TEMPORAL AND SALINITY IMPACTS ON THE MICROBIAL DIVERSITY AT THE EILAT, ISRAEL, SOLAR SALT PLANT

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EXTENDED ABSTRACT

A major solar salt works in Israel is located just north of the town of Eilat and close to the airport. This solar salt works has been in operation since 1977 and produces about 170,000 tons a year. A three-year study was initiated to examine the temporal and salinity impacts on the microbial community throughout this saltern. At the time this study was begun in the late 1990's, there were several paradigms about the microbial community:
1. there is greatly reduced diversity as the water reaches saturation;
2. because of the relative stability of the salt concentrations in the various pans, there is little change in the microbial community; and,
3. halophilic *Archaea* are not found in ponds with less than 15% salt and halophilic *Bacteria* would not be found in pans at saturation.
The first paradigm was developed based on a few studies using one medium and single observations of various salterns. The second paradigm was an assumption that had not been systematically tested, and the third paradigm, at least for the *Bacteria*, was shown in 1980 not to be true with the discovery of the genus *Halomonas*.
We examined these paradigms by systematically studying on a seasonal basis the saltern at Eilat, Israel, as well as the Cargill Solar Salt Plant in Newark, California. Both traditional and molecular techniques have been used throughout these studies.
Several different approaches were taken: plate counts were made on several different media, whole community carbon requirements were determined, molecular fingerprinting of the whole microbial community using the amplicon length heterogeneity approach was performed to determine the changes in the community composition over time, and partial characterization of the isolated pure cultures were all performed. Samples were taken at least twice a year corresponding to the cooler and hotter times of the year.
Based on this extensive study there were significant differences in the plate counts and in the ratios of the various peaks found during the fingerprinting. It is safe to say now that the microbial community in the waters of a solar saltern is variable and both domains can be found throughout the saltworks.

Keywords: bacteria, fingerprinting, community structure, lipid analysis, carbon sources, extracellular enzymes

1.INTRODUCTION

Over the years many solar saltworks have been investigated for specific or unusual

microorganisms. This is especially true for the Santa Pola saltern in Alicante, Spain. From these studies, new genera (*Salinibacter*) [1] and a large variety of *Bacteria* and *Archaea* have been isolated showing the diversity of these systems [2,3,4], and the square bacterium, Walsby's squares [5], has been recognized but, until recently, not cultured [6,7]. None of these studies, however, have been a systematic seasonal examination of a saltern over several years. We report here the results of our three-year investigation of the Eilat, Israel saltern.

2.MATERIALS and METHODS

2.1 Sampling Locations and Dates

All samples were collected over a three-year period from the Eilat, Israel, saltworks. The locations, dates, temperature, and density and pH are listed in Table 1. The plan of the saltworks showing the exact locations of the samples as it was configured during this study is shown in Figure 1. Sampling occurred at the inlet (100-level pans), in the evaporators (200-level pans), and the crystallizers (300- level pans).

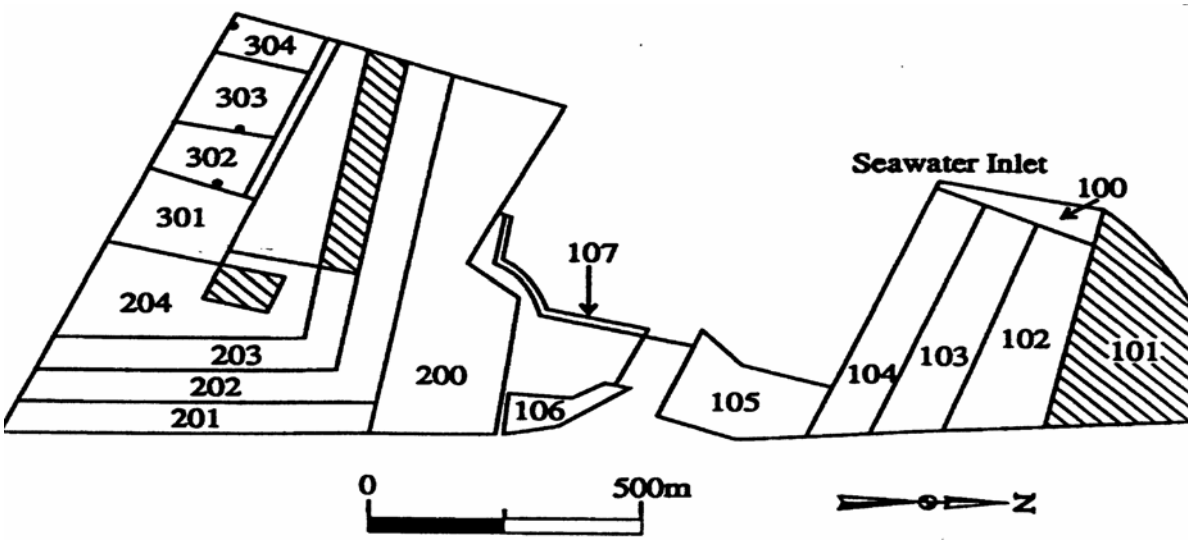

Figure 1: Plan of the Eilat, Israel saltworks

2.2 Isolation Media

Two types of media were used throughout this study. The first was R2A medium (Difco) which was modified to contain 20 g/L of $MgSO_4 \cdot 7H_2O$. The second medium was the Modified Casamino Acid Medium (MCAT) which contained per liter of tap water: casamino acids (7.5 g), sodium citrate (3 g), yeast extract (0.5 g), $MgSO_4 \cdot 7H_2O$ (20 g), and Bacto Agar (Difco) (20 g). The pH was adjusted to 7.2-7.5, and solar salt was added to the appropriate concentration. Both media were prepared at 0, 3-4, 8, 15, and 25 percent solar salt. All chemicals except for the solar salt were of analytical grade.

Table 1. Sampling information for the Eilat saltworks

Sample Location	Date Sampled	Water Temperature °C	Water Density	pH
E-1-302	96/08/16	33	1.238	NT
E-1-305	96/08/16	29.5	1.149	NT
E-1-300	96/08/17	27	1.187	NT
E-1-202	96/08/17	23	1.084	NT
E-1-100	96/08/17	25.5	1.026	NT
E-2-302	97/01/23	NT	1.124	NT
E-2-203	97/01/23	NT	1.190	NT
E-2-202	97/01/23	NT	1.110	NT
E-2-104	97/01/23	NT	1.080	NT
Sample Location	Date Sampled	Water Temperature °C	Water Density	pH
E-2-100	97/01/23	16	1.045	NT
E-3-100	97/11/8	33.9	10.26	6.9
E-3-200	97/11/8	37.3	1.100	7.5
E-3-201/2	97/11/8	37	1.130	7.6
E-3-203/204	97/11/8	41.5	1.170	7.7
E-3-304	97/11/8	40.2	1.241	7.0
E-4-100	98/02/16	22	1.026	8.18
E-4-200	98/02/16	21	1.110	7.85
E-4-202	98/02/16	21	1.160	7.79
E-4-304	98/02/16	21	1.226	7.39

2.3 Plating and Enumeration Methods

Samples were plated within 2 hours of collection and in duplicate using a modification of the spread-drop-plate method [8]. They were incubated at 37°C and counted every week for up to six weeks. Growth was scored according to the color of the colonies. Data are reported as the total colony-forming units per mL (TCFU/mL) and as the total pigmented colony-forming units per mL (TPCFU/mL).

2.4 Isolation and Partial Characterization of the Colonies

Colonies were picked from the plates in the same ratio in which they were grown. If 10% of the colonies were pink, 30% red, 5% yellow, and 55% white, then that was the ratio used for selecting the colonies for purification and further study. The cultures were purified on the same salt concentration and medium from which they were isolated. All tests were performed at that salt concentration until the salt/temperature studies were performed.

Standard bacteriological tests for extracellular enzyme production were performed for

lipase, DNase, gelatinase, amylase, and proteinase. Selected cultures were tested for other biochemical characteristics using the API system (bioMerieux, Inc.) or individually prepared tests.

2.5 Whole Community Analyses

2.5.1 Molecular Analyses

At each location a 2-3 L sample was centrifuged and the pellet frozen until analyzed at GMU. Molecular fingerprinting was accomplished using the amplicon length heterogeneity (ALH) procedure described by Suzuki et al. [9] with details of its application to halophiles in Litchfield and Gillevet [10]. Total genomic DNA was extracted using the Bio101 FastDNA Spin Kit for Soil (Qbiogen Inc., Vista, California). The 16S rRNA gene was amplified using a set of the universal bacterial primers 27F and 355R [11] or a set of consensus haloarchaea primers 1HKF and H305R (5'-ATTCCGGTTGATCCTGCCGG-3' and 5'-GTTACCCCACCGTCTACCT-3', respectively) (11) with the 5' ends of the forward primers labeled with the fluorochrome 6-carboxyfluorescein (6-FAM). Nonfluorescent PCR products for the *Archaea* were cloned using the TOPO TA cloning kit with pCR®2.1-TOPO (Invitrogen) and sequenced using the Big Dye Terminator kit (PE Applied Biosciences) on a SpectruMedix 9610 (SpectruMedix) 96-capillary Sequencer. Replicate extractions were analyzed to demonstrate the reproducibility of the method and peaks appearing in two of the three replicates and accounting for greater than 1% of the amplicons were included in the analyses.

2.5.2 Carbon Utilization

The BIOLOG GN plates were used to assess carbon utilization patterns in the samples with less than 15% salt. The 96-well microtiter plates were inoculated with 140 µL of sample and incubated for up to four weeks. The plates contain 95 different carbon sources with the first well serving as the control. Color change from clear to pink or purple indicated the organisms had grown and were able to use that particular carbon source.

3. RESULTS

3.1 Enumeration

The CFU on the plates were counted every week for up to six weeks. The results, Figure 1, indicate that MR2A was a superior medium for obtaining CFU from this saltworks. In fact, the counts on MR2A were from 0-12% higher at the inlet, from -10% to 100 % higher at 202, and from 13% to over six orders of magnitude higher at the crystallizer 304. The counts were extremely low during the first two weeks (data not shown) and the number only stabilized after four to six weeks of incubation. The
incubation temperature was approximately that of the environmental temperature so the organisms were not subjected to a significant temperature shock.

As expected there were essentially no pigmented cultures in the inlet samples as the major pink to red pigments occur in the more halophilic bacteria. On the MCAT medium, the highest counts were obtained in August 1997 in pan 202, while the highest counts on the MR2A medium were found in August 1996 and January 1997 for the crystallizer and August 1997 and February 1998 in the evaporation pond 202. The greatest variability between the TCFU and the TPCFU appeared routinely in the evaporator 202 where in the August 1996 and January 1997 samples the pigmented organisms equaled the total count, but there were major differences between the two types of enumerations in August 1997 and February 1998.

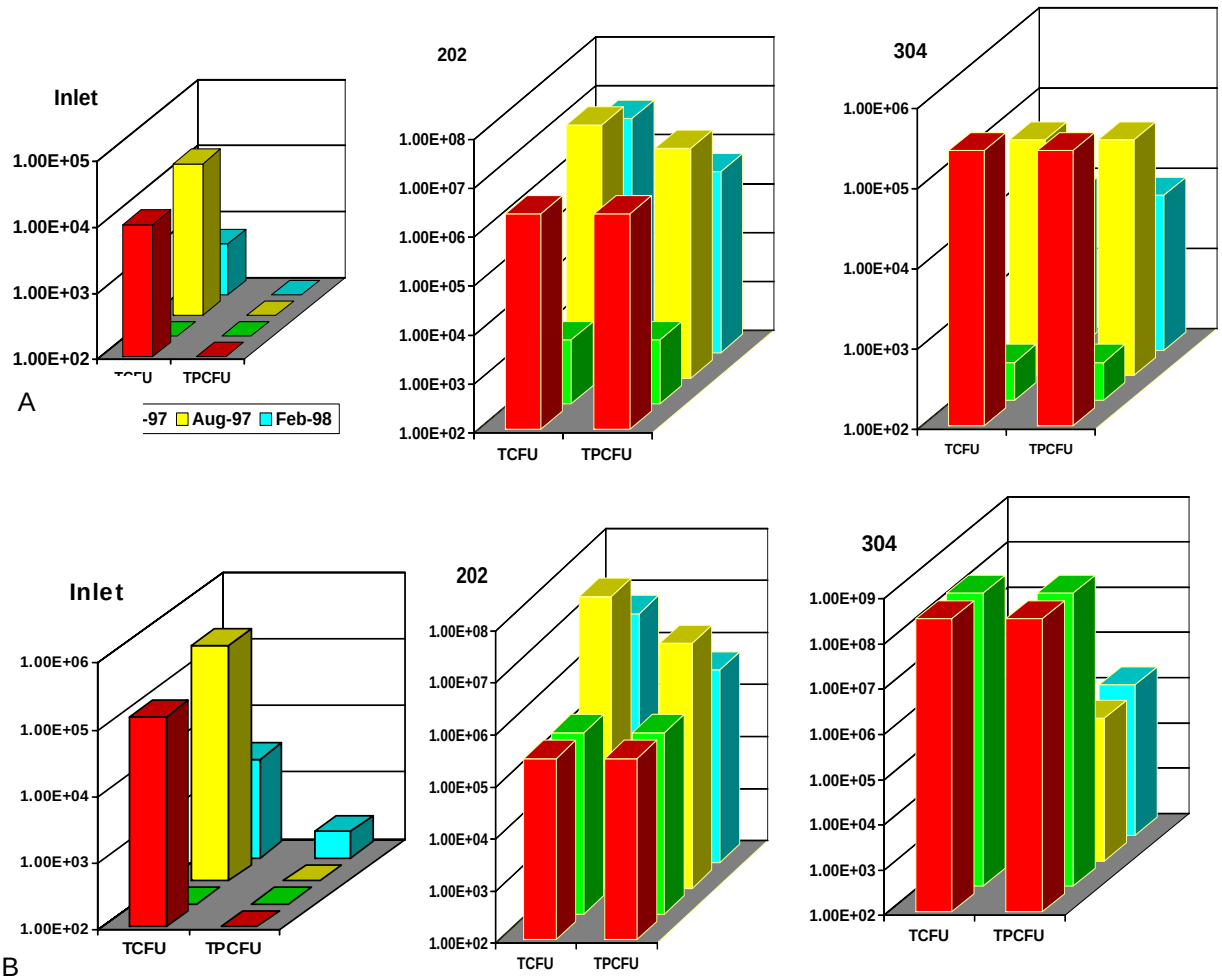

Figure 1. Enumeration of microorganisms growing on MCAT in Section A and on MR2A in Section B.

3.2 Characterization of Cultures

A total of 101 pure cultures were tested at their optimal salt concentrations for the production of extracellular enzymes. These enzymes could be important in obtaining nutrients for the microbes in this oligotrophic environment. The effect of increasing salt concentrations was to have generally fewer organisms producing extracellular enzymes (Table 2). In the majority of cases proteases were produced by the bacteria growing at 8% but those organisms growing at higher salt concentrations generally did not produce protease or even gelatinase, another type of protein-digesting enzyme. This same pattern was observed with the tests for the other extracellular enzymes.

Table 2: Effect of salt concentrations on extracellular enzyme production by pure cultures.

Sampling Period	Extracellular Enzyme Tested	Percent Salt Used in Media			
		8%	15%	20%	25%
	PROTEASE				
E-1		100	10	NT[a]	0
E-2		60	0	86	0
E-3		80	11	0	0
E-4		71	0	NT	0
	AMYLASE				
E-1		100	0	NT	0
E-2		60	0	43	0
E-3		60	44	25	0
E-4		57	25	NT	0
	LIPASE				
E-1		100	0	NT	0
E-2		80	0	100	8
E-3		100	22	75	0
E-4		100	38	NT	8
	DNASE				
E-1		0	20	NT	0
E-2		60	0	0	0
E-3		90	11	0	0
E-4		100	0	NT	0
	GELATINASE				
E-1		0	0	NT	0
E-2		80	0	0	0
E-3		10	33	0	0
E-4		57	63	NT	0

[a] NT= Note tested

3.3 Molecular Studies: Fingerprinting and Clone Identification

The seasonal effects on the community structure are shown in Figure 3. When one compares the fingerprints of the 200-level evaporators and crystallizers, it becomes clear that the uncultivated microbial community varies considerably with the seasons. In fact, the February samples in both E-4-200 and E-4-304 show the same operational taxonomic units (OTU) with peaks at 241, 245, and 247 base pairs while the August crystallizer samples contain 6 OTU's. The ratios of the various peaks also differ between the seasons and the sampling locations. When the products from the *Archaea* primers were cloned and sequenced, a very wide diversity of sequences was obtained which had high similarities to the following microorganisms: *Halobaculum gomorrense, Haloferax medeterranei, Haloferax volcanii, Halorubrum distributum, Halorubrum saccharovorum, Halobacterium* sp. strain ACM3911, *Halobacterium* AUS, *Halorubrum sodomense, Natronomonas pharaonis, Halococcus salifodinae,* and *Haloarcula mukohataei.*

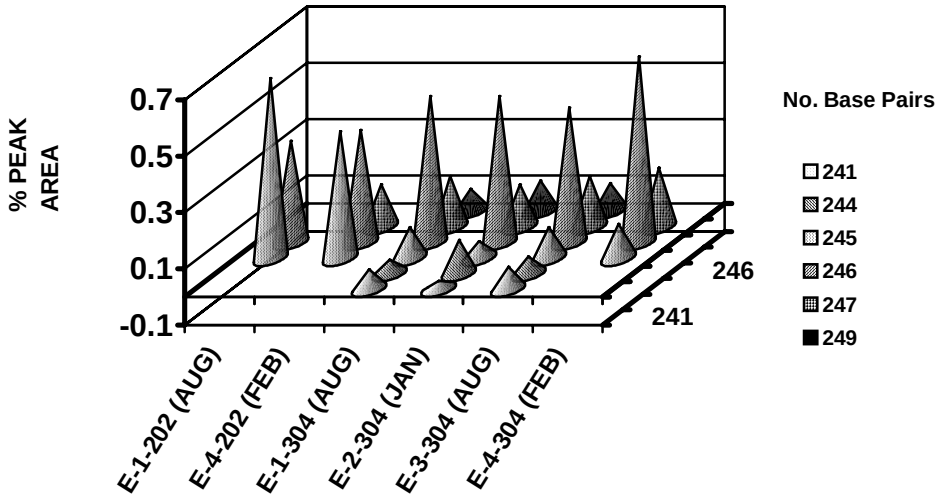

Figure 3: Seasonal comparison of the fingerprints for the 200-level evaporators and crystallizers

3.4 Whole Community Carbon Utilization

From the BIOLOG plates it was noted that no single carbon source was used by the community each time period. There were several that were commonly not used. The data were analyzed by the niche overlap indices (NOI) developed by Wilson and Ludlow [12]. The NOI is defined as the number of carbon sources that any two samples utilized in common as a proportion of the total number of carbon sources utilized by one strain. This comparison of the eight Eilat samples was done manually, and the result of the comparison of E-1 Inlet sample with all of the other samples is shown in Figure 4. The higher the score the greater similarity, so E-1 inlet had greatest similarity to the inlet sample from E-3 and E-4 and the E-1-200 sample, Figure 4.

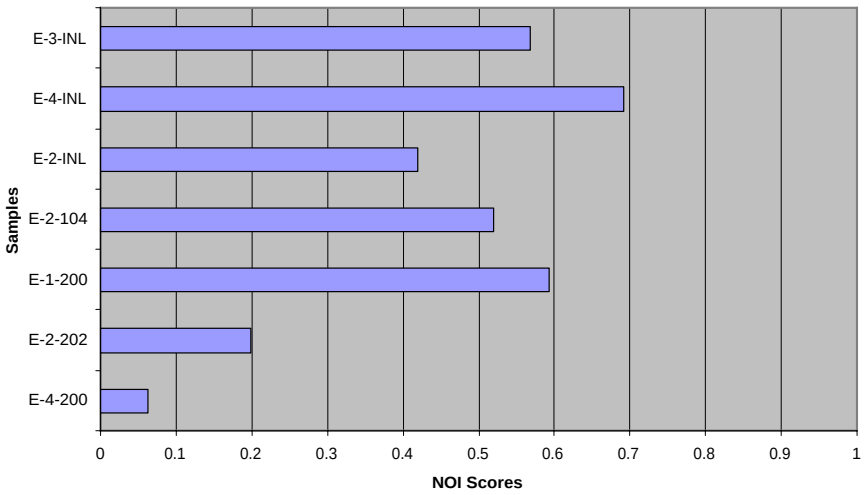

Figure 4. Niche Overlap Index (NOI) scores for the E-1 Inlet sample versus all of the other Eilat samples.

4.DISCUSSION

At the start of this study it was assumed that as the salinity increased there would be a reduced diversity in the microbial community [4][13]. Comparisons of the fingerprints between the crystallizer pond (304) and the evaporation pond (202), Figure 3, show that this is not necessarily true. In fact, there were more operational taxonomic units in 304 than 202. Each of the OTUs may, and probably does, represent several genera, and this was noted in the listing of genera identified from cloning the PCR products of the crystallizer. In a recent study, Burns et al. reported that there was little difference between the cultivated and molecular results for a crystallizer at the Cheetham Salt works, Victoria, Australia [14]. The relationship of salinity and seasonal impact on a hypersaline lake has been reported by Cytryn et al. [15]. These authors noted that in both stratified and mixing episodes, the predominant bacteria were *Archaea*. Meanwhile, Joint et al. [16] noted that both primary productivity and uptake rates for nitrate and ammonia decreased with increasing salinity except that at salinities greater than 22% there was more uptake of ammonium and nitrate than was expected based solely on primary productivity. Thus, the role of halobacteria and a diverse microbial community were implied.

Although the BIOLOG GN plates cannot be used with salinities higher than around 15% [10], the versatility of the inlet and 202 communities in regards to carbon utilization is surprising. That there were no compounds out of the 95 tested in the plates that were used by all of the samples was unexpected. Studies at the Cargill solar salt plant in San Francisco had revealed that 9 substrates were used 85% of the time by the microbial communities: glucose, L-alanine, L-asparagine, citric acid, d-glucosamininc acid, maltose, L-proline, sucrose, and D-trehalose [17]. Several of these compounds are known to be compatible solutes for members of the Domain *Bacteria*.

The dynamic nature of the microbial community is also confirmed by the cultivation experiments, Fig. 2. The January and February samples tended to have fewer CFU's than the two August samples. The evaporation pond 202 had salinity ranges during this period from 11.7 to 15.7% salt. It is thus unlikely that the pigmented organisms enumerated in 202 were *Salinibacter* spp. since the members of this genus require at least 15% salt for growth [18]. Recently, Purdy et al. reported the isolation of haloarchaea at salinities as low as 9% [19] which is well within the range of salt concentrations found in the evaporator 202. Thus, it is likely that some of the pink to red pigmented bacterial isolates from 202 will be haloarchaea that can grow at these lower salt concentrations. Further characterization of the over 200 isolates will help to resolve this question.

Although there have been many reports of the occurrence of extracellular proteases in the halophilic *Bacteria* and *Archaea,* there have been few studies screening the pure cultures for a variety of exo-enzymes. Lipolytic activity for haloarchaea strains was recently reported [20]. Twelve percent of the strains tested could hydrolyze olive oil in media containing approximately 20% salt. Amylase activity has also been detected in 16 Gram-positive cultures from Ethiopian soda lakes [21]. However, these organisms only tolerated salt up to 10% NaCl. We also found lipase, amylase and proteinase at 20% salt, but in the media containing 25% salt there was no extracellular enzyme activity. Thus our finding of amylase activity at 20% salt is unusual as relatively few haloarchaea have been reported to hydrolyze starch. We are currently studying amylase production by an Eilat culture that grows at 25 - 35% salt.

In summary, based on the data presented here, the microbial community in solar salterns is certainly not static but dynamic, responding not only to changes in salinity but also to their chemical environment, and this applies to the uncultivated community as well as the cultivated microbial community.

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