Community Composition and Carbon Utilization Profiles of Yodo River Microbes in Brackish and Freshwater Sediments

MEI YAMATO¹, JUNQIN PANG¹, SATOSHI SODA²*, and MICHIIHIKO IKE¹

¹Division of Sustainable Energy and Environmental Engineering, Graduate School of Engineering, Osaka University/ 2-1, Yamadaoka, Suita, Osaka 565-0871, Japan
²Department of Civil and Environmental Engineering Research Center for Biwako Sigma, Ritsumeikan University/ 1-1-1 Nojihigashi, Kusatsu, Shiga 525-8577, Japan

Abstract

This study was undertaken to obtain basic data related to the community composition and metabolic functions of estuarine microbes. Water and sediment samples were collected in 2014–2015 from Juso Lagoon (brackish area) and Shirokita Wando (freshwater area) along the Yodo River flowing to Osaka Bay. The genotypes and phenotypes of the estuarine microbes were evaluated, respectively, using eubacterial 16S rRNA genes based on terminal-restriction fragment length polymorphism (T-RFLP) and on carbon-utilization tests using Biolog GN2 plates containing 95 separate carbon sources. The microbial community structure and carbon utilization profiles of the brackish sediment differed greatly from those of freshwater sediment. Canonical correlation analysis revealed that the estuarine microbes could be characterized by conductivity (salinity) and temperature of their habitats. Deltaproteobacteria including sulfate-reducing bacteria were possibly dominant in the brackish sediment, suggesting effects of the high sulfate concentration in seawater on the microbial community. The heterotrophic population and the diversity index calculated from the T-RFLP analysis and the carbon source utilization tests in summer and autumn in vegetation zones were higher than those in non-vegetation zones of both brackish and freshwater areas, suggesting that river vegetation can stimulate microbial activity in the rhizosphere.

Keywords: brackish water, estuary, genotype, microbial ecosystem, phenotype

INTRODUCTION

Estuaries have high economic value. They provide various ecosystem services including the provision of biogeochemical cycles of carbon, nitrogen, phosphorus, sulfur, and other elements between the land and ocean systems⁴,⁸. Biochemical reaction rates generally increase with temperature⁹. Differences in the temperature response of microbes mediating organic matter decomposition in estuaries can result in decoupling between the production and consumption of organic intermediates⁰. Seawater intrusion also influences organic matter decomposition, particularly in low-salinity regions of estuaries⁵,⁶,⁸. Salinity affects biogeochemical processes by changing the ionic strength, pH, sulfate availability, and the inhibitory H₂S concentration of water⁴. In addition, salinity can affect vegetation, which can in turn influence the biogeochemical cycle because of the release of oxygen and organic exudates through plant roots.

Human activities have altered and degraded these regions directly and indirectly on a global basis⁸. For conserving estuary environments, relations between phylogenetic
composition and functional characteristics of microbes in estuaries should be fully elucidated. Our earlier study characterized nitrogen-cycling functional gene profiles of the sediment microbes according to nitrification activity, denitrification activity, conductivity, pH, temperature, and vegetation in Juso Lagoon (brackish area) and in Shirokita Wando (freshwater area) along the Yodo River in Osaka. Juso Lagoon is a habitat of macrobenthos that include *Corbicula japonica* and *Grandidierella japonica*. The Shirokita Wando is a nursery environment of Itasenpara bitterling, a nationally protected fish. As far as we know, sediment microbes in those biodiversity hotspots in the urbanized river have not been well characterized. The present study was undertaken to obtain basic data related to the community composition and carbon utilization of estuarine microbes. As same as nitrogen, carbon is a fundamental element affecting pollution and biodiversity in water environment. The bacterial community and carbon source utilization profiles of the sediment samples were analyzed, respectively, using terminal-restriction fragment length polymorphism (T-RFLP) analysis and Biolog assay. The former, T-RFLP analysis, is a robust molecular method used for rapid analysis of microbial community structures. The latter, Biolog assay, is a redox-based technique capable of characterizing the functional potential of microbial communities based on its ability to use 95 carbon sources.

**MATERIALS AND METHODS**

**Sampling sites** Figure 1 presents the sampling site locations. Water and sediment samples were collected from Juso Lagoon in a brackish area and Shirokita Wando in a freshwater area along the Yodo River flowing to Osaka Bay in July, October, November 2014, and April 2015.

Fig. 1  Sampling site locations. Water and sediment samples were collected from Juso Lagoon in a brackish area and Shirokita Wando in a freshwater area along the Yodo River flowing to Osaka Bay in July, October, November 2014, and April 2015.

Briefly, the water temperature decreased from 30.8\(^\circ\)C to 13.7\(^\circ\)C in winter. The pH values of the samples were neutral, except for those of the samples in summer. Conductivity of the brackish samples (0.76–3.43 S/m) was higher than that of the fresh water (0.11–0.16 S/m).

Sediment samples were taken as grab samples from 0 to 9 cm depth by the 5-point composite method at 50 cm intervals. The sediment samples of vegetation zones were taken at > 5 m far from non-vegetation zones. They were transported on ice to the laboratory, where they were subjected to physicochemical quality measurements. Sediment samples were sieved through a 2.0-mm screen. Then heterotrophic bacteria were enumerated using the R2A agar plate (Merck and Co. Inc., Darmstadt, Germany).

**Biolog assay** Carbon source utilization profiles were determined using Biolog GN2 plates (Biolog, Hayward, CA, USA). Each well of the Biolog GN2 plate contains one of 95 different carbon sources and tetrazolium dye. Sediment samples (10 g-wet) were homogenized with 0.9% NaCl solution (10 mL). Supernatant of the sediment suspension (4 mL) was diluted with 0.9% NaCl solution (36 mL). Aliquots (150 µL) of the solution were added to wells of a Biolog GN2 plate. Then the plates were incubated statically at 28\(^\circ\)C. The absorbance of each well at 595 nm (A\(_{595}\)) was measured periodically during a 48
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h period. Wells for which A595 was higher than 0.25 were judged as positive. The average well color development (AWCD)11) and the used carbon source number were adopted as indices.

**T-RFLP analysis** Microbial DNA was extracted from sediment samples using ISOIL for bead beating (Nippon Gene Co. Ltd., Tokyo, Japan). The DNA was then purified (MagExtractor-PCR&Gel Clean up; Toyobo Co. Ltd., Tokyo, Japan). Subsequently, T-RFLP analysis was conducted as described in an earlier report12). The conserved region of eubacterial 16S rRNA gene was PCR amplified using 27F, of which the 5’-end was labeled with phosphoramidite fluorochrome 5-carboxyfluorescein (6-FAM), and 1392R primer set13). were defined as positive peaks. The candidate phylum for each T-RF was identified related to a database (Microbial Community Analysis (MiCA) III, http://mica.ibest.uidaho.edu).

**Multivariate statistics** The microbial community diversity was evaluated using the Shannon–Weaver index ($H'$) as $H' = \frac{1}{H''}$ (T-RFLP)$^{14}$ and $H'$ (Biolog)$^{15}$, which was calculated respectively from T-RFLP and the carbon source utilization profiles. Canonical correspondence analysis (CCA) and cluster analysis were conducted using PAST ver. 1.34 software (http://folk.uio.no/ohammer/past/) to relate water quality and the community composition or carbon utilization of the sediment microbes.

**RESULTS**

Microbial community composition in the sediment

As Table 2 shows, populations of heterotrophic bacteria of the vegetation zone in summer and autumn were slightly higher than those in the non-vegetation zone. The T-RFLP analysis was applied to characterize the eubacterial 16S rRNA gene in the sediment samples. The T-RF fingerprint patterns of the samples digested with HhaI are portrayed in Fig. 2. Dominantly detected T-RFs are presented in Table 3. Results demonstrate that unique microbial communities had formed at each sampling site. The T-RF of 545 bp was detected frequently from 9 of the 16 sediment samples. In fact, 17 T-RFs were detected only from freshwater samples.
Table 2 Summary of T-RFLP analysis and Biolog assay results

| Date     | Site* | Bacteria              | T-RF number | \( H' \) (T-RFLP) | AWCD | Used carbon number | \( H' \) (Biolog) |
|----------|-------|-----------------------|-------------|---------------------|------|--------------------|------------------|
| 24 Jul 2014 (Summer) | BV    | 3.3 x 10^6             | 30          | 3.2                 | 0.62 | 62                 | 4.2              |
|          | BN    | 1.7 x 10^6             | 29          | 3.0                 | 0.51 | 44                 | 4.0              |
|          | FV    | 1.4 x 10^6             | 36          | 3.3                 | 1.04 | 55                 | 4.2              |
|          | FN    | 7.2 x 10^5             | 36          | 3.3                 | 0.55 | 43                 | 3.9              |
| 4 Oct 2014 (Autumn) | BV    | Nov x 10^6             | 27          | 3.0                 | 0.56 | 55                 | 3.9              |
|          | BN    | 8.5 x 10^5             | 25          | 3.0                 | 0.47 | 43                 | 3.3              |
|          | FV    | 1.5 x 10^6             | 37          | 3.4                 | 0.54 | 60                 | 4.1              |
|          | FN    | 6.3 x 10^5             | 27          | 3.1                 | 0.25 | 26                 | 3.5              |
| 28 Nov 2014 (Winter) | BV    | 1.4 x 10^6             | 28          | 2.8                 | 0.38 | 43                 | 3.8              |
|          | BN    | -                     | 28          | 2.9                 | 0.14 | 15                 | 2.8              |
|          | FV    | 1.2 x 10^6             | 39          | 2.9                 | 0.35 | 52                 | 4.1              |
|          | FN    | 1.6 x 10^6             | 30          | 2.9                 | 0.31 | 39                 | 3.8              |
| 18 Apr 2015 (Spring) | BV    | 1.2 x 10^6             | 28          | 3.2                 | 0.98 | 76                 | 4.4              |
|          | BN    | 1.6 x 10^6             | 27          | 3.1                 | 0.70 | 69                 | 4.3              |
|          | FV    | 2.4 x 10^6             | 33          | 3.4                 | 0.93 | 68                 | 4.3              |
|          | FN    | 2.9 x 10^6             | 32          | 3.3                 | 0.67 | 56                 | 4.1              |

*Brackish vegetation (BV), brackish non-vegetation (BN), freshwater vegetation (FV), and freshwater non-vegetation (FN).  
–, Not determined

Fig. 2 HhaI-digested T-RF profiles of the eubacterial 16S rRNA genes in sediment samples collected from the Yodo River in July, October, November 2014, and April 2015. Panels show results for brackish vegetation (BV), brackish non-vegetation (BN), freshwater vegetation (FV), and freshwater non-vegetation (FN).
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Table 3  Dominant T-RFs detected from sediment samples of Juso Lagoon in a brackish area and Shirokita Wando in a freshwater area in July, October, November 2014, and April 2015

| T-RFs size (bp) | Detected site* | Candidate phyla |
|----------------|----------------|-----------------|
| 57             | FV(Jul), FN(Jul) | No data         |
| 59             | FV(Jul)         | Firmicutes, Planctomyces, α-, δ-, γ-Proteobacteria |
| 60             | FV(Oct)         | Flavobacteria, Acidobacteria |
| 90             | BN(Oct), FN(Nov), BV(Apr) | Flavobacteria |
| 91             | BN(Oct), BN(Nov), BV(Apr) | δ-Proteobacteria |
| 92             | BN(Jul), BN(Oct), BV(Nov), FV(Apr), FN(Apr) | Bacteroidetes |
| 93             | BV(Oct), BV(Nov), BN(Nov), BV(Apr) | δ-Proteobacteria |
| 94             | BV(Jul), BN(Jul), BV(Oct), BV(Nov), BN(Nov), FV(Nov), BN(Apr) | Flavobacteria |
| 95             | BN(Apr)         | No data         |
| 97             | BN(Apr)         | δ-Proteobacteria |
| 196            | FN(Nov)         | No data         |
| 197            | FN(Oct)         | γ-Proteobacteria, Bacilli |
| 199            | FN(Apr)         | α-Proteobacteria |
| 202            | FN(Apr)         | Actinobacteria, Dehalococcoidetes |
| 205            | BN(Apr)         | β-Proteobacteria |
| 206            | BN(Oct)         | β-Proteobacteria |
| 207            | BV(Jul), BN(Jul) | γ-Proteobacteria |
| 370            | FV(Jul), FN(Jul), FN(Oct) | Actinobacteria |
| 373            | FN(Nov)         | γ-Proteobacteria |
| 375            | FV(Oct)         | γ-Proteobacteria |
| 380            | FN(Nov)         | No data         |
| 381            | BN(Apr)         | No data         |
| 383            | FV(Nov), FN(Nov) | No data         |
| 384            | BN(Jul)         | No data         |
| 469            | FV(Jul), FN(Jul) | Actinobacteria |
| 539            | BV(Apr)         | No data         |
| 545            | BV(Jul), BN(Jul), BV(Oct), BN(Oct), FN(Oct), BV(Nov), BN(Nov), FV(Nov), BN(Apr) | No data |
| 555            | FN(Apr)         | No data         |
| 556            | BV(Apr)         | Clostridia      |
| 557            | FN(Apr)         | No data         |
| 560            | FN(Oct)         | No data         |
| 561            | FV(Jul), FN(Jul), FN(Nov) | No data         |
| 563            | BV(Nov), BN(Nov) | No data         |
| 564            | BN(Oct), FV(Oct) | No data         |
| 565            | BN(Apr)         | γ-Proteobacteria |
| 566            | BV(Oct), BN(Nov), FV(Apr) | No data         |

*Brackish vegetation (BV), brackish non-vegetation (BN), freshwater vegetation (FV), and freshwater non-vegetation (FN).
than those in the non-vegetation zone. The logarithm of the viable counts of heterotrophic bacteria showed positive correlation with $H'(T-RFLP)$ of the brackish samples ($r = 0.86$) but showed little correlation with that of the freshwater samples ($r = 0.65$).

Phylogenic genera of the eubacteria in the samples were inferred from the T-RF sizes, although many T-RFs were not included in the database. Proteobacteria (59, 197, 199, 373, and 375 bp) and Actinobacteria (202, 370, and 469 bp) were dominant in the freshwater sediment. However, bacteria belonging to the phylum Proteobacteria were dominant in the brackish sediment (91, 93, 97, 205, 206, 207, and 565 bp). Two T-RFs of 93bp and 97bp identified as $\delta$-Proteobacteria were inferred as Desulfofobacterales.

Figure 3 presents ordination plots of CCA results for the T-RF profiles and environmental variables of the sediment samples. The first CCA axis explains 99.3% of the total variance; the second axis added 0.7%. The sediment samples were divided roughly into brackish and freshwater groups along the first axis. The strongest determinant for the community composition of the sediment microbes was conductivity. In each group, the sediment samples were shown along the second axis correlated with temperature. Little difference was found between the samples of the vegetation zone and those of the non-vegetation zone. The T-RFs with high absolute values of canonical loadings are shown in Table 4. The T-RFs of 551, 574, and 580 bp had positive loadings, whereas 204, 534, and 469 bp had negative loadings for Axis 1. The T-RFs of 214 bp, 407 bp, and 662 bp had positive loadings, although 571, 537, and 370 bp had negative loadings for Axis 2.

**Carbon source utilization profiles of sediment microbes**

Carbon source utilization profiles of the sediment samples are portrayed in Fig. 4. Results show that the profiles of the brackish samples differed from those of freshwater

![Fig. 3 Ordination plots of CCA results for Hha-digested T-RF profiles of the eubacterial 16S rRNA genes and environmental variables (temperature and conductivity) of sediment samples collected from the Yodo River in July, October, November 2014, and April 2015. Results are shown for brackish vegetation (BV), brackish non-vegetation (BN), freshwater vegetation (FV), and freshwater non-vegetation (FN).](image)

Table 4  T-RFs with high absolute values of canonical loadings of CCA

| T-RF (bp) (Assumed class) | Axis 1 Loading | T-RF (bp) (Assumed class) | Axis 2 Loading |
|--------------------------|---------------|--------------------------|---------------|
| 551 (-)                  | 2.07          | 214 (β-Proteobacteria)   | 2.04          |
| 574 (-)                  | 2.07          | 407 (-)                  | 1.93          |
| 580 (-)                  | 2.07          | 662 (-)                  | 1.78          |
| 563 (-)                  | 2.07          | 542 (-)                  | 1.75          |
| 557 (-)                  | 1.98          | 553 (-)                  | 1.75          |
| 204 (γ-Proteobacteria)   | -1.42         | 571 (-)                  | -2.78         |
| 534 (-)                  | -1.42         | 537 (α-Proteobacteria)   | -2.78         |
| 469 (Actinobacteria)     | -1.40         | 370 (Actinobacteria)     | -2.78         |
| 79 (β-Proteobacteria)    | -1.38         | 356 (Actinobacteria)     | -2.78         |
| 194 (δ-Proteobacteria)   | -1.37         | 218 (-)                  | -2.78         |
|                          |               | 62 (α-Proteobacteria)    | -2.78         |
Fig. 4  Carbon utilization profiles of sediment samples collected from the Yodo River in July, October, November 2014, and Apr 2015. Results are shown for brackish vegetation (BV), brackish non-vegetation (BN), freshwater vegetation (FV), and freshwater non-vegetation (FN).
sediment samples. Table 2 presents results of the biology assay of the sediment samples. The used carbon source numbers by the sediment samples in spring were higher than the other seasons. The AWCD, the used carbon source number, and $H'(\text{Biolog})$ of the vegetation sediment showed higher values than those of the non-vegetation sediments. The logarithm of the viable counts of heterotrophic bacteria were found to have positive correlation with $H'(\text{Biolog})$ of brackish samples ($r = 0.97$), but little correlation was found with that of freshwater samples ($r = 0.39$). No correlation was found between the $H'(\text{Biolog})$ and $H'(\text{T-RFLP})$ in brackish and freshwater samples.

Figure 5 portrays ordination plots of CCA results for the carbon source utilization profiles and environmental variables of the sediment samples. The first CCA axis explains 99.5% of the total variance; the second axis added 0.5%. Similarly to the results of the microbial community analysis, the sediment samples were broadly divisible into brackish and freshwater groups along the first axis. The strongest determinant for the carbon source utilization potential of the sediment microbes was conductivity. In each group, sediment samples are shown with seasonally driven variation along the second axis correlated with temperature fluctuation. Little difference was found between the samples of the vegetation zone and those of the non-vegetation zone.

Carbon sources with high absolute values of canonical loadings are shown in Table 5.

Table 5  Carbon sources with high absolute values of canonical loadings of CCA

| Carbon source                  | Axis 1 Loading | Carbon source                  | Axis 2 Loading |
|-------------------------------|---------------|-------------------------------|---------------|
| l-Threonine                   | 3.29          | α-Hydroxybutyric acid         | 5.69          |
| γ-Hydroxybutyric acid         | 2.76          | Sebacic acid                  | 4.90          |
| α-Cyclodextrin                | 2.58          | i-Erythritol                  | 3.18          |
| m-Inositol                    | 1.94          | β,γ-L-Carnitine               | 2.71          |
| α-Keto butyric acid           | 1.91          | Succinamic acid               | 2.63          |
| α-D-Glucose-1-phosphate       | −2.57         | Pyruvic acid methyl ester     | −2.44         |
| β-Hydroxybutyric acid         | −2.40         | L-Arabinose                   | −1.85         |
| l-Leucine                     | −2.33         | D-Mannose                     | −1.83         |
| d-Galactonic acid lactone     | −2.11         | D-Arabitol                    | −1.76         |
| Glycyl-l-aspartic acid        | −2.05         | α-Cyclodextrin                | −1.53         |
DISCUSSION

The microbial community composition and carbon source utilization profiles of the brackish sediments differed greatly from those of the freshwater sediments. The CCA for both T-RFLP analysis and biology assay showed that the estuarine microbes were characterized by the conductivity (salinity) and temperature of their habitats (Figs. 3 and 5). The brackish sediments were characterized by bacteria with the T-RFs of 551, 574, 580, 563, and 557 bp (Table 4). Microbes in the brackish sediments were characterized by utilization of the specific carbon sources such as L-threonine, γ-hydroxybutyric acid, and α-cyclodextrin (Table 5). In addition, the nitrogen-cycling functional gene profiles of the microbes were characterized by conductivity and temperature\(^{7}\). The mixing of freshwater and seawater communities and probable existence of a typical estuarine bacterial community are known to create high diversity and temporal and spatial variation\(^{16–18}\).

Therefore, sulfate is not present in large quantities in freshwater areas, methanogenesis is usually the dominant anaerobic degradation process of organic matter. However, salinity intrusion is likely to increase sulfate reduction process of organic matter. As reported for other estuaries, it is probable that factors such as nutrient concentrations and DO also determine the microbial community composition\(^{9,17,18}\).

The viable counts of heterotrophic bacteria, \(H'(T\text{-RFLP})\) and \(H'(\text{Biolog})\) of the vegetation zone in summer and autumn were slightly higher than those in the non-vegetation zone. As reported from an earlier study\(^{7}\), nitrification and denitrification activities were higher in the vegetation zone than in the non-vegetation zone. These results are likely to reflect that river vegetation can stimulate microbial activity in the rhizosphere. The exudates of common reed in the vegetation zone can activate the carbon utilization potential of sediment microbes\(^{20}\).

Salinity and temperature can change estuarine vegetation, yielding indirect effects on the biogeochemical processes. Salt marsh plant communities play an important role in the climate change adaptation of estuaries.

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