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Protistan grazing and viral lysis losses of bacterial carbon production in a large mesotrophic lake (Lake Biwa)

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Running title: Losses of bacterial carbon production in Lake Biwa

Key words: Bacterial growth, Grazing mortality, Viral-mediated mortality

Abstract

The grazing and lysis mortality of planktonic bacteria were estimated using the modified dilution method and respiratory quinone (RQ) analysis in mesotrophic Lake Biwa, Japan. The planktonic bacterial assemblages in the lake consisted of various RQs subgroups with different growth and mortality rates. The sum of total bacterial mortalities due to protistan grazing and viral lysis accounted for 96.6% (range; 89.0 - 107.2%) of daily total bacterial production. This is the first report which successfully demonstrates a balanced relationship between bacterial production and losses using the modified dilution method in a lake. The growth rates of ubiquinone (UQ)-containing bacteria were faster than those of menaquinone-containing bacteria. Especially, the dominant and fastest-growing bacterial groups in the present study were the bacterial groups containing UQ-8 or UQ-10. The sum of their production and loss accounted for 60% of carbon fluxes within the microbial loop. Thus, a large portion of the carbon cycling through the bacterial community in Lake Biwa can be explained by the carbon fluxes through dominant bacterial groups.

Introduction

In the pelagic environments of freshwater and marine systems, a significant fraction of primary production is consumed by heterotrophic bacteria via dissolved organic matter (DOM) released from phytoplankton (Cole et al. 1988; Ducklow 2000). Protistan grazing and viral lysis are two important determinants of the fate of bacterial biomass (Azam et al. 1983; Proctor and Fuhrman 1991), with different ecological and biogeochemical implications. Protistan grazing transfers bacterial biomass to higher trophic-level organisms via the microbial loop (Azam et al. 1983), whereas viral lysis leads to the recycling of carbon and nutrients, both of which are derived from lysed bacterial biomass and are re-supplied to bacteria (Bratbak et al. 1990; Gobler et al. 1997; Proctor and Fuhrman 1991).

Natural bacterial assemblages consist of various subgroups in terms of ecological and biogeochemical features. The relationship between dynamics of bacterial community structure and variations in carbon cycling within the microbial loop are not well understood in natural aquatic systems, though the results of some ecological models suggest that changes in bacterial community structure can affect the carbon fluxes through bacterial communities (Miki et al. 2008). Only limited attempts have so far been conducted to make simultaneous estimation of grazer-induced and virus-induced bacterial mortality for distinct bacterial groups (Šimek et al. 2001; 2007). Obviously, different mortalities among distinct bacterial groups can influence bacterial community composition. The ‘size-selective mortality’ for flagellates changes the size distribution of bacterial community (Pernthaler 2005), whereas the ‘host-specificity mortality’ for viruses changes the bacterial community structure (Thingstad 2000). It is, therefore, important to simultaneously estimate grazing-induced mortality and virus-induced mortality for better understanding on carbon fluxes through bacterial community.

Specific growth and mortality rates of microbial populations can be simultaneously estimated from observed differences in their rates of population growth in a series of

66 incubated diluted and undiluted natural water samples (Landry and Hassett 1982; Landry et al.
67 1984). The dilution technique, originally developed for the estimation of grazing pressure by
68 micro-zooplankton on phytoplankton (Landry and Hassett 1982), is valid to estimate protistan
69 bacterivory (Landry et al. 1984; Trremaine and Mills 1987). Evans et al. (2003) proposed a
70 modified dilution technique to estimate the impacts of protistan grazing and viral lysis on the
71 picoeukaryote *Micromonas* spp. Their technique was successfully applied to estimate the
72 impact of viral lysis on planktonic bacteria in freshwater lake (Tijidens et al. 2008) and ocean
73 (Taira et al. 2009).

74 Fluorescence *in situ* hybridization (FISH) technique as one of the most powerful
75 quantitative molecular approaches has been used for quantifying and visualizing bacterial
76 cells in freshwater and seawater (Pernthaler et al. 1998; Glöckner et al. 1999). The FISH
77 technique is suitable for targeting at specific phylogenetic group levels but less suitable for
78 analysis of the full bacterial community, because quantitative application for analysis of all
79 bacterial groups requires the use of many target-specific probes and also need to optimize its
80 protocol for each target groups (Bouvier and del Giorgio 2003). Despite the superiority of
81 FISH technique-based approaches in terms of phylogenetic identification, respiratory quinone
82 (RQ) analysis has been successfully used to quantify bacterial biomass and to overview
83 bacterial community composition in freshwater (Takasu et al. 2013). Respiratory quinone
84 (RQ), including ubiquinone (UQ) and menaquinone (MK), are electron-transporting
85 compounds in bacterial plasma membranes. Different types of RQ differ in their preference of
86 electron accepters for energy metabolism (Hedrick and White 1986). A bacterial phylum has
87 generally only one dominant molecular species of respiratory quinone (Collins and Jones
88 1981; Hedrick and White 1986). The RQ analysis provides a less laborious and accurate
89 method for simultaneously determining bacterial carbon biomass and community composition
90 because of the chemical analytical-based method with a standardized quantitative extraction
91 protocol (Hu et al. 1999; Takasu et al., 2013). The modified dilution technique combined with

RQ analysis provides us quantitative information about carbon fluxes through distinct bacterial groups in complex microbial food web.

Lake Biwa is a large (surface area, 674 km^2; water volume, 27.3 km^3; watershed area, 3848 km^2), deep (maximum depth, 104 m), mesotrophic and monomictic lake in Japan. Large lakes in the world are generally important freshwater resources (Herdendorf 1990). In addition, large lakes are a precious food production site (Constanza et al. 1997). Net bacterial production in Lake Biwa was estimated as 30% of primary production (Nagata et al. 1990), and their total consumption (net bacterial production plus respiration) was accounted for 50 to 100% of the primary production (Nagata et al. 2012). It has been regarded that carbon fluxes through bacterial community are highly active, and that bacteria are a potentially important basis of the pelagic food web in Lake Biwa (Nagata 1990). Thus, elucidating the carbon fluxes through bacterial subgroups is important for our better understanding about carbon cycling in ecosystem of Lake Biwa.

In the present study, we quantified the carbon fluxes through the bacterial community by estimating carbon production and losses calculated from growth and mortality rates of planktonic bacteria in Lake Biwa. We hypothesized that each bacterial group would have different ecological roles within microbial loop. To test this hypothesis, we determined the grazing and lysis mortalities of each bacterial group using the modified dilution technique and RQ analysis. Our data demonstrated that a large portion of the carbon cycling through the bacterial community in Lake Biwa can be explained by the carbon fluxes through dominant bacterial groups.

Materials and Methods

Dilution experiments

The dilution experiments were conducted in June and October 2011 and May, June, and July 2012, using lake water collected from St. Ie-1 (35º12’58”N, 135º59’55”E; maximum water

depth 73 m) in the pelagic area of the north basin of Lake Biwa, Japan. Approximately 100 L of lake water was collected from 5 m depth using a 10-L acryl water sampler. Enumeration of microbes was performed by placing 200 mL of the water sample in a polypropylene bottle and fixing immediately with glutaraldehyde at a final concentration of 1%. Water samples for the dilution experiments were poured into acid-washed 10-L polyethylene bags or 20-L polyethylene tanks. The lake water was gently filtered through 20 μm mesh to remove mesozooplankton, and 50 L of the filtrate was gravity filtered through 0.2 μm filter cartridges (PALL Acropak Supor membrane capsules) and collected into tanks. After the filtration, half of the 0.2 μm filtrate was passed through a 30 kDa tangential flow filtration system (Millipore PES membrane) to prepare a virus-free diluent. The 20 μm filtrate was diluted in 0.2 μm or 30 kDa diluents to dilution levels of 1.0, 0.8, 0.4, and 0.2, in 5-L polycarbonate bottles washed with 1.2M HCl before use. The bottles were then incubated for 36 to 48 hours at *in situ* temperatures, in dark conditions. Subsamples for the measurement of RQs were collected into clean polyethylene bags at the beginning (0 hours) and end of the incubations.

Enumeration of microbes

For the enumeration of bacteria, 1 or 2 mL of the fixed water sample was used. Bacterial cells were counted using an epifluorescence microscope (BX60, Olympus) under ultraviolet excitation by the DAPI (4,6-diamidino-2-phenylindole) method (Porter and Feig 1980) using 0.2 μm pore-size black polycarbonate filters (Advantec). At least 300 bacterial cells were counted, and a minimum of 20 fields were randomly selected. Fifteen milliliters of the fixed water sample were used for the enumeration of nanoflagellates, and 0.1 mL (1 mL from the 10x diluted samples with 0.02 μm filtered distilled water) was used for the enumeration of viral-like particles (VLP). Heterotrophic nanoflagellates (HNF) and pigmented nanoflagellates (PNF) were counted using epifluorescence microscopy under ultraviolet and green excitation respectively, using the primulin method (Caron 1983), using 0.8 μm pore-size black polycarbonate filters (Corning). Cells of PNF were enumerated by

144 autofluorescence using an epifluorescence microscope under green excitation. For HNF and
145 PNF counting, a minimum of 100 fields were randomly inspected. VLP were counted using
146 epifluorescence microscopy under blue excitation by the SYBR Green I method (Noble and
147 Fuhrman 1998; Patel et al. 2008), using 0.02 μm pore-size Anodisc filters (Whatman). More
148 than 300 VLP were counted and then a minimum of 20 fields were randomly examined.
149 *Chemical variables*
150 Samples for dissolved organic carbon (DOC) measurements were filtered through 0.2 μm
151 polycarbonate filters (Whatman) washed with 1.2M HCl before use. DOC concentrations
152 were determined using a total organic carbon analyzer (TOC-5000A; Shimadzu).
153 To determine chlorophyll *a* (chl. *a*) concentrations, 100 mL water samples were filtered
154 through 0.2-μm polycarbonate filters (Whatman) and analyzed using the *N*′
155 *N*-dimethylformamide method (Moran and Porath 1980) with a fluorescence spectrometer
156 (RF-5300PC; Shimadzu).
157 *Quinone analysis*
158 For RQ analysis, bacteria-sized particles in 5-L of the water samples were collected using 0.2
159 μm pore-size Teflon filters (Advantec) after passing through 2.0 μm nominal pore-sized
160 GMF-2UM glass fiber filters (Whatman) to remove large particles such as phytoplankton and
161 zooplankton. About 97.2–99.9% of bacterial cells passed through the GMF2UM filter
162 (Takasu et al. 2013). In the experiment in June 2011, the GMF-2UM glass fiber filter was not
163 used.
164 The RQ concentrations were determined using a modified method previously described
165 by Hu et al. (1999). Briefly, quinones were extracted from the filters with a
166 chloroform-methanol mixture (2:1, v/v) and re-extracted into hexane. UQs and MKs
167 contained in the crude extract were separated and purified using Sep-Pak® Plus Silica
168 (Waters). The molecular species and concentrations of quinones were determined using a high
169 performance liquid chromatography (HPLC) system equipped with an ODS column (pore size,

3.5 μm; Eclipse Plus C18, 3.0 × 150 mm; Agilent) and a photodiode array detector (SPD-M20A; Shimadzu). Details on the analytical conditions have been described by Takasu *et al*. (2013). The type of quinones was identified according to the UV spectrum of each peak observed in the photodiode array detector. The quinone species were identified by the linear relationship between the logarithm of the retention times of quinones and the number of their isoprene units, using the identification-supporting sheet (made by T. Kunihiro) based on the equivalent number of isoprene units (ENIU) of quinone components as described by Tamaoka *et al*. (1983).

In the present study, we express each RQ type as follows: ubiquinone, UQ-*n*; menaquinone, MK-*n*. The number (*n*) indicates the number of isoprene units in the side chain of the quinone. For example, UQ-10 represents a ubiquinone with 10 isoprenoid units, and MK-9(H_8) represents a menaquinone with 9 isoprenoid units where one of the nine units is hydrogenated with eight hydrogen atoms.

Calculations

The synthesis and destruction rates of each RQ are proxies of the growth and mortality of bacterial groups with different types of RQ. Thus, the apparent growth rates (μ_{app}, d^{-1}) of bacterial subgroups with different types of RQ were calculated from the concentrations of each RQ at the beginning and end of the incubation experiment, with the assumption that bacterial growth would follow an exponential model (Landry and Hassett 1982)

$$\mu_{app} = (1/t) \ln (N_t/N_0)$$

where t is the duration of the incubation (days), and N_0 and N_t are RQ concentrations (pmol L^{-1}) at the beginning and end of the incubation, respectively. Two dilution series were prepared: a 30 kDa dilution series to estimate the combined effects of protistan grazing and viral lysis rate (M_{g+v}, d^{-1}) and a 0.2 μm dilution series to determine the protistan grazing rate (M_g, d^{-1}) on bacteria. The slope of the regression lines from the 0.2 μm dilution series represents the grazing rate. The difference between the slopes of the regression lines

196 represents the bacterial mortality rate due to viral lysis (M_v, d^{-1}), and this difference was tested
197 using analysis of covariance (ANCOVA). The intercept of the 30 kDa dilution series gives the
198 instantaneous growth rate (μ, d^{-1}) of bacteria when neither grazing nor viral lysis occurs
199 (Evans et al. 2003).

200 Carbon fluxes through bacterial subgroups with different RQ types were determined by
201 combining the carbon conversion factor from RQ (Takasu et al. 2013) and data from the
202 dilution experiments. For each specific bacterial subgroup, the carbon production (*CP*, μg C
203 L^{-1} d^{-1}), losses to grazing (*GL*, μg C L^{-1} d^{-1}), and losses to viruses (*VL*, μg C L^{-1} d^{-1}) were
204 calculated using the formulas of Baudoux et al. (2008):

205 $$CP = \mu \times P_m$$

206 $$GL = M_g \times P_m$$

207 $$VL = M_v \times P_m$$

208 $$P_m = P_0 \times [e^{(\mu - M_{g+v})t} - 1]/(\mu - M_{g+v})t$$

209 where μ (d^{-1}) is the dilution-based specific growth (y-intercept of the 30 kDa regression), M_g
210 and M_v are the dilution-based grazing and viral lysis rates (in d^{-1}), respectively, P_0 (in μg C
211 L^{-1}) is the initial carbon biomass of bacteria, P_m (in μg C L^{-1}) is the geometric mean carbon
212 biomass of bacteria during the incubation, and *t* (in d) is the time of incubation.

213 *Statistical analysis*

214 Analyses with Student's t-test and ANCOVA were conducted using the free statistical
215 environment R (R Development Core Team 2011).

216

217 **Results**

218 *Physicochemical and biological conditions of the sampling site*

219 Water samples used for the modified dilution technique, covered wide ranges of
220 physicochemical properties: water temperatures ranged from 16.0 to 27.1°C; DOC
221 concentrations from 1.21 to 1.56 mg C L^{-1}; and chl. *a* concentrations from 3.09 to 31.4 μg L^{-1}

(Table 1). The HNF number (0.5×10^6 to 2.7×10^6 cells L^{-1}) was in most cases higher than the PNF number (0.3×10^6 to 0.6×10^6 cells L^{-1}). The bacteria:Total nanoflagellates (TNF) abundance ratios in Lake Biwa ranged between 725 and 2,417 (Table 1). The VLP number varied from 2.4×10^{10} to 4.1×10^{10} VLP L^{-1} (Table 1). The range of viruses to bacteria abundance ratios (range: 12.3 to 36.2) falls within the average reported for freshwater systems (Maranger and Bird 1995).

Estimation of growth and mortality rates

The RQ concentrations at a dilution level of 1.0 varied in the range of 32.4 to 90.9 pmol L^{-1} at the beginning of the dilution experiments (Fig. 1A). A total of 12 types of RQ were detected, and UQ-8, UQ-10, MK-8, MK-9, and MK-9(H_8) were detected as major RQs (Fig. 1B). In general, negative relationships between the dilution factor and apparent growth rate (RQ synthesis rate) were found in the <0.2 μm diluent (Table 2, Fig. S1). We regarded relationships with r^2>0.8 and significance levels of p<0.1 as statistically significant. We discussed growth and mortality rates using only the statistically significant data based on the criteria, though previous studies have used not only significant but insignificant growth and mortality values (e.g. Tijdens et al. 2008). Out of the 39 cases, 23 and 14 met these criteria for significance using the <0.2 μm diluents and the <30 kDa diluents, respectively (Table 2). In 5 out of the 39 experiments, both the <0.2 μm and <30 kDa dilution series were statistically significant, and the differences in the slopes of the two dilution series were also statistically significant (Table 2). Estimates of the growth and mortality due to grazing and lysis of UQ-8-containing bacteria in June 2011; total bacterial community (expressed as RQ in Table 2), UQ-, and UQ-8-containing bacteria in October 2011; and UQ-containing bacteria in May 2012 were statistically significant. However, most of the linear relationships between growth and the dilution factor were statistically insignificant (Table 2, Fig. S1). Rates could not be determined for UQ-10- and MK-containing bacteria in some experiments using the <30 kDa diluents because of the positive relationship (against theory) between the dilution factor and

apparent growth rate (Table 2, Fig. S1).

The growth rate of the total bacterial community (expressed as RQ in Table 3) varied from 1.05 to 2.42 d^{-1} (Table 3), and the grazing and lysis rates of total bacteria varied from 0.46 to 0.74 d^{-1} and 0.69 d^{-1}, respectively (Table 3). Growth and grazing rates varied among individual bacterial groups, and those of UQ-containing bacteria tended to be higher than those of MK-containing bacteria (Table 3). The sums of the grazing and lysis rates of individual groups had values close to their growth rates.

The changing patterns in the mortality due to grazing and lysis (M_{g+v}) of total bacteria and UQ-8- and UQ-10-containing bacteria were similar to the growth rates of those bacteria, even though grazing rates (M_g) on those bacteria were constant (Fig. 2).

Bacterial carbon production and losses

Daily carbon production (*CP*) in the present study ranged between 16.3 and 52.5 μg C L^{-1} d^{-1} (average: 37.4 μg C L^{-1} d^{-1}) (Table 4), close to the estimates from previous studies in Lake Biwa using tritiated thymidine uptake (5 to 59 μg C L^{-1} d^{-1}) (Nagata 1987) and the frequency of dividing cells (4.1 to 33 μg C L^{-1} d^{-1}) (Nagata 1987). Grazing loss (*GL*) was two times higher (average: 18.5 μg C L^{-1} d^{-1}) than lysis loss (*VL*) (average: 9.2 μg C L^{-1} d^{-1}) (Table 4). *GL* showed a pattern of change similar to that of initial carbon biomass (Fig. 3). Contributions of grazing loss and lysis loss to bacterial production in Lake Biwa fell into the ranges of previous estimates in other lakes (Table 5). Total losses (*TL*) of bacteria were almost the same (average: 36.2 μg C L^{-1} d^{-1}) as *CP*, and this tendency was found in individual bacterial groups (Table 4). The net carbon production of bacteria (CP_{net}: $CP-TL$) was calculated to estimate the remaining bacterial carbon biomass (Table 4). Estimates of total bacterial CP_{net} were positive on average (average: 1.2 ± 4.4 μg C L^{-1} d^{-1}), varying from negative (−3.1 μg C L^{-1} d^{-1}) to positive (5.8 μg C L^{-1} d^{-1}) values. Positive estimates of CP_{net} were mostly found in UQ-8-containing bacteria (average: 0.8 μg C L^{-1} d^{-1}, range: 0.21 to 1.78 μg C L^{-1} d^{-1}) (Table 4). Overall, the CP_{net} values of total bacteria and individual bacterial groups were less than

10% of each *CP* value.

Discussion

Grazing and lysis mortality of bacteria

The dilution technique is based on a critical assumption, which assumes that the plankton mortality rates resulting from predation are proportional to the dilution effect on predator (grazer and/or virus) densities (Landry & Hassett 1982). This assumption may not always meet because non-linear relationships between the dilution factor and apparent growth rate have been frequently reported in previous studies (e.g. Tijdens et al. 2008, Personnic et al. 2009). In the present study, the positive relationship between the dilution factor and apparent growth rate was found from some experiments using the <30 kDa diluent (Table 2, Fig. S1). It is known that viral lysis of bacterial cells leads to the recycling of carbon and nutrients, both of which are derived from lysed bacterial biomass and are re-supplied to bacteria (Bratbak et al. 1990; Gobler et al. 1997; Proctor and Fuhrman 1991). Thus, the possible interpretation could be that growth of bacteria was stimulated by the lysed bacterial cells in some of the <30 kDa diluent series.

In limnetic and oceanic systems, concentrations of organic substrates and inorganic nutrients are generally low, causing death of planktonic bacteria to starvation (Amy and Morita 1983). So, planktonic bacteria are required to have some physiological adaptation for efficient uptake and utilization of substrates as well as for long-term survival under carbon and energy limitation (del Giorgio and Gasol 2008). In Lake Biwa, however, the sum of total bacterial mortalities due to protistan grazing and viral lysis accounted for 96.6% (range; 89.0 - 107.2%) of daily total bacterial production in the present study. Thus, almost all bacterial production is efficiently consumed by protists and viruses before bacterial death due to other environmental factors in Lake Biwa within a day. In other words, fate of bacterial production in the epilimnion of Lake Biwa may be mainly determined through microbial interactions.

This is the first study which demonstrates a balanced relationship between bacterial production and losses in a freshwater lake using the direct estimation method. The modified dilution method was rarely applied to the estimation of protistan grazing and virus lysis of freshwater bacteria, and only two previous studies are so far available in freshwater systems (Personnic et al. 2009, Tijdens et al. 2008) (Table 5). In those studies, however, estimates of grazing losses frequently exceeded the sum of grazing plus lysis loss estimates. Personnic et al. (2009) suggested that some complex interactions among microbes such as synergistic and antagonistic effects of viral lysis and protistan grazing on bacterial production occurred in their incubation experiments. By contrast, the sum of the grazing and lysis losses of bacteria (*TL*) was almost equal to *CP* in the present study (Table 4). Thus, complex interactions among microbes might be negligible in our experiments, and almost all bacterial production is efficiently consumed by protists and viruses in Lake Biwa.

Bacterial abundances in the epilimnion of Lake Biwa were relatively constant and changed within the order of 10^9 cells L^{-1} (Nagata 1987; Nakano 1992; Nishimura et al. 2005; Takasu et al. 2013), whereas other microbes such as picophytoplankton, nanoflagellates, and viruses respectively showed large variations from 10^5 to 10^8 cells L^{-1} (Nagata 1988), from 10^2 to 10^5 cells L^{-1} (Nagata 1988), and from 10^9 to 10^{11} VLP L^{-1} (Nishimura and Nagata 2007; Pradeep Ram et al. 2010). In addition, bacterial abundance is generally less variable than bacterial production (Nagata 1987; Nakano 1992). In the present study, the percentages of remaining bacterial carbon production ($\%CP_{net} = (CP_{net} / CP) \times 100$) ranged from −7.2 to 11.0% (average ± SD, 3.4 ± 9.4). These estimates suggest that the less than 11% of daily bacterial production remains or excessively loses, and those small increase or decrease in net bacterial production does not change the whole bacterial abundance so much. This may be the major reason why the bacterial abundance in the epilimnion of Lake Biwa is relatively constant.

Owing to food limitation, nanoflagellates in lakes show clearance rates independent of

bacterial density (Bird and Kalff 1993). The bacteria:TNF ratios in Lake Biwa were low (average: 1346, Table 1) and similar to those of oligotrophic lakes and oceans (Sanders et al. 1992). Thus, nanoflagellates in Lake Biwa are also under food limitation. When we calculated the specific grazing loss (SGL = *GL* / initial TNF density), SGL estimates were relatively constant ($1.24 \pm 0.29 \times 10^{-5}$ μg C TNF $cell^{-1}$ d^{-1}) despite variations in the bacteria:TNF ratio (Table 1). The previous study suggested that nanoflagellates are clearing as much water as is physically possible independent of bacterial density when they are under food limitation (Bird and Kalff 1993). The results of the present study also indicate that individual TNF in Lake Biwa ingest as much bacteria as possible due to the chronically food limitation. The elimination mechanisms of bacteria should be different between protistan grazing and viral lysis, though bacterial production and the sum of grazing and lysis losses were almost equal in Lake Biwa (Table 4). In the present study, most of the viral lysis rates (M_v) were not statistically significant (Table 2), similar to those in previous studies (e.g. Tijdens et al. 2008; Personic et al. 2009). The sum of grazing and lysis mortality rates (M_{g+v}) seasonally changed, though grazing rates (M_g) were relatively constant during the study period (Fig. 2). Thus, the changing pattern of M_{g+v} is due to that of M_v. So, the changing pattern of M_{g+v} was similar to that of growth rate, suggesting that viral mortality is coupled with bacterial growth rate (Fig. 2). Similar results have been reported in previous studies (e.g., Weinbauer 2003). In Lake Biwa, the loss processes of bacterial production can be explained by a combination of protistan grazing pressure, which is independent of bacterial abundance, and viral lysis, which is dependent on bacterial growth (Fig. 2, RQ).

Carbon fluxes through major bacterial groups and differentiating the ecological traits of bacterial groups

The present study elucidated that planktonic bacterial assemblages in Lake Biwa consisted of various RQs subgroups with different growth and mortality rates (Table 3). The growth rates of UQ-containing bacteria were higher than those of MK-containing bacteria in Lake Biwa

(Table 3). Because of the large midpoint potential of UQs, UQs are thermodynamically favorable compounds to use oxygen as an electron acceptor compared to MKs, and this makes energy gains by UQs higher than those of MKs (Søballe and Pool 1999). The coupling between bacterial production and oxygen consumption (respiration) has been reported in aquatic systems (reviewed by del Girogio and Cole 1998; Robinson 2008). Thus, UQ-containing bacteria may have an advantage over other bacteria in the epilimnion of Lake Biwa because of the lake's oxygenated condition. UQ-containing bacteria showed higher GL than MK-containing bacteria (Table 3), probably due to higher abundance which would have higher encounter with nanoflagellates. In addition, there may be another reason why UQ-containing bacteria had higher GL. In freshwater planktonic bacterial community, UQ-8- and UQ-10-containing bacteria are generally gram-negative Proteobacteria, whereas MK-8, MK-9, and MK-9(H_8)-containing bacteria are gram-positive Actinobacteria (e.g. Hiraishi and Kato 1999). The consumption of gram-positive freshwater planktonic bacteria (mainly Actinobacteria) is selectively avoided by nanoflagellates due to various protection mechanisms of gram-positive bacteria such as cell surface charge and cell size reduction (reviewed by Pernthaler 2005). Thus, it is possible that UQ-containing bacteria are preferentially grazed by nanoflagellates.

UQ-8- and UQ-10-containing bacteria were the most dominant and fastest-growing bacterial groups during the study period (Fig. 1, Table 3). The average *CPs* of UQ-8- and UQ-10-containing bacteria were respectively estimated at 12.2 and 13.8 μg C L^{-1} d^{-1}, accounting for 31.7% (range: 28.4 to 35.2%) and 28.5% (range: 25.0 to 32.1%) of total bacterial *CP* (Table 4). The sum of the average *TL* of UQ-8- and UQ-10-containing bacteria (26.0 μg C L^{-1} d^{-1}) also accounted for 60% of total bacterial *TL* (Table 4). Hence, the fate of those two bacterial groups explained a large portion of the carbon fluxes within the microbial loop of Lake Biwa. Our estimation thus implies that the magnitude of carbon fluxes within the microbial loop can be regulated by the production of major bacterial groups.

R-BT065 cluster bacteria (a subcluster of Beta-proteobacteria) have been reported to be the most abundant (up to 50% of total bacteria) (Zwart et al. 2002; 2003) and have UQ-8 as their major RQ (e.g., Hahn et al. 2010a; 2010b; Kasalický et al. 2010). R-BT065 cluster bacteria have been reported to be the fastest-growing segment of bacterial communities in European freshwater lakes (Šimek et al. 2006; Salcher et al. 2008). These bacteria preferentially inhabit the oxygenated layer of oligo-mesotrophic lakes (Piburger See, Austria; Lake Zurich, Switzerland) (Salcher et al. 2008; 2011). The environmental conditions of the epilimnetic water in the north basin of Lake Biwa may be similar to those of the aforementioned European lakes (Nishimura et al. 2005; Kim et al. 2006; Takasu et al. 2013). UQ-10-containing bacteria have been classified as Alpha-proteobacteria (Hiraishi 1999). In the freshwater Alpha-proteobacteria, members of the LD12 clade are one of the most abundant ubiquitous lineages (Salcher et al. 2011). Previous study suggested that LD12 bacteria generally prefer the upper epilimnetic water layers during nutrient limited summer season (Salcher et al. 2011). This feature is well consistent with UQ-10-containing bacteria in Lake Biwa (Takasu et al. 2013).

The CP_{net} of UQ-8-containing bacteria showed positive estimates, whereas the CP_{net} of UQ-10 containing bacteria showed negative estimates in the present study (Table 4). In our previous study, UQ-8-containing bacteria dominated in Lake Biwa throughout a year, whereas biomass of UQ-10-containing bacteria was relatively low (Takasu et al. 2013). In addition, biomass of those bacterial groups showed different seasonal changing patterns (Takasu et al. 2013). Thus, it is likely that relatively low biomass of UQ-10-containing bacteria was due to higher loss of the bacteria by protistan grazing and/or viral lysis than that of UQ-8-containing bacteria in Lake Biwa.

Conclusion

Our study is the first to demonstrate a balanced relationship between bacterial production and losses in a freshwater lake using the modified dilution method. UQ-8- and UQ-10-containing

bacteria were the two dominant groups, and the sum of their production and losses explained 60% of the carbon fluxes within the microbial loop. Thus, a large portion of carbon fluxes through the bacterial community may be explained by the carbon fluxes of dominant bacterial groups.

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615

Table 1. Physico-chemical and biological variabes

Date	WT ($^{\circ}$C)	DOC (mg C L^{-1})	Chl. *a* (µg L^{-1})	Bacteria (10^9 cells L^{-1})	TNF (10^6 cells L^{-1})	HNF (10^6 cells L^{-1})	PNF (10^6 cells L^{-1})	VLP (10^{10} VLP L^{-1})	Bacteria:TNF	VBR
22 June 2011	22.0	ND	9.07	2.0	3.0	2.7	0.3	2.4	725	12.3
19 October 2011	20.0	ND	3.09*	1.2	1.1	0.5	0.6	3.1	2417	25.3
16 May 2012	16.0	ND	31.1*	0.9	1.4	1.1	0.3	3.1	759	36.2
25 June 2012	21.5	1.21	31.4	2.5	2.4	1.8	0.6	3.4	1389	13.3
24 July 2012	27.1	1.56	6.16	2.6	2.3	1.8	0.5	4.1	1441	15.7

WT, Water Temperature

ND, Not Determined

VBR, Viruses to bacterium ratio

*Water samples were filtered using different type of filter (GF/F glass fiber filter, Whatman) (I. Mukherjee and S.D. Thottathil, personal communication).

Table 2. Summary of growth (μ), grazing mortality (M_g), lysis mortality (M_v), and total mortality (M_{g+v}) from results of the dilution experiments.

Date	Bacterial RQ type	Diluent	Dilution level	Linear fit		Regression slopes	μ	M_g	M_V	M_{g+V}
				r^2	p limit	p limit				
22 June 2011	RQ	0.2 μm	4	0.993	<0.01	NS	1.128	**0.697**	0.123	0.820
		30 kDa	4	0.659	NS					
	UQ	0.2 μm	4	0.996	<0.01	NS	1.206	**0.777**	0.133	0.910
		30 kDa	4	0.727	NS					
	MK	0.2 μm	4	0.688	NS	NS	0.714	0.42	-0.082	0.338
		30 kDa	4	0.175	NS					
	UQ-8	0.2 μm	4	0.957	<0.05	<0.05	**1.943**	**0.578**	**1.095**	**1.673**
		30 kDa	4	0.965	<0.05					
	UQ-10	0.2 μm	4	0.989	<0.01	NS	0	**0.812**		
		30 kDa	4	0.004	NS					
	MK-8	0.2 μm	4	0.127	NS	NS	0.360	0.070	0.178	0.248
		30 kDa	4	0.304	NS					
	MK-9(H_8)	0.2 μm	3*	0.865	<0.1	NS	0	**-0.248**		
		30 kDa	4	0.464	NS					
19 October 2011	RQ	0.2 μm	4	0.980	<0.01	<0.01	**1.225**	**0.459**	**0.689**	**1.148**
		30 kDa	4	0.996	<0.01					
	UQ	0.2 μm	4	0.944	<0.05	<0.01	**1.767**	**0.268**	**1.471**	**1.733**
		30 kDa	4	0.978	<0.05					
	MK	0.2 μm	4	0.968	<0.05	<0.05	0	**0.602**		
		30 kDa	4	0.436	NS					
	UQ-8	0.2 μm	4	0.926	<0.05	<0.01	**2.188**	**0.543**	**1.554**	**2.097**
		30 kDa	4	0.998	<0.01					
	UQ-10	0.2 μm	4	0.440	NS	NS	0.192			0.161
		30 kDa	4	0.063	NS					
	MK-7	0.2 μm	4	0.986	<0.01	<0.1	0	**0.968**		
		30 kDa	4	0.069	NS					
	MK-8	0.2 μm	4	0.819	<0.1	<0.05	0	**0.533**		
		30 kDa	4	0.768	NS					
	MK-9	0.2 μm	4	0.873	<0.1	<0.1	0	**0.589**		
		30 kDa	3*	0.651	NS					
	MK-9(H_8)	0.2 μm	4	0.882	<0.1	<0.05	0	**0.351**		
		30 kDa	4	0.601	NS					
16 May 2012	RQ	0.2 μm	4	0.612	NS	<0.05	**2.424**	0.646	1.511	**2.157**
		30 kDa	4	0.948	<0.05					
	UQ	0.2 μm	4	0.829	<0.1	<0.05	**1.382**	**2.066**	**-0.936**	**1.13**
		30 kDa	4	0.999	<0.001					
	MK	0.2 μm	4	0.591	NS	NS	0.995	1.136	-0.859	0.277
		30 kDa	4	0.427	NS					
	UQ-8	0.2 μm	4	0.648	NS	<0.05	**2.962**	0.721	2.052	**2.773**
		30 kDa	4	0.952	<0.05					
	UQ-10	0.2 μm	4	0.710	NS	<0.05	**2.524**	0.552	2.012	**2.564**
		30 kDa	4	0.944	<0.05					
	MK-8	0.2 μm	4	0.559	NS	NS	0.481	1.027	-0.718	0.309
		30 kDa	4	0.198	NS					
	MK-9(H_8)	0.2 μm	4	0.098	NS	NS	0.253	0.290	-0.188	0.102
		30 kDa	3*	0.101	NS					

Table 2. Continued

Date	Bacterial RQ type	Diluent	Dilution level	Linear fit r^2	Linear fit p limit	Regression slopes p limit	μ	M_g	M_V	M_{g+V}
25 June 2012	RQ	0.2 μm	4	0.968	<0.05	<0.1	1.448	**0.644**	0.720	1.364
		30 kDa	3**	0.958	NS					
	UQ	0.2 μm	4	0.976	<0.05	NS	1.567	**1.368**	0.080	1.448
		30 kDa	3**	0.954	NS					
	MK	0.2 μm	4	0.831	<0.1	<0.05	1.349	**0.113**	1.183	1.296
		30 kDa	3**	0.963	NS					
	UQ-8	0.2 μm	4	0.985	<0.01	NS	1.245	**1.589**	-0.381	1.208
		30 kDa	4	0.961	NS					
	UQ-10	0.2 μm	4	0.958	<0.05	NS	1.816	**1.077**	0.522	1.599
		30 kDa	3**	0.937	NS					
	MK-8	0.2 μm	4	0.019	NS	NS	0	0.088		
		30 kDa	3**	0.911	NS					
	MK-9	0.2 μm	4	0.595	NS	NS	0.220	0.388	-0.126	0.262
		30 kDa	3**	0.259	NS					
	MK-9(H_8)	0.2 μm	4	0.003	NS	NS	**0.242**	0.046	0.277	**0.323**
		30 kDa	3**	0.983	<0.1					
24 July 2012	RQ	0.2 μm	4	0.862	<0.1	NS	**1.048**	**0.744**	0.379	**1.123**
		30 kDa	4	0.870	<0.1					
	UQ	0.2 μm	4	0.92	<0.05	NS	**1.446**	**1.007**	0.492	**1.499**
		30 kDa	4	0.897	<0.1					
	MK	0.2 μm	4	0.76	NS	NS	0.416	0.644	-0.24	0.404
		30 kDa	4	0.655	NS					
	UQ-8	0.2 μm	4	0.930	<0.05	NS	**1.202**	**0.926**	0.256	**1.182**
		30 kDa	4	0.891	<0.1					
	UQ-10	0.2 μm	4	0.911	<0.05	NS	**1.636**	**1.120**	0.647	**1.767**
		30 kDa	4	0.913	<0.05					
	MK-8	0.2 μm	4	0.393	NS	NS	0.026	0.392	-0.269	0.123
		30 kDa	4	0.222	NS					
	MK-9	0.2 μm	4	0.535	NS	NS	**1.398**	0.409	0.893	**1.302**
		30 kDa	4	0.841	<0.1					
	MK-9(H_8)	0.2 μm	4	0.923	<0.05	<0.1	0.056	**0.933**	-0.684	0.249
		30 kDa	4	0.455	NS					

Statistically meaningful values are shown in bold.

NS, Not significant

*20% Initial sample was under the detection limit.

**40% Initial sample could not be measured due to laboratory accident.

Table 3. Ranges and averages of daily growth (μ), grazing mortality (M_g), lysis mortality (M_v), and total mortality (M_{g+v})

Bacterial RQ type	μ (d^{-1})		M_g (d^{-1})		M_v (d^{-1})		M_{g+v} (d^{-1})	
	Range (Min - Max)	Average ± SD	Range (Min - Max)	Average ± SD	Range (Min - Max)	Average ± SD	Range (Min - Max)	Average ± SD
RQ	1.05 – 2.42	1.57 ± 0.75	0.46 – 0.74	0.64 ± 0.13	–	0.69	1.12 – 2.16	1.48 ± 0.59
UQ	1.38 – 1.77	1.53 ± 0.21	0.27 – 2.07	1.10 ± 0.67	-0.94 – 1.47	0.27 ± 1.70	1.13 – 1.73	1.45 ± 0.30
MK	–	–	0.11 – 0.60	0.36 ± 0.36	–	–	–	–
UQ-8	1.20 – 2.96	2.07 ± 0.73	0.54 – 1.59	0.91 ± 0.49	1.10 – 1.55	1.33 ± 0.33	1.18 – 2.77	1.93 ± 0.67
UQ-10	1.64 – 2.52	2.08 ± 0.63	0.81 – 1.12	1.00 ± 0.17	–	–	1.77 – 2.56	2.17 ± 0.56
MK-8	–	–	–	0.53	–	–	–	–
MK-9	–	1.40	–	0.59	–	–	–	1.30
MK-9(H_8)	–	0.24	-0.25 – 0.93	0.35 ± 0.59	–	–	–	0.32

SD, Standard Deviation

Table 4. Ranges and averages of daily carbon production (*CP*), grazing loss (*GL*), lysis loss (*LL*), total losses (*TL*) and net carbon production (CP_{net})

Bacterial RQ type	*CP* (µg C L^{-1} d^{-1})		*GL* (µg C L^{-1} d^{-1})		*LL* (µg C L^{-1} d^{-1})		*TL* (µg C L^{-1} d^{-1})		CP_{net} (µg C L^{-1} d^{-1})	
	Range (Min - Max)	Average ± SD	Range (Min - Max)	Average ± SD	Range (Min - Max)	Average ± SD	Range (Min - Max)	Average ± SD	Range (Min - Max)	Average ± SD
RQ	16.3 – 52.5	37.4 ± 18.8	6.1 – 30.9	18.5 ± 17.5	–	9.2	15.3 – 46.7	36.2 ± 18.1	-3.1 – 5.8	1.2 ± 4.4
UQ	8.0 – 27.0	19.7 ± 18.8	1.3 – 40.3	19.4 ± 19.7	-18.3 – 6.9	-5.7 ± 17.8	8.1 – 24.7	18.3 ± 8.9	-0.9 – 4.9	1.4 ± 3.1
MK	–	–	–	–	–	–	–	–	–	–
UQ-8	5.1 – 18.5	12.2 ± 5.5	1.3 – 9.5	4.9 ± 4.2	3.6 – 7.2	5.4 ± 2.5	4.9 – 17.3	11.3 ± 5.1	0.2 – 1.8	0.8 ± 0.8
UQ-10	10.9 – 16.8	13.8 ± 4.2	–	7.4	–	–	11.7 – 17.1	14.4 ± 3.8	-0.3 – -0.9	-0.6 ± 0.4
MK-8	–	–	–	–	–	–	–	–	–	–
MK-9	–	5.8	–	–	–	–	–	5.4	–	0.4
MK-9(H_8)	–	0.6	–	–	–	–	–	0.8	–	-0.2

SD, Standard Deviation

Table 5. Studies of simultaneous determination of grazing and viral lysis rates in freshwater systems

Method	Grazing % potential production	Lysis % potential production	Location and water layer	Trophic status	Reference
TC & FVIC	81.8 - 108.0	7.7 - 27.8	Lake Plußsee, Epilimnion	Eutrophic	Weinbauer & Hofle 1998
	2.9 - 27.6	19.6 - 46.8	Lake Plußsee, Metalimnion	–	Weinbauer & Hofle 1998
	5.0 - 8.9	38.4 - 97.3	Lake Plußsee, Hypolimnion	–	Weinbauer & Hofle 1998
FLB & FVIC	50	25	Římov Reservoir, Surface water	Meso-Eutorphic	Šimek et al. 2006
TC & FVIC	10.3	6.4	Lake Pavin, Epilimnion	Oligomesotrophic	Bettarel et al. 2003
	8.4	15.6	Lake Pavin, Epilimnion	–	Bettarel et al. 2003
Tritiated thymidine labeled bacteria	58.2	26.3	Lake Tanganyika, Upper water (Wet season)	Oligotrophic	Pirlot et al. 2007
	88.5	39.6	Lake Tanganyika, Upper water (Dry season)	–	Pirlot et al. 2007
CD & FVIC	78*	7.7*	Lake Erie, Surface and Deep water	Eutrophic	Gobler et al. 2008
FLMB & viral dilution & FVIC	18 - 63*	35 - 60*	Lake Bourget, Surface layer	Mesotrophic	Jacquet et al. 2005
MD	45.9	100.5	Lake Loosdrecht, Surface layer	Eutrophic	Tijdens et al. 2008
MD	37.3-76.5	ND	Lake Geneva, Surface layer	Mesotrophic	Personnic et al. 2009
	18.2 - 56.8	ND	Lake Bourget, Surface layer	Mesotrochip	Personnic et al. 2009
	5.3	ND	Lake Annecy, Surface layer	Oligotrophic	Personnic et al. 2009
MD	37.5 – 71.0	56.2	Lake Biwa, Epilimnion	Mesotrophic	This study

TC, theoretical caluculation; FVIC, frequency of visible infected cells; FLB, fluorescence labeled bacteria; CD, conventional dilution method; FLMB, fluorescence labeled micro-beads; MD, modified dilution method

*Grazing or lysis % standing stock d^{-1}

ND, not determined

Figure legends

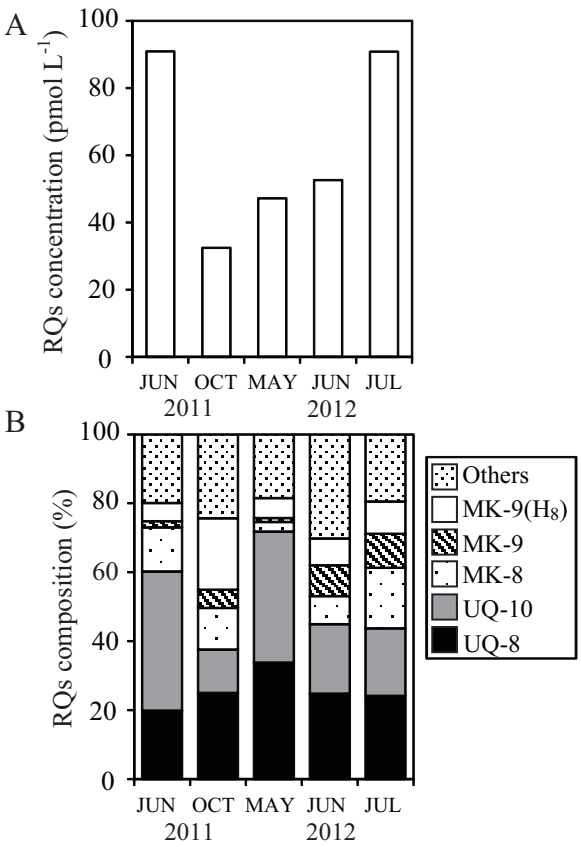
Fig. 1. RQs concentration (A) and composition (B) of 100% samples at the beginning experiments.

Fig. 2. Changes in growth (μ), grazing mortality (M_g), lysis mortality (M_v), and total mortality (M_{g+v}) rates of total bacteria (RQ) and major bacterial groups. An asterisk indicates that the rate is statistically meaningful (see Table 2).

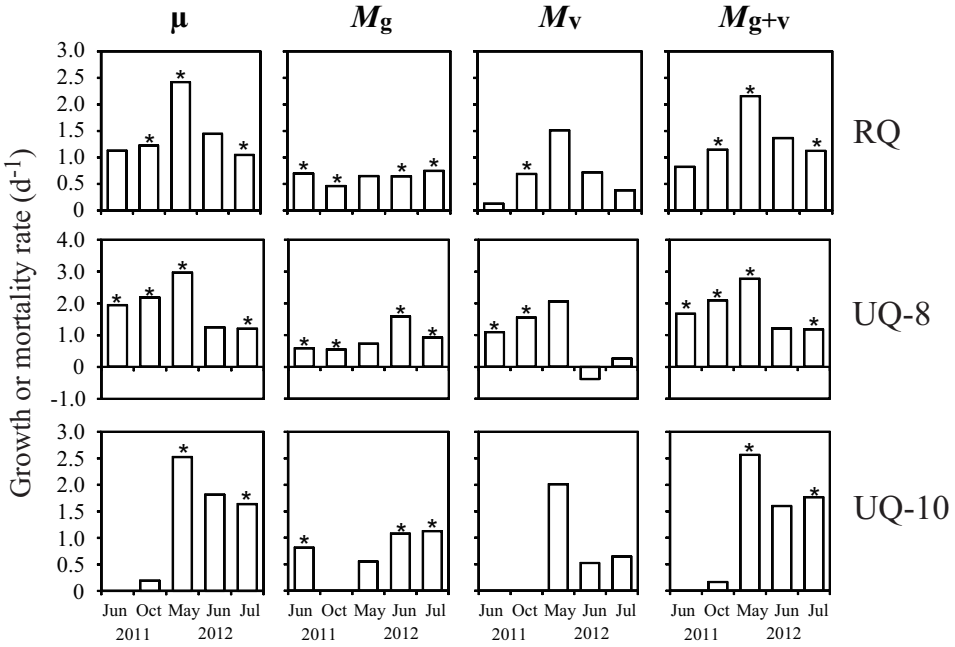
Fig. 3. Changes in carbon biomass (*CB*), production (*CP*), grazing loss (*GL*), lysis loss (*LL*) and total losses (*TL*) of total bacteria (RQ) and major bacterial groups. An asterisk indicates that the rate is statistically meaningful (see Table 2).

Appendix

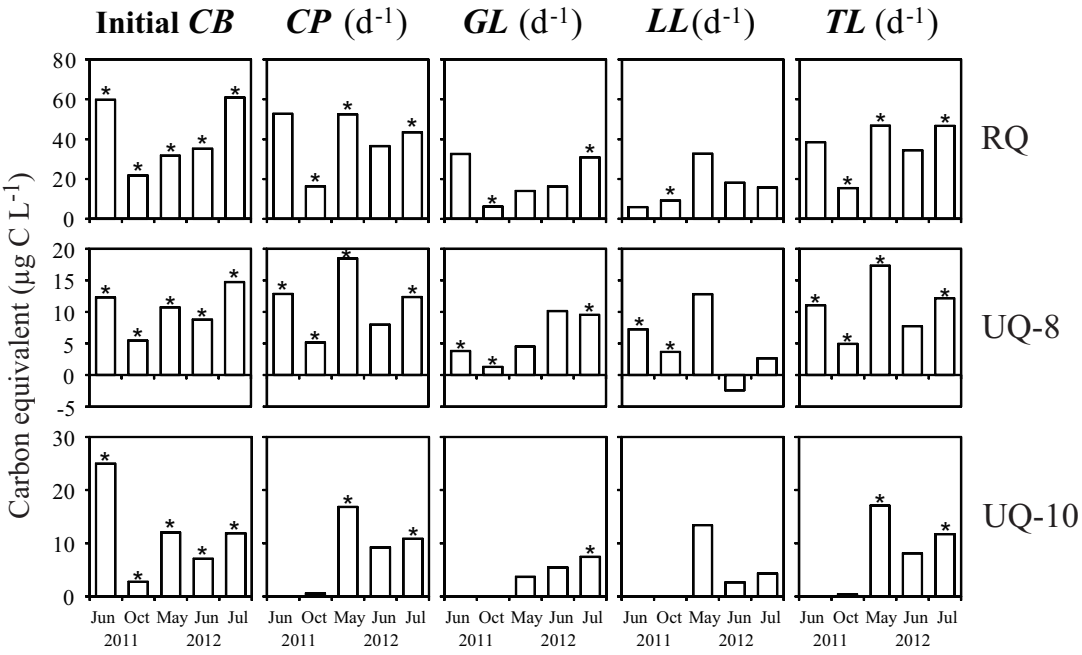
Fig. S1. Regression analyses of dilution experiments to estimate growth and mortality raty



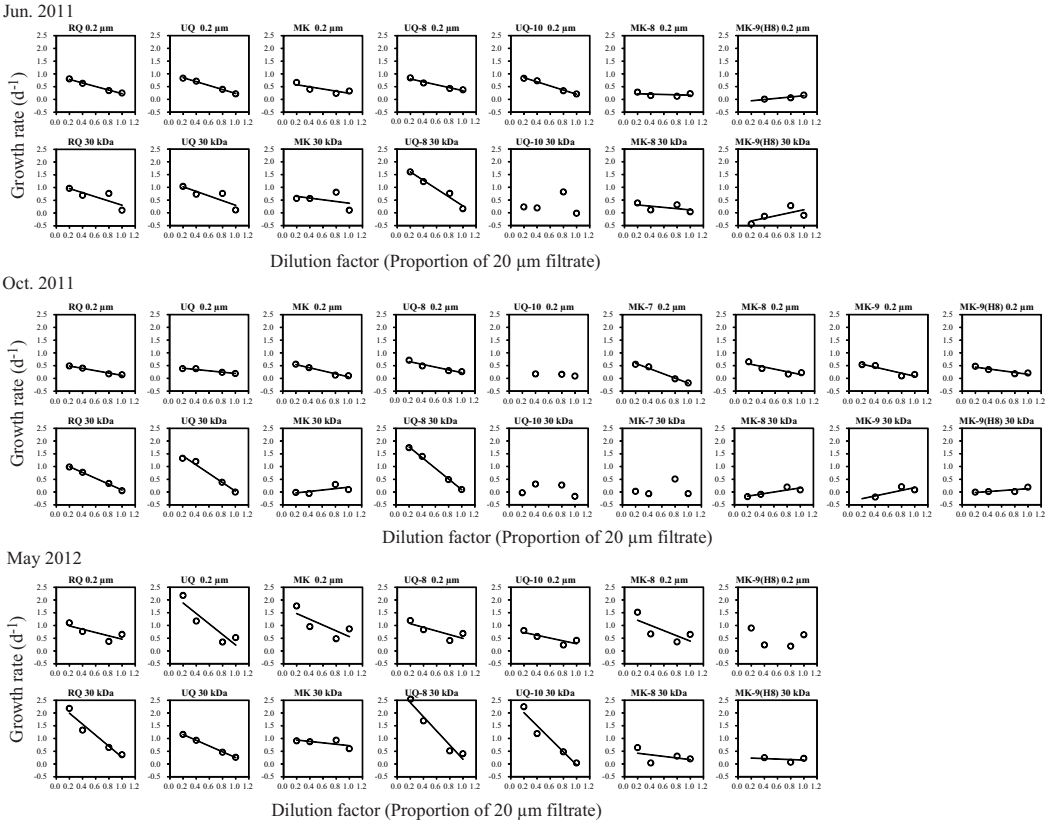
Takasu et al. Fig. 1



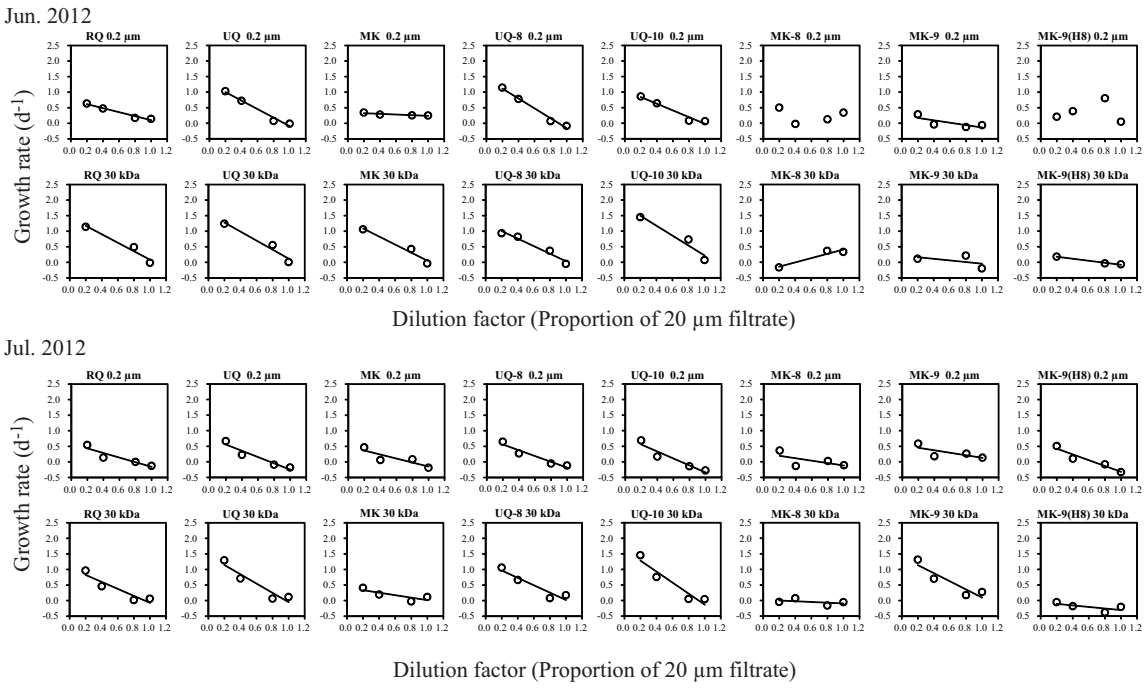
Takasu et al. Fig. 2



Takasu et al. Fig. 3



Takasu et al. Fig. S1



Takasu et al. Fig. S1 continued.