

Microbial and biochemical alterations due to storage of deep-sea sediments under ambient tropical conditions

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Response of indigenous bacteria to incubation at normal pressure and tropical temperature ($28 \pm 2^\circ\text{C}$), were examined in four deep-sea sediments from polymetallic-nodule fields in Central Indian Basin (CIB). Sediments were stored in sterile air-tight polythene containers. Changes in microbial and biochemical parameters were monitored once in every two months for a year. Bacterial counts and ATP decreased from $\sim 10^8$ to $\sim 10^7$ g^{-1} and $\sim 10^3$ to $\sim 10^1$ ng g^{-1} dry sediment respectively, within 8-10 months before stabilization. Protein: carbohydrate ratio fluctuated considerably. The four samples responded distinctively to storage, suggesting dependence of variations on inherent sedimentary properties. Though bacteriologically and biochemically altered, the integrity of metals in sediments changed only marginally. Sediment properties evolved from a non-steady phase to a steady phase.

[**Keywords:** Incubated sediments; deep-sea; ATP; microbe; biochemistry; Central Indian Basin].

Introduction

Deep-sea sediments harbour microbes with a total count of $\sim 10^8$ cells g^{-1} under extreme conditions such as $\sim 2^\circ\text{C}$ temperature, 500 bar pressure and no natural sunlight. In view of the possibility of deep-sea mining activities, to replenish diminishing resources of strategic metals on land, this study was launched with the aim of evaluating the alterations in natural microbial and biochemical characteristics of deep-sea sediment samples incubated under tropical conditions. Often precious deep sea samples are stored in repository either for reference or sample sharing for different analyses subsequently. These deep-sea sediments and mineral ore deposits are likely to be stored under ambient conditions until the process of extraction. Given this background, it is important to know microbial and biochemical changes due to storage, before undertaking further processing of the sample. This study was necessitated because sediments associated with deep-sea minerals such as the polymetallic nodules and ferro-manganese crusts, host a variety of microflora that could be 'mined' along with the minerals. The objective of the study is to evaluate whether any natural processes tend to alter samples

especially in tropical condition where temperature and moisture could contribute to accelerate sample deterioration. During the intervening period between lifting of ores at sea, transportation to land and the actual metallurgical processing; these sediments/mineral deposits would be stored under tropical conditions of 1 atm. pressure, $30 \pm 10^\circ\text{C}$ temperature. The possible microbial and biochemical changes as well as the quantity of extractable metal before and after storage were thus analyzed for four deep-sea samples from different sedimentary regimes of Central Indian Basin for a period of 1 year.

According to Ahmed and Oades¹ and Contin and Todd², long term incubation or "storage" affects microbial biomass, ATP concentrations and other chemical parameters besides an increase in water soluble/extractable nutrients like organic C^{3,4} and P^{5,6}, extractability of humic substances, cation exchange capacity and pH⁷. However, microbes like *Arthrobacter* and certain rod-shaped bacteria are capable of surviving for long periods in dry soil by forming endospores, cysts or other resistant structures^{8,9}. Rewetting of stored sediments increases microbial activity

attributed to faster turnover of microbial biomass¹⁰ and increased mineralization of soil organic matter, due to air-exposure¹¹⁻¹⁵. The present study shows how deep-sea sediment microbial communities undergo change during

the storage of wet sediments. Also, the nature of changes under normal tropical condition is governed by contrasting geochemical properties intrinsic to the sediment samples.

Table 1-Sample locations in Central Indian Basin, water depth, sediment type with elemental C and N composition.

Core	Location	Depth (m)	Sediment -type and setting	TOC (%)	TIC (%)	TOC/TN
SVBC 27	11°30'S, 75°30'E	5274	Siliceous ooze +nodules ⁴³	0.16±0.09	0.05±0.05	7.90±5.21
SVBC 19	13°00'S, 75°30'E	5104	Siliceous ooze + nodules ⁴³	0.58±0.54	0.05±0.03	25.00±13
SVBC 36	16°02'S, 75°29'E	5042	Red clay on a seamount flank neighbouring to hydrothermally altered site ^{44,45}	0.33±0.42	0.22±0.22	23.26± 6.57
SVBC 37	16°07'S, 75°25'E	3992	Carbonaceous ooze on seamount top ⁴⁵	0.26±0.06	9.51±0.52	65.00±40.45

Materials and Methods

The samples were collected during the 26th expedition of R.V. *Akademic Boris Petrov* (December 2006-January 2007), as a part of “Environmental studies for nodule mining in Central Indian Basin” (Polymetallic Nodules-Environmental Impact Assessment, PMN-EIA) programme, in the Central Indian Basin. Four samples from geologically distinct realms were collected for the present study (Figure 1 and Table 1).

The samples were collected with the help of standard United States Naval Electronics Laboratory (USNEL) – type box core and transferred into sterile plastic bags at ambient tropical conditions. Samples for the initial (month 0) observation were analysed immediately onboard after sample retrieval. The box-core penetrated up to 25-30 cms bsf on an average. The whole sampled sediment column was stored together. Sub-sampling was done after homogenisation. The sediment samples were then sealed and incubated in a closed plastic container of 40lt capacity at 1 atm and room temperature ($28 \pm 2^\circ\text{C}$). The seal was removed every 2 months under sterile conditions and sediments were analysed for different microbial and biochemical properties for a year. Sub-sampling was done as quickly as possible to minimize extraneous changes.

Total carbon and nitrogen was measured by NCS 2500 Elemental Analyser¹⁶ using standard procedures. Total inorganic carbon was analyzed by UIC CM 5014 coulometer.

The accuracy of measurement was verified using USGS standard reference material (MAG-1).

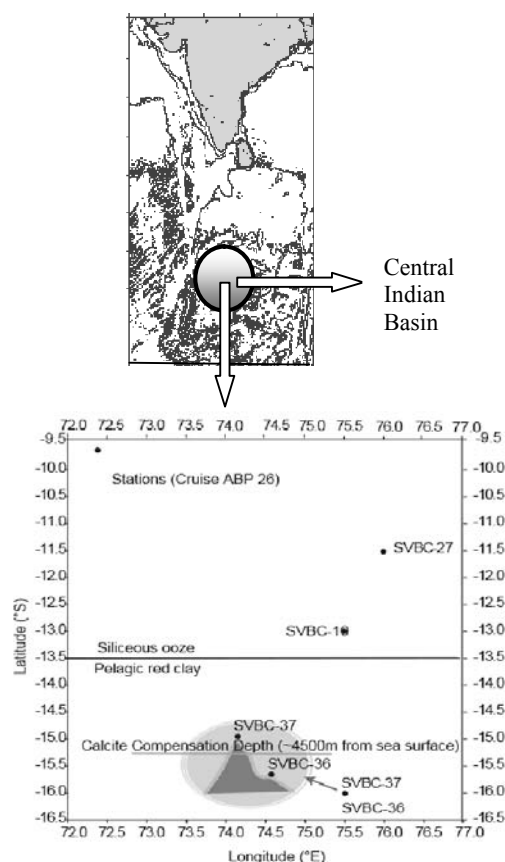


Fig. 1-Sampling locations in the Central Indian Basin showing siliceous cores SVBC 27 and SVBC 19 with inset showing schematic location of pelagic red clay core SVBC 36 and calcareous ooze SVBC 37 on seamount site at 75.5°E and 16°S.

Total organic carbon was determined by subtracting total inorganic carbon from total carbon. The C/N was calculated as the ratio between total organic carbon and total nitrogen. These parameters along with LOM were studied for defining the native quality of organic matter of the natural sample at the start of the experiment.

Total proteins of sediments were estimated by Lowry's Folin Ciocalteu method¹⁷, total carbohydrates by phenol-sulphuric acid method¹⁸ and total lipids by Bligh and Dyer method¹⁹. The nature and origin of the organic matter was estimated by the protein to carbohydrate and carbohydrate to labile organic matter (LOM= sum of total proteins, carbohydrates and lipids) ratios²⁰⁻²³.

Total ATP in deep-sea sediments was estimated by luciferin-luciferase reaction²⁴, using adenosine triphosphate disodium salt as standard (Sigma Chemicals) on a Perkin Elmer, Wallac 1409 DSA, Liquid Scintillation Counter²⁵.

Total count was estimated by fixing ten-fold sterile sea-water diluted sediment slurry with buffered formalin at 2% final concentration. The aliquot after sonication was stained with 0.01% acridine orange and filtered on 0.22µm black polycarbonate filter paper (Millipore, USA)²⁶. Samples were counted on a Nikon 80i epifluorescence microscope and normalized per gram of dry sediment.

Colony forming units on ZoBell Marine Agar (ZMA) were assessed using 20% concentration of ZoBell Marine broth in 1.5% agar²⁷ and enumerated within 8-10 days. Nitrifying bacteria were enumerated on modified Winogradsky's media for ammonium and nitrite oxidizers²⁸. Aerobic Sulphur oxidizers (*Thiobacillus denitrificans* like organisms, TDLO) were enumerated on modified Leiske's medium^{29, 30}. Nitrifiers and TDLO appeared within 10-35 days.

Representative sediments were analyzed for bulk metal content (Fe, Ni and Co) at month zero and 33rd month after incubation under normal room temperature and pressure. Total Fe in sediments was analysed voltammetrically (Metrohm Limited, Herisau, Switzerland)³¹. The concentration of Ni and Co in samples was determined using hanging mercury drop electrode (HMDE) in the differential pulse mode^{32, 33}.

In case of epifluorescence counts, 10-15 microscopic fields cumulating to more than 500 cells were considered for deriving standard deviations. Other parameters were analyzed in triplicates. All parameters were normalized per gram of sediment dry weight unless mentioned otherwise. One way analysis of variance (ANOVA) was used for confirming the significance in variations. Correlation between microbial and biochemical parameters were analyzed for each of the sediment samples by Pearson's correlation after log₁₀ (x+1) transformation, using Statistica 6.

Table 2-Summary of changes in microbial and biochemical parameters on storage of CIB sediment samples under normal tropical conditions. Percentage of change is calculated with respect to initial and final observations [Decrease (-); Increase (+)]. TC-Total bacterial counts; ATP - Adenosine triphosphate; ZMA -Heterotrophs on ZoBell Marine Agar; NI - ammonium oxidizers; NII- Nitrite oxidizers; TDLO- *Thiobacillus denitrificans*-like organisms; Prot-Proteins; CHO- Carbohydrates; LOM-Labile organic matter

	TC	ATP	ZMA	NI	NII	TDLO	Prot	CHO	Lipid	LOM
SVBC 27	-97.49	-99.47	-96.71	-96.75	-99.37	-15.85	+78.56	+22.54	+12.47	+38.84
SVBC 19	-73.40	-83.71	-99.48	-99.79	-98.84	-42.65	-35.83	+10.41	+6.70	-8.38
SVBC 36	-98.25	-94.07	-99.92	-99.97	-99.88	-99.60	-88.80	+68.30	+35.55	-8.40%
SVBC 37	-94.14	-99.83	-99.76	-99.23	-99.80	+43.33	+426.18	+572.50	+18.49	+165.41

Results

TOC, TIC and C/N ratio obtained from the homogenized top 25 cms bsf are presented in table-1 as background environmental information. The standard deviations represent the down-core heterogeneity. Total proteins

increased in SVBC 27 and SVBC 37 by 79 and 426% respectively and decreased in SVBC 19 and SVBC 36 by 36 and 89% within 10 months' time (Fig. 2a).

Table 3a-Microbial and biochemical variations of CIB sediments at ambient tropical conditions at the beginning of storage. Only statistically significant interrelationships are mentioned here.

Inter-relating pairs	SVBC-27	SVBC-19	SVBC-36	SVBC-37
ATP-TC	-0.961**	-0.993***	-0.999***	
ZMA-TC	-0.907*			
NI-TC	-0.904***			
ZMA-ATP	0.999*	-0.936*	-0.967**	
PROTEIN-NII	0.906**			
PROTEIN-TC				0.905*
PROTEIN-TDLO	0.999***	-0.887*		-0.995***
CHO-NII	0.954**			
CHO-TC				0.902*
CHO-TDLO	0.885*	-0.999**		-0.964**
CHO-PROTEIN	0.924*	0.882*		
LIPID-NII	-0.875*			
LIPID-CHO	-0.949*			
LIPID-TDLO				-0.964**
LOM-NII	0.92*			
LOM-TDLO	0.998***			-0.952*
LOM-PROTEIN	0.999***	0.997***	0.882*	0.936*
LOM-CHO	0.934*			0.947*
NI-ATP		-0.962**	-0.979**	
NI-ZMA		0.98*	0.998***	
NII-ATP		-0.993***	-0.975**	
NII-ZMA		0.893*	0.999***	
NII-NI		0.95*	0.999***	0.984**
TDLO-TC			0.974**	-0.893*
TDLO-ATP			-0.969**	
TDLO-ZMA			0.999***	
TDLO-NI			0.999***	0.987**
TDLO-NII			0.999*	0.993***

Table 3b-Microbial and biochemical alterations at the end of storage indicating sample stabilization and loss in inter-sample variability. Only statistically significant interrelationships are mentioned here.

Inter-relating pairs	SVBC-27	SVBC-19	SVBC-36	SVBC-37
ZMA-ATP	0.999***	0.999***	0.999***	0.999***
ZMA-TC				0.906*
NI-ATP	0.999***	0.999***	0.999***	0.999***
NI-TC				0.894*
NI-ZMA	0.999***	0.999***	0.999***	0.999***
NII-ATP	0.999***	0.999***	0.999***	0.999***
NII-TC				0.889*
NII-ZMA	0.999***	0.999***	0.999***	0.999***
NII-NI	0.999***	0.999***	0.999***	0.999***
ATP-TC				0.887*

*p < 0.05 **p < 0.01

***p < 0.001

Table 4- Ratio of heterotrophs: autotrophs along latitudinal gradient at the onset of the experiment.

Sediment core	Heterotroph: Ammonium oxidizers	Heterotroph: TDLO
SVBC-27	2.65	0.88
SVBC-19	2.18	0.92
SVBC-36	1.03	0.44
SVBC-37	2.56	2.51

Total carbohydrates showed marginal to large increase in all four sediments after 10 months in the order SVBC 19 < SVBC 27 < SVBC 36 < SVBC 37 with 10, 23, 68 and 572% increase when compared to 0 day observations (Fig. 2b and Table 2). Total lipids show marginal increase in all four sediments in 10 months in the

order SVBC 19 < SVBC 27 < SVBC 37 < SVBC 36 with 7, 12, 18 and 36% increase (Fig. 2c and Table 2). The dominance of proteins among the constituents of labile organic matter is characteristic of most of the CIB sediments [13]. Therefore, variation in LOM reflects the variations in proteins (Figs 2a, 2d and Table 2).

Samples SVBC-27 and 37 shows 39 and 165% increase in LOM respectively at the end of 10 months, whereas both SVBC 19 and 36 show 8% decrease in LOM respectively during the same time (Fig 2d). Protein to carbohydrate ratio (PRT/CHO) increased by 46% in SVBC 27 and decreased by 42, 93 and 22 in SVBC 19, SVBC 36 and SVBC 37 respectively (Fig. 3a). Carbohydrate to labile organic matter ratio (CHO/LOM) decreased by 11% in SVBC 27 and increased by 21, 84 and 153% in SVBC 19, 36 and 37 (Fig. 3b).

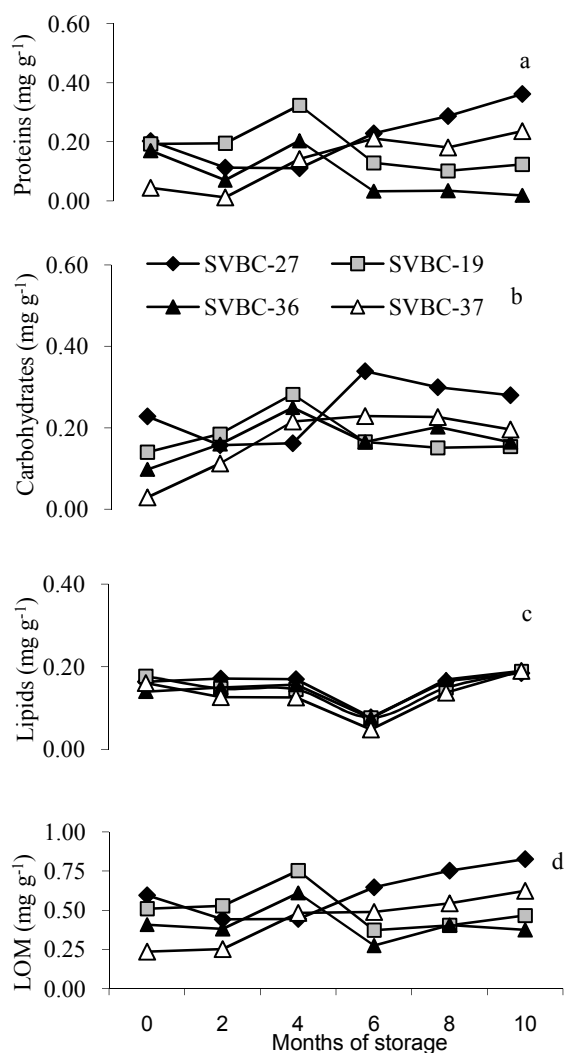


Fig. 2-Variation in labile biochemistry of the CIB samples over the storage period. a. Total proteins b. Total carbohydrates c. Total lipids d. Labile Organic Matter (LOM).

ATP content in all the four samples varied from 10² -10⁴ ng g⁻¹ dry sediment at the onset of the experiment. These variations narrowed down by an order and remained steady at 10³ - 10⁴ ng g⁻¹ dry sediment till 8 months. A drastic

fall in ATP was noted at the 10th month after which the samples stabilized between 10¹ -10² ng g⁻¹ dry sediment (Fig. 4a). The variation in ATP between the start and the end was significant at p<0.05.

Total counts of bacteria showed a decline by one order in SVBC 19 and nearly two orders in SVBC 27, SVBC 36 and SVBC 37 after a year (Fig. 4b). The variation was significant (p<0.05).

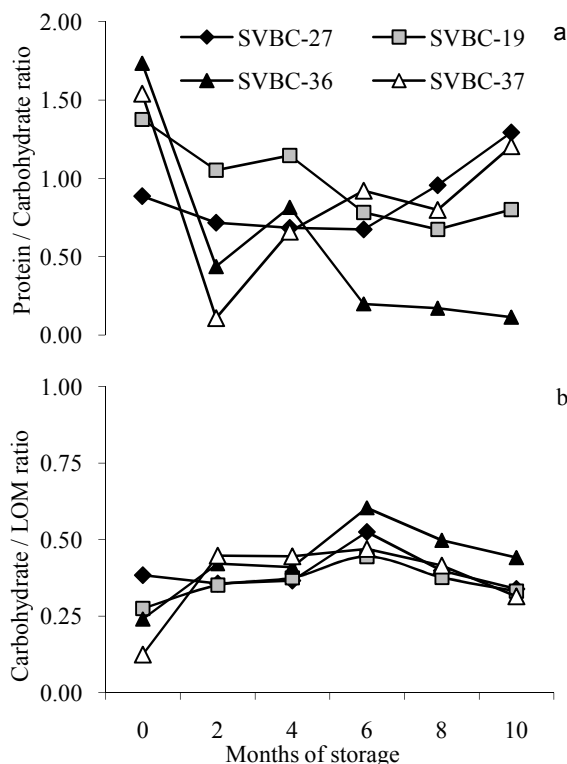


Fig. 3-Variation in biochemical ratios of CIB samples over the storage period. a. Protein/Carbohydrate ratio b. Carbohydrate/LOM ratio.

Both heterotrophs and the potential nitrifying autotrophs (ammonium oxidizers and nitrite oxidizers) showed a decline in culturable counts from 10⁵-10⁶ colony-forming units (cfu) to 10²-10³ cfu g⁻¹ dry sediment. The variation was significant at p<0.05. The TDLO were analyzed until the 8th month and showed a gradual decline from 10⁵-10⁶ to 10²-10³ cfu.g⁻¹ dry sediment (Fig 5).

The analyses of total metal content in the sediments did not show statistically significant difference between the values at month 0 and month 33 after storage at normal temperature and pressure. Total Fe was almost constant from 2.30±0.15% from month 0 to 2.28±0.51% in month 33 after incubation (n=5). Total Co

varied from 350.968 ± 55.73 ppm to 275.18 ± 95.58 ppm during the same time. Total Ni varied from 258.372 ± 10.64 ppm to 249.156 ± 23.97 ppm during the same time.

The variation of different parameters showed different patterns (Table 3) for each of the sediment samples. A summary of inter-relations between biochemical and microbial properties in the four sediment samples during the experimental time-course are presented in Table 3.

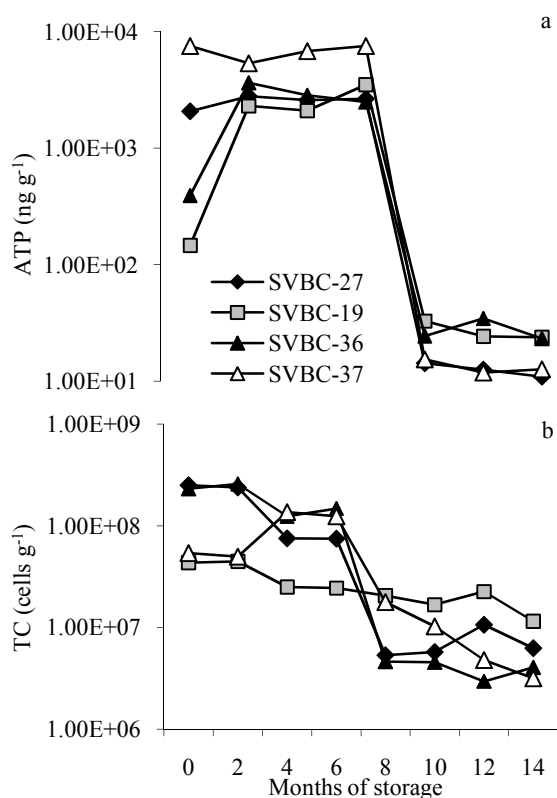


Fig. 4- Variation in microbial parameters of CIB samples over the storage period. a. ATP concentration b. Total bacterial counts.

Discussions

Archived soil and sediment samples from well-managed and carefully documented long-term field experiments are invaluable in monitoring changes due to different managements, environments and pollutant inputs³⁴⁻³⁸. Studies on stored or incubated sediments are rare in case of deep-sea sediment samples. The sediments of CIB form the substrate associated with deep-seabed polymetallic nodules rich in various metals like Fe, Mn, Ni and Co. To the best of our knowledge, this is the first attempt to study the effect of incubation at normal pressure and

ambient tropical temperature on deep-sea abyssal sediments.

It is hypothesized that samples being stored in repositories under ambient conditions of temperature may be sufficiently appropriate for studies related to ore quality, microbial succession pattern on storage, biochemical changes if any. The results of this study could be of interest to the researchers carrying out analysis of marine sediments, medical practitioners who may have to treat someone with allergy due to handling such samples as well as metallurgists involved in processing of ores associated with them.

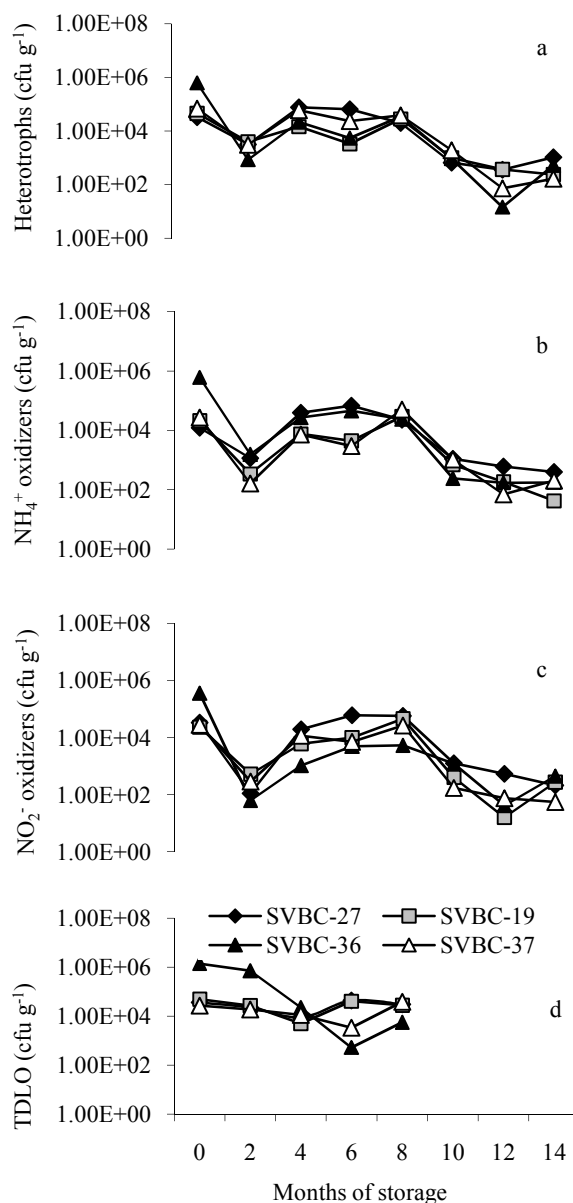


Fig. 5-Variation in the culturability of bacterial groups of the CIB samples over the storage period

The distribution of Labile organic matter (LOM) and protein/carbohydrate patterns in all the cores (Fig 2 and 3) indicate that initial non-steady biological reactions stabilize by eight to ten months, reiterating the normal behaviour of LOM in CIB sediments. The nature, dominance and distribution of LOM in CIB is determined by the relative recalcitrance and influx of fresher organic matter from terrestrial sources and export from detrital remains^{13,14,39}. Higher protein/carbohydrate ratio has been associated to fresher LOM²². Proteins form the dominant component of LOM in CIB, with the increasing dependence of LOM on protein shifting towards lipids as one proceeds from north to south.

Adenosine Triphosphate (ATP) decrease (Fig. 4a) is indicative of a fall in microbial biomass and activity and therefore increased stability of the stored samples. The results are consistent with previous work except that a four-time longer span is required for stabilization as compared to terrestrial organic-rich soils and sediment⁴⁰. The effect of water-trapped in the packets results in this two-order drop in ATP from the 0 month observation. Similar observations have also been noted earlier in terrestrial soils^{1,2,41}.

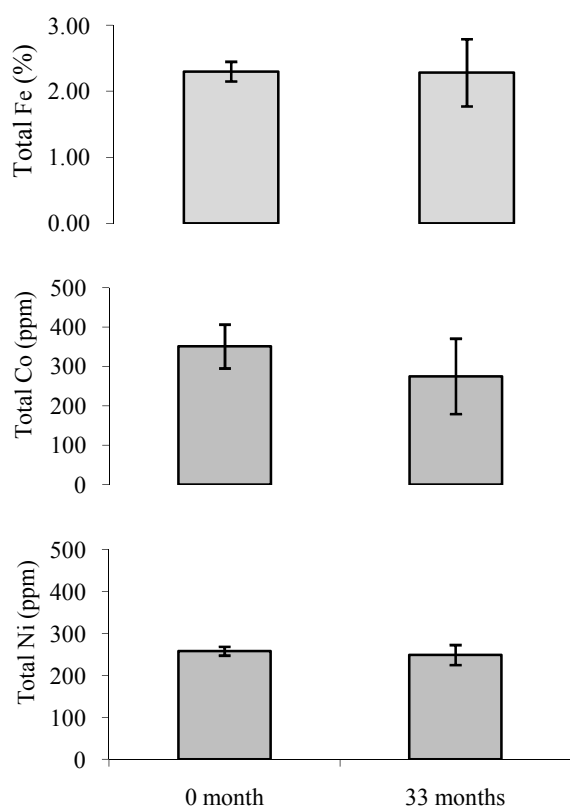


Fig 6- Variation of metal contents of the CIB samples over the storage period.

Both total counts (TC) and retrievable counts (RC) of bacteria are strongly influenced by storage and decrease by two orders at the end of the experiment. In contrast to this static storage, disturbance of sediments could however increase RC but decreases TC⁴². In the present experiment, interrelations between the analyzed parameters (Table 3) throw light on the nature of progressive changes in the biochemical and microbial properties. The close interrelationships of the different retrievable fractions to the changing LOM and ATP highlight the importance of retrievable bacteria as indicators of environmental and functional changes. Clear gradation of decreasing heterotrophy along latitudinal section (10°S to 16°S) is noted from the ratio of heterotrophs/ autotrophs at the onset of the experiment except for carbonaceous core SVBC 37 on top of the seamount which behaves similar to the northernmost siliceous core SVBC 27 (Table 4). Despite different sediment types, stabilization in different samples has occurred at the same time. The total metal contents have also remained constant over the period of storage.

The storage of these deep sea samples over a year does not alter the basic metal content of the sediment and are as suitable as fresh samples for the purpose of metal extraction. The total metal content remains same while the metal valency may change from reduced to oxidized forms on storage. In fact some of our other experimental studies under cold (4°C±2) showed enhanced metal mobilization/immobilization patterns³³ as compared to this experiment (at room temperature). This seems reasonable as the deep sediment microbial community is more active at the near zero temperatures.

The collection of polymetallic nodules or any other deep-sea minerals is challenging but will be the way of industrial output in the next decades. Keeping this socio-economic interest of the country and the world in mind, such details and mosaics of deep-sea sedimentary research are extremely important.

Conclusion

In the present study, the stabilization of the samples from non-steady microbially active phase to steady stabilized phase took at least 8-10 months under normal tropical conditions.

This stabilization of sediment microbial behavior is artificially brought about at a faster rate during storage making samples more suitable for geochemical analysis. The changes in the LOM and ATP are closely coupled to that of retrievable bacterial fractions rather than the total bacteria. This shows that the retrievable fraction could be a good proxy for observing functional changes in sediments over time. Present experiment recapitulates the intrinsic geochemical, biochemical and microbial nature of the sediments. The behavioral variations of culturable/retrievable bacteria in varying geological settings appear significant in deciding the distribution of organic matter and its labile components. Total metal contents remain constant over the period of storage. Increasing the time of observations or measuring the impact of humidity could throw more light on the effect of ambient storage conditions on the samples. Though the samples get altered both bacteriologically and biochemically over time, it is highly probable that the integrity of the bulk geochemical quality may be maintained as both oxidative and reductive processes of metals operate at temporal and spatial proximity.

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