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Effect Based Methods for Monitoring and Assessment of Aquatic Ecosystems: Applications and Future Perspectives

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“I always believed, and I still believe, that whatever good or bad fortune may come our way we can always give it meaning and transform it into something value”

Herman Hesse

Short Summary

The thesis is about the application of Effect Based Methods (EBM) for the monitoring and assessment of aquatic ecosystems. This research approach has been developed in the last years and has the aim to contribute to identify potential chemical risks for aquatic ecosystems and indirectly for human health. The identification of these risks is a key aspect for the application of prevention and policy measures needed to protect environment and human health.

The Effect Based Methods (Bioassays in vivo and in vitro, biomarkers) have been recommended in the context of the Common Implementation Strategy of the European Water Framework Directive that is an ambitious European legislative act with the key aim to achieve a good status for all water bodies in Europe. EBM are tools used for the monitoring of waterbodies and other environmental compartments with the aim to detect effect caused by pollutants or group of pollutants, these effects can be detected at molecular, cellular, individual or populational level.

During the 3 years of the project an extensive evaluation of these methods in Europe has been carried out and the potentiality to be implemented in the European and national legislation has been highlighted. Furthermore the possibility to prevent indirect effects on human health has also been considered. A specific case study has been dedicated to the FET test (Fish embryo toxicity test) that is a bioassay widely applied in this field.

EBM have been applied experimentally in the Tiber river basin, mainly in the urban part, to identify the presence of potential effects caused by mixtures of chemical pollutants and/or emerging substances. These methods have been applied also to investigate the causes of a specific event that happened in 2020 and that has caused a massive fish kills in Tiber river.

In conclusion, based on the research carried out, these methods could be considered mature enough to be included in the legislative framework for the protection of water resources.

CHAPTER 1

Introduction and Legislative Background

General Introduction

Human wellbeing and economic prosperity depend on the sustainable use of ecosystems. Over the last decades there has been increasing emphasis both on the sustainable use of natural resources and on the recognition that humans are dependent on ecosystems for their well-being. This dependence extends beyond the resources provided by ecosystems (water, food, fibre, minerals, energy) to benefits such as climate regulation, flood control, pest and disease regulation, clean air and recreation (1).

The Millennium Ecosystem Assessment (2) drew attention both to the reliance of human well-being on ecosystem services and to the widespread degradation of ecosystems and the services they provide. The Ecosystems services are the direct and indirect contribution of ecosystems to human well-being. For example, more than 60% of the Earth's ecosystem services have been degraded in the last 50 years and in the EU, 88% of fish stocks are fished beyond maximum sustainable yields and only 11% of protected ecosystems are in a favourable state (3).

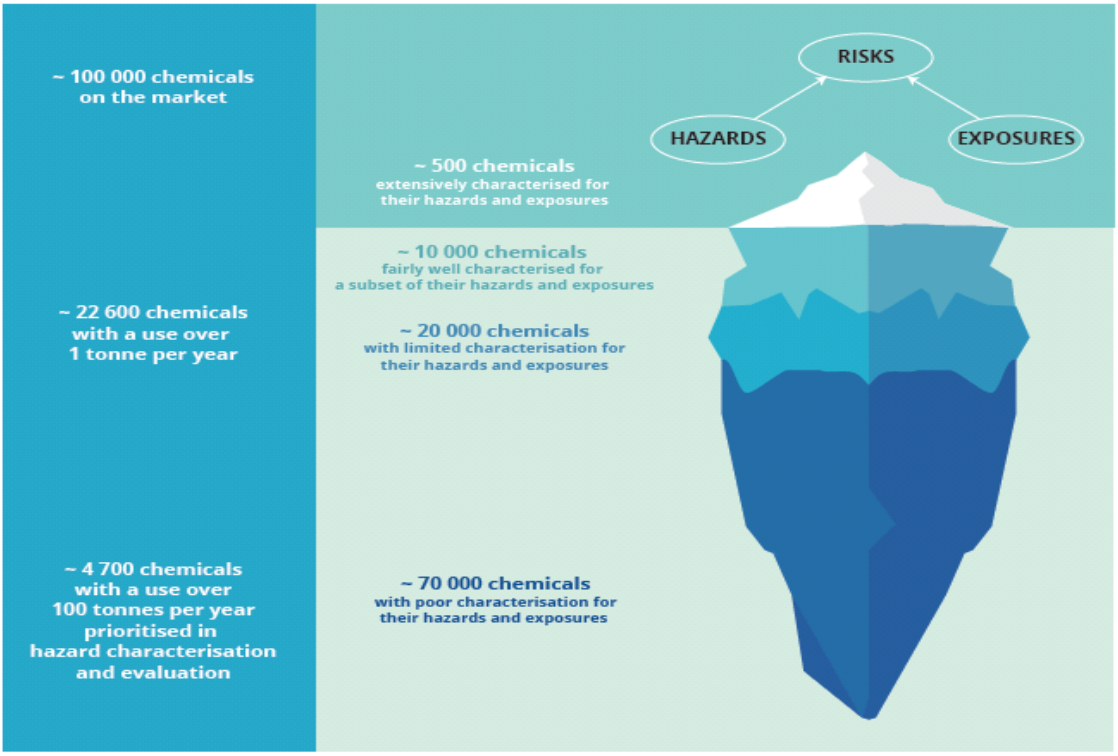
The EU is implementing a number of policies to enhance the sustainable use of natural resources and halt the degradation of ecosystem services. EU policies and guidelines for chemical environmental risk and impact assessment have consistent, high-level, aspirational goals for protecting the environment as a whole, including ecosystem structure.

The European Green Deal for a new growth strategy (4), has set the EU on a course to become a sustainable climate neutral and circular economy by 2050. It has also set a goal to protect better human health and the environment as part of an ambitious approach to tackle pollution from all sources and move towards a toxic-free environment.

Chemicals with hazardous properties can cause harm to human health and the environment. While not all hazardous chemicals raise the same concerns, certain chemicals cause cancers, affect the immune, respiratory, endocrine, reproductive and cardiovascular systems and increase vulnerability to diseases. For this reason the European Chemicals strategy for sustainability (4) will be based on protection of health and environment and innovation.

The EU is still lacking a comprehensive information base on all substances placed on the market and on their overall environmental footprint, including their impact on climate, and this hinders the proper management of chemicals and products and does not allow for a full sustainability assessment. Monitoring the presence of chemicals in humans and ecosystems is key to improve the understanding of their impact, and should be further promoted.

Figure 1: the unknown territory of chemical risk, EEA (5)



In particular the strategy mentioned that people and other living organisms are daily exposed to a wide mix of chemicals originating from various sources. Significant progress has been made in recent years to close some knowledge gaps on the impact of the combination effect of those chemicals. However, the safety of chemicals in the EU is usually assessed through the evaluation of single substances, or in some cases of mixtures intentionally added for particular uses, without considering the combined exposure to multiple chemicals from different sources and over time (6).

To adequately address the combination effect of chemical mixtures, legal requirements need to be consistently in place to ensure that risks from simultaneous exposure to multiple chemicals are effectively and systematically taken into account across chemicals-related policy areas. As it is currently not realistic nor economically feasible to specifically assess and regulate an almost infinite number of possible combinations of chemicals, scientific consensus is emerging that the effect of chemical mixtures needs to be taken into account and integrated more generally into chemical risk assessments. In parallel, targeted methodologies could be further developed and explored for specific policy areas.

The Thesis is about the application of Effect Based Methods (EBM) for the monitoring and assessment of aquatic ecosystems. This research approach has been developed in the last years and

has the aim to contribute to identify potential chemical risks for aquatic ecosystems and indirectly for human health. The identification of these risks is a key aspect for the application of prevention and policy measures needed to protect environment and human health.

The Effect Based Methods (Bioassays in vivo and in vitro, biomarkers) have been recommended in the context of the Common Implementation Strategy of the European Water Framework Directive (7) that is an ambitious European legislative act with the key aim to achieve a good status for all water bodies in Europe. During the 3 years of the project an extensive evaluation of these methods in Europe has been carried out and the potentiality to be implemented in the European and national legislation has been highlighted. Furthermore the possibility to prevent indirect effects on human health has also been considered. A specific case study has been dedicated to the FET test (Fish embryo toxicity test) that is a bioassay widely applied in this field.

Bioassays have been applied experimentally in the Tiber river basin, mainly in the urban part, to identify the presence of potential effects caused by mixtures of chemical pollutants and/or emerging substances. The Tiber river has been chosen in this research because has been selected as European pilot river basin with the aim to implement of the Water Framework Directive (Art.5) and leading to the development of river basin management plans (8).

Figure 2: Network of European Pilot River Basins in the context of the Common Implementation Strategy of the Water Framework Directive

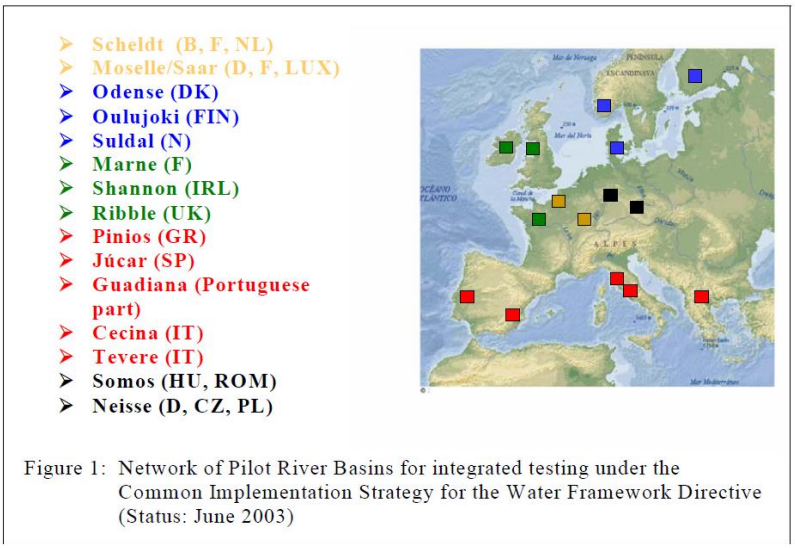


Figure 1: Network of Pilot River Basins for integrated testing under the Common Implementation Strategy for the Water Framework Directive (Status: June 2003)

The Water Framework Directive -A milestone for water protection

The European Water Framework Directive (WFD), which was published in the Official Journal of the European Community on 22 December 2000, is probably the most significant legislative instrument in the water field to be introduced on an international basis for many years. The WFD is based on an "Ecosystem approach", it means that the *Environmental management is based on the best understanding of the ecological interactions and processes necessary to sustain ecosystem composition, structure and function"* (9).

Figure 3: Sources of Pollution of a river basin (EEA 2018).

Source: EEA.

The Directive aims to achieve and ensure a "good ecological and chemical status" of all waterbodies throughout Europe by 2027 through the updating and implementation of management plans at the river basin level. The implementation is based on a Common Implementation Strategy (CIS) that involves all member states and stakeholders, included scientist, it has the aim to work towards a successful implementation of the core legislation on water at EU level. The main objectives of the CIS (Figure 4) are to ensure a better implementation of the water legislation and also to promote the integration of water-related issues in other environmental policies, as well as in other sectoral policies such as agriculture, transport or energy. Furthermore, as the River Basin Management Plans and Flood Risk Management Plans are key instruments for water management

in the EU, they are very relevant to the implementation of the commitments taken in the framework of the Sustainable Development Goals of the UNEP.

Figure 4: The CIS (Common Implementation Strategy) structure in 2019-2021

The WFD is based on an integrated approach to the monitoring and assessment of the quality of surface waterbodies.

The WFD requires three monitoring programmes for the chemical substances (10):

Surveillance monitoring: to supplement and validate the impacts analysis, to support the efficient and effective design of future monitoring programmes and to assess long-term changes in natural conditions and changes resulting from anthropogenic activity. The monitoring is performed at least once every management cycle (usually every 6 years).

Operational monitoring: to establish the status of those waterbodies identified as being at risk of failing to meet the WFD environmental objectives and to assess any changes in the status resulting from the programmes of measures.

Investigative monitoring: to determine the reasons for exceedances or predicted failure to achieve environmental objectives if the reasons are not already known and to determine the magnitude and impacts of accidental pollution.

The assessment of ecological status takes into account the effects at population and community level, based on the use of specific indices and ecological quality ratios. Good ecological status is defined in terms of the values of the biological quality elements (phytoplankton, macroalgae, angiosperms, benthic invertebrate fauna and fish), the hydrological and morphological conditions and the physico-chemical elements. Good ecological status (or potential) requires that the concentrations of the specific pollutants (also called river basin specific pollutants) do not exceed the environmental quality standards (EQSs) set at member state level. There is an indicative, not exhaustive, list in Annex VIII of the WFD of possible specific pollutants, which includes a wide range of substances and groups of substances that can often be detected in surface waterbodies.

The chemical status assessment and classification are based on the compliance with legally binding European environmental quality standards (EQSs) for selected chemical pollutants (priority substances) of EU-wide concern. EQSs for priority substances are set in the Directive 2008/105/EC, recently amended by the Directive 2013/39/EU (11).

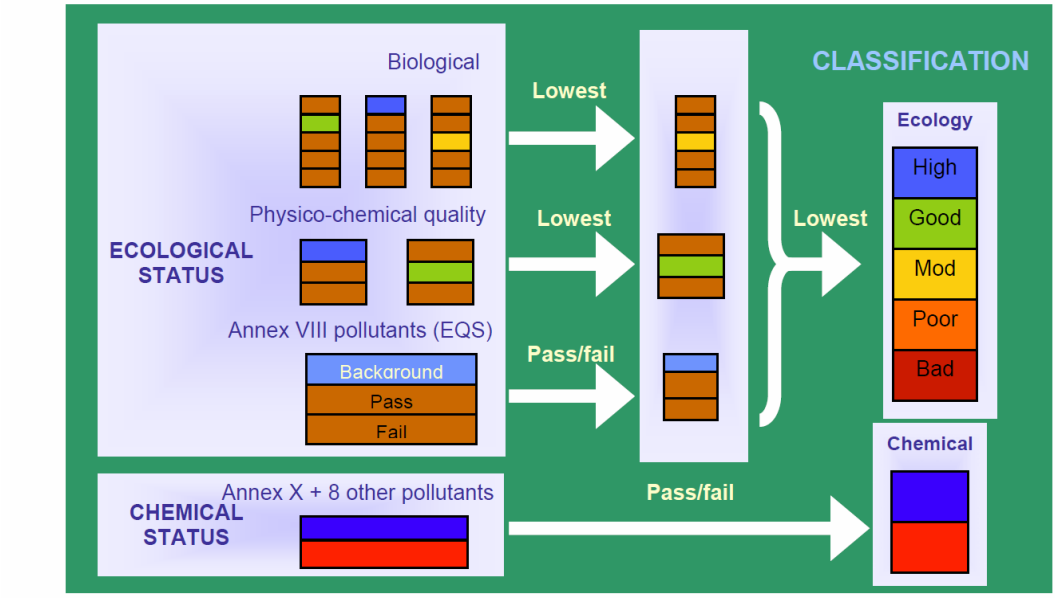
The EQSs (Figure 3) are designed to protect the environment and human health. Two types of water column EQSs are included for each priority substance:

• The annual average (AA) value or concentration of the substance concerned calculated over a one-year period: the purpose of this standard is to ensure the long-term quality of the aquatic environments. • The maximum allowable concentration (MAC) of the substance: the purpose of this second standard is mainly to limit short-term pollution peaks.

The EQSs are integrative limits designed to protect the aquatic environment (pelagic and benthic organisms), human health through dietary intake of fish and seafood or drinking water as well as birds and mammals that are exposed through aquatic food webs (“secondary poisoning”). In some cases, drinking water protection can also be the main driver of a water EQS, and applied for water bodies that are used for drinking water extraction. In Italy the Directive has been transposed with the legislative decree n. 172/2015 (12).

The methodology used to establish such EQSs for water, biota and sediment is described in detail in the TGD CIS guidance 27 (13). Sediment EQSs can be established by individual Member States and aim at protecting benthic organisms from substances accumulating in sediment. Biota EQSs are established when the main driver is to protect human health (when exposed to substances in fish and seafood) and/or predators (e.g. fish-eating birds) from risks of secondary poisoning from substances accumulating in prey (e.g. Mercury).

Figure 5: Use of EQS for Classification of the chemical and ecological status of surface waterbodies in the Water Framework Directive.



The Directive 2013/39/EU includes a revised (second) list of priority substances and provisions to improve the functioning of the legislation: the number of priority (and group of priority) substances is currently 53. The priority substances are selected on the basis of the procedure described in article 16 of the WFD that takes into account monitoring and modelling data, toxicological and ecotoxicological properties.

The Watch-List-How to deal with Emerging Contaminants

The monitoring data collected from Member States, although significantly improved over the past years, are not always fit-for-purpose in terms of quality and territorial coverage on European Union. Monitoring data are particularly lacking for many emerging pollutants, which can be defined as pollutants currently not included in routine monitoring programmes at EU level but which could pose a significant risk, depending upon their potential ecotoxicological and toxicological effects and on their levels in the aquatic environment. The Directive 2013/39/EU mentions that a new mechanism is needed to provide the Commission with targeted high-quality monitoring information on the concentration of substances in the aquatic environment, with a focus on emerging pollutants and substances for which available monitoring data are of insufficient quality for the purpose of risk assessment. In order to maintain monitoring costs at reasonable levels, the mechanism should focus on a limited number of substances, included temporarily in a watch list, and a limited number of

monitoring sites but should provide representative data that are fit for the purpose of the EU prioritisation process. The watch list must have a limited number of such substances and monitoring them EU-wide for up to 4 years. For the member states, the number of monitoring stations can be variable (in Italy, e.g. the total number will be 20). Frequent reviews of the list will ensure that substances are not monitored longer than necessary and that substances for which a significant risk at EU level is confirmed are identified as candidate priority substances with as little delay as possible. The first watch-list (14) has been published in 2015 (Figure 5) and includes pesticides, pharmaceuticals, anti-oxidants, suncreams, industrial chemicals.

The status of European waterbodies based on the WFD approach

In 2018 the EEA (European Environmental Agency) has published 2 report (15,16) about the implementation of the Water Framework Directive and on the quality status of European waterbodies; the results are based on the second river basin management plan adopted by the Member States.

Some key messages of the report are the following.

- Around 40 % of surface waters (rivers, lakes and transitional and coastal waters) are in good ecological status or potential.

- A total of 38 % of surface water bodies in the EU were in good chemical status. 46 % were not in good status and for 16%, the status was reported as “unknown”.

- Atmospheric deposition leads to contamination with mercury in over 45 000 water bodies failing good chemical status. Inputs from urban waste water treatment plants lead to contamination of over 13000 water bodies with polyaromatic hydrocarbons (PAHs), mercury, cadmium, lead and nickel.

- Chemical pollutants are or have been emitted into water bodies through a range of pathways and from a variety of sources, including industry, agriculture, transport, mining and waste disposal, as well as from our own homes. Significant levels of some priority substances have built up from historical use and this legacy pollution may persist in water bodies long after pollutant discharges and inputs have ended.

- Of the thousands of chemicals in daily use, relatively few are reported under the WFD. There is a gap in knowledge at European level over whether any of these other substances present a significant risk to or via the aquatic environment, either individually or in combination with other substances. In addition, information on the sources and emissions of many pollutants remains incomplete, limiting the scope for identifying and targeting appropriate measures.

In the report “Chemicals in European Waters” of 2018 (16) the European Environmental Agency in a specific chapter states that under the WFD, the assessment of surface water quality is separated into chemical and ecological status. Such separation is a practical solution for water regulation but is an artificial separation for the environment and concerns have grown about the 'cocktail effect', namely, mixtures of chemicals at low concentrations that, in combination, may cause harm. The separation of these statuses can be criticised, as the reported 'chemical status' of a water body may be remote from what is actually occurring in the water ecosystem.

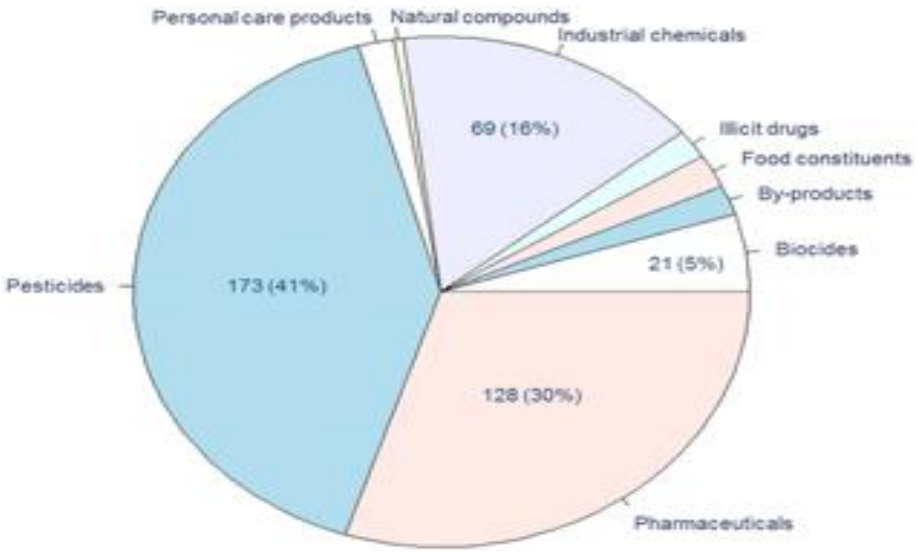
To establish causal relationships between chemical pollution and ecological effects, it has to be appreciated that, in the real world there are no cases where only a single substance occurs in the environment. Emissions data and research show that the aquatic environment has to deal with mixtures of chemicals, including many more substances than just priority substance, nutrients from urban point sources, agricultural diffuse pollution, metals from stormwaters and atmospheric deposition, as well as many potentially harmful organic chemicals from urban waste water and agriculture, have been shown to be present in freshwater systems simultaneously. Furthermore WFD assessment criteria for chemicals (EQSs) are generally developed substance-by-substance, based on laboratory studies, and usually do not consider the consequences of exposure to multiple chemicals or cumulative effects from several stressors or modifying factors. To derive EQSs and to establish monitoring programs for all these substances is highly challenging and for the RBSPs (River Basin Specific Pollutants) different Member States have so far frequently established in some cases quite different values for the same substance.

In a previous Communication of the European Commission of May 2012 (17) is stated that in relation to the effects on wild species and ecosystems, the situation is less clear and the possibility of combination/mixture effects should be considered both in the case of independently acting chemicals as well as for chemicals with similar modes of action. Methodologies for the identification of chemical mixtures of potential concern as well as for the assessment of chemical mixtures are available. However, there are extensive knowledge and data gaps (mainly related to the mode of action and exposure data) that limit the extent to which mixtures can be properly assessed.

Need for an innovative approach

The limitations of the current WFD approach to regulate toxic chemicals was evident from the previous section and the classical single-chemical risk assessment approach for the management of chemical pollution of water bodies has some limitations (18,19,20,21): It is not possible to analyse, detect and quantify all substances that are present in the aquatic environment.

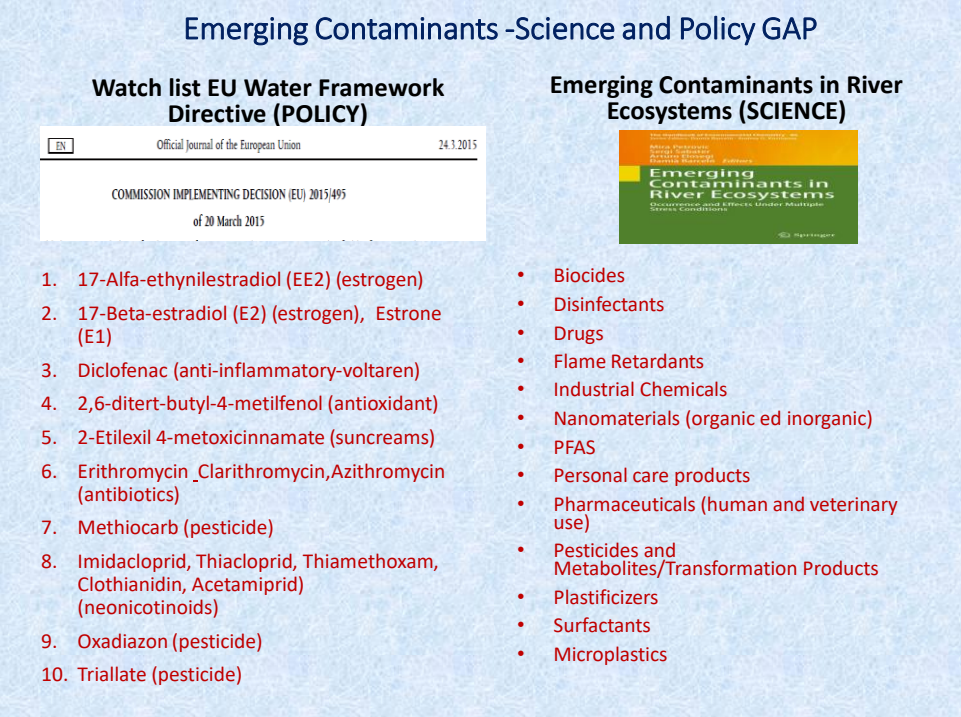
Figure 6: Figure taken by the EU Project Solutions related to the emerging contaminants in water bodies. https://www.solutions-project.eu/.



Currently, more than 700 emerging pollutants, their metabolites and transformation products, are listed as present in the European aquatic environments (22) by the Norman (Network of reference laboratories, research centres and related organizations for monitoring of emerging environmental substances) network www.norman-network.net.

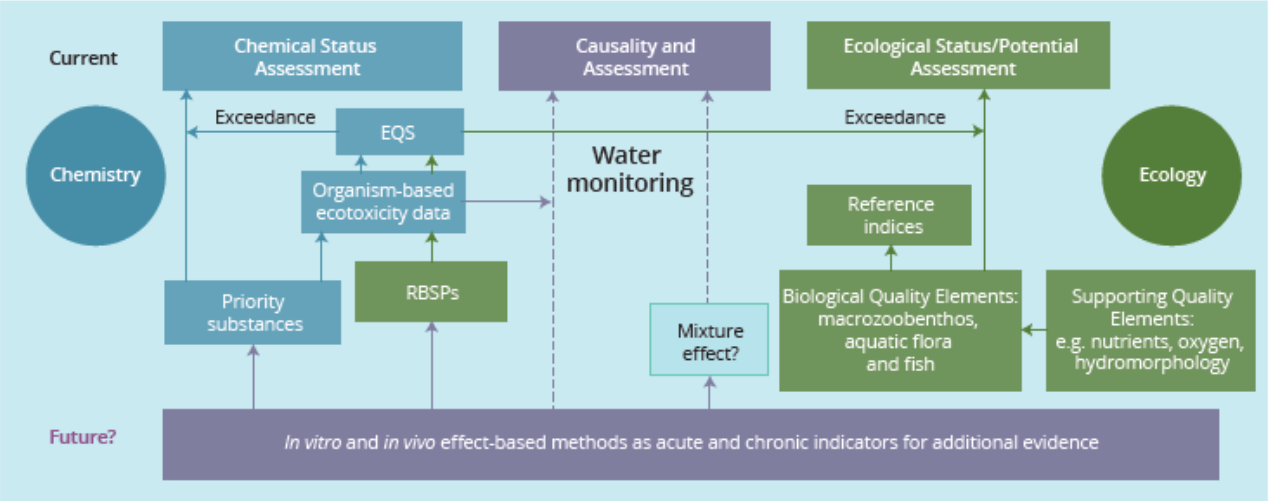
Emerging pollutants (EPs) are defined as synthetic or naturally occurring chemicals that are not commonly monitored in the environment but which have the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects. In Figure 7 it is showed the science-policy gap between the WFD legislative approach and the results of scientific studies and projects in relation to emerging contaminants.

Figure 7: Gap between Policy and Science in relation to the emerging contaminants



The European Reach (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation has registered more than 100.000 chemical substances (23). The effects from the mixture of substances present in the aquatic environment may not be predictable on the basis of chemical analyses alone. To reach the protection goal we also must understand the potential for effects caused by the sum of the chemical substances in the aquatic environment (including emerging pollutants, metabolites and transformation products) and to link the observed effects with cost-effective measures. It is important to know which are the real effects (24) caused by the sum of the chemical substances in the aquatic environment (including emerging pollutants, metabolites and transformation products) and to link the observed effects with cost-effective management objectives. The report of EEA of 2018 clearly states that **Effect Based Methods** could represent a great support for the identification of effects caused by mixtures of pollutants and not monitored substances (Figure 8) and also to link chemical and ecological status.

Figure 8: Use of effect based methods to link chemical and ecological status of the WFD proposed by the European Environmental Agency (EEA,2018).



CHAPTER 2

Effect Based Methods in Europe (overview)

Effect Based Methods-generalities

The history behind several "legacy" chemical substances show that they were first identified to be of major concern after observations of adverse effects were made in the environment. For instance, the effects from (tributyltin) TBT were documented about a decade before the effects could be linked to TBT (25,26). Also, effects from DDT and PCB were discovered through observations in the aquatic environment and on birds. Thus, these substances were not identified to be of concern through pro-active risk assessments but the risks with these chemicals were identified retroactively. Estrogenic effects have also been observed in the aquatic environment and several estrogenic substances, such as EE2, that can explain field-observations such as intersex in fish have been identified (27, 28).

A more systematic monitoring of effects would potentially be able to discover additional substances of concern that could pose potential threats to ecological systems and human health.

Effect Based Methods (EBM) are tools (29) used for the monitoring of waterbodies and other environmental compartments with the aim to detect effect caused by pollutants or group of pollutants, these effects can be detected at molecular, cellular, individual or populational level, they include:

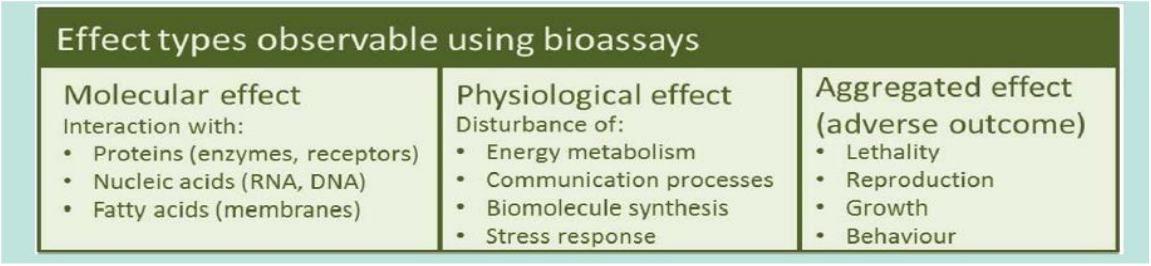
Bioassays in vitro and in vivo, which measure the toxicity of environmental samples under defined laboratory conditions, on cellular or individual levels.

Biomarkers that detect biological responses at the cellular or individual levels, measured in field exposed organisms.

EBM are designed to capture effects at different levels of complexity and specificity; they can measure either a specific response, a physiological response, or an unspecific response at the molecular, cellular, organ, organism, or population level. A specific effect is understood as the consequence of an interaction of a chemical with a specific group of biomolecules. This could be measured as an enzyme activity, an agonistic or antagonistic response indicating receptor binding of a chemical, an alteration of protein or gene expression, a protein or DNA adduct formation, or an

alteration of membrane integrity; or as aggregated effect (lethality, reproduction, growth, behavior) (30)

Figure 9: effect detected through bioassays



Bioassays are regularly used in the environmental risk assessment of pesticides, veterinary pharmaceuticals, biocides and also under the REACH regulation and there are hundreds of bioassays available only for toxicological use.

For monitoring purposes EBM are often included in regular monitoring programs or screening campaigns, they have since long been used to assess effluents (WEA, Whole Effluent Assessments) containing complex mixtures. As an example, the German waste water ordinance defines specific EBM, i.e. mostly in vivo biological test systems such as the algae test and fishio embryo test (FET) for the discharge of waste water. In the Directive 2010/75/EU on industrial emissions including Best Available Techniques (BAT), some BAT Reference Documents (BREFs) require the monitoring of emissions with EBM (E.G. Commission Implementing Decision (EU) 2016/902 of 30 May 2016 establishing best available techniques (BAT) conclusions, under Directive 2010/75/EU of the European Parliament and of the Council, for common waste water and waste gas treatment/ management systems in the chemical sector). EBM for example are also mentioned in relation to the HP14 criterion for the assessment of hazardous waste: the properties which render waste hazardous are laid down in Annex III of Directive 2008/98/EC and are further specified by the Decision 2000/532/EC. Primarily the assessment is based on the chemical composition of the waste. However, if the chemical composition is unknown EBM, i.e. ecotoxicological tests, are applied. EBM are commonly applied in the sediment monitoring programmes of marine Convention like OSPAR. Bioassays are used, for example, to support risk assessment and management of contaminated sediment and to provide decision support for reducing the release of toxic substances into the environment. In Italy EBM are also applied in the context of the health impact assessment that is a procedure aimed at protecting the health of the populations exposed to the impacts due to plans/programs/plants can determine on environment (31).

Bioassays are used in individual Member States to provide decision support to prohibit the release of toxic substances into the environment (e.g. WEA Whole Effluent Assessment in the permitting process and evaluation of dredged sediments that are considered for sea disposal). Other applications include for example the Dutch alarm system that directly triggers control measures (closing drinking water intake).

In the context of the CIS of the WFD a technical report on Effect Based Methods has been published in 2014 (27). The technical report aims at presenting the state of the art of aquatic effect-based monitoring tools and to describe in which way these tools can help EU member states to make more efficient monitoring programmes (including reduction of monitoring costs).

Some objectives for the use of effect-based tools in a WFD context are mentioned in the report:

• As screening tools, as part of the pressures and impact assessment to aid in the prioritisation of waterbodies

• To establish early warning systems

• To take the effects of chemical mixtures or chemicals that are not analysed into account (e.g. to support investigative monitoring where causes of a decline of specific species are unknown)

• To provide additional support in water and sediment quality assessment, though not as a replacement for conventional chemical and ecological monitoring under the WFD.

The use of EBM for monitoring in the WFD context can overcome some of the challenges identified in the previous chapter. They can be used for several purposes:

–For detecting the effects of mixtures of compounds in water resources and demonstrating their potential to affect aquatic organisms and human health,

– For detecting hot spots of contamination for investigative monitoring,

– For identifying risk drivers and prioritizing them for management measures,

– For linking chemical and ecological status.

Bioassays and Biomarkers

The EBM can be divided in 3 Groups:

- Bioassays in vitro
- Bioassays in vivo
- Biomarkers

Bioassays In vitro

In vitro bioassays are based mainly on cell lines (lower biological organisational level), responding to those compounds in a sample that have the same mode of action, such as binding to a specific cellular receptor or change in a specific DNA component (27). They have much in common with chemical analytical screening tools, but a "biological detector" is used and therefore these bioassays are often refered to also as "bioanalytical tools". More or less any type of sample can be analysed, and the results are frequently expressed on a chemical equivalent basis (32,33). However, they measure the cumulative effect from all substances in the sample having the same mode of action and not only that particular substance. In contrast to in vivo assays that capture the effect of chemicals on whole organisms, in vitro assays detect unwanted biological effects on a molecular level such as the activation of a cellular receptor or signaling pathway, the induction or inhibition of a specific enzymatic activity or the mutation of a DNA sequence. In vitro EBM are fast and have the potential for automation and thus allow a high throughput screening of samples. They are widely used for screening purposes in chemical risk assessment because at least in part they can serve as alternative test methods for animal testing. In vitro bioassays which measure the same endpoint and species employed as in vivo reference models may display different sensitivity for the same substance or chemical mixtures.

<u>Examples of Standardized Methods for in vitro methods</u>

- **Erod**: ISO/TS 23893-2, 2007. Water quality -- Biochemical and physiological measurements on fish -- Part 2: Determination of ethoxyresorufin-O-deethylase (EROD)

- **Ames**: (T98 and T100 strains): ISO 16240, 2005; Determination of the genotoxicity using the Salmonella/microsome test ISO 11350, 2012Determination of the genotoxicity of water and waste water – Salmonella/microsome fluctuation test (Ames fluctuation test)
- **UmuC**: ISO13829, 2000. Determination of the genotoxicity of water and waste water using the umu-test
- **Micronucleus test** (V79): ISO 21427-2: 2006 Water quality -- Evaluation of genotoxicity by measurement of the induction of micronuclei -- Part 2: Mixed population method using the cell line V79 OECD Test No. 487: In Vitro Mammalian Cell Micronucleus Test.
- **Vitellogenin induction test**: ISO 23893-3:2013- Water quality -- Biochemical and physiological measurements on fish - Part 3: Determination of vitellogenin
- **Estrogenicity(cell line:BG1Luc**): OECD 457, 2012: BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists (cell line: HeLa-9903) OECD 455, 2009: Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals

Possible Applications in the context of the EU Water Framework Directive (41)

• Allow the specific detection of relevant MoAs on a molecular level
• Allow for cost efficient high throughput measurements
• A number of in vitro EBM are standardised and thus mature for implementation
• Results can be used for a relative assessment, for prioritisation, source identification and investigative monitoring
• In vitro EBM with defined EBT (effect based trigger values)-values can be used for screening purposes and possibly even for a status assessment

In vivo Bioassays

In vivo bioassays are tests in which whole living organisms (including bacteria) are exposed to environmental samples (27,34,35,36) like surface water, sediment, waste water, dredge material or extracts from these samples. Tests are performed in the laboratory or, less frequently, in the field ("in situ" bioassays).

The "end point" is the type of effect that is measured in the in vivo bioassays, and some examples that are frequently used in this context are:

- Mortality
- Immobilization
- Effects on reproduction (i.e fertilization, hatching, embryo development)
- Effects on growth of individuals
- Effects on growth of populations
- Metabolic or physiological changes

- Behavioural changes
- Bioluminescence
- Molecular/Biochemical responses

In general, in vivo bioassays are broad spectrum assays, e.g an in vivo bioassay reacts on a variety of substances and on different types of toxicity. Nevertheless, it is important that the evaluation of toxic effects observed is based on the response observed in several species, because they can exhibit intrinsic differences in terms of sensitivity to various chemicals, also depending on the endpoint measured in the test. Both short and long term in vivo bioassays should preferably be carried out and at least three species of organisms belonging to different taxonomic groups and trophic levels (primary producer, decomposer/saprophytic, detritivore/filter feeder, consumer). Bioassays in vivo are in general acute or chronic. Acute toxicity means adverse effects that occur in a short time (not exceeding one third of the average time between birth and sexual maturity) while for chronic toxicity effects are measured in average after a period longer than 50% of the organism life time. According to these definitions, it is not possible to establish a time limit, e.g. 24, 48 or 96 h, to distinguish between acute, subacute or chronic assays. Bacteria, algae, invertebrates or other model species may have a very different average life time (37). For identification of the assays that make up the individual batteries, priority is given to those for which there are methodological protocols (standards). In vivo bioassay protocols have much in common with traditional toxicity test protocols, developed for chemical regulation purposes (such as the Daphnia magna test). In many cases the same test protocols can actually be used although, for chronic assays, the feeding and test-solution renewal schedule also may need to be adjusted.

The advantage of the use of in vivo assays is for example their broad implementation in pesticide regulation and effluent monitoring, monitoring programmes of Marine Coventions, sediment dredging. Many data on the impact of chemicals in the tests of REACH for example are available and their long-term application with standardised protocols (standards, guidelines) offers information on the precision of the procedures

In general, in vivo bioassays are broad spectrum assays, e.g. an in vivo bioassay reacts to a variety of substances and different MoAs. It is important that the evaluation of toxic effects of a sample is based on the response in several species, because they can exhibit intrinsic differences in terms of sensitivity to various chemicals and also depend on the endpoint measured in the test.

Figure 10: Example of In Vivo assays applied by Member States in aquatic monitoring programmes (27 Wernersson A.S., Carere M., Mancini L et al)

Organism	Test item	Endpoint	Species	Exposure
Bacteria	w, ws, e, p	Bioluminescence	*Aliivibrio fischeri* (f/m)	5 to 30 min.
		Enzyme activity	*Arthrobacter globiformis* (f)	2 h
Algae	w, e, p	Growth	*Phaeodactylum tricornutum* (m)	72 h
			Skeletonema costatum (m)	
		Growth	*Desmodesmus subspicatus* (f)	72 h
			Pseudokirchneriella subcapitata (f)	
		Growth	*Ceramium tenuicorne* (m)	7 days
Plants	w, e, p	Growth	*Lemna minor* (f)	7 days
	ws	Growth	*Myriophyllum aquaticum* (f)	10 days
Rotifera	w, e, p	Mortality	*Brachionus plicatilis*	24 to 48 h
Crustacea (amphipods)	ws	Mortality	*Corophium spp.* (and other amphipods) (m)	10 days
	w, e, p	Mortality	*Artemia franciscana* (m)	24 h, 14 days
		Mortality	*Acartia tonsa* (m)	96 h
			Tigriopus fulvus (m)	
		Mobility, mortality, reproduction	*Daphnia magna* (f)	24/48 h, 21 days
			Cerodaphnia dubia (f)	
Nematoda	ws	Mortality, fertility, reproduction	*Caenorhabditis elegans* (f)	96 h
Annelida	ws	Mortality, reproduction	*Lumbriculus variegatus* (f)	28 days
Insecta	w, ws	Mortality, reproduction	*Chironomus riparius* (f)	48 h, 28 days
Bivalvia	w, e, p	Development	*Crassostrea gigas* (m)	24 to 72 h
			Mytilus galloprovincialis (m)	
			Tapes philippinarum (m)	
Echinodermata	w, e, p	Fertilisation	*Paracentrotus lividus* (m)	≤72 h
		Development	*Sphaerechinus granularis* (m)	
Polychaeta	ws	Mortality	*Hediste diversicolor* (m)	10 days
Vertebrata (fishes)	w, e, p	Mortality and genotoxic damage	*Danio rerio* (and embryos of other species) (f)	96 h, 28 days
			Dicentrarchus labrax (m)	

w, water; e, elutriate; p, pore water; ws, whole sediment; f, freshwater; m, brackish and marine.

Possible Application in the context of the EU Water Framework Directive (41)

- Cover complex mixtures (of unknown composition) and perhaps even cumulative effects when combined with other stress factors
- Assess status and/or identify significant pressure
- Assess sediment quality
- Assess Metal bioavailability when water chemistry outside validation range
- Assess quality of effluents or leachates

Biomarkers

Biomarkers can be used to study effects such as biochemical, physiological, histological, or morphological alterations in field exposed individuals (27,38,39,40). They are sometimes divided into specific and general biomarkers. The latter respond to several types of substances and possibly also other stressors than hazardous substances. Specific biomarkers are generally related to a limited number of substances. Specific biomarkers can more easily be related to a particular pressure whilst general biomarkers have the capacity to integrate the response related to several stress factors and thus also toxicologically induced responses from contaminant mixtures.

Biomarkers are in turn often divided into those that are to be considered "effect biomarkers" in the sense that the response (endpoint) typically can be linked to negative health effects, whereas some biomarkers are categorised as "exposure biomarkers" in the sense that they are measuring presence of compound or its metabolites and interactions with receptors.

Imposex is for example considered to be an effect biomarker of very high ecological relevance since the effects observed are related to reproduction and measured on a high organisational level (tissue/organism). There were also extensive field effects observed, that were related to population decline. Another effect biomarker that can be considered to be of very high ecological relevance is reproductive success in eelpout, because it is related to reproduction and measured on high organisational level, and field effects have been observed in locally impacted areas.

Metallothionein (MT) induction can on the other hand be considered to be an exposure biomarker of low/moderate ecological relevance, because it is involved in the regulation of the intracellular concentrations of essential and non-essential metals and MTs provide protection against oxidative stress. Thus, if there is a response it is not straightforward to link it to a negative health impact.

The use of biomarkers in particular has a long tradition in some Member States and regional sea conventions. Within the regional sea conventions (OSPAR, HELCOM, UNEP-MAP and the Bucharest convention) and ICES (International Council for the Exploration of the Sea) several EBMs have since long been included in recommended or agreed monitoring programs although most are not considered mandatory methods to contracting parties.

They are also used in the context of the marine strategy framework directive (MSFD).(see Figure 11).

Figure 11: Biomarkers used in the context of the Marine Strategy Framework Directive

Mussels	Metallothionein (MT) content Acetylcholinesterase (AChE) activity Glutation-S-transferase (GST) activity Micronuclei (MN) formation Lysosomal membrane stability (LMS) Scope of growth (SFG) Glutation peroxidase (GPx) activity Catalase (CAT) activity Cell damage
Fish	EROD activity Fish disease index (FDI) Levels of bile metabolite 1-hydroxyoprene Intersex Formation of DNA adducts Liver tumours Liver pathologies Blood vitellogenin (Vtg) White blood cells alterations Activities of detoxication enzymes Gonad index % deformed larvae
Birds	Mass mortality Breeding success Egg shell thickness Contamination of eggs (coastal birds)
Other biota	Embryos malformations (amphipods) Imposex (gastropods)

Possible Applications in the context of the water framework directive (41)

1. Cover complex mixtures (of unknown composition) and perhaps even cumulative effects when combined with other stress factors – to assess status and/or identify significant pressure
2. Cover mixture effects from substances sharing the same MoA– to assess status and/or identify significant pressure
3. Identify relevant indicators
4. Assess sediment quality
5. Bioanalytical methods to assess status of regulated substances
6. Metal bioavailability when water chemistry outside validation range
7. Assess status where there are high natural metal concentrations (>EQS)

Modes of Action and EBM

The term mode of action (MoA) refers within the Adverse Outcome Pathway (AOP) (42) strategy to the specific mechanism by which the chemical compounds present in water produce their adverse effects on aquatic organisms. An AOP is an analytical construct that describes a sequential chain of causally linked events at different levels of biological organisation that lead to an adverse health or ecotoxicological effect.

The MoA is the process initiated by the interaction of the toxicant with the organisms, for example with a receptor, which progresses through molecular, biochemical, physiological and/or anatomical changes in the organism to result in sub-lethal and lethal effects.

Identification of the MoA can lead to an understanding of the molecular target (e.g. biological receptor) of a chemical and extrapolation to anticipated effects or biological responses. In this context, EBM offer the possibility to monitor the overall response from multiple chemicals in environmental samples and estimate their impact on different levels of biological organisation. For this reason, they have been proposed to complement the chemical analytical methods to provide a more holistic approach for the water chemical status assessment.

An interesting published JRC technical report (43) provides an overview of the MoA of the Priority Substances (PS) in the WFD and other substances of concern (from the first WL and the current exercise to prioritise candidates for the PS list substances). The purpose of that report was to present an overview of the MoAs reported in ecotoxicological studies. In the report, the substances of interest are grouped into categories based on their chemical structure and common use, e.g. herbicides, PAHs, insecticides; as well as common MoA and toxicological endpoints, e.g. photosynthesis inhibition, endocrine disruption, oxidative stress.

Furthermore, the available EBM linked to the MoA are identified. However, it is not possible to identify single EBM that account for all the relevant effects (including effects on different organisms) of each PS, alone or in combination. Furthermore, certain factors (e.g. toxicokinetics and toxicodynamics) other than the aqueous concentration may influence the toxicity of the substances, therefore even where an in vitro bioassay result might be expected to correlate with the results of field measurements, there may not be an exact correlation .

Common MoA/effects identified in the JRC technical report are: Photosynthesis inhibition, Endocrine disruption, Oxidative stress, Activation of metabolising/detoxifying pathways, Genotoxicity, Histopathology, Stress proteins, Unique pathway toxicity (e.g. acetylcholiesterase inhibition, imposex, presence of metallothioneins). However, for some classes of chemicals, such as the neonicotinoid and pyrethroid insecticides, for which MoA is well-characterised in their target organisms, there is limited information regarding the mechanism that causes toxicity in non target organisms including aquatic species.

Figure 12: JRC report 2018. MOAs of Priority Substances and Watch list substances of the EU Water Framework Directive

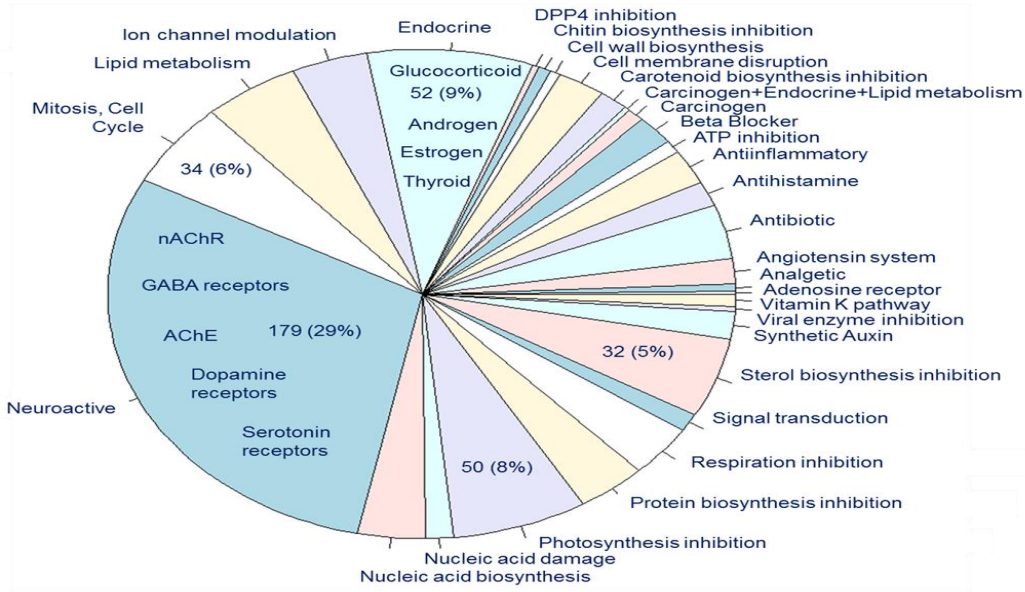
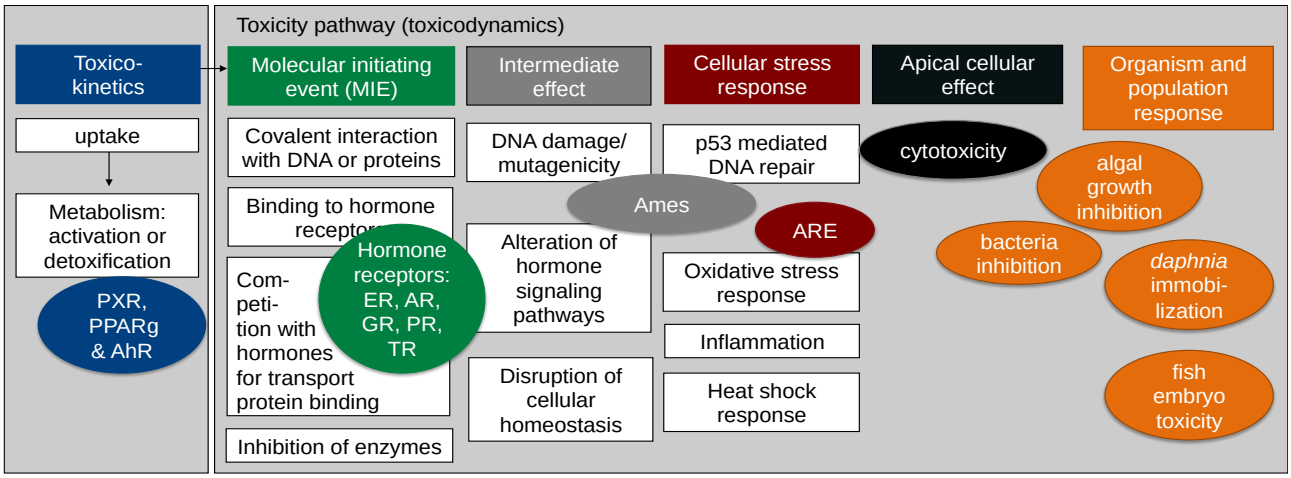


Figure 13. Schematic representation of the MoA, the process through which a chemical compound exerts its adverse effects and application of EBM (Effect Based Methods). Adapted from OECD



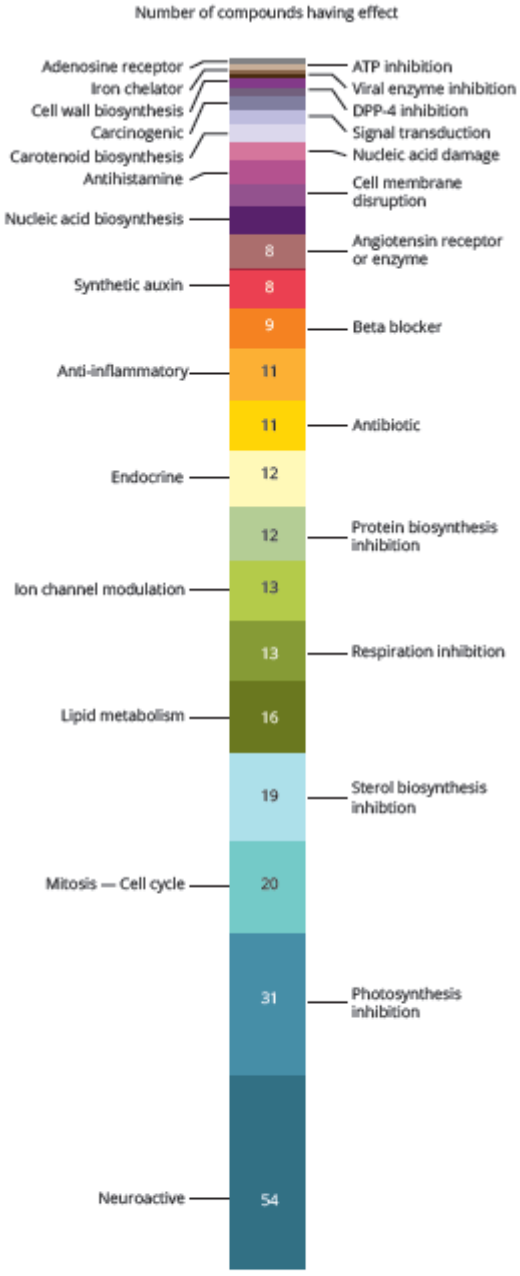
(http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathwaysmolecular-screening-and-toxicogenomics.htm)

Chemicals can exert independent, additive, synergistic or antagonistic effects (44). Additive and synergistic effects would lead to an increased toxicological effect. A better understanding of the MoA and potential interactions of chemicals is crucial for water quality assessments.

A relevant study performed by Busch et al. (30) described the diversity of potential molecular targets for contaminant-biosystem interactions. In this study, 426 organic chemicals were detected in three European rivers, including 173 pesticides, 128 pharmaceuticals, 69 industrial chemicals and 56 other compounds. For about two-thirds of these compounds, the interactions with biological systems are known. These compounds can interact with more than 100 different biological molecules known to exist in aquatic organisms. This complicated picture was simplified by building broader categories of modes of action, into which the chemicals could be sorted because of their known biological target molecules or key events.

For freshwater contaminants, 27 mode-of-action categories were identified (Figure 14); so even with a potentially unlimited number of chemicals, there was a limited range of adverse biological effects. While remaining aware of the fact that the development of toxicity is a complex process, with diverse events that might not be yet considered, this approach could serve as a starting point to simplify toxicity assessment.

Figure 14: Modes of Action of organic micropollutants in 3 european rivers



Notes: ATP, Adenosine triphosphate (energy carrier in the cells of all known organisms); DPP-4, dipeptidyl peptidase-4 (an enzyme).

Samples from sites in the Rhine, Danube and Mulde/Saale Rivers.

Source: Busch et al., 2016.

Neurotoxicity

Neurotoxicity was identified (41) as one of the most emerging modes of action in the aquatic environment. It has been estimated that up to 30% of all commercially used chemicals may have neurotoxic potential 30.000 chemicals (45). The largest group of organic micropollutants with a known mode of action identified in the mentioned Busch study (30) were neuroactive compounds, which affect or interact directly with the nervous system. Chemicals that affect the nervous system interact with different molecular targets, e.g. different insecticides either binding to the nicotinic acetylcholine receptor or inhibiting the enzyme named acetylcholine esterase . Both of these modes of action affect the signalling in the nervous system and mixtures of such chemicals will enhance the effects. Aquatic invertebrates might be particularly at risk owing to exposure to mixtures of different kinds of insecticides, while other species, such as fish, might be affected by the presence of antidepressant or antiepileptic pharmaceuticals that affect the nervous system of fish, possibly in combination with effects caused by insecticides. The numbers of potential neurotoxicants in the environment is raising and can pose a risk for humans and the environment. Considering the increasing numbers of environmental contaminants with potential neurotoxic potential, eco-neurotoxicity should be also considered in future risk assessments. In order to do so novel test systems are needed that can cope with species differences within ecosystems a selection of assays could be guided by Adverse Outcome Pathways (AOPs) relevant for eco-neurotoxicity. The German Federal Ministry of Education and Research (BMBF) founded the project NeuroBox and the EU NORMAN network is performing a ringtest with neurotoxic substances considering behavioral changes in *Danio rerio*. Moreover, EURL ECVAM of the Joint Research Centre (JRC) is working on in vitro approaches to detect developmental neurotoxicity (DNT) triggered by a single chemical or in mixture. An evaluation of neurotoxicity (including developmental stage) is also be performed using non-mammalian species since the mechanisms underlying the development and function of the nervous system are well conserved across the phylogenic tree. Many of the basic molecular processes are identical in mammals and in non-mammalian species. Therefore, several alternative species including *Danio rerio, Oryzias latipes* or *Xenopus laevis* are used as vertebrate non-mammalian models and complementary to in vitro approaches (46). The small size, transparency during embryogenesis and speed of development make these species suitable for chemical testing. The gathering of data from these multiple information sources, could be used to

develop Integrated Approaches to Testing and Assessment (IATA) designed in a fit-for-purpose manner for different regulatory purposes, including aquatic and human health protection. In the light of these ongoing developments a relevant selection of neurotoxicity assays for environmental assessments can be discussed at a later stage to advance the safety of assessments for neurotoxicity in the future.

Inventory of EBM-WFD Activity of the Subgroup EBM

In 2016 (Bratislava 28-29 November) the Water Directors endorsed the need for a new approach for the chemical status assessment explicitly stating that EBM should be used to elaborate a holistic approach for the evaluation of the surface water quality.

A specific sub-group was established with representatives from nine Member States (MS), Switzerland and several stakeholders. The sub-group (in total 54 experts) has elaborated the Terms of Reference (ToR) of the Activity after a long discussion at the WG Chemicals and a consultation with the Ecostat Group and the Marine Strategy WGs.

The Main Objective of the activity of the group was to examine and further document the possible implementation of effect-based methods (EBMs) for monitoring and assessment in the WFD context, alongside traditional chemical analysis, bearing in mind their possible application under the MSFD. It has built on all scientific evidence and practical knowledge available to-date, including the conclusions of the Effect Based Tools technical report.

The ToR finalized in 2016 was based on a series of specific objectives:

1. Identification of chemical modes of action (MoAs) (e.g. estrogenicity, Ah receptor binding, acetylcholinesterase inhibition, anti-cholinergic activity, photosynthetic inhibition, mutagenicity, immunotoxicity), considered to be of relevance in or via the aquatic environment for the protection of aquatic ecosystems and human health.

2. Perform an inventory of MoAs (if known) for currently regulated and/or monitored compounds (in particular priority and other WFD Annex X substances, watch-list substances, and river basin specific pollutants identified to be of concern).

3. Based on 1 and 2, identification and prioritisation of EBM (*in vivo* and *in vitro*) available for the detection of the relevant MoAs, in the different matrices of the aquatic environment. The prioritisation will consider the level of maturity of the methods, including whether they are available for routine use, and their robustness and reliability.

4. Development, where possible, of *in vivo and in vitro* effect-based trigger values, signaling a risk to or via the aquatic environment (including risks to human health from chronic exposure via consumption of drinking water or fishery products if possible), with the aim of making effect-based methods applicable (alongside chemical tools) in WFD chemical monitoring and assessment.

5. Based on objectives 3 and 4, selection of relevant EBM (*in vitro* and *in vivo*) that can be used alongside chemical methods for the evaluation of complex mixtures occurring in the different types of aquatic environments (e.g. freshwaters, coastal waters), and aiming at being able to identify significant pressures and water bodies at elevated risk (i.e. support the WFD assessment of pressures and impacts). This will include consideration of the comparability of the results given by the different methods, and as far as possible the definition of quality control criteria for these tools in the context of the WFD, on the lines of the criteria defined by the QA/QC Directive.

6. Evaluation of ecological methods that can be used to address also chemical pollution, including the metagenomics approaches.

7. Identification of a list of EBM to be considered for Marine Strategy application according to D8 criterion 8.2.1 (of Decision 2010/477/EU) and/or considered within the WFD, taking also harmonisation between the WFD and MSFD into account.

8. Assess the availability and suitability of investigative approaches for identifying the underlying causes contributing to the overall risks, to identify sources of emissions and facilitate measures.

9. Assess the practical feasibility and cost effectiveness of implementing at EU-scale possible strategies using EBM, to better take into account mixture risk assessment and mixture risk management under the WFD for relevant MoAs, as far as possible ensuring consistency with other legislations. In particular, this will include a comparison of the advantages/drawbacks of using effect-based tools alongside chemical tools, compared with using only chemical methods as in the current approach to chemicals under the WFD.

The final report of this activity has been approved at the Strategic Coordination Group of the CIS (Common Implementation Strategy) in 2019 (Carere, M., Lettieri, T., Wernersson, A.S., Hanson, N., Buchinger, S., Kase-Pasanen, R. et al. "Proposal for Effect-Based Monitoring and Assessment in the Water Framework Directive". *Report to the CIS WG Chemicals on the outcome of the work performed in the subgroup on effect-based methods (EBMs).*

An important result of this activity has been the collection of EBM at European level, in total 135 EBMs were included of which 57 could be categorised as in vitro assays, 44 as in vivo assays and 34 as biomarkers (see Addendum at the end of the Thesis). The inventory collected so far does not claim to be complete and would have to be further developed.
Together the EBMs collected cover the following MoAs and type of effects:

- Endocrine disruption of sex hormones (of relevance for e.g. reproduction):
  - Activation and antagonistic activity of the estrogen receptor (ER) *in vitro*
  - Neurosteroids *in vivo*
  - Vitellogenin induction (*in vivo* and as biomarker)
  - Spiggin induction (as biomarker)
  - Activation and antagonistic activity of androgen receptor (AR) *in vitro*
  - Activation and antagonistic activity of progestogenic receptor (PR) *in vitro*
  - Imposex (tissue level, as biomarker)
  - Intersex (tissue level, as biomarker)
- Endocrine disruption of glucocorticoids (of relevance to e.g. development, metabolism, immune system):
  - Activation and antagonistic activity of the glucocorticoid receptor (GR)
- Endocrine disruption of thyroid hormones (of relevance to development, growth, and metabolism of all vertebrates, major role in neurogenesis and brain function)
  - Binding assay to thyroid receptor (TR)
  - Activation and antagonistic activity of the thyroid receptor (TR)
- Genotoxicity and mutagenicity
  - DNA strand breaks (*in vitro*)
  - Reporter gene expression (+S9) (*in vitro*)
  - Mutagenicity (point mutation, clastogenic effect)
  - DNA damage (Comet assay) (*in vivo* at early life stage and as biomarker)
  - Gene transcriptions
- Immune response
  - KappaB (*in vitro*)
  - Fish disease (biomarker)
- Activation of metabolic enzymes

  - Activation of the peroxisome proliferator-activated receptor (PPARγ) (*in vitro*)
  - Activation of human pregnane x receptor (PXR) (*in vitro*)
- Oxidative stress
  - Reactive oxygen species (ROS, *in vitro*)
  - Stress proteins (biomarker)
  - Protein carbonylation (biomarker)
  - Gene transcriptions
- Internal regulation
  - Metallotionein induction (biomarker)
  - Ah receptor activation (of relevance to e.g. detoxification) (as *in vitro* and *in vivo* and biomarker - EROD)
  - PAH metabolites (biomarker)
  - Gene transcriptions (biomarker)
  - P-glycoprotein efflux (P-gp) (biomarker)
- Hemoglobin synthesis
  - ALA-D (biomarker)
- Lysosomal membrane stability (biomarker)
- Inhibition of photosynthesis
  - PSII-inhibition (algae, higher plants) (*in vitro*/*in vivo*)
- Neurotoxicity
  - Acetylcholinesterase (AChE) inhibition (overstimulation of neuromuscular junctions) (*in vivo* and as biomarker)
- Cytotoxicity (cell death)
  - In fish cell lines (*in vitro*)
  - In algae (inhibition of photosynthesis and loss in biomass/growth, *in vivo* but single cell organisms)
  - In bacteria (inhibition of bioluminescense, *in vivo* but single cell organisms)
  - Lipid peroxidation (biomarker)
- Embryotoxicity (*in vivo*)
- Spermiotoxicity (*in vivo*)
- Development (*in vivo*)
  - Molting
  - Growth
  - Larval development
- Histopathological changes
  - Fish Liver histopathology (LH) and liver macroscopic neoplasms (MLN) (biomarkers)
  - Mussels (gametogenesis, digestive gland and tube, biomarkers)
- Malformation (*in vivo*)
  - Embryo of amphipods, fish (*in vivo* and biomarkers)
  - Benthic diatoms (biomarker)
  - Mentum deformations in chironomids (biomarker)

- Behaviour (*in vivo*)
  - Immobilisation
  - Swimming behavior
  - Photomotor response
  - Feeding inhibition
- Reproduction (*in vivo*)
  - Invertebrates
  - Fish (also in viviparous organism, eelpout, as biomarker)
  - Pregnancy rate in marine mammals (biomarker/ecological level)
  - Egg shell thinning in predatory bird (biomarker)
- Lethality
  - *In vivo* assays on several trophic levels such as fish (early life stage), invertebrates (also benthic) and aquatic plants
  - Biomarker in mussels (aerial survival)
  - Survival of off spring (mammals and predatory birds, biomarker/ecological level)

In the previous sections related to Bioassays in vitro, in vivo and biomarkers the final WFD applications mentioned is a result of this WFD activity

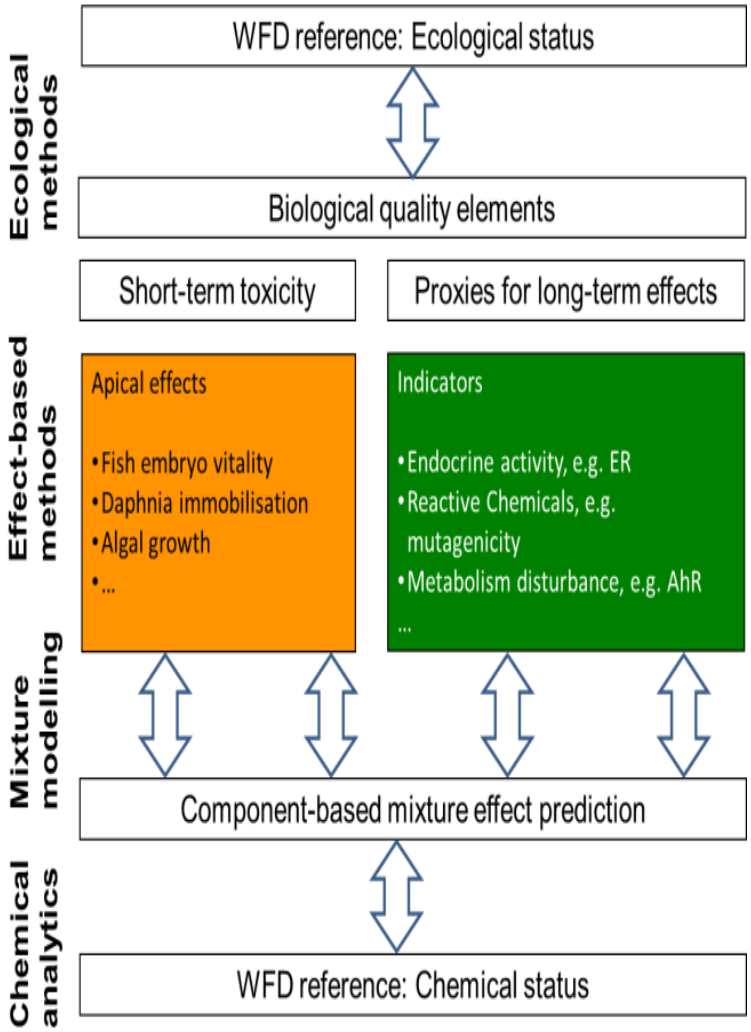
Battery of bioassays

For a broad scope, the battery of ecotoxicological tests should have (27) a sensitive and an overall discriminatory power responding to as many forms of contamination as possible.The most suitable approach is generally based on the choice of an adequate battery of tests and choice of species which should take into account different aspects: sensitivity, specificity, availability of organisms (for *in vivo* bioassays), the variability of the method, cost/effectiveness, ethics, as well as standardization and intercalibration of the methods.

In the context of the WFD 2019 Report (41) there is also a specific section dedicated to the possible battery of bioassay for water monitoring. The bioassay batteries of different projects have been reviewed and compared in order to identify and to suggest a common battery of bioassays. A recent NORMAN network interlaboratory study (ILS) verified whether a battery of miniaturised bioassays, conducted in 11 different laboratories following their own protocols, would produce comparable results when applied to evaluate blinded samples consisting of a pristine water extracts spiked with four emerging pollutants as single chemicals or mixtures (Assays evaluated effects on aquatic organisms from three different trophic levels (algae, daphnids, zebrafish embryos) and mechanism-specific effects using in vitro estrogenicity (ER-Luc, YES) and mutagenicity (Ames, Ames Fluctuation) assays (33).

Within the SOLUTIONS EU project a broad battery of in vitro bioassays based on human and fish cell lines as well as whole organism assays using bacteria, algae, daphnids and fish embryos were assembled for use in water quality monitoring. The selection of bioassays was guided by the principles of adverse outcome pathways in order to cover relevant steps in toxicity pathways known to be triggered by environmental water samples. In a proof-of concept study the effects of 34 water pollutants, which were selected based on hazard quotients, available environmental quality standards and mode of action information, were fingerprinted in the bioassay test battery. The proof-of-concept study not only demonstrated the utility of fingerprinting single chemicals for an improved understanding of the biological effect of pollutants, but also highlighted the need to apply bioassays for water quality monitoring in order to prevent underestimation of the overall biological effect. The recommended bioassay battery is also detailed in an upcoming Policy brief of the SOLUTIONS project (47). It is suggested to complement in vitro assays by apical bioassays representing at least fish (fish embryo testing), invertebrates (Daphnia) and algae (cell multiplication inhibition). Of the MoA-specific in vitro assays, priority should be given to endocrine disruption and mutagenicity. Dioxin-like effects should be analysed particularly in sediments, biota and equilibrium passive samplers since typical drivers of these effects are very hydrophobic and accumulate in these matrices.

Figure 15: Recommended test battery (Solutions Project) in the context of chemical and ecological status monitoring



Validation

Standardisation and intercalibration aspects are of particular importance if monitoring results are to be used in a regulatory context, also emphasised by the QA/QC Directive 2009/90/EC (see figure) in the WFD context. Internationally, the OECD (Organisation for Economic Co-operation and development) and ISO (International Organisation of Standardisation) are the most important bodies for development, validation and standardization of analytical as well as effect-based test methods. Whereas the Test Guidelines Programme, within the Environmental Directorate of the OECD is focused on test methods for single substance testing ("toxicity tests"), the Technical Committee (TC) 147 "Water Quality" of ISO is dedicated to the environmental aspects of water quality control. Another important body of validation and standardization of bioassays/toxicity tests is the US EPA. In general, the protocols for single substance tests can frequently be adapted to work also for complex environmental samples. However, environmental samples usually have much lower concentrations of toxic substances (see above) than the concentration ranges generally used in toxicity tests within chemicals testing. Further standardisation of effect-based methods such as *in vitro* bioassays for regulatory applications and use for surface water monitoring is needed. For investigative purposes such as screening however, non standardised methods could still be very valuable, and are sometimes the only option also within chemical analysis. A common validation framework that can also cover tools that are less established is therefore valuable to increase comparability of such data from different regions.

Art. 6 of the QA/QC Directive

1. Member States shall ensure that laboratories or parties contracted by laboratories apply quality management system practices in accordance with EN ISO/IEC-17025 or other equivalent standards accepted at international level.

2. Member States shall ensure that laboratories or parties contracted by laboratories demonstrate their competences in analysing relevant physico-chemical or chemical measurands by: (a) participation in proficiency testing programmes covering the methods of analysis referred to in Article 3 of this Directive of measurands at levels of concentrations that are representative of chemical monitoring programmes carried out under Directive 2000/60/EC, and (b) analysis of available reference materials that are representative of collected samples which contain appropriate levels of concentrations in relation to relevant environmental quality standards referred to in Article 4(1).

3. The proficiency testing programmes referred to in paragraph 2(a) shall be organised by accredited organisations or internationally or nationally recognised organisations which meet the requirements of ISO/IEC guide 43-1 or of other equivalent standards accepted at international level. The results of participation in those programmes shall be evaluated on the basis of the scoring systems set out in ISO/IEC guide 43-1 or in the ISO-13528 standard or in other equivalent standards accepted at international level.

EBM and Genotoxicity

The risks from genotoxic substances in water bodies is currently assessed in the WFD using a chemical-analytical, substance to substance approach (41). Some compounds with mutagenic properties, such as PAHs and benzene, are included in the EQS-Directive 2013/39/UE. Current regulatory context and use of EBMs Annex VIII of the WFD defines, amongst others, compounds "that possess carcinogenic or mutagenic properties" as main pollutants for European water bodies indicating the relevance of this biological effect. Several EBM permit to evaluate genotoxicity (Ref), i.e. damage of the genetic information within a cell through an interaction of a genotoxic substance with DNA sequence or structure which may lead to mutations (mutagenicity), and further to cancer (carcinogenicity). For the latter reason, the use of EBM specific for this MoA is fundamental also for the protection of human health, when exposed to e.g. drinking water. The added value of using Mutagenicity tests is that they are predictive of integral mutagenic/carcinogenic activity (48,49,50). They can evaluate the combined action of potentially hazardous compounds present e.g. in drinking water as complex mixtures and not only a specific compound. They are able to take into consideration the synergism, additivity or even antagonism of substances. The extraction method is also very important for this type of assay.

In vitro bioassays for the detection of mutagenic and clastrogenic potentials are used within REACH (Council Regulation (EC) No 440/2008). Mutagenicity tests are rapid, relatively cheap and have the potential for automation and thus high throughput screening. There are several EBM that can be used to assess genotoxicity in the presence or absence of an external metabolic activation, e.g. by the use of S9-mix, such as Ames, micronucleus test (MN), Comet assay, P53, SOS-umu test, SOS-chromo test and others. Below are described some examples.

Intercalibration of genotoxicity tests

In the context of an Interlaboratory test coordinated by Norman Network in which the Department Environment and Health of the Italian Institute of Health (R. Crebelli and M. Carere) has participated, several eco-genotoxicity methods have been intercalibrated in 2018. A report has been published in 2019 (51). We have performed the Ames MutagenicityTest.

The aim of the interlaboratory study was to explore the performance of different bioassays for genotoxicity and related mechanisms and to generate communication, discussion and inspiration on the use of bioassays that detect (potential) genotoxicity of mixtures of chemicals. Samples

contained a mixture of three genotoxic chemicals from different classes of compounds; polycyclic aromatic hydrocarbons, aromatic amines, and a pesticide precursor. The samples were dissolved in either sewage treatment plant (STP) effluent or dimethyl sulfoxide (DMSO). Samples in the STP effluent represented realistic environmental water samples, while the DMSO mixtures represented concentrates thereof. Participants were encouraged to use their in-house assays and analysis methods to test samples. As a result, there was great variety in the number and variation on the assays tested.

The methods used were the following

Ames Mutagenicity

The Ames test is one of the most commonly applied used bioassays for water quality (52). The Ames test uses strains of *Salmonella typhimurium* with mutations which inhibit the bacteria's production of histidine (auxotrophic mutants). The bacteria are therefore unable to grow without the addition of histidine to the growth medium. When the auxotrophic bacteria are exposed to test samples which contain mutagenic compounds, the bacteria can revert back to being able to grow on medium without histidine (prototrophic). Often (rat) liver enzyme is added to test the metabolic activation of test components. In the case of the fluctuation test this is performed entirely in liquid culture and revertant bacteria are often detected by a change in color of sample wells. The color change is a result of bacterial metabolism reducing the pH of the medium in the well (53). The Ames test has many advantages, it is a very versatile assay, its different modifications have been developed to determine mutagenic potencies, and it is recommended by several agencies

p53 CALUX

The p53 CALUX assay detects activation of the tumor suppressing gene, TP53. Increased p53 levels are indicative of genotoxicity, as the p53 protein responds to DNA damage, and is a transcription factor for genes related to DNA-damage repair, cell-cycle arrest and apoptosis (54). Chemical activated luciferase gene expression (CALUX) is a bioassay used to detect specific chemicals in a sample. This is done through a modified cell line with a luciferase reporter gene and response elements which induce transcription of the light generating enzyme (BDS, 2014). The p53 CALUX test uses human osteosarcoma cells (U2OS cells).

Micronucleus

The micronucleus test is used to identify the (chemical-induced) formation of micronuclei (small membrane bound DNA fragments) in the cytoplasm of cells. These micronuclei contain lagging chromosome fragments or whole chromosomes. The test often uses the Chinese hamster ovary (CHO) cell line and can be performed with and without metabolic activation (±S9). Cells are visually scored for the presence of micronuclei. Increased frequency of micronuclei is indicative of induced chromosomal damage (55).

ToxTracker

The ToxTracker test is a green fluorescent protein based genotoxicity assay consisting of different mouse embryonic stem (mES) reporter cell lines which are responsive to compounds which are genotoxic or induce oxidative stress (56). The ToxTracker assay is also able to provide insight into the primary toxic properties of compounds through integrated evaluation of the results from the different reporter cells in the test. In this trial, genotoxicity, oxidative damage, cellular stress and protein damage were assessed using the ToxTracker

SOS-Chromo

The SOS-Chromo test detects DNA damage by quantifying the expression of the sfiA gene, which is a part of the SOS repair system. In *Escherichia Coli* PQ37 the lacZ gene is controlled by the sfiA promoter (57). When DNA damage occurs due to genotoxic samples, the SOS repair system is activated, the lacZ gene is induced and the synthesis of β–galactosidase is quantified by a color change (optical density).

UMU-Chromo

The UMU-Chromo test detects DNA damage by quantifying the expression of the umuC gene, which is a part of the SOS repair system. In *S. typhimurium* TA 1535 [pSK 1002] the umuC gene is fused to the lacZ reporter gene (58). Similar to the SOS-Chromo test, when DNA damage occurs, the SOS repair system is activated, the lacZ gene is induced and the synthesis of β–galactosidase is quantified by a color change (optical density).

Comet

The Comet Test, also called the single cell gel electrophoresis (SGCE) test, detects DNA damage in cells (48). After exposure, cells are lysed removing all cellular protein so that only DNA remains. The DNA is allowed to unwind under alkaline conditions, then electrophoresis is applied. Under electrophoresis, smaller DNA fragment travel faster than larger, more intact DNA fragments, forming an image of a comet, with intact DNA at the head of the comet and a tail of DNA fragments. The extent of DNA damage is directly proportional to the size of the comet tail.

Overall, the interlaboratory study has generated a large and variable data set to begin to analyze the performance of different bioassays for genotoxicity and related mechanisms. The variability of the data meant that detailed analysis was not possible within the scope of this report, however, general conclusions have been presented along with suggestions for future research.

Case Study: Focus on FET (Fish embryo toxicity) –An example of EBM

Together with my colleagues of ISS we have performed a specific literature review with the aim to critically evaluate previous studies that adopted early life stages of zebrafish (i.e. embryos and early larvae) as innovative experimental models for identifying and investigating the ecotoxicological profiles of chemicals that are listed as priority or emerging substances under the WFD, the work has been published in Microchemical Journal in 2019 (59). An extract of the paper is reported below.

Introduction

This literature review focused on studies performed using bioassays with zebrafish embryo and early-larvae for the detection of toxic effects of chemical substances on the early development of this organism. All Priority substances listed in the WFD and all the chemicals included in the Watch List for emerging water pollutants have been considered in this review. Totally, we considered 59 chemicals or groups of chemicals as they are listed in the WFD and the WL. The literature studies considered in this literature review were collected using the online tool Google Scholar, by entering the name of one of the 59 substances or group of substances (e. g. "mercury") and "zebrafish embryos" or "zebrafish early larvae" or "*Danio rerio*" as keywords. Only the most cited literature studies compatible with our goals were considered, based on the results returned by the search engine. According to the Directive 2010/63/EU on animal welfare and scientific research, the principle of the 3Rs (Replacement, Reduction, Refinement) requires to minimise the number and suffering of tested animals. The earliest life stages of zebrafish (Danio rerio,zf) meet such principle and do not fall into this regulatory framework. Therefore, the use of embryos and early larvae is considered as a valid alternative to animal testing on adult individuals. Zebrafish embryos and early larvae were used as models in all the studies that we collected. The literature studies that have been reported in this work are associated to the different PSs and WL substances.

Results and Discussion

A list of all the effects reported collecting all the literature studies is shown in Table 1.

These effects include lethality endpoints and several sublethal aberrations at morphological, molecular, physiological and behavioural level. Each effect can be due to one or more MoA as they represent the detectable results caused by different toxicity mechanisms. We identified 10 MoA types that are able to cause different deleterious effects in zebrafish early stages (Fig. 16). The MoA types reported and their frequency emerged from the considered literature studies are: DNA toxicity (genotoxicity, mutagenicity and gene expression; 23.31%), carcinogenicity (3.07%), neurotoxicity (19.02%), cardiocirculatory toxicity (13.50%), endocrinotoxicity (6.13%), immunotoxicity (2.45%), reproductive system impairment (1.23%), oxidative stress (9.20%), cell cycle impairment (2.45%) and developmental toxicity (19.63%). Each MoA and the relative effects were then associated to the PSs and WL substances.

As reported above, the three main MoA that we found in this review are: DNA toxicity, neurotoxicity and developmental toxicity. About the experimental techniques to detect the different MoA, the developmental toxicity results in morphological aberrations on early stages of zebrafish that are clearly identified by optical microscopy. All the other MoA need instead more specific investigations, i.e. molecular, physiological and behavioural analysis. For instance, DNA toxicity, that is the most found MoA in this review, represents an important field of survey for understanding the deleterious effects at genetic level, that is also strictly connected to other toxicity mechanisms, such as carcinogenicity. Indeed, several studies demonstrated that many PSs affect the DNA of zebrafish embryos. Combination of methods using different multiple endpoint testing on this animal model could be important in the environmental chemical contamination assessment based on the EBMs. Zebrafish early stages are also a valid model for investigating complex mechanisms as the neurotoxicity. Among potential neurotoxicants, pesticides represent a large group of chemicals belonging to PSs and WL substances and are particularly hazardous for zebrafish. Performing behavioural analysis and recording alterations in locomotor activity allows revealing the potential neurotoxicity of chemicals and environmental water samples. Swimming behaviour of zebrafish larvae by 120 hpf is a useful tool in neurotoxic assessment. Neurotoxicants in the environment are increasing in amount and number, but neurotoxicity assessment is still not required for monitoring programmes and then they are little performed . Therefore, zebrafish early stages allow a very large spectrum of morphological, physiological, molecular and behavioural observations.

Fig. 16: MoAs reported

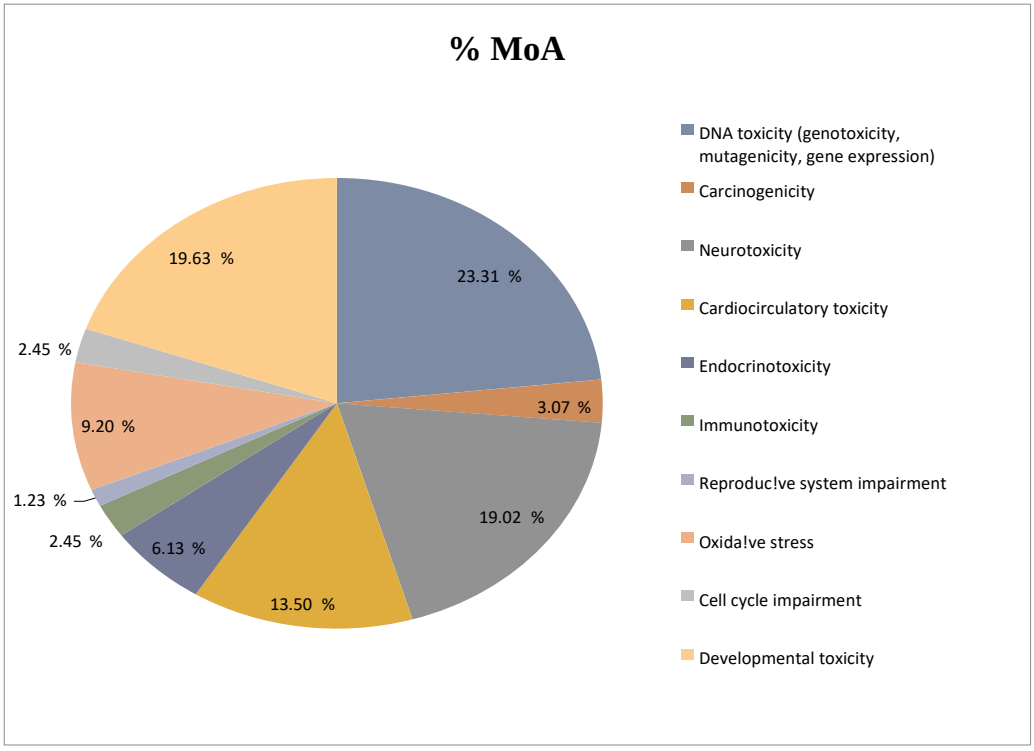


Table 1: Effects reported in the manuscript

Lack of somite formation	Non-detachment of the tail	Lack of heartbeat
Weak heart beat and blood flow	Endocrinological defects	Immunological diseases
Locomotor activity	Pericardial edema	Yolk sac edema
Phototactic response	Yolc sac deformities	Tail malformation
Notochord and column malformations	Eye malformations	Cranial/head/Brain malformations

Paralysis, reduced motility and touch response	Uncontrolled convulsions	Muscle structure
General underdevelopment and body size reduction	Premature organogenesis	Chondrogenesis inhibition
Fin malformations	Hypopigmentation or absence of pigmentation	Advance or delay in hatching
Developmental delay	Liver damages	Kidney damages
Upregulation/downregulation gene expression	Biochemical alterations	Coagulated Embryos

Conclusions

The zebrafish early stages model enables effect investigation on a wide range of biological aspects, from the molecular to the whole-organism level. The FET test is very precious to detect the effects of chemicals and mixtures included in environmental samples.

Indeed, this test allows a quality water assessment through the analysis of potential morphological aberrations occurring during the embryonic fish development into potentially polluted samples: it is not by chance that its use has been increasing also in environmental monitoring. Moreover, the use of zebrafish embryos has become very popular also because of their special properties ranging from the high fecundity and ease of maintenance to the rapid growth and transparency of the shell that allows optical observation during the ongoing development saving time, tools and meeting the aforementioned ethical objectives aiming to reduce the animal sufferance. The peculiarity of this test is exactly the possibility to treat zebrafish early stages like elements of in vitro experiments, but since they are developing organisms, we can use them as if they were models for an in vivo test. Furthermore, this species is set out in an extensive literature that eases several kinds of analysis and it shares a large part of its genome with humans (approximately 70% of human genes have at least one obvious zebrafish orthologue since several critical pathways that regulate vertebrate development are highly conserved. These features make zebrafish a good candidate to indirectly evaluate the effects on public health especially because it has been demonstrated by many experiments that zebrafish embryos represent promising models for predicting toxicity and teratogenicity of chemicals in mammals.

As an outcome of the study, the potentiality of the zebrafish embryo model to detect an extraordinary amount of effects after a short and premature exposure to WFD-relevant single toxicants was demonstrated. Fish embryotoxicity tests have been already used for effluent toxicity assessment and the European Commission has recently recommended this test among different toxicity bioassays for wastewater industrial treatment . Therefore, early life stages of fish represent attractive models for understanding toxic mechanisms and assessing the environmental risk of chemicals, both in water and sediment samples. The impressive and increasing number of studies using zebrafish early stages demonstrates the potentiality of this model in monitoring plans that need fast and preliminary screening on the effects of vertebrates exposed to chemicals, mixtures or environmental contaminated samples. Our recommendation is to use the EBM on indicator organisms such as zebrafish early stages as an integrative model for surveillance and investigative monitoring programmes within the WFD together with other bioassays with a view to screening water bodies, supporting risk assessment and prioritizing the practical measures that must be implemented.

CHAPTER 3

Experimental Application of EBM

Aims of the study and Site selection

The experimental application of Effect Based Methods has been carried out in the laboratories of the Unit Ecosystems and Health of the Italian Institute of Health (Istituto Superiore di Sanità).

The Area of the study was located along the Tiber River basin in the Lazio Region. The Tiber River basin has been previously selected in the context of the Water Framework Directive (8,60) as European pilot river basin with the aim to test the guidelines elaborated in the context of the Common Implementation Strategy and for this reason has been chosen also for the experimental part of the project.

The general aims of the experimental study were the following:

- Evaluation of the chemical pollution of the urban part of the Tiber river basin through the use specific EBMs (bioassays in vivo) with a support of chemical analysis
- Application of EBM as tools for investigative monitoring during specific events
- Recommendations to be implemented in future monitoring strategies useful for an update of the legislation

The Area of the study was located along the River Tiber basin in the Lazio Region, comprising two different sites along the main watercourse and one tributary (Figure 14), other 2 sites in the urban part of the River have been selected furing a fish-kill event happened in 2020. The Tiber river Basin has been selected as Pilot River Basin the context of the WFD (2005)

The Tiber River is the second largest river after the Po, rising on the slope of Monte Fumaiolo, a major summit of the Apennine Tosco-Emiliano, with a catchment area of 17,375 km^2. The water volume ranges from 60 $m^3 \cdot s{-}1$ to 3200 $m^3 \cdot s{-}1$, with a yearly average of 230 m3·s−1. The river is 405 km long, and runs through four administrative regions from Emilia-Romagna, Tuscany, Umbria e Latium. Its major tributaries are the Chiascio, Nestore, Paglia, Nera, Treja, Farfa and Aniene. The lower stretches the Tiber flows through the city of Rome and the Tiber branches out into a delta enters, the main channel being the Fiumara (Fiumicino) to the Tyrrhenian Sea.

The Tiber River has been classified based on the principle established in the context to by WFD as "Very Lage rivers" because its catchment area is more than 10.000 km² and River type is " RL2" because its water alkalinity is major than 0,5 meq/L (61). The anthropogenic activities that affect the waters quality are due to industrial settlements such as steel mills, chemicals and paper mills, are mainly located along Nera and Aniene tributaries. The uses of the water resources are multiple, first of all the direct use of the Appennine springs and the Lazio volcano lakes for the water supply of urban settlements and Rome in particular. A significant use of surface water is linked by irrigation and the most important withdrawals are those of the upper Tiber valley and those of the final strength of the river in Ostia and Maccarese areas. The water withdrawals for industrial uses are present in the middle valley of the river in Tivoli and along the industrial area of the Aniene. Furthermore the city of Rome the River receives the waste water of the treatment plants. The total human population living in this geographic area is approximately 4.7 million people.

The plan of the River Tiber Basin Authority (TRBA) reports a large land agricultural use of the basin covering about 53% of the surface, while approximately 39% is forested and 5% is urbanised.

Previous studies in the Tiber River found different organophosphate (OPPs) pesticides in the river and in its estuary, even if mostly in lower concentrations than the guideline values (62). Moreover, other environmental pollutants were found in the last stretch of the river. For instance, high concentrations of pharmaceuticals, such as the hydroxymetabolite of the mood-stabilising drug carbamazepine and the non-steroidal anti-inflammatory drug diclofenac, PAHs, pesticides, perfluorinated and polyfluorinated alkyl substances (PFAS), were detected (63). The occurrence of antibiotics was recorded (64) and also alkylphenols (65,66). Other studies have also detected mutagenic effects in the urban part of the river (67,68) and also PCB and organochlorine pesticides have been detected in another study (69). The data of the local environmental agency obtained following the current legislation show that some priority substances, e.g. nickel, exceeded the environmental quality standards (EQS) in the Tiber River (70).

The Tiber River basin, in general, has experienced prolonged dry periods. Major drought events that affected the entire basin occurred in 1955, 1971, 1987, 1990, 1993, 2003 and 2007. Floods are usual along the Tiber River. There were frequent floods in the first decade of the millennium and in the last years.

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The sampling strategy (figure 17) has included 2 sites along the urban river stretch within Rome, in both southern and in northern parts, and 1 sampling site located in the protected area of tributary River Farfa. Specifically, the upstream site Castel Giubileo (CG) is located in the northern part of Rome before the entrance in the main city (this site is likely affected by leaching of waste, agricultural and zootechnical discharges as well as the presence of small and medium factories). The downstream site Mezzocammino (MC) is located in the south of Rome, about twenty kilometres from the estuary in the Tyrrhenian Sea and is located right after a sewage treatment plant. The site River Farfa (FA) is located in a River Tiber tributary at around 50 km before Rome: it was chosen as a reference site for its good ecological status detected following the WFD requirements (71).

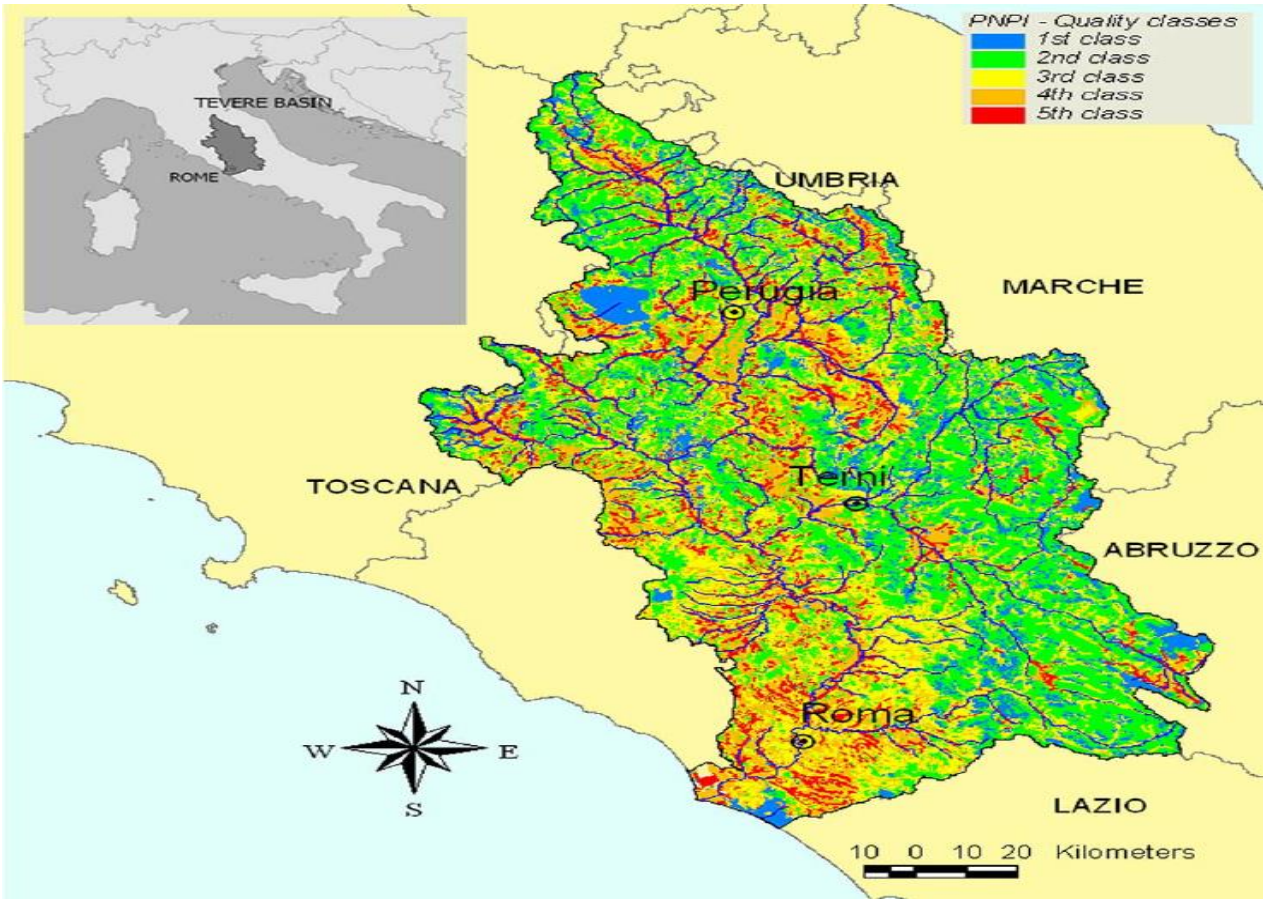
Three sampling campaigns were conducted in June/July 2018, December 2018 and July 2019, in each site.

Furthermore in May 2020 the FET test has been applied in a:

- sampling campaign to evaluate the effects of the Lockdown due to the Covid-19 (CG, MC and AN)
- sampling campaign during a fish kills event that happened in the urban part of the River Tiber, in this case have been taken the day after the event at Ponte Milvio (PM), Tiberina Island (IT) and Aniene river (AN).

Figure 17: Map of the 3 selected sites

Figure 18: Tiber River Basin



Analytical Methods

Analyses were carried out at the Unit of Ecosystem and Health of the Italian Institute of Health (Istituto Superiore di Sanità, ISS, Rome). The Unit works in quality according to the UNI CEI EN ISO/IEC 17025 standard and participates in different national and European interlaboratory comparisons. Water samples (2 L) were collected in each site between 0 and 20 cm from the

surface water and then they were quickly stored at +4 °C. Each sample was filtered at 0.45 µm in order to remove the suspended materials, analysis have been perfomed also on on filtered samples.

The Methods Applied are 2 EBM standardized widely used in Europe (72):

- **Daphnia Magna Acute OECD Test 202:2004**
- **Fish Embryo Acute Toxicity (FET) test (OECD 236/2013)**

Furthermore chemical analysis have been performed in the 3rd campaign about a wide range of emerging contaminants. Also the Comet assay has been applied (on zebrafish embryos), but the data are preliminary and are not included in the results.

Daphnia Magna OECD Test

Daphnia sp. Acute Immobilisation assay (OECD 202:2004). The Daphnia sp. Acute Immobilisation assay is a screening method using freshwater crustacean daphnid species. In this study, the test was performed starting from the resistant forms of D. magna, i.e. ephippia, that were included in the kit named as Daphtoxkit® and developed by the Laboratory for Environmental Toxicology and Aquatic Ecology (LETAE) at the University of Ghent, in Belgium. This test has been performed in 2018 and in the 3rd Campaign of 2019.

The experimental procedure application followed the OECD No. 202:2004 guideline (73). The tests lasted 48 hours. All the physicochemical parameters were measured at the start of tests and after 48 hours. Six independent tests were performed, one for each sample. Three tests were carried out during 2018, while the other three during 2019. Each test was performed in three replicates. Twenty daphnids per sample (no longer than 24 hours after the hatching) were exposed in multi-well plates and they were incubated at 21 ± 1 °C in the dark. Each well contained five daphnids in 10 mL of the water sample. A control was performed exposing twenty daphnids to the test medium. At the end of the experiment, the number of immobilised individuals was recorded. Daphnids were considered immobilised if no directed movement was observed within 15 s after gentle stirring.

Fish Embryo Acute Toxicity (FET) test (OECD 236/2013)

Wild type zebrafish embryos were used to perform the analysis. The bioassay was conducted according to the OECD No. 236:2013 guideline (74). The embryos were collected from the breeding groups at the Laboratory of

Breeding fish were maintained in tanks with a loading capacity of 1-L water per fish at 26 ± 1 °C and with a fixed photoperiod of 12:12 (light:dark). Independent tests were performed, one for each sample. Each test was performed in two replicates. Zebrafish eggs were exposed in 24-well plates at a developmental stage ranging from 32 to 128 cells of segmentation. Each well contained one egg in 2 mL of the sample. A plate control and internal control were prepared with the test medium. Embryos were kept in dark conditions for four days at 26 ± 1 °C. The morphological observations of the embryos were made at 96 hours post fertilisation (hpf).

Four apical observations were recorded as lethal endpoints indicating acute toxicity:

- coagulation of the embryo,
- non-detachment of the tail,
- lack of somite formation,
- lack of heartbeat.

Sublethal endpoints were also recorded in order to improve the evaluation of the sample toxicity with an enhanced level of detail. The investigated sublethal endpoints were: spine deformation, hatching delay, general underdevelopment, absence of pigmentation, eye deformation, tail deformation, fin deformation, low heartbeat, head skeleton malformation, edema.

Figure 19: morphology of normal embryo at 96h

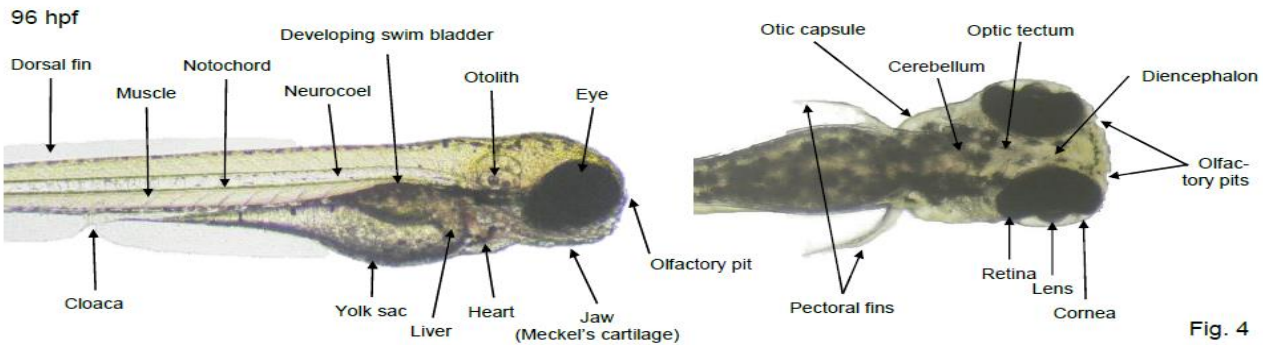
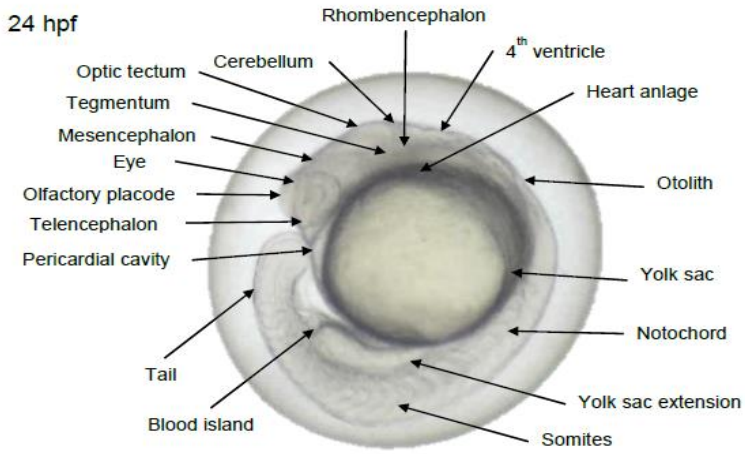


Figure 20: morphology of normal embryos at 24h



Danioscope

DanioScope is a software of the Noldus that use a video of one or more zebrafish embryos. DanioScope automatically recognizes each embryo (within their chorion). Then it measures their activity. If floating occurs you can simply adjust the location of the measurements during the experiment.

Besides dynamic measurements, DanioScope also allows you to easily monitor the morphology of your zebrafish. You can upload images or take snapshots from videos to monitor changes in morphology.With intuitive drawing tools you can define distances or areas you are interested in, and after calibration DanioScope will measure lengths and surfaces automatically. This way you can easily determine tail length, eye size, and pericardial area. Or any other measurement, because you can define your own! By images from different time point, you can easily monitor growth and malformations over time.

Photograph Instrumentation

The observations of the daphnids and the photographs of representative zebrafish individuals were performed using a Leica S8AP0 stereo microscope linked to a Basler acA 1300-60 gm camera. The

images were acquired thanks to the software Media Recorder™ 4.0 provided by Noldus Information Technology.

Comet Assay with Zebrafish embryos

This assay has been performed by the colleagues Francesca Marcon and Cristina Andreoli of the Unit Mechanisms of Toxicity, Modelling and Mutagenicity of ISS, the embryos have been selected and collected by Ines Lacchetti (Unit Ecosystems and Health of ISS). This test should be further implemented in future and the results are not discussed, but a short description is described below.

Zebrafish embryos (20-25 per experimental point) were exposed for 48 or 96 hours to water samples collected in three different sites of Tiber river, namely Castel Giubileo (CG), Farfa (FF) and Mezzocamino (MZ) in the first campaign. Negative and positive controls were included in each experiment, respectively represented by embryos exposed to fresh water and ionizing radiation; 4Gy from a ^{137}Cs source (0.8 Gy/min) was the dose selected for the positive control. Briefly, after anesthetization (10 minutes in ice), embryos were transferred in a glass potter containing cold PBS and gently homogenized to obtain a cell suspension that was cleared of debris by filtration through a 50 μm BD filter; cells were washed two times in cold PBS (centrifugation 10 minutes, 200 g, 4°C), counted and diluted in cold PBS to obtain the final concentration of $1{,}5x10^6$/ml. Alkaline version (pH>13) of Comet Assay was performed as described in Andreoli et al., (49) to evaluate the induction of DNA strands breaks. Cells ($3x10^5$ per experimental point) were embebbed with 0,7 % Low Melting Point Agarose (LMA) on 1% Normal Melting Point Agarose(NMA) precoated slides, in duplicate for each experimental point. Slides were kept on ice for 10 minutes and then immersed in the Lysis solution (10mM Tris-HCl, 2.5M NaCl, 100mM Na2EDTA, pH 10, with 1% Triton and 10% DMSO freshly added at 4°C) for 1 hour at 4°C. Slides were incubated in cold Electrophoresis buffer (1mM Na2EDTA, 300mM NaOH, pH 13) for 20 minutes for DNA denaturation. Electrophoresis was carried out for 20 min at 25 V and 300 mA (0,8 V/cm) at 4 °C with the same buffer. Furthermore, slides were neutralized in buffer (0.4M Tris-HCl, pH 7.5) and stained with ethidium bromide. The analysis was performed using a fluorescent microscope (Leica,Wetzlar, Germany) and DNA damage was quantitated as percentage of DNA in the tail by a dedicated image analysis system (IAS 2000 Delta Sistemi Italia), randomly scoring a total of 100 nucleoids (50 per slide) for each experimental point.

Chemical Analysis

The Chemical Analysis have been performed at the Helmoltz Centre for Environmental Research UFZ of Leipzig (Werner Brack and Riccardo Massei). A list of 504 compounds likely to occur in environmental samples (i.e. pesticides, pharmaceuticals, industrial products) were selected for chemical analyses in the 3rd Sampling campaign (July 2019). Water samples were directly injected in the LC instrument after filtration.

Analyses were performed on a quadrupole-Orbitrap MS (QExactive Plus, Thermo). A Thermo Ultimate 3000 LC system with a Kinetex 2.6 μm EVO C18 (50x2.1 mm) column equipped with a pre-column (C18 EVO 5.x2.1 mm) and an inline filter was used for chromatographic separation. For data evaluation the Tracefinder 3.2 Software (Thermo) was used. UFZ used an internal, method-matched calibration for quantification of the samples. For confirming identity of target compound peaks, accurate masses and retention times of the main adduct in the full scan runs (usually M+H+ or M-H-) were used as well as the match of experimental and theoretical isotope patterns fund the presence of one or two fragment ions from the MS/MS experiments. For quantification, the peak area of the main adduct in full scan mode was used. For prioritization purposes, the lowest Predicted No Effect Concentration (PNEC) values were retrieved from the NORMAN Ecotoxicology database (https://www.norman-network.com/nds/ecotox). PNEC values were predicted using QSAR models for freshwater and in different environmental matrices (sediments, marine water and biota).

Results

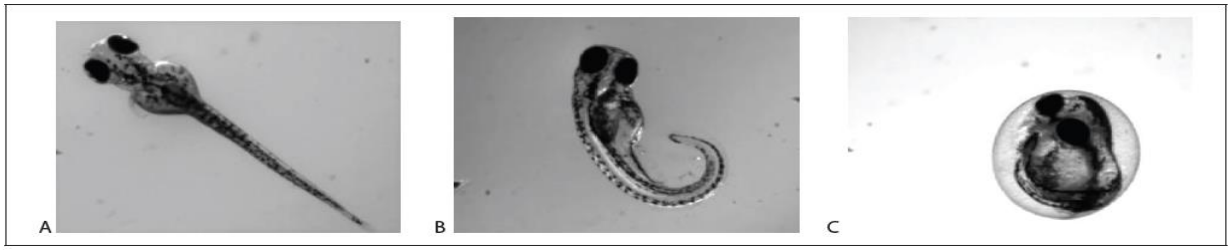
Fish embryo toxicity (FET)

Lethal and sublethal endpoints on zebrafish embryos were observed and recorded in all the tests. The mortality rate was ≤10% in the plate control and internal control. Mortality was observed in all the tested samples for each site, although in the third campaign of 2019 the percentage is much lower and a minimum number of lethal effects can be due to the mortality rate characteristic of this species. Indeed, only the embryo mortality percentage greater than 10% is considered significant according to the guideline. CG showed a difference in lethality between CG 1 and CG2 and CG3. Sublethal endpoints were also recorded in all the samples (see figure 21) and occurred as spine deformation, delay or absence in the hatching and general underdevelopment.

Table 2: Results of the FET test in the 2 campaigns of 2018 and the campaign of 2019.

	% Mortality	% Sublethal Effect
CG 1	5	5
FA 1	25	12,5
MC 1	27,5	5
C-	0	0
CG 2	27,5	10
FA 2	6,6	36,6
MC 2	27,5	12,5
C-	0	0
CG 3	12,5	2,5
FA 3	10	7,5
MC 3	5	7,5
C-	0	0

Figure 21: Sublethal effects detected by the FET test. All the images were obtained at 96 hpf. A) represents a normal developing hatched embryo; B) shows the spine deformity; C) is a non-hatched individual



Results of Campaigns of 2020 with FET

The FET test has been also applied to the samples detected in two different sampling campaigns carried out on the Tiber river in Rome. The first campaign was performed in order to check the effects of the Covid -19 lockdown in May and took place on three sites, Mezzo Cammino (MC4), Castel Giubileo (CG4) and Aniene (AN1).

The second campaign was performed on two different sites located in the urban stretch of the same river (Ponte Milvio (PM) and Isola Tiberina (IT) and on a third site located along Tiber's main tributary Aniene (AN2), concomitantly with a massive fish mortality that occurred on the 31 May 2020. The Aniene sampling site is located at the Montesacro quarter in proximity of the "Ponte Nomentano bridge".

For the first campaign the FET test was performed on the samples of MC4, AN1 and CG4 (all samples were filtered to 0.45μm). For all samples two different 24-wells plates were set up and a negative control plate was added to the test. The results showed a condition of general toxicity in all the filtered samples, with mortality values between 15 and 40%. In almost every plate sub-lethal effects were reported. In the PM sample a mean mortality of 28% was observed, with sub-lethal effects on two embryos.

For the second campaign the same test was performed on the samples of AN2, IT and PM (all samples were filtered to 0.45) and on the non-filtered IT and PM samples.

As for the previous test, for all samples two different plates were set up and a negative control plate was added to the test. The results showed a mortality rate between 15 and 26.3%, with sub-lethal effects observed.

Among the sub-lethal effects of the second campaign spinal cord deformities where frequently observed, with lordosis and scoliosis in most cases (see figure 23). This kind of malformation has generally no potential for regeneration. Moreover, some individuals affected by these deformities showed a partial lack of pigmentation, with pigmented eyes but little or no cromatophores along the body. The same condition has been observed even in individuals not affected by other forms of sub-lethal effects.

Table 3: Results of the FET test in the 2 campaigns of 2020 (the data are average of 2 readings)

Post lockdown	**% Mortality**	**% Sublethal Effect**
CG 4	**20**	**2,5**
MC 4	**30**	**7,5**
AN 1	**43**	**5**
Fish Kills		
PM (NF)	**28**	**5**
PM	**10**	**33**
PM*	**45**	**15**
IT (NF)	**23**	**10**
AN2	**22,5**	**0**

- repeated sample

Table 4: Results in detail of sample of Ponte Milvio (PM) collected after the fish kills

Ponte Milvio	**Mortality %**	**No Hatching rate %**	**sublethal**					
			P	**SD**	**H**	**E**	**U**	**SW**
A	55	10	4/9	2/9	1/9	1/9	1/9	3/9
B	35	20	2/13	5/13	0	0	0	2/13
Tot % ± s.dev	**45,0 ± 14,1**	**15,0 ± 7,1**	**17,7 ± 3,1**	**30,3 ± 11,5**	**5.6 ± 7.9**	**5.6 ± 7.9**	**5.6 ± 7.9**	**24.4 ± 12.7**

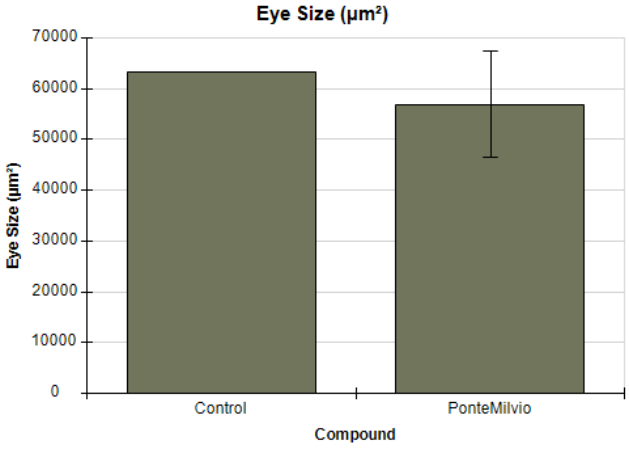
P, pigmentation; SD, spine deformation; HB, slow heartbeat; E, edema; U, underdeveloped; SW, swimming behavior unnormal

The results show high acute toxicity with a value of 45% of dead embryos at 96 hpf. Furthermore, many sublethal effects occurred after exposure; in particular, 30.3% of embryos looked deformed with spine deformation (scoliosis or lordosis) and 17.7% appeared with poor pigmentation, also the swimming behavior appeared not normal 24,4% of larvae had a trembling swim.

Table 5: variations of body length and eye size in the ponte milvio sample (Danioscope)

Compound	Body Length (µm)	Body Length (µm)	Body Length (µm)	Eye Size (µm²)	Eye Size (µm²)	Eye Size (µm²)
Compound	N samples	Mean	Standard Error	N samples	Mean	Standard Error
Control	1	4096.002	-	1	63287.91	-
PonteMilvio	10	3463.046	194.2168	10	56952.73	10438.75

Figure 22: Eye size and Body length reduction measured with Danioscope



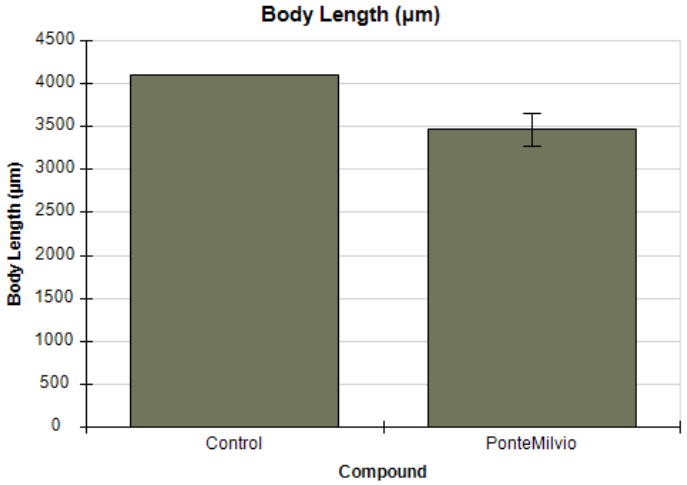


Figure 23: Embryos deformity (scoliosis) in Ponte Milvio sample

Daphnia *sp.* Acute Immobilisation assay

The bioassay with Daphnia has been applied only in the first (2018) and third sampling campaign (2019). The results (72) of *Daphnia* sp. Acute Immobilisation assay did not show any acute effect for the two Tiber River samples of MC and CG. The recorded percentage of mobility inhibition ranged between 0% and 20%, for the 2 campaigns. The percentage of immobilised individuals recorded was closed to 0% as regards the third campaign (MC2 and CG2 in table 6-to be noted that these are the samples of the 3rd campaign of 2019.). However, the first sample of Farfa River (FA1) weakly affected the motility of daphnids showing an acute toxicity effect, ranging from 15% to 35%, while the second sample (FA2-to be noted that this is the sample of the 3rd campaign of 2019) was not toxic for the crustacean (*Table 6*). Samples that show values ranging between 20% and 50% are considered low toxic on the basis of the scale of toxicity used by Regional Environmental Protection Agency of Lazio – ARPAL. Statistical analysis revealed that the differences between the two campaigns are significant (*Figure 25*). However, considering the low amount of the data, these conclusions have to be treated very carefully and further analyses are needed.

Table 6 (Cristiano et al, ref 72) **:** Results of daphnia magna test (2018 and 2019).

Sampling	Site	Replicate 1 (%)	Replicate 2 (%)	Replicate 3 (%)	μ	σ
2018	FA1	30	15	35	26.7	10.4
	CG1	10	10	15	11.7	2.9
	MC 1	5	20	5	10	8.7
	C-	3,3	0	0	1.1	1.9
2019	FA2	5	5	0	3.3	2.9
	CG 2	0	0	0	0	0
	MC2	0	0	0	0	0
	C-	0	0	0	0	0

FA2, MC2 and CG 2 are the samples of the third sampling campaign, in the other parts of the Thesis these are mentioned as FA3, MC3 and CG3.

Statistical Analysis

Some of the experimental data were analysed performing non-parametric tests for the campaigns of 2018 and 2019. The Kruskal-Wallis test allowed the comparison among the sampling sites in the two different years (2018 and 2019) in the same period (July) and the different time pattern showed by the Delta (values of 2019 – values of 2018). Moreover, the average and the standard deviation were calculated where possible. The statistical analysis was performed with the aid of the SAS® software. (see reference n. 72).

Figure 24: Wilcoxon Score Distribution showing the different time pattern (values of 2019 – values of 2018) indicated by the delta. Significance is expressed as Pr > Ch-square and it has be equal to ≤0.05. FA: Farfa River; CG: Castel Gandolfo; MC: Mezzocammino; C-: negative control.

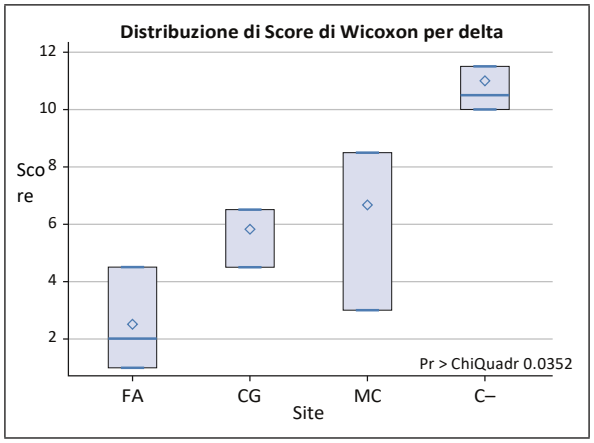
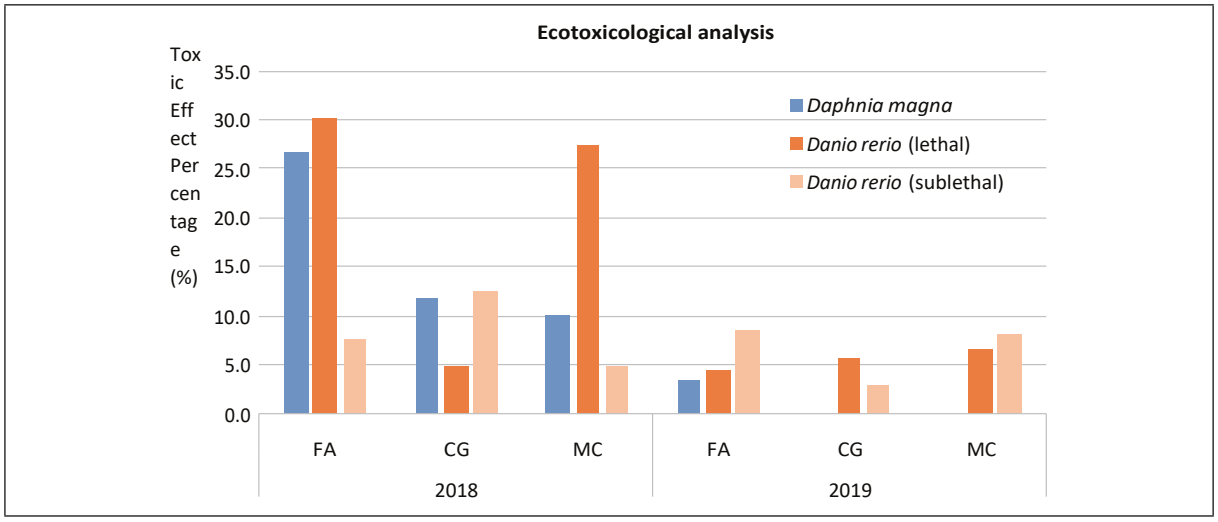


Figure 25: data of Daphnia and FET in the first (summer 2018) and third (summer 2019) sampling campaigns (see reference n.72).



Chemical Analysis

The Chemical analysis have been performed in the 3rd campaign of 2019.

Chemical analyses detected 34 chemicals out of a list of 504 chemicals. Among the 34 chemicals, 4 compounds were detected below the limit of quantification. Overall, concentration ranged from 4.9 ng/L (metalaxyl) up to 3.5 µg/L (di-n-butyl phosphate). The highest number of compounds were detected in the area of MC followed by GC and FA. Overall, UFZ detected in all the sites concentrations of industrial chemicals, plasticizers and flame retardant such as tri(butoxyethyl)phosphate, 2-naphthalene sulfonic acid, Di-n-butyl phosphate with a concentration ranging between 1 and 3 µg / L. In particular, the area of MC showed a higher incidence of pharmaceutical products (10) and pesticides (7) with respect to the other two sites. Furthermore, UFZ detected high concentrations of telmisartan (1.1 µg/L), the bronchodilator agent theophylline (747 ng/L) and the antibiotic erythromycin (538 ng/L). Moreover has detected high concentrations of the herbicide metazachlor (2.6 µg/L).

The area of GC was characterized by a lower incidence of pharmaceutical (3) and pesticides (4). While it was still possible to observe a high concentration of metazachlor (1.2 µg/L), the antimicotic ciclopirox was detected for the first time in this location at a concentration of 1.1 µg/L. Finally, the only pharmaceutical detected in FA was erythromycin at a concentration of 544 ng/L.

An overview on the chemical analyses is given in table 5

Table 7: Concentrations levels of emerging contaminants and PNEC (predicted no effect concentrations). In red substances for which PNEC has been exceeded**.**

Compounds	**Concentration levels (µg/L)**	**PNEC (predicted no effect concentration) (µg/L)**
Tri(butoxyethyl)phosphate	**0.084(FA) 1.109 (CG) 3.1 (MC)**	**0.14**
Di-n-butyl phosphate	**0.136 (FA), 3.578 (CG), 0,308 (MC)**	**5.8**
Benzyldimethyldodecylammonium	**0.334 (FA), 0.334(CG)**	**0,062**
Lauryl diethanolamide	**0.018 (FA) 0.044 (CG)**	**0.95**
Tetrabromobisphenol A	**0.258 (FA) 0.483 (CG), 0.371 (MC)**	**0.28**
Erythromycin	**0.544 (FA) 0.538 (MC)**	**0.2**
2-Aminobenzimidazole	**0.125 (MC) 0.147 (CG)**	**2.31**
5-Methyl-1H-benzotriazole	**0.125 (MC) 0.147 (CG)**	**150**
Propamocarb	**0.061 (MC) 0.058 (CG)**	**710**
Metazachlor	**2.662 (MC), 1.270 (CG)**	**0.02**
Carbamazepine	**0.028 (MC) 0.007 (CG)**	**0.05**
Gabapentin-Lactam	**0.349(MC), 0.266 (CG)**	**100**
DEET	**0.17 (MC) 0.014 (CG)**	**88**
Triethylcitrate	**0.182 (MC)**	**73.9**
N-Butylbenzenesulfonamide	**0.081 (MC)**	**21**
2-Naphthalene sulfonic acid	**2.221 MC)**	**34**
Caffeine	**1.353 (MC)**	**1.2**
Cotinine	**0.148 (MC)**	**10**
Metalaxyl	**0.005 (MC)**	**20**
Kresoxim-methyl	**0.051 (MC)**	**0.1**

Ethofumesate	**0.077 (MC)**	**3.1**
Dinoseb	**0,037 (MC)**	**0.41**
Metazachlor BH479-12	**0.278 (MC)**	**-**
N-Acetyl-4-aminoantipyrine	**0.034 (MC)**	**100**
Ketoprofen	**0.04 (MC)**	**2.1**
Ranitidine	**0.495 (MC)**	**3.1**
10,11-dihydroxycarbamazepine	**0.085 MC)**	**2.39**
Amitriptyline	**< LOQ**	**0.14**
Telmisartan	**1.143 (MC)**	**0.00055**
Theophyllin	**0.747 (MC)**	**14.8**
Terbuthylazine	**0.0075 (CG)**	**0.06**
Metolachlor	**0.02 (CG)**	**0.2**
Ciclopirox	**1.158 (CG)**	**8.15**

CHAPTER 4

Discussion and Future Perspectives

Discussion about EBM results and future perspectives

In all the sampling sites ecotoxicological effects have been detected, they have been found also in the site FA where there is not a strong antropogenic pressure being a natural protected area. Acute effects have been detected only in few samples indicating that the levels of the chemical contaminants is not high, but the impact is given by the number of contaminants present in the aquatic environments also at low levels.

The data confirm also the results of previous studies in which effects in the urban part of the river have been detected (68). The different results obtained with the Farfa River samples in the sampling campaigns for Farfa river could be due to specific seasonal environmental perturbations as well as different levels of chemical discharge into the watercourses or single pollution phenomena. For instance, the available rainfall data show that the summer of 2018 was much rainier than the summer of 2019 (75), especially in the area of the Farfa River. For Daphnia the positive results recorded in the tested samples of the first campaign were weak and contrasting with those emerged in the samples from the third campaign. These observations could reveal the presence of chemicals or chemical mixtures that may not be assessed with the only record of lethal endpoints, although Daphnia Magna effects have been detected in previous studies (76). Furthermore, most of the chemical substances might have been stuck to the organic material that was removed during the sample filtering [77].

For the FET test sublethal effects occurred as spine deformation, delay or absence in the hatching and general underdevelopment, and they were recorded in all the samples. Specifically, the application of the FET test has been useful in several scientific studies to detect the effects of chemical pollution on living organisms in surface water bodies, even for substances at very low exposure levels (78). Moreover, the detection of sublethal effects can be linked to some widespread classes of environmental pollutants and it allows the understanding of the main modes of action (MoAs) of these substances, e.g. DNA toxicity, neurotoxicity, developmental toxicity, cardio-circulatory toxicity (59).

The underdevelopment effect was observed in previous studies for many chemicals such as dioxins and pesticides (79). Two very common sublethal endpoints detected were spine deformities (80,81,82), that are shared effect by almost every group of dangerous substances, these effects are also linked to neurotoxic substances (83). The head portion is also injured along with eye malformations that could be potentially induced by the heavy metals (84) and biocides (85). Other

typical malformations involve fin deformation and tail defects as underlined at least in studies that investigated the effects of pesticides, organobromides, PFAS and heavy metals (86,87,88). Such effects are strongly connected to the alterations in locomotor activity and consequently to the survival rate.

Hatching rate was another common effect found. Certain substances are able to advance or delay the hatching time, such as organobromides (89), heavy metals (90), pesticides and the perfluorinated alkylated substances as the PFOS (91). Specifically for the metals a previous study conducted by ISS (92) published in 2005 in order to assess the presence of selected toxic trace elements (As, Cd, Hg and Pb) in muscle tissues of fish caught in different tracts of Tiber River (urban area of Rome and different rural areas upstream and downstream), remarked a general condition of low-level pollution of the area under study, although further studies should be performed on this aspect**.** Hypopigmentation or absence of pigmentation (93) were found in embryos exposed to different substances as alkylphenols, heavy metals and pesticides.

High peaks of toxicity were registered in the organisms exposed to the Farfa River samples. The outcomes of the FET test also showed a certain level of toxicity in the site of Mezzo Cammino as expected: this site is indeed located downstream to the city of Rome and it is affected by the pollution load typical for a big city, i.e. small enterprise discharges, waste pollution, personal care products, detergents, heavy metals. MC also receives the emissions of the urban wastewater treatment plant. Further studies integrating other ecotoxicological bioassays and chemical analyses are needed to improve our knowledge about the state of health of the study area.The results of the test were congruent with those shown in D. Magna Acute Immobilisation assay, although very weak.

The presence in the river basin of several contaminants that have been detected at levels sometimes above the PNEC (predicted no effect concentration-table 4) is the sign that the application of more stringent reduction measures are needed in the Tiber River basin. These data are in part expected, for example in a previous study conducted in the Tiber River with the contribution of the JRC (Joint Research Centre of EU) high concentrations of pharmaceuticals, such as the hydroxymetabolite of the mood-stabilising drug carbamazepine and the non-steroidal anti-inflammatory drug diclofenac in the urban part have been detected, furthermore PAHs, pesticides, perfluorinated and polyfluorinated alkyl substances (PFAS) have been also found (63).

The substances detected in our study included pharmaceuticals, pesticides, plasticizers, antibiotics, cosmetic product, personal care product, solvents.

Among these substances Benzyldimethyldodecylammonium (Industrial use - corrosion inhibitors and anti-scaling agents), Tri(butoxyethyl)phosphate (Primary plasticizer for most resins and

elastomers; floor finishes and waxes; flame-retarding agent), Tetrabromobisphenol A (Primarily used as a reactive flame retardant), Erythromycin (antibiotic), Coffein (It is predominantly used in the food sector and pharma sector),Telmisartan (pharmaceutical for hypertension), Metazachlor (pesticide) have exceeded the PNEC and for these substances a potential risk for aquatic ecosystems can be predicted.

It is difficult to link the effect detected with the FET and Daphnia with the chemical substances that have been discovered during the study, but in some cases this link can be hypothesized, in particular for specific substances that have a specific MoAs is it possible to argue that some of the chemical substances detected can have potentially caused the effects that have been detected with the methods applied, but further studies should be carried out to better reinforce this link.

The non steroidal pharmaceutical Ketoprofen has been detected in the MezzoCammino site. This compound is suspected mutagenic (94) and acts on nucleic acid biosyntesis and RNA polymerase. It would be relevant to perform eco-genotoxicity tests in the river Tiber and the Comet assay with zebrafish embryos that has been preliminarly applied should be carried out on more samples.

Other contaminants detected are neuroactive (e.g Dinoseb), cardiotoxic (e.g. Propamocarb-angiotensin receptor) or are endocrine distrupting chemicals (e.g. Telimisartan, Metholachlor), are respiratory (Ethofumosate) or photosynthesis (Ranitidine) inhibitors, they can act on cell mitosis (Metazachlor) and lipid metabolism (Ciclopirox). Some studies have highlighted that ketoprofen has a potential mutagenic activity.

It is important to highlight that the chemical analysis has been performed only during the 3rd sampling campaign. Most of the emerging contaminants have been detected in Mezzo Cammino site as expected considering that is the site located in the southern part of Rome and where all possible sources of pollution are integrated, Farfa (FA) site is the site with the minor number of chemical contaminants, although surprisingly some compounds have been also detected in this naturalistic site indicating the presence of possible sources of pollution also in this area. For example Lauryl diethanolamide that is a Foam stabilizer for liquid household detergents/ foam stabilizer for shampoos can be expected due to the presence of small urban areas, but the high levels of erythromicin are a sign of the possible presence of antibiotic for livestock. Erythromicin is one of the substances included in the EU Watch-List of the WFD (95) and has been detected at relevant concentrations, maybe it is used as veterinary pharmaceuticals in livestocks of the area.

In the Castel Giubileo site there is the presence of specific pesticides such as terbuthylazine a substance widely diffuse in Italy and for this reason included also in the national Italian decree Dgls

172/2015 (12) with a specific EQS (environmental quality standard), metholachlor and ciclopirox has been detected also only in CG.

The end of May 2020 a kill fish has been detected in the urban part of river Tiber. Thousands of fishes of different species have been found dead (e.g. *Barbus barbus – Barbo europeo, Liza sp. – Presumibilmente Cefalo calamita, Squalius squalus – Cavedano, Silurus glanis – Siluro, Bramis brama – Abramide commune, Cyprinus carpio – Carpa , Carassius sp. – Carassio*) in the trait between Ponte Milvio and the Ponte Marconi the 31th of May.

Figure 26: figures of dead fishes in the urban part of Tiber river

The preliminary analysis performed with the test FET (Fish Embryo Toxicity Test) have detected embriological and neurological effects. The results of the sample taken at Ponte Milvio in particular has showed high acute toxicity with a value of 45% of dead embryos at 96 hpf. Furthermore, many sublethal effects occurred after exposure; in particular, 30,3% of embryos looked deformed with spine deformation (scoliosis or lordosis) and 17,7% appeared with poor pigmentation, also the swimming behavior appeared not normal 24,4% of larvae had a trembling swim. Also in the Isola Tiberina sample similar effects have been reported.

As discussed in the previous section these effects can be potentially caused by chemical substances such as pesticides. In particular it is relevant to highlight that the chemical analysis performed by the regional local authority (ARPA LAZIO-96) have detected the pesticides cypermethrin (0.014 µg/L) and Clothianidin, a neonicotinoid substance (0.67 µg/L). In particular the data detected for Cypermethrin are higher in comparison at average levels. Both these substances are included in the EU WFD lists.

Cypermethrin is a synthetic pyrethroid which has been used widely as pesticides/insecticide. It has been classified as Priority Substance for the WFD and for this reason should be reduced by all sources of pollution. The EQS (environmental quality standard) included in the Directive 2013/39/UE for this substance are the following:

AA (Annual Average): 8×10^{-5} µg/L

MAC (Maximum Allowable Concentration): 6×10^{-4} µg/L

It is evident that the concentrations detected in the Tiber river of Cypermethrin are much higher also of the Maximum Allowable Concentration that should protect from acute effects.

In a specific study (97) Zebrafish embryo toxicity test was used to determine the toxic effects of cypermethrin in the present study. Zebrafish embryos were exposed to various concentration of cypermethrin (0.001, 0.003, 0.01, 0.03 and 0.05 µg/l) and the observations on the lethal, sub lethal

and sublethal continuous endpoints were recorded. The results showed that the sub lethal and lethal effects of the zebrafish embryos increased with respect to an increase in the concentration of the cypermethrin. It is evident from this study that even low levels of Cypermethrin contamination in the aquatic environment would affect the developmental stages of fishes inhabiting the aquatic systems. Cypermethrin in another study (98) induced a suite of abnormalities including bodyaxis curvature, pericardial edema and large yolk sac in zebrafish embryos. The teratogenic lesions induced by Cypermethrin in zebrafish embryos were in accordance with the results reported.

Clothianidin has been included in the European Watch-List of the WFD (see figure 6), it is a neonicotinoid and has adverse ecotoxicological effects (95). The PNEC (Predicted No Effect Concentration), based on a chronic study on chironomus riparius is 0.65 µg/L.

It is very probable that the overall effects detected by the EBM are caused also by the mixtures of all these pollutants because also before the "fish kills" event, in the May campaign of 2020, effects have been detected with the use of FET (see results-table 3). Furthermore the fish kills can be also caused by suspended material that has reduced the oxygen levels in the water, the day before the fish kill there has been a flash storm in the city of Rome that can have leached chemical substances and also wastes in the river.

The Tiber River could be used in the future as a reservoir for drinking water in the city of Rome, especially considering the global climate change effects, e.g. water scarcity (99,100,101). Water scarcity can also increase the concentration and ecological effects of pollutants with the reduction of water quantity. Tiber river is also affected by flooding that can give clogging of wastewater treatment plants urban, cross-contamination of rivers/soil/lands and increasing run-off, remobilization of sediment chemical contaminants. Therefore, in this context, every new result on the water quality of this river ecosystem should be carefully considered. Integrating EBM, e.g. FET test and D. Magna Acute Immobilisation assay, into investigative monitoring for water quality management is fundamental to evaluate the hazards of the whole chemical substances widespread in the water bodies.

In general in the Tiber river basin different ecotoxicological effects have been detected and the results can contribute to help the local authorities to apply the best measures to protect the aquatic ecosystem taking into account the future uses also for the consumption of drinking water. The study, will contribute also to improve in general the knowledge of the quality status of Tiber river and help to identify the sources of pollution and to detect effects of mixtures of pollutants. Furthermore it would be essential to combine the morphological observations detected in this study with the methods taking into account different MoAs (see Chapter 2), e.g. mutagenicity and

neurotoxicity, in order to increase the knowledge on the underlined toxicity mechanism. A better comprehension of the chemical pollution levels in the Tiber River basin, including emerging substances, e.g. pharmaceuticals and pesticides not included in the legislation, would be fundamental to prevent risks for human health when there is a reuse of water for agricultural, aquaculture and drinking purposes.

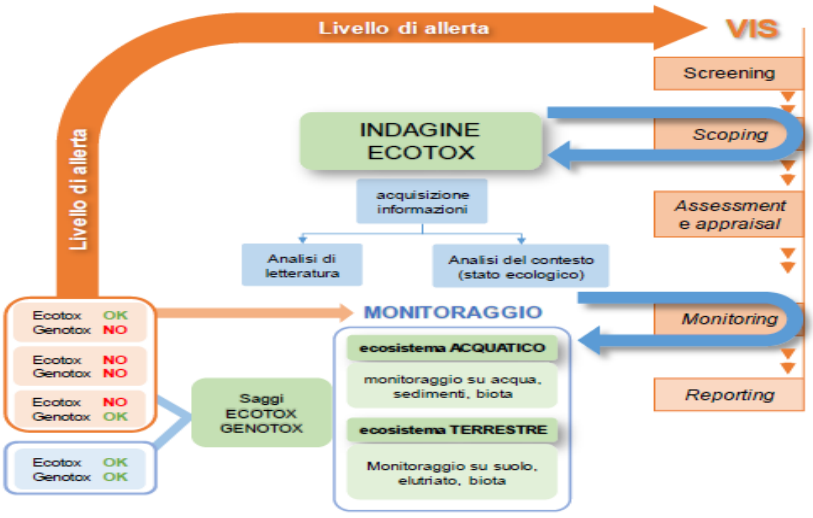
As a general comment, also related to the previous Chapter of the thesis EBM may reveal risks for natural communities working as environmental early warning systems. In general it is of major importance to discover effects related to chemical substances before significant effects on population level occur, because damage at the population and ecosystem level can take a long time to repair. Any warning signals would need to trigger investigative monitoring projects, such as effects directed analyses and regional studies upstream.

Most EBM and all categories (in vitro, in vivo and biomarkers) can be used, alongside other methods, to identify water bodies that are subject to significant pressures and thus risk failing the WFD objectives (102,103). If effects are observed, especially if being "severe", it is an indication of impact. EBM are also of interest if there is no obvious reason for an insufficient ecological status of a water body. The use of EBMs can provide insights into the role of chemical contamination. In case of negative test results (no observed toxicity) in spite of using a test battery and sensitive tests, the presence of chemical contaminants cannot be excluded but is less likely to be responsible for the observed ecological effects and viceversa.

The detection of effects by EBM indicate the presence of bioactive uninvestigated compounds. The selection of EBMs to use in a particular case needs to consider case-specific circumstances. If compounds present are largely unknown (not monitored), a battery of EBMs is normally needed. Also, knowledge about the source/s (type of pressure) is valuable in selecting a suitable EBM or battery of EBM.

In Italy (31) the application of EBM has been recently adopted for the Health Impact Assessment of large enterprises such as for example incinerators or refineries. The EBM in this context have a role of scoping (screening) and monitoring and can trigger further measures (Figure 27), in this case eco-genotoxicity methods (e.g.Ames and Comet) have an important role in relation to human health protection.

Figure 27: Application of effect based methods in the Health Impact Assessment (VIS) in Italy



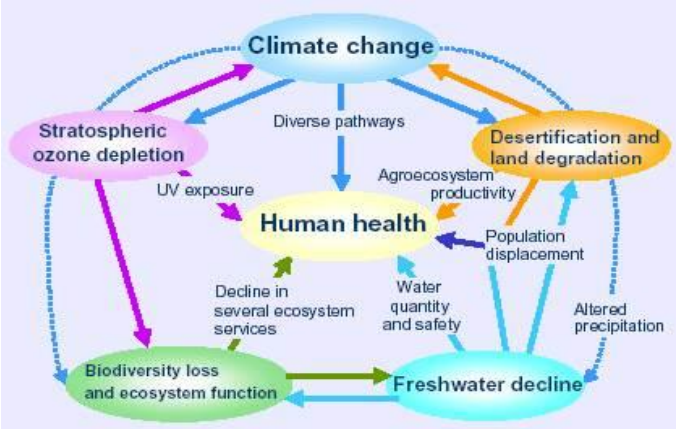
EBM are indeed useful also for identifying the need for abatement measures and assessing their efficiency. If EBM indicate unacceptable risks, decisions on measures can be taken without knowing the individual drivers of the risk. Examples are the observation of enhanced toxicity downstream of the discharge of effluents that may be reduced with improved treatment technologies using advanced oxidation processes or activated carbon or toxicity abatement downstream of agricultural areas by applying extended buffer strips along the stream. The comparative application of EBM upstream and downstream the discharge indicates the success of the measure in a cost-efficient way without the identification of individual chemicals. Moreover, the WFD suggests combining Lines of Evidence, whereby EBM results can be combined with other approaches such as emission inventories, pollutant concentration measurements and ecological monitoring data.

Overall Conclusions

The causal links between environmental change and human health are complex because they are often indirect, displaced in space and time, and dependent on a number of modifying forces (WHO-Figure 28). Human health ultimately depends upon ecosystem products and services (such as availability of fresh water, food and fuel sources) which are requisite for good human health and productive livelihoods. Significant direct human health impacts can occur if ecosystem services are no longer adequate to meet social needs. (https://www.who.int/globalchange/ecosystems/en/).

Human interventions are altering the capacity of ecosystems to provide their goods (e.g. freshwater, food, pharmaceutical products, etc) and services (e.g. purification of air, water, soil, sequestration of pollutants, etc). The introduction of contaminants into ecosystems caused by anthropic activities (Figure) and global and socio-economic environmental changes gives rise to "emerging problems" (104,105,106). Their spread, interaction and effects on environment and human health are still poorly understood and need to be identified. Therefore, the development of efficient and rapid methods (100) is fundamental to reduce their effects and for the implementation of preventive measures. Ecosystem approaches explore how ecosystem changes can have adverse impacts on human health and implement practical solutions to address these health challenges. The Water Framework Directive is a valid example of this approach, but it should be updated in relation to monitoring and assessment programmes.

Figure 28: WHO global change and ecosystems



http://www.who.int/globalchange/ecosystems/en/

The Project has included a review performed at European level in the context of my activity in the WFD implementation, an intercalibration exercise carried out on genotoxicity methods, a specific focus/case study on a literature review on FET (Fish embryo toxicity) and an experimental part carried out with the application of specific bioassays in the Tiber River Basin.

The activities performed collected during these 3 years have showed that there is the need to include the Effect Based Methods (EBM) in the legislation for the protection of water resources because of their potentiality to detect effects in the ecosystems relevant for the aquatic organisms and indirectly for human health.

In conclusion the EBMs in the legislative framework and at policy level could have the following great advantages:

- **Screening Function**: the detection of effects indicate the possible presence of detrimental contaminations caused by pollution and can thus trigger further activities such as source identification, analysis of the pressure and impacts, chemical monitoring. The case study on kill fish of river Tiber is an example of investigative monitoring.
- **Early Warning Role**: EBM are fundamental for Early warning of effects before impact at population levels occur (precautionary principle), this point is also particular relevant taking into account climate change effects on chemical contamination (e.g. flooding and water scarcity) and it is linked to human health aspects.
- **Mixture Effect Detector**: EBM can detect effect of mixtures of chemical pollutants in the ecosystems that is not possible to analyse or detect with chemical analysis.

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ADDENDUM

Lists of EBM in the European inventory

(work performed by EU group “Effect Based Methods” in the context of the WFD).

List of *in vitro* assays :

1. DR CALUX/DR
2. PAH CALUX
3. ERalfa CALUX/ER-Luc (agonistic/antagonistic)
4. ER-CALUX
5. T47D-Kbluc
6. BG1Luc4E2
7. ERa_Luc_BG1
8. AR CALUX (agonistic/antagonistic)
9. YES (Yeast Estrogen Screen)
10. YAS (Yeast Androgen Screen)
11. micronucleus assay
12. TTR binding assay
13. umu-Test
14. PPARg-GeneBLAzer
15. PPARy-CALUX
16. HG5LN-hPXR
17. PXR-CALUX
18. MELN
19. ER-GeneBLAzer
20. SSTA ERα-HeLa-9903
21. A-YES
22. 3d YES
23. ISO-LYES (Sumpter)
24. ISO-LYES (McDonnell)
25. Anti-ER-GeneBLAzer

26. Anti-ERa_Luc_BG1
27. Anti-A-YES
28. AR-GenBLAzer
29. MDA-kb2
30. A-YAS
31. anti AR-GenBLAzer
32. anti MDA-kb2
33. anti AR-CALUX
34. anti PR-CALUX
35. ZELH-zfERbeta2 and ZELH-zfERalpha
36. HELN-PRB
37. GR-GeneBLAzer
38. antiGR-GeneBLAzer
39. TTR RLBA
40. TTR FITC_T4
41. XETA
42. Anti-TR-LUC-TRE
43. Comet assay
44. SOS Chromotest
45. Ames Fluctuation Test (TA98)
46. Ames Fluctuation Test (TA100)
47. RT gill-W1
48. RTG2
49. SAF1
50. AREc32
51. anti HELN-PRB
52. PLHC-1 / EROD
53. AREGeneBLAzer
54. Nrf2-CALUX
55. P53 CALUX
56. kappaB CALUX
57. PSII-inhibition (algae and higher plants via Imaging-Pulse-Amplitude-Modulation)

List of *in vivo* assays and the respective endpoints included in the inventory:

58. EASZY (Cyp19a1b-GFP)

59. REACTIV (unspiked)
60. RADAR (unspiked)
61. anti-AR RADAR (spiked)
62. Vibrio Fischeri (Bacteria) bioluminescence
63. Lumistox
64. 72h Algal growth inhibition
65. 24h Synchronous algae reproduction
66. 24h Combined algae assay (growth)
67. 2h Combined algae assay (PSII)
68. 48h *Daphnia magna* immobilisation
69. *Daphnia magna* reproduction test
70. *Ceriodaphnia dubia,* survival/ reproduction test
71. FET (Danio rerio) Fish Embryo Acute Toxicity test – mortality and sublethal effects
72. Oryzias latipes (fish)
73. Oryzias melastigma (fish)
74. Oryzias mykiss (fish)
75. 14d (fish) *Danio rerio*, mortality
76. *Crassostrea gigas (Bivalvia)* embryo-larval development
77. Mytilus sp (mollusca) embryo larval development
78. 7d Gammarus sp. feeding (in situ assay)
79. 7d Gammarus sp. acetylcholinesterase (in situ assay)
80. Gammarus sp. reprotoxicity (in situ assay)
81. Gammarus sp. endocrine disrupting (in situ assay)
82. *Ceramium tenuicorne* (red macroalga) growth rate
83. *Nitocra spinipes* (harpactoid copepod) survival
84. *Potamopyros antipodarum* (snail) survival rate and reproductive output
85. *Nassarius reticulata* (snail)
86. *Hyalella azteca* (amphipod)
87. *Gmelinoides fasciatus* (amphipod)
88. *Corophium volutator* (amphipod)
89. Brachionus (rotifera)
90. *Artemia franciscana* (crustacea) mortality
91. 48h/7d *Acartia tonsa* (crustacea) mortality, larval development
92. *Tigriopus fulvus* (crustacea)
93. *Hediste diversicolor* (Polychaeta)

94. *Paracentrotus lividus (*echinodermata) fecundity, larval development
95. Heterocypris incongruens (Ostracoda) growth inhibition, mortality
96. Chironomus assay
97. Mussel larvae
98. Lumbriculus assay
99. Nitocra spinipes LDR test (larval development rate)
100. *Amphibalanus Amphitrite* (crustacea) mortality
101. Fetax (amphibians embryos)

List of biomarkers and the respective endpoints included in the inventory:

102. Imposex, VDSI index - Penis and Vas Deference development
103. Imposex, RPSI index - Relative Penis Size Index
104. LMS (Lysosomal Membrane Stability) - minutes destabilisation period
105. MT (metallothionein) induction - concentration of MT (common unit: ug/mg cytosolic protein)
106. ALA-D (delta-amino-leuvulinic acid dehydratase) - porphobilinogen (PBG) formed per unit time and protein (nmol/l PBG/mg protein/min)
107. Cytochrome P450 1A activity /EROD (resorufin production; pmol/min/mg protein)
108. DNA adducts - number of adducted nucleotides per number of undamaged nucleotides, but also analysed as diagonal radioactive zones, DRZs (composite of multiple overlapping DNA adducts)
109. PAH metabolites - e.g. 1-hydroxypyrense or 1-hydroxyphentanthrene (ng/mg)
110. LH (Liver Histopathology) - occurrence of changes
111. MLN (Macroscopic Liver Neoplasm) - visible tumors on the surface of fish livers
112. Externally visible fish diseases - different types; FDI (Fish Disease Index) is calculated based on EVD (externally visible diseases), MLN, LH.
113. Reproductive success in eelpout - mean prevalence malformed fry, late dead fry, early dead fry and total abnormal fry. Different malformation classes.
114. VTG (vitellogenin) - concentration in blood plasma (ng/ml), of different types; in male
115. VTG (vitellogenin) - concentration in blood plasma (ng/ml), in female
116. Intersex in male fish - intersex prevalence (presence/absence)
117. Spiggin
118. Micronucleus assay - permanent and hereditary double DNA strand breaks (frequency of MN (FMN%) and frequency Nucleus abnormalities (FNA) - need to compare samples with a blank)
119. Amphipod embryo malformation - number (ratio) of malformed embryos
120. Stress proteins (Hsp) - amount of protein (semi quantitative), relative density units
121. Acetylcholinesterase (AChE) assay - AChE inhibition (nmol/min and mg protein)
122. Comet assay - tail moment, % DNA tail, length

123. Mussel histopathology (gametogenesis) - cell type composition (digestive gland epithelium), digestive tube epithelial atrophy and thinning, lysosomal alterations and inflammation
124. Stress on stress - anoxic/aerial survival (LT50 and TMM, time to maximum mortality)
125. SfG, Scope for Growth - alterations in energy available for growth and reproduction
126. Benthic diatom malformation - number (frequency) of malformed valves
127. Egg shell thinning of bird eggs
128. Sea eagle productivity
129. Pregnancy rate in seal
130. Genes involved in xenobiotic biotransformation and regulation (e.g. cytochrome 1A, AhR, ugt, metallothioneins)
131. Genes involved in oxidative stress (e.g. gpx, cat, HSPs), apoptotic response (e.g bax, p53, caspase), DNA repair (e.g nucleotide-excision repair xpa and xpc genes)
132. Mentum deformation in chironomids
133. Lipid peroxidation (LPO)
134. Protein carbonylation
135. P-glycoprotein efflux (P-gp)

LIST OF DELIVERABLES and ACTIVITIES (Mario Carere)

Main Activities

Co-Chair of the WG Chemicals of the European Water Framework Directive- 2015- Praesent

Co-Chair of the European Drafting Group "Effect Based Methods" in the context of the EU Water Framework Directive. 2016-2019.

Member of the European Working Group on "Water Reuse".

Main Deliverables during the period of the Doctorate

L. Mancini, S. Marcheggiani, C. Puccinelli, I. Lacchetti**, M. Carere**, T.Bouley. G*lobal environmental changes and the impact on human health and ecosystems*. DOI 10.12910/EAI2017-057. In "Energia, Ambiente e Innovazione". Settembre 2017

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