CHAPTER 7

P MOBILIZATION BY BACTERIA IN NATURE AND IN MICROCOSM SEDIMENT
Phosphorus which is an integral component of every organism, from bacteria and plankton to fish and organisms of higher trophic level, is an essential macronutrient and often remain traces in freshwater ecosystems. To meet the P requirement, phosphate fertilizers are infrequently applied in aquaculture due to problem of uncontrolled eutrophication and fixation of major part of the fertilizer P in sediment (Jana, 2007). P liberating bacteria could be a way to combat the situation by mobilizing sediment-bound P and contributing to the available P pool. In agricultural system the P releasing bacteria, more specifically PSB, have been intensively studied and are commercially exploited for production enhancement. Thus an easy but yet unproven option would be to use biofertilizers developed for agricultural crops in aquaculture production systems. However, presuming better adaptability and hence higher performance, aquatic P releasing bacteria would be preferred over their terrestrial counterparts for application in aquatic ecosystems.

Besides sorption, sediment may also release and act as an important source of P to the overlying water. This internal P loading (i.e. P release from the sediment) has profound effect on trophic state and is a well studied phenomenon in wetlands, mostly in context of eutrophication (Boström et al., 1982; Søndergaard et al., 1999; Song et al., 2009). However, utilization of this mobilizable P pool for productivity enhancement in P limiting situation may be a topic of intense research. Microbial decomposition of sediment organic matter, microbe-mediated reductive dissolution of Fe(III)-oxide, and diffusion of microbial cellular polyphosphate under anoxic conditions are major biological P release mechanisms in lake sediment (Boström et al., 1988; Søndergaard et al., 1999; Tong et al., 2005). P dynamics in sediments with high inputs of reactive organic matter have been reasonably well studied (Mort et al., 2010; Jilbert et al., 2011).
However, neither Ca-P form nor role of PSB or other P releasing bacteria have been examined in terms of P release in aquatic sediment. Only few works have been conducted assessing the biofertilization potential of P releasing bacteria in aquatic systems.

The objectives of the study were to assess the natural mobilization of Ca–P pool in freshwater sediment, and to study the P releasing activity of the bacteria previously isolated from different freshwater sources in sediment for elucidating ecological significance, potential in P release, and relevance of the bacteria in freshwater environments.

### 7.2 Materials and methods

#### 7.2.1 Estimation of Ca-P mobilization in nature

For estimation of Ca-P mobilization in nature sediment samples were collected during August 2009 to October, 2011 from both the wetlands. Sampling procedure was same as described in Chapter 4. The sediment pH and water temperature were also measured during this period using multiparameter water analysis probe (WTW).

#### 7.2.2 Bacteria employed in the work

To examine the role of bacteria in sediment P release bacterial isolates with high \textit{in vitro} P releasing activity were initially tested in wetland sediment. However, assuming wide variation in culture and field condition, and bacteria showing better efficiency \textit{in vitro} might not be equally effective in nature and \textit{vice-versa}; all of the isolates were tested for the microcosm study later.

#### 7.2.3 Microcosm experiment set up

Since Bhomra wetland sediment had higher Ca-P content (result described in Chapter 4), the ability of PSB isolates in releasing P from sediment was assessed in a microcosm experiment set up with freshly collected Bhomra sediment. The experiments were
conducted in 5 occasions, involving both 50 ml and 250 ml flasks in each occasion. A 60\% sediment slurry was prepared with filtered (0.22 \mu m) sterile wetland water and 45 ml aliquots were placed in to 50 ml capacity tissue culture flasks (HiMedia Laboratories, India) (Fig. 7.1). Volume was made up to 50 ml with glucose (1.85 mM final concentration; as labile substrate which would presumably increase the PS activity of the bacteria), bacteria at 9.18 \times 10^9 - 1.04 \times 10^{10} \text{ CFU and filtered water; negative control bottles received no bacteria, ‘only sediment’ flasks received neither bacteria nor glucose. All the bottles were tightly capped, gently mixed and incubated in dark at 30^\circ C, 80 rpm shaking on a horizontal shaker with thorough manual shaking twice a day. At regular intervals (1 day, 7 day, 14 day), 9 ml sediment slurry was withdrawn, mixed with 36 ml NH}_4\text{Cl (1M), shaken for 2h on a horizontal shaker and available P (Avl.P) content was measured. Another similar set of experiments was set up, but in 250 ml flasks with 4.59-5.2 \times 10^{10} \text{ CFU bacteria from which 50 ml slurry was withdrawn at different incubation intervals, air dried, ground and P fractions were estimated by method described in Chapter 4. The experiments were established in live sediment to ensure that the target strains are capable of surviving in the presence of existing microbial communities, since our long term aim is to use them as a biofertilizer in live sediments.}
To rule out contributions of natural microorganisms in the microcosm experiment and to examine P release from the Ca-P fraction, bacteria were also tested in heat-sterilized sediment (autoclaved at 121°C for 15 min for 3 consecutive days) slurry and P was fractionated.

### 7.2.3 Microcosm experiment set up using cyanobacteria as bio-indicator

Two isolates performing well in sediment microcosm study in releasing P from sediment was further assessed in another microcosm experiment set up with freshly collected wetland sediment and primary producing organism to assess P release through their growth. A 40% sediment slurry was prepared with filtered (0.22 µm) sterile wetland water and 195 ml volume was dispensed in 250 ml conical flasks and the volume was made up to 200 ml by adding glucose (labile substrate for increasing the microbial activity; 1.85 mM final concentration). The preparations were autoclaved for destroying the native organisms of the sediment. The test bacteria were grown overnight in TSB, washed in normal saline (0.85%) by repeated centrifugation. Bacteria was inoculated @ $9.18 \times 10^9 - 1.04 \times 10^{10}$ CFU per flask. The flasks were incubated for 72 hours at 30°C, 80 rpm shaking on a horizontal shaker. After 3 days, cyanobacteria cultures ($Aphanocapsa$ sp. and $Oscillatoria$ sp.) were added @ 4.5 ml of suspension having transmittance set to 20 in BiOLOG turbidity meter ($\sim 3.2 \times 10^8 - 4.7 \times 10^{10}$ CFU ml$^{-1}$). Negative control flasks received no test bacteria, but the cyanobacterium only. All the flasks were cotton plugged, gently mixed and incubated in algal growth chamber with light (wavelength 450 nm) (Anderson and McIntosh, 1991). After an incubation of 10 days, the increase in primary productivity was measured by assessing cyanobacterial growth. The growth of $Aphanocapsa$ sp. was measured by estimating chlorophyll-$a$ as per Standard Methods, APHA (2005). However, for $Oscillatoria$ sp., which is a
filamentous cyanobacteria, the filaments could not be collected due to precipitation on the sediment bed, and productivity was measured qualitatively by visual estimation.

### 7.2.4 Statistical analysis

Seasonal variations in Ca-P level and pH in wetlands were examined by ANOVA and subsequent post-hoc analysis using SAS Enterprise Guide (4.2).

### 7.3 Results

#### 7.3.1 Ca–P mobilization in aquatic systems

In Akaipur wetland the water temperature ranged between 16.9-26.8°C in winter, 33.1-34.7°C in summer and 26.4-32.4°C in rainy season; corresponding seasonal temperatures in Bhomra wetland were 17.0-26.7°C, 31.8-34°C and 26.4-31.6°C respectively. There was an increase of 14°C in water temperature during peak summer over the peak winter. Round-the-year monitoring of Ca-P fraction in the wetlands showed a marked seasonal variation: Ca-P level gradually increased during rainy and winter seasons reaching a peak at the end of winter in Bhomra wetland, followed by a sharp fall in summer (P<0.01) (Fig. 7.2). In Akaipur wetland a similar, but statistically insignificant trend, was noted. There was a significant correlation (R= -0.325, P<0.05) between water temperature and sediment pH in Bhomra but not in Akaipur and between sediment pH and Ca-P content (R=0.82, P<0.01) for both the wetlands.
Microcosm experiments with 97 bacterial isolates were conducted in 5 batches in different months when the wetland sediment used for the study had different P content and microbial activity. This was reflected in Avl.P level in ‘only sediment’ flasks which varied between 0.02-0.07 mg l⁻¹ on the first day of experiment and progressively

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**Fig. 7.2** Seasonal variation in sediment Ca–P and pH in the studied wetlands. A Akaipur wetland. B Bhomra wetland. Mean ± S.E

**7.3.2 P release by bacterial strains in sediment**

Microcosm experiments with 97 bacterial isolates were conducted in 5 batches in different months when the wetland sediment used for the study had different P content and microbial activity. This was reflected in Avl.P level in ‘only sediment’ flasks which varied between 0.02-0.07 mg l⁻¹ on the first day of experiment and progressively
increased up to 0.14 mg l\(^{-1}\) indicating release of phosphorus by natural microorganisms and/or through redox mediated changes during incubation. To reduce this heterogeneity among different sets of experiment, Avl.P values in the ‘only sediment’ flasks in different days of incubation were subtracted from the respective control (Ctrl-Glu) (sediment+glucose) and bacteria (sediment+glucose+bacteria) values. This subtraction lead to negative values in some cases, however, this does not mean negative Avl.P levels in actuality. All the values presented are relative to the ‘only sediment’ Avl.P levels. Although we tested all isolates in sediment, to avoid complexity in presentation, Avl.P levels in different days of incubation are presented here (Fig. 7.3) for 10 representative isolates from different niches.

Addition of glucose (Ctrl-Glu) negated Avl.P increases observed in ‘only sediment’ flasks. For the majority of the isolates a progressive decline in Avl.P level was recorded.
with incubation, while an increase in Avl.P was detected for 43% isolates which we considered to be ‘P-releasing’. For these promising strains, mostly of sediment origin, the increase was evident from 3rd day and it reached peak in 6-9 days in most cases. The Avl.P levels on the 9th day of incubation showed an appreciable release of sediment P by some of the sediment isolates like CPSM3, CPSM8, CPSM10, AP14, BP11b, APh1 etc. and quenching or uptake of Avl.P by others including all those from water (Fig. 7.4a and b). Among the promising ones, isolates from the Churni river had the highest P release activity, followed by those from Bhomra and Akaipur wetland.

<table>
<thead>
<tr>
<th>Table 7.1</th>
<th>Effect of bacterial inoculation on soil P-fractions (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Live sediment</strong></td>
<td><strong>Avl.-P</strong></td>
</tr>
<tr>
<td><strong>Sediment</strong></td>
<td>1 d</td>
</tr>
<tr>
<td>Sediment</td>
<td>9.53</td>
</tr>
<tr>
<td>Sediment + Glu</td>
<td>8.03</td>
</tr>
<tr>
<td>Sediment + Glu + CPSM1</td>
<td>9.86</td>
</tr>
<tr>
<td>Sediment + Glu + CPSM2</td>
<td>7.83</td>
</tr>
<tr>
<td>Sediment + Glu + CPSM3</td>
<td>7.92</td>
</tr>
<tr>
<td>Sediment + Glu + CPSM7</td>
<td>9.65</td>
</tr>
<tr>
<td><strong>Sterile sediment</strong></td>
<td><strong>1 d</strong></td>
</tr>
<tr>
<td>Sediment</td>
<td>14.19</td>
</tr>
<tr>
<td>Sediment + Glu</td>
<td>12.38</td>
</tr>
<tr>
<td>Sediment + Glu + CPSM1</td>
<td>12.08</td>
</tr>
<tr>
<td>Sediment + Glu + CPSM2</td>
<td>12.92</td>
</tr>
<tr>
<td>Sediment + Glu + CPSM3</td>
<td>15.01</td>
</tr>
<tr>
<td>Sediment + Glu + CPSM7</td>
<td>14.64</td>
</tr>
</tbody>
</table>
Fig. 7.4a  Avl.P levels in sediment suspension in microcosm set-up with different bacterial isolates
Fig. 7.4b  Avl.P levels in sediment suspension in microcosm set-up with different bacterial isolates
The microcosm study with P-fractionation yielded indistinct results (Table 7.1). Autoclaving led to an increase in Avl.P level, may be due to the release of P from microbial cell lysis, or P release from organic form by increased temperature during sterilization. Addition of bacteria to sterile sediment resulted in initial rise, followed by a sharp decrease in Avl.P indicating initial bacterial release of P followed by its quick uptake. The microbial P uptake was more marked in live sediment amended with glucose. Inoculation of bacteria resulted in higher level of Avl.P, in comparison to ‘sediment+Glu’ indicating liberation of sediment-bound P by test strains, in excess of the P requirement of the microbial community, at least during initial days of incubation. This was more prominent, although statistically insignificant, for two of the four isolates tested. Ca-P was the dominant inorganic fraction both in live and sterile sediment. Additions of glucose or bacteria lead to short-term decline in Ca-P content in live sediment. This loss of the Ca-P fraction with bacterial inoculation was also visible in sterile sediment, but to a lesser extent. Compared to live sediment, the Fe-P content was low in sterile sediment; possibly heat sterilization removed O\textsubscript{2}, making the sediment anaerobic and thereby reducing the Fe-P content. Addition of bacteria to sterile sediment further lowered the Fe-P content nearly to zero. This was more prominent shortly after experiment set up, but Fe-P progressively increased over incubation, possibly due to availability of more air space (regular sample withdrawal created more air space in flasks). This repartitioning of P was evident with incubation for the Ca-P fraction also. Addition of bacteria to live sediment also led to similar results. Overall, there was higher level of Avl.P after 1 day of incubation in presence of bacteria and this correlated with low Ca-P and Fe-P levels. As none of our strains were effective in solubilizing Fe-P in culture medium, the decrease in Fe-P after addition of glucose or bacteria could be due to microbial oxygen consumption leading to solubilization of Fe-P. Al-P fraction showed a
minor decrease with incubation period, independent of autoclaving or glucose or bacteria addition (raw data not given), indicating its hard insoluble nature (Illmer et al., 1995).

### 7.3.3 Microcosm experiment using cyanobacteria as bio-indicator

The result showed different biofertilization properties of different bacterial strains. While strain CPSM8 induced profuse growth of both the cyanobacterial species, strain Aph1 containing flasks hardly showed such effect. The maximum growth of *Aphanocapsa* sp. was noticed at the level of 6.22 μg l⁻¹, with an average of 6.75 ± 0.99 μg l⁻¹ for strain CPSM8, while that was for Aph1 was only meager [highest 0.21 μg l⁻¹, average 0.27 ± 0.03μg l⁻¹ (Table 7.2)]. Qualitative assessment of growth of *Oscillatoria* sp. also gave almost similar pattern of result. For strain CPSM8, profuse growth of cyanobacteria was observed after 10 day (Figure 7.5), while moderate growth was observed for strain Aph1.

#### Table 7.2 Effect of bacterial inoculation on primary production

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Quantitative chlorophyll-a count (μg l⁻¹)</th>
<th>Qualitative chlorophyll-a count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>(Aphanocapsa</em> sp.)</td>
<td><em>(Oscillatoria</em> sp.)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>Average</td>
</tr>
<tr>
<td>Control</td>
<td>0.14 – 0.17</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>CPSM8</td>
<td>6.22 - 8.29</td>
<td>6.75 ± 0.99</td>
</tr>
<tr>
<td>Aph1</td>
<td>0.21 - 0.33</td>
<td>0.27 ± 0.03</td>
</tr>
</tbody>
</table>

*Fig. 7.5* Cyanobacterial growth in microcosm experiment; left, *Oscillatoria* sp., right, *Aphanocapsa* sp., T, test (with bacteria); C, control
There was very low (0.15 ± 0.04 μg l\(^{-1}\)) cyanobacterial growth in negative control flasks.

### 7.4 Discussion

#### 7.4.1 Ca-P mobilization in nature

As sediment is an important source of essential nutrients driving primary productivity, we sought to augment sediment P for the enhancement of aquaculture productivity in oligotrophic waters. The study showed that sediment Ca-P is being naturally mobilized and could be a potential reliable pool. There was a marked seasonal variation in sediment Ca-P content in wetland ecosystems with its increase or accumulation over rainy and winter seasons and mobilization in summer. Since dissolution of Ca-P is not critically regulated by redox potential changes (Ann et al., 2000), this summer release of Ca-P is most likely directly or indirectly mediated by temperature driven microbial activity and change in sediment pH. Although significantly higher proportion (~76%, P<0.01) of Ca-P was released from Akaipur sediment, the total amount of Ca-P mobilized was more in Bhomra (400-500 mg kg\(^{-1}\)) due to its higher Ca-P content. Logically, the larger the reservoir size, the higher the amount of expected release from a mobile pool and water bodies having optimum or high Ca-P reserve would probably be appropriate when biofertilization with P releasing bacteria for production enhancement is desired. Although the recorded Ca-P contents were more or less similar to that found in a South Indian river (Padma and Nair, 2010) or in eutrophic West lake and Xixi wetland in China (Qian et al., 2010), the levels were much lower than those found in many rivers, estuaries, coasts and sea (Ann et al., 2000; Xiaojiang et al., 2001; Padma and Naira, 2010) suggesting less significance of Ca-P in overall sediment P regeneration, at least in organic matter laden wetlands.

Apatite formation is a pH dependent process and organic acids may hinder in apatite formation. The low Ca-P reserve in Akaipur sediment could be explained, at least partly,
by its acidic pH. There was also a drop in sediment pH, more marked in Bhomra, during summer presumably due to organic acids produced from breakdown of organic matter accumulated in these wetlands. The presence of greater TOC contents (Result described in Chapter 4) in Bhomra may support the higher production of humic acid and other organic acids in sediment, since sediment TOC and humic acid correlates well (Sardessai, 1994). Although the influence of acid production on Ca-P pool mobilization might be a general contribution of microbial decomposition as a whole, it is natural for P releasing bacteria more specially PSB, to play a major role in Ca-P solubilization. The Ca-P mobilization could be related to the high abundance of PSB (data given in Chapter 5) in wetland sediments ($R^2=0.22$, $P=0.237$), decrease in sediment pH ($R^2=0.674$, $P<0.01$) in summer and substantiated by solubilization of Ca-P by the isolates in vitro. Introduction of P releasing bacteria in sediment proved that some of the isolates were effective but other were ineffective for P release, despite the fact that many of them had moderate-to-high P solubilizing or mineralizing activity in vitro culture medium. This failure of about 57% strains is either due to a lack of adaptation of these strains in sediment, an inability of the strains to release P in sediment or due to a high rate of P assimilation exceeding the rate of P regeneration. Preferential and rapid uptake of orthophosphate and its intracellular storage by microbes in aerobic aquatic environments is a widely known phenomenon (Gächter and Meyer, 1993). Microbial P assimilation with or without intracellular storage was also evident from decreasing level of available P when sediment microbial activity was stimulated with glucose and when bacteria amended sediment was incubated for more time. Despite this P uptake, some of the isolates regenerated P in excess of their own requirements and assimilation by the sediment microbial community, as evidenced by their ability to increase Avl.P levels up to 0.233 mg l$^{-1}$ in the sediment slurry. This regeneration was prominent in 1-2 weeks
period and thereafter Avl.P level decreased indicating cellular P assimilation in excess of
generation either due to death of inoculated bacteria, limitation in substrate availability,
also probably repartitioning of P. Strains from the Churni river were more capable of
regenerating P, than those from other sources. The average sediment C:P ratios (obtained
from results of Chapter 4) of the wetlands and river were low, only 5.39, 6.79 and by
mass in Akaipur, Bhomra and Churni river respectively, indicating P enrichment. The
C:P ratios of freshwater ponds and lakes ranges between 7 to 265 (Tong et al., 2005;
Geurts et al., 2010). Considering the sediment C:P ratio below 40:1 suitable for P release
(Gächter and Meyer, 1993), the wetland and river sediments having lower C:P ratios are
prone to P mineralization and bacteria releasing P in excess of their own and sediment
community requirements (Geurts et al., 2010), would be beneficial as a biofertilizer in
freshwater ecosystems. The live sediment slurry study indicated survival and adaptability
of the strains, evidenced by their P regeneration in sediment environment without need
of much extraneous requirements.

Fractionation of sediment P in microcosm study showed that test strains caused a
decrease in Ca-P level, indicative of P release from this form. However, the drop in Ca-P
level was short lasting and not very prominent, indicating that the Ca-P was not the sole
or preferred target of P releasing bacteria and, as such, Ca-P fraction might have limited
functions in overall internal P loading. Ca-P formed less than 10% of total sediment P
concentration of the systems under study. With lesser occurrence of Fe-P, it may be
assumed that a large share of the sediment P was organic in nature. Hence transformation
of organic P to soluble forms is likely to play an important role in explaining the
behaviour of P release in P releasing bacteria inoculated treatments. This was more so
because the release of P to available form was more pronounced during summer months
when the rate of decomposition of sediment organic matter was higher, especially under
narrow C:P ratios of the sediments. P releasing bacteria especially P mineralizers have often been detected with phosphatases, nucleotidase etc. involved in organic matter mineralization (De Souza et al., 2000; Mudryk, 2004; Sahu et al., 2007; Sri Ramkumar and Kannapiran, 2011). As P is an essential and often limiting macronutrient, some P release mechanisms are probably universal among aquatic microorganisms and the presence of more than one P release mechanism would be desirable for a biofertilizer at field level.

### 7.4.3 PSB in augmenting growth of primary producer

P is released from sediment to sediment water interface and from there to overlying water, which in turn promotes the growth of primary producers. P releasing bacteria acts as a driving force in this mechanism owing higher productivity (Song et al., 2009). In this study one strain out of 2 strains tested, augmented growth of cyanobacteria. The differential ability of strains augmenting primary production might be contributed by several factors like their ability for soil adaptability, rate of P uptake and accumulation, capability to use varied P resources in sediment and the most important their genetic profile of harbouring various genes involved in P mineralization and organic matter degradation. The genetic profiles of two strains, studied through whole genome sequencing are covered in Chapter 8.