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# Proteome response of fish under multiple stress exposure: Effects of pesticide mixtures and temperature increase

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ABSTRACT

Aquatic systems can be subjected to multiple stressors, including pollutant cocktails and elevated temperature. Evaluating the combined effects of these stressors on organisms is a great challenge in environmental sciences. To the best of our knowledge, this is the first study to assess the molecular stress response of an aquatic fish species subjected to individual and combined pesticide mixtures and increased temperatures. For that, goldfish (*Carassius auratus*) were acclimated to two different temperatures (22 and 32 °C) for 15 days. They were then exposed for 96 h to a cocktail of herbicides and fungicides (S-metolachlor, isoproturon, linuron, atrazine-desethyl, aclonifen, pendimethalin and tebuconazole) at two environmentally relevant concentrations (total concentrations of 8.4 μg $L^{-1}$ and 42 μg $L^{-1}$) at these two temperatures (22 and 32 °C). The molecular response in liver was assessed by 2D-proteomics. Identified proteins were integrated using pathway enrichment analysis software to determine the biological functions involved in the individual or combined stress responses and to predict the potential deleterious outcomes. The pesticide mixtures elicited pathways involved in cellular stress response, carbohydrate, protein and lipid metabolisms, methionine cycle, cellular functions, cell structure and death control, with concentration- and temperature-dependent profiles of response. We found that combined temperature increase and pesticide exposure affected the cellular stress response: the effects of oxidative stress were more marked and there was a deregulation of the cell cycle *via* apoptosis inhibition. Moreover a decrease in the formation of glucose by liver and in ketogenic activity was observed in this multi-stress condition. The decrease in both pathways could reflect a shift from a metabolic compensation strategy to a conservation state. Taken together, our results showed (1) that environmental cocktails of herbicides and fungicides induced important changes in pathways involved in metabolism, cell structure and cell cycle, with possible deleterious outcomes at higher biological scales and (2) that increasing temperature could affect the response of fish to pesticide exposure.



## 1. Introduction

Freshwater aquatic systems are the recipients of many pollutants, of which pesticides are of great importance. France is the leading European country in terms of agricultural area (Alim'agri, 2012) and the fourth largest consumer of pesticides in the world (Bonnefoy, 2012). Pesticides essentially contaminate freshwater surface waters through the leaching of soils and rainwater runoff from agricultural land; with momentary but important increases in pollution input during heavy rainfall events (Boithias et al., 2011; Debenest et al., 2008; Polard et al., 2011). The 15 most frequently detected molecules in surface waters are herbicides and fungicides, essentially used to limit weed production and control pathogen invasion in field crops and viticulture (Butault et al., 2010). In 2012, 24.7% of the French river monitoring sites showed a total pesticide concentration above 0.5 μg $L^{-1}$ with concentrations exceeding 5 μg $L^{-1}$ in some rivers (Statistiques Ministère Environnement, France).

In standard laboratory bioassays and commonly used toxicity tests, aquatic organisms are experimentally exposed to single target molecules at given dilutions (e.g. Minguez et al., 2016; Sumona et al., 2016). This might not reflect realistic exposure con-

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ditions of wild aquatic organisms that are often exposed to several toxicants and stressors at the same time. The vast majority of scientific studies on herbicide toxicity concern a few molecules at environmentally unrealistic concentrations, especially when studied organisms are aquatic vertebrates (e.g. fish or amphibians) (Solomon et al., 2013). Additive or synergetic effects between compounds in environmental pesticide mixtures may lead to a dangerous underestimation of their impacts on aquatic vertebrates (Coors and Frische, 2011; Hayes et al., 2006). A few studies on fish models have shown that environmental cocktails of herbicides and/or fungicides may increase genotoxicity (Polard et al., 2011), changes in gene expression (Evrard et al., 2010; Marchand et al., 2006), and decrease in resistance to pathogens (Fatima et al., 2007). In a previous work (Gandar et al., 2016), we found that a cocktail of herbicides and fungicides at environmental concentrations decreased the sediment reworking behavior of goldfish (Carassius auratus L.) and caused a metabolic compensation with a global depletion in energy reserve. These results showed that pesticide cocktails encountered by fish in their environment are likely to result in significant effects at the molecular, physiological and behavioural levels.

In addition, environmental factors such as temperature are likely to affect the pesticide's toxicity on aquatic organisms. Temperature changes may result from both natural (e.g. seasonal fluctuations) and anthropogenic factors (e.g. thermal pollution). In ectothermic species such as fish, temperature changes strongly affect the homeostasis and the physiological status of organisms (Pörtner and Knust, 2007; Pörtner et al., 2006), potentially making them more sensitive to pollutants (Sokolova et al., 2012; Sokolova and Lannig, 2008). An increased toxicity with raised temperature is commonly observed for metals and pesticides, particularly when chemical exposure occurs at temperatures close to the limits of the tolerance range (Heugens et al., 2001; Laetz et al., 2014; Laskowski et al., 2010; Sokolova and Lannig, 2008). Despite the importance of the environmental temperature effect in pollutant toxicity on aquatic organisms, very few studies have focused on combined effects between temperature and pesticide mixtures in fish (Gandar et al., 2016; Laetz et al., 2014). Most of these studies focus on organophosphate insecticides (Dietrich et al., 2014; Laetz et al., 2014).

For 30 years, protein expression has been used to measure the exposure of organisms to pollutants or to assess their toxic properties. However, the specificity of these biomarkers to a class of pollutants and their reproducibility between experiments and/or organism models are now widely questioned (Benninghoff, 2007). Toxicoproteomics allows to explore without a-priori knowledge the proteins and physiological pathways impacted by chemicals (Benninghoff, 2007; Denslow et al., 2005; Wetmore and Merrick, 2004). Proteomics help to identify potential effect biomarkers and to predict the main effects of pollutants at higher scales of organization. In fish, proteomics has been used to study the effects of pesticides (Biales et al., 2011; Chen and Huang, 2011; Laldinsangi et al., 2014; Sanchez et al., 2009), heavy metals (Dorts et al., 2011; Karlsen et al., 2011; Ling et al., 2009; Wang et al., 2013), perfluorooctane sulfonate (Roland et al., 2014), or brominated flame retardants (Kling and Förlin, 2009; Kling et al., 2008). This technique may also be used to study the effects of other environmental factors (e.g., hypoxia: Douxfils et al., 2012; osmotic stress: Kumar et al., 2009) or multiple stressors in fish (Wang et al., 2008).

Based on our previous observations on behaviour and metabolic status of the goldfish, we hypothesized that the exposure to pesticide cocktail and/or increased temperature leads to metabolic and physiological perturbations on the molecular scale. The aim of this study was to explore the proteome response of a freshwater fish, the goldfish, exposed to (1) a cocktail of herbicides and fungicides at environmental concentrations (2) to combined pesticide and thermal stress. For that, goldfish were acclimated to two different temperatures (22 and 32C) for 15 days. They were then exposed for 96 h at the two temperatures (22 and 32 °C) to a mixture of herbicides and fungicides (S-metolachlor, desethyl-atrazine, isoproturon, linuron, aclonifen, pendimethalin and tebuconazol) at Low Dose (LD) and High Dose (HD) for total concentrations of 8.4 $\mu g\,L^{-1}$ and 42 $\mu g\,L^{-1}$ respectively. To assess that, 2-D proteomic analysis was performed to identify the differentially expressed proteins in the liver of the exposed fish, as liver is an essential organ for the detoxification of pollutants and systemic metabolic regulation. Proteome profiles were then compared using pathway enrichment analysis software to determine the biological functions involved in the individual or combined stress response and to predict the potential deleterious outcomes at higher biological levels.

## 2. Materials and methods

### 2.1. Chemicals

Pesticides were obtained from Sigma-Aldrich (St. Louis, MO, USA): S-metolachlor (CAS-No: 87392-12-9, PESTANAL®, analytical standard, 98,4% pure), isoproturon (CAS-No: 34123-59-6, PESTANAL®, analytical standard, 99% pure), linuron (CAS-No: 330-55-2, PESTANAL®, analytical standard, 99,7% pure), atrazine-desethyl (CAS-No: 6190-65-4, PESTANAL®, analytical standard, 99,5% pure), aclonifen (CAS-No: 74070-46-5, PESTANAL®, analytical standard, 99,8% pure), pendimethalin (CAS-No: 40487-42-1, PROWL®, analytical standard, 98,8% pure), tebuconazol (CAS-No:107534-96-3, PESTANAL®, analytical standard, 99,3% pure). Acetone (CAS: 67-64-1, Fisher Chemical, HPLC solvent) was purchased from Fisher Scientific (Illkirch, France).

### 2.2. Fish model and thermal conditions

The goldfish (*Carassius auratus*) is an Asian species introduced in French lentic water areas during the twentieth century (e.g., ponds, backwaters of rivers, floodplain waterbodies) (Keith et al., 2011). In this study, the goldfish was chosen as a biological model because of (1) its large distribution worldwide, (2) its sensitivity to a wide range of stresses, (3) its resistance to thermal stress and hypoxia, (4) its easy maintenance and acclimatization in captivity and (5) its sufficient size to evaluate the effects of pollutants on organs without having to "pool" individuals. Consequently, its use in toxicological studies is growing fast (Fatima et al., 2007; Feng et al., 2013; Li et al., 2014; Wang et al., 2008; Xu et al., 2015; Zheng et al., 2013).

The temperature tolerance of the goldfish is large: between 0.3 °C and 12.6 °C for the lower limit and 30.8° C and 43.6° C for the upper limit. The high and low limits are a function of the acclimatization of the individuals (Ford and Beitinger, 2005). The optimum temperature is around 25–28 °C (Fry and Hart, 1946). The two different experimental temperatures (i.e. 22 and 32 °C) were chosen for two reasons; (1) They are in the tolerance range of the goldfish and on both sides of its thermal optimum (Fry and Hart, 1946), and (2) The highest temperature reflects the extreme thermal conditions observed in the Garonne river during heat waves (Croze et al., 2007). These phenomena, which last several days and are likely to induce physiological thermal stress on aquatic species, are predicted to increase in frequency and intensity with Climate change (IPCC, 2014).

Fish in the size range of 10–12 cm were purchased from the fish farm Carpio (Consac, France). They were first acclimatized for a minimum of two weeks in opaque tanks under controlled conditions (18 °C with a 12:12 h light regime). Water was aerated and dechlorinated prior to fish introduction. Half of the water was