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Combined effects from gamma irradiation and fluoranthene exposure on carbon transfer from phytoplankton to zooplankton

Francisco J.A.Nascimento[1]; Claus Svendsen[2] and Clare Bradshaw[1]*

[1]Department of Ecology, Environment and Plant Sciences, Stockholm University. 106 91 Stockholm, Sweden

[2]Centre for Ecology and Hydrology, Maclean Building, Benson Lane, Crowmarsh Gifford, Wallingford, OX10 8BB, Oxfordshire, United Kingdom

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* Corresponding author: e-mail: francisco.nascimento@su.se

Abstract

Risk assessment does not usually take into account mixtures of contaminants, thus potentially under- or overestimating environmental effects. We investigated how the transfer of carbon between a primary producer, *Pseudokirchneriella subcapitata,* and a consumer, *Daphnia magna,* is affected by the acute exposure of gamma radiation (GR) in combination with the PAH fluoranthene (FA). We exposed *D. magna* to five concentrations of FA and five acute doses of GR as single contaminants and in nine binary combinations. We compared the observed data for 3 endpoints – incorporation of carbon by *D. magna, D. magna* ingestion rates and growth – to the predicted joint effects of the mixed stressors based on the Independent Action (IA) concept. There were deviations from the IA predictions especially for ingestion rates and carbon incorporation by *D. magna*, where antagonistic effects were observed at the lower doses, while synergism was seen at the highest doses. Our results highlight the importance of investigating the effects of exposure to GR in a multi-stressor context. In mixtures of GR and FA the IA-predicted effects seem to be conservative as antagonism between the two stressors, possibly due to stimulation of cellular anti-oxidative stress mechanisms by GR, was the dominant pattern.

Introduction

Human population growth together with increased rates of industrialization and use of chemicals, have exposed both humans and ecosystems to an array of different contaminants and stressors. Among these, radionuclides and their impacts on ecosystems are a subject of rising concern from regulatory bodies, especially after the Fukushima Daiichi nuclear power plant accident in 2011. Radioactive isotopes release ionizing radiation (e.g., alpha-, beta or gamma radiation) that break bonds in biological molecules causing direct damage such as double-strand breakage in DNA [1] and genotoxic DNA alterations [2]. Furthermore, radiation ionizes water into reactive oxygen species that oxidise cellular structures, often provoking damage and toxic effects [3]. Such effects of ionizing radiation on a cellular level often translate into important direct effects at the individual and population level. A number of studies have showed that ionizing radiation can significantly decrease survival, reproduction, and growth of aquatic invertebrates [4,5]. Environmental radiation protection norms adopted by organizations such as the International Atomic Energy Agency (IAEA) or the International Commission on Radiological Protection (ICRP) mostly rely on data obtained from experimental studies where radiation was tested as the sole contaminant or stressor [6]. However, a number of toxic chemical compounds can ordinarily be found where radionuclides are abundant and a safety concern. Radioactive waste management methods often mix radionuclides with other toxic chemicals, e.g, waste containers contain Cr, Ni and Zn, while the over-packs contain Cr, Ni, Mn, Pd, To, Mo which may be released to the environment after disposal [7]. Waste water produced during the extraction and exploration of oil, gas and shale gas often contains enhanced levels of naturally occurring radionuclides together with a number of other chemicals including polycyclic aromatic hydrocarbons (PAHs) [8]. In addition, radionuclides in mixtures with other toxic compounds have also been detected in an analysis of U.S. Superfund Waste Sites,[6,9]. PAHs in particular, have been found to co-occur with radioactive contaminants at 67% of the contaminated sites managed by this program [9]. PAHs are organic contaminants pervasive in aquatic ecosystems. They occur naturally as a by-product of incomplete combustion of fossil fuels and from anthropogenic activities such as oil spills and urban runoff, which often results in contamination of ecosystems [10]. PAHs are toxic, genotoxic, carcinogenic, and bioaccumulative, constituting a serious pollution problem [11,12]. In addition, the toxicity of some PAHs, such fluoranthene (FA), pyrene and anthracene, to aquatic species has been found to increase severely in the presence of ultraviolet (UV) radiation [13], increasing the production of free oxygen radicals that induce oxidative stress through the destruction of tissues and the interference with biomolecular pathways [14]. Since oxidative stress is also one of the most important pathways through which ionizing radiation affects biological processes there is potential for synergistic effects between ionizing radiation and PAHs. This illustrates the relevance of studying ionizing radiation and PAHs as they often occur in nature – as mixtures.

Increasing numbers of studies provide strong evidence that the effects provoked by a mixture of stressors can be different from the sum of the effects when the stressors are tested in isolation due to synergistic or antagonistic effects [15,16]. The effects of chemicals in mixtures are caused by interactions that can occur at different levels: contaminants can (a) affect the availability of other contaminants to organisms; (b) decrease or enhance the uptake of other contaminants into the organism; (c) repress or stimulate detoxification mechanisms that organisms have evolved to cope with contaminants [17]. Within mixture toxicology a paradigm of predicting/estimating the joint effect of multiple non-interacting chemicals through "addition" has been developed and tested based on two underpinning concepts, namely Concentration Addition (CA) and Independent Action (IA). If chemicals have the same mode of action, their combined toxicities can be described by the CA model. When two stressors have different modes of action their combined effects can be described by the IA model. Deviations from the predictions of these two concepts including synergism or antagonism can be detected in mixtures [18].

Here we present a study that investigated how feeding-related endpoints in *Daphnia magna* were affected by the exposure to external gamma radiation in combination with the PAH fluoranthene. Daphnids are common zooplankton grazers in freshwater systems and are an important factor in controlling phytoplankton biomass and species composition [19]. We specifically focused on feeding-related endpoints such as incorporation of carbon, since these processes encompass interactions between two different trophic levels, while being ecologically relevant at both at the individual and population level. In addition, feeding assays are widely used in ecotoxicological assays and can be up to 50-fold more sensitive to stress than other endpoints such as survival [20]. We exposed *D. magna* to five different concentrations of FA and five different doses of gamma radiation as single contaminants and in nine binary mixtures. We then measured the assimilation of carbon from the microalga *Pseudokirchneriella subcapitata* by *D. magna.* Our goal was to test the specific null hypotheses:

a) ingestion rates, incorporation of carbon from phytoplankton by *D. magna* and *D. magna* growth are not decreased by exposure to either gamma radiation or fluoranthene and b) there is no interactive effect between these two contaminants.

Methods

Algae cultures

The green algae *P. subcapitata* was grown 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}. The algae were labeled by adding 1.22 GBq of $NaH^{14}CO_3$ (Amersham; specific activity 1.998 GBq mmol to the MBL medium). After 1 week of incubation, the algae were harvested by centrifugation at 3000 g for 10 min. Once centrifuged, the algae formed a pellet at the bottom and the supernatant was discarded. To remove non-incorporated ^{14}C present in the interstitial water between the algae cells, the pellet was rinsed and resuspended in MBL medium, centrifuged again, and the supernatant water was checked for radioactivity after the addition of 5 mL of Ultima Gold scintillation cocktail (Perkin Elmer). This procedure was repeated until the radioactivity of the rinsing water was below 0.05% of that incorporated in the algae. Shortly after the rinsing, samples of the concentrated algae suspension were taken to measure chlorophyll content (absorbance at 684 nm) and estimate biomass according to Rodrigues et al [21]. The concentrated algae suspension was then frozen at -20ºC. Before the start of the experiment the algae were slowly thawed at 4ºC. After thawing, samples of the concentrated *P. subcapitata* suspension were observed under a microscope to confirm that freezing and thawing did not affect *P. subcapitata* cell integrity. Samples from the same concentrated suspension were used to measure its radioactivity in a liquid scintillation counter (LKB Wallac Rackbeta 1214) after the addition of scintillation cocktail (Ultima Gold)[22]. The final radioactivity of the phytoplankton suspension was 54.0 ±1.4 Bq mgC^{-1}.

Zooplankton cultures

Daphnia magna adults were obtained from ITM (Stockholm University, Sweden) and reared in the laboratory for several weeks. Animals were kept in artificial freshwater (pre-aerated M7 medium at a pH of 8.1) prepared according to OECD protocols supplemented with vitamins, renewed every week. Cultures were maintained in 2 L beakers at 20 ^{0}C (±1 ^{0}C) on a 16:8 light:dark photoperiod at a light intensity of 0.4 µmol m^{-2} sec^{-1} and at a density of 1 animal per 25 ml. Daphnids were fed with the green algae *P. subcapitata*, at a daily ration of approximately 0.1-0.2 mgC/day/daphnid. Exposure experiments were performed with juveniles 2-3 days old.

Test compound and concentrations

An aqueous stock solution of FA (Aldrich Chemical Co., MW 202.26; 98% purity) was made by dissolving a known amount of FA in HPLC grade acetone. Different volumes of this FA solution were pipetted to four different 2000 ml beakers with 1500 ml M7 medium to achieve four different nominal FA exposure concentrations (20, 40, 80, 160 µg L^{-1}) in addition to a unexposed control. The FA concentrations were chosen to cover the range where effects on feeding-related endpoints had previously been observed [23,24] . The acetone was allowed to evaporate overnight.

Four additional beakers with the same nominal FA concentrations were prepared as described above, for determination of actual concentrations of FA in the M7 medium. Measured concentrations of FA in the *D. magna* media were assessed using high performance liquid gas chromatography (HPLC) at a commercial laboratory (ALS Scandinavia AB)

Exposure

D. magna individuals were added to the 5 different beakers for exposure to FA that lasted 24 h at 20 ^{0}C with a 16:8 light:dark photoperiod. After 24 hours *D. magna* individuals were collected from the FA beakers and picked into 5 different 60 ml plastic containers with M7 medium with the corresponding FA concentration. The plastic beakers were immediately taken to the irradiation facility and exposed to gamma radiation (Gammacell 1000, ^{137}Cs source). The radiation rate was 6.7 Gy min^{-1} and the radiation doses were 0, 25 Gy, 50 Gy, 100 Gy and 200 Gy, which corresponded to 0, 3.7, 7.5, 14.9 and 29.8 minutes in the irradiation source. These doses were chosen to include a range where an effect of gamma radiation on our endpoint could be expected based on previous pilot studies, since EC_{50} values from studies with comparable doses/dose rates and experimental duration are not available in the literature. In the environment, pure external gamma irradiation is seldom encountered, as organisms will take up radionuclides and receive additional internal dose. This experiment was therefore set up as a proof-of-concept to test mixture toxicity theory for radiation, rather than mimicking natural conditions.

The gamma dose distribution was homogenous throughout the containers containing the daphnids. This was verified by attaching Gafchromic film RTQA2 (ISP, USA) on the container. The measured values were within 0.12% of the nominal dose. One of the five containers with *D. magna* individuals did not receive any gamma radiation, but was otherwise handled in the same way as the other samples.

Feeding test

The irradiated *D. magna* were then divided into 57 experimental units (glass beakers) with 50 ml new M7 medium, each beaker receiving 5 individuals. In addition, 20 individuals were preserved in 70% ethanol to determine average initial size at the start of the experiment. The experiment had 19 treatments with 3 replicates per treatment each (see Fig 1) and started with the addition of 0.2 mg C of the ^{14}C-labeled *P. subcapitata* suspension to each replicate. Initial samples to estimate the number of microalgae cells present at the beginning of the experiment were collected and frozen at -20 ^{0}C. The daphnids were left to feed for one day.

After 24 h, the *D. magna* were collected from the experimental units, placed into new containers with fresh M7 medium for 20 min to clean their guts, picked out and preserved in 70% ethanol. The time between this step and the addition of the algae to each replicate was recorded and all endpoints were adjusted to a period of 24h. The contents of the experimental units (M7 medium +uneaten algae) were transferred to Falcon tubes and frozen at -20 °C for later estimation of ingestion rates.

After the termination of the experiment, each individual preserved in ethanol was photographed using a light microscope (WildM28 Leica, Switzerland) and a digital camera (Dino lite, Taiwan). The total 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 the length-weight relationship published by Kersting and van der Leeuw-Leegwater [25].

After length measurements the 5 daphnids from each replicate were pooled and solubilized in 1 mL of Soluene-350 for 24 h at 60 ^{0}C and left overnight to reduce chemiluminescence. After addition of 10 mL of Ultima Gold XR, radioactivity was measured in a liquid scintillation counter (LKB Wallac Rackbeta 1214) to calculate the incorporation of radiolabeled carbon in each treatment during the experiment.

The algae cell concentrations in the experimental media at the beginning and end of the 24h-feeding period were determined under a microscope using a hemocytometer. This data was used to calculate ingestion rates by *D. magna*, according to Frost [26]. Since there was no algae growth during our experiment, changes in average algae concentration (C) could be expressed as:

Equation 1:

$$C = Cf - Ci/[t2 - t1]$$

where C_f is the *P. subcapitata* cell density in each replicate at the end of the feeding test, C_i the *P. subcapitata* cell density added to each replicate at the beginning of the feeding test; and t2-t1 the duration of the feeding test.

In addition, the filtering rate (F) was calculated by the expression

Equation 2:

$$F = V/N$$

where, V is the volume of each experimental unit, N the number of daphnids in each replicate.

Ingestion rates (I) were then calculated using:

Equation 3:

$$I = C * F$$

Statistics

The ^{14}C radioactivity in *Daphnia magna* in each replicate was corrected for background radiation and recalculated to carbon incorporation in micrograms (µgC / µg dw *Daphnia/day*).

Analyses of the dose-response curves were done using R software version 3.2.0 (http://www.r-project.org) and the extension package *drc* (version 2.3-96 [27]). 16 different models were analyzed (including log-logistic, Weibull type I and II regression models, and the Cedergreen-Ritz-Streibig model) [28], and used to calculate EC_{50} and corresponding standard error and confidence intervals using the delta method [29]. Model selection was performed using Akaike's information criterion (AIC). The existence of a dose effect was tested by the *noEffect* test (*p* value), while goodness-of-fit was assessed by the *lack-of-fit test* (*p* value), both included in the *drc* package [27].

Analysis of predicted versus observed effects for mixtures:

As mentioned above, two models, CA and IA are commonly used to estimate the joint effect of multiple contaminants. Gamma radiation and FA present obvious dissimilarities in their modes of action, although both these stressors have the potential to cause oxidative stress. It would be theoretically informative to compare the observed data against both the CA and IA reference models regardless of mechanistic considerations. However, in our study it was not possible to calculate the predicted CA joint effects due to inability to fit a dependable dose-response curve to the FA single stressor data. While we could not derive the full predicted dose-response surface for IA either, it was nonetheless possible to calculate the predicted unaffected fractions for all mixture points of our experimental design, since a factorial design was employed (see below). For this reason we have here only compared the observed data to the IA model.

The independent action model assumes that the mixture components act dissimilarly (Bliss, 1939) and can be formulated as;

Equation 4:

$$Y = u_0 \prod_{i=1}^{n} q_i(c_i)$$

where Y is the measured biological response, u_0 the control response, and $q(c_i)$ denotes the probability of non-response (i.e. the unaffected fraction), functionally related to concentration c of compound i.

Usually, the prediction of joint effects would be made based on the single chemical dose-response curves to predict the effects from each single chemical at their concentration in the mixture. These individual chemical effects can then be converted to proportional effects compared to the controls or "unaffected fraction" (UAF), and allow calculation of the expected joint effect from that given mixture [30].

Factorial experimental designs were chosen to allow the application of point by point comparison of observed data against expected effects according to the IA concept, even in the case where a dose-response curve could not be fitted to one of the stressors (FA), not allowing for a full response surface analysis for IA or any CA prediction. As such, the prediction of the expected joint effects of the mixtures based on IA were estimated by simply calculating the observed UAF for each of the individual stressors for each dose, and multiplying these to derive the expected joint unaffected fraction. Standard errors of expected joint effects of mixtures were calculated by the expression:

Equation 5

$$SE = XY\sqrt{\left(\frac{Sex}{X}\right)^2 + \left(\frac{Sey}{Y}\right)^2}$$

where X and Y are the biological response for each stressor, Se_x and Se_Y the standard error of the biological response for stressor X and Y, respectively.

Robust statistical analysis of observed against expected values for carbon incorporation and ingestion rates was difficult, as splitting the data down to single treatments meant comparing three observed replicate values against the predicted effect. As such, the differences between the IA predicted and observed values for all endpoints were assessed in relation to the general pattern of the data.

Results and Discussion

There were no indications that freezing *P. subcapitata* affected *Daphnia* feeding in our controls. *P. subcapitata* cells were intact at the start of the experiment and the daphnids showed a normal feeding behavior. In addition, carbon incorporation by *D. magna* in our controls was within the range of the unexposed controls in other studies with similar experimental conditions (Nascimento et al, unpl).

Single toxicant exposures

Gamma radiation

No mortality was detected at any doses in the single stressor exposure or in the controls. The gamma doses used in this experiment were high, but within a range not unknown at contaminated sites. For example, in lakes in the Mayak area, Russia, used as nuclear waste ponds for decades, absorbed dose rates for zooplankton and phytoplankton are estimated as 3.8 and 40 Gy day^{-1}, respectively [31] In the Techa River, in the same area, doses to biota as high as 200-800 Gy were estimated after an accident in 1957 [32].

Exposure to gamma radiation had a significant effect on ingestion rates ($p<0.001$, Fig. 2A) with an EC_{50} of 146 ± 15 Gy (EC_{50} ± SE, see Table 1). Ingestion rates in *D. magna* increased slightly in individuals exposed to the lowest dose of gamma radiation (25 Gy). This increase in ingestion rates at 25 Gy dose suggests a response to increased energy requirements to deal with the stress provoked by exposure to radiation. The stress at this dose did not seem to induce significant harm to *Daphnia*, since individuals in this treatment showed an active feeding behavior, and growth similar to the controls. This was not the case for individuals in the 200 Gy treatment, where ingestion rates were depressed significantly.

In addition, our results show clearly that acute exposure to gamma radiation decreases the incorporation of carbon from phytoplankton by *D. magna* ($p= 0.001$, Fig. 2B). This endpoint showed a dose-dependent response to gamma radiation with the EC_{50} being calculated at 109 ± 54 Gy (Table 1). Carbon incorporation in daphnids decreased at every dose, more significantly at 100 and 200 Gy. The difference in response between ingestion rates and carbon incorporation was seen previously in *D. magna* exposed to alpha-emitters such as uranium-238 and americium-241 [33,34] where no effect on ingestion rates due to radiotoxicity of these radionuclides was found. These studies did, nonetheless, find a reduced scope for growth (SPG), defined as the difference between energy assimilated from food and energetic costs of metabolism, for *D. magna* exposed to radiation. This decrease in SPG was attributed mostly to increased metabolic costs that come with dealing with radiation, as ingestion rates (the proxy for energy intake used in that study) were not affected. The discrepancy seen in our study between the endpoints of incorporation of carbon and ingestion rates suggests otherwise; that even though ingestion rates are unchanged when exposed to high levels of ionizing radiation, energy intake is affected. These results also agree with Massarin et al [35] who found that uranium-238 exposure caused a reduction in carbon assimilation by *D. magna* that resulted in a lower SPG.

We observed that the mobility of *Daphnia magna* was reduced in our experiment in the 200 Gy treatment. This overall reduced activity as a result of exposure to this dose of gamma radiation likely contributed to the decrease in the ingestion rates and carbon incorporation by *D. magna*. In addition, exposure to high levels of uranium can induce severe damage to *D. magna* digestive tract and clear impacts on the amount of food assimilated [35]. It is possible that exposures to the high doses of gamma radiation used in our study produced similar damage in the digestive tract of *D. magna*. Decreased energy intake can have important consequences at both individual and population level. Massarin et al 2011 [36] using a modelling approach (DEBtox), were able to link uranium-induced decreased carbon assimilation to effects on both growth and reproduction. We observed such an effect of gamma radiation on growth in our experiment ($p=0.025$, Fig. 2.C), although this was only clear at the highest gamma radiation dose ($EC_{50\ growth} = 235 \pm 58$ Gy, see Table 1). This is in agreement with multiple other studies which have reported effects on growth and reproduction of zooplankton as a result of exposure to gamma radiation [37,38] or alpha-emitters radionuclides [33,34]. 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 [39,40] and retarded growth has been suggested to indicate a metabolic burden for detoxification or damage repair [41].

Fluoranthene

FA measured concentrations

The measured FA concentrations in water in the different treatments were close to the nominal concentrations previously mentioned. The measured FA doses were 0, 23, 44, 67, 147 μg L^{-1}. These concentrations are high but comparable to FA concentrations found in contaminated aquatic sites like groundwater samples from coal and oil gasification plants [42] or water from urban runoffs [43] that can reach concentrations of FA of 50 μg L^{-1} and 130 μg L^{-1}, respectively.

Exposure to FA did not result in any significant effects on carbon incorporation, growth or ingestion rates in daphnids. As such, it was not possible to calculate biologically relevant EC_{50} values for FA for any of these endpoints (Fig 2 D, 2 E, 2 F, and Table S1 in supplementary information). This lack of effect of FA at all the doses here tested was unexpected as Barata and Baird 2000 [23] observed EC_{50} for ingestion rates by *D.magna* at 38 μg L^{-1}, well below our highest tested dose, although with a longer exposure period. Several authors have reported FA and other PAHs to affect not only feeding and mortality in aquatic species [11,13,44], but also embryonic viability and resource acquisition [45].

Mixture toxicity

In general, the IA concept accurately predicted the effects of the mixtures for the endpoint of growth (Fig 3A). There were, however, consistent deviations from the IA predictions for the endpoint carbon incorporation by *D. magna*. More carbon was incorporated than predicted by the IA concept at lower dose combinations, and in some cases this difference was considerable (Fig. 3B). An example of this can be seen in the treatments 25 Gy+ 44 μg L^{-1}, 50Gy+ 44 μg L^{-1} and 50Gy+ 67 μg L^{-1} where carbon incorporation was on average 62%, 37% and 37% higher than the predicted, respectively (Fig. 3B). A similar, but less clear pattern was seen for ingestion rates in the mixture treatments with lower dose combinations (Fig 3C), with one exception (25Gy + 23 μg L^{-1}). With this exception, ingestion rates were generally higher than what was predicted for the lower doses in our study, particularly in the 25Gy+ 44 μg L^{-1} and in the 50 Gy+ 44 μg L^{-1}, that showed ingestion rates 26% and 40% higher than expected, respectively. The patterns seen for carbon incorporation and ingestion rates suggest that at the lower range of the tested exposures there were deviations to the IA concept that could be classified as antagonistic. One of the principal pathways through which PAHs such as FA and radiation can provoke effects on organisms is through the increase of the cellular production of reactive oxygen species (ROS), that studies have shown to be affected by contaminants [46]. To counter ROS production, organisms need to enhance antioxidant defenses to be able to maintain a balance and avoid oxidative stress. These defenses are often composed of proteins, enzymes and other compounds like ascorbic acid, glutathione and uric acid [46]. It is possible that the exposure to FA in our experiment, which started before the acute exposure to radiation, stimulated the anti-oxidant defense mechanisms that helped *D. magna* cope with some of the effects associated exposure to radiation, thus explaining the antagonism seen in the lower doses. In addition, the energy requirements to sustain these antioxidant defenses are likely to have stimulated *Daphnia* energy acquisition, as seen by the suggested antagonism found in most of the lower dose mixture treatments regarding daphnid ingestion rates and carbon incorporation.

On the other hand, at the doses of 200Gy + 66μg L^{-1} and 147μg L^{-1} FA the observed carbon incorporation and ingestion rates were lower than the predicted IA value (on average 27 and 33%, respectively), suggesting a synergistic behavior of the two stressors at these doses. Although, to our knowledge, no other published study has tested the effects of gamma in combination with PAHs, a significant number of studies on aquatic organisms have found synergism between PAHs when together with UV. Although the intensity and wavelength of gamma and UV radiation are different, its mode of action is in part similar underlining the relevance of the comparison. For example, Nikkilä et al. [47] found that toxicity of pyrene to *D. magna* was increased when present with UV-radiation. UV radiation in a mixture with other organic contaminants also increases oxidative stress in *D. magna* individuals in combined exposures when compared to the single stressor treatments [48]..Gamma radiation could potentially be acting in a similar way to UV radiation, increasing the toxicity of FA in the 200 Gy+ 66 µg/L and 200Gy+ 147 µg/L treatments where we observed this synergistic effect. The stress and damage caused by the combined exposure to these two stressors at such high doses was probably too much for the organism to cope with reducing daphnid mobility. In addition, exposure to high levels of ^{238}U and FA have been seen to cause extensive cellular damage in daphnids [49], and important histological effects on the digestive tract of *D. magna*. Among these histological effects is the reduction of microvilli in the intestine tract that can decrease the efficiency of the energy intake by organism[50]. Massarin et al. [36] observed increasing damage on the midgut structure with increasing uranium concentration, indicating that the decrease in food assimilation resulted from direct damage to the intestinal epithelium caused by exposure to uranium. Although our study does not present direct evidence of this, the sum of these direct effects on the digestive tract by both stressors at such high concentrations can help to explain the lower than expected incorporation of carbon by the daphnids. In addition, the decreased food acquisition, as show by the decreased ingestion rates would also reduce the capability of the daphnids to sustain the energy requirements of the repair mechanisms against ROS or

DNA damage, further enhancing the effects of the mixtures. However, it must be underlined that this synergism happened at a high acute gamma dose (200 Gy), only seen in nuclear accident sites such as in the Techa River near the Mayak Nuclear Materials Production Complex after the Kyshtym disaster in 1957, where biota was exposed to doses between 200-800 Gy [32]. In addition, this synergism was not seen for growth, probably due to the short duration of our experiment.

Our results suggest that there is limited potential for synergistic effects in mixtures of gamma radiation with FA, for the endpoints tested in our study. In fact, there seems to be antagonistic interactions in regards to ingestion rates and incorporation of carbon by *D. magna* at the lower spectrum of the doses we tested in the mixtures treatments with these 2 stressors (Fig. 3). Since feeding assays have been reported to be approximately 50X more sensitive than other standardized acute ecotoxicological endpoints [20] one might expect these results to be applicable to less sensitive parameters at the individual and population levels. Nevertheless, we did find indications of synergistic effects in mixtures of radiation with FA, although only at extreme levels of acute radiation. It would be important to investigate if the effects of the mixtures with radiation and PAHs observed here occur with chronic exposure to radiation, and if so at which doses.

One finding of this study concerns how different the interpretation of its data would look if only one stressor was assessed. Only assessing the effects of gamma radiation when in combination with FA, would markedly overestimate its impact on the feeding of *Daphnia,* leading to potentially erroneous conclusions. This reinforces how important it is to evaluate the joint effects of contaminants in mixtures. Environmental radiation protection guidelines and tools adopted by international organizations (e.g., IAEA[46]; ICRP[47]) are still based on studies that considered radiation as the sole contaminant, in isolation from other stressors. Our study shows that using mixture toxicity tools and assessment techniques that include radiation with other contaminants need to be taken into account in environmental protection legislation regarding radioactive elements.

In addition, we present a method to perform mixture analysis based on the IA concept when reliable dose-response curves are difficult to obtain for one or both stressors, which is often the case, particularly at environmentally relevant levels of the stressors. However, where such non-effects can be foreseen replication should be increased to allow statistical pairwise comparisons. This information can be very helpful for future studies investigating ecotoxicological effects of mixtures of contaminants/stressors.

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Supporting information

Includes Tables S1 and S2 with dose-response parameters for fluoranthene exposure as the single stressor and the raw data used in this experiment, respectively. This information is available free of charge via the Internet at http://pubs.acs.org/ .

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Fig 1- Experimental design outlining the treatments investigated in this study. Single contaminant exposure treatments on x-axis (fluoranthene) and on y-axis (gamma radiation)

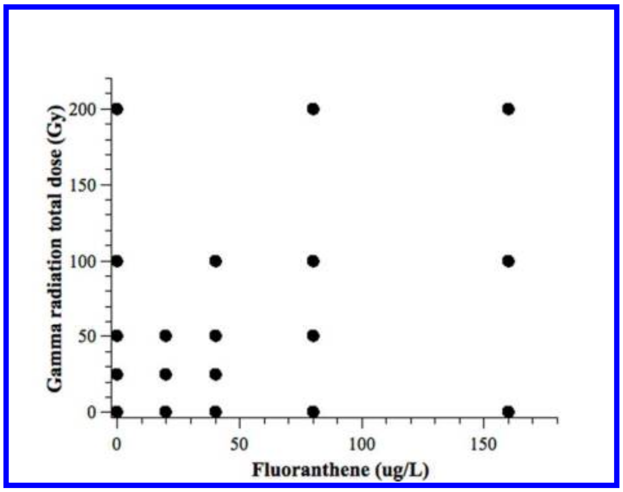


Fig 2. Changes in ingestion rates (A and B), incorporation of carbon by *D. magna* from *P. subcapitata* (C and D) and growth (E and F)) in relation to gamma (left column) and fluoranthene dose (right column) in the single contaminant treatments. Values are given as Unaffected fraction (UAF). Full circles represent observed data, while dashed lines show modeled predictions

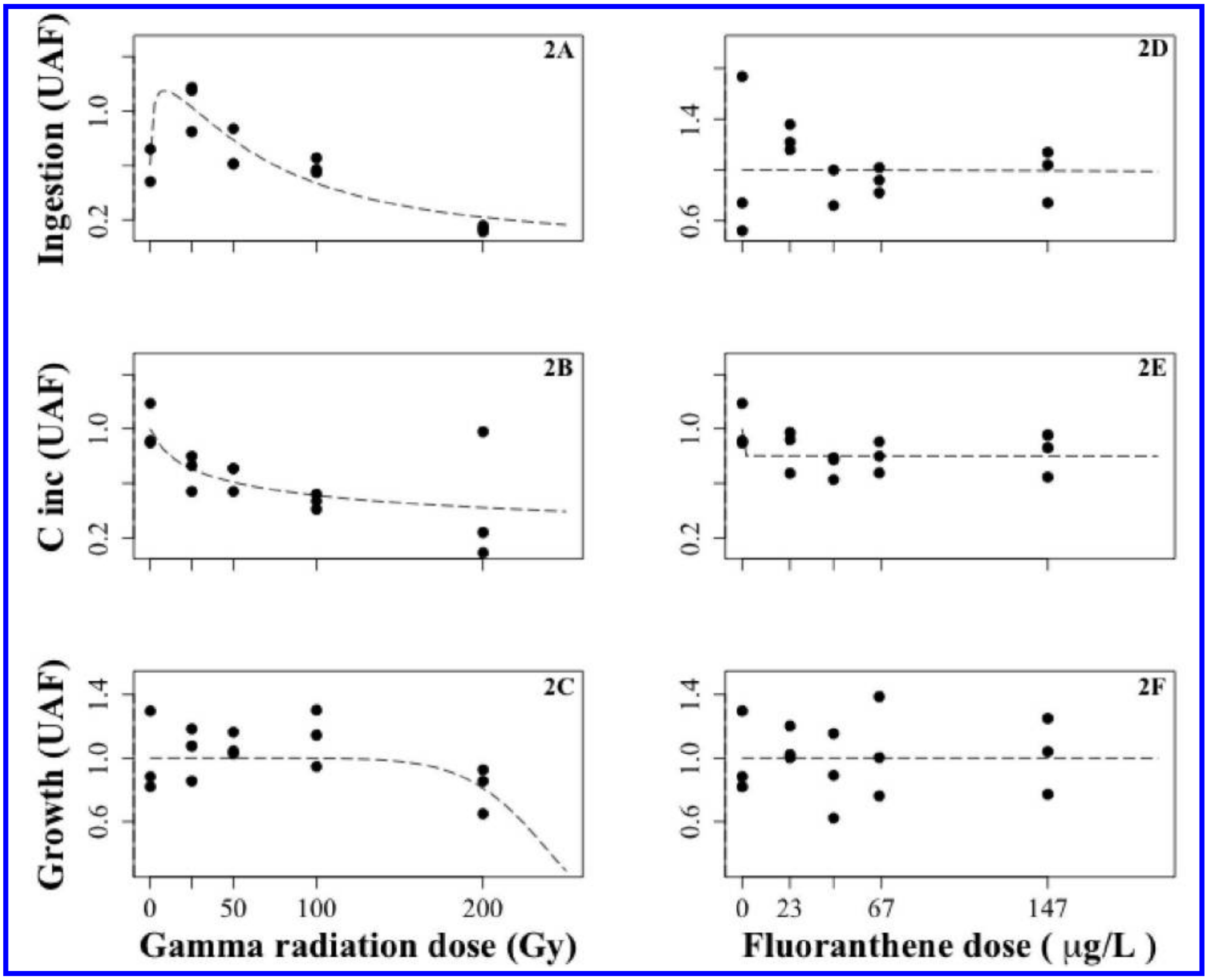


Fig 3. Shows the average±SD observed unaffected fractions (UAFs) for each mixture treatment (black squares) exposed to varying treatment combinations of Fluoranthene (FA) concentrations (µg/L) and Gamma radiation doses (total Gy) in each of the studied endpoints: A) Growth; B) Carbon incorporation and C) ingestion rates. Label next to each black square show treatment code. The solid line indicates the predicted UAFs for each joint FA x Gamma treatment based on the Independent Action concept (pairwise multiplications of the all the UAFs for the respective single FA treatment and single Gamma treatment), and the dashed lines the standard error of the expected joint effects of the mixtures.

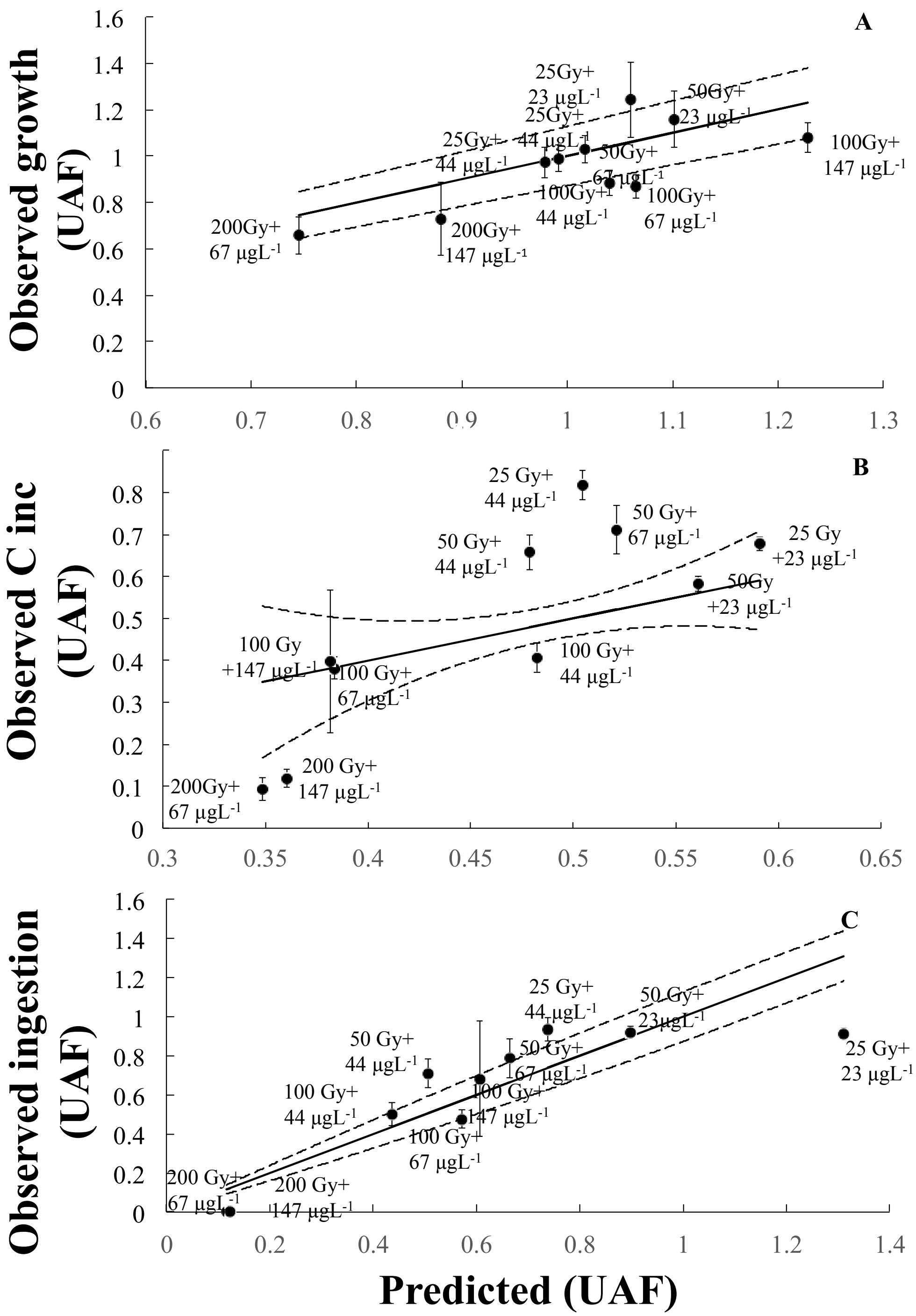
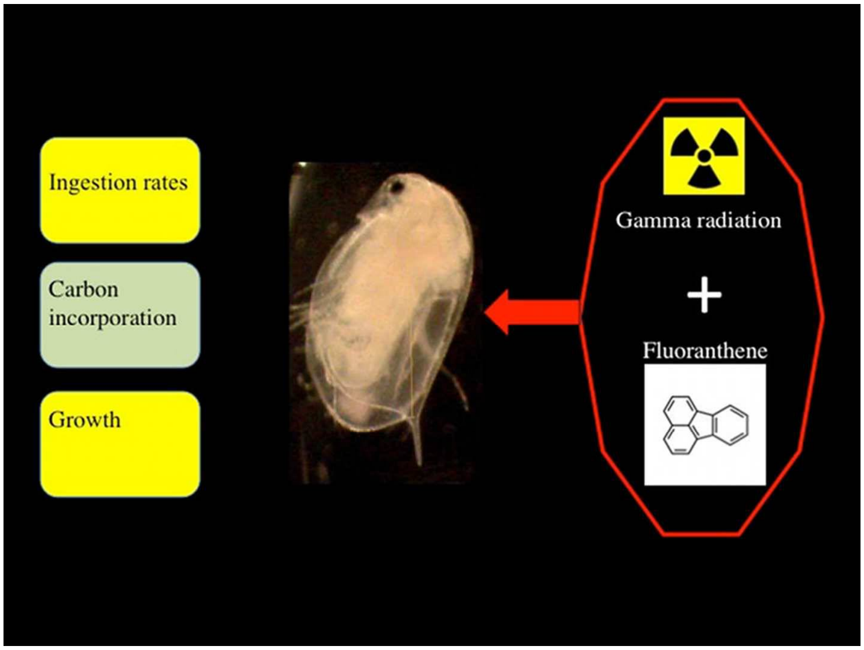

A
Observed growth (UAF)
25Gy+ 23 µgL-1
50Gy+ 23 µgL-1
25Gy+ 44 µgL-1
25Gy+ 44 µgL-1
50Gy+ 67 µgL-1
100Gy+ 147 µgL-1
100Gy+ 44 µgL-1
100Gy+ 67 µgL-1
200Gy+ 67 µgL-1
200Gy+ 147 µgL-1
B
Observed C inc (UAF)
25 Gy+ 44 µgL-1
50 Gy+ 67 µgL-1
50 Gy+ 44 µgL-1
25 Gy +23 µgL-1
50Gy +23 µgL-1
100 Gy +147 µgL-1
100 Gy+ 67 µgL-1
100 Gy+ 44 µgL-1
200Gy+ 67 µgL-1
200 Gy+ 147 µgL-1
C
Observed ingestion (UAF)
25 Gy+ 44 µgL-1
50 Gy+ 23µgL-1
50 Gy+ 44 µgL-1
50 Gy+ 67 µgL-1
25 Gy+ 23 µgL-1
100 Gy+ 44 µgL-1
100 Gy+ 147 µgL-1
100 Gy+ 67 µgL-1
200 Gy+ 67 µgL-1
200 Gy+ 147 µgL-1
Predicted (UAF)

Table 1- Best model, model fit tests, median effective concentration (EC50) values and respective slopes (beta) calculated from exposure to gamma radiation as the single stressor. Standard errors for beta and EC_{50} are show beside values in parenthesis.

Endpoint	**Best Model**		**Model fit**		**Model parameters**	
	Model	Model function	*Lack of fit* test	*noEffect* test	beta (±SE)	EC50 (±SE)
Ingestion	Cedergreen-Ritz-Streibig	*f(x) = c + \frac{d-c+f exp(-1/(x^{α}))}{1+exp(b(log(x)-log(e)))}*	p=0,052	p<0,001	4.5 (0,76) p=0,001	146 (15) p<0,001)
C inc	Weibull	*f(x) = \exp(-\exp(b(\log(x)-e)))*	*p= 0, 97*	p= 0, 001	0.43 (0.23) p=0,1	109 (54) p=0,2
Growth	Weibull	*f(x) = \exp(-\exp(b(\log(x)-e)))*	p=0,6	p= 0, 025	7 (10) p=0,4	232 (41) p=0,008

TOC Art


Ingestion rates
Carbon incorporation
Growth
Gamma radiation
+
Fluoranthene