Effects of changing salinity on the lipid composition of *Salinivibrio costicola* and potential applications as a salinity indicator

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Abstract

Throughout the world the salinity of lakes and lagoons is fundamentally related to the relative rates of precipitation and evaporation. Salinity proxies have recently been applied to reconstruct hydrologic changes in the geologic past from sediment cores taken in the ocean and in lakes. Here a new proxy for salinity based on changes to the lipid composition of *Salinivibrio costicola* is proposed. Analysis by column and gas chromatography and mass spectrometry of the lipid composition of *S. costicola* grown at a range of NaCl concentrations from 26 to 112 ppt reveal several ratios of lipid quantities that correlate well with salinity. R values up to 0.96 demonstrate a positive correlation between the ratios of some lipids and NaCl concentration. This suggests that some lipid ratios may describe salinity well enough to serve as a proxy for salinity.

Introduction

The D/H (deuterium/ hydrogen) and δ^{18}O salinity proxies have been successfully applied to paleoclimate reconstructions in regions where precipitation patterns constitute a major feature of climate variability (Sachs et al. 2007). During dry periods evaporation exceeds precipitation and salinity increases in lakes and lagoons. During wet periods precipitation exceeds evaporation and salinity decreases in these bodies of water. In the case of the D/H proxy these changes in salinity are recorded through physiological processes by the microorganisms which inhabit lakes and lagoons. When these organisms die, they become entrained within sediment, recording salinity at the time of death. A sediment core removed from a lake or lagoon can be analyzed for selected proxies, and wet and dry periods reconstructed. However proxies are often influenced by factors other than the target parameter (Fritz 2007). To avoid systematic errors during a climate reconstruction it is necessary to use multiple proxies. The development of supplemental proxies is therefore of great importance for accurate paleoclimate reconstructions.

Salinivibrio costicola, a moderately halophilic γ-Protobacteria (Mellado et al. 1996), is an ideal organism for studying a potential lipid indicator of salinity. This organism is ubiquitous in saline and hypersaline environments; it has been widely reported in North American (Haws 2005; Litzer and Caton 2006) and European (Romano et al. 2005; Sanchez-Porro et al. 2002) saline environments, as well as the marine environment (Franklin et al. 2005; Thompson and Polz 2006). Despite its ubiquitous nature

Salinivibrio is thought to be present only in low levels in these environments compared to the total microbial community (Thompson and Polz 2006).

The physiology of *Salinivibrio* has been described (Ventosa et al. 1998), although an investigation into the effects of salinity on the membrane lipids of this organism appears to be lacking. Such studies have been carried out on other bacteria (Haque and Russell 2004; Huflejt et al. 1990; Valderrama et al. 1998) and on algae (Xu et al. 1998), yeast (Prista et al. 1997; Turk et al. 2007), and Archaea (Asker et al. 2002; Tenchov et al. 2006). All studies point to significant changes in lipid composition with changing salinity. These changes allow halotolerant and halophilic organisms to resist the greater ionic strength and osmotic stress that comes with higher salinity (Valderrama et al. 1998).

Here a new proxy for salinity is proposed, based on a ratio of select lipids produced by *S. costicola* isolated from sediment obtained at the southern portion of the Great Salt Lake of Utah. This proxy would be analogous to the TEX_{86} proxy for temperature based on Crenarchaeotal membrane lipids (Brassell et al. 1986; Powers et al. 2004; Schouten et al. 2002; Schouten et al. 2003).

Methods

Culturing and isolation methods

Sediment was sampled from above the waterline but below the annual high water mark at the southern end of the Great Salt Lake in June, 2007 at 41° 4'46.58" N 112° 12'17.39" W. The sediment was immediately placed in a cooler on dry ice, and stored at -20° C within 48 hours. After 6 months a portion of the sediment was removed from the bag with a sterile spatula and placed in 10 mL of sterile $NaCl/Na_2SO_4$ solution (60g each in 1 L H_2O) in a sterile culture tube. The culture tube was vortexed and allowed to incubate for 24 hours. At that time 200 µL of the liquid was transferred with a pipette to a plate containing solid media (Table 1).

Colonies were isolated with respect to morphology by streaking onto fresh agar. Isolation was verified by cell morphology using epifluorescent microscopy on cells stained with DAPI and Acridine Orange. After a series of five streaks the fastest growing culture was selected for large scale culturing, 16S rRNA identification, and lipid analysis. Large volume cultures were obtained by growing the isolate in 600 mL of liquid media (identical to the media described in Table 1, but without agar) at NaCl concentrations of 26, 51, 75, 98, and 112 ppt (weight: weight) within a covered, sterilized 1 L glass media bottle. A sterilized magnetic stir bar and stirrer were used to keep the bottles continuously stirred at 200 rpm. Growth rates were monitored using a Beckman Coulter DU 800 spectrophotometer to measure transmittance at 550 nm. While still in the exponential growth phase cells were harvested by filtering onto preweighed Whatman 47 mm GF/F filters with a 0.7 µm particle retention. A maximum of 60 mL of culture was passed through each filter to prevent cracking of the filter or lysing of the cells. Each 600 mL culture produced 6 filters, these filters were paired for the remainder of the analysis allowing for analysis in triplicate. After harvest the filters were placed in combusted glass sample jars and stored at -20° C. For a blank, 60 mL of media were filtered and analyzed along with the samples.

Lipid extraction and analysis methods

Once frozen the filters were freeze-dried at 16 Pa and -45° C for 24 hours. Filters were weighed and lipid material extracted with dichloromethane at 1500 psi and 100° C using a Dionex ASE 200 (Accelerated Solvent Extractor). Extraction cells were packed with 1 g Na_2SO_4 and combusted quartz. Following extraction the solvent was removed from the vials by evaporation with nitrogen. The lipid

Table 1. Media composition for the isolation of *Salinivibrio costicola*. This media was suitable for the rapid growth of *S. costicola* and numerous other aerobic halotolerant or halophilic organisms from the Great Salt Lake. These organisms have not been identified, but their colonies are morphologically distinct from *S. costicola*. Media for the large scale cultivation of *S. costicola* was identical, except no agar was used and the NaCl concentrations were manipulated to 26, 51, 75, 98, and 112 ppt (weight: weight).

Component	Quantity in 1 L
NaCl	60 g
Na_2SO_4	60 g
Agar	10 g
$MgSO_4 \cdot 7H_2O$	10 g
Casein Hydrolysate	5 g
KCl	5 g
KNO_3	1 g
Yeast Extract	1 g
$CaCl_2 \cdot 6H_2O$	.2 g
Beef Extract	3 g
Peptone	5 g
Glucose	5 g

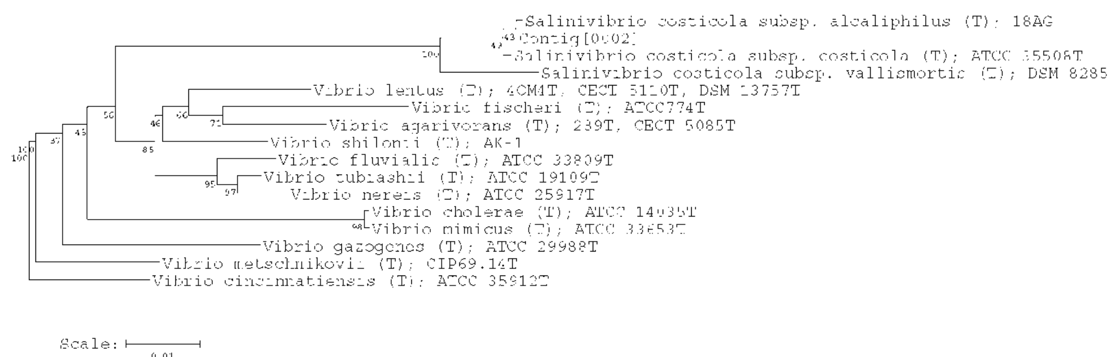

Figure 1: Phylogenetic tree of isolate and nearest matches. The isolate (Contig[0002]) was placed in a tree using the RDP Tree Builder tool (Cole et al. 2007). Vibrio cencinnatiensis was selected as an outgroup. The bootstrap value of 100 places the isolate within S. costicola.

material was stored at 40° C for further analysis.

Column chromatography using pipettes filled with 0.9 g 5% deactivated silica of mesh size 70-230 was used to separate the lipids into three fractions by polarity. The first fraction was eluted with hexane, the second with ethyl acetate, and the third with methanol. Each fraction was transferred to a GC (gas chromatography) vial equipped with a combusted 250 µL insert and all solvent was evaporated with nitrogen. The lipid material was re-suspended in 20 µL anhydrous toluene and 500 ng of C_{36} quantification standard was added. Immediately prior to analyzing by gas chromatography each fraction was silylated with 10 µL BSTFA+TMCS (N,O-bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane) and 10 µL pyridine. Care was taken to limit the exposure of BSTFA+TMCS to air. After the addition of

the silylating agents samples were vortexed and heated at 60° C for 60 min.

10 µL of each sample (20% total material) was injected on an Agilent 6890N GC coupled to a 7360B series FID (flame ionization detector). A J+W Scientific 19091J-413 HP-5 column of 30 m length, inner diameter 0.32 mm, and 0.25 µm film thickness was used. Anhydrous toluene was injected periodically throughout the sample run to insure that the instrument was running properly. Peaks of interest on the chromatogram were identified using an Agilent 6890N GC coupled to a 5975 inert mass selection detector and equipped with a 7360B series injector. The same model of column was used for both gas chromatographers.

All quantifiable peaks in the ethyl acetate and methanol fractions that appeared in four out of five sample sets were manually integrated using the Agilent Chemstation integration tool. Lipids eluted in the methanol fraction are identified by a '3' in their designator. For lipids from the ethyl acetate fraction all possible ratios were investigated for a correlation with NaCl concentration. For lipids in the methanol fraction only lipids with a Pearson Correlation Coefficient (R) value greater than 0.90 when quantities of the first sample set were plotted against the concentration of NaCl were placed in ratio. Statistically significant correlations were determined by consulting a statistical table (Triola 2007). For lipids eluted in the methanol fraction significance was determined at P=0.01 and 12 df, even when more than 14 samples were present within the sample set. Sample sets were larger for lipids eluted in the ethyl acetate fraction, resulting in the assignment of 25 df for the determination of statistical significance (P=0.01).

16S RNA identification

An agar plate containing pure colonies of the isolate was submitted to Laragen Inc. of Las Angeles, CA for extraction, amplification, and sequencing. A total of 985 base pairs were used for identification with the RDP (Ribosomal Database Project) (Cole et al. 2007) following alignment and trimming of forward and reverse sequencing runs. A phylogenetic tree of the isolate, several strains of *S. costicola*, and related *Vibrios* was created using the online RDP alignment and tree building tools (Fig. 1).

Results

Lipid Analysis

Within the lipid fraction eluted with ethyl acetate 8 quantifiable peaks were identified. All 28 possible two lipid ratios were analyzed. R values (Pearson Correlation Coefficient) for a linear regression between each ratio and NaCl concentration ranged from 0.61 (ratio A/C as illustrated in Fig. 2) to 0 as shown in Table 2. Averaged values for the ratio producing the only statistically significant correlation with salinity (A/C) produced an R value of 0.94 as shown in Fig. 2.

The fraction eluted with methanol produced 50 quantifiable peaks, 29 showed a statistically significant correlation ($R>0.96$ for $P=0.01$ and $df=3$) with salinity within the first sample set and were analyzed in two additional sample sets. All 403 possible two lipid ratios were analyzed. R values between each ratio and NaCl concentration ranged from 0.78 to 3.87×10^{-4} as shown in Table 3. Averaging all available values of 3AB/3AY returned the highest correlation with NaCl concentration of any calculated ratio, with an R value of 0.96 as shown in Fig. 3. Averaged ratios of the next six highest R values are shown in Fig. 4. Pearson correlation coefficients between these ratios and NaCl concentration range from 0.78 to 0.74. The six highest unaveraged correlations are shown in Fig. 5. Retention times for lipids A, C, 3K, 3M, 3R, 3U, 3V, 3AB, 3AK, 3AR, 3AW, and 3AY are shown in Table 4.

Table 2. R values for potential lipid ratios of all quantifiable peaks from the fraction eluted with ethyl acetate. Ratio numerators are shown at left, denominators across the top. The strongest correlation with NaCl concentration was found in the ratio of lipids A and C, shown in bold.

	B	C	E	F	I	J	K
A	-0.47	**-0.61**	-0.37	-0.38	-0.09	-0.33	-0.11
B		-0.47	-0.06	0	0	-0.36	-0.04
C			0.17	0.41	0.27	-0.17	0.23
E				0.01	0.06	-0.39	0.01
F					0.11	-0.37	0.07
I						-0.44	-0.4
J							-0.03

Table 3. R values for lipid ratios of all quantifiable peaks eluted with methanol against NaCl concentration. Ratio numerators are shown at left, denominators across the top. Ratios with an R value greater than |.66| are shown in bold. The lipid 3K can be seen to dominate the stronger correlations.

	3C	3D	3F	3I	3K	3L	3M	3N	3O	3R	3U	3V	3W	3AB	3AC	3AF	3AH	3AI	3AJ	3AK	3AL	3AP	3AQ	3AR	3AT	3AW	3AX	3AY
3A	-0.15	-0.34	0.02	0.4	**0.74**	0.17	0.59	0.12	0.02	-0.2	0.04	-0.09	-0.12	0.08	-0.05	-0.02	0.15	0.13	-0.09	0.09	-0.04	0.24	0.15	0.25	0.07	0	0.01	-0.24
3C		-0.32	0.07	0.16	**0.75**	-0.09	0.62	0.14	-0.01	-0.15	-0.06	-0.1	-.02	0.12	-0.07	-0.03	0	0.05	-0.33	-0.02	-0.09	0.27	-0.07	0.33	0.16	-0.01	-0.02	-0.27
3D			0.46	0.64	**0.74**	.21	0.65	0.41	0.33	0.37	0.46	0.29	0.15	0.55	0.19	0.39	0.46	0.41	0.06	0.27	0.41	0.45	0.26	0.44	0.45	0.3	0.21	0.11
3F				0.31	**0.7**	-0.05	0.62	0.02	-0.14	-0.43	-0.24	-0.21	-0.16	-0.07	-0.21	-0.17	-0.03	-0.04	-0.29	0.01	-0.24	0.19	-0.07	0.22	-0.03	-0.06	-0.15	0.21
3I					**0.78**	-0.22	**0.66**	-0.08	-0.3	-0.58	-0.23	-0.22	-0.16	0.21	-0.15	-0.06	0.31	-0.15	-0.21	-0.2	-0.23	0	0.04	0.03	-0.05	-0.33	-0.21	-0.55
3K						**-0.68**	-0.39	**-0.73**	**-0.72**	**-0.75**	**-0.73**	**-0.69**	**-0.66**	-0.61	**-0.71**	**-0.69**	-0.57	**-0.72**	-0.55	**-0.73**	**-0.72**	**-0.71**	-0.54	**-0.75**	**-0.67**	**-0.73**	**-0.73**	**-0.74**
3L							0.57	0.09	-0.01	-0.21	0.11	-0.07	0.09	0.4	0.05	0.03	0.19	0.01	-0.18	-0.15	-0.01	0.07	0.13	0.16	0.14	-0.21	-0.07	-0.29
3M								-0.51	-0.51	-0.54	-0.52	-0.49	-0.55	-0.46	-0.53	-0.5	-.32	-0.53	-0.44	-0.56	-0.53	-0.53	-0.42	-0.52	-0.53	-0.58	-0.55	-0.59
3N									-0.54	-0.29	0	-0.12	-0.11	0.31	-0.27	0.07	-.36	0.01	-0.04	0	-0.06	0.15	0.2	0.21	0.05	-0.1	-0.16	-0.28
3O										-0.15	0.21	0.08	-0.02	0.45	-0.01	0.23	.41	0.21	-0.01	0.09	0.15	0.29	0.22	0.34	0.22	0.01	0	-0.19
3R											0.27	0	-0.09	0.24	0.03	0.1	0.27	0.34	-0.03	0.17	0.14	0.38	0.22	0.39	0.25	0.14	0.11	-0.08
3U												-0.18	-0.15	0.12	-0.17	-0.07	0.18	0	-0.05	-0.02	-0.18	0.18	0.2	0.22	0.02	-0.08	-0.18	-0.3
3V													0.16	0.3	0	0.15	0.27	0.05	-0.03	0.02	0.06	0.17	0.18	0.23	0.15	0.01	-0.07	-0.09
3W														0.29	0.01	0.04	0	-0.07	-0.58	-0.05	0	-0.02	-0.29	0.01	0.12	0.01	-0.14	-0.04
3AC															-0.22	-0.24	-0.05	-0.28	-0.11	-0.19	-0.25	-0.17	0.08	-0.14	-0.2	-0.17	-0.34	-0.31
3AB																0.09	0.27	0.2	0.01	0.12	0.1	0.32	0.23	0.32	0.22	0.07	0.04	-0.09
3AF																	0.43	-0.07	-0.08	-0.05	-0.06	0.05	0.13	0.09	0.07	-0.05	-0.17	-0.17
3AH																		-0.34	0	-0.31	-0.28	-0.26	0.07	-0.19	-0.21	-0.31	-0.36	-0.33
3AI																			-0.07	-0.03	-0.16	0.28	0.17	0.24	0.07	-0.16	-0.26	-0.42
3AJ																				-0.14	-0.13	-0.09	0.6	-0.07	-0.11	-0.18	-0.15	-0.2
3AK																					0.06	0.2	0.15	0.2	0.13	-0.04	0	-0.39
3AL																						0.41	0.12	0.32	0.2	-0.01	-0.2	-0.21
3AP																							0.12	0.1	-0.12	-0.28	-0.47	-0.59
3AQ																								-0.26	-0.3	-0.33	-0.34	-0.36
3AR																									-0.13	-0.24	-0.37	-0.46
3AT																										-0.08	-0.27	-0.3
3AW																											0.01	-0.22
3AX																												-0.27

16S RNA identification

Analysis of the 16S rRNA sequence indicate a close relationship with *Salinivibrio costicola* subsp. *alcaliphilus* with an S_ab score of 0.994. The S_ab score is the number of oligomers shared between the test strain and the database strain divided by the lowest number of oligomers in either strain (Cole et al. 2007). The isolated strain of *S. costicola* also matched closely with *Salinivibrio costicola* subsp. *costicola* with an S_ab score of 0.994. Bootstrap value for placement within *S. costicola* is 100.

Discussion

Analysis of the lipid composition of *S. costicola* across a wide range of salinity suggests some potential for a lipid indicator of salinity. A

Table 4. Identifier and retention time on the GC-FID for each identified lipid. The 3 in the designator indicates that the lipid was eluted in the third (methanol) fraction during column chromatography.

Identifier	Retention Time (min)
A	14.48
C	21.1
3K	16.95
3M	17.22
3R	17.93
3U	18.51
3V	18.77
3AB	20.16
3AK	21.97
3AR	24.37
3AW	27.15
3AY	29.17

total of 25 statistically significant correlations between lipid ratios and salinity were found, with R values exceeding the threshold for significance of 0.661 for the methanol fraction and 0.479 for the ethyl acetate fraction. All but one of these ratios is derived from lipids eluted in the methanol fraction. This is not surprising, as the effect of salinity on polar lipids has been previously reported (Asker et al. 2002).

Within the ethyl acetate fraction (lipids of intermediate polarity) the single statistically significant correlation results from the ratio of lipids A and C. Fig. 2 shows the averaged and unaveraged values for this ratio. The wide scatter in unaveraged values is typical of the calculated ratios. It is unknown at this time whether this is an artifact of column or gas chromatography, or manual integration. Two separate cultures are represented in the A/C sample set, but these ratios from both cultures are equally dispersed across the range of values. A similar effect can be seen within the methanol fraction (Fig. 4), despite the fact that only samples from one single culture were used. Since each culture was homogenous, the variance in relative lipid quantities must result from some process downstream of the culture. This variation does present obvious challenges to the application of such a ratio to determining salinity.

When the ratios are averaged, very strong correlations with NaCl concentration result. In the case of A/C an R value of -0.95 is reached (Fig. 2). The ratio of 3AB/3AY produces an even better correlation, with an R value of -0.96 (Fig. 3). This is a strong correlation, equivalent to that of currently used paleoclimate proxies including TEX_{86}. In some cases the TEX_{86} proxy correlates with temperature at an R^2 value of 0.92 (Powers et al. 2004), corresponding to an R value of 0.96. Further correlations between averaged lipid ratios and NaCl concentration are shown in Fig. 5. In each of these cases there is a single outlier at 51 ppt concentration of NaCl disrupting the correlation. Removal of the outlier results in R values near 0.99 in all cases. Considerable further testing will determine whether averaged values of these ratios hold their correlation with NaCl concentration. If this is the case, then multiple gas and column chromatography runs averaged together from the same environmental sample should accurately represent salinity. However this would suggest that the scattering of data

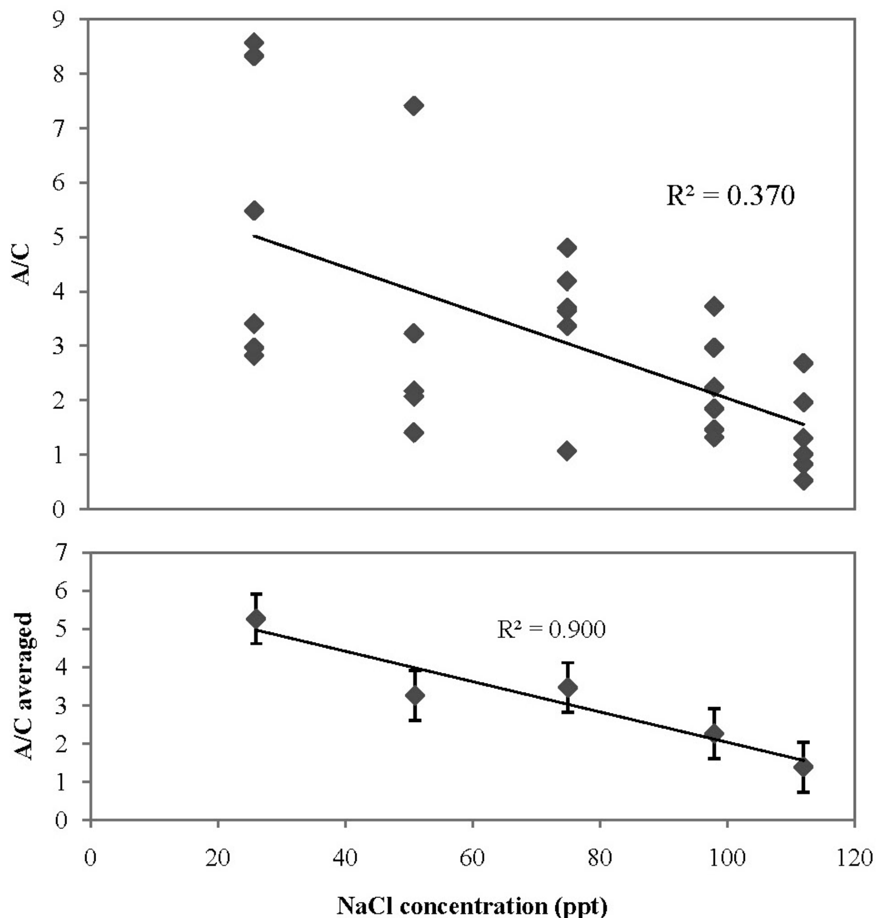

Fig. 2. The ratio of lipids A and C. Two sets of cultures are shown. A relatively poor correlation between the ratio and the concentration of NaCl can be seen for the unaveraged values. Error bars for the averaged values show standard error.

points must result from random error produced during analysis. A rigorous examination of experimental methods would then be necessary to identify the source of this random error.

Currently, attempts to identify the compounds of interest listed in Table 2 have been unsuccessful as a result of a poor noise to signal ratio caused by lipid material within the culture media. These interfering lipids also confuse the effect of salinity on the quantities of the lipids discussed here. Chromatogram peaks corresponding to lipids derived from components of the culture media obscure most of the peaks of interest derived from *S. costicola*. Although an effort was made to subtract peaks corresponding to media lipids from each sample chromatogram, it was not possible to do this with any degree of confidence. As a result the media was taken as a constant, and shifts in peak area with changing salinity were attributed to *S. costicola* lipids underlying the larger peaks of media lipids. It will be necessary to develop a lipid free media for the culture of *S. costicola* to verify the changes in lipid composition reported here, and to correctly identify compounds of interest.

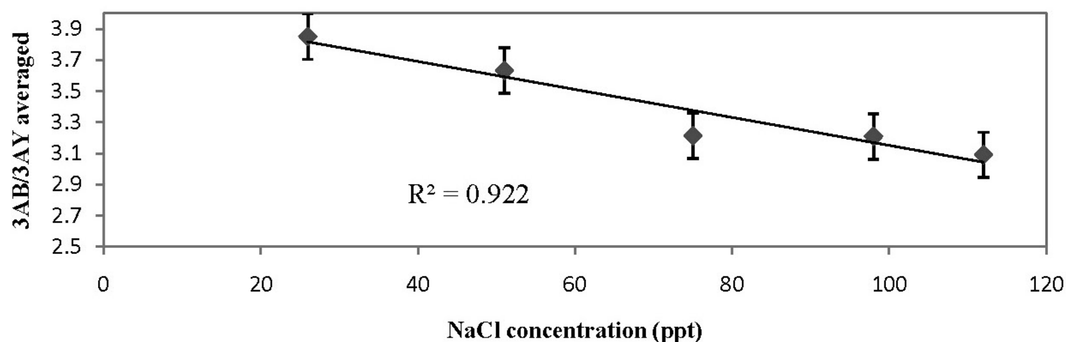

Fig. 3. The average ratios of lipids 3AB and 3AY. All triplicate repeat ratios from a single culture set were averaged and the average values plotted against NaCl concentration. The strong correlation with NaCl concentration can be seen. Error bars indicate standard error.

Conclusions

Lipids contained within *S. costicola* shift in composition as the concentration of NaCl changes. For some lipids, this shift is predictable enough that a ratio of the quantity of one lipid to another can be used to estimate NaCl concentration in culture. These ratios have potential applications as paleoclimate proxies for salinity, although considerable work remains to correctly identify these lipids and to verify their relationship with NaCl concentration.

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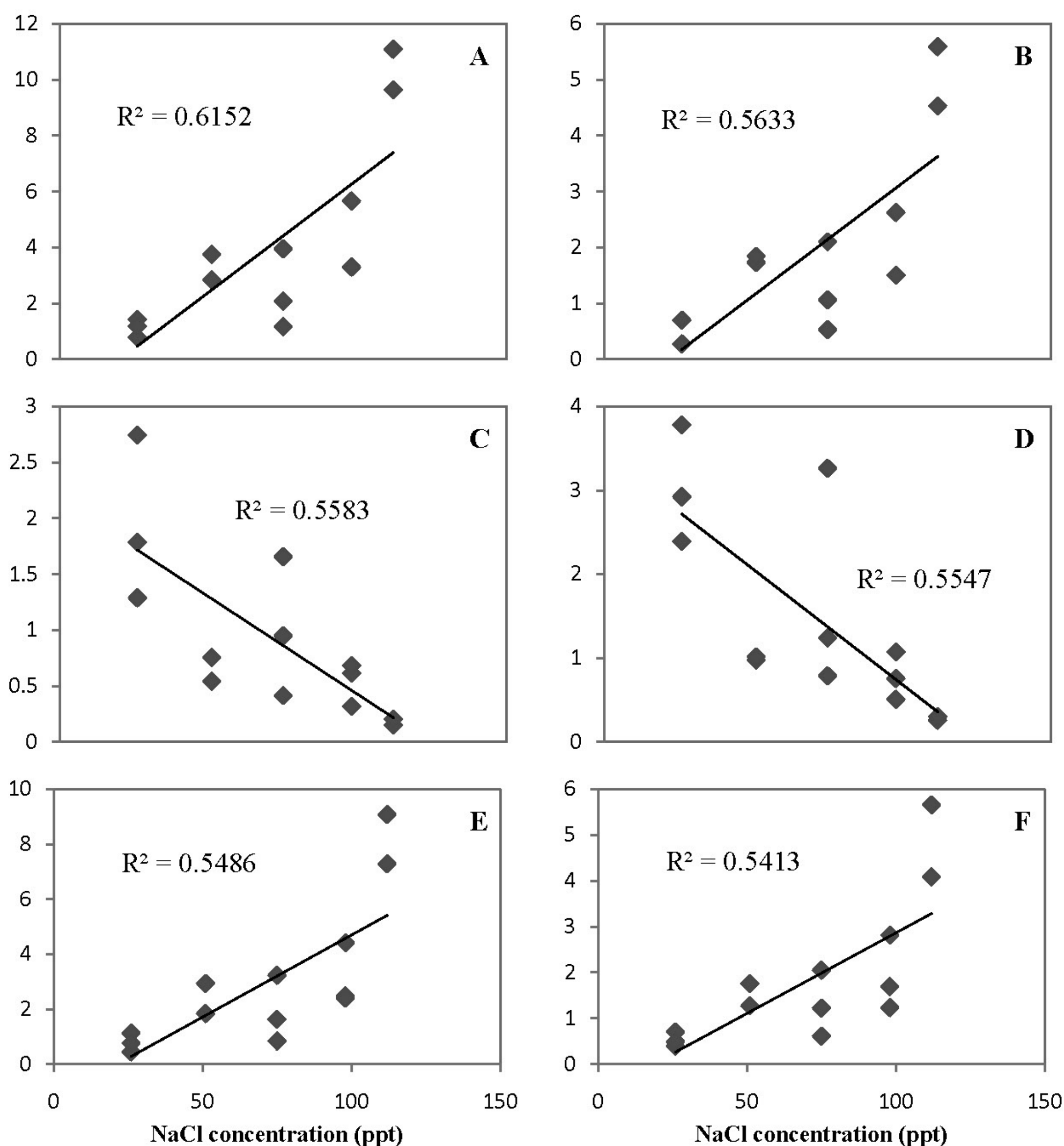

Fig. 4. Best correlations between NaCl concentration and ratios of lipids within the fraction eluted with methanol. A) 3I/3K B) 3C/3K C) 3K/3R D) 3K/3AY E) 3D/3K F) 3A/3K.

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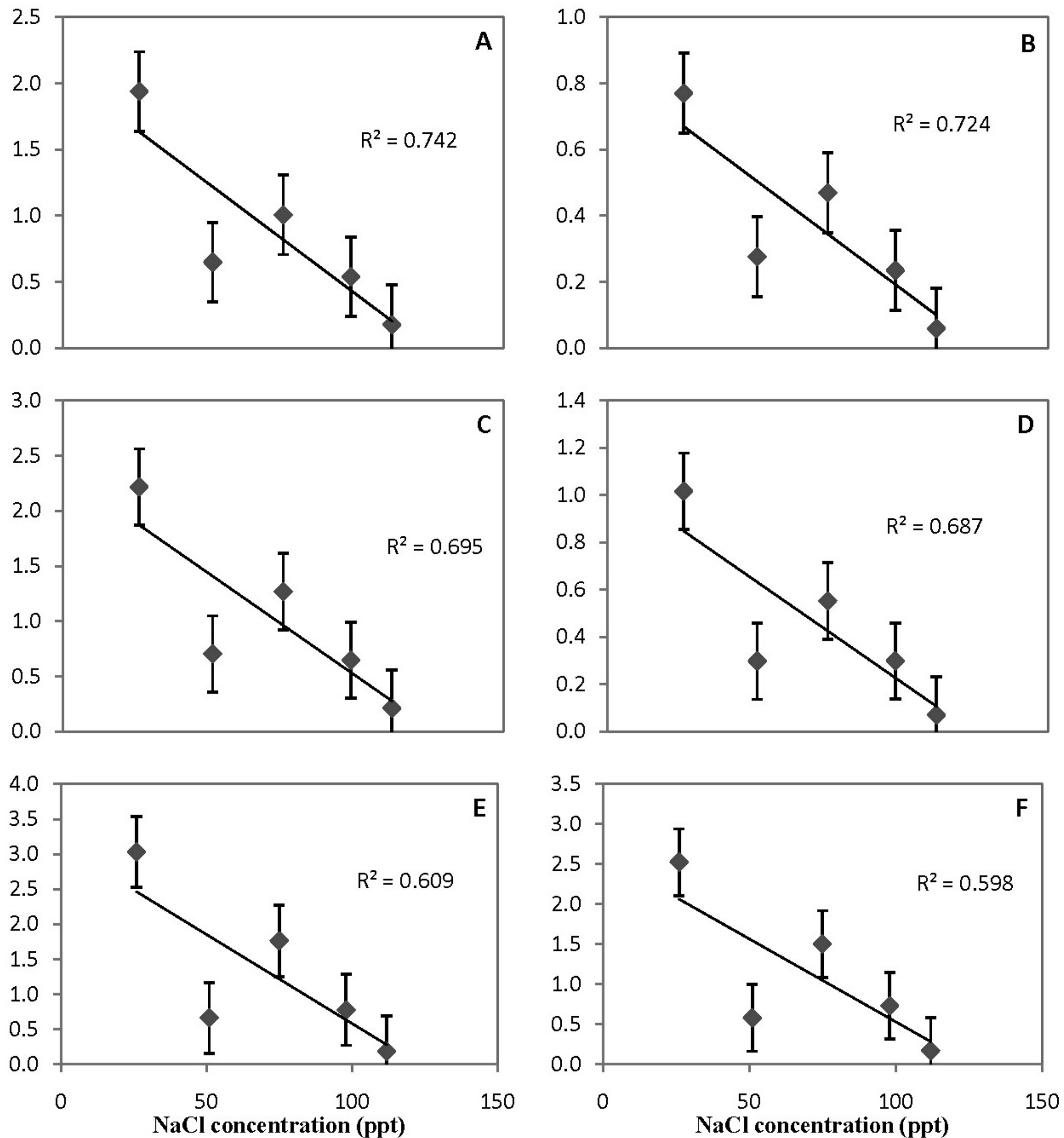

Fig. 5. Best correlations between NaCl concentration and averaged ratios of lipids within the fraction eluted with methanol. In all cases the 51 ppt data point is an outlier, falling well below the trend line. Removal of the 51 ppt data point results in R^2 values near .99 in all cases. Error bars show standard error. A) 3K/3R B) 3K/3AK C) 3K/3U D) 3K/3AR E) 3K/3AY F) 3K/3AW.

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