Chapter 6
Chapter 6: Microbial interaction with ilmenite

6.1. Introduction

Though ilmenite is known to be a recalcitrant mineral, the iron component is readily released by bacterial action which benefits the community in the ecosystem. This chapter highlights the important findings to elaborate this statement at the microbial level.

The results on the effects of ilmenite and iron are presented in this chapter and are divided into two sections.

Section 6.2.: Reductive phase
Section 6.3.: Oxidative phase

6.2. Reductive phase

6.2.1. Microcosm experiments with berm sediments: Abundance and activity at bacterial and primary level

6.2.1.1. Abundance and activity at bacterial level

6.2.1.1.1. Abundance of iron reducing bacteria as determined by MPN method: Seasonal and down core variation in the berm sediments

6.2.1.1.1. Results

Most probable number (MPN) estimates revealed that the population of iron reducing bacteria in the sediment decreased with depth in November and ranged from 7.5 cells g\(^{-1}\) at (20-40) cm layer to 9.3 x 10\(^3\) cells g\(^{-1}\) at (0-5) cm layer (Fig. 6.1). A sub-surface maxima was observed at (5-10) cm layer depth during August and the population ranged from 0.75 x 10\(^3\) cells g\(^{-1}\) at (20-40) cm layer to 2 x 10\(^3\) cells g\(^{-1}\) at (5-10) cm layer. However, there was no such definite trend during April and the population varied from 30 cells g\(^{-1}\) at (10-15) cm layer to 1.5 x 10\(^3\) cells g\(^{-1}\). Correlation analysis between the MPN numbers and ferrous iron [Fe(II)] concentration revealed that the Fe(II) and MPN influenced each other negatively (r = 0.42) during the monsoon season. On the other hand, during November, this relationship became positive and caused nearly 71% variation (Fig 6.2.).
Fig 6.1: Enumeration of iron reducers using ferrozine-MPN assay

Fig 6.2: Correlation analysis between Fe(II) concentration and MPN during (a) August, (b) November and (c) April

\[ y = -0.0134x + 1.8835 \]
\[ R^2 = 0.1778 \]

\[ y = 0.1404x - 3.5079 \]
\[ R^2 = 0.7093 \]

\[ y = 0.0105x + 0.3031 \]
\[ R^2 = 0.0825 \]
6.2.1.1.2. Iron released from ilmenite by natural flora in microcosms: Seasonal and down core variation in the berm sediments

6.2.1.1.2.1. Results
Iron release from ilmenite occurred both in native and heat- and azide treated control at RT. Sodium molybdate at a concentration of 20mM was used to inhibit sulfate reducing activity. Fe(II) indicated as a pink colouration due to the binding to ferrozine reagent, accumulated in the native as well as in both the controls i.e heat killed and azide amended medium. However, the levels of accumulation of Fe(II) in the native experiment sediments were considerably higher than the accumulation in the controls. The biological release of iron from ilmenite started from the 3d and peaked on 7d and was generally higher than the control. In the experimental tubes, the average rate of removal of iron in the form of Fe(II) was nearly three times higher than the corresponding control. The concentration of Fe(II) released from ilmenite in the experimental tubes varied from 1.19 to 145.89 mg-at.Fe g\(^{-1}\) (Fig. 6.3., Plate 6.1). However, in the azide control it varied from non detectable levels to 97.52 mg-at.Fe g\(^{-1}\) while in the heat killed control it varied from 2.47 to 190.83 mg-at.Fe g\(^{-1}\). In the experimental tubes, highest potential iron reduction of 146 mg-at.Fe g\(^{-1}\) was recorded during the monsoon season of August. Depth wise the maximum was the same and encountered at deeper depth of (15-20) cm layer. In the post-monsoon month of November, the highest potential iron reduction was recorded at shallower depth of (5-10) cm at 107.22 mg-at.Fe g\(^{-1}\). Similarly, in April the highest potential iron reduction was recorded at (5-10) cm at 74.43 mg-at.Fe g\(^{-1}\). The average potential iron reduction rates varied from 4.6 mg-at.Fe g\(^{-1}\) d\(^{-1}\) in April to 8.65 mg-at.Fe g\(^{-1}\) d\(^{-1}\) during August.

6.2.1.1.3. Discussion
Iron reduction and oxidation can be of prime importance for aerobic and anaerobic microbiological processes within sand. In the Kalbadevi beach sediments, the berm sediments usually had higher accumulation of ilmenite (Valsangkar, 2005). Hence this sediment along with the associated microflora was used in the present study to validate the hypothesis that natural consortia of microflora from this region could mobilize iron from the mineral ilmenite at
Fig 6.3: Removal of iron from ilmenite containing Kalbadevi beach sediment using native bacteria. IL- Ilmenite with native bacteria, IL- HKD: heat killed sediment, IL- AKD: azide killed sediment.
Plate 6.1: Fe(II) released from ilmenite rich Kalbadevi beach sediment by natural flora in microcosms (down core variation during post monsoon)
a) experiment using native bacteria
b) heat killed control

* Ferrozine binds with Fe(II) and gives a pink colouration
an appreciable rate. In the present study, microcosm experiments using native bacteria and ilmenite showed that these microbes were capable of releasing soluble iron from the mineral ilmenite into the medium. This was perhaps an inherent ability of the native bacteria since chemical removal of iron from mineral media containing heat and azide treated sample was relatively low. Similar trends were observed in the experiments that were replicated over three sampling months of August, November and April which define the monsoon, post-monsoon and pre-monsoon season, respectively. The average potential iron reduction rates in the Kalbadevi sediments ranged from 4 to 9 mg-at.Fe g$^{-1}$ d$^{-1}$. Generally, the maximum rates of microbial iron reduction reported for natural freshwater and marine sediments varies over several orders of magnitude ranging from 2-100 nmol Fe(III) cm$^{-3}$ h$^{-1}$ (Thamdrup, 2000; Roden and Wetzel, 2002; Jensen et al., 2003; Canavan et al., 2006).

Concentration of Fe(II) in the experimental sediments was generally higher than the controls especially at the surface layers. This indicated that the microbial rate of release of iron from the ilmenite was higher than the controls. Reductive dissolution is known to occur in natural ilmenites which contain significant amounts of Fe(III) (White et al., 1994). However, the microbial participation acts as a catalyst and increases the reaction several fold. At deeper depths, the chemical removal of Fe(II) seemed to prevail in cell free controls especially in the heat killed controls. The heat killed deep sediments overcame the effect of heat during the non monsoon months especially during the pre-monsoon. In this beach sediment, Firmicutes occurred at a frequency of nearly 41% (Chapter 7). It is quite possible that these spore formers may be resistant to heat and proliferate at later stages to participate in the release of iron from sediment. Bacilli spp have been known to be involved in solubilizing large amounts of iron from limonite, goethite and hematite (de Castro and Ehrlich, 1970). Studies have also shown that spore formers such as B. subtilis had incredible heat resistance that prevented the complete inactivation of the enzyme with temperature control (Gardner et al., 2006; 2008). It may also imply that free enzymes adhering to sediment particles are only temporarily disabled when heat killed.
MPN analysis offers a convenient culture-based method for enumerating respiratory (dissimilatory) iron reducing bacteria (DIRB). The number of iron reducing bacteria as estimated by Ferrozine-MPN method was high during the post-monsoon season of November suggesting that the proliferation of these group of bacteria are dependent on the higher bio-availability of organic carbon which generally increases during the post-monsoon months. MPN estimates of the iron reducers also indicated that this group of bacteria decreased with increasing depth again suggesting that they were constrained by availability of the organic carbon. It is well known that iron reducers do not require strongly reducing conditions and are well distributed in surficial sediments. Their tolerance of intermediately oxidized environments is reflected in their distribution. Water acts as a barrier to oxygen penetration, allowing its depletion and the generation of suboxic conditions (Ponnamperuma, 1972). Further, biological oxygen demand generated by organic matter accumulating at the deeper layers creates oxygen-reducing conditions and consequently, lowers the $E_h$. As other terminal electron acceptors are reduced, the redox potential continues to drop (Cummings et al., 2002).

The activity of iron reduction was the highest during the monsoon season and occurred at deeper depths. During post-monsoons, nearly 71% of the variation in the Fe influenced the MPN estimates positively suggesting its effect on the proliferation of bacteria. On the other hand, during monsoons, nearly 18% of the variation in the Fe influenced the MPN estimates negatively suggesting that the concentration of the released iron was beyond optimal level. Perhaps the dissolution of ferrous ions is facilitated more in fresh water thus enabling iron reduction to proceed at a faster rate. The trend depicts that the biological rate of release of iron in surface sediments is more than the deeper layers. As this process follows the trend in the distribution in organic matter, microorganisms can reduce ferric iron during the metabolism of organic matter (Lovley, 2000). In the previous chapters of 4 and 5 it had been shown that organic matter in the berm sediments is high (1.3 mg g$^{-1}$). Previous studies have also revealed that the availability of Fe(III) for microbial reduction is also an important factor controlling the extent of organic matter
decomposition with Fe(III) serving as the terminal electron acceptor (Lovley, 2000). In turn, the in situ dissimilatory iron reduction is influenced by a variety of factors, including the microbial community structure and biomass (Dollhopf et al., 2000), the quality and quantity of the organic matter (Chen et al., 2003), and the type and abundance of Fe minerals (Bonneville et al., 2004). Also, Fe(III) respiration is coupled to a substantial portion of organic matter remineralization in the surface sediments of marine and freshwater environments (Kostka et al., 2002). Under suboxic conditions, ferric iron is a potential electron acceptor for organic matter degradation (Froelich et al., 1979; Lovley and Phillips, 1988). Thus iron reducers in the berm sediments play a significant role in bringing Fe(II) into the solution and making it bio-available for other trophic levels.

Sodium molybdate at a concentration of 20 mM prevented sulfate reducing activity and therefore binding of iron to the sulphide does not occur. Molybdate is known to inhibit sulfate reduction (Oremland and Capone, 1988). It is well known that sulphide produced can chemically reduce ferric iron, and this will affect the measured iron reduction rate. The activity of sulphate reducing bacteria (SRB) was effectively inhibited by molybdate (Ito et al., 2002, Nielsen et al., 2002). Hence all the iron was available for iron reduction and not diverted for precipitation by sulphide. It is hence tempting to interpret the lack of any inhibitory effect of molybdate on iron reduction as evidenced by reduction of Fe(III) to Fe(II). It is thus possible that the native dissimilatory iron reducers contribute to the major part of iron reduced from ilmenite in Kalbadevi sediment.

6.2.1.2. Effect of varying concentration of ilmenite on heterotrophs

6.2.1.2.1. Results
Varying concentrations of ilmenite were added to elucidate the response of culturable heterotrophic bacteria in terms of their abundance on 1% strength nutrient broth in 1.5% agar. With the addition of ilmenite to the sediment in a ratio of 1:1 to 1F, CFU retrieved on day 3, 5, and 15 increased by 63, 44, and
31% respectively more than the control. CFU retrieved from the control flask was $1.12 \times 10^6$, $2.28 \times 10^6$, and $4.56 \times 10^6$ CFU g$^{-1}$ on 3$^{rd}$, 5$^{th}$ and 15$^{th}$ day respectively. Similarly when the quantity of the ilmenite was doubled i.e in the ratio of 2:1 in 2F, CFU retrieved at day 3 increased by nearly 50% more than the control. However, when the quantity of ilmenite added was in the ratio of 3:1, the retrievability increased >88% by the third day. This retrievability was maintained at level which was always 50% more than the control. Highest retrievability was recorded from 3F on all the days (Fig 6.4h).

Generic diversity increased with the increasing concentration of the ilmenite addition from 5 to 6 genera (Fig 6.4a-g). When different proportions of ilmenite ranging from namely 0, 50, 66, 75, 80% were used, the retrievability of different genera changed. The most dominant genus was *Pseudomonas* spp. It was retrieved on 50% ilmenite at $10^6$ CFU g$^{-1}$. Interestingly, the next maximum group was *Enterobacter* reaching $10^6$ at 0% and $10^5$ at 80%. Both *Alcaligenes*, *Aeromonas* and *Marinococcus* were retrieved at $10^6$ CFU g$^{-1}$ at 75%. *Aeromonas* was retrieved at $10^6$ CFU g$^{-1}$ at 75%. Least retrievable was *Acinetobacter* and was retrieved only on 80% concentration of ilmenite. Preliminary analysis of siderophore activity showed that all the isolates were actively producing siderophores at the end of the 15$^{th}$ day.

### 6.2.1.2.2. Discussion

Microbes inhabiting terrestrial or aquatic environments acquire iron directly from natural sources that are for the most part insoluble. Ferric minerals serve as a primary nutritional source of iron for a variety of microbes, including water-borne pathogens and environmental species. Their iron-scavenging activities has important geochemical and environmental repercussions, both for global iron cycling, trace metal mobilization as well as mineral instability over geological time (Cornell and Schwertmann, 1996; Hersman et al., 2001; Kraemer, 2004). Moreover, studies have shown that a number of bacterial pathogens require sufficient iron acquisition to enhance significantly their virulence capability (Expert et al., 1996; Genco and Desai, 1996; Mietzner et al., 1998; Schryvers and Stojilkovic, 1999; Vasil and Ochsner, 1999).
Fig 6.4: Effect of varying concentration of ilmenite on the different genera of bacteria, (a-g) different genera, h) total retrievability
Maximum culturability is at a ratio of one part of sand and three parts of ilmenite. Siddiquie et al., (1984) reported heavy minerals to range from 7-79% at Kalbadevi. Increases in bacterial cell abundance have been reported previously in Fe-amended bottle experiments from the equatorial Pacific (Price et al., 1994), Gerlache Strait, Antarctica (Pakulski et al., 1996), and coastal California (Hutchins and Bruland, 1998). The average abundance of bacteria in the control and experimental flasks was in the order of $10^6$ CFU g$^{-1}$. Most of the isolates were gram-negative. Previous study by Atlas and Barther, (1981) have found that most of the marine bacteria i.e. >95% were gram negative and motile.

*Pseudomonas* spp. were predominant in all the experimental flasks. *Alcaligenes* and *Marinococcus* were optimally retrieved at 75% while *Pseudomonas* was optimally retrieved at 50% suggesting that high concentration of ilmenite may be detrimental. Bacterial species of the genus *Bacillus, Arthrobacter, Clostridium* and *Pseudomonas* have been reported as Fe-reducing bacteria (Ottow and Glathe, 1971; Lovley, 1993). Likewise, *Acinetobacter* are also retrievable at 80% ilmenite suggesting that they could be potential pathogens. It is known that some *Acinetobacter* spp such as *Acinetobacter haemolyticus* is an opportunistic pathogen and produces siderophores such as acinetoferrin (Okujo et al., 1994). Some of the *Acinetobacter* are known to express siderophore-mediated iron-acquisition systems (Dorsey et al., 2004). Likewise *Aeromonas* spp is known to be involved in Fe(III) reduction (Knight and Blakemore, 1998). The culturability of *Vibrio* was not affected by ilmenite suggesting that this group of organisms do not respond to varying concentration of iron or are adaptable to wide concentration of iron released from ilmenite. It is known that some of *Vibrio* possess active transport system for iron and do not seem to be affected very much by concentration of ilmenite (Mazoy et al., 1997). With *Enterobacter* it is interesting to note that there are definitely two groups - those that grow at 80% ilmenite and those that grow at lower concentration.

Thus the effect of increasing concentration of ilmenite on retrievability showed that it improved by nearly 88% especially on 75% concentration of ilmenite.
6.2.1.3. Effect of iron released from ilmenite at bacterial level on abundance and activity at primary level

In order to appreciate the effect of the reduced iron released, experiments were conducted to monitor the changes at the bacterial and primary level i.e. changes in bacterial and phytoplankton abundance. Diversity at the phytoplankton level was examined. Also, net changes in Fe(II) and Fe(III) were monitored throughout the experiment.

6.2.1.3.1. Results

6.2.1.3.1.1. Change in iron concentrations with time

Generally, the concentration of Fe(II) and Fe(III) followed an irregular pattern of alternate increase and decrease in both the controls (C1-only seawater, C2-seawater + FeSO₄·7H₂O), while in the presence of ilmenite, these two components of Fe increased significantly with time (r >0.952, n = 11, p ≤ 0.001) (Fig 6.5, Table 6AT1, Appendix III).

6.2.1.3.1.2. Effect of FeSO₄·7H₂O and ilmenite on total living microbial biomass - ATP

The ATP concentration increased during the first few days of incubation, followed by a decrease. In the flasks containing seawater and ilmenite i.e. Exp-1, ATP was nearly 50 times higher than the C-2 and Exp-2. This concentration increased significantly with time (r = 0.836, n = 11, p ≤ 0.001) (Fig 6.6a, Table 6AT1, Appendix III).

6.2.1.3.1.3. Effect of FeSO₄·7H₂O and ilmenite on total bacterial counts (TC)

TC showed an increase with time. At the end of 84d the maximum cell numbers were recorded in C-2. The numbers increased from 2.9 x 10⁷ to 16.9 x 10⁷ cells mL⁻¹ in C-1, to 17.2 x 10⁷ cells mL⁻¹ in C-2, to 3.18 x 10⁷ cells mL⁻¹ in Exp-1, and to 5.13 x 10⁷ cells mL⁻¹ in Exp-2 flask. This increase in TC was significant only in controls [(r = 0.936(C-1), r = 0.654(C-2)]. TC also related positively with Fe(III) in C-1 (r = 0.599) (Fig 6.6b, Table 6AT1, Appendix III). However, in the experimental flasks, TC did not relate with any parameters and increase in TC was static at 10⁷ cells mL⁻¹.
Fig 6.5: Changes in concentration of (a) Fe(II) and (b) Fe(III) with time in control (C-1 - seawater, C-2 - FeSO₄·7H₂O) and experimental microcosms (Exp-1 - seawater + ilmenite, Exp-2 - seawater + ilmenite + FeSO₄·7H₂O).

Fig 6.6: Changes in (a) ATP concentration and (b) bacterial cell counts (TC) with time in control (C-1 - seawater, C-2 - FeSO₄·7H₂O) and experimental microcosms (Exp-1 - seawater + ilmenite, Exp-2 - seawater + ilmenite + FeSO₄·7H₂O).
6.2.1.3.1.4. Effect of FeSO$_4$.7H$_2$O and ilmenite on Chlorophyll a (Chl a)

Chl a concentration increased up to 14d and decreased thereafter. It peaked on 7d in control flasks and on 14d in Exp-1 and 2. Chl a related significantly to many parameters like phytoplankton abundance, ATP, algal abundance, Fe(II) and Fe(III) in Exp-1 (Fig 6.7a, Table 6AT1, Appendix III).

6.2.1.3.1.5. Effect of FeSO$_4$.7H$_2$O and ilmenite on phytoplankton

Phytoplankton cell abundance followed a varied growth pattern (Fig 6.7b). Initial cell number was 8.0(±0.4) x 10$^3$ cells L$^{-1}$. Maximum cell counts of 4.5 x 10$^4$ cells L$^{-1}$ was recorded on the 7d in C-1, and reduced thereafter. In C-2, the peak abundance was attained on the third day with 8.6 x 10$^4$ cells L$^{-1}$. It attained maximum counts on the 42nd day with 32.8 x 10$^4$ cells L$^{-1}$ in Exp-1. Here, there was a significant increase in cell abundance with time (r = 0.853, n = 11, p < 0.001) and it related positively with ATP, Fe(II) and Fe(III) (Table 6AT1, Appendix III). In the Exp-2, there was significant increase in abundance (r = 0.768, n = 11, p < 0.01), and maximum abundance was recorded on the 56th day with 27.8 x 10$^4$ cells L$^{-1}$ (Fig 6.7b). Surprisingly, the phytoplankton cell abundance followed the same increase in concentration of ATP. However, this relationship was not significant. In C-2, the cell abundance peaked within 3d at lower number of 8.61 x 10$^4$ cells L$^{-1}$. In Exp 1 and 2, the abundance peaked much later i.e. on 42d, which was >20times higher than control.

6.2.1.3.1.6. Effect of FeSO$_4$.7H$_2$O and ilmenite on distribution of different genera of phytoplankton

Diatoms dominated in terms of the number of genera and abundance. Initially, the phytoplankton assemblage comprised of 14 genera namely Asterionella spp., Biddulphia spp., Coscinodiscus spp., Ditylum spp., Grammatophora spp., Navicula spp., Nitzschia spp., Phaeocystis spp., Prorocentrum spp., Rhizosolenia spp., Synedra spp., Thalassionema spp., Thallassiothrix spp., and Triceratium spp. (Fig 6.8). During the course of the experiment, 29 genera were identified. Highest number of genera were recorded on 3d in C-1 (16 genera), C-2 (17 genera) and Exp-2 (28 genera) and 14d in Exp-1 (27 genera). Nitzschia spp. dominated followed by Navicula spp. Though Coscinodiscus spp. was present in large numbers in the controls, their
Fig 6.7: Changes in (a) Chl a concentration, (b) phytoplankton cell abundance, (c) algal abundance with time in control (C-1 - seawater, C-2 - FeSO₄·7H₂O) and experimental (Exp-1 - seawater + ilmenite, Exp-2 - seawater + ilmenite + FeSO₄·7H₂O) microcosms.
Fig 6.8: Ilmenite alone in microcosm (c) Exp-1 promotes higher abundance and diversity than (d) Exp-2, (a) C-1 and (b) C-2.
numbers were reduced sharply in Exp-1 and 2. *Eunotia* and *Pinnularia* spp. were found to increase in Exp-1 and 2. These species were conspicuously absent in control flask during the later stages of incubation. While Exp-2 showed highest species richness (\(d = 4.647\)), Exp-1 showed highest diversity index (\(H' = 3.156\)) and evenness (\(J' = 0.9576\)) (Table 6.1)

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There was massive development of green algae in Exp-1 and 2 (Fig 6.7c, Plate 6.2). Bubbles were seen attached to the mat at the end of 84d. However in C-2, the mats were translucent and distributed throughout the flasks. SEM images showed that diatoms as well as algal filaments apparently covered the ilmenite grains (Plate 6.3). Preliminary counts showed that ciliates numbered \(12 \times 10^2\) cells L^-1 in C-1, \(8.8 \times 10^2\) cells L^-1 in C-2, \(14 \times 10^2\) cells L^-1 in Exp-1 and \(12.8 \times 10^2\) cells L^-1 in Exp-2.
Plate 6.2: Effect of iron released from ilmenite on abundance and activity at primary level
a) C2 flask containing FeSO₄.7H₂O
b) Exp-1 flask containing ilmenite grains
c) Exp-2 flask containing ilmenite and FeSO₄.7H₂O

Plate 6.3: Colonization of ilmenite grains by diatoms and green algae in Exp-1 flask containing ilmenite grains
a) Image using light microscopy
b-d) SEM images
6.2.1.3.2. Discussion

It is well known that in marine environment, iron is an important micronutrient controlling primary production, the availability of which depends on the speciation of iron. Consequently, there are ecosystems that are limited by this micro-nutrient. The transportation of iron to the marine environment is usually through three pathways i.e. via fluvial input, atmospheric deposition and processes on seafloor such as diagenesis, sediment re-suspension and hydrothermal venting (Ussher et al., 2004). Thus iron containing placer minerals like magnetite, ilmenite released from terrestrial rocks and transported by rivers and streams from the hinterland into the adjoining seas could be a major source of iron especially in the near-shore environments.

The experiments conducted to simulate natural systems in microcosms show that iron released from ilmenite could be stimulatory. In Exp-1 flask, there was a significant accumulation of iron in the form of Fe(II) and Fe(III) though there was no added FeSO$_4$.7H$_2$O suggesting microbial participation. Interestingly, the $r$ values which highlight the release of iron with time were close to the values in Exp-2 where FeSO$_4$.7H$_2$O was added (Table 6AT1, Appendix III). This release of iron by indigenous bacteria has been dealt in section 6.2.1.1. The release of iron not only supports bacteria but other living organisms. This influence on living organisms could be measured in terms of ATP. It is well known that iron plays a major role in the reduction of oxygen for synthesis of ATP, photosynthesis as well as respiratory electron transport (Geider and La Roche, 1994; Neilands, 1995). This experiment clearly demonstrates that iron released from the ilmenite could contribute to the high biomass which in turn leads to high concentration of ATP. This is corroborated by high phytoplankton and benthic algal biomass in Exp-1 but not by bacterial counts.

Total bacterial counts were generally in the range of $10^{7-8}$ cells mL$^{-1}$. Surprisingly, the controls recorded bacterial numbers that were one order more than the experiment. Moreover, in the presence of ilmenite, this numbers seem to be more or less static at $10^{7}$ cells mL$^{-1}$. This lack of increase in bacterial biomass could be due to the grazing by the protozoans at the next
trophic level. It is quite possible that the presence of grazers in the size range of <200μm could contribute to these low bacterial numbers. Ciliates were generally encountered in the samples and they are also known to predate on the bacteria. Therefore the bacterial abundance remaining apparently at the same level and one order below control could be due to these grazers. The bacterial abundance was thus kept in a dynamically static state by the secondary grazers, that were more abundant in experimental than the control flasks. This lack of difference in Exp-1 and 2 suggests that bacteria are not inhibited by higher concentration of iron in Exp-2. These observations suggest that bacteria can tolerate higher concentrations than primary producers. Study by van Wambeke et al., (2008) has shown that after stimulation of heterotrophic bacteria with Fe, the number of heterotrophic flagellates also increased. However, this leads to enhanced bacterial predation. The stronger relationship noticed between bacterial numbers and Fe(III) concentration in the control C-1 flask could be due to the use of Fe(III) as an electron acceptor in respiratory cycles for maintenance. This relationship is uncoupled in experimental flasks especially Exp-2 where iron is non-limiting.

Iron is not only required in the respiratory but also in the photosynthetic cycles. Chl a was also used as a proxy for live phytoplankton biomass because of the difficulty in differentiating between live and dead phytoplankton. During the course of the experiment, both phytoplankton density and Chl a increased in the experimental flasks. It was much higher than C-1 and C-2 and peaked on 14d. In C-1 and C-2, the Chl a concentrations peaked by 3d, and there was a gradual decrease thereafter. The delayed peak in the Exp-1 and 2 is attributable to the slower release of iron from ilmenite. The earlier attainment of the peak in the controls could be due to more readily available iron but at much lower concentration. The concentration of Chl a is highest in Exp-2 due to increased iron released from ilmenite as well as due to the added FeSO₄.7H₂O. Thus the experiment clearly demonstrates that the iron released from the mineral ilmenite is used in photosynthesis and thus stimulate the growth of phytoplankton. In mesoscale experiments conducted elsewhere, phytoplankton biomass and productivity,
especially in large-celled phytoplankton such as diatoms, increased several fold after Fe enrichment in the surface mixed layer (Martin et al., 1994; Coale et al., 1996, 2004; Boyd et al., 2000; Tsuda et al., 2003). The relationship between iron and Chl a is interesting in the present set of experiments. A strong relationship between Chl a and iron is encountered only in Exp-1. It is low and insignificant in C-1, C-2 and Exp-2 for different reasons, iron being highly limiting in C-1 and C-2 and in excess in Exp-2. In Exp-1, the concentration of iron governs the growth and sustenance of phytoplankton for a longer period. Besides concentrations of Chl a related significantly to phytoplankton abundance suggesting high viability.

The average abundance of phytoplankton in Exp-1 and Exp-2 increased significantly with time (p ≤ 0.01) and recorded a high abundance which was >20 times more than C-1. SEM images clearly revealed the colonization of the ilmenite grains not only by the filaments of algae but also by diatoms (Plate 6.2 an 6.3). As mentioned earlier, cell abundance related positively with Chl a, ATP, Fe(II) and Fe(III) in Exp-1. Both Exp-1 and 2 supported 27 and 28 genera as compared to 16 and 17 in C-1 and C-2. The total number of phytoplankton count, diversity index and species evenness index was highest in Exp-1. Diatoms were dominant in the control as well as the experimental microcosms. Generally, in the natural marine ecosystems, the Bacillariophyta (diatoms) are found to be the most dominant phytoplankton group. Bottom dwelling genera such as Nitzschia spp and Navicula spp. proliferated at the later stages of the experiment in all the microcosms suggesting they had tolerance to a wider range in iron concentration. However, in the presence of ilmenite i.e. Exp-1 and 2, percentage occurrence of two genera namely Eunotia and Pinnularia spp. increased during the later stages of the experiment. Their percentage increased from non-detectable levels to >4% of the total genera at 85d. Both these species have been known to occur in iron enriched waters of moderately high conductivity (Czarnecki and Cawley, 1997), thus suggesting their preference for higher concentrations of iron. Site specific studies have shown that Pinnularia occurs as brown mucilaginous mats on wet soils and along the border of streams and pools commonly in
thermal and non-thermal sulfuric acid habitats having high concentrations of metals at pH 1.0-3.0 (Albertino, 1995). Strands of filamentous algae and bubbles were also predominantly present especially in Exp-1 indicating active algal and plankton growth (Plate 6.2 and Plate 6.3). In contrast, C-1 showed no visible growth throughout the experiment. Growth in C-2 was marked by white floccules of diatomite indicating early loss of viability. Such observations have been made by Sutherland et al., (1998). "Blister" or "bubble" mats have been usually attributed to oxygen trapped in the microalgal mucilage (Jørgensen et al., 1983; Yallop et al., 1994).

The present experiments with microcosms clearly highlight the influence of the bio-available iron released from ilmenite by microbial action on the abundance and diversity of phytoplankton. It also shows that in the presence of ilmenite alone, there is both increase in diversity and evenness that is better than in the presence of combination of FeSO₄·7H₂O and ilmenite. The health of the ecosystem is thus maintained by the diversity it promotes. The stimulatory effect of iron released from this mineral could be beneficial to the biocoenosis in Kalbadevi Bay.

6.2.2. Iron released from ilmenite by selected isolates

In order to discern the contribution from morphologically and numerically different types of bacteria for iron release, experiments were carried out to screen them for ability to release iron from ilmenite.

6.2.2.1. Results

Nearly 60% of the 100 isolates obtained on different media had the ability to release Fe from mineral ilmenite. The data presented here pertains to four of the best isolates which could effectively release iron from ilmenite.

6.2.2.1.1. Characterization of the studied bacterial isolates

Microbial dissolution of ilmenite was followed in the laboratory using bacterial isolates. Preliminary analyses revealed that the experiments on four bacterial isolates showed that the culture when grown in media amended with mineral ilmenite was capable of leaching out iron in the form of Fe(II).
6.2.2.1.2. Cell and colony morphology

The biochemical characterization of these isolates is given in the table 6.2. All the strains were gram negative and non motile. S3 and S11 were cocobacilli while S7 and S8 were coccoid. All the strains were oxidase positive.

6.2.2.1.3. Phylogeny of studied isolates

Potential isolates were subjected to 16S rDNA analyses for identifying them upto species level (Table 6.2).

Table 6.2: Biochemical and 16S rDNA characterization of the bacterial isolates

<table>
<thead>
<tr>
<th>Isolates number</th>
<th>Gram character</th>
<th>Motility</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>OF test</th>
<th>Amylase</th>
<th>Protease</th>
<th>Lipase</th>
<th>DNase</th>
<th>Nearest homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3</td>
<td>Gm –ve coccooid</td>
<td>Non motile</td>
<td>+</td>
<td>-</td>
<td>Oxidative</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>uncultured alpha proteobacterium B23; (GenBank entry: EU360292) (Homology: 99.1%)</td>
</tr>
<tr>
<td>S7</td>
<td>Gm –ve coccooid</td>
<td>Non motile</td>
<td>+</td>
<td>-</td>
<td>Oxidative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>bacterium CWISO21; (NCBI Accession no: DQ334359) (Homology: 99.5%)</td>
</tr>
<tr>
<td>S8</td>
<td>Gm –ve coccooid</td>
<td>Non motile</td>
<td>+</td>
<td>-</td>
<td>Oxidative</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>bacterium GB058; (Accession No: AB433333) (Homology: 99.4%)</td>
</tr>
<tr>
<td>S11</td>
<td>Gm –ve coccooid</td>
<td>Non motile</td>
<td>+</td>
<td>-</td>
<td>Oxidative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Yangia pacifica (T); type strain: DX5-10; AJ877265 (Homology: 99%)</td>
</tr>
</tbody>
</table>

Phylogenetic analysis of the 16S rDNA sequence of S3 isolate indicated that it belonged to class Alphaproteobacteria. The nearest known relative of S3 isolate is an uncultured Alphaproteobacterium B23 (GenBank entry: EU360292). S3 isolate has 99.1% sequence identity to uncultured Alphaproteobacterium B23 over 1200 unambiguously aligned positions (Fig 6AF1, Appendix IV). Isolate S7 was grouped within the gamma class of the
Proteobacteria and had close relationship of 99.5% with bacterium CWISO21, (NCBI Accession no: DQ334359) (Table 6.3, Fig 6.9).

<table>
<thead>
<tr>
<th>S_ab score</th>
<th>Organism Name</th>
<th>NCBI Accession No</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.979</td>
<td><em>Alteromonas</em> sp.</td>
<td>AJ391191</td>
</tr>
<tr>
<td>0.984</td>
<td><em>Alteromonas</em> sp.</td>
<td>AJ391192</td>
</tr>
<tr>
<td>0.983</td>
<td>biphenyl-degrading bacterium</td>
<td></td>
</tr>
<tr>
<td>0.987</td>
<td><em>Pseudoalteromonas</em> sp.</td>
<td></td>
</tr>
<tr>
<td>0.989</td>
<td>uncultured bacterium;</td>
<td></td>
</tr>
<tr>
<td>0.995</td>
<td>bacterium</td>
<td></td>
</tr>
<tr>
<td>0.980</td>
<td>uncultured <em>Alteromonas</em> sp.</td>
<td></td>
</tr>
<tr>
<td>0.980</td>
<td>uncultured bacterium;</td>
<td></td>
</tr>
<tr>
<td>0.995</td>
<td><em>Pseudoalteromonas</em> sp.</td>
<td></td>
</tr>
<tr>
<td>0.978</td>
<td>uncultured <em>Alteromonas</em> sp.</td>
<td></td>
</tr>
<tr>
<td>0.981</td>
<td>uncultured bacterium</td>
<td></td>
</tr>
<tr>
<td>0.982</td>
<td><em>Alteromonas mauleodi</em> 'Deep ecotype'</td>
<td></td>
</tr>
<tr>
<td>0.982</td>
<td><em>Alteromonas</em> sp.</td>
<td></td>
</tr>
<tr>
<td>0.980</td>
<td><em>Alteromonas alvinellae</em></td>
<td></td>
</tr>
<tr>
<td>0.980</td>
<td>uncultured bacterium;</td>
<td></td>
</tr>
<tr>
<td>0.980</td>
<td>uncultured bacterium</td>
<td></td>
</tr>
<tr>
<td>0.980</td>
<td>uncultured bacterium</td>
<td></td>
</tr>
<tr>
<td>0.982</td>
<td><em>Pseudoalteromonas</em> sp.</td>
<td></td>
</tr>
<tr>
<td>0.981</td>
<td><em>Alteromonas</em> sp.</td>
<td></td>
</tr>
<tr>
<td>0.982</td>
<td>uncultured bacterium</td>
<td></td>
</tr>
</tbody>
</table>

* S_ab: Sequence Match Score

S7 culture Aligned Sequence Data: (1411bp)

> S7

ACCATGCAAGTCCAGAACGGTAAACATTTTAGCTTGCTAATCGATGCGATGTCGGGACGGTGAGAATGTTGGAAGAAGGAGATACGTTGGAAACAGACTGC

Table 6.3: Alignment View and Distance Matrix Table (With S7 sequence taken as reference sequence)
Fig 6.9: Phylogenetic affinity of S8 isolate
16S rDNA analysis revealed that the S8 isolate was most closely related (99.4%; 1,214 nucleotide positions considered) to the Flavobacteriaceae bacterium GB058; (Accession No: AB433333) a member of the family Flavobacteriaceae within the Flavobacteria class of Bacteroidetes (Fig 6AF2, Appendix IV). The closest known relative of S11 isolate was Yangia pacifica (T), type strain: DX5-10 with 99% similarity in 1325 nucleotides considered (Accession No. AJ877265) (Fig 6AF3, Appendix IV).

6.2.2.1.4. Cell growth and reduction of ilmenite

The pH in the cell free control and the experiment flasks fluctuated between 4.5-7.0 during the entire experimental period for nearly all the tested isolates (Table 6.4, Fig. 6.10). Concentration of Fe(II) in the media of the test isolate, S7, was double that of the control. Cell numbers of S7 isolate showed an increasing trend in presence of increasing concentration of Fe(II) (Plate 6.4). The S8 and S11 isolates displayed a sinusoidal growth curve. It initially increased by day 13 than showed a decreasing trend. These numbers again increased by day 62. TEM analysis showed the presence of intracellular metal accumulation in S8 and S11 isolate (Plate 6.5).

<table>
<thead>
<tr>
<th>Isolates tested</th>
<th>Control</th>
<th>S3</th>
<th>S7</th>
<th>S8</th>
<th>S11</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH change during experiment</td>
<td>6.46</td>
<td>6.47-</td>
<td>5.11</td>
<td>6.48</td>
<td>6.18</td>
</tr>
<tr>
<td>Maximum cell yield (x 10^6 cells mL^-1)</td>
<td>8.72</td>
<td>382</td>
<td>20.48</td>
<td>60.48</td>
<td></td>
</tr>
<tr>
<td>Maximum amount of Fe(II) estimated in the solution (μM)</td>
<td>0.91</td>
<td>0.17</td>
<td>0.184</td>
<td>0.21</td>
<td>0.19</td>
</tr>
<tr>
<td>Rate of Fe(II) removal (μM d^-1)</td>
<td>-12.12</td>
<td>-2.52</td>
<td>-19.90</td>
<td>-1.25</td>
<td>-1.94</td>
</tr>
</tbody>
</table>

* initial in parentheses
Fig 6.10: Change in pH and cell nos during removal of Fe (II) from ilmenite by the selected isolates
Plate 6.4: SEM images of isolates involved in the release of iron from ilmenite
Plate 6.5: TEM images of S8 isolate (a-b) and S11 isolate (c-d)
Though the trends in the relationship with Fe(II) and cell numbers were visible, there was no significant correlation between the two parameters. Chemical rate of removal of Fe(II) in the solution fluctuated between -12.12 to 15.62 μM d^-1. However, the biological rate of removal of Fe(II) from ilmenite varied between -19.90 to 35.48 μM d^-1 and the S7 isolate displayed the highest rates of removal.

6.2.2.2. Discussion

Iron in sediments often exists as poorly crystalline Fe(III) oxides (Lovley, 1993; Nealson and Saffarini, 1994). Iron oxides and oxyhydroxides which may occur in marine environments are goethite (α-FeOOH), akaganéite (β-FeOOH), lepidocrocite (γ-FeOOH), feroxyhite (δ'-FeOOH), ilmenite (FeTiO₃), hematite (α-Fe₂O₃), magnetite (Fe₃O₄) and maghemite (γ-Fe₂O₃) (Burns and Burns, 1981). The extent of diversity among dissimilatory metal reducers has been reviewed by Lovley (1993) and Nealson and Saffarini (1994). It is well known that microorganisms can reduce Fe(III) (hydr)oxide minerals, hematite, and magnetite (Roden and Zachara, 1996; Fredrickson et al., 1998; Zachara et al., 1998). The four tested isolates belonged to Proteobacteria and Bacteroidetes. The experiment with the isolates is clearly suggestive of the diversity of the organisms in the Kalbadevi beach. Lonergan et al., (1996) in his study of phylogenetic analysis of dissimilatory Fe(III)-reducing bacteria indicated that the metal reduction capacity is a characteristic that is widely spread in the domain Bacteria.

All the isolates were capable of releasing Fe(II) from the mineral ilmenite. This rate of removal was the highest to S7 isolate which had a taxonomic affinity to Gammaproteobacteria. The negative rate of removal of Fe(II) in the solution suggested that the Fe(II) brought into the solution was re-oxidized to Fe(III). Growth rate and cell numbers can be caused by minor differences in the inoculum size, the viability of the cells in the inoculum, temperature, pH, etc and may have an implication in the variation in the rate of Fe(II) removal. This type of variability in reduction experiments with microorganisms have previously been noted (Fredrickson et al., 1998). It is probable that the use of
higher innocula of S7 isolate and perhaps more rapidly metabolizing cells may have established relatively more favourable redox conditions. This may also probably explain the higher rate of Fe(II) removal in the solution as well as the equally faster re-oxidation. Low accumulation of ferrous iron in medium containing other isolates i.e S3, S8 and S11 could indicate possibly that these isolates were capable of extracting the Fe(II) from ilmenite at a net rate that is slow.

Thus it may be inferred that ilmenite which was previously known as recalcitrant mineral (Page and Huyer, 1984; Page and Grant, 1987) and resistant to bacterial attack is possibly more accessible to bacterial reduction than hitherto believed.

6.3. Oxidative phase (Oxidation of iron released from ilmenite)

6.3.1. Effect of varying concentration of chemical form of iron, FeSO$_4$.7H$_2$O on a bacterial isolate.

6.3.1.1. Results

6.3.1.1.1. Ecological background

The total bacterial abundance in the Kalbadevi berm sediment ranged between $10^{5-6}$ cells g$^{-1}$. The morphotypes representing the isolate Fe13 were frequently encountered on the isolation medium and made up to 10% of the total iron utilizing bacteria retrieved on ferric ammonium citrate plates.

6.3.1.1.2. Characterization of the bacterial isolate

The biochemical characterization of this isolate, Fe13 showed that it is a gram-negative short rod belonging to Gammaproteobacteria member *Pseudomonas* sp. Results of 16S rDNA analysis confirmed its generic identity. The isolate was closely related to *Pseudomonas agarici* at a similarity index of 98%.
6.3.1.1.3. Bacterial cell growth in presence of increasing concentration of iron

Cell growth in terms of cell numbers was monitored to assess the response of the cells to increasing iron concentration. The initial inoculum of cells was $0.084(±0.022) \times 10^9$ cells mL$^{-1}$. Maximum growth was attained in 9d. In EM-E1 medium containing only seawater and glucose, the cell abundance increased to $0.78(±0.27) \times 10^9$ cells mL$^{-1}$ (Fig 6.11i). However, the cell numbers in EM-E2, EM-E3, EM-E4 and EM-E5 increased 25%, 30%, 32% and 6% more than the Fe-unamended EM-E1 medium. In EM-E2, the cell numbers reached a maximum of $0.97(±0.048) \times 10^9$ cells mL$^{-1}$ in 9d. Similarly, in EM-E3, the maximum cell numbers recorded was $1.02(±0.014) \times 10^9$ cells mL$^{-1}$ in 9d. In EM-E5 medium, the peak cell abundance was lower at $0.82(±0.09) \times 10^9$ cells mL$^{-1}$. The calculated instantaneous growth rate constant $μ_9$ increased with the increasing concentration of iron. Consequently, the corresponding mean generation time for cells was lower at 100 µM-Fe concentration (Table 6.5).

The time required for the cells to double in numbers was low when the medium was amended with Fe concentration ranging between 1 to 100 µM FeSO$_4$.7H$_2$O.

<table>
<thead>
<tr>
<th>Concentration of FeSO$_4$.7H$_2$O (µM)</th>
<th>$μ_9$</th>
<th>g</th>
<th>cell specific activity (M cell$^{-1}$ d$^{-1}$) Fe(II)$→$Fe(III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.354</td>
<td>2.811d</td>
<td>2.61</td>
</tr>
<tr>
<td>1</td>
<td>0.508</td>
<td>2.551d</td>
<td>8.73</td>
</tr>
<tr>
<td>10</td>
<td>0.758</td>
<td>2.503d</td>
<td>-10.3</td>
</tr>
<tr>
<td>100</td>
<td>0.992</td>
<td>2.503d</td>
<td>119.96</td>
</tr>
<tr>
<td>1000</td>
<td>1.133</td>
<td>2.737d</td>
<td>-221.9</td>
</tr>
</tbody>
</table>

Like the cell numbers, ATP concentrations and cellular proteins increased with time. Biomass yield measured in terms of cellular ATP concentration also increased with time. However the average concentration of cellular ATP did
Fig 6.11: Change in (i) cell nos. (ii) ATP content and (iii) protein concentration of isolate Fe 13 in presence of varying concentration of iron
(a) EM medium alone, (b) EM medium + 1 μM FeSO₄·7H₂O, (c) EM medium + 10 μM FeSO₄·7H₂O, (d) EM medium + 100 μM FeSO₄·7H₂O and (e) EM medium + 1000 μM FeSO₄·7H₂O
not differ between the Fe amended media and Fe unamended medium (Fig 6.11ii). Cell protein concentrations increased from 245.31(±13.61) μg mL\(^{-1}\) in the EM-E1 medium (Fig 6.11iii). Amendments of Fe in EM-E2, EM-E3, EM-E4 and EM-E5 medium resulted in maximal values of 930.47(±82.97) μg mL\(^{-1}\) in 15d, 878.22(±11.71) μg mL\(^{-1}\) in 9d, 877.11(±11.71) μg mL\(^{-1}\) in 15d, and 1735.31(±50.06) μg mL\(^{-1}\) in 15d respectively. pH measurements showed a decrease of the initial pH but no difference at the end of the incubation period (Fig 6.12).

6.3.1.1.4. Bacterial cell morphology in presence of iron amendment

The above biochemical changes were accompanied by morphological changes. Changes in cell size and aggregation behavior, biofilm and precipitate formation was evident in SEM under 8000x magnification (Plate 6.6). Cells from unamended medium were short rod-shaped bacteria measuring 1.0x0.4 μm (Plate 6.6b). Aggregation, biofilm and cluster formation was generally visible in amended experimental flasks and rarely in control. When the medium was amended with 1 μM-Fe (Plate 6.6c) the cell size decreased to 0.8 x 0.3 μm and was accompanied by the formation of a biofilm. At 10 μM-Fe amendment, the cell size further decreased to 0.7 x 0.2 μm and the cells aggregated in clusters (Plate 6.6d). The size of the biofilms formed was larger than those formed at 10 μM-Fe amendment. At 100 μM-Fe concentration, the cells seemed to increase marginally in size to 0.8 x 0.3 μm and were accompanied by precipitates and thicker biofilm formations (Plate 6.6e). However, cells from 1000 μM-Fe amendment were examined under lower magnification of 3000x using SEM. At 1000 μM, the cells became very large measuring 2.5 x 0.6μm, and were associated with biofilm and precipitates (Plate 6.6f).

6.3.1.1.5. Chemical nature of accumulated iron

EDS analysis further confirmed that the metal precipitate was iron oxide (Fig 6.13). The point x-ray analysis carried out on cells as well as on Fe precipitate in the microscope field revealed that the intensity of peaks for Fe on bacterial cell was 20% higher than the background precipitate. XRD analysis was
Fig 6.12: Change in pH of the medium during cell growth
(a) EM medium alone, (b) EM medium + 1 µM FeSO\textsubscript{4}.7H\textsubscript{2}O,
(c) EM medium + 10 µM FeSO\textsubscript{4}.7H\textsubscript{2}O,
(d) EM medium + 100 µM FeSO\textsubscript{4}.7H\textsubscript{2}O
and (e) EM medium + 1000 µM FeSO\textsubscript{4}.7H\textsubscript{2}O
Plate 6.6: SEM images of bacterial cells and matrix formation by Fe13 strain
(a) represents 0-d culture, (b) EM medium alone, (c) EM medium + 1 μM
FeSO₄·7H₂O, (d) EM medium + 10 μM FeSO₄·7H₂O, (e) EM medium + 100 μM
FeSO₄·7H₂O and (f) EM medium + 1000 μM FeSO₄·7H₂O
(Magnification for A to E-x8000, F-x3000)
Fig 6.13: SEM-EDS spectrum showing typical elemental composition in (A) precipitate in control flask, (B) precipitate in experimental flask, (C) cells in experimental flask

Fig 6.14: XRD analysis on precipitate in a) control b) experimental flask
performed to understand the mineral type in bacterial cells. The peaks for biogenic and abiogenic mineral phases were found to be overlapping each other. However, the intensity of the peaks in experiment (Fig 6.14b) was slightly lower than in control (Fig 6.14a). The diffractograms of the iron precipitate confirmed the oxidized form of iron to be Fe₂O₃ and α-Fe₂O₃. Based on the “d” values, the mineral form of Fe was identified as hematite (Fe₂O₃) and goethite (α-Fe₂O₃). The d values for corresponding mineral hematite was 1.692, 1.838 and for goethite was 2.692 and 2.51 (Fig 6.14).

6.3.1.1.6. Change in Fe(II) and Fe(III) rate constant by Fe₁³

The optimal growth concentration of iron was at 100 μM where increase in soluble iron was accompanied by maximum increase in cell numbers. The rate constants for converting soluble Fe to insoluble increased with increasing Fe concentration (Table 6.6). On 9d when the cell numbers peaked, the rate constant “µFe(II)” for soluble iron was negative up to the 10 μM-Fe concentration and became positive at higher concentrations of 100 and 1000 μM (Table 6.6). The result showed that the cell specific activity for the Fe(II)→Fe(III) reaction generally increased with increasing iron-amendment except for some cases which did not follow the trend. The maximum cell specific activity for soluble Fe was recorded at 100μM FeSO₄.7H₂O concentration. It ranged from -221 x 10⁻²⁰ M cell⁻¹ d⁻¹ in EM-E5 to 120 x 10⁻²⁰ M cell⁻¹ d⁻¹ in EM-E4 medium.

<table>
<thead>
<tr>
<th>Concentration of FeSO₄.7H₂O (μM)</th>
<th>µFe(II) control</th>
<th>µFe(II) experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-0.326</td>
<td>-0.556</td>
</tr>
<tr>
<td>1</td>
<td>-0.290</td>
<td>-0.193</td>
</tr>
<tr>
<td>10</td>
<td>-0.254</td>
<td>-0.006</td>
</tr>
<tr>
<td>100</td>
<td>0.254</td>
<td>0.737</td>
</tr>
<tr>
<td>1000</td>
<td>0.000</td>
<td>4.372</td>
</tr>
</tbody>
</table>
6.3.1.1.7. Change in Fe(II)/Fe(III) ratio by Fe13

The average Fe(II)/Fe(III) ratio in the control series ranged from 0.11±0.06 in EM-C1 to 2.96±0.71 in EM-C4. On the other hand, the average ratio in the experimental series during the course of the experiment was 0.64±0.89 in EM-E1, 0.1±0.03 in EM-E2, 0.12±0.04 in EM-E3, 2.72±1.67 in EM-E4 and 2.22±1.14 in EM-E5 (Fig 6.15). The trend in the change over time in ratio of Fe(II)/Fe(III) using voltammetry were comparable to the change in ratio using ferrozine assay at lower concentration of ≤100uM (Fig 6.16).

6.3.1.2. Discussion

The influence of increasing concentration of iron on an isolate from placer rich sediments was studied in order to monitor the increase in cell number and to determine its participation in the changes in Fe(II)/Fe(III) ratio.

Total bacterial cell abundance in the Kalbadevi beach sediments varies from 10^6-6 cells g^-1 and is generally low compared to other sandy intertidal beaches. The natural abundance of bacteria in the present study area is generally an order of magnitude less than reported elsewhere (Schmidt et al., 1998; Luna et al., 2002; Moreno et al., 2006). Bacteria are found commonly attached to the grains in sandy beach sediment and their abundance is controlled by various factors having complex relationships (Meadows and Anderson, 1968; Yamamoto and Lopez, 1985). The isolate was able to grow in the presence of the inorganic iron (FeSO_4·7H_2O) at circumneutral pH and can bring about a net fall in the Fe(II) concentration alternatively thus demonstrating the metabolic ability of this dominant group of *Pseudomonas* from ilmenite rich placer sediments. *Pseudomonas* spp. are generally known for their metabolic versatility.

The growth of the isolate was accompanied by increase in ATP and protein concentrations confirming active participation (Riemer et al., 2004). ATP concentrations which are similar to that of control without iron could be due to the effect of glucose. The scanning electron micrographs demonstrate that the
Fig 6.15 Change in Fe (II)/Fe(III) ratio during cell growth
(a) EM medium alone, (b) EM medium + 1μM FeSO$_4$.7H$_2$O,
(c) EM medium + 10μM FeSO$_4$.7H$_2$O,
(d) EM medium + 100μM FeSO$_4$.7H$_2$O
and (e) EM medium + 1000μM FeSO$_4$.7H$_2$O
Fig 6.16: Comparative study of Fe(II)/Fe(III) ratio using ferrozine assay by spectrophotometry (a & c) and modified Metrohm method by voltammetry (b & d)
bacterial cell size decreases with the increasing iron concentration. The cells in the unamended medium appear to be dividing and hence appear large. However, at the highest concentration of iron tested, the cells appear very elongated (2.5x0.6μ, Plate 6.6f). At 1 and 10 μM amendment, the aggregation of the cells and the formation of biofilm suggest that this physiological adaptation could be towards improvement in iron scavenging ability. At a still higher concentration of 100 μM the matrices enlarge perhaps bypassing the necessity for aggregation. The findings complement the previous work, which, show that iron-deficient cells were smaller than iron-sufficient cells (Page and Grant 1987). This study highlights that when the cells are actively scavenging (in this case ambient 1 to 100 μM) they tend to decrease in size till a threshold is reached.

Generally, the quantity of iron recovered in various fractions do not account for the total iron added initially at the start of the experiment especially at higher concentrations. This is because some of the iron gets converted into hematite and goethite which may not be amenable to reduction by hydroxylamine. Moreover, at higher concentrations some of the iron gets lost as a film on the walls of the vessels. Hence for measuring ferrous iron production, some researchers have used ratio of Fe(II):total Fe, in order to better account for potential error which may inherently be introduced by sampling of a suspended solid phase (Langley et al., 2009). Yet others have used some other ratios such Fe(II)/Fe(III), [Fe(II)+ Fe(III)]/Fe(II) ratio (Sand et al., 1992; Propp and Propp, 2001). Studies on the cell interiors have revealed that the higher the concentration of iron, the lower the Fe(II)/Fe(III) ratio. Further, when the oxygen was less in the medium, the ratio was higher. (Kot and Bezkorovainy, 1993; Kot et al., 1994). In this study, the Fe(II)/Fe(III) ratio was used to monitor changes. Comparison between live experimental and abiotic controls showed definite trends (Fig 6.15).

The Fe(II)/Fe(III) ratios were counterchecked using the voltammeter. Samples containing initial concentration of 100 and 1000 μM of Fe were re-analyzed using voltammetry since the ferrozine method is said to have its limitations of
detecting all the iron in the solution (Anastacio et al., 2008). Figure 6.16 elaborates the results obtained by ferrozine assay and compares it with that obtained by voltammetry using pyrophosphate method. Though the behavior of controls is different, the trend in the experimental curves is comparable. From 0 to 21d, there is a fall in the ratio suggesting oxidation. Subsequently, the trend is either maintained or there is slight increase in the ratio suggesting relative increase in respiration and relative decrease in use of iron as an energy source. Higher average ratios were recorded for concentrations of 100 and 1000 µM indicating that the pool of soluble iron in the medium was more at these concentrations. Also, these ratios were higher in the control medium indicating that most of the iron was in the soluble phase. Similarly, higher ratios of Fe(II)/Fe(III) were recorded using the ferrozine method. This was due to hydroxylamine based extraction method which extracted only the hydroxylamine extractable iron. This is the inherent drawback of the method. However, in this experiment the amount of iron that the bacteria could oxidise to Fe(III) was assessed.

Measurement of release of reduced iron showed that the change is rarely steady suggesting of net oxidation and reduction phases both in control and experiment. However, the changes in the experiment are always more than in the control with the final trend being more oxidative. Like total activity, specific activity (activity normalized for cell growth) followed the same pattern. It is perhaps at this concentration the bacterium used the iron optimally for growth. At higher concentration, the precipitation is higher but growth rate decreases.

Studies have shown that microorganisms have the capability to couple between Fe(II) oxidation and Fe(III) reduction in a localized iron cycle and this can influence the biogeochemistry of other elements including carbon, phosphorus and sulfur (Neubauer et al., 2002). Some researchers opine that this oxidation is more passive than active. They argue that the probability in many cases of the so-called “biological iron oxidation” by these bacteria is confined to absorption of chemically oxidized iron by the sheath or slime layer surrounding the sheaths (Bridge and Johnson, 1998). However, these studies
clearly demonstrate that the oxidation is not passive but active. In this study, the bacterial isolate did not form a sheath, but a biofilm, which probably entrapped the oxidized form of iron. EDS images analyzed revealed that the precipitated form as well the cells were rich in Fe and O. Further, XRD analysis indicated the presence of haematite and goethite. The XRD diffractogram clearly show the similarity in iron oxidation productions between both the control and experiment. However, semi quantitative analysis suggests that these products could be relatively higher in the experiment than in the control. Mean generation time (g) calculated from the increase in cell numbers was higher for concentrations between 1 and 100 μM. At these concentrations, the bacterial isolate oxidizes the soluble iron for mixotrophic growth, thus attaining maximum cell abundance at 100 μM.

The foregoing experiments show that this isolate, Fe13, from ilmenite rich sediment is capable of scavenging the reduced iron and use it as an energy source. It generally expresses optimal mixotrophic growth when the ambient concentration is 100 μM. It is therefore suggested that the ambient concentration of iron in the environment governs the contribution of bacteria like Fe13 to iron oxidation in microaerophic to anaerobic conditions in beach sediments. At very high concentration it accelerates oxidation with reduced cell yield. The experiment elucidates the metabolic capability of the isolate. It could act as an efficient geochemical agent that could mitigate iron concentrations, by oxidizing or precipitating it when the concentrations are high. Thus this bacterial strain may be conveniently harnessed for bioremediation under iron-replete conditions.