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Joint toxicity of cadmium and ionizing radiation on zooplankton carbon incorporation, growth and mobility

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Abstract

The risk of exposure to radioactive elements is seldom assessed considering mixture toxicity, potentially over- or underestimating biological and ecological effects on ecosystems. This study investigated how three endpoints, carbon transfer between phytoplankton and *Daphnia magna, D. magna* mobility and growth, responded to exposure to γ-radiation in combination with the heavy metal cadmium (Cd), using the MIXTOX approach. Observed effects were compared with mixture effects predicted by concentration addition (CA) and independent action (IA) models and with deviations for synergistic/antagonistic (S/A), dose-level (DL) and dose-ratio (DR) dependency interactions. Several patterns of response were observed depending on the endpoint tested. DL-dependent deviation from the IA model was observed for carbon incorporation with antagonism switching to synergism at higher doses, while the CA model indicated synergism, mainly driven by effects at high doses of γ-radiation. CA detected antagonism regarding acute immobilization, while IA predicted DR-dependency. Both CA and IA also identified antagonism for daphnid growth. In general, effects of combinations of γ-radiation and Cd seem to be antagonistic at lower doses, but synergistic at the higher range of the doses tested. Our results highlight the importance of investigating the effects of exposure to γ-radiation in a multi-stressor context.

Introduction

The impact of radionuclides on the environment is a concern for scientists, managers and legislators. Although tightly regulated, radionuclides are routinely released into the environment as an operational practice by nuclear facilities, military activities, mining and research facilities. In addition, radioisotopes are also released into the biosphere as a result of nuclear accidents like those at Chernobyl and more recently Fukushima. Radioactive isotopes release ionizing radiation (α, β or γ-radiation) and exposure to ionizing radiation can have important biological effects both directly, since it can provoke double-strand breakage in DNA molecules [1], or indirectly through increased production of reactive oxygen species that oxidize cellular structures, causing cell damage and other deleterious effects [2]. Ionizing radiation can negatively impact survival, reproduction and growth of aquatic invertebrates [3,4] and these effects may extend to populations and subsequent generations [5]. The assessment of the risks that the release of radionuclides pose to the environment is often built on experimental data from scenarios where radiation was tested as the only stressor [6]. However, contaminants rarely occur in the environment in isolation[7] and radionuclides are no exception [6,8], creating a difficult challenge for regulators. This has prompted the development of different models and tools to predict how contaminants act in mixtures and how they affect biological systems. These models have been tested with good results on both aquatic [9,10] and terrestrial [11,12] ecosystems. The models of concentration addition (CA) and independent action (IA) are an example of tools used to predict quantitatively the joint effects of mixtures based on the behavior of the components as single contaminants. Deviations from the predictions of these two models can thus be detected and provide useful predictive information to managers [13]. Although these models often produce accurate predictions of the effects of mixtures[7] there are a significant number of studies that show deviations from the models, where the effects of the mixture are higher or lower than those expected based on the single contaminant effects. Furthermore, mixtures with individual component concentrations below their No Observed Effect Concentrations (NOEC) can cause significant effects in ecological systems [14,15]. As such, how stressors interact in mixtures to provoke effects on species and ecosystems is a central question in ecotoxicology.

Possible interactive effects between contaminants and radioactive elements are particularly poorly understood [6]. Many other toxic chemicals often coexist with radionuclides in scenarios where they pose a risk to the surrounding environment [8]. For example, anthropogenic activities, such as mining for coal, phosphate, metals and uranium, and oil and shale exploration increase concentrations of naturally occurring radionuclide (including gamma-emitters) and metals (including Cd) to concentrations that can create potential ecological risks [16]. In addition, radioactive waste management methods often mix radionuclides with other toxic chemicals including metals [17]. An analysis of U.S. Superfund Waste Sites found metals like cadmium (Cd) to co-occur often with radioactive contaminants at these contaminated sites [8]. Cd is a metal with widespread use in a number of industries, including oil exploration, refining and chemical fertilizers production [18]. Since it is often present in industrial and municipal effluents and urban runoff [19], Cd is found frequently in aquatic ecosystems, where it is known to be toxic to aquatic organisms at low concentrations. Exposure to Cd affects several biological processes, provoking structural and functional disruption at a cellular level to a wide range of organisms [20,21]. In addition, Cd and other metals can affect food intake and energy supply in zooplankton, which often results in decreased swimming activity, growth and reproduction [22,23].

The co-occurrence of Cd and gamma-emitting radionuclides in the environment demonstrates that studies concerning the effects of exposure to contaminants as mixtures in aquatic ecosystems are of high ecological relevance. To our knowledge no published studies have focused on the interactions between γ-radiation and Cd in a mixture toxicity context. Only recently have efforts started to be made to evaluate interactions between γ-radiation and Cd in a mixture toxicity context, within the framework of an EU-funded project, STAR, of which this study is a part. Here we report on a study that looked at how the transfer of carbon between a primary producer, *Raphidocelis* (formerly *Pseudokirchneriella*) *subcapitata,* and a consumer, *Daphnia magna,* was affected by exposure to external gamma radiation and Cd, both in isolation and as mixtures. *D. magna* is an abundant and important species in freshwater ecosystems, mediating phytoplankton biomass and community structure [24]. Carbon transfer is a feeding-related endpoint that is particularly relevant from an ecological perspective as it relates to the flow of energy between primary producers and consumers in ecosystems. In this study we exposed *D. magna* to 7 different concentrations of cadmium and 8 different doses of γ-radiation as single contaminants and in 25 binary mixtures. We then measured three endpoints: i) assimilation of carbon from the microalga *R. subcapitata* by *D. magna*, ii) *D. magna* growth and iii) and *D. magna* mobility. We tested the following null hypotheses:

a) Incorporation of carbon from phytoplankton by *D.magna*, *D.magna* growth and mobility are not reduced by γ-radiation or Cd

b) both the CA and the IA model describe, without deviations, the interactive effect between these two contaminants.

Methods

Algae culture

The green algae *R. subcapitata* was cultured continuously in MBL medium with added nutrients (SNV, 1995), at a temperature of 19 ºC under a 16 : 8 h light : dark cycle with a light intensity of approximately 75 µmol m^{-2} sec^{-1}. *R. subcapitata* were labeled with ^{14}C with the addition of 1.42 GBq of $NaH^{14}CO_3$ (Amersham; specific activity 1.998 GBq $mmol^{-1}$) to 3 L of the culture in MBL medium. Following a 2-week incubation period, the algae were harvested by centrifugation at 3000 g for 10 min and washed with distilled water to remove non-incorporated radioactivity in the water between the algae cells. This washing was repeated until the radioactivity of the rinsing water was below 0.05% of that incorporated in the algae. After the rinsing, the absorbance at 684nm of the concentrated algae suspension was measured and its biomass calculated from the absorbance following Rodrigues et al[25]. Samples were also taken to estimate how much ^{14}C label was incorporated by *R. subcapitata* by measuring their radioactivity in a liquid scintillation counter (LKB Wallac Rackbeta 1214) after the addition of scintillation cocktail (Ultima Gold). The final activity concentration of the phytoplankton suspension was 6.4 ± 0.35 Bq µg C^{-1}.

Zooplankton cultures

Daphnia magna neonates were obtained from Antwerp University. Belgium and reared in the laboratory in bio-filter treated tap water (pH 8.4–8.5, conductivity 513 µS cm^{-1}) at 20 °C under a constant light-dark cycle (14 h light: 10 h dark). Water was substituted three times a week and after each water exchange the daphnids were fed with 4×10^5 algae cells ml^{-1} (*R. subcapitata* and *Chlamydomonas reinhardtii* in a

3:1 ratio).

Test compounds and concentrations

Stock solutions of $CdCl_2$ (Aldrich Chemical Co.. MW 183.32; 98% purity) were prepared by dissolving a known amount of $CdCl_2$ in deionized water. Different volumes of these $CdCl_2$ solutions were added to the experimental *D. magna* medium to achieve the required 8 different nominal doses of Cd (0.062-2.4 μM). The Cd concentrations were chosen to cover the range where effects on the endpoints here used were previously observed [20]. One extra replicate for each of the 8 Cd concentrations was prepared. These extra replicates were sent for analyses to determine actual concentrations of Cd in the *D. magna* experimental medium, using atomic emission spectroscopy (ICP-AES) at the commercial laboratory ALS (ALS Scandinavia AB).

Exposure

Ten *D. magna* juveniles (2-3 days old) were added to each experimental unit containing 50 ml of the medium with varying concentrations of Cd. The daphnids were then transferred to the irradiation unit where they were irradiated for 68.7 hours with gamma radiation from a ^{137}Cs source. Light levels in the exposure room were uniform across the experiment (1-1.5 μmol m^{-2} s^{-1}, 16 h light: 8 h dark) and the temperature was 19.4 ±0.1 °C (average ±SD). The experimental units were placed in 8 concentric rows around the central 360° gamma source, taking care that rows nearer the source did not shield the rows behind. Cd concentrations were arranged randomly within each row. γ-radiation dose rates were measured through thermoluminescent dosimetry by attaching a thin film dosimeter to the front an experimental unit in each row. On the first and last rows, corresponding to the lowest and highest gamma radiation dose, an additional dosimeter was attached to the back of the experimental tubes to determine the attenuation of gamma radiation dose through the tube and medium. The control treatment plus the Cd-only treatments were placed in the same room under the same experimental conditions, but were protected by a lead wall to avoid exposure to gamma radiation.

The experiment had 40 treatments (Fig S1), each with 4 replicates. A fully factorial design was used, with two factors – Cd and γ-radiation exposure. There were six Cd concentrations (measured concentrations – 0, 0.10, 0.20, 1.05, 2.10 and 3.95 μM Cd^{2+}) and six gamma doses (measured dose rate – 0, 36, 72, 175, 273 and 417 mGy h^{-1}). In addition, extra single factor treatments were included in order to establish robust dose-response curves for both Cd and γ-radiation when in isolation (0.54 and 2.96 μM Cd^{2+} and 107, 209 and 404 mGy h^{-1}, respectively). Concentrations and doses were chosen to cover as much of the dose-response curve as possible, based on previous experiments and practical constraints of the gamma exposure set-up. During the 68.7 hours of exposure, *D. magna* in each replicate were fed with an unlabelled algae suspension of *R. subcapitata* (0.08 mgC / Daphnia / day).

Feeding test

After the 68.7 h of exposure, the medium in all replicates was replaced in order to remove all unlabelled algae and faecal pellets produced during the experiment. A 24 h feeding experiment was then performed where 350 μL of ^{14}C-labelled algae (6.4 Bq μgC^{-1}) was added to each replicate. The feeding experiment was carried out in a fume cupboard at a temperature 20.4 ± 0.4 °C (average ± SD). Approximately 24 h later (exact times were recorded), the daphnids were sieved out and allowed to empty their guts for 20 mins in clean medium and their mobility was recorded. Following this, the *D. magna* were collected and preserved in 75% ethanol.

Carbon incorporation

After the termination of the experiment, each preserved individual *D. magna* was rinsed thoroughly with distilled water and photographed using a light microscope (WildM28 Leica, Switzerland) connected to a digital camera (Dino lite, Taiwan). The body length of each *D. magna* was measured with the software DinoCapture, and compared to average initial size to estimate growth in each treatment. In addition, the weight of each individual was calculated from existing length-weight relationships [26].

Animals were pooled together into scintillation vials for ^{14}C analysis. The number pooled varied, depending on how many individuals were recovered, but was never less than two in order to obtain a clear ^{14}C signal. Tissues were solubilized in 1 ml Soluene at 60°C for 6-10 h. Following this, 10ml Ultima Gold LL was added to each sample and the samples left in the dark for at least 24 h before analysis to reduce chemoluminescence. Radioactivity was measured in a liquid scintillation counter (LKB Wallac Rackbeta 1214) to determine the incorporation of radiolabeled carbon in each treatment during the experiment. The ^{14}C radioactivity was standardised to the dry weight of *D. magna* individuals in each replicate and feeding time (in hours), corrected for background radioactivity and recalculated from dpm to $\mu g\ C^{-1}$ *Daphnia* $dw^{-1}\ day^{-1}$

Data analysis

R software version 3.2.0 (http://www.r-project.org) and the extension package *drc* (version 2.3-96) [27] were used to perform the analysis of the dose-response curves. Growth data for gamma radiation was Box-Cox transformed to comply with the assumption of homogeneity of variance.

Sixteen different models belonging to 4 model classes were analyzed: log-logistic; Weibull type I, and II regression models; and the Cedergreen-Ritz-Streibig model [28]. Dose effects in the single contaminant treatments were tested using the *noEffect* function (*p* value), and goodness-of-fit by the *lack-of-fit* test (*p* value), both included in the *drc* package [27]. Model selection was conducted using Akaike's information criterion (AIC). Among the models with equal fit, the function that estimated EC_{50} with lowest standard error was preferred.

Mixture modeling

The observed toxicity of the mixtures was compared against both the alternative reference models of CA and IA using the MIXTOX approach described by Jonker et al [13]. Deviations between the data and these reference models were explored for general patterns by stepwise incorporation of additional parameters describing relevant interactions between the effects of the two contaminants; synergistic/antagonistic (S/A), concentration-ratio–dependent (DR) and dose level–dependent deviation (DL). The improved fit and description of the data attained with these parameter additions were then tested to see if the improvement was significantly better taking into account the extra parameters and reduced degrees of freedom. Briefly, the S/A models were fitted to our data using the starting parameters produced by the CA and IA models, with an additional parameter, *a*, set to zero. If these S/A models produced a statistically better fit (tested with the *Chi-square* test), the parameters were used as starting values for the DR and DL models that included an additional variable set to zero (*b1* and B_{DL} for the DR and DL models, respectively). If the fit to the observed data improved statistically with these extra parameters, the best model was selected. Based on a pilot experiment (Fig S2) the EC_{50} parameter for the endpoint immobility for gamma radiation was constrained to a maximum of 912 mGy h^{-1} to be able to maintain more realistic EC_{50} parameters and better run the models. The interpretation of the statistically significant parameters generated by the extended models was done according to Jonker et al. [13], where a detailed description of this interpretation is available.

Results

Single contaminant exposures

γ-radiation

Our results show clearly that 3-day exposure to γ-radiation decreases both the incorporation of carbon from phytoplankton by *D. magna* (Fig. 1A, *noEffect* test $p <$ 0.001) and *Daphnia* growth (Fig. 1B, *noEffect* test $p = 0.005$. Carbon incorporation showed a dose-dependent decrease with an EC_{50} of 534 ± 231 mGy h^{-1} (EC_{50}±SE; Table S1). γ-radiation effects on daphnid growth were less pronounced, resulting in an EC_{50} of 404 ± 11 mGy h^{-1} (Table S1). The effects of γ-radiation on these endpoints were nevertheless significant and it is possible that these would be clearer in an experiment with a longer duration. On the other hand, γ-radiation did not have a significant effect on acute immobility of *Daphnia* (Fig. 1C; *noEffect* test $p = 0.23$)

Cadmium

There was a significant effect of Cd on all endpoints (Fig. 1 D-F), showing clearly that cadmium is more toxic to *D. magna* than exposure to γ-radiation at the doses tested. Daphnid mobility was significantly decreased by exposure to Cd (*noEffect* test, p<0.001), with the EC_{50} of 0.64 ± 0.12 μM Cd^{2+} (Fig. 1F, Table S2). A similar pattern was seen for other endpoints; incorporation of carbon by *D. magna* decreased with increasing exposure to cadmium, showing a dose-dependent response with an EC_{50} calculated at 0.12 ± 0.01 μM Cd^{2+} (*noEffect* test, p<0.001, Table S2). *D. magna* growth was also strongly affected by Cd (Fig. 1E) with an EC_{50} of 0.12± 0.03 μM of Cd^{2+} (Table S2).

Binary-Mixture toxicity

The parameters that resulted from the fitting of the MIXTOX models to our data, together with the corresponding statistical tests that compare if the models were statistically different from each other, are presented in Table 1. Statistical comparisons between the reference CA and IA models and the corresponding extended models with deviation parameters revealed statistically significant deviations for most of the endpoints tested, indicating interaction between γ-radiation and cadmium.

The CA and IA reference models fitted our data for the incorporation of carbon by *D. magna* relatively well, explaining 76% and 80% of the variation, respectively (Table 1). Introducing an extra MIXTOX parameter (*a*) that accounts for synergy or antagonism (S/A), significantly improved the fit to the observed carbon incorporation data (*Chi-square* test; p = 0.017 and p = 0.0004, for $CA_{S/A}$, Table 1). This parameter is negative in $CA_{S/A}$, indicating synergism, i.e, lower carbon incorporation than that predicted by the CA model (Table 1). In Figure 2A we can see that the carbon incorporation in *D. magna* in the treatments exposed to the higher doses of γ-radiation (empty symbols) is driving this synergism. In these treatments the joint effect of the mixture (represented as effective mean concentration) is generally higher than that predicted by the CA model. Introducing additional parameters to the $CA_{S/A}$ model did not improve its fit.

In contrast, *a* is positive for $IA_{S/A}$, suggesting antagonism, i.e. higher carbon incorporation than expected with the IA reference model. Adding another parameter ($b_{D/L}$) to the $IA_{S/A}$ model showed that $IA_{D/L}$ described the carbon incorporation data significantly better than $IA_{S/A}$ (Table 1, *Chi-square* test, p = 0.031), indicating that the interaction between cadmium and γ-radiation could be more complex. The positive *a* and the low b_{DL} parameter indicates that we see antagonism at low level doses that weakens and changes to synergism at dose levels higher that the EC_{50}, with the magnitude of the antagonism/synergism being dose level dependent (increasing away from the EC_{50}). This antagonism at lower doses and synergism at higher doses is visible on Fig. 3A. Observed EC_x in treatments exposed to lower dose-rates of γ-radiation are generally lower than the corresponding EC_x predicted by the IA models, particularly in the treatments exposed to 36 and 72 mGy h^{-1} (full circles and triangles in Fig. 3A). However, this changes at higher exposure to γ-radiation, as for example in the treatment exposed to 273 mGy h^{-1}, where observed EC_x were consistently higher than predicted, indicating synergism. Therefore, the observed data shows different deviations from the effects predicted by the CA and IA models, with different interactions between γ-radiation and Cd for incorporation of carbon; while the data shows synergism compared to the CA prediction, there is less observed effect than IA predicts at low doses (antagonism), a deviation that switches to synergism with increasing dose levels (Table 1).

The fit of the predicted effects by the IA and CA models against the observed effects of our binary mixtures on *D. magna* growth was statistically significant; despite only explaining a low percentage of the variation in the *D. magna* growth data set (31% and 13%, for CA and IA, respectively, Table 1). Introducing additional deviation parameters significantly improved the fit for both reference models, and $CA_{S/A}$ and $IA_{S/A}$ were the best fitting models, explaining 41 and 23% of the variability for this endpoint. Both $CA_{S/A}$ and $IA_{S/A}$ described significant antagonistic interactions between the two mixture components (*Chi-square* test $p < 0.001$ for both comparisons, Table 1). This general pattern of lower observed EC_x than predicted EC_x is represented in Figures 2B and 3B.

Approximately 75% and 77% of the variability in the acute immobilization data set was described by the CA and IA reference models, respectively (Table 1). The introduction of further parameters also provided significant improvements to the fit of the CA deviation models (*Chi-square* test, $p < 0.001$) with CA_{DR} being the model that best fitted the acute immobilization data, identifying antagonism where the effects were mainly caused by γ-radiation (Fig. 2C). Deviations from the IA model were also detected, as the addition of supplementary parameters improved the fit of the models (*Chi-square* test, $p = 0.004$). IA_{DL} was the best fit, describing dose level dependent antagonism at low doses that switched to synergism at dose levels higher than the EC_{50} (Fig. 3C).

Although in general the extra deviation parameters added to the reference CA and IA models only marginally improve the fit (ie. the r^2) of the models to the whole dose-response surface, particularly for the endpoints carbon incorporation and immobility, these improvements were still statistically significant. The general pattern of the deviations from CA/IA is important to recognize since it highlights potentially important and biologically significant interactive effects of the combined stressors

Discussion

Single contaminant toxicity

γ-radiation

No observable effects were seen in the mobility of the daphnids exposed to the single stressor treatments with γ-radiation alone (Fig. 1C, *noEffect* test $p = 0.19$). Conversely, our results show clearly that 3-day exposure to gamma radiation decreases the incorporation of carbon from phytoplankton by *D. magna* (Fig. 1A) and has effects on its growth (Fig, 1B). Nascimento et al.[29] found a similar dose-dependent decrease in carbon incorporation in daphnids exposed to high acute doses of γ-radiation. These authors found carbon incorporation to be more sensitive to γ-radiation than other feeding-related endpoints such as ingestion rates that only showed a response at high doses. These findings are in accordance with Alonzo et al. [30] who found no effect of low chronic exposure to γ-radiation on *D. magna* feeding rates.

These effects of γ-radiation on carbon incorporation can be related to interference with the acquisition of energy by the digestive system of the daphnids. Experiments using uranium-238 (an alpha emitter) have shown damage to the digestive tracts of *D. magna*[31] and the earthworm *Eisenia fetida*[32], decreasing the energy (carbon) incorporated by the animals [31]. Furthermore, exposure to similar doses of γ-radiation here used can increase the production of ROS and oxidative stress in aquatic invertebrates, resulting in a metabolic cost for damage repair and detoxification processes [4,33]. Metabolic cost theory predicts that organisms activate energy-consuming defense and repair mechanisms under stress conditions that compete for energy resources with processes as growth and reproduction [34,35] and retarded growth has been suggested to indicate a metabolic burden for detoxification or damage repair [36]. Indeed, reduced incorporation of carbon as a result of exposure to radionuclides later translated to negative effects on both growth and reproduction of *D. magna* in other experiments [31]. This is in agreement with other studies which have reported effects on growth and reproduction of zooplankton as a result of exposure to γ-radiation [5,37,38] or α-emitting radionuclides [39,40].

Cadmium

All 3 endpoints investigated here, incorporation of carbon, growth and acute immobility, were severely affected by Cd already at the low end of our tested concentrations (Fig. 1 D-F). Exposure to cadmium affects feeding-related endpoints in a number of cladoceran species [41–43]. This reduction in feeding can be a result of behavioral responses, such as decreased mobility, food avoidance and diminished filtration rates[20], orphysiological responses, such as gut poisoning and impairment of the digestive system[41,44]. This reduced energy acquisition can translate to effects on growth. Furthermore, Cd competes with the metabolism of essential nutrients with similar atomic numbers, such as calcium (Ca) [45]. Cd not only decreases Ca uptake due to its toxicity to Ca channels and its interference with the Ca-ATPase metabolism [46], but also competes with Ca in target sites where both elements are preferentially taken up, such as the midgut diverticula. This interference with Ca metabolism affects digestion and gut physiology [41,44],. As a non-essential metal, Cd will also stimulate energetically costly detoxification mechanisms, such as repair of biomolecules[47], metallothionein production[48], and Cd storage in granules in order to reduce its bioavailability[49]. These processes, together with the marked decreases in energy acquisition (carbon incorporation) by *D. magna,* can explain the strong effects of Cd in all of the endpoints studied in our experiment.

Binary mixture toxicity

The comparisons of observed results against both the CA and IA model predictions showed some consistent patterns. The results for incorporation of carbon by *D. magna* suggest that there are synergistic interactions between Cd and γ-radiation regarding the transfer of carbon between *R. subcapitata* and *D. magna* at least at the higher range of the doses tested. The IA extended model indicated significant dose-level dependent deviation from both reference models, with antagonism at low mixture doses and synergism at high mixture doses, while CA, the more conservative model, detected generally synergistic deviations across the whole dose-response surface. Synergistic effects between contaminants are often explained by one contaminant increasing the uptake or the activity of the other, or by interfering with the detoxifying or repair processes [50]. As mentioned previously, both γ-radiation and Cd can cause damage to the digestive tract of daphnids and interfere with digestive processes [31,41,44] , and it is possible that simultaneous exposure to these two stressors increases the severity of these effects, impacting endpoints as incorporation of carbon by *D. magna.*

Repair mechanisms activated when organisms are exposed to stress can also be affected by exposure to γ-radiation and Cd. In order to minimize oxidative damage, organisms have developed a number of anti-oxidative mechanisms that consist mostly of enzymes and metabolites to neutralize oxidants such as ROS [53]. However, the activity and effectiveness of both antioxidant compounds can be reduced due to exposure to Cd [55]. Cd interference with catalase and peroxidase activity and reduced metallothionein effectiveness [53-56] may be an explanation for the reduction in feeding and energy acquisition by *D. magna* and for the synergism seen in incorporation of carbon and acute immobilization at high doses. It is important to note that this potential synergism seems to occur at the higher doses/dose rates of γ-radiation. The γ-gamma radiation doses used in this exposure can generally be considered high, particularly at the doses where synergism is observed, but are in a range of what can be found at contaminated sites. For example, in lakes in the Mayak area, Russia, that have been used as nuclear waste ponds for decades, absorbed dose rates for zooplankton and phytoplankton have been estimated as 3.8 and 40 Gy per day, respectively [58]. Similarly, Cd concentrations used in our study were high but within the range of values found in contaminated sites [59].

The pattern seen in our study at lower and more environmentally realistic doses was antagonism, with the exception of $CA_{S/A}$ for carbon incorporation. Some studies have suggested that low level disturbances in the cellular redox balance induced by Cd can also exert a positive influence [55]. Depending on the dose of exposure, the tissue and the organism exposed, ROS can increase cell growth and stimulate biological repair mechanisms for both oxidative stress and exposure to metals [53,55]. Indeed, our results indicate higher daphnid growth than predicted by both the CA and the IA models, which might be a short-term consequence of this biological stimulation. However, as these models fitted the data for this endpoint less well, these results should be interpreted with care.

Increased antioxidant defenses and repair mechanisms also increase energy demand by the organisms, explaining the higher than expected carbon incorporation and mobility at the lower range of the doses tested. As such, the antagonisms indicated by the MIXTOX models, mostly at the lower end of our exposure doses, can be related to this stimulatory role of ROS species. However, it should be noted that our results are based on a relatively short exposure (68h). The energetic costs related to the maintenance of stimulated defense mechanism associated with chronic exposure to these stressors can carry important long-term ecological consequences. It would be important to assess if these antagonistic effects are present in longer exposures to both of these stressors.

Taken as a whole, our findings indicate that the interactions between γ -radiation and cadmium follow an antagonistic pattern when compared to the mixture reference models. Nevertheless, overall synergism when compared to the CA- predicted carbon incorporation, should warrant caution and be taken into account when assessing the ecological risk of exposure to radionuclides and γ–radiation when in mixtures with metals. Feeding-related endpoints are more sensitive than other endpoints, and are considered as more appropriate endpoints for studies of relatively short duration such as ours [60]. Longer duration studies, preferably multi-generational, with lower exposure doses, would provide valuable additional information.

Our results emphasize the value of assessing the joint effects of contaminants in mixtures. Most risk management tools for radioactive substances implemented by international organizations, such as the International Atomic Energy Agency (IAEA) or the International Commission on Radiological Protection (ICRP), are still built on evidence from studies where radiation is in isolation from other stressors. Our study provides compelling evidence that the use of mixture toxicity tools and assessment techniques to evaluate the risk posed by radiation with metals can be important in the development of improved environmental protection legislation regarding radioactive elements.

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Supporting information

Includes Figure S1 and S2 with the experimental design outlining the treatments investigated in this study, and the dose response curve from the pilot used to constrain the gamma radiation EC50 regarding the endpoint *D. magna* immobility. Tables S1 and S2 with dose-response parameters for γ -radiation and cadmium exposure as the single stressor, respectively. Table S3 shows the Observed experimental data together with both CA and IA model predictions (reference + best performing model) This information is available free of charge via the Internet at http://pubs.acs.org/ .

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Fig 1. Changes in incorporation of carbon by *D. magna* from *R. subcapitata* (A and D), growth (B and E) and acute immobilization (C and F) in relation to γ-radiation (left column) and cadmium dose (right column) in the single contaminant treatments. Values are given as Unaffected fraction (UAF), ie., relative to the control. Full circles represent observed data, while dashed lines show modeled predictions.

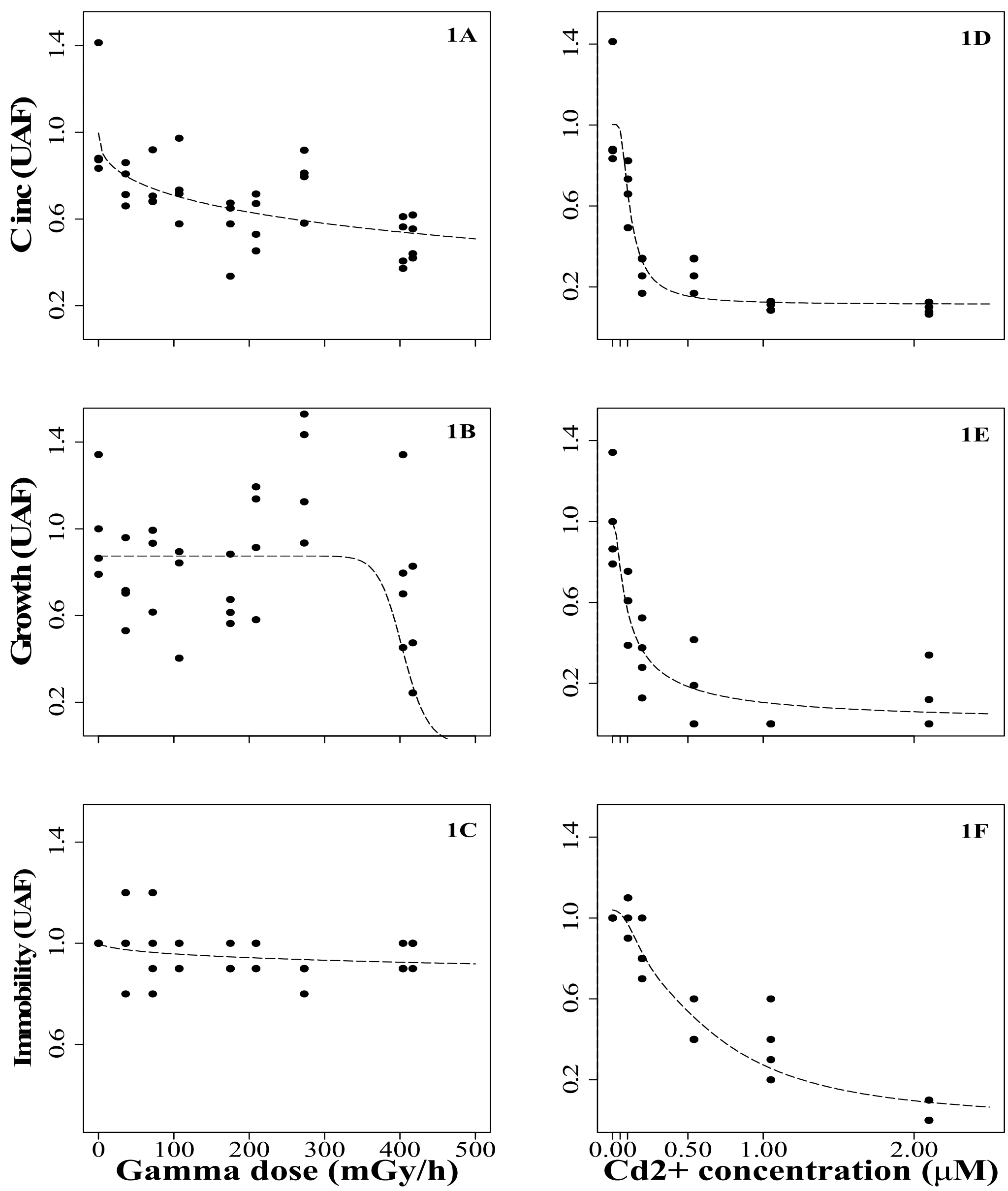
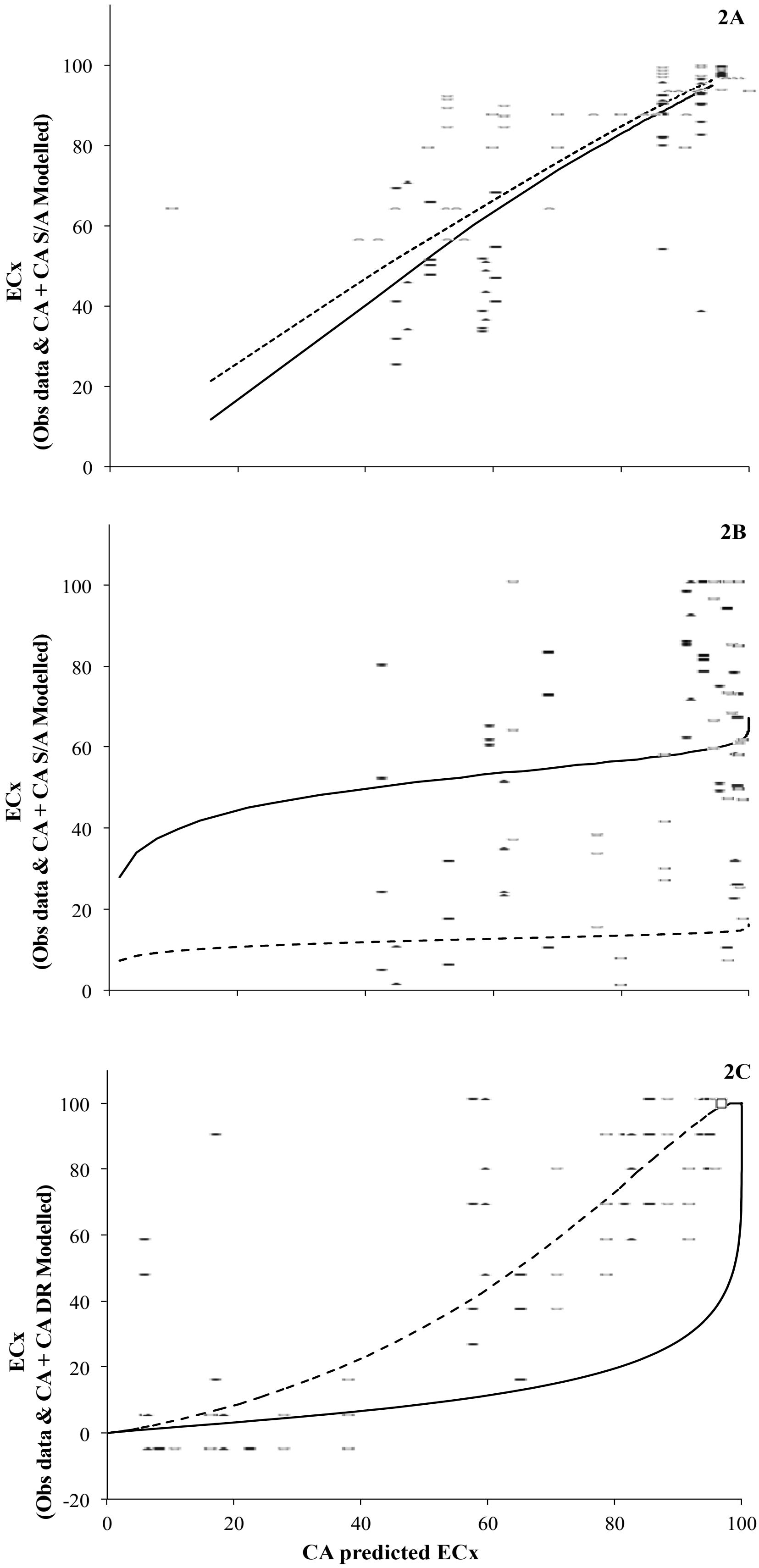
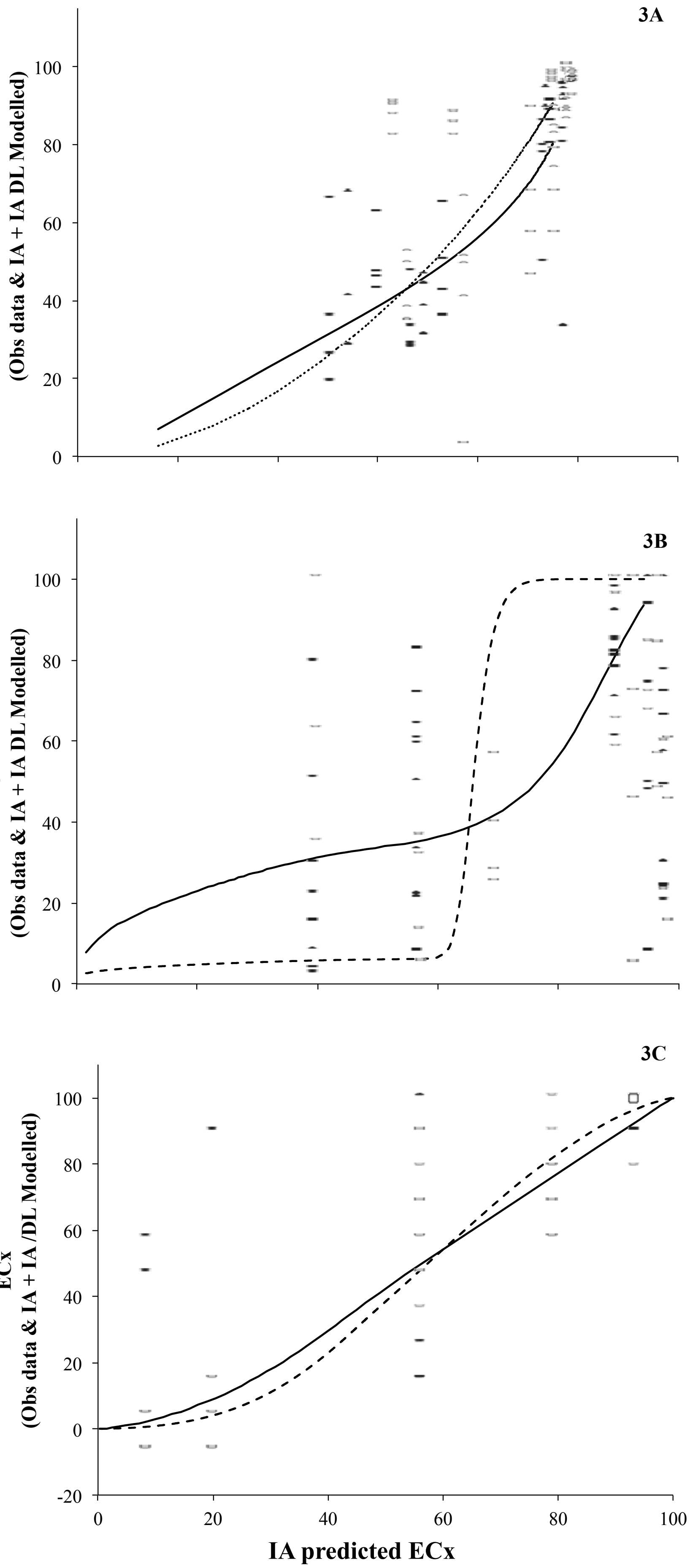


Fig 2. The joint effects (expressed as effective concentration, ECx) for (A) carbon incorporation, (B) growth and (C) acute immobility, in experiments exposing Daphnia magna to γ -radiation and cadmium. All figures show observed data and best concentration addition (CA) model fits, including those from significant deviation functions. All are plotted against an x value of the expected ECx values, based on CA of joint effects using parameters from the best-fit model. The dashed line represents the CA model prediction, while the full line represents the ECx values modelled by the best-fit model from significant deviation functions (S/A for carbon incorporation and growth and DR for acute immobility). The difference between the observed ECx and the best fit represent the degree to which the whole data surface can be explained based on the CA model. The remaining differences between predicted and experimental ECx values reflect the interactions occurring between the two stressors. Filled circles, triangles and squares show treatments exposed to 36, 72 and 175 mGy h-1 of γ –radiation, respectively, and empty circles and squares represent treatments exposed to 273 and 417 mGy h-1 of γ –radiation, respectively..



2A
ECx
(Obs data & CA + CA S/A Modelled)
0
20
40
60
80
100
2B
ECx
(Obs data & CA + CA S/A Modelled)
0
20
40
60
80
100
2C
ECx
(Obs data & CA + CA DR Modelled)
-20
0
20
40
60
80
100
0
20
40
60
80
100
CA predicted ECx

Figure 3. The joint effects (expressed as effective concentration, ECx) for (A) carbon incorporation, (B) growth and (C) acute immobility, in experiments exposing *Daphnia magna* to γ -radiation and cadmium. All figures show observed data and best Independent action (IA) model fits, including those from significant deviation functions. All are plotted against an x value of the expected ECx values, based on IA of joint effects using parameters from the best-fit model. The dashed line represents the IA model prediction, while the full line represents the ECx values modelled by the best-fit model from significant deviation functions (S/A for carbon incorporation and growth and DR for acute immobility). The difference between the observed ECx and the best fit represent the degree to which the whole data surface can be explained based on the IA model. The remaining differences between predicted and experimental ECx values reflect the interactions occurring between the two stressors. Filled circles, triangles and squares show treatments exposed to 36, 72 and 175 mGy h-1 of γ –radiation, respectively, and empty circles and squares represent treatments exposed to 273 and 417 mGy h-1 of γ –radiation, respectively..

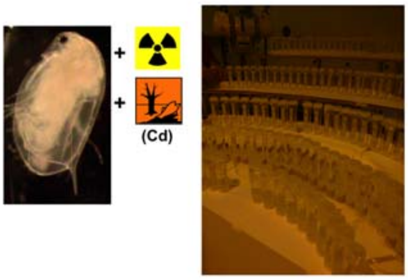


3A
ECx
(Obs data & IA + IA DL Modelled)
0
20
40
60
80
100
3B
ECx
(Obs data & IA + IA DL Modelled)
0
20
40
60
80
100
3C
ECx
(Obs data & IA + IA /DL Modelled)
-20
0
20
40
60
80
100
0
20
40
60
80
100
IA predicted ECx

Table 1. Summary of the analysis of the effect of γ-radiation and cadmium on i) carbon incorporation, ii) growth and iii) acute immobilization of *Daphnia magna*. Parameters constrained during fitting due to poor single compound effect data are indicated in italics. **β** is the slope of the individual dose–response curve; $\mathbf{EC_{50}}$ (in mGy h^{-1} for γ-radiation and Cd^{2+}μM for Cd) is the median effect concentration; *a*, b_{DL}, and b_{DR} represent the parameters in the deviation functions; while *p* shows the significance of the reference model's fit to the data, and $p(\chi^2)$ indicates the result of the *Chi-square* test for improvement of fit. S/A means synergism/antagonism and DL dose level dependent deviation from the reference model. The abbreviation NA means that the quantity is not applicable.

	Carbon incorporation		Growth		Acute immobilization	
	Reference	Best model	Reference	Best model	Reference	Best model
CA	CA	S/A	CA	S/A	CA	DR
Max	0.99	0.99	1.26	0.88	0.95	0.94
β_{gamma}	0.65	0.43	0.26	133	54	54
β_{Cd}	0.92	0.95	0.40	0.20	1.98	1.75
$EC50_{gamma}$	423	735	912	413	*912*	*912*
$EC50_{Cd}$	0.16	0.19	0.09	0.04	0.65	0.44
a	NA	-2.88	NA	65.29	NA	0.8
r^2	0.76	0.77	0.31	0.41	0.75	0.78
b_{DR}	NA	NA	NA	NA	NA	8.8
p/p(χ^2)	<0.0001	0.017	<0.0001	<0.0001	<0.0001	<0.0001
IA	IA	DL	IA	S/A	IA	DL
Max	0.82	0.93	0.85	0.86	0.94	0.94
β_{gamma}	0.90	0.76	19	121	41.53	41.53
β_{Cd}	1.18	0.99	0.51	0.18	2.18	1.93
$EC50_{gamma}$	*898*	536	468	415	898	*912*
$EC50_{Cd}$	0.31	0.19	0.51	0.03	0.47	0.04
a	NA	0.03	NA	11.7	NA	3.8
b_{DL}	NA	-90.88	NA	NA	NA	1.4
r^2	0.80	0.82	0.13	0.23	0.77	0.81
p/p(χ^2)	<0.0001	<0.0001	0.0005	<0.0001	<0.0001	0.004

TOC Art



Joint toxicity of cadmium and ionizing radiation on zooplankton carbon incorporation, growth and mobility

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Table S1- Best model, model fit tests, median effective concentration (EC_{50}) values and respective slopes (beta) calculated from exposure to γ-radiation as the single stressor. Standard errors for beta and EC_{50} are shown beside values in parentheses. Nr indicates absence of response to gamma radiation.

Endpoint	**Best Model**		**Model fit**		**Model parameters**	
	Model	Model function	*Lack of fit* test	*noEffect* test	b (±SE)	EC50 (±SE)
C inc	Weibull 1	*f(x) = \exp(-\exp(b(\log(x)-e)))*	p=0.23	p<0.001	0.43±0,17 (p=0.016)	534±231 (p=0.11)
Growth	Log logistic	*f(x) = 0 + \frac{d-0}{1+\exp(b(\log(x)-\log(e)))}*	p= 0.39	p= 0.005	24.3±30 (p=0.4)	404±11 (p<0.001)
Immobility	Weibull 2	*f(x) = \exp(-\exp(b(\log(x)-e)))*	p=0.7	p= 0.23	-0.13±0.13 (p=0.33)	nr

Table S2- Best model, model fit tests, median effective concentration (EC_{50}) values and respective slopes (beta) calculated from exposure to cadmium as the single stressor. Standard errors for beta and EC_{50} are show beside values in parentheses.

Endpoint	**Best Model**		**Model fit**		**Model parameters**	
	Model	Model function	*Lack of fit* test	*noEffect* test	b (±SE)	EC50 (±SE)
C inc	Weibull 2	*f(x) = \exp(-\exp(b(\log(x)-e)))*	p=0.23	p<0.001	-1.88±0.87 (p=0.04)	0.12±0.013 (p<0.001)
Growth	Weibull 2	*f(x) = \exp(-\exp(b(\log(x)-e)))*	p= 0.7	p<0.001	-0.87±0.32 (p=0,01)	0.121±0.03 (p=0.001)
Immobility	Log logistic	*f(x) = 0 + \frac{d-0}{1+\exp(b(\log(x)-\log(e)))}*	p=0.26	p<0.001	1.42±0.26 (p<0.001)	0.64±0.12 (p<0.001)

Table S3- Observed unaffected fraction (Average + SD, n=4) and IA and CA predicted effects (Reference + best performing model predictions) of γ-radiation and cadmium in isolation and in mixtures on i) carbon incorporation, ii) growth and iii) mobility of *Daphnia magna*. S/A means synergism/antagonism, DR dose ratio dependent deviation, and DL dose level dependent deviation from the reference model.

		Carbon incorporation					Growth					Mobility				
Gamma (mGy/h)	Cd (nM)	Obs (UAF)	CA (UAF)	CA-S/A (UAF)	IA (UAF)	IA-DL (UAF)	Obs (UAF)	CA (UAF)	CA-S/A (UAF)	IA (UAF)	IA-S/A (UAF)	Obs (UAF)	CA (UAF)	CA-DR (UAF)	IA (UAF)	IA-DL (UAF)
0	0	1.00±0.28	0.99	0.99	0.82	0.92	1.00±0.24	1.26	15244105	0.85	0.86	1.00±0.0	0.94	0.95	0.95	0.95
36	0	0.76±0.09	0.82	0.78	0.78	0.82	0.73±0.18	0.88	0.88	0.85	0.86	0.95±0.1	0.94	0.95	0.95	0.95
72	0	0.75±0.12	0.75	0.73	0.75	0.76	0.85±0.2	0.83	0.88	0.85	0.86	0.93±0.1	0.94	0.95	0.95	0.95
107	0	0.75±0.16	0.7	0.69	0.72	0.71	0.71±0.27	0.8	0.88	0.85	0.86	0.95±0.06	0.94	0.95	0.95	0.95
175	0	0.56±0.13	0.65	0.66	0.69	0.66	0.68±0.14	0.76	0.88	0.85	0.86	0.93±0.05	0.94	0.95	0.95	0.95
209	0	0.59±0.12	0.61	0.63	0.66	0.63	0.96±0.28	0.75	0.88	0.85	0.86	0.95±0.06	0.94	0.95	0.95	0.95
273	0	0.78±0.14	0.56	0.6	0.63	0.58	1.26±0.28	0.73	0.88	0.85	0.86	0.88±0.05	0.94	0.95	0.95	0.95
404	0	0.49±0.12	0.5	0.56	0.57	0.51	0.82±0.38	0.7	0.82	0.83	0.82	0.93±0.05	0.94	0.95	0.95	0.95
417	0	0.52±0.09	0.5	0.56	0.57	0.51	0.39±0.35	0.7	0.39	0.82	0.52	0.98±0.05	0.94	0.95	0.95	0.95
0	0.102	0.68±0.14	0.6	0.64	0.64	0.6	0.59±0.15	0.61	0.4	0.59	0.39	1.03±0.1	0.93	0.91	0.92	0.87
0	0.196	0.28±0.08	0.45	0.49	0.51	0.46	0.33±0.17	0.53	0.37	0.53	0.36	0.83±0.13	0.89	0.81	0.85	0.76
0	0.54	0.28±0.08	0.25	0.27	0.27	0.24	0.15±0.2	0.41	0.33	0.42	0.32	0.45±0.1	0.6	0.44	0.46	0.38
0	1.05	0.10±0.02	0.15	0.17	0.16	0.15	0.0±0	0.34	0.31	0.35	0.3	0.38±0.17	0.26	0.2	0.18	0.17
0	2.1	0.09±0.03	0.09	0.09	0.08	0.08	0.11±0.16	0.27	0.28	0.28	0.27	0.03±0.05	0.07	0.07	0.05	0.07
0	2.96	0.08±0.04	0.06	0.07	0.05	0.06	0.17±0.34	0.24	0.27	0.25	0.26	0.0±0.0	0.04	0.04	0.03	0.04
0	3.95	0.11±0.06	0.05	0.05	0.04	0.04	0.10±0.2	0.22	0.26	0.22	0.25	0.0±0.0	0.02	0.03	0.02	0.03
36	0.102	0.59±0.19	0.57	0.56	0.61	0.58	0.53±0.29	0.61	0.49	0.59	0.5	0.68±0.26	0.93	0.92	0.92	0.93
36	0.196	0.61±0.08	0.44	0.45	0.49	0.44	0.34±0.02	0.53	0.42	0.53	0.42	0.68±0.39	0.89	0.82	0.85	0.83
36	1.05	0.25±0.15	0.15	0.16	0.15	0.14	0.16±0.13	0.34	0.31	0.35	0.31	0.4±0.32	0.57	0.43	0.46	0.4
36	2.1	0.12±0.06	0.09	0.09	0.07	0.07	0.28±0.22	0.27	0.28	0.28	0.28	0.25±0.1	0.24	0.19	0.18	0.17
36	3.95	0.03±0.01	0.05	0.05	0.04	0.04	0.74±0.4	0.22	0.26	0.22	0.26	0.03±0.05	0.06	0.06	0.05	0.06
72	0.102	0.45±0.18	0.54	0.51	0.59	0.58	0.90±0.09	0.6	0.57	0.59	0.59	0.98±0.05	0.93	0.93	0.92	0.94
72	0.196	0.56±0.06	0.43	0.42	0.47	0.45	0.60±0.12	0.53	0.47	0.53	0.47	0.95±0.06	0.88	0.84	0.85	0.86
72	1.05	0.08±0.03	0.15	0.16	0.14	0.13	0.08±0.12	0.34	0.32	0.35	0.32	0.25±0.21	0.55	0.43	0.46	0.41
72	2.1	0.21±0.28	0.09	0.09	0.06	0.06	0.0±0	0.27	0.28	0,00	0,00	0.28±0.15	0.22	0.18	0.18	0.17
72	3.95	0.02±0.01	0.05	0.05	0.16	0.17	0.41±0.3	0.22	0.25	0.22	0.26	0.0±0.0	0.06	0.06	0.05	0.06
175	0.102	0.47±0.08	0.49	0.44	0.51	0.54	0.87±0.29	0.59	0.7	0.59	0.75	1.00±0.0	0.92	0.94	0.92	0.94
175	0.196	0.48±0.12	0.4	0.37	0.35	0.38	0.53±0.38	0.52	0.58	0.53	0.6	1.00±0.0	0.87	0.89	0.85	0.9
175	1.05	0.13±0.04	0.15	0.15	0.11	0.11	0.13±0.09	0.34	0.34	0.35	0.35	0.6±0.14	0.48	0.44	0.46	0.44
175	2.1	0.09±0.02	0.09	0.09	0.05	0.05	0.43±0.53	0.27	0.29	0.28	0.3	0.18±0.15	0.18	0.16	0.18	0.17
175	3.95	0.03±0.01	0.05	0.05	0.15	0.16	0.41±0.19	0.22	0.25	0.22	0.27	0.08±0.1	0.05	0.05	0.05	0.06
273	0.102	0.12±0.04	0.45	0.4	0.47	0.53	0.45±0.38	0.59	0.77	0.59	0.81	1.00±0.0	0.92	0.94	0.92	0.94
273	0.196	0.14±0.02	0.37	0.33	0.32	0.38	0.61±0.1	0.52	0.65	0.53	0.7	0.95±0.06	0.84	0.91	0.85	0.92
273	1.05	0.03±0.01	0.15	0.14	0.1	0.11	0.18±0.18	0.34	0.37	0.35	0.38	0.48±0.19	0.4	0.47	0.46	0.46
273	2.1	0.02±0.02	0.09	0.09	0.05	0.05	0.22±0.08	0.27	0.3	0.28	0.31	0.13±0.13	0.13	0.15	0.18	0.17
273	3.95	0.04±0.03	0.05	0.05	0.13	0.15	0.61±0.34	0.22	0.25	0.22	0.27	0.05±0.1	0.04	0.04	0.05	0.06
417	0.102	0.53±0.08	0.41	0.37	0.42	0.5	1.05±0.24	0.58	0.81	0.57	0.81	0.98±0.05	0.9	0.94	0.92	0.94
417	0.196	0.45±0.1	0.35	0.3	0.29	0.37	0.55±0.13	0.51	0.72	0.51	0.68	0.9±0.08	0.77	0.92	0.85	0.92
417	1.05	0.16±0.06	0.14	0.14	0.09	0.12	0.39±0.35	0.34	0.4	0.33	0.28	0.33±0.17	0.28	0.53	0.46	0.48
417	2.1	0.11±0.02	0.08	0.08	0.05	0.05	0.24±0.21	0.27	0.31	0.27	0.21	0.3±0.08	0.08	0.14	0.18	0.17
417	3.95	0.03±0.01	0.05	0.05	0.03	0.03	0.52±0.2	0.22	0.25	0.21	0.17	0.0±0.0	0.02	0.04	0.05	0.06

Fig S1- Figure 1- Experimental design outlining the treatments investigated in this study.

Single contaminant exposure treatments on y-axis (cadmium) and on x-axis (γ-radiation)

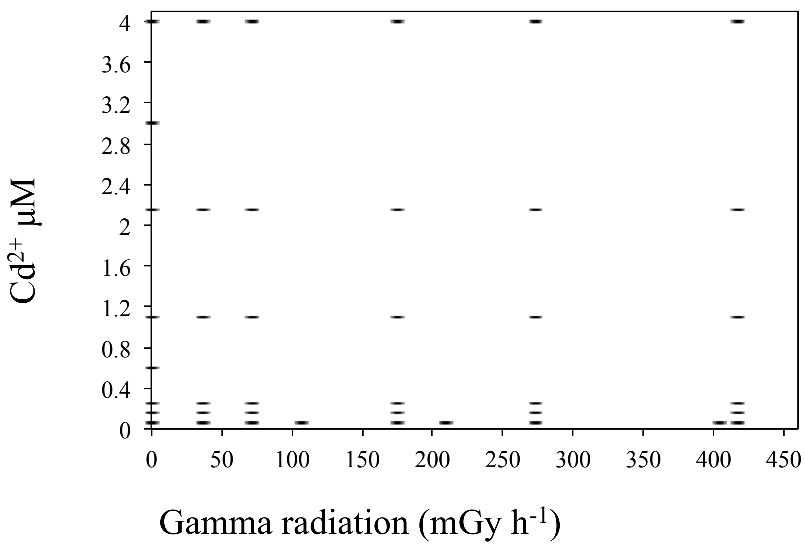

Fig S2 - Changes in incorporation of carbon by *D. magna* from *R. subcapitata* , in relation to γ-radiation from a pilot experiment performed with daphnids from the same origin and of the same age that were exposed to gamma radiation in the same setup as our experiment. Values are given as Unaffected fraction (UAF), ie., relative to the control. Full circles represent observed data, while the dashed lines shows modeled predictions.