EUROPEAN COMMISSION

Community research

STAR

(Contract Number:Fission-2010-3.5.1-269672)

DELIVERABLE (D-N°4.3)

Tools for mechanistic understanding of induced effects for mixed exposure

Author(s): Rodolphe Gilbin, Claus Svendsen, Nele Horemans, Clare Bradshaw, Hans-Christian Teien, Dag Anders Brede, Cécile Dubois, Sandrine Frelon, Louise Kiel Jensen, Catherine Lecomte-Pradines, Adrien Margerit, Francisco Nascimento, Deborah Oughton, Dave Spurgeon, Brit Salbu, Hildegarde Vandenhove

Editors: Rodolphe Gilbin, Claus Svendsen, Nele Horemans, Clare Bradshaw, Hans-Christian Teien, Laureline Février

Reporting period: 01/02/2014– 31/07/2015

Date of issue of this report: 6/07/2015

Start date of project : 01/02/2011 Duration : 54 Months

STAR

STRATEGY FOR ALLIED RADIOECOLOGY

EURATOM

DISTRIBUTION LIST

Name	Number of copies	Comments
André Jouve, STAR EC Project Officer	1	Electronically (pdf file)
Laureline Février, STAR Co-ordinator; WP-1, IRSN	1	Electronically (pdf file)
STAR Management Team members WP-2; T. Ikaheimonen, STUK WP-3; A. Liland, NRPA WP-4; H. Vandenhove, SCK•CEN WP-5; F. Alonzo, IRSN WP-6; L. Skipperud, NMBU WP-7; B. Howard, NERC	1 per member	Electronically (pdf file)
STAR Steering Committee M. Steiner, BfS A. Real, CIEMAT J-C. Gariel, IRSN T. Ikaheimonen, STUK H. Vandenhove, SCK•CEN C. Bradshaw, SU A. Liland, NRPA B. Howard, NERC B. Salbu, NMBU N. Fisher, SUNY J. Nishikawa, Tokai Univ.	1 per member	Electronically (pdf file)
STAR Wiki site		Electronically (pdf file)
STAR's External Advisory Board	1 per member	Electronically (pdf file)
ALLIANCE members	1 per member	Electronically (pdf file)

Project co-funded by the European Commission under the Seventh Euratom Framework Programme for Nuclear Research &Training Activities (2007-2011)		
Dissemination Level		
PU	Public	PU (after 01/07/2017)
RE	Restricted to a group specified by the partners of the	RE (before 01/07/2017)
CO	Confidential, only for partners of the [STAR] project	

Executive Summary

This document is the final Deliverable for the third task of the STAR Work Package 4 "Environmental Radiation Protection in a Mixed Contaminant Context". The overarching goal of this task was to provide new experimental data for the assessment of the combined toxic effects of radioactive and stable substances to the ecosystems. The first aim was to show that the joint toxicity of such mixtures is predictable from single substance toxicity data, according to the additivity concepts now established in the literature for stable contaminants. Then, the objective was to identify the exceptions from additivity, *i.e.* cases where interactions (synergism or antagonism) occur in mixtures including radioactive substances.

A few exemplary binary mixtures were considered (gamma irradiation or uranium as radioactive substance; cadmium or fluoranthene as stable substance), on the basis of their modes of action, with theoretically similar and dissimilar toxic modes of actions. Five different species groups (the nematode worm *Caenorhabditis elegans*; the aquatic plant *Lemna minor*; the fish *Salmo salar*; the crustacean *Daphnia magna*; the unicellular algae *Pseudokirchneriella subcapitata*) were chosen to assess the toxic effects of those binary mixtures. Selecting different species groups was expected to aid in the extrapolation process and the generalization of conclusions for a whole ecosystem (i.e. additive effects or interactions). The study of interaction mechanisms was also implemented at different biological organization levels (sub-cellular/cellular, individual, species life cycle, and community) in support to the identification of underlying mechanisms at the origin of the interactions (i.e. whether in the exposure media, at toxicokinetic and/or toxicodynamic levels).

The experimentally observed toxic effects were analysed against the joint effect predictions according to the Concentration Addition (CA) or Independent Action (IA) concepts, as it is now established in the literature. Possible synergistic or antagonistic effects were subsequently investigated by statistical tests comparing measured mixture toxicities with the predicted values. For a generalization of the description of combined effects over time and among endpoints, this approach was also applied for the nematode *C. elegans* to more complex, but biologically relevant toxicokinetic and toxicodynamic models (DEBtox).

This work showed that the joint effects of radioactive and stable substances could be predicted in a robust way from single substance toxicity data, according to CA or IA concepts. Moreover, the results showed that interactions at different levels may result in deviation of mixture effects from the reference model predictions. Those interactions were further assessed for two binary mixtures:

- Despite their different toxic potent between species, an overall antagonism between uranium and cadmium was identified for all tested organism and almost

all toxicity endpoints. This antagonism was further explained by interactions in the exposure media, and/or interactions for bioavailability and bioaccumulation. Focusing on bioavailable and internal cadmium and uranium concentrations allowed revealing potential toxicodynamic interactions.

- The combined effects of gamma irradiation and cadmium showed also a mostly antagonistic interaction pattern, although less clear among species. An antagonistic interaction was observed for both *Lemna minor* and *Daphnia magna* at low cadmium toxic concentrations, whereas at higher irradiation dose rates levels or high cadmium concentrations synergistic interactions might occur. On the other hand, *Salmo salar* and *Caenorhabditis elegans* exposure to gamma irradiation and cadmium mixture did not reveal any obvious synergistic or antagonistic effects. Those contrasted patterns may be the result of complex toxicodynamic interactions.

The experimental and modelling practice developed within this task is important since a review of past experiments demonstrated that mixture experiments involving radioactive and stable substances were not always performed according to optimal experimental set-up and robust interpretations. The tools proposed in this document will direct scientists to adequate experiment set-up, experiment execution and data assessment.

Most of the obtained results demonstrated interactive effects. Although mostly antagonism, some synergistic interactions were identified. The number of scenarios, test organisms and mixture combinations that could be tested in the frame of this project was limited and conclusions should be confirmed by additional experiments. Possible interactions at low toxic doses remain a question. Moreover, interactions may remain at higher level of organization (trophic/population) and long term exposures that were not address in the performed experiments.

List of Acronyms

CA	Concentration Addition
DEB	Dynamic Energy Budget
DL	Dose Level dependant interaction
DNA	Deoxyribo Nucleic Acid
DR	Dose Ration dependant interaction
EAB	External Advisiory Board
EC	European Commission
EQS	Environmental Quality Standard
ERA	Ecological Risk Assessments
FL	Fluoranthene
IA	Independent Action
NEC	No Effect Concentration
NOECs	No Observed Effect Concentrations
NORM	Naturally-Ocuring Radioactive Materials
PBPK	Physiologically Based Pharmacokinetic
PBTK	Physiologically Based Toxicokinetics
PNEC	Predicted No Effect Concentration
PNEDR	Predicted No Effect Dose Rate
TD	Toxicodynamic
TK	Toxicokinetic
TU	Toxic Unit

Table of Contents

Foreword

The overarching goal of the STAR Work Package 4 "Radiation Protection in a Mixed Contaminant Context" is to determine if radiation protection criteria for wildlife are robust, even within a mixed contaminant context.

To achieve this goal, four specific objectives were pursued:

1. Critically review existing approaches, methods and tools developed in ecotoxicology for assessing exposures, effects and risks in a mixed contaminant context and evaluate their applicability for radioecological research and radioecological risk assessments (task 1; D-N°4.1, Vandenhove et al., 2012a).
2. Test and improve selected ecotoxicological approaches and tools for reliable radionuclide (bio)availability and exposure assessment under mixed contaminant conditions, and improve the understanding of underlying mechanisms and processes (task 2).
3. Apply selected approaches developed in ecotoxicology to assess the impact of mixed contaminant conditions on radiation induced effects, and improve the understanding of underlying mechanisms and processes (task 3; D-N°4.3 – this document).
4. Integration of all research and technology development results for a critical evaluation on how mixed contaminant conditions may affect radiation protection standards (task 4).

This document is the final Deliverable for the third task. The goal of task 3 was to provide new experimental data in order to evaluate: (1) if the experimental approach selected allows for the assessment of multiple contaminant interactions with radioactivity; (2) if toxicity of such mixtures is predictable from single substances toxicity data; (3) the species sensitivity to multiple contaminant conditions; and (4) the uncertainty associated with the use of existing mixture toxicity models.

This work benefited from the already existing expertise in the various partner laboratories for experimentation on different model species: the nematode *Caenorhbditis elegans* (IRSN, NERC), the plant *Lemna minor* (SCK•CEN), the fish *Salmo salar* (NMBU) and plankton communities with *Daphnia magna* and *Pseudokirchneriella subcapitata* (SU, IRSN).Through the research activities performed, we endeavoured to favour integration between the STAR partners by (1) selecting similar approaches (experimental conditions, data treatment), (2) by knowledge and expertise exchanges, and (3) scientific collaborations:

- NERC and IRSN provided a web-based course to teach the mathematical methodology to assess mixture effects so the involved partners use the same approach.

- Two mixtox courses were organized at SCK•CEN together with NERC and IRSN (common with WP5).
- IRSN was involved in experiments for *C. elegans* (in interaction with NMBU) with the support of NERC for experimental design and interpretation of obtained results, as well as to establish the D-R curves for Cd and fluoranthene.
- SCK•CEN performed the experiments with *L. minor* with the support of NERC for experimental design and interpretation of obtained results.
- NMBU (with NRPA) worked with *S. salar* with the support of NERC for experimental design and interpretation of obtained results
- SU worked with plankton communities with the support of NERC for experimental design and interpretation of obtained results
- SU and IRSN performed part of their experiments at SCK•CEN irradiation facilities (which has the added benefit of promoting integration and sharing of facilities).
- NERC assisted with the experimental design and data treatment for all partners. They shared their expertise on effects studies with organics.

Note that many of the results presented in this report are preliminary and still unpublished. In some cases, further data analyses, experiments and interpretation may actually change slightly the conclusions given in this document. The definitive conclusion of each series of experiments will be given in the peer-reviewed papers already submitted (see list page 85) or to be submitted.

The diffusion of this document is Restricted,
only for partners of the [STAR] project, during 2 years.
It will be publically available after 01/07/2017.

1 Introduction

Increased industrialization and population densities have led to humans and the environment being exposed to a multitude of contaminants, for which little is known about their combined health and ecological consequences. The issue of multiple contaminants has been addressed in a number of international projects (e.g. NoMiracle (Lokke, 2009), BEAM (Backhaus et al., 2010), PHIME (2011)) and reviews (Kortenkamp et al., 2009; Van Gestel et al., 2011). However, these approaches still do not consider radioactive contaminants, nor integrate the recent derivation of environmental radiation protection criteria by international organizations (e.g. IAEA, 1992; ICRP, 2008; UNSCEAR, 2008; EU, 2014) and EURATOM projects (ERICA, Larsson, 2008; PROTECT, Howard et al., 2010). In the framework of radiological protection of the environment, recent consideration has been given to the mixture issue under the umbrella of the IUR (2011) and IAEA EMRAS II (IAEA, 2011) working groups on Multiple Stressors. Although about three-quarters of the papers reviewed suggested some form of interaction of effects existed among the stressors, this review (Vanhoudt et al., 2012) highlighted that conclusions were mostly based on the incorrect principle of effect summation or on own judgment of the authors. In many cases this stems from the fact that the studies were not designed specifically to investigate mixture or interacting effects (dose-response curves not fully covered, confounding environmental factors, lack of systematic quantitative assessment of exposure concentrations/doses, lack of mechanistic understanding…) and from misunderstandings (or misuses) of concepts for the description of combined effects.

Below, as a justification of the present research, the main conclusions of the critical review of existing approaches, methods and tools for mixed contaminant effect assessment in ecotoxicology, and their usefulness for radioecology (D-N°4.1, Vandenhove et al., 2012a) are summarized. Then, the general objectives, hypotheses and the applied strategy to assess the effects of mixtures including radioactive substances, and to improve the understanding of underlying mechanisms and processes, are presented.

Co-occurrence of radioactive substances and stable contaminants: a reality!

Radionuclides occur together with other contaminants in many situations such as routine liquid releases from nuclear power plants (Garnier-Laplace et al., 2008), high-level radioactive waste disposal (Harju-Autti and Volckaert, 1995), uranium mining and milling (Geletneky et al., 2002; Salbu et al., 2011), NORM industry (Tayibi et al., 2009; Müller et al., 2000). In addition to the above controlled and planned releases of radionuclides by industries, radionuclides have been released to the global environment following a series of historic events (nuclear weapon tests, use of depleted uranium ammunition, nuclear weapons accidents, nuclear reactor accidents, dumping of nuclear waste at sea). Adding to the list is the use of radionuclides for medical purposes, research, or specific uses in the industry. This shows that radionuclide releases in the

environment are expected to occur at extremely varying situations where other non-radioactive contaminants are present.

General concepts are available for the description of combined effects.

Effect characterization in support of Ecological Risk Assessment under mixed contaminant exposure conditions is a major challenge (Eggen et al., 2004). In the case of very complex mixture or specific situations, the only way is to study the entire mixture as a whole, without identifying the individual chemicals and their possible interactions (Groten et al., 2001). However, those results can hardly be generalized, or be used for an *a priori* ERA. Another way to consider mixtures is to use mathematical models for the prediction of combined effects, based on the known individual effects of contaminants (Groten et al., 2001). This approach has the advantage to allow the use of knowledge on single contaminants ecotoxicology, as well as being compatible with most of the ERA frameworks (EC, 2011). Two mathematical reference models, "concentration addition" (CA) and "independent action" (IA), are generally accepted for the prediction of the combined effects of contaminants.

The review performed at the beginning of STAR WP4 project (D-N°4.1, Vandenhove et al., 2012a) concluded that there is no theoretical or conceptual limit that would prevent the application of the general concepts already proposed for the handling of mixtures in ecotoxicology. Particularily, CA and IA models are scientifically validated approaches in support to component-based Cumulative Risk Assessments under the assumption of no interactions between the stressors, and provide a basis for the consideration of mixtures with radioactive substances. However, from an experimental point of view, the exploration of mixtures including radioactive substances may be challenging (non-monotoneous effect patterns of gamma irradiation at low doses, scarcity of irradiation facitities…) as well as incorporating external radiation doses into the existing conceptual framework (e.g TUs).

The underlying assumption of CA and IA models is additivity of the individual stressor effects, i.e. no interaction between the contaminants. There is considerable evidence from research on non-radioactive contaminants that the effect of multiple contaminants are frequently additive, with some exceptions however, that jeopardize the robustness of mixtures ERA methodologies (Kortenkamp et al., 2007; Baas et al., 2010a,b).

The challenge remains to identify the exceptions from additivity, *i.e.* cases where interactions (especially synergistic interactions) occur in mixtures including radioactive substances. In those cases, a need for mechanistic understanding of interactions at different process levels (interactions in the exposure media, interactions at uptake sites, toxicokinetic, or toxicodynamic interactions) is needed. In most cases such needs arise from an ERA perspective, where both quantifying the interaction amplitude and identifying their origins are required for the development of alternative mechanistic models (*e.g.* PBTK and dynamic models) in support to the refinement of Risk Assessment.

2 Objectives, Tested hypotheses and Experimental strategy

In this context, the overarching goal within STAR - Work Package 4 was to determine if radiation protection criteria for wildlife are robust enough, even within a mixed contaminant context. An experimental strategy was established, using (adapted) approaches applied in ecotoxicology, to evaluate whether additive joint action (CA or IA) alone could describe the effects observed or if interactions would be highlighted among several plausible binary mixtures including radioactive substances.

If no interaction occurs for different organisms (eg. plants, invertebrates, vertebrates), then consideration of mixtures using an additive joint action approach may be considered acceptable (though it should be recognized that it may be difficult to make firm conclusions based on a low number of experiments). If, however, significant interactions do exist, then additional consideration may be required when evaluating protection criteria for radiation in the presence of other contamination.

2.1 Tested hypotheses and overall approach

The experimental strategy was chosen in regard to two hypotheses to be tested:

H1: The effects of chemical contaminants and ionising radiation are additive.

H2: If there are interactions between the effects induced by ionising radiation and chemical contaminants, these interactions will be independent of test organism.

The general approach was to attempt to derive dose-response curves for each of the single components in the mixture, and then to apply the general concepts of CA/IA to mixtures including ionising radiation or radionuclides, both to make predictions on mixture effects addition as well as to assess deviations from addition. This approach is also possible to apply to more complex, but biologically relevant toxicokinetic and toxicodynamic models (eg. DEBtox, Jager et al., 2010 – see §3 for details). This would allow a generalization of the description of combined effects over time and among endpoints, as well as extrapolations to other mixtures, other species, and other exposure situations. The identification of underlying mechanisms at the origin of the interactions (ie. whether in the exposure media, at toxicokinetic and/or toxicodynamic levels) are then required in support to its proper description and generalization.

In regard to the two above hypotheses, a choice was made to experimentally testing a limited number of binary mixtures, and on a limited number of species.

2.2 Choice of binary mixture

We considered a few exemplary binary mixtures1 (a radioactive contaminant - either external irradiation or internal contamination - and a stable contaminant), chosen on the basis of modes of action, and representative of existing or planned situations. An effort was also made to select co-contaminants with theoretically similar and dissimilar modes of action compared to radioactive substances. An effort was also made to understand the mechanisms underlying the interactive effects. The selected binary mixtures for effects-directed research are presented in Table 1. The Table 2 describes in more detail the reasons for the selection.

Table 1: Binary mixtures tested for effects directed research within STAR-WP4

stable / *radioactive*	**Cadmium (Cd)**	**Fluoranthene (FL)**
Gamma (γ) irradiation	x	x
Uranium (U)	x	x

[1] The final experimental plan is the result of STAR partners consensus, and followed the recommendationsrecommandations from the External Advisory Board (STAR workshop (November 2011); WP4 workshop (May 2011); STAR-expert consultation workshop (January 2012)). See MS43 (Vandenhove et al., 2012b) for details.

Table 2: Summary of the information in support to the selection of radioactive and stable stressors for their binary mixture effect testing

	Ionizing radiation *(external gamma)*		**Uranium** *(as uranyl)*			**Cadmium**			**Fluoranthene**		
General occurrence	natural background with primordial (U and Th-series, K-40) and cosmogenic (C-14, H-3, Be-7) radioisotopes and cosmic radiation; artifically produced radionuclides (routine releases from nuclear industries (from mining to waste) and accidents; military uses…).		naturally occuring, levels increased by nuclear industries; long half-life			ubiquitous in all NORM contaminated sites, levels increased by mining, smelting, refining, fuel combustion, etc.; Priority substance under the Water Framework Directive			one of the priority list PAH (FL is usually maximum 1/20 of total (16 USEPA) PAHs concentration), used as a model PAH and indicator of other more dangerous PAH; formed from coal/oil combustion, persistent in the environment, high potential for biomagnification.		
Mode of action	oxidative damages of biomolecules (DNA, proteils, lipids), oxidative stress, genotoxicity		low radiotoxicity of natural/depleted uranium; high uranyl affinity for carboxilic and phosphate groups, interaction with essential ions (eg. Ca, Mg) through binding to membrane transport and cytosolic proteins; oxidative damages, genotoxicity			high affinity for sulfhydryl groups, replaces essential ions (Ca, Mg), inactivates functional protein (ROS regulation, DNA repair) with genotoxic and oxidative stress consequences; detoxification by metallothionein induction			non-polar narcotic, non-specific binding binding to cell membranes; readily metabolized by fish; suspected mutagenicity; interaction with plant hormone metabolism and photosynthesis processes; similar toxicity for different plant and animal species		
Basis for the selection	reference for the study of radiation-induced effects; controlable exposure (continuous, homogeneous, easily quantifiable)		importance of uptake, toxicokinetic process, and interaction with other cations; ecologically relevant; delivers full dose-response at realistic exposure			same as for uranium + extensive literature on its ecotoxicity			relatively high solubility and low volatility, ease of handling in a lab-based experiments; extensive literature on animal toxicity		
Typical level of exposure	*µGy/h (internal+external)*	*ref*	*µg/L(µg/kg)*	*µmol/L*	*ref*	*µg/L(µg/kg)*	*µmol/L*	*ref*	*µg/L(µg/kg)*	*µmol/L*	*ref*
Unaffected ecosystems											
Soil	7.10^{-2} - 6.10^{-1} (up to 6.10^{1})	a, b	5.10^{2} - 5.10^{4}	2.10^{0}-2.10^{1}	d	3.10^{2}	3.10^{0}	g	< 5.10^{2}	2.10^{0}	i
Seawater	1.10^{-1} - 6.10^{0} (up to 3.10^{1})	c	2.10^{1}	1.10^{-2}	e	1.10^{-3} - 4.10^{-2}	1.10^{-5} - 4.10^{-4}	h	1.10^{-3}	5.10^{-6}	w
Freshwater	4.10^{-1} - 4.10^{0} (up to 6.10^{1})	b	1.10^{-1} - 2.10^{1}	4.10^{-4}- 10^{-1}	e, f	1.10^{-1}	1.10^{-3}	g	2.10^{-2}	8.10^{-5}	w
Contaminated ecosystems											
Soil	2.10^{2} - 4.10^{2} (up to >5.10^{4})	j, k, l	1.10^{4} - 1.10^{6}	4.10^{1} -4.10^{4}	n	7.10^{3}	7.10^{1}	p	9.10^{1} – 1.10^{5}	4.10^{-1} - 5.10^{2}	r
Seawater	1.10^{2}										
Freshwater	3.10^{1} – $4. 10^{2}$	m	2.10^{1} - 5.10^{2}	10^{-1}-8.10^{1}	o	2.10^{1}	2.10^{-1}	q			
Screening value											
Soil	1.10^{1} (2.10^{0} to 2.10^{2})	s	1.10^{5}	4.10^{2}	t	1.10^{3} – 2.10^{3}	1.10^{1} – 2.10^{13}	v	1.10^{3}	7.10^{0}	w
(Fresh)water	1.10^{1} (2.10^{0} to 2.10^{2})	s	3.10^{-1} – 5.10^{-1}	10^{-3} – 2.10^{-3}	u	8.10^{-2} – 3.10^{-1}	7.10^{-4} – 2.10^{-3}	v	1.10^{-2}	5.10^{-5}	x

a calculated total weighted absorbed dose rates to small mammals lungs in radon-rich soils (Macdonald and Laverock, 1998)

b calculated total weighted whole-body absorbed dose rates (Beresford et al., 2008)

c calculated total weighted whole-body absorbed dose rates (Brown et al., 2004)

d Ribera et al. (1996)

e Ragnarsdottir and Charlet (2000)

f concentrations measured in the vicinity of uranium mines in Kazakhstan (Uralbekov et al., 2011)

g regional (European) background (EC, 2007)

h baseline concentrations in European coastal waters (Santos-Echeandía et al., 2012)

i sediment-core record of past pyrogenic PAHs deposition in pre-industrial times (Musa Bandowe et al., 2014)

j calculated total weighted whole-body absorbed dose rates to soil invertebrates (nematodes)) in some areas of the Chernobyl Exclusion Zone 25-year after the accident (Lecomte-Pradines et al., 2014)

k estimated dose rates during the early phase (1986) and long term (2008) in some areas of the Chernobyl Exclusion Zone (Geras'kin et al., 2008)

l early phase maximum total whole-body dose rate calculated for marine fish 5 months after the Fukushima accident (2011) (Vives i Batlle et al., 2014)

m early phase (1957) and long term (1992) maximum total whole-body dose rate calculated for aquatic organisms in lakes of the southern Uralsafter the Mayak accident (Kryshev et al., 1996)

n Carvalho et al. (2007), Gongalsky (2006), Lottermoser et al. (2005)

o concentrations measured in the vicinity of uranium mines (Ragnarsdottir et Charlet, 2000 ; Uralbekov et al., 2011)

p soil concentration in the vicinity of the former lead Metaleurop Nord smelter (Bernard et al., 2010)

q predicted environmental concentrations downstream NiCd battry recycler or Cd producing/processing sites (EU, 2007)

r 16 USEPA priority PAH concentration in urban soils of Beijing (Peng et al., 2010)

s generic (and organism group-specific) predicted no-effect dose rate (Andersson et al.; 2008)

t ecotoxicity thresholds for uranium (Sheppard et al., 2005)

u interim EQS in France (MEDE, 2007) and EQS in The Netherlands (RIVM, 2014)

v Predicted No Effect Concentrations depending on water hardness and pH (SCA, 2011)

w EU (2008)

x EQS (EU, 2006)

2.3 Choice of organisms and toxicity endpoints

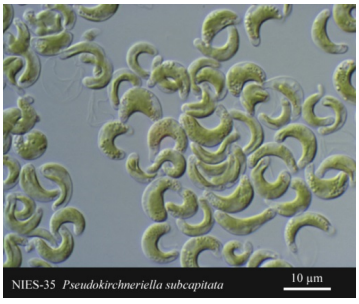
Different species groups and levels of biological organization were chosen to assess the effects of binary mixtures (Figure 1). Selecting different species groups was expected to aid in the extrapolation process and the generalization of conclusions (i.e. additive effects or interactions). We also selected organisms most suitable for the study of interaction mechanisms at different biological organization levels (sub-cellular/cellular, individual, species life cycle, and community). In addition, test conditions were established from existing standardized protocols and guidelines, with some adaptations (specific details are given in Section 4).

Caenorhabditis elegans
(photo: IRSN)

Lemna minor
(photo: SCK.CEN)

Salmo salar
(photo: NMBU)

Daphnia magna
(photo: Wikimedia commons)



Pseudokirchneriella subcapitata
(photo: http://www.shigen.nig.ac.jp)

Figure 1: organisms chosen in support to STAR WP4 experiments.

- Invertebrate: nematode *Caenorhabditis elegans*

  The soil nematode *Caenorhabditis elegans*, widely used in ecotoxicology, was chosen due to its experimental convenience (short life cycle and ease of growth and reproduction measurement over time) and because its sensitivity to single U, Cd and gamma irradiation has already been documented (Dutilleul et al., 2014; Swain et al., 2010; Buisset-Goussen et al., 2014). The nematode *C. elegans* was used to conduct (partial) life-span experiments with single, then binary mixtures for a mechanistic energy-based analysis perspective (DEBtox, see §0). For the experiments, the life

traits (growth, reproduction, lifespan) were recorded on individually-exposed organisms. *C. elegans* is commonly cultured and used in ecotoxicological assays on NGM-agar. Within this design, The wild-type N2 nematode is maintained at 20°C, 70% RH and in darkness, in Petri dishes filled with modified[2] nematode growth medium (NGM) and seeded with *Escherichia coli* as food source.

- Plant: *Lemna minor*

  Lemna minor, a free floating macrophyte, is an easy to culture and handle vascular plant that is relatively sensitive to different toxicants and hence suitable for ecotoxicological testing (Fenske et al. 2006; Moody and Miller 2005). It is a common, relatively simple structured freshwater plant belonging to the *Lemnoideae* (duckweed subfamily). Macrophytes have an important ecological role in aquatic ecosystems. They form food and shelter for freshwater invertebrates and fish and are considered to be storage reservoirs for nutrients and a certain trace elements (Newman 1991; Pandit 1984; Lahive et al. 2011. In a regulatory context *Lemnaceae* are the only freshwater plants required for ecotoxicity testing of chemicals including metals (Brain and Solomon 2007; Lewis 1995; Mohan et al. 1999).

  Lemna minor growth inhibition test is a well-established aquatic plant ecotoxicological test with standard endpoint parameters related to growth and photosynthesis e.g. chlorophyll a and b levels. For the experiments we used a 7-day growth inhibition test as described in OECD guideline 221(OECD, 2006) with some modifications[2].

- Vertebrate: fish *Salmo salar*

  Atlantic salmon (*Salmo salar*) is a salmonid fish species which have been demonstrated to be one of the most sensitive fish species and is very important environmentally as well as economically. Complete life-cycle studies with salmonid fish are impractical since it takes two to five years for these fish to reach maturity (Environment Canada, 1998). The early developmental stages (i.e. embryo, larvae and early juveniles) are, however, considered to be equally or more sensitive to contaminant exposure than adults (EC, 1998).

  Toxicity of binary mixtures was assessed using (1) acute toxicity experiments (96 h) of *S. salar* juvenile parr, based on a standardized protocol OECD guidelines 203 (Environment Canada, 1998; OECD 1992) and approved in advance by the Norwegian Animal Research Authority (NARA ID: 4615); or (2) toxicity experiments of Atlantic salmon embryos based on OECD guidelines 210 (OECD 2013).

[2] Modifications were made on the composition of the exposure media due to the tendency of U to form precipitating complexes with different ions (phosphates, carbonates) leading to changes in bioavailability and toxicity.

Toxicity experiments using the juveniles life stage are advantageous as information can be obtained on elemental transfer from water to gills and subsequently to different organs and tissues, and the accumulation can be correlated with different responses; from physiological changes in blood (e.g., glucose, plasma ions) to mortality. In contrast, in toxicity experiments using long-term exposure (90 days) of embryos - from fertilization of the egg to hatching – responses associated with development, growth and mortality are investigated. Interaction mechanisms in exposed embryo were explored by using RNA-Seq gene expression profiling.

- Community: plankton (algae *Pseudokirschneriella subcapitata* – daphnid *Daphnia magna*)

  A simple (2 species) mixture of phytoplankton and zooplankton species was chosen. Working with such a combination increases the complexity of the experiments, but adds a level of ecological realism to the other single species tests by investigating the effects of mixtures on a species interaction. The species chosen were the cladoceran *Daphnia magna* and the chlorophyte *Pseudokirchneriella subcapitata* (though preliminary experiments with gamma irradiation were also performed on another chlorophyte *Dunaliella tertiolecta*). Considerable optimization had to be done since this two-species system is not a standard ecotoxicology test system and there are limited existing effects data for gamma irradiation, Cd and fluoranthene.

  D. magna were maintained in M7 medium and *P. subcapitata* in MBL medium. The main endpoints chosen were feeding rate (algal cells eaten per hour) and carbon assimilation (^{14}C) from the alga to the cladoceran (ie. trophic transfer). In addition, specific endpoints for the two species were also measured for *D. magna (*growth, respiration, number of eggs, oxidative stress (catalase) and mobility). *D. magna* individuals (3 days old) were exposed to contaminants for 24-72 hours, either at the same time as they were irradiated or immediately before or after. *D. magna* individuals were then allowed to graze on ^{14}C-labelled *P. subcapitata* for 24 hours.

  For algae, a range of different endpoints were measured in the different experiments, covering different levels of biological organization (e.g., at subcellular level: oxidative stress, pigment concentrations; at cellular level: individual cell size and biomass; at population-level: population biomass and cell density).

3 Tools for the description of combined effects and identification of interactions

In order to test the driving hypothesess of this work, the choice was made to analyse observed effects against the joint effect predictions according to the Concentration Addition (CA) or Independent Action (IA) concepts, as it is now established in the literature. Where possible this was done based on full response surface modelling, and where not then expected mixture effects were calculated according to CA and IA based on the full or partial single stressor dose-response curve data, and observed joint effects compared to expected CA and IA mixture toxicity predictions. Possible synergistic or antagonistic effects are subsequently investigated by statistical tests comparing measured mixture toxicities with the predicted values. The first section (§3.1) summarises the general CA and IA concepts as well as their use as reference models for the identification of deviations and description of interactions (§3.2). Those concepts may also be implemented within the mechanistic DEBtox modelling (see §3.3).

The main outlines of the analysis of combined effects are given in section 3.2: first, the different experimental designs for the study of binary mixtures are described followed by different ways to present the results. Finally an overview is given of associated data treatment and statistics as performed with the MixTox tool or alternatives, especially when full dose-response curves cannot be obtained.

Additionally understanding the kinetics of toxic effects, and inter-connections between endpoints, may allow a better characterization of possible interactions and can lead to generalization for all endpoints and exposure times. Therefore the last section (§3.3) presents the most recent attempts for an integrated and dynamic mechanistic modeling of combined effects using the DEBtox framework.

3.1 CA and IA concepts

Concentration Addition (CA) and Independent Action (IA) are concepts based on pharmacological assumptions about sites and modes of actions of substances (similar mode of action for CA and dissimilar for IA). Their theoretical basis is summarized below.

3.1.1 Concentration Addition

In **Concentration Addition**, all components in the mixture behave as if they were simple dilutions of each other. The joint effect is equal to the sum of the concentrations of each chemical expressed as a fraction of their own individual toxicity (Greco et al., 1992; Warne, 2003; Backhaus et al., 2004). It is written mathematically as follows:

$$\sum_{i=1}^{n} \frac{c_i}{ECx_i} = 1$$

with c_i the exposure concentration of chemical i in the mixture, ECx_i the concentration of chemical i alone which would elicit x % effect (e.g. EC_{50} when x = 50 %).The ratio c_i/ECx_i is called a toxic unit (TU) and was introduced by Sprague in 1970, when he measured water pollutants. One toxic unit (1 TU) is the concentration of a chemical that corresponds to the selected toxic effect (e.g. x = 50 %).

Hence, the joint load or strength of the mixture (M) given in a common unit can be rewritten as follows:

$$M = \sum_{i=1}^{n} TU_i = TU_{mix}$$

From this and knowing or estimating the shape of a typical dose-response curve for the stressors involved in an organism or a system so EC values can be derived, an effect estimate of the ΣTU for any given effect level (e.g. EC_{50}) can be made. Where the calculated value of M (or sum of TUs) equals 1 the mixture would be expected to follow CA additivity, meaning if the TUs are based in EC_{50}'s then any mixture with a M=1 would be expected to give 50% effect. If the observed toxic effect is lower than 50% for a mixture where the TUs sum to 1, or the sum of TUs is higher than 1 in a mixture observed to cause 50% effect, then an antagonistic deviation from CA has occurred. Conversely, if the observed toxic effect is higher than 50% for a mixture where the TUs sum to 1, or the sum of TUs is lower than 1 in a mixture observed to cause 50% effect, then a synergistic deviation from CA has occurred.

In terms of applying CA to external gamma irradiation which is not measured in "concentration", concentrations are replaced with doses or dose rates. This might seem strange and in princible contravention of the CA principle of the stressors acting as dilutions of each other, but has been applied here to test if CA could be applied. Mathematically there is no issue as the TU is a unitless number based calculation of the ratio of the level in which a stressor is present over the level needed to cause a certain effect. (i.e. the units "divide out").

3.1.2 Independent Action

The concept of Independent Action is based on the probability of the chemicals involved having an effect on an individual or target being statistically independent. For a binary mixture this would mean that the mixture effect of chemical A and B is the sum of the individual effects (E) of A and B minus the portion of the population in which toxicities overlap:

$$E_{A,B} = E_A + E_B - (E_A * E_B)$$

This means, if chemical A causes 20 % mortality and chemical B 70 % mortality, the mixture effect of these two chemicals is not 90 %, but 76 %. For a multicomponent mixture this relationship can be expressed as follows:

$$E_{mix} = 1 - (1 - E(c_1))\,(1 - E(c_2))\ldots(1 - E(c_n))$$

$$E_{mix} = 1 - \prod_{i=1}^{n}[1 - E(c_i)]$$

with E_{mix} being the expected effect of the mixture, n the number of mixture components, and E(ci) the effect of the ith mixture component if applied alone in the concentration (Backhaus et al., 2004; Altenburger and Greco, 2009).

This becomes simpler to apply if expressed in terms of the unaffected fractions (UAF) or probability of non response ($q(c_i)$) of each chemical, as the probability of not being affected by a given mixture can be summarised as the joint probability of not being affected by all the individual chemicals involved, and calculated as a product of these:

$$\text{UAFmix} = \text{UAFc1} * \text{UAFc2} * \ldots \text{UAFcn} = \prod_{i=1}^{n}[UAF(c_i)]$$

And the response in the mixture (Ymix) for a biological response restricted between Umax and 0 then calculated as follows (Bliss, 1939):

$$\text{Ymix} = \text{Umax} \prod_{i=1}^{n}[q(c_i)]$$

3.1.3 *Use of CA/IA as reference models, identification of deviations and description of interactions*

Whether considering CA or IA the simplest way to define the main deviations of "synergy" and "antagonism" is to consider what an "undesirable effect" is. Thus, where a mixture is observed to induce a higher level of "undesired effects" than predicted by the reference model it is defined as synergism, and *vice versa* where a lower level of "undesired effects" are observed than was expected it is termed antagonism. Keeping this in mind avoids possible confusion when endpoints like mortality and growth is analysed in the same test. Where for mortality a higher than expected response value would be termed synergy, then for growth a higher than expected response value would be termed antagonism (see Figure 2).

For IA the comparison of the predicted and observed response values is simple, while for CA the observed response levels need to judged against whether or not the sum of

single stressor TUs (based on the given effect level) present in the mixture sum to 1 (additivity), >1 (antagonisms) or < 1 (synergy), as outlined in section 3.1.1 above (Groten et al., 2001). Jonker and co-workers (2005) presented a MIXTOX model based on CA and IA being able to characterize mixture interaction effects by quantifying the degree and pattern of deviations of the data from either reference model (see section 3.2.3.1 below for a detailed description).

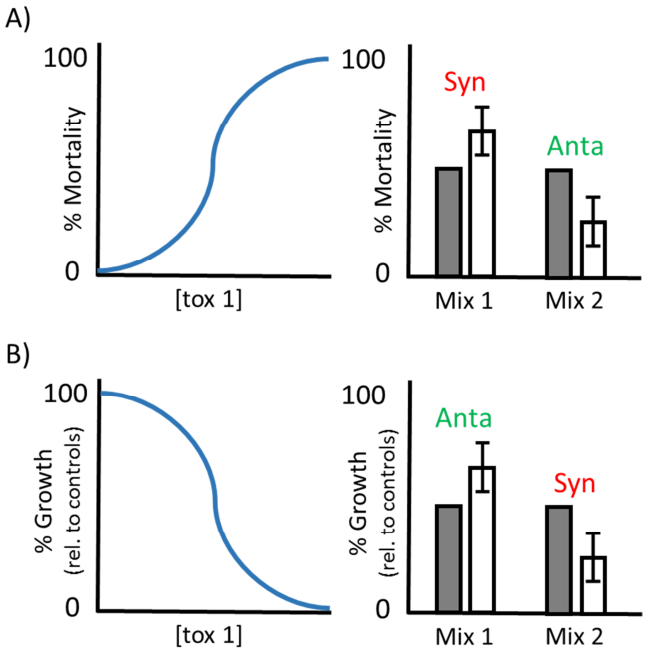
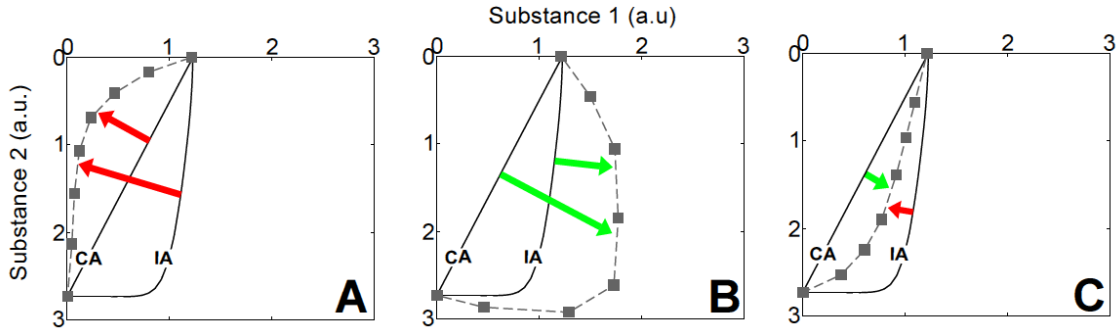


Figure 2: shows how the terms Synergy and Antagonism are best defined agaist parameters where the response parameter is either increasing with dose (panel A - Mortality) or decreases with increasing dose (panel B – growth relative to controls), and how they differ.

In toxicology and ecotoxicology, knowledge of modes of actions of substances is often missing. When measuring endpoints at the organism level such as mortality, reproduction or growth rate, only the net effect of the toxicity is assessed, and this does not always relate to a single specific mode of action. Hence, the choice between CA and IA as a reference model is often difficult to make, and it is recommended that both concepts should be used.

However, the comparison of data against both the CA and IA concepts can lead to apparently ambiguous conclusions. For example, a mixture response judged as antagonistic when assessed against CA, may well, at the same time, be synergistic with respect to IA. Therefore, the minimum requirements when reporting on synergistic/antagonistic interactions, is that the reference model with which it is compared is clearly identified. To validate experimental results and to allow for precautious assessments, Drescher and Boedeker (2003) suggested that data should be

related to both CA and IA, and the relatoionship between the relations should be considered. They have shown that the relationships between CA and IA depend on the distribution functions, the corresponding slope parameters, and on the concentration of the mixtures (an example is given in Figure 3).



The relative position of CA and IA isoboles* (continuous lines) is determined by the slopes of the dose-response curves (DRC) of substance 1 and 2 (in this example, slopes are > 1.3 and CA and IA isoboles are non-superimposable).
(A) & (B): The deviation of observed effects (squares and dotted line) is large, so the conclusions compared with both CA and IA reference isoboles are the same (in **A** = synergism; **B** = antagonism).
(C): The observed effects are within the "prediction envelope", and contrasting conclusions are reached depending on which reference model is considered (CA: antagonism; IA: synergism). It is worth noting that when the slopes of the DRCs are lower than 1.3 the predicted IA isobols will lie below or inside the CA isobols.

*an isobole is an isoeffective line through the concentration-response surface, defined by all the concentration combinations of the components of the mixture that produce an identical mixture effect.

Figure 3: examples of the possible contrasted interpretations of combined effects of a binary mixture, as a function of reference additive models Concentration Addition (CA) or Independent Action (IA).

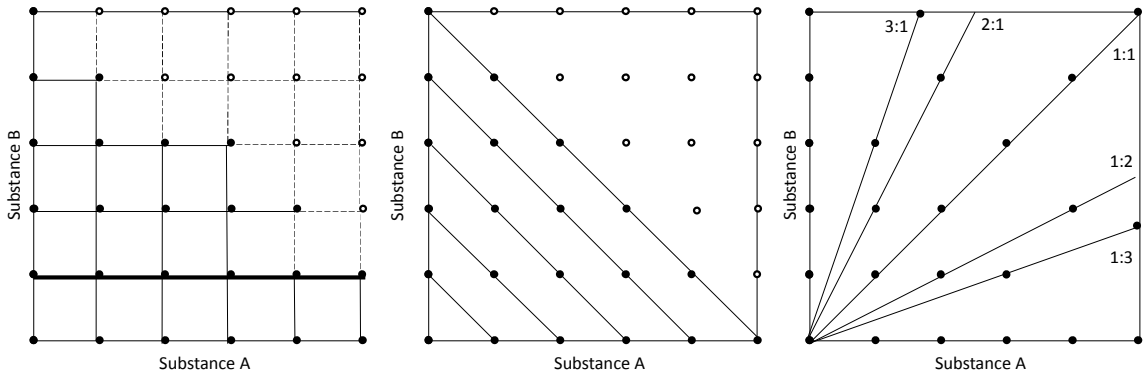
3.2 MixTox tool and alternative analysis of combined effects

3.2.1 Experimental designs used for mixture exposure

The strength of CA/IA models to identify interacting effects as deviations from the predictions relies on the reproducibility of the (binary) mixture toxicity experiments. Reproducibility depends on the variance of the endpoint and the tested species, both within and between experiments (Cedergreen et al., 2007). Mixture experiments naturally expand in numbers of treatments very fast as complexity is increased. The optimisation of the experimental design therefore becomes a critical balance between the number of treatments required to address questions at different levels of complexity and achieving sufficient replication to draw reliable conclusions. As the number of experimental units that can be handled is the locigal and unmovable limiting factor and

the number of replicates can be estimated from the experience of the operators for established test systems, then the key element is to be realistic and limit the complexity of the questions asked to ensure a reliable answer can be delivered rather than stretch it too far and thin.

Several specific designs of varying complexity have been described in the literature to analyse whether the toxicity of a binary mixture deviates from the predicted mixture effect by CA/IA (Figure 4). The final choice of the experimental set up is limited amongst other things by the number of experimental units that can be handled (see D-N°4.1 (Vandenhove et al., 2012a) for a full overview of commonly used experimental designs). Of the below mentioned designs, the more frequently used are isobole designs, point and fixed-ratio design, while full or fractionated factorial designs are rarely applied due to their higher experimental unit demand.



Factorial design
full (n=31) or partial (n=24)

A in presence of B
bold line (n=10)

Isobolic design
5 isoboles (n=21)

Ray design
5 ratio B:A tested (n=24)

Figure 4: type of experimental designs used for the study of binary mixture toxicity (example of 5 concentrations tested of substances A and B + 1 control)

When only few experimental units are possible, a point design can be chosen enabeling the assessment of possible synergy or antagonism for the point(s) used. The ability to cope with more experimental units allows more complex isobole and single ray designs to be used, allowing dose/effect level- or ratio-dependent deviations from the reference models to be addressed. When many units can be used simultaneously (>10 of experimental units), factorial designs are possible and more robust as even if one stressor fails to produce a response curve it is at least still possible to calculate expected IA effects to compare the observed data against all points. A fixed-ratio design is less resource-demanding and still provides valuable data (Jonker et al., 2005), and can if designed carefully (see ray design in Figure 4) still be made to be partially factorial and thus deliver the advantage mentioned above.

3.2.2 *Representation of binary mixture effect data*

The important thing for any presentation of mixture data is to communicate the difference between the reference model prediction (or best fit to this) and the actual observed joint effects data. There is a number of ways to present the data (see Figure 5). These include, but are not limited to: concentration response relationships for different series (factorial designs); three dimensional response surface plots (ray or factorial designs); isobolograms (ray or factorial designs); and observed against expected plots (all designs). Each of these plots has their respective value and use. Response surface plots can provide an overall summary, but can be difficult to interpret especially when presenting both model and raw data in one plot. Isoboles can be simple to interpret, but can be difficult to draw when there are only relatively few data points, and there are issues with graph software spline functions "inventing patterns" where there is no data. Observed vs expected plots can provide a simple summary and are also intuitive, but can obscure the detailed patterns across mixture ratios unless these can be ploted as separate series (Ok for ray designs, but difficult for fully factorial design).

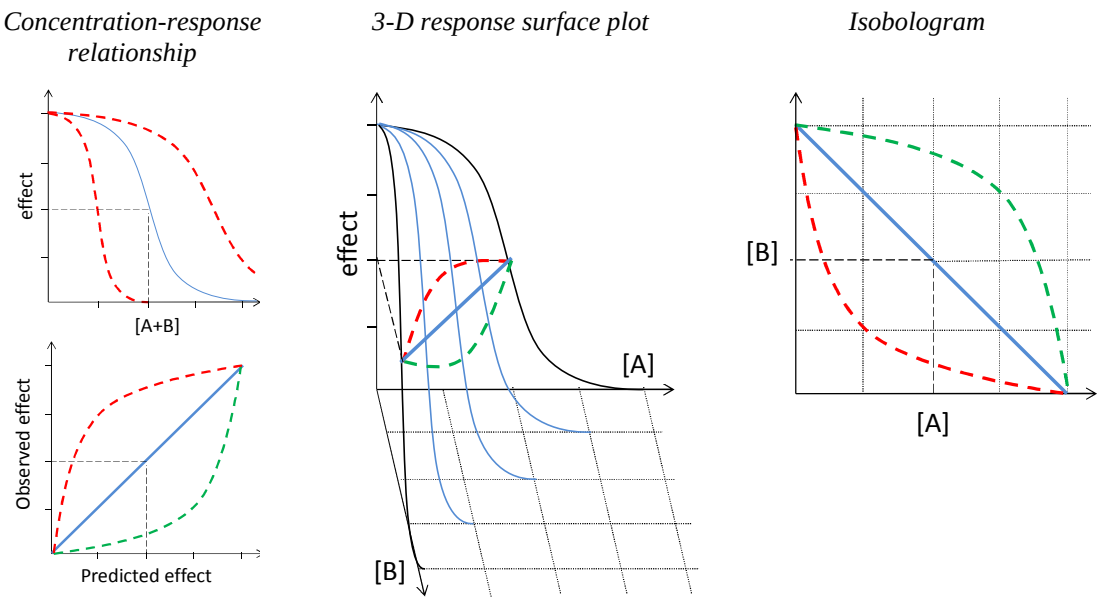


Figure 5: example of graphical representation of binary mixtures (substance A + B) and the deviation from additivity.

3.2.3 *Data treatment and associated statistics*

3.2.3.1 *MixTox tool*

Jonker et al. (2005) proposed an approach (MixTox) to statistically evaluate if data follow CA or IA or if possible interacting effects are present. In this approach CA and IA are considered as two equally valid reference models for predicting additive effects of non-interacting chemicals. The approach enables the combined analysis of entire dose-response surfaces of mixture and single stressor data, and deviation from CA and IA additivity can be described either by simple (i.e. synergism or antagonism), or complex (with deviations dependent on mixture ratios, or effect levels) interaction models.

A full description of the MixTox tool, used in this study, can be found in Jonker *et al.* (2005). Briefly, MixTox provides statistical comparison between the CA and IA additive reference models and a set of nested deviation models. The simplest deviation model, Synergism/Antagonism (S/A), requiring one additional parameter, *a*. Dose-Ratio (DR) and Dose-Level (DL) dependent deviation models required a second additional parameter, b_i (specific to one substance of the mixture) and b_{DL} respectively, and with the values of *a* and *b* parameters describing the nature of the interaction (Jonker *et al.*, 2005). First, a 3-parameter logistic dose-response function is used to describe the single contaminant effects. The parameter values obtained are then used as a starting point to fit first the CA or IA reference models and then the deviation models.

The statistical quality of improved description provided by adding the extra parameters of the deviation models can in the MixTox tool assessed via the χ^2-test as the models are nested (Jonker *et al.*, 2005).

Estimation of Confidence Intervals of the parameters

The confidence intervals (CIs) of the parameters of the model fits are not available from the MixTox Excel sheet, mainly for technical reasons, but also for the reason that they are interlinked when fitting the response surfaces and therefore difficult to interpret. This is especially the case for the a and b deviation parameters, where one should not assume the model does not provide significantly better description of the mixture data even if one or both of the individual 95 % confidence intervals of parameters a and b overlap zero as their significance is based on how the parameters jointly affect the response surface. The significance testing of the nested deviation models of the excel tool covers this fact. However, if desired such CIs can derived either through bootstrapping or by coding the MixTox model equations into various proprietary statistical software (e.g. SAS, Matlab, Genstat and potentially R) where the nonlinear regression functions usually produce error estimates.

While bootstrapping does allow CI values to be obtained for a and b parameters and for the dose-response curve (DRC) parameters which are useable and technically correct for publication it sometimes presents surprisingly large 95 % confidence intervals. This is because all parameters interact to form the shape of the dose response surface, and hence bootstrapping will put in fits where one parameter takes one extreme and another

counters with an “opposite acting” extreme. It can therefore be recommended to derive the DRC parameters and associated CIs for the single chemical from DRCs fitted in isolation of the mixture data using any commercial statistical graph fitting software (e.g. Sigmaplot) as this gives the audience a representation of the quality of the experimental data they are used to interpret.

Selection of the best fit model

While the selection of the best fit model is generally straight forward within the nesting of the models (i.e. reference model (CA or IA) > Synergism/Antagonism -> DL or DR) there are two additional choices people generally want to make that are not supported by the statistics of the nested models, namely: CA vs IA or DL vs DR. While the output of the MIXTOX sheets will provide information on whether the reference models provide a significant fit to the data there is no direct way to compare the fits of the CA and IA models based on these MIXTOX outputs. Such relative ranking of model fits can be undertaken with the Akaike information criterion, but this provides not absolute test of the models fit to the data, but simply just rank the two models even if both fits are poor. For the selection of DR vs DL within either reference model one can of course look to the R^2 value, which if very different gives a good suggestion. However, when both the DR and DL deviation models are significantly better than Syn/Anta it is worth plotting the isobols of both the DL and DR best fits in the same isobologram. Usually one then finds that they are not actually that different (at least in the area of the dose response surface that is covered by actual data). The researcher should use this as a guide to describe the main important features of where the observed data deviates from the reference model(s).

Rather than trying to force a selection between the CA and IA as the “best reference model” it is most useful to treat them as two equally relevant reference models against which to compare observed data (especially as there is often little scientific knowledge or reason to guide/force such a selection). It is therefore simplest and most informative to view the two models as equally relevant reference alternatives and the best fitting deviation option as a summary describtion of the data to each models different mathematical connotation. Indeed, the IA model is a statement about relationships between probabilities of response, whereas the CA model is a statement about relative toxicities and their combinations. Considered as such the two models form the outer bounds of what has been termed the “prediction envelope”, where normally (given reasonable DRC slopes i.e. >1.3 for the 3para log logistic equation) the IA model will predict the least joint effects from a mixture and the CA model will give the most conservative prediction of the possible joint effect. Often the observed data will be observed lying between the two models and as such in some cases even be best described by significant antagonism in relation to CA and significant synergy in relation to IA (again see Figure 3).

3.2.3.2 *Alternatives to the MixTox tool when full dose-response curve(s) are not available for one or both single stressors*

For CA there is no alternative and analysis is not advised (although it is mathematically possible if enough reliable prior information (from other data) is available to fix the EC_{50} and beta for the missing DRC). In contrast, for IA there is an alternative for mixture experiments that use a truly factorial design (i.e. where the exact single chemical exposures used in the mixtures were also tested in isolation). For such factorial mixture data a simpler data analysis method is possible, based on calculating the values that would be expected assuming IA using the unaffected fractions of each single stressor according to the equations set out in section 3.1.2 above. However, while this will allow comparison of observed mixture data against IA expectations there will most often be too few data points (e.g. 3 replicate mixture responses) to make any meaningful statistical testing of significance of the the point by point differences. The most basic semi statistical approach possible would be raking the residuals between observed and expected values and showing that all the negative or positive residuals were from a certain area of the response surface. Alternatively a "biological significance" approach can be taken where an acceptable level of deviation (e.g. 10, 20 or 30%) in response from the predicted IA effect is defined, and "biologically significant" synergy or antagonism definedoutside that margine of tolerance.

3.3 Use of Dynamic Energy Budget (DEB) theory for a dynamic modelling of combined effects

The CA/IA approaches on single endpoint dose response surfaces are generally accepted and used, there are associated limitations. The CA and IA models themselves provide a prediction of the mixture based on single chemical dose-response curve relationships assuming no interaction, or a systematic deviation (i.e. synergism or antagonism throughout the whole data set). The MixTox tool and similar approaches are perfectly adapted for 'classical' ecotoxicological studies, focused on the description of the effect of one single endpoint (e.g. growth or reproduction) as a function of exposure concentrations, for a fixed exposure time and on describing any patterns of deviation from CA and IA including DR and DL dependent ones (Svendsen et al., 2010a). Neither of these approaches allow explicit analysis of how mixture effects develop in time (Baas et al., 2007), vary between the endpoint considered (Cedergreen and Streibig, 2005), or indeed of how such effects integrate to the level of population performance or fitness.

To overcome these shortcomings a biology-based approach such as a DEBtox model can be used to estimate the toxic effects of mixtures on growth, reproduction and survival over the life cycle of exposed organisms (Baas et al., 2009a, b). A general description of DEB-tox concepts is given in D-N°5.4 (Horemans et al., 2014) as well as an in depth mathematical description of the model. In the following, we only focus on the adaptation of the CA and IA concepts within a DEBtox framework, and their consideration at the level of metabolic processes (Jager et al., 2010).

With CA, contaminants are considered as similarily acting on the same biological target and a same DEBtox metabolic process (Figure 6): each contaminant has its own TK module, but share the same TD module. Internal concentrations of the contaminant, weighted by their toxic potential are added. This implementation allows the analysis of effects without necessarily getting the full dose-response curves of single substances (Jager et al., 2010).

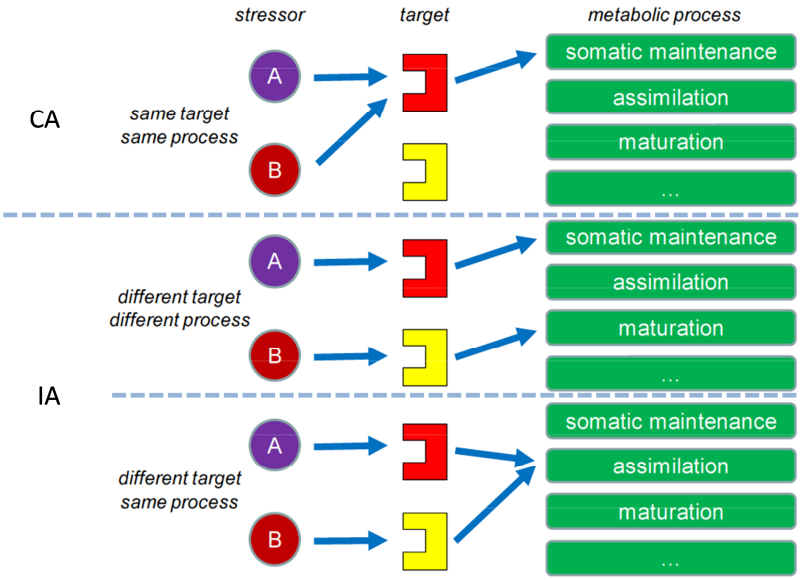


Figure 6: DEBtox representation of the combined effects of two stressors (A and B) – from Jager (2014)

In the case of IA, the mixed contaminants are considered to act on different biological targets. They have their own TK and TD modules. Indeed, they can affect the same or different DEBtox metabolic process (see Figure 6). Stress functions of each individual contaminant can be applied to the same or different DEB metabolic process.

4 Main results on the combined effects of binary mixtures

This chapter summarizes the results obtained for each of the binary mixtures tested (Table 3). Available knowledge in the literature on the toxicity of each single contaminant was used to support the choice of the mixture concentrations tested. If no information was available, preliminary studies of single dose-response relationship were also performed for some contaminants and tested organisms (results not shown), as described in D-N°5.1 (Garnier-Laplace et al., 2011). Mixture experimental designs were set up based on the shape of the single stressors dose-response curves, for further data analysis according to the CA or IA concept. When possible, dose-response relationships for single contaminants were produced at the same time as running mixture toxicity experiments, mainly because of difficulties of reproducibility between experiments done at different times. This issue of reproducibility is even more prominent in the case of community experiments, by having two species that can vary subtly in age and condition between experiments.

Table 3: Tested combined effects on the different species

Radioactive substance	***Uranium***		***Gamma irradiation***	
Stable substance	***Cadmium***	***Fluoranthene***	***Cadmium***	***Fluoranthene***
C. elegans	*Partial factorial* *[U]=0.95-1.3 mM* *[Cd]=6-40 µM*	*Partial factorial* *[U]=0.95-1.3 mM* *[FL]=6-250 µM*	*Full factorial* *γ =1-1500 mGy/h* *[Cd]=0.1-100 µM*	*Not tested*
L. minor	*Ray design* *[U]=3-75 µM* *[Cd]=3-67 µM*	*Not tested*	*Full factorial* *γ=26 -1500 mGy/h* *[Cd]= 4-32 µM*	*Not tested*
S. salar	*Partial factorial* *[U]=4.2-14.7µM* *[Cd]=0.9-35 nM*	*Not tested*	*Partial factorial* *γ=0.4-40 mGy/h* *[Cd]= 2.7-267 nM*	*Not tested*
D. magna *+ P. subcapitata*	*Not tested*	*Not tested*	*Full factorial* *γ =2.5-100 Gy* *[Cd]=0.09-8.9 µM*	*Factorial/Ray* *γ =25-200 Gy* *[FL]=0.1-0.8 µM*

The type of design and nominal tested concentrations in the exposure media are given.

For each tested species group, an extended summary of results is provided in the following chapters, after a brief reminder of the specific experimental conditions and methods. For two of the tested binary mixtures (U + Cd and gamma irradiation + Cd), several species have been tested, which allowed to address hypothesis H2 (interactions are independent of test organism). Two summary tables (Table 4 and

Table 5, for UxCd and gammax Cd binary mixtures, respectively) are provided to support the discussion on the (dis)similarities of interactions as a function of the species tested.

Briefly, for animals species (nematode, fish), single Cd toxicity was 48 to 623 times higher than U (ration of EC50 expressed as actual external concentrations), that indicates that U is less potent than Cd. In contrast, EC_{50} values of U and Cd for plants were within the same order of magnitude. Gamma irradiation EC_{50}s were obtained at a continiuous exposure to 10 to 800 mGy/h (30 to 60 Gy for acute daphnids exposure), i.e. 2 to 3 orders of magnitudes higner than the background level.

Despite the differences in U *vs.* Cd toxic potent between species, an overall antagonism between U and Cd was identified for all tested organism and almost all endpoints, when the results were explained as a function of external exposure concentrations. This antagonism was further explained by a U/Cd interaction in the exposure media, bioaccumulation or interactions at the toxicokinetic steps (see §5.1 for a detailed discussion). Focusing on bioavailable and internal Cd and U concentrations (eg. bacterial lawn of nematodes, accumulated concentrations in plants and fish tissues) allowed reavaelling interactions that may occur at toxicodynamic steps.

The combined effects of gamma irradiation x Cd showed also a mostly antagonistic interaction pattern. However, this was less clear among species. An antagonistic interaction was observed for both plants and daphnids at low Cd toxic concentrations, whereas at higher irradiation dose rates levels or high Cd concentrations synergistic interactions might occur. On the other hand, fish and nematode exposure to gamma irradiation and Cd mixture did not reveal any obvious synergistic or antagonistic effects. Those contrasted patterns may be the result of complex toxicodynamic interactions (e.g. one stressor enhancing the antioxidant capacity or DNA repair of the cells).

Table 4: Summary table of the conclusions provided by the Mixtox analysis of the combined effects of Uranium and Cadmium

** Best fitting model (Reff: additive model; S/A: Synergism/Antagonism; DR: Dose-Ratio dependent interaction; DL: Dose-Level dependent interaction)*

				EC50 (µM)			Concentration Addition				Independent Action			
Species	***Endpoint***	***Design***	***Exposure measurement***	***U***	***Cd***	***U/Cd ratio***	***Best* Model***	***p-value***	***R^2***	***Conclusion***	***Best* Model***	***p-value***	***R^2***	***Conclusion***
Caenorhabditis elegans	Growth 11-days	fractional factorial	actual in agar	1.10^{3}	2.10^{1}	48	Reff	3.10^{-43}	0.76	**Additivity**	DL	2.10^{-3}	0.81	**Synergism at low doses**, changing below EC50 level
			actual in bacterial lawn	4.10^{4}	8.10^{3}	5	DR	4.10^{-2}	0.82	**Antagonism** where Cd dominates effects	Reff	7.10^{-52}	0.82	**Additivity**
	Reproduction 11-days	fractional factorial	actual in agar	1.10^{3}	1.10^{1}	83	Reff	1.10^{-37}	0.71	**Additivity**	DR	1.10^{-4}	0.87	**Antagonism** where Cd dominates effects
			actual in bacterial lawn	4.10^{4}	4.10^{3}	8	DR	4.10^{-2}	0.88	**Antagonism** where Cd dominates effects	DL	$3\ 10^{-2}$	0.88	**Synergism at low doses**, changing above the EC50 level
Lemna minor	Growth 7-days	Fixed ray	nominal in water	2.10^{1}	1.10^{1}	1	DR	1.10^{-7}	0.97	**Antagonism** where Cd dominates effects	DR	5.10^{-8}	0.97	**Antagonism** where Cd dominates effects
			actual in water	1.10^{1}	1.10^{1}	1	Reff	6.10^{-48}	0.94	**Additivity**	Reff	2.10^{-47}	0.94	**Additivity**
			accumulated	1.10^{4}	6.10^{2}	23	Reff	2.10^{-3}	0.27	**Additivity**	DR	7.10^{-13}	0.84	**Antagonism** where Cd dominates effects
Salmo salar	Survival 3-days	Full factorial	actual in water	1.10^{1}	2.10^{-2}	623					DR	3.10^{-5}	0.73	**Synergism** where U dominates effects
			accumulated in gills	1.10^{3}	5.10^{1}	19					DL	4.10^{-2}	0.26	**Antagonism** at low doses, changing above the EC50 level
			accumulated in liver	8.10^{0}	6.10^{0}	1					DR	8.10^{-3}	0.71	**Synergism** where U dominates effects

Table 5: Summary table of the conclusions provided by the Mixtox analysis of the combined effects of Gamma irradiation and Cadmium

** Best fitting model (Reff: additive model; S/A: Synergism/Antagonism; DR: Dose-Ratio dependent interaction; DL: Dose-Level dependent interaction) ; ** point by point IA analysis*
All exposure concentrations are nominal.

		EC50		Concentration Addition				Independent Action			
Species	***Endpoint***	***Gamma irradiation***	***Cd (µM)***	***Best* Model***	***p-value***	***R^2***	***Conclusion***	***Best* Model***	***p-value***	***R^2***	***Conclusion***
*Caenorhabditis elegans** (4-9 days)*	Survival (liquid SSPW, 4 days)	2-7.10^2 mGy/h	1.10^1					**Additivity**			
	Growth& Reproduction (liquid SSPW, 4 days)	2-7.10^2 mGy/h	3.10^0					**Additivity**			
	Reproduction (agar, 9 days)	>5.10^1 mGy/h	2.10^1					**Slight potentiation** (increased Cd toxicity under gamma irradiation)			
Lemna minor (7 days)	Growth	8.10^2 mGy/h	2.10^1					DL	1.10^{-2}	0.94	**Antagonism** at low doses, changing above the EC50 level
	Re-growth (+ 7 days)	6.10^2 mGy/h	2.10^1					S/A	4.10^{-2}	0.96	**Antagonism**
Salmo salar (92 days)	Embryo mortality	1-4.10^1 mGy/h	3.10^{-1}	**Additivity** (no obvious synergistic or antagonistic effects on mortality or development)							
Daphnia magna (1 day)	Growth	3.10^1 Gy (acute dose)	9.10^{-2}	S/A	1.10^{-6}	0.41	**Antagonism**	S/A	1.10^{-5}	0.22	**Antagonism** *From first experiment:* **Antagonism** in 6/9 of combined treatments (none significant)
	Immobility	6.10^1 Gy (acute dose)	4.10^{-1}	DR	4.10^{-2}	0.79	**Antagonism** where gamma dominates effects	DR	5.10^{-3}	0.79	**Antagonism** where gamma dominates effects
	Carbon incorporation	4.10^1 Gy (acute dose)	4.10^{-3}	S/A	2.10^{-2}	0.77	**Synergy**	DL	3.10^{-2}	0.82	**Antagonism** at low doses, with dose level dependent magnitude

Table 3 *(cont.)*

		EC50		Concentration Addition				Independent Action			
Species	***Endpoint***	***Gamma irradiation***	***Cd (µM)***	***Best* Model***	***p-value***	***R^2***	***Conclusion***	***Best* Model***	***p-value***	***R^2***	***Conclusion***
Pseudokirchneriella subcapitata ** *(3 days)*	%TDP										**Antagonism** in all combined treatments; significant for highest Cd conc (1000 µg/L)
	Catalase activity										**Antagonism** in all combined treatments; more pronounced at higher dose combinations, but rarely significant
	MDA										**Antagonism** in 8/9 of combined treatments; significant in 4 of these (no pattern)
	Pigments (Zeaxanthin+Antheraxanthin) : Violaxanthin										**Synergism** in the 5Gy treatments changing to an antagonistic interaction at 50 and 100 Gy combinations, but only the two highest dose combinations were significant
	cell size										**Antagonism** at all combined treatments
	cell biomass										**Antagonism** in all combined treatments; significant in 7/9 of these
	population biomass										**Antagonism**, but this was rarely significant.
	population density										**Antagonism**, significant for all 1000 µg/L Cd treatments and significant or nearly so for all 100 µg/L Cd treatments

4.1 Combined effects of Uranium and Cadmium

4.1.1 Caenorhabditis elegans

In this series of experiments, the objective was to address thoroughly the combined toxicity of U and Cd, in order to identify -if any interaction occurs- their evolution over time and their consistency among endpoints (eg. growth, reproduction). In this aim, *Caenorhabditis elegans* was selected for its short life cycle. It allowed covering whole dose–response relationships, as well as endpoints and times that are relevant for a species life-cycle. The objective was also to provide a dataset for a further DEBtox modeling of the combined effects (see §5.4).

More specifically, we aimed at providing indications on the origin of interaction, either in the exposure media or due to more complex toxicokinetics or toxicodynamics processes. For the first possible interaction (in the exposure media), U and Cd concentrations in the food source (bacterial lawn) were assumed to relate better to the bioavailable concentrations than the concentrations the spiked media (agar). Then, the differences in interactions, as a function of time or endpoints, were considered as indications for a toxicokinetic/dynamic interaction.

Material & Methods

The wild-type *C. elegans* strain (Bristol N2) was maintained at 20.4±0.4 °C, 72±10% relative humidity in darkness, on nematode growth medium (NGM) agar seeded with *Escherichia coli* OP50 strain. In NGM, the KPO_4 buffer was replaced with HEPES buffer to avoid U precipitation. Seeded plates were exposed to UV to suppress bacterial activity during the experiment. The agar was supplemented with HNO_3, NaOH and $NaNO_3$ to ensure constant pH (pH=4.8), as well as Na and NO_3 concentrations, for all exposure conditions.

Numerous U/Cd ratios were tested to cover the dose–response relationship of both compounds. Age-synchronized nematodes (±2 h) were exposed individually in 35 mm Petri dishes to seven U concentrations (0.95, 1.05, 1.16, 1.19, 1.23, 1.26, and 1.30 mM) and seven Cd concentrations (0.006, 0.009, 0.012, 0.016, 0.022, 0.029, and 0.040 mM) combined in a fractional factorial design (a control condition, n=10, and 48 exposed conditions, n=3) during 11 d. After 3 d, the nematodes were transferred daily to fresh equivalent condition plates.

The effects were measured as length increase and brood size of *C. elegans*, from hatching to the end of growth and reproduction, in order to determine the onset and kinetics of possible interactions. Hatched juveniles (within 24 h post-egg-laying) were considered as offspring. Length increase was determined daily by calculation of the difference between measured length and the average measured length at hatching (220±14 µm).

In a separate experiment, bacterial lawns were collected for the measurement of U and Cd bacterial lawn concentrations. Initial actual U and Cd concentrations in NGM-agar medium were verified to be close to nominal concentrations.

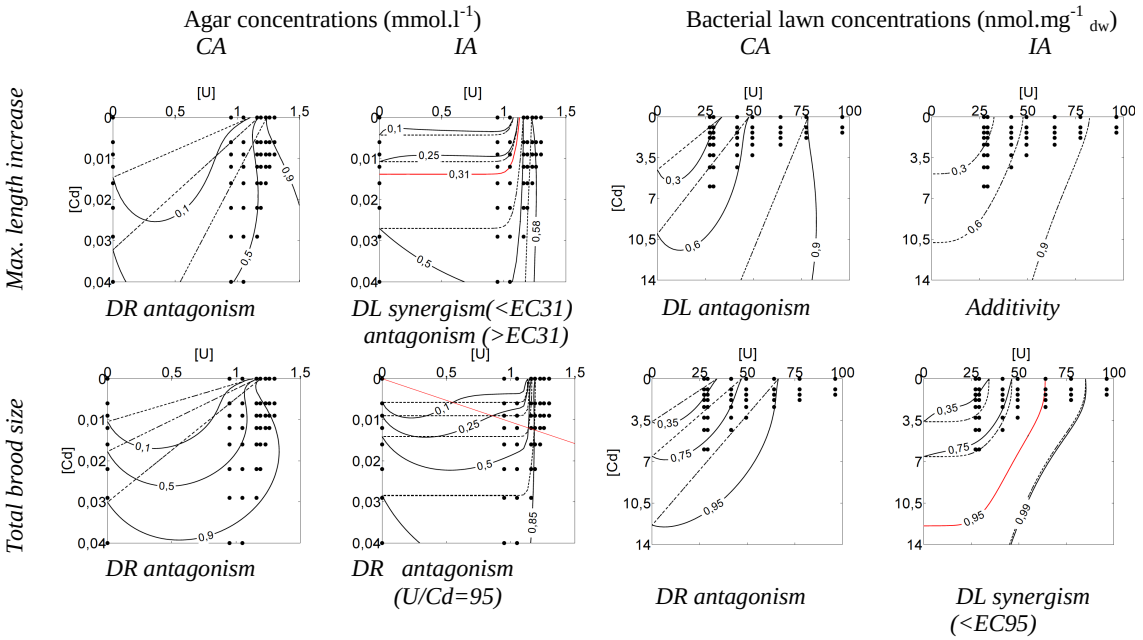
Results

Data were analyzed simultaneously using both CA and IA as reference additivity models to highlight possible interactions between U and Cd. The best fits were obtained with IA models.

On the basis of U/Cd agar concentrations, a strong antagonism was identified for most of the concentration combinations and this interaction persisted over time. Complex deviation models (i.e. DR and DL) described the data significantly better than the simple synergism/antagonism: eg., for total brood size and maximum length increase and using RA as a reference model, synergism was identified at low dose levels and increasing antagonism at high dose levels (Figure 7, left).

Chemical measurements in bacterial lawn showed that the transfer of U to bacteria was not affected by the presence of Cd. Surprisingly, the transfer of Cd from agar to bacteria lawn in the presence of U was consistently reduced by almost a factor of 2.

Based on these findings, data were re-analyzed on the basis of U/Cd bacterial lawn concentrations (i.e. expected bioavailable concentrations). By this way, an important part of the strong antagonism could be explained (Figure 7, right). The results were included in the additivity window defined by the two reference additivity models (CA and IA): compared to IA additivity, a mild transient synergy on reproduction, and additivity on growth, was identified. Compared to CA, a mild but systematic antagonism was identified for both endpoints. The nature of the combined effects appeared somewhat variable over time (not shown). This suggests that modulations in connection with the kinetics of appearance of effects could occur.



Results are presented on the basis of U and Cd agar (left) or bacterial lawn (right) concentration, considering concentration addition (CA) or independent action (IA) as reference model. Dotted lines represent the additive model, while solid lines indicate the most parsimonious significant model; values indicate the effect level of each isobol (e.g. 0.5 indicates that the mixture produce 50% effect); black dots represent the tested conditions

Figure 7: isobolograms of the most parsimonious significant models describing the U and Cd combined effects on *C. elegans* on maximal length increase and total brood size (11d post-hatching).

Main conclusions & Highlights

A strong antagonism between U and Cd was identified for growth and reproduction of *Caenorhabditis elegans*. This antagonism was mainly explained by a U/Cd interaction in the exposure media (U hindered the transfer of Cd from agar to the bacterial lawn, then to the nematode).

Assuming bacterial lawn concentrations as a proxy for the actual U and Cd bioavailable concentrations, milder -but significant- interactions were still identified, developing slightly over time. Overall additive effects, without interaction, were identified to follow IA additivity.

Different deviation patterns (i.e. simple, DR, or DL deviations) were identified over time, possibly due to experimental data variability. It underlines the limit of descriptive MIXTOX approach to analyze the joint effects of chemicals over time.

In order to describe the joint effects over time, and to provide a generic conclusion on the presence or absence of interaction (independent of the considered time and endpoint), the use of mechanistic models such as DEBtox may provide an interesting framework.

4.1.2 *Lemna minor*

L. minor was used to study possible interacting effects of Cd and U in freshwater plants. In this respect in addition to the single dose response curves, a mixture experiment with Cd and U being present in different concentrations and ratios (ray design) was set up. According to the OECD guidelines 221 describing the *Lemna* growth inhibition test, effect of U and Cd mixture was evaluated on different growth related endpoints including frond area, frond number, fresh and dry weight. The implications of interactions of the metals in the medium and at the site of uptake in terms of the predication of the mixture effect are being considered.

Material & Methods

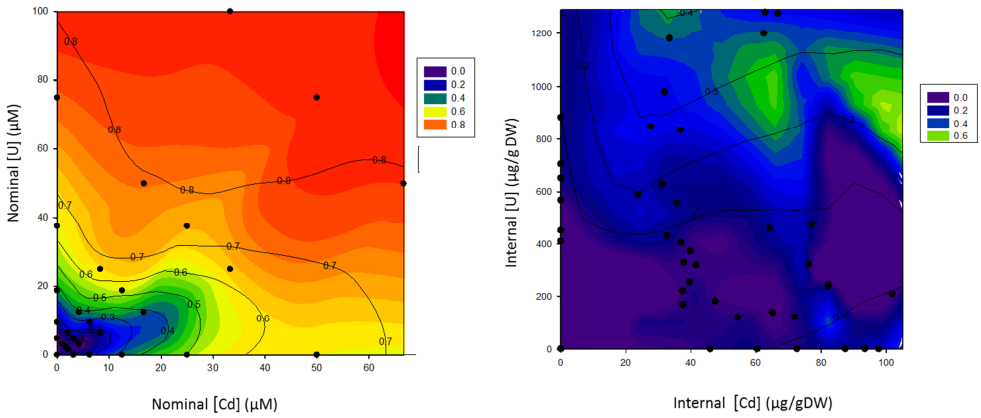
Lemna minor cv. Blarney plants were obtained from Dr. M. Jansen (University College Cork, Ireland) and cultured aseptically in 250 mL glass erlenmeyers containing half-strength Hütner medium (Brain and Solomon, 2007) under continuous light (Osram 400W HQI-BT daylight, 80-100 µmol/m^2.s) at 24°C. Plants were sub-cultured every 10-12 days by transferring three plants to 100 mL of fresh growth medium. Exposure of *L. minor* to the a combination of U and Cd was essentially performed as described (Horemans et al. 2015) following the OECD guidelines for a *Lemna* growth inhibition test (OECD 2006) using as a test medium K-medium (Cedergreen et al. 2007) with phosphate concentrations lowered to 0.5 mg/L. To stabilise pH during toxicity tests, 5 mM filter-sterilised (0.22 µm) MES (2-(N-morpholino)ethanesulfonic acid) was added. For the experiments three plants (between 9 to 12 fronds) were aseptically transferred to polycarbonate-pots containing 100 mL of the modified K-medium. A 1 cm, surface-sterilised floating ruler was added for calibration of images. Pots were covered with a 9 cm plastic petridish and experiments were run for 7 days under the same light and temperature conditions as used for normal plant culture. Uranium and Cd were administered to the different media as a filter-sterilised solutions of $UO_2(NO_3)_2$6H2O (SPI chemicals, USA dissolved in 100mM HCl) or $CdCl_2$ (dissolved in deionised water) in a final concentration ranging from 0-150 µM for U and 0-75 µM for Cd. Six replicas were used for control conditions and at least three for each of the U and Cd concentrations applied. To evaluate *Lemna* growth pictures were taken every 2 days and the frond number and frond area was determined by picture analysis (ImageJ software). After 7 days fresh and dry weight of the plants was measured.

The mixture experiment followed a classical ray design based on U and Cd being present in 0U:3/3Cd, 1/3U:2/3Cd, 1/2U:1/2Cd, 2/3U:1/3Cd or 3/3U:0Cd ratio's, based on their respective IC_{50}s, and the concentration ranges were chosen based on initial single metal dose response experiments. U and Cd were administered to the plants as a filter sterilised solution of $UO_2(NO_3)_2.6H_2O$ or $CdCl_2$, respectively in a concentration range varying from 0 to 200µM. Prior to addition of the plants the pH was readjusted by addition of filter sterilised NaOH. To ensure a stabile pH during the 7-day test 5 mM MES (or Tris for higher pH values) was included in the test medium. At the end of the growth inhibition test pH and conductivity of the medium was measured. Samples for U

and Cd analysis were taken at the beginning and end of the growth inhibition test at the level of the roots of the *Lemna* plants (half way the pot). Samples were subsequently acidified and analysed for the presence of U or Cd using ICP-MS (Perkin-Elmer, elan 500). The plants were harvested per pot and after dry-weight was determined, further ashed and the U and Cd concentration was determined in the plants. As plant material was limited and exposure to higher metal concentrations resulted in fragile, damaged or dead plants this procedure could only be done for plants treated with up to 20 µM of Cd or 25 µM of U.

Results

Average growth rate of *L. minor* was followed through different endpoints (frond area, frond number, chlorophyll a/b concentration, fresh weight and dry weight). From these different endpoints frond area seemed to be the most robust as well as a sensitive endpoint to measure the effect of Cd and U. Hence, it was chosen to assess growth inhibition based on frond area data.
As expected both Cd and U induced a dose dependent decrease in plant growth. For the growth inhibition induced by Cd and based on nominal Cd concentrations an EC10 value of 1.5 ± 0.2 µM and an EC50 value of 24.1 ± 1.4 µM was estimated. The derived EC10 and EC50 values for U were 6.5 ± 0.9 µM and 29.5 ± 1.9 µM, respectively. Hence toxicity of Cd and U for *L. minor* is in the same order of magnitude. Comparing the slope of the two dose response curves shows that the one of Cd is less steep than the one of U.



Level of growth inhibition is presented as a contour plot in different colours as indicated by the legend. For each datapoint n=3 except for control conditions without U or Cd added where n=6. Solid lines represent the growth inhibition predicted by MIXTOX implementing dose-ratio dependent interactions compared to independent action. Black dots represent the tested conditions.

Figure 8: gowth inhibition induced in *L. minor* exposed for seven days to different U and Cd mixtures expressed on (left) nominal and (right) internal U and Cd concentrations.

Possible interacting effects of U and Cd in the binary mixture were assessed using the MIXTOX model as described by Jonker et al. (Jonker et al. 2005). It was shown that independent of the chosen reference model (concentration addition (CA) or independent action (IA)) a strong dose ratio dependent antagonism was observed when the toxicity data were expressed on the nominal U and Cd concentrations. Expressing effects against the measured medium U and Cd concentrations, however, increased the variation in the data. In the MIXTOX analysis this higher varitation in the data resulted in the interactive effect between U and Cd no longer being significantly present. Possible interactions were further studied on internal U and Cd levels. For these data we were obliged to limit ourselves to the lowest concentrations as at higher Cd or U concentrations plants were too fragile to apply a washing procedure (see materials and methods). When internal U and Cd levels were used to express toxicity a significant dose ratio specific antagonism reappeared.This indicates that interaction of U and Cd in the plants can, at least partly, occurred internally after the metal uptake.

Although test circumstances and organisms are very different these results bear similarities to the results obtained with *C. elegans* and *Salmo salar*. These similarities but also differences between the response of the three organisms will be further discussed in paragraph 0.

Main conclusions & Highlights

Frond area was found to be the most sensitive and robust growth endpoint to express the toxicity of both metals.

The EC_{50} values of U and Cd inducing *L. minor* growth inhibition lie within the same order of magnitude indicating both metals are as potent to this macrophyte. However, the shape of the dose response curve of U is steeper than that of Cd.

Using the MIXTOX approach to assess possible toxic interactions between U and Cd a clear dose ratio dependent antagonism compared to either CA or IA was present when the data were expressed on nominal U and Cd concentrations.

While in the medium measured Cd concentrations were in agreement with expectations according to the nominal concentrations, measured U concentrations were lower than nominal. Using measured Cd and U concentrations in the MIXTOX approach a possible antagonistic led to higher variability in the data and possible interactions could no longer be significantly indicated based on the current data.

However, when focusing on internal Cd and U concentrations a significant dose ratio/level dependent interaction is still present indicating that toxicodynamic explainable interactions between Cd and U might be present.

4.1.3 Salmo salar

In this series of experiments, the objective was to address the combined toxicity of U and Cd, to identify possible interaction effects on the toxicokinetic (uptake in gills and liver) and toxicodynamic (mortality, physiological changes). Atlantic salmon (*Salmo salar* L.) was selected due to its high sensitivity, simplicity of sampling internal organs and to ensure that one environmentally as well as economically relevant vertebrate species was included in the study. The design using *S. salar* allows using environmental realistic concentrations and enables in-depth understanding of underlying mechanisms of interactions.

More specifically, we aimed to identify the mechanisms of interaction. First, possible toxicokinetics interaction between U and Cd in uptake from water to different tissues (gills and liver) were in focus. Then, the toxicodynamic responses related to internal concentrations were evaluated.

Material & Methods

Toxicity of binary mixtures of U and Cd to Atlantic salmon juvenile parr were studied using US EPA very soft water, at 8.7±0.3 °C and pH 6.7±0.4. Fish (13.6±5.5 g and 11.5±1.3 mm) were exposed in groups (n=7) to the different U/Cd concentration waters contained in 100 l tanks lined with plastic bags for 96h. A range of water quality parameters such as pH, O_2, CO_2, NH_4, temperature were determined to ensure that the general water quality were within normal range for fish. The exposure protocol was based on the standardized OECD guidelines 203 (Environment Canada, 1998; OECD 1992) and approved in advance by the Norwegian Animal Research Authority (NARA ID: 4615).

Partial factorial design was used with seven nominal U concentrations (0, 4.2, 8.4, 9.9, 11.3, 12.6 and 14.7 µM) and ten nominal Cd concentrations (0, 0.9, 1.8, 3.6, 5.3, 7.1, 8.9, 12.5, 17.8 and 35.5 nM). U was added from stock solution (223 mM) prepared by dissolving $UO_2(NO_3)_2.H_2O$ in Type 2 water 24 h prior to use. Cd was added from stock solutions (31.1 µM Cd) prepared by dissolving CdCl in Type 2 water.

Size fractionation of water was performed to obtain information about U speciation in the exposure water. Membranefilters (0.45µm) and utrafiltration (Pall hollow fiber, cutoff of 10 kDa) were used to exclude particles and colloids, respectively. U and Cd in water samples were determined using ICP-MS (8800 ICP-MS Triple Quad, Agilent Technologies). Actual U and Cd concentrations in exposure waters were verified by measurements to be close to nominal concentrations. U and Cd were predominatly present as < 10 kDa species.

Exposed fish were dissected to collect different tissues and organs according to the EMERGE protocol (Rosseland et al., 2001).

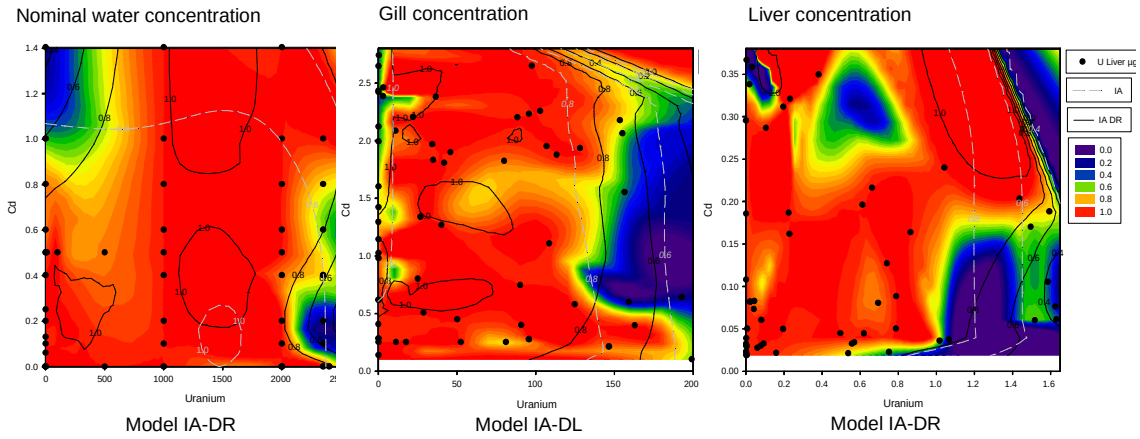
Collected tissue of gills, kidney and liver were freeze dried prior to digestion (Ultraclave, Milestone) and determination of U and Cd by ICP-MS, and bioaccumulation of U and Cd (mg element organ/mg/L water) estimated.

The effect end-point was mortality, but to understand underlying mechanisms other physiological parameters were examined. These included blood samples (plasma ions and blood glucose), and gill and liver samples (gene expression performed on selected samples) (§0).

Results

Dose-response relationship/curves were established for U and Cd individually using mortality as endpoint (Figure 9). U caused no mortality at 8.4 µM and 100 % mortality at 12.5 µM. The LC_{50} value value of 11.0 ± 1.4 µM was estimated based on three different experiments. For Cd 100% mortality was not gained at the highest concentration tested (35.5 nM), but LC_{50} values for Cd were observed to be 8.9 nM. Thus, the toxicity of Cd is around a factor of 1000 higher than of U for *S. Salar*. The slope of the Cd dose response curve is less steep than the one of U. Exposure to the two compounds had different effects on the glucose and blood plasma Cl levels in the fish. The fish exposed to U showed a significantly increased glucose level and low blood plasma Cl levels, while no changes in glucose or Cl levels were detected until mortality for Cd exposed fish. These differences in sub-lethal effects indicate different mode of action of U and Cd.

Based on the findings indicating different mode of actions of the two compounds, possible interacting effects of U and Cd in the binary mixture were assessed using the independent action (IA) model and deviations from this model. The deviation model was used to test deviation from the IA prediction. Results indicating joint effect of U and Cd on survival appeared antagonistic where Cd dominates the effect, not reaching the effect that was predicted by IA, independent using the water concentrations, gill concentration or liver concentration of U and Cd as input to the deviation model.



The color represents the observed data (red 100 % survival and purple 0 % survival). The solid black isobols describe the most significant deviation model (values indicate effect levels), black dots represent concentrations in the tested mixtures. The IA models based on water concentration, gill concentration and liver concentration of U and Cd describe 67 %, 11 % and 64 %, respectively, of the variance of survival and the deviation models 73 %, 26 % & 71 %.

Figure 9: isobolograms describing the U and Cd combined effects on survival of Atlantic salmon juvenile parr on the basis of U and Cd concentration in water, gills and liver of exposed fish using independent action (IA) as reference model (gray isobols).

The antagonistic effect appeared to act on the toxicokinetics, as the uptake of Cd in the liver of the juvenile parr was significantly lower in the presence of U, as well as on the toxicodynamic as demonstrated by the deviation model using the internal concentrations.

The deviation model indicate also a significant synergistic effect of U and Cd on survival where U dominates the effects (close to LC50 of U), as joint effect are higher than predicted by IA, using water or liver concentration of U and Cd as input to the model.

Main conclusions & Highlights

The LC_{50} values of U of *S. salar* juveniles is a factor of 1000 higher than of Cd, and indicate that *U* is less potent than Cd for salmonid juveniles. The shape of the dose response curve of U is, however, steeper than the one of Cd.

U and Cd showed a clear antagonistic interaction using water concentrations or bioaccumulation in gills or liver of the exposed fish. The antagonistic effect appeared mainly on the transfer of Cd from water to gills, as the uptake of Cd in liver of the juveniles parr was significantly lower in presence of U.

The antagonistic effect appeared, however also on the toxicodynamic taken into account the accumulated internal concentrations (U and Cd in Liver), as demonstrated by using deviation models.

4.2 Combined effects of Uranium and Fluoranthene

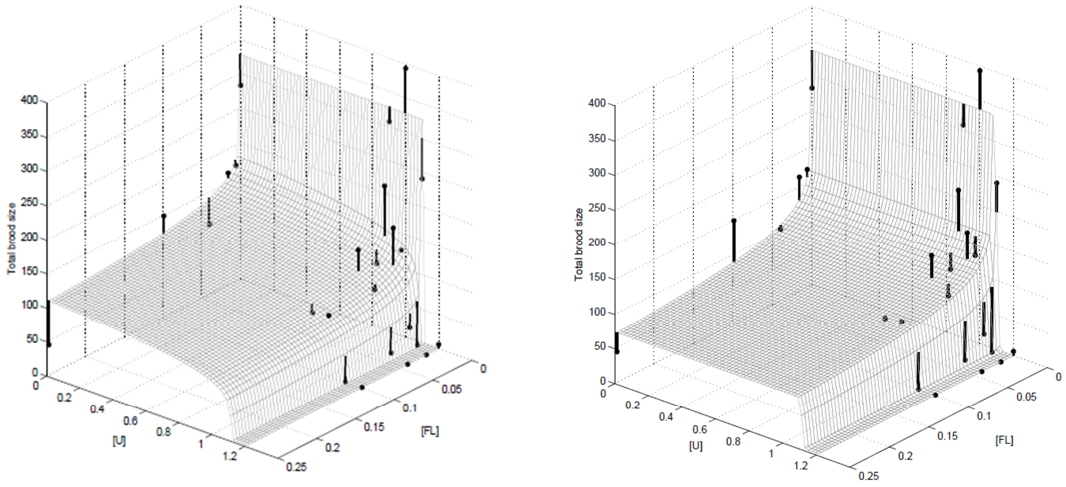
The combined effects of uranium (U) and fluoranthene (FL) were only tested on nematode. In this series of experiments, the objective was to provide a dataset to allow a MixTox tool modeling and identify possible synergistic or antagonistic interactions between U and FL. First, we verified the toxicity of FL exposure alone in our exposure conditions (from 0.004 to 0.25 mM FL): an EC50-96h of 0.06[0.02-0.15] mM (nominal concentration) on *C. elegans* fecundity was obtained, consistent with the literature. Then, a fractional factorial design was used for the U/FL mixture toxicity assay.

Material & Methods

Toxicity of binary mixtures of U and fluoranthene (FL) was assessed on *Caenorhabditis elegans* with a similar protocol to the U+Cd mixture. Nematodes were individually exposed (n = 84) at 28 exposure conditions (n = 3 per condition), including two controls (with and without 0.05% acetone, the solvent used to dissolve FL). Six concentrations of FL (0, 6, 16, 40, 99 and 247 µM) and U (0, 0.9, 1.0, 1.1, 1.2, 1.3 mM) were selected to cover the full dose/response curve. Growth and reproduction of each individual was measured after 96-h exposure

Results

Both Concentration Addition (CA) and Independent Action (IA) additivity models gave relatively good correspondance to the observation (R^2 = 0.82 and 0.78, respectively - p. value <0.05), despite high experimental variability (Figure 10). The fits were not improved using a Synergy / Antagonism deviation model. We can therefore conclude to an additive effect of the U + FL mixture.



Concentration Addition **Independent Action**

black dots represent the measurments, the grid surface represents the modeled values and the vertical black bars represent the distance between the data and model predictions

Figure 10: uranium and fluoranthene combined effects on *C. elegans* brood size (96h post-hatching) and their prediction using CA or IA additivity.

Main conclusions & Highlights

Fluoranthene precipitates beyond 0.25 mM in the agar, while the very flat slope of the FL dose-response curve required covering a wide range of concentrations (eg. on reproduction, the EC50 is 0.056 mM, and the EC70 at 0.5 mM).

These results are to be considered carefully (high experimental variability, actual concentrations could not be measured).

However, despite these experimental flaws, the interaction between U and FL on the growth and reproduction of the nematode, if it exists, is probably not very intense.

4.3 Combined effects of gamma irradiation and cadmium

4.3.1 Caenorhabditis elegans

In this series of experiments, the objective was to test the effect of gamma irradiation on the toxicity of Cd to the growth and reproduction of *C. elegans*. In a separate experiment performed at IRSN (Buisset-Goussen et al., 2014), gamma radiation showed an absence of significant effect on growth and reproduction (dose rate ranging from 6.6 to 42.7 mGy h(-1)). Thus, the first objective was to identify the differences in Cd toxicity in presence or in absence of gamma irradiation exposure. Two Cd

concentrations were selected, at a moderate (ca. EC20) and high (ca. EC50) toxicity level to reproduction.

Then, a second experiment has been conducted during 2014 at NMBU. The objective was to enlarge the range of Cd exposure (two-fold serial dilutions ranging between 0.1 to 100 µM) and gamma exposure (nominal dose rates: 1, 10, 40, 100, 200, 750, 1500 mGy/h.

Material & Methods

The wild-type *C. elegans* strain (Bristol N2) was propagated at 20±1 °C, in darkness, on nematode growth medium (NGM) agar seeded with *Escherichia coli* OP50 strain.

For the first experiment at IRSN, toxicity of Cd in the presence or absence of gamma irradiation was assessed on *Caenorhabditis elegans* with a similar protocol to the U+Cd mixture. Isolated organisms (n=7 per condition) were exposed to external gamma in containers perpendicular to the source to allow homogeneous dose rate at the surface of each experimental unit (Buisset-Goussen et al., 2014). Dose rates were determined by Monte Carlo N-Particle Model (MCNP) and RPL dosimeters. Reproduction and body length were observed daily. Nematodes were exposed continuously to 0, 23.7 and 47.3 mGy/h, with and without Cd (at 8 and 17 µM).

For the second experiment at NMBU, toxicity assessment was performed in an aqueous exposure scenario (standard soil pore water, SSPW) (Tyne et al., 2014), with initial pH 6.7. Synchronized populations of larvae (L1) were exposed at 20±1 °C in darkness in microtitreplates, using circa 15 animals pr 0.5 mL SSPW in each well. The toxicity of cadmium and gamma irradiation was assessed using a full factorial design (Cd were 0.1, 1, 10, 100µM; gamma nominal dose rates were 1, 10, 40, 100, 200, 750, 1500 mGy/h). At 96 h the number of surviving nematodes was counted. The experiments were then terminated, and animals stained with Rose Bengal red and incubated at 60°C for 30 minutes. The samples were analyzed using a LEICA M205 stereomicroscope using LAS image analysis software. For each replicate, length of individual nematodes were measured (n=7). Reproduction was assessed by counting of number of offspring per adult in each well. In addition, fecundity was scored by ratio of pregnant adults. All experiments were performed as independent triplicates. Total Cadmium concentrations were determined by ICP-MS analysis, and the gamma nominal dose rates were 1, 10, 40, 100, 200, 750, 1500 mGy/h.

Results

The results of the first experiment at IRSN confirmed that gamma irradiation at 23.7 to 47.3 mGy/h did not affect significantly the growth and reproduction of nematodes. No difference was observed between Cd-only and Cd+irradiation conditions at the lower tested dose-rate (23.7 mGy/h), on both growth and reproduction. However, the results obtained on reproduction showed a slight potentiation of Cd toxicity at 17 µM for the highest tested dose rate. (Figure 11)

.

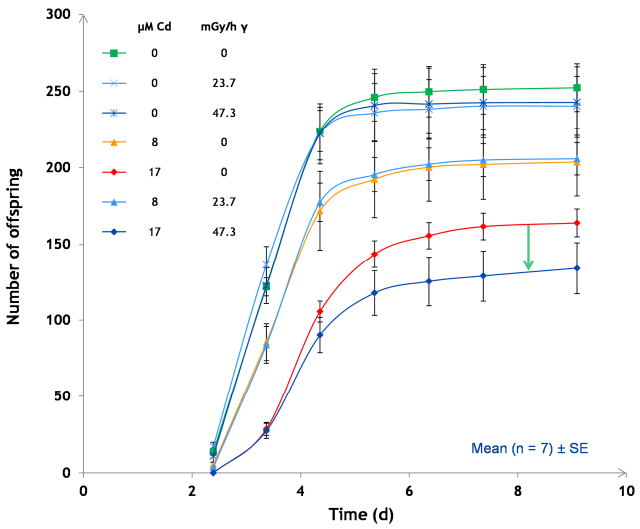


Figure 11: gamma irradiation and Cd combined effects on *C. elegans* reproduction in time (2 to 9d post-hatching).

In the second experiment at NMBU, dose dependent effects were observed for all endpoints following Cadmium exposure (LD50 of 12.5 µM, EC50 was at 6 µM and 3 µM for growth and reproduction, respectively). Gamma irradiation did not result in mortality at any of the tested doses, but reduced growth and abnormalities were observed in certain individuals at 750 mGy/h and 1500 mGy/h. Reproduction was significantly affected at all doserates ≥ 100 mGy/h, however 11% reproduction was observed even at 72 Gy. At 144 Gy reproduction was reduced by 99%, but all nematodes that had reached adult stage carried eggs.

Results from the binary mixtures of cadmium and gamma did not reveal any obvious synergistic or antagonistic effects on mortality, growth or reproduction at any of the tested combinations (Figure 12).

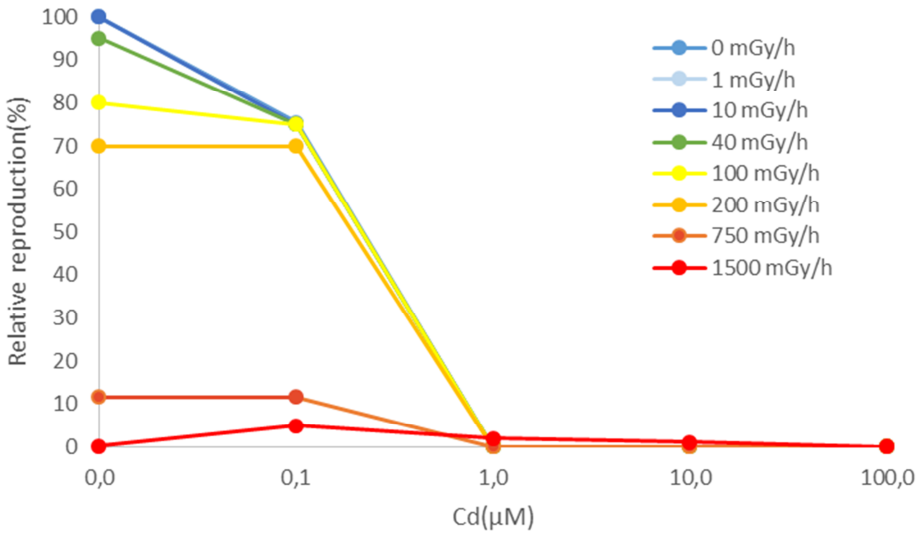


Figure 12: effect on reproduction of binary combination of gamma radiation with cadmium. Reproduction measured as number of offspring per adult, presented as % relative to unexposed controls.

Main conclusions & Highlights

The results confirmed the low sensitivity of *C. elegans* to gamma irradiation up to 47 mGy/h. Reproduction was significantly affected only at dose rates ≥ 100 mGy/h.

The toxicity of Cd was dependent from the exposure protocol (EC50 on reproduction at 13 µM in NGM agar vs. 3 µM in liquid SSPW). A comparison based on internalized concentrations would be necessary to compare properly the results.

A slight potentiation of Cd toxicity was shown in the first experiment (agar NGM protocol), but the second experiment (liquid SSPW protocol) did not reveal any obvious synergistic or antagonistic effects.

The interaction between Cd and gamma irradiation, if it exists, may be low.

4.3.2 Lemna minor

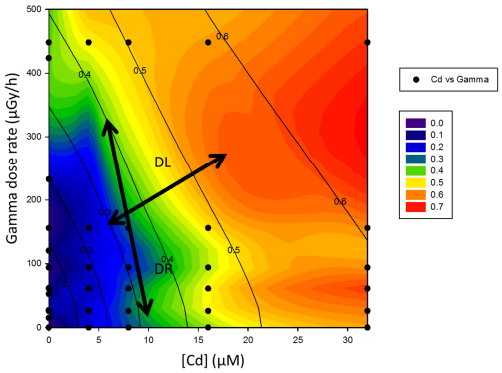
It was the objective of this series of experiments to study possible interacting effects between gamma radiation and Cd exposure in plants. To study this *L. minor* plants were exposed to different dose rates of gamma and or Cd in a full factorial design based upon the results of single exposures. Growth inhibition was measured using frond area as a growth endpoint as this was indicated previously (see §4.1.2) as a robust and sensitive growth parameter.

Material & Methods

Growth and treatment of *L. minor* plants is essentially similar as described in §4.1.2. Briefly, asceptically grown *Lemna minor* cv. Blarney were cultured in 250 mL glass erlenmeyers containing half-strength Hütner medium (Brain and Solomon, 2007) under continuous light (Osram 400W HQI-BT daylight, 80-100 µmol/m^2.s) at 24°C. Plants were sub-cultured every 10-12 days by transferring three plants to 100 mL of fresh growth medium. To expose *L. minor* to gamma radiation in combination with Cd plants were transferred to the irradiation facility of SCK•CEN and placed at different distances from a ^{137}Cs source for 7 days. As such plants were exposed to gamma dose rates varying from 26 to 1500 mGy/h. Six different gamma levels were obtained in this way. In a full factorial design foud different Cd concentration, added as $CdCl_2$ dissolved in deionised water, varying from 4 to 32 µM were applied to each but the highest gamma dose rate. Control plants for gamma radiation exposure were placed in a growth cabinet under similar conditions as the irradiation facility. The *Lemna* growth inhibition test followed OECD guidelines (OECD 2006) with some modifications such as using as a test medium K-medium (Cedergreen et al. 2007) with phosphate concentrations lowered to 0.5 mg/L. To stabilise pH during toxicity tests, 5 mM filter-sterilised (0.22 µm) MES (2-(N-morpholino)ethanesulfonic acid) was added. For the experiments three plants (between 9 to 12 fronds) were aseptically transferred to polycarbonate-pots containing 100 mL of the modified K-medium. A 1 cm, surface-sterilised floating ruler was added for calibration of images. Pots were covered with a 9 cm plastic petridish and experiments were run for 7 days under the same light and temperature conditions as used for normal plant culture. Six replicas were used for control conditions and at least three for each of the U and Cd concentrations applied. To evaluate Lemna growth pictures were taken every 2 days and the frond number and frond area was determined by picture analysis (ImageJ software). After 7 days, fresh and dry weight of the plants was measured. After the 7 day exposure three plants from each exposure condition were taken aseptically and put again in 100 mL fresh half strength Hütner medium for 7 days under control conditions to evaluate recovery capacity. Growth during this recovery experiment was again followed taking pictures at day 2, 5 and 7 of the recovery period and by sampling plants at the end of the period for fresh weight and dry weight analysis.

Results

Both Cd and gamma irradiation induced growth related effects in *L. minor*. When exposing the plants solely to Cd an EC10 value of 1.5 ± 0.2 µM and an EC50 value of 24.1 ± 1.4 µM were estimated. Gamma irradiation alone resulted in dose dependent growth inhibitions starting from approximately 27 mGy/h onwards. A ten-percentage growth inhibition (EDR10) of frond area gave an estimated dose rate of 95 ± 7 mGy/h and the EDR50 was estimated to be around 852 ± 41 mGy/h. As for Cd and U (see §4.1.2) also for gamma irradiation frond area was the most sensitive area compared to the other growth related endpoints tested.



For each data point n=3 except for control conditions without U or Cd added where n=6. In (A) solid lines represent the growth inhibition predicted by MIXTOX according to Independent action. Black arrows represent the area dominated by dose-level (DL) or Dose-Ratio (DR) dependent interactions Black dots represent the tested conditions.

Figure 13: levels of growth inhibition measured in *L. minor* plants exposed to different gamma/Cd mixtures for 7 days and presented as a contour plot in different colours as indicated by the legend

For the mixture experiment it was a disadvantage that gamma effects on growth were only observed for the highest dose rates tested. Using the MIXTOX approach we could only apply IA when the highest dose rate of 1500 mGy/h was added in the evaluation so a good description of the Gamma dose response curve needed to be included. However, while independent action fitted this data set pretty well (explaining 93% of the total variation), there is (see Figure 13) a general antagonism at lower doses and a small area of synergy where Gamma doses are low and Cd is starting to have an effect. It is therefore not surprising that SA, plus both DL and DR dependent interactions both provided significant better fit of the data than IA indicating significant interactions between Cd and gamma radiation were present.

From Figure 13 it is clear that for the biggest part of the surface DR and DL dependent predictions are close to the IA predictions. The DR dependent function is mainly trying to explain the overall antagonistic interactions seen compared to IA in the lower than EC50 part of the mid mixture ratio part of the surface. The interaction switched to synergism at very high gamma dose rates and also, for the lower dose rates of gamma combined with the higher Cd concentrations. Like the DR dependent function the the DL one is also mainly describing the antagonism present at low effect levels. This decreases in magnitude as the EC50 level is approached and final switches to mild synergy at dose levels above the EC50/EDR50. As high gamma dose rates above 800 mGy/h (EDR50 value) are not encountered in the environment even at accident scenario's (Table 2), this high effect level synergistic interaction is unlikely to occur in

the environment, while the synergy seen at combinations of lower Gamma (~30mGy/h) and middle range Cd (10-20 µM) may realistically be observed during accidental releases.

The exposure of *L. minor* plants to gamma radiation or to Cd induced distinct morphological effects in the plants. After seven day exposure plants exposed to the highest gamma dose rates showed a growth arrest but seemed to have no further morphological damage while Cd treated plants were showed chlorosis, colony breakage and morphological abnormalities in addition to reduced growth. To further study this a recovery experiment was performed that allowed plants to regrow in control conditions for an additional seven days. For the highest applied dose rates, toxicity was more severe after a seven-day regrowth period (see MS 4.7). In contrast, when plants exposed to different Cd concentrations were put again in control conditions an almost complete recovery was obtained after 7 days for all Cd concentrations tested. For the plants recovering from a combined gamma and Cd exposure a possible synergistic effect compared to IA seemed to be present. At this moment a final experiment is being run in which the exposure is being shifted in time. Plants are first being exposed to different gamma dose rates and then in the revovery period different Cd concentrations are being added. The experiment is being completed with a final recovery period (without Cd or gamma).

Main conclusions & Highlights

Both Cd and gamma adversely impact *L. minor* growth but with distinct morphological features. Although for gamma radiation, within the tested dose rate range, a clear effect was only visible at the highest dose rates at the end of the experiment. During an additional seven days recovery experiment it was clear that *L. minor* are able to recover from the Cd induced stress whereas gamma effects worsened.

Simultaneous exposure of plants to Cd and gamma radiation results in a clear joint effect that cannot be explained solely by the presence of either stressor.

In addition compared to IA an antagonistic interaction (DL and DR dependent) seems to be present at low dose levels or Cd concentrations whereas at higher dose levels or high Cd concentrations synergistic interactions might occur. These synergistic interactions are however unlikely to occur at environmental concentrations (Table 2).

4.3.3 Salmo salar

In this series of experiments, the objective was to test the combined toxicity of gamma and Cd to the development of Atlantic salmon (*Salmo salar L.)* embryos. The study focused on the effects on development and mortality during 92 days exposure, from fertilization of the egg to hatching.

Material & Methods

Combined toxicity of gamma irradiation and Cd towards Atlantic salmon embryos was studied using US EPA very soft water, at 5.9±0.3 °C and pH 6.7±0.4. Dry stripped eggs (200) from three different female were dry fertilized with sperms from one male, and placed in three separate exposure units according to Kallqvist et al. (2003), as parallels. Each exposure unit received water from a reservoir in a recycling system. Eggs were exposed for 92 days, from the time of fertilization until hatching. A range of water quality parameters such as O_2, CO_2, NH_4, temperature were determined to ensure that the general water quality were within normal range for fish. The exposure protocol was based on the standardized OECD guidelines 210 (OECD 2013).

Half factorial design was used with five nominal gamma doses (0, 0.4, 1, 10 and 40 mGy/h) and five Cd nominal concentrations (0, 2.7, 26.7, 89.0, 267 nM). A cobalt 60 source at NMBU was used for gamma exposure and different dose rate was obtained by placing the exposure chambers at different distances from the source. Total dose for gamma exposure was determined using Inlight Nanodot (Landauer). Cd was added from stock solutions (31.1 µM Cd) prepared by dissolving CdCl in Type 2 water.

Cd concentrations in the reservoir were measured regularly, and exposure solutions were changed if the deviation for Cd concentration was more than 10 % compared to nominal concentration. To obtain information about size distribution of Cd in water, membranfilters (0.45µm) were used prior to the determination of Cd using ICP-MS (8800 ICP-MS Triple Quad, Agilent Technologies). Concentrations of Cd in unfiltered and filtered samples were similar and verified to be close to the nominal concentrations.

Bioaccumulation of Cd in eggs was followed during the exposure period. To determine the uptake of Cd, six eggs were randomly collected from each replicate at fixed time intervals during the experimental periode. Eggs were sampled individually in the form of total egg (n=3) and egg contents (n=3), emptied by a syringe. Whole eggs and eggs contents were weighted, digested using ultraclave (Milestone) and analysed of Cd using ICP-MS.

The effects end points were degree of swelling, time of hatching and mortality. To understand the underlying mechanisms gene expression were performed on limited selection of embryos (§0).

Results

Results demonstrated limited transfer of Cd into the eggs as only a small fraction, about 3 %, was measured within the eggs and the larger part would have been associated with the chorion. Cd exposure affected the swelling at all Cd concentrations tested, while a slight increase in mortality was observd at exposure concentrations 26.7, 89.0 and 267 nM Cd (Figure 14). Delayed hatching was only detected at the highest exposure concentration (267 nM). Gamma irradiation caused a slight increase in mortality at 10 mGy/h and 100 % mortality at 40 mGy/h. It was not possible to verify interacting effects of gamma and Cd using the current effect end-points and tested concentrations

and doses. Additional effect parameters should be assessed before effects interactions can be assessed.

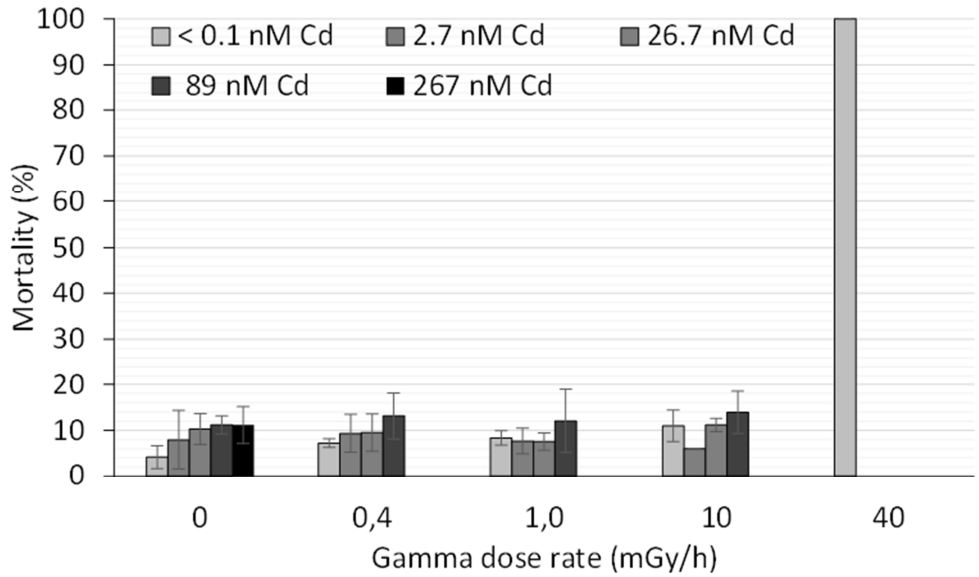


Figure 14: Effect on mortality (%) of binary combination of gamma radiation with cadmium towards Atlantic salmon (*Salmo salar* L.) embryos.
Main conclusions & Highlights

The LC_{50} values of gamma for *S. salar* juvenile embryos was between 10 and 40 mGy/h when exposing the eggs from fertilization until hatching, while for Cd the LC_{50} concentration was higher than 267 nM. The EC_{50} for time of hatching was not possible to evaluated from the gamma radiation experiments, while Cd the EC_{50} was lower than 267 nM.

Results from the binary mixtures of gamma and Cd did not reveal any obvious synergistic or antagonistic effects on mortality or development at any tested combinations up to 10 mGy/h gamma dose rates and 89 nM Cd.

4.3.4 Plankton community

In this series of experiments, the objective was to determine whether there was an additive or interactive effect of gamma irradiation and Cd on a range of endpoints measured in zooplankton and phytoplankton. The main focus was to determine effects on the transfer of carbon from the primary producers to the consumers, but other endpoints were also measured in order to try and understand potential mechanisms.

Material & Methods

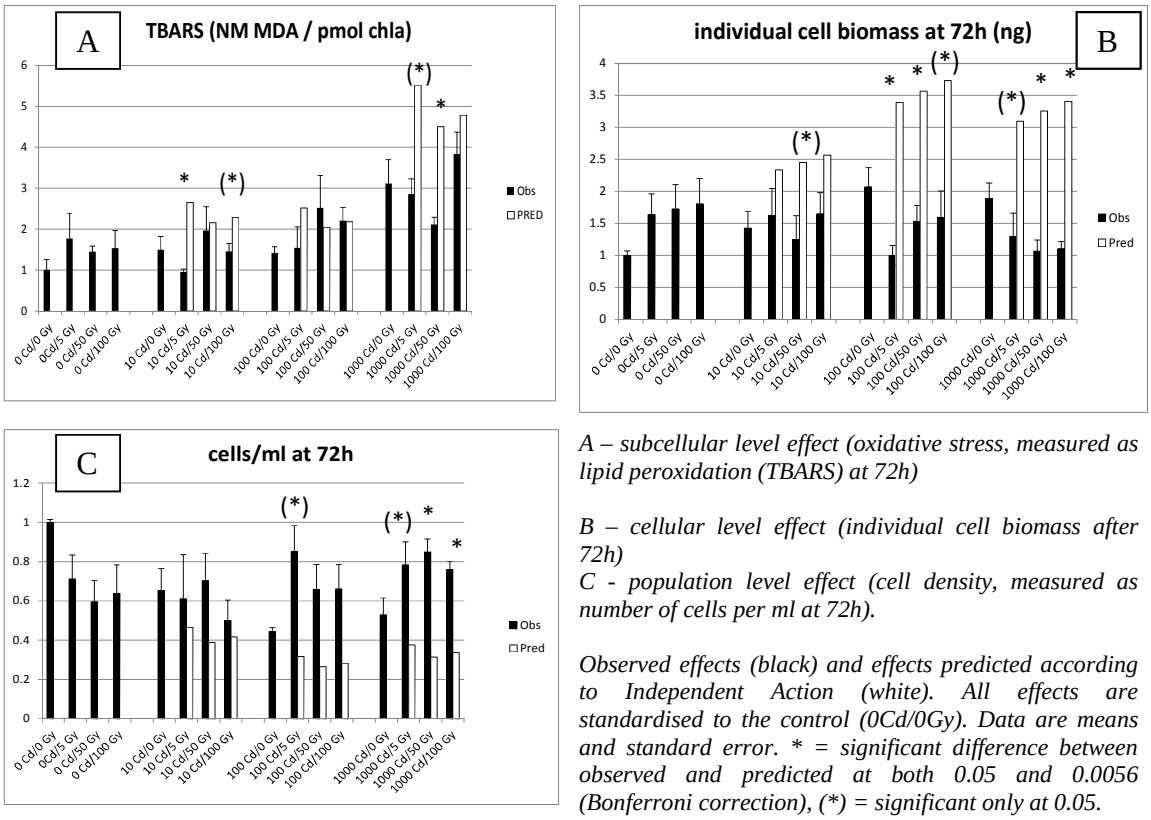
A first experiment exposed both test species (*P. subcapitata* and *D. magna*) separately to the same doses/concentrations of gamma/Cd (0, 5, 50, 100 Gy at 6.7 Gy min^{-1} and 0, 10, 100, 1000 µg Cd L^{-1} (0, 0.09, 0.89, 8.9 µM, respectively) in a fully factorial experimental design. Both species were subjected to an acute exposure of gamma irradiation followed immediately by exposure to Cd (72h for the alga, 24h for the cladoceran). *D. magna* endpoints were: growth, respiration, number of eggs, oxidative stress (catalase) and mobility. *P. subcapitata* endpoints were oxidative stress (lipid peroxidation; TBARS and catalase; CAT), pigment composition (neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, chlorophyll a and b), vitamin B1 content, individual cell size and biomass, population biomass and population cell density.

Observed effects were compared to the predicted joint effects of the mixed stressors based on the Independent Action (IA) concept using point by point mixture toxicity statistics (see 3.2.3.2).

Two further experiments were performed with chronic gamma exposures and a more extensive experimental design. There were six Cd concentrations (measured concentrations – 0, 11.5, 22, 117, 236, 444 µg L^{-1} (0, 0.1, 0.2, 1.0, 2.1, 3.95 µM, respectively) and six gamma doses (total measured dose over 3 days – 0, 2.5, 5, 12, 19, 28.6 Gy), as well as extra single factor treatments in order to establish better single factor dose-response curves. The main endpoint was assimilation of carbon from *P. subcapitata* by *D. magna*. Full MixTox analysis was performed. Of these the second experiment was most conclusive and is summarized here.

Results

The first experiment showed that for *D. magna*, the clearest effects were seen for respiration, where there was a strong antagonistic response in all binary combinations (ie. decrease in respiration was not as much as predicted by IA). There was a slight antagonistic response for *D. magna* growth, but many of the *D. magna* died at the highest gamma dose (100 Gy). Interactive effects for other endpoints (number of eggs, oxidative stress) were not possible to calculate due to lack of data. For nearly all *P. subcapitata* endpoints, combined effects were antagonistic compared to values predicted by IA, though the level of significance varied (Figure 15).



A – subcellular level effect (oxidative stress, measured as lipid peroxidation (TBARS) at 72h)

B – cellular level effect (individual cell biomass after 72h)
C - population level effect (cell density, measured as number of cells per ml at 72h).

*Observed effects (black) and effects predicted according to Independent Action (white). All effects are standardised to the control (0Cd/0Gy). Data are means and standard error. * = significant difference between observed and predicted at both 0.05 and 0.0056 (Bonferroni correction), (*) = significant only at 0.05.*

Figure 15: Selected combined effects of gamma irradiation and cadmium in *P. subcapitata*

The results of the second experiment are summarized in Table 6. For ^{14}C assimilation by *D. magna*, the SA (synergism) model was best when testing deviation from CA, though the synergism at lower dose combinations should be treated with caution due to the large influence of a few data points. Testing deviation from IA suggested the DL model was best (change from antagonism to synergism at dose levels > EC50). For *D. magna* immobility, the best model fits were CA S/A (antagonism) and IA D/L (antagonism at low levels and synergism at >EC50 relative to the IA model). Significant antagonism was seen for *D. magna* growth compared to both CA and IA predictions (S/A model best in both cases) – ie the *D. magna* grew more than expected.

Table 6: Summary of results from chronic gamma and Cd experiments with *Daphnia magna*.

Endpoint	Best model CA	CA Interaction	Best model IA	IA Interaction
Incorporation of C	S/A	Synergism*	DL	Antagonism at low doses; synergism at high.
Acute immobility	S/A	Antagonism	DL	Antagonism at low doses; synergism at doses >EC_{50}
Growth	S/A	Antagonism	S/A	Antagonism

*this result should be treated with caution, see text.

4.4 Combined effects of gamma irradiation and fluoranthene

The combined effects of gamma irradiation and fluoranthene were only tested on the simple (2 species) community of phytoplankton and zooplankton species. An experiment was carried out to investigate how the transfer of carbon between the primary producer *Pseudokirchneriella subcapitata*, and the consumer *Daphnia magna* was affected by the acute exposure of gamma radiation in combination with the PAH fluoranthene (FL).

Material & Methods

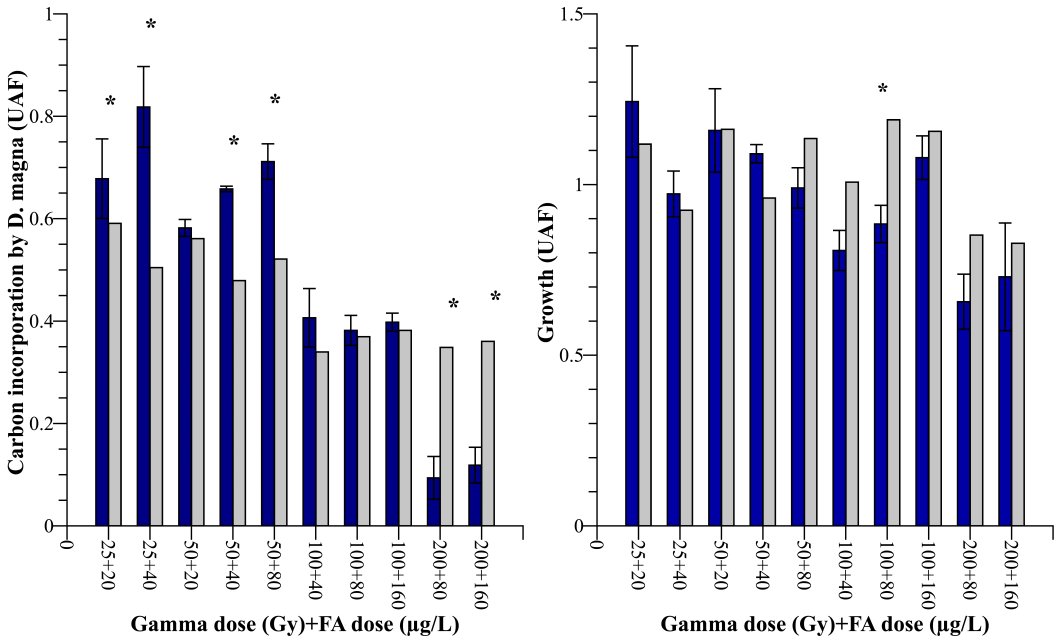
D. magna were exposed to five concentrations of FL (0, 23, 44, 67, 147 µg L^{-1} (0, 0.11, 0.22, 0.33, 0.73 µM, respectively)) and then five acute doses of gamma (0, 25, 50, 100, 200 Gy at 6.7 Gy min^{-1}) as single contaminants and in nine binary combinations in a combined factorial and ray design. FL exposures were for 24h, followed immediately by the acute gamma exposures. A 24h feeding test was then performed on the *D. magna* in clean media.

The observed data for 3 endpoints – incorporation of carbon (^{14}C) by *D. magna* from ^{14}C-labelled *P. subcapitata*, *D. magna* ingestion rates (number of cells consumed during 24h) and growth – were compared to the predicted joint effects of the mixed stressors based on the Independent Action (IA) concept. Since it was not possible to obtain single stressor dose-response curves for fluoranthene, point by point mixture toxicity statistics were used (see 3.2.3.2).

Differences between observed and predicted values were tested with independent one sample Student's T-tests.

Results

There were significant deviations from the IA predictions especially regarding the carbon incorporation by *D. magna*, where antagonistic effects were observed at the lower dose combinations (ie. more C was incorporated than predicted by IA) (Figure 16A). In contrast, synergism was seen at the highest dose combinations (200 Gy + 66 and 147 µg/L FL). A similar pattern was seen for ingestion rates in the mixture treatments with lower dose combinations. The same trends were seen for *D. magna* growth but few of the observed differences were significantly different to the expected values (Figure 16B).



*Values on the x-axis represent gamma radiation+FL exposure. Blue bars represent observed data while grey bars are predicted values according the IA concept. Error bars represent standard error. * indicate observed treatments that are statistically significantly different from the predicted values.*

Figure 16: Carbon incorporation by *D. magna* from *P. subcapitata* (right) and *D. magna* growth (left) exposed to gamma irradiation and fluoranthene.

Main conclusions & Highlights

There were significant deviations from the IA predictions, especially regarding the carbon incorporation and ingestion rates by *D. magna*, where antagonistic effects were observed at the lower doses.

In contrast, synergism was seen at the highest doses.

In mixtures of gamma and FL the IA-predicted effects seem to be conservative as antagonism between the two stressors was the dominant pattern.

A possible mechanism for this antagonism is the stimulation of cellular anti-oxidative stress mechanisms by exposure to gamma irradiation.

5 Discussion on the underlying mechanisms of interactions

There is still a clear need for a comprehensive classification scheme for stressors on a mechanistic basis, to support application of CA/IA models for ERA. In support to the application of those concepts to mixtures including radioactive substances, two areas of particular mechanistic understanding have been shown in this work to be crucial:

- For radioactive substances, the radiotoxic effects seem non-specific (e.g. DNA and protein alteration, oxidative stress) and should theoretically best relate to Independent Action, more than Concentration Addition.
-
- On the other hand, chemical toxicity and specific tissue/cellular distribution (and internal dose) of some alpha or beta emitters would need specific consideration (e.g. uranium), in the same way than trace metals (e.g. Cu, Cd, Pb…) where bioavailability, bioaccumulation and bioconcentration in specific tissues or organs are key processes to link exposure and effect.

Moreover, the results presented in this document also highlight that the identification of an interaction also depends on the chosen endpoints (level of observed biological organization) for one species, and also differs between species as a function of the considered binary mixture. This had a strong influence on the conclusions we could draw regarding our initial hypotheses (Table 7).

Table 7: Final conclusions on the initial hypotheses

H1: The effects of chemical contaminants and ionising radiation are additive.
H2: If there are interactions between the effects induced by ionising radiation and chemical contaminants, these interactions will be independent of test organism.

	U+Cd	U+FL	γ+Cd	γ +FL
H1	**rejected**	**accepted**	**rejected**	**rejected**
H2	**accepted**		**rejected**	

In the following discussion, we focus mainly on the two binary mixtures where both hypotheses could be tested (U+Cd and gamma+cadmium), and contrasting observations were made: for U+Cd, a common antagonistic pattern was shown, with consistent underlying explanations (see §0), whereas it was not the case for gamma+cadmium (see §4.3) or other mixtures.

Variable conclusions, among different species or tested endpoints, calls for further biological (mechanistic) investigations. Four different approaches were tested in order to addres the mechanistic understanding of interactions and their variations:

- the study of toxicants bioaccumulation and uptake in support to the understanding of uranium and cadmium interactions (§0);
- the study of gene expression in support to the understanding of single and combined effects of U and gamma irradiation (§0);
- the study of complex links between subcellular, cellular and population-level effects in phytoplankton, as well as trophic interactions between phytoplankton and zooplankton (§5.3);
- the use of a toxicokinetic and toxicodynamic models (DEBtox) for a generalization of the description of combined effects of cadmium and uranium over time and among endpoints. (§5.4).

5.1 A common pattern for the combined effects of U and Cd among species groups: understanding bioaccumulation and toxicokinetics is required for explaining/predicting mixture effects

An overall antagonistic interaction in the combined effects of U and Cd was identified. This interaction was remarkably consistent regardless of the tested species (*C. elegans*, *L. minor* and *S. salar*) and raises the question of a common mechanism, independent of the organism.

Here, we propose to reconsider the results obtained on the combined effects of U and Cd on the three species tested (see §4.1), paying special attention to exposure concentrations in the exposure media, and in light of uptake and bioaccumulated concentration toxicokinetics in organisms. We then propose to conclude on the possible origin (shared or not among organisms) of this antagonistic combined effects of U and Cd.

5.1.1 An interaction between U and Cd in the exposure media of C. elegans

The re-analysis of U/Cd combined effects on *C. elegans* on the basis of bacterial lawn concentrations (i.e. expected bioavailable concentrations) showed that an important part of the strong antagonism could be explained by an interaction between U and Cd on bacteria (§4.1). Those results indicated that an interaction was occurring in the exposure media between U and Cd, as U hindered the transfer of Cd from agar to the bacterial lawn.

A possible explanation for this interaction could be that the presence of U in much higher molar concentrations decreased the number of available adsorption sites for Cd on the bacteria. In a separate experiment, we showed that the internalized Cd fraction in nematodes was reduced by a factor close to 2 in the presence of U (Figure 17), similar

to the transfer of Cd from agar to bacterial lawn. These results were consistent with the assumption that feeding is the main uptake route of metals in *C. elegans* and may suggest that the U/Cd interaction in exposure media explains, at least partly, the strong antagonism identified in length increase and brood size data on the basis of U/Cd agar concentrations.

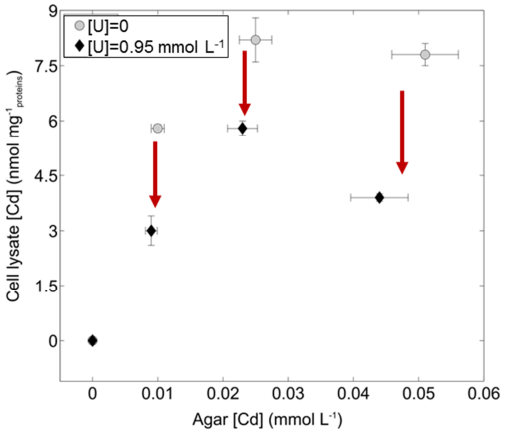


Figure 17: Cd bioaccumulation by *C. elegans* larvae after 10h exposure in the presence or absence of U in agar

Interactions between metals occurring in exposure media have already been documented in the literature. For example, the simultaneous presence of copper and zinc in standard soil was found to result in synergistic effects on population growth of *C. elegans* (Jonker et al., 2004).

A number of studies have also reported antagonistic interactions between the bioavailable form of Cd (Cd^{2+} free ions) and other divalent cations in different organisms (Groten et al., 1991, Odendaal and Reinecke, 2004 and Zidar et al., 2009). One of the major reported bioavailable forms of U is uranyl cation UO_2^{2+} (Hogan et al., 2005). In the present study, the difference in concentration levels between U and Cd favours a competitive decrease in Cd internalization, because there was between 5 and 85 times more U than Cd in bacterial lawn. Such competition would be consistent with a net antagonistic interaction regarding toxicity.

5.1.2 *Bioaccumulation measurement in* Salmo salar *tissues reveals toxicokinetics interaction between U and Cd*

To study the possible toxicokinetic interacting effects of U and Cd in *Salmo salar* the concentrations were measured in water, in gills and liver of exposed juveniles (§4.1.3) and in eggs of exposed embryos (§4.3.3). Concentration of both U and Cd in tested water were close to nominal concentrations. Fractionation of exposure waters demonstrated that U was predominantly present as dissolved species, as more than 90 % was present as U species less than 10 kDa in size independent upon pH. At highest

concentration tested, however, a small colloidal 1-2 nm in size was observed using FFF-ICP-MS decreasing with increasing pH (7.3-7.7). More than 90 % of Cd was also present as dissolved species (<0.45µm) and as LMM species (<10 kDa).

U accumulated in fish gills, and the accumulation was positively correlated with the U concentration in the water, and strongly dependent on pH with higher accumulation at lower pH (pH range 5.5-6.0) and lower accumulation at higher pH (7.3-7.7). The U gill concentration correlated with changes in blood glucose and plasma Cl as well as with mortality independent of pH. In fish with more than 210 nmol U/g dw gill, blood samples showed reduced plasma Cl concentration and increased blood glucose. This indicated ion regulation problems and also the induction of stress responses in fish. Mortality was observed at concentration levels >340 nmol U/g gill dw. A positive correlation between U concentration in gills and in the liver and kidney of fish showed that U was also crossing the blood barriers of the gills and was actively taken up in fish.

Cd also accumulated in gills and liver of the fish, and the accumulation was also positively correlated with the Cd concentration in water. In addition, a significant correlation was observed between Cd- liver concentration and mortality. Mortality was observed at concentration higher than 15 nmol Cd/g dw gill and 3 nmol Cd/g dw liver. Thus, the observed effect on survival of both U and Cd exposed fish were correlated with the accumulated concentration of the elements.

The uptake of U and Cd in fish eggs was also examined (see method description in §4.3.3). This experiment demonstrated a high uptake of U (30 %) and 100 % mortality at 8.4 µM. However, fish eggs exposed to Cd showed relative low uptake (about 3 %) and limited mortality at the highest concentration tested (267 nM). These results demonstrated a lower transfer of Cd compared to U into the eggs and the Cd measured in the egg samples was mainly associated with the chorion. The low accumulation of Cd into the eggs also explained the apparent low sensitivity to Cd in the early life stages (embryos) compared to juvenile stage (Cd LC_{50} values of embryos and juveniles, was >267 nM and 8.9 nM, respectively). On the opposite, the large accumulation of U in the eggs may explain the apparent higher sensitivity of U to the early life stages (eggs) compared to juvenile stage (U LC_{50} values of embryos and juveniles was <8.4 µM and 11.0 ± 1.4 µM, respectively).

When exposing Atlantic salmon juveniles to different concentrations of U and Cd in mixtures (see §4.1.3), the Cd uptake in fish was lowered in the presence of U, as demonstrated by the significantly lower Cd concentrations in liver (Figure 18). This indicates that U blocks the uptake of Cd into the fish. However, the mechanism is one way only as the presence of Cd had no influence on the accumulation of U in fish. The results show interaction effect of U and Cd on the toxicokinetic level. Since concentration of Cd in the liver of the fish exposed to both Cd and U never reached levels associated with Cd mortality (>3 nmol/g dw liver), further interactions on the toxicodynamics was demonstrated also to be antagonistic by the deviation model using the internal concentrations (see §4.1.3). Results indicate also a significant synergism where U effects dominates.

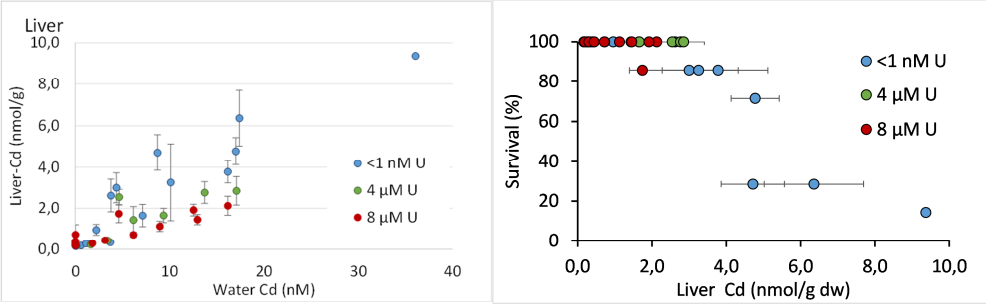


Figure 18: Correlation between concentrations of dissolved Cd in water and Cd in liver (left), and correlation between concentration of Cd in liver and survival of Atlantic Salmon (*Salmo salar*) at different U water concentrations (right).

5.1.3 Bioaccumulation measurements in Lemna minor *reveal toxicokinetics interaction*

For studying the possible interacting effects of U and Cd in *L. minor* the metal concentrations were measured in the medium and in the plants after seven days exposure. Test solution concentration for Cd were close to nominal concentrations, with between 86% and 95% of the Cd retrieved in the filtered medium sample with an exception for the lowest two concentrations of 0.5 and 1 µM where only 66% and 75% of the nominal Cd concentration was retrieved, respectively. In contrast the concentrations of U retrieved in filtered medium after seven days were lower than expected. For the four lowest U concentrations used only between 49 and 69 % of the metal could be measured in filtered fraction. For the three highest concentrations between 84 % and 89 % of the nominal concentration was found in the filtered (filtration pore size of 0.45 µm) medium. In the mixture set up it was shown that within the tested concentration range the presence of one metal did not influence the total amount retrieved in the medium of the other metal. As expected, the uptake of Cd and U into the plants increased with increasing concentrations of the metals in the medium. Within the tested concentration range the presence of Cd had no influence on the accumulation of U into the *L. minor* plants. Contrastingly, Cd uptake into the plants was significantly lowered in the presence of U, indicating that U blocks the uptake of Cd into the plants. In the *L. minor* experimental setup U and Cd are present in similar molar concentrations. Hence, in contrast to the experiments with *C. elegans* described in §5.1.1 the decrease in Cd uptake by U in the plants cannot be explained by U outcompeting the Cd adsorption. Modelling the speciation of U and Cd within the test solution did not show significant changes in the calculated free ion concentrations of either metal indicating that it is not an effect of changed bioavailability of Cd.

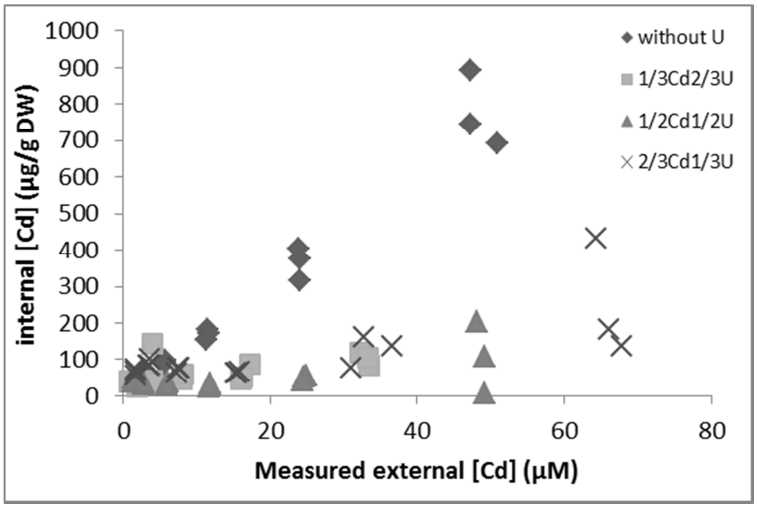


Figure 19: Bioaccumulation of Cd into *L. minor* plants exposed for seven days to different Cd concentrations in the presence or absence of different U concentrations. The ratio between the Cd and U is given in the legend.

When expressing the toxicity of U and Cd data on internal metal concentrations it is clear that toxicity of one of the metals alone could not explain the data and hence joint effect was present that followed both IA as CA. Additionally it was shown that at low internal U and Cd concentrations a clear dose-ratio dependent antagonism was present compared to the reference models. These results indicate that the interacting effect of U and Cd in *L. minor* occurs at the toxicodymamic rather than the toxicokinetic part of the toxicity.

5.1.4 *Main conclusions & Highlights*

Of these three studies on *C. elegans*, *L. minor* and *S. salar*, an overall antagonism was observed, but different conclusions can be drawn.

Our results showed that the combined effects of U and Cd are highly dependent on how the exposure is assessed. For plants and fish, the verification of actual exposure concentrations in the water, and in the case of salmon the element speciation, allowed us to assume that no chemical interaction occurred (eg. due to changes in calculated chemical speciation). Changes in the exposure conditions however (eg. pH for fish) were at the origin of changes in bioavailability, which can be possibly considered through bioavailability modelling approaches (see D4.2 for an illustration of such approaches). Despite the absence of interactions in the water, U and Cd bioaccumulation in plants and fish were affected in mixtures in comparison to single exposure indicating potential toxicokinetic interactions (eg. at uptake sites).

On the other hand, the apparent antagonism observed in the nematode was largely explained by an interaction in the exposure medium (on bacteria that serve as food),

tuhs the bioaccumulated concentrations were rather correlated with levels in ingested feed than the exposure medium (agar).

Our results showed also that the presence of Cd had a negligible influence on the accumulation of U. Inversely, Cd uptake was significantly lowered in the presence of U, indicating that U blocks the uptake of Cd into the plants and fish, as well as the the transfer of Cd from media (agar) to food (bacterial lawn) of nematodes. This trend is probably due to the intrinsic properties of U and Cd, with bioavailable ions (ie. free cationic ions) that may compete for sorption sites and uptake. Moreover, this trend was observed not only when U was in excess (eg. nematode experiments and fish experiment), but also at equimolar U/Cd concentrations (eg. plants).

Lastly, once such interactions in the medium and at toxicokinetic level are considered, our results showed also that interacting effect of U and Cd may also occur at toxicodynamic part of the toxicity, and may change in time or as a function of the considered endpoints.

5.2 How gene expression (pathway level) can help in the understanding of single and combined effects of U and gamma irradiation

Gene expression analyses of Atlantic salmon (*Salmo salar* L.) tissues (gills, liver and embryo) have been applied to identify differentially expressed genes (DEGs) after exposing juvenile parr to U, gamma and Cd separately and in combination. Specific toxicological and/or biological processes could be determined after mapping the salmon DEGs to mammalian orthologs and subsequently to protein-protein network and pathway analysis.

Interaction at pathway level in gills and liver of Atlantic salmon juveniles was studied using microarrays in combination with quantitative real-time reverse transcription polymerase chain reaction (qPCR). The juveniles were exposed to the stressors 48 hrs prior to sampling and RNA extraction. The interaction at pathway level was studied by RNA seq of embryo collected after 159-degree day exposure. Individual Atlantic salmon eggs with wet weight 0.12±0.01 g (mean ± SD) were sampled and immediately frozen by liquid nitrogen prior to storage at -80 ^{o}C and RNA extraction. Total RNA was extracted using Direct-zol™ RNA MiniPrep kit (Zymo Research Corp., Irvine, CA, USA) according the manufacturer's instructions.

Uranium exposure

Gene expression in fish gills

The microarray gene expression analysis identified 142, 573 and 1688 differentially expressed genes (DEGs) in the gills of salmon after 96 h exposure of juveniles to 0.5, 2 and 4.0 mg U/L (pH 7.2), respectively These DEGs were associated with known gene ontology (GO) functions such as immune response, DNA/protein damage, apoptosis and sodium ion transport. The results showed that various toxicity pathways involved in

oxidative stress, mitochondrial dysfunctions (electron transport chain), DNA/protein damage, immune system and ion transport were affected by the U deposition in gills. This is also supported by the fact that fish exposed to 4 mg U/L at pH 7.2 showed signs of impaired ion regulation with significantly reduced blood plasma-Cl and -Na. The massive increased expression of glycolysis, glucogeogenesis and insulin like receptors genes reflected the observed high plasma glucose levels caused by the uranium.

Gene expression in fish liver

The microarray gene expression analysis identified 847, 891 and 766 differentially expressed genes (DEGs) in the liver of salmon after 48 h exposure of juveniles to 0.25, 0.5 and 1.0 mg U/L, respectively (Figure 20). These DEGs were associated with known gene ontology functions such as generation of precursor metabolites and energy, carbohydrate metabolic processes and cellular homeostasis. The salmon DEGs mapped to mammalian orthologs and the protein-protein network and pathway analysis showed that various toxicity pathways involved in mitochondrial functions, oxidative stress, nuclear receptor signalling, and organ damage were commonly affected by all U concentrations (Figure 21). However, no significant formation of micronuclei in the red blood cells or alterations of plasma stress variables were identified (Song et al., 2014).

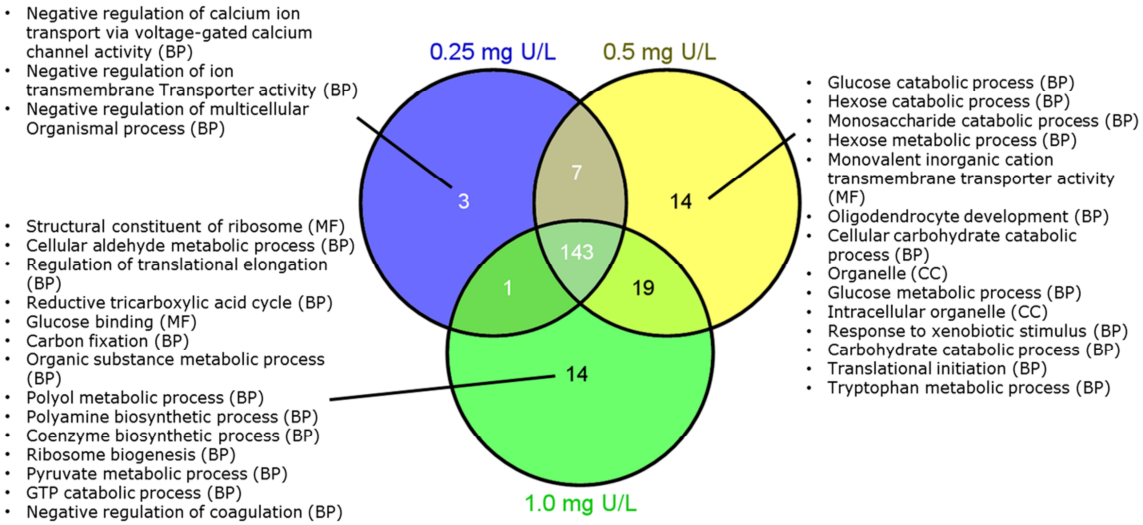


Figure 20: Gene Ontology (GO) functions. A Venn diagram analysis of GO terms that were significantly overrepresented ($p < 0.05$) in the liver of Atlantic salmon (*Salmo salar*) after 48 h waterborne exposure to 0.25, 0.5 and 1.0 mg/L nominal concentrations of depleted uranium (DU). The results were related to up-regulated genes. Lists of descriptions were corresponding to the GOs that were uniquely regulated by different concentrations of U. BP: biological process; MF: molecular function; CC: cellular component (after Song et al., 2014).

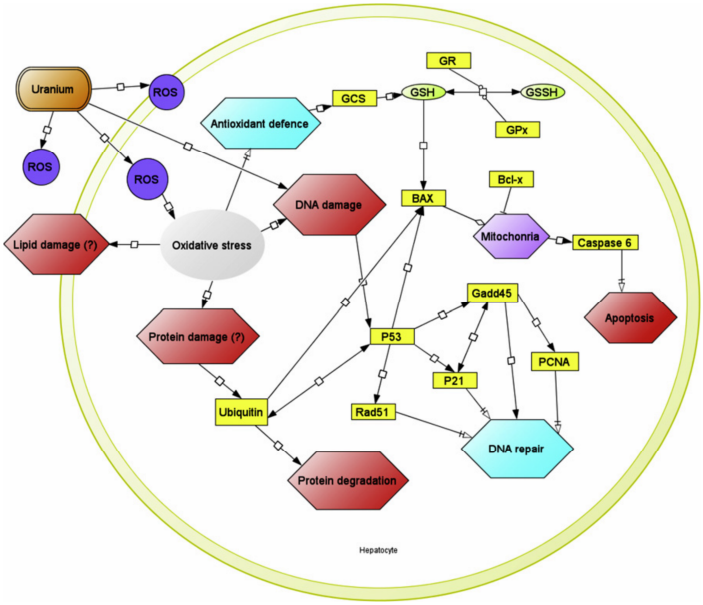


Figure 21: Proposed toxicological pathways and potential adverse effects induced by uranium in the liver of Atlantic salmon (*Salmo salar*) after 48 h exposure (Song et al., 2014).

Thus, for gene expression of both gills and liver tissues from Atlantic salmon juvenile parr exposed to uranium, results showed a concentration dependent change in number of expressed genes; correlation with increased concentration of U in the tissues. Consistent with the observed impaired ion regulation in fish accumulating high U gill concentration, the gene expression analysis identified pathways involved in ion regulation. It is conceivable that these are involved in the uptake of U.

Gamma irradiation

Gene expression in liver of Atlantic salmon juvenile parr

The microarray gene expression analysis identified 222, 495 and 909 differentially expressed genes (DEGs) in the liver of salmon juvenile parr after 48 h gamma radiation (^{60}Co) exposure to 0.3, 1.5 and 5.8 mGy/h, respectively. The results clearly showed that the key toxicity pathways regulated were dependent on the radiation exposure doses. The low gamma dose affected inorganic phosphate homeostasis, while the medium gamma dose modulated the mitochondrial transmembrane potential, fatty acid metabolism, nuclear receptor signalling and hypoxic responses The highest gamma dose regulated P53 signalling, anti-oxidative response, mitochondrial dysfunction and cell death (Figure 22).

Two pathways which were related to cell cycle checkpoint regulation and cardiovascular disorder were common between medium and high gamma treatments. No toxicity pathway was found to be commonly regulated across all radiation exposure doses (Song et al., 2014).

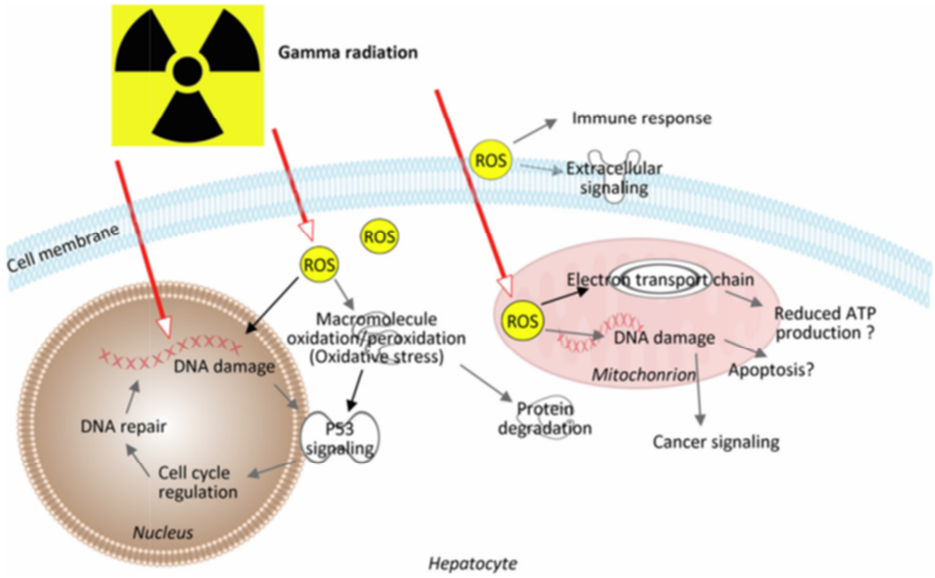


Figure 22: Putative toxicological mechanisms of low-dose gamma radiation in the liver of Atlantic salmon (*Salmo salar*) after short-term exposure (Song et al., 2014)

Gene expression Atlantic salmon embryo

Preliminary gene expression analysis using RNAseq identified 5833, 16 and 0 differentially expressed genes (DEGs) in embryos of Atlantic salmon after 162 degree days (about 90 days) exposure to 40, 10 and 1 mGy/h, respectively. Thus, results showed a dose dependent change in number of expressed genes. Gene ontology analysis showed that the DEGs were associated with functions related to the developmental program of the embryogenesis. Specifically, this involved both stem cell differentiation, neuron, mussel, brain and eye development. This is agreement with the fact that embryos exposed to 40 mGy/h, were significantly delayed in development, were affected by deformities and died before 450-degree days and never reached hatching.

Exposure to Cd was associated with transfer of Cd to the egg interior, with reduced swelling and delayed hatching. Preliminary gene expression of Cd toxic effects indicated a much less pronounced effect at the gene expression level. However, the RNAseq identified 167, 0 and 19 differentially expressed genes (DEGs) in embryos of Atlantic salmon after 162 degree days exposure to 30, 10 and 3 µg/L Cd, respectively. Gene ontology analysis showed that the DEGs were associated with neurotoxicity, cell

membrane integrity, fatty acid metabolism and maintenance of protein function, all consistent with known toxic mechanisms of Cd.

Gene expression analysis was also performed on embryos exposed to the combination of gamma and Cd to identify potential interactive effects. Preliminary results indicate, however, minor differences from additive effects.

Combined effect of U and gamma irradiation

The microarray gene expression analysis identified differentially expressed genes (DEGs) in the liver of salmon juvenile parr after 48 h exposure to gamma separately (14 mGy/h), uranium (depleted uranium) separately (250 µg/L), and U + gamma in combination. Dramatic hepatic transcriptional changes were found after 48 h exposure, with 2303 (DU), 3122 (Gamma) and 3460 (Combi) genes being differentially expressed compared to the control. DEGs related to combined functions were identified in addition to DEGs induced by uranium and gamma separately. Pathway analysis showed that various toxicity pathways involved in oxidative stress, DNA damage, mitochondrial ETC alteration were also potential mode of actions of the combination of U and Gamma, and that a number stress responses were dissimilar between single and combined stressors**.**

Identification of interactive genes using the independent action (IA) model results demonstrated both emerging and disappearance of genes, i.e. synergistic and antagonistic effects, respectively. A further evaluation was carried out to quantitatively predict the combined transcriptional changes using the IA model and identify potential interactive effects. Based on the whole-array expression data, totally 1675 genes were predicted to have significant deviations from response addition (IA), with 625 showing potentiation (synergism) and 1050 showing suppression (antagonism). Results indicated for example that DEGs associated with Cell Cycle, DNA damage and oxidative stress caused additive effects, DEGs associated with p53 signalling, immune response, regulation of mitochondrial membrane disappeared from Gamma due to interactions (antagonism), while DEGS associated with Cell Cycle emerged (synergism) in combination exposure due to interactions (Song et al., in prep). Thus, combined exposure to U and Gamma affected the expression of a number of regulator and effector genes involved in several key pathways showing potentiation or repressive effects.

Main conclusions & Highlights

As illustrated in the present work, the gene expression (pathway level) analysis can improve our knowledge and understanding of the underlying mechanisms of single and combined effects. Transcriptional responses was also successful linked to physiological changes at higher organismal levels such as blood plasma glucose and blood plasma ion correlating with bioaccumulation (e.g., uranium in gills). Thus, dose response relationship could be evaluated based early responses, physiological changes and adverse outcome.

Gene expression helps for the identification of 'mode of action (MoA)' (toxicological profile of each contaminants), and is highly useful in characterizing common and unique MoA linked to combined exposure and thereby identifying transcriptional changes as a consequence of their combined toxicity. Induction of oxidative stress, DNA damage, mitochondrial ETC alteration were proposed as potential mode of actions of U, gamma and their combination.

Identification of interactive genes using independent action model (IA) demonstrated both emerging (synergism) and disappearing (antagonism) of genes following combination of U and Gamma. Based on the whole array expression data, interactive genes could be identified and used to characterise interactive effects. Results illustrated that DEGs (e.g., associated with DNA damage and oxidative stress) caused additive effects followed combination of U and gamma, DEGs (e.g. associated with regulation of mitochondrial membrane) disappeared from Gamma due to interactions, and DEGs (e.g. associated with Cell Cycle) emerged in combination exposure due to interactions. Thus, the gene expression increased our understanding of single and combined effects by merging information from several toxic pathways.

5.3 Interactions at integrated population-community level

A complex link between subcellular, cellular and population-level effects in phytoplankton

In general, cadmium and gamma applied as single stressors had the same type of effect (negative or positive) on all endpoints – e.g. a positive effect on most subcellular endpoints and a negative effect on population level endpoints. Although mechanistic investigations were not performed, examination of all the endpoints together and correlations between these endpoints suggest the following interpretation, connecting effects at different levels of biological organization. At subcellular level, there was increased production of protective photoprotective and antioxidant molecules (e.g. pigments, catalase), but also increased oxidative damage (lipid peroxidation). This may cause the observed decrease in cell growth, and decrease in population biomass and cell number (ie. population density), possibly through interference with the cell cycle and delayed cell division.

When cells were exposed to both gamma and Cd, nearly all interactions were antagonistic (although not all were significantly so, see §0). Thus, increased biomolecule production was not as great as expected, leading to a less than expected decrease in cell size and increase in cell biomass and the negative impacts at population-level were in turn smaller than expected. Since both Cd and gamma irradiation are known to cause antioxidant responses and/or oxidative stress, the antagonistic responses seen may be partly explained by each stressor enhancing the antioxidant capacity of the cells to deal with either stressor.

Trophic interactions between phytoplankton and zooplankton

Cd is known to reduce feeding through e.g. decreased filtration rates, reduced digestive efficiency from Cd through gut poisoning and/or competition of Cd ions with essential

nutrients at the gut wall. All of these will lead to increased energy demands and thus reduced energy available for growth and mobility. Few studies have been done on the effects of radioactive substances or gamma irradiation on feeding-related endpoints in *Daphnia*, and mechanisms for reduced feeding rate or C-assimilation are unknown. However it is probable that radiation-induced oxidative stress causes increased damage and energy expenditure that can lead to decreased activity, feeding and growth. For both of the single stressor exposures in our experiment (and other pilot studies we have performed, not reported here), these three endpoints are nearly always linked; increased concentrations/doses lead to less mobile individuals that incorporate less C and grow less.

In our two studies with combined Cd and gamma exposures, the generally antagonistic effects seen for ^{14}C-assimilation (ie. the decrease in ^{14}C uptake is less than expected) and for immobility and growth (ie. greater than expected mobility and growth) are logical, since these three endpoints are linked. In the study where respiration was also measured, this also showed an antagonistic response. Just as for the algae (previous paragraph), it may be that antioxidant responses induced by both Cd and gamma exposure enable the daphnids to cope better with the combined stressors than either stressor in isolation. In addition, the energy requirements to sustain these antioxidant defenses could have stimulated *D. magna* energy acquisition. At higher dose combinations, there are indications of synergistic interactions (cf. IA) (e.g. less ^{14}C taken up than expected, more immobility than expected). At these exposures, *D. magna*'s ability to cope with the induced stress is probably overwhelmed.

5.4 Use of the DEBtox approach for the integrated modelling of mixture toxicity on multiple endpoints over a species life cycle

In the above Section (§4.1), the use of reference additivity models (CA and IA) was shown to be very useful to describe the additive effects of binary mixture, and to identify deviations from additivity. However, these approaches only enabled an analysis of the joint effects of toxicants at one single endpoint and one exposure time. They do not allow interpretations at toxicokinetic or toxicodynamic level, and finally cannot provide a clear and generic conclusion on the absence/presence of an interaction when different deviation patterns from the reference additivity are identified as a function of the considered endpoints, life stages or exposure times.

The emergence of energy-based toxicokinetic-toxicodynamic models offers alternatives to the commonly used but limited descriptive approaches (MixTox) for the analysis of mixture toxicity data. However, such models have only been put into practice in a limited number of mixture toxicity studies and still have to be confronted with experimental data. To our knowledge, only three attempts considering sublethal endpoints were made (Péry et al., 2008; Jager et al., 2010, 2014). All of them were conducted with the TK/TD DEBtox model (see §3.3).

We propose here a re-interpretation of the dataset obtained on the joint toxicity of U and Cd on the growth and reproduction of *C. elegans* (see §4.1), over the entire growth and

reproduction period of the exposed nematodes. On the basis of U and Cd concentrations in food, joint effects were shown to be close to the independent action reference model predictions but slight deviations were identified over time. Thus, our objective is to assess the ability of the DEBtox model, incorporating CA and IA additivity models, to describe simultaneously the growth and the reproduction of *C. elegans* exposed to single or mixed U and Cd.

Material & Methods

The experimental data analyzed in the present study are described above (§4.1) and in Margerit et al. (2015). A DEBtox model including the specific adaptations proposed for *C. elegans* was used (Goussen et al., 2015; Jager et al., 2005) with the integration of the CA and IA additivity models to predict the joint effects of non-interacting chemicals (Jager et al., 2010). In addition, as proposed by Goussen et al. (2015), a supplementary mode of action producing direct effects on the male gamete reserve (hazard to male gamete model) was considered, in order to improve the description of total brood size of exposed nematodes.

Besides, an effect of U and Cd on the length at first reproduction (*Lp*) of *C. elegans* was observed. The simplified DEBtox model is not able to mechanistically deal with such an effect (Jager et al., 2012). Thus, the relationship between metal exposure and *Lp* was taken into account in a descriptive way.

As in other simplified DEBtox models, the uptakes of U and Cd were estimated using a one-compartment diffusion model where the compartment size may change over time as organisms grow (Kooijman and Bedaux, 1996). Internal concentrations were then linked to effects on growth and reproduction through a toxicodynamics (TD) module assuming a specific physiological mode of action. In the case of a U+Cd exposure, when concentration addition was assumed, internal concentrations of U and Cd, scaled by their relative toxic potencies, were added to estimate a unique stress level through

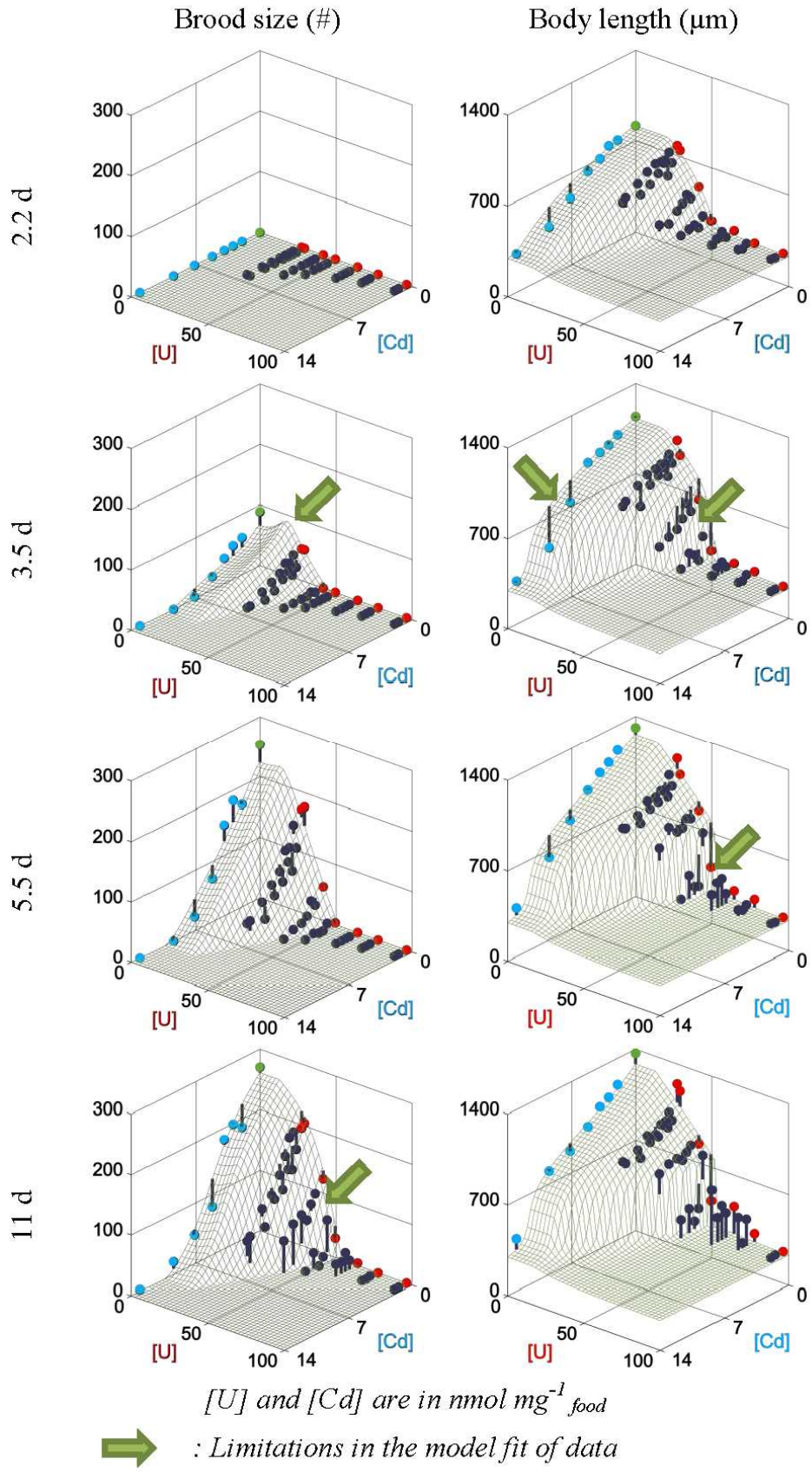


Figure 23: DEBtox modeling of the control, U-, Cd-, and U+Cd-exposed *C. elegans* over time on the basis of the DEBtox-integrated response addition.

the same TD module (Jager et al., 2010 - see Figure 6). Alternatively, when response addition was assumed, internal U and Cd concentrations were used to estimate the stress level induced by each ETM through their own TD module and joint effects were calculated by multiplying both stress levels (Figure 6).

Modeling was conducted using Matlab® 8.2.0.701 software (MathWorks), and parameters were estimated by maximizing the normal log-likelihood function (ℓ).

Results

Both trace metals were shown to affect the energy assimilation of *C. elegans*, as in previous DEBtox modeling of the single U and Cd toxicity (Alda-Alvarez et al., 2005; Swain et al., 2010; Jager et al., 2014; Goussen et al., 2015). Contrasted TK/TD parameters were obtained for U and Cd (a rapid U uptake *vs.* a moderate Cd uptake kinetics is predicted). A very low No Effect Concentration (NEC) was estimated for Cd (NEC=3 10-5 nmol mg-1 food) in comparison to U (NEC=12 nmol mg-1 food), indicating that very low amount of Cd may exert physiological effects on *C. elegans*.

The DEBtox modeling of the joint effects provided a rather accurate description of most of the U+Cd-exposed *C. elegans* data over time (
Figure 23). The best fit was obtained using the IA additive reference model. Those results are consistent with the previous conclusions using the MixTox model (§4.1), and definitely conclude that an interaction between U and Cd occurring at organism level, during the toxicokinetics or toxicodynamics steps, is unlikely or has only minor consequences on the growth and reproduction of nematodes. This study also underlines that even if the compounds of a mixture share the same DEBtox physiological mode of action, the IA reference model may still provide a better fit of joint toxicity data than the CA reference model.

However, some limitations of the DEBtox model were shown (
Figure 23, green arrows). Principally, the model was shown to overestimate the toxicity for nematodes exposed to U/Cd combinations close to the observed EC50. This unsuccessful prediction was unlikely due to complex TK/TD interactions, but rather suggests that some simplification assumptions in the DEBtox model for *C. elegans* are not entirely fulfilled (i.e. use of a specific size-dependent ingestion limitation function). This calls for an improvement of the simplified DEBtox model for *C. elegans* to better account for the physiological effects of (mixed) toxicants.

Main conclusions & Highlights

The model correctly fitted the growth and reproduction of control *C. elegans*, with some adaptations of the simplified DEBtox model.

A decrease in energy assimilation combined with direct effects on reproduction was the best physiological mode of action for single U and Cd, in agreement with the literature. Considering those modes of action and some adaptations of the simplified model (ie.

effect of toxicants on the length at first reproduction), the DEBtox model correctly fitted single U- and Cd- exposed *C. elegans* data.

The integration of Independent action (IA) in the DEBtox model allowed a rather accurate description of mixture toxicity data, better than using Concentration Addition (CA). This modeling exercise confirms that TK/TD interactions between U and Cd is unlikely or has only minor consequences on the growth and reproduction of nematodes.

Some limitations of the DEBtox model, specific to the *C. elegans* DEBtox model, were highlighted. An improvement of the model would be necessary to enhance its predictive capacities (eg. to provide a mechanistic description of U and Cd effect on the Length at puberty).

6 Conclusion

This document delivered a synthesis of the different experimental datasets provided by the common work of several research group participants to STAR WP4. This increased integration of experimentalists and modellers was important since mixture experiments are very demanding, require a multidisciplinary approach, and require shared infrastructure (chemistry, (molecular)biology, geochemical modelling, effects assessment models, irradiation facilities and facilities to work with radioactivity, …).

The experimental and modelling practice developed within this WP4 (experimental and modelling tool box) is important since a review of past experiments demonstrated that mixture experiments involving radiation or exposure to radionuclides were not always performed according to optimal experimental set-up and robust interpretations. The tools proposed in this document will direct scientists to adequate experiment set-up, experiment execution and data assessment.

We showed that processes interacting at different levels may result in deviation of mixture effects from the reference model (CA, IA) predictions:

(1) Interactions in the media that change the environmental availability of one or more chemicals;

(2) Interactions at site of uptake and/or elimination of the chemical from the organisms that result in modulation of the total accumulated internal concentration of one or more mixture components;

(3) Interactions at the target site that affect the binding of one or more chemicals to a receptor through which toxicity is (partly) mediated.

This is important since it shows that exposure conditions, biological receptors and organisms physiology are important in the understanding of the combined effects of toxicants.

Mixture toxicity should best be assessed by dynamic and biology based methods (eg. DEBtox). For *C. elegans*, the U and Cd toxicity dataset was used to explore the underlying mechanism of interaction with the support of DEBtox modelling. Simulations have successfully described the toxicity of U and Cd alone, consistent with previous DEBtox modelling for U and Cd. To describe the combined effects of U and Cd, an interaction term was considered in the various physiological parameters of the model.

Testing efforts should also be directed towards more molecular mechanistic understanding. In this way, the gene expression data have demonstrated to increase our understanding of single and combined effects by merging information from several toxic pathways.

For the scenarios tested and based on the presently available data, we could demonstrate that effects observed could be predicted using CA/IA or deviations thereof. In all cases, taking account of the combined effects of the multiple radionuclide/radiation and other

chemical stressor present provided a better prediction of observed hazard than consideration of any single stressor in isolation. This implies that we can predict mixture toxicity from single compound data based on the mixture impact models that were developed in the domain of ecotoxicology. Our work confirms that for sites containing mixtures of pollutants including radionuclides, regulation on a case for case basis for the single chemicals present may underestimate the ecosystem effects on multiple stressor exposures.

With our results we demonstrated interactive effects. Although we found mostly antagonism, some synergistic interactions were however also found. The number of scenarios, test organisms and mixture combinations that could be tested in frame of this project were limited and conclusions should be confirmed by additional experiments (e.g. experiments were performed at quite high exposure levels; we mainly observed joint effect, where both stressors are toxic). Possible interactions at low toxic doses remain a question. Moreover, interactions may remain at higher level of organization (trophic/population) and long term exposures that were not address in the performed experiments.

7 References

Alda Álvarez O., Jager T., Kooijman S. A. L. M., Kammenga J. E. 2005. Responses to stress of *Caenorhabditis elegans* populations with different reproductive strategies. Func. Ecol. 19(4):656-664.

Andersson P., Garnier-Laplace J., Beresford N.A., Copplestone D., Howard B.J., Howe P., Oughton D., Whitehouse P. 2009. Protection of the environment from ionising radiation in a regulatory context (protect): proposed numerical benchmark values. Journal of Environmental Radioactivity 100(12):1100-1108

Baas J, B.P. van Houte, C.A.M. Gestel, S.A.L.M.Kooijman, 2007. Modeling the effects of binary mixtures on survival in time. Environ Toxicol Chem; 26:1320–7.

Baas J, J. Willems, K. Kraak, T. Vandenbrouck, T. Jager, S.A.L.M. Kooijman, 2009a. Prediction of daphnid survival after in situ exposure to complex mixtures. Environ Sci Technol; 43:6064–9.

Baas J, T. Jager, S.A.L.M. Kooijman, 2010a. A review of DEB theory in assessing toxic effects of mixtures. Sci Total Environ; 408:3740–3745

Baas, J., A.M. Stefanowicz, B. Klimek, R. Laskowski, and S.A.L.M. Kooijman. 2010b. Model-based experimental design for assessing effects of mixtures of chemicals. Environ. Pollut. 158, 115-120.

Baas, J., T. Jager, S.A.L.M. Kooijman, 2009b. A model to analyze effects of complex mixtures on survival. Ecotoxicology and Environmental Safety, 72 (3), pp. 669-676.

Backhaus, T., H. Blanck, M. Faust. 2010. Hazard and Risk Assessment of Chemical Mixtures under REACH: State of the Art, Gaps and Options for Improvement Swedish Chemicals Agency report PM 3/10. pp 74

Beresford N.A., Barnett C.L., Jones D.G., Wood M.D., Appleton J.D., Breward N., Copplestone D. 2008. Background exposure rates of terrestrial wildlife in England and Wales. J Environ Radioactiv 99(9): 1430-1439.

Bernard F., Brulle F., Douay F., Lemière S., Demuynck S., Vandenbulcke F. 2010. Metallic trace element body burdens and gene expression analysis of biomarker candidates in Eisenia fetida, using an "exposure/depuration" experimental scheme with field soils. Ecotoxicology and Environmental Safety 73(5):1034-1045.

Bliss C.I. 1939. The toxicity of poisons applied jointly. Ann Appl Biol 26:585–615.

Brain RA, Solomon KR (2007) A protocol for conducting 7-day daily renewal tests with Lemna gibba. Nat Protoc 2 (4):979-987. doi:10.1038/nprot.2007.146

Brown J.E., Jones S.R., Saxén R., Thørring H., Vives i Batlle J. 2004. Radiation doses to aquatic organisms from natural radionuclides. J Radiological Prot 24(4 A):A63-A77.

Buisset-Goussen, A., Goussen, B., Della-Vedova, C., Galas, S., Adam-Guillermin, C., Lecomte-Pradines, C. (2014). Effects of chronic gamma irradiation: A multigenerational study using *Caenorhabditis elegans*. Journal of Environmental Radioactivity 137:190-197.

Carvalho F. P., Madruga M. J., Reis M. C., Alves J. G., Oliveira J. M., Gouveia J., Silva L., 2007. Radioactivity in the environment around past radium and uranium mining sites of portugal. J. Environ. Radioact. 96(1–3):39-46.

Cedergreen N, Christensen AM, Kamper A, Kudsk P, Mathiassen SK, Streibig JC, Sorensen H (2008) A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. Environ Toxicol Chem 27 (7):1621-1632

Cedergreen N, Kudsk P, Mathiassen SK, Sorensen H, Streibig JC (2007) Reproducibility of binary-mixture toxicity studies. Environ Toxicol Chem 26 (1):149-156

Cedergreen N., J.C. Streibig. 2005. Can the choice of endpoint lead to contradicting results of mixture toxicity experiments? Environmental toxicology and Chemistry24, 1676-1683.

Dutilleul M., Bonzom J.-M., Lecomte C., Goussen B., Daian F., Galas S., Réale D. 2014. Rapid evolutionary responses of life history traits to different experimentally-induced pollutions in *Caenorhabditis elegans*. BMC Evolutionary Biology, 14(1)252

EC (European Commission). 2006. Proposal for a Directive of the European Parliament and of the Council on Environmental Quality Standards in the Field of Water Policy and Amending Directive 2000/60/EC. COM(2006) 397 final. Brussels (BE): EC.

EC (European Commission). 2007. European Union Risk Assessment Report. Cadmium oxide and Cadmium metal. Part I – Environment. Series: 3rd Priority List Volume: 72, 678pp.

EC (European Commission). 2008. European Union Risk Assessment Report. Coal-Tar Pitch, High Temperature. Draft report R323_0805_ENV_FINAL_ECB.DOC. 427pp.

EC (European Commission). 2011. Technical Guidance For Deriving Environmental Quality Standards (TGD-EQS). Guidance Document n°27. Common Implementation Strategy for the Water Framework Directive. Technical report-2011-055, 203 p.

EC (European Commission). 2014. COUNCIL DIRECTIVE 2013/59/EURATOM of 5 December 2013 laying down basic safety standards for protection against the dangers arising from exposure to ionising radiation, and repealing Directives 89/618/Euratom, 90/641/Euratom, 96/29/Euratom, 97/43/Euratom and 2003/122/Euratom. Official Journal of the European Union L13, vol.57, 17 Januray 2014.

Eggen, R.I.L., R. Behra, P. Burkhardt-Holm, B.I. Escher, and N. Schweigert. 2004. Challenges in ecotoxicology. Environ. Sci. Technol. 38:58a–64a.

Environment Canada. 1998. Biological test method - toxicity tests using early life stages of salmonid fish (rainbow trout). Report EPS 1/RM/28 -1E.

Fenske C, Daeschlein G, Gunther B, Knauer A, Rudolph P, Schwahn C, Adrian V, von Woedtke T, Rossberg H, Julich WD, Kramer A (2006) Comparison of different biological methods for the assessment of ecotoxicological risks. Int J Hyg Environ Health 209 (3):275-284. doi:10.1016/j.ijheh.2005.12.004

Garnier-Laplace J, Gilbin R, Horemans N (eds). 2011. F. Alonzo, C. Bradshaw, C. Della-Vedova, C. Lecomte, J. Garnier-Laplace, R. Gilbin, T. Hertel-Aas, T.G. Hinton, N. Horemans, D.H. Oughton, G. Rudolfsen, B. Salbu, H. Vandenhove. Plans for laboratory radiation effects studies. STAR Deliverable D-N°5.1, 127pp.

Garnier-Laplace, J., K. Beaugelin-Seiller, R. Gilbin, C. Della-Vedova, O. Jolliet, and J. Payet. 2008a. A Screening Level Ecological Risk Assessment and ranking method for liquid radioactive and chemical mixtures released by nuclear facilities under normal operating conditions. International Conference on Radioecology and Environmental Radioactivity, Jun 2008, Bergen. Proceedings – Oral and Oral Poster Presentations, part 2 (Eds Strand et al.), Norwegian radiation protection Authority, Østerås, Norway, pp 11-14.

Geletneky, J.W., G. Büchel, and M. Paul. 2002. Impact of acid rock drainage in a discrete catchment area of the former uranium mining site of Ronneburg (Germany). In: *Tailings and Mine Waste 2002, Swets and Zeitlinger*, 67-73.

Geras'kin S.A., Fesenko S.V., Alexakhin R.M. 2008. Effects of non-human species irradiation after the Chernobyl NPP accident. Environ Int 34(6):880-897.

Gongalsky K. B. 2006. Bioaccumulation of metals by soil-dwelling insects in a uranium production area. Eur. J. Soil Biol. 42(S1):180–185.

Goussen B., Beaudouin R., Dutilleul M., Buisset-Goussen A., Bonzom J. M., Péry, A. R. R. (2015). Energy-based modelling to assess effects of chemicals on *Caenorhabditis elegans*: A case study on uranium. Chemosphere 120:507-514.

Groten, J.P., V.J. Feron, and J. Sühnel. 2001. Toxicology of simple and complex mixtures.Trends Pharmacol. Sci. 22, 316-322.

Harju-Autti, P., and G. Volckaert. 1995. Evaluation of the chemical-toxic consequences of geological disposal of radioactive waste. R-3051, SCK•CEN, Mol, Belgium

Horemans N, Alonzo F, Zimmer E, Hinton T (eds). 2012. F Alonzo, E Zimmer, D Plaire, A Buisset-Goussen, C Adam-Guillermin, C Lecomte-Pradines, N Horemans. Understanding the “metabolic” mode of actions of two different types of radiation using biokinetics/DEB-tox models. STAR Deliverable D-N°5.4, 697pp.

Horemans N, Van Hees M, Van Hoeck A, Saenen E, De Meutter T, Nauts R, Blust R, Vandenhove H (2015) Uranium and cadmium provoke different oxidative stress responses in *Lemna minor* L. Plant Biol 17:91-100. doi:10.1111/plb.12222

Howard, B.J., N.A. Beresford, P. Andersson, J.E. Brown, D. Copplestone, K. Beaugelin-Seiller, J. Garnier-Laplace, P.D. Howe, D. Oughton, and P. Whitehouse. 2010. Protection of the environment from ionising radiation in a regulatory context – an overview of the PROTECT coordinated action project. J. Radiol. Prot. 30, 195-214.

http://ec.europa.eu/environment/chemicals/pdf/report_Mixture%20toxicity.pdf. (checked 30/11/2011)

IAEA (International Atomic Energy Agency). 1992. Effects of Ionizing Radiation on Plants and Animals at Levels Implied by Current Radiation Protection Standards, Technical Reports Series No. 332

IAEA (International Atomic Energy Agency). 2011. EMRAS-II working group on biological effects (http://www-ns.iaea.org/projects/emras/emras2/default.asp?s=8&l=63). (checked 30/11/2011)

ICRP (International Commission on Radiological Protection). 2008. Environmental protection: the concept and use of reference animals and plants. ICRP-108.Volume 38, Issues 4-6, Pages 1-242.

IUR (Interantional Union of Radioecology). 2011. http://www.iur-uir.org/en/task-groups/id-3-radioecology-in-a-multiple-stressor-environment. (checked 30/11/2011)

Jager T. 2014. Making sense of chemical stress. Applications of Dynamic Energy Budget theory in ecotoxicology and stress ecology. version 1.1 of 15 October 2014, available at www.debtox.info/book.php.

Jager T., Alda Álvarez O., Kammenga J. E., Kooijman S. A. L. M. 2005. Modelling nematode life cycles using dynamic energy budgets. Func. Ecol. 19(1):136-144.

Jager T., Gudmundsdóttir E. M., Cedergreen N. 2014. Dynamic modeling of sublethal mixture toxicity in the nematode *Caenorhabditis elegans*. Environ. Sci. Technol. 48(12):7026-7033.

Jager T., T.Vandenbrouck, J. Baas, W.M. De Coen, S.A.L.M. Kooijman. 2010. A biology-based approach for mixture toxicity of multiple endpoints over the life cycle. Ecotoxicology 19: 351-361.

Jager T., Zimmer E. I.. 2012. Simplified Dynamic Energy Budget model for analysing ecotoxicity data. Ecol. Model.225:74-81.

Jonker MJ, Svendsen C, Bedaux JJM, Bongers M, Kammenga JE. 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. Environ Toxicol Chem 24 (10):2701-2713

Kallqvist T., Rosseland B.O., Hytterød S, Kristensen T. 2003. Effect of Zinc on early life stages of brown trout (*Salmo trutta*) at different levels of water hardness. Norwegian institute for Water research, Report No.O-21279, Serial No. 4678-03, 2003; 34.

Kooijman S. A. L. M., Bedaux J. J. M. 1996. Analysis of toxicity tests on *Daphnia* survival and reproduction. Water Res. 30:1711-1723.

Kortenkamp, A., M. Faust, M. Scholze, and T. Backhaus. 2007. Low-level exposure to multiple chemicals: reason for human health concerns? Environ. Health. Persp. 115, 106-114.

Kortenkamp, A., T. Backhaus, and M. Faust. 2009. The state of the art on mixture toxicity. Project: 070307/2007/485103/ETU/D.1

Kryshev I.I., Romanov G.N., Isaeva L.N., Kholina Yu.B. 1996. Radioecological state of lakes in the Southern Ural impacted by radioactivity release of the 1957 radiation accident. Journal of Environmental Radioactivity 34(3):223-235.

Lahive E, O'Callaghan MJA, Jansen MAK, O'Halloran J (2011) Uptake and partitioning of zinc in Lemnaceae. Ecotoxicology 20 (8):1992-2002. doi:10.1007/s10646-011-0741-y

Larsson, C.M. 2008. An overview of the ERICA integrated approach to the assessment and management of environmental risks from ionising contaminats. J. Environ. Radioact. 99, 1364-1370.

Lecomte-Pradines C., Bonzom J.-M., Della-Vedova C., Beaugelin-Seiller K., Villenave C., Gaschak S., Coppin F., Dubourg N., Maksimenko A., Adam-Guillermin C., Garnier-Laplace J. 2014. Soil nematode assemblages as bioindicators of radiation impact in the Chernobyl Exclusion Zone. Sci Total Environ 490:161-170.

Lewis MA (1995) Use of freshwater plants for phytotoxicity testing - a review. Environ Pollut 87 (3):319-336. doi:10.1016/0269-7491(94)p4164-j

Lokke H. 2010. NoMiracle: Novel Methods for Integrated Risk Assessmentof Cumulative Stressors in Europe.: Publisheable final activity report. http://ec.europa.eu/research/endocrine/pdf/nomiracle_final_report.pdf (checked 30/11/2011)

Lottermoser B. G., Ashley P. M., Costelloe M. T., 2005. Contaminant dispersion at the rehabilitated Mary Kathleen uranium mine, Australia. Environ. Geol. 48(6):748–761.

Macdonald C.R., Laverock M.J. 1998. Radiation exposure and dose to small mammals in radon-rich soils. Arch Environ Contam Toxicol 35(1):109-120.

Margerit A., Lecomte-Pradines C., Svendsen C., Frelon S., Gomez E., Gilbin R. 2015. Physiological response of the nematode *Caenorhabditis elegans* exposed to binary mixture of uranium and cadmium. *Ecotoxicol. Environ. Saf.* 118:139-48.

MEDE (Ministère de l'Ecologie et du Développement Durable). 2007. Circulaire définissant les « normes de qualité environnementale provisoires (NQEp) » des 41substances impliquées dans l'évaluation de l'état chimique des masses d'eau ainsi que des substances pertinentes du programme national de réduction des substances dangereuses dans l'eau. Circulaire 2007/23, DE / MAGE / BLPDI, n° 23, 7 mai 2007.

Mohan BS, Hosetti BB, PLAGIAAT (1999) Aquatic plants for toxicity assessment. Environ Res 81 (4):259-274. doi:10.1006/enrs.1999.3960

Moody M, Miller J (2005) *Lemna Minor* Growth Inhibition Test. In: Blaise C, Férard J-F (eds) Small-scale Freshwater Toxicity Investigations. pp 271-298

Müller, J., H. Ruppert, Y. Muramatsu, and J. Schneider. 2000. Reservoir sediments – a witness of mining and industrial development (malter Reservoir, eastern Erzgebirge, Germany). Environmental Geology 39(12), 1341-1351.

Musa Bandowe B.A., Srinivasan P., Seelge M., Sirocko F., Wilcke W. 2014. A 2600-year record of past polycyclic aromatic hydrocarbons (PAHs) deposition at Holzmaar (Eifel, Germany). Palaeogeogr Palaeoclim Palaeoecol 401:111-121.

Newman RM. 1991 Herbivory and detritivory on freshwater macrophytes by invertebrates - a review. J N Am Benthol Soc 10 (2):89-114. doi:10.2307/1467571

OECD (Organisation for Economic Cooperation and Development) 2006. Test No. 221: Lemna sp. growth inhibition test. In. Guidelines for the Testing of Chemicals, Section 2. Effects on Biotic Systems. ISSN: 2074-5761 (online) DOI: 10.1787/20745761

OECD (Organisation for Economic Cooperation and Development) 2013. Test No. 210: Fish, Early-life Stage Toxicity Test. In. Guidelines for the Testing of Chemicals, Section 2. Effects on Biotic Systems. ISSN: 2074-5761 (online) DOI: 10.1787/20745761

Pandit AK. 1984. Role of macrophytes in aquatic ecosystems and management of fresh water resources. Journal Name: J Environ Manage; (United States); Journal Volume: 18:1:Medium: X; Size: Pages: 73-88

Peng C., Chen W., Liao X., Wang M., Ouyang Z., Jiao W., Bai Y. 2011. Polycyclic aromatic hydrocarbons in urban soils of Beijing: Status, sources, distribution and potential risk. Environmental Pollution 159(3):802-808.

Péry A. R. R., Geffard, A., Conrad, A., Mons, R., Garric, J. 2008. Assessing the risk of metal mixtures in contaminated sediments on *Chironomus riparius* based on cytosolic accumulation. Ecotoxicol. Environ. Saf. 71(3):869-873.

PHIME. 2011. – PHIME: Public health impact of long-term, low-level mixed element exposure in susceptible population strata. http://www.phime.org/ (checked 30/11/2011).

Ragnarsdottir K.V., Charlet L. 2000. Uranium behaviour in natural environments. In Cotter-Howells, J. D., Campbell, L. S., Valsami-Jones, E., et Batchelder, M. (Eds.), Environmental mineralogy: microbial interactions, anthropogenic influences, contaminated land and waste management, Mineralogical Society Series 9, pp. 245–289. The Mineralogical Society of Great Britain and Ireland.

Ribera D., Labrot F., Tisnerat G., Narbonne J. F. 1996. Uranium in the environment: Occurrence,transfer, and biological effects. Rev Environ Contam Toxicol 146:53–89.

RIVM (Rijksinstituut voor Volksgezondheid en Milieu). 2014. Water quality standards for uranium: Proposal for new standards according to the Water Framework Directive. RIVM Report 270006003, van Herwijnen R, Verbruggen EMJ (eds.), 92pp.

Rosseland B.O., Massabuau J-C., Grimalt J., Hofer R., Lackner R., Raddum G., Rognerud S., Vives I. 2001. Fish ecotoxicology, The EMERGE fish sampling manual for live fish. The EMERGE Project (European Mountain lake Ecosystems: Regionalisation, diaGnostic and socio-economic valuation). (http://www.mountain-lakes.org/emerge/methods/29.pdfl).

Salbu B, Stegnar P, Strømman G, Skipperud L, Rosseland BO, Heier LS, Lind O, Oughton D, Lespukh E, Uralbekov B, Kayukov P. 2011. Legacy of Uranium Mining Activities in Central Asia–Contamination, Impact and Risks. UMB report.

Santos-Echeandía J., Caetano M., Brito P., Canario J., Vale C. 2012. The relevance of defining trace metal baselines in coastal waters at a regional scale: The case of the Portuguese coast (SW Europe). Marine Environ Res 79:86-99.

SCA (Swedish Chemical Agency). 2011. Risk assessment of Cadmium in the Swedish environment. Bilaga 5, Parkman H. (ed), 30pp.

Sheppard S.C., Sheppard M.I., Gallerand M.-O., Sanipelli B. 2005. Derivation of ecotoxicity thresholds for uranium. J Environl Radioactiv79 (1):55-83.

Song Y., Salbu B., Teien H-C., Heier S.L., Rosseland B.O., Tollefsen K.E. Dose-dependent hepatic transcriptional responses in Atlantic salmon (*Salmo salar*) exposed to sublethal doses of gamma radiation. Aquatic Toxicology 156 (2014) 52–64

Song Y., Salbu B., Teien H-C., Heier S.L., Rosseland B.O., Høgåsen T., Tollefsen K.E. 2014. Hepatic transcriptomic profiling reveals early toxicological mechanisms of uranium in Atlantic salmon (*Salmo salar*). BMC Genomics 2014, 15:694

Svendsen, C., Siang, P., Lister, L. J., Rice, A., et Spurgeon, D. J., 2010. Similarity, independence, or interaction for binary mixture effects of nerve toxicants for the nematode *Caenorhabditis elegans*. Environ. Toxicol. Chem., 29(5), pp. 1182–1191

Swain, S., J. Wren, S.R. Stürzenbaum, P. Kille, T. Jager, M.J. Jonker, P.K. Hankard, C. Svendsen, J. Chaseley, B.A. Hedley, M. Blaxter, and D.J. Spurgeon. 2010. Linking toxicants mechanism of action and physiological mode of action in *Caenorhabditis elegans*. BMC Systems Biology, 4, 32.

Tayibi, H., M. Choura, F.A. López, F.J. Alguacil, and A. López-Delgado. 2009. Environmental impact and management of phosphogypsum. Journal of Environmental Management 90, 2377-2386.

Tyne W, Lofst S, Spurgeon D J, Jurkschat K, Svendsen C (2014). A new medium for *Caenorhabditis elegans* toxicology and nanotoxicology studies designed to better reflect natural soil solution conditions. Environ Toxicol Chem 32(8):1711-1717

UNSCEAR (United Nations Scientific Committee on the Effects of Atomic Radiation). 2008. Effects of ionizing radiation on non-human biota**,** Fifty-sixth session, Vienna, 10 to 18 July 2008, A/AC.82/R.672, pp. 134.

Uralbekov B. M., Smodis B., Burkitbayev M. 2011. Uranium in natural waters sampled within former uranium mining sites in Kazakhstan and Kyrgyzstan. J Radioanal Nucl Chem 289(3):805–810.

van Gestel, C.A.M., M. Jonker, J.E. Kammenga, R. Laskowski, C. Svendsen. (Eds.). 2011. Mixture Toxicity: Linking Approaches from Ecological and Human Toxicology, CRC Press/Taylor and Francis Group, ISBN: 978-1-4398-3008-6, Boca Raton, London, New York. pp 320.

Vandenhove H, Horemans N, Gilbin R, Lofts S, Bradshaw C, Février L, Spurgeon D, Oughton D, Thørring H, Salbu B, Steiner M, Vaaramaa K, Jensen LK. 2012b. Integrated Research and Experimental Plan under STAR-WP4. STAR WP4 Milestone MS43.

Vandenhove H, Horemans N, Gilbin R, Lofts S, Real A, Bradshaw C, Février L, Thørring H, Brown J, Oughton D, Mora J-C, Adam C, Alonzo F, Saenen E, Spurgeon E, Salbu B. 2012a. Critical review of existing approaches, methods and tools for mixed contaminant exposure, effect and risk assessment in ecotoxicology and evaluation of their usefulness for radioecology. STAR Deliverable D-N°4.1, 244pp.

Vanhoudt, N., H. Vandenhove, A. Real, C. Bradshaw, K. Stark. 2012. A review of multiple stressor studies that include ionising radiation. Environmental Pollution 168:177-92.

Vives i Batlle J., Aono T., Brown J.E., Hosseini A., Garnier-Laplace J., Sazykina T., Steenhuisen F., Strand, P. 2014. The impact of the Fukushima nuclear accident on marine biota: Retrospective assessment of the first year and perspectives. Science of the Total Environment, 487 (1), pp. 143-153.

8 Annex - List of papers and presentations issued from this work

Submitted and published papers in national and international journals

The Results and Discussion chapters of this report (§ 4 and 5) are syntheses of the work performed within STAR WP4. A more detailed presentation of the results and discussion may be found in the papers corresponding to each chapter.

	Chap.
Horemans N., Van Hees M., Saenen E., Van Hoeck A., Smolders V., Blust R., Vandenhove H. 2015. Influence of nutrient medium composition and growth related endpoints on uranium toxicity in Lemna minor. Journal of Environmental Radioactivity, in press, 10.1016/j.jenvrad.2015.06.024	***4.1.2***
Horemans N., Van Hees M., Van Hoeck A., Saenen E., De Meutter T., Nauts R., Blust R., Vandenhove H. 2015. Uranium and cadmium provoke different oxidative stress responses in *Lemna minor* L. Plant Biol 17:91-100.	***4.1.2***
Margerit A., Lecomte-Pradines C., Svendsen C., Frelon S., Gomez E., Gilbin R. 2015. Nested Interactions in Uranium and Cadmium Combined Toxicity to the nematode *Caenorhabditis elegans*. *Ecotoxicol. Environ. Saf.* 118:139-48.	***4.1***
Nascimento F.J.A., Svendsen C., Bradshaw C. Combined effects from gamma irradiation and fluoranthene exposure on carbon transfer from phytoplankton to zooplankton. Submitted to Environmental Science & Technology	***5.3***
Song Y., Salbu B., Teien H-C., Heier S.L., Rosseland B.O., Høgåsen T., Tollefsen K.E. 2014. Hepatic transcriptomic profiling reveals early toxicological mechanisms of uranium in Atlantic salmon (*Salmo salar*). BMC Genomics 2014, 15:694	
Song Y., Salbu B., Teien H-C., Heier S.L., Rosseland B.O., Tollefsen K.E. Dose-dependent hepatic transcriptional responses in Atlantic salmon (*Salmo salar*) exposed to sublethal doses of gamma radiation. Aquatic Toxicology 156 (2014) 52–64	
Van Hoeck A., Horemans N., Van Hees M., Nauts R., Knapen D., Vandenhove H., Blust R. Characterizing dose response relationships: chronic gamma radiation in *Lemna minor* induces oxidative stress and altered polyploidy level. Submitted to Journal of Environmental Radioactivity	***4.3.2***

Presentations at National and International scientific congress

Bradshaw C., Meseh DA., Alasawi H., Qiang M., Nascimento F. 2014. Combined effects of gamma irradiation and cadmium on cellular and population-level endpoints of the microalga *Pseudokirchneriella subcapitata*. Oral presentation, ICRER 3rd International Conference on Radioecology & Environmental Radioactivity, September 2014, Barcelona, Spain.

Gilbin, R., Margerit A., Lecomte-Pradines C., Beaugelin-Seiller K., Garnier-Laplace J. 2013. Prise en compte des expositions combinées à des substances stables et radioactives pour l'évaluation du risque écologique. Oral presentation, Séminaire de Toxicologie Nucléaire Environnementale et Humaine, September 2013, Paris, France.

Horemans N., Van Hees M., Smolders V., Vandenhove H. 2012. Dose dependent effects induced by uranium in the freshwater macrophyte *Lemna minor*. Oral presentation, International symposium on Environmental Radioactivity, September 2012, Plymouth, UK.

Horemans N., Van Hees M., Smolders V., Vandenhove H. 2012. Speciation, bioavailability and toxicity of uranium in different *Lemna minor* growth media. Poster presentation SETAC May 2012 Berlin, Germany.

Horemans N., Van Hees M., Van Hoeck A., Vandenhove H. 2013. Use of the *Lemna minor* growth inhibition test to study dose dependent effects of uranium in plants. Oral presentation, SETAC, May 2013, Glasgow, UK.

Horemans N., Van Hees M., Van Hoeck A., Vandenhove H. 2014. The *Lemna minor* growth inhibition test as basis to evaluate radiation or radionuclide-induced effects on freshwater plants. Oral presentation, ICRER 3rd International Conference on Radioecology & Environmental Radioactivity, September 2014, Barcelona, Spain.

Horemans N., Van Hees M., Van Hoeck A., Vandenhove H., Svendsen C. 2015. Metal uptake and toxic growth effects in *Lemna minor* exposed to varying mixtures of uranium and cadmium. Oral presentation SETAC, May 2015, Barcelona, Spain.

Horemans N., Van Hees M., Willrodt C., Turtiainen T., Lofts S., Vandenhove H. Influence of pH and cations on the speciation, bioavailability and toxicity of uranium in the freshwater macrophyte *Lemna minor*. Oral presentation, ICOBTE, July 2015, Fukuoka Japan

Horemans N., Van Hees M., Willrodt C., Turtiainen T., Vandenhove H. 2014. Influence of pH and cations on the speciation, bioavailability and toxicity of uranium in *Lemna minor*. Oral presentation SETAC, May 2014, Basel, Switserland.

Horemans N., Van Hoeck A., Van Hees M., Nauts R., Wannijn J., Vandenhove H. 2013. Dose dependent stress effects induced by gamma radiation in the macrophyte *Lemna minor*. Poster presentation, Society of Experimental Biology, June 2013, Florence, Italy.

Margerit A., Lecomte-Pradines C., Svendsen C., Frelon S., Gomez E., Gilbin R. 2014. Combined effect of uranium and cadmium on physiological parameters of the

nematode *C. elegans*. Oral presentation, SETAC North America 35th Annual Meeting, November 2014, Vancouver, Canada.

Margerit A., Lecomte-Pradines C., Svendsen C., Frelon S., Gomez E., Gilbin R. 2014. Physiological response of the nematode *Caenorhabditis elegans* exposed to binary mixture of uranium and cadmium.. Short oral presentation and poster, ICRER 3rd International Conference on Radioecology & Environmental Radioactivity, September 2014, Barcelona, Spain.

Margerit A., Lecomte-Pradines C., Svendsen C., Frelon S., Gomez E., Gilbin R. 2013. Étude expérimentale et modélisation des effets combinés de l'uranium et du cadmium, en mélange, sur la croissance et la fécondité du nématode *C. elegans*. Oral presentation, Séminaire de Toxicologie Nucléaire Environnementale et Humaine, September 2013, Paris, France.

Nascimento F., Bradshaw C. 2013. Effects of γ irradiation in a mixture context on the transfer of carbon between phytoplankton and zooplankton: preliminary results. Invited talk at the Swedish Radioecology Society's Annual Meeting, 24 April 2013, Stockholm, Sweden.

Nascimento F., Svendsen C., Bradshaw C. 2014. Interactive effects of gamma irradiation and the PAH fluoranthene on the transfer of carbon between phytoplankton and zooplankton. Short oral presentation and poster, ICRER 3rd International Conference on Radioecology & Environmental Radioactivity, September 2014, Barcelona, Spain.

Teien H.C 2015. Combined toxicity of cadmium, uraniumand gamma in Atlantic salmon - role of toxicokinetics and toxicodynamics. Oral presentation, CERAD Conference, Februray 2015, Oslo, Norway.

Teien H.C., Salbu B. 2014. Speciation, uptake and toxicity of uranium in Atlantic salmon (*Salmo salar*) juveniles. Poster, ICRER 3rd International Conference on Radioecology & Environmental Radioactivity, September 2014, Barcelona, Spain. Teien H.C 2015. Uranium model experiments Atlantic salmon. Oral presentation, CERAD Conference, Februray 2015, Oslo, Norway.