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# Reconciling standard procedures and environmental realism in ecotoxicology: conceptual and practical challenges

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SIReNa



Mémoire présenté par

**Davide Anselmo Luigi VIGNATI**

En vue de l'obtention de l'

**Habilitation à Diriger des Recherches**

Spécialité : Ecotoxicologie, Biodiversité, Ecosystèmes

Université de Lorraine

Ecole Doctorale Sciences et Ingénierie des Ressources Naturelles (SIReNa)

Laboratoire Interdisciplinaire des Environnements Continentaux (LIEC)

UMR CNRS 7360

## Reconciling standard procedures and environmental realism in ecotoxicology: conceptual and practical challenges

Travaux présentés le 07 juillet 2021

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# List of abbreviations

ASTM: American Society for Testing Material (now ASTM International)

AVS: Acid Volatile Sulphides

BLM: Biotic Ligand Model

BSA: bovine seroalbumine

CBR: Critical Body Residue (CBR)

CIPAIS: Commissione Internazionale per la Protezione delle Acque Italo-Svizzere  
(International Commission for the Protection of Italian Swiss Waters)

CMR: carcinogenic, mutagenic or reprotoxic

CNRS: Centre National de la Recherche Scientifique

DET: Diffusive Equilibration in Thin films

DGT: Diffusive Gradient in Thin films

DYMBAM: Dynamic Bioaccumulation Models

EC: European Commission (used for in-text references)

EC2CO: Ecosphère Continentale et Côtière (project initiative co-funded by CNRS and other French institutions)

ECHA: Environmental Chemicals Agency

EC<sub>x</sub>: effect concentration eliciting a  $x$  percentage response in exposed organisms

EDA: effect directed analysis

EEA: European Environment Agency

EQC: environmental quality criteria

EqP: equilibrium partitioning approach

EQS: environmental quality standard

ERA: environmental risk assessment

EU: European Union

FIAM: free-ion activity model

GLP: good laboratory practices

ISO: International Organization for Standardization

LabexR21: Laboratory of Excellence Ressources 21 “Strategic metals in the 21<sup>st</sup> century”

MSFD: Marine Strategy Framework Directive

NOEC: non-observable effect concentration

NTA: nanoparticle tracking analysis

OCDE/OECD : Organisation de Coopération et de Développement Economiques /  
Organization for Economic Cooperation and Development  
PEC and PEC-Q: probable effect concentration (quotient)  
PNEC: probable no-effect concentration  
RA: risk assessment  
SDG: sustainable development goals  
SEF: Sediment Enrichment Factors  
SPC: single particle counting  
SPM: suspended particulate matter  
SQG: sediment quality guidelines  
TBMF: toxicity and bioavailability modifying factors  
TIE: toxicity identification evaluation  
TRA: tissue residue approach  
UN: United Nations  
UPBTs: ubiquitous persistent bioaccumulative toxic substances  
WFD: Water Framework Directive

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# Résumé

## Présentation et cadre du document

Ce projet d'Habilitation à Diriger des Recherches (HDR) s'intéresse à certains défis conceptuels et pratiques auxquels l'écotoxicologie est confrontée afin d'assurer la protection des milieux naturels soumis aux pressions anthropiques. Le document est organisé en trois parties : le curriculum vitae du candidat (Partie I), la présentation de ses activités de recherche passées et en cours (Partie II) et la description détaillée de son projet de recherche (Partie III). Cette dernière partie discute d'abord des aspects choisis de la discipline de l'écotoxicologie et décrit ensuite les approches que le candidat propose de développer pour contribuer à l'étude de ces défis sur un horizon de cinq à six ans.

## Activités de recherche (Partie II)

Comme pour beaucoup des collègues m'ayant plus ou moins récemment précédé, la soutenance d'HDR constitue un moment d'évaluation rétrospective, et peut-être introspective, de sa propre carrière pour en identifier les fils conducteurs et proposer un programme de recherche structuré et cohérent pour les années à venir. Ainsi, la presque totalité de mes recherches passées et en cours peuvent être reliées à l'étude du devenir, de la biodisponibilité et des effets biologiques des éléments traces métalliques (ETM) dans les milieux aquatiques continentaux. Cette cohérence au niveau de grands axes de recherche ne m'a pas empêché de toucher à des problématiques très différentes (voir le tableau 1). A ce propos, je me suis interrogé sur les possibles risques de dispersion dans mes centres d'intérêt scientifique et donc sur la possibilité de retombées négatives en terme de qualité pour mes recherches. Au final, je préfère considérer la diversité de mes activités comme un effort pour atteindre une vision la plus possible interdisciplinaire de l'écotoxicologie ou des sciences de l'environnement en général ; tout en étant conscient du fait que, dans la meilleure des hypothèses, je n'arriverai à franchir qu'un tout petit bout du chemin vers l'interdisciplinarité. Quant à la qualité de mes recherches, je m'en remets à l'évaluation de mes articles et de mes projets par les pairs ainsi qu'à l'évaluation périodique de mes activités par le CNRS. C'est tout de même réconfortant de constater que certaines de mes affirmations dans un *viewpoint* publié en 2007 dans la revue *Environmental Science & Technology* semblent être toujours valables plus de 10 ans après (voir Partie II, section 1). Le reste de cette section 'Activités de recherche' décrira brièvement les thématiques principales sur lesquelles j'ai travaillé jusqu'ici.

*Evaluation de la qualité des sédiments.* Les sédiments constituent à la fois des sites d'accumulation et de potentielles sources secondaires pour plusieurs contaminants. C'est en m'intéressant à la qualité des sédiments dans la rivière Pô (le principal fleuve italien) que j'ai appris les techniques et les premières connaissances nécessaires à l'étude du comportement des ETM dans l'environnement. Les résultats de mes recherches ont permis d'identifier les zones les plus impactées par les activités anthropiques au long de la rivière Pô ainsi que dans ses affluents et d'étudier les effets de crues sur la contamination sédimentaire dans le même bassin (Partie II, section 2.2). Des recherches similaires ont ensuite pu être effectuées dans le Delta du Danube (Roumanie/Ukraine) en montrant que les ETM retrouvés dans cette importante zone

humide proviendraient principalement des sources anthropiques situées en amont du Delta (Partie II, section 2.3). Ces études ont aussi démontré que les teneurs en Cr et Ni dans les sédiments peuvent être fortement influencées par la présence de roches mafiques et ultramafiques (naturellement riches en ces éléments) dans les bassins versants des fleuves étudiés. Il est donc nécessaire de prendre en considération ces facteurs géologiques pour éviter des conclusions erronées quant à la qualité de leurs sédiments. Plus généralement, l'utilisation des analyses chimiques comme outils pour l'évaluation du risque environnemental liés à la contamination de sédiments doit se faire en prenant en considération les limitations pratiques et conceptuelles de ce type d'approches (Partie II, section 2.4).

*Biodisponibilité et effets des contaminants dans le compartiment sédimentaire.* Les analyses chimiques constituant la base des études sur la qualité des sédiments permettent l'identification des zones majoritairement impactées par des activités anthropiques, mais ne fournissent que peu d'information sur les risques environnementaux associés à la contamination. En effet, seule une fraction des contaminants présents dans les sédiments peut interagir avec le biote. La connaissance de cette fraction, dite *biodisponible*, est indispensable pour appréhender la possibilité et l'ampleur des éventuels effets néfastes au niveau des individus, des populations et des communautés benthiques. L'évaluation de la fraction biodisponible et des effets biologiques y étant associés peut se faire en laboratoire (Partie II, sections 3.1 et 3.2) ou directement sur le terrain (Partie II, section 3.3). Dans les études en laboratoire, j'ai participé à des recherches qui ont montré l'importance d'une caractérisation détaillée du comportement des contaminants dans les systèmes expérimentaux utilisés ; surtout pour des outils relativement complexes du type microcosmes (Partie II, section 3.1). De plus, une série de projets menés sur presque six ans dans un système aquatique impacté par des rejets de tannage du cuir aura permis de démontrer que les tests standardisés de bioaccumulation et d'écotoxicité en laboratoire peuvent négliger la présence d'importantes voies d'exposition aux contaminants sur le terrain (Partie II, section 3.3).

*Travaux sur le mercure.* Le mercure possède trois caractéristiques uniques parmi les ETM dont les cycles biogéochimiques ont été modifiés par l'activité humaine: i) une forte capacité de bioaccumulation, ii) une forte tendance à la bioamplification au long de chaînes trophiques, et iii) l'existence de formes volatiles qui en permettent le transport atmosphérique sur des longues distances. Ces caractéristiques en font une substance prioritaire dangereuse et ubiquiste au sens de la réglementation européenne (Directive 2013/39/EU). J'ai commencé à m'intéresser à cet élément en participant aux travaux de la Commission Internationale pour la Protection des Eaux Italo-Suisses. Mes recherches ont permis de clarifier quelques aspects géochronologiques de la contamination mercurielle dans plusieurs lacs sud-alpins en Italie du nord ainsi que de proposer des mécanismes contrôlant la distribution spatiale du mercure dans les sédiments du Lac Majeur (Italie/Suisse) (Partie II, section 4.1). D'autres travaux en collaboration avec l'Agence Régionale de l'Environnement du Piedmont (Italie), section de la province du Verbano-Cusio-Ossola, auront démontré qu'une partie du mercure présent dans les sédiments du même Lac pouvait être remobilisée dans les chaînes trophiques suite à sa bioaccumulation dans les organismes benthiques (Partie II, section 4.3). Au cours de ces recherches j'ai eu aussi l'occasion de m'intéresser aux discussions liées à l'adoption des nouvelles normes de qualité

environnementale pour le mercure au niveau national (Italie) et international (Partie II, section 4.2). Entre 2012 et 2016, j'ai ainsi contribué à l'animation d'un groupe de travail dédié au sein de la SETAC (Society of Environmental Toxicology and Chemistry) pour défendre la validité scientifique de la nouvelle norme de qualité environnementale basée sur la mesure du mercure dans les matrices biologiques.

*Répartition des éléments traces métalliques.* Le devenir environnemental, la biodisponibilité et les effets écotoxiques des ETM dépendent fortement de leur spéciation (sans oublier l'importance de l'écologie des organismes et d'autres facteurs que je discute dans mon projet de recherche à la Partie III du document). De façon simplifiée, le terme *spéciation* indique la totalité des formes chimiques et physiques dans lesquelles un ETM, mais la définition est applicable à d'autres substances, peut se retrouver dans l'environnement. Plus spécifiquement, le mot *répartition* définit la distribution des ETM entre la matière en suspension (MES), les colloïdes et la solution proprement dite (ou solution vraie). La distinction entre ces trois phases est opérationnelle et a fait l'objet des nombreuses études méthodologiques. Ainsi, la séparation entre MES vs. colloïdes + solution vraie par filtration à 0,45 µm est regardée comme une opération de routine ne présentant pas de difficultés particulières. Toutefois, le choix d'un seuil de filtration de 0,45 µm a des raisons purement historiques et peut amener à une sous-estimation de la fraction colloïdale de certains ETM (Partie II, section 5.1). De plus, pour un même seuil de filtration, les porosités des différents dispositifs varient et certains filtres peuvent retenir des particules beaucoup plus petites par rapport au seuil nominal (Partie II, section 5.1). En revanche, j'ai pu constater que différents dispositifs d'ultrafiltration permettent d'obtenir, sous certaines conditions des résultats similaires quant à la distribution de plusieurs ETM entre les colloïdes et la solution vraie. L'utilisation des dispositifs d'ultrafiltration à petite échelle pour l'étude de la répartition du mercure et du méthylmercure dans les eaux interstitielles de sédiments complète mes contributions méthodologiques sur le sujet de la répartition. Les techniques d'ultrafiltration présentant de nombreuses contraintes logistiques, j'ai aussi proposé, avec des résultats satisfaisant, des modèles simples pour estimer les concentrations dans la solution vraie à partir des concentrations filtrées (Partie II, section 5.3).

Mes autres recherches sur le sujet ont porté sur l'étude de la répartition des ETM dans des milieux aquatiques contrastés afin d'identifier les mécanismes généraux pouvant contrôler leur distribution parmi la MES, les colloïdes et la solution vraie. Ainsi, les éléments dits terrigènes (par exemple Al et Ti) sont principalement associés aux particules (MES et colloïdes) avec des concentrations extrêmement faibles dans la solution vraie. D'autres éléments pouvant former des espèces anioniques dans les eaux naturelles (As, Sb et U) se retrouvent principalement dans la solution vraie alors que la répartition des éléments divalents (Cu, Co, Zn, Pb) et du Cr varie selon les systèmes étudiés. La validité de ces observations est bien évidemment limitée aux zones d'études (Partie II, section 5.2). Une étude plus approfondie a permis de déterminer les mécanismes contrôlant le comportement des ETM à la confluence entre la rivière Pô et un affluent pollué : la rivière Lambro (Partie II, section 5.2). Lors du mélange de deux masses d'eau, il est en effet possible d'estimer la fraction de chacune d'elles en utilisant la concentration d'un traceur soluble tel que le sodium. Après quelques calculs, la comparaison des teneurs des ETM en amont de la confluence, en aval de la confluence et dans l'affluent permet d'identifier les mécanismes pouvant affecter la répartition des ETM ; notamment

l'adsorption des éléments en solution vraie sur la MES (ou leur désorption à partir de la MES), l'agrégation/coagulation des colloïdes ou une combinaison des deux phénomènes.

*Etudes spécifiques sur le devenir et l'écotoxicité du chrome.* Les recherches sur le chrome sont discutées dans une section à part à cause de particularités de cet élément. Dans les eaux de surfaces, le Cr se retrouve principalement dans deux états d'oxydation, le Cr(VI) et le Cr(III), présentant un comportement environnemental, une biodisponibilité et une écotoxicité très différents. Très brièvement, le Cr(VI) forme des espèces chimiques anioniques (chromates) ayant une forte mobilité dans l'environnement et pouvant facilement pénétrer dans les cellules à cause de leur analogie structurale avec les ions sulfates. En revanche, le Cr(III) tend plutôt à former des espèces chimiques cationiques qui s'adsorbent sur les colloïdes ou sur la MES. De plus, les mécanismes d'acquisition cellulaire du Cr(III) sont moins bien connus et cet état d'oxydation est normalement réputé comme étant peu biodisponible et peu toxique pour le biote. Sur la période 2000–2008, j'ai contribué à plusieurs études de terrain dans deux systèmes aquatiques impactés par des rejets de tanneries ; une activité qui utilise des grandes quantités de Cr(III) pour traiter les peaux avant leur utilisation. Des études dans les eaux de ces systèmes aquatiques ont permis de quantifier la distribution du Cr(III) entre colloïdes et vraie solution, mais aussi de détecter la présence du Cr(VI) dans la vraie solution. D'autres investigations ont confirmé la présence du Cr(VI) aussi dans les eaux interstitielles suggérant la possibilité d'une oxydation du Cr(III) au niveau de sédiments. Selon un modèle conceptuel développé à partir de l'ensemble des résultats, le Cr(III) est rapidement transféré de la colonne d'eau aux sédiments par un mécanisme de pompage colloïdal alors que le Cr(VI), y inclus sa fraction formée par oxydation du Cr(III) dans les sédiments, peut être transporté sur de longues distances (Partie II, section 6.1). Comme mentionné plus en haut dans le volet *Biodisponibilité et effets des contaminants dans le compartiment sédimentaire*, des investigations sur la bioaccumulation du Cr à partir des différents voies d'exposition ont été effectuées pendant les mêmes campagnes.

A partir de l'année 2005, j'ai commencé à m'intéresser aussi à l'écotoxicologie du Cr vis-à-vis des organismes modèles de laboratoire (principalement des algues d'eau douce) afin de mieux évaluer ses risques potentiels dans les différentes situations de terrain. Ces recherches ont généré des résultats innovants en rupture avec le consensus qui considère le Cr(VI) comme étant beaucoup plus écotoxique que le Cr(III). En particulier, j'ai pu constater que l'ajout de Cr(III) aux milieux standardisés utilisés en écotoxicologie (ajout initial sous forme des sels solubles de type chlorure ou nitrate de Cr) génère rapidement des hydroxydes de Cr(III) qui se caractérisent par une faible solubilité. Leur présence provoque donc une diminution très rapide et très marquée de la teneur en Cr par rapport à la quantité initialement ajoutée au milieu écotoxicologique. Par exemple, une diminution de la concentration filtrée d'au moins 50% en 24 h et supérieure à 80% en 72 h (durée standard d'un test avec des algues de laboratoire) a été observée dans un milieu pour les algues d'eau douce. Ces phénomènes sont liés aux propriétés chimiques du Cr et peuvent se vérifier dans n'importe quelle matrice aqueuse ayant un pH entre 6 et 9 ; ce qui est normalement le cas pour les milieux utilisés en écotoxicologie. En conclusion, il est très probable que l'écotoxicité du Cr(III) vis-à-vis d'organismes d'eau douce soit fortement sous-estimée.

Des recherches plus récentes effectuées à partir de 2012 (date de mon recrutement au CNRS) ont montré que, une fois ajouté dans un milieu standardisé pour algues d'eau douce, le Cr(III)

ionique disparaît dans 5 heures pour laisser la place à des formes nanoparticulaires dont le potentiel écotoxique reste à vérifier. Des expériences de bioaccumulation du Cr(III) et du Cr(VI) avec l'algue modèle *Chlamydomonas reinhardtii* ont aussi démontré que les deux formes redox se distribuent de façon très similaire parmi les composantes cellulaires ; en confirmant que le potentiel écotoxicologique du Cr(III) ne doit pas être négligé. A partir de ces résultats, j'ai donc pensé à une approche permettant d'exposer simultanément les organismes aux Cr(III) et au Cr(VI) dont les résultats préliminaires sont discutés dans la Partie II (section 6.3) et les applications à venir dans le projet d'HDR (Partie III, section 2.5).

### **Projet de recherche**

*Cadre conceptuel.* Afin de réconcilier les procédures standardisées de laboratoire avec la réalité du terrain il est avant tout nécessaire de connaître les champs d'application, les forces et les limites de ces mêmes procédures. Pour ce faire, la distinction entre *fiabilité* et *pertinence* peut constituer un cadre conceptuel très utile. La *fiabilité* des résultats d'un test écotoxicologique correspond à leurs valeurs intrinsèques en terme de justesse, précision et reproductibilité. A l'opposé, la *pertinence* indique le degré d'applicabilité des mêmes résultats à une situation spécifique telle qu'une évaluation du risque sur un site bien identifié. Les procédures standardisées sont généralement conçues pour assurer un maximum de fiabilité mais l'appréciation de leur pertinence revient à leurs utilisatrices et utilisateurs.

Ce focus sur la fiabilité est très utile pour classer les (nouvelles) substances en fonction de leur potentiel écotoxique et de leurs modes d'action (par exemple, génotoxicité, mutagénicité ou reprotoxicité). Ces informations permettent ainsi d'identifier les substances les plus dangereuses, dont l'utilisation doit être réglementée, ou encore d'interdire la mise sur le marché des substances avec des propriétés indésirables (conception bénigne de produits). En revanche, les procédures standardisées peuvent s'avérer non pertinentes pour évaluer les risques des substances autorisées sur la bases des études en laboratoire lors de leur utilisation volontaire (herbicides, pesticides) ou introduction accidentelle (résidus médicamenteux) dans l'environnement. Ces limitations de la pertinence des procédures standardisées découlent de certaines hypothèses qui en accompagnent le développement et l'utilisation, à savoir, à titre d'exemple :

- a) la réponse des organismes à une substance est principalement contrôlée par l'exposition à travers la voie aqueuse,
- b) la composition des milieux standardisés utilisés en laboratoire garantit un maximum de biodisponibilité de substances testées (c'est-à-dire que les résultats obtenus incorporent une marge de sécurité par rapport aux situations de terrain),
- c) la sensibilité d'un organisme vis-à-vis d'une substance et proportionnelle à la durée d'exposition.

Ces types d'hypothèses ne sont pas forcément valides dans des situations réelles où l'assimilation d'une substance peut avoir lieu principalement par la voie alimentaire et sa concentration montrer des fluctuations plus ou moins marquées dans le temps. Les tests standardisés ignorent aussi de nombreux facteurs, typiques des conditions de terrain, pouvant radicalement modifier la sensibilité d'un organisme à une certaine substance : la présence des mélanges de substances, les fluctuations de température, la dégradation d'habitat, la prédation, la compétition intra- et interspécifique ou encore la présence de parasites pour n'en citer que

quelques-uns. A partir de ces considérations, j'ai donc essayé de résumer les limitations de tests standardisés en termes de pertinence et, le cas échéant de fiabilité, afin de pouvoir : i) identifier des problématiques générales pour mieux réconcilier les tests standardisés avec la réalité de terrain et ii) proposer des questionnements scientifiques pouvant apporter des éléments de réponse à ces problématiques.

*Problématiques générales retenues pour le développement du projet scientifique.* En partant du cadre conceptuel présenté ci-dessus, j'ai retenu six grands problématiques méritant, à mon avis, davantage d'études pour augmenter la pertinence des approches standardisées sans en compromettre la fiabilité : i) l'amélioration des connaissances sur la biodisponibilité de substances dans les milieux standardisés utilisés en laboratoire, ii) la mesure de concentrations accumulées par les organismes exposés aux substances, iii) l'étude des effets de mélanges de substances, iv) l'étude de l'importance de différentes voies d'exposition et des effets de matrices, v) l'importance du facteur temps en relation aux réponses biologiques et vi) le liens entre effets de substances et changements climatiques globaux. Pour chaque problématique j'ai essayé de présenter un état de l'art succinct dans la section 1 de la partie III du document. A la fin de la même section, j'ai inclus des réflexions très personnelles sur les possibles dérives de la science et leurs conséquences potentielles sur mon programme de recherche. J'utilise ici le mot 'dérive' pour indiquer les pressions auxquelles les scientifiques sont soumis·es pour augmenter constamment leur productivité (articles, rapports, communications de tous types, engagement avec les pouvoirs publics et le secteur industriel) ainsi que leurs capacités d'entrepreneurs scientifiques (acquisition de fonds sur la base d'appels à projets compétitifs, promotion de son champ/infrastructure de recherche auprès de financeurs). Ces considérations ne relèvent pas strictement de mon champ disciplinaire, mais elles trouvent, j'en suis persuadé, toute leur place dans un projet d'HDR. En effet, une personne habilitée à diriger des recherches sera amenée à encadrer directement des doctorant·e·s et devra leur transmettre non seulement du savoir-faire mais aussi du savoir-être scientifique pour leur permettre de poursuivre avec succès leurs carrières académiques ou dans d'autres secteurs professionnels. Plus particulièrement, ces nouvelles recrues doivent apprendre à reconnaître et combattre les dérives dans leur domaine de recherche afin de préserver l'éthique et la rigueur nécessaires au vrai progrès scientifique.

#### *Questionnements scientifiques et approches de recherche*

La dernière partie de ce document (Partie III, section 2) se compose d'une synthèse de quelques-uns des défis conceptuels et pratiques devant être considérés pour réconcilier les pratiques écotoxicologiques standardisées avec la complexité des situations réelles. Il est avant tout nécessaire de comprendre que 'réconcilier procédures standardisées et réalisme environnemental' ne revient pas à 'extrapoler des résultats obtenus en laboratoire à des situations de terrain'. En effet, l'extrapolation laboratoire-terrain se base sur l'utilisation optimale des connaissances disponibles (obtenues en conditions contrôlées de laboratoire) pour l'évaluation et la gestion du risque dans des situations spécifiques. Elle ne questionne donc pas la validité des hypothèses sous-jacentes aux tests standardisés ni se propose d'en améliorer la pertinence en y apportant en amont des modifications dûment justifiées et documentées. Au besoin, l'extrapolation laboratoire-terrain peut toutefois utiliser des approches plus complexes

et non standardisées. En revanche, la ‘réconciliation entre procédures standardisées et réalisme environnemental’, qui constitue l’objectif de cette HDR, s’attaque directement à l’étude et, il est souhaitable, à la résolution des limites conceptuelles et pratiques des procédures en question.

Cette discussion introductive est suivie par la présentation de cinq questions scientifiques que j’ai retenues pour mon projet. Pour chaque question, je présente des considérations générales suivies d’une vision personnelle sur le sujet (vision s’appuyant tout de même sur la littérature scientifique) et des propositions concrètes de recherche. Dans ce résumé, je me limiterai à une brève description des activités de recherche proposées, en demandant aux lecteurs et aux lectrices de bien vouloir se référer au texte en anglais pour le cadre scientifique de chaque question. Les questionnements que je propose peuvent s’appliquer à toutes substances, mais les recherches envisagées en pratique sont axées sur les ETM qui constituent mes contaminants modèles dans la plupart des cas.

Question 1. *Choix des métriques pour la mesure des effets d’une substance.* Un des objectifs primaires de l’écotoxicologie consiste dans la définition de relations concentration–réponse entre une substance et un paramètre biologique. Très souvent, les concentrations d’exposition sont mesurées en termes de concentrations totales et les réponses au niveau apicale (mortalité ou croissance de l’organisme, ou de la population d’organismes dans le cas des cultures d’algues, par exemple). Dans le cas des ETM, seulement une fraction de la concentration totale, dite biodisponible, contribuera toutefois aux réponses biologiques observées. Sur la base des recherches précédentes, je propose d’approfondir ultérieurement la caractérisation détaillée de la spéciation des ETM dans les milieux écotoxicologiques utilisés en laboratoire. Quant aux métriques de réponse, je pense tourner mon attention vers l’utilisation de l’efficacité photosynthétique pour mieux appréhender les effets des ETM vis-à-vis des algues d’eau douce. Finalement, j’espère aussi pouvoir élargir mon champ d’investigation à des modèles biologiques autres que les algues ; par exemple le crustacé *Daphnia magna* et l’insecte *Chironomus riparius*.

Question 2. *Aspects temporels des tests écotoxicologiques en relation aux situations réelles.* La plupart de tests standardisés préconise la mesure des réponses biologiques après un temps d’exposition fixe (normalement établi par une norme) à la substance d’intérêt. Cette approche néglige complètement la variabilité de la durée d’exposition dans des situations de terrain ; avec plusieurs conséquences quant à la pertinence des résultats obtenus. Un organisme pourrait en effet tolérer une concentration donnée d’une substance sur un temps  $x$  (correspondant à la durée du test standardisé) mais en être affecté suite à l’exposition pour un temps  $5x$  sur le terrain. Plusieurs expositions répétées à des concentrations sans effets lors d’une exposition continue en laboratoire pourraient aussi déclencher des réponses biologiques non prévisibles sur la base des tests standardisés. En pratique, je compte me servir de microplaques équipées avec des membranes filtrantes (type Multiscreen™ distribuées par Merck) pour mieux appréhender l’importance du facteur temps sur l’écotoxicité du Cr(III) et du Cr(VI) vis-à-vis des algues d’eau douce. Ces dispositifs permettent de renouveler périodiquement le milieu d’exposition et donc d’envisager de scénarios très variés en termes de durée d’exposition à une substance.

Question 3. *Ecotoxicité de mélanges.* Dans la plupart de cas, les tests standardisés examinent les effets biologiques d’une substance à la fois ; une situation très éloignée de la réalité où les organismes sont exposés à des mélanges très complexes de substances ainsi qu’à

d'autres facteurs de stress. Avec le nombre de substances existantes, il n'est évidemment pas envisageable d'en étudier toutes les combinaisons possibles. J'ai donc décidé de focaliser ma réflexion sur des ETM qui possèdent plusieurs états d'oxydation pouvant être rencontrés simultanément dans l'environnement ; par exemple arsenic, antimoine, fer, sélénium et chrome. Comme déjà discuté dans la présentation de mes activités de recherche, les deux états d'oxydation du Cr normalement retrouvés dans l'environnement, Cr(III) et Cr(VI), ont des caractéristiques très différentes en termes de biodisponibilité et écotoxicité. Afin de pouvoir étudier les effets des mélanges de ces deux formes, je propose d'utiliser des solutions isotopiquement enrichies en  $^{50}\text{Cr(III)}$  et  $^{53}\text{Cr(VI)}$ . L'utilisation d'une approche isotopique permet de quantifier, par une simple analyse ICP-MS, la concentration de chaque état d'oxydation tant dans le milieu d'exposition tant, qui plus est, dans les organismes exposés. Sachant que le Cr(VI) est rapidement réduit en Cr(III) en milieu intracellulaire, l'approche proposée est la seule permettant ce type d'analyses. La possibilité d'interconversions entre les deux états d'oxydation dans le milieu d'exposition constitue un point de vigilance qui devra être suivi avec des analyses par couplage chromatographie ionique ICP-MS.

Question 4. *Effets de matrices et voies d'exposition.* La composition simplifiée des matrices utilisées lors de tests standardisés modifie la biodisponibilité de contaminants par rapport aux matrices naturelles. De plus, l'absence des particules environnementales (matière en suspension, colloïdes organiques et inorganiques) dans les milieux de laboratoire ne permet pas d'apprécier l'importance des différentes voies d'exposition aux substances vis-à-vis, par exemple, des organismes filtreurs ou détritivores. L'approche isotopique proposée pour la question 3 peut aussi être utilisée pour améliorer la compréhension des rôles des voies aqueuse et trophique dans l'accumulation du Cr par des organismes modèles comme *Daphnia magna* ou *Chironomus riparius*. En pratique, ces organismes pourront être exposés à des algues (*D. magna*) ou à des sédiments (*C. riparius*) préalablement contaminés avec du Cr(III) et, en même temps, à une phase aqueuse dopées avec du Cr(VI). Ce scénario peut être considéré comme réaliste dans beaucoup de situations. En effet, le Cr(VI) ne montre qu'une faible tendance à s'adsorber sur les particules environnementales (hors certains hydroxydes de Fe à des pH supérieurs à 8–8,5) alors que le Cr(III) est la forme prédominante à l'intérieur des organismes ou adsorbée sur les particules. L'ajout de phases porteuses colloïdales aux milieux aqueux standardisés peut aussi permettre d'en rapprocher la composition à celle des eaux naturelles et donc d'appréhender avec plus de pertinence la biodisponibilité des ETM pour les organismes.

Question 5. *Écotoxicologie, changement climatique et continuum terre-mer.* A l'heure actuelle, je n'ai pas encore développé de projets de recherche précis sur cette question. Toutefois, il n'est pas réaliste de négliger les possibles conséquences du changement climatique sur l'écotoxicité des substances ni la variabilité des effets d'une même substance dans le continuum terre-mer. A titre d'exemple, des sécheresses prolongées peuvent s'accompagner à une dilution moins efficace des rejets de station de traitement des eaux usées ; ce qui peut augmenter les concentrations des certaines substances dans les eaux naturelles avec des possibles conséquences au niveau écotoxicologique. En relation (très générale) à ce sujet, j'ai collaboré avec des ingénieurs chimistes qui développent des matériaux innovants pour la dégradation des molécules récalcitrantes. Ce type de travaux présentent un intérêt écotoxicologique car l'effluent purifié de la molécule cible peut tout de même avoir un potentiel écotoxique suite à la formation des produits de dégradation toxiques et non identifiés. Compte

tenu de la demande croissante à laquelle la ressource hydrique est soumise, je réfléchis sur la possibilité de poursuivre des collaborations autour de ces thématiques.

En plus de la discussion des cinq questions mentionnées ci-dessus, la partie III du document contient aussi des recommandations pratiques sur les stratégies pouvant être appliquées afin de se protéger contre les dérives scientifiques le plus communes et contribuer à l'avancement des connaissances en écotoxicologie de la meilleure façon possible. Cette dernière partie s'adresse en premier lieu (et peut-être exclusivement) à moi-même, mais j'espère qu'elle pourra stimuler des discussions sur des aspects très présents dans notre quotidien de scientifiques mais souvent très peu ou pas du tout discutés entre collègues ou, encore moins, avec nos étudiant·e·s.

# Executive summary

The present HDR project deals with the challenges that fundamental and applied ecotoxicology faces to protect natural environments against potential damage arising from the use of chemicals in our everyday lives. Being based on my research experience, ongoing projects and current thoughts for future endeavours, the project mainly focuses on knowledge gaps related to the environmental behaviour and ecotoxicity of trace elements. However, the discussion is organized around general issues and scientific questions that bear relevance also to other types of chemicals. Only the content of the proposed *Scientific Research Project* (Part III of the document) is summarized below.

To begin with, the strength and weaknesses of standardized procedures are examined using a reliability vs. relevance framework. Reliability indicates the intrinsic quality of experimental data (*i.e.*, precision, accuracy, reproducibility), while relevance considers how fit-for-purpose are such data when assessing specific concerns in specific environmental settings. Using this framework, I discuss why standardized tests are used, what they actually can (and cannot) assess, and how their limitations can (and do) actually drive progress in ecotoxicological research. Following these general considerations, I then examine a few broad issues in some depth: the correct quantification of exposure to chemicals, the importance of chemical mixtures, the presence of toxicity-modifying factors and different routes of exposure, the inclusion of temporal issues in ecotoxicology and some links between ecotoxicological research and climate change. A personal view on the risks of bias in ecotoxicological research (and on its possible causes) is also provided. With regard to bias, I am particularly concerned with how the functioning of the modern research industry and its focus on quantitative indicators may push *bona fide* scientists to indulge in questionable research practices without even realizing it.

The last part of the document presents five broad *research questions* to which I would like to bring some contribution in my future projects:

- the choice of the most appropriate metrics of exposure and response,
- a better consideration (or rather a rediscovery) of the importance of temporal aspects in ecotoxicity testing,
- the study of a special type of chemical mixture, notably the combination of different redox states of the same trace element,
- testing the applicability of laboratory results in the real-world (*i.e.* , matrix effects) and the importance of different routes of exposure for filter- and deposit-feeders,
- and exploring some of the links between ecotoxicology, climate change and protection of the river-to-sea continuum.

The sections related to questions one to four include background considerations, a personal (hopefully sufficiently documented) view on the subject, and plans or proposals for future research activities. Question five is discussed in terms of its overarching importance in determining future priorities in ecotoxicology. In addition to this hard-science topics, I have included a few practical proposals to help me in staying on the lookout for possible bias in my research and in relation to my mentoring activities.

# Acknowledgments

Science is a collective endeavour. Although I did my fair share of efforts, any achievement in my career would have never been possible without the mentoring, advice, vision and support of many people. Properly and specifically thanking them all would easily increase the size of the present document by about one-fourth. I will therefore explicitly mention the few people who may end up browsing this dissertation (and this section thereof). All the unnamed ones may rest assured that I have not forgot them and what they did for me.

*My sincerest thanks to:*

- the members of the HDR committee for accepting to devote their valuable time to review the present document. I hope it has been an interesting, if not enjoyable, reading and I am looking forward to their questions and remarks,
- the current and past LIEC board-of-directors for their support to my activity and for their everyday commitment in the difficult tasks of managing research (and researchers); with specific mentions to Laure Giambérini for her tireless determination in moving us forward, Pascale Bauda and Fabien Thomas for their support and enthusiasm while serving during the first 5 years of LIEC, and Jean-François Férard for his invaluable help during my recruitment process at CNRS and LIEC (and for establishing contact in the first place),
- the colleagues who made themselves available to serve as members of the present HDR committee and whom I could eventually not invite: Dr. Arno Gutleb at LIST (Luxembourg), Prof. Susanne Heise at the Hamburg University of Applied Sciences and Prof. Vera Slaveykova at the University of Geneva,
- the colleagues whom I contacted when I first thought of defending an HDR in 2014; a plan that had to be revised and postponed until now due to circumstances beyond my control: Dr. Inge Werner, Dr. Rodolphe Gilbin, Prof. Catherine Mouneyrac, Dr. Jérôme Rose, Dr. Jean-Luc Loizeau, Dr. Eric Meux, and Dr. Villieras Frédéric,
- my team-leader Carole Cossu-Leguille for bearing with my tendency to browse into (too) many topics and being always available to guide me back on track,
- the colleagues of the team ‘Toxicology of the Environment’ at LIEC for the fruitful discussions of ideas and opinions,
- the directress of the Ecole Doctorale Sirena, Elmira Arab Tehrany-Kahn, and the LIEC representative Elisabeth Maria Gross for assisting in the process of enrolment and making it smooth,
- the personnel of the Ecole Doctoral Sirena, especially Isabelle Maréchal and Stéphane Lebreton, for their professional and timely assistance,
- all the colleagues at LIEC for being available to exchange ideas or to plan and carry out experiments with...

*Additional thanks to (in random order):*

- Vincent Felten for putting up with my cumbersome presence in the same office and teaching me determination in the difficult exercise of pursuing scientific rigour,
- Jean-François Masfaraud for his scientific and personal consideration,
- Sylvie Cotelte for her engagement in favour of environmental responsibility in research,
- Eric Battaglia for always finding time to discuss common projects despite his numerous obligations,
- Philippe Usseglio-Polatera for his help and advice in the DANUBIUS-PP project,
- Marie-Noëlle Pons for her energy and pragmatism in properly finalizing complex research endeavours,
- my master and PhD students (also outside LIEC) for surviving me and my shifting management styles and schedules, in particular Imad Aharchaou for teaching me how to mentor and to Pauline Louis for her patience in discussing feedbacks,
- the other members of the ‘despicable-me minions team at LIEC’, Laetitia Minguez and Justine Flayac, along with Anne Vicente for bringing some salt and pepper into everyday lab-life and helping in surviving its occasional downsides,

*Outside LIEC, CNRS and University of Lorraine, my heartfelt thanks to:*

- my thesis supervisor and friend Janusz K. Dominik (BdV), formerly ‘Professeur extraordinaire’ at the University of Geneva for sharing his boundless love of science,
- Benoît J.D. Ferrari (BdL), currently director of the Swiss Centre for Applied Ecotoxicology, for opening to me the doors of ecotoxicology and honouring me with his friendship,
- the one and only Adrian *Pongo* Stanica (GBR), director of GeoEcoMar and soul of the DANUBIUS Research Infrastructure for all he is doing and how he does it,
- the people in the DANUBIUS-PP consortium,
- the colleagues at the Luxembourg Institute of Technology within and outside the ANR project ECOTREE, particularly Christophe Hissler, Loïc Martin (now at IRD) and Sébastien Cambier,
- Marina Camusso for initiating me to the ropes of trace element analysis,
- the former colleagues at the Institut F.-A. Forel (now Department F.-A. Forel for environmental and aquatic sciences) who may end up browsing this document (likely Jean-Luc Loizeau, Frédéric Gimbert, Stéphane Guédron, Andrea Garcia Bravo, Montserrat Filella) but also to all the others whose names, help and office locations in Versoix I could still mention on the spot,

- the core of the Polish Dunajec-Czorsztyn team (Ewa Szalinska, Anna Czaplicka and Michal Krzyszkowski – extra points for non-native Polish speakers reading this out aloud) for some of my most tiring (and most rewarding) days in science,
- the colleagues at the CNR-ISMAR in Venice for the campaigns in good and stormy weather and for the many stimulating summer schools,
- the colleagues at the CNR-IRSA in Brugherio and Verbania-Pallanza for sharing passion and knowledge during our projects on sud-alpine lakes, in particular Piero Guilizzoni, Andrea Lami, Aldo Marchetto, Stefano Polesello, Sara Valsecchi and Angela Boggero,
- all the colleagues inside and outside the mentioned institutions who offered, or are offering, me the opportunity and pleasure of working with them.

*And outside the professional realm, my personal, sincerest and heartfelt thanks to:*

- my wife Mariya for putting up with me on a daily basis and 24/7 during the Covid-19 pandemic (and possibly more during the preparation of this HDR),
- my parents who allowed me the luxury to pursue my own goals (and make my own mistakes) while they were taking care of everyday life's problem,
- my Italian and Ukrainian families (dogs and cats included, in particular my feline HDR co-author Yushka) for the much-needed breaks and distractions,
- my life-long friends Luigi and Matteo for reminding me that there is a (sometimes interesting and gratifying) life outside science.

## **Part I – Curriculum vitae**

## 1. Personal information

Davide Anselmo Luigi VIGNATI

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## 2. Working experience

01/02/2012–present      Researcher  
Université de Lorraine, CNRS, LIEC UMR7360, Metz (France)

01/04/2008–31/01/2012      Researcher  
National Research Council of Italy-Water Research Institute  
(CNR-IRSA), Brugherio (MB), Italy

01/01/2000–31/03/2008      Doctoral and post-doctoral fellow  
University of Geneva, Institut F.-A. Forel<sup>1</sup>, Versoix (Switzerland)

30/09/1996–31/12/1999      Junior researcher  
National Research Council of Italy– Water Research Institute  
(CNR-IRSA), Brugherio (MB), Italy

## 3. Education

2003      Doctorat ès Science (PhD), University of Geneva, Switzerland  
1998      Italian state exam for professional biologists  
1996      Master in Biology, Università degli Studi di Milano, Italy  
1989      Diploma, Scientific High School Galileo Galilei, Legnano, Italy

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<sup>1</sup> Currently Department F.-A. Forel for Environmental and Aquatic Sciences, University of Geneva, Geneva

#### 4. Teaching and administration activities

##### *Teaching*

Having always occupied research positions, my teaching duties have mainly consisted in contributions to specific subjects within larger modules (master level and continuous learning), block courses tailored to specific audiences at PhD student level, or participation in summer schools (master level).

2017–ongoing. Yearly contribution of 6 hours to the module *Devenir et effets de polluants dans les matrices air–eau/sediments*, Université de Lorraine, UE806

2013–ongoing. Contribution to the continuous learning course *Les tests d'écotoxicité au service de l'évaluation des risques*. Université de Lorraine, organized by Dr. S. Cotelle, LIEC

2019. Block course of 12 h, Università degli Studi dell'Insubria (Como, Italy), PhD level: *Trace element speciation and its implications for environmental behaviour and ecotoxicological effects*

2010–2017. Scientific communication and writing for PhD students. Block course (25 hours). Università degli Studi di Milano, Italy

2004–2016. Teaching (lectures, practical work, leading discussions) during summer schools in coastal and transitional environments for the students of the University of Geneva (several editions in Venice – Italy, Faro – Portugal, Uzlina – Romania, Primorskoie – Ukraine)

##### *Administration*

- Co-creation and co-organisation of an internal group for celebrating the 50 years of ecotoxicology (with VASSEUR Paule – Emeritus Professor, LIEC; MASFARAUD Jean-François – Maître de Conférence, LIEC; MINGUEZ Laetitia – CNRS researcher, LIEC; FLAYAC Justine – Assistant engineer, LIEC; VICENTE Anne – freelancer in scientific communication): <https://www.echosciences-grandest.fr/dossiers/l-ecotoxicologie-fete-ses-50-ans>
- Moderator of the LIEC internal project group Soil-Water Continuum (Continuum Sol-Eau)
- Co-correspondent of INEE for international activities
- Co-organizer of weekly internal seminars at LIEC (currently on-hold due to health restriction measures due to Covid-19 pandemic)
- Member of LIEC internal steering groups on communication and international activities
- 2012–2017. Member of LIEC scientific committee
- 2012–2017. Co-chair of the group 'Ecodynamics and ecotoxicology of contaminants' at LIEC (about 20 person/equivalent in research time)
- 2012–2016. Co-chair of an interest group on mercury contamination for the SETAC (Society of Environmental Toxicology and Chemistry) including organization of dedicated congress sessions, workshops and a special science symposium in 2015

## 5. Research activities

### 5.1. Bibliometry (as of 30<sup>th</sup> April 2021)

Total number of peer-reviewed publications in Web of Science journals: 53

h-index: 18 (Web of Science)

Total number of citations: 995 (Web of Science)

Publications in other journals: 10

Books: 2 (as co-editor)

Reports and book chapters: 11

Invited seminars and talks: 15

Platform presentations at international congresses: 41

Poster presentations at international congresses: 63

### 5.2. Research projects

2021–present. Bioavailability and ecotoxicity of redox sensitive elements to biota: a stable isotope approach (B-Rex-Ist). CNRS EC2CO initiative.

Project leader

2017–present. Ecotoxicology of Rare Earth Elements in Aquatic Systems (ANR ECOTREE; <https://anr.fr/Projet-ANR-16-CE34-0012>).

Work-package co-leader “Origin and ecotoxicology of REE”

2019–present. Programme PON RI (Programma Operativo Nazionale Ricerca) 2014/2020: Advanced photocatalytic processes using polymer functionalized nanocomposites activated by solar radiation for the removal of emerging contaminants from wastewaters in view of their reuse (industrial doctorate program in cooperation with the University of Salerno, Italy).

Reference person for LIEC

2016–2019. Preparatory phase for the “International Centre for Advanced Studies on River-Sea Systems” (H2020 DANUBIUS-PP; [www.danubius-pp.eu](http://www.danubius-pp.eu); [www.danubius-ri.eu](http://www.danubius-ri.eu)).

Reference person for Université de Lorraine and CNRS

2015–2017. Action on Ni and associated elements, subproject: Impact Environnemental sur les écosystèmes aquatiques : Approche écotoxicologique. (ANR Labex Ressources 21; <https://ressources21.univ-lorraine.fr/>).

Participant

2015–2017. Action on Rare Earth Elements. Subproject: Predicting and understanding REE effects at the sediment water interface. (ANR Labex Ressources 21; <https://ressources21.univ-lorraine.fr/>).

Participant

2016. Development of a non-lethal indicator (fish scales) for monitoring metallic contamination in biological matrices (DECAMET). (OTELo – Observatoire Terre et Environnement Lorraine).

Project leader

2015–2016. Excellence projects – action 86. Understanding Cr biological fate in freshwater bivalves using isotopically-enriched solutions. (ANR Labex Ressources 21; <https://ressources21.univ-lorraine.fr/>).

Project leader

2014. Excellence projects – action 67. Uptake and intracellular distribution of Cr(III) in freshwater algae. (ANR Labex Ressources 21; <https://ressources21.univ-lorraine.fr/>).

Project leader

2012–2013. INSOKINTOX: Insoluble elemental species in algal culture medium: a kinetic approach to their formation and ecotoxicological relevance. (Project EC2CO ; CNRS, INSU).

Project leader

2009. Partitioning of total mercury and methylmercury between colloids and true solution in aqueous matrices. Bilateral exchange project funded by SNF (Swiss National Science Foundation) and CNR (National Research Council, Italy).

Project co-leader

2008–2012: Investigation on DDT and dangerous substances in the ecosystem of Lake Maggiore. (Programs of the CIPAIS — International Commission for the Protection of Italian-Swiss Waters; [www.cipais.org](http://www.cipais.org)).

Work package leader “Mercury and trace elements contamination”

2007–2010. Mobility and bioavailability of metals in continental waters; an integrated approach – Part 4: narrowing the laboratory vs. field gap. (SNF, Swiss National Science Foundation).

Project co-proponent

2006. Mobilité et biodisponibilité de métaux dans les eaux continentales: études détaillées sur le mercure. Accepted request for a contribution to the purchase of an AFS mercury analyzer to ‘Société Académique de Genève’ and ‘Fondation Schmidheiny’.

Project leader

2001–2007. Mobility and bioavailability of metals in continental waters; an integrated approach – Parts 1, 2, 3. (SNF, Swiss National Science Foundation).

Participant

## 6. Mentoring activities

### 6.1. PhD theses and PhD committees

NAVARRA Wanda (ongoing). Combined processes of adsorption and solar-light activated photocatalysis for the removal of contaminants of emerging concern in wastewater in view of their reuse. Università degli Studi di Salerno (Italy); LIEC acts as a partner for providing ecotoxicological training to the PhD candidate.

Director: CAROTENUTO Maurizio; Tutor LIEC: VIGNATI Davide A.L.

Contribution to mentoring: 20%

LOUIS Pauline (2021). Origin, behaviour and fate of rare earth elements in wastewater treatment plants and associated receiving environments. Université de Lorraine, France.

Director: PONS Marie-Noëlle; Co-director: VIGNATI Davide A.L.

Contribution to mentoring: 50%

AHARCHAOU Imad (2017). Ecotoxicity of trivalent elements: early markers, trophic transfer and importance of insoluble species. Université de Lorraine, France.

Director: VIGNATI Davide A.L.; Co-director: BATTAGLIA Eric.

Contribution to mentoring: 50% (Thesis Director – *avec dispense d'HDR*)

PONTI Benedetta (2012). Ecotoxicological assessment of freshwater environments. XVI PhD Course in Environmental Sciences, Università degli Studi dell'Insubria, Como (Italy)

Direction: Prof. BETTINETTI Roberta

Contribution to mentoring: 10%

KOTTELAT Régis (2008). Physico-chemical characterization of flow-through microcosms and their use in the study of route of exposure for Cd and Cr(III) in *Daphnia magna*. University of Geneva, Switzerland.

Director: Prof. DOMINIK Janusz; Co-director: Dr. FERRARI Benoît J.D.

Contribution to mentoring: 25%

### *Thesis committees*

BURGA Karen (2012). Développement et validation de microbiotests en phase solide pour l'évaluation écotoxicologique de sédiments. Université de Lorraine, France.

### 6.2. Master students

#### 6.2.1. Master 2 (or equivalent if outside France)

AMBIAUD Antonin (2021). Bioavailability and ecotoxicity of Cr(III) and Cr(VI) mixtures vis-à-vis of the freshwater green alga *Raphidocelis subcapitata*. Université de Lorraine, France.

Director: VIGNATI Davide A.L.

LODATO Ilaria (2019). Assessment of the toxicity of Cr(III) and Cr(VI) using the green alga *Raphidocelis subcapitata*. Università degli Studi di Salerno, Italy.

Director: BALDANTONI Daniela; Tutor at LIEC: VIGNATI Davide A.L.

FUMAGALLI Sonia (2010). Analysis of mercury content in fish products and media communication on the risks vs. benefits of fish consumption. University of Insubria Como, Italy.

Director: PASQUARÈ Federico A.; Co-director: VIGNATI Davide A.L.

POLA Annalisa (2011). Mercury contamination in the abiotic and biotic components of Lake Maggiore and comparison with other sudalpine lake. University degli Studi dell'Insubria (Italy). University of Insubria Como, Italy.

Director: BETTINETTI Roberta; Co-director: VIGNATI Davide A.L.

MARCHESE Gabriele (2010). Mercury contamination in Lake Maggiore: present situation, temporal evolution and comparison with other subalpine lakes. Università degli Studi di Milano, Italy.

Director: TREMOLADA Paolo; Co-director: VIGNATI Davide A.L.

RADCHENKO Inna (2008). The importance of colloidal P forms and their bioavailability in aquatic ecosystems. Collaboration funded by 'Bourse Lombard 2006' between the University of Geneva (Switzerland) and the CNR-ISMAR (Venice, Italy).

Director: DOMINIK Janusz; Co-directors: ZONTA Roberto and VIGNATI Davide A.L.

BRIGHI Alice (2008). Université de Genève and CNR-ISMAR (Venice, Italy). Importance of sediments and pore-waters as secondary sources of phosphorus to the water column in the Venice lagoon. Collaboration funded by 'Bourse Lombard 2006' between the University of Geneva (Switzerland) and the CNR-ISMAR (Venice, Italy).

Director: DOMINIK Janusz; Co-directors: ZONTA Roberto and VIGNATI Davide A.L.

SIMO Placède (2006). Metal bioaccumulation by *Chironomus* spp. Exposed to sediments from the Vidy Bay: relationships with survival and growth. University of Geneva, Switzerland.

Director: DOMINIK Janusz; Co-directors: FERRARI Benoît J.D. and VIGNATI Davide A.L.

BEYE Mamadou Lamine (2005). Toxicity and bioavailability of chromium to the alga *Pseudokirchneriella subcapitata* in standard and enriched ISO medium. University of Geneva, Switzerland.

Director: DOMINIK Janusz; Co-directors: FERRARI Benoît J.D. and VIGNATI Davide A.L.

#### 6.2.2. Master 1

SIMONIN Cécile (2014). Genotoxicity of chromium to freshwater algae. Université de Lorraine, France.

Director: COTELLE S.; Co-director: VIGNATI Davide A.L.

YON Bérengère (2014). Ecotoxicité et biodisponibilité du chrome chez une algue verte d'eau douce (*Pseudokirchneriella subcapitata*). Université de Lorraine, France.

Director: BATTAGLIA E.; Co-director: VIGNATI Davide A.L.

ARAB Mohammed (2014). Chromium ecotoxicity to the green alga *Pseudokirchneriella subcapitata*. Université de Lorraine, France.

Director: VIGNATI Davide A.L.; Co-director: MASFARAUD Jean-François

AIT-BENAMARA Nassim (2014). Chromium ecotoxicity to a freshwater green alga: optimization of an experimental protocol based on flow-cytometry. Université de Lorraine, France.

Director: VIGNATI Davide A.L. Co-director: BATTAGLIA Eric

ADA-FOUDA Rufine (2014). Study of chromium genotoxicity to the alga *Pseudokirchneriella subcapitata*. Université de Lorraine, France.  
Director: VIGNATI Davide A.L. Co-director: COTELLE Sylvie

### 6.3. Post-doctoral researchers and others

MARTIN Loïc (2018–2020). Study of Rare Earth Elements behaviour.  
Post-doctoral researcher at LIST (Luxembourg), supported by the project ANR Ecotree.  
Co-mentoring with HISSLER Christophe, researcher at LIST (Luxembourg Institute of Technology).

ROMERO-FREIRE Ana (2016–2017). Chemistry and ecotoxicity of Rare Earth Elements.  
Post-doctoral researcher at LIEC, supported by LabexR21.  
Co-mentoring with GIAMBERINI Laure

GONZALEZ Veronica (2012–2014). Ecotoxicity of Rare Earth Elements.  
Post-doctoral researcher at LIEC supported by LabexR21.  
Co-mentoring with GIAMBERINI Laure and LEYVAL Corinne

ACANFORA Floriana (Feb 2017–Jul 2017). Ecotoxicity of chromium to freshwater algae.  
Erasmus post-degree mobility.

## 7. Collaborations with visiting scientists<sup>2</sup>

VALSECCHI Sara, researcher, National Research Council of Italy-Water Research Institute (CNR-IRSA), Brugherio (MB), Italy.

Subject of the visit: Spiking procedures for sediment toxicity and bioaccumulation tests.

Period: July 2016

LIBRALATO Giovanni, associated professor, Università Ca' Foscari, Venezia (Italy).

Subject of the visit: Ecotoxicity of nano-iron to freshwater algae.

Period: May–June 2015

LOFRANO Giusy, researcher, Università degli Studi di Salerno, Fisciano (SA), Italy.

Subject of the visit: Use of nano-zerovalent iron for chromium toxicity reduction.

Period: May–June 2015

LIBRALATO Giovanni, associated professor, Università Ca' Foscari, Venezia (Italy).

Subject of the visit: Toxicity of nano-zero-valent iron to freshwater algae.

Period: June 2014

LIBRALATO Giovanni, associated professor, Università Ca' Foscari, Venezia (Italy).

Subject of the visit: Toxicity of various fresh and aged silver nanoparticle suspensions to *Pseudokirkneriella subcapitata* (Korshikov).

Period: June 2013

SZALINSKA van OVERDIJK Ewa, professor, Cracow University of Technology, Cracow, Poland.

Subject of the visit: Chromium bioavailability to aquatic organisms.

Period: August 2013

SANTIAGO MORALES Javier, PhD student, Universidad de Alcalà, Madrid, Spain.

Subject of the visit: Ecotoxicological assessment of TiO<sub>2</sub> nanoparticles in aquatic ecosystems.

Period: June – August 2012

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<sup>2</sup> This includes the definition of the scientific program for the visit and direct cooperation during the visit

## **8. Scientific visits to foreign institutions**

September 2020–September 2021. Associated researcher, National Research Council of Italy – Water Research Institute (CNR-IRSA), Verbania-Pallanza branch. Research program: Geochronology and behaviour of mercury in sud-alpine lakes.

3–28<sup>th</sup> June 2019. Invited researcher and teaching (block course) at the doctoral level. Università degli Studi dell’Insubria (Como, Italy).

14<sup>th</sup> January–4<sup>th</sup> February 2017. Short-term scientific mobility, COST action 1205 (ENTER– Engineered Nanomaterials from Wastewater Treatment and Stormwater to Rivers). Project : Understanding the dynamics of Cr nanoparticles dissolution and formation in aqueous freshwater matrices. University of Agricultural Sciences (Uppsala, Sweden).

12–29<sup>th</sup> April 2016. Short-term scientific mobility, COST action 1205 (ENTER– Engineered Nanomaterials from Wastewater Treatment and Stormwater to Rivers). Project: Characterization and hazard assessment of effluents treated by photocatalytic processes using ZnO nanoparticles. Università degli Studi di Salerno (Italy).

## 9. Invited seminars and talks

Vignati D.A.L. Physico-chemical and biological processes and interactions with contaminants. Course on ‘Evaluation of ecotoxicological sediment quality’ organized by the Swiss Ecotox Centre, Bern, 14–15<sup>th</sup> November 2019. (In French and in German)

Vignati D.A.L. Biota-based environmental quality standards for mercury – a challenge for river-sea system research and management. EUSDR (European Strategy for the Danube Region), Synergies and collaboration opportunities in advanced research for river-sea systems. Bucharest, 3<sup>rd</sup> October, 2019.

Vignati D.A.L. Trace element speciation and its implications for environmental behaviour and ecotoxicological effects. Università degli Studi dell’Insubria, Como, Italy, 4–6 juin 2019.

Vignati D.A.L., Masson G. DECAMET – Développement d’un indicateur tissulaire non légal (écailles de poisson) pour le suivi de la contamination métallique dans les matrices biologiques. *Séminaire de l’Observatoire Terre et environnement de Lorraine*, 5<sup>th</sup> July 2018, Nancy, France.

Vignati D.A.L. The principles of sound ecotoxicology in the regulatory context for trace elements, 1<sup>st</sup> March 2016, Università degli Studi dell’Insubria, Como, Italy.

Battaglia E., Vignati D.A.L. Ecotoxicity of a (slightly) neglected toxicant: trivalent chromium (In French). Scientific seminar of ‘Observatoire Terre et Environnement de Lorraine’. Vandoeuvre-lès-Nancy, France, 8 July 2013. (Présentation conjointe / joint presentation)

Vignati D.A.L., Battaglia E. Effects of Cr on algae and daphnids (in French). *1<sup>st</sup> Atelier Nickel Ressource 21*. 21<sup>st</sup> March 2013, Nancy, France. (Présentation conjointe / joint presentation).

Vignati D.A.L., Montargès-Pelletier E., Rotureau E., Parant M., Féraud J.-F., Battaglia E., Sohm B., Cossu-Leguille C. Ecotoxicity of trivalent elements: appreciating the role of insoluble species. Réseau LorLux, 1<sup>st</sup> Workshop. Axis 4: Metals, PAHs and nanoparticles. Centre de Recherche Public – Gabriel Lippmann, Belvaux, Luxembourg. 5<sup>th</sup> February 2013.

Vignati D.A.L. Balancing pragmatism and realism in environmental sciences. Programme of re-training and requalification of Iraqi experts in the field of biochemistry and technology for peaceful application. 29<sup>th</sup> July 2010, Milan, Italy.

Ferrari B., Vignati D. Environmental realism and relevance of bioaccumulation and ecotoxicological tests. Symposium ‘Frontiers in Lake Research’ celebrating 30 years of the Institut F.-A. Forel. 17<sup>th</sup> September 2010, Geneva, Switzerland. (Joint presentation).

Vignati D.A.L. Potential risks from sediment-bound elements during dredging works: an example from the Danube Delta. Maîtrise Universitaire en Sciences de l’Environnement (University of Geneva, Switzerland). 21<sup>st</sup> May 2010, Geneva, Switzerland.

Vignati D.A.L. Mercury contamination in Lake Maggiore: a synthesis of the activities of the International Commission for the Protection of Swiss-Italian Waters. Workshop on ‘Mercury : management, health and ecological challenges for the XXI century’. 10<sup>th</sup> March 2010, Monza, Italy. (in Italian).

Vignati D.A.L. The expert in environmental sciences between pragmatism and environmental realism. Doctoral School in Environmental Sciences of Università dell'Insubria. 29<sup>th</sup> January 2010, Como, Italy.

Vignati D.A.L., Berlinksy M. The importance of environmental realism in 21<sup>st</sup> century ecological monitoring and risk assessment. International expert conference on 'The safety of navigation and environmental security in a transboundary context in the Black Sea Basin'. 24–26<sup>th</sup> June 2008, Odessa, Ukraine.

Dominik J., Vignati D. Microelements and forms of phosphorus in sediments of the Ukrainian part of the Danube Delta. International seminar 'Analysis of monitoring results of realization of the first stage of DWNC – Danube-Black Sea – restoration works'. 27–28<sup>th</sup> April 2005, Odessa, Ukraine.

## 10. Other activities and responsibilities

2019: expert report on the ecotoxicity of trivalent Cr for the ‘Fondation Scientifique Rovaltain’

2016–2019. Preparation of the DANUBIUS-RI Science and Innovation Agenda (contributor).  
<https://danubius-pp.eu/results/>

2012–2016. Co-chair of the SETAC (Society of Environmental Toxicology and Chemistry) mercury group

2014. Guest editor of a special series of papers on Hg in Environmental Toxicology and Chemistry. Editorial contribution: *Environmental Toxicology and Chemistry* 33, no. 6 (2014): 1199–1201

2014. Participant (by invitation only) in the workshop ‘FP7 Dancers’ (Danube macroregion: capacity building and excellence in river systems (basin, delta and sea). 17–18 March 2014. Bucharest, Romania

2011–2014. Co-chair of scientific sessions at the annual SETAC Europe congresses in Milan, Italy (2011); Berlin, Germany (2012); Glasgow, United Kingdom (2013); and Basel, Switzerland (2014)

2012 and 2014. Co-chair of scientific sessions at annual SETAC North America congress in Long Beach, CA, USA (2012) and Vancouver, BC, Canada (2014)

2013. Co-organizer of the International Workshop ‘Managing mercury pollution in the 21<sup>st</sup> century: bridging science and policy’. 6<sup>th</sup> October 2013, Kumamoto (Japan)

2013. Café des Sciences. Co-animateur. Pollution cachées: alimentation à risque? Metz, 21<sup>st</sup> May

2012. Co-organizer of the ‘NEAR 4 International summer school. Water related issues in the Odessa region – Danube Delta, Ukraine’. Odessa and Vilkovo (Ukraine), 10–18<sup>th</sup> September

2011. Qualified to the ‘Fonctions de professeur des Universités’, section 67

2010. Co-organizer of the workshop ‘Mercury: regulatory, health and ecological challenges for the 21<sup>st</sup> century’ (working language : Italian). Milan (Italy), 10<sup>th</sup> March

2008 to 2012. Participation in the annual workshop of CIP AIS (Commissione Internazionale per la Protezione delle Acque Italo-Svizzere; International Commission for the Protection of Italian-Swiss Waters – [www.cipais.org](http://www.cipais.org))

Project evaluation for Région des Pays de la Loire (1) and for the Swiss National Science Foundation (1)

## 11. Publication list

### 11.1. Publication in Web of Science peer-reviewed journals

Vignati D.A.L., Lofrano G., Libralato G., Guida M., Siciliano A., Carraturo F., Carotenuto M. 2021. Photocatalytic ZnO-assisted degradation of spiramycin in urban wastewater: degradation kinetics and toxicity. *Water*, 13(8): 1051.

Louis P., Messaoudene A., Jrad H., Abdoul-Hamid B.A., Vignati D.A.L., Pons M.-N. 2020. Understanding Rare Earth Elements concentrations, anomalies and fluxes at the river basin scale: The Moselle River (France) as a case study. *Science of the Total Environment*, 742, 140619.

Pons M.-N., Louis P. and Vignati D. 2020. Effect of lockdown on wastewater characteristics: a comparison of two large urban areas. *Water Science and Technology* 82(12), 2813-2822.

Vignati D.A.L., Bettinetti R., Boggero A., Valsecchi S. 2020. Testing the use of standardized laboratory tests to infer Hg bioaccumulation in indigenous benthic organisms of Lake Maggiore (NW Italy). *Applied Sciences*, 10(6), 1970.

Romero-Freire, A., Joonas, E., Muna, M., Cossu-Leguille, C., Vignati, D.A.L., Giamberini, L. Assessment of the toxic effects of mixtures of three lanthanides (Ce, Gd, Lu) to aquatic biota. *Science of the Total Environment*, 661 : 276–284.

Vignati D.A.L., Ferrari B.J.D., Roulier J.-L., Coquery M., Szalinska E., Bobrowski A., Czaplicka A., Kownacki A., Dominik, J. 2019. Chromium bioavailability in aquatic systems impacted by tannery wastewaters. Part 1: understanding chromium accumulation by indigenous chironomids. *Science of the Total Environment*, 653: 401–408.

Ferrari B.J.D., Vignati D.A.L., Roulier J.-L., Coquery M., Szalinska E., Bobrowski A., Czaplicka A., Dominik J. 2019. Chromium bioavailability in aquatic systems impacted by tannery wastewaters. Part 2: new insights from laboratory and *in situ* testing with *Chironomus riparius*. *Science of the Total Environment*, 653: 1–9.

Aharchaou I., Py J.-S., Cambier S., Loizeau J.-L., Cornelis G., Rousselle P., Battaglia E., Vignati D.A.L. 2018. Chromium hazard and risk assessment: new insights from a detailed chemical speciation study in a standard test medium. *Environmental Toxicology and Chemistry* 37: 983–992.

Bettinetti R., Kopp-Schneider A., Vignati D.A.L. 2018. The European water-based environmental quality standard for pentachlorophenol is NOT protective for benthic organisms. *Science of the Total Environment*, 613–614: 39–45.

Romero-Freire A., Minguez L., Pelletier M., Cayer A., Caillet C., Devin S., Gross E.M., Guérolde F., Pain-Devin S., Vignati D.A.L., Giambérini L. 2018. Assessment of baseline ecotoxicity of sediments from a prospective mining area enriched in light rare earth elements. *Science of the Total Environment*, 612, 831–839.

Aharchaou I., Rosabal M., Liu F., Battaglia E., Vignati D.A.L., Fortin C. 2017. Bioaccumulation and subcellular partitioning of Cr(III) and Cr(VI) in the freshwater green alga *Chlamydomonas reinhardtii*. *Aquatic Toxicology*, 182, 49–57.

Vignati D.A.L., Bettinetti R., Marchetto A. 2016. Long-term persistence of sedimentary copper contamination in Lake Orta: potential environmental risks 20 years after liming. *Journal of Limnology*, 75(s2), 107–119.

Guédron S., Devin S., Vignati D.A.L. 2016. Total and methylmercury partitioning between colloids and true solution: from case studies in sediment overlying and pore waters to a generalized model. *Environmental Toxicology and Chemistry*, 35, 330–339.

Gonzalez V., Vignati D.A.L., Pons M.-E., Montarges-Pelletier E., Bojic C., Giamberini L. 2015. Lanthanide ecotoxicity: first attempt to measure environmental risk for aquatic organisms. *Environmental Pollution*, 199: 139–147.

Gonzalez V., Vignati D.A.L., Leyval C., Giamberini L. 2014. Environmental fate and ecotoxicity of lanthanides: are they a uniform group beyond chemistry? *Environment International*, 71: 148–157.

Ponti B., Bettinetti R., Dossi C., Vignati D.A.L. 2014. How reliable are data for the ecotoxicity of trivalent chromium to *Daphnia magna*? *Environ. Toxicol. Chem.* 33(10): 2280–2287.

Bank M.S., Vignati D.A.L., Vigon B. 2014. United Nations Environment Programme's Global Mercury Partnership: Science for successful implementation of the Minamata Convention. *Environ. Toxicol. Chem.* 33(6): 1199–1201.

Ferrari B.J.D., Vignati D.A.L., Dominik J. 2014. Bioaccumulation kinetics and effects of sediment-bound contaminants on chironomids in deep waters: new insights using a low-disturbance *in situ* system. *Environmental Technology* 35(4): 456–469.

Vignati D.A.L., Secieru D., Bogatova Y.I., Dominik J., Céréghino R., Berlinsky N.A., Oaie G., Szobotka S., Stanica A. 2013. Trace Element Contamination in the Arms of the Danube Delta (Romania/Ukraine): Current State of Knowledge and Future Needs. *J of Environmental Management* 125: 169–178.

Vignati D.A.L., Polesello S., Bettinetti R., Bank M.S. 2013. Mercury environmental quality standard in Europe: Opportunities and challenges. *Integrated Environmental Assessment and Management* 9: 167–168.

Pasquarè F.A., Bettinetti R., Fumagalli S., Vignati D.A.L. 2013. Public health benefits and risks of fish consumption: current scientific evidence versus media coverage. *Public Health Nutrition* 16 (10): 1885–1892.

Piscia R., Guilizzoni P., Fontaneto D., Vignati D.A.L., Appleby P.G., Manca M. 2012. Dynamics of rotifer and cladoceran resting stages during copper pollution and recovery in a subalpine lake. *International J. Limnology* 48: 151–160.

Camusso M., Polesello S., Valsecchi S., Vignati D.A.L. 2012. Importance of dietary uptake of trace elements in the benthic deposit-feeding *Lumbriculus variegatus*. *Trends Anal. Chem.* 36: 103–112.

Guédron S., Huguet L., Vignati D.A.L., Liu B., Gimbert, F., Ferrari B.J.D., Zonta R. Dominik J. 2012. Tidal cycling of mercury and methylmercury between sediments and water column in the Venice Lagoon (Italy). *Marine Chemistry* 130–131: 1–11.

Guilizzoni P., Levine S.N., Manca M., Marchetto A., Lami A., Ambrosetti, W. Brauer A., Gerli S., Carrara E.A., Rolla A., Guzzella L., Vignati D.A.L. 2012. Ecological effects of multiple stressors on a deep lake (Lago Maggiore, Italy) integrating neo and palaeolimnological approaches. *J. Limnology* 71 (1): 1–22.

Bobrowski A., Kapturski P., Zarębski J., Dominik J., Vignati D.A.L. 2012. A Catalytic adsorptive stripping voltammetric determination of hexavalent chromium in overlying and interstitial waters isolated from sediments contaminated by tannery wastes. *Analytical Letters* 45: 495–507.

Kottelat R., Vignati D.A.L., Garcia-Bravo A., Dominik J., Ferrari B.J.D. 2010. Characterization of trace element geochemistry in continuous flow-through microcosms: a preliminary step to environmentally meaningful ecotoxicological experiments. *Chemosphere* 81: 1407–1415.

Vignati D.A.L., Dominik J., Beye M.L., Pettine M., Ferrari B.J.D. 2010. Chromium (VI) is more toxic than Cr(III) to freshwater algae: a paradigm to revise? *Ecotoxicology and Environmental Safety* 73: 743–749.

Szalinska E., Dominik J., Vignati D.A.L., Bobrowski A., Bas B. 2010. Seasonal transport pattern of chromium (III and VI) in a stream receiving wastewater from tanneries. *Applied Geochemistry* 25: 116–122.

Bettinetti R., Galassi S., Ciceri F., Neri M.F., Falandysz J., Camusso M., Vignati D.A.L. 2009. Sediment quality assessment in the Gulf of Gdansk (Baltic Sea) using complementary lines of evidence. *Environmental Management* 43: 1313–1320.

Vignati D.A.L., Valsecchi S., Polesello S., Patrolecco L., Dominik J. 2009. Pollutant partitioning for monitoring surface waters. *Trends Anal. Chem.* 28: 159–169.

Casale F., Nieddu G., Burdino E., Vignati D.A.L., Ferretti C., Ugazio G. 2009. Monitoring of submicron particulate matter concentrations in the air of Turin city, Italy. Influence of traffic-limitations. *Water, Air, Soil Pollut.* 187: 141–149

Szalinska E., Czaplicka-Kotas A., Vignati D.A.L., Ferrari B.J.D., Dominik J. 2008. Bioavailability of sedimentary chromium for chironomids in the upper Dunajec river (southern Poland). *Verh. Internat. Verein. Limnol.* 30(4): 534–536.

Vignati D.A.L., Mamadou L.B., Dominik J., Klingemann A.O., Filella M., Bobrowski A., Ferrari B. 2008. Temporal decrease of trivalent chromium concentration in a standardized algal culture medium, experimental results and implications for toxicity evaluation. *Bull. Environ. Contam. Toxicol.* 80: 305–310.

Vignati D.A.L., Burdino E., Congiu A.M., Cicala F., Pardos M., Nieddu G.F., Ugazio G. 2008. Screening of sediment quality in 24 tributaries of the Po river (Italy). *Water, Air, Soil Pollut.* 190: 129–141.

Kottelat R., Vignati D.A.L., Chanudet V., Dominik J. 2008. Comparison of small- and large-scale ultrafiltration systems for organic carbon and metals in freshwater at low concentration factor. *Water, Air, Soil Pollut.* 187: 343–351.

Dominik J., Vignati D.A.L., Koukal B., Pereira de Abreu M.-H., Kottelat R., Szalinska E., Bas B., Bobrowski A. 2007. Speciation and environmental fate of chromium in rivers contaminated with tannery effluents. *Eng. Life Sci.* 7: 155–169.

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## **Part II – Past research activities**

## 1. Sketching a coherent picture of twenty years of research

My past and current research focuses on the environmental behaviour, bioavailability and biological effects of trace elements in (continental) aquatic systems. Although trace elements occur naturally in the environment, their biogeochemical cycles have been disrupted by anthropogenic activities at various times during human history. Their concentrations are therefore enriched above natural levels in practically all ecosystems, including in remote areas (Hong et al., 1996; Schwikowski et al., 2004). Whether concentration enrichments translate into increased environmental risks actually depends on a large number of environmental, biological and ecological factors. Understanding the interplay and the joint outcome of these factors requires:

- a continuum of investigations, ranging from small-scale, short-term laboratory experiments to long-term field observations,
- modelling tools to reconstruct (past) drivers of environmental change and exploring possible future scenarios, and
- an interdisciplinary approach to extrapolate laboratory results to real-field situations or, conversely, to interpret phenomenological observations in natural environments within coherent theoretical frameworks.

Starting from these bases, I have strived to adopt an integrative and issue-driven approach to research, while harmonising my own priorities with the scientific policies of the host institutions and the project-based functioning of the scientific enterprise.

A broad summary of my previous activities is presented below according to the different topics that I have approached during my career (Table 1). All of them contributed to my current scientific vision and, hence, to the development of the HDR project described in part three of this document. Rather than ‘dispersion’ across a number of research fields, the variety of topics illustrated in Table 1 testifies a constant effort to enlarge one’s scientific vision to different environmental compartments, model organisms, contaminants, techniques and disciplines.

With the benefit of hindsight, I am much pleased to notice a career-long, substantial coherence with the concepts exposed in the viewpoint ‘*Laboratory-to-field extrapolation in aquatic sciences*’ (Vignati et al., 2007); boldly stating that “Narrowing laboratory-versus-field gaps appears to be a conceptual rather than a methodological challenge”. This strong statement apparently retains all its validity considering that, in their recent editorial on the emergence of microplastics as global pollutants, Borja and Elliott (2019) wrote “...we have many of the tools at our disposal, but we are particularly poor at using this”. Such statements do not however imply that all the necessary methods and techniques to solve today’s environmental problems are already available (or already existed more than 10 years ago). Rather, they invite us to consider a different way of planning and doing research. In his analysis of the 2019 Global Sustainable Development Report (available at: <https://www.uved.fr/fiche/ressource/le-rapport-mondial-sur-le-developpement-durable-gsdr-de-2019-analyse>, starting from 22m05s), Prof. Jean-Paul Moatti (President of the IRD, Institut de Recherche pour le Développement, France at the time of recording) stresses that achieving the goals of sustainable development requires to embrace a scientific approach that is issue-driven rather than discipline-driven. In this approach, the continued development of the fundamental science remains a key factor in building sustainable societies. However, individual disciplines (including human and social sciences) must be mobilised as needed to tackle the systemic environmental challenges that originate from our lifestyles and consumption patterns (EEA, 2015). Reconciling laboratory-based studies to field observations will be one important conceptual and practical step toward such an ambitious objective.

Starting from the general considerations outlined above, I have reviewed the main topics and results of my past research (Table 1) and tried to assess their current validity in terms of approaches, conclusions, and recommendations. Although the topics listed in Table 1 apply to

many chemicals, most of the discussion will focus on issues linked to contamination by trace elements; in agreement with my specific expertise. On the other hand, I have strived to avoid bias in the selection of the cited references and have acknowledged studies supporting both my own results or alternative hypotheses.

Table 1. Positioning of the major topics of my previous and ongoing research within broader research areas.

Research areas	Specific research topic(s)	Activity period
Sediment quality evaluation and bioavailability of sediment bound contaminants	Assessment of the extent and effects of sediment contamination	1996 – ongoing
Use of sedimentary archives as a tool for long-term monitoring and risk assessment	Geochronology of Hg contamination and Hg risk assessment	2008 – 2015
Environmental behaviour of trace elements and its potential implications for ecotoxicity	Trace metal partitioning in contrasting aquatic environments (including methodological studies)	2000 – ongoing
Chemical issues in contaminant ecotoxicology	Elemental speciation in ecotoxicological media including its implications for hazard/risk assessment	2005 – ongoing
Contaminant bioaccumulation	Chromium internalization and distribution inside organisms	2014 – ongoing

## 2. Sediment quality evaluation

### 2.1. Overview

During the 1990s, bed sediments became increasingly recognized as a major sink, and potential secondary source, for several contaminants of concern. Important conceptual developments in the use of sediments for environmental quality assessment actually gained momentum during those years, including:

- the sediment quality TRIAD approach, based on the simultaneous and integrated collection of chemical, ecotoxicological and ecological information for assessing sediment quality (Canfield et al., 1994; Chapman, 1990; Leslie et al., 1999);
- the Acid-Volatile Sulfide (AVS) approach, postulating that AVS control trace element toxicity to benthic organisms (Di Toro et al., 1992);
- the definition of Sediment Quality Guidelines (SQG) as benchmark values for triggering further assessment of sediment quality at specific locations (Long et al., 1998; MacDonald et al., 2000).

These approaches have been progressively refined, integrated, or constructively criticized to better account for trace element bioavailability (Fairbrother et al., 2007; McCarty and MacKay, 1993; Simpson and Batley, 2007a).

For many contaminants, sediments integrate inputs from the overlying water column over periods of weeks to months and can therefore provide a much more representative snapshot of ‘environmental quality’ than the corresponding water analyses. Furthermore, particle-reactive contaminants represent a greater risk to sediment-dwellers that can derive much of their contaminant burden from the ingestion of contaminated sediment. Indeed, the assumption that a water-based quality criterion for a given substance is protective of benthic biota is being

increasingly challenged and current European legislation leaves room for regulatory approaches based on contaminants' concentrations in sediments (EC, 2010).

Although sediment quality assessment is useful to identify the river stretches most impacted by anthropogenic contaminants (or other stressors), results must be interpreted by giving due consideration to basin geology, river hydrology and morphology, and knowledge of present and past existing human pressures. It is also important to remember that chemical measurements alone will hardly ever provide definite hints on the actual contaminant bioavailability. In particular, caution is mandatory when comparing measured contaminant concentrations with SQG. Concentrations below SQG may be assumed to reflect limited risk (from chemical contamination), but concentrations above SQG do not necessarily imply the presence of a risk linked to a given chemical (Allen Burton, 2017). Furthermore, individual SQG do not consider the possible interactions among contaminants, although approaches to integrate mixture effects of a few chemicals (e.g., selected trace elements, PAH, PCBs and a few organic pesticides) have been proposed (Long et al., 2006). Despite these limitations, SQG remain useful to identify critical areas requiring specific research efforts in terms of risk assessment; as illustrated in the two following case studies.

## *2.2. Assessment of sediment quality in the river Po basin (Northern Italy)*

The river Po is the largest Italian river (basin area over 70,000 km<sup>2</sup>) and flows west to east across one of the most populated and industrialized areas of Italy. During the 1990s, three major scientific projects were carried out to assess the sediment quality in the main river bed and its tributaries. The first project was coordinated by the Italian Water Research Institute (IRSA-CNR, Italy) and included two major campaigns (September 1996 and March 1997) in the river Po main channel under stable low flow conditions. The second project was led by the Institut F.-A. Forel (IFAF, University of Geneva, Switzerland; currently Department F.-A. Forel for Environmental and Aquatic Sciences) and took place in September 1994 (during high flow conditions) and October 1996 (during normal flow conditions) again in the river Po main channel. The third project, also led by IFAF, collected samples in 24 tributaries along the course of the river Po in October 1996 and April 1997. I was involved in the data elaboration and interpretation for all projects; which allowed me to cross-examine the different findings and contribute to characterize rather exhaustively the state of sediments in the River Po basin at the time of sampling.

All studies were planned with a general knowledge of the human pressures existing in the river basin (IRSA, 1991). While confirming that the cities of Turin and Milan were major sources of contaminants to the river Po, the results also highlighted the presence of diffuse sources of trace elements, PCBs, and DDTs in the lower course of the river. In the case of trace elements, the geological peculiarities of the river Po catchment, consisting in the presence of mafic and ultramafic rocks in specific areas (Amorosi et al., 2002), turned out to be an important point for the correct interpretation of the results in terms of sediment quality. Mafic and ultramafic rocks have naturally high Cr and Ni content in their crystalline lattice so that such high concentrations are usually accompanied by little bioavailability of these elements. An uncritical comparison of measured concentrations with the corresponding SQGs would have led to conclude that Cr and Ni posed a potential threat to biota in the whole catchment of the river Po; including those stretches situated close to its springs and upstream of known anthropogenic sources of contamination. After properly considering the available information, it was possible to conclude that the high levels of Cr and Ni in river Po sediments mostly originated from natural sources and contributed only marginally to sediment toxicity (Camusso et al., 2002). These conclusion agreed with the subsequent ecotoxicological evaluations performed by Viganò et al. (2003).

Comparison of elemental content in bed sediment (and suspended particulate matter) during low and high flow conditions (Vignati et al., 2003) further highlighted that floods leave behind sediments with coarser grain size and a lower degree of contamination. For example, the fraction of leachable vs. total Pb and Zn (taken as representative of elements subject to anthropogenic impact) was lower during floods than under normal flow condition. These observations may bear relevance when evaluating the risks of legacy contaminants that can be remobilized from floodplains during extreme high-flow events.

The study of sediment contamination in the major tributaries (Vignati et al., 2008b) showed that only a minor number of them could clearly be identified as sources of polluted particulate material to the main river bed. Otherwise stated, especially in the lower stretches of the river Po, diffuse sources from the basin appeared as the main driver of sediment contamination, although a few, local critical situation were found for some tributaries.

### 2.3. Assessment of sediment quality in the Danube river Delta (Romania/Ukraine)

The Danube river is one of the major European water courses. Its Delta is an important biosphere reserve under the Ramsar Convention, but also a major waterway for sea-going vessels and an economically important area for both Romania and Ukraine. The Danube Delta comprises three major branches (Chilia, Sulina and Sfantu Gheorghe); the Chilia branch forming a large secondary delta before entering the Black Sea. The state of the Danube river is regularly monitored by the International Commission for the Protection of the Danube River (ICPDR). Interesting additional research on sediment contamination and ecotoxicity in Ukrainian estuaries, including the Danube Delta, has also been published by Burgess et al. (2009) and Burgess et al. (2011). However, the synthesis I realized thanks to the Network for Environmental Assessment and Remediation (NEAR, a program coordinated by the University of Geneva between 1997 and 2014) offers the most complete and detailed snapshot of trace element contamination in the whole Danube Delta for the period 2004–2007 along with a comparison with earlier data from 1995–1997 (Vignati et al., 2013b).

In that work, an evaluation of sediment quality was performed using the PEC-Q (Probable Effect Concentration Quotient) approach (Long et al., 2006):

$$PEC - Q = \frac{\sum_{i=1}^n \frac{[Me]_i}{PEC_i}}{n}$$

where  $[Me]_i$  is the concentration of the  $i_{th}$  element measured in a given sample,  $PEC_i$  is the corresponding PEC value (MacDonald et al., 2000), and  $n$  is the total number of elements taken into consideration and for which PEC values are available. While PEC-Qs remain empirically based, they are superior to comparison among measured concentrations and PEC for individual elements, because they try to account for possible mixture effects of sediment-bound contaminants. Two critical PEC-Qs values equal to 0.25 and 0.34 have been proposed for evaluating the potential risk associated with sediment contamination (Long et al., 2006). The former value identifies situations where ecotoxicological response from benthic organisms in laboratory toxicity tests is likely to exceed a threshold of 20 % when compared with control organisms. The latter value identifies sites where reduction in the abundances of specific taxonomic groups and of the total abundance of benthic organisms are likely to occur. As in the case of the river Po, Cr and Ni concentrations in Danube Delta sediments could originate from the weathering of rocks naturally rich in these elements. Without consideration of this peculiarity, sediments in the Danube Delta would be considered as posing a serious risk to benthic organisms at most locations (Fig. 1).

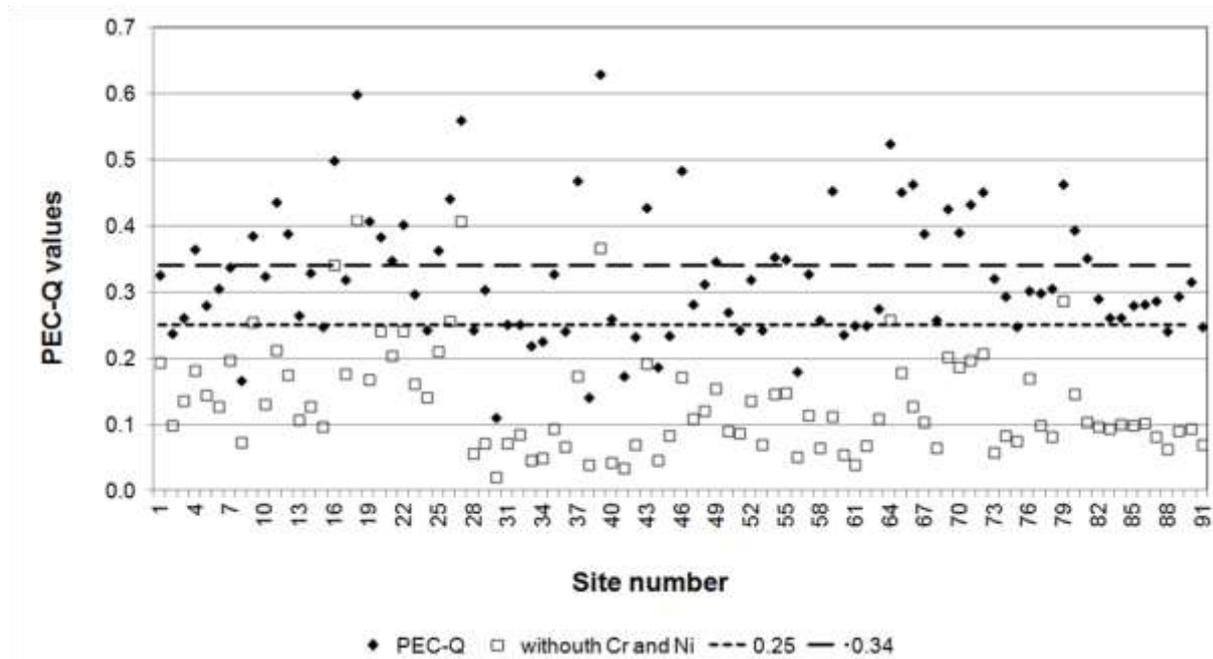


Figure 1. Values of PEC quotients (PEC-Qs) for sediment samples collected in different parts of the Danube Delta and calculated with (black triangles) or without (white squares) Cr and Ni concentrations. Horizontal dashed lines correspond to the PEC-Q criteria of 0.25 (expected ecotoxicity in laboratory standard tests) and 0.34 (expected reduction in abundances of indigenous benthic organisms). See text for more details (source: Vignati et al., 2013b).

In this specific case, I was even able to estimate the fraction of Cr and Ni originating from the presence of Cr and Ni bearing rocks located in the Danube catchment by using empirical relationships between concentrations of Cr vs.  $TiO_2$  and Ni vs.  $Fe_2O_3$  (Vignati et al., 2013b). Overall, at the time of sample collection, most of trace elements inputs to sediments of the Danube Delta seemed to originate from upstream sources, with only a limited number of contamination hot-spots possibly affected by local activities. The extensive spatial cover of the study allowed to put forward some management recommendations that could not be made on the basis of previous studies. In particular, monitoring sediment contamination at the mouth of the Sulina and Sfantu Gheorghe branches should be enough to obtain a reliable picture of the overall sediment quality in both branches. On the other hand, the complex morphology of the secondary Chilia Delta requires a larger monitoring network for an appropriate evaluation of its sediment quality.

#### 2.4. The sediment quality guideline approach in risk assessment frameworks

The analysis of contaminant levels in sediments provides useful indications as to the presence of anthropogenic inputs of contaminants into a given water body. Following the development of the TRIAD approach (see section 2.1), the need for benchmark values against which to evaluate the measured contaminant concentration became apparent and consensus-based SQGs were proposed for selected trace elements, total PCBs, PAHs, and a few organochlorine pesticides (MacDonald et al., 2000). Ideally, comparing measured concentration and benchmark values allows to draw conclusion as to the potential risks of sediment contamination. The PEC-Q approach (section 2.3) took this rationale a step further by recognizing the need to consider the combined effects of co-occurring contaminants. However, the number of contaminants capable of accumulating in sediments is much larger than those for which SQGs are available and identifying the precise contaminant(s) responsible for adverse ecotoxicological effects is not at all straightforward. The existence of different sets of SQGs

derived for different purposes (e.g., environmental protection vs. disposal or reuse of dredged material) can potentially lead to variable assessment outcomes. Furthermore, my contributions to sediment quality evaluation in the river Po and the Danube Delta showed that some SQGs values may simply be inappropriate for application in areas with peculiar geological settings.

All this considered, exceedance of SQGs (preferably considered collectively with the PEC-Q approach) should simply serve as a first tier to trigger more detailed evaluations of the bioavailability of sediment-bound contaminants and, hence, their possible contribution to sediment ecotoxicity. In a work in the Gulf of Gdansk (Baltic Sea), Bettinetti et al. (2009) showed that assessing contaminant bioaccumulation by benthic communities could bring useful complementary information as to the actual exposure of benthic organisms in situations of moderate contamination. A similar rationale underlies risk assessment frameworks that examine the use contaminant concentrations accumulated by benthic organisms as a better indicator of potential environmental risk. However, the applicability of such an approach may be further complicated by the need to consider the distribution of the contaminant burden among pools of different bioavailability at both the organism and cellular level (Rainbow, 2002).

Allen Burton (2017) urged for renewed caution against the use of SQGs as a stand-alone tool for making decisions about remedial action or other policy options and proposed to adopt improved approaches for SQG derivation. Among the caveats against an uncritical use of SQGs, Allen Burton (2017) includes the consideration of background levels of trace elements and the temporal variability and spatial patchiness of contaminants distribution. Research needs to go beyond the current limitations of SQGs are diverse, but remind us to better consider the ecological context in which a risk assessment study (with or without associated management options) is being conducted.

As a personal contribution to the importance of a proper conceptual use of SQGs or similar benchmark values, I would like to caution against using exclusively chemical approaches to make inferences about contaminant bioavailability and potential ecotoxicological risks. Examples of such approaches (Atibu et al., 2016; Bhuiyan et al., 2014) include (but are not limited to):

a) The geochemical index ( $I_{geo}$ ):  $\log_2(C_z)/1.5(C_b)$

b) The concentration factor (CF):  $C_n/C_b$

c) The sediment enrichment factor (SEF):  $SEF = \frac{\frac{C_z}{Al_z} - \frac{C_b}{Al_b}}{\frac{C_b}{Al_b}}$

where:  $C_z$  = content of an element in the sample  $z$

$C_b$  = content of an element in world mean river bed sediment / suspended matter

$Al_z$  = content of Al in sample  $z$

$Al_b$  = content of Al in world mean river bed sediment / suspended matter

Elemental content in world mean river bed sediments or suspended matter are obtained from literature sources such as Turekian and Wedepohl (1961) or Salomons and Förstner (1984). Normalisation to aluminium content can be introduced to compensate for differences in particle grain size (higher Al concentration corresponds to a higher clay content and, hence, a finer grain size) and should provide better estimates of trace metal enrichment compared with the simpler “concentration factor – CF”. The mathematical formulation for SEF calculations can vary

slightly and different elements (e.g., Al, Sc, Ti) can be chosen for normalization. In any case, as seen for SQGs, such approaches can at best indicate that a given area or site is enriched in a given element. However, they do not provide any indication about its bioavailability and potential for adverse effects on resident biota or risks linked to its environmental mobility. Several sediment characteristics may modulate contaminant bioavailability at a given site (Campana et al., 2013; Simpson and Batley, 2007b) and non-chemical stressors such as altered sediment balance or habitat destruction can be the main drivers of adverse ecological effects (Burton and Johnston, 2010). Conclusions drawn on the basis of  $I_{geo}$ , CF, SEF or other similar approaches should therefore not be used as a final decision-making tool to trigger management actions.

Additional caution is required not to draw wrong conclusions about the actual level of elemental enrichment in a given sediment. Such errors are very easily made when geochemical contamination indexes are used without considering the actual purpose for which they were originally created. For example, the SEF was proposed by Kemp et al. (1976) to evaluate *temporal enrichment* of trace element in *sediment cores* from the Great Laurentian Lakes. In such circumstances, it could be reasonably assumed that no major changes in the geological background level of trace elements occurred at the sampling location during the time span covered by a given core. Furthermore, normalization to a geogenic element such as Al (considered unaffected by anthropogenic activities), accounted for possible temporal changes in sediment grain-size and composition; both of which can modify background elemental concentrations. All these assumptions are almost certainly violated when the SEF (or any other geochemical index) is applied across sites to evaluate the *spatial enrichment* of trace elements along the course of a large river or over extended areas. In such circumstances, geological differences between the sediment in the studied area and the material used to establish the reference value (or the reference site) can artificially increase the contamination ‘factor’. Calculation of SEF using average element concentrations for ‘world mean river’ bed sediments (Salomons and Förstner, 1984) overestimated the potential contamination in river Po sediments; even at sites which were free of contamination when a local reference sites was used (Table 2). These differences in the calculated SEF values simply reflect the naturally higher aluminium (Al) and trace element content of river Po sediments compared with ‘world average’ values; at least at the time of sampling and at the sites taken into consideration.

Table 2. Calculation of Sediment Enrichment Factors (SEF) for sediments collected at three sites in the river Po (Italy) using world average (WA) concentrations for bed sediments (Salomons and Förstner, 1984) or concentrations measured at a local reference sites (RS) upstream of known anthropogenic influence. All concentrations (Conc) are in mg kg<sup>-1</sup> dry weight. Data for the river Po are from Vignati et al. (2003) .

	Al	Cd			Pb			Ni		
	Conc	Conc	SEF (WA)	SEF (RS)	Conc	SEF (WA)	SEF (RS)	Conc	SEF (WA)	SEF (RS)
WA	72,000	0.17	---	---	19	---	---	52	---	---
RS	102,726	0.51	---	---	43	---	---	133	---	---
Site A	86,795	0.64	2.1	0.4	39	0.7	<0.1	133	1.1	0.1
Site B	88,595	1.51	6.2	2.3	72	2.1	0.85	99	0.6	<0.1
Site C	85,314	1.02	4.1	1.3	50	1.2	0.34	102	0.7	<0.1

Such issues become particularly relevant when using geochemical indexes to evaluate sediment quality in (prospective) mining areas. In such settings, using ‘world average’ values

to calculate geochemical indexes must be done with extreme caution and ought to be associated with some measures of bioavailability or biological endpoints. While an uncritical use of SEF may overestimate enrichment factors, high elemental concentration due to the presence of geological peculiarities should not be systematically dismissed as risk-free either. In terms of ecotoxicology, the origin of the contamination is immaterial, albeit management and remediation option will clearly differ between situations of documented anthropogenic impact and those of linked to natural factors. Some aspects related to these topics are discussed in section 3.

### **3. Bioavailability and effects of sediment bound contaminants**

Measurements of total contaminant concentrations in sediments and, where available, comparison with the corresponding SQGs can identify areas potentially affected by anthropogenic activities. However, such measurements do not allow a precise assessment of the risk posed by those contaminants to resident biota because only a fraction of the total contaminant concentration, the so-called bioavailable fraction, will be able to interact with organisms and elicit biological responses. Determining the bioavailable fraction of sediment-bound contaminants remains a challenge for the scientific community and I will further discuss it in the context for my HDR project. In all cases when information on contaminant bioavailability is missing or insufficient, ecotoxicological evaluation of sediment quality offers the opportunity to understand if increased contaminant concentrations may be associated with adverse biological effects.

Ecotoxicological tools for assessing sediment quality are diverse and include, among the others, observations of physiological and/or behavioural effects in resident biota (e.g., biomarkers of exposure and effects), laboratory ecotoxicity testing using micro- and mesocosm approaches and *in situ* ecotoxicity testing. Taken in isolation, each of these approaches is unlikely to provide a definite answer as to the actual environmental risk linked to the presence of contaminated sediments. On the other hand, their combined results can provide different lines of evidence to pinpoint one or more contaminant(s) as the likely cause of the observed biological effects. Indeed, as observed by Wang (2011), ecotoxicology requires an integration between the study of contaminant environmental behaviour, exposure and bioaccumulation, and ecotoxicological response. While I cannot yet claim to have performed a study fully integrating these three aspects, previous work allowed me to make some contributions to this research field.

The results presented below are part of collaborative research efforts in which I was involved during my post-doctoral years at the F.-A. Forel Institute (University of Geneva, Switzerland; currently Department F.-A. Forel for Environmental and Aquatic Sciences) and while being on duty at the Water Research Institute of the Italian National Research Council (IRSA-CNR) from 2008 to 2012. More recent work, performed at my current institution, is also discussed in the present section because it represents a conceptual and practical continuation of these earlier efforts (Table 1). These more recent results were also obtained thanks to collective work and credit will be given as necessary in the following subsections.

#### *3.1. Assessing bioavailability of sediment-bound contaminants in the laboratory*

Sediment-dwelling aquatic organisms accumulate contaminants via multiple pathways including aqueous uptake via the gills or dermal routes and ingestion of contaminated sediments or preys. The Dynamic Multipathway Bioaccumulation Model (DYMBAM) represents an interesting approach to determine the relative importance of different routes of exposure for contaminant bioaccumulation (Luoma and Rainbow, 2005).

Bioaccumulation tests with the oligochaete *Lumbriculus variegatus* (Camusso et al., 2012) showed that the predominant route of exposure may differ among trace elements. Experiments were performed using field collected sediments and the dietary uptake route was shown to be predominant for As, Cd, Cu, Zn, while Ni was accumulated mainly from pore water and Pb from a combination of pore water exposure and sediment ingestion. These findings support the repeated calls to better consider dietary exposure pathways when assessing the bioavailability and effects of sediment-bound contaminants (Allen Burton, 2017; Simpson and Batley, 2007a). To further complicate matters, subsequent work showed that simple manipulations such as sediment homogenisation before starting laboratory experiments could change the quantity of elements accumulated by the midge *Chironomus riparius* (Ferrari et al., 2014). In particular, accumulation of Cr and Cu was increased in laboratory exposure conditions (i.e., after sediment mixing) compared with larvae of *C. riparius* caged in the field (see section 3.3 for more details). While the biological traits of *L. variegatus* and *C. riparius* differ, both works showed that simple homogenization of field collected material before laboratory test, which is usually recommended in standardized procedures, can alter contaminant bioavailability with respect to field conditions.

The importance of characterizing the geochemical behaviour of contaminants in laboratory exposure systems was specifically investigated using a flow-through microcosm developed by Dr. Benoît Ferrari and Régis Kottelat (Kottelat et al., 2010). The microcosms consisted of a layer of uncontaminated reference sediments covered by filtered natural water continuously spiked with Cd, Cr(III), Cu and Pb (Fig. 2). Both sediments and filtered water were collected from Lake Geneva. This technically complex device was shown to possess several advantages in creating well-characterized exposure conditions for ecotoxicity testing with both pelagic and benthic organisms. In particular, a steady state partitioning of trace elements between the colloidal and truly dissolved phases, operationally defined as 3kDa–1.2µm and < 3kDa respectively, was rapidly achieved and maintained for the entire experiment duration (about 1 month). Elemental concentrations in pore waters (PW) collected at three different depths within the sediments (0–0.5cm, 0.5–1cm, 1–1.5cm) decreased by less than a factor of two (five for Pb) between the beginning and the end of the experiment. This level of variability in PW concentrations could be considered as very low compared with conventional (at that time) spiking procedures (Lee et al., 2004; Simpson, 2004). Finally, elemental concentrations in the surface sediment layer increased in a predictable way during the experiment; *i.e.* the (total) exposure concentrations could be modelled at any time without the need to interrupt an experiment or to perturb the experimental system. Because setting up flow-through microcosms is time and labour intensive, the use of such medium-complexity tools will be justifiable only in high-tier assessments to validate results obtained with simpler methods or before starting expensive remedial operations. Overall, we concluded that detailed knowledge of contaminant dynamics in microcosm systems before ecotoxicological investigations can be very helpful for a better extrapolation of laboratory findings to field situations. Indeed, characterizing contaminant behaviour in laboratory experimental settings does not eliminate potential artefacts, but allows to better interpreting ecotoxicological results and avoiding wrong conclusions about their actual environmental relevance.

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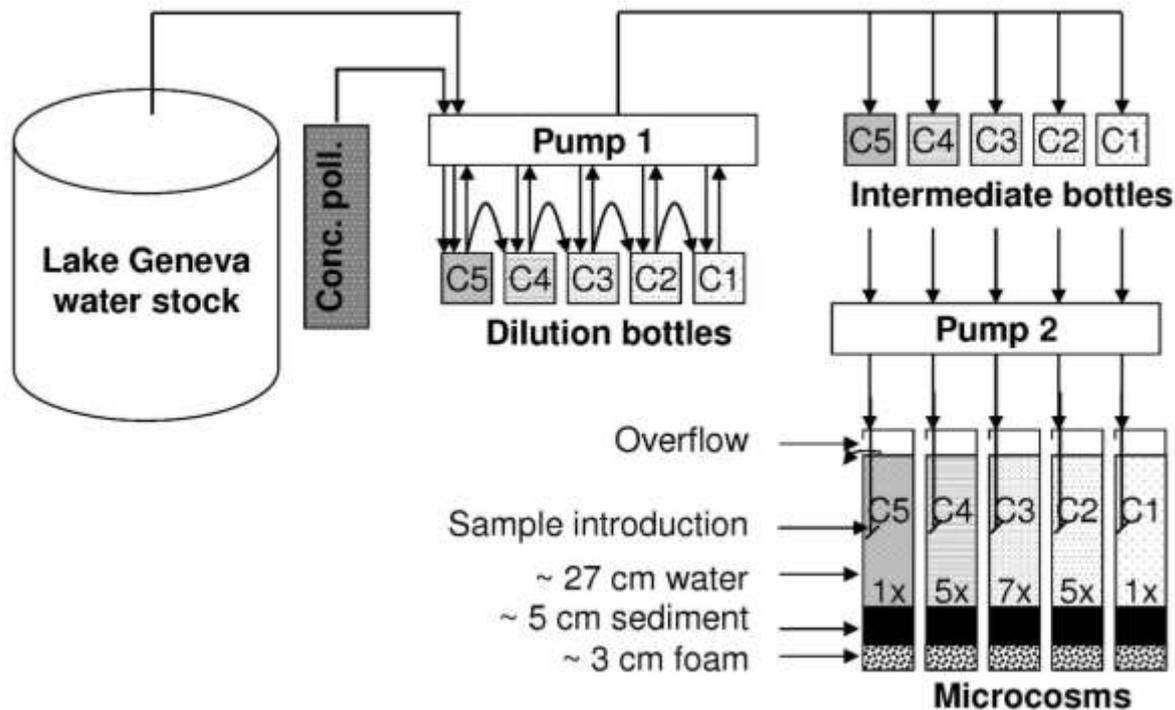


Figure 2. Schematic diagram of the microcosms' layout and operation used by Kottelat et al. (2010). A peristaltic pump (Pump 1) prepares the five dilutions of spiked metals (C1–C5) in the “dilution bottles” by mixing the stock of Lake Geneva water and the concentrated pollutant solution (Conc. poll.). The same pump transfers the “dilutions” into the “intermediate bottles” from where a second pump (Pump 2) continuously feeds the different microcosms (from 1 to 7 depending on the dilution). Excess water is eliminated by an overflow and collected for proper disposal.

In simpler experimental settings, sediments are usually spiked with the contaminant(s) of interest and left to equilibrate for a fixed time before starting ecotoxicological investigations. Simpson and Batley (2007b) cautioned about the importance of verifying that the adopted spiking procedure results in stable exposure concentrations in the various matrices (overlying waters, sediment and pore waters) from which organisms can accumulate contaminants. Standard procedures (OCDE, 2007; 2010) acknowledge that equilibration time after spiking is sediment- and chemical-specific and can take up to five weeks. However, the same procedures recommend equilibration periods between 48h and 7 days; essentially to avoid excessive degradation of the test substance in the case of organic compounds.

During a research visit from Dr. Sara Valsecchi (CNR-IRSA, Italy) in 2016, we examined the behaviour of Co, Ni and Cr(III) spiked into synthetic sediments with variable content of organic carbon, Fe and Mn. This experiment was carried out in the framework of a LabexR21 subproject (<http://ressources21.univ-lorraine.fr/>) on ultramafic environments under the coordination of Prof. Laure Giambérini and with significant contributions from Dr. Laetitia Mínguez (chargée de recherche at LIEC) and Dr. Ana Romero-Freire (post-doctoral fellow at LIEC at that time). Total concentrations of Co, Ni and Cr measured in sediments 6, 14, 35 and 70 days after spiking agreed well with expected values and did not show any temporal trends or differences as a function of the sediments' OC, Fe and Mn content. On the other hand, water parameters evolved differently depending on sediment composition. Element concentrations in overlying waters initially fluctuated and achieved stable values after 14 to 70 days of equilibration, depending on the element and on the sediment characteristics. Concentrations in pore waters initially showed decreasing trends and stabilized only after 40 days of equilibration. In practice, the incubation times suggested by the OCDE/OECD norms for sediment toxicity

testing with *L. variegatus* (OCDE, 2007) and *C. riparius* (OCDE, 2010) do not appear appropriate if stable exposure conditions have to be maintained over the entire duration of such experiments. Once again, the purpose is not to decry standard practices (which actually make some provisions for checking exposure concentrations), but to underline the importance of characterizing laboratory exposure systems to the best possible extent. This chemical step appears invaluable to interpret the subsequent ecotoxicological results, including bioaccumulation measurements, in a reliable and environmentally relevant way.

### 3.2. Assessing effects of sediment-bound contaminants in the laboratory

The evaluation of sediment ecotoxicity in several tributaries of the river Po (see section 2.2) identified those sites where the presence of the analysed contaminants could be responsible for the observed effects (Vignati et al., 2008b). I would consider the results of this study as reliable, but of moderate relevance according to the definitions provided by Moermond et al. (2016) and Roth and Ciffroy (2016) (see part III, section 1.2 for more details on reliability and relevance in ecotoxicology). These experiments were performed following accepted and scientifically valid procedures, but the effects of sediment-bound contaminants were assessed by exposing organisms to sediment organic extracts. Such an approach poorly mimics real environmental conditions and neglects the possibility of dietary uptake in real field situations. Furthermore, no quantification of contaminants was performed in the extracts. Despite this self-criticism, I still consider these results as capable of providing some indications as to the tributaries with the greatest ecotoxicological potential at the time of sampling. Furthermore, this perfectible past experience has been useful to plan more relevant studies in which I took part in recent years; specifically a collaborative research to assess the possible effects of trace element mobilization in a prospective mining area for lanthanides (Romero-Freire et al., 2018).

In this research, funded by the LabexR21 project (<http://ressources21.univ-lorraine.fr/>) and coordinated by Prof. Laure Giambérini, sediment aliquots were extracted using aqueous ecotoxicological media of variable composition. The extracts were then tested following standardized procedures and assayed for their content of lanthanides and other trace elements (Romero-Freire et al., 2018). The conceptual strength of this approach is that all lanthanides were simultaneously extracted into the test solutions according to their partitioning behaviour between field-collected sediments and the chosen aqueous matrices. Test organisms were therefore exposed to a cocktail of lanthanides that simulated possible real-field scenarios much better than experiments testing the effects of one or few lanthanides (Gonzalez et al., 2014; Herrmann et al., 2016). Because such approach reproduces the relative environmental mobility of the different lanthanides, it also has improved relevance compared with tests using lanthanides' equimolar mixtures (Tai et al., 2010). These strengths are accompanied by some pitfalls, though. In ecotoxicological tests using fluorescence or absorbance measurements as an endpoint, the simple presence of sediment particles can cause false positives and must be carefully considered in result interpretation (Romero-Freire et al., 2018). Elements (or compounds) other than lanthanides are also extracted from the sediments into the aqueous matrix and can contribute to the observed effects in test organisms. As it appeared early in ecotoxicology, a major part of the contaminants responsible for observable biological effects is often difficult to identify when working with natural matrices (Camusso et al., 2000; Hendriks et al., 1994). In such cases, research combining the use of natural matrices with more controlled experiments can help in better identifying the role of target contaminants in causing the observed effects. Indeed, previous knowledge of effect concentrations of selected lanthanides (Ce, Gd and Lu) in standardized conditions (González et al., 2015) allowed a better appreciation of their possible contribution to the overall ecotoxicological effects of the sediment extracts.

In another study, a battery of three standard tests with benthic organisms (*L. variegatus*, *C. riparius* and *Tubifex tubifex*) was used to show that the current European environmental water

quality standard for pentachlorophenol (PCP) is not protective of benthic organisms accumulating the bulk of their contaminant burden via sediment ingestion rather than uptake from the aqueous phase. The assumption that water exposure is protective of other routes of exposure is often embedded in many European environmental quality standards (EQS), even if the debate is open as to its validity (Allen Burton, 2017). Starting from the water based EQS of 0.4 µg/L for PCP, we applied the equilibrium partitioning approach used by European Commission (EC, 2005b) and estimated at 119 ng g<sup>-1</sup> d.w. the corresponding EQS for PCP in sediments. This theoretical value exceeds the quality standard of 30–40 ng g<sup>-1</sup> calculated on the basis of experimental results and highlights the potential for possible shortcomings in the EU legislation. On the other hand, PCP monitoring in sediment is not regularly performed in Europe and the environmental significance of the results remains to be evaluated (Bettinetti et al., 2018).

### 3.3. Assessing bioavailability and effects of contaminants in the field

Important note: full credit for the technical developments of the *in situ* exposure devices used in this research goes to Dr. Benoît J.D. Ferrari (Ferrari et al., 2014). Work described in this section was carried out from 2002–2008 under the direction of Prof. Janusz Dominik and in strict cooperation with Dr. Ferrari at the F.-A Forel Institute (currently Department F.-A. Forel for Environmental and Aquatic Sciences, University of Geneva). Dr. Ferrari is currently director of the Swiss Centre for Applied Ecotoxicology (Ecotox Centre). My contribution to this research effort has been in term of investigation, formal analysis and writing of manuscript drafts (see CRediT roles at <https://casrai.org/credit/>).

All the work described in this section was carried out in the Dunajec river/Czorsztyn reservoir system (southern Poland) where chrome-based, artisanal leather tanning is an important economic activity (Pawlikowski et al., 2006; Szalinska et al., 2010). Leather tanning uses large quantities of Cr(III), usually as basic chromium sulphate Cr(OH)(SO<sub>4</sub>)<sub>2</sub>, to increase the durability of leather and to confer other desirable characteristics such as softness and thermal stability to treated hides (Dixit et al., 2015; Lofrano et al., 2013). Although the composition of tannery wastewater is very complex (Dixit et al., 2015), the work to which I contributed was specifically dedicated to understanding the bioavailability and possible effects of chromium to benthic organisms.

Initial field investigations simply compared the total chromium content in sediments and indigenous chironomids assemblages collected from various sites in the Dunajec/Czorsztyn system. Chromium accumulation by chironomids was inversely proportional to Cr sediment content at sampling locations without or with low impact from tannery activities, while the two variables did not show any simple relationship at the most contaminated sites (Szalinska et al., 2008). These contaminated sites, named Waksmund and Maniowy, had contrasting characteristics and were retained for detailed investigations. The site of Waksmund was located in a lotic environment and directly impacted by the discharge of a small artisanal tannery, while Maniowy was representative of a shallow (5–7 m) lentic environment locally influenced by a small creek receiving the effluent of a wastewater treatment plant. Tanneries were also present in the region of Maniowy, but not all of them discharged their waste to the treatment plant.

Work carried out at the two sites included:

- a) Quantification of Cr bioaccumulation in indigenous chironomids,
- b) Estimation of the bioavailable fraction of sediment-bound Cr using a biomimetic approach (Box 1),
- c) Characterization of Cr speciation within sediments and at the sediment-water interface using DGT/DET probes and catalytic adsorptive stripping voltammetry (Bobrowski et al., 2012),
- d) *In situ* exposure of laboratory-reared specimen of *Chironomus riparius* along with on-site controls,



contaminated Waksmund site, suggesting that Cr bioavailability was lower at the latter site (*i.e.* at the site most directly influenced by tannery discharges). However, the biomimetic extraction with BSA (see box 1) estimated a similar percentage of Cr (about 0.1% of the total) as being available for potential assimilation by biota at both sites. According to these results, differences in Cr bioaccumulation by indigenous chironomids could therefore not be explained by differences in the bioavailability of sediment-bound Cr. Concentrations of filterable Cr measured in porewaters, Cr concentrations measured by DGT/DET and Cr(VI) levels were also systematically higher at Waksmund than at Maniowy; contrary to what was observed in chironomids. Many factors not directly examined during our field campaigns could have contributed to the observed discrepancies between Cr levels in sediments and in indigenous chironomids; *e.g.* horizontal and vertical heterogeneity of sediment Cr content, temporal variability of Cr concentrations in pore- and overlying waters, differences in species composition between the two sites. However, we surmised that chironomids actually accumulated a significant part of their Cr body burden by ingesting suspended matter freshly deposited at the sediment-water interface. This hypothesis was validated by studying chromium accumulation in specimen of laboratory-reared *Chironomus riparius* using *in situ* exposures and standardized laboratory bioaccumulation tests.

Three exposure scenarios were set-up to identify the contribution of different exposure pathways to Cr accumulation by chironomids:

- a) laboratory exposure using field-collected sediment (plus clean sand as a control),
- b) on-site exposure in chambers containing clean artificial sand, but with free exchange with the overlying water column, and
- c) *in situ* exposure to field sediment using the S-WISS apparatus (Ferrari et al., 2014).

The on-site chambers served as controls for the *in situ* exposures, while the laboratory experiments were performed to evaluate how well a standardized bioaccumulation test could reproduce a situation in the field using the same species of invertebrate. The unique design of the S-WISS apparatus allowed for feeding of the chironomids during *in situ* and on-site exposures, thus eliminating the possible confounding influences of food availability. Laboratory exposure also allowed to establishing the possible ecotoxicological effects of sediments onto the exposed organisms.

Laboratory results essentially conformed to the expected outcome with controls showing negligible Cr accumulation compared with field-collected sediments. Chromium accumulation by organisms exposed to field-collected sediments in the laboratory was in the same order of magnitude ( $77 \pm 5 \mu\text{g g}^{-1}$ ) as accumulation by organisms exposed *in situ* ( $128 \pm 33 \mu\text{g g}^{-1}$ ;  $n=3$  in both cases, errors being expressed as one standard deviation); albeit the difference was statistically significant (Tukey HDS test on log-transformed data,  $p < 0.05$ ). On the other hand, control chironomids exposed on site (*i.e.*, growing on clean sand, but being exposed to ambient water and suspended particles) accumulated  $101 \pm 21 \mu\text{g g}^{-1}$  ( $n=3$ ) of Cr, a value comparable to chironomids exposed *in situ* via all possible exposure routes. Accumulation of Cr from the Tetramin® food added to all exposure conditions could clearly be neglected and the very low levels of Cr in laboratory controls agreed with the low Cr content reported for Tetramin® (Habashy, 2005). Negligible Cr accumulation in laboratory control chironomids also indicated that the clean sand used as a growing substratum did not contribute appreciably to Cr burdens. Furthermore, calculations using biodynamic modelling (see details in Ferrari et al., 2019) showed that Cr accumulation by chironomids exposed *in situ* could not be accounted for by Cr uptake via pore water or via sediment ingestion. In the end, ingestion of freshly deposited suspended particulate matter appeared as a highly plausible additional exposure route under field condition. This hypothesis is supported by experimental data, compatible with chironomids' ecology (McLachlan, 1977; Stief and de Beer, 2006) and confirmed by other recent experiments using a customized *in situ* emergence platform (Ferrari and Faburé, 2017).

Survival of chironomids during bioaccumulation tests was almost complete for laboratory controls ( $19.6 \pm 0.7$  individuals,  $n = 12$  replicate exposures), slightly reduced for laboratory exposure to field-collected sediments ( $18.1 \pm 2.2$  individuals,  $n = 12$ ) and on-site controls ( $16.3 \pm 3.7$ ,  $n = 12$ ) and clearly affected for *in situ* exposures ( $9.8 \pm 2.7$  individuals,  $n = 11$ ). Chironomids exposed *in situ* and on-site controls also showed reduced growth compared with laboratory controls and laboratory exposed chironomids. Data elaboration by Dr. Benoît Ferrari estimated a critical body burden (CBR) of  $77 \mu\text{g Cr g}^{-1}$  d.w. for effects on growth. The proposed CBR value is in reasonable agreement with the results of Méndez-Fernández et al. (2013) who calculated a CBR of  $34 \mu\text{g Cr g}^{-1}$  tissue d.w. for the oligochaete *Tubifex tubifex* exposed to natural sediments spiked with hexavalent Cr as  $\text{K}_2\text{Cr}_2\text{O}_7$ . On the other hand, Norwood et al. (2007) estimated at  $7.9 \mu\text{g g}^{-1}$  d.w. the lethal body concentration leading to 25% mortality in specimen of *Hyalella azteca* exposed for 4 weeks to aqueous solutions spiked with Cr(VI). Considering that Cr(VI) is rapidly converted into Cr(III) inside living cells (Zhitkovich, 2005), a comparison of CBR values remains meaningful despite the possible differences in Cr speciation in the exposure matrix. Although sediments impacted by tannery wastewaters receive many other contaminants along with Cr (Dixit et al., 2015), the CBR approach deserves further study as a tool to assess the risk of Cr contamination in real-field situations. Furthermore, chironomids may withstand pollution levels capable of killing more sensitive organisms without apparent harm (Bervoets et al., 2016).

#### 3.4. A critique of my previous research on the bioavailability and effects of sediment-bound contaminants

In retrospective, I am pleased to note that the work described in the previous subsections agrees very well with a conceptual framework for laboratory-to-field extrapolation described in Vignati et al. (2007). This framework postulates that a combination of field and laboratory work (supported by adequate theoretical knowledge and testable working hypotheses) constitutes an optimal approach to develop the necessary knowledge to understand and protect our environment (Fig. 3). It would be inappropriate to claim that I have systematically and successfully implemented this whole approach over the past 15 years. However, I am quite satisfied with my research evolving from the use of simple and more or less standardized tests (Vignati et al., 2008b) to approaches trying to understand the importance of different routes of exposure or the actual contribution of the contaminants of interest to the observed effects (Bettinetti et al., 2018; Ferrari et al., 2019; Romero-Freire et al., 2018; Vignati et al., 2019). Efforts to reconcile chemical results (e.g., use of the biomimetic extraction) with observed bioaccumulation in living organisms are also worth mentioning (Ferrari et al., 2019; Vignati, 2004; Vignati et al., 2019). Chemical extraction procedures, starting from Tessier et al. back in 1979, provide useful indications on the relative mobility of trace elements associated with sediments. However, their results in terms of bioavailability and potential ecotoxicity require validation by measurements in biological matrices or by correlation with observed ecotoxicological effects (see part III).

The specific study of Cr accumulation by benthic organisms in aquatic systems contaminated by tannery discharges (section 3.3) allowed to directly investigate the uptake of this element in environmental settings where it is expected to occur mainly in its trivalent form (Pereira et al., 2012; Rosales et al., 2017). The experimental findings (Ferrari et al., 2019; Vignati et al., 2019) partly questions the conclusions of previous research on Cr(III) bioavailability and ecotoxicity. Berry et al. (2004) and Besser et al. (2004) concluded that Cr(III) is poorly bioavailable and of little ecotoxicological concern based on study performed using formulated sediments spiked with soluble Cr(III) salts. Becker et al. (2006) reached similar conclusions when studying contaminated sediments in areas impacted by chromium ore processing residues – COPR. These contrasting results make perfect sense when considering the difficulties linked with

spiking aqueous and sedimentary matrices with trivalent Cr (Kottelat et al., 2010 and my unpublished results briefly described in section 3.1). Furthermore, Cr speciation in sediments impacted by COPR is likely extremely different from the Cr species in sediments impacted by discharges from tanning activities. In the latter case, organic Cr forms may occur (Walsh and O'Halloran, 1996). Such Cr species likely have higher bioavailability than the Cr oxyhydroxides that are formed when spiking aqueous or solid matrices with Cr(III) salts or the mineral Cr forms accompanying COPR discharges. All this research has contributed to the reflection leading to the scientific questions developed in the HDR scientific project (part III).

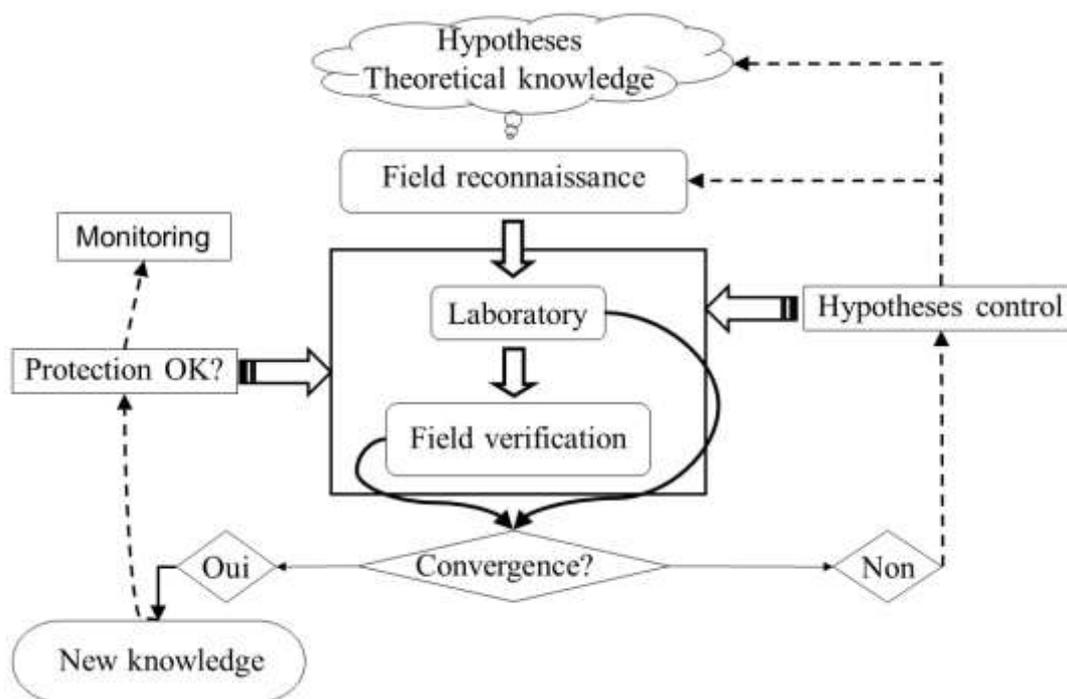


Figure 3. Schematic representation of a possible conceptual strategy for better reconciling laboratory and field investigations (modified from Vignati et al. 2007).

Equally appreciable is the critical interpretation of results from standardized tests to foster further reflection on basic assumptions of contaminant bioavailability. The need to pay more attention to dietary exposure in (sediment) risk assessment has been repeatedly stressed (Allen Burton, 2017; DeForest and Meyer, 2014). The recent collaborative work on PCP (Bettinetti et al., 2018) supports this recommendation by showing that the water-based EQS for PCP may fail to protect sensitive benthic organisms. More importantly, these results question the accepted paradigm that water-based EQS are conservative for other exposure routes and call for more attention to the contaminants' environmental behaviour and the ecology of the resident organisms. The validity of such recommendations is further increased considering that EQS or consensus-based guidelines may be underprotective as to their ability to preserve healthy community structures under WFD requirements (Bervoets et al., 2016; Van Ael et al., 2015).

From a pragmatic point of view, the objective of risk assessment is not to bring all ecosystems back to a pristine state (although this may remain an ideal ultimate goal), but to ensure that impacts are kept within a range that does not affect ecosystem structure and functioning (Meybeck, 2003). The detailed characterization of exposure concentrations for different uptake routes, a clear understanding of the temporal aspects of laboratory exposure scenarios and a combination of laboratory and field approaches represent a valid strategy to

reconcile standard procedures and environmental realism for improved environmental protection (Fig. 3). In this sense, the conclusions from my previous work in this research field seem, until now, to conceptually stand the test of time, albeit some specific investigations might have become of limited environmental relevance in view of the later scientific developments.

#### **4. Geochronology of Hg contamination in sud-alpine lakes and implications for risk assessment**

Mercury is a very toxic metal that biomagnifies along food webs and poses serious risks to top predators and to humans, mainly via consumption of contaminated preys, fish and sea food (Evers and Sunderland, 2019). The tendency of Hg to biomagnify derives from the transformation of Hg into organic mercury forms (mainly methylmercury – MMHg), while the existence of Hg gaseous forms enhances long-distance transport and makes Hg a contaminant of global concern (Mason, 2013). Mercury is included in the European list of priority dangerous substances of Directive 2013/39EC and a global treaty, the [Minamata Convention on Mercury](#), has been into force since October 2013 to restrict and, where possible, eliminate its use. Despite the past and current efforts to curb Hg emissions, legacy mercury (i.e., mercury emitted into the environment from various activities in the past) still remains a problem in several European countries and worldwide (EEA, 2018; Evers and Sunderland, 2019).

From 2008 to 2012, I was involved in the activities of CIPAIS (Italian acronym for International Commission for the Protection of Italian Swiss Waters) on the contamination of Lake Maggiore (Italy/Switzerland) by DDTs, Hg and other contaminants. Specifically, I have been the principal investigator for all aspects regarding pollution by Hg and other trace elements (only mercury will be discussed in the present document). I also took part in a working group coordinated by Italian regional water authorities to manage Hg pollution in Lake Maggiore, studied Hg contamination in others sud-alpine lakes in Northern Italy and co-chaired an interest group on Hg under the auspices of SETAC from 2010 to 2016 (Bank et al., 2014).

##### *4.1. Geochronology of Hg pollution in Lake Maggiore and other sud-alpine lakes*

Lake Maggiore is located south of the Alps along the border between Italy and Switzerland. The lake has been affected by anthropogenic Hg inputs since ancient times because of artisanal and, during the 19<sup>th</sup> century, industrial-scale gold mining in some parts of its catchment. However, the major contribution to Hg contamination has originated from a chlor-alkaly plant that has been in operation for most of the 20<sup>th</sup> century in the catchment of the river Toce, the second most important tributary of the Lake (Damiani and Thomas, 1974; Guilizzoni et al., 2012). Sampling of sediment cores from various part of Lake Maggiore has allowed a rather clear reconstruction of the geochronology of Hg contamination and of its spatial extent. The work presented in this section has benefited from inputs by several colleagues from CNR-IRSA (Brugherio and Pallanza branches).

By examining a core of about 110 cm collected in the area most directly impacted by Hg discharges, we concluded that: 1) no geogenic anomalies leading to high background Hg levels exist in this part of the lake, 2) the presence of gold-mining activities in the catchment increased Hg levels in lake sediments between 1550 and 1850 AD, and 3) maximum Hg contamination (with increases > 200-fold above background levels) occurred in the 1920s and 1940s; in correspondence with the peak activities of the chlor-alkaly plant (Vignati and Guilizzoni, 2011). Other cores from the southern part of the Lake showed similar temporal variation of Hg levels, albeit with lower concentration, suggesting that Hg inputs were transported to this part of the Lake by the predominant water circulation (Ambrosetti et al., 2012). Analysis of selected samples by Multi-Collector ICP-MS (in collaboration with Prof. Chris Metcalfe and Prof. Holger Hintelmann at the Trent University, Ontario, Canada) also supported this hypothesis,

although further research would be needed to reconstruct the exact transport mechanisms of Hg in Lake Maggiore. These findings retained however a certain policy-relevance because they showed that Hg contamination extended beyond the priority area that Italian authorities had identified for monitoring and, possibly, remediation programs.

In addition to these basin-specific efforts, I built a cooperative network to get information on the chronology of Hg contamination in other sud-alpine lakes. While Lake Maggiore has a known history of industrially-derived Hg contamination, this is not the case for other lakes in the same region. Observations for different lakes showed that Hg natural background levels were similar (0.05–0.1 mg kg<sup>-1</sup> dry weight) in all the examined water bodies. On the other hand, marked differences existed as to the levels of current contamination and, when multiple cores were available, between different parts of the same water body. These observations are also policy relevant because Hg is considered as a Ubiquitous Persistent Bioaccumulative Toxic substance (UPBTs) under Directive EU 2013/39. For such substances, EU legislation stipulates that, when reporting the chemical status of a water body according to the WFD provisions, data for UPBTs can be presented separately so that improvements in quality obtained for other substances are not obscured. The rationale is that the global origin of UPBTs makes it impossible for a single Member State to control Hg sources that can be located outside its own territory. While this is a sensible approach, my sedimentological data show that local situation can be independent from global Hg sources and that due consideration must constantly be given to potentially unknown specific situations requiring site-specific interventions.

#### 4.2. Risk assessment and regulatory aspects

As for other elements, the actual risks linked to Hg contamination depend on its bioavailability to organisms rather than on its total concentration in sedimentary (or aqueous) matrices. In the case of Hg, things are further complicated by the bacterially-mediated reactions leading to the formation of the highly bioavailable methylmercury that easily enters and biomagnifies along food chains. The current EU legislation (Directive 2013/39EC) has recognized these peculiarities and proposed Hg concentrations for regulatory purposes to be directly monitored in biota. At present, the environmental quality standard (EQS) for mercury is 20 µg kg<sup>-1</sup> wet weight of fish, without further specification neither for the species nor for specific tissues. The use of other matrices (i.e., organisms other than fish or abiotic matrices) is in principle allowed, provided that the Member States show that an equivalent level of protection is achieved.

Monitoring of Hg concentrations in the zebra mussel (*Dreissena polymorpha*) and in three species of fish is part of the CIP AIS program in Lake Maggiore. As to the period 2008–2012 during which I took part in this program, monitoring of mussels collected at eight different locations in Lake Maggiore showed that mussels living in the zone more directly impacted by past Hg discharges accumulated higher Hg levels than elsewhere. In the case of fish, we consistently observed that the accumulated levels were higher than the proposed EQS of 20 µg kg<sup>-1</sup> in all the three monitored species (landlocked shad — *Alosa fallax lacustris*; whitefish — *Coregonus lavaretus* and the common roach — *Rutilus rutilus*). These monitoring results can be found in the annual CIP AIS reports available on the Commission's website ([www.cipais.org](http://www.cipais.org)) and have been used to get a general long-term overview of Hg contamination in the lake biota (Guzzella et al., 2018). Thanks to voluntary efforts with fellow colleagues, I also analysed Hg concentration in specimen of landlocked shad collected in other sud-alpine lakes and observed that the proposed EQS was systematically exceeded even in lakes without any history of industrial Hg contamination. Mercury contamination actually remains a primary cause of regulatory non-compliance in EU water (EEA, 2018).

Considering the management problems arising from such situation, it may be worth discussing if the present Hg EQS is overprotective (and thus needs revision) or if the state of EU water bodies is actually highly compromised with respect to Hg contamination. The current

EQS was initially proposed using a deterministic approach, i.e., by dividing the Non Observable Effect Concentration for oral toxicity obtained from a single critical study by a safety factor of 10 (EC, 2005a). Nonetheless, calculations based on a probabilistic approach (WCA, 2014) resulted in similar numerical values, suggesting that there are no scientific grounds to dismiss the current EQS as erroneous. This does not rule out that the EQS might be too conservative with respect to background Hg levels in fish inhabiting European water bodies. Hope and Louch (2014) modelled the likely Hg levels in fish before the beginning of marked human influence in North American freshwater environments. The modelled median Hg concentrations ranged from 7 to 47  $\mu\text{g kg}^{-1}$  wet weight in fish fillet, but the corresponding 5<sup>th</sup> and 95<sup>th</sup> percentile concentrations ranged from 1 to 554  $\mu\text{g kg}^{-1}$ . On the other hand, Depew et al. (2012) showed that levels of 40  $\mu\text{g kg}^{-1}$  Hg can have adverse effect on the reproduction of some fish species. In summary, the approach of assessing mercury regulatory compliance using Hg concentration in fish tissue is scientifically sound, but examination of further evidence is required to understand the reasons of such widespread non-compliance (Vignati et al., 2013a). The apparent ability of fish species to cope with high Hg body burdens without apparent adverse effects may be linked to the interactions between Hg and Se in living tissues. Selenium can strongly complex Hg and prevent its interaction with biological targets (Ralston et al., 2007), so that it may be important to consider the co-occurrence of Hg and Se when evaluating mercury-related environmental and health risks (Ralston et al., 2015). This approach is however far from being met with a full consensus by the scientific community (Gerson et al., 2020)

It is also important to note that the EU EQS of 20  $\mu\text{g kg}$  wet weight (w.w.) is meant for the protection of fish-eating birds (and mammals) and hence, at least in my opinion, it is not directly comparable with standards for the protection of human health. Aquatic birds and mammals feed almost exclusively on fish; which is not the case for the general human population. In agreement with these observations, regulation 1881/2006 fixes a maximum Hg level of 0.5 or 1  $\text{mg kg}^{-1}$  w.w. in fish for human consumption; the level of 1  $\text{mg kg}^{-1}$  w.w. being applicable to certain species listed in the same regulation. The existence of multiple environmental quality standards applicable for different purposes complicates appropriate risk communication to the general public. For example, Pasquaré et al. (2013) showed that two major Italian newspapers supported different views as to the possible risks of excessive Hg intake from fish consumption. It appears that more cooperation among scientists, public health nutritionists and dietitians is necessary to help consumers to make the best choices to maximize the benefits of fish consumption while reducing the risk from consuming Hg-contaminated species.

#### *4.3. A critique of my previous research on Hg-related issues*

My contribution to this field has been either of regional interest (section 4.1) or dealing with policy-related aspects (section 4.2). In such context, I remember with particular satisfaction the numerous meetings at the regional environmental agency of the Verbano-Cusio-Ossola province (Piedmont, Italy). The agency personnel was in charge of managing the monitoring and, if applicable, remediation activities in relation to Hg and DDT contamination in Lake Maggiore. As already mentioned, mercury and DDT contamination in Lake Maggiore mostly originated from industrial activities located at a priority national site in the basin of the river Toce, a major tributary of Lake Maggiore. Thanks to the results and approaches described in sections 4.1 and 4.2, my colleagues and I advised important changes in the monitoring program performed by the environmental consultant hired by the company owning the industrial installation on the priority site. In particular, we were able to rebut the consultant's claims of naturally high levels of Hg in Lake Maggiore sediments and of Hg pollution being of concern only in a limited zone of the Lake. We also suggested to the regional environmental agency to ask that Hg and DDT bioaccumulation tests, in addition to ecotoxicity tests, should be performed by the company's consultant. Indeed, in the absence of gross recent contamination,

the chronic effects of Hg and DDT are often linked to their possible remobilisation from sediments by benthic organisms and subsequent biomagnification via dietary uptake rather than to direct ecotoxicity to benthic organisms. Our suggestion was accepted and the CNR-IRSA was given the responsibility to verify the results obtained by the consultant.

I am equally pleased to note that my being in favour of the use of a biota-based EQS (Vignati et al., 2013a) has stood the test of time. Back a decade, the European Directive 2008/105 allowed Member States to use an EQS based on total filterable Hg concentrations in water or to opt for a biota-based EQS. Discussions to make the biota-based criterion the default approach soon followed and Directive 2013/39 eventually confirmed this specific approach. At present, a consensus has developed as to the choice of *fish* as the most appropriate matrix for a biota-based monitoring of Hg contamination, although additional work remains necessary with regard to fish species selection and tissue choice, in particular whole fish vs. fillet (Fliedner et al., 2018). Similarly, plans are being developed for monitoring mercury in fish (and wildlife) at a global scale in application of the Minamata convention on mercury (Evers and Sunderland, 2019). On the other hand, I regret that several results have not yet been made public via peer-reviewed articles or open access databases. The results for Hg bioaccumulation tests have eventually been published in a special issue of *Applied Sciences* (Vignati et al., 2020) and I hope to organize the results about Hg geochronology in the different lakes in a scientific publication over the course of 2020–2021.

## **5. Partitioning of trace elements and its implications for elemental behaviour, bioavailability and ecotoxicity**

Environmental behaviour, bioavailability and ecotoxicity of trace elements strongly depend on their speciation; i.e., the full range of chemical and physical forms in which an element exists in the environment. This simple definition is appropriate to describe my past activities, albeit more inclusive and refined definitions of speciation are far from trivial (Templeton et al., 2000) and subject to criticisms and need for revision (Lespes et al., 2016). My specific interests in this research field included, and still includes, the study of metal partitioning between the particulate, colloidal and truly dissolved phases, the distribution of elements between the sediment solid matrix and associated pore waters, and the speciation of Cr in natural and synthetic matrices. This last topic will be dealt with separately in section 6.

The distinction among particulate, colloids and true solution is operational and error prone (Horowitz, 2013; Town and Filella, 2002), but provides a conceptual approach to better understand the environmental fate and ecotoxicology of trace elements in the environment. Particulate-bound elements will tend to accumulate in sediments, while the truly dissolved fraction will be transported over long distances along with the moving water masses. The behaviour of the colloidal fraction will vary depending on the actual environmental conditions. The mechanisms of colloidal pumping (Gustafsson and Gschwend, 1997; Honeyman and Santschi, 1989) can remove colloiddally-bound contaminants from the water column to bottom sediments. On the other hand, in the absence of coagulation and flocculation, contaminants associated with colloids will move along with the truly dissolved fraction.

### *5.1. Methodological contributions to the study of partitioning*

The study of elemental partitioning is generally based on fractionation of raw water samples by techniques such as filtration or centrifugation. Filtration is commonly regarded as a routine procedure in both scientific and regulatory contexts. However, filter performance varies greatly (Horowitz, 2013 and references therein) and the risk of filtration artefacts should be systematically checked prior to large monitoring programs or when comparing data sets obtained by different institutions. Filtration at 0.45µm is by far the most widespread approach

following the early work of Goldberg et al. (1952). However, according to Town and Filella (2002), filtration at 0.45 $\mu\text{m}$  provides no useful information to understand behaviour and fate of trace elements. Personally, I would suggest a more elaborate and milder form of this statement in that filtration at 0.45 $\mu\text{m}$  *alone* does not provide *all the necessary information* to understand the behaviour and fate of trace elements. Environmental particles actually form a continuum from small colloids to large suspended particulate matter (SPM) and any fractionation based on the use of filters or other techniques will be *de facto* operational. Indeed, particle size is not the only criterion to distinguish between particulate and colloidal matter. Large organic aggregates of very low density (*e.g.*, marine snow) would be classified as suspended particulate matter according to their size. However, such aggregates would not settle solely under the action of gravity, which makes them eligible to be classified as colloidal material (Gustafsson and Gschwend, 1997). Given the natural complexity of the particle-size continuum, methodological contributions are constantly needed to advance our understanding of filtration and ultrafiltration artefacts along with possible way to minimize them.

With regard to filtration, I showed that the use of 0.45 $\mu\text{m}$  membrane filters in research or monitoring programs can overlook the existence of significant pools of trace elements associated with coarse colloids (operationally defined as particles between 0.45 and 1.2  $\mu\text{m}$ ) (Vignati and Dominik, 2003). Another study compared the performance of membrane filters and filtration cartridges to show that very different devices can fractionate water samples in a similar way except for some elements of geogenic origin and for highly turbid waters (Vignati et al., 2006). This information is important considering that the use of filtration cartridges (which should be less artefact-prone due to their large filtration surface) is not always logistically possible. A similar comparison between small- and large-scale ultrafiltration systems showed that they provided similar results with regard to element partitioning between colloids and true solution (Kottelat et al., 2008). Such results are useful considering that large-scale systems can be used when working with surface waters, for which sample availability is usually not limiting, but not for sediment pore waters or other situations where sample volume can be very small.

Small-scale ultrafiltration was successfully applied to the study of Hg and methylmercury (MMHg) partitioning in overlying and interstitial sediment waters. This study constituted an important methodological application given that sediments are a privileged site for Hg accumulation and methylation. Accurate partitioning of Hg and MMHg between colloids and true solution could be obtained from sample volumes as low as 50 mL (Guédrón et al., 2016). By applying the methodology to contrasting aquatic systems, we showed that Hg and MMHg distribution between the truly dissolved and colloidal fractions could be modelled from the corresponding filterable concentrations (see section 5.3).

In the PhD thesis of Régis Kottelat, to which I contributed about one quarter of the tutoring, we also made direct use of small-scale ultrafiltration to study the distribution of trace elements in laboratory microcosms. The characterization of element behaviour in microcosms is of critical importance to ensure an adequate interpretation of the biological responses observed with such experimental set-ups. We showed that the partitioning between colloids and true solution of selected elements (Cd, Cr(III), Cu, Pb) rapidly reached a steady state in the water column of the microcosms (Kottelat et al., 2010). This is a highly desirable feature for the further study of elemental ecotoxicity to pelagic organisms considering that element complexation by colloids can markedly alter their bioavailability and effects (Koukal et al., 2003).

Finally, I also devoted some attention to continuous flow centrifugation (CFC); a technique that allows to recover larger amounts of suspended particulate matter (SPM) with respect to filtration or laboratory centrifugation. Suspended particulate matter is an important carrier of trace elements and can represent a route of exposure to metals for *e.g.*, filter feeders (Weltens

et al., 2000) or surface deposit feeders (Ferrari and Faburé, 2017; Ferrari et al., 2019). The SPM compartment is currently not considered in the regulatory context, but it will likely receive attention as our understanding of contaminant accumulation following SPM ingestion increases (Schubert et al., 2012). The efficiency of CFC to recover SPM having been previously demonstrated (Burrus et al., 1989; Santiago et al., 1990), I focussed on the possibility of using the CFC effluent (i.e., water without SPM but still containing colloids) for the determination of colloidal and truly dissolved elemental fractions. Characterization of trace element content and partitioning in the CFC effluent would allow direct comparison with results for the SPM retained in the centrifuge and also provide an integrated signal on element partitioning in the water phase. However, our results showed that the CFC effluent was unsuitable for further characterization of elemental partitioning. While passing through the CFC system, a fraction of SPM was broken into smaller, colloidal-sized particles, thus artificially increasing the metal content of the effluent. We therefore concluded that CFC should be used only for recovery of SPM and that characterization of colloidal + truly dissolved element concentration must be accomplished using other techniques (Rossé et al., 2006).

### *5.2. Partitioning in specific and contrasting aquatic systems*

Despite the methodological limitations associated with fractionation of water samples by filtration and ultrafiltration, the use of both techniques can help to better understand and predict the biogeochemistry of trace elements and other contaminants. In my early career, I was charged with the analysis of data collected during a major flood event in the river Po and clarified the importance of high flows for trace metal loads in this basin. The available data also showed that, for certain elements, the partition coefficients between suspended particulate matter (SPM) and the corresponding filterable fraction (less than 0.45  $\mu\text{m}$ ) became constant at SPM concentrations above 500  $\text{mg L}^{-1}$ . These observations do not conform to the *particle concentration effect*; i.e., the tendency of partition coefficients to decrease with increasing SPM concentration (Benoit and Rozan, 1999). Indeed, for very high flow conditions, the particle population transported by river waters is likely dominated by material originating from surface soil run-off, thus creating an exceptional situation as to the distribution of trace elements between the particulate and the filterable fraction. These results have been published in Italian journals and presented as case studies during the works of the Network for Environmental Monitoring and Assessment (Camusso et al., 1999; 2001; Vignati et al., 2005a).

By examining the partitioning of trace elements in a range of contrasting aquatic systems, I managed to determine general tendencies in their environmental behaviour. Highly particle reactive elements such as Al, Ti, and Pb tended to associate with SPM and colloids, with their truly dissolved concentrations often being below the analytical detection limits of ICP-MS. Divalent elements (Co, Cu, Zn) showed variable distribution among SPM, colloids and true solution, depending on environmental variables such as pH, conductivity and oxygen saturation. Elements such as As, Sb and U would apparently occur as anionic species and were found mainly in the truly dissolved phase. All these observations are valid for the range of aquatic systems that were studied, but do not necessarily have universal validity. For this reason, the same results were examined also in terms of elemental partition coefficients to evaluate if they could provide more generally valid findings.

As explained in chapter 7 of my PhD thesis (Vignati, 2004), the added value of partition coefficients with respect to the simple examination of element concentrations in different fractions is that partition coefficients provide an indication of the relative affinity of trace elements for a given fraction. The  $K_d$  expresses the affinity of an element for SPM with respect to the total filterable phase (i.e., colloids + true solution):

$$K_d(L\ kg^{-1}) = \frac{[Me]_{SPM}}{[Me]_{TF}}$$

where  $[Me]_{SPM}$  is the concentration of the element (contaminant) in the particulate phase expressed in  $mg\ kg^{-1}$  and  $[Me]_{TF}$  is the concentration in the total filterable phase in  $mg\ L^{-1}$ . In practice, an element with a  $K_d$  of  $10^5$  will have 100,000 times more affinity for SPM than for the total filterable phase. Note however the same element will occur mainly in the filterable phase at low SPM concentrations ( $1-2\ mg\ L^{-1}$ ) typical of *e.g.* oligotrophic lakes, but for more than 90% in associations with the particulate phase at SPM concentrations between 10 and 100  $mg\ L^{-1}$  which are often found in rivers.

Ideally, the value of the partition coefficient  $K_d$  should reflect the outcome of the various processes regulating the adsorption of an element onto a given solid phase. Adsorption/desorption processes are controlled by several environmental variables (pH, ionic strength, temperature, composition of the sorbing phases); which explains the within- and between-systems variability of partition coefficients. On the other hand, the existence of the particle concentration effect (see above) is unexpected because a  $K_d$  should not vary with SPM concentrations when all other environmental variables are held constant. One possible explanation for the phenomenon is the presence of colloids, which constitute an additional phase for the adsorption of trace elements and whose concentration tends to increase nonlinearly with that of SPM (Gustafsson and Gschwend, 1997). This increase in the colloidal mass will be unaccounted for in the  $K_d$  formula (where colloids are considered as part of the filterable phase) and may provoke the observed decrease in  $K_d$  values as a function of SPM. Explicit consideration of the colloidal material as an additional binding phase for trace element was therefore explored as a means to eliminate the particle concentration effects (Benoit and Rozan, 1999); in formulas:

$$K_p^c(L\ kg^{-1}) = \frac{[Me]_{SPM} + [Me]_{Coll}}{\frac{SPM + Coll}{[Me]_{TD}}}$$

The  $K_p^c$  expresses the affinity of a given element for the total available solids (SPM + Colloids) with respect to the truly dissolved phase, with  $[Me]_{TD}$  being the concentration of the contaminant in the truly dissolved phase in  $mg\ L^{-1}$ ,  $[Me]_{coll}$  the concentrations in the colloidal phase (in  $mg\ kg^{-1}$ ), SPM and *Coll* the concentration of suspended particulate matter and colloids in  $mg\ L^{-1}$ . The  $K_p^c$  coefficient was successfully used to account for the particle concentration effect in four rivers with different background chemistries and degree of anthropogenic pressure in the same region (Benoit and Rozan, 1999). Application of the same approach to contrasting aquatic environments in different countries during my own research showed that the ability of the  $K_p^c$  to eliminate the particle concentration effect was actually metal and system dependent. These results do not detract from the conceptual validity of the  $K_p^c$ , but point to additional research needs to understand the factors controlling elemental partitioning in different real field conditions (Tang et al., 2002). Further theoretical basis and discussion of these issues can be found in my PhD thesis.

Examining the distribution of trace elements between SPM, colloids and true solution also proved a valuable tool to clarify the importance of different mechanisms in controlling elemental behaviour during the mixing of two different water masses. Samples were collected at two sites in the River Po (see section 2.2), upstream and downstream of its confluence with the River Lambro, one of its most polluted tributaries (at least at the time of sampling; July 2001). One sample was also collected in the River Lambro itself just upstream of its confluence with the River Po. Because the Lambro waters had a higher conductivity than those of the River

Po, it was possible to estimate the fraction of Lambro waters in the mixing zone using sodium (Na) as a conservative tracer (Pettine et al., 1996):

$$X_{Lambro} = \frac{([Na^+]_{site2} - [Na^+]_{site1})}{([Na^+]_{Lambro} - [Na^+]_{site1})}$$

Where:  $X_{Lambro}$  is the fraction of Lambro waters in the mixing zone

*Site1* indicates waters samples in the river Po upstream of the confluence with the River Lambro

*Site 2* indicates waters samples in the river Po downstream of the confluence with the River Lambro.

$[Na^+]$  is the measured concentration of filterable sodium.

For the specific field campaign and associated measurements (Vignati et al., 2005c), the calculated values for  $X_{Lambro}$  was 0.17. Assuming conservative behaviour, the trace element concentrations (Conc) at site 2 could therefore be estimated by rearranging equation the equation for  $X_{Lambro}$  as follows:

$$Conc_{site2} = (Conc_{Lambro} \cdot X_{Lambro}) + (Conc_{site1} \cdot (1 - X_{Lambro}))$$

Significant differences between measured and calculated concentrations for site 2 would indicate non conservative behaviour in the Lambro-Po mixing zone.

Major elements preferentially transported in the truly dissolved phase (Ca, Mg, K) and trace elements showing minor association with colloids (As, Mo, Sb, U and V) showed conservative behaviour in the mixing zone; i.e., the experimentally measured concentrations in the truly dissolved and colloidal phases agreed within calculation errors with expected concentrations. The other elements considered in the study (Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Ti and Zn) behaved non-conservatively and knowledge of their partitioning between colloids and true solution allowed to speculate about the possible mechanisms controlling their behaviour in the mixing zone (Vignati et al., 2005c):

- Adsorption of truly dissolved elements onto SPM; i.e., only the truly dissolved concentrations behaved non-conservatively,
- Aggregation/coagulation of colloids; i.e. only colloidal concentrations behaved non-conservatively,
- A combination of both mechanisms described above; i.e. both truly dissolved and colloidal concentrations behaved non-conservatively.

Overall, the results obtained from this study confirmed that colloidal pumping could contribute to the removal of trace elements from the water phase (true solution + colloids) to SPM and ultimately to the sediment compartment, which had been previously identified as a sink for Lambro-born contaminants after its confluence with the river Po (Camusso et al., 2002; Vignati et al., 2003).

The same study also showed that colloid-bound contaminants could reduce the average number of offsprings in the rotifer *Brachionus calyciflorus* exposed for 48h to the colloidal fractions of samples from the three sites (Vignati et al., 2005c). These results showed that colloids isolated from natural aquatic system contributed to the toxic burden in filter-feeding organisms, which cautions against the tendency to consider toxicity mainly as the result of exposure to truly dissolved contaminants. However, the role of natural colloids in controlling ecotoxicity can be site-specific (Gueguen et al., 2004) and, where relevant, still calls for a specific study.

As a logical ramification of these research efforts, I have examined the importance of partitioning in relation to the regulatory monitoring of contaminants in surface waters. In the current regulatory context, monitoring of priority trace elements (Cd, Ni, Pb, Hg)<sup>3</sup> requires the analysis of samples filtered at 0.45µm. No mention is made, or consideration given, to the presence of colloids, the potential artefacts of filtration, or the possible contribution of SPM as a route of exposure to contaminants. Neglecting the importance of partitioning and SPM has no scientific foundation with regard to the environmental behaviour and bioavailability of contaminants (Fig. 4). On the other hand, policy makers clearly need to balance the available scientific knowledge with practical constraints, including economical aspects (Fig. 4). In this sense, historical reasons (i.e., regulation of chemicals has been traditionally focussed on the water phase) and unsolved methodological challenges (i.e., how to define a shared methodology for SPM collection and analysis) may well explain the current state of European legislation for monitoring priority trace elements. Nonetheless, as detailed in the conclusions of Vignati et al. (2009), neglecting partitioning has several potential pitfalls for environmental security (i.e., accumulation and toxicity of elements via SPM ingestion are not considered) and environmental management (potential underestimation of current contamination following filtration artefacts or unmonitored accumulation of SPM-bound elements into bed sediments).

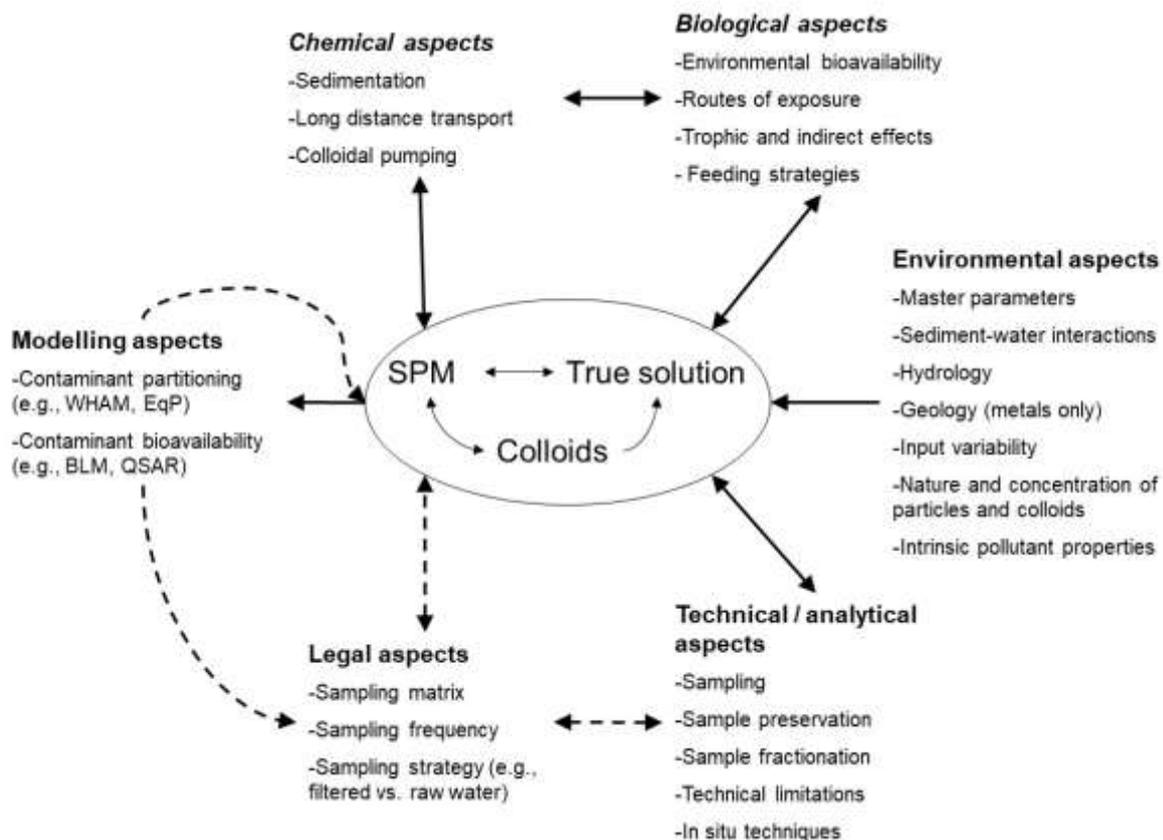


Figure 4. Importance of contaminant partitioning in relation to contaminant behaviour and its implications for regulatory chemical monitoring strategies. Full arrows indicate well-established or well-considered issues. Dotted arrows indicate areas where more research or better consideration in the regulatory context are needed (based on the original drawing from the author as published in Vignati et al., 2009).

<sup>3</sup> Note that the most recent legislation (Directive 2013/39 EU) stipulates that Hg monitoring must be carried out in biological matrices. Monitoring in the water phase is still acceptable, in principle, provided that the Member State shows that it offers the same degree of protection. Personally, I support Hg monitoring in biota (section 4).

These above considerations granted the following recommendations. In the short term, standardized protocol for filtration must receive more attention by regulatory bodies. Factors usually considered as trivial in routine application (e.g., volume of filtered water, speed of filtration, type of filter) can markedly affect the actual metal fraction that passes through filter pores for subsequent analytical quantification. Such factors should be explicitly mentioned in field and laboratory protocols to ensure long-term comparability of results and compilation of meaningful temporal trends (Hall et al., 1996; Horowitz et al., 1992; Horowitz et al., 1996). In the medium, long term, the filtration technical problems are likely to be less relevant as new techniques will hopefully allow for more accurate determination of the bioavailable fraction of a given element. At present, filterable element concentrations are considered as representative of their bioavailable fraction. Except for Hg, it is assumed that protecting organisms from water-borne exposure will protect them also from exposures via other routes. However, SPM bound elements can contribute to accumulation (Ferrari et al., 2019; Weltens et al., 2000) and will increasingly require attention by regulators. Luckily, filtration artefacts have only a minor effect on the fraction of trace elements bound to SPM and the major challenge will be to exactly determine how much of the SPM-bound concentration will be bioavailable during the passage through the digestive tract of the organisms (see section 3.3). Interestingly, the opposite situation exists for priority organic microcontaminants for which concentrations must be determined in raw water samples (Vignati et al., 2009). For these substances, the regulatory provisions may result in poorly reproducible results considering that the measured concentrations may change depending on the extraction protocols applied to the raw water samples (Ademollo et al., 2012). In particular, the extraction efficiency of a specific substance using a given protocol can vary depending on SPM concentrations in the water sample and according to the substances to be measured. Furthermore, the various agencies will not be pushed to collect information on the actual partitioning of such contaminants which would facilitate the acquisition of new knowledge on their environmental behaviour and bioavailability via different exposure routes.

### *5.3. Modelling partitioning between colloids and true solution*

Determination of element partitioning between colloids and true solution using tangential flow ultrafiltration is a rather time-consuming process. Membrane preparation (acid and base cleaning), blank verification, system pre-conditioning and sample fractionation may easily take a full day of work for one operator in the laboratory, thus limiting experimental output and increasing costs. Because ultrafiltration requires raw waters to be preliminarily filtered, I examined whether the truly dissolved fraction of an element could be modelled by the corresponding filterable ones; the colloidal fraction being then amenable to calculation by difference. Nowadays, commercially available centrifugal ultrafiltration units (e.g., Amicon® Ultra-15 Centrifugal Filter Units) have shortened the time needed for separating true solution and colloids from several hours to tens of minutes. However, modelling possibilities retain their interest considering the comparatively high cost (above 10 EUR per unit) of such single-use devices.

By combining my original data with previously published ones, I develop simple linear models effectively allowing the prediction of the truly dissolved concentrations of Cd, Cu, Ni and Zn from the corresponding total filterable ones (Vignati et al., 2005b). The agreement between measured and modelled truly dissolved concentrations was within a factor of 1.5 for 78% of the data points in the case of Cd (n=65), 82% for Cu (n=133), 83 for Ni (n= 93), and 87% for Zn (n=64). Interestingly, differences in the nominal cut-off sizes of filters and ultrafilters among studies did not seem to have any influence on the validity of the linear relationships. This observation probably follows from the actual filter cut-offs being usually different from the nominal ones (She et al., 2008; Vignati et al., 2006). Analogous linear

regression models for total and trivalent Cr could be obtained for two aquatic systems impacted by tannery wastewaters with contrasting characteristics (Dominik et al., 2007). The slope of the two models were very similar (0.336 for total Cr and 0.333 for trivalent Cr), in line with our findings that colloidal Cr could comprises almost exclusively Cr(III) (Bobrowski et al., 2004). Other studies also confirmed the validity of this approach to model truly dissolved Hg and MMHg concentrations in the water column and in sediment pore waters from various aquatic systems (Guédron et al., 2016).

#### *5.4. A critique of my previous research on environmental behaviour and availability of trace elements*

The sections from 5.1 to 5.3 essentially presented biogeochemical results and discussed their possible contribution to the understanding of trace element behaviour in continental surface waters. It is however interesting to examine the possible implications of partitioning with respect to trace element bioavailability to aquatic organisms. The details of this strategy are described in chapter 9 of my PhD thesis (Vignati, 2004). Variations in master environmental parameters that affect elemental partitioning (see Fig. 4) will also affect, directly or indirectly, their bioavailability. For example, a decrease in pH will modify the net surface charge of environmental particles and cause trace element desorption into the truly dissolved phase, thus potentially increasing elemental bioavailability. However, freshly desorbed trace elements will distribute among the ligands present in the truly dissolved phase and the net ecotoxicological outcome will be determined by the combination of several processes.

Explicit consideration of the presence of colloids in filtered waters can also assist in reconciling laboratory ecotoxicological results with the potential for ecotoxicological effects in natural waters. Because colloids are not present in standardized ecotoxicological media, laboratory-derived effect concentrations are, in principle, more comparable with truly dissolved than filterable levels measured in natural waters. The modelling possibilities described in section 5.3 are therefore interesting to obtain such kind of information in the absence of experimental measurements. However, the effect of colloids on elemental ecotoxicity, though often protective, depends on the composition of the colloids themselves (Koukal et al., 2003; Koukal et al., 2007) and colloiddally-bound contaminants may be available to filter-feeders via ingestion. These considerations are of interest considering that the European regulatory framework is moving towards environmental quality standards based on calculated bioavailable fractions using empirically-based, simplified biotic ligand model tools (Merrington et al., 2016; Peters et al., 2018). These models estimate the bioavailable concentration of an element starting from its filterable ( $< 0.45 \mu\text{m}$ ) concentration and the values of pH plus DOC and Ca concentrations in the corresponding aqueous sample. Apart from the possible limitations of the biotic ligand model (Duval and Rotureau, 2014; Mebane et al., 2020), approaches neglecting contaminant partitioning can both overlook ecotoxicity linked to the ingestion of colloiddally-bound contaminants or overestimate the actual concentration of available elements by considering the total filterable concentrations instead of the truly dissolved ones. That said, such bioavailability-based approaches to environmental regulations are a huge improvement compared with fixed-value environmental quality standards. It remains to be ascertained to what extent a better consideration of elemental partitioning would improve their predictive abilities.

This critique would be incomplete without questioning the actual capabilities of filtration and ultrafiltration to provide the biogeochemical information that is expected from them. Both techniques are operationally defined and artefact prone (see sections 5.1 and 5.2) and a distinction between particles and colloids based on nominal (ultra)filter pore-sizes neglects the much more complex nature of the difference between the two (Gustafsson and Gschwend, 1997). However, the operational distinction among particulate, colloids and true solution has

helped (and can still help) in understanding contaminant transport and behaviour in continental, estuarine and marine settings (Benoit et al., 1994; Honeyman and Santschi, 1988; Millward, 1995; Powell et al., 1996; Turner and Millward, 2002). The presence of colloids can also facilitate the transport of otherwise poorly mobile radionuclides in groundwater aquifers (Kersting et al., 1999). Finally, significant fractions of the so-called technologically critical elements (Nassar et al., 2015) can occur in the colloidal phase (Kulaksız and Bau, 2013), which bears consequences for correctly understanding their increasingly disrupted biogeochemical cycles. As anticipated in section 5.3, centrifugal ultrafiltration devices capable of rapidly separating colloids from true solutions are now commercially available and there is little reason to limit research on partitioning to the simple separation of particulate and filterable fractions. Personally, I would however invite to be critical about the use of such devices which, contrary to other ultrafiltration apparatuses (Kottelat et al., 2008), do not allow to check for the presence of ultrafiltration artefacts and can suffer from sorptive losses (Kocman et al., 2013). On the other hand, results from a yet unpublished contribution (Martin et al., submitted-a) suggest that centrifugal ultrafiltration unites can provide useful information on the partitioning of rare earth elements in natural waters, although further methodological studies are needed. Despite these limitations, filtration/ultrafiltration procedures offer logistical advantages compared with more complex techniques such as Flow Field Flow Fractionation that rely on non-portable, complex laboratory instrumentation. Indeed, colloid characterization is constantly facing potential artefacts arising from rapid changes that take place in the colloidal size distribution after sampling (Chen and Buffle, 1996a; b; Filella and Buffle, 1993). In conclusion, the study of contaminant partitioning to understand elemental behaviour still remains a worthy scientific objective.

On the other hand, I am less certain about the relevance of partitioning in relation to contaminant bioavailability. From an ecotoxicological point of view, partitioning is just one of the many facets of speciation controlling elemental bioavailability. Even if a perfect separation between e.g., colloids and true solution could be achieved, the bioavailable contaminant fraction in the latter will still vary depending on master environmental parameters and composition of the aqueous matrix. Furthermore, the bioavailability of particle bound contaminants will depend on the ecology of the exposed organisms and on the composition of colloidal particles. Discussing all the techniques and theoretical frameworks necessary to understand contaminant bioavailability goes beyond the purpose of this short critique and reference can be made to e.g. Mason (2013). Furthermore, there are intrinsic limitations in trying to understand bioavailability solely based on the study of contaminant behaviour in environmental matrices outside living organisms. In the end, biological effects usually require an interaction between the contaminant and some biological target. The Critical Body Residue approach (McCarty and MacKay, 1993; Meador et al., 2010) postulates that contaminant effects are better understood on the basis of the concentrations actually accumulated inside living organisms rather than (a fraction of) concentrations measured outside organisms. The approach works reasonably well for chemicals that act by narcosis, but the situation is more complicated for trace elements some of which can be essential or can be accumulated in biologically inactive forms inside an organisms (Rainbow, 2002). Searching for the most appropriate metric to express the ecotoxicity of a contaminant is actually one of the research questions of this HDR project and it will be further discussed in part three of the present document.

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## 6. Environmental behaviour of chromium and its ecotoxicity to freshwater organisms

In surface water environments, chromium occurs in two main redox forms with contrasting environmental behaviour and different toxicity (Gorny et al., 2016; Viti et al., 2014). Chromium (VI) is highly mobile in the environment, easily crosses biological membranes, and is a known carcinogenic and mutagenic in humans. Chromium (III) is particle-reactive and thus less mobile in the environment because it tends to bind to suspended particulate matter (and colloids) and accumulate in bed sediments. Chromium (III) is also considered less toxic to aquatic organisms, the main environmental risk being considered the possible oxidation of sediment-bound Cr(III) to Cr(VI) in the presence of Mn oxides at the sediment-water interface.

From 2000 to 2008, I devoted substantial efforts to cooperative projects dealing with the study of Cr behaviour, bioavailability and ecotoxicity in aquatic systems impacted by tannery wastewaters. The tanning process uses large quantities of trivalent Cr that may be discharged to the environment along with other chemicals (Dixit et al., 2015; Lofrano et al., 2013). Most of the work was performed in the Dunajec river/Czorsztyn reservoir system (southern Poland). My interest in Cr ecotoxicity to freshwater organisms actually took off in 2004 as a side project for some master students and eventually served as the foundation of a large part of my activity upon joining the CNRS. These activities have been funded by a EC2CO project from the CNRS, contributions from LabexR21 and overheads obtained from projects on other research topics and collaborations. Other contributions to this research field based on the use of *in situ* exposure devices have been described in section 3.3.

### 6.1. Environmental behaviour of chromium

The peculiarities of Cr geochemistry introduce additional challenges in the study of its partitioning between colloids and true solution (see section 5). In particular, the actual concentrations of both Cr(III) and Cr(VI) must be quantified in the total filterable, colloidal and truly dissolved fractions of a water sample to correctly understand the environmental behaviour and fate of this element. By combining tangential flow ultrafiltration and catalytic adsorptive stripping voltammetry, we showed that about two thirds of Cr(III) were associated with colloids of variable molecular weight, while Cr(VI) was detectable only in the truly dissolved phase (Bobrowski et al., 2004). These observations are in agreement with theoretical knowledge on the behaviour of Cr(III) and Cr(VI), but the detailed study of Cr speciation allowed to develop a conceptual model of Cr behaviour in aquatic systems impacted by tannery discharges (Dominik et al., 2007). It appears that Cr(III) is rapidly removed from the water column to the sediment compartment via colloidal pumping (Honeyman and Santschi, 1989), while Cr(VI) remains in the water column and can be transported over longer distances. Chromium(III) showed a similar behaviour in flow-through laboratory microcosms (Fig. 2) where it reached a steady-state distribution among colloids and true solution and showed a time-dependent accumulation in the sediment compartment (Kottelat et al., 2010). The accumulation of Cr(III) into the sedimentary compartment highlighted two critical areas for understanding Cr fate and risk in the presence of tannery discharges: the bioavailability of sediment-bound Cr and the likelihood of Cr(III) oxidation to Cr(VI) within sediments and at the sediment-water interface. My contributions to the former issue are described in section 3 of this part of the document, but I did not go beyond exploratory studies with regard to the latter.

Bobrowski et al. (2012) experimentally confirmed the presence of Cr(VI) in interstitial waters extracted from a sediment core collected close to wastewater discharges from tanneries. The main objective of this study was to develop a suitable method to specifically quantify Cr(VI) in the presence of high concentrations of dissolved organic carbon and total Cr (up to 260 mg L<sup>-1</sup> and 450 µg L<sup>-1</sup>, respectively). Apart from method validation, the maximum Cr(VI)

concentration was observed at 1.5 cm below the sediment-water interface, suggesting the possibility of a diffusional transport of Cr(VI) from sediments interstitial water to river water. The Cr(VI) levels never exceeded 2% of the total Cr concentration measured in interstitial waters, but the results confirmed the potential for some remobilization of Cr from contaminated sediment. The combined use of Diffusive Gradient in Thin films (DGT) and Diffusive Equilibration in Thin films (DET) in another study also indicated that the concentration of ionic trivalent Cr was less than 1% of the total Cr concentration (Vignati et al., 2019) in interstitial waters. These data collectively suggests that, in sediment interstitial waters, most of Cr occurs under complexed, possibly colloidal, forms whose bioavailability to sediment-dwelling organisms remains to be assessed. Finally, hydrological conditions were also shown to affect the mobility and distribution of Cr(III). In particular, Cr(III) accumulated in river sediments close to tannery discharges was periodically remobilized during high-flow conditions and transported to otherwise uncontaminated areas (Szalinska et al., 2010).

The chromium concentrations measured in water and sediments from the Dunajec/Czorsztyn systems were often close to, or above, values likely to cause adverse effects in living organisms. In another study carried out in Morocco (Koukal et al., 2004), we actually observed acute toxicity of filtered waters collected in a river impacted by tannery discharges and containing up to 214  $\mu\text{g L}^{-1}$  of total Cr. In these samples, Cr was mainly in the form of Cr(III) as shown by Pereira De Abreu (2006). However, the same samples contained substantial amounts of Cu (up to 40  $\mu\text{g L}^{-1}$ ), Zn (up to 130  $\mu\text{g L}^{-1}$ ), and Ni (up to 10  $\mu\text{g L}^{-1}$ ), had low oxygen contents (some being anoxic at the time of sampling) and high conductivity. To understand the actual contribution of trivalent chromium to the observed ecotoxicological effects, it was therefore necessary to start studying Cr(III) ecotoxicity and bioavailability in controlled laboratory conditions (see the conceptual strategy described in figure 3).

## 6.2. Chromium ecotoxicity to aquatic freshwater organisms

The study of the ecotoxicity of an element in (standardized) laboratory settings aims at establishing a relationship between exposure concentrations and (a range of) biological responses. A precise knowledge of the actual exposure concentrations is therefore a prerequisite to any meaningful interpretation of ecotoxicological effects. In the case of chromium, obtaining such knowledge must address two major challenges: the simultaneous occurrence, in natural waters, of both Cr(VI) and Cr(III) and the complex chemistry of trivalent chromium that can markedly modify exposure concentrations over the typical duration of ecotoxicological tests. In this section I will focus on the latter aspect; the simultaneous assessment of Cr(VI) and Cr(III) ecotoxicity being an objective of my HDR project.

At circumneutral pH, a typical situation in many ecotoxicological test media, trivalent Cr rapidly undergoes hydrolysis and forms poorly soluble Cr hydroxides (Rai et al., 1989). Starting from these theoretical considerations, I showed that Cr concentrations in test medium the standard test medium ISO 8692, hereinafter ISO medium (ISO, 2012), for freshwater algae amended with Cr(III) decreased in a time- and concentration-dependent manner (Vignati et al., 2008a; Vignati et al., 2010). Otherwise stated, testing of trivalent chromium should be carried out following protocols for sparingly soluble substances (ISO, 2006). This recommendation is not as trivial as it may seem upon a first reading. Chromium(III), like all other test substances, is usually added to the test medium in the form of soluble chloride, nitrate or sulphate salts, which means that insoluble Cr(III) chemical species are formed directly in the test medium (in a time- and concentration-dependent manner). These findings have far reaching consequences because, except for Chapman et al. (1980), the studies currently considered in risk assessment documents on trivalent Cr (WHO, 2009) completely neglect the phenomenon. In all such cases, the actual ecotoxicity of Cr(III) has been grossly underestimated and most likely requires a complete re-assessment (Aharchaou et al., 2018; Vignati et al., 2010).

However, the composition of a standard laboratory test medium is not fully representative of a natural water. In particular, standard media often do not contain dissolved organic carbon that is ubiquitous in natural waters and has a strong complexing capacity for trace elements (Mason, 2013; Pourret et al., 2007). By comparing the ecotoxicity of Cr(III) and Cr(VI) in a modified culture medium containing dissolved organic carbon (i.e., filtered Lake Geneva water mixed with ISO medium), I was nonetheless able to confirm that Cr(III) concentrations were not stable even in the presence of organic carbon. In addition to this, Cr(III) was more toxic than Cr(VI) to the model freshwater alga *R. subcapitata* in both standard and modified ISO medium (Vignati et al., 2010).

Other experiences with the crustacean *Daphnia magna* confirmed that medium composition had a strong influence on Cr(III) behaviour in laboratory aqueous matrices and, hence, on its ecotoxicity (Ponti et al., 2014). In well-buffered media (alkalinity 4.9 meq L<sup>-1</sup>), total filterable Cr concentrations (added as trivalent Cr) decreased to 0 mg L<sup>-1</sup> over 48h; i.e. the duration of an acute test with *D. magna*. In such instances, the absence of acute ecotoxicological effects may well reflect an absence of actual exposure to the toxicant during most of test rather than a low ecotoxicity of Cr(III). In poorly buffered media (alkalinity 0.8 meq L<sup>-1</sup>), the addition of Cr(III) lowered the pH of the test medium, thus preventing or slowing down the formation of insoluble Cr hydroxides. In these circumstances, precisely characterizing Cr speciation by analytical measurement or modelling can be extremely helpful for comparing different studies and extrapolating laboratory results to real field situations. One feature common to all these examples was the importance of kinetics in determining the actual exposure concentration and the associated ecotoxicological effects. A significant effort to better characterize Cr(III) speciation in ecotoxicological media, specifically in the ISO medium for freshwater algae, was carried out during the PhD thesis of M. Imad Aharchaou (Aharchaou, 2017).

Considering the foreseeable complexity of the necessary analytical measurements, the relative ecotoxicity of Cr(III) and Cr(VI) to the green alga *R. subcapitata* were first studied using a standardized microplate test performed according to the ISO 8692 norm (Wang et al., 2012) and the EC<sub>50</sub> were estimated for both Cr redox forms. These experiments confirmed that Cr(III) was more ecotoxic than Cr(VI) in the specific experimental conditions (no culture, operator or material effect compared with previous study performed in another laboratory). Further characterization of Cr chemical behaviour was then performed using medium aliquots amended with Cr(III) or Cr(VI) using Erlenmeyer flasks. Total and total filterable (0.45 and 0.22 µm) concentrations remained stable in medium aliquots amended with Cr(VI), but filterable concentrations decreased by 40–90 % in 6 to 8 hours in medium aliquots amended with Cr(III). The simultaneous determination of both total and total filterable Cr concentrations was necessary to verify that the observed concentration decrease was not due to adsorptive losses onto vessels' walls, but to the actual formation of Cr-bearing particles in the exposure medium initially spiked with soluble Cr(III) salts.

A second series of experiments showed that no redox interconversion between Cr(III) and Cr(VI) did not occur neither in the absence nor in the presence of algae, which guarantees that the higher ecotoxicity of Cr(III) is a real observation and not an artefact due to the oxidation of Cr(III) to Cr(VI) during exposure. However, analyses of medium aliquots spiked with Cr(III) using IC-ICP-MS (Ion Chromatography Inductively Coupled Plasma Mass Spectrometry) revealed that the changes in Cr(III) speciation were extremely rapid, with free ionic Cr(III) concentration dropping below analytical detection limit in about 5 hours; i.e., less than one tenth of the test duration (72 hours). The disappearance of ionic Cr(III) was accompanied by the formation of other chemical species (Fig. 5a) whose exact ecotoxicological effects remain unknown.

Other measurements using Nanoparticle Tracking Analysis (NTA), Single Particle Counting (SPC), and single-particle ICP-MS confirmed that Cr-containing particles were formed within

minutes after spiking of the exposure medium with trivalent Cr (Fig. 5b). Overall, the results from such detailed study allowed to defining three possible scenarios for the ecotoxicity of Cr(III) to freshwater algae (Aharchaou et al., 2018):

- Cr(III) effects at 72 hours (the duration of a standardized algal test) are controlled by an initial short-time (pulse) exposure to ionic trivalent Cr,
- The residual concentration of ionic Cr(III), less than  $1 \mu\text{g L}^{-1}$ , is enough to cause the observed growth inhibition after 72 hours of exposure,
- Chemical species other than ionic Cr(III) contribute to the observed ecotoxicological effects.

Understanding such issues and, more in general, the kinetics of chemical speciation and ecotoxicological responses is part of the specific research questions of my HDR project.

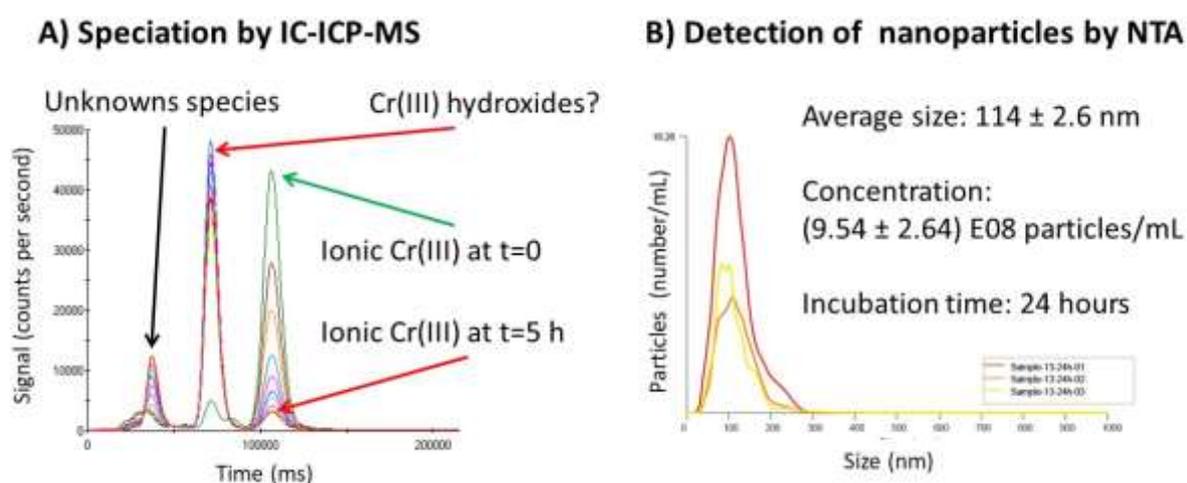


Figure 5. Panel A: Chromatograms of the temporal evolution of ionic Cr(III) concentrations and other Cr species in ISO medium initially amended with  $60 \mu\text{g L}^{-1}$  of Cr(III). Panel B: Number and size of nanoparticles formed after 24 hour of incubation in ISO medium initially amended with  $60 \mu\text{g L}^{-1}$  of Cr(III).

### 6.3. Chromium bioaccumulation in freshwater algae and mussels

Section 6.2 sketched a quick picture on the possible difficulties in interpreting the results of Cr ecotoxicity on the basis of exposure concentrations measured in the test medium, especially in the case of trivalent Cr. Other fundamental questions deal with the uptake and behaviour of Cr accumulation in living organisms, notably whether Cr(III) and Cr(VI) have similar fate inside cells and comparable biochemical effects in tissues.

In surface waters, Cr(VI) usually occurs as anionic chromate species which easily enter cells because of their structural similarity with the essential sulphate ions (Gorny et al., 2016; Viti et al., 2014). How Cr(III) cationic or neutral species interact with cells is less clear, albeit iron transport mechanisms may be involved (Beyersmann and Hartwig, 2008; Viti et al., 2014). However, Cr(VI) is a very strong oxidant and is quickly reduced to Cr(III) in intracellular environments (Zhitkovich, 2005). Otherwise stated, only Cr(III) is expected to occur within cells. Starting from these observations, cells of *Chlamydomonas reihardtii* were exposed to Cr(VI) or Cr(III) to investigate differences in their uptake kinetics and in their intracellular distribution. These experiments required the use of radiolabelled Cr salts and were performed in collaboration with Prof. Claude Fortin (INRS, Québec, Canada) during a scientific visit of Dr. Imad Aharchaou at the INRS and with the financial help of LabexR21.

The corresponding results (Aharchaou et al., in preparation) showed a linear uptake of both Cr(III) and Cr(VI) over 60 minutes as well as an absence of Cr efflux. Uptake rates generally

increased with concentration (exposure range  $10^{-9}$ – $10^{-5}$  M, approx. 0.05–500  $\mu\text{g L}^{-1}$ ) and, in the case of Cr(III), also as a function of pH, which suggests the possibility of competition for cellular uptake between the  $\text{Cr}^{3+}$  and  $\text{H}^{+}$  ions. Chromium(VI) exhibited higher uptake rates than Cr(III) for low exposure concentrations (1 and 10 nM), while the opposite was observed for algae exposed to levels of 10  $\mu\text{M}$ . On the other hand, cellular uptake rates for the two redox forms were similar in cells exposed to 100nM or 1 $\mu\text{M}$  (approx. 5–50  $\mu\text{g L}^{-1}$ ), which covers the range of several natural environments (McClain and Maher, 2016; Saputro et al., 2014).

Chromium accumulation in cells of *C. reinhardtii* exposed to 100nM of Cr(III) or Cr(VI) for 1 h and 72 h showed some differences in terms of total accumulated elemental burdens and intracellular distribution. However, accumulation in the metal-sensitive fractions (cellular organelles and heat-denaturable proteins) after 72 hours was remarkably similar for both redox forms (Aharchaou et al., 2017). Furthermore, Size Exclusion Chromatography ICP-MS (SEC ICP-MS) showed that intracellular Cr bound to the same biomolecules irrespective of the redox form initially added to the exposure medium. These observations agree with the hypothesis that Cr(III) is the main oxidation state of this element in the intracellular environment due to reduction of Cr(VI) by reaction with cytoplasmic organic molecules such as cysteine and glutathione (Valko et al., 2005). Overall, Cr(III) and Cr(VI) seem to have a similar intracellular fate in *C. reinhardtii*, confirming the need for more comparative studies on the bioavailability and effects of both redox forms.

The main limitation of comparative studies on the behaviour of Cr(VI) and Cr(III) is that they cannot account for neither their simultaneous occurrence in natural waters nor their possible combined effects. These challenges can be elegantly addressed by considering that Cr has four stable isotopes:  $^{50}\text{Cr}$  (natural abundance 4.31%),  $^{52}\text{Cr}$  (83.76 %),  $^{53}\text{Cr}$  (9.55 %), and  $^{54}\text{Cr}$  (2.38 %). Isotope fractionation is often used in geochemistry to understand the behaviour of Cr during weathering or other environmental processes (Wu et al., 2017). In an ecotoxicological context, it is however the possibility to create isotopically enriched, speciated Cr solutions that bears the most exciting opportunities. Solutions containing more than 95% of  $^{50}\text{Cr}$ (III) or  $^{53}\text{Cr}$ (VI) are actually commercially available. It is therefore possible to expose organisms to  $^{50}\text{Cr}$ (III) and  $^{53}\text{Cr}$ (VI) and then follow the biological fate of both isotopes at the same time. Although Cr(VI) will be reduced to Cr(III) following cellular uptake, it will still be possible to distinguish the two isotopes by a simple analysis by ICP-MS. The technical feasibility of this approach with the freshwater mussel *Corbicula fluminea* has already been verified (Aharchaou, 2017) and its development will be presented in the HDR scientific project (part III of the present document).

#### 6.4. A critique of my past work on chromium ecotoxicity and bioavailability

Most of my research on chromium behaviour and biological effects has challenged the paradigm ‘Cr(VI) is much more (eco)toxic than Cr(III)’ that bears a strong consensus in the scientific community. Considering the results presented in sections 6.2 and 6.3, I have sufficient grounds to consider such statements as an oversimplified representation of a much more complex situation.

Back in 2005, we unexpectedly observed that Cr(III) nitrate was more toxic than potassium dichromate to the green alga *R. subcapitata*. The first observations were initially treated as the result of some experimental error, e.g., mistakes in dilution or weighing while preparing the Cr(III) solutions. Following further testing, we were however forced to admit that Cr(III) was indeed more ecotoxic than Cr(VI) in our experimental conditions. The subsequent attempts to understand Cr behaviour in the ISO 8692 medium for freshwater algae alerted us about the complexity of Cr(III) chemistry and prompted us to examine the temporal stability of Cr concentrations in medium aliquots amended with trivalent Cr. The task was not complex, but it required a painstaking cleaning of all the material, including microplates and pipet tips, to

ensure that results would stand scrutiny from the scientific community. Yet, our first submission on the topic (*circa* April 2007) faced upfront rejection from the editor of Environmental Toxicology and Chemistry on the ground that ‘we all know that exposure concentrations must be quantified to reliably interpret the outcome of ecotoxicological test’; a problem that was subsequently recognized as far from solved (Harris et al., 2014). A similar struggle took place when publishing the results of ecotoxicological experiences showing the possible shortcomings of the paradigm ‘Cr(VI) is more toxic than Cr(III)’. While such difficulties are a normal aspect of the scientific enterprise, I cannot but wonder whether cognitive bias played any role in the difficulties we faced to get those first results published. Fast forward 15 years, other researchers have questioned the putatively low ecotoxicity of Cr(III) and we have successfully published our results. Have they been worth the time, efforts, and money, and do they justify further commitment to this research topic?

From a fundamental point of view, the answer is ‘yes’ to all the questions. Most of the early studies on Cr(III) ecotoxicity simply do not provide reliable results (my own review article in preparation) and the accumulation of experimental evidence on Cr(III) being of potential ecotoxicological concern cannot be questioned any longer (Chow et al., 2018; Dazy et al., 2008; do Nascimento et al., 2018; Jasso-Chávez et al., 2010; Speranza et al., 2007; Thompson et al., 2002). There is clearly enough available evidence to invite the scientific community to wholly reconsider the hazard assessment of Cr(VI) vs. Cr(III). It remains however open to debate, and I ask such question myself, if Cr(III) actually poses a risk in real environmental settings. Recently published results (Ferrari et al., 2019; Vignati et al., 2019) suggest such possibility, but more research is needed on this specific aspect.

For the time being, I am satisfied with these research efforts having contributed to adopt similar approaches for other trace elements, notably lanthanides, whose chemical properties also cause the formation of insoluble, (nano)particulate species in standardized ecotoxicological media (Gonzalez et al., 2014; González et al., 2015). As a general concluding caveat, I am not against doubting the actual effectiveness of ‘my approach’ (consisting in an extremely detailed characterization of exposure conditions in ecotoxicological test media). With one or two exceptions, I still stand behind the results that I have so far published. On the other hand, I wonder how much sense it makes to take a lot of trouble to follow the behaviour of a contaminant in a standardized medium with limited environmental relevance and at concentrations that are usually much higher (sometimes by orders of magnitude) than the corresponding environmental ones. I am asking these questions in the most serendipitous manner. Indeed, only a critical evaluation of laboratory results against real-field conditions will allow us to reach the correct balance between environmental protection from chemical contamination and rationale use natural resources. This HDR project is a personal attempt to contribute to the collective efforts that will be required to achieve this overarching objective.

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## **Part III – Scientific research project**

## 1. Context

### 1.1. Preamble

The primary focus of (eco)toxicology is the study of the harmful effects of chemicals, or more in general stressors, across levels of biological organization ranging from genes to ecosystems and across different spatial and temporal scales (Van den Brink, 2008; Walker et al., 1996). This is not equivalent to advocate a zero concentration policy for all xenobiotics or background-like levels for naturally occurring, although both can serve as ultimate ideal targets. Considerations linked to chemicals' bioavailability, homeostatic capabilities of organisms and resilience of ecosystems also come into play. The practical objective of ecotoxicology is therefore to help in keeping chemicals' levels below threshold concentrations that, if exceeded, would disrupt individual health, population viability or community/ecosystem functioning (Fig. 6). Some authors suggest to use the word contaminant for substances that are enriched above background levels and pollutants for substances that cause an adverse effect on biota. The considerations in the introduction of Walker et al. (1996) are however grounded enough to discourage such use. Lack of consensus also exists in relation to the appropriate terminology for metals and metalloids (Pourret and Bollinger, 2018). I will therefore stick to the generic terms *chemical* or *substance* and use the term *stressors* when referring to non-chemical factors that can modulate biological responses to chemicals or cause by themselves adverse effects on biota.

With the exception of critically endangered species, ecotoxicology focusses on avoiding undesirable impacts of chemicals and stressors at the population, community and ecosystem levels rather than on ensuring the survival of each individual within a given population (Fig. 6). This approach is illustrated by, e.g., the sustainable management of fish stocks, where a healthy, stable, or increasing, population must be maintained to ensure that individuals from the same population can be caught (i.e., killed) for human consumption. These considerations are extremely important considering that chemicals have become an essential part of our everyday lives and we constantly strive to achieve maximum benefits from their use while minimizing the potential associated risks (EEA, 2019). However, as stated by (Batley et al., 2005) in relation to the development of sediment quality guidelines:

“The need to assess and manage risks from chemicals is generally driven by societal and legal mandates that do not provide the luxury of delaying decision making until all complexities are resolved”.

As a consequence, ecotoxicologically-based regulatory frameworks continuously evolve following new scientific and methodological developments; including the correction of errors or simplifying assumptions.

Quantifying risks necessitates an adequate knowledge of hazard and exposure characteristic of each substance or family of substances (Fairbrother et al., 2007). Hazard is usually assessed using standardized laboratory tests to rank the intrinsic ecotoxicological potential of substances under the same, controlled conditions. Exposure assessment requires not only knowledge of the concentration of the chemical of interest in the appropriate environmental matrix, but also of its inputs (including duration and periodicity), speciation and environmental behavior (Connon et al., 2012; Schwarzenbach et al., 2006; Wang, 2011). Thoroughly understanding exposure is not only a key factor in real-world risk assessment, but also in the correct interpretation of responses observed during standardized ecotoxicological studies. Indeed, establishing a clear concentration-response relationship can be particularly challenging when dealing with sparingly soluble substances (ISO, 2006), volatile substances (Bettinetti et al., 2013), trace elements forming insoluble species in test media (Santore et al., 2018; Wilson et al., 2017), nanomaterials (Handy et al., 2012) or microplastics (Borja and Elliott, 2019); even in controlled and well-defined laboratory settings.

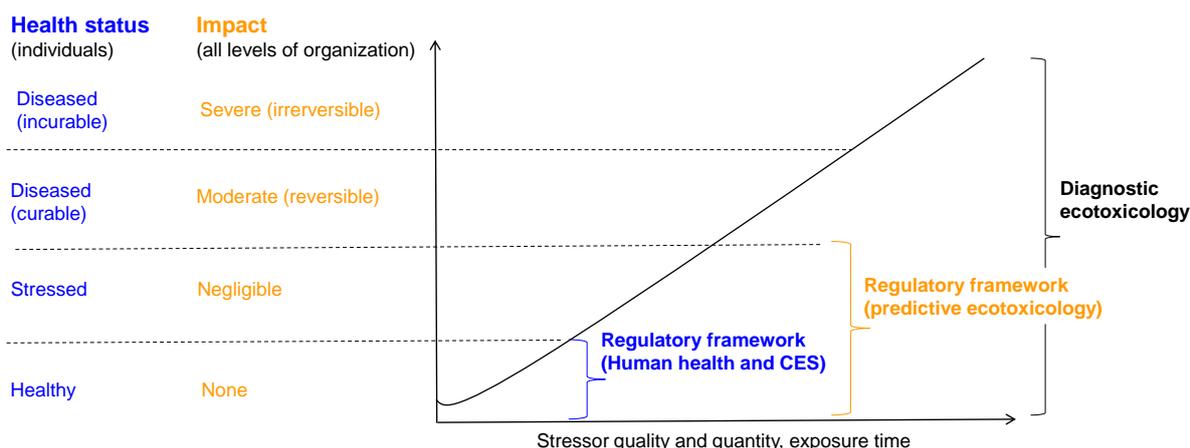


Figure 6. Conceptual relationship between stressors (chemicals or other factors) vs. individual health status or possible impacts at other levels of biological organization including subcellular (e.g., biomarkers), individual or community/ecosystem-level responses. CES, Critically Endangered Species. (Created by the author based on Walker et al., 1996 and Meybeck, 2003).

Despite significant research efforts, it is estimated that hazard and exposure are extensively characterized for only 500 of the approx. 100,000 chemicals currently on the market, with information being classified as *limited* or *poor* for over 75% of them (EEA, 2019). Furthermore, even determining the actual risks of well-characterized chemicals often remains challenging. Several factors (e.g., physical habitat degradation, altered temperature or pH ranges, competition or predation) can modify biological responses to chemicals in real-field conditions compared with controlled and simplified laboratory-settings (Liess et al., 2016; Posthuma et al., 2016). Extrapolating biological responses obtained at low level of biological organization to the population, community and ecosystem levels, that are the ultimate target of environmental protection measures, also presents inherent challenges (Schwarzenbach et al., 2006; Van den Brink, 2008; Van Straalen, 2003).

Overall, the European Union is not on track to meet its 2020 policy objectives to minimize the release and effects of hazardous chemicals on the environment and human health, nor to achieve good environmental status for all continental and marine waters (EEA, 2018; 2019). The 2030 outlook is also negative mainly because of the substantial lack of information for many chemicals that are released into the environment and the overall projected increase in the use of chemicals (EEA, 2019). Malaj et al. (2014) showed that the acute and chronic ecotoxicological risks in EU waters increase with data availability; suggesting that current knowledge may underestimate the chemical risks in EU waters. According to the report on the state and outlook of the European environment (page 249, section 10.4.1), it usually takes from 7 to 9 years for a substance to be evaluated for potential restriction measures under the REACH legislation, so that a lot of unmonitored exposure may occur for a long time before any corrective action is undertaken (EEA, 2019). The monography 'Late lessons from early warnings' (EEA, 2013) confirms that unwanted damage often occurs in such situations of unregulated risks or even when regulation of dangerous chemicals by e.g., substitution is not endorsed rapidly enough (Gabbert et al., 2014).

The overarching challenges of managing chemicals at the European or global level are linked to our consumption patterns and lifestyle and outside the scope of the present HDR project. On the other hand, chemical management strategies will likely remain grounded in standardized ecotoxicological tests to assess the intrinsic hazard properties of (new) chemicals. Such knowledge is essential to avoid environmental releases of new substances without sufficient

hazard information (preventive regulation) and to confidently extrapolate laboratory results to real field conditions when *e.g.* a substance is considered as a substitute for another one subject to regulatory restrictions (phasing out). It is therefore useful to understand the limitations and purposes of standardized ecotoxicological procedures in order to effectively manage chemicals.

In the remainder of this *Context* section, I will first discuss the reasons that justify the need for a certain degree of standardization in ecotoxicology (section 1.2) and then examine some of the issues associated with properly characterizing exposure–response relationships (sections 1.3 and 1.4). Sections 1.5 to 1.7 will deal with some of the complexities that are encountered when moving from the laboratory scale to real situations; namely the simultaneous exposure to multiple substances (section 1.5), the relative importance of different routes of exposure (section 1.6) and the ecotoxicology of climate change (section 1.7). Finally, section 1.8 will examine how the current scientific context may contribute to scientific bias and, among the others, to maintaining the laboratory *vs.* field gap in ecotoxicology. This context section is however not meant to provide a thorough state-of-the-art about each issue, but rather to summarize the different factors that should be kept in mind when planning and performing ecotoxicological research.

### *1.2. Reliability vs. relevance: the dual nature of ecotoxicology*

Ecotoxicology is both a fundamental discipline with its own, evolving, and sometimes poorly defined code of practices (Ågerstrand et al., 2013; Ågerstrand et al., 2011) and an applied discipline with a key role in the risk assessment of chemicals (Fairbrother et al., 2007). An ecotoxicological investigation can therefore have different objectives, including (Fig. 7):

- a) understanding the effects of chemicals on living organisms at the (sub)cellular, tissue, organ or individual level (toxicology oriented),
- b) understanding the interactions between chemicals and organisms under variable environmental conditions (ecology oriented and risk-assessment oriented),
- c) providing support for decision-making to prevent unwanted damage from chemicals (hazard- and risk-assessment oriented), and,
- d) understanding how responses to chemicals at the organism level can be extrapolated to higher levels of biological organization (ecology, hazard- and risk-assessment oriented).

It is important to note that support to decision-making can consist in using the information to derive (legally binding) environmental quality criteria or to actually perform risk assessment in relation to an existing real situation (Solomon et al., 2008b).

As any other scientific discipline, ecotoxicology promotes research practices toward maximum ‘data reliability’, where reliability defines the intrinsic quality of the data independent of the possible contexts for their application and use (Roth and Ciffroy, 2016). On the other hand, the use of ecotoxicological data for risk assessment and management is equally concerned with ‘data relevance’ which is context-dependent and considers to what extent a given set of data is suitable for a specific, applied purpose (Roth and Ciffroy, 2016). When unravelling the mechanisms by which a substance affects a specific biochemical pathway, considering how laboratory conditions compare with real field situations (*i.e.*, data relevance) may be of less concern than in cases when ecotoxicological results are called upon to inform the decision of risk assessors and managers. Cannon et al. (2012) define risk as the combination of hazard and exposure with hazard assessment consisting in:

“...the determination of the toxicological effects, including the analysis of adverse effects at different exposure concentrations”,  
and exposure assessment in:

“...the determination or prediction of the extent, frequency and duration at which biota are being exposed to a chemical in nature”.

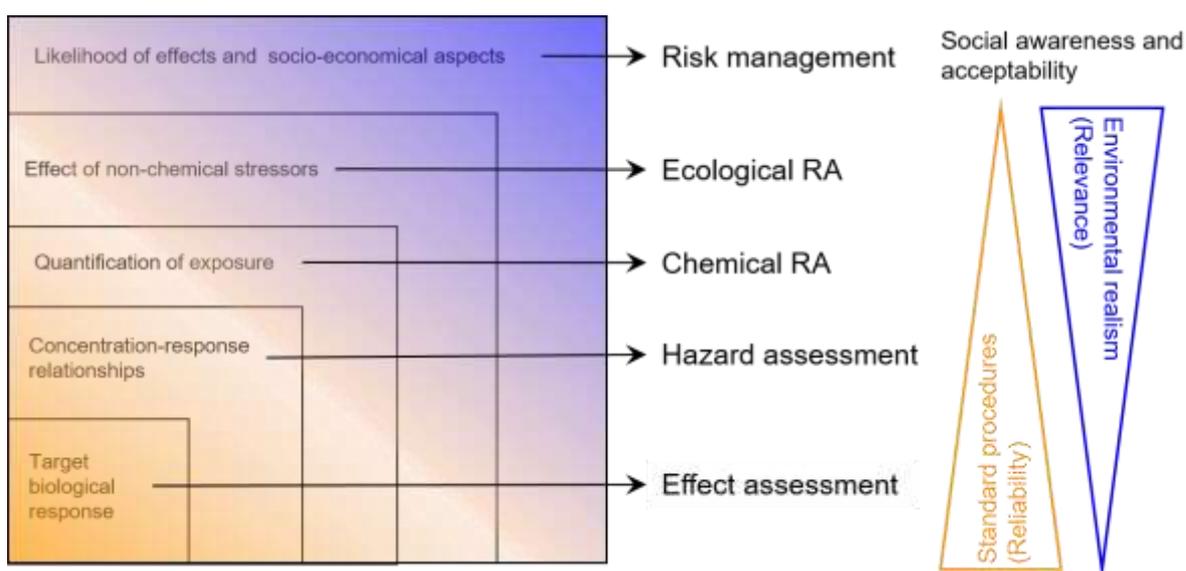


Figure 7. Schematic overview of the increasing complexity of an ecotoxicological investigation in relation to scientific (fundamental) and applied objectives and to ‘reliability’ and ‘relevance’ issues. Risk management adds levels of complexity (i.e., social awareness and acceptability; economic considerations) beyond environmental disciplines related to ecotoxicology. RA, Risk Assessment.

In the context of hazard and risk assessment, ecotoxicology therefore integrates the three research fields of chemical environmental fate, organisms exposure to chemicals and toxicological responses of organisms (Wang, 2011). Risk management adds two more dimensions to this framework: striking the balance between the benefits and the costs of a given intervention (Forbes and Calow, 2012) and appreciating the joint outcome of ‘the likelihood of an effect’ and ‘its consequences’ (Burkett et al., 2014). Given such broad scope and decidedly interdisciplinary nature, it is no wonder that the ecotoxicological community is constantly striving to reconcile the highly reliable knowledge it generates using standardized procedures in the laboratory with the more complex and variable environmental settings it desires to protect (Attanasio, 2016).

In his historical overview on the development of ecotoxicology, Féraud (2013) reports 13 different definitions of ecotoxicology provided by various authors between 1971 and 2008. All the proposed definitions contain the word ‘effect’ (on biota), reflecting the general objective of ecotoxicology, while only six of them contain the word or the notion of ‘fate’ of substances. In any case, none of them states, explicitly or implicitly, that ecotoxicological research must rely exclusively on laboratory standardized tests. Indeed, the ecotoxicological community presently uses a variety of tools that are much more comprehensive and diversified than standardized laboratory tests (Burton Jr et al., 2012; Crane et al., 2007a; Ferrari and Faburé, 2017; Ferrari et al., 2014; Rosen et al., 2012). However, much of the hazard and risk assessment framework ultimately relies, at least in part, on laboratory (standardized) tests to derive acceptable exposure values to chemicals or to screen new chemicals for unwanted effects. It is therefore necessary to clearly understand and agree on why standardized tests are used and what they can and cannot tell about the hazard and exposure associated with different chemicals or combinations thereof. Apart from the quest for scientific progress, knowing the strength and the limitations of the available tools prevents parties with vested interests to exploit current limitations in the scientific state-of-the-art to downplay uncomfortable findings in regulatory or public debates.

### 1.2.1. Why are standard tests used?

The first acute ecotoxicity tests can be traced back to the second half of the 19<sup>th</sup> century and the first standardized method for testing the ecotoxicity of industrial wastes and chemicals to freshwater fishes was published in 1945 (Hoffman et al., 1995)<sup>4</sup>. While standard tests are now routinely used to assess and rank the hazard of chemicals, it is noteworthy that some of them were initially developed for different purposes. In particular, the original purpose of the US EPA Algal Assay Procedure published in 1971 arose from the need to develop "...acceptable standardized algal growth tests...as a tool in controlling eutrophication" (USEPA, 1971). On the other hand, more recent standardized test procedures with freshwater algae explicitly mention the assessment of the chronic toxicity of (mixture of) substances contained in effluents and receiving waters as a part of their scope (ISO, 2012; OCDE, 2011) The OECD guideline 201 for testing chemicals clearly states "The purpose of this test is to determine the effect of a substance on the growth of freshwater microalgae and/or cyanobacteria". Overall, ecotoxicological testing of complex effluents seems to have preceded its use for assessing the effect of individual substances or known mixtures thereof (Hoffman et al., 1995), which is not at all surprising considering the efforts, time and resources required to develop and validate a standardized tests (Cotelle et al., 2015; De Cooman et al., 2015; Persoone et al., 2009). From a more general perspective, the need for ecotoxicological testing became prominent from the 1960s onwards when society realised that technological fixes would not have been able to completely compensate the environmental pressures linked to human activities (Jørgensen, 1995; Meybeck, 2003; Vasseur et al., 2020). Thus, the development of ecotoxicology and of the associated regulatory context (Figs. 6 and 7) ultimately follows from the collective failure of the human society to achieve a scenario of zero-discharge for chemicals.

Despite their limitations (see section 1.2.2 for a more detailed discussion), standardized tests are instrumental for the Mutual Acceptance of Data (MAD) on the possible effects of chemicals on selected model organisms. Given the challenges linked with the foreseeable increase in the use of chemicals (EEA, 2019; UNEP, 2019), MAD is necessary to establish a shared knowledge basis among scientists, stakeholders, regulators and the general public to implement scientifically sound and socio-economically acceptable environmental policies. With the mutually accepted procedures (MAPs) of standardized tests, experiments can be replicated and data compared and accepted across laboratories worldwide. Good laboratory practices (GLPs) are probably the most developed example of MAPs. Adherence to GLPs is regarded as particularly important by industries and stakeholders that need to protect themselves against potential liability issues. Indeed, GLPs have been the basis to judge the reliability of ecotoxicological studies for risk assessment for a long time (Klimisch et al., 1997). It is however increasingly accepted that ecotoxicological studies not following GLPs can be perfectly suitable for hazard/risk-assessment purposes if they provide enough information about test organisms, methods and experimental procedures (Moermond et al., 2016). In the end, standardized tests provide industry, stakeholders and regulators with a basis of shared, trusted data to rank the hazard of chemicals, albeit for a limited number of them.

Standardized tests can be based on whole-organisms responses (*e.g.*, algal growth inhibition test — OCDE (2011) —, acute and chronic tests with the crustacean *Daphnia magna* — OCDE (2012) — or the midge *Chironomus riparius* — OCDE (2004), or on methods to identify whether a given chemical exhibits one or more specific mode/mechanism of action of particular concern such as carcinogenicity, reprotoxicity or estrogenic effects (OCDE, 2018). Knowledge about the apical effects and mode of action of a chemical can then be combined with knowledge

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<sup>4</sup> These author erroneously state that the term 'ecotoxicology' was first coined by René Truhaut in 1969. The term was actually jointly developed by R. Truhaut and J.-M. Jouany. See Féraud (2013) and Vasseur et al. (2020) for a revised history about these pioneers of ecotoxicology.

on its physico-chemical properties to estimate its most likely environmental fate and potential risks from accidental or deliberate release into the environment. Standardized tests usually allow to define exposure vs. response relationships (Figs. 6 and 7) and the corresponding data (ideally from several tests using different organisms and endpoints) can then be used to derive environmental quality standards (EQS) or guidelines according to established procedures (EC, 2018). Such standards and the procedure for their derivation can be revised or updated as new data are generated, more sophisticated and user-friendly tools become available, *e.g.*, the simplified biotic ligand model tools (Peters et al., 2011), site-specific studies in areas of potential concern performed (Chen et al., 2016), or the advancement of scientific knowledge makes some of their characteristics obsolete or too narrow-focussed (Reiley, 2007; Warne et al., 2014). Standardized tests are also attractive in that they can be modified or adapted to better mimic environmental situations; *e.g.*, by test using (uncontaminated) field-collected matrices instead of fully synthetic ones. The use of ‘modified’ standard procedures preserves reliability and, to a certain extent, increases the relevance of these methods. However, environmental matrices usually contain multiple chemicals and identifying those contributing to the observed adverse effects is still a long-standing challenge in ecotoxicology (Escher et al., 2020; Hendriks et al., 1994; Schwarzenbach et al., 2006). Compared with standard laboratory matrices, the composition of natural water and sediments is also more complex and heterogeneous, possibly providing multiple route of exposure to chemicals. Mixture effects and issues related to route of exposure will be discussed in section 1.6.

#### 1.2.2. What can standardized tests assess?

Standardized tests provide reliable information as to the possible range of biological *responses* to contaminants, but leave on the concerned parties the burden to evaluate whether such effects will result in *impacts* (immediate or delayed, acute or chronic) in real world situations. In an ecological sense, *impacts* refer to the presence of some kind of adverse effect on population abundance, distribution or dynamics (Gaylord et al., 2015). Thus, *responses* (and not *impacts*) measured in standardized tests (*e.g.*, decreased growth and reproduction, changes in the levels of biomarkers or modifications in the patterns of gene expression) are environmentally relevant only if they can be interpreted in an ecological context (Gaylord et al., 2015; Godbold and Calosi, 2013; Russell et al., 2012). Such considerations are most useful considering that results from standardized tests are used to establish environmental quality standards (or criteria; *i.e.* proactive use) or for risk assessment and management purposes (*i.e.*, retrospective use) (Solomon et al., 2008b). Proactively testing new chemicals allows to rank them according to their hazard potential and, ideally, to completely avoid the use of the most problematic ones. On the other hand, standardized tests alone cannot provide definite information about the actual risks associated with chemicals already circulating in the environment. In real field conditions, the biological effects of a chemical are modulated by the likelihood, extent and duration of exposure, the environmental behaviour and fate of the chemical and the presence of multiple stressors and confounding factors (see sections 1.3 to 1.7); none of which is accounted for by standardized tests. Furthermore, the assumptions behind standardized tests will become embedded in the numerical criteria derived from their results. Such assumptions collectively define the conceptual possibilities of the standardized tests and provide a framework of validity for the corresponding results (Table 3).

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Table 3. Overview of some conceptual assumptions underlying standardized test procedures along with possible field-relevant issues neglected under such assumptions. The list is not exhaustive and not all assumptions apply across different tests. For example, sediment toxicity tests can account for multiple route of exposures.

<b>Assumptions</b>	<b>Untested Possible alternatives (non-exhaustive list)</b>	<b>Examples</b>
<i>Toxicity from aqueous exposure controls the organisms' response</i>	Exposure occurring via multiple routes depending on the chemical's properties and the organism's ecology	Dietary uptake from preys or via sediment ingestion
<i>Direct toxic effects are the primary ecological and regulatory concern</i>	Biomagnification along trophic chains is the main issue	Mercury biomagnification and use of biota-based EQS
<i>Simplified test matrices maximize chemical bioavailability and provide conservative criteria</i>	Chemical speciation of the chemical lowers exposure concentrations resulting in an underestimation of toxicity (if such phenomena not accounted for)	Formation of insoluble chemical species of metals in standardized test media
<i>Test media provide optimal growth conditions so that the observed responses can be attributed solely to exposure to the chemical</i>	The configuration of standardized test forces unrealistic maximum exposure to chemicals by preventing temporal and spatial variability in their concentrations and not allowing organisms to adopt behavioural responses to reduce their exposure	Discontinuous exposures, patchy contamination in sediments
<i>Effects on model organisms are representative of the potential ones at higher levels of biological organization (populations, community, ecosystems)</i>	Predator-prey interactions cannot be predicted from standardized single-organism tests, sensitive functional endpoints may be missed	Production of fine particulate matter by gammarids (Andreï et al., 2016)
<i>(Chronic) responses are caused by continuous exposure to chemicals</i>	Patterns of exposure depend on the chemical of interest and its uses and can vary following changes in environmental conditions	Daily variation in e.g., wastewater discharges, contamination spikes related to flood events
<i>Resistance to chemicals is the main parameter controlling responses and impacts</i>	Responses and impacts have a resilience component; i.e., the ability of organisms and populations to recover from a temporary stress or to progressively adapt to it	Acclimation or genetic adaptation following chronic exposure to chemicals

Considering the limitations of standardized tests has already led to major regulatory advances for some priority chemicals at the EU level. Specifically, the European Union now adopts biota-based standards for Hg, PAH, brominated diphenylethers, hexachlorobenzene, hexachlorobutadiene, dioxin and dioxin-like compounds (Directive 2013/39/EC), thus

explicitly accounting for their environmental behaviour and the risk of secondary poisoning of top predators following their biomagnification along trophic chains. This approach is a major change compared with the traditional use of water-based criteria. Analogous conclusions on how addressing the limitation of standard methods can improve environmental quality criteria for Se, ammonia and tributyltin can be found in Suter (2018). Along with such regulatory success-stories, understanding the actual contribution of dietary exposure to the ecotoxicity of chemicals remains an active field of research (Campana et al., 2013; Croteau et al., 2017; Weltens et al., 2000) and it is to be hoped that further regulatory improvements will be implemented as more information becomes available.

The temporal scales of standard tests also deserve attention because, even in the case of chronic tests, they may not be able to detect acclimation/adaptation responses that can make organisms more tolerant to a given chemical (Fischer et al., 2013; Postma and Davids, 1995). Laboratory organisms exposed to a chemical for the first time may show a higher level of response compared with analogous ones that have been exposed to sub-lethal levels of a stressor over several generations. Bossuyt and Janssen (2004) tested the tolerance to copper of different cultures of the crustacean *Daphnia magna* after 18 months of acclimation. Those authors observed that Cu-acclimated daphnids were significantly more tolerant to copper during acute exposure test, but differences between acclimated and non acclimated organisms were not significant in chronic tests. Otherwise stated, acclimation can occur in the laboratory, but may be of little consequence in risk assessment terms because risk assessment is based, preferentially, on chronic exposure data (provided that they are available).

Indirect effects are also an important aspect that cannot be properly assessed using standardized ecotoxicological tests. Standard procedures focus on direct effects of a stressor on a specific model organism, but do not consider the role of the organism, or more precisely its population, in the field. Fleeger et al. (2003) reviewed 150 papers on indirect toxicant effects and reported that contaminant-induced changes in trophic interactions were observed in 60% of the studies. In the most frequent situation, a predator was more sensitive than its prey to a given contaminant so that the prey population increased. However, Clements et al. (1989) reported that sublethal Cu exposure made preys less efficient at avoiding predators, resulting in population decline of the prey.

Standard ecotoxicological tests usually provide optimal growing conditions to model organisms, so that observed effects can be univocally ascribed to the contaminant of interest. In this sense, exposed organisms can divert all excess energy to the defence against the added contaminant and are likely more resistant to that contaminant than they will be in the field. Otherwise stated, laboratory tests may underestimate the hazard and risk of a stressor compared with natural conditions. On the other hand, during laboratory exposures in confined environments, organisms cannot resort to responses such as avoidance of contaminated matrices (including contaminated food presented to organisms in some tests) that allow them to simply not be where the contaminant is.

This short overview of some conceptual assumption of standardized tests confirms that they can perform well to define the hazard of a stressor, but must be integrated with other tools and disciplines to correctly assess the actual risks of the same stressor in nature. According to Mebane et al. (2019): “Adherence to GLP [*good laboratory practices*] does not therefore mean that a study is well-designed. It only means that a study is well-documented, quality assured, and that the study design protocol was expressly followed. But if the study design wasn’t appropriately relevant and rigorous for the study questions, the well documented and reproducible results will doubtfully be meaningful”. It is therefore necessary to strike a balance between the reliability of standardized procedures and

the need for relevance in fundamental ecotoxicological studies as well as in hazard and risk assessment. A few considerations in this sense are presented in the next subsection.

### 1.2.3. Current frameworks to ensure reliability and relevance

The importance of increasing the environmental realism (i.e., the relevance) of standardized ecotoxicological experimentation is now clearly recognized. Recommendations in this sense include conceptual and theoretical frameworks to assist appropriate test choices and experimental design (Bednarska et al., 2013; Harris et al., 2014) as well as detailed lists of reporting criteria to evaluate the reliability and relevance of ecotoxicological studies in view of their use for risk assessment (Ågerstrand et al., 2011; Binet et al., 2018; Isigonis et al., 2015; Moermond et al., 2016). Web-based tools have also been developed for the purpose ([www.scirap.org](http://www.scirap.org)). Reporting of metadata accompanying the effect concentration for the chemical of interest is indeed invaluable to assess if a given set of data may be considered relevant for data-poor situations and also to allow the development of broadly applicable modelling tools.

The strengths and weaknesses of eleven frameworks to assess and improve reliability and relevance of ecotoxicological studies have been reviewed by Roth and Ciffroy (2016). Overall, it appears that, in environmental (and human) risk assessment, there is no substitute for producing data from carefully planned, reliable and relevant studies in the first place. Indeed, all the frameworks to assess reliability and relevance not only give risk assessor the possibility to evaluate the quality of existing data, but also provide scientists with guidance on how to maximize the reliability and relevance of their experiments in order to make them user-relevant in risk assessment (should that be the purpose, or one of the purposes, of a specific research). The increasing attention to data reliability and relevance reflects the conceptual challenges, methodological difficulties and knowledge gaps of the three components of ecotoxicology as defined by Wang (2011): environmental fate, exposure and toxicological response which are briefly discussed in sections 1.3 and 1.4.

Thanks to the emergence of nano(eco)toxicology, it has become clear that even trivial aspects such as the time elapsed between the preparation of a solution/suspension of a chemical or the type of labware used in a test can strongly affect the final response of the test organism (Malysheva et al., 2015; Sørensen and Baun, 2015). Ensuring reliability may thus require a much closer attention to reporting even simple and putatively irrelevant aspects of one's research, so that cross-comparisons can be as accurate as possible and differences in the outcome between apparently similar studies can be understood and accounted for. Other points requiring more attention in the future (Roth and Ciffroy, 2016) include the wider adoption of frameworks for simultaneously evaluating collections of studies on a certain chemical and improved delimitation of reliability vs. relevance criteria.

While supporting all the possible measures to ensure that standardized ecotoxicological testing achieves the highest possible levels of reliability and relevance, it is important to remember that the biological responses observed in such tests will then be used to establish criteria for the protection of ecosystem structure and functioning in the long-term. As such, extrapolation and associated uncertainties will probably remain unavoidable. Much will also depend on the conceptual framework in which the results of (reliable and relevant) ecotoxicity tests are interpreted and used. If a pollution-prevention principle approach is adopted, the most conservative tests will be given the highest consideration during risk assessment. On the other hand, adopting Ecological threshold or Recovery principles may tolerate higher concentrations of a given chemical (or group of chemicals) in the environment provided that their effects are transient and/or do not affect ecosystem structure and functioning (Meybeck, 2003; Solomon et al., 2008b) (see also Fig. 6). The selection of one conceptual approach over the other will

also depend on societal and economic issues that go beyond reconciling standard procedures and realisms in ecotoxicological research (Figs. 1 and 2).

1.2.4. How criticisms drives the development of ecotoxicology  
Discussions on the limitations and potential pitfalls of standardized ecotoxicological procedures are not new as exemplified by *e.g.*, the critique of algal toxicity tests by Janssen and Heijerick (2003) or by earlier concerns on the actual usefulness of such tests (Lewis, 1990). Much of the past and current criticisms is actually scientifically grounded and remains a powerful driver to develop more relevant approaches and tools in fundamental and applied ecotoxicology. At the same time, the societal relevance of ecotoxicological research will very likely increase considering that our reliance on chemicals will continue to rise in the future (see section 1.1) and that deteriorating trends dominate the 2030 outlook at the EU level as to chemical emissions, impacts and risks (EEA, 2019). While continuously improving ecotoxicological research, care is therefore required not to convey to non-ecotoxicologists, decision makers, stakeholders and the general public the idea that standard tests are useless, flawed or completely irrelevant for environmental protection.

Most of the criticisms to standardized tests can be addressed by recognizing their actual aims and scopes along with a careful consideration of the experimental and methodological factors that can affect the test outcome (Janssen and Heijerick, 2003; Manakul et al., 2017; Olkova et al., 2018). Standardized tests aim at providing reliable, repeatable (refers to intra-laboratory variability) and reproducible (refers to inter-laboratory variability) information for hazard assessment of different substances to derive generally applicable and conservative EQS or to assess environmental samples for ecotoxic effects. A standardized procedure therefore provides guidance on several methodological aspects that could potentially affect the organism survival and sensitivity and the overall reliability of the test itself (De Cooman et al., 2015; Persoone et al., 2009). On the other hand, a standardized procedure does not address the inherent limitations of a test with regard to its relevance which is by definition context-dependent and is accompanied by several specific research needs (Table 3).

While ecotoxicological data of inadequate quality exist in the scientific record (Harris et al., 2014), reaching consensus and taking action on complex environmental issues is a complex and time consuming process (EEA, 2013). However, the recent evolution of the European regulatory framework confirms that policy can be receptive to new ways of looking at the risk assessment of chemicals, which constitutes a powerful driver for the advancement of ecotoxicological research. Directive 2013/39 on priority and hazardous substances contains legally enforceable EQS expressed in terms of bioavailable concentrations for two trace elements (Ni and Pb) or established for environmental matrices other than water, *i.e.* biota. In practice, the directive introduces two major scientific tenets into legislation: the consideration that EQS based on total elemental concentration neglect bioavailability issues (possibly leading to inappropriate decision-making as to the management of these chemicals) and the recognition that water-based EQS may be inadequate when the main environmental concerns arise from dietary intake and biomagnification rather than direct effects (Table 3).

In a broader context, Directive 2000/60/EC (the so-called Water Framework Directive – WFD) (EC, 2000) has overturned the way in which we manage the environment by putting the priority on the structural and, increasingly, functional integrity of ecosystems as the ultimate objective of European environmental policy. Although achieving good ecological status goes beyond managing chemicals, major scientific advances in ecotoxicology, monitoring and hazard/risk assessment have actually been driven by the challenges associated with putting the WFD principles into practice (Wernersson et al., 2015). Reaching the ultimate WFD objective of good ecological and chemical status for all European waters will continue to provide impetus to address unsolved issues such as the presence of many potentially ecotoxic substances that

still go unmonitored (EEA, 2019), the development of data for the ecotoxicity of emerging pollutants or the representativeness of model laboratory species as surrogates for natural biological communities. In parallel, the periodical revision process of priority (and hazardous) substances in EU legislation will continue to identify substances that may be, or may become, of high concern due to (possible) widespread use and documented hazards; thus contributing to the societal relevance of ecotoxicology (Gomez Cortes et al., 2020).

Table 4. Overview of possible criticisms as to the lack of relevance of standardized ecotoxicological tests along with suggestions for future research and possible solutions.

<b>Criticisms</b>	<b>Caveats against an excessively direct interpretation</b>	<b>Research needs and possible solutions</b>
<i>Unrealistic exposure concentrations</i>	<ul style="list-style-type: none"> <li>➤ Concentration is only one factor in determining the response of organisms</li> <li>➤ Total concentration is not representative of the bioavailable fraction</li> <li>➤ Marked, often rapid, decreases in the added contaminant concentration can occur over test duration due to <i>e.g.</i>, hydrolysis or volatilisation</li> <li>➤ High concentrations include safety margins to account for subtler or long term effects not directly assessed by the standardized test (EEA, 2013, p. 30)</li> <li>➤ The test can still serve to rank the hazard of different chemicals when searching for alternatives to chemicals with restrictions (future bans) or to evaluate the suitability of a chemical for developing benign-by-design products</li> </ul>	<ul style="list-style-type: none"> <li>➤ Determine contaminant behaviour and speciation in the standard medium</li> <li>➤ Verify if mode of actions/mechanism of action are the same at different concentration levels</li> <li>➤ Modify test matrix to increase its environmental relevance</li> <li>➤ Verify if EC<sub>50</sub> or EC<sub>10</sub> are within the concentration range observed in the environment</li> </ul>
<i>Unrealistic exposure matrices</i>	<ul style="list-style-type: none"> <li>➤ Standardized matrices ensure data reliability and intercomparability for hazard assessment purposes. This helps in prioritization of substances in environmental regulation</li> <li>➤ Results from a standardized ecotoxicological test are only one component of risk assessment processes and frameworks</li> <li>➤ Testing of natural samples for ecotoxicity is a routine task, but remains of limited use in the absence of a clear identification of the chemicals causing the observed effects</li> </ul>	<ul style="list-style-type: none"> <li>➤ Standardized norms allow for flexibility for site-specific evaluation purposes or for substances, other than nanoparticles/nanomaterials, with poor water solubility (ISO, 2006)</li> <li>➤ Presence of organic complexes in natural waters may increase bioavailability (i.e. laboratory tests are not necessarily conservative)</li> <li>➤ Speciation models for trace elements can help to account for differences across matrices</li> <li>➤ Generally valid models (<i>e.g.</i>, BLM)<sup>a</sup> can be developed to account for the main toxicity-modifying factors related to matrix composition</li> </ul>

<sup>a</sup> Biotic ligand model

Table 4. Continued

<i>Non consideration of mixture effects</i>	<ul style="list-style-type: none"> <li>➤ Contaminants may share a similar mode of action or a class of contaminant may be driving ‘chemical ecotoxicity’ in a given environment</li> <li>➤ Difficult/impossible to re-create complex real-world mixtures in the laboratory, anyway</li> <li>➤ Standard exist only for a limited number of substances</li> <li>➤ There are between 40,000 and 100,000 chemicals on the market with 6000 representing 99% of the total volume (SOER 2020; Global chemical outlook)</li> </ul>	<ul style="list-style-type: none"> <li>➤ Examine pressures at a given location (or at a basin scale) and try to establish priority components of the relevant mixtures</li> <li>➤ Continue to improve the dialogue between monitoring agencies and ecotoxicological research to define priority mixtures</li> <li>➤ Perform EDA<sup>b</sup> or TIE<sup>b</sup> procedures as needed</li> <li>➤ Integrate traditional ecotoxicological approaches with effect directed analysis to develop <i>good ecotoxicological status</i> guidelines for EU waters (see Fig. 8)</li> </ul>
<i>Temporal and spatial variability unaccounted for</i>	<ul style="list-style-type: none"> <li>➤ The severest responses may be driven by short, pulse-like exposures to peak concentrations rather than by continued exposure to approximately constant concentrations</li> <li>➤ Avoidance responses may occur for mobile organisms</li> <li>➤ Heterogeneity in contamination (especially in sediment and soils) can provide clean micro-habitats even in polluted environments</li> </ul>	<ul style="list-style-type: none"> <li>➤ Pay more attention to the kinetics aspects of ecotoxicological responses</li> <li>➤ Reconsider metrics such as the ET<sub>50</sub> (effect time) that have fallen out of use with no actual scientific reason</li> <li>➤ Develop approaches to simulate time-variable exposures and assess their effects compared with continuous exposure to the same average concentration</li> <li>➤ Use <i>in situ</i> validation when relevant</li> <li>➤ Use modelling tools</li> </ul>
<i>Ecologically irrelevant routes of exposure</i>	<ul style="list-style-type: none"> <li>➤ All routes may be relevant in a life-cycle perspective</li> <li>➤ An irrelevant route for the adult stage may be controlling ecotoxicity during organism development</li> </ul>	<ul style="list-style-type: none"> <li>➤ Respect the ecology of the test organisms</li> <li>➤ Use micro/mesocosm approaches</li> <li>➤ In risk assessment, privilege data that are both reliable and relevant</li> </ul>
<i>Use of unrepresentative species</i>	<ul style="list-style-type: none"> <li>➤ Some response mechanisms are conserved across species</li> <li>➤ Some species can be tested in representative conditions (i.e. temperate species in tropical conditions) even if they are from different areas/regions</li> <li>➤ Test species tend to be resistant (to handling stress for example), so that their responses may alert against possible severer effects on more sensitive species</li> </ul>	<ul style="list-style-type: none"> <li>➤ Develop/adapt tests to species of relevant in local, regional or specific climate contexts</li> <li>➤ Consider the possibilities of trait-based ecotoxicology</li> <li>➤ Use multiple line of evidence to make decisions</li> </ul>
<i>Importance of non-chemical stressors and biological interactions (smothering, predation, parasitism)</i>	<ul style="list-style-type: none"> <li>➤ None, but these issues are outside the current scope of RA for chemicals</li> </ul>	<ul style="list-style-type: none"> <li>➤ Further research is needed</li> <li>➤ Confirm laboratory results with <i>in situ</i>/on-site experiments</li> <li>➤ Evaluate the importance of the different factors in the field</li> <li>➤ Consider ecotoxicology and climate change interactions</li> </ul>

<sup>a</sup> Effect Direct Analysis, Toxicity Identification Evaluation

Table 4. Continued

<p><i>Contaminant bioaccumulation and handling by organisms is not considered (e.g., high total bioaccumulation with no effect due to storage in nontoxic forms)</i></p>	<ul style="list-style-type: none"> <li>➤ None, this issue is normally outside the scope of standardized ecotoxicological tests</li> <li>➤ Tests to estimate bioaccumulation are available</li> <li>➤ Substances can be classified as potentially bioaccumulative based on their properties</li> </ul>	<ul style="list-style-type: none"> <li>➤ Integrate bioaccumulation measurements to effect measurements when relevant</li> <li>➤ Use techniques to study the intracellular distribution of a chemical or its distribution among organs</li> <li>➤ Consider that effects may originate at the cellular surface without the need for internalization</li> </ul>
<p><i>In vitro tests neglect the repair mechanisms that a whole organisms can activate in response to a chemical and overestimate the hazard/risk</i></p>	<ul style="list-style-type: none"> <li>➤ <i>In vitro</i> tests provide important information with regard to intrinsically undesirable properties such as carcinogenicity, mutagenicity and reprotoxicity</li> <li>➤ Avoiding chemicals with intrinsically undesirable properties is key for systemic ameliorations such as benign-by-design products and circular economy</li> <li>➤ A single chemical is part of a complex exposome and ‘something from nothing’ effects have been documented for mixtures of chemical with the same mode of action</li> </ul>	<ul style="list-style-type: none"> <li>➤ Further develop our understanding of the mechanisms of action/mode of action of chemicals to achieve a more mechanistic-driven management of chemicals</li> <li>➤ For a legacy substance (or for a chemical already in use), absence of <i>in vivo</i> effects may indicate low bioavailability or degradation and hence a low regulatory priority</li> </ul>

As documented in volume 2 of the EEA report *Late lessons from early warning* (EEA, 2013), only a combination of observation and solid evidence from (eco)toxicological research will allow appropriate management of environmental problems. In this sense, standardized tests can be combined with monitoring activities (and I include EDA under the definition of monitoring) to provide the best knowledge as to the hazards associated with substances known to occur in the environment (Koschorreck et al., 2015). In the end, issue-driven research will invariably reconcile standard laboratory procedures with environmental reality provided that fundamental research will continue to develop more refined tools with the specific objective to improve our ability to assess risks in real-world settings.

#### 1.2.5. A personal view on the current context

Although the time from discovery to application can be long, conclusions from (eco)toxicological research invariably have strong impacts on economic and social aspects of our lives; as exemplified by the phasing out of leaded petrol starting from 1970s, the entry into force of the Minamata Convention on mercury in 2017, or the development of REACH legislation. With so much at stake, ecotoxicologists can easily find themselves between Scylla and Charybdis; most times being no Ulysses. On the one hand, economic actors and (part of) the general public may become suspicious of the flux of information continuously uncovering possible new environmental and health threats. On the other hand, environmental advocacy

groups and citizens' groups could fear that important risks do not receive enough attention (both in terms of extent of the response and swiftness of the response) because of vested interests from powerful lobbying groups. Ecotoxicology must therefore contribute to properly manage real environmental issues, help to identify *false positives* and minimize *false negatives*. False positives refer to situations where regulation is introduced, but turns out to be unnecessary at a later stage; while false negatives represent cases of unrecognized and unregulated real risks (EEA, 2013). Most interestingly, out of 88 putatively false positives examined in the 2013 EEA report, only 4 were real false positives.

Indeed, results from standardized ecotoxicity tests are deemed conservative with respect to natural waters, *i.e.* they quantify the hazard potential of a chemical in (laboratory) conditions that are expected to maximize its bioavailability. Under such assumptions, risk management based on EQS derived using standardized tests minimizes the likelihood of false negatives, while accepting a possible increase in false positives. It is however important not to fall into the misconception of considering EQS and, much more so, other non regulatory-binding guidelines as a pass/fail threshold (Allen Burton, 2017) or the presence/absence of hazard in laboratory tests as a definite proxy for the actual risk in the field. Comparison of laboratory-derived EQS or other criteria with field data can support the hypothesis that compliance with such values offer an appropriate degree of protection to *in situ* populations or communities (Crane et al., 2007b; Iwasaki and Ormerod, 2012). However, sensitive endpoints or species may be outside the scope of standard tests (Andreï et al., 2016) and assumptions such considering water-based criteria protective for sediment-ingesting benthic organisms may turn out to be invalid (Bettinetti et al., 2018). Consequently, ecotoxicological paradigms also need to be evaluated and, if necessary, reconsidered depending on protection objectives, availability of new knowledge and the need for cost-effective approaches in environmental management (Brack et al., 2017; Merrington et al., 2014). In the long run, standardized tests may eventually become a sort of certified ecotoxicological reference material for ecotoxicology to ensure that effects observed in more complex experimental settings are not due to fluctuations in the sensitivity of model organisms following modified test conditions.

As already mentioned, it is particularly important to remember that only a fraction of the substances currently in use have been characterized sufficiently well from an ecotoxicological point of view. In the future, we should probably move toward situations where ecotoxicological testing of environmental samples becomes an important, if not the primary, criterion to determine whether a sample (or a water body) can be classified as having an overall good chemical status. An ecotoxicological evaluation of chemical status can be more relevant, and probably more cost-effective, in identifying water bodies under chemical stress rather than the analysis of a number, no matter how large, of (priority) selected chemicals. Such ecotoxicologically-based water quality assessment can find its place in a tiered approach (Fig. 8) that partly mirrors the process used in hazard assessment of chemicals under the REACH regulation by combining whole-organisms test for apical effects and *in vitro* tests for the carcinogenic, mutagenic or reprotoxic (CMR) potential of waters (or other environmental matrices such as sediments and soils). Suggestions in this sense are embedded in EDA strategies (Brack et al., 2017; Burgess et al., 2013), but standardizing such framework for regulatory use will be far from trivial.

An ecotoxicologically-based framework could in principle assume that an absence of effects in tier 1 indicates a chemically safe situation, *i.e.* a regulatory compliance, in the field (Fig. 8). However, the presence of chemicals in environmental matrices varies in time and space, meaning that several batch samples will have to be collected to reach reliable conclusions about the absence of chemical risks. Exclusive reliance on off-site laboratory tests could therefore suffer from the same limitations as chemical monitoring with regard to sampling strategies. At the opposite, ecotoxicological monitoring remains superior to chemical monitoring in spotting

effects linked to *e.g.* mixtures of priority substances (each occurring at concentrations below the corresponding probable no-effect concentrations – PNEC) or unmonitored chemicals (Fig. 8 tiers 2 and 3). Active biomonitoring strategies can complement ecotoxicological tests in the laboratory by providing time-integrated measurements of the type of effects occurring at a given site. Overall, an ecotoxicological evaluation of chemical status will likely be more effective in screening situations where chemical contamination is the main cause of environmental impairment from those in which non-chemical stressors are the drivers of impact. In the presence of adverse effects in tier 1, the following tiers should allow to establish causality (Fig. 8). Several important caveats must however be mentioned not to miss situations of potential concern.

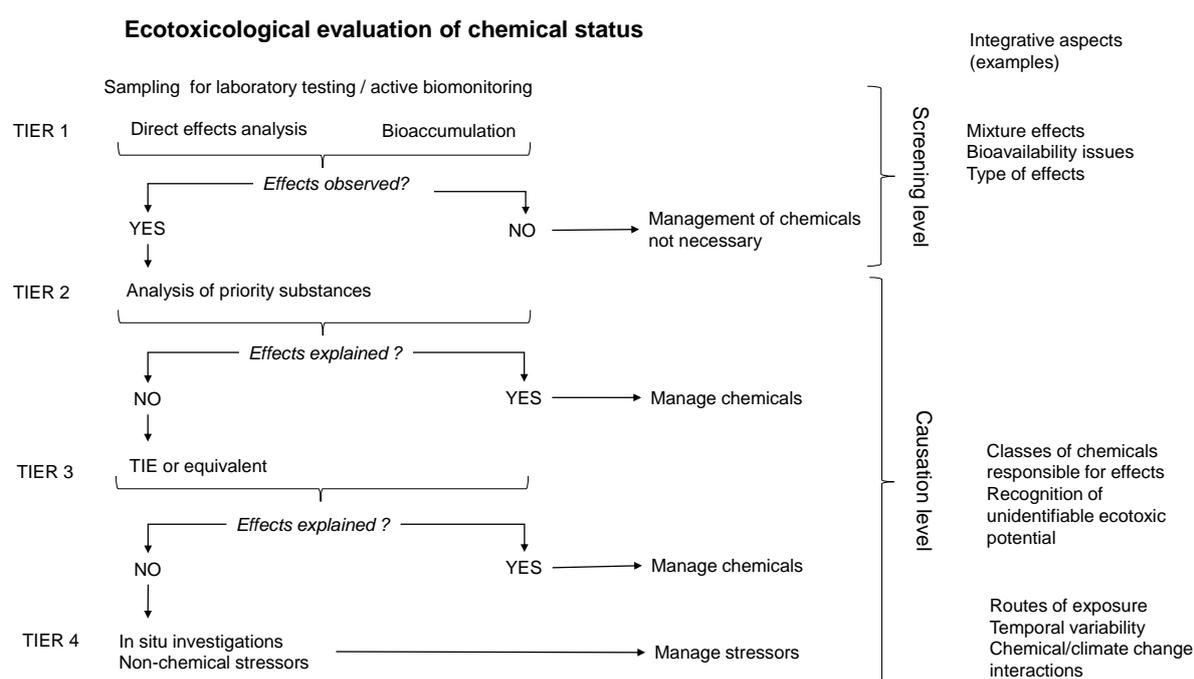


Figure 8. Example of a possible tiered approach for using ecotoxicological tools to evaluate the chemical status of water bodies. Integrative aspects (right-hand side of the figure) highlight the advantages of different types of ecotoxicological testing or approaches compared to chemical analyses.

*First, there is no one-fits-all approach in establishing causality.* As pointed out by Chapman and Hollert (2006) in relation to sediment quality evaluation, establishing causality will require investigators to choose specific lines of evidence depending on the actual chemicals of concern and protection targets. This principle is generally valid.

*Second, ecotoxicology can be a proxy for good chemical status, but not for good ecological status.* Discrepancies between chemical and ecological status should trigger more complex investigations to ensure that laboratory testing does not miss chemical effects *in situ* (Fig. 8); particularly in relation to routes of exposures that are unaccounted for in batch laboratory tests (see section 1.6). Several micro and mesocosms tools along with devices for *in situ* testing can provide useful bridges to better understand how complex and variable environmental situations affect organisms' response to chemicals in real situations (Crane et al., 2007a; Ferrari et al., 2014).

*Third, as it is the case for chemical analysis, issues of sampling frequency and representativeness will have to be addressed.* The presence of a given chemical may be linked

to seasonal discharges from artisanal, industrial or agricultural activities (Hogan et al., 2013; Szalinska et al., 2013). Temporal variability in chemical inputs obviously influences the ecotoxicological potential of a given sample. Random collection and testing of a relatively clean sample may therefore classify as compliant a water body suffering from severe, but periodic pollution. On the other hand, the presence of effects in a punctual sample will not allow to assess the possibility of recovery from toxic effects or the higher resilience of a community compared to the laboratory model organisms.

*Fourth, the choice of the environmental matrix to test will be an added criticality in an ecotoxicology-driven regulatory framework for water-body quality.* Saved for the few chemicals that are regulated in biota, an approach also coming with specific practical challenges (Fliedner et al., 2018), compliance with EQS for chemicals is often based on the analysis of bulk or filtered water samples. Selecting bed sediments as a test matrix would provide a more time-integrated signal for those chemicals that tend to accumulate in this compartment and a more holistic assessment of the chemical risks in a given water body. However, quality criteria for sediments do not yet have regulatory value due to the difficulties in identifying the bioavailable chemical fraction against fluctuating total background values; especially for trace elements and ubiquitous substances (Campana et al., 2013; Simpson and Batley, 2007a). Furthermore, standardized ecotoxicological tests with benthic organisms are skewed towards species inhabiting fine-grained sediments (*e.g.*, chironomids and oligochaetes) that are irrelevant in the case of fast flowing habitats where hard substrates dominate. Considering this short and non-exhaustive list of caveats when attempting an ecotoxicological evaluation of chemical status, it is not surprising that regulatory hazard assessment remains largely based on the routine chemical monitoring of priority substances despite the conceptual superiority of ecotoxicological approaches. However, regulatory, conceptual and technological developments in safety testing for new chemicals will almost certainly increase the importance of ecotoxicological approaches in the routine assessment of chemical risks in the future (Basu et al., 2019; Nel and Malloy, 2017; Suter, 2018)

The almost complete reliance of the current regulatory framework on effect concentrations derived from fixed exposure times (assumed to represent acute or chronic exposures) is equally noteworthy. As a matter of fact, not enough is known on the lethal exposure times or on the effect of (repeated) transient exposures for many elements and substances, although the importance of both issues is being increasingly documented (see section 2.4). While monitoring of exposure concentration in the field is moving towards the adoption of passive samplers capable of integrating measurements (Brack et al., 2017; Merrington et al., 2014; Posthuma et al., 2016), the temporal dimension of ecotoxicological effects remains largely unexplored for many chemicals. It may be sensible to stimulate more research on this temporal aspects of ecotoxicology by using semi-static exposures or pulse-exposure of limited duration to see how long the effects of repeated exposure, or of a pulse exposure, are likely to last and what is their extent. In general, I would argue that much of the standard test framework is concerned with an *ecotoxicology of resistance* (*i.e.*, how much of a chemical an organism can take before being adversely affected) rather than with an *ecotoxicology of resilience*; *i.e.*, up to which level of exposure organisms can recover from an exposure and how long it will take them to recover. The ecotoxicology of resilience is partly embedded in risk assessment using micro/mesocosms experiments that can consider latent and indirect effects as well as system recovery (Solomon et al., 2008b)

Finally, in the current context of systemic environmental challenges (EEA, 2015), hazard from chemicals must be weighed against other stressors that may be the major drivers of impacts in the field (Belletti et al., 2020; Burton and Johnston, 2010; Posthuma et al., 2016). Without specific modifications, standardized ecotoxicological tests are not designed to assess the influence of variables linked with land use, climate change and socio-economic drivers. It

is however noteworthy that remediation action focussing on habitat restoration can drastically fail because of the unsuspected presence of a single, previously unidentified chemical from diffuse source (Tian et al., 2021).

#### 1.2.6 Rationale for structuring the present research project

As I argued, among many others, in a former viewpoint (Vignati et al., 2007), there is no single approach when assessing the relevance of laboratory standard results. Following my past experience and future research plans, I have selected six issues that can contribute to reconciling the reliability of standardized practices with the need for environmental realism in (aquatic) ecotoxicology: correctly quantifying *exposure to chemicals* in environmental matrices (section 1.3) or directly in exposed organisms (section 1.4), assessing the *effect of chemical mixtures* (section 1.5), understanding *matrix effects* and the importance of *routes of exposure* (section 1.6), increasing knowledge about the importance of *exposure duration* in controlling ecotoxicological responses (discussed in details in section 2.4) and evaluating the relevance of standard conditions in relation to specific *issues linked to climate change* (section 1.7). Last but not least, I will briefly discuss how and why scientific bias can creep into ecotoxicological research hindering its advancement or creating knowledge biases (section 1.8). The objective of each section is to illustrate the overall significance a given issue for ecotoxicological research and hazard/risk assessment, without any pretention to provide an exhaustive coverage on the literature for each subject. Specific aspects will be examined more in depth in relation to the research proposal section.

Section 1.3 to 1.7 define some aspects of the overarching framework that must be considered when attempting to reconcile ecotoxicological standard procedures and environmental realism. Biological availability (defining the fate of chemicals inside organisms; *i.e.*, effect or response assessment) and bioavailability (defining the fraction of a chemical that can effectively interact with an organism; *i.e.*, exposure assessment) are directly related with the primary objectives of ecotoxicology (Fig. 9). For a given chemical, knowledge of biological availability and bioavailability, which may be prevented by technical limitations, would be enough to guarantee the reliability of the results from standardized procedures. On the other hand, understanding the environmental relevance of the same results must consider a larger set of biological and environmental factors that collectively control the chemical bioaccessibility and whose study belongs to several environmentally-related disciplines such as ecology, hydrology, limnology, population biology and so on (Fig. 9). All these considerations apply to individual chemicals that mostly occur as mixtures in natural environments and collectively define the exposome (Vermeulen et al., 2020; Wild, 2005) (Fig. 9). Finally, effects of chemicals and chemical mixtures can be modified by other stressors such as temperature fluctuations, habitat degradation, interspecific relationship (e.g., competition or predation). These non-chemical stressors can both reflect natural fluctuations and originate from anthropogenic drivers such as agriculture, energy production and land use that generate specific pressures including habitat fragmentation, alteration of sediment transport and introduction of alien species (Fig. 9). The study of these non-chemical aspects of environmental stress is covered only marginally by standardized ecotoxicological procedures and can limit their environmental relevance as defined in section 1.2.

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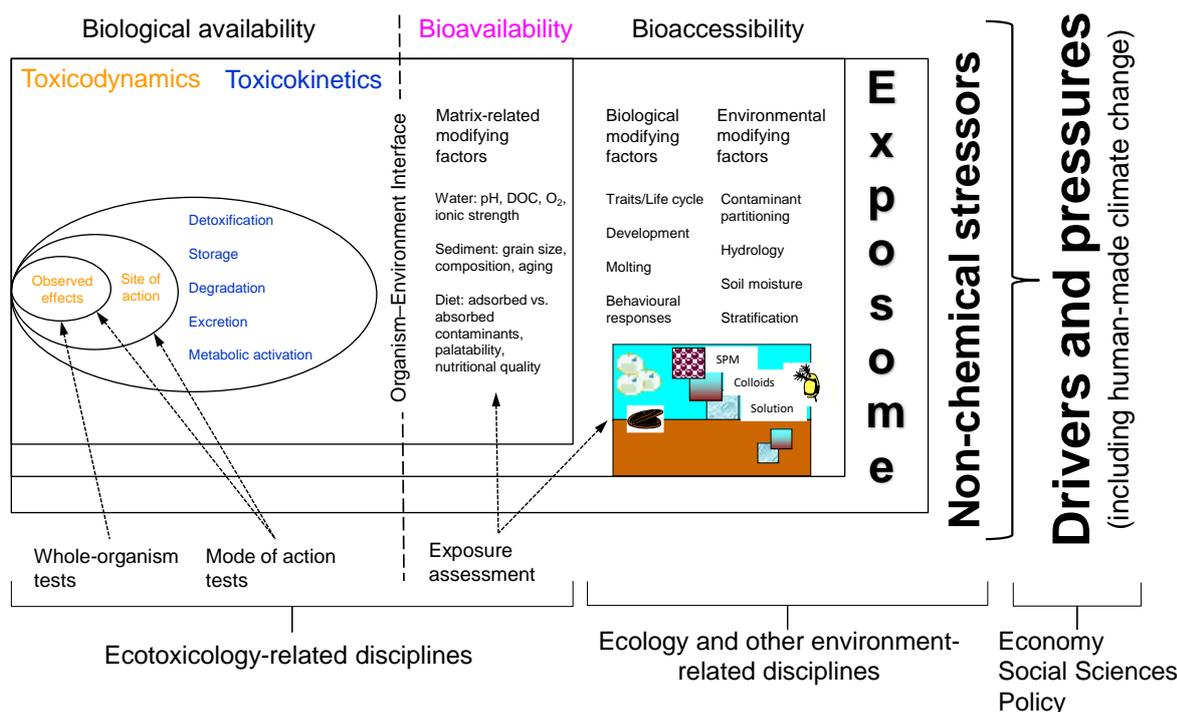


Figure 9. Schematic view of the overarching issues to be considered in reconciling standard procedures and environmental realism in ecotoxicology.

### 1.3. Quantifying exposure to chemicals from measurements in environmental matrices

Establishing concentration–response relationships is one of the fundamental tenets of ecotoxicology to establish the ‘safe’ concentration for any given chemical. Concentration–response relationships allow to estimate Effect Concentrations ( $EC_x$ ) that define the quantity of a chemical resulting in a percentage effect  $x$  on a given biological endpoint.  $EC_x$  can be accompanied by confidence intervals and are therefore preferable to unbound metrics, e.g. NOECs (Non Observable Effect Concentrations), because they can provide an indication of the uncertainty associated with the expected effects (Fox and Landis, 2016).  $EC_x$  can refer to various biological endpoints ranging from lethality (which is a specific case referred to as lethal concentration  $x$  –  $LC_x$ ) to growth and reproductive output or other measures of biological stress/fitness. According to Suter (2018), benchmark values used for hazard and risk assessment, which include  $EC_x$ , should:

- a) refer to endpoints worthy of protection,
- b) define a threshold level in terms of magnitude of effect,
- c) have a clear temporal dimension,
- d) be accompanied by a specific exposure metric (e.g., total or filterable chemical concentration in water),
- e) have well-defined limits of applicability.

For any given chemical, the same exposure metric (point *d* of the list) should ideally be measurable in both laboratory and field settings, thus facilitating the extrapolation of results from controlled conditions to real case scenarios. In standardized ecotoxicological tests, concentration–response curves are usually established in terms of total chemical concentration in the exposure matrix (e.g., water, sediment or soil depending on the test organism). However, chemical exists in a variety of forms (Fig. 10), especially in field conditions, and a wide consensus stipulates that only a fraction of their total concentration will contribute to elicit

adverse effects in test organisms (Fig. 9). Standardized tests do not necessarily put organisms into contact with all the possible forms in which a chemical occurs in the environment. For example, standardized aqueous tests are usually limited to testing of soluble forms of a chemical (Fig. 10), although the presence of colloidal particles can markedly modify the ecotoxic potential of *e.g.*, trace elements (Koukal et al., 2003; Koukal et al., 2007; Slaveykova et al., 2003). Nanoecotoxicology has markedly increased the attention of ecotoxicologists to the correct determination of substance behaviour during laboratory tests, including consideration of apparently trivial issues such as the choice of plastic materials and filter types that may affect organisms' response (Malysheva et al., 2015; Mitrano et al., 2012; Sekine et al., 2015). It is therefore surprising that similar issues do not systematically receive the same scrutiny in tests used to establish the hazard (and risk) of trace elements. For example, compliance with environmental quality standard for trace elements is based on field measurements of the corresponding filterable concentrations. The potential pitfalls of filtration in trace element analysis are numerous and well-known (Hall et al., 2002; Horowitz, 2013; Horowitz et al., 1992; Vignati et al., 2006), but remain unheeded in most studies.

Establishing safe levels of chemicals in sedimentary (and soil) matrices brings about additional complexity because of the presence of multiple routes of exposure (*e.g.*, interstitial waters, overlying waters and particles in the case of sediments; section 1.6) and the heterogeneous chemical composition of the solid matrix. In the case of trace elements in anoxic sediments, the Acid Volatile Sulphides (AVS) framework for aquatic sediments postulates that only the elemental fraction occurring in the interstitial waters would contribute to sediment ecotoxicity toward benthic organisms (Batley et al., 2005). The Equilibrium Partitioning Approach (EqP) provides a similar, but more general framework for all chemicals and solid matrices. The EqP approach assumes that sediment (soils) actually act as a complexing phase in equilibrium (or steady-state) with the interstitial water that contains the ecotoxicologically active fraction of a chemical (Batley et al., 2005). These theoretical frameworks resulted in an extensive evaluation on the possible use of interstitial water for ecotoxicity testing instead of bulk sediments (Carr and Nipper, 2003). The EqP approach was actually used at the European level to estimate guideline values of priority chemicals in sediments as it can be seen in the substance data sheets for priority chemicals. Data sheets can be found on the public [CIRCABC interface](#) — log in required — and one example for Hg can be found [here](#). A thorough critique of the AVS and EqP approaches can be found in *e.g.*, Batley et al. (2005). What is of interest in the context of the present project is that dismissing solid matrices as a potential source of biologically active contaminants is neither scientifically nor conceptually justified (Ortega-Calvo et al., 2015; Peijnenburg and Jager, 2003).

Several non-predatory benthic organisms (or terrestrial invertebrates) rely on ingesting particulate material to meet their energetic and nutritional needs. Their digestive tracts are therefore chemical reactors whose role is to desorb nutrients from the ingested solids (Penry and Jumars, 1987). It has been demonstrated that toxic substances can also be desorbed from sediments during this process (see box 1 in Part II, section 3). This desorption of chemicals from ingested solids implies that dietary exposure can contribute significantly to the chemical body burden of an organism (Casado-Martinez et al., 2010b; Croteau et al., 2013; Wang et al., 1997; Weston and Mayer, 1998). From an ecotoxicological perspective, it is therefore necessary to identify the bioavailable fraction of a chemical that is bound to environmental particles, *i.e.*, suspended particulate matter, bed sediments or soils (colloids having some peculiar properties that require different approaches). This can be done by resorting to the use of weak and mild reagents that are supposed to provide a better indication of the mobility and bioavailability of a given chemical. Sequential extraction procedures for trace elements are a popular example of such an approach (Quevauviller et al., 1997; Tessier et al., 1979), but remain operationally based, cannot identify the actual chemical species present in the solid matrix and should be used

with an understanding of their scope and limitations (Bacon and Davison, 2008; Kersten, 2002). The kinetic aspects of bioaccumulation linked to the feeding strategy of each organisms also remain unaccounted for when using these approaches (see section 1.6).

#### 1.3.1. Quantifying exposure to trace elements: the importance of speciation

Simply put speciation comprises all the physico-chemical forms of an element that can be encountered in a given environment (Gagnon and Vigneault, 2013; Lespes et al., 2016), although much more formal and complex definitions are possible (de Paiva Magalhães et al., 2015). Metal speciation includes the existence of different chemical forms in the true solution and in association with environmental particles as well as their distribution among different environmental compartments (Fig. 10). Its importance in controlling elemental fate and bioavailability is well established (Gagnon and Vigneault, 2013; Mason, 2013) and theoretical frameworks such as the free ion activity model (FIAM) and the biotic ligand model (BLM) provide practical possibilities to link speciation with metal bioavailability, especially in aqueous matrices (Campbell and Fortin, 2013). While the term speciation is most often encountered with reference to trace elements (and I will stick to this class of chemicals in this section), concepts such as the distribution among compartments and the presence of different chemical forms in solution or in environmental particles are in principle applicable to any substance. Speciation is therefore a key factor for assessing the ecotoxicity of chemicals because it controls their bioavailability and, along with other biological and environmental factors, bioaccessibility (Fig. 9).

This subsection will specifically focus on trace element speciation in aqueous matrices because of both my research interests and of the increasing regulatory consideration that speciation is receiving in setting EQS for these chemicals. Current consensus in ecotoxicity testing requires that  $EC_x$  values are determined on the basis of analytically measured chemical concentrations in the exposure matrix (Harris et al., 2014; Moermond et al., 2016). The analytical verification step is important to ensure that actual exposure concentrations agree with expected ones and may alert the experimenter about contamination problems or concentration changes linked to elemental speciation during the tests. In this sense, studies relying on measurements of total exposure concentrations can still be informative about elemental ecotoxicity (Blinova et al., 2018; Galdiero et al., 2019). However, explicit consideration of speciation in ecotoxicity testing offers a superior approach to account for factors modifying bioavailability and hence ecotoxicity (Fig. 9). Because only a fraction of the total elemental concentration will be bioavailable, its measurement (or estimation) during ecotoxicological testing will facilitate the extrapolation of laboratory results to field situation or provide a basis to understand the differences among different studies (Gonzalez et al., 2014).

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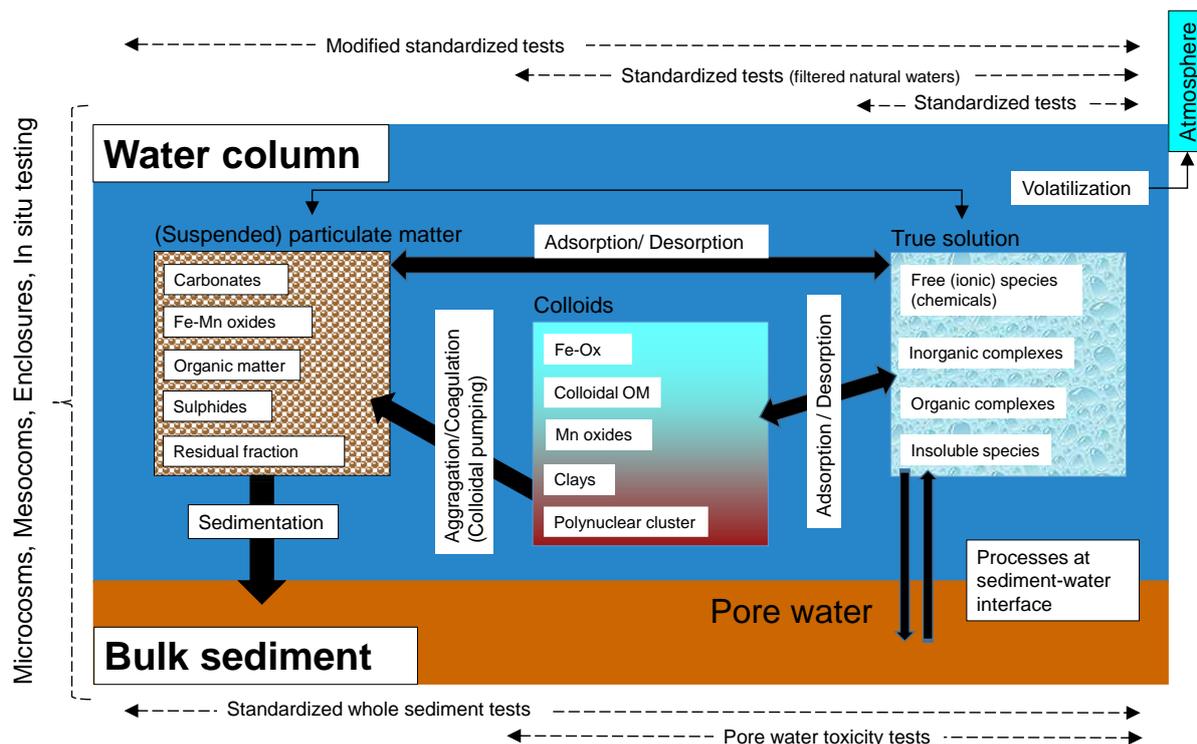


Figure 10. General overview of the distribution of different forms of a chemical (a.k.a. speciation) across environmental compartments, selected dynamic processes among compartments and the corresponding ecotoxicological approaches to assess chemical's effect according to their distribution among compartments. Compartments are not drawn to scale and arrow thickness is not proportional to the relative importance of the corresponding process. The list of possible chemical species and carrier phases is not exhaustive.

In standardized ecotoxicological media that do not contain colloidal or particulate matter, the bioavailable fraction usually includes free ionic species and, possibly, other species such as  $\text{AgCl}_0$ , Ag thiosulphate or Al hydroxides (Crémazy et al., 2013a; Fortin and Campbell, 2001; Santore et al., 2018). In an ideal situation, analytical tools would be available to accurately quantify the chemical species interacting with living organisms and establish the corresponding concentration–responses relationships. Some analytical techniques such as DGTs may be good proxies of the bioavailable elemental fraction, but do not necessarily measure a unique elemental species (Slaveykova et al., 2004; Zhang and Davison, 2015). Alternatively, it is possible to develop models of variable complexity to predict the bioavailable fraction and ecotoxicity of elements starting from relatively simply routine measurements (e.g., filterable elemental concentration) and a number of known toxicity and bioavailability modifying factors (TBMF) (Fig. 9). The neglected pitfalls of the membrane filtration technique have been described by several authors (Hall et al., 1996; Horowitz, 2013; Horowitz et al., 1996; Vignati et al., 2006) and could require consideration in large research programs to avoid the associated methodological artefacts. The effect of TBMF onto elemental bioavailability have also been recognised as early as the 1930s and hardness-corrected quality criteria for most metals were introduced by US EPA already during the 1980s (Adams et al., 2020). At present, the biotic ligand model (BLM) is the most widely used tool to incorporate TBMF derived from ecotoxicological research into environmental regulations on trace element ecotoxicity, although other approaches are in use or under consideration (Brix et al., 2020; Mebane et al., 2020).

The BLM includes chemical, physiological and toxicological components that allow the prediction of the bioavailable fraction at the site of action or, which is equivalent, of an

ecotoxicological threshold values specific for a given exposure matrix with specific hydrochemistry. It is however important to note that BLM reposes on the assumption of a chemical equilibrium in the exposure solution and, implicitly, at the biological interface that constitutes the biological ligand (Paquin et al., 2002). Addressing these aspects from a mechanistic point of view requires much more complex conceptual approaches and mathematical formalisms to understand elements dynamics at the interface between biological membranes and the exposure matrix (Duval and Rotureau, 2014). The prediction of elemental complexation by natural organic matter often constitutes another limit of the chemical speciation calculation performed during BLM applications (Town et al., 2019). Although much improvement is needed, it is important to acknowledge that BLM or other bioavailability model have unquestionably brought progress in hazard and risk assessment of trace elements. In particular, they allowed EQS to move from a generally fixed-value approach for all waters (apart from the above-mentioned hardness-based, empirical corrections) to the explicit consideration of the effects of several bioavailability and toxicity modifying factors.

Mechanistically-based or empirical bioavailability model are already available for a rather limited number of metals, namely: Ag, Al, Cd, Cu, Ni, Pb and Zn. Mebane et al. (2020) formulated twelve recommendations to develop and apply such models, ranging from attention to non-equilibrium conditions in ecotoxicological matrices to the existence of underappreciated toxicity modifying factors such as P in tests with algae. My own work on the ecotoxicity of trivalent Cr shows that speciation in standardized ecotoxicological test medium can rapidly change and lead to situations in which model organisms are simultaneously exposed to free ions, inorganic complexes and Cr-bearing nanoparticles (Aharchaou et al., 2018; Vignati et al., 2010). Allowing sufficient time for a test system to reach equilibrium can therefore be necessary in order to correctly characterize the ecotoxicity of elements forming insoluble species (Wilson et al., 2017) or in the presence of ligands such as DOC that can require several hours to equilibrate with the element of interest (Mebane et al., 2020). On the other hand, chemical discharges into the environment are not necessarily continuous and can show diel variability, notably due to wastewater treatment plant operation, or sudden changes due to extreme hydrological conditions. Regardless of the chosen approach, relying on measurements of total concentrations at the beginning of an ecotoxicological test appears insufficient to properly characterize the exposure-response relationship between chemicals and test organisms.

#### *1.4. Exposure to chemicals: a view from inside organisms*

When living in a given environment, each organism will ‘see’ different fractions of a chemical depending on the several factors controlling their bioaccessibility and bioavailability (Fig. 9). Even admitting that these fractions can be precisely known (Fig. 10), their actual measurement may remain a challenge. Viewing chemical bioaccessibility and bioavailability from the point of view of the exposed organisms rather than of chemical forms in exposure matrices represents a conceptual approach that may address some of these difficulties.

As a matter of fact, a chemical will exert an adverse biological effect only after reaching a critical accumulation threshold at a (specific) biologically active site (Fig. 9). Based on this hypothesis, McCarty and MacKay (1993) discussed the possibility of using a Critical Body Residue (CBR) approach or a Tissue Residue Approach (TRA) to establish safe concentrations of chemicals. In practice, safe levels and, where applicable, environmental quality standards are defined as concentrations accumulated by the organism(s) instead of concentrations (or forms) in environmental matrices (Fig. 10). Internal concentrations may then be used to derive safe levels of chemicals in the various environmental compartments; in line with the most used regulatory approaches (Escher and Hermens, 2004).

Sappington et al. (2010) reviewed the use of TRA in risk assessment and listed the following advantages: integration of exposure pathways, integration of temporal and spatial changes in

exposure levels, easier development of mixture models for chemicals with similar mode of action, easier analytical detection of chemicals occurring at very low concentrations in water, direct consideration of factors related to external bioavailability, and elimination of confounding factors for different environmental conditions. However, TRA does not necessarily account for differences in sensitivity among species and its success varies depending on factors such as the chemical properties of a substance and the actual possibility of establishing relationships between tissue residue and organism responses (Sappington et al., 2010). Application of a TRA makes most sense for those chemicals that enter organisms mainly via dietary exposure (section 1.6) and/or acting as nonspecific baseline toxicants. However, as it can be evinced from Fig. 9, chemical concentrations measured in whole organisms remain surrogates of the true effective dose present at the actual site of action and causing the observed response (McCarty et al., 2010).

In the case of trace elements, the TRA approach has been successfully applied for Se and methylmercury — MeHg (Adams et al., 2010). Indeed, Hg in the EU legislation and Se in the US legislation are regulated (or can be regulated) on the basis of internalized levels in the most relevant biological compartments of the food chain (EC, 2013; Suter, 2018). However, generalized application of TRA to other elements is hindered because organisms often resort to various strategies to control their internal concentration of essential or non-essential trace elements (Meador et al., 2008; Rainbow, 2002). Essential trace elements are required for normal metabolic processes and can occur at high concentration in critical cellular compartments as non-toxic complexes with proteins or other biomolecules (Finazzi et al., 2015). Organisms maintain a metabolically active pool of such elements and can either excrete their excess concentrations or store them element in detoxified forms. Non-essential elements can also be stored in detoxified form and excreted subsequently. The possible presence of inactive elemental forms inside one organism explains why high bioaccumulation does not necessarily result in ecotoxicity. In practical terms, this means that measuring the accumulated element concentration in the whole organisms is not necessarily indicative of metabolic exposure to that element. Adams et al. (2010) conclude that, at present, TRA application remains species- and site-specific; i.e., it suffers from the same limitations as the traditional approaches based on quality criteria in the other environmental matrices. However, Adams et al. (2010) also suggest research avenues for further general development of the approach, including the use of kinetic modelling to determine the uptake and excretion rate of elements.

Kinetics approaches are both interesting and promising for several reasons. First, comparison of measured accumulation with theoretical kinetic models can provide indications of the main route of exposure to a given metal for a given organism. For example, Camusso et al. (2012) showed that the oligochaete *Lumbriculus variegatus* accumulated Ni mainly from pore water, As, Cd, Cu, and Zn via sediment ingestion and Pb via both routes. Second, the use of DYMBAM (Dynamic Bioaccumulation Models), a typical kinetic approach, can allow the prediction of element accumulation by different organisms and explain while metal accumulation varies among organisms for similar exposure conditions (Luoma and Rainbow, 2005). Third, the rate of element uptake, rather than the tissue concentration, may ultimately determine the toxicity of an element toward (aquatic) organisms (Casado-Martinez et al., 2010a).

Another approach to improve the relationship between chemical accumulation and observed biological responses relies on experimental protocols for fractionating the total contaminant body burden into biologically active and inactive fraction(s). The degree of complexity of such approaches varies markedly. In the simplest cases, washing of the biological material to remove adsorbed contaminants (with *e.g.*, EDTA in the case of several trace elements) allows to quantify the internalized fraction of the contaminant that could reach the biological target site(s). However, as previously explained, the intracellular environment is a very complex

matrix where contaminants can be prevented from interacting with a critical biological target by a variety of mechanisms. More refined strategies try to identify the subcellular fractions containing the metabolically active (i.e., potentially ecotoxic) fraction of the total accumulated concentration (Aharchaou et al., 2017; Crémazy et al., 2013b; Gimbert et al., 2016). The approach can be further refined by identifying the macromolecules that interact with a given element contained in one of the metabolically active fractions (Aharchaou et al., 2017). It is particularly noteworthy that measurement of the elemental pool in the cytosolic fraction apparently allows a better estimation of the concentration that can be available to predators via trophic transfer (Cardon et al., 2020; Dumas and Hare, 2008; Reinfelder and Fisher, 1991).

Combining data from chemical speciation in the exposure matrices with internal accumulation in biologically active fractions (and better still at the site of action) clearly allows a better understanding of how concentration resulting in biological effect vary among organisms with different traits. Studies combining a detailed characterization of external exposure and internal accumulation are therefore necessary, especially for those elements (or chemicals) whose effects are not yet successfully modelled using BLM or other conceptual frameworks. However, establishing environmental quality standards or guidelines in biological matrices using CBR/TRA does not eliminate the need to know what are the safe concentrations of a chemical in the environment. Even in the best case of very similar TRA across a large number of organisms, the concentration in the environment resulting in that TRA will vary across aquatic ecosystems because of the presence of modifying factors such as those described in Figs. 9 and 10. In other words, application of TRA for regulatory purposes can potentially simplify compliance monitoring by integrating temporal and spatial variability of chemical contamination in water or sediment into a single measurement in biota. On the other hand, TRA does not completely eliminate the need for a detailed study of chemical's speciation in the exposure medium. In cases of regulatory non-compliance, it may indeed be necessary to estimate the bioavailable environmental concentration of the chemical resulting in the observed TRA. Such estimated external concentration can then be used to maintain chemical releases at a safe level (or as a basis to enforce a ban on that chemical).

The example of Hg under the directive EU 2013/39 is particularly instructive. The strength of the biota-based EQS is that it provides a scientifically sound way to simplify the routine monitoring of a priority substance by automatically taking into account (albeit as a black box) the issues linked to bioaccessibility, bioavailability and partly biological availability (Fig. 9). Adoption of a biota-based EQS also offers methodological advantages because it eliminates the need for the accurate and precise determination of total Hg and MeHg which can be particularly challenging at the (sub)nM levels typically found in natural environments (Coquery et al., 2005; Hall et al., 2002). However, the Hg concentration that will result in an exceedance of the EQS value ( $20 \mu\text{g kg}^{-1}$ ) will be strongly dependent on the Hg fraction that is methylated, because methylmercury is much more bioavailable than inorganic Hg. Unluckily, the degree of Hg methylation depends on a large number of environmental factors such as pH, trophic states and microbially-mediated processes (Fitzgerald et al., 2007). Furthermore, compliance with the biota-based EQS for Hg remains extremely problematic across Europe (EEA, 2018). Whether this is indicative of widespread Hg contamination in EU waters or of the need to refine the current EQS is a matter of debate (Gerson et al., 2020; Vignati et al., 2013a). Overall, understanding exposure to chemicals from the point of view of the organisms is as important as studying chemical behaviour in the environment (Wang, 2011). Both approaches are therefore necessary to further increase the reliability and relevance of ecotoxicological data for both research and regulatory purposes.

### 1.5. What is your favourite cocktail?

Ecotoxicological research is often performed on individual chemicals which is partly linked to the need to regulate some of them (the priority substances) via specific environmental quality criteria (although some mixtures with a typical composition — *e.g.*, PAH, PCB, TCDD — are actually regulated using a single standard for the whole mixture). Under real-field conditions, situations in which one single substance drives the organisms' responses are possible. However, effects originating from the simultaneous exposure to mixtures of chemicals and, possibly, non chemical stressors are often the norm. Standardized ecotoxicological tests can identify the possible overall biological effects of complex mixtures of chemicals (*e.g.*, toxic effects effluents or contaminated sediments), but without providing information on the actual chemicals contained in the tested matrix and their contribution to the observed effects. Such information can be gathered using Toxicity Identification Evaluation (TIE) and Effect Directed Analysis (EDA) to identify the specific (group of) chemicals of concern (Brack et al., 2017; Burgess et al., 2013). Nonetheless, a definite evaluation of the risk associated with the mixture will still require knowledge of the corresponding concentration–response relationships for the chemicals of concern, implying that: i) all substances of concern contained in the effluent can be identified and quantified analytically, and ii) their individual effects in standard conditions are known. This is often not the case, given that only a fraction of the chemicals on the market is sufficiently characterized for the corresponding (eco)toxicological properties (EEA, 2019) although substantial progress is being made in this sense (ECHA, 2021).

The study of mixture effects can be undertaken with at least three different purposes: the mechanistic understanding of the interactions among chemicals, the prognostic risk assessment of specific mixtures or the diagnostic (retrospective) risk management of mixtures encountered in real field situations (Posthuma et al., 2008). A series of reviews recently discussed the state-of-the-art and the future challenges in diagnostic mixture risk assessment and management (Escher et al., 2020; Johnson et al., 2020; Vermeulen et al., 2020; Zimmerman et al., 2020). Non-target chemical analysis of environmental matrices by high-resolution mass spectrometry can be used in combination with EDA and TIE to estimate mixture toxicity and apportion the observed effects between identified and unidentified chemicals (Escher et al., 2020). Depending on the selected biological response, the fraction of toxicity linked to unidentified chemicals may be larger than 90% (Neale et al., 2017). This inability to identify the actual chemicals causing toxicity may be surprising at first; considering that back in 1994 (when the number of chemical in the environment can be reasonably assumed to have been lower), Hendriks and co-authors already reported that a major part of compounds responsible for the toxicity of river Rhine waters remained unidentified (Hendriks et al., 1994). It is therefore important to acknowledge that, when used in an appropriate framework, the available tools can successfully track a specific substance responsible for large-scale effects. Specifically, Tian et al. (2021) used TIE/EDA to successfully demonstrate that a single, highly toxic ( $EC_{50} = 1 \mu\text{g L}^{-1}$ ) chemical derived from the wearing of tire rubber is jeopardizing costly habitat restoration efforts to restore Coho Salmon population in US waters. Although mixture ecotoxicity often deals with similar situations involving a large (and increasing) number of organic chemicals, their metabolites and degradation products, exposure to mixtures of naturally occurring trace elements can also be a matter of concern for human and environmental health (Gwenzi, 2020).

The most important distinction in mixture assessment is whether chemicals share a similar mode of action and whether they show interactive effects or not. The answer to these two questions will determine the model approach to be used when interpreting mixture effects (Table 5). In addition, we should consider that mixtures can be asynchronous in time and space, both aspects requiring more attention during laboratory studies. Nowadays it remains logistically unfeasible to go beyond the study of quaternary mixtures (Nys et al., 2018) and the criteria to choose which mixtures to characterize for their ecotoxicity become therefore

extremely important. Even accepting that only 5000 chemicals may be of global concern (Landrigan et al., 2018; Vermeulen et al., 2020), a direct assessment of all their possible combination is obviously impossible. Unluckily, the choice of model chemical (mixtures) for ecotoxicological research is not always done based on objective criteria of environmental relevance, but can be guided by novelty and scientific popularity. ‘Newly released substances’ for which there is a lack of information will usually see a surge in corresponding ecotoxicological studies (Johnson et al., 2020), although ecotoxicological research is resilient against such type of bias in the long-term. This phenomenon may however affect the priorities of funding agencies that are, at least in part, willing to support research perceived as relevant to answer pressing societal expectations. I do not mean to imply that concern over *popular substances* is systematically unjustified, but societal expectations can be driven by extra-scientific factors, notably the bandwagon effect (Daughton, 2014), that should be taken into account. (Data-poor) substances getting a good media-coverage are indeed more likely to get funding and *popular substances* may end up being overly studied (at least with regard to some aspects) with the loss of scientific time and resources. For example, the editorial authored by Borja and Elliott (2019) in the Marine Pollution Bulletin argued that papers reporting the occurrence of microplastics could already be considered uninteresting (from a fundamental research point-of-view) given the ubiquitous distribution of such chemicals. At the same time, more research efforts should be carried out to rapidly characterize the extent and likelihood of their impacts in natural environments (see Figs. 6 and 7).

Table 5. Possible frameworks to interpret mixture effects of chemicals according to the existence of interactive effects and knowledge of their mode of actions. Based on Posthuma et al. (2008).

Effect	Mode of action	
	Similar	Dissimilar
<i>Non interactive</i>	Simple similar action	Simple independent action
	Model: concentration addition	Model: response addition
<i>Interactive</i>	Complex similar action	Dependent action
	Model: empirical	Model: empirical

Relatively well-characterized legacy chemicals for which validated models exist are attractive to scientists because of the possibility to bring more or less incremental contributions to the current body of knowledge. This is driven by a genuine desire to contribute research outputs of the highest possible quality, but partly also by the need to maintain the level of (sometimes unreasonably high) productivity required by the current research industry (see section 1.8). As a consequence, research on typical, but less popular, mixtures and their components can be slowed down even when it has potentially worldwide implications (Gwenzi, 2020; Johnson et al., 2020). Finally, understanding mixture ecotoxicity often assumes the availability of solid data for the individual components of the mixture (Nys et al., 2018). Erroneous or insufficiently documented information for a mixture component will therefore affect the assessments of the mixture itself. In the case of trace elements, improper characterization of their ecotoxicological potential can often arise from an insufficient consideration of their speciation during standardized ecotoxicological tests (Aharchaou et al., 2018; Gonzalez et al., 2014). In the context of the present HDR, I will specifically focus on a representative case of a particular mixture linked to elements that simultaneously occur under more than one redox state in natural environments (section 2.5)

### 1.6. Do you live here? Understanding the importance of routes of exposure

In the current regulatory context, most testing is carried out using aqueous exposures in media of known standard composition. For risk assessment purposes, results from these tests are then extrapolated to aqueous matrices of variable composition. This approach has led to major advances in the incorporation of important scientific principles into environmental regulations; notably the development of bioavailability-based EQS for Ni and Pb in the EU legislation and of the corresponding user-friendly tools for their application (Peters et al., 2011; Peters et al., 2018). Depending on an organism's ecological traits, exposure to chemicals in the field can however occur via routes other than the aqueous phase; e.g., ingestion of suspended particles for filter feeders, bed sediments for benthic organisms in soft substrates, plant debris for organisms living in headstreams with rocky substrates and preys. At a larger scale, the transfer of chemicals from aquatic to terrestrial food-webs via the emergence of aerial insects with an aquatic larval stage could also be considered as a particular route of exposure (Becker et al., 2018; Tremblay et al., 1998). Given the possible developments of this HDR project, the present section will mainly focus on the role of (suspended) sediment ingestion as an additional source of exposure to chemicals for filter and deposit feeders. Particle ingestion satisfies the nutritional requirements of these organisms, but potentially exposes them to sediment-bound chemicals during the same process. It is therefore necessary to understand when considering sediment/particulate exposure becomes necessary to guarantee sufficient environmental realism in ecotoxicological studies and risk assessment evaluations. The following two examples using Hg and PCP as model chemicals well illustrate the fundamental and practical importance of such considerations.

In the EU, Hg is currently regulated using a biota-based environmental quality standard (EQS). I have already explained and supported the scientific superiority of this approach when discussing my past research activities (Part II, section 4). However, a sediment-based approach to Hg monitoring and assessment is also possible. The [substance data sheet for mercury](#), which constitutes the basis for deriving the EQS proposed at the European levels in Directives 2008/105 later superseded by 2013/39, determines a water-based EQS using a species sensitivity distribution approach. The water-based EQS is then combined with a partition coefficient of  $10^5$  and an assessment factor of ten to derive a tentative quality standard for sediments of  $670 \mu\text{g kg}^{-1}$  dry weight. This value appears sufficiently protective when compared with the few standard ecotoxicological tests available for benthic organisms exposed to  $\text{HgCl}_2$  (NOEC range 2–930  $\text{mg kg}^{-1}$  d.w.), but is close to potentially toxic levels recently determined for the oligochaete *Tubifex tubifex* ( $\text{EC}_{20} = 1.12 \text{ mg kg}^{-1}$ ) and for sensitive benthic taxa in Northern Spain rivers (threshold value  $0.9 \text{ mg kg}^{-1}$ ). This information can be found in Méndez-Fernández et al. (2019). Overall, the tentative EQS for Hg in sediments may provide a good 1<sup>st</sup> tier screening for adverse Hg effects on sediment-ingesting benthic organisms, but could require adjustment to protect sensitive species. In any case, I must again stress that the possible direct toxic effects must not turn the attention away from the bioaccumulation/biomagnification issue. Standardized sediment toxicity tests with benthic organism often offer the possibility to assess Hg accumulation; a practice that should be strongly encouraged to provide two environmentally relevant outputs with one single standardized test.

The [case of PCP](#) tells a different story. Application of the EqP approach as described above for Hg provides an indicative sediment-based EQS of  $119 \mu\text{g PCP per kg}$  of sediment on a dry weight basis. Bettinetti et al. (2018) tested the ecotoxicity of PCP to three representative benthic organisms (*C. riparius*, *L. variegatus* and *T. tubifex*) and found that an EQS of  $30 \mu\text{g kg}^{-1}$  may be required to protect the most sensitive species; *i.e.*, the tentative standard derived from aqueous values using the EqP may be underprotective. These examples are not meant to disqualify the scientific robustness of current risk assessment procedures, but simply to stress the need for a minimum of experimental verification of model predictions; especially when

extrapolating between different environmental media such as the water column and the bed sediments.

### 1.6.1. Bioaccessibility as a driving concept in the study of routes of exposure

For a given chemical, the relative importance of different routes of exposure depends on the physico-chemical properties of the substance itself and on biological parameters including organism ecology, physiology and life stage (Fig. 11). The ecotoxicological relevance of biological parameters to reconcile standardization and environmental realism is well summarized by the concept of *bioaccessibility* (Dong et al., 2016; Semple et al., 2004). *Bioaccessibility* defines the pool of a chemical that is *potentially* available to living organisms. As stated by Semple et al. (2004) “Bioaccessibility encompasses what is actually bioavailable (here and) now plus what is potentially bioavailable”. Thus, the bioaccessible fraction cannot be reached by the organism here and now, but may become bioavailable following changes in environmental or biological factors (Fig. 9). The bioaccessibility concept also considers kinetics and dynamic aspects that are not strictly included in the bioavailability concept (Fig. 9). Finally, and broadly speaking, bioaccessibility also encompasses the possible differences in exposure to chemicals among life stages of organisms that have a planktonic larval stage and a benthic adult stage (e.g., *Dreissena polymorpha* and many bivalves) or a benthic larval stage and a terrestrial/aerial adult stage (e.g., chironomids and many aquatic insects).

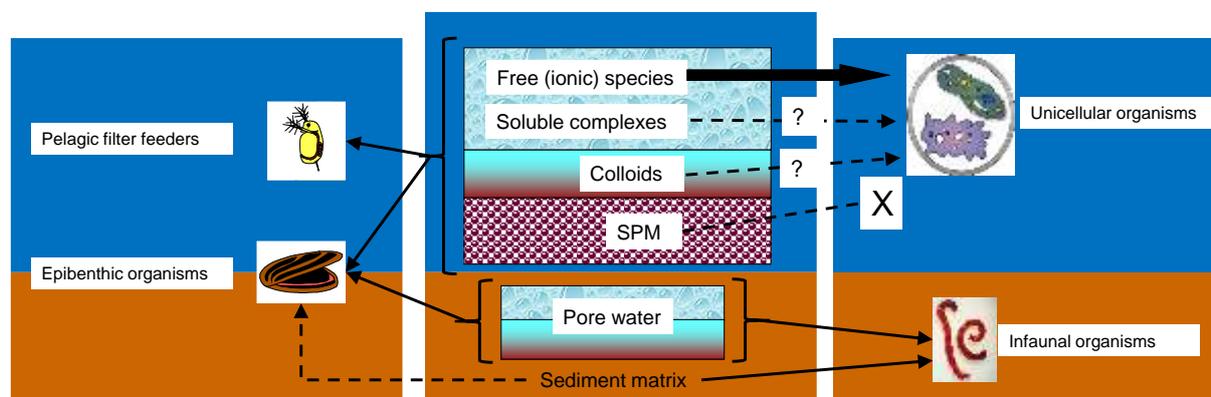


Figure 11. Overview of the bioaccessible fractions of a chemical for different types of aquatic organisms. Full arrows indicate that a given fraction is usually bioaccessible for the type of organism under consideration. Dotted lines with a question mark indicate that a fraction may be more or less bioaccessible depending on environmental conditions. SPM (suspended particulate matter) is not bioaccessible to unicellular organisms. See figure 10 for more details about the chemical heterogeneity of the different compartments.

In terms of ecotoxicological relevance, bioaccessibility acknowledges that, under field conditions, organisms may be able to reach contaminant pools that are simply not provided during standardized tests or to escape contaminated sediment patches to avoid exposure to contaminants. Some bioaccessibility issues in standardized tests can be easily addressed by considering the ecology of the biological model used in the laboratory, thus avoiding erroneous experimental set-ups (Harris et al., 2014). However, understanding bioaccessibility (and associated bioavailability) in the case of exposure to particle-bound contaminants remains

challenging due to the heterogeneous nature of the particles themselves (Fig. 10). Considering both filter and deposit feeders, bioaccessible pools may originate from the (anoxic) pore waters, oxic (and sub-oxic) overlying waters, the water column, a fraction of the total chemical concentration bound to the oxic and anoxic particulate matrix and, where applicable, ingestion of prey organisms (Batley et al., 2005) (Fig. 11). The relative contribution of each pool to the chemical uptake by an organisms can also vary following fluctuations in master variables such as pH and degree of oxygen saturation, hydrological and limnological factors (Fig. 9).

#### 1.6.2. Challenges in integrating bioaccessibility issues in standardized tests

Standardized whole-sediment ecotoxicological tests can reproduce only very partially the complex bioaccessibility pattern typical of real-world situations. The classical approach of spiking artificial sediments with the chemical of interest, albeit viable in terms of mutually accepted procedures and reliability, does not always correctly reproduce contaminant distribution between the solid phase and interstitial waters with possible repercussions on responses of the exposed organisms (Simpson et al., 2004; Wang et al., 2004). The use of natural sediments instead of artificial ones, albeit attractive, is suitable for specific case studies, but not for the derivation of guidelines to be adopted at the national or international level. Furthermore, simple handling procedures such as homogenization of field-collected sediments before splitting into test vessels can render more bioaccessible and bioavailable some chemical pools that were not necessarily so under the original field conditions (Ferrari et al., 2014; Ferrari et al., 2019). In the specific case of trace elements, this may be due to re-oxygenation of anoxic pore waters and sediments during sampling and handling (Williamson and Burgess, 2003). More generally, in many field situations, relative clean, recent sediments may have topped older sediments with higher levels of several legacy chemicals (Guilizzoni et al., 2012). Grab sampling and homogenization before laboratory tests can mix layers with a different degree of chemicals content and put model laboratory organisms in contact with chemicals that are *de facto* not bioaccessible in the field. The patchy distribution of contaminants in contaminated sediments can also provide zones of suitable living conditions even in areas characterized by an overall high level of contamination. Such situations strongly contrasts with the typical experimental set-up of laboratory sediment toxicity tests where a single spike of contaminant is added to sediments that undergo stabilization and homogenization before addition of test organisms. Finally, depending on the relative time scales of contaminant accumulation in sediments and generation time of organisms, (multigenerational) acclimation and adaptation can occur in the field (Fischer et al., 2013). Despite all these complexities, approaches are available to incorporate bioaccessibility (and bioavailability) issues in the hazard and risk assessment of sediment-bound chemicals.

Bioaccessibility issues also apply to some aspects of aqueous (metal) speciation that are not addressed by the current approaches based on filterable concentrations (section 1.3). For example, bioaccessible colloidally-bound elements may become bioavailable to unicellular organisms following desorption of chemicals due to *e.g.*, changes in ionic strength after mixing of different water masses (Fig. 10 and 11). Changes in ionic strength are not necessarily limited to estuarine environments, but can be relevant at the confluence between a major river and a polluted tributary (Vignati et al., 2005c) or even downstream effluent discharges. The reverse process of adsorption of soluble chemicals onto colloids (or particles) can obviously occur (Fig. 10). In this case, a fraction of the bioavailable chemical pool is transferred to the bioaccessible pool for a variable time. Colloidal pumping (Honeyman and Santschi, 1989; 1991) can also actively transfer elements from the truly dissolved phase to the particulate phase and thence to sediments, thus modifying the pool of bioaccessible contaminants for both aquatic and benthic organisms. For practical purposes, the BLM seems to be able to predict sufficiently well the

effect of dissolved organic carbon on the bioavailability of trace elements (Peters et al., 2011), but it does not explicitly consider the presence of colloidal and soluble pools of chemicals. The presence of colloids affects the bioaccessibility of chemicals in different ways for different organisms including unicellular algae (Koukal et al., 2003; Slaveykova et al., 2003) and filter feeders (Vignati et al., 2005c). Overall, properly addressing bioaccessibility in the standardized laboratory context appears as an important step to increase the relevance of laboratory tools and reconcile them with real field situations.

Biodynamic modelling approaches (DYMBAM) are a conceptually convenient way to estimate the contribution of aqueous and dietary routes, addressing some bioaccessibility issues. This approach usually integrates aqueous routes with exposure via ingestion of suspended particulate matter of bottom sediments, but could in principle apply to the ingestion of colloids provided that they can be isolated and characterized for the relevant parameters. One mathematical formulation of the DYMBAM is as follows:

$$C_{biota} = \frac{k_u \cdot C_w}{k_{ew} + g} + \frac{AE \cdot IR \cdot C_{sed}}{k_{es} + g}$$

Where,  $C_{biota}$  is the chemical concentrations in biota at steady state (in  $\mu\text{g g}^{-1}$  d.w.);  $k_u$  is the uptake rate constant from the aqueous phase (in  $\text{L g}^{-1} \text{d}^{-1}$ );  $C_w$  is the aqueous filterable concentration (in  $\mu\text{g L}^{-1}$ ),  $k_{ew}$  and  $k_{es}$  are the efflux rate constants of the chemical following uptake from the aqueous filterable phase and from sediments (or food), respectively (both in  $\text{d}^{-1}$ );  $AE$  is the chemical assimilation efficiency from particles (*e.g.*, sediments; in % of the total content);  $IR$  is the food ingestion rate (in  $\mu\text{g g}^{-1}$ ),  $C_s$  is the chemical content in particles ( $\mu\text{g g}^{-1}$ ) and  $g$  is the growth rate constant (in  $\text{d}^{-1}$ ). Model parameters can be determined by exposing filter- or deposit feeders to radiolabelled chemicals via both exposure routes (Baumann and Fisher, 2011; Casado-Martinez et al., 2010b; Wang and Fisher, 1999; Wang et al., 1997). Once model parameters are known, chemical accumulation by the organisms can be estimated from the simple measurements of the chemical concentrations in water and sediments. On the other hand, model parameters are usually organisms-specific and it is clear that all the possible organism/chemical combinations cannot be covered using this model. Nonetheless, DYMBAM provides a theoretical framework to support the importance, or even the predominance, of dietary exposure to contaminants for particle-ingesting organisms (Baumann and Fisher, 2011; Croteau et al., 2017).

### 1.7. Where will you be in 2100? Ecotoxicology in the context of climate change

The 2020 World Economic Forum Report classifies ‘climate change action failure’ as the second global risk in terms of likelihood and as the first one in terms of impacts. Related environmental global risks such as extreme weather, biodiversity loss, natural disasters and water crisis consistently figure among the corresponding top 5 since 2016 or earlier (World Economic Forum, 2020). Climate change-related risks can have several socio-economical consequences that fall outside the realm of ecotoxicology. However, related phenomena such as changes in hydrological patterns, more marked temperature extremes, species migration, (sea)water acidification and altered global carbon cycle can all potentially affect the chemicals’ environmental fate and ecotoxicology. According to the 5<sup>th</sup> assessment report of the Intergovernmental Panel on Climate Change (IPCC), the likelihood of changes in climate systems phenomena is virtually certain (99–100% likelihood) for *e.g.*, an increase in global surface air temperature, the number of hot days and night as well as ocean acidification (Burkett et al., 2014). Given such trends, organisms (will) more frequently experience previously unknown environments or living conditions (Chapman, 2011; Woolway et al., 2021) in which they have to cope with combined stress from non-chemical and chemical factors (Fig. 9).

Depending on the actual rate of changes, some components of ecosystems may be able to adapt to the ongoing or forthcoming changes over the course of the 21<sup>st</sup> century. However, the increase in the amplitude of daily fluctuation for some master variables (*e.g.*, temperature) may already expose organisms to conditions that are at the limits of their physiological tolerance.

Altered temperature, precipitation and salinity patterns also have important and variable consequences on chemicals' behaviour and fate (Noyes et al., 2009). Higher temperatures may facilitate the degradation of persistent organic chemicals, but may also release legacy pollutants such as DDT from mountain glaciers, polar ice caps and permafrost back into the local and global biogeochemical cycles (Bettinetti et al., 2016; Bettinetti et al., 2008). In the case of intentionally applied pesticides, higher degradation rates are in principle beneficial, provided that additional applications for pest control purposes will not be required and that applied quantities will be adjusted to avoid excess residues on edible crops (Noyes et al., 2009; Xi et al., 2021). On the other hand, increased degradation of dissolved organic carbon at higher temperatures could make chemicals more bioavailable. Higher temperatures will also shift the thermodynamic equilibria of chemicals, thus modifying their partitioning and, in fine, their bioaccessibility and bioavailability (Figs. 9, 10 and 11). The biological outcome of such physico-chemical processes will ultimately depend on the interaction between changing contaminant behaviour and organism-specific metabolic changes at higher temperatures (Zhang et al., 2020).

Higher salinities linked to increased evaporation, drought and seawater intrusion are also foreseeable consequences of climate change and may lead to changes in their bioavailability following salting out (Turner, 2003). At the opposite, extreme precipitation and increased ice/snow melt can cause salinity to decrease; at least temporally and locally. Changes in salinity also influence the stability of colloids also with possible consequences on chemicals' bioaccessibility and bioavailability (Filella and Buffle, 1993; Koukal et al., 2007). The net outcome of these competing processes in terms of chemicals' behaviour and fate may be predicted by evaluating the conservative *vs.* non conservative behaviour of a chemical along a salinity (or conductivity) gradient (Millward and Turner, 1995; Vignati et al., 2005c). However, the corresponding ecotoxicological outcome will equally depend on the effects of such salinity changes on organisms' physiology and requires further investigation.

Ocean acidification is another virtually certain effect of climate change (Burkett et al., 2014). Most interestingly, it appears that continental waters could also experience a certain degree of acidification (Hasler et al., 2017; Phillips et al., 2015), although its extent will vary depending on the buffering capacity of the rocks constituting a given catchment. Changes in pH can alter the speciation and ecotoxicity of chemicals in several ways. In the case of trace elements occurring as cationic species, a decrease in pH is expected to cause desorption from environmental particles (suspended particulate matter and colloids) toward the truly dissolved phase along with an increase in the concentration of free metal ions (Millero et al., 2009; Stockdale et al., 2016). Free metal ions are considered as the most bioavailable chemical species so that, in principle, water acidification could lead to an increase in toxicity. Once again, the interplay between elemental speciation in the bulk solution, processes at the biological interfaces and the possible direct effects of pH changes on the organisms' physiology will determine the ability of the different species to cope with chemical stressors under a changing climate.

Increased temperatures will decrease oxygen solubility in water, with potential strong effects on the Fe, Mn and organic carbon cycles during the early diagenetic processes at the sediment-water interface. Reductions in O<sub>2</sub> levels may become particularly marked in large reservoirs, eutrophic water bodies and deep lakes due to high primary productivity or incomplete water mixing. Dissolution of Fe oxyhydroxides or Mn oxides in anoxic conditions can release trace elements back into the water column and change their environmental mobility (Jaiswal and

Pandey, 2020). The released elements may become partly available to biota due to the spatial redox heterogeneity of the sedimentary environment.

Given all these considerations, incorporating climate-change issues into risk assessment frameworks for chemicals should become a mandatory step, especially when management decisions can have long-term (>50 years) consequences; *e.g.*, dam construction (or removal) and mining activities (Landis et al., 2013). With specific regard to the interactions between climate change and chemicals, Landis et al. (2013) also highlight the need to develop approaches that better consider the probabilistic nature of both stressors and effects in environmental risk assessment programs (ERA). Standardised ecotoxicological procedures are just one of the many components of ERA, but they can be framed to answer specific questions linked to changing risks from chemicals during prolonged droughts or following an increase in the number and intensity of chemical pulses associated with run-off during storm events.

During extended drought periods (and assuming constant discharges from point sources), dissolved chemicals discharged via wastewater treatment plants or industrial effluents may occur at higher concentrations for longer periods (Fig. 12). Because  $EC_x$  for many chemicals decrease with increasing exposure time until a threshold concentration is reached (Walker et al., 1996), prolonged droughts may induce chemical stress to biota even at concentrations normally considered as safe under a typical hydrological pattern. Furthermore, droughts are usually associated with additional stressors due to changes in master variables such as T, pH, water dissolved oxygen and salinity; all of which are usually kept constant (and at optimal physiological values) during standard ecotoxicological testing. Calculation procedures for chemical EQS or benchmarks do include safety factors to account for non-optimal conditions in the field compared with standard laboratory exposure. However, such factors may prove inadequate under the increasingly fluctuating stress conditions that organism will encounter in the field. Establishing threshold concentrations for chemicals under reasonably probable future field scenarios therefore requires attention by both scientists and stakeholders.

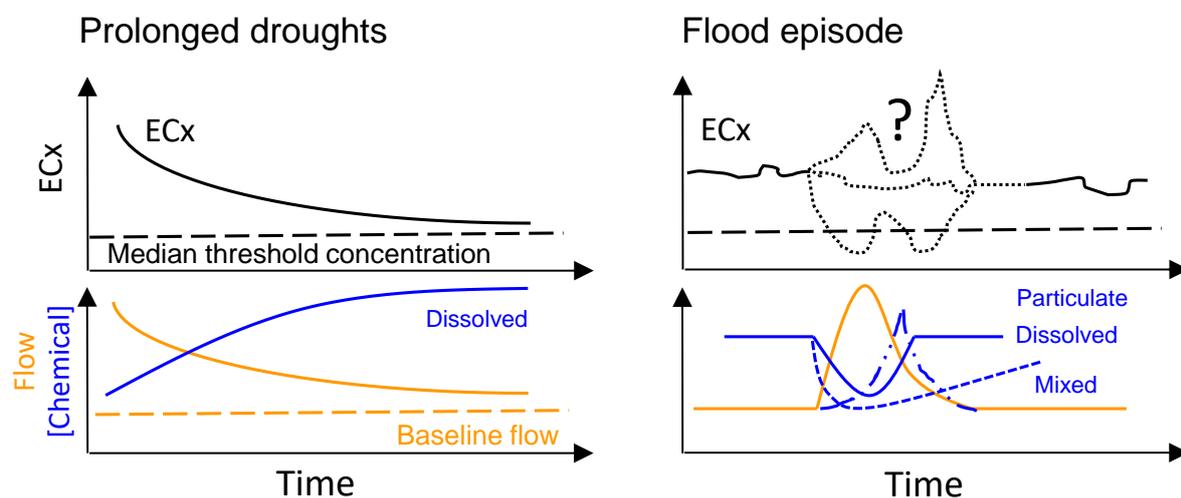


Figure 12. Bottom panels: relationship between flow and chemical concentrations during drought periods (left) or a flood pulse (right). Top panels: corresponding possible changes in  $EC_x$  values under the two hydrological conditions (see text for more details).

Extreme precipitation events can also markedly increase the concentration of several chemicals in water and soils (Camusso et al., 1999; Noyes et al., 2009), thus resulting in a higher number of contamination pulses linked to run-off from contaminated land or sites. More

frequent pulse-exposure to chemicals may not allow to biota sufficient time to recover between two subsequent peak-exposures. As reviewed by Luís de Sá Salomão et al. (2020), simulating pulse exposures is already a reality in ecotoxicology and may be a useful tool in evaluating chemical risks under fluctuating chemical levels (Fig. 12). Chemical behaviour during floods is however less clear cut than during droughts (Fig. 12) and includes dilution of dissolved chemicals originating from point sources, linear or exponential increase of particle-bound chemicals, or again complex patterns for chemicals with mixed sources (Davis and Zobrist, 1978). Furthermore, changes in contaminant partitioning and, consequently, bioavailability during the rapidly changing hydrological conditions typical of floods can be chemical-specific and may require case-by-base studies

As it is often the case in reconciling laboratory and field results, understanding the changes in chemicals' effects during flood will require a careful combination of field and laboratory work (Vignati et al., 2007). Standardized tests may not necessarily be the most relevant tool to evaluate the combined effects of several temporally and spatially fluctuating variables. However, once the most likely exposure scenarios are identified, solid ecotoxicological data for chemicals of known or foreseeable concern can be generated and the chemicals ranked according to the associated potential risks. Identifying the most problematic stressors under a certain climate scenario also helps in prioritising chemicals (and stressors) needing 'mixture' assessment (section 1.5).

### *1.8. Reliable and relevant ecotoxicological research in the current scientific context*

"It is not uncommon for honest individuals to suppress memories of inconvenient facts" (Bishop, 2020).

According to the Official Journal of the French Republic, the delivery of a HDR diploma recognizes the high scientific level of the candidate, the originality of his/her research activity in a sufficiently broad scientific domain, and his/her ability to develop an independent research strategy and to effectively tutor junior researchers ([Arrêté du 23 novembre 1988](#)). These qualifying attributes assume that the HDR candidate personally follows, shares with colleagues, and teaches to students and junior scientists a certain type of research ethics that has become increasingly under threat. Apart from serious cases of research misconduct (see for example the blog Retraction Watch, [www.retractionwatch.com](http://www.retractionwatch.com)), the use of questionable research practices such as omitting variables from statistical analysis or switching statistical methods to reach a predefined threshold of significance seems to be on the rise (Fraser et al., 2018). It is telling that qualitative comments from respondents to the questionnaire of Fraser et al. (2018) were unanimous in pointing out how the current research industry is leading scientist to increasingly succumb to the use of these practices.

In the present document, I define the research industry as the present functioning of Science in terms of its expectations for regular, innovative, high-impact scientific output and constant (successful) applications for additional research funding. Important collateral tasks such as engagement with stakeholders and the general public, research management meetings, tutoring and science vulgarisation also add to an ever increasing list of expected *industrial scientific products* to be included in periodic individual and institutional evaluation. It is not at all inappropriate to include the obtainment of an HDR diploma among the expected research products' of individuals and institutions. At the individual level, the HDR determines who can officially direct a PhD thesis and, officially or unofficially, be eligible for career advancements. At the institutional level, the total number of HDR-holders determines for example the number of PhD scholarships that a laboratory can receive from the Ministry of Research and Education each year. I would be pleased to invite the interested members of the present committee to write

about our opinions on the actual strength and weaknesses of this *de facto* mandatory career step for French researchers.

Back to the rising incidence of questionable practices, it is obviously rejoicing to see that cases of serious misconduct dictated by pure self-interest remain comparatively rare. However, the continuous piling up of different responsibilities takes more and more time away from the core task of the researcher: testing hypotheses to develop or correct (existing) theories capable of explaining natural and social phenomena. Scientist with teaching duties pay an additional tribute to the research industry in that they are paid to teach, but are evaluated (mainly) on the basis of their research output. It is therefore no wonder that some people may be tempted to take shortcuts to fulfil their quota of industrial research production, especially when their career or scientific survival are at stake (*e.g.*, tenure-track review or renewal of — the umpteenth — temporary contract are approaching fast). On the other hand, it is equally important to recognize that honest mistakes do occur, especially when people are under pressure to deliver products as quickly as possible for the above-mentioned reasons. In such cases, the best thing is simply to admit errors and openly discuss their [causes and consequences](#). On the other hand, apparently innocent or widely accepted research practices can also contribute to create an unhealthy research climate in laboratories or cause bias in research. A [special issue on laboratory health](#) published by Nature in 2018 illustrates that criticism of the productivity mantras embedded in the research industry (and alas ever increasingly in institutional culture) and an objective and honest appraisal of our own shortcomings as humans are both necessary to avoid conscious and unconscious bias.

Practices such as salami slicing (*i.e.*, publishing a complex research in a series of least-publishable units) or p-hacking are examples of scientific misconduct linked to the perverse functioning of the research industry; in particular its expectations in terms of both quantity and perceived quality (*i.e.*, journal impact factors) of overall productivity. Welcome to the *publish or perish* mantra with its upgraded versions considering output frequency (*Publish frequently or Perish*) and/or perceived impact (factor) (*Publish frequently in high impact journal or Perish*). In a chapter of her humorous yet disenchanting [monography on the malfunctioning of the scientific enterprise](#), Elodie Sabin-Teyssier proposes a more radical way to describe this race to who ‘sells’ more science: “*Rubbish or Perish*”. Ms. Sabin-Teyssier specifically describes how the proliferation of predatory journals is swamping readers with poor quality articles and, above all, allowing scientists and institutions to manipulate their metrics. Mario Biagioli spoke about the ‘Impact or Perish’ culture, suggesting that any numerical indicator to allegedly evaluate scientific quality is bound to be abused (Biagioli, 2016). Why people keep inventing new indicators instead of finding ways to get rid of them remains a difficult question to answer, though, and alternatives are not necessarily easy to find (Tregoning, 2018).

Moving a step farther, Smaldino and McElreath (2016) warn about the natural selection of bad science that is being fuelled by the above-mentioned productivity mantras. Because most of the scientific endeavour is evaluated using productivity indexes, those who produce more eventually get to have more of everything, including (under)graduate students and post-docs, valuable and increasingly-rare *commodities*, who will adopt similar strategies for their own survival. (And who are we, tenure middle- or advanced-career fellows to blame them?). Following all these considerations, my personal conclusion after more than 20 years in research is that this productivity mantras are ultimately rooted in well...bullshit. Based on the eponymous essay of Harry Frankfurt ‘On Bullshit’ (Frankfurt, 2005) and on McCarthy et al. (2020), I define bullshit as a statement (or a series of statements) that cannot be checked for its grounds into reality and whose sole purpose is to advance someone’s agenda and/or to protect someone’s interests. At the time of writing (early 2021), the new law that will shape the French research landscape for the next 10 years is the perfect example on how a purely ideological positions (approximately: Science will function better on a per project basis carried out by

temporary staff) is being pushed forward without any evidence to ground this assumption and even less to confirm its potential benefits.

I am not actually objecting to requirements for a project to be written and evaluated before granting money to a research team. Projects structure ideas, verify their feasibility, identify potential bottlenecks (thus providing contingency plans) and allow a certain degree of financial and temporal planning. However, there is absolutely no proof that making the survival of researchers and research units conditional to funding acquired via projects will result in better science. Chances are that it will not even result in a better productivity. The idea that (French) researchers are underperforming (read: taking advantage of their quality of state servant) is not only offensive; it is patently wrong.

The analysis of Courtioux et al. (2019) is formal and I reproduce it here *verbatim*:

- The French research system exhibits, at first order, an optimal productivity given the funding granted by the government;
- There is no reason to claim it performs poorly and thus needs to be reformed.

The direct implication of these two observations is that the only way to increase the performance of the (French) research system is to put more money into public research. Not taking clues about this and pushing through an agenda that takes exactly the opposite path is, simply and plainly put, to pave the way to a ‘Bullshit or Perish’ mantra where research choices are totally guided by the need to comply with specific indicators. McCarthy et al. (2020) propose a 4-step strategy for dealing with workplace (and institutional) bullshit: the C.R.A.P. framework (Comprehend, Recognize, Act, Prevent). I daresay the acronym also hints to the word *crap* which, according to the Oxford English dictionary, is coarse slang for *nonsense* or *rubbish*. A working group on how to use the C.R.A.P. framework to improve research politics, policy and administration would be most useful. In the meanwhile, those shaping science policies would do society (and their) best interest by heeding Benjamin Franklin advice:

“An investment in knowledge pays the best interest”  
‘The Way to Wealth’ (1758).

Think about it, folks higher up.

Other important shortcomings of the current research system are, at least in my opinion, much less depressing because originate from a lack of culture again scientific bias which, in turn, is rooted in how our own brains are primed for self-deception. Nuzzo (2015) used the term *cognitive fallacies* to define four self-deception strategies:

- hypothesis myopia (focussing exclusively on evidence in favour on one hypothesis and ignoring other explanations),
- the Texas sharpshooter (a metaphor for p-hacking based on the fable of the person who drew a target around bullet holes left in a wall after shooting),
- asymmetric attention (closely scrutinizing unexpected results, while uncritically accepting expected ones as being reliable),
- and just-so storytelling (finding a coherent story to explain findings even when there is no real story behind the findings).

Other practical advice to avoid bias or reduce its impact consist in defining sets of criteria to assess the reliability of scientific findings and hence the scientific integrity of the associated publications (Grey et al., 2020; Moermond et al., 2016; Sutherland et al., 2013). What’s most important, however, is that addressing cognitive fallacies calls for authors and peers to slow down and take time to assess the actual significance (no statistical meaning intended) of their findings; in open contrast with the functioning principles of the research industry that puts a premium on speed and the associated quantitative indicators.

Discussing such non-technical issues in a HDR project is surely unusual and may seem out of purpose. Furthermore, I am clearly venturing far outside my research realm, which exposes me to the very risk of building a biased opinion on subjects on which my knowledge of the

literature is superficial at most. Nonetheless, the functioning of the research industry has a huge impact on the way I organize and conduct my work; including the scientific quality of my publications and, most importantly, the scientific quality of the publications of those whom I will hopefully directly mentor in the future. At this point of my career, it may be tempting to take for granted such soft-skills related to avoiding bias when performing research and interpreting results. The many comments and blogs about this subject in top journals just prove that one is probably never good enough at this, though. The efforts of universities to include such ‘scientific culture’ aspects in their curricula for the future generations of students and academics are therefore to be commended. Nonetheless, scientists from my generation received little or no training on such issues during their graduate studies, but must also strive to keep up with them. All these reasons prompted me include some reflections on how to handle my own self-bias in the practical part of my project (section 2.8).

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## 2. Detailed research proposal

### 2.1. A synthesis of conceptual and practical challenges in reconciling standard procedures and environmental realism

Reconciling standard procedures with environmental realism is conceptually different from laboratory-to-field extrapolation of ecotoxicological knowledge in the regulatory context, although the two are obviously related. The former aims at verifying the scientific underpinnings of standardized procedures against environmental realisms, gathering new knowledge to correct wrong assumptions and developing more relevant procedures or approaches without compromising data reliability. The latter is more concerned with how to use available knowledge (from standardized tests) to obtain reasonably accurate predictions of risks in real field situations. In both cases, it is important to clearly define what questions are being asked and to what extent the available tools can provide adequate answers to those question. Broad questions such as “What will be the ecosystem evolution in the long term under the current and foreseeable anthropogenic pressures?” will need to be dissected into several and more directly answerable ones (*e.g.*, what chemicals are introduced into the ecosystem by each pressure, what is the expected effect of each chemical). All considered, reconciling standard procedures (*i.e.*, data reliability) with environmental realism (*i.e.*, data relevance) is a complex issue cutting across the conceptual, regulatory, management and methodological frameworks of fundamental and applied ecotoxicological research (Fig. 13).

The book ‘Extrapolation Practice for Ecotoxicological Effect Characterization of Chemicals’ (Solomon et al., 2008a) lists six necessary aspects to reconcile reliability and relevance in ecotoxicology: i) matrix and media extrapolation, ii) (Quantitative) Structure Activity Relationship and extrapolation, iii) extrapolation across levels of biological organization, iv) mixture extrapolation, v) temporal extrapolation, and vi) spatial extrapolation. The importance of these practices does not seem to have decreased over time (at least conceptually) as confirmed, *inter alia*, by the review from Luís de Sá Salomão et al. (2020). However, the relevance of each practice will depend on the specific conceptual framework of any given investigation. The conceptual framework, also referred to as problem definition in environmental risk assessment, will define the corresponding management and regulatory frameworks which, in turn, will drive the development and application of the necessary tools framework (Fig 13). The relative importance of reliability and relevance can vary depending on the specific objectives of the investigation.

Simple conceptual frameworks such as ‘Is substance A more ecotoxic than substance B?’, which apply when searching for substitutes of priority dangerous substances, are more concerned with reliability to generate data to accurately rank the ecotoxicity of substances A and B (Fig. 13 A,B). Despite their simplicity, such questions are vital for the sound management of chemicals, because they ensure that a minimum set of reliable data is generated and [substances needing further risk assessment are identified](#) (ECHA, 2020). More conceptually complex questions will plot farther along the relevance axis; usually accepting some decrease in the associated reliability (*i.e.*, an increased uncertainty) (Fig. 13 A). Nonetheless, Tian et al. (2021) successfully answered a very complex question (*e.g.*, why Coho salmon population fail to respond positively to massive habitat restoration efforts) by using EDA/TIE by progressively collecting the necessary information along the relevance/reliability continuum. It is therefore possible to enter any framework (Fig. 13) at the most appropriate point and fill the gaps in knowledge until reliable and relevant conclusions can be achieved (Cairns, 2003; Munkittrick and McCarty, 1995).

For example, the regulation of new chemicals begins with data generation (Fig. 13 B) to establishing reliable, but often scarcely relevant, acute environmental quality criteria (Fig. 13 C) and moves toward more inclusive and complex approaches when these basic criteria support the need for further investigations. At the opposite, overarching legislations or agreements such

as the Water Framework Directive – WFD, Marine Strategy Framework Directive – MSFD or the United Nations millennium development goals strive for as much relevance as possible by moving well beyond the regulation of individual chemicals and ensure the long-term conservation of functional socio-ecosystems. It is however noteworthy that even such overarching frameworks ultimately need reliable criteria for their enforcement. Thus, the evaluation of chemical status under the WFD relies on the daughter directive 2013/39/EU and associated standards. Overall, ecotoxicologists already possess a big set of tools covering different levels of reliability and relevance (Fig. 13 D), although this does not eliminate the need for new methodological developments or improvement of existing approaches (Baillard et al., 2020; Escher et al., 2020; Larras et al., 2018; Major et al., 2020). When using any tool, the most strategic issue in reconciling standard procedures with environmental realism is probably to be as wary as possible about type III errors (Landis et al., 2013). Type III error occur when the correct procedures are used to answer the wrong fundamental or applied scientific questions.

Type III errors can be particularly frequent in retrospective risk assessment following ecosystem degradation or repeated exceedance of quality guidelines or standards. I have personally participated in a working group involving regulators, stakeholders, consultants and researcher to assess the possible risks associated with legacy Hg contamination in Lake Maggiore (Italy/Switzerland) (see also Part II, section 4). The investigation should have focussed from the beginning on issues such as the potential remobilization into the food chain of the legacy Hg contained in bed sediments; *e.g.*, Vignati et al. (2020). Instead, the stakeholder’s consultant invested a considerable amount of money to test for acute toxicity the elutriates of hundreds of sediment samples collected at water depths > 150 m. Contaminants other than Hg could have been present (notably, DDTs, PCBs and dioxin-like compounds) and toxicity testing may have been justified on a somewhat smaller scale. However, neglecting the key questions of bioaccumulation and biomagnification in relation to Hg was clearly a type III error because the simple absence of direct toxic effects would not have been enough to dismiss bioaccumulation/biomagnification issues that are the most important in managing long-term mercury contamination. The error was much more regrettable considering that, at the time of the investigation, the European Union had already recognized the need to embrace a biota-based environmental quality standard for Hg

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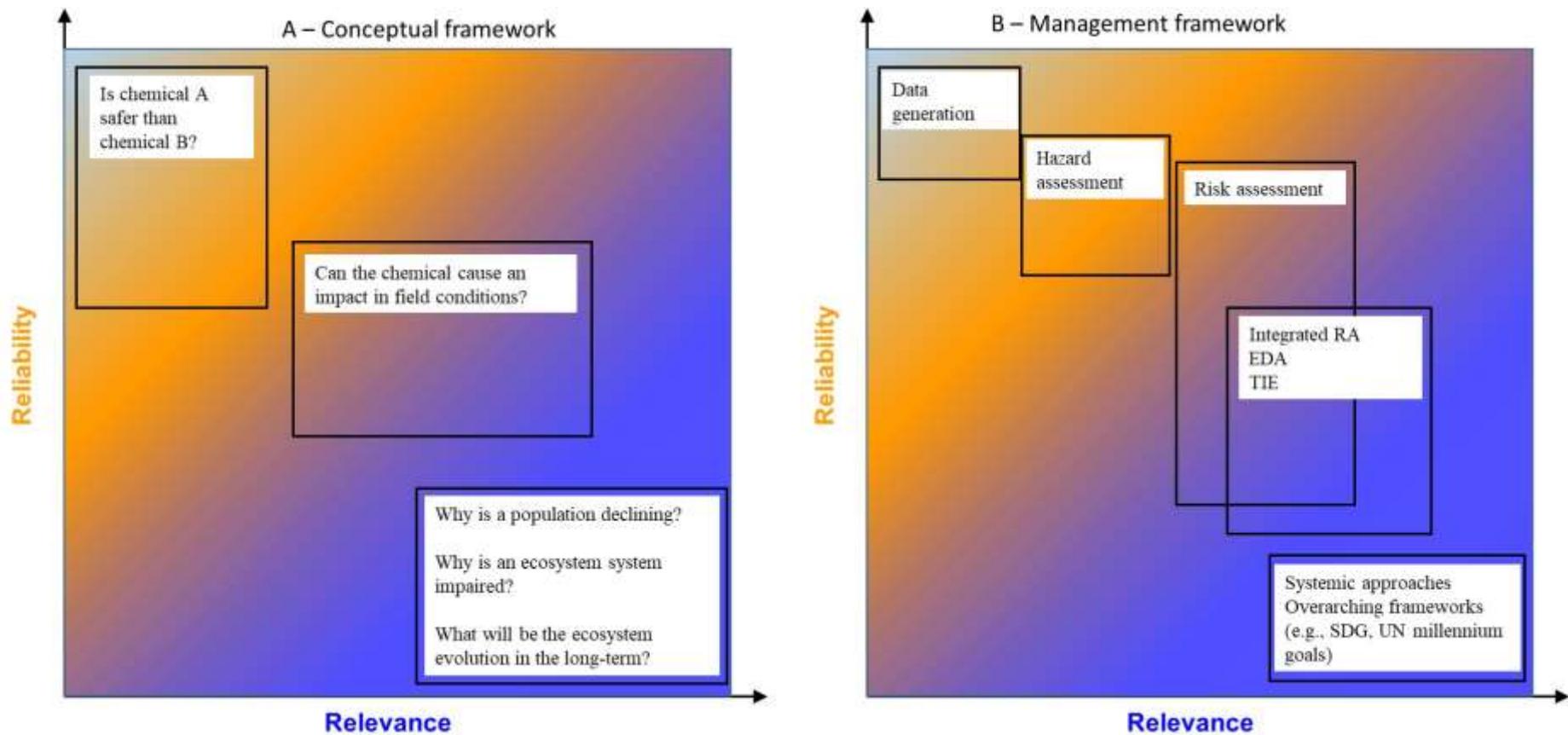


Figure 13. Examples of the possible positioning of selected conceptual (A), management (B), regulatory (C) and tool (D) frameworks along reliability and relevance axis in ecotoxicology. RA, risk assessment; EDA, Effect Directed Analysis; TIE, Toxicity Identification Evaluation; SDG, Sustainable Development Goals; UN, United Nations.

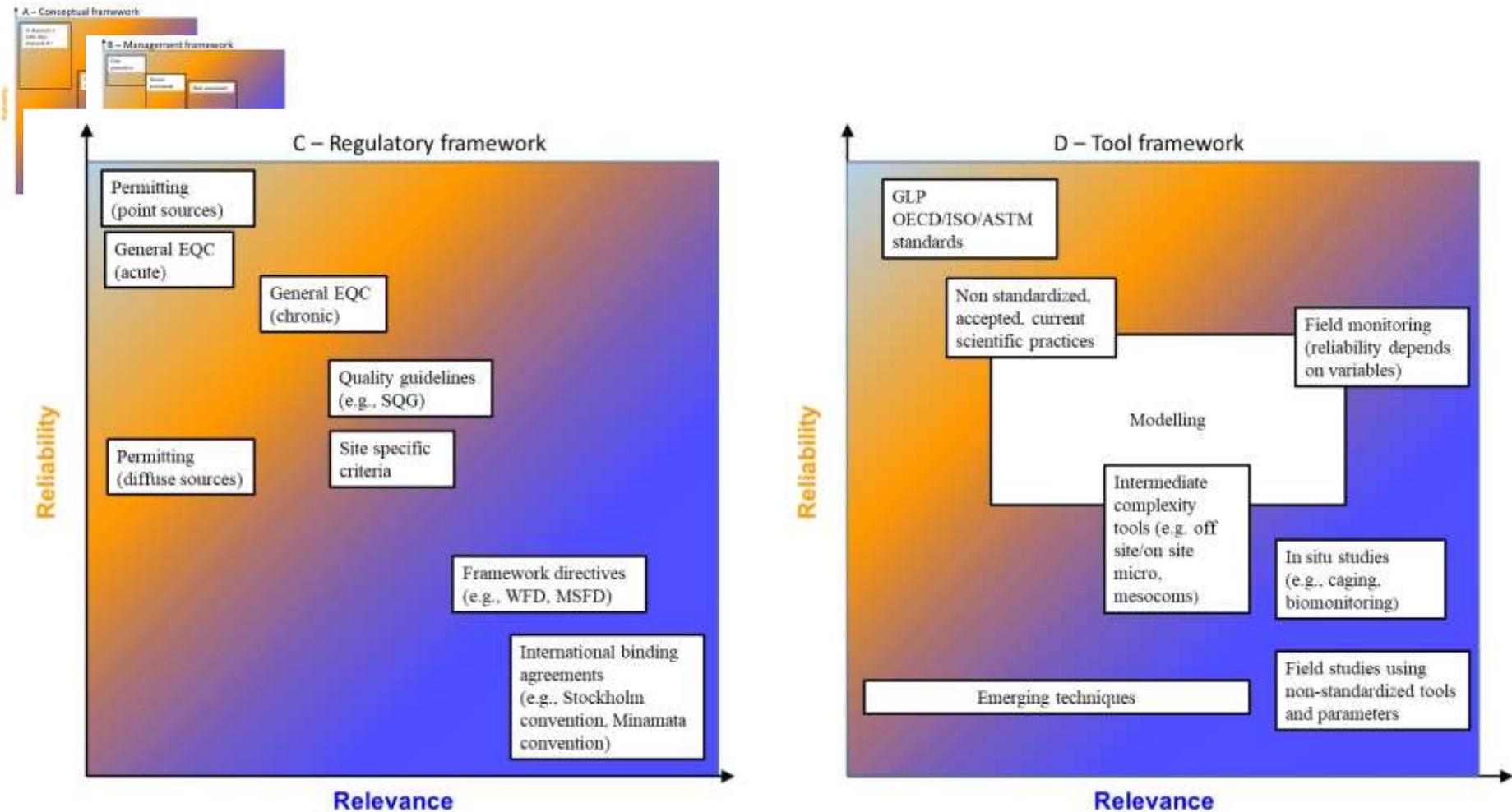


Figure 13 (continued). EQC, Environmental Quality Criteria; SQG, Sediment Quality Guidelines; WFD, Water Framework Directive; MSFD, Marine Strategy Framework Directive; GLP, Good Laboratory Practices; OECD, Organization for Economic Co-operation and Development; ISO, International Organization for Standardization; ASTM, American Society for Testing Material (now ASTM International).

In the same vein, Allen Burton (2017) showed that exclusive reliance on Sediment Quality Guidelines as a decision tool for taking remedial actions of contaminated sediments was likely to result in costly mismanagement strategies. Type III errors may also occur in data generation for new chemicals. This was notably the case for some early ecotoxicological studies on the effects of nanoparticles and nanomaterials that overlooked the physico-chemical peculiarities of such substances. Such studies were likely examples of *bona fide* errors partly dictated by the present functioning of research (Part III, section 1.8), but nonetheless produced data of both doubtful reliability and scarce relevance (Petersen et al., 2014). Paraphrasing the warning of Stout (1993) about inappropriately constructed monitoring programs, type III errors may reduce technically correct ecotoxicological studies to “an enchanting but unproductive effort of data collection”.

The examples listed in Tables 3 and 4 show that the practical challenges of reconciling standard procedures with environmental realism, which include avoiding type III errors, are multiple and diverse. Several of those challenges may apply simultaneously, highlighting the interdisciplinary nature of relevant ecotoxicological research. The outstanding diversity of the available ecotoxicological tools and approaches can be appreciated by consulting the entries of the Encyclopaedia of Aquatic Ecotoxicology (Férard and Blaise, 2013). The 104 entries of the Encyclopaedia span several research fields, including chemistry, biochemistry, ecology, immunology, parasitology and many more. At first, it may seem surprising to observe that only a minor part of the available ecotoxicological knowledge is being used to establish the legal framework for protecting the environment against unwanted effects of chemicals. However, many approaches have not yet reached the degree of reliability that is needed to be explicitly considered in the development of environmental quality criteria (Fig. 13 C). On the other hand, all the approaches can contribute to increase data relevance and must be considered when searching for answers to broad conceptual questions and management needs (Fig. 13 A,B,C). It is therefore the task of the ecotoxicologist to warn stakeholders and regulators about the limitations or, in worst-case scenarios, inadequacy of standard procedures and suggest ways to correctly assess environmental risks.

## 2.2. Project rationale

Given the breadth and complexity of the *reliability vs. relevance framework* (Fig. 13), research to reconcile standard procedures and environmental realism can be carried out in several ways. This project will specifically focus on possible approaches to increase the relevance of standard ecotoxicological procedures while preserving their reliability. Relevance is often assessed retrospectively when using laboratory-derived knowledge during real-world risk assessment/risk management programs. This approach may be understandable and work well for legacy chemicals that have been extensively characterized over the years, although it does not necessarily eliminate the need to collect more relevant data during the process. However, prospective risk assessment for new chemicals should rather start by asking how ‘reliable methods’ can be made as relevant as possible to real-world scenarios by considering the properties (expected environmental behaviour and ecotoxicity) and planned uses of the chemicals. The same strategy can be used to effectively fill existing knowledge gaps for any chemical.

Modifications to standard tests are actually acceptable provided that they are properly justified and described in detail. The norm ISO 14442 explicitly discusses possible modifications to the standardized protocol for freshwater algae to test “poorly soluble materials, volatile compounds, metals and waste water” (ISO, 2006). Similarly, De Schampelaere et al. (2014) modified the standard test medium for algae to avoid the formation of insoluble Pb complexes that could have hindered the interpretation of ecotoxicological data. Such modifications can solve potentially important methodological problems and improve the

reliability of the concentration vs. response data, although they do not necessarily increase the data environmental relevance.

Criticisms to standardized ecotoxicological tests essentially points to their inadequacy to properly simulate realistic environmental conditions (Table 4), even when their conceptual limitations are properly taken into account (Table 3). On this basis, the use of laboratory-derived knowledge to assess field situations can be questioned and, ultimately, the overall validity of current risk assessment procedures and environmental policies doubted upon. For example, the results (and eventually the EQS) obtained from standardized aqueous toxicity tests are usually considered conservative (third assumption in Table 3); *i.e.*, they are believed to offer some sort of intrinsic security factor by creating conditions of maximum chemical bioavailability and, hence, minimizing the risk for false negatives in hazard/risk assessment. There are however several instances in which this will not be the case including, but not limited to (Tables 3 and 4):

- Water-borne exposure is not the main exposure route,
- Biomagnification issues rather than (or along with) direct toxic effects are the main concern,
- Exposure concentrations are not stable during laboratory testing,
- field situations are characterized by repeated pulse-exposures of variable amplitude and duration as opposed to the continuous exposure scenarios used in the laboratory,
- Toxic effects of chemicals are increased by additional (non-chemical) stressors including climate change related issues,
- Standard endpoints miss more sensitive ones in real field situations.

Careful consideration of these and similar shortcomings can be used to identify general research questions to directly improve the relevance of data obtained from standardized tests. With this purpose in mind, I have identified a few specific research questions for the present HDR project (Table 6). While not claiming that the proposed questions possess absolute novelty, they are all pertinent to encourage research in understudied or not completely understood aspects of ecotoxicology. Some of the questions in table 6 have indeed been on the research agenda for quite a long time. However, they remain far from being completely answered or their focus regularly changes the following acquisition of new knowledge or the appearance of new (classes) of chemicals in the ecotoxicological agenda. Other questions have arisen more recently; notably the ecotoxicology of climate change and difficulties connected with bias in data generation and reporting. The proposed questions are just a small subset of all those that are actually needed to reconciling standard procedures with environmental realisms. In this sense, they simply reflect personal research priorities. On the other hand, they are sufficiently broad to serve as generators for further conceptual and methodological development.

The member of the present HDR committee will easily notice that the depth of my reflexion is not the same for each one of the questions. This situation depends on the links (or absence of links) between each question and my previous research and on the ongoing curve of my interdisciplinary learning. For each question, I provide some general background context followed by a few personal, and absolutely non-exhaustive, considerations on the subject before discussing perspective research activities to be implemented in future projects.

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Table 6. Overarching questions in reconciling standard procedures and environmental relevance.

Overarching questions	Possible research approaches	Practical suggestions (including gaps)
<i>What is the most appropriate metric to evaluate the effects of a given substance or stressor?</i>	<ul style="list-style-type: none"> <li>➤ Exposure metrics:               <ul style="list-style-type: none"> <li>• Determine the bioavailable fraction in test media,</li> <li>• Use internal contaminant burdens.</li> </ul> </li> <li>➤ Response metrics:               <ul style="list-style-type: none"> <li>• Select different types of response from organisms,</li> <li>• Identify early responses related to later individual- or population-level responses.</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>➤ Verify the temporal stability of exposure concentrations over test duration,</li> <li>➤ Combine external and internal exposure metrics,</li> <li>➤ Consider endpoints with a different response ‘dynamics’ if feasible.</li> </ul>
<i>How to reconcile the timing of laboratory testing with that of field exposure?</i>	<ul style="list-style-type: none"> <li>➤ Investigate how temporal aspects affect the outcome of ecotoxicological tests,</li> <li>➤ Monitor field situation of interest to identify real-world scenarios to be studied in controlled conditions.</li> </ul>	<ul style="list-style-type: none"> <li>➤ Use different exposure scenarios (pulses, continuous),</li> <li>➤ Adjust exposure duration based on field monitoring information.</li> </ul>
<i>How to account for mixture exposures?</i>	<ul style="list-style-type: none"> <li>➤ Identify field-relevant mixtures of contaminants including non-chemical factors,</li> <li>➤ Verify the environmental viability of emerging treatment options for contaminated effluents.</li> </ul>	<ul style="list-style-type: none"> <li>➤ Ecotoxicological geology,</li> <li>➤ Consider simultaneous exposure of different redox species of the same element,</li> <li>➤ Test effluents for potential effects linked with unknown toxic, by-products.</li> </ul>
<i>How to account for matrix effects and uptake routes?</i>	<ul style="list-style-type: none"> <li>➤ Include relevance-enhancing modifications into the composition of standardized test media</li> <li>➤ Consider organism ecology and contaminant properties in choosing test procedures and experimental set-up.</li> </ul>	<ul style="list-style-type: none"> <li>➤ Add representative colloidal carrier phases to test media,</li> <li>➤ Choose a (clean) natural matrix as a reference (important in large programmes),</li> <li>➤ Consider dietary exposure for filter and deposit feeders.</li> </ul>
<i>How to integrate issues such as climate change and river-to-sea continuum in ecotoxicological research?</i>	<ul style="list-style-type: none"> <li>➤ Examine the different drivers of responses in each situation.</li> </ul>	<ul style="list-style-type: none"> <li>➤ Test the most relevant drivers for their effects,</li> <li>➤ Include continuum aspects (longitudinal, lateral, vertical, temporal and level-of-organization) in the problem statement to aid test design.</li> </ul>
<i>How to properly report results and avoid bias?</i>	<ul style="list-style-type: none"> <li>➤ Contribute to ensure a verifiable, lasting record of raw data to allow future checks by peers and third-parties,</li> <li>➤ Embrace procedures such as electronic laboratory workbooks as per institutional policies.</li> </ul>	<ul style="list-style-type: none"> <li>➤ Store raw data on institutional repositories,</li> <li>➤ Use on-line tools such as Scirap to guide reporting results of scientific studies,</li> <li>➤ Be open in admitting alternate hypotheses</li> <li>➤ Evaluate alternative publications modes when possible</li> </ul>

### 2.3. Question 1. What is the most appropriate metric to evaluate the effects of a given substance or stressor?

#### 2.3.1 Background considerations

Finding reliable relationships between the level of exposure to a chemical (stressor) and one or more biological response belongs the fundamental objectives of ecotoxicology. Such relationships require the measurement of two components: the applied quantities of the stressor and the extent of the biological response at each level of exposure to the stressor. In some standardized experiments, the range of exposure concentrations chosen for a given stressor is expected to cover the range between *no response* and *complete inhibition* of the selected biological endpoint (see section 2.3.2). However, it is also possible to effectively model biological responses in term of Benchmark concentrations (*i.e.*, a statistically significant deviation from the control response) without the need to attain a 100% response nor to have monotonic concentration–response patterns (Baillard et al., 2020; Larras et al., 2018). In any case, an ecotoxicological investigation typically yields a series of paired exposure *vs.* response metrics each being, ideally, both reliable and relevant (see section 1.2). The relevance criterion may initially be of secondary importance in fundamental investigations concerned with elucidating mode or mechanisms of actions, but is a key feature for applied ecotoxicology and chemical (stressor) management (see section 2.1). In the latter case, we should be able to measure the same metric (or reasonably related proxies of the same metric) in both standardized laboratory settings and in specific real-world contexts.

It is hardly a surprise that not all combinations of exposure *vs.* response metrics will be equivalent in terms of reliability and relevance as defined in section 1.2. Being able to identify and use reliable and relevant metrics is a key factor to eventually “...deliver meaningful environmental metrics (Merrington et al., 2014)” ensuring the desired type and level of environmental protection. For the sake of completeness, it is important to remember that the term metric can be used to indicate (legally binding) guidelines for toxicants (Warne et al., 2014), the actual measurements selected in field monitoring programs for comparison with such guidelines (Merrington et al., 2014) and the actual ways of measuring the concentration and response variables to derive an EC<sub>x</sub> or benchmark values from a (standardized) test. The third meaning is the most relevant to this part of the present document.

Given the sheer (and growing) number of chemicals, it is unlikely that we will ever be able to establish environmental quality benchmark values for all of them or to measure all of their concentrations during monitoring operations. In this sense, using an ecotoxicological approach to evaluate the chemical status of a water body (section 1.2.5 and Fig. 8) retains a considerable interest for assessing whether exposure to a real-world matrix can cause damage to organisms and ecosystem’s functions. Biomonitoring with indigenous organisms may usefully complement the ecotoxicological evaluation by showing if they are actually experiencing effects due to long-term exposures directly in the field (Devin et al., 2014). On the other hand, once the potential for effects has been shown, it becomes essential to identify their causes and, if appropriate, to estimate by how much the level of specific chemicals will have to be lowered to restore the ecosystem to a situation without chemical-stress or within a level of stress compatible with its functioning (see Fig 6). Once again, standardized (or controlled) tests will be required for this purpose because society is likely to be unwilling to accept the cost of remediation in the absence of clear causal links between the present of specific chemicals and the observed biological effects.

Research on nanoparticles has definitely shown the importance of characterizing as best as possible the actual exposure level of the target biological model to the chemical of interest (Handy et al., 2012; Petersen et al., 2014). In the same period, a panel of ecotoxicologists explicitly wrote to “have become progressively more concerned about the quality of some published ecotoxicology research (Harris et al., 2014)” and defined twelve principles

of Sound Ecotoxicology. It turns out that the way in which exposure concentrations and biological responses are expressed are critical for their environmental relevance; i.e. to their amenability to laboratory-to-field extrapolation. Inappropriate choices of exposure metrics can therefore make laboratory results both irrelevant and unreliable. Furthermore, all metrics of exposure currently used in the regulatory context and in much ecotoxicological research are in reality approximations for the actual concentration of the chemical present at the biological target site (Fig. 9). Developing methods to determine the bioavailable fraction of a chemical indeed try to identify the fraction of total concentration most closely associated with the biologically active fraction and, hence, with the observed responses.

Several biological responses can actually be measured in both laboratory and field conditions; thus fulfilling, in principle, the ideal combination of reliability and relevance. Biomarker measurements are a good example of responses that can be reliably measured in the laboratory on individuals exposed to known chemicals as well as in indigenous organisms exposed to a complex array of stressors. Reconciling them then becomes a (far from trivial) effort of exactly identifying the contribution of individual stressors to the observed biomarker expression. The use of in situ ecotoxicity testing (Burton and Nordstrom, 2004; Crane et al., 2007a; Ferrari et al., 2014) also allows *to bring to the field* reliable biological endpoints (*e.g.*, grown inhibition and emergence of model organisms) for relevant measurements in specific real-world situations. Yet, most of the reliable endpoints borrowed from the laboratory to the field are measured at relatively low levels of biological organization (*e.g.*, biochemical or individual). Such metrics deal with the health status of the individual rather than with the impacts of chemicals at the higher levels of organization (population, community, ecosystems) that risk assessors and risk managers want to protect (Figs. 6 and 7). However, new tools are constantly being developed to improve our ability in linking individual and population level responses (Charles et al., 2018; Coulaud et al., 2015; Larras et al., 2020).

### 2.3.2. Some personal considerations on selected metrics of exposure and response

Reliable ecotoxicological experiments should always include an analytical verification of the actual exposure concentration to derive meaningful concentration–response relationships (Harris et al., 2014; Moermond et al., 2016). Relevant ecotoxicological experiments should (ideally) use metrics that provide direct information as to the bioavailability and bioaccessibility of a chemical and are equally measurable in both laboratory and field settings. Because exposure metrics are usually expressed as (a fraction of) the chemical’s content in the test matrix, although the quantity of chemical accumulated by the test organism can be a valid alternative (see section 1.7), it is important to understand what kind of information can be provided by the metric of choice (Fig. 14).

When quantifying chemicals in standardized laboratory test media, measurements of total concentrations simply provide a check against random experimental errors, losses via adsorption to the walls of test vessels, volatilization or degradation of the test substance. This may be satisfactory in terms of reliability. On the other hand, total concentrations are poor proxies for the bioavailable (and bioaccessible) fraction of a chemical (Fig. 9). The use of total concentrations can overlook other potential problems linked to the properties of certain chemicals; notably sparingly soluble organic chemicals or trace elements forming chemical species with limited solubility (Blinova et al., 2018; Riedl and Altenburger, 2007; Weyman et al., 2012). In the latter case, the formation of species with low solubility (*e.g.*,  $\text{Cr}(\text{OH})_3$ ,  $\text{Gd}_2(\text{CO}_3)_3$ ) results in the presence of colloiddally-sized particles in the exposure medium possibly decreasing the bioavailable elemental concentration (Aharchaou et al., 2018; El-Akl et al., 2015; González et al., 2015; Wilson et al., 2017). In such instances, using total concentrations as exposure metrics would underestimate the potential ecotoxicity of a chemical,

provided that the precipitate does not elicit itself some kind of ecotoxic response in analogy with engineered nanoparticles.

Filterable or ultrafilterable concentrations may be better proxies for the bioavailable fraction of chemicals, although the potential pitfalls of both techniques must be clearly understood (see section 1.3). Further refinement of exposure metrics is possible by measuring (or modelling) specific chemical forms such as the free ion (Heringa et al., 2004; Leguay et al., 2016) or specific redox states (*e.g.*, Cr(VI) measurements in addition to total Cr determination). In conclusion, total concentrations can provide a first check as to the presence of methodological issues, but will need to be integrated by more refined metrics of exposure to:

- a) correctly interpret biological responses,
- b) ensure reliable conclusions from ecotoxicity testing (*i.e.*, consistent results across laboratories and test media), and
- c) provide the necessary level of relevance for comparison with real field situations.

Additional issues linked with temporal variability of exposure concentrations during the tests will be discussed in section 2.4. Furthermore, for organisms that actively ingest environmental particles, the contribution of different routes of exposure to chemicals will also have to be considered (section 2.6).

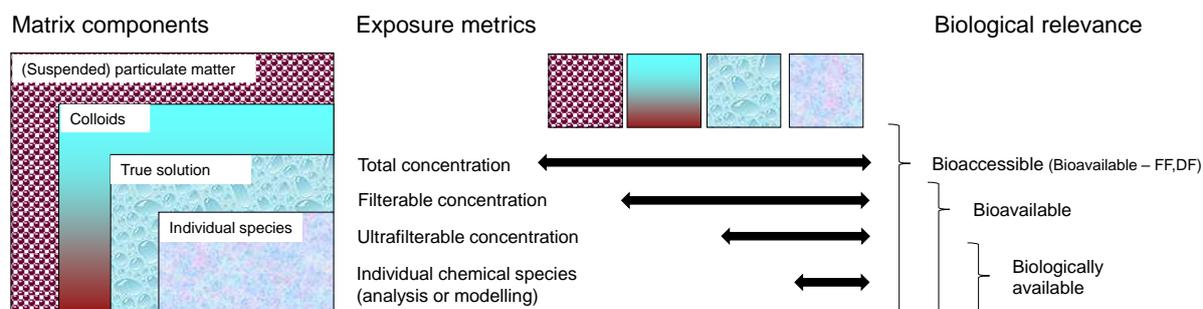


Figure 14. Example of possible metrics for measuring exposure to chemicals in ecotoxicological tests with aquatic organisms in relation to matrix components (simplified operational representation) and to the biological relevance of each metric. FF, filter-feeders; DF, deposit feeders.

Direct measurements of chemical accumulation by organisms used in standardized test can also be adopted as metrics of exposure. The conceptual advantages of this approach have been briefly discussed in section 1.4. By analogy with measurements in exposure matrices (Fig. 14), total accumulated concentrations simply prove that a given chemical has interacted with the organism, but provide absolutely no clue as to the chemical fraction responsible for any observed response. It is also worth noting that the more complex the organism, the less relevant the total accumulated concentration will be in terms of explaining the observed biological response. Indeed, the study of chemical bioaccumulation in complex organisms (*e.g.*, fish and mussels) is often done on a per-organ basis. On the other hand, total accumulated concentrations in *e.g.*, whole fish can be useful to estimate chemical transfer to predators ingesting whole preys; a regulatory relevant issues for substances such as mercury that are regulated via biota-based EQS (Zuliani et al., 2019).

At a finer scale, the biologically active and inert fractions of the accumulated chemical (see section 1.4) can be distinguished using operationally defined procedures; which can have several advantages. Gimbert et al. (2016) showed that the kinetics of Hg accumulation by larvae of the midge *Chironomus riparius* could be better understood in terms of the accumulated cytosolic fraction (*i.e.*, excluding exoskeletons, gut content and cellular debris from the pool of

accumulated Hg) rather than in terms of total Hg concentrations in whole larvae. The use of the cytosolic fraction also appears more related to the potential for accumulated chemicals to undergo trophic transfer from *e.g.*, marine algae to copepods (Reinfelder and Fisher, 1991). This observation suggests that only a fraction of the total accumulate concentration may be available for assimilation following the ingestion of contaminated preys; a consideration which may have also have regulatory implications (see the previous paragraph). In the case algae, a distinction between adsorbed and absorbed fractions may also allow to better understand the concentration *vs.* response relationships; although chemicals may be further fractionated into biologically inert and active pools inside the cells (Aharchaou et al., 2017; Crémazy et al., 2013b).

The diversity of metrics of exposure is mirrored by the variety of metrics of response. The response metric measured during standardized tests should provide suitable answers for the objectives of the investigation. This criterion may not be automatically fulfilled in applied investigation aiming to establish whether a chemical poses a potential threat under real-world conditions. Standardized tests often provide response metrics at the biochemical, cellular and individual level of organization; while ecotoxicology is concerned with the protection of populations, communities and functional ecosystems (Fig. 6). The Adverse Outcome Pathway (AOP) framework provides a conceptual construct to link responses across levels of biological organization. While establishing all causal linkages across all levels of organization requires substantial research efforts and often remains elusive, populations models are being successfully developed to bridge individual and population-level responses for representative species (Baudrot et al., 2018; Coulaud et al., 2015)

In general, a biological response can be aspecific (*i.e.*, it is expressed in the same way regardless of the chemical or stressor causing it), indicative of a certain general mode of action (*e.g.*, mutagenicity), or specific to a peculiar (class of) chemicals (*e.g.*, interaction with a specific receptor). Whole-organisms effects such as mortality, growth, reproductive output or time to emergence in the standardized test with the midge *Chironomus riparius* are good examples of aspecific apical responses. The sensitivity of these apical responses varies among chemicals, which usually have different mechanism of action at the molecular level, and among species for a given chemical (Nys et al., 2019). However, these responses can often be scaled from zero to one hundred percent provided that an adequate range of exposure is selected. On the other hand, behavioural, biochemical and omics responses are (often transient) expressions of a chain of events possibly leading to effects at the whole organism level according to the AOP framework. As a consequence, it is usually not possible to scale them on a 0–100% range and, in relation to unexposed controls, the response of such metrics can be either positive or negative. These metrics can however provide useful early warning signs of deteriorating environmental conditions as well as insights into the mode of action of different substances (Larras et al., 2018).

### 2.3.3. Perspective research activities

A considerable part of my previous and current research is concerned with improving metrics of exposure for trace elements potentially forming insoluble chemical species in standardized ecotoxicological test media. Past results specifically showed that, for trivalent Cr and lanthanides, measurements of total elemental concentration at the beginning of an ecotoxicological test is simply a quality control procedure to ensure that no contamination, losses or experimental artefacts have occurred (see section 2.3.2). Its use as the sole metric of exposure should therefore be considered both unreliable and irrelevant until otherwise experimentally demonstrated. Unreliability essentially arise from the progressive decrease of exposure concentration over the test duration, resulting in the underestimation of actual ecotoxicity when only total initial concentrations are used (Simpson et al., 2003). Furthermore,

colloidal-sized species (e.g.,  $\text{Cr}(\text{OH})_3$  or  $\text{Ln}_2(\text{CO}_3)_3$ ,  $\text{Ln}$  being any element along the lanthanide series) can occur even in the absence of visible precipitates or concentration decrease. The presence of such species will go undetected by total concentration measurements. Irrelevance follows, in turn, from the different speciation of elements in standardized test matrices vs. natural waters. The BLM or other model constructs can practically address such limitations (Mebane et al., 2020), but do not provide a direct measurement of the actual elemental species eliciting biological responses in laboratory and field settings. Furthermore, the few attempts to use BLM to explain Cr(III) ecotoxicity have so far been unsuccessful (Song et al., 2014), while limitations exist in its application in the case of lanthanides (Aharchaou et al., 2020; Zhao and Wilkinson, 2015). Refining exposure metrics in standardized tests therefore remains a worthy scientific objective.

In practical terms, the ideal exposure metric should combine reliability in standardized test (*i.e.*, limited variability among test media and across organisms) with environmental relevance (*i.e.*, being measurable in the field). Specific objectives for my future research include:

- a) Enhanced characterization of the truly dissolved elemental fraction in test media,
- b) Direct determination of free ionic species (when analytically feasible) for comparison between laboratory and field settings,
- c) Use of tools for integrating exposure information over time.

The pursuit of such objectives is already technically feasible thanks to the commercial bioavailability of small-volume ultracentrifugal units (objective a) and diffuse gradients in thin films, DGTs (objective c) (Davison and Zhang, 2012; Martin et al., submitted-b). DGTs actually accumulate various chemical species of a given element, but also provide a degree of versatility that makes them interesting for comparing laboratory and field situations. Objective 'b' is not technically hindered in the case of Cr; for which ion-chromatography ICP-MS provides a suitable technique (Aharchaou et al., 2018; Seby et al., 2003); while further analytical developments are necessary in the case of lanthanides. Most interestingly, DGTs are available for trivalent and hexavalent Cr species and have been explored for lanthanides (Gao et al., 2019; Pan et al., 2015; Yuan et al., 2018). Attention to elemental speciation in test media should be accompanied by a survey of the temporal evolution of exposure concentration over the test duration (see section 2.4). Considering that the proposed specific objectives are not limited to Cr and lanthanides, I hope that they will generate attention to similar issues for other elements or chemicals.

Another feature of my past and current research is its strong focus on the model freshwater alga *Raphidocelis subcapitata*, although I participated in studies using the freshwater crustacean *Daphnia magna*, the midge *Chironomus riparius* and the mollusc *Corbicula fluminea*. My interest in filter- (*D. magna*, *C. fluminea*) and deposit-feeders (*C. riparius*) stems from the desire to better clarify the importance of different routes of exposure to chemicals, including dietary exposure, for such organisms (see section 2.6). Furthermore, in the case of daphnids, the presence of colloidal-sized species in standard test media can also contribute to elicit the observed biological responses. Kottelat (2008) showed that specimen of *D. magna* exposed to trivalent Cr accumulated a significant amount of Cr-containing material in their digestive tracts; a situation that can reasonably be expected for other elements forming precipitates in standardized test media such as lanthanides. Whether such precipitates may elicit biological responses remains to be ascertained, but confirms the need for an appropriate choice of exposure metrics during ecotoxicological testing.

Thermodynamic modelling can clearly provide useful indications about the expected speciation of trace elements in both aqueous and heterogeneous media as reviewed by Groenenberg and Lofts (2014). The problem with these models is their limited ability to account for the kinetics of the various reactions controlling elemental speciation. Experimental verification of model predictions is therefore desirable to confirm the applicability of model

results in a given (laboratory) setting. Good agreement between actual measurements (when possible) and model predictions opens the possibility toward an improved extrapolation of laboratory results to real field situations. Under the simplifying but often reasonable assumption that the free element concentrations would control biological effects, ecotoxicological metrics expressed in terms of free element in the test medium may provide a reliable and more relevant metric. On the other hand, modelling elemental speciation in natural waters still presents substantial challenges, especially in relation to the exact complexing capabilities of natural organic matter (Town et al., 2019).

In conclusion, one major challenge in reconciling standardized studies and environmental realism originates from the markedly different composition of exposure matrices in laboratory vs. field conditions. With comparable information on element speciation in both settings, it should be easier to establish whether any risk from the presence of an element can be expected under realistic scenarios. More relevant exposure metrics can also answer the recurring criticisms about standardized tests using chemical concentrations much higher than corresponding levels in the field (Table 3). Indeed, while using high concentrations in laboratory settings is not advisable, the organisms' response will be determined only by a fraction of the contaminant added to the standard test medium. Laboratory vs. field comparison should indeed be made based on the concentration of such fraction or its best possible proxy.

With regard to metrics of response, I took few 'fishing trips' that made me curious about the possibility to examine the inhibition of photosynthetic activity along with the apical endpoint (*e.g.*, algal growth factor) that I have used so far. In particular, the inhibition of the activity of photosystem II (PSII) constitutes an interesting and easy-to-measure metrics of response for a key functional process in algae and plants (Rodríguez et al., 2007; Suresh Kumar et al., 2014). What makes PSII attractive is its potential use as an early-warning metric for more long-term responses, including growth inhibition. Early warning responses are particularly interesting when studying chemicals whose concentrations (and speciation in the case of trace elements) vary strongly over the test duration. In such cases, shortening exposure time may improve the reliability of the associated exposure metric. So far, I observed that PSII inhibition at 24 hours and algal growth inhibition at 72 hours seem to provide comparable  $EC_{50}$  when the exposure metric is expressed in terms of  $\mu\text{g}$  of Cr per cell (Fig. 15). This follows from methodological constraints that require an initial algal inoculum of  $2 \cdot 10^5 \text{ cell mL}^{-1}$  for reliable measurement of PSII activity at  $t = 0$  as compared to the standard inoculum of  $2 \cdot 10^4 \text{ cell mL}^{-1}$  for the growth test at 72h (ISO, 2012). Further research is however necessary considering that Cr(III) concentration in standard ISO 8692 test medium for algae can already decrease by more than 60% over 24h. Possible areas for future investigations include pH adjustments and the use of simplified exposure medium to better control Cr(III) speciation and to elicit measurable PSII responses at shorter exposure time.

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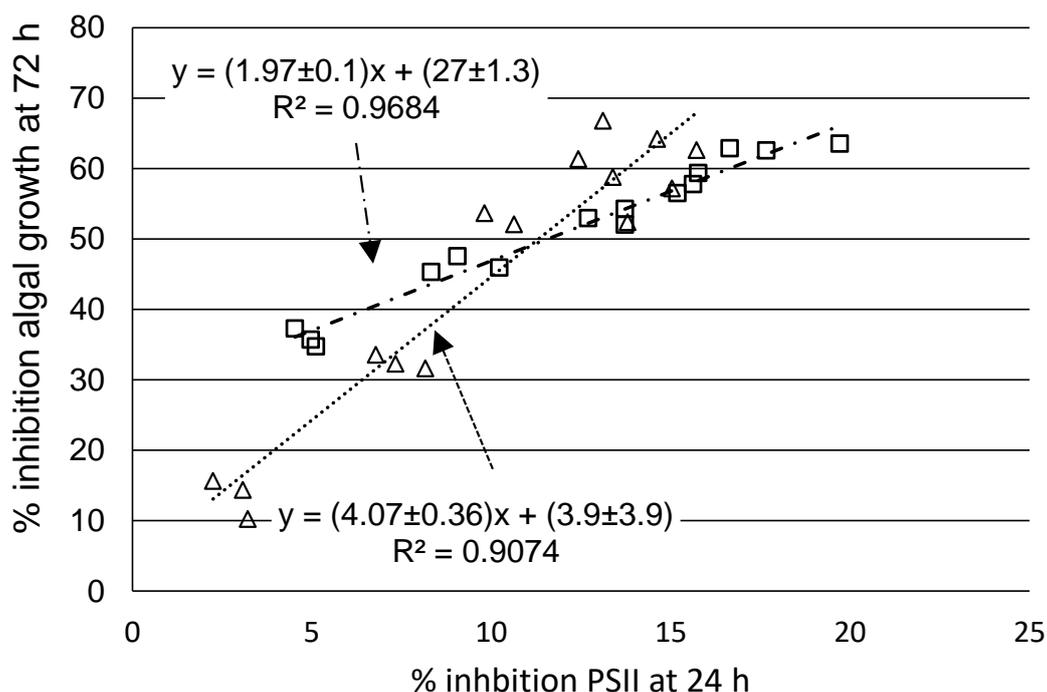


Figure 15. Example of possible correlations between inhibition of algal growth at 72 h and of photosystem II (PSII) at 24 h for cultures of *Raphidocelis subcapitata* exposed to trivalent Cr(III) in standardized ISO 8692 test medium. Inhibition of algal growth was calculated from the decrease in cell number compared with control algae. (Unpublished data from the author).

## 2.4. Question 2. How to reconcile the timing of laboratory testing with that of field exposure?

### 2.4.1. Background considerations

The simple existence of acute and chronic standardized tests shows the importance of time when assessing the responses of organisms to chemicals. It is therefore somewhat surprising to observe how the bulk of ecotoxicological research has become entangled in the use of  $EC_x$  values established after fixed exposure times, especially in relation to hazard- and risk-assessment purposes. The duration of standardized procedures is actually optimized to ensure that, in the absence of added stressors, the test medium provides optimal survival and growth conditions for the test organisms throughout the test. Such choices serve test-reliability well, but come at a cost in terms of relevance.

A first untested assumption of fixed-time test configurations is that the selected endpoints reflect biological responses caused by continuous exposure to approximately constant concentrations of a chemical over the whole test duration. However, changes in exposure concentration over time scales relevant to test duration may be important in the case of volatile or easily degradable compounds or for trace element undergoing rapid hydrolysis reactions (see section 2.3). If exposure concentrations vary with time, biological responses can reflect either the exposure to the average chemical concentration over the entire test, partly in line with the *standard* assumption, or the effect of the initial concentration-pulse before the decrease in chemical concentration. The behaviour of Cr(III), whose filterable concentrations in some standardized test media approach zero over the test duration, is a good practical example of this kind of conceptual issues (Aharchaou et al., 2018).

An second consideration concerns the possibility that the similar responses can be recorded at the end of the standard test duration following a time-invariant, reduced performance of the exposed organisms throughout the test or an initial period of stasis followed by a comparable performance of exposed and control organisms. Recording biological responses only after a

fixed period (*i.e.*, at the end of the test) will not allow to distinguish between the two situations. Let us consider a practical example based on standardized ecotoxicological tests for algae (*e.g.*, ISO 8692) that require responses to be recorded after 72h of exposure.

If an initial stasis in algal growth during *e.g.* the first 24 h is followed by a growth phase at a rate comparable to or higher than negative controls, the potential of the chemical to induce a short-term response will be overlooked (full growth recovery) or underestimated (partial recovery) (Fig. 16). On the contrary, growth inhibition may start to appear after a lag phase of *e.g.*, 24h so that recording responses only at 72h will miss the potential tolerance of the algae for exposures up to a few hours. All such situations may be relevant not only for those chemicals whose concentrations are not stable over the entire test duration, but also for discontinuous exposures in real field conditions. It is also noteworthy that the exponential growth of control algae over the entire test duration may be the net outcome of more complex growth patterns requiring a high-resolution measurements to be detected (Altenburger et al., 2008).

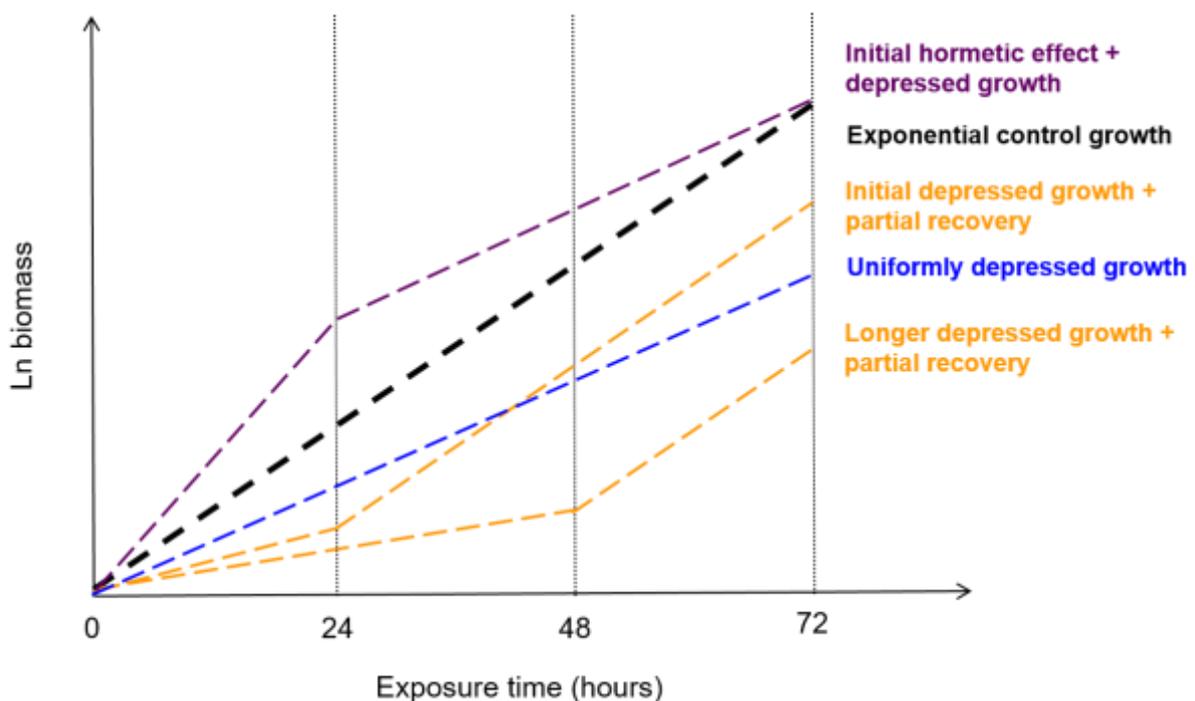


Figure 16. Theoretical examples showing how assessment of biological responses solely at the end of a standardized ecotoxicological tests can miss more complex response patterns with potential environmental relevance and implications for hazard and risk assessment. The selected biological response (natural logarithm of the biomass) is for a hypothetical exposure of an algal population to a chemical over 72 h; *i.e.* the standard duration of algal tests according to norms ISO 8692 (ISO, 2012) and OECD 201 (OECD, 2011).

A third aspect related to test time is the actual correspondence between temporal exposure patterns in the standardized test *vs.* real-world situations (Boesten et al., 2007). Under field conditions, continuous exposure to constant concentrations is just one possible scenario among several other options involving intermittent or pulsed exposure (Luís de Sá Salomão et al., 2020). Pulsed exposures consist in one or more concentration peaks of a chemical followed by periods of no exposure or, better, periods in which the chemical is below analytical detection limits. The latter option may result in undetected exposure if analytical tools do not yet offer sufficient sensitivity compared with concentration of potential ecotoxicological concern.

Intermittent exposures represent periodical fluctuations in the concentration of a chemical that remains always measurable over the period of interest. Under such exposure patterns, organisms may have time to recover between two subsequent concentration peaks whose amplitude, incidentally, does not have to be the same. For single-pulse exposures, knowing the resilience of a population to a chemical may be more important than its resistance to the same chemical. The ability of standardized test of fixed duration and, theoretically, with constant exposure concentration to predict biological responses in such conditions requires careful verification under typical scenarios or even on a case-by-case basis depending on the extension and ramifications of the associated research program.

#### 2.4.2. Some personal considerations on temporal aspects in ecotoxicity testing

The link among time, concentration and toxicity is axiomatic in ecotoxicology; *i.e.* the same concentration of a chemical will usually elicit a stronger response as exposure time increases (Walker et al., 1996). From a fundamental point of view, there is therefore little reason to neglect the temporal dimension of ecotoxicity. From a practical point of view, the generalized use of  $EC_x$  at fixed time of exposure is however easier to understand. Comparing  $EC_x$  for different chemicals after a fixed time of exposure allows their ranking in terms of ecotoxic potency, provided that comparable metrics are used (see section 2.3). However, Baas et al. (2010) caution that such rankings make sense only for individual model species and for chemicals showing similar patterns of response in time. Ranking of chemicals based on  $ET_x$  (effect time; *i.e.*, the time necessary for a given concentration to elicit a  $x$ -percent response) would require the ecotoxic potency of each chemical to be defined by the combination of concentration and time. Such time dependency of toxicity could however be easily accounted for by ranking chemicals on 3-dimensional surfaces rather than on 2-dimensional plots; in analogy to figure 1 of Fox and Billoir (2013) explaining how to incorporate a time component in species-sensitivity distributions (SSD). In a simpler way, the product of concentration and time (*i.e.*, the dose) could also be used to rank chemicals on 2-dimensional plots, while better accounting for the temporal dimension of exposure *vs.* response relationships. However, this approach requires some assumptions to be satisfied because the observed influence of exposure duration on biological responses reflects the overall outcome of toxicokinetic and toxicodynamic processes.

Toxicokinetics describes the distribution and metabolism of chemicals inside the organism and their elimination from the organism, while toxicodynamics studies the link(s) between internal concentration (at the target receptor) and effects (Tennekes and Sánchez-Bayo, 2013). Most importantly, toxicodynamics determines if a given chemical possesses or not a threshold concentration that corresponds to a level of exposure below which no biological response will be elicited no matter how long the exposure time. However, chemicals that bind irreversibly (*i.e.*, for times longer than the organism's lifespan) to their biological receptors do not have a threshold concentration or, otherwise stated, have a threshold concentration of zero. Depending on the reversibility of the chemical-receptor bond and of the corresponding biological responses, concentration–response relationships will have one of the following characteristics:

- i. The response is solely concentration dependent. This happens when both binding to the receptor and the biological response are quickly reversible,
- ii. The product of the concentration and time is a constant (Haber's rule). This situation can reflect both the irreversible binding of a chemical to its biological receptor or the presence of irreversible responses after the chemical-receptor interaction occurred (and even if such interaction is reversible),
- iii. The response to a given concentration of a chemical is reinforced by time. This occurs when both the binding to the biological receptor and the associated response are irreversible.

These characteristics apply only for chemicals that act via receptor binding, but not to those acting by narcosis. These simple considerations, based on the review by Tennekes and Sánchez-Bayo (2013), provide the theoretical basis to understand the importance of the time factor in increasing the relevance of standardized tests. Most interestingly, all the different relationships can be described using the Druckrey-Küpfmüller equation:

$$C \cdot t^n = \text{constant}$$

The values of  $n$  describe the characteristics of the concentration–response relationship: time independence for  $n < 1$ , compliance with the Haber’s rule for  $n = 1$  and reinforcement of effect by time for  $n > 1$  (Tennekes and Sánchez-Bayo, 2013). Even without a more complex mathematical treatment, it is clear that considering how time affects the organisms’ response to chemicals is essential in reconciling standard procedures with environmental realism.

Other temporal issues are more linked to real-field situations in which pulses or intermittent inputs, notably of herbicides and pesticides, rather than continuous exposures are the norm (Ashauer et al., 2007; Luís de Sá Salomão et al., 2020). Understanding pulse exposures is of interest because it allows not only to establish the resistance of organisms to a pulse (*i.e.*, how strong a pulse an organism can stand for given duration before getting impaired), but also their resilience (*i.e.*, how much and how fast they can recover from a pulse). The speed of recovery also determines the frequency of pulse that an organism or a population can withstand without being temporarily or permanently. Copin and Chèvre (2015) found that pulse duration, rather than pulse intensity was the controlling factor inhibiting the growth of two algal species exposed to herbicides inhibiting the activity of photosystem II (PSII). However, the same authors acknowledged that the situation may be different for herbicides showing delayed recovery phases. Furthermore, Laviale et al. (2011) showed that the responses of periphyton communities to pulses of PSII inhibiting herbicides could vary depending on the chosen response metrics, although complete recovery was observed in all cases for pulse exposures of 1, 3 or 7h. The appearance of delayed effects or the absence of recovery following pulse exposures have also been reported, though. Beketov and Liess (2008) observed delayed effects of thiacloprid on aquatic insects following a 24h pulse exposure. Ashauer et al. (2017) showed that sequential exposure to pulses of two insecticides (carbaryl and chlorpyrifos) elicited different responses depending on the exposure sequence. Alonso and Camargo (2015) observed that the freshwater planarian *Polycelis felina* responded differently to ammonia under continuous *vs.* discontinuous treatments, with the former affecting more severely survival and the latter inducing stronger behavioural responses. Such variable response patterns confirm the need for further studies on the temporal aspect of ecotoxicology. Although continuous exposure could result in higher lethality among test organisms in the laboratory, the sublethal effects of chemical pulses may be more environmentally relevant in the long run.

#### 2.4.3. Perspective research activities

The study of pulse exposures seems particularly popular for pesticides and herbicides (see section 2.4.2), in agreement with their discontinuous application and release during conventional agricultural practices. Pulsed releases of trace elements can however occur in relation to *e.g.*, artisanal tannery activities (Szalinska et al., 2013) or weathering of exposed mine stockpiles (Gunkel-Grillon et al., 2014). Hogan et al. (2013) showed that Mg pulses could elicit ecotoxicological responses in different freshwater organisms, although to a lesser extent than continuous exposures. At the opposite, neglecting pulsed-exposure could underestimate the risk of Se to aquatic biota (Hoang and Klaine, 2008). Overall, there is plenty of scope to better consider temporal aspects in assessing the ecotoxicology of trace elements; especially considering their variable concentration and speciation as a function of numerous

environmental factors such as river hydrology and hydrochemistry or seasonal cycles in lentic water bodies.

Understanding temporal effects of chemicals has already led to the development of several models and to strong criticisms of standard ecotoxicological metrics (Fox and Landis, 2016; Jager, 2011; Tennekes and Sánchez-Bayo, 2013). At present, I do not have the necessary level of proficiency to incorporate meaningful suggestions for the improvement of such models in my reflexions for future activities. There are however some aspects directly linked to my research that are unquestionably relevant to the temporal aspects of ecotoxicology; notably the complex behaviour of those elements whose speciation (and concentration) can show marked variations during standardized ecotoxicological tests.

One strategy to handle such issues would be to let the test system to equilibrate and then eliminate the insoluble component before testing (Wilson et al., 2017) or simply engage in research to develop BLMs applicable for such elements (Aharchaou et al., 2020; Crémazy et al., 2013a; El-Akl et al., 2015). On the other hand, one may argue that natural systems are hardly ever at equilibrium. They may be considered at a biological steady state when chemical discharges are continuous and the speciation processes in the recipient environmental compartment are fast compared to the biological responses caused by a chemical. However, discontinuous or pulsed exposure can lead to dynamic situations in which the time scales of chemical speciation and biological responses are comparable. Finally, variations in pH, conductivity and other master variables (all of which usually happen at the confluence between two rivers, downstream point-source dischargers or extreme weather events) will cause dynamic changes that affect elemental speciation and consequently bioavailability and effect (see section 1.3). In such dynamic scenarios, a few questions become of interest in reconciling standard procedures and environmental realism:

- i. How long does it take for a given concentration of a chemical to induce an x-percent response in model organisms under continuous exposure conditions?
- ii. Do repeated pulses of a chemical lead to increased ecotoxicity compared with continuous exposure?
- iii. How long do the responses induced by a pulsed or continuous exposure last or, otherwise stated, how fast and how much can an organism recover when the chemical stressor is removed?

When using algae as model organisms, simulating pulsed exposure may be difficult because the algal suspension will need to be (periodically) separated from the exposure medium to create the desired pattern of chemical pulses. Copin and Chèvre (2015) used centrifugation to transfer cultures of *Scenedesmus vacuolatus* and *Raphidocelis subcapitata* to clean test medium after exposures of different duration to two pesticides. Weber et al. (2019) used a chemostat to maintain an algal suspension at steady state and study the possible effects of the pesticide chlorotoluron under simulated pulse + continuous exposures. Personally, I have thought of employing commercially available multiscreen microplates to provide some answers to questions i–iii above with regard to Cr ecotoxicity to model algae. Multiscreen microplates resemble standard 96-well microplates except that the bottom of each well is equipped with a membrane filter that allows for medium-renewal while retaining algae on the membrane surface inside each well (Radetski et al., 1995). These experimental devices offer some interesting possibilities; notably the three scenarios described below using Cr(III) as an example (Fig. 17).

In a first scenario, algae can be exposed to Cr(III) under semi-static conditions with daily renewal of the exposure solutions. In this way, the concentration of ionic Cr(III) can be reset to its initial value at  $t=24\text{h}$  and  $t=48\text{h}$  (remember that Cr(III) rapidly forms  $\text{Cr}(\text{OH})_3$  by hydrolysis — Aharchaou et al., 2018). At each renewal time and at the end of the test ( $t = 72\text{ h}$ ), exposure solutions can be recovered for analysis of filterable Cr concentration and a time-weighted mean of concentration exposure can be calculated. This configuration provides hints as to possible

intermittent exposure scenarios in real field conditions, although it does not account for differences in Cr speciation between the test medium and natural waters. One additional caveat is that the  $\text{Cr}(\text{OH})_3$  formed in the exposure medium will be (partly) retained on the microplate filters. It is currently not known if  $\text{Cr}(\text{OH})_3$  are ecotoxicologically inert or not. In a second scenario, Cr(III)-spiked solutions can be left to age for different times, recovered by filtration and then used for exposing algae. For sufficiently long aging times, one can assume that the spiked solution has reached a steady state with regard to Cr speciation in the medium. In particular, aging for 72 h followed by filtration before testing would eliminate most of the insoluble  $\text{Cr}(\text{OH})_3$  nanoparticles, thus providing hints as to role of such particles in causing the algal responses observed using standard experimental configuration (see above). A third scenario consists in simulating a pulse exposure by starting a test according to the standard procedure, removing (and analyse) the exposure solutions at the end of the established pulse period and then follow algal growth in clean medium to study the dynamics of algal recovery. Similar configurations can be planned for elements other than Cr(III) or to study the temporal evolution of toxic mixtures of selected elements (section 2.5).

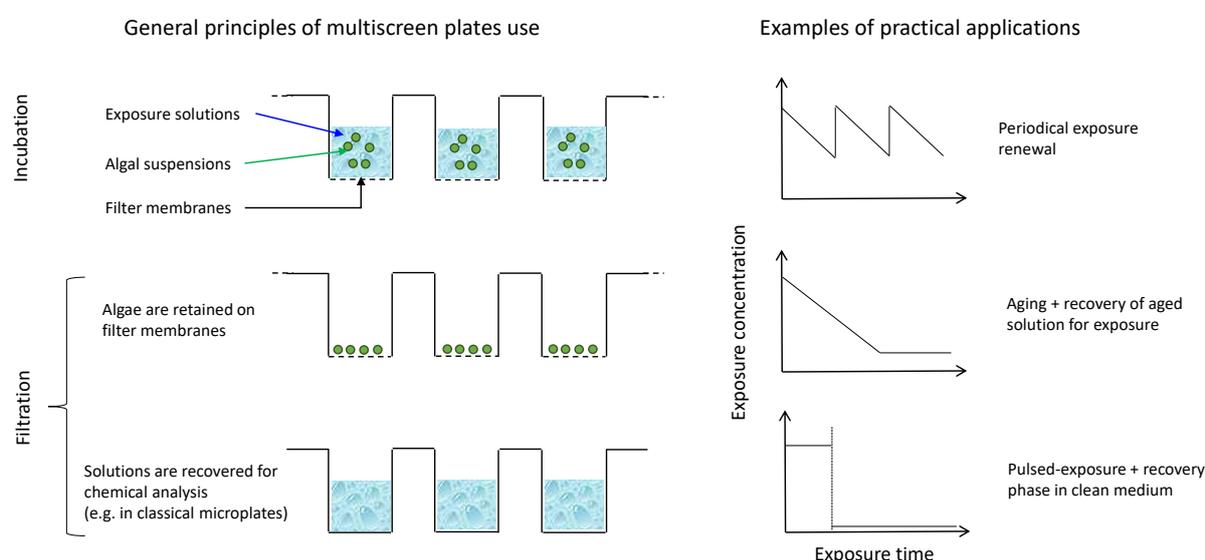


Figure 17. Characteristics of multiscreen plates equipped with membrane filters at the bottom of each plate-well and examples of experimental exposure scenarios for algal suspension.

Questions i–iii can obviously be applied to model organisms other than algae, to communities of primary producers or even using microcosms configurations with several organisms. Such questions have a fundamental value and, in combination with appropriate modelling tools, can improve our ability to incorporate time in the ecotoxicological evaluation of chemicals. Development of standardized approaches to handle temporal aspects of ecotoxicology could equally improve the outcome of large efforts to classify the hazard and risks of chemicals; *e.g.*, the ‘chemical universe’ plan of the European Chemical Agency (ECHA, 2020). In the case of widely used chemicals (or in the case of new chemicals) it is indeed not enough to know how much of the chemical can be safely used, but also how long and how often. In this sense, we should have access to more information on the exposure regimes occurring in real field situations to identify representative ecotoxicologically relevant scenarios for in-depth study (Boesten et al., 2007).

## 2.5. Question 3. How to account for mixture exposures?

### 2.5.1. Background considerations

Standardized procedures have been mostly used to characterize the effect of one chemical at a time on one organism at a time, which results in several limitations (see table 3). Although more investigations on multiple species exposed to individual chemicals are equally required (Baillard et al., 2021), this section will focus on issues related with the study of chemical mixtures. In many real-world situations, organisms will indeed be simultaneously exposed to several of chemicals with changing relative abundances and for variable times. Pesticides formulations are a peculiar case of mixtures that can be assessed as such, because the whole formulation rather than the simple active substance will be purposely introduced into the environment. Intensive research efforts can eventually identify an individual chemical responsible for effects at the population levels in the field (Tian et al., 2021), but resolving mixture ecotoxicity beyond the identification of its modes of action still remains elusive (Escher et al., 2020). Regulation of mixture ecotoxicity may also need improvement as to the development of ecotoxicological information for several individual substances (Kortenkamp and Faust, 2018).

The fundamental understanding of mixture ecotoxicity reposes on a solid knowledge of the ecotoxicity of individual components; the objective being to establish whether combinations of chemicals induce additive, more than additive or less than additive responses. How to model the contribution of each chemical to the overall mixture effect will depend on the mode of action of individual components and their potential interactions (Table 5). Detailed discussions on the strength and limitations of the different models to describe mixture effects can be found in Heys et al. (2016), Kortenkamp et al. (2007) and Lasch et al. (2020). The something from nothing effect for mixtures of estrogenic chemicals (Silva, 2002) or other substances (Carvalho et al., 2014) are good examples of the environmental relevance of mixture ecotoxicology. Such studies confirm that concentration (or dose) addition can be used to predict the effects of mixtures of several chemicals with similar mechanisms of action when each one individually occurs at levels considered as safe. From a regulatory perspective, Directive 2013/39 already uses a component-based approach to account for mixture effects of dioxins and dioxin-like compounds or PAH. In component-based approaches, the toxicity of each component of the mixture is expressed in terms of relative potency referred to the most toxic compounds. A single criterion can then be derived for a group of chemicals with similar mechanism of action (Heys et al., 2016). However, legislation does not yet address mixtures of chemicals belonging to different groups (Kortenkamp and Faust, 2018), despite evidence that mixtures of substances with different mechanisms of action, each occurring at levels considered as safe, may collectively elicit adverse biological responses (Carvalho et al., 2014; Faust et al., 2003; Kortenkamp et al., 2007). Challenges linked to temporal issues, matrix issues, routes of exposure and representativeness of model organisms obviously apply to mixture assessment and should be the focus of further research (Heys et al., 2016).

### 2.5.2. Some personal considerations on mixture exposures

Compared with the plethora of organic chemicals, the number of existing trace elements could seem by far more manageable for mixture assessment. However, using simple combinatorial calculus, we can easily calculate the number of possible binary mixtures of the 92 natural trace elements taken in pairs (*i.e.*, the simplest mixture situations):

$$C(92,2) = \frac{92!}{2! \cdot (92 - 2)!} = \frac{92 \cdot 91 \cdot 90!}{2! \cdot 90!} = \frac{92 \cdot 91}{2} = 4186$$

Where,  $C(92,2)$  is the number of combinations without repetition of 92 variables taken in pairs;  $!$  is the factorial symbol and 4186 is the number of theoretical experiments to be carried out. Such number is both impractical and unrealistic, besides assuming that the bioaccessible, bioavailable and biologically available fractions of each element can be known precisely (see Fig. 9).

Because elements occur naturally, all of them would in principle co-exist in any matrix according to their planetary abundances. The presence of anthropogenic enrichments with possible ecotoxicological consequences could therefore be easily identified and the mixture of enriched elements selected for investigation. Similar approaches would be applicable to elemental enrichments linked to natural causes (Gwenzi, 2020; Minguez et al., 2021). In general, the choice of elemental mixtures to be studied must therefore be made based on their environmental relevance (*i.e.*, two or more elements are frequently enriched in situations of particular environmental, social and economical interest), environmental co-occurrence and enrichment (*i.e.*, lanthanides), known similarities in mode of action, or known antagonistic effects (*e.g.*, Se vs. Hg or Cr(VI)). Note however that the study of elemental mixture can be somehow biased by the availability of detailed information on few individual elements (Nys et al., 2017).

### 2.5.3. Perspective research activities

Following my long-term efforts to better characterize the ecotoxicity of Cr(VI) and Cr(III) toward a few model freshwater organisms (Aharchaou et al., 2018; Ferrari et al., 2019; Ponti et al., 2014; Vignati et al., 2010; Vignati et al., 2019), I have become concerned with considering that these two valence states actually constitute a peculiar case of a binary elemental mixture. The mechanisms by which Cr(III) enters cells are not completely understood and may include internalization of hydrophobic complexes, endocytosis of Cr-bearing particles and transport mechanisms involving transferrin (Beyersmann and Hartwig, 2008). Chromium(III) can also affect membrane integrity (Filice et al., 2019). On the other hand, Cr(VI) easily crosses biological membranes thanks to its structural analogy with sulphate anions (Viti et al., 2014) and gets rapidly reduced to Cr(III) in the intracellular environment (Viti et al., 2014; Zhitkovich, 2005). Besides generating oxidative stress, such reduction creates a situation in which Cr(III) is the dominant redox form occurring in intracellular environment (Fig. 18) and available for interaction with cellular components including DNA. In agreement with the predominance of a single redox state within cells, we have previously shown that Cr has a similar intracellular distribution and interacts with the same biomolecules in populations of the alga *C. reinhardtii* exposed to Cr(VI) or Cr(III) (Aharchaou et al., 2017). Considering that both Cr(VI) and Cr(III) may be of ecotoxicological concern, understanding the ecotoxicity of their mixtures becomes a justifiable fundamental and applied scientific objective.

Unpublished research carried out under my supervision showed that commercially available Isotopically Enriched Solutions (IES) can be successfully used to simultaneously follow the fate of Cr(III) and Cr(VI) in both exposure media and model organisms (Aharchaou, 2017). The use of IES allows an easy analysis of each redox form by conventional ICP-MS; at least in the absence of major interconversion reactions between them. Under this assumption, model organisms can be simultaneously exposed to Cr(III) and Cr(VI) using solutions isotopically enriched in  $^{50}\text{Cr}$  and  $^{53}\text{Cr}$ (VI). Such solutions are commercially available and can help to answer two important questions:

- i. What is the combined (mixture) ecotoxicity of Cr(III) and Cr(VI) to a model organism?
- ii. What is the relative contribution of Cr(III) and Cr(VI) to the total Cr body burden in the model organisms and how does this relate to the observed ecotoxicity?

Simultaneous exposure to both redox forms of Cr is a huge step forward to properly understand how this element exerts its effects in real field situations. Indeed, Cr(III) and Cr(VI)

often occur together in natural environments, albeit in variable proportions (Saputro et al., 2014; McClain and Maher, 2016) and both forms can contribute to Cr body burdens in biota (Semeniuk et al., 2016; Aharchaou et al., 2017) and, eventually, to Cr ecotoxicity in real field conditions. Complementary research activities in this sense are presented in section 2.6.

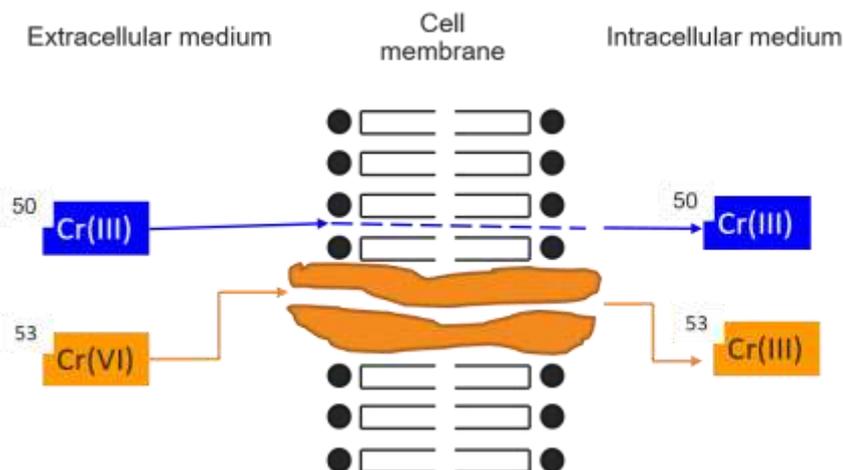


Figure 18. Overview of the isotopic approach proposed for studying the effects and bioaccumulation of Cr(VI) and Cr(III) mixtures on biota. The extracellular medium is spiked with  $^{50}\text{Cr(III)}$  and  $^{53}\text{Cr(VI)}$ . The latter undergoes reduction to  $^{53}\text{Cr(III)}$  in the intracellular environment. Note that Cr(VI) enter cells via sulphate transporters, while the uptake routes of Cr(III) are less understood.

## 2.6. Question 4. How to account for matrix effects and uptake routes?

### 2.6.1. Background considerations

Standardized laboratory test media have a well-defined composition, at least at the beginning of the experiment, to provide optimal growing conditions for the selected model organism. The controlled medium composition also ensures the necessary reliability of the tests for regulatory uses, but is a major trade-off in term of environmental realism. For example, aqueous media for testing with freshwater organisms are hardly representative of natural waters in terms of major and minor elements content, pH, ionic strength, alkalinity and organic carbon content; all of which markedly influence chemical behaviour in real environment. Investigation of such matrix-issues (Heijerick et al., 2005) eventually led to the optimization and regulatory use of BLM for some trace elements (Peters et al., 2011; Peters et al., 2014). Nonetheless, any ecotoxicological model reposes on the availability of good quality and artefact-free ecotoxicological data. Obtaining such data for model development and calibration still remains a challenge for many elements (Gonzalez et al., 2014; Ponti et al., 2014; Song et al., 2014; Vignati et al., 2010; Wilson et al., 2017).

The second major simplification of aqueous standardized media is the absence of environmental particles, despite substantial evidence that ingestion of colloids and suspended particulate matter contribute to contaminant uptake by filter- or deposit-feeders organisms (see section 1.6). Issues of aqueous vs. dietary contaminant uptake also apply in the case standardized whole-sediment tests (Camusso et al., 2012; Ferrari et al., 2019) where the use of formulated sediments may miss some sedimentary characteristics (*e.g.*, organic carbon content, Fe content) that influence contaminant behaviour and bioavailability (Campana et al., 2013; Simpson and Batley, 2007a).

### 2.6.2. Personal considerations on matrix effects and uptake routes

As mentioned in section 2.6.1, one of the most conspicuous differences between aqueous standardized test media and natural waters is the absence colloids and organic ligands in the former. The absence of colloidal or truly dissolved organic matter, which is an important controlling factor for the bioavailability of trace elements (and other chemicals) under field conditions (Twiss et al., 2001; Worms et al., 2006), has important implications. In some cases, it does create conditions of maximum element bioavailability during the tests, in line with the assumption that laboratory tests are conservative (Table 3). Although such situation may lead to overestimate the hazard of a chemical, it automatically applies the precautionary principle and tries to account for differences in species sensitivity, time-scales and combined effects during laboratory to field extrapolation; see *inter alia* Kostich et al. (2017). However, lack of organic matter in test media can favour the formation of insoluble chemical species (e.g., Cr hydroxides, lanthanides phosphates and carbonates) whose occurrence in real-field conditions may be limited by the presence of organic ligands. As a consequence, a major fraction of the element concentration added to the test medium can quickly transform into poorly soluble complexes whose bioavailability is poorly characterized (Aharchaou et al., 2018). In the presence of such complexes, the results of standardized tests can underestimate the actual elemental ecotoxicity (Gonzalez et al., 2014; Ponti et al., 2014; Vignati et al., 2010). At the opposite, the presence of organic matter can increase the solubility of some elements (McClain and Maher, 2016) making them more mobile in the environment and modifying their ecotoxicity (Koukal et al., 2003; Twiss et al., 2001). A detailed characterization of the bioavailable element fraction in standard test media and natural waters would clearly facilitate comparison between laboratory results and field monitoring measurements (see section 2.3).

As to multiple routes of exposure, the relative importance of each route will also depend on the ecology of (model) organisms. For example, the model species *C. riparius* is a surface deposit feeder that can actively accumulate contaminants from freshly deposited particles at the sediment-water interface (Ferrari et al., 2019). Other model species such as *L. variegatus* bury head down in sediments and their chemical body burden may be more directly related to concentration in bulk sediments (Vignati et al., 2020). Finally, ingestion of suspended particles by filter feeders may be an important pathway of contaminants along trophic chains.

### 2.6.3. Perspective research activities

Once again, my practical research plans concerning the effect of matrix composition and route of exposures in reconciling standard investigations and environmental realisms are concerned with improving our understanding of the bioavailability and ecotoxicity of Cr. Specifically, I would like to investigate:

- i) how Cr ecotoxicity is affected by the presence of a few standard colloidal phases,
- ii) to what extent laboratory results in fully standardized media can be extrapolated to natural situations, and
- iii) how ingestion of particle-bound Cr can control its accumulation by filter feeders (e.g., *D. magna*) and deposit feeders (e.g., *C. riparius*).

These perspectives activities also exploit the advantages of the isotopical approach described in section 2.5.; at least for objectives ‘i’ and ‘iii’.

Research on activity ‘i’ is included in a just-funded project on the study of Cr bioavailability to algae. Standardized test medium will be amended with known amounts of selected colloidal carrier phases (goethite, a form of colloidal organic carbon, and Mn oxides) to evaluate how such particles modify algal responses to Cr(VI), Cr(III) and their mixtures. Ideally, the concentration of each carrier phase should be similar to those found in representative natural waters. The occurrence of possible adverse effects of the colloidal on algae and of

Cr(VI)/Cr(III) redox interconversions in the presence of colloids will be experimentally verified. For the latter aspect, the use of isotopically enriched solutions will allow to distinguish between Cr(III) originally added as such, e.g.,  $^{50}\text{Cr(III)}$ , and Cr(III) eventually formed following reduction of Cr(VI) by organic matter or goethite, e.g.  $^{53}\text{Cr(III)}$ . Note that the absence of such interconversions in the standard ISO medium for algae has already been confirmed (Aharchaou et al., 2018).

Activity 'ii' jointly implements question 1 (section 2.3) and question 5 (this section) in relation to the importance of matrix-effects on elemental speciation and bioavailability. One of the conclusions from my previous research is that reconciling standardized test and environmental relevance for Cr(III) ecotoxicity requires a much better consideration of its speciation in ecotoxicological test media to ensure meaningful comparisons with field situations. *In situ* speciation should also be characterized to the highest possible level of detail. Options in this sense can include the use of DGTs, which are also commercially available for the specific detection of Cr(VI) (Pan et al., 2015), and of filtration/ultrafiltration procedures. The ecotoxicity of natural matrices can also be directly tested with *e.g.* algae (for filtered and ultrafiltered waters) and daphnids (for raw, filtered and ultrafiltered waters). The co-occurrence of high level of Ni in natural ultramafic waters may represent an interesting additional challenge in relation to mixture ecotoxicity (question 4, section 2.5). At the end of 2019, before the first Covid-19 outbreak and the following pandemic, we carried out some preliminary work in the Pluhův Bor stream; an ultramafic environment located in the Slavkov Forest, Czech Republic (Krám et al., 2012). Being naturally enriched in Cr and Ni, ultramafic waters provides suitable situations to study the bioavailability and ecotoxicological potential of these elements and their mixture in environmentally realistic conditions.

The use of isotopically enriched solutions can also help in clarifying the importance of different routes of exposure for Cr(III) and Cr(VI), including its trophic transfer from *e.g.*, algae to daphnids or other filter feeders. Because, Cr(III) is particle reactive and Cr(VI) mainly occurs in soluble forms (Bobrowski et al., 2004; Gorny et al., 2016), it is possible to imagine experimental set ups where organisms are simultaneously exposed to  $^{50}\text{Cr(III)}$ -bearing particles and  $^{53}\text{Cr(VI)}$ -enriched solutions. Under the assumption of no redox interconversions between the two valence state (subject to experimental verification), both isotopes can be quantified in exposed organisms after variable time intervals. Such experiments would provide a clear indication on how much Cr(III) and how much Cr(VI) contribute to the total Cr body burden of filter feeders. Analogous experiments can be conceived for deposit feeders such as *C. riparius* (Fig. 19).

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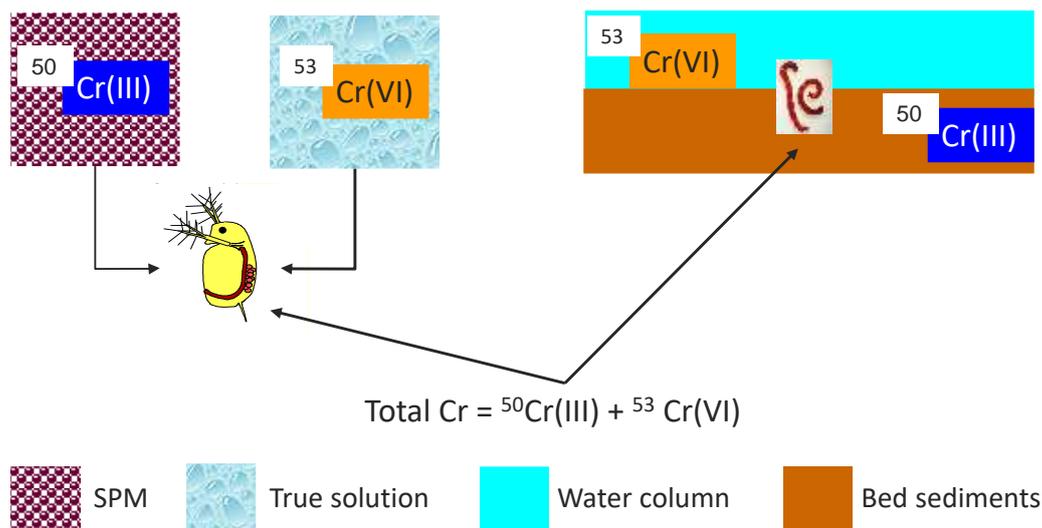


Figure 19. Possible uses of isotopically enriched solutions to distinguish between Cr uptake from the ingestion of contaminated particles (SPM, suspended particulate matter) or directly from true solutions in filter- and deposit-feeders.

Other intriguing possibilities arise when considering that intracellular Cr occurs mostly as Cr(III) (see section 2.5). Let us consider algae exposed to  $^{50}\text{Cr(III)}$  and  $^{53}\text{Cr(VI)}$ , either individually or as a mixture (and always assuming that no interconversions between the two occur in the exposure medium). The isotopical approach allows a clear distinction between the direct accumulation of Cr(III) from the exposure medium ( $m/z = 50$  in the algal biomass) and the accumulation of what was originally present as Cr(VI) in the same medium ( $m/z = 53$  in the algal biomass). Feeding algae with such patterns of Cr accumulation to filter feeders will allow to investigate how both Cr forms pass from aqueous matrices to primary producers to primary consumers. In other scenarios, filter feeders could be simultaneously exposed to algae enriched in  $^{50}\text{Cr(III)}$  and to solutions spiked with  $^{53}\text{Cr(VI)}$ , simulating real-field situation where Cr(III) is taken up by food ingestion and Cr(VI) by uptake from solution (Roditi and Fisher, 1999; Wang et al., 1997). On the other hand, I have to acknowledge that Cr(III) has been often considered as an inert tracer of digestion (Roditi and Fisher, 1999; Wang and Fisher, 1996), but the issue seems to deserve further study (Aharchaou, 2017). Furthermore, the proposed isotopic approach does not eliminate the problems connected with Cr(III) hydrolysis and the associated changes in speciation and exposure concentrations in standard media (see section 2.3). Finally, colloidal Cr(VI) may also occur in the presence of *e.g.* Fe oxyhydroxides that are capable of binding anions at pH values high enough to give them a net positive charge (Warren and Haack, 2001). Even with these caveats, the use of speciated stable isotope solutions does offer exciting opportunities that are surely worth exploring.

## 2.7. Question 5. How to integrate issues such as climate change and river-to-sea continuum in ecotoxicology

### 2.7.1. A few preliminary thoughts for the future

The structure of this section differs from the previous ones simply because my vision on such broad issues is still too limited to propose defensible perspective research activities. Nonetheless, the synthetic context provided in section 1.7 leaves no doubt about the need to incorporate climate change-related issues in future ecotoxicological research. In the same vein, the overarching challenges toward a healthy environment (as related to European strategies such as living well within the limits of the planet or the European Green Deal) clearly have a

systemic character; i.e. they are direct consequences of the presence of humans on the planet and, above all, of their consumption patterns and associated uses of natural resources (EEA, 2015). Along with global-scale investigations, the river-to-sea continuum therefore represents an adequate scale to understand and manage the combined impact of several, simultaneous anthropogenic pressures. The river-to-sea continuum is a socio-ecological concept in which the societal and economical uses of natural spaces are superimposed onto the spatial (longitudinal, lateral and vertical) and temporal ‘natural dimensions’ of a river at the catchment scale. More details on the importance of river-to-sea continua can be found in the scientific agenda of the [Danubius-RI project](#) or in [this synthesis](#) prepared by the CNRS (in French).

The ecotoxicological challenges in preserving the socio-ecological integrity of such continua are easily understood using the DPSIR approach (Driver, Pressure, State Change, Impacts, Response) (Gari et al., 2015). Typical *Drivers* include energy production, agriculture, industry, navigation, and impoundment (e.g., dam construction); each one resulting in several associated *Pressures* such as habitat fragmentation, alteration of sediment budgets and inputs of chemicals. Excessive pressure lead to *State changes* (shifts from lotic to lentic conditions, modification of trophic and chemical status) and, eventually, to unwanted *Impacts*: habitat losses, impaired fish migration, accumulation of chemicals in biota, loss of aesthetic values and so on. Appropriate human *Responses* can reverse trends and restore desirable features. Within the DPSIR framework, climate change represents a global driver superimposed to regional or local ones. In the same framework, the release of chemicals into the environment is just one of the possible pressures contributing to the observable impacts (Burton and Johnston, 2010), although a better global governance of chemicals remains a priority (Wang et al., 2021). Along with many other scientific issues, the conceptual questions discussed in the present HDR therefore bear a certain significance with regard to both climate change and the river-to-sea continuum.

Increases in median temperatures and temperature extremes, water acidification, lower oxygen saturation, increased low-flow duration (and many other state changes) can all modify biological responses to chemical stressors by either impacting the physiological status of biota, changing the bioaccessibility and bioavailability of chemicals or both. These phenomena and many others are already occurring and will almost certainly be further amplified during the 21<sup>st</sup> century (Burkett et al., 2014). A steep learning curve awaits ecotoxicologists aiming at evaluating chemical hazard and risks in fluctuating scenarios of different duration and complexity. In such context, developing a better *ecotoxicology of resilience* (section 1.2.5) may help in appropriately handling chemical risk in the long run.

The ecotoxicology of river-to-sea continuum also includes many aspects that I have not explicitly considered in the present document (e.g., terrestrial environments, atmospheric circulation of chemicals, parasitology, immunology). Recognizing the limited scope of this HDR and to avoid trivial considerations on such complex issues, I will limit myself to a brief discussion to illustrate how ecotoxicology can be a key discipline in cross-cutting challenges using safe water reuse as an example.

The United Nations World Water Assessment Programme estimates that nearly half of the world population is already affected by water scarcity, with a worsening outlook to 2050 (UN-Water, 2018). In practice, huge improvements in water recycling and reuse will be necessary to guarantee safe water for all as well as enough water for all human and natural uses. The changing river hydrological patterns may lead more frequently to situations where the anthropogenic loads from WWTP undergo only little dilution (e.g., possible incipient EC<sub>50</sub> can be reached, Fig. 12) or situations where excess rain brings more pulses of untreated sewage, urban and agricultural run-off to natural waters. It would therefore be necessary that most molecules contained in such anthropogenically impacted waters are properly degraded, although treating diffuse inputs is inherently more complex than handling point sources and may require other strategies. In particular, effluents from wastewater treatment plants contain

several recalcitrant chemicals (e.g., pharmaceuticals, personal care chemicals, hormone-like substances) that can contribute to long-term mixture effects and limit the safe re-use of water for irrigation and drinking purposes. The development of advanced oxidation processes using photocatalysis is one of the strategies being currently explored to tackle this problem (Lofrano et al., 2016; Lofrano et al., 2018), although some challenges remain to ensure that the sector will deliver its promised results (Loeb et al., 2018).

What is of interest in the context of the present HDR project is that developing safe technologies for water reuse implies three distinct steps: the availability of safe-by-design materials with adequate photocatalytic properties, the development of efficient and economically viable degradation processes, and the production of environmentally-safe effluents. While all the three steps are necessary for safe water reuse, ecotoxicology is clearly most concerned with the third step and, indirectly, with the first one. The focus of process optimization has traditionally been the full degradation of the parent compound. However, increasing evidence shows that the elimination of the target molecule from an effluent does not necessarily result in the elimination of its toxicity because of the possible presence of hazardous degradation by-products or of the nanophotocatalytic material added for treatment purposes (Lofrano et al., 2018; Vignati et al., 2021). Ecotoxicology therefore has an extremely important role in ensuring that policy development on water reuse will be informed by the best available science and that policy-relevant research will be carried out timely and effectively (Wang et al., 2021).

## 2.8. Handling sources of bias

2.8.1. On the challenges of correctly positioning one's research  
The present HDR project identifies a few broad scientific questions to reconcile standard procedures and environmental realism (Table 6) and proposes a number of related research topics and possible methodological approaches. However, it leaves many relevant issues uncovered: intraspecific competition, predator-prey interactions, parasitism and commensalism, variable geographical settings, very short and very long time scales, just to mention a few. Because the relevance of any given ecotoxicological investigation is context-dependent (section 1.2), it is vital that all the available information (and all the information gaps) are properly considered when evaluating the fitness-for-purpose of a specific study. Such issues has become well recognised and recommendations to properly deal with them already exist (Ågerstrand et al., 2011; Moermond et al., 2016). On the other hand, we may still need to develop better frameworks to avoid bias in data framing and interpretation. How to handle exceedance of Environmental Quality Standards, or other non-regulatory benchmarks, provides a good example of how bias can quickly and easily creep into (reliable and relevant) ecotoxicological results.

One inherently wrong approach to handle ecotoxicological results, an approach sometimes used in news outlet, is to automatically assume that a risk situation is occurring and management/remedial action is required following the detection of chemical at a level above the corresponding environmental quality criterion. Considering all the assumptions behind the numerical values of EQS and benchmarks, such situations simply call for further research. In data-rich situations, the frequency of the exceedance, its duration, its amplitude and the presence of other chemicals (possible mixture effects) or stressors can be properly evaluated and integrated with biomonitoring, (*in situ*) toxicity testing and other approaches by establishing an appropriate risk assessment framework. In data poor situation, monitoring programs or modelling efforts can be started to collect more information on the issue.

Statement in the line “dangerous substances are detected everywhere” might be useful to raise awareness and promote more responsible (*i.e.*, limited) consumption of chemicals; but will not assist in establishing priorities for fundamental research on, and environmental

management of, chemicals. As often reminded in the present document, standardized tests, on which benchmark values are based, provide a rather good way to assess the inherent hazard of a chemical. However, a lot more is required to understand if the same chemical, alone or in mixtures, poses a risk in real world conditions and, if so, what kind of action is required. While many critical situations exist and (chemical) stress is a worldwide concern (EEA, 2019; UNEP, 2019), the ecotoxicological literacy must be increased among the general public and non-ecotoxicological researchers concerned with the biological implications of environmental contamination.

#### 2.8.2. On some hidden reasons of scientific bias

The advancement of ecotoxicological knowledge and its practical application to the management of chemicals and other anthropogenic stressors require the availability of reliable data and an unbiased interpretation of their relevance, implications and ramifications. As I briefly discussed in section 1.8, the modern research industry can lead some of us to indulge in questionable practices, thus creating knowledge biases that can slow down scientific progress and, left unchecked, result in erroneous risk management decisions (Allen Burton, 2017) and possibly public distrust in science.

Because none of us is 100% immune to the pressures of the research industry and its bias-inducing practices, it seems appropriate to conclude this HDR project by acknowledging my own vulnerability to such pressures and presenting some thoughts on how to protect my research from them. Here, I am particularly concerned with the logical fallacies described by Nuzzo (2015) (see section 1.8) and the potential risks to i) produce biased research and ii) promote bias among fellow scientists and students. The few *check points* presented below, some of them being the subject of specialized research, can be considered as a personal commitment to keep an open eye on issues that are critical to maintain an ethical scientific career and to teach future ecotoxicologists how to protect themselves against some pitfalls of the scientific enterprise.

Check point 1. *Keep up-to-date with scientific progress.* I know this seems a no brainer. After all, it is what we are paid for and also great intellectual fun. Yet, it is a daily fight to protect time for this basic task against a multitude of other obligations generated by research administration, management meetings, evaluation procedures, scholarly writing to obtain extramural funding, teaching and mentoring as well as being mentored (i.e., developing necessary new skills). The sheer number of articles published every week also adds the challenge to select those that one may want to read and understand in depth. It becomes all too natural to progressively narrow reading efforts to a specific question of a given subdiscipline. In today's complex scientific arena, being specialized is necessary, but it's also a short step away from seeing complex environmental problems through a bias-generating subdisciplinary filter.

Check point 2. *Remain humble and critical about the actual implications of your research.* Innovation has become an abused word in the research world. Research agencies, journal editors and the public seem to be expecting ground breaking discoveries to stem from virtually any single experiment. It is therefore tempting to overhype (and it is actually easy to overhype) both the implications of a project proposal and the significance of the associated results. Disagree? How about the extra-terrestrial importance of my research on trivalent Cr? (Trivalent) chromium behaviour and ecotoxicity are obviously key issues for a safe agriculture on Mars, where ultramafic regolith (which is Cr and Ni rich) seems to abound (Vithanage et al., 2019).

A good way to keep this check-point in action would be to advertise the Cha-Cha-Cha theory of science according to which discoveries can arise by Charge, Challenges or Chance (Koshland

Jr., 2007). While Chance indeed implies stumbling on innovative unexpected findings (provided that one has had the time to intellectually train to catch their significance), Charge and Challenge are based on long-term efforts of a whole research community. Wanna dance?

Check point 3. *Do not think you are on a crusade.* It happens that some results do not fit into current theories or go against the current consensus and, yes, I do have my stories on this in relation to my research on Cr ecotoxicity. So, how do I react when I see a Cr paper that blindly supports current consensus? Do I have the right to jeopardize the paper simply because it does not consider aspects that I know well? Would my remarks help in improving the paper or provide a biased opinion on the importance of a marginal issue? Otherwise stated, am I an innovator or a fossil? In his ‘Dictionnaire amoureux de la Science’, at the entry ‘Communauté Scientifique’, Claude Allègre mentions the following quote from Max Plank: “One never convinces anybody about new theories, but people die and the young impose new theories” (Allègre, 2005). In the meanwhile, keep reading (see check point 1).

Check point 4. *Do not forget the real complexity of the big picture and the practical challenges in addressing it.* No matter how interdisciplinary one thinks of his research or his vision, we all still see overarching scientific issues somehow filtered through the lens of the discipline (or the few disciplines) we know better. This is perfectly normal, but should remember us to remain objective when interpreting the significance and the ramifications of our latest results. It should also keep us honest when proposing our approaches to address practical needs or answering project calls. Thorough documents published by the European Environmental Agency or other international organization can provide a reality check for positioning our ideas and findings within cross-boundary issues such as ensuring water sufficiency (quantity and quality) in a changing climate. At the other hand of the spectrum, policy makers, funding agencies and institutions should also become more germane as to the objectives and expectations of specific calls on the same issues. High-risk, innovative approaches to systemic challenges come with no guarantee of results or, otherwise stated, the certainty of obtaining usable results will decrease the permissible level of innovation within a research project.

Check point 5. *Remember that you cannot do it all right (especially alone) even if it may be risky and difficult.* In this increasingly competitive research world, it is easy to become wary of discussing one’s most original scientific thoughts for fear of being scooped by somebody else. The case of Mr. Jouany and Mr. Truhaut, the two French pioneers of ecotoxicology, is emblematic (Vasseur et al., 2020) and it probably just one example among many others. Congress presentations featuring complete (and sometimes published) results may indicate a creeping climate of mistrust where we fear that our yet-to-be-tested ideas may be stolen from us. My recent experience in the European project DANUBIUS-RI for the creation of a pan European research infrastructure on river-sea systems ([www.danubius-ri.eu](http://www.danubius-ri.eu)) has helped me to understand the benefits of large cooperative efforts and of the resilience that it is needed to keep them on track. All in all, it is always worth to search for help. It is therefore regrettable that inequality in access to funding and opportunities may lead to increased competition instead of fostering cooperation, in line with similar risks for other societal issues (World Economic Forum, 2020).

Check point 6. *Deeply thank colleagues for their bluntest comments and teach yourself to ask for them.* Strange one; isn’t it? Consider the following. Number 1: The comment is on your science, not on your person<sup>5</sup>. Number 2: you will often have an explanation to counter the comment. Number 3: better to know it now from a colleague than later from a reviewer, a project evaluator or, worst of all, a tenure-track committee.

<sup>s</sup>Should it really be or sound like an *ad hominem comment*, discuss it straight away with your colleague, your hierarchy and the human resources department; in this order.

### *Check points 7. Stay up-to-date with the trends (and possible pitfalls) of the modern research industry*

The modern research industry, with its emphasis on quantitative production and evaluation, pushes the scientific community toward reckless competition and, alas, promotes and perpetuates suboptimum science and research practices (Edwards and Siddhartha, 2017; Smaldino and McElreath, 2016). It is therefore up to concerned scientists to stay aware of such perverse mechanisms, try not to get caught in them and take corrective action when they do. This kind of concern takes time and commitment (and likely puts the concerned individual at a competitive disadvantage). Edwards and Siddhartha (2017) argue, in my opinion convincingly that increasing focus on productivity beyond a certain optimum actually results in loss of ‘true scientific productivity’. The same authors draw a very bleak picture of how the modern research industry fuels unethical behaviour at both individual and institutional levels. Extreme cases of misconduct such as the submission of scientific articles produced by paper factories (i.e., without any actual intervention from researchers) remain, for the moment, a limited phenomenon (Else and Van Noorden, 2021). However, quantifying even serious misconduct including data fabrication and falsification is difficult and the situation may further worsen for borderline questionable practices including a large range of statistical manipulations of experimental data (Fanelli, 2009; Fraser et al., 2018).

Another phenomenon I want to briefly discuss is the increasing concentration of the scientific citation counts into the hands of a small number of elite scientists. According to Nielsen and Andersen (2021), the top 1% most cited scientist across 118 disciplines got 21% of the total global citations in 2015 (up from 14% in 2000). Two contrasting hypotheses are put forward to explain the phenomenon: i) a worsening situation in which opportunities for new scientific developments are lost or, ii) a self-improving situation where the excessive number of scientists on the intellectual market can be reduced without consequences on the scientific progress. I cannot provide objective evidence to support none of the hypotheses. From a subjective point of view, I do however see a discomfiting analogy with the tendency of global wealth to become progressively concentrated into the hands of 1% of the world population. At the end of 2019, 1% of the world population held 43.4% of the total world wealth, with similar differences occurring within countries (Shorrocks et al., 2020). If science is a highly stratified social system, as stated in the incipit of Nielsen and Andersen (2021), increasing such social inequalities is unlikely to do any good to the scientific community as a whole. This (untested) opinion is not meant to advocate a system without any competition; a system that will probably result in a path where scientific productivity is not at its optimum in both terms of quantity and quality. On the other hand, excessive competition will select r-strategist, opportunistic scientists capable of adapting to rapidly changing scientific fashions and political priorities at the expenses of those who may be well capable of building their own innovative scientific niche if allowed the necessary time and resources.

Many less competitive scientist often have short scientific careers (*i.e.*, are forced to leave academia). However, they often make significant contributions to the research priorities of other scientists without being ever given an opportunity to develop a research agenda of their own. Although many of them find other rewarding professional paths, we could wonder why universities are forced to churn out as much as PhD as possible to maintain their status, ranks and resource levels, while perfectly knowing that they will be unable to provide even temporary jobs for most of those PhD. Embarking in a PhD will provide the concerned individual with a range of soft skills that are highly useful in the job market as a whole. Nonetheless, a high number of unemployed PhD also creates a buyer’s market with an excess offer of highly qualified individuals whose intellectual capital may be lost to jobs that require less

qualifications. Begging the pardon of the committee members and or the école doctorale, the pressure to increase the number of HDR in laboratories to keep (or increase) resource level may go down the same slippery slope. In particular, it remains to be seen whether the number of available PhD scholarships will increase quickly enough to allow each HDR to be paired to at least one PhD student. I refrain from linking all these potential pitfalls of the modern research industry to the new [French law on research](#) for the simple reason that I have not read the law in full at the time of writing.

Check point 8. *Master citation indexes*

When a measure becomes a target,  
it ceases to be a good measure (Goodhart's Law).

It is probably correct to assume that highly-cited scientists are those making the highest impact in their fields. It is equally correct to caution that less-cited scientists may be working in smaller research communities, publishing in outlets other than scholarly journals, or in languages other than English. No matter how you look at them, some bottom questions about citation indexes remain however unanswered: are high citation counts identifying the best science or the best scientific competitors? All other things being equal, is a scientist with 100 publications and an h-index of 40 more impactful than a scientist with 35 publications and an h-index of 35? How do you accurately weight the contribution of the n<sup>th</sup> author in a list of >10 co-authors? And, above all, how can we make sure that citations are appropriate? Are citation counting the impact of an idea or are they counting something else?

According to Werner (2015), citations can have different purposes: provide context and background, make reference to previously published methods, credit previous results, propose improvements. They can also be out of purpose or manipulated in a variety of ways (Biagioli, 2016; Van Noorden and Chawla, 2019). In January 2019, I became curious to evaluate who, and to what purpose, had been citing one of my papers that I consider sufficiently thought-provoking (i.e., Vignati et al., 2010). The main message of the paper was (or should have been) that 'Cr(III) can be more toxic than Cr(VI) and the issue deserves further study'. At that time, the paper had 60 citations according to Google scholar. The question is not if the paper is highly cited (which actually is when you choose the correct index; see below), but rather if the paper managed to be cited for its main message or inspired further research on the topic (including criticisms and results opposing its conclusions).

Based on my very informal analysis, almost half of the citations turned out to be irrelevant (i.e., the paper was cited out of purpose in the introduction of the citing article). Some citation actually made reference to my paper in support of the hypothesis that Cr(VI) is more toxic than Cr(III) (Fig. 20). This may reflect my inability to properly structure the message of the paper or, more optimistically, the good review of the previous literature contained in the paper (credit to Simon Devin, Professor of ecotoxicology at LIEC for this interpretation). Considering the number of irrelevant citation I see when reviewing articles before publication, this personal case is unlikely to be exceptional and points to the need to better verify the relevance of the cited literature during the peer review process.

Clarivate™ ([www.clarivate.com](http://www.clarivate.com)) has recently developed a new tool called 'beamplots' capable of taking into account the weight of a given publication in its specific field. Clarivate™ hails it as a new tool for *responsible research evaluation*. According to such beamplots, Vignati et al. (2010) ranks in the top 13% most cited paper in the specific field (as I write in April/May 2021); which is not too bad considering my overall scientific significance. On the other hand, given my simple personal citation analysis for this paper (Fig. 20), there is not necessarily a

reason to rejoice about my research on Cr being that influential, but I leave the readers to develop their own conclusions.

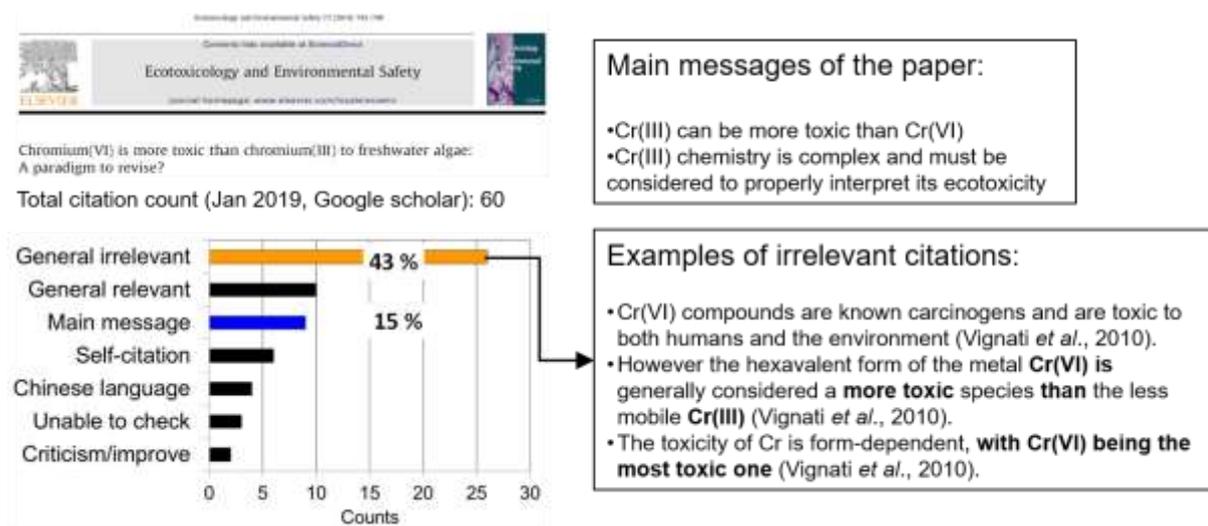


Figure 20. Self-appreciation of the citation pattern for Vignati et al. (2010). The total number of citations was split into sub-categories to verify if reference to the work could be considered as appropriate with respect to the main messages of the paper (see upper box on the right). *General irrelevant*, inappropriate reference in the introductory part of the citing paper (see lower box on the right), *General relevant*, appropriate reference in the introductory part of the citing paper, *Main message* and *Criticism/improve*, most impactful citation either supporting the main message or constructively using the limitation of the paper to justify new research, *Chinese language*, references in Chinese language (unable to evaluate), *Unable to check*, no access could be obtained to the full text.

Check point 9. *I cannot stop them, but I won't stop trying* [Warning, explicit thoughts].

Considering what follows, it is important to remind that these thoughts are primarily addressed to myself and open to discussion. Some statements may reflect personal bias due to an incomplete knowledge of issues that are outside my research field (although they are very present in my everyday scientific life).

In the modern research arena, it is impossible to be bullshit-free, but one can constantly improve at being bullshit-wise (see section 1.8 for a definition of bullshit in the content of this document). Here is a very incomplete list of some tips that should accompany my research years as a (hopefully) HDR holder:

- When chairing a selection or evaluation committee, avoid making any decision based on numerical indicators of any kind. This is a way to reinforce a culture of productivity, not a culture of scientific excellence.
- When submitting an article, take time to make things easy for the reviewers. Preparing a good cover letter and abstract to avoid upfront rejection is a service to yourself and the community. However, actually submitting an advanced draft of a manuscript just to 'give it a try and I will fix it if the reviewers ask for it' unduly shifts your own writing burden onto *bona fide* colleagues. Remember that submitting a paper with shortcomings you have spotted (but really do not have time to fix now) is a questionable practice. And both attitudes are ways to reinforce one's scientific productivity while staying on the wrong side of the bullshit/no-bullshit divide.

- When reviewing an article, take time to make things easy for authors by providing detailed and constructive comments. In the absence of specific suggestions, comments in the vein ‘The results section should be revised for clarity’ are utterly useless. Making a cursory review potentially leaves important shortcomings uncorrected, delays scientific progress and is a way to indirectly contribute to the natural selection of bad science (Smaldino and McElreath, 2016).
- When responding to the reviewer #2 who sent 6 pages of comments packed with practical suggestions and references (not his own), think that s/he has devoted his/her (week-end) time with a sincere desire to help improving the paper. Dismissing the reviewer’s concerns (and resubmitting the same manuscript without correction to another journal) because of the need to publish it quickly, is a way to...well...being unprofessional besides ungrateful. It may also contribute to increasing the circulation of bad science if the paper manage to skip through the peer-review filter with its shortcomings uncorrected.
- When acting as editor, take time to make things easy for authors and reviewers (and I know that many editors are scientists devoting time in a service to the community). Checking compliance with journal formatting guidelines is not the reviewer’s job. Most importantly a reviewer may indeed suggest to shorten a paper to eliminate redundancies. On the other hand, screening initial submissions against a word limit is a way for publishing companies to promote salami slicing, encourage submission of the least publishable units and get more article processing charges (long-life Plan S).
- When discussing where to publish the results with your students, think about the match between results and the journal’s target audience. Choosing a publishing outlet on the primary basis of impact factor reinforces a culture of evaluation based on scientifically meaningless numerical indicators that can be tampered with (Biagioli, 2016).

This list of checkpoints could continue for much longer and quickly get tedious. I therefore prefer to draw a couple of ‘morals’ from all this. The first one is that changing the research industry starts with admitting that its function is not optimal and with discussing what needs to be changed in the interest of science. The second one is that getting unconsciously caught into questionable research practices (shall we call it bullshitting?) may be easier than most of us think; as opposed to serious misconduct. It is therefore important to keep an open eye on such practices, practice self-correction when needed and preach the no bull\*\*\*\* path to fellow colleagues when necessary; none of which is easy and all of which is time-consuming. A third point is that we are becoming aware of the perverse incentives of science industry, but often do not yet have practical alternatives; as pointed out by Tregoning (2018) in relation to the use of impact factors by selection committees who are often forced to apply quantitative criteria as per institutional requirements.

To conclude this politically incorrect portion of text, it is dutiful to admit that I am pretty much satisfied with the way these career issues are handled at my current institution; especially at the laboratory level. I am very sympathetic with all those who strive daily to reconcile the advancement of science with a set of criteria trying to kill it. This does not mean that things do not need further improvement and my personal checkpoints might somehow contribute to kick-off discussions in this sense.

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### 3. Concluding remarks

Considering the pervasiveness of chemicals in our everyday life (EEA, 2019; UNEP, 2019), the ecotoxicological research will continue to play a central role in their effective management for a long time. The last annual report on the Integrated Regulatory Strategy of the European Chemicals Agency has identified nearly 300 chemicals as candidates for regulatory action and many others in need of more ecotoxicological information (ECHA, 2021). At the same time, the steady demand for raw materials will continue to sustain the need for additional research on the biogeochemistry of several trace elements. The scientific questions proposed in the present HDR project should therefore retain their pertinence in the medium- and long-term, especially when it comes to temporal patterns of exposure (Question 2), mixture effects (Question 3) and the importance of different uptake routes (Question 4). These three issues are also necessary to understand and manage more comprehensive challenges such as the ecotoxicology of climate change and of river-to-sea continua (Question 5). Finally, improving metrics of exposure and response (Question 1) is one of the core objectives of fundamental ecotoxicological research. Given such context, I have reasonable hopes for considering the questions proposed in the present HDR (Tables 6 and 7) as appropriate for the development of future research projects.

Each one of the proposed question is actually broad and complex enough to sustain a career-long research on its own. However, only their combination can provide a minimum understanding of the factors that govern the geochemistry and ecotoxicology of chemicals in real field conditions. This does not mean that research funding should be directed almost exclusively toward the solution of known practical, and often pressing, environmental issues. Rather, laboratory results ought to be systematically questioned for their actual relevance in typical real-world situations, so that advances in fundamental ecotoxicological knowledge can be rapidly used to tackle societally relevant problems. Because a good 50% of my research activity at LIEC has revolved around the ecotoxicology of chromium, I plan to start the implementation of the present HDR project by applying some of the proposed research questions to advance current knowledge on the actual hazard and risk of this element (Table 7). However, the proposed questions and the experimental approaches can serve as generators to address similar problems for other elements and chemicals.

From an ecotoxicological point of view, chromium can be considered a binary mixture of its two most commonly occurring redox states, namely Cr(III) and Cr(VI). The study of the Cr(III)/Cr(VI) mixtures has never, to the best of my knowledge, been attempted so far. This knowledge gap may partly follow from Cr(III) having long been considered of scarce ecotoxicological concern (Aharchaou et al., 2018; Ponti et al., 2014). In the just-funded EC2CO project B-Rex-Ist (Bioavailability and ecotoxicity of redox sensitive elements to biota: a stable isotope approach), I propose to use solutions enriched with the speciated stable isotopes  $^{50}\text{Cr(III)}$  and  $^{53}\text{Cr(VI)}$  to start investigations on this subject. The B-Rex-Ist project addresses questions Q3 and Q1a (Table 5) related to exposure metrics in terms of aqueous matrix concentrations and in terms of accumulated Cr by the freshwater alga *Raphidocelis subcapitata*. I hope that this project will serve as a basis for more comprehensive investigations in the future. A project using Cr isotopically enriched solutions to study the mechanisms of toxicity and uptake of Cr(III) and Cr(VI) mixtures using cell culture obtained from the zebrafish (*Danio rerio*) has been submitted to the ANSES (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail). The project is led by Vincent NORMANT (Maître de Conférence at LIEC) whom I will assist for all the aspects specifically related to the use of stable isotopes.

The stable isotope approach could also be used to understand how uptake from dietary and dissolved routes contribute to the total Cr body burden of filter- and deposit-feeders such as the crustacean *Daphnia magna* or the midge *Chironomus riparius*, respectively (Table 5, Q4). While employing stable isotopes to elucidate element uptake by organisms is not unprecedented

(Croteau et al., 2017; Croteau et al., 2007), the (future) commercial availability of isotopically enriched solutions for different redox states of the same element could open new possibilities of investigation. Indeed, several elements (*e.g.*, As, Fe, Sb, Se) have more than one oxidation state of ecotoxicological relevance (Mason, 2013; Schiavon et al., 2017) although the approach may not be viable in the case of As which has only one stable isotope; an unfortunate occurrence given that the differences in the ecotoxicity of As(III) and As(V) (Wang et al., 2015).

The binary Cr(III)/Cr(VI) mixture can be extended to include Ni (Table 5, Q3), thus creating controlled exposure scenarios most relevant to ultramafic environments where both Cr and Ni can occur at levels of ecotoxicological concern (Gwenzi, 2020; Minguez et al., 2021). A new cooperation with the Czech Geological Survey (CGS) will allow easy and regular access to a representative ultramafic environment (Krám et al., 2012; Novak et al., 2017) for validation of laboratory findings using speciation measurements *in situ* and natural aqueous matrices for ecotoxicological testing (Table 5, Q4). In this vision, questions Q1a, Q3 and Q4 can ensure an appropriate combination of continuity and innovation in my research during the first post-HDR years. Question Q1b could find its place as a side project in this general program, especially considering that I have already done some preliminary work on the inhibition of photosynthesis as a metric of response (section 2.3.3).

Table 7. Summary of the scientific questions discussed in the present HDR document along with possible funding sources and tentative timelines. Some questions (*e.g.*, Q1a and Q3) are currently being implemented and will serve as the basis for related research in the medium-term. In some cases, additional brainstorming efforts are still necessary to choose the best way to actually implement selected aspects of a given question.

Scientific questions (see Table 7.1)	Ongoing research	Possible funding sources	Timeline					
			2021	2022	2023	2024	2025	2026
Q1a : metrics of exposure	Yes	EC2CO B-Rex-Ist, ANR Ecotree, Future ANR project	■	■	▨	▨	▨	▨
Q1b : metrics of response	No	Future ANR project		▨	■			
Q2 : temporal aspects in ecotoxicology	No	To be defined (related to IEA; see Q4)		▨	■	■	■	■
Q3 : mixture exposures	Yes	EC2CO B-Rex-Ist, ANSES project (submitted)	■	■	▨	▨	▨	▨
Q4 : matrix effect and routes of exposure	Yes	eLTER funding for field trips, IEA, Future ANR project		■	■	■	■	■
Q5 : climate change, river-to-sea continuum	No	MSREI + MSCA		▨	▨	■	■	■



(Planned) Implementation



Brainstorming



Further application

EC2CO, project call ‘Ecosphère continentale et côtière’ from the CNRS; IEA, International Emerging Action funded by CNRS; ANSES, Agence nationale de sécurité sanitaire de l’alimentation, de l’environnement et du travail; eLTER, Long-Term Ecosystem Research in Europe; MRSEI, Montage de Réseaux Scientifiques Européens ou Internationaux; French ANR); MSCA, Marie Skłodowska-Curie Actions.

Question Q2 can be approached from both a standardized and environmental-realism perspective (Table 5). In the first option, exposure times and pulse-patterns would be chosen based on practical constraints (*e.g.*, daily renewal of solutions) or scientific curiosity. For the second option, information on the temporal changes of ambient chemical levels and speciation will be required. Two master thesis have already confirmed the experimental feasibility of using multiscreen plates for periodic medium renewal or for testing aged solutions using freshwater

algae in standardized laboratory conditions. Similarly, a preliminary field campaign in the ultramafic Pluhův Bor basin (Krám et al., 2012) has confirmed that DGT for Cr(III) and Cr(VI) can be successfully deployed to obtain information of Cr speciation in real-field conditions. A proposal for two field trips to the same site has just been funded under the umbrella of the European Research Infrastructure eLTER and I do hope that it can serve as a basis for an International Emerging Action (IEA) funded by the CNRS to be submitted for 2022–2023.

More complex questions related to the ecotoxicology of climate change and river-to-sea continua (Table 5, Q5) are presently at the brainstorming stage. It is however appropriate to mention both issues because they provide the general framework to address long-term, ecotoxicity-modifying factors (*i.e.*, temperature increase or water acidification related to climate change) or the changing pressures to which organisms are exposed along a river-sea continuum. Furthermore, framing specific ecotoxicological questions in broader frameworks will help in identifying ecotoxicological issues and situations of general interest. In practice, I am currently contributing to the discussions of the LIEC internal group ‘soil-to-water continuum’. At the time of writing, we have identified a couple of research priorities for further discussion: i) extreme events (floods and extended droughts) related to climate change as modifying factors of chronic pressures and ii) contaminant *vs.* organisms interactions along the soil-water continuum. The ecotoxicological problems linked to the removal of recalcitrant compounds from the effluents of municipal wastewater treatment plants (see section 2.7) could constitute another interesting research axis related to Q5. Whether it will be advisable to submit research proposals to calls such as MRSEI (Montage de Réseaux Scientifique Européens ou Internationaux) or MSCA (Marie Skłodowska-Curie Actions) also depends on the research priorities of other researchers with whom I am currently working and requires further discussion.

Finally, the topic ‘How to handle sources of bias (section 2.8)’ will hopefully span the remainder of my research career and, for this reason, has not been assigned a specific timeline in table 5. In response to my own criticism to the modern research industry, I have decided to join the newly created ‘[Peer Community — PCI — in Ecotoxicology and Environmental Chemistry](#)’. This initiative (along with other analogous PCI groups) tries to break away from some problems of the current publishing system, notably the lack of transparency of the peer review process and the need to publish in (high-impact factor, pay-per-view or pay-per-publish) journals to get credit for a given piece of work. In the PCI system, any submission will undergo an open and interactive peer-review process and eventually receive a unique digital object identifier (doi). In this way, an article pre-print and the associated data initially uploaded onto open access repositories (e.g., HAL; <https://hal.archives-ouvertes.fr>) will become a fully-citable item having undergone a full peer-review as it is the case for journals owned by publishing companies. Due to career considerations, the authors retain a full right to submit the reviewed manuscript, as ‘recommended’ by the PCI, to a traditional scientific journal. However, this step would not be mandatory under the PCI rules.

Without being too naïve about the changes that this approach may bring forward, I do hope that it will attract some attention. Junior researchers will probably have to weigh the pressure for open-access publication against the need to comply with recruitment or tenure-track criteria based, among the others, on journal impact factors. On the other hand, senior authors having already achieved a solid scientific reputation may choose such new publication outlets to publish inspirational review papers and pave the way for further changes in the way we publish and evaluate our research. The following quotation of the *Extended Epstein-Heisenberg Principle* seems an appropriate way to conclude this document and to protect my credibility against changes and delays in the research schedule proposed in table 5:

In an R & D (*Research and Development*) orbit, only 2 of the existing 3 parameters can be defined simultaneously. The parameters are: task, time, and resources (money).

1) If one knows what the task is, and there is a time limit allowed for the completion of the task, then one cannot guess how much it will cost.

2) If the time and resources are clearly defined, then it is impossible to know what part of the R & D task will be performed.

3) If you are given a clearly defined R & D goal and a definite amount of money which has been calculated to be necessary for the completion of the task, one cannot predict if and when the goal will be reached.

4) If one is lucky enough to be able to accurately define all three parameters, then what one is dealing with is not in the realm of R & D.

This version of the Epstein-Heisenber principle has been copied from the freely accessible [intranet page](#) of the School of Mathematical Sciences of the University of Nottingham).

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