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Estimation of carbon biomass and community structure of planktonic bacteria in Lake

Biwa using respiratory quinone analysis

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Running title: Carbon biomass and community structure of bacteria in Lake Biwa

23

24 **Abstract**

25 The relationship between bacterial respiratory quinone (RQ) concentration and biomass was

26 assessed for Lake Biwa bacterial assemblages to evaluate the utility of bacterial RQ

27 concentration as an indicator of bacterial carbon. Biomass estimated from RQ concentration

28 correlated well with that from cell volume, indicating that RQ concentration is an appropriate

29 indicator of bacterial biomass. The estimated carbon content per RQ content (carbon

30 conversion factor) of bacteria was 0.67 mg C nmol RQ^{-1}. Bacterial carbon biomass, which

31 was estimated from RQ concentration using the conversion factor, ranged between 0.008 and

32 0.054 mg C L^{-1} (average: 0.025 mg C L^{-1}) at 5 m depth and between 0.010 and 0.024 mg C

33 L^{-1} (average: 0.015 mg C L^{-1}) at 70 m depth. Ubiquinone-8-containing bacteria dominated the

34 epilimnion and hypolimnion. Compared to the conventional image analysis, the bacterial RQ

35 analysis is a less laborious way for simultaneous determination of bacterial biomass and

36 community.

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Introduction

Bacteria are numerically important components in the water columns of freshwater and marine systems. Many studies have demonstrated that the efficiency of recycling of matter depends on the bacterial metabolic activity, growth rate, and biomass (reviewed by Ducklow 2000; Azam and Malfatti 2007). As a result, bacteria are thought to be the major players in mineralization and biogeochemical organic matter transformations. Previous studies have reported that natural bacteria differ in terms of size distributions (Nagata 1986), metabolic state (Gasol et al. 1999), dissolved organic matter (DOM) utilization (Kirchman et al. 2004; Yokokawa and Nagata 2010), and growth rate (Yokokawa et al. 2004).

Since natural bacterial assemblages consist of various subgroups in terms of ecological and biogeochemical features, the phylogenetic and functional diversity of bacteria has been investigated in marine and freshwater environments. A majority of previous studies have discriminated bacterial communities on the basis of the 16S rRNA gene and with dramatic development of molecular biological techniques, the phylogenetic and functional diversity of bacteria has been revealed (Glöckner et al. 1999, 2000; Kirchman et al. 2004; Yokokawa et al. 2005). However, phylogenetically distinct bacterial groups do not always correspond to functional groups in matter cycling (Langenheder et al. 2005, 2006). The information about physiological discrimination of bacterial groups such as cellular components and nucleic acid content is quite limited, and the utility of physiological discrimination in bacterial community in the carbon cycling has not yet been fully understood.

Bacterial biomass is the fundamental parameter that directly links estimations of production, growth efficiency, and bioenergetics of the bacteriovores. Image analysis of bacterial cell volume is the most common tool used to estimate bacterial biomass, and this method has improved over several decades (Nagata and Watanabe 1990; Blackburn et al.

63 1998; Posch et al. 2009). However, image analysis is time-consuming, laborious, and involves

64 many technical difficulties such as staining with fluorescent dyes (Posch et al. 2001; Straza et

65 al. 2009) and artificial errors in cell volume measurement (Nagata and Watanabe 1990). A

66 less time-consuming, less laborious, and more accurate method for bacterial carbon biomass

67 measurement is needed to evaluate bacterial contributions to matter cycling in aquatic

68 systems.

69 Respiratory quinone (RQ), including ubiquinone (UQ) and menaquinone (MK), are

70 components of the electron transport chain located in the bacterial plasma membranes.

71 Bacteria have diverse respiratory systems, and they can use more than 20 inorganic or organic

72 redox pairs for energy production (Li 2010). Individual RQs differ in their preference for the

73 electron acceptor for energy metabolism. In general, UQs are mainly produced for the oxygen

74 and nitrate respiratory types because of the large midpoint potential between UQs and UQH_2

75 (+0.122 V), whereas MKs (-0.074 V) are mainly produced for the respiratory types with

76 low-potential electron acceptors. As individual bacteria has only 1 dominant RQ type, RQs

77 are potentially useful as specific biomarkers for discriminating between the biomass of

78 bacterial subgroups with different types of energy metabolism (Collins and Jones 1981;

79 Hedrick and White 1986; Villanueva et al. 2007). RQs have been shown to be appropriate

80 biomarkers for tracing the bacterial biomass in various environments (Hedrick and White.

81 1986; Hiraishi 1999), as they are membrane lipids and not storage lipids, and undergo rapid

82 degradation within hours or days after cell death (Hiraishi and Kato 1999). As these

83 biomarkers directly indicate the bacterial biomass, improvement in the chemical

84 determinations of bacterial biomass may provide a less time-consuming and more accurate

85 method for estimating bacterial biomass in aquatic systems.

86 RQ typing, which discriminates bacterial subgroups based on differences in energy

87 metabolism, may be appropriate for identifying useful functional units in ecological

4

88   matter-cycling studies. However, there is not much clarity on whether bacterial RQ

89   concentration can be used as an indicator of bacterial biomass in water columns, although

90   bacterial quinone concentrations in soil environments have displayed linear relationships with

91   bacterial carbon concentration (Saitou et al. 1999) and cell abundance (Hiraishi et al. 2003).

92   Thus, our major goal was to examine the utility of planktonic bacterial RQ analysis as a

93   reliable method for carbon biomass estimation in Lake Biwa. Moreover, we also evaluated the

94   reliability of RQ-based bacterial discrimination as a tool for planktonic bacterial community

95   analysis.

96

97   **Materials and Methods**

98   *Study site and sampling*

99   Lake Biwa is a large (surface area, 674 km^2), deep (maximum depth, 104 m), monomictic,

100   and mesotrophic lake located in the central part of Honshu Island, Japan. The sampling station

101   for the present study was a pelagic station (35º 12′58″ N, 135º 59′55″ E; maximum depth 73

102   m), in the north basin of the lake. Samples were collected every month from June 2010 to

103   March 2011. Depth profiles of water temperature were determined using a CTD probe (SBE

104   911 plus; Sea Bird Electronics). Samples were collected from 2 distinct layers at depths of 5

105   m (the epilimnion) and 70 m (the hypolimnion) with a 10-L acrylic water sampler and poured

106   into 5- or 10-L polyethylene bags for RQ analysis. The samples were also collected with 5-L

107   Niskin X bottles (General Oceanics) and poured into 500-mL polycarbonate bottles washed

108   with 1.2 M HCl for analyses of chlorophyll *a*, dissolved organic carbon (DOC), and

109   particulate organic carbon (POC). Bacterial enumeration was performed by taking 200 mL of

110   the water sample in a polypropylene bottle and fixing immediately with glutaraldehyde at a

111   final concentration of 1%.

Bacterial enumeration

We used 2 mL of the fixed water sample for enumerating the bacteria. Bacterial cells were stained with DAPI, filtered through black polycarbonate filters having a pore size of 0.2-µm (Millipore), and counted using an epifluorescence microscope (BX60, Olympus) under ultraviolet excitation (Porter and Feig 1980). At least 20 fields were randomly inspected in triplicate, and more than 300 bacterial cells were counted for each replicate. The length and width of each bacterial cell were measured for more than 200 bacterial cells in each sample with image analysis software (Image J; National Institute of Health). Images were captured at a magnification of 1000× with a CCD camera (ORCA-ER; Hamamatsu) equipped with an epifluorescence microscope. Bacterial cell volume was calculated as described by Nakano and Kawabata (2000).

Chemical variables

Samples for DOC were filtered through 0.2-µm polycarbonate filters (Whatman) that had been washed with 1.2 M HCl. DOC concentrations were determined using a total organic carbon analyzer (TOC-5000A; Shimadzu).

On the other hand, to determine chlorophyll *a* concentration, 100 mL to 200 mL of water samples were filtered through 0.2-µm polycarbonate filters (Whatman) and analyzed with the *N′ N*-dimethylformamide method (Moran and Porath 1980) using a fluorescence spectrometer (RF-5300PC; Shimadzu).

POC measurements were carried out between September 2010 and March 2011. Glass fiber filters of 0.3 µm nominal pore size (GF-75, Advantec) were used. About 68.7–84.9% of the bacterial cells were retained on the GF75 filter (data not shown). One to 2 L of water samples were filtered through precombusted (450°C, 5 h) GF75 filters. POC concentrations were measured using a CN corder (MT-700; Yanako). The contribution of calcium carbonate to the particulate carbon concentration was ignored because particulate calcium concentrations are

137   typically low in the study area (Mito et al, 2002).

138   *RQ analysis*

139   Water samples were filtered through the GMF2UM glass fiber filters (Whatman) to remove

140   large particles such as phytoplankton and zooplankton, and the filtrates were filtered again

141   through 0.2-μm Teflon filters (Advantec) to retain bacteria-sized particles. About 97.2–99.9%

142   of bacterial cells passed through the GMF2UM filter (data not shown).

143   The RQ concentrations were determined using a modified method as previously described

144   by Kunihiro et al. (2008, 2011). Briefly, quinones were extracted from the filters with a

145   chloroform-methanol mixture (2:1, v/v) and re-extracted into hexane. UQs and MKs

146   contained in the crude extract were separated and purified on a Sep-Pak® Plus Silica (Waters).

147   The molecular species and concentrations of quinones were determined with a high

148   performance liquid chromatography (HPLC) system equipped with an ODS column (pore size,

149   3.5 μm; Eclipse Plus C18, 3.0 × 150 mm; Agilent) and a photodiode array detector

150   (SPD-M20A; Shimadzu). A mixture of 20% isopropylether in methanol was used as the

151   mobile phase, at a flow rate of 0.5 mL min^{-1}. The column oven temperature was maintained at

152   35°C. UQs and MKs were quantified at wavelengths of 275 nm and 270 nm, respectively.

153   UQ-10 (Sigma) was used as a quantitative standard. The quinones were identified according

154   to their column retention times and the UV spectrum of each peak was observed in the

155   photodiode array detector (Hiraishi and Kato 1999).

156   In the present paper, we refer to the RQ types using the following abbreviations:

157   ubiquinone, UQ-*n*; menaquinone, MK-*n*. The number (*n*) indicates the number of isoprene

158   units in the side chain of the quinone. For example, UQ-10 represents a ubiquinone with 10

159   isoprenoid units, and MK-9(H_2) represents a menaquinone with 9 isoprenoid units where 1 of

160   the 9 units is hydrogenated with 2 hydrogen atoms.

161 *Cluster analysis based on the RQ profiles*

162 Quantitative evaluation of the changes in the microbial community during the study period

163 was carried out by calculating a dissimilarity index (*D*) based on the quinone profiling data

164 using the following equation (Hiraishi et al. 1991):

165

166
$$D(i,j) = \frac{1}{2} \sum_{k=1}^{n} \left| f_{ki} - f_{kj} \right|$$

167

168 where f_{ki} and f_{kj} are the mole fractions of the *k* quinone component in the *i* and *j* samples,

169 respectively. The distance matrix was used for cluster analysis.

170 The between-groups linkage method was used for cluster formation with the aid of the

171 KyPlot 5.0 program (KyensLab Inc.).

172 *Cultures*

173 Bacterial strains and a mixture of culturable bacteria were used to analyze the differences in

174 carbon yields of RQs between UQ- and MK-containing bacteria. Surface water of the

175 sampling station aged over 6 months in dark following GF/F filtration was used to prepare

176 PYG agar plates (5 g L^{-1} peptone, 2.5 g L^{-1} yeast extract, and 1g L^{-1} glucose) and 10 times

177 diluted PYG agar plates. One-hundred-microliter aliquots of water samples were spread on

178 these plates and the plates were incubated at 20ºC for 2 weeks. 4 bacterial strains, namely, O,

179 P, Y1, and Y2 (Table 2) were isolated. While colonies of the strains O and P were orange and

180 pink, respectively, the strains Y1 and Y2 were yellow with different colony morphologies

181 (Table 2). The isolates were grown axenically in diluted PYG liquid medium at 20ºC and

182 harvested in the exponential growth phase by filtration (GF75 filter; Advantec).

183 The culturable bacterial mixture was obtained by inoculating diluted PYG liquid medium

184 with 100 µl of water sample collected from the 5 m depth layer on February 2011. The

185 culturable bacteria were harvested in the same manner as the isolates. Harvested cells were

used for measuring the concentrations of quinones and POC (see above). The cell number and cell volume were estimated by fixing several milliliters of the culture medium with glutaraldehyde (final concentration, 1%). Cell-specific carbon content (CSCC), volume-specific carbon content (VSCC), and RQ-specific carbon content (RSCC) were calculated for each culture according to the following equations:

$$\text{CSCC (fg C cells}^{-1}) = \frac{\text{POC in culture}}{\text{Bacterial cell number in culture}}$$

$$\text{VSCC (pg C } \mu m^{-3}) = \frac{\text{POC in culture}}{\text{Total bacterial cell volume in culture}}$$

$$\text{RSCC (mg C nmol}^{-1}) = \frac{\text{POC in culture}}{\text{RQ yield from culture}}$$

Incubation experiments

Incubation experiments were conducted to determine bacterial RQ and carbon contents at the sampling site. A water sample was collected at a depth of 5 m at the sampling site on June 22, 2011, and filtered through GF/C glass fiber filters having a nominal pore size of 0.2-μm or 1.2-μm (Whatman). The 0.2-μm filtrate (microorganism free) and the 1.2-μm filtrate (bacterial grazer free) were mixed in a ratio of 9:1 to reduce bacterial density for avoiding depletion of carbon and nutrient source during the incubation and poured into a 6-L polyethylene bag. Glucose was added to each bag at a final concentration of 25 μmol L^{-1} to stimulate bacterial growth. The experiment was run in triplicate, and the experimental bags were incubated in the dark at *in-situ* temperature. After 5 days of incubation, samples were removed to measure POC concentration, bacterial quinone concentration, bacterial abundance, and cell volume. Samples for POC analysis were collected on precombusted (450°C, 5 h)

210 GF75 filters, and the amount of carbon was measured using a CN corder (MT-700; Yanako).

211 CSCC, VSCC, and RSCC were also calculated from the incubation experiment.

212 *Statistical analysis*

213 Analyses using the Student's t-test and Pearson's coefficient were performed with

214 Microsoft Excel.

215

216 **Results**

217 *Variation in physico-chemical parameters*

218 Water temperature at 5 m varied from June to December 2010, whereas water temperature

219 at 70 m was low and relatively constant throughout the study period (Table 1). Because the

220 differences in water temperature between the 5 m and 70 m depths from January to March

221 2011 were not more than 0.4°C, June to December 2010 was regarded as the stratification

222 period and January to March 2011 as the mixing period. During the stratification period, DOC,

223 POC, and chlorophyll *a* concentrations varied at 5 m, whereas at 70 m, all the parameters

224 were relatively low with constant values (Table 1).

225 *Seasonal variation in bacterial number, biovolume, and RQ concentration*

226 During the stratification period, bacterial number and bacterial cell volume at 5 m varied

227 from 1.4×10^9 to 4.3×10^9 cells L^{-1} and from 0.16 to 0.77 mm^3 L^{-1}, respectively (Fig. 1A, 1B).

228 In contrast, the bacterial number and bacterial cell volume at 70 m were less variable (7.5 $\times$

229 10^8 to 1.1×10^9 cells L^{-1}, 0.10 to 0.22 mm^3 L^{-1}, respectively). During the late stratification

230 period (November or December) and the mixing period, the differences in bacterial number

231 and cell volume between the 5 m and 70 m depths diminished (Fig. 1A, 1B).

232 At 5 m, the RQ concentration exhibited a variable pattern similar to that of bacterial

233 number throughout the study period (Fig. 1C). RQ concentration at the 5 m depth gradually

234 decreased from 101.5 pmol L^{-1} to 13.7 pmol L^{-1} during the stratification period, whereas that

235 of the 70 m depth fluctuated between 23.0 and 42.4 pmol L^{-1}. During the mixing period, RQ

concentration of the 5 m and 70 m depths showed similar changing patterns, ranging from 14.0 to 33.0 pmol L^{-1} and from 14.7 to 25.6 pmol L^{-1}, respectively.

Significantly, the RQ concentrations at 5 m and 70 m depths showed fairly linear relationships with bacterial number ($r^2 = 0.74$, $p < 0.001$) and bacterial cell volume ($r^2 = 0.94$, $p < 0.001$) (Fig. 2). When we individually used the data of 5 m or 70m depth, we found a significant correlation only for bacterial number ($r^2 = 0.82$, $p < 0.001$) and bacterial cell volume ($r^2 = 0.98$, $p < 0.001$) at 5 m depth was observed (Fig. 2). However, there were no significant correlations at 70 m depth (Fig. 2).

Seasonal variation in UQ and MK concentrations

At 5 m, UQ and MK concentrations were the highest in July 2010 and then gradually decreased (Fig. 3A, B). At 70 m, UQ concentrations fluctuated from 9.8 to 18.9 pmol L^{-1} during the stratification period, whereas the MK concentration was relatively stable. Relative concentration of UQ was generally higher than that of MK at the hypolimnion, whereas the opposite trend was found at 5 m (Fig. 3C, D).

The major RQ concentrations at both depths showed similar changing patterns with some exceptions (Fig. 4A–F). Generally, the highest values were observed in July or August at 5 m and tended to decrease toward the mixing period. UQ-8 (Fig. 4A) and MK-9(H$_8$) (Fig. 4F) were the dominant RQs at 5 m, varying from 3.4 to 24.0 pmol L^{-1} and from 2.4 to 17.6 pmol L^{-1}, respectively. At 70 m, most of the dominant quinone species did not exhibit any seasonal variability, although UQ-8 highly fluctuated throughout the study period (6.0 to 17.0 pmol L^{-1}) (Fig. 4A).

During the stratification period, the relative concentrations of UQ-8 at 70 m (36% to 57%; average, 48%) was much higher than that at 5 m (24% to 33%; average, 29%) (Fig. 4G). In contrast, the relative concentrations of MK-9(H$_8$) at 5 m was higher (15% to 32%; average, 23%) than that at 70 m (16% to 17%; average, 17%) during the stratification period (Fig. 4L).

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261 During the mixing period, the relative concentrations of UQ-8 and MK-9(H_8) were not

262 significantly different between the 5 m and 70 m depths (Fig. 4G, L).

263 The cluster analysis based on the dissimilarity of the RQ profiles divided the bacterial

264 communities into 3 different groups: Group I (5 m, stratification period), Group II (70 m,

265 stratification period), and Group III (5 m and 70 m, mixing period) (Fig. 5). Dissimilarity

266 values less than 0.1 are not recognized as different quinone profiles (Hu et al. 2001). Based on

267 these criteria, bacterial communities of Group I consist of various groups with different types

268 of RQs (>0.1).

269 The contributions of UQ-8 to the total RQ concentration of Group I, Group II, and Group

270 III were 29%, 48%, and 38%, respectively (Fig. 6). Contributions of MK-9 (H_8) to the total

271 RQ of Group I, Group II, and Group III were 23%, 7.3%, and 16%, respectively (Fig. 6).

272 UQ-9, MK-8(H_2), and MK-9(H_6) were the dominant RQs in Group I, Group II, and Group III,

273 respectively (Fig. 6).

274 *Variations in carbon content per RQ content from cultures and incubation experiment*

275 UQ-10 was detected in strains O and P, whereas strains Y1 and Y2 contained MK-6 as the

276 sole RQ (Table 2). UQ-7, UQ-8, UQ-9, and MK-7 were detected in the mixed culture at molar

277 ratios of 3:276:1:1.5 (Table 2). The average CSCC, VSCC, and RSCC were 120 fg C cell^{-1},

278 0.17 pg C μm^{-3}, and 0.38 mg C nmol^{-1}, respectively (Table 2). The coefficients of variation

279 (CV) for CSCC, VSCC, and RSCC were 60%, 30%, and 27%, respectively (Table 2). In

280 terms of CV, the RSCC in cultures was less variable than CSCC and VSCC. The RSCC of

281 UQ-10-containing strains (average, 0.48 mg C nmol^{-1}) was high relative to those of

282 MK-6-containing bacteria (average, 0.33 mg C nmol^{-1}). However, the RSCC of the mixed

283 culture with a predominance of UQ-8 (98%) exhibited a similar value (0.30 mg C nmol^{-1}) as

284 that of MK-6-containing bacteria. UQ-8, UQ-10, MK-6, MK-7, and MK-10 were detected in

285 the incubation experiment at molar ratios of 27:5:52:7:1. The average CSCC, VSCC, and

286　RSCC from the incubation experiment were 42 fg C cell^{-1}, 0.17 pg C μm^{-3}, and 0.67 mg C

287　nmol^{-1}, respectively.

288

289　**Discussion**

290　*Bacterial biomass estimation*

291　　The coefficient of determination (r^2) for the relationship between bacterial biovolume and

292　RQ concentration was higher (0.94) than that between bacterial number and RQ concentration

293　(0.74), suggesting that RQ content is a better indicator of bacterial biovolume than bacterial

294　cell number. Thus, RQs can be used for bacterial biomass estimation in Lake Biwa. However,

295　on using the data of 70 m depth individually, RQ concentration did not correlate with cell

296　number or biovolume (Fig. 2). The annual variation of cell number at 70 m was much lower

297　(CV, 28%) than that at 5 m (CV, 45%), while the annual variation of cell-specific RQ

298　concentration at 70 m was higher (CV, 40%) than that at 5 m (CV, 32%). A lack of

299　correlation between cell abundance and RQ concentration at 70 m was probably due to the

300　high variations of cell specific RQ concentration (Fig. 2A). The annual variation of cell

301　volume specific RQ concentration at 70 m was also much higher (CV, 32%) than that at 5 m

302　(CV, 14%). In the hypolimnion of Lake Biwa, the amounts of available nutrients for bacteria

303　are much higher than that of the epilimnion during the stratification period, whereas the

304　supply of labile DOM is limited (Nishimura et al. 2005). Bacterial nucleic acid content is

305　affected by phosphorus concentration in the hypolimnion of Lake Biwa (Nishimura et al.

306　2005). The bacterial RQ content may be regulated by both the supply of organic substrates

307　and electron acceptors such as oxygen, nitrate, and nitrite, though it is not generally

308　understood.

309　　The reliability of RQs as a tool for estimating bacterial carbon biomass was evaluated by

310　examining RSCC variations in bacterial cells (Table 2). It has been reported previously that

13

cell-specific RQ content varies between strains, whereas strong positive correlations between RQs and total cell number have been reported in soil and aquatic environments (Hiraishi et al. 2003). However, our results indicate that RQ concentration was a better indicator of bacterial cell volume than cells number (Fig. 2). Thus, it is likely that the large variation in cell-specific RQ content in previous studies was due to the variability of cell volumes.

The VSCC of freshwater bacteria in previous studies varied depending on environmental conditions and bacterial activity (Nagata 1986; Bjørnsen 1986; Nagata and Watanabe 1990; Kroer 1994; Loferer-Krößbacher et al. 1998). As differences in measurement techniques and representativeness of the average cell volume derived from a limited number of cell size measurements may result in different estimates, in contrast, the variation in RSCC in the present study was small (Table 2). The RQ measurement can predict bacterial carbon biomass with at least equally good precision, compared to image analysis-based measurement of bacterial biovolume.

However, In contrast to estimations by image analysis, bacterial RQ analysis by HPLC provides a less laborious way for simultaneous determination of bacterial biomass and composition. About one day could be sufficient to analyze about 20 samples if the HPLC is equipped with an autosampler, though the sample preparations are needed before the HPLC analysis.

Bacterial carbon concentration estimated by RQ concentration

Individual strains grown in an artificial medium showed wide ranges of both VSCC and RSCC (Table 2). As far as our knowledge goes, only a single report on RSCC value in natural bacterial community: 0.43 mg C nmol RQ^{-1} (Hu et al. 2001) is available to date. However, this value was derived from activated sludge samples, and the RSCC values of bacterial communities in lakes have never been estimated. The RSCC value in the present study, therefore, is the first estimation for planktonic bacterial biomass. The RSCC value calculated

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336   from the incubation experiment was 0.67 mg C nmol RQ^{-1} (see Results). This RSCC value is

337   the highest amongst the values from the strains and previous estimates, and bacterial carbon

338   biomass estimation based on RQ concentration would carry an error ranging from 0.27 to 0.67

339   mg C RQ^{-1} (Table. 2). However, larger variations of VSCC estimated from image

340   analysis have been reported in a previous study and exhibited 5 fold differences within 9

341   analyses from natural bacterial assemblages in Lake Biwa (Nagata 1986). Such a large

342   variation of VSCC from natural bacteria has been reported in many studies (Bjørnsen 1986,

343   Nagata and Watanabe 1990, Kroer 1994). Thus, the estimated range of RSCC in this study is

344   rather small, compared to the range displayed by VSCC determined by image analyses. The

345   bacterial community composition in our incubation experiment was dominated by UQ-8,

346   similar to natural bacterial community in the lake. Hence, the RSCC value from the natural

347   bacterial community thus determined was used as the conversion factor for estimating

348   planktonic bacterial carbon biomass according to the following equations: Carbon biomass

349   (mg C l^{-1}) = 0.67 × RQ concentration (pmol L^{-1}). During the study period, the carbon

350   biomasses of bacteria ranged from 0.008 to 0.054 mg C L^{-1} at 5 m, and from 0.010 to 0.024

351   mg C L^{-1} at 70 m (Table 3). Contributions of bacterial biomass to the total POC concentration

352   at the 5 m and 70 m depths were from 3.1% to 7.1% (average: 4.7%) and from 1.9% to 10%

353   (average: 7.4%), respectively (Table 3). Using D-amino acids as bacteria-specific biomarkers,

354   Kawasaki et al. (2011) estimated the living bacterial contribution to POC in surface waters of

355   North Pacific Gyre as 5.2–8.2%, which is close to the value estimated in the present study.

356   There are potential sources of error in the current estimation of carbon biomass. The

357   conversion factor determined in the present study may still be an overestimation due to the

358   presence of nonliving colloidal and submicron particles (Koike et al. 1990; Kaiser and Benner

359   2008), and bacterial release of nonliving detrital particles in the bacterial size fraction

360   (Kawasaki and Benner 2006; Kawasaki et al. 2011). Further examination of the relationship

361 between bacterial carbon content and RQ content is needed for more reliable bacterial carbon

362 estimation.

363 *Succession of bacterial community structure*

364   As there was no significant difference between RSCC of UQ-containing bacteria and that

365 of MK-containing bacteria (Table 2), it could be assumed that RSCC would be fairly constant

366 independent of the RQ types, and the relative concentration of individual RQ could be used as

367 an indicator of relative biomass of individual RQ containing bacteria. Moreover, since RQs

368 can be regarded as a specific biomarker for discriminating bacterial subgroups with different

369 types of energy metabolism, Group I comprised highly diverse communities (>0.1) in terms

370 of metabolic state. Group II could be further divided into several groups, although

371 dissimilarity values were less variable than that of Group I. Bacterial communities of Group

372 III fell into a small cluster (<0.1), except for the bacterial community at the 5 m depth in

373 March 2011. Thus, bacterial communities of Group III are relatively uniform assemblages,

374 suggesting that the metabolic state of bacterial communities in Group III at both the depths

375 came similar during the mixing period.

376   The relative abundances of UQ-8 and MK-9 (H_8) varied among the groups (Fig. 4G, 4L)

377 and there appear to be 2 major determinants for dividing the 3 groups in the dendrogram (Fig.

378 5). Bacteria with these RQs may be susceptible to changes in certain physicochemical

379 parameters caused by water mixing and may become opportunistically dominant (Fig. 6).

380 Chemical and biological parameters, such as DOC and chlorophyll *a* (Table 1), were almost

381 uniformly distributed by vertical water mixing (Table 1). RQ compositions at the 2 depths

382 also became similar during the mixing period (Fig. 5). These results suggest that bacterial

383 groups with different types of RQs shifted due to changes in physicochemical parameters

384 caused by vertical water mixing.

385   The predominant UQ-8-containing bacteria exhibited a unique oscillation pattern

386 particularly at 70 m throughout the study period (Fig. 4A, 4G). A broad corresponding

387 relationship was observed between phylogenetic assignment of bacteria and dominant RQ

388 (Collins and Jones 1981; Hiraishi 1999). *Betaproteobacteria*, which is one of the major

389 phylogenetic bacterial groups in freshwater systems (Glöckner et al. 1999), mostly contains

390 UQ-8 as the dominant RQ (Hiraishi 1999). UQ-8-containing bacteria have been associated

391 with nitrogen dynamics (Sinha and Annachhatre 2007; Hamada et al. 2010), and most

392 nitrifying, ammonia-oxidizing- and/or nitrate-oxidizing bacteria belong to this group (Lim et

393 al. 2004; Sinha and Annachhatre 2007). A good correlation between UQ-8 concentration and

394 nitrite + nitrate concentration ($r^2 = 0.84$, $n = 8$, $p < 0.01$) was found in the present study,

395 although only limited data on nitrite and nitrate concentrations were available from December

396 2010 to March 2011 (S.D. Thottathil, unpublished data). In general, UQs are preferably

397 produced by the nitrate respiratory types with relatively high potential electron acceptors.

398 Particularly, UQ-8-containing bacteria may preferentially use nitrate as the final electron

399 acceptor. However, it remains unclear whether UQ-8 is preferentially used for nitrate and

400 nitrite respiration under oxygenated conditions, as in the hypolimnion of Lake Biwa where the

401 annual minimum concentration of hypolimnetic dissolved oxygen is up to 3.2 mg L^{-1} (Kim et

402 al. 2006).

Conclusion

404 To the best of our knowledge, this is the first study that demonstrates the utility of RQ

405 analysis in planktonic bacterial biomass estimation. The results yielded reliable values for

406 carbon concentration and valuable information regarding bacterial contributions to POC.

407 Although the relative abundance of each RQ shifted due to the changes in physicochemical

408 parameters caused by vertical water mixing, UQ-8 dominated the total RQ concentration

409 throughout the study period and accounted for up to 57% of the total RQ concentration.

410 Elucidation of the relationship between biomass of the major bacterial groups, such as UQ-8

411   containing bacteria, and environmental variables, along with estimation of the growth and

412   mortality of those bacterial groups in future studies may provide insights into the regulation of

413   carbon cycling by bacteria.

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572 Table 1. Summary of variability in physico-chemical parameters

Parameters	Stratification (5 m)	Stratification (70 m)	Mixing (5 m & 70 m)
Water temperature (°C)	22.5 ± 6.6	8.2 ± 0.1	7.7 ± 0.6
DOC (mg C L^{-1})	1.18 ± 0.07	0.93 ± 0.06	0.93 ± 0.01
POC (mg C L^{-1})	0.45 ± 0.30[a]	0.20 ± 0.04[b]	0.22 ± 0.02[c]
Chlorophyll *a* (µg L^{-1})	4.92 ± 3.81	0.30 ± 0.10	0.31 ± 0.88

573 Abbreviations: DOC, dissolved organic carbon; POC, particulate organic carbon.

574 [a] Values are from October to December 2010 (n = 3).

575 [b] Values are September, November and December 2010 (n = 3).

576 [c] Values from 70 m on March 2010 were not available due to a laboratory accident.

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Table 2. Carbon yields of bacteria and RQs from culturable bacteria

Culture	Colony features	Average cell sizes (μm^3)	Detected RQs	CSCC (fg C $cell^{-1}$)	VSCC (pg C μm^{-3})	RSCC (mg C $nmol^{-1}$)
Strain O	Orange, Small	0.62	UQ-10	150	0.24	0.43
Strain P	Pink, Small	1.3	UQ-10	220	0.17	0.52
Strain Y1	Yellow, Small	0.54	MK-6	54	0.10	0.27
Strain Y2	Yellow, Large	0.71	MK-6	120	0.17	0.39
Mix	—	0.28	UQ-7: 8: 9: MK-7 = 3: 276: 1: 1.5	46	0.17	0.30
Average				120	0.17	0.38
SD				70	0.05	0.10
CV (%)				60	30	27

588 Abbreviations: CSCC, cell-specific carbon content; VSCC, volume-specific carbon content;

589 RSCC, RQ-specific carbon content.

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Table 3. Ranges of the estimated contributions of bacteria to POC

	5 m	70 m
Bacterial C (mg C L^{-1})^a	0.008 – 0.054 (0.025)	0.010 – 0.024 (0.015)
Contribution to total POC (%)^b	3.1 – 7.1 (4.7)	1.9 – 10 (7.4)

606 Abbreviations as in Table 1.

607 The values provided in parentheses are the average values.

608 ^{a} Values are from June 2010 to March 2011 (n = 10 [5 m] and 11 [70 m]).

609 ^{b} Values are from September 2010 to March 2011 (n = 7 [5 m] and 6 [70 m]).

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Figure legends

Fig. 1. Seasonal variations in (A) bacterial number, (B) bacterial biovolume, and (C) respiratory quinone concentration.

Fig. 2. Relationships between (A) bacterial number and RQ concentration and (B) bacterial biovolume and RQ concentration.

Fig. 3. Concentrations of (A) UQ, (B) MK, relative concentrations of (C) UQ and (D) MK at water depths of 5 m and 70 m.

Fig. 4. Changes in the concentrations of (A) UQ-8, (B) UQ-10, (C) MK-7, (D) MK-8, (E) MK-9, and (F) MK-9(H_8). Changes in the relative concentrations of (G) UQ-8, (H) UQ-10, (I) MK-7, (J) MK-8, (K) MK-9, and (L) MK-9(H_8). Plots at July 2010 are modified from Takasu et al. (2012).

Fig. 5. Cluster analysis of the dissimilarity value matrix data from mole fractions of RQs.

Fig. 6. RQ compositions of Groups I, II, and III.
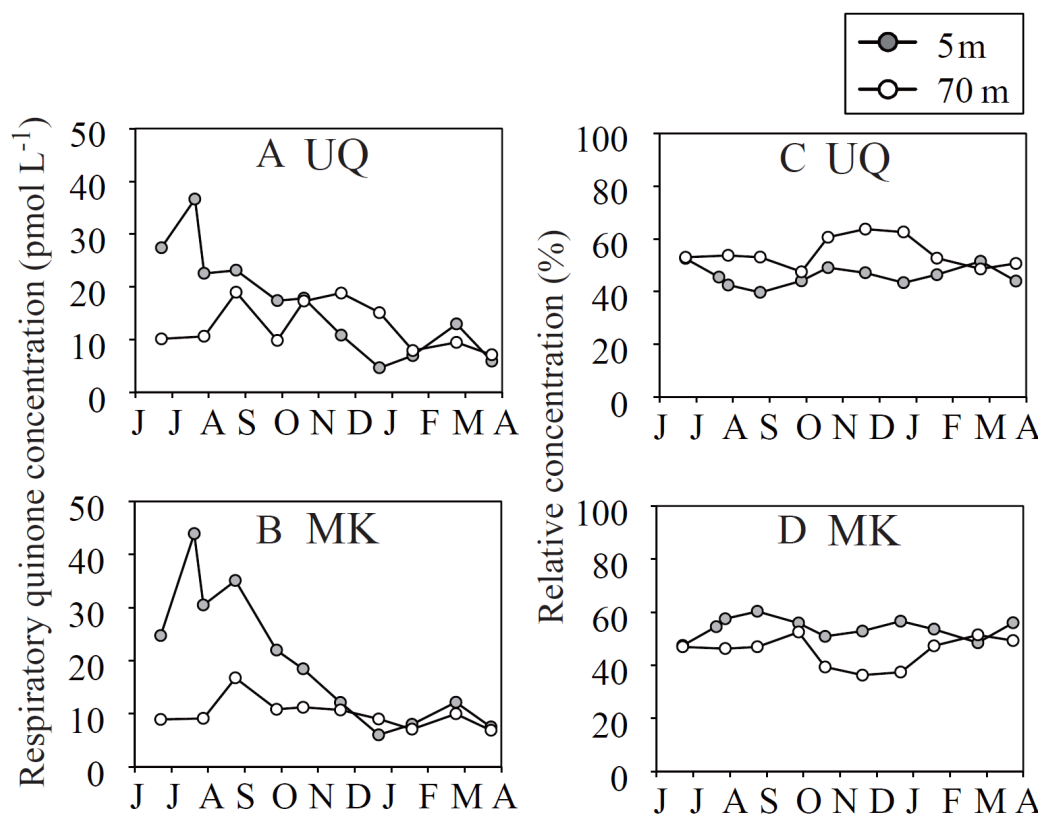
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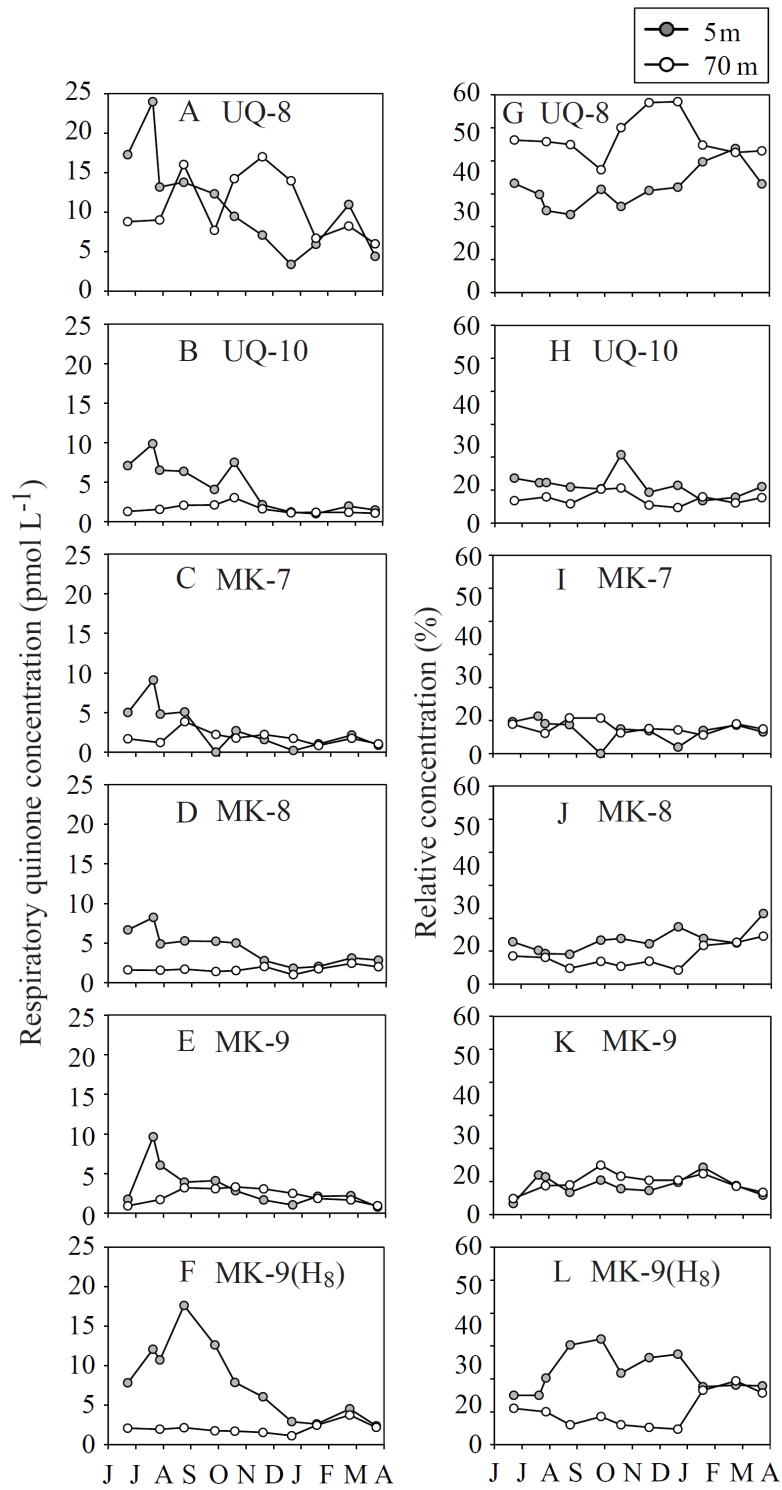632

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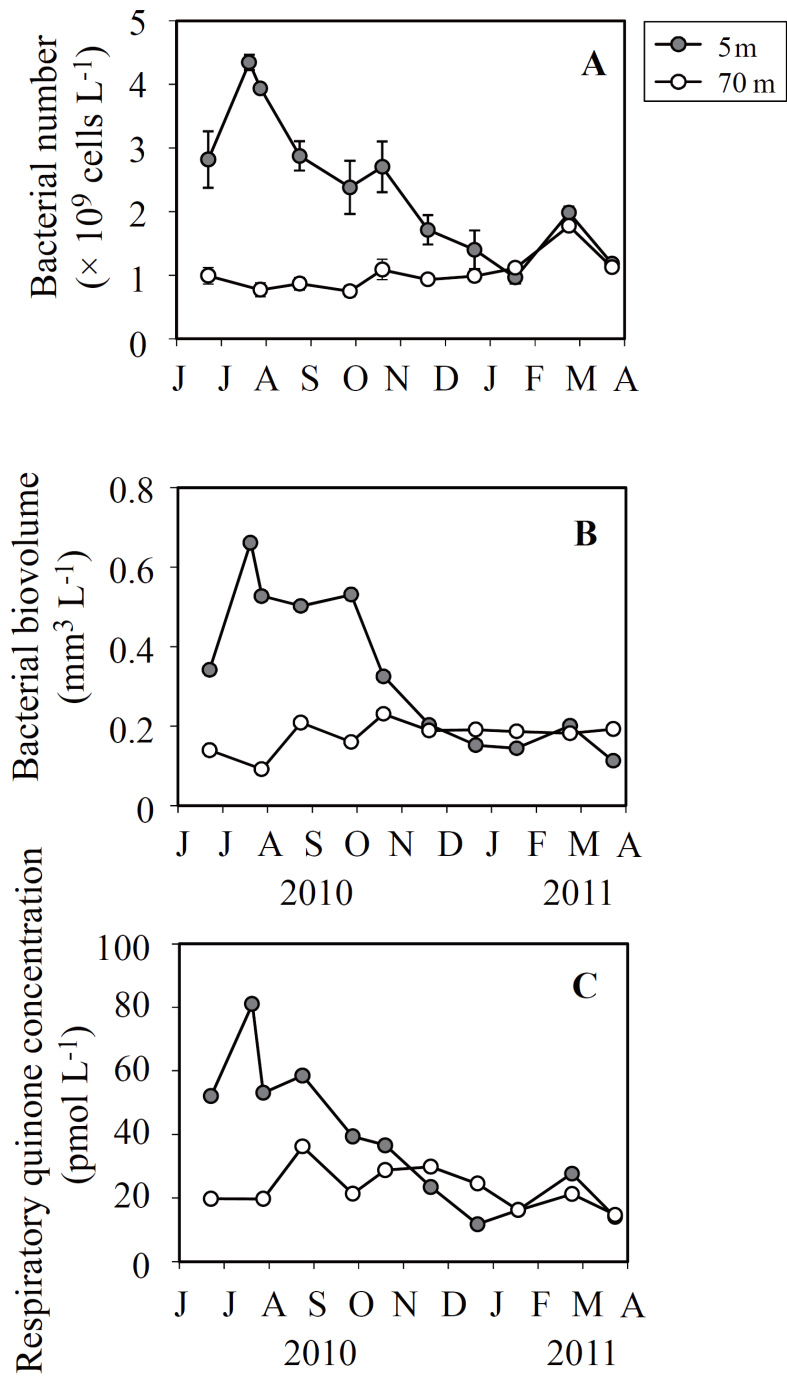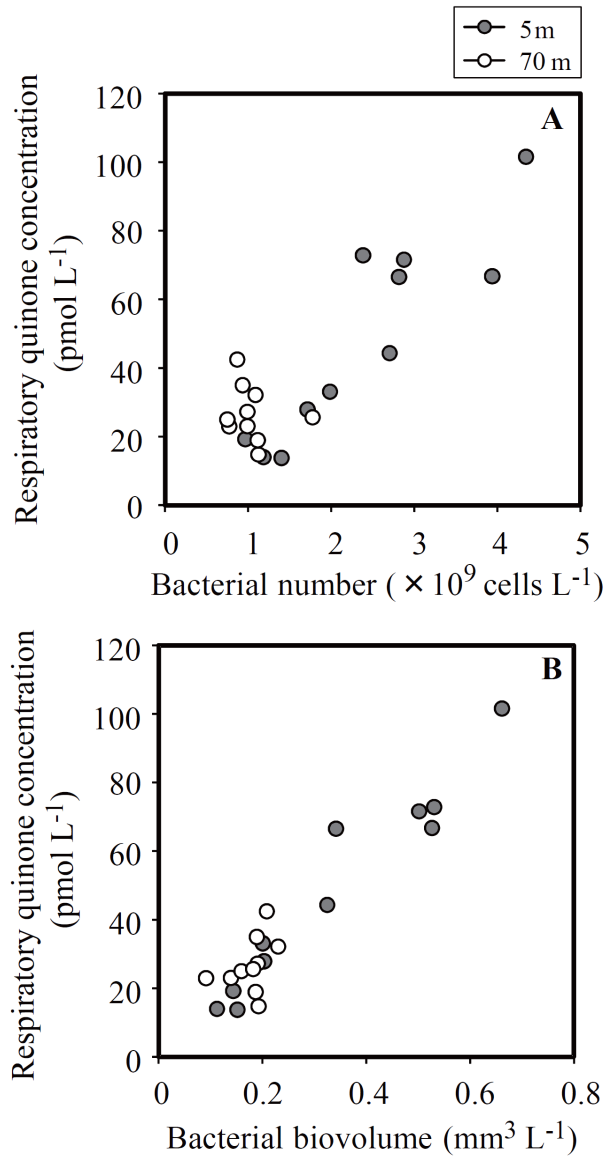
Fig. 2.

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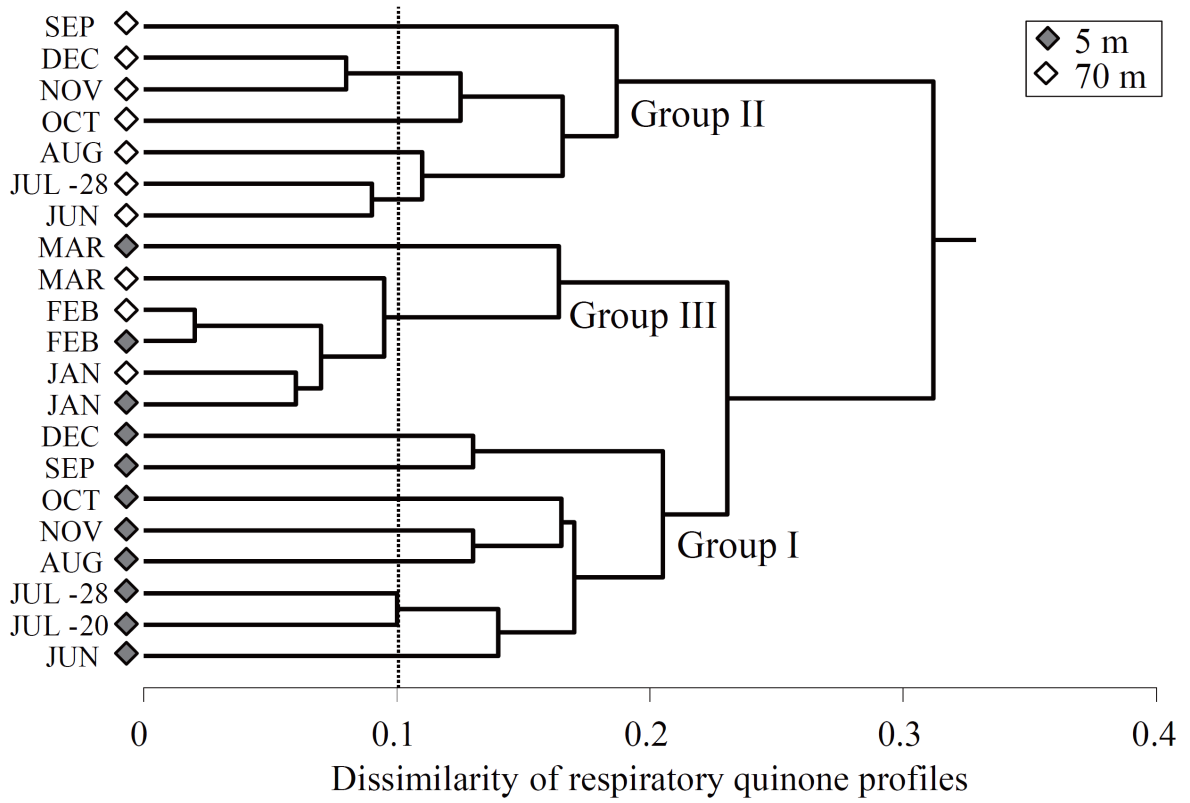
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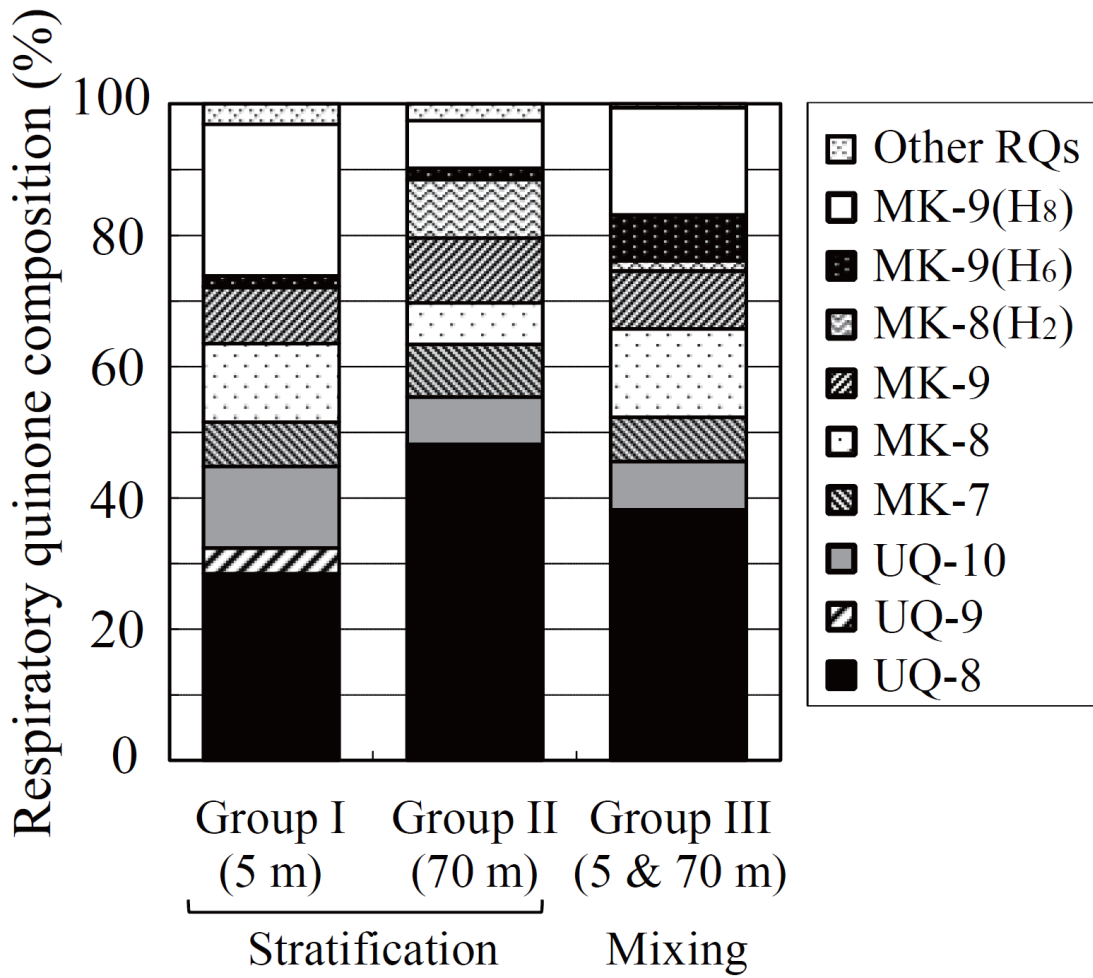
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Fig. 6.

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