Chemosphere 205 (2018) 24–30

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Implications in studies of environmental risk assessments: Does culture medium influence the results of toxicity tests of marine bacteria?

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HIGHLIGHTS

- Negative effects were found in *Roseobacter* sp. and *Pseudomonas litoralis* under different concentrations of Zn and Cd.
- Metal sensitivity or tolerance is influenced by the culture medium used.
- Results showed a possible interaction between Zn and Cd and the components of a marine full-nutrient culture medium (MB).
- New culture medium (MB_{sw}) is proposed for toxicity tests in marine bacteria for environmental risk assessments.

ARTICLE INFO

Article history:
Received 30 January 2018
Received in revised form
10 April 2018
Accepted 12 April 2018
Available online 14 April 2018

Handling Editor: Shane Snyder

Keywords:
Pseudomonas litoralis
Roseobacter sp.
Bacterial population
Metal toxicity
Zinc
Cadmium
Culture medium
Risk assessment

ABSTRACT

Two marine bacterial populations (*Roseobacter* sp. and *Pseudomonas litoralis*) were exposed to different concentrations of zinc (300, 625, 1250, 2000, 2500 and 5000 mg L^{-1}) and cadmium (75, 250, 340, 500 and 1000 mg L^{-1}) using two culture media (full nutrient Marine Broth 2216 "MB" and 1:10 (vol/vol) dilution with seawater of Marine Broth 2216 "MB_{SW}"), in order to assess population responses depending on the culture medium and also potential adverse effects associated with these two metals. Different responses were found depending on the culture medium (Bacterial abundance (cells·mL^{-1}), growth rates (μ, $hours^{-1}$), and production of Extracellular Polysaccharides Substances (EPS) (μg glucose·$cells^{-1}$). Results showed negative effects in both strains after the exposure to Zn treatments. Both strains showed highest metal sensitivity at low concentrations using both culture media. However, different results were found when exposing the bacterial populations to Cd treatments depending on the culture medium. Highest toxicity was observed using MB at low levels of Cd concentrations, whereas MB_{SW} showed toxicity to bacteria at higher concentrations of Cd. Results not only showed adverse effects on *Roseobacter* sp. and *Pseudomonas litoralis* associated with the concentration of Zn and Cd, but also confirm that depending on the culture medium results can differ. This work suggests MB_{SW} as an adequate culture medium to study metal toxicity bioassays in order to predict realistic effects on marine bacterial populations.

1. Introduction

Trace metals are one of the most common pervasive and persistent pollution problems in aquatic systems (Naser, 2013). Cadmium (Cd) is a transition metal widely used in industrial processes (Naik et al., 2012). The toxic potential of Cd lies in its capacity to interact with essential components in cells, altering cellular functions (Bruins et al., 2000; Vogel and Fisher, 2010) and it may be accumulated in the food chain causing a toxic effect on the marine environment (Naser, 2013). Zinc (Zn) is an essential metal required at low concentrations for growth and metabolism activities (Gadd, 2010; Matyar et al., 2008). However, when Zn concentration exceeds the requirements, it may exert an adverse effect on microorganisms (Banerjee et al., 2015). Moreover, Zn is able to inhibit proteases and enzymes of marine bacteria, respiratory electron

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https://doi.org/10.1016/j.chemosphere.2018.04.066
0045-6535/

transport systems and could also induce an inhibition of essential cell processes (Bong et al., 2011, 2010). Despite the environmental problems derived from metal pollution, marine bacteria are able to adapt to many environmental conditions by resistance mechanisms against metals (Rial et al., 2011; Dash et al., 2013). Indeed, several metal tolerant species of bacteria have been reported in the literature: *Pseudomonas sp., Roseobacter sp., Streptomyces sp., Salinobacter sp., Vibrio sp., Alteromonas sp.* (Matyar et al., 2008; Selvin et al., 2009). Marine bacteria are considered key specie in diverse ecosystems, as they are involved in several marine biogeochemical cycles (Dash et al., 2013).

So far, in order to examine the effects of metals on marine bacteria, ecotoxicological studies have been carried out using different full-nutrient culture media (Bauvais et al., 2014; Rial et al., 2011). There is a lack of standardisation regarding growth culture media used for marine bacterial toxicity assays (Borrero-Santiago et al., 2016a). This fact may lead to misleading conclusions on metal tolerance or sensitivity of tested species. The culture medium used to perform metal toxicological assays might change the metal toxicity because of its potential metal-media component interactions (Li et al., 2011; Chen et al., 2006). Furthermore, full-nutrient culture media might not be the most suitable to predict the real impacts of such toxicants in natural environments since its components might interact with metals (Chen et al., 2006). Moreover, this culture media do not mimic the natural oligotrophic marine bacterial environment. Authors such as Ammerman et al. (1984) reported positive results using seawater as a culture medium for growth assays without any nutritional supply. However, it might be possible that the bacteria population did not growth under optimal conditions. For these reasons, it is essential to study and improve the existing culture media in order to obtain realistic results. In this context, a diluted culture medium with seawater is one of the best options in order to avoid nutritional problems, while still being close to realistic conditions and avoiding metal-media interactions. In fact, other authors already used diluted culture media for toxicity assays (Takeuchi et al., 1997; Teira et al., 2012). However, the dilution selection differs from one author to another. The idea of using the same culture medium in toxicity test with bacteria has been considered recently by authors such as Borrero-Santiago et al. (2016a, b), Díaz-García et al. (2017) or Moreno-Andrés et al. (2018).

Therefore, taking into account all these previous issues mentioned, this paper will address the lack of standardisation on the culture media under laboratory conditions. The present work compares two culture media: a) full nutrient Difco Marine Broth 2216 "MB" and b) 1:10 (vol/vol) dilution with seawater of Difco Marine Broth 2216 "MB_{SW}", already proposed by Borrero-Santiago et al. (2016a) to perform marine bacteria toxicity tests. Our hypothesis is that MB_{SW} could be also an appropriate culture medium for metal toxicity assays in order to avoid metal-nutrient interactions. This study was carried out using two different metals, Zn (essential metal) and Cd (non-essential metal), on two bacterial populations (*Roseobacter* sp. and *Pseudomonas litoralis*). In order to accomplish this aim, bacterial abundance (cells·mL^{-1}), growth rates (μ, $hours^{-1}$), and production of Extracellular Polysaccharides Substances (EPS) (μg glucose·$cells^{-1}$) were analysed under different concentrations of Zn and Cd, and using two different culture media.

2. Materials and methods

2.1. Microorganisms, culture medium and laboratory conditions

Two representative species of marine bacteria from the marine ecosystem were studied: *Roseobacter sp.* (CECT 7117), and *Pseudomonas litoralis* (CECT 7669) (Hagström et al., 2000; Nair et al., 2008; Köchling et al., 2011; Pascual et al., 2012). These bacterial populations have been already selected for CO_2 ecotoxicological assays (Borrero-Santiago et al., 2016a, b; Díaz-García et al., 2017) and UV disinfection studies (Moreno-Andrés et al., 2018). Both strains were obtained from the Spanish Culture Collection (www.cect.org). Dried cells were cultured in Difco Marine Broth 2216 (MB) and incubated on a vertical orbital shaker (Orbital shaker "Orbit" Selecta) (12 rpm) at 26 ± 2 °C for at least 48 h in order to reach an optimal concentration of cells (1×10^{12} cells·mL^{-1}). MB was prepared as per the commercial instructions and sterilized at 121 °C for 20 min. Culture media selected for the toxicity assays were: MB (full-nutrients culture medium) and diluted MB in sterile seawater (MB_{SW}) (semi-natural culture medium) (Borrero-Santiago et al., 2016a, b). MB_{SW} was prepared according to Borrero-Santiago et al. (2016a). Seawater was filtered through 0.2 μm pore membrane filters (Millipore) and sterilized at 121 °C for 20 min (Harrison and Berges, 2005). Seawater was collected from an area relatively free of anthropogenic metal pollution in the Bay of Cádiz (SW Spain) as a reference station (Blasco et al., 2010). Water was collected from surface waters at ± 1 m depth during high tide (salinity 31 ± 1) and kept cool and in the dark for no more than 24 h (Harrison and Berges, 2005). To avoid metal contamination, all materials were acid-washed (HNO_3 10%) prior to use for 24 h and rinsed using deionized distilled water obtained from a Milli-Q system (Merck Millipore).

2.2. Experimental set-up

Bioassays were performed in an isolated chamber in order to keep the sterile condition and temperature constant, avoiding contamination and temperature variations. Metal-free flasks in triplicates were used to perform the bioassays. The same concentration of cells were inoculated in each replicate (1×10^{11} cells·mL^{-1}) and they were exposed to a metal stress conditions of Cd and Zn on a vertical orbital shaker (Orbital shaker "Orbit" Selecta) (12 rpm) at 26 ± 2 °C. Experiments under MB were run for 120 h and under MB_{SW} were run for 72 h. Differences in the bioassay duration were due to nutrient features of each culture. However, 72 h is enough to allow performing a toxicological assay and bacterial population accomplished the necessary growth both in MB and MB_{SW} as culture media (Borrero-Santiago et al., 2016a).

Samples were taken at predefined times intervals of 24 h to study the effects of Zn and Cd in tested strains.

2.3. Metal stocks and concentrations

Concentrated metal stock solutions were prepared by diluting $CdCl_2$ (Fluka Analytical) and $ZnCl_2$ (Panreac) in deionized distilled water. Stock solutions were sterilized at 121 °C for 8 min. The bacterial resistance to Zn and Cd was screened using the plate diffusion method proposed by Hassen et al. (1998). Central wells were made in Petri dishes and 500 μL of different metal solution concentrations was poured in to the wells.

The h0% growth inhibition for eachologia, lo quito????ay (Borrero-Santiago et al., 2016a). establecido asi y en Rdos y discusiPetri dishes were later incubated at 26 ± 2 °C for 48 h, creating a concentration gradient around the well. Thereafter, 100 μL of the bacterial stock was inoculated and incubated at 26 ± 2 °C for 48 h based on the culture conditions of the strains. Duplicate dishes of each strain were prepared for every concentration level. After the incubation time, the area of growth inhibition (inhibition zone) was measured (in mm) as the distance from the edge of the well to the limit edge where colonies start to grow (Selvin et al., 2009). Bacteria were identified as metal sensitive when the inhibition zone was