EURION

European Cluster to
Improve Identification
of Endocrine Disruptors

EURION Progress Report

Public summary, November 2020

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1. Introduction

European Cluster to Improve Identification of Endocrine Disruptors, EURION, is a cluster of eight European research projects. The projects aim to develop new test methods for identification of endocrine disruptors (EDs) as the current testing tools, including regulatory in vivo tests and novel in vitro assays, do not appropriately identify ED-induced effects related to certain less studied endocrine-mediated pathways and health outcomes. EURION was established in January 2019 in order to optimise synergies and avoid overlaps between the projects selected for funding from the call SC1BHC-27-2018 'New testing and screening methods to identify endocrine disrupting chemicals'.

The projects are:

- ATHENA - Assays for the identification of thyroid hormone axis-disrupting chemicals: elaborating novel assessment strategies.
- EDCMET - Metabolic effects of endocrine disrupting chemicals: novel testing methods and adverse outcome pathways
- ENDpoiNTs - Novel testing strategies for endocrine disruptors in the context of developmental neurotoxicity
- ERGO - Breaking down the wall between human health and environmental testing of endocrine disrupters: EndocRine Guideline Optimisation
- FREIA - Female reproductive toxicity of EDCs: a human evidence-based screening and identification approach
- GOLIATH - Beating Goliath: Generation of novel, integrated and internationally harmonised approaches for testing metabolism disrupting compounds
- SCREENED - A multistage model of thyroid gland function for screening endocrine-disrupting chemicals in a biologically sex-specific manner
- OBERON - An integrative strategy of testing systems for identification of EDs related to metabolic disorders

The cluster projects will:

- deliver novel/improved ED assay candidates for regulatory use (pre-validated);
- provide support for the OECD work on testing and assessing chemicals for ED identification, especially feeding into the OECD Endocrine Disrupter Screening and Testing Programme.
- contribute to enhanced international cooperation;
- contribute to the development of an international strategy and guidelines for testing EDs and assessing associated hazard and risk

As new and improved approaches are needed to increase the quality, efficiency and effectiveness of existing methods to meet demanding and evolving regulatory requirements, EURION enables effective collaboration between the projects to reach, and contribute towards, the main goal of improving test methodologies of ED identification. Being part of the cluster facilitates sharing of knowledge, data and expertise as well as allows joining forces for common dissemination and communication activities in order to maximize impact, both at project and cluster-level.

The cluster is coordinated by rotating teams consisting of two projects at a time to cover the 5-year life span of the projects. For the first 15 months, the EURION cluster was coordinated by GOLIATH and OBERON. Since 1 April 2020, ENDpoiNTS and ERGO have held the chairmanship and will continue coordinating the cluster until June 2021. After that, ATHENA and SCREENED will take over the coordinatorship while EDCMET and FREIA will act as coordinators during the last quarter of the funding period.

This report summarises the progress and activities of the EURION cluster and the eight projects during the 18-month EC reporting period, from 1 January 2019 to 30 June 2020. The first part of the report is focused on providing a progress overview of the individual projects. The project-specific public summaries are followed by an overview of test methods to be developed, cluster highlights and working group summaries.

2. Project summaries

2.1 ATHENA project summary

Assays for the identification of Thyroid Hormone axis - disrupting chemicals: Elaborating Novel Assessment strategies - ATHENA

Summary of the context and overall objectives of the project

Endocrine disrupting chemicals (EDCs) are found to be ubiquitous in the environment; these chemicals are added to various materials and products such as plastics, pesticides and flame retardants but ultimately end up in the environment and food chain and can accumulate in the human body, causing negative health consequences. EDCs disrupt the normal functioning of hormonal systems by mimicking hormones or blocking their natural action in the body.

From amongst the different hormonal systems, the ATHENA project is interested in the thyroid hormone system and how brain development *in utero* is affected by EDCs which act on the thyroid system, called thyroid hormone system disrupting chemicals (THDCs).

For the first few weeks of gestation, the foetus is unable to produce thyroid hormone and relies on the mother for its essential supply. It is well-established that maternal hypothyroidism (thyroid under-function) can lead to impaired psychomotor development and lowered IQ in offspring. It has been shown that THDCs in the maternal system (for example certain pesticides and flame-retardant chemicals) can decrease circulating thyroid hormone, with lower levels reaching the foetus and leading to the same negative outcome. Over-production of thyroid hormone also has detrimental effects. There are several points within the thyroid hormone system where THDCs can act to disrupt the system; partners within the ATHENA consortium will focus on different areas within the thyroid hormone system to develop test methods to identify THDCs and their mechanism of action. An improvement of test methods is urgently required; the available test methods only provide a minimal assessment of thyroid hormone system disruption which is inadequate and leaves several gaps in the available test methods for protection of human health against THDCs.

The overall objective of the ATHENA consortium is to develop test methods for the identification of chemicals that disrupt the thyroid hormone system, thus increasing the testing repertoire to include in the OECD test guidelines. The focus of test development will be to capture the consequences of maternal thyroid hormone deficiency or over-production on the developing brain.

Work will focus on understanding disruption of local maternal thyroid hormone by inhibition of enzymes which are involved in thyroid hormone synthesis and metabolism and cell membrane transporters, by development of predictive methods and screening of compound libraries. The consequence of maternal hypothyroidism on the foetus due to interference of the delivery of thyroid hormones across the placenta, the blood brain barrier (BBB) and the blood cerebrospinal fluid barrier (BCSFB), will also be studied.

The results of experimental work will allow us to construct a network of the thyroid system that can explain how maternal exposure to THDCs can lead to adverse neurodevelopmental effects. It will allow us to formulate a comprehensive testing strategy which will be positioned for international harmonisation to protect against chemicals that are harmful to brain development in foetal life.

Overview of progress and main results achieved so far

In the first 18 months of the project, the ATHENA consortium achieved the following aims:

- Using existing epidemiological data from two large studies, found that exposure to thyroid system disrupting chemicals in pregnant women is associated with disruption of hypothalamic-pituitary-thyroid axis and exploited these results as a published manuscript (Derakhshan et al Environment International 133 (2019) 105123). There are further manuscripts in preparation.

- Progress in the development of 3D cell models of brain development and neural stem cell division: a human organoid model with a defined panel of neuronal markers covering different stages of maturation and the various cell populations within the organoids, and mouse neurospheres, for which pharmacological assessment of specific TH-dependent endpoints and neural stem cell proliferation has been studied.

- Smaller studies of several thyroid system disrupting compounds have been performed in rats and mice. These studies were dose-selection studies performed to ascertain thyroid hormone system disruption and clarify potential toxicities when compounds were given to pregnant animals. The results from these studies form the basis for large developmental toxicity studies where effects on the thyroid hormone system and brain development will be examined in detail. One major endpoint in these studies will be the formation of heterotopia - the occurrence of misplaced neurons in a defined structure. Heterotopia are linked to thyroid hormone system disruption in rats and in humans they have been correlated to seizure and epilepsy.

- Progress in test method development for a non-radioactive high throughput assay for screening interactions at two non-receptor targets, which has also generated data for QSAR modelling contributing to the development and validation of new QSAR models for inhibition of deiodinases and cell transporter (DIO2, DIO3, DEHAL1 and OATP1C1). A model is also being generated for another transporter (NIS) involved in thyroid hormone synthesis.

- Work has progressed towards robotisation of the Xenopus Eleutheroembryonic Thyroid Assay (XETA), an existing method in the OECD test guidelines. The assay has been refined and validated for medium throughput and will progress to high throughput format. There has also been progress towards development of strategies for establishing the mode of action of THDCs to elucidate their targets in the body and provide information about specific molecular initiating events (MIE).

- Cellular models are being established for study of the disruption of thyroid hormone transport between mother and foetus for placenta, BBB and BCFB. A placenta perfusion model has also been set up to test transplacental T4 transport to identify the important TH transmembrane transporters that may be inhibited by THDCs. One of these transporters is undergoing development for a nonradioactive high throughput assay to allow screening of inhibitors.

Progress beyond the state of the art and expected potential impact

Exposure of pregnant women to thyroid system disrupting chemicals can manifest as learning and developmental problems in the child. This is due to disruption of the maternal thyroid hormone system, which the foetus relies on in the first few months in the womb. Certain chemicals have been identified as thyroid system disruptors, leading to a decrease in thyroid hormone, however they are not necessarily

recognised as such, due to their mechanism of action. The current status quo of regulatory test guidelines has a very limited definition of what constitutes a THDC, which means that harmful chemicals are not recognised and their negative impacts to human health cannot be prevented.
The aim of the ATHENA project is to provide an improved understanding of the thyroid hormone system with regards to endpoints and targets of THDCs, this will allow the development of a testing strategy to incorporate into regulatory guidelines at international level. In the first 18 months of the project the ATHENA consortium has worked towards all the expected deliverables and milestones of the project (although there has been some delay due to COVID-19) and are well positioned to continue to meet the goals of the project.

Neurodevelopmental problems, which include; learning difficulties, low IQ and autism, require medical and social support and can impact the ability of a child to lead an independent life. At the level of the population, this fundamentally has a negative socio-economic outcome considering the cost of care, treatment and burden to society. Exposure to THDCs is avoidable and it is vital that pregnant women (and other vulnerable members of society) are provided with correct, timely advice and this requires restricted usage of products containing THDCs or of the chemicals themselves. The ATHENA project aims to identify and improve the testing strategy for THDCs and ultimately to contribute to regulatory guidelines to protect against chemicals that can cause neurodevelopmental changes in the foetus.

Further information about ATHENA:
Website: https://athenaedctestmethods.net
Cordis: https://cordis.europa.eu/project/id/825161
Twitter: @ATHENAprojectEU

2.2 EDCmet project summary

Metabolic effects of Endocrine Disrupting Chemicals: novel testing METhods and adverse outcome pathways – EDCMET

Summary of the context and overall objectives of the project

Endocrine disruptors (EDs) are defined as exogenous chemicals that alter functions of the endocrine system, thereby causing adverse health effects in an organism or its progeny. Historically, the field of ED research has focused on reproductive endocrinology and related hormones, which is reflected in the regulatory test methods assessing endocrine effects of xenobiotics. However, Recent evidence links increased incidence of metabolic syndrome - a cluster of metabolic risk factors including abdominal obesity, dyslipidaemia, elevated blood pressure, and elevated fasting glucose - to ED exposure, further increasing the incidence of atherosclerosis and type 2 diabetes. However, the current testing tools do not appropriately identify effects related to less-studied endocrine-mediated pathways and health outcomes, such as disruption of lipid and glucose metabolism, in humans. Activation of nuclear receptors (NRs) has been linked to the development of several metabolic and liver-related diseases. While public databases, such as ToxCast, contain numerous assays for the assessment of NR modulation, important metabolism-related NRs have not been included in the analyses or the information available is misleading due to lack of performance information of the assays. In addition to the NRs, EDs may alter metabolic functions by other mechanisms, such as impairment of mitochondrial respiration, which has been linked to the development of insulin resistance and non-alcoholic fatty liver disease. New and improved approaches are needed to increase the quality, efficiency and effectiveness of existing methods to evaluate the effects of EDs and to meet the demanding and evolving regulatory requirements worldwide.

The “Metabolic effects of Endocrine Disrupting Chemicals: novel testing METhods and adverse outcome pathways” (EDCMET) project focuses on developing novel or improved computational and *in vitro* methods, such as non-cellular assays and cell culture systems, as well as standardized *in vivo* animal models to assess the metabolic effects of EDs, with a focus on NRs regulating these processes. Further, unbiased omics techniques will be used to investigate tissue and plasma markers *in vivo*. Epidemiological and field monitoring data is used to gain information regarding the human exposure to EDs and related metabolic effects. EDCMET will also apply the adverse outcome pathway (AOP) paradigm to identify molecular initiating events (MIEs) and predict the emerging adverse biological phenotype. EDCMET comprises of academic and non-academic partners and experts in various research fields, including systems toxicologists, experimental biologists with a thorough understanding of the molecular mechanisms of metabolic disease and comprehensive methodological skills and ultimately, epidemiologists linking environmental exposure to adverse metabolic outcomes. The interdisciplinary approach and complementary expertise of the participants will aid in the identification of novel mechanisms of action and the development of novel or improved validated test methods for regulatory purposes.

Overview of progress and main results achieved so far

EDCMET teams have been exploring and collecting publicly available resources and data, including NR proteins and ligands, ED compounds and omics data for the action of EDs in hepatic cell models and *in vivo*. An initial analysis pipeline for prediction of chemicals with ED potential has been generated. Liver transcriptomics data from mice has been produced and plasma and liver metabolomics from wildtype and NR knockout mice have been planned. Metabolomics data is already available from selected cohort

samples and possibilities for further metabolomics analyses are explored. The *de novo* omics data will be used to further develop and validate the omics analysis pipelines.

To evaluate a battery of NR-cofactor interaction assays, a small set of ED chemicals has been screened across a panel of relevant NRs and further assays are under development. Reporter gene assays for predicting the activation of NRs involved in the regulation of metabolic pathways have been largely optimized and detailed evaluation of assay performance is on-going. The NCATS and OECD Framework guidance on standardized *in vitro* test systems and scientific assay validation have been followed and implemented in the development and scientific pre-validation of the NR assays to ensure robustness and reproducibility of results as well as transferability of the assays. These, together with established standard operating procedures (SOPs), will ensure the applicability of the protocols also in the international regulatory context. Mitochondrial respiration assays are under development to enable assessment of functional effects of EDs on cellular level in hepatic cell models in high-throughput format. High-throughput, fluorescence-based AdipoRed assay is available to evaluate the steatotic effects of EDs.

SOPs have been developed for performing insulin tolerance and glucose tolerance tests and for inducing obesity, insulin resistance, and non-alcoholic fatty liver disease using high-fat diet in mice. Standard protocols for glucose and insulin tolerance tests and liver steatosis have been developed in humanized mice. Experiments with ED reference compounds are on-going. Multiple approaches, including traditional risk assessment, guideline values and toxicological cut-off points, toxicokinetic modelling and WHO risk assessment methodology and health effects based on epidemiological studies, have been considered. Analyses of EDs from cohort samples have been started and further analysis methods are under development.

Progress beyond the state of the art and expected potential impact

EDCMET will provide an array of new or improved testing tools for risk assessment of metabolism disrupting EDs. These tools are expected to identify novel disrupted pathways in human liver and adipose tissues and to increase human relevancy in testing and risk prediction and further reduce the use of laboratory animals. The methods and models will be coupled with human exposure data, linking the levels of EDs with metabolic endpoints and health outcomes. The developed methods undergo preliminary experimental validation and can contribute to the current OECD test systems. All information concerning the EDs and protocols, will be provided for the regulatory use and the regulatory implementation will be done in collaboration with contacts in national and EU-level. EDCMET will lay the groundwork for future testing and ED toxicity assessment and contribute to a better understanding of human exposure to chemicals and the associated burden of metabolic diseases. EDCMET will also deliver methods to be commercialised and used by the SMEs involved in the project.

Endocrine-related diseases represent a high cost for healthcare systems in the EU countries and metabolic effects of EDs are poorly understood. EDCMET will increase the knowledge on metabolic diseases by characterization of the affected critical pathways in liver and adipose tissues using the AOP approach. EDCMET will also aid in the development of novel biomarkers for metabolic diseases and provide a relevant base for assessing adverse human health effects of environmental chemicals. The availability of fast and cheaper test systems can stimulate research around environmental chemicals and contribute to safer marketed products and thereby have a positive impact on the environment. The international team of EDCMET will increase awareness of the new testing methods and information on the metabolic effects of EDs among scientists and stakeholders in their respective countries. International collaboration within the EURION cluster and with further research groups or the enterprise sector, as well as contacts with authorities, will further facilitate the flow of important data and other information, leading to potential

new initiatives. All partners are active in making the public and scientific communities aware of the project's results and targets through scientific conferences and publications as well as project website, social media and workshops. Collaboration within EURION will further increase the quality, efficiency and effectiveness of the test methods to meet the evolving regulatory requirements and contribute to the development of international strategy and guidelines for ED testing and risk assessment.

Further information about EDCMET:
Website: www.uef.fi/edcmet
Cordis: https://cordis.europa.eu/project/id/825762
Twitter: @edcmet_eu

2.3 ENDpoiNTs project summary

Novel Testing Strategies for Endocrine Disruptors in the Context of Developmental NeuroToxicity – ENDpoiNTs

Summary of the context and overall objectives of the project

ENDpoiNTs is developing a new testing strategy for developmental neurotoxicity to meet the regulatory as well as scientific and societal needs for improved hazard and risk assessment of endocrine disrupting chemicals (EDCs). Globally, serious concerns have arisen about the exposure to anthropogenic chemicals that can produce adverse health effects via disruption of the body's endocrine (hormone) system, known as EDCs. During a lifetime, people are exposed to numerous EDCs via food, water, air as well as various products and materials. From a scientific point of view, there is no doubt that exposure to EDCs can adversely affect the endocrine system, but current chemical screening and testing tools need to be improved and harmonized to meet regulatory requirements worldwide.

A significant knowledge gap is how EDCs affect neurodevelopment, and endocrine disruption (ED)-induced developmental neurotoxicity (DNT) is hardly covered by the testing tools in regulatory use. The brain is among the most vulnerable organs with respect to toxic insults, particularly during development, and EDCs have indeed been shown to target the (developing) brain in animal models. Even in humans, several EDCs at low exposure levels have been associated with adverse effects on neurodevelopment in children in large epidemiological studies. The adverse effects are manifested in changes in cognition, behaviour, and other brain functions, and even by an increased risk for neurodevelopmental disorders.

The main objective of ENDpoiNTs is to generate new scientific knowledge on how endocrine disruption is linked to developmental neurotoxicity at the molecular, cellular, tissue, and organism level. Based on this new knowledge, the project will:

- Develop predictive computational tools for chemical screening, using machine learning and combination of evidence;
- Develop and validate cellular testing and screening tools that are linked to a clearly defined and empirically validated endocrine mode of action;
- Develop novel molecular endpoints for existing animal-based test guidelines by integrating experimental and human omics data with cellular as well as cognitive and behavioural outcomes;
- Ensure human relevance by linking experimental and epidemiological evidence with regard to chemical exposures and molecular markers;
- Develop an integrated approach to identify endocrine disrupting chemicals inducing DNT based on the developed battery of computational, cellular, and animal-based assays and molecular markers;
- Ensure regulatory relevance by engaging with key stakeholders and, together, develop and integrate novel strategies for EDC testing and assessment into European and international chemical regulatory frameworks.

Overview of progress and main results achieved so far

A number of cellular and animal-based models exist to study DNT endpoints, addressing cellular key events and neurodevelopmental outcomes. Some of these models are already at relatively high readiness for, or

even integrated into, regulatory testing. However, the predictive value of these models to detect ED-induced DNT is largely unknown. Thus, in ENDpoiNTs, we are assessing the responsiveness of these DNT endpoints to endocrine interference and the underlying mechanisms and pathways that correlate DNT effects with EDC exposure. So far, results from several models, both human and rodent, show that EDC targets are involved in key cellular processes during brain development. Furthermore, first experimental results indicate that some of these endpoints may also be affected by low doses of EDCs, e.g., bisphenol A, some phthalates, pesticides and perfluorinated compounds, and that some effects are species-specific. For several of the hormone-depended endpoints, standard operating procedures (SOPs) have been established, which is a crucial first step towards test method establishment.

For the management and handling of the experimental data, a standardised database platform has been developed. It includes data capture and curation procedures as well as data treatment and statistical analysis approaches that are relevant for ENDpoiNTs. Moreover, it serves as platform for all data integration approaches. Furthermore, complementing the experimental results, 12 high confidence Quantitative Structure Activity Relationships (QSARs) have been developed for predicting the agonistic and antagonistic modes of action of several hormone receptors. This will be the basis for computational models identifying chemicals that can induce DNT via these interactions. Using data from the public domain and produced in ENDpoiNTs, the molecular interactions, cellular effects and adverse outcomes will be integrated using the Adverse Outcome Pathway (AOP) framework. To enable this, relevant literature is being reviewed to identify brain regions, neurotransmitter systems, and endocrine systems of interest. For establishing human relevance of the test methods developed in ENDpoiNTs, doses producing an adverse effect in test systems will be compared with human exposure data from the cohorts included in the project and other international data sets for both single reference EDCs and for their mixture. For comparison, the metrics have been evaluated using established regulatory values translated to biomonitoring equivalent concentrations.

Finally, to engage with key stakeholders and the public at large, ENDpoiNTs has set up a webpage (https://endpoints.eu/), produced project flyers and an animated short film about the aims of the project. Additionally, within the EURION cluster, ENDpoiNTs has generated a stakeholder list and initiated a survey to receive inputs from key stakeholder regarding methods development.

Progress beyond the state of the art and expected potential impact

ENDpoiNTs will significantly advance the scientific knowledge on how EDCs exert their negative effects on the developing brain. Based on the promising preliminary results, ENDpoiNTs has successfully started developing endpoints, tools, and methods that are based on mechanistic understanding. A large focus is put on developing new approach methodologies (NAMs), i.e. computational and cellular tools, but also on guidance on how novel molecular DNT endpoints can be amended into existing animal-based testing guidelines. Integration of experimental and epidemiological data and systematic inter-species comparisons will ensure human relevance of the developed methods.

Hence, ENDpoiNTs will advance the current foundations of regulatory science in this area clearly beyond the state of the art by i) enhancing human-relevance of screening methods, and ii) developing an integrated platform of testing strategies and methods to test and assess ED-induced DNT. The development of a strategy for integrating the battery of testing tools in the Integrated Approach to Testing and Assessment (IATA) framework and integrating epidemiological research and data in risk assessment of EDCs will also advance the scientific basis for risk management. Moreover, the impact of ENDpoiNTs will reach beyond the EDC field, since the in silico, in vitro and in vivo models, methods and procedures developed in the project can be applied to any chemical or chemical mixture. Ultimately, this will have impact on

policymaking and regulations and enable better protection of vulnerable populations and particularly, the developing brain.

Further information about ENDpoiNTs:
Website: https://endpoints.eu/
Cordis: https://cordis.europa.eu/project/id/825759
Twitter: @ENDpoiNTs_EU

2.4 ERGO project summary

Breaking down the wall between human health and environmental testing of endocrine disrupters: EndocRine Guideline Optimisation - ERGO

Summary of the context and overall objectives of the project

ERGO project will break down the wall between mammalian and non-mammalian vertebrate regulatory testing of endocrine disrupting chemicals (EDCs) by identifying, developing and aligning thyroid-related biomarkers and endpoints (B/E) for linkage of effects between different vertebrate classes. To achieve this, an adverse outcome pathway (AOP) network covering various modes of thyroid disruption (TD) in multiple vertebrate classes will be developed. An AOP starts from a molecular initiating event (MIE) and outlines the sequence of key events (KE) leading to a relevant adverse outcome at the organism or population level. The AOP network will provide the scientifically plausible and evidence-based foundation for the selection of B/E and assays in lower vertebrates predictive of human health outcomes. These assays will be prioritised for validation in ERGO.

ERGO will re-think ED testing strategies from in silico methods to in vivo testing and develop, optimise and validate existing in vivo OECD guidelines and (if required) new in vitro protocols with novel TD endpoints and consequently reduce requirements for vertebrate animal testing by preventing duplication of testing in mammals and non-mammalian vertebrates. The ERGO Integrated Approaches to Testing and Assessment (IATA) strategy will increase the screening capacity to enable more chemicals to be tested for ED properties.

To achieve its goals of providing stakeholders and businesses with better tools and strategies needed to ensure improved management of EDCs, ERGO has four overarching objectives:

- Investigate, develop and validate thyroid B/E predicting effects across vertebrate classes for inclusion in new in vitro and existing in vivo OECD test guidelines (TGs) for improved identification and safer assessment of thyroid disrupting chemicals.
- Develop an AOP network across vertebrate classes for identification of thyroid B/E applicable for assessment of cross-class thyroid disrupting KE.
- Transform new data, tools and understanding into a harmonised IATA testing strategy for regulation of EDCs by inclusion of stakeholders at the global level in the incorporation of the cross-vertebrate class testing approach.
- Publish a guidance document on extrapolation of TD effects across mammalian, fish and amphibian OECD TGs.

Overview of progress and main results achieved so far

The “Chemical Properties Estimation Software System” named ChemProp has been adapted to be used as the ERGO database system. Furthermore, the chemical selection was made for the in vitro test battery as well as for the in vivo assays. The experts forming the User Reference Group (URG) members have been agreed upon and will be convened in autumn 2020. Also, the scope for AOP development within ERGO has been established. The AOP development progress has been outlined. Knowledge gaps has been identified based on the current status of the AOPs and relevant to achieving the goals of ERGO.

Data mining has been started, and decisions on amount and type of data to be looked up have been made. For in silico bioavailability triggers, model candidates are under evaluation. Concerning In-vitro-to-in-silico biotransformation, the experimental setup for S9 enzyme mix assays including reference systems has been started. Based on the S9 assay according to literature, respective variants employing fish and rat derived S9 fractions have been established and validated. Finally, 1107 potential EDCs were extracted from the EDC DataBank. Multiple partners have performed intensive literature review work to contribute to existing data. First experiments have been performed. Part of the results of these experiments have been critically evaluated. From January-April 2020, SDU coordinated the preparation of an open-access peer reviewed publication in a special issue of Int. J. Mol. Sci. (IJMS) with all WP5 partners contributing. The publication was published as a "project report" outlining the main objectives of ERGO and can be downloaded from: https://www.mdpi.com/1422-0067/21/8/2954

In the context of selection of in vivo and in vitro reference compounds for long-term fish studies, several in vitro assays, literature and available mammalian studies have been evaluated to give an overview of mechanistic apical data of screened substances. For evaluation of data from mechanistic studies in mammals with reference compounds, a list of compounds to be tested has been provided. In order to perform genomic analyses, a cohort of mice exposed to control and test substances have been produced and a large collection of samples collected. For the establishment of an AOP based strategy for epidemiological and human exposure studies, a database is being developed on the interactions of pollutants commonly detected in epidemiological studies with priority MIEs for TD identified within ERGO. Furthermore, proposals have been prepared for the development and validation of fish-based thyroid biomarkers.

A portfolio of communication and dissemination resources and tools was developed in the first year of the project to facilitate promotion and widespread awareness of the project. Dissemination activities and external strategic communication is carried out on an ongoing basis by all partners across the full project duration. An ERGO Introductory Video has been created, showing the impact of endocrine disruption on everyday life, and is being promoted at events and on social media. The ERGO Project website (https://ergo-project.eu/) has been launched, carrying out general dissemination of ERGO results, news, events and progress and with a dedicated section for 'Outreach'. ERGO social media has been introduced, the project Twitter page (https://twitter.com/ERGO_EU; @ERGO_EU), disseminating through LinkedIn, promoting ERGO activities and results as well as connecting with sister cluster group projects (EURION) Partners have attended high profile conferences and events representing the ERGO project and promoting its results and the EURION cluster. The ERGO Data Management Plan has also been developed, including a data inventory table, and the ERGO Innovation Task Force (ITF) was activated.

Progress beyond the state of the art and expected potential impact

ERGO is a coordinated attempt to contribute filling the gaps in the field of TD. It will allow to identify both disturbance of the thyroid axis and its potential adverse effects in different vertebrate classes. ERGO is expected to improve methodologies for using cell tests and fish and amphibian assays for early screening of substances and to develop new in silico models for predicting internal dose of TDCs to design physiologically based toxicokinetic modelling (PBTK) models and to link MIEs with AE within an AOP network.

The overarching impact of ERGO's products is improved health of the citizens of Europe due to lower exposures to EDCs at home and in the workplace. Citizens will remain confident that their environment is protected from EDCs and a sustainable circular economy based on EDC-free products is promoted. ERGO

will provide practical solutions to support more effective EDC testing and screening in Europe over the next decades and economically, EU will benefit from reduced health care costs and European industries will prosper due to faster and cheaper development of new products. This will be a consequence of ERGO's fast, effective and efficient EDC testing and screening approach enabling better prediction of chemical risks for humans and the environment in line with regulatory requirements, with the long-term vision of an EDC-safe world.

The direct key end-users of ERGO's products are regulators and industry; all constrained by the scarcity of practical EDC test and screening tools for fast, cheap and robust hazard and risk assessment of goods feeding a growing sustainable European circular economy. With thyroid disruption (TD) as object, the proof of concept will be delivered with industry and regulators integrated up-front in the project (co-design). ERGO is a need- and application-driven project that combines excellent science with a full commitment to deliver novel testing strategies for EDCs and more efficient and effective hazard- and risk assessments.

ERGO is expected to increase basic knowledge on the detailed role of TH disturbances. The ERGO approach would be of significant interest for the safety assessment of existing chemicals lacking endocrine and developmental toxicity data and new chemicals at an early stage of their industrial development. This methodology should allow:

- To simultaneously screen chemicals for their potential human TD effects, as well as their environmental impact with similar negative effects on fish and amphibians.
- Significantly reduce the requirement of vertebrate animal testing, with respect to animal welfare.
- A relevant assessment at the vitro scale opens the door to automation and higher throughput screening of chemicals, which would further reduce the cost of their assessment.

Further information about ERGO:
Website: ergo-project.eu
Cordis: www.cordis.europa.eu/project/id/825753
Twitter: @ERGO_EU

2.5 FREIA project summary

FREIA: Female Reproductive toxicity of Endocrine disrupting chemicals (EDCs): a human evidence-based screening and Identification Approach

Summary of the context and overall objectives of the project

The FREIA project aims to provide better test methods to identify human-made chemicals that disturb hormones and their actions on development and function of the reproductive system in women. Currently available test methods do not work well, which is partly the reason why the effects of such endocrine disrupting chemicals (EDCs) on female reproductive health are often overlooked in regulatory chemical safety assessments. This means that women's reproductive health is at risk globally. Our EU-funded project FREIA (Grant Agreement number 825100) will increase our understanding of how EDCs can harm female reproductive health. We will use this information to provide better test methods that enable fit-for-purpose chemical regulation. We will also share our knowledge widely to improve the reproductive health of women globally.

Overview of progress and main results achieved so far

In the FREIA project, the development of better test methods consists of two phases. In the first, "Discovery phase" we will look for biological characteristics (biomarkers) for female reproductive toxicity and develop a test strategy using existing knowledge as well as new data collected from our laboratory studies. For this, we will use two well understood EDCs, diethylstilbestrol (DES, a potent estrogen receptor activator) and ketoconazole (KTZ, a blocker of steroid hormone production). In the second, "Testing phase" of the FREIA project, we will assess how well our testing strategy works.

Cells from human fetal, child and adult ovarian tissues have been cultured in the lab to identify which cell types are present in the human ovary and delineate harmful effects of DES and KTZ in human ovaries at various life stages. Fragments of human fetal ovaries (6-12 weeks after conception) were cultivated with various concentrations of KTZ for one week. We observed that KTZ decreased the percentage and the total number of immature germ cells, irrespective of the age of the ovary. Similar initial experiments with DES showed no obvious toxic effect on ovarian cells. We will study the effects of KTZ and DES better by assessing specific ovarian cellular markers, screening the hormones produced by the ovarian cells and by analysing ovarian gene expression profiles. Methods were established to measure known and suspected EDCs, e.g. perfluorinated chemicals, phthalates, bisphenols and parabens, and to measure biomarkers in biological fluid that surrounds the developing oocyte in the adult ovary. These follicular fluid samples were retrieved from women undergoing fertility treatment in Sweden and Estonia. In the samples of the Swedish women, we already have discovered associations between chemical exposure and the number of oocytes retrieved from the patient in the fertility treatment, as well as the chance of the treatment resulting in a live birth. Together these data from human tissues will pinpoint changes in biological characteristics in human ovarian cells at various life stages upon exposure to EDCs.

A rat study was conducted to define biomarkers that predict the onset of female reproductive toxicity. Female rats were orally exposed to three dose levels of DES or KTZ from day 7 of pregnancy until 22 days after giving birth (postnatal day - PND 22). Both DES and KTZ caused endocrine disrupting effects in the pups. The onset of puberty was delayed in the female pups in all DES groups and the middle KTZ group, which was demonstrated by a delay in vaginal opening. No influence on ovary weights at PND 14, 22 or 90

was detected. The pulsatory release of Gonadotropin Realising Hormone (GnRH), a hallmark for puberty, was studied in the brain of the female pups at PND 22, 42 and 90. At PND 22, the two highest doses of DES and KTZ induced a delay in the time between GnRH pulses. At PND 42, but not PND 90, this was also observed for DES. Interestingly, our finding of delayed GnRH pulses confirms the delay in onset of puberty indicated by examination the age at vaginal opening. A much lower number of animals were needed to see effect on GnRH release than on age at vaginal opening, which provides an important focus for improving test methods for female reproductive toxicity assessment.

Several cell-based and computational methods are being (further) developed that may help the identification of EDCs that are toxic to female reproduction. A sensitive reporter gene assay for modulation of estrogen receptor (ER)-beta activity and a G-coupled protein estrogen receptor (GPER) activity assay are being developed that allow fast screening of potential EDCs on these receptors. An assay looking at multiple cellular markers in human ovarian cell lines showed distinct results on mitochondrial function and oxidative stress upon DES and KTZ exposure. Clear harmful effects were observed on the maturation of bovine oocytes exposed to DES in a culture dish, which lead to the decreased ability to develop into an embryo after fertilisation. A sensitive analytical method to measure steroid hormones is being optimised that will be applied to blood from the rat studies and cell culture media. Computational quantitative structure-activity relationship (QSAR) models to predict the effects of EDCs on aromatase inhibition and peroxisome proliferator-activated receptor (PPAR)-gamma activation are being improved using existing data sets. How these methods can be used in a test strategy to predict female reproductive toxicity will be explored further. Through thorough review of the available literature, we have now constructed ten possible pathways to explain how EDCs can cause female reproductive disorders. These so-called putative adverse outcome pathways (pAOPs) have been published (doi: 10.1007/s00204-020-02834-y). These pAOPs together with the results from the ovarian cell studies and rat studies will help us to pinpoint which test methods are needed to predict a chemical's effect on female reproductive development and health and improve test strategies for future use in regulatory chemical safety assessment.

On the FREIA website, general background information on EDCs and female reproductive health can be found as well as project specific information, including webinar recordings, peer-reviewed scientific publications and the FREIA factsheet and infographic (both available in multiple languages).

Progress beyond the state of the art and potential impacts

FREIA provides the unique possibility to investigate hormonal processes in human ovaries from fetal to adult age to improve scientific knowledge on the causes of female reproductive toxicity. Our committed collaborators for policy, advocacy and communicating actions to promote women's health and a healthy society allow FREIA to have a huge societal impact. The FREIA approach will strongly support the work of European regulatory agencies, or even globally through the EURION cluster activities. The tools we are developing perfectly fit the needs of modern-day toxicity testing with a clear regulatory application in mind. Together, the FREIA outcomes will support testing, identification and assessment of EDCs that are toxic for female reproduction.

Further information about FREIA:
Website: www.freiaproject.eu
Contact: info@freiaproject.eu
Twitter: @freiaprojectEU

2.6 GOLIATH project summary

Beating Goliath: Generation Of NoveL, Integrated and Internationally Harmonised Approaches for Testing Metabolism Disrupting Compounds

Summary of the context and overall objectives of the project

The incidence metabolic disorders such as obesity, diabetes and metabolic associated fatty liver disease has reached 'Goliathan' proportions. The worldwide increase in metabolic disorders cannot be explained by lifestyle and genetic factors alone; the role of environmental factors in these disorders has been increasingly acknowledged. Exposure to endocrine disrupting chemicals (EDCs) that disrupt metabolism – chemicals collectively referred to as 'metabolism disrupting chemicals' (MDCs) – is an environmental risk factor that urgently requires more attention. MDCs are natural and anthropogenic chemicals that have the ability to promote metabolic changes that can ultimately contribute to the development of obesity, diabetes and/or fatty liver in humans. Considering the important role these metabolic alterations can play in the global epidemics of metabolic disorders, it is essential that international chemicals regulations require the identification of MDCs and the assessment of the risk associated with exposure.

Within European chemicals regulations, criteria to identify EDCs have been proposed that require information on a chemical's endocrine mode of action (MoA) and related adverse effects relevant for human health. However, currently no regulatory *in vivo* or *in vitro* tests exist to identify the potential metabolism disrupting effects of chemicals. The need for these tests has been internationally recognized, as without them, comprehensive hazard and risk assessment of chemicals for potential metabolism disrupting activity is virtually impossible.

The overall aim of the GOLIATH project is to improve hazard and risk assessment of EDCs by generating novel, optimised, integrated and internationally harmonised approaches for testing metabolic disruption. The GOLIATH project spans the entire spectrum of testing, from *in silico* predictive modelling and high throughput screening, to the development of robust ready-to-use *in vitro* assays and optimisation of current *in vivo* testing guidelines. By incorporating novel omics technologies to translate *in vitro* and *in vivo* assay outcomes into human health effects, GOLIATH will generate new testing strategies for MDCs, and provide novel insights in the mechanisms by which MDCs disrupt metabolic pathways and induce adverse effects on human health. With a consortium comprised of world-leading experts in endocrinology, physiology, molecular biology, endocrine disruption, toxicology, epidemiology, bioinformatics, test method development, validation and chemical regulation, GOLIATH will be pivotal in the development of an internationally harmonised strategy for testing MDCs.

The overall objectives of the project are:

1. To improve the understanding of the endocrine modes of action of metabolism disrupting chemicals.
2. To develop assay candidates for metabolic disrupting chemicals based on confirmed MoA and key biological effects in target tissues.
3. To select and develop assay candidates into (pre-)validated test methods, in collaboration with OECD, ensuring test method definition, transferability, inter-laboratory testing and assessment of predictivity, which are prerequisites for their regulatory use.
4. To develop an internationally harmonised, integrated approach to testing and assessment (IATA) of MDCs, using an Adverse Outcome Pathway (AOP) conceptual framework.

Overview of progress and main results achieved so far

The first period of the GOLIATH project was dedicated to several main tasks, namely:

1) A review of current state of the science and overview of human exposure to MDCs (published in the International Journal of Molecular Sciences (https://www.mdpi.com/1422-0067/21/10/3480)), to be further elaborated in collaboration with OECD
2) Identification of suitable model chemicals for the selected assays within GOLIATH
3) Establishment of strategies for *in silico* kinetic modelling and performance of *in silico* analysis, including crystallization experiments and molecular docking via the publicly available platform EDmon (http://atome.cbs.cnrs.fr/ATOME_V3/SERVER/EDMon_v3.html)
4) Initial high throughput screening of chemicals for activation of key nuclear receptors
5) Developments dedicated to the augmentation of the CYP induction assay from pharmaceuticals to MDCs
6) Further development of *in vitro* assays and zebrafish in vivo model to assess metabolic disruption in liver, pancreas and adipose tissues, including creation of standard operating procedures and test definitions for pre-validation purposes
7) Development of research plans for human cohort analysis
8) Establishment of standardized protocols to be used by all GOLIATH partners for multi-omics (transcriptomics, metabolomics, lipidomics) analyses.
9) Development of AOP and IATA frameworks.
10) International outreach via EURION's International Advisory Board and various dissemination activities

Progress beyond the state of the art and expected potential impact

In order to progress beyond the state of the art, the expected results of the GOLIATH project are to:

- Generate a set of robust, well-characterised and ready-to-use in vitro test methods for the effects of MDCs on key target cells in metabolic disorders that will be (pre-)-validated and further implemented beyond the duration of the project
- Realize an integrated approach for testing and assessment of MDCs, that will exceed the duration of GOLIATH, by bringing together new and existing test methods, in a framework that will be internationally harmonised and relevant for regulatory purposes
- Describe systematically in an AOP framework the mechanisms by which MDCs disrupt metabolism and contribute to metabolic disorders in humans, thereby providing information on the endocrine mode of action which is essential for defining ED criteria
- Generate a significant knowledge base on the exposure to and effects of MDCs relevant to the European population, providing a substantial contribution to the weight of evidence for the role of chemicals in metabolic disorders

Further information about GOLIATH:
Website: https://beatinggoliath.eu
Cordis: https://cordis.europa.eu/project/id/825489
Twitter: @beating_goliath

2.7 OBERON project summary

An integrative strategy of testing systems for identification of EDs related to metabolic Disorders - OBERON

Summary of the context and overall objectives of the project

Despite the high incidence of metabolic diseases, most available mammalian in vivo assays do not address ED-related mechanisms of action, and the specific endpoints associated with metabolic disorders are missing. Furthermore, metabolic pathway dysregulations are not only involved in the development of metabolic disorders as such (diabetes, obesity, etc.), but they are also critical for the development of a number of other outcomes such as cancer, immune diseases, and neurological diseases. Thus, the availability of relevant tests for metabolic dysregulation may have impacts that are much wider than those related to metabolic diseases. The OBERON project aims at providing a series of new effective and validated tests, which are essential for industries and regulatory needs, to support risk assessment of putative ED chemicals in relation to metabolic disorders. By its multidisciplinary strategy and the establishment of an integrated approach for testing and assessment (IATA), the ultimate aim of OBERON is to provide more accurate predictive testing by developing a battery of novel, easy to use, effective and validated test systems, combining different experimental and computational strategies. The battery will include the most relevant developed/improved test systems in a decisional tree and should ultimately be used for regulatory purposes regarding ED assessment in relation to metabolic disorders. In order to develop such a test system, the OBERON vision relies on a multidisciplinary approach supported at all stages by data analysis and computational studies. The different tests will be built on 'realistic exposure to chemical substances' using data from well-characterized on-going epidemiology and human biomonitoring studies (biomarkers concentrations in cord blood, peripheral blood or urine samples) as well as the literature.

OBERON tests will be developed based on a panel of ten EDs, selected because of their widespread use, potential toxicity and production in large quantities worldwide (millions of tons annually). Most of them are of regulatory interest in Europe (e.g., BPA, di(2)ethylhexyl)phthalate (DEHP)) and others are well-known EDs that can be used as "known-controls" (heavy metals, dichlorodiphenyldichloroethylene (DDE)). Substituents to these compounds have also been selected (e.g., BPS and BPF) because, although they have been considered safer than the original compounds, they have in fact similar properties and their health effects are still largely unknown.

Data generated within OBERON, combining cell biology, zebrafish, omics technologies, and systems biology, will allow improving knowledge on EDs. Novel mechanistic information will be established, allowing the development of uncharacterized adverse outcome pathways (AOPs), as well as AOP networks, and identification of novel biomarkers to improve risk assessment frameworks for human health effects. To reflect a 'real life' exposure, birth cohorts and other epidemiological studies' information will be used to determine a set of case studies that will be applied to a set of new easy-to-implement combined complementary methods (in vitro, in vivo, in silico). Therefore, to reach its aims and establish an innovative of integrated approach for testing and assessment (IATA), OBERON has identified several objectives: 1) Integration of Epidemiology and Human Biomonitoring Studies with ED Test Systems for Metabolic Disorders; 2) Development of Whole Organism Test Systems to Identify EDs Implicated in Metabolic Disorders; 3) Development of Human-Relevant In Vitro Test Systems to Identify EDs Involved in Metabolic Disorders; 4) Providing Computational Models to Help Prioritization of EDs; and 5) Establishment of an ITS and Capture of Mechanistic Effects of EDs on

Metabolic Disorders.

Overview of progress and main results achieved so far

During the first period, and taking advantage of the expertise diversity of the 11 partners from the OBERON project we started the establishment and harmonization of several experimental tests across the different EU laboratories (in vitro and zebrafish). Some experimental protocols have been already produced for this purpose. For human studies, data compilation was performed for the ten studied EDCs, for different age groups and time periods, by sex and country from the different European biomonitoring studies and population based cohort studies, that will serve to characterize EDC concentrations to be used for in vitro and in silico studies. Moreover, some chemical measurements have started for two French cohorts and follow-ups have been initiated for two adult cohorts. Regarding in silico modeling, a review paper on human pregnancy PBPK models was published (Codaccioni et al. Comp.Tox. 2019;12:100111).

An important activity in OBEON is the data infrastructure and bioinformatics, as many data will be generated. All bioinformatics results will be used for computational modeling to create the final tiered tests, and to develop new adverse outcome pathway (AOPs) for metabolism. We created an internal working group dedicated to omics studies as these ones will be performed across the different WPs and activities (human, in vitro and zebrafish). By now we are working on the design of a pilot study that is to decide how many samples will be tested, which type of sample, etc. A computational network science-based model was also established, using existing knowledges, which was published (Taboureau et al. Toxicol. Appl. Pharmacol., 2020; 405:115210).

During this first period, expected deliverables including ethics were achieved. An introductory OBERON project publication was done (Audouze et al. Int. J. Mol. Sci. 2020; 21(8):2988), a website (https://oberon-4eu.com/), two leaflets (Presentation of Oberon, Why should we assess metabolic effects of endocrine disruptors?, both available on https://oberon-4eu.com/), various national press-releases, and social media (@OBERON_4EU) were also achieved for dissemination of OBERON.

The COVID-19 crisis slowed down the full project, but we took the opportunity to explore endocrine disrupting chemicals and COVID-19 relationships by a computational systems biology approach (Wu et al. Environment International. 2020:106232).

Progress beyond the state of the art and expected potential impact

In epidemiological studies on the metabolic effects of EDs, we will not only use traditional phenotypic markers but also a set of relevant molecular markers to improve the links between exposure and effects. A variety of non-animal experimental approaches with a large set of biological outcomes will be integrated using computational approaches to further improve the value and predictive capacity of these sets of tests. Improving the quality of the testing strategy will prevent the dissemination of toxic compounds and will have health, social and economic benefits.

Further information about OBERON:
Website: https://oberon-4eu.com/
Cordis: https://cordis.europa.eu/project/id/825712/fr
Twitter: @OBERON_4EU

2.8 SCREENED project summary

A multistage model of thyroid gland function for screening endocrine-disrupting chemicals in a biologically sex-specific manner - SCREENED

Summary of the context and overall objectives of the project

Some chemicals used to produce materials commonly found in everyday life, such as plastics, tin cans, cosmetics, pesticides, among others are in a class of chemicals known as endocrine disruptors (EDs). EDs are not without danger: these molecules interfere with the endocrine system, disrupting the physiological production and the target effects of hormones. In particular, EDs have proven effects on the reproductive system and impact on the occurrence of obesity, type 2 diabetes and cardiovascular diseases during aging. There is also a growing evidence that EDs interfere with the functioning of the thyroid. EDs cause changes in thyroid hormone concentrations, the peripheral metabolism of these hormones and signalling by their receptors. The mechanism by which they act on the thyroid axis is, however, still far from clear. Current tests to measure the effect of EDs on the thyroid are limited by the availability of adequate quantities of human thyroid tissue, the authenticity with which current thyroid model systems (cells in culture) recapitulate the complexity of the native gland and by the inability to measure or predict the effects of EDs after low dose exposure. The EU project SCREENED aims to develop three-dimensional (3D) cell-based *in vitro* tests to better characterize the effects of EDs on the thyroid. The solutions SCREENED will develop will overcome the limitations of existing tests including by being more sensitive at low doses of exposure to potential EDs and supporting the prediction of their toxicity on human health in a sex-specific manner.

Overview of progress and main results achieved so far

During the project's initial 18 months, we designed and fabricated the first working bioreactor prototypes. Current prototypes enabled culture of enriched thyroid follicles in a microphysiological setting that closely resembles the native tissue. Optical sensors were successfully integrated into the bioreactor housing to measure and monitor oxygen levels throughout culture. We have improved our stem cell derived thyroid functional *in vitro* model using embryonic stem cells to allow it to be an efficient tool to test the EDs effect on the function of the thyroid gland.

Five decellularization protocols suitable for removal of all cell types from the adult male and female, rat thyroid gland have been developed without hampering the main structural and geometrical 3D organisation of the thyroid lobe matrix: each showing a preferential retention of specific matrix molecules (collagen types, structural proteins, growth factors, glycosaminoglycans). In addition, isolation, expansion, enrichment and characterisation of adult male rat, thyroid stem cells / progenitor (TSC /P) using a simple, reliable and reproducible methodology has been achieved, based on long-term, semi-starvation low-density monolayer subcultures.

We also focused on the optimisation of microfluidic bioprinting for the generation of the bioprinted thyroid model. We evaluated different hydrogel based bioink formulations to assess their printability and the parameters for an optimal bioprinting in terms of fibre dimension and deposition. We verified that bioprinted cells remain viable and metabolically active inside the construct. We also increased bioprinted constructs' robustness by post crosslinking the construct with a $CaCl^2$ base solution. Finally, we exploited a new core-shell bioprinting technique that allowed to produce hollow fibers containing thyroid and endothelial cells at the same time. As a further method to provide better control of cell spatial positioning

for the 3D cellular assays to be developed in SCREENED, we also optimized a method to magnetize cells while maintaining a high viability.

The current lack of available proteomic and transcriptomic information on the thyroid drove us to initiate the first combined proteomic and transcriptomic analysis of ED effects on (non-cancerous) thyroid cells, using state of the art technologies to identify thousands of gene and protein thyroid signatures. Preliminary data on recent research on the *in vivo* effects of a mixture of polychlorinated biphenyls on the hypothalamic-pituitary-thyroid (H-P-T) axis of adult male rats have provided groundwork for eventual development of theoretical modelling of *in vivo* EDs action on the rat thyroid gland.

Progress beyond the state of the art and expected potential impact

One of the project aims is to focus on the development of reversibly sealed microfluidic bioreactors for long-term culture of thyroid cells. Our bioreactors are expected to enable prediction of thyroid disruption while being compatible with high-throughput platforms, which are two major requirements for industrial applicability. As such, the bioreactors have the potential to advance the organs-on-chip field and foster its adoption by the pharmaceutical industry to reduce or even replace animal models in pre-clinical testing for drug screening and drug discovery. The establishment of a human thyroid *in vitro* model also constitutes a major breakthrough in the field of thyroid research and will be extremely useful to study thyroid gland diseases. These models bring a fast, cheaper and animal-free alternative to screen a list of EDs toxic effect, using a system that resembles what happens in humans. Changes in matrix composition due to different decellularization protocols, that we develop, provide different molecular environments ideal to test the role of different matrix proteins in driving differentiation of seeded adult TSC/P from either male or female rats. Differential and sex-specific matrix composition may represent a reference for eventual bioprinting of matrix replica as engineered surrogates of native matrices in a developing microbioreactor. In addition, evidence that adult male TSC/P can give rise to clonogenic colonies with molecular markers of native TSC/P enlighten their usefulness as an innovative experimental tool for developing 3D functional thyroid organoids to be used for the project purposes, promising to be of relevant commercial impact in the field of *in vitro* assays.

We also produced a bioprinted 3D model able to mimic the thyroid architecture and functionality. The main models currently available for thyroid studies are based on 2D cell cultures or on the use of animal models. Either of these two solutions do not represent the human thyroid complexity. With bioprinting, we could produce a 3D model able to closely mimic the human thyroid and to offer different advantages over the classical 2D model. At the same time, the bioprinted model could reduce the use of animal models for drug screening and discovery purposes, while offering a new platform for studying thyroid physiology and physiopathology. The potential addition of magnetized cells as a further method to control cell spatial deposition in 3D would offer the capacity to precisely locate thyrocytes and endothelial cells in the 3D cellular assays of SCREENED. This method would allow an exquisite control in organoids and decellularized extracellular matrix constructs, as well as a further level of spatial control in bioprinted constructs.

Since there is no existing state of the art for (normal) thyroid cell transcriptomics and proteomics, we will establish a baseline transcriptome and proteome of human thyroid cells and the impact of ED's on them. By the end of the project we expect to be able to comprehensively collect and combine comparative and reliable data on the effects of a list of EDs on the in vitro 3D thyroid bioprinted systems developed by SCREENED for comparison with *in vivo* data from rodent models (male and female) used as a control system that includes the entire H-P-T axis.

Further information about SCREENED:
Website: https://www.screened-project.eu/
Twitter: https://twitter.com/ScreenedH
LinkedIn: https://www.linkedin.com/in/screened-project-8625611b4/
Cordis: https://cordis.europa.eu/project/id/825745

3. Overview of test methods to be developed

The EURION projects are developing and (pre-)validating methods and novel tools for better hazard identification of chemicals, focusing on thyroid system, developmental neurotoxicity, metabolic effects and female reproduction.

Table 1: Overview of test methods to be developed

	ATHENA	EDCMET	ENDpoiNTs	ERGO	FREIA	GOLIATH	OBERON	SCREENED
Definitive tests								
Addition of:								
Molecular readouts to existing test guidelines			x	x		x		
Endpoints to existing test guidelines	x			x	x	x		
More sensitive endpoints to existing test guidelines	x		x	x	x	x		
Biomarkers to existing test guidelines	x		x	x	x	x		
Screening tests								
Cell-based assays	x	x	x	x	x	x	x	x
Cell membrane transporters assays	x							
3D cell models							x	x
Stem cell assays	x		x			x		x
Reporter gene assays	x	x	x	x	x	x		
Non-cellular assays		x						
In vitro biotransformation				x				
Whole organism embryo		x	x	x	x	x	x	
Computational tools for screening (QSAR or other)	x	x	x	x	x	x	x	
Blood cerebrospinal fluid barrier (BCSFB) assay	x							
Blood brain barrier (BBB) assay	x							
High throughput assays	x	x	x	x	x	x	x	x
Molecular mechanism based assays (omics readouts)		x	x	x		x	x	x

4. Cluster highlights

The European Cluster to improve Identification of Endocrine Disruptors, EURION, was launched by DG Research and Innovation in Brussels, Belgium, 31 January 2019 with representatives from the eight projects as well as the International Advisory Panel (IAP) and observers from various EU agencies. GOLIATH and OBERON chaired the cluster for the first 15 months and organised bi-monthly virtual meetings with the project Coordinators, as well as representatives from DG-RTD and JRC. The chairmanship activities included the formation of thirteen different working groups (WG) with representatives from each of the projects in each WG. These WGs have met multiple times in the first 18 months to establish collaboration between projects.

Further EURION activities have included liaising with the IAP, with the goal of (i) providing a bridge to other European and international initiatives and regulatory bodies; (ii) bringing understanding of international stakeholder needs, issues, concerns, drivers, and opportunities; (iii) providing independent assessment and feedback on the regulatory relevance of the test methods (under the different regulatory frameworks); (iv) promoting communication with main stakeholders in the field. In October 2019, GOLIATH and OBERON organized a first virtual meeting with the IAP. The purpose of the meeting was to discuss first achievements of the projects and introduce the cluster working groups.

As cluster coordinators, GOLIATH and OBERON organised the annual cluster meeting in Paris, France, 5 February 2020, with around 170 representatives, including project participants, IAP members, and stakeholders from public and private organisations. In addition to disseminating EURION to the outside world (e.g. through a EURION video and fact sheet), the cluster meeting facilitated face-to-face meetings with participants in the working groups, promoted collaboration and sharing of knowledge between the projects. The cluster meeting was preceded by a workshop (4 February 2020) on the topic of (pre)validation, organized by JRC and supported by GOLIATH and OBERON.

In April 2020, the coordination of the cluster was transferred to ENDpoiNTs and ERGO which have continued cluster activities according to the mandate, including the bi-monthly coordinator meetings.

Some key achievements of the Cluster include so far:

- Publication of all eight projects in the International Journal of Molecular Sciences special Issue “Advances in the Research of Endocrine Disrupting Chemicals 2.0” (https://www.mdpi.com/journal/ijms/special_issues/EDC_2).
- Instigating stakeholder interaction, including launch of an initial survey of stakeholders’ interests, needs and priorities in relation to the development of endocrine disruptor (ED) test methods.
- Achieving synergies in rodent studies, including tissue sharing between projects, resulting in fewer animals being used and thus contributing to the principles of 3Rs (Replacement, Reduction and Refinement).
- Providing general Adverse Outcome Pathway (AOP) training to all EURION projects.

5. Working groups

Since the start of the EURION Cluster, 13 Working Groups (WGs) have been established, each with a special thematic focus in accordance with the cluster mandate to optimise synergies and avoid overlaps between the projects. The WGs have leaders from the eight projects as well as from the Joint Research Centre, JRC.

The EURION Cluster WGs are:

- ADVERSE OUTCOME PATHWAYS (AOPS) WG
- ANIMAL STUDIES: AQUATIC ORGANISMS WG
- ANIMAL STUDIES: RODENTS WG
- CHEMICALS WG
- DATA MANAGEMENT WG
- DISSEMINATION AND COMMUNICATION WG
- EPIDEMIOLOGY WG
- IN SILICO WG
- INTEGRATED APPROACHES TO TESTING AND ASSESSMENT (IATA) WG
- IN VITRO MODEL STUDIES WG
- OMICS WG
- REGULATORY AFFAIRS AND POLICY (RAP) WG
- VALIDATION WG

5.1 ADVERSE OUTCOME PATHWAYS (AOPs) WG

AOPs (WG chair: Dries Knapen, ERGO)

Purpose and objectives of the WG:

- To support and facilitate AOP-related activities in the EURION projects by
    - Providing general AOP training
    - Organising discussion groups and meetings focused on specialised topics
- To bring together AOP-structured information and data across projects by
    - Providing generic templates for data entry and sharing
    - Organising AOP development workshops

Progress to date: WG membership has been established and the mission and overall planned activities of the WG have been defined. Two teleconference meetings have taken place and the AOP WG convened at the EURION annual cluster meeting in Paris. A survey to identify AOP training needs within EURION projects was carried out on which the AOP training scheme for 2020 has been based. Three introductory AOP training webinars have taken place. The training webinars were well attended, and recordings have been made available for those who were unable to attend. Using the EURION-wide Microsoft Teams infrastructure, a dedicated AOP WG channel including file sharing capabilities has been made available for future AOP development. Overall, the WG current and planned activities are anticipated to stimulate collaborative AOP development which could significantly improve the application of results obtained in EURION.

Activities in near future: The EURION AOP training webinar series will be concluded in the fall with one or two final webinars that will include time for Q&A.
In 2021, an AOP workshop will be organised (face-to-face or online, depending the evolution of the coronavirus pandemic) and aligned with the 2021 EURION cluster meeting. The workshop will consist of two parts:

a. Advanced AOP training
b. Collaborative AOP development (entering data into the AOP-wiki, etc.)

5.2 ANIMAL STUDIES: AQUATIC ORGANISMS WG

Animal studies: aquatic organisms (WG chair: Lisa Baumann, ERGO and Jessica Legradi, ENDpiNTs)

Purpose and objectives of the WG:

- Insight in models and outcomes used across projects
- Share and compare protocols and chemicals
- Explore cross-project collaboration in ring studies

Progress to date: This working group has had two meetings in the first year of EURION: on May 24, 2019, and September 24, 2019. The first meeting was of an introductory nature, in which we shared information on our models and our goals within each of our projects. On the second meeting, we shared protocols and information on test chemicals, and discussed the possibility to select one chemical to be used by all partners, in order to integrate endpoint measurements across projects for a common publication on zebrafish: neurotoxicity, thyroid hormone disruption, steatosis, adipogenesis, insulin regulation, etc. We did not meet at the EURION cluster meeting in February 2020 due to limited time and space. Plans to meet after February 2020 were put on hold due to other priorities following COVID-19 lockdown.

5.3 ANIMAL STUDIES: RODENTS WG

Animal studies: rodents (WG chair: Julie Boberg and Majorie van Duursen, FREIA)

The WG objectives are to:

- Share experience and expertise on *in vivo* rodent studies,
- Share tissues among the EURION projects and beyond, whenever possible.

Progress to date: The WG has held three teleconferences (04/2019, 07/2019, 06/2020). In the first meeting, the objectives of the WGs were discussed and people introduced their projects. All participants agreed that the added value of this working group initially lies in sharing of experience and expertise as well as the opportunity to share tissues among projects. As projects proceed, more in-depth discussions will be held focusing on a specific project and its outcomes. The second meeting was held to discuss the table with project information. At the TC with the EURION IAP (11 October 2019), an update of the WG activities was presented by the WG chair. At the EURION cluster meeting (5 Feb 2020), a break-out session was dedicated to this WG and all projects gave a brief update. Several members of the IAP attended this break-out session. The next WG meeting was held in June 2020, to update each other on the project progress and any derivations from the planning due to the COVID-19 pandemic. A table was drafted in which all participants have added details on the planning of the experiments and design (regularly updated via Teams. The table also denotes tissues that are not primarily of use for the project who performs the study, and thus can potentially be used by other EURION projects and beyond. Already tissues have been shared among projects, e.g. ovaries and uteri from ENDpoiNTs were harvested for FREIA. Livers from FREIA have been sent to partners in EDC-MET project.

5.4 CHEMICALS WG

Chemicals (WG chair: Pim Leonards, ENDpoiNTs)

The purpose and of objectives of the WG Chemicals are:

- to provide an actual overview of EDCs studied by the projects,
- to provide an overview of EDCs used by multiple projects, and
- to exchange information on the analytical quality and protocols of the studied EDCs.

Progress to date: The WG has updated the list of chemicals tested in the cluster (currently n=115). Twenty-two chemicals are tested in multiple projects and for these chemicals, information on the quality of test chemicals has been collected and the companies and batches of the highest quality have been indicated. The aim is to use the same chemical batch for all projects in the pre-validation phase.

Activities in near future: The WG chemicals is continuously collecting information on the stability of the test chemicals, and protocols for preparation of bioassay solutions.

5.5 DATA MANAGEMENT WG

Data Management (WG chair: Anna-Liisa Levonen, EDCMET)

Purpose and objectives of the WG:

- To discuss and evaluate data management solutions and approaches, including data documentation and databases
- To provide templates and/or instructions how to make data findable and interoperable
- To provide support in data management for projects and aid in the development of project specific data management plans
- Integration and harmonization of data infrastructure of EURION – EURION data management roadmap for environmental exposure /ED health effects

Progress to date: The working group has had two teleconferences in 2019 and one face-to-face meeting during the yearly cluster meeting in Paris 2020. Current status of data management and DMPs of projects, sharing of data and use of common databases as well as the functions of the working group have been discussed in the meetings.

Activities in near future: Next teleconference for further discussions on the working group functions and plans will be organized after all projects have shared their DMPs through the EURION collaboration platform, to facilitate further discussions on the functions of the working group. Participants from other working groups (especially *in silico* and *omics*) will be invited to discuss their data sharing and management approaches.

5.6 DISSEMINATION AND COMMUNICATION WG

Dissemination and communication (WG chair: Cluster chairmanship team)

Purpose and objectives of the WG:

- Promote the activities and results beyond EURION to regulators, scientific community, policy makers and the general public, employing a range of communication and dissemination tools;
- Ensure timely and efficient knowledge management and sharing, while safeguarding that suitable IP management strategies and processes are applied to EURION;
- Capture key messages and outcomes, based on knowledge generated through EURION to ensure effective sharing of knowledge outputs to end users;
- Maximise post-project uptake by developing thorough and forward-thinking plans that clearly outline the potential end-users of the project. Optimise post-project uptake by knowledge transfer activity required to ensure objective and measurable short and long-term impacts of the cluster.

Progress to date:

- A EURION joint Communication & Dissemination Plan has been elaborated and agreed upon from all of the EURION project coordinators.
- The EURION website has been launched. The website is being monitored with an ongoing update and development of features and content.
- A Twitter account for EURION has been launched
- Joint visual identity had been developed and launched:

    - Logo & brand guidelines
    - Template for documents and presentations
    - Template for documents and presentations
- The cluster leaflet had been created and launched
- A stakeholder list for communication/dissemination (via a 'subscribe to news' button on the EURION website)

Activities in near future: For the ED Forum 2020 to be arranged by the European Commission, EURION will be organising a 1-hour session at the forum.

5.7 EPIDEMIOLOGY WG

Epidemiology (WG chair: Carl-Gustaf Bornehag, ENDpoiNTs)

The purpose and objectives of the Epidemiology WG, EWG: The overall aim is to compile information on epidemiological studies in the cluster, and more specifically:

- to coordinate the involved epidemiological studies within the cluster
- to establish a data management plan for how to share information/results from different epidemiological studies.

Progress to date: The group has had three meetings so far: EWG web-meeting, September 30th, 2019, EURION cluster web-meeting, October 11, 2019, and the EURION cluster face-to-face meeting in Paris, February 5th, 2020. In general, the group considers that it is highly important to collaborate between the studies and labs within the cluster, but also more specifically between different epidemiological studies. The group has also expressed the need to focus on exposures for which we do not have a lot of data and to look into both single substances and mixtures. Efforts are ongoing to see how data and results from different epidemiological studies could be shared.

Activities in near future: Another question to be decided upon is if epidemiological studies outside of Europe could be included, as well as HBM4EU. The EWG plans to have a web-based meeting during 2020, and hopefully a face-to-face meeting in the spring 2021.

5.8 IN SILICO WG

In silico (WG chair: Nick Plant, EDCMET)

Purpose and objectives of the WG:

- To share analysis approaches, intelligence around test chemicals etc. and to provide a platform for scientific discussion and inter-laboratory validation
- To generate joint publications exploring the utility of in silico methodologies for risk assessment within the ED space

Progress to date: The working group met during the EURION meeting in Paris (02/2020). Synergies (chemicals, techniques, expertise etc.), aims of the working group, sharing of data and approaches as well as possibilities for joint publications (e.g. review articles) were discussed.

Activities in near future: Gather information on existing methods and pipelines as well as the needs of different projects and data gaps throughout the cluster. Setting up a common glossary and establishing synergies (experimental data, integration etc.).

5.9 INTEGRATED APPROACHES TO TESTING AND ASSESSMENT (IATA) WG

IATA (WG chair: Miriam Jacobs, GOLIATH)

Purpose and objectives of the WG:
To build understanding of IATA development, and develop collaborative approaches amongst the EURION cluster members, IATA's are a main deliverable for all EURION projects.

- To review and understand existing IATA guidance
- To review and learn from i) case studies of relatively simple IATAs: e.g eye and skin irritation ii) case studies of more complex IATAs: Non-Genotoxic carcinogenicity
- To agree common understanding of terminology and applications
- To build harmonised bridges across the EURION projects

Progress to date: A first TC was held on 4 October 2019, with participation from GOLIATH, ERGO, FREIA, ATHENA, EDCMET and the JRC. The call began with an information exchange on projects, and relevant OECD IATA information links were discussed. It was acknowledged that IATA harmonisation for specific endpoints, are core to the success of IATA development and utility, and noted that IATA work will increase as projects develop their assays, it is too early in the project progress now. Commonalities were noted between the metabolic disruption projects in EURION, between the thyroid, DNT and brain with external IATA initiatives (e.g. EC DNT IATA work, EC thyroid validation, OECD thyroid scoping document). FREIA, is the only project focusing on female reproduction. The second TC was held on 2 December 2019 with participation from GOLIATH, OBERON, ERGO, FREIA, ATHENA and JRC. The objective was to become more familiar with the IATA guidance provided previously and to look at recent IATA development experience at the OECD: specifically, case studies on skin corrosion and irritation, eye corrosion and irritation, and skin sensitisation.

From the early experiences at the OECD there are several lessons that can be learned to help EURION harmonisation work:

- Lesson 1: Multitudes of different IATAs for the same endpoint are a minefield for the Mutual Acceptance of Data at the OECD.
- It is important to clarify and agree definitions: commonly understood starting points are crucial.
- Lesson 2: Conceptual development work first needs to be established the diverse views (all stakeholders) can 'live with'.
- Lesson 3: This takes time and lots of to and from discussion.

Whilst the early OECD IATA work has been focused on hazard identification and categorisation, using the Mode of Action methodology, this is evolving to use a more AOP structured approach with IATA development. Currently incorporation of exposure assessment /risk assessment is not so clear.

It was agreed that: This WG could be used to identify harmonisation commonalities between the EURION projects to facilitate uptake at the OECD level, this will probably come at later stages of the projects, after assay development work has progressed further. However, it is good to start building this early on. Furthermore, good communication is needed with the EURION AOP WG in particular to identify common nodes for AOP-IATA work (and there is an overlap in WG membership). At the EURION meeting in February

2020, the progress described above was reviewed and the chair strongly encouraged greater active collaboration between the consortia, to ensure that we all produce IATAs that can be taken forward for harmonised regulatory applications.

Activities in near future: A third TC will provide an opportunity for updates from EURION members on their IATA development work, so that synergies can start to be identified. There will also be a presentation and discussion on the more complex ongoing OECD IATA work on Non Genotoxic Carcinogenicity. Subsequent discussions will focus on establishing common definitions, and active collaborative work, particularly where there are project overlaps. It is likely that combined endpoint specific TCs with the EURION AOP WG will facilitate the progress.

5.10 IN VITRO MODEL STUDIES WG

In vitro models (WG chair: Lorenzo Moroni, SCREENED)

Purpose and objectives: The working group has gathered online and in person in the annual meeting of Paris 2020. Initial activities aim at generating a database of cells used in the different projects of EURION to individuate possible synergies and additional collaborations in the context of *in vitro* models for ED screening.

Progress to date: In the Paris meeting, a few important items were further discussed. As in tissue culture plasticware there are additives that are known to be EDs, attention has been raised by members of the group to make sure that this is considered while designing toxicology experiments. A protocol was shared with the WG to extract from cell culture plasticware most of such EDs before cell culture use. This will be applied as much as possible across EURION. Additional discussion was centred around the use of alternative supplements to culture media than Fetal Bovine Serum (FBS). For as much as all members of the working group agreed that this is a very important item, it falls beyond the tasks of each project of the EURION cluster, so it will not be practically implemented as many of the cellular assays currently under development would have to be completely redesigned in an *ad hoc* focused project targeting FBS alternatives.

5.11 OMICS

OMICS (WG chair: Karine Audouze, OBERON)

Purpose and objectives: The main objective is to harmonize the data in order to be able to use them across the different projects, and to establish an 'EURIONOMICS protocol' to have comparable data.
The OMICS working group led by K. Audouze (OBERON), includes 32 participants from six of the seven other ED projects (the project ATHENA does not plan any omics activity), and Jukka Sund, JRC will be part of the working group as EU representative.

Progress to date: A first meeting took place in June 2019 with 5 of the 8 projects. During that meeting, it appeared that various OMICS activities were planned (different technologies). Therefore, four sub-groups were set up in order to facilitate methodological discussions. A second meeting was held during the EURION annual meeting in Paris in January 2020, where all sub-groups had the possibility to discuss with all partners. Several email exchanges were done during the COVID-19 health crisis.

The 4 sub-groups (and subgroup leaders) are:

- metabolomics/lipidomics (F. Jourdan, GOLIATH)
- transcriptomics (F. Chalmel, FREIA)
- proteomics (S. Pennington, SCREENED)
- epigenetics (D. Sarigiannis, OBERON)

Development of a GitHub directory (https://github.com/orgs/EURION-OMICS/) has been set up to centralize the analysis pipelines developed and used by each EURION cluster member, independently of the technology used (transcriptomics, metabolomics, proteomics, etc.). The objective is both to capitalize on the experience of each team in data pre-processing and analysis, and to make these standardized procedures available for the community. This is a crucial step in producing FAIR data. A GitHub Guidelines document has been prepared and will be updated when necessary.

Activities in near future: A series of webinars and trainings is planned in 2020-2021. An online survey was sent to all participants from the WG omics, and K. Audouze has contacted external potential speakers from the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST) from the OECD. The first webinar is planned on November 13th, and it will focus on 'OMICS based approaches in toxicology for informing regulatory decisions'. Three speakers have agreed to present the current status on the standardization and harmonization of OMICS data reporting. J. Harill (US EPA) and C. Yauk (University of Ottawa) will introduce the transcriptomics reporting framework (TRF), and M. Viant (University of Birmingham, UK) will present the metabolomics reporting framework (MRF). Other webinars are expected to be run in 2021, including two e-training: F. Jourdan to present MetExplore, and F. Chalmet to present the TOXsIgN repository. Also, creation of a leaflet for 'non-OMICS' scientists is planned, with the participation of all projects, by the end of 2020, that will be available on the EURION webpage. Other issues are still under discussion such as data sharing between projects and data management (has to be discussed with other WG as the WG data management).

5.12 REGULATORY AFFAIRS AND POLICY (RAP) WG

Regulatory Affairs and Policy (WG chair: Andreas Kortenkamp, ATHENA)

The purpose and objectives of the WG: To elaborate proposals for improving the implementation of ED testing in relevant EU regulations and directives. This overall aim is broken down into several short-term and medium-term work steps, such as (but not limited to):

- How are triggers for further ED testing currently interpreted across regulations?
- Which OECD-validated *in vitro* and *in vivo* tests can / should be implemented in the short term to improve ED testing?
- Which methods should be implemented to capture ED in wildlife and non-target organisms (e.g. invertebrates)

In the long run, the group will also examine:

- How ED test data are used further, e.g. for establishing ADIs or TDIs
- How improvements in the EU can impact on ED testing beyond the EU

Progress to date: The group has held two meetings and agreed on a detailed work plan. The group is in the process of documenting current activities at European Commission level on updating testing requirements for EDCs. Contacts with the French PEPPER initiative have been made.

5.13 VALIDATION WG

Validation (WG chair: Sharon Munn, JRC)

Purpose and objectives of the WG:

- To provide a platform for cross-cluster discussion on issues relevant to validation through identification of issues and sharing of best practices
- To develop and provide relevant training

Progress to date: WG membership was established and the first teleconference took place in May 2019. The first validation training took place prior to the EURION cluster meeting on 4th February 2020 in Paris conducted by JRC. There were around 50 participants. The focus of the training was on establishing an assay in-house using the OECD guidance on Good *in vitro* methods Practice (GIVIMP) recently established as a template to follow with emphasis on good documentation and inclusions of all relevant parameters in the SOP to facilitate transfer of the method to another laboratory.
The process of validation leading to regulatory acceptance at OECD level was also presented and break-out groups facilitated discussion on the aspects presented in the plenary lectures.

Activities in near future: The aim is to hold the next teleconference before the end of the year to discuss further needs and future activities. The course evaluation feedback forms from the training will provide a useful starting point with respect to future training needs.

Contact

EURION Coordination 1 April 2020- 30 June 2021

Joëlle Rüegg (ENDpoiNTS), email: joelle.ruegg@ebc.uu.se

Henrik Holbech (ERGO), email: hol@biology.sdu.dk

Main editors of the report:

Elina Drakvik (ENDpoiNTs) and Helle Lyngborg (ERGO)

Design:

AquaTT