AQUATIC MICROBIAL COMMUNITY RESPONSES TO STRESS: COMPARISON OF NONTAXONOMIC AND TAXONOMIC INDICES

by

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(ABSTRACT)

Three nontaxonomic indices; ATP/Chlorophyll a (ATP/Chla), ATP/ADP, and Chlorophyll a/Pheopigment (Chla/Pheo) were compared to the taxonomic measures of species diversity ($\bar{d}$) and species richness as indicators of stress in aquatic environments. Field and laboratory microcosm responses of indigenous microbial communities exposed to municipal sewage treatment plant (STP) effluent were monitored. The STP effluent produced increased adenylate concentrations, ATP/ADP and ATP/Chla ratios, and decreased Chla, Chla/Pheo, $\bar{d}$, and species richness relative to upstream reference communities. Nontaxonomic responses were consistent in four separate field tests.

Significant differences in responses were discernible in 3 d when communities were transferred from reference to polluted sites. Chla/Pheo decreased more rapidly than other measurements. The predictive capability of laboratory flow-through microcosm tests was examined by simultaneously transferring communities from upstream reference sites to downstream field sites and to various dilutions of field effluent in the laboratory.

Microcosm adenylate and Chla concentrations and Chla/Pheo increased with effluent exposure, ATP/ADP and species richness were constant with effluent concentration. Laboratory ATP/Chla and $\bar{d}$ were higher in controls than effluent exposed communities. Adenylate and Chla concentrations and Chla/Pheo were higher in microcosm than field studies probably because of higher temperatures and decreased flow rates. Diatoms dominated the reference field site. Achlorophyllous flagellates, coccoids and bacteria dominated downstream field sites. Oscillatoria dominated the laboratory control communities. Diatoms increased and Oscillatoria decreased with increasing laboratory effluent concentrations. ATP and Chla concentrations were reliable estimators of total and plant biovolumes respectively except when Oscillatoria dominated.

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CHAPTER I

INTRODUCTION

In recent decades much emphasis has been placed on detecting, predicting, monitoring for and preventing environmental perturbations. Methods have evolved from environmental chemical and physical measurements to qualitative and quantitative taxonomic biological characterizations, single species toxicity tests, multispecies and community level toxicity tests. The changing methods have coincided with the recognition of the need for ecologically realistic data. The impact of pollution on complex ecosystem level interactions that occur in situ are difficult to predict from chemical measurements or single species toxicity tests (Cairns, 1981, 1983). Community level tests may incorporate these interactions by including energy exchange through several trophic levels and therefore, may be better indicators and predictors of ecosystem level responses.

Naturally occurring microbial communities, as opposed to gnotobiotic assemblages, contain many complex interacting trophic levels: algal primary producers, protistan and metazoan herbivores, primary and secondary carnivores, and detritivores. Their small size results in an intimate contact and potentially large interaction with the abiotic environment (Karl, 1980). Numerous investigators have proposed the use of microbial communities as indicators of stressed ecosystems and/or

characterizers of ecosystem types (Cairns, 1978, 1983; Cairns, et al., 1979; Karl, 1978, 1980, et al.; Karl and Holm-Hansen, 1978; Holm-Hansen, 1970, 1973; Falkowski, 1977; Weitzel, 1979; and others). Recently, investigators have examined the applicability of microbial tests as predictive devices of ecosystem stress (Matthews et al., 1982; Buikema et al., 1983). These communities appear to model and respond to stress as higher-level communities both structurally and functionally (Cairns, 1982; Matthews et al., 1980, 1982).

The objective of this research was to compare several nontaxonomic and taxonomic measurements of microbial responses to environmental stress and evaluate the predictive capability of laboratory microcosm studies utilizing the same measures. To increase ecological realism, indigenous communities and site-specific water was used. The primary hypothesis was that natural microbial communities exposed to stress will undergo structural changes because sensitive organisms will be replaced by more tolerant forms and the metabolic status or growth potential of the community will decrease. These structural and functional responses are detectable through measures of ATP/Chlorophyll a, ATP/ADP, and Chlorophyll a/Pheopigment, and are predictable from laboratory microcosm tests.

Ratios of ATP/Chlorophyll a (ATP/Chla), ATP/ADP, and Chlorophyll a/Pheopigment (Chla/Pheo) have been proposed as potential indicators and monitors of environmental stress. ATP/Chla is a structural measure relating the total biomass to

plant biomass. Weber (1973), Matthews et al. (1980, 1982a, 1982b), Chiovdani and Pagnotto (1978), and APHA (1980) term this ratio the autotrophic index and describe high values associated with communities exposed to organically enriched environments. This occurs because tolerant heterotrophs such as bacteria, fungi, and protozoans predominate under these conditions. Clean water communities are characterized by low ATP/Chla ratios. Matthews et al. (1980, 1982b) demonstrated correlations between ATP/Chla responses of microbial communities and macroinvertabrate responses exposed to the same stress and Buikema et al. (1983) correlated field and laboratory ATP/Chla responses with protozoan invasion rates as assessors of pollution. The relation between high ATP/Chla ratios and organic enrichment has been examined in the euphotic zone of lotic environments and may not hold for aphotic and/or lentic habitats.

Adenylate ratios of microbial communities and larger organisms have been used to evaluate the metabolic status of the cell (Karl, 1978, 1980;; Karl and Holm-Hansen, 1978, Holm-Hansen, 1970, 1973; Falkowski, 1977; Witzel, 1979; Ivanovici, 1980a, Wiebe and Bancroft, 1975, Giesy and Dickson, 1981; and others). The adenylate energy charge ((ATP + 1/2 ADP)/(ATP + ADP + AMP), Atkinson and Walton, 1967), ATP/ADP, and ATP/AMP ratios are most frequently used. ATP is an energy storage compound that drives many cellular reactions and the relative

abundance of ATP, ADP, and AMP regulate many catabolic and anabolic processes (Atkinson, 1969). High adenylate ratios indicate stored energy, the potential for growth, and metabolic well being. Low ratios resulting from relatively high levels of ADP and/or AMP are considered indicative of an energy deficit and metabolic stress. Adenylate measurements of an entire community assess the average metabolic status of the individuals within that community.

Chla/Pheo ratios are indicative of the physiological status of algae (APHA, 1980; Bastardo, 1980; Yentsch, 1965) separate from the heterotrophic portion of the community. Pheopigments are the primary degradation products of chlorophylls and may result from stressed algal cells and/or from heavy grazing pressure. Hallegraeff (1981) found high pheopigment concentrations associated with large numbers of zooplankton grazers and hypothesized that phytoplankton chlorophyll was converted to pheopigment when acidified by the digestive systems of the zooplankters.

This discussion will define microbes as organisms less than 200 um in greatest dimension including bacteria, fungi, yeasts, acellular and multicellular algae and protozoans, and metazoans. Environmental stress is considered as any toxin, alteration, or nutrient enrichment incurred from anthropomorphic activities that alters a community's natural structure or function.

Few, if any, studies have examined these three nontaxonomic indicators simultaneously and compared them to the more traditional measures of species diversity and species richness under lotic field conditions and microcosm simulation tests. Through this research a better understanding of relationships of community and ecosystem responses to stress and the predictive capabilities of microbial microcosm studies may be gained.

CHAPTER II

GENERAL METHODS

ARTIFICIAL SUBSTRATES

The use of artificial substrates for the collection of microbial communities is widely accepted (Cairns, 1982; Weitzel, et al. 1979). Artificial substrates are colonized in a fashion similar to natural substrates and aid the collection of replicable and comparable samples within and between treatments. Artificial substrates are not all equally suitable for a given situation. Weitzel et al. (1979) found significant differences between diatomaceous species associations on replicate glass microscope slides. They concluded that the placement of glass slide samplers is critical for reliable results that are indicative of water quality and not microhabitat conditions. Important factors regulating microbial growth that must be considered are current flow, temperature, and solar incidence.

Cairns et al. (1979) found polyurethane foam units (PFUs) best suited for assessing environmental affects to complex microbial assemblages. These substrates were used in this study. Preliminary experiments utilized 5 cm x 6 cm x 7.7 cm PFUs while latter experiments had 5 cm x 6 cm x 3.75 cm PFUs. The results from large and small PFUs were compared. Trends were similar but absolute concentrations of adenylates and chlorophyll were higher in the small PFUs.

The actual placement of artificial substrates in all studies is critical. Ideally each substrate is exposed to the exact physical conditions as the others. All PFUs were tied to small native rocks and positioned in slow moving pools so they remained approximately 5 cm below the water's surface. The position of an individual PFU relative to the others was determined before through random number generation or drawing numbers from a hat. This included which PFUs would be transferred from the upstream reference site to a particular downstream or laboratory treatment and its position at this new site. The specific locations of PFUs at field sites were chosen so all PFUs were exposed to similar current velocities and illumination.

In translocation experiments, the PFUs transferred to the downstream sites were always placed downstream of those PFUs already at the site. This was done to avoid introducing organisms from the reference site to downstream PFUs via the transferred communities. In these experiments the PFU communities originally downstream were compared with those transferred there. All PFUs were collected by cutting their anchor lines and gently removing from the water. They were either processed at streamside or placed in individual ziplock bags on ice in a dark container and returned to the laboratory for extraction or further experimentation. A microbial sample was obtained by squeezing the PFU over a 600 ml beaker, mixing thoroughly and then removing a subsample with an autopipette.

ADENYLATES

The use of ATP as a biomass estimate and adenylate ratios as indicators of cellular metabolic status has been widespread since introduced by Holm-Hansen and Booth (1966) and Atkinson and Walton (1967). Atkinson defined the adenylate energy charge (EC) as: (ATP + 1/2 ADP)/(ATP + ADP + AMP), and a measure of the metabolic energy available to an organism. The ratio is dimensionless and theoretically ranges from 1 (all ATP) to 0 (all AMP). This measurement has been proposed as a potential sublethal stress indicator for a variety of aquatic and marine organisms (Karl, 1980; Karl and Holm-Hansen, 1978; Ivanovici, 1980a, 1980b; Witzel, 1979; Dickson et al., 1982; Wiebe and Bancroft, 1975; and others).

Drawbacks of EC measurements are the ability of the cell to maintain a relatively constant EC over a wide range of conditions and total adenylate concentrations (Karl, 1980), and the lack of response to ATP/ADP and ATP/AMP ratios changes which are more important regulators of certain metabolic activities than the EC (Lowery et al., 1971). A large fluctuation in ATP/ADP or ATP/AMP may result in minor changes to the EC. The EC tends to mask drastic changes of ATP and/or ADP concentrations relative to AMP and variability between measurements. The nature of the EC calculation makes the influence of AMP much greater than the other two adenylates. The variability of AMP measurements is the greatest of the three due to low concentrations and the difficulties involved with its enzymatic conversion to ATP prior

to analysis (Karl and Holm-Hansen, 1978; Campbell, 1980). Most investigators use the EC as a metabolic indicator; however, several have examined the use of ATP/ADP or ATP/AMP ratios as indicative of the same (Ivanovici, 1980b; De Filippis et al., 1981; Zaroogian et al., 1982; and Lowery et al., 1971). Ivanovici (1980b) and Zaroogian et al. (1982) found good correlations between EC and ATP/ADP measurements but less precision with the ATP/ADP ratio. I have examined published data from several investigators ATP, ADP, and AMP concentrations or EC and found a significant correlation between the two ratios ($r = 0.857$, $p = 0.0001$; Figure 1 and Table 1). Correlations within studies had a mean of 0.736 and a range of 0.103 to 1.0. These data represent a variety of organisms, analytical techniques and environmental conditions. Microbial organisms have less variability about the regression than larger multicellular organisms. One advantage of the ATP/ADP ratio over EC analyses is a smaller resource requirement. ATP and ADP measurements take about the same time as AMP measurements alone and use fewer and lower quantities of enzymes. In light of these considerations and the significant correlation between ATP/ADP:EC, ATP/ADP ratios were used for this study rather than EC measurements.

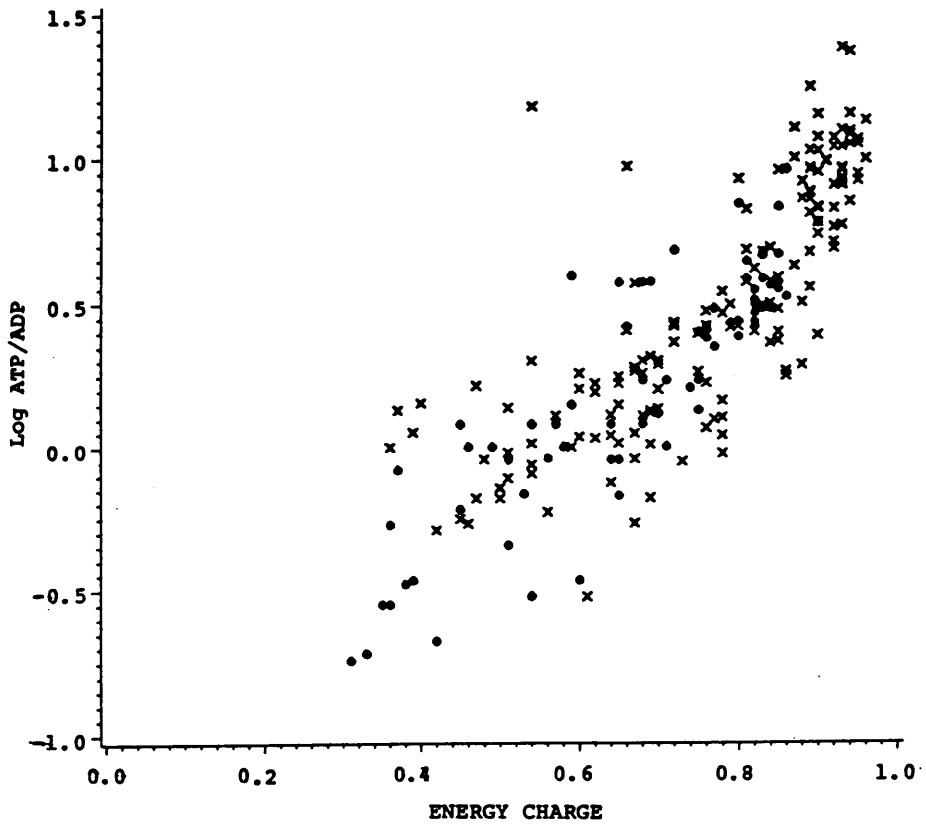


Figure 1. Log_{10} ATP/ADP versus energy charge, • indicates microbial studies (N = 76); X indicates studies of larger multicellular organisms (N = 162).

Table 1. References used in Figure 1.

Reference	Organism
Microbial Studies	
Dietzler et al., 1974	*E. coli*
Harrison, 1976	bacteria
Knowles, 1977	microbes
Montague and Dawes, 1974	bacteria
Bornefeld and Weis, 1981	bacteria
Upchurch and Mortenson, 1980	bacteria
Witzel, 1979	microbes
Multicellular Studies	
Ballantine et al., 1978	nematodes
Ching, 1976	soybeans
Dickson and Giesy, 1982	crayfish
Dickson et al., 1982	crayfish
Giesy and Dickson, 1981	clams
Ivanovici, 1980b	molluscs
Karl et al., 1978	metazoa
Skjoldal and Bamstedt, 1977	zooplankton
Vetter and Hodson, 1982	fish
Zaroogian et al., 1982	oysters

The utilization of ATP as a biomass indicator and adenylate ratios as indicators of metabolic status rely on several assumptions described by Holm-Hansen (1973), Karl (1980), and Campbell (1980):

1. adenylates are synthesized only by living organisms and are ubiquitous in all organisms
2. the ratio of ATP to biomass is relatively constant and predictable
3. cellular levels of ATP do not change drastically with normal changes in environmental conditions
4. energy-containing adenylates (ATP and ADP) are rapidly degraded when life ceases
5. interferences from non-cellular (or from dead cells) adenylates are not significant
6. stressful environmental conditions induce a change in the relative concentrations of adenylates in the cell
7. adenylates can be quantitatively extracted and measured.

These assumptions are approached in some laboratory cultures, but natural systems may pose some problems (Karl, 1980; Campbell, 1980).

The ubiquity of adenylates is accepted (Lehninger, 1965); however, it is possible to produce adenylates abiologically from some precursors through ultraviolet irradiation (Ponnamperuma et al., 1963) and under conditions thought to be present in the primitive atmosphere (Oro, 1960). Abiotic production of adenylates should be negligible in most naturally occurring environments. Under normal environmental conditions, cellular

ATP concentrations are relatively stable and predictable and do not fluctuate significantly with changing conditions (Holm-Hansen, 1970, 1973; Paerl and Williams, 1976). This has been established for several types of organisms but should not be universally applied to all organisms (Karl, 1980).

The assumption that adenylates are rapidly degraded at death is questionable. Hodson and Azam (1977) and Riemann (1979) have found appreciable amounts of dissolved ATP in sea and fresh waters. Adenylates are probably not secreted by living cells but are thought to be released by feeding predators (Karl, 1980). This release may interfere with adenylate estimates under certain conditions, e.g., high productivity.

Normal fluctuations in environmental conditions do not significantly affect adenylate ratios; however, drastic or stressful conditions do (Karl, 1980; Ivanovici, 1980a). This is the rationale for using the ratios as stress indicators. Adenylates can be extracted and measured quantitatively (Karl, 1980; Holm-Hansen, 1969; Campbell, 1980; and others) but appropriate methods must be rigorously followed. For example, concentrating a sample by filtration may stress the organisms and shift adenylate ratios, or adenylates may be lost by rupturing delicate organisms (Jones and Simon, 1977), while direct injection of a sample will include dissolved adenylates (Karl, 1980). Precautions must also be made to insure complete extraction and to prevent the back conversion of AMP to ADP and ATP during the analysis (Karl and Holm-Hansen, 1978).

Despite the questionable validity of all assumptions and precautions necessary to establish reproducible analytical techniques, the use of ATP is the most convenient and reliable method for estimating total microbial biomass (Karl, 1980). Karl also describes the use of adenylate ratios as having great potential as metabolic indicators especially for assessing sub-lethal effects of toxins to organisms.

In this study adenylates were extracted with boiling TRIS and assayed with the luciferin-luciferase bioluminescence technique. These methods are modifications of those used by Holm-Hansen and Booth (1966), Karl and Holm-Hansen (1978), and Matthews (1981) and were developed from several preliminary investigations with Cedar Run samples (described later). Successful extraction requires rapid cell death, lysis, complete nucleotide release, complete and irreversible inactivation of enzyme activity, and long-term stability of the extracted nucleotides (Karl, 1980).

TRIS was prepared by diluting 0.602 g $MgSO_4$, 0.731 g EDTA, and 2.422 g trisma base (Sigma T1503) to 1.0 L with distilled deionized (DIDW) water. This was adjusted to a pH of 7.70 with HCl and autoclaved. The buffered TRIS solution should maintain a relatively constant pH. TRIS was pipetted into 50 ml test tubes, covered with aluminum foil and stored at $4^{o}C$ until extractions were initiated (usually within 24 h).

Shortly before sample collection, several sealed test tubes of TRIS were placed in beakers of water and brought to a boil

on a hot plate. Immediately after squeezing the PFU over a 600-ml beaker and thoroughly mixing the sample a 1.0 ml subsample was transferred with an autopipette into a test tube of boiling TRIS in the water bath. After three subsamples/PFU were injected, each into a separate test tube, the extraction tubes were removed momentarily and mixed with a vortex mixer. This resuspended the sample and rinsed down any material clinging to the sides of the test tube. After five min. in the extraction bath, the samples were remixed, placed in an ice bath to cool then sealed with parafilm and frozen at -20°C until they could be assayed. It is imperative that the TRIS temperature remain near boiling for efficient and reproducible extractions. This was accomplished by sealing the test tubes with aluminum foil and piercing a small hole in the foil with the tip of the autopipette at injection; this also minimized evaporation of the TRIS. Pre-injection TRIS temperatures were 99+°C while post-injection temperatures fell briefly to approximately 98°C and then returned to the former temperature. A direct injection method was used to minimize stress from concentration technique (Jones and Simon, 1977) and because most PFU samples had substantial adenylate concentrations and did not require further concentration.

Adenylate standards were prepared by diluting 0.100 g of desiccated ATP (Sigma A5394) or ADP (Sigma A6646) to 100.0 ml with sterile TRIS. These stock solutions were frozen in sealed autoclaved 2 ml polyethylene vials. Working standards were

prepared from the stocks in concentrations ranging from 0 to 5000 ug/L depending on the anticipated concentrations in the PFU extracts. The standards were treated exactly as the PFU extracts; that is, 1.0 ml volumes of standard solutions were injected into TRIS, mixed, extracted for 5 min. remixed, cooled, and frozen until assayed.

Extraction efficiencies were estimated by spiking PFU extracts with 1.0 ml of a standard solution. This was done in several early experiments and produced a mean extraction efficiency of approximately 90%. The addition of the spikes along with the PFU sample lowered the extraction temperatures about 5 to $10^{o}C$ for approximately 2 min. This was judged unacceptable and spiking was discontinued. Extraction efficiencies were assumed to be similar for all samples. Matthews (1981) calculated similar extraction efficiencies for a variety of samples from Cedar Run.

Each adenylate assay required two subsamples; one for ATP alone and one for ATP+ADP enzymatically converted to ATP. These were prepared as follows. Fifty ul of a TRIS solution containing $MgCl_2$ (15 mM), Na_2HPO_4 (75 mM), phosphoenolpyruvate (PEP, 0.5 mM; Sigma P7002), and pyruvate kinase (PK, 20 ug; Sigma P9136) was injected with an autosyringe into 500 ul polyethylene centrifuge tubes and capped. These tubes would be for analysis of ATP + ADP. A second set of tubes for analysis of ATP alone received 50 ul of TRIS containing the $MgCl_2$ and Na_2HPO_4 but not PEP or PK. A 200 ul portion of each PFU extract

or adenylate standard was pipetted into each of the tubes. All extracts and solutions were allowed to reach room temperature before volumetric transfer. The mixtures were incubated at 30°C for 30 min., placed in a 200°C oven for 5 min. to denature the PK and allowed to reach room temperature before assay. The $MgCl_2$ and Na_2HPO_4 solutions were adjusted to pH 7.40 units before use. Empty centrifuge tubes were sealed prior to being autoclaved and remained so throughout these steps to minimize contamination. They were opened briefly to inject enzyme mixtures and extracted samples. The samples were managed in batches of about 40 (one test tube rack at a time) to minimize incubation period differences between the first and last samples.

The luciferin-luciferase enzyme assay depends on a quantitative reaction with ATP to produce luminescence. The general reaction occurs as follows:

$$\text{ATP + reduced luciferin + } O_2 \xrightarrow{\text{luciferase}}$$

$$\text{AMP + inorganic } PO_4 + CO_2 + \text{light.}$$

The physical and chemical properties of firefly luciferase and reaction mechanism have been described by DeLuca (1976).

All adenylate assays were made with a Labline 9140 ATP Photometer. Purified firefly luciferin-luciferase (Analytical Luminescence Labs Inc., San Diego, CA) was diluted with TRIS according to the manufacturer's recommendations. The concentrated solution was either frozen in sealed 2 ml polyethylene tubes for later use or diluted further and allowed to age, then

used directly. Stock solutions of luciferase were diluted to produce background light emissions of 200 to 500 cpm (a 5 to 10x dilution).

The actual ATP assay was performed as follows: a 400 ul volume of luciferase was pipetted into a polystyrene (8 mm x 50 mm) test tube and a background luminescence reading made. Immediately after the background reading, 100 ul of extract were pipetted into the luciferase solution still in the photometer. Exactly 20 s later the luminescence of the extract mixture was recorded. Karl and Holm-Hansen (1978) found a 4:1 ratio of enzyme to sample volume provided reliable mixing. Two replicate injections were assayed from each centrifuge tube.

A 6 s integrated counting period was used. Preliminary tests comparing repetitive readings of background fluorescence of two 400 ul luciferin-luciferase samples showed lower variability with 6 s integrated counts than with peak height counts. A variance ratio test (Zar, 1974) was used to compare the variances of each technique. Integrated counting had a mean variance of 120 cpm while peak counting had a mean variance of 540 cpm ($N = 20$/sample; $p < 0.01$). Extreme care was used to inject each extract sample similarly. Repetitive injections of a standard ATP solution were used to develop an optimal and replicable injection technique. Net luminescence from replicate sample injections seldomly varied by more than 10%.

A series of 5 ATP and/or ADP standards were assayed between every 9 to 15 PFU subsamples. Two sets of standards were used

to calculate the adenylate concentrations of the samples they bracketed. Several ATP+ADP mixtures were also assayed to check for interferences between adenylates; none occurred. Linear regression correlation coefficients for standard curves were > 0.98.

The difference between background and sample lumination was used to calculate ATP concentrations. ADP concentrations were determined from the difference between the ATP and ATP+ADP concentrations. The original volumes of TRIS extracts were measured by pouring the remainder of the extract into a 25-ml burette. The volume used for analysis (usually 0.4 ml) was added to this value. The slightly different extract volumes could then be accounted for in the final calculation.

All unspecified chemicals were reagent grade from Sigma Chemical Company. All glassware was washed with phosphate-free Liqui-nox detergent (1%), rinsed at least 10 times with tap water, 10 times with distilled water, 5 times with distilled deionized water, dried, sealed with aluminum foil and autoclaved. The polystyrene photometer tubes were not autoclavable.

CHLOROPHYLL

Chlorophyll was extracted with acetone and assayed with a fluorometer (Holm-Hansen and Riemann 1978; Matthews, 1981; APHA, 1980). Fluorometry was used because of its increased sensitivity over spectrometric analyses. While the adenylates of a particular PFU sample were being extracted, 1.0 ml subsamples of PFU extract were injected directly into 9.0 ml of cold 100% reagent grade acetone. Direct injection of PFU extracts into 100% acetone was more efficient and reproducible than filtration and/or grinding with 90% acetone or dimethylsulfoxide (Matthews, 1981). The chlorophyll extracts were vigorously mixed and placed in a dark container at $4^{o}C$ for 24 h. During this time period, the samples were remixed by swirling the entire container at approximately 1, 3, and 23 h. Samples were assayed in the same order as extracted to minimize extraction time differences.

Chlorophyll *a* was measured using a Turner Designs Series 10 Fluorometer with the following filter and lamp specifications:

Emission filter 10-051 color specification 2-64
Excitation filter 10-050 color specification 5-60
Reference Filter 10-052 color specification 3-66
Lamp 10-045, blue.

Five ml of the supernate were assayed before and after acidification with 3 drops 1.45 N HCl. Matthews found this ratio kept the pH above 2.5 (H^{+} concentration below $3x10^{-3}$ M) as recommended by Riemann (1978). Acidification corrects for pheophytin *a*.

The fluorometer was calibrated with a series of pure chlorophyll a standards (APHA, 1980; Sigma C5753). Chlorophyll content of the standards was determined spectrophotometrically (Gilford 250). To maintain reproducible results at various sensitivities, the fluorometer was also calibrated with a coproporphorin standard (Matthews, 1981). The fluorometer was calibrated several times during the study; however, no instrument adjustments were necessary. Chlorophyll a and pheopigment concentrations were determined according to APHA (1980) as were the values for T and F_s used in the calculations. A value of 2.0454 for T and 1.988×10^{-4} for F_s were used.

CHEMICAL ANALYSES

Field water samples were collected in 500 ml dark polyethylene containers and stored on ice or refigerated until analyzed. Laboratory samples were placed directly in beakers and analyzed or stored in 30 ml polythelylene containers and refrigerated until analysis. Dissolved oxygen (YSI 54A, air calibrated) conductivity (YSI 32) and pH (Orion Research 399A) were measured according to APHA (1980). Hardness was measured by the EDTA titrametric method (0.1 M EDTA) and alkalinity by the potentiometric method (0.025 N H_2SO_4) described by APHA (1980). Temperature and dissolved oxygen were measured directly and the other parameters within 12 h of collection. The samples were allowed to reach room temperature before analysis.

Thirty ml volumes were preserved with 6 drops of pure HNO_3 (Baker instrument analyzed) and later analyzed with atomic flame

asborption spectrophotometry (Perkin-Elmer 460) for Ca, Cd, Cu, Fe, K, Mg, Na, Pb, and Zn (USEPA, 1983). Chloride, NO_3, NO_2, PO_4, and SO_4 were measured with a Dionex model 14-U ion chromatograph (APHA, 1985) with a 300 ul sample loop and the following columns: P/N 030827 anion separator; P/N 030825 guard; and P/N 030828 suppressor. Samples were spiked with a 50x eluant (1/50 dilution into sample) prepared from the same stock as the working eluant to remove the water dip. A combined standard was assayed after every third sample; bracketing standards were used for each set of three samples. The standards contained 0.8 mg/l F^-,4.0 mg/l Cl^-, 8.0 mg/l NO_2^-, 20.0mg/l $PO_4^{\equiv}$, 4.0 mg/l Br^-, mg/l $NO_3^{=}$, and 20.0 mg/l $SO_4^{\equiv}$ or twice these concentrations. Fluoride and bromide were not detected in any samples. Anion measurements were completed within one week of collection. Ammonia was measured with the phenate method (APHA, 1980) within 2 d of collection. The USEPA (1982) recommends unpreserved water samples of domestic sewage be stored in polyethylene bottles at 4^oC and analyzed within 3 d of collection.

SITE DESCRIPTION

The field portion of this research was conducted on Cedar Run, a small 2nd order stream in Montgomery Co., Virginia. The headwaters of the stream are located just south of Blacksburg (Figure 2). Most of the stream overlies limestone and flows through open agricultural (livestock) areas and some mixed hardwood forests. The mean current velocity is approximately 20 -

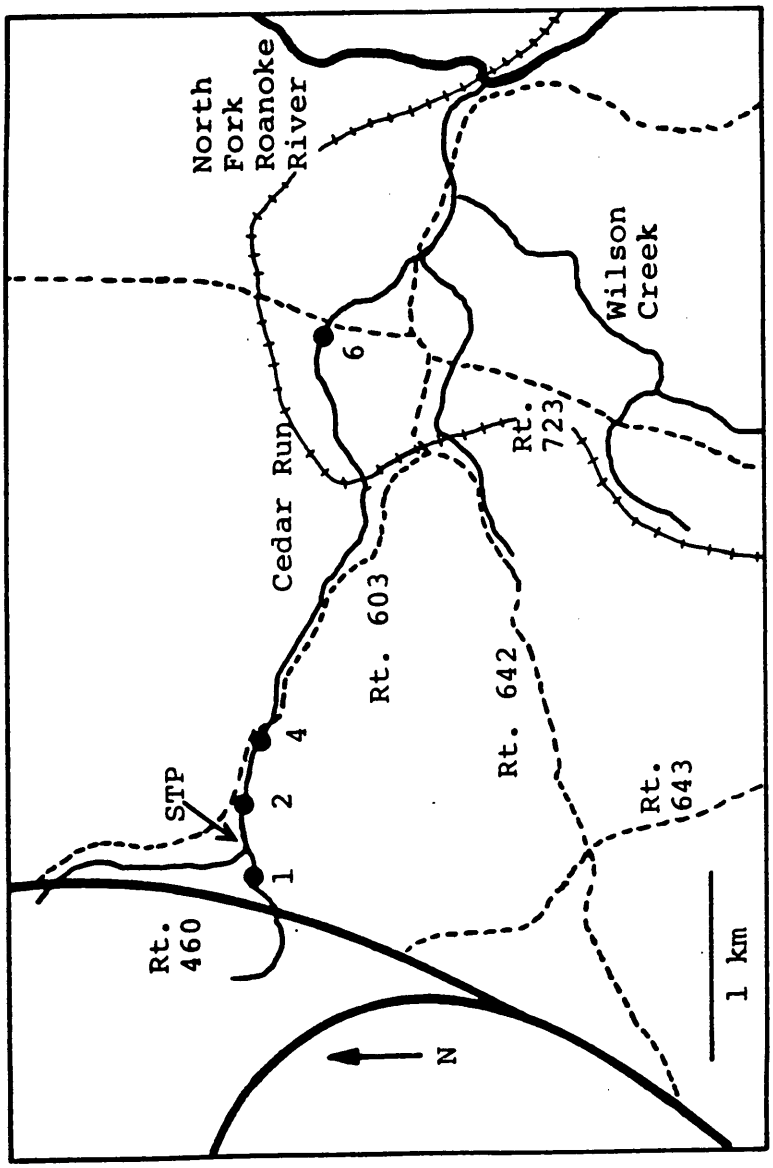


Figure 2. Cedar Run site locations, STP indicates sewage treatment plant.

35 cm/s with a mean volume discharge of 0.0025 to 0.034 m^3/s (Matthews, 1981).

During these studies the stream received approximately $1.3x10^6$ L/d ($3.5x10^5$ g/d; Blacksburg-VPI Sanitation Authority) of domestic sewage effluent from a sewage treatment plant (STP) located at the confluence of the North and South branches of the stream. The treatment plant provides secondary treatment with a trickling filter and activated sludge processing unit. Effluent chlorine residuals are approximately 1 to 1.5 mg/L. Just below the STP is an industrial effluent containing low levels of heavy metals and an approximate discharge of 0.2 to 1.1 x 10^6 L/d (0.5 to $3.0x10^5$ g/d; B. Fender, Federal Mogul, pers. comm.). Between Sites 4 and 6 the stream received some runoff from Rt 603, a railroad bed with coal accumulations, and occasional cattle activities. These effluents and runoffs provided the stress to downstream test sites.

All field sites were in or adjacent to open fields and received direct sunlight from approximately 9 am till dusk. Specific sampling sites were slow moving pools. Solar radiation in bright sunlight was about 5000 ft-c and 1200 ft-c in open shade. Openings were left for the possible addition of two more sampling sites (3 and 5); however, these were not used. Site 2 was near Matthews' Site CR3; Site 3 near Matthews' Site CR4; and Site 6 near Matthews' Site CR5. Each site is characterized in Table 2.

Table 2. Site Descriptions

Site	Elev. (m)	Stream Order	Loc.*	Width (m)	Flow (m/s)	Depth (m)	Substrate
1	604	1	0.1k above	1.5	0.15	0.15	silt, cobble
2	595	2	0.3k below	2.5	0.20	0.30	gravel
4	590	2	0.6k below	2.0	0.25	0.25	gravel, sand
6	454	2	3.0k below	3.0	0.20	0.30	gravel, sand

* Location relative to sewage treatment effluent.

LABORATORY STUDIES

Two experiments entailed transferring precolonized PFUs from the field reference site to a flow-through laboratory system which delivered different dilutions of stream water (effluent). The delivery system consisted of a headbox (100 L) where precolonized PFUs from the field reference site were maintained. This served as a source for invading organisms to the PFUs in the test chambers.

Test chambers were constructed of glass (45 cm x 29.5 cm x 15 cm) and contained 12.5 $\pm$ 0.2 L of test water. Three PFUs/chamber were anchored to plastic hooks secured with silicon caulk and remained just below the water's surface. Test water was delivered to the chambers by simultaneously pumping (peristaltic) equal volumes of headbox water and premixed dilutions of stream water.

The laboratory diluent water was prepared in 45 L volumes by adding the following salts to dechlorinated tap water: $NaHCO_3$, 300 mg/L; $MgCl_2 \cdot 6H_2O$, 250 mg/L; KCl, 12 mg/L. $CaSO_4 \cdot 2H_2O$, 180 mg/L; This mixture was based on recommendations of the USEPA (1975) for hard water that were modified to match Cedar Run water at the upstream site. $NaHCO_3$, $MgCl_2$, and KCl were added to the water as stock solutions while crystalline $CaSO_4 \cdot 2H_2O$ was dissolved during diluent preparation while it was thoroughly mixed and aerated.

Water from Site 4 was collected twice a day (90 L/trip) and stored temporarily in plastic carboys. This water was used undiluted or mixed with diluent in 1:1 or 1:3 ratios. Diluent and test water were stored in 45 L covered containers lined with plastic bags before being pumped through the system. The plastic liners were soaked for 24 h in dechlorinated tap water before use to leach any compounds that might contaminate the water.

The highest possible concentration of effluent delivered by this system is 50% due to the dilution with headbox water. The final concentrations of effluent from Site 4 in the test chambers were 0%, 12%, 25%, and 50% in the combined laboratory-field study (three test chambers/conc.) and 0%, 25%, and 50% in the pilot laboratory study (two test chambers/conc.). Water delivery occurred by premixing equal volumes of headbox and test water in 300 ml beakers resting in small plastic boxes. The mixed water overflowed the beakers and drained by gravity through capillary tubes in the bottom of the plastic boxes. The water then flowed through Tygon tubing to the test chambers. The mean delivery rate for all test chambers was 34 $\pm$ 0.4 ml/min (measured twice/exp.) for an approximate turnover rate of four times/d.

Six - 40 watt Vita lite (Durotest Corp., North Bergen, NJ) fluorescent lights were suspended 13 cm from the water's surface above each bank of three chambers. This provided 1140 $\pm$ 24 ft-c of lumination. These measurements were made at the water's

surface, three readings/chamber. A 12 h photoperiod was synchronized with the natural photoperiod by turning the lights on and off at sunrise and sunset respectively.

BIOLOGICAL ENUMERATIONS AND IDENTIFICATIONS

Algae, diatoms, protozoans and bacteria were quantified in several experiments to characterize community structure and to compare biovolumes with ATP and chlorophyll estimates. Only cells with protoplasts were considered. Rotifers and fungi were not included in these counts.

Bacteria were quantified using a method similar to Porter and Feig (1980). The compound 4'6-diamindino-2-phenylindole (DAPI) is a highly specific fluorescing stain for DNA. When excited with 365 nm wavelength light the DNA-DAPI complex fluoresces bright blue while DAPI bound to non-DNA material and unbound DAPI fluoresces yellow. This allows bacteria to be easily distinguished from detrital material.

A sample was preserved for analysis by pipetting a 0.5 ml sample from each thoroughly mixed PFU extract into 9.5 ml of 2% formaldehyde while adenylates were extracted. The preserved sample can be examined up to six months later with no ill effects (Porter and Feig, 1980).

DAPI staining was carried out as follows. All water was distilled deionized water (DIDW) autoclaved as was all glassware. Nucleopore filters (NP, 0.2 um, SN: 110606) were presoaked at least 10 min. in Irgalan black solution (Baker, 2 g/L plus 20 ml of acetic acid). This provides a dark background

which improves the visibility of fluorescing cells. The NP filter was rinsed in two baths of DIDW, placed on a wetted glass fiber backing filter and clamped into a glass suction filtration unit. A sample was vigorously shaken before a 3.0 ml subsample was pipetted onto the NP filter, along with 0.7 ml of DIDW and 0.1 ml DAPI (0.01 ug/ml; Sigma D1388). This solution was mixed gently and allowed to stand 5 min. The solution was then filtered under a 5 psi vacuum. The vacuum was released and 2.0 ml of DIDW pipetted onto the NP filter and gently mixed. The sample was filtered again at 5 psi and then the filter was air dried. A thin layer of low fluorescing immersion oil (Cargille type B) was applied to an acetone rinsed microscope slide. The dry NP filter was placed on the oil and another small drop of oil placed over the filter followed by a # 1 coverslip.

The prepared slide was examined under oil immersion (1000x) on a fluorescent microscope with a 365 nm light source. There was a problem with aggregation of bacterial cells in about 10% of the samples. To lessen the bias this could create, 10 random fields were counted but the highest and lowest counts were disregarded, leaving 8 fields/slide. Three slides/sample from each of three randomly selected PFUs/treatment were examined (9 enumerations/treatment). Biovolume conversions were carried out as described later in this section.

The remaining organisms were quantified by pipetting a 1.0 ml sample from each thoroughly mixed PFU extract into 9.0 ml of 12% Lugols solution (APHA, 1980). A stronger than normal solu-

tion was used due to high cellular concentrations. From this preservate, a 0.5 ml sample was diluted to approximately 5 ml, settled and observed with a phase contrast inverted microscope (Lund, et al. 1958). One sample/PFU from field sites (3 PFUs/site) and one sample/test chamber from laboratory treatments (3 chambers/treatment) were randomly selected for analysis.

The enumeration of microbial communities required several different magnifications to estimate all size fractions equally. Low magnifications tend to underestimate the numbers of small organisms while high magnifications tend to underestimate large organisms. To minimize these tendencies several magnifications and scanning techniques were used with each sample. Each individual taxa was counted with just one technique. The following magnifications and techniques were used, the values in parentheses were used for general characterizations for the immediate and delayed laboratory extractions and the second field translocation study.

100x - entire slide is scanned.

250x - transect near center of slide 780 um wide is scanned a minimum of 15 (10) mm

400x - minimum of 100 (50) - 190 um square random fields are counted

1000x - transect near center of slide 200 um wide is scanned a minimum of 5 mm or until 1000 organisms are counted

When low numbers of organisms were encountered longer transects were examined. Most 5 mm transect scans at 1000x included 100 to 300+ cells.

Many organisms were grouped into three taxa to lessen the difficulties involved with identifying distorted and/or ruptured individuals, separating sexual from asexual stages, and separating coccoid forms from deflagellated flagellates. These groups and their respective organisms are listed below.

Protozoans - sarcodines, ciliates, achlorophyllous flagellates and coccoids > 20 um and ruptured cells > 50 um (smaller cells could not be differentiated from detritus)

Achlorophyllous - cells < 20 um without chlorophyll, includes flagellates and coccoids

Chlorophyllous - cells < 20 um with chlorophyll

Most cells could be identified to a specific genus or classified in one of the above groups.

Biovolumes of the most common organisms were estimated by measuring the dimensions of 15 to 30 organisms/taxa from field and laboratory samples. The average measurements were converted to volumes by using geometric shapes that best fit the organisms. The volumes of field and laboratory organisms were quite similar so one volume/taxa was used for the conversions. Table 3 lists the magnification used for enumeration, counting unit, geometric shape, and volume estimates for the most common

Table 3. Enumeration methods for dominant taxa.

Taxon	Magnification	Count Unit	Shape	Unit Vol. (um^3)
Achnanthes spp.	400	cell	rod	50
Cymbella spp.	400	cell	2 cones	650
Gomphonema spp.	400	cell	1 cone	500
Melosira varians	400	cell	rod	3500
Navicula cuspidata	100	cell	ellipsoid	83800
Navicula spp.	400	cell	ellipsoid	1800
Nitzschia spp.	400	cell	ellipsoid	1800
Surirella ovata	400	cell	ellipsoid	1000
Characium spp.	400	cell	2 cones	650
Ankistrodesmus spp.	400	cell	2 cones	80
Scenedesmus spp.	400	cell	2 cones	80
Spirogyra spp.	100	40 um seg	rod	12500
Oscillatoria spp.	400	40 um seg	rod	9000
Chlorophyllous<20 um	1000	cell	sphere	900
Achlorophyllous<20 um	1000	cell	sphere	900
Protozoans	250	cell	sphere	4000
Bacteria	1000	cell	ellipsoid	0.8

taxonomic groups. Organisms comprising less than 1% of the total count do not appear in this table but were used for diversity calculations.

The protozoans and Nitzschia spp.were difficult to characterize with an average volume. The protozoan group included sarcodines in the 10 to 30 um range, large cilliates over 100 um such as Vorticella spp. and ruptured cells. The Nitzschia group includes several common species, the most abundant size class (N. palea) was used for volume estimates. A volume slightly larger than the most abundant size class in the protozoan group was used to compensate for larger but uncommon cells.

The general formula converting counts to biovolumes/ml of PFU extract is:

$$V = \frac{A \times B \times D \times F}{C \times E}$$

where; V = biovolume/ml PFU extract

A = area of entire settling chamber

B = number of individuals counted

C = area counted

D = dilution factor (20 for bacteria, 10 for others)

E = volume filtered (0.3 ml) or settled (0.5 ml)

F = volume of individual counting unit

Occurrence data were collected as follows:

Algae - from settled samples used for enumeration

Diatoms - from cleaned samples from PFU extracts

Protozoans - from live wet mounts from PFU extracts examined shortly after collection.

Diatom samples were prepared by pouring approximately 5 ml of PFU extract into a 600-ml beaker and adding 80 ml of H_2O_2 and several crystals of $K_2Cr_2O_7$. The contents were allowed to settle after digestion. The supernatant was then carefully poured off and the beaker filled with DIDW. The cleaned diatoms were allowed to resettle (24 h minimum). This rinsing-settling process was repeated until the solution was colorless. The colorless supernatant was poured off and the remaining diatom frustules saved for mounting. The diatoms were mounted by placing a drop of the diatom sample on a # 1 coverslip and allowing the water to evaporate. The coverslip was then inverted onto a drop of Hyrax (Custom Research & Development, Auburn, CA) mounting medium on a microscope slide and heated at 300° C to drive off the solvent.

Taxonomic references include: Hustedt, 1930; Kudo, 1966; Patrick and Reimer, 1966, 1975; Prescott, 1962; Smith, 1950; Tiffany and Britton, 1952; and Van Heurck, 1880 - 1885.

STATISTICAL ANALYSES

Treatment differences (sites or techniques) were analyzed with one-way ANOVAs using the SAS 1982 package at VPI&SU (Statistical Consulting Facilities, VPI&SU). Error associated with PFUs and replicate measurements of PFUs was pooled together as sampling error. This was used as the error term for F-ratio tests. A significance level of a = 0.05 was used for multiple comparisons (Student-Newman-Keuls test; Zar, 1974) while specific p-values are provided for two-way comparisons.

Biological data (taxa occurrence) were characterized through multivariate cluster analyses (Pielou, 1984) utilizing Pinkham and Pearson's coefficient of association B (Pinkham and Pearson, 1976). This coefficient is defined as:

$$B = 1/k \sum \frac{\text{Min}(X_{ia}, X_{ib})}{\text{Max}(X_{ia}, X_{ib})}$$

where; X_{ia} = number (or presence) of individuals in the ith taxon for sample a

X_{ib} = number (or presence) of individuals in the ith taxon of sample b

K = number of comparisons or different taxa in the two samples.

This coefficient of similarity accounts for the occurrence of individuals in a taxa and also maintains the integrity of each taxon. This pairwise comparison is repeated for all possible

combinations. The B values are then clustered in dendrogram fashion according to Pielou (1984). With occurrence data, mutual absence of a taxon in two samples is ignored. This is commonly done with this type of data because it is likely that rare taxa are overlooked in one or more samples. These analyses were conducted with the computer program of Bonham-Carter (1972) as modified by Pinkham, et al.

Shannon-Wiener (H) diversity and relative diversity (J) indices (Brower and Zar, 1977) were determined from count data with the following formulae:

$$H = -\Sigma\, p_i \log_{10} p_i$$

$$J = H/H_{max}$$

$$H_{max} = \log_{10} s$$

where; p_i = the proportion of the total number of individuals occurring in taxon i

s = the total number of taxa in the sample.

This index was used to compare the relative abundance of organisms occurring in the combined laboratory and field study treatments. A brief summary of each experiment and initiation date is presented in Table 4.

Table 4. Summary of experiments.

Experiment	Date Initiated	Tests
Acid and TRIS extractions	9-23-83	Nontaxonomic
Field and laboratory extractions, Site 2	3-18-84	Nontaxonomic
TRIS extraction volumes	4-24-84	Nontaxonomic
Field and laboratory extractions, Sites 1 and 4	10-17-84	Nontaxonomic
Immediate and delayed laboratory extractions	12-7-84	Nontaxonomic and taxonomic
Timed field translocation	7-19-84	Nontaxonomic
Field translocation	9-18-84	Nontaxonomic and taxonomic
Pilot laboratory	8-20-84	Nontaxonomic
Combined field and laboratory	11-14-84	Nontaxonomic and taxonomic

CHAPTER III

EXTRACTION STUDIES

ACID AND TRIS EXTRACTION

A problem that may occur when using adenylates as biomass and stress indicators is possible shifts in adenylate concentrations due to handling and extraction techniques (Karl, 1980; Campbell, 1980). The purpose of this experiment was to test the hypothesis that different adenylate extraction techniques yield similar ATP concentrations and ATP/ADP ratios. There are several generalized techniques available for nucleotide extractions. These are: extraction with organic solvents at or above room temperature; acid extraction at or near $0^{o}C$; and extraction with boiling buffered solutions. Karl (1980) includes a summary of studies comparing extraction efficiencies of various techniques with a range of sample types. However, no single extraction procedure is universally applicable to all sample types. Two commonly employed techniques were compared for use with Cedar Run PFU samples; cold acid extraction (Karl and LaRock, 1975) and boiling TRIS (tris(hydroxymethyl) aminomethane hydrochloride, Holm-Hansen and Booth, 1966; Karl and Holm-Hansen, 1978).

METHODS

Three PFUs were placed at Site 2 for 3 d. The PFUs were collected and squeezed over 600-ml beakers in the field. Four 1.0 ml samples from each PFU were injected into 4.0 ml of cold

0.6 N H_2SO_4, thoroughly mixed and placed on ice. After 10 min., one of the four samples from each PFU received a 1.0 ml spike of 100 ng/ml ATP, the other three samples received 1.0 ml of TRIS to correct for the TRIS in the spike. These spikes were used to estimate recovery efficiencies. Six drops of 10 N NaOH and 0.7 ml of 0.048 M EDTA were added to each extract. The PFU extracts in the beakers were then transported to the laboratory on ice in a dark ice chest.

In the laboratory, the acid extraction procedure was repeated and TRIS extractions were conducted as described in the general methods section except the samples were not vortexed during extraction. Three replicates and a spike were extracted from each PFU for each of the three techniques (acid-field, acid-lab., and TRIS-lab.) The acid extracts were adjusted to a final pH of 7.7 with NaOH. All extracts were frozen until assayed. After assay all extract volumes were standardized as previously described. Extract differences were tested with a one-way ANOVA using PFU(EXT) (PFUs nested within extraction technique) as the error term in F-ratios.

RESULTS

The summarized results are presented in Table 5. Boiling TRIS extractions yielded higher ATP concentrations, ATP/ADP ratios, and extraction efficiencies than cold acid extractions. There was no difference between field and laboratory acid extraction procedures. TRIS extracts had the lowest mean coefficient of variation (C.V. = std. dev./mean) for the four variables.

DISCUSSION

The boiling TRIS extraction procedure appears to be the least variable and most efficient technique. The higher variances of acid extracts may be associated with adjusting the small volumes to pH 7.7. Acid extracts must be pH adjusted individually after extraction, while the TRIS buffer is pH adjusted prior to use in the actual extractions. The luciferin-luciferase reaction is very sensitive to pH (Seliger and McElroy, 1960).

There appears to be no shift of ATP to ADP in acid extracted adenylates due to the delay between field and laboratory; however, this experiment is not cogent due to the high variability of the acid extracts. Acid extraction may cause an adenylate shift from ATP to ADP relative to TRIS extraction. This is suggested by lower ADP/ADP ratios and higher ADP estimates with acid extractions.

Table 5. Mean adenylate values from TRIS and acid extractions, different letters indicate significant differences within each parameter (a = 0.05), * (S.D.).

Parameter	TRIS Lab.	Acid Lab.	Acid Field
ATP ug/L	87 A (34)*	35 B (17)	35 B (25)
ADP ug/L	59 A (28)	84 A (40)	94 A (52)
ATP+ADP ug/L	145 A (46)	119 A (43)	128 A (57)
ATP/ADP	1.76 A (1.09)	0.59 B (0.57)	0.48 B (0.41)
% Spiked Recovery	0.88 A (0.10)	0.81 A (0.08)	0.79 A (0.06)

All extraction procedures had relatively high C.V.s. This may be the result of slightly different (PFU) squeezing techniques or positioning in the stream for individual PFUs. Adenylate concentrations increased significantly with successive PFU samples (randomized blocked ANOVA, blocks = PFUs, $p = 0.0001$). From this it is evident that greater care must be utilized in the placement and sampling of PFUs in future experiments.

The TRIS extraction procedure was utilized in succeeding experiments. The next experiment addressed possible adenylate shifts with TRIS extractions due to the delay between field collection and laboratory extraction.

FIELD AND LABORATORY TRIS EXTRACTIONS

Handling stress may shift adenylate concentrations and interfere with measurements intended to characterize environmental stress (Jones and Simon, 1977; Karl and Holm-Hansen, 1978). These experiments were designed to assess the effect of the delay between field collection and laboratory extractions to adenylate, chlorophyll, and pheopigment concentrations. The first experiment compared field and laboratory extractions of field PFUs. The second was similar but utilized paired treatments at two different sites, and the third experiment compared immediate and delayed extractions of laboratory incubated PFUs. The latter two experiments compared the responses of two different community types to the same handling procedures.

Short term community stress should be determined by two different techniques to evaluate any effects due to handling. By utilizing the ATP/ADP ratio as the only stress indicator, stress was then defined by this measure. An alternative short term stress indicator for the entire community was sought but no reasonable technique could be found. Long term stress effects were assessed through several measures.

PAIRED FIELD AND LABORATORY EXTRACTIONS

METHODS

Six PFUs were incubated at Site 2 for 3 d. Three of the PFUs were used for field adenylate and chlorophyll extractions. The other three PFUs were placed in sealed ziplock bags on ice in the dark and extracted in the laboratory within two hours of collection. Extraction methods are described in the adenylate and chlorophyll general methods section. For both field and laboratory adenylate extractions a 1.0 ml sample was injected into 9 ml of TRIS and extracted without vortexing. Spikes were not used. A small gas camp stove was used to heat the boiling water baths in the field. Extraction differences were compared with a one-way ANOVA with PFU(EXT) as the error term for F-ratios.

RESULTS

The summarized results of the two extraction techniques are presented in Table 6. ADP concentrations and, therefore ATP/ADP ratios differed between techniques; the other measurements did

Table 6. Mean adenylate and chlorophyll values from field and laboratory extractions, * (S.D.), ** mean coefficient of variation (C.V.) for each extraction technique.

Parameter	Field	Lab.	p-value
ATP ug/L	19 (7)*	22 (4)	0.432
ADP ug/L	20 (9)	8 (5)	0.040
ATP+ADP ug/L	39 (13)	30 (6)	0.231
ATP/ADP	1.08 (0.59)	3.66 (2.88)	0.010
ATP/Chl a	65 (23)	83 (18)	0.169
Chl a mg/L	0.31 (0.12)	0.27 (0.04)	0.624
Pheo mg/L	1.06 (0.35)	1.12 (0.27)	0.855
Chl a+Pheo mg/L	1.38 (0.45)	1.39 (0.29)	0.965
Chl a/Pheo	0.29 (0.06)	0.25 (0.05)	0.218
Mean C.V.**	36.8	30.1	

not. Variability was quite high and thus limited the probability of detecting differences. TRIS temperatures may drop when the sample is injected due to the relatively small 9 ml extraction volumes. This may be the cause of both the variability and the observed adenylate differences.

COMPARISON OF TWO EXTRACTION RATIO VOLUMES

The high variability associated with previous TRIS extractions may have been due to temperature depressions at sample injection. High extraction temperatures are necessary to ensure quick and complete adenylate extractions. The purpose of this experiment was to compare the variability of 1:9 ml to 1:20 ml sample to TRIS ratios.

METHODS

A series of ATP and ADP standards (0, 10, 50, 100, and 200 ug/l) were prepared and stored on ice to simulate iced PFU samples. One ml volumes of the standards were injected into 9 ml and 20 ml portions of hot TRIS and extracted for five minutes without vortexing. Adenylate assays were conducted as described earlier. The standard deviation (S.D.) about the regression line for each set of adenylate standards and volume ratio was used to determine which method had the lowest variability.

RESULTS

Standard curves of adenylate concentrations versus luminescence for both extraction ratios were compared. Extraction ratios of 1:20 resulted in lower standard deviations about the regression lines and higher regression coefficients than 1:9 ml

ratios. Results of linear regressions are presented in Table 7. Post-injection temperatures dropped to approximately 90°C in the 9 ml TRIS extractions; while 20 ml volumes dropped to approximately 95°C and recovered more quickly. These results supported the interpretation of the first field and laboratory extraction experiment and indicated the need for a follow up study using 20 ml extraction volumes.

FIELD AND LABORATORY EXTRACTIONS AT SITES ONE AND FOUR

This experiment was designed to test three hypotheses: 1) field and laboratory extractions are similar when 1:20 ml extraction ratios are used; 2) upstream and downstream communities respond similarly to the delay between field collection and laboratory extraction; and 3) upstream and downstream communities have similar adenylate and chlorophyll concentrations.

METHODS

Three PFUs were incubated at both Site 1 and Site 4 for 3 d before analyses. Variation due to PFUs within extraction technique was limited by cutting each PFU in half with scissors in the field; one half extracted immediately at the field site, the other half extracted in the laboratory. A small portion of the PFU sample was lost while cutting the PFU in half. Methods were the same as for the first field-laboratory extraction study except 20 ml of TRIS were used and the extractions mixed during the procedure as described in the adenylate methods section.

Table 7. Regression coefficients from linear regression analysis of standard curves (r^2) and standard deviations about the regression lines (S.D.) for 1:9 and 1:20 ml extraction volume ratios.

	1:9 ml		1:20 ml	
	r^2	S.D.	r^2	S.D.
ATP	0.849	88	0.951	26
ADP	0.912	543	0.964	19

Extraction techniques were compared within sites with blocked ANOVAs utilizing PFUs as blocks. F-tests used PFUxEXT variability as the error term. Site differences were compared by grouping extraction techniques within sites and using PFU(SITE*EXT) variability as the error term for F-tests.

RESULTS

Chemical and physical measurements are presented in Table 8. All cations and anions (except Mg and Ca) were higher at Site 4. Temperature and conductivity were higher at Site 4 while dissolved oxygen, pH, alkalinity, and hardness were higher at Site 1.

Adenylate and chlorophyll results are presented in Table 9. Laboratory extractions yielded significantly higher ATP and ATP+ADP relative to field extractions at Site 1. Adenylate concentrations were also higher in laboratory extractions from Site 4 but not significantly. The ATP/ADP ratios of laboratory extractions at Site 4 were higher than field extractions. The remaining measurements were similar between both extraction techniques within sites. Maintaining high field extraction temperatures was difficult as in the previous experiment.

Differences between the two sites are apparent (Table 10). Site 4 had higher adenylate, ATP/Chla, and pheopigment values, slightly higher ATP/ADP ratios and lower Chla/Pheo ratios than Site 1.

Table 8. Mean physical and chemical parameters at Sites 1 and 4 from field and laboratory extraction study, * (S.D.).

Parameter	Site 1	Site 4	Parameter	Site 1	Site 4
Temp. (^{o}C)	17.5	20.0	NH4 (mg/L)	0.3	4.0
	-	-		(0.2)	(0.9)
D.O. (mg/L)	10.2	8.8	Ca "	46.6	19.8
	-	-		(1.0)	(2.6)
pH	8.06	7.66	Mg "	20.1	7.8
	(0.03)*	(0.05)		(1.9)	(1.6)
Cond. (us)	580	1487	Na "	29.0	198.0
	(5)	(6)		(1)	(22)
Alk. ($CaCO_3$ mg/L)	230	163	K "	5.8	9.2
	(2)	(2)		(2.7)	(1.4)
Hard. "	300	100	Pb "	0.0	0.0
	(10)	(5)		-	-
Cl (mg/L)	73.4	181.9	Cd "	0.0	0.0
	(9)	(13)		-	-
PO4 "	0.3	13.9	Ni "	0.0	tr
	(0.5)	(1.7)		-	-
NO3 "	2.7	51.1	Cu "	0.0	tr
	(1.1)	(2.0)		-	-
SO4 "	31.0	160	Zn "	0.0	0.06
	(2)	(1)		-	(0.01)
NO2 "	0.4	tr	Fe "	0.08	0.18
	(0.1)	-		(0.01)	(0.07)

Table 9. Mean adenylate and chlorophyll values from field and laboratory extractions; * (standard deviation); ** mean coefficient of variation (C.V.) for each technique.

	UPSTREAM SITE 1		
	Field	Lab.	p-value
ATP ug/L	100 (22)*	153 (20)	0.024
ADP ug/L	88 (17)	141 (22)	0.086
ATP+ADP ug/L	188 (27)	295 (30)	0.010
ATP/ADP	1.18 (0.41)	1.27 (0.29)	0.842
ATP/Chl a	100 (42)	77 (11)	0.427
Chl a mg/L	1.08 (0.30)	1.99 (0.21)	0.090
Pheo mg/L	0.35 (0.09)	0.54 (0.18)	0.354
Chl+Pheo mg/L	1.43 (0.34)	2.54 (0.37)	0.133
Chl a/Pheo	3.17 (1.01)	3.97 (0.96)	0.386
Mean C.V.**	26.8	17.8	

Table 9. (cont.)

	DOWNSTREAM SITE 4		
	Field	Lab.	p-value
ATP ug/L	463 (194)	700 (376)	0.432
ADP ug/L	335 (82)	409 (220)	0.588
ATP+ADP ug/L	797 (258)	1109 (574)	0.476
ATP/ADP	1.36 (0.44)	1.83 (0.79)	0.017
ATP/Chl a	374 (96)	517 (186)	0.235
Chl a mg/L	1.19 (0.23)	1.27 (0.27)	0.759
Pheo mg/L	1.15 (0.26)	1.47 (0.34)	0.457
Chl+Pheo mg/L	2.34 (0.49)	2.75 (0.61)	0.565
Chl a/Pheo	1.04 (0.09)	0.87 (0.04)	0.138
Mean C.V.**	25.4	34.3	

Table 10. Mean adenylate and chlorophyll values by site from extraction experiments at Sites 1 and 4; * (S.D.), ** mean coefficient of variation (C.V.) for each site.

Parameter	Site 1	Site 4	p-value
ATP ug/L	126 (34)*	582 (319)	0.013
ADP ug/L	115 (33)	372 (168)	0.005
ATP+ATP ug/L	241 (61)	953 (466)	0.010
ATP/ADP	1.15 (0.35)	1.60 (0.67)	0.056
ATP/Chl a	89 (32)	446 (163)	0.001
Chl a mg/L	1.53 (0.53)	1.23 (0.25)	0.114
Pheo mg/L	0.45 (0.17)	1.31 (0.34)	0.001
Chl a+Pheo mg/L	1.98 (0.66)	2.54 (0.58)	0.114
Chl a/Pheo	3.57 (1.05)	0.96 (0.12)	0.001
Mean C.V.**	31.5	34.3	

IMMEDIATE VERSUS DELAYED LABORATORY EXTRACTIONS

Previous field and laboratory experiments were inconclusive in determining if the delay between field collection and laboratory extraction was affecting adenylate results because actual extraction techniques differed. Although extractions were carried out as similarly as possible, it was difficult to be as meticulous in the field as in the laboratory. High TRIS temperatures, crucial for complete and replicable extractions, were especially difficult to maintain in the field because of more harsh ambient conditions. Effects due to different extraction temperatures between field and laboratory were inseparable from those attributable to the delay.

One final extraction experiment was conducted using PFU communities incubated in the laboratory system. Some PFU communities were removed from the system and extracted immediately, while others were removed and stored in bags on ice in dark before extraction to simulate the delay following field collection. Differences resulting between the two treatments should be attributable to the delay and storage on ice since variations in extraction techniques were minimized.

METHODS

Twelve PFUs were incubated for three days at field Site 1. Six colonized PFUs were transferred from the field to one laboratory test chamber receiving diluent (0% effluent), the other six to a test chamber receiving 50% effluent. The test

chambers had been used in a previous experiment and had test water flowing through them for twenty days prior to this study. After 7 d, 3 PFUs from each test chamber were extracted immediately upon removal. The remaining 6 PFUs were treated as if collected in the field, being stored on ice for about 1 1/2 h before extraction. TRIS volumes were 20 ml and the samples were vortexed during extraction. Dominant organisms were determined by microscopic examinations of one sample from each of two PFUs/concentration as described earlier.

The results were analyzed with two different statistical designs. First, a one-way ANOVA treating each combination of concentration and extraction technique as a separate treatment was used to test for differences between effluent concentrations. The mean square errors of PFUs nested within the concentration by extraction interaction (PFU(CONCxEXT)) were used as the denominators in F-ratio tests. This compared effluent concentration differences while accounting for extraction differences. No interaction between concentration and extraction technique occurred. Secondly, results were compared by examining each extraction technique within each concentration. The F-ratios of these comparisons had PFU within extraction technique as the error term. Significant differences are assumed when $p < 0.05$.

RESULTS

Extraction techniques are compared within concentration exposure in Table 11. There were no significant differences between immediate and delayed extractions with communities from 0% effluent. Delayed extractions of communities exposed to 50% effluent had significantly lower chlorophyll a concentrations. No differences occurred between the other measurements.

Effluent concentrations are compared in Table 12. The 50% effluent exposed communities had significantly higher values for every measure except Chla/Pheo and ATP/Chla, which were significantly lower than those in 0% effluent. Biovolume concentrations of the dominant organisms (excluding bacteria) and their relative abundance are presented in Figures 3 and 4 respectively. Communities in 50% effluent had total biovolumes of approximately 2.4×10^6 um^3/ml and were about 75% chlorophyllous organisms. The 0% effluent communities had total biovolumes of 4×10^5 um^3/ml and were approximately 60% chlorophyllous organisms. A list of the dominant organisms biovolumes is presented in the appendix (Table A1).

DISCUSSION

Adenylate and chlorophyll concentrations may be altered by the delay between field and laboratory and/or by differences between field and laboratory extractions. These changes are not significant when treatment (site) differences are large but may mask more subtle differences.

Table 11. Mean adenylate and chlorophyll values from immediate and delayed extractions for 0% and 50% effluent concentrations in the laboratory; * (standard deviation), ** mean coefficient of variation (C.V.) for each extraction technique.

	0% Effluent		
	Immediate	Delayed	p-value
ATP ug/L	130 (25)*	170 (29)	0.063
ADP ug/L	110 (19)	118 (33)	0.505
ATP+ADP ug/L	240 (35)	289 (41)	0.110
ATP/ADP	1.23 (0.31)	1.56 (0.53)	0.080
ATP/Chl a	398 (80)	445 (102)	0.452
Chl a mg/L	0.33 (0.05)	0.44 (0.12)	0.419
Pheo mg/L	0.41 (0.01)	0.06 (0.02)	0.368
Chl+Pheo mg/L	0.37 (0.05)	0.46 (0.13)	0.407
Chl a/Pheo	8.23 (1.84)	7.65 (2.07)	0.718
Mean C.V**	18.5	26.7	

Table 11. (cont.)

	50% Effluent		
	Immediate	Delayed	p-value
ATP ug/L	846 (232)	825 (78)	0.906
ADP ug/L	617 (275)	410 (154)	0.343
ATP+ADP ug/L	1463 (498)	1236 (161)	0.545
ATP/ADP	1.49 (0.37)	2.28 (0.83)	0.120
ATP/Chl a	261 (66)	307 (32)	0.370
Chl a mg/L	3.22 (0.18)	2.69 (0.17)	0.026
Pheo mg/L	0.68 (0.12)	0.59 (0.15)	0.426
Chl+Pheo mg/L	3.91 (0.22)	3.28 (0.29)	0.058
Chl a/Pheo	4.85 (0.92)	4.84 (1.11)	0.976
Mean C.V	22.7	19.0	

Table 12. Mean adenylate and chlorophyll values from 0% and 50% effluent concentrations in the labortory; * (S.D.), ** mean coefficient of variation (C.V.) for each effluent concentration.

Parameter	0%	50%	p-value
ATP ug/L	150 (34)*	836 (171)	<0.001
ADP ug/L	114 (27)	514 (243)	0.003
ATP+ADP ug/L	264 (45)	1350 (382)	<0.001
ATP/ADP	1.39 (0.46)	1.89 (0.75)	0.049
ATP/Chl a	421 (94)	284 (56)	0.006
Chl a mg/L	0.37 (0.09)	2.96 (0.32)	<0.001
Pheo mg/L	0.05 (0.02)	0.63 (0.15)	<0.001
Chl a+Pheo mg/L	0.42 (0.11)	3.59 (0.41)	<0.001
Chl a/Pheo	7.94 (1.96)	4.85 (1.00)	0.006
Mean C.V.**	26.0	25.6	

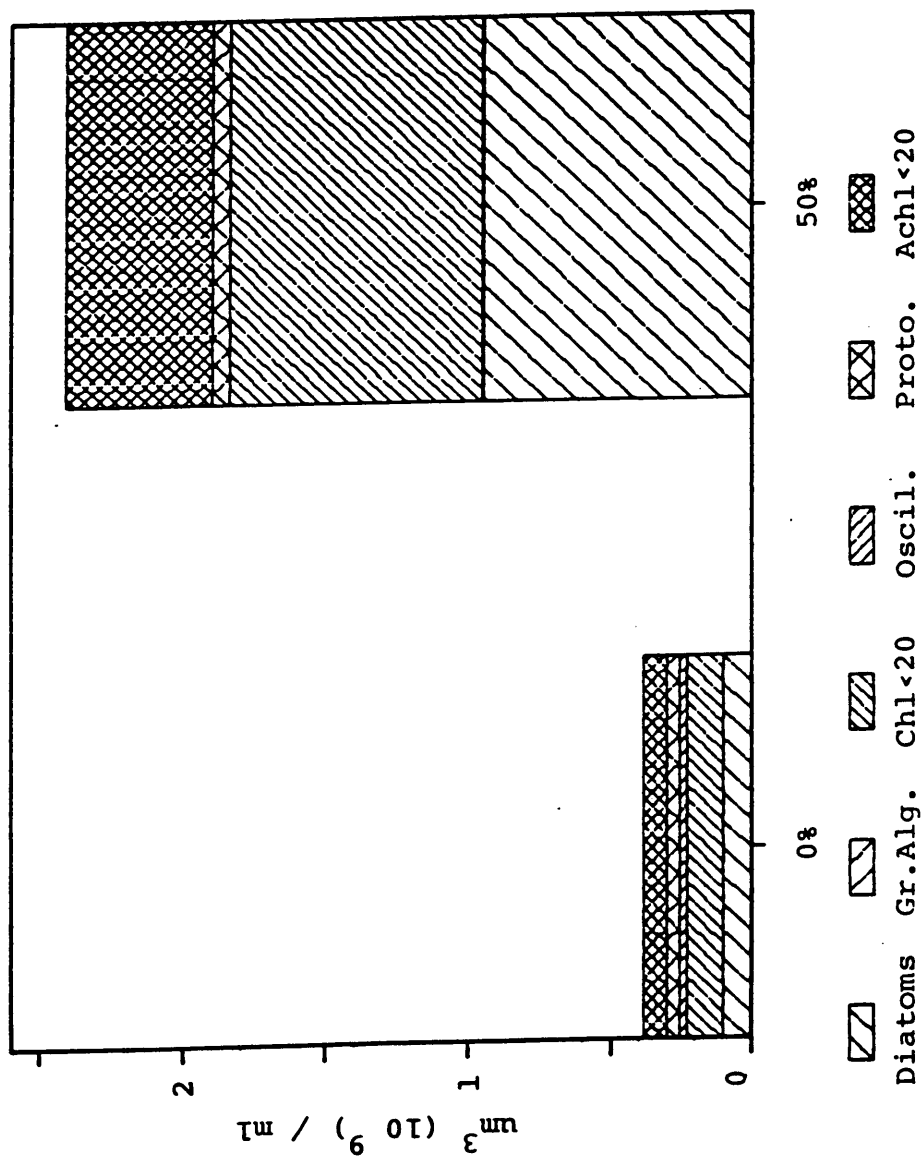


Figure 3. Biovolume concentrations per ml PFU of the dominant organisms from 0 and 50% effluent exposures. Gr.Alg. = green algae < 20um, Chl<20 = chlorophyllous org. < 20um, Oscil. = Oscillatoria, Proto. = protozoans, Ach<20 = achlorophyllous org.< 20um.

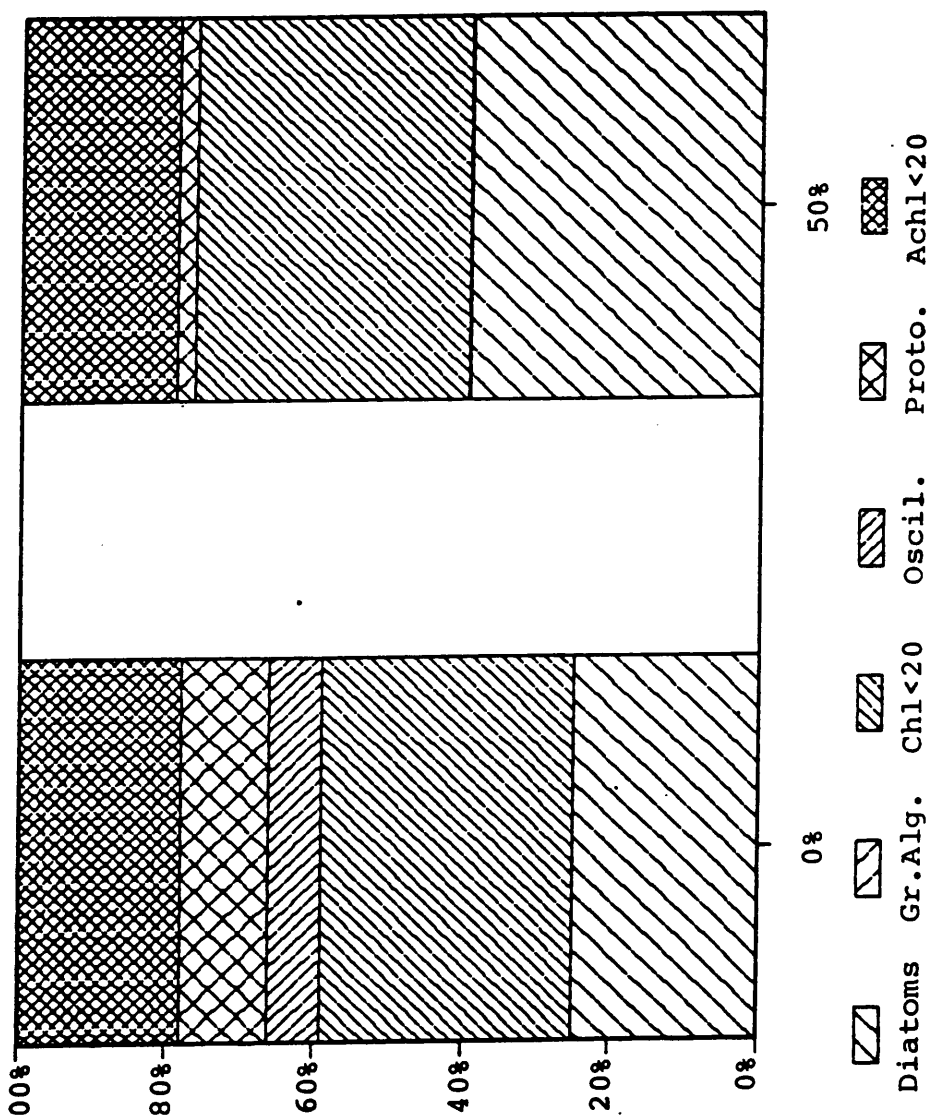


Figure 4. Relative concentrations of the dominant organisms from 0 and 50% effluent exposures. Gr.Alg. = green algae > 20um, Chl<20 = chlorophyllous org. < 20um, Oscil. = Oscillatoria, Proto. = protozoans, Ach<20 = achlorophyllous org.<20um.

In four of five comparisons, ATP/ADP ratios increased with delayed extractions ($p < 0.12$). This increase was probably due to more efficient extractions in the laboratory. Cool and windy field conditions made it difficult to maintain high extraction temperatures. Low extraction temperatures may result in incomplete adenylate extractions and/or a shift of ATP to ADP (Karl, 1980). This is supported through higher ATP recoveries and ATP/ADP ratios with laboratory extractions.

Delayed extractions did not affect chlorophyll concentrations of 0% effluent communities in the laboratory but resulted in lowered concentrations from communities exposed to 50% effluent. The different responses may be due to differences in community structures; organisms in the effluent exposed communities may be more sensitive to the delay on ice or the delay may affect extraction characteristics.

The 1:20 ml TRIS extraction ratios resulted in less variable data and are probably more efficient than 1:9 ml ratios. Vortexing the extracts during extraction seemed to increase extraction efficiencies and reduce variability (experiments 2 and 3). This was not specifically tested and could also be the result of seasonal affects.

Some comparisons should be noted between the second and third extraction experiments. Even though these experiments were conducted under different conditions some patterns are common to both. Effluent exposed communities had much higher adenylate concentrations, slightly higher ATP/ADP ratios and

lower Chla/Pheo ratios than reference communities. This suggests that effluent exposed communities have larger growth potentials overall; but algae have more chlorophyll a relative to pheopigments when not exposed to the effluent. The pigment differences are probably significant even if the chlorophyll and pheopigment estimates are in error due to chlorophyll b and pheophytin b interferences. In contrast to these similarities, field effluent exposure produced increased ATP/Chla ratios while laboratory exposure produced lower ratios relative to 0% effluent. This difference resulted from higher chlorophyll a concentrations in laboratory treatments.

The laboratory experiment was the final one of the entire study and was conducted to determine if the delay significantly alters extraction results and thus lead to inaccurate conclusions. The previous extraction experiments suggested that laboratory extractions were more practical than field for the following reasons: 1) PFUs could be collected from all sites within 20 min. minimizing diel interferences; 2) the boiling TRIS solutions could be ready upon return to the laboratory so all extractions could be completed within one to two hours after collection; and 3) higher extraction temperatures could be maintained. These help minimize within treatment variability and between treatment variability due to time and extraction efficiency differences.

Disadvantages regarding possible shifts of adenylate and pigment concentrations were outweighed by the aforementioned advantages. These shifts appear negligible when compared to treatment differences.

CHAPTER IV

FIELD TRANSLOCATION EXPERIMENTS

Previous studies have shown that upstream and downstream communities at Cedar Run have different adenylate and chlorophyll concentrations. In order to determine the predictive capabilities of microcosm simulations the *in situ* responses of translocated communities must be assessed. Two experiments were designed to determine if communities transferred from upstream to downstream respond to stress through shifts of physiological status and/or structural changes due to extinction of sensitive species and invasion of tolerant organisms indigenous to the stressed site. These experiments were concerned primarily with non-taxonomic responses.

TIMED TRANSFER STUDY

METHODS

The first experiment was designed to determine the time period necessary to detect a response in transferred communities. Sixteen PFUs were placed at Site 1 and eight at Site 2. After 3 d, six PFUs were transferred from Site 1 to Site 2. Two PFUs from Sites 1, 2, and the transferred group were collected for analysis at the time of transfer 0 h and at 5, 24, and 72 h after translocation.

To eliminate possible differences between different adenylate assay sessions that might interfere with treatment differences at a given time after transfer, all samples from

each collection time were grouped and assayed together. In all remaining experiments, adenylates were assayed randomly to minimize possible effects due to differences between analytical sessions.

RESULTS

The ATP, ATP+ADP, ATP/ADP, ATP/Chla and Chla/Pheo responses over time are presented in Figure 5. Transferred communities were noticeably different after 72 h. The Chla/Pheo ratio changed rapidly while adenylate and pigment concentrations responded more gradually. Adenylate concentrations remained relatively constant in upstream communities and increased with time in downstream and transferred communities. The ATP/ADP ratios remain stable except for a decrease in all treatment ratios at 5 h. Downstream communities maintained higher adenylate concentrations and ATP/ADP ratios than the other communities. The Chla/Pheo ratio of the transferred communities dropped sharply with transfer and remained at the low levels typical of the downstream communities.

The ATP/ADP ratio depressions at 5 h was due to uncharacteristically high ADP concentrations detected during the analytical session for this sample group. ADP levels were approximately twice those of 0 and 24 h for a given treatment; however, ATP concentrations were stable within treatments through the first 24 h after transfer. ATP concentrations should vary with ADP concentrations if this shift is indeed due to microbial responses. In light of this information, the high

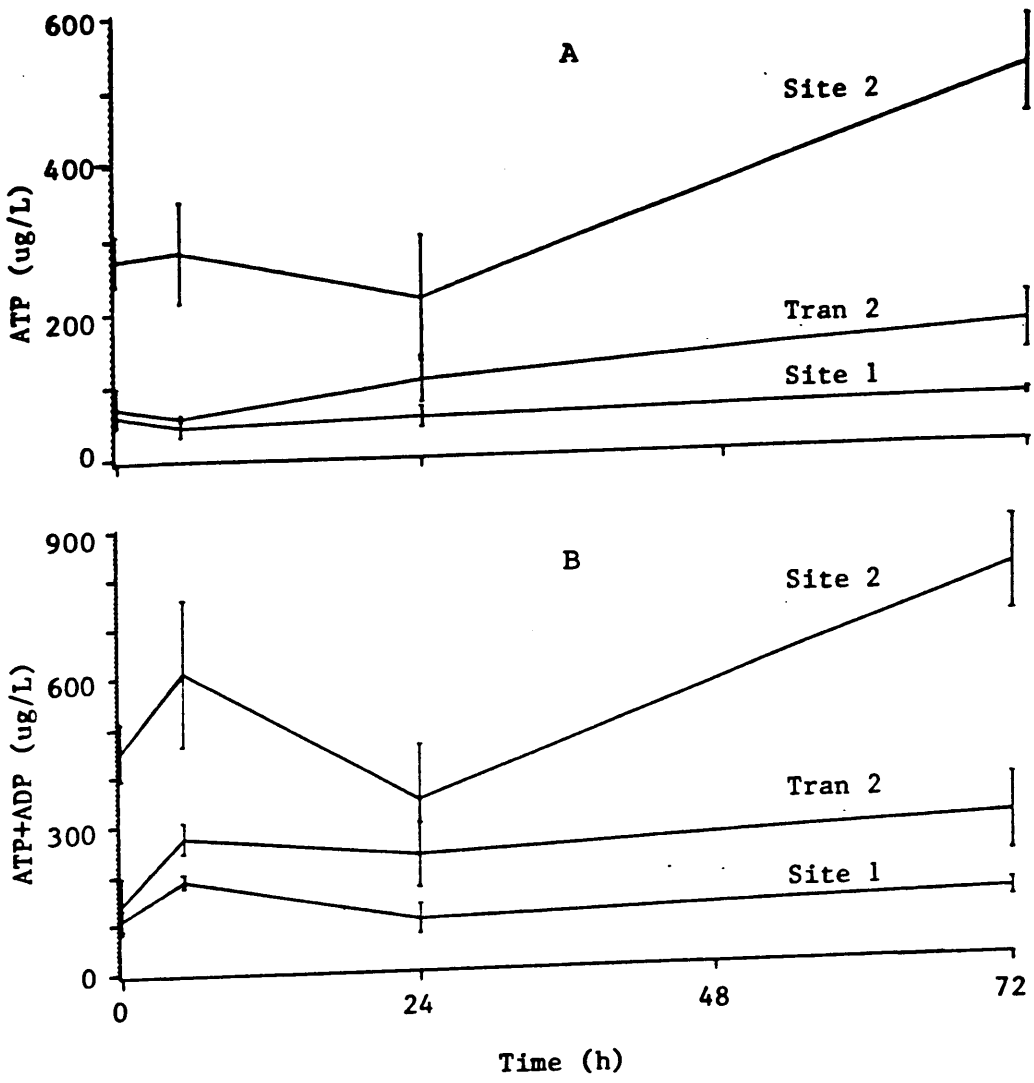


Figure 5. Adenylate and chlorophyll responses over time for Site 1 and 2 communities and transferred communities. One S.D. either side of mean indicated by bars.

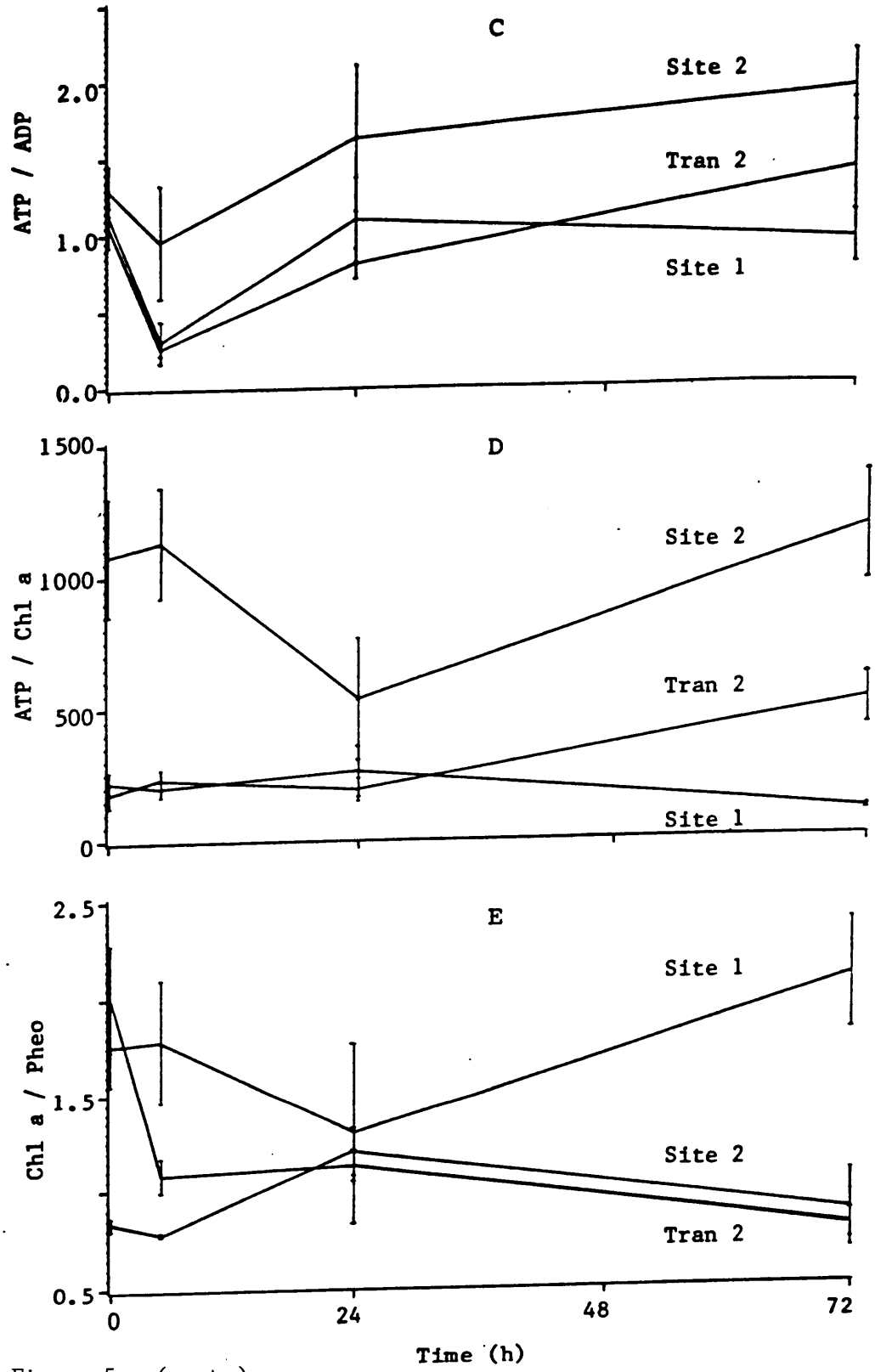


Figure 5. (cont.)

ADP concentrations may have resulted from analytical error. An examination of all remaining data sets rendered no other recurring errors of this type. Minor differences between analytical sessions would add to overall experimental variability and not bias a particular treatment because adenylate samples were assayed randomly throughout all experimental treatments.

FIELD TRANSLOCATION STUDY

METHODS

A second translocation experiment was conducted to verify the results of the timed response study. Site 4 was used as the downstream site because water could be easily collected from this site for succeeding laboratory studies. Eight PFUs were placed at Site 1 and four at Site 4 for 5 d afterwhich 4 PFUs were transferred from upstream to downstream. (Five d incubations were used because the PFUs were sparsely colonized after 3 d.) All PFUs were analyzed 3 d after the transfer. Water samples were collected for analysis on d 1, 6 and 9 d. Treatment differences were analyzed with a one-way ANOVA utilizing the PFU within treatment mean square error for F-ratio tests. Biovolumes of the dominant organisms of each community were estimated by examining one subsample from each of two PFUs/treatment. Enumeration methods were described in an earlier section.

RESULTS

Mean values of chemical and physical measurements are presented in Table 13. Nitrogen and phosphate enrichment occurred downstream, temperature increased, and conductivity, alkalinity, and hardness decreased. Copper, zinc, iron, and possibly nickle concentrations increased downstream. Treatment comparisons for adenylates and chlorophyll responses are summarized in Table 14.

Trends in this experiment were similar to those of the earlier time response study. Responses of transferred communities tended towards those of downstream reference communities. Adenylate concentrations and ATP/ADP ratios increased while chlorophyll concentrations and Chla/Pheo ratios decreased relative to upstream communities.

Biological enumerations are presented in Figure 6 with relative abundance in Figure 7. Upstream communities had the lowest total biovolume concentrations and were dominated by chlorophyllous organisms. Downstream and transferred communities had approximately twice the biovolume concentrations of upstream communities and were dominated by achlorophyllous organisms. Transferred communities and downstream communities have lower diatom biovolumes and higher biovolumes of organisms < 20 um. Total biovolume/chlorophyllous biovolume ratios follow the trend of ATP/Chla ratios. A more detailed account of the dominant organisms is presented in the appendix (Table A2).

Table 13. Mean physical and chemical parameters for translocation study Sites 1 and 4, * (S.D.).

Parameter	Site 1	Site 4	Parameter	Site 1	Site 4
Temp.(^{o}C)	18.5	20.8	NH_4 (mg/L)	<.1	3.2
	(2.6)*	(1.3)		-	(0.5)
D.O.(mg/L)	9.9	9.0	Ca "	25.7	9.0
	(0.6)	(0.4)		(9.3)	(5.8)
pH	7.59	7.50	Mg "	13.5	5.3
	(0.4)	(0.4)		(3.2)	(1.7)
Cond.(us)	637	300	Na "	36.5	216.5
	(34)	(140)		(6)	(144)
Alk.(CaCO	240	174	K "	10.7	16.1
mg/L)	(3)	(26)		(5.7)	(3.4)
Hard. "	307	192	Pb "	0.0	0.0
	(16)	(52)		-	-
Cl (mg/L)	109.2	271.8	Cd "	tr	tr
	(13)	(209)		-	-
PO_4 "	0.0	10.8	Ni "	0.0	tr
	-	(0.16)		-	-
NO_3 "	2.1	49.6	Cu "	0.0	0.10
	(1.0)	(13)		-	(0.05)
SO_4 "	33.0	116.0	Zn "	tr	0.09
	(3)	(52)		-	(0.02)
NO_2 "	0.9	3.5	Fe "	0.02	0.07
	(0.1)	(1.6)		(0.01)	(0.02)

Table 14. Mean adenylate and chlorophyll values from the field translocation study, * (standard deviations), ** mean coefficient of variation (C.V.) for each treatment, different letters indicate significant differences within each parameter (a = 0.05).

Parameter	Site 1	Trans 4	Site 4
ATP ug/L	159 A (37)*	1936 B (422)	1800 B (876)
ADP ug/L	202 A (28)	626 B (234)	745 B (391)
ATP+ADP ug/L	360 A (50)	2562 B (533)	2546 B (1232)
ATP/ADP	0.79 A (0.20)	3.56 B (1.56)	2.64 B (0.94)
ATP/Chl a	88 A (23)	1543 B (405)	2625 C (1355)
Chl a mg/L	1.84 A (0.35)	1.28 B (0.18)	0.70 C (0.06)
Pheo mg/L	0.64 A (0.18)	1.08 B (0.21)	0.74 A (0.08)
Chl a+Pheo mg/L	2.44 A (0.53)	2.36 A (0.39)	1.44 B (0.13)
Chl a/Pheo	3.27 A (0.76)	1.21 B (0.12)	0.95 B (0.06)
Mean C.V.**	21.9	23.8	30.1

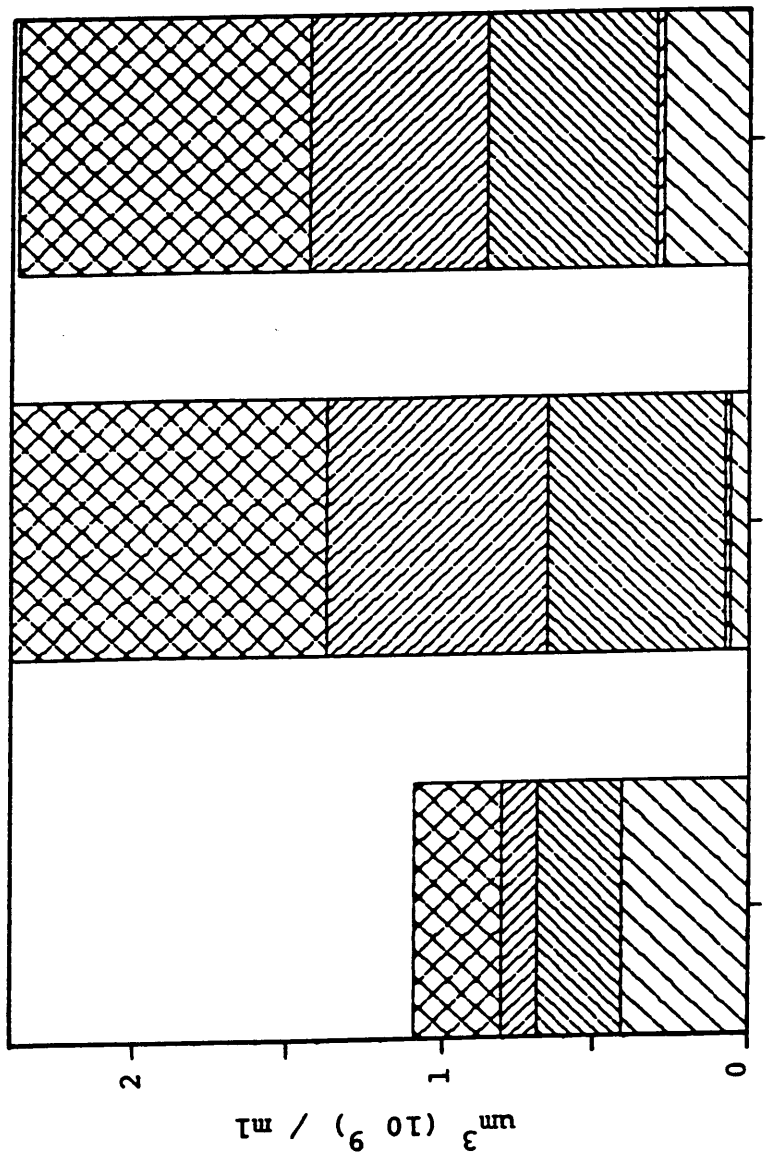


Figure 6. Biovolume concentrations per ml PFU for the dominant organisms from the translocation study. Gr.Alg. = green algae > 20um, Chl<20 = chlorophyllous org. < 20um, Proto. = protozoans, Achl<20 = achlorophyllous org. < 20um.

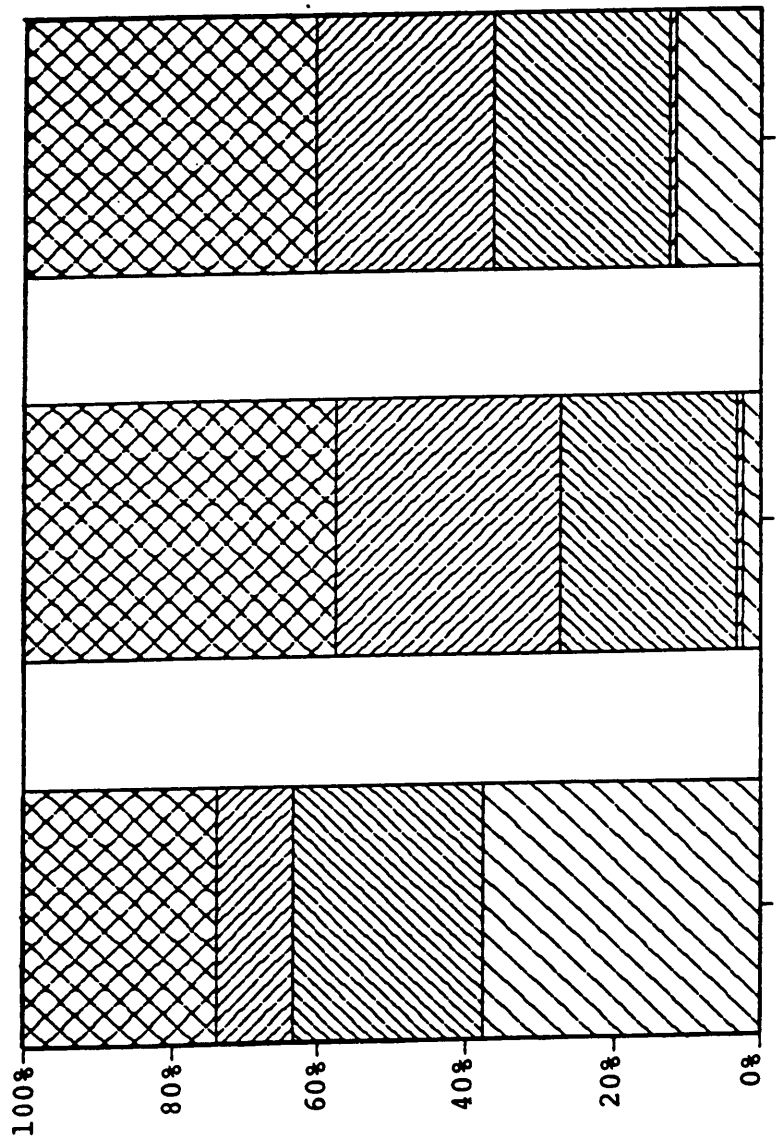


Figure 7. Relative concentrations for the dominant organisms from the translocation study. Gr.Alg. = green algae > 20um, Chl<20 = chlorophyllous org. < 20um, Proto. = protozoans, Achl<20 = achlorophyllous org. < 20um.

DISCUSSION

Upstream and downstream communities have dissimilar adenylate, chlorophyll and structural characteristics. Transferred communities lost diatoms and acquired protozoans, bacteria, and chlorophyllous and achlorophyllous organisms < 20 um. These structural characteristics are exemplified by the nontaxonomic measures. ATP/Chla ratios agree with total biovolume/chlorophyllous biovolume ratios as do ATP and total biovolumes estimates.

Chlorophyllous biovolumes are similar for all three communities yet the Chla/Pheo ratios shift. The decreased values of transferred communities may result from a combination of two factors: 1) decreasing Chla/Pheo ratios of transferred algae or loss of algae, and 2) low Chla/Pheo ratios of invading indigenous algae. Decreasing diatom biovolumes from upstream to communities transferred to downstream suggest that conditions downstream are detrimental to diatom growth. If this true the Chla/Pheo ratios of these organisms might decrease. The small indigenous algae downstream appear to be characterized by low Chla/Pheo ratios. The biovolumes of these organisms are similar in both transferred and downstream communities. The average Chla/Pheo of transferred and invading organisms may result in are intermediate value associated with transferred communities. Chlorophyll + pheopigment concentrations of transferred communities seem to be maintained by the diatom communities.

Increased ATP/ADP ratios of transferred and downstream communities suggest that the dominant indigenous organisms have large growth potentials. The responses of these abundant organisms may mask those of smaller populations with low energy reserves and low growth potentials.

Upstream responses suggested that these communities were dominated by algae with high chlorophyll a concentrations yet were nutrient-limited. High Chla/Pheo ratios indicated low levels of algal degredation products and the low ATP/ADP ratios suggested some form of stress. Chapman et al. (1971) and Karl (1980) relate low EC and ATP measurements with nutrient-limited organisms.

Transferred communities in both studies developed significant differences relative to upstream communities within 3 d. The responses were consistent and are probably due to both structural and physiological changes. The ATP/Chla ratio was a reliable nontaxonomic indicator of microbial community structure and increased with organic input. The Chla/Pheo ratio appeared to indicate the physiological condition of algal populations and decreased with environmental stress.

The ATP/Chla and Chla/Pheo ratios seem to indicate environmental perturbation, while ATP/ADP ratios reflect environmental conditions relative to the dominant organisms exposed to that environment. High adenylate ratios may indicate communities with large growth potentials, but large potentials for growth are not necessarily indicative of unperturbed environments.

CHAPTER V

LABORATORY AND FIELD STUDIES

PILOT LABORATORY STUDY

Field studies have shown that unstressed upstream and stressed downstream communities have different ATP, ATP+ADP, chlorophyll concentrations and different ATP/ADP, ATP/Chla and Chla/Pheo ratios. Communities transferred from upstream to downstream develop values similar to the downstream communities or intermediate values. The objective of this study was determining if the responses observed under field conditions could be predicted from laboratory microcosm tests.

METHODS

Eighteen PFUs were incubated at Site 1 for 72 h. They were then transferred to the laboratory and placed in a flow-through system. Six colonized PFUs were placed in the headbox, and two in each of six test chambers. Two control chambers received diluent only, two received 25% effluent and two received 50% effluent. The effluent was collected twice daily from Site 4 at mid-morning and near dusk. After 72 h, the 6 PFUs from the test chambers and two from the headbox were removed for extraction. Water samples for chemical analyses were collected on day 1 from the upstream field site when the PFUs were collected and on days 1, 2, and 3 from the laboratory test chambers. The diluent water was collected just prior to entering the headbox.

RESULTS

Mean chemical and physical measurements are listed in Table 15. The laboratory diluent was similar to the upstream reference water with the exceptions of Cl, SO_4, and hardness. Phosphate, NO_3, and NH_4 levels were highest in the 50% effluent treatment indicating nutrient enriched conditions. Analyses were not conducted on the water from Site 4 during this experiment.

Mean values and statistical comparisons of adenylates and chlorophyll measurements are presented in Table 16. Adenylate and chlorophyll concentrations increase with increasing effluent concentrations. The ATP/ADP ratio decreased slightly, but not significantly, with increasing effluent concentration. All Chla/Pheo ratios were high; however, the Chla/Pheo ratios of the communities in 50% effluent was higher and more variable than the others. ATP/Chla ratios remain constant over effluent concentrations. Variability within treatments was greater relative to field studies.

DISCUSSION

The overall community responses from effluent exposure in the laboratory were less dramatic than field responses. Adenylate concentrations increased with effluent concentration; however, this increase is due entirely to algae and not heterotrophs as in the field. In contrast to field results, chlorophyll concentrations and Chla/Pheo ratios increased with effluent levels indicating that algae became more abundant and had low levels of pheopigments when exposed to effluent in the

Table 15. Mean physical and chemical parameters for the pilot laboratory study, * (S.D.)

Parameter	Site 1	Diluent	50%	25%	0%
Temp.(^{o}C)	20.0	21.8	23.0	23.0	22.9
	-	(0.5)	(0.1)	(0.1)	(0.2)
D.O.(mg/L)	7.6	7.9	8.6	8.8	8.9
	-	(1.1)	(0.9)	(0.9)	(1)
pH	7.92	8.15	8.20	8.14	8.06
	(0.06)	(0.01)	(0.05)	(0.09)	(0.03)
Cond.(us)	765	746	731	765	816
	(6)	(19)	(20)	(15)	(6)
Alk.($CaCO_3$ mg/L)	222	180	168	157	150
	(0)	(3)	(7)	(7)	(5)
Hard. "	280	385	280	238	250
	(0)	(10)	(67)	(10)	(1)
Cl (mg/L)	69.3	10.8	46.7	34.1	11.3
	(5.1)	(0.2)	(5.2)	(10.2)	(1.0)
PO_4 "	0.0	1.0	4.1	2.3	1.1
	-	(0.1)	(0.5)	(0.6)	(<.1)
NO_3 "	4.9	2.8	27.3	15.1	4.0
	(0.1)	(1.3)	(6.2)	(3.1)	(1.8)
SO_4 "	28.9	174.9	192.9	207.5	196.7
	(0.1)	(50)	(22)	(10)	(62)
NO_2 "	tr	0.2	1.0	0.0	0.0
	-	(0.1)	(0.1)	-	-

Table 15. (cont.)

Parameter	Site 1	Diluent	50%	25%	0%
NH_4 (mg/L)	0.3	0.0	1.3	0.6	0.0
	(0.1)	-	(0.1)	(<.1)	-
Ca "	19.6	14.6	15.4	26.3	26.3
	(<.1)	(3.4)	(5.7)	(11.0)	(12.0)
Mg "	11.3	11.5	11.7	16.7	19.5
	(0.7)	(2.6)	(3.5)	(1.7)	(4.3)
Na "	46.8	45.7	63.5	55.8	43.8
	(1.1)	(5.3)	(8.0)	(2.6)	(6.7)
K "	15.7	14.6	14.0	12.9	12.9
	(2.2)	(4.4)	(3.8)	(3.8)	(2.1)
Pb "	0.0	0.0	0.0	0.0	0.0
	-	-	-	-	-
Cd "	0.0	0.0	tr	0.0	0.0
	-	-	-	-	-
Ni "	0.0	0.0	tr	tr	0.0
	-	-	-	-	-
Cu "	0.0	0.0	0.05	0.04	0.0
	-	-	(0.01)	(0.01)	-
Zn "	tr	tr	0.08	0.04	tr
	-	-	(0.02)	(0.03)	-
Fe "	0.03	tr	0.03	tr	tr
	(0.04)	-	(0.02)	-	-

Table 16. Mean adenylate and chlorophyll values from the pilot laboratory study, different letters indicate significant differences (a = 0.05), * (S.D.) ** mean coefficient of variation (C.V.) for each treatment.

Parameter	Headbox	0%	25%	50%
ATP ug/L	232 A (32)*	183 A (62)	326 AB (101)	444 B (159)
ADP ug/L	139 A (50)	119 A (69)	216 AB (91)	321 B (179)
ATP+ADP ug/L	371 A (58)	302 A (124)	541 AB (177)	765 B (322)
ATP/ADP	2.09 A (1.46)	1.76 A (0.69)	1.66 A (0.61)	1.56 A (0.53)
ATP/Chla	107 A (21)	110 A (39)	105 A (39)	117 A (38)
Chl a mg/L	2.20 AB (0.28)	1.94 A (1.05)	3.62 AB (1.94)	3.96 B (1.17)
Pheo mg/L	0.23 A (0.06)	0.41 A (0.29)	0.58 A (0.42)	0.41 A (0.35)
Chla+Pheo mg/L	2.43 A (0.31)	2.35 A (1.31)	4.19 A (2.35)	4.37 A (1.49)
Chla/Pheo	9.99 AB (2.00)	5.54 A (2.33)	7.04 AB (2.28)	16.22 B (9.82)
Mean C.V.**	25.0	51.4	44.0	45.5

laboratory. This may be due a number of factors including lower chlorine residuals and lower flow rates in the laboratory. Chlorine residuals from the STP should decrease while the effluent is stored prior to use (12 h maximum). The physical scouring effect of moving water currents in the field were also lacking under laboratory conditions.

Headbox and reference (0%) communities were compared to determine if the smaller test chambers, diluent storage or delivery equipment effected reference communities. No significant differences were detected between headbox and reference community responses. Adenylate and chlorophyll concentrations and ATP/Chla ratios of headbox and reference communities were similar to those of upstream field communities. This suggests that laboratory conditions do not adversely affect the communities over short time periods.

Results of this preliminary laboratory study did not closely predict field responses. Effluent exposures produced increased ATP/Chla and ATP/ADP ratios in the field but not in the laboratory. Several design changes were implemented in the next laboratory experiment in an attempt to enhance predictive capability.

In this study, the headbox and test communities were introduced to laboratory conditions simultaneously. This meant that PFUs transferred to test chambers were exposed to relatively barren surroundings compared to field exposures where resident communities existed. Colonization of the test chambers

may have been hindered by acclimation of headbox communities. An acclimation period of several days would allow the headbox community to stabilize under laboratory conditions and for precolonization of each test chamber with a resident community before colonized sponges from the field were incorporated. This would be a better representation of field conditions.

Low effluent exposures resulting from low maximum flow rates obtainable with the laboratory system may be compensated for by using smaller PFUs and longer exposure periods. This might increase effluent exposure to the communities by increasing circulation around the PFUs and exchange between the inner portion of the PFU and surrounding water.

Detection of treatment differences in this study was hampered by large within treatment variability and small sample sizes. Increased sampling effort should decrease within treatment variability thus increasing the probability of detecting significant differences.

COMBINED FIELD AND LABORATORY STUDY

The objective of this study was to determine if field responses of transferred microbial communities were similar to those exposed in laboratory microcosm tests. Results of the pilot laboratory study did not follow those of field studies. Several changes were made in an effort to enhance predictive capabilities. This study compared the responses of field and laboratory treatments while simultaneously exposing the same communities to the effluent. Taxonomic community analyses were

compared to non-taxonomic analyses to determine if ATP and chlorophyll measures agree with biovolume estimates.

METHODS

Several changes were made in this study based on the results of the pilot laboratory study. The lack of response in the pilot study may have been due to several reasons discussed earlier: lack of acclimation period, insufficient exchange between PFU and environment, low effluent turnover rates, low flow conditions and insufficient exposure period.

Changes made to improve this study are described below. The delivery system was operated for several days before placing transferred PFUs into the test chambers. This allowed the communities in the headbox to acclimate to laboratory conditions and the colonization of test chambers by organisms suited to each. A longer exposure period and smaller PFUs (5 cm x 6 cm x 3.75 cm) were used to increase flow around the PFUs and exchange between the interior of the PFUs and the environment.

Laboratory flow rates were lower than in the field because: 1) the laboratory delivery system limited maximum flow rates to less than 100 ml/min., which would not simulate field flow rates, and 2) logistical limitations made it impractical to prepare the volumes of diluent and effluent dilutions necessary to maintain delivery rates above 40 ml/min. Consequently, the physical effects of field flow rates could not be simulated. Laboratory temperatures could not be maintained as low as field temperatures.

Replicate water samples for chemical analyses were collected between 10:00 and 11:00 h on d 1, 4 and 7 from field sites and from each test chamber on days 10 and 15. Subsamples were examined for algal, protozoan and bacterial composition as described previously.

The manner in which PFUs were handled is described below and illustrated in Figure 8.

Day 1	Place sixteen PFUs at Site 1 three each at Sites 4 and 6
Day 2	Start effluent flow through test chambers at 0, 12, 25 and 50% concentrations, three chambers per concentration
Day 4	Transfer three PFUs from Site 1 to Site 4 and three from Site 1 to Site 6 Transfer seven PFUs to laboratory headbox Place forty PFUs at Site 1 for later transfer to laboratory
Day 7	Remove all PFUs from Sites 4 and 6 and the three day 1 PFUs remaining at Site 1 for analysis
Day 8	Transfer forty PFUs from Site 1 to laboratory system, three PFUs per test chamber and four PFUs added to headbox
Day 16	Remove all laboratory treatment PFUs for analysis

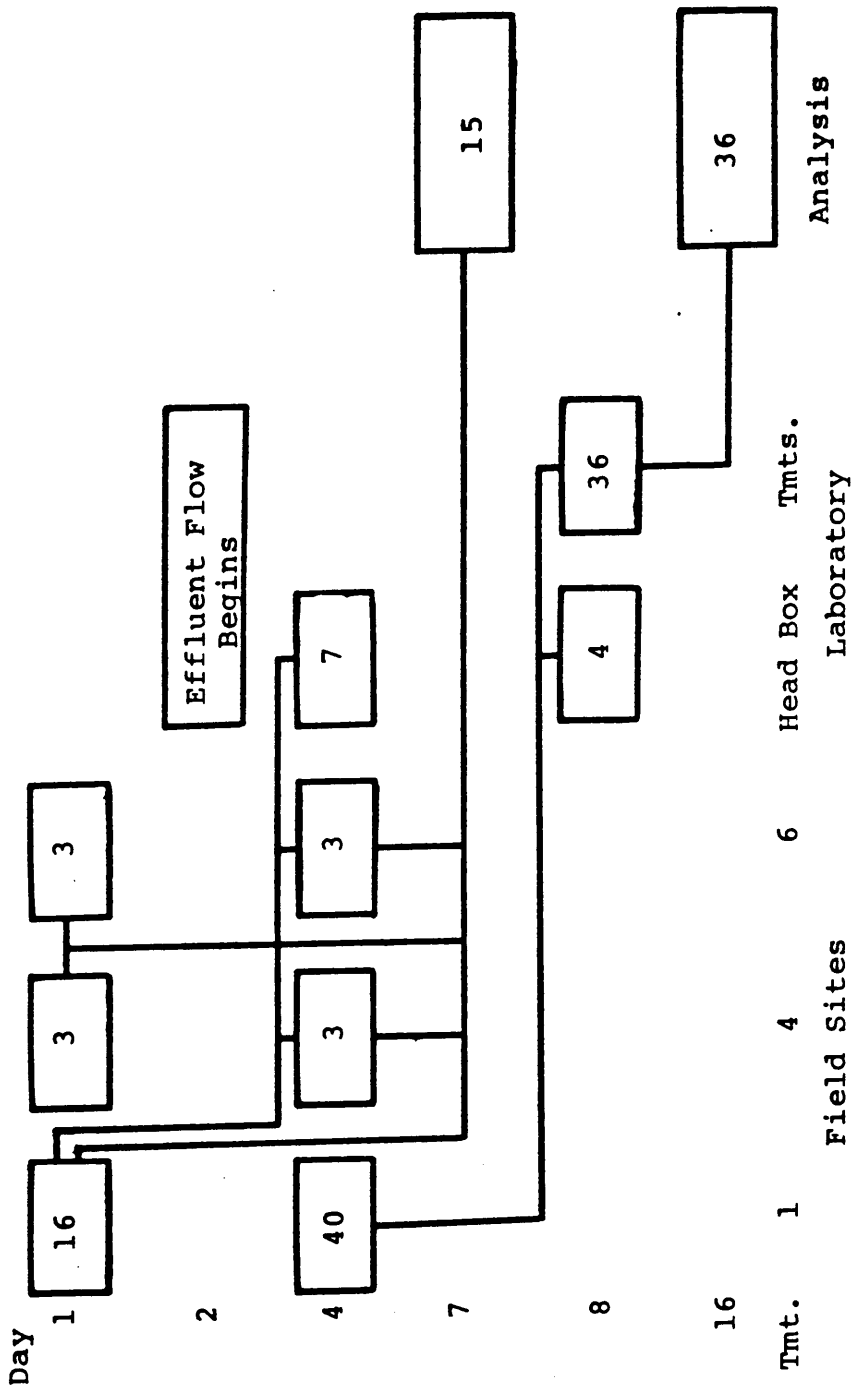


Figure 8. PFU handling scheme for combined field and laboratory study, number of PFUs handled indicated in each box, transfers indicated by lines.

RESULTS

Mean values for chemical and physical water analyses are presented in Table 17. Chloride concentrations were highest at Sites 1 and 4. Mean daily chlorine residuals released from the STP were 1.6 mg/l (pers. comm., C. Vaught, Plant Supervisor, Blacksburg-VPI-Sanitation Authority). Phosphate, nitrate, sulfate, ammonia, sodium, and metal concentrations were lowest at Site 1 and in 0% effluent test chambers, while Ca and Mg were slightly higher in these treatments. Laboratory temperatures were 5 to 13^{o} C higher than field temperatures. Dissolved oxygen and pH were higher in laboratory treatments. Conductivity, alkalinity and hardness levels were comparable between field and laboratory treatments.

Mean daily STP discharge for d 1 through 3 was $1.3x10^{6}$ 1/d ($3.3x10^{5}$ g/d) and $1.2x10^{6}$ 1/d ($3.1x10^{5}$ g/d) for d 4 through 8. The mean daily discharge for September through October was $1.4x10^{6}$ 1/d ($3.6x10^{5}$ g/d) (pers. comm. C. Vaught). Discharge during the exposure period for transferred PFUs was approximately $2x10^{5}$ 1/d ($5.5x10^{4}$ g/d) below average.

Table 18 lists mean adenylate and chlorophyll values with comparisons from a blocked ANOVA test for field treatments. Letters indicate significant differences (a = 0.05); Trends of previous field experiments for upstream, downstream, and transferred communities were again evident. Adenylate concentrations increased at the downstream site and perturbation

Table 17. Mean physical and chemical parameters for combined field and laboratory study, * (S.D.).

Parameter	Site 1	Site 4	Site 6	0%	50%	25%	12%
Temp.(^{o}C)	7.3	12.5	7.3	19.8	19.6	19.6	20.6
	(0.3)*	(0.3)	(0.6)	(1.3)	(1.3)	(1.4)	(1.1)
D.O.	12.1	10.6	11.8	12.3	14.8	15.8	17.0
(mg/L)	(2.3)	(3.6)	(2.9)	(1.8)	(3.1)	(4.0)	(4.6)
pH	8.13	7.86	8.02	8.64	8.75	8.76	8.99
	(0.04)	(0.12)	(0.10)	(0.17)	(0.38)	(0.29)	(0.39)
Cond.(us)	680	1040	751	662	740	696	340
	(37)	(800)	(209)	(11)	(111)	(60)	(2)
Alk.($CaCO_3$	22	160	178	179	160	170	174
mg/L)	(23)	(45)	(14)	(19)	(34)	(11)	(13)
Hard. "	298	153	192	267	205	242	245
	(8)	(13)	(13)	(12)	(8)	(8)	.(9)
Cl (mg/L)	161.4	206.0	83.7	108.6	97.7	100.6	101.0
	(30)	(250)	(50)	(3.4)	(12.2)	(14.3)	(9.5)
PO_4 "	0.0	10.7	3.8	0.0	4.8	2.4	0.7
	-	(2.1)	(1.3)	-	(0.6)	(0.4)	(0.2)
NO_3 "	1.9	42.2	28.5	0.8	17.6	10.2	4.8
	(0.5)	(19.7)	(10.3)	(0.2)	(3.7)	(3.4)	(1.7)
SO_4 "	31.5	139.2	178.5	114.3	153.3	135.3	125.9
	(2.1)	(59)	(116)	(5.6)	(48.6)	(31.6)	(6.1)
NO_2 "	0.0	0.1	1.0	0.1	2.4	1.9	0.1
	-	(0.2)	(0.5)	(0.2)	(0.1)	(0.1)	(0.2)

Table 17. (cont.)

Parameter		Site 1	Site 4	Site 6	0%	50%	25%	12%
NH_4	(mg/L)	0.1	3.5	2.0	(<0.1)	2.5	1.2	0.5
		(<0.1)	(0.5)	(0.6)	(<0.1)	(0.2)	(0.2)	(0.2)
Ca	"	48.7	22.3	29.6	37.2	32.5	36.0	36.3
		(4.1)	(1.4)	(4.5)	(2.1)	(5.8)	(3.4)	(3.6)
Mg	"	21.8	9.2	14.4	22.3	16.4	19.5	20.3
		(1.9)	(0.8)	(2.3)	(1.4)	(2.1)	(1.5)	(2.6)
Na	"	60.6	160.4	97.5	69.0	101.4	79.1	79.5
		(13.7)	(154)	(39.2)	(6.8)	(38.0)	(16.8)	(9.2)
K	"	4.9	8.0	6.9	10.3	10.1	9.4	9.4
		(0.7)	(0.7)	(1.3)	(0.7)	(1.6)	(0.7)	(0.7)
Pb	"	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		-	-	-	-	-	-	-
Cd	"	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		-	-	-	-	-	-	-
Ni	"	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		-	-	-	-	-	-	-
Cu	"	0.0	0.09	tr	0.0	tr	tr	tr
		-	(0.01)	-	-	-	-	-
Zn	"	0.0	0.07	0.06	0.0	0.03	tr	0.0
		-	(0.02)	(0.03)	-	(0.02)	-	-
Fe	"	0.08	0.21	0.11	0.0	0.10	tr	tr
		(0.02)	(0.04)	(0.08)	-	(0.02)	-	-

Table 18. Mean field adenylate and chlorophyll values from combined field and laboratory study, different letters indicate significant differences within each parameter (a = 0.05), * (S.D.), ** mean coefficient of variation (C.V.) for each treatment.

Parameter	Site 1	Site 4	Trans 4	Site 6	Trans 6
ATP ug/L	50 A (28)*	346 B (52)	179 AB (31)	1032 C (191)	907 C (143)
ADP ug/L	106 A (34)	317 B (80)	264 B (56)	584 C (107)	550 C (157)
ATP+ADP ug/L	156 A (60)	664 B (98)	444 AB (76)	1616 C (255)	1456 C (250)
ATP/ADP	0.45 A (0.14)	1.17 B (0.41)	0.70 A (0.16)	1.82 C (0.46)	1.77 C (0.56)
ATP/Chla	33 A (11)	743 B (190)	256 A (44)	858 B (156)	1078 B (188)
Chl a mg/L	1.44 A (0.37)	0.49 C (0.11)	0.72 BC (0.15)	1.20 AB (0.01)	0.92 ABC (0.27)
Pheo mg/L	0.70 A (0.19)	0.64 A (0.11)	0.88 A (0.15)	0.79 A (0.03)	0.58 A (0.20)
Chla+Pheo mg/L	2.13 A (0.53)	1.12 A (0.21)	1.59 A (0.36)	1.99 A (0.03)	1.50 A (0.46)
Chla/Pheo	2.13 A (0.43)	0.76 B (0.08)	0.82 B (0.05)	1.53 C (0.06)	1.61 C (0.23)
Mean C.V.**	32.1	20.6	12.9	11.9	24.5

was indicated by high ATP/Chla and low Chla/Pheo ratios (Figures 9, 10 and 11). High ATP/Chla ratios suggest a perturbed situation at Site 6 yet rebounding Chla/Pheo ratios indicate partial recovery of the algal community. ATP/ADP ratios increased below the STP and were highest at Site 6 (Figure 12). Chlorophyll a concentrations were highest at Site 1 and lowest at Site 4 (Figure 13). Transferred community responses were more similar to those of downstream communities than upstream communities. Chlorophyll + pheopigment concentrations were similar with all treatments.

Mean adenylate and chlorophyll values for laboratory treatments are presented in Table 19. Laboratory results were similar in some respects but opposite in others when compared to field results. Adenylate and chlorophyll concentrations increased in effluent exposed communities (Figure 14). ATP/ADP, ATP/Chla, and Chla/Pheo results are presented in Figure 15. ATP/ADP ratios were similar with all levels of exposure and similar to those of the pilot laboratory study (Table 16). Reference communities (0%) had higher ATP/Chla ratios than effluent exposed communities which were similar to each other (Figure 15). Laboratory exposure resulted in higher adenylate concentrations, Chla/Pheo ratios and chlorophyll concentrations (except 0%) compared to field exposures.

Plots comparing ATP and chlorophyll a concentrations with organism biovolumes are presented in Figures 16 and 17 for all study treatments. Chlorophyll a is a good biovolume estimate

Table 19. Mean adenylate and chlorophyll values from laboratory portion of combined field and laboratory study, different letters indicate significant differences within each parameter (a = 0.05), * (S.D.), ** mean coefficient of variation (C.V.) for each treatment.

Parameter	0%	50%	25%	12%
ATP ug/l	1002 A (475)*	1378 AB (150)	1903 C (376)	1604 BC (613)
ADP ug/l	686 A (196)	849 A (255)	1314 B (422)	1116 AB (629)
ATP+ADP ug/l	1688 A (730)	2226 AB (256)	3217 C (707)	2720 BC (1192)
ATP/ADP	1.47 A (0.51)	1.80 A (0.70)	1.57 A (0.48)	1.74 A (0.83)
ATP/Chla	906 A (470)	179 B (19)	277 B (55)	309 B (100)
Chl a mg/l	1.13 A (0.24)	7.72 B (0.45)	6.89 C (0.62)	5.18 D (0.97)
Pheo mg/l	0.13 A (0.06)	0.80 B (0.11)	1.74 C (0.42)	1.25 D (0.42)
Chla+Pheo mg/l	1.26 A (0.25)	8.52 B (0.46)	8.63 B (0.59)	6.43 C (1.22)
Chla/Pheo	11.50 A (8.36)	9.78 AB (1.54)	4.20 B (1.07)	4.60 B (1.60)
Mean C.V.**	44.6	15.8	21.1	36.1

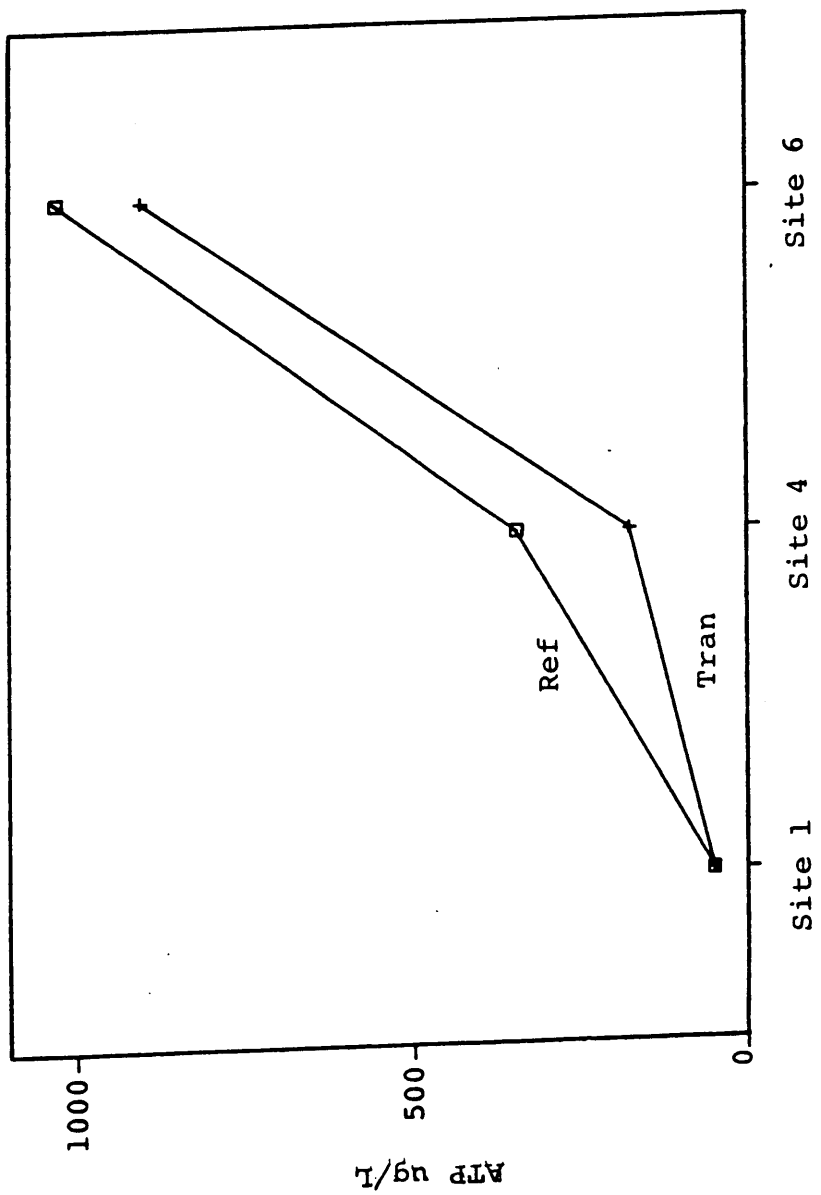


Figure 9. ATP concentrations for reference and transferred communities.

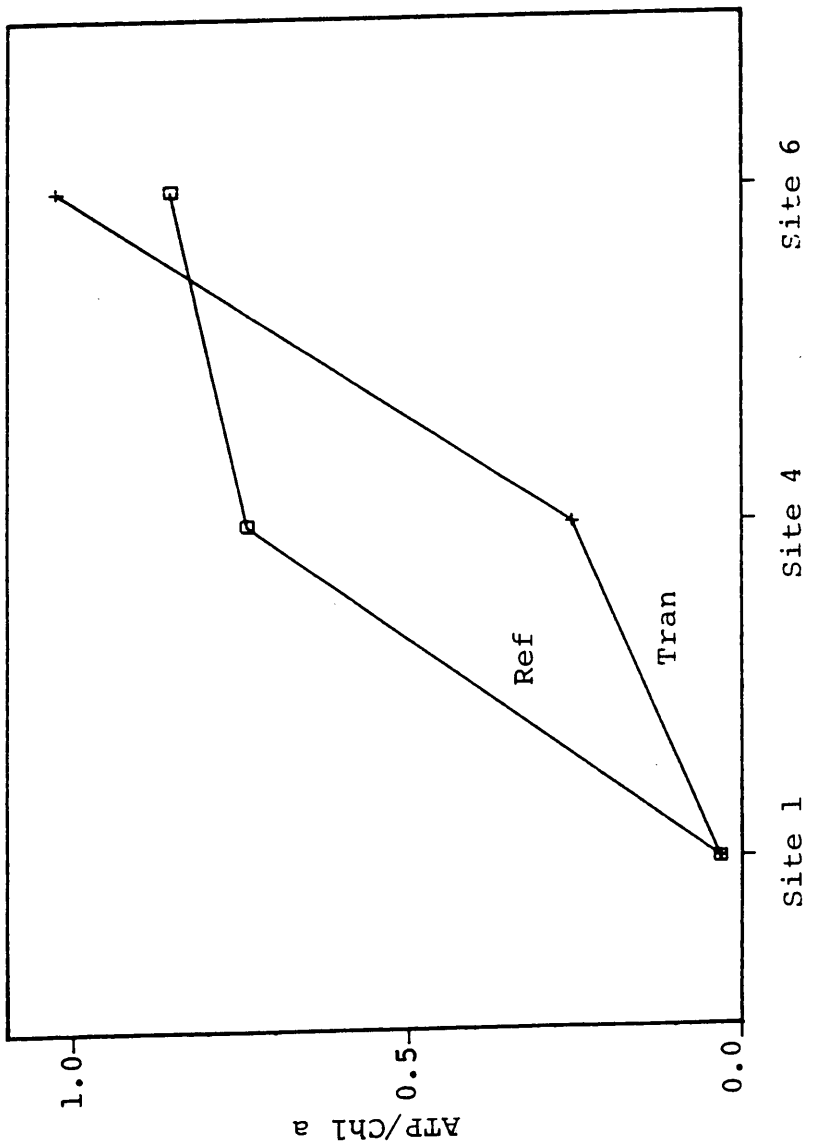


Figure 10. ATP/Chla values for reference and transferred communities.

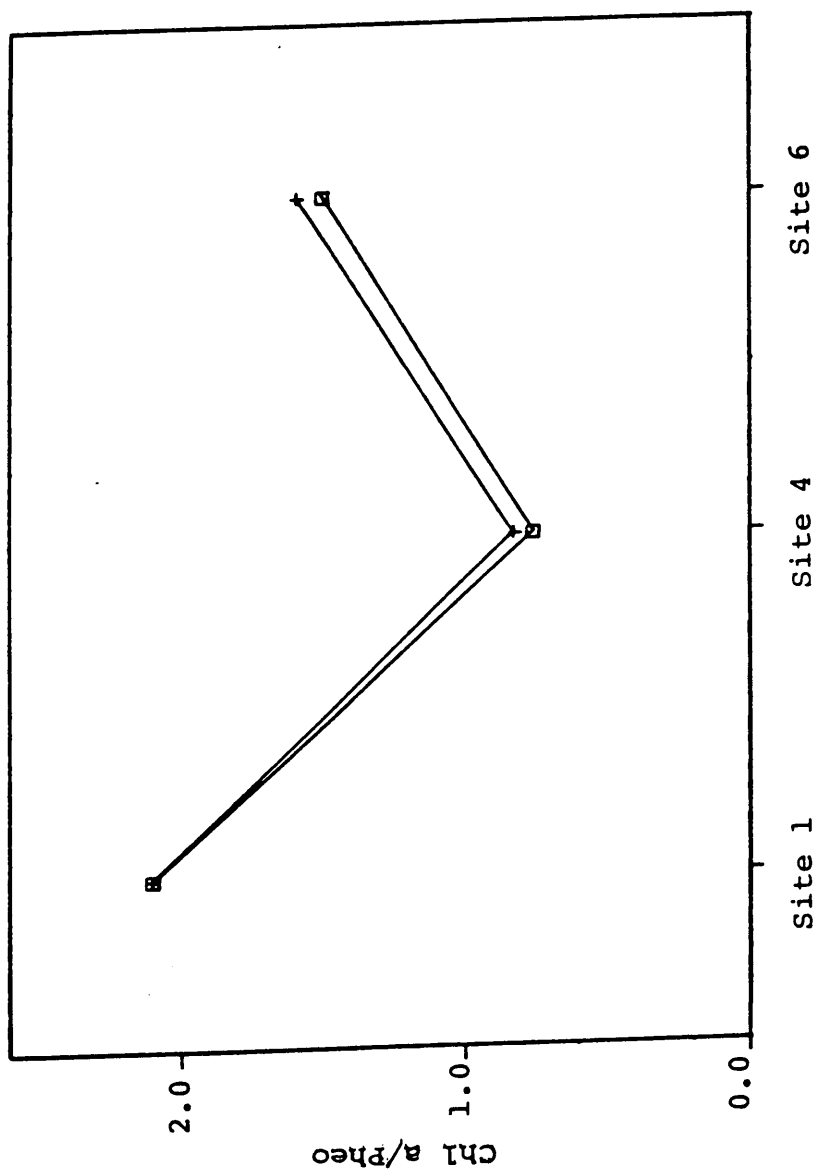


Figure 11. Chla/Pheo values for reference and transferred communities.

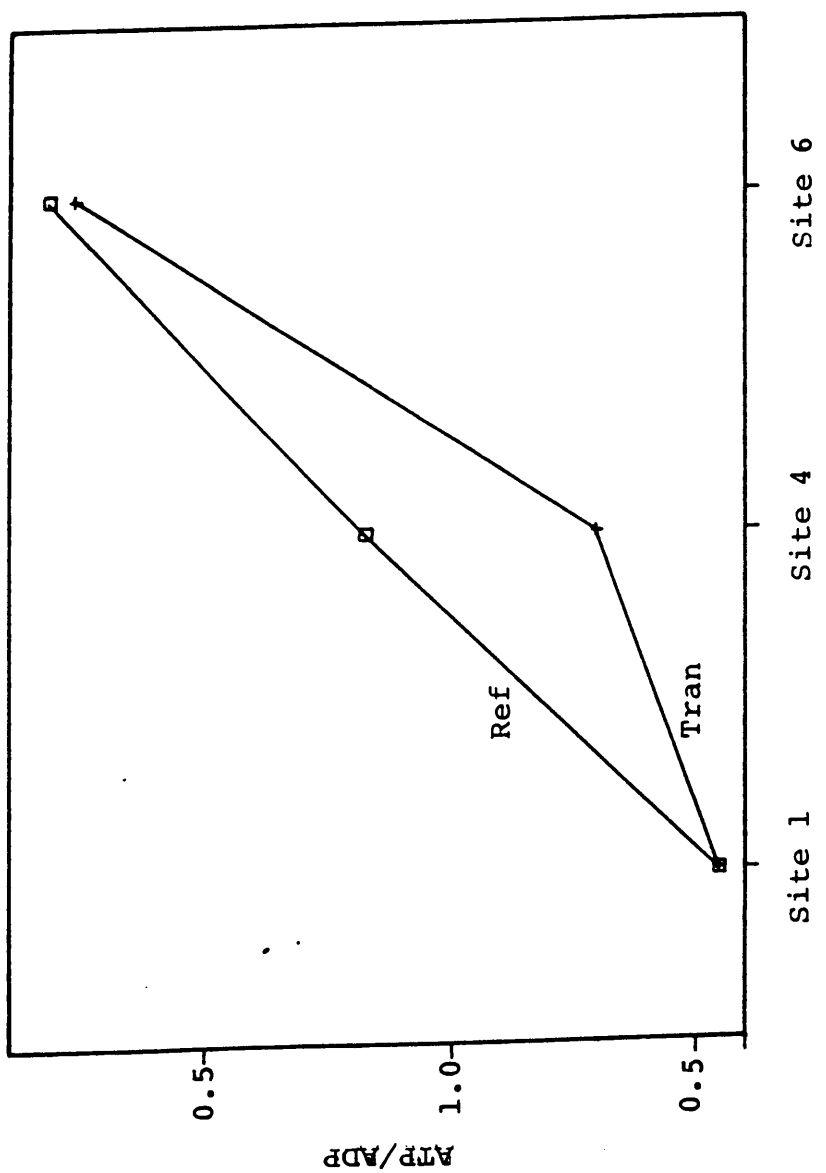


Figure 12. ATP/ADP values for reference and transferred communities.

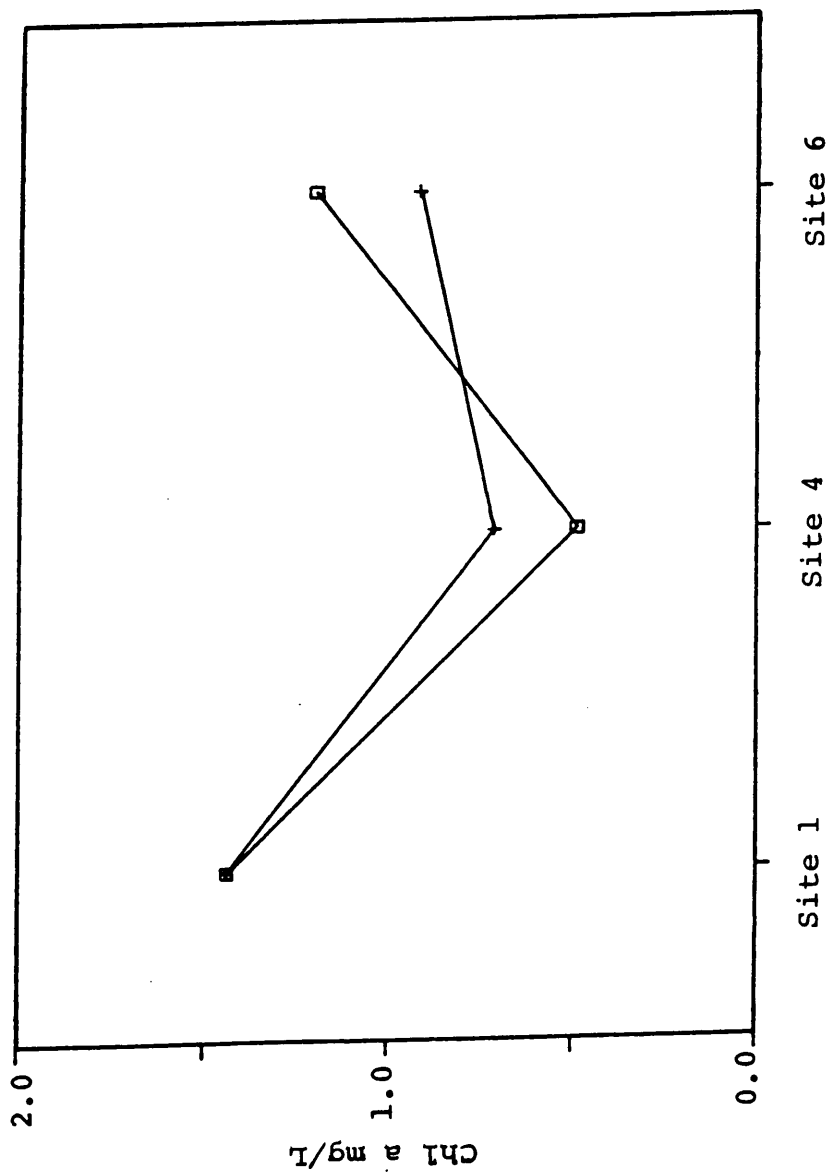


Figure 13. Chlorophyll a concentrations for reference and transferred communities.

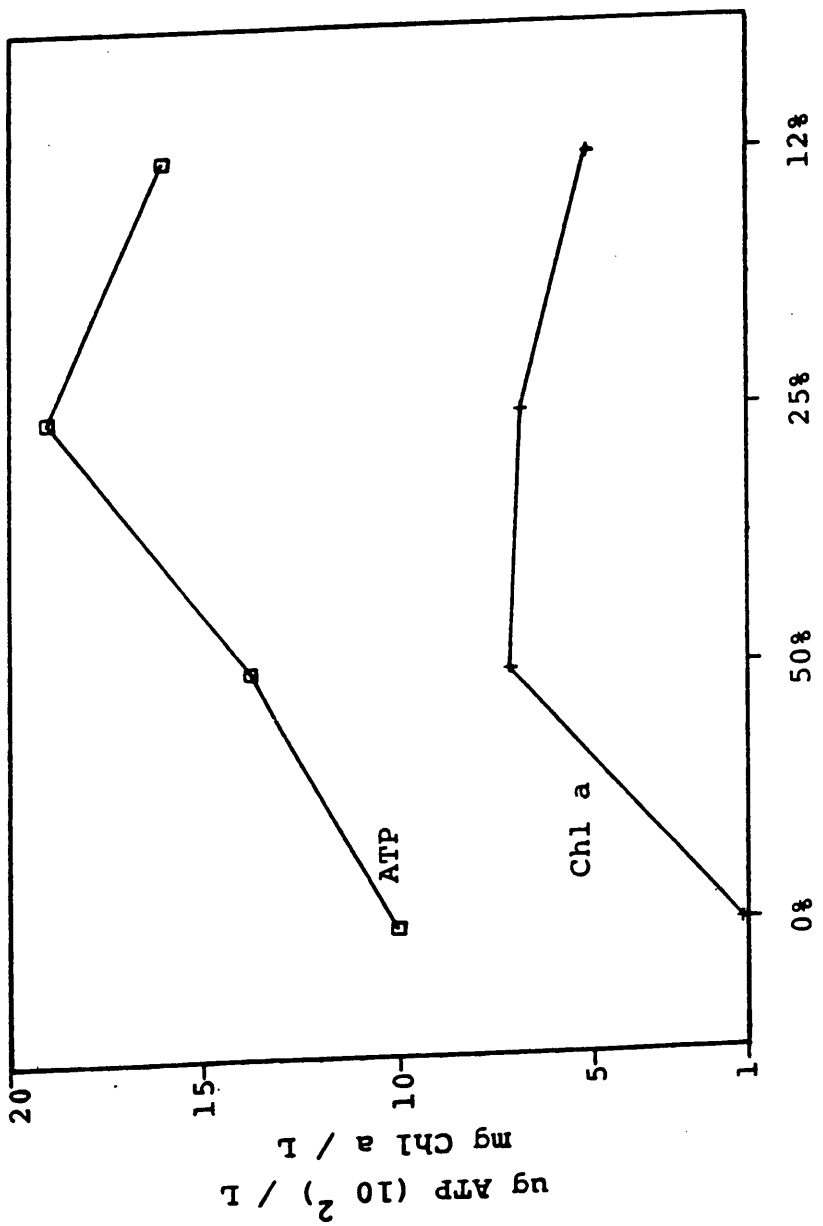


Figure 14. ATP and chlorophyll a concentrations for laboratory treatments.

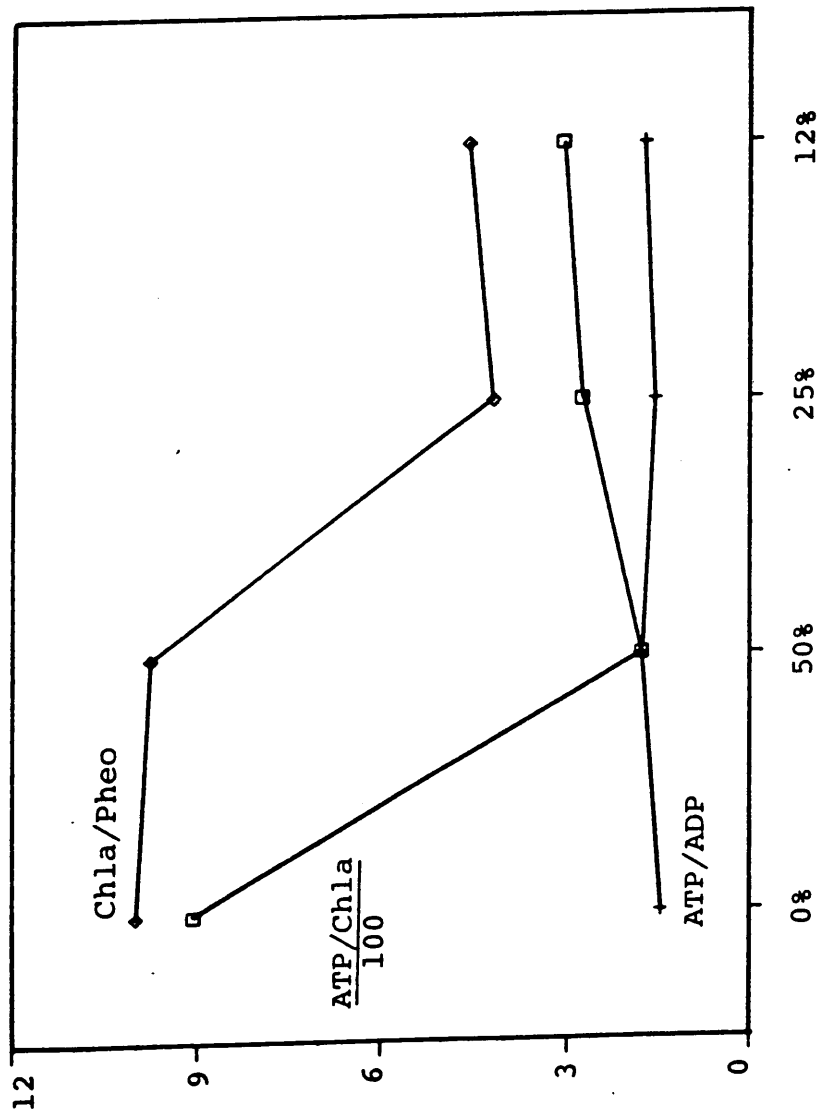


Figure 15. ATP/Chla, ATP/ADP, and Chla/Pheo values for laboratory treatments.

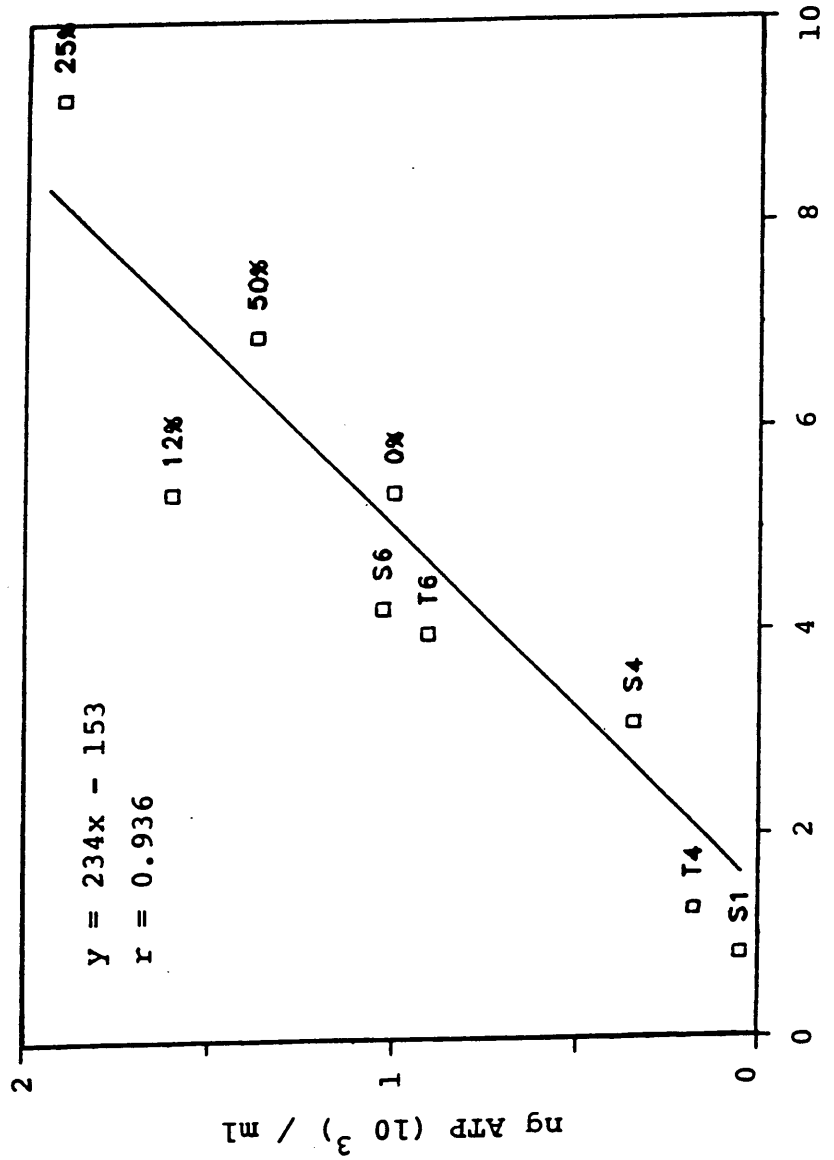


Figure 16. ATP concentrations and total organism biovolume concentrations for all treatments in combined field and laboratory study.

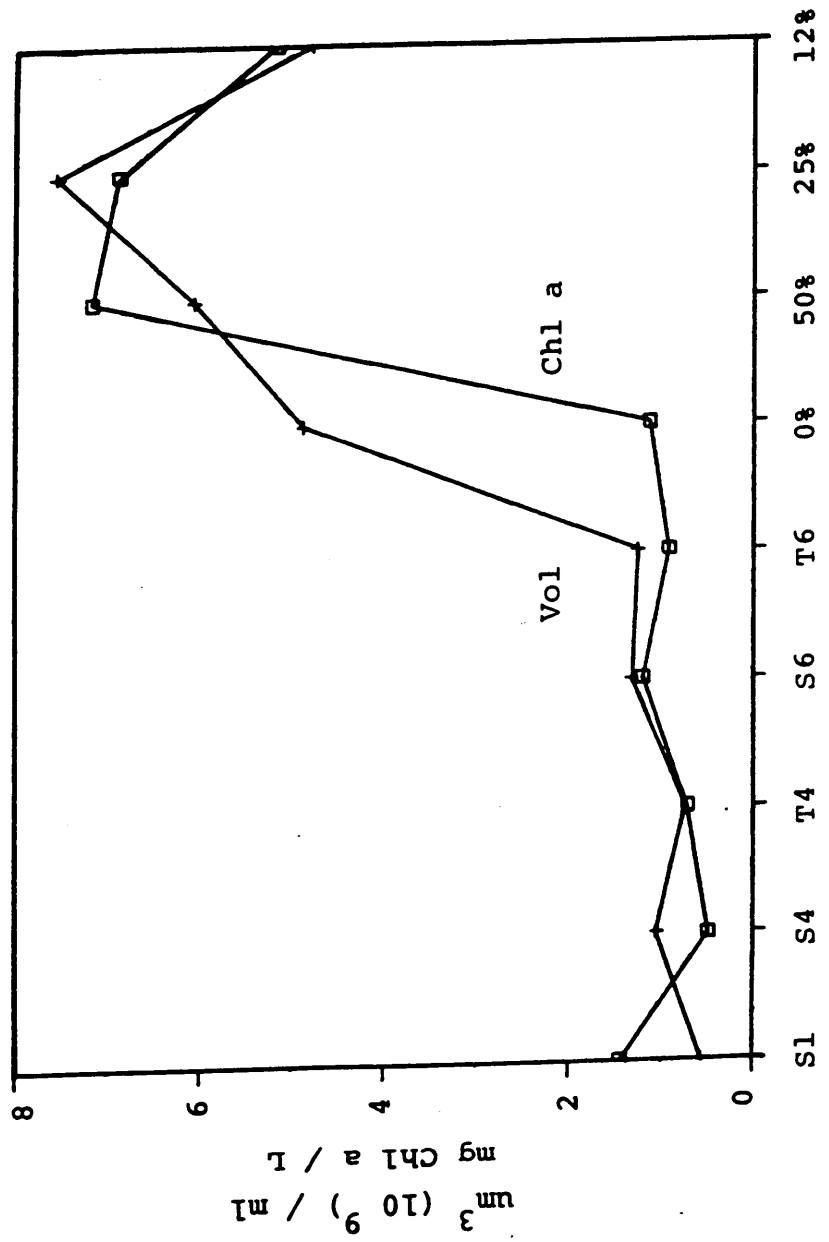


Figure 17. Chlorophyll a concentrations and chlorophyllous organism biovolume concentrations for all treatments in combined field and laboratory study.

for chlorophyllous organisms in all treatments except 0% laboratory exposures. ATP/Chla ratios are compared to total biovolume/chlorophyllous biovolumes in Figure 18. Again, these relationships for 0% laboratory exposures are anomalous.

Biovolume concentrations of the dominant organisms in field communities (except bacteria) are presented in Figure 19. Upstream communities exhibited the greatest diversity and lowest density, while the opposite occurred at Site 6. Organisms less than 20 um in diameter comprised the largest portion of downstream and transferred communities; of these, the achlorophyllous organisms were more abundant than chlorophyllous ones. Protozoans are more common downstream than upstream. The dominant achlorophyllous organism at Site 4 was Bodo putrificans and at Site 6, Monas spp. Oscillatoria comprised approximately 8% of the upstream biovolume, was absent in downstream reference and rare in transferred communities. The relative abundance of dominant organisms in field communities are presented in Figure 20.

Concentrations of dominant green algae and diatoms in field PFUs are presented in Figures 21 and 22, respectively. Chlorophyllous organisms less than 20 um (coccoids and flagellates) are the dominant algal form in all field communities (note that these organisms are expressed in 1/100th their actual concentrations). Characium spp. were the next most abundant. Diatoms were most abundant at the upstream site.

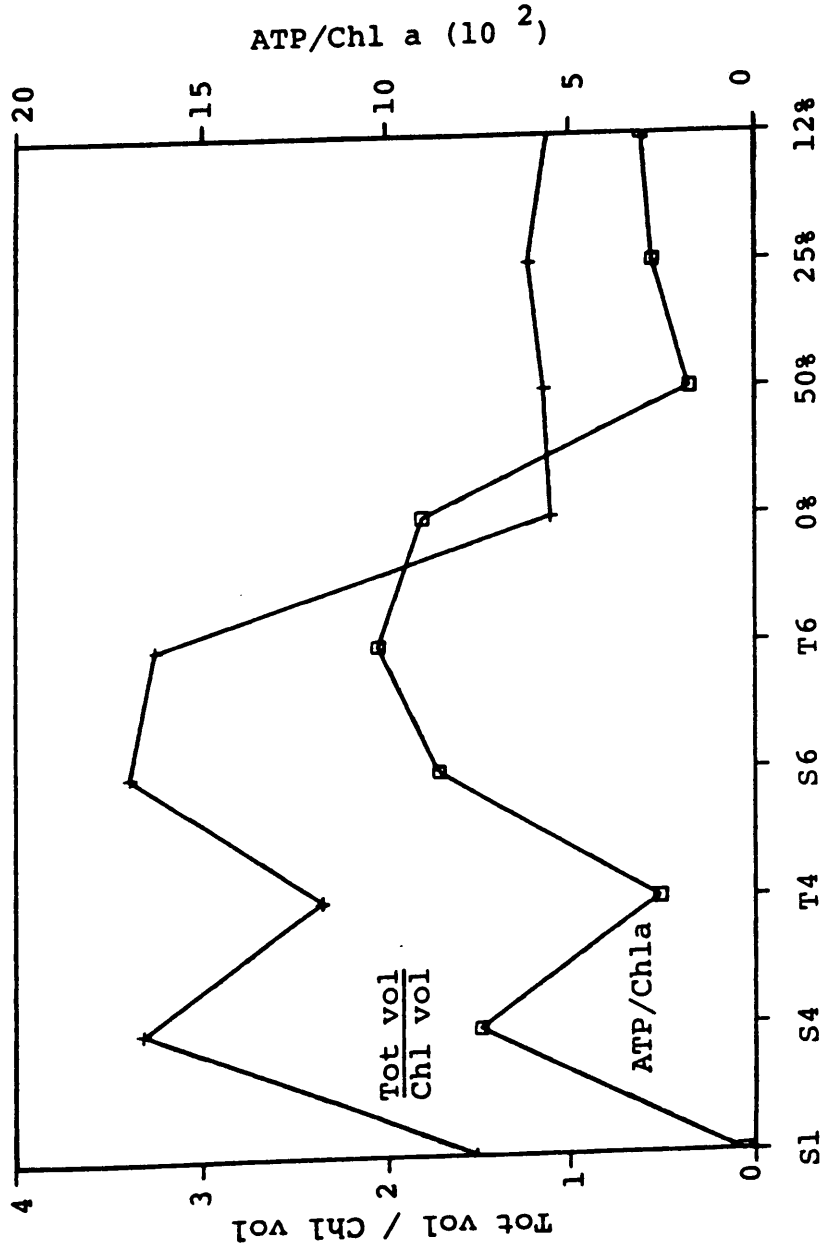


Figure 18. ATP/Chla and total biovolume/chlorophyllous biovolume for all treatments in combined field and laboratory study.

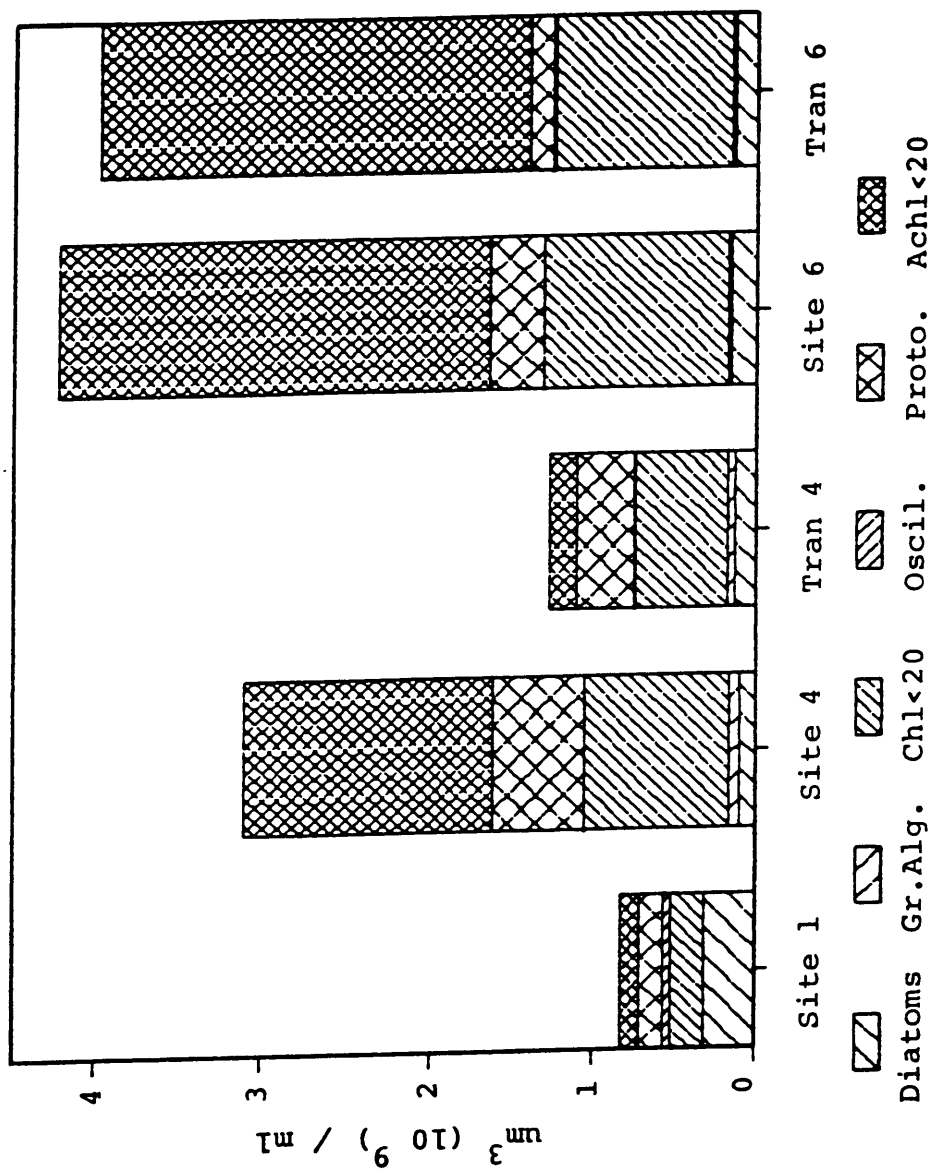


Figure 19. Biovolume concentrations of the dominant organisms (excluding bacteria) from field communities of combined field and laboratory study. Gr.Alg. = green algae > 20 um, Chl<20 = chlorophyllous org. < 20um, Oscil. = Oscillatoria, Proto. = protozoans, Achl<20 = achlorophyllous org. < 20um.

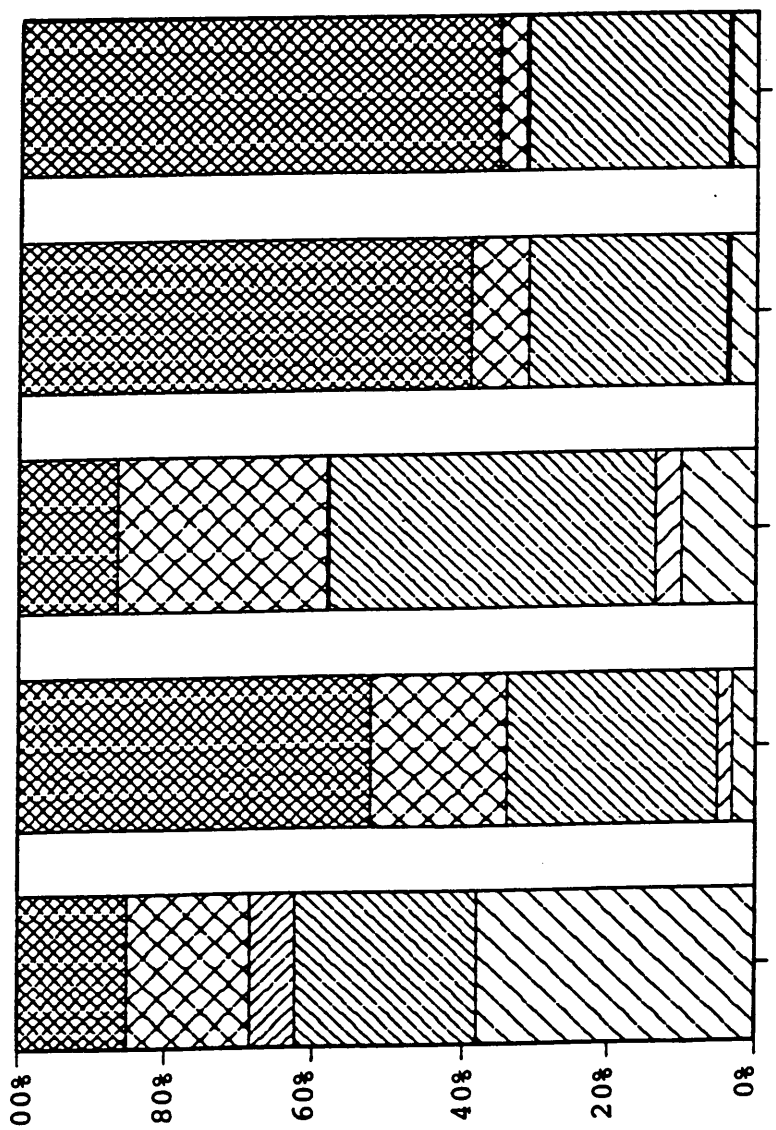


Figure 20. Relative concentrations of the dominant organisms (excluding bacteria) from field communities of combined field and laboratory study. Gr.Alg. = green algae > 20um, Chl<20 = chlorophyllous org. < 20um, Oscil. = Oscillatoria, Proto. = protozoans, Achl<20 = achlorophyllous org. < 20um.

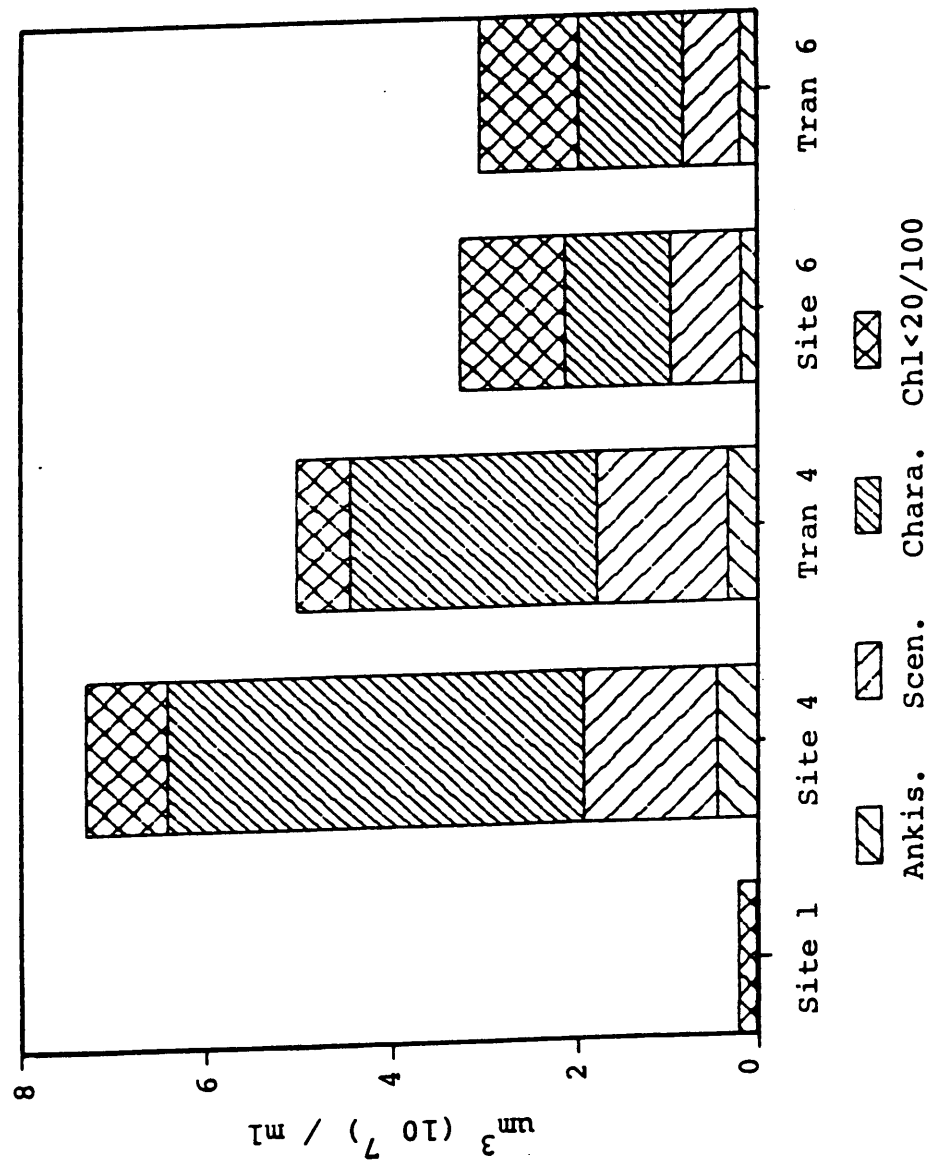


Figure 21. Biovolume concentrations of the dominant green algae from field communities of combined field and laboratory study. Ankis. = Ankistrodesmus, Scen. = Scenedesmus, Chara. = Characium, Chl<20/100 = 1/100th the concentration of chlorophyllous org. < 20um.

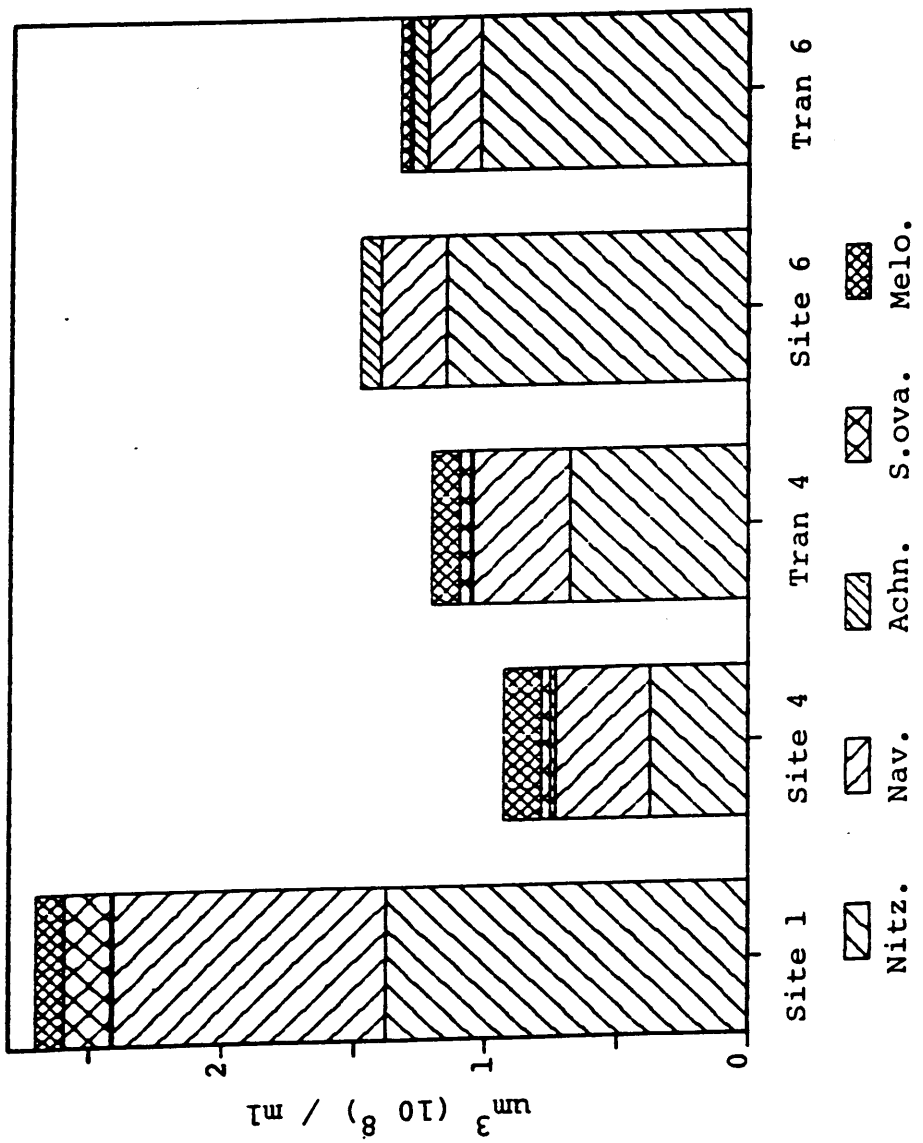


Figure 22. Biovolume concentrations of the dominant diatoms from field communities of combined field and laboratory study. Nitz. = Nitzschia, Nav. = Navicula, Achn. = Achnanthes, S.ova = Surirella ovata, Melo. = Melosira.

Nitzschia spp. (primarily N. palea) and Navicula spp. were the two most common genera. Bacteria were most populous in Site 4 communities (Figure 23).

Shannon-Weiner diversity indices are plotted in Figure 24. Low diversity at Sites 4 and 6 is due in part to high abundance of the flagellates Bodo and Monas, respectively. The diversity of transferred communities dropped quickly during the three day exposure period. Upstream PFUs were an olive brown color, while downstream and transferred PFUs were grey. A more detailed account of diatom, algal and protozoan concentrations for both field and laboratory treatments are presented in the appendix (Tables A3 and A4).

Cluster analyses incorporating the Pinkham and Pearson similarity coefficient, B (Pinkham and Pearson, 1976) for diatom and protozoan occurrence are presented as dendrograms in Figure 25. These results agree with nontaxonomic trends. Communities transferred to each downstream site are most similar to resident communities. Upstream reference communities are separate from downstream and transferred communities. Both diatom and protozoan communities respond similarly. Occurrence data for these analyses for diatoms and protozoans are presented in the appendix (Tables A5 and A6).

Concentrations of the dominant groups of laboratory exposed communities are presented in Figure 26. Oscillatoria was most common in 0% exposures and least common in 50% exposures in which diatoms were most abundant. Chlorophyllous organisms less

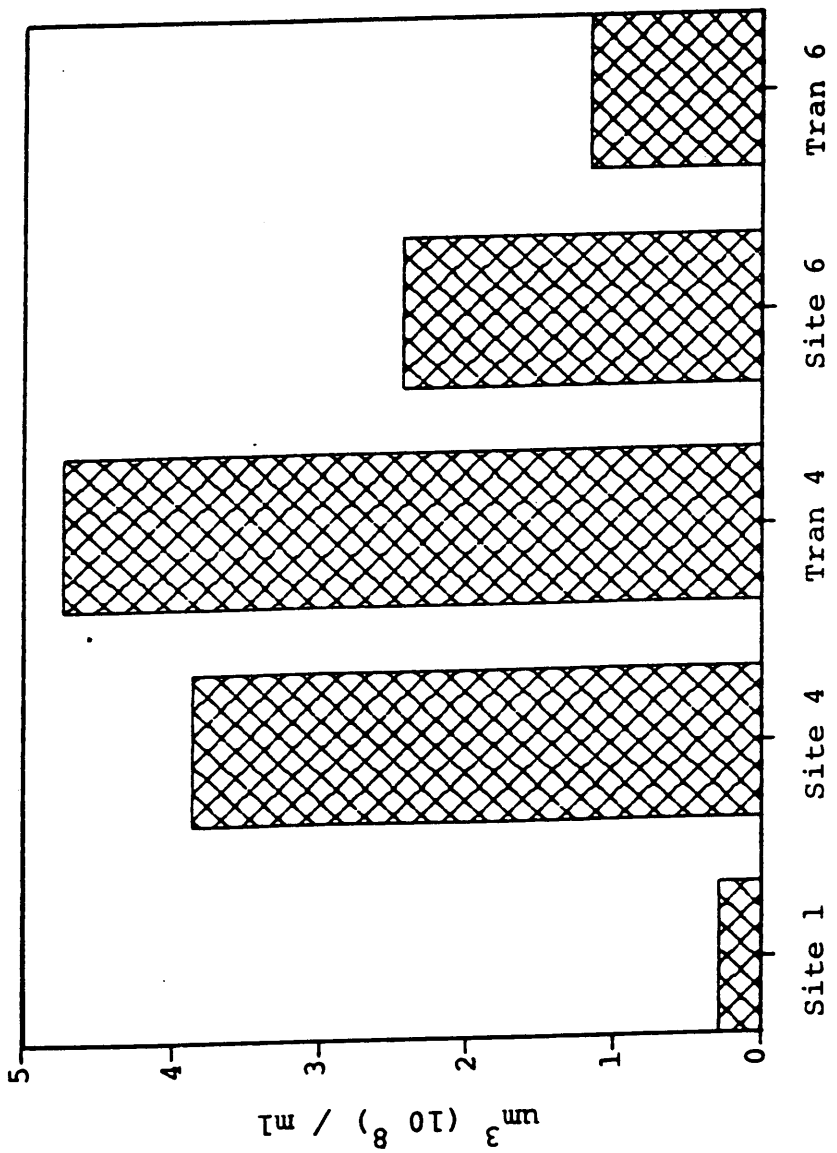


Figure 23. Bacterial biovolume concentrations from field communities of combined field and laboratory study.

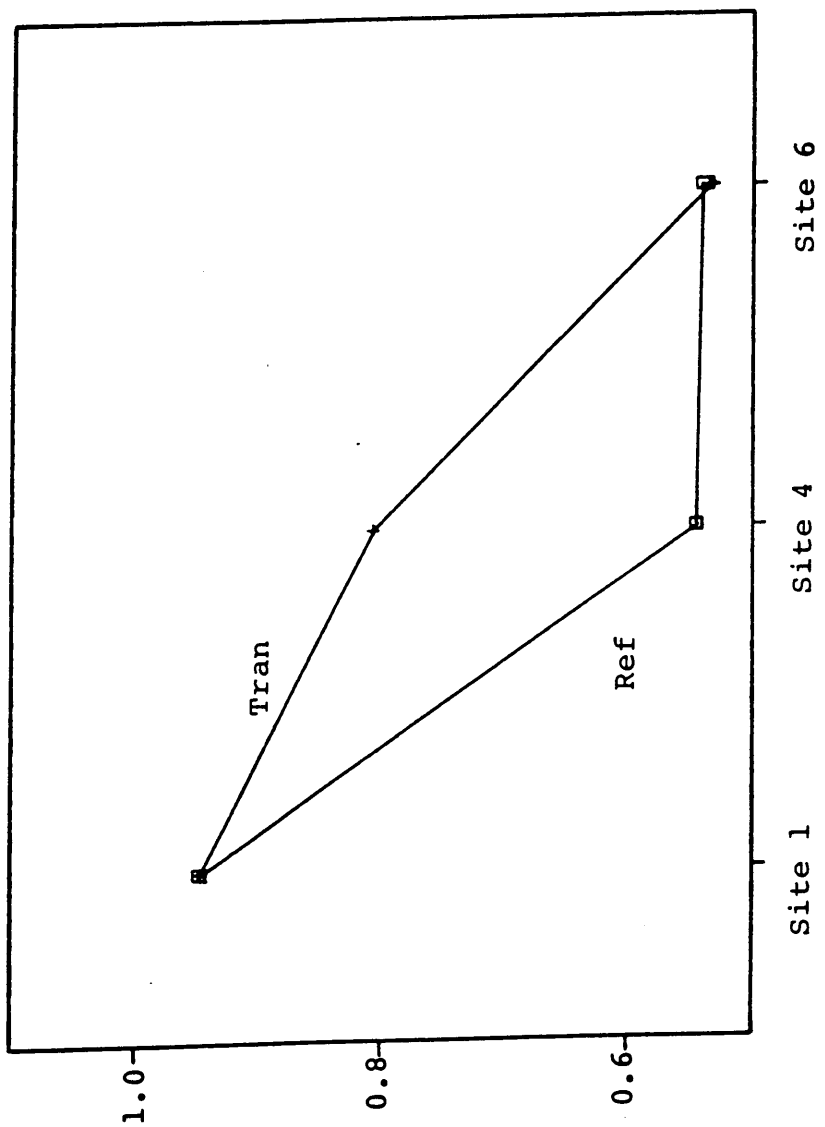


Figure 24. Shannon-Weiner diversity indices from reference and transferred communities of combined field and laboratory study.

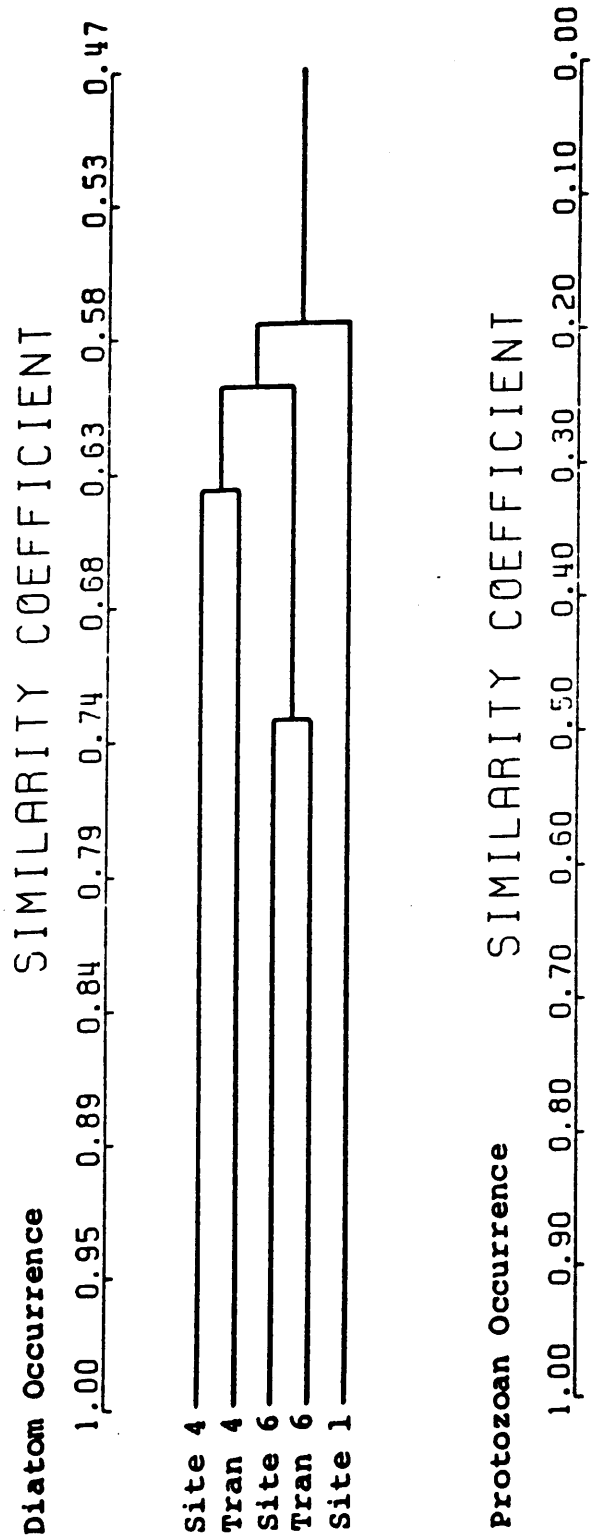


Figure 25. Cluster analyses for diatom and protozoan occurrence from field portion of combined field and laboratory study.

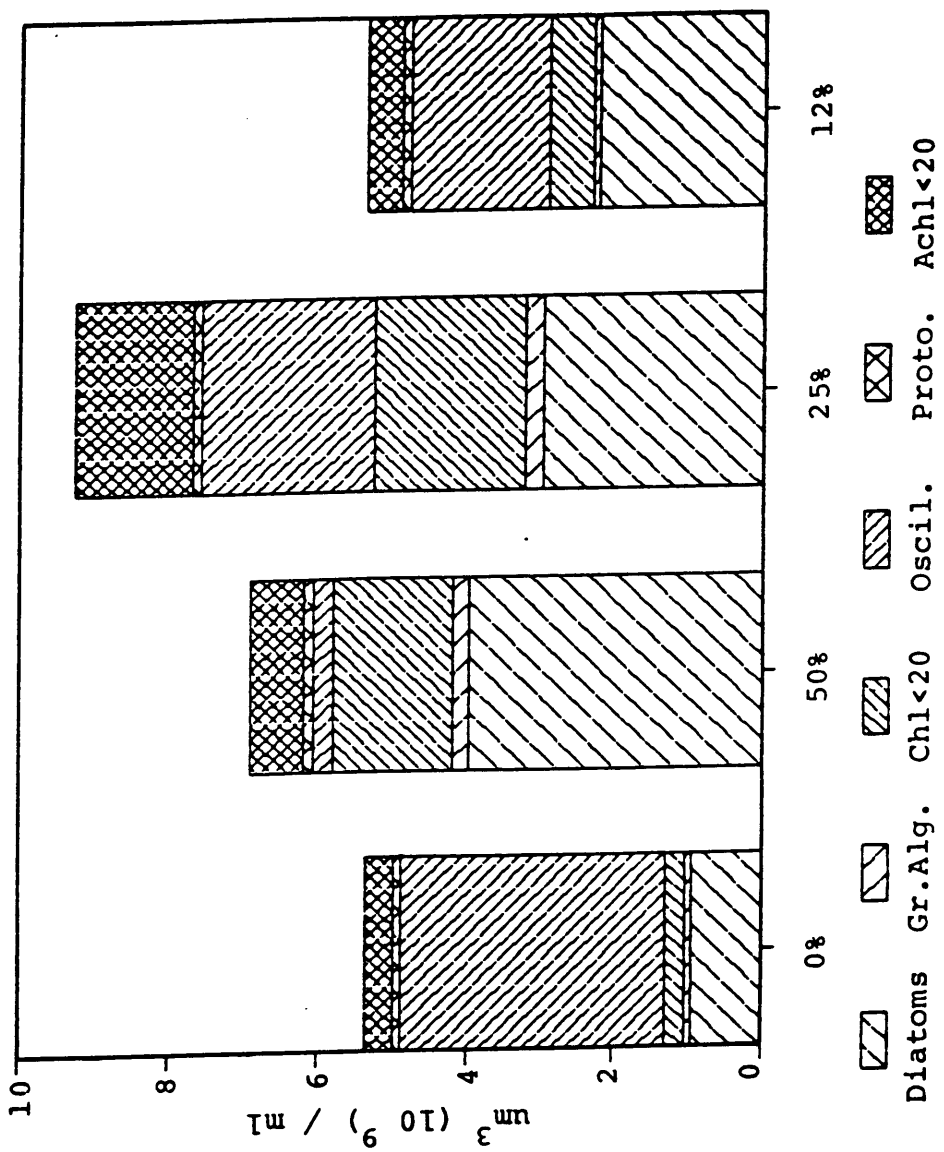


Figure 26. Biovolume concentrations of the dominant organisms (excluding bacteria) from laboratory communities of combined field and laboratory study. Gr.Alg. = green algae > 20um, Chl<20 = chlorophyllous org. < 20um, Oscil. = Oscillatoria, Proto. = protozoans, Achl<20 = achlorophyllous org. < 20um.

than 20 um were most common in 25% and 50% effluent exposures. The relative abundance of dominant organisms in laboratory communities are presented in Figure 27. Concentrations of dominant green algae and Oscillatoria appear in Figure 28. Dominant taxa in descending overall abundance were Oscillatoria spp., coccoids and flagellates (less than 20 um), Scenedesmus spp. and Ankistrodesmus spp. Diatom concentrations increased with effluent exposure (Figure 29). The most common genus was Nitzschia (primarily N. palea). The 0% reference communities had slightly higher diversity than effluent exposed communities (Figure 30). Bacterial concentrations in effluent exposed communities were similar to each other and significantly higher than thoses of reference communities.(Figure 31.)

Visual characterizations of laboratory PFU communities were: 0%, pale olive-brown color; 12%, olive-brown with dark bluegreen filaments; 25%, dark green overall with many bluegreen filaments; and 50%, bright green with few bluegreen filaments.

Laboratory exposures resulted in increased adenylate and chlorophyll concentrations and corresponding biovolumes compared to field exposures. Total biovolume concentrations were $5x10^9$ to $9x10^9$ um^3/ml in laboratory communities and $1x10^9$ to $4x10^9$ um^3/ml in field communities. Achlorophyllous and chlorophyllous coccoids and flagellates less than 20 um dominated downstream field communities where diversity was low. Laboratory effluent exposures produced slightly more diverse communities in which

Figure 27. Relative concentrations of the dominant organisms (excluding bacteria) from laboratory communities of combined field and laboratory study. Gr.Alg. = green algae > 20um, Chl<20 = chlorophyllous org. < 20um, Oscil. = Oscillatoria, Proto. = protozoans, Achl<20 = achlorophyllous org. < 20um.

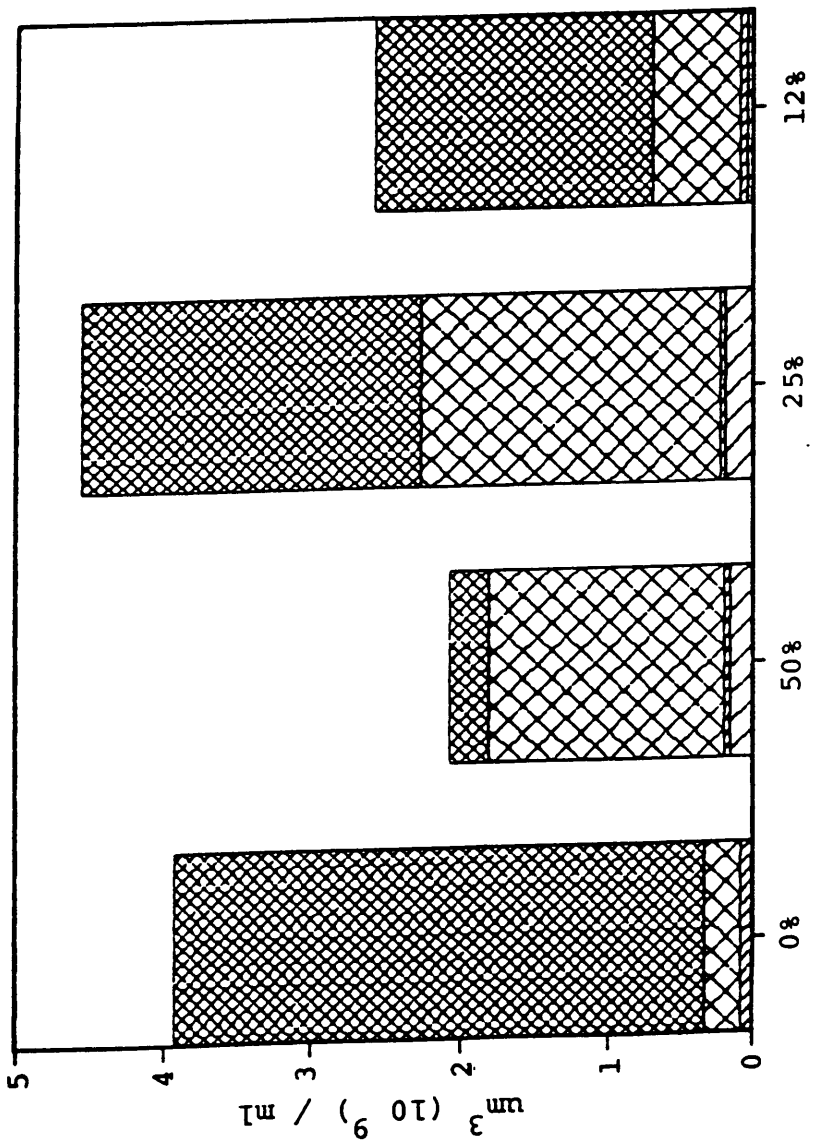


Figure 28. Biovolume concentrations of the dominant green algae from laboratory communities of combined field and laboratory study. Scen. = Scenedesmus, Spiro. = Spirogyra, Chl<20 = chlorophyllous org. < 20um, Oscil. = Oscillatoria.

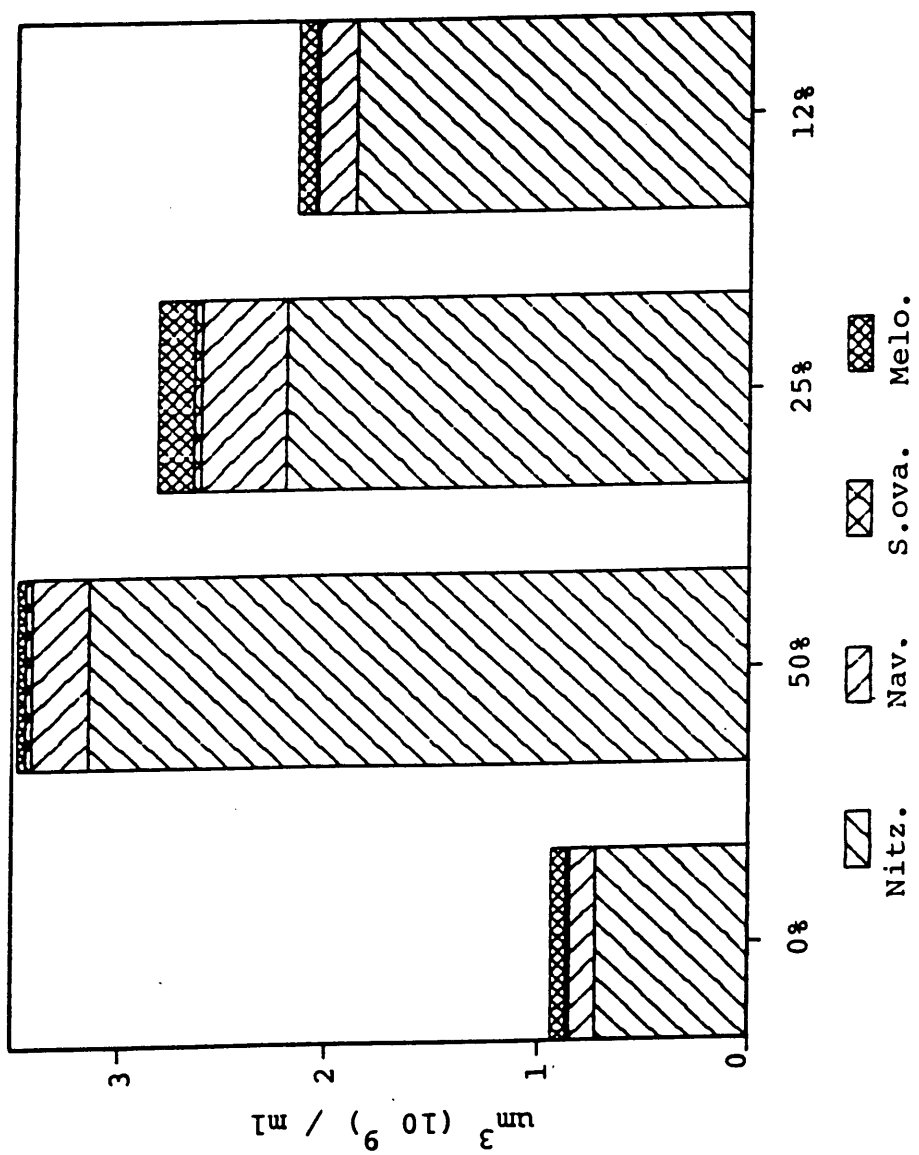


Figure 29. Biovolume concentrations of the dominant diatoms laboratory communities of combined field and laboratory study. Nitz. = Nitzschia, Nav. = Navicula S.ova. = Surirella ovata, Melo. = Melosira.

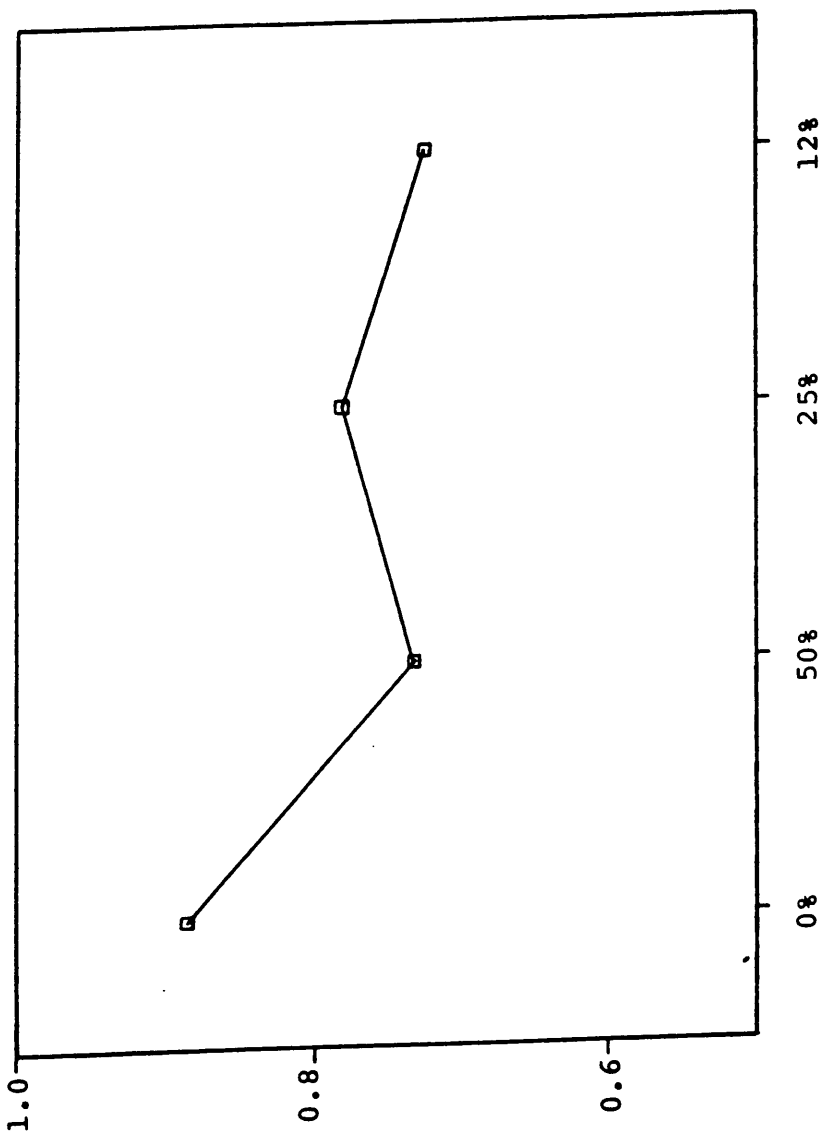


Figure 30. Shannon-Weiner diversity indices from laboratory communities of combined field and laboratory study.

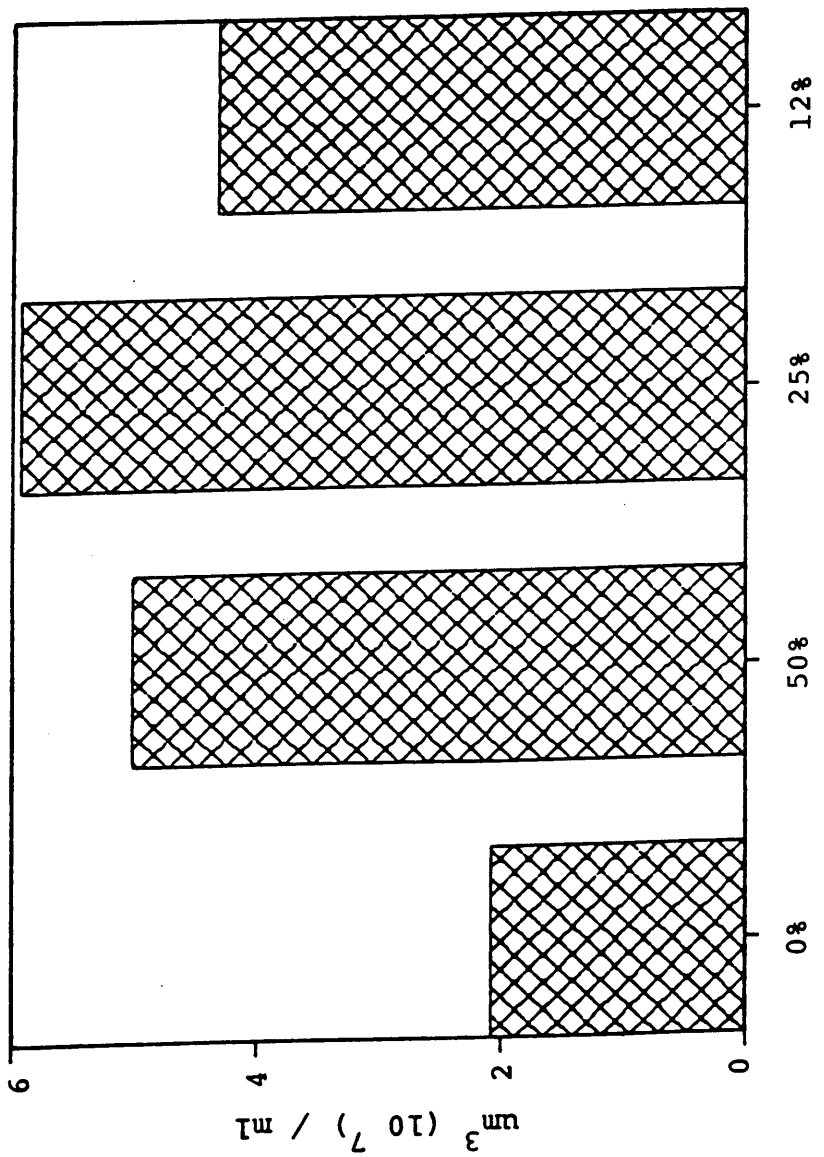
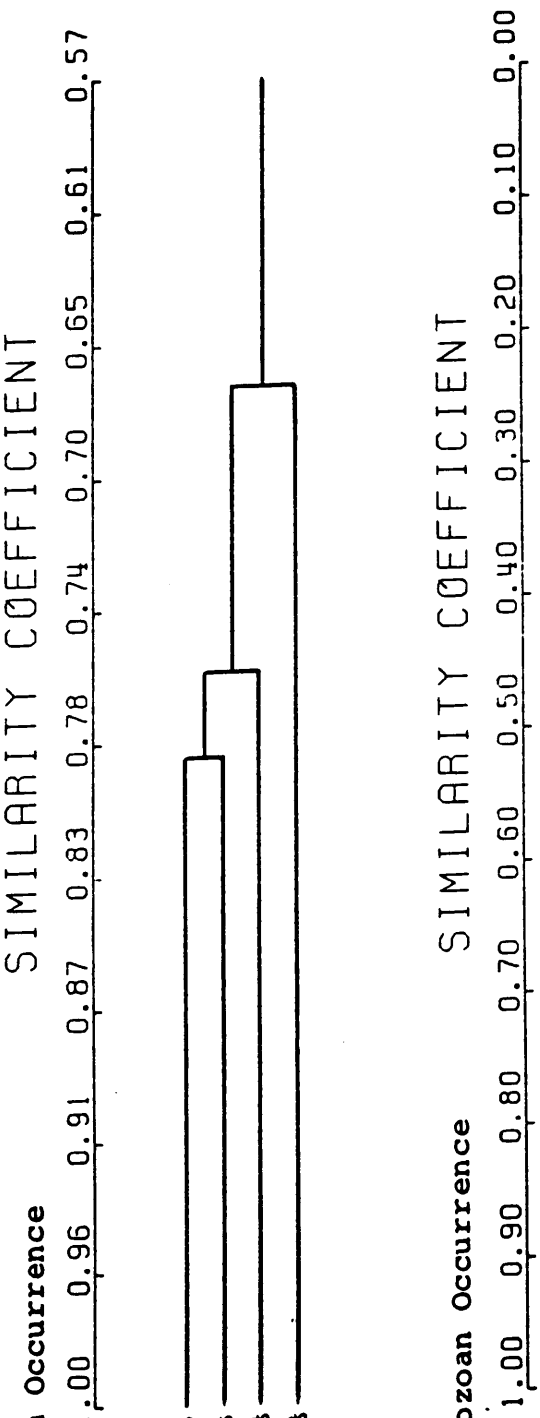


Figure 31. Bacterial biovolume concentrations from laboratory communities of combined field and laboratory study.

diatoms and Oscillatoria became more abundant. Protozoans (ciliates, sarcodines and flagellates greater than 20 um) and bacteria were more common under field conditions.

Cluster analyses for diatom and protozoan occurrence are presented in Figure 32. Diatom communities are most similar between 12% and 25% effluent exposures and these two communities are more similar to those from 0% than 50% effluent exposures. Protozoan communities are most similar between 25% and 50% effluent exposures and these communities are more similar to 12% than 0% exposed communities. Occurrence data used in these analyses for diatoms and protozoans are presented in the appendix (Tables A5 and A6).

Shannon-Wiener species diversity indices, the number of taxa and the total number of taxa identified for protozoans and diatoms for all treatments are presented in Table 20. Species diversities were calculated using biovolume data as described in the general metheds section. Reference communities in both laboratory and field treatments had the highest species diversity. The number of protozoan taxa dropped from upstream to Site 4 and increased again at Site 6 but remained relatively constant in laboratory treatments. Similar numbers of diatom taxa were identified in all treatments.



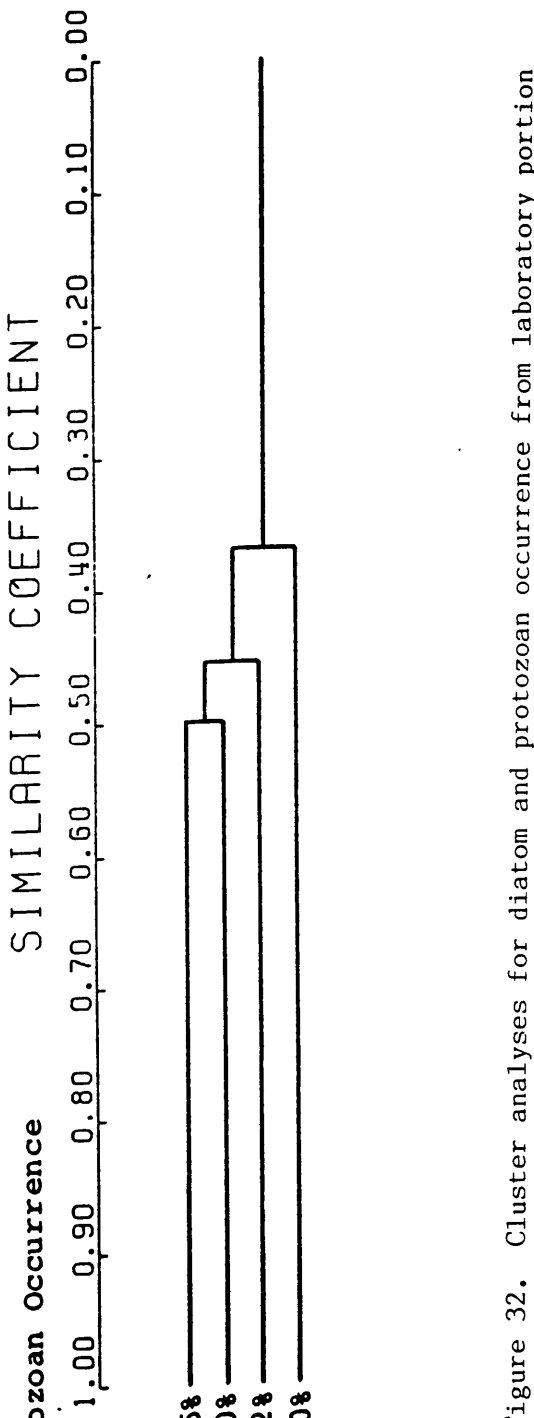


Figure 32. Cluster analyses for diatom and protozoan occurrence from laboratory portion of combined field and laboratory study.

Table 20. Numbers of protozoan and diatom taxa, and species diversity for field and laboratory treatments.

Site	S1 *	S4	T4	S6	T6	0%	50%	25%	12%	TOTAL
Protozoa	49	16	13	40	38	41	43	44	49	119
Diatoms	83	89	88	74	77	88	86	86	80	136
Sp. Div.	0.949	0.546	0.807	0.541	0.533	0.886	0.733	0.782	0.733	

* S1 = Site 1, S4 = Site 4, T4 = Trans 4, S6 = Site 6, T6 = Trans 6.

DISCUSSION

Field responses at Sites 1 and 4 were very similar to those of previous studies; adenylate concentrations and ratios increased and chlorophyll concentrations and Chla/Pheo ratios decreased at Site 4. The lower adenylate and chlorophyll concentrations at Site 4 in this study relative to former studies are probably due to lower water temperatures and decreased STP discharges. These factors may also account for the fact that transferred communities did not change as much as in previous studies. Lower discharge rates resulted when the Blacksburg student population (1/2 the area population) left town for Thanksgiving vacation.

The nontaxonomic measures of ATP/Chla and Chla/Pheo agree with the taxonomic stress indicators of species diversity and numbers of protozoan taxa as indicators of environmental stress. The number of diatom taxa did not change with varying degrees of pollution; however, the actual composition of diatom communities did as indicated by cluster analysis. Both diatom and protozoan community cluster analyses indicated a different community composition at each site and that transferred communities became like resident indigenous communities.

Interpreting community responses at Site 6 was complicated by the appearance of approximately 40 cattle directly upstream of the site between days 4 and 6 of the study. Communities at Site 6 show some signs of recovery but also suggest that this site was receiving organic pollution. Partial recovery is indi-

cated by increased chlorophyll concentrations and numbers of protozoan taxa. High ATP/Chla and low species diversities also suggest organic enrichment. The high ATP/Chla suggests more enrichment than the nutrient analyses did. The communities responded to episodic nutrient inputs from the cattle that were not detected in water samples. The cattle were not in the stream when water samples were collected.

These results are similar to Matthews (1981). During a year long study at Cedar Run, she observed decreased ATP/Chla ratios at this site relative to Site 4 (Matthews' Sites CR4 and CR5) throughout the year except from August through October. During this time, ATP/Chla increased from Site 4 to Site 6 as in this experiment. The ciliates Epistylis and Vorticella were common at this site and rare or absent at Sites 1 and 4. Weber (1973) describes these two genera as indicative of moderate organic pollution.

Laboratory results of nontaxonomic indices were similar to those of the pilot laboratory study except for reference communities. ATP/Chla and chlorophyll concentrations of reference communities were markedly different from exposed communities. This probably resulted from high Oscillatoria concentrations. Oscillatoria comprised approximately 60% of the biovolume in reference communities and was inversely proportional to effluent concentrations (Figure 20). This may have resulted from a nitrogen or micro-nutrient deficiency. The ability to fix nitrogen (Stewart, 1973) gives Oscillatoria a

distinct advantage in 0% effluent exposures where nitrate concentrations were below 1 mg/l and nitrite and ammonia were undetected (Table 17). This genus was present in upstream reference communities yet undetected in downstream reference communities in field tests. This agrees with Weber (1973) who found filamentous cyanobacteria dominated communities upstream of an STP effluent and occurred in low numbers downstream.

Nutrient limitation (Healey, 1975) and high light intensities decrease cellular chlorophyll concentrations (Fogg, et al., 1973). The olive-brown color of reference communities and the corresponding low chlorophyll concentrations support this. The Oscillatoria in effluent-exposed communities were dark blue-green. Most cyanobacteria assume a yellow-brown color with nitrogen deficiencies (Fogg, et al., 1973). Compounding this are the difficulties involved with extracting chlorophyll from cyanobacteria (Riemann and Ernst, 1982; Marker, 1972; and Rai, 1973). Reference communities were the only ones deviating from chlorophyll a to biovolume comparisons (Figure 17) and from agreement of ATP/Chla to total biovolume/chlorophyllous biovolume (Figure 18). The general structure of reference communities was similar to effluent exposed communities if biovolume ratios were substituted for ATP/Chla.

Increased chlorophyll concentrations in effluent exposed communities probably results from increased diatom biovolumes, which are easily extracted (Marker, 1972), and decreased numbers of Oscillatoria. The Oscillatoria component of 25% effluent

communities increased the chlorophyllous biovolume to approximately 30% more than in 50% communities (Figure 27); yet chlorophyll concentrations were similar. In this study, errant conclusions regarding community structure would result if only ATP/Chla was considered.

Oscillatoria concentrations were proportional to measurement variability. This occurred because the entangled filaments were difficult to homogenize without increasing handling stress.

Higher adenylate and chlorophyll concentrations in laboratory tests relative to field tests may be due to increased temperatures, lower chlorine residuals, lower flow rates and lack of grazers in laboratory treatments. Microcosm temperatures and adenylate concentrations were more similar to those of the second field translocation study. Even though adenylate concentrations were similar, effluent exposed communities were dominated by heterotrophs in the field and autotrophs in the laboratory. Reduced laboratory flow rates preclude the physical scouring effects of field flow conditions and encouraged growth of lentic forms. Water movement is an important factor limiting the accumulation of cells unable to attach to a substrate (Weitzel, 1979).

Manuel and Minshall (1978) described similar yet opposite results due to current and/or grazing differences between a stream and streamside microcosms enriched with nitrogen. Scouring in the stream minimized algal biomass accumulations and differences between biomass/chlorophyll ratios. In contrast,

both enriched and unenriched (control) microcosms supported abundant periphyton growth. The primary difference between real and artificial streams was current velocity and grazing. Nutrient enrichment increased the abundance of Nitzschia spp. as occurred in Cedar Run laboratory studies.

Metal chelation and lower chlorine residuals may have resulted during storage of effluent prior to delivery. Organic compounds in the effluent should chelate heavy metals, reducing potential toxicity. Nutrient enrichment significantly reduced the toxic effects of cadmium in microcosm studies (Hendrix et al., 1981). They found densities of taxonomic groups to be the most consistent indicators of cadmium effects and biomass and plant pigments the least sensitive. They suggested the use of biomass accumulations and pigment ratios in future studies. Metal chelation might explain the lower Chla/Pheo of communities in 12% and 25% effluent concentrations. It is possible that metals were not chelated as completely in these treatments and detrimentally affected the algae.

Close agreement between ATP and biovolumes, and ATP/Chla and biovolume ratios suggests that the nontaxonomic indicator, ATP/Chla is a good measure of community structure. However, inaccuracies may result if environmental conditions induce aberrant chlorophyll or adenylate concentrations within a dominant portion of the community or extraction efficiencies vary widely between community types. Comparing ATP/Chla to

biovolume ratios may be useful in detecting conditions which alter chlorophyll to biovolume relationships.

Laboratory simulations of lotic environments may result in more accurate predictions if the diluent was supplemented with essential nutrients and increased flow rates were provided. Simulations of lentic environments might provide more accurate predictions.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The objectives of these studies were:

1) to determine if adenylate and chlorophyll concentrations of microbial communities shift from handling stress incurred with the delay between field collection and laboratory analyses and to optimize extraction techniques,

2) to compare ATP/Chla, ATP/ADP and Chla/Pheo responses of stressed microbial communities to each other and to taxonomic responses,

3) to determine if ATP/Chla ratios accurately assess community structure by comparing with biovolume estimates and,

4) to determine if field responses are predictable through laboratory microcosm studies.

Adenylate and chlorophyll concentrations may shift with handling stress and the magnitude of shift appears to depend on the community in question; however, the shifts detected in this study were minor when compared to differences between treatments. Adenylate extractions were optimized by sealing the extraction tubes and vortexing during the extractions. Field extractions were less efficient and more variable than laboratory extractions because high extraction temperatures were difficult to maintain in the field. Acid extractions were less efficient and more variable than TRIS extractions. ATP/ADP ratios correlate with energy charge measures for a variety of organisms, experimental conditions and analytical techniques.

The non-taxonomic measures of microbial responses, ATP/Chla, Chla/Pheo, and ATP/ADP were consistent under different field treatments as were taxonomic responses. Communities exposed to STP effluent had higher ATP/Chla and ATP/ADP and lower Chla/Pheo, diversity and richness than reference communities. ATP/Chla measures agreed with total biovolume/chlorophyllous biovolume ratios as indicators of community structure with all communities except those dominated by Oscillatoria. Protozoan structural responses were most definitive of environmental conditions; however, diatom communities may be equally so if living organisms were distinguished from empty frustules.

The ATP/Chla ratio is a structural measure like species diversity but describes the relative abundance of heterotrophs to autotrophs, i.e., high values are indicative of heterotrophic dominance. However, this measure can be biased by errant adenylate and/or chlorophyll extraction efficiencies. Microscopic examinations (however brief) should be utilized to determine if a community is dominated by organisms that have extraordinary extraction characteristics. ATP/Chla measures were not indicative of community structure when Oscillatoria dominated because these organisms had either low chlorophyll concentrations and/or were difficult to extract. In this case ATP/Chla estimates did not agree with biovolume estimates.

ATP/ADP ratios indicate the metabolic status of a cell. Community measurements using this ratio indicate how that particular community responds to its environment. In these studies several taxa were well suited to a perturbed environment; these groups florished at the expense of others and developed high adenylate ratios indicating stored energy and a large growth potential. Communities in an unperturbed environment had low ATP/Chla and high Chla/Pheo ratios and species diversities. Competition for limited resources produced a community with lower energy reserves and thus lower potential for growth than one dominated by a few species under conditions stressful to most species. Therfore, communities with high adenylate ratios are not necessarily associated with unperturbed environments. This is not to be interpreted to mean that communities with low growth potentials are associated with unperturbed conditions. In this study, adenylate ratios of indigenous communities exposed to organic pollution were not good indicators of environmental stress. This measure may be useful for assessing microbial responses to short-term exposures of toxic compounds and comparing the responses of different communities to the same stress.

Responses of communities transferred from upstream to downstream of the STP tend toward those of endogenous downstream communities. Chla/Pheo responses were quick while ATP/Chla and ATP/ADP responded more gradually. Responses of transferred communities were not entirely predictable from laboratory

microcosm studies. Further investigations are necessary to determine which factors are critical for laboratory predictions.

The low predictive capability of these microcosm studies may have been due to low flow conditions, chemical changes of the effluent during storage, and lack of essential nutrients in the diluent. Predictive capability may be enhanced by correcting these problems. Laboratory simulations utilizing the techniques of this study may be more suitable for predicting the effects of short-term exposures to toxic compounds rather than nutrient enrichment.

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APPENDIX

TAXONOMIC SUMMARIES

Table A1. Biovolume concentrations of dominant organisms from 0% and 50% laboratory effluent exposures from immediate and delayed laboratory extractions.

	0%	50%
Taxa	um^3 (10^5) / ml PFU	
Cymbella spp.	23	0
Gomphonema spp.	0	0
Melosira varians	105	0
Achnanthes spp.	0	16
Navicula spp.	225	378
N. cuspidata	0	0
Nitzschia spp.	603	9000
Surirella ovata	2	45
Ankistrodesmus spp.	0	0
Characium spp.	0	0
Spirogyra spp.	0	0
Scenedesmus spp.	3	57
Oscillatoria spp.	270	0
Achlorophyllous < 20um	843	5022
Chlorophyllous < 20um	1305	8890
Protozoans	460	600
Total	3839	24009
% of Total		
% Diatoms	24.9%	39.3%
% Green algae > 20 um	0.1%	0.2%
% *Oscillatoria*	7.0%	0.0%
% Achlorophyllous <20um	22.0%	20.9%
% Chlorophyllous <20um	34.0%	37.0%
% Protozoans	12.0%	2.5%

Table A2. Biovolume concentrations of dominant organisms from reference and transferred communities from field translocation study.

Taxa	Site 1	Site 2	Tran 4
	um^3 (10^5) / ml PFU		
Cymbella spp.	13	0	0
Gomphonema spp.	0	0	0
Melosira varians	140	0	0
Achnanthes spp.	10	6	21
Navicula spp.	1814	414	1620
N. cuspidata	0	0	0
Nitzschia spp.	2075	153	1152
Surirella ovata	53	0	0
Ankistrodesmus spp.	0	0	0
Characium spp.	0	0	0
Spirogyra spp.	0	0	0
Scenedesmus spp.	5	209	229
Oscillatoria spp.	0	0	0
Achlorophyllous < 20um	2853	10188	9414
Chlorophyllous < 20um	2808	5796	5634
Protozoans	1160	7200	5720
Bacteria	608	680	1168
Total	11541	24646	24958
% of Total			
% Diatoms	35.6%	2.3%	11.2%
% Green algae > 20 um	0.0%	0.8%	0.9%
% Oscillatoria	0.0%	0.0%	0.0%
% Achlorophyllous < 20 um	24.7%	41.3%	37.7%
% Chlorophyllous < 20 um	24.3%	23.5%	22.6%
% Protozoans	10.1%	29.2%	22.9%
% Bacteria	5.3%	2.8%	4.7%

Table A3. Biovolume concentrations of dominant organisms from field portion of combined field and laboratory study.

	Site 1	Site 4	Tran 4	Site 6	Tran 6
Taxa		um^3 (10^5) / ml PFU			
Cymbella spp.	58	26	39	13	13
Gomphonema spp.	10	10	15	35	15
Melosira varians	105	140	105	0	35
Achnanthes spp.	8	20	5	77	60
Navicula spp.	1040	360	366	252	198
N. cuspidata	377	16	27	0	10
Nitzschia spp.	1377	378	684	1152	1026
Surirella ovata	170	31	43	0	11
Ankistrodesmus spp.	0	45	32	17	18
Characium spp.	0	448	266	117	117
Spirogyra spp.	0	0	0	0	0
Scenedesmus spp.	2	147	144	78	62
Oscillatoria spp.	504	0	45	0	126
Achlorophyllous <20um	1224	14733	1710	25740	25758
Chlorophyllous <20um	2007	8901	5643	11439	10908
Protozoans	1388	5720	3600	3320	1440
Bacteria	292	3872	4728	2438	1170
Total	8563	34849	17456	44679	40969
% of Total					
% Diatoms	36.7%	2.8%	7.4%	3.4%	3.3%
% Green algae >20um	0.0%	1.8%	2.5%	0.5%	0.5%
% Oscillatoria	5.9%	0.0%	0.3%	0.0%	0.3%
% Achlorophyllous <20um	14.3%	42.3%	9.8%	57.6%	62.9%
% Chlorphyllous <20um	23.4%	25.5%	32.3%	25.6%	26.6%
% Protozoans	16.2%	16.4%	20.6%	7.4%	3.5%
% Bacteria	3.4%	11.1%	27.1%	5.5%	2.9%

Table A4. Biovolume concentrations of dominant organisms from laboratory portion of combined field and laboratory study.

Taxa	0%	50%	25%	12%
	um^3 (10^5) / ml PFU			
Cymbella spp.	29	26	32	6
Gomphonema spp.	3	40	10	35
Melosira varians	728	336	1715	875
Achnanthes spp.	6	94	55	7
Navicula spp.	1220	2646	4194	1800
N. cuspidata	39	4916	1424	553
Nitzschia spp.	7317	31392	22068	18810
Surirella ovata	160	340	350	120
Ankistrodesmus spp.	0	172	176	40
Characium spp.	0	0	0	0
Spirogyra spp.	825	500	375	500
Scenedesmus spp.	8	1481	1882	335
Oscillatoria spp.	36000	2610	22950	18810
Achlorophyllous < 20um	3762	7101	15669	4599
Chlorophyllous < 20um	2502	16101	20403	6084
Protozoans	1000	1240	1240	1160
Bacteria	209	500	590	430
Total	53800	69500	93100	54200
% of Total	99.6%	99.3%	99.4%	99.2%
% Diatoms	17.7%	57.3%	32.0%	41.0%
% Green algae > 20um	1.6%	3.1%	2.6%	1.6%
% Oscillatoria	66.9%	3.8%	24.6%	34.7%
% Achlorophyllous < 20um	7.0%	10.2%	16.8%	8.5%
% Chlorophyllous < 20um	4.6%	23.2%	21.9%	11.2%
% Protozoans	1.9%	1.8%	1.3%	2.1%
% Bacteria	0.4%	0.7%	0.6%	0.8%

Table A5. DIATOM OCCURRENCE

DUPERF 4	DTNSTS 4	DTARNS 6	DTNRAS 6	LAB 0 %	LAB 05 %	LAB 52 %	LAB 21 %	
-	X	X	- -	-	-	-	-	Achnanthes affinis
-	X	-	- -	-	-	-	-	A. deflexa
-	X	X	X -	-	-	-	-	A. hauckiana
-	X	X	X X	-	-	-	-	A. hustedii
X	X	-	- -	-	-	-	-	A. linearis
X	X	X	X X	X	X	X	X	A. lanceolata
-	X	-	X X	-	X	-	X	A. lanccelata v. omissa
-	-	X	- -	X	X	X	-	A. microcephala
X	X	X	X X	X	X	X	X	A. minutissima
-	-	-	- -	-	X	-	X	Amphora normanii
X	-	X	X X	X	-	-	X	A. ovalis
-	-	X	- X	X	-	-	X	A. ovalis v. pediculus
-	X	X	X X	-	-	X	-	A. perpusila
X	X	X	X X	X	X	X	X	A. submontana
X	X	-	X -	X	-	X	X	Caloneis bacillum
-	-	-	- -	X	-	-	-	C. bacillaris v. themalis
-	-	-	- -	X	-	-	-	C. lewisii
-	-	-	- -	X	-	X	-	Campylodiscus hibernicum
X	X	X	X X	X	X	X	X	Cocconeis pediculus
X	X	X	X X	X	X	X	X	C. placentula
X	X	X	X X	-	-	-	-	Cyclotella meneghinana
X	X	X	X X	X	X	X	X	Cymatopleura solea
-	-	-	- X	X	X	X	X	Cymbella affinis
X	X	X	X X	X	X	X	X	C. amphicephala
-	X	-	- -	-	-	-	-	C. gibba
-	-	-	- X	-	-	X	X	C. microcephala
X	X	X	X X	X	X	X	X	C. minuta
X	X	X	X X	X	X	X	X	C. minuta v. silesiaca
X	X	X	X X	X	X	X	X	C. prostata
X	X	X	X X	X	X	X	X	C. sinuata
X	X	X	X X	X	X	X	X	C. tumida
-	X	-	- -	-	-	-	-	C. triangulum
X	-	-	- -	X	-	X	-	C. turgidula
X	-	-	- -	X	X	X	X	Diatoma tenue
X	X	X	X X	X	X	X	X	D. vulgare
X	-	X	- -	X	X	X	X	D. vulgare v. breve
X	-	-	X X	X	X	X	-	Denticula tenuis

LEGEND: X present, - absent

Table A5. DIATOM OCCURRENCE (cont.)

UPREF	DNSTR4	TRANS4	DNSTR6	TRANS6	LAB 0%	LAB 50%	LAB 25%	LAB 12%	
X	X	X	-	-	X	X	X	X	*Diploneis elliptica*
X	X	X	X	X	X	X	X	X	*D. oblongella*
-	X	-	X	-	-	-	-	-	*Epithemia gibberula*
X	-	-	-	-	-	-	-	X	*Eunotia arcus*
-	X	X	X	X	-	X	X	-	*E. curvata*
-	X	X	-	-	-	-	X	X	*E. pectinalis* v. *minor*
-	X	X	-	-	-	-	-	-	*E. pectinalis* v. *recta*
-	-	-	X	-	-	-	-	-	*E. pectinalis* v. *ventricosa*
-	-	-	X	X	-	-	-	X	*E. vanheurickii* v. *intermedia*
-	X	-	-	-	X	X	X	-	*Fragilaria capucina* v. *mesolepta*
-	X	-	-	-	-	-	-	-	*F. leptostauron* v. *dubia*
-	-	-	X	-	-	-	-	-	*F. pinnata* v. *lanceolata*
X	X	X	X	X	X	X	X	X	*Frustulia rhomboides*
-	X	-	-	-	-	-	-	-	*Gomphonema abreviatum*
-	X	X	-	X	-	X	-	-	*G. acuminatum*
X	X	X	-	-	-	-	-	-	*G. acuminatum* v. *pusilla*
-	X	X	-	-	-	-	-	-	*G. affine*
X	X	X	X	X	X	X	X	X	*G. angustatum*
X	X	-	X	-	X	-	-	X	*G. angustatum* v. *sarcophagus*
-	-	-	X	-	X	-	-	-	*G. angustatum* v. *citeria*
X	-	X	-	-	X	X	X	X	*G. clevei*
X	X	-	X	X	X	X	X	-	*G. gracile*
-	-	-	X	X	-	-	-	-	*G. intricatum*
X	X	X	X	X	X	X	X	X	*G. olivaceum*
X	X	X	X	X	X	X	X	X	*G. parvulum*
-	X	X	X	X	-	-	X	-	*G. sphaerophorum*
-	X	-	X	X	X	X	X	-	*G. subclavatum*
-	X	X	-	-	-	-	-	-	*G. subclavatum* v. *mexicanum*
-	X	X	X	X	X	X	X	X	*G. tenellum*
X	-	X	-	-	-	-	-	-	*G. tergestinum*
X	X	X	-	X	X	X	X	X	*G. truncatum*
X	X	X	-	-	X	X	X	-	*Gyrosigma scalproides*
-	-	-	X	X	-	-	-	-	*Hantzschia amphioxys*
X	X	-	X	X	X	X	X	-	*H. amphioxys* f. *capitata*
X	X	X	X	X	X	X	X	X	*Melosira varians*
X	X	X	X	X	X	X	X	X	*Meridion circulare*
-	X	-	-	-	X	-	-	-	*M. circulare* v. *constricta*
-	-	-	-	-	X	X	X	X	*Navicula cuspidata*
X	X	X	-	X	X	X	X	X	*N. cuspidata* v. *ambigua*

Table A5. DIATOM OCCURRENCE (cont.)

UPREF	DNSTR4	TRANS4	DNSTR6	TRANS6	LAB 0%	LAB50%	LAB25%	LAB12%	
X	-	X	-	-	-	-	X	-	Navicula cocconeiformis
X	X	X	X	X	X	X	X	X	N. cryptocephala
X	-	-	-	-	X	-	-	X	N. exigua v. capitata
-	X	X	-	-	-	X	X	X	N. greggaria
-	X	-	-	-	-	-	-	X	Navicula grimmei
-	-	X	-	-	-	X	-	X	N. mutica
-	X	X	X	X	-	X	-	-	N. mutica v. stigma
X	X	X	X	X	X	X	-	-	N. mutica v. tropica
-	-	X	X	-	X	X	-	X	N. pupula
X	X	-	-	-	-	-	-	X	N. pupula v. capitata
X	X	-	-	-	-	-	-	-	N. pupula v. mutata
X	X	X	-	X	-	-	-	X	N. pupula v. rectangularis
X	-	X	-	X	X	X	X	X	N. pygmea
X	X	X	X	X	X	X	X	X	N. radiosa
X	X	X	X	X	X	X	X	X	N. rynchocephala
X	-	X	X	-	-	-	X	-	N. salinarum
X	X	X	X	X	X	X	X	X	N. tripunctata
X	X	X	X	X	X	X	X	X	N. viridula
-	-	-	-	-	-	X	X	-	Nedium iridis
X	-	X	-	-	X	X	X	X	N. iridis v. ampliatum
X	-	-	-	-	-	-	-	X	N. temperei
X	X	X	X	X	X	X	X	X	Nitzschia acicularis
X	X	X	X	X	X	X	X	X	N. amphibia
X	-	X	-	X	X	X	X	X	N. angustata
X	X	X	X	X	X	X	X	X	N. apiculata
X	X	X	-	-	X	X	X	X	N. denticula
-	-	X	-	-	-	-	-	-	N. denticula v. tabellariae
X	X	X	X	X	X	X	X	X	N. dissipata
X	-	-	-	X	X	X	X	-	N. fasiculata
X	X	X	X	X	X	X	X	X	N. filiformis
X	X	X	X	X	X	X	X	X	N. fonticula
X	X	X	X	X	X	-	-	-	N. gracilis
X	X	X	X	X	X	X	X	X	N. hungarica
X	X	X	X	X	X	X	X	X	N. linearis
X	X	X	X	X	X	X	X	X	N. palea
X	X	X	X	X	X	X	X	X	N. paradoxa
X	-	X	-	X	X	X	X	X	N. parvula
X	-	-	-	-	X	X	X	X	N. sigma
X	-	X	-	-	X	X	X	X	N. sigmoidea

Table A5. DIATOM OCCURRENCE (cont.)

UPREF	DNSTR4	TRANS4	DNSTR6	TRANS6	LAB 0%	LAB50%	LAB25%	LAB12%	
-	X	-	-	-	-	-	-	-	*Nitzschia sinuata* v. *tabellarae*
X	-	-	X	X	X	X	X	X	*N. triblionella*
-	X	X	X	-	-	X	-	-	*Peronia intermedium*
X	X	-	X	X	-	-	-	-	*Pinnularia biceps*
-	-	X	X	X	X	X	X	-	*P. brebissonii*
-	-	-	-	-	-	X	X	-	*Pinularia maior*
-	-	-	-	-	X	X	-	-	*P. obscura*
X	-	X	-	X	X	X	X	X	*P. torta*
X	X	X	X	X	X	X	X	X	*Rhoicosphenia curvata*
X	-	-	-	X	X	X	X	X	*Rhopaloidia gibba*
-	-	X	-	-	X	X	X	-	*R. gibberula*
-	-	-	-	-	-	-	X	-	*Stauroneis smithii*
X	X	X	X	X	X	X	X	X	*Surirella angustata*
X	X	X	X	X	X	X	X	X	*S. ovalis*
X	X	X	X	X	X	X	X	X	*S. ovata*
-	-	-	-	-	X	X	X	X	*S. robusta*
X	X	X	X	X	X	X	-	-	*Synedra goulardii*
X	X	X	-	-	X	X	X	X	*S. parvula*
X	X	X	X	X	X	X	X	X	*S. pulchella* v. *lanceolata*
X	X	X	X	X	X	X	X	X	*S. ulna*
-	X	X	X	X	-	-	-	-	*S. ulna* v. *spathulifera*

Table A6. PROTOZOAN OCCURRENCE

T R L E V	U P R F	D N S T E R 4	T R A N R S 4	D N S T R S 6	T R A N S 6	L A B 0 %	L A B 0 %	L A B 5 %	L A B 2 %	
N*		-	-	-	X		-	-	-	Acanthocystis sp.
R		-	-	-	-		X	X	-	Acineta sp.
N		X	-	-	-		-	-	X	Actinophrys sp.
N		-	-	-	-		X	X	X	Actinosphaerium sp.
N		-	X	-	X		-	X	-	Amoeba dubia
B		X	-	-	-		-	X	-	Anisonema acinus
B		X	-	X	X		-	-	-	A. pusillum
B		X	-	-	X		X	X	X	Aspidisca costata
B		-	-	X	X		-	-	-	A. lynceus
S		X	-	X	-		-	-	-	Astasia klebsi
B		X	-	-	-		-	X	-	Balladyna elongata
B		-	-	X	X		-	-	-	B. parvula
B		X	X	-	X		X	-	X	Bodo edax
B		X	X	-	X		X	-	X	B. globosa
B		X	-	X	-		X	X	X	B. minimus
B		-	X	-	-		-	-	-	B. putrinus
B		X	X	X	X		X	X	X	B. variabilis
B		-	-	X	-		-	-	-	Cercomonas crassicauda
B		-	X	X	-		-	-	-	C. longicauda
B		-	-	-	-		-	X	-	Chilodenela caudata
B		X	-	-	-		X	X	X	C. nana
B		-	-	-	-		-	X	-	C. cucullus
B		X	-	-	-		X	X	-	C. uncinata
P		X	-	-	-		X	-	X	Chlamydomanas cigar
P		X	-	-	-		-	-	-	C. globosa
P		X	-	-	-		-	-	-	C. monadina
P		-	-	X	X		-	X	X	C. reinhardi
P		X	-	X	X		X	-	-	Chlamydomonas spp.
P		-	-	-	-		-	-	X	Chromulina caudata
P		-	-	-	-		X	-	-	C. globosa
P		X	X	-	-		X	X	X	Chroomonas nordstedii
P		X	-	-	X		-	-	-	C. reflexa
B		X	-	-	X		X	X	X	Cinetochilum margaritaceum
B		X	-	-	X		-	-	-	Cochliopodium sp.
B		-	-	-	-		-	X	X	Coleps uotospinus

LEGEND: X Present, - Absent

Table A6. PROTOZOAN OCCURRENCE (cont.)

TROPHIC LEVEL	UPSTREAM FROM DAM 4	DOWNSTREAM FROM DAM 4	UPSTREAM FROM DAM 6	DOWNSTREAM FROM DAM 6	LAB 0%	LAB 5%	LAB 25%	LAB 100%	
B	-	X	X	X	X	-	-	-	-	Colpidium campylum
B	X	-	-	-	-	-	-	-	-	Colpoda aspera
B	-	-	-	-	-	-	X	-	-	C. cucullus
P	X	-	-	-	-	X	X	X	X	Cryptomonas ovata
P	X	-	-	-	-	-	-	-	-	C. platyuris
B	X	-	-	-	-	-	-	-	-	Ctedoctema acanthocry
B	-	-	-	X	X	-	-	-	-	C. ovalis
B	X	-	-	-	-	X	X	X	X	Cyathomonas truncata
B	X	-	-	X	X	X	X	X	X	Cyclidium glaucoma
B	X	-	-	-	-	-	-	-	-	Cyphoderia sp.
B	-	-	-	-	-	X	-	-	-	Cyrtolophosis major
B	-	-	-	-	-	-	-	-	X	Difflugia sp.
B	-	-	-	X	-	-	-	-	-	Enchelydium fusidens
B	-	-	-	-	-	-	X	X	-	Enchelys sp.
B	-	-	-	-	-	X	X	X	-	Entosiphon sp.
B	-	-	-	X	X	-	-	-	-	Epistylis sp.
P	X	X	X	X	X	-	-	-	-	Euglena acus
P	-	-	-	-	-	-	-	-	X	E. deses
P	X	-	-	-	-	X	X	X	X	E. spirogyra
A	-	-	-	-	-	-	X	-	-	Euplotes patella
B	-	-	-	X	-	-	-	-	-	Filamoeba sp.
A	X	-	-	-	-	X	-	-	X	Frontonia lecus
B	-	-	-	-	-	-	X	-	-	Glaucoma pyriformis
B	-	-	-	-	-	X	X	X	X	Halteria grandinella
B	X	X	-	X	X	-	X	X	X	Hartmanella sp.
B	-	-	-	-	-	-	-	X	-	Hemiophrys agilis
B	-	-	-	-	-	-	-	X	-	H. plearosigma
B	-	X	-	-	-	-	-	-	-	Heteronema sp.
B	-	-	-	-	X	-	X	-	-	Holophra sp.
A	-	-	-	-	-	X	-	X	X	Lembandion bullinum
P	X	-	-	-	-	-	-	-	-	Lepocinclis ovum
B	-	-	-	-	-	-	X	X	-	Litonotus cygnus
B	-	X	-	-	X	X	X	X	X	L. fasciola
B	-	-	-	-	X	-	X	X	X	L. hirundo
B	-	-	-	-	-	X	-	-	-	Loxodes vorax

Table A6. PROTOZOAN OCCURRENCE (cont.)

TRLEV	UPREF	DNSTR4	TRANS4	DNSTR6	TRANS6	LAB0%	LAB50%	LAB25%	LAB12%	
B	-	-	-	X	X	-	X	X	-	Mayorella sp.
B	-	-	-	-	-	-	X	-	X	Metopus sp.
B	X	-	X	X	X	-	-	-	-	Monas sociabilis
B	X	X	X	X	X	X	X	X	X	Monas sp.
B	-	-	X	X	X	X	X	X	X	Naegleria gruberi
A	-	-	-	-	-	-	-	X	X	Nassala sp.
B	X	-	-	-	-	-	-	-	-	Notosolenus apocamptas
B	X	-	-	-	-	X	-	-	-	N. sinuatus
P	X	-	-	-	-	-	-	-	-	Ochromonas sp.
B	X	X	X	X	X	X	X	X	X	Oikomonas sp.
B	X	-	-	-	-	-	X	-	-	Oxytricha fallax
B	-	X	-	X	-	-	X	X	X	O. ludibunda
B	-	-	-	-	-	-	X	X	-	O. ovalis
B	-	X	-	-	X	-	-	-	-	O. similis
P	-	-	-	-	-	-	X	-	X	Paramecium bursaria
P	-	-	-	-	-	-	X	-	-	P. caudata
B	-	-	-	-	-	X	-	-	X	Pelomyxa
B	-	-	-	-	X	-	-	-	-	Peranema grandulifer
B	X	X	-	-	-	X	-	X	-	P. trichophor
B	X	-	-	-	-	-	-	-	-	Petalomonas mediocanel
B	-	-	-	-	-	-	X	-	-	P. mira
B	X	-	-	X	X	X	-	X	-	P. pussilum
B	X	-	-	X	-	X	X	-	X	Physamonas vestita
B	X	-	-	X	X	X	-	X	X	Pleuromonas jaculans
B	-	-	-	X	-	-	-	-	X	Pseudodifflugia sp.
S	-	-	-	X	-	-	-	-	-	Rhabdomonas spiralis
B	X	-	-	X	X	-	-	-	-	Rhynchomonas nasuta
B	-	-	-	-	-	X	-	-	-	Spathidium sp.
A	-	-	-	X	-	-	-	-	-	Strobidium sp.
A	X	-	-	-	-	-	-	-	X	Strobilidium gyrans
N	-	-	-	-	X	X	-	X	X	Stylonchia mytilus
B	-	-	-	-	-	-	X	-	-	S. putrina
B	X	-	X	X	X	X	X	-	-	Sterromonas sp.
R	-	-	-	X	-	-	-	-	-	Trachelius ovum
B	-	-	-	-	X	-	X	X	X	Trachelophyllum pussilum

Table A6. PROTOZOAN OCCURRENCE (cont.)

TRLEV	UPREF	DNSTR4	TRANS4	DNSTR6	TRANS6	LAB 0%	LAB 50%	LAB 25%	LAB 12%	
B	-	-	-	-	-	-	X	-	-	*Trichopelma* sp.
B	-	-	-	-	-	-	-	X	X	*Trochilia minuta*
B	X	-	-	-	-	-	-	-	-	*T. paulustris*
B	X	-	-	-	-	-	-	-	X	*Thecamoeba* sp.
B	-	-	-	-	X	-	-	-	-	*Trichamoeba* sp.
B	-	-	-	-	-	X	-	-	-	*Uroleptus limnetis*
B	-	-	-	-	-	X	-	-	-	*Urotricha* sp.
B	-	-	-	-	X	-	-	-	-	*Urostyla gracilis*
B	-	-	-	-	-	X	-	-	-	*Urostyla* sp.
B	-	-	-	-	-	-	X	-	-	*Vahlkampfia* sp.
B	-	-	-	X	-	-	-	-	-	*Vanella* sp.
B	-	-	-	X	X	X	X	X	X	*Vorticella microstoma*
B	-	-	-	X	X	X	X	X	X	*V. picta*
B	-	-	-	-	-	-	X	-	-	*Vorticella* sp.

* Trophic levels:
A = algae eater B = bacteriovore N = non-specific
P = producer R = raptor S = saprobe

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