APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1978, p. 349-355
0099-2240/78/0036-0349$02.00/0

Vol. 36, No. 2
Printed in U.S.A.

Occurrence and Ecological Significance of GTP in the Ocean and in Microbial Cells

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Received for publication 5 April 1978

A comparison between the ATP concentrations based on peak height light emission values (0 to 3 s) and integrated light flux determinations (15 to 75 s) for a variety of seawater samples revealed that the integrated method of light detection consistently yielded higher ATP concentrations, ranging from 1.38 to 2.35 times larger than the corresponding peak ATP values. A significant correlation ($r = 0.923$) was observed for a plot of ΔADP (i.e., integrated ATP − peak ATP) versus GTP + UTP, suggesting that the analytical interference on the ATP assay was the result of the presence of non-adenine nucleotide triphosphates. Size-fractionation studies revealed an enrichment of the non-adenine nucleotide triphosphates, relative to ATP, in the smallest size fraction analyzed (<10 μm). Investigations were conducted with 20 species of unicellular marine algae to determine their intracellular nucleotide concentrations, and these determinations were compared to the levels measured in lab cultures of the marine bacterium *Serratia marinorubra*. These results indicated that the intracellular GTP/ATP ratios in *S. marinorubra* increase in direct proportion to the rate of cell growth, and that the GTP/ATP ratios in bacteria are much greater than in growing algae, presumably due to the differences in rates of cellular biosynthesis. It is concluded that quantitative determinations of GTP/ATP ratios in environmental sample extracts may be useful for measuring microbial growth.

The study of marine microbial ecology has been plagued by difficulties surrounding the measurements of biomass and metabolic activity. Much of the data that have been reported are based upon biochemical estimates. The techniques most widely used are the measurement of ATP (9, 10), muramic acid (13, 16, 17), and lipopolysaccharide (23) for biomass and heterotrophic potential determinations (26), activity of the electron transport system (18), and rates of lipid biosynthesis (24) for estimating metabolic activity. Biomass determinations based on ATP measurements have received wide attention and use and nearly universal acceptance in oceanographic work.

During a recent investigation of the distribution of ATP in a variety of intertidal ecosystems, Karl (submitted for publication) observed a serious disparity when comparing the results obtained for the two most commonly employed methods of environmental ATP analysis, peak height of light emission and integrated light flux determinations. Thin-layer chromatographic separation procedures revealed the occurrence of GTP and UTP at levels high enough to interfere significantly with quantitative ATP determinations (submitted for publication). Many important biosynthetic reactions are coupled to the hydrolysis of nucleotide triphosphates (NTPs) other than ATP, and since the intracellular concentrations of non-adenine NTPs fluctuate as a function of the growth rate, it was suggested that quantitative determinations of GTP (expressed as GTP/ATP ratios) may be useful for measuring microbial growth rates in nature (submitted for publication).

Concurrent with this investigation of intertidal marine sediments, a study was initiated to determine the general occurrence of GTP and UTP in environmental samples. In addition, laboratory studies were initiated using pure cultures of marine bacteria and unicellular algae to examine and compare their intracellular NTP concentrations. This communication presents the results of these laboratory and field investigations.

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MATERIALS AND METHODS

Sample collection and nucleotide extraction. (i) Bacteria. The marine bacterium, *Serratia marinorubra* ZoBell and Feltham, was grown in continuous aerobic culture on a medium consisting of 1.5 g of

peptone, 0.05 g of yeast extract, and 25 mg of $FePO_4$ per liter in 80% seawater. Under steady-state conditions, the growth rate was exactly equal to the dilution rate of the chemostat vessel (D = input of new medium [milliliters per hour]/culture volume [milliliters]), and ranged in these experiments from 0.16 to 1.85 h^{-1}. At predetermined sampling intervals, a small volume of cells (1 ml) was removed from the growth vessel, and duplicate subsamples (50 μl) were immediately extracted by injecting the cells into 5 ml of boiling tris(hydroxymethyl)aminomethane buffer (0.02 M, pH 7.7). All samples were extracted for 5 min (at 100°C) and immediately frozen (−20°C) for subsequent analysis.

(ii) Algae. Twenty species of unicellular algae representing most major phytoplankton groups (i.e., Bacillariophyta, Pyrrophyta, Chrysophyta, Haptophyta, Cryptophyta, Chlorophyta, Prasinophyta, and Rhodophyta) were obtained from the Food Chain Research Group Culture Collection courtesy of J. Jordan. The cells were sampled and extracted as described above.

(iii) Seawater. Seawater samples were collected at various stations (see tables and figure legends for exact geographical locations) using 5-liter and 30-liter Niskin bottles (General Oceanics) and were processed immediately upon shipboard arrival. For nucleotide extraction, between 50 ml and 2 liters of seawater was filtered through Nitex filter disks or microfine glass fiber filters (Reeve-Angel 984-H), and the concentrated particulate material was extracted in 5 ml of boiling tris(hydroxymethyl)aminomethane buffer as described by Holm-Hansen (9).

Quantitative nucleotide determinations. ATP was measured by the firefly bioluminescent reaction procedure. For each sample extract or standard ATP solution, both the peak height of light emission (0 to 3 s) and the integrated light flux (15 to 75 s) were measured on the same injection using an ATP photometer (SAI Technology, La Jolla, Calif.) interfaced with a strip chart recorder. The peak height of light emission was measured directly from the recorder traces, and the integrated light values were obtained from the photometer. The firefly luciferase enzyme mixture was prepared by reconstituting each vial of FLE-50 (Sigma Chemical Co., St. Louis, Mo.) with 5 ml of distilled water. After an aging period of 3 to 4 h (at 25°C), the preparation was diluted to 25 ml with 10 ml of 0.04 M $MgSO_4$ and 10 ml of 0.1 M sodium arsenate buffer (pH 7.4) and allowed to age for an additional 30 to 60 min before use.

GTP and UTP were determined using the nucleoside diphosphokinase-firefly luciferase coupled reaction described by Karl (Methods Enzymol., in press; Anal. Biochem., in press). The sum of GTP + UTP was taken as the total light emission following the complete enzymatic hydrolysis of ATP, using GTP as the primary standard. GTP was measured following the enzymatic hydrolysis of ATP and UTP as described by Karl (in press).

RESULTS AND DISCUSSION

Quantitative ATP determinations. A series of seawater samples was collected at stations located within the Southern California Bight, and the particulate material contained within these samples was extracted and analyzed for ATP. Table 1 presents the results of these determinations. Each sample extract was analyzed for peak and integrated light emissions. These data indicated that the integrated method of light detection resulted in a consistently higher estimate of ATP than did the corresponding peak emission data. The differences between the two measured values (i.e., integrated ATP − peak ATP) is referred to as ΔATP (see Table 1). When ΔATP is normalized to the appropriate peak ATP concentrations (i.e., [ΔATP/peak ATP] × 100%), it is apparent that the integral determinations result in "overestimates" of between 33 and 135% of the peak ATP concentrations. There was no consistent pattern between ΔATP and peak ATP with increasing water depth or with distance from shore (Table 1). This observed disparity between the two most commonly used methods for ATP detection is similar to the results presented by Karl (submitted for publication) for extracts of intertidal sediments. A subsequent examination of the kinetics of the light emission reactions catalyzed by selected sample extracts revealed an altered pattern of reactivity relative to standard ATP solutions. The altered kinetics were particularly apparent in the portion of the curve following the peak height of light emission (i.e., >5 s), thereby resulting in an overestimate of the integrated light emission values. This altered reactivity has been shown to be the result of non-adenine NTPs (particularly GTP and UTP) in the environmental sample extracts (Karl, submitted for publication).

Quantitative determinations were made of ΔATP and GTP + UTP for several of the seawater extracts. A Spearman rank difference correlation analysis revealed a significant correlation (r = 0.923; n = 16) between these two measured values, suggesting that the measurement of ΔATP (i.e., integrated ATP − peak ATP) may be used as a relative estimate of the GTP + UTP concentrations in seawater sample extracts. Although crystalline firefly luciferase is specific for ATP (4, 6), it is well known that commercial reagents react with a number of non-adenine NTPs to produce light. Even though a number of extensive laboratory investigations have been conducted on the nonspecificity of crude firefly reagents (15, 19, 20), the actual quantitative significance of this additional reactivity has not been previously demonstrated for environmental nucleotide extracts.

Size fractionation of GTP + UTP. Table 2 presents the results of an experiment conducted to determine the nucleotide levels of various

TABLE 1. *ATP concentrations in the Southern California Bight*[a]

Station[b]	Depth (m)	Peak ATP (ng/liter)	Integrated ATP (ng/liter)	ΔATP[c] (ng/liter)	ΔATP/peak ATP (×100%)
101	1	210	494	284	135
		389	553	164	42
	5	203	428	225	111
		211	385	174	82
	13	364	538	174	48
		458	695	237	52
102	1	222	390	168	76
		150	325	175	117
	7	150	267	117	78
		126	225	99	79
	16	470	753	283	60
		372	494	122	33
103	1	139	236	97	70
		179	348	169	94
	22	132	233	101	77
		136	234	98	72
	55	62	84	22	35
		45	80	35	78
105	1	117	220	103	88
		102	221	119	116
	27	154	257	103	67
		93	167	74	80
	62	68	110	42	62
		65	126	61	94
106	1	85	166	81	95
		78	156	78	100
	32	123	200	77	63
		131	195	64	49
	56	28	53	25	89
		97	181	84	87

[a] Samples were collected between 20 and 27 October 1976.

[b] Station 101: 0.9 km offshore, total depth, 20 m; 32°57.4'N, 117°16.5'W. Station 102: 1.9 km offshore; total depth, 38 m; 32°57.3' N, 117°17.3'W. Station 103: 5.6 km offshore; total depth, 230 m; 32°56.8'N, 117°18.9'W. Station 105: 41 km offshore; total depth, 1,000 m; 32°52.2'N, 117°37.8'W. Station 106: 107 km offshore; total depth, 1,850 m; 32°31.6'N, 118°07'W.

[c] ΔATP equals integrated ATP − peak ATP.

sized particles in the ocean. The results indicated that there was no significant difference between the (GTP + UTP)/ATP ratios in the >33 μm, <33 μm, >10 μm, or <10 μm size fractions of surface seawater. The (GTP + UTP)/ATP ratios ranged from 0.187 to 0.415, with a mean of 0.294 (Table 2, station 1). Table 2 (station 2) compares the results obtained for the (GTP + UTP)/ATP ratios of the total particulate material in seawater (filtered onto a microfine Reeve Angel glass fiber filter) with that of the <10 μm fraction for samples of increasing water depth. At all three depths sampled (10, 100, and 500 m), there was an enrichment of GTP + UTP relative to ATP in the smallest size fraction. For the water sample collected from 500 m, 75% of the total GTP + UTP appeared in the <10 μm size fraction, as compared to only 40% of the total ATP (Table 2).

GTP + UTP in algae. In an attempt to interpret these results, an investigation was initiated in the laboratory to determine the range of (GTP + UTP)/ATP ratios found in a variety of marine microorganisms. Twenty species of logarithmically growing unicellular algae were extracted, and their (GTP + UTP)/ATP ratios were determined. The results indicated a range from 0.192 to 0.680 with an overall mean of 0.377. This value was similar to the ratios calculated for the surface samples and the total particulate material for the vertical profile collected in the Southern California Bight (Table 2). Furthermore, the GTP concentrations for the algal cultures and for the seawater samples averaged 53% of the GTP + UTP values.

GTP in *S. marinorubra*. Although most cel-

TABLE 2. *Nucleotide concentrations for various samples collected in the Southern California Bight*[a]

Station[b]	Sample depth (m)	Sample type	ATP (pmol/liter)	GTP + UTP (pmol/liter)	(GTP + UTP)/ATP	
1	Surface	33 μm Nitex	284	53	0.187	0.227
			274	73	0.266	
		<33 μm[c]	290	95	0.328	0.336
			323	111	0.344	
		10 μm Nitex	245	53	0.216	0.316
			217	90	0.415	
		<10 μm[d]	331	100	0.302	0.297
			361	105	0.291	
2	10	Total[e]	312	105	0.337	
		<10 μm	58	41.1	0.709	
	100	Total	227	58.9	0.259	
		<10 μm	16.5	11.9	0.721	
	500	Total	11.8	4.8	0.405	
		<10 μm	4.5	3.6	0.807	

[a] Samples were collected on 29 October 1976.
[b] Station 1: Surface water sample collected at 32°47.5'N, 117°17.3'W; total water depth, 26 m. Station 2: water column samples collected at 32°43.2'N, 117°30.1'W; total water depth, 1,100 m.
[c] 33 μm filtrate collected onto a 984-H Reeve Angel filter.
[d] 10 μm filtrate collected onto a 984-H Reeve Angel filter.
[e] Water filtered onto a 984-H Reeve Angel filter.

lular metabolic reactions are coupled to the formation or hydrolysis of ATP, many endergonic anabolic sequences are coupled to the hydrolysis of non-adenine NTPs. GTP and UTP are both required for the activation and interconversion of carbohydrate precursors for cell wall biosynthesis and for RNA transcription. In addition, GTP is required for the initiation, amino acyl tRNA binding, and translocation processes of protein biosynthesis. At least two molecules of GTP are hydrolyzed for each peptide bond that is formed during the polypeptide elongation cycle of protein synthesis (14).

A series of experiments was conducted to examine fluctuations in the intracellular GTP/ATP ratios of the marine bacterium *S. marinorubra* as a function of growth rate (Fig. 1). It is apparent from these data that the intracellular GTP concentrations increase in direct proportion to the cellular growth rate (generations per hour) when normalized to cellular ATP levels. These results are similar to the results previously presented for bacteria (22), protozoa (5), algae (11), and fungi (3). The mean GTP/ATP ratios for *S. marinorubra* ranged from 0.39 (μ = 0.18 h^{-1}) to 1.70 (μ = 1.87 h^{-1}) and decreased to 0.3 in early stationary cultures. The ratios of GTP to ATP in eucaryotes are much lower than in procaryotes (3, 5, 11, 21), presumably due to their much lower specific rates of growth and protein biosynthesis. Since the GTP/ATP ratio in microorganisms appears to be a function of the cellular growth rate, determinations of GTP/ATP ratios in environmental samples may be a useful parameter for estimating the mean growth status of microbial assemblages in nature.

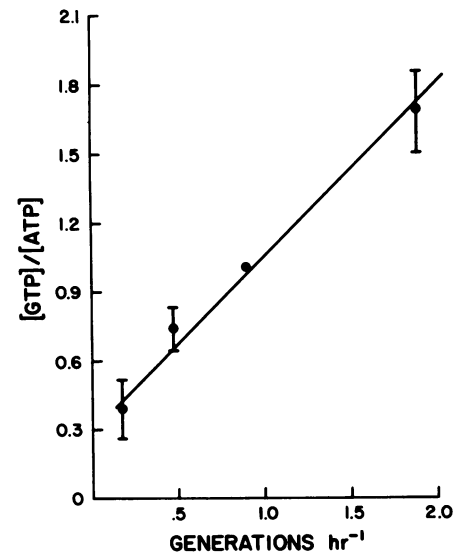

FIG. 1. *GTP/ATP ratios as a function of the growth rate of the marine bacterium S. marinorubra, growing in chemostat cultures.*

GTP/ATP ratios in seawater. Figure 2 presents the vertical distributions of ATP and GTP and the corresponding GTP/ATP ratios at a hydrostation located in the Southern California Bight. Both concentration profiles exhibited similar vertical trends, with peak values occur-

ring within the upper 100 m of water. The GTP/ATP ratios ranged from 0.10 to 0.19 and compared favorably with the data presented in Table 2 (station 2) for the total seawater samples (assuming GTP = 0.53 × [GTP + UTP]). Bacteria (Fig. 1), on the other hand, as well as oceanic particles <10 μm in diameter (Table 2, station 2), have much higher GTP/ATP ratios, indicating greater rates of cellular biosynthesis. Figure 3 presents a similar depth profile that was obtained from samples collected in the Black Sea. The results indicate that although the GTP/ATP ratios in the upper portion of the water column (0 to 100 m) were comparable to the samples collected in the Southern California Bight (i.e., GTP/ATP, 0.11 to 0.19; Fig. 3), the samples collected from the remainder of the water column had significantly higher ratios, ranging from 0.22 to 0.56 with an overall mean of 0.36. The water column of the Black Sea is characterized by a steep pycnocline, induced by salinity, which serves to isolate the lower portion

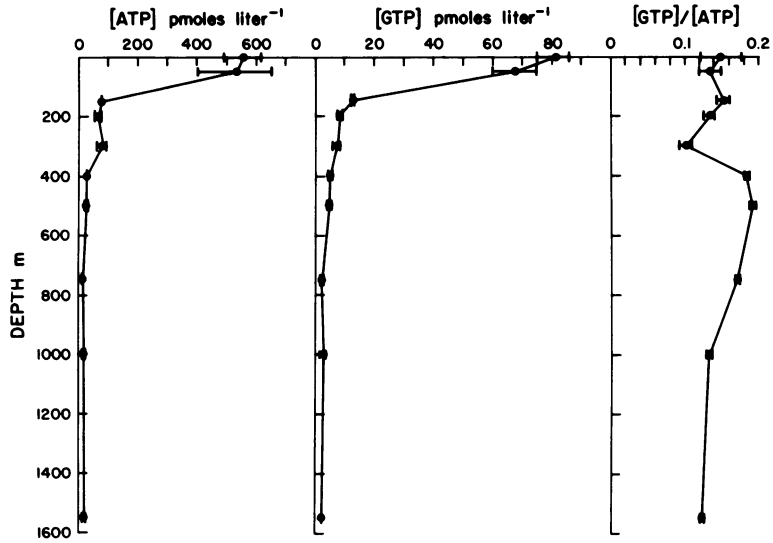

FIG. 2. *Vertical distributions of ATP and GTP and the corresponding GTP/ATP ratios for water samples collected in the Southern California Bight. Station location was 33°30.1'N, 119°20.0'W; total water depth was 1,650 m. Samples were collected on 3 August 1977.*

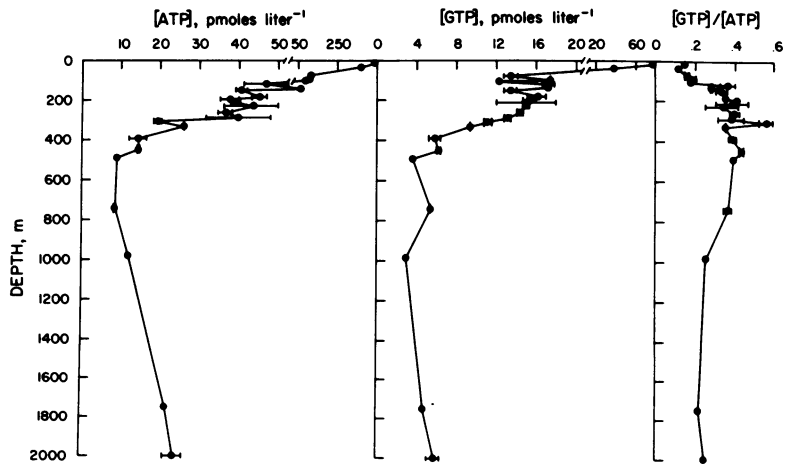

FIG. 3. *Vertical distributions of ATP and GTP and the corresponding GTP/ATP ratios for water samples collected at a station located in the western basin of the Black Sea. Station location was 42°50.0'N, 33°00.0'E; total water depth was 2,200 m. Samples were collected during April 1975. These data were obtained from the same water samples as data previously reported by Karl (Limnol. Oceanogr., in press) for station 1355.*

of the water column from exchange with the oxygen-rich surface waters. As a result, the Black Sea is permanently anoxic from 100 m to the bottom (see D. M. Karl, Limnol. Oceanogr., in press). Since procaryotes are the only organisms known to survive under prolonged anoxic conditions, this observed increase in the GTP/ATP ratios might reflect the natural enrichment of bacteria that are capable of higher rates of biosynthesis. No sample from the oxygenated portion of the water column had a GTP/ATP ratio greater than 0.19, and, by comparison, no sample from the anoxic portion had a ratio less than 0.22.

In the past, estimates of microbial activity were generally inferred from biomass (ATP) determinations. In more recent years, measurements of ADP and AMP have been made in addition to the ATP determinations to estimate the metabolic or growth potentials of the naturally occurring microbial populations (12, 25; Karl, in press). Although GTP/ATP ratios cannot be used to calculate absolute growth rates, the data presented in this report suggest that the determination of GTP/ATP ratios in seawater samples may be useful for detecting areas of microbial growth and for comparing relative rates of community biosynthesis within natural microbial populations. After more extensive laboratory investigations of the nucleotide levels in various marine microorganisms have been conducted, it may be possible to translate GTP/ATP ratios directly into absolute rates of protein synthesis; however, these interpretations await more extensive laboratory studies.

ATP determinations in seawater, biomass estimates, and C/ATP ratios. As discussed previously, the nonspecificity of crude luciferase preparations is well documented (2, 19). Although all NTPs tested in our laboratory (i.e., CTP, GTP, ITP, and UTP) reacted with crude luciferase preparations (FLE-50), the purine nucleotides (GTP and ITP) reacted to a much greater extent than the pyrimidine nucleotides (CTP and UTP) on an equimolar basis, due to the reaction specificity of firefly nucleoside diphosphokinase. Furthermore, the kinetics of the NTP-coupled light emission reaction are dependent upon the concentration of the crude luciferase preparations. The more concentrated the enzyme mixture (i.e., FLE-50 made up to 5 ml versus 50 ml), the greater the concentrations of ADP and nucleoside diphosphokinase in the preparations, and therefore the faster the NTP-dependent light emission reaction (i.e., shifted towards the ATP peak). Additional experiments conducted in our laboratory indicated that, for a given enzyme preparation, the kinetics of the GTP-dependent reaction was concentration dependent, whereas the kinetics of the reactivity towards ATP was concentration independent. Therefore, the "error" due to non-adenine NTPs will be dependent upon the NTP/ATP ratio and the absolute concentrations of nucleotides in the extracts, as well as on the particular enzyme preparation used. Holm-Hansen and Booth (10) estimated the quantitative significance of this nonspecificity to be between 5 and 35%. This determination was based upon data presented by Iwamura et al. (11) for the nucleotide levels of the green alga *Chlorella*. It is evident from the data presented in this report (Table 1 and Fig. 1) that the error is much greater than originally expected (i.e., 33 to 135%). In addition, the error will vary considerably depending upon the structure (i.e., numerical importance of procaryotes) and the growth status of the particular microbial communities.

Concerning biomass estimates, Holm-Hansen and Booth (10) reasoned that the nonspecificity of crude luciferase was of no great importance since environmental ATP determinations are ultimately related to C/ATP values obtained with laboratory cultures of marine microorganisms. The conversion factor most commonly used is ATP × 250 = biomass carbon (9), but the original laboratory data indicated a much wider range in C/ATP values for bacteria (7) than for unicellular algae (8). These results could have been caused by large fluctuations in the GTP/ATP ratios with changing growth rates (as seen in Fig. 1), rather than absolute concentrations of intracellular ATP. Therefore, the true C/ATP ratios in these microorganisms were probably much greater than 250. In fact, Ausmus (1) has reported C/ATP ratios of 500 for a variety of soil bacteria; her methods involved peak height analyses which we now know significantly reduce the interferences due to non-adenine NTPs. In more recent years, many modifications and improvements have been introduced to the original techniques described for the analysis of ATP in seawater. The more common applications of peak height measurements and the use of partially purified reagents (which are available commercially from Sigma Chemical Co. and DuPont) for analyzing environmental samples result in serious difficulties in comparing and interpreting data obtained in different laboratories.

Summarizing, the significant results of this study are as follows. (i) The measurement of ATP in seawater sample extracts using integrated light flux determinations results in variable overestimates (33 to 135%) of the true ATP concentrations. (ii) The difference between the calculated ATP concentrations based on integrated versus peak height determinations,

termed ΔATP, is significantly correlated (r = 0.932) with the GTP + UTP concentrations in the same sample extracts. (iii) Measurements of the (GTP + UTP)/ATP ratios in seawater indicate that the non-adenine NTPs are generally "enriched" in the fraction of particle size <10 μm, suggesting that this size fraction represents the principle growth component of seawater. (iv) The intracellular GTP/ATP ratios in the marine bacterium *S. marinorubra* increase in direct proportion to the cellular growth rate, and the GTP/ATP ratios of unicellular algae, protozoans, and fungi are lower than in bacteria, presumably due to lower rates of biosynthesis. (v) The GTP/ATP ratios have been measured and compared in water samples collected from the Southern California Bight and from the western basin of the Black Sea; the natural enrichment of bacteria in the anoxic portion of the Black Sea is reflected as an increase in the GTP/ATP ratios. (vi) Quantitative determinations of the GTP/ATP ratio in environmental samples may provide insight into questions concerning microbial growth in nature.

ACKNOWLEDGMENTS

I thank a number of colleagues for their contributions to this investigation. J. R. Beers (R/V AGASSIZ), O. Holm-Hansen (R/V E.B. SCRIPPS), H. W. Jannasch (R/V CHAIN), and M. M. Mullin (R/V AGASSIZ) served as cruise leaders during the collection of the various seawater samples. Lisa Campbell helped in sample collection and ATP analyses. A. F. Carlucci and O. Holm-Hansen critically reviewed the manuscript and made helpful comments for its improvement.

This work was supported by Energy Research and Development Administration contract EY-76-C-03-0010 P.A. 20 and by a travel grant awarded to the author by Scripps Institution of Oceanography.

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