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Further evidence of elemental composition as an indicator of the bioavailability of humic substances to bacteria

Abstract—Batch culture experiments examined the ability of an isolated bacterial community to utilize four humic substances with similar molecular size but variable elemental composition. Univariate and multivariate linear regression analysis of the results provided evidence of a significant positive relationship between the N : C ratio and bacterial concentrations. In contrast, neither H : C nor O : C ratios were significant predictors of humic substances bioavailability, and their inclusion in the multivariate model provided no further explanatory power compared with the univariate model using N : C as the single independent variable. These findings suggest that N : C ratios provide the best indicator of bioavailability for complex, recalcitrant carbon moieties typical of many aquatic systems.

The most important source of organic matter used by bacteria is often considered to be algal in origin in the form of photosynthetic exudates and cell breakdown products. However, recent research has highlighted the importance of humic substances (HS) as an organic carbon source when algal productivity is low. Despite this possibility, it is still unclear what determines HS availability for bacterial utilization. Many studies have focused on molecular size as an important factor determining microbial utilization of HS (Amador et al. 1989; Tulonen et al. 1992; Amon and Benner 1996). The accepted model of bioavailability is that the degree of recalcitrance to bacterial breakdown is positively correlated with the size of the organic moiety (Saunders 1976). Although this conceptual model is still widely accepted, recent studies indicate that some high molecular weight compounds (>1 kDa) are rapidly utilized by bacteria (Amon and Benner 1994, 1996). This suggests that molecular size may not be the sole factor controlling bioavailability of dissolved organic matter (DOM). Sun et al. (1997) found evidence of an association between elemental ratios and bioavailability of DOM to bacteria. Interestingly, they emphasized that bioavailability was positively correlated with the percentage of aliphatic carbon in a sample (indicated by H : C ratio and carboxyl content). However, there are strong indications that N : C ratios may also play an important role in HS utilization by bacteria (Meyer et al. 1987; Kroer 1993). The present study used controlled laboratory systems to investigate further the role of elemental composition in predicting the bioavailability of humic substances to bacteria. Humic substances with similar molecular size distributions but very different elemental ratios were used. This presented an opportunity to assess the potential importance of elemental ratios in determining bioavailability while holding the effect of molecular weight constant.

Pretreatment of equipment—To minimize carbon and microbial contamination, all equipment was either precombusted in a muffle furnace at 450°C (12 h) or soaked in Decon 80 (48 h), rinsed in Milli-Q water (48 h), and autoclaved at 121°C (20 min).

Isolation of humic substances and preparation for use—Three sources of humic substances were used in the experiment. Aldrich humic acid (AHA) is extracted from peat and was supplied as granules. Aquatic DOM was extracted by lyophilization from 27 liters of GF/F filtered humic rich stream water collected in winter from Whitray Beck, North Yorkshire, U.K. (Lead et al. 1994). Frequently, aquatic HS is operationally defined as material that adheres to XAD-8 resin (Thurman 1985). In Whitray Beck, approximately 95% of the DOM adheres to XAD-8 resin during the winter (Lead unpubl. data). Thus, it is reasonable to assume that the majority of the extracted DOM comprised humic substances, and as such is termed aquatic HS (AqHS) for the purpose of the study. Finally, peat HS (PHS) was extracted under nitrogen from a sample of peat from the Duddon catchment, Cumbria, U.K., using 2 M NaOH.

Subsamples of the HS were suspended in Milli-Q water and dialyzed (12–14-Da molecular weight cutoff) against Milli-Q water for 78 h to remove loosely attached molecules and ions. Although the molecular weight cutoff of the dialysis membrane was large compared with the molecular weights subsequently determined for the HS samples, visually, there was minimal increase in the brown coloration in the dialysate compared with the color of the humic material retained by the dialysis tubing. Hence, it was concluded that relatively little humic material was lost during the process. This may be attributed to a kinetic control of the movement of the HS because of the relatively slow diffusion of HS across the membrane. The HS were lyophilized and stored in a desiccator in the dark until use. When needed, 1 g l^{-1} suspensions of AHA, AqHS, PHS, and nondialyzed AHA (whole AHA [wAHA]) were prepared in autoclaved pH 5.5 buffered Chalkley's medium (CM, 1.7×10^{-3} M NaCl, 5.0×10^{-5} M for both KCl and $CaCl_2$; Needham et al. 1937). Because of incomplete dissolution of the HS in CM, the suspensions were centrifuged at 25,894 × *g* for 10 min to sediment particles with a diameter of >0.047 μm (assuming particles have a spherical geometry and a density of 1.8 g cm^{-3}; Aiken et al. 1985). The supernatant was removed and stored in the dark at 4°C and was used within 2 h of preparation.

Characterization of the humic substances by size exclusion chromatography and elemental analysis—Prefiltered (0.2-μm filters, Whatman) samples were applied to a column (3.2 by 22.5 cm) of Sephadex G75 (Pharmacia Fine Chemicals) that has a fractionation range for globular proteins of 3,000 to 80,000 Da and for dextrans, 1,000 to 50,000 Da. It

was considered that this molecular size range encompassed the majority of molecular size distributions encountered in humic substances (Thurman et al. 1982; De Nobili et al. 1989; Lead et al. 1994). The eluant used for all experimental runs was degassed CM. The eluant was pumped around the system at 0.25 ml h^{-1} using a 2120 Varioperpex peristaltic pump (LKB Bromma). The total bed volume of the column (V_t) was 119.34 cm^3, while the void volume (V_0) of the gel bed was 47.5 cm^3. For all runs, the sample volume was less than 1% of the total bed volume. The eluate was measured for ultraviolet absorbance using a Pharmacia optical unit LKB UV1 with 280-nm filters. In order to relate size exclusion chromatography (SEC) output to actual molecular weight, the column was calibrated using HS standards of known molecular weight (Lead et al. 1994). The results of SEC analysis of the HS are presented as weight-averaged molecular weights (WAMw; Shaw unpubl. data).

A Carlo Erba EA1108 elemental analyzer was used to measure carbon, hydrogen, and nitrogen contents of the HS. Ash content of the HS was determined gravimetrically as the residue after combustion at 450°C. Oxygen content was calculated from the percentages of C, H, and N, after correction for ash content (Lead et al. 1994; Sun et al. 1997).

The bacterial inoculum—Stream water aliquots of 0.2 ml taken from a humic stream (Duddon Valley, Cumbria, UK) were applied to the surface of CM agar (1.5% Lab M agar No. 1) with 1 g L^{-1} glucose as the carbon source. Plates were then incubated at 25°C for 20 d to allow for the appearance of slow-growing bacteria. The entire spread plate community was scraped from the agar and suspended in 50 ml of CM supplemented with 1 g L^{-1} glucose as carbon source. The cultures were incubated until bacteria reached the late exponential phase of growth. The cells were then harvested and washed with CM three times to remove residual glucose.

Bacterial concentrations—Samples for measuring bacterial concentrations were fixed immediately using 0.2-μm filtered phosphate buffered glutaraldehyde (2% final concentration). Bacterial abundance was determined by epifluorescence microscopy using 4′,6-diamidino-2-phenylindole (DAPI) to stain cells (Porter and Feig 1980). Appropriate aliquots of sample were added to 5 ml of sterile Milli-Q water to give Whipple grid counts typically containing 20 to 80 bacteria on 0.2-μm black polycarbonate membrane filters (25 mm in diameter). DAPI was then added (0.1 μg ml^{-1} final concentration) and left to stain for 5 min. For each filter, more than 400 cells were counted (Jones 1979).

Experimental procedure—The bacterial inoculum was added to give an initial concentration of 8.0×10^5 cells ml^{-1} to triplicate flasks containing 50 ml of CM. These flasks were treated with either 50 mg L^{-1} wAHA, AHA, AqHS, or PHS, or no C source (control). The flasks were incubated at 20°C in the dark. Samples were taken at 14.5, 24, 38.5, 62.5, and 117 h (approximately 5 d) and fixed with glutaraldehyde before concentrations of bacteria were determined using epifluorescence microscopy.

Peak (approximately 3 d) bacterial concentrations in treatment flasks were calculated as the difference between concentrations of bacteria in treatment and control flasks.

Table 1. Elemental ratios and weight averaged molecular weights (WAMw) of the four HS used in the bacterial incubations ($n = 3$). For elemental ratios, the relative standard deviation between replicates was less than 5%.

Sample	N:C	H:C	O:C	WAMw (Da)
wAHA	0.015	0.137	0.538	3,625
AHA	0.016	0.116	0.309	4,200
AqHS	0.089	0.165	0.646	4,300
PHS	0.031	0.089	0.732	4,200

Statistical analyses—Data were tested for normality using the method of normal plotting (Croxton et al. 1988), while heteroscedasticity was assessed using the F_{max} test (Sokal and Rohlf 1995). Where necessary, log transformation of the data was undertaken prior to any statistical analysis. Analysis of variance (ANOVA) was used for statistical analysis of bacterial concentrations and weight-averaged molecular weight of humic substances. In addition, ordinary least-squares regression was used to examine the association between elemental ratios and HS bioavailability. The Vuong likelihood ratio test (Vuong 1989) was used to compare the explanatory power of alternative regression models.

Elemental ratios and weight-averaged molecular weights of the four HS—Results of elemental analysis and WAMw for the four HS are presented in Table 1. SEC results indicated no significant differences in the WAMw of the HS, with mean values of 3,625 Da for wAHA, 4,200 Da for PHS and AHA, and 4,300 Da for AqHS (ANOVA; $F = 1.16$, $P > 0.05$, $n = 3$). Although there is some debate on the applicability of SEC as a determinant of molecular weight (Swift and Posner 1971; Wershaw and Aiken 1985; Lehto et al. 1986), maintenance of correct and constant conditions under which the experiments were conducted ensured that the SEC results provided a comparative measure of molecular weight and size for the HS. As such, these findings provided an opportunity to examine the role of elemental composition when molecular size was held constant.

In contrast to the SEC results, elemental analysis indicated differences in the elemental composition of the HS (Table 1). N:C ratios increased in the order wAHA, AHA, PHS, and AqHS (0.015, 0.016, 0.031, and 0.09, respectively), while H:C increased in the order PHS, AHA, wAHA, and AqHS and O:C increased in the order AHA, wAHA, AqHS, and PHS. These results suggest intrinsic differences in the elemental composition of the three HS that may give relevant information concerning their diagenetic age and structure. For example, Sun et al. (1997) postulated that high N:C ratios may be indicative of less diagenetic alteration of DOC and hence enhanced bioavailability to bacteria. This is consistent with the conclusions of Amon and Benner (1996), who conjectured that HS with higher N:C ratios were utilized more efficiently by bacteria. Further, Sun et al. (1997) suggested that H:C ratios reveal information on the relative

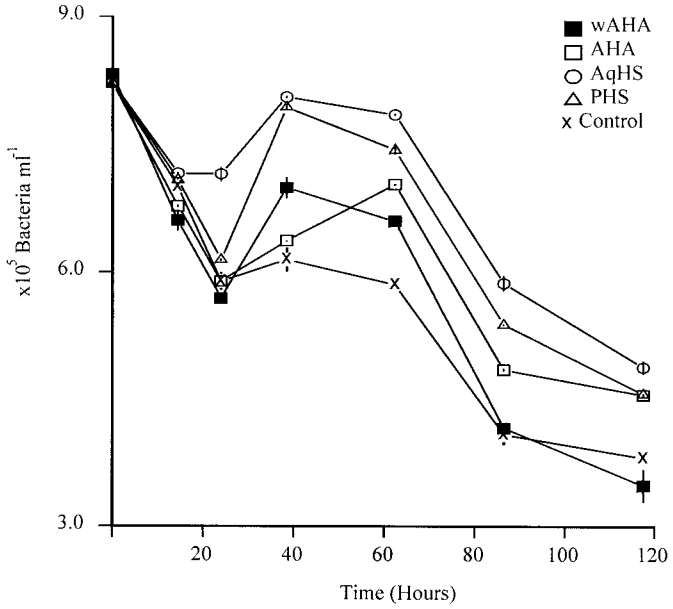

Fig. 1. Changes in the concentration of bacteria incubated with the four HS and the control. Vertical lines indicate the standard error of the mean of the data.

proportions of aliphatic and aromatic carbon moieties in a compound. A low H:C ratio is indicative of a greater proportion of aliphatic carbon moieties in a compound, leading to increased bioavailability (Moran and Hodson 1990). Finally, high O:C ratios may indicate a greater COOH content in organic carbon moieties and suggest a greater degree of diagenetic change than a low O:C ratio (Sun et al. 1997). Thus, we can hypothesize that an ideal bacterial substrate would have high N:C and low H:C and O:C ratios. None of the four substrates used in the present study match this definition of an ideal energy source, possibly suggesting that bioavailability of these HS may be more complex than first thought.

Changes in bacterial concentrations—During the first 24 h, bacterial concentrations in both the treatment and control flasks decreased because of the change from a labile substrate (glucose) to the relatively recalcitrant HS or no C source (Fig. 1). However, after 24 h, bacterial concentrations increased in all four treatments compared with the bacterial concentrations in the controls. Concentrations reached maxima for all four HS treatments between 38.5 and 62.5 h. However, there were significant differences in the maximum bacterial concentrations in the treatments with levels decreasing in the order AqHS, PHS, AHA, wAHA ($F = 8.71$, $p < 0.005$). Bacterial concentrations in all four treatments and the control then declined significantly after 62.5 h.

Model of bioavailability—Similar to Sun et al. (1997), regression methods were used to investigate the relationship between bacterial concentrations and the elemental ratios of the four HS (Table 2). After assessing the normality of the data and their residuals, ordinary least-squares regressions were estimated. The dependent variable in all four models was net maximum bacterial concentration (maximum treatment bacterial concentration minus control bacterial concentration), while the explanatory variables were N:C, H:C, and O:C. Both univariate and multivariate models were estimated to explain bacterial concentrations. The four models used were as follows:

$$\text{Model 1:}\quad \text{Max. concentration} = \alpha_0 + \beta_1 \text{N:C}$$

$$\text{Model 2:}\quad \text{Max. concentration} = \alpha_0 + \beta_1 \text{H:C}$$

$$\text{Model 3:}\quad \text{Max. concentration} = \alpha_0 + \beta_1 \text{O:C}$$

$$\text{Model 4:}\quad \text{Max. concentration} = \alpha_0 + \beta_1 \text{N:C} + \beta_2 \text{H:C} + \beta_3 \text{O:C}$$

The Vuong test (Vuong 1989) was used to compare the explanatory power of the alternative models (Table 2, panel C). The results of the univariate models indicated that model 1 had significantly greater explanatory power for the data

Table 2. Summary statistics for regression of maximum net bacterial concentrations on elemental ratios. Figures in parentheses denote significance levels. NS, not significant.

	Intercept	N:C	H:C	O:O	Adjusted R^2 value
Panel A. Univariate models					
Model 1	0.76×10^5 (0.0001)	16.6×10^5 (0.0001)			0.914 (0.0001)
Model 2	0.18×10^5 (NS)		9.4×10^5 (NS)		0.145 (NS)
Model 3	0.18×10^5 (NS)			2.0×10^5 (0.05)	0.192 (0.05)
Panel B. Multivariate model					
Model 4	0.52×10^5 (0.05)	15.3×10^5 (0.0001)	1.05×10^5 (NS)	0.28×10^4 (NS)	0.903 (0.0001)
Panel C. Relative explanatory power					
	Model 1 vs. model 2	Model 1 vs. model 3	Model 1 vs. model 4		
Vuong's Z statistic	−6.44 $P < 0.01$	−5.69 $P < 0.01$	0.93 NS		

than models 2 and 3 (Table 2, panel C; Vuong's test: $Z = -6.44$ and -5.69, $P < 0.01$ for N:C compared with H:C and O:C, respectively). The results indicate that there is no significant difference between the univariate model 1 and the multivariate model 4 (verified by the Vuong's test statistic, Table 2, panel C). However, while parameter estimates for N:C in model 4 are positive and significant at the $P < 0.0001$ level, the estimated coefficients on H:C and O:C are not significant at conventional levels. Therefore, the optimum regression model (model 1) omits these explanatory variables. However, both the HS and bacterial isolation methods may have altered the structure and composition of both these variables, and hence their representation of the natural environment should be used with some caution. Nevertheless, although the estimated regression parameters themselves are unique to this study and do not have any predictive connotation, the model does illustrate the significant control of N:C ratio on the bioavailability of HS to bacteria.

Within the present study, increased bacterial concentrations were positively correlated with the N:C ratio of the HS. This is consistent with the conclusions of both Amon and Benner (1996) and Kroer (1993), who reported highest growth efficiencies on DOC with high N:C ratios. Previous research suggests that the ratio between N:C for bacteria ($N:C_B$) and N:C for substrate ($N:C_S$) is important in the regulation of bacterial gross growth efficiency (Parnas 1975; Fenchel and Blackburn 1979; Lancelot and Billen 1985). Goldman et al. (1987) reported highest concentrations of bacteria on a range of substrates with $N:C_B:N:C_S$ ratios of approximately 2:1. Unfortunately, it was not possible to measure the $N:C_B$ in the present study. However, $N:C_B$ values found in the literature range from 1:3.9 to 1:5.6 for nutritionally fit bacteria (Luria 1960) and from 1:3.9 to 1:8.7 for naturally occurring bacteria (Nagata 1986). Thus, if a realistic theoretical $N:C_B$ ratio of 0.2 (1:5) is used, it can be seen that the system containing AqHS had a $N:C_B:N:C_S$ ratio of 2.22:1, which is close to the ideal value suggested by Goldman et al. (1987). However, the remaining systems diverged from this ideal with $N:C_B:N:C_S$ ratios of 6.45:1, 12.5:1, and 13.33:1 for PHS, AHA, and wAHA, respectively. Thus, while the systems in the present study were likely to have been nitrogen limited, AqHS is relatively rich in N compared with wAHA, AHA, and PHS. However, the nitrogen and carbon content of the whole HS does not necessarily give an indication of the relative availability of C and N to bacteria (Kroer 1993); hence, the results may have been driven by carbon rather than nitrogen limitation. This is considered unlikely in the present study, since at the beginning of the experiment, labile inorganic nitrogen concentrations in the flasks were low (approximately 1 μM). In addition, at 117.5 h, 10 μM KNO_3 (final concentration; Lindell et al. 1995) was added to the flasks (data not shown). After N addition, bacterial concentrations increased again to significantly higher values than were observed in the flasks prior to the addition. Taken together, these facts would suggest that N and not C limitation was being observed in the present study.

In contrast to the results for N:C, the H:C and O:C ratios in the present study do not fit the model of bioavailability proposed by Sun et al. (1997). Their study concluded that DOC bioavailability was negatively correlated with the O:C ratio. In the present study, the opposite result was obtained: a positive correlation between O:C ratio and bacterial concentrations was observed (model 3). However, this relationship was insignificant when compared with the N:C ratio. In addition, no significant relationship between H:C ratio and bacterial concentrations was observed either in the univariate or multivariate models.

There may be several reasons why the findings of the present study are not comparable to those of Sun et al. (1997). First, we used net maximum bacterial concentrations as our bioavailability indicator rather than bacterial biomass. This assumption does not allow for the fact that the bacteria may have been entering dormancy that can also be associated with an increase in bacterial numbers. However, we consider the increase in bacterial concentrations to be because of HS utilization, since the control systems devoid of a carbon source did not exhibit an increase in bacterial concentrations of similar magnitude (Fig. 1). Future studies should aim to include more robust measurements of bacterial growth and carbon utilization, including bacterial respiration and production, and carbon uptake. Secondly, Sun et al. (1997) utilized DOC from a range of locations that may have included highly labile compounds such as autotroph photosynthates. In contrast, our study utilized highly recalcitrant HS as the carbon source, which may have influenced the results of regression analysis. In particular, although our ratios were similar to values found in the literature (e.g., Visser 1983; Aiken et al. 1985; Lead et al. 1994) and O:C ratios in the present study lie within the range reported by Sun et al. (1997) (0.19–0.80), H:C were considerably lower than those reported in the last-named study. The low H:C ratios presented here may indicate that the HS used may be more complex than the isolated DOC used by Sun et al. (1997), which may have reduced the effect of H:C and O:C on bioavailability in the present case. Our statistical analysis of the data published by Sun et al. (1997) lends weight to this argument and reveals strong similarities to our own findings. We undertook multivariate analysis using their data and discovered that including N:C, H:C, and O:C as explanatory variables gave significant t statistics for N:C and O:C ($P < 0.005$), but H:C was insignificant. In addition, by removing leaf leachate data (which is likely to have undergone the least diagenetic change and included the greatest proportion of aliphatic carbon moieties) from the data set, O:C also became insignificant and only N:C had any significant explanatory power ($t = 3.25$, $P < 0.01$). These results would suggest that the data of Sun et al. (1997) actually provide strong evidence of the importance of N:C ratios in determining the bioavailability of recalcitrant DOC in natural water samples and would therefore support our findings.

In conclusion, the results presented here support the hypothesis that elemental composition may be used to predict

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DOC bioavailability for bacterial uptake. However, the influence of the various elemental ratios may vary depending on the type of DOC to be considered. Thus, when the majority of DOC is composed of highly complex, weathered recalcitrant molecules such as HS, N : C ratios have a significant effect on the bioavailability of these moieties for bacterial uptake. However, when DOC samples have undergone minimal diagenesis and have a more variable molecular complexity, other elemental ratios such as O : C may influence bacterial utilization.

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