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Specific Proteomic Response of *Unio pictorum* Mussel to a Mixture of Glyphosate and Microcystin-LR

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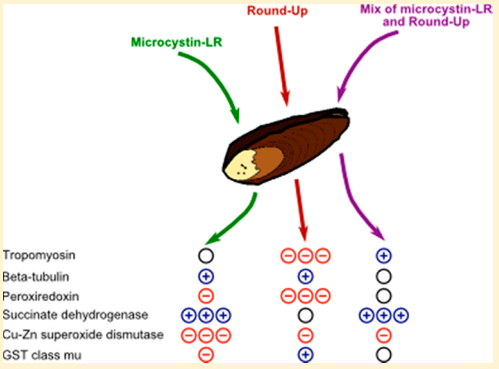
Supporting Information

ABSTRACT: Cyanobacterial toxins and pesticides regularly impact freshwaters. Microcystin-LR is one of the most toxic and common cyanobacterial toxins whereas glyphosate is the active ingredient of a widely use herbicide. As filter feeders, freshwater mussels are particularly exposed. Like many native bivalve species, *Unio pictorum* suffers from a continuous decline in Europe. In order to get a deeper insight of its response to contaminants, *U. pictorum* was exposed to either 10 μg L^{-1} of microcystin-LR or 10 μg L^{-1} of glyphosate or a mixture of both. Proteins of the digestive glands were extracted and analyzed by DIGE. Gel analysis revealed 103 spots with statistical variations, and the response seems to be less toward glyphosate than to microcystin-LR. Specific spots have variations only when exposed to the mixture, showing that there is an interaction of both contaminants in the responses triggered. The proteins of 30 spots have been identified. They belong mostly to the cytoskeleton family, but proteins of the oxidative pathway, detoxification, and energetic metabolism were affected either by glyphosate or microcystin-LR or by the mixture. These results demonstrate the importance to study contaminants at low concentrations representative of those found in the field and that multicontaminations can lead to different response pathways.



KEYWORDS: *DIGE, cyanobacteria, Roundup, proteomics, bivalve, Unionid*

1. INTRODUCTION

For many anthropogenic pollutants, either freshwater systems are the temporarily transport system or their sediments provide the long-term if not final sink.[1] Pesticides, polycyclic aromatic hydrocarbons, heavy metals, pharmaceutical compounds, and natural cyanobacterial toxins can be found at low concentrations and in various mixtures in both water and sediments, depending on the physicochemical properties of the substance.[2−5] Hence, organisms in contaminated waters are continuously exposed to a mixture of different contaminant varieties. Assessing the impact of those mixtures on organisms includes questions about prediction of potentially harmful effects from single to mixed exposure, as well as the possible interactions of different compounds at low concentrations. Indeed the toxicity of a mixture is usually different from that of the components.[6]

Rivers and lakes adjacent to agricultural fields suffer from the introduction of pesticides and surplus nutrients, causing eutrophication. Among the pesticides, glyphosate is one of the mostly used herbicides worldwide.[7] In 2007, almost 90,000 tons were sold in the U.S.,[8] of which almost 90% were applied as postemerge herbicides within the agricultural sector for wheat, mice, soja, and other crops. Glyphosate is one of the most common pesticides found in freshwater, with concentration values reaching up to 430 μg L^{-1}.[2,9] This herbicide acts by inhibiting the amino acids synthesis through the shikimic acid pathway, thus leading to an inhibition of protein synthesis in plants.[10] Specifically, it blocks the 3-phosphoshikimate 1-carboxyvinyltransferase, which exists only in plants, fungi, and bacteria, but it is not a selective herbicide. Indeed, concerning vertebrates, acute toxicity of glyphosate is relatively low; for example, in rats the LD_{50} is around 5000 mg kg^{-1}.[11] However, in commercial formulations, adjuvants and surfactants improve absorbance of glyphosate, thus increasing toxicity.[12−14] Moreover, aquatic animals, such as fishes, mussels, and amphibians, are more sensitive than terrestrial animals.[12,15−17] Harmful effects of glyphosate include oxidative stress[18−22] and DNA damage;[23−25] it alters glycogen reservoirs[13,18,26] and inhibits acetylcholine esterase.[27−29] Interestingly, activation of antioxidant and detoxification processes may also reduce its effects.[20,21,30−32] Taking all this into account, in Europe the

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maximal threshold allowed for a single pesticide is 0.1 μg L^{-1} in drinking water and 2 μg L^{-1} in freshwater.[33]

The eutrophication of freshwaters favors the phytoplankton community to shift toward cyanobacteria, which may form dense and recurrent blooms. Those blooms pose a natural, but evident risk for ecosystem and human health due to the capacity of many cyanobacteria to produce toxic or bioactive metabolites.[34] One of the most common cyanotoxins are microcystins: they are cyclic heptapeptides with uncommon modified amino acids and two variable amino acids, leading to more than 80 structural variants.[35] In temperate regions, microcystin-LR is the most common and quite toxic variant.[36] Its main target in vertebrates is the liver, as it accumulates, thereby entering hepatocytes via an organic anion transport polypeptide.[37] The effect however is the same in all organisms (apart from cyanobacteria themselves): it binds and inactivates protein phosphatase of types 1 and 2A, which are involved in regulation pathways.[38] Noxious consequences include deregulation of the MAPK pathway, oxidative stress, depletion of glycogen, and damage of DNA, visible by a disruption of the cytoskeleton (reviewed in refs 39 and 40). Depending on the exposure concentration and duration, microcystin-LR can lead to cell death, death of the organism, or proliferation of initiated cells.[41,42] Thus, the WHO established a guideline value of maximum 1 μg L^{-1} in drinking water.[33]

However, within exposure concentration and duration limits, microcystins can be detoxified in aquatic organisms via conjugation to glutathione by the glutathione *S*-transferase (GST) enzyme system.[43] This enhances water solubility, decreases the binding to protein phosphatases, and aids excretion, e.g. by P-glycoprotein.[44,45] Enzymatic and nonenzymatic antioxidant mechanisms quench the oxidative stress caused by cyanobacterial toxins (reviewed in ref 46).

In waters contaminated with anthropogenic pollutants or cyanobacteria, organisms, in particular those with a limited spatial activity range, have to develop physiological adaptation strategies to live within those exposure scenarios. Those adaptations may include increased detoxification and excretion pathways, enhanced quenching for oxidative stress molecules (ROS, reactive oxygen species) or metals, and, moreover, activated repair systems for cellular damage at protein, lipid, and nuclear acid levels.[47]

One organism group with limited spatial displacement is mussels, living either attached on hard substrates or buried within soft sediments. While buried, they may encounter being exposed to higher concentrations of pollutants in the sediment pore water via their foot, simultaneously feeding by filtering the water for small particles, hence also bioaccumulating contaminants from the water.[48,49] Both habitat and feeding add to a risk of high exposure for several mussel species. Hence, pollution of freshwater environments may contribute to the worldwide decline of freshwater bivalves, in particular of the Unionidae superfamily, of which several species are close to extinction.[12,50,51]

Unionid species are native in Europe and are listed as endangered in some countries. Nevertheless, several Unionid species have been used for assessment of water quality, e.g. for bioaccumulation studies of metals[52] or cyanobacterial toxins.[53] Biomarker reactions of *Unio* sp. indicated responses in polluted waters by altered antioxidant and biotransformation enzymes, increased metallothionines, oxidative damage, or affected glutathione pool or DNA damage.[54−58]

Instead of investigating a single biomarker reaction, proteomics and particularly 2D-gel based resolution provide the possibility to quantify variations of thousands of proteins at once, to better understand the response pathway to a pollutant or to identify specific patterns of reactions.[59] However, whereas genetic information is limited, identification of the proteins may become a challenge and depends on available information in protein databases about the species of interest or relatively closely related species.[60] 2D-gel based proteomic techniques have been successfully applied in fish, investigating proteome response to cyanobacteria and their toxins.[61−65]

Due to their restricted action radius and filter feeding, mussels are valued biomonitoring organisms, for which proteomics is increasingly applied; however, marine species are more frequently investigated than freshwater ones.[66,67] Very few studies so far investigated effects of cyanobacteria and their toxins using a proteomic approach in mussels, e.g. revealing changes of structural and metabolic proteins.[68,69]

Concerning the pesticide Roundup or its active ingredient glyphosate, proteomic studies focused on crop plants (e.g. refs 70 and 71), and to our knowledge, a single study was performed on mice,[72] whereas none of the investigated animals could be exposed in the environment. As glyphosate is used as a postemerge pesticide, recommended for corn until 30 cm, it may occur in lakes simultaneously with cyanobacterial blooms. In the present study, we studied the influence of exposure to microcystin-LR and Roundup or a mixture of both contaminants on protein expression in the digestive glands of *Unio pictorum*. Digestive glands accumulate microcystin-LR to a higher extent than other tissues, and hence, we expected these to be most affected.

A seven day exposure should mimic the environmental situation during a bloom decay; however, to exclude the influence of the manifold other bioactive cyanobacterial compounds, the pure toxin was chosen for the experiments. The DIGE technology was applied to study the protein profile in order to identify proteins that are either induced or suppressed when mussels are exposed to these contaminants singly or as a mixture.

2. MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma Aldrich and of the highest purity, if not stated specifically.

Mussels Sampling and Acclimation

Unio pictorum specimens with a shell length between 8 and 9.6 cm were sampled in March 2012 in Étang de Boulet in Feins (Ille-et-Vilaine, France). Mussels were acclimatized under a 14/10 light cycle for five days at 15 °C, five days at 18 °C, and then five days at 20 °C. Water was progressively changed from water of the sampling site to artificial freshwater (AFW) containing 0.1 g of sea salt, 0.103 g of $NaHCO_3$, and 0.2 g of $CaCl_2$ in deionized water. They were fed daily *ad libitum* with *Spirulina* sp. lyophilized powder.

Experimental Setup

Roundup Flash was used as the commercial formulation of glyphosate with 450 g L^{-1} active ingredient, isopropylamine salt of glyphosate, and 90 g L^{-1} etheramines. Microcystin-LR (ENZO Life Sciences) stock solution was prepared to be 1 g L^{-1} in methanol. Mussels were exposed for 7 days to either 10 μg L^{-1} of microcystin-LR, 10 μg L^{-1} of glyphosate, or a mix of

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10 $\mu g\ L^{-1}$ of microcystin-LR and 10 $\mu g\ L^{-1}$ of glyphosate, all diluted from the stock in AFW. To exclude effects biased by methanol, the control, AFW, was enriched with 0.001% methanol, corresponding to the amount of the microcystin-LR treatment. Each individual mussel was placed in 1 L of aerated media with daily renewal. Six biological replicates for each condition were conducted. Mussels were fed daily with 12 mg of *Spirulina* sp. lyophilized powder per specimen in all treatments to exclude food related effects. Mussels were sacrificed about 24 h after the last feeding to ensure all food was processed. Concentrations of microcystin-LR in the exposure medium were confirmed at medium renewal (mean and STD of all renewals: 10.34 ± 1.64 $\mu g\ L^{-1}$) according to Dahlmann et al.,[73] separating on an Agilent 1200 HPLC (Zorbax Eclipse XDPC C_{18}, at 40 °C), increasing acetonitrile 0.1% FA from 25% to 75% in 5.5 min followed by a 1 min washing step and equilibrating for 6 min. Identification of microcystin-LR was conducted on an Agilent 6410 TripleQuad LC/MS (at 300 °C, gas flow 10 L/min, nebulizer: 50 psi, capillary 4500 V, fragmentor 190, and collision energy at 85), using the m/z of 995.5 to 135.

Digestive Gland Extract Preparation

At the end of exposure, mussels were rinsed briefly and opened by cutting the shell adductor muscle, and digestive glands of mussels were dissected, immediately frozen in liquid nitrogen, and kept at −80 °C until protein extraction. For protein extraction, ca. 60 mg of digestive gland tissue was homogenized in 300 μL of 7 M urea, 2 M thiourea, and 2% CHAPS and centrifuged twice at 25000*g* for 15 min at 4 °C to remove any cell debris. Total protein concentration was determined using the Bradford Protein Assay Kit (BioRad) according to the manufacturer's instructions. Absence of protein degradation was confirmed by SDS-PAGE (NuPAGE Novex 12% Bis-Tris gels ran in MOPS buffer, Life technologies). As the SDS-PAGE resulted in two different patterns, which we assume to be related to the gender of the mussels, we selected for further analysis three out of the six biological samples producing very similar protein patterns per condition (Supporting Information Figure 2).

DIGE

Fifty micrograms of protein extracts from individual biological replicates of control and exposed animals were minimally labeled with 400 pmol of cyanine dyes Cy3 or Cy5 (GE Healthcare), in a reciprocal manner (i.e., dye swapping) according to a standardized protocol.[74] Fifty micrograms of combined protein extracts derived from a mix of all samples were labeled with 400 pmol of Cy2 and used as internal standard for the normalization of spot abundances. Individual Cy2, Cy3, and Cy5 labeling reactions were mixed and incubated in a solubilization buffer (DeStreak Rehydration solution; GE Healthcare) containing 0.5% IPG buffer pH 4−7 (GE Healthcare) in a 450 μL final volume, for 1 h at room temperature.

Isoelectric focusing (IEF) was performed with pH 4−7 NL 24 cm IPG strips (GE Healthcare) using an IPGphor isoelectric focusing apparatus (GE Healthcare) at 20 °C and with 50 μA/strip to reach a total of 60 kVh. Following IEF, the IPG strips were equilibrated for 15 min at room temperature in SERVA IPG-strip equilibration buffer (Serva Electrophoresis) containing SDS and 54 mM DTT, and then for 15 min at room temperature with the same buffer containing 112.6 mM iodoacetamide. Equilibrated IPG strips were transferred onto a 26 cm × 20 cm 12.5% acrylamide gel cast onto nonfluorescent gel support (Serva Electrophoresis). The protein separation was carried out in anodal and cathodal buffers (Serva Electrophoresis) at 1 W/gel for 1 h and 2.5 W/gel overnight. After electrophoresis, gels were scanned at a resolution of 200 μm (pixel size) using a Typhoon 9400 imager (GE Healthcare) in fluorescence mode using appropriate excitation and emission wavelengths, filter, and photomultiplier (PMT) sensitivity for each dye (PMT values: 415, 400, and 430 for Cy2, Cy3, and Cy5, respectively).

The global fluorescence intensities of the scanned images were normalized by adjusting the exposure times to the acquired average pixel values. For each biological replicate, one gel was conducted.

Gel Image and Multivariate Analysis

The gel image analysis was performed using the DeCyder software (version 5.01). For each protein, the mean normalized abundance (n = 3) was used to calculate fold changes among treatments. Cutoff values of >1.5-fold in absolute value[74] together with p-value = 0.05 (two-way ANOVA) were used for the selection of differentially modulated spots of interest. PCA (principal component analysis) and PLS-DA (partially least squared discriminant analysis) analyses were performed using the R software (version 2.15.1) and mixomics package.

Preparative Gel, Spot Picking, and Digestion

Four hundred micrograms of a mix of protein extracts from all analyzed samples (i.e., internal standard) were loaded on preparative gels and run in the same experimental conditions as for the analytical gels. Gels were stained with LavaPurple and scanned using a Typhoon 9400 imager (GE Healthcare) in fluorescence mode using 532 nm excitation and 560 nm emission wavelengths. Gel images were analyzed using Decyder software and matched against the spots referenced in the picking list created after the detection of the significantly up- or down-regulated protein signals in the analytical gels. The picking list was exported to Screen Picker (Proteomics Consult) for spot picking. In gel digestion was performed as previously described[75] with minor modifications. Briefly, gel pieces were washed twice in Milli-Q water, dehydrated for 15 min in 100% acetonitrile, and dried at 37 °C during 20 min. Gel pieces were then rehydrated at 4 °C for 15 min in a 50 mM NH_4HCO_3 digestion buffer containing 12.5 ng/μL of trypsin (modified, sequencing grade, Promega). Remaining digestion buffer was removed, and 30 μL of 50 mM NH_4HCO_3 was added for overnight incubation at 37 °C. Digested peptides were extracted from gel pieces by several incubations: 20 min in 70% acetonitrile/0.1% formic acid (FA), 5 min in 100% acetonitrile, and 15 min after adding the same volume of 70% acetonitrile/0.1% FA. At each step the supernatant was collected and pooled with the previous one. Pooled supernatants were evaporated in a vacuum centrifuge to reach a final volume of 40 μL.

Mass Spectrometry

The MS measurements were done with a nanoflow high-performance liquid chromatography (HPLC) system (Dionex, LC Packings Ultimate 3000) connected to a hybrid LTQ-OrbiTrap XL (Thermo Fisher Scientific) equipped with a nanoelectrospray ion source (New Objective). The HPLC system consisted of a solvent degasser nanoflow pump, a thermostat column oven kept at 30 °C, and a thermostat autosampler kept at 8 °C to reduce sample evaporation. Mobile

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phases A (99.9% Milli-Q water and 0.1% formic acid, FA (v/v)) and B (99.9% acetonitrile and 0.1% FA (v/v)) were delivered by the Ultimate 3000 nanoflow LC system (Dionex, LC Packings). Eighteen microliters of prepared peptide mixture was loaded onto a trapping precolumn (5 mm × 300 μm i.d., 300 Å pore size, Pepmap C18, 5 μm) for 3 min in 2% buffer B at a flow rate of 25 μL/min. This step was followed by reverse-phase separations at a flow rate of 0.25 μL/min using an analytical column (15 cm × 300 μm i.d., 300 Å pore size, Pepmap C18, 5 μm, Dionex, LC Packings). The gradient ranged from 2% to 35% buffer B for the first 30 min, increased to 60% buffer B until minute 40, and to 90% buffer B in minute 43. Finally, the column was washed with 90% buffer B for 9 min and equilibrated with 2% buffer B for 21 min prior to loading of the next sample.

The peptides were detected by directly eluting them from the HPLC column into the electrospray ion source of the mass spectrometer. An ESI voltage of 1.5 kV was applied to the HPLC buffer using the liquid junction provided by the nanoelectrospray ion source, and the ion transfer tube temperature was set to 200 °C. The MS instrument was operated in its data-dependent mode by automatically switching between full survey scan MS and consecutive MS/MS acquisition. Survey full scan MS spectra (mass range 400−2000) were acquired in the OrbiTrap section of the instrument with a resolution of R = 60000 at m/z 400; ion injection times are calculated for each spectrum to allow for accumulation of 10^6 ions in the OrbiTrap. The ten most intense peptide ions in each survey scan with an intensity above 2000 counts (to avoid triggering fragmentation too early during the peptide elution profile) and a charge state = 2 were sequentially isolated at a target value of 10000 and fragmented in the linear ion trap by collision induced dissociation (CID). Normalized collision energy was set to 35% with an activation time of 30 ms. Peaks selected for fragmentation were automatically put on a dynamic exclusion list for 60 s with a mass tolerance of ±10 ppm. The maximum injection time was set to 500 and 300 ms for full MS and MS/MS scan events, respectively, and for an optimal duty cycle the fragment ion spectra were recorded in the LTQ mass spectrometer in parallel with the OrbiTrap full scan detection. For OrbiTrap measurements, an external calibration was used before each injection series, ensuring an overall error mass accuracy below 5 ppm for the detected peptides. MS data were saved in RAW file format (Thermo Fisher Scientific) using XCalibur 2.0.7 with tune 2.5.5 SP1.

Protein Identification

The Proteome Discoverer 1.2 software was used to submit MS/MS data to the following database: NCBInr version of July 2012 (17701311 sequences, 6073447286 residues) using the Mascot search engine (Mascot server v2.2; http://www.matrixscience.com). Parameters were set as follows: trypsin as enzyme with one allowed miscleavage, carbamidomethylation of cysteins as fixed modification, methionine oxidation, and phosphorylation of serine, threonine, and tyrosine as variable modifications. Mass tolerance for MS and MS/MS was set at 10 ppm and 0.5 Da, respectively. Identified peptides were filtered based on Xcorr values and the Mascot score to obtain a false discovery rate of 1% and a false positive rate of 5%. As the genome of *U. pictorum* is not sequenced and hence identification efficiency was low, a de novo approach was tried with PEAKS software (version 6.0, Bioinformatics solution) with only sequences of proteins from NCBI of Protostomia (downloaded in September 2012). The settings were trypsin cleavage, parent mass error tolerance of 15 ppm, fragment mass error tolerance of 0.5 Da, carbamidomethylation as fixed modification, and oxidation of methionine as variable modification. Protein identification was validated if protein −10 log P was above 20, peptide −10 log P was above the threshold allowing the false discovery rate to be under 0.1%, and at least two different peptides were identified.

3. RESULTS

After 7 days of exposure to 10 μg L^{-1} of microcystin- LR or 10 μg L^{-1} of glyphosate or a mixture of both (confirmed in both exposures with 10.34 ± 1.64 μgL^{-1}), *U. pictorum* mussels showed no mortality and were still filtering. Mussels were clearing 0.096 μg microcystin-LR per gram of fresh weight each day in the single exposure and 0.125 μg of microcystin-LR per gram of fresh weight in the combined exposure with glyphosate. After 7 days of exposure, they cleared from the medium 0.669 ± 0.145 μg MC g fw^{-1} respectively 0.875 ± 0.179 μg MC g fw^{-1} (Supporting Information Table 1).

Spots Variations

Protein profiles of the digestive gland revealed around 2200 spots on each gel (Figure 1), of which 103 spots have

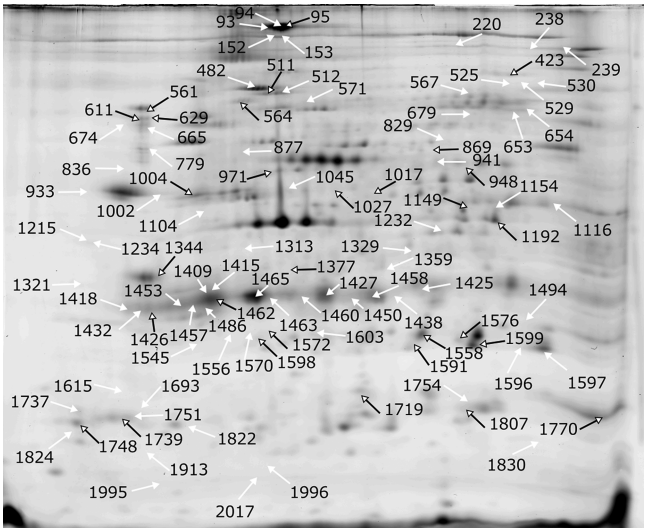

Figure 1. Localization of spots on one representative DIGE gel. The 103 spots having statistical variations are shown. The identified spots are shown by black arrows.

significant variations using two-way ANOVA due to microcystin-LR, Roundup, or an interaction of both. Only spots changed at least above 1.5-fold or below 0.67-fold among the six ratios calculated (microcystin-LR/control, Roundup/control, mix/control, Roundup/microcystin-LR, mix/microcystin-LR, mix/Roundup) have been kept. Proteins are down- or up-regulated between a 0.09-fold and 11.83-fold change. Seven spots have significant variations only for Roundup, 38 only for microcystin-LR, and 30 reacted to an interaction (the mixture) of both compounds (Figure 2). Seven spots have significant variations in the three categories. Each group of treatment is well separated by PCA when using the first three components, evidencing a specific response pattern for each exposure (Figure 3), confirmed by PLS-DA analysis (data not shown). The first component of the PCA is mostly negatively correlated to spots 1598, 1462, 1426, 1234, 1215, and 1045 and positively

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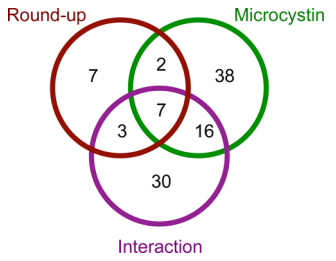

Figure 2. Number of spots statisticaly differentially expressed.

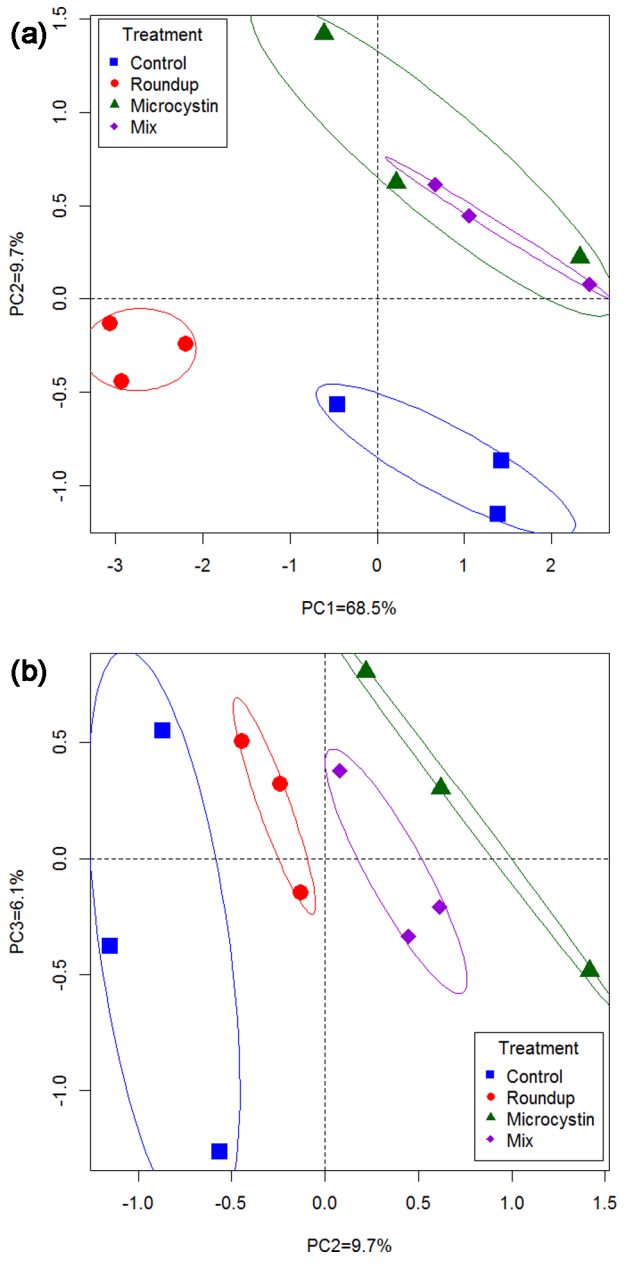

Figure 3. PCA diagrams along the first and second component (A) and the second and third component (B).

correlated with spots 95, 93, 94, 512, 511, 564, 561, 629, 665, 511, and 779 (Supporting Information Figure 1A). This axis mostly contributes to separate Roundup-treated samples. The second component is negatively correlated to spot 1576 and positively correlated to spot 971. The axis highlights separations between control and MC-treated samples (alone or in the mix, Supporting Information Figure 1A). In the relation between the second and third components, spots 869, 829, 836, and 1576 are mostly correlated to the second component, thus participating in the separation of the four exposures along the second component axis (Figure 3B and Supporting Information Figure 1B).

Identification

A protein amount of 54 of the changed spots was sufficient for analysis by LC-MS/MS in order to identify proteins. As only three spots could be recognized with Mascot software, *de novo* sequencing with PEAKS software was conducted to identify proteins. Using the NCBInr database and restricting it to protein sequences of Protostomia, 30 spots could be identified with at least two different peptides (Table 1). Most of these proteins were identified several times in different species, confirming the annotation. Only the top score proteins are shown in Table 1. The identified proteins are mainly from other bivalves and, hence, are relatively closely related (oyster *Crassostrea gigas*, *Saccostrea kegaki*, mussels *Mytilus galloprovincialis*, *Cristaria plicata*, *Hyriopsis cumingii*, *Hyriopsis schlegelii*, scallop *Mizuhopecten yessoensis* or *venerids*, *Pseudocardium sachalinensis*, and *Mercenaria mercenaria*). Most of the proteins functions are related to cytoskeleton (myosins, actins, tubulins) but also to oxidative stress (peroxiredoxins, ferritin, copper–zinc-superoxide dismutase) and detoxification (μ-glutathione-S-transferase, Table 2). Some discrepancies were found between theoretical and experimental molecular weight, either due to phylogenetic differences between the mussel of origin and *U. pictorum* or due to only fragments of proteins that were recovered on the gel due to the technique.

Microcystin-LR effects

In total, 63 spots have significant variations due to microcystin-LR exposure (Figure 2) compared either to control, Roundup, or the mix. Most of the 17 identified protein spots are cytoskeleton proteins and belong to the myosin family, but also actins occurred (Table 1). Some myosins are slightly down-regulated (around 1.1: spots 95 and 561) or moderately up-regulated (around 1.3: spots 511, 564, 629, and 1344). One actin is up-regulated by a factor 1.8 (spot 971), and another is down-regulated by a factor 1.4 (spot 1192). Other proteins are involved in the carbohydrate metabolism, particularly glycolyse (phosphoglycerate kinase, down-regulated by factor 1.3) and Krebs cycle (succinate dehydrogenase up-regulated by factor 2.1, malate dehydrogenase down-regulated by factor 1.1).

The oxidative stress related enzyme copper–zinc superoxide dismutase (decreased by factor 3.7) and the detoxification enzyme GST of the μ-class (decrease by 1.3 factor) are identified. Proteins of the spots 1576 decrease 1.6-fold and increase in spot 1598 1.1-fold but a mix of two proteins in those spots prevented the clear assignation. Moreover, the chaperone T complex protein 1 (TCP 1) is slightly down-regulated by a factor 1.2.

Roundup Effects

In total, 19 spots show significant variations (ANOVA) due to Roundup exposure, out of which only 7 were specific for Roundup alone (Figure 2). Most of them are down-regulated when mussels were exposed to Roundup compared to control: some of them greatly (spots 93, 665, 1597, 1737, 1739, and 1995) from 2.3-fold to 11.1-fold and some to a lesser extent, from 1.1 to 1.7 (spots 674, 1232, 1329, and 1603). The others (423, 1045, 1313, 1321, 1450, 1556, 1822, and 2017) are

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Table 1. Protein Spots Identified with PEAKS

spot	protein	NCBI id	species	score (−10 log P)	no. of unique peptides	coverage (%)	MW (kDa)	ANOVA
95	paramyosin	405966986	*Crassostrea gigas*	207.35	18	14	98	Inter, MC
482	catchin	6682323	*Mytilus galloprovincialis*	139.63	6	6	113	Inter
511	myosin heavy chain isoform B	353351562	*Doryteuthis pealeii*	160.65	4	2	221	Inter, MC
561	tropomyosin	219806592	*Scapharca broughtonii*	197.61	10	24	33	Inter, MC
564	myosin heavy chain isoform B	353351562	*Doryteuthis pealeii*	188.17	5	3	221	Inter, MC
611	tropomyosin	4468224	*Helix aspersa*	74.39	2	8	33	Inter
629	tropomyosin	11177139	*Mizuhopecten yessoensis*	105.57	4	15	33	Inter, MC
869	succinate dehydrogenase	268581093	*Caenorhabditis briggsae*	78.65	2	6	70	MC
948	T-complex protein 1 subunit α	383855040	*Megachile rotundata*	95.68	3	6	60	MC
971	actin	5881788	*Artemia franciscana*	77.71	2	7	42	MC
1004	β-tubulin	194068375	*Saccostrea kegaki*	173.54	12	26	50	Inter
1017	F1 ATP synthase α	346473631	*Amblyomma maculatum*	61.17	2	3	60	Inter
1027	β-tubulin	510354	*Onchocerca gibsoni*	100.58	2	5	50	Inter
1149	phosphoglycerate kinase	195161406	*Drosophila persimilis*	106.45	2	7	44	MC
1192	β-actin	68138000	*Callinectes sapidus*	161.13	6	21	42	MC
1344	tropomyosin	219806598	*Pseudocardium sachalinensis*	162.19	9	12	33	MC
1377	malate dehydrogenase	405952134	*Crassostrea gigas*	78.39	2	13	30	Inter, MC
1426	14−3−3 epsilon protein	357618137	*Danaus plexippus*	158.49	6	15	30	Inter
1462	actin	558673	*Limulus polyphenus*	83.61	2	7	42	Inter
1558	glutathione S-transferase class mu	152926607	*Cyphoma gibbosum*	111.23	3	10	25	MC
1572	ferritin	405978589	*Crassostrea gigas*	93.39	2	10	20	Inter
	glutathione S-transferase class mu	152926607	*Cyphoma gibbosum*	81.44	2	18	25	
1576	peroxiredoxin	306451460	*Cristaria plicata*	218.84	14	67	22	MC
	glutathione S-transferase class mu	152926607	*Cyphoma gibbosum*	82.14	2	9	25	
1591	peroxiredoxin	306451460	*Cristaria plicata*	85.53	4	31	22	Inter
1598	proteasome subunit β type 4	405969077	*Crassostrea gigas*	115.39	10	17	78	Inter
	eukaryotic translation initiation factor 3 subunit K	389611863	*Papilio xuthus*	80.54	2	10	25	
1599	peroxiredoxin	306451460	*Cristaria plicata*	96.66	3	14	22	Inter
1719	hypothetical protein	405969168	*Crassostrea gigas*	84.13	2	5	18	MC
1739	myosin essential light chain	404435710	*Hyriopsis cumingii*	183.79	8	53	17	Inter, RU
1748	myosin regulatory light chain	228390	*Mercenaria mercenaria*	168.34	5	17	18	Inter
1770	cyclophilin A	295824573	*Hyriopsis schlegelii*	99.44	5	50	17	Inter
1807	Cu−Zn superoxide dismutase	208431891	*Cristaria plicata*	92.26	2	17	16	Inter, MC

moderately up-regulated, from 1.1-fold to 2.5-fold. Only one of the spots has been identified; it is spot 1739, which is a myosin light chain, a cytoskeleton component. This myosin is down-regulated by a factor 8.3 in the Roundup exposure compared to the control.

Interaction Effects

ANOVA test indicated 56 spots with significant variations related to an interaction response of combined microcystin-LR and Roundup exposure. These spots have a moderate up- or down-regulation in the mixed exposure compared to the control, ranging from −1.5-fold to 1.6-fold change. They are −2.1 down-regulated to 2.7 up-regulated in the mix when compared to the microcystin-LR exposure. Interestingly, when comparing to the Roundup exposure, their fold change is multiplied by −2.2 to 11.8. Most of these are up-regulated by more than 1.5 (28 out 56) and even 24 by more than a factor of 3. Most of the identified spots are cytoskeleton related (myosins, actin, tubulins). When comparing the exposure of the mix to the control, myosins are slightly down-regulated (spots 482, 1739, and 1748: around 1.1) or moderately up-regulated (spots 95, 511, 512, 561, 564, 611, and 629: 1.2 to 1.6). When comparing the mix to the microcystin-LR exposure, myosins are up-regulated (from 1.1 to 2.7 for catchin) except for the light chains (1739 and 1748), which are slightly down-regulated (around 1.1). The most prominent changes are seen when comparing the mix to the Roundup exposure, as all the myosin family is greatly up-regulated by a factor 3.6 to 11.8. For the two β-tubulins (1004 and 1027), they are down-regulated comparing the mix to the other exposures, and the greatest change occurs when comparing to the Roundup exposure (−1.6 for 1004 and −1.4 for 1027). There is only a slight up-regulation for 1027 when comparing the mix to the control.

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Table 2. Statistically Significative Variations of Proteins Spots[a]

Functions	RU/C	MC/C	Mix/C	RU/MC	Mix/MC	Mix/RU	
Cytoskeletton	**Paramyosin, catchin, myosin (2), tropomyosin (4), myosin LC (2)**	**Catchin**		**Paramyosin, catchin,myosin (2), tropomyosin (4), actin, myosin LC (2)**		Beta-tubulin, beta-actin, actin	**Downregulated**
	Beta-tubulin (2) , actin	Actin	Paramyosin, myosin, actin	beta-actin	Paramyosin, **catchin**, tropomyosin	**Paramyosin, catchin, myosin (2), tropomyosin (4),** actin, **myosin LC**	**Upregulated**
Oxidative stress	**Peroxiredoxin (2)**	Peroxiredoxin, **superoxide dismutase**		**Peroxiredoxin (2)**		Ferritin	**Downregulated**
	Ferritin			Ferritin, **superoxide dismutase**	**Superoxide dismutase**	**Peroxiredoxin (2)**	**Upregulated**
Detoxification		GST				GST (2)	**Downregulated**
	GST			GST (2)			**Upregulated**
Carbohydrate metabolism				**Succinate dehydrogenase**		Phosphoglycerate kinase, malate dehydrogenase	**Downregulated**
		Succinate dehydrogenase	**Succinate dehydrogenase**	Phosphoglycerate kinase		**Succinate dehydrogenase**	**Upregulated**
Protein maturation	**Cyclophilin**			**Cyclophilin**		TCP1	**Downregulated**
	Proteasome, eIF3					**Cyclophilin**	**Upregulated**
Others						F1 ATP synthase, 14-3-3, hypothetical protein	**Downregulated**
	F1 ATP synthase			Hypothetical protein			**Upregulated**

[a]Only proteins having fold change above |1.5| are indicated. Proteins in bold have a fold change above |2|. eIF3, eukaryotic translation initiation factor 3; GST, glutathione S-transferase; myosin LC, myosin light chain; TCP1, T complex protein 1.

Only one actin (1462) shows a significant variation associated with an interaction pattern. This is also always down-regulated when comparing the mix to the other exposures and mostly with the Roundup exposure by a 1.8 factor. Three proteins involved in oxidative stress have been identified: two peroxiredoxins and superoxide dismutase. The two peroxiredoxins are slightly down-regulated when comparing the mix to the control (around 1.1) and moderatly up-regulated compared to microcystin-LR exposure (1.3). But a radical change is observed with Roundup exposure comparison: they are up-regulated by a factor 3.1 (for spot 1591) to 5.4 (spot 1599). The superoxide dismutase is also down-regulated in the mix compared to control exposure by a factor 1.3, but the biggest variations are relative to microcystin-LR exposure: it is enhanced by a factor 2.6 whereas no change is observed in comparison with Roundup.

Cyclophilin A has been identified in spot 1770, which is up-regulated when comparing the mix exposure to the other treatments, the greatest in comparison to Roundup exposure (by a factor 3.1).

Similarity of Variations

When clustering the protein spots along their variation pattern in the different samples (Figure 4), a group of 25 spots stand out. They have a common pattern of being severely down-regulated in the exposure with Roundup when comparing to control and a slight up-regulation in the exposure to microcystin-LR and in the mix. Most of these proteins are of the myosin family (paramyosin, catchin, tropomyosin, myosin heavy chain, myosin light chain). The effects of Roundup, which are quite dramatic, decreasing these proteins by a factor of 2.4 to 11.1, completely vanished in the mix. In this group of proteins, there are also two peroxiredoxins showing the same pattern. All of these spots have at least a significant variation by two-way ANOVA related to interaction except for spot 1344.

4. DISCUSSION

A proteomic approach using the DIGE technology was applied to follow changes in protein patterns and identify the most prominent ones in mussels *U. pictorum* exposed for 7 days to cyanobacterial toxin microcystin-LR, the pesticide Roundup with glyphosate as active ingredient or a mixture of both. No mortality was observed throughout our experiment, and the mussels continued to filtrate for food; hence, all exposures were of low toxicity. The 7-day exposure is comparable to a semichronic exposure in the field, for example, during a cyanobacterial bloom lysis. Both toxicants may occur simultaneously during early cyanobacterial blooms in eutrophicated water bodies and postemergent application of the herbicide in early summer. The herbicide also affects the cyanobacteria community, and in Lake Erie, Planktothrix sp. was found to be resistant, whereas Microcystis sp. blooms were decreased.[76] However, both cyanobacteria were able to endure higher concentrations (IC_{50} of growth for *P. agardhii* about 200 μM, and *M. aeruginosa* about 90 μM) compared to the concentrations used in the mussel's exposure (10 μg, ca. 0.06 μM).

Effects of Microcystin-LR

Most of the proteins that significantly varied in the digestive gland of *U. pictorum* following exposure to microcystin-LR are cytoskeleton proteins, confirming the well-known feature of

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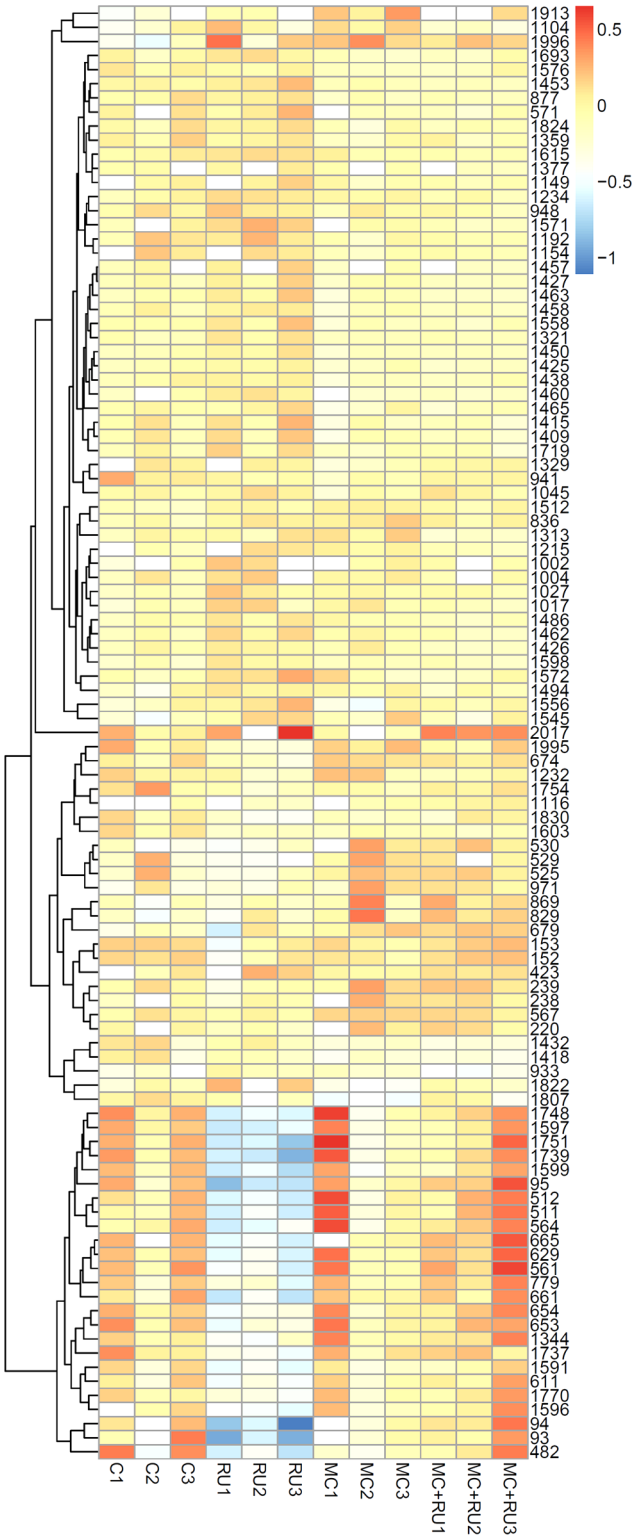

Figure 4. Heat map of differentially expressed spots. The reference is the internal standard of the DIGE. The values shown are the values of the spot volumes normalized and standardized.

microcystins disrupting the cytoskeleton.[77,78] One mechanism facilitating the disruption of microtubules is by disabling the activity regulation of MAPK proteins via their phosphorylation state, partly controlled by PP2A, which itself is inhibited by microcystins.[79] Changes in cytoskeleton proteins due to toxic stresses have frequently been reported,[59] but also more specifically due to microcystin-LR treatment, which can be directly via the PPAse inhibitions or indirectly via generation of oxidative stress, which is also a known effect of microcystins.[68] In our study, the different components of the cytoskeleton and also their various isoforms responded differently in *U. pictorum* digestive glands to microcystin-LR exposure: myosins were slightly down-regulated (paramyosin and one tropomyosin), and some (two myosin heavy chain isoform B and two tropomyosins) were up-regulated, and the two actins also reacted in both directions.[68] Identified actin and myosin chains (namely actin-3, unconventional myosin heavy chain 6, and dynein beta chain) up-regulated in digestive glands of the mussel *Corbicula fluminea*, whereas, similarly to our study, both up- and down-regulation occurred in gills. Contrary to our results, they found the metabolic enzyme phosphoglycerate kinase up-regulated in *C. fluminea* digestive glands, whereas it was slightly down-regulated (factor 1.3) in *U. pictorum*. Previous studies highlighted glycogen usage in digestive glands *U. tumidus* following exposure to microcystin-LR or cyanobacterial extract.[58] However, the pathway for further breakdown of glucose to form pyruvate for the Krebs cycle seems to be slowed down in *U. pictorum*, whereas the up-regulated succinate dehydrogenase indicated an increased Krebs cycle activity.

Similar cytoskeletal changes have been observed in mussels, exposed to the cyanobacteria *Cylindrospemopsis raciborskii* or its toxin cylindrospermopsin.[69] In addition, up-regulation of the ATPase β subunit in digestive systems of both *C. fluminea* and *M. galloprovincialis* indicated that more energy was required. In accordance with our study, they also found the glycolysis enzyme triosephosphate isomerase down-regulated in gills of *M. galloprovincialis* exposed to toxin-producing *C. raciborskii*. Another similarity is the down-regulation of chaperones, in our case the TCP 1, and in *M. galloprovincialis* the heat shock protein 60.[69] To date no study has shown that TCP 1 is deregulated upon MC-LR treatment. However, chaperones, particularly of the hsp family, should increase during stress scenarios and were affected by microcystin exposure in fish.[63,64,80] Indeed, reactive oxygen species can alter protein, notably by oxidation,[81] which in turn could trigger chaperon expression.

Detoxification of microcystin starts by conjugation to glutathione by GST isoenzymes.[43] GST activities increase due to exposure to cyanobacteria or their extracts in some mussel species, as *D. polymorpha* or *Diplodon chilensis patagonicus*, but were not modified in *Unio tumidus*.[58,82] The slight decrease of μGST in *U. pictorum* observed in the present study supports these findings by indicating that unlike other mussel species both Unionids do not seem to increase activity of this detoxification pathway. However, as several GST isoenzymes facilitate detoxification to different degrees, further investigations are necessary to confirm the capacity of Unionids to cope with this toxin.[83] Another way of regulating activity of an enzyme may be facilitated by, for example, phosphorylation status, which we did not investigate in our study. Comparably, in *M. galloprovincialis* and *C. fluminea*, activities of the detoxification enzyme GST and the antioxidant enzyme GPx were affected, but no changes of the corresponding protein levels were observed using 2D gel analysis,[69] which could be due to modification of the activation state of the enzyme, which is already present in the cell.

We could evidence changes in some oxidative stress defense proteins, but in contrast to our expectation, the antioxidant enzyme Cu−Zn superoxide dismutase was strongly down-

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regulated during the exposure to microcystin-LR. Other antioxidant enzymes such as catalase or glutathione peroxidase were not among the identified proteins. Superoxide dismutase enhanced its activity in gills and mantle in *Unio tumidus* following seven days exposure to 50 $\mu g\ L^{-1}$ of microcystin-LR but not at 10 $\mu g\ L^{-1}$ nor in the digestive gland at both concentrations.[58] On the contrary, the other species investigated, the invasive *Dreissena polymorpha*, responded with activated biotransformation and antioxidant enzymes in the same exposure scenario.[58] In Zebrafish, activities of both antioxidant enzymes superoxide dismutase and glutathione peroxidase were activated only up to 1 $\mu g\ L^{-1}$ microcystin-LR or -RR; before, at higher toxin concentration, and with longer exposure, they became exhausted.[84]

Effects of Roundup

It seems that *U. pictorum* is less affected by Roundup alone than by the mix or microcystin-LR alone, as less spots display significant variations (19 spots were significantly altered compared to 56 and 63 and only 7 specific for Roundup; Figure 2). Only one spot has been identified, which is a myosin essential light chain. It is strongly down-regulated in exposure to Roundup. It has been shown that glyphosate can impair cytoskeleton, and particularly microtubules and actin filaments in cell lines.[85,86]

Multiexposure vs Single Exposure

Proteins were not affected identically when *U. pictorum* was subjected to microcystin-LR or Roundup or when exposed to the mix of both. ANOVA revealed 56 spots with significant variations related to an interaction of the two compounds and 30 spots with only significant variations specific to this interaction. Up-regulation occurred more often in mussels exposed to the mixture, compared to single exposures.

Some proteins were regulated in opposite ways in the single exposures compared to the mixture: for the spot 95, a myosin, there was a slight down-regulation when exposed to microcystin-LR by a factor 1.1, a down-regulation by a factor 7.7 in the Roundup exposure, and an up-regulation in the mix by a factor 1.5 when compared to the control, indicating the stronger effects of microcystin-LR

Other proteins were affected by either microcystin-LR or Roundup treatment alone whereas the combination of both caused the effect to vanish. As an example, the spot 482, a protein of the myosin family, was decreased by factor 3 when comparing microcystin-LR exposure to control and by factor 6 when comparing Roundup exposure to control, and almost no decrease was observed in the mix when compared to control (by a factor of 1.1). For another spot, 1004, which is a tubulin, we observed a slight up-regulation when comparing the microcystin-LR exposure to the control (by a factor of 1.2), a greater up-regulation in the exposure to Roundup compared to controls (by a factor of 1.6), and no effects when exposed to the mix compared to the control. This is the same case as for 482, which means that, in the mix, the effects of both compounds are annihilated except that there is an up-regulation when having the compounds alone.

When looking at the variation pattern of all spots (heatmap, Figure 4), 25 spots seemed down-regulated when comparing exposures to Roundup alone to microcystin-LR alone or the mixture. But the two-way ANOVA detects only significances of the interaction. This is due to the fact that two-way ANOVA compares all samples containing Roundup (e.g., Roundup and mix exposures) to samples not having Roundup (control and microcystin-LR exposures). A one-way ANOVA may have shown that there was a significant variation between control and Roundup exposure for these spots. These proteins have an opposite variation in Roundup treated samples (decreased expression), but in the mixture, most of them increased due to the microcystin-LR effect. Most of these proteins belong to the myosin family, and unlike in the microcystin-LR treatments, the actin proteins were not identified. Down-regulation of myosin proteins may impair intracellular transport processes. Down-regulation of peroxiredoxin may indicate lower oxidative stress or an impaired or changed antioxidant activity, as peroxiredoxin function is to reduce organic peroxides; however, other measures of oxidative damage would need to undermine this. If cyclophilin A has the same function in invertebrates as in vertebrates, namely within the immune system, protein folding and in response to oxidative stress, we assume that some of its protective functions were required in the mussels due to the pesticide and cyanotoxin stress.

5. CONCLUSION

Freshwater mussels, such as Unionids, may suffer in the environment from multiple contaminants exposures, from which we selected an herbicide (Roundup with the active ingredient glyphosate) and the cyanobacterial toxin MC-LR. A semichronic (7 days) exposure to low concentrations both singly and as a mixture was conducted with *Unio pictorum*. This is the first study of digestive glands proteomic profiles in *U. pictorum* using the DIGE method. The lack of genetic information on the studied species limited the protein recognition; nevertheless, 30 spots were identified using *de novo* sequencing by mass spectrometry. We evidenced effects at low concentrations for both substances. In several cases, contrarily to the single exposure reaction, the effect of the mixture was more complex and not additive. In addition to the expected cytoskeleton related proteins, enzymes related to antioxidant and detoxification processes were identified. As they were often down-regulated, we assume an impairment of the mussel capacity to react to environmental stress.

■ ASSOCIATED CONTENT

Supporting Information

Table showing cumulated amounts of microcystin-LR cleared from the media per gram of mussel fresh weight over 7 days exposure period, and figures showing variable representations of PCA, and SDS-PAGE showing the two different patterns distinguished for the subsampling. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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