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„Enrichment and characterization of anaerobic phototrophic Fe(II)-oxidizing prokaryotes“

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Abstract

Iron is the fourth most abundant element in the Earth's crust and an essential element for most organisms. One of the largest iron deposits in the world is found in the banded ironstone, known as BIFs (banded iron formation), which are distributed in the ancient cratons, such as Australia and Canada. These alternating layers of iron-rich and silicate layers were mainly formed in the Archeaen (3.8 - 1.8 Ga).

Early on there was a theory that bacteria were responsible for this oxidation of Fe(II) to Fe(III). It was discussed that cyanobacteria caused the formation of BIFs, by producing oxygen required for the oxidation of Fe(II). Some decades ago the theory of bacteria relevant for the formation of this iron stones changed because scientists discovered a new group of bacteria which do not need oxygen for life and could oxidize Fe(II) into Fe(III). The so-called Fe(II)-oxidizing anoxygenic phototrophic bacteria that obtain their energy from sunlight and fix CO_2 using Fe(II) as a sole electron donor form solid, usually poorly crystallized iron minerals such as ferrihydrite.

In the last twenty years, much research has been conducted in the field of anoxygenic phototrophic Fe(II)-oxidizing bacteria in marine and freshwater sediments. To evaluate the significance and spread of Fe(II) oxidation by phototrophic Fe(II)-oxidizing bacteria and to find out which kind of bacterium is responsible for this reaction, twelve samples were collected in the countries of Austria and Switzerland. Moreover, eight more enrichments were cultivated. In the end, one pure culture was obtained from one enrichment culture and characterized in detail.

First, anoxic enrichment cultures, with bicarbonate-buffered freshwater medium and 10 mM of $FeSO_4$, were prepared. The Fe(II) was used as sole electron donor. Cultivation conditions were similar for all experiments: bottles and test tubes were horizontally incubated under a light bulb (25 W) at approximately a 45 cm distance at room temperature (~23°C).
There is a possible bias by enriching in medium, so this could be the reason for the few positive results with the freshwater medium.
In addition, six sets of original sample material, where no freshwater medium was used, were also incubated in the light at room temperature. One tube of each sample set was left without additives; a second tube was supplied with 10 mM of $FeSO_4$, and to a third tube 10 mM of $FeSO_4$; and cells of two *Geobacter* strains were added.

Growth experiments and iron measurements were conducted by applying the ferrozine-photometer method and showed an average of 10-21 days for iron oxidation and that iron oxidation did indeed take place in the enrichments without freshwater medium.
A relatively widespread occurrence of phototrophic iron oxidation was observed. Only three out of twenty samples showed no Fe(II)-oxidizing activity in the end.

To isolate this sort of bacterium from enrichments cultures agar dilution series and agar plates were prepared.
The isolate ViMi23 was a microaerophilic phototrophic Fe(II)-oxidizing bacterium with a growth temperature optimum near 25°C. The pH optimum for the growth of ViMi23 was approximately 6.8. It formed red colonies on agar plates and in agar dilution series. Analysis of 16S rRNA gene sequences revealed an affiliation with *Rhodopseudomonas pseudopalustris* DSM 123^T.
Cells of strain ViMi23 were elongated to club-shaped and contained bacteriochlorophyll *a*. This isolate showed no fermentation metabolism, but was able to grow photoheterotrophically with many other substrates, for example, acetate, succinate or cysteine. Strain ViMi23 grew also photoautotrophically with H_2 or sulfur compounds such as thiosulfate or elemental sulfur.

Zusammenfassung

Eisen ist das vierthäufigste Element in der Erdkruste und ein wichtiges Element für die meisten Lebewesen. Eines der größten Eisenvorkommen der Erde findet sich in den Bändereisenerzen, den sogenannten BIFs (Banded Iron Formation), welche in den alten Kratonen, wie Australien und Kanada, verteilt sind. Diese Wechsellagerungen von Silikat- und Eisen-reichen Lagen entstanden unter anderem im Archäikum (3,8-1,8 Ga).
Schon früh gab es eine Theorie wonach Bakterien für die Oxidation von Fe(II) zu Fe(III) zuständig waren. Wurde früher von Cyanobakterien gesprochen, die den benötigten Sauerstoff für die Oxidation zu Verfügung stellten, gibt es seit einigen Jahren die Theorie von Eisenoxidierenden Bakterien welche keinen Sauerstoff fürs Leben brauchen und für die Reaktion von Fe(II) zu Fe(III) zuständig sind. Die sogenannten phototrophen Eisenoxidierenden Bakterien, die ihre Energie aus dem Sonnenlicht erhalten und CO_2 fixieren verwenden Fe(II) als Elektronendonor und bilden aus Fe(II) welches im Wasser gelöst ist, festes meist schlecht kristallisierte Eisenminerale wie Ferrihydrit.

Diese Arbeit beschäftigt sich einerseits mit der ökologischen Verbreitung der Eisenoxidation durch phototrophe Eisenoxidierende Bakterien im Gebiet von Österreich und der Schweiz. Andererseits mit der Isolierung und Charakterisierung eines Bakteriums welches für die Reaktion von Fe(II) zu Fe(III) zuständig ist. Zu diesem Zweck wurden 20 Proben aus unterschiedlichen Ökosystemen wie Eisenquellen, Teichen oder Flüssen genommen.
Mit diesen Proben wurden anschließend im Labor anoxische Anreicherungen mit Bikarbonat gepufferten Frischwassermedium angelegt. Diese wurden, genauso wie alle anderen Versuche, in Glasgefäßen horizontal unter einer Glühbirne (25 W) in etwa 45 cm Entfernung bei Zimmertemperatur (~23°C) inkubiert.
Zusätzlich wurden von sechs Proben Grobanreicherungen ohne Medium angelegt. Je ein Röhrchen der Probe blieb ohne Zusätze, einem weiteren wurden 10 mM $FeSO_4$ zugesetzt und dem dritten Ansatz wurden 10 mM $FeSO_4$ und Zellen zweier *Geobacter*-Arten zugegeben.

Es wurden Wachstumsversuch und Eisenmessungen mittels der Ferrozin-Methode durchgeführt. Diese zeigten, dass die Oxidationen von Fe(II) in Anreicherungen durchschnittlich 10-21 Tage dauerten und dass es in den Grobanreicherungen tatsächlich ebenfalls zu einer Eisenoxidation kam.
Zusammenfassend konnten bei 17 von 20 Proben in einer oder mehreren Anreicherungsmethoden eine phototrophe Eisenoxidation festgestellt werden.

Der zweite Teil der Arbeit beschäftigt sich mit der Isolierung und Charakterisierung eines phototrophen Fe(II)-oxidierenden Bakteriums. Zur Isolierung wurden Agar-Verdünnungsreihen und Agar-Platten verwendet. Das Isolat ViMi23 wurde phylogenetisch, morphologisch und physiologisch charakterisiert. Es handelte sich dabei um ein Bakterium welches aufgrund seiner 16S rDNA nahe mit *Rhodopseudomonas pseudopalustris DSM 123*^{T} verwandt war. Stamm ViMi23 bildete in Agar-Verdünnungsreihen oder auf Agarplatten rote Kolonien.

Die Zellen von Stamm ViMi23 zeigten eine keulenförmige Zellform und beinhalteten Bakteriochlorophyll *a*. Bei einer Temperaturspanne von 4-35°C lag das Temperaturoptimum bei 25°C. Wachstum konnte in einem pH-Bereich von 6.4-7.9 erfolgen, wobei das pH-Optimum bei 6.8 lag. Das Isolat zeigte keinen Gärungsstoffwechsel, konnte aber Sauerstoff als Elektronenakzeptor im Dunkeln und andere Elektronendonatoren zum photoautotrophen oder photoheterotrophen Wachstum nutzen (unter anderem H_2, Thiosulfat, S^0, Acetat, Succinat, Propionat). Liegt neben Fe(II) eine organische Elektronenquelle wie Acetat vor, wird Acetat von Stamm ViMi23 bevorzugt verwendet.

1 Introduction

Iron is the most common element by weight in the Earth's crust and the second most abundant metal in the lithosphere after aluminum (Lutgens &Tarbuck, 1998).
Iron is an essential nutrient for most organisms except for *Lactobacillus* ssp. (Archibald, 1983). Depending on the prevailing environmental physicochemical parameters (examined pH, redox potential and oxygen concentration), iron oxidation states occur twice (+2 and +3) (Stumm & Morgan, 1996). Fe(III) is stable under oxic conditions, while Fe(II) will oxidize rapidly with molecular oxygen in the air or water (Cornell & Schwertmann, 2003; Rose & Waite, 2007).

Iron occurs in different phases in the environment, among other things, as oxides, carbonates, sulfides and silicates, with different solubilities. While Fe(II) is very soluble at the pH of 7, Fe(III) is poorly soluble. Different processes, like adsorption, precipitation and dissolution, are responsible for the concentration of dissolved Fe(II) (Canfield, 2005).
The most important minerals in Fe-rich sediments are goethite [α-FeOOH], hematite [Fe_2O_3], magnetite [Fe_3O_4], siderite [$FeCO_3$], chamosite [$(Fe^{2+},Mg)_5Al(AlSi_3O_{10})(OH)_8$] and in special cases, also pyrite [FeS_2]. When there is a deficiency in dissolved oxygen, Fe(II) is present in the form of ferrous salts, most commonly as carbonate, chloride and sulfate (Okrusch & Matthes, 2005).

The largest accumulations of iron (90% of the world's iron supply) are the Banded Iron Formations (BIFs) (Nealson, 1983; Lunine, 1999). BIFs are Pre-Cambrian-age sedimentary deposits. In the period between the Late Proterozoic and early Archean (2.7-1.9 Ga), the largest part of the BIFs were deposited.
These deposits show a lamination of alternating Fe-rich and Si-rich layers. An oxide-rich BIF typically consists of hematite [Fe_2O_3], magnetite [Fe_3O_4], siderite [$FeCO_3$] and iron silicates (James, 1966; Walter, 2011). The total iron concentration makes up about 20 to 40 wt% (Klein, 2005). The dominant iron species is Fe(III) (Ehrlich & Newman, 2009).
This lamination occurs in different sizes, from millimeters to several meters thickness (Trendall and Blockley, 1970; Trendall and Blockley, 2002; Klein, 2005). The major iron sources was the mid-ocean ridge or hotspots (eg Jacobsen and Pimentel-Klose, 1988; Holland, 1973; Bau & Möller, 1993; Barley et al. 2005; Canfield, 1998).

The origin of these BIFs is not yet fully clarified (Konhauser et al., 2002). It is believed that for the chemical oxidation of Fe(II) oxygen had to be produced by cyanobacteria (Fig. 1;

Cloud, 1968; Holland, 1984). This oxygen is said to have reacted with the Fe(II) in the water column and consequently making for a deposit.
A second hypothesis states that there may have been photo-oxidative processes showing evidence that Fe(II) might have absorbed light at a wavelength of 200-400 nm, been oxidized to Fe(III) and consequently deposited (Cairns-Smith, 1978; Braterman et al., 1983; Francois, 1986; Konhauser et al., 2007a).
Another hypothesis argues that Fe(II) was removed from water with the help of sulfides (Canfield, 1998; Poulton et al. 2004).

According to the latest hypothesis, anoxygenic iron-oxidizing phototrophic bacteria were responsible for the deposition of BIFs (Fig. 1). Different isotopic analyses show that the average temperature 3.5 Ga ago was between 26 and 35°C (Blake, 2010), thus making the life of bacteria possible. Different studies also demonstrate that nutrients were present in sufficient concentrations in the oceans at that time (Konhauser et al., 2007b).

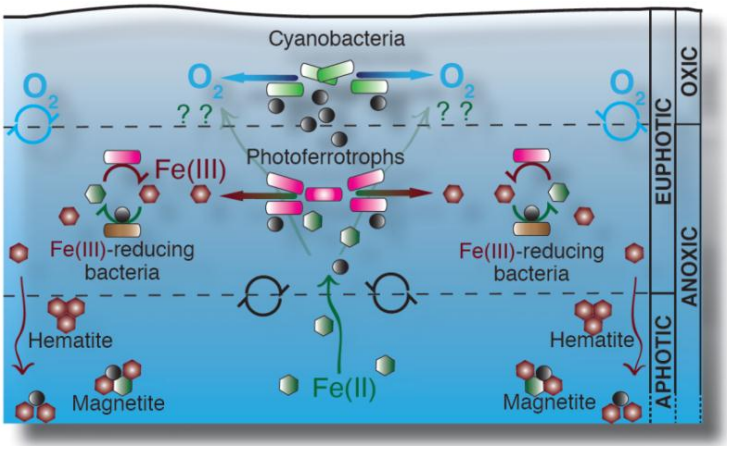

Figure 1 Hypotheses on the development of BIFs: Ferrous iron was oxidized either directly by photoferrotrophs in anoxic zones or indirectly by oxygen that was produced by cyanobacteria. Ferric iron was then either reduced by the activity of Fe(III)-reducing bacteria or deposited.
(adapted from Xavier, 2011).

The 'iron bacteria' were among the first prokaryotes to be observed and recorded by pioneer microbiologists, namely Ehrenberg and Winogradsky, in the 19th century. Bacteria that catalyze the oxidation of iron can be subdivided into four main physiological groups: (I) acidophilic, aerobic iron oxidizers, (II) neutrophilic, aerobic iron oxidizers, (III) neutrophilic, anaerobic (nitrate-depending) iron oxidizers; (IV) anoxygenic phototrophic iron oxidizers (Hedrich et al., 2011).

The first evidence that microorganisms were able to oxidize Fe(II) in anaerobic environments came through observations with phototrophic purple Proteobacteria. After the pioneering work of Widdel et al. (1993) and Ehrenreich & Widdel (1994), several iron-oxidizing phototrophs from a variety of freshwater and marine environments were isolated

and characterized (Croal et al., 2004b; Heising and Schink, 1998; Jiao et al, 2005; Straub et al, 1999).
Iron oxidization at circumneutral pH can be coupled to photosynthesis by phototrophic purple bacteria, since the midpoint potential of photosystem I is ~450 mV (Widdel et al., 1993; Dutton et al.,1978). The redox pair Fe(III)/Fe(II) in an bicarbonate-containing environment has an E'0 of about +0.2 V.

Anoxygenic phototrophic Fe(II)-oxidation occurs according to the following equation (Ehrenreich & Widdel, 1994):
$4\ Fe^{2+} + CO_2 + 11\ H_2O \rightarrow$ biomass $[CH_2O] + 4\ Fe(OH)_3 + 8\ H^+$

Anaerobic bacteria live in the absence of O_2. Fe(II) can be used by this group of bacteria as a source of reductant for carbon dioxide-fixation. During this process, Fe(II) is transformed to Fe(III), precipitating as an iron mineral. At circumneutral pH diverse microorganism form various kinds of poorly to well-crystallized iron minerals such as FeOOH or $Fe(OH)_3$ (Heising & Schink, 1998; Benz et al., 1998; Kappler et al., 2005; Straub et al., 1996; Straub & Buchholz-Cleven, 1998).

To date, seven cultures of phototrophic Fe(II) oxidizers are known. The freshwater strains include *Rhodobacter ferrooxidans* strain SW2 (Ehrenreich & Widdel, 1994), *Rhodomicrobium vannielii* strain BS-1 (Heising & Schink, 1998), *Chlorobium ferrooxidans* strain KoFox (Heising et al., 1999), *Thiodictyon sp.* strain F4 (Croal et al., 2004) and *Rhodopseudomonas palustris* strain TIE-1 (Jiao et al., 2005). The known marine strains are *Rhodovulum iodosum* and *Rhodovulum robiginosum* (Straub et al., 1999). The different types of iron oxidizing bacteria show different morphological and physiological characteristics.

Rhodobacter ferrooxidans strain SW2 is a non-motile, small (0.5 by 1 to 1.5 µm) and rod-shaped phototrophic iron oxidizing bacterium. In addition to Fe(II), it can utilize H_2 as well as monocarboxylic acids, glucose and fructose as electron donors (Ehrenreich & Widdel, 1994). The bacterium *Rhodomicrobium vannielii* strain BS-1 is able to oxidize Fe(II), but the produced Fe(III)-hydroxides precipitate as a solid crust on the cell surface. In the stationary phase of growth this type of bacterium forms polyhedral exospores. The size of this strain is about 0.7 µm wide and 2 µm long. It is able to grow with different organic substrates but is unable to reduce Fe(III) in the dark (Heising & Schink, 1998). The phototrophic green sulfur bacterium *Chlorobium ferrooxidans* strain KoFox is able to oxidize ferrous iron in co-culture with "*Geospirillum*" strain KoFum. It was not possible to

obtain a pure culture of the phototrophic bacterium in dilutions in liquid or in agar medium. Microscopic analyses showed a ratio of 1,000 phototrophic KoFox cells to 1 heterotrophic KoFum cell. Strain KoFum probably produces essential but unknown growth factors for *Chlorobium ferrooxidans* strain KoFox. The absorption spectrum showed bacteriochlorophyll *c* was present in these green phototrophic bacteria. Strain *KoFox* could not utilize sulfide as electron source, which is atypical for green phototrophic bacteria (Heising et al., 1999). The phototrophic bacterium *Thiodictyon sp.* strain F4 looks different from the other iron oxidizing bacteria. Cells of this strain are 1.5 to 2 µm wide and 5 to 7 µm long. They contain gas vacuoles and partly form long chains. When grown with acetate, a purple-violet pigmentation was observed (Croal et al., 2004). The fifth freshwater representative is *Rhodopseudomonas palustris* strain TIE-1 that grows photoautotrophically with Fe(II), H_2, or thiosulfate and photoheterotrophically with different organic substrates. It was also able to grow chemoheterotrophically in the dark (Jiao et al., 2005).

It is currently not known how and where Fe(II) oxidation takes place at the cellular level. There are many possible places, such as on the surface of the outer membrane, in the cytoplasm or in the periplasm of the cell (Croal et al., 2007). Different strains of iron-oxidizing bacteria probably use different kinds of oxidation and transport mechanisms, which lead to interaction between the produced iron oxides and the cells (Schaedler et al., 2009). This may lead to encrustation of the cell with Fe(III) during the iron oxidation and the consequent death of the cells (Schaedler et al., 2008; Hegler, 2010; Schaedler et al., 2009).

One goal of this thesis was to find out in which environments anoxygenic phototrophic bacteria catalyzed the oxidation of Fe(II). Different isolation studies had been done in the last decades. This thesis looks at the geographical and ecological distribution of anaerobic phototrophic bacteria in parts of Austria and Switzerland.
The second goal of this thesis was to determine which types of bacteria carry out this process. Different enrichment and isolation methods were applied and the morphological, physiological and phylogenetic features of one isolate were determined.

2 Material and Methods

2.1 Sources of organisms

Sediment for the enrichment cultures were collected in different areas of Austria and Switzerland (Fig. 2 and Table 1). All samples were sediment-water mixtures from a depth of about 2-4 cm. The sample locations represented different environments at different altitudes. Samples were either collected in sterile flat bottles (50 ml) or in sterile glass jars without headspace.

In addition to my own twelve samples, eight more enrichments (labeled WM, SN, MG, MK, SB 610, BS 610, BS 45, BW 45) had been provided by Dr. Kristina Straub.
The enrichment culture from which strain ViMi23 was isolated was started with a mixture of different sediments of ponds and streams in the metropolitan area of Vienna (Weidlingbach, Pond near Klosterneuburg, Hirschgartenteich bei Mauerbach).

After setting up the enrichments, sample material was stored in the refrigerator at about 4°C.

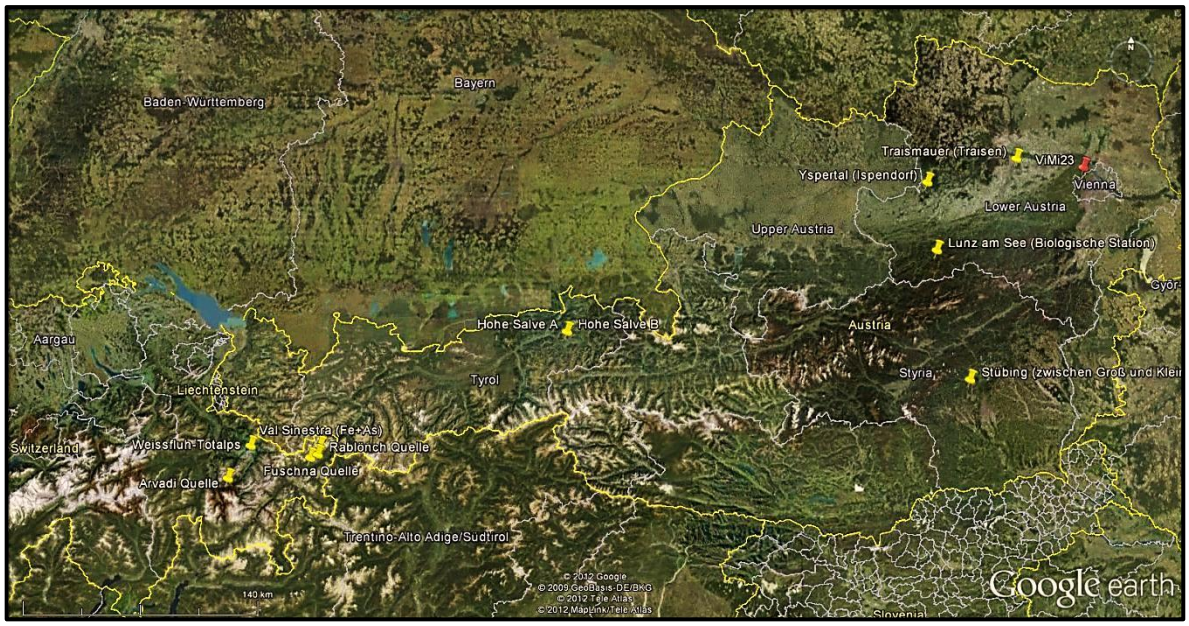

Figure 2 Locations of sample material: Twelve samples taken July and August 2011 (yellow); sampling location for strain ViMi23 (red).

Table 1 Sample Points

Sample Nr.	Sample short	Place	Coorinates		dates	pH	Iron - oxidation
1	TM	Traismauer (Traisen) (209 m) (AUT)	N 48° 18,987'	E 15° 43,596'	2011/07	6,6	**+**
2	YT	Yspertal (Isperdorf) (286 m) (AUT)	N 48° 12,457'	E 15° 00,633'	2011/07	5,2	**+**
3	LS	Lunz am See (Biological Station) (614 m) (AUT)	N 47° 51,134'	E 15° 04,022'	2011/07	6	**+**
4	S	Stübing (between Groß und Kleinstübing) (451 m) (AUT)	N 47° 10,585'	E 15° 17,439'	2011/07	5,9	**+**
5	HSA	Hohe Salve A (1593 m) (AUT)	N 47° 27,631'	E 12° 12,356'	2011/08	5,8	**+**
6	HSB	Hohe Salve B (1566 m) (AUT)	N 47° 27,651'	E 12° 12,178'	2011/08	6,5	**+**
7	AQS	Arvadi Quelle (955 m) (CH)	N 46° 39,845'	E 09° 38,260'	2011/08	6,7	**+**
8	WTS	Weissfluh-Totalp (2446 m) (CH)	N 46° 50,279'	E 09° 48,230'	2011/08	5,2	**-**
9	ROS	Rablönch Spring Top (1117 m) (CH)	N 46° 48,332'	E 10° 19,338'	2011/08	6,6	**+**
10	RUS	Rablönch Spring (1196 m) (CH)	N 46° 48,328'	E 10° 19,358'	2011/08	6,8	**-**
11	FQS	Fuschna Spring (1230 m) (CH)	N 46° 47,255'	E 10° 15,868'	2011/08	n.d	**+**
12	VSS	Val Sinestra Spring (Fe+As) (1466 m) (CH)	N 46° 51,074'	E 10° 20,249'	2011/08	6,5	**+**
13	WM	Enrichment - Vienna surroundings (AUT)	n.d	n.d	2008/10	n.d	**+**
14	SN	Enrichment - Neusiedler See (AUT)	n.d	n.d	2008/11	n.d	**+**
15	MG	Enrichment - Ditch close to Constance (GER)	n.d	n.d	2009/04	n.d	**+**
16	MK	Enrichment - Town ditch in Bremen (GER)	n.d	n.d	2008/10	n.d	**+**
17	SB 610	Enrichment - Sediment of a biotope in Leopoldsdorf (AUT)	n.d	n.d	2011/03	n.d	**+**
18	BS 610	Enrichment - Town ditch Vienna (AUT)	n.d	n.d	2011/03	n.d	**+**
19	BS 45	Enrichment - Stream sediments of Gatringsbach (AUT)	n.d	n.d	2011/03	n.d	**+**
20	BW45	Enrichment - Sediment of a Biotope of Wallern (AUT)	n.d	n.d	2011/03	n.d	**-**

AUT…Austria, GER… Germany, CH…Switzerland; n.d = not determined

2.2 Methods

2.2.1 Medium and Cultivation

Enrichments of anoxygenic phototrophic bacteria were prepared in 50 ml flat glass bottles with a freshwater medium modified by Ehrenreich & Widdel (1994) containing 0.6 g L^{-1} of potassium phosphate (KH_2PO_4), 0.3 g L^{-1} of ammonium chloride (NH_4Cl), 0.025 g L^{-1} of magnesium sulfate ($MgSO_4 \times 7H_2O$), 0.4 g L^{-1} of magnesium chloride ($MgCl_2 \times 6H_2O$) and 0.1 g L^{-1} of calcium chloride ($CaCl_2 \times 2H_2O$). The heat-stable salts were dissolved in ultra-pure water and autoclaved at 121°C for 25 minutes (Widdel, 1980). The medium was buffered at pH 6.8-6.9 with 30 mmol L^{-1} of a bicarbonate solution that was autoclaved separately under a N_2/CO_2 atmosphere (90:10).

The partially finished medium was cooled to room temperature in an ice-water bath under an N_2/CO_2 atmosphere (90:10). At the end, a 1 mL L^{-1} trace element solution (Tschech & Pfennig, 1984) and a 1 mL L^{-1} selenate- tungstate solution (Widdel, 1980) were added. Both the trace mineral solution and the selenite-tungstate solution were separately autoclaved in sealed glass bottles with airspace. This medium was filled with no head space in 50 ml sterile flat bottles, using a sterile filling bell (Widdel & Bak, 1992).

For enrichments, freshwater medium was inoculated with 10% sample material and 10 mM of $FeSO_4$ was added from an anoxic stock solution kept under nitrogen gas. These bottles were then incubated horizontally under a light source (25 W light bulb) at a distance of about 50 cm and at a room temperature of about 23°C.

2.2.2 Enrichments without medium

Test tubes were filled two third with original sample material (water-sediment mixtures). A set of three different test tubes for experiments were carried out. One test tube was left without an additive; and as for the second test tube, 10 mM of $FeSO_4$ was added. Concerning the third tube, 10 mM of $FeSO_4$ and a 1% inoculum of Fe(III)-reducing bacteria, *Geobacter bremensis* and *Geobacter pelophilus*, were added (Straub & Bucholz-Cleven, 2001). The air space was flushed with N_2 gas for 30 seconds and closed with rubber septa and screw caps to establish anoxic conditions. For incubation, the tubes were placed under light at room temperature (~23°C). The tubes had been slightly shaken every other day.

2.2.3 Oxic Medium

For phosphate-buffered freshwater medium, 4.0 g L^{-1} of potassium phosphate (KH_2PO_4), 1.0 g L^{-1} of ammonium chloride (NH_4Cl), 0.2 g L^{-1} of magnesium sulfate ($MgSO_4 \times 7H_2O$), 0.1 g L^{-1} of calcium chloride ($CaCl_2 \times 2H_2O$) and 1.0 g L^{-1} of sodium chloride (NaCl) were dissolved in ultra-pure water. The medium was then dispensed into 70 ml portions into 100 ml glass bottles and autoclaved at 121°C for 25 minutes. To complete the buffer, 100 g L^{-1} of K_2HPO_4 was dissolved in ultra-pure water and distributed in 50 ml portions in 100 ml glass bottles. Subsequently, these bottles were autoclaved. After autoclaving and cooling, the K_2HPO_4 solution was complemented with 1 ml of the stock solution of trace elements and selenium-tungsten. Finally, 70 ml of the medium was mixed with 7 ml of the modified K_2HPO_4 solution.

2.2.4 Biogenic ferric iron

Biogenic ferric iron was pooled from old enrichment cultures that had completely oxidized ferrous iron. After sedimentation of iron minerals, the supernatant was replaced with distilled water. This was repeated two times. Then, the water-iron mineral suspension was divided evenly into two centrifuge tubes. These two vials had been centrifuged for ten minutes at 8,000 revolutions per minute in the HiCen® 21C centrifuge. Afterwards, the supernatant liquid was decanted. As a further step, both vessels were filled at the same weight with ultra-pure water then centrifuged. This step was repeated three times.

After the final centrifugation step, the supernatant was discarded and the iron minerals were suspended in a small volume of ultra-pure water to obtain a solution with a high iron concentration. The solution thus obtained was applied to three test tubes (each 8 ml) and divided with a small offset magnetic stirrer. The test tubes were sealed with rubber septa and screw caps. To make the iron suspension anoxic, they were alternately vacuumed and gassed with N_2 three times and then sterilized by autoclaving for 15 minutes at 121°C.

2.3 Isolation

2.3.1 Agar dilution method

For the isolation of individual colonies in agar, 3.3 g of agar was melted in 100 ml of ultra-pure water in an Erlenmeyer flask and then 3 ml portions were transferred into test tubes, sealed with an aluminum cap and autoclaved for 15 minutes at 121°C. The agar was re-boiled and kept warm at 60°C in a water bath before utilization. Eight ml of a pre-heated medium was mixed with 5 mM of acetate and added to the agar. In addition, 10 mM of $FeSO_4$ was added and the agar mixture was subsequently cooled in a water bath to 40°C.
About 50 µL of sample was added to the first of the six tubes and sealed with butyl rubber stoppers. After light shaking, a small amount was transferred into the next dilution step and resealed with a butyl stopper; this procedure was repeated for the following tubes. The finished tubes were cooled in an ice bath. Subsequently, the gas phase of the test tubes was replaced with a N_2/CO_2 (90:10) gas mixture and incubated in the dark overnight. Then tubes were incubated upside down under light (25 W bulb) (Widdel & Bak, 1992).
In addition, some agar dilution series were supplied with reduction agents. For this purpose, 2 mM of cysteine or 4 mM of ascorbate were added to the freshwater medium with acetate and $FeSO_4$. As for the remaining steps, see above.

Moreover, the homogeneity of strain ViMi23 was checked in agar dilutions with acetate as electron donor.

2.3.2 Agar plates

Agar plates were prepared for the streak plate method. For this purpose, 3 g of agar were autoclaved with 300 ml of ultra-pure water. After autoclaving and cooling on a magnetic stirrer 5 mM of NH_4Cl, 2 mM of $MgSO_4$, 1 mM of $CaCl_2$, 5 mM of acetate and a buffer were added. Three different buffer were applied: 10 mM KPP (potassium phosphates), 10 mM MOPS (3-(N-morpholino) propaesulfonic acid) or 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid).

Cultures or single colonies were streaked our by the 12-line method on agar plates. These plates were incubated with the agar side up in anoxic jars with a N_2 atmosphere, which was replaced two times on the following days. After incubation overnight in the dark, the anoxic jars were placed under light. For further isolation, individual colonies were then re-streaked using the same technique.

The same number of plates was incubated under aerobic conditions at room temperature in the dark for the same period of time to see whether bacteria were also able to grow with oxygen as electron acceptor.

Additionally this method was also used to control the purity of strain ViMi23. Dilutions of this strain were spread on plates with acetate or nutrient broth and then incubated at 22°C in the dark. Both types of plates showed no growth of other types of bacteria.

2.3.3 Bottle Plates

In this method agar (prepared in the same way as described above with KPP as buffer) was poured into flat glass bottles instead of petri dishes. In the first attempt, flat bottles (50 ml) were filled with 4 ml of agar, so that one side of the bottle lay covered. The bottle was filled, cooled and the gas phase was replaced with N_2 and sealed with a butyl rubber stopper. A gas exchange was carried out two times.

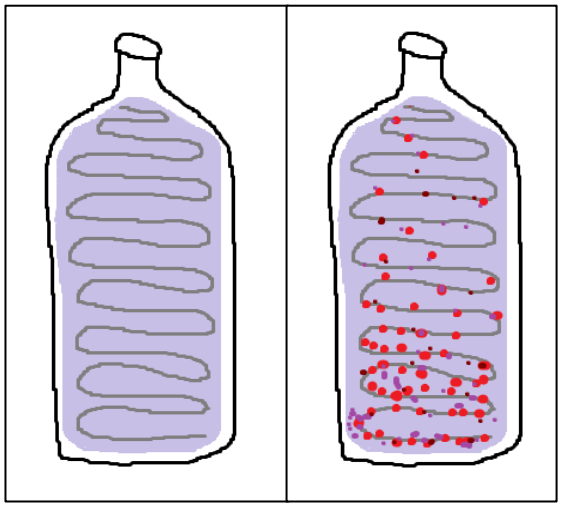

Figure 3 Schematic images of bottle plates. Streaking out separates microbial cells which grow into colonies.

The streak of colonies was done with an inoculation loop in a narrow zigzag pattern. Instead of distributing the inoculating with the loop, some drops of the sample were placed in the middle of the agar with a long cannula. Then the bottle was held at an angle to spread the sample over the agar surface and to prevent the carving of the agar.

Consequently, the gas phase was exchanged with N_2 and sealed with a rubber stopper. After an overnight incubation in the dark, bottles were incubated with the agar side up under light.

For easier handling of the next step, 17 g/L agar was chosen to gain a firmer agar. 100 mL flat bottles were filled up with 10 mL of the new agar mixture. The other steps remained the same.

This technique was used for the isolation of phototrophic bacteria that formed purple blooms and didn't grow in agar dilution series. These blooms were withdrawn from enrichment cultures with long cannulas and subsequently transferred to the bottle plates. To liberate cells from aggregates samples were mixed in the presence of glass beads or incubated for about 25 seconds in an ultrasonic bath.

2.3.4 Liquid dilution series

Another technique was the isolation via liquid dilution series, which was used for phototrophic bacteria that formed purple blooms and didn't grow in agar dilution series. Dilutions were inoculated from enrichment culture YT247. Here, in the first approach, two parallel dilution series were prepared with a series of six dilutions steps (10^{-2}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}). Ten ml of a freshwater medium was filled in test tubes and 10 mM of $FeSO_4$ was added. The atmosphere of these tubes was exchanged with N_2/CO_2 gas for about 30 seconds and then sealed with a septum and a screw cap. Subsequently, the first tube was inoculated with 1% of the sample. This tube was then mixed well and served as a sample for the next dilution step. Every dilution step was carried out twice. Subsequently, the test tubes were incubated in the light at room temperature.

2.4 Analytical techniques

2.4.1 Iron Measurement

Iron was quantified spectroscopically with ferrozine (Stookey 1970) with a modified protocol as described by Hegler et al. (2008). This technique was needed for the determination of Fe(II) and Fe(III) concentrations in enrichments as well as for the growth experiments with enrichment cultures or strain ViMi23.

Samples were taken using syringes through the septum. About 200 µL had been transferred to Eppendorf tubes. For Fe(II) quantification, 100 µL of the sample were quickly mixed with 900 µL of 1 mol L^{-1} HCl to prevent oxidation of Fe (II). Then two Eppendorf tubes with 500 µL of 1 mol L^{-1} HCl and 500 µL of ferrozine solution (10% w / v ammonium acetate, 0.1% w / v ferrozine) were prepared and 10 µL of the fixed sample was added to each tube.

To measure the total iron concentration, 10 µL of the sample was thoroughly mixed in the first step with 500 µL of a hydroxylamine hydrochloride solution (50% w / v in 1 M HCl).
In the beginning, the time needed to reduce Fe(III) with hydroxylamine hydrochloride was tested with one sample and different incubation times: After 15, 30, 45, 60 and 120 minutes the iron concentration was measured. It turned out that all Fe(III) was already reduced after 30 minutes. Accordingly, all experiments were incubated for 30 minutes before 500 µL of ferrozine solution was added. Two subsamples were analyzed per sample for Fe(II) and two for total iron. The ferrozine solution was allowed to react with the Fe(II) for a short time before measuring the iron concentration with a UV-visible spectrophotometer (Model: Varian, Cary 50 Conc) at 562 nm. For the Fe(II) measurement, water was used as a zero value and for the total iron measurement, a mixture of 500 µL and 500 µL of hydroxylamine ferrozine solution was prepared.

The measurement of iron in the enrichment with a freshwater medium was similar to that of enrichments without medium. However, the measurements had been conducted at specific time intervals (24, 48 or 72 hours). From the resulting Fe(II) and total iron values, the values for the Fe(III) concentration were able to be calculated. For calibration 2.5, 5, 10, 20, 30; or 40 mmol of Fe(II)SO_4 was used.

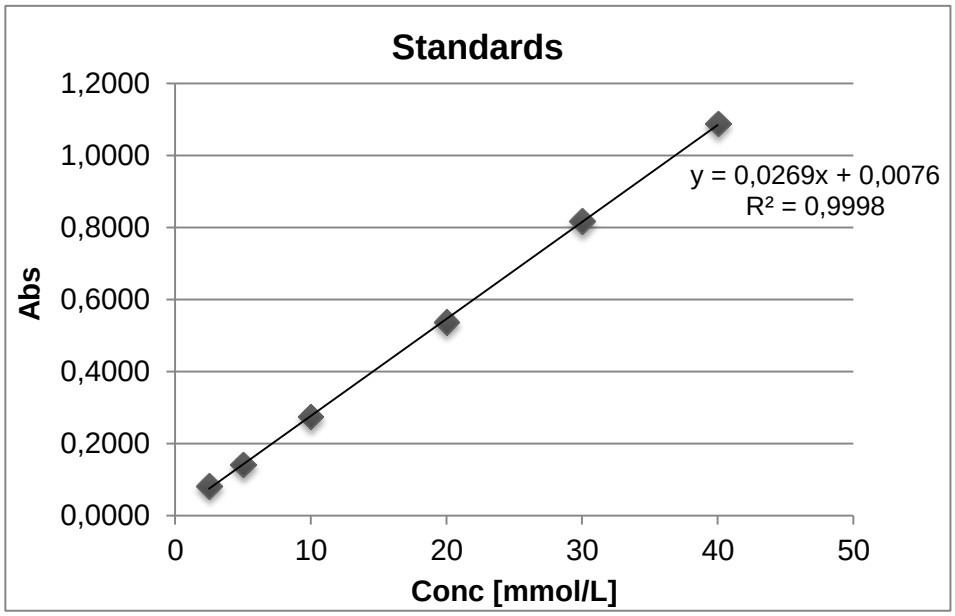

Figure 4 Calibration curve for iron measurements with ferrozine.

2.4.2 Absorption spectrum

About 1 ml of culture was centrifuged in an Eppendorf tube. Pelleted cells were re-suspended in 500µl liquid and mixed with 500µL of a concentrated sugar solutions consisting of 5 g of granulated sugar and 3 ml of potassium phosphate. The sugar solution had been prepared by heating and shaking the mixture of sugar and buffer over a Bunsen burner until a complete solution occurred. The cell suspension was placed in a cuvette made of special optical glass. Sugar solution was used as blank. The subsequent measurements were performed from 200 to 1000 nm.

2.4.3 Microscopy

A light microscope (Leica DME 400x) was used to inspect enrichment cultures and isolates. To determine cell sizes, the samples were colored with two different dyes: methylene blue or safranine red. 25 µl dye solution was mixed with 25 µl of the sample (Bast, 2001). Photos were taken (Leica DM4500P 630x) from dyed cells.

2.5 Characterization

2.5.1 pH range

To determine the pH range for growth, two approaches were made, one with acetate as electron donor and the second one with Fe(II) as electron donor. To obtain a range of pH values different volumes of NaOH or HCl were added to medium prior inoculation. To test-tubes with 10 ml of freshwater medium supplied with acetate, 50, 100, 250 or 300 µl of 1M NaOH were added. Similarly, 50, 100 or 150 µl of 1M HCl were added. For pH testing with iron, tubes contained 10 ml of medium and 50, 100, 150, 250 and 300 µL of 1M NaOH or 25 and 50 µL of 1M HCl. For equilibration reactions, all test tubes were incubated for about two hours before pH values were actually determined.

The pH electrode was calibrated with buffers 4 and 7 so that all samples tubes with HCl, the zero value and those without additives could be measured. To measure the tubes with NaOH, the electrode was adjusted to buffer solutions 7 and 10. In two sets of tubes was the pH measured and two further sets of tubes were inoculated with 1% of an acetate-grown culture and incubated in the light.

2.5.2 Temperature range

The determine the temperature range and optimum temperature for strain ViMi23, two parallel test-tubes with 10 ml of fresh water medium, 5 mM of acetate and a 1% inoculum were incubated at different temperatures. The experiments for 25°C, 28°C, 35°C, 40°C and 45°C were carried out in water baths set at these temperatures. The incubation took place under a light source (25 W light bulb). For control, some tubes were put in the light at room temperature of 23°C.

To test for growth at 4°C (refrigerator), cells were incubated in oxic medium with 5 mM of acetate in a 100 ml Erlenmeyer flask with 2% inoculums. Furthermore, two Erlenmeyer flasks were filled with oxic medium, 5 mM of acetate and 2% inoculum and incubated in a water bath cooled by a cooling unit in a dark room. The temperature was adjusted to 10°C. Controls were incubated in the dark at 22°C.

2.5.3 NaCl-Tolerance

To determine the tolerance of NaCl, test tubes containing 10 ml of freshwater medium and 5 mM of acetate were used. Here, a 1% inoculum was taken from an acetate-grown culture. Two of these tubes were supplied with 0.5% NaCl. For the third tube, a concentration of 1% NaCl was selected. The NaCl stock solution was autoclaved separately before it was used.

After the full growth of the experiment with 1% NaCl, new tubes with 2 and 3% NaCl were made. These tubes were inoculated from the culture that grew in the presence of 1% NaCl. All tubes were incubated at room temperature in the light.

2.5.4 Electron donors

Photoautotrophic and photoheterotrophic growth was tested with a variety of electron donors. In each case, the test tubes were filled with 10 ml of freshwater medium gassed for 30 seconds and sealed with a rubber septum and a screw cap. Most electron donors were tested in different concentrations: 2 mM and 4 mM sulfide, 2.5 mM and 5 mM thiosulfate, 2 mM and 4 mM cysteine, 1.5 mM FeS and 1 and 2 mM of polysulfide. In two tubes, a few flakes of elemental sulfur were added. Organic substrates were added from sterile stock solutions, which were either autoclaved or filter sterilized. Growth with H_2 as electron donor was tested in Erlenmeyer flasks in an anaerobic chamber with a gas mixture that contained hydrogen (95% N_2 / 5% H_2).

2.5.5 Electron acceptors

To test growth with different electron acceptors, 10 ml of anoxic freshwater medium was placed in test tubes. In addition, 5 mM of acetate as the electron donor was added. Nitrate, sulfate, biogenic Fe(III) and Fe-citrate were added thereto at a final concentration of 2.5 mM and 5 mM. Little elemental sulfur was as described above (2.5.4). All test tubes were incubated anoxically in the dark at 22°C. Oxygen as an electron acceptor had already been tested in an early step during the isolation on oxic agar plates. For this experiment, the plates had been incubated in the dark under ambient air.

2.5.6 Fermentation metabolism

Growth by fermentation with glucose, pyruvate, yeast extract or fumarate was tested in anoxic tubes with 10 ml of freshwater medium. For each substrate two tubes (containing either 2.5 mM or 5 mM substrate) were prepared. Yeast extract was supplied with a final concentration of 0.1 and 0.05%. The experiments were inoculated with 1% and incubated in the dark at 22°C.

2.5.7 Influence of reduction agents

In order to assess the influence of reducing agents on the growth of strain ViMi23, two tubes with 10 ml of anoxic medium, 5 mM of acetate and 2 mM of cysteine or 4 mM of ascorbate were applied. These test tubes were inoculated with 1% of the culture. Furthermore, a similar test series commenced, with the addition of 10 mM of $FeSO_4$. For controls, two tubes

containing 5 mM of acetate as well as two tubes with 5 mM acetate and 10 mM of $FeSO_4$ were prepared.

2.5.8 Growth experiments

Three different types of growth experiments were made with strain ViMi23.
In two types of experiments the oxidation of Fe(II) was monitored. For one experiment, two tubes with 10 ml and 10 mM of $FeSO_4$ were prepared and inoculated with 10% of an iron-grown culture. The second approach was performed with three test tubes filled with 10 ml of freshwater medium and 10 mM of $FeSO_4$. The 10% inoculum was from an acetate-grown culture. The concentrations of Fe(II) and the total iron were measured every 48 or 72 hours with the ferrozine method (see 2.4.1).

Photoheterotrophic growth with acetate was monitored by measuring the optical density (OD) at 660 nm with the UV-visible spectrophotometer. Initially, freshwater medium with 5 mM of acetate was used as blank, but was later replaced by water which showed the same absorption value. This experiment was done with three tubes filled with 10 mL of freshwater medium and 5 mM of acetate. Experiments were inoculated with 10% of an acetate-grown culture. For control, two tubes were inoculated with 10% of a pasteurised sample. This sample was pasteurised for 10 minutes in a water bath at 80°C.

2.5.9 Full phylogenetic study by complete 16S rDNA sequence analysis

A complete 16S rDNA gene sequence analysis including a phylogenetic analysis was done by the DSMZ (The Leibniz-Institute DSMZ - German Collection of Microorganisms and Cell Cultures). For this analysis, four test tubes with acetate-grown cells of strain ViMi23 were sent to Germany.

3 Results

3.1 Characterization of sampling sites and sample material

Six samples were taken in the middle and eastern parts of Austria (July and August 2011). Different types of soil were chosen to examine a variety of environments for the presence of anoxygenic Fe(II)-oxidizing phototrophic bacteria. Sample TM was collected from the river Traisen in the area of Traismauer. It is a wide, but only 30-cm deep river with gravelly, sandy and light-gray sediment. The pH value at the sampling time was 6.6. The second sample (YT) was taken in the upper part of Yspertal/Isperdorf, also in Lower Austria. The big Ysper showed a red coloration of the water over the entire running of the gorge, with a measured pH value of 5.2 at the sampling point. A gravelly and sandy sediment was taken from the upper three centimeters of the sediment (Fig. 5A). Sample LS was obtained from the Biological Station in Lunz am See. The sample was collected from a small pond with a measured pH of 6.0. The sediment was very muddy, fine and dark-gray to black with a high proportion of organic material (Fig. 5B). Sample S was taken from the river between the towns of Kleinstuebing and Großstuebing in Styria. Again, the pH was about 6.0. This soil was fine-sandy to clay with a light brownish-gray color. The last two samples in Austria were taken in Tyrol on the Hohe Salve (HAS and HSB). These were upland moor samples from a cow-paddock. Despite their geographic proximity, both samples showed different colors and types of soil. Sample HSA (Fig. 5C) exhibited a dark brown color approaching black. On the contrary, sample HSB (Fig. 5D) showed an extremely small grain size with a brownish orange color. A high organic content in the form of grass and wood was visible in both samples. The pH value was different in the two samples. In sample HSA a pH of 5.8 and at HSB a pH of 6.5 was measured.

Six additional samples were collected in the Swiss Alps (August 2011). At that time all sample sources showed temperatures below ten degrees and pH values below 7. Sample AQS was obtained from the Arvadi spring (Fig. 5E), an iron-sulfur spring near Davos. At the first part of this spring, the precipitation of white sulfur minerals was visible, while precipitation of iron minerals with an intensive orange color took place in the lower part of the spring from where the sample was taken. The soil was fine with an orange-brown color. The pH value of water at the sampling point was 6.7. A lake in the area of Weissfluh Totalp (WTS) represented the highest sampling point (2446 m). Sample WTS was a gray, fine-grained sediment-water sample with a pH value of 5.2. The samples RUS and ROS originated from the same location but from different sites of the spring area. Sample ROS (Fig. 5F) was taken near the source of the Rabloench spring. The second sample RUS (Fig. 5G) was taken a few meters away, where the foothills of the source flow down a little

hill. Both soils showed an orange-red color and consisted of very fine sediment. The Rabloench spring is composed of calcium-bicarbonate chalybeate water. The pH value of the spring changed, owing to CO2 outgassing and precipitation of different minerals, from acidic to basic. The pH of the two samples were 6.6 for sample ROS and 6.8 for sample RUS.
Another sample came from the Fuschna spring (FQS), a source of very highly mineralized water. It is a calcium-carbonate spring with an average temperature of 6-9°C. The sample contained a strong orange to red color and was taken shortly beyond the source of the spring. In the near surrounding of this spring cyanobacteria mats occurred. The 6th sample from Switzerland came from a river just below the Val Sinestra spring (Fig. 5H), an arsenic-iron one. This sample showed a deep orange color and was composed of fine sediment particles and few little stones.

The microscopic picture of most samples was very similar. Microorganism with different cell shapes (cocci, rods, spirilla) and sizes could be observed. Some of the microorganisms were motile. In addition cyanobacteria that formed chains and morphologically different diatoms were present. In sample FQS, also structures resembling stalks described for *Gallionella spp.* or sheets resembling those of *Leptothrix spp.* were visible.

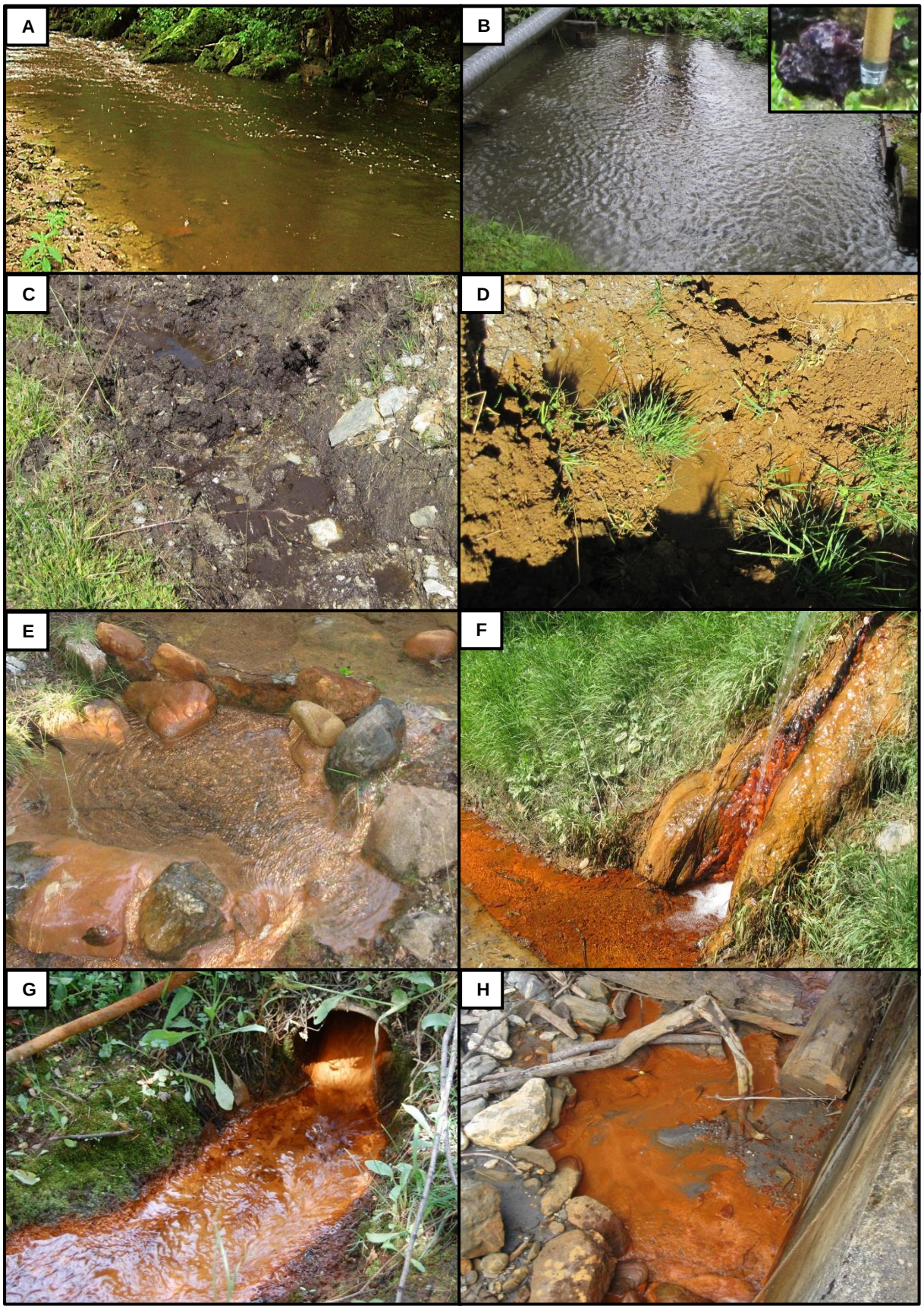

Figure 5 Sample points: Yspertal/Isbendorf [A], : Lunz am See [B], Hohe Salve A [C], Hohe Salve B [D], Arvadi Spring [E], Rabloench Spring Top [F], Rabloench Spring [G], Val Sinestra Spring [H]

3.2 Enrichments and Isolation

3.2.1 Enrichments with medium

The addition of $FeSO_4$ to the anoxic and bicarbonate-buffered medium led to the formation of a fluffy white precipitate of mostly $FeCO_3$ or iron phosphates (Ehrenreich & Widdel, 1994). The enrichments with freshwater medium were incubated in the light (25 W bulb) at room temperature. After the complete oxidation of Fe(II), subcultures were prepared and incubated under the same conditions.
After about three weeks of incubation, three enrichments (YT247, HSA28 and S247) showed an iron oxidation which became visible with the change of the color of the sediment. All three sediments turned from a gray-brownish color into an orange color. With the addition of iron, a white precipitate was formed, which went through a grayish-green color before it turned orange in the end. These iron oxides became deposits on the bottom and on the walls of the vessels (Fig. 6).

The enrichment YT247 showed the first signs of Fe(II)-oxidation after three weeks: the sediment began to turn orange. The complete Fe(II)-oxidation in this enrichment took about nine weeks; only then the first passage was created. The Fe(II)-oxidation in passages of YT247 was much faster and took only about ten days. Because the rate of Fe(II)-oxidation became faster, the size of the inoculum was reduced from 10% to 1%, starting with the eighth passage. With 1% inoculum, it took about eight to eleven days for the full oxidation of the ferrous iron. After complete iron oxidation, purple blooms formed on the surface of the liquid (Fig. 11, 12). These purple blooms appeared in a light violet color first and became darker with longer incubation in the light. Under the microscope these bacteria showed a growth in size in this phase. These purple blooms consisted of rod-shaped, vacuole-forming bacteria, which resembled cells of *Thiodictyon* strain L7 (Widdel et al., 1993; Fig. 13). Although the enrichment culture was dominated by *Thiodictyon*-like cells, other types of bacteria present in these samples. Similar purple blooms also occurred in enrichment cultures SN, HSA28 and BS610.

First signs of Fe(II)-oxidation of the enrichment HSA28 were already seen after eleven days. After the third passage of enrichment culture HSA28, subcultures were inoculated with 1%. The passages showed nearly the same oxidation time in as in passages of YT247. Additional to the purple blooms which occurred in the passages, some green algae appeared on the surface of the iron minerals formed after two month of incubation. These algae grew after full oxidation of the sediment in the first enrichment of sample HSA. Under the microscope algae were confirmed with the help of UV - light, where the algae turned intensively red.

Enrichment S247 showed a complete oxidation of ferrous iron after eight weeks. However, already in the first subculture of S247 Fe(II)-oxidation ceased.

The enrichment of sample FQS showed an oxidation of iron after about four months. The other enrichments TM247, LS247, HSB28 and ROS318 showed a more or less pronounced Fe(II)-oxidation after five months of incubation. After the completion of iron oxidation, the enrichment of sample LS247 showed growth of algae on the surface of iron minerals. None of these slow enrichment cultures were subcultured.

Subcultures of already existing enrichment cultures (BS610, BS45, SB610, MG, MK, SN, WM) oxidized iron rapidly within 7-15 days.

Growth experiments were done to describe the Fe(II)-oxidation in enrichment cultures in more detail. For these experiments three tubes with 10 mL freshwater medium and 10 mM $FeSO_4$ were prepared for each enrichment culture tested. All experiments were inoculated with ten percent of fully grown enrichment cultures. Two tubes of each series were incubated in the light. The third tube of each series was placed in the dark for control. The concentrations of Fe(II) and total iron were measured regularly every 24 hours. The course of Fe(II)-oxidation was similar in enrichment cultures HSA28, YT247 and MK (Fig. 7).
After a short lag phase of two (HSA28, MK) to three (YT247) days, all cultures oxidized iron in about seven to eight days. In all samples the dark control showed no Fe(II)-oxidation. The iron oxidation of all enrichments was nearly complete, but a small amount of remnant Fe(II) (approximately five to seven percent) was probably not accessible for the bacteria.
Similar growth experiments were done with enrichments SN and BS610, which showed basically the same results (data not shown).

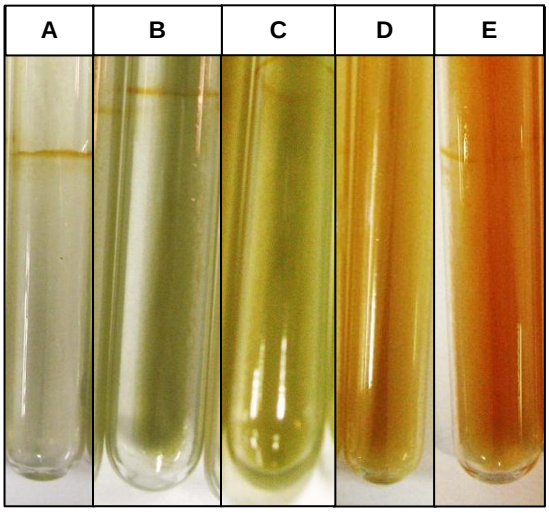

Figure 6 Growth experiment with enrichment culture HSA28:
t=0, white precipitates after the addition of $FeSO_4$ to the freshwater medium [A]; t=1 d, greenish- grey coloration of precipitates [B]; t=3 d, greenish to light orange color of precipitates [C]; t=5 d, iron oxidation is visible [D]; t=7 d, complete iron oxidation [E]

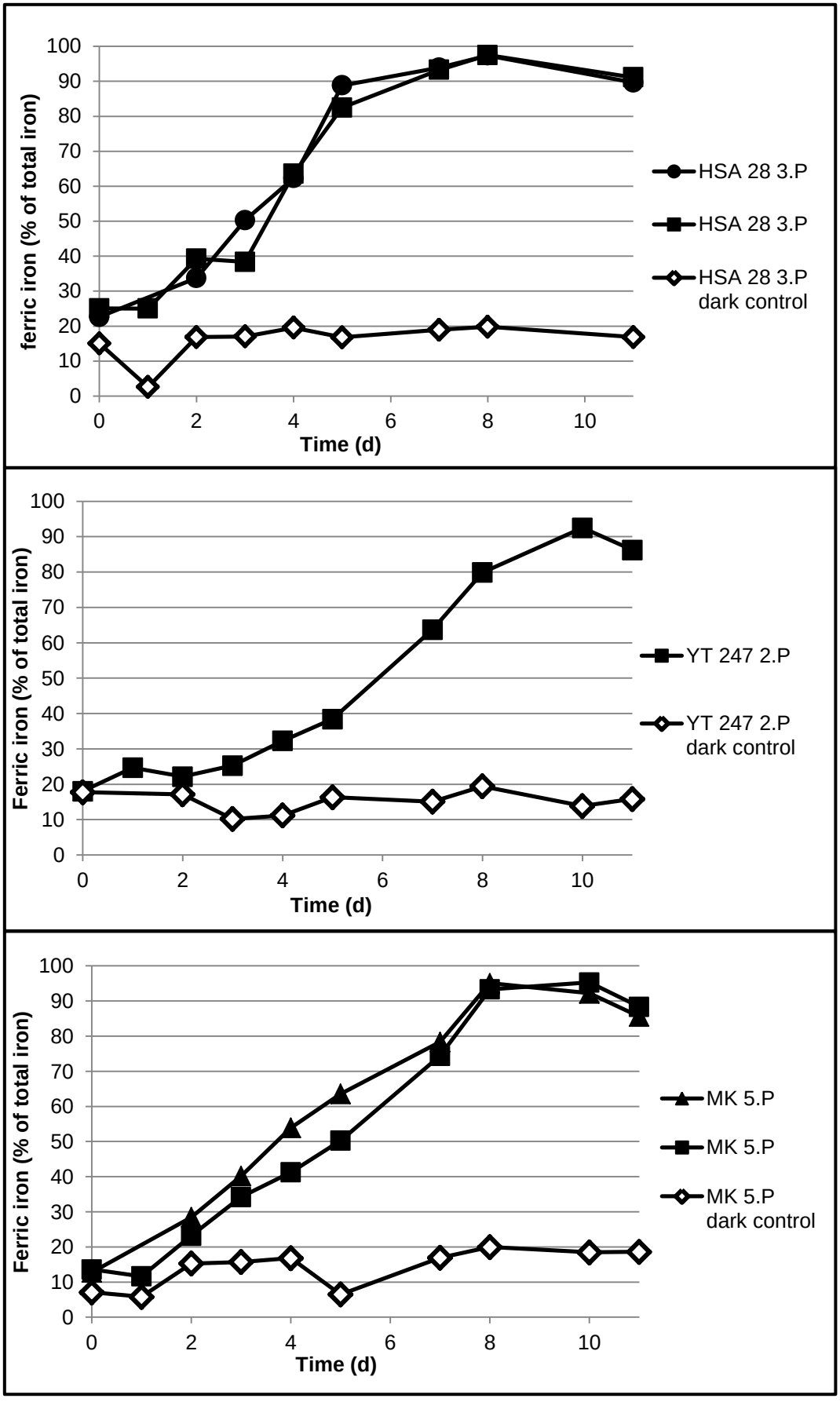

Figure 7 Growth of three enrichment cultures with 10 mM $FeSO_4$ as sole electron donor in bicarbonate-buffered freshwater medium.

3.2.2 Incubation of sample material

For the enrichments without medium, the original material of samples VSS, FQS, AQS, S, HSB, and a mixture of ROS and RUS (ROUS) were chosen because they showed no iron oxidation in the classical enrichments with medium after about four months of incubation.

From the beginning, sample VSS showed an intensive bright orange color (Fig. 8A), making a simple observation of the Fe(II)-oxidation process impossible. The sample supplied with cells of *Geobacter* species and $FeSO_4$ showed only a slight darkening of the sediment during the incubation.
The sample HSB also showed a strong brownish orange color in the beginning. The two pure enrichments with $FeSO_4$ or $FeSO_4$ and cells of *Geobacter* species showed a significant iron oxidation after three week in the light. In addition, green algae had also grown on the iron deposits and on the glass walls of the test tubes after two month of incubation.
In all three enrichments of sample S, an oxidation of iron was observed, which was the lowest in the tube with $FeSO_4$ and cells of *Geobacter* species. This enrichment also showed a significant blackening of the sediment as well as in the pure enrichment without adding iron. In the other two tubes, an oxidation layer was formed on the glass wall of the tubes and on the border between the sediment and gas atmosphere.
Sample AQS showed an intense black coloration of the sediment after addition of iron and cells of *Geobacter* species. The tube without the addition of iron also showed a darkening of the sediments. The sample with more Fe(II) showed a slight iron oxidation. In the pure enrichments of FQS, no visible iron oxidation was observed, with the exception of sample in which cells of *Geobacter* species were added, the sediment changed to a dark brown color. Even with the mixed sample of ROS and RUS (ROUS), no visible Fe-oxidation occurred during the three month of incubation.

After three months of incubation, concentrations of Fe (II) and total were determined. The iron measurements of VSS confirmed a high content of Fe(III), which had been suggested by the orange color before. The Fe(II) concentration of in the pure sample and the sample that was supplied with $FeSO_4$ was nearly zero, thus indicating that Fe(II) was oxidized during incubation in the light. In the sample with cells of *Geobacter* species and $FeSO_4$, a higher concentration of Fe(II) was measured.
The results of sample FQS showed no iron oxidation. In the sample with cells of *Geobacter* species and the supply of Fe(II), a little more Fe(II) and Fe(III) was measured.

Sample ROUS showed no iron oxidation over this time period. Sample AQS from the sulfur-iron fountain was rapidly blackening in the enrichments without any addition and with cells of

Geobacter species and iron. The approach with the addition of $FeSO_4$ showed no blackening of the sediment, but little signs of iron oxidation. However, iron measurements didn't confirm this macroscopic observation but suggested that no Fe(II)-oxidation occurred in these three series of enrichments.

The pure enrichments of sample S initially had a grayish color in the sediment. The approaches with and without $FeSO_4$ showed a pronounced iron oxide layer at the sediment-water interface (Fig. 8B, 8C). The sample of both $FeSO_4$ and cells of *Geobacter* species showed a slight oxidation of ferrous iron. In contrast to the visible oxidation in the tubes, there weren't any indications for iron oxidation in the results of the photometric measurements.

The strongest and fastest iron oxidation of the enrichments with original sample material showed enrichments of HSB. The approaches with $FeSO_4$ and $FeSO_4$ and cells of *Geobacter* species showed both a strong oxidation of iron at the sediment surface and on the glass walls of the test tubes. Also, algae formed on the iron minerals (Fig. 8D). As for enrichment where no iron was added no changes could be observed. The results of the iron measurements showed no Fe(II) oxidation in the tube with additional iron and additionally, less Fe(III) in the sample with cells of *Geobacter* species than that of the other approaches.

Table 2 Results of iron measurement after three month

	Sample	Mean Fe(II) [mmol]	Mean Σ Fe [mmol]	mean Fe(III) [mmol]
1	VSS - pure	0,2082	250,9108	250,7026
2	VSS + 10mM Fe(II)	1,3253	194,8885	193,5632
3	VSS + 10mM Fe(II) + *Geobacter*	13,6691	247,1375	233,4684
4	FQS - pure	15,4071	26,4796	11,0725
5	FQS + 10mM Fe(II)	25,3086	40,7435	15,4349
6	FQS + 10mM Fe(II) + *Geobacter*	38,1970	51,2416	13,0446
7	ROUS - pure	17,6264	38,6766	21,0502
8	ROUS + 10mM Fe(II)	23,1190	45,6636	22,5446
9	ROUS + 10mM Fe(II) + *Geobacter*	40,6506	71,5372	30,8866
10	ROUS + Geos	37,7045	39,6673	1,9628
11	AQS - pure	8,4963	15,5911	7,0948
12	AQS + 10mM Fe(II)	7,6357	11,4647	3,8290
13	AQS + 10mM Fe(II) + *Geobacter*	10,7862	11,4628	0,6766
14	S - pure	3,9572	76,6227	72,6654
15	S + 10mM Fe(II)	14,6022	64,1822	49,5799
16	S + 10mM Fe(II) + *Geobacter*	28,1933	91,2342	63,0409
17	HSB - pure	93,8587	265,0186	171,1599
18	HSB + 10mM Fe(II)	84,7602	258,4201	173,6599
19	HSB + 10mM Fe(II) + *Geobacter*	134,0242	214,2007	80,1766

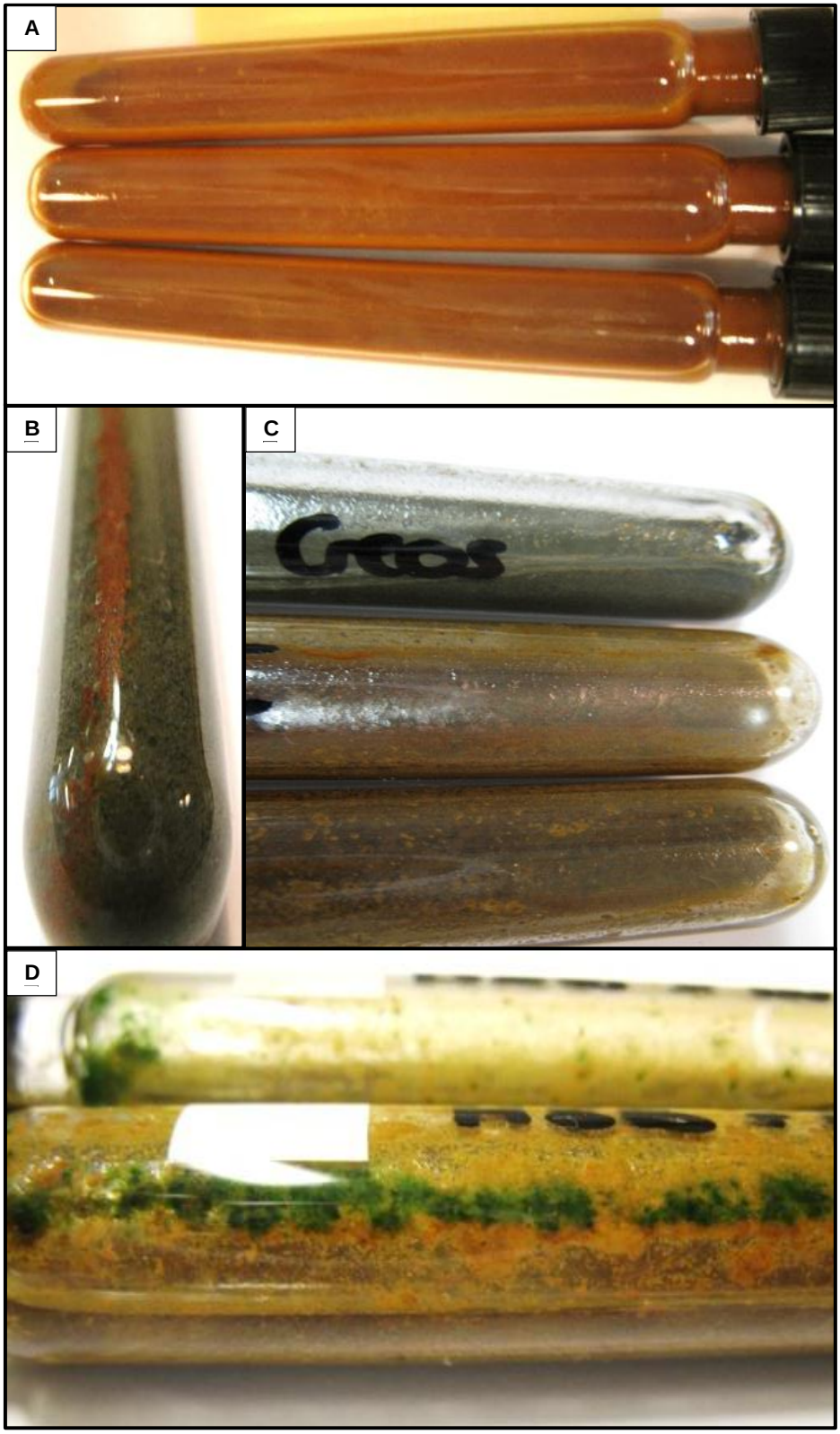

Figure 8 Enrichments with original sample material; strong orange color of sample VSS before and after iron oxidation [A]; oxidation layer of sample S [B, C], strong Fe(II)-oxidation of sample HSB with algae formed on the iron minerals [D]

3.3 Isolation

3.3.1 Agar dilution series

Several agar dilution series with 10 mM of $FeSO_4$, and 5 mM of acetate or 5 mM of $FeSO_4$ and with reducing agents such as ascorbate (4 mM) or cysteine (2 mM) were prepared for enrichments and passages of sample SN, MK, BS610, MK, HSA28 and YT247. After approximately three weeks, light to dark red colonies of different shapes and sizes were visible in all agar tubes supplied with $FeSO_4$ independent from the type of enrichment culture used as inoculum. The colonies sometimes reached a size up to 3 mm.
In dilution series inoculated from enrichments MK and SN, in addition to small red UFO-shaped colonies (Fig. 9A), some red cloudy shaped colonies (Fig. 9B) grew. In samples YT247 and HSA28, very small dark red UFO-shaped and branched colonies (Fig. 9C) were formed in the same period of time.
In the lower dilutions of sample HSA28, a satellite growth occurred. Here, several small red colonies grew around larger red colonies. Also, this phenomenon was observed in some other lower dissolution steps.
Little black colonies grew in some of the first dilutions of different samples.
"Ghosts" also occurred in all samples in the low dilutions: these were white to translucent irregular colonies which were only able to be seen in certain instances with binoculars where backlighting was available.

The colonies in the dilution series with 4 mM of ascorbate or 2 mM of cysteine were much smaller than the colonies in the samples without a reducing agent. The tubes with cysteine showed a higher number of colonies than those supplied with ascorbate. In tubes with cysteine, some black colonies were observed. After six to seven weeks of incubation a black skin formed around many red colonies in the agar dilution series with cysteine (Fig. 9D, 9E). These colonies showed a slight iron oxidation after about five weeks. When these colonies were picked, this black skin burst and the red cells of the colony became visible. Some of these red-black colonies were examined with the microscope. They showed a different picture than the other purely red colonies. The black skin formed some kind of very long and fine filaments. Some short rods were also able to be seen.

Most of the tube inoculated with enrichment cultures of YT247 showed high growth in the first centimeter of the agar. On the contrary, there were only a few single and small colonies in the lower part of the tubes. After several months of incubation, some colonies (e.g. YT247) showed an orange-brown crusting of the colonies which had been found in the upper part of the agar (Fig. 9F, 9G).

In order to verify whether the different kind of colonies were able to oxidize iron, the tubes were broken and the individual colonies were picked with a drawn-out Pasteur pipette. These colonies were transferred to a test tube with 10 mM of FeSO4 in freshwater medium. These tubes were incubated horizontally in the light. From these picked colonies, only the sample of YT247 showed signs of iron oxidation after several weeks. Some of the other picked colonies of samples SN and HSA28 showed signs of iron oxidation, but the capability to oxidize Fe(II) ceased in the first passage. Phototrophic bacteria that had formed purple blooms (from enrichment HSA28) were unable to form colonies in agar dilution series with 5 mM of acetate and 4 mM of ascorbate.

Many more colonies were picked and tested for their ability to oxidize ferrous iron from diverse agar dilution series. Unfortunately, none of these colonies which were green, red or pink in color was able to oxidize iron.

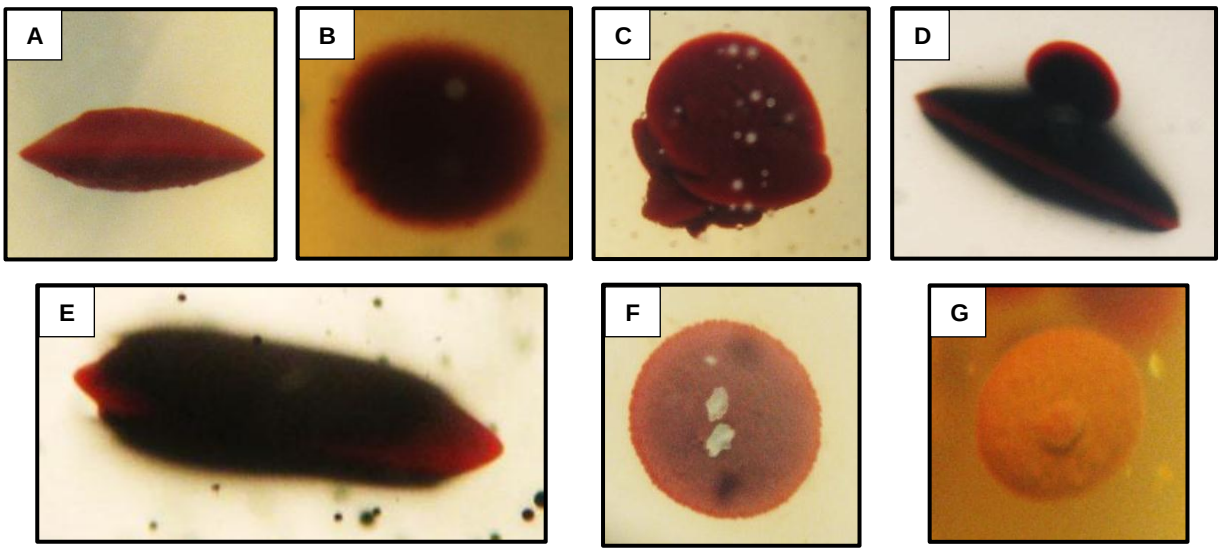

Figure 9 Colonies from different agar dilution series: very common red UFO-shaped colony [A]; red cloudy colony [B]; branched red colony [C]; red colonies with a black skin from the agar dilution series with cysteine [D,E]; encrusted red colonies after five month of incubation [F,G];

3.3.2 Agar plates

Three weeks after spreading cells of enrichment culture WM on agar plates with different buffers (KPP, MOPS, HEPES), colonies were formed. The different plates with various buffers showed similar types of colonies. But the number and the size of the colonies differed significantly. Cells grew better with HEPES or MOPS while on plates with KPP as buffer only low numbers of small colonies grew. On all the plates with HEPES or MOPS, red colonies grew about 1 mm in diameter. Some of these colonies were streaked on new agar plates. Another three weeks later, the grown colonies were able to be picked. These colonies were

either transferred to tubes with 10 mM of $FeSO_4$ or into tubes with 5 mM of acetate. All of these picked colonies showed an oxidation of iron after about two or three weeks, or in the case of acetate, a strong growth which was visible in a strong red coloration. The colonies that had been transferred into medium with acetate had grown much faster than those transferred into medium with $FeSO_4$ as sole electron donor.
Unfortunately, cells from enrichment culture HSA28 that had formed purple blooms were unable to grow on agar plates.

3.3.3 Bottle Plates

Two types of bottle plates were utilized. For the first set of bottles (50 mL), a low agar concentration was chosen. In these bottles, the purple blooms from enrichment cultures HSA28 and BS610 were spread out. After about two weeks of incubation, the first colonies were picked from the bottles of enrichment HSA28, in order to test the capability of iron oxidation in test tubes with medium supplied with 10 mM of $FeSO_4$. On the agar surface, the colonies showed two different types of colonies, the more reddish-colored colonies and the colonies, which were reddish-purple. Under the microscope, both colonies showed a very similar picture; mostly rod-shaped cells were to be seen.

Figure 10 Bottle Plate of the fished purple booms. Three different types of colonies could be seen. The big purple colonies, the middle sized red and the brown small colonies.

One of the picked colonies showed iron oxidation within two weeks. As the next step, this sample was again spread out in bottles with a higher concentration of agar (17 g L^{-1}). The grown colonies in this passage were able to be picked three weeks later. In the last two passages, the size of the colonies was reduced. Smaller reddish-brown colonies were formed on the agar. Even colonies picked later showed iron oxidation.

The bottle plates inoculated from enrichment YT247 (Fig. 10) which had directly been incubated in the light showed three different kinds of colonies that were able to be best seen with the binoculars. There were small red to dark red colored colonies like in the plates of sample HAS28, very small orange-brownish colonies and big light red to purple colonies. Later, these big colonies showed an orange coloration which had to be an encrustation of the colony with iron. The colonies in the bottle plates which had been incubated with less light showed smaller colonies. These colonies had grown much slower than those in the direct light. All three types of these colonies had been picked and tested to their ability to oxidize iron. About half of the picked colonies showed a slight iron oxidation after four weeks. Under

the microscope they showed the same rounded iron minerals as the picked colonies of sample HSA28.

3.3.4 Liquid dilution series

The liquid dilution series was inoculated with cells from the purple blooms (Fig.11, 12) that occurred in enrichment YT247 because it was not possible to isolate this type of cells via agar dilution series. After about eleven days, the oxidation was complete in the low dilution levels (10^{-2}, 10^{-3}). The highest dilution showed no Fe(II)-oxidation (10^{-8}) anymore. After about four weeks, the second dilution series was inoculated from the fully oxidized dilution (10^{-6}). The growth time for these dilutions took the similar time as those in the first dilution series. The oxidation took about seven weeks until dissolution step 10^{-7} was completely oxidized.

F ollowing the complete oxidation, purple blooms were formed simultaneously in all dilution steps. The highest dilution (10^{-8}) again showed no iron oxidation. Under the microscope, it was observed that the population unify with each isolation step. The purple blooms consisted of oval to round shaped cell which contained gas vesicles, resembling cells of *Thiodictyon sp.* strain F4 (Croal et al., 2004). Cells of enrichment culture YT247 were 1.5 to 2 µm in diameter and didn't show the formation of aggregates (Fig. 13). In the first dilution series, small very fast moving short rods and oval to round gas vesicles containing bacteria were seen. The majority of cells were irregular rods that were motile. The iron minerals that had formed in all dilution series consisted mainly of crumbly aggregates, but rounded to sharp iron grains were also observed in some tubes. In the second dilution series, the short motile rods dominated. The final dilution series was inoculated from dilution step 10^{-7}. The first sign of iron oxidation in the first dilution step (10^{-2}) could be seen after five days of incubation.

Figure 12 Purple bloom in enrichment culture YT247 formed after complete Fe(II)-oxidation

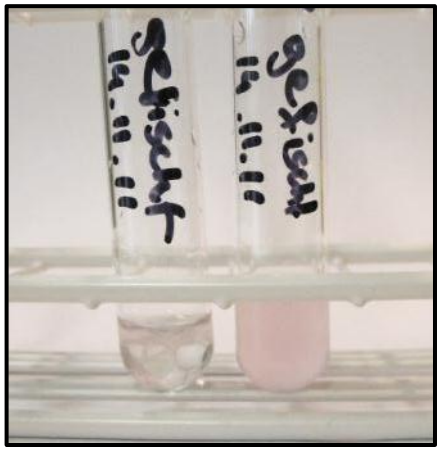

Figure 11 Pooled cells from purple blooms of enrichment YT247; with (left) and without (right) glass pearls to destroy cell aggregates.

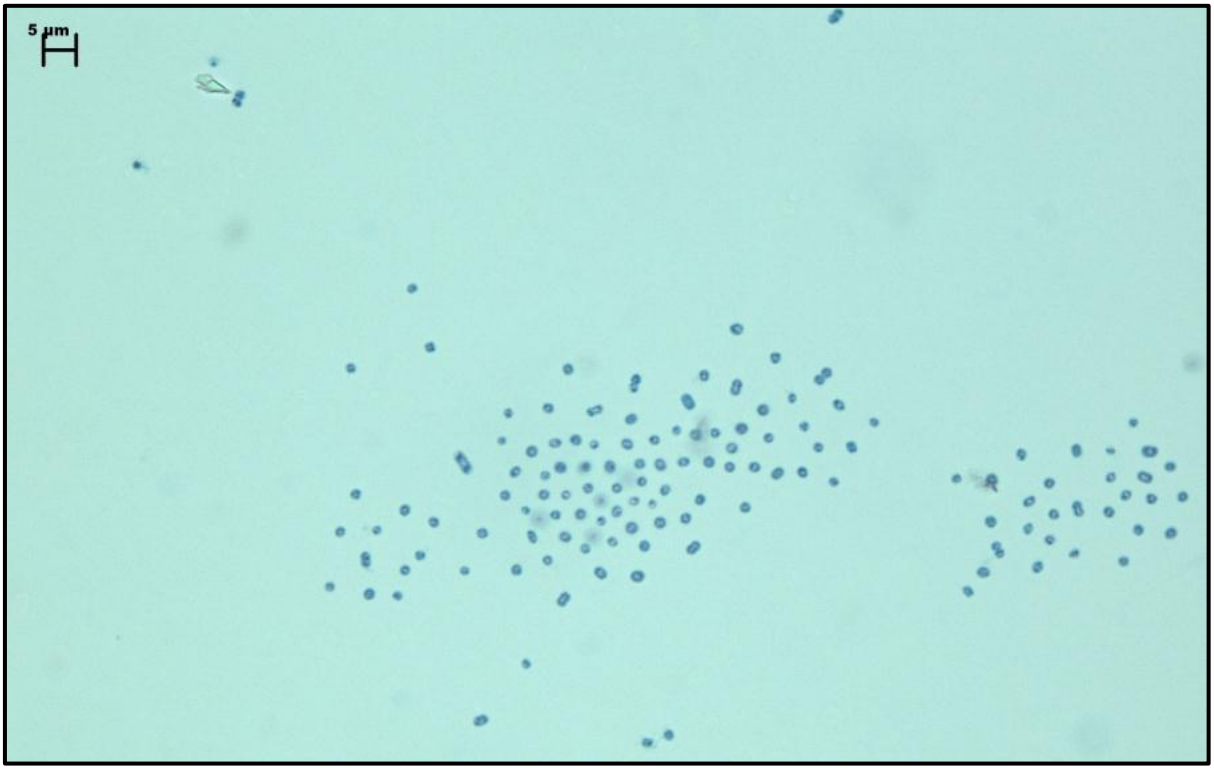

Figure 13 Microscopic image of cells that formed the purple blooms in enrichment culture YT247 after the second liquid dilution series.

3.4 Characterization of ViMi23

3.4.1 Way of isolation ViMi23

One enrichment culture was inoculated with a mixture of samples that were collected in the metropolitan area of Vienna. This enrichment developed very well and passages oxidized 10 mM ferrous iron in the light within seven to ten days. The isolation of strain ViMi23 from this enrichment culture involved different methods. First, an agar dilution series with 5 mM of acetate was inoculated from the fifth passage of the enrichment. Red colonies that grew in these agar dilution series were picked and successfully tested for the oxidation of ferrous iron. This was followed by the streaking of cells on agar plates with 5 mM of acetate and the subsequent incubation in jars under anoxic conditions in the light. Within three weeks red colonies developed and several isolated colonies were streaked out a second time on fresh agar plates and incubated as described before. Finally, several isolated colonies were transferred into liquid medium with ferrous iron. From twelve cultures that showed Fe(II)-oxidation in the light, strain ViMi23 was chosen for characterization.

3.4.1 Morphology and color

Cells of strain ViMi23 were 0.6 µm in diameter and 2.5 to 3.0 µm in length. A good motility was observed under all incubation conditions. Cells were elongated to club shaped and showed no gas vesicles (Fig. 15). Next to isolated cells, cells in chains or in aggregates were visible. Aggregates were formed particularly in the late phases of incubation. The iron oxidation of the isolate occurred through a grayish green intermediate state, as it had already been observed in the enrichments before (Fig. 6). The cells were not encrusted in iron minerals, but rather loosely attached to the formed iron minerals.

The ferric precipitates formed by ViMi23 were finely grained and fluffy. The observed color of the iron precipitate of ViMi23 showed a darker and not so intensive orange color as the original enrichment from the beginning. Ferromagnetic precipitates were never observed. The color of the incubated culture with other substrates than Fe(II) showed a red color resembling the color of raspberries. The in vivo absorption spectrum of strain ViMi23 exhibited absorption maxima in the long wavelength range characteristic of bacteriochlorophyll *a* (Fig. 14) with the main peaks at 807 and 865 nm (Pfening & Tuempler, 1991).

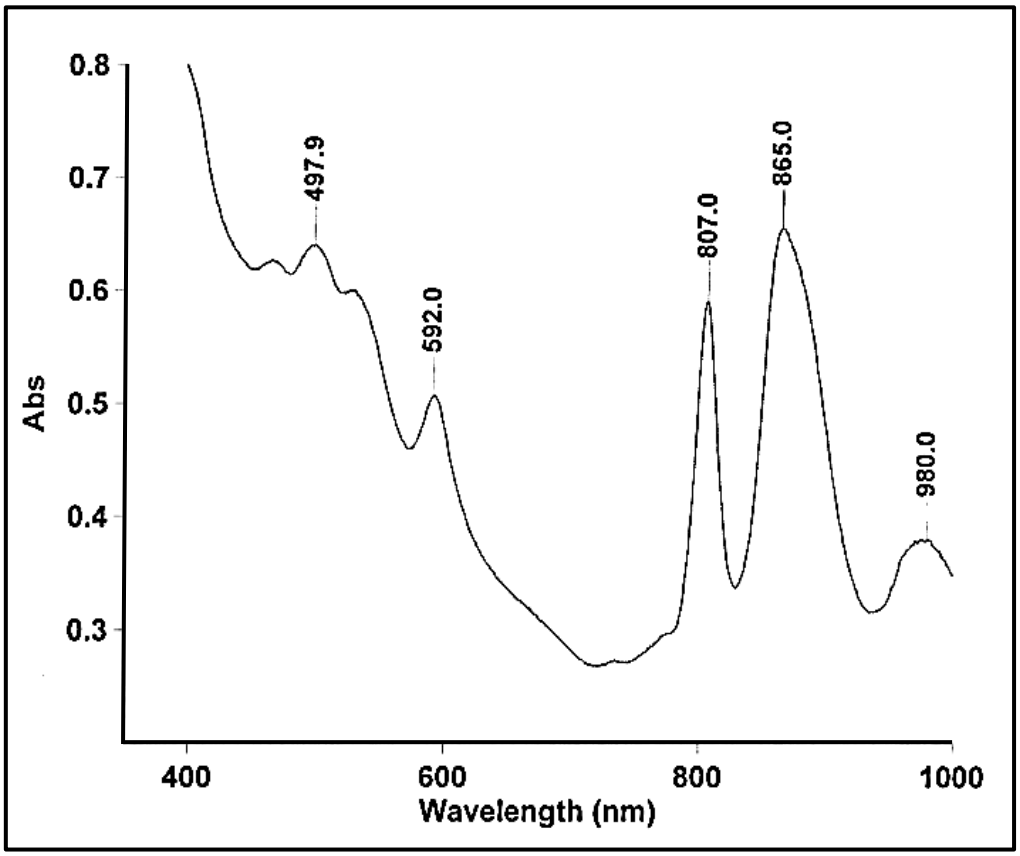

Figure 14 In vivo absorption spectrum of strain ViMi23 with absorption maxima at 807 and 865 nm that are characteristic for bacteriochlorophyll *a*.

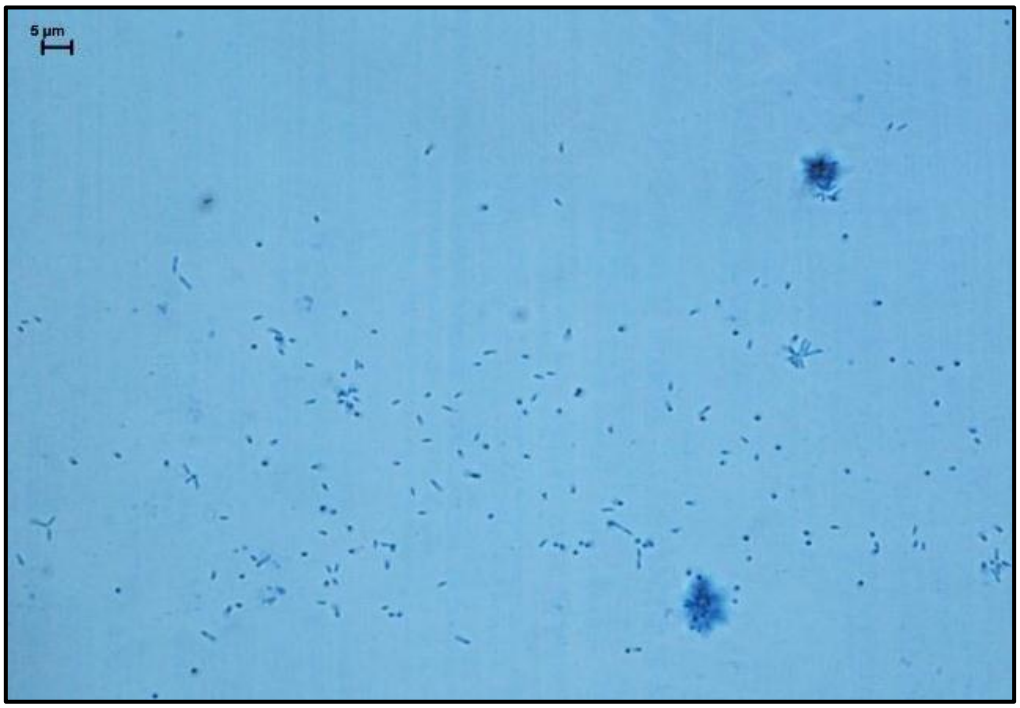

Figure 15 Morphology of ViMi23: Cells of strain ViMi23 were 0.6 µm in diameter and 2.5 to 3 µm in length. ViMi23 cells were elongated to club shaped. Only few cell aggregates were formed.

3.4.2 Physiology of ViMi23

3.4.2.1 pH range

The pH range for growth of strain ViMi23 with acetate as electron donor was 6.2 - 7.9, with an optimum at 6.8. The addition of $FeSO_4$ lowered the pH value of the freshwater medium. The growth took much longer than with acetate. The experiments with $FeSO_4$ as electron donor showed a pH range for growth between 6.0 and 7.1.

Table 3 pH range of growth for strain ViMi23 with acetate

pH- value	growth
9.2	-
9.0	-
7.9	++
7.0	++
6.8	++
6.4	+++
6.2	+
5.8	-

Table 4 pH range of growth for strain ViMi23 with Fe(II)

pH-value	growth
8.7	-
8.2	-
7.1	+
6.5	+
6.4	+
6.4	+
6.2	+
6.0	+

3.4.2.2 Temperature range

The temperature range for growth of strain ViMi23 was tested with acetate as electron donor. Optimal growth was observed at 25°C and ceased at temperatures above 35°C. Incubations at 40 and 45°C showed no growth, cells were nonmotile and had lost their typical shapes. Within one week of incubation growth was observed at 10°C and it took about four weeks to detect growth even at 4°C. Thus, strain ViMi23 had a relatively wide temperature range for growth, from about 4 to 35°C, with an optimum temperature at 25°C.

3.4.2.1 NaCl Tolerance

Growth experiments with acetate that were additionally supplied with 0.5% NaCl showed growth already after four to five days of incubation while addition of 1% NaCl delayed growth; cultures with 1% NaCl were fully grown only after 12 days of incubation. In the presence of 2% NaCl, only very weak growth was observed and with 3% NaCl no growth occurred within eight weeks of incubation.

3.4.2.2 Influence of reduction agents

This series of tests showed an influence of reducing agents (ascorbate or cysteine) on the growth rate of strain ViMi23. The fastest growth with acetate as electron donor was observed in the presence of 2 mM of cysteine. Growth in the presence of 4 mM of ascorbate was also faster than in experiments without reducing agent. The influence of reducing agents was visible macroscopically and with the microscope. For each reducing agent, two test tubes were made, which showed different growth and different pictures under the microscope. The two tubes with ascorbate as the reducing agent showed two different microscope pictures. For the tube with the intense color, the microscopic image showed typical elongated to club shaped cells. The other tube with weaker coloration, showed smaller and rod-shaped cells that were highly motile. A similar observation was recorded for experiments with cysteine as the reducing agent. With cysteine, some of the smaller rod-shaped cells had also been seen in the better grown culture.

The tubes with cysteine as the reducing agent and Fe(II) as electron donor showed two different colors. One of the tubes formed black aggregates and the second tube exhibited aggregates that were orange in the beginning. In the end of incubation both tubes were orange. Nevertheless, under the microscope the two samples showed similar pictures.

3.4.2.3 Electron donors

Photoautotrophic growth was tested with different electron donors other than Fe(II). Within the first 14 days only the test tubes containing 2 or 4 mM thiosulfate showed signs of growth. Also polysulfide could be utilized as electron donor. The microscopic picture was similar to growth with acetate in the presence of reducing agents like cysteine. Tubes with elemental sulfur showed also growth. Growth experiments in an anaerobic chamber with 3 to 4 percent hydrogen showed weak growth of strain ViMi23. No growth was observed with 2 or 4 mM sulfide, 1.5 mM FeS and 5 or 10 mM Mn^{2+} during eight weeks of incubation.

Photoheterotrophic growth of strain ViMi23 was possible with a variety of electron donors. Good growth was observed with 2.5 mM and 5 mM of succinate, acetate, propionate, pyruvate, malate, butyrate or 2 and 4 mM cysteine within the first four to five days. Growth with 2.5 mM and 5 mM fumarate was delayed and only weak growth was recorded with 2.5 mM ethanol as electron donor which was confirmed with the microscope: only few motile cells were detected. No growth occurred with 5 mM ethanol, glucose or citrate within eight weeks of incubation.

3.4.2.4 Electron acceptors and fermentation metabolism

Strain ViMi23 was only able to use oxygen as an electron acceptor for respiratory growth in the dark with 5 mM acetate as electron donor. No respiratory growth was observed with nitrate, sulfate, S_0, Fe-citrate or biogenic Fe(III). Furthermore, strain ViMi23 didn't grow by fermentation with glucose, pyruvate, fumarate or yeast extract.

3.4.2.5 Growth

The photoheterotrophic growth of strain ViMi23 with 5 mM acetate under anaerobic conditions was monitored by measuring the absorbance at 660 nm at regular time intervals (24 h, 72 h). For inoculum, 10% of an acetate grown culture was taken.

After four days of incubation, the cultures with acetate were fully grown. This test showed an intense raspberry color and the OD_{660} data showed no further changes after four days.

The doubling time of strain ViMi23 was about 32 hours (Fig. 16).

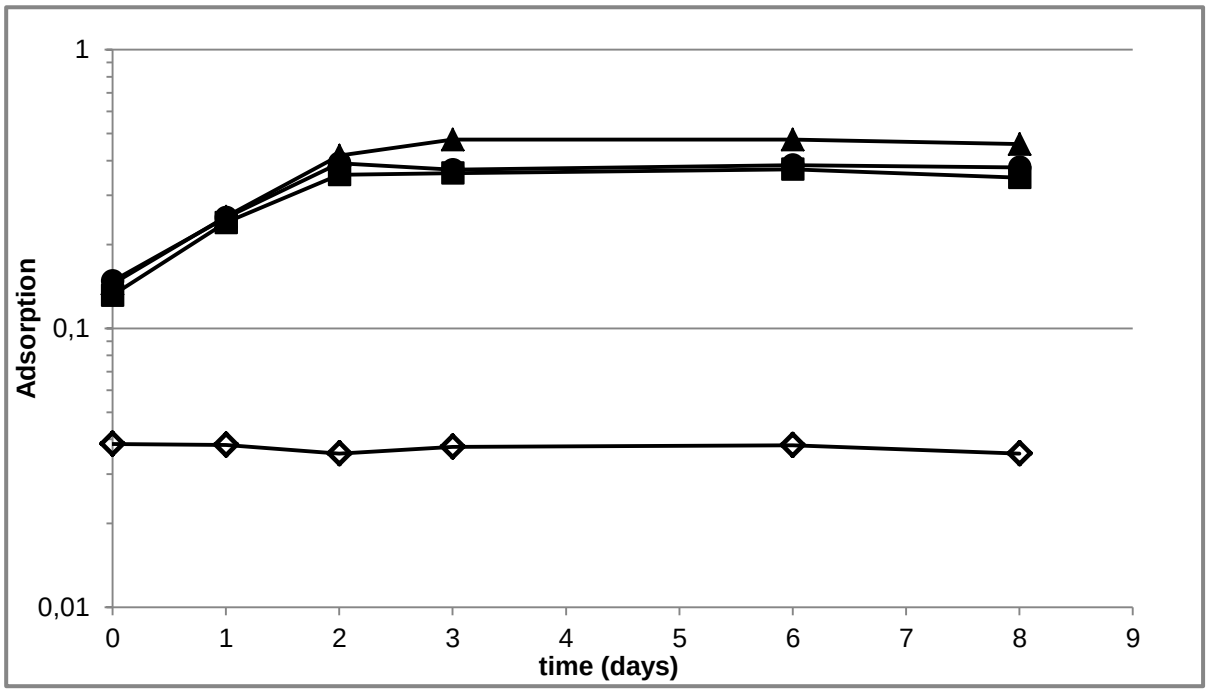

Figure 16 Growth of strain ViMi23 with acetate. Average doubling time was determined at the steepest part of the growth curve. Filled symbols represent growing cultures, open symbols represent a pasteurized control.

A second growth experiment with Fe(II) was done, to measure the time of growth with iron as the sole electron donor. Two different series were prepared. The first series was inoculated with 10% of an acetate grown culture; the second series with 10% of a culture grown with Fe(II). It turned out that the Fe(II)-oxidation by strain ViMi23 was significantly faster in experiments that had been inoculated from an iron grown culture (Fig. 17).

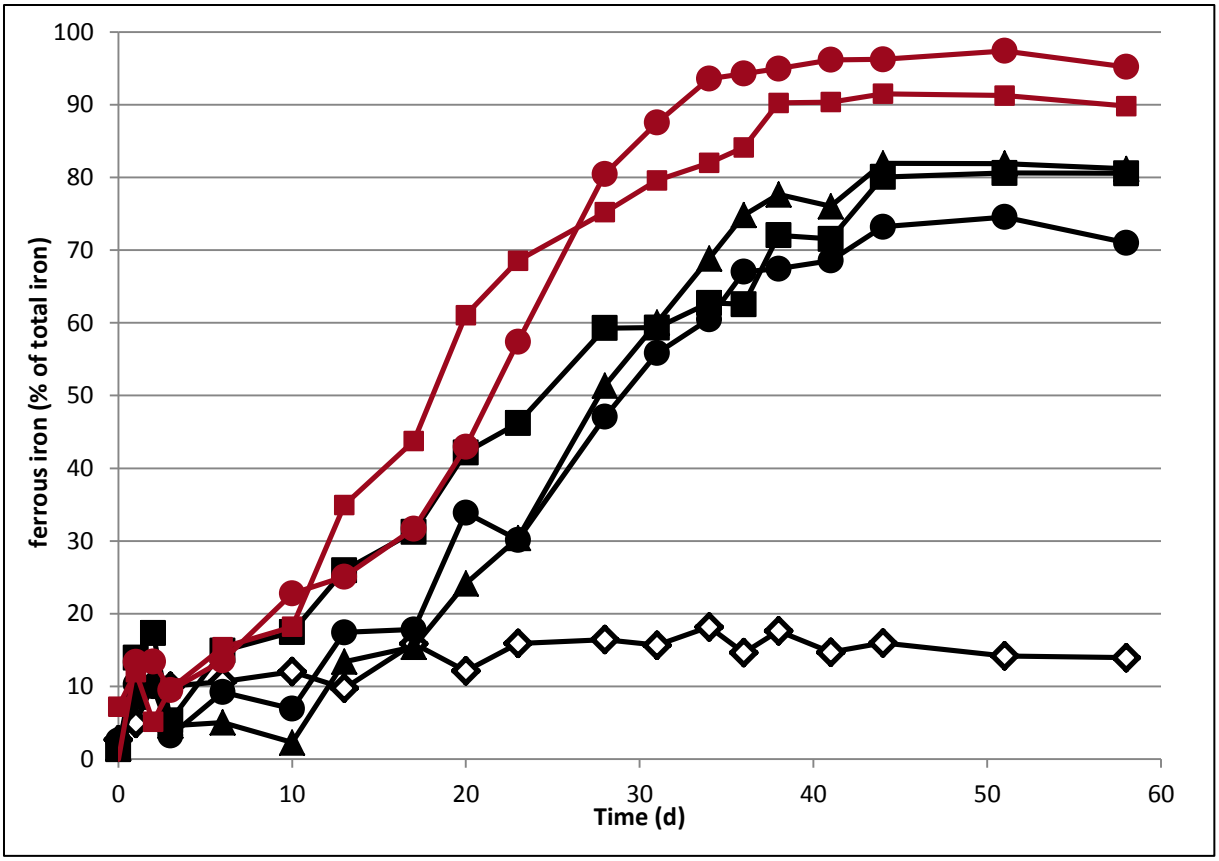

Figure 17 Growth of strain ViMi23 with 10 mM Fe(II) as sole electron donor. Ferric iron in a pasteurized control (◊). Ferric iron in experiments that were inoculated from an Fe(II)-grown culture (■, ●) or from an acetate-grown culture (▲, ■, ●).

3.4.3 Phylogeny of ViMi23

A full phylogenetic study by complete 16S rDNA sequence analysis was done at the DSMZ. For this analysis, four test tubes with acetate-grown cells of strian ViMi23 were sent to Germany. The results of this analysis showed, that strain ViMi23 is closely related to *Rhodopseudomonas pseudopalustris* strain DSM 123^T (Fig.14; Table 5).

Table 5 Phylogenetic relationship of strain ViMi23.

Strain	% similarity of the 16S rRNA gene sequence to strain ViMi23
Rhodopseudomonas palustris DSM 126^T	97.5
"Rhodopseudomonas harwoodiae" JA351^T	96.4
Rhodopseudomonas parapalustris JA310^T	99.0
***Rhodopseudomonas pseudopalustris* DSM 123^T**	**100.0**
Rhodopseudomonas julia DSM 11549^T	89.1
Rhodopseudomonas rhenobacensis DSM 12706^T	97.8
Rhodopseudomonas faecalis JCM 11668^T	98.4

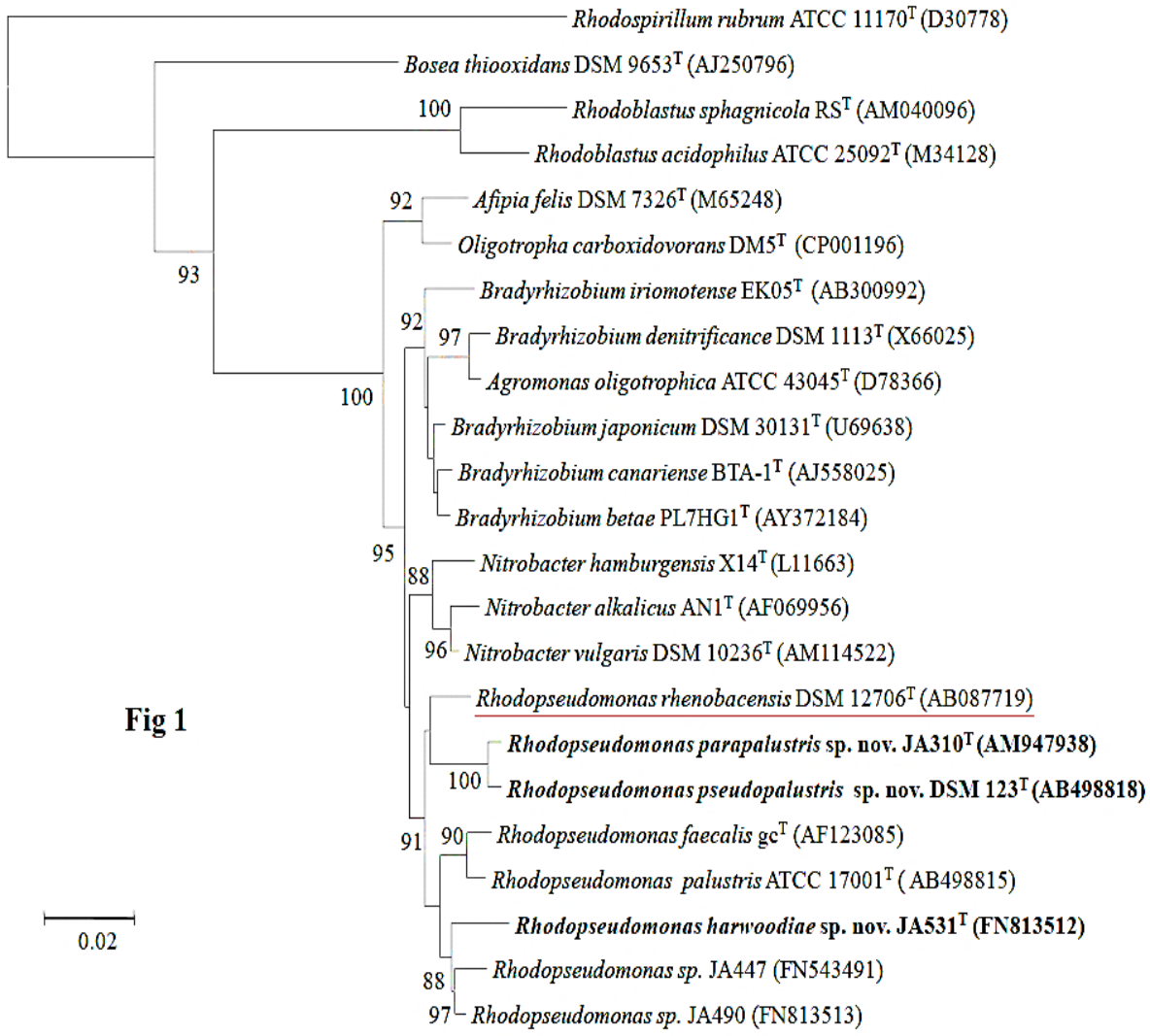

Fig 1

Figure 18 Dendrogram showing the phylogenetic relationships of members of the genus *Rhodopseudomonas*. The tree was constructed (1350 gap-free sites were compared) by the neighbor joining (NJ) method using the MEGA4 software. Bar: two nucleotide substitutions per 100 nucleotides (Ramana et al., 2012)

4 Discussion

4.1 Geographic distribution of phototrophic Fe(II)-oxidizing bacteria

Some literature about the isolation of phototrophic Fe(II)-oxidizing bacteria has been published in the last two decades but the distribution of this physiological type of phototrophic bacteria in different environments has not been investigated. The task of this thesis was therefore the collection of samples from different environments in Austria and Switzerland (Fig. 2), the enrichment and subsequent isolation of iron-oxidizing bacteria from these samples.

All enrichment cultures except those inoculated with sediment from the lower part of the Rabloench fountain, the sample WTS in Switzerland and enrichment BS45 formed rusty-brown precipitates during five months of incubation. One reason for this observation may be the temperature, since many of the collected samples were taken at locations where the temperatures were below 10°C. The temperature during laboratory incubations was around 23°C. This dependence of temperature was observed before by Hegler et al. (2008). They found out that the optimal temperature for three tested iron(II)-oxidizing strains was between 23°C and 26°C. Another reason may be an unknown growth factor, which was not supplied with the medium. For all enrichments, a bicarbonate buffered freshwater medium without vitamins was used. So bacteria that depend for growth on vitamins could not grow. Yet another reason may be the nearly neutral pH value (6.8-6.9) of the medium because pH measurements during sampling showed that some samples had slightly acidic pH value.
The iron speciation could be another reason for the lack of iron oxidation in some enrichment cultures. It could be possible that these bacteria were not able to utilize the offered $FeSO_4$. Also the iron concentration is an important point. Hegler et al. (2008) determined an optimal Fe(II) concentration of 8 mM. At higher concentrations the oxidation decreased (Hegler et al., 2008).

Hegler et al. (2008) also pointed out that for this type of bacteria there is a light dependency which had not been tested during this thesis. All experiment had been incubated in the light of a 25W bulb in about 45 to 50 centimeter distance. But different Fe(II)-oxidation rates could be observed dependent on the light intensity. For some enrichment it made a difference if it was incubated close or at greater distance from the light source. Some enrichments preferred being incubated under the light source like samples HSA28. Cells of enrichment culture YT247 also grew with less light.
Finally, it could also be possible that there were no phototrophic iron-oxidizing bacteria present in these samples.

Because of the few positive enrichments with freshwater medium, enrichments with original sample material were prepared. Six samples were chosen for this experiment which didn't show an iron oxidation after three months of incubation. These enrichments without medium showed more and faster positive results in the phototrophic oxidation of iron. But outgassing of CO_2 and H_2S and especially the iron deposits of minerals on the glass wall of test tubes made it difficult to interpret the iron measurements with the ferrozine-photometer method. The formation of algae on the iron minerals, formed at the end of the iron oxidation, falsified the results of measurements of the iron as well. Some of the pure enrichments clearly showed signs of iron oxidation macroscopically. However, this observation couldn't be confirmed by iron measurement. This method was successfully applied for analyzing enrichments with sample VSS, because the intense orange color of the original sample material masked additional iron oxidation processes in the light. As for the iron measurement, some fine sediment was needed. In some samples the sediment was to coarse-grained, so the iron measurement lacked precision.

The time needed to oxidize iron was very different in the beginning and ranged from two weeks to five months. Finally, all subcultures need about the same time of 7 to 21 days for complete oxidation of 10 mM Fe(II). The rate of Fe(II)-oxidation changed as well for one sample during the different enrichment and passage steps. For most enrichments the time for oxidation became much shorter which could be explained by a better adaptation of the bacteria to the incubation conditions. Small variations in Fe(II)-oxidizing rates in the same culture were due to the differences in culture age or slightly different inoculum sizes which were added, leading to a variation of cell numbers.

Some growth experiments were made to describe the phototrophic Fe(II)-oxidation in greater detail (Fig. 7). The progress of iron oxidation looked similar for all enrichments. The lag phase lasted 2-4 days, and then the iron oxidation lasted up to 14 days. Over time, the iron oxidation of the enrichments became much faster. One explanation for this observation could be the better adaptation of the cells to the incubation conditions.

4.2 Isolation of phototrophic Fe(II)-oxidizing bacteria

Agar dilution series had been prepared for some of the twenty enrichments. These showed not only differences in growth, color and size of colonies but also in the distribution of bacteria within the test tubes. In most of the dilution series, red to dark red UFO-shaped to round and cloudy colonies had been observed (Fig. 9A, 9B). The size of these colonies differed from tube to tube. Most of these red colonies were about 0.5 mm to 2.5 mm in diameter, but the size was mostly less than 1 mm in diameter.

Especially in the agar experiments with cysteine as a reducing agent, additional types of colonies grew. One form of the colonies had a round shape and a black color. The initially formed red colonies formed a black outer layer over time, which burst open when picking the colonies as a capsule. The reason why this layer is formed is as yet unclear. Under the microscope, there were different images between the picked colonies and the later incubated culture. Shortly after the picking, the colony showed a small rod-shaped organism and additionally long fine filaments. In the image of the incubated culture, the fine filaments were not observed any longer, so it might be possible that these filaments built up this black skin around the red colonies.

Some of the red colonies formed in the agar dilution series with acetate and $FeSO_4$ showed a light orange to orange color after five to six months of incubation. This color turned out as a crust of iron minerals. Inside this crust the red colony still existed. In addition green colonies occurred, which turned out to be encrusted red colonies. None of such colonies showed iron oxidation when transferred into liquid medium.

To simplify isolation, cells were streaked out on agar plates and incubated in anoxic jars in the light. Here bacteria of enrichment culture HSA28 grew better than in the agar dilution series. For further simplification, bottle plates were prepared. For bottle plates, a thin agar layer on one flat side of the bottle was utilized to spread out cells. The agar concentration was increased to 17 g/l and the bottle volume from 50 ml to 100 ml for easier handling. This method turned out to be a good substitution for agar plates in the anoxic jars, because it facilitated observing the growth of bacteria.

It had been observed that the time for growth of some enrichments differed between liquid medium, agar dilutions and agar plates. For enrichments YT247 and HSA28, the full oxidation of iron in liquid medium took about nine days, but in solidified medium the growth took three weeks or longer. The growth rates also differed in different phases of isolation. The size of the colonies of enrichment culture HSA28 decreased with each isolation step. In

the third set of bottle plates, the colonies had been too small and there were no real single colonies for further transfer. Also the time to form colonies took longer with each step.

Some of the picked colonies from the agar dilution series and agar bottle plates showed a further difference to the original enrichments. The cultures formed Fe(III) precipitates of a light orange to green color while enrichments formed Fe(III) minerals of an intensive orange color. These macroscopic differences were reflected by differences in the microscopic picture. On the one hand, the enrichments showed fluffy, fine and irregular formed iron minerals; but on the other hand round, bigger and compacter iron minerals were formed in cultures of isolated colonies. How these round particles were formed could not be analyzed during this thesis.

4.3 Characterization of ViMi23

One isolate, strain ViMi23, was obtained in the course of the experiments from sample material collected in the metropolitan area of Vienna. According to 16S rDNA analysis, the isolate was affiliated with purple nonsulfur bacteria of the genus *Rhodopseudomonas* sharing 100% sequence similarity with *Rps. pseudopalustris* strain DSM 123^T which was recently re-classified by Ramana et al. (2012). Despite the high similarity in 16S rDNA sequences, strain ViMi23 shows significant morphological and physiological differences to *Rps. pseudopalustris* strain DSM 123^T as will be described below.

The color of photoheterotrophic grown cultures of strain ViMi23 were intensively red and the color resembled the one of raspberries. In cultures grown with Fe(II) as the sole electron donor, the brown to orange color of the produced iron minerals masked the color of the cells.
The cell size determined for strain ViMi23 differs significantly from the cell size reported for *Rps. pseudopalustris* strain DSM 123^T. While strain ViMi23 showed on average a cell size of 0.6 µm in diameter and 2.5 to 3.0 µm in length, its relative was 0.5 to 1.0 µmin in diameter and 2.0 to 6.0 µm in length. Only *Rps. palustris* showed a similar size. Like all strains of the genus *Rhodopseudomonas*, cells of strain ViMi23 produce bacteriochlorophyll *a*. The carotenoids of strain ViMi23 were not analyzed.

The physiological characterization of strain ViMi23 included experiments with various electron donors, electron acceptors and possible fermentation substrates. Furthermore, physicochemical parameters such as salinity tolerance, pH range, temperature range and sensitivity towards oxygen were determined. In general, strain ViMi23 showed greater physiological diversity than close relatives.

Strain ViMi23 did not need NaCl for growth, but it could tolerate NaCl up to a concentration of 2%. In contrast *Rps. pseudopalustris* DSM 123^T also did not require NaCl for growth, but had only a tolerance up to the concentration of 0.5% NaCl.

Strain ViMi23 grew photoheterotrophically between pH 6.0 and 7.9 with an optimum at pH 6.4. The three closest relatives grew in a similar pH range, i.e. from pH 6 to 8.5 (Ramana et al., 2012). Photoautotrophic growth with Fe(II) occurred in the pH range from 6.0 to 7.1. Other strains of Fe(II)-oxidizing bacteria showed a little higher tolerance for different pH values; for example *Rhodobacter ferrooxidans* strain SW2 oxidized Fe(II) in the range of pH 5.2 to 7.5 (Hegler et al., 2008).

Strain ViMi23 grew in a temperature range from 4°C to about 35°C. In contrast, *Rps. pseudopalustris DSM 123^T* showed a temperature range from 20 to 35°C with the optimum at 30°C. Other close relatives of *Rps. pseudopalustris* also prefer higher temperatures for growth: *Rps. palustris DSM 126^T* grew in a temperature range from 25 to 40°C (optimum 30°C) and *Rps. parapalustris JA310^T* grew at temperatures between 25 and 35°C (optimum 30°C).

Obligate Fe(II)-oxidizing bacteria live in anoxic environments. The absence of oxygen is necessary because otherwise dissolved Fe(II) will react with molecular oxygen to Fe(III). Like some other phototrophs, strain ViMi23 prefers low concentrations of oxygen during photoheterotrophic growth. Accordingly, strain ViMi23 showed a specific distribution of colonies in agar dilution series which were incubated in the light with acetate (Fig. 19). In the first centimeters, the colonies were very small, deeper in the agar the size of the colonies increased. If the anaerobic bacteria do not tolerate oxygen, they can lower their requirements for light, so that they live deeper in the sediment (Hegler et al., 2008). Additionally, strain ViMi23 showed respiratory growth with oxygen in the dark. No other electron acceptors tested were utilized by strain ViMi23.

Figure 19
Agar dilution serie of ViMi23

Strain ViMi23 showed preferential growth with organic substrates as electron donors. Many different substrates were tested and compared with the results of strain *Rps. pseudopalustris* DSM 123^T (Ramana et al., 2012).
Best growth of strain ViMi23 was observed with acetate, succinate, or butyrate. Also *Rps. pseudopalustris* could grow with different organic substrates, for example, acetate, lactate or pyruvate. Strain ViMi23 as well as its relatives could not utilize citrate as electron donor. No

growth by fermentation was observed for strain ViMi23 or *Rps. pseudopalustris* (Ramana et al. 2012).

Ramana et al. (2012) observed no photoautotrophic growth of *Rps. pseudopalustris DSM 123^T*. Several different sulfur species and H_2 were tested to check if strain ViMi23 could grow photoautotrophically with other electron donors than Fe(II). Indeed, strain ViMi23 showed slow but significant growth with different sulfur species as well as with H_2. Growth with diverse sulfur species or molecular hydrogen is also known from other Fe(II)-oxidizing strains such as *Rhodobacter ferrooxidans* strain SW2 (Ehrenreich & Widdel, 1994 or *Rhodopseudomonas palustris* strain TIE-1 (Jiao et al., 2005). *Thiodictyon* strain L7 could oxidize Fe(II) better in the presence of H_2 (Ehrenreich & Widdel, 1994).

Growth of strain ViMi23 was with acetate always faster than with Fe(II) as electron donor (Fig. 16, 17). It was additionally observed in growth experiments that the source of the inoculum had an influence on the rate of Fe(II)-oxidation (Fig. 17). Growth experiments inoculated from acetate-grown cultures had a significantly longer lag phase indicating the preference for organic electron donors. Early on, Ehrenreich & Widdel (1994) mentioned this phenomenon for one isolate. *Rhodobacter* strain SW2 started the oxidation of iron only after the consumption of the organic substrate acetate.

5 Conclusion and Outlook

Iron oxidation by anoxygenic phototrophic bacteria possibly takes place in several different environments in Austria and Switzerland. Only three out of twenty samples showed no signs of iron oxidation in laboratory incubations. The time for Fe(II)-oxidation in stable enrichment cultures differed with the source of the initial inoculum, but took in general about 7 to 21 days.

Strain ViMi23 showed that at least some anoxygenic Fe(II)-oxidizing bacteria can cope with different salinities, temperatures, pH values and light conditions. The metabolic versatility allows furthermore photoautotrophic and photoheterotrophic growth with a variety of electron donors as well as respiratory growth with oxygen in the dark. Due to this metabolic versatility it can be speculated that strain ViMi23 occurs widespread in nature.

This thesis has shown significant potential and a call for future work. Research will be priority driven. High priority has the characterization of isolate HSA28 and the finishing of the isolation of strain YT247 plus it is subsequent characterization. Another issue concerns the round Fe(III) precipitates that were formed by enrichment cultures HSA28 and YT247. In addition, the red colonies with the black skin out of the agar dilution series with cysteine should be isolated and studied in more detail.

A correlation between this type bacterium and the banded iron formations was not directly dealt with in this master thesis. There remains the possibility that Fe(II)-oxidizing bacteria were involved in the genesis of BIFs.

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Appendix

Table 6 Comparison of strain ViMi23 to the closest relatives (characteristics)

	ViMi23 *Rhodopseudomonas pseudopalustris*	***Rhodopseudomonas pseudopalustris sp. nov.* DSM123^{T*}**	***Rhodopseudomonas parapalustris sp. nov.* JA310^{T}***	***Rhodopseudomonas palustris* ATCC17001^{T}****
Characteristic				
Isolation source	Soil/pond water	soil/pond water	soil	soil/sludge/pond water
Cell shape	Elongate to club	rods	rods	rods with rounded ends
Cell Size	0.6 X 2.5-3.0	0.5-1.0 X 2.0-6.0	0.8-1.2 X 2.0-4.0	0.4-1.0 X 1.5-3.0
Color of culture	red-brown	red-brown	red-brown	red-brown
Optimal pH (range)	6.8 (6.0-7.9)	7 (6-8)	6,5-7 (6-9)	6,5-6.9 (6.5-7.5)
Temperatur optimum(range; °C)	25 (4-35)	30 (20-35)	30 (15-35)	30 (25-40)
aerobic dark growth	+	+	+	+
Photoautotrophic growth with	H_2,S^0, thiosulfate	-	-	-
Growth factors	non	PABA	Thiamine, PABA	PABA
NaCl tolerance	>2%	>0.5%	>0.5%	n.d

n.d=no data; PABA= 4-Aminobenzoic acid;

*data from Ramanaet al., 2012; **data from Jiao et al., 2005 and Ramana et al., 2012

Table 7 Comparison of strain ViMi23 to the closest relatives (substrates)

	ViMi23 *Rhodopseudomonas pseudopalustris*	*Rhodopseudomonas pseudopalustris sp. nov.* DSM123^{T*}	*Rhodopseudomonas parapalustris sp. nov.* JA310^{T}*	*Rhodopseudomonas palustris* ATCC17001^{T}**
Acetate	+	+	+	+
Benzoate	n.d	+	-	+
Butyrate	+	+	-	+
Caproate	n.d	+	-	(+)
Caprylate	n.d	+	-	-
Citrate	-	-	-	-
Ethanol	-	+/-	-	+
Fe(II)	+			
Fe(III) biogenic iron	-	n.d	n.d	n.d
Formate	+	+	-	-
Fructose	n.d	-	-	-
Fumerate	+	+	+	+
Glucose	-	+/-	(+)	+
Glutamate	n.d	+	-	-
Glycerol	n.d	+	+	+
Glycolate	n.d	+	-	n.d
Lactate	n.d	+	+	+
Malate	+	+	+	+
Propanol	+	+	-	n.d
Propionate	+	+	-	+
Pyrovate	+	+	+	+
Succinate	+	+	+	+

n.d=no data; *data from Ramana, 2012; **data from Jiao et al., 2005 and Ramana et al., 2012

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die vorliegende Masterarbeit selbstständig und ohne fremde Hilfe verfasst, andere als die angegebenen Quellen und Hilfsmittel nicht benutzt bzw. die wörtlich oder sinngemäß entnommenen Stellen als solche kenntlich gemacht habe.
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I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Wien, am 1.Juni.2012

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