

Phosphate Solubilizing Bacteria and Alkaline Phosphatase Activity in Coastal Waters off Trivandrum^{**}

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Abstract

Phosphorus is a key nutrient in marine environment. Phosphate solubilising bacteria (PSB) have the ability to solubilise ionic forms of orthophosphoric acid to free form of phosphorous in the water column. Both PSB and alkaline phosphatase activity (APA) were estimated to appreciate their pattern of distribution and expression of activity off Trivandrum. Abundance of PSB (MPN and CFU) is more at near-shore stations (2.54×10^5 and 1.65×10^4) when compared to off-shore stations (2.13×10^5 and 0.90×10^4). However, PSA was more at off-shore stations (1.78 uM/l/hr) than at near-shore locations (1.32 uM/l/hr). Surface waters recorded higher activity (3.18 uM/l/hr) than bottom (2.90 uM/l/hr). Alkaline phosphatase responds to increase in temperature measured in these waters. Our studies suggest that the activity off coastal waters is stimulated by fall in levels of ambient phosphate concentrations. Detailed studies would help us to establish the threshold concentration of phosphorous that governs the alkaline phosphatase.

Introduction

The phosphate solubilizing bacteria (PSB) are capable of dissolving the insoluble inorganic phosphorus into soluble organic phosphorus. Microorganisms especially bacteria play a vital role in the biogeochemical cycling of nutrients in the marine environment. Most of the phosphate and nitrate incorporated into the aquatic plants, animals and sediments are due to the action of the microbes. They are also responsible for decomposing dead organic matter and recycling of phosphorus (Ayyakkunnu and Chandramoha, 1970; De Souza *et. al.*, 2000). Bacterial

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action and autolysis as well as excretion of living zooplankton are believed to account for the degradation of organic phosphorus (Illmer and Schinner, 1995; Ahamed and Shahab, 2011). While inorganic phosphate is generally regarded as the most bioavailable form of phosphorus, its concentration in the surface waters of the open ocean is much smaller than that of dissolved organic phosphorus (DOP) (Das *et. al.*, 2007, Dyhrman *et. al.*, 2007).

Phosphorus is an essential nutrient to all life, being a structural and functional component of organisms. Large quantities of elemental phosphorus are immobilized in living organism, and are locked up in the sediments as insoluble organic and inorganic compounds (De *et. al.*, 2011; Paytan and McLaughlin, 2000) mostly released by alkaline phosphatase activity (APA).

Alkaline phosphatase (AP) is a key-enzyme in the marine environment which catalyzes the liberation of orthophosphate from organic phosphorus compounds. The total phosphatase activity in seawater results from a mixture of phosphatases localized on the cell surfaces of bacteria and from dissolved enzymes (Hoppe, 2003). Phosphatases are inducible catabolic ectoenzymes of aquatic micro-organisms (Boavida, 1990; Vazquez, 2000). The distribution of phosphatase activity among the particulate and the dissolved fractions is highly variable. Dissolved fraction can contribute up to 70% of the total APA to release bioavailable phosphorous (Siuda, 1984).

Phosphorus is the base of the marine food chain, and its availability can directly impact the structure and function of marine ecosystems. Recent results have shown that phosphorus limits primary productivity in some coastal waters (Huang and Hong 1999). Phosphatases play a prominent role in the avoidance of phosphorous limitation in the sea (Sundareshwar, *et. al.*, 2003; Vidal *et. al.*, 2003; Zohary and Robarts, 1998). Thus, high phosphatase activities have been measured in eutrophic or deep water in the presence of relatively high phosphate concentrations (Cooper *et. al.*, 1991). Information of such activities in our waters is limited. Hence, the present study was undertaken to elucidate the contribution of PSB and their activity, in coastal waters during monsoon season off Trivandrum, south west coast of India. Our study showed that APA is lower in bottom

waters but was triggered off-shore when dissolved inorganic phosphate (DIP) was low.

Materials and methods

Sampling locations: Trivandrum

The water samples were collected on board FRV Sagar Sampada 276 from different stations at depths 30, 50, 100, 200, near-shore and 500 and 1000 m off-shore off Trivandrum during June 2010.

Table 1

	Lat	Long	Max. depth	Depths.
St1	8°28.808	76°51.133	30	5,15, 25
Stn 2	8°30.45	76°42.198	50	5, 15, 25,45
Stn 3	8°27.064	76°29.981	100	5, 25, 50, 75, 100
Stn.4	8°26.903	76°26.182	250	5, 25, 50, 75, 150, 250
Stn.5	8°26.134	76°20.734	500	5, 25, 50, 75, 150, 250, 420
Stn.6	8°26.007	76°06.909	1000	5, 25, 50, 75, 150, 250, 500, 750, 950

Details of samples collected from different depths – off Trivandrum June 2010.

Station 30 to 200m depth-near shore
and rest stations denoted as off shore.

Salinity and temperature were derived from the Sea Bird CTD connected to the rosette Niskin sampler that was operated vertically across all stations. Water sample from pre-selected discrete depths were collected using 10 L Niskin bottles (Table 2). Sub-samples were taken for analysis of various parameters like dissolved oxygen (DO), nutrients (nitrate, nitrite, ammonia, phosphate and silicate), Chlorophyll *a* (Chl *a*). Seawater sub-samples for microbiological analyses were collected into sterile containers immediately after retrieval of the bottle. Dissolved oxygen (DO) and nutrients such as ammonium, nitrite, nitrate, phosphate and silicates in the seawater samples were measured on board by titrimetric methods and SKALAR auto analyzer as described by Wurl (2009) respectively.

Table 2

Parameters	PSB-CFU	PSB-MPN	APA
Whole transect (n=35)			
Depth	-0.3774 [*]	-	-
Temp.	0.4081 [*]	-	-
pH	0.4297 ^{**}	-	-
Near-shore waters (n=19)			
DO	-	-	-0.400
PO ₄	-0.412	-	-
silicate	-	-	0.381
NH ₄	-	-	0.422
Chl a	-	-	-0.381
Phaeopigment	-	-	-0.380
Off-shore waters (n=16)			
Temp	0.508 [*]	-	-
pH	0.5697 [*]	-	-
Chlorophyll	0.530 [*]	-	-
NO ₃	-0.529	-	-
Surface waters (n=16)			
Depth	-	-	-0.4927
pH	0.5747 [*]	-	-
PO ₄	-	-0.5913 [*]	-
NH ₄	0.5452 [*]	-	0.6050 ^{**}
chlorophyll	-	-	-0.7106 ^{**}
Phaeopig	-	-	-0.5259 [*]
Bottom layers (n=18)			
Temp	0.4621 [*]	-	-
PO ₄	-	-	0.4932 [*]

Pertinent relationships of APA and PSB to environmental parameters.

Without star-almost significant, with star significant (* p<0.05 and ** p<0.01)

Abundance of phosphate solubilizing bacteria

Phosphate Solubilizing Bacteria were enumerated by both most probable number and as colony forming units (CFU) on different selective media. MPN of PSB was estimated by microtitre plate method using paranitrophenol phosphate (PNP) as substrate (Gautm et. al.,2003). PSB were also enumerated by surface spread plating onto hydroxide apatite medium according to De Souza et. al., (2000). After inoculation, the plates were incubated for 4-5 days at room temperature ($30 \pm 1^\circ\text{C}$). Phosphate solubilization was indicated by the formation of a solubilization or a clear zone around their colonies.

Alkaline phosphatase activity

The alkaline phosphatase activity (EC 3.1.3.1) was estimated by a method of Taga and Kobori (1978) and as elaborated by De Souza et. al., (2000). The samples were incubated with para-nitro phenol phosphate (PNPP) in appropriate salinities. The phosphatase activity was indirectly measured by the amount of phenol released. Suitable controls were maintained in all analyses and the results represent the average of replicates.

Biological data was log transformed for statistical analysis. Inter-relationships between biological and environmental parameters were statistically examined using Statistica ver. 6.0 and analysis tool pack in Microsoft Excel. Analysis of variance (ANOVA) was carried out to indicate significant variation between the parameters.

Results and discussion

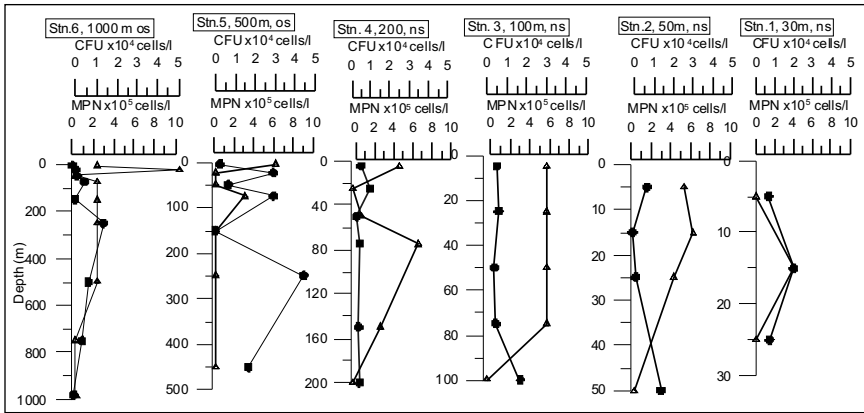
Spatial variation in PSB_{CFU} and PSB_{MPN} distribution -off Trivandrum

PSB- CFU and MPN did not show any depth dependent variation. They ranged from non detectable levels to 3.5×10^4 cells l^{-1} and 4×10^5 cells l^{-1} respectively (Fig 1.). They followed similar trends in distribution along all transects. Abundance of both PSB -CFU and MPN (1.65×10^4 and $2.54 \times 10^5 \text{ l}^{-1}$) is marginally more near-shore than off-shore stations (0.90×10^4 and $2.13 \times 10^5 \text{ l}^{-1}$).

Abundance of PSB-CFU and MPN were more near-shore than off-shore. This difference may be due to rains and land run-off during monsoon season. The monsoonal rain enriches the coastal environment with essential nutrients. Subsequently, bacteria grow rapidly and increase their population density by utilizing the nutrients. Kannirapan and

Ravindran (2012) also found higher PSB population density during monsoon season.

Fig 1



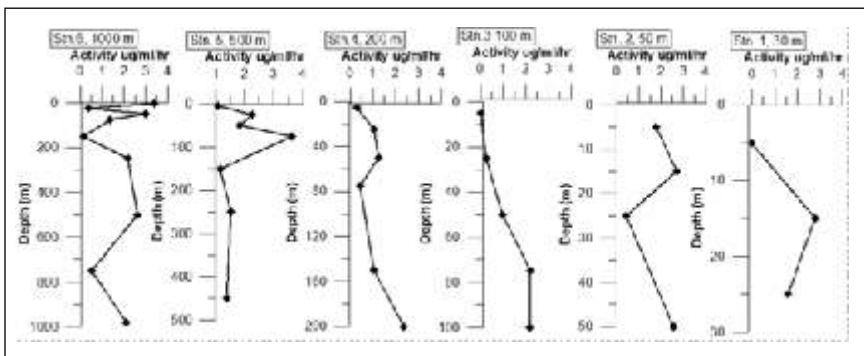
(●PSB MPN and ΔPSB-CFU)

Distribution of PSB-MPN and PSB-CFU-off Trivandrum (-PSB MPN and-PSB-CFU)

Variation in APA distribution-off Trivandrum

APA ranged from non detectable levels to 3.61 $\mu\text{M} / \text{l/hr}$ (Fig 2). APA did not show depth dependent variation. Unlike PSB, the average APA was more in off-shore (1.78 $\mu\text{M} / \text{l/hr}$) than near-shore (1.32 $\mu\text{M} / \text{l/hr}$). APA recorded higher activity (3.18 $\mu\text{M} / \text{l/hr}$) at the surface than bottom (2.90 $\mu\text{M} / \text{l/hr}$). Interestingly, the off-shore PSB apparently show higher efficiency of solubilizing inorganic phosphate.

Fig 2



(●PSB MPN and ΔPSB-CFU)

Alkaline phosphatase activity -off Trivandrum

However, close to the bottom the production is more than the uptake of phosphate. In off-shore waters, though the activity is high the ambient phosphate is low suggesting that the uptake of phosphorous is higher than production. These observations suggest that the activity is governed by ambient phosphate concentrations. Low ambient concentrations due to faster phytoplankton uptake stimulate the activity in off-shore waters. APA may be considered to regulate the productivity in coastal regions through contribution of available phosphorous to primary production. Similar observations have been made by Know *et. al.*,(2011) in the northern part of Gamak Bay, Korea.

Variation in environmental parameters related to PSB and APA

Both PSB abundance and APA are related to most environmental parameters notably depth, temperature, pH, phosphate and chlorophyll. The near-shore sea surface temperature (SST) off Trivandrum was lower at 25.20°C than the off-shore at 29.08°C. Salinity also showed significant variation during sampling varying from 34.30 ± 0.02 near-shore to 35.1 ± 0.12 off-shore; pH showed no particular trend and varied from 7.70 to 8.26. Dissolved oxygen content in the surface water increased from 1.323 near-shore to 4.335 ml l⁻¹ off-shore. Phosphate concentration lack particular patterns and varied from 0.850 to 1.557 uM l⁻¹. The chlorophyll varied from a low 0.004 off shore to a high 3.474 ug l⁻¹ near shore.

In this study, PSB-CFU decreases with depth ($r = -0.3774$, $p < 0.05$) but increases with temperature ($r = 0.4081$, $p < 0.05$) and pH ($r = 0.4297$, $p < 0.01$) measured. Decrease of PSB-CFU due to depth is indirect as it is linked to phytoplankton abundance. PSB-CFU increased with increase in pH and temperature as the enzyme responds to increase in temperature in the alkaline range. However, Kannirapan and Ravindran (2012) showed that during monsoon season, phosphatase activity was negatively related to pH. Unlike PSB-CFU, PSB-MPN and APA did not show any relation with any parameter when considered wholly.

However, when near-shore and off-shore stations were examined separately, interesting relationships emerged. PSB-CFU, MPN and APA from near-shore stations showed almost near-significant relationships. PSB-CFU decreases with phosphate concentration ($r = -0.41$) suggesting that the nutrient is already available. APA decreases with dissolved oxygen ($r = -0.40$), chlorophyll ($r = -0.38$) and phaeopigment ($r = -0.38$) but

increases with silicate ($r=0.38$) and ammonia (0.42). The results indicate that phosphate solubilisation is an aerobic reaction and phytoplanktons are also responsible for the production of major fraction of APA. Both phytoplankton and bacteria are known to contribute to the hydrolysis of DOP to DIP in seawater. The amount hydrolyzed by phytoplanktons is higher, as their biomass is much higher than that of bacteria in the open-ocean and coastal areas (Know *et. al.*,2011). When chlorophyll degrades, dissolved phosphate get released into the environment and indirectly suppresses the APA. Dissolved AP can also be liberated into the environment through the lysis of dead phytoplankton cells (Chrost, 1991).

In off-shore, PSB-CFU increase with temperature ($r=0.508$, $p<0.05$), pH ($r=0.5697$, $p<0.05$) and chlorophyll ($r=0.53$, $p<0.05$) but decrease with nitrate concentration ($r=-0.529$, $p<0.05$). Solubilization of phosphate is favored by high temperatures with high organic phosphorus content. The density of PSB and mineralization increases when the temperature increases (Sri Ramkumar *et. al.*,2011). In the present study about 28% of the variance in PSB abundance is because of variations in chlorophyll a in off-shore waters. The same observation was also made by Know *et. al.*, (2011) in his study. The study suggests that the physiological adjustment of phytoplankton and bacteria to changing environment causes temporal changes in the dissolved phosphate. The decrease of PSB-CFU along with ammonia may be due to phosphorous dissolution occurring from the pool of calcium-bound phosphorous through oxidation of ammonium to nitrite through nitrate by the nitrifiers. The result obtained in this study was also observed by Date, (2000) Khan *et. al.*,(2009) when the crops inoculated with bacteria, phosphate and nitrate concentration also increased.

Like in near-shore waters, all the surface samples examined showed that, APA decreased with increase in chlorophyll ($r=-0.7106$, $p <0.01$) and phaeopigment ($r=-0.5259$, $p < 0.05$). Unlike near-shore where APA related to increase in ammonia, in surface waters both PSB-CFU ($r=0.5452$, $p<0.05$) and APA ($r=0.6050$, $p<0.01$) are related. As in whole water samples, PSB-CFU related to increase in pH ($r=0.5747$ $p<0.05$). PSB-MPN and phosphate related to each other only in surface waters negatively ($r=-0.5913$, $p<0.05$) suggesting that at the time of sampling there was enough phosphate to prevent proliferation of PSB.

Similar to whole waters and off-shore waters, PSB-CFU related with increase in temperature ($r= 0.46$, $p<0.05$) in the bottom layers. Though

APA activity increases with phosphate concentration in both surface and bottom, the relationship is more significant in the bottom layers ($r= 0.49$, $p<0.05$) suggesting a tighter coupling between the two parameters. In surface waters high APA was associated with low phosphate concentration but in bottom waters high APA related with high phosphate concentration. The activity is mostly governed by the availability of total phosphate. De Souza *et. al.*, (2001) also demonstrated that in sediment and seawater samples around the Indian Peninsula, APA was high wherever the total phosphate content was high. Phosphatase activity has therefore been used as a phosphorus deficiency indicator in bacterial populations and phytoplankton abundance.

Our studies thus suggest that though PSB are less off-shore than near-shore, the APA off coastal waters is stimulated by fall in levels of ambient phosphate concentrations. Detailed studies would help us to establish the threshold concentration of phosphorous that governs the alkaline phosphatase.

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