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Copper-induced metabolic variation of oysters overwhelmed by salinity effects

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HIGHLIGHTS

- Cu and salinity affected osmotic regulation, energy and glycerophospholipid metabolism.
- Cu exposure resulted in synthesis of dimethylglycine to cope with severe osmotic stress.
- Combined effects of Cu and salinity were consistent with the singular salinity effects.
- Salinity should be considered in predicting Cu toxicity in estuarine organisms.

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ABSTRACT

In estuarine environments, Cu (copper) contamination is simultaneously coupled with salinity variation. In this study, ^{1}H NMR was applied to investigate the metabolic disturbance of estuarine oysters *Crassostrea hongkongensis* under both Cu and salinity stresses. Oysters were exposed to dissolved Cu (50 μg L^{-1}) at different salinities (10, 15 and 25 psu) for six weeks, and the Cu accumulation in the oyster tissues was higher at lowered salinity. Based on the NMR-metabolomics results, disturbances induced by Cu and salinity was mainly related to osmotic regulation, energy metabolism and glycerophospholipid metabolism, as indicated by the alteration of important metabolic biomarkers such as alanine, citrate, glucose, glycogen, betaine, taurine, hypotaurine and homarine in the gills. At lower salinity, oysters accumulated higher energy related compounds (e.g., glucose and glycogen) and amino acids (e.g., aspartate, dimethylglycine and lysine), with the enhancement of ATP/ADP production and accumulation of oxidizable amino acids catabolized from protein breakdown. With Cu exposure, the synthesis from glycine to dimethylglycine was observed to cope with severe osmotic stress, together with the elevation of lysine and homarine. The effects induced by Cu were much similar for each salinity treatment, but the combination of Cu and salinity turned out to be consistent with the singular salinity effects. Therefore, salinity played a dominant role in affecting the metabolites of oysters when combined with Cu exposure. This study indicated that salinity should be taken into consideration in order to predict the Cu toxicity in estuarine organisms.

1. Introduction

In estuarine environments, the potential exposure and toxicity of metals to aquatic organisms may be greatly elevated with the direct discharges of anthropogenic wastewaters from industrial activities, consequently influencing the living status of local organisms. In southern Chinese estuaries, blue-colored oysters were discovered as a result of severe Cu contamination (Pan and Wang, 2012). Oysters are the hypo-accumulators of Cu, and their tissue Cu concentrations can reach phenomenally high, e.g., 14,000 μg g^{-1} (equivalent to 1.4% of the dry weight) in *Crassostrea hongkongensis* (Wang et al., 2011). Although Cu is an essential element for aquatic organisms, it can induce reactive hydroxyl radicals with an excessive concentration, and enhance the reactive oxygen species (ROS) catalysis, causing oxidative damage to lipids, proteins and DNA (Ringwood et al., 1998; Gaetke and Chow, 2003; Zhang et al., 2011a, 2011b). Unlike other bivalves, oysters have relatively low elimination of Cu, and can detoxity metals by binding with metallothionein-like proteins (MTLP), which may explain why

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oysters accumulate Cu to such high levels.

Cu toxicity can be influenced by various environmental stressors such as temperature, salinity, *p*CO2, and oxygen content (Adeyemi and Klerks, 2012; Deruytter et al., 2015; Gaetke and Chow, 2003), among which salinity is one of the dominant stressors and can closely affect Cu bioavailability. An increase in salinity may provide certain protection against Cu (Lauer et al., 2012). Previous studies demonstrated that metal accumulation increased at a lower salinity (Wang and Dei, 1999), likely caused by a higher available free metal ions. Deruytter et al. (2014) also found that salinity had a nonlinear effect on Cu, and exposure at high salinity reduced the Cu accumulation in mussel larvae.

The estuarine oysters, *Crassostrea hongkongensis*, are widely distributed in Southern China estuaries, and frequently used to monitor the health status of estuarine environment due to their abundance, sessile habit, long life span, large size, and tolerance towards pollution (Abbe et al., 2000; Guo et al., 2015; Ji et al., 2015a, 2015b; Gupta and Singh, 2011). Meanwhile, oysters *C. hongkongensis* can tolerate a wide range of salinity in waters from 5 psu to 25 psu (Liu and Wang, 2013). These oysters can adapt to tidal dynamic environment by accumulating or releasing osmolytes accordingly, or even closing valves to avoid severe osmotic disturbance. However, only a few physiological (i.e. clearance rate and heart rate) or enzymatic parameters have been measured to reveal the biological and physiological responses induced by Cu on oysters, with a lack of an overview of the whole organism or tissue effects (Al-Subiai et al., 2011; Jing et al., 2006).

Proton Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy-based metabolomics approach can provide a powerful tool to study the small molecular chemical compounds (<1000 Da) in cells, tissues, and organisms (Hurley-sanders et al., 2015; Watanabe et al., 2015). Application of metabolomics has been widely adopted in multiple fields, including disease diagnosis and health monitoring (Ji et al., 2015a; Liu et al., 2011; Watanabe et al., 2015), and can reveal the global biological profiles and toxicity of specific pollutants in an effective and efficient way (Campillo et al., 2015). Bivalves are the preferred experimental subjects for metabolomics study to reflect the influence of environmental pollution (Carregosa et al., 2014; Wu and Wang, 2010, 2011). Numerous studies have demonstrated that this approach can effectively provide a global analysis of the metabolites in bivalves. Wu and Wang (2010) compared the metabolite profiles of marine mussel *Perna viridis* exposed to Cd and Cu, and found that the metabolite profiles in metal-exposed samples were significantly separated from that of the control group. Both Cu and Cd can lead to the disturbance in neurotoxicity, osmotic regulation as well as energy metabolism. Taking advantage of ^{1}H NMR, the alteration of important metabolites can be readily detected.

The objective of this study was therefore to elucidate the effects of salinity on Cu-induced metabolic changes in *C. hongkongensis* by ^{1}H NMR. Very few studies have examined the physiological effects of salinity on Cu toxicity during chronic exposure. In this study, oysters were exposed at three levels of salinity (10, 15 and 25 psu) with or without Cu (50 μg L^{-1}) for three and six weeks. In bivalves, gills have both large surface areas and short diffusive distances to balance ion or gas exchange, and are regarded as the main target of waterborne pollutants. The setting of salinities corresponded to the real environment of oyster's living waters. We specifically tested whether salinity or Cu or the combination of both could induce different metabolic profiles of oysters' gills. In biomonitoring study, metal accumulation in the biomonitors is used to indicate the bioavailable metals in the aimbient environment. In our study, three different salinities were employed to mimic the estuarine environment influenced by tidal actions.

2. Materials and methods

2.1. Sample collection

Around 400 oysters *C. hongkongensis* (shell length of 3–4 cm) were collected from an estuary near Jiaomei, Fujian Province, in Southern China, where the average surface salinity varied over the range of 14–26 psu depending on the tidal action (Weng and Wang, 2014). The gender of oysters were not identified since experiments were conducted during the non-reproductive period. The sizes of oyster were kept similar (3–4 cm). After collection, oysters were immediately transported to the Coastal Marine Laboratory in the Hong Kong University of Science and Technology. Shells were carefully cleaned and the oysters were acclimated in aerated seawater (25 °C, 18 psu) for 10 days. Algal powders were fed to oysters every day at a daily rate of 2% of the average (n = 5) tissue dry weight. After acclimation, oysters were divided into three groups, and maintained in seawater of 10, 15 and 25 psu for 7 days, respectively. The salinity was adjusted by mixing filtered seawater and distilled water. The gradient of salinity was reduced or increased by 2 psu daily until reaching the final targeted salinity.

After acclimation, oysters from each salinity treatment were separated into two groups. In total, there were six tanks containing 80 L water for culturing oysters (n = 60). Three groups were exposed to Cu at a concentration of 50 μg L^{-1}, while other salinity groups served as the control without Cu exposure (10, 15 and 25 psu). This exposed Cu concentration was chosen based on our previous study in a nearby contaminated estuary, in which the dissolved Cu concentrations varied between 2.8 and 12.5 μg L^{-1} (Weng and Wang, 2014), while the total Cu concentration in water could reach up to 80 μg L^{-1} during the effluent release periods (Wang and Wang, 2016). Cu was pumped into the static experimental system tank continuously from a stock flask to maintain relatively constant concentration of Cu in the water. Water parameters (pH, salinity) were stable during the whole exposure period. The water pH in the six treatments was comparable. Seawater was replaced every two days and oysters were fed with algae powders at a rate of 1% of average dry weight during the intervals of replacing water in clean seawater. At three and six weeks of exposure, oysters from each group were collected and rinsed with MilliQ water for three times, and immediately dissected and stored at −80 °C for further Cu concentration measurement ($n = 5$) in the digestive gland and gills, as well as ^{1}H NMR detection in gills ($n = 7$).

2.2. Cu concentration measurements

Oysters ($n = 5$) from each treatment were dissected for digestive gland and gill tissues. They were homogenized first and freeze dried for four days. Then, dry tissues were weighed and microwave digested with 2 ml of 68% nitric acid for 12 h. For quality assurance/quality control purpose, the standard oyster 1566b (National Institute of Standards and Technology) tissue samples were used as the reference and digested following the same procedure. Based on the certified metal concentrations of 1566b samples, the recovery rate ranged between 90% and 110%. After 12 h of digestion, the tissue samples were diluted with MilliQ water and measured by inductively coupled plasma-atomic emission spectroscopy (ICP-OES, Optima 700DV).

2.3. Metabolite extraction and NMR detection

Polar metabolites were extracted according to a previously modified extraction protocol (Bligh and Dyer, 1959; Liu et al., 2011). Approximately 100 mg of gill tissues were homogenized in a tube