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By William M. Dunstan and David W. Menzel

Reprinted from Limnology and Oceanography
Vol. 16, No. 4, July 1971
pp. 623–632
Made in the United States of America

CONTINUOUS CULTURES OF NATURAL POPULATIONS OF PHYTOPLANKTON IN DILUTE, TREATED SEWAGE EFFLUENT[1]

William M. Dunstan and David W. Menzel[2]
Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

ABSTRACT

Seawater diluted with secondary-treated sewage effluent provides excellent enrichment for the maintenance of mixed natural populations of marine phytoplankton in continuous culture. Treated effluent, sampled over 1 year, was consistent in the ratios of plant nutrients and similar in its properties of plant growth stimulation and level of toxicity. The heterogeneous continuous culture system produced large quantities of plant carbon with the concomitant removal of nitrogen and phosphorus from sewage effluent. The plant species that grew in the continuous cultures were common to the typical coastal phytoplankton and the selection and elimination of species was gradual considering the chemical complexity of the sewage effluent enrichment.

INTRODUCTION

Effluents from municipal sewage treatment plants have exerted an increasingly important influence in the coastal marine environment. Sewage outfalls into estuaries and coastal waters have increased sharply in the past 15 years (Cronin 1967) and rivers bring increasingly larger quantities of municipal waste into the coastal zone. For example, it has been estimated that 16% of the summer flow of the Hudson River at Yonkers, before effluents from New York City enter the river, is municipal sewage effluent (Howells et al. 1970). Comparable estimates in the Potomac River (Shapiro and Ribeiro 1965) show that effluent loads vary seasonally from 5 to 40% of the river volume.

Several studies have been made of the *in situ* effects on marine populations of sewage outfalls (Allan Hancock Foundation 1965; Hume et al. 1962). The information concerning the phytoplankton is often inconclusive since data on unpolluted baseline conditions is limited and our understanding of phytoplankton ecology in unpolluted waters is far from complete. Even more discouraging in assessing *in situ* alterations is the combination of both municipal and industrial effluents so that ecological effects cannot be assigned specifically to domestic sewage or any other single factor. In Tampa Bay for example, phosphate shipping, developer landfill, municipal sewage and other influences are all imposed on the same area. In the New York Bight it is difficult to distinguish any one factor as influencing planktonic organisms when effluents from the Hudson River, sewage sludge from New York City, and industrial wastes must all be taken into account (Ketchum 1969; Pierce 1969).

Another approach to the problem is to examine the biological effects of controlled sewage additions to large impoundments or artificial estuaries. Odum and Chestnut (1970) initiated experiments on two large ponds artificially seeded with organisms, one treated with sewage effluent. Skulberg (1968) and Oswald and Golueke (1967) described similar experiments on large ponds. Although these pilot studies are important for their realism, they are quite costly and require long-term study.

In the experiments described here the problem of sewage influence on phytoplankton was investigated in large-scale heterogeneous continuous cultures (Her-

[1] Contribution No. 2648 from the Woods Hole Oceanographic Institution. The investigation was supported by National Science Foundation Grant GB-15103.

[2] Present address: Skidaway Institute of Oceanography, Savannah, Georgia 31406.

TABLE 1. *Plant nutrient concentrations (in μg-atom/liter) in treated sewage effluent, surface seawater, and phytoplankton growth medium*

	NH_4-N	NO_3-N	PO_4-P	Si
Sewage effluent, secondary treatment plant. Cranston, R.I. (diluted to 10%)	157.7	1.65	16.9	17.1
Sewage effluent, secondary treatment. National average (10%) (Weinberger et al. 1966)	118.0	24.2	26.4	83.4
Surface water at the Narrows, N.Y.C. (40° 36.4′ N, 74° 03′ W) Sep 1968	41.9	8.6	0.76	17.3
Sargasso Sea surface water (38° 06′ N, 70° 43.5′ W) Sep 1968	3.9	0.1	0.09	1.3
Seawater enrichment medium (Guillard and Ryther 1962) diluted to 10%	177.1	*	7.3	20.0

* NO_3-N or the NH_4-N are used in marine medium.

bert 1961). We felt that more information would be provided by the type of laboratory experimentation which, on one hand, was not hampered by the expense and operational difficulty of artificial impoundments but, on the other hand, was still representative of the natural situation. Large-volume continuous cultures, inoculated with 15 liters of seawater containing its natural phytoplankton population, were enriched with the secondary-treated sewage effluent from a representative municipal plant. The system was monitored for carbon production, chlorophyll, species, cell numbers, and nutrient uptake. Controls were provided by parallel systems with artificial media containing matched levels of nitrogen and phosphorus or with untreated seawater. We thank E. M. Hulburt for his identification and cell counts of the phytoplankton and J. H. Ryther for his valuable criticism of this manuscript.

SEWAGE EFFLUENTS

If the concentration of plant nutrients in a 10 : 1 mixture of seawater and sewage effluent is compared with a "typical" artificial medium used for the growth of marine phytoplankton (Table 1) it is obvious that comparable proportions of nitrogen, phosphorus, and silicon are available for plant growth. Furthermore sewage effluents contain thiamin, biotin, and B12 (Vallentyne 1957) as well as a variety of chelated metals (Bargeman and Parkhurst, unpublished). Some investigators have reported the presence of hormonal plant growth substances (Stirn 1970). But deleterious effects on the natural flora or outright mortality might be expected if significantly high levels of substances such as cyanide, copper, lead, arsenic, cobalt, manganese, zinc, and other toxicants are present in treated sewage (North, Stephens, and North, unpublished). The effluent from a secondary sewage treatment plant in Cranston, Rhode Island, was used for our studies because it is comparable in composition to the national average of secondary sewage effluent (Table 1). The Cranston plant handles a population of 60,000, processes on the average 21.6 million liters/day and has only a small industrial load (3–6%) from a chemical company and a fabric manufacturer. This industrial fraction is considered representative since recent directives require industries either to process their own special wastes or to certify that their waste does not interfere with the treatment process (Chem. Eng. News 1970). Concentrations of some heavy metals in one batch of the undiluted effluent, determined by atomic absorption spectrometry, were (in ppm): Cu, 0.005;

TABLE 2. *Variations in plant nutrient concentrations (µg-atom/liter) in Cranston, Rhode Island, secondary-treated effluent*

Sample date	NH_4-N	NO_3-N	NO_2-N	PO_4-P	Si	Total-N : P
Oct 1969	1,173	41.5	1.5	151	101	8.1
Feb 1970	807	19.3	4.7	95	90	8.8
Apr 1970	1,153	5.6	2.4	124	168	9.4
Jun 1970	2,310	3.8	1.2	234	316	9.9
Nov 1970	2,019	19.0	1.6	183	127	11.2
Jan 1971	1,998	10.1	6.9	227	224	8.9
Mean	1,577	16.5	3.1	169	171	9.4

Fe, 0.01; Mn, 0.02; Pb, <0.10; Hg, 0.0015 (by vapor method); Sb, not detectable; Ni, 0.50; Zn, 0.05.

The major nutrients (N, P, and Si) in the Cranston effluent varied in concentration but, as shown in Table 2, their relative proportions were quite consistent over the sampling period. Experiments were designed using the NH_4-N level as a means of matching different batches and relating the sewage effluent to artificial medium.

EXPERIMENTAL PROCEDURE

Cultures were grown in 15-liter Plexiglas vessels with a circulating-water jacket for temperature control. Light was provided by four 40-W "cool white" fluorescent lights (ca. 10,000 lux incident) on a 12-hr light–dark cycle. Cultures were agitated by a magnetic stirring bar and the vessel was scraped daily with a polypropylene spatula. The continuous flow system was controlled by micropumps (Buchler Instr. Inc. 2-6000) which provided fresh medium; a siphon-gravity system was used for outflow (Fig. 1). Chlorinated sewage effluent was collected directly from the outfall and kept frozen in polycarbonate bottles. Experiments using culture media were based on dilutions of f-medium of Guillard and Ryther (1962). The natural populations used for inocula were collected from Vineyard Sound off Woods Hole and filtered through a 100-µ net to remove the larger animals. The seawater with its remaining plankton population was added to the culture vessel and the pumping of the sewage-enriched filtered seawater or diluted f-medium was immediately begun. The analytical methods used were: particulate carbon (Menzel and Vaccaro 1964), chlorophyll (Yentsch and Menzel 1963; Lorenzen 1966), ammonia (Solórzano 1969), nitrate (Wood et al. 1967), nitrite (Bendschneider and Robinson 1952), phosphorus (Murphy and Riley 1962), silicon (Strickland and Parsons 1968). Cells were concentrated by centrifugation for identification (Hulburt 1970) and in dense cultures counted directly by hemocytometer.

RESULTS

The typical development of the continuous culture under the above conditions

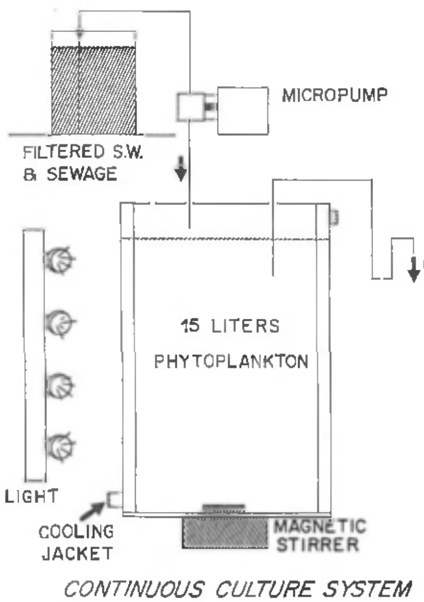

FIG. 1. Continuous culture system.

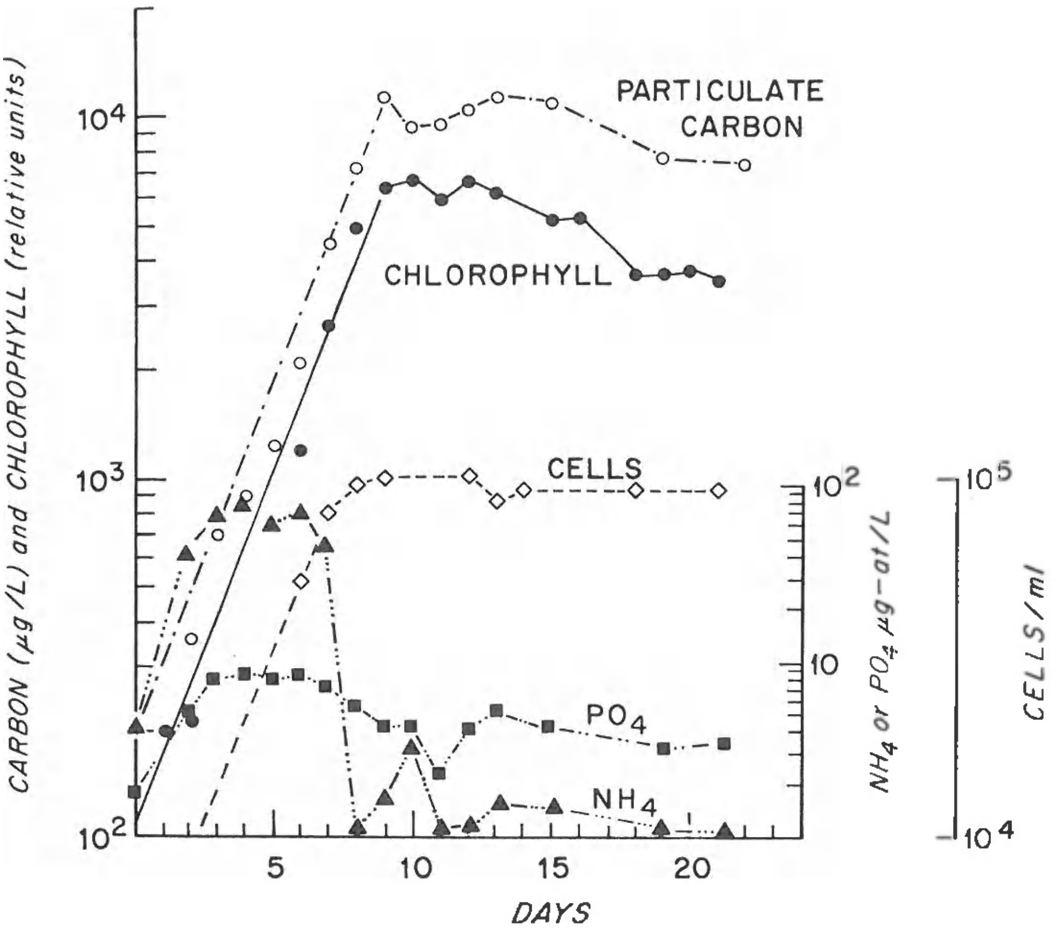

FIG. 2. The change in monitored factors in a typical experiment over a 25-day period.

is shown in Fig. 2. After a period of 3–5 days the system reached an equilibrium in which nutrient uptake and algal production were stable. The mean levels of particulate carbon during this period were used to calculate optimum algal production potential, sewage concentration, and turnover rates (turnover rate, here and elsewhere, refers to the fraction of the culture harvested and replaced with new medium each day). We held the turnover rate constant at 25% of the vessel volume per day to determine the influence of different sewage concentrations on algal production (Fig. 3). The efficiency of production was reduced at sewage concentrations over 10%.

Figure 4 summarizes the results of experiments in which different turnover rates were used with a constant concentration of 10% sewage. The total yield of algal carbon was directly related to turnover rates of from 10–50%. Experiments using rates over 50% indicate that yield is maximal at or near 50%. Even at 150% the cultures were not washed out.

The rate of utilization of nitrogen and phosphorus by the system varied directly with increasing sewage concentration (Fig. 5). The algal population attained a level at each concentration of sewage that efficiently removed all of the nitrogen from the system. Although phosphorus uptake also increased with increasing nutrient

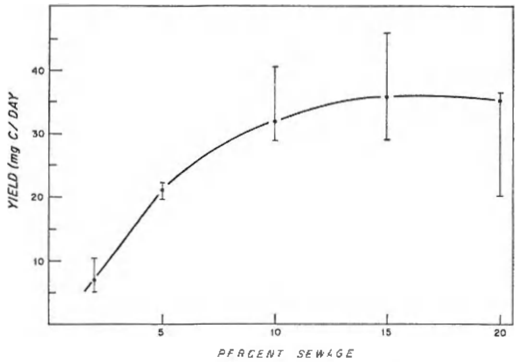

FIG. 3. Sustained particulate carbon production at a turnover rate of 25% with different percentages of sewage. Each point represents the mean of daily particulate carbon measurements over a 15-day period (temperature = 16C).

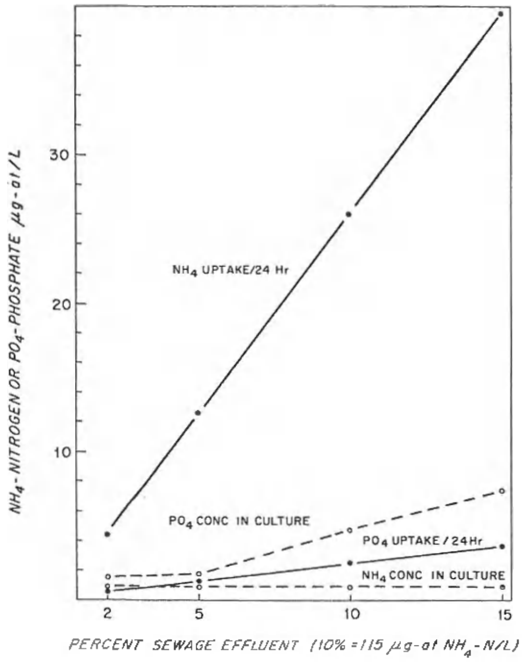

FIG. 5. The nitrogen and phosphorus concentrations maintained in the culture vessels and the rate of uptake/day at different percentages of sewage. Each point represents the mean of daily measurements over a 15-day period.

concentration, all of the phosphate in the medium obviously was not being used and there was a gradual increase of that element in the culture medium with increasing concentrations of sewage (Fig. 5).

Although the continuous cultures reached an equilibrium with respect to phytoplankton biomass and nutrient uptake, the system was certainly not stable in species composition. Selective pressures on the natural populations are exerted by such experimental conditions as light, temperature, culture volume, and containers as well as by the complex sewage mixture, sewage concentration, and turnover rates. In this initial study a variety of conditions was used so that several natural populations could be subjected to a broad range of experimental environments. Fifteen-liter samples from Vineyard Sound taken between November and May were used as starting populations and these were subjected to sewage concentrations of from 1–15%, at temperatures from 10–26C, and at turnover rates of 10–50% of the culture vessel per day. The five most prevalent species, by cell count, were defined in each experiment as the dominant organisms and considered to represent the bulk of the plant biomass. The natural population was often composed of only

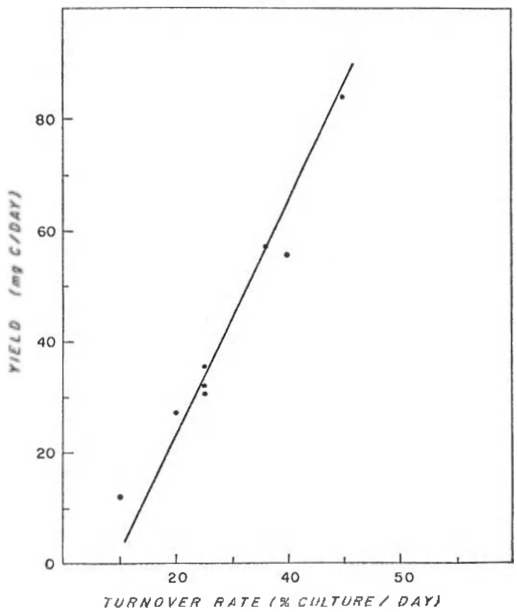

FIG. 4. Sustained particulate carbon production at different turnover rates using 10% sewage (temperature = 16C).

TABLE 3. *Changes in species composition of natural phytoplankton populations after reaching equilibrium in sewage-enriched continuous cultures (summary of 12 experiments)*

	% of experiments in which species were:			
Species	Initially dominant*	Initially and finally dominant	Initially dominant and finally present	Initially dominant and finally absent
Skeletonema costatum	75	67	8	0
Thalassionema nitzschioides	75	75	0	0
Asterionella japonica	58	33	17	8
Pennate sp.	58	17	42	0
Cylindrotheca closterium	42	42	0	0
Licmophora lyngbei	33	8	25	0
Thalassiosira nordenskioldii	33	8	8	17
Paralia sulcata	25	0	0	25
Chaetoceros debilis	25	17	8	0
Leptocylindrus minimus	17	0	8	8
Thalassiosira levanderi	8	0	0	8
Guinardia flaccida	8	0	8	0
Nitzschia seriata	8	0	8	0
Eucampia zoodiacus	8	0	0	8
Rhizosolenia fragilissima	8	0	0	8
Pleurosigma angulatum	8	0	0	8
Chaetoceros compressus	8	0	8	0

* Dominant species are defined as the five most prevalent organisms by cell count.

two or three species which represented 90% of the biomass (Hulburt 1970).

A summary of 12 experiments is shown in Table 3. The cells prevalent in the natural population generally tended to remain dominant and grow in the sewage-enriched continuous cultures. Only *Paralia sulcata*, *Thalassiosira levanderi*, *Eucampia zoodiacus*, *Rhizosolenia fragilissima*, and *Pleurosigma angulatum* did not thrive in the continuous cultures after having been one of the five dominant species in the initial population.

Certain organisms not abundant in the natural population might be expected to be stimulated by sewage effluent and become dominant. This happened in only 2 of the 12 experiments where six species became dominant in culture that were not observed in initial counts of the natural population. These species were *Leptocylindrus minimus*, *Leptocylindrus danicus*, *Cylindrotheca closterium*, *Rhizosolenia delicatula*, *Chaetoceros* sp., and an unidentified dinoflagellate. The first four are not unusual in coastal diatom populations and were, in other experiments, noted in the initial as well as the final population.

Figure 6 shows two representative examples of the changes in species with time in the sewage-enriched cultures and in controls with artificial medium having matched nitrogen and phosphorus concentrations. Also shown (Fig. 6A) are the results of an experiment at a low level of sewage enrichment (1%) with matched artificial medium and filtered seawater as controls.

DISCUSSION

A great deal has been learned about nutrient uptake and growth kinetics of heterotrophic microorganisms through use of the chemostat (Herbert 1961; Jannasch 1967). Eppley and Dyer (1965), Taylor (1960), and Porcella et al. (1970), among others, have applied the principle of the chemostat to the study of unicellular algal populations. These continuous flow systems require that all variables save that being studied be rigidly controlled so that the quantitative effects on population growth kinetics be described mathematically. Although the validity of this approach cannot be questioned, its limitations in the study of ecological problems must be recognized.

A study of the effects of treated sewage

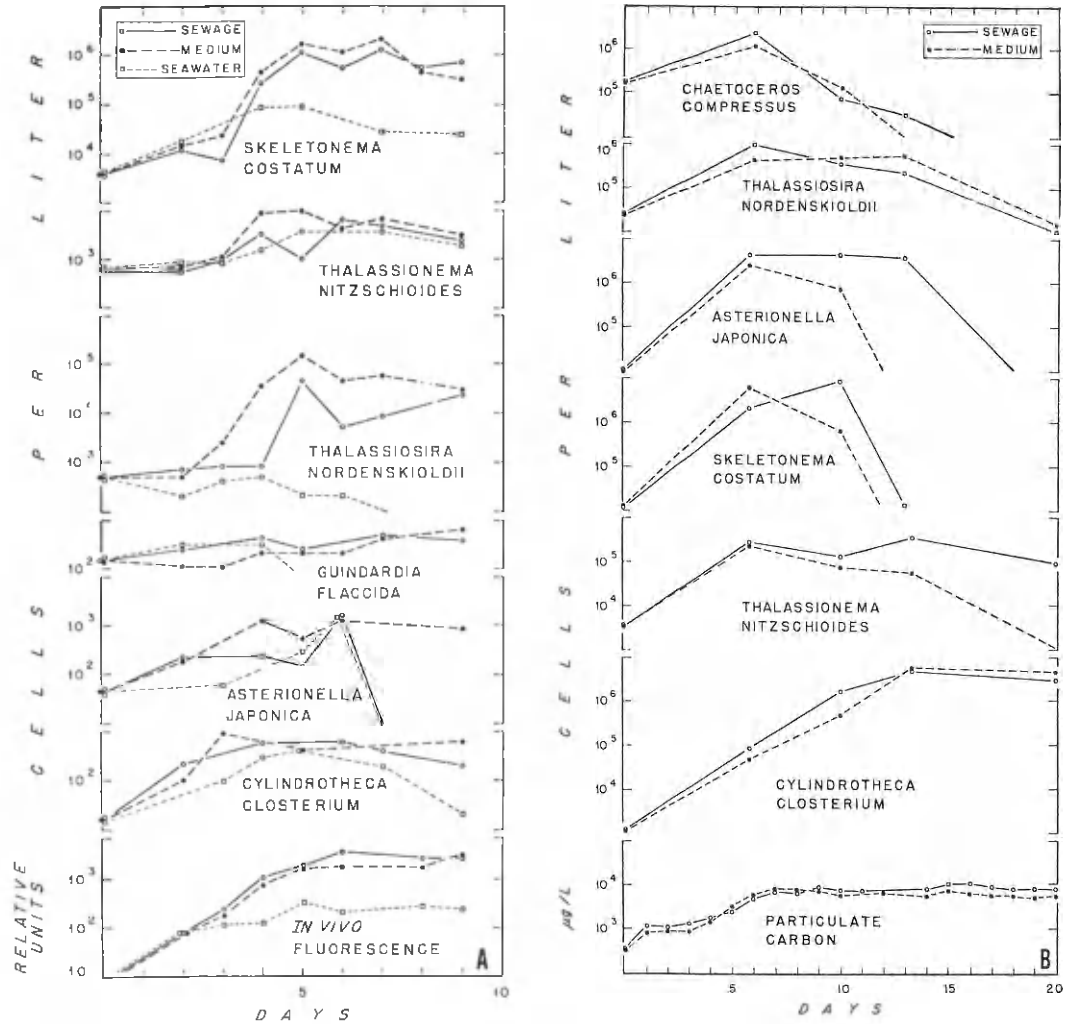

FIG. 6. The succession of the six most prevalent phytoplankton species. A. With 1% sewage enrichment compared to nitrogen-equivalent f-medium and unenriched seawater. November initial population. B. With 10% sewage enrichment and nitrogen-equivalent f-medium. April initial population.

effluent, a variable and partially undefined enrichment (Painter and Viney 1959), on the growth of the heterogeneous plankton community under the highly variable conditions of the estuarine or coastal environment is possible at this time only through an empirical approach. Studying the problem in nature, however, as is usually done, adds another dimension of uncertainty to the fugitive nature of the plankton itself. To circumvent this, we developed an experimental system that would combine the mechanics of a chemostat with the physical, chemical, and biological variability of the natural environment and that would thereby permit the study of a definable plankton population under quasi-natural conditions.

Under these experimental conditions, a continuous or flowing culture could be established in a 15-liter illuminated culture vessel using diluted effluent from a secondary sewage treatment plant as a culture medium. Concentrations of algae in the culture vessel and yields of algae, computed as the output of cells from the continuous culture system, were roughly proportional to the concentration of sewage effluent in the enriched seawater medium up to concentrations of about 20% sewage and 80% seawater. In terms of major nutrients, 20% sewage contains some 300 μmoles of NH_4-N, 30 μmoles of PO_4-P, and 30 μmoles of SiO_3-Si per liter. The lower growth rates and yields at higher levels of sewage may have resulted from toxicity of one or more of the major plant nutrients or simple dilution of the seawater. As the phytoplankton consisted predominantly of a mixture of marine diatoms that have rather high salinity requirements, dilution to a salinity of ca. 22‰ is suspected to be the inhibiting factor at sewage : seawater mixtures above 20 : 80. Also, concentrations over 100 μmoles of NH_4-N have been reported to be inhibitory to certain species of phytoplankton in culture (Guillard 1963).

When a constant sewage : seawater ratio of 10 : 90 was used, the yield of algae from the flowing system increased with increasing rate of flow of medium through the system up to a turnover rate of 50% of the culture volume per day. The yield of the system under these conditions was limited as indicated by the fact that at all concentrations of sewage used up to inhibitory levels, and at all flow rates, the inorganic nitrogen (ammonia) in the culture vessel was reduced to levels that were within the limits of analytical accuracy. Phosphate, on the other hand, gradually increased in the medium until, at any given turnover rate, it reached an equilibrium concentration that varied in direct proportion with the concentration of sewage in the medium. In other words, nitrogen was the limiting nutrient in the system and phosphorus was present in excess of the need for it.

Ryther and Dunstan (1971) showed that this is the normal situation in estuaries and coastal marine areas affected by domestic pollution, because the ratio of N : P by atoms in sewage is about 5 : 1, while that in marine phytoplankton, though variable, usually averages about 10 : 1. Thus nitrogen, in an available inorganic form, is usually the limiting factor to phytoplankton production in coastal marine waters, and it is seldom found in the surface layers of such regions in greater than trace amounts. Phosphorus, on the other hand, is normally present in significant quantities after all the available nitrogen has been used.

The Cranston sewage with an N : P ratio by atoms of about 9.4 : 1 contains relatively more nitrogen or less phosphorus, as the case may be, than does the national average (Table 2). In spite of this, nitrogen still appears to be limiting and surplus phosphorus is present relative to the proportions of these elements assimilated by the particular phytoplankton communities that grew in our experiments. A series of analyses of the algae grown in several experiments in a 10% sewage medium, a mixed diatom population dominated by *Skeletonema costatum*, gave an N : P ratio by atoms of 9.85 : 1.

It was possible to reach a steady-state equilibrium in the sewage-enriched continuous flow cultures with respect to total biomass, algal yield, and nutrient uptake, but the qualitative aspects of the phytoplankton populations did undergo continual change. However the changes in species composition and diversity were surprisingly and unexpectedly slow. Theoretically, in a continuous flow system there should be a rapid and complete selection for the one species whose growth rate is most favored by the conditions (Powell 1958). Mixed steady-state populations have been achieved only when two or more microorganisms are nutritionally associated and noncompetitive (Contois and Yango, unpublished). In our mixed populations of phytoplankton one would expect that those species whose growth rates were inhibited or reduced by the conditions imposed would be rapidly washed out of the system. In addition to sewage effluent these conditions include light, temperature, agitation, vessel effects, and turnover rates. Thus it was unexpected that the phytoplankton species found in the natural population in most cases grew together and survived for periods of 15–20 days before one or two species became overwhelmingly dominant (Table 3 and Fig. 6). At high turnover rates of 100% and over, the domination of one or two species occurs more rapidly. But at turnover rates of 50% and below, gradual enrichment with sewage effluent apparently raises the whole phytoplankton biomass and then selection at this higher population level slowly takes place. Furthermore, at no time during the course of these experiments did blue-green algal or exotic phytoplankton species grow to dominate the cultures.

The data from larger-scale experiments in ponds enriched with sewage in general show the same increase in many species rather than rapid selection for one or a few species (Kuenzler 1970; May 1970). The experiments of Antia et al. (1963), using a large-volume plastic sphere showed no obvious succession of species but an increase in the species prevalent in the initial population. Similarly, during the brief spring phytoplankton bloom in the Sargasso Sea off Bermuda, a dozen or more species of diatoms appeared simultaneously and grew together, equally sharing dominance during the brief existence of this ephemeral community (Hulburt et al. 1960). These various examples suggest that when environmental conditions, primarily nutrient levels, become favorable for the rapid growth of phytoplankton, an entire community of organisms having similar growth potentials may develop simultaneously with little or no selective pressure on one another. Rather, it is in the quantitatively stabilized population, where further growth depends on recycling of a growth-limiting nutrient, that competition and selection appear to take place.

The continuous culture system used in the experiments reported here would appear to be particularly useful in studying algal succession. In addition to basic information on this subject, however, the experiments also show that sewage per se does not exert a strong selective influence on the species composition and diversity of the coastal marine phytoplankton. Species changes that occur in the sewage-enriched algal community appear to result from the quantitative rather than the qualitative aspects of the enrichment, are slow to develop, and are little more than the readjustment of the community to a new equilibrium.

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