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Heterotrophic Utilization of Glucose and Glutamate in an Estuary: Effect of Season and Nutrient Load

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Uptake of ^{14}C-labeled glucose and glutamate was studied at several sites in Baltimore Harbor, Eastern Bay, and the Rhode River. Levels of uptake of ^{14}C-labeled glutamate, measured over a period of 1 year during September, December, April, May, and June to estimate seasonal effects on heterotrophic utilization of selected nutrients, were highest in May and lowest in December. In a comparison of visibly polluted and unpolluted sites, the greatest amount of incorporation of glucose or glutamate and the highest V_{max} values were observed at those sites where the microorganisms were exposed to varied and higher levels of pollutants, suggesting that V_{max} may function as an indicator of relative pollution. Mineralization values ranged from 30 to 43%. A range of 0.44 to 2.32 μmol of C/liter per day was calculated for glutamate uptake.

Since estuaries and coastal regions are used increasingly as sinks for various pollutants and waste products (11), it is important to measure the biochemical abilities of estuarine and marine microorganisms involved in recycling processes (7, 8). Information is needed about not only the range of compounds utilized but also the rate of microbial substrate conversion to cellular material or carbon dioxide.

In this study, selected sites in the Rhode River subestuary of the Chesapeake Bay were examined for the ability of the in situ microbial flora to incorporate glucose or glutamate. The seasonal effects on heterotrophic uptake were determined and various sites were compared to assess the effects of visible pollution. Based on these studies, the rates of mineralization were determined at two sets of sites, paired on the basis of visible sources of pollution, such as oil slicks, and population density.

MATERIALS AND METHODS

Sampling methods and sites. Water samples were collected with a sterile Niskin sampler at a depth of 1 m. Volumes of water samples required for analysis were measured into sterile graduated cylinders.

Locations of the Rhode River sampling sites are shown in Fig. 1. Included in the study of the Rhode River were: a marsh, situated in Muddy Creek and referred to as station 5.4; a populated area in Cadle Creek, station CCO.6; relatively deep water centrally located in the Rhode River and referred to as station 3.38 (Big Island); and the junction of the Rhode River, West River, and Chesapeake Bay, station 0.0 (mouth of Rhode River). Other sites in Chesapeake Bay included in the study were Colgate Creek, an oil-polluted site in Baltimore Harbor, and two areas in Eastern Bay, an open water area in Chesapeake Bay that supports a highly productive shellfishery.

Relative incorporation of labeled substrates. The relative incorporation of various labeled substrates was estimated by using the method of Williams (17). This method was employed in the preliminary experiments to determine filter size and to select substrates, as well as in the seasonal study of glutamate uptake and the comparison of glucose uptake at stations in the Rhode River and Chesapeake Bay. Carbon dioxide evolution was measured by a modification of the technique of Thompson and Hamilton (14). For each substrate tested, two 400-ml samples were placed in sterile stoppered bottles previously covered with tire tubing to prevent light penetration. Controls were provided by adding 40 ml of a 36% formaldehyde solution to 360 ml of sample.

Radioactively labeled substrates were obtained from New England Nuclear Corp. Specific activities for the substrates were as follows: L-[U-^{14}C]glutamic acid, 234 mCi/mmol; and D-[U-^{14}C]glucose, 196 mCi/mmol; [1-^{14}C]palmitic acid, 12.5 mCi/mmol; [U-^{14}C]glycerol, 7.4 mCi/mmol; [^{14}C]urea, 4.5 mCi/mmol.

In each case, an amount equal to a final concentration of 0.1 μg of labeled substrate per liter was added to appropriate flasks at intervals of 5 min. Samples were withdrawn at 10- or 15-min intervals over a period of 1.5 to 2 h. A series of membrane filters (10, 5, 1.2, and 0.45 μm; Millipore Corp.), prewashed with unlabeled substrate, was employed in preliminary experiments. Subsequently, a 0.45-μm filter was used. Samples were also collected from controls at each sampling to determine whether counts were attributable to particulate entrapment. At T = 1, a 10-ml sample was removed and gently filtered into a rubber-capped scintillation vial. Im-

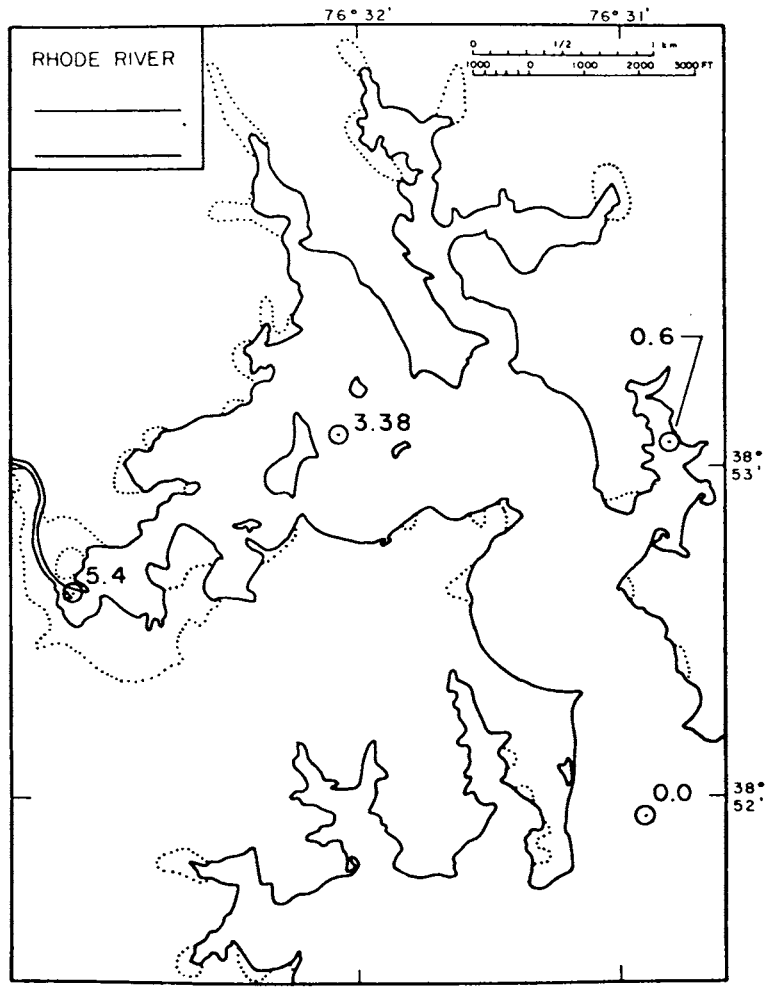

FIG. 1. *Map of the Rhode River subestuary showing the four sampling stations: 0.0, 3.38, 5.4, and CCO.6.*

mediately thereafter, 0.2 ml of 1.0 N H_2SO_4 was injected to terminate uptake. The filters were washed with 5 ml of sterile Rhode River water and 5 ml of air. The filters were separated and placed in individual scintillation vials containing a standard Omnifluor dioxane mixture. Samples were returned to the laboratory, where the CO_2 was pumped through a closed system into Woehlers solution A, diluted with an equal amount of solution B, and counted. Counting, with quench correction, was done using an Intertechnique liquid scintillation counter, model no. SL40.

Methods for identification and classification of bacteria isolated from samples collected at the sites are provided in a separate communication (J. F. Carney, V. Y. Yang, and R. R. Colwell, manuscript in preparation).

Glutamate mineralization at paired stations. A modification of the Hobbie and Crawford (8) technique was followed in this study. Triplicate samples were used to determine incorporated substrate, and a second set of replicates in triplicate were used for metabolized, cell pool, and cell-associated substrates. Cell-associated and pooled substrates were calculated by determining the difference between the filtered cells and the acid-treated cells. Carbon dioxide evolved was also measured.

Sample volumes (12 ml) in 50-ml serum bottles were examined. Glutamate concentrations ranged from 1 to 10 μg/liter, and formaldehyde-treated controls were included for each concentration. Samples were shielded from the light during incubation in situ for 1 h. Reactions were terminated by addition of H_2SO_4 or by filtration, as described above, and evolved carbon dioxide was entrapped.

RESULTS

Preliminary experiments. In a study using filters of several pore sizes, approximately 70 to 94% of the labeled substrate passed through the 5-μm filters and was trapped on the 1.2-μm

filters. Consequently, 0.45-μm filters were used in subsequent experiments to maximize entrapment of the microorganisms.

The uptake of several substrates was studied to select one to be used in the seasonal survey. Comparison of glutamate, palmitate, urea, and glycerol at station 3.38 during September 1973 showed that glutamate was taken up to a greater extent than the other compounds. Little uptake of palmitate was detected. Consequently, glutamate was selected as the substrate for a seasonal survey.

Seasonal uptake of glutamate. Very little uptake of glutamate was noted in December, whereas maximum uptake occurred in May (Table 1). April was unusually cold; hence uptake was only 2.5 times greater than that measured in December. Uptake data for June and September indicated activity intermediate between the winter minimum and late spring maximum.

Comparative uptake of glucose at selected sites. Comparison of results obtained for samples collected at sites in the Rhode River stations with other Chesapeake Bay stations showed that, with increased pollution loads, microbial populations demonstrated greater heterotrophic potential (Table 2). That is, the nutrient load on the ecosystem, via seepage from septic tanks, marinas, and gas stations in Cadle Creek and from ship traffic and oil spills from tankers in Colgate Creek, was reflected in the increased heterotrophic uptake measured at these sites relative to the lower uptake data recorded for samples collected at the visibly less polluted sites, such as Eastern Bay, Big Island (station 3.38), and the Rhode River mouth (station 0.0).

There are several techniques available for measurement of labeled CO_2 (8, 9, 14, 18). In the study reported here, when the work was initiated, samples were transported to the laboratory before undertaking measurement of CO_2. Erratic results were obtained in the first set of experiments, which suggested that CO_2 was lost during transport, since problems are not encountered when the CO_2 is pumped off immediately upon acidification. Moreover, recent work by Griffiths et al. (6) demonstrated that the method used to terminate uptake can affect results; in particular, acidification causes release of cell-associated and internally pooled substrate. Thus, results are presented here as relative incorporation, i.e., the ratio of the uptake at one station to the lowest amount of uptake observed, for the purposes of seasonal and spatial comparison.

These incorporation studies were complemented with an analysis of the generic composition of the microbial populations at each of the locations included in the study. Fifty isolates were selected from samples collected at each of the sites. The pure cultures were subjected to taxonomic analysis, and identification to the genus level was accomplished.

Although the total numbers of heterotrophic bacteria observed for stations 3.38 and CC0.6 were similar, approximately 10 times greater uptake was observed for samples collected at Cadle Creek, as compared with station 3.38 samples (Table 3). The generic composition for the two sites revealed dominance of the same two genera, *Vibrio* and *Pseudomonas*. However, at station 3.38 the genera *Pseudomonas*

TABLE 1. *Seasonal comparison of glutamate incorporation at station 3.38 (Big Island)*

Month	Temp (C)	Relative incorporation
September	29	10.0
December	4	1.0
April	15	2.4
May	23	30.0
June	25	10.0

TABLE 2. *Relative glucose incorporation at stations in the Rhode River subestuary and in Chesapeake Bay (July 1974)*

Location	Description	Temp (C)	Relative incorporation
0.0	Open water	25	2.5
3.38	Open water	29	1.4
5.4	Marsh	31	5.0
CC0.6	Populated	27	10.5
Eastern Bay	Unpolluted	27	1.0
Colgate Creek	Oil polluted	28	12.5

TABLE 3. *Generic distribution of bacterial isolates at stations 3.38 (Big Island) and CC0.6 (Cadle Creek) (July 1974)*[a]

Bacterial isolates	Generic distribution	
	Station 3.38	Station CC0.6
Achromobacter	1.9	0
Aeromonas	0	2.2
Coryneform	1.9	0
Enterobacteriaceae	0	2.2
Moxarella-Cytophaga-Flavobacterium	7.7	4.4
Pseudomonas	46.1	52.2
Vibrio	32.7	39.1

[a] The relative incorporation at stations 3.38 and CC0.6 was 1.4 and 10.5, respectively.

and *Vibrio* accounted for 79% of the population, with *Moraxella-Cytophaga-Flavobacterium*, *Achromobacter*, and coryneforms making up the remainder of the population, whereas, at Cadle Creek, *Pseudomonas* and *Vibrio* spp. comprised 91% of the population, with *Moraxella-Cytophaga-Flavobacterium*, *Enterobacteriaceae*, and *Aeromonas* comprising the remaining 9% of the colony-forming units.

Glucose mineralization of paired stations. Mineralization of glucose was measured in May 1975, employing the Wright-Hobbie-Crawford kinetic approach, thus providing further study of the observed correlation between nutrient load and heterotrophic potential. Sets of stations were paired; i.e., two stations recognized as being polluted and two as unpolluted were chosen. Pairing of stations also included consideration of proximity so that the effects of physical parameters would be minimized. Thus, the paired stations were Cadle Creek and Big Island in the Rhode River and Colgate Creek and Eastern Bay in the Chesapeake Bay. Data presented in Table 4 show that a higher V_{max} was calculated for samples measured at the two polluted sites, Colgate Creek and Cadle Creek.

Analyses of ammonia, combined nitrate and nitrite, and total dissolved phosphorous are given in Table 5. Colgate Creek water had a higher inorganic nutrient content than did Eastern Bay at all of the sampling periods. However, the concentration of inorganic nutrients in the Rhode River varied with the time of sampling, and no pattern was immediately apparent. However, there was a correlation between rates of ^{14}C uptake and rates of labeled phosphorous uptake. The micrograms of P per liter per hour for Big Island and Cadle Creek were as follows: May, 10.1, 28.2; June, 6.4, 16.4; and July, 5.3, 6.8 (D. Correll, personal communication). In each case, uptake was more rapid at Cadle Creek.

DISCUSSION

Preliminary experiments. Based on our studies and similar work by Williams (17), it was concluded that the observed uptake was that of organisms smaller than 5 μm. Uptake experiments were combined with autoradiography by Paerl (12), who provided convincing evidence that observed uptake was the result of bacterial and fungal heterotrophic activity. Consequently, 0.45-μm filters were employed to maximize the accuracy of the method.

Burnison and Morita (3) reported that, of sixteen ^{14}C-labeled amino acids, glutamate, asparagine, and aspartate were respired to the greatest extent, indicating these to be preferential energy sources for microorganisms in the natural environment. In the comparative study reported here, glutamate was taken up to a greater extent than urea or glycerol, whereas relatively little palmitate uptake occurred. Andrews and Williams (1) reported no uptake of either palmitic acid or stearic acid for seawater samples. Thus, glutamate was selected as the substrate for the seasonal study.

Seasonal uptake of glutamate. A seasonal effect on glutamate uptake was demonstrated with temperature a predominant factor in the winter. Studies by other investigators have shown that low temperatures reduce rates of nutrient uptake (13). Hence, the results of this study concerning seasonal effects are in good agreement with the published data. However, nutrient concentration, salinity, and other factors may have an effect on uptake and respiration, as observed in the June and October

TABLE 4. *Kinetic parameters for uptake of ^{14}C-labeled glutamate in May 1975*

Area	Type	T_t	V_{max}	% Respired	μmol of C/liter per day
Colgate Creek	Cells	5.6	2.25		
	CO_2	6.9	0.38		
	Total	3.0	2.27	38	1.86
Eastern Bay	Cells	4.0	0.40		
	CO_2	9.0	0.16		
	Total	2.7	0.56	30	0.46
Cadle Creek	Cells	3.2	1.4		
	CO_2	4.6	1.5		
	Total	1.8	2.9	43.4	2.32
Big Island	Cells	2.0	0.32		
	CO_2	9.0	0.19		
	Total	1.6	0.53	36.3	0.44

TABLE 5. *Comparative nutrient data for Rhode River and Chesapeake Bay stations in 1974*

Station	NH_4 (μg/liter)	NO_3 + NO_2 (μg/liter)	Total dissolved phosphorous (μg/liter)
April			
0.0	112	362	
3.38	25	36	9
5.4	32	191	30
CC0.6	35	58	10
May			
0.0	36	234	
3.38	39	54	5
5.4	28	28	25
CC0.6	46	10	6
E.B.	3	41	0.4
Col. C.	43	65	0.6
June			
0.0	28	84	
3.38	37	2	27
5.4	14	5	75
CC0.6	24	2	8
E.B.	2	18	0.5
Col. C.	42		1.4
July			
0.0	27	1	
3.38	34	7	13
5.4	31	4	70
CC0.6	55	6	5
E.B.	9	3	1.5
Col. C.	52	36	1.3

sampling periods. It should be emphasized that cultures can be selected for, or adapt to, reduced temperatures, as in the case of microorganisms in antarctic waters. Interestingly, Morita et al. (10) found that the indigenous antarctic microflora demonstrated metabolic activities comparable to those of microorganisms in temperate zones.

Relationship between nutrient load and observed heterotrophic activity. The relative uptake of glucose, determined at several sites in the Rhode River and Chesapeake Bay, showed correlations between visible levels of pollution and increased relative uptake, with higher values noted at Colgate Creek and Cadle Creek. Lower values were observed for Eastern Bay, Big Island, and the mouth of the Rhode River.

The relationship was examined further in the study of glutamate mineralization rates for sites paired on the basis of proximity and differing levels of observed pollution. Results obtained corroborated the earlier work. Higher V_{max} values were observed for samples collected at the polluted sites, Colgate Creek and Cadle Creek, compared with the relatively unpolluted areas, i.e., Eastern Bay and Big Island. The rate of uptake of labeled phosphorous was also higher for the Cadle Creek samples compared with those collected at the Big Island site.

Respired CO_2 varied between 30 and 43%. Glutamate respiration, determined from published data, was as follows: 61% for pond water (8) and a variation of 38% in August to 63% in February for upper Klamath Lake (3). Williams (17) reported very low respiration values for open ocean water, ranging from 1.6 to 8.2%, when amino acids served as substrate. Calculations of production (micromoles of C per liter per day) showed higher productivity for samples collected at Colgate and Cadle Creeks than reported by Burnison and Morita for upper Klamath Lake (3). Productivity for Eastern Bay and Big Island samples were significantly lower.

In considering V_{max}, the number of organisms at each site should be taken into account since V_{max} is dependent on not only the type but also the number of organisms present (19). Microbial populations at Cadle Creek were 1.9×10^3 CFU/ml and at Big Island, 2.1×10^3 CFU/ml, both relatively similar in total numbers. However, the aerobic heterotrophic population at Cadle Creek demonstrated a higher V_{max} than that of Big Island. The microbial population at Colgate Creek was observed to be approximately 10 times larger compared with Eastern Bay, 2.0×10^4, suggesting either a lower rate of activity per cell or that only a fraction of the viable cells were active under the in situ conditions.

Studies by Gill and Ratledge (5) showed that hydrocarbons inhibit metabolism of glucose by microorganisms, with the indication being that only hydrocarbon-utilizing organisms are affected. Hydrocarbon-utilizing bacterial populations in Colgate Creek range from 0 to a maximum of 10% of the aerobic heterotrophic population (15). The glucose effect may also prove to be of a wider spectrum than only for hydrocarbon-utilizing microorganisms. This point obviously requires further study. Walker and Colwell (16) have published preliminary evidence showing that glucose respiration can be inhibited in Colgate Creek in January, a time at which oil-degrading bacteria are most active (15).

The taxonomic and quantitative data obtained in these comparative studies were interesting in that it demonstrated that the types, and not only the numbers, of bacteria are important. Although the same genera were found

at both Big Island (station 3.38) and Cadle Creek (station CCO.6), eightfold greater uptake was noted at Cadle Creek (Table 3). The observed increased uptake may, indeed, reflect either a greater representation of *Vibrio* and *Pseudomonas* spp. at Cadle Creek (12% higher than at Big Island) or different species within these genera as has been noted in earlier work (4), or both. The total number of bacteria at the two stations was similar. Thus, the total colony-forming units or total heterotrophic numbers, although useful indexes, do not reveal sufficient information concerning microbial populations, especially when ecological questions are asked.

Comparison of termination methods. Griffiths et al. (6) and Baross et al. (2) demonstrated that the intracellular pooled substrate and cell-associated substrate in the process of transport are removed from cells when reactions are terminated by the addition of acid. Since the acid used to release carbon dioxide in one set of flasks also fixes the cells, comparative V_{max} values were calculated for both systems (Table 6). The V_{max} values should be higher and the turnover times should be shorter where filtration was used for termination. Such results were obtained, thus substantiating the assertion that reduced values are obtained when acidification is used for termination. These data indicate that use of acidification to terminate uptake leads to underestimation of the amount of substrate incorporated, by 29 to 43% in this case.

Variation in control values. Thompson and Hamilton (14) suggested that variances in the control values at different substrate concentrations can occur. In this study, separate (blank) controls were run for each substrate concentration. The average values for the four concentrations were 80, 314, 1,144, and 1,586 counts/min per 10 ml. An inexplicably high count of 4,687 counts/min per 10 ml was obtained at the lowest concentration. This value was eliminated as being spurious. However, it is concluded that one or more controls should be included for each substrate concentration.

In summary, the greatest amount of heterotrophic activity observed in the Rhode River samples examined occurred in the late spring months. Relatively similar rates of uptake were observed in both early summer and fall. Maximum incorporation and highest V_{max} values were observed at sites where the nutrient load was judged to be higher. It is concluded that V_{max} may provide a useful index of relative pollution, i.e., nutrient load, in natural waters.

TABLE 6. *Effect off reaction termination methods on apparent uptake*

Area	V_{max}		T_t	
	Acid	filtration	Acid	Filtration
Colgate Creek	1.3	2.25	7.2	5.6
Eastern Bay	0.28	0.4	12.0	4.0
Cadle Creek[a]	0.9	1.4	4.0	3.2
Big Island[b]	0.23	0.32	5.0	2.0

[a] Station CC0.6.
[b] Station 3.38.

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