Occurrence of extremely halophilic Archaea in sediments from the continental shelf of west coast of India

Marine environments range from 3 to 3.5% in salinity in the open ocean waters[1] and shorelines[2] with the exception of certain regions like the Dead Sea and the Great Salt Lake, which have salinity[3,4] in the range of 15–20%.

Microorganisms classified under the domain Archaea are gaining tremendous attention for their ecological distribution, biochemistry, physiology, molecular biology and biotechnological applications[3–6]. Extreme halophiles classified under Archaea are reported from hypersaline regions containing 3.5–4.5 M (20–30%) NaCl concentrations, such as natural econiches like the Dead Sea and the Great Salt Lake[3,4] and man-made salt pans. Two recent reports have indicated the presence of such extreme halophiles in econiches with salinity as low as 3.5–5% NaCl concentrations in Spain and Japan[7,8]. The presence of extremely halophilic Archaea from offshore marine (continental shelf) sediments from the west coast of India using a simple method of Most Probable Number (MPN) estimation, is reported for the first time.

Sediments were collected during the pre-monsoon seasons of 1999 and 2001, between March and April, from five stations of the ONGC, namely, Panna (PPA), Mukta (MAA), Tapti (TPP) and Bombay High (B121), located in the continental shelf region, off the Mid-West Coast of India between Goa, Mumbai and Gujarat, while on cruises on board the research vessel *Sagar Paschimi* (Dept. of Ocean Development [DOD], Govt. of India), hired by the IPSEM (Institute for Petroleum Safety and Environment Management, Betul, Goa, India), for the ONGC. The average distance of sampling sites was 40–100 miles from the shore, at an average depth of 21–75 m; five to ten sites per station were demarcated for the sediment sample collection (Figure 1), in the vicinity of the offshore oil rig installations of the ONGC. Sediment samples from each site were collected using a Vee Wan grab, immediately wrapped in aluminium foil, stored at –70°C and transported to our laboratory at Goa University, Goa. Portions of each sediment sample, amounting to 5 g was suspended, separately in 100 ml of sterile 25% NaCl solution, mixed vigorously, allowed to stand for 30 min. The overlaying sediment-free suspension was estimated for haloarchaeal counts by the MPN method. The MPN method, conventionally used for estimating coliform counts[9], was modified to specifically enumerate haloarchaea by using nutrient-rich sodium chloride tryptone yeast extract (NTYE) medium consisting of (g/l) NaCl – 250; $MgSO_4$ – 20, KCl – 5, $CaCl_2$ – 0.2, Tryptone – 5, Yeast Extract – 3; pH was adjusted to 7, using 1N NaOH. All the chemicals used were from HiMedia, India.

The MPN enumeration for each sample was carried out separately using a total of 15 tubes, out of which the first 5 tubes contained 5 ml of double strength NTYE medium, while each of the remaining 10 tubes contained 5 ml of single strength NTYE medium. Each of the first 5 tubes containing 5 ml of double strength NTYE medium was inoculated with 5ml of the inoculum (i.e., the sediment-free suspension mentioned above); each of one set of 5 tubes containing 5 ml of single strength NTYE medium was inoculated with 0.5 ml of the inoculum and each of the remaining 5 tubes were inoculated with 0.05 ml of the inoculum. All the tubes, however, had 25% of NaCl to ensure selective growth of haloarchaea (extreme halophiles). pH indicator was not used in the medium; hence only the changes in turbidity within each set of tubes was monitored visually; the MPN deduction was done as per McCrady's table[10] and back-calculated to obtain the MPN of haloarchaea per gram sediment (MPN/g). The MPN tubes of different sites within a station showed varying turbidity. The index of turbidity also varied from station to station, corresponding to an average of (MPN/g) 1.15×10^2 (PPA), 85 (TPP), 1.14×10^2 (MAA) and 1.80×10^2 (B121) – indicating growth of microorganisms that require/thrive on 25% NaCl – this average count is exclusive of the sites PPA-1, 5, 10, 11 and B121N, all of which showed MPN/g in excess of 5×10^2 (with an upper limit in excess of 1.4×10^3). Thus, a wide range of MPN/g, from a count of 7, at the site MAA-4, to that in excess of 5×10^2, at PPA-1, 5, 10, 11 and B121N (Figure 2 *a–c*) was observed.

All earlier studies have dealt with heterotrophic eubacteria in marine sediments from Indian coastal and offshore sediments. In one study, occurrence of a very high population of 10^7–10^9/g of heterotrophic eubacteria in marine sediments from the West and South-East Coast of India, from Dabol–Ratnagiri to Trivandrum–Tuticorin was reported[11]. In another study, restricted to offshore and nearby shore sediments off Mangalore Coast in South-West Coast of India, marine sediments were found to have a high count 10^5–10^7/g of halotolerant eubacterial microorganisms[12]. The presence of haloarchaea, observed in the MPN experiments conducted by us using offshore sediments that are non-hypersaline, itself is a significant finding for the Indian subcontinent. Of the four offshore stations, viz. PPA, MAA, TPP and B121 that were sampled, the sediment samples from TPP, which were coarse, sandy, showed the lowest average count of 85/g, while those from PPA (with 5 of the sampling sites showing identical counts of haloarchaea, in excess of 5×10^2/g) and B121 (showing the highest average count of 1.80×10^2/g) were clayey in nature. Clayey sand or clay has been reported to have a higher percentage of organic matter and higher count of eubacteria than sandy sediments[13]. The higher population of heterotrophic eubacteria, encountered with clay and clayey-sand sediments than with fine or coarse sand sediments, has been inferred as a negative relationship of bacterial population with particle size and a significant direct relation with organic matter[14]. Thus, the results of our study follow the pattern of the negative relationship between particle size and haloarchaeal population, similar to that observed for eubacterial population in earlier studies[12–14]. It may be noted that, in terms of consistency of MPN counts, samples from 4 sites (PPA-1, 5, 10, 11) out of the 11 sampling sites of PPA station showed consistency of MPN (5×10^2/g), while in the case of TPP-station, with 11 sampling sites, the samples from sites TPP-1,

Station name and No.	Latitude	Longitude	Bearing	Depth (m)
Panna-PPA				
PPA-1	19′19.071N	72′01.658E	150	44
PPA-2	19′19.508N	72′01.505E	150	43
PPA-3	19′19.782N	72′01.432E	150	43
PPA-4	19′20.173N	72′01.374E	60	45
PPA-5	19′20.362N	72′01.617E	60	43
PPA-6	19′20.582N	72′01.920E	60	41
PPA-7	19′20.863N	72′00.441E	30	40
PPA-8	19′20.550N	72′00.870E	330	41
PPA-9	19′20.200N	72′00.818E	330	42
PPA-10	19′19.899N	72′00.924E	240	42
PPA-11	19′19.775N	72′00.676E	240	42
Mukta-MAA				
MAA-R	19′21.304N	71′47.304E	270	59
MAA-1	19′21.800N	71′51.450E	270	58
MAA-2	19′21.200N	71′50.800E	270	56
MAA-3	19′21.290N	71′50.400E	270	56
MAA-4	19′20.543N	71′50.281E	360	58
MAA-5	19′20.139N	71′50.444E	360	58
Tapti-TPP				
TPP-R	20′33.540N	72′02.060E	360	24
TPP-1	20′34.915N	72′02.060E	360	24
TPP-2	20′35.610N	72′02.330E	360	22
TPP-3	20′35.821N	72′02.287E	360	22
TPP-4	20′36.258N	72′02.598E	270	22
TPP-5	20′36.247N	72′02.790E	270	21
TPP-6	20′35.973N	72′03.312E	270	21
TPP-7	20′36.178N	72′00.978E	90	21
Bombay High-B121				
B121North-N	19′02.718N	71′32.501E	121	73
B121East-E	19′01.700N	71′33.000E	211	72
B121South-S	19′00.810N	71′32.560E	301	73
B121-R	18′59.568N	71′32.744E	301	74

Figure 1. Sampling stations in continental shelf region between Goa and Gujarat; (i) Tapti (TPP)-1, (ii) Mukta (MAA-2), (iii) Panna (PPA)-3, (iv) Bombay High (B121)-4; Sampling pattern followed at each station shown at '*' 'R' refers to a central reference point and • refers to the sampling sites, viz. 1, 2, 3...10, 11.

2, 3, 6 and 8 showed a consistent low count of 26–28/g. On the other hand, in the case of MAA and B121 stations, where sampling was restricted to 4–5 sites, counts of no two sites were identical (Figure 2 *c*). From this, it is evident that, the more the sampling sites, the higher would be the accuracy of the MPN count, particularly when MPN studies are carried out for microorganisms in econiches, in which they are not expected to thrive/survive. Therefore, our study recommends the necessity of sampling of sediments from several sites within a given station for future studies on enumeration of microorganisms in econiches, in which they are not expected to thrive/survive, and extreme halophiles from marine sediments, in particular, as in this case.

In order to confirm that the MPN is a consequence of growth of true haloarchaea in TYE with 25% NaCl, the contents of the MPN tubes for each sample were pooled together aseptically, and then plated on TYE with 25% NaCl agar, incorporated with 750–1000 IU/ml benzyl penicillin (Hi Media, India), since extremely halophilic archaea are known to be resistant to antibiotics like penicillin[15].

A total of 13 cultures were obtained that were not only growing at 25% salt concentration, but were resistant to high concentrations of benzyl penicillin. Only two of the cultures, one each from PPA (PPA-1) and TPP (TPP-1), showed the orange-red pigmentation characteristic of halophilic archaea; one culture from MAA showed the biochemical and gram staining characteristics of *Flavobacterium* sp., whereas the rest of the cultures, viz. 5 from PPA (PPA-2, 3, 4, 5, 6), 2 from TPP (TPP-2, 3), 1 from MAA (MAA-2) and 2 from B121 (B121E, B121S) stations were not pigmented.

Cells of halophilic archaea, which require a high concentration of NaCl in their environment to maintain the cell membrane integrity, lyse when subjected to hypotonic shock. When the cells of the pigmented cultures from TPP and PPA were exposed to water (i.e., no NaCl), the cells were observed to lyse, further

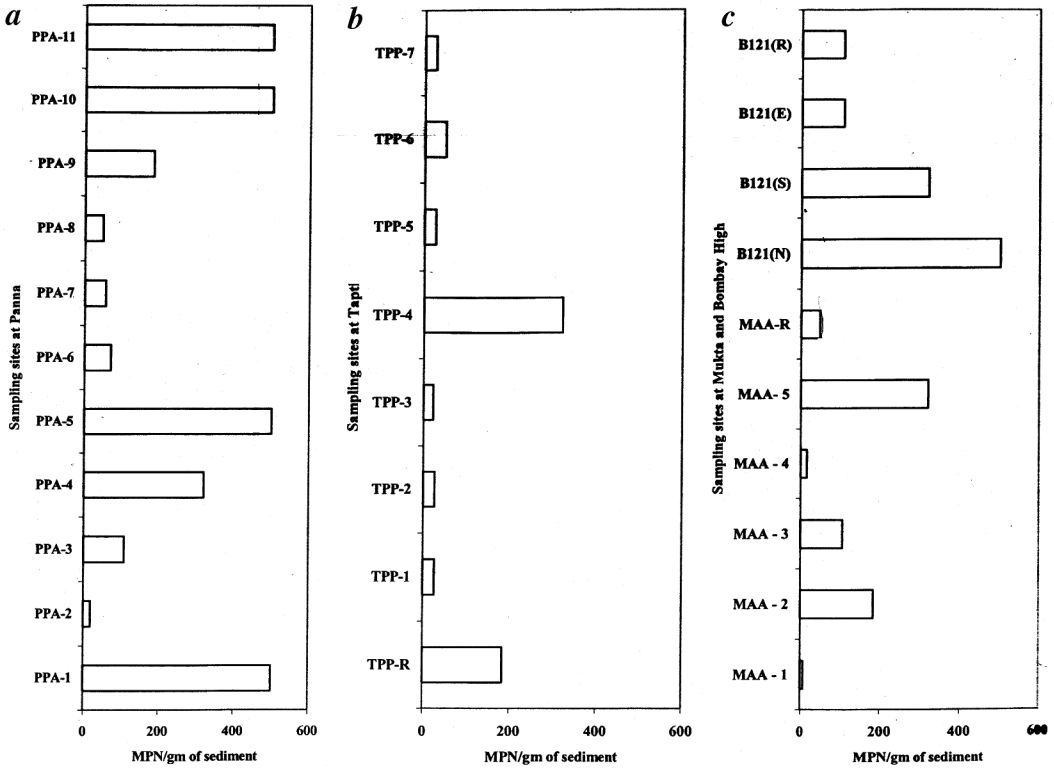

Figure 2. Most probable number estimation per g of sediment samples collected from (*a*), Panna, (*b*), Tapti and (*c*) Mukta and Bombay High station during premonsoon.

confirming their haloarchaeal nature. All the 14 isolates were routinely maintained on NTYE (25% NaCl), incorporated with high concentrations of penicillin.

The relatively low average count of 7 to 5×10^3 of extreme halophiles in offshore sediments, observed in our study, in contrast to the very high count of 10^5–10^9 marine eubacteria, reported in earlier studies[12,13], is perhaps illustrative of the severe limitations on haloarchaeal distribution in non-hypersaline environments. Nevertheless, the presence of haloarchaea in marine sediments (with a salinity level of 3%–3.5% NaCl), an econiche in which they are not expected to survive, is a significant finding that reflects the physiological and ecological complexities of halophilic archaea, and furthermore, raises the question pertaining to the survival mechanism employed in overcoming adverse hyposaline conditions of such an environment.

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T. M. RAGHAVAN
I. FURTADO*

Department of Microbiology,
Goa University,
Goa 403 206, India
**For correspondence.*
e-mail: ijfurtado@rediffmail.com