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## Toxicometabolomics as a tool for next generation environmental risk assessment

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### Abstract

Traditionally applied methodology in environmental risk assessment (ERA) has fallen out of step with technological advancements and regulatory requirements, challenging effectiveness and accuracy of the assessments. Extensive efforts have been focused towards a transition to a more data-driven and mechanistically-based next generation risk assessment. Metabolomics can produce detailed and comprehensive molecular insight into affected biochemical processes. Combining metabolomics with environmental toxicology can help to understand the mechanisms and/or modes of action underlying toxicity of environmental pollutants and inform adverse outcome pathways, as well as facilitate identification of biomarkers to quantify effects and/or exposure. This Technical Report describes the activities and work performed within the frame of the European Food Risk Assessment Fellowship Programme (EU-FORA), implemented at the section 'Environmental Chemistry and Toxicology' at the Department of Environmental Science at Aarhus University in Denmark with synergies to an ongoing H2020 RIA project 'Endocrine Guideline Optimisation' (ERGO). In accordance with the 'training by doing' principles of the EU-FORA, the fellowship project combined the exploration of the status of scientific discussion on methodology in ERA through literature study with hands-on training, using the metabolomics analysis pipeline established at Aarhus University. For the hands-on training, an amphibian metamorphosis assay (OECD test no.231) was used as a proof-of-concept toxicometabolomics study case. Both a targeted biomarker – and an untargeted metabolomics approach was applied.

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**Table of contents**

Abstract..... 1

1. Introduction..... 4

2. Description of the work programme..... 5

2.1. Aims..... 5

2.2. Activities/methods ..... 5

2.2.1. Theoretical introduction to practices and challenges in environmental risk assessment..... 5

2.2.2. Practical introduction to environmental toxicometabolomics- case study ..... 6

2.2.2.1. Background..... 6

2.2.2.2. Sample preparation – thyroid hormone and metabolite extraction ..... 7

2.2.2.3. Data acquisition and thyroid hormone quantification ..... 7

2.2.2.4. Data acquisition untargeted metabolomics..... 8

2.2.2.5. Preprocessing of untargeted data ..... 9

3. Conclusions..... 10

References..... 10

Abbreviations..... 12

## 1. Introduction

EFSA performs environmental risk assessments (ERAs) as part of its evaluations of 'regulated products', including pesticides, genetically modified organisms and additives in animal feed. Traditionally, the ERAs of these products have been performed on a single-substance basis, single exposure route and for a specific type of use (Sousa et al., 2022). These traditional approaches bear several limitations, challenging accuracy, efficiency and relevance of the risk assessments. Driven by significant advancements in scientific knowledge, analytical techniques and computational capabilities, which have expanded the understanding of toxicology, biology and exposure assessment, in addition to ethical considerations and a desire to reduce animal testing, several initiatives have been established in Europe to support a shift towards next generation risk assessment in order to overcome limitations and improve the quality of ERAs (Cozigou et al., 2015; Moné et al., 2020; Miccoli et al., 2022; Sousa et al., 2022; Marx-Stoelting et al., 2023).

Based on the current framework, traditional ERAs often focus on a limited set of endpoints (e.g. mortality, immobility) and may not capture the full range of potential hazards and risks associated with chemicals and environmental stressors. Next generation risk assessment seeks to provide a more comprehensive evaluation of potential adverse effects and exposure scenarios. Consequently, a special emphasis is placed on improving the understanding of underlying mechanisms of toxicity and adverse outcomes.

High-throughput technologies, such as omics techniques, allow for the rapid screening of large numbers of samples or chemicals, but the application of omics can also support a mechanistically-based approach (Brockmeier et al., 2017). Omics produce detailed and comprehensive molecular insights into the biochemical processes occurring in stressed microbes, plants and animals, enabling us to understand more about how these organisms are responding to environmental stresses.

In the past two decades, metabolomics has emerged as a promising alternative and/or supplementary tool to traditional toxicological assays, focusing on the qualitative and quantitative study of small molecules (< 1,500 Da) in biological samples or organisms to identify key metabolites involved in various biological processes. As metabolic changes can be influenced by environmental factors, but also diet, sex and disease, the high degree of controlled conditions in toxicological models present suitable platforms for metabolomics analyses with diverse applications (da Silva et al., 2021). Environmental toxicometabolomics, a subfield of metabolomics, aims to provide insights into how environmental stressors and chemical contaminants perturb the metabolic pathways of organisms, leading to altered physiological responses and potential adverse effects. The identification and quantification of specific metabolites or patterns of metabolites that serve as biomarkers of effect and/or exposure provides valuable information for risk assessment, regulatory decision-making and pollution management (Fowler, 2012), while global profiling of metabolic changes in response to chemical exposures allows for the characterisation of the metabolic networks and biological processes affected. As metabolomics captures the end products of cellular processes, it provides direct information on metabolic responses and the physiological state, and therefore gives the closest reflection of the phenotype of an organism or biological system. This knowledge helps in understanding the mechanisms of toxicity and adverse effects induced at sub-lethal environmental doses. Sublethal effects may not be readily apparent through traditional toxicological endpoints but can have long-term consequences for organisms and ecosystems.

Adverse outcomes resulting from chemical toxicity are rarely caused directly by dysregulation of individual molecules or pathways; rather, they are often caused by system-level perturbations that occur in networks of molecular events (Ravichandran et al., 2022). Recognising the interactions of molecules, pathways and biological processes within networks is fundamental for gaining a comprehensive understanding of the mechanism of action of chemical toxicity in complex biological systems. The adverse outcome pathway (AOP) concept, developed by the US Environmental Protection Agency in 2011, provides a framework to collect, organise and evaluate relevant information on biological and toxicological effects of chemicals (Ankley et al., 2010; Villeneuve et al., 2014). Existing mechanistic knowledge is organised and used to link a molecular-level perturbation of a biological system triggered by a chemical (a molecular initiating event; MIE) through a sequence of causally linked key biological events (key event; KE) to an adverse health or ecotoxicological outcome of regulatory concern (adverse outcome) including population-level responses (Ankley et al., 2010, 2023; Kramer et al., 2011; Villeneuve et al., 2014). Recent advancements in various omics technologies, and integrative multi-omics approaches, have facilitated the identification of MIEs and KEs within AOPs, offering a more comprehensive understanding of toxicity pathways and the connections between

different levels of molecular organisation (Bedia, 2022). Metabolites affected by chemical exposure can provide valuable data to support the development of AOPs. On one hand, metabolite levels can offer critical insights into mechanisms underlying KEs, helping to define the mode of action of an environmental toxicant. On the other hand, particular metabolites may directly represent KEs within an AOP, causally linking the components of the pathway to an AO. Furthermore, metabolomics data can be compared across different species and even extrapolated to predict the effects of stressors in other organisms. Cross-species applicability is essential for the development of AOPs with relevance to multiple species and ecological contexts (Ankley et al., 2010; Brockmeier et al., 2017; Haigis et al., 2023). Metabolomics-based read-across and grouping approaches, utilising structural similarity, have been shown to successfully support evidence through reducing uncertainty in the characterisation of the toxicity profile of analogue chemicals (e.g. phenoxy herbicides (van Ravenzwaay et al., 2016; Sperber et al., 2019).

Despite the growing agreement on the potential metabolomics offer for informing risk assessments when applied as a part of an integrated systems biology approach or when considered in the context of the AOP framework (Brockmeier et al., 2017; EFSA, 2018), it is still not clear how omics datasets can be used in regulatory applications in the risk assessment of chemicals (Viant et al., 2019). While the validation of metabolomics studies still needs to overcome a number of challenges in order to be widely implemented in routine ERAs, the potential of omics techniques to produce new evidence and facilitate the development of alternative testing methods, collectively referred to as new approach methodologies (NAMs), has been recognised (EFSA, 2022a,b; Otto et al., 2023). Progress towards a harmonised reporting framework has been made to support regulatory acceptance of metabolomics data (Buesen et al., 2017; Viant et al., 2019; Harrill et al., 2021; Miccoli et al., 2022). EFSA aims to adopt omics and associated bioinformatic approaches as routine tools in relevant RAs by 2030 (EFSA, 2022b).

## 2. Description of the work programme

### 2.1. Aims

The aim of the work programme was for the fellow to gain insights into the current scientific discussion in ERA methodology through literature study. Additionally, the fellow received hands-on training in toxicometabolomics as a potential tool for next generation systems-based ERA approaches, using the analysis pipeline established at the Environmental Metabolomics lab at the Department of Environmental Science (ENVS) at Aarhus University (AU; Denmark) on a case study.

### 2.2. Activities/methods

The fellow was integrated into the work at AU through a work programme consisting of four defined modules covering both a theoretical and practical introduction to environmental toxicometabolomics and its potential applications in ERA. The work programme was based on on-going project work and previous research interests at the Environmental Metabolomics lab. Between January and July 2023, the fellow spent a total of 3-months physically present at the hosting site. Further data processing and analysis could be performed remotely at the sending site using cloud-based infrastructure.

#### 2.2.1. Theoretical introduction to practices and challenges in environmental risk assessment

As a part of the working group, the fellow participated in weekly lab meetings, learning about the different applications of environmental metabolomics through the various ongoing research projects of the group members.

As an integral part of the group's activities within an on-going HORIZON 2020 project at the host institution ('Endocrine Guideline Optimisation' (ERGO) project; <https://ergo-project.eu/>), which aims to investigate mechanisms of endocrine disrupting chemicals (EDCs) and improve identification and hazard assessment of EDCs using standardised test guidelines based on optimised approaches, the fellow participated in an ERGO project meeting, held in Amsterdam in January 2023. Moreover, the preceding annual meeting of the European Cluster to improve identification of endocrine disruptors (EURION cluster) provided a broad overview over current research efforts to support the development and improvement of test systems for EDCs. Further insights into challenges with current ERA practises

with respect to data requirements were gained and deepened through a collaboration with a PhD candidate on a manuscript discussing suitability of standard tests, and the potential of a mechanistic approach using NAMs with respect to chemical properties of the test compound, taking cationic polymers as an example (A. M. B. Hansen et al., 2023, Manuscript submitted for publication).

In addition, the fellow attended focused workshops and meetings on relevant topics:

- EFSA Risk Assessment Research Assembly (7.12.22, Berlin, Germany)
- EURION omics working group meeting (15.3.23, online)
- ENVS Research seminar on Emerging environmental pollutants and public health evaluations (19.4.23, AU campus Risø, Roskilde, Denmark)
- International Summer School on Non-Targeted Metabolomics (21–25.8.23, Copenhagen, Denmark)

The training was complemented through the 3-weeks EU-FORA induction training (September 2022), and three completed training modules (December 2022, March 2023 and June 2023), covering also topics around ERA, the application of omics in risk assessment and the AOP framework (Training module 2). The fellowship training will be concluded with a last training module taking place in August 2023.

### 2.2.2. Practical introduction to environmental toxicometabolomics- case study

For hands-on experience with metabolomics approaches, an amphibian metamorphosis assay (AMA; Organisation for Economic Co-Operation and Development (OECD) test guideline 231) was used as a proof-of-concept toxicometabolomics study case. The study was made in close collaboration with academic partners within the ERGO project. Both a targeted and untargeted approach was applied.

#### **Disclaimer**

Detailed results obtained from the metabolomics analyses are not included in this report, as the study is blinded, and parts of the analyses are still on-going. Further collaboration on the study and data is planned subsequent to the finalised fellowship-programme, with the aim to publish the results in peer-reviewed scientific journals. The EU-FORA fellowship and funding will be acknowledged in any manuscript submitted for peer-reviewed scientific journals.

#### 2.2.2.1. Background

Endocrine disrupting chemicals are substances that can interfere with the hormonal system of living organisms, potentially leading to adverse effects on human health and the environment. Over the past decade, the European Union (EU) has taken significant measures to address the issue of EDCs (EC, 2018), and specific provisions have been included in the legislation on pesticides (EC 1107/2009<sup>1</sup>), biocides (EC 528/2012<sup>2</sup>), chemicals in general ('REACH Regulation', EC 1907/2006<sup>3</sup>), medical devices<sup>4</sup> and for aquatic environments according to the 'Water Framework Directive' (2000/60/EC<sup>5</sup>), in line with the different requirements laid down in the relevant legislation.

Based on the definitions for EDCs proposed by the WHO in 2002 and 2009 (WHO/IPCS, 2002, 2009), the EU introduced specific criteria for the classification of EDCs, particularly for pesticides and biocidal products in 2017 (EC, 2017), allowing for more stringent regulation and monitoring of these substances. The specific scientific criteria for identification of EDCs address three key elements: (i) chemical-induced adverse effects on the endocrine system of humans or non-target organisms (adversity), (ii) chemical-specific endocrine modes of action (MOAs) and (iii) the scientifically plausible causal link between the adverse effects observed and the endocrine activity of the substance (causality/plausibility). According to EDC criteria, all available scientific data must be considered in the assessment, and a weight of evidence approach should be applied (EC, 2017, 2018; ECHA/EFSA, 2018).

#### **The amphibian metamorphosis assay**

The AMA is a screening test, identifying substances that interfere with thyroid-mediated pathways or the function of hypothalamic–pituitary–thyroid (HPT) axis in vertebrates (OECD, 2009). The test is

<sup>1</sup> OJ L 309, 24.11.2009, pp. 1–50.

<sup>2</sup> OJ L 167, 27.6.2012, pp. 1–123.

<sup>3</sup> OJ L 396, 30.12.2006, pp. 1–849.

<sup>4</sup> OJ L 117, 5.5.2017, pp. 1–175.

<sup>5</sup> OJ L 327, 22.12.2000, pp. 1–73.

conducted with larval stages (tadpoles) of the African clawed frog, *Xenopus laevis*. The assay is designed as a dose–response, exposing tadpoles at Nieuwkoop and Faber (NF) developmental stage 51 (<http://www.xenbase.org/>, RRID:SCR\_003280) to a minimum of three different concentrations of a test chemical in addition to a control for 21 days. The developmental stage, hindlimb length, snout to vent length measurement and wet weight are recorded as apical endpoints on day 7 and day 21 of the assay, in addition to thyroid gland histopathology at test termination.

Anuran metamorphosis is triggered by thyroid hormones and highly regulated by the HPT axis. The regulation of thyroid hormone-dependent molecular and physiological processes during metamorphosis, which occurs following a precise sequence and timing, is highly susceptible to disruptions caused by environmental and chemical factors (ECHA/EFSA, 2018). As the development of *X. laevis* is well-characterised, with distinct stages from egg to tadpole to adult (Zahn et al., 2022; Fisher et al., 2023), it is considered a validated test species for the AMA, providing a standardised framework for assessing effects (adversity) of chemicals on amphibian metamorphosis.

However, although the relevance of the AMA for tier 1 identification of thyroid-disruptive chemicals is largely recognised (ECHA/EFSA, 2018), the assay is exclusively based on morphological endpoints, and specificity of the thyroid responsive endpoints with respect to thyroid activity of chemicals has been questioned (Dang, 2019). While the AMA is not designed to indicate a molecular target of a chemical, the incorporation of biochemical or molecular biomarkers, such as thyroid hormone or expression of thyroid hormone related genes, into the guideline as mechanistic endpoints has been proposed, informing the MoA criterion and facilitating the development of a targeted test strategy (Dang, 2022).

#### 2.2.2.2. Sample preparation – thyroid hormone and metabolite extraction

Two types of anuran tissues from the AMA were chosen for the study: Thyroid tissue was chosen for the analysis to characterise and explore thyroid-related metabolite changes. Moreover, experimental evidence suggests that alterations in the thyroid hormone system can influence eye development in vertebrates, but the chain of events from the molecular interaction of thyroid hormone system disruption to adverse outcomes to eye morphology and vision (the AOP) are not yet fully understood (Gölz et al., 2022). To further explore a potential relationship between thyroid- and eye metabolism, also eye tissue was included for the study. The samples were prepared for both targeted and untargeted metabolomics.

For each tissue, three individual samples of each of the four treatment replicates ( $n = 4$  replicate tanks per treatment  $\times$  three replicates per tank; developmental stages at day 21 between NF 58–62) were taken and kept ( $-80^{\circ}\text{C}$ ) for the additional metabolomics analyses. Treatment groups were labelled using an unknown colour code to provide blinding of all experimenters during endpoint recording, tissue sampling, sample preparation and analyses. Metabolites were extracted from the samples in random order, as previously reported (Pannetier et al., 2023), following a modified version of the Matyash method (Matyash et al., 2008; Sostare et al., 2018), which is based on a methyl-tert-butyl ether (MTBE)/methanol/water (2.6/2.0/2.4) biphasic solvent system to extract both polar and non-polar compounds separately. To enhance the extraction of thyroid hormones to the polar phase, 1% ammonium hydroxide (v/v) was added to the polar solvents (methanol and water). Procedural blanks were included from the start of the extraction protocol.

Both samples and procedural blanks were spiked with isotopically labelled  $^{13}\text{C}$ -thyroid hormone internal standards. The polar compounds (methanol/water phase) were enriched using solid-phase micro-extraction and reconstituted in 5% methanol containing an instrument control standard. As an additional quality control (QC) measure, a pool of all study samples was prepared (QC sample) to monitor stability throughout the run.

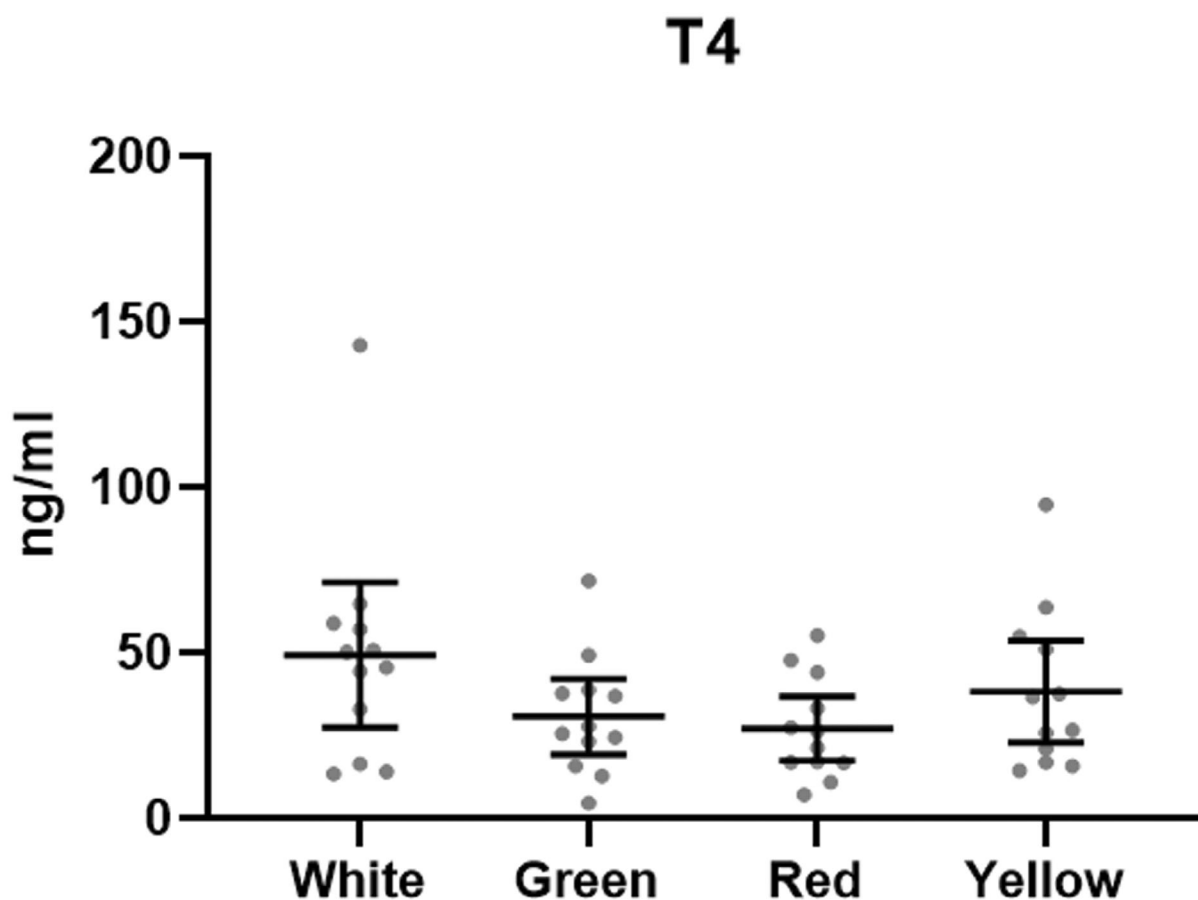
The extracts of the polar phase were used for both targeted TH determination and untargeted metabolomics. For metabolomics analyses, a mix of metabolite standards prepared in QC sample was added to the sequence. The separated non-polar phase (MTBE) was evaporated to dryness and reconstituted in 8Bu + solvent (Danne-Rasche et al., 2018) containing a mix of 14 isotopically labelled internal standards of all major lipid classes to enable quantification and/or normalisation.

#### 2.2.2.3. Data acquisition and thyroid hormone quantification

Following a previously developed protocol, the targeted analysis of THs was performed on an Agilent 6495c triple-quadrupole system with a hyphenated Agilent 1290 Infinity II ultra-high performance liquid chromatography (UHPLC) system (Agilent Technologies, Santa Clara, CA USA) as described by (Hansen et al., 2016; Pannetier et al., 2023). Twelve THs were included into the targeted

analysis. The targeted THs were quantified in reference to external standard multi-point calibration curves, constructed using a serial dilution of an equimolar mix of neat standards. The data were analysed using the MassHunter Quantitative Analysis software (Agilent Technologies, Santa Clara, CA USA).

Detection and quantification of several THs was successful in the anuran tissues (e.g. thyroxine (T4); Figure 1). Incorporation of TH measurements with the results of standard AMA apical endpoints, will help to confirm whether observed effects on metamorphosis (development of limbs and tail) are indeed due to alterations in TH signalling pathways and not caused by general toxicity, secondary effects or unrelated mechanisms of action. Also, the quantification of targeted compounds allows for determining the potency and concentration at which a chemical affects thyroid function, as well as comparison of the potency across chemicals. Thus, the generated data may give valuable additional information, which minimises uncertainties around ambivalent results on standard assay endpoints and can be used in a regulatory context. With amphibians representing sensitive indicators for ecosystem health and changes, EDC screening with the AMA has environmental relevance. However, the thyroid system is highly conserved among vertebrates. Therefore, a more accurate identification of thyroid-related mechanisms through the AMA may also be relevant for hazard identification for human exposures.



**Figure 1:** Thyroxine (T4) concentrations in tissue of tadpoles exposed to three different doses of the test compound for 21 days or unexposed. The experimental groups are colour-coded to maintain experimenter blinding regarding the treatment until the analysis is concluded

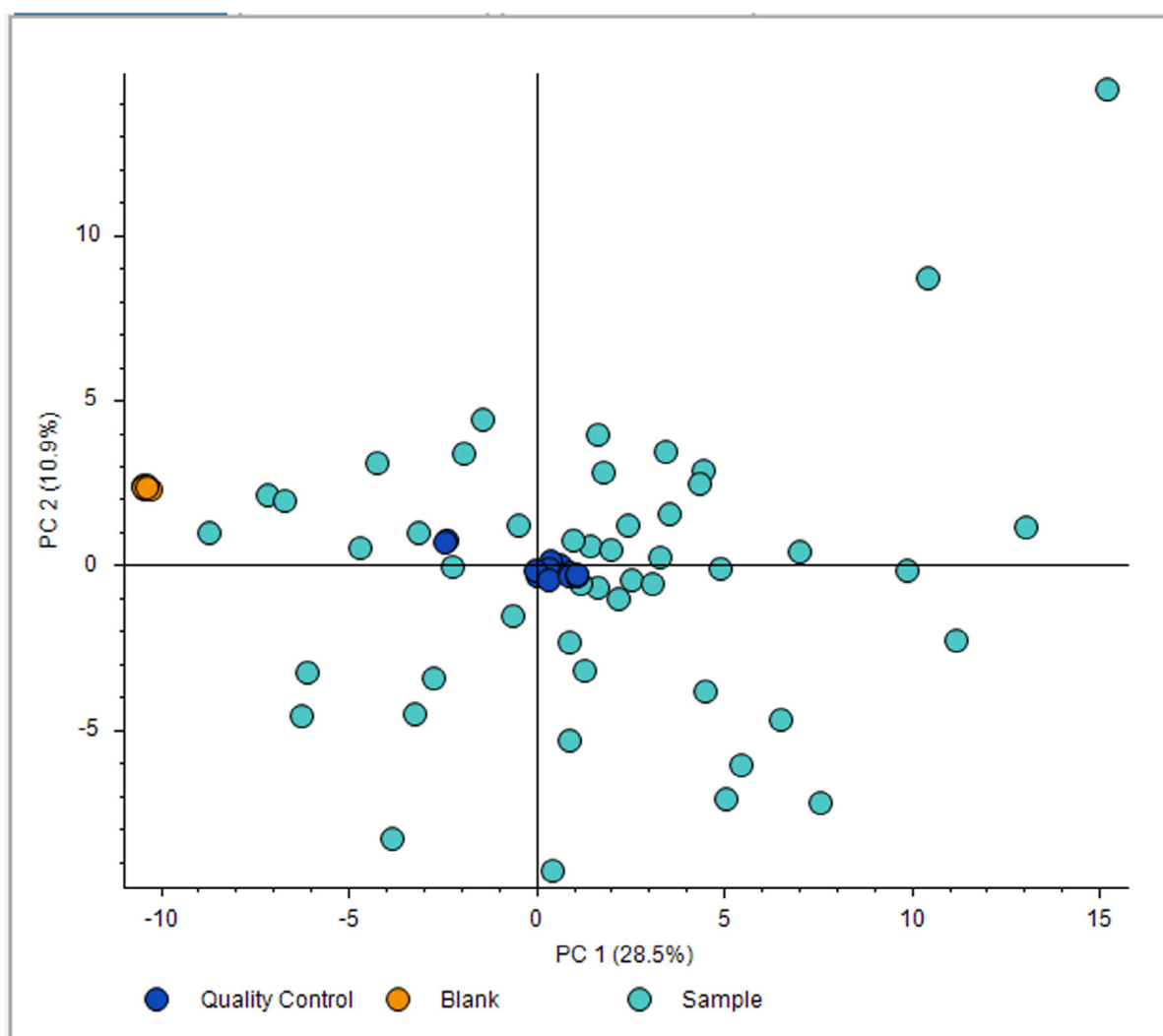
#### 2.2.2.4. Data acquisition untargeted metabolomics

To further explore sub-lethal effects on the thyroid and a potential relationship between thyroid and eye metabolism, in a second step, untargeted metabolomics and lipidomics were performed using UHPLC Orbitrap high resolution tandem mass spectrometry system (Q Exactive HF, ThermoFisher Scientific). The samples were analysed in a randomised order. To account for potential instrument fluctuations and detect systematic errors, a pool of all study samples was interspersed as a QC regularly between individual samples during the injections.



### 2.2.2.5. Preprocessing of untargeted data

Four untargeted metabolomics datasets were generated, to explore lipid-changes and general metabolic pathways. The fellow became acquainted with the different steps necessary for the data processing from raw data files to a list of features, and learned how to compose, customise and run an identification workflow using the Compound Discoverer software (version 3.3; Thermo Scientific). The identification workflow was based on a pre-existing workflow template, including retention time alignment and unknown peak detection, compound detection and grouping, gap filling and merging of features, background correction based on instrument and procedural blanks, and normalisation based on the included QC samples. Initial compound identification was based on formula and accurate mass data from both custom mass lists and publicly available databases, as well as mass spectral library matching. The pre-processed datasets, each containing more than 1000 detected features, were preliminarily explored using the statistical tools available in the Compound Discoverer software (Figure 2).



**Figure 2:** Principal component analysis filtered by sample type. To validate stability and reliability of the analytical method used, a pool of all included samples (Quality Control (QC); dark blue circles) was injected repeatedly throughout the acquisition of tadpole samples (light blue circles). Solvent blanks (orange circles) were also included. Apart from an additional QC sample containing a mix of standards (also dark blue), the QC samples show close clustering.

Correct identification of the large number of compounds detected in the individual datasets is essential for accurate and reliable interpretation of metabolic pathways and organismal responses.

Identification and annotation of the compounds from the untargeted metabolomics datasets is still ongoing at the time of writing. Finalising the work programme, the fellow will participate in the Summer School for non-targeted metabolomics data mining, which is co-organised by the section Environmental Chemistry and Toxicology and hosted by Statens Serum Institute in August 2023. The collaboration between the fellow and the hosting site will continue subsequent to the fellowship programme, in order to conclude the statistical and pathway analyses, and biological interpretation of the generated datasets.

### 3. Conclusions

During the EU-FORA fellowship programme, the fellow was introduced to the current issues in ERA methods, challenges arising from traditional approaches, as well as the potential of metabolomics as a tool to support the transition to a more data-centric, mechanistically based next generation ERA. Being fully integrated into the Environmental Chemistry and Toxicology section at the Department of Environmental Science at AU, synergies between the fellowship working programme and on-going research activities were enabled and established, allowing for direct application of new knowledge and greatly enhancing the 'training by doing' character of the fellowship programme.

Moreover, the physical placement at AU provided a unique opportunity for the fellow to gain very valuable first-hand experience, and to familiarise herself with environmental toxicometabolomics applying both a targeted and untargeted metabolomics workflow, based on the protocols established at the hosting site. In addition to the scientific insights from the studies, the fellow learned best practice approaches for quality assurance and omics-based data analysis, and strengthened personal skills related to laboratory techniques, mass spectrometry technologies, application of bioinformatic computational tools and data management. The practical work provided transferable knowledge, which can be incorporated to add value to on-going research into food and feed safety at the fellow's home institution, the Institute of Marine Research (IMR), Norway, and built a foundation for future collaboration between the IMR and AU.

Both through the training modules, exchange among the fellows as well as opportunities created by the fellow's supervisor Martin Hansen during the implementation of the working programme, participation in the EU-FORA programme allowed to expand the scientific network within (eco) toxicology research, food and environmental safety and risk assessment, and provided a very valuable personal and professional experience.

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## Abbreviations

AMA	amphibian metamorphosis assay
AOP	adverse outcome pathway
AU	Aarhus University
EDCs	endocrine disrupting chemicals
ENVS	Department of Environmental Science, Aarhus University
ERA	environmental risk assessment
EU-FORA	European Food Risk Assessment Fellowship Programme
HPT	hypothalamic–pituitary–thyroid
KE	key event
MIE	molecular initiating event
MOA	mode of action
MTBE	methyl-tert-butyl ether
NAMs	new approach methodologies
NF	Nieuwkoop and Faber
OECD	Organisation for Economic Co-Operation and Development
QC	quality control
UHPLC	ultra-high performance liquid chromatography