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# Simulating $CO_2$ leakage from sub-seabed storage to determine metal toxicity on marine bacteria

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ABSTRACT

$CO_2$ storage in sub-seabed marine geological formations has been proposed as an adequate strategy to mitigate high $CO_2$ concentration from the atmosphere. The lack of knowledge about the potential risks of this technology on marine bacteria population in presence of metals has lead us to perform laboratory-scale experiments in order to evaluate its consequences. Thus, the effects of Zn and Cd were studied under acid conditions on *Roseobacter* sp. and *Pseudomonas litoralis*. Bacterial abundance (cells $mL^{-1}$), growth rates ($\mu$, $h^{-1}$), relative inhibitory effects of $CO_2$ ($RI_{CO2}$), and production of Extracellular Polysaccharides Substances (EPS) (μg Glucose $cells^{-1}$) were evaluated. A decreasing exopolysaccharides (EPS) production was found under low pH. Bacterial abundance as well as growth rates showed negative effects. Data obtained in this work are useful to determine the potential effects associated with enrichment of $CO_2$ and metals on the marine ecosystem.



## 1. Introduction

The increase of $CO_2$ emissions associated with the use of fossil fuels such as coal and gas as energy sources has become a concerning issue as it is considered the main contributor to global warming and climate change (Leung et al., 2014). Oceans absorb the excess of anthropogenic $CO_2$ due to the differences in the partial pressures between seawater and atmosphere. Thus, the increase of dissolved $CO_2$ in the oceans entails an acidification (Hofmann and Schellnhuber, 2010). As a result, the scientific community has focused attention on Carbon Capture and Storage (CCS) due to its potential use to mitigate the negative effects of anthropogenic $CO_2$. This technology consists of capture of $CO_2$ from emission sources, transport and finally injection of such gas into stable geological formations (Steeneveldt et al., 2006). The storage of $CO_2$ in stable geological formations (either onshore or offshore) is considered the most viable approach to reducing levels of carbon dioxide in the atmosphere (IPCC, 2013). Given that oceans are known to have the greatest storage capacity, sub-seabed stable geological formations were proposed as potential areas for $CO_2$ sequestration (Herzog, 2001). Despite being a feasible technique, the storage of $CO_2$ in those formations is uncertain since this technology has risks associated that need to be assessed (Damen et al., 2006). $CO_2$ stored may present a potential risk to the marine environment in case of accidental leakage. Such events may change seawater chemistry, leading to ocean acidification, with devastating consequences for the marine environment (Lohbeck et al., 2012). Several studies simulating $CO_2$ leakage has been conducted in order to evaluate its effects on the marine ecosystems. These investigations showed adverse effects in a wide range of marine organisms, from primary producer such bacteria (Borrero-Santiago et al., 2016a, 2016b), microalgae (Bautista-Chamizo et al., 2016) and macrofauna (Almagro-Pastor et al., 2015). A $CO_2$ leakage may not only provoke impacts on marine fauna but also on trace metal mobilization and bioavailability. $CO_2$ seepage may release metals from marine sediments increasing their solubility in the water column (Rodríguez-Romero et al., 2014). In fact, a combination of acid conditions and trace metals in the sediment are known to have lethal effects in a broad range of marine organisms (Bautista-Chamizo et al., 2016; De Orte et al., 2014). However, there is a lack of knowledge related to the possible consequences on marine bacteria due to a synergy between metals and acidification (Borrero-Santiago et al., 2016a). Bacteria play an essential role in the marine ecosystem as they are involved in several biogeochemical cycles such as carbon flow, organic matter decomposition and nutrient cycles (Dash et al., 2013). *Roseobacter* sp. and *Pseudomonas litoralis* were selected in this study as tested strains. These species belong to two of the major groups of bacteria in the marine ecosystem, α—and γ—proteobacteria, respectively. According to Hagström et al. (2000), *Roseobacter* and *Pseudomonas* are very representative genuses in a total of 126 bacterial isolates. *Roseobacter* clade is a broadly distributed lineage. In surface waters of the open sea, 1 out of 10 bacterial cells belongs to this clade, and 1 out of 5 bacteria in coastal waters (Moran et al., 2007). This clade is characterized by their capacity to adapt to environmental disturbances (Dash et al., 2013). On the other hand, *Pseudomonas* is one of the most studied γ—proteobacteria because of the

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huge metabolic diversity and biotechnological benefits of this genus (Palleroni, 2010).

$CO_2$ storage sites should be carefully selected and the possible risk associated with these areas should be previously assessed. In this sense, sub-seabed geological formations from the Bay of Cádiz were proposed as a possible $CO_2$ storage site because of the storage capacity and the stability of the geological formations in this area (BOE, 2008). For this reason, this area was chosen as a theoretical place to assess the consequences of the implementation of CCS technology in a metal contaminated area. The main source of contamination in the area is related to the intense ship traffic activity (Rodríguez-Romero et al., 2013). Therefore, the aim of this study was to evaluate the response of two marine bacteria populations against the combined effects of $CO_2$ leakage event in a metal polluted area previously described by Rodríguez-Romero et al. (2013). Laboratory-scale $CO_2$ leakage was simulated using the concentrations of Zn (204.1 mgZn $L^{-1}$) and Cd (0.247 mgCd $L^{-1}$) found in sediments of the Bay of Cádiz at different pH treatments (7.8, 7, 6.5, 6 and 5.5).

## 2. Materials and methods

### 2.1. Microorganisms, culture medium, reagents and incubation conditions

Two different strains (*Roseobacter* sp. CECT 7117, and *Pseudomonas litoralis* CECT 7669) were used for toxicological assessment. They were obtained from the Spanish Culture Collection (www.cect.org).

Initially, the strains were grown in Marine Broth 2216 (Difco) (MB) to reach exponential phase at 22 ± 1 °C for 48 h. Both strains were incubated in an orbital shaker (Orbital shaker "Orbit" Selecta). A 1:10 (v/v) dilution of MB with seawater ($MB_{sw}$) proposed by Borrero-Santiago et al. (2016a), was used for the toxicity test.

Seawater was collected from San Pedro River (SPR), a non-metal polluted area from Bay of Cádiz (SW Spain) (Blasco et al., 2010), filtered through 0.20 μm membrane pore filters (Millipore) and sterilized at 121 °C for 20 min. Lab material was previously acid-washed ($HNO_3$ 10%) 24 h, soaked with Milli-Q water (Merck Millipore system), and sterilized at 121 °C 20 min.

Concentrated solutions of metals were separately prepared using $ZnCl_2$ (Panreac) and $CdCl_2$ (Fluka Analytical) and later sterilized at 121 °C for 8 min. Metal concentrations were selected based on contaminated sediments of the Bay of Cádiz (204.1 mg $L^{-1}$ for Zn and 0.247 mg $L^{-1}$ for Cd) (Rodríguez-Romero et al., 2013).

### 2.2. Experimental set-up

Bioassays were performed in 100 mL Erlenmeyer flasks using $MB_{SW}$ as culture medium. The same concentration of cells ($1 \times 10^{10}$ cells $mL^{-1}$) was exposed to Zn and Cd under 5 different pH treatments (7.8, 7, 6.5, 6 and 5.5). The highest pH (7.8) was run as a control, which suggested the furthest location from the leakage point and pH 5.5 as the closet area of the leakage point (Borrero-Santiago et al., 2016b). Each flask was amended with the metal solution and buffered with MES (50 mM) for pH 6.5, pH 6 and pH 5.5 and HEPES (100 mM) for pH 7 to avoid pH fluctuations (Wang et al., 2002). Two controls in triplicates without $CO_2$ supply used were: negative control (without metal) and positive control (with metal). The values of pH in the control were measured daily using a desk pH meter (Hi 22216 Hanna Instruments) and the values of pH in the treatments were monitored using pH sensors as part of the $CO_2$ injector system (Fig. 1).

### 2.3. $CO_2$ injection system

An adapted $CO_2$ injection system used was described by Borrero-Santiago et al. (2016a) for bacteria toxicity. This system was designed for keeping the sterile conditions required for bacterial assays (Fig. 1). Briefly, the system mimics enrichment of $CO_2$ in aquatic systems by bubbling $CO_2$. The $CO_2$ system allows simulating different scenarios of a $CO_2$ leakage controlled by the electronic control system (AT-Control system). Once the pH sensors detect the desired pH, the solenoid valves are closed. Thus, if a variation in pH is recorded, the solenoid valves are opened to inject $CO_2$ in order to keep the pH constant.

### 2.4. Sampling and analytical determinations

#### 2.4.1. Bacterial growth

Cell abundance was calculated using calibration curves reported by Borrero-Santiago et al. (2016b) for the tested strains. The optical density (OD) at 660 nm was measured at pre-established times (0, 6, 12, 24, 48 and 72 h) to obtain bacterial growth curves and growth rates. Bacterial specific growth rates were calculated according to Widdel (2010). The inhibitory effects of $CO_2$ was calculated using growth rates (μ) by Enfors and Molin equation (Enfors and Molin, 1981):

$$RI = 100 \cdot \frac{\mu c - \mu CO_2}{\mu}$$

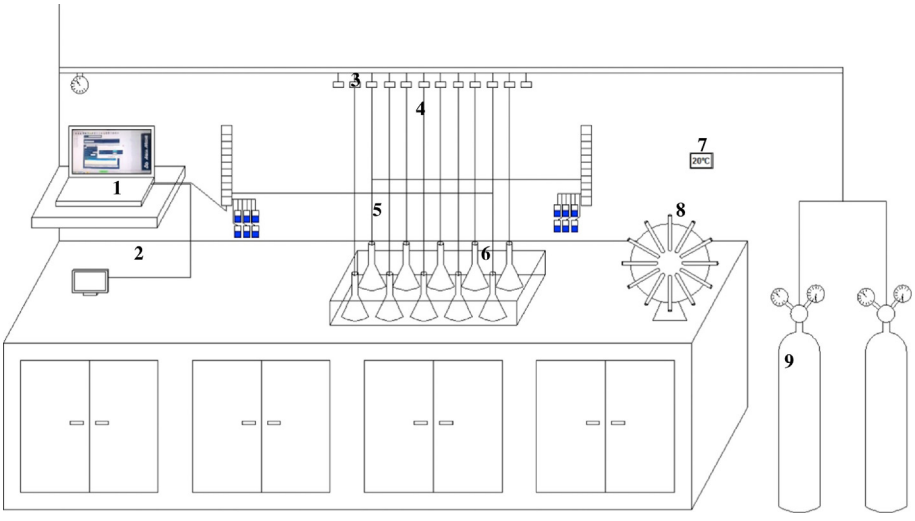


**Fig. 1.** Scheme of the $CO_2$ injection system adapted to perform marine bacteria toxicity assays used in this study (modified from Borrero-Santiago et al., 2016a) (Patent request form P201500918). 1) Laptop to control $CO_2$ injection through Aquamedic 8.0 software; 2) AT-Control system; 3) Solenoid valves which allow and regulate $CO_2$ injection; 4) $CO_2$ injectors; 5) pH sensors which are connected to AT-control system; 6) Erlenmeyer flasks; 7) Temperature regulator; 8) Rotatory orbit with started cultures; 9) $CO_2$ gas bottle.

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