Environmental Pollution 237 (2018) 442–451

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Ecotoxicological effects of the herbicide glyphosate in non-target aquatic species: Transcriptional responses in the mussel *Mytilus galloprovincialis*☆

M. Milan [a,*,1], G. Dalla Rovere [a,1], M. Smits [a,b], S. Ferraresso [a], P. Pastore [c], M.G. Marin [d], S. Bogialli [c], T. Patarnello [a], L. Bargelloni [a,e,1], V. Matozzo [d,1]

[a] Department of Comparative Biomedicine and Food Science, University of Padova, Viale dell'Università 16, 35020 Legnaro, PD, Italy
[b] Marine Environmental Science Laboratory (LEMAR), Université de Bretagne Occidentale -Rue Dumont d'Urville, 29280 Plouzané - IUEM Technopole Brest-Iroise, France
[c] Department of Chemical Sciences, University of Padova, Via Marzolo 1, 35131 Padova, Italy
[d] Department of Biology, University of Padova, Via Ugo Bassi 58/B, 35131 Padova, Italy
[e] CONISMA - Consorzio Nazionale Interuniversitario per le Scienze del Mare, Roma, Italy

ARTICLE INFO

Article history:
Received 20 November 2017
Received in revised form
26 January 2018
Accepted 16 February 2018
Available online 15 March 2018

Keywords:
Glyphosate
Herbicide
Mytilus galloprovincialis
Bivalve
Transcriptomics

ABSTRACT

Glyphosate has been the most widely used herbicide worldwide over the last three decades, raising increasing concerns for its potential impacts on environmental and human health. Recent studies revealed that glyphosate occurs in soil, surface water, and groundwater, and residues are found at all levels of the food chain, such as drinking water, plants, animals, and even in humans. While research has demonstrated that glyphosate can induce a broad range of biological effects in exposed organisms, the global molecular mechanisms of action still need to be elucidated, in particular for marine species. In this study, we characterized for the first time the molecular mechanisms of action of glyphosate in a marine bivalve species after exposure to environmentally realistic concentrations. To reach such a goal, Mediterranean mussels *Mytilus galloprovincialis*, an ecologically and economically relevant species, were exposed for 21 days to 10, 100, and 1000 μg/L and digestive gland transcriptional profiles were investigated through RNA-seq. Differential expression analysis identified a total of 111, 124, and 211 differentially regulated transcripts at glyphosate concentrations of 10, 100, and 1000 μg/L, respectively. Five genes were found consistently differentially expressed at all investigated concentrations, including SERP2, which plays a role in the protection of unfolded target proteins against degradation, the anti-apoptotic protein GIMAP5, and MTMR14, which is involved in macroautophagy. Functional analysis of differentially expressed genes reveals the disruption of several key biological processes, such as energy metabolism and Ca^{2+} homeostasis, cell signalling, and endoplasmic reticulum stress response. Together, the results obtained suggest that the presence of glyphosate in the marine ecosystem should raise particular concern because of its significant effects even at the lowest concentration.

1. Introduction

Glyphosate, which represents the primary active constituent of the commercial pesticide Roundup (RD), is a broad-spectrum, nonselective, systemic herbicide, currently among the most widely used agricultural chemicals on a global scale (Annett et al., 2014; Cattani et al., 2014; Myers et al., 2016). This organophosphorus compound is highly effective because of its ability to reduce protein synthesis through specific inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway that governs the synthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan in plants, algae, fungi, and some microorganisms (Barry et al., 1992; Devine et al., 1993; Franz et al., 1997; Shehata et al., 2013).

Due to its widespread use in agriculture, forestry, urban areas,

☆ This paper has been recommended for acceptance by Dr. Harmon Sarah Michele.

* Corresponding author.
E-mail address: massimo.milan@unipd.it (M. Milan).
[1] Equal contribution.

https://doi.org/10.1016/j.envpol.2018.02.049
0269-7491/

and aquaculture as a plant growth regulator, glyphosate can easily spread throughout ecosystems, including surface waters, thus reaching plants, animals, and the food chain. Despite the fact that glyphosate is generally considered to have a low contamination potential in surface waters or groundwater (Vereecken, 2005), recent investigations on glyphosate and amino-methylphosphonic acid (AMPA), the microbial transformation product of glyphosate, have raised increasing concerns about the potential risks to the aquatic environment. Glyphosate concentrations ranging between 10 and 45 μg/L have been measured in rivers (e.g. Struger et al., 2008), while much higher concentrations (>320 μg/L) were detected in wetland environments and ponds after glyphosate application to nearby fields (e.g. Battaglin et al., 2009). Kemp et al. (1983) estimated that up to 2% of the herbicides applied to agriculture fields end up in coastal waters, and persistence of glyphosate in seawater has been demonstrated to be closely related to light exposure and temperature, with a half-life ranging between 47 and 315 days (Mercurio et al., 2014). In Italy, the Italian National Institute for Environmental Protection and Research (ISPRA) highlighted that glyphosate has been responsible for exceeding the environmental quality standards of water in 76 cases (25.2%) out of 302 surface water monitoring stations (ISPRA, 2016). Regarding marine environments, Wang et al. (2016) measured glyphosate concentrations ranging from 13 μg/L to 1377 μg/L in seawater samples collected during a cruise in the Western Pacific in April 2015. In water samples from ten German Baltic estuaries, glyphosate concentrations ranged from 28 to 1690 ng/L and decreased along the salinity gradient, demonstrating the transport of this compound to the Baltic Sea (Skeff et al., 2015). A survey of emerging contaminants in sediment samples from the estuarine receiving environment around Auckland (New Zealand) demonstrated the presence of relatively high levels of glyphosate (up to 950 ng/g) at residential sites (Stewart et al., 2014). In addition, the biodegradation of glyphosate was quantified using standard “simulation” flask tests with native bacterial populations and coastal seawater from the Great Barrier Reef. The half-life for glyphosate at 25 °C in low-light was 47 days, 267 days in the dark at 25 °C and 315 days in the dark at 31 °C (the longest persistence reported for this herbicide) (Mercurio et al., 2014). The authors concluded that the long persistence of glyphosate in flask experiments was indicative of reduced degradation in particular conditions such as flood events and suggested the release of dissolved and sediment-bound herbicide into the lagoon (Mercurio et al., 2014).

While the mode of action of glyphosate is specific to plants, several studies have demonstrated a wide range of toxicological effects in vertebrate and invertebrate species, including aquatic organisms. Glyphosate and its commercial formulation Roundup have been shown to increase oxidative stress in fish species by enhancing ROS generation and inhibiting antioxidant systems, as demonstrated in *Prochilodus lineatus* exposed for 6, 24 and 96 h to 10 mg/L of Roundup (Cavalcante et al., 2010). In addition, exposure to 10 mg/L Roundup was shown to inhibit acetylcholinesterase (AChE) activity in brain and muscle tissues from *Prochilodus lineatus* (Modesto and Martinez, 2010). Genotoxicity of glyphosate formulation was demonstrated in the goldfish *Carassius auratus* (Çavaş and Könen, 2007) while DNA damage was found in *Anguilla anguilla* exposed to Roundup (58 and 116 μg/L) and in the catfish *Corydoras paleatus* after treatment with a Roundup concentration of 6.67 μg/L, corresponding to 3.20 μg/L glyphosate (Guilherme et al., 2010, 2012; De Castilhos Ghisi and Cestari, 2013). Potential effects on reproduction through the disruption of steroid hormone synthesis and increased oxidative stress were also reported in zebrafish (*Danio rerio*) after exposure to 10 mg/L of glyphosate and Roundup (Uren Webster et al., 2014). The same authors also demonstrated the altered expression of several transcripts coding for components of the antioxidant system, stress-response proteins, energy metabolism, and pro-apoptotic signalling molecules in the brown trout *Salmo trutta* exposed to 0.01, 0.5 and 10 mg/L of glyphosate and Roundup (Uren Webster and Santos, 2015). Several studies on the biological effects of glyphosate and other herbicides have also been carried out in aquatic invertebrates. Among the most interesting results, the Pacific oyster *Crassostrea gigas* showed changes in xenobiotic detoxification activity, energy production, and immune system response (Tanguy et al., 2005; Epelboin et al., 2015), while increased glutathione-s-transferase (GST) and alkaline phosphatase (ALP) activities and lipid peroxidation were observed in the mussel *Limnoperna fortunei* treated with nominal glyphosate concentrations of 1, 3 and 6 mg/L (Iummato et al., 2013).

Despite the aforementioned studies, which represent only a fraction of the current literature, there is still little information concerning the molecular mechanisms of action in marine organisms, particularly in bivalves. Transcriptional analyses may represent a powerful tool for the investigation of molecular responses to chemicals, as well as provide valuable data to predict potential adverse health effects on organisms and ecosystems. Recently, several studies performed using DNA microarrays in bivalve species demonstrated that transcriptional data provide extremely useful information regarding the main metabolic pathways involved in the response to environmental pollutants (Milan et al., 2013a, 2013b, 2015, 2016; Avio et al., 2015; Mezzelani et al., 2016). The recent development of Next Generation Sequencing (NGS) technologies has made it possible to conduct transcriptional analyses on any environmentally relevant species through tools such as RNA sequencing (RNA-seq), providing detailed information on the global molecular mechanisms involved in toxicity. To date, these tools are still scarcely used in ecotoxicology, though their potential is increasingly acknowledged by the scientific community.

This study was aimed at evaluating, through a RNA-seq approach, the mode of action of glyphosate in the mussel *M. galloprovincialis*, an edible bivalve species of great commercial interest that is widespread in marine coastal areas and extensively used as a model organism in ecotoxicological studies. To this end, transcriptional profiles were investigated in mussels after a long-term exposure (21 days) to environmentally realistic concentrations of glyphosate (10, 100, 1000 μg/L).

2. Materials and methods

2.1. Mussel exposure to glyphosate and tissue sampling

Specimens of *M. galloprovincialis* (6.5–7 cm shell length) were collected from a reference site located in the southern basin of the Lagoon of Venice (Italy). Before glyphosate exposure, mussels were acclimated in the laboratory for 5 days using large aquaria with aerated natural seawater (salinity of 35 ± 1 psu, temperature of 18 ± 0.5 °C); they were fed daily with microalgae (*Isochrysis galbana*, at an initial concentration of about 500,000 cells/L). Only mussels that showed signs of good health status (secretion of new byssal threads and reattachment to aquarium surface) were subsequently selected for exposure to glyphosate.

A stock solution (0.1 g/L) of glyphosate ($C_3H_8NO_5P$; CAS: 1071-83-6; molecular weight: 169.07; log K_{ow}: −4.59 to −1.70) (Sigma-Aldrich, Milano, Italy) was prepared in distilled water, whereas working solutions were prepared by diluting the stock solution in seawater.

In order to avoid spawning and to reduce possible additional stress, the exposure was performed far from the sexual maturity period of the mussels (June 2016). Animals (in total 70 per condition) were exposed for 21 days to 0 (control), 10 μg/L (LG: low concentration), 100 μg/L (MG: medium concentration) or 1000 μg/L