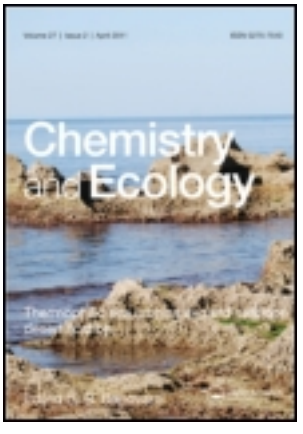

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Immobilisation of manganese, cobalt and nickel by deep-sea-sediment microbial communities

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Box core samples BC26 and BC36 from geologically different settings were examined to test the hypothesis that autochthonous microbial communities from polymetallic-nodule-rich Central Indian Basin sediments actively participate in immobilising metal ions. The bottom water dissolved oxygen concentration was reported to be 4.2–4.3 $mL \cdot L^{-1}$ in the northern siliceous ooze (BC26) and 4.1–4.2 $mL \cdot L^{-1}$ in the southern pelagic red clay (BC36); the sedimentation rates for these regions were 0.834 and 0.041 $cm \cdot kyr^{-1}$, respectively. An onboard experiment, conducted under oxic and sub-oxic conditions with 100 μmol of Mn, Co and Ni, showed that microbial immobilisation under sub-oxic conditions was higher than in azide-treated controls in BC26 for Mn, Co and Ni at 30, 2 and 4 cm below sea floor (bsf), respectively, after 45 days. The trend in immobilisation was BC26 > BC36, Co > Mn > Ni under oxic conditions and Mn > Co > Ni under sub-oxic conditions. The depth of maximum immobilisation for Co in BC26 under sub-oxic conditions coincided with the yield of cultured Co-tolerant bacteria and Ni only with organic carbon at 4 cm bsf. This study demonstrates that the organic carbon content and bioavailable metal concentrations in sediments regulate microbial participation in metal immobilisation.

Keywords: metals; microbes; immobilisation; sediment; Central Indian Basin

1. Introduction

Sediments are complex mixtures of Fe- and Mn-oxide mineral phases, detrital organic matter and several other organic and inorganic phases. Sediments support different groups of metal-oxidising and -reducing microorganisms [1]. Microbes in sediments can interact with dilute metal ions in solution, including sequestration, and aid in concentrating these ions in the sediment. The ions either remain in sediments as bio-metal complexes or undergo a series of microbial or chemical transformations. This might lead to the recycling of metals into the overlying water column or to their immobilisation in sediments as authigenic mineral phases [2].

The microbial immobilisation of metals can be catalytic or non-catalytic; it proceeds via four different mechanisms, namely biosorption, bioaccumulation, redox reaction and complex formation [3]. Immobilisation may be through cellular sequestration and accumulation, or through extracellular precipitation [4,5]. Marine bacteria produce large amounts of organic surface material that interacts with metal ions. The exopolysaccharides are subsequently involved in the precipitation

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of soluble metal cations and the formation of oxide species on bacterial walls, which chemically react with the residual metals in solution [6–8].

A significant portion of the metals and their various organic derivatives in nature are contained within the living biomass. For example, Razzell and Trussell [9] addressed the interaction of *Thiobacillus* spp. with sulphide-bearing ores and their role in the dissolution of oxides and the release of valuable metals. Likewise, Fortin et al. [10] and Basnakova and Macaskie [11] highlighted the contribution of bacteria to the formation of sulphide and phosphide minerals by chemical complexing with available constituent sulphide and phosphide groups. A few studies also explain the intracellular sequestration [12,13] and extracellular precipitation [14,15] of metals by a group of metal-resistant bacteria, each affecting metal mobility in the environment.

The deposition of organic carbon and the availability of oxygen from bottom waters are primary factors that affect the geochemical and biological processes in the sediment–water system [16]. The availability of nutrients and oxygen therefore determines the sensitivity and adaptation of an organism to metals. Sediments are the ultimate repositories for environmental particles, and microbial processes within the sediment can also control chemical changes as a response to metal exposure. Previous studies have shown the immobilisation of metals in sterile sediments by individual isolates [17,18]. This study aimed to investigate the immobilisation of metals by autochthonous microbial communities.

The study area covers two regions of the Central Indian Basin (CIB) (Figure 1) [19]. The region for box core (BC) 26 is located at 10°S latitude and 75.5°E longitude in the northern region of the ocean. The sampling site has a water depth of 5339 m, the environment is relatively less oxidising [20] and receives more clay from rivers [21,22]. BC36 is located at the southern region of the basin at 16°S latitude and 75.5°E longitude. The region has a water depth of 5042 m, the

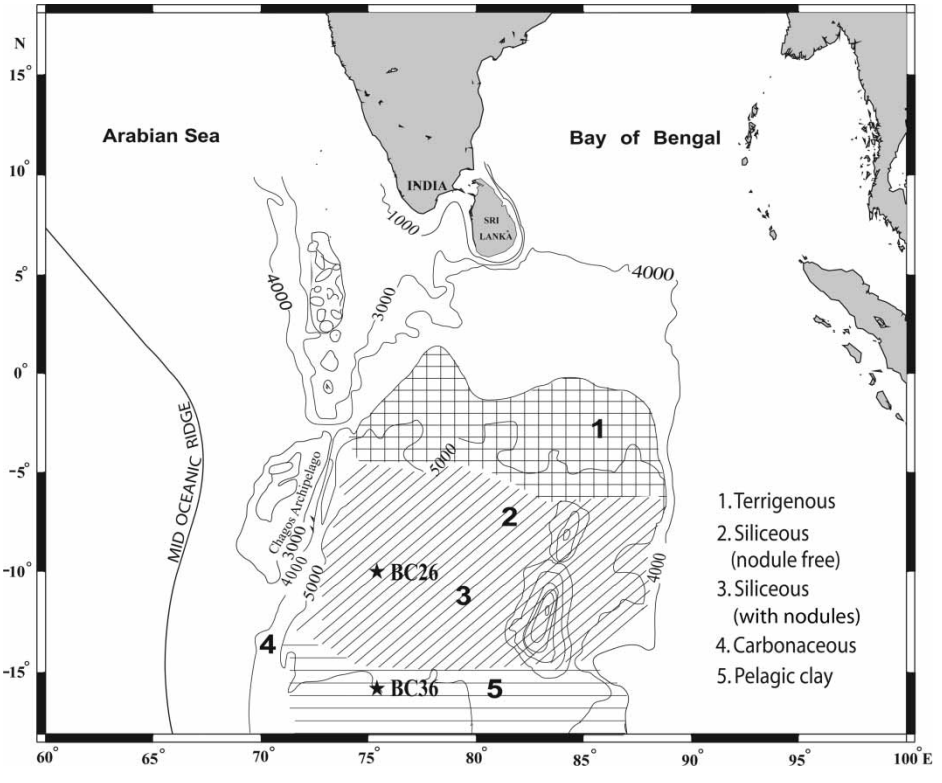

Figure 1. Area map shows sediment types and sampling locations for BC26 and BC36. Modified from Mascarenhas-Pereira et al. [19].

environment is more oxidising [20] and is known for the slower rate of sediment deposition [21]. The formation and dissolution of authigenic (oxyhydr)oxides of Fe and Mn influence the cycling of trace metals in oxic/sub-oxic surface sediments [23]. In view of that, we performed our experiment under oxic and sub-oxic incubation conditions to test the hypothesis that autochthonous microbial communities from CIB sediments actively participate in metal immobilisation. This study assesses the contribution of redox conditions, microbial abundance and activity to the immobilisation of Mn, Co and Ni.

2. Materials and methods

2.1. *Onboard sampling*

The samples were collected and processed on board Akademik Boris Petrov cruise number 26. Sediment cores were retrieved using a United States Naval Electronics Laboratory (USNEL) box corer of dimensions 50 × 50 × 50 cm. BC26 was collected from a siliceous ooze area and BC36 from a pelagic red clay region. The box cores were subsampled by inserting acrylic core liners of 6.3 cm diameter into the sediment. The sediment cores were further subsectioned at 2 cm intervals up to a depth of 10 cm and thereafter at 5 cm intervals up to a depth of 35 cm. Sediment samples were collected in sterile polyethylene bags and stored at 4 °C until further investigations.

2.2. *Total bacterial counts*

The sediment dilution for total bacterial counts (TBC) was prepared by suspending ∼2 g of homogenised sediment in 9 mL of sterile seawater. A small portion of the above dilution was fixed with buffered formalin to a final concentration of 2% and was stored at 4 °C until analysis [24]. The samples were sonicated at 15 Hz for 15 s, the slurry was allowed to settle for 30 s and 1 mL of the supernatant was filtered through a 0.22 μm black Millipore polycarbonate filter. Samples were then stained with 0.01% acridine orange for 3 min prior to microscopic observation. Approximately 10–15 microscopic fields were counted for each sample at ×1500 magnification using a Nikon 80i epifluorescence microscope (Nikon, Tokyo, Japan). Counts were normalised per gram of dry sediment.

2.3. *Plate counts*

Bacteria resistant to Mn, Co, Ni and heterotrophic bacteria were enumerated in triplicate using the spread plate method from the above dilution. The heterotrophs were grown on 20% ZoBell marine agar (ZMA). Metal-resistant bacteria were cultured on seawater agar (SWA) (1.5% bacto agar in natural seawater with no other additional nutrients) amended with 100 μmol of metal salts $MnCl_2{\cdot}4H_2O$, $CoCl_2{\cdot}6H_2O$ and $NiCl_2{\cdot}6H_2O$ (Sd. Fine Chem. Ltd). To isolate bacteria resistant to more than one metal, 100 μmol of each metal was added to SWA medium of the above composition. The inoculated plates were incubated at 3 ± 1 °C for 4–10 days and the colony forming units (cfu) were counted and normalised for gram dry weight of sediment.

2.4. *Eh measurements*

Eh measurements were made at the beginning and end of the experiments. Before the measurements, the Eh electrode was rinsed with distilled water and calibrated with oxidation reduction potential (ORP) standard solution to ensure accurate readings. For measurements, the electrode

was dipped slowly into the sediment slurry in tubes and allowed to stabilise for 60 s. The direct reading obtained by mV meter was taken in triplicate and the mean values were determined. Eh was measured following the user's guide (Redox/ORP Electrodes, Thermo Electron Corporation 2005).

2.5. *Determination of total organic carbon*

Total inorganic carbon (TIC) was analysed using a UIC CM 5014 Coulometer with $CaCO_3$ as the standard and total carbon with an NCS 2500 elemental analyser [25], cross-checked with a UIC CM 5014 Coulometer. Total organic carbon (TOC) was determined indirectly by subtracting the values of TIC from total carbon [26].

2.6. *Solid-phase concentration of metal ions in sediments*

The concentration of metal ions in the sediment was determined by voltammetry after total decomposition of 50 mg of lyophilised sediments using the closed vessel digestion method [27]. The Mn concentration in aqueous samples was determined following the method of Colombini and Fuocco [28] with two standard additions of $MnCl_2{\cdot}4H_2O$ ($4\,mg{\cdot}L^{-1}$). The concentration of Ni and Co in samples was determined using the modified method of Herrera-Melian et al. [29]. Analysis of the metals was carried out using a voltammeter in interface with 797VA computrace (Metrohm, Switzerland). The potential was measured against an Ag/AgCl reference electrode and a platinum rod as a counter electrode. Ni and Co determination was carried out with a hanging mercury drop electrode (HMDE) in the differential pulse mode. The determination was carried out at pH 9 in a measuring cell containing 10 mL of supporting electrolyte (ammonia buffer, pH 9.5) and 100 μL of dimethylglyoxime (0.1 mol) in ethanol. A sample volume (1 mL), depending on the concentration, was appropriately diluted with Milli-Q water (18.2 Ω resistance) prior to analysis. The sample was added directly to the measuring vessel containing the electrolyte and was degassed with 99.9995% nitrogen (Medgas and Equipments) for 300 s prior to deposition. The analysis was carried out with a deposition time of 30 s, an equilibration time of 5 s and a voltage step time of 0.3 s. The potential was scanned in replications of 3 from -0.699 to -1.2 V with sweep rate of $6\,mV{\cdot}s^{-1}$. The analysis was quantified with two standard stock additions of Ni^{2+} ($NiCl_2{\cdot}6H_2O$, $1\,mg{\cdot}L^{-1}$) and Co^{2+} ($CoCl_2{\cdot}6H_2O$, $1\,mg{\cdot}L^{-1}$).

2.7. *Demonstration of metal immobilisation*

Box core sediments collected from two distinct regions of CIB were used to set up the laboratory experiments onboard. Portions of sediments from each subsection of the cores were inoculated using sterile spatula into 15 mL screw-capped tubes containing 100 μmol of metal chloride salts dissolved in sterile seawater. Suitable azide (10 mmol) poisoned controls to check for metal adsorption and autoclaved natural seawater amended with metal salts without any inocula (sterile controls) for abiotic precipitation were maintained. The incubation was carried out in triplicate at $3 \pm 1\,^{\circ}C$ (to simulate *in situ* temperatures) under oxic and sub-oxic conditions. The oxic incubation was assured by directly inoculating 1.5 ± 0.5 g wet sediment in half-filled tubes and sub-oxic in completely filled tubes.

2.8. *Metal analysis*

The supernatant (1 mL) from inoculated and uninoculated tubes after centrifugation (8000 rpm for 10 min at 4 °C) were analysed on day 0 and at the end of 45 days to determine the change in metal concentration. The residual concentration of metals in each tube was analysed using

a spectrophotometric (Multiskan Thermo Spectrum) method. Any samples that were delayed in analysis were acidified with 1 N HCl and stored at 4 °C. The Mn concentration in the sample was determined using the 1-(2-pyridylazo)-2-naphthol method at 560 nm [30]. Determination of Ni with dimethylglyoxime and Co with nitroso-R-salt were carried out according to the scheme of Chester and Hughes [31] at 460 and 500 nm, respectively. Mean and SD were calculated for microbially (biotic) and non-microbially (abiotic) promoted metal immobilisation. Values were corrected for chemical precipitation in both the experimental setups. One-way analysis of variance (ANOVA) and correlation analysis including the levels of significance were done for data points $n = 10$ using Statistica.

2.9. *Determination of sediment dry weight*

The sediment slurry from each tube was mixed and poured onto a preweighed filter positioned in a filtration set-up at the end of analysis. The filter with sediment was dried at 105 °C and reweighed until constant. The filter weight was subtracted from the sediment weight to derive the actual dry weight of the sediment. The concentration of the immobilised metal was deduced and normalised for gram dry weight sediment after correcting for corresponding controls.

3. Results and discussion

3.1. *Characteristics of the two sampling stations*

Two sediment cores BC26 and BC36 were collected along the north–south transect of the CIB and were analysed for immobilisation of Mn, Co and Ni by the sediment microbial communities. The sediment slurry was found to support the growth of both heterotrophic and metal-resistant bacteria. The yield of cultured heterotrophic bacteria varied from below detectable levels (BDL) to $5.4 \pm 0.73 \times 10^3$ cfu·g^{-1} dry sediment in BC26 (Table 1) and $8.0 \pm 0.18 \times 10^4$ to $2.4 \pm 0.37 \times 10^5$ cfu·g^{-1} sediment in BC36 with maximum culturability at 8 cm depth (Table 2). The metal-resistant bacteria count ranged from BDL to $9.0 \pm 0.11 \times 10^2$ for Ni at 10 cm depth, $4.3 \pm 0.31 \times 10^4$ for Co and Mn at 2 and 20 cm depth, respectively. In mixtures of Mn, Co and Ni (100 μmol each), the culturability of bacteria was $1.4 \pm 0.33 \times 10^3$ cfu·g^{-1} at 10 cm depth in BC26 (Table 1). With BC36 it was $2.0 \pm 0.1 \times 10^4$ to $2.8 \pm 0.04 \times 10^5$ cfu·g^{-1} for Co, with maximum culturability at 10 cm depth, $3.9 \pm 0.06 \times 10^4$ to $2.4 \pm 0.31 \times 10^5$ for Ni, 1.2 ± 0.24 to $3.9 \pm 0.02 \times 10^5$ for Mn and $5.0 \pm 0.02 \times 10^4$ to $2.2 \pm 0.18 \times 10^5$ cfu·g^{-1} for metals in combination with maximum culturability at 4 cm depth (Table 2).

Although the total bacterial abundance did not show a definite trend with the depth of the cores (Figure 2a,b), the distribution of organic carbon did. The organic carbon in sediments varied from 0.175 to 0.485% in BC26 (Figure 2c) and 0.09 to 0.19% sediment dry weights in BC36 (Figure 2d) with maximum carbon concentration at 4 and 2 cm depth, respectively. The TOC content in seawater was 21.78 mg C·L^{-1} in BC26 and 9.18 mg C·L^{-1} in BC36. The abiotic precipitation of metal ions in uninoculated sterile controls was negligible. Eh as a measure of oxidising and reducing conditions showed that BC26 could support sub-oxic and BC36 the oxic processes better (Figure 2e,f). The Eh in BC26 experimental tubes decreased from +80.2 mV to +11.98 mV (± 2.6, $n = 3$) in the oxic and +6.24 mV (± 3.2, $n = 3$) in the sub-oxic incubations at 35 and 30 cm bsf, respectively. The Eh in BC36 decreased from +79.96 mV to +28.36 mV (± 2.6, n = 3) in the oxic and +25.52 mV (± 3.2, n = 3) in the sub-oxic incubations at 2 and 10 cm, respectively, for Mn (Table 3).

The maximum solid-phase concentration of Mn (14.93 μmol·g^{-1}) and Ni (4.55 μmol·g^{-1}) in BC26 was observed at 8 cm depth and Co (6.24 μmol·g^{-1}) at 30 cm depth. The concentration of Mn

Table 1. Plate counts of heterotrophic and metal-resistant bacteria in 1.5% bacto agar-amended seawater with and without metal chlorides (100 μmol) in BC26.

Depth		SWA				
bsf (cm)	ZMA	No metal	Ni	Co	Mn	Ni–Co–Mn
2	$1.5 \pm 0.18 \times 10^2$	BDL	BDL	$4.3 \pm 0.31 \times 10^4$	$2.5 \pm 0.22 \times 10^4$	BDL
4	$2.0 \pm 0.40 \times 10^2$	BDL	BDL	$2.7 \pm 0.15 \times 10^4$	$1.5 \pm 0.25 \times 10^4$	BDL
6	BDL	BDL	BDL	$1.5 \pm 0.10 \times 10^3$	$6.0 \pm 0.05 \times 10^2$	BDL
8	$5.4 \pm 0.73 \times 10^3$	BDL	BDL	$1.7 \pm 0.32 \times 10^4$	$4.1 \pm 0.61 \times 10^4$	BDL
10	$5.0 \pm 0.28 \times 10^1$	$1.5 \pm 0.57 \times 10^2$	$9.0 \pm 0.11 \times 10^2$	$6.6 \pm 0.60 \times 10^3$	$3.8 \pm 0.72 \times 10^4$	$1.4 \pm 0.33 \times 10^3$
15	BDL	BDL	BDL	$1.7 \pm 0.13 \times 10^4$	$3.9 \pm 0.23 \times 10^4$	BDL
20	BDL	BDL	BDL	$1.2 \pm 0.24 \times 10^4$	$4.3 \pm 0.45 \times 10^4$	BDL
25	$1.4 \pm 0.11 \times 10^3$	BDL	BDL	BDL	BDL	BDL
30	$5.0 \pm 0.02 \times 10^1$	BDL	BDL	BDL	BDL	BDL
35	BDL	BDL	BDL	BDL	BDL	BDL

Note: All the values in the table represent colony forming units (cfu)·g^{-1} dry weight of sediment. The data are given as mean ± SD, $n = 3$. ZMA, ZoBell marine agar; SWA, seawater agar; bsf, below sea floor; BDL, below detection level. For details, see text (Section 2.3).

Table 2. Plate counts of heterotrophic and metal-resistant bacteria in 1.5% bacto agar-amended seawater with and without metal chlorides (100 μmol) in BC36.

Depth		SWA				
bsf (cm)	ZMA	No metal	Ni	Co	Mn	Ni–Co–Mn
2	$8.0 \pm 0.18 \times 10^4$	$6.8 \pm 0.70 \times 10^4$	$1.6 \pm 0.02 \times 10^5$	$2.6 \pm 0.23 \times 10^5$	$1.8 \pm 0.10 \times 10^5$	$1.9 \pm 0.13 \times 10^5$
4	$1.0 \pm 0.11 \times 10^5$	$6.1 \pm 0.06 \times 10^4$	$2.4 \pm 0.31 \times 10^5$	$1.7 \pm 0.07 \times 10^5$	$3.9 \pm 0.02 \times 10^5$	$2.2 \pm 0.18 \times 10^5$
6	$8.8 \pm 0.09 \times 10^4$	$5.4 \pm 0.42 \times 10^4$	$9.1 \pm 0.46 \times 10^4$	$1.3 \pm 0.35 \times 10^5$	$3.3 \pm 0.21 \times 10^5$	$1.6 \pm 0.09 \times 10^5$
8	$2.4 \pm 0.37 \times 10^5$	$3.5 \pm 0.51 \times 10^4$	$2.1 \pm 0.10 \times 10^5$	$1.5 \pm 0.16 \times 10^5$	$2.4 \pm 0.03 \times 10^5$	$6.8 \pm 0.42 \times 10^4$
10	$1.6 \pm 0.24 \times 10^5$	$5.0 \pm 0.02 \times 10^4$	$1.6 \pm 0.05 \times 10^5$	$2.8 \pm 0.04 \times 10^5$	$1.3 \pm 0.09 \times 10^5$	$5.0 \pm 0.02 \times 10^4$
15	$1.2 \pm 0.08 \times 10^5$	$1.2 \pm 0.07 \times 10^4$	$3.9 \pm 0.06 \times 10^4$	$1.4 \pm 0.05 \times 10^5$	$1.2 \pm 0.24 \times 10^5$	$6.2 \pm 0.18 \times 10^4$
20	$1.4 \pm 0.15 \times 10^5$	$7.1 \pm 0.15 \times 10^4$	$5.1 \pm 0.31 \times 10^4$	$6.6 \pm 0.02 \times 10^4$	$1.7 \pm 0.11 \times 10^5$	$1.6 \pm 0.31 \times 10^5$
25	$1.6 \pm 0.26 \times 10^5$	$2.5 \pm 0.11 \times 10^4$	$4.2 \pm 0.17 \times 10^4$	$2.0 \pm 0.10 \times 10^4$	$1.7 \pm 0.07 \times 10^5$	$7.9 \pm 0.01 \times 10^4$
30	$2.0 \pm 0.24 \times 10^5$	$3.1 \pm 0.32 \times 10^4$	$9.9 \pm 0.06 \times 10^4$	$1.0 \pm 0.08 \times 10^5$	$2.2 \pm 0.05 \times 10^5$	$1.5 \pm 0.11 \times 10^5$
35	$1.7 \pm 0.09 \times 10^5$	$3.0 \pm 0.13 \times 10^4$	$9.5 \pm 0.22 \times 10^4$	$4.8 \pm 0.15 \times 10^4$	$2.4 \pm 0.21 \times 10^5$	$7.2 \pm 0.45 \times 10^4$

Note: All the values in the table represent colony forming units (cfu)·g^{-1} dry weight of sediment. The data are given as mean ± SD, $n = 3$. ZMA, ZoBell marine agar; SWA, seawater agar; bsf, below sea floor; BDL, below detection level. For details, see text (Section 2.3).

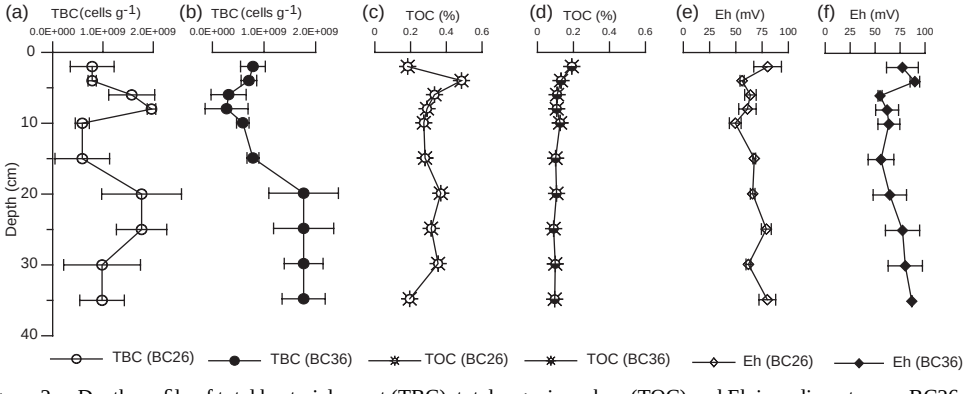

Figure 2. Depth profile of total bacterial count (TBC), total organic carbon (TOC) and Eh in sediment cores BC26 and BC36 (mean ± SD, $n = 10$ for TBC and $n = 3$ for Eh).

Table 3. Maximum immobilisation of metals at various depths below sea floor (bsf) compared with maximum in plate counts (colony forming units; cfu).

		Core BC26								
		Biotic				Abiotic				
		Oxic[a]		Sub-oxic[b]		Oxic[c]		Sub-oxic[d]		
Metal	Parameters	Value	Depth (cm)	Value	Depth (cm)	Value	Depth (cm)	Value	Depth (cm)	Inference
Mn	Immobilisation (μmol)	34.4 ± 0.053	35	85.6 ± 0.047	30	11.6 ± 0.011	35	22.5 ± 0.047	35	a. Suggests that oxidised Mn is less prone to dissolution at this depth
	Plate counts (cfu)									b. Suggests bacterial participation
										c. Suggests that 86% of the variation in biotic oxidation could
	i) Seawater agar	–		–		–		–		be due to the variation in abiotic
										d. Suggests passive immobilisation on Mn-oxide phase
Co	Immobilisation (μmol)	35 ± 0.33	2	46.3 ± 0.29	2	8.45 ± 0.055	35	6.97 ± 0.027	10	a. Immobilisation of Co by other metal-tolerant bacteria in the sediments
	Plate counts (cfu)									b. Suggests bacterial participation
										c. Only passive process could be involved
	i) Seawater agar	–		–		–		–		d. Suggests passive immobilisation on Mn oxide phase
	ii) Co	4.3×10^4		4.3×10^4		–		–		
Ni	Immobilisation (μmol)	6.3 ± 0.055	25	6 ± 0.08	4	0.98 ± 0.008	25	0.65 ± 0.012	4	a. Ni immobilisation may be catalysed more by bacteria resistant to Ni or Co
	Plate counts (cfu)									b. Energy-dependent low-affinity uptake process
										c. Complexation of Ni with organic carbon in sediments
	i) Seawater agar	–		–		–		–		d. Complexation of Ni with organic carbon in sediments could
	ii) ZoBell marine agar	–		–		–		–		contribute to its immobilisation at a much lower rate

(*Continued*)

Table 3. Continued.

		Core BC36								
		Biotic				Abiotic				
		Oxic[e]		Sub-oxic[f]		Oxic[g]		Sub-oxic[h]		
Metal	Parameters	Value	Depth (cm)	Value	Depth (cm)	Value	Depth (cm)	Value	Depth (cm)	Inference
Mn	Immobilisation (μmol)	19.76 ± 0.41	2	13.44 ± 0.04	10	25.33 ± 0.04	6	22.89 ± 0.03	6	e. Suggests mixotrophic mode of nutrition f. Suggests that high background Mn concentration along with the amended Mn could be toxic g. Could be regulated by concentration of the metal and the availability of particle-binding sites in sediments h. Active process of immobilisation is inhibited by azide and not the passive
	Plate counts (cfu)									
	i) Seawater agar	6.8×10^4		–		–		–		
Co	Immobilisation (μmol)	45 ± 0.028	2	27.5 ± 0.537	6	6.43 ± 0.011	8	7.18 ± 0.024	8	e. Could be by process in which Ni-immobilising bacteria catalyse Co immobilisation and vice versa f. Suggests co-oxidation of Co by Mn-oxidising microorganisms g. Could be regulated by concentration of the metal and the availability of particle-binding sites in sediments h. Could be by a metabolism-independent process like cell surface adsorption/precipitation
	Plate counts (cfu)									
	i) Seawater agar	6.8×10^4		–		–		–		
	ii) Co	–		–		–		–		
Ni	Immobilisation (μmol)	7.03 ± 0.09	2	6.6 ± 0.075	8	0.95 ± 0.009	25	0.703 ± 0.01	4	e. Suggests that the same microbial communities are capable of immobilising both the metals f. Suggests that the same microbial communities are capable of immobilising both the metals g. Could be regulated by concentration of the metal and the availability of particle-binding sites in sediments h. Could be by a metabolism-independent process like cell-surface adsorption/precipitation
	Plate counts (cfu)									
	i) Seawater agar	6.8×10^4		–		–		–		
	ii) ZoBell marine agar	–		2.4×10^5		–		–		

Note: All the values in the table correspond to gram dry weight of sediment. The data in the table are given as mean ± SD, $n = 3$. For details, see text (Sections 2 and 3).

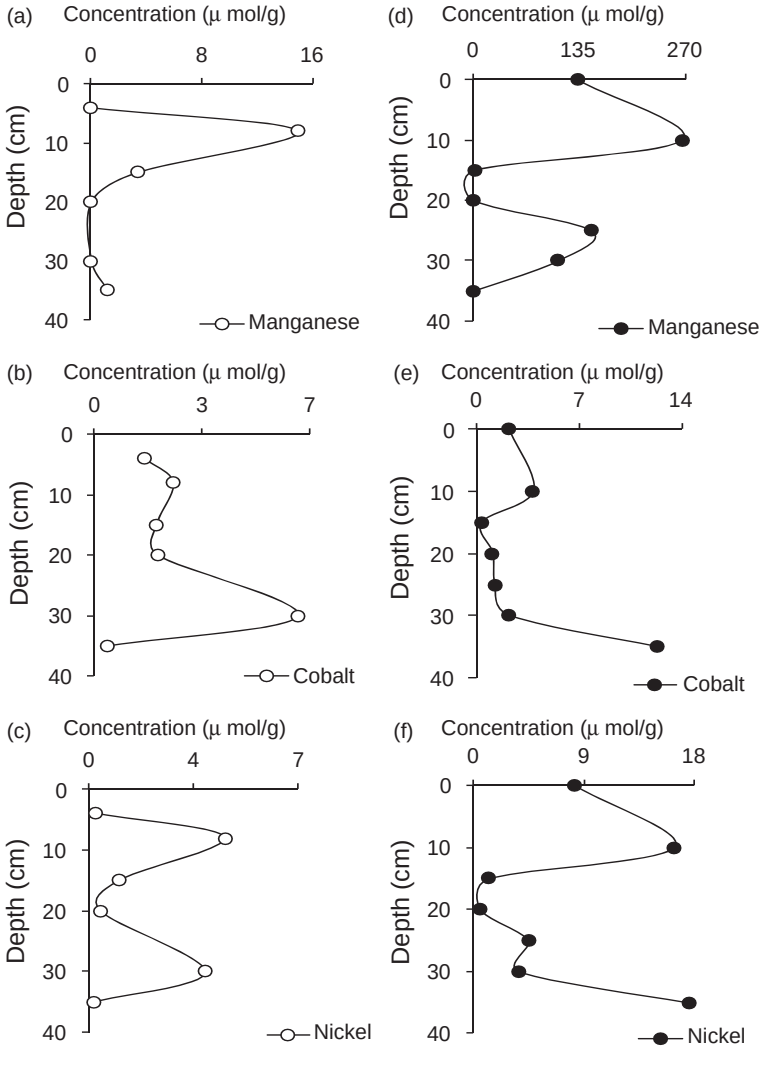

Figure 3. Solid-phase concentrations (single measurements) of Mn, Co and Ni in sediment cores collected from the Central Indian Basin. (a–c) Metal concentrations in BC26, (d–f) metal concentrations in BC36.

in BC36 sediment core was maximum (265.8 μmol·g^{-1}) at 10 cm depth and Co (12.29 μmol·g^{-1}) and Ni (17.52 μmol·g^{-1}) at 35 cm depth (Figure 3). The concentration of the above metal ions was found to be unevenly distributed in the sediment cores. The maximum concentration for all the three metals was observed towards the surface, decreased with depth and showed a secondary maximum towards the subsurface in both the sediment cores. Our results agree with the report of Zhang et al. [32] on the double maxima for solute profiles.

3.2. *Immobilisation under oxic condition in BC26*

3.2.1. *Biotic immobilisation*

The results showed higher Mn immobilisation in the subsurface sediment (Figure 4a). The immobilisation of the metal was 34.4 ± 0.053 μmol·g^{-1} dry sediment at 35 cm bsf. Manganese

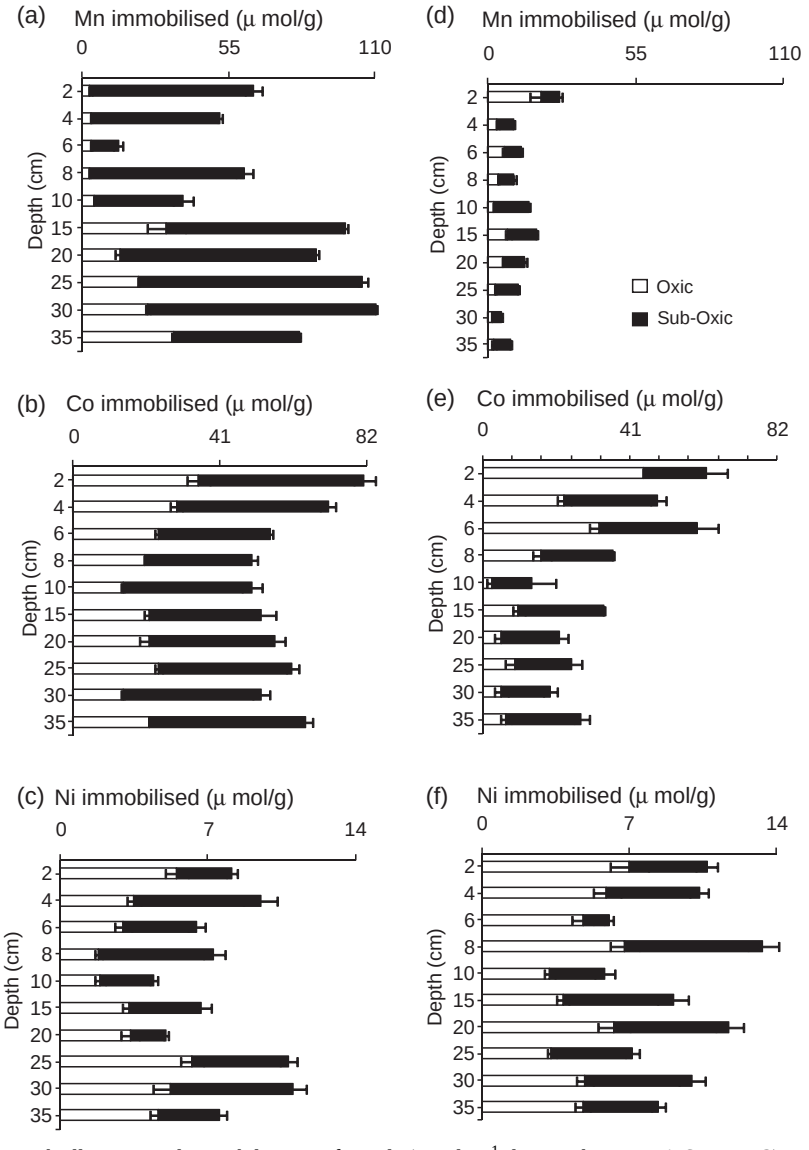

Figure 4. Microbially promoted immobilisation of metals ($\mu mol \cdot g^{-1}$ dry weight, mean $\pm$ SD, $n = 3$) in experiments as a function of depth. (a–c) The immobilisation of metals in BC26, (d–f) the immobilisation of metals in BC36. The error bars are shown on the left-hand side for oxic incubation and on the right-hand side for sub-oxic incubation.

immobilisation increased with depth ($r = 0.852$, $p < 0.001$). The yield of cultured heterotrophic bacteria co-varied with TBC at an r-value of 0.602 ($p < 0.05$) suggesting that the variation in the former is responsible for 36% of the variation in the latter. The immobilisation maximum observed at the deeper depth suggests that oxidised Mn is stable at this depth and is less prone to dissolution. The subsurface manganese maximum observed at 30–35 cm depth [33–35] in 13 different sediment cores collected from CIB substantiates the present finding.

By contrast to Mn, higher Co immobilisation ($35 \pm 0.328\ \mu mol \cdot g^{-1}$) was recorded at the sediment surface (Figure 4b). Interestingly, although the immobilisation of this metal was maximum at the surface and coincided with the maximum yield of cultured Co tolerant bacteria (Table 3), this process showed a negative relationship with the yield of cultured Co tolerant bacteria by an

r-value of 0.745 ($p < 0.01$). This probably suggests that immobilisation of Co is mediated by other metal-immobilising bacteria in the sediments. The association of Co with Mn in the surface sediment is one process in which Mn-oxidising bacteria participate indirectly in Co immobilisation. The results agree with the observations of Sundby et al. [36] and Glasby [37] on Co enrichment in the surface sediments. Our observations also support the earlier findings [38,39] on the co-oxidation of Co with Mn in the aquatic systems. Likewise, immobilisation of Co is known to co-occur with iron reduction in bacteria [40].

Like Mn, higher immobilisation of Ni ($6.3 \pm 0.055\,\mu mol{\cdot}g^{-1}$) was observed at the deeper 25 cm layer. The immobilisation of Ni negatively related with the yield of Mn tolerant bacteria by r-value of 0.643 ($p < 0.05$). This relation suggests that Ni immobilisation may not be catalysed by Mn-oxidising bacteria but more by bacteria resistant to Ni or Co. Further it is also suggested that in certain microorganisms, the resistance to one metal can also confer resistance to the other metals depending on the bioavailability and combination of metals. Interestingly, a study by Stoppel and Schlegel [41] has shown that bacteria tolerant to Ni are also tolerant to Co and posses determinants that resemble each other.

3.2.2. *Abiotic immobilisation*

The abiotic immobilisation was comparatively less than the biotic immobilisation because only the passive process could be involved. The difference could be as much as three to four times in the case of Mn, four to six times in the case of Co, and six times in the case of Ni. The maximum immobilisation of Mn (11.61 ± 0.011) and Co ($8.45 \pm 0.06\,\mu mol{\cdot}g^{-1}$) occurred at 30–35 cm bsf and Ni ($0.98 \pm 0.08\,\mu mol{\cdot}g^{-1}$) at 25 cm bsf (Figure 5). The Mn immobilisation showed depth dependence with an r-value of 0.928 ($p < 0.001$) and was also related to biotic Mn oxidation with an r-value of 0.930 ($p < 0.001$), suggesting that 86% of the variation in biotic Mn oxidation could be due to the variation in abiotic oxidation. The BC26 sediments are composed of siliceous ooze and have low concentrations of metal ions (Figure 3). Our results shows that abiotic metal immobilisation proceeds slowly and the rate depends upon the oxygen concentration, redox condition prevailing in the sediments, and the rate of the reverse activity ie dissolution. The results agree with the reports of Muller et al. [42] and Glasby [37] on surficial diagenesis and the regeneration rate of Mn in the sediments with that of accretion rate of the metal in the associated polymetallic nodules. The passive sorption of metal ions on sediment and mineral particles occurs by physical attraction and/or chemical precipitation.

3.3. *Immobilisation under sub-oxic condition in BC26*

3.3.1. *Biotic immobilisation*

As mentioned above, biotic immobilisation is definitely greater than abiotic immobilisation. Sub-oxic immobilisation is 2.4 times greater with Mn and 1.3 times greater with Co than oxic.

Under sub-oxic conditions, the microbial immobilisation of Mn ($85.6 \pm 0.047\,\mu mol{\cdot}g^{-1}$) was maximum at 30 cm bsf. Maximum Co ($46.3 \pm 0.29\,\mu mol{\cdot}g^{-1}$) immobilisation was at 2–4 cm bsf and is synchronised with the maximum yield of cultured Co-tolerant bacteria ($4.3 \pm 0.31 \times 10^4\,cfu{\cdot}g^{-1}$) and organic carbon (0.485%), suggesting bacterial participation. Our results agree with previous studies [43–47] which reported a positive correlation between heavy metal concentrations and the percentage of bacteria resistant to metals in different soils and sediments. However, maximum Ni immobilisation ($6 \pm 0.08\,\mu mol{\cdot}g^{-1}$) was evident at 4 cm depth (Figure 4c) and was synchronised with the organic carbon concentration (0.485%) in sediment, suggesting an energy-dependent low-affinity uptake process. The results are in agreement with Jasper and Silver [48] and

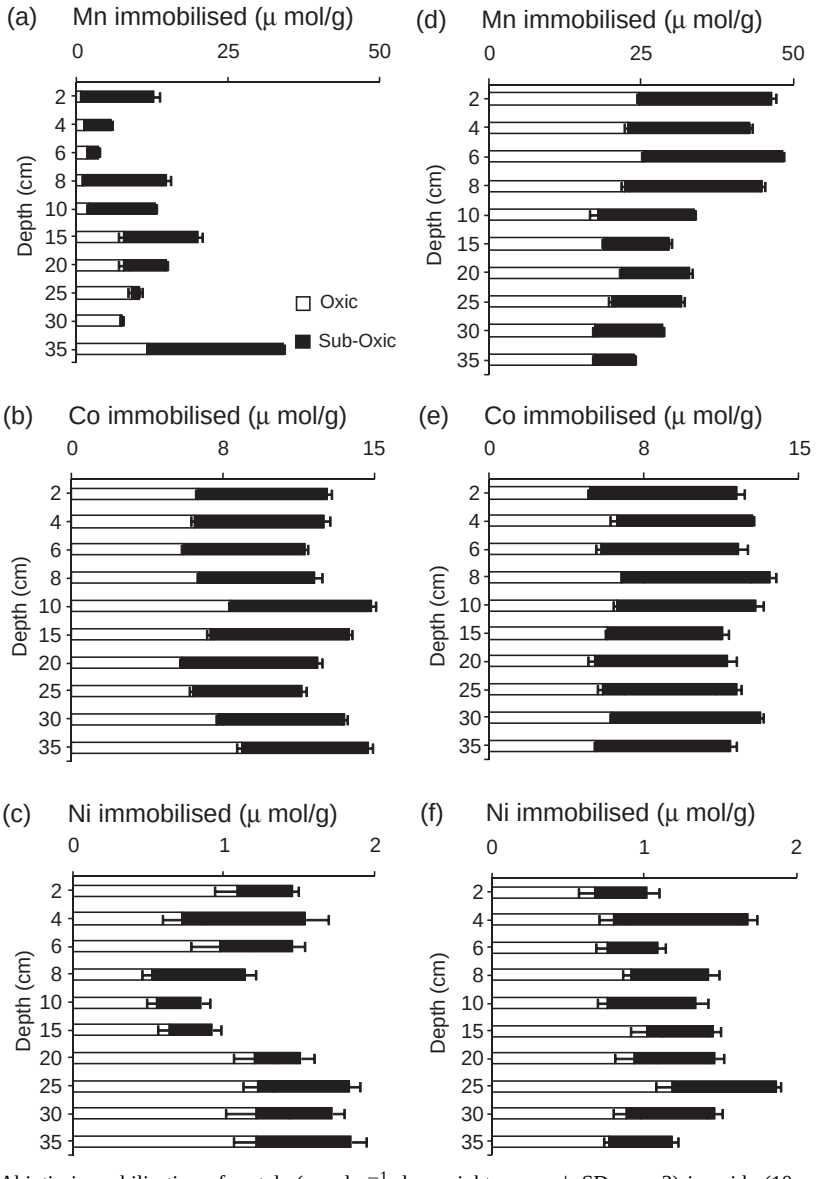

Figure 5. Abiotic immobilisation of metals ($\mu mol \cdot g^{-1}$ dry weight, mean ± SD, $n = 3$) in azide (10 mmol)-treated sediments as a function of depth. (a–c) The adsorption of metals in BC26, (d–f) the adsorption of metals in BC36. Error bars are shown on the left-hand side for oxic incubation and on the right-hand side for sub-oxic incubation.

Bryson and Drake [49] on energy-dependent Ni transport in bacteria and the chemo-organotrophic mode of nutrition in microorganisms in the presence of nutrients.

3.3.2. *Abiotic immobilisation*

It is again emphasised that abiotic immobilisation is less than biotic immobilisation, especially under sub-oxic conditions. The immobilisation profile of Mn was similar to that occurring under oxic conditions with a maximum of $22.5 \pm 0.047\ \mu mol \cdot g^{-1}$ at 35 cm bsf (Figure 5a). Peaks in Co and Ni immobilisation of 6.97 ± 0.027 and $0.65 \pm 0.012\ \mu mol \cdot g^{-1}$ were at 10 and 4 cm bsf,

respectively (Figure 5b,c). The distribution of Mn and Co did not match the organic carbon content of the sediment. The immobilisation detected at low levels in poisoned sediments suggests passive immobilisation on the Mn oxide phase. Our results agree with earlier reports on such trapping of metal ions by manganese oxides [50,51]. However, the immobilisation of Ni was less than Co and corresponded with the organic carbon (0.485%) content of the sediment. Thus, organic carbon in sediments might also complex with Ni and contribute to its immobilisation at a much lower rate than under biotic conditions. Such an association of Ni with organic carbon in sediments has been observed previously by Turner et al. [52] and Xue et al. [53].

3.4. *Immobilisation under oxic condition in BC36*

3.4.1. *Biotic immobilisation*

In this core, biotic immobilisation is greater than abiotic immobilisation, except in the case of Mn where it is the other way round. Under oxic conditions, immobilisation of all three metals was maximum at the sediment surface (2 cm bsf) and was lower than in BC26. The trend in the immobilisation was Co ($45 \pm 0.028\,\mu mol{\cdot}g^{-1}$) > Mn ($19.8 \pm 0.41\,\mu mol{\cdot}g^{-1}$) > Ni ($7 \pm 0.09\,\mu mol{\cdot}g^{-1}$), as shown in Figure 4d–f. The solid-phase concentrations of metal ions were Mn > Ni > Co. Thus, the depth at which maximum immobilisation occurred did not match with the depth that showed a maximum for the solid-phase concentration of metal ions. This suggests that the gradient of the metal concentration was more optimal for immobilisation at the surface than at the subsurface. The organic carbon content of the core was maximum (0.19%) at the surface and is related to the immobilisation of Mn with an r-value of 0.835 ($p < 0.01$), suggesting a mixotrophic mode of nutrition. The culturability of bacteria tolerant to Mn–Co–Ni was maximum (1.9 to $2.2 \times 10^5\,cfu{\cdot}g^{-1}$) within the 0–4 cm depth in multiple metal-amended plates. These observations suggest that microbially mediated immobilisation of the above metals in BC36 sediments is driven by the nutritive status of the sediment and the predominance of metal-tolerant bacteria. It is consistent with the observation of Dean-Ross and Mills [54], that groups of microbial communities that are relatively abundant in the sediment can utilise one or more aromatic compounds and are resistant to one or more heavy metals.

The microbial immobilisation of Co was synchronised with the maximum in Mn immobilisation. The yield of cultured Co-tolerant bacteria was positively related to TOC and yield of Ni-tolerant bacteria with r-values of 0.766 ($p < 0.01$) and 0.606 ($p < 0.05$), respectively. These relations suggest that Ni and Co immobilisation are mediated by the same microbes to a certain extent (36%) and 58% variation in both is dependent on the variation in TOC. The ability of Ni-resistant bacteria to immobilise Co is evident from previous experimental records [55]. However, Co and Ni immobilisation can be independent of Mn immobilisation and might occur by a process in which Ni-immobilising bacteria catalyse Co immobilisation and vice versa.

The immobilisation of Ni was comparatively lower than the other metals. However, the yield of cultured Ni-tolerant bacteria might correlate with the yield of Co-tolerant bacteria ($r = 0.606$, $p < 0.05$), suggesting that the same microbial communities are capable of immobilising both metals. These results agree with our recent reports [4,56] on Ni and Co immobilisation by Mn-oxidising bacterial isolates from the Indian Ridge system. TBC correlated positively ($r = 0.868$, $p < 0.001$) and TOC negatively with depth ($r = 0.666$, $p < 0.05$). These observations suggest that microbial abundance, the diversity of their metabolic activities and the number of metal-resistant bacteria in the sediment direct the immobilisation of Ni in BC36 sediments. Nonetheless, TOC in sediments were also found to influence the bioavailability of the metal in sediments. It is clear from the report of D'Hondt et al. [57] that organisms that depend on electron-accepting

pathways with higher standard free energies may have higher energy requirements than organisms that depend on pathways with lower standard free-energy yields.

3.4.2. *Abiotic immobilisation*

Although the prevailing environmental conditions in these sediments are appropriate for microbial immobilisation of Mn, abiotic immobilisation of the metal was higher than biotic immobilisation. It is suggested that processes that occur in these sediments could be more passive than active. The maximum immobilisation of Mn ($25.33 \pm 0.04\,\mu mol{\cdot}g^{-1}$) occurred at 6 cm depth (Figure 5d). Co and Ni immobilisation continued to remain higher under biotic conditions and showed maximum abiotic immobilisation of $6.43 \pm 0.011\,\mu mol{\cdot}g^{-1}$ for Co and $0.95 \pm 0.009\,\mu mol{\cdot}g^{-1}$ for Ni at 8 and 25 cm depth, respectively (Figure 5e,f). The immobilisation of metal ions did not relate with organic carbon or the natural concentration of the metal ions in sediments. The results suggest that immobilisation by abiotic processes is directed to changes with concentration of the metal and the availability of charged particle binding sites in sediments. Our results agree with a previous report [58] on trace metal binding to reactive sediment surfaces.

3.5. ***Immobilisation under sub-oxic condition in BC36***

3.5.1. *Biotic immobilisation*

Unlike under oxic conditions, the immobilisation of Mn was maximum ($13.5 \pm 0.035\,\mu mol{\cdot}g^{-1}$) at the greater depth of 10 cm bsf. Co and Ni continued to show maximum immobilisation of 27.5 ± 0.54 and $6.6 \pm 0.075\,\mu mol{\cdot}g^{-1}$ at shallower depths of 6 and 8 cm bsf, respectively (Figure 4e,f). Thereafter, a gradual decrease in immobilisation was observed towards the bottom of the core. Earlier investigations [54] on bacterial-community composition and function along a heavy-metal gradient provide the explanation that bacteria are not exposed to the same concentrations of heavy metal *in situ* as in laboratory-prepared media. However, our study did not use laboratory-prepared organic media. Moreover, it simulates deep-sea conditions by avoiding any organic amendments and maintaining the temperature at $3 \pm 1\,^{\circ}C$. Therefore, it is suggested that our results might reflect *in situ* trends to a certain extent. The maximum immobilisation of Mn and Ni coincided with peak culturability of heterotrophic bacteria and the solid-phase sediment concentration of metal ions.

The immobilisation of Mn showed a negative relationship with the yield of cultured Mn-tolerant bacteria ($r = 0.609$, $p < 0.05$). This suggests that the background concentration along with the amended Mn might be beyond the tolerance limit. The toxicity of excess Mn is evident from earlier studies [14, 59]. By contrast, the depth of the greatest Co immobilisation was synchronised with the maximum culturability of multiple metal-tolerant bacteria and the organic carbon content of the sediments. The immobilisation of Co showed correlation with the yield of cultured Mn-tolerant bacteria ($r = 0.677$, $p < 0.05$). The association of Co with Mn and its co-oxidation by Mn-oxidising microorganisms result in immobilisation. Our results agree with those of Lienemann et al. [60] on the oxidation of Co in association with Mn in aquatic systems. It is therefore inferred that the metal concentration in the sediment, its organic carbon content and the microorganisms present might govern the active immobilisation of metal ions.

3.5.2. *Abiotic immobilisation*

At this sampling site, abiotic immobilisation of metal ions is higher than the biotic immobilisation. Maximum immobilisation of Mn ($22.89 \pm 0.03\,\mu mol{\cdot}g^{-1}$) occurred at 6 cm, Co

($7.18 \pm 0.024\,\mu mol{\cdot}g^{-1}$) at 8 cm and Ni ($0.70 \pm 0.01\,\mu mol{\cdot}g^{-1}$) at 4 cm bsf (Figure 5). The depth of maximum immobilisation of Co matched that of maximum culturability of heterotrophic bacteria from the biotic samples. Also, the passive rates with the azide-treated sediments were much lower for Co and Ni than untreated samples. Similarly, depth of immobilisation of Ni coincided with the yield of cultured Ni-tolerant and Mn–Co–Ni-tolerant bacterial numbers in biotic samples. Results suggest that when used as a poison, azide restricts the active process of metal immobilisation by bacteria, but does not inhibit passive metal ion binding to the bacterial cell surfaces. Our results agree previous reports [61] that surface adsorption is possible even in non-viable cells, albeit at low levels. Heavy metals can be immobilised by metabolism-independent process on cell-surface components and charged particles in sediment, depending upon the milieu.

3.6. *Immobilisation under oxic vs. sub-oxic conditions*

Microbial immobilisation of metals under sub-oxic conditions was significantly higher ($p < 0.001$) in BC26 than in BC36 and was higher than in azide-treated controls. The trend in immobilisation was Co ($35 \pm 0.328\,\mu mol{\cdot}g^{-1}$) > Mn ($34.4 \pm 0.053\,\mu mol{\cdot}g^{-1}$) > Ni ($6.3 \pm 0.055\,\mu mol{\cdot}g^{-1}$) under oxic conditions, and Mn ($85.6 \pm 0.047\,\mu mol{\cdot}g^{-1}$) > Co ($46.3 \pm 0.29\,\mu mol{\cdot}g^{-1}$) > Ni ($6 \pm 0.08\,\mu mol{\cdot}g^{-1}$) under sub-oxic conditions. In BC36, the metal immobilisation was higher under oxic conditions. The trend in immobilisation was Co ($45 \pm 0.028\,\mu mol{\cdot}g^{-1}$) > Mn ($19.8 \pm 0.41\,\mu mol{\cdot}g^{-1}$) > Ni ($7 \pm 0.09\,\mu mol{\cdot}g^{-1}$) under oxic conditions and Co ($27.5 \pm 0.54\,\mu mol{\cdot}g^{-1}$) > Mn ($13.44 \pm 0.035\,\mu mol{\cdot}g^{-1}$) > Ni ($6.6 \pm 0.075\,\mu mol{\cdot}g^{-1}$) under sub-oxic conditions. The immobilisation rates of metals in these sediments were mostly Mn > Co > Ni. Generally, the immobilising rates of Mn and Co were greater at BC26 than at BC36, with the rates under sub-oxic conditions being higher than under oxic conditions. Such discernible differences were not seen in the case of Ni.

4. Summary and conclusions

From this study it is clear that the higher immobilisation of metals under sub-oxic conditions in the subsurface sediment was not greatly influenced by organic carbon, but rather by the higher availability of reduced metal ions. The significant differences in metal immobilisation between the experiment and poisoned controls probably indicate the involvement of both active and passive process of metal immobilisation in the experiment and the latter process only in the case of control. This study demonstrates that organic carbon content and the concentration of the bioavailable metals in sediments regulate microbial participation in metal immobilisation.

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