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DESCRIPTION OF AN EXPERIMENTAL SET UP FOR THE CULTURE OF BENTHIC FORAMINIFERA IN CONTROLLED pH CONDITIONS

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ABSTRACT

Acidification of the oceans is one of the consequences of ongoing increasing atmospheric CO_2 concentrations. The effects on organisms that build their shells of calcium carbonate are not sufficiently studied and might be detrimental. Simulating ocean acidification scenarios in the laboratory is a reasonable way to study their response to decreased pH and carbonate ion concentrations. In this study we describe in detail an experimental system to carry out ocean acidification experiments with non-symbiotic benthic foraminifera. We test the performance of the designed experimental set up by running a long-term experiment (90 days) using a potentially suitable benthic foraminiferal species for culturing *(Miliolinella* spp.*)*. Although foraminifera did not survive the experimental period likely due to ciliates infestation, seawater pH measurement results indicate that the design is suitable for carrying out ocean acidification experiments.

Key words: ocean acidification, benthic foraminifera, culture, experimental set up, seawater pH.

RESUMEN

La acidificación oceánica es una de las consecuencias del aumento progresivo de la concentración de CO2 en la atmósfera. Los efectos en los organismos que construyen sus conchas con carbonato cálcico no están suficientemente estudiados, pero podrían ser perjudiciales. La simulación en laboratorio de diferentes escenarios de acidificación oceánica es una forma de estudiar la respuesta de esos organismos al descenso de pH y de la concentración del ion carbonato. En este estudio se describe minuciosamente un sistema experimental diseñado para realizar experimentos de acidificación oceánica con foraminíferos bentónicos sin simbiontes. La viabilidad del diseño experimental se ha probado con un experimento de larga duración (90 días) utilizando una especie de foraminífero bentónico potencialmente apta para ser cultivada (*Miliolinella* spp.). Aunque los foraminíferos no sobrevivieron durante todo el experimento debido a contaminación por ciliados, los resultados de las medidas del pH marino ponen de manifiesto que el diseño es adecuado para llevar a cabo experimentos de acidificación oceánica.

Palabras clave: acidificación oceánica, foraminíferos bentónicos, cultivos, diseño experimental, pH marino.

INTRODUCTION

The atmospheric CO_2 concentrations have been continuously rising over the last decades and have led to a large CO_2 uptake by the oceans. This ocean CO_2 uptake has caused a reduction in seawater pH and carbonate ion concentrations (Caldeira and Wickett, 2003; Pérez *et al.*, 2013). Ocean acidification has already impacted marine calcifying organisms (Orr *et al.*, 2005), including foraminifera (Moy *et al.*, 2009), and it is expected to observe additional decrease in the pH of the ocean over the next hundred years. During the last decade intense research has been conducted to investigate the effect of acidification on marine calcifying organisms. Studies reveal varying responses between taxa with some organisms being affected positively, negatively or not having an impact at all (e.g., Iglesias-Rodriguez *et al.*, 2008; Ries *et al.*, 2009) and, within the same taxonomic range, variations may occur between species (reviewed in Doney *et al.*, 2009). In the case of foraminifera, experiments with symbiotic free foraminifera reveal that simulated ocean acidification caused dissolution and decreased calcification rates (Dissard *et al.*, 2010; Haynert *et al.*, 2011; Khanna *et al.*, 2013) whereas studies on symbiont-bearing foraminifera reveal more variable responses. Some species seem unaffected by high pCO_2 levels (i.e., *Amphistegina gibbosa*, McIntyre-Wressing *et al.*, 2013), others show decreased shell weight and shell growth (*Marginopora* spp., Kuroyanagi *et al.*, 2009; Sinutok *et al.*, 2011) and others (species of *Amphisorus*) show no-linear response to increasing pCO_2 levels (Fujita *et al.*, 2011). Comparing the results of ocean acidification experiments is difficult because of the different methodologies and experimental conditions used by different authors. Yet, all these studies suggest that the impacts of ocean acidification on marine calcifying

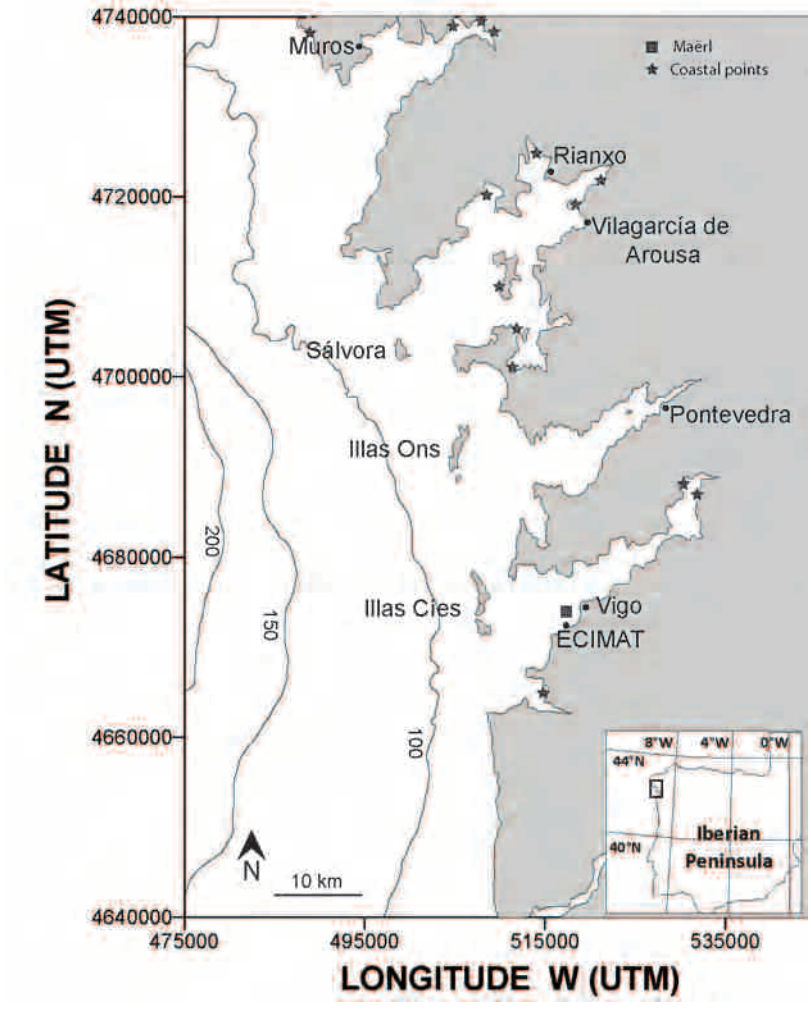

Figure 1:

Surveyed areas for live benthic foraminifera in the Galicia Rías Baixas (stars) and location of the sampling station (maërl bed, square) to collect the living benthic foraminifera. This map shows also the location of the Estación de Ciencias Mariñas de Toralla (ECIMAT) in the Ría de Vigo.

organisms remain still poorly known and that more research should be carried out to better understand to which extent and which organisms will be affected by decreasing pH. Those investigations are not only relevant for near future ocean conditions, but also for particular intervals from the past characterized by widespread deep-sea acidification, such as the Paleocene-Eocene thermal maximum (PETM, ~55 Ma, Zachos *et al*., 2005).

Laboratory experiments under controlled environmental conditions have been commonly used for empirical calibration of foraminiferal proxy records (Hintz *et al*., 2004; McCorkle *et al*., 2008; Barras *et al*., 2010; Diz *et al*., 2012). A large part of the success of these experiments depends on finding a suitable experimental set up capable of maintaining stable physico-chemical conditions. One of the goals on ocean acidification experiments is keeping the seawater pH constant during experimentation, which might be especially difficult on long-term experiments (see Haynert *et al*., 2011; McIntyre *et al*., 2013) mainly because of the processes concomitant to culture living organisms (degradation of food remains, excretions, etc.). Designing a suitable experimental set up is not a simple task because it requires taking into account multiple methodological and ecological aspects (see Riebesell *et al*., 2010). Seawater culturing systems dedicated to the culture of benthic foraminifera for short term ocean acidification experiments have been described recently (Kuroyanagi *et al*, 2009; Haynert *et al*., 2011; McIntyre-Wressnig *et al*., 2013), however a detailed description of an experimental set up for ocean acidification experiments is not yet available. Here, we describe and test the performance of an experimental system to carry out long-term culture experiments in pH controlled conditions with non-symbiotic benthic foraminifera. The system has the potential to be modified to accommodate other organism's requirements.

MATERIAL AND METHODS

Laboratory culture of benthic foraminifera

The performance of the experimental set up should be tested with live benthic foraminifera. The first issue was to select an area where live benthic foraminifera were collected easily and then to select the foraminifera species to perform better in a long-term experiment. First, we surveyed several intertidal areas of the Galician Rías Baixas (NW Iberian margin) in February 2011 (Fig. 1, stars). Live (and dead) benthic foraminifera were scarce at the time of sampling. However, live benthic foraminifera are comparatively abundant in subtidal beds of the Ría de Vigo colonized by red coralline algae (*Lithothamnion corallioides* and *Phymatolithon calcareum*, collectively known as maërl; Peña and Barbara, 2008). A maërl bank located near the marine laboratory (8 meters water depth, Fig. 1 square) was selected as a sampling station to collect living foraminifera. Samples were always collected by scuba divers using aqualung and the help of a small boat. Upon arrival to the ECIMAT, the sediment was stored in an open circuit with running seawater until further analysis (within days of collection).

Benthic foraminifera in maërl beds are highly diversified and the most common species are characterized by an attached or free living mode of life (Diz *et al*., 2004), including low magnesium calcite benthic foraminifera *Elphidium complanatum*, *Rosalina globularis*, *Cibicides refulgens*, *Bolivina pseudoplicata*, and several species that build their shells of high-Mg calcite such as *Quinqueloculina* spp. and *Miliolinella* spp. In order to determine which of these species were more suitable for experimentation, we observed the behavior of the foraminifera for several weeks in laboratory cultures. Observations were used to evaluate qualitatively their reproduction, growth rates and survival rates, the most important parameters to take into account in long term experiments aiming to obtain calcite born and grown in experimental conditions. Thus, foraminifera with bright orange cytoplasm and evidence of movement were picked from the sediment (maërl beds) from different size fractions (63-125 µm and >125 µm) and placed in petri dishes with seawater at room temperature (~18-20ºC). Foraminifera were frequently fed with fresh marine algae (e.g., *Tetraselmis suecica*, *Pavlova lutheri, Rhodomonas lens*) and the seawater was renewed once every one to two weeks.

The fluorescent pattern of these species after calcein incubation (10-15 days) was also examined in order to have a rough estimation of the growth rate. The fluorescent compound, calcein, is used in foraminiferal culturing to discriminate the pre-existing foraminiferal calcium carbonate from the precipitated during experiments (Barras *et al*., 2009, Diz *et al*., 2012). Calcein binds with calcium and incorporates into the mineral structure, so that chambers formed during calcein incubation fluoresce green when observed under epifluorescent microscope (excitation at 470 nm, emission at 500 nm). Chambers formed after incubation are non fluorescent (Bernhard *et al*., 2004). Even though it was not indicated in the original study of Bernhard *et al*. (2004), we realized that dissolving calcein in seawater (10 mg calcein L^{-1} seawater) changes the initial seawater carbonate chemistry. The seawater pH decreases about 0.2 pH units in the first two hours and an additional decrease of 0.1 units might happen in the next few days until it stabilizes. The calcein pH should be carefully checked in order to ensure that incubation does not take place near calcite undersaturation.

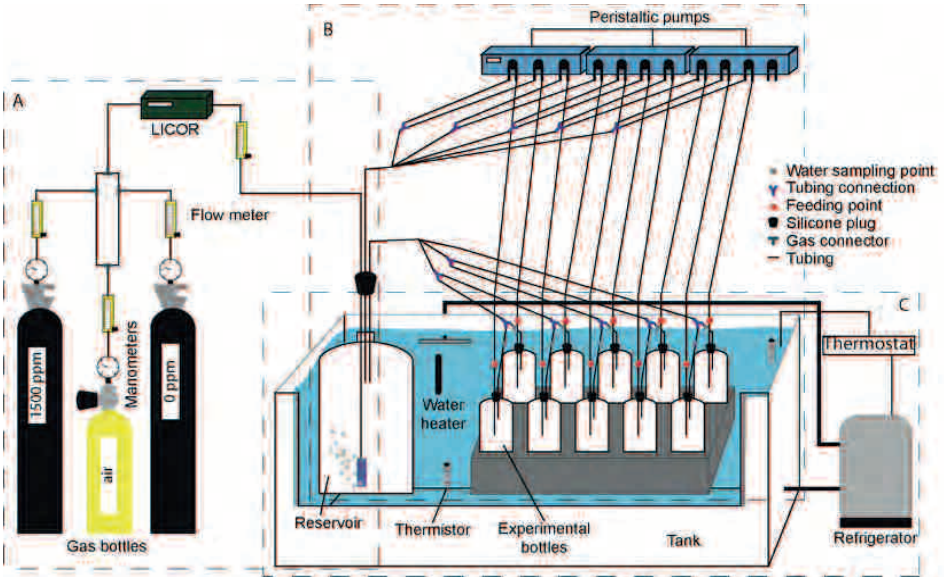

Figure 2:

Outline of the experimental set up designed to culture benthic foraminifera under stable pH conditions. A large reservoir of seawater (50L) is bubbled with a mixture of gases (A) to simulate a particular ocean acidification scenario. The CO_2 concentration of the mixture is monitored with a LICOR instrument (A). The seawater circulates from the reservoir to the experimental recipients and back to the reservoir by means of peristaltic pumps. The tubing is impermeable to gas exchange (Tygon © R3603). The experimental recipients consist of 500 mL bottles filled with water from the reservoir (leaving almost not head space), and capped with a two holes (inlet and outlet tubes) silicone plugs. Connectors were incorporated to inlet and outlet tubes experimental bottles to allow adding food and sampling experimental seawater respectively without disturbing the circuit (B). An external thermostat connected to a refrigerator/heating unit maintains the temperature within ± 0.1ºC (C).

Description of the experimental set up

In this section we describe the components of the experimental set up. This consists of a closed recirculation system in which natural seawater is bubbled with air at a particular concentration of CO_2 as to simulate a particular ocean acidification scenario. Seawater re-circulates from a large reservoir through the experimental bottles containing live benthic foraminifera by means of multichannel peristaltic pumps. This closed recirculation system runs in dark conditions (absence of natural or artificial light) and at a constant temperature.

• Experimental seawater

Natural seawater was the preferred option because its chemical properties are more similar to the natural environment than artificial seawater.

In our experiment, the natural seawater was supplied by the marine Laboratory Estación de Ciencias Mariñas de Toralla (ECIMAT) and it was collected directly from a marine area located outside the lab, in the Ría de Vigo (Fig. 1). After collection, the seawater was aged for about two weeks in order to exhaust the nutrients present in the water. Later, the water was filtered through a 0.20 µm cellulose filter and then stored in an autoclaved 50 L tank (called reservoir thereafter).

• Bubbling of seawater with gases

The manipulation of the carbonate system is carried out by changing dissolved inorganic carbon (DIC) at constant total alkalinity (TA) (Riebesell *et al*., 2010). This method better simulates the changes in seawater carbonate chemistry that might take place in case of continuous increasing levels of atmospheric CO_2. Thus, DIC was modified by bubbling the experimental seawater with air at a particular CO_2 concentration (Schulz *et al*., 2009). The theoretical pCO_2 concentration required to obtain a target seawater pH could be calculated from the TA, temperature and salinity values of the experimental seawater using the CO2SYS freeware software (Lewis and Wallace, 1998; Pierrot *et al*., 2006, http://cdiac.ornl.gov/ftp/co2sys/CO2SYS_calc_XLS_v2.1/). Likewise the degree of calcite saturation (Ω factor) could be calculated from measured pH (see below) and TA. For ocean acidification scenarios, the desired CO_2 concentration in the bubbling air could be obtained by mixing different proportions

of a) compressed air (local atmospheric [CO_2 value] compressed in a diving tank) and b) standard gas with a particular concentration of CO_2. A mixture of compressed air and synthetic air (without CO_2) could be used in case of simulating a scenario of atmospheric pCO_2 values lower than average present values (Fig. 2).

In our experiment, the experimental seawater was bubbled with compressed air from the marine laboratory ECIMAT. The [CO_2] in the bubbling air was monitored with a LICOR instrument and the readings logged every 5 minutes. The LICOR was calibrated every two/three days using a gas blank (synthetic air with no CO_2) and a standard gas of 1500 ppm of CO_2 (Fig. 2).

• Seawater pH

The seawater pH was measured spectrophotometrically on the total hydrogen ion concentration pH scale following the method described in Clayton and Byrne (1993). This method consists of adding 75 µL of m-cresol purple to the seawater sample (20 mL) and measuring its absorbance at 3 wavelengths, i.e., λ_{HI}=434 nm; λ_I=578 nm and $\lambda_{non\text{-}abs}$=730 nm. The total hydrogen ion concentration can be determined by $pH=pK_2+\log_{10}[I^{2-}]/[HI^-]$. The pH was measured at 25ºC with a Cecil VIS spectrophotometer and referred in total scale at the temperature of the experiments (15.7ºC).

• Temperature

Experiments were run at constant temperature to minimize the influence of this parameter on seawater pH. In order to do that, the reservoir and the experimental bottles were covered in tap water and placed in a large tank. The water of the tank was homogenized to prevent vertical temperature gradients. An external thermostat controls the functioning of a refrigerator that maintains the temperature within a ± 0.1ºC range (Fig. 2). Temperature is logged every 10 minutes with a thermistor.

• Experimental recipients

The experimental recipients are 500 mL autoclavable Nalgene © bottles. The bottles are filled with water from the reservoir (leaving almost not head space), and capped with two holes silicone plugs were inlet and outlet tubes are inserted. The relatively large volume of water compared to the size of the foraminifera and complete daily renewal of the water should minimize potential geochemical gradients. A tube Tygon © R3603, which is impermeable to gas exchange was used to connect the reservoir, the peristaltic pumps and the experimental bottles. Selected benthic foraminifera species are placed at the bottom of the recipients (see Results section). A detailed scheme of the experimental set up and its components is displayed in Fig. 2.

• Food addition

In the described experiment, foraminifera were fed with green marine microalgae. Fresh food was the preferred option because previous studies indicated that benthic foraminifera perform better than with freeze-dried algae (Barras *et al.*, 2009). Microalgae delivered from National Center for Marine Algae (NCMA), were cultured in a Walne-medium and maintained at 20ºC in and Erlenmeyer flask with a 12 h dark-light cycle. In order to prevent contamination, samples were taken directly from culture recipients (no replication of cultures). The amount of food added to the experiments ($3x10^6$ cells of *Tetraselmis suecica* experimental bottle every 5 days) was quantified by counting the concentration of individual algae cells using a Neubauer counting chamber following standard procedures (Coutteau, 1996). An aliquot containing the amount established of food we wish to add to the experimental vessels was centrifuged for 20 minutes at low speed (4500 rpm) and at ambient temperature. The supernatant containing the algae medium was discharged and cells were re-suspended with culturing seawater and added to the experimental recipients.

• Summary of the conditions during experimentation period

The performance of the described set up was tested by running an experiment between 05/12/2011 and 02/03/2012 in the ECIMAT marine laboratory. Temperature of the experiment was 15.7 ±0.1ºC. A temperature of about 15ºC can be considered representative of the average mean ocean surface seawater conditions.

The pH of this seawater at the temperature of the experiment was $8.11_{t=15.7}$. This seawater was bubbled with compressed air from the ECIMAT (local CO_2 concentration) for the duration of the experiment and the air flux was maintained a 10 L/hour. In order to test the differences in the seawater pH between the reservoir and the experimental seawater, the pH were measured every 2-3 days. The complete volume of seawater of the experimental recipients was renewed daily at a rate of 20.8 mL/hour. The experiment was run with fifteen live benthic foraminifera per each of the experimental bottles. Foraminifera were fed every 5 days with $3x10^6$ cells of *Tetraselmis suecica* per experimental bottle (Fig. 2).

Table 1:

Summary of qualitative observations on the behavior of several species of benthic foraminifera in laboratory cultures. Foraminifera were collected from maërl beds.

Qualitative criteria used for survival rate in this experiments are as the following
Low: individuals remain active (form cyst, show pseudopodial emission, present colored cytoplasm) for less than 15 days.
Moderate: individuals remain active between 15-30 days.
High: individuals remain active more than 30 days.

Species	Sieve Size	Max. duration of observations	Food	Reproduction	Survival rate
Elphidium complanatum	> 125 µm	36 days	*Rhodomonas lens* *Pavlova lutheri*	No	Moderate
Bolivina pseudoplicata	63-125 µm	25 days	*Tetraselmis suecica*	No	Low
Cibicides refulgens	63-125 µm > 125 µm	90 days	*Pavlova lutheri*	No	High
Rosalina globularis	63-125 µm > 125 µm	90 days	*Pavlova lutheri* *Tetrasemis suecica*	No	High
Miliolinella spp. */Quinqueloculina* spp.	63-125 µm > 125 µm	45 days	*Tetraselmis suecica* *Pavlova lutheri*	Yes	High

RESULTS AND DISCUSSION

Selection of benthic foraminiferal species for long-term experiments

Observations of the behavior of the five selected benthic foraminiferal species in laboratory cultures are summarized in Table 1. These observations, together with information about the fluorescent pattern were used to evaluate qualitatively the suitability of each of them for long-term culture experiments. Because obtaining foraminifera born and grown during experimental period would be an advantage to analyze the effects of ocean acidification on the geochemistry of the shells, we prefer foraminifera that reproduce easily and that show high survival and growth rates in laboratory cultures. *Bolivina pseudoplicata* is the species with lowest survival rate. *Elphidium complanatum* remained alive for longer in cultures, however very few individuals (and none of *B. pseudoplicata*) show fluorescent chambers indicating very low growth rates (pattern not shown). These results made these two species potentially unsuitable for culturing. On the other hand, *Cibicides refulgens* and *Rosalina globularis* incorporate calcein during incubation period (see examples in Fig. 3C-F) and showed evidence of activity (cyst formation and pseudopodial emission) for two months, however they did not reproduce. On the contrary, *Quinqueloculina* spp./*Miliolinella* spp. showed moderate survival rates but reproduction occurred after two weeks in the laboratory. These results make these species good candidates for long-term culturing. Finally, individuals of *Miliolinella* spp. from the 63-125 µm fraction were the species used for the long-term experiment.

Evaluating the performance of the experimental set up

• Seawater pH during experimentation

The seawater bubbled with compressed-air with a CO_2 concentration ranging between 442 and 460 ppm during the first 11 days of the experiment evidenced a logarithmic pH decrease from the initial value of $8.11_{t=15.7}$ to 7.99 (Fig. 4A). The CO_2 concentrations in the air were particularly high from the day 11th to the 17th days of the experiment, reaching average values as high as 555 ± 32 ppm (Fig. 4B). As a result of that, the seawater pH decreased 0.1 units in 6 days reaching the lowest values of the experiment (7.89, Fig. 4A). Later, the pH recovered and remains stable (pH= 7.95 ±0.012, n=29) from day 23rd to the end of the experimental period (Fig. 4A), when the CO_2 concentrations in the compressed air decreased and showed much less variability (430 ± 25 ppm, Fig. 4B). The

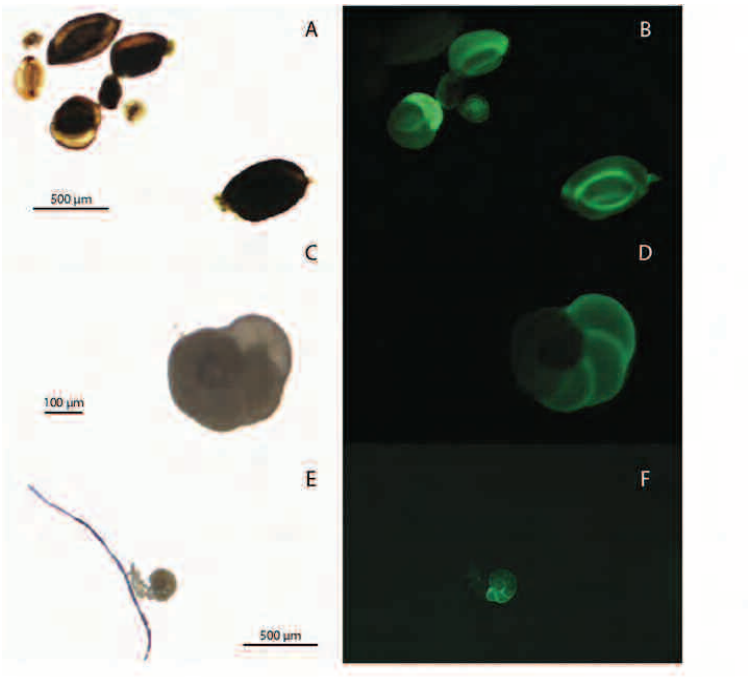

Figure 3:

Selected photographs of the fluorescent pattern of studied species (right panel) and same image under normal light (left panel). Individuals were incubated in calcein (10 mg/L of seawater) for 10-15 days following Bernhard et al. (2004). Photographs A-B show that the fluorescent pattern in porcelaneous species is complex since some individuals showing fluorescence in all chambers or last chamber and sutures within chambers (B). Photographs C-D and E-F show the fluoresce pattern of marked chambers of Rosalina globularis and Cibicides refulgens respectively. Photographs were converted from color to black and white.

differences of the seawater pH between the reservoir and the experimental bottles at each seawater sampling event were always low, ranging from 0.09 (maximum difference) and 0.00 (no difference) with average values of 0.015 ± 0.013 pH units. These results indicate that experimental set up and the conditions applied to run this experiment were suitable for maintaining the differences of seawater pH between the reservoir and the experimental bottles at minimum. This is especially remarkable given that this was a long-term (90 days experiment) and food was added frequently.

• Benthic foraminifera

The benthic foraminifera (*Miliolinella* spp.) introduced at the beginning of the experiment were all recovered from the experimental bottles at the end of the experimental period. All were dead. Reproduction did not occur during the course of the experiment. Mortality was likely caused by ciliates infestation. Ciliates were observed in the seawater of all experimental recipients at the end of experiment.

The ciliates proliferation is a problem no exclusive of benthic foraminiferal culturing but also affects major aquaculture production. Identifying the sources of contamination and maintaining cultures free of microorganisms requires considerable routine effort. We cannot completely disregard that some ciliates could be brought into the system by the microalgae added as food or by the foraminifera themselves. However, we considered (after carrying out several trials) that most likely ciliates come with the natural seawater and that later proliferate in the presence of organic carbon. Thus, aging and microfiltrating might not be sufficient to prevent ciliates proliferation during experimental period, and seawater might need autoclaving. This is a clear disadvantage in relation to natural seawater because autoclaving changes the physical and chemical properties of the seawater.

The ecological success of ocean acidification experiments with benthic foraminifera relies on a benthic foraminiferal species that grow fast and that are able to reproduce easily as well as to build theirs calcium carbonate shells in a broad range of calcium carbonate saturation states or pHs. Reproduction of benthic foraminifera in laboratory conditions has been documented (McCorkle *et al*., 2008; Barras *et al*., 2010; Saraswat *et al.,* 2011, Diz *et al*., 2012; Seears and Wade, 2013) however there is no guaranty that takes place when running an experiment. This explains why most

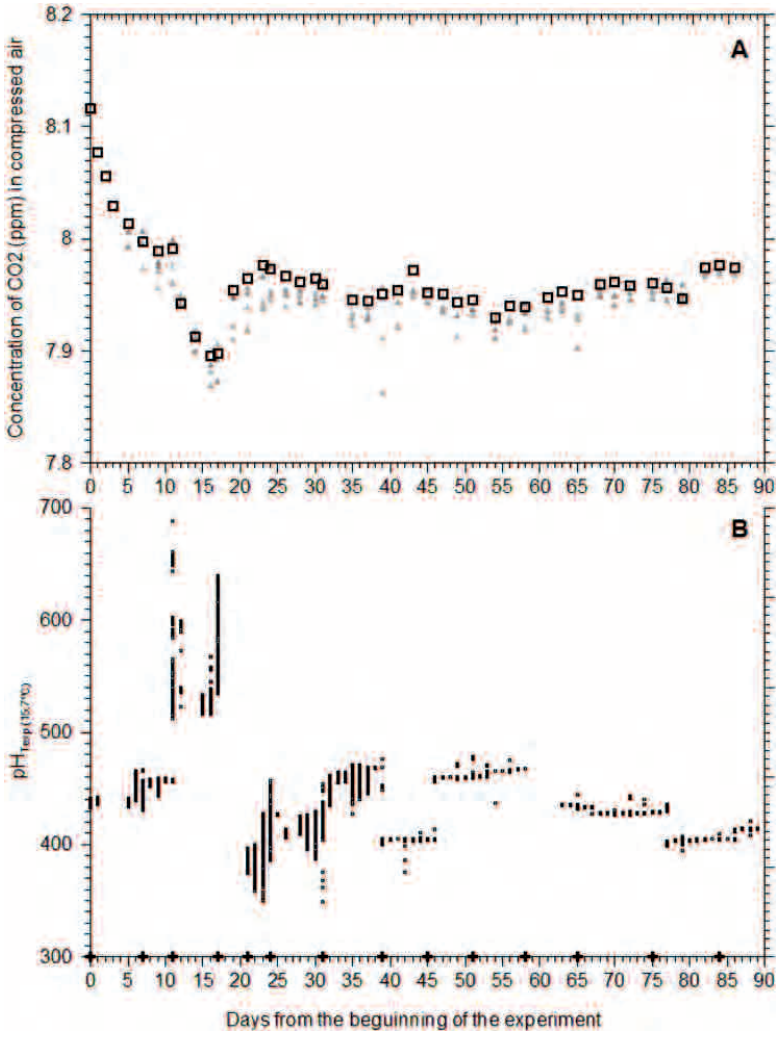

Figure 4:

Seawater pH and CO_2 concentrations for the duration of the experiment:

A) evolution of the experimental pH (total scale) in the reservoir (empty squares) and the experimental bottles (triangles).

B) Single measurements of CO_2 concentrations recorded by the LICOR instrument on the air used to bubble the experimental seawater.

Missing data correspond to computer failure or electricity cuts. Crosses at the bottom axis indicate when the compressed air bottle was refilled with new air.

of the information of the effect of ocean acidification on benthic foraminiferal shells comes from chambers (not whole individuals) formed under controlled experimental conditions (e.g., Kuroyanagi *et al.*, 2009; Allison *et al.*, 2010; Haynert *et al.*, 2011).

CONCLUSIONS

An experimental system to carry out ocean acidification experiments with non-symbiotic benthic foraminifera is described in detail. In addition to that, an experiment was run to test its performance. The designed experimental set up and the conditions applied to run this experiment were suitable for maintaining the differences of seawater pH between the reservoir and the experimental bottles at minimum. This suggests that the proposed design will be suitable to maintain stable pH conditions in long-term ocean acidification experiments. The experimental design could be modified to accommodate other organism requirements such as symbiotic foraminifera (light required) or other hard-shelled small calcifying organisms such as small gastropods or echinoderms.

The benthic foraminifera species selected for running the experiment (*Miliolinella* spp.) did not survive the experimental period. We attributed death to unexpected ciliates proliferation during culturing. Maintaining culturing seawater free of undesired microorganisms is an issue to take into account in experimental procedures.

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