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DATASET BRIEF

Improving the proteome coverage of *Daphnia magna* - implications for future ecotoxicoproteomics studies

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Abstract

Aquatic pollution is an increasing problem and requires extensive research efforts to understand associated consequences and to find suitable solutions. The crustacean *Daphnia* is a keystone species in lacustrine ecosystems by connecting primary producers with higher trophic levels. Therefore, *Daphnia* is perfectly suitable to investigate biological effects of freshwater pollution and is frequently used as an important model organism in ecotoxicology. The field of ecotoxicoproteomics has become increasingly prevalent, as proteins are important for an organism's physiology and respond rapidly to changing environmental conditions. However, one obstacle in proteome analysis of *Daphnia* is highly abundant proteins like vitellogenin, decreasing the analytical depth of proteome analysis. To improve proteome coverage in *Daphnia*, we established an easy-to-use procedure based on the LC-MS/MS of whole daphnids and the dissected *Daphnia* gut, which is the main tissue getting in contact with soluble and particulate pollutants, separately. Using a comprehensive spectral library, generated by gas-phase fractionation and a data-independent acquisition method, we identified 4621 and 5233 protein groups at high confidence (false discovery rate < 0.01) in *Daphnia* and *Daphnia* gut samples, respectively. By combining both datasets, a proteome coverage of 6027 proteins was achieved, demonstrating the effectiveness of our approach.

KEYWORDS
aquatic pollution, *Daphnia*, data-independent acquisition (DIA), ecotoxicoproteomics, proteomics

Anthropogenic activities severely impact aquatic ecosystems. Substances originating from a wide variety of sources, like sewage, nutrients, and terrigenous materials, petroleum, heavy metals, and microplastics, have negative effects on aquatic ecosystems [1]. During the last years, comprehensive biomonitoring and exposure studies on the effects of various pollutants were done in many aquatic ecosystems with different organisms [1–3]. Several of these studies addressed morphological and life-history effects as well as underlying molecular mechanisms. In this context, proteomics has become a powerful tool, and the term "ecotoxicoproteomics" was introduced by Bjornstad et al. [4] and Gouveia et al. [5]. The cladoceran *Daphnia*, which inhibits a central role in lacustrine ecosystems [6], has been studied intensely and is an established test organism in ecotoxicology [7]. Although the overall number of proteomics studies on *Daphnia* is still limited, most of them focus on the effects of various toxins [8–11]. When investigating *Daphnia* in ecotoxicoproteomic studies, besides interfering gut

Abbreviations: GPF, gas-phase fractionation; DIA, data-independent acquisition

proteases [12], a common drawback are highly abundant proteins like egg-yolk vitellogenin, preventing the identification of lower abundant proteins. To increase the analytical depth of LC-MS/MS-based proteome analysis, we tested a procedure based on the separate analysis of whole daphnids and dissected daphnid guts combined with a data-independent acquisition (DIA) strategy [13]. A similar approach was followed, in the study of Gouveia et al. [14] in *Gammarus fossarum* where proteome and microbiome studies of the intestine (INT) and the hepatopancreatic caeca (HC) were performed.

For sample generation, the *D. magna* clone BL2.2 was cultured in groups of 20 female daphnids in 1.5 L jars containing 1 L M4 medium [15] at 20 ± 0.5°C and a 16 h:8 h light:dark regime. Daphnids were fed ad libitum with the unicellular green algae *Acutodesmus obliquus* three times a week. After the daphnids had released their third brood, gut samples and whole organism samples were taken, with five replicates each. For the dissection of the guts, daphnids were washed three times in phosphate buffer (25 mM, pH 7.4), and placed in phosphate buffer (25 mM, pH 7.4) containing protease inhibitor cocktail (complete Ultra tablet mini, Roche, Germany) under a stereomicroscope (Leica M50 with cold light source Leica KL 300 LED, Leica Microsystems, Germany) equipped with a digital camera for microscopy (Olympus DP26, 5 Megapixel, Olympus Corporation, Japan; software cellSens Dimension, Olympus Corporation, Japan). Dissected guts were transferred individually into 1.5 mL vials, the remaining buffer was removed, and then snap-frozen in liquid nitrogen. To prepare the whole animal samples, daphnids were washed three times in phosphate buffer (25 mM, pH 7.4), transferred individually into 1.5 mL vials, and then snap-frozen in liquid nitrogen. Daphnids were homogenized by ultrasonication in 40 μl of lysis buffer (8 M urea, 50 mM ammonium bicarbonate) supplemented with protease inhibitors (one Ultra tablet mini per 10 mL buffer (Roche, Germany)). *Daphnia* guts were lysed in 20 μl of lysis buffer using the same procedure. Protein quantification was done using the Pierce 660 nm Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA) and protein concentrations were adjusted. After reduction and alkylation, sequential digestion, first with Lys-C (4 h, 37°C) followed by trypsin (16 h, 37°C), was performed for all samples.

For LC MS/MS analysis, 1 μg of peptides were loaded on a trap column (PEP-Map100 C18, 75 μm × 2 cm, 3 μm particles (Thermo Fisher Scientific, USA)) and separated on a reversed-phase column (PepMap RSLC C18, 75 μm × 50 cm, 2 μm particles, Thermo Scientific, U.S.A) at a flow rate of 250 nl/min with an 80 min gradient of 5%–20% solvent B followed by 9 min increase to 40%. After separation, the column was washed with 85% solvent B for 9 min. Solvent A consisted of 0.1% FA in water and solvent B of 0.1% FA in acetonitrile. Measurements of the two sample sets (whole *Daphnia* and *Daphnia* guts) were performed sequentially, each in the following order adopted from Pino et al. [16]. First, three samples of a set were measured, then six gas-phase fraction runs (GPF), followed by the last two samples of a set. To minimize carryover, we run blanks between samples. DIA MS analysis was performed with a Q Exactive HF-X mass spectrometer (Thermo Scientific, U.S.A), acquiring 75 × 8 m/z wide DIA spectra in the range of 400–1000 m/z, using a staggered window scheme optimized by skyline [17]. For DIA library construction, instrument settings were adjusted based on Pino et al. [16], and pooled samples of *D. magna* and *D. magna* guts were measured six times, using staggered narrow 4 m/z isolation windows (GPF1: 400–500 m/z; GPF2: 500–600 m/z; GPF3: 600–700 m/z; GPF4:700–800 m/z; GPF5: 800–900 m/z; GPF6: 900–1000 m/z) as described in Searle et al. [18]. Mass spectra were acquired using a collision energy of 27, resolution of 15 K, maximum inject time of 20 ms, and an automatic gain control (AGC) target of 1e6. The built-in deep learning-based spectrum and retention time prediction of the software DIA-NN [19] in combination with the *D. magna* UniProt database (08/2021) were used for library generation and data analysis. For the analytical runs, the precursor range was set to m/z = 390–1010. For the peptide intensity calculation, a fixed number of data points were integrated around the peak apex (DIA-NN setting: robust LC) to minimize the effect of noise on the data [20, 21]. Raw intensities were normalized using MaxLFQ algorithm, as described in Cox 2014 [22]. For further parameters, the default settings were used. The "Genes" column was used for benchmarking IDs to count unique proteins as described by Demichev et al. [23]. For further data evaluation in Perseus (1.5.3.2) [24], the unique genes matrix containing data filtered at FDR < 0.01 was used. Additionally, we screened for similar protein sequences within our dataset using CD-HIT [25] with a sequence identity cut-off of 80%. To obtain graphical illustration, R Studio (4.1.1) was used with the tidyverse and psych packages. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [26] with the project accession: PXD029198. To obtain further information including GO and protein class annotations of the identified *D. magna* proteins, a DIAMOND [27] search against the UniProt *Daphnia pulex* database was performed and the best matched orthologues (identity > 75%) in this search were mapped to GO terms and protein classes using PANTHER [28].

Applying the above-mentioned workflow, based on DIA combined with a comprehensive *D. magna* spectral library, we were able to identify 31,952 unique peptides from entire *Daphnia* samples, which could be assigned to 4621 proteins (FDR < 0.01). In the *Daphnia* gut samples, 46,091 unique peptides could be identified and assigned to 5233 proteins (FDR < 0.01). A CD-HIT analysis, at a sequence identity cut-off of 80%, revealed only 93 similar sequences in the entire daphnid dataset and 94 similar entries in the *Daphnia* gut dataset, demonstrating the low degree of redundancy within the list of identified proteins. The corresponding proteins are marked in Table S1 (ST_1, ST_2) and corresponding clusters are listed in Table S1 (ST_5, ST_6). Unsupervised hierarchical clustering (Figure 1A), as well as a principal component analysis (PCA) (Figure 1B), shows a clear separation between the

Statement of significance

The presented workflow significantly improves the proteome coverage in *Daphnia magna* and will be particularly useful for future ecotoxicoproteomics studies in *Daphnia*.

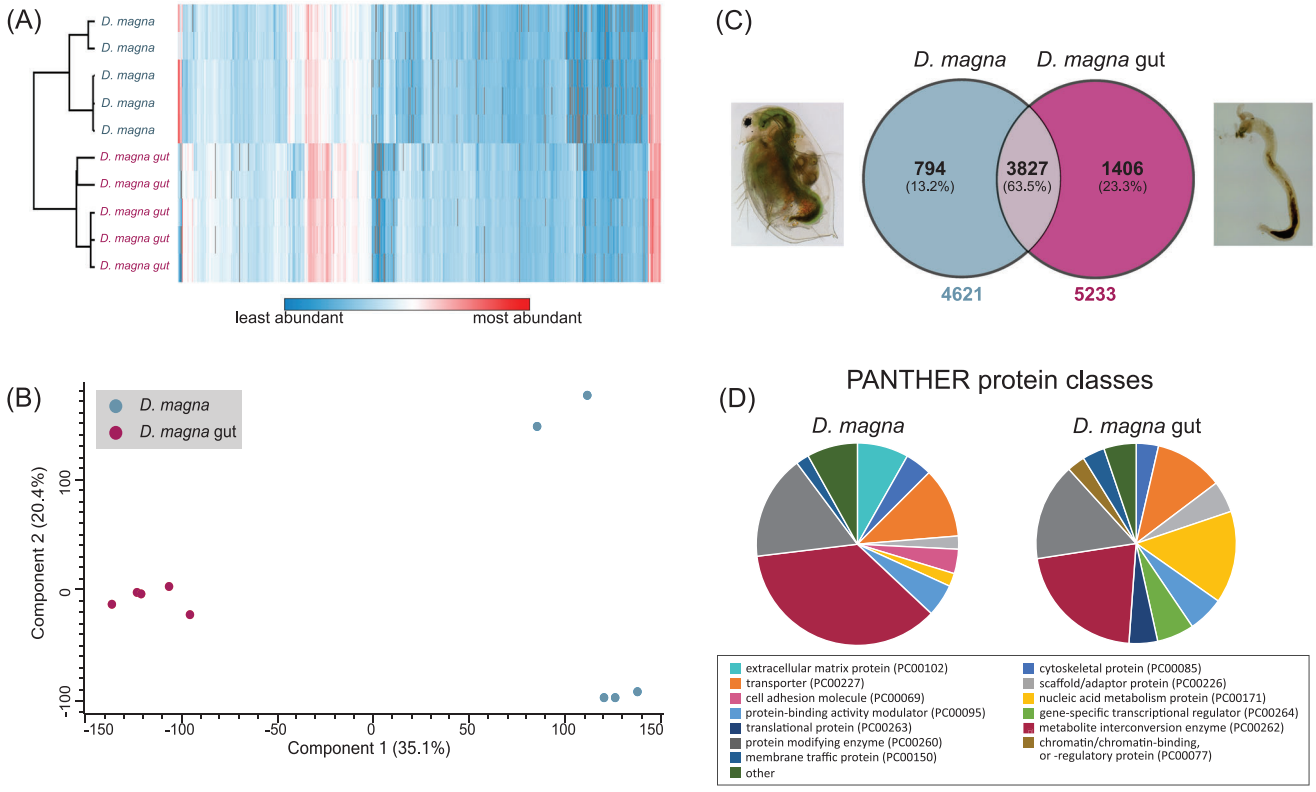

FIGURE 1 Heatmap (A) and PCA (B) of intensity values of entire *D. magna* and *D. magna* gut proteins. (C) Venn diagram showing the overlap of identified proteins within the two groups *D. magna* and *D. magna* gut. On the sides, pictures of a whole *D. magna* and the dissected gut of *D. magna*. (D) PANTHER functional classification of protein classes, for proteins found exclusively in *D. magna* and exclusively in the *D. magna* gut. "Other" includes all protein classes with a percentage < 2%

sample types. Between the identified proteins from the whole daphnids and the dissected guts, 3827 were shared. Thus, while only 794 proteins were identified exclusively in the whole *Daphnia*, 1406 proteins were identified exclusively in the *Daphnia* guts (Figure 1C). A functional classification (PANTHER) of proteins found exclusively in the *Daphnia* and the *Daphnia* gut samples respectively, revealed strong differences concerning the protein classes between both sample sets (Figure 1D and Table S1 (ST_3, ST_4)). The results for the GO classification can be seen in Figure S1 and Table S1 (ST_1, ST_2). The top 10 most abundant proteins for *Daphnia* and *Daphnia* guts (Table 1) are listed below. The list of all identified and quantified proteins can be found in Table S1 (ST_1, ST_2). In the whole daphnid samples, the most abundant protein is the Vitellogenin domain-containing protein (APZ42_030366), followed by Gastrotropin (APZ42_014823) and other vitellogenin related proteins (APZ42_007246, APZ42_034044, and dmagvtg1). In the gut, the two most abundant proteins belong to the family of fatty acid-binding proteins (FABPs; Gastrotropin (APZ42_014823) and Myelin P2 protein (APZ42_014822). Furthermore, several serine protease family members like trypsin and chymotrypsin-like proteins were detected in high abundance. We additionally performed a Panther overrepresentation test for the gut samples (results can be found in Table S2) and found GO terms, connected to some digestion related proteins listed in Table 1 (e.g., Gastrotropin and Trypsin) to be overrepresented. More specifically, we found the GO term proteolysis (GO:0006508) to be enriched (adjusted *p*-value: 1.01E-15) in which trypsin and the Chymotrypsin-like proteins are involved. Furthermore, we found the term lipid binding (GO:0008289) to be overrepresented with an adjusted *p*-value of 2.89E-15, to which Gastrotropin is connected. The reproducibility of the individual sample preparations was evaluated with scatter plots, showing high Pearson correlation coefficients > 0.87 for all replicates (Figure 2). Strikingly, the correlation within the gut samples appeared significantly higher, indicating that the proteomes of entire daphnids showed a higher interindividual variance than the individual gut proteomes.

The advantage of analyzing dissected guts and entire daphnids separately is underlined by the fact that 1406 proteins could be identified exclusively in the gut samples. This is particularly relevant since the gut is the body part that is directly exposed to toxic substances and is therefore predestined for ecotoxicological studies using soluble or particulate compounds. Our experimental approach may therefore be useful to investigate the effects of aquatic micro-and nanoplastic pollution on daphnids [29–32], since *Daphnia*, as filter-feeder, is particularly exposed to particulate contaminants. Compared to other proteomic studies on *D. pulex* [9, 33, 34] as well as studies on *D. magna* from our lab [29, 35–37], our dataset and spectral library, containing data from 58186 unique *D. magna* peptides and

TABLE 1 List of the ten most abundant proteins found in the entire *Daphnia* and the *Daphnia* gut samples, searched against the UniProt database and sorted by descending intensities

Protein name	*D. magna* Accession	*D. magna* Gene name	Intensity	
Vitellogenin domain-containing protein	A0A164NU47	APZ42_030366	3.47E+09	Entire *Daphnia*
Gastrotropin	A0A162NXP2	APZ42_014823	3.20E+09	
VWFD domain-containing protein (Fragment)	A0A164FEL7	APZ42_007246	2.88E+09	
Superoxide dismutase[a]	A0A164KEM1	APZ42_034044	2.54E+09	
Superoxide dismutase[a]	Q766D3	dmagvtg1	2.09E+09	
Myelin P2 protein	A0A162NXQ0	APZ42_014822	1.93E+09	
Myosin heavy chain	A0A162DGC9	APZ42_024143	1.93E+09	
Uncharacterized protein (Fragment)	A0A162NPJ9	APZ42_016145	1.80E+09	
Superoxide dismutase[a]	A0A164KEC2	APZ42_034043	1.49E+09	
VWFD domain-containing protein	A0A164KJQ5	APZ42_033978	1.48E+09	
Gastrotropin[a]	A0A162NXP2	APZ42_014823	2.02E+10	*Daphnia* gut
Myelin P2 protein	A0A162NXQ0	APZ42_014822	1.01E+10	
Gelsolin	A0A162REA6	APZ42_012792	2.94E+09	
Gastrotropin[a]	A0A162NXN3	APZ42_014825	2.64E+09	
Chymotrypsin-like protein	A0A164XNK5	APZ42_019721	2.19E+09	
C-type lectin ctl-mannose-binding	A0A0P5Z2V5	APZ42_022561	1.94E+09	
Uncharacterized protein	A0A164ZPB9	APZ42_017126	1.27E+09	
Uncharacterized protein	A0A164QBY8	APZ42_028735	1.22E+09	
Trypsin	A0A162CGT5	APZ42_018874	1.21E+09	
Prostaglandin D2 synthase-like protein	A0A164LEK4	APZ42_033122	1.12E+09	

[a]Corresponding sequence alignments can be found as Supplementary Text Files 1 and 2.

6027 proteins, represents one of the largest libraries and may be the base for future studies addressing the proteomes of daphnids. Due to the lack of comparable, publicly available *D. magna* proteomic datasets, no further in-depth meta-analysis of other datasets was performed. Nevertheless, our results strongly suggest that analyzing proteomes of entire daphnids and their guts separately, combined with a sophisticated DIA method, significantly improves the overall proteome coverage.

In conclusion, the presented approach is easy to perform and knowledge on molecular changes in the gut is particularly interesting due to its direct interactions with environmental pollutants. Further, the advancement of ecotoxicoproteomics may foster our understanding on

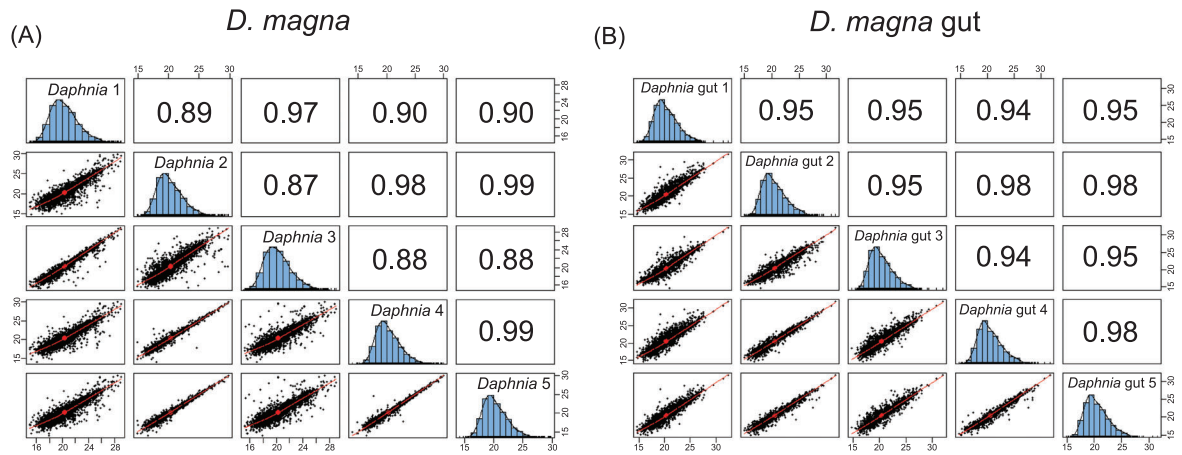

FIGURE 2 Multiscatter plot of log2 normalized intensity values of DIA measurements in (A) *Daphnia* and (B) *Daphnia* guts. The Pearson correlation is shown in the upper right corner of the scatter plots. Histograms show the distribution of log2 normalized intensity values for each sample

the molecular mechanisms underlying adverse effects of soluble and particulate contaminants in freshwater ecosystems.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the PRIDE repository, dataset identifier PXD029198. Project Name: Improving the proteome coverage of Daphnia magna.

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SUPPORTING INFORMATION

Additional supporting information may be found online https://doi.org/10.1002/pmic.202100289 in the Supporting Information section at the end of the article.

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