

Precipitation of Iron in Windowpane Oyster Shells by Marine Shell-Boring Cyanobacteria

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Abstract Shells of windowpane oyster (*Placuna placenta* (L.)) in the intertidal zone of the Zuari estuary, Goa, were often found to be black in color. Microscopical observation of partially decalcified shells showed the presence of cyanobacterial filaments encrusted with black precipitate. Microchemical test (Prussian blue reaction) and wavelength dispersive x-ray analysis confirmed this precipitate to be of iron. Mineralogical studies of this black precipitate, using x-ray diffraction and scanning electron microscopy, suggested the presence of iron as iron oxides. The cyanobacteria from such black shells were cultured in enriched seawater medium. In this medium also they precipitated iron as confirmed by Prussian blue reaction. They were identified as *Plectonema terebrans* Born et Flah and *Phormidium* sp.

Keywords Iron, windowpane oyster shell, shell-boring cyanobacteria, marine.

Introduction

Biological metal accumulation by a wide variety of organisms, ranging from prokaryotes to eukaryotes, is a well-known phenomenon (Lowenstam 1981). In some cases reduced metals are used as an energy source, as in iron bacteria, or as electron donors or acceptors (Ehrlich 1978). In others, they are acted upon to detoxify the environment and precipitate the excess toxic metals (Murphy and Levy 1983) or to concentrate iron for growth and eliminate competition (Murphy et al. 1976). Deposition of iron as ferric hydroxide in nodules and sheaths in species of green algae (Nicholls and Fung 1982) and in mucilaginous sheaths of freshwater cyanobacteria (Hindak 1982; Hickel and Polingher 1988) has been reported. Magnetite (Fe_3O_4) biomineralization by freshwater and marine magnetotactic and nonmagnetotactic anaerobic bacteria (Frankel and Blakemore 1979; Lovley et al. 1987; Bazylinski et al. 1988) has aroused considerable interest in magnetism and magnetoreception by organisms.

Biogenic iron deposition by endolithic cyanobacteria and fungi in rocks has been reported by Hessland (1949) and Krumbein and Jens (1981). We are reporting here on marine shell-boring filamentous cyanobacteria, responsible for deposition of iron in the shells of *Placuna placenta* (L.), commonly called windowpane oyster.

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Materials and Methods

Collection and Preparation of Samples

Infested black and healthy white shells were collected from intertidal zones of the Zuari estuary, Goa (15°33' N and 73°50' E). The shells were thoroughly washed with sterile seawater and decalcified using 0.5 N HCl or 5% EDTA solution.

Culturing of Cyanobacteria

The cyanobacterial filaments, isolated from shells decalcified with EDTA, were washed with sterile seawater and inoculated in enriched seawater-ES medium (McLachlan 1973) in a 12-h dark/light cycle.

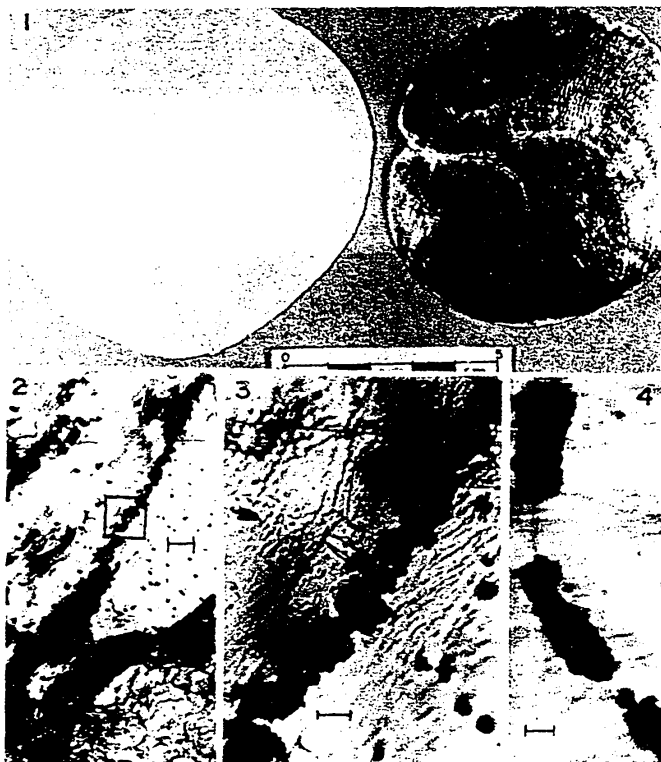
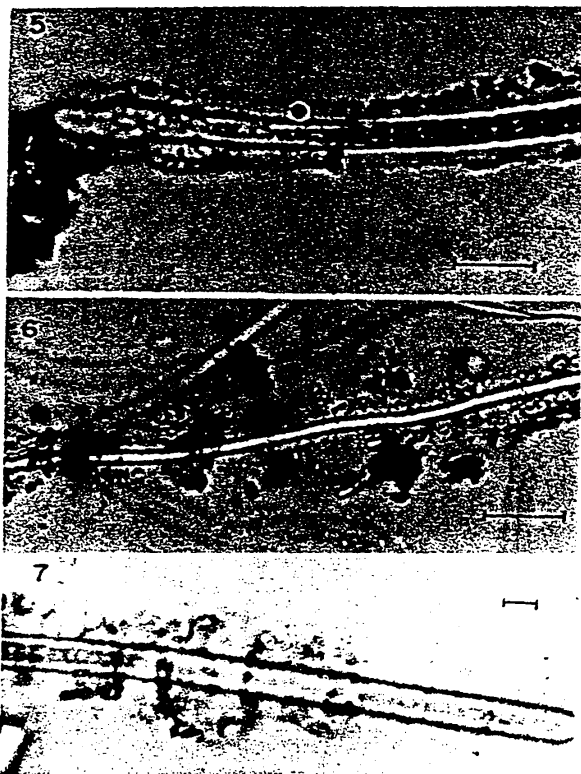


Figure 1. Healthy and infested shells. Figures 2-4. Partially decalcified shells; (2) general view, bar = 50 μm ; (3) enlarged, bar = 10 μm ; (4) filamentous organisms encrusted with black precipitate as revealed by partial decalcification of shell with 0.5 N HCl, bar = 10 μm .



Figures 5 and 6. Cyanobacterial filaments removed after dissolving the black shell in HCl. Deposits around and on the filaments showed positive Prussian blue reaction, bar = 10 μm . Figure 7. Cyanobacterial filament isolated from decalcified shell and stained for iron by the Prussian blue reaction; bar = 20 μm .

Microchemical Test

To detect the presence of iron on the surface of cyanobacterial filaments in cultures and thin pieces of decalcified shells, a Prussian blue reaction was applied (Shermann, 1967) using aqueous potassium ferrocyanide in 1% HCl. Ferrocyanide produces a deep blue color with ferric iron.

Scanning Electron Microscopy

For SEM preparation, the decalcified thin pieces of black shell and the cyanobacterial cultures were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.5 for 1–2 h, dehydrated in a graded series of acetone, and critical-point freeze-dried in a CPD apparatus (Polaron) for 1 h. They were coated with carbon and gold and examined in an electron microscope with scanning attachment (JEOL 100 CX, Japan).



Figure 8. Iron deposits in the culture of *Phormidium* sp., bar = 10 μ m.

Wavelength Dispersive x-Ray Analysis

Cyanobacterial filaments from the culture as well as thin slices of black and white shells and acid-insoluble residue from black shells were air-dried and coated with carbon and gold. A scanning electron microscope equipped with a wavelength dispersive spectrometer (WDS) (Camebax-Micro, 571 Model, Cameca) together with two programs (R. Quali and R. Quanti programs, Cameca software) for qualitative elemental checks was used.

Quantitative Estimation of Iron

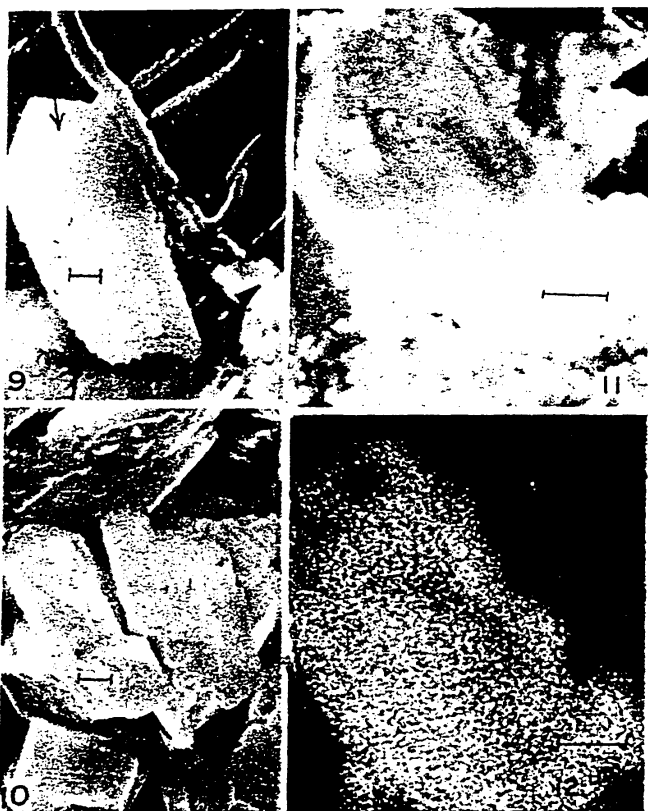
Black and white shells (10 replicates each) and cyanobacterial cultures (five replicates) were digested with nitric acid at 100°C for 12 h. Toward the end, 1 mL of perchloric acid was added and the mixture taken to dryness. The residue was dissolved in double-distilled water and diluted to 10 mL. Iron concentration was estimated with a double-beam atomic absorption spectrophotometer (GBC Scientific Equipment, model 902).

For preliminary estimations, a spectrophotometric method using the TPTZ (2,4,6-tripyridil 1,3,5,5-triazine) reagent was found satisfactory (Grasshoff et al. 1983).

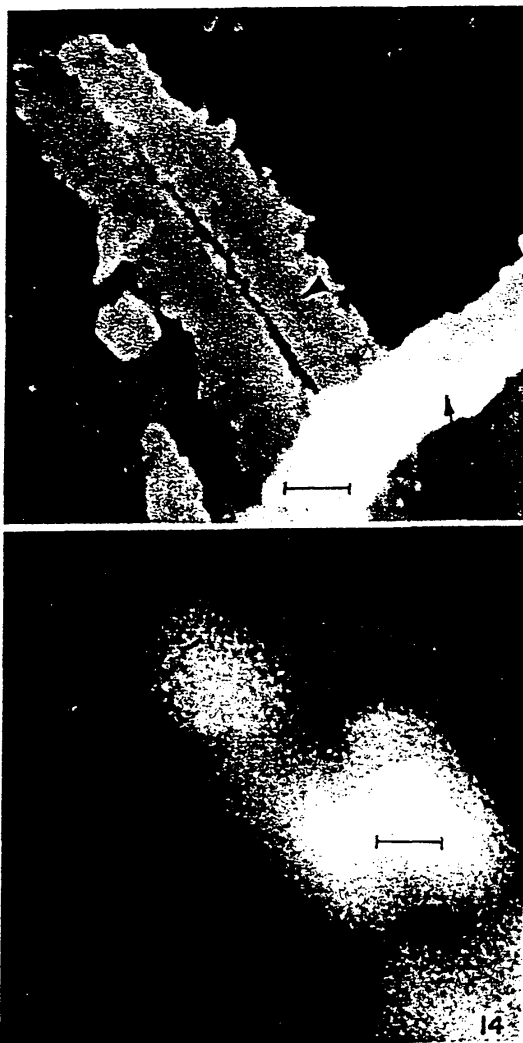
Results and Discussion

Placuna placenta occurs buried in mud in the subtidal zone in the Zuari estuary, Goa. Shells of dead animals are washed ashore and found distributed from the low- to the high-tide watermarks in the intertidal zone. The shells at the low-tide water mark are heavily infested with shell-boring green algae and cyanobacteria and are green in color as a result (Raghukumar et al. 1988). The shells at the high-tide water mark are not infested by shell-boring microorganisms and are therefore white in color. Many shells in the intertidal region are found to be greenish black to completely black in color (Fig. 1).

Microscopic observation of partially decalcified black shells, using 0.5 N HCl, revealed the presence of filamentous shell-boring cyanobacteria encrusted with heavy black granular deposits (Figs. 2-4). Red autofluorescence of these filaments under an



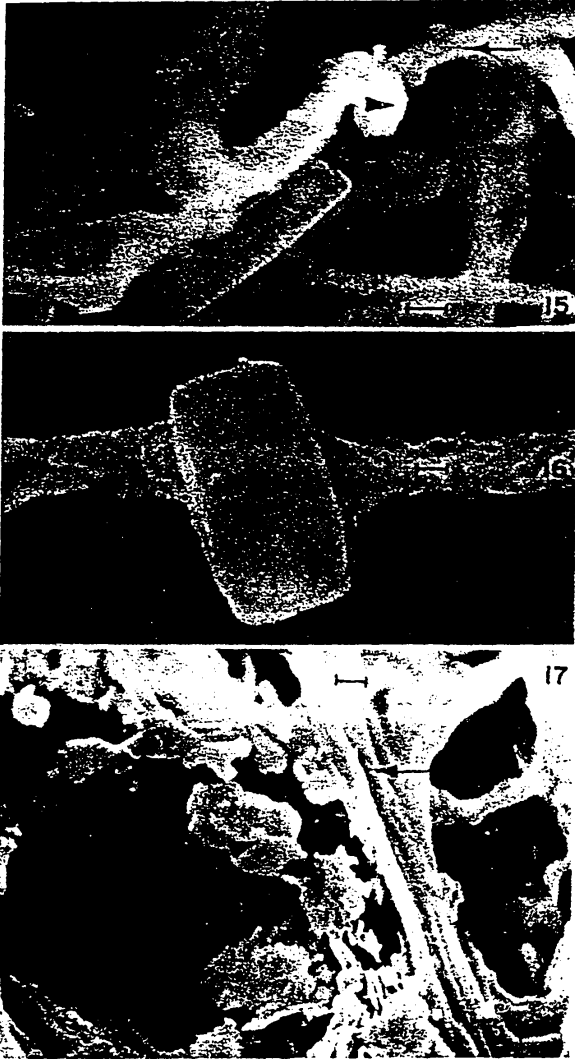
Figures 9-11. SEM photomicrographs. (9) Crystalline deposit (arrow) near a cyanobacterial filament and amorphous (arrowhead) deposits on the same in a partially decalcified shell, bar = 1 μ m. (10) Crystal structure from the acid-insoluble residue from a black shell, bar = 1 μ m. (11) Crystal on a partially decalcified black shell (near a cyanobacterial filament), bar = 25 μ m. Figure 12. Distribution of iron as white spots in the same crystal using wavelength dispersive analysis system, bar = 25 μ m. The material in Figs. 9-12 gave a positive reaction for iron by microchemical test.



Figures 13 and 14. Cyanobacterial filaments. (13) SEM of filament (arrow) with iron precipitate (arrow-head), bar = 0.66 μm . (14) Distribution of iron as white spots in the same precipitate using wavelength dispersive analysis, bar = 0.66 μm .

epifluorescence microscope confirmed them to be shell-boring cyanobacteria. Such filaments were separated from the shell by further dissolution of the shell with dilute HCl. However, such a treatment also removed and disturbed part of the black deposits around the filaments. The deposits were present both within the mucilaginous sheath of the filaments and outside. These deposits proved positive for iron using the Prussian blue

Iron Precipitation in Oyster Shells



Figures 15–17. SEM photomicrographs of cyanobacterial iron deposits. (15) Cyanobacterial filamen: encrusted with amorphous deposits, with arrow indicating naked cyanobacterial filament and arrowhead indicating iron deposits on the same filament, bar = 1 μm . (16) Crystalline deposits in critical-point freeze-dried culture of *Phormidium* sp., bar = 1 μm . (17) Amorphous granular deposits in critical-point freeze-dried culture of *Plectonema terebrans*, with arrow indicating bundles of cyanobacterial filament and arrowhead the iron deposits, bar = 2 μm .

(PB) reaction (Figs. 5-7). Only the black deposits, and not the white region in the shell, showed positive PB reaction. The white shells did not show a positive PB reaction. The greenish black shells showed the presence of chlorophyll as well as iron.

The cyanobacteria from the black shells were isolated in culture using enriched seawater medium (Es) (McLachlan 1973) and were identified as *Plectonema terebrans* Born et Flah and *Phormidium* sp. Black or orange-brown deposits formed around the cyanobacterial filaments in such cultures (Fig. 8). Prussian blue reaction confirmed such precipitate to be iron. This trait of precipitation has been retained by these organisms on repeated subculturing in ES medium over a year.

Scanning electron microscopy of a black shell (Fig. 9) showed bundles of cyanobacterial filaments encrusted with amorphous (arrowhead) and crystalline deposits (arrow). An SEM photomicrograph of acid-insoluble residue from a black shell (Fig. 10) revealed the presence of similar crystals. Such crystals were octahedral and rhombohedral in shape, morphologically resembling magnetite and hematite (Kostove 1968). Wavelength dispersive x-ray analysis of the crystals present around the filaments in black shells revealed a high concentration of iron (> 3000 counts) (Figs. 11-14).

Scanning electron microscopy of critical-point freeze-dried samples from cultures (Figs. 15-17) revealed bundles of cyanobacterial filaments associated with amorphous (Figs. 15 and 17) or crystalline (Fig. 16) deposits. Microchemical tests of the same freeze-dried samples showed such deposits to be iron.

The x-ray diffraction studies of the black acid-insoluble residue showed the presence of iron oxides. Dilute HCl completely dissolved away calcium from the shell and also a major part of the black precipitate. The cyanobacterial filaments remained as part of the organic debris in this. Less than 5% of iron remained in this precipitate and thus did not give a smooth diffractogram. The white shell powder did not show the presence of iron oxides.

Quantitative estimation of iron, first by spectrophotometry and later by atomic absorption spectrophotometry (Table 1), showed an absence of iron in the white shells and 10-12 mg total iron in black shells. The iron contents of waters in the Zuari estuary at the collection sites vary from 15 to 75 $\mu\text{g/L}$ (Anonymous 1979). Thus there appears to be a 700 to 1000-fold increase in iron in shells from ambient concentration caused by the shell-boring cyanobacteria *Plectonema terebrans* and *Phormidium* sp.

The cyanobacteria appeared to accumulate iron from the flowing waters. Other shell-boring cyanobacteria, namely, *Hyella* sp. and *Mastigocoleus testarum* Lagerh., were normally present in the greenish black shells of windowpane oyster (Raghukumar

Table 1
Quantitative Estimation of Iron in Various Substrates

Substrate	Iron Concentration	Method
White shell	0.3 (\pm 0.01) mg/g shell ^a	Atomic absorption
Black shell	11.0 (\pm 4.0) mg/g shell ^a	Atomic absorption
<i>Plectonema terebrans</i>	4.3 (\pm 1.5) mg/g dry wt. ^b	Atomic absorption
Ambient seawater	15 to 75 $\mu\text{g/L}$ during a year ^c	Atomic absorption

^aMean of 10 replicates.

^bMean of 5 replicates.

^cAnonymous (1979).

Table 2
Summary of Methods Employed for Characterization of Iron in Various Substrates

Tests	Application	
	Black Shell	Culture
Microchemical test (Prussian blue reaction)	Examination of thin pieces of black shell and acid-insoluble residue from black shells	Examination of live cultures of <i>Plectonema terebrans</i> and <i>Phormidium</i> sp.
Wavelength dispersive x-ray analysis	Examination of thin pieces of black shell and acid-insoluble residue from black shells	Examination of dried cultures of <i>Plectonema terebrans</i>
Scanning electron microscopy	Examination of thin pieces of black shell and acid-insoluble residue from black shells	Examination of critical-point freeze-dried cultures of the cyanobacteria
x-Ray diffraction	Examination of acid-insoluble residue from black shell	

et al. 1988). However, they were seldom present in the black shells. When they occurred, they were devoid of chlorophyll and appeared unhealthy.

The various methods (Table 2) employed during this study indicated that the black coloration of shells was due to the presence of iron. This accumulation was brought about by the associated shell-boring cyanobacteria. This was further confirmed by the precipitation of iron by these two organisms in culture. The white shells, on the other hand, lacked the cyanobacteria and the associated iron precipitate.

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References

- Anonymous. 1979. Master plan for pollution control of the rivers Zuari and Mandovi. Technical Report No. 02/79. National Institute of Oceanography, Dona Paula, Goa, India.
- Bazyliński, D. A., R. B. Frankel, and H. W. Jannasch. 1988. Anaerobic magnetite production by a marine magnetotactic bacterium. *Nature* 334:518-519.
- Ehrlich, H. L. 1978. Inorganic energy sources for chemolithotrophic and mixotrophic bacteria. *Geomicrobiol. J.* 1:65-83.
- Frankel, R. B., and R. P. Blakemore. 1979. Magnetite in freshwater magnetotactic bacteria. *Science* 203:1355.
- Grasshoff, K., M. Ehrhardt, and K. Kremling. 1983. *Methods of Seawater Analysis*. Verlag Chemie, Weinheim.
- Hessland, I. 1949. Lower Ordovician penetrative and enveloping algae from the Siljan District. *Bull. Geol. Inst. Univ. Uppsala* 33:409-427.
- Hickel, B., and U. Pollinger. 1988. Mass development of an iron precipitating cyanophyte

- (*Cyanodicyron imperfectum*) in a subtropical lake (Lake Kinneret, Israel). *Phycologia* 27:291-294.
- Hindak, F. 1982. On some planktonic coccoid blue greens and characteristics of iron precipitates. *Arch. Hydrobiol. (Suppl.)* 63:241-258.
- Kostov, I. 1968. *Mineralogy*. Oliver and Boyd, Edinburgh, 580 pp.
- Krumbein, W. E., and K. Jens, 1981. Biogenic rock varnish of the Negev desert (Israel): An ecological study on iron and manganese transformation by cyanobacteria and fungi. *Oecologia* 50:25-38.
- Lovley, D. R., J. F. Stolz, G. L. Nord, and E. J. P. Phillips. 1987. Anaerobic production of magnetite by a dissimilatory iron reducing microorganism. *Nature* 330:252-254.
- Lowenstam, H. A. 1981. Minerals formed by organisms. *Science* 211:1126-1131.
- McLachlan, J. 1973. Growth media—marine. In E. D. Stein, Jr. (Ed.), *Handbook of Phycological Methods*. Cambridge University Press, Cambridge, pp. 25-52.
- Murphy, R. J., and J. F. Levy. 1983. Production of copper oxalate by some copper tolerant fungi. *Trans. Br. Mycol. Soc.* 81:165-168.
- Murphy, T. P., D. R. S. Lean, and C. Nalewajko. 1976. Blue-green algae: Their excretion of iron-selective chelators enables them to dominate other algae. *Science* 192:900-902.
- Nicholls, K. H., and D. Fung. 1982. Accumulation of iron on the cell walls of the two monospecific freshwater genera, *Catena* and *Dichotomosiphon* (Chlorophyceae). *Arch. Protistenkd.* 125:209-214.
- Raghukumar, C., S. Pathak, and D. Chandramohan. 1988. Biodegradation of calcareous shells of the windowpane oyster by shell-boring cyanobacteria. In M. F. Thompson, R. Sarojini, and R. Nagabhushanam (Eds.), *Marine Biodeterioration*. Oxford and IBH Publishing, New Delhi, pp. 693-701.
- Skermann, V. B. D. 1967. *A Guide to the Identification of the Genera of Bacteria*. Williams & Wilkins, Baltimore.