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Innovative use of foraminifera in ecotoxicology: a marine chronic bioassay for testing potential toxicity of drilling muds

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Abstract

Benthic foraminifera are traditionally used as bioindicators of anthropogenic impact in marine environments. We are increasing their bioindication potential by developing a chronic bioassay method.

This chronic test is elaborated during 30 days on two types of drilling muds with different concentrations in a view of measuring the pseudopodal activity and the presence of new built chambers on foraminifera. The principle is to use the capacity of foraminifera to construct a calcareous shell around his organic cell. This test is therefore conducted in a solution of calcein/seawater to observe fluorescent new built chambers with an epifluorescent microscope.

The first results are promising: foraminifera react physiologically to a 30-day incubation with high concentrations of drilling muds. They are evidently sensitive for long term contact with such chemical mixtures. Moreover, it appears that NABM (non aqueous based mud) are more toxic than WBM (water based mud).

1. Introduction

Benthic foraminifera, marine unicellular organisms protected by a shell, are traditionally used as bioindicators of anthropogenic impact in marine environments (e.g. Watkins, 1961; Seiglie, 1968; 1971; Setty, 1976; Rao and Rao, 1979; Schafer, 1982; Setty and Nigam, 1984; Bhalla and Nigam, 1986; Nagy and Alve, 1987; Schafer et al., 1991; Alve, 1995; Coccioni, 2000; Bergin et al., 2006).The study of their assemblages (standing stocks, species composition and diversity) has shown to be a reliable tool to assess the environmental impact of industrial activities, and the recolonization of the affected areas after cessation of the activities (Mojtahid et al., 2006; Denoyelle et al., 2010). We are trying to improve the bioindication potential of foraminifera by developing a chronic bioassay method, comparable to those based on sea scallops (Cranford et al., 1999) or daphnia (Ecetoc, 1980). In order to develop this method, a series of laboratory experiments has been performed, in which foraminifera were exposed to various concentrations of two different types of drilling fluids for 30 days.

Foraminiferal applications to pollution monitoring are usually based on the assemblage characteristics: density (total number of foraminifera), species diversity (total number of species) and faunal composition. Culture studies where foraminifera are incubated with pollutants are needed to validate field based pollution studies. Such studies have already been conducted on mixed pollutants, such as oil (e.g. Ernst et al., 2006), and on specific chemicals, such as Tri-n-butyltin (TBT) (Gustafson, 2000) or copper (Alve, 1999; Le Cadre, 2005).

Drilling muds are complex mixtures containing several chemical substances. The major fluid component of a drilling mud may be water (water based mud, WBM) or an alternative fluid with oil-like physical properties (non aqueous based mud, NABM). These drilling fluids are necessary to lubricate the drilling bit and to maintain the pressure of the well. The drilling mud also transports pieces of sedimentary rocks, so-called cuttings, upward to the platform. Once on the platform, the cuttings are treated with special devices (shale shakers, centrifugation, etc.) in order to separate the cuttings from the surrounding drilling mud, which is recovered, and as much as possible used again. However, cuttings still contain small quantities of adherent mud. Depending on the regulations of specific countries, there may or may not be restrictions on the discharge of those cuttings into the marine environment (Dalmazzone et al., 2004).

Particular attention has been given to these drilling fluids because of their potential toxicity for the marine fauna (Neff et al., 1983; Conklin et al., 1983). Acute lethal effects of NABM have been reported for a variety of marine organisms and are consequently a concern for oil companies. These bioassay tests usually give an assessment of the toxicity of chemical contaminants after a 96h exposure period. However, such bioassays can not predict the consequences of long term exposure on the benthic fauna. Long term exposure not only leads to prolonged contact with toxic substances, but also to physical disturbances and/or organic enrichment.

We decided to use foraminifera as a new test organism because it is known that different levels of species strongly vary in their sensitivity towards chemical substances. In fact, it is difficult to set standards for the protection of marine fauna by extrapolating ecotoxicological results from a single, or a very limited number of species. The relative sensitivities of the investigated taxa may be very different, and not the same for all pollutants. It is evident that in such a context, the use of a single species for an ecotoxicological evaluation (as it is often the case) is strongly reductive. Foraminiferal bioassays could give valuable additional information, because these organisms represent lower trophic levels than the commonly investigated macrofaunal organisms.

The purpose of this study is:

- To examine the long term sensitivity of foraminifera with respect to chemical substances in NABM and WBM
- To evaluate the toxicity of 2 different drilling muds during long term exposure

Our chronic test is based on a 30 days incubation of foraminifera in solutions with different concentrations of non aqueous based mud (NABM) and water based mud (WBM). To these solutions, we added the fluorescent tracer calcein, which allows us to recognize newly built chambers, which were formed during the exposure period. The parameters measured after the exposure are 1) the activity of each individual by the observation of pseudopodal activity, and 2) the growth rate of foraminifera by observing fluorescent newly built chambers.

2. Material and methods

Culture conditions

Sediment samples containing *Ammonia tepida* were collected at 2 different times in August 2009 and May 2010 at the intertidal area in the Bay of Aiguillon, located on the West coast of France. During low tide, the 5 first millimeters of the sediment were sampled under diatom assemblages (presenting as green spots on the muddy sediment), where foraminifera are abundantly present, and stored carefully in rectangular plastic bottles (5cm*5cm*10cm). The same day, in the laboratory, each sediment sample was washed over sieves of 600 µm, to remove most of the polychaetes, gastropods and algal waste, and 150 µm, to concentrate adult specimens of *Ammonia tepida.* We added seawater sampled at 250m in the Bay of Biscay until the bottles were half full. The sea water was previously microfiltered through a 0.45 MicronSep Cellulosic membrane and stored in plastic containers at the same temperature as the culture.

To keep the foraminiferal specimens alive, 4 millimeters of rehydrated *Chlorella* sp. (green algae) solution and 2 millimeters of a diatom solution were added every 3 days, and 2/3 of the water was replaced weekly. Salinity was checked several times, and was always around 36‰. The temperature and light cycles were not rigorously controlled, but bottles were stored in the culture room where the temperature was always around 22°C and light followed the natural cycle.

Selection of living individuals

To conduct bioassays based on mortality rates, it is essential to choose alive and active specimens of *Ammonia tepida*. In order to do so, the first step was to pick with a fine brush green/brown colored individuals surrounded by sticky algal debris. For shallow water foraminifera, the coloration of the cytoplasm is frequently used as an indication of viability (e.g., Goldstein and Corliss, 1994, Berhnard , 2004, LeCadre et al., 2005), just as the accumulation of organic particles around the aperture (e.g., Goldstein and Corliss, 1994). However this method of observation is not efficient to distinguish accurately dead from living foraminifera (Bernhard, 2000). Therefore we decided to control on a second step the vitality of each individual by observing the extension of pseudopods. The foraminifera were placed in groups of 30 individuals on small Petri dishes, and were after 10 to 20 minutes observed under an inversed microscope (Axiovert25, Zweiss) to detect the presence of pseudopods. All

Ammonia tepida with extended pseudopods were picked and stored in a final Petri dish, prior to the experimental incubation. This method guarantees that the selected specimens are alive and avoids false positives. (e.g., Lecadre et al., 2005).

Marking of foraminifera with calcein

The Fluorescent marker calcein has been used to label the shell of foraminifera. It binds with calcium and incorporates into the mineralized structure, and therefore all chambers formed during calcein incubation fluoresce green when viewed with an epifluorescence microscope (Bernhard et al., 2004).

The calcein solution had been previously prepared in a glass bottle with a concentration of 10 mg/L of microfiltered seawater. This solution was prepared the day before the beginning of the 30 days tests, because it needs a night of agitation at 340 rpm to dissolve the calcein. At the beginning of the contamination tests, 100 ml of this solution was added to each beaker.

Preparation of the toxicity tests

The effects of drilling muds were determined by comparing foraminifera that were kept in treated solutions for 30 days with foraminifera that lived in untreated solutions. 2 different types of drilling muds, NABM and WBM, were used; they were stored in the laboratory at 15°C before the test. The first series of bioassays on NABM was conducted in August 2009 and the second series on WBM in May 2010.

We used glass beakers, previously washed with acid, to conduct our chronic tests. In order to study the effect of drilling muds on foraminifera, six sets of different culture mediums were prepared with various concentrations of muds, between 0 and 10 000 mg/L for NABM and between 0 and 100 000 mg/L for WBM (Tab. 1). The desired solutions of contaminants were prepared by adding directly different amounts of drilling mud (in grams) to 100 mL of seawater with added calcein.

One experimental set was used without the addition of mud, and served as a control.

To all sets, 30 living *Ammonia tepida* were added and the beakers were hermetically closed with a piece of PARAFILM “M”.

Control of the conditions

To avoid at maximum evaporation of volatile components from the muds, the feeding and the bubbling of the test media were done as quickly as possible, at the same time.

Every 3 days, 100 µL of food (*Chlorella* sp. and diatoms) were added and 20 minutes of bubbling ensured the oxygenation of the media. The pH and the salinity were controlled at the beginning and at the end of the test.

End of the tests

At the end of the tests, after 30 days of exposition, all foraminifera were washed with caution to get rid of all adhering material, and were observed under an inversed microscope during 30 minutes to inspect the emission of pseudopods.

Following, they were observed under an OLYMPUS SZX12 stereomicroscope equipped with epifluorescence optics (OLYMPUS U-RFL-T, excitation at 470 nm, emission at 500 nm). All green labeled chambers correspond to new chambers built during the exposure period. Their number gives an approximate idea of the growth of individuals.

3. Results

After 30 days of exposure, each foraminiferal individual was picked out of the culture medium, washed and prepared to be studied. Not all of the 30 individuals were found at the end of the test (Tab. 2). The percentage of individuals with pseudopodal activity (Fig. 1) and the percentage of individuals which have built one or more new chambers (Fig.2) were determined.

With increasing concentrations of both NABM (non aqueous based mud) and WBM (water based mud), the percentage of individuals showing pseudopodal activity decreases (Fig.1).

After 30 days in the control of the NABM experiment, 75% of the individuals showed pseudopodal activity. In the bottles with 100 and 500 mg/L of NABM, about 60% showed pseudopodal activity ($p < 0.05$). At a level of 1 000 mg/L, still 45% of the foraminifera were active ($p < 0.05$). For the highest concentration, of 10 000 mg of NABM per liter of seawater, only 10 % of the individuals showed pseudopodal activity after 30 days of exposure.

For WBM, 97% of the individuals from the control show pseudopodal activity. With increasing drilling mud concentrations, 85% of the population is active at 100 mg/L ($p < 0.05$) and 92% at 500 mg/L ($p > 0.05$). For concentrations of 1 000 mg/L and 10 000 mg/L the percentage of individuals emitting pseudopods decreases to about 40% ($p < 0.05$). At the

highest WBM concentration of 100 000 mg/L, only 1 individual (3%) showed pseudopodal activity.

The percentage of individuals which have built one or more new chambers was also determined (Fig.2).

For NABM, in the control 75% of the individuals have built chambers after 30 days of incubation. In the test with a mud concentration of 100 mg/L, this percentage was about 55% ($p < 0.05$). Next, in the bottle of 500 mg/L, only 30% of all the foraminifera have built chambers ($p < 0.05$). This decrease was confirmed by the 1 000 mg/L experiment, in which 20% of the individuals added one or more new chambers ($p < 0.05$). In the highest concentration of NABM, of 10 000 mg/L, only 5% of the tested individuals built one or more chambers at the end of the 30 days bioassay.

For WBM, 70% of the individuals have built chambers in the control. Almost the same percentage has been observed in mud concentrations of 100, 500 and 1 000 mg/L ($p > 0.05$). For the concentration of 10 000 mg/L this percentage decreases until 55% ($p < 0.05$). No individuals have shown chamber addition at the highest concentration of 100 000 mg/L.

4. Discussion

We tested the viability of the foraminifera at the end of our experiments in 2 different ways, by observing pseudopodal activity for about 30 minutes, and by determining whether the foraminifera had added new chambers. No observed parameters suggest that the foraminifer is inactive, but it does not necessarily imply that it is dead.

In fact, it is, at present, extremely difficult to determine in our laboratory whether a foraminifer is dead or alive; no simple methods with 100% reliable results exist presently. Bernhard (2000) tried to assess and develop vitality tests useable with foraminifera. However pseudopodal observations stay the more accurate method.

This study has been undertaken to characterize the specific response of one foraminiferal species *Ammonia tepida* incubated with different concentrations of pollutants coming from drilling activities. It appears that this species is influenced by a 30-day incubation in both the two types of drilling muds. Our chronic bioassays using foraminifera show that these

organisms have a clear sensitivity in terms of pseudopodal activity and absence or presence of newly built chambers.

After 30 days of incubation, the pseudopodal activity seems to be equally affected by NABM or WBM incubation. The threshold concentration seems to be 1 000 mg/L for the 2 types of mud: foraminifera appear to have difficulties to extend pseudopods after 30 days of incubation in this concentration. This suggests that the 2 muds have a similar long term effect on foraminifera in terms of pseudopodal activity, which reflects the status of the cytoplasm.

For increasing NABM concentrations, there is a very gradual decrease of the percentage of foraminifera which have added new chambers, until only 1 specimen (5%) showed chamber addition at 1 000 mg/l of NABM. For WBM, an entirely different trend is observed: at all concentrations until 1 000 mg/l, more than half of the foraminifera added new chambers, as in the control. At 100 000 mg/l of WBM, however, not a single specimen has added one or more new chambers. This strongly suggests that NABM has a more negative impact on foraminiferal growth and/or chamber addition than WBM.

Finally, our results indicate that for NABM some foraminifera have a pseudopodal activity at the highest concentration without having added new chambers. The contrary is observed for WBM incubation, where some foraminifera have added chambers but do no longer extend pseudopods after the end of the experience.

Unfortunately, it is impossible to compare on data from the NABM and WBM controls. The 2 series have been conducted at 2 different periods. This has an impact on the physiological state of the used community of foraminifera in the culture and in the bioassay.

5. Conclusion

It is clear that foraminifera react physiologically to a 30-day incubation with high concentrations of drilling muds. They are evidently sensitive for long term contact with such chemical mixtures. It appears that NABM are more toxic than WBM, this is especially suggested at lower concentrations where NABM start to inhibit chamber addition, whereas this is not observed for WBM.

The advantages of using foraminifera for evaluating the toxicity of chemicals are their short life-cycle, easy and cheap cultivation, and the presence of a plasma membrane resembling the membrane of cells of higher organisms (Twagilimana et al., 1998). However, foraminiferal bioassays are new and still present problems, such as the development of an adequate vitality test. Consequently, there is a need to conduct additional experiments with several replicas, specific contaminants and different periods of incubation to confirm the suitability of foraminifera for assessing potential toxicity. These data have to be compared with the ones existing on other organisms to confirm that foraminifera are a sensitive species adapted to ecotoxicological studies.

Tables and Figures

Type of Mud	Concentration (mg/L)
NABM	0; 100; 500; 1 000; 10 000
WBM	0; 100; 500; 1 000; 10 000; 100 000

Tab. 1 List of the drilling muds concentrations

	Mud Concentration (mg/L)	No. of specimens at the beginning	No. of specimens found at the end
NABM	0	30	30
	100	30	30
	500	30	28
	1 000	30	28
	10 000	30	28
WBM	0	30	29
	100	30	30
	500	30	25
	1 000	30	30
	10 000	30	27
	100 000	30	30

Tab. 2 Description of the 2 series of bio-assays on NABM and WBM

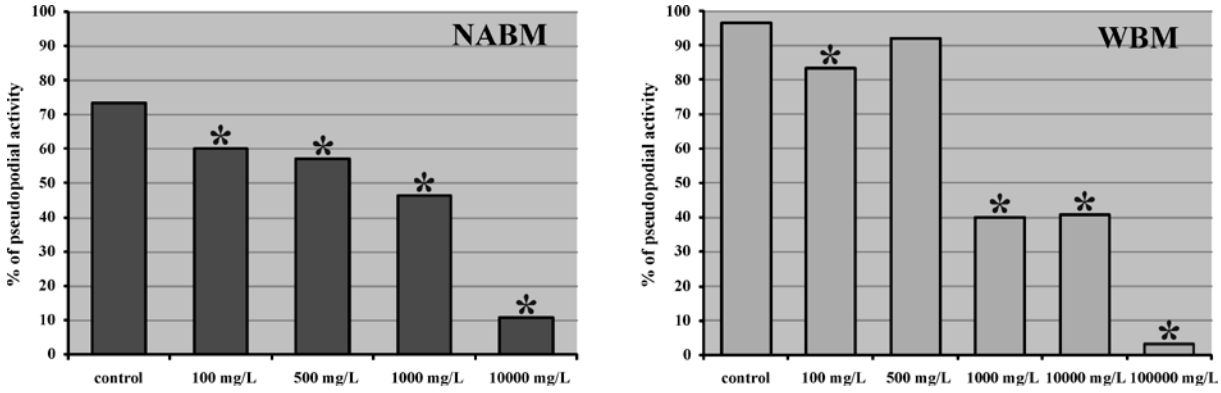

Fig. 1 Percentage of individuals with pseudopodal activity after 30 days of exposure in control set and in contaminated sets with NABM and WBM

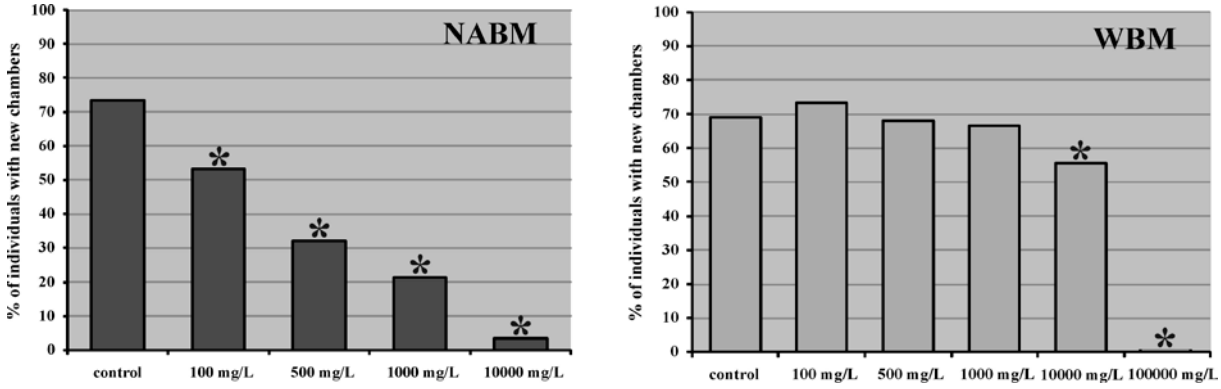

Fig. 2 Percentage of individuals which have built one or more new chambers after 30 days of exposure in control set and in contaminated sets with NABM and WBM

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