aqueous phase was similarly extracted once with 0.5 volumes of phenol, 0.5 volumes of chloroform:isoamyl alcohol (24:1), and twice with one volume of chloroform:isoamyl alcohol. The nucleic acids were then precipitated with ethanol as described above.

The concentrated samples were adjusted to 0.5 M potassium acetate and stored on ice for 2 hours. The resulting brown precipitate was then centrifuged at 16,000 x g in a GSA rotor for 30 minutes, and the supernatant saved for further purification.

#### Cesium Chloride-Ethidium Bromide Gradient Centrifugation

After potassium acetate precipitation of some of the organic carbon in the samples, the supernatants were initially purified either

by CsCl-EtBr (cesium chloride-ethidium bromide) gradient centrifugation and then further purified by hydroxyapatite chromatography, or put directly through hydroxyapatite chromatography. For CsCl-EtBr centrifugation (55), one g/ml CsCl and 600  $\mu$ g/ml ethidium bromide were added to the samples, which were then centrifuged in a vertical Ti 50 rotor at 40,000 rpm for 40 hours at 20°C. Ethidium bromide was removed by extraction with n-butanol. Samples were then dialyzed against four changes of 0.12 M NaP, pH 6.8, prior to hydroxyapatite chromatography.

#### Hydroxyapatite Chromatography

DNA grade hydroxyapatite (Bio-rad, Rockville Centre, NY) was prepared by boiling in 0.01 M NaP, pH 6.8, for 10 minutes, centrifuging for 2 minutes at 5000 rpm, and then pouring off the supernatant. The hydroxyapatite was mixed with 8 M urea, 0.12 M NaP, pH 6.8, and then centrifuged again as before. Samples were mixed with approximately 5 grams of hydroxyapatite by gentle intermittent swirling at room temperature for one hour. This mixture was poured into a column made of a 60 ml disposable syringe with siliconized glass wool as a support base and an 18 gauge needle as an outlet. The starting buffer was allowed to flow out by gravity, and some organic contaminants were eluted by passing enough 8 M urea, 0.12 M NaP, pH 6.8, through the column to reach an effluent absorbance of 0 between 320 and 220 nm. This typically required between 100 and 300 ml. DNA was then eluted with several fractions of the appropriate NaP buffer. Purity and concentration of these fractions were monitored by

spectroscopic scans from 320 to 220 nm on a Beckman DU-40 Spectrophotometer.

#### Isolation and Purification of Extracellular DNA

Prior to lysis of sediment samples, extracellular DNA was extracted with four sequential 2:1 (buffer:sediment) extractions with 0.12 M NaP, pH 8. The samples were then concentrated by precipitation with ethanol and purified by hydroxyapatite chromatography as described above.

In an attempt to measure the likelihood that cells were being lysed during the extracellular DNA extraction procedure, E. coli DH1 cells containing the plasmid pEPA 90 were added at a rate of  $4 \times 10^7$  cells per gram sediment to 100 g Ft. Loudon sediment suspended in 200 ml 0.12 M sodium phosphate buffer, pH 8. The extracellular and intracellular DNA fractions were extracted and purified. The innoculated plasmid, pEPA 90, is a pUC 18 vector which contains an Eco RI, Pst I restriction fragment of the napin storage protein gene cloned from Brassica napus (16) (generously provided by Steven Cuskey). The concentrations of the napin gene were measured by quantitative hybridization probing with an XhoI-EcoRI restriction fragment of the gene. This approximately 200 bp fragment was isolated by two passages through 0.7% agarose electrophoresis and recovered by electroelution.

#### Measurement of Extracellular DNA Concentrations by HPLC

Samples were collected from the Singleton terminal area of Ft.

Loudon Reservoir, Knoxville, TN, by core sampler at depths of 0-5 cm, 5-10 cm, and 10-15 cm, placed in sterile Whirl-Pak bags (NASCO, Inc.), and immediately stored on ice. Samples were then transported back to the laboratory, where extracellular DNA was immediately extracted as above from 2 gram samples, three replicates per sample. These samples were not concentrated further. Parallel samples were removed for determination of water content. Samples to be analyzed by high performance liquid chromatography (HPLC) were filtered through a 0.2  $\mu$ m Acrodisc disposable filter assembly (Gelman Sciences, Ann Arbor, MI).

Samples were fluorescently labelled by Hoescht 33258 as described above and analyzed on a Series 410 HPLC with an LCI-100 laboratory computing integrator (Perkin-Elmer, Norwalk, CT), a Model 157 Fluorescence Detector (Beckman Instruments, Fullerton, CA). The column used was a Mitsui Toatsu HCA hydroxyapatite column (7.6 mm ID x 10 cm L analytical column with a 4 mm ID x 1 cm L guard column, Rainin Instrument Co., Emeryville, CA). Samples were injected into a 20 ml fixed volume injection loop, with the column equilibrated with 0.1 M potassium phosphate buffer, pH 6.8, 8 M urea, and stepped up to 0.4 M potassium phosphate buffer, 8 M urea, upon injection. All buffers were filtered through a 0.2  $\mu$ m filter and degassed by sparging with helium. Standard curves were made using calf thymus DNA concentrations that bracketed the concentration of the samples, and the concentrations related to the areas of the peaks. These

concentrations were then expressed as ug extracellular DNA per dry gram of sediment.

### Hybridizations

All DNA:DNA hybridizations were performed using a slight modification of the protocol suggested by the vendor for use with GeneScreenPlus (New England Nuclear, DuPont, Inc., Wilmington, DE). DNA was spotted onto GeneScreenPlus with the aid of a Dot Blot vacuum manifold (Schleicher and Schuell, Keene, NH). The membrane was then prehybridized for 6 to 24 hours, as per the manufacturer's suggestion at 65°C with 1% SDS, 1M NaCl, and 10% dextran sulfate, plus the addition of between 1 - 2 mg non-homologous calf thymus DNA. DNA labelled with  $^{32}\text{P}$  by nick translation with  $^{32}\text{P}$ -dCTP was added, and the hybridization allowed to continue for 24 hours at 65°C. The membranes were washed according to the manufacturer's suggestion, with the inclusion of a higher stringency wash than was called for by the standard protocol. The final wash was at 65°C in 0.1X SSC and 1% SDS for 1 hour. The autoradiograms were then developed on x-ray film (X-OMAT AR, Eastman Kodak Co., Rochester, NY). After a sufficient time of exposure, the x-ray films were developed.

## CHAPTER 5

### RESULTS

#### DNA Adsorption to Soils and Sediments

DNA adsorption to five soils, a lake sediment, and an acid-washed sand were analyzed using standard batch slurry methods and by fitting either the Freundlich or the Langmuir model to the data. The range of DNA concentrations used in this study were believed to be environmentally relevant based on previous studies. Maeda and Taga (53) reported total DNA concentrations of up to 35  $\mu\text{g/g}$  of ocean sediment, and Torsvik and Goksoyr (92) found up to 90  $\mu\text{g}$  of bacterial DNA per gram of dry soil. We have used DNA concentrations ranging between 10  $\mu\text{g}$  per gram of soil to 200  $\mu\text{g}$  per gram in an attempt to describe the sorptive behavior of DNA within an environmentally relevant range.

As can be seen from Table 4, soils and sediments which contain a significant amount of montmorillonite adsorb all of the DNA added to the system, up to at least 200  $\mu\text{g/g}$ . The single exception is the Memphis soil (Figure 4), which adsorbs a maximum of 15.4  $\mu\text{g/g}$ , as described by the Langmuir equation. The pH of this soil in SSC is relatively high (7.2), and it is likely that not all of the DNA was in the molecular (unionized) form. That DNA which was not ionized was probably intercalated between the clay interlayers, whereas the remaining ionized DNA was not adsorbed. Further, when the solution was adjusted to pH 10 with NaOH, no DNA was sorbed, and when the pH

Table 4. DNA Sorption Model Characteristics to Soils and Sediments<sup>a</sup>

| Soil/<br>Sediment   | DNA,<br>Molecular<br>weight | pH  | $K (\mu\text{g/g}) -$<br>$(\mu\text{g/mL})^{-n}$ | n    | r <sup>2</sup> |
|---------------------|-----------------------------|-----|--------------------------------------------------|------|----------------|
| Freundlich Fit      |                             |     |                                                  |      |                |
| Eustis              | high                        | 6.8 | 2.36                                             | 0.95 | 0.87           |
|                     | low                         | 6.8 | 1.51                                             | 0.86 | 0.95           |
| acid-washed<br>sand | high                        | 6.8 | 2.82                                             | 0.68 | 0.90           |
|                     | low                         | 6.8 | 3.15                                             | 0.42 | 0.99           |
| Langmuir Fit        |                             |     |                                                  |      |                |
| Memphis             | high                        | 7.2 | 1.02                                             | 0.95 |                |

<sup>a</sup>All DNA added to Maury (pH 6.1), Iberia (pH 6.3), Ft. Loudon (pH 6.5), and Memphis (pH 5.8) was adsorbed. No DNA added to Lily (pH 6.9) or to Memphis (pH 10) was adsorbed.

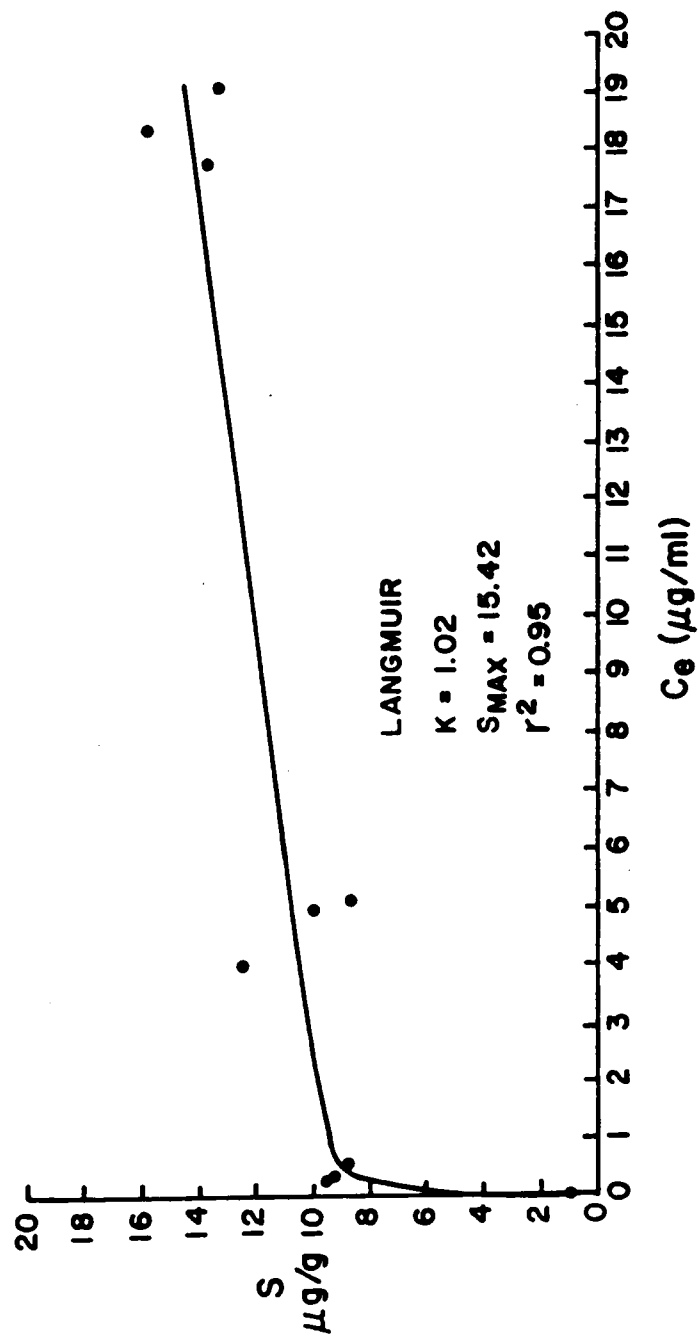


Figure 4. Memphis Soil DNA Adsorption Isotherm at pH 7.2.

was adjusted to pH 5.9 with HCl, all of the DNA was adsorbed. This indicates that the surface pH of some natural soils and sediments may be near the pKa of DNA (pH 5), and that significant amounts of DNA could be present in the aqueous phase even in the presence of montmorillonite.

The adsorption of DNA to sand was studied at two different shear sizes. Adsorption of sheared and unsheared DNA by Eustis soil and acid-washed sand was described satisfactorily by the Freundlich equation (Figure 5). In both sands, unsheared DNA was sorbed to a greater extent than the sheared DNA, as well as showing a more linear isotherm ( $n$  closer to 1) in the range of concentrations studied.

The adsorption isotherm for acid-washed sand (Figure 6) is somewhat different from the Eustis soil in that the  $K$  values for the unsheared versus the sheared are very close. The sheared isotherm resembles that of the unsheared at low concentrations, but more sheared DNA is sorbed at equilibrium concentrations greater than 20  $\mu\text{g/ml}$ . It should be noted that both the  $n$  and the  $K$  values of the Eustis isotherms are higher than those for the acid-washed sand, indicating a higher sorptive capacity. This may be due to the presence of clay minerals, such as small amounts of montmorillonite (Table 2, page 48) in the Eustis that were not present in the sand. Divalent cations may also be present in Eustis which may have participated in cation bridging to the surface of the soil. The fact that higher molecular weight DNA sorbs to a greater extent than the low molecular weight DNA is attributed to more adsorptive moieties

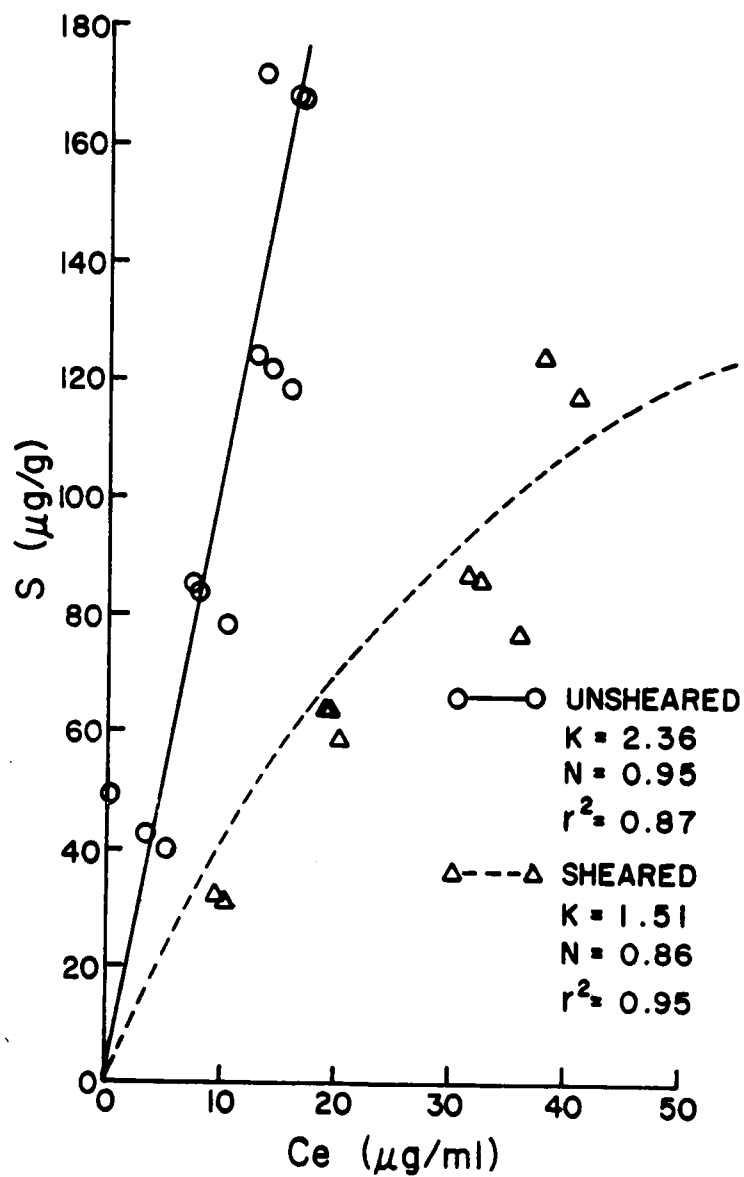


Figure 5. Adsorption of High (Unsheared) and Low (Sheared) Molecular Weight DNA to Eustis Soil.

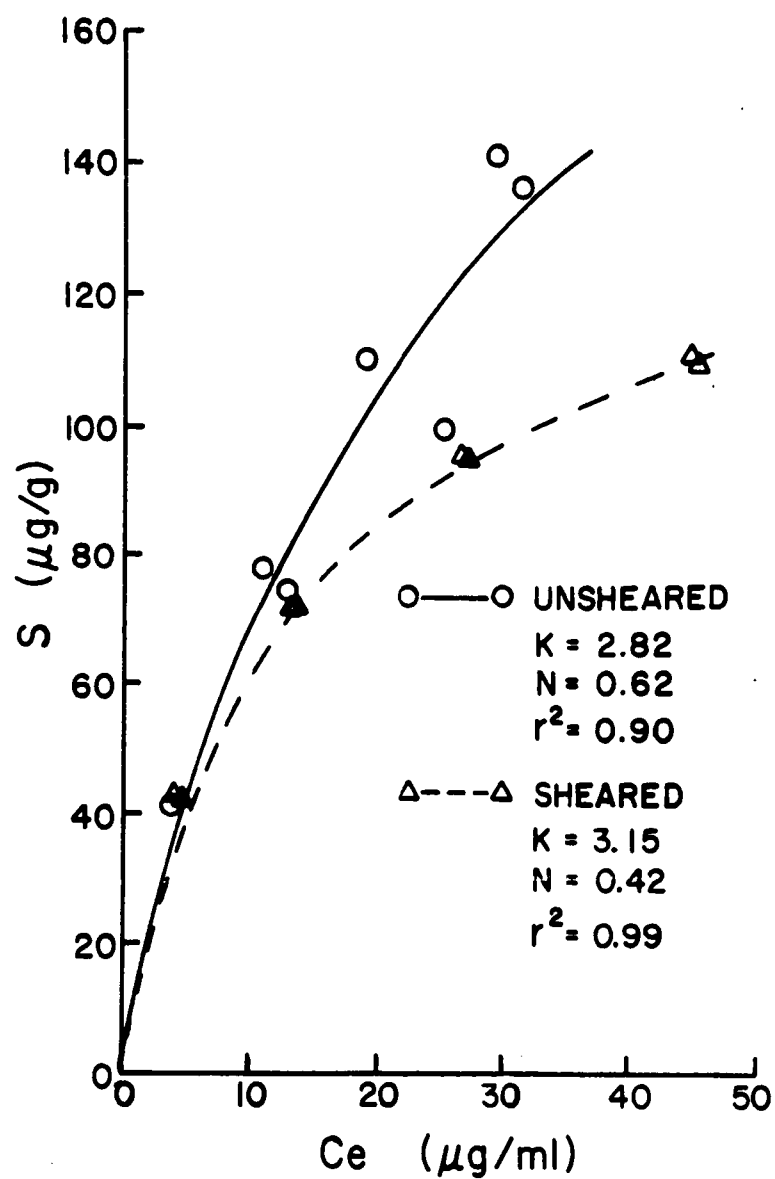


Figure 6. Adsorption of High (Unsheared) and Low (Sheared) Molecular Weight DNA to Acid-Washed Sand.

(primarily the phosphate backbone) per fragment in a large DNA fragment than there are in a small one.

DNA adsorption at a single initial concentration, but over a range of molarities of pH 6.8 sodium phosphate buffer (NaP) were conducted for Ft. Loudon sediment, Iberia soil, and acid-washed sand to examine the effects of phosphate concentration on adsorption. The mechanism of adsorption of DNA to the mineral hydroxyapatite is by exchange of the phosphates within the mineral with the phosphates in the DNA, and a similar effect might have been operating with adsorption in or on natural soils. As can be seen from Figure 7, the relative effects of ionic strength are quite different in each case, with acid-washed sand being affected the least. Over the range of 0.012 M NaP to 0.12 M NaP, the adsorption decreased only 1.6%, while the Iberia soil decreased 23% and Fort Loudon sediment decreased 43% over the same range. This difference in the sensitivities to changes in NaP concentrations may be related to the different sorption mechanisms available in each case. The Iberia soil shows a linear decrease increasing NaP concentration, and this may be due to a simple competition for phosphate sorption sites, analogous (although not necessarily homologous) to the case described above for hydroxyapatite. Adsorption to sand is relatively insensitive to the addition of phosphate, and it is quite possible that another mechanism for adsorption is being expressed in this case.

The contribution of organic carbon to DNA sorption appears minimal at the pH values of the soils studied. Hartsell soil, which contains 1.98% organic carbon, but no montmorillonite (Table 2, page

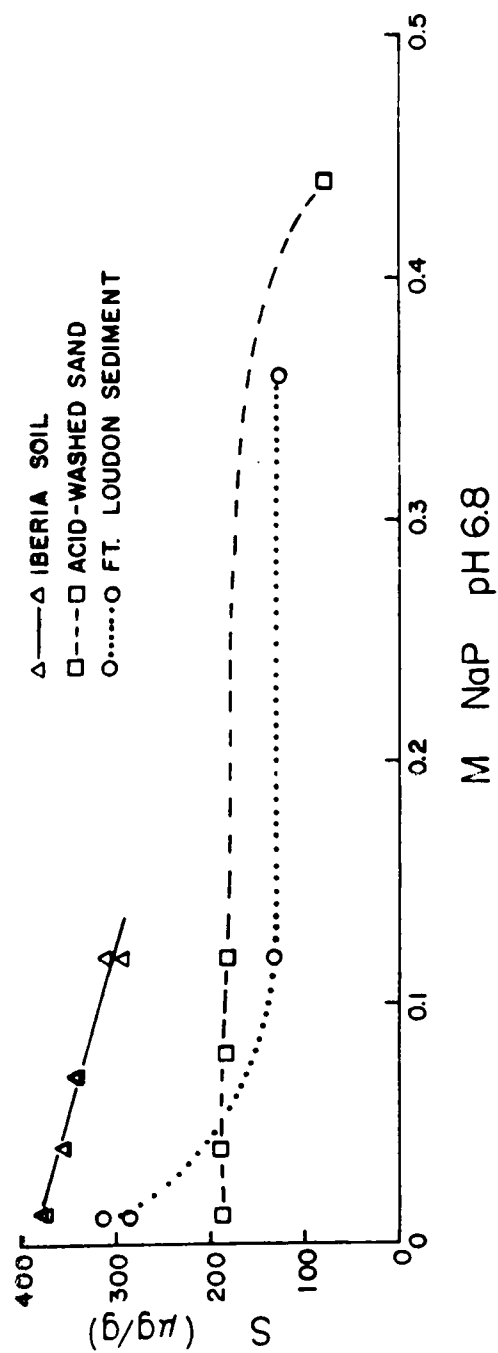


Figure 7. The Influence of Sodium Phosphate Buffer Molarity on the Adsorption of Unsheared DNA.

49) adsorbed no DNA. It may be that small amounts of DNA may adsorb to organic carbon through cation bridging to phenolic or carboxylic acid groups, but in levels too low to detect using this method. When 0.4 grams of activated charcoal (pH 9.5) were challenged with 250 µg DNA, all DNA was adsorbed.

#### Extraction of DNA from Soils and Sediments

A procedure for the extraction of adsorbed DNA was developed using Iberia soil as the model sorbent. Iberia was chosen as the model soil because it was believed that it would be the most difficult of the available soils from which to extract DNA due to its high cation exchange capacity (CEC) and montmorillonite contents. Since DNA adsorption is pH dependent in soils containing montmorillonite, a procedure based on alkaline extractions was developed. This is different from the extraction technique developed by Craven and Karl for use with sands. They extracted DNA from silt and noncarbonate sands with one 1:5 sediment volume:volume of 0.5 M  $\text{H}_3\text{PO}_4$ , and dissolved carbonate sands with 1 M HCl. The efficiencies of recovery for these extractions was not presented, and it may be assumed that the DNA was not unaffected by exposure to these mineral acids due to acid depurination.

A high pH buffer was needed for extraction from Iberia soil in order to overcome the natural buffering capacity of the soil. When SSC was adjusted to pH 10 with sodium hydroxide, the pH of the solution after extraction was 7.3 and no DNA was removed. It should be noted that the pH of a natural clay particle may be two to three pH

units below that of the bulk solution (R. Lewis, personal communication). When the soil slurry was adjusted to pH 9 with sodium hydroxide, a significant amount of DNA was released. The most efficient extraction procedure for Iberia soil used sequential extractions with pH 10 sodium carbonate buffer. A single extraction by addition of sodium hydroxide to pH 10.6 followed by sonication proved to be only partially successful. It was hoped that sonication would improve mixing within the clay, thereby ionizing all DNA and releasing adsorbed DNA. This was not completely successful; in fact, the longer the sonication time (ranging from 0-15 minutes at 40% maximum power), the lower the amount extracted. Five sequential extractions with pH 10 sodium carbonate buffer were sufficient to remove 98% of  $^{32}\text{P}$ -labelled exogenous DNA added to the soil (Table 5). The extraction of DNA seemed to correlate with the extraction of other soil organic matter as evidenced by the presence of color in the extracts.

A similar extraction scheme was developed for Ft. Loudon sediment, except that only four extractions with 0.12 M NaP, pH 8, were required to remove 95% of the added DNA (Table 6). An extraction buffer with a lower pH was used in this system because the buffering capacity of the sediment was lower (both montmorillonite and organic carbon contents were lower) than of the Iberia soil.

To test whether or not adsorption to Ft. Loudon sediment could be completely prevented by lysis of cells in pH 8 buffer, adsorption of DNA was measured in pH 6.8 and pH 8 NaP. Of 400  $\mu\text{g}$  DNA added to one

Table 5. Extraction of DNA from Iberia Soil Using pH 10 Buffer<sup>a</sup>

| Extraction | % Removed<br>Per Extraction (CV) <sup>b</sup> | % Removed<br>Cumulative (CV) <sup>b</sup> |
|------------|-----------------------------------------------|-------------------------------------------|
| 1          | 6.42 (0.10)                                   | 6.42 (0.10)                               |
| 2          | 18.06 (0.09)                                  | 24.48 (0.05)                              |
| 3          | 32.32 (0.02)                                  | 56.80 (0.03)                              |
| 4          | 24.67 (0.02)                                  | 81.48 (0.02)                              |
| 5          | 16.45 (0.05)                                  | 97.93 (0.02)                              |

<sup>a</sup>Sequential extractions made with 2:1 (buffer:sediment, w/w) extractions with 0.025 M NaHCO<sub>3</sub>, 0.025 M Na<sub>2</sub>CO<sub>3</sub> buffer.

<sup>b</sup>CV = coefficient of variation based on three replicates.

Table 6. Extraction of DNA from Ft. Loudon Sediment Using 0.12 M NaP Buffer, pH 8<sup>a</sup>

| Extraction | % Removed<br>Per Extraction (CV) <sup>b</sup> | % Removed<br>Cumulative (CV) <sup>b</sup> |
|------------|-----------------------------------------------|-------------------------------------------|
| 1          | 5.59 (0.09)                                   | 5.59 (0.09)                               |
| 2          | 48.31 (0.03)                                  | 53.9 (0.03)                               |
| 3          | 24.89 (0.08)                                  | 78.79 (0.04)                              |
| 4          | 17.59 (0.07)                                  | 96.4 (0.05)                               |

<sup>a</sup>Sequential extractions using 2:1 (buffer:sediment, w/w) were made.

<sup>b</sup>CV = coefficient of variation for three replicates.

gram of sediment, only 135  $\mu\text{g}$  were adsorbed in pH 6.8 buffer and 145  $\mu\text{g}$  in pH 8 buffer. This is considerably less adsorption than was seen in the SSC buffered system (greater than 200  $\mu\text{g/g}$ ), and may be due either to the presence of phosphate in these buffers (note the effect of phosphate on adsorption to this sediment shown in Figure 7), or the greater buffering of the sodium phosphate buffer over SSC. The observation that slightly less adsorption occurred in pH 6.8 than in pH 8 buffer may indicate that adsorptive mechanisms other than intercalation or phosphate exchange are at work in these systems. More work is needed in this area to truly identify all adsorptive mechanisms present.

A more efficient buffer to prevent the adsorption of DNA to sediments is 0.1 M sodium pyrophosphate, 0.12 M sodium phosphate. When DNA was added at a rate of 333  $\mu\text{g}$  per gram of Iberia soil in this buffer, only 90  $\mu\text{g}$  per gram was adsorbed, as opposed to 160  $\mu\text{g}$  being adsorbed in 0.12 M NaP, pH 6.8. While this was more efficient at preventing adsorption, it was also much more efficient at extracting humic acids than other buffers, resulting in the extracts being black colored. It was believed that this would add considerable difficulty to purifying the DNA, and work with this buffer was discontinued.

#### Purification of DNA from Activated Sludge

A procedure for the isolation of DNA from activated sludge was developed as a preliminary exercise in isolating DNA from the environment. Activated sludge contains humic substances that may interfere with purification, but it does contain high densities of

cells (approaching  $10^{10}$  cell/ml) which would increase the likelihood of isolating DNA. It is also an important milieu for the biodegradation of hazardous wastes, and the frequencies of specific catabolic genes present in activated sludge would be of great interest in defining the potential that the sludge may have for degradation of these wastes.

Two replicate samples of one liter sludge each were lysed by freeze-thawing, lysozyme treatment, and finally SDS treatment followed by homogenization in a Bead-Beater. Preliminary studies showed that DNA could be purified by standard phenol:chloroform:isoamyl alcohol extractions followed by isopropyl alcohol precipitation, RNase digestion and final purification by hydroxyapatite. The amount of cellular contaminants such as protein and lipids were high due to the high level of biomass present in the sludge, and a large number of phenol:chloroform extractions, eight, were necessary in order to remove them from the samples. The samples were still not completely clean at this point, and a batch hydroxyapatite method was necessary to purify the sample completely.

By adding a sodium acetate precipitation on ice before the solvent extractions, a significant amount of the contaminants were removed, and the number of extractions required was decreased to three. Hydroxyapatite chromatography was not necessary, and the yield averaged 2 mg DNA per liter of sludge from two samples. The significant characteristics of this DNA are listed in Table 7.

Table 7. Pertinent Characteristics of DNA Isolated from Activated Sludge

| $A_{260/280}$ | $A_{260/230}$ | Yield<br>(mg/L) | Size<br>Range<br>(kb) |
|---------------|---------------|-----------------|-----------------------|
| 2.0           | 1.94          | 2.65            | 9.5-0.5               |

## Purification of DNA from Sediments

### Cell Lysis

Three sediments representing a range of sediment types were selected for the development of the procedure for purification of sediment DNA (Table 3, page 52). A multiphase procedure was developed for the lysis of cells in the presence of these sediments consisting of one freeze-thaw cycle, treatment with 1% SDS at 65-70°C, followed by completion of lysis in the Bead-Beater. Efficiencies of lysis for these sediments are presented in Table 8, with the Bayou Texar and Range Point samples being evaluated by acridine orange direct counts (AODC) (34), and lysis in the Ft. Loudon sample evaluated by plate counts on YEPGA (1 g dextrose, 2 g polypeptone, 0.2 g yeast extract, 0.2 g  $\text{NH}_4\text{NO}_3$ , 18 g agar per liter). Colonies formed at the highest dilution in the Ft. Loudon case were characterized by Gram stain, and the results are presented in Table 9. The lysate was enriched in small Gram positive cells having a white mucoid colony morphology.

### Concentration of Extracts

Concentration of samples following extraction was necessary due to the large sample volumes incurred, and two basic procedures, ethanol precipitation and precipitation by polyethylene glycol (PEG) were investigated.

Concentration by ethanol precipitation was investigated using three different methods: with the addition 0.3 M sodium acetate; with the addition of 2.5 M ammonium acetate; and with no added salts. The addition of salts to an ethanol precipitation is primarily used to

Table 8. Lysis Efficiency and DNA Recovery from Sediments

| Sediment    | Lysis<br>Efficiency<br>(%) | DNA<br>Recovered<br>( $\mu$ g/g) |
|-------------|----------------------------|----------------------------------|
| Ft. Loudon  | 90                         | 8                                |
| Bayou Texar | 90                         | 26                               |
| Range Point | 99                         | 16                               |

Table 9. Evaluation of Lysis in Ft. Loudon Sediment

| Treatment  | Total<br>Plate Counts<br>(Per gram) <sup>a</sup> | Cell<br>Type <sup>b</sup>                                         |
|------------|--------------------------------------------------|-------------------------------------------------------------------|
| Pre-Lysis  | $4.67 \times 10^6$                               | Gram (+) cocci (2)<br>Gram (-) rods (3)<br>Gram (+) rod<br>Fungus |
| Post-Lysis | $4.65 \times 10^5$                               | Gram (+) cocci                                                    |

<sup>a</sup>Cells grown on YEPGA for 2 weeks at room temperature, 2 replicates.

<sup>b</sup>Cell types found in highest dilution.

separate proteins from DNA, leaving proteins in solution while DNA is precipitated (17). It was assumed that the addition of salts above the 0.12 M sodium phosphate already present in the extract would not affect the efficiency of precipitation, and only the ease of purification was compared. Only precipitation for Ft. Loudon sediment was compared in this way. Regardless of the treatment, the same amount of further purification, i.e. hydroxyapatite chromatography, was required. The addition of either salt resulted in large amounts of salts being precipitated, which increased the difficulty of sample manipulation.

The use of polyethylene glycol has the advantage of not expanding the already rather large sample volume as much as ethanol precipitation; only 0.5 volumes of PEG are added while two volumes of ethanol are required. Ethanol precipitation with 0.3 M sodium acetate was compared with PEG precipitation in the Range Point sediment, and it was immediately apparent that ethanol precipitation is much easier. A series of phenol:chloroform:isoamyl alcohol extractions was required to remove the PEG, and this increased the time required significantly. This series of phenol:chloroform:isoamyl alcohol extraction removed a significant amount of the contaminants, however. The yield of pure DNA recovered from hydroxyapatite chromatography following ethanol precipitation was 11.5  $\mu\text{g}$  per gram of Range Point sediment, while 16.2  $\mu\text{g/g}$  were recovered from precipitation by PEG. It should be noted that this increase in yield is probably due to a higher degree of purification and not to a higher efficiency of precipitation. A comparison of fractions taken from hydroxyapatite is presented in

Table 10, which shows the greater number of fractions of acceptable purity taken from the PEG precipitated sample than from the ethanol precipitated sample. The technique used in hydroxyapatite chromatography was improved considerably over that used in this comparison, and it is not known whether these same results would still be valid with the newer technique.

Some contaminants in the samples were precipitated out by the addition of 5 M potassium acetate to a final concentration of 0.5 M prior to concentration by ethanol precipitation. This removed a significant amount of organic contaminants as qualitatively judged by the difference in color after the precipitation.

Another means of purification investigated was the use of polyvinylpolypyrrolidone (PVPP) to remove humic substances from the extracts. PVPP is an insoluble polymer that can be mixed with soil extracts to adsorb humics, which are then removed by centrifugation (38). The main concern with its use in DNA extracts is that DNA as well as humics may be adsorbed to the PVPP, thereby limiting its usefulness. To examine this possibility, five ml of 20  $\mu$ g DNA per ml spiked with  $^{32}$ P-DNA was added to 0.5 grams PVPP saturated with 0.12 M NaP, pH 8 buffer and the mixture shaken. The amount of DNA adsorbed to PVPP was determined and a partition coefficient of 3.39 ml/g was calculated. While a great deal of the color was removed from this solution, so was 75% of the added DNA. This level of loss was held to a minimum if only 0.1 g. PVPP were added to ~ 30 ml extract, and a significant improvement in purity was seen.

Table 10. Comparison of Yields of Hydroxyapatite Purified DNA Recovered from Range Point Sediment Concentrated by Ethanol and Polyethylene Glycol (PEG) Precipitation

| UV<br>Wavelength (nm) | Fraction* |       |       |      |      |
|-----------------------|-----------|-------|-------|------|------|
|                       | 1         | 2     | 3     | 4    | 5    |
| Ethanol Precipitation |           |       |       |      |      |
| 260                   | 0.06      | 0.56  | 0.59  | 0.43 | 0.35 |
| 280                   | 0.01      | 0.275 | 0.31  | 0.23 | 0.19 |
| 230                   | 0.02      | 0.32  | 0.40  | 0.32 | 0.29 |
| 260/280               | 6         | 2.04  | 1.90  | 1.87 | 1.82 |
| 260/230               | 3         | 1.75  | 1.48  | 1.34 | 1.19 |
| Mass Recovery (ug)    | --        | 140   | 147.5 | --   | --   |
| PEG Precipitation     |           |       |       |      |      |
| 260                   | 0.30      | 0.77  | 0.55  | 0.39 | 0.20 |
| 280                   | 0.15      | 0.40  | 0.28  | 0.23 | 0.11 |
| 230                   | 0.15      | 0.39  | 0.30  | 0.26 | 0.14 |
| 260/280               | 2.0       | 1.93  | 1.96  | 1.70 | 1.82 |
| 260/230               | 2.0       | 1.97  | 1.83  | 1.50 | 1.43 |
| Mass Recovery (ug)    | 75        | 193   | 138   | --   | --   |

\*5 ml fractions of 0.25 M NaP, pH 6.8, taken from hydroxyapatite chromatography. This represents yields from 25 grams of sediment.

The primary routes for purification of the DNA were through CsCl gradient centrifugation and hydroxyapatite chromatography. The efficacy of CsCl gradient centrifugation was investigated with the Bayou Texar sediment. Ultracentrifugation did purify the sample significantly, but the sample still had contaminants present and further purification was necessary. A separate sample by-passed preliminary purification by CsCl and purification was attempted directly by hydroxyapatite chromatography. No DNA of acceptable purity was recovered by this treatment even with the extraction of samples with phenol:chloroform, and it was believed that CsCl centrifugation was required before hydroxyapatite chromatography. Subsequent development of the hydroxyapatite procedure allowed DNA from both the Range Point sample (with a higher organic carbon content than Bayou Texar samples) and Ft. Loudon sediment to be recovered without CsCl gradient centrifugation. It may be that the inclusion of the CsCl gradient centrifugation is necessary for some sediment, but not for all. This improvement in the hydroxyapatite procedure will be discussed in the following section.

The primary technique used in purification of the DNA was hydroxyapatite chromatography. This involves the binding of the DNA and contaminants to hydroxyapatite, the selective elution of contaminants by 8 M urea, 0.12 M NaP, displacement of the urea by 0.012 M NaP, and finally elution of the DNA with a higher phosphate buffer. Most contaminants are eluted with the 8 M urea, 0.12 M NaP prior to elution of the DNA, but some remain adsorbed to the hydroxyapatite and may coelute with the DNA if the molarity of the

eluent is too high. This decreases the number of acceptable fractions, and therefore the yield of pure DNA recovered is decreased. Torsvik (88) had recommended elution with 0.4 M NaP, but as mentioned before this did not yield sufficiently clean DNA from the Bayou Texar sample. The molarity of sodium phosphate buffer was lowered to 0.28 M in the purification of the Range Point sample (Table 10), which was slightly higher than the 0.238 M NaP recommended by Bio-rad for the elution of double stranded DNA. The molarity of phosphate may be decreased below that recommended by Bio-rad, with the effect that the DNA peak is spread out over several fractions rather than all eluting in the first few milliliters. There was no apparent difference in the DNA eluted with increasing fractions, i.e. the molecular weight range was constant with increasing fraction number (Figure 8).

The purified DNA from Ft. Loudon sediment had a size range of between 9.5 and 0.5 kb (Figure 9), and a sample ultraviolet spectrophotometric scan is shown in Figure 10. DNA isolated from Ft. Loudon was digested by the restriction enzymes Eco RI, Hind III and Sau 3AI (Figure 11), as well as being nick translatable. DNA was nick translated and incorporation of the labelled nucleotide was confirmed by separation of the larger labelled DNA from the unincorporated nucleotides by Sephadex G-50 chromatography. Repeated attempts to duplicate the restriction digests have failed. It is not known at this time whether the DNA is consistently restrictable or not.



Figure 8. The Molecular Weight Distribution of Ft. Loudon DNA in Successive Fractions Eluted from Hydroxyapatite.

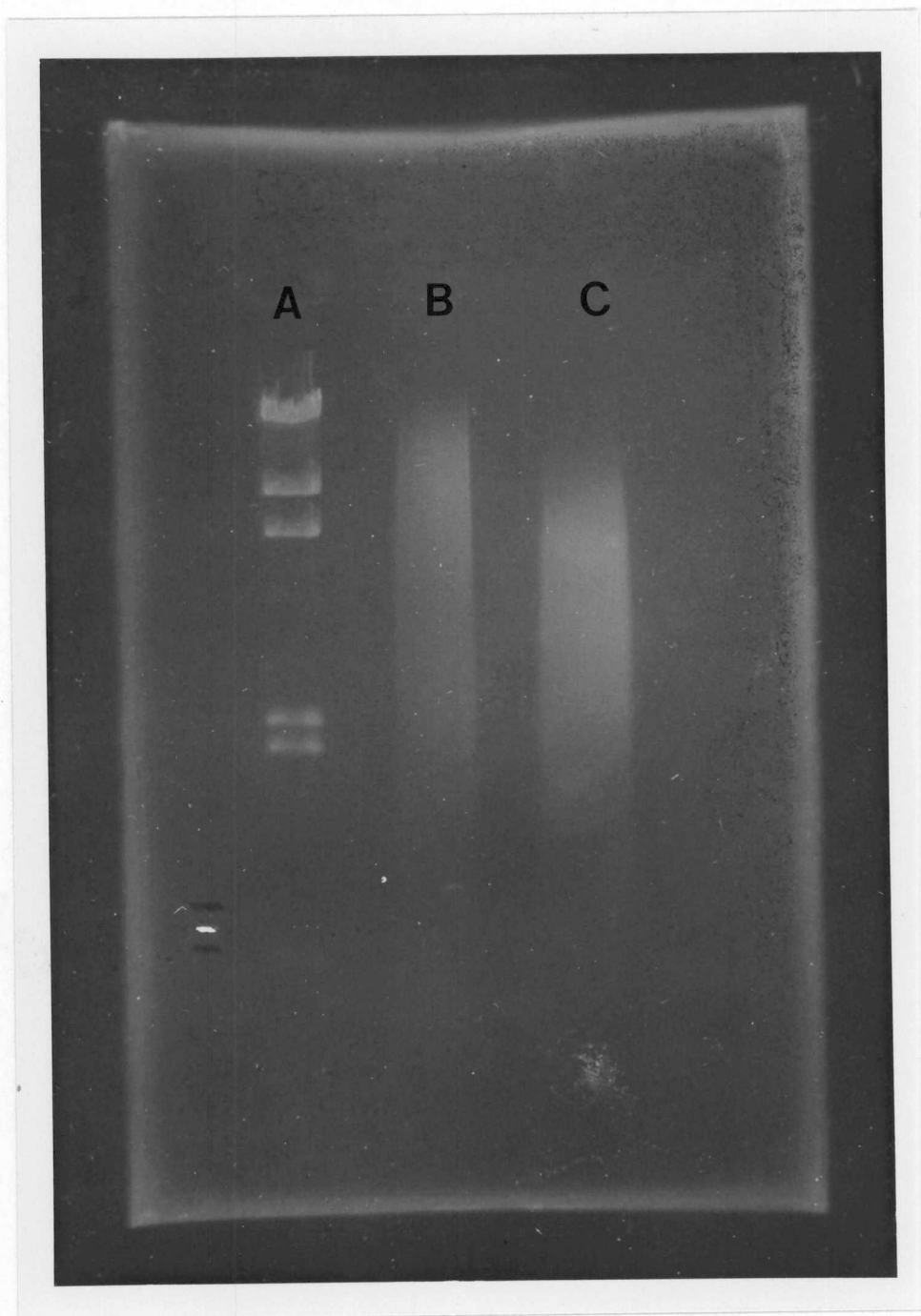


Figure 9. Agarose Electrophoresis Gel of Intracellular and Extracellular DNA Isolated from Ft. Loudon Sediment. Lane A: Bacteriophage  $\lambda$  x Hind III, with Band Sizes of 23.1, 9.4, 4.4, 2.3 and 2.0 kb. Lane B: Extracellular DNA. Lane C: Intracellular DNA.

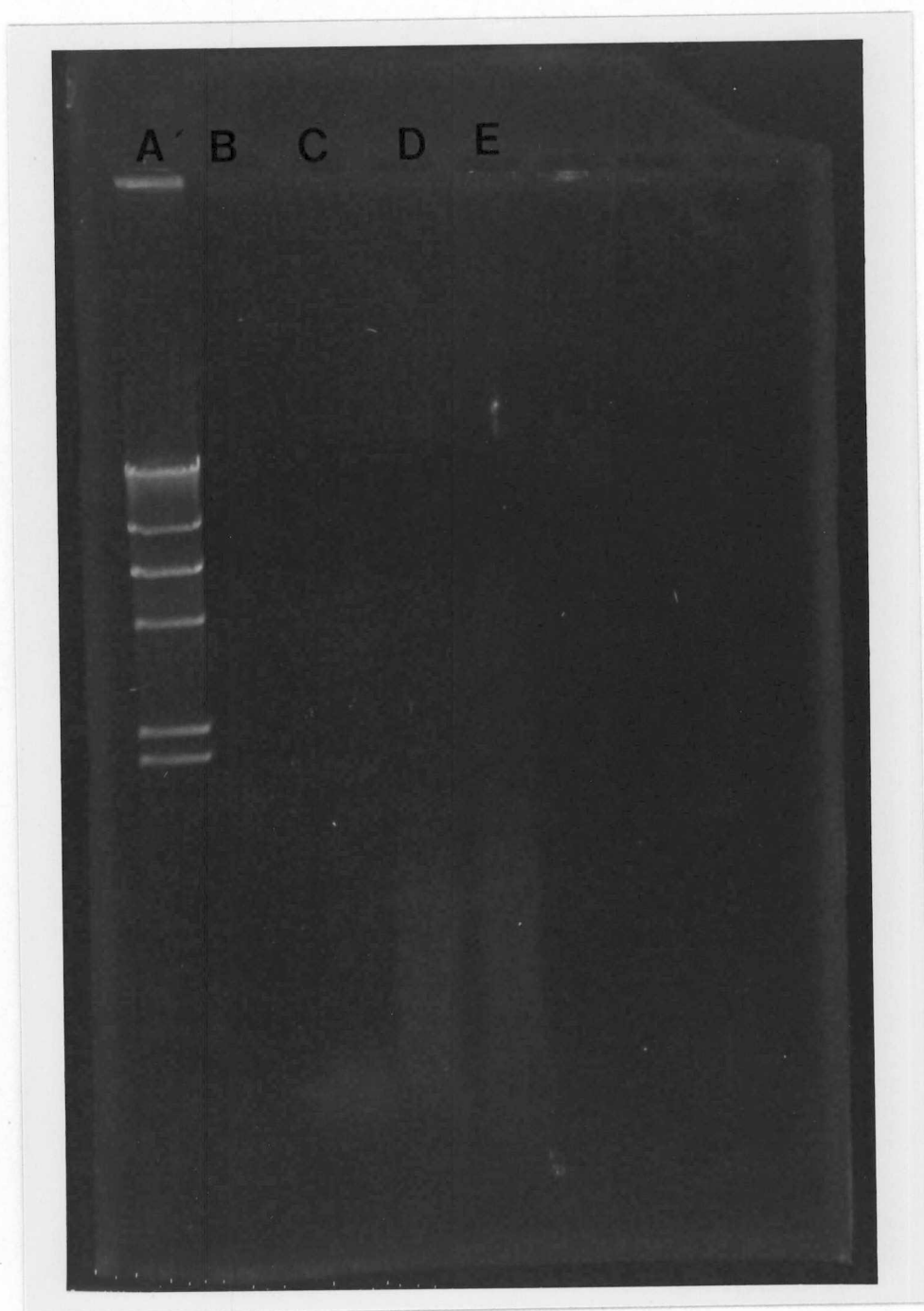


Figure 11. Restriction Digests of Intracellular DNA Isolated from Ft. Loudon Sediment. Lane A:  $\lambda$  x Hind III. Lane B: Eco RI Digestion. Lane C: Hind III Digestion. Lane D: Sau 3AI Digestion. Lane E: Unrestricted DNA.

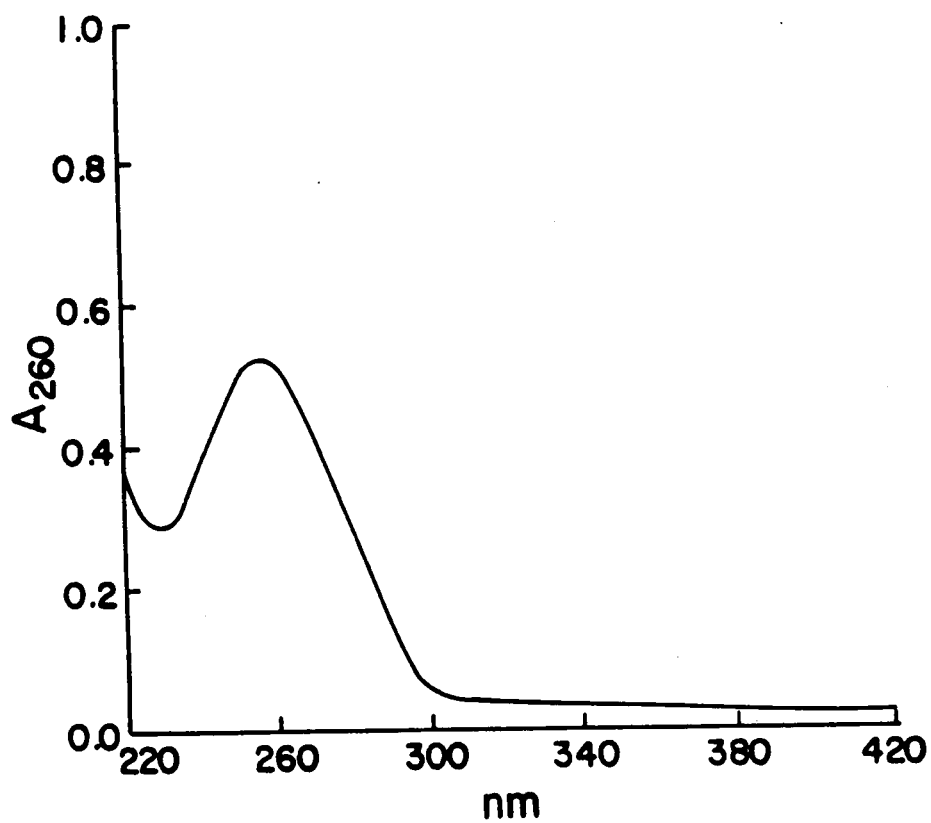


Figure 10. Ultraviolet-visible Spectrogram of DNA Isolated from Ft. Loudon Sediment.

### Efficiency of Purification

The efficiency of the purification technique was evaluated with Ft. Loudon sediment.  $^{32}\text{P}$ -labelled DNA was added to three replicates of lysed sediments, extracted with alkaline sodium phosphate buffer and concentrated by ethanol precipitation. The samples were then dialyzed and purified by hydroxyapatite chromatography. Samples were taken after each step, and the amount of radioactivity remaining calculated. These data are presented in Table 11 and the efficiency of hydroxyapatite chromatography is shown in Table 12. Both the ethanol precipitation step and the hydroxyapatite chromatography resulted in large losses of DNA, with only 38.9% of the DNA being recovered as pure. The loss of DNA at during ethanol precipitation seems to result from DNA not being transferred from the centrifuge tubes used to pellet the precipitation rather than from the DNA not being precipitated. Only 3.2% of the total radioactivity was left in the supernatant after centrifugation, but scanning the centrifuge tubes with a geiger counter following removal of the precipitate revealed a significant level of radioactivity remaining. The largest losses from hydroxyapatite chromatography (Table 12) were in the urea wash (9.6%) and in the loss due to insufficient purification (9.15%).

### Extraction and Purification of Extracellular DNA

By extracting DNA from sediments prior to lysis of the sediment, extracellular DNA may be extracted. These extracts were precipitated by ethanol and purified by hydroxyapatite chromatography. The

Table 11. Recovery Efficiency of Purified DNA from Ft. Loudon Sediment

| Step                                                           | Percentage (CV)* |
|----------------------------------------------------------------|------------------|
| Extraction                                                     | 93 (0.06)        |
| Postassium Acetate Precipitation                               | 86.1 (0.03)      |
| Amount Remaining in Supernatant after<br>Ethanol Precipitation | 3.3 (0.29)       |
| After Dialysis                                                 | 62.3 (0.07)      |
| Hydroxyapatite Chromatography<br>(as pure DNA)                 | 38.9 (0.040)     |

\*CV = coefficient of variation based on three replicates, except for hydroxyapatite where n = 2.

Table 12. DNA Recovery Efficiency from Hydroxyapatite Chromatography

| Step                                                   | Percentage (CV)* |
|--------------------------------------------------------|------------------|
| Not Adsorbed to Hydroxyapatite                         | 4.15 (0.80)      |
| Eluted in 8 M Urea, 0.12 M NaP                         | 9.6 (0.38)       |
| Recovered as Pure DNA                                  | 64.3 (0.05)      |
| Eluted as Impure DNA or Remaining<br>on Hydroxyapatite | 9.15 (0.10)      |
| Total                                                  | 90.00 (0.08)     |

\*CV = coefficient of variation based on two replicates.

molecular weight distribution of extracellular DNA is somewhat larger than that of intracellular DNA due to the shearing of the DNA by the the Bead-Beater (Figure 9). A sample ultraviolet spectrophotometric scan of extracellular DNA isolated from Ft. Loudon sediment is presented in Figure 12.

The assumption that no cells are lysed during the extraction of extracellular DNA was checked by the addition of E. coli DHI cells containing a rapeseed storage protein structural gene. It was believed that this gene is not present in the indigenous DNA pool, and therefore any sequences homologous to this gene present in the extracellular DNA would have come from cells that had been lysed during the extraction process. It can be seen from Figure 13 that there is a small amount of positive hybridization with the rapeseed gene fragment in the extracellular DNA fraction. This positive hybridization in the extracellular DNA is likely to be due to contamination of the probe with the vector, pUC18. Noninnoculated DNA also showed hybridization with the probe, as did the negative control, pUC18. A separate experiment revealed a high level of sequences homologous to pUC18 present in the noninnoculated DNA, indicating that any contamination of the probe with the vector would result in false positive hybridization.

Concentrations of pSS 50 in extracellular and intracellular DNA isolated from Ft. Loudon reservoir at various times over the course of a year were compared by dot blot hybridizations (Figure 14). The samples were taken from an area of approximately 100 square meters and a statistically valid design was not implemented, so it is not possible

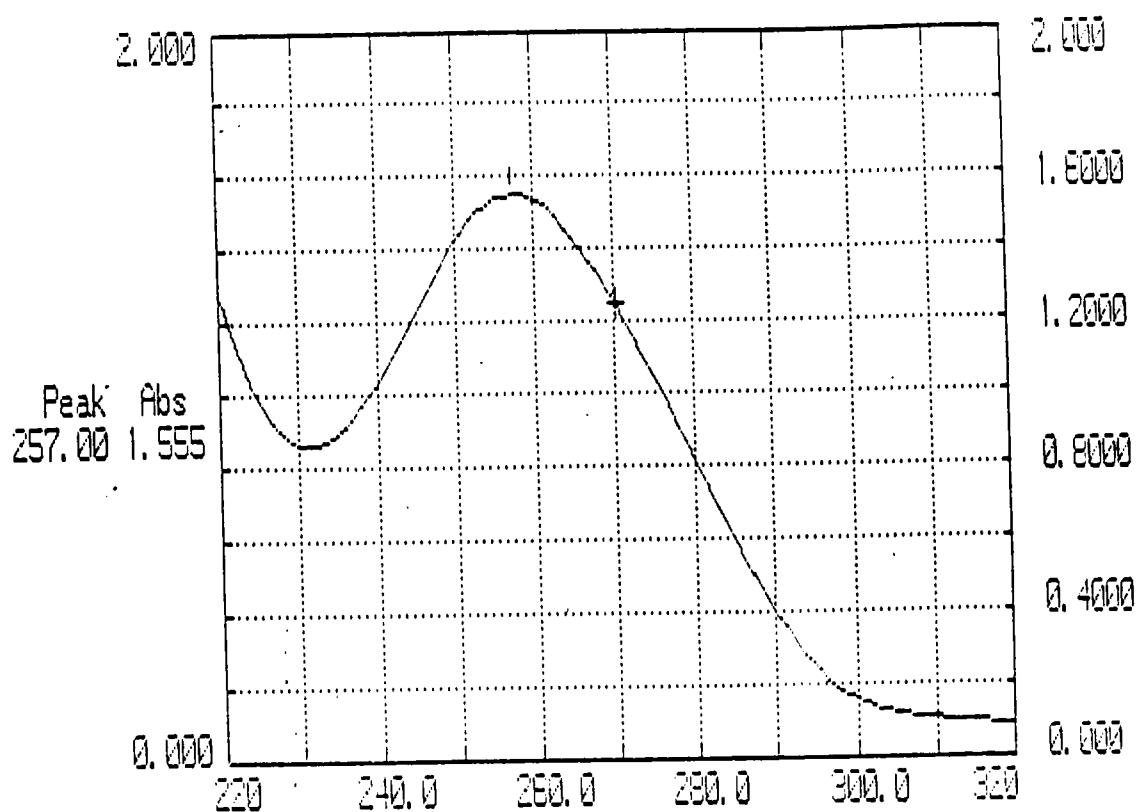


Figure 12. Ultraviolet-visible Spectrogram of Extracellular DNA Isolated from Ft. Loudon Sediment.

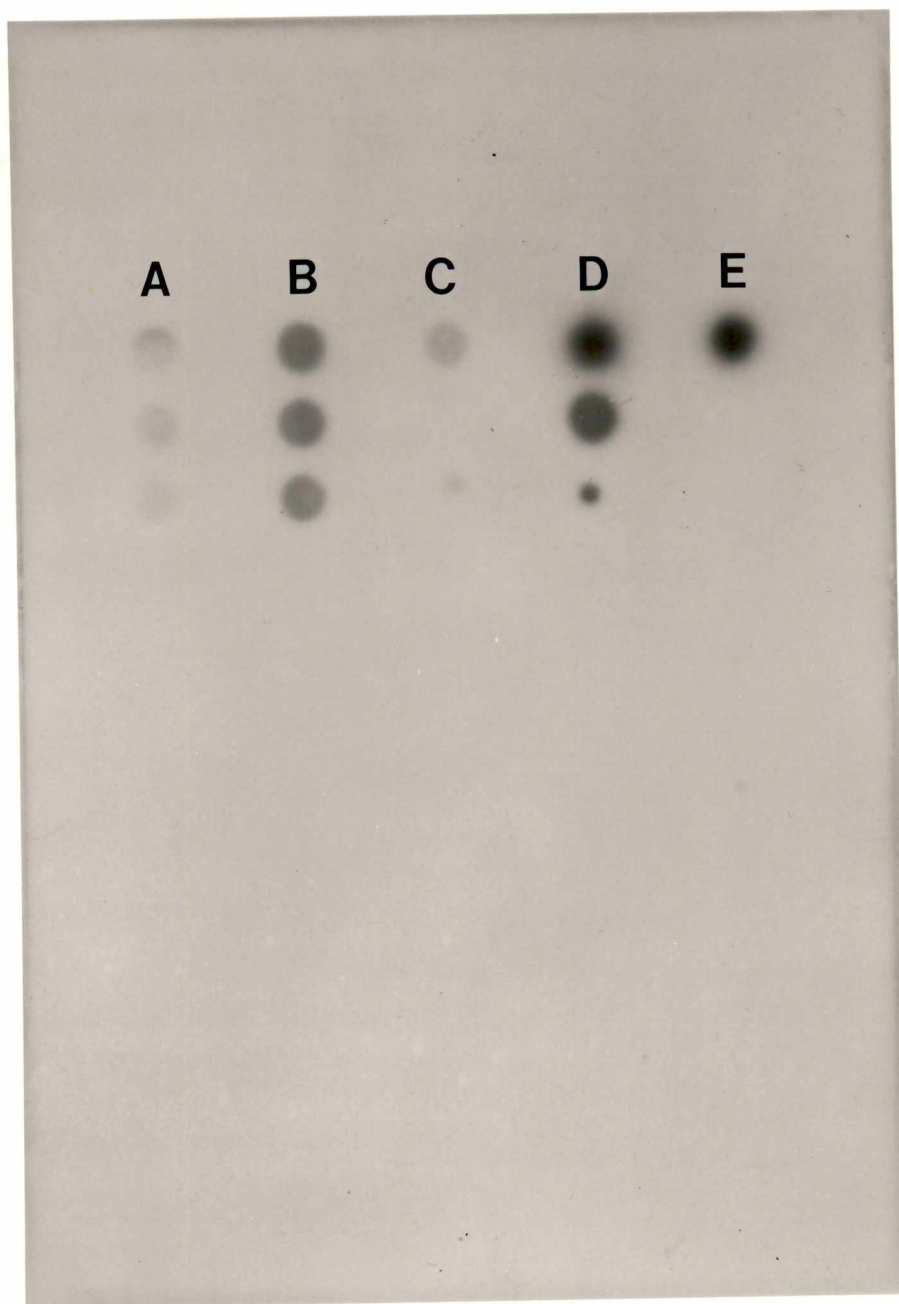


Figure 13. Dot Blot, Hybridization of Napin Gene Sequences in Extracellular and Intracellular DNA Innoculated with E. coli DHI Harboring pEPA90. A: Extracellular DNA; 5  $\mu$ g, 3  $\mu$ g, 1  $\mu$ g. B: Intracellular DNA; 5  $\mu$ g, 3  $\mu$ g, 1  $\mu$ g. C: Non inoculated Intracellular DNA; 2  $\mu$ g. D: Napin protein storage gene restriction fragment;  $10^{-4}$   $\mu$ g,  $10^{-5}$   $\mu$ g,  $10^{-6}$   $\mu$ g. E: pUC18, 0.001  $\mu$ g.

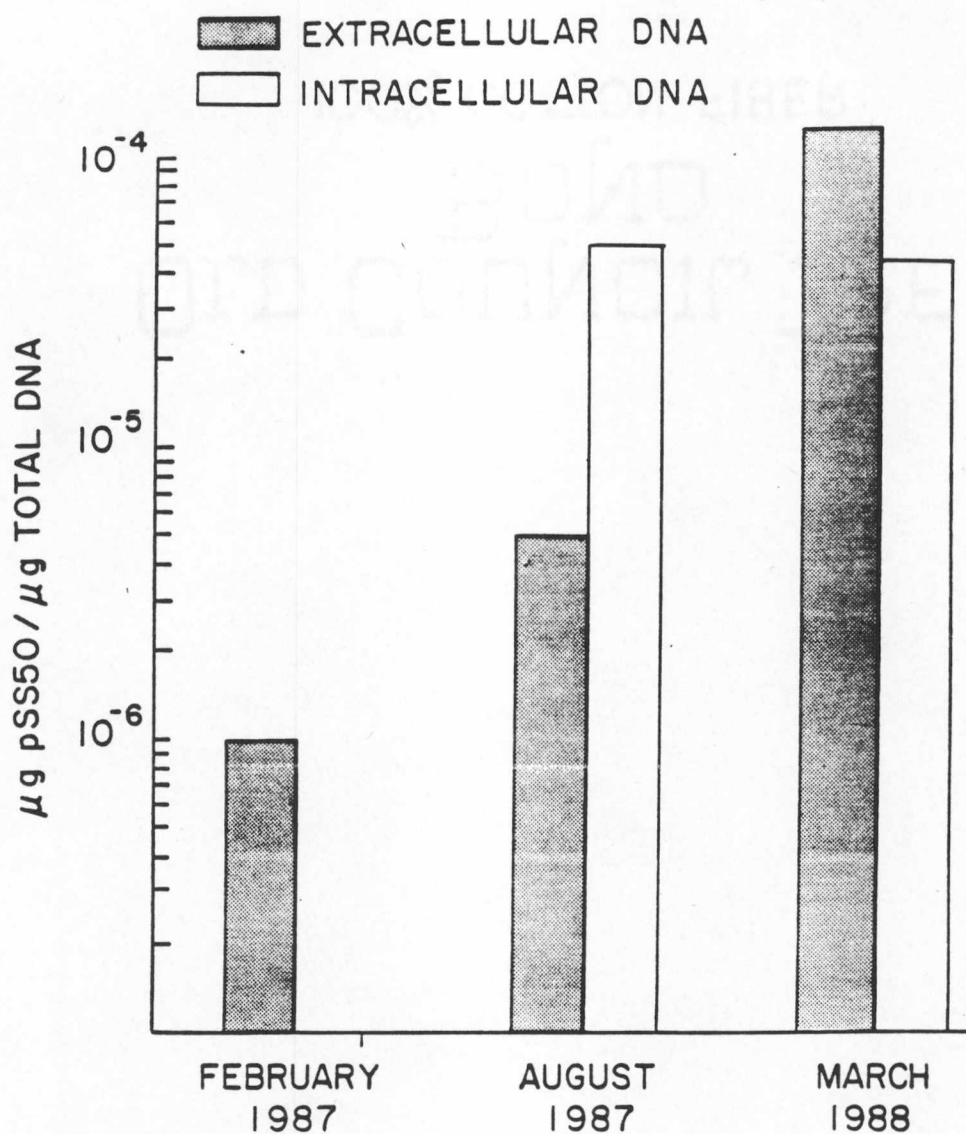


Figure 14. Comparison of Sequences Homologous to pSS 50 in Extracellular and Intracellular DNA Isolated from Ft. Loudon Sediment.

to make any conclusions concerning the fluctuations of pSS 50 in these fractions over time. The difference in the concentrations within each time point may be compared, however, and there is a clear difference within each of these timepoints. Of particular interest is the February, 1987, sample point, where no pSS 50 was detectable in the intracellular fraction but was detected at a low concentration in the extracellular fraction.

#### Measurement of Extracellular DNA Concentrations by HPLC

A procedure for the rapid and accurate measurement of extracellular DNA concentrations was developed utilizing an hydroxyapatite HPLC column with a fluorescent detection system (Figure 15). This procedure by-passes both the ethanol precipitation step and dialysis necessary for large scale purification with open column chromatography, thereby reducing sample manipulation and time required for analysis. A sample chromatogram is presented in Figure 16, showing organic contaminants eluting in a large peak at around 4 ml elution volume and DNA eluting around 10 ml elution volume. Core samples were taken from the Singleton terminal area of Ft. Loudon reservoir, the extracellular DNA extracted, and the DNA labelled with Hoescht 33258. The results of these measurements are presented in Figure 17.

## **Quantitative Analysis of Extracellular DNA**

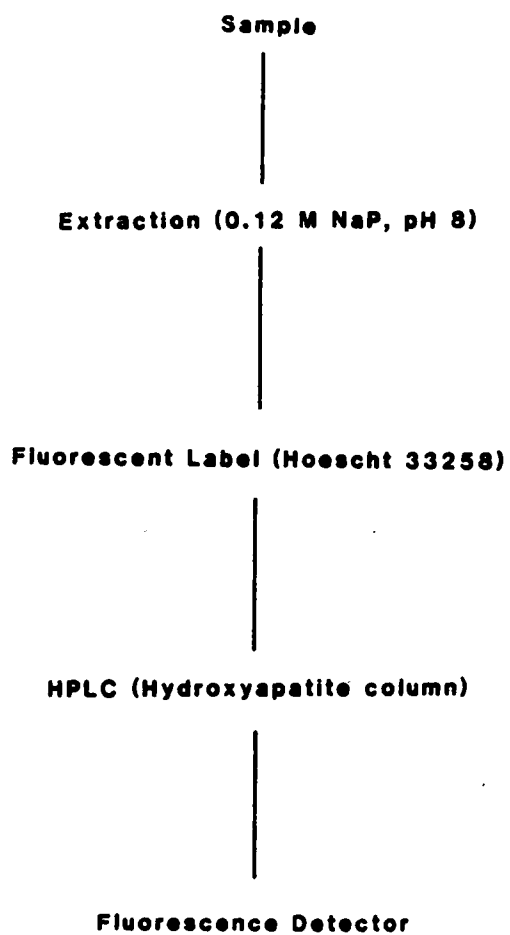


Figure 15. Optimized Protocol for the Extraction and Measurement of Extracellular DNA by High Performance Liquid Chromatography.

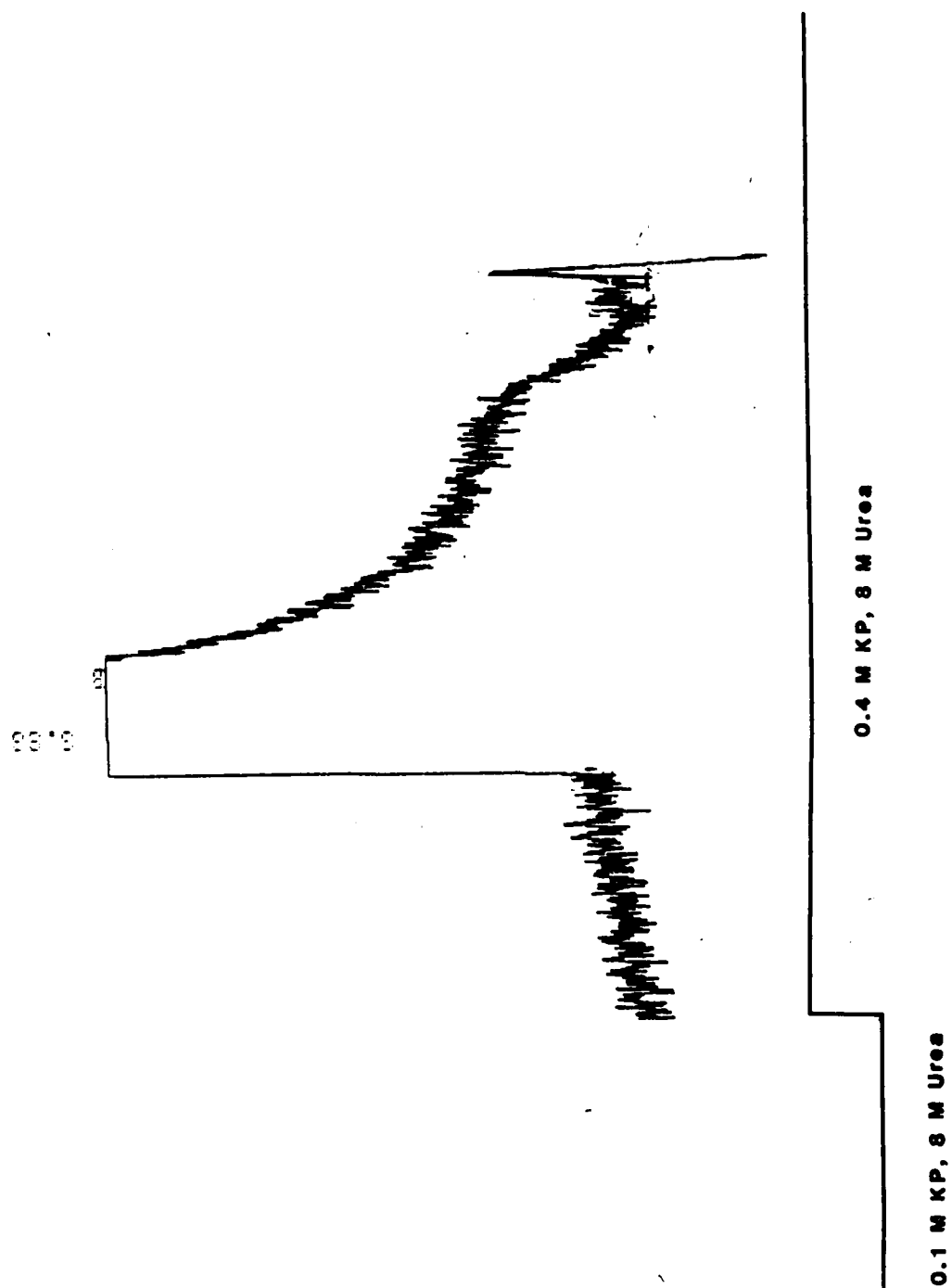


Figure 16. Chromatogram of Extracellular DNA with Elution Profile.

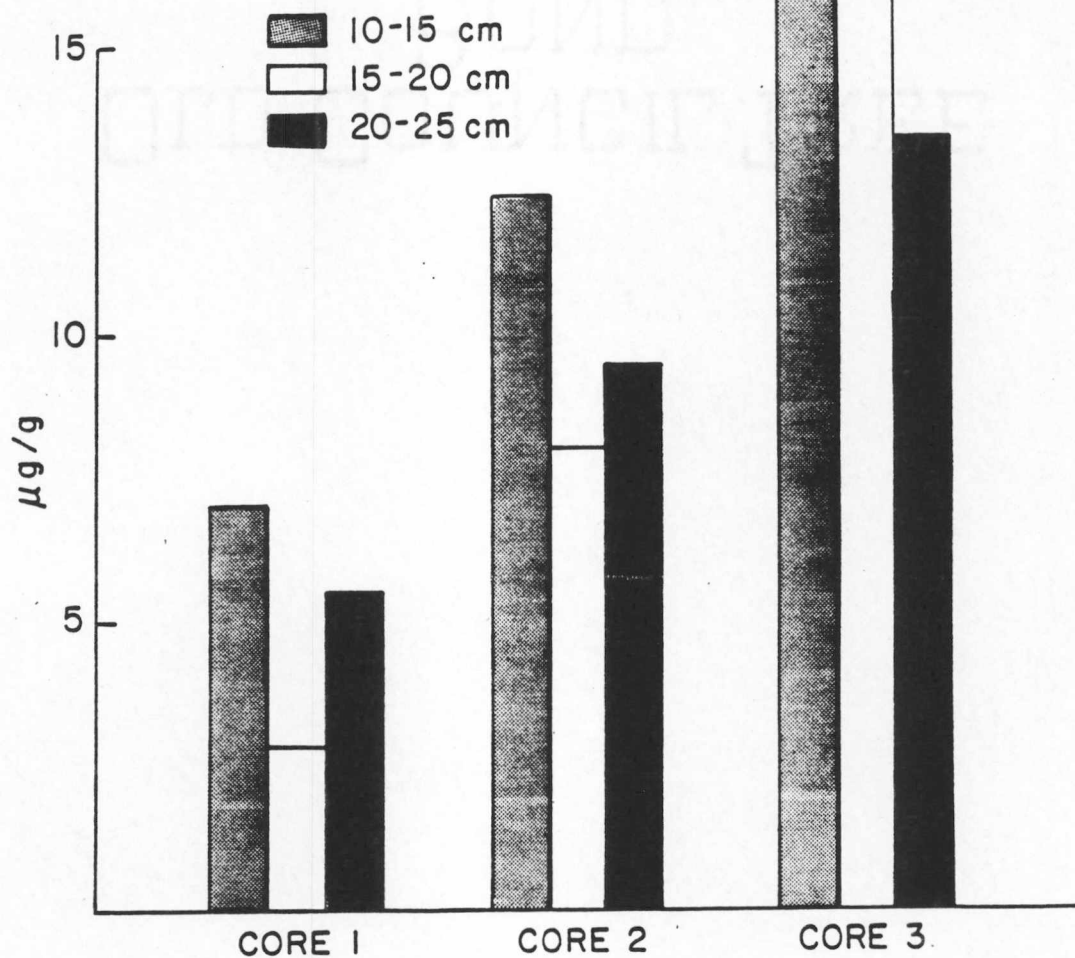


Figure 17. Sediment Extracellular DNA Concentrations at Three Depths in Ft. Loudon Reservoir. Three Core Samples were Taken within One Square Meter.

## CHAPTER 6

### DISCUSSION

This dissertation describes the development and evaluation of a new method for the extraction and purification of microbial DNA from sediments. This technique yields DNA of suitable purity and quality for most molecular biological applications that are essential in the emerging field of molecular microbial ecology.

The technique is based upon the direct lysis of microorganisms in the presence of the sediment, followed by the extraction of the released DNA from the sediment and its subsequent purification (Figure 18). It was believed that a more representative sample of microbial DNA could be obtained by direct lysis and extraction of DNA rather than by first separating cells from the sediment. Microbes are lysed by a combination of heat and detergent treatment followed by ballistic disintegration in a Bead Beater. Over 90% of the cells present are lysed by this method and the efficiency of lysis may be dependent upon the physical characteristics of the sediment. Of the three sediments investigated in this study, those containing more than 8% sand (Ft. Loudon and Bayou Texar) yielded 90% lysis, while the more finely textured sediment (Range Point) yielded 99% lysis. Not enough data has been gathered for anything more than speculation about the effects of sediment texture on lysis, but it may be that more coarse sediments afford indigenous cells a higher degree of protection than more finely textured ones.

The accurate measurement of lysis efficiency is hampered by the

## Purification of Extracellular and Intracellular DNA

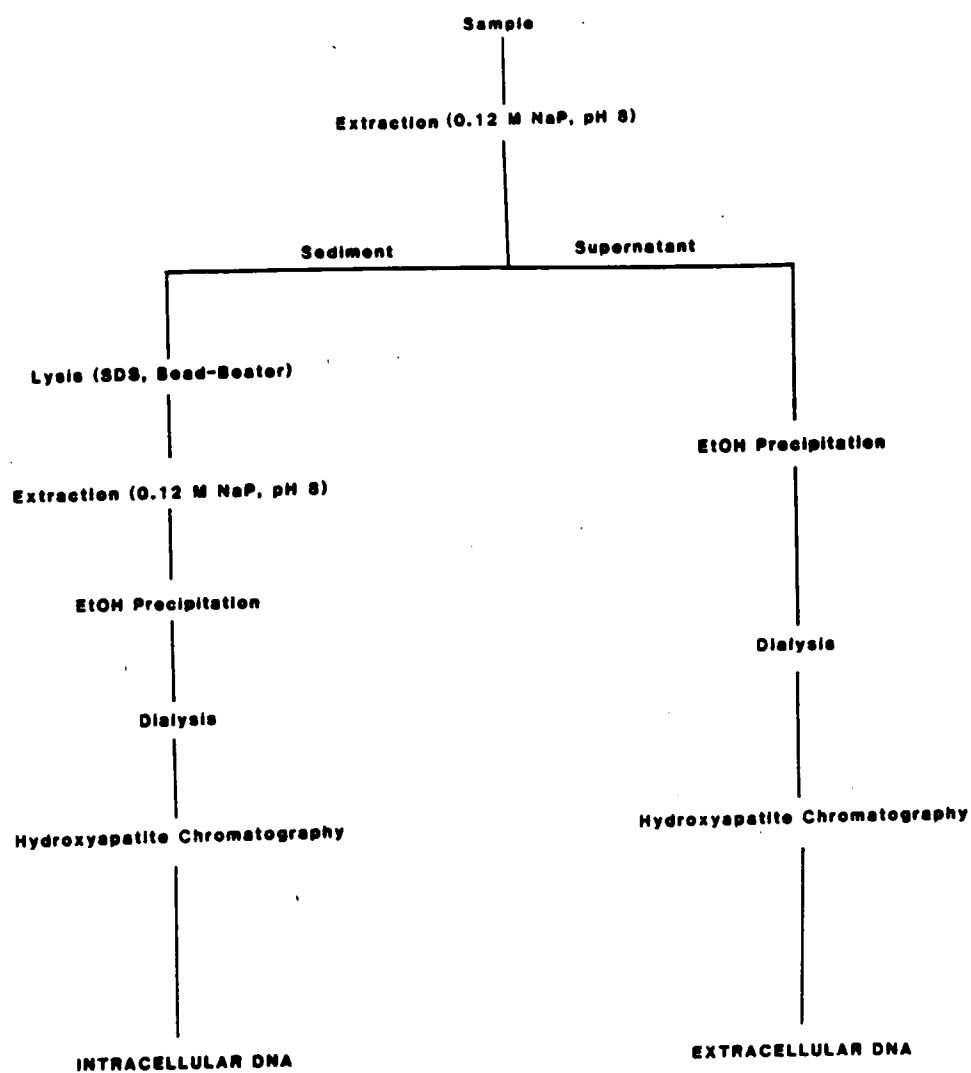


Figure 18. Optimized Protocol for the Direct Isolation of Extracellular and Intracellular DNA from Sediment.

inability to quantitatively remove cells from the surface of the sediment particles and by the additional requirement that the cells be culturable if plate counts are to be used. It is unlikely that all of the cells present in the system were accounted for in either the pre-lysis or the post-lysis cell counts, and it is not known what biases were added into the determination of lysis efficiency as a result of these unknowns. Because of the harsh nature of the detergent treatment and ballistic disintegration, it is likely that more cells become detached from the particles during the lysis procedure than prior to the lysis procedure. This would result in cells previously unavailable for counting due to irreversible attachment becoming free for counting following lysis. Efficiency determinations would therefore be more likely to underestimate lysis than to overestimate lysis.

The main criticism of existing procedures for environmental DNA isolation has been that they probably do not yield representative samples. Since these procedures are based on the removal of whole cells from the environmental matrix with successive washings with buffer, it is likely that only those species that are easily dislodged are isolated. The efficiency of recovery of cells reported by Holben et al. (38) is only 35%, considerably less than the 90 - 99% lysis efficiency reported in this study. The lysis efficiency of existing procedures has not been reported, and it may be that more bias is introduced in the resistance of some species to lysis. The lysis procedure described in this study is less efficient at lysing small Gram positive cocci in the Ft. Loudon sediment. This cell type

represented less than 30% of the culturable cells prior to lysis, but were present at over 90% in the lysate. This represents a reduction of approximately 30% of Gram positive cocci as opposed to a 90% reduction in total cell counts. A bias is introduced in the lysis step, and more work needs to be done to improve the total efficiency of lysis.

Once cells are lysed in the presence of the sediment, DNA is subject to adsorption to sediment particles. By identifying those factors that control the adsorption of DNA to sediments, a more efficient extraction procedure could be developed. To this end, DNA adsorption isotherms were conducted on a range of soil and sediment types. Sediments and soils containing a significant amount of montmorillonite adsorbed all DNA presented at environmentally relevant concentrations. This adsorption was sensitive to pH, with much less adsorption occurring at alkaline pH's than at acidic pH's. Greaves and Wilson (31) reported similar results with clean montmorillonite. Sands were also shown to adsorb DNA, although to a much lower extent than those containing montmorillonite. The adsorption of DNA to sands was described by the Freundlich model, which usually implies a dynamic adsorption-desorption equilibrium.

The soils and sediments tested also were sensitive at varying degrees to the amount of phosphate present in the system. The major adsorptive moiety in DNA is likely to be the phosphate backbone, and if so, an increase in phosphate concentration may aid in the extraction of DNA. This type of response is seen in elution of DNA

from hydroxyapatite. Iberia soil, with the highest montmorillonite and organic carbon contents of the sorbents tested, showed a linear decrease in adsorption of DNA with increasing phosphate concentration. Ft. Loudon sediment showed an initial precipitous drop in adsorption with increasing phosphate concentration, a phenomenon which may be indicative of oxidized iron complexing the DNA. Adsorption to acid washed sand was the least sensitive to increasing phosphate concentration.

The primary factors controlling DNA adsorption to soils and sediments were shown to be the mineralogy and pH of the sorbent, and to a lesser extent, phosphate ion concentration. An extraction scheme taking these factors into account was developed. Sequential extractions with an alkaline sodium phosphate buffer was shown to remove 95% of exogenous DNA added to Ft. Loudon sediment. It is highly unlikely that either adsorption or this extraction procedure are influenced by DNA base sequence or composition, and is therefore considered to yield an unbiased sample of free DNA present in the sediment.

The use of sodium phosphate as an extractant is not universally recommended, particularly in soils and sediments that have high cation exchange capacities. Because of the high organic matter and clay contents of Iberia soil, this soil was highly buffered and phosphate buffer at pH 8 was not an effective extractant. An extractant was needed that would raise the pH of the bulk solution sufficiently so that the pH would be above the pKa of DNA. Sodium carbonate buffer at

a pH of 10 was sufficient for this task, and efficiently extracted over 95% of exogenous DNA added.

Once the DNA has been extracted from the sediment, the samples must be concentrated. Concentration by polyethylene glycol precipitation and by ethanol precipitation were compared, with neither procedure appearing to be more efficient than the other. Since ethanol precipitation is considerably easier to perform than polyethylene glycol precipitation (ethanol precipitation doesn't require phenol:chloroform extractions, as does precipitation by polyethylene glycol), it was the method of choice. A drawback to ethanol precipitation is that it expands sample volumes to what may be an unmanageable size. Extraction of 100 grams of sediment typically yields close to one liter of extractant. The addition of two volumes of ethanol for the precipitation brings the total volume up to three liters, a volume that takes hours to centrifuge. Future studies may investigate the efficacy of precipitation by cetyltrimethylammonium bromide, a technique that does not appreciably expand sample size.

The samples must be purified by contaminants following concentration. These samples are typically colored dark brown to black as a result of the extraction of humic substances along with the DNA. These highly polymerized organic acids are difficult to separate from DNA, and the most effective means of separation found to date has been by hydroxyapatite chromatography. Humic and fulvic acids are adsorbed to hydroxyapatite along with the DNA, but most are removed by elution with 8 M urea and a low molarity phosphate buffer. After these contaminants are eluted, purified DNA is eluted by a buffer with

increased phosphate concentration. Contaminants held more strongly by the hydroxyapatite begin to elute after most of the DNA has been eluted. The choice of phosphate concentration in these buffers is crucial to the isolation of purified DNA. Each batch of hydroxyapatite varies with respect to the phosphate molarity at which DNA elutes, and care must be taken not to elute DNA with the contaminants. The vendor for hydroxyapatite (Bio-Rad, Richmond, CA) recommends molarities at which single and double stranded DNA will elute from each batch of hydroxyapatite, but these may not be the best for environmental purifications. During this study, it was found that DNA would elute at lower phosphate molarities than recommended by Bio-Rad (0.20 M phosphate as opposed to the recommended 0.261 M). Use of the lower phosphate concentration ensured that most DNA would be eluted ahead of the contaminants remaining on the hydroxyapatite.

Most losses in efficiency come during the purification by hydroxyapatite chromatography. Losses came from some DNA not being adsorbed to the hydroxyapatite, some being lost during the elution of contaminants with 8 M urea, and some not being completely purified. It is possible that some of these problems may be overcome through the use of high performance liquid chromatography (HPLC). Alternative stationary and mobile phases may be explored that may prevent the loss of DNA during elution of contaminants. Preliminary results with an analytical hydroxyapatite HPLC column were encouraging, indicating that a preparative scale hydroxyapatite HPLC column may yield a higher efficiency of purification. The only preparative scale hydroxyapatite

column currently available is produced by Mitsui-Toatsu and is marketed in the United States by Rainin Instruments (Emeryville, CA) at the rather prohibitive price of \$5000. Since copious amounts of DNA are currently available by open column chromatography, a preparative scale HPLC column may not be cost effective at this time. It would, however, allow for a faster purification as well being adaptable to automation.

The isolation, and even the existence, of extracellular DNA in the environment is of great interest at the moment due to its importance as a potential source of genetic exchange. This technique allows the discrimination to be made between extracellular and intracellular DNA. Extracellular DNA is first extracted from the sediment by alkaline phosphate buffer and then concentrated by ethanol precipitation and purified by hydroxyapatite chromatography (Figure 18). Since the existence of extracellular DNA is subject to debate (R. Atlas, personal communication), a control was devised to ensure that the extraction procedure was truly isolating extracellular DNA and not lysing a significant amount of cells in the process. E. coli DH 1 containing a plasmid carrying the gene coding for the napin storage protein gene of rapeseed (Brassica napus) (16) was inoculated into sediment, and the extracellular and intracellular DNA fractions isolated. This rapeseed gene is totally exogenous to the sediment, and if any sequences homologous to it appear in the extracellular fraction, it may be assumed that it was due to lysis during the extraction procedure. This was not the case, and no napin gene sequence was detected in the extracellular DNA fraction. This was

considered to be a good control for lysis since the host for the napin gene was a rather fragile laboratory strain more likely to lyse than the heartier environmental strains present in the sediment.

This represents the first extraction and purification of extracellular DNA from sediments, as well as the first molecular analysis. The concentrations of the catabolic plasmid, pSS 50, were measured in the extracellular and the intracellular fractions at different times over the course of a year, and it was found that the composition of the two fractions varied considerably. While no conclusions can be drawn concerning the dynamics of pSS 50 with time in the sediment due to a lack of statistically valid design, it can be seen that the concentration of pSS 50 may vary by as much as three orders of magnitude from the extracellular fraction to the intracellular. This indicates that DNA may persist in the environment for significant lengths of time following the death and lysis of the host organism. Free DNA has been observed to be protected by adsorption to montmorillonite (32), a clay present in these sediments.

A rapid and accurate method for measuring extracellular DNA concentrations was also developed which utilizes HPLC and a fluorescence detection system. This system is a significant improvement over existing methods since it measures pure rather than contaminated DNA. Deflaun and Paul (21) have reported that their measurements of dissolved DNA by labeling with Hoescht 33258 in marine and estuarine samples are interfered with by nonspecific fluorescence. This problem is by-passed by using

HPLC to separate fluorescence due to contaminants, from fluorescence due to DNA.

The DNA isolated by this procedure is of high purity and quality, which makes it available to any number of molecular biological techniques being used in ecological analysis. The isolated DNA is pure as judged by ultraviolet spectrophotometry between 220 and 330 nm, a region which would indicate the presence of contaminating humic substances or proteins. It has also been shown to be of sufficient quality for digestion with two commonly used restriction endonucleases, Eco RI and Hind III.

The most commonly used application at this time is quantitative hybridization analysis, which allows the frequency of specific genetic sequences in the DNA samples to be measured. This type of analysis has yielded quantitative data on the presence of pSS 50 in Ft. Loudon sediments, present in concentrations too low to be detected by the colony hybridization procedure (Janet Packard, personal communication). DNA isolated by this technique has also been used to determine the potential for nitrogen fixation in the Bayou Texar and the Range Point sediments by hybridization analysis with the nif K, D, and H genes (67).

One drawback to this technique is that sequences in low abundance may not be detected. Two techniques are currently being adapted for use in environmental samples that may alleviate this problem. Steffan and Atlas (84) have recently reported the use of polymerase chain reaction (PCR) for the amplification of specific gene sequences in DNA extracted from a fresh water sediment, and have increased the

sensitivity of detection of an added gene by three orders of magnitude. Specific sequences are amplified by first denaturing the target DNA, hybridization of the target with synthetic oligonucleotide primers followed by extension of the primers by the addition of polymerase. This cycle may be repeated numerous times until the desired degree of amplification has been achieved.

Another method that has been proposed for the detection of sequences in low abundance is the use of reassociation kinetics to isolate low copy sequences. DNA that has been denatured and is allowed to reassociate will reassociate according to second order kinetics, with those sequences in highest abundance reassociating first (11). By monitoring the reassociation reaction, those sequences present in high copy may be separated from the less concentrated sequences by stopping the reaction at a desired point. The unreassociated DNA may then be separated from the more abundant double stranded DNA by hydroxyapatite chromatography and subjected to quantitative hybridization probing. This technique is still in the developmental stages and has not been thoroughly tested.

Since the DNA recovered by this technique may be cut with common restriction endonucleases, it can probably be cloned. Shot gun cloning of environmental DNA is currently being used as a means of studying the population genetics of sulfate reducing bacteria in marine sediments (22). Clones containing DNA coding for rRNA regions unique to sulfate reducers are identified and their genetic sequences determined and compared for the purpose of constructing molecular

phylogenies. DNA isolated by the technique described in this dissertation could be subjected to this type of analysis for a multitude of applications, such as studying the variation in genes coding for the catabolism of specific pollutants.

### Summary

This dissertation describes the development of a new technique for the isolation of environmental DNA. The procedure is based upon the direct lysis of microbial cells in the presence of the sediment, followed by the extraction of the DNA from the sediment particles and its subsequent purification by hydroxyapatite chromatography. The efficiency of purification has been evaluated, and while copious amounts of DNA have been isolated (up to 26  $\mu\text{g/g}$ ), the procedure may be only 40% efficient. Most losses come during the purification by hydroxyapatite chromatography. The DNA recovered is of high quality and may be restricted by common restriction endonucleases.

The procedure also allows the discrimination between extracellular and intracellular DNA to be made, and a method for the accurate measurement of extracellular DNA concentrations has been developed.

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## VITA

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