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Nested interactions in the combined toxicity of uranium and cadmium to the nematode *Caenorhabditis elegans*

Adrien Margerit [a,*], Catherine Lecomte-Pradines [b], Claus Svendsen [c], Sandrine Frelon [a], Elena Gomez [d], Rodolphe Gilbin [a]

[a] Biogeochemistry, Bioavailability and Radionuclide Transfer Laboratory (PRP-ENV/SERIS/L2BT), Institute of Radioprotection and Nuclear Safety (IRSN), Cadarache, Building 183, BP3, 13115 St-Paul-lez-Durance Cedex, France
[b] Laboratory of ECOtoxicology (PRP-ENV/SERIS/LECO), Institute of Radioprotection and Nuclear Safety (IRSN), Cadarache, Building 183, BP3, 13115 St-Paul-lez-Durance Cedex, France
[c] NERC Centre for Ecology and Hydrology, Maclean Building, Benson Lane, Crowmarsh-Gifford, Wallingford, Oxfordshire OX10 8BB, United Kingdom
[d] UMR Hydrosciences- Université Montpellier 1, DSESP-Faculté de Pharmacie, BP 14491, no 15 Av Charles Flahault, 34093 Montpellier Cedex 05, France

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ABSTRACT

Uranium is a natural, ubiquitous radioactive element for which elevated concentrations can be found in the vicinity of some nuclear fuel cycle facilities or intensive farming areas, and most often in mixtures with other contaminants such as cadmium, due to co-occurrence in geological ores (*e.g.* U- or P-ore). The study of their combined effects on ecosystems is of interest to better characterize such multi-metallic polluted sites. In the present study, the toxicity of binary mixture of U and Cd on physiological parameters of the soil nematode *Caenorhabditis elegans* was assessed over time. Descriptive modeling using concentration and response addition reference models was applied to compare observed and expected combined effects and identify possible synergistic or antagonistic interactions. A strong antagonism between U and Cd was identified for length increase and brood size endpoints. The study revealed that the combined effects might be explained by two nested antagonistic interactions. We demonstrate that the first interaction occurred in the exposure medium. We also identified a significant second antagonistic interaction which occurred either during the toxicokinetic or toxicodynamic steps. These findings underline the complexity of interactions that may take place between chemicals and thus, highlight the importance of studying mixtures at various levels to fully understand underlying mechanisms.

1. Introduction

Uranium is a naturally occurring radioactive element for which environmental concentrations may be considerably magnified by anthropogenic activities in the vicinity of nuclear fuel cycle facilities (*e.g.* mining, waste management) (Hinck et al., 2013) or, for example, in intensive farming areas (phosphorus fertilizer application) (Schipper et al., 2011). Despite the radioactive properties of U, the risks to the environment in relation to its chemotoxicity generally outweigh those in relation to its radiotoxicity (Mathews et al., 2009).

In ecosystems, toxic trace metals are invariably found associated with numerous other organic or inorganic compounds. For example, the presence of other toxic metals such as arsenic, cadmium, chromium, or zinc, sometimes in high amounts, have been reported in U contaminated areas (Hinck et al., 2013; Pereira et al., 2008). However, because the assessment of mixture toxicity is often complex and labor intensive, only a few studies have dealt with the combined toxicity of U and other trace elements. Two studies were conducted on plants. In the first study, an antagonistic interaction was identified in the combined toxicity of U and Cu on the growth rate of *Lemna aequinoctialis* (Charles et al., 2006). The second study by Horemans et al. (2011) found no interaction between U and Cd in their joint effects on the growth of *Arabidopsis thaliana*. However, at the molecular level, the authors demonstrated an antagonism between U and Cd in the H_2O_2 production of treated plants. Another study was conducted on a fish species where the acute toxicity of a complex mixture of U and

Abbreviations: CA, concentration addition; CI95, confidence interval 95%; DL, dose level dependent deviation model; DR, dose-ratio dependent deviation model; NGM, nematode growth medium; RA, response addition; RSS, residual sum of square; SA, simple synergism/antagonism deviation model; TD, toxicodynamic; TK, toxicokinetic

* Corresponding author. Fax: +33 442 199 151.
E-mail addresses: adrien.margerit@gmail.com (A. Margerit), rodolphe.gilbin@irsn.fr (R. Gilbin).

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other trace metals and metalloids on survival was assessed after 4 days exposure (Hamilton and Buhl, 1997). No interaction was reported by the authors.

When the issue of mixture is addressed by regulators, the additive joint effects are generally predicted using two reference models for non-interaction, namely, concentration addition (CA), for mixtures of similarly acting toxicants, or response addition (RA, also termed independent action), for mixtures of dissimilarly acting toxicants (Kortenkamp et al., 2009). However, as reported for U and other trace metals, the toxicity of such mixtures to organisms may also result in unexpected synergistic or antagonistic interactions, that is, more or less effects than the additive joint effects predicted from CA or RA reference models (Greco et al., 1995). The inclusion of chemical interactions in the risk assessment of mixtures has become of great interest (Backhaus et al., 2013; Ragas et al., 2011).

In the literature, contrasting joint effects can be identified depending on the approach chosen to study chemical mixtures, namely, (i) the choice of the reference additive model, CA or RA (Backhaus et al., 2010), (ii) the endpoint and time of exposure considered (Cedergreen and Streibig, 2005; Kamunde and MacPhail, 2011; Van Gestel and Hensbergen, 1997), and (iii) the different concentration combinations tested (*e.g.* single combination, single, or multiple concentration ratio(s)).

In ecotoxicology, existing knowledge on mixed contaminants is generally insufficient to make a strong statement on their (pharmacological) (dis)similarity. The choice between CA and RA reference models is thus not straightforward. Alternatively, Jonker et al. (2005) proposed an approach (MIXTOX) in which CA and RA are considered as two equally valid reference models for predicting the additive effects of non-interacting chemicals. This approach enables the analysis of the entire dose–response surface of mixtures. Deviation from CA and RA 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.

In addition, when assessing the joint effects of metals, interactions may occur at various levels. In exposure media, interactions between metals may modulate their bioavailable fractions, while at the organism level, they may modulate their uptake and depuration processes (toxicokinetics, TK) and/or their reaction with biological target(s) (toxicodynamics, TD) (Spurgeon et al., 2010). Thus, in metal mixture studies, the assessment of bioavailable concentrations may aid in highlighting the possible step at which an interaction occurs in the toxicity mechanism (Jonker et al., 2004; Posthuma et al., 1997; Qiu et al., 2011).

In view of the above considerations, the aim of the present study was to thoroughly address the combined toxicity of U and Cd on the growth and reproduction of *Caenorhabditis elegans*. The soil nematode *C. 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 and Cd has already been documented (Dutilleul et al., 2013; Swain et al., 2010). A broad approach was applied. First, the combined effects of U and Cd on length increase and brood size were analyzed on the basis of both CA and RA reference additivity. Secondly, numerous U/Cd ratios were tested to cover the dose–response relationship of both compounds. Thirdly, the analysis was performed from hatching to the end of growth and reproduction of *C. elegans* in order to determine the onset and kinetics of possible interactions; Lastly, joint toxicity data were analyzed on the basis of both total and bacterial lawn U/Cd concentrations. In the present study, U and Cd concentrations in bacterial lawn (the food source used) were assumed to relate better to the bioavailable concentrations than the concentrations in agar (the spiked media used) as Höss et al. (2011) demonstrated that feeding was the main uptake route of Cd in *C. elegans*.

2. Materials and methods

2.1. Materials

Uranyl nitrate ($UO_2(NO_3)_2 \cdot 6H_2O$, >98.5% purity), cadmium chloride ($CdCl_2 \cdot 2.5H_2O$, >99% purity), HEPES buffer (pH 5.5) and other *C. elegans* maintenance chemicals were purchased from Sigma-Aldrich (France).

2.2. Strain and maintenance

The wild-type *C. elegans* strain (Bristol N2) used in this study was provided by the *Caenorhabditis* Genetics Center (MN, USA). The nematodes were cultured at 20 °C, 70% relative humidity in darkness, on nematode growth medium (NGM) agar seeded with *Escherichia coli* OP50 strain, according to the standard method (Stiernagle, 2006). Bacteria were prepared as mentioned in Supporting materials and methods. Seeded plates were exposed to UV (20 min, Bio-Link Crosslinker; 254 nm, 200 μW m^{-2}) to suppress bacterial activity (Goussen et al., 2013).

2.3. Mixture toxicity assay

Nematodes were exposed individually in 35 mm Petri dishes filled with modified NGM-agar, in which KPO_4 buffer was replaced with HEPES buffer to avoid U precipitation (Dutilleul et al., 2013; Goussen et al., 2013). Stock solutions of uranyl nitrate in demineralized water (62 mM) and of cadmium chloride in 0.2% HNO_3 (4 mM) were used to spike NGM-agar before pouring into the plates. Acidification of medium due to uranyl solution was compensated with the addition of NaOH. Due to the extra addition of H^+ with Cd solution and of NO_3^- with Cd and uranyl solution, the agar was supplemented with HNO_3 and $NaNO_3$ to ensure the same amount of these compounds in all conditions including the control (pH=4.8; $[NO_3^-]$=2.9 mM). Nematodes were exposed 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 (Fig. 1). A total of one control condition (n=10) and 48 exposed conditions (n=3) were assayed. The tested U/Cd concentration ratios ranged between 24 and 217. Concentrations were chosen in accordance with previous knowledge on the effects of each substance alone on

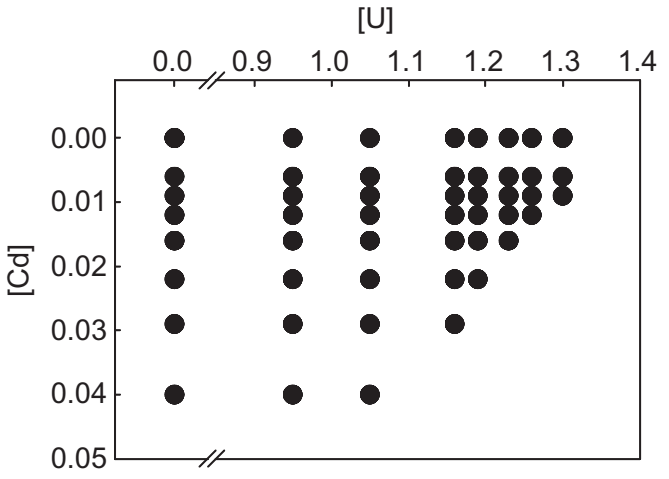

Fig. 1. Fractional factorial design used for the U/Cd mixture toxicity assay with *Caenorhabditis elegans*. Concentrations are expressed in mM.