# PHYSICOCHEMICAL AND MICROBIOLOGICAL STRATIFICATION OF A MEROMICTIC, ACIDIC MINE PIT LAKE (SAN TELMO, IBERIAN PYRITE BELT)

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## Abstract
The 11 year-old, acidic pit lake formed in San Telmo mine shows at present a permanent stratification, with two layers of distinct aqueous composition and redox conditions which are separated by a chemocline at a depth of 29 m. The upper layer is physically lighter, has a lower content of dissolved solids, and shows oxygenic conditions and mostly oxidised dissolved Fe. The lower layer is denser, shows a higher content of sulphate and metals, and is completely anoxic, with Fe(II)>Fe(III). The microbial habitat of the pit lake is also stratified, with a general pattern of decreasing biomass (~$10^8$ cells $mL^{-1}$ at the water surface, and ~$10^6$ cells $mL^{-1}$ at depth) and increasing biodiversity of extremophilic microorganisms with depth.

## Introduction
In 1989, and after decades of exploitation for the recovery of metals (Cu, Zn, Pb, Ag, Au) and S from the massive sulphide mineralizations during the XX century, the large San Telmo mine pit (Iberian Pyrite Belt, Huelva, SW Spain), with final dimensions of 580 m × 375 m × 100 m, was abandoned, and the mine workings and pumping operations were stopped. An important inflow of acid mine drainage and groundwater to the pit (at an approximate rate of around 3,600 $m^3$/day) provoked the progressive flooding of the mine void between 1989 and 1995. As the water rose, it reacted with pyrite and the rest of sulphides present in the pit walls, thus gaining large quantities of acidity, sulphate and metals. At present, the pit lake formed in San Telmo mine is highly acidic (pH 2.7-2.8) and contains high concentrations of dissolved solids. Further, with a surface area of 143,600 $m^2$, a maximum depth of 100 m, and a volume estimated to be around 7-8 $Hm^3$, the San Telmo pit lake represents the largest volume of standing acidic water in the Iberian Pyrite Belt.
These physical and chemical characteristics make the San Telmo pit lake an attractive and extreme aqueous environment in which to perform detailed research from several scientific perspectives (physical limnology, hydrogeochemistry, extremophilic microbiology), but also a suitable, large-scale model for the interpretation of the interactions between acidophilic microbes and their aqueous environment, specially concerning the Fe and S cycles in acidic pit lake systems. This work describes preliminary results obtained during three sampling campaigns carried out between 2005 and 2006. These initial studies were aimed at unravelling possible physical, chemical and microbiological vertical gradients in the water column of the pit lake.

## Methods
The field measurements and water sampling were carried out on December 2005, March 2006 and July 2006. Vertical profiles of pH, Eh, temperature (T), dissolved $O_2$ (DO), and electric conductivity (EC) were performed with a Hydrolab® Quanta multi-parametric probe. Quantitative measurements of Fe(II)/Fe(III) concentrations were performed on site using a digital titration method (HACH Instruments company). Water samples were taken at different depths with an opaque, 2.2 L-capacity, BetaPlus® PVC bottle (Wildlife Supply Company). All samples were filtered on site with 0.45 μm membrane filters (Millipore), stored in 125 ml-polyethylene bottles, acidified and refrigerated at 4ºC during transport. Water samples were analyzed by AAS for Na, K, Mg, Ca, Fe, Cu, Mn, Zn and Al, ICP-AES for Ni, and ICP-MS for As, Cd, Co, Cr, P, Pb, Th, U and Y.
In addition, aliquot samples were taken for the identification and quantification of the microbial community in the water column. These samples were fixed on site in plastic tubes with formaldehyde 5%, and subsequently studied in the laboratory by Catalyzed Reporter Deposition Fluorescence in situ Hybridization (CARD-FISH). Hybridization and microscopy counting of hybridized and 4',6'-diamidino-2-phenylindole (DAPI)-stained cells were performed as described previously (Pernthaler et al., 2002). Both generic and specific probes were used for the microbiological study, including: EUB338 (domain *Bacteria*), ALF968 (*α-Proteobacteria*), ACD638 (genus *Acidiphilium*), BET42a (*β-Proteobacteria*), GAM42a (*γ-Proteobacteria*), THIO1 (genus *Acidithiobacillus*), LEP154+LEP634+LEP636 (genus *Leptospirillum*), FER656+TMP654 (*Ferroplasma* and *Thermoplasma archaea*), and NON338 (negative control probe). Probe sequences and hybridization conditions were described previously (González-Toril et al., 2006).

## Results and Discussion

### 1. Physicochemical stratification

The vertical profiles obtained from the three sampling campaigns (Figs. 1 and 2) indicate that the San Telmo pit lake is clearly stratified throughout the entire year. The lake shows a vertical distribution with a well defined layer boundary at around 29 m depth, which separates an upper, oxygenated and lower conductivity layer (called *mixolimnion*), from a lower, anoxic and higher conductivity layer (called *monimolimnion*). This lake is meromictic in nature, as the boundary layer at 29 m is permanent and stable all the year round. The transition between the mixolimnion and the monimolimnion represents a chemocline in which significant compositional and redox changes take place. The internal configuration is homogeneous in both layers, although the mixolimnion is temporally stratified from early spring to late autumn, when a warmer and lighter *epilimnion* is formed in the upper 2 meters of the water column, overlying an intermediate, transition layer (*metalimnion*) between 2 m and 5 m depth, and a lower and cooler *hypolimnion* between 5 m and 29 m depth (Fig. 1).

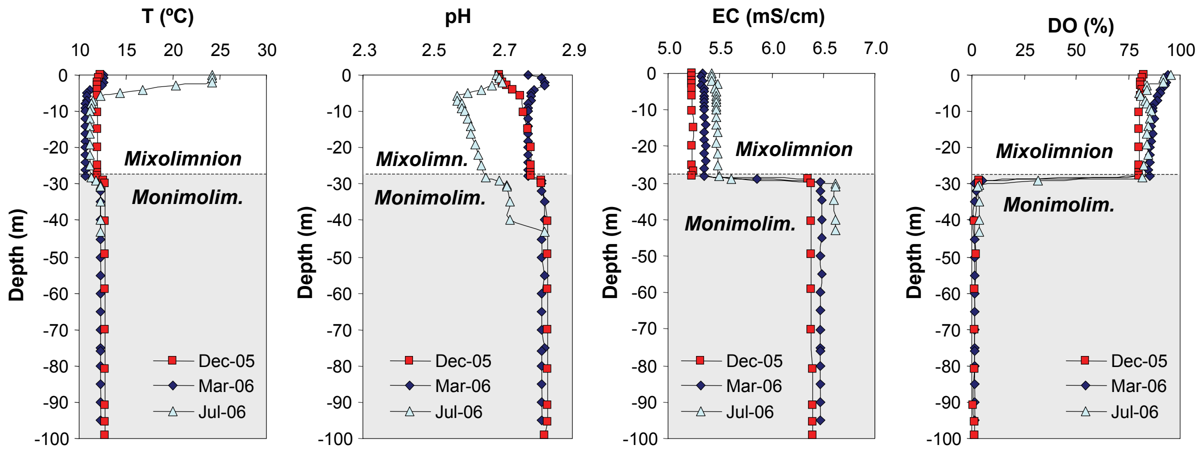


**Figure 1. Vertical profiles showing the variation of temperature (T), pH, electric conductivity (EC) and dissolved oxygen (DO) content with depth in the San Telmo pit lake on several seasons.**

The uppermost layers are either in direct contact with (epilimnion) or under influence of (metalimnion) atmospheric oxygen and solar radiation, also receiving variable water inputs of either direct rainfall and/or runoff from the pit walls and surrounding waste piles, which usually consist in highly acidic and metal-rich drainage waters with abundant dissolved and particulated Fe(III) (Table 1). Therefore, both layers are influenced by a number of physical and geochemical processes (e.g., cooling/warming depending on the season, followed by stages of mixing and/or stratification, evapoconcentration at near-surface conditions, photosynthetic production of oxygen by algae, dissolution of incoming Fe(III) colloids, precipitation of Fe(III) phases, photoreduction of dissolved and/or particulated Fe(III), bacterial re-oxidation of Fe(II), etc.) which may temporally alter factors like T, pH, DO or Fe(II)/Fe(III) ratio (Diez-Ercilla et al., 2006; Sánchez-España et al., *in press*).

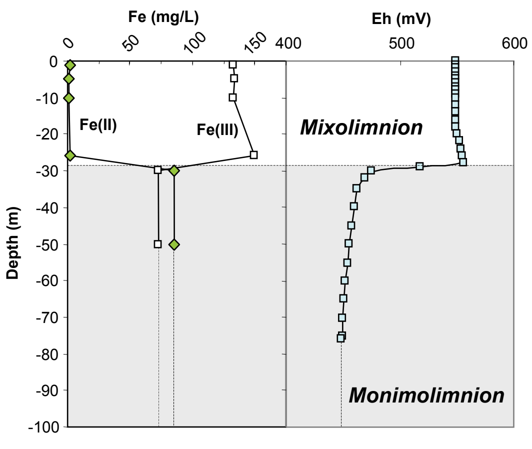


**Figure 2. Vertical profiles of Fe(II), Fe(III) and Eh in the San Telmo pit lake (March 2006).**

**Table 1. Water chemistry of the AMD inflow to the San Telmo pit lake (March 2006).**

| Q | pH | Eh | CE | OD | Na | K | Mg | Ca | Fe(II) | Fe(III) | $SO_4$ | Al | Mn | Cu | Zn | Ni | As | Cd | Co | Cr | Pb | Th | U |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *L/s* | | *mV* | *mS/cm* | *mg/L* | *mg/L* | *mg/L* | *mg/L* | *mg/L* | *mg/L* | *mg/L* | *g/L* | *mg/L* | *mg/L* | *mg/L* | *mg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* |
| 2.8 | 3.17 | 403 | 7.49 | 8.8 | 22.2 | 3.4 | 791 | 246 | 152 | 40 | 5.8 | 234 | 72.4 | 22.5 | 129 | 736 | 32 | 293 | 1,750 | 33.4 | 41.9 | 3.45 | 35.5 |

The bottom of the mixolimnion (hypolimnion) is slightly cooler than the monimolimnion, but this slight density difference provoked by the thermic gradient (around 1.5ºC, equivalent to a density difference of around 0.0003 g/cm$^3$) is not important enough as to compensate the density contrast introduced by the difference in dissolved solids concentration as indicated by the electric conductivity (around 1 mS/cm, equivalent to a density difference of around 0.001 g/cm$^3$). Consequently, the chemocline represents a permanent physicochemical barrier which impedes mixing of both layers. This fact strongly determines the redox conditions at both sides of the chemocline, with the upper mixolimnion showing higher redox potential (~550 mV) and dissolved iron chiefly in its oxidised form (Fe(III)>95% $Fe_{total}$), and the lower monimolimnion exhibiting more reduced conditions (Eh~450 mV) and a more significant presence of ferrous iron (Fe(II)>55-60% $Fe_{total}$; Fig. 2). Such distribution of the Fe(II) to Fe(III) ratio is also homogeneous along the 71 m deep-monimolimnetic layer and is independent of the season of the year, suggesting some kind of redox equilibrium.

As regards to the major ion and trace element content of the pit lake, a clear difference between the mixolimnetic and the monimolimnetic layer exists, as shown by the obtained profiles (Table 2, Fig. 3). Most elements (including sulphate as major anion, Na, K, Mg and Ca as alkaline and alkaline earth metals, in addition to Fe, Al, Mn, Cu and Zn as major metals, and Cd, Ni, Co, P and Y as relevant trace elements) behave in a similar manner as does the electric conductivity, with significant increases of their respective concentrations (e.g., 15% for Fe and Al, 33% for Cu and Zn, 55% for Ca, etc.) when passing from the upper to the lower layer.

However, the opposite trend is shown by some other elements such as As and Pb, which exhibit a higher concentration in the mixolimnetic layer (Fig. 3). The distribution of these two elements in the AMD waters of the Iberian Pyrite Belt (IPB) has been shown to be closely associated with sorption processes on the mineral surfaces of nanocristalline particles of Fe(III) hydroxysulphates such as schwertmannite, which usually act as efficient adsorbant(s) at low pH (Sánchez-España et al., 2006). In consequence, the observation that both elements display a gradual increase towards the water surface suggest that such relative enrichment could be intimately associated with the presence of Fe(III) colloids (<0.45 µm) in the water column (more abundant near the water surface, where the oxidation and the precipitation reactions are more widespread than at depth), although this hypothesis deserves further demonstration. The higher As and Pb contents at the surface could be alternatively explained by a combination of (i) influence of the AMD inflow to the pit lake (Table 1), which contributes with dissolved and particulated Fe(III), As and Pb, and (ii) reconcentration by evaporative processes.

**Table 2. Water chemistry of the San Telmo pit lake at different depths (December 2005 and March 2006).**

***December 2005***

| Depth | Na | K | Mg | Ca | Fe | $SO_4$ | Al | Mn | Cu | Zn | As | Cd | Co | Cr | Ni | P | Pb | Th | U | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *m* | *mg/L* | *mg/L* | *mg/L* | *mg/L* | *mg/L* | *g/L* | *mg/L* | *mg/L* | *mg/L* | *mg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* |
| *0* | 17 | 2.15 | 460 | 225 | 158 | 3.97 | 142 | 48 | 25 | 89 | 92 | 180 | 922 | 63 | 458 | 408 | 38 | 6 | 20 | 320 |
| *-10* | 17 | 1.84 | 451 | 222 | 152 | 4.00 | 142 | 46 | 23 | 89 | 76 | 172 | 917 | 62 | 475 | 440 | 36 | 8 | 19 | 327 |
| *-25* | 17 | 1.87 | 450 | 219 | 154 | 3.75 | 137 | 46 | 24 | 89 | 65 | 173 | 911 | 59 | 469 | 395 | 38 | 5 | 19 | 326 |
| *-35* | 24 | 0.97 | 612 | 346 | 174 | 4.88 | 156 | 68 | 30 | 117 | 39 | 225 | 2,357 | 55 | 649 | 492 | 10 | 14 | 26 | 474 |
| *-55* | 24 | 0.97 | 605 | 342 | 178 | 4.84 | 156 | 67 | 30 | 118 | 35 | 232 | 2,408 | 65 | 632 | 501 | 13 | 7 | 26 | 459 |
| *-75* | 24 | 0.97 | 591 | 340 | 175 | 5.01 | 161 | 68 | 30 | 117 | 36 | 243 | 1,307 | 66 | 652 | 531 | 13 | 7 | 25 | 470 |

***March 2006***

| Depth | Na | K | Mg | Ca | Fe | $SO_4$ | Al | Mn | Cu | Zn | As | Cd | Co | Cr | Ni | P | Pb | Th | U | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *m* | *mg/L* | *mg/L* | *mg/L* | *mg/L* | *mg/L* | *g/L* | *mg/L* | *mg/L* | *mg/L* | *mg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* | *µg/L* |
| -1 | 22 | 2.00 | 508 | 223 | 153 | 3.91 | 138 | 44 | 23 | 89 | 89 | 196 | 1,205 | 29 | 512 | 654 | 68 | 7 | 22 | 314 |
| -5 | 20 | 1.98 | 494 | 220 | 153 | 3.57 | 138 | 44 | 23 | 87 | 123 | 192 | 1,171 | 29 | 501 | 532 | 58 | 7 | 22 | 301 |
| -10 | 24 | 1.95 | 494 | 212 | 154 | 3.88 | 137 | 44 | 23 | 88 | 140 | 194 | 1,149 | 28 | 496 | 610 | 56 | 7 | 23 | 304 |
| -26 | 21 | 2.04 | 490 | 219 | 156 | 3.87 | 136 | 43 | 23 | 88 | 74 | 192 | 1,132 | 28 | 488 | 569 | 56 | 7 | 23 | 301 |
| -30 | 26 | 1.03 | 622 | 337 | 167 | 4.63 | 145 | 64 | 29 | 114 | 90 | 245 | 1,640 | 31 | 675 | 878 | 21 | 10 | 35 | 435 |
| -50 | 26 | 1.09 | 622 | 340 | 166 | 4.6 | 144 | 64 | 30 | 114 | 66 | 245 | 1,595 | 30 | 644 | 732 | 22 | 10 | 34 | 416 |

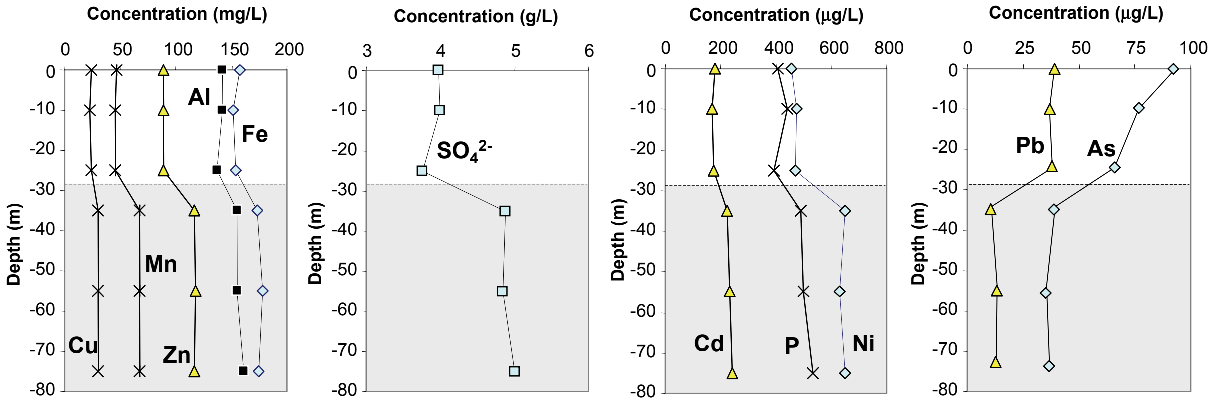


**Figure 3. Vertical profiles showing the variation in the concentration of major metals, trace elements and sulphate with depth in the San Telmo pit lake (Data from December 2005).**

Calculation of the saturation index (SI) for selected Fe(III) and Al minerals (carried out with the Phreeqc 2.7 geochemical code; IGME, 2006, *unpublished report*) indicates that the San Telmo pit lake is invariably saturated with respect to a number of Fe(III) phases such as schwertmannite (SI~14-17), K-jarosite (SI~8-10), Na-jarosite (SI~5-7) and goethite (SI~2-3) along the entire water column, although the calculated SI's for the mixolimnion are statistically higher than those of the monimolimnion. These mineral phases could be playing a role in the control of the aqueous/solid phase distribution of Fe(III) in the water column and at the water/sediment interface, although at the measured conditions of pH-Eh, schwertmannite is the most common precipitating mineral (Sánchez-España et al., 2006 and *in press*). With respect to the Al phases, the water shows undersaturation (and therefore tendency for dissolution) in the more common minerals in AMD systems (e.g., basaluminite, alunite, gibbsite; SI~-1 to -12), although jurbanite is near equilibrium. Finally, the pit lake seems to be under equilibrium with respect to gypsum, which commonly controls the aqueous distribution of Ca.

## 2. Microbiological stratification

From a microbial perspective, the acidic pit lake of San Telmo is also characterized by three different layers which correlate excellently with the vertical distribution shown by the physicochemical parameters (Table 3). Thus, the first 5 m of the water column, which correspond to the upper and warmer epilimnion and metalimnion, are characterized by a high biomass (~$10^8$ cells $mL^{-1}$), and a low biodiversity which is mainly characterized by α-proteobacteria (~78%). The second layer, comprised between 5 m and 26 m and corresponding to the rest of the mixolimnion, is characterized by a considerably lower biomass (~$10^6$ cells $mL^{-1}$) but a higher biodiversity in which, in addition to *α-proteobacteria* (~47%), both *γ-proteobacteria* (8-15%) and *Leptospirillum* (17-22%) are also common, and small numbers of archaea (~3%) have been also detected. Finally, a third layer representing the deeper and anoxic monimolimnion is recognized, in which both the biomass (~$3.7\times10^6$ cells $mL^{-1}$) and the biodiversity increase again with respect to the lower mixolimnion.

**Table 3. Bacterial cells identified at different depths in the San Telmo pit lake (Data from March 2006). Total DAPI counts (cell $mL^{-1}$) and fraction (%) of cells detected with the different probes.**

| | Total cells ($10^6$) | % EUB338 | % ALF968 | % ACD638 | % BET42a | % GAM42a | % THIO 1 | %LEP154 + LEP634 + LEP636 | % Total bacteria | % FER656 + TMO654 |
|---|---|---|---|---|---|---|---|---|---|---|
| - 1m | 109.4 | 88.0 | 76.7 | 0.00 | BDL[1] | < 1% | BDL | < 1% | 76.7 | BDL |
| - 5m | 97.0 | 87.9 | 78 | - | BDL | BDL | BDL | < 1% | 78 | BDL |
| - 10m | 1.9 | 54.4 | 47.1 | BDL | 0.8 | 8.5 | < 1% | 17.7 | 74.1 | 3.16 |
| - 26m | 0.8 | 67.5 | 47.3 | 1.7 | BDL | 15.4 | 0.70 | 22.1 | 86 | < 1% |
| - 30m | 3.7 | 53.1 | 6.2 | - | 6.7 | 1.9 | BDL | 1.6 | 16.3 | < 1% |
| - 50m | 3.5 | 52.1 | 5.0 | - | 1.7 | 0.9 | BDL | 1.8 | 9.5 | BDL |

[1] BDL= Below Detection Limit

The described vertical zonation of the microbial habitat in the San Telmo pit lake is in agreement with that described for the majority of thermally stratified lakes, in which bacterial biomass and productivity are commonly highest in the epilimnion, decrease to a minimum in the metalimnion and upper hypolimnion, and increase in the lower hypolimnion (Wetzel, 2001). Further, the notably higher numbers of bacterial cells detected near the surface with respect to those observed at depth correspond to a pattern which has already been described in comparable acidic mine pit lakes like the Berkeley pit, Montana, USA (University of Montana, *unpublished report*) and even in acidic mine drainage pools of much smaller scale (e.g., Malki et al., 2006). However, it is worth noting that the observed bacterial biomass near the surface is notably higher than those reported in other acidic pit lakes like Berkeley (~$10^5$ cells $mL^{-1}$; University of Montana, *unpublished report*), in natural lakes of Germany (~$10^5$ cells $mL^{-1}$; Wetzel, 2001), or in the adjacent and highly acidic Tinto river system (mainly $10^5$ to $10^7$ cells $mL^{-1}$; González-Toril et al., 2003). Growth of microorganisms is controlled by nutrient availability and environmental factors such as temperature, and is closely correlated with primary production (photosynthetic and chemolithotrophic; Wetzel, 2001; González-Toril et al., 2003). Therefore, the high density of living bacteria in the upper 5 meters of the water column is coherent with recent studies carried out in San Telmo (Diez-Ercilla et al., 2006) which showed that UV-A radiation is fastly attenuated at depth (first 3-4 cm of the water column) whereas the photosynthetically active radiation (PAR) can penetrate up to depths of around 5 meters. Therefore, it is hypothesized that a close correlation exists in San Telmo between solar radiation, photosynthetic activity of phytoplankton (i.e., oxygen and nutrient availability) and numbers and diversity of bacterial cells.
Additionally, a number of questions are still to be addressed, as for example, the distribution of *Leptospirillum* (restricted to the lower mixolimnion), or the exact nature of the interactions between microorganisms and the aqueous environment, specially concerning the Fe and S cycles in the water column and at the water-sediment interface (oxidation of Fe(II) by iron-oxidising bacteria under oxic and/or anoxic conditions, possible reduction of Fe(III) and $SO_4$ compounds by Fe(III)- and sulphate-reducing bacteria, etc.).

## Conclusions

The present work demonstrates that a combination of different physical and geochemical processes accounted during the flooding phase of a pit lake (including entrance of groundwater, run-off and rainfall, oxidative dissolution of pyrite and other sulphides, dissolution of secondary sulphate salts, etc.) can lead to strong density gradients which may eventually result in a permanent and stable stratification of the water column. In the case of the young San Telmo pit lake, this stratification is observable not only from a merely hydrochemical viewpoint, but also from a microbiological perspective. Both the density and diversity of the bacterial communities has been shown to vary with depth, as a response to the changing physico-chemical conditions prevailing at each layer.

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