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# Transcriptomic approach: A promising tool for rapid screening nanomaterial-mediated toxicity in the marine bivalve *Mytilus edulis*—Application to copper oxide nanoparticles

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ABSTRACT

The extensive development of nanotechnologies will inevitably lead to the release of nanomaterials (NMs) in the environment. As the aquatic environments represent the ultimate sink for various contaminants, it is highly probable that they also constitute a reservoir for NMs and hence aquatic animals represent potential targets. In a regulatory perspective, it is necessary to develop tools to rapidly screen the impact of NMs on model organisms, given that the number of NMs on the market will be increasing. In this context High Throughput Screening approaches represent relevant tools for the investigation of NM-mediated toxicity. The objective of this work was to study the effects of copper oxide nanoparticles (CuONPs) in the marine bivalve *Mytilus edulis*, using a transcriptomic approach. Mussels were exposed in vivo to CuONPs (10 $\mu g \cdot L^{-1}$ CuO NPs) for 24 h and analysis of mRNA expression levels of genes implicated in immune response, antioxidant activities, cell metabolism, cell transport and cytoskeleton was investigated by qPCR on hemocytes and gills. Results showed common effects of CuONPs and its ionic counterpart. However, greater effects of CuONPs on GST, SOD, MT, Actin, ATP synthase gene expressions were observed compared to ionic form indicating that toxicity of CuONPs is not solely due to the release of $Cu^{2+}$. Even though *M. edulis* genome is not fully characterized, this study provides additional knowledge on the signaling pathways implicated in CuONP-mediated toxicity and demonstrates the reliability of using a qPCR approach to go further in the cellular aspects implicated in response to NPs in marine bivalves.

## 1. Introduction

The large-scale of nanomaterials (NMs) producted for nanotechnologies leads to their potential release into the environments. Aquatic compartments are the ultimate sink for many conventional contaminants and can represent a potential receptacle for NMs, for which no comprehensive regulatory policy currently exists. Copper oxide nanoparticles (CuONPs) are extensively used in many industrial products such as wood preservation, inks, skin products, textiles as well as electronic circuits and batteries due to their antimicrobial properties and elevated thermal and electrical conductivity (Vance et al., 2015). In normal physiological conditions, copper (Cu) is an essential metal involved in cell homeostasis as it participates in many enzymatic reactions as a co-factor (cytochrome oxidase, superoxide dismutase) but Cu can also be toxic at high concentrations (da Silva and Williams, 2001; Gaetke and Chow, 2003). Many studies have shown effects of Cu at different levels of the biological organization (molecular, cellular, individual, population) (Maria and Bebianno, 2011; Buffet et al., 2011). However, even though toxicity of Cu is supposed to be mainly due to the fact that it generates oxidative stress and the production of reactive oxygen species (ROS), no clear link has been demonstrated between Cu-induced oxidative stress and toxicity at higher levels of biological organization.

For decades, bivalve mollusks have been used as sentinel organisms for investigating the toxicity of many pollutants. Mussels are ideal organisms for environmental risk assessments as they are filter feeders, with a large geographic distribution, that have been shown to bioaccumulate a large range of contaminants (Balbi et al., 2017; Banni et al., 2016; Châtel et al., 2010, 2011). The interest of this invertebrate group for studying the impact of NMs has grown since recent years (Canesi et al., 2015; Canesi and Corsi, 2016) as they have been demonstrated to participate to NP transfer in the food chain (Baun et al., 2008). Mussels have also been shown to possess highly developed mechanisms for cellular internalization of nano and micro-scale particles by endocytosis and phagocytosis, respectively (Moore, 2006). NMs have also been shown to accumulate in gills and hepatopancreas as well as induce immune system impairments in *Mytilus* spp. (Canesi et al., 2012, 2015; Rocha et al., 2015). In the Mediterranean mussel *M. galloprovincialis*,

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CuONPs have been shown to decrease antioxidant defenses, induce lipid peroxidation and metallothionein (MT) both in gills and digestive gland (Gomes et al., 2011). CuONPs also enhance DNA damage in hemocytes of the same species (Gomes et al., 2013). Although < 1% of $Cu^{2+}$ dissolution from the nanoparticulate form appeared, this observed genotoxicity was due to the nano form itself, as also demonstrated in another bivalve specie *Scrobicularia plana* (Buffet et al., 2011).

There is also an urgent need to develop more integrative tools to predict ecosystem impacts for risk assessment (Gottschalk et al., 2013). Even though conventional biomarkers provide sensitive indicators for risk evaluation, it is likely that studies using these assays minimize the observed response by preselecting proteins/mechanisms endpoints from well-known cell signaling pathways whereas other specific proteins/genes pathways may be activated/expressed following nano exposure (Handy et al., 2012; Moore, 2006). Moreover, the extensive number of NMs used in industry makes it difficult to test them all. In this context, there is an urgent need to develop methods that increase the rate in which products can be tested, such as High Throughput Screening (HTS), which allows for rapid toxicity evaluation of many substances and allows for the investigation of the mode of action of these substances, minimizing the "a priori" responses for future definition of new biomarkers of NM exposure. Proteomics have recently been used to investigate AgNPs and CuONPs toxicity (Gomes et al., 2013, 2014) in *M. galloprovincialis* and results demonstrated that expression of proteins, implicated in cytoskeleton, cell adhesion and mobility were modified following exposure.

Among HTS techniques, transcriptomic approaches are of great interest as they allow the screening of a battery of genes in which expression is upregulated or down regulated following exposure. However, the lack of a well-described genomic database for many aquatic species limits the selection of techniques that can be applied.

In this context, this study implements a transcriptomic approach through the use of quantitative PCR in the marine bivalve *M. edulis* to evaluate tissue-specific differences in gene expression for gills and hemocytes after a short-term (24 h) mussel exposure to CuONPs (10 $\mu g{\cdot}L^{-1}$). Gills, the first tissue exposed to pollutants, and hemocytes, immune cells in the mussel, represent interesting target tissue for chemical toxicity assessment.

There is a lack of standardized analytical methods to estimate real concentrations of NPs present in complex matrices (water, sediment, biota) of the environment. Thus since few years, modeling approaches based on emission scenarios are employed to predict NPs concentrations in different compartments of the environment (e.g. Gottschalk et al., 2013). Environmental concentrations of Cu NPs in water were estimated using models in the range from approximately 0.1 to 0.7 $\mu g{\cdot}L^{-1}$ in water (Keller and Lazareva, 2013) which is lower than the dose used in the present study. However, the concentration used in the study is representative of environmentally realistic concentration of Cu (10 $\mu g{\cdot}L^{-1}$) that has been found in highly contaminated areas (Bryan and Langston, 1992). In addition to this, the concentration selected has been used in previous studies and will allow for the comparison of the present results to data from the literature (Gomes et al., 2012, 2014; Buffet et al. 2011, 2013). Furthermore, a 24 h exposure was chosen as previous studies using *Mytilus* species have shown responses to NM exposure for this time period (Gagné et al., 2008; Canesi et al., 2010). A battery of genes implicated in metabolic activity, cell transport, immune response, oxidative stress, cytoskeleton and cell cycle control were selected as they are partly or fully sequenced in *M. edulis*. This study allowed for insight into novel pathways involved in CuONP toxicity. This tool may be applicable for rapid screening of a battery of NMs to help in developing comprehensive regulatory policies on the use of these chemicals.

## 2. Material and methods

### 2.1. Preparation of test solutions

Test solutions were prepared using a previously described method (Buffet et al., 2011). Briefly, $CuNO_3$ was first dissolved in 2% $HNO_3$ (w/w) at a concentration of 1 $g{\cdot}L^{-1}$. A stock solution of CuONPs (Intrinsiq Materials Limited - 10–100 nm) (25 $mg{\cdot}L^{-1}$) was prepared in ultra-pure water and dispersed using sonication (Branson sonicator S-450) for 5 min at 4 °C. The solution was then left at 4 °C for 10 min to normalize the temperature prior to exposure. The stability of the CuO NP in the stock solution and in the experimental media (containing CuONPs) were characterized using dynamic light scattering (DLS) and zeta potential, at the start and end (24 h) of the experiment.

### 2.2. Animal exposure

Twelve mussels (*M. edulis*) 4–5 cm in size, anterior-posterior, were separated into 4 glass aquaria filled with artificial sea water (Tropic Marine ® - Tropicarium Buchshlag Dreieich Germany) (30 psu) at 15 °C (1 mussel/L) for 3exposure conditions: (*i*) artificial sea water (control); (*ii*) 10 $\mu g{\cdot}L^{-1}$CuO NPs; (*iii*) 10 $\mu g{\cdot}L^{-1}$ dissolved $Cu^{2+}$. This concentration (10 $\mu g{\cdot}L^{-1}$) was chosen to conform to a previous study on CuO NPs (Buffet et al., 2011). After 24 h, a scalpel was gently inserted into the ventral-anterior side of the animal and a 2 mL syringe containing 0.1 mL of Alseve (ALS) buffer (20.8 g/L glucose, 8 g/L sodium citrate, 3.36 g/L EDTA, 22.5 g/L NaCl, pH 7.0) was used to extract the hemolymph. After aspirating hemolymph from organisms, the needle was removed from the syringe and the contents were filtered through a 70 μm filter into a falcon tube maintained at 4 °C. The gills were excised from the organisms after extraction of the hemolymph. Both gills and hemolymph were then stored at −80 °C prior to conduct real time qPCR. In order to avoid interferences between food and the fate of NPs, invertebrates remained unfed during the whole duration (24 h) of the experiment.

### 2.3. RNA extraction, RT-PCR and qRT-PCR analysis

Total RNA from control and treated mussels was extracted using TRIzol Reagent as described by Châtel et al. (2017). RNA concentration and purity were measured with a NanoDrop 2000 (Thermo Scientific). First strand cDNA synthesis was carried out on 1 μg of total RNA extract with oligo-dT primers according to Improm II Reverse Transcriptase kit (Promega). Preparations of hemocytes, and gills cDNA were used to quantify specific transcripts in CFX Connect™ Real-Time PCR Detection System (Biorad) using SYBR Green Power Master Mix (Invitrogen) with the primers pairs listed in Table S1. Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (Cp). Cp values for target genes (TG) were compared to the corresponding values for a reference gene (elongation factor gene) to obtain ΔCp values (ΔCp = Cpref − CpTG). PCR efficiency values for reference and tested genes were calculated as described (Navarro et al., 2011), and assumed to be around 100 ± 5% from these calculations.

### 2.4. Statistical analysis

The measured values were compared among different groups using the non-parametric test Mann–Whitney (XL-Stat software). Statistical significance was accepted at $P < 0.05$.

## 3. Results

### 3.1. Characterization of CuONP preparation

Average size distribution of CuO NP (25 mg/L) in DIW was 183 nm