SCIENTIFIC REPORT
EXCHANGE VISIT GRANT

EUROPEAN SOIL FOUNDATION
MOLTER (Natural molecular structures as drivers and traces of terrestrial C fluxes)

Reference number:	2701
Project title:	*Effect of soil management in the microcompartmentation patterns of stabilized C and N forms in soils*
Duration:	4 weeks
Starting date:	23/01/2010
Applicant's name:	Dr. Ana PIEDRA BUENA DÍAZ IRN, CCMA-CSIC, Madrid, Spain
Host researcher/institute:	Dr. Marie-France DIGNAC BioEMCo, Thiverval-Grignon (France)
	Dr. Cornelia RUMPEL BioEMCo, Thiverval-Grignon (France)

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1. Purpose of the stay

The objective of the proposed work was to combine the high specialization of the host research team in the study of soil organic matter (SOM) functions and dynamics and the candidate's experience on the proposed analytical techniques to develop an innovative approach for the study of SOM stabilisation mechanisms. In particular, the activity consisted of the molecular characterization of the lipid fraction of soil organic matter (SOM) in undisturbed and cultivated soils, in order to determine its speciation within the different aggregate sizes, and to assess its stabilisation degree and preservation mechanisms.

The project focused on the study of the lipid patterns obtained by GC/MS and GC/FID, with particular attention to the prevailing chain lengths and carbon preference indices (CPI) of alkanes and fatty acids, as well as to the identification of terpenoids and steroids. Studies supplying additional information on SOM turnover and stabilisation into the soil, such as elementary analyses and isotopic characterization ($\delta^{13}C$) were also included.

The soil lipid fraction was selected because of the biomarker value (Simoneit and Mazurek, 1982; Tissot and Welte, 1984; Killops and Killops, 1993; Zancada et al., 2004), but also due to the increasing interest of studying the aliphatic soil fraction. Whereas studies on SOM have been traditionally addressed in terms of C mineralization, hence related to C short-term turnover, recent research is mainly focusing on C stabilisation processes (Poirier et al., 2006; Rasse et al., 2005, 2006), which in fact seeem to be more related to long-term soil C storage. Hence, the classical assumption that intrinsic chemical recalcitrance of certain organic compounds, such as lignins, was strongly related to SOM stabilisation is now being substituted by a long-term, wider approach. This new approach takes into account the association of SOM with soil minerals, the physical and physico-chemical protection of SOM by microaggregates and the role of soil microbial communities (Rasse et al., 2005, 2006; Marschner et al., 2008;

Rumpel et al., 2010). In the particular case of soil lipids, it has been reported that cyclic lipids can condense into macromolecules or incorporate into structures such as humic substances or lipidic polymers. These condensed lipids would be preserved from microbial and enzymatic degradation within the smaller soil microaggregates, leading to their accumulation in soils under highly biodegradation-resistant forms (Amblès et al., 1991; Eglinton and Logan, 1991).

The information derived from this collaboration aims to enlarge the present knowledge on SOM dynamics, and in the type and extent of the effects of changes in soil use and management. Also, the molecular data obtained from this work could be used in further studies on C-fluxes model development.

2. Activities carried out before the stay (laboratories of the CCMA-CSIC, Madrid, Spain)

2.1. *Soil sampling*

Soil samples were collected from the upper horizon (0-30 cm) of a cultivated soil and an adjacent non cultivated site at Buenavista del Norte (Tenerife, Canary Islands, Spain; Fig. 1). Both sites had similar slope (1%) and altitude (110 m.a.s.l).

The cultivated soil had been under banana crop for more than 50 years, receiving continuous organic amendment (about 40 t year-1) with semi-composted cow or poultry manure with lignocelullosic material (straw, wood debris).

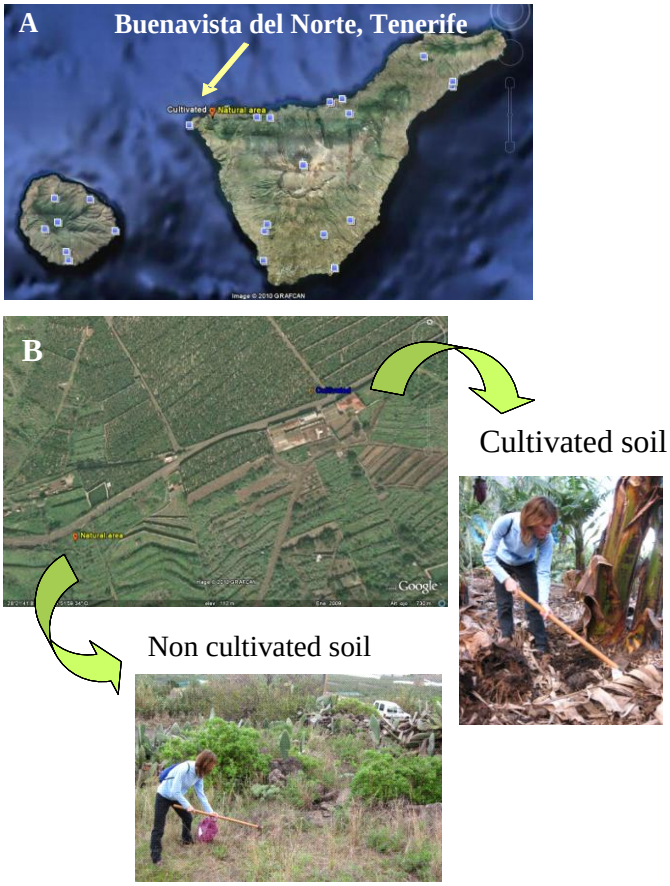

Figure 1.

Sampling sites. *A.* General localisation. *B.* Specific area

In the case of the non-cultivated site the organic matter inputs mainly derive from the characteristic vegetation of the area, consisting mainly of plants from the Asteracea and Euphorbiacea families (Table 1). This plants are considered as secondary vegetation, in substitution of the originary one represented by *Euphorbia balsamifera* and *E. canariensis.*

Table 1
Vegetation at the non-cultivated site: Buenavista del Norte, Tenerife, Canary Islands (Spain)

Family	Genus and species
Asteraceae	*Artemisa thuscula* *Kleinia neriifolia* *Launaea arborescens*
Cactaceae	*Opuntia maxima*
Euphorbiaceae	*Euphorbia canariensis*
Leguminosae	*Bituminaria bituminosa*
Poaceae	*Hyparrhenia* sp.
Polygonaceae	*Rumex lunaria*
Rubiaceae	*Rubia fruticosa*

The soil samples were air dried and sieved to 2 mm. Fractionation by aggregate size was performed into three cathegories: 1-2 mm, 0.5-1 mm and <0,5 mm.

2.2. *Soil lipids extraction and determination of soil organic carbon*

Soil lipids were extracted with petroleum ether (40-60ºC) in a Soxhlet device and methylated with trimethylsilyldiazomethane (Aoyama and Shioiri, 1990) for the chromatographic analyses. The organic C content in the soil samples was estimated by wet chemical oxidation (Walkley and Black, 1934).

3. Activities carried out during the stay at the host institute

3.1. *Chemical characterization*

The C and N contents, as well as the isotopic composition of soils were determined with a Carlo Erba elemental C and N analyzer NA 1500 coupled to a VG Sira 10 mass spectrometer , using tyrosine (59.89%C) as standard reference.

3.2. *Chromatographic analyses*

The separation and identification of the main compound groups in the soil lipid fraction was performed by GC/MS. The studied lipid groups included alkanes, fatty acids, steroids and terpenoids, because of the biomarker value of these compounds both of their (plant or microbial) origin and of the dominant transformation processes of SOM (Wiesenberg et al., 2004). Also, they give information about preservation and/or degradation of vascular plant material in soils (Otto and Simpson, 2005) and, consequently, on the dynamics of the biogeochemical cycle.

For fatty acids and alkanes, also a quantitative analyses by GC/FID was performed, using a commercial mixture of bacterial acid methyl ester (BAME, Supelco, Sigma-Aldrich Co.) for fatty acids and a solution of C_{11}, C_{12}, C_{15} and C_{17} alkanes (undecane, dodecane, pentadecane and heptadecane) for alkanes, as standard references.

4. Main results obtained

4.1. *Aggregate distribution*

The relative amounts of the different aggregate-size categories were quite different between the studied soils (Fig. 2). A conspicuous reduction on the proportion of bigger aggregates and a consequent increase in the smaller aggregates contribution in the cultivated soil was observed. These changes in soil aggregate distribution suggest an effect of mechanical destruction of macroaggregates in the cultivated soil derived from tillage practices.

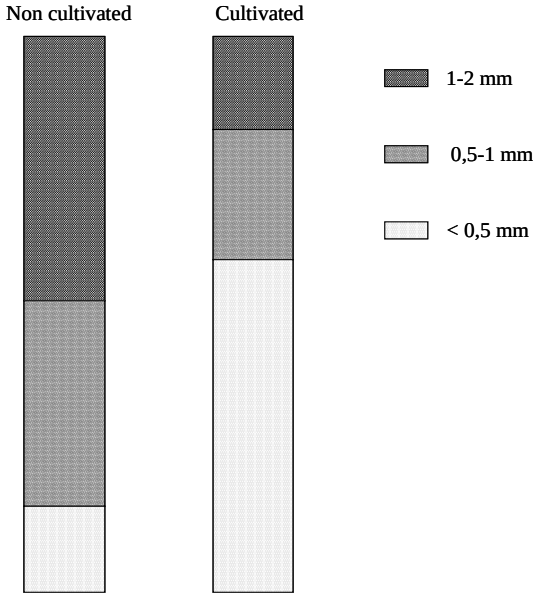

Figure 2.
Soil aggregate distribution in the studied soils.

4.2. *Chemical characterization*

The **organic C content estimated by wet chemical oxidation** was higher in the cultivated soil (Fig. 3), indicating accumulation of organic matter in the soils subjected to organic amendment, even under the subtropical climatic conditions of the sampling area, in which microbial activity is regular and constant all along the year. Concerning the aggregate size, higher C contents were found in the bigger aggregates of the non cultivated soil and in the smaller aggregates of the soil under banana crop. These results contrasted with the data obtained from the elementary analyses.

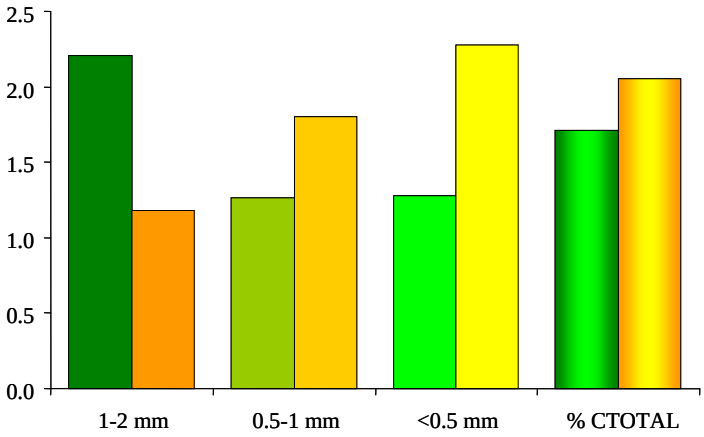

Figure 3.
Organic C content in the different aggregate-size cathegories of soil, as determined by wet chemical oxidation (Walkley and Black, 1934). Green columns: non cultivated soil; yellow columns: cultivated soil.

The **C content in the extracted lipid fra**ction was higher in the cultivated, amended soil, especially in soil aggregate fractions <1 mm (Fig. 4). These results betray the effect of manuring, although the possible contribution of waxes from banana leafs should also be taken into account. On the other hand, the effect of such an increase of the soil lipid fraction on soil physical properties such as water repellency and aggregate stability would be of interest.

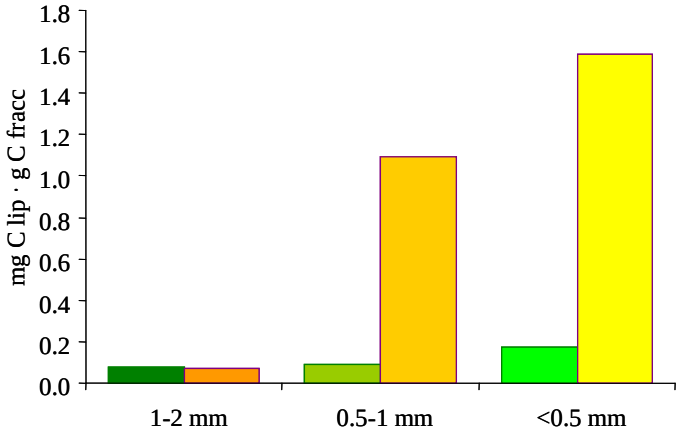

Figure 4.
Organic C content of soil lipids in the different aggregate-size cathegories as determined by wet chemical oxidation (Walkley and Black , 1934). Green columns: non cultivated soil; yellow columns: cultivated soil.

The **C content as determined by the elementary analysis** was in agreement with the results from wet chemical oxidation for bulk soils: the higher values were observed in the cultivated soil Fig. 5). However, and in contrast with the data from the classical method, the elementary analysis showed the values of C content increased in both soils as the aggregate size decreased. This preservation of lipids could be interpreted as the effect of the reduced accesibility of enzymes and microorganisms to soil organic matter in the smaller aggregates.

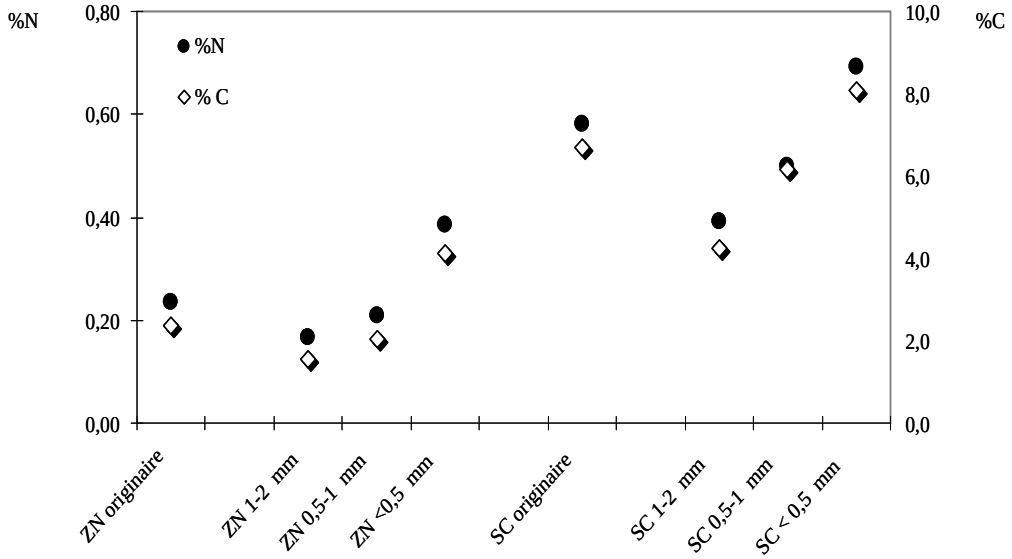

Figure 5.
Soil C and N content in the different aggregate-size categories as determined by elementary analysis. ZN: non cultivated soil; SC: cultivated soil.

The **N content** showed a similar pattern than the C content (Fig. 5), leading to **C/N ratios** quite similar for both sites (Fig. 6). This information could be of interest to adjust the amounts of N fertilizing allowed by European regulations in order to avoid the leaching of nitrates. At present, these levels have been established taking into account the results of studies carried out under environmental conditions and management practices very different than the ones in the area under study. Local, or at least regional, studies on this subject would be necessary, not only as regards the amounts but also on the N sources (synthetic vs. organic) to be considered.

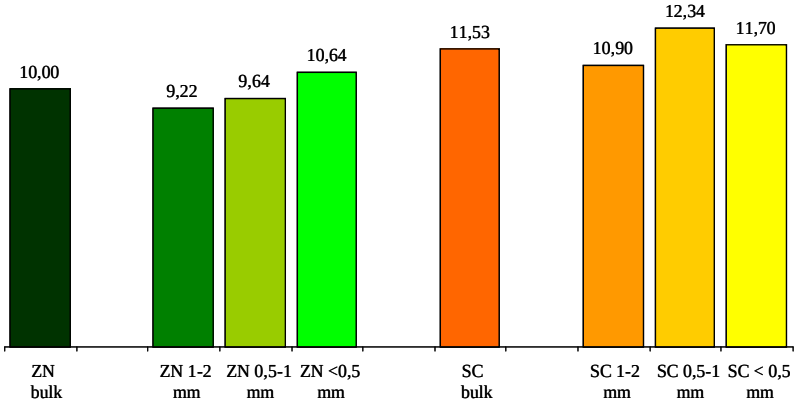

Figure 6.
Values of C/N ratio in the different aggregate-size cathegories. ZN, green: non cultivated soil; SC, yellow-orange: cultivated soil.

Concerning the calculated values of $\delta^{13}C$, it was observed that there were very similar within the different soil fractions and slightly (but statistically significant) higher in the cultivated soil compared to the non cultivated one. It is unclear the reason of this increased isotopic C ratio, but the presence of CAM plants in the non-cultivated site might have some influence. Crasulaceae (CAM plants) usually show C isotopic ratios more similar to C4 than to C3 plants, but these values are highly variable (Osmond et al., 1973; Hirst, 2010), and some authors have indicated that under subtropical temperatures such as those in the Northern region of Tenerife, CAM plants use preferably the C3 photosynthesis metabolic pathway (Deleene et al., 1985). This observation would be corroborated by the lower $\delta^{13}C$ values found in the studied non-cultivated site.

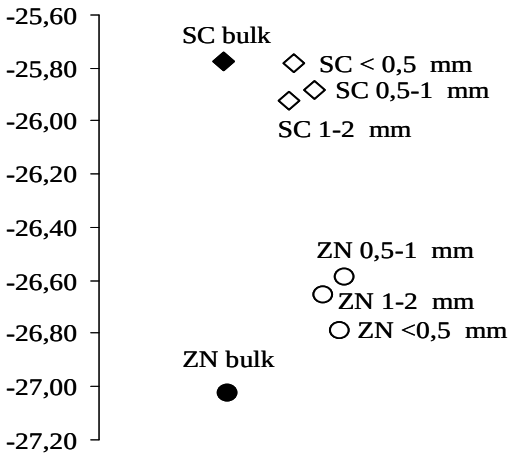

Figure 7. Values of $\delta^{13}C$ in the different aggregate-size cathegories. ZN: non cultivated soil; SC: cultivated soil.

4.3. *Chromatographic analyses*

The **fatty acids** represented 20–20.5% of the lipids identified in the mass spectra, both in number de compounds (19 of 78 of the identified compounds) and as a part of the total chromatographic area measured. The fatty acids pattern (abundances of the individual compounds) was very similar both in the cultivated and non-cultivated sites. The Carbon Preference Indices (even/odd C) indicated a marked predominance of fatty acids with even C number in both sites, with 2.86–5.75 CPI in the non-cultivated site and 7.26–9.15 CPI in the cultivated site. The Preference Indices, i.e. ratio between long chains (≥20C) and short chains (<20C), was 0.53–0.55 in the non-cultivated site and 1.03–1.36 in the cultivated site. These indices indicate that vegetation is the major source of the lipidic fraction of SOM in both sites, with some contribution of microbial metabolism in the non-cultivated site, betrayed by their lower CPI and PI values.

The **alkanes** were the group which most amounted to the lipidic fraction: whereas the number of identified alkanes corresponded to 18% of the total identified compounds, they represented about 60% of the total chromatographic area measured. The alkanes pattern showed more differences between the cultivated and non cultivated sites than that observed for fatty acids. The Carbon Preference Indices (odd/even C) indicated prevalence of alkanes with odd C number in both soils, with 3.41–4.66 CPI in the non-cultivated site and 5.73–8.00 CPI in the cultivated site. The Preference Indices (ratio ≥20C chains/<20C chains) were more indicative of the changes from the non cultivated to the cultivated site, with 1.51–2.74 and 7.97–13.45 values, respectively. In fact, in the cultivated site a dramatic depletion of the short chain alkanes (from microbial origin), and in particular of the C_{17} alkane, typical of algae and bacteria, was observed. The ratios calculated for alkanes were in agreement with those for fatty acids, indicating plants as the main contributors of soil lipids, and more microbial inputs in the non-cultivated site.

The identified **steroids** (around 5% of the lipids identified) consisted in phytosterols: campesterol (24-methylcholest-5-en-3β-ol), ergost-5-en-3-ol,(3,beta, 24R), stigmasterol and sitosterol (cholest-5-en-3-ol, 23-ethyl-(3,beta-ol)). These compounds are the most common constituents of epicuticular waxes of higher plants (Huang and Meinschein, 1979; Baker, 1982; Brassell et al., 1983; Harwood and Russell, 1984; Bianchi, 1995; Huang et al., 1995) and could also be found in soils amended with herbivorous animal manures that have been mixed with straw. In fact, the steroid distribution in soils amended with these types of manures is usually more comparable to the manure-straw mixture than to the initial soil (Ibáñez et al., 2000).

Concerning to **terpenoids** (about 15% of the lipids identified), no monoterpenes or sesquiterpenes were found, whereas the presence of diterpenes, which are typical biomarker compounds of gymnosperm vegetation (Otto and Simoneit, 2001; Otto and Wilde, 2001; Otto et al., 2003), was only represented by ferruginol methyl ether. Other diterpenes, such as abietanes, pimaranes or labdanes, were absent. However, several types of triterpenes were identified, mainly pentacyclic triterpenes of the oleane, ursane and lupane classes (Table 2), as well as their precursor molecule, squalene. These molecules are widespread and typical of angiosperm vegetation (Hill and Whitehead, 1966; Pant and Rastog, 1979; Simoneit, 1986; Ekweozor and Udo, 1988; Ekweozor and Telnaes, 1990; Woodhouse et al., 1992; Kosmowska-Ceranowicz et al., 1993; Rullkötter et al., 1994; Mahato and Sen, 1997; Jacob et al., 2005, 2007; Sonibare and Sojinu, 2009). Also, some hexacyclic triterpenes of the gammacerane class (A'-neogammacer-22(29)-en-3-ol,acetate,(3.beta,21.beta)) were found, as well as lower amounts of seco-triterpenes (methyl-roburate).

Table 2.
Pentacyclic triterpenes identified in the studied soil lipids

Class	Compound
Oleane	Beta-amyrene (olean-12-ene) Beta-amyrin (olean-12-en-3beta-ol) Beta-amyrin acetate (olean-12-en-3beta-ol,acetate) Germanicol (olean-18-en-3beta-ol) Methyl-moronate (olean-18-en-28-oic acid,3-oxo,methylester) Olean-18-en-28-oic acid,3 (acetyloxy), methylester Epifriedelinol (d:a friedooleanan-3-ol,(3.beta)) Taraxerol (d-friedoolean-14-en-ol,(3.,beta)) Taraxerol methyl ether (D-friedoolean-14-en-3-methoxy,(3.beta)) = sawamilleti Taraxerol acetate (d-friedoolean-14-en-3-ol,acetate,(3.beta))
Lupane	Lup-20(29)-en-3-one Lupeol (lup-(20,29)-en-3-ol (3.beta)) Lup-(20,21)-en-3-ol, acetate, (3.beta) Acetyl-betulinic acid (lup-20(29)-en-28-oic acid,3-(acetyloxy),(3.beta))
Ursane	Alpha-amyrin (urs-12-en-3-ol,(3.beta)) Taraxasterol (urs-20(30)-en-3-ol) Pseudotaraxasterol (urs-20-en-3-ol,(3.beta,18.alpha,19.alpha)) d:a-friedoursan-3-one I 3-keto-urs-12-ene I

Conclusions

The analysis performed showed the influence of cultivation -with high inputs of organic amendments- on the lipidic fraction of soil organic matter.

- The microbial contribution to lipid constituents was limited at both sites but slightly more pronounced in the non-cultivated site.
- The change in vegetation in the cultivated soil compared to the non-cultivated one was evidenced by the lipidic biomarkers, in particular alkanes and carboxylic acids.
- Variations in ^{13}C amounts could be attributed to the change in the plant debris ^{13}C contents in the cultivated compared to the non-cultivated site.
- The terpenoids identified in this study showed low biomarker value, due to their widespread distribution and unspecific origin, since they are frequent constituents of angiosperms.

Other comments

The data obtained in this work will be the subject of a publication involving researchers from both institutions in the next future. At present, the results are being thoroughly studied and compared to the literature, in order to better interpret the differences and similarities observed in the aggregate-size categories from each soil.

During the stay the applicant actively participated in the lab scientific activities, such as paper and work seminars. In particular, a seminar presenting the aim and preliminary results of this work was performed by the applicant in the research team meeting of 12th February.

It must be highlighted that the general organization and friendly behaviour of the group provided a context very favourable for the development of the proposed project. In this sense,

both the scientific and human aspects were developed during the stay. The applicant had the opportunity of interacting with pre-doctoral students, post-doctoral and institution researchers, technicians, engineers and administrative personnel, both from the host team and related research groups. Some of these contacts opened the possibility of future collaborations in multidisciplinary research lines.

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