

Multiple carbon substrate utilization by bacteria at the sediment–water interface: seasonal patterns in a stratified eutrophic reservoir

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Abstract Sediment–water interfaces (SWIs) in lakes and reservoirs harbor diverse assemblages of heterotrophic bacteria that influence organic matter cycling. Seasonal reservoir mixing and stratification affect the rate and type of dissolved organic carbon (DOC) utilized by SWI bacteria, however these seasonal carbon utilization dynamics remain poorly understood. We conducted an exploratory multi-seasonal study using Biolog EcoPlates to measure SWI bacterial carbon substrate utilization rates (CSURs) and patterns in a monomictic eutrophic reservoir. Principal components analysis (PCA) was used to elucidate seasonal CSUR variation. Carbohydrate utilization was greatest during early (onset of) stratification and also was high during winter mixing. Amino acid utilization was greatest during late (prolonged) stratification. Carboxylic acids had greatest utilization during late stratification, but also had moderate to high utilization during all other seasons. Amines and polymers exhibited moderate utilization during all seasons. We related seasonal variation in these CSURs to SWI bacterial abundance, temperature, dissolved

oxygen, and redox potential. Collectively these environmental variables accounted for 49.4–62.3% of the total substrate utilization variance by season. Several individual substrate CSURs were significantly ($P < 0.05$) positively and/or negatively correlated with individual environmental variables, with L-asparagine and putrescine exhibiting significant positive and negative correlations with every measured environmental variable. Lastly, seasonal changes in CSURs corresponded to various changes in SWI bacterial assemblage composition, with highest similarities among early stratification and autumnal overturn assemblages and lowest similarities among late stratification and winter mixing assemblages.

Keywords Sediment–water interface · Bacteria · Carbon substrates · Stratified reservoir · Biolog EcoPlates

Introduction

Secondary production via heterotrophic bacterial uptake and metabolism of dissolved organic carbon (DOC) substantially contributes to organic matter cycling in aquatic ecosystems (Sinsabaugh et al., 1997; Chin-Leo, 2002). Sediment–water interfaces (SWIs) of lakes provide ample habitats for these heterotrophic bacteria (Liikanen & Martikainen, 2003; Bloesch, 2004).

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High and low molecular weight DOC (HM-DOC and LM-DOC) as well as particulate organic carbon (POC) and other dissolved and particulate organic matter (DOM and POM) not utilized by pelagic microbes collects and concentrates at the SWI (Dean, 1999; Vreča, 2003; Heinen & McManus, 2004). The DOC fraction contributes a substantial portion of total organic matter in these freshwater sediments (O'Loughlin & Chin, 2004). Bacteria that blanket the SWI incorporate DOC into their biomass and oxidize DOC into inorganic carbon using a variety of electron acceptors in respiratory pathways, releasing chemically reduced compounds into the water column (Vreča, 2003; Bloesch, 2004). Further, *in situ* studies and theoretical mixing models have shown that SWI bacteria exhibit high activities (Butorin, 1989; Gantzer & Stefan, 2003). However, seasonal stratification and mixing events and their effects upon changes in SWI bacterial populations and DOC utilization remain unknown.

Physically, the SWI is the transition layer from a fluid (water) to solid (sediment) matrix (Danovaro et al., 1998). This includes the water layer near the sediment surface that contains a steep gradient of physical and chemical dynamics (Boudreau & Jørgensen, 2001). As SWI bacteria incorporate and oxidize organic carbon, electron acceptors are utilized in order of decreasing free energy yield. Dissolved oxygen, being the energetically favored electron acceptor, is quickly depleted resulting in the active SWI bacterial consortia shifting to assemblages that can utilize less energetically favored acceptors (e.g., nitrate, sulfate). This process lowers redox potential and increases concentrations of chemically reduced nutrients (e.g., ammonium, sulfides) (Liikanen & Martikainen, 2003). The chemically reduced SWI rapidly disintegrates during episodes of hypolimnetic overturn (Gantzer & Stefan, 2003). The reintroduction of dissolved oxygen restores oxidizing redox potentials and shifts active SWI bacterial assemblages towards species that favor aerobic respiration, halting anaerobic respiration and production of reduced nutrients (Stumm, 2004). These cycles of thermal stratification and overturn also alter the quantity, and possibly the type, of DOC substrates present at the SWI (Dean, 1999).

Various methods have been used to study bacterial DOC utilization in aquatic ecosystems including culture-dependent (e.g., incubation cultures, selective plating) and culture-independent (e.g., respiration rates, biomass production) methods (Jahnke & Craven, 1995; Rosenstock et al., 2005). For our investigation we utilized Biolog EcoPlates, a phenotypic assay, to observe SWI seasonal bacterial utilization of LM-DOC (<1 kDa) substrates at the SWI of a monomictic, eutrophic reservoir. Biolog EcoPlates contain 96 wells, each containing a distinct DOC substrate and redox-sensitive tetrazolium dye. Thirty-one different carbon substrates in triplicate and three carbon-free control wells provide intraplate replication. These substrates include various carbohydrates, amino acids, carboxylic acids, amines, and small polymers (Table 1). Carbon substrate utilization rates (CSURs) are generated by inoculating unamended bacterial samples into the plate wells, incubating the plates, and spectrophotometrically measuring the optical density (OD₅₉₀) of purple formazan dye formation from tetrazolium reduction, which is proportional to carbon substrate oxidation rate (Mills & Garland, 2002).

While Biolog assays suffer from the same inherent biases as selective culturing, they are a valuable and inexpensive way to elucidate functional potential changes in various microbial communities (Choi & Dobbs, 1999; Mills & Garland, 2002; Christian & Lind, 2006). Also, recent studies have addressed and minimized problems that have historically plagued Biolog assays including inoculum size, incubation temperature effects, and incubation of anaerobic bacterial communities (Christian & Lind, 2006). While the carbon sources in Biolog assays may not represent the DOC compounds found *in situ*, oxidation of these substrates may serve as a proxy for understanding various classes and patterns of substrates that are preferred under various physicochemical conditions (Grover & Chrzanowski, 2000).

Using Biolog EcoPlates, we measured SWI bacterial community CSURs from samples taken during autumnal overturn, winter mixing, early (onset of) summer stratification, and late (prolonged) summer stratification. We also measured

Table 1 List of Biolog EcoPlate substrates, their corresponding classes, and plate code number

Substrate	Plate code
<i>Amino acids (n = 6)</i>	
L-Arginine	A4
L-Asparagine	B4
Glycyl-L-Glutamic Acid	F4
L-Phenylalanine	C4
L-Serine	D4
L-Threonine	E4
<i>Carbohydrates (n = 10)</i>	
D-Cellobiose	G1
i-Erythritol	C2
D-Galactonic acid γ -lactone	A3
N-Acetyl-D-glucosamine	E2
Glucose-1-phosphate	G2
β -Methyl-D-glucoside	A2
D,L- α -Glycerol phosphate	H2
α -D-Lactose	H1
D-Mannitol	D2
D-Xylose	B2
<i>Carboxylic acids (n = 9)</i>	
γ -Hydroxybutyric acid	E3
α -Ketobutyric acid	G3
D-Galacturonic acid	B3
D-Glucosaminic acid	F2
Itaconic acid	F3
D-Malic acid	H3
Pyruvic acid methyl ester	B1
2-Hydroxybenzoic acid	C3
4-Hydroxybenzoic acid	D3
<i>Amines (n = 2)</i>	
Phenylethylamine	G4
Putrescine	H4
<i>Polymers (n = 4)</i>	
α -Cyclodextrin	E1
Glycogen	F1
Tween 40	C1
Tween 80	D1

corresponding bacterial abundance and physicochemical variables (e.g., temperature, dissolved oxygen, redox potential). Our objectives were to: (1) determine substrate classes preferentially used by SWI bacterial consortia during each seasonal mixing and stratification event; (2) determine the amount of variation in seasonal substrate utilization explained by corresponding physicochemical variables; (3) detect correlations among individual substrate CSURs and individual physicochemical variables; and (4) analyze similarities and differences in seasonal community-level physiological profiles (CLPPs) derived from the CSURs.

Materials and methods

Field sampling

Lake Belton, Bell County, Texas, a monomictic, eutrophic reservoir, served as the sampling location. It thermally stratifies in late spring, maintains an anaerobic hypolimnion throughout summer, and overturns in late autumn (Christian et al., 2002). Five sampling stations representing the depth gradient below the photic zone were chosen (Table 2). Depth (m), water temperature ($^{\circ}\text{C}$), dissolved oxygen (mg l^{-1}), and redox potential (mV) of the SWI were measured using a YSI 600QS sonde by lowering the sonde to the sediment surface and waiting for it to stabilize. Two dates were sampled per mixing event: autumnal overturn, winter mixing, early (onset of) stratification, and late (prolonged) stratification (Table 3). The late stratification and autumnal overturn samples were collected over 2 years due to the short time span of stratification and overturn events.

Samples were retrieved from the SWI via a 3.2 l horizontal PVC Alpha water sampler (Wildlife Supply Company, Buffalo, New York, USA). The sampler was lowered to the sediment, raised approximately 0.5 m, moved 2 m horizontally and gently lowered to the sediment surface. This technique minimized disruption of the SWI. Two samples were taken from each sample site, with the second sample collected approximately 10 m from the first as measured via GPS. This minimized sediment resuspension of the first sample affecting the second sample. This sampling

Table 2 Morphometric characteristics of Lake Belton and corresponding average depth of each sampling site located within the reservoir

Location	31°07' N, 97°29' W
Surface area (km^2)	49.8
Volume ($\times 10^8 \text{ m}^3$)	5.45
Secchi depth (m)	1.2–2
Mean depth (m)	
Whole lake	10.9
Sampling site 1	13.7
Sampling site 2	16.4
Sampling site 3	20.5
Sampling site 4	22.8
Sampling site 5	25.8

Table 3 Physicochemical data and bacterial abundance at the sediment–water interface for all sampling sites and dates. Dates are classified based on mixing characteristics of the lake

Classification	Date	Site	Temperature (°C)	Dissolved oxygen (mg l ⁻¹)	Redox potential (mV)	Bacteria × 10 ⁶ ml ⁻¹
Late stratification	16-Oct-03	1	23.7	3.1	225	1.2
		2	17.1	0.6	276	1.0
		3	17.0	0.0	93	5.0
		4	15.5	0.0	38	1.9
		5	14.9	0.0	4	0.6
Autumnal overturn	6-Nov-03	1	21.1	3.0	296	1.5
		2	21.0	4.8	364	1.3
		3	21.1	5.5	219	3.0
		4	21.1	5.1	342	1.2
		5	18.6	1.2	103	2.5
Winter mixing	31-Jan-04	1	11.4	9.7	348	10.5
		2	11.3	10.0	347	5.0
		3	11.4	10.1	367	13.1
		4	11.4	10.6	392	4.0
		5	11.5	10.6	383	6.3
Winter mixing	12-Mar-04	1	11.8	7.6	384	1.4
		2	11.4	7.1	380	1.3
		3	11.2	7.7	394	1.0
		4	10.9	9.9	391	1.3
		5	10.9	8.6	383	2.2
Early stratification	6-May-04	1	19.4	8.0	217	1.1
		2	18.8	6.0	231	1.1
		3	17.9	6.9	409	0.7
		4	16.4	2.5	293	0.5
		5	15.1	1.5	255	0.6
Early stratification	1-Jul-04	1	25.8	4.4	381	1.2
		2	22.9	1.1	258	1.5
		3	21.0	0.4	186	1.0
		4	19.6	0.2	155	1.6
		5	19.4	0.0	120	2.5
Late stratification	9-Sep-04	1	27.0	2.9	230	1.7
		2	26.8	1.3	157	2.4
		3	23.7	0.8	108	1.9
		4	20.6	0.0	37	1.7
		5	20.1	0.0	15	1.2
Autumnal overturn	14-Oct-04	1	24.6	7.4	431	1.1
		2	24.6	7.2	405	1.5
		3	24.6	5.3	411	2.3
		4	24.6	6.8	415	2.2
		5	24.5	6.6	341	3.1

scheme was used to sample the benthic boundary layer. This layer is a component of the sediment–water interface, often defined as an area of high discontinuity in wet bulk density and high rates of sinking particles, thus differing from the water column and sediment column (Austen et al., 2002; Hulbert et al., 2002). Thus the samples were turbid, yet liquid in consistency. Equal volumes of the duplicate samples from each station (water with sediment particles) were pooled in a 300-ml dark dissolved oxygen bottle

and capped to maintain in situ dissolved oxygen concentration. The sampler was rinsed with 0.45- μ m filtered and/or deionized water between samples. Samples were held at collected temperature until returned to the laboratory. Samples were processed within 5 h of collection.

Laboratory analyses

Biolog EcoPlates (Biolog, Inc., Hayward, California, USA) were inoculated with 150 μ l of

pooled sample per well. A single EcoPlate was inoculated for each pooled sample, with one pooled sample per station. EcoPlates contain each of the 31 substrates in triplicate, thus allowing intraplate replication for each pooled sample. For samples with dissolved oxygen concentrations less than 0.2 mg l^{-1} , anaerobic inoculation, processing, and incubation techniques were used as outlined in Christian & Lind (2006).

Plates were incubated at 22°C and read at OD_{590} (i.e., absorption spectrum of the formazan precipitate) once daily for 5 days using a Biolog MicroStation 2 plate reader. About 22°C was chosen for the incubation temperature, not as an optimum, but to maintain consistency throughout the experiment. Varying incubation temperatures instead of a pre-selected incubation temperature may confound spatial and temporal CSUR variation with differences due to incubation temperature variation (Christian & Lind, 2006).

For each plate, mean OD_{590} for each carbon substrate at each reading time was corrected by subtracting the mean OD_{590} of the control (no substrate) wells at the same reading time. Subtraction of control wells eliminated color and turbidity effects due to varying amounts of sediment (clay) particles in the samples. The corrected OD_{590} for each substrate was plotted against time. Logistic bacterial growth rates were observed; therefore a 3-parameter sigmoidal curve was fitted to each substrate for each sample using Sigma Plot 2000. The rate of exponential growth determined by the curve was the CSUR metric (Lindstrom et al., 1998; Christian & Lind, 2006).

In addition to Biolog assays, aliquots of each pooled sample were preserved in formalin (2% final concentration) for total bacterial enumeration. The bacteria were stained with DAPI fluorochrome ($1 \mu\text{g ml}^{-1}$ final concentration), filtered onto $0.2\text{-}\mu\text{m}$ blackened polycarbonate filters, and viewed under UV excitation at $1,500\times$ magnifications. Total bacteria ml^{-1} were estimated by counting 20 fields or 300 bacteria per filter (Porter & Feig, 1980). For bacteria attached to clay particles, a correction factor of $2\times$ was applied (Lind & Dávalos-Lind, 1991).

Statistical analyses

Summary and univariate statistics (i.e., correlation/regression, One-way ANOVA, Student's *t*) were performed using JMP 5.0 and Microsoft Office Excel 2003. A significance level of $\alpha = 0.05$ was used. CANOCO 4.5 was used for all multivariate analyses. CanoDraw for Windows, a graphical analysis package included with CANOCO 4.5, was used for graphical analyses of the multivariate data.

The complete multivariate data set consisted of four subsets, each subset including all sites and dates corresponding to a specific season (mixing event). Each subset consisted of two parts: (1) response (dependent) variables (CSURs) for the 31 substrates (listed in columns) for each corresponding date and site (listed in rows); and (2) supplemental environmental (independent) variables (i.e., temperature, dissolved oxygen, redox potential, bacterial abundance) (columns) for each corresponding date and site (rows). This grouping by subset allowed individual analyses for each mixing event, and partitioned out the covariable effects of sample site. All CSUR data were centered to a mean of zero and standardized to unit variance before conducting the multivariate analyses.

Preliminary data analysis using detrended correspondence analysis (DCA) on the response (CSUR) data for each subset, detrended by segments, established first gradient lengths of 1.4–2.9 standard deviation units. Gradients less than 2.0 indicate relatively low beta diversity; hence these data should be subjected to linear ordination methods (e.g., principal components analysis) rather than unimodal ordination methods (e.g., correspondence analysis). However, gradients ranging from 2.0 to 4.0 work well with either ordination method (Lepš & Šmilauer, 2003). For consistency and comparison among subsets, principal components analysis (PCA) was used. This analysis extracted orthogonal linear combinations of variables (principal components) that corresponded to the maximum amount of variation in the subset. PCA produces as many principal components as there are variables. However, only significant components are retained for analyses (ter Braak & Šmilauer, 2002).

The environmental variables were projected onto the PCA axes a posteriori to assess the

amount of variation in the PCA data that could be attributed to the environmental variables. While this approach was indirect, it did not require stringent assumptions as does direct gradient analyses (e.g., Redundancy Analysis, Canonical Correspondence Analysis) and therefore precludes the use of significance tests (Grover & Chrzanowski, 2000).

Results

Seasonal carbon substrate utilization patterns

Figure 1(a–d) shows loadings of SWI bacterial CSURs onto the first two PCA axes for each

seasonal mixing event. Only utilizations that had greater than 50% fit (i.e., significant loadings) onto the two axes collectively were included for interpretation. Additionally, only the first two PCA axes were retained for interpretation as the other axes did not account for a significant percentage of the total variance as determined by application of the broken-stick model (Lepš & Šmilauer, 2003; Peres-Neto et al., 2003).

During early summer stratification, the first two PCA axes accounted for 68.2% of the total substrate utilization variance. Significant loadings were observed for 15 of the 31 substrates, all strongly positively associated with the first axis and weakly, but significantly associated with the

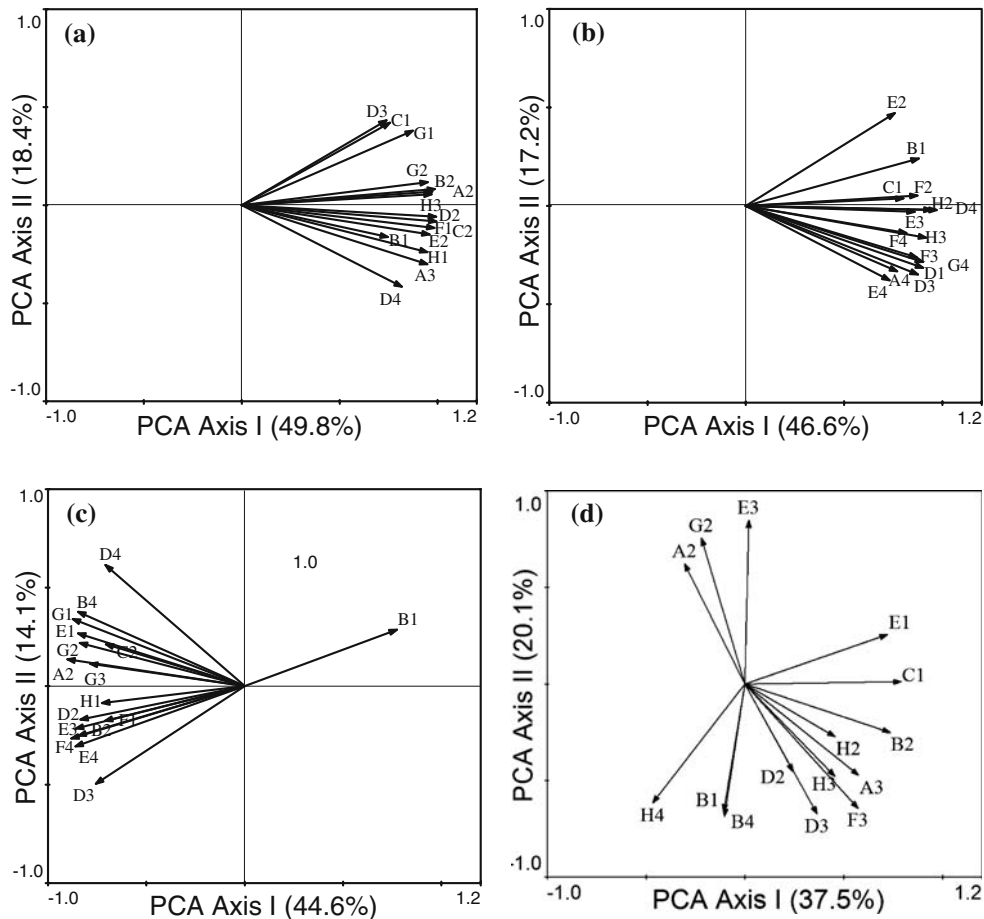


Fig. 1 (a–d) Principal components analyses showing loadings on principal components axes I and II for substrates that exhibited greater than 50% fit to the axes for early stratification (a), late stratification (b), autumnal overturn

(c), and winter mixing (d), respectively. Percent variance explained by each axis is given. Arrows connect at origin. Code numbers correspond to substrates given in Table 1

second axis (Fig. 1a). These loadings included 9 carbohydrates, 3 carboxylic acids, 2 polymers, and 1 amino acid. Hence, carbohydrates contributed the highest relative utilization by SWI bacteria during early stratification.

During late season (prolonged) stratification, measured during two consecutive years, the first two PCA axes accounted for 63.8% of the total substrate utilization variance. Significant loadings of 15 substrates were observed, all strongly positively loading onto the first axis and weakly onto the second axis (Fig. 1b). These loadings included 6 carboxylic acids, 4 amino acids, 2 polymers, 2 carbohydrates, and 1 amine. Opposite of early stratification, late season stratification was characterized by the high SWI bacterial utilization of amino acids and carboxylic acids and low utilization of carbohydrates.

At the onset of autumnal overturn, as measured during two consecutive years, significant loadings for 17 substrates were observed, all except pyruvic acid methyl ester explained by high negative loadings on PCA axis I and moderate loadings on axis II (Fig. 1c). The first two PCA axes accounted for 58.7% of the total variance. Substrates exhibiting significant loadings included 7 carbohydrates, 4 amino acids, 4 carboxylic acids, and 2 polymers. Because of the negative loadings on the first axis (which explained 44.6% of the substrate utilization variance), these substrates contributed little to SWI bacterial carbon substrate utilization during overturn, specifically most carbohydrates and amino acids.

During winter mixing, significant loadings were observed for 15 substrates. The first two PCA axes accounted for 57.6% of the total variance (Fig. 1d). These substrates exhibited diverse loadings on both PCA axes. Three carbohydrates and 2 polymers exhibited positive loadings on PCA axis I, while 2 carbohydrates and 1 carboxylic acid exhibited positive loadings on PCA axis II. Four carboxylic acids, and a single carbohydrate, amino acid, and amine display negative loadings on PCA axis II. These data indicate that during cold, mixing conditions carbohydrates and polymers contributed the highest utilization responses, yet trends were not as defined as during other seasons.

Carbon substrate utilization variation attributed to environmental variables

While only the first two PCA axes were significant for each seasonal data set, there are a total of as many PCA axes as there are substrates ($n = 31$), in total accounting for 100% of the substrate utilization variance. Of this total variance, linear combinations of measured environmental variables (i.e., bacterial abundance, SWI temperature, dissolved oxygen, redox potential) were fitted onto the PCA axes a posteriori to determine the percentage of total variance attributable to the environmental variables (Table 4). For each seasonal mixing event, environmental variables accounted for no less than 50% of the total variance, with the largest percentage accounted for during autumnal overturn (62.3%). Temperature was the largest attributable variable during early stratification (19.2%) and autumnal overturn (40%). The largest amount of variation accounted for by dissolved oxygen and redox potential individually was during autumnal overturn (32.4% and 30.8%, respectively) and the least during late stratification (7.3% and 11%, respectively). The relationship for dissolved oxygen and redox potential individually also held for the combined effect of dissolved oxygen and redox potential. Their combined effect is more ecologically interpretable than either variable individually due to their high correlation and codependence. The sum of the variances of the individual variables was greater than the total variance explained by the variables collectively due to significant correlations among several of the variables (Lepš & Šmilauer, 2003).

During late season stratification and winter mixing, bacterial abundance accounted for the largest proportion of the CSUR variance (20.5% and 24%, respectively), while during autumnal overturn it accounted for little variance (5.7%). A significant difference was observed among seasonal bacterial abundance (One-way ANOVA, $F_{3,36} = 4.36$, $P < 0.01$). However, the only pairwise difference was greater abundance during winter mixing than during early stratification (Tukey's HSD, $q = 2.69$, $\alpha = 0.05$). While higher bacterial abundance during winter mixing explained a high percentage of variance, this trend

Table 4 Percent variance in seasonal CSUR data explained by individual environmental variables and all variables collectively. Sum of percentages of individual

variables are greater than percentage of all variables collectively due to correlation of the individual variables

	Winter mixing	Early stratification	Autumnal overturn	Late stratification
All variables	49.8	54.0	62.3	62.1
Bacteria ml ⁻¹	24.0	14.4	5.7	20.5
Temperature (°C)	6.4	19.2	40.0	16.4
Dissolved oxygen (mg l ⁻¹)	15.1	14.3	32.4	7.3
Redox potential (mV)	16.1	14.6	30.8	11.0
Oxygen * redox	13.6	19.9	38.6	6.7

did not hold for all seasons. There was no significant difference between bacterial abundance during late stratification and autumnal overturn (Student's $t_{0.05,9} = 0.06$, $P = 0.81$), yet variance explained by bacterial abundance was 20.5% and 5.7%, respectively.

Carbon substrate utilization and environmental variable correlations

Because a large percentage of variance in SWI bacterial substrate utilization was explained by the measured environmental variables, we explored relationships among those environmental variables and individual substrates (Table 5). These correlations were derived from data of all seasons inclusive. Six significant correlations occurred for temperature, all of which were negative. These

included 3 carboxylic acids, 1 amino acid, and both amines. For these substrates, all exhibited higher utilization as SWI temperature decreased. Six significant correlations also occurred for bacterial abundance, however these correlations were positive. These included 1 amino acid, 1 carbohydrate, 2 carboxylic acids, and both amines. For dissolved oxygen, two positive significant correlations occurred, 1 amino acid and 1 amine. No significant correlations occurred for redox potential alone, but 3 significant positive correlations occurred for the combined effect of dissolved oxygen and redox potential. These included 1 amino acid, 1 carboxylic acid, and 1 amine. Only L-asparagine (amino acid) and putrescine (amine) utilization showed significant correlation with every environmental variable, indicating strong utilization of these substrates by all seasonal SWI bacterial communities.

Table 5 Pearson product–moment correlation coefficients between environmental variables and substrates among a total of 40 samples. Only significant correlations areshown. Data are inclusive of all seasons. Single, double, triple, and quadruple asterisks indicate P -values less than 0.05, 0.01, 0.001, and 0.0001, respectively

Temperature (°C)		Bacteria ml ⁻¹	
Substrate	Correlation coefficient (r)	Substrate	Correlation coefficient (r)
L-Asparagine	-0.46**	L-Asparagine	+0.52***
γ -Hydroxybutyric acid	-0.33*	N-Acetyl-D-glucosamine	+0.33*
D-Glucosaminic acid	-0.46**	Itaconic acid	+0.43**
Itaconic acid	-0.58****	Pyruvic acid methyl ester	+0.33*
Phenylethylamine	-0.48****	Phenylethylamine	+0.48**
Putrescine	-0.41**	Putrescine	+0.84****
Dissolved Oxygen (mg l ⁻¹)		Dissolved Oxygen \times Redox (mV)	
Substrate	Correlation coefficient (r)	Substrate	Correlation coefficient (r)
L-Asparagine	+0.41**	L-Asparagine	+0.39**
Putrescine	+0.46**	Pyruvic acid methyl ester	+0.30*
		Putrescine	+0.42***

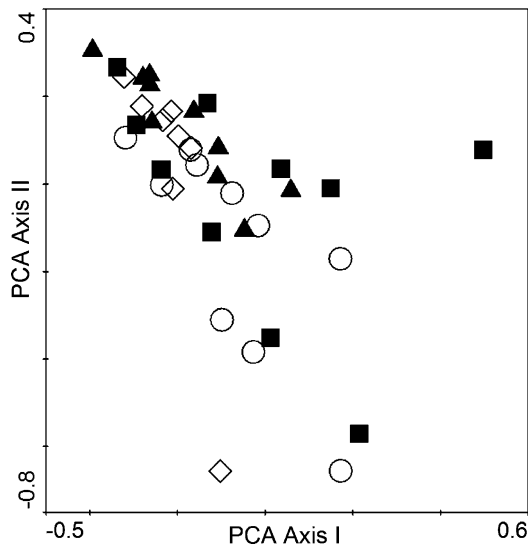


Fig. 2 Sample loadings (CLPPs) on PCA axes I and II for all seasons. Sample loadings are derived from principal components scores of all substrates. Symbols close together imply sample similarity. Graph is a composite overlay of four separate PCA graphs, therefore percent variance is not listed. Two samples from early stratification were removed from the graph, but not analyses, due to their extreme outlying values. ◇ = early stratification, ■ = late stratification, ▲ = autumnal overturn, ○ = winter mixing

Community-level physiological profiles

Figure 2 shows sample loadings for each sampling date and site (as classified by season) used in the study (Table 3) projected onto PCA axes I and II. Each sample point was derived from linear combinations of all substrate utilization scores for a specific site and date. The graph is a composite overlay of all seasons; therefore percent variance is not listed for the axes. The distance between points is proportional to similarity among individual samples (i.e., further apart is less similar). Highest similarity among samples occurred during early stratification, with all but one sample clustered together with low eigenvalues along PCA axis I and high eigenvalues along PCA axis II. The two furthest outliers from early stratification were excluded from the graph, but not from analyses, due to their extreme distance away from other samples. High similarity also occurred during autumnal overturn, even though these samples were collected during two different years. Lowest similarity was observed during late stratification, with a wide range of eigenvalues spread along PCA axes I

and II. Low similarity was also observed during winter mixing, explained primarily by PCA axis II.

Discussion

Organic carbon utilization by heterotrophic bacteria strongly influences internal nutrient cycling in freshwater ecosystems and impacts eutrophication processes (Sinsabaugh et al., 1997; Qu et al., 2005). Specifically, bacterially mediated sediment nutrient releases often input nutrients into the water column at greater quantities than allochthonous sources (Heinen & McManus, 2004; Song et al., 2004). Organic carbon utilized by heterotrophic bacteria is commonly classified into three groups: particulate organic carbon (POC), high molecular dissolved organic carbon (HM-DOC), and low molecular dissolved organic carbon (LM-DOC) (Wirtz, 2003). Our investigation specifically explored SWI bacterial utilization of the LM-DOC via the use of Biolog EcoPlates. CSUR data generated from the EcoPlates represents bacterial community functional potential and has been used to assess functional ability and changes in various environmental bacterial communities (Garland, 1997; Preston-Mafham et al., 2002). By considering the limitations of EcoPlates (i.e., inoculum density, incubation temperature, oxygen contamination) and modifying our protocol to avoid these limitations (i.e., using growth rate metrics, incubation temperature standardization, anaerobic techniques), results were robustly interpreted (Christian & Lind, 2006).

Seasonal carbon substrate use

Few explanations for preferential seasonal use of DOC substrates by freshwater (specifically, SWI) bacteria have been proposed (Pettine et al., 1999; Brugger et al., 2001) therefore this investigation served an exploratory, not confirmatory purpose. Future investigations (i.e., in situ chemical measurements; stable isotope analysis) into the presence or absence of these substrates at the SWI will aid the results obtained in this study. Most substrates in the Biolog EcoPlates were used at various extents during all seasons; however the first two PCA axes for each seasonal data subset shows only those substrates that contributed to

significant variation in increased and/or decreased substrate utilization. Therefore different seasonal patterns of SWI bacterial substrate use were elucidated from the PCA graphs (Fig. 1a–d).

During the onset of spring stratification, characterized by warming temperatures and depletion of dissolved oxygen and decreasing redox potential, SWI bacterial assemblages preferentially used carbohydrates over carboxylic acids, amino acids, and amines. As summer stratification progressed (i.e., late season stratification), indicated by warm temperatures and depleted dissolved oxygen and reduced redox potential, substrate preference shifted to amino acids, carboxylic acids, and amines with little use of carbohydrates. At the onset of autumnal overturn marked by decreasing temperatures, replenishment of dissolved oxygen, increasing (oxidized) redox potentials and presence of turbulent mixing, SWI bacteria decreased use of amino acids and continued little use of carbohydrates. Throughout winter mixing, SWI bacteria had high preference for carbohydrates and polymers.

Highest carbohydrate use during early stratification, along with high carbohydrate use during winter mixing may suggest that SWI bacterial communities prefer carbohydrate substrates under oxic conditions and pre-anoxia, under a wide variety of temperatures. Indeed, the preferred catabolic pathway of many aerobic and facultative heterotrophs involves oxidation of a simple or complex carbohydrate and oxygen as a terminal electron acceptor (Madigan et al., 1997; Rosenstock & Simon, 2003). In addition, fixed carbon in the form of carbohydrates synthesized via phytoplankton photosynthesis and carbohydrate-rich allochthonous organic matter is flushed in and sinks to sediments during lake mixing (Vreča, 2003; Heinen & McManus, 2004); hence conditions present during winter mixing may select for SWI bacterial populations that readily utilize carbohydrates. Lowest carbohydrate utilization during late season stratification and onset of autumnal overturn are possibly attributed to SWI carbohydrate depletion from lack of mixing combined with high hypolimnetic bacterial activity that oxidizes sinking carbohydrates before they can reach the sediments (Cole & Pace, 1995; Seiter et al., 2005).

Amino acids were preferentially used during late stratification. Amino acids were used to a

much lesser extent during autumnal overturn, winter mixing, and onset of stratification. Unlike carbohydrates and carboxylic acids, amino acids are nitrogen-rich (Madigan et al., 1997). Thus SWI bacteria may utilize amino acids as a nitrogen, in addition to a carbon, source by assimilating the ammonium side-chain (Pettine et al., 1999). In turn, the ammonium is incorporated into organic molecules such as other amino acids and proteins (Hollibaugh & Azam, 1983). High use of amino acids suggests that bacteria may be nitrogen deprived during late stratification, even though during this time ammonium is produced from bacterial nitrate reduction. Thus nitrogen deprivation at the SWI during late stratification may result from decreased sinking of nitrogen-rich organic matter due to lack of reservoir mixing (Hodell & Schelske, 1998). Because of the opposing catabolic and anabolic processes of amino acid utilization, the breakdown of these extracellular amino acids and incorporation into cellular amino acids may be performed by separate bacterial taxa in a synergistic interaction rather than both processes conducted simultaneously by the same taxon (Atlas & Bartha, 1998). In addition, previous studies on Lake Belton have indicated that larger bacteria are present during late summer stratification and their size is a function of anoxia (Christian et al., 2002); hence the larger bacteria may require a greater number of enzymes and proteins for metabolic functions, which can be synthesized via uptake of ammonium from amino acid breakdown in the environment.

Carboxylic acid utilization was greatest during late season stratification, but also had marked utilization during other seasons. Of the EcoPlate substrates, the carboxylic acids are the most diverse substrates in terms of molecular weight and chemical configuration. Little is known about free carboxylic (organic) acids in aquatic and sediment systems. Naturally occurring organic acids such as carboxylic acids are often a product of bacterial fatty acid catabolism, photochemical degradation of HM-DOC, or as an end product of fermentative metabolism, which occurs independent of dissolved oxygen and redox potential (Bertilsson & Tranvik, 2000; Ding & Sun, 2005). Studies at SWIs in marine systems have shown

that organic acids degrade more quickly under aerobic than anaerobic conditions and their concentrations are unrelated to bacterial abundance (Ding & Sun, 2005). However, our results show highest utilization of carboxylic acids during anaerobic, reducing conditions of late stratification. Two possible reasons for this phenomenon are suggested: (1) increased photoperiod during late stratification increases photodegradation of HM-DOC and POC in the photic zone, increasing the amount of organic acid-rich DOC that sinks to the SWI, possibly selecting for organic acid-utilizing bacteria (Tranvik et al., 1999); (2) fermentative bacteria may be more prevalent during anoxic conditions, which require the use of an organic compound, such as a carboxylic acid as an electron acceptor in a fermentative pathway (Madigan et al., 1997).

Selective pressures on SWI bacterial assemblages

We measured several SWI environmental (physicochemical and biological) variables that exhibited seasonal change, including: temperature, dissolved oxygen, redox potential, and bacterial abundance. We invoked Beijerinck's adage, "everything is everywhere, the environment selects" when assessing these variables affects on CSUR variance, hence the environmental selective pressures upon SWI bacterial assemblages. While the EcoPlates were themselves selective, the measured environmental variables collectively were associated with at least 50% of the total CSUR variance each season, thus were influential environmental selectors for the SWI bacterial assemblages (Table 4).

During seasons marked by high variance explained by bacterial abundance, not just abundance, but also, bacterial per-cell activity (i.e., ability to utilize substrate) may attribute to this variance. Recent studies on Lake Belton SWI bacterial communities showed that specific per-cell activity varies on a seasonal basis, supporting this evidence (Christian & Lind, 2007).

The SWI exhibited wide seasonal temperature variation. The highest amount of CSUR variance explained by temperature was during autumnal overturn, while the lowest was during winter

mixing. CSUR variance due to temperature differences was proportional to the temperature range exhibited during that season; with the large CSUR variance during autumnal overturn corresponding to a wide temperature range (18.6–24.6°C) and the small variance during winter mixing corresponding to a small temperature range (10.9–11.8°C). All plates were incubated at 22°C to maintain consistency throughout duration of the experiment (Christian & Lind, 2006). However this incubation temperature may have affected SWI bacterial growth rates, but presumably not pattern (Grover & Chrzanowski, 2000). Therefore CSUR variance due to temperature was a function of collected temperature as well as the normalized temperature of incubation (22°C).

At the SWI, dissolved oxygen depletion was coupled with lowered redox potential; therefore their effects upon CSUR variance are best understood when considered in tandem. The combined effect accounted for the largest percentage of CSUR variance during autumnal overturn. It also accounted for a large percentage of CSUR variance during early stratification. These seasons had the greatest ranges of dissolved oxygen and redox potential. Large redox potential changes are defined by bacteria that utilize various electron acceptors, implying tremendous changes in SWI bacterial community composition (Sweerts et al., 1991). Autumnal overturn and early stratification had the combined effect of dissolved oxygen and redox potential working in reverse for their respective seasons. Therefore it was not surprising that CSUR variance also showed an opposite relationship (Fig. 1a, c).

Individual substrate utilization and environmental variable correlations

We assessed individual substrates whose CSUR was significantly positively or negatively correlated with the individual physicochemical variables, all seasons inclusive. Carbohydrates showed strong utilization during two seasons marked by a wide variation in physicochemical variables (early stratification and winter mixing); therefore no individual carbohydrate CSUR was significantly correlated with any environmental

variable. Most significant correlations occurred with carboxylic acids (Table 5), possibly due to their highest utilization during a single season marked by similar physicochemical conditions (late stratification). Interestingly, two substrates were correlated with all measured environmental variables. These substrates were L-asparagine (amino acid) and putrescine (amine). Positive correlations of these substrates CSURs occurred with bacterial abundance, dissolved oxygen, and the combined effect of dissolved oxygen and redox potential. Negative correlations occurred with temperature. These two substrates are characterized by low C/N ratios. However, a significant trend was not observed with every low C/N ratio substrate. Yet a recent study utilizing EcoPlates demonstrated a preferential utilization of high nitrogen-containing substrates by bacteria in marine aquatic environments (Sala et al., 2006).

Community-level physiological profiles

In addition to individual CSURs, Biolog plates have been used to profile total bacterial community similarities (Mills & Garland, 2002). Applying this to our data (Fig. 2), highest SWI bacterial community similarities occurred during early stratification and autumnal overturn, while lowest similarity occurred during winter mixing and late stratification. High similarity in the autumnal overturn samples was unexpected because sampling events were conducted during two separate years. This evidence suggests possible similar SWI bacterial community succession occurs during overturn. However, late stratification samples were also collected during consecutive sampling years and exhibited high dissimilarity. Yet, some late stratification samples from the first year were more similar to samples collected during the second year than samples collected during the same year. Such seasonal succession in aquatic bacteria has been observed among differing temperate lakes (Grover & Chrzanowski, 2000).

Conclusions

This investigation explored differences among SWI bacterial CSURs, CLPPs, and several related

environmental variables throughout seasonal mixing, stratification, and anoxia in a eutrophic lake. Using Biolog EcoPlates, distinct seasonal shifts in CSURs were observed. Seasonal differences in SWI bacterial abundance, temperature, dissolved oxygen, and redox potential accounted for a large percentage of CSUR variance. Preferential use of amino acids during late summer stratification suggests seasonal SWI nitrogen limitation. Seasonal similarity of CLPPs from two separate years suggest a possible predictable succession of SWI bacterial communities, a phenomenon still poorly understood among aquatic bacterial communities. While many steadfast conclusions cannot be obtained from Biolog assays, these data contribute to the ever expanding knowledge of carbon cycling in aquatic ecosystems and relays the importance of SWIs and their associated biota to the understanding of lake ecosystem nutrient dynamics and processes. Future investigation into reservoir SWI bacterial dynamics can integrate our findings into developing specific carbon cycling models and establishing in situ carbon measurements utilizing a variety of other tools.

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