It can be concluded from this study that the 281 bp *Mbo*I-digested genomic DNA segment can be used as an RFLP marker to distinguish the closely related ecoraces of tropical tasar silk-producing insect *A. mylitta* D.

1. Sinha, A. K., Sinha, R. K., Goel, A. K., Sinha, B. R. R. P. and Thangavelu, K., A review on the breeding and genetic aspects of tropical tasar silkworm, *Antheraea mylitta*. D. In Proc. of the conference on cytology and genetics, 1994, vol. 4, pp. 7–16.
2. Yasukochi, Y., A dense genetic map of the silkworm, *Bombyx mori*, covering all chromosomes based on 1018 molecular markers. *Genetics*, 1998, **150**, 1513–1525.
3. Tan, Y. D. *et al.*, An amplified fragment length polymorphism map of the silkworm. *Genetics*, 2001, **157**, 1277–1284.
4. Reddy, K. D., Abraham, E. G. and Nagaraju, J., Microsatellites of the silkworm, *Bombyx mori*: abundance, polymorphism and strain characterization. *Genome*, 1999, **42**, 1057–1065.
5. Nagaraju, J., Kathirvel, M., Muthulakshmi, M., Subbiah, E. V. and Kumar, L. D., FISSR–PCR: a simple and sensitive assay for high throughput genotyping and genetic mapping. *Mol. Cell. Probes*, 2002, **16**, 67–72.
6. Cheng, T. C. *et al.*, Mining single nucleotide polymorphisms from EST data of silkworm, *Bombyx mori*, inbred strain Dazao. *Insect Biochem. Mol. Biol.*, 2004, **34**, 523–530.
7. Prasad, M. D. and Nagaraju, J., A comparative phylogenetic analysis of full-length *mariner* elements isolated from the Indian tasar silkmoth, *Antheraea mylitta* (Lepidoptera: Saturniidae). *J. Biosci.*, 2003, **28**, 443–453.
8. Datta, A., Ghosh, A. K. and Kundu, S. C., Differential expression of fibroin gene in developmental stages of silkworm *A. mylitta* (Saturniidae). *Comp. Biochem. Physiol. Part B*, 2001, **129**, 197–204.
9. Padhi, B. K., Ghosh, S. K. and Mandal, R. K., Characterization of *Mbo*I satellites in *Cirrhina mrigala* and *Clarias batrachus* (Pisces). *Genome*, 1998, **41**, 34–39.
10. Sambrook, J. and Russell, D. W., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001, 3rd edn.
11. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J., Gapped BLAST and PSI–BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, 1997, **25**, 3389–3402.
12. Rohrmann, G. F. and Karplus, P. A., Relatedness of baculovirus and gypsy retrotransposon envelope proteins. *BMC Evol. Biol.*, 2001, **1**, 1.
13. Thompson, J. D., Higgins, D. G. and Gibson, T. J., ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, 1994, **22**, 4673–4680.
14. Wright, J. M., DNA fingerprinting in fishes. In *Biochemistry and Molecular Biology of Fishes* (eds Hochachka, P. and Mommsen, T.), Elsevier, Amsterdam, 1993, vol. 2, pp. 57–91.
15. Swofford, D. L., PAUP* Phylogenetic Analysis Using Parsimony (* and other methods), Ver. 4.0b 10. Sinauer Associates, Sunderland, Massachusetts, 2003.

ACKNOWLEDGEMENTS. Labanyamayee Das Molecular Genetics R&D Laboratory and Bioinformatics facilities of this Institute have been used for this work. Financial support was obtained from CSIR and the Ministry of Human Resources and Development, New Delhi. B.M. obtained Senior Research Fellowship from CSIR. We thank Central Tasar Research and Training Institute, Ranchi, its research stations, and Directorate of Sericulture, Midnapore, Govt of West Bengal for providing silkworm ecoraces.

Received 4 June 2005; revised accepted 21 September 2005

Effect of carbon source concentration and culture duration on retrievability of bacteria from certain estuarine, coastal and offshore areas around peninsular India

Reshma C. Goltekar, K. P. Krishnan, Maria-Judith B. D. De Souza, A. L. Paropkari and P. A. Loka Bharathi*

National Institute of Oceanography, Dona Paula, Goa 403 004, India

Improved culture-based approaches efficiently compliment the limitations encountered in molecular methods to delineate the diversity and functions of uncultured organisms. The present study endeavours to reduce the anomaly between total counts (TC) and retrievable plate counts as colony forming units (CFU). Retrievability was improved by decreasing nutrient concentration in the culture medium. Maximum retrievability of 10^4 CFU ml^{-1} was obtained on 0.1% nutrient strength (100% corresponds to 8 g l^{-1} nutrient broth with 1.5% agar-agar) with samples from the Bay of Bengal offshore and coastal waters. However, in the Arabian Sea, retrievability was maximum (10^2 CFU ml^{-1}) on 33 and 100% nutrient strength. By varying the nutrient concentration, retrievability could be enhanced to 24% of TC in the estuaries, 3–14% in coastal waters and 5% in offshore waters. Bacteria from relatively more dynamic estuarine systems seemed less resilient compared to the coastal and offshore populations, as they were best retrieved (10^5 CFU ml^{-1}) only on 1% nutrient strength.

Keywords: Bacterial retrievability, coastal and offshore waters, colony forming units, estuaries, nutrient strength.

MANY decades ago it was realized that nutrient concentration in the commonly used media is several fold higher than that present in the environment[1–3]. For oligotrophic environments, plate counts on nutrient-poor media are several fold higher than those obtained on conventional media[4,5]. In the last decade, a number of molecular techniques have been employed to assess the bacterial diversity independent of cultural methods. Estimates on data compiled for culturability indicate that in sea water and unpolluted estuarine water, percentage culturability can reach up to 0.1 and 3% of total counts (TC) respectively, whereas from activated sludge[6] it can go up to 15. However, studies generally reveal that 99% or more of the bacterial diversity remains uncultured and unexplored[7].

Given that the molecular approach can only tell us about the existence and potential functions of microorganisms, it is of utmost importance to be able to retrieve, cultivate and

*For correspondence. (e-mail: loka@darya.nio.org)

maintain the ecologically diverse uncultured forms[8]. Therefore, the culture-based approach could prove to be better, if successful[9]. Attempts with extinction dilution techniques to culture the uncultured forms[10] have yielded a high retrievability of 2–60%, but here an effort has been made with decreasing strength of nutrients in the media and varying periods of incubation. The present work is an attempt to maximize retrieval of bacteria from oceanic, coastal as well as estuarine sea water in the form of Colony Forming Units (CFU) by simulating oligotrophic to eutrophic conditions of growth. Since the estuaries, coastal seas and offshore regions represent contrasting regimes, the retrievability data could reflect the availability of Dissolved Organic Carbon (DOC) and perhaps its relative dynamics in the environment.

The marine, coastal and estuarine water samples were collected from the following areas:

(i) Arabian Sea coastal waters (15°20′N and 72°17′E) – Cruise SK172 on *ORV Sagar Kanya*.
(ii) Bay of Bengal offshore waters (12°58′N and 80°35′E) – Cruise SK145B on *ORV Sagar Kanya*.
(iii) Bay of Bengal coastal waters (13°00′N and 80°25′E) – Cruise SK145B on *ORV Sagar Kanya*.
(iv) Zuari estuary (Goa) (15°10′–15°30′N and 74°25′E).

All the samples were collected using Niskin's water sampler attached to a CTD rosette. The sub-samples were collected into sterile containers through pre-rinsed tygon tubing and analysed on-board within 1–2 h. The water column up to 2000 m was sampled at predetermined intervals for location 1, whereas only surface waters were analysed from locations 2 to 4.

Estuarine samples were subjected to three-fold dilution (10^{-1}, 10^{-2} and 10^{-3}) using 50% aged, autoclaved coastal sea water and pre-filtered to remove large suspended materials. Coastal and offshore samples were plated directly without diluting. The inoculum size for plating the estuarine samples was restricted to 50 µl whereas for coastal and offshore samples it was 100 µl. The samples were surface-plated on nutrient agar prepared in 50% sea water of 0, 0.1, 1, 10, 25, 33, 50 and 100% nutrient strength (100% corresponds to 8 g l^{-1} nutrient broth (NB) with 1.5% agar-agar (A) from Himedia Laboratories Pvt Ltd, Mumbai, India. Henceforth, the various strengths would be represented as '%NB + A'. The plates were incubated at room temperature and CFU were counted at intervals of 24 h. Un-inoculated sterile nutrient plates were used as controls for extraneous contamination during incubation.

Samples for total bacterial abundance were fixed using buffered formalin (0.4% final concentration) and stored in refrigerator (4°C) till further analysis. Bacteria were estimated using the Acridine Orange Direct Count (AODC) method[11]. The filters were mounted onto a glass slide and viewed under a BHF-342 epifluoresence microscope.

In the estuaries, maximum retrievability of 10^5 CFU ml^{-1} was on 1% NB + A (Figure 1). This was followed by 10 and then 0.1%, whereas in 0, 33 and 50% NB + A, CFU was of the order of 10^3 ml^{-1}. Though there is high variation of DOC and nutrients in the estuaries[12] which could induce nutritional flexibility, it was observed that lower nutrient strengths yielded higher retrievability. There was a difference of two orders between higher and lower nutrient strengths. This may be due to the heavy organic flux from the estuary into the adjoining coastal waters, reducing the residual time for degradation of organic carbon. A short residential time can be accounted by the large seasonal influx of freshwater into the estuary, with unchanged tidal amplitude over large distances[13]. Cumulative increase in numbers on 1% NB + A showed a steady increase from day 1 to 12, that later stabilized. Similarly, the increase stabilized after 5 days on 10 and 0.1%. Though the nutrient requirements are low, estuarine bacteria appear to have a short lag phase compared to coastal and offshore waters.

In coastal waters of the Bay of Bengal, the maximum retrievability (10^4 CFU ml^{-1}) was on 0.1% NB + A (Figure 2). The next best was on 1% NB + A with no difference in order. Cumulative growth stabilized at the end of five days in nutrient strengths was greater than 10%. It was clear that though the lower strengths showed reduced growth at the

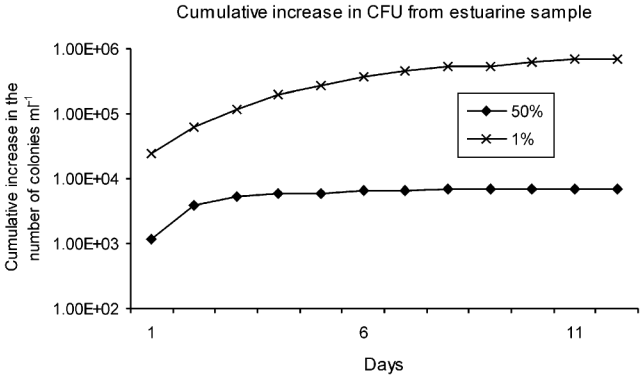

Figure 1. Percentages refer to '%NB + A'. Retrievability on 33 and 10% was of the same order as 50%, while 0.1 and 0% showed retrievability of the same order as 1%.

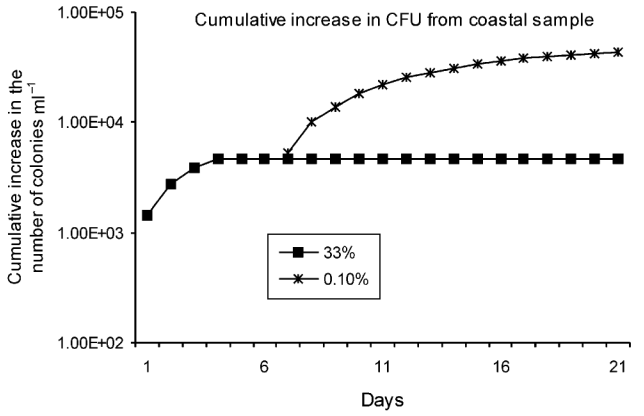

Figure 2. Percentages refer to '%NB + A'. Retrievability on 50, 10, 1 and 0% was of the same order as 33%.

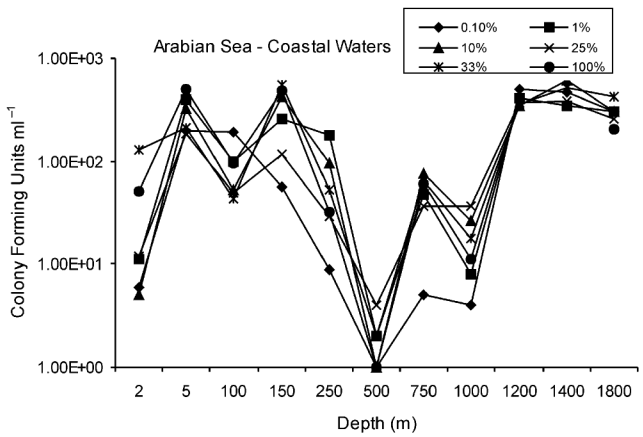

Figure 3. Percentages refer to '%NB + A'. Depth-wise retrievability pattern in the Arabian Sea. Incubation period: 7 days.

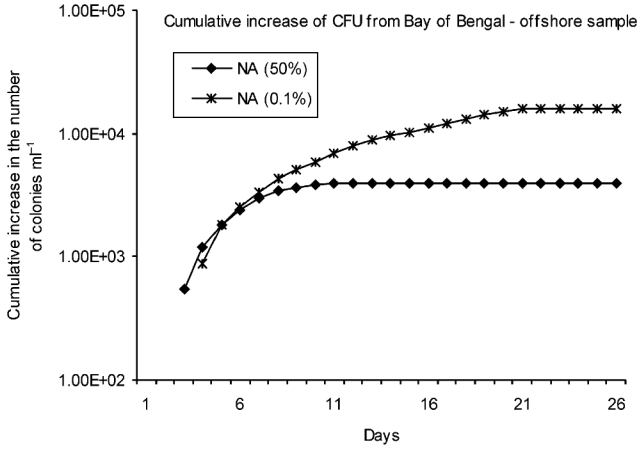

Figure 4. Percentages refer to '%NB + A'. Retrievability on 33, 10, and 0% was of the same order as 50%, whereas that on 1% was of the same order as 0.1%.

initial period (3–7 days), the cumulative increase exceeded the higher strengths, which showed active increase only up to a period of five days. In general, maximum retrievability needed an incubation period of 21 days. In the coastal Arabian Sea, 100% NB + A recorded the maximum CFU (10^2 ml^{-1}) at a depth range of 0–150 m (Figure 3). In the depth ranges 250–1000 m and 1200–1800 m, 1 and 33% showed retrievability maxima (10^3 CFU ml^{-1}) respectively. Higher yield in higher strengths could reflect the nutrient status of the sampled location. The Arabian Sea is known to be highly productive[14,15], with restricted circulation[16,17] and concomitant high oxygen consumption rates at the surface. The oxygen consumption rates (~ 400 Tg/y) were about four times higher than in the Bay of Bengal[18]. This is because the organic matter entering the Bay of Bengal from the rivers is adsorbed onto mineral particles and therefore made less available to the microbes. Perhaps the occurrence of a well-stratified oxygen minimum zone[17] is also responsible for lower retrievability from these layers. On the whole, the retrievability in coastal waters was lower by one to three orders (10^{2-4}) compared to the estuary (10^5).

Comparing the retrievability pattern in coastal waters of the Arabian Sea and Bay of Bengal, it might be possible to assess the potential bioavailability of organic carbon in these waters. In the Arabian Sea, intense remineralization of organic matter takes place predominantly in the surface layers, due to a high heterotrophic carbon demand (1203 Tg C yr^{-1})[19]. This is because of the occurrence of a well-stratified oxygen minimum zone (OMZ), which leads to preservation of organic carbon in these layers[20]. Hence the prevailing organic loading supports increased retrievability at higher nutrient strengths in these waters.

The retrievability maximum (10^4 CFU ml^{-1}) in the Bay of Bengal offshore waters was on 0.1%, and not much affected by varying the nutrient strength (Figure 4). By lowering the strength from 50 to 0.1%, CFU retrievability improved by an order from 10^3 to 10^4. The offshore sample did not show any marked difference from the Bay of Bengal coastal sample, perhaps suggesting that there is little decrease in availability of DOC from the coastal to the offshore region in the Bay of Bengal.

The foregoing studies demonstrated that the nutritional flexibility of bacteria was more enhanced in oligotrophic conditions. The retrievability of bacteria from estuarine sources is not uniformly high across a range of carbon concentrations, as might have been expected from the dynamic nature of the estuary. This could be because of the estuarine diurnal cycles that provide the right concentrations required for a particular group. On the other hand, in the unchanging oligotrophic marine environment, the supply of nutrients could be sporadic and adaptation of the microbes instantaneous[21]. This could be the reason for enhanced retrievability of microbes on varied nutrient strengths from oligotrophic offshore waters.

Hence regardless of the sample type, nutrient levels could be reduced and incubation prolonged to maximize retrievability of slow growers, as enhanced nutrients induce metabolic imbalance resulting in cell death[22]. Up to 50-fold decrease in media strength could bring about over 100-fold increase in retrievability (Figure 1). By varying the nutrient levels, retrievability could be enhanced to 24% of total counts in the estuaries, 0.1–13% in coastal waters and 5% in the offshore waters (Table 1). Repeated sampling in these locations as well as in new regimes will give us a more definite understanding of the retrievability patterns in these regions and help us elucidate the subtle links between available DOC pools and retrieval characteristics. Detailed phenotypic analyses of fast and slow growers would throw light on their physiological differences and help enhance their retrievability further.

Thus our study indicates that retrievability of bacteria from estuarine sources is not uniformly high across a range of carbon concentrations, as might have been expected from the dynamic nature of the estuary. This suggests that estuarine bacteria are nutritionally less resilient

Table 1. Percentage retrievability of bacteria

Station	TC (ml^{-1})	Medium strength used			Altered medium strength used		
		Strength of medium (%)	Retrievable counts (ml^{-1})	Per cent retrievable	Strength of medium (%)	Retrieval counts (ml^{-1})	Per cent retrievable
Estuary	2.9×10^6	33	1.3×10^3	0.0045	1	7.02×10^5	24.21
Bay of Bengal coastal	3.2×10^5	33	4.6×10^3	1.4375	0.1	4.32×10^4	13.50
Arabian Sea coastal (0–150 m)	3.4×10^5	33	2.1×10^2	0.0618	100.0	4.98×10^2	0.15
Arabian Sea coastal (250–1000 m)	5.3×10^5	33	6.4×10	0.0121	1.0	1.82×10^3	0.34
Arabian Sea coastal (1200–1800 m)	2.2×10^5	33	5.2×10	0.2364	10.0	6.16×10^3	2.80
Bay of Bengal offshore	3.0×10^5	33	6.4×10^3	2.1333	0.10	1.6×10^4	5.33

than offshore forms. Retrievability patterns could perhaps reflect the bioavailable DOC of the sampled waters. Conversely, this information could be effectively coupled to the existing extinction dilution techniques to obtain maximum retrievability, to reduce the 'great plate count anomaly'[23].

1. Roszak, D. B. and Colwell, R. R., Survival strategies of bacteria in natural environment. *Microbiol. Rev.*, 1987, **51**, 365–379.
2. Zobell, C. E. and Grant, C. W., Bacteria utilization of low concentration of organic matter. *J. Bacteriol.*, 1943, **45**, 555–564.
3. Moore, E. B. R., Wittch, R. M., Fortnagel, P. and Timmis, K. N., 16S ribosomal RNA gene sequence characterisation and phylogenetic analysis of a dibenzo-p-dioxin-degrading isolate with a new genus *Sphingomonas*. *Appl. Microbiol.*, 1993, **17**, 115–118.
4. Akagi, Y., Taga, N. and Simidu, U., Isolation and distribution of oligotrophic marine bacteria. *Can. J. Microbiol.*, 1977, **23**, 981–987.
5. Kuznetsov, S. L., Dubinina, G. A. and Lapteva, N. A., Biology of oligotrophic bacteria. *Annu. Rev. Microbiol.*, 1979, **33**, 377–387.
6. Amann, R. L., Ludwig, W. and Schleifei, K., Phylogenetic index and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.*, 1995, **59**, 143–169.
7. Fuhrman, J. A. and Campbell, L., Microbial diversity. *Nature*, 1998, **393**, 410–411.
8. Bull, A. T., Goodfellow, M. and Ward, A. E., Search and discovery strategy for biotechnology: The paradigm shift. *Microbiol. Mol. Biol. Rev.*, 2000, **64**, 573–606.
9. Watve, M. *et al.*, The 'K' selected oligotrophic bacteria: A key to uncultured diversity? *Curr. Sci.*, 2000, **78**, 1535–1542.
10. Button, D. K., Schut, F., Quang, P., Martin, R. and Robertson, B. R., Viability and isolation of marine bacteria by dilution culture: Theory, procedures, and initial results. *Appl. Environ. Microbiol.*, 1993, **59**, 881–891.
11. Hobbie, J. E., Daley, R. J. and Jasper, S., Use of nucleopore filters for counting bacteria by epifluorescence microscopy. *Appl. Environ. Microbiol.*, 1977, **33**, 1225–1228.
12. De Souza, M. J. B. D., Studies on particle-associated bacteria in a tropical estuarine system. Ph D thesis, Goa University, 2000.
13. Unnikrishnan, A. S., Shetye, S. R. and Gouveia, A. D., Tidal propagation in the Mandovi–Zuari estuarine network, west coast of India: Impact of freshwater influx. *Estuarine Coastal Shelf Sci.*, 1997, **45**, 737–744.
14. Brock, J., Sathyendranath, S. and Platt, T., Modeling the seasonality of subsurface light and primary production in the Arabian Sea. *Mar. Ecol. Prog. Ser.*, 1993, **101**, 209–221.
15. Brock, J., Sathyendranath, S. and Platt, T., A model study of seasonal mixed-layer primary production in the Arabian Sea. *Proc. Indian Acad. Sci. Earth Planet. Sci.*, 1994, **103**, 163–176.
16. Yoder, J. A., McClain, C. R., Feldman, G. C. and Esaias, W. E., Annual cycles of phytoplankton chlorophyll concentrations in the global ocean: a satellite view. *Global Biogeochem. Cycles*, 1993, **7**, 181–193.
17. Olson, D. B., Hitchcock, G. L., Fine, R. A. and Warren, B. A., Maintenance of the low-oxygen layer in the central Arabian Sea. *Deep-Sea Res. II*, 1993, **40**, 673–685.
18. Sarma, V. V. S. S., An evaluation of physical and biogeochemical processes regulating perennial suboxic conditions in the water column of the Arabian Sea. *Global Biogeochem. Cycles*, 2002, **16**, 29.1–29.11.
19. Sarma, V. V. S. S. *et al.*, Carbon budget in the eastern and central Arabian Sea: An Indian JGOFS synthesis. *Global Biogeochem. Cycles*, 2003, **17**, 13.1–13.13.
20. Paropkari, A. L., Prakashbabu, C. and Mascarenhas, A., New evidence for enhanced preservation of organic carbon in contact with oxygen minimum zone on the western continental slope of India. *Mar. Geol.*, 1993, **111**, 7–13.
21. Pointdexter, J. S., Oligotrophy: feast or famine. *Adv. Microbiol. Ecol.*, 1981, **5**, 63–89.
22. Bloomfield, S. F., Steward Gordan, S. A. B., Dodd, C. E. R., Booth, I. R. and Power, G. M., The viable but non-culturable phenomenon explained? *Microbiology*, 1998, **144**, 1–2.
23. Staley, J. T. and Konopka, A., Measurements of *in situ* activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annu. Rev. Microbiol.*, 1985, **39**, 321–346.

ACKNOWLEDGEMENTS. We thank the scientists and crew on-board *ORV Sagar Kanya* for cruises 145B and 172. Constructive suggestions of two anonymous reviewers are acknowledged. K.P.K. thanks the University Grants Commission, New Delhi for award of Senior Research Fellowship. This is NIO contribution number 4049.

Received 2 April 2005; revised accepted 28 September 2005