Ecotoxicology and Environmental Safety 149 (2018) 1–9

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Larval development ratio test with the calanoid copepod *Acartia tonsa* as a new bioassay to assess marine sediment quality

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ARTICLE INFO

Keywords:
Ecotoxicology
Chronic test
Development inhibition
Larval mortality

ABSTRACT

The copepod *Acartia tonsa* was used as a model species to assess marine sediment quality. Acute and chronic bioassays, such as larval development ratio (LDR) and different end-points were evaluated. As a pelagic species, *A. tonsa* is mainly exposed to water-soluble toxicants and bioassays are commonly performed in seawater. However, an interaction among *A. tonsa* eggs and the first larval stages with marine sediments might occur in shallow water environments. Here we tested two different LDR protocols by incubating *A. tonsa* eggs in elutriates and sediments coming from two areas located in Tuscany Region (Central Italy): Livorno harbour and Viareggio coast. The end-points analyzed were larval mortality (LM) and development inhibition (DI) expressed as the percentage of copepods that completed the metamorphosis from nauplius to copepodite. Aims of this study were: i) to verify the suitability of *A. tonsa* copepod for the bioassay with sediment and ii) to compare the sensitivity of *A. tonsa* exposed to different matrices, such as water and sediment. A preliminary acute test was also performed. Acute tests showed the highest toxicity of Livorno's samples (two out of three) compared to Viareggio samples, for which no effect was observed. On the contrary, LDR tests with sediments and elutriates revealed some toxic effects also for Viareggio's samples. Results were discussed with regards to the chemical characterization of the samples. Our results indicated that different end-points were affected in *A. tonsa*, depending on the matrices to which the copepods were exposed and on the test used. Bioassays with elutriates and sediments are suggested and LDR test could help decision-makers to identify a more appropriate management of dredging materials.

1. Introduction

The planktonic copepod *Acartia tonsa* (Dana) is a cosmopolitan species living in temperate and subtropical marine and brackish water environments. This species is an important member of the zooplankton community and represents a fundamental link between the primary and the secondary production in estuarial and coastal areas (Sei et al., 1996).

In the Mediterranean Sea *A. tonsa* was reported for the first time in 1980s in the Northern Adriatic lagoons, where it became dominant in the zooplankton displacing native congeneric species (Comaschi et al., 2000; Sei et al., 1996). As for others calanoid copepods, *A. tonsa* develops within six different naupliar stages (NI-NVI) (larvae) and five copepodite stages (CI-CV) (juvenile) before moulting into adults (Mauchline, 1998). At temperate latitudes eggs hatch to nauplii within 48 h and adults become sexually mature after 10–12 days, depending on the temperature range, the quality and the quantity of food supplied (Marcus and Wilcox, 2007).

This species is largely used as a model organism in ecotoxicological studies and its sensitivity to pollutants is generally greater than that of other crustaceans (Sverdrup et al., 2002). Different end-points were proposed for risk assessment of chemical substances released in marine environment. In particular, in a 48 h-acute test (Gorbi et al., 2012; ISO, 1999) end-points considered were adult or naupliar mortality, whereas in a 7-days semichronic bioassay end-point was nauplii immobilization or mortality (Gorbi et al., 2012; UNICHIM, 2012). Furthermore, the easily detectable morphological change from nauplii to the first copepodite stage was also considered an end-point in larval development ratio (LDR) test (ISO, 2015). More recently, other chronic end-points, such as the reduction of fecundity of *A. tonsa* mature females (number of eggs) during 4-days exposure test and daily egg hatching success were considered useful to evaluate the environmental risk associated with nanomaterials in seawater (Zhou et al., 2015, 2016).

The ecotoxicological analyses carried out on a combination of organisms are necessary to weight the biological relevance of the measured effects (Pane et al., 2008 and reference herein), therefore, protocols for the evaluation of sediment toxicity should include organisms belonging to different trophic levels. Two methodological approaches

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https://doi.org/10.1016/j.ecoenv.2017.10.062
Received 5 April 2017; Received in revised form 24 October 2017; Accepted 30 October 2017

were generally used to test sediment quality: a direct exposure of the organisms to whole sediments or an indirect exposure to elutriates (ISO, 2015; Nendza, 2002). Classically, benthic organisms such as amphipods (Simpson and Spadaro, 2011) or harpacticoid copepods, such as *Nitocra spinipes, Tisbe tenuimana, Robertgurneya hopkinsi,* and *Halectinosoma sp.* (Perez-Landa and Simpson, 2011; Ward et al., 2011) were used as model organisms in sediment quality assessment tests. A further bioassay, proposed by Anderson et al. (1996) considered the flux of contaminants out of sediments and adopted protocols to measurement of solid-phase toxicity at the interface sediment-water which can affect both benthic or pelagic organisms.

As a pelagic species living in the water column, *A. tonsa* is potentially exposed to water-soluble toxicants and therefore, bioassay protocols were carried out incubating copepods in seawater or elutriates deriving from the sediments. However, eggs of pelagic copepod can also settle on the bottom, especially in shallow waters, remaining in contact with marine sediments.

The calanoid copepod *Acartia tonsa* was recently included in the battery of organisms to be used for the assessment of sediment toxicity, as a key species in the Italian legislation (DM 173/2016; IMO, 2017). Depending on the quality classification of sediments, different managements were then suggested according to dumping legislation. To the best of our knowledge, there is no standardized protocol providing a direct exposure of *A. tonsa* copepods to sediments.

Moreover, the use of a single filter-feeding organism cultured in laboratory to assess the quality of both sediments and water condition, could be extremely advantageous in terms of time and costs necessary to collect wild organisms.

In our study two LDR protocols were performed: in the first *A. tonsa* eggs were incubated in seawater (elutriate) obtained from marine sediments (LDR-E) following ISO protocol (ISO, 2015), in the second eggs were directly incubated in the interface sediment-water (LDR-S) to mimic a shallow water interaction between early life copepod stages and sediments.

Aims of the study were: i) to verify the suitability of *A. tonsa* copepod for the bioassay with sediment ii) to compare the sensitivity of *A. tonsa* exposed to elutriates or sediments. Two end-points, larval mortality (LM) and development inhibition (DI), were considered. Sediments, dredged from differently polluted areas located in the Tuscany Region, Livorno and Viareggio (Central Italy) were tested. A preliminary acute, short-term bioassay was also performed to verify the toxicity of the elutriates in terms of egg hatching success and naupliar mortality (Gorbi et al., 2012). Results were also discussed considering chemical characterization of the sampling sediments and elutriates.

2. Materials and methods

2.1. Sampling sites and matrices prUSEPAration

In 2016 marine sediments were collected, during dredging operations, in two different areas located in Tuscany (Central Italy); the largest and most industrial area of Livorno harbour and the touristic, coastal area of Viareggio (Fig. 1). Three sites were situated in Livorno harbour (L1: 43°34′15″ N, 10°18′27″ E; L2: 43°34′25″ N, 10°18′22″ E; L3: 43°34′40″ N, 10°18′19″ E) and five along Viareggio coast (V1: 43°53′49″ N, 10°13′0″; V2: 43°50′44″ N, 10° 14′ 29″ E;V3: 43°52′6″ N, 10°13′44″ E; V4: 43°52′8″ N, 10°14′14″ E; V5: 43°53′8″ N, 10°13′26″ E) in the Southern Ligurian Sea (Italy).

Sediments were collected with a mechanic grab at a depth ranging from 3 to 8 m depending on the sites and stored in the dark at 4 ± 2 °C for a maximum of one month, before their use in ecotoxicological tests with *A. tonsa* copepod. A commercially available high quality sediment composed of quartz sand was used as control (Rassasie srl, Sala Bolognese, Bologna, Italy).

The elutriates were prUSEPAred by mixing sediments with seawater (30 g/L salinity, pH 8.2 ± 0.1) collected in an unpolluted area of Livorno (1:4 weight:volume) and filtered through a 0.22 μm mesh net filter (FSW) (USEPA, 2001). The mixture was stirred for 1 h using an orbital shaker (Yellow Line OS 2 basic) and centrifuged at 5100 × *g* for 20 min at 4 °C (Thermo Scientific, SL 16R, Rodano, Italy) (USEPA/COE US Army Corps of Engineers, 1991, 1998). Water phase, sUSEPArated from the sediment, was used for acute and LDR-E test.

2.2. Chemical analysis

Marine sediments collected in Livorno harbour and along the Viareggio coast were analyzed for trace metals (heavy metals, HMs) Cadmium (Cd), Copper (Cu), Nickel (Ni) and Lead (Pb), and for total polycyclic aromatic hydrocarbons (PAH's).

For metal analysis, sediments were dried at 60 °C in an oven and disaggregated in a mortar, before chemical treatment. For each sample a known quantity of sediment (0.3 g) was digested with a solution of HNO_3/HCl and ultrapure water in a microwave oven (USEPA, 2001). Subsequently the acid material was diluted to 25 mL by adding ultrapure water and treated with the methods reported by EPA (2007a). Trace metals Cu, Cd, Ni and Pb present in seawater (FSW) and in the elutriate obtained by the control sediment and by L1 (L1-E) were analyzed following the method described by Morelli and Fantozzi (2008) and measured with inductively coupled plasma mass spectrometer (ICP-MS, Agilent Technologies 7900, Santa Clara, CA, USA). Suitable internal standards were used for each analyte to calibrate the instrument following the manufacturer instructions. Trace element recoveries in the samples were ≥ 98% for HMs (EPA, 1998) and a quantitative limits was < 0.1 μg/L.

Total PAH's in elutriates and sediments were analyzed by gas chromatography (GC7890 Agilent Technologies Santa Clara, CA, USA) connected to mass spectroscopy (Triple Quadrupole GC-MS 7000C, Agilent Technologies) after: extraction of water insoluble organic compounds from the samples (EPA, 2007b, 2014). The calibration standards were prred following EPA procedures (EPA, 2014) through dilution of the stock solution with dichloromethane and the recovery data was > 85%. All reagents were purchased by Sigma-Aldrich (Milan, Italy).

2.3. Culture of Acartia tonsa and egg collection

Acartia tonsa copepods were reared through multiple generations at the ISPRA laboratory in Livorno (Italy) in 20 L tanks containing FSW at 30 g/L salinity. Copepods were fed, three times a week, with a mixed algal diet of *Isochrysis galbana*, *Rhinomonas reticulata* and *Rhodomonas baltica* provided at a final concentration > 1500 μg Carbon/L. The algal strains were cultured as described in Zhang et al. (2013) and fed to copepods during their exponential growth phase. Copepod cultures were maintained in a climate-controlled chamber at 20 ± 1 °C and with 14:10 h light:dark photoperiod. Water in the tanks was partially renewed every month.

Mature adult copepods were sorted from the main culture by filtering the water through a 300 μm mesh net filter. Selected organisms were then incubated at a density ≤ 40 ind/L in a 800 mL beaker containing *R. reticulata* algae in the exponential growth phase, at concentration > 1500 μg Carbon/L. After 18–24 h culture medium was filtered through a 155 μm mesh net filter to separate eggs from adults, the filter was gently rinsed with FSW and eggs were sorted under a Leica stereomicroscope (Milan-Italy). For the following 3 days adults were then transferred to the beakers with new medium, in order to collect eggs. Eggs, collected every day, were then stored for a maximum of one month at 3 ± 1 °C in 50 mL vials, or immediately used for the bioassays (Vitiello et al., 2016).

2.4. Acartia tonsa acute test

The acute test with *A. tonsa* was performed following the methods