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Dynamic Multiple Reaction Monitoring of amphipod *Gammarus fossarum* caeca expands molecular information for understanding the impact of contaminants

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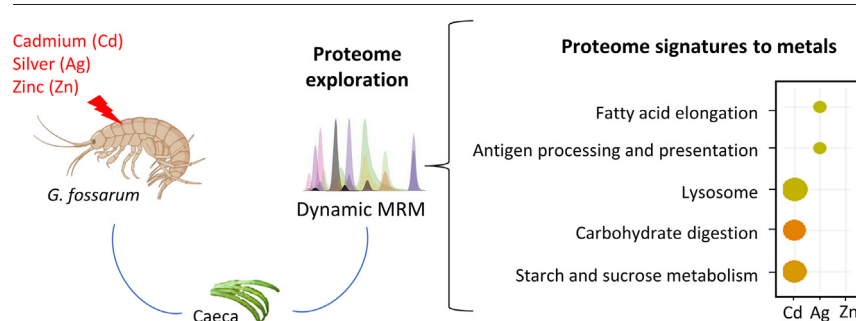
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HIGHLIGHTS

- Development of a dMRM method for comprehensive analysis of organ proteomes
- Impacts of sublethal concentrations of metals on the caecal proteome of *G. fossarum*
- Metal-specific proteomic signatures, shedding light on affected molecular pathways
- Innovative strategy for the discovery of molecular biomarkers in sentinel species

GRAPHICAL ABSTRACT



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ABSTRACT

Mass spectrometry in multiple reaction monitoring (MRM) mode is a powerful technique that can provide highly selective, multiplexed, and reproducible quantification of peptides derived from proteins. Ideal for the application of molecular biomarkers in biomonitoring surveys, MRM tools have been recently developed to quantify sets of pre-selected biomarkers in freshwater sentinel species. Still limited to the validation and application phase of biomarkers, dynamic MRM (dMRM) acquisition mode has increased the multiplexing capacity of mass spectrometers, expanding opportunities to explore proteome modulations in sentinel species. This study evaluated the feasibility to propose dMRM tools for investigating sentinel species proteomes at the organ level and demonstrated its potential for screening contaminant effects and discovering new protein biomarkers. As a proof of concept, a dMRM assay was developed to comprehensively capture the functional proteome of the caeca of *Gammarus fossarum*, a freshwater crustacean, commonly used as a sentinel species in environmental biomonitoring. The assay was then used to assess the effects of sub-lethal concentrations of cadmium, silver, and zinc on gammarid caeca. Results showed dose-response and specific metal effects on caecal proteomes, with a slight effect of zinc compared to the two non-essential metals. Functional analyses indicated that cadmium affected proteins involved in carbohydrate metabolism, digestive and immune processes, while silver affected proteins related to oxidative stress response, chaperonin complexes and fatty acid metabolism. Based on these metal-specific signatures, several proteins modulated in a dose-dependent manner were proposed as candidate biomarkers for tracking the level of these metals in freshwater ecosystems. Overall, this study highlights the potential of dMRM to decipher the specific modulations of proteome expression induced by contaminant exposure and pinpoints specific response signatures, offering new perspectives for the *de novo* identification and development of biomarkers in sentinel species.

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1. Introduction

Mass spectrometry (MS)-based proteomics technologies offer new insights into the molecular responses of sentinel species to pollution and environmental changes (Gouveia et al., 2019). Proteins, as the informational, enzymatic and structural units of cells, can be proposed as indicators of biological and physiological alterations in animals (Nguyen and Alfaro, 2020). As such, certain proteins may be promising biomarkers for detecting the adverse effects of contaminants on the biology of sentinel species used to gauge the overall health of an ecosystem (Trapp et al., 2014; Bjørnstad et al., 2006; López-Pedrouso et al., 2020). By combining data from RNA sequencing and shotgun mass spectrometry analyses, proteogenomic methodologies made the identification of protein sequences possible in species without sequenced genomes (Armengaud et al., 2014). In the field of aquatic ecotoxicology, this strategy is now routinely used to characterize and improve the annotation of proteomes of sentinel species (Trapp et al., 2016; Leprêtre et al., 2019). Specifically, this approach provided a catalogue of the reproductive proteome of the crustacean *Gammarus fossarum* and the immune proteome of the bivalve *Dreissena polymorpha* (Leprêtre et al., 2019; Trapp et al., 2016). Shotgun methodologies currently provide the semi-quantitative measurements of thousands of proteins (Wilde et al., 2022), and are typically used to compare proteome changes in organisms exposed or not to pollutants and to identify potential ecotoxicological biomarkers (Trapp et al., 2018). For example, comparative shotgun analyses conducted on gammarids exposed in the laboratory to xenobiotics resulted in the identification of potential biomarkers linked to the endocrine perturbation of *G. fossarum* (Koenig et al., 2021). Shotgun proteomics has traditionally relied on data-dependent acquisition (DDA) to identify peptides in complex biological samples. However, the increasing scanning speeds of high-resolution mass spectrometry have paved the way for a newer approach known as data-independent acquisition (DIA) (Doerr, 2015). The DIA mode involves fragmenting all peptides, which offers superior accuracy, reproducibility, and more exhaustive peptide sampling than DDA methodologies (Doerr, 2015). While DIA shotgun proteomics has been successfully employed in ecotoxicology to improve the proteome coverage of the crustacean *Daphnia magna* (Wilde et al., 2022), it remains highly challenging in terms of data analysis, and applying it to non-sequenced organisms may introduce biases for protein identification and quantitation (Searle et al., 2020).

Once discovered by shotgun proteomics, targeted proteomics approaches are then often used for validation and deployment of candidate biomarkers (Gouveia et al., 2019). In particular, the multiple reaction monitoring mode of mass spectrometry (MRM) offers a highly selective, multiplexed, reproducible and accurate quantitation of peptides derived from proteins (Vidova and Spacil, 2017). Cost- and time-effective in terms of sample preparation and data processing, targeted proteomic approaches are particularly suitable for the high-throughput quantification of protein biomarkers in large-scale biomonitoring surveys (Shi et al., 2016; Vidova and Spacil, 2017; Leprêtre et al., 2022). New methodologies have been notably designed for the rapid detection of peptides identified from proteogenomic studies, significantly reducing the time required for the development of MRM assays in sentinel species (Leprêtre et al., 2020). Additionally, new acquisition modes such as scheduling and spike-in triggered methods have greatly enhanced the multiplexing capacity of mass spectrometers, increasing the number of peptides and related proteins monitored in a single analysis (Bentum and van Bentum and Selbach, 2021). Taking advantages of these advances, highly multiplexed MRM assays were recently designed to monitor hundreds of immune proteins in the hemolymph of *D. polymorpha*, and 277 peptides derived from 157 proteins were monitored in a whole organism of male gammarids (Faugere et al., 2020; Leprêtre et al., 2020). For small sentinel species such as *G. fossarum*, targeted proteomic assays have been until now developed from whole bodies, despite the fact that proteins measured at the organ level may respond more specifically to contaminants and may provide refined information on the biological functions altered by pollution exposure. Moreover, MRM assays are still limited to the measure of tens of proteins

preselected from upstream comparative shotgun experiments and linked to specific biological processes, limiting this approach exclusively to the validation phase of molecular biomarkers.

Ongoing advances in MRM multiplexing capabilities offer the possibility to considerably increase the number of targeted proteins and cover the diversity of molecular pathways currently addressed by shotgun proteomics approaches. Given the analytical advantages of MRM assays (high reproducibility, selectivity and accuracy) over shotgun proteomics, such comprehensive MRM assays hold great potential for efficient screening of the impact of multiple contaminants on sentinel species proteomes with no *a priori* selection of candidate molecular targets. Such a tool may be particularly effective for the discovery of novel protein biomarkers among those in lower abundance but physiologically highly relevant. The use of comprehensive MRM assays for the discovery phase of biomarkers also represents an opportunity to improve the traditional pipeline of protein biomarker development (Gouveia et al., 2019). Indeed, this innovative strategy has the potential to significantly reduce the time and resources required for the development of protein biomarkers in sentinel species by skipping the conventional shotgun proteomic approach for the discovery phase of biomarkers and using the technical platform already implemented for the application of biomarkers in biomonitoring surveys.

For the first time, this study aims to evidence the feasibility to propose MRM tools for exploring the proteomes of sentinel species at the organ level and demonstrate the value of comprehensive MRM assays to decipher the effects of contaminants and support the development of new molecular biomarkers. To prove this concept, multiplexing performance of MRM tools were fully exploited to develop an MRM assay capable of screening a broad spectrum of proteins and molecular pathways of the *G. fossarum* caeca. Hepatopancreatic caeca are a key organ of detoxification, digestion, production and secretion of enzymes in crustaceans (Cervellione et al., 2017; Gismondi et al., 2017). Moreover, it has been recently shown that it is involved in metal bioaccumulation and depuration in *G. fossarum* (Gestin et al., 2023). This innovative MRM assay was then applied on gammarid caeca exposed to sublethal concentrations of two non-essential metals (cadmium and silver) and one essential metal (zinc) to assess their specific effects on the caecal proteome and propose new candidate biomarkers of metal contamination.

2. Material and methods

2.1. Sampling and maintenance of gammarids

Gammarids were collected from a well-established population of *G. fossarum* living in an organic watercress farm located in Saint-Maurice-de-Réments (France). Organisms were acclimatized to the laboratory for 10 days in tanks supplied with drilled groundwater, under a constant temperature (12 °C), controlled aeration and a photoperiod of 16/8 h light/dark. During the whole acclimatization period, organisms were fed *ad libitum* with alder leaves (*Alnus glutinosa*). For the development phase of MRM assays, caeca from 24 calibrated (average body size of 2 cm) male gammarids were sampled, pooled and stored at −80 °C until the protein extraction step. Dissection was performed as previously described (Gestin et al., 2022). Briefly, the dissection process was carried out meticulously under a binocular microscope. Firstly, the cephalon was carefully separated from the body. Then, the urosome was separated from the abdomen to reveal the caeca and intestine. The caeca were then delicately extracted from the intestine and placed into 2 mL microtubes and then snap-frozen in liquid nitrogen. The other non-dissected gammarids were proceed to metal exposure experiments.

2.2. Experimental exposure of gammarids to metals

Two metal exposure experiments were conducted on male gammarids. The first ‘dose finding’ experiment was designed to identify and select sublethal concentrations of Cd, Ag, and Zn. The second experiment aimed to evaluate the effects of the three metals on the caecal proteome of

Table 1

Survival and feeding inhibitions of gammarids after exposure for 7 days to different concentrations of cadmium, silver and zinc metals tested in the dose finding experiment. The three concentrations (C1, C2 and C3) selected for proteomic investigations are noted in the table. *P*-values of survival and feeding rate differences in comparison to the control condition were retrieved from one-sided proportion tests and Student's *t*-tests, respectively. Significant *p*-values (<0.05) are highlighted in bold, and "NA" means Not Available.

Condition	Nominal concentrations (µg/L)	Survival (%)		Feeding inhibition (%) ^a	
		Mean ± SD	p-Values	Mean ± SD	p-Values
Control	0	95 ± 5	NA	0 ± 21	NA
Cadmium (Cd)	0.2	95 ± 5	5.00E-01	-11 ± 17	4.96E-01
	0.6 (C1)	100 ± 0	7.63E-01	-11 ± 11	4.84E-01
	1.8 (C2)	100 ± 0	7.63E-01	10 ± 13	5.22E-01
	5.4 (C3)	90 ± 11	3.36E-01	17 ± 26	3.86E-01
	16.2	75 ± 13	1.42E-02	38 ± 18	5.63E-02
	48.6	57 ± 12	1.18E-04	75 ± 9	6.84E-03
Silver (Ag)	0.06 (C1)	92 ± 9	5.00E-01	-13 ± 10	3.34E-01
	0.2 (C2)	92 ± 9	5.00E-01	14 ± 13	3.34E-01
	0.6 (C3)	85 ± 20	1.32E-01	35 ± 22	7.01E-02
	1.8	37 ± 20	9.87E-08	73 ± 29	8.96E-03
	5.4	0	2.20E-16	NA	NA
	16.2	0	2.20E-16	NA	NA
Zinc (Zn)	6.6 (C1)	NA	NA	NA	NA
	20 (C2)	97 ± 5	5.00E-01	-54 ± 26	2.09E-02
	60 (C3)	87 ± 15	2.14E-01	-3 ± 21	8.47E-01
	180	92 ± 5	5.00E-01	-1 ± 30	9.32E-01
	540	45 ± 19	1.78E-06	48 ± 19	1.45E-02
	1620	12 ± 15	3.60E-13	95 ± 7	1.44E-03
	4860	0	2.20E-16	NA	NA

^a Negative values for feeding inhibition indicate a feeding stimulation compared to the control condition.

G. fossarum. The day before the experiments, 1000 and 500 calibrated male gammarids (average size of 2 cm) for the first and second experiment, respectively, were sorted and placed overnight in a mixture (50/50) of drill and Evian mineral water. Then, gammarids were randomly placed in 500 mL plastic beakers (10 gammarids per beaker, 4 replicate beakers per experimental condition) containing Evian water with constant aeration, photoperiod and temperature (12 ± 0.5 °C). For each experiment, mediums were completely renewed every day. The renewal of the medium consisted of adding to 500 mL of Evian water, 100 µL of stock solutions (X 10 concentrated) of Cd chloride (CdCl₂), Ag nitrate (AgNO₃) and Zn sulfate (ZnSO₄) prepared in demineralized water acidified to 0.5 % (v/v) nitric acid. For the control condition, the stock solution was composed of demineralized water acidified to 0.5 % (v/v) nitric acid (65 % Suprapur). After 1 and 3 days of exposure, 3 replicates of 25 mL water were collected from three beakers of each experimental condition from the 'dose finding' experiment (Table 1), before and after media renewal. Water samples were acidified to 0.5 % (v/v) nitric acid (65 % Suprapur). Cadmium, Ag and Zn concentrations were measured by inductively coupled plasma mass spectrometry with triple quadrupole technology (ThermoFisher Science iCAP™ TQ ICP-MS), according to NF EN ISO 17294-2 (AFNOR, 2016). The limit of quantification was 0.005 µg/L for Cd and Ag, and 0.5 µg/L for Zn (Supplementary Table S1). Quality controls included systematic use of blank tests and standard solutions.

2.2.1. Identification of sublethal concentrations of Cd, Ag and Zn for proteomic investigations

The effect of metals on survival and feeding rate (FR) of *G. fossarum* was evaluated on gammarids exposed for 7 days to 7 concentrations of Cd (0; 0.2; 0.6; 1.8; 5.4; 16.2; 48.6 µg/L), Ag (0; 0.06; 0.2; 0.6; 1.8; 5.4; 16.2 µg/L) and Zn (0; 20; 60; 180; 540; 1620; 4860 µg/L). To evaluate the survival of gammarids, dead organisms were counted and removed from the experiment every day. Using the R package 'stats', one-sided proportion tests were performed to compare mortalities between the control and exposed conditions to establish the presence of significant mortality effects (*p*-values <0.05). Feeding tests were performed as described in Coulaud et al. (2011) to evaluate the effect of metal levels on the feeding behavior of gammarids. Briefly, prior to the experiment, 10 alder leaves

discs of the same surface (21 mm) were placed in each experimental beaker. After 7 days of exposure, remaining leaf discs were scanned and remaining leave surfaces were determined with the software ImageJ. For each experimental condition, feeding rates (FRs) of gammarids, expressed as consumed surface per day per gammarid, were calculated as detailed in Coulaud et al. (2011). Then, feeding inhibitions, were calculated as the ratio between FRs calculated for metal-exposed organisms and those calculated for control organisms and were expressed as percentages. Student's *t*-tests were conducted with the R package 'stats' to compare the FRs of gammarids from control and exposed conditions and identify significant effects of metals on feeding inhibitions or stimulations (*p*-value <0.05). The median lethal concentrations (LC50) and the median effective concentrations on feeding inhibition (EC50) of metals were estimated using the MOSAIC web interface (Charles et al., 2018). Based on survival and feeding inhibition rates, three concentrations of each metal were selected for proteomic investigations. These concentrations were chosen to ensure that the gammarids remained in the sublethal range during metal exposures (with a mortality rate below 20 %) to prevent that proteome modulation was more affected by lethal effects than metal exposure. The highest sublethal concentrations were chosen to maximize the potential impact of the metals on gammarid proteome. Specifically, when possible, the highest concentrations were selected to exceed 15 % of feeding inhibition, a marker known to be sensitive to metal exposure, as previously described in Coulaud et al. (2011).

2.2.2. Caecum sampling in gammarids exposed to sublethal concentrations of metals

To assess the effect of sublethal concentrations of metals on the caeca proteome of *G. fossarum*, gammarids were exposed for 7 days to three concentrations of Cd (C1: 0.6; C2: 1.8; C3: 5.4 µg/L), Ag (C1: 0.06; C2: 0.2; C3: 0.6 µg/L) and Zn (C1: 6.6; C2: 20; C3: 60 µg/L). Based on the results obtained from the range-finding experiment, we added a new concentration for Zn exposure (6.6 µg/L). A control condition was also performed where gammarids were not exposed to any metal. Throughout the experiment organisms were fed *ad libitum* with leaves (*A. glutinosa*). After 7 days of exposure, 15 gammarids per experimental condition were randomly sampled from the four replicate

beakers. The caeca were collected from amphipods under stereomicroscopic magnification using fine forceps. For each experimental condition, five pools of three caeca were collected and stored at -80°C until dMRM investigations.

2.3. Selection of candidate proteins, peptides, and MRM transitions

Protein sequences were extracted from a proteogenomic database constructed from tandem mass spectrometry acquisitions on 48 caeca of *G. fossarum*. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD037805 and <https://doi.org/10.6019/PXD037805>. Based on this proteogenomic database, we identified 804 proteins systematically detected in the 48 caeca. To favor the outcome of the functional investigation, 105 proteins without functional annotation (see Section 2.8) or annotated as being ribosomal proteins were not retained for further analysis, leading to the final selection of 699 proteins. Three reporter peptides per protein were selected for the MRM search so as to (1) be unique to the target protein (proteotypic), (2) not exhibit chemical modifications such as spontaneous deamination or uncontrolled oxidations, and (3) not exhibit missed cleavage events that could induce quantitation bias (Gerber et al., 2003). Short (<5 residues) and long (>25 residues) peptides were also avoided. Whenever possible, peptide sequences containing methionines were excluded due to their possible oxidative modifications (Chiva and Sabidó, 2017). The most intense peptides with the highest number of spectral counts found in the proteogenomic analysis were considered for the selection of a reporter peptide. As described in Lepître et al. (2020), the raw shotgun data were analyzed using Protein Prospector software (<http://prospector.ucsf.edu>) to identify the six most intense fragment ions of each peptide and define them as MRM transitions for the detection phase of peptides in MRM. The Q1 and Q3 masses as well as the collision energy values were predicted using the software Skyline 21.1.0.146 to produce lists of MRM transitions (MacLean et al., 2010).

2.4. Sample preparation for proteomic investigations

2.4.1. Protein extraction

Pools of caeca were resuspended in 500 μL of TRI-Reagent (Thermo Fisher Scientific). After adding 100 μL of glass beads (1:1:1 ratio: 0.1 mm glass; 0.1 mm silica and 0.5 mm glass), caeca were ground under FastPrep (MP Biomedicals) with two series of 30 s and 4 movements per second. Samples were then centrifuged at 12,000 $\times g$ for 2 min at 4°C to remove cellular debris and beads. After transferring the supernatant to new 1.5-mL microtubes, 100 μL of chloroform was added to separate RNA from protein. Samples were then vortexed for 15 s and incubated for 15 min at room temperature before centrifugation for 15 min at 12,000 $\times g$ and 4°C . The colorless supernatant containing RNA was removed and 150 μL of absolute ethanol was added in samples to precipitate the remaining DNA. Samples were mixed by inversion and incubated at room temperature for 3 min. After centrifugation for 5 min at 2000 $\times g$ and 4°C , the supernatant containing the proteins was transferred to 2 mL microtubes containing 1.5 mL of iced acetone (3 times the volume). Samples were incubated at -20°C for 30 min to favor protein precipitation. After centrifugation for 15 min at 12,000 $\times g$ and 4°C , protein pellets were washed twice in 1 mL of a solution of 95 % ethanol containing guanidine hydrochloride (0.3 M) followed by incubation steps at room temperature and centrifugation at 8000 $\times g$ at 4°C for 5 min. A final wash was performed by resuspending the protein pellet in 900 μL of absolute ethanol. Then, samples were vortexed for 15 s and incubated for 10 min at room temperature before centrifugation at 8000 $\times g$ for 5 min at 4°C . The supernatant was removed and the pellet was dried at room temperature for 10 min before being resuspended in 100 μL of Protein Solubilization Buffer (PSB, Bio-rad). Total protein concentrations were determined using Bradford assay (Bradford, n.d.). For each sample, an aliquot containing 150 μg of extracted proteins was conserved for further analyses.

2.4.2. Tryptic digestion

Samples of 150 μg total protein were transferred to 2 mL Eppendorf tubes containing 1.2 mL of 50 mM ammonium bicarbonate (AMBIC). Dithiothreitol (DTT) at 150 mM diluted in AMBIC was added to the samples to a final concentration of 15 mM. Samples were incubated for 40 min at 60°C and after cooling to room temperature, iodoacetamide (IAM) at 150 mM diluted in AMBIC was added to a final concentration of 20 mM. After 40 min of incubation at room temperature, 75 μL of a 2 mg/mL TPCK trypsin solution (Fisher Scientific, France) diluted in 50 mM AMBIC was added to initiate protein digestion with a ratio of 1:1 (trypsin/protein). Samples were kept at 37°C for 1 h with 500 rpm agitation and the digestion was stopped with 20 μL of formic acid (FA). Then, 10 μL of an internal standard (ISTD) solution containing 9 heavy labelled peptides at a concentration of 4 $\mu\text{g}/\text{mL}$ were spiked in samples to check analytical shifts during MS analyses. Finally, to remove any solid particles, samples were centrifuged for 5 min at 10,000 $\times g$ (room temperature) before solid phase extraction (SPE) steps.

2.4.3. Solid phase extraction (SPE)

The SPEs were performed using 60 mg Oasis HLB 3 mL reverse phase cartridges. Cartridge columns were first conditioned with 1 mL of methanol and 1 mL of water containing 0.5 % FA. Samples were then loaded into the cartridges before being washed with 2 mL of a water/methanol solution (95/5, v/v) containing 0.5 % FA. Peptide elution was performed with 1 mL of methanol containing 0.5 % FA in new 1.5 mL microtubes containing 100 μL of 10 % glycerol in methanol. Eluted samples were then placed under nitrogen flow to evaporate the methanol and retain only the peptides contained in the remaining 10 μL of glycerol. After evaporation, peptides were resuspended in 50 μL of water/acetonitrile (90/10) with 0.5 % formic acid (FA). Samples were then centrifuged for 5 min at 12,000 $\times g$ and transferred in vials, ready for MRM analyses.

2.5. Liquid chromatography and mass spectrometry

Peptide solutions were analyzed using an Agilent 1290 Infinity II HPLC (High Performance Liquid Chromatography) system coupled to an Agilent 6495B triple quadrupole mass spectrometer. For each MRM analysis, 20 μL volume of sample containing 50 μg of total protein were injected into the system. Liquid chromatographic separation was performed using Waters Xbridge BEH C18 column of size 100 mm \times 2.1 mm \times 3.5 μm coupled to a symmetry XBridge BEH C18 guard column of size 2.1 mm \times 5 mm \times 3.5 μm (Waters, Milford, MA, USA). Solvent A was 0.1 % AF water and solvent B was 0.1 % AF acetonitrile. The temperature of the auto-sampler and column were kept at room temperature and 40°C , respectively.

The mass spectrometer was interfaced to an Agilent ESI JetStream (AJS) electrospray ion source operating in positive ionisation mode with an ion sputtering/spray voltage of 4500 V. The nebuliser gas flow rate was set at 12 L/min with a pressure of 40 psi using nitrogen (N_2). The ion source was set at 250°C at the nebuliser and 210°C at the transfer capillary. A gas flow rate (N_2) of 17 L/min was applied in the transfer capillary. The collision gas (N_2) was set to a value of 4 V. Ion funnel parameters were set at 145 V for High Pressure RF and 115 V for Low Pressure RF. Q1 and Q3 were set to a unit resolution of plus or minus 1 Da. Instrument control and data acquisition was performed using MassHunter Data Acquisition B.09.00 software.

2.6. Development of a single Multiple Reaction Monitoring assay

The search of peptides by MRM was performed on peptides extracted from a pool of 24 caeca. MRM analyses were performed with a flow rate set as 300 $\mu\text{L}/\text{min}$ and the following HPLC gradient (time (min), % of B): (0, 5 %); (2, 5 %); (38, 35 %); (38.1, 100 %); (43, 100 %); (43.1, 5 %); (48, 5 %) for a total time of 48 min. First, only 200 MRM transitions selected from peptides found abundant and well spread over the chromatographic gradient in shotgun analyses were monitored using the classical

mode of MRM. Thanks to the retention times (RTs) defined in shotgun experiments, a linear correlation line with the RTs of peptides detected by MRM could be established (Supplementary Fig. S1). The equation of the linear correlation was used to predict the RTs of all remaining peptides. Based on their theoretical RTs, remaining peptides were searched using the dynamique MRM (dMRM) mode with 6-minute acquisition windows per transition. MS data were visualized using the Skyline software. Peptides were considered detected when at least three of their MRM transitions were aligned and when at least one of its transitions was detected with a signal-to-noise ratio higher than three.

To monitor all detected peptides in a single dMRM analysis, chromatographic elution of peptides were reconsidered and optimized. For this purpose, the HPLC gradient was extended to 90 min with the following elution conditions: (time (min), % of B): (0, 5 %); (2, 5 %); (16, 9 %); (75, 20 %); (85, 35 %); (85.1, 100 %); (88, 100 %); (88.1, 5 %); (90, 5 %). A flow rate of 400 $\mu\text{L}/\text{min}$ was retained to obtain a minimum of 10 points per chromatographic peak and ensure a reliable quantitation of peptides. The same methodology described above was applied to identify RTs of peptides under the new elution gradient. First, 200 MRM transitions of peptides, well detected and well spread over the HPLC gradient, were monitored by MRM with the new HPLC gradient. Linear correlations between RTs (48 vs 90 min gradient) were used to predict the RTs of peptides along the new elution gradient (Supplementary Fig. S1B). Thanks to these predicted RTs, experimental RTs of peptides were searched using the dMRM mode with acquisition windows of 6 min. Finally, a unique comprehensive dMRM assay was established for monitoring all peptides in a single analysis. This final dMRM assay included the monitoring of 4 MRM transitions per peptide with acquisition windows ranging from 2 to 4 min and was applied to investigate the modulation of caeca proteomes in gammarids exposed or not to metals.

The final dMRM method and MS data acquired from the metal exposure experiments were deposited at the PeptideAtlas SRM Experiment Library (PASSEL) under the dataset identifier PASS03807 (Farrah et al., 2012).

2.7. Data processing and differential proteomics

The MRM chromatographic peaks were integrated using the Skyline software. Peptide levels were measured from the area of their most intense and least interfered transition ($\text{CV} < 20\%$). Only transitions detected with a signal-to-noise ratio >3 in $>80\%$ of samples were considered for the differential proteomic analysis. Peptide levels were \log_2 transformed to perform normalization. Using the dataprocess function of the MSstats R package, peptide intensities were normalized by a set of 5 housekeeping peptides (HKPs) (supplementary Table S2), identified with the methodology described in Lepître et al. (2022). The data normalized by HKPs and used for the differential proteomic analysis are available in the supplementary Table S3.

Peptide levels from metal-exposed gammarids were compared to control gammarids to evidence proteins modulated by the different concentrations of Cd, Ag and Zn in gammarid caeca. For each experimental condition, peptide fold changes were calculated as the ratio between the mean levels of peptides measured in metal-exposed gammarids to the mean levels of peptides measured in control gammarids. Using the webtool StatsPro dedicated to differential proteomic analyses (Yang et al., 2022), significant analysis of microarrays (SAM) tests, derived from the Student's *t*-test, was performed to highlight peptides significantly modulated in metal-exposed gammarids compared to control samples. Significant differences were accepted with a false discovery rate (FDR) lower than 0.05 and a fold change (FC) threshold higher than $|1.3|$, which has been widely used in the literature to consider protein modulations (Liu et al., 2017; Sequeiros et al., 2017; Uozie et al., 2017). Finally, proteins were considered as differentially expressed (DEPs) when at least one of their reporter peptides was considered significantly modulated.

2.8. Functional analysis

Proteomic sequences derived from the proteogenomic database were annotated using different databases. BLAST annotations and Gene

Ontology Biological processes (GO-BP) were performed with the BLASTp module of OmicsBox software (version 2.0). The sequence similarity search was performed against the SWISSPROT database without taxonomic restriction. KEGG (*Kyoto Encyclopedia of Genes and Genomes*) pathways were predicted using the EggNOG-mapper tool, taking into account the default parameters (Huerta-Cepas et al., 2017). All functional annotations were validated with an expected value (*E*-value) threshold set at 1×10^{-3} .

Functional enrichment analyses of DEPs were performed with the over representation analysis (ORA) proposed by the R package WebGestalt (Liao et al., 2019). Based on the KEGG GO-BP annotations, customized functional databases of *G. fossarum* caecum proteome were generated for the enrichment tests (Supplementary Table S2). All proteins detected in at least 80 % of samples with a S/N ratio >3 were defined as a reference list (background). DEPs from gammarids exposed to Cd, Ag, Zn, and cocktail conditions (all concentrations combined) were tested for ORA analysis. KEGG and GO-BP annotations were considered enriched with a minimum of 3 DEPs and a *p*-value lower than 0.05 according to the ORA test. To remove redundancy between functional categories, the enriched annotations were clustered by the weighted set cover algorithm (Liao et al., 2019; Savage et al., 2019). A Fold Enrichment (FE) score was also calculated as the ratio between the number of DEPs annotated to a functional category and the total number of proteins annotated to this category in the background list. The significantly enriched functional annotations were then visualized and displayed with bubble plots produced with the R package ggplot2.

3. Results and discussion

3.1. Comprehensive investigation of the caecal proteome by dynamic multiple reaction monitoring

Based on the proteogenomic database, a list of 699 functionally annotated proteins from gammarid caeca was initially selected for the development of the MRM assays (Fig. 1). Whenever possible, up to three reporter peptides per protein were targeted to capture the levels of these selected proteins. In total, 1919 peptides derived from the 699 proteins were retained as candidate peptides for the development of MRM assays (Fig. 1). To optimize the detection of peptides in MRM analyses, MS/MS spectra generated from shotgun analyses were analyzed and the six most intense fragment ions were set as MRM transitions, totaling the selection of 11,514 MRM transitions for the 1919 targeted peptides (Fig. 1).

As discussed by Chen et al. (2019), methodology transfer from the shotgun to targeted proteomics often represents the bottleneck of proteomics, especially when a large number of peptides are queried. This challenge is typically time and resource consuming, not only during the development phase of the assay, but also throughout the sample analysis. Different strategies can be implemented to transfer methodologies from shotgun proteomics to targeted proteomics. The classical acquisition mode MRM enables to scan peptides and transitions throughout the full analysis but remains limited to a maximum number of concurrent transitions. In this study, scanning of the 1919 peptides and 11,514 MRM transitions targeted in the caecum of *G. fossarum* would require >57 MRM analyses and method developments, demanding a costly and time-consuming effort. Unlike the conventional MRM mode, the dMRM assay tracks analytes during a given retention time window, reducing the number of concurrent MRM transitions and offering a high level of multiplexing. For this reason, the dMRM mode is particularly attractive for the rapid detection of a large number of peptides but requires first the definition of RT windows specific to each targeted analyte. Using similar chromatographic conditions to the present study (HPLC gradients and columns), Faugere et al. (2020) observed a linear correlation between RTs of *G. fossarum* peptides detected in shotgun and MRM experiments. Therefore, we took advantage of the linear correlation to predict RTs of peptides searched by dMRM. First, about 30 abundant peptides covering the entire shotgun HPLC gradient were monitored using the MRM mode to establish the linear correlation ($R^2 > 98\%$) between RTs (Supplementary Fig. S1). Thanks to the linear correlation equation, peptide RTs were predicted, and the 1889 remaining

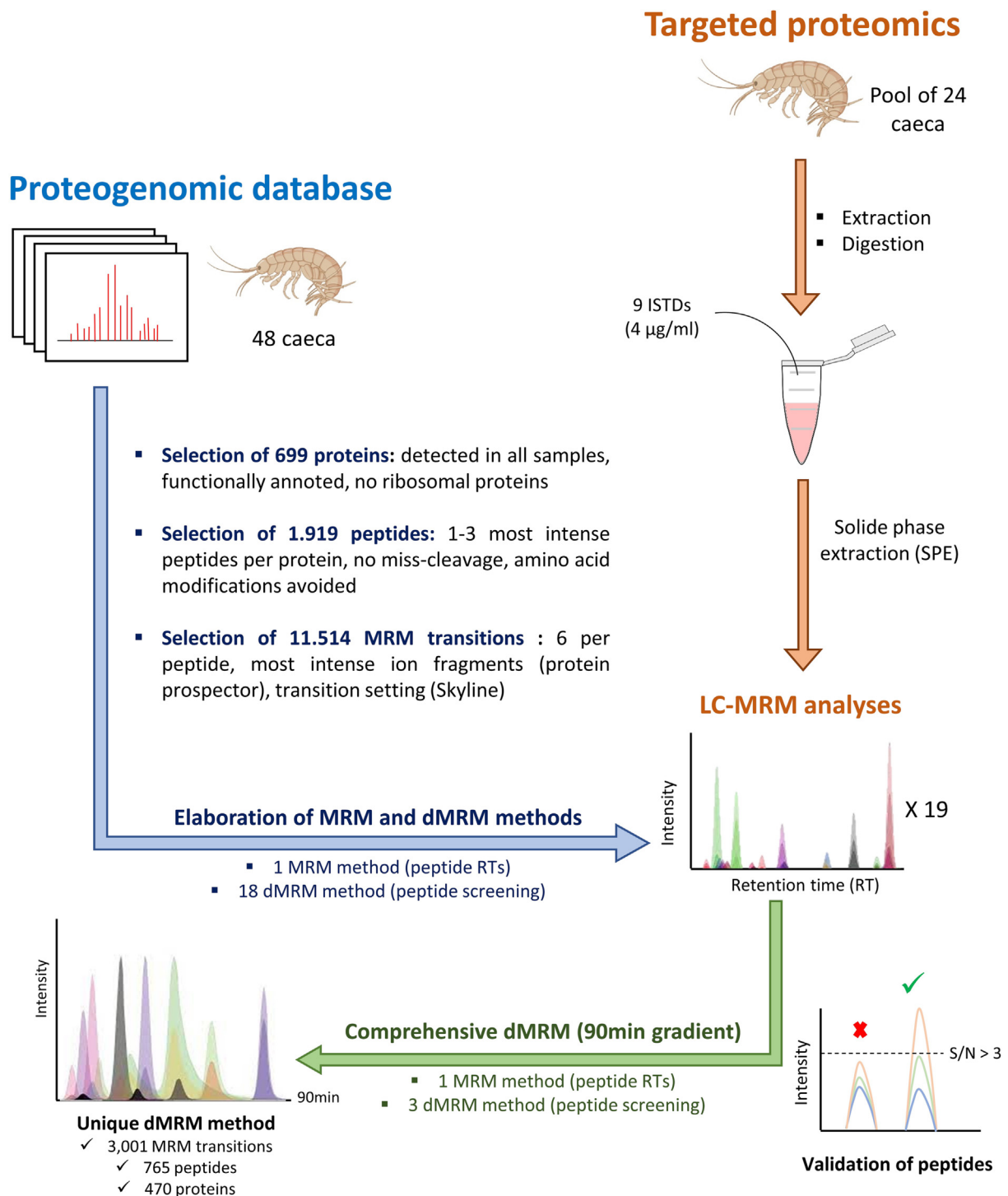


Fig. 1. Schematic representation of the workflow used to develop an unbiased dynamic multiple reaction monitoring (dMRM) approach for a comprehensive investigation of the caecal proteome of *Gammarus fossarum*.

peptides were scanned in only 18 dMRM runs. Overall, 765 peptides related to 470 proteins were detected with at least three aligned MRM transitions and one transition measured with a S/N ratio >3. More specifically, 72 proteins were detected with 3 reporter peptides, 151 proteins were detected with 2 peptides and 247 proteins were measured with a single reporter peptide. Because the dMRM mode is limited to a maximum of 200 concurrent transitions, 3 dMRM methods of 48 min would be required for monitoring the 3060 MRM transitions selected from the 765 detected peptides (4 transitions per peptide). To save biological material, reduce time analyses, and avoid MS contaminations (Kang et al., 2017; Leprêtre et al., 2022),

chromatographic conditions were optimized and extended to 90 min and a unique comprehensive dMRM method was established for tracking all peptides in a single run.

To get insight into the biological functions of the caecal proteome investigated by dMRM, proteins were annotated using GO-BP and KEGG pathways. >70 % of targeted proteins were annotated with GO-BP and KEGG pathways. Indeed, 2787 GO-BP and 284 KEGG pathways were represented by the proteins followed in the dMRM method. These GO-BP and KEGG pathways represent >80 % of the annotations observed for proteins initially selected from shotgun data. The comparison of the KEGG

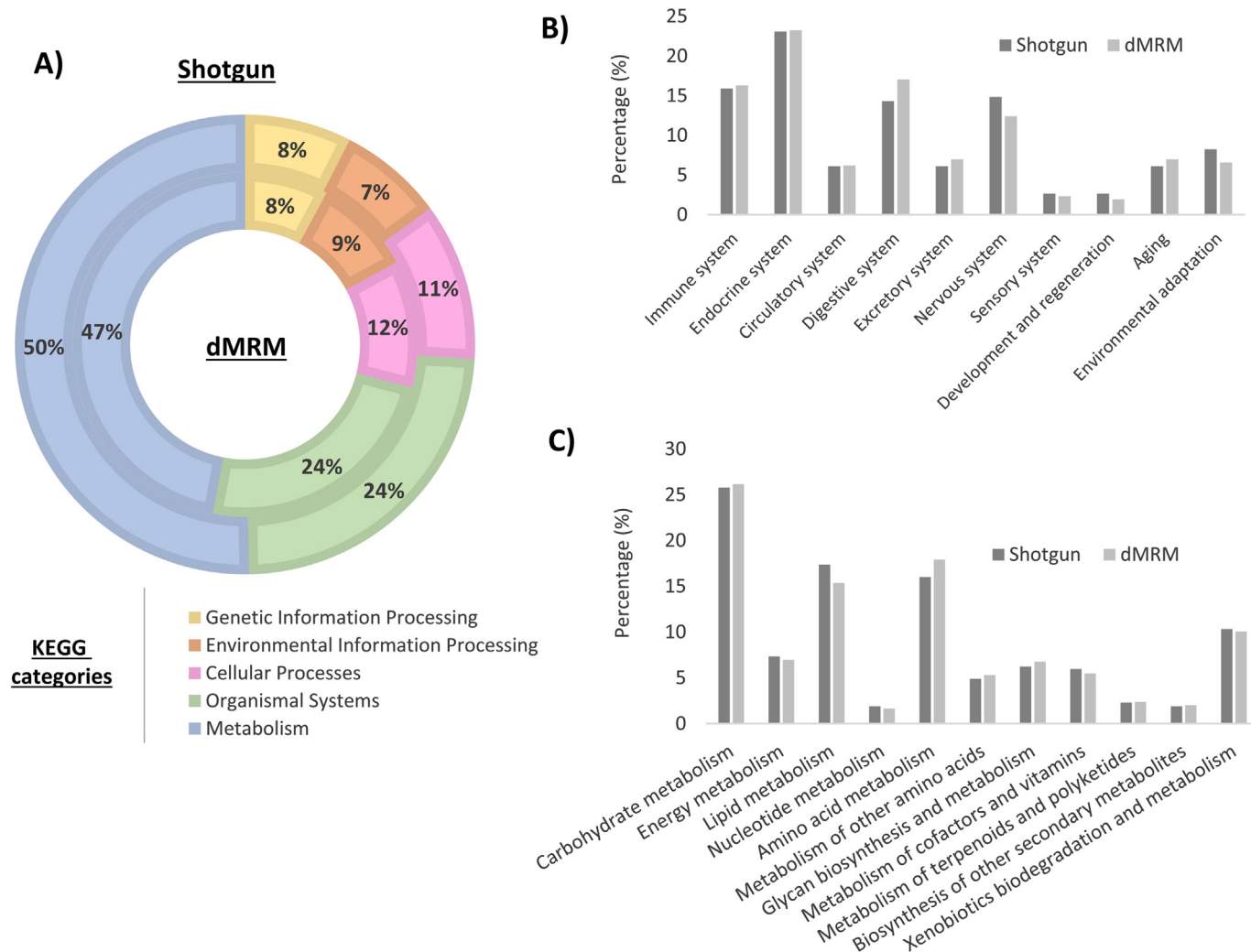


Fig. 2. Description of KEGG categories and subclasses annotated to proteins measured by shotgun or dMRM. **Fig. 2A** compares the percentage of proteins annotated to KEGG categories in the shotgun proteome and in the dMRM proteome. **Fig. 2B** and **C** compare the percentage of proteins annotated to subclasses of KEGG annotations within the categories 'Organismal system' (B) and 'Metabolism' (C).

annotation of the proteins investigated by both the shotgun and dMRM assay revealed no significant differences in the distribution of KEGG pathway frequency (Fig. 2). This suggests that the proteins included in the dMRM assay have a similar coverage of biological functions without any obvious selection bias. As a result, the final dMRM method provides the ability to investigate most of the biological pathways found in the caeca proteome of *G. fossarum*. To our knowledge, this is the first study in which a dMRM method was developed to comprehensively investigate molecular pathways of a sentinel species' organ proteome. Given its highly analytical performance and the range of proteins and biological functions monitored, the dMRM method developed in this study should provide a reliable way of identifying changes in the proteome exposed to a variety of experimental conditions, opening up new possibilities for deciphering the effect of contaminants on gammarid caeca. Following the same methodology established in this study, the development of comprehensive dMRM assays could be easily extended to other organs of *G. fossarum*, or other sentinel species for which proteogenomics data are available.

3.2. Screening the effect of metal exposure on the proteome of *G. fossarum* caeca

A first dose-finding experiment was performed to evaluate the effect of seven concentrations of Cd, Ag and Zn on survival and feeding rate of gammarids. The aim of this experiment was to identify Cd, Ag, and Zn concentrations that did not induce mortality but altered the feeding behavior

(indicated as C3). These concentrations and two lower concentrations (C1 and C2) were then used to expose the organisms for the proteomic studies. During this experiment, concentrations of metals measured in media after renewals confirmed that gammarids were exposed to the expected nominal concentrations (Supplementary Table S1). However, concentrations measured before renewals were lower than nominal concentrations, pointing to a probable bioaccumulation of metals in *G. fossarum* tissues and/or metal absorption on alder leaves or experimental beakers. As reported in the supplementary Table S4, LC50 values were estimated at 56.6 µg/L for Cd, 1.61 µg/L for Ag and 558 µg/L for Zn. For feeding inhibition EC50 were estimated at 18.78, 0.8 and 349 µg/L for Cd, Ag and Zn, respectively (Supplementary Table S5). Moreover, statistical tests performed on survival and feeding rates in the exposed condition compared to the control condition indicate that increasing concentrations of Cd, Ag and Zn have a dose-dependent effect on survival and feeding rates of gammarids, with a transient stimulation at low dose for Zn (Table 1). Based on these results, three nominal concentration levels (C1, C2 and C3) of Cd, Ag and Zn, below LC50 and EC50 values, were selected for proteomic investigations (Table 1). The highest non-lethal concentrations were chosen based on survival data (no significant effect on mortalities; survival rate higher than 80 %). In terms of feeding inhibition, the highest concentration (C3) selected for Cd was below the EC25, while the highest concentrations selected for Ag and Zn exposures were between the EC25 and EC50 (supplementary Table S5). Mean feeding inhibition was 17 % and 35 % compared to the

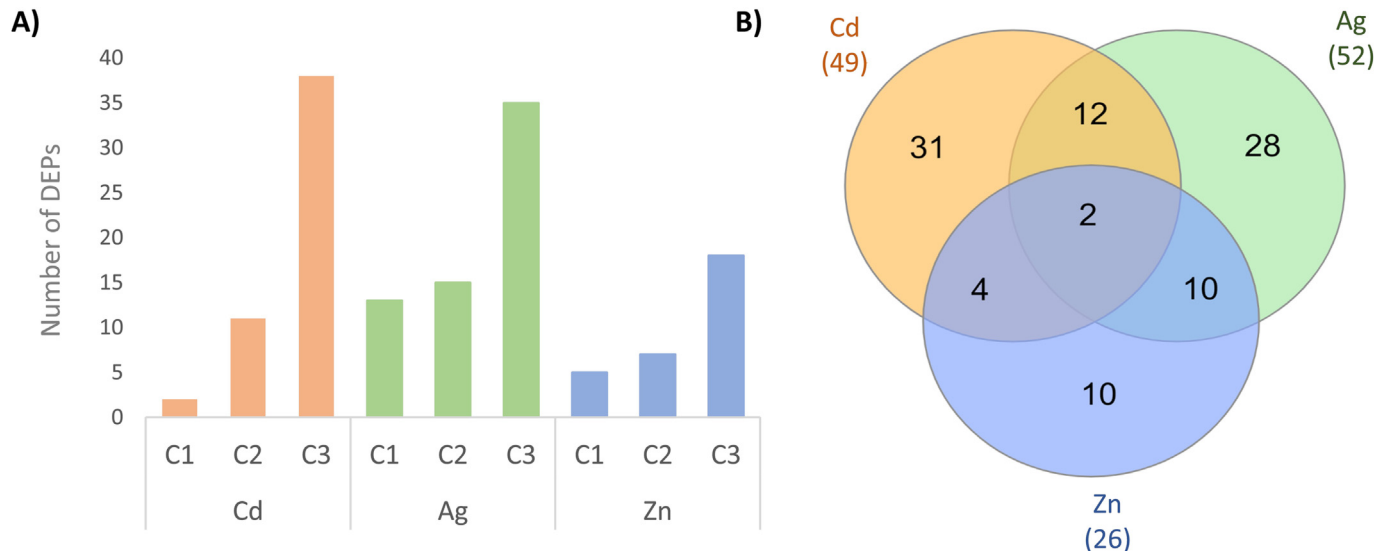


Fig. 3. Differentially expressed proteins (DEPs) in the caeca of gammarids exposed to Cd, Ag and Zn. Panel A shows the number of DEPs affected in each concentration of metals. The Venn diagram (Panel B) shows the number of DEPs common or specific to Cd, Ag, and Zn conditions, all concentrations merged.

control condition for the highest selected concentrations (C3) for Cd and Ag respectively (Table 1). For Zn, as feeding inhibitions were observed only in concentrations that induced high mortality rates (Table 1), we shifted the concentration range C1 C2 C3 to surround the concentration stimulating the feeding activity. Hence, the C3 concentration presents an inhibition rate difference of 51 % compared to the C2 condition.

In order to test the ability of the dMRM assay to identify different patterns of proteome modulation induced by the exposure to different contaminants, the dMRM method was applied to investigate the effects of three sub-lethal concentrations of Cd, Ag and Zn on the proteome of *G. fossarum* caeca. A total of 649 peptides linked to 416 proteins were detected with an S/N ratio >3 in at least 80 % of the samples and were considered for the differential proteomic analysis performed on HKP normalized data. For each metal, the number of DEPs increased with the exposure concentration of Cd, Ag and Zn (Fig. 3A), indicating a dose-dependent effect of each metal on the proteome. For the lowest concentration (C1), few proteins were modulated in the caeca of gammarids exposed to Cd (2 DEPs) and Zn (5 DEPs), while 13 DEPs were observed in gammarids exposed to Ag (Fig. 3A). Conversely, the most significant modulations were observed at the highest concentrations (C3), with higher numbers of DEPs observed in the caeca of gammarids exposed to Cd (38 DEPs) and Ag (35 DEPs) compared to those exposed to Zn (18 DEPs). Similar dose-related effects were observed for the feeding inhibition at these three concentrations (Table 1), confirming a biological response to increasing concentrations of the three tested metal ions. For each metal, the majority of differentially expressed proteins (DEPs) were specific to the tested concentrations, with only a limited number of DEPs found to be common across conditions (Supplementary Fig. S2). Particularly, greater overlaps of DEPs were observed between the C2 and C3 concentrations of Cd (2 DEPs) or Ag (4 DEPs).

When all tested concentrations are considered, a higher number of DEPs was observed in gammarids exposed to Ag (52 DEPs) and Cd (49 DEPs) compared to gammarids exposed to Zn (26 DEPs) (Fig. 3A). Different proteomic signatures were observed between gammarids exposed to Cd, Ag or Zn. Indeed, only two proteins were modulated by the three metals and most of DEPs were modulated specifically in gammarids exposed to Cd (31 DEPs), Ag (28 DEPs) or Zn (10 DEPs) (Fig. 3B). Subsequently, functional enrichment analyses of DEPs were conducted to get insight into the biological functions specifically affected by the three metals (Fig. 4). A higher number of GO-BPs and KEGG pathways were considered enriched in gammarids exposed to Cd (8 GO-BPs, 3 KEGG pathways) and Ag (5 GO-BPs, 2 KEGG pathways) compared to gammarids exposed to Zn (1 GO-BP), confirming a relatively weaker biological effect of Zn on the

caeca proteome of *G. fossarum* in our testing conditions. The functional analyses of DEPs also confirmed the specificity of the proteomic responses to metals, since only 2 GO-BPs were shared between Cd and Ag conditions. In gammarids exposed to Cd, 6 GO-BPs and 3 KEGG pathways were specific to this metal. Most of the functional annotations specifically enriched in Cd-exposed gammarids were linked to metabolic processes, mainly carbohydrate metabolism (Fig. 4). These included the GO-BPs 'metabolic process' (5 DEPs) and 'oligosaccharide metabolic process' (3 DEPs) enriched with a Fold Enrichment score (FE) higher than 60 %, as well as the KEGG pathways 'starch and sucrose metabolism' and 'carbohydrate digestion and absorption', both enriched with >7 DEPs and FEs >60 %. Other functional annotations specifically enriched in Cd-exposed gammarids included the GO-BPs 'regulation of biological quality', 'immune system processes', 'cellular response to starvation' and the KEGG pathway 'lysosome'. Similarly, several biological functions and pathways were exclusively affected in gammarids exposed to Ag. In particular, the GO-BPs 'cellular response to oxygen-containing compound', 'chaperone-mediated protein complex assembly' and the KEGG pathway 'antigen processing and presentation' were enriched with 3 DEPs and FE scores of about 50 %. The KEGG pathway 'fatty acid elongation' was also enriched specifically in gammarids exposed to Ag with 3 DEPs. In gammarids exposed to Zn, only the GO-BP 'glutathione metabolic process' was considered enriched with 3 DEPs and low FE score (20 %).

Overall, these results demonstrate the strength and relevance of using dMRM tools to get insight into the biological effect of contaminants on sentinel species proteomes. By targeting proteins belonging to an unbiased and comprehensive set of the biological pathways annotated in gammarid caeca by shotgun proteomics, our dMRM assay revealed dose-dependent effects and distinct proteomic profiles in the caeca of gammarids exposed to sublethal levels of Cd, Ag and Zn. Specifically, dMRM investigations indicated that sublethal concentrations of Zn induced a weak effect on the proteome. While many studies and our results showed that excessive levels of Zn can induce toxic effects on animals, Zn remains an essential trace mineral that regulates key biological functions required for growth and health. In this study, sublethal concentrations of Zn selected for proteomic investigations induced few significant changes in the proteome of gammarid caeca. This was supported by the fact that the tested concentrations of Zn did not affect the feeding behavior of gammarids. In contrast, dMRM investigations revealed that sublethal concentrations of Cd and Ag impacted around 12 % of the investigated proteome. In particular, distinct proteomic signatures indicate different adverse effects of Cd and Ag on biological functions of gammarid caeca. Specifically, the functional analysis revealed that

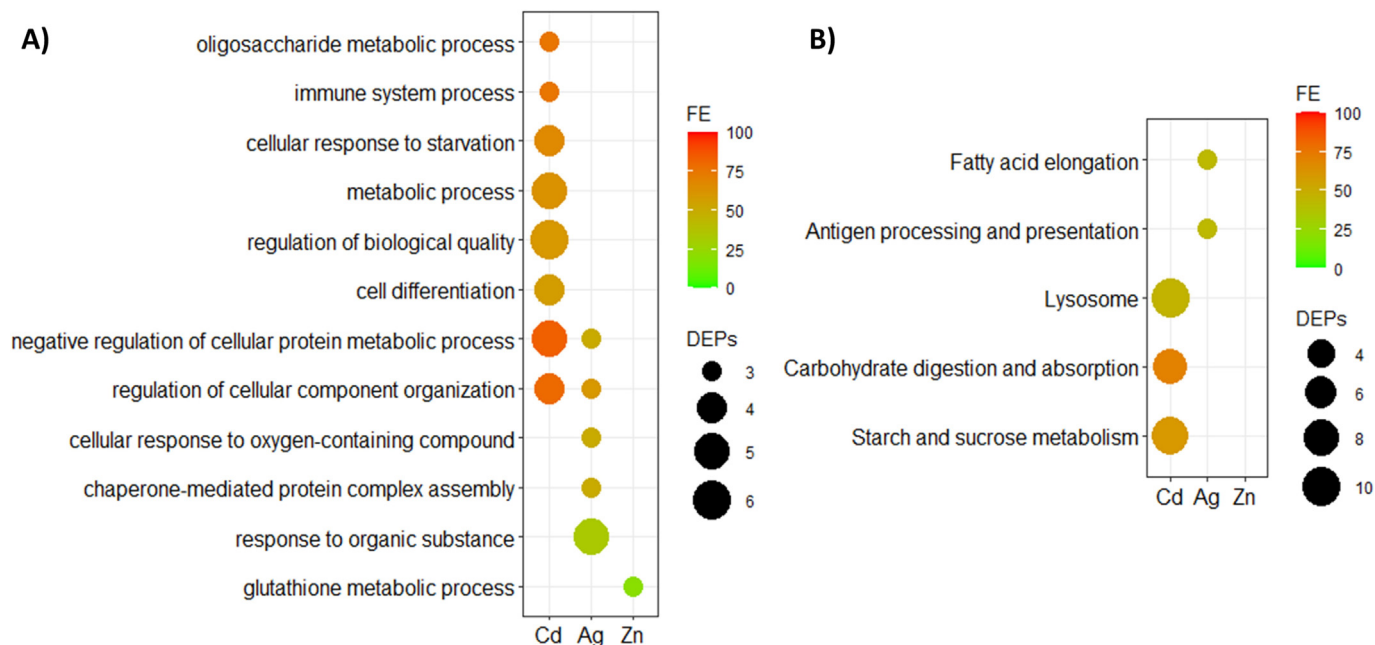


Fig. 4. Gene-ontology biological processes (A) and KEGG pathways (B) enriched in DEPs identified from the caeca of gammarids exposed to cadmium (Cd), silver (Ag) or Zinc (Zn). Functional annotations were considered enriched with a minimum of 3 DEPs and a p -value lower than 0.05 according to the ORA test. Fold enrichment scores (FEs), expressed as a percentage, were calculated as the ratio between the number of DEPs related to a category and the total number of proteins related to the same category.

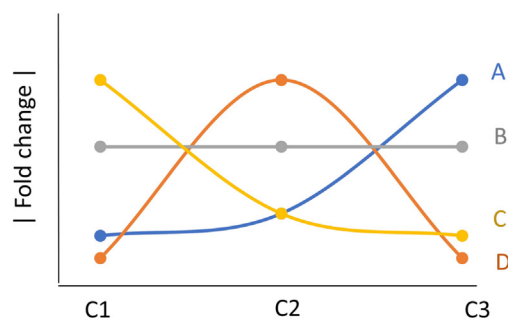
biological processes related to the carbohydrate metabolism, immune system and digestive processes involving lysosomal proteins were specifically impacted in *G. fossarum* exposed to Cd. Such results are consistent with previous shotgun proteomics and transcriptomic analysis performed on freshwater crustaceans exposed to Cd. Proteins involved in protein carbohydrate metabolism and innate immunity were found more abundant in *Gammarus pulex* populations sampled from sites naturally contaminated to cadmium (Cogne et al., 2019). Using DNA microarrays, Soetaert et al. (2007) observed an up-regulation of genes linked to digestive processes of carbohydrate metabolism in *Daphnia magna* exposed to sub-lethal concentrations of Cd. In gammarids exposed to Ag, specific DEPs were linked to the response to oxygen-containing compounds, chaperonin-complexes and fatty acid metabolism. Numerous molecular toxicological studies, conducted at the molecular level, showed that Ag ions and nanoparticles (NPs) lead to the generation of reactive oxygen species (ROS) that cause cell damages (Poynton et al., 2012; Gomes et al., 2015). Transcriptomics and/or proteomics studies conducted on Ag-exposed invertebrates, such as the sentinel worms *Eisenia fetida* and *Enchytraeus crypticus* or the crustacean *Daphnia magna*, also revealed a modulation of chaperonin-related proteins that may participate actively in the recovery or degradation of proteins damaged by cellular stress induced by silver ions and nanoparticles (Poynton et al., 2012; Novo et al., 2015; Maria et al., 2021). The effect of Ag on the fatty acid metabolism observed in the caecum of *G. fossarum* is also consistent with previous results reported in the literature. Indeed, fatty acid alterations have been detected in many organisms exposed to Ag ions or Ag-NPs, including in invertebrate species (Xiang et al., 2020; Seyedi et al., 2021; Maria et al., 2021). In the same way as non-targeted proteomic and transcriptomic techniques, dMRM analyses allowed to decipher and discriminate the effects of three metals on the biological functions of the caecal proteome, without prior assumptions. Successfully applied for revealing specific signature of metal contamination, the dMRM assay developed in this study could be readily deployed to screen the effects of other contaminants on gammarid caeca.

3.3. Discovery of candidate biomarkers specific to metal contaminations

Finally, we explored the dMRM results of gammarid caeca proteomes exposed to multiple concentrations of Cd, Ag or Zn to test the ability of

this screening approach to provide new candidate biomarkers of metal contamination. To accurately assess both the presence and the toxic effects of contaminants, biomarkers should meet several criteria (Forbes et al., 2006). Ideally, biomarkers should be (1) specific enough to differentiate between different types of contaminants, (2) modulated in a dose-dependent manner to reflect the level of contamination in the environment, and (3) provide information about the biological functions impacted by contaminants to better understand the health status of organisms.

In this study, dMRM investigations revealed unique effects of Cd, Ag, and Zn on the caecal proteome of *G. fossarum*. The functional analyses indicated that DEPs signatures of each metal were linked to different molecular pathways, making this dataset a valuable resource for identifying specific biomarkers of metal contamination. To evaluate the interest of these proteins to reveal the level of metal contamination, DEPs linked to biological functions affected by the 3 metals were classified into four patterns of modulation based on their fold change values across the different concentrations tested as shown in Fig. 5. Among the 24 DEPs linked to biological functions enriched specifically in the Cd exposure, 14 DEPs were modulated in a dose-dependent manner (pattern A, Fig. 5) and 1 DEP was sensitive to all concentrations of Cd (pattern B, Fig. 5), making them particularly relevant for assessing the level of Cd-induced stress on *G. fossarum*. These proteins included digestive and lysosomal enzymes (e.g., glucosylceramidase, endoglucanase, glucosidase, amylase), which have consistently demonstrated high expression either in gammarids sampled from Cd-contaminated sites or in fishes exposed to Cd in laboratory conditions (Cogne et al., 2019; Xie et al., 2019; Hani et al., 2018; Dedourge-Geffard et al., 2009). However, 9 DEPs were modulated exclusively at the C2 concentration (pattern D, Fig. 5) and were not suitable for tracing a dose-related response to Cd. A total of 14 DEPs were related to GO-BPs and KEGG pathways specifically affected in gammarids exposed to Ag. As with gammarids exposed to Cd, the majority of DEPs affected by Ag followed pattern A (7 DEPs) or B (3 DEPs), while 2 DEPs were exclusively modulated at concentration C1 (pattern C, as shown in Fig. 5), and another 2 DEPs followed pattern D. Notably, the HSP90 and the Stress-induced phosphoprotein 1 (STIP1), a co-chaperone of heat shock proteins (An et al., 2023), emerged as a key proteins of interest among the DEPs following the pattern A in Ag-exposed gammarids. Previous studies have reported the modulation of several HSP-associated genes, including HSP90, in response to Ag particles and Ag ions in various organisms



Pattern of modulation	Cd	Ag	Zn
A	14	7	0
B	1	3	2
C	0	2	1
D	9	2	0

Fig. 5. Pattern of modulations of DEPs defined according to their fold change values observed across the different concentrations tested. The table shows the number of DEPs linked to enriched functional annotations following the patterns A, B, C or D in gammarids exposed to cadmium (Cd), silver (Ag) and zinc (Zn).

(Poynton et al., 2012; Novo et al., 2015; Maria et al., 2021). Finally, among the 3 DEPs linked to the GO-BP 'glutathione metabolic process' in gammarids exposed to Zn, 2 DEPs followed the pattern B and 1 DEP followed the pattern C. Glutathione-S-transferases (GSTs) are often impacted in invertebrate species exposed to metals, including Zn, which reinforces the potential of these DEPs as biomarkers for monitoring the effect of metal contamination (Radwan et al., 2010; Rhee et al., 2007; Kafel et al., 2003). Based on these observations, a panel of DEPs that follow the pattern of modulation A could be proposed as candidate biomarkers for monitoring the effect of metal contaminants on *G. fossarum* (Supplementary Table S4). Giving their modulations at the lowest concentrations of metals, DEPs following the pattern B may also be of great interest for assessing biological effects of contaminants at low doses. The utilization of these candidate biomarkers for diagnosing the effects of metal contamination may complement direct chemical analysis in aquatic environments. In fact, biomarkers enhance our understanding of the organisms' health effects of metal contamination by providing insights into early molecular changes that can lead to toxicological effects, and overcoming limitations associated with direct chemical analysis (Milinkovitch et al., 2019; Hook et al., 2014). Our results confirm the interest of incorporating biomarkers into biomonitoring programs to improve risk assessment strategies and facilitate the early warnings of chemical exposure.

In contrast to previous studies that employed shotgun approaches for the discovery of biomarkers, these candidate biomarkers were identified directly with the tools implemented for their deployment in biomonitoring surveys (Gouveia et al., 2019). In this regard, comprehensive dMRM investigations offer the advantage of shortening the time and investment devoted to the development process of biomarkers, by discovering new biomarkers technically ready to use in biomonitoring surveys. The strategy used for the rapid development of comprehensive MRM methods could easily be applied to other organs and/or sentinel species. Once established, these MRM methods could be used to propose candidate biomarkers that are sensitive and specific to various contaminants. In this study, normalizing peptide abundances with a customized set of housekeeping peptides showed the relevance of dMRM to detect modulations in biological pathways and identify potential biomarkers. In order to evaluate the relevance of candidate biomarkers by absolute quantification, the same dMRM method platform could be used by spiking standard peptides. It is important to note that the candidate biomarkers of metal contamination identified in this study should be validated under more realistic environmental conditions. Indeed, these biomarkers were identified in gammarids exposed to controlled laboratory conditions, which does not take into account the complexity of the environment. Therefore, it seems necessary to evaluate the dynamics of these biomarkers in active biomonitoring campaigns in sites contaminated or not with metals to validate their relevance for diagnosing metal contamination in aquatic ecosystems.

4. Conclusion

In summary, this study proposes for the first time a dMRM method able to accurately track the abundance of 470 proteins, thus analyzing most of the key biological functions of an organ in a small crustacean used as

sentinel species. The comprehensive dMRM assay was highly effective for deciphering the biological effects of three metals on the proteome of *G. fossarum* caeca. Specifically, the dMRM analyses revealed dose-response effects and specific protein signatures for each tested metal, indicating distinct modes of action for Cd, Ag, and Zn on the caecal biology. Overall, these results demonstrate the strength of comprehensive dMRM approaches in screening the effects of multiple contaminants on the entire proteome, making it an ideal method for the *de novo* development of ecotoxicological biomarkers.

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CRedit authorship contribution statement

Maxime Leprêtre: Supervision, Conceptualization, Methodology, Formal analysis, Writing – original draft. **Arnaud Chaumot:** Supervision, Writing – review & editing. **Rayan Aboud:** Methodology, Formal analysis. **Nicolas Delorme:** Methodology. **Anabelle Espeyte:** Methodology, Writing – review & editing. **Arnaud Salvador:** Writing – review & editing. **Sophie Aycirix:** Methodology, Writing – review & editing. **Jean Armengaud:** Methodology, Writing – review & editing. **Marina Coquery:** Methodology, Writing – review & editing. **Olivier Geffard:** Supervision, Conceptualization, Methodology, Funding acquisition, Writing - review & editing. **Davide Degli-Esposti:** Supervision, Conceptualization, Methodology, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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