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Abstract

To date, most of the enrichment methods aiming at the growth of an efficient electroactive biofilm are based on the hypothesis that anode respiring bacteria (ARB) are mostly dissimilatory metal-reducing bacteria (DMRB). Thus, this work evaluated the use of a culture enriched in DMRB as a strategy to enrich ARB on anodes. DMRB were enriched with Fe(III) as final electron acceptor and then transferred to a potentiostatically-controlled system with an anode as sole final electron acceptor. Three successive iron-enrichment cultures were carried out. The first step of enrichment revealed a successful selection of the high current-producing ARB *Geoalkalibacter subterraneus*. After few successive enrichment steps, the microbial community analysis in electroactive biofilms showed a significant divergence with an impact on the biofilm electroactivity. Enrichment of ARB in electroactive biofilms through the pre-selection of DMRB should therefore be carefully considered.

Keywords:

Microbial bioelectrochemical systems; Dissimilatory metal-reducing bacteria; Anode respiring bacteria; *Geoalkalibacter subterraneus*.

1 Introduction

Bioelectrochemical systems (BES) such as microbial fuel cells (MFC), microbial electrolysis cells (MEC) or, more recently, microbial electrosynthesis (MES) reactors rely on the ability of electroactive bacteria to transfer/accept electrons to/from a solid external electron acceptor/donor such as an anode or a cathode, respectively. The transferred electrical current can be used for the direct production of electricity in MFCs or the production of valuable molecules via MES.

In previous studies, it has been demonstrated that ARB can be enriched from many different environmental sources such as freshwater, marine sediments, salt marshes, anaerobic sludge, wastewater treatment plant sludge and mangrove swamp sediments (Miceli et al., 2012). In natural environments such as aquatic sediments, submerged soils and the terrestrial subsurface, dissimilatory metal-reducing bacteria (DMRB) use a variety of extracellular electron acceptors like iron and manganese (Lovley, 1993). Interestingly, many DMRB are as well capable of using a conductive material as final electron acceptor. Thus, such ability in DMRB has opened a window of opportunities in the specific enrichment of ARB (Wang et al., 2010).

Different enrichment techniques have been used to form high current-producing biofilms such as the 1) use of effluents from continuously operated BESs (Sleutels et al., 2011), 2) scraping and dispersion of an already well formed electroactive biofilm (Liu et al., 2008) and 3) placement of electrodes with developed electroactive biofilms in the vicinity of a new electrode material (Kim et al., 2005). Although the previous enrichment of DMRB as inoculum for BES has been occasionally tested, the lack of information regarding the analysis

of the microbial communities prohibited a detailed discussion (Kim et al., 2005; Wang et al., 2010).

A recent study by Feng et al. (2012) has reported the improvement of current densities by pure cultures of ARB within the *Shewanellaceae* family when such cultures have been ammended with iron. On the other hand, using mixed cultures as original inoculum, only a few studies have focused on enriching DMRB bacteria in order to get a stable and efficient biofilm consortium mainly composed of ARB.

Zhang et al. (2014) enriched ARB by adding Fe(III) in the electrolyte solution of BESs experiments. This strategy permitted to i) select iron-reducing bacteria from wastewaters while anaerobically oxidizing organic matter and ii) a current production three times higher than the one obtained without the iron addition. Aditionally, Kim et al. (2005) previously tried to enrich mixed cultures in DMRB to be further used as inoculum for BES. They conducted enrichments of DMRB contained in sludge by exposing certain amount of its biomass to a ferric iron medium. However, neither the improvement in performance or the succesful enrichment of DMRB were demonstrated.

A more detailed study that reported the previous enrichment of DMRB as inoculum for BES is the work by Wang et al. (2010). They exposed an already developed electroactive biofilm to an iron-rich medium. Once iron reduction took place, they used such DMRB-enriched biomass as inoculum for BESs. As expected, the electrochemical performances were improved for the BESs inoculated with previous iron-enriched biomass (power density: 226 mW/m^2, coulombic efficiency: 34%), that was higher than the original electroactive biofilm (209 mW/m^2, 23%). Unfortunately, no details were presented regarding the microbial selection ocurred therein.

Until now, most of the ARB enrichment strategies have consisted in the use of an existing electroactive biofilm (Kim et al., 2005; Miceli et al., 2012; Wang et al., 2010) or the use of

effluent from a running BES (Sleutels et al., 2011). These techniques imply to run a BES before in order to obtain an efficient electroactive biofilm. Thus, an iron-enrichment strategy requires less energy and is a less time consuming ARB pre-selection technique.

The objective of the present work was to evaluate whether an enrichment procedure of DMRB is necessarily linked to the presence ARB in terms of electron transfer performances. An iron enrichment method was developed to specifically select DMRB capable of further colonize the electrode of a potentiostically-controlled sytem. The effect of successive iron-reducing enrichment culture stages on microbial selection and electrochemical performance of both DMRB and ARB in biofilms was then characterized. The novelty of this work is the focus on the description of microbial communities during both types of enrichments. This analysis is ignored in several studies that mainly focuse on improving the overal performance of the system, without the detailed understanding of the microbial community characterization.

2 Materials and methods

The enrichment of both dissimilatory metal-reducing bacteria (DMRB) cultures and anode-respiring bacteria (ARB) embeded within biofilms was performed under moderate halophilic conditions (35 g_{NaCl}/L) i) due to the salinity measured in the sediment sampling location (see inoculum details in supplementary material) and ii) to provide enough conductivity in BES. Sediments from a salt plant already adapted to high salt concentrations were used to i) inoculate a medium containing Fe(III) as sole electron acceptor in order to favor the selection of DMRB (E1) and ii) inoculate an electrochemical system equipped with a three-electrode setup to form an electroactive biofilm (B0) on a graphite anode (Fig. S1). The first DMRB-enrichment culture named E1 was then used as inoculum for further DMRB- (E2) and ARB-enrichments (B1). This procedure was reiterated three times in four replicates to observe the effect of the succession of enrichment culture stages on both

bioelectrochemical performance and microbial selection. Further details on the three electrode system arrangement, DMRB and ARB enrichment cultures, chemical analyses and electrochemical data analysis are presented in the Supplementary Material.

Molecular analyses of bacterial communities were performed after acetate consumption in each biofilm reactor and enrichment steps. PCR products were separated by capillary electrophoresis single-strand conformation polymorphism (CE-SSCP), previously described (Pierra et al., 2015). With this technique, DNA fragments of the same size but with different DNA compositions are separated**.** Molecular biology protocols are detailed in the Supplementary Materials.

3 Results and discussion

3.1 DMRB-enrichment cultures

3.1.1 Iron conversion

In Fig. 1A, the electron transfer from acetate to Fe(III) in DMRB-enrichments is shown as moles of iron reduced per mole of acetate. Under the tested conditions, this ratio progressively increased from 4.7 up to the stoichiometric conversion rate of 8.0 $mol_{Fe(II)}/mol_{acetate}$ in DMRB-enrichments E1 and E3, respectively. Wang et al. (2010) and Roden & Lovley (1993) reported similar maximal rates in DMRB-enrichments (about 8.0 $mol_{Fe(II)}/mol_{acetate}$). Such conversion rate is close to the maximal theoretical value of electrons transferred after complete acetate oxidation, which means that Fe(III) was the only electron acceptor available for acetate oxidation. During DMRB-enrichment steps neither biomass, liquid or gas end products were measured. Thus, it cannot be firmly excluded the existence of other metabolic pathways. The increase in acetate conversion ratio for Fe(III) reduction into Fe(II) is likely due to the progressive depletion of alternative electron acceptors that are initially present in the sediment used as inoculum.

-Please insert **Figure 1** here-

3.1.2 Microbial community analysis of enriched DMRB

A significant bacterial selection occurred along the DMRB-enrichment steps with the predominance of few bacterial species, sub-dominant in the original microbial community (Fig. S3). Their relative percentage abundance is shown in Fig. 1A. As an overall trend, *Geoalkalibacter subterraneus* (henceforth *Glk. subterraneus*) and a microorganism within the family *Geobacteraceae* were selected with a decrease of the abundance of *Clostridiaceae* species along the enrichment steps. The selection of *Glk. subterraneus* is not surprising since it is a well-known ARB (Carmona-Martínez et al., 2013; Miceli et al., 2012) and DMRB (Greene et al., 2009). This is in agreement with the expected effect of the DMRB-enrichment strategy. Moreover, *Glk. subterraneus* rather than other ARB was likely selected due to the saline conditions employed.

3.2 ARB-enrichment in anodic biofilms

3.2.1 Maximum electron transfer rate (V_{max})

V_{max} represents the maximum rate of electrons transfer from the biofilm to the anode per unit of time as described in the Supplementary Material. It is worth to notice that the parameter V_{max} is estimated from the Gompertz model for the first time to describe the electrochemical performance of anodic biofilms. Therefore, no comparison is possible for this parameter with previous studies. It can be clearly seen that V_{max} increased along the ARB-enrichment steps up to 300 C/d in B2 (Fig. 1B). However, for biofilm B3 formed from the DMRB-enrichment E3, V_{max} significantly decreased to 150 C/d. This was likely due to the selection of less number of ARB or non-ARB at all after several enrichment steps as indicated in Fig. 1. Such non-ARB might have outcompeted ARB within the biofilm for substrate

consumption. The lower V_{max} observed for E3 compared to E2 might also be due to a dilution of ARB present in the sediment throughout the DMRB-enrichment steps.

3.2.2 Coulombic efficiency (CE) as an indicator of overall performances

The fraction of electrons available in the substrate that ends up as electrical current in the system is denominated as Coulombic efficiency (CE) (Sleutels et al., 2011). CE is an indication of the actual amount of coulombs harvested from substrate oxidation and transferred by the biofilm to the anode. The increase of CE is one of the most important challenges in BESs, since most of the available electrons contained in the organic substrate are expected to be transferred to the anode to create an economically feasible process. In our study, CE gradually increased from 30±4% (B0) until it reached a plateau at ≥ 100 % (B1-B3). It is worth noticing that a single step of DMRB-enrichment and the use of its biomass as inoculum for B1 was enough to achieve a high CE.CE values >100% were very likely due to the oxidation of H_2 produced in the system at the cathode since a significant percentage of H_2 was detected in the gas phase at the maximum of current production (CO_2 8.4 %, H_2 42.12 %, N_2 33.26 % and CH_4 11.30 %). The overestimation of the electrons recovered at the anode was probably due to H_2 recycling, a well-known phenomenon occurring in single chamber BES (Lee & Rittmann, 2010). The evolution of H_2 at the cathode in membrane-less BES can thus negatively affect the evaluation of the BES's performances in terms of CE due to the presence of H_2-oxidizing ARB able to convert H_2 into electrons. Such conversion clearly produces a flow of electrons that not necessarily comes from the substrate fed to the BES which in turn increases CE values.

3.2.3 Average maximum current density

Although no increase in maximal current density (j_{max}) was observed after the first ARB-enrichment step (the highest j_{max} was obtained for B0: 4.5 A/m^2), CE increased through enrichment steps from B0 to B2 as discussed above. The lack of gradual improvement of j_{max}

might suggest the presence of essential nutrients or perhaps mediators in the sediments that were required to achieve higher j_{max} values. The removal of such nutrients could have hindered further improvement of j_{max} in B1, B2 and B3. Moreover, a diluting effect of essential requirements for proper biofilm growth or electron mediators might have occurred along the DMRB-enrichment step chain. There are similarities between this phenomenon shown with successive iron-reduced enrichment cultures and previous studies where current densities had not been recovered among successive batch cycles of the same biofilm. Kim et al. (2005) tried a similar approach to improve the performance of electroactive biofilms by inoculating electrodes with biomass previously exposed to an iron-containing medium. Interestingly, they did not observe either significant performance improvement. The latest indicates that not all the DMRB can behave as ARB for the formation of electroactive biofilms, a finding only demonstrated for a few ARB (Richter et al., 2007). Although such diluting effect is not extensively reported in the literature, we suggest that a dilution of ARB might have caused the loss of activity in the formation of an electroactive biofilm.

3.2.4 Microbial community analysis of anodic biofilms

3.2.4.1 High microbial selection in ARB-enrichments caused by previous DMRB-enrichments

Interestingly, the CE-SSCP profiles obtained for ARB-biofilms were composed of only 1 or 2 dominant species (Fig. S3 B0-B3). This finding is well in agreement with previous studies in which BES inoculated with sediments presented a high anodic microbial simplification (Pierra et al., 2015). On the contrary, several studies report a highly diverse microbial community with bacteria (ARB and non-ARB), especially for BES inoculated with non-saline sources such as domestic wastewater. The high selection observed could be the result of specific experimental conditions such as pH 7.0, temperature at 37°C, synthetic medium composition , the fixed potential, and mostly the saline conditions applied.

3.2.4.2 *Geoalkalibacter* vs. *Geobacteraceae* in ARB-enrichments

In B0 (Fig. 1C) the most dominant microorganisms colonizing the anodes were affiliated to *Desulfuromonas* spp. and *Glk. subterraneus* (ARB within the δ-*Proteobacteri*a class). From B1 to B3 *Glk. subterraneus* was selected as the dominant bacteria. This finding is consistent with previous reports where a majority of δ-*Proteobacteri*a was found in electroactive biofilms inoculated with sediments (Miceli et al., 2012, Pierra et al., 2015, refer to supplementary materials). In this study, the selection of *Glk. subterraneus* from the DMRB-enrichment E1 permitted to increase the performances of the ARB-enrichment B1 when compared to the biofilm directly developed from the initial sediment (B0) (Fig. 1). Besides, it is shown here that only one DMRB-enrichment step is required to select *Glk. subterraneus* under saline conditions. Indeed, the use of the first DMRB-enrichment as inoculum (E1) provided both an ARB enriched community B1 and a DMRB community (E2). The most abundant bacterium in B1 was 99% similar to *Glk. subterraneus* at 16S rDNA sequence level and the most abundant bacterium in E2 was an unknown species belonging to the *Geobacteraceae* family with 89% similarity to *Glk subterraneus*. This bacterium belonging to *Geobacteraceae* family presented abilities to reduce Fe(III) as many *Geobacteraceae* do. Nevertheless, it has probably a low capacity to transfer this property to graphite anode respiration, as previously described for other DMRB (Richter et al, 2007). Therefore, the emergence of this *Geobacteraceae* in the detriment of *Glk. subterraneus* can be explained due to the decrease of electroactive efficiency of biofilms after the first enrichment step.

3.2.4.3 Convergence of bacterial communities correlates with electroactive performances

Fig. 2 presents the results of a principal component analysis (PCA) showing the differences in the microbial community structures of the bacterial communities during the

successive DMRB- and ARB-enrichments. CE-SSCP profiles are compared by using the Euclidian distances between CE-SSCP patterns as detailed elsewhere (Pierra et al., 2014). According to the PCA analysis, the closer the points from each other, the more similar the microbial communities are. Both DMRB-enrichment cultures and ARB-enriched biofilms in comparison to the sediment are presented. A positive correlation between the optimum performance (Fig. 1) and the similarity between DMRB-reducing and ARB-reducing microbial communities was observed (Fig. 2). The best performing ARB-enrichment (B2) (in terms of electrons recovered as CE calculated) and its corresponding DMRB-enrichment (E2) were closely related.

One unanticipated finding was a divergence in the microbial community composition between DMRB- and ARB-enrichments especially between E3 and B3. This might suggest a difference between extracellular electron transfer mechanisms between DMRB- and ARB-enrichments. Mechanisms employed by DMRB and ARB probably required slightly different physiologies. Thus, the selection of bacteria on DMRB-enrichments surely conducted the emergence of a specific consortium specialized on Fe(III) respiration, not necessarily able to use an electrode material as final electron acceptor, as previously observed by Richter et al. (2007). Nevertheless, here one step of ARB-enrichment (B0) was enough to achieve successful substrate conversion in terms of CE (see Fig. 1).

-Please insert **Figure 2** here-

4 Conclusion

This study reported the effect of employing a previously grown culture enriched in iron-reducing microorganisms (DMRB-enrichment) as inoculum to develop electroactive biofilms (ARB-enrichment). One single step of DMRB-enrichment was enough to favor the selection of the ARB *Glk. subterraneus*. This recently characterized ARB provided interesting electroactive skills considering the saline conditions used. Then, the divergence in microbial

250 community composition, associated with a decrease of electroactive efficiency, suggests that
251 respiration on Fe(III) and on anode presumably require dissimilar skills.

252 **5 Acknowledgements**

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Figure captions

Fig. 1 - A) Relative abundance of main bacteria in the Sediment used as inoculum and DMRB-enrichments E1, E2 and E3 (columns). Conversion rate of acetate into Fe(II) in DMRB-enrichments (empty squares). B) Electron transfer dynamics for ARB-enrichments. Maximum charge production rate (empty squares) and Lag phase (full squares). C) Relative abundance of main bacteria (columns) and Coulombic efficiencies (CE in %) (full squares) . Note: Rel. abundance values are reported for those ARB found at more than 9 % in at least 1 bacterial community.

309 **Fig. 2** - Principal Component Analysis (PCA) biplot of CE-SSCP profiles. For each biofilm
310 (B0, B1, B2, B3), an average of 4 replicate profiles is presented (◊). CE-SSCP profiles of the
311 sediment originate inoculum E0 (●) and of iron-enrichments microbial communities (E1, E2
312 E3) are also presented (○). The first two principal components (Axis1 and Axis2) explained
313 61.1% of the genetic variation.

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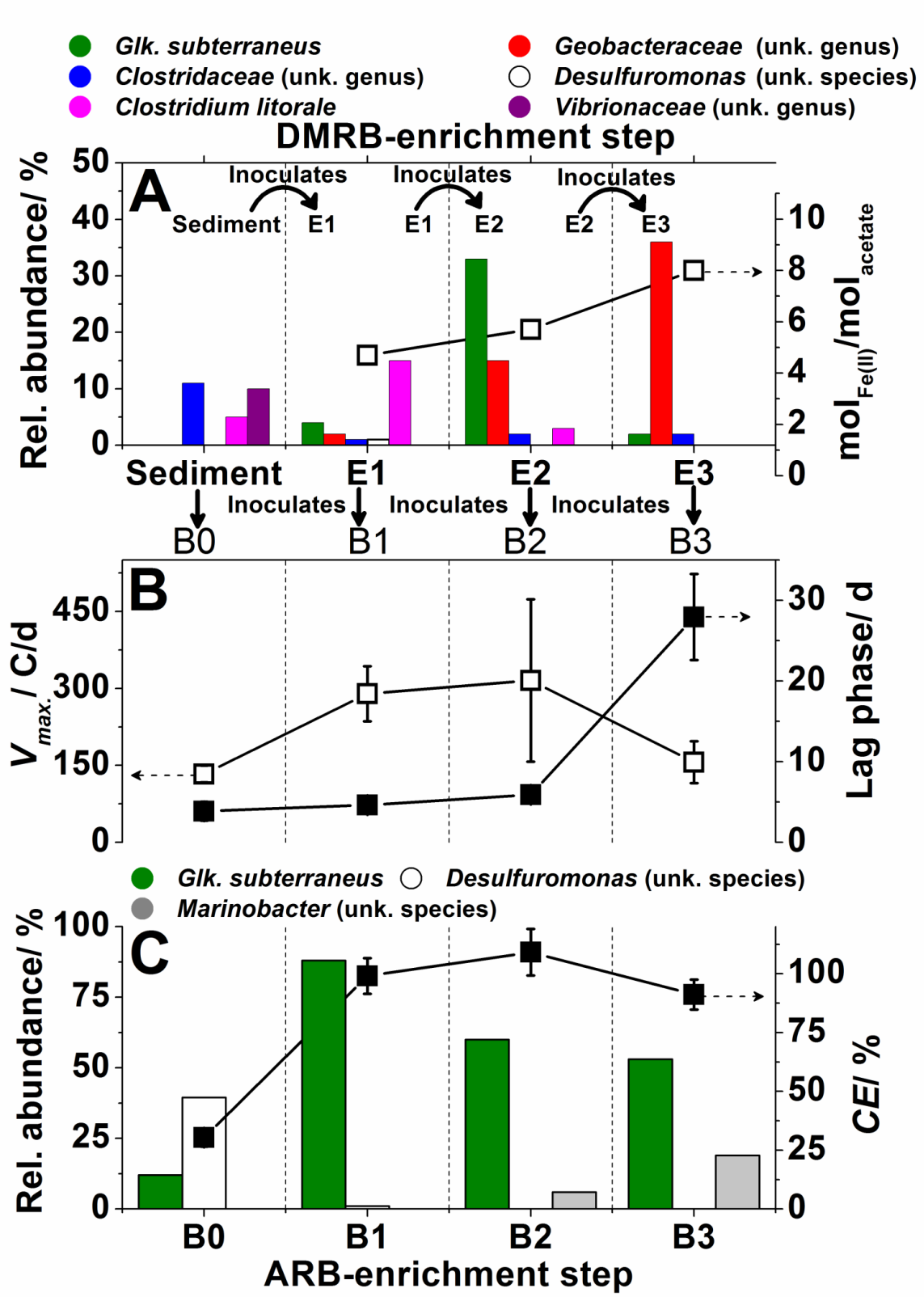
315 **Fig. 1:**



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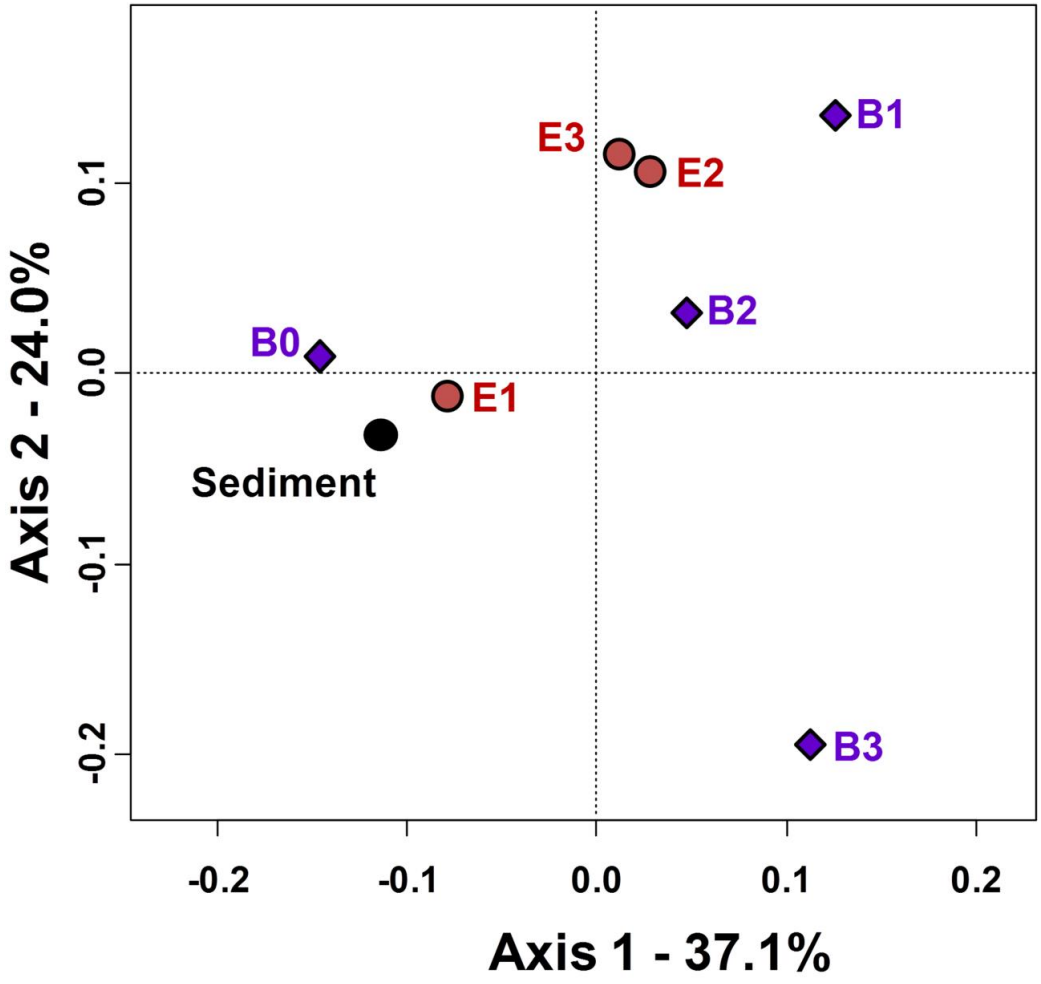
318 **Fig. 2 :**


B1
E3
E2
B2
B0
E1
Sediment
B3
0.1
0.0
-0.1
-0.2
Axis 2 - 24.0%
-0.2
-0.1
0.0
0.1
0.2
Axis 1 - 37.1%

319