Research Article

Exploratory Analysis of the Application of Animal Reduction Approaches in Proteomics: How Much Is Enough?

Laura M. Langan and Bryan W. Brooks

Department of Environmental Science, Institute of Biomedical Studies, Baylor University, Waco, TX, USA

Abstract

Animal testing has long been the cornerstone of chemical safety assessments, but fish embryo assays represent an alternative. Omics studies allow the examination of early molecular responses of organisms to environmental stressors, but reduction of animal use within this context has been overlooked. For proteomics, there is significant disparity and variability in the organismal pool size used for studies, ranging from 1-1500 embryos per replicate for zebrafish alone. However, it is unknown if varying sample pool size results in differences in protein identifications. To examine whether the detected proteome changes depend on this variable, 3 pool sizes (5, 10 or 20 embryos or larvae per replicate) were compared using the two most common fish models with an appropriate biological replicate number determined by power analysis (n = 7). Data was acquired using MS^e, resulting in 1,946 and 3,172 protein groups identified (1% false discovery rate) for fathead minnow and zebrafish, respectively. Proteins were not differentially expressed among pool sizes, and no significant difference was observed among the identified protein groups. However, for the fathead minnow, a decrease in the number of identified proteins was observed with increasing pool size, while a trend towards an increase in protein identifications was observed in zebrafish between the lowest and highest pool size. Taken together, our observations suggest that a proteome characterization experiment using these fish models can achieve comparable protein identifications using pool sizes of less than 5 organisms per replicate, assuming a protein requirement of 50 µg or less.

1 Introduction

Globally, the use of animals for scientific purposes has been increasing (Taylor and Alvarez, 2019). Similarly, the use of fish in scientific research has also been increasing worldwide due to a rapid expansion of the fish farming industry and the increasing use of fish as models for the assessment of ecotoxicity of chemicals, as transgenic model organisms for human diseases, and in the pharmaceutical industry. Russell and Burch (1959), who originally proposed the 3Rs principle, envisaged its hierarchical application where priority should be placed on replacing, followed by reducing, and lastly refining animal experiments. 3Rs methods are often less costly than traditional animal tests and, in some cases, provide better information to inform risk assessment. Fish as non-mammalian vertebrates provide researchers different opportunities to implement the 3R principles (Schaeck et al., 2013; UK Home Office, 2015), e.g., the use of fish instead of rodents is regarded as a refinement in the sense of the 3Rs (Bert et al., 2016).

It is important to note that there are varying stances regarding the protection of fish for use as experimental animals in different countries (Halder et al., 2010). Full reporting and break-down of animals per country is rare, although within the European Union (EU), where fish used for experimental purposes are protected under Directive 2010/63/EU (as amended by Regulation (EU) 2019/1010), the Commission makes all EU-level statistical information on the use of animals for scientific purposes publicly available through regular reports and in the ALURES[1] statistical

Received July 21, 2021; Accepted November 15, 2021;
Epub November 24, 2021; © The Authors, 2022.

ALTEX 39(2), 258-270. doi:10.14573/altex.2107212

Correspondence: Laura M. Langan, PhD
Department of Environmental Science
Institute of Biomedical Studies
Baylor University, Waco, TX, USA
(Laura_Langan@Baylor.edu)

[1] https://ec.europa.eu/environment/chemicals/lab_animals/alures_en.htm

EU database. In the United States, cold-blooded animals like fish are not covered by the Animal Welfare Act and therefore are not counted in the annual US Department of Agriculture statistics[2]. However, even regulatory jurisdictions that do not protect fish as experimental animals provide indications that they support their replacement, reduction or refinement based on their acceptance of certain assays or encouragement to develop and use new assays and approaches (e.g., Japan and the United States).

The use of fish as experimental models has increased in the EU from 13% in 2015 (EU28) to 26% in 2018 (EU28 and Norway)[1] of all animal use. It is important to note that the changes in reported use over time are based on juvenile and adult fish, since procedures on immature forms (embryos and larvae) are not protected and therefore not counted in the EU unless they have reached the free-feeding stage (> 5 dpf for zebrafish reared at 28°C).

To address the critical need to determine acute toxicity of an ever-increasing list of chemicals, the OECD adopted the fish embryo acute toxicity test (FET) in 2013 as OECD TG 236 (OECD, 2013). Zebrafish embryos, which are used for FET assays to replace the acute fish toxicity (AFT) test (Rawlings et al., 2019), have also been assessed to replace larval growth and survival tests (Jeffries et al., 2015) and shown to be a useful surrogate for other fish species used in the AFT (Su et al., 2021).

While FET tests already play an important role in identifying potential hazardous substances, the use of omics technologies, i.e., integrative molecular approaches (DNA, RNA, protein), within these models can provide a more comprehensive understanding of the molecular effects of toxins at different levels of biological organization and are predicted to be among the most plausible solutions to the international chemical safety crisis (Campos et al., 2018; Becker, 2019; Sauer et al., 2017). The use of omics technologies is expanding, and the global metabolomics market was estimated to have a worth of over 2 billion USD in 2021 while the proteomics market was estimated to reach a projected worth of > 55 million USD by 2026 (Meigs et al., 2018), which is primarily driven by an increase in research activity[3].

Proteomics, the large-scale study of proteins, is already an indispensable technology for the characterization of complex biological systems, particularly for observing changes caused by chemical agents. The current potential for using proteomics in toxicological research has developed concurrently with advances in the technology to separate and identify thousands of proteins in complex mixtures, including single cell proteome characterization in complex tissues (Lombard-Banek et al., 2019; Aballo et al., 2021). Nevertheless, in contrast to the established use of proteomics in other disciplines such as animal production (Almeida et al., 2021), it remains unclear how best to make use of proteomics information in a regulatory context. Of relevance to the current study, proteomics was proposed to harbor the potential to significantly reduce and refine the use of laboratory animals (Kroeger, 2006).

While comparatively new, the use of proteomics in aquatic organisms is highlighted in several excellent reviews (López-Pedrouso et al., 2020; Gouveia et al., 2019; Knigge, 2015; Sanchez et al., 2011). For over 20 years, proteomics has advanced an understanding of basic physiological and developmental processes (Purushothaman et al., 2019; Ge et al., 2017; Wu et al., 2017; Kwon et al., 2016) and tissue-specific protein expression patterns (Lavelle et al., 2018; Martyniuk and Alvarez, 2013), and further enhanced efforts to define responses of fish to environmental stressors such as hypoxia (Dhillon and Richards, 2018; Delcourt et al., 2015), endocrine disrupting chemicals (Ayobahan et al., 2020; Smith et al., 2018; Wit et al., 2010; Martyniuk et al., 2009), harmful algal blooms (Chen et al., 2017; Frøyset et al., 2016), perfluorinated chemicals (Hagenaars et al., 2013; Zhang et al., 2012), nanomaterials, and PAHs (Della Torre et al., 2018; Gündel et al., 2012).

Next to zebrafish embryos, which are the species used in OECD TG 236 (OECD, 2013), other species with varying developmental timelines are also used in the context of embryo fish bioassays (Stieglitz et al., 2016). For example, Japanese medaka hatch after approximately 7 days, while fathead minnow hatch after approximately 4-5 days at water temperatures between 23-30°C, and after up to 13 days at cooler temperatures (13°C). In this respect, the different physiology and behavior of fish species makes it necessary to define species-specific criteria for assessing animal welfare. However, given that there are over 30,000 different teleost fish species, which differ profoundly in anatomy, physiology, etc., focus must be placed on model organisms commonly used for experimental purposes.

With a view towards implementation of the reduction approach in toxicological studies, the initial aim of this study was to establish what number of embryos or larvae is required for proteomic characterization, given the sensitivity of the method and the lack of a universal current definition of protected life stages for different teleost species. Proteome characterization using a shotgun/global approach was undertaken using the two most common larval fish models, the fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*), with the goal of understanding whether variations in organism pool size significantly and specifically impact the number of protein identifications.

Considering previously reported issues for regulatory bioinformatics in food and drug safety (Healy et al., 2016), including the need for standards and validation processes, the use of standardized internationally recognized toxicity testing methods specific to each species for the experiment were employed. For proteomics, statistical power depends on specific factors including variance in protein expression, effect size (the change in protein expression), number of replicates, and the α selected by the researcher. Too many replicates may be a waste of time and resources, whereas a study with low power may not detect biologically important protein changes. Depending on the experimental protocol, three biological replicates are typically used in aquatic proteomic studies although prior research in human plasma pro-

[2] https://bit.ly/3raEcWt

[3] https://www.marketsandmarkets.com/Market-Reports/proteomics-market-731.html

teomics has demonstrated that six samples per group/treatment provide sufficient statistical power for most proteins with fold changes > 2 (Zhou et al., 2012). Given that the current study examines whether animal number affects the quantity of proteins identified per pool size, balancing replicate number is critical.

A primary goal of proteomics is to comprehensively identify all proteins in each sample and further to determine how the proteome is altered in response to a modifier, stressor, or toxin. Most proteomic studies using larval fish do not explicitly justify the use of the number of individual organisms that are pooled prior to analysis, although exceptions exist (Lee et al., 2021). We hypothesized that proteomic characterization would be best achieved with a minimal pool size to avoid highly abundant proteins obscuring the detection of sensitive response indicators usually expressed at lower levels. The organismal pool size was chosen by balancing how many embryos/larvae are needed to obtain 50 µg of protein for sample digestion with the minimum pool size range reported in the literature for zebrafish (see Tab. S1[4]). Based on the literature and the outlined experimental parameters, the required protein content is equivalent to ~6-13 larvae (3-5 dpf) (Lemeer et al., 2007), whereby zebrafish are considered in the larval stage following the development of the protruding-mouth at 72 h according to Kimmel et al., (1995).

2 Animals, materials and methods

Experimental design and animal husbandry

An *a priori* power analysis was carried out to calculate the minimum number of samples such that results are reproducible using the pwr package in R, with the power of the experiment set at 0.8, group set to 3, α set at 0.05, and presuming that proteome coverage of differing pool sizes would have a medium to large effect (0.5-0.8) (Cohen, 1988). This resulted in an n value of 6-14. Balancing power with available resources for this initial study, an n of 7 was chosen for proteomic characterization.

Culture and experimental conditions followed Institutional Animal Care and Use Committee protocols approved at Baylor University. Tropical 5D wildtype zebrafish were maintained under standard culture conditions at Baylor University as previously described (Kristofco et al., 2015, 2018; Steele et al., 2018). Adult zebrafish (12-14 months) were spawned under natural breeding conditions the evening prior to egg collection, and embryos were collected in a sieve within two hours of the onset of daylight. Embryos were subsequently washed with culture water to remove feces, rinsed with hydrogen peroxide (~3%) to minimize fungal contamination, and washed with fresh culture water before being transferred into a collection petri dish. Organisms were staged (Kimmel et al., 1995) and placed in 100 mL beakers with clear culture water volume varying based on OECD TG 236 (OECD, 2013) guidelines. Embryos were maintained at 28°C with a 14 h light/10 h dark photoperiod and received daily water changes. At 96 hpf (hours post fertilization), larvae were immobilized by submersion in ice water (~5-10 min), and snap frozen in liquid nitrogen.

Fathead minnow larvae (96-120 h old) were obtained from Environmental Consulting and Testing (Superior, WI, USA). Fish larvae were acclimated in aged, dechlorinated tap water before being transferred to individual beakers according to standardized EPA methods (US EPA, 2002), where each larval fish received 5 mL of tap water. Fish larvae were cultured under a 16:8 h light:dark photoperiod and fed *Artemia* sp. nauplii (Pentair AES, Apopka, FL, USA) twice per day by administering 50-150 µL aliquots (density dependent) in an incubator held at 25°C for the duration of the experiment (96 h). Daily water renewals (~80%) occurred for the duration of the experiment. Following 96 h of growth, larvae were immobilized by submersion in ice water and snap frozen in liquid nitrogen.

Fish were randomly partitioned at a density of 5, 10 and 20 embryos/larvae per experimental unit (beaker), with seven replicates (beakers) of each experimental unit for both fish species.

Proteomic characterization

A complete standard operating procedure[5] is provided. Briefly, frozen homogenates were thawed on ice, washed twice with PBS, and then lysed using buffer described by Wiśniewski (2016) for zebrafish or RIPA buffer supplemented with 2% SDS for fathead minnow. Protein concentration was quantified using the Pierce BCA assay (Thermo Fisher). For rapid evaluation of quality and reproducibility among biological and technical replicates and starting pool sizes, 3 samples for both zebrafish and fathead minnow were first visually inspected using SDS-PAGE (100 µg) followed by Coomassie blue staining (Fig. S1[4]).

Samples were then analyzed using a Waters Synapt GS-S (Waters, Milford, MA) quadrupole time-of-flight (Q-TOF) mass spectrometer tuned to a resolution of 20,000 (FWHM) controlled by MassLynx software (Version 4.2, Waters). For each sample, 1-7 µL (species dependent), equivalent to ~500 ng peptides, were injected with loading kept low to avoid detector saturation, although MS^e has been reported to produce consistent identifications in the range of 0.25-1 µg (Shliaha et al., 2013). Peptides were separated using a nanoAcquity Ultra Performance LC system (Waters, Milford, MA) equipped with 75 µm x 100 mm BEH C18 column with a particle size of 1.7 µm and a nanoAcquity UPLC Symmetry C18 Trap column (Waters, Milford, MA). The mobile phase A consisted of 0.1% formic acid in water, and B consisted of 0.1% formic acid in 100% acetonitrile. Each sample was first retained on a trapping column and then washed using 99.5% A for 3 min at a flow rate of 10 µL/min. Peptides were separated using a run time of 95 min gradient with a 0.6 second scan time and a mass range of 50-2000 Da. Mass spectra were acquired in MS^e mode, alternating between low energy scan [Func 1] (6 eV) to acquire peptide precursor data and higher energy scan [Func 2] (ramping from 27 to 50 eV) to acquire fragmentation data. An auxiliary pump delivered a [Glu1]-fibrinopeptide B (GFP) (m/z

[4] doi:10.14573/altex.2107212s

[5] doi:10.17504/protocols.io.btyvnpw6

785.8426) as an external calibrant (lockmass) at a concentration of 100 fmol/µL at a rate of 0.5 µL/min. Quality controls (QC), prepared by pooling equal volumes of each sample, were injected at the beginning and thereafter every 7 samples to provide a measurement of the system's stability and performance, and the reproducibility of the sample preparation and analysis method.

Data analysis

In this study, n refers to the number of replicate beakers with varying stocking densities of individual organisms for each species. Statistical analysis was carried out in R (Version 4.0.2)[6]. Data (total protein content) was tested for normality using the Shapiro-Wilk (SW) test and homogeneity of variance using the Bartlett test (B), and when assumptions were met, a t-test was performed, and results presented following Bonferroni correction. Box and whisker plots were used to display the data giving a summary of the relevant variables in the form of median values, quartiles, range, and possible extreme values (outliers).

For proteomics, Waters MS[e] files were converted to .mzML file format using msconvert software (Chambers et al., 2012) to import them into the R project environment. The conversion parameters, R scripts, packages, and commands are supplied in Table S2[4]. Briefly, all files were centroided using MSNbase (Gatto et al., 2020), with a preliminary experiment using internally spiked enolase (644.8595 m/z; 100 fmol) used to determine centroiding parameters.

Following centroiding, peak picking, retention time alignment, and grouping were carried out using the xcms package (Benton et al., 2010; Tautenhahn et al., 2008; Smith et al., 2006) with study parameters chosen using a combination of IPO and patRoon packages (Helmus et al., 2021; Libiseller et al., 2015) using QC samples consisting of aliquots from all samples combined (n = 3-4) for zebrafish and fathead minnow, respectively. Extracted peaks were exported, searched using MSGFplus, and rescored by Percolator (Käll et al., 2007; Spivak et al., 2009; The et al., 2016), a combination that has been shown to outperform popular peptide identification tools for numerous data sets (Kim and Pevzner, 2014). As Q-TOF is a relatively new spectral type, a new scoring parameter was trained and utilized for any subsequent data analysis. MS1 mass tolerance was constrained to 50 ppm, and the fragment ion mass tolerance was set to 0.5 Da (determined after running MSGF+ scoring param on the data; full search parameters Tab. S2[4]). False discovery rate (FDR) was set at 1% using a target decoy approach (TDA). Decoy protein sequences were generated from the zebrafish fish proteome UP000000437 (UNIPROT; 46,849 sequences) or from the fathead minnow proteome (EPA FHM 2.0; 47,578 protein sequences (Martinson et al., 2021) using the de Bruijn decoy generation tool (Moosa et al., 2020) and the target-small decoy search strategy (Kim et al., 2019). Parsimony principle was applied for protein grouping, and the level of confidence for peptide identifications was estimated using the Percolator node with decoy database search. Statistical post-processing was performed with Percolator and peptide spectrum matches filtered by q-value (1% FDR). Biological replicates were filtered to keep only identifications that were observed in 4 out of the 7 replicates in both species, with normalization carried out using variance stabilization normalization (Vsn) (Välikangas et al., 2018) and missing values imputed using KNN (k nearest neighbors, biological replicates) and QRILC (for species datasets) (Wei et al., 2018; Lazar et al., 2016). Differential expression was carried out

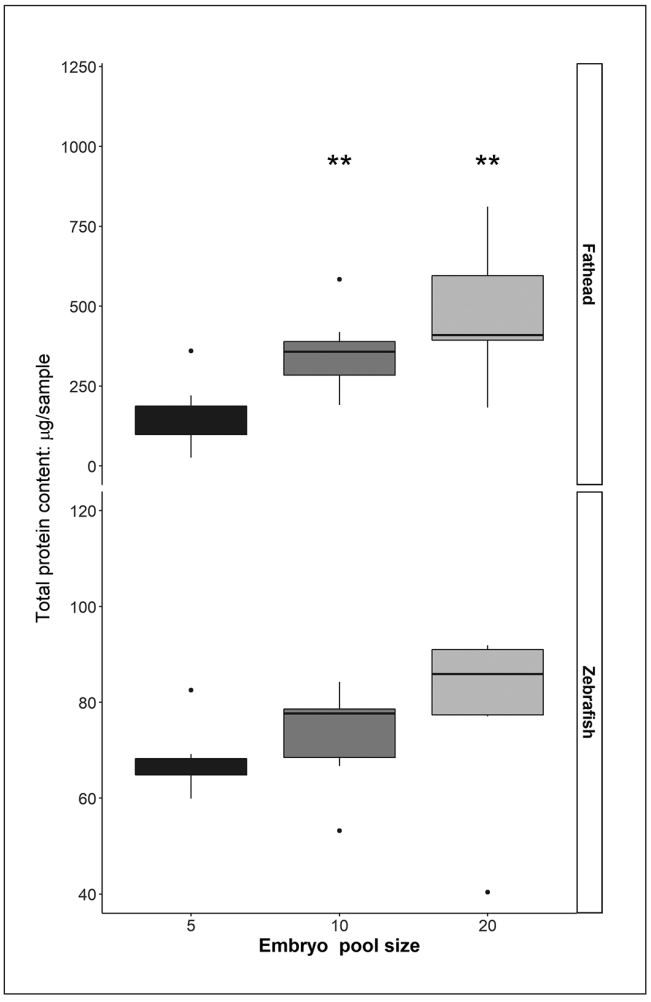

Fig. 1: Comparison of total protein concentration in lysates from two species and three pool sizes

5, 10 or 20 fathead minnow larvae or zebrafish embryos were snap frozen and lysed in TRIS-HCL or RIPA buffer, respectively. Protein content was determined by the Pierce BCA assay. Total protein is calculated based on the precipitated sub-sample (200 μL for fathead minnow and 100 μL for zebrafish) for both species. Note the difference in scale between fathead minnow and zebrafish. (n = 7; **, $p < 0.01$).

6 http://www.r-project.org/

7 Gregori, J., Sanchez, A. and Villanueva, J. (2016). msmsEDA and msmsTests: R / Bioconductor packages for spectral count label-free proteomics data analysis. (Version 1.28.0)

Tab. 1: Protein group identifications at two precursor mass tolerances for both zebrafish and fathead minnow
Protein abundance was estimated following percolator post-processing using spectral counting (SC) whereby the total number of fragments that map to peptides of a given protein are counted. For each pool size and species, n = 7.

		20 ppm tolerance			50 ppm tolerance		
Species	**Pool size**	**IDs**	**1% FDR**	**5% FDR**	**IDs**	**1% FDR**	**5% FDR**
Zebrafish	5	3705	2770	3161	5689	3512	4158
	10	3710	2814	3149	5721	2258	2701
	20	3706	2825	3181	5716	3633	4551
	All	4179	3172	3593	7619	3906	4644
Fathead minnow	5	3426	1574	2348	5426	2951	3763
	10	3040	1320	2072	4483	2312	2981
	20	3179	1455	2144	4887	2438	3289
	All	3978	1946	2817	7044	4034	5098

using msmsTests[7] with p-value < 0.05 and a q-value [Benjamini Hochberg] < 0.01 (FDR = 1%) considered significant. However, as each sample represents pools of the same organism grown under the same conditions, we did not expect any differentially expressed proteins to be identified. For both species, the end of the experiment fell at a time that both fish would be considered in the larval stage of development (> 72 hpf [zebrafish]; > 5 dph [fathead]) although the experiment was initiated while the fish were both in the embryo development phase. Therefore, results are presented based on the developmental stage at the initiation of the experiment for both species (embryos).

3 Results

3.1 Variation in total protein content

Determination of total protein concentration provides important information on the quality and reproducibility of the initial extraction process. It further feeds into the proteomic pipeline where an average protein amount is used as an estimate for the starting value in the mass spectrometry protein digestion protocol. For both species, an increase in total protein content was observed that was proportionate to the increasing initial sample pool size. Statistically significant differences (t-test, $p < 0.05$, $n = 7$ per pool size for each species) in protein content were observed only in fathead minnow samples and only in comparison to the 5-embryo pool size (Fig. 1). Total protein content per embryo was 17 ± 7 µg for zebrafish and 28 ± 3 µg for fathead minnow.

3.2 Protein group identification and expression profiles

For the fathead minnow model, 1,946 total protein groups were identified using a search tolerance of 20 ppm and filtered at 1% FDR (~4% of the total searched database), with the highest number of identifications in the 5-pool size (Tab. 1). Variability was visualized using a boxplot, with a non-significant ($n = 7$, $p > 0.05$) decrease in identifications observed with increasing pool size (Fig. 2A).

When identifications were examined, the highest proportion of unique proteins was identified in the 10-pool size, with a mean overlap of 29% observed among the pool sizes (Fig. 2B). Relaxation of the FDR criteria to 5% resulted in an increase in overlap among the pool sizes to 52%, with a change in unique protein allocation (Fig. S3[4]). Taken together, observations for fathead minnow revealed a modest and non-significant increase of ~8% identifications at the smaller pool size over the larger pool size, supporting the hypothesis that using a small pool size for proteomics studies is feasible with this species using the applied criteria.

In zebrafish embryos, 3,172 total proteins were identified in the full experiment (~7% of the total searched database) (Tab. 1). No significant difference in protein identifications was found ($n = 7$, $p > 0.05$; Fig. 2C), though there was a modest increase in identifications from the 5 to the 20 embryo pool size. Although mean protein group identifications appear close in the 10- and 20-embryo pool size, they overlapped only ~30% (1% FDR), with the 20-embryo pool size resulting in the highest number of unique protein identifications at 20% (Fig. 2D; see Fig. S2[4] for more detailed breakdowns).

To investigate the impact of this consideration further, unique proteins identified by pool size in zebrafish were grouped according to gene ontology using the clusterProfiler package in R (Yu et al., 2012), specifically focusing on biological processes and molecular function, with the top 3-4 rankings of gene counts per pool size reported (Tab. S3[4]). For molecular functions, the highest proportion of gene counts was associated with the larger pool size; however, this observation switched for biological processes whereby the 5-embryo pool size had the higher proportion of unique gene counts. This proportional result suggests that pool size plays an important role in protein groupings in the later stages of the proteomics pipeline.

Protein quantification for shotgun proteomics is a complicated process where errors can be introduced in each of the steps. Due to the hypothesis tested in the present study, a stringent FDR of 1% was chosen to provide confidence in observed trends. How-

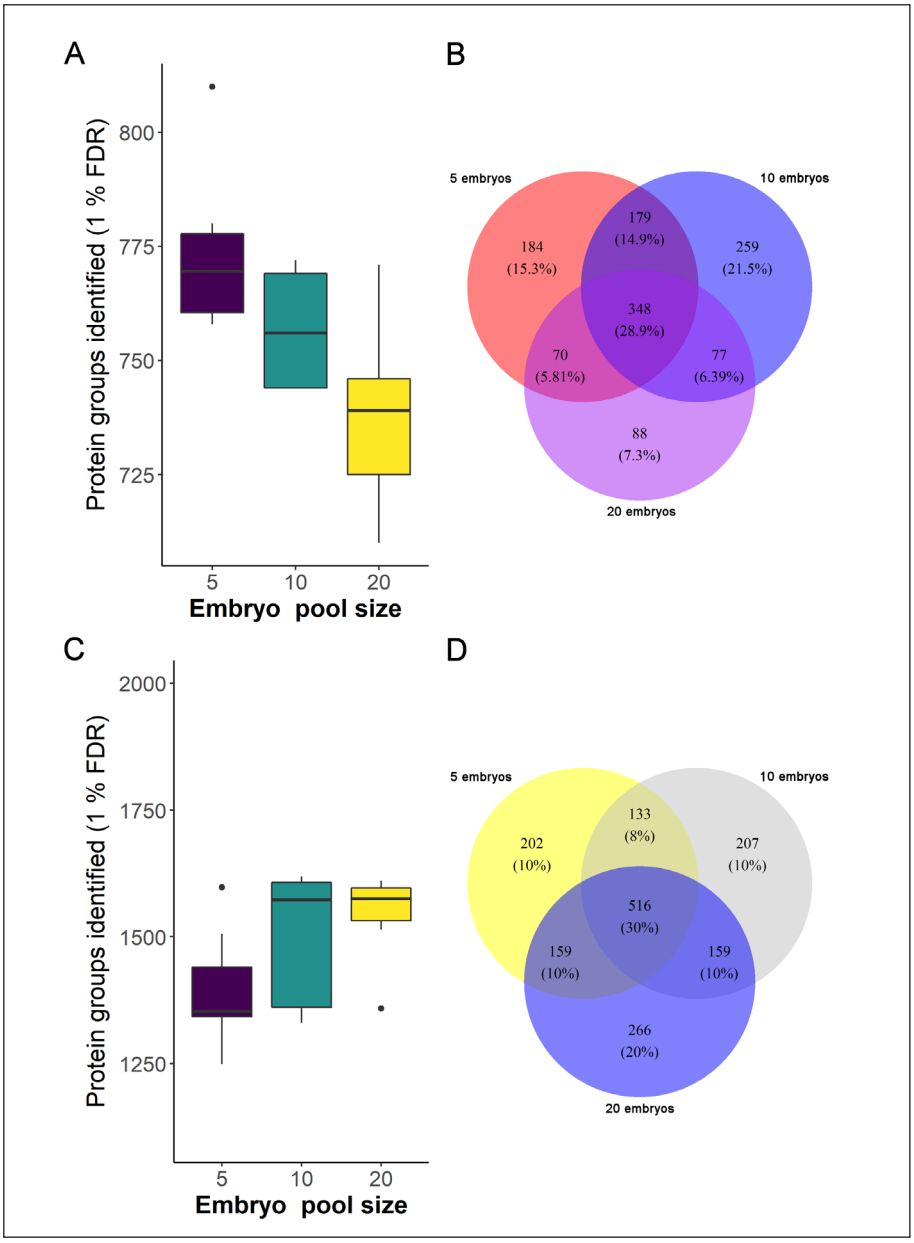

Fig. 2: Comparison of total protein identification differences between fathead minnow (A,B) and zebrafish (C,D) with different pool sizes
Number of protein groups identified by Q-TOF mass spectrometer and searched at 20 ppm and post-filtered using Percolator (1% FDR) in pool sizes of 5, 10 and 20 fathead minnow larvae (A) and zebrafish embryos (C). Co-efficient of variation between identification of replicates for both zebrafish and fathead minnow was approximately 3%. ($n = 7$; $p > 0.05$). Number of unique proteins and overlap between pool sizes in fathead minnow larvae (B) and zebrafish embryos (D). It should be noted that overlap increases when the filtering is set to a less stringent criterion (5 % FDR) for both species.

ever, it is important to note that the application of less stringent FDR filtering criteria, specifically 5%, resulted in an overlap of 70% and 52% among pool sizes for zebrafish and fathead minnow. Likewise, unique proteins associated with each pool size consistently averaged 3-4%.

3.3 Variability in search parameters

The relation between the number of identifications and false discovery rates was examined after searching the respective organisms' databases with post-processing by Percolator. Analysis of the search results at two precursor tolerances with results are presented in Table 1. When total protein identifications were compared between the 5-embryo and 20-embryo pool size at 1% FDR, a modest increase of 2% was observed at 20 ppm, increasing to 3% at the larger precursor tolerance of 50 ppm for zebrafish. Under similar comparisons, as pool size increased, total protein identifications for fathead minnow decreased by 8% and 17% at the 20 and 50 ppm precursor tolerances, respectively. Comparisons of the identifications between the two precursor tolerance windows indicated a large proportion of identifications are shared (> 38%; 1% FDR) for both species, with the larger precursor tolerance resulting in higher proportions of unique identifications for both zebrafish (Fig. 3A) and the fathead minnow (Fig. 3B). Taken together, search parameters and filtering criteria play a critical role in the final number of identifications.

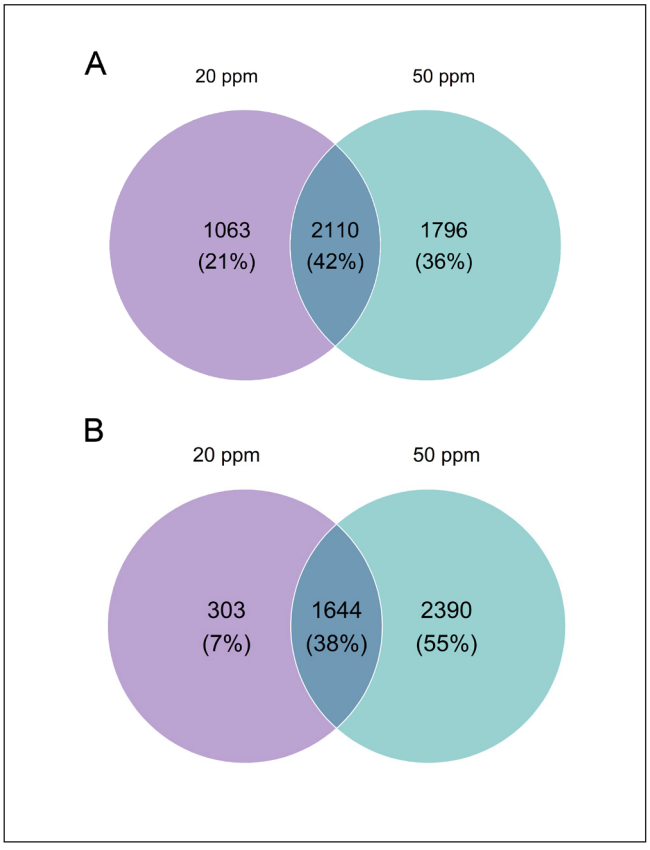

Fig. 3: Overlap of total protein identifications based on 20 and 50 ppm search tolerances for zebrafish (A) and fathead minnow (B) following post-processing with Percolator (1% FDR)

4 Discussion

While advances in omics technologies have led to increased knowledge and applications, the potential of such technologies within toxicology remains unfulfilled. Omics methods can provide mechanistic information, detecting the smallest changes at the molecular level, which precede threshold responses of traditional morphological and clinical endpoints, and therefore can inform adverse outcome pathways (AOPs; Ankley et al., 2010) for chemical hazard and risk assessment (Brockmeier et al., 2017) while potentially reducing animal use. Establishment of standardized protocols and good laboratory practice (GLP) for proteomics studies is challenging but can be achieved and will be essential especially for their use in the context of regulatory toxicology (Bouhifd et al., 2015).

In the present study, proteomic characterization of different pool sizes of the two most common laboratory embryo and larval fish models was established. For zebrafish, total proteins identified in previous papers (non-exhaustive list) are outlined in Table S1[4]. These were highly variable, ranging from fewer than 100 to 8363 proteins, with pool sizes between 1 and 1000 embryos (< 120 hpf) used for data-dependent acquisition (DDA) methods. The total number of proteins was mostly not reported in the papers using zebrafish larvae (> 120 hpf), but one study found 4466 proteins using a pool size of 200. For data-independent acquisition (DIA) methods, total protein identifications for zebrafish adults have been reported as 10,405 (Blattmann et al., 2019), while 8,120 proteins were identified in deyolked fish up to 72 hpf using a pool size of 20 (van der Plas-Duivesteijn et al., 2014).

Using a widely available DDA-based database and without deyolking, the current study using DIA identified between 3,172-4,644 proteins in total (1% FDR, 20 ppm tolerance and 5% FDR, 50 ppm tolerance, respectively, Tab. 1) in zebrafish, with comparable identifications among the three chosen pool sizes. This observation is noteworthy, given that even higher identification and quantification is possible when a spectral library is employed (Fernández-Costa et al., 2020). While previous proteomic studies of fathead minnow larvae are comparatively limited, Moreton et al. (2020) reportedly identified 1,303 proteins using a 5-embryo pool size, which is comparable but lower than the number of proteins identified in this study (1,946 with 1% FDR, 20 ppm tolerance and 5,098 with 5% FDR, 50 ppm tolerance, Tab. 1). In both species, while FDR and precursor tolerance play a key role in protein identifications, it is critical to note that there was no significant difference among the various pool sizes, although trends were observed: While a small increase in protein identifications was observed in zebrafish, a contrasting decrease was observed in the fathead minnow, suggesting that each model and pool size should be characterized individually.

Unique proteins identified per perturbation, or proteins that are found to be differentially expressed, are typically investigated further in proteomics studies, with the first step being associating a group of proteins or disparate data into a scientifically meaningful context, which can drive novel insight. In this way, ontologies can provide standard descriptors of data, and their incorporation within modern toxicology in the context of advancing toxicological knowledge has been recently discussed (Boyles et al., 2019). Expanding the zebrafish data in the current study, unique proteins were linked to unique genes, and differences in grouping were examined. Differences among the pool sizes were typically in the region of 2-6%. These may be associated with highly abundant proteins obscuring less abundant protein signals, or the identified unique proteins may be involved in distinct steps in the identified functional group. This requires further investigation, but it suggests that studies investigating specific pathways or responses to perturbations may be improved by a step-wise reduction in organism pool size to obtain a more comprehensive map of responses. Furthermore, studies aiming to map a particular biological process may also benefit from reducing organism pool size as a first step, prior to expansive animal-based studies.

Taken together, the current comparative analysis of the influence of pool size on protein identifications appears clear for larval fish, namely that reducing pool sizes of zebrafish and the fathead minnow to 5-10 embryos may be beneficial both for proteome characterization and pathways analysis. This reduction of individuals within sample pooling steps for two different developmental stages of two common fish models is consistent with the 3Rs ap-

proach. Although a non-protected life stage of zebrafish was used in the current study, we expect a similar result would be obtained if an experiment were extended beyond the non-protected life stage, which has direct relevance within the context of the 3Rs.

In establishing that it is possible to reduce animal numbers in aquatic proteomics studies without impacting the number of proteins identified, it is also clear that many variables can influence such results. Proteomics data processing is far from routine, and analysis pipelines are diverse, although significant improvements in depth and reproducibility of results have been achieved when experimental parameters were optimized (Révész et al., 2021; Bruderer et al., 2017). While it may not be possible to fully standardize every aspect of the proteomics workflow, it is important to optimize and document as many variables as possible. In being methodologically transparent, future researchers are better able to critically assess research, minimize animal usage through avoidance of repeated studies, and increase power and confidence in the proteomic results. In this respect, we discuss some of the novel outcomes and limitations of the current study and how they are being addressed.

Pooling individuals for sufficient biomass is not a new practice, and in omics techniques it is usually associated with sample availability, variability, experimental time, and cost. Experimental designs using pooled samples are often chosen out of necessity, to reduce the effects of biological variation, and to reduce costs of high-content methods (Kendziorski et al., 2005). However, limitations are also associated with this approach, as low-abundance proteins may be lost, even when represented in most individual samples (Sadiq and Agranoff, 2008), and it is only theoretically acceptable if the pool represents the biological average of the individual samples, which is not always the case (Molinari et al., 2018).

Considering that the replacement of animal experiments is increasingly becoming a priority topic in research toxicology, the lack of research into standardization of starting material for omics methods may reflect the relative newness of this approach, such that analysis strategies research labs are regularly using are arbitrarily employed until new information is presented. While an underlying assumption in proteomics has been that substantial amounts of tissue are needed to perform comprehensive characterizations, with certain signaling or method development studies that employ deyolked embryos requiring even more starting material (e.g., Kwon et al., 2016; Lemeer et al., 2008, 2007), the current study demonstrates that this is not always necessary. Indeed, for the fathead minnow model, smaller pool sizes improved identifications.

In the present study, we chose pool sizes based on the requirement of 50 µg protein. We further optimized the method recognizing the importance of extraction and homogenization on protein variability (Piehowski et al., 2013), the use of sonication to efficiently lyse cells, and maximized sample concentration of protein and minimized loss to surfaces (Feist and Hummon, 2015). In addition to recognizing that while previously published studies on the same type of starting material or organisms may form the rational basis for sample preparation method selection, caution must be used when starting from this perspective. Critical to any mass spectrometry-based proteomics analysis workflow is the selected sample preparation strategy, which is a key determinant in the information that will be obtained, since preparation methods influence the subset of proteins that can be reliably identified and/or quantified. Nevertheless, the corresponding selection is often not based on a fit-for-purpose evaluation. For example, the initial lysis step should be selected based on the cell source, with lysis in tissue culture much easier than lysis of cells in a tissue or with a high level of contractile proteins such as skeletal muscle. The buffers differ in their ability to solubilize proteins, with those containing sodium dodecyl sulfate (SDS) or other more ionic detergents considered to be the harshest and therefore most likely to give the highest yield. In this respect, TRIS-HCL has been reported as the lysis buffer of choice for cytoplasmic bound proteins, while RIPA is recommended for proteins that are membrane, nuclear or mitochondria bound. For the current study, TRIS-HCL was chosen since this buffer has been reported most frequently in the literature for zebrafish, while RIPA was reported most frequently for the fathead minnow model. It is possible that other lysis buffers may return even larger identification yields. The distinct lack of consistency in lysis buffer used for larval fish, while potentially reflecting methodologies or analysis strategies that research labs regularly use (Eng et al., 2011), may also reflect the need for a universal preparation method. In examining human tissue originating from the head and neck area, Klont et al. (2018) argued that it may not be possible to agree on a universal sample preparation method for proteome analysis, and indeed it may also be necessary to employ method-specific (e.g., in-gel digestion, in-solution digestion, on-filter digestion, etc.) preparation methodologies for specific tissue types. Nonetheless, multiple tissue types and organs are inherently pooled during a typical larval fish study, therefore it is possible that a universal species-specific sample preparation method for proteome analysis could be chosen. Further investigations are required to identify whether a universal lysis and sample preparation methodology is feasible in the future for fish embryo toxicity proteomics studies.

Most experimental variability is presumed to be biological, with increasing biological replicates recommended over technical replicates. In the present study, we used a comparatively high number of 7 biological replicates per treatment, compared to the typically reported 1-3 for proteomic studies with fish models. Importantly, with the reduction in the number of larval fish required for proteomics characterization, results in the current study also benefited from increasing biological replicates without substantially changing experimental design. Experimental sample size number was critically chosen to produce both reliable and reproducible results based on total biological variation as recommended for quantitative proteomics (Levin, 2011). For quality identifications, proteins not represented in a certain fraction of biological replicates were removed, reducing overall identifications by 47-65%; these identifications were organism- and pool size-dependent. However, this loss could potentially be corrected through the incorporation of technical replicates (> 2), which have been shown to increase reproducibility and repeatability in protein identifications. The incorporation of both technical and biological replicates is possible within the current construct of

this experimental approach without substantially increasing costs and could increase both experimental precision and efficiency of the statistical analysis.

Further, most search engines require a long list of user-supplied parameters, with appropriate values for these chosen based on instrumentation or prior search parameters and not with respect to the organism, experiment, or sample preparation, all of which can contribute significantly to the final proteome coverage. MS/MS spectra with inaccurate mass assignments have been reported to be easily identified by a database search with large precursor tolerance windows (Chick et al., 2015; Weng et al., 2013) or, alternatively, using an open search strategy allowing for larger precursor tolerance (Avtonomov et al., 2019; Li et al., 2018), in some instances resulting in a 300% increase in the number of identified spectra (Kong et al., 2017). This was tentatively investigated in the current study, where a precursor tolerance of 50 ppm (1% FDR) resulted in an increase of 19 and 52% identifications for zebrafish and fathead minnow (Tab. 1), respectively, highlighting its importance.

Until recently, assessing experimental error and deriving optimized search parameters for LC-MS/MS was time-consuming and labor-intensive; however, new tools have emerged which will greatly benefit experimental design and choice. One such tool that is under investigation to further enhance proteome characterization in fish studies is the application of Param-Medic, which is reported to be particularly beneficial for Q-TOF data and which reported over 52% more peptide spectrum matches than the original article when optimized (May et al., 2017). Further, the combination of several search engines has been reported to markedly increase the number of protein identifications compared to a typical single database search tool (Searle et al., 2008); this approach continues to be investigated by our research team.

It cannot be ignored that there are pros and cons to the use of the DIA approach that may be particularly beneficial to reducing animal usage. DIA is superior to DDA approaches in quantification, reproducibility, specificity, sensitivity, and accuracy, and, importantly, in the quantification of low protein amounts (Barkovits et al., 2020; Tsou et al., 2016), with prior studies demonstrating this for fathead minnow embryos where 180 proteins were quantified although 1,946 proteins were identified (Moreton et al., 2020). Equally critical, however, is that DDA technologies are the method of choice for mass spectrometry-based proteomics discovery experiments, and, as such, the current results require validation across platforms to widen applicability, which is currently underway.

Finally, and perhaps most importantly, while this study has clearly established that proteomic characterization in both zebrafish and fathead minnow is possible and beneficial in terms of proteins identified and associated linkages to ontologies and genes, it is based on control samples. It is important that this trend is validated with a broad spectrum of compounds as recommended by Kroeger (2006) in reviewing how omics can contribute to the 3Rs. Proteomics is predicted to contribute significantly to the ongoing development of AOPs (Ives et al., 2017). The outlined study clearly demonstrates that a step-wise decrease in pool size can increase the coverage of genes associated with certain protein groups. Such an increase in the identification of genes associated with specific pathways could feed directly into the creation of structured AOP knowledgebases via ontology-based annotation using AOP terms and enhance understanding of key events leading to adverse outcomes.

As summarized by Lippolis et al. (2019), omics data sets have not yet achieved all the proposed goals, including reduction and refinement of laboratory animals. In this context, while the role of omics has been evaluated and reviewed in various contexts, including in the development of AOPs (Brockmeier et al., 2017) and chemical risk assessment (Buesen et al., 2017), the current study represents the first examination of the number of animals necessary to carry out proteomics characterization based on a defined starting concentration of protein. The elegance of the study design allowed for a combination of scientific progress in technological sensitivity and proteome coverage while simultaneously incorporating ethical and animal welfare considerations that demand animal usage is minimized, while critically demonstrating that reducing embryo pool size may be beneficial for total proteome characterization.

While recognizing that the zebrafish embryos used in the present study currently represent a non-protected life stage, guidelines for the ethical use of animals are dynamic and must be reviewed in line with technological developments and the appearance of new ethical issues. In this respect, the development of new approach methodologies (NAMs) in chemical risk assessment creates opportunities for the use of more sensitive methodologies. In the USA, for the purposes of the TSCA, the EPA recognizes NAMs as encompassing "*any alternative test method and strategies to reduce, refine, or replace vertebrate animals*" (US EPA, 2017), including AOP development, which significantly incorporates omics-based approaches. Indeed, the ongoing OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST) is dedicated to the topic "*omics-based technologies for toxicological evaluations, which supports AOP development and MoA assessment*" (Buesen et al., 2017). While not necessary under current requirements, the proactive implementation of reduction on a method that is already considered a refinement or reduction approach will benefit animal research in general, while increasing understanding of chemical compounds, financially allowing expensive compounds to be assessed, and simultaneously respecting the use of laboratory animals.

Based on the collected information, a proteome characterization experiment using fish embryos requiring 50 µg of total protein per sample would require less than 5 embryos per replicate for either species. Crucially, if less protein is required, which can be envisaged, even fewer organisms will be required. Such an experimental design could allow for increased biological replicates, with the application of the filter aided sample preparation (FASP) method providing sufficient volume for the addition of technical replicates, thereby increasing the power of the experimental design in addition to the statistical interpretation. Furthermore, our observations highlight that the use of conventional and internationally recognized standardized testing procedures for both common fish species examined here resulted in protein identifications within the range reported in the literature. While proteomics, and indeed other omics technologies, are still in the

growth phase, the research community has the historical opportunity to shape this discipline for years to come, standardizing both the proteome field, and potentially other omics, in line with the principles of the 3Rs for species used in an aquatic toxicology context and in alternative vertebrate studies. Proteomics with the animal reduction approach outlined here represents a new testing strategy that will need to be further validated before being accepted through the characterization of a broad spectrum of compounds, in addition to consistent approaches for performing omics studies (Sauer et al., 2017; Kroeger, 2006). To achieve this aim in balancing the adoption of omics techniques and concurrently minimizing increases in animal usage, results from the current study provide a starting point to standardize proteomic studies using already established regulatory accepted study criteria while also incorporating framing to reduce animal usage during validation processes.

References

Aballo, T. J., Roberts, D. S., Melby, J. A. et al. (2021). Ultrafast and reproducible proteomics from small amounts of heart tissue enabled by Azo and timsTOF Pro. *J Proteome Res 20*, 4203-4211. doi:10.1021/acs.jproteome.1c00446

Almeida, A. M., Ali, S. A., Ceciliani, F. et al. (2021). Domestic animal proteomics in the 21st century: A global retrospective and viewpoint analysis. *J Proteomics 241*, 104220. doi:10.1016/j.jprot.2021.104220

Ankley, G. T., Bennett, R. S. and Erickson, R. J. (2010). Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem 29*, 730-741. doi:10.1002/etc.34

Avtonomov, D. M., Kong, A. and Nesvizhskii, A. I. (2019). DeltaMass: Automated detection and visualization of mass shifts in proteomic open-search results. *J Proteome Res 18*, 715-720. doi:10.1021/acs.jproteome.8b00728

Ayobahan, S. U., Eilebrecht, S., Baumann, L. et al. (2020). Detection of biomarkers to differentiate endocrine disruption from hepatotoxicity in zebrafish (Danio rerio) using proteomics. *Chemosphere 240*, 124970. doi:10.1016/j.chemosphere.2019.124970

Barkovits, K., Pacharra, S., Pfeiffer, K. et al. (2020). Reproducibility, specificity and accuracy of relative quantification using spectral library-based data-independent acquisition. *Mol Cell Proteomics 19*, 181-197. doi:10.1074/mcp.RA119.001714

Becker, R. A. (2019). Transforming regulatory safety evaluations using new approach methodologies: A perspective of an industrial toxicologist. *Curr Opin Toxicol 15*, 93-98. doi:10.1016/j.cotox.2019.07.002

Benton, H. P., Want, E. J. and Ebbels, T. M. D. (2010). Correction of mass calibration gaps in liquid chromatography-mass spectrometry metabolomics data. *Bioinformatics 26*, 2488-2489. doi:10.1093/bioinformatics/btq441

Bert, B., Chmielewska, J., Bergmann, S. et al. (2016). Considerations for a European animal welfare standard to evaluate adverse phenotypes in teleost fish. *EMBO J 35*, 1151-1154. doi:10.15252/embj.201694448

Blattmann, P., Stutz, V., Lizzo, G. et al. (2019). Generation of a zebrafish SWATH-MS spectral library to quantify 10,000 proteins. *Sci Data 6*, 190011. doi:10.1038/sdata.2019.11

Bouhifd, M., Beger, R., Flynn, T. et al. (2015). t^4 workshop report: Quality assurance of metabolomics. *ALTEX 32*, 319-326. doi:10.14573/altex.1509161

Boyles, R. R., Thessen, A. E., Waldrop, A. et al. (2019). Ontology-based data integration for advancing toxicological knowledge. *Curr Opin Toxicol 16*, 67-74. doi:10.1016/j.cotox.2019.05.005

Brockmeier, E. K., Hodges, G., Hutchinson, T. H. et al. (2017). The role of omics in the application of adverse outcome pathways for chemical risk assessment. *Toxicol Sci 158*, 252-262. doi:10.1093/toxsci/kfx097

Bruderer, R., Bernhardt, O. M., Gandhi, T. et al. (2017). Optimization of experimental parameters in data-independent mass spectrometry significantly increases depth and reproducibility of results. *Mol Cell Proteomics 16*, 2296-2309. doi:10.1074/mcp.RA117.000314

Buesen, R., Chorley, B. N., da Silva Lima, B. et al. (2017). Applying 'omics technologies in chemicals risk assessment: Report of an ECETOC workshop. *Regul Toxicol Pharmacol 91, Suppl 1*, S3-S13. doi:10.1016/j.yrtph.2017.09.002

Campos, B., Colbourne, J. K., Brown, J. B. et al. (2018). How omics technologies can enhance chemical safety regulation: perspectives from academia, government, and industry. *Environ Toxicol Chem 37*, 1252-1259. doi:10.1002/etc.4079

Chambers, M. C., Maclean, B., Burke, R. et al. (2012). A Cross-platform toolkit for mass spectrometry and proteomics. *Nat Biotechnol 30*, 918-920. doi:10.1038/nbt.2377

Chen, L., Hu, Y., He, J. et al. (2017). Responses of the proteome and metabolome in livers of zebrafish exposed chronically to environmentally relevant concentrations of microcystin-LR. *Environ Sci Technol 51*, 596-607. doi:10.1021/acs.est.6b03990

Chick, J. M., Kolippakkam, D., Nusinow, D. P. et al. (2015). A mass-tolerant database search identifies a large proportion of unassigned spectra in shotgun proteomics as modified peptides. *Nat Biotechnol 33*, 743-749. doi:10.1038/nbt.3267

Cohen, J. (1988). Statistical power analysis for the behavioral sciences. 2nd edition. New York, USA: Lawrence Erlbaum Associates. doi:10.4324/9780203771587

Delcourt, N., Quevedo, C., Nonne, C. et al. (2015). Targeted identification of sialoglycoproteins in hypoxic endothelial cells and validation in zebrafish reveal roles for proteins in angiogenesis. *J Biol Chem 290*, 3405-3417. doi:10.1074/jbc.M114.618611

Della Torre, C., Maggioni, D., Ghilardi, A. et al. (2018). The interactions of fullerene C60 and benzo(α)pyrene influence their bioavailability and toxicity to zebrafish embryos. *Environ Poll 241*, 999-1008. doi:10.1016/j.envpol.2018.06.042

Dhillon, R. S. and Richards, J. G. (2018). Hypoxia induces selective modifications to the acetylome in the brain of zebrafish (Danio rerio). *Comp Biochem Physiol B Biochem Mol Biol 224*, 79-87. doi:10.1016/j.cbpb.2017.12.018

Eng, J. K., Searle, B. C., Clauser, K. R. et al. (2011). A face in

the crowd: Recognizing peptides through database search. *Mol Cell Proteomics 10*, R111.009522. doi:10.1074/mcp.r111.009522

Feist, P. and Hummon, A. B. (2015). Proteomic challenges: Sample preparation techniques for microgram-quantity protein analysis from biological samples. *Int J Mol Sci 16*, 3537-3563. doi:10.3390/ijms16023537

Fernández-Costa, C., Martínez-Bartolomé, S., McClatchy, D. B. et al. (2020). Impact of the identification strategy on the reproducibility of the DDA and DIA results. *J Proteome Res 19*, 3153-3161. doi:10.1021/acs.jproteome.0c00153

Frøyset, A. K., Khan, E. A. and Fladmark, K. E. (2016). Quantitative proteomics analysis of zebrafish exposed to sub-lethal dosages of β-methyl-amino-L-alanine (BMAA). *Sci Rep 6*, 29631. doi:10.1038/srep29631

Gatto, L., Gibb, S. and Rainer, J. (2020). MSnbase, efficient and elegant R-based processing and visualization of raw mass spectrometry data. *J Proteome Res 20*, 1063-1069. doi:10.1021/acs.jproteome.0c00313

Ge, C., Lu, W. and Chen, A. (2017). Quantitative proteomic reveals the dynamic of protein profile during final oocyte maturation in zebrafish. *Biochem Biophys Res Commun 490*, 657-663. doi:10.1016/j.bbrc.2017.06.093

Gouveia, D., Almunia, C., Cogne, Y. et al. (2019). Ecotoxicoproteomics: A decade of progress in our understanding of anthropogenic impact on the environment. *J Proteomics 198*, 66-77. doi:10.1016/j.jprot.2018.12.001

Gündel, U., Kalkhof, S., Zitzkat, D. et al. (2012). Concentration-response concept in ecotoxicoproteomics: Effects of different phenanthrene concentrations to the zebrafish (Danio rerio) embryo proteome. *Ecotoxicol Environ Saf 76*, 11-22. doi:10.1016/j.ecoenv.2011.10.010

Hagenaars, A., Vergauwen, L., Benoot, D. et al. (2013). Mechanistic toxicity study of perfluorooctanoic acid in zebrafish suggests mitochondrial dysfunction to play a key role in PFOA toxicity. *Chemosphere 91*, 844-856. doi:10.1016/j.chemosphere.2013.01.056

Halder, M., Léonard, M., Iguchi, T. et al. (2010). Regulatory aspects on the use of fish embryos in environmental toxicology. *Int Environ Assess Manag 6*, 484-491. doi:10.1002/ieam.48

Healy, M. J., Tong, W., Ostroff, S. et al. (2016). Regulatory bioinformatics for food and drug safety. *Reg Toxicol Pharmacol 80*, 342-347. doi:10.1016/j.yrtph.2016.05.021

Helmus, R., ter Laak, T. L., van Wezel, A. P. et al. (2021). patRoon: Open-source software platform for environmental mass spectrometry based non-target screening. *J Cheminformatics 13*, 1. doi:10.1186/s13321-020-00477-w

Ives, C., Campia, I., Wang, R.-L. et al. (2017). Creating a structured adverse outcome pathway knowledgebase via ontology-based annotations. *Appl In Vitro Toxicol 3*, 298-311. doi:10.1089/aivt.2017.0017

Jeffries, M. K. S., Stultz, A. E., Smith, A. W. et al. (2015). The fish embryo toxicity test as a replacement for the larval growth and survival test: A comparison of test sensitivity and identification of alternative endpoints in zebrafish and fathead minnows. *Environ Toxicol Chem 34*, 1369-1381. doi:10.1002/etc.2932

Käll, L., Canterbury, J. D., Weston, J. et al. (2007). Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat Methods 4*, 923-925. doi:10.1038/nmeth1113

Kendziorski, C., Irizarry, R. A., Chen, K.-S. et al. (2005). On the utility of pooling biological samples in microarray experiments. *Proc Natl Acad Sci U S A 102*, 4252-4257. doi:10.1073/pnas.0500607102

Kim, H., Lee, S. and Park, H. (2019). Target-small decoy search strategy for false discovery rate estimation. *BMC Bioinformatics 20*, 438. doi:10.1186/s12859-019-3034-8

Kim, S. and Pevzner, P. A. (2014). MS-GF+ makes progress towards a universal database search tool for proteomics. *Nat Commun 5*, 5277. doi:10.1038/ncomms6277

Kimmel, C. B., Ballard, W. W., Kimmel, S. R. et al. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn 203*, 253-310. doi:10.1002/aja.1002030302

Klont, F., Bras, L., Wolters, J. C. et al. (2018). Assessment of sample preparation bias in mass spectrometry-based proteomics. *Anal Chem 90*, 5405-5413. doi:10.1021/acs.analchem.8b00600

Knigge, T. (2015). Proteomics in marine organisms. *Proteomics 15*, 3921-3924. doi:10.1002/pmic.201570213

Kong, A. T., Leprevost, F. V., Avtonomov, D. M. et al. (2017). MSFragger: Ultrafast and comprehensive peptide identification in shotgun proteomics. *Nat Methods 14*, 513-520. doi:10.1038/nmeth.4256

Kristofco, L. A., Du, B., Chambliss, C. K. et al. (2015). Comparative pharmacology and toxicology of pharmaceuticals in the environment: Diphenhydramine protection of diazinon toxicity in Danio rerio but not Daphnia magna. *AAPS J 17*, 175-183. doi:10.1208/s12248-014-9677-5

Kristofco, L. A., Haddad, S. P., Chambliss, C. K. et al. (2018). Differential uptake of and sensitivity to diphenhydramine in embryonic and larval zebrafish. *Environ Toxicol Chem 37*, 1175-1181. doi:10.1002/etc.4068

Kroeger, M. (2006). How omics technologies can contribute to the '3R' principles by introducing new strategies in animal testing. *Trends Biotechnol 24*, 343-346. doi:10.1016/j.tibtech.2006.06.003

Kwon, O. K., Kim, S. and Lee, S. (2016). Global proteomic analysis of lysine acetylation in zebrafish (Danio rerio) embryos: Proteomics and 2-DE. *Electrophoresis 37*, 3137-3145. doi:10.1002/elps.201600210

Lavelle, C., Smith, L. C., Bisesi, J. H. et al. (2018). Tissue-based mapping of the fathead minnow (Pimephales promelas) transcriptome and proteome. *Front Endocrinol 9*, 611. doi:10.3389/fendo.2018.00611

Lazar, C., Gatto, L., Ferro, M. et al. (2016). Accounting for the multiple natures of missing values in label-free quantitative proteomics data sets to compare imputation strategies. *J Proteome Res 15*, 1116-1125. doi:10.1021/acs.jproteome.5b00981

Lee, H., Sung, E. J., Seo, S. et al. (2021). Integrated multi-

omics analysis reveals the underlying molecular mechanism for developmental neurotoxicity of perfluorooctanesulfonic acid in zebrafish. *Environ Int 157*, 106802. doi:10.1016/j.envint.2021.106802

Lemeer, S., Ruijtenbeek, R., Pinkse, M. W. H. et al. (2007). Endogenous phosphotyrosine signaling in zebrafish embryos. *Mol Cell Proteomics 6*, 2088-2099. doi:10.1074/mcp.M600482-MCP200

Lemeer, S., Jopling, C., Gouw, J. et al. (2008). Comparative phosphoproteomics of zebrafish Fyn/Yes morpholino knockdown embryos. *Mol Cell Proteomics 7*, 2176-2187. doi:10.1074/mcp.M800081-MCP200

Levin, Y. (2011). The role of statistical power analysis in quantitative proteomics. *Proteomics 11*, 2565-2567. doi:10.1002/pmic.201100033

Li, D., Lu, S., Liu, W. et al. (2018). Optimal settings of mass spectrometry open search strategy for higher confidence. *J Proteome Res 17*, 3719-3729. doi:10.1021/acs.jproteome.8b00352

Libiseller, G., Dvorzak, M., Kleb, U. et al. (2015). IPO: A tool for automated optimization of XCMS parameters. *BMC Bioinformatics 16*, 118. doi:10.1186/s12859-015-0562-8

Lippolis, J. D., Powell, E. J., Reinhardt, T. A. et al. (2019). Symposium review: Omics in dairy and animal science – Promise, potential, and pitfalls. *J Dairy Sci 102*, 4741-4754. doi:10.3168/jds.2018-15267

Lombard-Banek, C., Moody, S. A., Manzini, M. C. et al. (2019). Microsampling capillary electrophoresis mass spectrometry enables single-cell proteomics in complex tissues: Developing cell clones in live Xenopus laevis and zebrafish embryos. *Anal Chem 91*, 4797-4805. doi:10.1021/acs.analchem.9b00345

López-Pedrouso, M., Varela, Z., Franco, D. et al. (2020). Can proteomics contribute to biomonitoring of aquatic pollution? A critical review. *Environ Poll 267*, 115473. doi:10.1016/j.envpol.2020.115473

Martinson, J., Bencic, D. C., Toth, G. P. et al. (2021). De novo assembly and annotation of a highly contiguous reference genome of the fathead minnow (Pimephales promelas) reveals an AT-rich repetitive genome with compact gene structure. *bioRxiv*, 2021.02.24.432777. doi:10.1101/2021.02.24.432777

Martyniuk, C. J., Alvarez, S., McClung, S. et al. (2009). Quantitative proteomic profiles of androgen receptor signaling in the liver of fathead minnows (Pimephales promelas). *J Proteome Res 8*, 2186-2200. doi:10.1021/pr800627n

Martyniuk, C. J. and Alvarez, S. (2013). Proteome analysis of the fathead minnow (Pimephales promelas) reproductive testes. *J Proteomics 79*, 28-42. doi:10.1016/j.jprot.2012.11.023

May, D. H., Tamura, K. and Noble, W. S. (2017). Param-Medic: A tool for improving MS/MS database search yield by optimizing parameter settings. *J Proteome Res 16*, 1817-1824. doi:10.1021/acs.jproteome.7b00028

Meigs, L., Smirnova, L., Rovida, C. et al. (2018). Animal testing and its alternatives – The most important omics is economics. *ALTEX 35*, 275-305. doi:10.14573/altex.1807041

Molinari, N., Roche, S., Peoc'h, K. et al. (2018). Sample pooling and inflammation linked to the false selection of biomarkers for neurodegenerative diseases in top-down proteomics: A pilot study. *Front Mol Neurosci 11*, 477. doi:10.3389/fnmol.2018.00477

Moosa, J. M., Guan, S., Moran, M. F. et al. (2020). Repeat-preserving decoy database for false discovery rate estimation in peptide identification. *J Proteome Res 19*, 1029-1036. doi:10.1021/acs.jproteome.9b00555

Moreton, M. L., Lo, B. P., Simmons, D. B. D. et al. (2020). Toxicity of the aquatic herbicide, reward®, on the fathead minnow with pulsed-exposure proteomic profile. *Comp Biochem Physiol Part D Genomics Proteomics 33*, 100635. doi:10.1016/j.cbd.2019.100635

OECD (2013). Test No. 236: Fish Embryo Acute Toxicity (FET) Test. *OECD Guidelines for the Testing of Chemicals, Section 2*. OECD Publishing, Paris. doi:10.1787/9789264203709-en

Piehowski, P. D., Petyuk, V. A., Orton, D. J. et al. (2013). Sources of technical variability in quantitative LC-MS proteomics: Human brain tissue sample analysis. *J Proteome Res 12*, 2128-2137. doi:10.1021/pr301146m

Purushothaman, K., Das, P. P., Presslauer, C. et al. (2019). Proteomics analysis of early developmental stages of zebrafish embryos. *Int J Mol Sci 20*, 6359. doi:10.3390/ijms20246359

Rawlings, J. M., Belanger, S. E., Connors, K. A. et al. (2019). Fish embryo tests and acute fish toxicity tests are interchangeable in the application of the threshold approach. *Environ Toxicol Chem 38*, 671-681. doi:10.1002/etc.4351

Révész, Á., Milley, M. G., Nagy, K. et al. (2021). Tailoring to search engines: Bottom-up proteomics with collision energies optimized for identification confidence. *J Proteome Res 20*, 474-484. doi:10.1021/acs.jproteome.0c00518

Russell, W. M. S. and Burch, R. L. (1959). The Principles of Humane Experimental Technique. London, UK: Methuen.

Sadiq, S. T. and Agranoff, D. (2008). Pooling serum samples may lead to loss of potential biomarkers in SELDI-ToF MS proteomic profiling. *Proteome Sci 6*, 16. doi:10.1186/1477-5956-6-16

Sanchez, B. C., Ralston-Hooper, K. and Sepúlveda, M. S. (2011). Review of recent proteomic applications in aquatic toxicology. *Environ Toxicol Chem 30*, 274-282. doi:10.1002/etc.402

Sauer, U. G., Deferme, L., Gribaldo, L. et al. (2017). The challenge of the application of 'omics technologies in chemicals risk assessment: Background and outlook. *Regul Toxicol Pharmacol 91*, S14-S26. doi:10.1016/j.yrtph.2017.09.020

Schaeck, M., Van den Broeck, W., Hermans, K. et al. (2013). Fish as research tools: Alternatives to in vivo experiments. *Altern Lab Anim 41*, 219-229. doi:10.1177/026119291304100305

Searle, B. C., Turner, M. and Nesvizhskii, A. I. (2008). Improving sensitivity by probabilistically combining results from multiple MS/MS search methodologies. *J Proteome Res 7*, 245-253. doi:10.1021/pr070540w

Shliaha, P. V., Bond, N. J., Gatto, L. et al. (2013). Effects of traveling wave ion mobility separation on data independent acquisition in proteomics studies. *J Proteome Res 12*, 2323-2339. doi:10.1021/pr300775k

Smith, C. A., Want, E. J., O'Maille, G. et al. (2006). XCMS: Processing mass spectrometry data for metabolite profiling

using nonlinear peak alignment, matching, and identification. *Anal Chem 78*, 779-787. doi:10.1021/ac051437y

Smith, L. C., Lavelle, C. M., Silva-Sanchez, C. et al. (2018). Early phosphoproteomic changes for adverse outcome pathway development in the fathead minnow (Pimephales promelas) brain. *Sci Rep 8*, 10212. doi:10.1038/s41598-018-28395-w

Spivak, M., Weston, J., Bottou, L. et al. (2009). Improvements to the Percolator algorithm for peptide identification from shotgun proteomics data sets. *J Proteome Res 8*, 3737-3745. doi:10.1021/pr801109k

Steele, W. B., Kristofco, L. A., Corrales, J. et al. (2018). Comparative behavioral toxicology with two common larval fish models: Exploring relationships among modes of action and locomotor responses. *Sci Total Environ 640-641*, 1587-1600. doi:10.1016/j.scitotenv.2018.05.402

Stieglitz, J. D., Mager, E. M., Hoenig, R. H. et al. (2016). A novel system for embryo-larval toxicity testing of pelagic fish: Applications for impact assessment of Deepwater Horizon crude oil. *Chemosphere 162*, 261-268. doi:10.1016/j.chemosphere.2016.07.069

Su, T., Lian, D., Bai, Y. et al. (2021). The feasibility of the zebrafish embryo as a promising alternative for acute toxicity test using various fish species: A critical review. *Sci Total Environ 787*, 147705. doi:10.1016/j.scitotenv.2021.147705

Tautenhahn, R., Böttcher, C. and Neumann, S. (2008). Highly sensitive feature detection for high resolution LC/MS. *BMC Bioinformatics 9*, 504. doi:10.1186/1471-2105-9-504

Taylor, K. and Alvarez, L. R. (2019). An estimate of the number of animals used for scientific purposes worldwide in 2015. *Altern Lab Anim 47*, 196-213. doi:10.1177/0261192919899853

The, M., MacCoss, M. J., Noble, W. S. et al. (2016). Fast and accurate protein false discovery rates on large-scale proteomics data sets with Percolator 3.0. *J Am Soc Mass Spectrom 27*, 1719-1727. doi:10.1007/s13361-016-1460-7

Tsou, C.-C., Tsai, C.-F., Teo, G. C. et al. (2016). Untargeted, spectral library-free analysis of data-independent acquisition proteomics data generated using Orbitrap mass spectrometers. *Proteomics 16*, 2257-2271. doi:10.1002/pmic.201500526

UK Home Office (2015). Statistics of Scientific Procedures on Living Animals Great Britain 2014. https://bit.ly/3G1sMbB

US EPA (2002). Method 1000.0: Fathead Minnow, Pimephales promelas, Larval Survival and Growth; Chronic Toxicity. Environmental Protection Agency. http://www.epa.gov

US EPA (2017). Alternative Test Methods and Strategies to Reduce Vertebrate Animal Testing. https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/alternative-test-methods-and-strategies-reduce

Välikangas, T., Suomi, T. and Elo, L. L. (2018). A systematic evaluation of normalization methods in quantitative label-free proteomics. *Brief Bioinform 19*, bbw095. doi:10.1093/bib/bbw095

van der Plas-Duivesteijn, S. J., Mohammed, Y., Dalebout, H. et al. (2014). Identifying proteins in zebrafish embryos using spectral libraries generated from dissected adult organs and tissues. *J Proteome Res 13*, 1537-1544. doi:10.1021/pr4010585

Wei, R., Wang, J., Su, M. et al. (2018). Missing value imputation approach for mass spectrometry-based metabolomics data. *Sci Rep 8*, 663. doi:10.1038/s41598-017-19120-0

Weng, R. R., Chu, L. J., Shu, H.-W. et al. (2013). Large precursor tolerance database search – A simple approach for estimation of the amount of spectra with precursor mass shifts in proteomic data. *J Proteomics 91*, 375-384. doi:10.1016/j.jprot.2013.07.030

Wiśniewski, J. R. (2016). Quantitative evaluation of filter aided sample preparation (FASP) and multienzyme digestion FASP protocols. *Anal Chem 88*, 5438-5443. doi:10.1021/acs.analchem.6b00859

Wit, M. D., Keil, D., van der Ven, K. et al. (2010). An integrated transcriptomic and proteomic approach characterizing estrogenic and metabolic effects of 17 α-ethinylestradiol in zebrafish (Danio rerio). *Gen Comp Endocrinol 167*, 190-201. doi:10.1016/j.ygcen.2010.03.003

Wu, Y., Lou, Q.-Y., Ge, F. et al. (2017). Quantitative proteomics analysis reveals novel targets of miR-21 in zebrafish embryos. *Sci Rep 7*, 4022. doi:10.1038/s41598-017-04166-x

Yu, G., Wang, L.-G., Han, Y. et al. (2012). clusterProfiler: An R package for comparing biological themes among gene clusters. *OMICS 16*, 284-287. doi:10.1089/omi.2011.0118

Zhang, W., Liu, Y., Zhang, H. et al. (2012). Proteomic analysis of male zebrafish livers chronically exposed to perfluorononanoic acid. *Environ Int 42*, 20-30. doi:10.1016/j.envint.2011.03.002

Zhou, C., Simpson, K. L., Lancashire, L. J. et al. (2012). Statistical considerations of optimal study design for human plasma proteomics and biomarker discovery. *J Proteome Res 11*, 2103-2113. doi:10.1021/pr200636x

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

Research reported in this publication was supported by the National Institute of Environmental Health Sciences of the National Institutes of Health under award number 1P01ES028942 to BWB. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Additional support was provided by Baylor University. The authors acknowledge Baylor University Mass Spectrometry Center (BU-MSC) for support during this work, and specifically Dr Chinthaka Seneviratne for technical assistance.