Fate and Impact of Oil in Mangrove Ecosystem: PRISME Experiment.

Ronan Jézéquel[1], Karine Duboscq[1], Léa Sylvi[2], Emma Michaud[3], Lise Millera Ferriz[2], Robert Duran[4], Cristiana Cravo-Laureau[4], Valérie Michotey[2], Patricia Bonin[2], Franck Gilbert[5], Marc Tedetti[2], Cécile Militon[2], Philippe Cuny[2]

[1]CEDRE (CEntre de Documentation, de Recherche et d'Expérimentations sur les pollutions accidentelles des eaux), Brest, France

[2] MIO (Mediterranean Institute of Oceanology), OSU Institut Pytheas, UM110 CNRS IRD-AMU, Marseille, FRANCE

[3] Institut Universitaire Européen de la Mer, LEMAR UMR 6539 CNRS-IRD-UBO, Plouzané, FRANCE

[4] Université de Pau et des Pays de l'Adour, UMR CNRS 5254 IPREM – EEM. Pau, FRANCE.

[5] Université Paul Sabatier, ECOLAB UMR 5245 CNRS-UPS-INPT. Toulouse, FRANCE.

ronan.jezequel@cedre.fr

Abstract

Mangroves are among the most sensitive marine ecosystems to oil pollution due both to the oil sensitivity of mangroves species and to the high persistence of oil hydrocarbons in these environments. Despite their ecological and socio-economic value, the potential effects of an oil spill on French Guiana mangroves remain unknown so far. Yet, there is an increasing transboundary risk of oil spill due to Brazilian offshore oil exploitation (in mid-April 2013, there were 122 offshore drilling rigs in Brazil, including 29 under construction – Brazilian Amapá region located in the FG border area is thought to become an important world oil production area in the years to come). As a matter of fact, due to the intense and persistent North Brazil current that flows northwestward, oil spills originated in the Brazilian Equatorial Margin may reach the coast not only in Brazil but also along other South American countries, particularly in French Guiana coast. A set of questions then arises: How benthic communities of mangroves would cope with an oil spill? What are the bioremediation and resilience capacities of the benthic system? The objective of the PRISME project was to assess the fate of oil and its impact on benthic species during a one-month experiment performed in French Guiana both *in situ* (Sinnamary estuary) and *ex situ* (laboratory). A controlled oil spill was simulated in young mangrove sediments according to an experimental protocol based on the use of plastic corers which prevent any contamination of the surrounding environment (Cuny et al., 2014; Miralles et al., 2007). Oil hydrocarbons dynamics as well as benthic communities (micro, meio and macrobenthos) response to oil contamination were analysed at the end of the incubation. Results and knowledge gained from the experimental work will be used to develop an approach for assessing coastal vulnerability for prevention and readiness regard to oil spills.

1 Introduction

Mangrove forests, which grow at the interface between land and sea in the intertropical areas, play a fundamental role in the functioning of coastal ecosystems and provide different ecosystem services (nurseries, coastal stabilization, natural attenuation,

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biogeochemical cycling) and socio -economic values (fishing, tourism, logging, landscape) estimated at 200 000 USD/ha per year (Costanza et al. 1997). Mangroves, considered "outstanding communities" by the French National Alliance for Environmental Research (AllEnvi), cover an area of about 698 square kilometers in French Guiana (FG) representing 70% of French mangroves (4th place in South America). Mangroves of FG have, moreover, a specific dynamic compared to most other mangroves in the world. The Guiana coast is indeed a unique entity, characterized by a particularly active morpho- sedimentary dynamics which is under the direct influence of the massive suspended-sediment discharge of the Amazon river (754 106 tons yr− 1 ± 9%) (Anthony et al., 2010). This dynamic is accompanied by a heterogeneous remodeling of coastline characterized by marked deposition (formation of mud banks) but also of erosion phases affecting the coastline. It induces high and changing turbidity, also controlled by coastal and tidal currents. The advance of the Amazon freshwater plume and the freshwater inputs of Guiana rivers, coupled with coastal hydrodynamics and seasonal regimes also induce high variability of salinity of coastal waters. Because they cope with the varying salinities and thanks to fast-colonizing and fast-growing capacities, mangrove forests constitute the only adapted natural system to this unstable environment. Annually around ~300 million m3 of sediment from the Amazon are transported by ocean currents forming mud banks that migrate along the coast from east to west toward the Orinoco River. Mangrove dynamics follow the one of mud banks. Mangroves quickly colonize the banks as soon as mud is sufficiently consolidated (Gratiot et al., 2007). Five successive steps in the development and maturation of the mangroves can be distinguished: mud bank above water level, mangrove pioneer (propagules settlement), young, mature, and senescent forest (Fromard et al., 1998).

Oil spills may represent a major threat to the mangrove communities with a sensibility to oil pollution that may vary according to the development stage of mangrove. Previous studies carried out on different mangroves around the world have shown that the effects and persistence of an oil contamination on these ecosystems is extremely variable. Depending on the oil characteristics but also on the physico-chemical characteristics of the coastal area and of the mangrove communities, spilled oiled may be rapidly degraded (e.g. Burns et al., 2000; Farias et al., 2008) or, on the contrary, impact the mangrove communities for several years (e.g. Burns et al., 1993; Kingston, 2002, Baca et al., 2014). Whatever the case, due to the peculiarities of FG mangrove, from what we know so far, it is extremely difficult to predict the fate and effects that would induce a massive oil spill on the FG mangroves.

The present paper describes the protocols used during a field experiment conducted in a young mangrove of Sinnamary estuary (French Guiana). After one month in natural environment, contaminated and non-contaminated samples of sediment were collected and sent to laboratories for analyses. The objectives were to:

- Assess the natural degradation of oil through chemical analyses;
- Study the response of microbial communities to oil pollution (diversity and abundance);
- Assess the effects of oil on macrofaunal activity (bioturbation).

2 Materials and Methods

2.1 Site location

A young mangrove forest of *Avicennia germinans* located in the Sinnamary estuary was selected for PRISME experiment (Figure 1). In case of an oil spill, this successional stage of mangrove colonization is supposed to be one of the first to be impacted by oils slick

coming ashore. In addition, this site has already been studied and fully characterized in the framework of a multiyear project called BIOMANGO (2013 – 2016) (http://www-iuem.univ-brest.fr/biomango/fr).

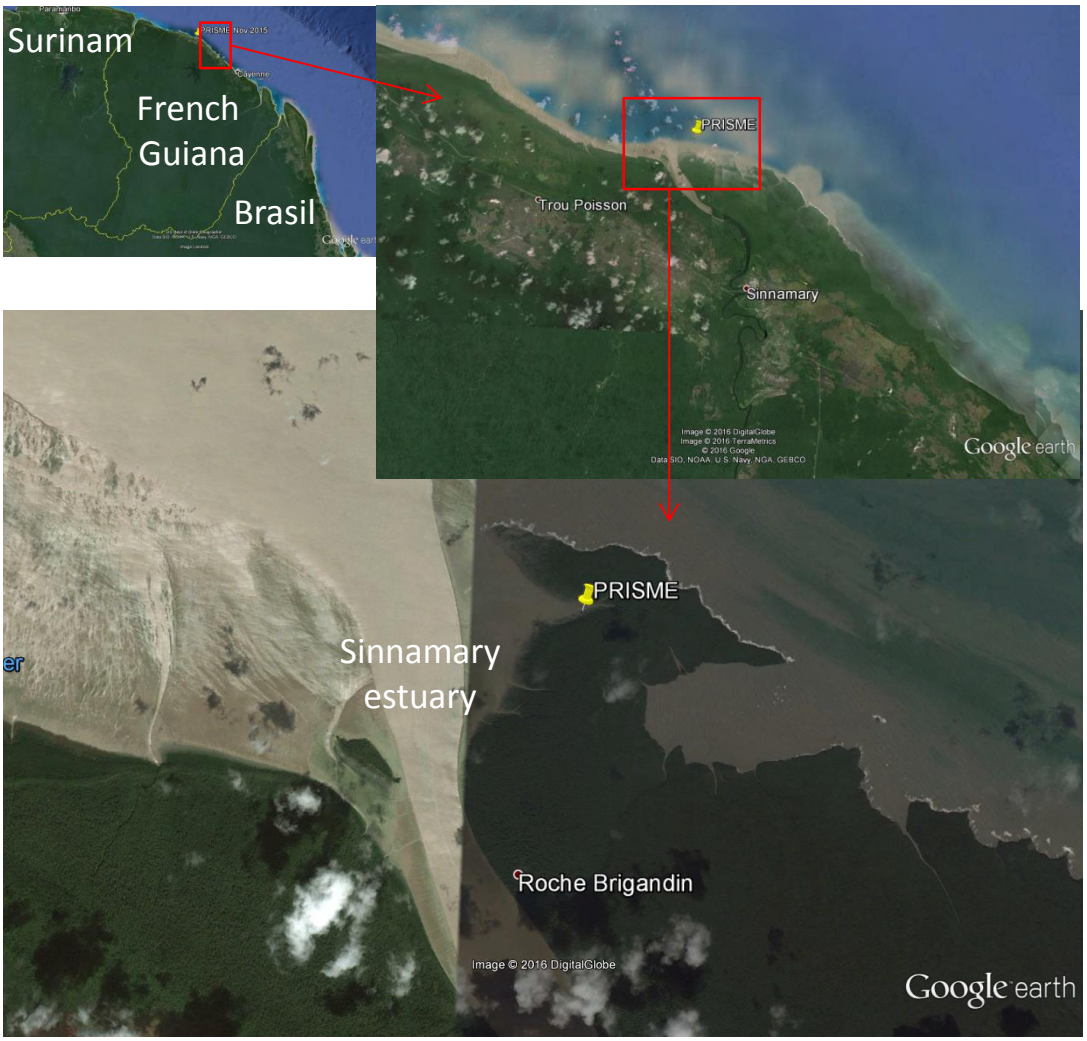

Figure 1 Location of PRISME experimental station in the Sinnamary estuary in French Guiana.

2.2 Experimental Protocol

Experimental units used for the experiment consisted in eight plastic cores made of 30 cm length and 10 cm diameter PVC tubing. These PVC cores, left open at each end, were inserted in October 2015 into the natural sediment at two locations (four cores in each) separated by 10 meters and located under the shadow of mangrove trees (Figure 2): one for the incubation of the contaminated cores (HC+) and the other for the incubation of control, uncontaminated cores (HC-). In order to simulated the oil spill, sediments were contaminated by the addition at the surface of a mixture of contaminated sediments.

Two liters of surface sediment (0-2 cm depth) were collected close to the inserted cores in both locations. These sediments were manually mixed during 20 minutes with, for the preparation of the contaminated mixture, 70 mL of a light crude oil previously topped at 200°C in order to simulate 12 hours of natural weathering. A volume of 12 mL of the

contaminated mixture was transferred at the surface of each of the four cores to be contaminated in the corresponding incubation location (Figure 3B). In order to quantify the macrofaunal reworking activity, 1 mL of a microsphere solution (diameter = 9,8 µm ± 0,553; Fluoresbrite® Fluorescent Microspheres, Polysciences Inc.) was added in each core over the 1.5 cm thick layer of the deposited mixture (Fig. 3C). Finally, in order to stabilize the microspheres, a volume of 5 mL (corresponding to a sediment deposit layer of 0.5 cm thick) of the contaminated mixture was added at the surface of each HC+ core (Fig. 3D). The uncontaminated, control cores (HC-), were prepared similarly but using an uncontaminated sediment mixture instead of the contaminated one. The PVC cores protruded about 2 cm above the sediment/water interface but did not induced observable trapping of detritus within the cores after one month of *in situ* incubation.

Figure 2 Photography of the control incubation area. The experimental site was located in a sheltered area colonized by a young mangrove forest of *A. germinans* trees of about 2 to 4 meters height.

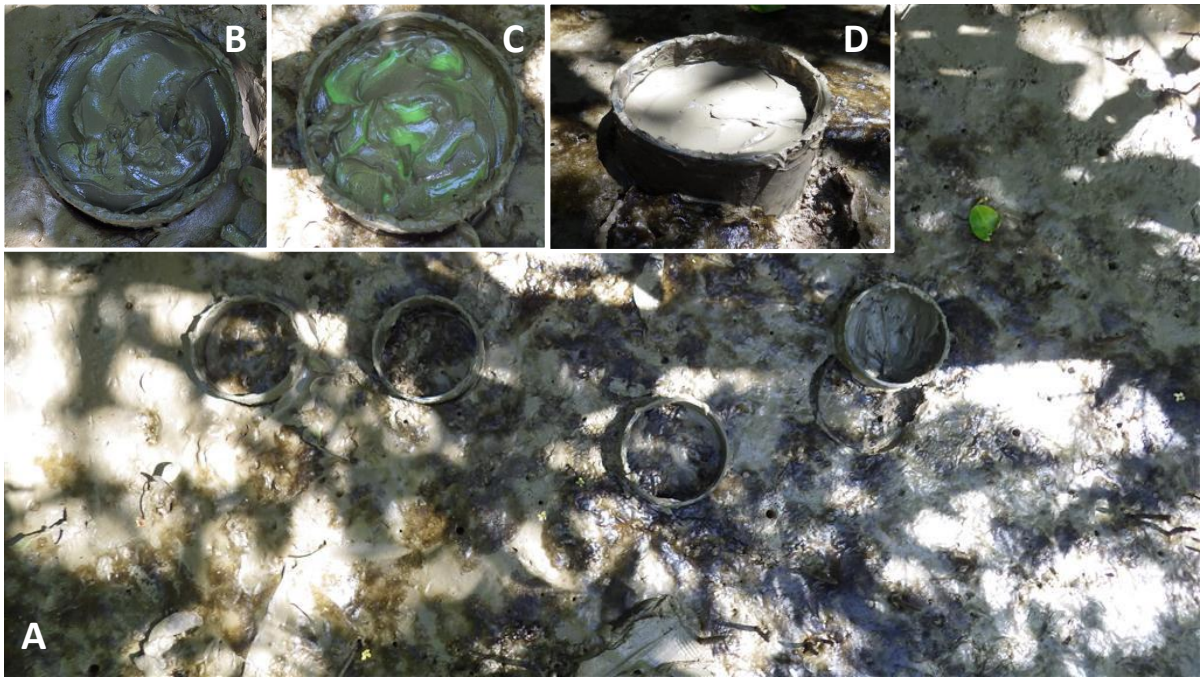

Figure 3 (A) Top view of the inserted cores in contaminated incubation area. Top view of a core after the sequential steps of addition of: (B) the contaminated mixture, (C) the microspheres and, (D) the microspheres' stabilization sediment layer.

2.3 Sediment sampling

After one month of experiment, cores were collected and immediately sliced on site into 0.5 cm layers, the first 2 cm, and then into 1 cm layers until the bottom of the core (Figure 4A) . From each of these layers 3 mL of sediment subsamples were collected for the analysis of the depth distribution of the microspheres. Several composite subsamples of sediments of the 0 – 2 cm, 2 – 4 cm, 4 – 6 cm and >6 cm core horizons were furthermore sampled for the different analyses to be done:

- Chemical analyses, 5g immediately frozen in dry ice box and stored at -20°C in glass bottles in the laboratory until analysis;
- Microbial analyses, 1.5 mL immediately frozen in liquid nitrogen, stored in a dry ice box on site then at -80°C in Eppendorf® tubes until analysis;
- Meiofauna analyses, 4 mL (Figure 4B) fixed with formaldehyde (4% final concentration) and stored at ambient temperature (Figure 4C);
- macrofauna analyses: sediments remaining after the subsampling steps were fixed with formaldehyde (5% final concentration) and stored at ambient temperature (Figure 4C).

Figure 4 (A) Sampling of the sediments for the microbial analyses. (B) Sampling of the sediments for the analysis of the meiofauna. (C) Fixation with formaldehyde (4% final concentration) of the sediment samples for the macrofauna and meiofaune analyses.

2.4 Analyses

2.4.1 Chemical Analyses

The sediment samples were split into three aliquots for moisture analyses (50°C for 24 hours), oil analyses and storage for complementary analyses, if needed.

Sediment samples were spiked with deuterated aliphatic (d42-eicosane) and aromatic hydrocarbons (d8-naphthalene, d10-biphenyl, d10-phenanthrene, d12-chrysene and d12-benzo(a)pyrene) surrogate standard (LGC standard, France). Oil was extracted from sediment samples using an ASE 350 (Accelerated Solvent Extraction, Dionex) (Table 1). Organic extracts were dried over Na_2SO_4 (activated at 400°C for 4 hours) and concentrated to approximately 2 mL using a Syncore (Büchi, Germany).

Table 1 Settings of the ASE 350.

Weight of sample (g)	2
Solvent	Methylene chloride
Temperature (°C)	100
Pressure (psi)	1700
Cycles	2
Heating (min)	5
Static (min)	5
Flush volume (%)	75
Purge (s)	60

Prior to GC/MS analyses, extracts were fractionated using a SPE (Solid Phase Extraction) cartridge (silica/cyanopropyl (SiO2/C3-CN; 1.0/0.5 g, 6 mL; Interchim, France) (Alzaga et al., 2004). Saturate and aromatic fractions were eluted simultaneously with 8 mL of methylene chloride/pentane (80:20, v/v) and concentrated to 2 mL.

Alkanes and aromatics were quantified using gas chromatography coupled with mass spectrometry (GC/MS). The GC/MS was an HP 6890N equipped with a split/splitless injector (Splitless time: 1 min, flow 50 mL/min) coupled to an HP 5973 Mass Selective Detector (MSD) (Electronic Impact: 70 eV, voltage: 1200 V). The injector temperature was maintained at 300°C. The interface temperature was 300°C. The GC/MS temperature gradient was from 50°C (1 min) to 300°C (20 min) at 5°C/min. The carrier gas was Helium at a constant flow of 1 ml/min. The capillary column used was an HP-5 MS: 30 m×0.25 mm ID×0.25 µm film thickness. *n*-Alkane and PAH semi-quantifications were performed using Single Ion Monitoring (SIM) mode with the most representative fragment (saturates) or the molecular ion (PAHs) of each compound at a minimum of 1.4 cycles/s.

Total Petroleum Hydrocarbon quantification was performed with chromatograms obtained in Scan mode. Different concentrations of the crude oil used for the experiment diluted with methylene chloride were used to calibrate the method and then to quantify the TPH extracted from the sediment samples.

2.4.2 Microbial Community Analyses

The sediment cores were separated in four horizons (0–2, 2-4, 4-6 and 6–10 cm). DNA was extracted from 0.25-0.30 g of sediment using the PowerSoil DNA isolation kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's recommendations. DNA was eluted in 100 µL water, quantified using a Shimadzu BioSpec-nano UV-Vis Spectrophotometer and stored at −20 °C until use.

For the analysis of the microbial diversity, PCR amplification was carried out using the primer set 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3') for the V4 region of 16S rRNA gene. The 25 µL PCR mixture contained 1U of Pfu DNA polymerase, 0.4 µM of each primer, 0,4 mM of dNTP, 1X of Pfu DNA Polymerase 10X Buffer with $MgSO_4$ (Promega), and 2 µL of template DNA (dilution 1/10). The reactions were performed in an iCycler thermocycler (Bio-Rad) under the following thermocycling steps : initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 20 s, and elongation at 72°C for 25 s, with a final extension at 72°C for 10 min. PCR products were visualized and quantified by

agarose gel electrophoresis. Equal amounts of the PCR products were polled together and sequenced on an Illumina MiSeq plateform (Genotoul, Toulouse, France). Raw data were processed using QIIME v1.8 (Caporaso et al., 2010) according to the following workflow: (1) quality filtering step removing reads according to Bokulich et al. (2013) recommendations, (2) chimera checking using usearch61, (3) de novo Operational Taxonomic Units (OTUs) picking step using –m uclust parameter (Edgar, 2010) and similarity threshold 0.97; (4) assignation taxonomy step based on the Greengenes taxonomy and Greengenes reference database v13.8 (MacDonald et al., 2012) and (5) alpha diversity index (Chao1 richness estimator, Shannon diversity index), rarefaction curves and beta diversity calculation step. The coverage percentage was estimated by Good's method (Good, 1953). Multivariate analysis were performed using the function ordinate from the phyloseq package in RStudio (version 0.99.491 ; http://www.rstudio.com/).

The bacterial and archaeal abundance were mesured by the determination of bacterial and archaeal 16S rRNA gene copy numbers, respectively. Q-PCR of the 16S genes was performed using the GoTaq® qPCR Master Mix (Promega) and a CFX96 Real Time System (C1000 Thermal Cycler, Bio-Rad Laboratories, CA, USA). For the determination of the bacterial 16S rRNA gene copy numbers, the primer set 300F (5'- GCC TAC GGG AGG CAG CAG-3') and Univ516R (5'- GTD TTA CCG CGG CKG CTG RCA-3') was used. The real time PCR cycles consisted of a denaturation step of 5 s, a hybridization step of 10 s at 55 °C with an elongation step of 12 s. For the determination of the archaeal 16S rRNA gene copy numbers, the primer set 931F (5'- AGGAATTGGCGGGGGAGCA-3') and 1100R (5'- YGGGTCTCGCTCGTTRCC-3') was used. The real time PCR cycles consisted of a denaturation step of 10 s, a hybridization step of 10 s at 62 °C with an elongation step of 20 s. Standard curves were constructed with $4{,}4.10^8$ to $4{,}4.10^2$ copies of a pGEMT-easy plasmid containing a Pseudomonas stuzeri 16S rRNA insert (bacterial 16S rRNA gene) and $3{,}2.10^8$ to $3{,}2.10^1$ copies of a pGEMT-easy plasmid containing an euryarchaeote insert (archaeal 16S rRNA gene). The resulting conditions lead to a Q-PCR efficiency higher than 99% (R^2=0.99). 16S rRNA genes abundances were standardized by the mass of DNA recovered per g dry sediment and log 10 transformed before statistical analysis performed with R software. The analysis of variance (ANOVA) was performed in order to investigate the effects of treatments on bacterial and archaeal abundances.

2.3.3. Hydrocarbonoclastic strains numeration

Sediments from the first 2 cm of a contaminated and uncontaminated core were sampled using an end-cut sterile 50-ml syringe, mixed with 20 mL of synthetic sterilized seawater ($g.L^{-1}$ in distilled water: Tris, 2; NaCl, 23; KCl, 0.75; NH_4Cl, 1; $MgSO_4 \cdot 7H_2O$, 6.16; $MgCl_2 \cdot 6H_2O$, 5.08; and $CaCl_2 \cdot 2H_2O$, 1.5 ; pH 7.7) and kept at 30°C in sterile tubes until use. Sediment sample (25 mL) was mixed with 75 mL of synthetic sterilized seawater and 0,1 mL of oil (Sahara Blend) in a 250-ml Erlenmeyer flask and was agitated in the dark on a reciprocal shaker (100 rev min^{-1}) at 30 ± 1°C for 1 week. Two-millilitre aliquots were transferred four times from the mixed culture to fresh medium under the same conditions. Serial dilutions of mixed culture (1 mL) were streaked onto agar plates prepared with synthetic sterilized seawater at the beginning and at the end of the incubation. Oiled patches made of Whatman© qualitative filters drenched with Sahara Blend were set by capillarity force on each Petri-dish lid in order to allow the diffusion of gaseous hydrocarbons.

After 2 weeks of incubation at 30°C, the colonies with different morphologies were selected, purified and identified (Corsellis et al., 2016).

2.4.3 Macrofauna and meiofauna Analyses

The fixed macrofauna samples were sieved with a 250 µm mesh to retain macroorganisms which were further preserved in formaldehyde (4%) for the counting and sorting of the organisms. The macrofauna was identified to the major taxonomic level possible with stereoscopic and optic microscopes using reference keys (Gilbert et al., 2015). Meiofaunal samples were sieved through 50 µm before staining with rose Bengal and observation under a binocular loupe (Christine et al., 2015). All meiofauna were counted and allocated to major taxonomic groups.

2.4.4 Macrofauna reworking activity measurement

The effect of the oil addition on the sediment reworking was estimated by using the deposited fluorescent particulate tracers (microspheres) as previously described (Stauffert et al. 2013; Ferrando et al. 2015). The subsampled sediments were used to quantify microspheres at the different depth using a microplate reader (Biotek Synergy Mx) at λex/λem: 441/486 nm The biodiffusion-like coefficient Db describing particle transport in the whole sedimentary column was quantified by applying a one-dimensional diffusion model to the data (Quintana et al. 2007).

3 Conclusion / Perspectives

The PRISME project had two main objectives: first of all, conduct a field experiment in the French Guiana mangrove in order to assess the short term behavior and impact of oil in this sensitive ecosystem. The second main objective was to gain knowledge and experience in order to prepare a major project which will include a larger-scale field experiment.

Among the perspectives discussed between the partners involved in PRISME project, the impact of oil on flora will be probably included in the final project: field site selected for PRISME project was characterized by the presence of a large number of young *A. germinans* trees and propagules which had the local peculiarity of a very rapid growth (10-20cm / month). This peculiarity leading to a rapid change in the structure of this young mangrove forest is one of the important lessons learned thanks to PRISME experiment. Special attention will therefore have to be paid to this specific characteristic of the French Guiana mangrove in order to set-up a successful long-term survey of the effects that would have of an oil contamination on this ecosystem. The behavior of oil within the mobile muds along the coast of this area certainly also deserves particular consideration for future experimental work.

At the time this paper was being prepared, analyses were still in progress. Preliminary results of chemical analyses indicated a migration of oil in the sediment column as oil was detected in the four layers of sediment cores (0-2 cm / 2-4 cm / 4-6 cm and 6 cm – bottom) with TPH concentrations decreasing with depths. Moreover, first results of *n*-alkanes analyses highlighted a decrease of *n*-C_{17}/pristane and *n*-C_{18}/phytane ratios, suggesting an effective oil biodegradation during the one month experiment. This biodegradation activity was further confirmed by the molecular identification of 16S DNA sequences belonging to well-known oil-degrading bacterial genera such as *Alcanivorax* or *Cycloclasticus*. Several other hydrocarbonoclastic bacteria, that are currently being identified, were also isolated from the superficial sediments of the experimental area.

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