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Thibaut Dumas, Bénilde Bonnefille, Elena Gomez, Julien Boccard, Hélène Fenet, Céline Roques, Frédérique Courant

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# Metabolomics approach reveals disruption of metabolic pathways in the marine bivalve *Mytilus galloprovincialis* exposed to WWTP effluent

Dumas T.<sup>1\*</sup>, Bonnefille B.<sup>1</sup>, Gomez E.<sup>1</sup>, Boccard J.<sup>2</sup>, Fenet H.<sup>1</sup>, Roques C.<sup>3</sup>, Courant F.<sup>1</sup>

<sup>1</sup>HydroSciences Montpellier, Université de Montpellier, 15 avenue Charles Flahault, 34093 Montpellier, France

<sup>2</sup>School of Pharmaceutical Sciences, University of Geneva, 1 rue Michel Servet, 1211 Geneva, Switzerland

<sup>3</sup>Institut des Biomolécules Max Mousseron, Université de Montpellier, 15 avenue Charles Flahault, 34093 Montpellier, France

\*Corresponding author: thibaut.dumas@umontpellier.fr

## Abstract

### 1. Introduction

The development of high resolution mass spectrometry (HRMS) has enabled to screen, in an untargeted way, hundreds of compounds at trace levels in any environmental matrices (e.g. water, sediment, organism). The power and sensitivity of this technology make it relevant and suitable to answer ecotoxicological issues, and especially through environmental metabolomics (Lankadurai et al., 2013). Indeed, HRMS-based chemical profiles collected on fluids or biological tissues of exposed organisms are likely to reflect exposure by the direct non-targeted detection of parent compounds and their metabolites (exposome). They may also reflect effects by detection and characterization of the modulation of endogenous metabolites (endometabolome) that may lead to physiological disturbances induced by the chemical exposure (Courant et al., 2014).

The aim of the present study was to perform an untargeted metabolomics approach to highlight endogenous metabolites disrupted by an urban wastewater treatment plant (WWTP) effluent exposure and then elucidate the biological effects of such exposure on Mediterranean mussels (*Mytilus galloprovincialis*). An untargeted analysis was also conducted on the WWTP effluent extract in order to characterise the exposure. Since pharmaceutical and personal care products consumption has been rising during the last decades, these compounds may constitute a large class of contaminants present in the urban WWTP effluent (470,000 inhabitants equivalent). Hence, the following WWTP effluent preparation was focused on the extraction of dissolved hydrophilic compounds.

### 2. Materials and methods

The WWTP effluent was prepared as follows. 15L of effluent was deposited on 30 cartridges (Oasis HLB® 500 mg, 6 cc) conditioned with 5 mL MeOH and 5 mL distilled water. Cartridges were then washed (5 mL Milli Q water), dried and eluted each with 2 x 5 mL of MeOH. The eluate was evaporated until 3 mL of extract was obtained.

The exposure was performed in controlled laboratory conditions. After seven days of acclimatisation, mussels from a treated group (n=25; 5 tanks containing 5 mussels each per 2L seawater) were exposed to 100 µL of the WWTP effluent extract corresponding to an environmental dilution of the effluent of 5%. Mussels from the control group (n=25) were exposed to solvent (100µL MeOH into each tank of 2L of seawater). During the acclimatisation and exposure periods, filtered seawater was continuously aerated and renewed daily (static renewal). Mussels were fed once daily with the marine green alga *Tetraselmis suecica* at constant density (10,000 cells/mL). Seven days following the exposure, mussels were dissected and their sex determined. No mortality was observed during the exposure period.

Metabolic fingerprints were generated from digestive gland (control group: n=9 males and n=10 females; exposed group: n=10 males and n=10 females) by LC-HRMS analysis (reverse phase PFPP column, Exactive Orbitrap) operating in positive and negative ionisation mode. Afterwards, univariate and multivariate tests were applied to highlight disrupted metabolites in male and female following exposure. A gender specific response was then assessed quantitatively and qualitatively by an ANOVA Multiblock OPLS (AMOPLS). This method takes advantage from the structured design of multifactorial experiments to decompose and quantify the sources of variability, and investigate main effects (exposure, gender) or interaction(s) (exposure×gender) using dedicated components (Boccard and Rudaz, 2016).

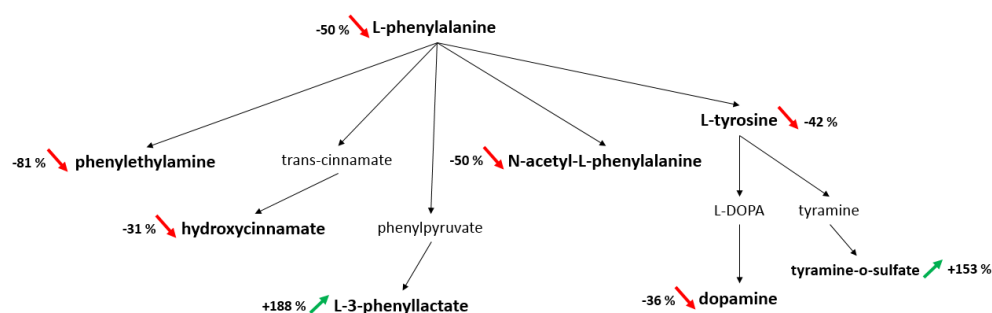
### 3. Results and discussion

#### 3.1 Characterisation of the WWTP effluent extract

Untargeted analysis of the WWTP effluent extract revealed numerous signals in both negative (ESI-; n=8194) and positive (ESI+; n=5773) electrospray ionisation modes. Among those signals, the Toxin and Toxin Targeted Database (Wishart et al., 2015) allowed the chemical annotation of 2912 and 1622 signals in ESI- and ESI+ respectively, based on an accurate mass tolerance of 0.002 Da. Majority of annotated compounds belonged to the classes “Drug”, “Pesticide” or “Household compound”, confirming the main urban nature of the WWTP effluent. Raw formulae of chlorine or sulphur containing contaminants were confirmed on the basis of chlorine or sulphur isotope ratio in mass spectrometry (e.g. sulfamethoxazole, nordazepam, fenofibric acid, fipronil, etc). When available in the laboratory, annotations were confirmed by the injection of the corresponding analytical standards in the same analytical conditions (diclofenac, venlafaxine, ofloxacin, oxazepam, 4-nonylphenol, etc.).

### 3.2 Disruption of male mussel endometabolome

The study of the endometabolome showed numerous metabolites modulated on treated male mussels in response to WWTP effluent exposure. The highlighted metabolites belong mainly to amino acids metabolism (e.g. tyrosine, phenylalanine, proline, etc.), neurohormones (dopamine and a serotonin metabolite), purine and pyrimidine metabolism (e.g. adenosine, guanine, uracil etc.), citric acid cycle intermediates (e.g. malate, fumarate), and a component involved in oxidative stress defence (oxidised glutathione). Figure 2 shows an example of altered metabolic pathways, i.e. phenylalanine and tyrosine metabolism. Modulation of these metabolites could have effects on several biological processes, which may lead to a negative impact of organism fitness, such as energy metabolism, DNA, RNA and



**Figure 2:** Partial map of phenylalanine and tyrosine metabolism in the response of male mussels to WWTP effluent exposure. Metabolites modulated by exposure are shown in bold. Red arrows correspond to down-modulations and green arrows to up-modulations (exposed compared to control). Direct interactions between metabolites are indicated with full arrows. protein synthesis, osmoregulation, byssus formation and adhesion, and reproduction.

### 3.3 Do males and females respond identically?

Female mussels also showed many impacted metabolites. Some of them were common to male, while many others seemed to be specific of female response. Thanks to the AMOPLS analysis, we were able to decompose the variability: the exposure effect (independently of gender) represented 7% ( $p < 0.01$ ) of the total variability, and the exposure $\times$ gender interaction, i.e. the gender specific response to WWTP effluent exposure, represented 4% ( $p < 0.05$ ) of the total variability. Meaningful biochemical information was obtained from the component related to each effect. The former highlighted metabolites modulated in response to exposure whatever the gender. Those relevant metabolites may constitute a set of biomarkers after further validation processes. The latter highlighted key metabolites involved in gender specific response (for example dopamine modulation was only observed in male in response to the WWTP exposure). This may provide a better understanding of response variabilities to the exposure.

## 4. Conclusion

Our work demonstrates the relevance of an untargeted metabolomics approach to characterise both exposome and endometabolome. We highlighted key molecular events triggered by chemical exposure, which could lead to adverse outcomes on individual fitness. In addition, it underlines that gender has to be taken into account for further research on response of organisms to stress. AMOPLS is a promising approach to better characterise multiple factors of variability in response to a stress.

*Keywords:* metabolomics, LC-HRMS, wastewater treatment plant effluent, metabolic pathways, *Mytilus galloprovincialis*

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