Marine Environmental Research 122 (2016) 59–66

Contents lists available at ScienceDirect

Marine Environmental Research

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

High sensitivity of embryo-larval stage of the Mediterranean mussel, *Mytilus galloprovincialis* to metal pollution in combination with temperature increase

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ARTICLE INFO

Article history:
Received 1 July 2016
Received in revised form
11 September 2016
Accepted 17 September 2016
Available online 19 September 2016

Keywords:
Copper
Silver
Global warming
Mediterranean mussel
Embryotoxicity

ABSTRACT

The present work aimed to assess the effects of two widespread metallic pollutants, copper and silver, along with environmentally-realistic temperature increases, on embryo-larval development of the Mediterranean mussel *Mytilus galloprovincialis*. First, mussel embryos upon fertilization were exposed for 48 h to increasing concentrations of Cu (0.5–500 μg/L) and Ag (0.1–100 μg/L) at different temperatures (18, 20, 22 or 24 °C) in order to characterize toxicity of each toxicant at the different tested temperatures. Increasing concentrations of a Cu-Ag mixture were then tested in order to assess the mixture effect at different temperatures (18, 20 or 22 °C). Embryotoxicity was measured after 48 h of exposure (D-larvae stage) considering both the percentage of abnormalities and developmental arrest in D-larvae. The results suggest that the optimum temperature for mussel larvae development is 18 °C (12.65± 1.6% malformations) and beyond 20 °C a steep increase of abnormal larvae was observed up to 100% at 24 °C. Ag was more toxic than Cu with a 50% effective concentration (EC50) at 18 °C of 6.58 μg/L and 17.6 μg/L, respectively. Temperature increased the toxicity of both metals as proved with the EC50 at 20 °C at 3.86 μg/L and 16.28 μg/L for Ag and Cu respectively. Toxic unit calculation suggests additive effects of Cu and Ag in mixture at 18 and 20 °C. These results highlight a possible impairment of *M. galloprovincialis* reproduction in the Mediterranean Sea in relation to increase of both pollutants and water temperature due to global warming.

1. Introduction

Marine invertebrates living and reproducing in polluted coastal areas affected by different types of chemicals - including heavy metals - may be impacted at different levels of biological organization (Devi et al., 1996). Copper is a natural and essential element which play an important role in organism functions (Szczypkas et al., 1994). However, elevated concentrations of this metal can be highly toxic (Viarengo, 1989; Negri et al., 2013). Anthropogenic uses of copper are primarily in fungicides (Reichelt-Brushett and Harrison, 2005) and in antifouling paints (Alsterberg et al., 2007), thus increasing copper concentrations in the marine environment, especially in coastal ecosystems. In aquatic ecosystems, copper concentration range from 0.2 to 30 μg/L in the water column, but is generally below 5 μg/L (Willis and Bishop, 2016).

The marine ecosystem is also contaminated with non-essential trace metals such as silver (Ag). Ag ion has been shown to be one of the most toxic heavy metals for aquatic invertebrates (Lam and Wang, 2006). Silver salts are used extensively as antimicrobial agents, particularly silver nitrate ($AgNO_3$) (Maillard and Hartemann, 2012). It has been used for decades in a number of anthropogenic activities, such as mining and photographic processing, as well as being employed in biocides and antimicrobial treatments (Bianchini et al., 2005; Suárez et al., 2010; Behra et al., 2013; Zhang et al., 2014). Silver concentration in water is up to 0.01 μg/L in unpolluted areas and 0.01–0.1 μg/L in urban and industrialized areas. Much higher concentrations were found in seawater from Galveston Bay, USA (8.9 μg/L) and 260 μg/L near photographic manufacturing waste discharges (Howe and Dobson, 2002).

Studies on toxic interactions of heavy metals indicated the occurrence of additive effects, synergistic effects, as well as

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http://dx.doi.org/10.1016/j.marenvres.2016.09.007
0141-1136/

antagonistic effects (Wu et al., 2008).

In addition, global warming represents an additional stress to coastal ecosystems. Surface water temperatures are expected to increase notably in the next decades between 1.4 °C and 3.1 °C and between 2.6 °C and 4.8 °C according to respectively IPCC's simulations RCP 6.0 and RCP 8.5 (IPCC, 2014) and will impact marine life if thermal stress is approaching or exceeding the limits of thermal tolerance (Hofmann and Todgham, 2010). Reproduction of marine invertebrates is highly sensitive to temperature, which is a key factor for spawning and development of invertebrate gametes and larvae (Thorson, 1950). Aquatic invertebrates are particularly sensitive to temperature fluctuations because of their ectothermic biology (Pat et al., 2000). Among marine species, the Mediterranean mussel *Mytilus galloprovincialis*, is an important candidate in environmental monitoring programs. Due to their wide distribution, sedentary lifestyle, filter feeding behavior, and tolerance for a large range of environmental conditions, this species has been widely used as a sentinel organism for marine pollution biomonitoring (Banni et al., 2007; Viarengo et al., 2007). Life stages of *M. galloprovincialis*, have different tolerances to chemical pollutants but embryo-larval stages are particularly sensitive to pollutant exposure (His et al., 1999; Byrne et al., 2008). Biochemical, physiological and molecular responses of *M. galloprovincialis* adults or juveniles to environmental stressors have been well documented (LeBlanc et al., 2005; Attig et al., 2014; Banni et al., 2014a and 2014b). While the acute bivalve embryo-larval assay is extensively used for evaluating chemical toxicity and water or sediment quality (His et al., 1999; Geffard et al., 2001; Beiras and Albentosa, 2004; Quiniou et al., 2005; Mai et al., 2012; Fabbri et al., 2014), data on impacts of pollutants in combination with other physical-chemical stressors on early developmental stages of bivalves are more limited (Deruytter et al., 2015; Gamain et al., 2016). This study aims to investigate the impacts of increasing water temperatures and metal pollution on *M. galloprovincialis* embryo-larval development. The *M. galloprovincialis* embryo larval assay was used to (1) evaluate the effect of a moderate increase in water temperature on embryonic development, (2) to determine the embryotoxicity of copper and silver alone and in mixture and (3) to investigate the developmental impact of combined exposure to both metals and temperature increase.

2. Material and methods

2.1. Test chemicals and sea water

Copper sulfate ($CuSO_4 \cdot 5H_2O$, CAS 7758-99-8, 99.999%) and silver nitrate ($AgNO_3$, CAS 7761-88-8, 99.999%) were of analytical grade and were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Seawater was collected from the "Banc d'Arguin" (Arcachon Bay, SW of France). Immediately after sampling, seawater was filtered (FSW) using a 0.22 μm pore membrane filter and stored at 4 °C in the dark.

2.2. Physical and chemical analysis

Dissolved oxygen level, salinity and pH were measured in FSW for each set of replicated conditions at T0 and T48h. Dissolved oxygen concentration (Do) was measured using an oxygen electrode coupled to a FIBOX3 PRESSENS and using PST3v 602 software. Salinity was measured using a WTW Conductivity Meter. pH was measured using an epoxy-body combination electrode, coupled to a Microprocessor pH meter, (pH 537) and calibrated with standard pH buffer solutions (HANNA Instruments, Romania).

The working solutions were chemically analyzed to confirm metal concentrations. Stock solutions, aliquots solutions and FSW, 50 ml each, were acidified with 5% final nitric acid (Nitrick acid 65%, Fluka). All the water samples were analyzed by inductively-Coupled Plasma Optic Emission Spectrometry (ICP-OES 720, Agilent Technologies) except the reference FSW and the lowest tested dose of Ag and Cu which were analyzed by atomic absorption spectrophotometer (Varian SpectrAA 240Z, Agilent Technologies, Santa Clara, USA). Method blanks were added to each set of samples. The validity of the method was periodically checked with Dolt-5 certified materials (Dogfish Liver Certified Reference Material for Trace Metals and other Constituents, NRCC-CNRC, CANADA) and values obtained were within the certified ranges. Concentrations were expressed in μg/L dissolved Cu^{2+} or Ag^{+}. For ICP-OES, detection limit (DL) was 0.4 μg/L and 2.26 μg/L for silver and copper respectively and for atomic absorption spectrophotometer DL was 0.04 μg/L and 0.5 μg/L for silver and copper respectively.

2.3. Toxicity assay

Stock solutions of copper (250 mg/L) and silver (100 mg/L) were prepared in Milli-Q water and stored at 4 °C. Two independent sets of experiments were performed: for single chemicals, aliquots of copper (0.5, 5, 15, 50 and 500 μg/L) and silver (0.1, 1, 3, 10, 30 and 100 μg/L) were prepared by diluting the stock solution in FSW. In a second set of experiments, binary equitoxic mixtures were prepared by mixing Ag and Cu at their effective concentrations EC5, EC10, EC25 and EC50. Unspiked FSW was used as negative control.

Mature *M. galloprovincialis* from Spain (farmed mussels) were induced to spawn by thermal stimulation alternating immersion in 0.2 μm FSW at 15 °C and 20 °C for 30 min each. Spawners were isolated in glass beakers containing 250 ml of 0.2 μm FSW. Egg quality and sperm motility were assessed using a microscope at 200× magnification. After spawning, sperm and eggs were sieved separately through 50 μm and 100 μm meshes, respectively, to remove debris. For each experiment, eggs and sperm from two individuals were selected according to their quality and counted under microscope. Egg suspension of one female was fertilized by sperm suspension of one male in the ratio of approximately 1:10. The embryo toxicity assay used in the present study was described in details by His et al. (1999), Quiniou et al. (2005) and AFNOR (2009). Fertilization success was checked briefly under a microscope (10–15 spermatozoa should be attached to each egg membrane) and only batches of fertilized eggs with fertilization success > 90% were used. Sixteen minutes after fertilization, volumes corresponding to 300 eggs were transferred into one well of a 24-well microplate (Cellstar, Greiner Bio-one) containing 2 mL of the contaminant solution. For single chemical toxicity, these microplates were incubated in a temperature-controlled chamber (Snijder Scientific) at 18; 20; 22 or 24 °C for 48 h in the dark and at 18, 20 or 22 °C for mixture toxicity test. During the experiment, the average salinity value was 31.2 ± 0.16 (u.s.i), oxygen saturation levels ranged from 99% to 103.3% and pH varied from 7.9 to 8.01. At the end of the 48 h-incubation, buffered formalin was added at 1% final concentration. The percentage of abnormal D-shaped larvae was determined by direct observation of one hundred larvae under an inverted microscope at magnification 400× equipped with a digital camera and an image acquisition software (Nikon eclipse). Assays were validated if the percentage of normal D-shaped larvae was above 80% for the controls (Quiniou et al., 2005) except for the treatment conditions at 22 °C and 24 °C with or without metals. Developmental arrest (embryos that have not reached the D-shaped larval stage) and larvae exhibiting developmental defects such concaved hinge, malformed or damaged shell, protruded mantle were recorded on 100 individuals per replicate. Four analytical replicates were performed for each condition and each experiment was replicated three times using three different pairs of genitors.