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Growth Response of Bacteria to Extracellular Products of Bloom Algae

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The growth response of native bacteria isolated from Maizuru Bay to extracellular organic carbon (EOC) of *Asterionella glacialis* (Bacillariophyceae) and *Chattonella marina* (Rhaphidophyceae) was examined. *A. glacialis* EOC selectively stimulated and inhibited the bacterial growth. Growth of a dominant strain *Pseudomonas* 022 during the bloom of *A. glacialis* was strongly stimulated by *A. glacialis* EOC, but Vibrios strains growth were not. *Psudomonas* 022 utilized selectively not only dissolved free amino acid (DFAA) but also dissolved combined amino acid (DCAA) in the algal EOC. It is suggested that bacteria change the concentration and composition of dissolved amino acids in the bloom period.

We previously described that the algal blooms are correlated with changes in the bacterial flora in Maizuru Bay.[1] As in general dissolved organic carbon (DOC) seems to originate directly or indirectly from phytoplankton, it was suggested that the bacteria utilizing more efficiently extracellular organic carbon (EOC) of the dominant phytoplankton would become predominant during the bloom.

Andrew and Williams,[2] and Lee and Bada[3] showed the low concentrations of free amino acids in seawater of about 58 to 120 nmol/*l*, which must be mainly derived from EOC. In spite of such a low concenttration these amino acids may play an essential role for growth of native bacteria in marine environment.

This paper describes the effects of extracellular organic carbon (EOC) of *Asterionella glacialis* and *Chattonella marina* on growth of native bacteria isolated from Maizuru Bay, and then referred to the utilization and uptake of dissolved free and combined amino acids of EOC by a dominant bacteria during the *A. glacialis* bloom.

Materials and Methods

Organisms

Twenty three bacterial strains for this study were isolated from the seawater during in Maizuru Bay in different seasons and identified according to Shewan's scheme.[4] Strains number 022, 23, S4, S6, S9, S10 and S11 belong to genus *Pseudomonas*, strains number 24, 3, S5, S8, SA4 and SA7 to genus *Acinetobacter*, strains number 26, 27, 13 and AL5 to Chromogenic bacteria, and strains number VOI, VOII, A5, M4, M18 and M19 to genus *Vibrio*. *Vibrio alginolyticus* ATCC 17749 was also tested.

Algal strains used were a monoxenic clonal culture of *A. glacialis* which occurred a bloom in October in Maizuru Bay[1], and an axenic culture of *Chattonella marina* (NIES-121) which was provided by National Institute of Environmental Sciences, Japan. The algae were maintained in f/2 medium[5] prepared with artificial seawater (ASW)[6] at 20°C and 48 μE/m^2/s on a 14: 10 LD cycle.

Preparation of EOC

Extracellular products (EOC) of *A. glacialis* and *C. marina* were prepared as follows: The algal cultures at a late log phase were first filtered by gravity filtration through a precombusted Whatman GF/C glass fiber filter, and then were filtered through sterilized GS Millipore filter (0.22 μm prore size) which was prewashed with sterile doubly-distilled water for eliminating organic contaminants from the filter. The filtrate was stored frozen until use.

Dissolved organic carbon (DOC) was determined with a total carbon analyser (Shimadzu, TOC-500).

Analysis of Amino Acids

EOC of *A. glacialis* was divided into two subsamples for the analysis of dissolved free and

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combined amino acids (DFAA and DCAA) separately. For desalting and concentrating EOC a Dowex 50W×4 cation exchange resin was used, which had been previously cleand with 2N NaOH, regenerated to H^+ form with 2N HCl, and rinsed to neutrality with doubly-distilled water. A subsample for DFAA was applied to the column followed by rinsing with doubly-distilled water. Thereafter the amino acids were eluted from the column with 3N NH_4OH. The elute was evaporated, and amino acids in it were separated with a Shimadzu high performance liquid chromatograph LC-6A, amino acids analysis system, and detected fluorometrically with o-phthalaldehyde. A subsample for DCAA was evaporated to dryness and the residue was hydrolysed with 6N HCl at 100°C for 24 h. The hydrolizate was evaporated to dryness. After being free of HCl the residue was redissolved in doubly-distilled water and desalted on a cationic exchange resin. Thereafter, the procedure was the same as for the dissolved free amino acids. DCAA concentrations were obtained by subtracting the DFAA from dissolved total amino acids. The analysis was perfomed with duplicate samples.

Amino acids in EOC of *C. marina* were analysed by the same procedure as described above.

Bacterial Assay

All glasswares were cleaned with acid and combusted at 400°C for 4 h prior to use. Chemicals used were analytical reagent grade. Each bacterial strain grown in modified ST10^{-1} medium[7] containing 0.5 g trypticase (BBL), 0.05 g yeast extract (Difco) and 25 μg ferric citrate, was aseptically harvested by centrifugation at 7000×g for 5 min and the pellet was washed twice with sterilized ASW. A 1 m*l* aliquot of the final suspension was inoculated in 25 m*l* of EOC medium or a control medium on Schott bottles and incubated in 20°C. F/2 medium containing glucose or trypticase at the same carbon concentration as EOC was used as control. Initial cell densities of 10^2–10^3 ml^{-1} in media were used. Bacterial growth was followed by the direct count method after staining with 4′–6′ diamidino-2 phenylindole (DAPI).[8] Their growth responses were expressed as growth rate and maximun cells density.

Determination of Amino Acids Utilization of Pseudomonas sp. strain 022

The inoculum of cells, which prepared as described above, was inoculated in flasks with 1 l of EOC and incubated in 20°C. After 30 h the culture was filtrated through GS Millipore filter (0.22 μm pore size) and the filtrate was used for analysis of amino acids.

Results

The growth responses (growth rate and maximum cells density) to extracellular products of *A. glacialis* and *C. marina*, of twenty three bacterial strains isolated from Maizuru Bay and *Vibrio alginolyticus* were examined and compared with the growth responses to glucose (Fig. 1). *Pseudomonas* strains 022 and 23 emerged in October showed higher growth rate about 0.18 h^{-1} in *A. glacialis* EOC. *Pseudomonas* 022 among twenty four strains produced the highest maximun cell densities (1.2×10^8 cells·ml^{-1}) in *A. glacialis* EOC. Other *Pseudomonas* strains did not show any distinct difference between their growth responses to EOC and to glucose. Bacteria of genus *Vibrio* showed the lowest response to *A. glacialis* EOC, but good response to *C. marina* EOC and glucose. *Vibrio alginolyticus* showed the same response as most Vibrios strains tested. Bacterial of genus *Acinetobacter* showed various patterns in their response. In the next experiment growth responses of several representative strains (022, 23, 24, 26, 27 and VOI) to *A. glacialis* EOC, *C. marina* EOC, glucose and trypticase peptone were examined (Fig. 2). The results showed that *Vibrio* VOI was strongly stimulated by trypticase peptone, and reached maximum cells densities over 10^8 cells·ml^{-1}. *Pseudomonas* 022 and 23 were also stimulated by trypticase peptone, but their growth rates and maximum cells densities were higher in *A. glacialis* EOC than in trypticase peptone. In Fig. 3. utilizations of *A. glacialis* EOC and *C. marina* EOC by *Pseudomonas* 022 and *Vibrio* VOI were compared. The results show that *Pseudomonas* 022 causes a decrease of both algal EOC as DOC for 24 h incubation. The *Vibrio* strain did not grow for 24 h and did not utilize DOC in *A. glacialis* EOC, but slowly in *C. marina* EOC.

Considering that the growth response of *Pseudomonas* 022 to trypticase peptone (max. cells dens. 5×10^7 cells·ml^{-1}; $\mu=0.16$ h^{-1}) was higher than that to glucose (max. cells dens. 1×10^6 cells·ml^{-1}; $\mu=0.1$ h^{-1}). It is suggested that some amino acids and pepetides in the EOC are promoting the growth of *Pseudomonas* 022. Utilization by *Pseudomonas* 022 of total dissolved free amino acids (DFAA) and dissolved combined

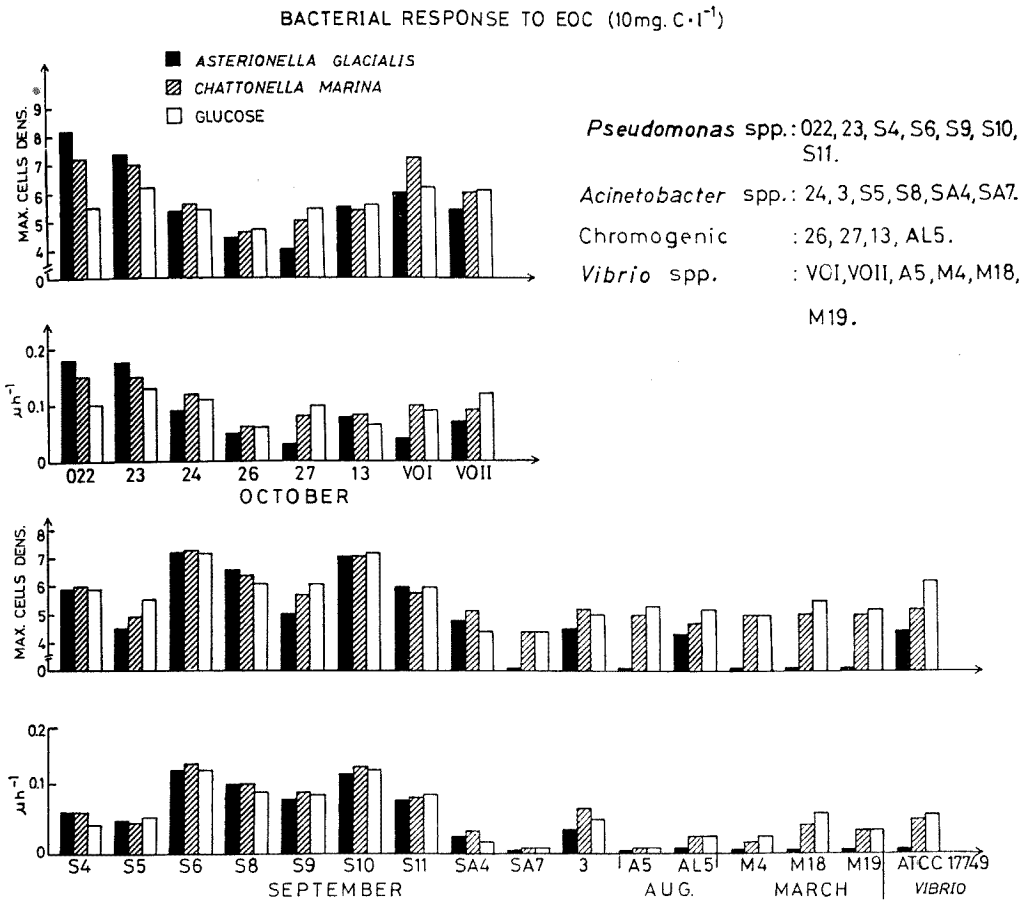

Fig. 1. Growth response of native bacteria to extracelluar organic carbon of *A. glacialis* and *C. marina*.

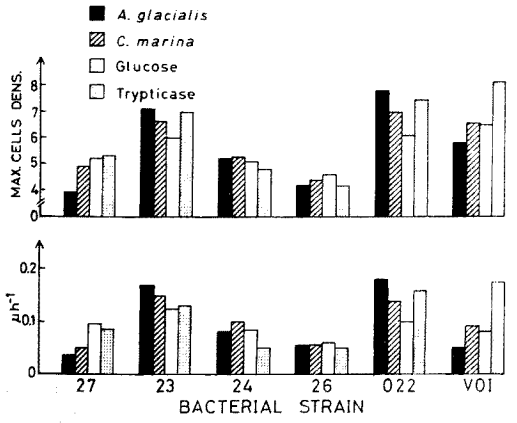

Fig. 2. Growth response of several representative bacteria to *A. glacialis* EOC and *C. marina* EOC.

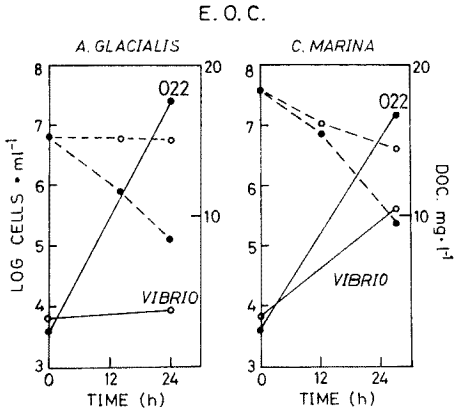

Fig. 3. Growth of *Pseudomonas* 022 (●—●) and *Vibrio* VOI (○—○) in *A. glacialis* EOC and *C. marina* EOC, respectively, and their utilization of the EOCs. (●--●) DOC utilization of *Pseudomonas* 022. (○--○) DOC utilization of *Vibrio* VOI.

Table 1. Changes of the total concentrations of dissolved free and combined amino acids (DFAA and DCAA) in extracellular products of marine phytoplankton, during incubation with a marine *Pseudomonas* strain 022

	Time (h)	DFAA (μ mole·l^{-1})	DCAA (μ mole·l^{-1})	Total (μ mole·l^{-1})
A. glacialis	0	0.54	17.56	18.10
	30	0.21	1.80	2.01
C. marina	0	0.77	4.29	5.06
	30	0.34	1.43	1.77

amino acids (DCAA) in *A. glacialis* EOC and *C. marina* EOC was investigated (Table 1). After 30 h incubation of *Pseudomonas* 022 in these two algal EOC, separately, decrease of DFAA and DCAA in these EOC was determined. Initial concentration of dissolved amino acids in *A. glacialis* EOC was 3.5 fold higher than that of *C. marina*. DCAA occupied a large portion of dissolved amino acids in these algae, and reached about 97% and 84% of total dissolved amino acids in *A. glacialis* EOC and *C. marina* EOC, respectively. *Pseudomonas* 022 utilized dissolved amino acids of both algal EOCs but the extent of its utilization differed. Its utilization of dissolved amino acids of the *A. glacialis* EOC was about 90%. Decrease of DFAA of *A. glacialis* was not so much (about 60%), as compared to about 90% decrease of DCAA. The utilization of DFAA and DCAA in *C. marina* EOC by *Pseudomonas* 022 showed a different pattern from that in *A. glacialis*; that is, bacterial utilization of *C. marina* DCAA was not so high (67%), as compared to that of *A. glacialis* DCAA.

In Figs. 4 and 5 are shown the utilization by *Pseudomonas* 022 of DFAA and DCAA in *A. glacialis* EOC and *C. marina* EOC, respectively. *Pseudomonas* 022 selectively utilized dissolved amino acids, and completely utilized ala, val, ile and leu in DFAA, and pro, cys, val, tyr, phe and arg in DCAA of *A. glacialis* (Fig. 4). And also it almost utilized thr, glu, ile, leu, his and lys. It was, however, observed that the utilizations of both free and combined gly and combined ala were poor. As shown in Fig. 5, *Pseudomonas* 022 showed also poor utilization of free and combined gly in *C. marina* EOC. Ala, ile, leu, his and lys in DFAA were completely utilized by it.

In Figs. 6 and 7 are illustrated the changes in mole composition of DFAA in *A. glacialis* EOC and *C. marina* EOC after 30 h incubation with *Pseudomonas* 022. Composition of DFAA in both algae EOC changed remarkably. DFAA in *A. glacialis* EOC was mainly composed of asp,

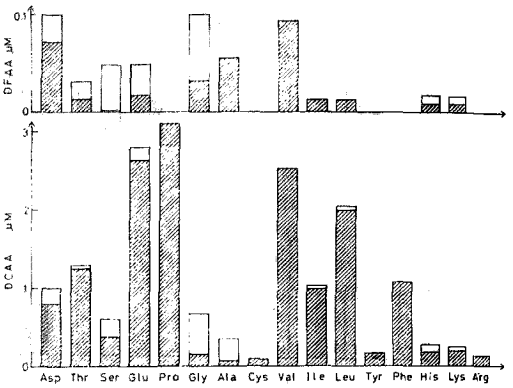

Fig. 4. Utilization by *Pseudomonas* 022 of dissolved free amino acids (DFAA) and combined amino acids (DCAA) of *A. glacialis* EOC.
▨ utilized fraction; □ remained fraction.

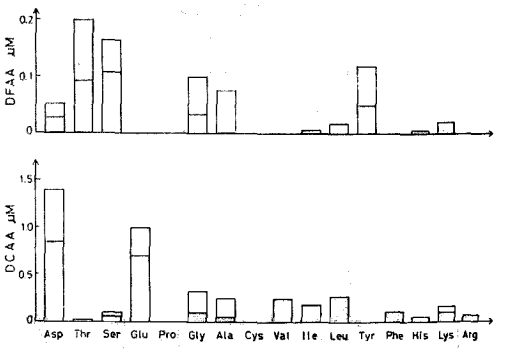

Fig. 5. Utilization by *Pseudomonas* 022 of DFAA and DCAA of *C. marina* EOC.
▨ utilized fraction; □ remained fraction.

gly and val, and DFAA in *C. marina* EOC was composed of thr, ser and tyr. In *A. glacialis* after 30 h incubated with *Pseudomonas* 022 increase of ser and gly was remarkably observed (Fig. 6). In *C. marina* gly, thr and tyr increased (Fig. 7).

Figs. 8 and 9 show the changes of DCAA compositions of *A. glacialis* EOC and *C. marina* EOC after 30 h incubation with *Pseudomonas* 022. It was also noted that DCAA composition in both algal

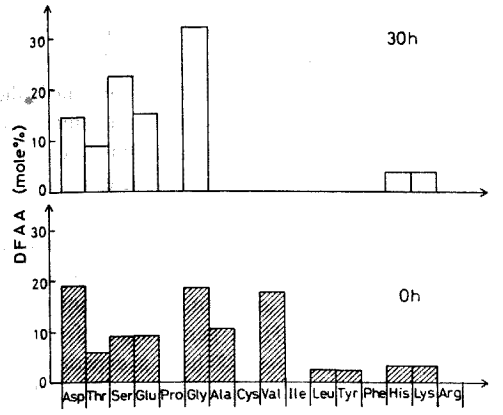

Fig. 6. Changes in amino acids mole composition of DFAA in *A. glacialis* EOC after 30h incubation with *Pseudomonas* 022.

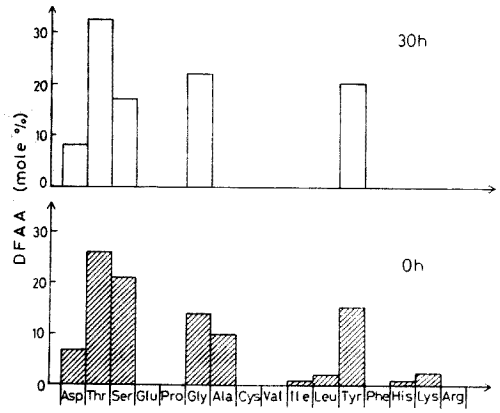

Fig. 7. Changes in amino acids mole composition of DFAA in *C. marina* EOC after 30h incubation with *Pseudomonas* 022.

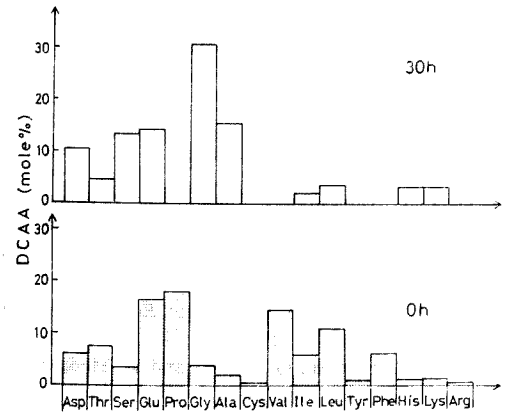

Fig. 8. Changes in amino acids mole composition of DCAA in *A. glacialis* EOC, after 30h incubation with *Pseudomonas* 022.

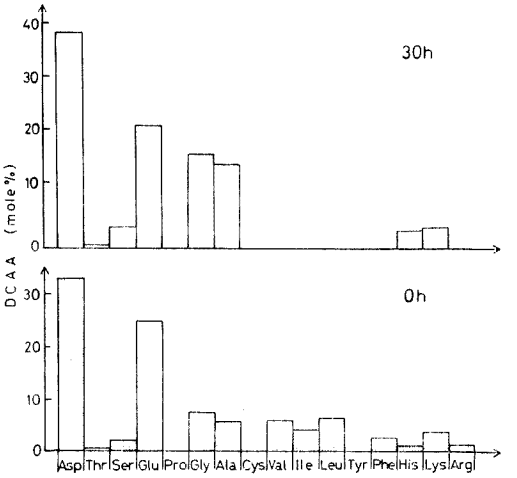

Fig. 9. Changes in amino acids mole composition of DCAA in *C. marina* EOC, after 30h incubation with *Pseudomonas* 022.

EOC was mainly glu, pro, val and leu in *A. glacialis* EOC, and was asp and glu in *C. marina* EOC. *Pseudomonas* 022 caused a drastic change in main DCAA composition in *A. glacialis* EOC after the incubation, resulting in the change from glu, pro, val and leu to gly, ser, glu and ala as dominant combined amino acids. This phenomenon was not observed with DCAA in *C. marina* EOC.

Discussion

In this study it was demonstrated that *A. glacialis* EOC selectively stimulated and inhibited the growth of native bacteria isolated in Maizuru Bay. The stimulative effect of *A. glacialis* EOC was not related to bacterial genera. Among 23 strains isolated through the year only two strains of *Pseudomonas* 022 and 23 were strongly stimulated in growth (Fig. 1). These strains were isolated as dominant culturable flora during the bloom of *A. glacialis*.[1] The inhibitory effect of *A. glacialis* EOC was remarkable to genera of *Vibrio*. The antagonism between *Vibrio* and phytoplankton was previously discussed.[9]

However, *C. marina* EOC showed a clear stimulatory effect on the growth of Vibrios strains. Such positive effect of algal EOC on genus *Vibrio* was reported by Dhevendaran *et al.*[10] who showed that *Vibrio* spp. reached 80% of the bacterial flora during a bloom of *Coscinodiscus* sp. It is probable that in the phytoplankton blooms the algal-bacterial interaction is species-specific.

Difference of growth response of algal EOC to

native bacteria may depend on difference in chemical composition of algal EOC. In fact the chemical compositions of dissolved free and combined amino acids in *A. glacialis* EOC and *C. marina* EOC was clearly different. Differences in composition of DFAA in two marine blue-green algae was reported.[11] In *Calothrix scopulorum* ala, thr and leu were the dominant DFAA. In *Nostoc entophytum* basic amino acids and thr were predominant. Determinations of *in situ* DFAA in a outdoor tank by Brockmann *et al.*[12] showed strong statistical correlations between *Nitzchia longissima* and val and ile, and also between *Thalassiosira rotula* and vale, leu and ile. Hammer and Eberlein[13] in a outdoor tank experiments observed that *in situ* DFAA concentrations increased during a bloom of *Thalassiosira rotula*, but decreased at the stationary phase of the algae.

Pro was observed as dominant, in DCAA in *A. glacialis* EOC, but was not found in *C. marina* EOC. The data of DCAA in algal EOC are scarce in the literatures published up to now. Fogg[14] and Jones and Stewart[15] reported that peptides released by *Anabaena cylindrica* were low molecular weight, and two peptides were composed of only ser and thr.

This research and the literatures suggested that composition of DFAA and DCAA released by microalga are dependent on the algal species involved.

The present study confirmed the previous results of Williams,[16] Whittle,[17] Brockmann *et al*[12] and others, that microalgae are a significant source of dissolved amino acids in the marine environment.

Pseudomonas 022 selectively utilized dissolved amino acids of algal EOC. The amino acids in both algal EOCs that this bacterium preferred to utilize were asp, glu, ala, ile, leu, and phe. Selective utilization of glu was reported by Williams and Yentsch.[18] Glu was observed in maximum concentration in a early stage of a *Chaetoceros* bloom, but rapidly decreased to minimum levels possibly due to bacterial uptake.[19]

Bacterial utilization of amino acids in algal EOC after 30 h incubation produced drastic changes in the composition of dissolved amino acids in algal EOC. Ser and gly were observed as dominant DFAA in both algal EOC after the the bacterial incubation, in addition to thr and tyr in *C. marina* EOC (Fig. 6 and 7). Lee and Bada[3] reported that in open ocean the amount of DCAA was about 10 times more than that of DFAA, and ser, ala and probably gly were the major components among them. Eguchi and Ishida (personal communication) obtained the same results as Lee and Bada[3] and Ishida and Kadota[20] found that gly, ser and glu were favorable substrates for oligotrophic bacteria in open ocean by kinetic study. In Klamath Lake, Oregon, ser, gly in addition to lys as dominant were found.[21] In three lakes in Denmark were founded ser, gly, ala and orn as dominant within 25 amino acids.[22] High percentages of ser and gly were observed after 48 h of a experiment of decomposition of *Skeletonema coastatum* cells by one *Pseudomonas* strain.[23] The dominance of ser and gly as free amino acids did not correspond with the amono acid composition of plankton proteins.[24]

It was observed in this study that bacteria themselves introduced changes in conscentration and composition of dissolved amino acids released by microalga. The fact of a common occurrence of a few free amino acids as dominant may not mean that it is important nutrient for bacteria. On the contrary other amino acids detected in lowest concentrations may be have a high turn-over rate.

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