Utjecaj dva oblika selena (selenit i selenat) na imunološki sustav gujavice Eisenia andrei

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Effect of two different forms of selenium (selenite and selenate) on the immune system of the earthworm *Eisenia andrei*

Master thesis

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Kratak sadržaj: Selen je element u tragovima, potreban u malim količinama za normalno funkcioniranje animalnih organizama. Iako esencijalan, ukoliko je u organizmu prisutan u previsokim koncentracijama postaje toksičan te dovodi do razvoja bolesti. Unatoč tome, toksični utjecaj na organizme iz tla nije dovoljno istražen. Cilj ovog rada je stoga bio istražiti utjecaj dva oblika selena, selenita i selenata, na imunološki sustav gujavice *Eisenia andrei*, koristeći celomocite, efektorske stanice njezina imunološkog sustava. Kako bi se provjerila sposobnost obrane od patogena nakon izlaganja selenu, učinjen je test fagocitoze. Osim toga, izmjereni su sadržaji ROS-a i NO-a. Rezultati pokazuju da selen ne utječe negativno na sposobnost fagocitoze gujavica pri koncentraciji selenita, tj. selenata od 5 $\mu g/cm^2$ te da ne utječe na promjenu u razini NO-a. Štoviše, pri toj koncentraciji selenat je utjecao na smanjenje sadržaja ROS-a u celomocitima.

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Effect of two different forms of selenium (selenite and selenate) on the immune system of the earthworm *Eisenia andrei*

Magdalena Grgić

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Supervisor: Mirna Velki, PhD, Assistant Professor
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Short abstract: Selenium is a trace element, required in small amounts for normal functioning of animal organisms. Even though essential, if present in concentration higher than necessary it becomes toxic and results in development of serious conditions. Despite this fact, researches concerning its toxicity effects on soil animals are scarce. The aim of this study was to investigate effects of two forms of selenium, selenite and selenate, on the immunological system of the earthworm *Eisenia andrei*, using coelomocytes, effector cells of its immune-system. To assess earthworm's ability of defence against pathogens after exposure to selenium, phagocytosis assay was done. Additionally, ROS and NO levels were measured. Results showed that selenium does not impair earthworm's capability of phagocytosis or cause significant changes in NO levels. Moreover, at concentration of 5 μg/cm^2 selenate causes decrease in ROS content in coelomocytes.

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1 INTRODUCTION

1.1 Selenium

Selenium (Se) is non-metallic element with atomic number of 34, set in group 16 of periodic table of elements called the chalcogens or the oxygen family group. It intermediates between sulphur (S) and tellurium (Te) with which it can be chemically compared. Se was discovered by accident in 1817 by Swedish chemist called Jöns Jacob Berzelius, while investigating red-brown sediment contaminating sulfuric acid. With additional examination, Berzelius found that this newly discovered element was behaving similarly to sulphur and was present in tellurium samples giving them their distinctive smell. One of the noted points by Berzelius was also that element was being absorbed in the contact with skin (McDowell, 2017).

Se is trace element essential for almost every living organism (Birringer et al., 2002). It is not required in large quantities but it is necessary for normal functioning of organism as a constituent in number of different proteins (Brown et al., 2000). Even though it is essential, if amount of Se in diet exceeds the needs of organism it easily becomes toxic and causes selenosis (Raisbeck, 2000). There is very thin line in concentration range between deficiency and toxicity of Se. For humans that range is between <40 μg per day and >400 μg per day (WHO1996; Lavender and Burk, 2006). Maybe the best explanation of nature of Se and its dual effect in organisms gave Jukes (1983) calling it an "essential poison".

Existing in multiple allotropes with different molecular forms results in different physical properties of Se. It appears as red or black powder and red or grey crystalline structure where latter is the most stable allotrope of all. Se ranks among the least common elements found (69^{th}). Its incidence in Earth's crust is estimated to be 90 parts per billion (Web2, Lakin 1972) and was among trace elements found in lunar samples (Haskin et al.,1970). Although it appears in nature, it is distributed broadly throughout the crust and rarely found in elemental form. Instead, it occurs combined with heavy metals, such as a copper or sulphide minerals, and hence its' main commercial source is a by-product in different refining processes, primarily refining of copper (Dhillon et al., 2018).

Although resembles sulphur (S) in chemical properties, Se is more metallic so it differs from S in electricity conducting properties. Unlike S, which has no conducting properties, Se is semiconductor and photoconductor and therefore often used in photocells

and solar cells, among many other things (Butterman and Brown Jr., 2004). It is also used in production of glass, both to decolorize and to give colour, depending on the compound being used (Fordyce, 2007).

Selenium appears in over 30 isotopes but only 6 of them are stable: ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se and ^{82}Se. Also, short-lived radioactive isotope ^{75}Se with half-life of 120 days is used in medical diagnostics, in the form of seleno-methionine to study digestive enzymes production (Web1). Se occurs in 5 oxidation states: -1, −2 (selenide), 0 (elementary selenium), +2, +4 (selenous acid/ selenite) and +6 (selenic acid/ selenate). It is mostly available in inorganic, water soluble oxyanions, selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), but it can also be found as selenide, elementary Se, in metal salts or bind to numerous organic moieties. Organo-Se compounds are divided into two classes: discrete molecules that have one unique chemical structure and where Se is bound with one carbon atom and natural organic matter (NOM) that derives from tissues containing discrete organo-Se (NOM-Se) with chemical structure differing from any other NOM-Se molecule (Wallschläger and Feldmann, 2010). Two most common Se organic species are dimethylselenide (DMSe, CH_3-Se-CH_3) and dimethyldiselenide (DMDSe, CH_3–Se-Se-CH_3).

Primary sources of selenium in nature are weathering of rocks containing selenium (McDowell, 2017) and volcanic releases and discharges including metallic sulphides. Secondary sources are biological sinks with accumulated selenium (National Research Council, 1974). Besides that, Se distribution throughout the environment is a result of number of various processes involving groundwater movements, weathering of rocks, precipitation, metabolic circulation of Se across the food chains and different anthropogenic activities as shown in Figure 1 (Winkel et al., 2012).

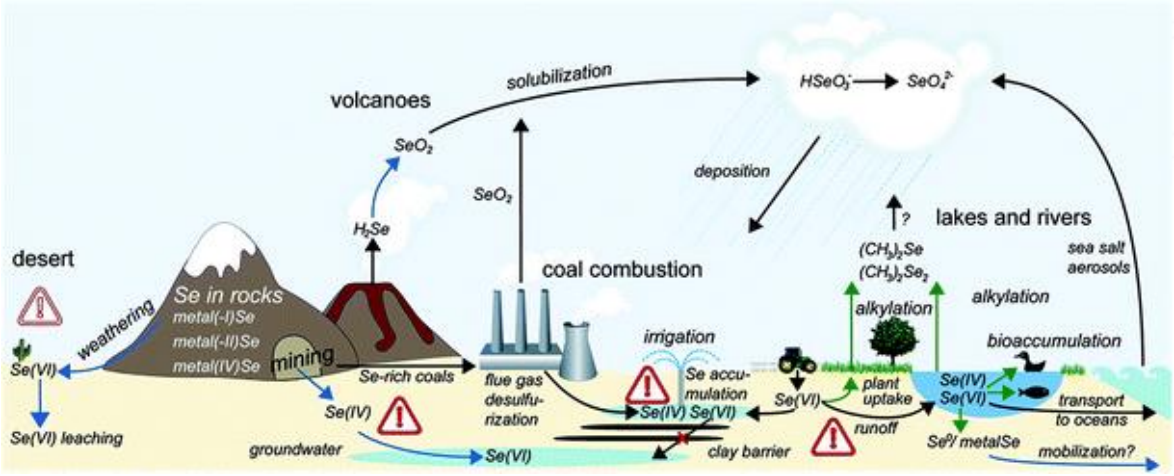

Figure 1. Global cycle of Se. Blue arrows show oxidation and green arrows show reduction, warning symbols indicate possibility of cumulation (shaded warning symbol) or shortage of Se (open warning symbol). (Taken from Winkel et al., 2012).

Emission of Se in atmosphere comes from two different sources. First one is natural, counting for 50-65% of total Se, and is a result of erosion of crust, volcanic activities and aerosol particles from sea (Nriagu, 1989). Second source is anthropogenic doings, with main redistribution source being the fossil fuel combustion and metal refining. Under atmospheric conditions Se species - particulate Se, volatile inorganic Se (S^0, SeO_2, and H_2Se), and organic Se (DMSe, DMDSe) are being transformed (Sharma et al., 2015). Lifetime of volatile organo-Se compounds in presence of O_3, $OH\cdot$ and $NO_3\cdot$ oxidants is between 5 minutes and 6 hours (Meija et al., 2002) and products of their reduction are CH_3-Se(O)-CH_3 and ionic methylates (Rael and Frankerberger, 1996). It was demonstrated by Guo and co-workers (2003) that volatile methylated and ethylated species Se species could also be produced by ultraviolet radiation from inorganic Se in the presence and low molecular weight organic acids.

Total Se concentrations in ambient waters are generally below 0.1 μg/L and in sea and fresh water 0.05 μg/L, where most of total Se is present as organic-Se: volatile DMSe, MDMSe and DMSeS (Wallschläger and Feldmann, 2010). High or toxic concentrations of Se in surface waters are relatively rarely found, although, irrigation of seleniferous soils can affect downstream concentrations of Se (McDowell, 2017). Groundwater concentration of Se is usually higher and is controlled by primary source, mobilization and retention at water-soli interface, transport in solution and concentration in groundwater under semiarid and arid conditions (Nicolli et al., 2012).

Soil Se content varies depending on the main parent material of the soil, with extrusive volcanic rocks (120 μg/g) and sedimentary rocks, such as in shales, and sandstone uranium deposits (1000 μg/g) having the highest content of selenium (Shamberger, 1981). Concentration of Se usually varies between 0.01 and 2 mg/kg with a world mean average of 0.4 mg/kg (Sharma et al., 2015). The highest concentration of Se in soil ever reported was probably in Ireland being around 1200 mg/kg (Fleming, 1962) and lowest in soils of New Zealand, Finland and Sweden with as low as 0.005 mg/kg (Underwood and Suttle, 1999). If soil contains enough Se for vegetation growing on it to accumulate it and become toxic to animals or humans it is considered seleniferous (Dhillon et al., 2019). When assessing and

labelling soils as seleniferous factors besides total Se content must be taken into consideration (Winkel et al., 2012).

Bioavailability of Se in soil is the most important parameter for Se entering the food chain and factors as pH, redox conditions and other ions, soil texture and other properties of soil all control its bioavailability. For example, in sandstones >80% of total Se is in water soluble form and hence available to plants whilst in pyritic phosphoric rocks that number is only 0.6% (Malisa, 2001). Presence of soil organic matter (SOM) is one of the factors that can negatively effect and reduce bioavailability of Se (Li et al., 2017). In most soils, water soluble selenate and selenite are the primary form of Se (McNeal and Balistrieri, 1989). If soil pH is alkaline or circumneutral and conditions are highly oxidizing, selenate is favoured form of Se (Geering et al., 1968). At acidic pH with the presence of iron oxide and organic matter selenite is less available than selenate because it has greater affinity of adsorption onto soil particles and becoming immobilized (Geering et al., 1968). In anoxic and reducing environment of soil, with high levels of organic matter both selenite and selenate can be reduced to Se^0 and Se (-2) precipitates (e.g., FeSe, $FeSe_2$) (Sharma et al., 2015) and thus become unavailable to plants. If organic matter in soil is scarce Fe/Al oxy-hydroxides will usually control Se adsorption (Floor and Román-Ross 2012).

1.1.1 Selenium in living organisms

1.1.1.1 Selenium in plants

Se is not an essential element for higher plants (Terry et al., 2000), but it can stimulate growth at low levels (Pilon-Smits et al., 2009). At high levels, Se is toxic for plants because it gets incorporated in proteins impairing their normal function, and it has also been suggested that it causes oxidative stress (Stadtman, 1990; Van Hoewyk, 2013).

Since Se and S have chemical similarities they are associated in the metabolism of plants, although Se atoms are larger so the Se-Se bond is longer and weaker than that of S-S (Sors et al., 2005). Se is taken into plants mostly in a form of selenate (Sors et al., 2005), but, besides chemical form, uptake also depends on soil pH and rhizosphere processes (Blaylock and James, 1994). It is thought that uptake of selenate and organic-Se is an active process (Shrift and Urlich, 1969), while selenite enters plant through passive diffusion (Arvy, 1993) and unlike selenate does not accumulate in roots (Läuchli, 1993). Selenate is taken into plant with high-affinity sulphate transporters in root epidermal cells (Shibagaki et

al., 2002). Se concentrates in different ways in different plants. After uptake, selenate has to be reduced, first to selenite and selenid and then assimilated into selenocysteine (SeCys), selenomethionine (SeMet) or other organic-Se compounds as it can be seen in Figure 2 (Pilon-Smits, 2014). By volatilizing SeMet into DMSe and breaking down SeCys into Se^0 and alanine plants can avoid toxic effects of Se (Terry et al., 2000; Van Hoewyk et al., 2005).

It is thought that plants ability of tolerating high levels of Se lays in diverting seleno-amino acids, SeCys and SeMet from incorporating proteins by changing them to non-protein amino acids - Se-methylselenocysteine (MeSeCys), γ-glutamyl-Se-methylselenocysteine (GGMeSeCys) and selenocystathionine (Dunnill and Fowden 1967; Brown and Shrift 1981; Burnell 1981) (Figure 2). Hyperaccumulator species of plants can additionally methylate SeCys into methyl-SeCys and avoid incorporation in proteins (Neuhierl and Böck, 1996).

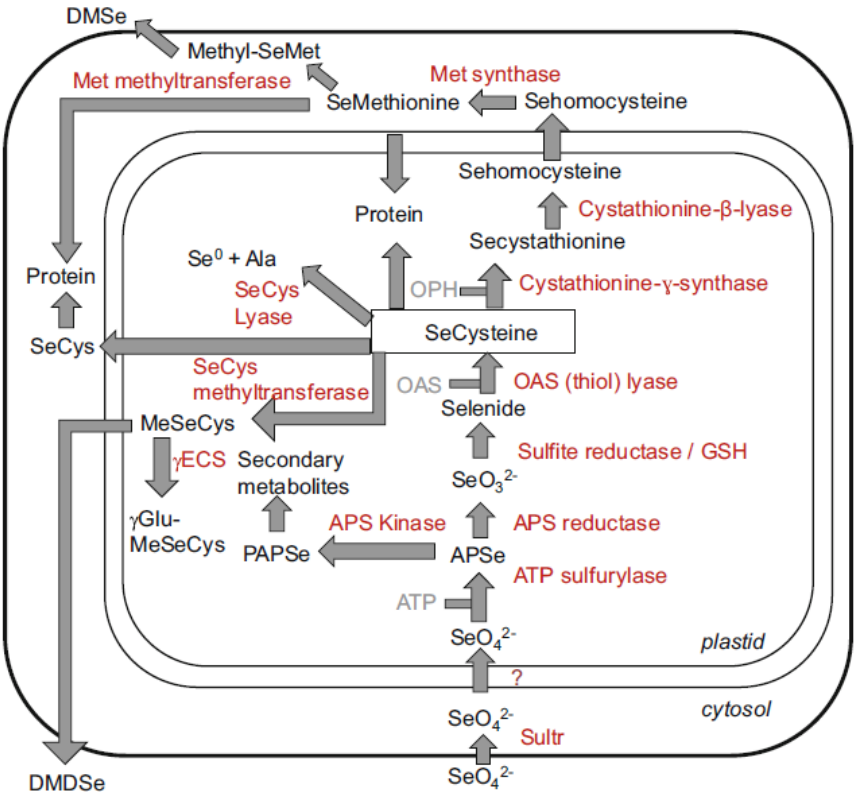

Figure 2. Assimilation of Se in plants (Taken from Pilon-Smits, 2014).

1.1.1.2 Selenium in other organisms

First written symptoms of effect of Se on animals were reported in 13th century by Marco Polo in Western China, about disease syndrome in horses after grazing (Dhillon et al., 2019). In 1857 Dr.T.C. Madison described clinical signs of so-called "alkali disease" after horses were grazing near Fort Randall (USA). Almost 80 year later toxicity symptoms described by Polo and Madison were attributed to Se. It was confirmed that levels of 5-12 ppm of Se in wheat samples are toxic (Robinson, 1933) and that grains grown on certain soils and containing high concentration of Se caused development of "alkali disease" in livestock (Franke, 1934; Franke and Potter, 1935). Few years later, perception of Se as toxic and undesirable started changing with reports about its beneficial and essential role in organisms. Poley and co-workers in 1941 discovered that chicks fed with diet with 2 ppm of Se gained more weight than the ones without Se supplementation. In 1957, two laboratories discovered independently that, what was until then called "Factor 3" and prevented laboratory rats from developing liver necroses and exudative diathesis in chicks, was actually Se (Schwarz and Foltz, 1957; Patterson et al., 1957). In 1973 it was discovered that Se was the component of the enzyme glutathione peroxidase (GSH-Px), and essential for its function (Rotruck et al., 1973) and selenoprotein-A was found in bacterium *Clostridium sticklandii* (Turner and Stadtman, 1973). Year later, it was discovered that 0.1 ppm of Se was required to maximize GSH-Px activity and any concentration of Se above that did not had any additionally effect. In 1978, selenium in GSH-Px was identified as amino-acid SeCys (Forstrom et al., 1978).

Later on, more and more proteins that contained Se were being discovered: formate dehydrogenase (Zinoni et al., 1987), iodothyronine 5′-deiodinase (Behne et al., 1990) which catalyses deionization of thyroxine (T4) to triiodothyronine (T3), selenoprotein-P (Read et al., 1990), selenoprotein W (Vendeland et al., 1995), thioredoxin-reductases (Zhang and Gladyshev, 2009) which reduces oxidized thioredoxin and selenoprotein-S (Papp et al., 2007) are among some. Overall, there are 25 proteins in human proteome that have Se in their structure (Papp et al., 2007) and 24 in rat and mouse (Barnes et al., 2009). Most of them are enzymes and directly included in antioxidant defence of organism and elimination of reactive oxygen species (ROS), but some proteins have other functions like selenoprotein-K which is involved in calcium flux in immune cells (Verma et al., 2011) or selenoprotein-V and H which have parts in signalling pathways (Roman et al., 2014).

Selenium availability for organism directly depends on its content in food sources and diet. Primary form of selenium ingested by humans is SeMet, followed by SeCys, selenoneine, MeSeCys, GGMeSeCys, sodium selenite and selenate (Rayman, 2012). After digestion, different small molecular weight Se-compounds are formed (Avery and Hoffman, 2018). Each one of these compounds has very distinct profile of biological activity and reactivity and can act as an electrophile, nucleophile or radical and hence have antioxidant or prooxidant effect in cell (Alhasan et al., 2019). SeCys, also called the 21st amino-acid, is incorporated in proteins involving insertion sequence called SECIS (Zinoni et al., 1990), while SeMet is randomly incorporated into proteins instead of methionine (Lazard et al., 2017). SeCys and SeMet have selenol group (-SeH) in their active site which is responsible for their function (Pilon-Smits and Quinn, 2010) and all selenoproteins, except selenoprotein-P, contain at least one SeCys residue (Roman et al., 2014). Physiologically relevant oxidation states of Se range from -2 to +4 so reduced species, as H_2Se and organic selenols (RSeH) with reducing capabilities, and oxidised SeO_3^{2-} and organic seleninic acids (RSe(O)OH) with oxidising capacities, can be found. Some Se species such as selenol(ate)s are capable of complexing metal ions like Cu^{2+}, Hg^{2+}, Cd^{2+}, Ag^{2+}(Aaseth et al., 2016). It was discovered that selenite and selenocystamine induce apoptosis and are cytotoxic (Stewart et al., 1999). In 2010, Se compound called selenoneine was identified in blue tuna (*Thunnus orientalis)* (Yamashita and Yamashita, 2010). It is described as analogue of ergothioneine, derivative of the amino acid L-histidine, and exhibits excellent ability of scavenging free radicals (Yamashita and Yamashita, 2010). Because of effectiveness of Se-compounds and their beneficial role in organisms, design and development of synthetic Se compounds has been gaining attention. One of them is ebselen, GPx-like antioxidant which can act as a neuroprotector and have role in protection against cardiovascular diseases (Azad et al., 2014).

When it comes to selenium, animal models show “U”-shaped efficacy curve, meaning that both deficiency and redundancy of Se in organisms will have negative consequences (Ferguson et al., 2012). It was shown in 1996 that abnormalities in genes coding selenoproteins are producing non-functional proteins and can result in embryonic lethality (Matsui et al., 1996). Beck has proven in 1997 that in hosts deficient in selenium or vitamin E coxsackie virus B3 induced change in viral phenotype so that anti-virulent strain became virulent and virulent strain became more virulent. Se deficiency can also result in development of Keshan disease, lethal cardiomyopathy, and can promote osteochondral

disease (Avery and Hoffman, 2018). Disrupt levels of Se intake are associate with numerous other conditions including cancer, diabetes, different cardiovascular and immune disorders and thus increased risk of mortality. On the other hand, if supplementation with Se exceeds the needs of organism it might increase risk of developing other conditions, like type-2 diabetes (Rayman, 2012).

1.2 Earthworms

1.2.1 Biology and ecology of earthworms

Earthworms belong to Annelida phylum, Clitellata class and Oligochaeta subclass. In Latin, anellus means “little rings”, which describes earthworms’ appearance with visibly segmented body of up to 150 segments. The most anterior, mouth segment, is called prostomium or acron, and the last, anal segment, is called pygidium. Both prostomium and pygidium are not considerate annulets, since they are not developed from annulet-growth area placed ahead of pygidium, and both of them do not have coelom. Each segment has four pairs of bristles with 1-25 chaete in each. Chaete are produced by follicle in epidermis and are mostly made of chitin. Epidermis in earthworms is single-layered and contains many mucus glands which help in covering epidermis with thin cuticle. There are two types of muscles under epidermis: circular muscles and longitudinal muscles (Figure 3). Additionally, underneath those two types, there is also layer of traverse muscles. Peristaltic contractions of muscles enable earthworms moving both forward and backwards, and completely separated segments with constant volume ensure diameter change is followed by change in length. Chetae on the sides help fix the animal during moving (Habdija et al., 2011).

Space between muscles and gut is called coelom (Figure 3). Coelomic cavities in segments are separated from each other by vertical partitions or dissepiments and connected only with perforations, allowing flow of coelomic fluid between segments. Dorsal pores (coelomopores) located between segments connect coelom with the environment. Coelomic cavities are coated with cilia covered mesothelium (Habdija et al., 2011).

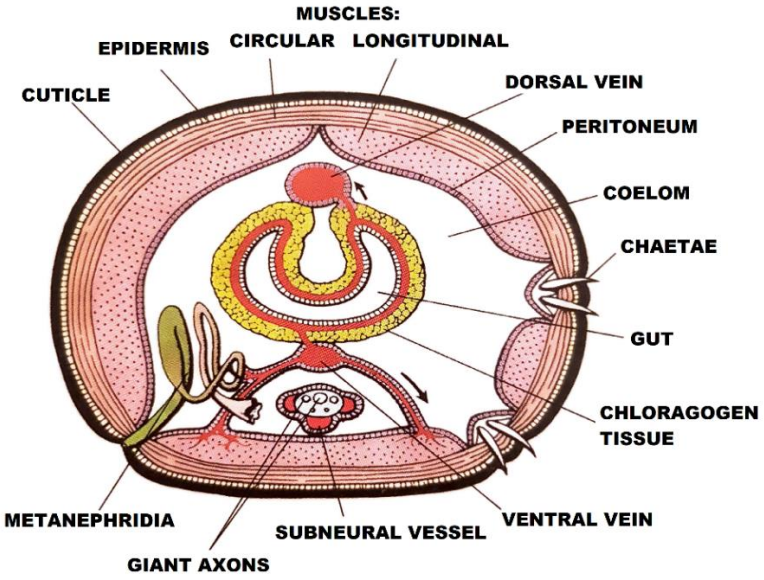

Figure 3. Cross-section of earthworm *Lumbricus terrestris* (Taken and modified from Habdija et al., 2011).

Nervous system of earthworms is segmented like the rest of the body. Cerebral pear-shaped ganglia pair is located above pharynx in third segment. From them to the front protostomial nerves stretch-out, and to the back two connectives forming ring around pharynx, which connects to the pair of ventral nerve fibres. Nerve cord consists of five giant axons – three on dorsal and two on ventral side. Additionally, every segment has a pair of ganglia from which periphery nerves emerge. Earthworms have photo-receptors in epidermis dispersed all over their body, but especially concentrated on the front and on the dorsal side. The other receptors they have are chemoreceptors which are amass above cuticle, forming small lumps placed in three rings around every segment. They are most numerous on the front part of the body (Habdija et al., 2011).

Most species of earthworms are detritivores and their gut is simple tube. One of the features they have is calcite-crystals-excreting Morren gland located in oesophagus. These crystals most likely take part in eliminating carbon dioxide (CO_2) accumulated in organism during feeding. Typhlosole, wilting on the dorsal side of the gut, enlarges gut surface. Chloragogen tissue is placed around the gut and from it chloragogen cells are detached from and released into coelom. Circulatory system of earthworms is made out of few pairs of side-hearts derived from veins, and respiration is done through skin. Adult earthworms have metanephridial excretion system with nephrostome in every segment opening to the outside. Clitellum, by which the class got its name, is reproductive structure made of 2-60 segments.

It has mucus glands, glands excreting cocoon and glands excreting albumin to feed embryo (Habdija et al., 2011).

Earthworms are ubiquitous organisms, classified as macrofauna of soil. Order Oligochaeta includes more than 8000 species in 800 genera (Edwards, 2004). Earthworms can be found living in most types of soils, except in soils of extreme climates, such as deserts. Lumbricidae and Megascolecidae are, ecologically, the most important families in North America, Europe, Australia and Asia (Coleman and Wall, 2015). Earthworms can range between few millimetres to two meters in length and between ten milligrams to a one kilogram in weight, with larger species usually found in southern latitudes (Edwards, 2004). The record species was, what was believed to be *Microchaetus* sp., 7 meters long and 75 mm in diameter (Lungström and Reinecke, 1969, reported by Edwards, 2004). Life cycle of earthworms can be as long as 10-12 years, but because of wide range of predators they usually live only 1-2 seasons (Edwards and Bohlen, 1996).

In temperate regions earthworms are active mainly in the spring and autumn. During winter and dry periods of summer they penetrate deeper into soil in order to hide from adverse temperatures. Cocoon production is usually seasonal with the most cocoons produced in spring or early summer and with one smaller peak in autumn. Number of cocoons varies from 1 to 20. Cocoon production and growth of earthworms are positively corelated with temperature, but hatching percentage and number of hatchlings per cocoon are negatively corelated with temperature (Edwards and Shipitalo, 1998). Also, some species can produce cocoons parthenogenetically (Edwards, 2004).

Lee (1985) and Bouché (1977) grouped earthworms into functional categories based on their morphology, behaviour, feeding ecology and their microhabitats in soil. The three primary functional categories of earthworms are (Figure 4):

- Epigeic - these species live in the litter and produce casts at soil surface. They usually have darker pigments and are smaller than earthworms of the other two groups. Some species from this group are *Eisenia andrei, Dendrobaena octaedra* and *Lumbricus rubellus.*
- Anecic - earthworms of this group live in vertical burrows connected with the soil surface and are less pigmented than the epigeic ones. Representatives include *Aporrectodea rosea, Aporrectodea caliginosa* and *Octolasion lacteum.*

- Endogeic – this category includes species that live in randomly oriented burrows and are bigger in size and pigmented on the dorsal side of the body, like *Lumbricus terrestris* or *Aporrectodea longa*.

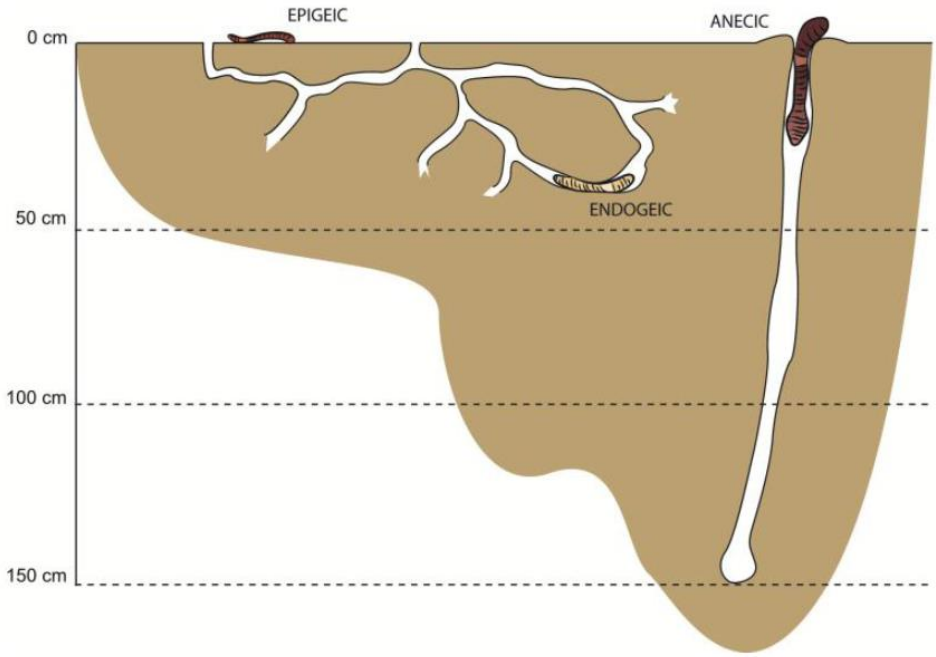

Figure 4. Illustration of earthworm ecology categories based on their microhabitat in soil (Taken from Schelfhout, 2010 as read in Schelfhout et al., 2017).

Earthworms have the most abundant animal biomass in most terrestrial ecosystems (Lavelle and Spain, 2001) but diversity of species in any site rarely exceeds ten species, with usual average of three to five species (Edwards, 2004). One of the first to raise attention to importance and beneficial effect of earthworms on soil was Darwin (1881) in his book *The Formation of Vegetable Mould through the Actions of Worms, with Observations of Their Habits,* where he noted that earthworms move large amounts of soil from deeper parts to surface. Today, earthworms are recognised as one of the most important of all soil fauna, and are considered ecosystem engineers affecting soil in numerous ways. Some of the effects include changing mineral and organic composition, affecting nutrient cycling, altering hydrology regimes and drainage and affecting plant population dynamics and community composition by burrowing, mixing and casting (Jones et al., 1994).

Earthworms are included in soil formation and turnover, organic matter breakdown and its' incorporation into soil. They help aerate the soil by creating macropores of 1-10 mm

in size (Coleman and Wall, 2015). By ingesting soil and organic matter, they colonize it with microbes from their guts which help break down soil organic matter (SOM). After that, they excrete casts in burrows in the soil or on the soil surface, and create "drilosphere" – zone in which soil is enriched with nutrients compared to the rest of the soil (Coleman and Wall, 2015). Drilosphere system influences soil microbial community and nutrient dynamics (Lavelle et al., 1998). Turnover rate of soil through casting ranges between 40-70 t/ha/y and 500-1000 t/ha/y (Bouché, 1983; Lavelle et al., 1992). Furthermore, earthworms can affect availability of nutrients (Edwards, 2004). Although most of the ways earthworms affect soil are beneficial, some of them might be undesirable. These include transmitting pathogens, being a vector for the spread of diseases, removing protective soil surface residues resulting in loss of N and C and enhanced microbial respiration (Coleman and Wall, 2015).

1.2.2 Immune system of earthworms

1.2.2.1 Cellular defence mechanisms

The first defence barrier of earthworms against pathogens is their skin. Besides supporting cells, it contains secretory and basal cells, among which latter are included in wound healing and exert phagocytic activity. They are considered to be homologous to coelomocytes (Burke, 1974a-c). Skin cuticle serves as an antimicrobial barrier (Rahemtulla and Løvtrup 1974) and mucus secreted by different types of secretory cells and epidermal glands contains antibacterial factors (Valembois et al. 1984, 1986, 1988).

Interest for earthworm's leukocytes, i.e. coelomocytes, started in 19th century (Kükenthal, 1885; Rosa, 1896, as read in Liebmann, 1942). Rosa (1896) did a comprehensive morphological study on the lumbricid coelomocytes. Coelomic fluid of earthworms contains different types of coelomocytes. Stein et al. (1977) differentiated 7 types in *Lumbricus terrestris* earthworm and segregated them based on their morphology and phagocytic properties in: basophils, type I and II acidophils, granulocytes, type I and II chloragogen cells and transitional cells. They concluded that basophils were predominant cells in coelomic fluid and all types of cells, except the chloragogen cells, express phagocytic abilities. A year later, Stein and Cooper (1978) did a cytochemical characterization of the coelomocytes to determine biochemical composition of coelomocyte types. Basophils were characterized as heterogenous regarding the size and inclusions in cells. Inclusions characteristic for basophils include glycogen-containing vacuoles, lipid-filled vesicles and

phagocytic vacuoles, but their number is individual for each cell. Glycogen in basophils is contained in large vacuoles, while in neutrophils and chloragogen cells it is found in smaller amounts and in small granules or dispersed through cytoplasm. Basophils and neutrophils contain lysosomes filled with enzymes acid-phosphatase and β-glucuronidase (De Duve et al., 1962). Even though similar to basophils, neutrophils contain smaller amounts of lipid, less cytoplasmic RNA than basophils and their nucleus is larger and less heterochromatic. They have also been characterized as having role in destruction of foreign tissue graft (Linthicum et al., 1977). Granulocytes and type I and II acidophiles all contain neutral glycoprotein in granules. Both types of chloragogen cells contain vesicles abundant with lipids, and some granules contain ferrous and ferric iron. Their cytoplasm contains glycogen, protein and mucopolysaccharides.

In later years, coelomocytes were put into three main subpopulations: eleocytes (chloragogen cells), hyaline amoebocytes (basophils and neutrophils) and granular amoebocytes (acidophils, granulocytes) which all have phagocytic ability (Figure 5). Detailed coelomocyte functions are presented in Figure 6 (Cooper, 1996). In recent years approach to characterization of subpopulations of coelomocytes is based on immunological markers (Engelmann et al., 2004, 2005), which has confirmed previously identified subpopulations.

General name	Light microscopy designation (Wright's stain)	Electron microscopy designation
Hyaline amoebocytes	1. Basophils	Lymphocytic coelomocytes of 2 types
	2. Neutrophils	Type I granulocytes
Granular amoebocytes	3. Acidophils (2 types)	Inclusion-containing coelomocytes
	4. Granulocytes	Not identified
	5. Not identified	Type II granulocytes
Eleocytes	6. Chloragogen cells (eleocytes) (2 types)	Chloragogen cells (eleocytes) (1 type only)

Figure 5. Coelomocytes present in *Lumbricus terrestris* (Taken from Cooper, 1996).

Function	Coelomocyte type	Species studied
Phagocytosis	Amoebocytes	Species from many families
	Eleocytes	*Eisenia foetida*
Encapsulation and formation of "brown bodies"	Amoebocytes	Many species
	Eleocytes[a]	(various families)
Wound closure (formation of wound plug)	All coelomocytes	Lumbricidae
Wound healing and regeneration (general)	Amoebocytes	*Eisenia foetida*
	Eleocytes	*Eisenia foetida*
Formation of substratum for regenerating epidermis during wound healing	Hyaline amoebocytes	*Eisenia foetida*
Graft rejection	Amoebocytes	*Lumbricus terrestris*
	"Splanchnopleural macrophage"[b]	*Eisenia foetida*
	Type I granulocyte[b]	*Lumbricus terrestris*
	Hyaline amoebocytes	*Eisenia foetida*
	"Small basophil"[b]	*Eisenia foetida*
Cytotoxicity	Unspecified	*Lumbricus terrestris*
		Eisenia foetida
	Eleocytes[c]	*Eisenia foetida*
	Amoebocytes[d]	
Possible blood cell precursors	Amoebocytes	Lumbricidae (several species)
		Pheretima indica
Possible precursor cells for epidermal basal cells and inter-muscular granule-containing cells	Hyaline amoebocytes	*Eisenia foetida*
Hemoglobin production	Hemoglobin producing cell	*Eisenia foetida*
Hemopoiesis	Eleocytes	*Eisenia foetida*
Nutritive ("trephocytic")	Eleocytes	Species from many different families
Association with developing eggs (possible nutritive function	Lamprocytes	*Enchytraeus fragmentosus*
Excretory	Eleocytes	Species from many families

[a]Probably passively involved.
[b]Subtype of hyaline amoebocytes.
[c]Nonspecific, naturally occurring.
[d]Specific, acquired.

Figure 6. Functions of different coelomocyte types (Taken from Cooper, 1996).

Microorganisms can enter coelomic cavity via dorsal pores (coelomopores). Number of bacteria naturally occurring in coelom is 0.9 x 10^5 per worm of average size. Their outgrowth is prevented by phagocytotic cells whose number is ten times higher than the number of microorganisms. If the number of bacteria is too high for effective removal exotoxins excreted by them additionally reduce earthworm's fitness and induce swellings (Dales and Kalaç, 1992). Earthworms have a few nonspecific reactions to foreign particles (Cooper, 1996) and their fate in coelomic cavity depends on their size – small particles such as bacteria are phagocytosed by coelomocytes in coelomic cavity, and larger particles, such as agglutinated bacteria or large foreign bodies are encapsulated, creating, what is called, the brown body. Its formation was described by Valembois and colleagues (1992) in experiment on thousand *Eisenia fetida andrei* earthworms. Formation of the brown body depends on the volume of particles and minimal volume is required to start the process initiated by the contact of particle with coelomocytes. Initial nodule of aggregated bacteria, gregarines, graft fragments and self-structures, such as setae or necrotic muscle cells, increases in volume because of the aggregation of coelomocytes and waste particles around it. When its size reaches 1-2 mm it loses adhesiveness towards free coelomocytes and its pigments, lipofuscin and melanin (Valembois et al., 1994), get darker, and from that, brown body got its' name. In the end, it gets eliminated by autotomy of posterior segments (Keilin, 1925). Besides this type of removal, microorganisms can also be eliminated by excretion via nephridia (Cameron, 1932), be engulfed by nephrostome or middle tube cells (Villaro et al., 1985).

1.2.2.2 Humoral defence mechanisms

Besides coelomocytes, coelomic fluid is comprised of other molecules with different biological activities, like lysozyme (Josková et al., 2009) and antimicrobial peptides (Cho et al., 1998; Liu et al., 2004). Lysozyme is bacteriolytic enzyme that catalyses hydrolysis of β-(1,4)-glycosidic bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (GlcNAc) of peptidoglycan of bacterial cell wall (Imoto et al., 1972). Lysozyme from earthworm *Eisenia andrei* (EALys) has high homology with other invertebrate lysozymes, among which highest with medicinal leech. Increase in its mRNA levels in coelomocytes are induced after challenge with both gram-positive and gram-negative bacteria (Josková et al., 2009). Antimicrobial peptides are diverse group of molecules in invertebrates, vertebrates and plants (Bilej et al., 2010). First earthworm antimicrobial peptide was isolated from *Lumbricus rubellus* and was named lumbricin I. This proline-rich peptide of 62 amino

acids is expressed constitutively, regardless of presence of bacterial infection, but only in adult individuals (Cho et al., 1998). Similar peptide was found in Asian earthworm *Pheretima tschiliensis,* and named PP-1. It shares 77,6% homology with lumbricin I and is synthetized only in body wall and localized in the mucus of the epidermis. Immunohistochemical studies suggested its role in mucosal defence. Bodó et al. (2018) identified lumbricin and lumbricin-related mRNA in *Eisenia andrei,* which are phylogenetically close to other annelid lumbiricns. Antimicrobial peptide called OEP3121 was found in the earthworm *Eisenia foetida* (Liu et al., 2004).

Haemolytic activity of coelomic fluid was first described in 1968 in form of hemolytic factor called *Eisenia andrei foetida* factor - EFAF. It was suggested that it has lipoproteinic composition and that it is released by chloragogen cells and eleocytes (Du Pasquier and Duprat, 1968 as reported by Roch et al., 1981). It was later found that EFAF consists of 2 glycosylated proteins of 40 kDa and 45 kDa and that two proteins constitute polymorphic system (Roch et al., 1981). 45-kDa protein is monomorphic while the 40-kDA one can exist in 4 isoforms (Roch et al., 1981). These proteins bind to sphingomyelin in plasma membranes and then polymerize forming open channels in membrane (Roch et al., 1989). They can agglutinate red blood cells (Roch et al., 1984) and are an important part of coelomic fluid cytotoxicity (Bilej et al., 1995). In *Eisenia fetida,* six different haemolytic isoforms were found which are result of polymorphism (Roch et al., 1987). In later years, EFAFs were named fetidins and it was found that cDNA of 40-kDa fetidin contains peroxidase signature and that fetidins from coelomic fluid have peroxidase activity (Lassegues et al., 1997).

In 1996 two proteins from coelomic fluid were isolated. One, size of 41 kDa, was called lysenin and it was discovered it can cause contraction of isolated rat aorta and kill rats upon intravenous injection. The other was size of 42 kDa and resembled lysenin but has shown no contractive activity (Sekizawa et al., 1996). Lysenin is expressed in large coelomocytes and free large chloragocytes present in the lumen of typhlosole but not in peripheral chloragocytes (Ohta et al., 2000). Immunohistochemical and immunofluorescent staining confirmed expression of lysenin in free-floating chloragocytes (Opper et al., 2013). In research with red blood cells and artificial liposomes it was shown that lysenin recognizes sphingomyelin and assembles to oligomers which leads to formation of pores with ~3 nm diameter (Yamaji-Hasegawa et al., 2003). Since bacteria do not have sphingomyelin in their membranes, its antibacterial activity must have different mechanism of action than the one

of its cytolytic activity (Bruhn et al., 2006). Lysenin has a common ancestry with prokariotic and eukariotic proteins which are responsible for pore-forming (De Colibus et al., 2012). Another pore-forming protein called eiseniapore was found in *Eisenia fetida.* Similar as lysenin it requires sphingomyelin or galactosylceramide to induce lysis by forming oligomers and creating pores (Lange et al., 1999). Eue et al (1998) discovered 3 hemolytic proteins from coelomic fluid of *Eisenia fetida* called H_1, H_2 and H_3, with hemagglutinating (H_3) and lytic activity (H_1, H_2, H_3). Koenig et al. (2003) isolated two proteins from coelomocyte lysate called CL_{39} and CL_{41} and has shown the identity of CL_{39} and $H_{1\text{-}3}$ hemolysins with fetidin and CL_{41} with lysenin.

In experiments with TNF-sensitive tumor L929 cell line, lytic protein called coelomic cytolytic factor – CCF was isolated. CCF acts as a pattern-recognition molecule contributing to non-self-recognition by identifying surface molecules of pathogens. It displays analogy with mammalian tumor necrosis factor (TNF) and is responsible for 40% of the cytolytic activity of coelomic fluid against L929 cell line (Bilej et al., 1995). Although similar in functional way, TNF and CCF do not have gene or amino acid sequence homology (Beschin et al., 1999). CCF binds to cell wall components of gram-negative bacteria (O-antigen of lipopolysaccharide), gram-positive bacteria (the peptidoglycan constituents muramic acid and muramyl dipeptide) and yeast (b-1,3-glucans and N, N0 - diacetylchitobiose) (Šilerová et al., 2006). Binding to cell wall components triggers activation of prophenoloxidase cascade and formation of cytotoxic and antimicrobial compounds (Bilej et al., 2010). It results in melanin production and formation of aforementioned brown body, and highly reactive and toxic quinone intermediates (Cerenius and Soderhall, 2004). CCF molecules were identified in 6 different Lumbricidae species, both on protein and mRNA level, and it was found that CCF from *Eisenia fetida* has the broadest saccharine binding specificity (Šilerová et al., 2006). That characteristic can be attributed to the environment it lives in – rich in organic matter and consequently in microorganisms.

1.3 Effect of selenium on earthworms

Currently, there is not much research on effect of selenium on the earthworms or the soil animals in general. One of the first studies was by Serda and Furst (2002), as said in Furst (2002) who reported LD50 values of selenite and selenate in earthworms of 31 and 60 mg/kg, respectively. It was proven that *Eisenia fetida* can tolerated 100 mg/kg of selenite without lethal effect but juvenile mass gain and adult cocoon production are affected.

Maximal accumulated capacity measured at sublethal concentration in long term study for Se was 337 mg/kg. Reported loss of accumulated Se content after 8-week recovery period was 5-6% (Fischer and Koszorus, 1992). Xiao et al. (2018) examined the differences of accumulation of selenite between *Pheretima guillemi* (anecic species) and *Eisenia fetida* (epigeic species) after 28-day exposure to soil spiked with 0.5, 5, 50, and 200 μg of Se per g of soil. Earthworms Se concentrations were increasing with increase of soil Se concentrations with maximum dry weight concentrations at 54.6 μg/g for *Pheretima guillemi* and 83.0 μg/g for *Eisenia fetida*, at highest Se soil concentration. They also report inhibition of earthworm growth and species-specific response to exposure to Se, with BAFs (bioaccumulation factors - ratio of Se concentration in earthworm to that measured in soil) of *Pheretima guillemi* higher in low-dose Se-spiked soil while in *Eisenia fetida* it was the other way around. Earthworms may concentrate Se from soil up to a factor of about 100 times, and even though it may not be lethal for them, it presents hazard to animals on the higher levels of food chain feeding on earthworms (Beyer (1987) as said in Nielsen and Gissel-Nielsen (1975)).

Examining effect of municipal sewage sludge on concentration of different elements in earthworm *Aporrectodea tuberculata* it was found that earthworms taken out of soil with sludge contained from 15-22 mg Se/kg dry weight, which was decrease in Se concentrations comparing to earthworms taken from control field, while concentrations of cadmium (Cd), copper (Cu) and zinc(Zn) increased (Helmke et al., 1979). Possible explanation for this is antagonistic effect between Se and other heavy metals (Schrauzer et al., 1977). Antagonising effect of Se on the acute toxicity was also confirmed for mercury and cadmium (Furst, 2002). In experiments on the distributions of metals (Cd, Zn, Cu, Fe) in tissues of earthworm *Allolobophora caliginosa* it was shown that metals mostly accumulate in encapsulating chloragogenous tissue (Carpenè et al., 2006).

Chronic toxicity test of selenate and selenite on the potworm *Enchytraeus albidus* (Annelida: Enchytraeidae) indicates selenate as more toxic form of Se in mortality and reproduction test, with LC50 of 5.69 mg/kg dry weight for total Se and 4.74 mg/kg dry weight for available Se in average, in comparison with selenite where LC50 was 22.5 mg/kg dry weight for total Se and 8.10 mg/kg dry weight for available Se in average. The reproduction was more sensitive to Se toxicity than the response in mortality with EC50 of selenate about one order of magnitude lower (0.41 mg/kg dry weight for total Se and 0.28 mg/kg dry weight for available Se in average) than that of selenite (7.3 mg/kg dry weight for

total Se and 2.46 mg/kg dry weight for available Se in average). In juvenile test, both forms of Se had a fewer juveniles produced than the control, with selenate being more toxic form (Somogyi et al., 2007). In later research of adult mortality (LC50) and juvenile production (EC50) on the same species, same group of researchers report *Enchytraeus albidus* as most sensitive animal species to selenate and selenite status of the soil ever tested before. When total concentrations were considerate, selenate has proven to be more toxic than selenite, while no difference was observed for available concentrations (Somogyi et al., 2012). In experiment on effects of selenite and selenate on plant-soil-earthworm system, selenite reduced activities of acetylcholinesterase (AChE) at concentration of 0.1 mg of Se per kg, while selenate did not cause any changes. At lowest concentration, both forms of Se induced AChE, although the difference was not significant. At concentration of 0.1 mg/kg, both concentrations of Se caused decrease in catalase (CAT) activity, while no significant changes in activity of glutathione-S-transferase (GST) were observed (Štolfa et al., 2017). Petek (2017) confirmed previously discovered fact that selenite is more toxic form of Se for *Eisenia andrei* species, and that exposing earthworms to Se results in changed oxidation status in earthworms. Both forms of Se induce glutathione reductase (GR) activity while reducing the one of superoxide dismutase (SOD). Selenite and selenate affect reduced and oxidized glutathione (GSH/GSSG) ration differently, indicating different activity mechanisms of two forms of Se.

1.4 Oxidative stress and ROS

Reactive oxygen species (ROS) are a by-product of every aerobic metabolism and biochemical reactions in cells. They are small, but very reactive, oxygen-containing molecules. At small concentrations their role in cells is beneficial, since they are included in normal physiological processes, such as intracellular signalling pathways and response to growth factor stimulation (Finkel, 2011). When their concentration in cell reaches certain point, cells' antioxidant mechanisms cannot effectively remove them and ROS accumulate in cell. This shift in dynamics of their formation and their removal by antioxidant system causes oxidative stress, which is thought to be basis of many pathological conditions in organisms (Birben et al., 2012). Source of ROS can be endogenous and exogenous. The first one is from all cellular components including mitochondria, lysosomes, nucleus, membranes and cytosol. Processes in which most of ROS are created is electron transport systems, inactivation of flavins, thiols and other small molecules and activity of different oxidases.

Exogenous sources include exposure to different environment pollutants, hyperoxic environment, pesticides, certain medications, radiation, metals and many other things (Stohs and Bagchi, 1995; Machlin and Bendich, 1987).

ROS can be divided into free radicals and non-radicals. Free radicals are species with at least one free electron, capable of independent existence. Free electron makes this species very reactive, although their reactivity can vary depending on type. Radicals can be created in a few different ways. First way is losing electron from non-radical which results in unpaired electron and positively charged radical cation. Contrary to that is creation of radical anion where non-radical gains an electron. One more way of creating radicals is homolytic fission - breaking of covalent bond. This requires a lot of energy, so consequently these types of radicals are produced by ultraviolet light, ionizing radiation and high temperature, making combustion (tobacco smoke, fire smoke) one of the many sources of ROS (Halliwell and Gutteridge, 1999). Physiologically, most important ROS are free radicals: superoxide anion ($O_2^{\cdot}$) and hydroxyl radical ($OH\cdot$) and non-radical: hydrogen peroxide (H_2O_2). Superoxide anion is formed when an electron is added to molecular oxygen, mostly in electron transport chain in mitochondria, from electrons leaked from process of reduction of oxygen to water (Birben et al., 2012). H_2O_2 is generated from superoxide anion shortly after its formation in mitochondria by superoxide dismutase (SOD) (Schieber and Chandel, 2014), but can also be produced by NAD(P)H oxidase, amino acid oxidase, cyclooxygenases, and xanthine oxidase (Birben et al., 2012). Hydroxyl radical is extremely reactive type of ROS. It oxidizes lipids in membranes, proteins and causes severe damage to DNA, including single and double strand breaks (Dizdaroglu and Jaruga, 2012). $OH\cdot$ is usually produced from H_2O_2 in the presence of metals like Fe^{2+} or Cu^{2+} in, what is called, Fenton reaction (Fenton, 1984). Basics of ROS are shown in Figure 7.

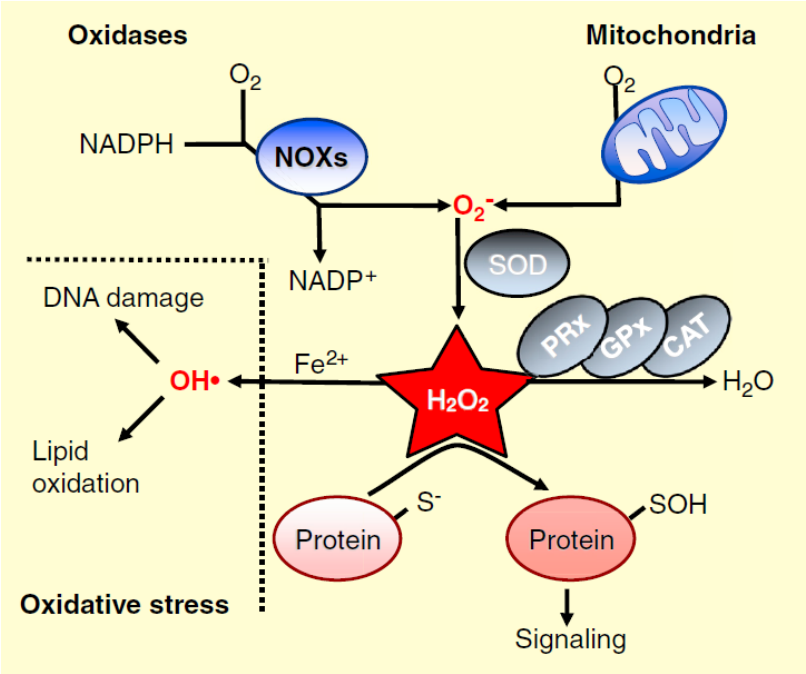

Figure 7. Basics of ROS (Figure taken and modified from Scheiber and Chandel, 2014).

Place of formation, nature of ROS and target molecules determine interaction characteristics of ROS. All cellular parts can be affected by them, but some are more susceptible than the others. These include enzymes with Fe-S clusters, 6-phosphogluconate dehydrogenase, fumarate hydratase and enzymes with thiol groups. Oxidized cysteine and methionine residues in proteins affected by ROS can be repaired to some extent, depending on the damage (Lushchak, 2011). ROS can modify DNA in several ways include degradation of bases, strand breaks, mutations, deletions, translocations cross-linking with proteins, instability of microsatellite region, methylation and other epigenetic changes. ROS can induce lipid peroxidation in lipid bilayer which disrupts membrane stability and increases membrane permeability (Girotti, 1985). Malondialdehyde (MDA) and unsaturated aldehydes created as a by-product of peroxidation can additionally damage cell components; for instance, they can inactivate proteins (Birben et al., 2012).

Antioxidant system protecting cells from damaging effect of ROS is consisted of enzymes and small molecular weight molecules (Machlin and Bendich, 1987). Enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and other redox proteins, such as thioredoxins (TRXs) and peroxiredoxins (PRXs). SOD is ubiquitous metal-enzyme, found in many cell compartments. To this day, four families of SOD have been discovered, classified depending on the metal in its centre:

Cu/Zn-, Ni-, Mn- and Fe-SODs. SOD reduces superoxide anion to produce less reactive hydrogen peroxide, which is later eliminated by different enzymes (Bafana et al., 2011). This way chance for $O_2^{\cdot}$ to react with nitric oxide to form peroxy nitrite is eliminated (Maritime et al., 2003). Catalase is a tetramer and each of its components contains heme in its active site. It destroys H_2O_2 by formation of catalase-H_2O_2 complex (compound I), and then reacting with another H_2O_2 molecule (compound II) (Halliwell, 1974). GSH-Px reduces $H_2O_2$and lipid hydroperoxides. It contains selenocysteine in active site and reduces peroxides in the presence of substrates – thiols to corresponding alcohol or water. Important for GSH-Px activity is glutathione (GSH), amino-acid tripeptide which accepts electron from ROS in this reaction, creating oxidized glutathione disulphide (GSSG). GSSG is later reduced back to GSH by glutathione reductase (GR), with NADPH as a reducer. Besides serving as a substrate for peroxidases, GSH can scavenge free radicals itself (Hayes and McLellan, 1999). Other small molecules that are part of antioxidant system, besides GSH, can be cytosolic or bound to membranes, such as vitamins. Vitamin E, tocopherol, which is lipid soluble, is part of the membrane defence mechanism from damage caused by oxidants. It adds electron to products of lipid peroxidation, that way stopping its propagation. Vitamin C also has high capacity for scavenging free radicals, especially oxygen ones (Birben et al., 2012).

Besides already mentioned ROS, there are also other types of free radicals generated *in vivo*: carbon-centred radicals which are intermediates in lipid peroxidation process, reactive nitrogen species (RNS), reactive bromine species (RBS) reactive sulphur species (RSS) and reactive chlorine species (RCS) (Halliwell and Gutteridge, 1999).

2 RESEARCH AIMS

The aims of this research were to determine the effects of two different forms of selenium on the immunological system of the earthworm *Eisenia andrei*. Main target of the investigation were coelomocytes, cells involved in primary immunological response to the pollutants and toxicants. In order to detect possible changes of the immunological system, following objectives were set:

- Determine if there is a difference in phagocytic ability of coelomocytes obtained from earthworms exposed to selenate and selenite
- Determine whether exposure to selenate and selenite causes changes in ROS production in coelomocytes
- Determine if exposure to two forms selenium cause changes in nitric oxide (NO) production.

3 MATERIALS AND METHODS

3.1 Experimental organism

Table 1. Taxonomic classification of the earthworm *Eisenia andrei*.

Kingdom:	Animalia
Phylum:	Annelida
Class:	Clitellata
Order:	Haplotaxida
Family:	Lumbricidae
Genus:	Eisenia
Species:	*Eisenia andrei*

Figure 8. *Eisenia andrei* earthworm (Photo: Magdalena Grgić).

In this research earthworm *Eisenia andrei* (Figure 8) was used as a model organism. It is recommended for use in this type of experiments by OECD (1984) if *Eisenia fetida* is not available. *Eisenia andrei* is a compost-living organism, commonly used in ecotoxicological research because of broad accessibility and low effort maintenance in laboratory. It is species with short life cycle and high production of offspring in ~5 cocoons per week (Haimi, 1990).

Earthworms were obtained from the Department of Zoology and Developmental Biology, Faculty of Sciences, University of Pécs and are acclimated to the laboratory conditions. Adult earthworms with visible clitellum were chosen for the experiment. They were taken out of the soil 24 hours before the experiment to purge/release content of their

guts and kept in clear plastic container on dampen paper, in the dark cabinet at the room temperature.

3.2 Filter paper contact test

To test acute toxicity of both selenium forms, selenite and selenate, filter paper contact test (OECD, 1984) was performed. For that purpose, flat-bottomed glass vials were used, with their total area of 105 cm^2 and entirely covered with filter paper without overlapping. Filter paper in each vial was moistened with 2 mL of selenite (SI) or selenate (SA) (both obtained from Sigma-Aldrich, USA) solution of certain concentration suspended in distilled water, or with 2 mL of distilled water for the control. Earthworms were rinsed in water, dried on paper towel and placed individually in the vials (Figure 9), covered with a metal lid, perforated to allow air flow, and placed into the dark cabinet at room temperature for duration of test, i.e. 48 hours. Exposed earthworms were inspected after 24 hours for mortality and to remove the vials with dead earthworms if any.

Figure 9. Filter paper contact test (Photo: Magdalena Grgić).

To determine concentrations of selenite and selenate which would be used in experiment a preliminary mortality test was conducted on two different occasions, with range of concentrations of 100 $\mu g/cm^2$, 50 $\mu g/cm^2$, 25 $\mu g/cm^2$, 12.5 $\mu g/cm^2$, 12.5 $\mu g/cm^2$, 6.25 $\mu g/cm^2$ for both selenite and selenate and 0 $\mu g/cm^2$ for control respectively. Number of exposed earthworms, per concentration, was 5.

3.3 Annexin V staining

After the preliminary test, additional Annexin V staining was done to examine the suitable concentration of selenate which will be used later on. Additional concentrations of selenate that were tested were 10 and 5 μg/cm² with 0 μg/cm² (distilled water) for the control. After filter paper contact test, coelomocytes of exposed earthworms were isolated, washed in Annexin V binding buffer (10mM HEPES, 140 mM NaCl, 2.5mM $CaCl_2$, pH: 7.4, Biotium) and stained with 7-aminoactiomycin D (7-AAD) in concentration of 1 μg/mL (Biotium). After incubation for 30 minutes at room temperature, 350 μL of PBS was added to cells and samples were measured/analysed with flow cytometry

Following preliminary concentration tests, annexin staining and information from previous research, concentration of 5 μg/cm² of selenite and selenate was chosen as a definite concentration to be used further on.

3.4 Coelomocyte isolation

After 48 hours of filter paper contact test with exposure of earthworms to 5 μg/cm² of selenite, selenate or water, earthworms were taken out of the vials, washed in water and dried on paper towels. After drying they were placed one by one into a Petri-dish containing 2 mL extrusion buffer (71.2 mM NaCl; 5 mM EGTA; 50.4 mM guaiacol-glycerol ether; 5% ethanol; pH: 7.3). In contact with extrusion buffer earthworms extruded yellow coloured coelomocytes through dorsal pores (Figures 10 and 11). If initially extrusion did not occur, they were gently squeezed with tweezers to stimulate release of coelomocytes. All 5 of the exposed earthworms of each concentration were placed one after another in the same Petri-dish so in the end each sample (SI, SA and control) contained coelomocytes of 5 earthworms. After isolation, 4 mL of *Lumbricus* balanced salt solution (LBSS, 71.5 mM NaCl; 4.8 mMKCl; 4.2 mM $NaHCO_3$; 1.1 mM $MgSO_4x7H_2O$; 0.4 mM KH_2PO_4; 0.3 mM NaH_2PO_4; pH 7.3) was added to coelomocytes, and consequently centrifuged for 5 minutes at 1000 rpm (Hettich Universal K2S, Germany). Coelomocyte pellet was kept and resuspended in 1 mL of LBSS.

Figure 10. Earthworm coelomocyte isolation (Photo: Magdalena Grgić).

Figure 11. Yellow coloured coelomocytes during isolation (Photo: Magdalena Grgić).

3.5 Counting of cells

To determine number of coelomocytes in each sample Trypan blue exclusion method was used. Trypan blue dye (0.4%) was added to 10 µL cells to make 20-fold dilution.10 µL of cell was placed in haemocytometer (Improved Neubauer) and cells were counted under inverted light microscope (Olympus CK-2 or CARL ZEISS ID 03). Number of cells in 1 mL of each sample was calculated by counting average number of cells in 4 outer squares of haemocytometer counter, each containing 16 smaller squares, and multiplied by dilution.

3.6 Phagocytosis assay

After cell counting, RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma-Aldrich, USA) and heated to 37 ºC was added to cell suspensions so that for every mL of the RPMI there was approximately $3x10^6$ coelomocytes. Mixture of coelomocytes and RPMI was divided into 3 wells of sterilized 24-well plate (VWR® Standard Multiwell Cell Culture Plate). 990 µL of cell suspension was added into two wells with 10 µL of FITC-coupled, heat-activated bacteria, *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (OKI II2001). In wells containing bacteria, cell to bacteria ratio was 1 to 10. Third well contained only 1 mL of cell suspension as it served as a control. Plate with coelomocytes and bacteria (Figure 12) was incubated during 24 hours, at room temperature and in the dark. After incubation, content from wells was carefully collected, washed in 3 mL phosphate-buffered saline (PBS) and centrifuged for 5 minutes at 1000 rpm (Hettich Universal K2S, Germany). Coelomocyte pellet was kept and 350 µL of PBS was added to it to prepare samples for flow cytometry. Phagocytosis assay was repeated 4 times, with 3 replicates for each measurement, to obtain sufficient number of replicates.

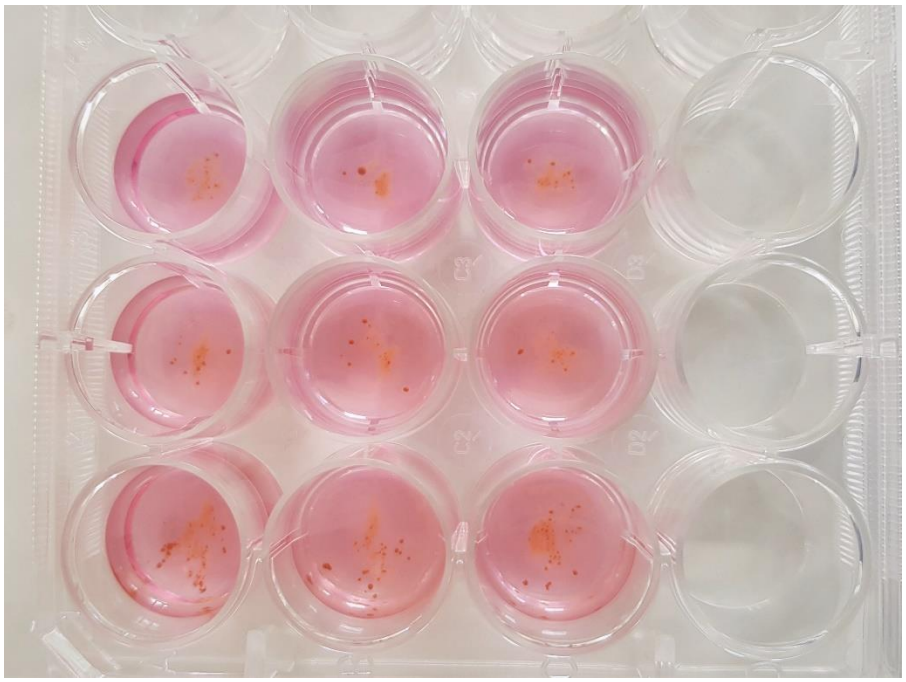

Figure 12. Incubation of coelomocytes with bacteria in RPMI (Photo: Magdalena Grgić).

3.7 DCF staining

H_2DCF-DA or 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) is a fluorescent dye used to detect ROS in living cells. This technique relies on oxidation of DCFDA to fluorescent 2',7'-dichlorofluorescein (DCF) with non-fluorescent intermediate. After isolation and counting of coelomocytes, to approximately 10 x 10^6 of coelomocytes that were obtained in each treatment sample (SI, SA and control) DCFDA (Sigma-Aldrich, USA) was added. DCFDA was prepared from stock solution containing DCFDA dissolved in dimethyl sulfoxide (DMSO). For working concentration of 10 μM which will be added to cells, DCFDA was diluted in LBSS. Suspension of cells and dye was then separated into replicates, each having 100 μL, and left for incubation for about 20 minutes in the dark at room temperature. After incubation, suspension was washed in 1 ml of LBSS and centrifuged for 5 minutes at 1000 rpm (Hettich Universal K2S, Germany). Pellet was resuspended in 350 μL of LBSS and was ready for flow cytometry analysis. Measured fluorescence of the cells was proportional to the amount of produced DCF, with dye emission at 529 nm. To obtain sufficient number of replicates, measurement was done three times.

3.8 DAF-FM staining

DAF-FM diacetate (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate) is reagent used to quantify low concentrations of nitric oxide (NO). It passively diffuses across cellular membranes until it reacts with NO and forms fluorescent benzotriazole with excitation/emission of 495/515 nm. Measured fluorescence is proportional to the amount of NO in cell.

After incubation, suspension was washed in 1 ml of LBSS and centrifuged for 5 minutes at 1000 rpm (Hettich Universal K2S, Germany). Pellet was resuspended in 350 μL of LBSS and was ready for flow cytometry analysis. Measurement was done on three occasions.

3.9 Flow cytometry

Flow cytometry after Annexin V staining, phagocytosis assay and DCF staining was performed with FACS Calibur flow cytometer (BD Biosciences, USA). In each sample which was analysed 30.000 events were counted. Flow cytometry data analysis was performed by FCS Express software (De Novo, USA).

3.10 Statistical analysis of data

Statistical analysis of data collected from this experiment was conducted in R statistical program, version 3.5.3. Normality of distribution of data was tested with Shapiro-Wilk test.

For phagocytosis assay and DAF-FM staining results data distribution was not normal and therefore non-parametric statistical tests followed. Homogeneity of variances was tested with Fligner test and differences between groups were tested with Kruskal-Wallis test, followed by pairwise Wilcox test to see the differences between the groups.

For DCF staining data, Shapiro-Wilk test was followed by Bartlett's test for variance homogeneity. Since variances were homogenous Analysis of Variance (ANOVA) was done to check for differences between tested groups. After the existence of difference was confirmed, *post hoc* Tukey HSD test was done.

All tests were conducted with significance level of 5% and results are expressed as a mean with standard deviation. Graphic representation of results was done in Microsoft Excel program.

4 RESULTS

4.1 Flow cytometry results and choosing coelomocyte population

Coelomocyte population gating for further analysis of data was based on physical parameters of cells – size and granularity, i.e. forward (FSC-H) and side (SSC-H) scatter, and fluorescence (Figure 13). Flow cytometry measurements were concentrated only on amoebocytes, to avoid eleocytes with high autofluorescence.

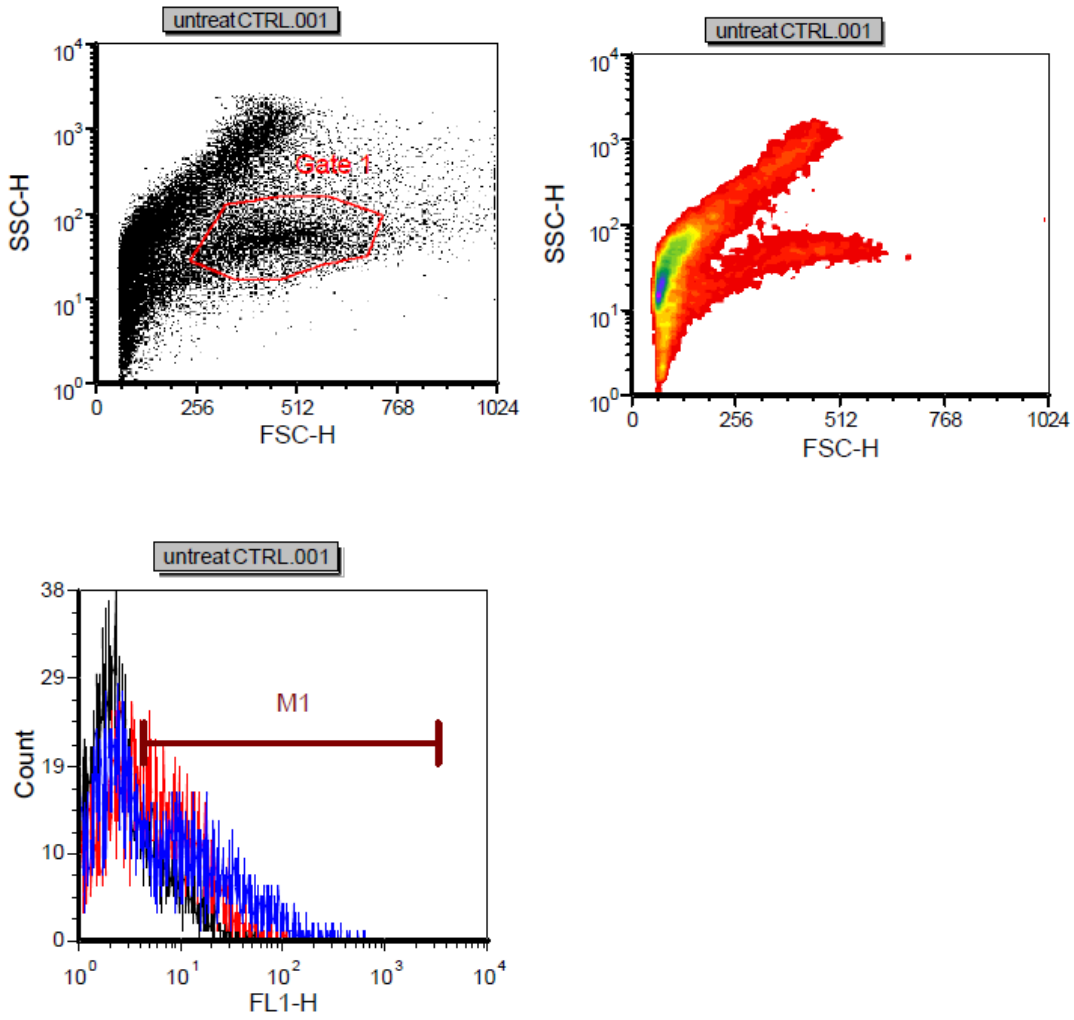

Figure 13. Analysis of extruded coelomocytes by flow cytometry; example given in this figure are coelomocytes from untreated control obtained after phagocytosis assay.

4.2 Effect of selenite and selenate on phagocytotic activity of coelomocytes in *Eisenia andrei*

Phagocytotic activity of coelomocytes exposed to 5 µg/cm² of selenite, selenate or water is presented as a percentage of gated cells. Results (Figure 14) show no significant difference in phagocytotic activity of coelomocytes after exposure to any form of selenium when compared to control group, for both *Escherichia coli* and *Staphylococcus aureus*. Furthermore, there was no significant difference in phagocytosis between two types of bacteria in any treatment. Even though there was no significant difference, phagocytotic activity of coelomocytes on *Staphylococcus aureus* bacteria was slightly higher in all treatments compared to the one on *Escherichia coli*. The differences visible in graph refer to differences between samples which contained bacteria and sample without bacteria of each treatment (CTRL, SI or SA).

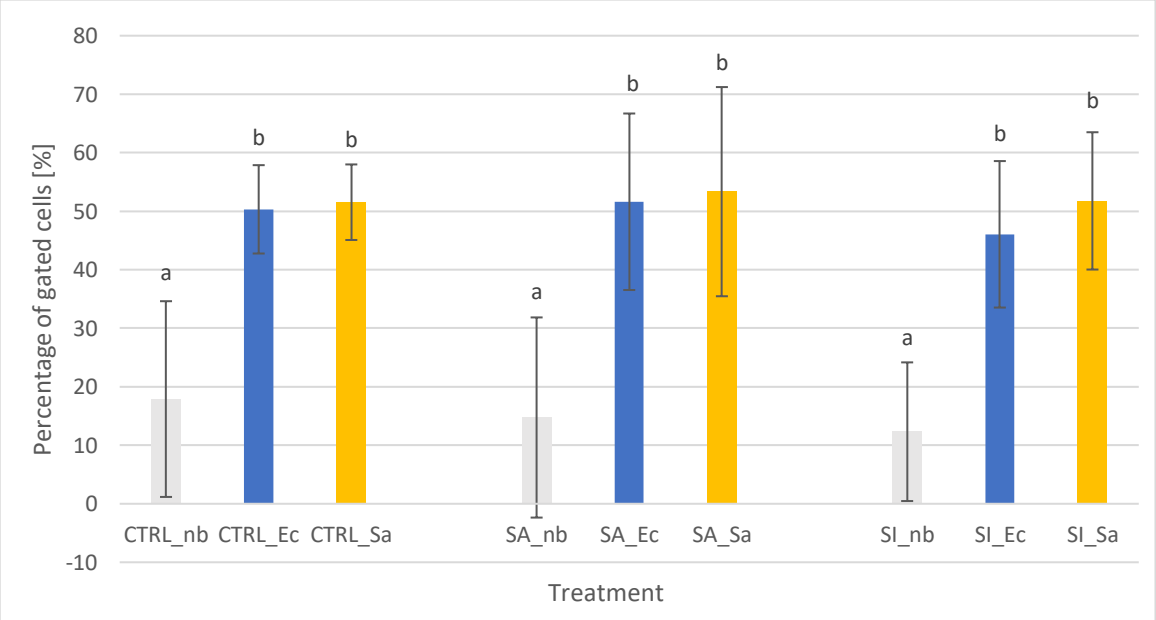

Figure 14. Percentage of gated cells in *Eisenia andrei* coelomocytes measured after 48-hour exposure to selenite and selenate and 24-hour long phagocytosis assay. Graph represents four measurements and shows mean ± standard deviation. Different letters mark statistical difference between groups ($p < 0.05$).

CTRL_nb – group exposed to water in filter paper contact test, and without bacteria in phagocytosis assay,
CTRL_Ec – group exposed to water in filter paper contact test, and to *Escherichia coli* in phagocytosis assay,
CTRL_Sa - group exposed to water in filter paper contact test, and to *Staphylococcus aureus* in phagocytosis assay,
SA_nb – group exposed to selenate in filter paper contact test, and without bacteria in phagocytosis assay,
SA_Ec – group exposed to selenate in filter paper contact test, and to *Escherichia coli* in phagocytosis assay,
SA_Sa - group exposed to selenate in filter paper contact test, and to *Staphylococcus aureus* in phagocytosis assay,
SI_nb – group exposed to selenite in filter paper contact test, and without bacteria in phagocytosis assay,
SI_Ec – group exposed to selenite in filter paper contact test, and to *Escherichia coli* in phagocytosis assay,
SI_Sa - group exposed to selenite in filter paper contact test, and to *Staphylococcus aureus* in phagocytosis assay

4.3 Effect of selenite and selenate on ROS content in coelomocytes of *Eisenia andrei*

ROS content in coelomocytes after exposure to two forms of selenium was measured by staining cells with DCFDA and measuring fluorescence, which was proportional to DCF production, i.e. ROS content. When compared to control group, treatment of 5 µg/cm^2 of selenate and selenite caused decrease in ROS content in coelomocytes (Figure 15). While both treatments triggered reduction, only the one caused by selenate significantly differed from the control group.

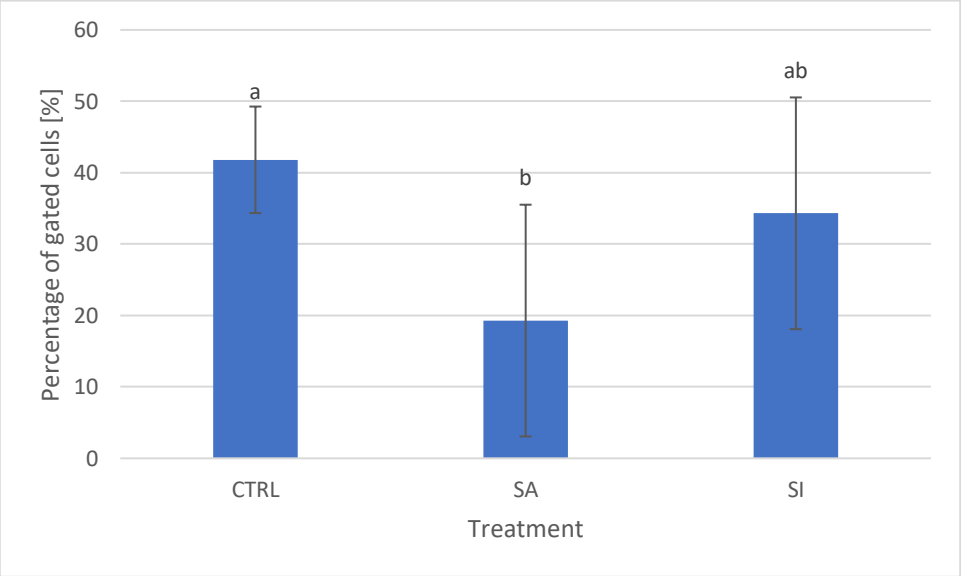

Figure 15. Percentage of gated cells (coelomocytes) after 48-hour long exposure to selenite and selenate and DCF staining. Graph represents three measurements and shows mean ± standard deviation. Different letters mark statistical difference between groups ($p < 0.05$).

CTRL – group exposed to water in filter paper contact test
SA – group exposed to selenate in filter paper contact test
SI - group exposed to selenite in filter paper contact test

4.4 Effect of selenite and selenate on phagocytotic nitric oxide (NO) levels in coelomocytes of *Eisenia andrei*

NO content in coelomocytes after exposure to two forms of selenium was measured by staining cells with DAF-FM and measuring fluorescence which was proportional to NO content in cells. Both selenate and selenite treatment caused increase in NO levels in cells, but neither of treatments caused significant change when compared to the control group (Figure 16). Percentage of gated cells are shown as relative values.

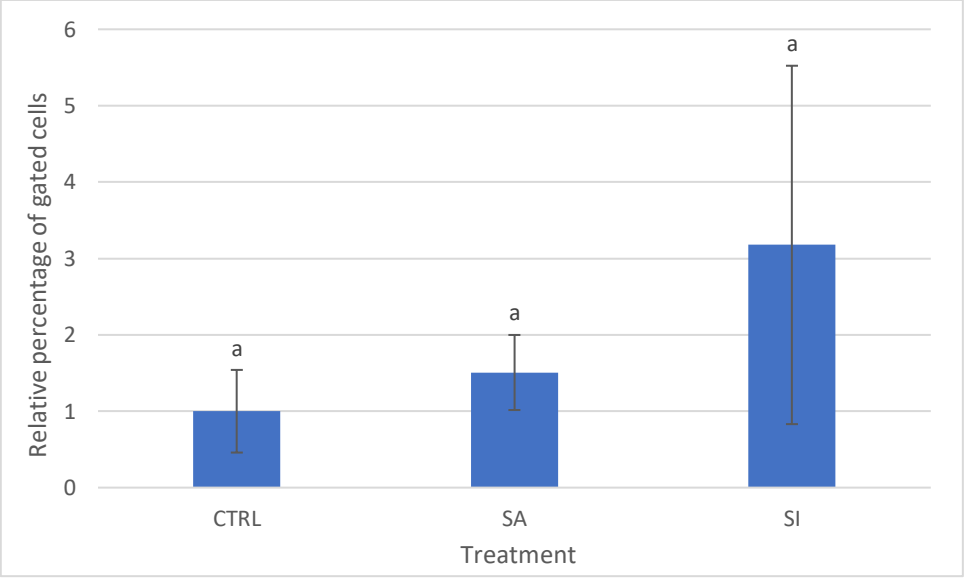

Figure 16. Relative percentage of gated cells (coelomocytes) after 48-hour long exposure to selenite and selenate and DAF staining. Graph represents three measurements and shows mean ± standard deviation. Different letters mark statistical difference between groups ($p < 0.05$).

CTRL – group exposed to water in filter paper contact test
SA – group exposed to selenate in filter paper contact test
SI - group exposed to selenite in filter paper contact test

5 DISCUSSION

Selenium is metal with “U”-shaped efficacy curve, affecting organisms in negative way, both when its concentration in environment is too high and too low (Ferguson et al., 2012). Since problem of low concentrations of selenium in soil and consequently in plants, especially grains used for food production, can be solved with biofortification, humans have resorted to this method hoping to solve the problem (Broadley et al., 2006). Biofortification is enhancing microelement accumulation in grains by fertilization of soil with, in this case selenium, or adding it by foliar spray, where both ways elevate Se soil concentration (Zhao and McGrath, 2009). With resolution of insufficient levels of selenium in grains, another problem could potentially be rising. It is not yet clear in which way elevated selenium concentration affects other organisms habituating the soil. In study using earthworm *Eisenia andrei* as a model organism, Ečimović et al. (2018) have extensively examined its sensitivity to the elevated selenate and selenite concentrations in soil. *Eisenia andrei* has shown to be more sensitive to selenite exposure in terms of acute toxicity, but it accumulated selenate in higher rate. Selenite has caused decrease in mRNA of antimicrobial factors and stress-related genes, and selenate apoptotic-like cell death in the coelomocytes. Efflux pump activity and oxidative status were also negatively affected.

In this research emphasis was on assessing effects of selenium on earthworm’s immunity, using earthworm immune cells - coelomocytes. Coelomocytes are significant constituent of earthworm’s immunity. One of their most important features is ability to phagocytise foreign particles and invading organisms, including bacteria that enter coelomic cavity through dorsal pores. Number and composition of coelomocytes can be changed after dermal exposure to immune-stimulators, phorbo-12-myristate-13-acetate (PMA), lipopolysaccharide (LPS) and concavaline A (ConA) (Homa et al., 2013). Stein et al. (1977) found all coelomocytes to be phagocytic, with the exception of chloragogen cells. In an experiment with FITC-labelled 2-hydroxyethylmethacrylate synthetic particles on *Eisenia foetida*, Bilej et al. (1990) have shown that phagocytotic activity can be increased after *in vivo* stimulation by protein antigen (arsanylated human serum albumin) and after pre-incubation of same particles in cell-free coelomic fluid, suggesting on opsonizing effect of coelomic fluid and its importance for defending organism from pathogens. Effect of selenium on phagocytosis in earthworms up until now has not been assessed. The results obtained in this study show that *in vivo* exposure to selenite and selenate does not cause

significant changes in earthworms' ability to phagocyte *Escherichia coli* or *Staphylococcus aureus*, at least at concentration of 5 μg/cm^2 of selenium which was used in the experiment. Even though there were no significant changes, phagocytosis of *Staphylococcus aureus* was slightly higher than the one of *Escherichia coli*. Since the exact effect of both forms of selenium on the earthworm immune system has not been clarified, it is hard to say what part of the phagocytic process could be affected by selenium, if it does affect it in any way, since other immunological constituents can be involved in defence against pathogens, including antimicrobial factors, such as lysenins and lumbricins. Conclusion could be that dermal exposure to selenium does not have additional immune-stimulating effect on earthworm, at least not at concentration of 5 μg/cm^2 of selenium. Additional research should be done, with wider range of concentrations, and especially with long-term exposure to low concentration of selenium which would represent environment-like conditions similar to the ones in selenium-enriched soil. Other metals, like copper, do cause changes in coelomocytes phagocytic activity. Nusetti et al. (1999) showed that exposure of the tropical earthworm *Amynthas hawayanus* to sublethal copper levels causes lower phagocytic activity on nitroblue tetrazolium (NBT) dye. They explained this change with alterations of cellular structures impairing cells capability to interact with antigens, changes in production of agglutinating factors, changes in phenotypes of phagocytic cell populations or perturbing the biochemical mechanism underlying phagocytic activity. Heavy metals also affect coelomocyte phagocytic activity in a negative way. In an experiment on *Lumbricus terrestris* species Cd, Hg and Zn caused increased cell mortality and impairment of coelomocyte phagocytic activity. Conversely, Pb was marked as less toxic and relatively well tolerated by coelomocytes (Fugère et al., 1996). Results from the present research indicate difference in effect of selenium compared to other metals on cellular defence mechanisms in different earthworm species.

When it comes to effect of selenium on oxidative status of earthworms, there is more research done than there is on the effect of selenium on phagocytosis in earthworms (Ečimović et al., 2018; Štolfa et al., 2017; Petek, 2017). Indicator of ROS production used in this research was DCFDA staining. Results show that, when applied in concentration of 5 μg/cm^2 in filter paper contact test, selenate caused significantly lower levels of ROS when compared to both control- and selenite- exposed earthworms. ROS level in group exposed to selenite was also lower than control but not statistically significant. These results are opposite from earlier results from Petek (2017) who explained higher levels of thiobarbituric

acid reactive substances (TBARS), marker of oxidative stress, in whole body homogenate samples from earthworms exposed to selenate (at 5 μg/cm^2) and selenite (2.5 μg/cm^2) with elevated ROS production. The difference in response to selenium in coelomocyte extract vs. whole body homogenate could be in sample type and difference in cells. Selenium does not cause oxidative stress to coelomocytes and they could be stimulated by selenium to enhance ROS defence and elimination mechanisms. In research by Štolfa et al. (2017) *Dendrobaena veneta* earthworms were exposed to selenite and selenate in 15-day soil test at concentrations of 0.01, 0.1, and 1 mg per kg of soil and results show reduced CAT activity at 0.1 concentration for both forms of selenium, indicating activation of antioxidant mechanism by selenium. Contrary to that, previously mentioned research by Ečimović et al. (2018) discovered no changes in CAT activity after exposure to selenium, but the species used in an experiment was *Eisenia andrei*, and test used was filter paper contact test. When everything is taken into consideration, it can be concluded that type of test and earthworm species used as a model organism can cause different response to selenium exposure. Additionally, selenium exposure probably affects different systems of organism, in this case earthworm, in a different manner. To completely understand effect of two forms of selenium on oxidative status of earthworms, enzymes and other segments of antioxidant defence system, total selenium concentration, in both whole body homogenate and coelomocyte extract, should be tested in comprehensive study.

The last research aim of this study was to asses NO levels in coelomocytes. Besides phagocytosis, antimicrobial peptides and encapsulation, synthesis of NO, as a response to parasite infection, is fourth invertebrate effector mechanism important in studies of ecological immunology (Rivero, 2006). NO is described as inducible, ubiquitous mechanism for direct elimination of pathogens and its response to infection is non-specific (Rivero, 2006). In invertebrates it is included in chemosensory and information processing, but has also been found to induce cellular and humoral immune response (Foley et O'Farrell, 2003). NO is a free-radical gas which, besides being toxic by itself, can additionally react with oxygen related reactive intermediates and yield other toxic species (Colasanti et al., 2001). Cook et al. (2015) gave evidence for antimicrobial role of NO in earthworms. They investigated NO production in coelomocytes of *Eisenia hortensis* after 16h incubation with soil bacteria. Results showed significantly elevated NO levels in all earthworms, no matter the bacteria used, with most prominent increase after exposure to gram-positive bacteria. It is also known that CCF induces NO production by macrophages, probably by interacting

with cell-surface ion channels (Bilej et al., 2006). NO in *Eisenia fetida* is produced as a neuromodulator by a ventral nerve cord (Kitamura et al., 2001). Results of the present study are, according to available literature, the first to investigate effect of some metal on NO production in earthworms. Even though there was no statistical difference in NO levels between control group of earthworms and groups exposed to selenite and selenate, results indicate higher levels of NO in the latter groups, compared to control. If this is the case, selenium could have immune-stimulating effect on coelomocytes. This finding is interesting, especially when kept in mind that ROS content measured in coelomocytes indicates decrease after exposure to selenate. The possible explanation could also be that selenium exposure could induce signalling pathways that rely on NO as a signalling molecule, since phagocytosis levels remained unchanged after selenium exposure. However, in order to be able to draw a conclusion on effect of selenium on NO production, additional repetition of this test should be performed.

6 CONCLUSION

To obtain insight into effects of two forms of selenium, selenite and selenate at concentration of 5 μg/cm^2, on immunological changes in earthworms, *Eisenia andrei* species was used as a model organism. Primary target of investigation were coelomocytes, which were used for measurement of phagocytic activity, ROS content and NO content in cells after *in vivo* selenium exposure of earthworms in filter paper contact test. Main findings of this research are:

1. *In vivo* exposure to any form of selenium in concentration of 5 μg/cm^2 does not cause changes in phagocytic activity on *Escherichia coli* and *Staphylococcus aureus* bacteria.
2. ROS levels in coelomocytes are reduced after exposing earthworms to 5 μg/cm^2 of selenate, indicating its positive effect on earthworm's antioxidant system.
3. Content of NO, an effector mechanism in invertebrate immunology, does not change in coelomocytes after *in vivo* selenium exposure, however the trend of increase in NO was evident and should be additionally investigated.

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