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The combined effect of depleted uranium, sodium arsenite and gamma radiation on survival, growth and reproduction of Caenorhabditis Elegans

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Abstract

The purpose of this work was to study the effect of depleted uranium, sodium arsenite and gamma radiation on three biological endpoints of *Caenorhabditis Elegans*. The three endpoints studied were survival, growth, and reproduction.

The nematodes were exposed to the stressors individually and in combination. Six different concentrations of uranium and arsenic were used. For uranium, the molar concentrations used were 100 μM (U1), 50 Mm(U2), 25 Mm(U3), 12.5 μM (U4), 6.25 μM (U5), and 3.1 Mm (U6). The concentrations used for arsenic were 1 mM (As1), 0.5 mM (As2), 0.25 mM (As3), 0.125 mM (As4), 0.63 mM (As5), and 0.315 mM (As6). The gamma radiation doses studied were 9.61 Gy and 19.22 Gy (~ 100 mGy/h and ~ 200 mGy/h dose rate during the 96 hours exposure).

In most of the cases all the nematodes survived. Only at the highest concentration of sodium arsenite there were cases of 100% mortality in the populations exposed; however this was not necessarily the rule. There were many cases in which the nematodes survived, although their growth was compromised.

All the stressors had detrimental effects on growth, except exposure to gamma radiation alone. There was a reduction in growth in that case but it was not statistically significant. The detrimental effects are particularly strong when exposed to sodium arsenite or combinations of sodium arsenite and uranium.

Reproduction was affected by all the stressors. The effects are always led by arsenic, in the statistical sense, but when mixed plates were exposed to gamma radiation, at the lowest dose of 9.61 Gy, no statistical significant for detrimental effects could be established, as opposed to the higher dose of 19.22 Gy, for which statistically significant results were found on the effects on growth and reproduction when the populations were exposed to gamma radiation at that dose and the mixture of depleted uranium and arsenic

1. Introduction

Exposure to contaminants or stressors rarely happens in isolation; instead, organisms and humans as well are primarily exposed to multi-component chemical (and other stressors) mixtures via food, water or surrounding environment. However, most of the studies in the field of toxicology focus on assessing chemical risk considering single substances [1, 2].

In the field of Radioecology, the topic of multiple stressors has become particularly important, considering that radionuclides usually occur in combination, depending on the source, and also combined with other contaminants such as metals and organic pollutants, which may act on the same end points; conversely, it is possible as well for a single stressor to induce multiple biological effects, for example in the case of interaction with multiple target sites [3]. A particular challenge is the fact that there are relatively few available documents which address the problem of mixture toxicity including radioactive contaminants [4], and therefore there is a consensus regarding the need to conduct focused studies including radioactive contamination in the context of mixed contaminants.

There is evidence that chemicals interact in such ways that the combined effect is quite different from the individual effects [2]. Most of the chemicals with similar modes of action produce combined effects which are larger than those of the individual components acting alone; this is referred to as synergism. Additivity occurs when the components act independently from each other, and the mixture's effect is such that it does not enhance neither diminish the single components' effects. Antagonism, on the other hand, is the case in which the result of the mixture components' interaction produces a weaker effect than that expected from the additive case, i.e. both cases of interaction, synergy and antagonism can be understood as deviations from additivity [2].

The issue of mixtures, nonetheless, is not new. The basic ideas of combined actions had already been introduced in 1939 by Bliss [5], and further developed in the 1950s by Hewlett and Plackett, and other researchers [6]. Their basic mathematical models are derived from a set of biological models of 'joint action', defined as similar or dissimilar depending on the sites of primary action in the organism were the same or different, and as interactive or non-interactive depending on whether on drug influenced or did not influence the biological action of the other.

The formulation used nowadays accounts for the first case, of similar modes of action, or similar joint action of the old jargon, by defining dose/concentration addition. The mixture dose/concentration is the sum of the adjusted doses/concentrations of the individual components, given by

$$D_{mix} = \sum_{i=1}^{n} aD_i \tag{1.1}$$

where D_{mix} is the mixture dose/concentration, a is a scaling factor to account for the differences in potency of the individual substances, and D_i represents the dose/concentration of each individual substance [2]. In this case, dose additivity is assumed over the whole range, including

dose/concentrations below the no observed adverse effect levels/concentrations (NOAEL/Cs). As mentioned above, antagonism or synergism will be regarded as a deviation (negative in the first case, positive in the second case) from the relationship (1).

Independent action, on the other hand occurs when the components of the chemical mixture exert their effect independently from each other usually acting by different modes of action which do not have influence on each other. The probability of an organism or individual of being affected by the mixture is given by

$$E(C_{mix}) = 1 - \prod_i^n (1 - E(C_i)) \quad (1.2)$$

where $E(C_{mix})$ represents the combined effect produced by the mixture concentration C_{mix} and $E(C_i)$ the effect of the individual component I, with concentration C_i.

Alternatively, a mathematical model can be fitted to the experimental dose-response curves by means of non-linear regression analysis; when there is a good fit, reliable effect concentrations (EC_x) can be determined together with the uncertainties [4]. Based on this, two approaches are used to predict the effects of combined toxicity, concentration addition (CA), and independent action (IA) [7]. Concentration addition is formulated in a slightly different way than equation (1), and both cases assume no interacting effects. The CA model is for mixtures the components of which have similar modes of action and it is described by

$$\sum_{i=1}^n \frac{Ci}{EC(Xi)} = 1 \quad (1.3)$$

where n is the number of components in the mixture, ECx_i the concentration of the i*th* component responsible for the x% effect when applied alone, and C_i is the concentration of that component in the mixture. The fraction C_i / ECx_i defines the so called toxic unit of the element i, and it represents the concentration of a mixture component scaled by its relative toxicity. These concepts are illustrated in figure 1.1.

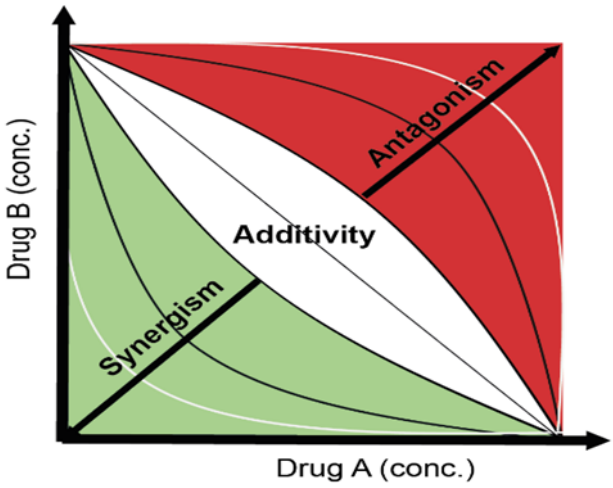

Figure 1.1. Concepts of additivity, synergism, and antagonism. The white region includes considers uncertainties, and synergism and antagonism are deviations from the additivity case. SOURCE: Rodea-Palomares *et al.* [7].

This thesis is an attempt to study the combined effect of Uranium, Arsenic and gamma radiation on three different end points, namely growth, reproduction and survival, using as a model the nematode *Caenorhabditis Elegans*. The next sections will introduce the general aspects about each stressor used in the study and also a description of the biological model used.

1.1 Depleted Uranium

Uranium is a ubiquitous radioactive element which occurs naturally in the Earth's crust at a concentration of approximately 3 mg/Kg, while in seawater it occurs at a concentration of approximately 3 µg/L, it is also found in groundwater and as a trace element in certain foods and drinking water; the human body contains on average 56 µg of Uranium, distributed mainly in the skeleton and muscles [8, 9]. It belongs to the actinide series (part of the f block), in fact, it was the first of the actinides to be discovered in the late 1700s in pitchblende ore. Uranium oxidizes easily in air, hence in its natural form is found usually oxidized. In ores, it occurs more commonly as uraninite (UO_2^{2+}), pitchblende ($U_3O_8^{+3}$) and also as secondary minerals, such as phosphates, silicates and complex oxides [9, 10].

Three isotopes, namely ^{238}U, ^{235}U and ^{234}U, form natural Uranium, with isotopic abundances of 99.2745%, 0.7200% and 0.0055% respectively. Of the three natural decay series, two originate from Uranium isotopes, namely, the Uranium series (^{238}U) and the Actinium series (^{235}U) [11] which are shown in figure 1.2 together with the Thorium series.

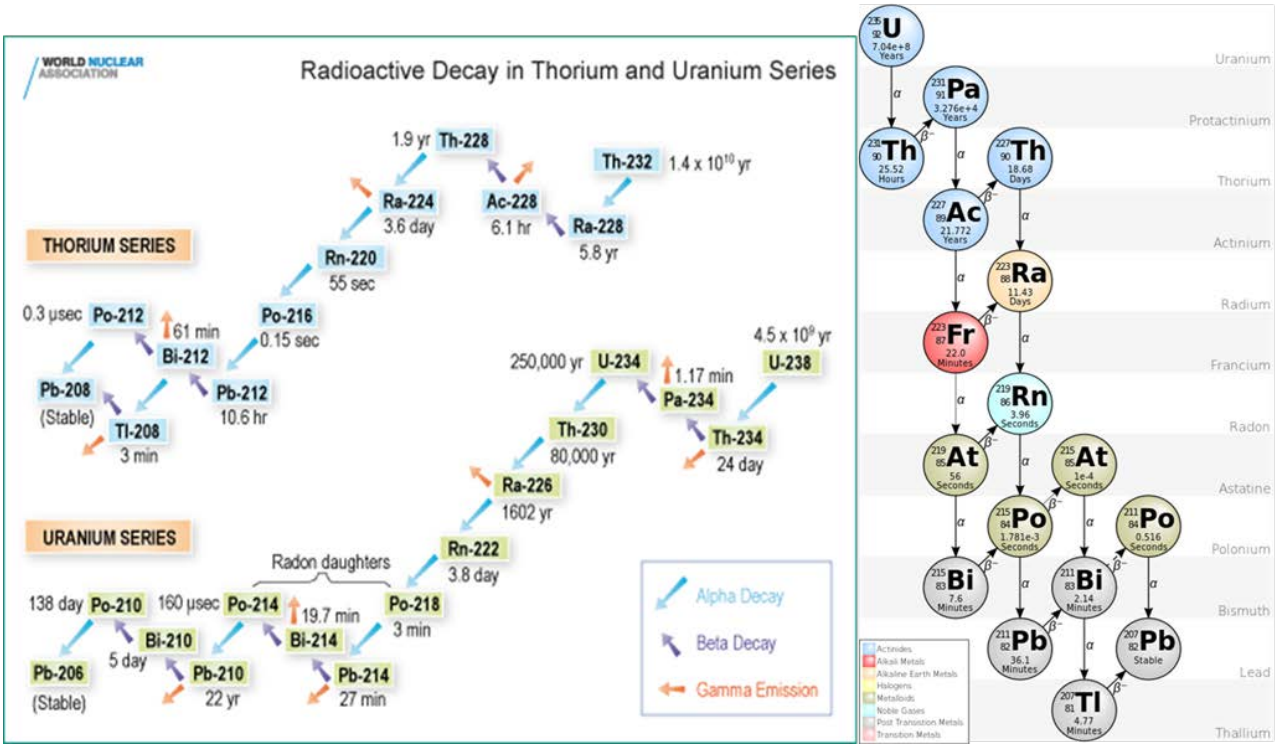

Figure 1.2. Naturally occurring decay series. On the left, thorium and uranium series; the actinum series is shown on the right. SOURCES: World Nuclear Association, and © Creative Commons (CC BY 3.0).

Table 1.1 summarizes the most important characteristics of natural Uranium and its main isotopes (i.e. half-lives, isotopic abundance and specific activity).

Table 1.1. The main Uranium Isotopes. Source: Blaise *et al.* [9].

Isotope	Half-life (years)	Relative mass (%)	Specific Activity (Bq/g)
^{238}U	$4.47x10^{9}$	99.3	12.455
^{235}U	$7.04x10^{8}$	0.72	80.011
^{234}U	$2.46x10^{5}$	0.006	$231x10^{6}$

The decay mode of the Uranium isotopes is alpha radiation, but some of their daughters undergo beta decay and some emit gamma radiation. In its natural form, it is in secular equilibrium with its daughters, and therefore in combination it is much more radioactive than pure Uranium [11].

Its main use is as fuel for nuclear reactors and in nuclear weapons. In the case of nuclear fuel, commonly UO_2 is produced using enriched ^{235}U in the form of UF_6 [11]. Depleted Uranium (DU) is a byproduct of the enrichment process, such that its ^{235}U content is 0.1-0.3% of its original value and its activity is approximately 60% of that of naturally occurring Uranium [11].

DU is used primarily in the military industry due to its valuable properties such as high density of 19.07 g/cm^3 comparable to that of Tungsten, pyrophoricity (which Tungsten lacks), and relatively low melting point (1132 °C) [9]. The DoD of the United States uses DU in their tank armor and munition which allows them to penetrate easily, especially steel, igniting on impact [12].

Three armed conflicts in the 1990, Gulf War, Bosnia & Herzegovina, and Kosovo, in which DU ammunition was used, raised concern and interest in researching the potential effects of environmental contamination by DU [9]. Studies based on soil samples collected from Kuwait and Kosovo have been able to identify the presence of DU particles, their isotopic $^{235}U/^{238}U$ ratios, and the size distribution. In the Kosovo case, it was found that more than 50% of the particles were had a diameter < 1.5 µm [13]. Their characteristics vary according to the release scenario and weathering conditions; for example, DU particles released during a fire at an ammunition storage facility contained oxidized U(+6) and crystalline structures containing Uranium, in this case the particles were easily dissolved in 0.15 M HCl, indicating they might be potentially bioavailable. On the other hand, particles originated from ammunition impact or from corrosion of unspent ammunition contained, as expected, Uranium in less oxidizes states, and were dissolved at a much slower rate [14], indicating that speciation plays a major role in any study regarding the behavior of radionuclides in the environment.

The IUPAC defines speciation as the chemical species distribution of an element in a sample [15]. In other words, speciation refers to the different physico-chemical forms in which radionuclides can be present in the environment, such as oxidation state, valence, molecular mass, crystallographic structure, magnetic properties and others [16-18]. Radionuclide species can be categorized according to size: species of size larger than 2 mm are regarded as fragments, while particles are defined as a localized aggregation of radioactive atoms with diameters larger than 0.45 µm (up to 2

mm), that create an inhomogeneous distribution of radionuclides significantly different from the distribution found in the matrix background [17-19]; species within the 0.001 µm- 0.45 µm size range are regarded as colloids and pseudo-colloids.

Low molecular mass radionuclides (LMM), which include cations, hydrated ions, charged or neutral complexes and organometallic compounds with diameters less than 1 nm, or molecular mass less than 1 kDa, are expected to be mobile and bioavailable, because of their potential ability to penetrate biological membranes, while high molecular mass forms (HMM), such as colloids and particles are expected to be inert for biological uptake [16, 17], however the 2011 IAEA report [20] points out that in general little attention is given to the ecosystem transfer and potential biological effects caused by the presence of radioactive particles. An illustration of the different forms and examples of their size distribution (taken from Salbu et al. 2004 [17]) is shown in figure 1.3.

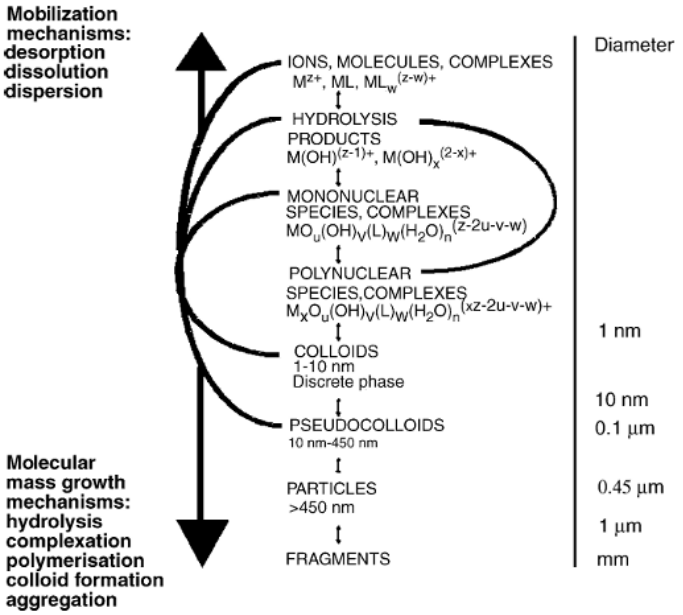

Figure 1.3. Examples of species size distribution and transformation processes which change the distribution of species. SOURCE: Salbu *et al*. 2004 [17].

In aquatic systems uranium can be found as metal ion (U^{4+} or UO_2^{+}), complexes with inorganic ligands, such as uranyl carbonate and uranyl phosphate, or as humic substances, like uranyl fulvate or humate [21].

1.2 Arsenic

Arsenic is a naturally occurring element, classified as 'metalloid' due to its intermediate physical and chemical properties between metals and non-metals. The most common oxidation states are Arsenite (As^{III}), and Arsenate (As^{V}), under reducing and oxygenated conditions; however it exists, in its natural form, in four oxidation states: -3 (arsine), 0 (elemental arsenic), +3, and +5; it can also exist as oxyanions, such as AsO_4^{-3} and AsO_3^{-3} [22]. It is present in the Earth crust at an average concentration of 2 mg/Kg. Volcanic activity is the main natural source of Arsenic, while the main

anthropogenic sources are mining, burning of fossil fuels and smelting of non-ferrous metals [23]. In nature, it is found mainly in its sulfide form, usually associated with complex minerals containing copper, nickel, lead, silver, and iron. It is present in more than 200 mineral species; of them, the most common is arsenopyrite [22, 23]. In well oxygenated water and sediments, most of the arsenic is present as arsenate, which is a thermodynamically stable (pentavalent) state. In some cases, depending on redox potential, Ph, and biological processes, arsenite and arsenate can interchange oxidation state. Its toxicity depends highly on its oxidation state and speciation in general, and inorganic species are considered more toxic than organic species; for example arsenobetaine (AsB) and arsenocholine (AsC) are considered nontoxic, and methylated species are considered only moderately toxic, but arsenate (As^{V}) is 70 times more toxic than methylated species, monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA), and in turn, arsenite (As^{III}) is 60 times more toxic than arsenate [24].

Typically, arsenic is present in relatively low concentrations in natural waters, however, weathering and leaching of arsenic-rich geological formations and mining waste may results in local elevated concentrations. Higher concentrations are found in groundwater, instead of surface waters; this could be due to aerobic oxidation of As and therefore attenuation of oxidic minerals, and also surface recharge and runoff [24]. In the environment, three major modes of biotransformation have been found: 1) redox transformation between arenite and arsenate, 2) reduction and methylation of arsenic, and 3) biosynthesis of organoarsenic compounds. The compounds which result from these processes are subject to biogeochemical cycling [23].

Chemically, arsenic is very similar to phosphorous (its neighbor in the periodic table), therefore, biological uptake of arsenate is similar to that of o-phosphate and they may compete for absorption sites [24]. For animals (and humans), bioavailability is the extent to which a substance can be absorbed by a living organism and reach the systemic circulation. For environmental risk assessments (including sediments and soils), the definition also considers the extent to which a substance dissociates from its environmental medium to become available for absorption [25].

1.3 Gamma Radiation

Soon after the discovery of X-rays, Wilhem Röntgen observed, in 1896, that X-rays were capable of producing skin burns which were difficult to heal [26]; the same year a useful treatment for nevus based on x-rays was proposed by Freund. These were the first observations which gave clues about the potential effects of radiation on matter (particularly on biological matter). Similarly, soon after the discovery of radioactivity, Piere Curie found that radium was also capable of producing hard healing skin wounds and even though radium was linked to skin cancer, it was also shown that it could be used to cure cancer [11]. These first observations probably constitute the birth of the field of Radiobiology, which is the study of the effects of ionizing radiation on biological systems.

Gamma radiation is a common mode of isomeric transition from an upper energy state of a nucleus to a lower energy state by emission of quanta of electromagnetic radiation, called γ-rays, which are

high energy photons [27]. These transitions normally follow other type of decay, such as alpha and beta decay; the isomeric transition requires a metastable state, but in other cases de-excitation may happen very rapidly (less than 10^{-12}s). They can also be produced by other means; γ-decay does not necessarily mean reaching the ground state; it is possible to have transitions between excited states; they can also be obtained by activation of nuclei by electromagnetic and particle bombardment (neutrons for example) [28].

Since photons are uncharged, they are capable of traveling long distances without interacting with matter [29, 30]. There are three relevant modes of interaction between photons and matter:

1) Photoelectric Effect. An incident photon transfer all of its energy E_γ to an orbital electron; as a result, the electron is ejected with kinetic energy

$$K = E_\gamma - E_e \tag{1.4}$$

where E_e is the binding energy of the electron. Photoelectric effect occurs more efficiently at low incident energy E_γ, and it is proportional to the atomic number Z, such that the probability of a photoelectric interaction is proportional to Z^5/ E_γ^3 [29].

2) Compton Scattering. This is the case when the incident photon only transfers a fraction of its energy to an electron, which is ejected while the photon is scattered with energy $E_\gamma' < E_\gamma$. The relationship between the energy of the incident photon, the energy after scattering (in MeV) and the scattering angle can be found from the momentum and energy conservation laws [27, 30]:

$$E_\gamma' = E_\gamma/[1 + (E_\gamma/0.511)(1-\cos\theta)] \tag{1.5}$$

Compton scattering is not as strongly dependent on Z (~Z/ E_γ) as the photoelectric effect, and it is most relevant in the energy range [0.1-1.0 MeV].

3) Pair Production. Photons with energies greater than 1.02 MeV can interact with the strong electromagnetic field in the vicinity of the nucleus via a process that creates a positron-electron pair from the photon's energy. The minimum energy required is at least twice the rest energy of an electron or positron, $m_e c^2$ = 0.511 MeV [31]. Since the positron is an antiparticle, it annihilates very soon after encountering an electron, creating in the process two photons of energy 0.511 MeV.

1.3.1 Chemical Basis of Radiation-induced Damage

A typical mammalian cell can be described roughly as containing ~ 70-85 % water, ~ 10-20 % protein, ~ 10% carbohydrates, and ~ 2-3% lipids [29]. This means that the effect of ionizing radiation on biological material is dominated by its interaction with water. When radiation is absorbed in biological material, it may interact directly with DNA (critical target), or it may interact with other molecules and atoms in the cell, of which most are conformed by water. If radiation interacts with DNA, its own atoms can become ionized or excited, and initiate a chain of events that lead to biological change; this is referred to as *direct action* of ionizing radiation and it is a dominant process for radiation of high linear energy transfer, such as neutrons or alpha particles. Linear energy

transfer (LET) is the amount of energy deposited per unit path length (keV/μm). LET is relevant for determining the biological consequences of radiation exposure; α-particles, neutrons and protons correspond to high LET radiation, and hence they are, potentially, much more damaging than low LET radiation (β^+, β^-, γ-rays, x-rays) [26, 27].

If radiation does not interact with DNA, but with other molecules instead (i.e. mainly water), free radicals will be produced, which are capable of diffusing and reaching the critical target, causing damage; this type of damage is called *indirect action* of radiation.

The early effects of radiation on water include the creation of ionized molecules H_2O^+, excited molecules H_2O^*, and free subexcitation electrons equal in number to the total number of ions produced [29]. Subexcitation electrons refer to electrons with kinetic energies smaller than the smallest electronic excitation energy E_0 of the molecule [32]. These species are produced in the vicinity of a track within ~ 10^{-15} s. Following the creation of these three species, more changes take place; within ~ 10^{-14} s ionized water molecules interact with neighboring, non-ionized molecules to form hydronium ions and hydroxyl radicals. In the case of the excited water molecules, these can get rid of the extra energy in two ways, by losing and electron, becoming ionized, or by molecular dissociation, which is also characterized by the vibrational periods of the water molecule ~ 10^{-14} s These reactions are represented by the following equations [26, 29, 33]:

$$H_2O \xrightarrow{radiation} H_2O^+ + e^- \qquad (1.6.a)$$

$$H_2O \xrightarrow{radiation} H_2O^* \qquad (1.6.b)$$

$$H_2O^+ + H_2O \longrightarrow H_3O^+ + \bullet OH \qquad (1.7)$$

$$H_2O^* \longrightarrow H_2O^+ + e^- \qquad (1.8.a)$$

$$H_2O^* \longrightarrow H + OH \qquad (1.8.b)$$

The subexcitation electrons migrate, depositing their energy as vibrational and rotational excitation of other water molecules, becoming thermalized in ~ 10^{-12} s. The effect of the thermalized electrons on local water molecules electrons is to orient their dipole moments, forming clusters called *hydrated electrons* [29]. These electrons, in the hydrated form, can subsequently interact with water molecules or with hydrogen ions to form atomic hydrogen, and with oxygen to form radicals called *superoxide ions*, O_2^- [33]:

$$e_{aq}^- + O_2 \longrightarrow \bullet O_2 \qquad (1.9)$$

where e_{aq}^- is the electron in the hydrated form (aqueous solution). Hydrated electrons react rapidly with many species having more positive reduction potentials (-2.29 V for e_{aq}^-), and the mode of reaction is generally written as a one-electron transfer process:

$$e_{aq}^- + S^n \longrightarrow S^{n-1} \qquad (1.10)$$

where n is the positive charge on the solute. The dominant kinetic parameter is likely the availability of a vacant orbital of the solute into which the electron can transfer [34].

Of the first species created right after the initial reaction, three are free radicals, i.e. chemical species with unpaired electrons (OH, e_{aq}^-, and H). The reactants migrate due to thermal motion in the vicinity of their creation site, diffusing in water and reacting chemically as individual pairs approach. The main reactions along the track of a charged particle in water during this stage are

$$OH + OH \longrightarrow H_2O_2 \quad (1.11)$$

$$OH + e_{aq}^- \longrightarrow OH^- \quad (1.12)$$

$$OH + H \longrightarrow H_2O \quad (1.13)$$

$$H_3O^+ + e_{aq}^- \longrightarrow H + H_2O \quad (1.14)$$

$$e_{aq}^- + e_{aq}^- + 2H_2O \longrightarrow H_2 + 2OH^- \quad (1.15)$$

$$e_{aq}^- + H + H_2O \longrightarrow H_2 + OH^- \quad (1.16)$$

$$H + H \longrightarrow H_2 \quad (1.17)$$

All of these reactions, except (1.14), remove chemically active species, since none of the products on the right-hand side consumes additional reactants (except H). These reactions continue until the remaining reactants diffuse far from each other and the probability for reaction becomes very small. The time scale of this stage is ~ 10^{-6} s.

1.4 Caenorhabditis Elegans

Caenorhabditis Elegans is a ubiquitous free living nematode. Typical adults measure approximately 1 mm long and have short life cycles, reaching adulthood in 3 days [35]. It is a relatively simple organism, of which, all of the ~ 1000 cells, including neurons and networks, have been completely mapped. It has become an important biological model for research in subjects such as gene regulation, ageing, and apoptosis, among many others [36, 37]. Figure 1.4 illustrates the anatomy of this nematode.

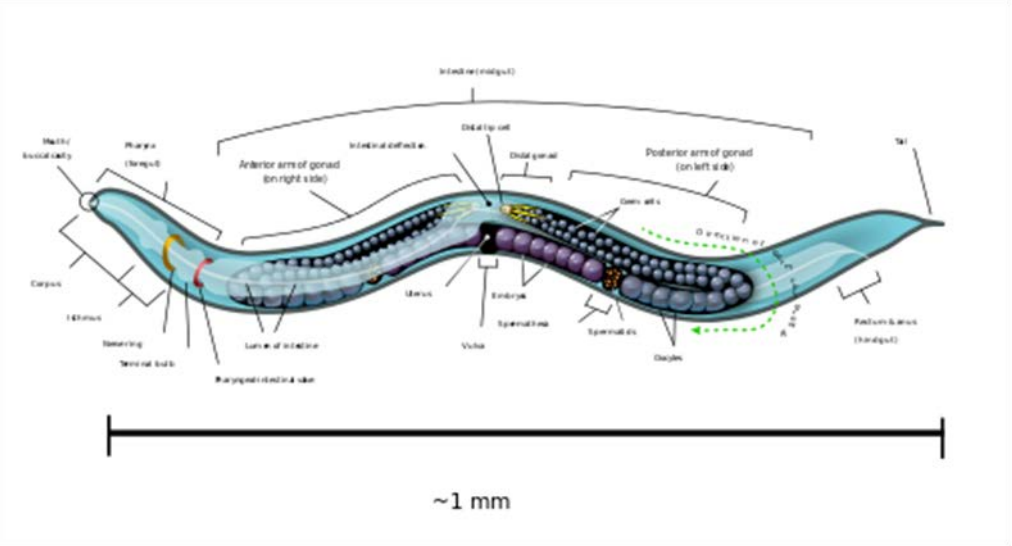

Figure 1.4. Anatomical diagram of an adult hermaphrodite *C. elegans*. SOURCE: K.D. Schroeder ©CC BY-SA 3.0.

Wild-type *C. elegans* has two sexes, there are females and males; however, most of the individuals are female hermaphrodites. Self-fertility originates in the third larval stage (L3), and it is achieved by a period of spermatogenesis, early in the development, followed by a period of oogenesis [38, 39].

The nematodes develop through four larval stages, referred to as L1, L2, L3, and L4. There is also an alternative larval stage denominated dauer state, which arises under stressed conditions. The L1 lasts approximately 16 hours, and the other stages last approximately 12 hours [35]. During the transition from one stage to the next, a period of inactivity named lethargus takes place, which resembles a sleep-like state. During lethargus, a new external collagenous layer (cuticle) is created, and this period finishes with the molting of the old cuticle. Adult hermaphrodites begin reproducing approximately 12 hours after the L4 molt [35]. Figure 1.5 describes graphically the larval stages

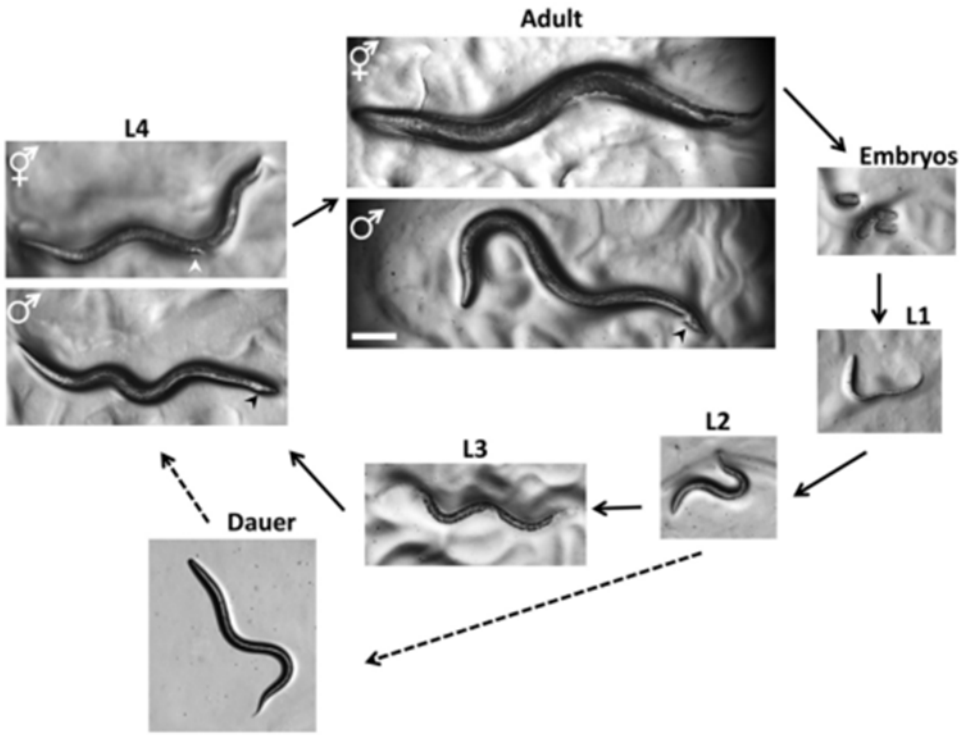

Figure 1.5. The four larval stages of *C. elegans.* Besides the stages L1-L4, under stress condition, dauer larvae might develop. SOURCE: [35].

One of the fields in which *C. elegans* plays an outstanding role as a biological model is environmental toxicology. One of the many reasons for its success as a model, aside its relative simplicity, is that many processes observed in higher organisms are conserved in *C. elegans* [40]. In particular, it has been used to investigate the toxicity and mechanism of toxicity of several metals, heavy metals and metalloids, such as Aluminum, Arsenic, Cadmium, Lead, Mercury and Uranium, among others [41-44].

The purpose of this study is to assess the effects of arsenic, uranium, gamma radiation and their combinations, using *C. elegans* as a model. The combination chosen is relevant, since both, uranium and arsenic are ubiquitous, and can be found together (particularly in groundwater) [45, 46]. Moreover, both (individually) are a reason of concern, regarding the environment and potential health effects. Arsenic is a known carcinogenic [47] and numerous studies show that depleted uranium is chemotoxic [48, 49].

The effects of each individual stressors have been studied previously, using this nematode. In the case of uranium, studies have found that in general it causes toxicity in a dose-dependent fashion; Jiang *et al.* report that metallothioneins have a protective effect [50]. Goussen *et al.* performed a multi-generational study (16 generations), of populations exposed to different uranium concentrations; they reported as well an increase of adverse effects such as reduced growth and fertility, as a function of uranium concentrations [51].

Regarding the effects of arsenic on *C. elegans*, Sahu *et al*. [52] tested two concentrations: 0.03% and 0.003% w/v. Positive dose-response correlation was found with stronger global gene expression

changes at the highest dose as compared to the effects at the lowest dose. Liao & Yu [53] found that pretreatment of nematodes with GSH, when exposed to arsenic, increased their survival rates, as compared to the nematodes exposed without pretreatment.

2. Materials and Methods

The model chosen to carry out this study was the nematode *Caenorhabditis Elegans*. The strain used was wild type N2 obtained from Caenorhabditis Genetic Center (Minneapolis, MN). Two methods for maintaining the nematode population were used; the first method requires preparing petri dishes with Nematode Growth Medium (NGM) agar, the recipe of which is described in detail together with other recipes in Appendix A. The agar is seeded with *Escherichia coli* OP50 (obtained from the same center), which is the food source of the nematodes, and then a 1 mm x 1mm chunk of agar from a healthy population is transferred to a fresh plate, under sterile conditions; the plates are kept in the dark at 21 °C. After approximately 3-4 days, under the right conditions, the plates should have plenty of gravid nematodes, which can be used to extract eggs, or to start a new culture [54].

The second method uses a liquid growth medium [Appendix A], and it is the preferred method when large amounts of gravid nematodes need to be used, therefore this was the default method used here to prepare the experiments. 100 mL of liquid medium is prepared with E. coli in an Erlenmeyer flask; 5 mL of nematode-rich medium from an existing culture is transferred to start a new culture and it is left shaking in the incubator, in the dark at 21 °C.

2.1 Nematode Synchronization Protocol

The exposure experiment requires synchronized nematodes at the L1 stage. This is achieved by extracting eggs from gravid adult nematodes and allowing them to hatch in the absence of food such that the larvae are halted at the L1 stage [55]. The following steps must be followed in order to obtain synchronized L1 nematodes:

1) The culture is allowed to grow for 80-96 hours (3-4 days) to guarantee enough gravid adults, and not much longer than that to prevent a stressed culture, with abundance of dauer nematodes [54].

2) 20 mL of the liquid culture is transferred to a centrifuge glass tube; a pellet containing nematodes is obtained by centrifuging at 3000 g for 2 minutes. It is important to use glass and not plastic at this stage, due to the tendency of the nematodes and eggs to adhere to plastic. A normal exposure experiment needs two such pellets; larger experiments may need more pellets.

3) After centrifuging, the supernatant is carefully removed, and 15 mL of bleaching solution is added to each pellet [Appendix A] and vortexed at 1800 rpm for 6 minutes, after which the nematodes should have dissolved leaving intact the eggs. Before centrifuging to obtain a pellet containing the eggs, the solution must be checked in the microscope to verify the bleaching efficiency.

4) The tubes are centrifuged at 3000 g for two minutes. A very faint pellet containing the eggs should be visible at the bottom. The supernatant must be carefully removed, to avoid disturbing the pellet and the risk of losing eggs. The pellets are washed 3 times with M9 [Appendix A] centrifuging at 3000g for two minutes every time, to remove all traces of bleaching solution that might damage the eggs

5) The pellets are resuspended in 1 mL of M9 each and transferred to 30 mm NMG plates using glass Pasteur pipettes. Approximately 30 droplets are transferred to each NMG plate and they are incubated for 18-24 hours at 21 °C shaking gently.

2.2 Exposure Protocols

Several experiments described in the next sections were carried out. The nematodes were exposed to different concentrations of depleted uranium, sodium arsenite, sodium arsenite and depleted uranium mixtures, gamma radiation, sodium arsenite and depleted uranium mixtures in the presence of gamma radiation, and control experiments for each case. All of the exposures lasted 96 hours and were prepared in 24-well microtiter plates using Simulated Soil Pore Water (SSPW), following a recipe prescribed Tyne *et al*., 2013 [56] to simulate soil solution conditions.

2.2.1 Simulated Soil Pore Water (SSPW)

SSPW was prepared with the recipe used by Tyne *et al*. 2013 [56], described in Appendix A. Immediately after preparing, the solution is allowed to shake with the lid open for 2-3 days, to allow CO_2 equilibrium. The solution has the tendency to become alkaline; therefore aliquots of nitric acid were used to adjust the pH, for the purpose of this work, chosen at ~ 6.6.

The equipment used for pH adjustment was a pH electrode SenTix® 81 and Hamilton Duracal calibration buffer solutions of pH 4.01 and 7.00.

2.2.2 Exposure to Depleted Uranium

$UO_2(NO_3)_2 \bullet 6H_2O$ solutions in SSPW were prepared at different molar concentrations. The Uranium exposure wells are prepared such that a total volume of 0.5 mL contains:

-0.25 mL of Uranium solution at molar concentrations of 200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM, or 6.25 µM, depending on the final exposure concentration wanted (100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, or 3.1 µM)

-0.2 mL SSPW

-0.025 mL of E. coli pellet resuspended to 2 mL volume solution with M9. The instructions to prepare a pellet of E. coli are given in appendix A.

-0.025 mL of nematode larvae suspended in SSPW and diluted to 10 larvae per droplet.

24 wells were prepared per experiment, with triplicates of each of the 6 concentrations, referred to as U1, U2, U3, U4, U5, and U6, from highest to lowest concentration. Also, control wells were prepared, replacing the 0.25 mL volume of Uranium solution with SSPW. Figure 2.1 illustrates the exposure wells.

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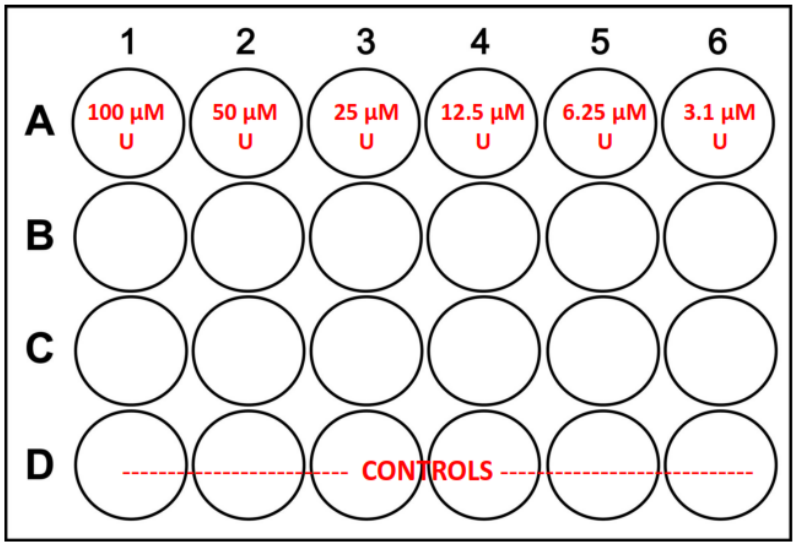

Figure 2.1 Illustration of a 24-well microtiter plate prepared with depleted uranium at 6 different concentrations. Source: R. Contreras.

2.2.3 Exposure to Sodium Arsenite

$NaAsO_2$ and SSPW solutions at 6 different molar concentrations were prepared. The final concentrations used for the experiment were 1 mM (As1), 0.5 mM (As2), 0.25 mM (As3), 0.125 mM (As4), 0.63 mM (As5), and 0.315 mM (As6). The wells were prepared to volume 0.5 mL exactly the same way as the wells exposed to Depleted Uranium, replacing the uranium solutions for Arsenic solutions of the right concentration, as shown in figure 2.2.

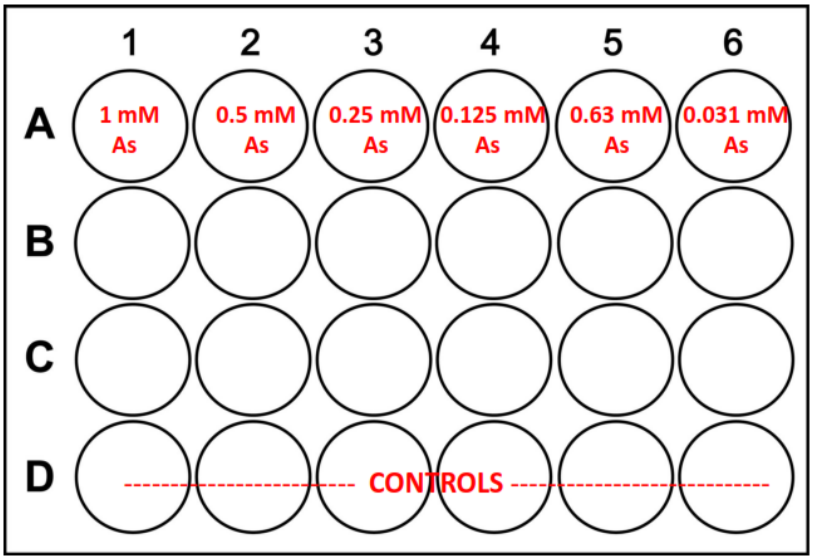

Figure 2.2 Illustration of a 24-well microtiter plate prepared with sodium arsenite at 6 different concentrations. Source: R. Contreras

2.2.4 Exposure to Depleted Uranium and Sodium Arsenite Mixtures

The exposure wells were prepared in a similar way as the exposures to single contaminants; however, intermediate molar concentrations were used. A total of 16 mixtures were prepared per experiment (three replicate experiments were performed for the mixed toxicity). The following combinations were used:

1) As2 + U2 (0.5 mM As + 50 µM U)
2) As2 + U3 (0.5 mM As + 25 µM U)
3) As2 + U4 (0.5 mM As + 12.5 µM U)
4) As2 + U5 (0.5 mM As + 6.25 µM U)
5) As3 + U2 (0.25 mM As + 50 µM U)
6) As3 + U3 (0.25 mM As + 25 µM U)
7) As3 + U4 (0.25 mM As + 12.5 µM U)
8) As3 + U5 (0.25 mM As + 6.25 µM U)
9) As4 + U2 (0.125 mM As + 50 µM U)
10) As4 + U3 (0.125 mM As + 25 µM U)
11) As4 + U4 (0.125 mM As + 12.5 µM U)
12) As4 + U5 (0.125 mM As + 6.25 µM U)
13) As5 + U2 (0.063 mM As + 50 µM U)
14) As5 + U3 (0.063 mM As + 25 µM U)
15) As5 + U4 (0.063 mM As + 12.5 µM U)
16) As5 + U5 (0.063 mM As + 6.25 µM U)

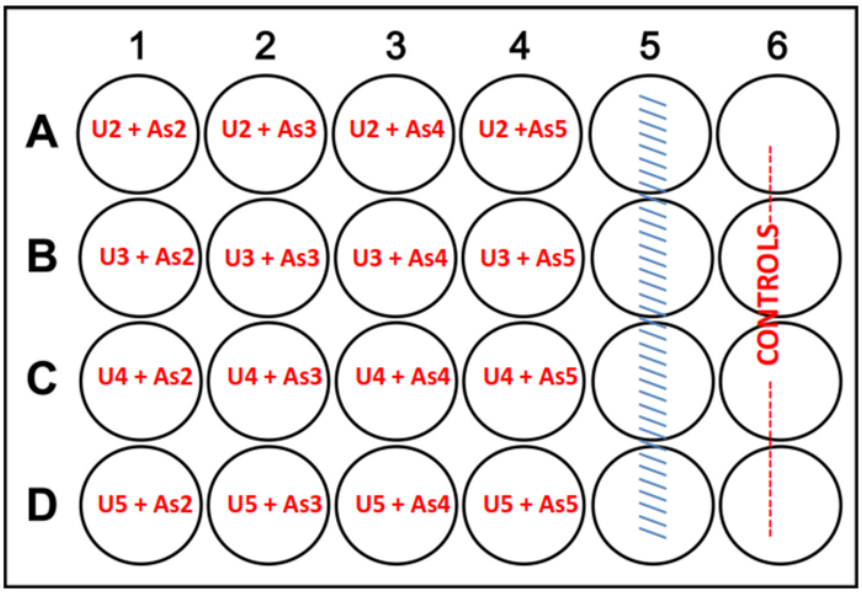

Figure 2.3 Illustration of a 24-well microtiter plate prepared with 16 mixtures of depleted uranium and sodium arsenite at different concentrations. Column 5, marked with blue lines, was left empty and column 6 was used for controls. Source: R. Contreras.

2.2.5 Exposure to Gamma radiation

The nematodes were exposed at the Figaro facility of NMBU, with the ^{60}Co gamma source, capable of providing a continuous dose rate from 2.5 Gy/h to 300 µGy/h [57]. An image of the FIGARO source is shown in figure 2.4

Figure 2.4. FIGARO ^{60}Co gamma irradiation source at NMBU. Source: CERAD annual report 2014 [57].

The nematodes were exposed during 96 hours to target dose rates of 1, 40, 100, and 200 mGy/h. Control experiments were done simultaneously as well. However, the samples irradiated at 40 mG/h were destroyed accidentally during transportation from the source to the laboratory. The samples irradiated at 1 mGy/h remain preserved in the cold room of the Isotope laboratory at NMBU. Complete set of data are available for 0 (control), 100 mGy/h, and 200 mGy/h exposures. The wells were prepared the same way the control wells for the previous exposures were prepared, with SSPW only, besides *E. coli* and nematode larvae.

The actual dose was obtained from a calibration report provided by the Norwegian Radiation Protection Authority (NRPA) [58]. The corrected dose comes from air kerma measurements with optically stimulated luminescent dosimeters (nanoDOT OSLD) placed in the positions of exposure plates. The reference measurements can be read from table 2.1.

Table 2.1. Corrected dose measurements with calibration at the FIGARO facility. The third column corresponds to the total dose during the 96-hour experiment. Source: NRPA.

Target Dose Rate (mGy/h)	Corrected Dose Rate (mGy/h)	Corrected Total Dose (mGy)
1	1.039	99.744
40	39.772	3818.112
100	100.127	9612.192
200	200.235	19222.56

2.2.6 Exposure to Gamma- Irradiated Samples of Depleted Uranium and Arsenite Mixtures

This experiment was designed identically to the mixed exposure experiment described in section 2.2.4, and those plates were exposed to the same doses described previously in section 2.2.5. Since they are independent experiments, control samples were prepared for this case as well. One control sample consists of no exposure at all, and a second control consists of mixtures only.

2.3 Endpoints

Three biological endpoints were studied: survival, growth, and reproduction. Survival was estimated by counting the larvae at the moment of exposure (t = 0), and counting the adults after 96 hours of exposure, using the HD Leica microscope camera MC170 HD, available at the Isotope Laboratory of NMBU. A prototype of such microscope is shown in figure 2.5.

Figure 2.5. A prototype of the HD Leica microscope camera MC170 HD used during this project. SOURCE: http://www.leica-microsystems.com/products/microscope-cameras.

Growth was measured with the Leica Application Suite (LAS) system after stopping the exposure (section 2.4). Reproduction was measured by counting the offspring after the exposure; this is an extremely time consuming procedure which is prone to errors, therefore in order to minimize the sources of error, each well was marked and divided into 8 sections, and each section was counted twice to average the number counted. The border between sections can be large as compared to the size of the offspring; hence, they were counted over these lines as well (twice and averaged). It is possible to count only a few sections and estimate the total amount of offspring assuming they are evenly distributed, but they are usually distributed in clusters of different sizes, therefore it was chosen to count each section. Even though this method seems inaccurate, in all the cases the difference was estimated in less than 3 offspring per adult nematode.

2.4 Samples Preservation after the 96-hour Exposures

The nematodes were stained with Rose Bengal and then they were killed by heat, at 60°C for 30 minutes; after cooling at room temperature, they can be stored at 4 °C for approximately 2 months. During the heating processes sometimes worms 'break' (figure 2.6) and their length cannot be measured properly, therefore those worms are not included in the data analysis.

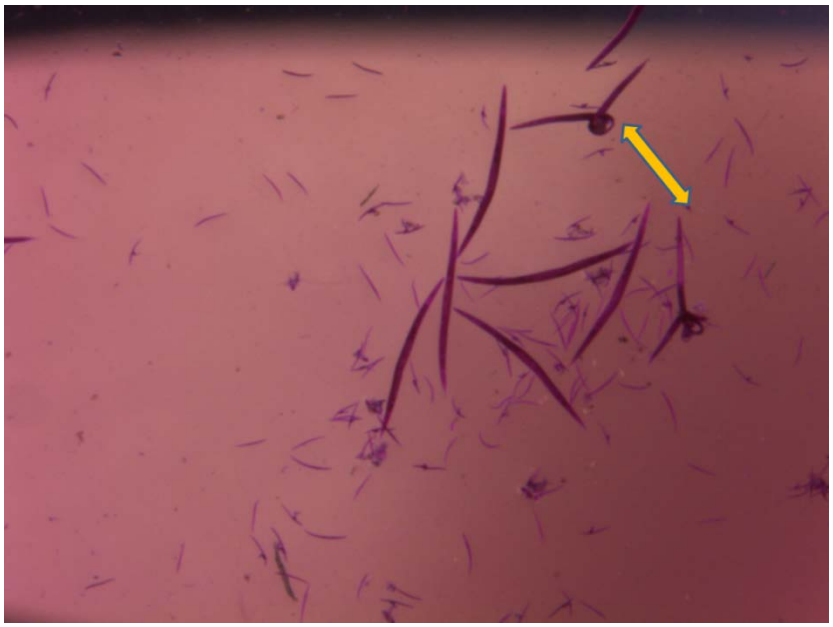

Figure 2.6. Stained nematodes after killing by heat. The typical adult measures approximately 1.2 mm. The yellow arrow points to two nematodes which suffered a noticeable body structure modification after the heat treatment, and therefore were not considered in the data analysis. SOURCE: R. Contreras.

2.5 Speciation Analysis

A speciation analysis was performed to determine the distribution of species in the wells. Since the number of combinations is enormous, and triplicates are required, a limited number of cases were chosen for the analysis: U1, U3, AND U6 for the samples containing depleted uranium only; As1, As3, and As6 for the samples containing sodium arsenite only; and U2+As2, U4+As2, and U5+As5 were chosen for the samples containing the mixtures. Three whole 24-well plates were used for each case. The following protocol was used: 0.5 mL wells were prepared, as described in sections 2.2.2, 2.2.3, and 2.2.4.

0.1 mL was extracted from the wells to account for the whole sample. Acrodisc® Syringe Filters with GHP filter (figure 2.7) were used to account for the <0.45 fraction; 0.2 mL of filtrate was extracted.

The volumes of total and filtrate samples were stored in 15 mL Nunc tubes and diluted with ultra-pure HNO_3 to a final volume of 5 mL, and placed in a heat sand bath at 90° C ,as preparation to be

analyzed with ICP-MS. The total number of samples analyzed with ICP-MS was 114, including triplicates of the total and filtrate at t = 0, and t = 96 h, blank triplicates and SSPW triplicates.

Figure 2.7. Acrodisc syringe filters. SOURCE: http://www.pall.com

2.6 Statistical Analysis

Standard statistical analyses were performed. The choice of test varies according to the type of data. For single contaminants, one-way ANOVA and Tukey's multiple comparison test (post-hoc) was performed in the cases when the p value was significant (statistical significance chosen at 99% confidence). In the case of mixtures, two-way ANOVA was used; when the data is not balanced (different number of subjects per case), unbalanced two-way ANOVA is needed, combined with Tukey's multiple comparison test, when $p < 0.01$. The calculations were performed with Minitab 15, GraphPad Prism ©, scripts written in R and Scilab, and in the case of unbalanced two-way ANOVA, the online tool provided by http://vassarstats.net/ (website for statistical computation) was used, because such test is not available from Minitab nor Graphpad Prism.

3. RESULTS

3.1 Exposure to Depleted Uranium

L1 stage larvae were exposed to 6 different doses of depleted uranium. $UO_2(NO_3)_2 \bullet 6H_2O$ was added in wells containing artificial soil solution and *E. coli.* The molar concentrations used to study the effect of uranium on survival, growth, and reproduction of *C elegans* were 100, 50, 25, 12.5, 6.25, and 3.125 µM.

The experiment was planned to be carried out at a target pH of ~ 6.7 by adjusting the pH of the solution containing uranium and SSPW already mixed, to avoid precipitation of uranium. A control experiment to measure pH during the exposure was used; the values are shown in table 3.1 and figure 3.1.

Table 3.1. pH values of the exposure wells as a function of time, during the experiment. The control well is prepared with SSPW only (no uranium); U6, U5, U4, U3, U2, and U1 refer to the molar concentrations of uranium in the wells: 3.125, 6.25, 12.5, 25, 50, and 100 µM.

t (hours)	0	24	48	72	96
pH Control	6.82	6.86	6.90	6.97	7.05
pH U6	6.96	7.04	7.05	7.06	7.09
pH U5	6.97	6.99	7.01	7.03	7.09
pH U4	6.96	6.95	6.95	6.91	7.09
pH U3	6.94	6.97	6.97	6.98	7.18
pH U2	6.85	6.95	6.96	6.97	7.10
pH U1	6.80	6.84	6.88	6.91	7.03

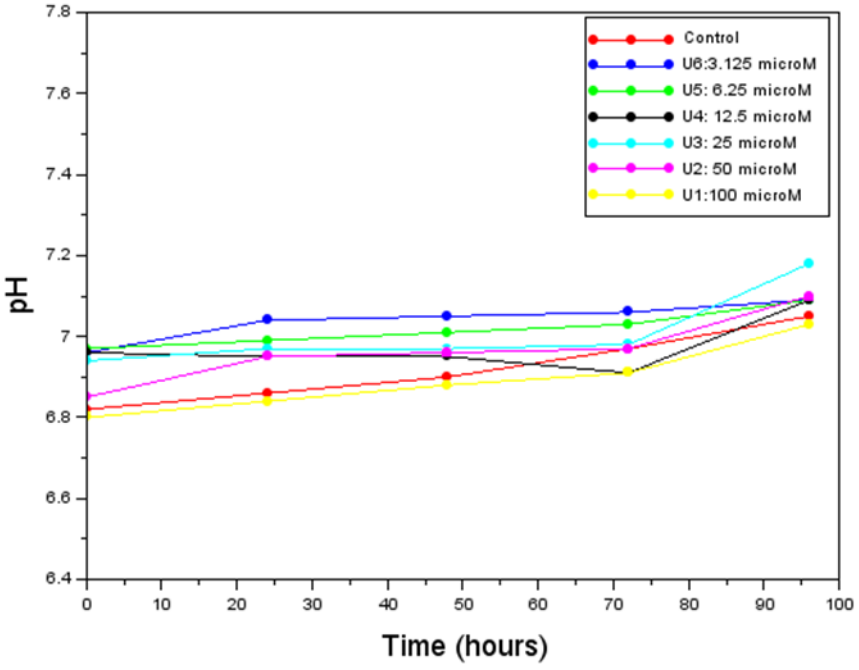

Figure 3.1. pH values as a function of time. In most cases, the values increase steadily as the experiment progresses.

The pH increases during the experiment, but it remains below 7.1; one of the reasons for this increase is the physiological pH of *E. coli*, with cytoplasmic pH in the range 7.2-7.8 [1, 2]. A short experiment to measure the evolution of the pH of the *E. coli* pellet alone was carried out. Table 3.2 shows the values measured during the 4-day experiment.

Table 3.2. pH values of the E. coli pellet diluted in SSPW, to volume 2 mL.

pH	7.24	7.25	7.28	7.42	7.31
time (hours)	0	24	48	72	96

The following sections will present the results found when investigating the effects of depleted uranium on different end points.

3.1.1 Survival

Survival was estimated by counting the larvae in the beginning of the exposure and by counting the adult worms at the end of the exposure. This method is inaccurate because the nematodes must be observed and counted under the microscope, but at this stage they are very small and often they remain still; hence, it is easy to underestimate their number. Nonetheless, they were counted and it was found, in approximately 10% of the wells observed, the number of adults was exactly the same as the number of larvae counted. In all the other cases the number of survivors was larger than the original number counted, suggesting no mortality. However since the method is not accurate, it can only be stated that no mortality was observed with the method used.

3.1.2 Growth

Growth was determined by measuring the length of the nematodes after the end of the exposure. The nematodes are killed by heat and stained as described in Chapter 2. Table 3.3 shows the results from three independent measurements, including average length, the median length and standard deviation.

Table 3.3. Adults' lengths measured with Leica Application Suite

LENGTHS (mm)	**Control**	**U6**	**U5**	**U4**	**U3**	**U2**	**U1**
mean	1.3551	1.3125	1.2452	1.2348	1.3037	1.3291	1.3061
median	1.379	1.339	1.251	1.237	1.298	1.3395	1.303
standard dev	0.0597	0.0808	0.0844	0.0687	0.0669	0.0908	0.0949

One-way ANOVA analysis was performed, using GraphPad Prism©, to compare the results at the different concentrations of uranium, and Tukey's multiple comparison test (post-hoc) was performed when the p value was significant (statistical significance chosen at 99% confidence, $p < 0.01$).

The ANOVA test performed determined that the differences in the mean values between the different uranium exposures are significant ($p < 0.0001$, $F = 14.20$). Tukey's multiple comparison tests gave the following results. The only two groups significantly different from the control measurements, with $p < 0.0001$, were U5 and U4 (12.5 µM and 6.25 µM). On the other hand, the

difference between U6 and U5 gives $p < 0.05$ (we use here $p < 0.01$ to be considered significant), U6 vs U4, $p < 0.001$, and no significant difference between U6 and the rest of the groups. The comparisons U5 vs U4 and U5 vs U3 are not significant, while U5 vs U2 has $p < 0.01$ ad U5 vs U1 has $p < 0.05$. The lengths measured at 12.5 µM (U4), are the ones which show the most significant difference with the rest (except with U5), with U4 vs U3 $p < 0.01$, U4 vs U2 and U4 vs U1 $p < 0.001$. The comparisons U3 vs U2, U3 vs U1, and U2 vs U1 are not significant, according to this test.

These results can be visualized in figure 3.2, which shows the mean values of the lengths with their standard deviations (error bars).

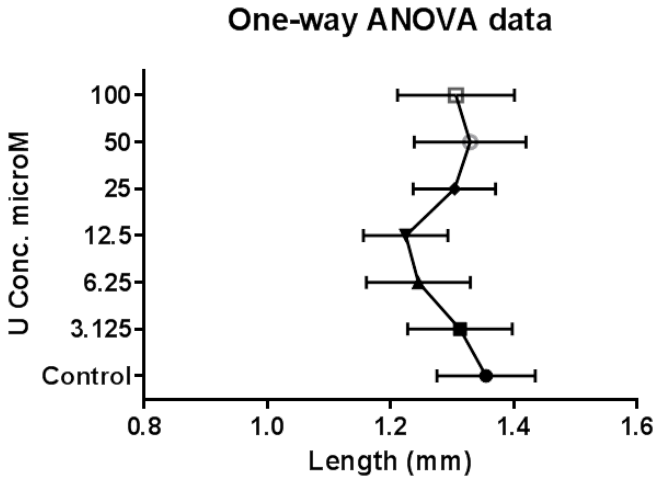

Figure 3.2. Mean values with the corresponding standard deviation. The maximum effect on growth seems to occur at a concentration of 12.5 µM (U4).

The results of this test suggest that intermediate concentration values, such as 12.5 µM have a larger effect on growth than the other doses. It is worth noting that all the data sets passed the normality test; also, the median follows a similar trend as the mean values. The median is shown in table 3.3, because this statistic is less sensitive to outliers than the mean, but here, it is possible to observe that both statistics follow a similar trend.

3.1.3 Reproduction

After staining the nematodes and exposing them to heat, the offspring were counted under the microscope. Table 3.4 summarizes the results, showing them as number of offspring per adult nematode.

Table 3.4. Number of offspring per adult nematode, as a function of uranium concentrations.

Molar Concentration (mM)	Control	0.0031	0.0063	0.0125	0.025	0.05	0.1
mean	34.38	50.25	43.10	45.13	41.67	42.13	30.75
median	32.50	50.00	43.00	44.75	38.00	41.75	32.25
standard deviation	6.42	4.09	3.78	3.07	6.79	6.69	12.16

Figure 3.3 represents the mean values of offspring per adult nematode, normalized by the control values. The error bars correspond to the standard deviation.

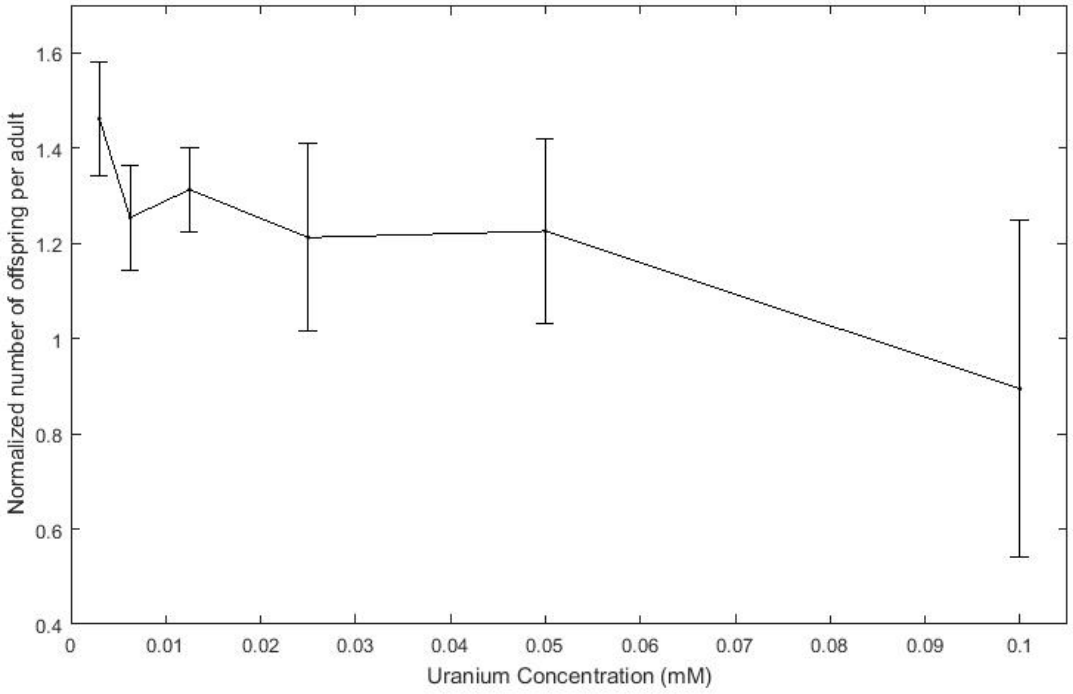

Figure 3.3. Number of offspring per adult nematode; the error bars represent the standard deviation. The numbers have been normalized by the control mean value.

Figure 3.3 shows a mild trend of decreasing number of offspring as the uranium concentration increases; however, the standard deviation is particularly large in the case of U1, the highest concentration. Also it is possible to observe, from table 3.4 that the average number of offspring is smaller for the control case than for the exposure cases, except for the highest concentration case; this is reflected by values larger than 1.0 on the vertical axis representing the normalized value (relative to the control case).

One-way ANOVA test was performed; $p = 0.009$, $F = 3.897$. But Tuckey's multiple comparisons test only shows a significant difference between the lowest concentration (U6, 3.125 µM), and the highest concentration (U1, 100 µM) with $p < 0.01$. Comparison between control and U6 results in $p < 0.05$, which in some cases is considered significant, but here it has been chosen to prefer $p < 0.01$ to be considered significant.

3.2 Exposure to Sodium Arsenite

Six different concentrations of $NaAsO_2$ were added in the wells containing SSPW, *E. coli*, and the nematodes (1 mM, 0.5 mM, 0.25 mM, 0.125 mM, 0.063 mM, and 0.0315 mM). The same end points as in the previous case were studied (survival, growth and reproduction); the results are presented in the next following sections.

$NaAsO_2$ is a strong base; the pH of the SSPW and $NaAsO_2$ mixture had to be lowered with nitric acid. Table 3.5 summarizes the pH controlled during the arsenic exposure, shown in figure 3.4.

Table 3.5. pH evolution during the exposure.

t (hours)	0	24	48	72	96
pH Control	7.10	7.18	7.19	7.40	7.38
pH As6	7.40	7.48	7.52	7.56	7.63
pH As5	7.28	7.35	7.42	7.43	7.53
pH As4	7.23	7.25	7.37	7.46	7.57
pH As3	7.21	7.28	7.36	7.37	7.40
pH As2	7.26	7.29	7.30	7.37	7.42
pH As1	7.28	7.29	7.30	7.33	7.34

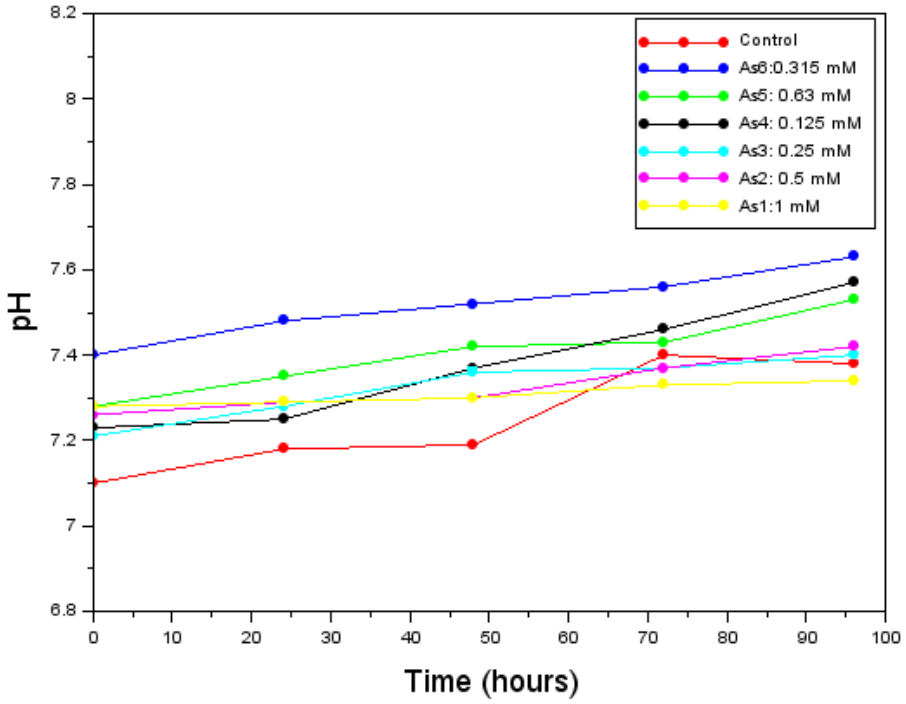

Figure 3.4. pH evolution towards higher values during the exposure to sodium arsenite.

3.2.1 Survival

L1 nematodes were counted in the beginning of the experiment, right after introducing them in the plate wells. After 96 hours, when the survivors have reached already adulthood, and after staining and killing them, they are counted again. As explained in section 3.1.1, the counting method is quite rudimentary, and it is only useful to provide a broad idea about survival.

In the case of Arsenite, however, an interesting phenomenon takes place. It was observed an apparent all-or-nothing mode of survival, in which at the highest dose of 1 mM, either all the nematodes survive (with adverse effects as described in the next sections), or they all die, almost immediately after starting the exposure. In 8 independent exposure experiments, 3 had survivors (in all the wells, in all the plates), while 5 of those experiments reported 100% mortality. For lower doses (0.5 mM and below), no mortality effects were observed; indeed, in most of the cases, the number of adult nematodes counted was higher than the number of offspring counted in the

beginning of the experiment (reflecting the inaccuracy of this method). In some cases, the numbers coincide; if there is a source of systematic error introduced by counting this way, then it would be expected that approximately the same surplus of adult nematodes are counted in all the plates, but in some cases the numbers coincide; this might indicate (very indirectly) that there is indeed mortality, but to prove such hypothesis, much larger samples and dedicated statistical analysis would be needed, the former being beyond the scope of this work.

3.2.2 Growth

As it was mentioned in the previous section, sodium arsenite has a noticeable effect at the highest concentration tested (1mM). Of the nematodes which survived at 1mM, all were apparently in an arrested state of development, as shown in figure 3.5. The lengths measured are summarized in table 3.6, including the retarded nematodes of the wells at highest concentration.

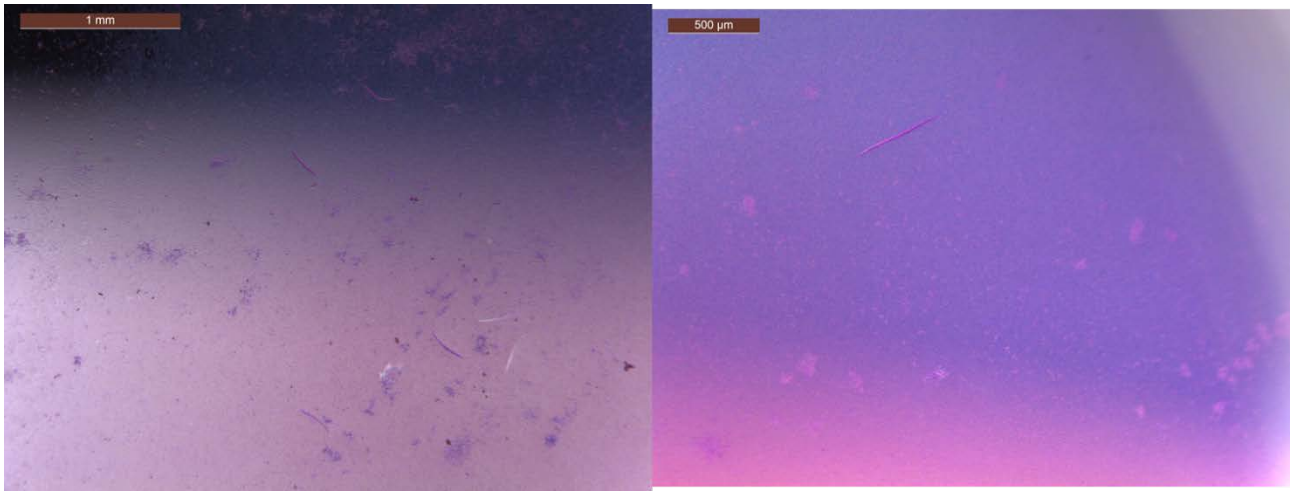

Figure 3.5. Survivor nematodes at the highest sodium arsenite concentration tested (0.1 mM). On the left, several specimens are visible, the scale bar is 1 mm (their size is a fraction of a millimeter). On the right, a single specimen is visible, the scale bar is 500 µm, which is the approximate length of this nematode. SOURCE: R. Contreras.

Table 3.6. Adult nematodes' lengths. The numbers shown for As1 correspond to survivors which show arrested development. The lengths were measured with the LEICA Application Suite.

LENGTHS (mm)	Control	As6	As5	As4	As3	As2	As1
mean	1.2775	1.3131	1.2729	1.2242	1.1418	1.2035	0.3363333
median	1.314	1.317	1.28	1.239	1.144	1.2075	0.2625
standard dev	0.0782	0.0528	0.0775	0.0943	0.0848	0.0586	0.14702

One-way ANOVA analysis was performed, using GraphPad Prism ©, and Tukey's multiple comparison test (post-hoc) was performed when the p value was significant (statistical significance chosen at 99% confidence). The lengths of the nematodes which survived at 1 mM (As1) were not included in this analysis, due to the obvious differences with the rest of the groups; the purpose is to identify subtle differences in the mean values.

The test, considering all the values of the control groups, and the exposed groups, As6 through As2, revealed a statistically significant difference between them ($p < 0.0001$, $F = 15.71$). Post-hoc Tukey's multiple comparisons test, revealed which groups were statistically different (comparing pairs each time). The difference between the control group and As3 is extremely significant ($p < 0.0001$), while there is no significant difference between control and As6, As5, and As4; $p < 0.05$ for the comparisons control vs As2 (i.e not significant for the level chosen here). The rest of the comparisons have the following p values: As6 vs As5 (not significant), $p<0.0001$ for all the other comparisons with As6 (As4, As3, and As2); $p<0.05$ (not significant) for As5 vs As4, $p<0.001$ for As5 vs As3, and $p<0.01$ (significant) for As5 vs As2; $p <0.01$ for As4 vs As3, and not significant for As4 vs As2 and As3 vs As2.

Figure 3.6 shows the mean values and error bars. It is easy to visualize that the As3 group (0.25 mM) is particularly different from the rest of the groups.

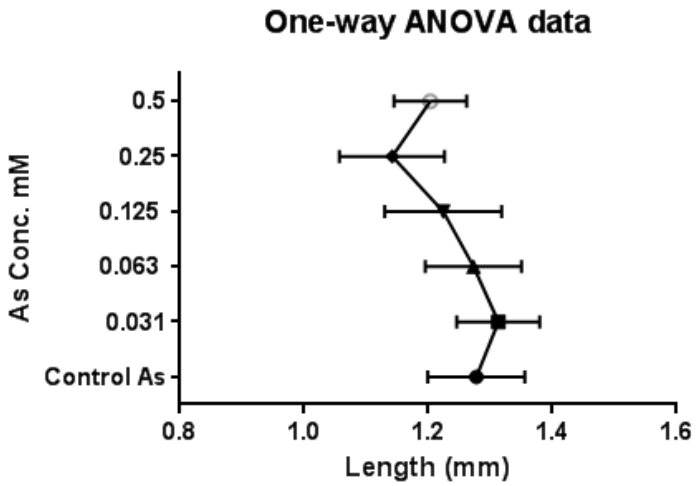

Figure 3.6. Mean values with the corresponding standard deviation (error bars). The maximum effect on growth occurred at a concentration of 0.25 mM, if the highest concentration of 1 mM is not taken in consideration.

The results support the hypothesis that arsenic has a detrimental effect on growth. The average length at 0.5 mM, appears slightly bigger than that at 0.25 mM, but the difference between those two groups is not statistically significant. If we observe the median (table 1) the trend is very clear; it was mention before that the median is a robust measure because it is not too sensitive to outliers, but it is good to have consistent results after the Tukey's multiple comparison test.

3.2.3 Reproduction

To count the offspring, the same procedure used in the case of depleted uranium was used. Nematodes were stained and killed by heat. The results are summarized in table 3.7.

Table 3.7. Number of offspring per adult nematode, as a function of arsenic concentration.

Molar Concentration (mM)	Control	0.031	0.063	0.125	0.25	0.50	1.0
mean	33.25	28.50	35.50	29.60	27.80	7.00	0.00
median	34.25	28.25	35.50	30.00	27.00	6.50	0.00
standard deviation	4.05	5.52	3.29	3.34	6.72	2.29	0.00

The statistical analysis used was one-way ANOVA ($p < 0.0001$, $F = 15.52$). The significant differences were given by Control VS As2 ($p < 0.0001$), As6 vs As2 ($p < 0.0001$), As5 vs As2 ($p < 0.0001$), i,.e As2 has the largest effect (aside As1) on reproduction. The other comparisons did not show any significant difference in the statistical test.

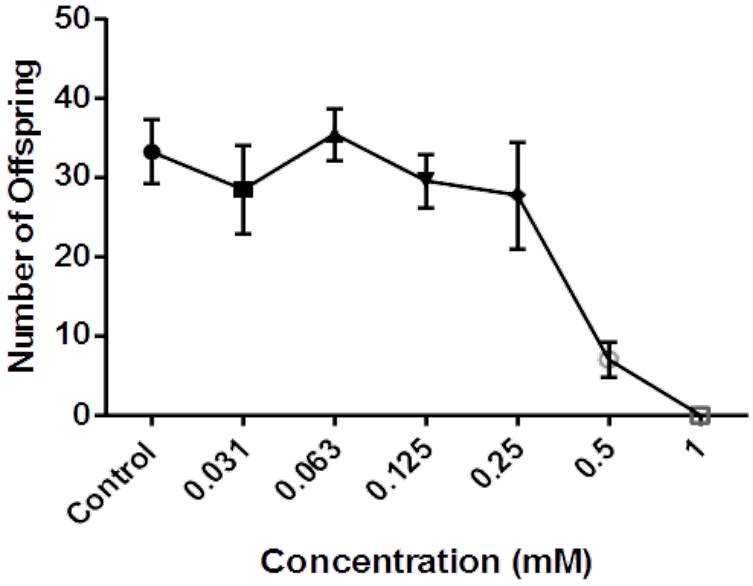

Figure 3.7. Number of offspring per adult nematode. There is a clear tendency to reduce the number of offspring at higher concentrations. This is particularly clear at 0.5 mM (only 7 offspring per adult nematode) and 1 mM (no reproduction).

Figure 3.8 is similar to 3.7, but it is normalized by the number of offspring of the control experiments (mean value).

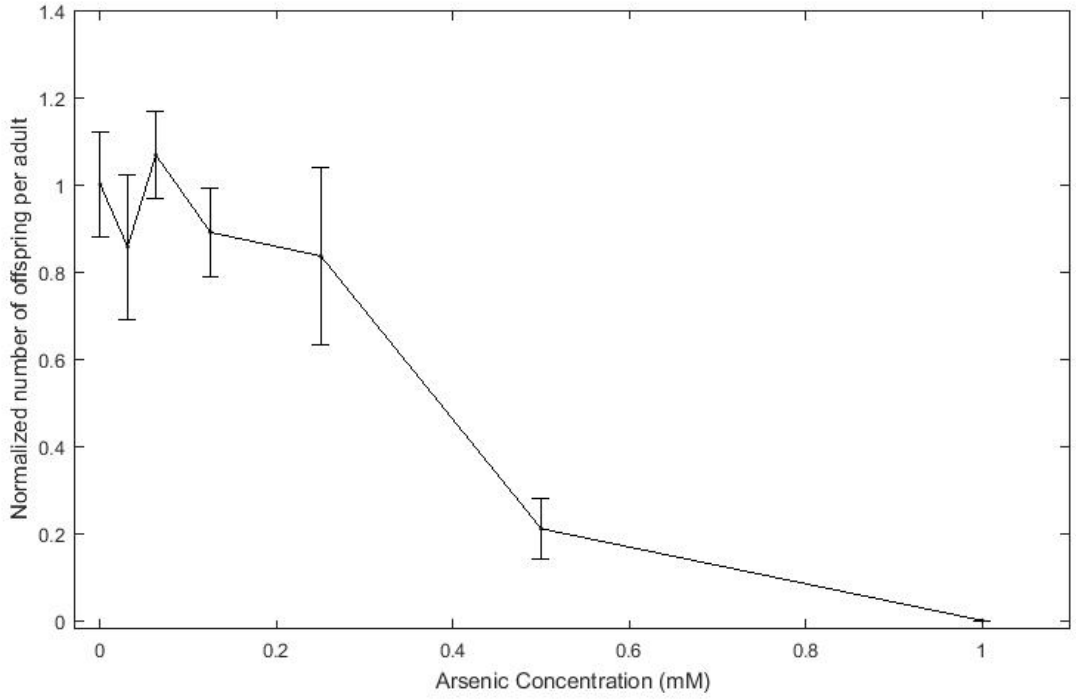

Figure 3.8. Normalized number of offspring per nematode. The error bars represent the standard deviation.

3.3 Exposure to a Mixture of Depleted Uranium and Sodium Arsenite

Several very large experiments were done in order to assess the combined effects of depleted uranium and sodium arsenite. These experiments also included measurements in the Figaro gamma source, but the results referred to gamma radiation will be reported in section 3.4, to avoid confusion. The concentrations used in the combinations were similar to the ones used in the single exposure experiment, but only intermediate cases were consider; the highest and the lowest concentrations were not included in the mixtures (there are 16 combinations or cases).

Table 3.8 compiles the pH measurements (average numbers); the mixtures are denoted U_iAs_j, with i,j = 2,3,4,5 and refers to the second highest doses used in the previous experiments, through the second lowest doses.

Table3.8. Average pH values in the exposure wells, as a function of time. Standard deviations are included.

t (hours)	0	24	48	72	96
U2As2	6.86 +/- 0.06	6.89 +/- 0.13	7.02 +/- 0.02	7.05 +/- 0.04	7.14 +/- 0.01
U2As3	6.79 +/- 0.06	6.92 +/- 0.02	7.06 +/- 0.14	7.07 +/- 0.09	7.20 +/- 0.06
U2As4	6.79 +/- 0.06	6.91 +/- 0.03	7.13 +/- 0.20	7.12 +/- 0.19	7.16 +/- 0.16
U2As5	6.85 +/- 0.02	6.93 +/- 0.05	7.08 +/- 0.17	7.12 +/- 0.17	7.25 +/- 0.05
U3As2	6.88 +/- 0.10	6.92 +/- 0.09	7.15 +/- 0.08	7.15 +/- 0.04	7.24 +/- 0.01
U3As3	6.83 +/- 0.09	6.87 +/- 0.07	7.10 +/- 0.14	7.12 +/- 0.15	7.19 +/- 0.05
U3As4	6.81 +/- 0.03	6.89 +/- 0.05	7.12 +/- 0.18	7.14 +/- 0.19	7.20 +/- 0.08
U3As5	6.81 +/- 0.05	6.91 +/- 0.04	7.11 +/- 0.17	7.10 +/- 0.13	7.18 +/- 0.03
U4As2	6.89 +/- 0.12	6.95 +/- 0.09	7.13 +/- 0.02	7.19 +/- 0.03	7.20 +/- 0.04

U4As3	6.86 +/- 0.08	6.82 +/- 0.14	7.11 +/- 0.13	7.12 +/- 0.13	7.19 +/- 0.06
U4As4	6.84 +/- 0.05	6.87 +/- 0.06	7.06 +/- 0.22	7.07 +/- 0.19	7.10 +/- 0.03
U4As5	6.84 +/- 0.03	6.91 +/- 0.07	7.11 +/- 0.17	7.11 +/- 0.17	7.22 +/- 0.05
U5As2	6.91 +/- 0.10	6.97 +/- 0.05	7.16 +/- 0.12	7.21 +/- 0.08	7.18 +/- 0.06
U5As3	6.84 +/- 0.03	6.92 +/- 0.03	7.10 +/- 0.19	7.15 +/- 0.15	7.10 +/- 0.13
U5As4	6.87 +/- 0.03	6.92 +/- 0.06	7.01 +/- 0.12	7.13 +/- 0.22	7.04 +/- 0.16
U5As5	6.90 +/- 0.04	6.88 +/- 0.05	7.00 +/- 0.11	7.01 +/- 0.08	7.02 +/- 0.07
Control	6.86 +/- 0.07	6.95 +/- 0.04	7.06 +/- 0.12	7.14 +/- 0.21	7.17 +/- 0.17

Figure 3.9 shows the pH values summarized in table 3.8. It can be observed that the pH values are stable, but with a moderate tendency to increase during the exposure.

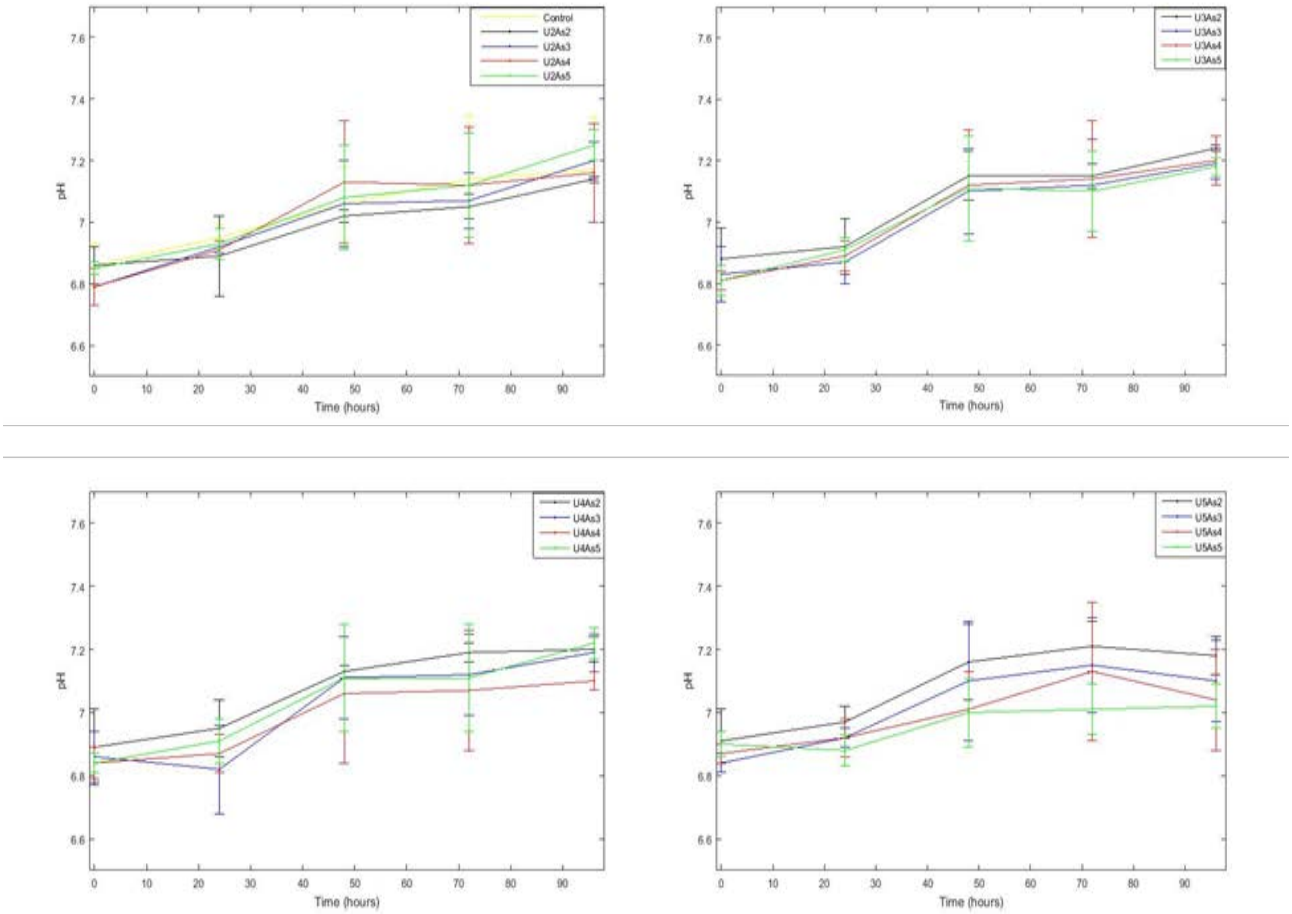

Figure 3.9. pH evolution during the experiment. On the upper left corner, the mixtures U2As2, U2As3, U2As4, U2As5 are included, together with the control pH. On the upper right corner, mixtures U3As2 through U3As5 are included. On the lower left corner, the pH values correspond to U4As2 through U4As5, and on the lower right corner, U5As2 through U5As5.

3.3.1 Survival

No mortality was observed (at least, not directly). In all the cases, the number of adult nematodes counted after finishing the experiment was either higher or the same as in the beginning. Therefore it can be assumed that all the nematodes survived, in all the cases.

3.3.2 Growth

The measurements done to estimate the size of the adult nematodes are shown in tables 3.9.a, 3.9.b, and 3.9.c. The average number of adult nematodes per well, counted in all the experiments was 7.1 (the target was to have 7 nematodes per well), however, as it was mentioned before, some nematodes broke during the heating process and could not be measured. The mean length of the control groups is 1.2399 mm, the median 1.2375 mm, and the standard deviation 0.0512 mm.

Table 3.9.a. Combined mean values of the lengths at different uranium and arsenic mixture concentrations.

Lengths mean (mm)	U5	U4	U3	U2
As5	1.2475	1.25789	1.23495	1.25726
As4	1.2569	1.24324	1.23927	1.2399
As3	1.25667	1.20406	1.2325	1.25169
As2	1.198	1.14792	1.19713	1.22683

Table 3.9.b. Combined median values of the lengths at different uranium and arsenic mixture concentrations.

Lengths median (mm)	U5	U4	U3	U2
As5	1.274	1.270	1.24	1.272
As4	1.272	1.257	1.228	1.239
As3	1.265	1.239	1.243	1.259
As2	1.205	1.198	1.201	1.232

Table 3.9.c. Combined standard deviation values of the lengths at different uranium and arsenic mixture concentrations.

Standard dev. (mm)	U5	U4	U3	U2
As5	0.0838	0.0574	0.0504	0.068
As4	0.0694	0.0691	0.0622	0.057
As3	0.0529	0.1700	0.0491	0.043
As2	0.0558	0.1975	0.0514	0.061

In two combination cases nematodes in arrested development state were found, namely U4As2, and U4As3. The nematode lengths measured for the whole combinations of samples were analyzed by means of unbalanced 2-way ANOVA, since there are two factors acting at the same time and at different combinations, and the number of measures in each case are not necessarily identical. This type of analysis is not available with common software such as Minitab or GraphPad Prism, but it can be written in R, and also can be done with online tools such as the one provided by http://vassarstats.net/ (website for statistical computation).

The results indicate that the effect of the interaction is led by sodium arsenite ($p < 0.001$, $F = 7.72$), while the effect of uranium on growth, as compared to that of arsenic, would not be significant (with $p = 0.06$, $F = 2.44$). Post hoc Tuckey's multiple comparisons test indicates that the combination (U4 + As2) is the main source of variation among groups. Three such comparisons have $p < 0.001$: Control vs (U4 + As2), (U4 + As5) vs (U4 + As2), and (U5 + As3) vs (U4 + As2). Three pair comparisons have $p < 0.01$: (U5 + As5) vs (U4 + As2), (U2 + As5) vs (U4 + As2), and (U5 + As4) vs (U4 + As2). Four pair comparisons have $p < 0.05$: (U3 + As5) vs (U4 + As2), (U4 + As4) vs (U4 + As2), (U2 + As4) vs (U4 + As2), and (U2 + As3) vs (U4 + As2).

It is worth highlighting that it was found, in section 3.1.2 that when exposed to depleted uranium only, U4 (which corresponds to a molar concentration of 12.5 μM) had the biggest effect on nematode growth. Here, combined with sodium arsenite, at the two highest molar concentrations, again this concentration yields a significant effect, including the presence of adults which did not develop fully.

3.3.3 Reproduction

Table 3.10 below shows the number of offspring per adult nematode normalized by the number of offspring found in the control wells, for each experiment.

Table 3.10. Average number of offspring per adult nematode, normalized by the number of offspring per adult nematode of the controls.

Average Number	U5	U4	U3	U2
As5	0.96 +/- 0.11	1.09 +/- 0.29	0.95 +/- 0.25	0.96 +/- 0.11
As4	0.86 +/- 0.14	0.87 +/- 0.09	0.85 +/- 0.31	0.82 +/- 0.22
As3	0.74 +/- 0.23	0.66 +/- 0.18	0.80 +/- 0.32	0.62 +/- 0.18
As2	0.51 +/- 0.30	0.34 +/- 0.22	0.40 +/- 0.07	0.24 +/- 0.06

Two-factor ANOVA test with repeated measures was performed. Similarly to the case with lengths, here, the dominant factor is sodium arsenite, with $p = 0.0004$, $F = 32.36$. For the uranium columns, $p = 0.36$ and $F = 1.28$. Post hoc multiple comparisons test showed that the pairs of set of data showing the most difference ($p < 0.01$) were U_2As_2 vs U_4As_5 and U_4As_5 vs U_4As_2. Other 4 pairs comparisons found $p < 0.05$ (not significant, but worth mentioning):

- U_2As_2 vs U_2As_5
- U_2As_2 vs U_3As_5
- U_2As_2 vs U_5As_5
- U_3As_2 vs U_4As_5

Figure 3.10 shows the average number of offspring per nematode (normalized) as a function of increasing combined concentrations, led by sodium arsenite. The combinations have been indexed from 1 to 16, such that 1 corresponds to U_5As_5, 2 corresponds to U_5As_5…, and 16 corresponds to U_2As_2.

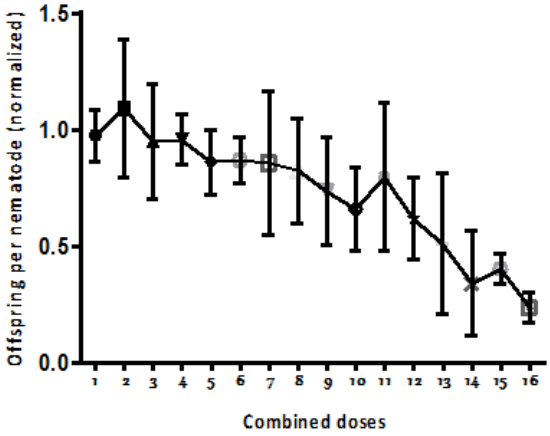

Figure 3.10. The effect of increasing concentrations of depleted uranium and sodium arsenite on the reproduction of *C. elegans*. The numbers on the vertical axis correspond to the normalized average number of offspring per adult nematode, and the bars correspond to the standard deviation (from table 3.9).

The combined effect of sodium arsenite and depleted uranium on reproduction can be easily visualized with a grey map, as shown in figure 3.11, where the scale on the right shows the correlation to normalized number of offspring, ranging from the lowest (black) to the highest (white). The red arrows point in the direction of increasing molar concentration of each compound.

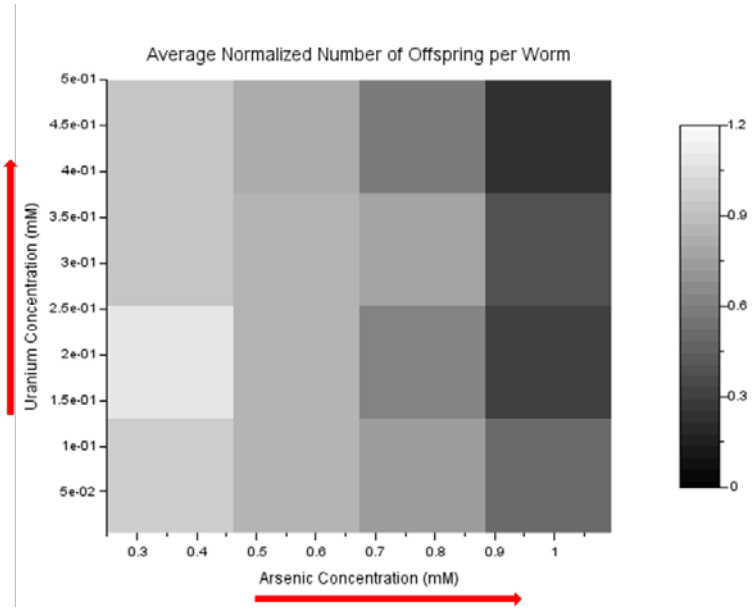

Figure 3.11. Grey map indicating the average (normalized) number of offspring. Darker colors indicate fewer offspring.

This figure is consistent with the results of statistical tests; the higher the concentration of both compounds, the lower the amount of offspring; this is particularly marked at the highest concentration of sodium arsenite.

3.4 Exposure to Gamma Radiation

The nematodes were exposed to gamma radiation in experiments done simultaneously with the mixtures of depleted uranium and sodium arsenite. The purpose is to study the effects of gamma radiation on *C elegans*, alone and in the presence of the mixture of compounds, with the same combinations reported in the previous section.

No mortality effects were observed. Adult nematodes were counted in the usual way, and in most cases the final number was larger than the original number. Only a few cases reported the same number of counted survivors and larvae.

Data of two dose rates are available; 100.13 mGy/h and 200.24 mGy/h. The microtiter plates were prepared in an identical fashion to those of the previous sections. To assess the effects caused by gamma radiation alone, plates were prepared with SSPW only, and placed at a distance from the source, which had been previously calibrated to give such doses. To assess the effects of gamma radiation in the presence of mixed compounds, plates were prepared identically to those of section 3.3, and they were placed at distances from the source providing the required dose.

3.4.1 Growth

Table 3.11 contains the length measurements of the control experiments, that is, no exposure, exposure to dose rate of 100.13 mGy/h, and exposure to dose rate of 200.24 mGy/h.

Table 3.11. Length measurements for 3 control cases. The first one, with no radiation, the second and third cases are controls in the sense they are not exposed to the chemical mixture, but to gamma radiation only.

LENGTHS (mm)	Control γ = 0	Control γ =100.13 mGy/h	Control γ =200.24 mGy/h
mean	1.24	1.20	1.20
median	1.24	1.22	1.21
standard dev	0.05	0.15	0.11

One-way ANOVA was performed on the set of data; $p = 0.02$, $F = 3.96$. The effect of the exposure at dose rates of 100.13 mGy/h and 200.24 mGy/h on growth does not show any difference between them, which can be read directly from table 3.10. But both are slightly different than control, with $p < 0.05$ (Tuckey's multiple comparisons). This can be appreciated in figure 3.12, which shows the mean values and their standard deviations

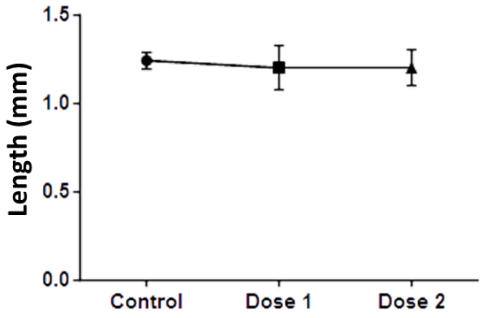

Figure 3.12. Length mean values with standard deviation. Dose 1 corresponds to 100.13 mGy/h and Dose 2 to 200. 24 mGy/h.

Tables 3.12 and 3.13, below, contain the length measurements of adult nematodes exposed to the depleted uranium and sodium arsenite mixture of section 3.3, and exposed to Dose 1 and Dose 2, respectively.

Table 3.12.a. Combined mean values of the lengths at γ = 100.13 mGy/h, in the presence of different depleted uranium and sodium arsenite mixture concentrations.

Lengths mean (mm)	U5	U4	U3	U2
As5	1.27	1.27	1.21	1.19
As4	1.23	1.23	1.19	1.25
As3	1.24	1.19	1.23	1.23
As2	1.23	1.19	1.23	1.16

Table 3.12.b. Combined median values of the lengths at γ = 100.13 mGy/h, in the presence of different depleted uranium and sodium arsenite mixture concentrations.

Lengths median (mm)	U5	U4	U3	U2
As5	1.27	1.28	1.27	1.22
As4	1.26	1.24	1.26	1.28
As3	1.24	1.22	1.23	1.23
As2	1.23	1.20	1.23	1.23

Table 3.12.c. Combined standard deviation values of the lengths at γ = 100.13 mGy/h, in the presence of different depleted uranium and sodium arsenite mixture concentrations.

Standard dev. (mm)	U5	U4	U3	U2
As5	0.07	0.06	0.15	0.11
As4	0.13	0.10	0.21	0.11
As3	0.09	0.20	0.15	0.07
As2	0.08	0.17	0.09	0.23

The mixture exposure wells which were also exposed to gamma radiation, presented a much higher rate of underdeveloped nematodes than the previous experiments. Approximately 3.5% of the nematodes exposed only to gamma radiation at a rate of ~ 100 mGy/h presented an abnormal growth (size less than 1.00 mm). In the case of mixture wells exposed to the same dose, some of them presented a high percentage of nematodes in arrested development while others presented none. The proportion of undeveloped adults (relative to the whole population of each mixture dose) is shown in table 3.13.

Table 3.13. Proportion of nematodes which presented abnormally small body size (below 1.00 mm).

% of the population	U5	U4	U3	U2
As5	0	0	10.34%	4.20%
As4	4.55%	3.13%	9.09%	8.00%
As3	3.13%	6.06%	2.94%	0
As2	0	7.41%	0	9.09%

The purpose of this table is only to report these findings. However, reporting the fraction of nematodes the body sizes of which are lower than 1.00 mm, is somewhat arbitrary here.

The data were analyzed similarly like in the previous cases, by means of unbalanced 2-way ANOVA. The results show no statistical difference between the lengths of the nematodes exposed to a total dose of 9.61 Gy and the chemical mixture, simultaneously ($p = 0.33$, $F = 1.16$ for uranium; $p = 0.19$, $F = 1.59$ for arsenic).

Table 3.14 summarizes the length measurements of the mixtures exposed to Dose 2 (19.22 Gy).

Table 3.14.a. Combined mean values of the lengths at γ = 19.22 Gy, in the presence of different depleted uranium and sodium arsenite mixture concentrations.

Lengths mean (mm)	U5	U4	U3	U2
As5	1.26	1.24	1.25	1.28
As4	1.28	1.24	1.23	1.26
As3	1.22	1.25	1.21	1.24
As2	1.18	1.18	1.18	1.15

Table 3.14.b. Combined median values of the lengths at γ = 19.22 Gy, in the presence of different depleted uranium and sodium arsenite mixture concentrations.

Lengths median (mm)	U5	U4	U3	U2
As5	1.26	1.26	1.24	1.3
As4	1.29	1.24	1.26	1.28
As3	1.24	1.27	1.24	1.26
As2	1.18	1.18	1.20	1.19

Table 3.14.c. Combined standard deviation values of the lengths at γ = 19.22 Gy, in the presence of different depleted uranium and sodium arsenite mixture concentrations.

Standard dev. (mm)	U5	U4	U3	U2
As5	0.08	0.07	0.06	0.07
As4	0.06	0.09	0.08	0.09
As3	0.12	0.06	0.15	0.06
As2	0.10	0.08	0.09	0.16

The nematodes exposed to a total dose of 19.22 Gy (200.24 mGy/h) also presented an increased percentage of individuals with smaller than normal body size, but the proportion of such individuals, overall is lower than in the previous case. The percentage in the population which was only exposed to gamma radiation at this dose is 2.86%. The percentages of smaller than 1.00 mm nematodes in the mixture wells are summarized below in table 3.15.

Table 3.15. Proportion of nematodes which presented abnormally small body size (smaller than 1.00 mm).

% of the population	U5	U4	U3	U2
As5	0	0	0	0
As4	0	3.33%	0	0
As3	3.33%	0	3.33%	0
As2	6.25%	5%	4%	8.70%

Unbalanced two-way ANOVA done on the length measurements at different combinations, revealed that in the case of 19.22 Gy exposure, the effect on this endpoint is led by arsenic ($p < 0.0001$, $F = 15.73$, the case for uranium had $p = 0.20$, $F = 1.54$), just like in the case of the populations exposed to the mixtures only but not to gamma radiation.

The most important pair comparisons were given by:

- U2As5 vs. U2As2 ($p < 0.0001$)
- U5As4 vs. U2As2 ($p < 0.0001$)
- U2As5 vs Control (200.24 mGy/h) ($p < 0.01$)

Other pair comparisons with $p < 0.05$:

- U2As5 vs U4As2 and U2As5 vs U3As2
- U5As4 vs U4As2, U5As4 vs U3As2, and U5As4 vs Control (200.24 mGy/h)
- U2As4 vs U2As2

The rest of the pair comparisons were not significant. Figure 3.12 is a plot of the mean values of the nematodes size with their standard deviation.

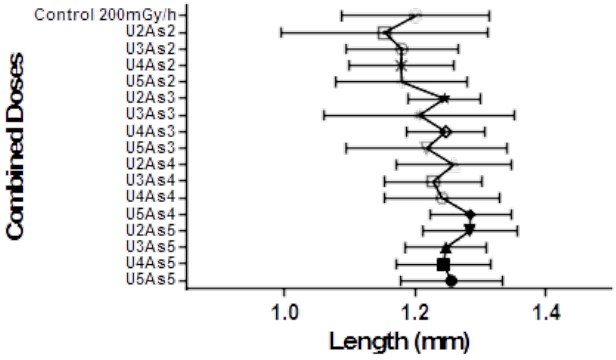

Figure 3.13. Mean values of the nematodes' lengths together with the standard deviation.

The effects of the mixtures of different concentrations of uranium and arsenic can be visualized with figure 3.14, which is a compilation of the three gamma radiation cases (no radiation, 9.61 Gy and 19.22 Gy).

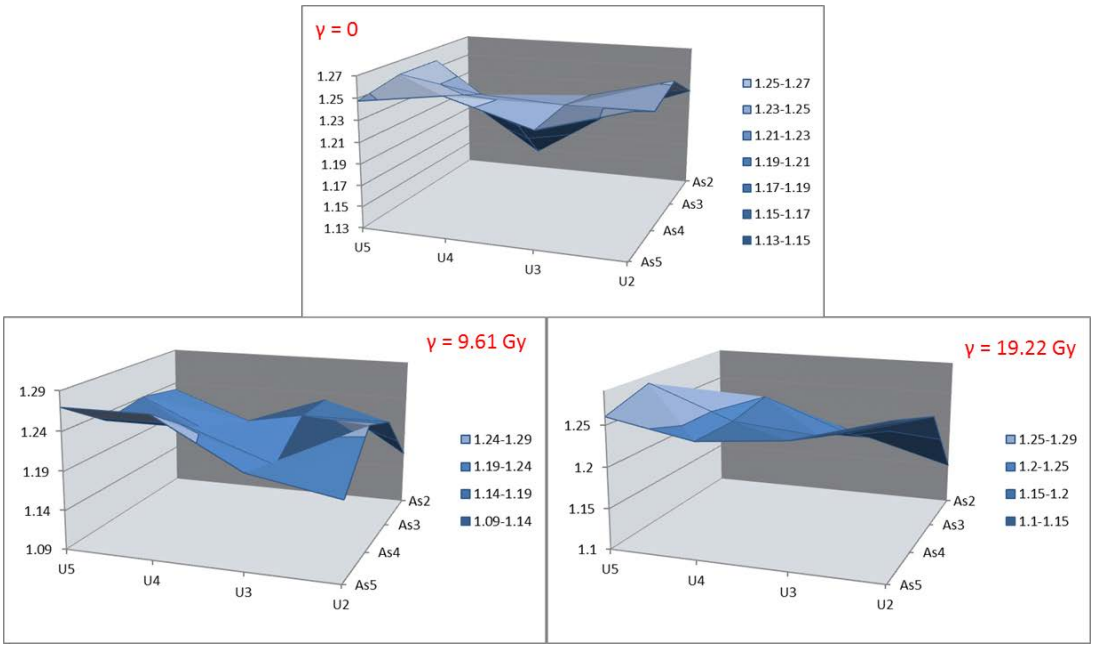

Figure 3.14. Comparison of the combined effects of uranium and arsenic on growth. On top, the effect of the mixture alone. Left bottom, the effects of exposure to a gamma radiation dose of 9.61 Gy together with the mixture. Right bottom, the mixture with a dose of 19.22 Gy

3.4.2 Reproduction

The average normalized (by the control) number of offspring per adult nematode is 0.92 +/- 0.02 in the case of exposure to 9.61 Gy, and 0.80 +/- 0.04 in the case of exposure to 19.22 Gy. Numbers smaller than 1 indicate less offspring per nematode, relative to the control case.

Tables 3.16a and 3.16b contain the number of offspring per adult nematode for both gamma exposures, normalized by the controls.

Table 3.16.a. Average number of offspring per adult nematode, normalized by the number of offspring per adult nematode of the controls, γ = 9.61 Gy.

Average Number	U5	U4	U3	U2
As5	0.91 +/- 0.20	0.94 +/- 0.22	0.94 +/- 0.20	0.81 +/- 0.27
As4	0.85 +/- 0.20	0.90 +/- 0.18	0.95 +/- 0.25	0.79 +/- 0.23
As3	0.59 +/- 0.13	0.65 +/- 0.16	0.70 +/- 0.20	0.52 +/- 0.07
As2	0.38 +/- 0.06	0.36 +/- 0.19	0.42 +/- 0.14	0.46 +/- 0.08

Table 3.16.b. Average number of offspring per adult nematode, normalized by the number of offspring per adult nematode of the controls, γ = 19.22 Gy.

Average Number	U5	U4	U3	U2
As5	0.94 +/- 0.25	0.77 +/- 0.10	0.86 +/- 0.06	0.96 +/- 0.20
As4	1.00 +/- 0.38	0.76 +/- 0.21	0.79 +/- 0.26	0.71 +/- 0.25
As3	0.58 +/- 0.20	0.57 +/- 0.08	0.54 +/- 0.20	0.56 +/- 0.08
As2	0.32 +/- 0.11	0.28 +/- 0.07	0.28 +/- 0.16	0.20 +/- 0.09

Two-factor ANOVA test with repeated measures on both factors, shows that in the case of the 9.61 Gy dose, the dominant factor, like in the previous case (no gamma radiation) is sodium arsenite, with $p = 0.006$, $F = 8.08$; while the variation due to depleted uranium is not relevant. The case for the 19.22 Gy exposure is very similar; $p = 0.008$, $F = 7.49$ for arsenic and not significant for uranium. However, the post hoc pair comparisons test did not find significant results in the case of gamma radiation exposure of 9.61 Gy. The only pairs with $p < 0.05$, for the first gamma radiation dose, were As5U4 vs As2U5, As5U4 vs As2U4, As5U3 vs As2U5, As5U3 vs As2U4, As4U4 vs As2U4, As4U3 vs As2U5, and As4U3 vs As2U4, i.e. basically the comparison between low arsenic doses and high arsenic doses.

The case of the highest gamma radiation exposure is slightly different. The trend is the same but a few of the pairs comparisons are statistically significant:

- As2U2 vs As4U5 ($p < 0.001$)
- As4U5 vs (As2U3, As2U4, As2U5) ($p < 0.01$)
- As5U2 vs (As2U2, As2U3, As2U4) ($p < 0.01$)

Figure 3.15 shows the normalized number of offspring as a function of increasing concentration, led by arsenic.

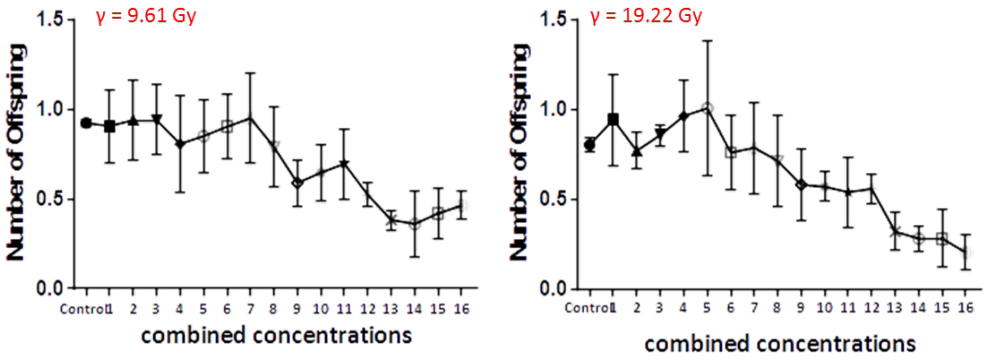

Figure 3.15. The effect of combined exposure to gamma radiation, sodium arsenite and depleted uranium.

3.5 Speciation Analysis

Samples were analyzed by ICP-MS, as described in Chapter 2. In order to find the actual amount of each element present in the samples (mg/L), first, the total amount of moles of each molecule is found from the known molarity at which the solutions were prepared. The following calculations were made for the samples which were prepared for analysis.

- **$UO_2(NO_3)_2 \bullet 6H_2O$** (total 36 samples, triplicates for total and triplicates for filtrate, at t = 0 and t = 96)

1) 100 µM sample ("U1")

The 0.5 mL solution in the original well at 100 µM concentration of $UO_2(NO_3)_2$ contains a total amount of moles found by proportion:

$$10^{-4} \text{ moles } UO_2(NO_3)_2 \text{ / 1 L solution} = X \text{ moles } UO_2(NO_3)_2 \text{ /5x}10^{-4} \text{ L solution}$$

$X = 5 \times 10^{-8}$ moles of $UO_2(NO_3)_2$ (total amount of $UO_2(NO_3)_2$ present in the 0.5 mL volume well).

To find the total amount of $UO_2(NO_3)_2$ molecules in 5×10^{-8} moles, the Avogadro's proportion can be used:

$$X \text{ molecules, } _{UO2(NO3)2} \text{ / 5x}10^{-8} \text{ moles} = 6.022\text{x}10^{23} \text{ molecules/1 mole}$$

$X = 3.01 \text{ x}10^{16}$ molecules

The number of grams of Uranium present in the original volume of solution can be calculated from its molar mass, 238.03 g/mol (and this is the amount of Uranium contained as well in 1 mole of $UO_2(NO_3)_2 \bullet 6H_2O$ molecule):

$$X \text{ grams U / } 3.01 \text{ x}10^{16} \text{ molecules} = 238.03 \text{ g/}6.022\text{x}10^{23} \text{ molecules}$$

X = 1.19×10^{-5} g of Uranium in 0.5 mL of solution

Nominal Concentration: 2.38×10^{-2} g/L

The 'total' samples correspond to 20% of the original sample taken directly into a tube (i.e 0.1 mL), by proportion, this should contain as well 20% of the Uranium: 2.38×10^{-6}g. This sample is diluted in ultrapure HNO_3 acid, to a volume of 5 mL, therefore the nominal concentration of the diluted sample is 4.76×10^{-4}g/L =2.38×10^{-6}g/5×10^{-3}L.

Nominal Concentration #2: 4.76×10^{-4}g/L

2) 25 µM sample ("U3")

The calculations are identical as in 1), except that the molarity is ¼ of the original, i.e. in the 0.5 mL well of solution at 25 µM there are 7.53×10^{15} atoms of Uranium, and the nominal concentrations of the original and diluted samples are:

Nominal Concentration: 5.96×10^{-3} g/L

Nominal Concentration #2: 1.19×10^{-4} g/L

3) 3.1 µM sample ("U6")

The molarity is 1/32 of U1. In the 0.5 mL volume of solution there are 2.35×10^{14} atoms of Uranium, and the nominal concentrations:

Nominal Concentration: 1.86×10^{-4} g/L

Nominal Concentration #2: 3.72×10^{-6} g/L

- $NaAsO_2$ (total 36 samples, triplicates for total and triplicates for filtrate)

1) 1 mM solution ("As1")

Similar calculations as in the Uranium case. Total amount of sodium arsenite, in moles, present in the 0.5 mL volume of SSPW is found by:

$$1 \times 10^{-3} \text{moles}/1 \text{ L} = x \text{ moles}/5 \times 10^{-4} \text{L}$$

x = 5.0×10^{-7} moles

Total number of molecules of $NaAsO_2$ in the 0.5 mL well:

$$X_{\#\text{molecules}}/5.0 \times 10^{-7} \text{ moles} = 6.022 \times 10^{23}\ _{\#\text{ molecules}}/1 \text{ mole}$$

X = 3.02×10^{17} molecules of sodium arsenite

The molar weight of Arsenic is 74.92 g/mol. To find the amount of Arsenic (grams) present in the well:

$$X \text{ g As}/3.02\text{x}10^{17} = 74.92 \text{ g As}/6.022\text{x}10^{23}$$

$X = 3.76\text{x}10^{-5}$ g of As

$$3.76\text{x}10^{-5} \text{ g}/5\text{x}10^{-4} \text{ L} = 7.50 \text{ X } 10^{-2} \text{ g/L}$$

The "total" sample (0.1 mL) should contain 20% of the whole amount, $7.5 \text{ x } 10^{-6}$ g of Arsenic. The nominal concentration of the 5 mL final solution is $1.50 \text{ x } 10^{-6}\text{g}/5\text{x}10^{-3}$ L = $3.01\text{x}10^{-4}$ g/L

Nominal Concentration: $7.52\text{x}10^{-2}$ g/L

Nominal Concentration #2: $1.50\text{x}10^{-3}$ g/L

2) 0.25 mM sample ("As3")

Using the same calculations, but at ¼ of the concentration:

Nominal Concentration: $1.88\text{x}10^{-2}$ g/L

Nominal Concentration #2: $3.76\text{x}10^{-4}$ g/L

3) 0.032 mM sample ("As6")

Nominal Concentration: $2.36\text{x}10^{-3}$ g/L

Nominal Concentration #2: $4.70\text{x}10^{-5}$ g/L

-As+U Mixtures (total 36 samples, triplicates for total and triplicates for filtrate)

1) As2 + U2: because both solutions are sharing the volume the actual molarity for each is half of what it was with the solution containing arsenic or uranium only. Hence, this sample is at 0.5 mM As and 50 µM U. The calculations are identical to the previous sections.

Nominal Concentration: $1.19\text{x}10^{-2}$ g/L Uranium and $3.76\text{x}10^{-2}$ g/L Arsenic

Nominal Concentration #2: $2.38 \text{ x}10^{-4}$ g/L Uranium and $7.52\text{x}10^{-4}$ g/L Arsenic

2) As3 + U4. The actual molarities of the initial solution (0.5 mL) are 0.25 mM arsenic and 12.5 µM uranium.

Nominal Concentration: $2.98\text{x}10^{-3}$ g/L Uranium and $1.88\text{x}10^{-2}$ g/L Arsenic

Nominal Concentration #2: $5.95\text{x}10^{-5}$ g/L Uranium and $3.76\text{x}10^{-4}$ g/L Arsenic

3) As5 + U5. The initial molarities are 0.062 mM Arsenic and 6.25 µM Uranium.

Nominal Concentration: $1.49\text{x}10^{-3}$ g/L Uranium and $4.70\text{x}10^{-3}$ g/L Arsenic

Nominal Concentration #2: $2.98\text{x}10^{-5}$ g/L Uranium and $9.40\text{x}10^{-5}$ g/L Arsenic

The report of the ICP-MS measurements of the samples is attached in Appendix 2.

4. Discussions and Conclusions

This work was focused on studying the effects of depleted uranium, sodium arsenite, gamma radiation and combinations of those on three end points of *Caenorhabditis Elegans*. The endpoints analyzed were survival, growth and reproduction.
Uranium was applied to plates containing nematodes and *E.* coli in six different molar concentrations, ranging from 100 µM to 3.1 µM, in exposure experiments that lasted 96 hours. Similar type of exposure experiments were carried out with sodium arsenite, but the molar concentrations used were approximately 1 order of magnitude higher than those of uranium, in the range 1 mM to 0.031 mM. The main topic of this thesis was combined toxicity; therefore, different combinations of depleted uranium and sodium arsenite were used, preparing the exposure experiments the same way were done for single contaminant. Four intermediate concentrations of each were chosen for this task, in the range 50 µM to 6.2 µM for uranium and 0.5 mM to 0.062 mM for arsenic. The effects of gamma radiation and mixture of compounds was also studied. Data available from two doses were used: to 9.61 Gy and 19.22 Gy (~ 100 mGy/h and ~ 200 mGy/h dose rate during the exposure). The results are summarized and discussed in the next sections.

4.1 Summary of Results

The study with depleted uranium alone was meant to be a follow up on a previous master thesis, from the same university [59]. In that work, mortality was reported for lower doses than those used here. However, the results of the experiments done for this work, did not agree with the former. One reason for this could be that the method for counting is very inaccurate. First, the larvae are counted under the microscope at the moment of starting the exposure, and then the survivor adults are counted after killing them. One way to overcome this inaccuracy would be by performing massive experiments and doing a statistical count. Here the trend found, for this experiment with depleted uranium only, and for the others as well, is that there is a 'surplus' of survivors, i.e., the number is largely underestimated in the beginning, but after staining, when the nematodes are fully grown, they can be counted quite precisely. Then, a variation of nematode surplus as a function of increasing concentration would give an estimate of mortality. In fact it was observed here a similar trend, but it could not be concluded that mortality had taken place. It would be worth doing measurements with larger numbers.

Evidence was found of detrimental effect of uranium on growth. The minimum lengths were measured for nematodes exposed at concentrations of 12.5 µM and 6.25 µM. ANOVA test found statistically significant differences between those two populations and the rest. Reduction of growth has been observed before in nematodes exposed to uranium [51, 60], however the doses are different, or the experiment is different (multi-generation, for example); but the results found here agree with those, at least for the two intermediate concentrations of 12.5 µM and 6.25 µM.

Uranium also showed effects on reproduction. There is a clear trend of reducing the number of offspring as the concentration increases, however this could only be established with $p < 0.01$ when comparing the maximum concentration (100 µM) with the lowest concentration (3.125 µM).

The effects are much more noticeable for the case of sodium arsenite (but the concentrations used were ~ 1 order of magnitude larger). Only in this case, at the highest molar concentration tested, 1mM, it was observed clear signs of mortality. However, this happened in an all-or-nothing fashion, in which, in some experiments, all the nematodes died, or they all survived at that molarity. This

agrees with other researchers' results on the effects of sodium on *C. elegans* [52], however their exposure protocols and doses are quite different. The effects of sodium arsenite on growth are much stronger than those of depleted uranium. The nematodes which survived at 1 mM, were all in a state of arrested development (their size resembles that of a larvae). Statistical test had very significant results for several of the pairs compared, more so at higher doses.

The effects of arsenic on reproduction are also quite clear; at higher doses they have very few offspring per adult nematode. At intermediate concentrations, the mean and median number of offspring per adult nematode has decreased by approximately 1/3, and at the second highest concentration (0.5 mM), only ~ 7 offspring per nematode in comparison to ~ 34 offspring per nematode of the control case.

The combination of depleted uranium and sodium arsenite at four different concentrations (intermediate cases of the single-component exposure), showed trends, but less straight forward than in the single-component case. The effect of the mixture on growth was dominated by the action of sodium arsenite, and the minimum average size was found for the combination of (As2 + U4), which corresponds to the molar concentration of 0.5 mM in arsenic and 12.5 µM in uranium. The statistical tests were significant ($p < 0.01$) and the most significant effect on growth reduction was due to this combination.

Similarly to the results for growth, the main component leading the detrimental effects on reproduction was arsenic. The statistical tests yield $p < 0.001$, however that result is influenced only by 2 of the combination pairs, namely (As2 + U2) vs (As5 + U4) and (As2 + U4) vs (As5 + U4). The rest of the comparisons only yield $p < 0.05$, which some researchers consider significant, but here it was chosen to prefer $p < 0.01$ for statistical significance.

The mixture plates were placed in the gamma facility as well. The previous experiment, with the same mixtures was replicated at doses of 9.61 Gy (~ 100 mGy/h for 96 hours), and 19.22 Gy (~ 200 mGy/h for 96 hours). First, the effects of gamma radiation alone were assessed and it was found no statistically significant differences between control and those two doses, on growth ($p = 0.02$), moreover, the mean lengths were identical for both doses, and slightly smaller than the control cases. When the nematodes were exposed to those gamma doses and the mixtures, the results were unexpected, in the sense that the mixture and 9.61 Gy dose did not have any significant effect on growth, as opposed to the case of the mixture in the absence of gamma radiation. However, the mixture with the second dose, of 19.22 Gy did have a statistically significant effect on growth ($p < 0.0001$), dominated by the highest combined concentrations of As2 + U2 (0.5 mM, 50 µM).

The effects on reproduction were also less clear than the effects of the mixtures alone. In both cases the whole comparison (2-way ANOVA) yields statistically significant results, but for the 9.61 Gy dose, there was no significant difference in the post hoc tests; only a mild trend with $p < 0.05$ for the highest arsenic concentrations only. For the 19.22 Gy dose, both tests were significant and the main component affecting reproduction is arsenic. However, figure 3.15 shows a very clear trend, of decreasing number of offspring as the combined dose increases. It can be observed a 'period 4' of bouncing values (which have the appearance of steps). That effect is due to uranium; it could not be proved statistically that uranium is interacting in a way other than additive with arsenic, but this trend suggests that it would be worth to design larger experiments to check for reproducibility.

The speciation analysis results (Appendix 2) show that at t=0, most of the uranium is in the <0.45µm fraction, at t=96 hours, a similar trend occurs, but the difference between both fractions is even

larger (or the amount which was found in the 'total' sample was even smaller). The amount that is present in the 'total' sample is approximately 2 orders of magnitude lower than that of the <0.45µm fraction at t=0, and closer to 3 orders of magnitude at t = 96 hours. The same is true for arsenic at t = 0, but at t = 96 hours, only half of it is present as 'total' (i.e double the concentration in the <0.45µm fraction).

But in the mixture wells, the behavior of uranium reverses. Arsenic maintains the same proportions in the mixture and as a single component, but uranium when mixed with arsenic is mainly present in the 'total' fraction, approximately double the concentration of the < 0.45 µm.

The differences in behavior could be due to the interaction of *E.* coli with uranium; it was mentioned before that the pellet of *E.* coli has the tendency to raise the pH in the well, and the uranium solution has the tendency to precipitate at pH above 6.7. Figure 4.1 shows an image taken of precipitate.

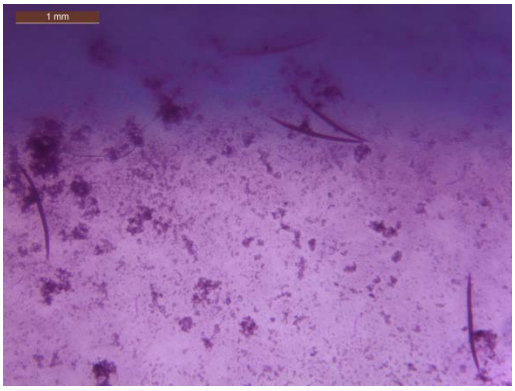

Figure 4.1. Uranium has the tendency to precipitate in the presence of *E. coli*. SOURCE: Roxana Contreras.

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