

## Acid Mine Water Treatment Using Novel Acidophilic Iron-Oxidizing Bacteria of the Genus “*Ferrovum*”: Effect of Oxygen and Carbon Dioxide on Survival

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### Abstract

Acid mine waters are characterized by low pH and high loads of iron and sulfate, resulting from the oxidative dissolution of sulfide minerals due to the mining activities. Owing to the effect of these contaminations on the environment, mine waters have to be treated before discharging into the rivers. At the open-cast pit Nochten (Germany) acid mine waters were remediated biotechnologically in a pilot plant by immobilization of ferrous iron resulting from microbial iron oxidation with subsequent precipitation of the iron-oxyhydroxysulfate schwertmannite. The microbial community was dominated by “*Ferrovum*”, which is an acidophilic autotrophic iron-oxidizing bacterium and was not yet been validly described. Since the sub-cultivation of enrichment cultures containing “*Ferrovum*” encountered difficulties and resulted in loss of strains, oxidative stress was considered to be a possible reason for this. Therefore, the culture JA12 containing “*Ferrovum*” and *Acidiphilium* was incubated under different oxygen and carbon dioxide concentrations, as well as under anaerobic conditions. Compared to atmospheric conditions, microaerobic conditions enhanced bacterial survival. Incubation of the culture under increased carbon dioxide and decreased oxygen content affected the bacterial survival positively, while the incubation under anaerobic conditions was not favorable. Information about the novel genus “*Ferrovum*” regarding physiological characteristics will facilitate the handling of the bacteria. The knowledge about optimal storage conditions may make it more simple to provide active cultures for use in mine-water treatment.

Key words: Mine water treatment, acidophilic iron-oxidizing bacteria, *Ferrovum* spp, survival, microaerobic

### Introduction

In active and abandoned mining sites sulfide mineral ores are exposed to oxygen and water resulting in effluents characterized by low pH and high loads of heavy metals, especially iron, and sulfate. These acidic mine waters have to be remediated before discharging into rivers to reduce the impact on the environment. The treatment of the waters can occur conventionally by neutralization with subsequent chemical iron oxidation or alternatively by biotechnological processes.

At the open-cast pit Nochten (Lusatia, Germany) mine waters were biotechnologically treated in a pilot plant. Ferrous iron was immobilized by microbial iron oxidation with subsequent precipitation of the iron-oxyhydroxysulfate schwertmannite. Molecular genetic studies of the microorganisms occurring in the mine water treatment pilot plant revealed the dominance of betaproteobacteria of the genus “*Ferrovum*” (Heinzel et al., 2009).

The novel genus “*Ferrovum*” has been detected in several acid mine waters using culture-independent methods (Hallberg et al., 2006; Hao et al., 2010; Kimura et al., 2011). Cultivation attempts of this genus resulted in one isolate (Johnson et al., 2014) and in several cultures containing “*Ferrovum*” and *Acidiphilium* or *Acidithiobacillus* (Tischler et al., 2013). The isolate “*F. myxofaciens*” P3G has been described as an acidophilic, psychrotolerant obligate autotrophic bacterium, which uses ferrous iron as electron donor and oxygen as electron acceptor (Johnson et al., 2014).

Since ferrous iron seems to be the sole energy source of the genus “*Ferrovum*” it is assumed that oxidative stress is increased for the bacterial cell (Ferrer et al., 2016). Due to the presence of ferrous iron and oxygen, reactive oxygen species (ROS), including hydrogen peroxide, superoxide radicals, and hydroxyl radicals, are formed in the cells via the Fenton and Haber-Weiss reactions. ROS damage most cellular component like DNA, RNA, and proteins (Cabiscol et al., 2000).

The long-term storage of “*Ferrovum*” spp. proved to be difficult. The storage as cryo culture, a common method in microbiology, failed (Johnson et al., 2014). Due to the increased oxidative stress level for acidophilic iron-oxidizing bacteria under atmospheric conditions, it was hypothesized that reduced oxygen concentrations may reduce the stress level. Simultaneously elevated carbon dioxide concentrations may increase the microbial growth, since carbon dioxide, the carbon source of “*Ferrovum*” spp., is only poorly soluble in acidic aqueous solution. Therefore, a culture containing “*Ferrovum*” and *Acidiphilium* was cultivated under different gas phases containing reduced oxygen and increased carbon dioxide levels.

## Material and Methods

### *Culture JA12*

Culture JA12 was obtained from mine waters of a treatment pilot plant at the open cast pit Nochten in Lusatia (Saxony, Germany) (Tischler et al. 2013). Analysis using terminal restriction length polymorphism (T-RFLP) revealed the presence of “*Ferrovum*” sp. and *Acidiphilium* sp. in the culture. The bacteria in the culture have recently been genome sequenced (Ullrich et al., 2015; Ullrich et al., 2016). To maintain the culture JA12 it was incubated at room temperature in artificial pilot plant water (APPW, pH 3.0) containing 5 mM ferrous iron and was transferred periodically into fresh medium (Tischler et al., 2013).

### *Incubation under different gas phases*

After the iron concentration, which was determined with the ferrozine method (Lovley & Philips, 1986), had reached approximately 2 mM, 50 µl of the culture JA12 were plated on overlay plates (Johnson et al., 1991; Tischler et al., 2013). After visible growth had occurred, the various overlay plates were incubated at room temperature under different gas phases. Gas phases chosen were (i) atmospheric conditions (21% O<sub>2</sub>, 0.05% CO<sub>2</sub>), (ii) anaerobic conditions (0% O<sub>2</sub>, 0% CO<sub>2</sub>), (iii) microaerobic conditions containing 1% O<sub>2</sub>, 1% CO<sub>2</sub> (gas mixture provided by PRAXAIR), and (iv) 2% O<sub>2</sub>, 5% CO<sub>2</sub> (adjusted by mass-flow regulators). 16 equal-sized small colonies were picked every week and streaked out on new overlay plates. Afterwards the plates were incubated under atmospheric conditions at room temperature. After about 10 days growth of colonies was evaluated to verify the survival under the various gas phases.

## Result and Discussion

Members of the genus “*Ferrovum*” have previously been shown to be difficult to sub cultivate for long periods and some strains have been lost (Johnson et al., 2014; Ullrich et al., 2016). Acidophilic bacteria in general face the problem that after use of the substrate and stop of respiration the proton gradient across the membrane is endangered by high external bacterial proton concentrations. Autotrophic acidophilic iron oxidizers in addition face the problem that on the one hand little energy is available from the oxidation of iron, but that on the other hand a lot of energy is needed to reduce CO<sub>2</sub> to generate biomass. In this paper we consider oxidative stress generated by reactive oxygen species as another reason for poor culturability of “*Ferrovum*”.

From the plates containing JA12 under different atmospheric conditions every week material from 16 previously untouched colonies was streaked out on new plates. The new plates were incubated under normal aerobic condition for ca. 10 days. Then it was recorded in how many cases of the 16 tests growth on the new plates had occurred. Growth on the new plates showed that at least some cells had survived on the original plates under the respective atmospheric conditions, while absence of growth suggested that many cells had lost the ability to divide (and therefore were considered “dead”). Thus, from the growth or the lack of growth on the new plates a “survival ratio” was determined for each atmospheric condition and each incubation period of the original plates (data between 16/16=1 and 0/16=0).

It was observed that especially under anaerobic conditions “*Ferrofum*” sp. JA12 lost its ability to divide pretty fast i.e. with 4 weeks (Fig. 1). For the colonies incubated under normal aerobic conditions, in contrast, it took about 9 weeks until none of the restreaked colonies resulted in any growth. In colonies kept under 1% O<sub>2</sub>, 1% CO<sub>2</sub> or 2% O<sub>2</sub>, 5% CO<sub>2</sub> at least some cells could survive until the 11<sup>th</sup> or 12<sup>th</sup> week (Fig. 1) and thus survived two or three weeks longer than those under normal atmospheric oxygen concentration (21%). By repeating the experiment six times basically similar results as those shown in Fig. 1 were obtained. Thus, microaerobic condition and/or increased CO<sub>2</sub> concentration seems to be favorable for strain JA12. However, it still needs to be elucidated which effect (decreased O<sub>2</sub> or elevated CO<sub>2</sub>) is responsible for the longer survival.

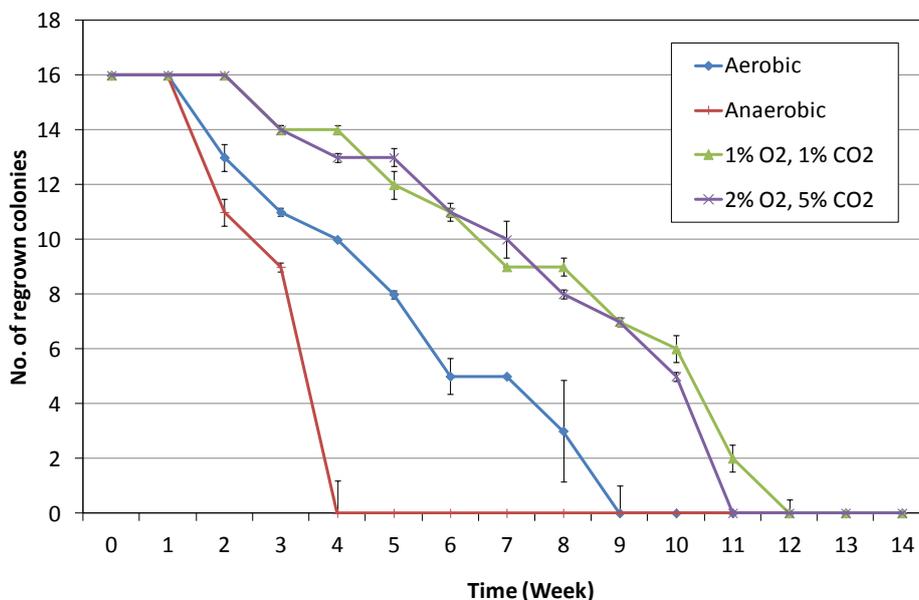


Figure 1 Effect of different gas phases on the survival of the “*Ferrofum*”-containing culture JA12.

Plates with *Ferrofum* sp. JA12 were incubated under various gas conditions. After the respective time, material from colonies was streaked out on new plates and the number of plates which showed growth was assessed after few weeks.

**Conclusions**

The experiment described here shows that the survival of “*Ferrofum*” sp. JA12 on plates strongly depends on the gas atmosphere. Microaerobic conditions or increased CO<sub>2</sub> concentration seem to have a favorable effect on survival. Accordingly, future studies will be focused on more details on the individual effect of O<sub>2</sub> or CO<sub>2</sub> each in separate experiments.

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