## D-1.2.2 Development of Analytical Methods for Marine Microbial Diversity

**Contact Person** Makoto M. Watanabe
Leader
Environmental Biology Division,
National Institute for Environmental Studies
Environment Agency
16-2 Onogawa, Tsukuba, Ibaraki 305-0053, Japan
Tel:+81-298-50-2555 Fax:+81-298-50-2577
E-mail:mmw@nies.go.jp

**Total Budget for FY1996-FY1998 17,412,000 Yen (FY1997; 5,739,000 Yen)**

**Abstract** The East China Sea is located at the mouth of the Yangtze River. The huge Three Gorges Dam, being constructed in the middle of the river, is expected to lead to changes in river flow that will affect East China Sea water quality. Our aim in this study was to observe the microbial community structure of the East China Sea as a baseline for comparison with future changes. We studied bacterial diversity in East China Sea surface water based on both phenotypes and genotypes. Bacteria from three common marine groups, *Alpha-proteobacteria*, *Gamma-proteobacteria* and CFB, were dominant. Some other groups, including Low G+C gram posi., High G+C gram posi. and relatives of *Verrucomicrobium*, were also observed. Dominant species in phenotype and genotype analysis were close relatives of *Alteromonas macreodii*. Phytoplankton diversity were determined using ultrastructural observation method using electro microscope, and flowcytometryanalysis (FCM) method. From ultrastructural observation, 4 different picophytoplankton, *Micromonas pussila* (Prasinophyceae), *Nannochloris*-like species (Chlorophyceae), *Imantonia rotunda* (Haptophyceae) and *Synechococcus* spp. (Cyanophyceae) were recognized. From FCM analysis, distribution of picophytoplankton were determined as follows; at stations A1, B1 and C1, close to the shore, the cell density ranged 200-700 cells/ml, while at stations B3, B5 and C5, furthest from the shore, the maximum number reached 16,800 cells/ml. From these results, the microflora seems to bedifferent in each site, and FCM and PCR methods could be useful for the routine study of microbial communities and biodiversity in the East China Sea.

**Key Words** Flow cytometry, Picoplankton, PCR, Phylogenetic tree

1. Introduction

The East China Sea is located offshore from the mouth of the Yangtze River. The huge Three Gorges Dam, height 190 m, width 2 km, being constructed in the middle reaches of the River will create a lake 600 km long, and is expected to lead to changes in river flow that will affect East China Sea water quality (Fig. 1). It is important to evaluate changes in microbial community structure with changes in the environment, since the microbial community functions as the bottom line in marine ecosystems. Our aim in this study was to observe the microbial community structure in the East China Sea as a baselinefor comparison with future changes. In the past, detection and analysis of bacteria in the environment have mainly been done by methods based on cultures. However, it is difficult to culture most bacteria in the environment. Recently, bacterial community-structure analyses that do not depend on cultivation have been widely carried out, especially using PCR for targeting 16S rRNA genes for non-culturable bacteria. In this study, we used both a culture-dependent method based on the pattern of carbon utilization and a culture-independent method based on the molecular technique of

targeting 16S rRNA sequences. On the other hand, phytoplankton play an important roll of biological production in marine ecosystem, while picophytoplankton are normaly difficult to detect and identify by microscope observation, because of their small size (around 1 $\mu$ m). In order to detect such species, we applied an ultrastructural observation method of enriched culture samples and flowcytometry analysis.

2. Research Objective

In this study, we evaluated several new methodologies, in addition to a conventional method, to determine the structure of the microbial community in the East China Sea.

3. Research Method

(1)Sampling

Samplings were carried out in October 1997 and May 1998, just before construction began on the Three Gorges Dam. We took 9 samples in total: from the surface (S), midwater (M), and bottom (B) at 3 stations, C1, C3 and C5 (Fig. 1-D).

(2)Comparison of bacterial community based on PCR-RFLP pattern

Bacterial community structures in the 9 samples were compared based on PCR-RFLP patterns. Seawater samples (100 ml) were filtered with a 0.2-$\mu$ m pore size filter. Total DNA from each filter was extracted with a Fast DNA kit (BIO101), the physical extraction method using glass beads, from bacteria cellstrapped on the filter. The 16S rRNA genes were amplified by PCR with bacterial universal primer sets and digested with a restriction enzyme. Digested DNAs were separated on 2.5% agarose gel and the patterns were analyzed by the PDI fragment analysis system.

(3)Phenotypic analysis of bacterial clusters

Eighty bacterial clones randomly isolated from marine agar plates on which the surface seawater sample at St. C1 was spread, were applied to Biolog GN plates, and the utilization patterns were analyzed by the Microlog MLclust software packages.

(4)Genotypic analysis based on 16S rRNA sequences

Bacterial diversity of the surface seawater at St. C1 was analyzed by comparing the partial sequences of 16S rRNA genes obtained by PCR. Amplified 16S rRNA genes were recovered from agarose gel, and then cloned into pCR. 2.1 vectors using a TA cloning kit. We obtained sequences for 150 clones of 16S rRNA genes with a minimum 400 bp from PCR primer 1494 reverse primer for all clones. All sequences were compared with similar sequences of reference organisms by BLAST search. A phylogenetic tree was constructed by the neighbor-joining method with the CLUSTAL W software package.

(5) Enriched culture method

In order to detect picophytoplankton which are normaly difficult to detect in field samples because of their small size (around 1 $\mu$ m), an enriched culture method was used. After 2-3 weeks incubation of enrichment cultures, developed picoplankton were fixed and identified by ultratructural observation.

(6) Flowcytometry analysis

For determining the distribution of picophytoplankton, a flow cytometry system (Becton Deckinson, FACSCalibur) were used. Seawater samples were fixedwith gluitaraldehyde (2.5%

final concentration) immediately after collection and stored in freeezer. Samples were illuminated with an argon laser beamat 488 nm, and their green, orange and red fluorescences (FL1, FL2 and FL3), and forward and side-angle light scatters (FSC and SSC) were etermined by FCM.

4. Results and Discussion

(1)Comparison of bacterial community structure by PCR-RFLP

The phylogenetic tree constructed from the PCR-RFLP pattern showed that the bacterial community structure at St. C1 was different from that of the othersites; also at all stations the structure at the surface was different from that in midwater and the bottom (Fig. 2). This suggested that freshwater from the Yangtze River affects the structure of bacterial communities in the East China Sea. We regarded St. C1 as a suitable site for evaluation of changes in the outflow of the Yangtze River.

(2)Bacterial concentration at St. C1

The density of bacteria at St. C1 as estimated by CFU on marine agar platesand by 4', 6-diamino-2-phenylindol (DAPI) direct count was $2.8x10^4$ $ml^{-1}$ and $4.2x10^5$ $ml^{-1}$, respectively, indicating that only some of the bacteria were culturable (Fig. 3).

(3)Phenotypic analysis of bacteria isolated from East China Sea

From phenotypic analysis of 80 isolates using the Biolog test system, we found 4 groups (Fig. 4): relatives of *Alteromonas macleodii*, relatives of *Roseobacter gallaeciensis*, a *Vibrio* group, a Tiny colony group. The Tiny colony group (Fig. 5), which comprised 70% of the isolates, were close relatives of *Roseobacter litoralis*, which is reported to have photosynthetic ability (Fig. 6). This may be related to the fact that the Tiny colony groups were mainly obtained from surface water, where light isat a maximum (data not shown).

(4)Genotypic analysis

From genotypic analysis, bacteria from 3 common marine groups, *Alpha-proteobacteria*, *Gamma-proteobacteria*, and C/F/B, were dominant. Some other groups,including low G+C Gram positive, high G+C Gram positive and relatives of *Verrucomicrobium*, were observed. Further unidentified groups and SAR and OCS clusters (non-culturable groups) were also observed (Fig. 7). These findings suggest that bacterial diversity in the East China Sea is immense.

(5) Enriched culture method

From ultrastructural observation, 4 different picophytoplankton were recognized. They were *Micromonas pussila* (Prasinophyceae), *Nannochloris*-like species (Chlorophyceae), *Imantonia rotunda* (Haptophyceae) and *Synechococcus* spp.(Cyanophyceae). All of them were known as cosmopolitan species in coastal and pelagic areas, although further studies for *Nannochloris*-like species and *Synechococcus* species are needed to describe the species name. Application of an enriched cultures of size fractionated seawater samples seems to bean efective method to detect picophytoplanktons.

(6) Flowcytometry analysis

The results of FCM analysis showed the differences in the biomass between collecting sites (Fig. 8). At stations A1, B1 and C1, close to the shore, thecell density ranged 200-700 cells/ml, while at stations B3, B5 and C5, furthest from the shore, the maximum number reached 16,800 cells/ml. Furthermore, the number of cells /ml in surface water samples tended to be higher

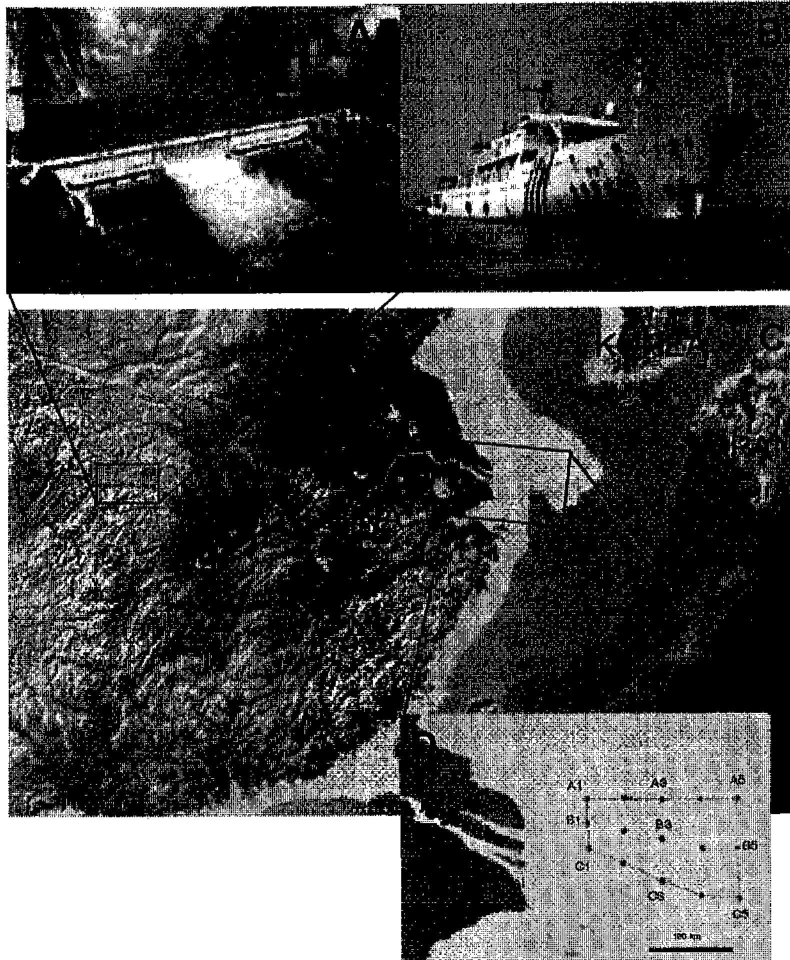

Figure 1. (A) Conceptual drawing of the Three Gorges Dam at its completion. (B) Kaikan-49, the survey vessel. (C) Map of the area including the Yangtze River. (D) Location of the survey stations.

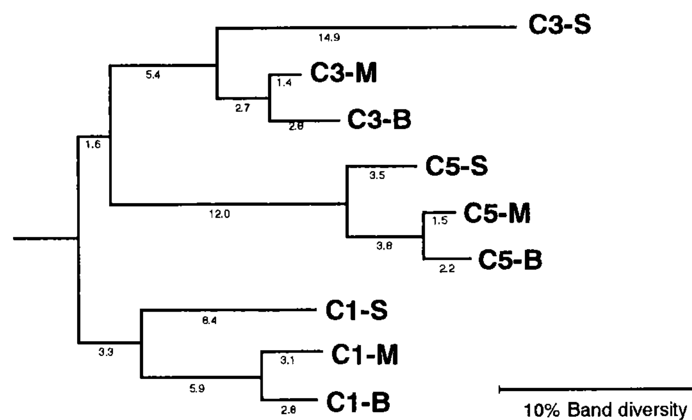


Figure 2. Phylogenetic tree based on PCR-RFLP patterns digested with *Hae* III.

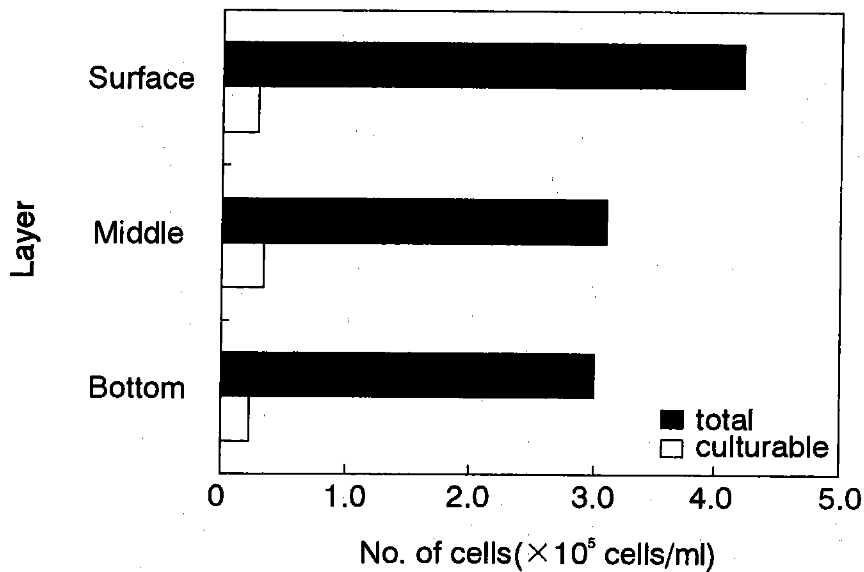


Figure 3. Comparison of bacterial numbers estimated from CFU on marine agar plates and by DAPI direct counts.

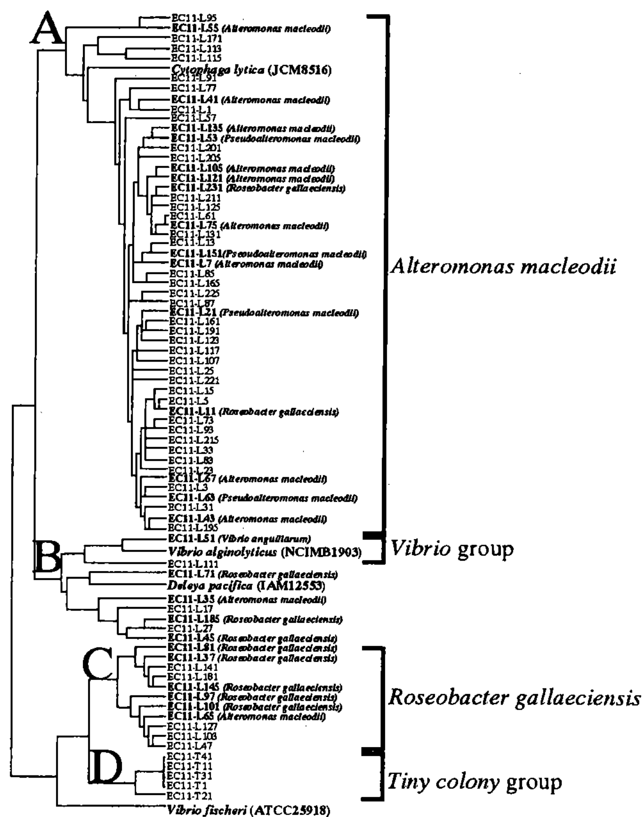


Figure 4. Phylogenetic tree based on carbon source utilization patterns obtained with the Biolog system for 80 isolates from surface waters at St. C1.

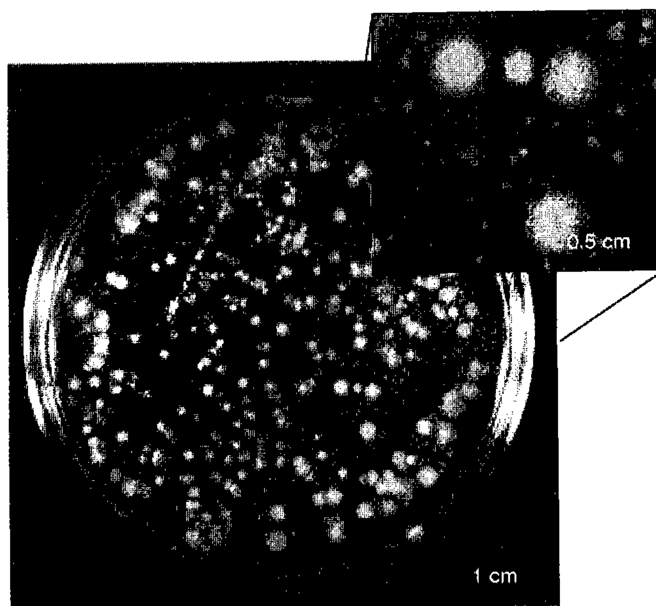


Figure 5. Bacterial colonies growing on a marine agar plate.

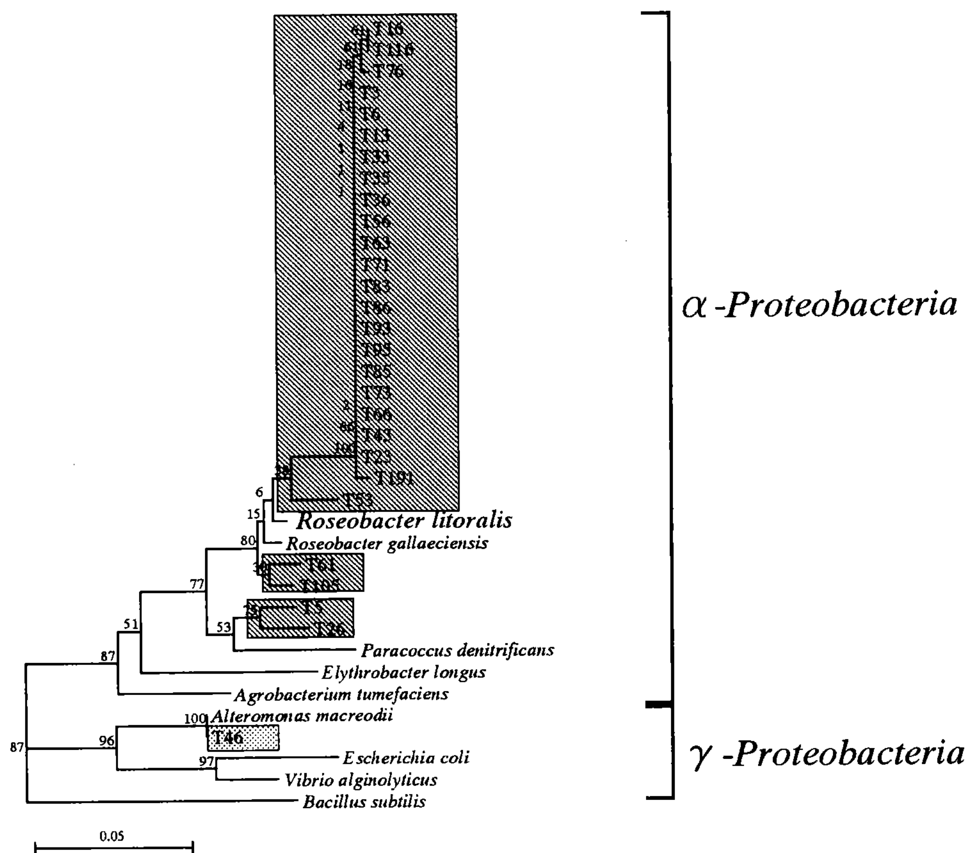


Figure 6. Phylogenetic tree based on partial sequences of 16S rRNA genes for the Tiny colony group.

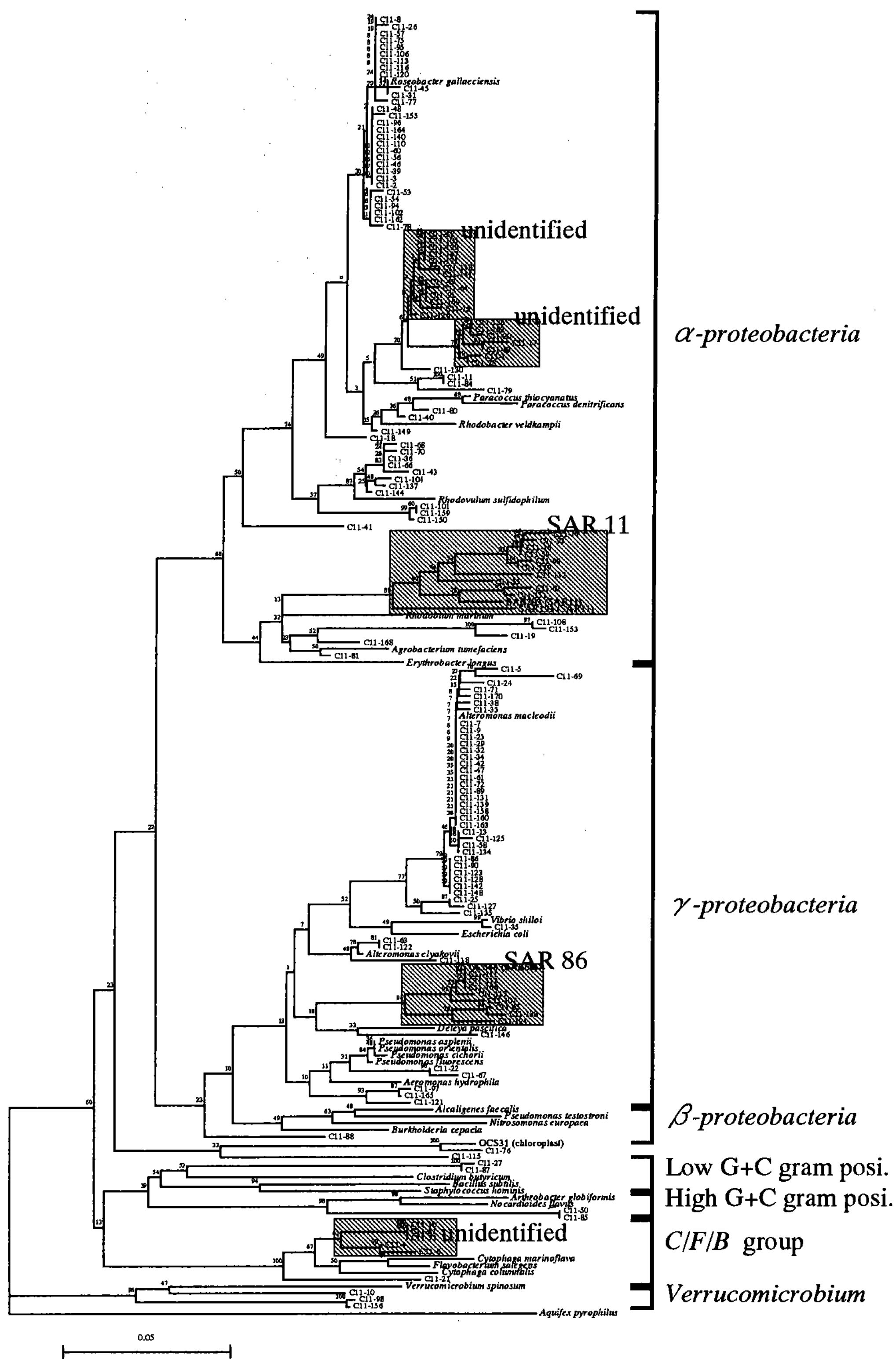


Figure 7. Phylogenetic tree based on partial sequences of 16S rRNA genes obtained from surface waters at St.C1by PCR with bacterial universal primer sets.

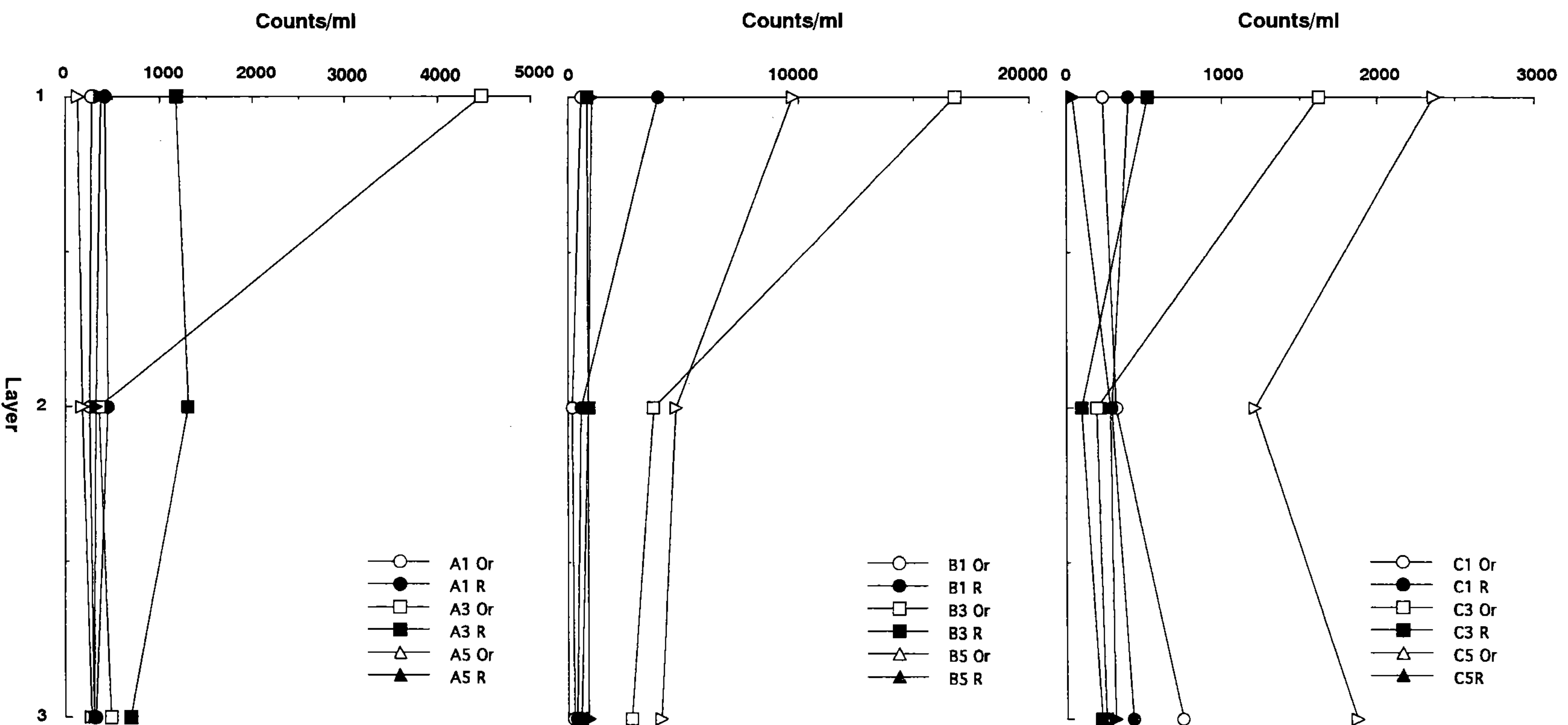


Fig. 8. Distribution of picophytoplankton determined by FCM analysis.
"Or" means cyanobacterial phytoplankton with orange fluorescences, and "R" means eucaryotic phytoplankton with red fluorescences.