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PRIMARY RESEARCH PAPER

Differential utilization of carbon substrates by aggregate-associated and water-associated heterotrophic bacterial communities

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Abstract The capacity to utilize carbon substrates is fundamental to the functioning of heterotrophic microbial communities in aquatic environments. Carbon-source utilization within the water column, however, is not a bulk property because microbial communities are patchily distributed on suspended organic aggregates (i.e., marine snow, marine aggregates, river aggregates, organic detritus, and bioflocs). In this study, Biolog Ecoplates were used to evaluate the metabolic capacity of heterotrophic bacterial communities associated with aggregates compared to communities in the surrounding water. Overall, aggregate-associated microbial communities demonstrated higher levels of metabolism, metabolic versatility, and functional redundancy, and a more consistent pattern of carbon-source utilization compared with water-associated communities. In addition, aggregate-associated communities more effectively exploited available resources, including representatives from several biochemical guilds and nitrogen-containing carbon sources. Within the aggregate-associated microbial community, metabolic activity was significantly higher in the presence of polymers, amino acids, and carbohydrates relative to amines and carboxylic acids. In comparison, metabolic activity of water-associated communities exceeded a threshold value for only two of the five guilds (polymers and carbohydrates) evaluated. These results suggest that compared with their free-living counterparts, aggregate-associated communities have a greater capacity to respond to a wider array of carbon inputs. Results also underscore the importance of targeting organic aggregates to better understand the role of microbial processes in ecosystem functioning.

Keywords Organic aggregates · Attached bacteria · Metabolic capacity · Functional redundancy · Metabolic plasticity

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Introduction

The microscale distribution of aquatic communities has been recognized as critical to the overall understanding of microbial ecology and the ability to predict larger, climate-scale changes (Azam & Long, 2001; Karl, 2007). Aquatic microbial communities are patchily distributed partly because of the presence of organic aggregates suspended in the water column. Silver et al. (1978) originally described these conglomerations of organic and inorganic particles (i.e., marine snow) as "physically distinct benthic-like microenvironments." Immediately thereafter, aggregates were identified as small-scale patches of relatively higher biomass and productivity compared to the surrounding water (Alldredge & Cohen, 1987;

Caron et al., 1986; Herndl, 1988). Subsequently, molecular analyses suggested that bacterial communities associated with aggregates were fundamentally different from their free-living counterparts in terms of structure, diversity, and phylogeny (DeLong et al., 1993; Rath et al., 1998; Crump et al., 1999).

Laboratory research on the ecology of organic aggregates has demonstrated that aggregates function as hotspots for microbial processes (reviewed by Simon et al., 2002) such as enzyme activities. For example, Karner & Herndl (1992) reported increased levels of hydrolytic activity (i.e., activity of α- and β-glucosidase and 1-aminopeptidase) in aggregate-associated bacteria. Likewise, Smith et al. (1992) documented that protease activity in aggregates was even higher than the activity of polysaccharidases, both of which were elevated relative to the surrounding water. Grossart et al. (2007) further demonstrated that aggregate-associated bacterial assemblages had significantly higher rates of bacterial protein production and protease activity relative to free-living counterparts. More recently, Ziervogel et al. (2010) reported hydrolytic activity in seawater containing aggregates to be two to three orders of magnitude higher than in seawater in which aggregates had not been generated. Accordingly, we hypothesized that these contrasts between aggregate-associated and water-associated communities would manifest as differential carbon-source utilization and set out to test and describe any intrinsic differences.

In addition, we integrated a dilution-regrowth design with the methods for generating organic aggregates to evaluate metabolic functional redundancy within the two types of communities. The systematic dilution of a relatively diverse community should remove its rarer members. Other researchers have successfully used a similar approach to evaluate microbial community functional responses to environmental stresses (Morales et al., 1996; Garland & Lehman, 1999; Franklin et al., 2001). Coupled with a regrowth period during aggregation, this design was used to create communities with potentially varying degrees of species richness.

Organic aggregates made in the laboratory simulate natural ones and have been used extensively to evaluate microbial processes (Shanks & Edmonson, 1989; Unanue et al., 1998). We assessed community-level metabolic capacity of heterotrophic bacteria with Biolog Ecoplates, designed to measure utilization of an array of environmentally relevant organic molecules (Insam, 1997). Although initially criticized for problems with interpretation (Preston-Mafham et al., 2002; Christian & Lind, 2006), Ecoplates have been shown to effectively discriminate among microbial communities in comparative studies (e.g., Choi & Dobbs, 1999; Leflaive et al., 2008; Tiquia, 2010). Using laboratory incubations to provide constant conditions, we provide examples of small-scale patterns in microbial metabolism that may, in turn, engineer water chemistry. The effects of in situ temperature, salinity, oxygen, nutrients, and dissolved organic material are all expected to influence metabolic patterns (e.g., Christian & Lind, 2007), but were beyond the scope of this project. Our primary objective was to assess the microscale distribution of microbial metabolic capacity (i.e., metabolic activity, metabolic versatility, and functional redundancy) through evaluation of carbon-substrate utilization.

Methods

Water source

Twenty liters of surface saltwater (14 PSU) were collected using a sterile container from the Smithsonian Environmental Research Center's dock on the Rhode River (tributary of the Chesapeake Bay) in Edgewater, Maryland, USA. Approximately 5 l were used as the source of water to seed the experimental microcosms. The remaining portion of 15 l was boiled for 10 min to kill vegetative bacterial cells, then cooled to room temperature, and mixed vigorously to break up particulate matter (both naturally occurring and that formed during boiling). Aliquots of heat-treated water plated on LB agar verified that no culturable heterotrophic bacteria remained. This unfiltered, heat-treated water was used as a supply of "bacteria-free" water in the dilution-regrowth design because filtered water lacks the particles necessary for aggregation.

Experimental design

Organic aggregates were generated according to the methods of Shanks & Edmonson (1989), using 1-l microcosm jars rolled at 12 rpm. To evaluate metabolic activity of the source water's heterotrophic

bacterial (hereafter referred to as "microbial") communities, three jars were filled (labeled 100%, Fig. 1). To assess community-level metabolic functional redundancy, four additional treatments (three jars each) were concurrently established by varying the percentage of source water (100 ml = 10%, 10 ml = 1%, 1 ml = 0.1%, 0.1 ml = 0.01%) added to jars otherwise filled with heat-treated water. Jars were well mixed to evenly distribute their microbial communities and abiotic particles, and then rolled for 24 h to produce organic aggregates and allow the seeded microbial communities to repopulate the microcosms. After 24 h, the jars were removed and placed upright for 10 min during which time all the visible aggregates settled to the bottom. Water was gently siphoned off and collected, thereby separating each microcosm into operationally defined aggregate- and water-fractions (Lyons et al., 2005). Settled aggregates were then homogenized into a slurry for analysis.

Carbon-source utilization

The aggregate-associated communities and the operationally defined macro-aggregate-free water communities (hereafter referred to as "water-associated") were evaluated for their capacity to oxidize 31 carbon substrates on Biolog Ecoplates. Ecoplates consist of three replicate sets of 31 carbon substrates (listed in Table 1) plus 3 control wells individually arrayed in a 96-well format. Each well also contains a minimal growth media and tetrazolium violet dye. This redox dye turns purple in the presence of electron transfer, indicating utilization (oxidation) of the substrate by

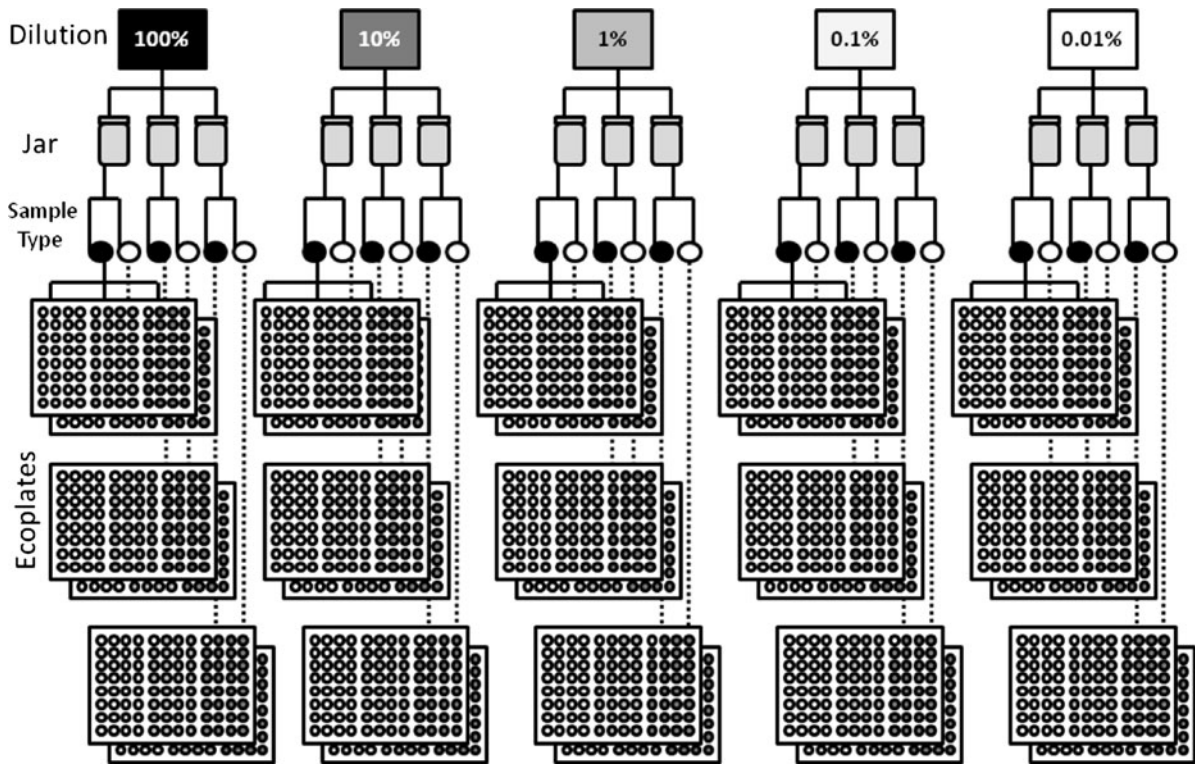

Fig. 1 The experimental design included five treatments ranging from 100% (i.e., undiluted; 10^0) to 0.01% (10^{-4}) unfiltered environmental water rolled for 1 day to produce organic aggregates. For each treatment, there were three replicate jars. For each jar, there were two sample types (i.e., aggregates and water, indicated by *black* and *white circles*, respectively). Aliquots from each sample type were loaded onto their own Ecoplate. Each 96-well Ecoplate contains three replicates of 31 carbon substrates plus three replicates of control wells. This fully nested design yielded 930 independent readings based on 2,790 readings per time point. Plates were read immediately (time zero) and once a day for 7 days to evaluate carbon-source utilization in the microbial community

Table 1 Microbial communities associated with aggregates and water differed in their utilization of carbon substrates

Substrate	AGGREGATE-ASSOCIATED COMMUNITY Percent Raw Water					WATER-ASSOCIATED COMMUNITY Percent Raw Water				
	100	10	1	0.1	0.01	100	10	1	0.1	0.01
POLYMERS										
α-Cyclodextrin	4.58	4.79	4.14	4.57	5.49	10.53*	13.65*	14.69*	13.89*	10.03
Tween 80	4.90	4.99	4.14	4.16	4.21	2.41	3.12	3.77	2.45	2.14
Tween 40	3.07	3.27	2.94	2.98	3.21	8.80	10.50	8.88	7.85	11.81
Glycogen	3.36	3.80	3.69	4.15	4.31	6.35	9.99	12.09	12.68	18.60*
AMINO ACIDS										
L-Asparagine (N)	5.00	5.02	4.38	4.47	4.78	1.40	1.81	0.86	1.38	0.35
L-Serine (N)	3.85	4.96	4.43	4.40	4.69	0.87	2.12	0.64	1.18	2.17
L-Arginine (N)	4.76	4.36	3.96	3.86	4.28	1.47	1.38	1.10	0.65	1.06
L-Threonine (N)	3.80	3.57	4.04	3.99	4.51	1.39	1.08	1.44	1.06	0.59
Glycyl-L-Glutamic Acid (N)	3.33	3.03	3.13	3.25	3.49	2.16	2.19	2.48	2.73	4.58
L-Phenylalanine (N)	2.80	2.02	3.59	3.43	3.70	-0.15	-0.38	-0.42	-0.55	-2.01
AMINES										
Phenylethylamine (N)	4.94	4.84	4.23	3.56	2.21	-1.13	1.65	0.05	-0.91	-3.81
Putrescine (N)	2.25	2.00	1.91	2.20	2.48	0.84	2.03	1.99	2.56	3.21
CARBOHYDRATES										
D-Mannitol	5.11*	5.38	4.69	5.40*	6.01*	7.40	7.91	7.34	9.33	15.52
D-Cellobiose	4.77	5.35	4.81*	5.00	5.11	10.29	7.07	11.22	11.46	10.15
N-Acetyl-D-glucosamine (N)	4.26	4.61	4.08	4.49	5.07	6.21	8.21	10.26	11.94	12.10
α-D-Lactose	4.01	4.28	4.70	4.29	3.64	5.28	2.84	1.03	1.18	2.03
D-Xylose	3.70	5.70*	4.71	3.49	4.56	4.06	3.80	3.35	1.36	0.09
I-Erythritol	3.58	3.93	4.78	4.58	2.97	1.82	1.37	1.13	1.63	0.45
β-Methyl-D-lucoside	2.19	2.46	2.78	3.48	3.88	7.55	2.45	3.82	3.15	4.45
D-Galacturonic Acid-γ-lactone	2.57	2.01	2.58	2.32	1.73	2.79	2.33	0.78	-0.76	-5.33
Glucose-1-phosphate	2.73	1.64	2.50	2.64	2.97	1.54	0.71	-0.50	-0.41	-2.57
D,L-α-Glycerol Phosphate	0.90	1.25	1.54	1.55	1.43	1.35	1.31	2.32	2.36	2.88
CARBOXYLIC ACIDS										
Itaconic Acid	4.58	3.60	3.43	3.85	3.02	3.43	3.55	3.01	1.55	1.21
D-Galacturonic Acid (N)	2.86	3.31	3.32	3.58	3.63	9.09	5.97	6.47	8.25	15.15
Glucosaminic Acid	3.60	2.46	3.46	2.78	-0.02	2.84	2.56	1.81	1.39	-2.49
4-Hydroxy Benzoic Acid (ph)	1.70	3.03	3.02	2.93	3.47	0.50	0.67	1.44	1.63	1.65
Pyruvic Acid Methyl Ester	2.68	1.72	2.10	1.78	1.91	5.30	3.56	1.62	3.96	4.76
D-Malic Acid	2.21	2.09	1.83	1.92	2.46	0.06	0.49	0.16	0.49	0.03
α-Ketobutyric Acid	2.29	1.42	1.83	1.71	1.37	-0.11	0.54	0.75	0.79	1.50
2-Hydroxy Benzoic Acid (ph;i)	-0.33	-0.71	-0.70	-0.87	-0.71	-2.87	-3.16	-2.63	-3.30	-7.31
γ-Hydroxybutyric Acid (i)	-0.07	-0.18	-0.06	0.06	0.13	-1.50	-1.31	-0.93	-0.99	-2.98
Number over 2% utilization	**27**	**25**	**25**	**25**	**24**	**16**	**18**	**13**	**13**	**15**

Numbers within the matrices correspond to the percentage of total utilization (i.e., plate average) for each substrate ($n = 3$ readings per dilution per community type). Bold values <2%; italicized values = 2–4%; bold italic values = 4–6%; underlined values >6%). Total number of substrates used (defined as usage > 2%) is listed in last row. Negative values result when average color development for a particular substrate was less than that of the corresponding control wells. Superscripts denote substrates used for N-use index (N), phenolic substrates (ph); substrates inhibitory to both communities (i), and the highest percent utilization (*) for both communities at each dilution

inoculated microbes (Bochner, 1989). Replicate control wells ($n = 3$) contain no additional carbon substrate; thus, any color development in them represented background metabolism caused by the microbial community and was subtracted from corresponding well readings on the same Ecoplate (Garland & Mills, 1991). For each sample type ($n = 2$: aggregate and water) from every jar ($n = 3$) of all treatments ($n = 5$), well-mixed aliquots of 150 µl (aggregate-slurry or siphoned water) were

loaded into individual wells on an Ecoplate (total of 30 Ecoplates). The fully nested design yielded 930 independent readings (5 dilution treatments × 3 jars × 2 sample types × 31 substrates) based on 2,790 individual readings ($n = 3$ per Ecoplate) per time point. All Ecoplates were incubated in the dark at room temperature (20°C). Optical density ($\lambda =$ 590 nm) of the liquid in each well was determined immediately (time = 0 h) as well as every day for at least 7 days using a BioTek plate reader, model ELX800 (BioTek Inc.).

Average well color development (AWCD) for each Ecoplate, a measure of overall community metabolic activity, was calculated in accordance with Garland & Mills (1991) after subtracting the starting values (i.e., time zero) of each Ecoplate from its subsequent readings (ΔAWCD) to account for inherent differences in the absorbance of the carbon substrates (Insam & Goberna, 2004) and natural turbidity differences between sample types (Christian & Lind, 2007).

Average substrate color development (ASCD), a measure of carbon-source utilization for individual substrates, was calculated according to Sala et al. (2005), except that negative values were maintained (i.e., not converted to zero). Negative values occurred when color development for a particular substrate was less than that in the corresponding control wells, indicating reduced (i.e., inhibited) metabolic activity. ASCD values were also used to evaluate (1) metabolic activity with respect to biochemical guild (e.g., polymers, amino acids, amines, carbohydrates, and carboxylic acids); (2) metabolic activity with respect to nitrogen content; and (3) identify preferred, usable, and inhibitory compounds (see definitions in "Results"). Metabolic versatility within each biochemical guild was calculated as the number of substrates contributing to at least 2% of total color development.

To evaluate utilization of low-molecular-weight, dissolved organic nitrogen compounds, a nitrogen use (N-use) index was calculated and expressed as the proportion of total substrate utilization due to substrates that contain nitrogen (Sala et al., 2006a). If all N-containing substrates ($n = 10$; i.e., 6 amino acids, 2 amines, 1 carbohydrate, and 1 carboxylic acid; Table 1) contributed equally to total substrate utilization ($n = 31$), then the summed contribution of these 10 substrates would be approximately 32% (i.e., 10/31 × 100).

Statistical analyses

Minitab and Analyse It (Excel) software packages were used for descriptive statistics and hypothesis testing. First, data were assessed for normality (Shapiro–Wilk W test) and homogeneity of variances (Levene's test). When data were normally distributed and variances were homogeneous (e.g., data from control wells), ANOVA was used to assess differences among treatment means. Otherwise, Kruskal–Wallis tests (nonparametric substitution for ANOVA) were used to evaluate differences in treatment medians. Results were considered significant when P values were less than $\alpha = 0.050$.

Results

Analyses focused on readings from day 7 post inoculation of the Ecoplates to (1) facilitate detection of maximum carbon-source utilization (i.e., saturation of utilization rates; Salomo et al., 2009) and (2) avoid potential confounding effects of inoculum size observed in the first 3 days post inoculation (Christian & Lind, 2006). Readings from the control wells of aggregate-associated communities ($n = 90$; distribution normal, Shapiro–Wilk W test, $P = 0.200$ with equal variances, Levene's test, $P = 0.925$) were not higher than those from water-associated communities (data not shown) as would be expected if a higher concentration of starting inoculum in the aggregate samples drove patterns in substrate utilization.

Overall community metabolic activity

To evaluate microscale differences between aggregate-associated and water-associated communities generated from the same starting waters, ΔAWCD readings were compared. Since ΔAWCD readings were not normally distributed (Shapiro–Wilk, $W = 0.94$, $P < 0.001$) and their variances were unequal (Levene's test $P < 0.001$), nonparametric tests were employed to assess differences among treatments. The variable "sample type" (aggregate or water) exerted a significant effect (Kruskal–Wallis 394.88, $P < 0.001$), such that average values for carbon-source utilization of the aggregate-associated communities were significantly greater than those

from water-associated ones (Fig. 2), but the variable "dilution" (100, 10, 1, 0.1, and 0.01%) did not have a significant effect on overall ΔAWCD readings (Kruskal–Wallis 9.32, $P = 0.053$). Within each sample type, dilution was not significant for aggregate-associated communities (Kruskal–Wallis 5.53, $P = 0.238$), but it was significant for water-associated communities (Kruskal–Wallis 18.44, $P = 0.001$). Within water-associated communities, median values from the microcosms with the smallest volume of seeded raw water (100 µl = 0.01%) were significantly lower than that of the undiluted (100% raw water) and the two least diluted (10 and 1%) microcosms (Fig. 2).

Metabolic activity with respect to biochemical guild

To evaluate whether the type of substrate available affected utilization patterns, substrate usage from all dilutions were grouped by biochemical guild (Table 1; Fig. 3). Data grouped by guild were not normally distributed (Shapiro–Wilk, $W = 0.95$, $P < 0.001$), and so nonparametric tests were selected. ASCD in the presence of polymers ($n = 4$ substrates; 120 readings), amino acids ($n = 6$ substrates; 180 readings), amines ($n = 2$ substrates; 60 readings), carbohydrates ($n = 10$ substrates; 300 readings), and carboxylic acids ($n = 9$ substrates; 270 readings) was significantly higher for aggregate-associated communities compared with water-associated ones (Fig. 3). Within aggregate-associated communities, the metabolic activity for each of the 5 guilds was above a standard threshold value for utilization (0.250, Zak et al., 1994; horizontal line in Fig. 3). Mean ranks of ASCD values were significantly higher (Kruskal–Wallis 130.13; $P < 0.001$) in the presence of polymers, amino acids, and carbohydrates as compared with amines and carboxylic acids (Fig. 3). In comparison, metabolic activity within water-associated communities was above the threshold value for only two of the five guilds (polymers and carbohydrates) evaluated. Metabolic activity in the presence of polymers was significantly higher (Kruskal–Wallis 117.32, $P < 0.001$) than that for carbohydrates, which in turn was significantly higher than values for the remaining three guilds.

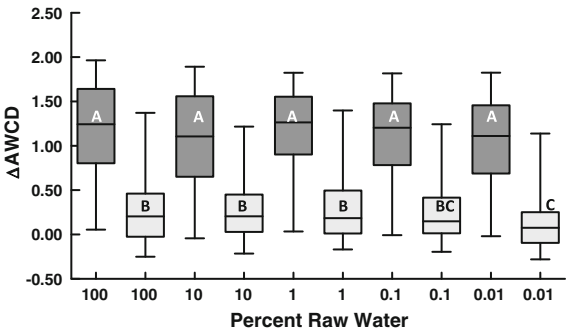

Fig. 2 Overall community metabolic activity (ΔAWCD) caused by aggregate-associated microbial communities (*dark boxes*) was significantly greater than that of water-associated microbial communities (*light boxes*). *Boxes marked by the same letters* denote median values not significantly different from each other. *Boxes* represent inter-quartile (25 and 75%), and *whiskers* represent (5 and 95%) ranges of 93 readings each (i.e., 31 substrates from each of three replicate jars)

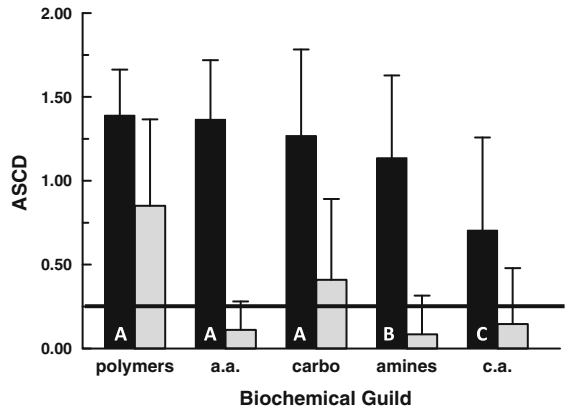

Fig. 3 Mean (and SD) utilization of carbon sources grouped by biochemical guild: polymers, *a.a.* amino acids, *carbo* carbohydrates, amines, and *c.a.* carboxylic acids. Utilization was significantly greater for the microbial communities associated with aggregates (*darker bars*) than for microbial communities associated with aggregate-free water (*lighter bars*). Water-associated communities only utilized two guilds (utilization: polymers > carbohydrates) above threshold values (0.250; *horizontal line*), whereas aggregate-associated communities were capable of using all the five guilds above the threshold level (utilization: polymers, amino acids, and carbohydrates > amines > carboxylic acids; *capital letters on darker bars*)

Metabolic activity with respect to nitrogen-containing compounds

To assess the degree to which nitrogen-containing carbon substrates contributed to overall metabolic

activity of each community type, N-use indices were calculated for each jar ($n = 15$). Data were not normally distributed (Shapiro–Wilk, $W = 0.82$, $P < 0.001$). Overall, the N-use indices were significantly higher for aggregate-associated communities compared with water-associated ones (Kruskal–Wallis 21.00, $P < 0.001$; Fig. 4). Within the aggregate-associated communities, the N-use index for the microcosms with the smallest starting inoculum (0.01%) was significantly lower (Kruskal–Wallis 9.63, $P = 0.047$) than for all other dilutions. For water-associated communities, the differences were not significant (Kruskal–Wallis 7.79, $P = 0.099$). Compared with the idealized threshold, the N-use index was greater than 32% for all aggregate-associated communities and less than 32% for all water-associated ones (Fig. 4).

Preferred, usable, and inhibitory compounds

To evaluate the importance of specific carbon sources, a substrate-by-substrate analysis was conducted on ASCD values and measures were expressed as a percentage of total utilization (Table 1). The carbon substrate having the highest ASCD value (one measure of "preference") differed between aggregate-associated and water-associated communities. In the former, the highest value in each treatment was associated with a carbohydrate: 100% = D-mannitol (5.11%); 10% = D-xylose (5.70%); 1% = D-cellobiose (4.81%); 0.1% = D-mannitol (5.40%); and 0.01% = D-mannitol (6.01%). In comparison, the highest values for water-associated communities were associated with polymers; for the first four treatments, it was α-cyclodextrin (10.53, 13.65, 14.69, and 13.89% respectively), whereas for the 0.01% dilution it was glycogen (18.60%).

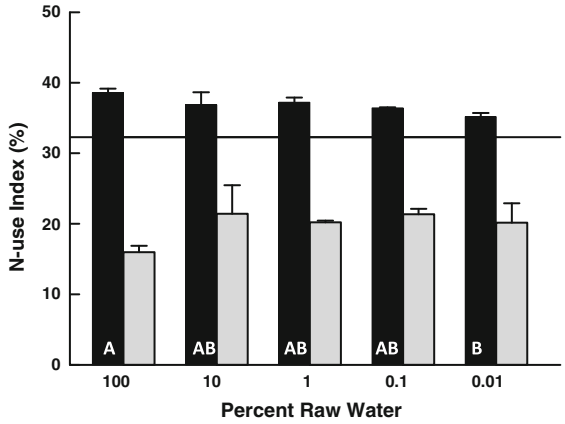

Fig. 4 Average nitrogen use (N-use) index expressed as the percent of total carbon substrate utilization attributable to substrates that also contain nitrogen ($n = 6$ for each dilution). If all N-containing substrates contributed equally to total substrate utilization, then the summed contribution of these 10 substrates would be 32.3% (*horizontal line*). The N-use index was higher than this level for all the aggregate-associated communities, whereas it was lower for all water-associated communities. Compared directly, the mean ranks of N-use index were significantly higher for the aggregate-associated communities. Within aggregate-associated communities, only the 100 and 0.01% dilutions were significantly different from each other (*letters on dark bars*)

To determine which substrates were used first (another measure of "preference"), optical-density data from earlier time points (days 1–3) were also evaluated, depending on the day (post-inoculation of an Ecoplate) when the first wells surpassed threshold values for carbon-source utilization (i.e., control- and time zero-corrected optical densities >0.250). For aggregate-associated communities, this criterion always occurred on day 1, but for water-associated communities, it was typically reached after 2–3 days of the loading of Ecoplates, likely reflecting different cell densities in the starting inoculum. Even so, the substrates first reaching this criterion differed between aggregate-associated and water-associated communities (Table 2). Five substrates (two polymers and three carbohydrates) were always used first by aggregate-associated communities in all dilutions. In contrast, a carboxylic acid was the only substrate type consistently used first by water-associated communities. There were no instances of amino acids or amines being used first by either community.

Comparing the "most-used" (Table 1) substrates to the "first-used" (Table 2) substrates identified which carbon sources were rapidly degraded and which ones required relatively longer periods of time to be fully utilized. For example, in water-associated communities, substrates showing the highest percent utilization were the polymers: α-cyclodextrin, Tween 40, and glycogen, the carbohydrates: D-mannitol, D-cellobiose, and *N*-acetyl-D-glucosamine, and one carboxylic acid, D-galacturonic acid. Since the first substrates to exceed threshold values also included the polymer (Tween 40), carbohydrate (D-mannitol), and carboxylic acid (D-galacturonic acid), results suggest

Table 2 List of first substrates used by each type of microbial community for each of the five treatments (100, 10, 1, 0.1, and 0.01%)

100	10	1	0.1	0.01
Aggregate-associated community				
Tween 40	Tween 40	Tween 40	Tween 40	Tween 40
α-Cyclodextrin	α-Cyclodextrin	α-Cyclodextrin	α-Cyclodextrin	α-Cyclodextrin
N-Acetyl-D-glucosamine	N-Acetyl-D-glucosamine	N-Acetyl-D-glucosamine	N-Acetyl-D-glucosamine	N-Acetyl-D-glucosamine
D-Mannitol	D-Mannitol	D-Mannitol	D-Mannitol	D-Mannitol
D-Cellobiose	D-Cellobiose	D-Cellobiose	D-Cellobiose	D-Cellobiose
β-Methyl-D-glucoside	D-Galacturonic acid	4-Hydroxy Benzoic Acid		4-Hydroxy Benzoic Acid
Water-associated community				
Pyruvic Acid Methyl Ester	D-Galacturonic acid	D-Galacturonic acid	D-Galacturonic acid	D-Galacturonic acid
Tween 40	Tween 40	D-Mannitol		Tween 40
	D-Mannitol			

For aggregate-associated communities, 5–6 substrates registered as utilized (i.e., readings over threshold values of 0.250) including 2 polymers (bold entries), 3–4 carbohydrates (italic entries), and 2 carboxylic acids (bold italic entries). For water-associated communities, only 1–3 substrates were registered as used, but included representatives from the same three guilds

that the other preferred substrates (α-cyclodextrin, glycogen, D-cellobiose, and *N*-acetyl-D-glucosamine), required more time to be fully utilized. Likewise, for aggregate-associated communities, utilization of amines and amino acids required relatively longer periods of time compared with representatives from other guilds.

In addition to being higher in aggregate-associated communities, carbon-source utilization was more evenly distributed across the array of 31 substrates compared to water-associated communities. The greater number of substrates utilized above the 2% level (Sala et al., 2005; bottom row of Table 1) indicated greater metabolic versatility in aggregate-associated communities. Accordingly, the pattern of carbon-source utilization was more limited for water-associated communities with fewer substrates (bottom row of Table 1) contributing relatively higher percentages (seven instances >6%) to an overall lower ΔAWCD (Fig. 2). In comparison, there was only one example of >6% utilization in aggregate-associated communities (i.e., carbohydrate, D-mannitol, in the 0.01% treatment). With respect to biochemical guild, metabolic versatility was greater for aggregate-associated communities metabolizing amino acids, amines, carbohydrates, and carboxylic acids. For the polymer guild, both communities metabolized four of the four substrates available.

Maintaining negative values in the analysis, rather than setting them to zero, facilitated classifying a substrate as "not used" as opposed to "inhibitory." For example, in aggregate-associated communities, the carbohydrate, D,L-α-glycerol phosphate, was utilized below threshold values (0–2%), whereas the carboxylic acids, 2-hydroxyl benzoic acid and γ-hydroxbutyric acid, inhibited metabolic activity in eight of 10 instances (Table 1). In water-associated communities, results from three of the amino acids were below threshold values for utilization (i.e., L-asparagine, L-arginine, and L-threonine), whereas one amino acid inhibited metabolic activity (e.g., L-phenylalanine). Furthermore, comparing patterns between the two types of communities revealed examples of substrates exploited by the aggregate-associated communities, but inhibiting to the water-associated ones (e.g., L-phenylalanine; Table 1).

Discussion

Data from Ecoplates were evaluated at several hierarchal levels. At the most general level, ΔAWCD for the 31 substrates was used as a metric for comparing overall community metabolic activity (1) between aggregate-associated and water-associated communities and (2) among communities re-grown from

varying volumes of seed water. For more specific analyses of patterns in differential carbon-source utilization, substrates were grouped according to their (A) biochemical guild and (B) nitrogen content. Finally, a substrate-by-substrate analysis was used to evaluate metabolic activity, versatility, and functional redundancy, and to identify preferred, usable, and inhibitory compounds for each type of community.

Overall community metabolic activity

Overall utilization of carbon substrates by aggregate-associated communities was significantly higher than utilization by water-associated communities, despite their origination from a single collection of environmental water. In other words, during the 24-h when aggregates formed in microcosms, microbial communities associated with organic aggregates were able to metabolize more number of carbon sources, at a higher level of activity, than microbial communities remaining in the water. In a similar study, Grossart et al. (2007) demonstrated that bacterial strains were quickly capable of up-regulating protease activity upon attachment to model aggregates (agar spheres).

Within each of the two community types, there was either no (aggregate-associated) or only one (water-associated) detectable difference in the total utilization of carbon sources across a dilution series of five orders of magnitude after 7 days of incubation (Fig. 2). Based on random sampling and probability, the dilution-regrowth design was expected to systematically remove rarer members from the original community (Franklin & Mills, 2005). If utilization patterns were driven solely by species diversity, then a corresponding decrease in the metrics of metabolic activity would be expected along the dilution gradient. If utilization patterns were determined by functional redundancy, however, then, as species were removed from the community, other members would nonetheless metabolize those substrates, and no decrease in metabolic activity would be expected. Results of this experiment imply that functional redundancy, and not strictly species diversity, was responsible.

In our earlier study, it was demonstrated that organic aggregates function as microscopic islands for microbial communities by evaluating four tenets of the Theory of Island Biogeography (Lyons et al., 2010). In this experiment, the dilution-regrowth design was used to ascertain if a fifth tenet (i.e., "distance from a source" relationship) would be observed. Assuming this relationship is in part a function of contact rate, we systematically decreased the volume of inoculum to mimic an increasing distance between an aggregate and its source of colonizers (i.e., smaller volume = further away). Surprisingly, the metabolic activity on aggregates formed from the original community (i.e., microcosm with 100% raw water) was the same as that of communities re-grown in 24 h from an inoculum of only 100 µl of raw water, equivalent to 0.01% dilution (Fig. 2). Although these results support the idea that evenness and functional redundancy in the initial community must have been relatively high (Franklin et al., 2001; Comte & Del Giorgio, 2010), no pattern predicted by island biogeography was observed. Similarly, but over larger spatial scales, Bell (2010) found little evidence for a relationship between geographic distance and community composition in aquatic bacterial communities, concluding that environmental factors (i.e., resource gradients) played an important role. In contrast, our experiment was designed to minimize environmental differences among microcosms, with the exception of inoculum volume. Interestingly, among water-associated communities, we did observe a slight decrease in metabolic activity, but only in microcosms with the smallest inoculum size (100 µl; Fig. 2). Overall, the results of this study indicate that aggregates provide better and more consistent habitat resources for attached bacteria than those available to free-living bacteria.

Metabolic activity with respect to biochemical guild

Aggregate-associated communities utilized most representatives from all the biochemical guilds (amines, amino acids, carboxylic acids, carbohydrates, and polymers), whereas water-associated communities metabolized mostly carbohydrates and polymers (Fig. 3). These results imply that aggregate-associated communities were more versatile than their freely suspended counterparts, an idea also supported by the former's overall higher use of substrates at levels of 2% or greater. Higher levels of metabolic versatility suggest aggregate-associated communities are more likely to capitalize on unexpected and potentially ephemeral inputs of dissolved organic carbon sources

in the environment. Consequently, we infer that the aggregate-associated communities in our study were more plastic than water-associated ones.

Metabolic activity with respect to nitrogen-containing compounds

The N-use indices for aggregate-associated communities were approximately 37%, indicating a disproportionately higher level of utilization of these nitrogen-containing substrates relative to overall metabolic activity (Fig. 4). In contrast, the indices were closer to 21% for water-associated communities, suggesting their members were unable to exploit many of these substrates. The difference between the two communities resulted principally from negligible to low metabolism of amino acids and amines by the water-associated communities.

Based on the observation that aggregate-associated communities were able to utilize all nitrogen-containing compounds, we hypothesize that they were more likely to have originated in a habitat with diverse dissolved organic nitrogen compounds having favorable concentrations. In contrast, utilization of these compounds was limited in water-associated communities, with the two notable exceptions of the nitrogen-containing carbohydrate (*N*-acetyl-D-glucosamine) and carboxylic acid (D-glucosaminic acid). Leflaive et al. (2008) documented preferential use of nitrogen-rich substrates in nitrogen-replete mesocosms, supporting the concept that utilization of Ecoplate substrates reflects recent exposure to similar compounds. Such exposure may then allow members of the microbial community to quickly adapt metabolic machinery to similar substrates available in Ecoplate wells. Given that organic aggregates are considered by some as small-scale chemical "patches" (Alldredge & Cohen, 1987; Kaltenbock & Herndl, 1992; Alldredge, 2000) in aquatic environments, aggregate-associated communities likely would be exposed to higher concentrations of dissolved nutrients, including the dissolved free amino acids (Muller-Niklas et al., 1994), than water-associated communities.

Alternatively, mere attachment of microbial communities to an aggregate may have triggered a metabolic capacity that was subsequently maintained in aggregate-associated communities but diminished in freely suspended ones. Such was the case shown by Grossart et al. (2007) and Taylor & Gulnick (1996), who demonstrated increased bacterial activity in response to a physical substrate, despite the absence of an organic one. In addition, the formation of aggregates has been shown to trigger significant enzyme production by the associated communities (Ziervogel et al., 2010). Our results demonstrate that communities associated with aggregates possess greater metabolic capacity than those which remain freely suspended. Experiments are ongoing to distinguish between these alternative interpretations of our results (Lyons & Dobbs, in prep.).

Preferred, usable, and inhibitory compounds

Microbial communities associated with organic aggregates are generally more abundant, productive, and diverse than their free-living counterparts (Alldredge & Silver, 1988; Herndl, 1988; Grossart et al., 2006). Our results demonstrate that compared with free-living microbial communities, aggregate-associated communities used more and a greater variety of Ecoplate substrates, used them more quickly and to a greater extent, and were inhibited by fewer of them.

Lack of substrate utilization has been reported by other researchers. For example, in an analysis of metabolic diversity and its links to pollution levels, Tiquia (2010) reported all but two Ecoplate substrates (i.e., carboxylic acids: D-malic acid and glucosaminic acid) were metabolized by river-water and groundwater aquatic microbial communities. In a seasonal study in Mediterranean waters, Sala et al. (2006b) compared aquatic microbial communities from coastal waters to those of harbors and also found differential carbon substrate utilization. Substrates not metabolized by microbial communities from coastal waters included one carbohydrate (β-methyl-D-glucoside) and one carboxylic acid (4-hydroxy benzoic acid). Those not used from harbor waters included four carboxylic acids (D-glucosaminic acid, itaconic acid, α-ketobutyric acid, and 4-hydroxy benzoic acid), one amino acid (L-phenylalanine), and one amine (phenylethylamine). In addition, Sala et al. (2005) listed two carbohydrates and three amino acids as not used by the springtime microbial communities from Antarctic seawaters and reported that the phenolic carboxylic acids (i.e., 2-hydroxy benzoic acid and 4-hydroxy benzoic acid) were the most intensely used substrates. By way of comparison, in our study using coastal estuarine water, 2-hydroxy benzoic acid was

inhibitory to both aggregate-associated and water-associated microbial communities. In total, these differences suggest that local physicochemical conditions and resources available in wide-ranging environments contribute to the specific patterns of metabolic activity observed.

Although substrates on an Ecoplate may not replicate the dissolved organic compounds at any given environmental site, they nonetheless have been identified as environmentally relevant (Insam, 1997) and successfully used as proxies for understanding different classes and patterns of carbon-source utilization under various conditions (Grover & Chrzarowski, 2000). Since our results illustrate differences in carbon-source utilization between the aggregate-associated and water-associated microbial communities, they indicate that the two communities differed in their abilities to exploit specific environmental resources. Should these results extend to the environment, the concentration of aggregates would become increasingly important with regard to the biogeochemical impact of aggregate-associated communities. Concentrations of large aggregates range from <1 to >5,000/l (Table 1 in Simon et al., 2002) and have been shown to vary with location, season, and tidal stage (Pilskaln et al., 2005; Lyons et al., 2007). In some environments, therefore, the chemical cycling of organic compounds linked to suspended aggregates would be profound.

Conclusion

Aggregate-associated communities demonstrated higher levels of metabolism, higher metabolic versatility, and functional redundancy, and more consistent substrate utilization compared with water-associated communities, even though both originated from the same source of water and were produced under the same conditions. In laboratory microcosms, aggregates provided better habitat resources for associated microbes. By inference, the aggregate-imposed, microscale distribution of aquatic microbial communities in nature has relevance for measuring, monitoring, and modeling metabolic processes. The results reported here enhance our understanding of aquatic microbial communities by providing an in-depth analysis of patterns of carbon- and nitrogen-substrate utilization.

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