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Combined effects of salinity, temperature and hypoxia on *Daphnia magna* metabolism

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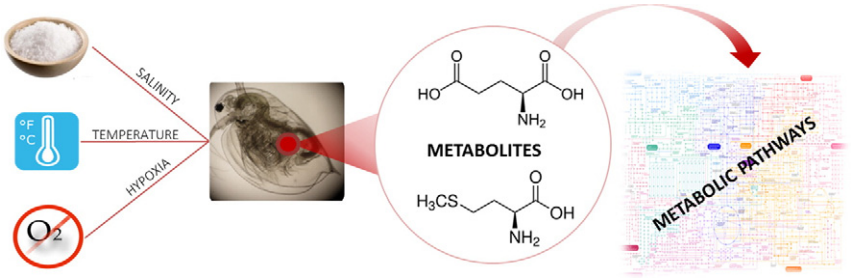
HIGHLIGHTS

- Study of the effects of salinity, temperature and hypoxia on *D. magna* metabolism
- Evaluation of changes on *D. magna* metabolome by chemometric methods
- Salinity was the most influential factor on *D. magna* metabolome.
- Energy metabolism pathways were altered by salinity.

GRAPHICAL ABSTRACT



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ABSTRACT

Metabolic changes of *Daphnia magna* pools due different abiotic factors linked to global climate change (salinity, temperature and hypoxia) were investigated using untargeted GC–MS and advanced chemometric strategies using a three factors two-level full factorial experimental design (DoE). Effects of these three factors and identity of the metabolites whose concentrations changed because of them were investigated. The simultaneous analysis of GC–MS data sets using Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) allowed the resolution of the elution and mass spectra profiles of a large number of *D. magna* metabolites. Changes in peak areas of these metabolites were then analyzed by Principal Component Analysis (PCA), by ANOVA-Simultaneous Component Analysis (ASCA) and by Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA), and the combined effects of the three investigated stressors were assessed. Results confirmed the strong influence of increasing environmental salinity levels on the *D. magna* metabolome. This impact was specially highlighted by changes on the cellular content of carbohydrates, fatty acids, organic acids and amino acid molecules. In contrast, these effects were less significant for the other two factors (temperature and hypoxia) at the moderate stressing experimental conditions investigated in this work when they were not combined with salinity.

1. Introduction

The ecological impact of stressors related with climate change is a recognized fact, although there is little information on the effects of some abiotic factors towards the organism and ecosystems integrity. The general effects of climate change on freshwater systems will likely increase water temperatures, change salinity contents, decrease dissolved oxygen levels, and increase toxicity of pollutants (Ficke et al., 2007). In the last years, the exposure of organisms to natural stressors is receiving attention in risk assessment studies (Bergman Filho et al., 2011).

Apart from the interaction between organisms and environmental systems, it is necessary to understand the interactions between

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stressors, and how individual and combinations of factors affect organisms. Statistical design of experiments (DoE) is a common tool to maximize the information obtained. Hence, the main goal of this approach is to generate the most useful information focusing on the investigation of the effects by means of their interaction. Using a full-factorial design every possible combination of factors at the designated levels is performed, resulting L^k possible combinations of k factors at L levels (Hibbert, 2012).

Metabolomics can provide a snapshot of the molecular events triggered by a variation of an environmental condition. The study of the variation in metabolome permits to obtain information regarding the status of the organism. Characterizing the metabolic responses of organisms to climate change stressors will highlight the power of metabolomics to reveal the interaction of biological organisms with their natural environment (Viant, 2008). To fully determine the metabolome of a biological organism, non-targeted analytical approaches allow the determination of a large number of metabolites, collecting as much information as possible (Alonso et al., 2015).

The crustacean *Daphnia magna* is a model organism in ecotoxicology, because of its easily culture, rapid growth and defined constant genetic background with a clonal reproduction. Changes in the metabolome of *D. magna* and related species have been studied across metals, polycyclic aromatic hydrocarbons, pharmaceuticals, pesticides, flame-retardants, perfluorate compounds and nanoparticles using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS), nuclear magnetic resonance (NMR), GC-Q-TOF/MS and LC-Q-TOF/MS (Kovacevic et al., 2016; Li et al., 2015; Nagato et al., 2013; Nagato et al., 2016; Poynton et al., 2011; Scanlan et al., 2015; Taylor et al., 2016a; Taylor et al., 2016b; Taylor et al., 2009; Taylor et al., 2010; Toyota et al., 2016; Vandenbrouck et al., 2010; Wagner et al., 2016; Zhang et al., 2017). In most studies reported above, changes in metabolomic profiles alone or in combination with transcriptomic ones, allowed to identify pollutant-specific biomarkers and hence differentiate modes of toxicity across the studied chemicals. The study of metabolic changes along the life-cycle or across natural stressors, such as low food supply, high salinity, high temperature and low oxygen levels, allowed to identifies common and dissimilar metabolic pathways across natural occurring stressors (Garreta-Lara et al., 2016; Jones et al., 2012; Wagner et al., 2015; Zhang et al., 2017). In only one study, effects of mixtures were addressed although interactive effects were not statistically tested (Vandenbrouck et al., 2010). Recently, multivariate ASCA methods were applied on the lipidomic profile of *D. magna* exposed to TBT during its reproductive cycle. This allowed testing statistically that TBT and the reproductive cycle affected independently and additively the lipidome of *D. magna* (Malik et al., 2016).

In our previous study, we assessed metabolomics changes of *D. magna* pools exposed acutely to sublethal levels of high salinity and temperature and low dissolved oxygen levels, which produced disturbances in the energy metabolic pathways (Garreta-Lara et al., 2016). However, the combination of these factors was not evaluated. Following this line of research a new study was proposed in order to achieve broader perspectives on the impact of these abiotic factors and about how their combination can affect the metabolome. The present work will evaluate the combined effects of salinity, temperature and hypoxia on *D. magna* metabolome, to anticipate the effects of these physical stressors on their metabolism and to identify the metabolites affected by these environmental stressors.

2. Materials and methods

2.1. Chemicals and standard solutions

D-glucose (U-13C6, 99%), used as the Internal Standard (IS), was supplied by Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Pyridine (anhydrous, 99.8%), derivatizing agents: methoxyamine hydrochloride (98%) (MeOX), chlorotrimethylsilane (TMCS) and *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (>98.5%) (MSTFA), and the saturated Alkane standard mixture for the performance test of GC systems from C_7 to C_{30} were also obtained from Sigma-Aldrich (St. Louis, MO, USA). Hexane, methanol and chloroform were analytical reagent grade, and sodium chloride (NaCl) salt was supplied by Merck (Darmstadt, Germany).

2.2. Sample preparation and experimental design

2.2.1. Experimental animals

All experiments were performed using a well characterized clone F of *D. magna* maintained indefinitely as pure parthenogenetic cultures (Barata and Baird, 1998). Photoperiod was set to 16 h light: 8 h dark cycle and temperature at 20 ± 1 °C. Bulk cultures of 10 animals L^{-1} were maintained in ASTM International hard water at high food ration levels (5×10^5 cells mL^{-1} of *Chlorella vulgaris*, respectively), as described in Barata and Baird (1998). The culture medium was changed every other day, and neonates were removed within 24 h.

2.2.2. Design of experiments (DoE)

Four days old *D. magna* juveniles were used. Animals were obtained from bulk cultures of 100 individuals reared in 10 L of ASTM as described above ("Experimental animals"). A two-level full factorial experiment with 3 factors (salinity (0 and 5 g L^{-1} NaCl), temperature (20 and 25 °C) and hypoxia (9 and 2 mg L^{-1} O_2)), 5 treatment replicate samples at each factor level and their combination was designed statistically (DOE) (Hibbert, 2012). These levels were selected to not affect the survival of the organisms. All treatment replicate samples implied independent *D. magna* sample pools, independent sample extractions and independent GC–MS analysis. Treatments with all factors at a low-level: no-salt with low levels of temperature and oxygen represent the control samples (C).

In the last 50 years, global average surface temperature increased by 0.10–0.16 °C per decade and, it is projected to rise by 1.8–4.0 °C by 2099 (relative to 1999) (IPCC, 2007). Recent advancing industrialization and urbanization have increased salt concentrations in formerly freshwater habitats. *D. magna* is mainly recognized as a freshwater cladoceran, but there are some strains that grow in brackish waters having salinities up to 12.5 g L^{-1} (Arnér and Koivisto, 1993). Natural populations of *D. magna* often migrate to deep hypoxic waters during the day to avoid fish predation, and then swim to surface water to graze on microalgae during the night (Cousyn et al., 2001). Thus, *daphnids* are physiologically well adapted to abrupt changes of oxygen levels (Paul et al., 1998; Zeis et al., 2009). This means that the selected temperature, increase of 5 °C, is close to that predicted from global climate change. Also, the tested levels of salinity (5 g L^{-1}) and low oxygen levels are similar to those that field *D. magna* populations may encounter.

Animals were exposed to individual factors: high salinity, 5 g L^{-1} NaCl (S), high temperature, 25 °C (T), and hypoxia conditions, around 2 mg O_2 L^{-1} (O), and to their binary combinations at high levels: high salinity with high temperature (ST), high salinity with hypoxia (SO) and high temperature with hypoxia conditions (TO). Finally, a tertiary mixture of the three factors was also tested at their high levels: high salinity with high temperature and hypoxia conditions (exposed samples (STO)). Randomly collected animals were exposed to different treatments according to the experimental design in groups of 10 individuals in 1.2 L glass jars completely filled with ASTM hard water. Based on a two-level three factors full factorial design (2^3), a total number of 8 independent treatments were done.