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The Interaction of Sodium Fluoride and Sodium Selenate on the Developmental Toxicity to Xenopus Laevis and Ambystoma Maculatum Embryos

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The Interaction of Sodium Fluoride and Sodium Selenate on the Developmental Toxicity to *Xenopus Laevis* and *Ambystoma Maculatum* Embryos

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By

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Abstract

Amphibians play critical roles in the environment's wellness. Fluoride (F) is a widely existing environmental pollutant. It is commonly known for being added to drinking water and as a topical dose on teeth. Selenium (Se) can be found in organic and inorganic forms in nature. Many different fertilizers, pesticides, insecticides, and fungicides contain sodium selenate in them. The range selenium has between deficiency and toxicity is narrow, meaning excess exposure could cause various adverse effects in aquatic organisms. *Xenopus laevis* is a standard model for developmental toxicity due to being minimal maintenance, cost efficient, and having transparent embryos. *Xenopus* makes an acceptable model for estimating the developmental effects of chemicals on native amphibians. *Ambystoma maculatum* is a non-endangered native species to Alabama making it an acceptable salamander to compare to *Xenopus.* The objective in this study is to determine the interactions effects on developmental toxicity of sodium fluoride, sodium selenate, and 3 mixtures combinations on amphibian embryos. The assay uses serial dilutions to expose various concentrations to frog embryos. The assay lasts 96-hours holding 20 embryos in small petri dishes per replicate. Assays for *Ambystoma* lasted 12-days holding 10 embryos in large, deep petri dishes per replicate. Assays were also performed to determine the developmental effects to different exposure times and lengths to 100 mg/L sodium selenate. Mortalities were counted for each day. Mortalities and malformations were counted on the last day of the assay and embryo length were measured. Means, standard error, probit analysis (LC50 and EC50(malformation)), ANOVA and Bonferroni's post hoc test were calculated using Systat. The teratogenic potential was calculated using the formula LC50/ EC50(malformation). Isobole diagrams were used to determine if the chemicals showed synergism, antagonism, response

addition, or concentration response. In *Xenopus*, the overall LC50, EC50 for NaF and Na_2SeO_4 was 603.37, 819.42 and 30.41, 15.13 respectively. The mixtures showed a combination of response addition and synergism at different combinations. In *Ambystoma*, the overall LC50, EC50 for NaF and Na_2SeO_4 was 540.39, 862.26 and 53.06, 69.21 mg/L respectively. The mixture showed response addition. In both *Xenopus* and *Ambystoma*, malformations such as stunted growth, edemas, loose gut, hemorrhages, bent notochords, and kinked tails were seen in both selenate, fluoride, and mixtures. Overall, these assays show the usefulness of the *Xenopus* embryos as model species for native amphibians. *Xenopus* embryos showed to be more sensitive to 100 mg/L sodium selenate at different developmental stages than accumulated exposure.

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Introduction

Amphibians in all types of ecosystems play critical roles to the wellness of the environment by providing important links in an ecosystem's food web and moving energy and nutrients across terrestrial-wetland boundaries (Homan et al., 2018). Amphibians are also known as an indicator species which signals whether an environment is healthy (Homan et al, 2018). Amphibians have always been the main vertebrate group that are at risk of exposure to pollution and contaminants in ephemeral systems (Mann & Bidwell, 2000). About half of frog species and one-third of salamander species in North America rely on ephemeral wetlands to breed and to lay their eggs (Turtle, 2000). Biologists express concern over the global loss of amphibian populations over the past 15 years due to habitat loss, pollution, invasive species, pathogens, and man-made chemicals (Baxter, 2015; Olivier, 2010). Most amphibians are vulnerable to contaminants during their larval stages because they can absorb many types of chemicals through their skin, gills, lungs, and digestive tract (Lanctot et al, 2017).

Amphibians have been adopted as the model of choice for applied ecological research into issues such as habitat loss, pollution, disease, and global climate change (Brod, 2019; Hopkins, 2007). Frogs and salamanders have been used in scientific research, such as developmental toxicology, due to their ability to undergo metamorphosis (O'Rourke, 2007) as well as their trophic importance, environmental sensitivity, research tractability, and impending extinction (Hopkins, 2007). Over the years, scientists have used amphibian embryos to research the effects of toxins, mutagens, and teratogens (O'Rourke, 2007).

Animals of all types have provided significant contributions to the understanding of modern medicine and advancement (Robinson et al., 2019). Scientists have made huge strides in

developmental science using model animals which are less expensive to maintain compared to using primates and well suited towards experimental manipulation (Toxicology, 2000). When scientists look at developmental mechanisms, all animals are very similar to one another (Toxicology, 2000). Using multiple species in studies has increased over the last couple of decades (Kleunen et al, 2014). Comparing the data collected from the native species (*Ambystoma maculatum*) and the model species (*Xenopus laevis*) will help scientists understand the similar developmental damage that could occur because the chemicals being tested can be found in the environment.

Chemicals of Interest

Sodium Fluoride

The chemical fluoride (F) is a widely existing environmental pollutant (Zhao, et al., 2022) that normally occurs in sedimentary formations that contain fluoride-bearing minerals (Ozsvath, 2009). They are derived from the parent rock, fluoride-rich clays, or fluorapatite (Ozsvath, 2009). Fluoride can be found in tea, seafood that has edible bones, medicinal supplements, and fluoridated toothpaste (Aoun, 2018). For some time, fluoride was thought to be beneficial to people's teeth (Aoun, 2018). Placing a topical dose of fluoride on teeth helps the remineralization of enamel and makes the teeth stronger (Aoun, 2018). When fluoride is exposed to teeth, it inhibits demineralization as the fluorapatite crystals form by the reaction with enamel apatite crystals (Arifa, 2019). Ingesting too much fluoride can be detrimental and can cause dental fluorosis, appearing early as enamel surface opacities (Martinez-Mier, 2012). Serious cases of dental fluorosis can lead to enamel fracture resulting from compromised enamel porosity (Martinez-Mier, 2012).

The enamel apatite crystals are more resistant to acid attack compared to hydroxyapatite (HAP) crystals (Arifa, 2019). Fluoride then enhances remineralization and speeds up the growth of new fluorapatite crystals by bringing calcium and phosphate ions together. It also stops acid producing bacteria by interfering with the production of phosphenol pyruvate (PEP) (Arifa, 2019). This is the key intermediate of the glycolytic pathway in bacteria (Arifa, 2019). Using fluoride-containing products like toothpaste and mouthwash help mix the fluoride with saliva giving teeth continuous exposure to the chemical preventing cavities (Aoun, 2018). New research has demonstrated the importance and advantages of the prevention or treatment of tooth decay and dental caries (Aoun, 2018). Previous studies have shown that water fluoridation has reduced tooth decay by 20 to 40 percent (Fluoride: Nature's tooth decay fighter, 2009).

In 1945, community trials allowed fluoridated water flow in thousands of public water systems, reaching to two-thirds of the United States' population (Pollick, 2004). Depending on the geographical location and source, the amount of fluoride in drinking water could range from 0.01 ppm to a maximum of 100 ppm (Aoun, 2018), with an optimal fluoride level established as 0.7 to 1.2 ppm (Fluoride: Nature's tooth decay fighter, 2009). The mineral fluorine is found naturally in the soil and aquifer sediments that accumulate in freshwater sources, particularly groundwater (Podgorski & Berg, 2022).Fifty percent of the world population has been reported to utilize ground water for drinking and household purposes (Zhao et al., 2022). This makes fluoride the greatest inorganic contaminant of drinking water worldwide giving 200 million people in 25 countries fluoride toxicity (Zhao et al., 2022). While ingesting small amounts of fluoride in one's diet could help prevent dental caries and strengthen bones, there are many adverse effects that ingestion at high doses can cause human health decline (Ozsvath, 2009).

Ingesting high levels can cause dental fluorosis and skeletal fluorosis, increased rates of bone fractures, decreased birth rates, increased rates of urolithiasis (kidney stones), impaired thyroid function, and lower intelligence in children (Ozsvath, 2009). Fluoride toxicity has been shown to cause damage to major systems in the body and cause reproductive issues such as reducing the number, density, vitality, and motility of sperm in rats (Zhao et al., 2022). The experiments showed that 100 mg/ L NaF destroyed the normal physiological structure of the rat's testis, which resulted in abnormal sperm morphology and the increase of sperm deformity rate (Zhao et al., 2022).

In the 1940's, shortly after water fluoridation was initiated, fetal malformations were noted in tadpoles, including head-tail length malformations and dysfunction of the neuromuscular system (Goh, 2003). When high doses of sodium fluoride were induced into *Schistosoma* infected snails, lipid peroxidation was enhanced, and antioxidant capacity of the cell decreased leading to the reduced disposal of oxygen-free radicals and peroxides (Koriem et al, 2016). In another study, unanesthetized canines, were administered high doses of sodium fluoride leading to hyperemia, acute focal hemorrhages, and resultant death (Leone et al, 1956). FETAX has shown a high degree of success in identifying mammalian teratogens (Goh, 2003). Through FETAX experiments, teratogenic action of sodium fluoride has been observed on frog embryos which indicate a strong possibility that the chemical may act directly on developing mammalian fetuses to cause malformation (Goh, 2003). The values for LC50, EC50 (malformations), and minimal concentration to inhibit growth (MCIG) of sodium fluoride met the limits established for a teratogen in frog embryos, showing that the chemical is a direct acting teratogen on developing embryos (Goh, 2003). In aquatic organisms, fluoride has shown

negative effects in fish and crabs (Pal et al, 2018). There have been tests reported on rats at the cellular, molecular, and organismal levels (Pal et al, 2018). Three to 10 hours is the terminal plasma elimination half-life from the ingestion of fluoridated drinking water (Goh, 2003). The half-life for sodium fluoride in community waters is 120 weeks for adults and 70 weeks for children (Martinez-Mier, 2012). When you isolate the half-life of sodium fluoride in the bones, it would be twenty years (Cope, 2017).

Sodium Selenate

Selenium (Se) is found in organic and inorganic forms (Sobolev et al., 2020) and is essential to animal physiology. Studies show selenium is best absorbed by plants and grains in its inorganic form and best absorbed by mammals in an organic form (Pecoraro et al., 2022). In soils worldwide, the average selenium content is 0.4 mg/kg but can be higher in different regions (Balint, 2021). When humans have chronic oral exposure to high concentrations of selenium, these compounds can produce a disease called selenosis which can lead to hair loss, nail brittleness, and neurological abnormalities such as numbness and other odd sensations in the extremities (National Center for Biotechnology Information 2023). Selenium has a biological half-life of 1.2 days and is eliminated mostly through urine output (Safety data sheet according to WHS regulations (hazardous chemicals) Amendment 2020 and ADG requirements 2021). Selenium has a narrow therapeutic index meaning small excesses in exposure could cause adverse effects in aquatic organisms (Lanctot et al, 2017). Selenium contamination is difficult for ecosystems to recover from because its ability to bioaccumulate and biomagnify in the food chain (Lanctot et al, 2017). When exposed to selenium at low levels, it caused developmental abnormalities within *Xenopus* embryos and tadpoles (DeYoung, 1991). Selenium in the form of

sodium selenate is toxic to aquatic organisms such as *Xenopus laevis* embryos and tadpoles (Browne & Dumont, 1979).

Sodium selenate (Na_2SeO_4) is colorless, rhombic crystals that are highly soluble in water (National Center for Biotechnology Information 2023). It is a salt of selenic acid that is used as a mineral supplement which is produced by Sigma-Aldrich chemical Co. (Hassen et al, 2016). Veterinarians have used sodium selenate on chicks to prevent exudative diathesis, sheep for white muscle disease, and ewes for infertility (National Center for Biotechnology Information 2023). There have been cases where it prevented pneumonia in premature lambs and calves (National Center for Biotechnology Information 2023). Studies on tomato plants treated with sodium selenate showed that the plants had less bacterial growth occur on them acting as an antimicrobial defense (Moretti et al, 2022).

Sodium selenate is considered an acute hazard and is extremely toxic to aquatic life (National Center for Biotechnology Information 2023). Studies demonstrated that rats and mice that consumed water with greater than 15 ppm expired (NTP Toxicity Studies of Sodium Selenate and Sodium Selenite (CAS Nos. 13410-01-0 and 10102-18-8) Administered in Drinking Water to F344/N Rats and B6C3F1 Mice, 1994). Additional research exposing *Xenopus* embryos to sodium selenate at concentrations of 2 ppm and above showed severe developmental abnormalities and increased mortality (Browne & Dumont, 1979). Many different fertilizers, pesticides, insecticides, and fungicides have sodium selenate in them (Stephen et al, 1989). Experiments were performed on wheat by coating the seeds in sodium selenate to see how much growth occurred (Stephen et al, 1989). When exposed to sodium selenate at concentrations

greater than 12mg/L, *Xenopus* embryos had edemas and malformations of the gut, heart, and face (DeYoung, 1991).

Test Animals

Xenopus laevis

When performing toxicity experiments, it is important to have a continuous supply of healthy unstressed test specimens (Foss & Rayburn, 1997). Most amphibians are oviparous, egg laying, as opposed to humans which are viviparous and give birth to live young (Fainsod & Kot-Leibovich, 2018). Amphibian embryos have been considered attractive as an assay system because it has remained the classical model for experimental embryological studies (Dumont et al, 1983). *Xenopus laevis*, the African Clawed Frog, is a commonly used experimental animal in many fields of science (Horn, 2006), including embryology, molecular biology, genetics, immunology, and toxicology (Böswald, 2022), because of large robust size, abundant eggs and embryos, and cost-effectiveness (Ishibashi et al, 2017). *Xenopus* embryos have been used successfully to assess teratogenic risk and toxicity of several mixtures and compounds (Gardner, 2017).

Xenopus laevis is relatively easy to take care of in a laboratory setting because they have a short generation interval, can be bred throughout the year, and have a lifespan of 15 to 30 years in captivity (Böswald, 2022). *Xenopus* females are significantly larger than the males with a nose to rump length of 9 to 14 cm compared to males at 6 to 10 cm (Bantle et al, 1991). Males are marked by dark arm pads and lack cloacal lips found in females of the species (Bantle & Sabourin, 1991). Frogs release oocytes outside their body which makes experiments easier to conduct compared to mammals due to ethical and practical reasons possible (Böswald, 2022).

Since the frogs are injected to express hormonal stimulation, this makes it possible for more oocytes to be obtained (Böswald, 2022). In one mating, *Xenopus* can produce over 1,000-5,000 eggs, and they can reproduce year-round (Wilzla et al, 2018). The embryonic development of *Xenopus* is rapid and occurs outside the mother's body (Galdiero, 2017). The embryos and tadpoles are excellent for experiments because their bodies are transparent, allowing malformations inside the body to be observed under a dissection microscope (Galdiero, 2017). When looking at the different developmental stages of the embryos, they are easy to observe and score (Dumont et al, 1983).

Ambystoma maculatum

Ambystoma maculatum, the Spotted Salamander, can have up to fifty round, light yellow and orange spots that run irregularly along the sides of their back, from eye to tail tip (Conant & Collins, 1998). The dorsal side of their body is a ground color black, slate, or bluish black while the ventral side is slate-gray (Conant & Collins, 1998). *Ambystoma* can grow to 4-7 inches in length (Conant & Collins, 1998). They live in forested areas throughout the eastern United States and parts of Canada (Gardner, 2017). They can be found in hardwood and mixed deciduous forests that have semipermanent pools within them (Gates & Thompson, 1981). Adults do have a high survivorship in the field and can live up to 32 years under the right conditions (Heuring & Mathis, 2014). They are part of the mole salamander family, living underground in burrows that they make or use burrows that other animals have made like rodents (Gardner, 2017). A reason why this species was used in this assay was that they are a native species to Alabama (Conant & Collins, 1998), and *Ambystoma maculatum* have a 50% mortality above pH 5 while other amphibian species have a higher acid tolerance (Ireland, 1991).

Ambystoma begin their breeding season in early spring (January to March), and breeding season can extend from a few days to three months (Andrews & Talmage, 2021). When the correct cues are met for this species, they will come out of their burrows during winter rains and find suitable pools to breed in (Gardner, 2017). Observations were made that the salamanders preferred pools that had low water hardness and low turbidity (Gates & Thompson, 1981). Salamanders are used in developmental biology frequently due to their large, accessible embryos (Gomez & Echeverri, 2021). *Ambystoma* are known for laying large globular egg masses that can be easily seen and identified in nature (Andrews & Talmage, 2021). The gelatinous egg masses can be either a clear or white color which have their own benefits (D'Errico, 2020). White egg masses are not preyed upon as much while clear egg masses allow more algal growth to occur inside the eggs (D'Errico, 2020). The eggs masses usually have been colonized by green alga commonly referred to as *Oophila amblystomatis* (Baxter, 2015). Studies have shown that the increased oxygen concentrations that the green algae produce is beneficial to the salamander embryos (Kerney, 2011). These baseball-sized egg masses can have 50-250 eggs when completely expanded (Andrews & Talmage, 2021). To make sure their eggs have a higher success rate of surviving, the adults find vernal pools that have little to no fish activity in them to reduce predation on the eggs (Andrews & Talmage, 2021). It takes about forty days for the eggs to hatch and seventy to hundred days for the larval period to conclude (Whitford & Vinegar, 1966).The problem that spotted salamanders have compared to other species is that they return to natal pools to breed (Turtle, 2000). The eggs are still at risk of predation by aquatic invertebrates and conspecifics such as cannibalism (Hossie et al, 2018).

FETAX

FETAX (Frog Embryo Teratogenicity Assay—*Xenopus*) is a most commonly used protocol for identifying potential developmental hazards in aquatic organisms (Bantle et al, 1990). It is used as a rapid test to identify developmental toxicants (Bantle et al, 1991). Using the system for FETAX has shown great potential in detecting teratogenicity of both individual compounds and complex mixtures (Dawson & Bantle, 1987). This kind of test is used for embryonic sensitivity in their early life stages (Bantle et al, 1991). It has been validated as a screening assay for not just pure chemical compounds but complex environmental mixtures as well (Fort et al., 1998). FETAX assays are 96-hour exposure standardization test and has an advantage of evaluating many parameters in a single study (Martini et al, 2012). Since this bioassay utilizes amphibians as its test species, evaluating the importance of environmental contaminants in the phenomenon of global amphibian decline, FETAX has become very useful (Mann & Bidwell, 2000). FETAX is used specifically with *Xenopus laevis* but can be used with other amphibians (Bantle et al, 1991). These assays are used on fertilized *Xenopus* embryos that are mid-blastula stage (Mouche et al, 2017). When the test is completed, compound teratogenic potential can be determined after analysis for mortality, malformations, and larva length (Mouche et al, 2017).

When a chemical is teratogenic, it means that the test material can cause abnormal morphogenesis (malformations) in test subjects (Bantle & Sabourin, 1991). The Teratogenic Index (TI) is used to measure the developmental hazard caused by the test material (Bantle & Sabourin, 1991). The TI can be determined by taking the 96-h LC50 and dividing it by the 96-h EC50 (Bantle & Sabourin, 1991). Since identifying mammalian teratogens is highly important,

FETAX is used with *Xenopus* embryos because of its high degree of success in indicating a strong possibility that chemicals or metals that may also cause developing malformations on mammalian fetuses (Goh, 2003).

Table 1

Definition terms

EC50	**The effective concentration of a substance that causes fifty percent malformations in a population**
LC50	The lethal concentration of a substance that causes fifty percent mortality in a population
LOEC	Lowest observed effect concentration: first sign of significant difference occurred at the concentration
MCIG	Minimal concentration that inhibits growth
NOEC	No observed effect concentration: no significant difference occurred at the concentration
Teratogen	A substance that interferes with normal fetal development and causes congenital disabilities or low birth weight
Teratogenic Index	LC50/EC50: An estimation of teratogenic risk; 1.5 or higher indicates the chemical can cause mortality and malformations

Performing FETAX experiments does have significance to developmental toxicology because it prioritizes samples for future testing with mammals (Bantle & Sabourin, 1991). FETAX is commonly used in studies because of its low cost (Dumont et al, 1983). They provide useful information that could be used in chronic toxicity estimations to aquatic organisms (Bantle & Sabourin, 1991). When working with aquatic species, FETAX use in developmental toxicity is important to measure because of embryo mortality, malformations occurring, and growth inhibition can occur at concentrations that normally would not influence adult organisms (Bantle & Sabourin, 1991). These tests can be useful when deriving water quality criteria for aquatic organisms in harsh environments (Bantle & Sabourin, 1991). Lastly, the results from

FETAX experiments can be useful for studying bioavailability and for studying structure-activity relationships (Bantle & Sabourin, 1991).

Interaction Studies

Interaction studies are important to developmental toxicology because they investigate the interactive effects of concurrent exposures to a chemical and physical agent (Nelson, 1994). When defining synergism and antagonism, they often have relation to the model of concentration addition (Sorensen et al, 2007). Each type of interaction in developmental toxicology has its own accepted definition: additivity (the combined effect of two or more developmental toxicants approximates the sum of the effects of the agents administered separately); antagonism (the combined effect of two or more agents, one or more of which are present at doses that would be developmentally toxic if given individually, is significantly less than the sum of the effects of the agents administered separately); synergism (the combined effect of two or more developmental toxicants is significantly greater than the sum of the effects of each agent administered alone) (Nelson, 1994). We can determine if the chemicals are synergistic, antagonistic, or have a response addition by making an Isobole diagram graph which plots the toxic units of the chemicals (Moser & Rayburn, 2007). The quality of performing interaction studies can be highly variable (Nelson, 1994).

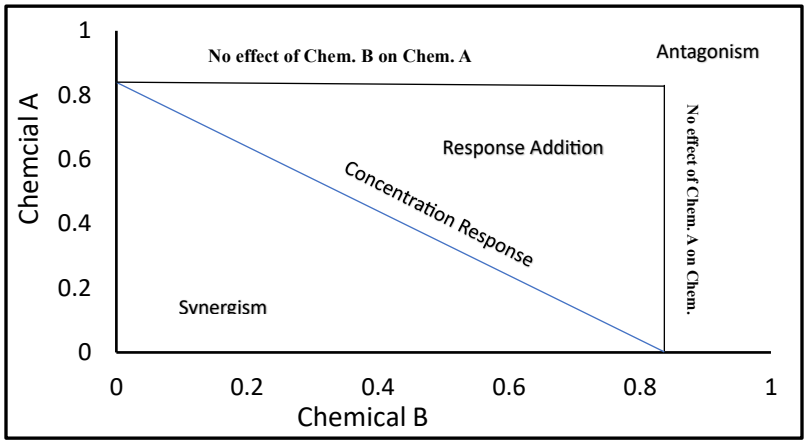
Isobole Diagram

Isobole diagrams combine dose-response curves for each mixture in the experiment that describe any possible deviations from concentration addition (Sorensen et al, 2007). See Figure 1. Toxic units (TU) in the project are defined as either the 96-hr LC_{50} or the 96-hr EC_{50} (Moser & Rayburn, 2007). Isobole diagrams have an x- and y-axis that represent a chemical A and

chemical B (Huang et al., 2019). The x- and y-axis will go up to one for a single toxic unit. An additive line will be drawn from the 1 TU of sodium fluoride to the 1 TU of sodium selenate to see which chemicals and which mixtures are synergistic, antagonistic, and have additive responses (Huang et al., 2019).

Figure 1

Generic Toxic Unit Graph



Hypotheses and Objectives

We hypothesize that the sodium selenate would be more toxic than the sodium fluoride in the experiments. Sodium fluoride was going to reduce the effects of sodium selenate in the interaction studies. The experiments are determining if *Xenopus* make an acceptable model for estimating the developmental effects of chemicals on native amphibians, including *Ambystoma* species therefore the *Ambystoma* embryos will have similar ranges of LC_{50}, EC_{50} (malformation), and growth impacts as *Xenopus* embryos. *Xenopus* embryos will be more

sensitive to accumulated exposure to 100 mg/L sodium selenate than embryos exposed for only 24 hours at different developmental times.

There are several objectives that will be performed in the experiment: 1) determine the LC_{50}, EC_{50} (malformations), and the minimal concentration to inhibit growth on treated *Xenopus laevis* and *Ambystoma maculatum* embryos; 2) compare the interactions between species; 3) compare the three interactions used with *Xenopus* to discover synergism and/or antagonism by creating Isobole diagrams. Lastly, determine the level of toxicity between the two chemicals.

Material and Methods

Chemicals and Materials

When preparing the FETAX solution, 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4$, $2H_2O$, and 75 mg $MgSO_4$ were dissolved with deionized or distilled water per liter (Bantle & Sabourin, 1991). The chemicals that were used in the assay are reagent-grade or better (Bantle & Sabourin, 1991). Chemicals purchased from Fisher Chemical were sodium bicarbonate, sodium chloride, and sodium fluoride with composition/purity ≥ 99.7%, ≥ 99%, ≥ 99%, respectively. Chemicals purchased from Sigma-Aldrich were calcium sulfate dihydrate, magnesium sulfate, potassium chloride, calcium chloride, sodium selenate, human chorionic gonadotropin (HCG), L-cysteine, and tricaine methane sulfate (MS222) with composition/purity ≥ 99%, ≥ 99.5%, 99%, ≥ 93%, ≥ 95%, 100%, ≥ 98%, and 100%, respectively.

Small petri dishes (60mm X 15mm) were purchased from Fisher Scientific. The deep petri dishes (100mm X 25mm) were also purchased from Fisher Scientific. The 3mL, large bulb transfer pipets were purchased from Samco Scientific. The 10mL serological pipets were purchased from OXFORD Lab Products.

Xenopus Methods

Xenopus Husbandry

Adult *Xenopus* were used in the assays and purchased from *Xenopus* I (Shirey & Rayburn, 2013). The animal care for the *X*enopus was performed in accordance with Jacksonville State University (JSU) Institutional Animal Care and Use Committee (IACUC), National Institutes of Health (NIH) number 001-07-05-23. Two pairs were held in 20-gallon glass aquariums that had water recirculating throughout them at air temperature (Parker & Rayburn, 2017). The temperature was kept at 23 ± 3°C daily (Bantle & Sabourin, 1991). Water quality tests were performed on a weekly basis to ensure the health of the xenopus (Bantle & Sabourin, 1991). Every day the frogs were fed protein-rich food pellets for their diet (Shirey & Rayburn, 2013). They were given a 12-h day/12-h night cycle (Parker & Rayburn, 2017).

Egg collection

A single pair of adults were injected with 500 mL of human chorionic gonadotropin (HCG) with a 1-mL tuberculin syringe that had a 1/2 inch long 26-gauge needle to induce breeding behavior and then they were placed in a false bottom breeding chamber for the night (Parker & Rayburn, 2017). Once egg production was confirmed, the eggs were checked for fertilization (Parker & Rayburn, 2017). Fertilized eggs were exposed to 2% L-cysteine prepared in FETAX solution for 1-3 minutes to remove the gelatinous covering on the eggs (Parker & Rayburn, 2017). Eggs were then rinsed for 2-3 minutes with FETAX solution to prevent L-cysteine from killing the eggs (Moser & Rayburn, 2007).

Embryo Sorting

The eggs were sorted twice to ensure healthy, normal embryos were being used (Bantle & Sabourin, 1991). Each petri dish had 8mL of different solutions pipetted in them and held 20 embryos that were in the mid blastula stage of development (Parker & Rayburn, 2017). Four petri dishes were used as controls while each concentration had two petri dishes (Bantle & Sabourin, 1991). Embryos were assigned to each dish randomly to keep the assay unbiased (Bantle & Sabourin, 1991).

Maintaining Assays

Embryos were placed in an incubator set to 24°C $\pm$ 1 throughout the experiment (Fort et al., 1998). Stock solutions of sodium fluoride, sodium selenate, and mixtures were made fresh every day in toxic units of 3:1, 1:1, and 1:3 ratio ($NaF:Na_2SeO_4$; Note for understanding: These are labels for the mixtures). The binary solutions were prepared at concentrations that had a range of 3% to 100% with two replicates, with each percentage under 100% diluted down with FETAX solution (Moser & Rayburn, 2007). Embryos were exposed to the test materials continuously throughout the assay (Bantle & Sabourin, 1991). Everyday pH levels of the controls and highest concentrations were observed and mortality for every dish (Bantle & Sabourin, 1991). On the last day of the experiment, embryos were anesthetized with a few drops of MS222 to more easily evaluate malformations and mortality (Parker & Rayburn, 2017). Embryos were photographed and measured for length (snout to tail) using ImageJ (Bantle & Sabourin, 1991). Two more definitive tests would be performed afterwards to get more accurate data (Bantle & Sabourin, 1991).

FETAX Methods (Experimental Design)

All FETAX experiments were performed accordingly to ASTM's standard guide for conducting the frog embryo teratogenesis assay-*Xenopus* (Bantle & Sabourin, 1991). Each experiment lasted 96 hours allowing observations and evaluations of developmental toxicity with each test material (Bantle & Sabourin, 1991).

Each assay had four replicated controls and six serial dilutions with two replicates per concentration. Since five experiments were performed simultaneously, 1,600 eggs were needed. Daily, dead embryos were removed from the dishes, and live embryos were counted (Moser & Rayburn, 2007). On the last day of the experiment, the final dead/alive count was recorded for mortality (Moser & Rayburn, 2007). Embryos were anesthetized with tricaine methane sulfate (MS222) to prevent them from moving (Bantle et al, 1991)

The number of malformations and types of malformations were recorded on the scoresheet of malformations at 96-h as described in the FETAX Atlas of Abnormalities, malformations were recorded (Bantle et al, 1991). The embryos were photographed over a light source and head-tail lengths were measured using ImageJ (Parker & Rayburn, 2017). The embryos were humanely euthanized once the experiment was completed (Moser & Rayburn, 2007). Two more definitive concentration-response experiments were performed using the same clutch of eggs per experiment (Parker & Rayburn, 2017).

Serial dilutions were made fresh everyday of 3:1, 1:1, and 1:3 mixtures. Each mixture had the same concentration percentages; 3.125%, 6.25%, 12.5%, 25%, 50%, and 100%. The percentages were broken down to calculate the amount of sodium fluoride and sodium selenate in the concentration for each mixture. By creating the Isobole diagram (e.g. Figure 1), the

mixtures will be compared to one another to determine which ones are concentration response, response addition, synergistic, or antagonistic.

Time/Exposure to Sodium Selenate

For each experiment, 640 embryos were used from different pairs of adults to show genetic variation. There were seven treatments and a negative control. With each treatment having four dishes, 20 embryos were used per dish. Treated embryos were exposed to 100 mg/L of sodium selenate. This concentration was used because previous experiments have shown that 100% mortality occurs.

The developmental time for all embryos was from small cell blastula to 96 hours or Stage 46 embryos. Exposure to sodium selenate was variable, with FETAX solutions used when not exposed to sodium selenate. The exposure times to sodium selenate were 96 hours (day 0-4), 72 hours (day 1-4), 48 hours (day 2-4), and 24 hours, (day 0-1, day 1-2, day2-3, and day 3-4). Until the treatments were exposed to sodium selenate, they were in FETAX solution. Once the 24-hour exposure time was complete, the embryos were placed back into FETAX solution.

Ambystoma Methods

Animal collection

Ambystoma egg masses were collected from the wetland area located at Henry Farm Park (33°46’ 59.0” N, 85°46’6.8” W) found approximately three miles away from Jacksonville State University (Gardner, 2017). Each visit 7-10 egg masses were gathered depending on size and stage of development. Both clear and white egg masses were used in the assays. Egg collection occurred early in the morning throughout early January to early March 2023 after a rain because the salamanders breed during the rainy conditions (Douglas & Monroe, 1981). Egg masses were

placed in a 5-gallon bucket with 1-gallon of water from the vernal pool (Gardner, 2017) and were brought back to the lab.

Embryo sorting

At the lab, the eggs were transferred from the bucket into watch glasses filled with FETAX solution (Bantle & Sabourin, 1991). To ensure that egg masses were randomized due to males fertilizing the same clutch of eggs from one female (Vitt & Caldwell, 2013), the embryos were removed from the surrounding gelatinous masses using a dissection probe. Double sorting was performed to find neural plate development or Harrison stage 19 embryos (Harrison, 1969). Due to the embryos large size, ten embryos were placed in large petri dishes (100mm X 25mm) filled with 50mL of solution.

Experimental design

The *Ambystoma* embryos go through forty early developmental stages slower making experiments last twelve days compared to *Xenopus* experiments which are only 96 hours (Harrison, 1969; Agudelo et al, 2021). Stock solutions of sodium fluoride, sodium selenate, and a mixture were made fresh every other day in toxic units of 1:0, 0:1, and a 20:1 ratio (Note for understanding: These are labels for the mixtures). The 20:1 ratio binary solution were prepared at concentrations that had a range of 3.125% to 100% with two replicates, with each percentage under 100% diluted down with FETAX solution (Moser & Rayburn, 2007). Each assay had four replicated controls and six serial dilutions with two replicates per concentration. Since the three experiments were performed at the same time, 480 eggs were needed.

Live/dead counts were still taken and removed every day of the assay (Gardner, 2017). The pH levels were conducted for the controls and highest concentrations of assay every other

day when the serial dilutions were changed. The pH levels from controls and highest concentrations were taken as a mixed sample from both replicates in each experiment rather than individual samples. On day 12, the final live/dead count was recorded for mortality (Gardner, 2017). Embryos were anesthetized with tricaine methane sulfate (MS222) to prevent them from moving (Gardner, 2017). The number of malformations and types of malformations were recorded on the scoresheet of malformations at 96-h as described in the FETAX Atlas of Abnormalities, malformations were recorded, but for a 12-d assay (Bantle & Sabourin, 1991). The embryos were photographed over a light source and head-tail lengths were measured using ImageJ (Bantle & Sabourin, 1991). The embryos were then humanely euthanized once the experiment was completed (Moser & Rayburn, 2007). Four more definitive concentration-response experiments were performed using the randomized clutch of eggs per experiment (Parker & Rayburn, 2017).

Data Analysis

Xenopus and *Ambystoma* data analyses were the same. Recorded data was placed in Systat Software for analysis (Shirey & Rayburn, 2013). To determine the 96-h LC_{50}, 96-h EC_{50} (malformations) for the *Xenopus* and the 12-d LC_{50}, 12-d EC_{50} (malformations) for the *Ambystoma*, probit analysis was used (Shirey & Rayburn, 2013). The Systat Software calculated the standard errors for the mortality, malformations, and length measurements of embryos from each concentration (Gardner, 2017). Using the Bonferroni's test, the minimal concentration to inhibit growth (MCIG), NOEC (no observed effects occurred), and LOEC (lowest concentration with observed effect) were determined for multiple comparisons (Moser & Rayburn, 2007). Using the formula LC50/EC50, the teratogenic index was calculated (Moser & Rayburn, 2007).

The teratogenic index (TI) was obtained from each of the experiments to determine any development risk from sodium fluoride and sodium selenate (Bantle et al, 1991).

Interaction Comparison

To determine if synergism and antagonism was present, an Isobole diagram was created (Moser & Rayburn, 2007). The pure chemicals, sodium fluoride and sodium selenate, have toxic units of 1.0, landing them on the X and Y axis of the diagram. The mixtures will land in places that will show if they are concentration response, response addition, synergistic, or antagonistic (See figure 1). The toxic units data points were placed on the Isobole diagram and if they fall under the concentration response line, they were considered synergistic, over the concentration response line and below the no effect lines was considered response addition and outside the no effect line was considered antagonistic (Moser & Rayburn, 2007)

Results

Xenopus Results

Sodium Fluoride

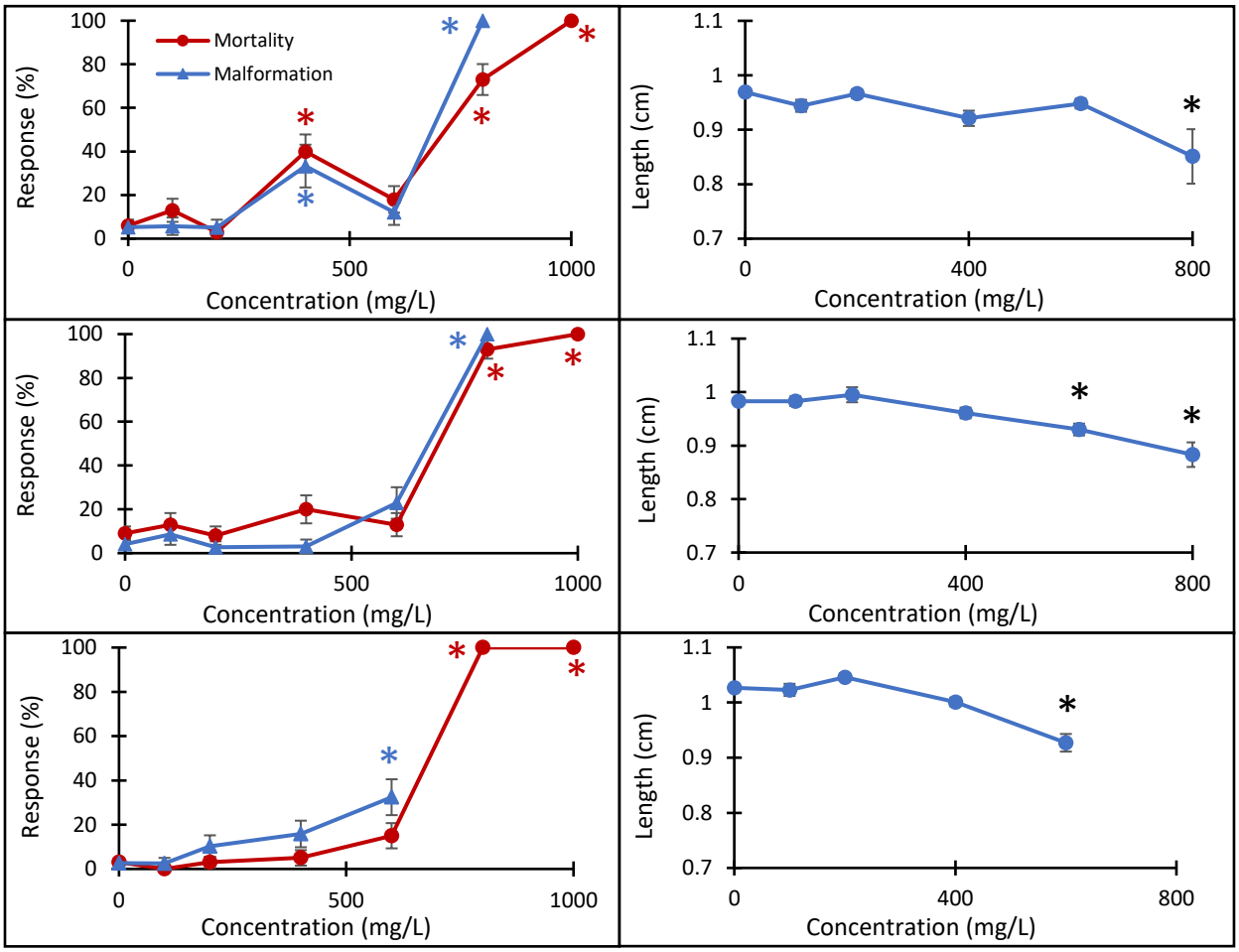
(Mixture designation Sodium fluoride : Sodium selenate 1:0)

Test 1's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 6.25% and 5.30%, respectively. The average pH measurement of FETAX was 7.73 ± 0.62, and the average pH measurement for sodium fluoride solution was 8.44 ± 0.46. The pH measurements that were taken for FETAX and sodium fluoride solutions were within the tolerance levels for this species. (Bantle & Sabourin, 1991). The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to sodium fluoride was 605.11 mg/L, with lower and upper Fieller bounds being 544.10 mg/L and 675.74 mg/L, respectively (Table 2). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to sodium fluoride was 743.02 mg/L, with lower and upper Fieller bounds being 609.10 mg/L and 993.64 mg/L, respectively (Table 2). When compared, malformation was similar with mortality until a change occurred at 800 mg/L showing 100% malformation. Mortality increased after 600 mg/L and hit 100% at 1,000 mg/L (Figure 2). The types of malformations that occurred in this test were loose guts, cephalic deformities, brain deformities, tail kinks, notochord bending, and several severe deformities (Figure 3). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 0.81. The mortality and malformation's NOEC and LOEC for the developing *Xenopus* embryos were 200 mg/L and 400 mg/L, respectively. The minimum concentration to inhibit growth (MCIG) was found to be 400 mg/L. The graphs indicate good consistency between the three-replicate test. The spacing

between the mortality and malformation lines are close together and are very steep. From controls to highest concentration, there was about a 10% reduction in growth.

Figure 2

Concentration-response graphs and growth graphs for ***sodium fluoride*** *: sodium selenate 1:0*



The top row is experiment 1, middle row is experiment 2 and the third row is experiment 3. The left graphs are mortality and malformation concentration-response graphs, and the right graphs represent growth. The * indicate significant difference from controls at $p \leq 0.05$.

Test 2's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 8.75% and 4.17%, respectively. The average pH measurement of FETAX was 7.39 ± 0.08, and the average pH measurement for sodium fluoride solution was 7.81 ± 0.15.

The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to sodium fluoride was 584.77 mg/L, with lower and upper Fieller bounds being 525.74 mg/L and 652.70 mg/L, respectively (Table 2). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to sodium fluoride was 989.94 mg/L, with lower and upper Fieller bounds being 736.23 mg/L and 1,758.21 mg/L, respectively (Table 2). When compared to test 1, similar results showed malformation was similar with mortality until a change occurred at 800 mg/L showing 100% malformation. Mortality increased after 600 mg/L and hit 100% at 1,000 mg/L (Figure 2). The types of malformations that occurred in this test were loose guts, edema, tail kinks, notochord bending, and a few severe deformities (Figure 3). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 0.59. The mortality and malformation's NOEC and LOEC for the developing *Xenopus* embryos were 600 mg/L and 800 mg/L, respectively. The minimum concentration to inhibit growth (MCIG) was found to be 600 mg/L.

Table 2

Results from three assays of Xenopus laevis embryos exposed to sodium fluoride.

Overall results for toxicity assays (mg/L)							Mortality		Malformation		MCIG
Assay	Total embryos	LC50	(95% Fieller)	EC50	(95% Fieller)	T.I.	NOEC	LOEC	NOEC	LOEC	
Test 1	320	605.11	544.10-675.74	743.02	609.10-993.64	0.81	200	400	200	400	400
Test 2	320	584.77	525.74-652.70	989.94	736.23-1,758.21	0.59	600	800	600	800	600
Test 3	320	628.74	582.77-678.08	783.48	623.67-1,150.08	0.80	400	600	400	600	600

TI = teratogenic index, NOEC = no observed effect concentration, LOEC = lowest observed effect concentration, MCIG = minimal concentration that inhibits growth

Test 3's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 2.50% and 2.56%, respectively. The average pH measurement of FETAX was 7.35 ± 0.36, and the average pH measurement for sodium fluoride solution was 7.90 ± 1.0. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to sodium fluoride was 628.74 mg/L, with lower and upper Fieller bounds being 582.77 mg/L and 678.06 mg/L, respectively (Table 2). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to sodium fluoride was 783.48 mg/L, with lower and upper Fieller bounds being 623.67 mg/L and 1,150.08 mg/L, respectively (Table 2). Mortality increased after 600 mg/L and hit 100% at 800 mg/L (Figure 2). The types of malformations that occurred in this test were loose guts, edemas, notochord bending, and a couple severe deformities (Figure 3). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 0.80. The mortality and malformation's NOEC and LOEC for the developing *Xenopus* embryos were 400 mg/L and 600 mg/L, respectively. The minimum concentration to inhibit growth (MCIG) was found to be 600 mg/L.

Figure 3

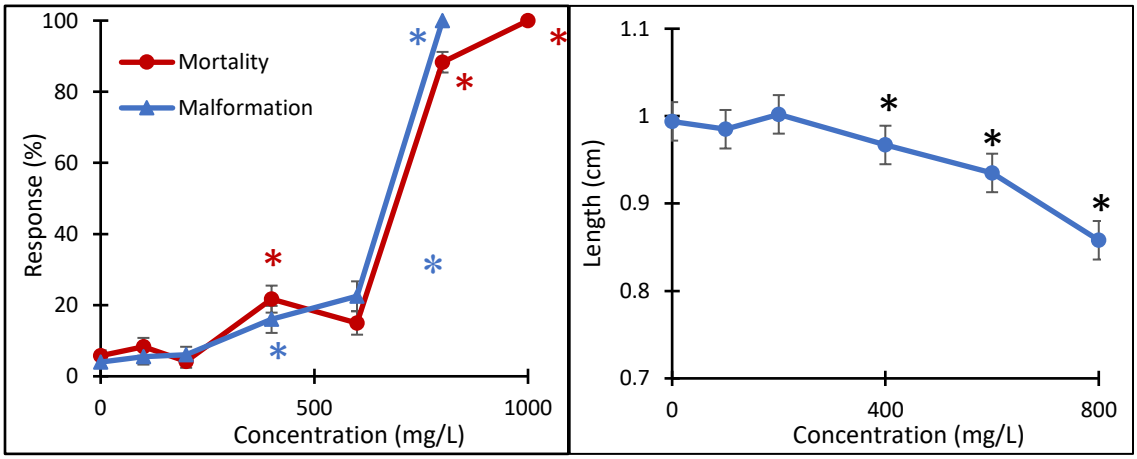
Malformations that appeared with Xenopus laevis that were exposed to sodium fluoride

(Top left) Test 1-healthy control *Xenopus* tadpole. (Top middle) Test 1 100 mg/L-loose gut malformation. (Top right) Test 2 600 mg/L-stunted with edemas, loose gut, and kinked tail. (Bottom left) Test 1 800 mg/L-stunted with bent notochord and loose gut malformation. (Bottom middle) Test 1 800 mg/L-stunted with optic, cephalic, edemas, loose gut, and bent notochord malformations. (Bottom right) Test 3 600 mg/L-stunted with edemas, loose gut, bent notochord and kinked tail.

The overall mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 5.83% and 3.98%, respectively. The overall average pH measurement of FETAX was 7.49 ± 0.21, and the overall average pH measurement for sodium fluoride solution was 8.05 ± 0.34. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to sodium fluoride was 603.36 mg/L, with lower and upper Fieller bounds being 570.58 mg/L and 638.61 mg/L, respectively (Table 8). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to sodium fluoride was 819.42 mg/L, with lower and upper Fieller bounds being 710.10 and 989.60 mg/L, respectively (Table 8). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 0.74. The mortality and malformation's NOEC and LOEC for the developing *Xenopus* embryos were 200 mg/L and 400 mg/L, respectively. The minimum concentration to inhibit growth (MCIG) was found to be 400 mg/L which can be observed in Figure 4.

Figure 4

Overall concentration-response graph and growth graph for sodium fluoride : sodium selenate 1:0

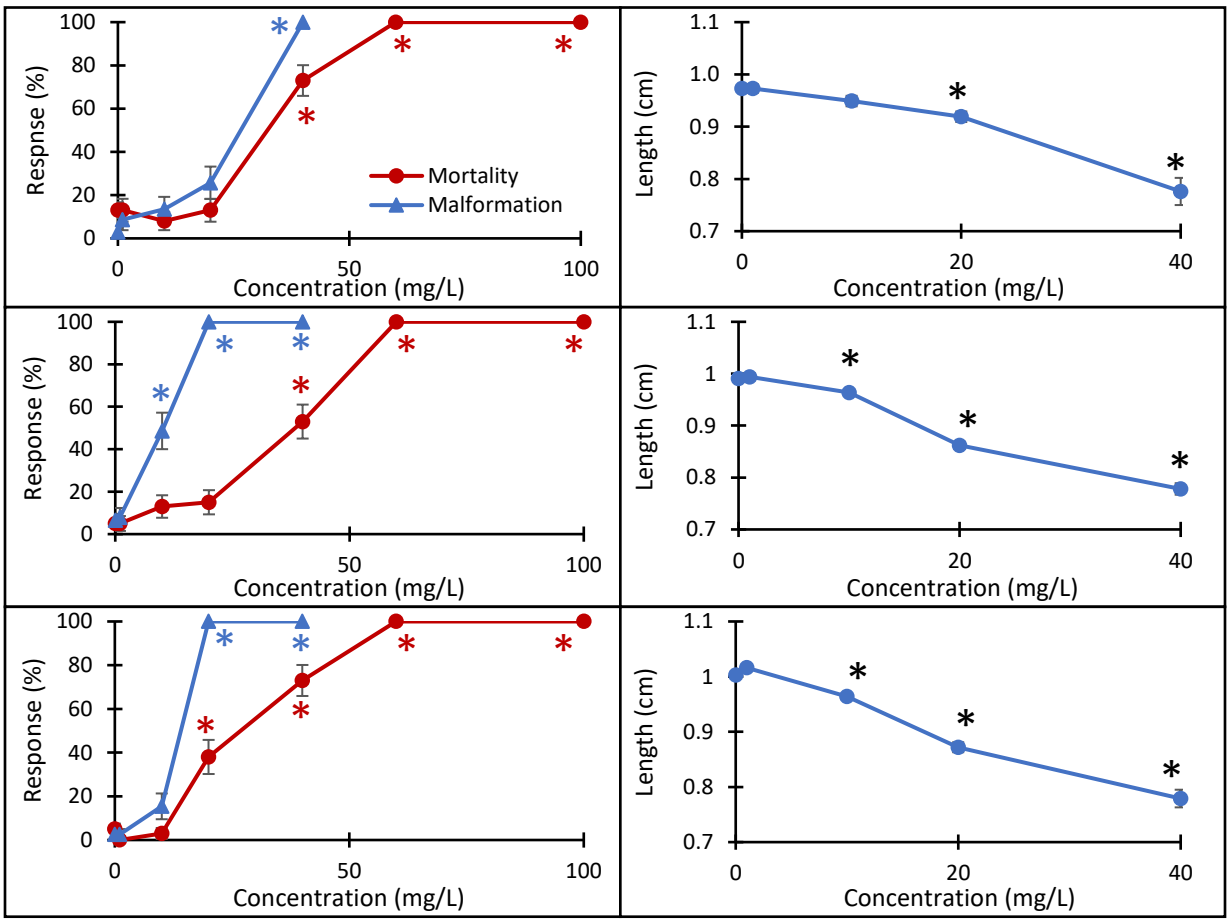


The left graph is a mortality and malformation concentration-response graph, and the right graph represents growth. The * indicate significant difference from controls at $p \leq 0.05$.

Sodium Fluoride : <u>Sodium Selenate</u> 0:1

Figure 5

*Concentration-response graphs and growth graphs for sodium fluoride : **sodium selenate** 0:1*



The top row is experiment 1, middle row is experiment 2 and the third row is experiment 3. The left graphs are mortality and malformation concentration-response graphs, and the right graphs represent growth. The * indicate significant difference from controls at $p \leq 0.05$.

Test 1's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 12.50% and 2.86%, respectively. The average pH measurement of FETAX was 7.45 ± 0.47, and the average pH measurement for sodium selenate solution was 7.20 ± 0.45. The pH measurements that were taken for FETAX and sodium selenate solutions were within the tolerance levels for this species. (Bantle & Sabourin, 1991).

The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to sodium selenate was 29.05 mg/L, with lower and upper Fieller bounds being 25.16 mg/L and 32.28 mg/L, respectively (Table 3). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to sodium selenate was 24.98 mg/L, with lower and upper Fieller bounds being 20.59 mg/L and 32.28 mg/L, respectively (Table 3). The types of malformations that appeared in this test were loose gut, edemas, cephalic deformities, tail kinks, notochord bending, and a few severe deformities (Figure 6). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 1.16. The mortality's NOEC and LOEC for the developing *Xenopus* embryos was 20 mg/L and 40 mg/L, and the NOEC and LOEC for malformations was 10 mg/L and 20 mg/L. The minimum concentration to inhibit growth (MCIG) was found to be 20 mg/L which can be observed in Figure 5. The spacing between the mortality and malformation lines are widening apart. The malformation lines are very steep while the mortality lines steadily increase over concentrations. From controls to highest concentration, there was over a 20% reduction in growth.

Test 2's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 5.00% and 6.58%, respectively. The average pH measurement of FETAX was 7.38 ± 0.10, and the average pH measurement for sodium selenate solution was 6.76 ± 0.31. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to sodium selenate was 33.66 mg/L, with lower and upper Fieller bounds being 29.72 mg/L and 38.39 mg/L, respectively (Table 3). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to sodium selenate was 9.10 mg/L, with lower and upper Fieller bounds being 7.55 mg/L and 11.05 mg/L, respectively (Table 3). The types of malformations that appeared in this test were loose gut, edemas, hemorrhages, and an optic deformity (Figure 6). The teratogenic index (96-h LC_{50} / 96-h EC_{50})

was 3.70. The mortality's NOEC and LOEC for the developing *Xenopus* embryos was 20 mg/L and 40 mg/L, and the NOEC and LOEC for malformations was 1 mg/L and 10 mg/L. This showed that mortality occurred at a higher concentration than malformations did. The minimum concentration to inhibit growth (MCIG) was found to be 10 mg/L which can be observed in Figure 5.

Table 3

Results from three assays of Xenopus laevis embryos exposed to sodium selenate

Overall results for toxicity assays (mg/L)							Mortality		Malformation		MCIG
Assay	Total embryos	LC50	(95% Fieller)	EC50	(95% Fieller)	T.I.	NOEC	LOEC	NOEC	LOEC	
Test 1	320	29.05	25.16-32.28	24.98	20.59-32.28	1.16	20	40	10	20	20
Test 2	320	33.66	29.72-38.39	9.10	7.55-11.05	3.7	20	40	1	10	10
Test 3	320	28.56	25.23-32.46	12.86	11.08-15.19	2.22	10	20	10	20	10

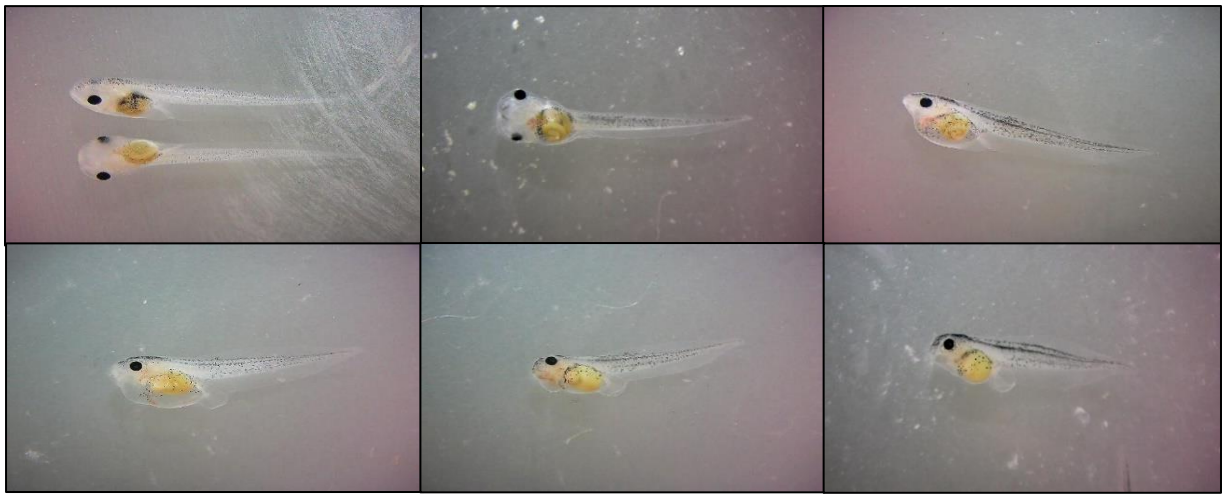
TI = teratogenic index, NOEC = no observed effect concentration, LOEC = lowest observed effect concentration, MCIG = minimal concentration that inhibits growth

Test 3's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 5.00% and 2.63%, respectively. The average pH measurement of FETAX was 7.17 ± 0.37, and the average pH measurement for sodium selenate solution was 7.16 ± 0.21. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to sodium selenate was 28.56 mg/L, with lower and upper Fieller bounds being 25.23mg/L and 32.46 mg/L, respectively (Table 3). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to sodium selenate was 12.86 mg/L, with lower and upper Fieller bounds being 11.08mg/L and 15.19 mg/L, respectively (Table 3). The types of malformations that appeared in this test were loose gut, edemas, notochord bending, hemorrhages, and brain deformities (Figure 6). The teratogenic index (96-h

LC_{50} / 96-h EC_{50}) was 2.22. The mortality and malformation's NOEC and LOEC for the developing *Xenopus* embryos was 10 and 20 mg/L. This showed that mortality and malformations occurred at the same concentration level. The minimum concentration to inhibit growth (MCIG) was found to be 10 mg/L which can be observed in Figure 5.

Figure 6

Malformations that appeared with Xenopus laevis that were exposed to sodium selenate



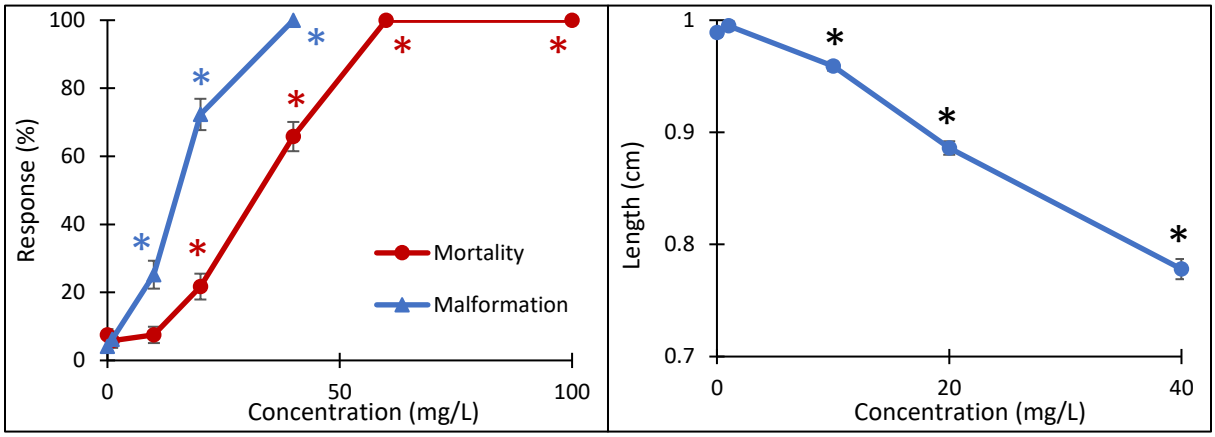
(Top left) Test 1-healthy control. (Top middle) Test 1 20 mg/L-loose gut. (Top right) Test 2 10 mg/L- loose gut and edema. (Bottom left) Test 3 40 mg/L-stunted with loose gut, edema, and hemorrhage. (Bottom middle) Test 2 20 mg/L-stunted with loose gut, edema, and hemorrhage. (Bottom right) Test 1 10 mg/L-stunted with loose gut, edema, cephalic, and bent notochord.

The overall mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 7.50% and 4.10%, respectively. The overall average pH measurement of FETAX was 7.33 ± 0.14, and the overall average pH measurement for sodium selenate solution was 7.04 ± 0.24. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to sodium selenate was 30.41 mg/L, with lower and upper Fieller bounds being 28.20 and 32.85 mg/L, respectively (Table 8). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to sodium selenate was 15.13 mg/L, with lower and upper Fieller bounds being 13.72 and 16.82 mg/L, respectively (Table 8). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 2.01. The

mortality's NOEC and LOEC for the developing *Xenopus* embryos was 10 and 20 mg/L and the NOEC and LOEC for malformations was 1 and 10 mg/L. The minimum concentration to inhibit growth (MCIG) was found to be 10 mg/L which can be observed in Figure 7.

Figure 7

Overall concentration-response graph and growth graph for sodium fluoride : ***sodium selenate 0:1***



The left graph is a mortality and malformation concentration-response graph, and the right graph represents growth. The * indicate significant difference from controls at $p \leq 0.05$.

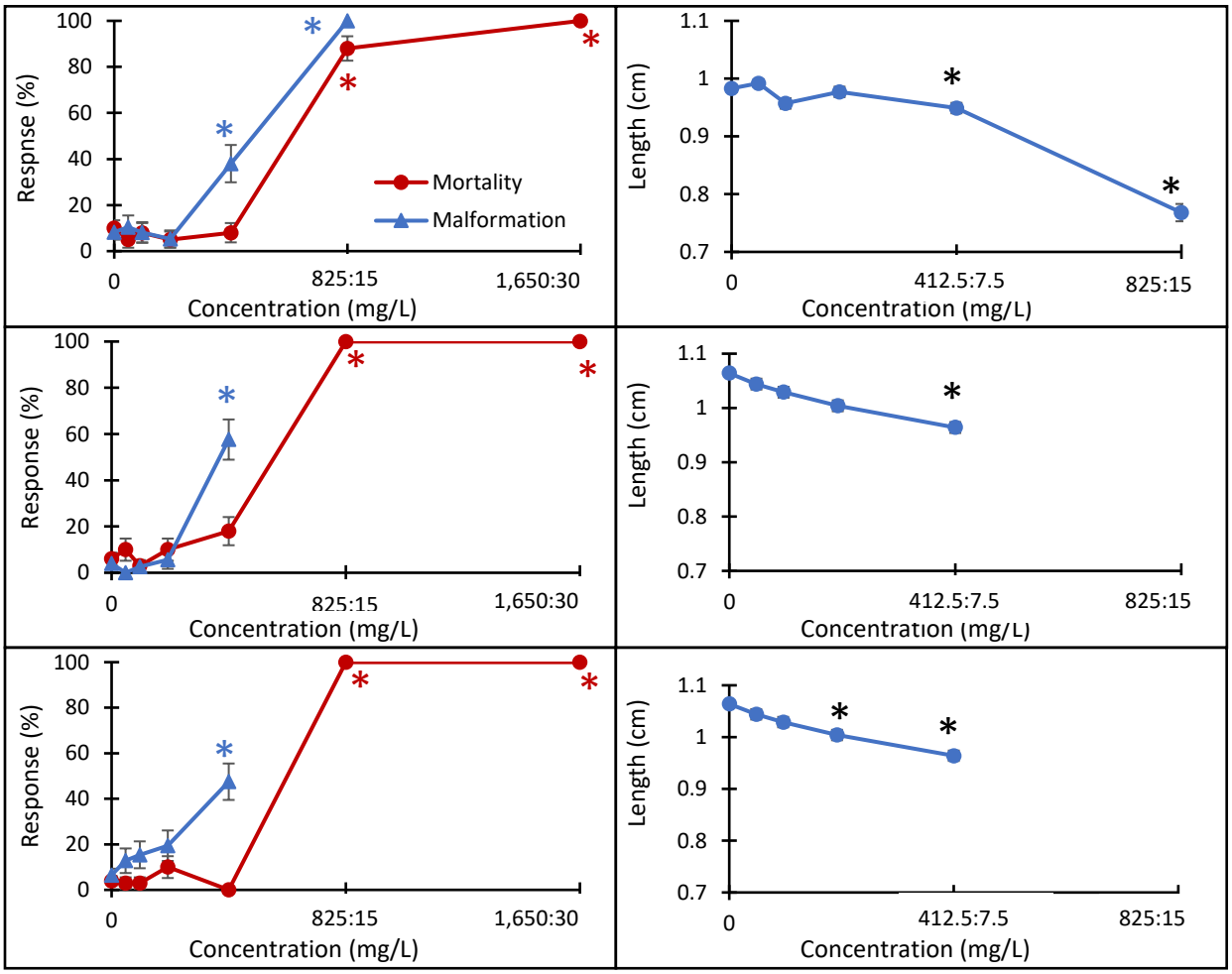
Sodium Fluoride : Sodium Selenate 3:1

Test 1's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 10.00% and 8.33%, respectively. The average pH measurement of FETAX was 7.79 ± 0.64, and the average pH measurement for the 3:1 (1,650:30) mixture solution was 8.55 ± 0.41. The pH measurements that were taken for FETAX and the 3:1 mixture solutions were within the tolerance levels for this species. (Bantle & Sabourin, 1991). The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to the 3:1 mixture was (594.66 mg/L_{NaF}:10.81 mg/$L_{Na2SeO4}$), with Fieller bounds (514.64 mg/L_{NaF}-9.36 mg/$L_{Na2SeO4}$:701.91 mg/L_{NaF}-12.76 mg/$L_{Na2SeO4}$), respectively (Table 4). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to

the 3:1 mixture was (520.41 mg/L_{NaF}:9.46 mg/L $_{Na2SeO4}$), with Fieller bounds (411.84 mg/L_{NaF}-7.49 mg/L $_{Na2SeO4}$:736.56 mg/L $_{NaF}$ -13.39 mg/L $_{Na2SeO4}$), respectively (Table 4).

Figure 8

Concentration-response graphs and growth graphs for ***sodium fluoride : sodium selenate*** *3:1*



The top row is experiment 1, middle row is experiment 2 and the third row is experiment 3. The left graphs are mortality and malformation concentration-response graphs, and the right graphs represents growth. The * indicate significant difference from controls at $p \leq 0.05$.

The types of malformations that appeared in this test were loose guts, edemas, kinked tails, bent notochords, eye, brain, cephalic and hemorrhages (Figure 9). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 1.14. The mortality's NOEC and LOEC for the developing *Xenopus*

embryos was (412.5_{NaF}:$7.5_{Na2SeO4}$ mg/L) and (825_{NaF}:$15_{Na2SeO4}$ mg/L), and the NOEC and LOEC for malformations was (198_{NaF}:$3.6_{Na2SeO4}$ mg/L) and (412.5_{NaF}:$7.5_{Na2SeO4}$ mg/L). This showed that mortality occurred at a higher concentration than malformations did. The minimum concentration to inhibit growth (MCIG) was found to be (412.5_{NaF}:$7.5_{Na2SeO4}$ mg/L) which can be observed in Figure 8. The spacing between the mortality and malformation lines are close together and are very steep. From controls to highest concentration, there was about a 10% reduction in growth.

Test 2's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 6.25% and 4.00%, respectively. The average pH measurement of FETAX was 7.28 ± 0.26, and the average pH measurement for the 3:1 (1,650:30) mixture solution was 8.06 ± 0.09. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to the 3:1 mixture was (487.74 mg/L_{NaF}:8.87 mg/$L_{Na2SeO4}$), with Fieller bounds (425.04 mg/L_{NaF}-7.73 mg/$L_{Na2SeO4}$:569.25 mg/L_{NaF} -10.35 mg/$L_{Na2SeO4}$), respectively (Table 4). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to the 3:1 mixture was (403.26 mg/L_{NaF}:7.33 mg/$L_{Na2SeO4}$), with Fieller bounds (344.03 mg/L_{NaF}-6.26 mg/$L_{Na2SeO4}$:496.49 mg/L_{NaF} -9.03 $_{Na2SeO4}$), respectively (Table 4). The types of malformations that appeared in this test were loose guts, edemas, and bent notochords (Figure 9). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 1.21. The mortality's NOEC and LOEC for the developing *Xenopus* embryos was (412.5_{NaF}:$7.5_{Na2SeO4}$ mg/L) and (825_{NaF}:$15_{Na2SeO4}$ mg/L), and the NOEC and LOEC for malformations was (198_{NaF}:$3.6_{Na2SeO4}$ mg/L) and (412.5_{NaF}:$7.5_{Na2SeO4}$ mg/L). This showed that mortality occurred at a higher concentration than malformations did. The minimum concentration to inhibit growth (MCIG) was found to be (412.5_{NaF}:$7.5_{Na2SeO4}$ mg/L) which can be observed in Figure 8.

Table 4

Results for three assays Xenopus laevis embryos exposed to mixture ratio 3:1 (1,650:30)

Overall results for toxicity assays (mg/L)							Mortality		Malformation		MCIG $NaF:Na_2SeO_4$
Assay	Total embryos	LC50 $NaF:Na_2SeO_4$	(95% Fieller) $NaF:Na_2SeO_4$	EC50 (Malformation) $NaF:Na_2SeO_4$	(95% Fieller) $NaF:Na_2SeO_4$	T.I.	NOEC $NaF:Na_2SeO_4$	LOEC $NaF:Na_2SeO_4$	NOEC $NaF:Na_2SeO_4$	LOEC $NaF:Na_2SeO_4$	
Test 1	320	594.66:10.81	514.64:9.36-701.91:12.76	520.41:9.46	411.84:7.49-736.56:13.39	1.14	37.5:7.5	825:15	198:3.6	37.5:7.5	37.5:7.5
Test 2	320	487.74:8.87	425.04:7.73-569.25:10.35	403.26:7.33	344.03:6.26-496.49:9.03	1.21	37.5:7.5	825:15	198:3.6	37.5:7.5	37.5:7.5
Test 3	320	546.15:9.93	482.30:8.77-627.33:11.41	433.79:7.89	343.86:6.25-613.80:11.16	1.26	37.5:7.5	825:15	198:3.6	37.5:7.5	198:3.6

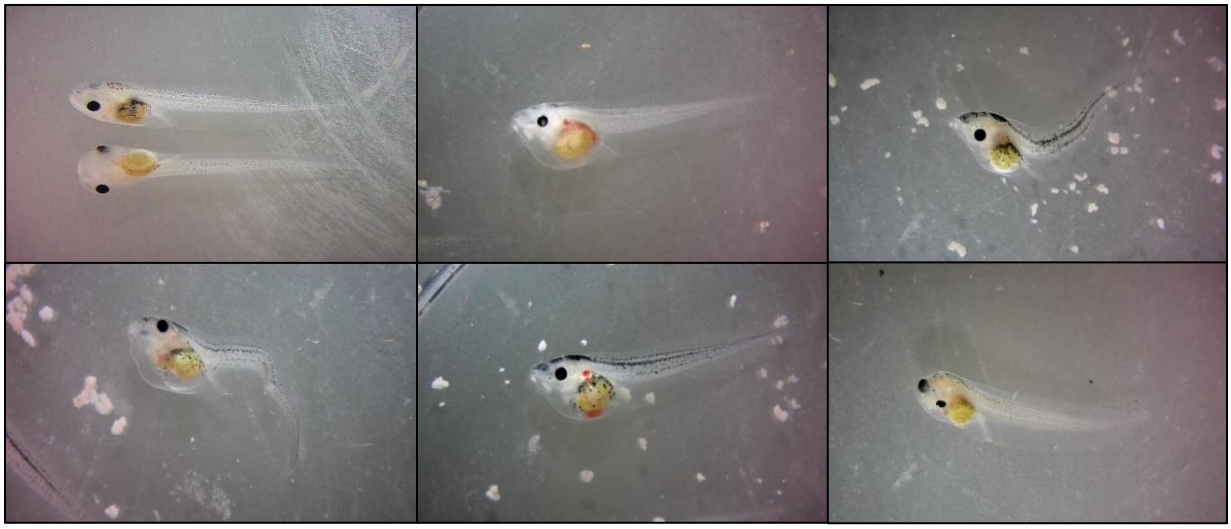
TI = teratogenic index, NOEC = no observed effect concentration, LOEC = lowest observed effect concentration, MCIG = minimal concentration that inhibits growth

Test 3's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 3.75% and 6.49%, respectively. The average pH measurement of FETAX was 7.40 ± 0.39, and the average pH measurement for the 3:1 (1,650:30) mixture solution was 8.03 ± 0.05. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to the 3:1 mixture was (546.15 mg/L_{NaF}:9.93 mg/L $_{Na2SeO4}$), with Fieller bounds (482.30 mg/L_{NaF}-8.77 mg/L $_{Na2SeO4}$:627.33 mg/L $_{NaF}$ -11.41 mg/L $_{Na2SeO4}$), respectively (Table 4). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to the 3:1 mixture was (433.79 mg/L_{NaF}:7.89 mg/L $_{Na2SeO4}$), with Fieller bounds (343.86 mg/L_{NaF}-6.25 mg/L $_{Na2SeO4}$:613.80 mg/L $_{NaF}$ -11.16 mg/L $_{Na2SeO4}$), respectively (Table 4). The types of malformations that appeared in this test were loose guts, edemas, kinked tails, bent notochords, and cardiac (Figure 9). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 1.26. The mortality's NOEC and LOEC for the developing *Xenopus* embryos was (412.5$_{NaF}$:7.5$_{Na2SeO4}$ mg/L) and (825$_{NaF}$:15$_{Na2SeO4}$ mg/L), and the NOEC and LOEC for malformations was (198$_{NaF}$:3.6$_{Na2SeO4}$ mg/L) and (412.5$_{NaF}$:7.5$_{Na2SeO4}$ mg/L). This showed that mortality occurred at a higher concentration than malformations did. The minimum

concentration to inhibit growth (MCIG) was found to be (198 $_{NaF}$:3.6$_{Na2SeO4}$ mg/L) which can be observed in Figure 8.

Figure 9

Malformations that appeared with Xenopus laevis that were exposed to ***sodium fluoride : sodium selenate*** *3:1*



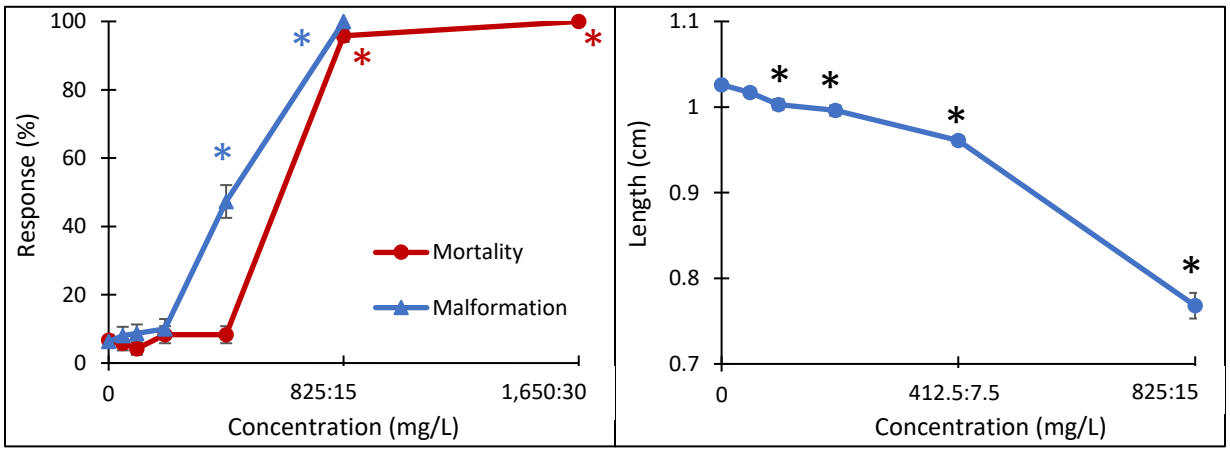
(Top left) Test 1-healthy control. (Top middle) Test 1 412.5:7.5 mg/L-loose gut, edema, and hemorrhage. (Top right) Test 2 412.5:7.5 mg/L-stunted with loose gut, kinked tail, and bent notochord. (Bottom left) Test 1 825:15 mg/L-stunted with loose gut, edema, bent notochord, and kinked tail. (Bottom middle) Test 3 412.5:7.5 mg/L-stunted with edema, loose gut, and hemorrhage. (Bottom right) Test 2 49.5:0.9 mg/L-stunted with eye, cephalic, brain, and loose gut.

The overall mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 6.67% and 6.23%, respectively. The overall average pH measurement of FETAX was 7.49 ± 0.27, and the overall average pH measurement for the 3:1 (1,650:30) mixture solution was 8.21 ± 0.29. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to the 3:1 mixture was (542.85 mg/L_{NaF}:9.87 mg/L $_{Na2SeO4}$), with Fieller bounds (501.60 mg/L_{NaF}-9.12 mg/L $_{Na2SeO4}$:590.87 mg/L $_{NaF}$ -10.74 mg/L $_{Na2SeO4}$), respectively (Table 8). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to the 3:1 mixture was (451.94 mg/L_{NaF}:8.22 mg/L $_{Na2SeO4}$), with Fieller bounds (396.99 mg/L_{NaF}-7.22 mg/L $_{Na2SeO4}$:529.98 mg/L $_{NaF}$ -9.64 mg/L

$_{Na2SeO4}$), respectively (Table 8). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 1.20. The mortality's NOEC and LOEC for the developing *Xenopus* embryos was (412.5$_{NaF}$:7.5 $_{Na2SeO4}$ mg/L) and (825$_{NaF}$:15 $_{Na2SeO4}$ mg/L), and the NOEC and LOEC for malformations was (198$_{NaF}$:3.6 $_{Na2SeO4}$ mg/L) and (412.5$_{NaF}$:7.5 $_{Na2SeO4}$ mg/L). This showed that mortality occurred at a higher concentration than malformations did. The minimum concentration to inhibit growth (MCIG) was found to be (99$_{NaF}$:1.8 $_{Na2SeO4}$ mg/L) which can be observed in Figure 10.

Figure 10

Overall concentration-response graph and growth graph for sodium fluoride : sodium selenate 3:1



The left graph is a mortality and malformation concentration-response graph, and the right graph represents growth. The * indicate significant difference from controls at $p \leq 0.05$.

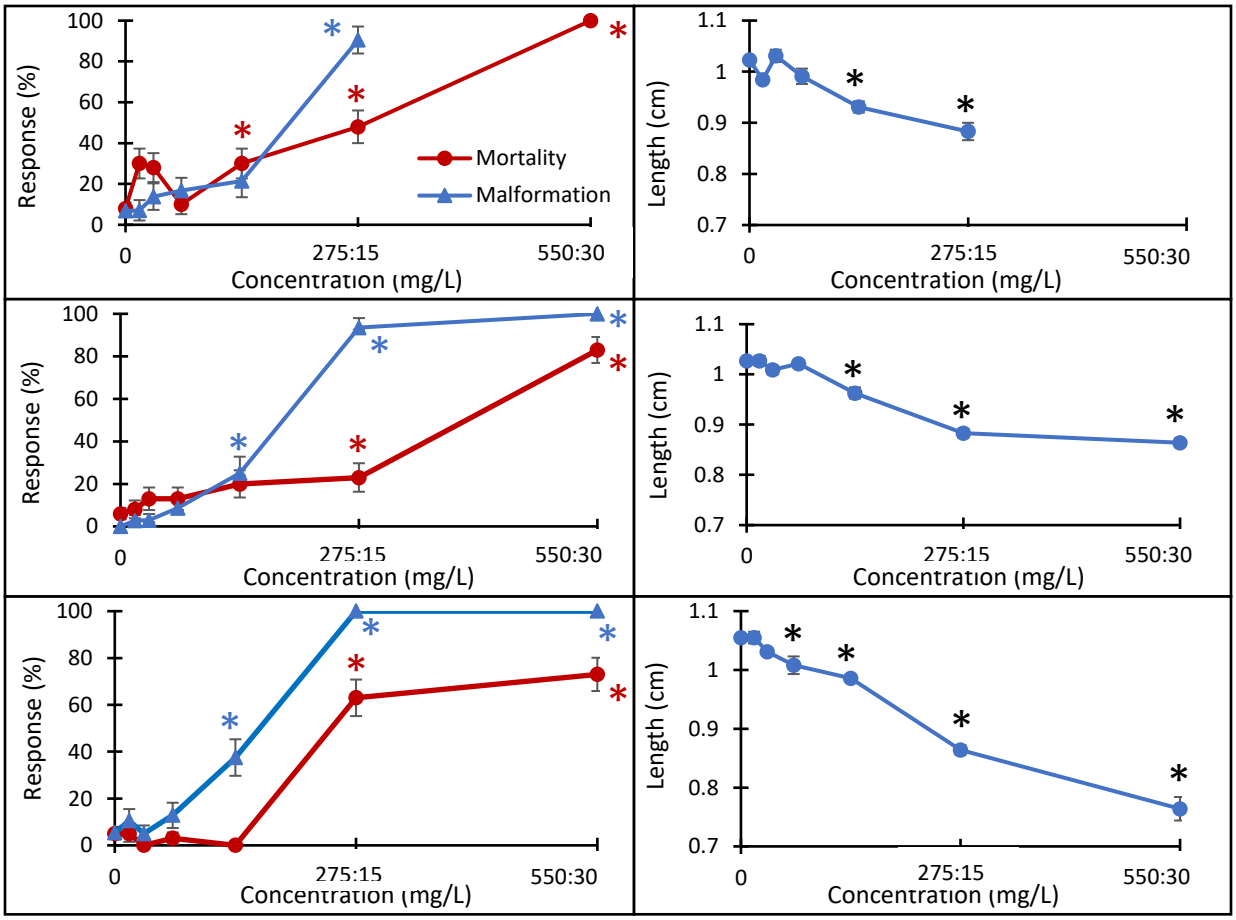
<u>Sodium Fluoride : Sodium Selenate</u> 1:1

Test 1's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 7.50% and 6.76%, respectively. The average pH measurement of FETAX was 7.76 ± 0.63, and the average pH measurement for the 1:1 (550:30) mixture solution was 8.04 ± 0.64. The pH measurements that were taken for FETAX and the 1:1 mixture solutions were within the tolerance levels for this species. (Bantle & Sabourin, 1991).

The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to the 1:1 mixture was 227.03 mg/L_{NaF}:12.38 mg/L $_{Na2SeO4}$, with Fieller bounds (190.61mg/L_{NaF}-10.40 mg/L $_{Na2SeO4}$:275.62 mg/L_{NaF}-15.03 mg/L $_{Na2SeO4}$), respectively (Table 5). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to the 1:1 mixture was (174.47 mg/L_{NaF}:9.52 mg/L $_{Na2SeO4}$), with Fieller bounds (144.01mg/L_{NaF}-7.86 mg/$L_{Na2SeO4}$:221.46 mg/L_{NaF}-12.08 mg/L $_{Na2SeO4}$), respectively (Table 5).

Figure 11

Concentration-response graphs and growth graphs for ***sodium fluoride : sodium selenate*** *1:1*



The top row is experiment 1, middle row is experiment 2 and the third row is experiment 3. The left graphs are mortality and malformation concentration-response graphs, and the right graphs represent growth. The * indicate significant difference from controls at $p \leq 0.05$.

The types of malformations that appeared in this test were only loose guts and edemas (Figure 12). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 1.3. The mortality's NOEC and LOEC for the developing *Xenopus* embryos was (66_{NaF}:$3.6_{Na2SeO4}$ mg/L) and (137.5_{NaF}:$7.5_{Na2SeO4}$ mg/L), and the NOEC and LOEC for malformations was (137.5_{NaF}:$7.5_{Na2SeO4}$ mg/L) and (275_{NaF}:$15_{Na2SeO4}$ mg/L). This showed that mortality occurred at a lower concentration than malformations did. The minimum concentration to inhibit growth (MCIG) was found to be (137.5_{NaF}:$7.5_{Na2SeO4}$ mg/L) which can be observed in Figure 11. The spacing between the mortality and malformation lines are widening apart. The malformation lines are very steep, and the mortality lines steadily increased over concentrations. From controls to highest concentration, there was about a 25% reduction in growth.

Test 2's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 6.25% and 0.00%, respectively. The average pH measurement of FETAX was 7.42 ± 0.10, and the average pH measurement for the 1:1 (550:30) mixture solution was 7.61 ± 0.12. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to the 1:1 mixture was (365.75 mg/L_{NaF}:19.95mg/L $_{Na2SeO4}$), with Fieller bounds (312.53 mg/L-17.05 mg/$L_{Na2SeO4}$:439.64 mg/L $_{NaF}$ -23.98 mg/L $_{Na2SeO4}$), respectively (Table 5). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to the 1:1 mixture was (174.31 mg/L_{NaF}:9.51 mg/L $_{Na2SeO4}$), with Fieller bounds (152.93 mg/L_{NaF}-8.34 mg/$L_{Na2SeO4}$:201.72 mg/L_{NaF}-11.00 mg/L $_{Na2SeO4}$), respectively (Table 5). The types of malformations that appeared in this test were only loose guts, edemas, notochord bending, hemorrhages, and severe deformities (Figure 12). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 2.1. The mortality's NOEC and LOEC for the developing *Xenopus* embryos was (275_{NaF}:$15_{Na2SeO4}$ mg/L) and (550_{NaF}:$30_{Na2SeO4}$ mg/L), and the

NOEC and LOEC for malformations was (66_{NaF}:$3.6_{Na2SeO4}$ mg/L) and (137.5_{NaF}:$7.5_{Na2SeO4}$ mg/L). This showed that mortality occurred at a higher concentration than malformations did. The minimum concentration to inhibit growth (MCIG) was found to be (137.5_{NaF}:$7.5_{Na2SeO4}$ mg/L) which can be observed in Figure 11.

Table 5

Results for three assays Xenopus laevis embryos exposed to mixture ratio 1:1 (550:30)

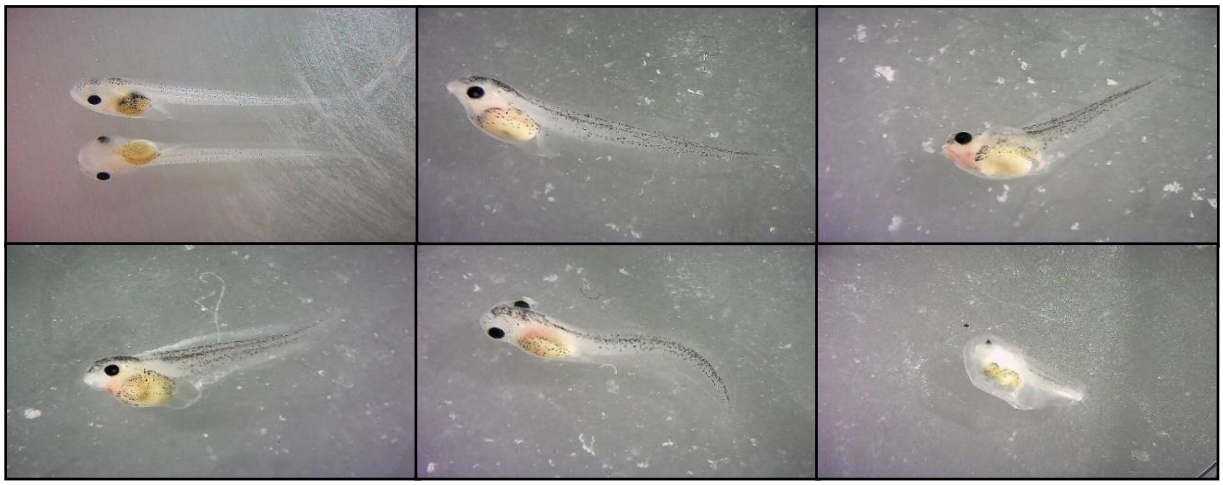
Overall results for toxicity assays (mg/L)							Mortality		Malformation		MCIG
Assay	Total embryos	LC50 $NaF:Na_2SeO_4$	(95% Fieller) $NaF:Na_2SeO_4$	EC50 (Malformation) $NaF:Na_2SeO_4$	(95% Fieller) $NaF:Na_2SeO_4$	T.I.	NOEC $NaF:Na_2SeO_4$	LOEC $NaF:Na_2SeO_4$	NOEC $NaF:Na_2SeO_4$	LOEC $NaF:Na_2SeO_4$	$NaF:Na_2SeO_4$
Test 1	320	227.03:12.38	190.63:10.40-275.61:15.03	174.47:9.52	144.05:7.86-221.43:12.08	1.30	66:3.6	137.50:7.50	137.50:7.50	275:15	137.50:7.50
Test 2	320	365.75:19.95	312.51:17.05-439.67:23.98	174.31:9.51	152.93:8.34-201.72:11.00	2.10	275:15	550:30	66:3.6	137.50:7.50	137.50:7.50
Test 3	320	366.18:19.97	321.42:17.53-423.56:23.10	152.66:8.33	128.78:7.02-189.05:10.31	2.40	137.50:7.50	275:15	66:3.6	137.50:7.50	66:3.6

TI = teratogenic index, NOEC = no observed effect concentration, LOEC = lowest observed effect concentration, MCIG = minimal concentration that inhibits growth

Test 3's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 5.00% and 5.26%, respectively. The average pH measurement of FETAX was 7.39 ± 0.38, and the average pH measurement for the 1:1 (550:30) mixture solution was 7.49 ± 0.30. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to the 1:1 mixture was (366.18 mg/L_{NaF}:19.97 mg/L $_{Na2SeO4}$), with Fieller bounds (321.39 mg/L_{NaF}-17.53 mg/L $_{Na2SeO4}$:423.53 mg/L $_{NaF}$ -23.10 mg/L $_{Na2SeO4}$), respectively (Table 5). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to the 1:1 mixture was (152.66 mg/L_{NaF}:8.33 mg/L $_{Na2SeO4}$), with Fieller bounds (128.78 mg/L_{NaF}-7.02 mg/L $_{Na2SeO4}$:189.05 mg/L_{NaF}-10.31 mg/L $_{Na2SeO4}$), respectively (Table 5).

Figure 12

Malformations that appeared with Xenopus laevis that were exposed to ***sodium fluoride : sodium selenate*** *1:1*



(Top left) Test 1-healthy control. (Top middle) Test 1 16.50:0.90 mg/L-loose gut malformation. (Top right) Test 1 275:15 mg/L-loose gut, edema, cephalic, and hemorrhage. (Bottom left) Test 2 550:30 mg/L-stunted with loose gut, edema, and hemorrhage. (Bottom middle) Test 1 66:3.6 mg/L-stunted with loose gut, edema, bent notochord, kinked tail, and hemorrhage. (Bottom right) Test 3 550:30 mg/L-severely stunted with loose gut, edema, cephalic, optic, kinked tail, and bent notochord.

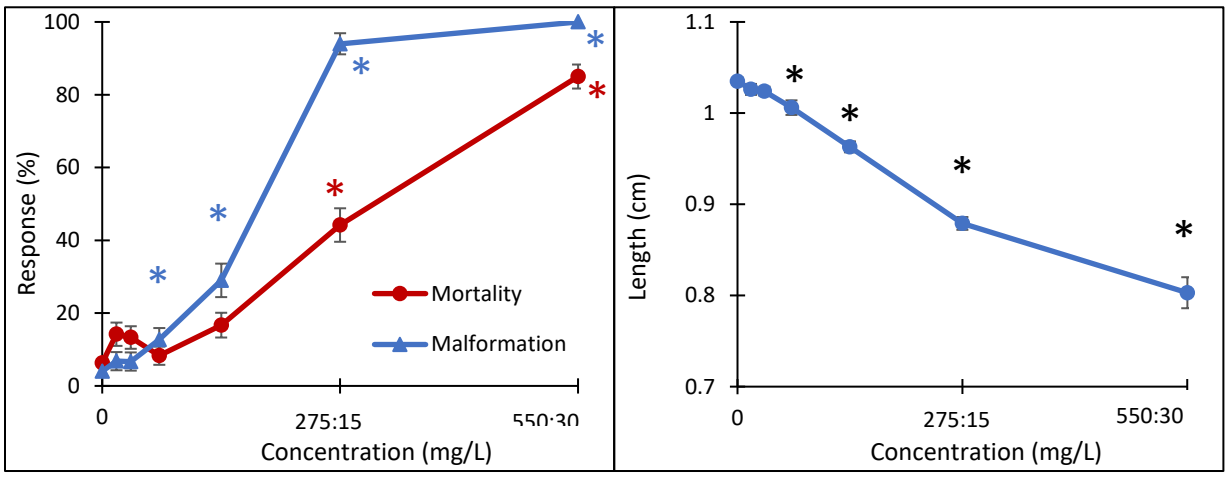
The types of malformations that appeared in this test were only loose guts, edemas, notochord bending, tail kinks, hemorrhages, and optic (Figure 12). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 2.40. The mortality's NOEC and LOEC for the developing *Xenopus* embryos was (137.5_{NaF}:$7.5_{Na2SeO4}$ mg/L) and (275_{NaF}:$15_{Na2SeO4}$ mg/L), and the NOEC and LOEC for malformations was (66_{NaF}:$3.6_{Na2SeO4}$ mg/L) and (137.5_{NaF}:$7.5_{Na2SeO4}$ mg/L). This showed that mortality occurred at a higher concentration than malformations did. The minimum concentration to inhibit growth (MCIG) was found to be (66_{NaF}:$3.6_{Na2SeO4}$ mg/L) which can be observed in Figure 11.

The overall mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 6.25% and 4.00%, respectively. The overall average pH measurement of FETAX was 7.52 ± 0.20, and the overall average pH measurement for the 1:1 (550:30) mixture

solution was 7.71 ± 0.29. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to the 1:1 mixture was (319.97 mg/L_{NaF}:17.45 mg/L $_{Na2SeO4}$), with Fieller bounds (292.36 mg/L_{NaF}-15.95 mg/L $_{Na2SeO4}$:352.50 mg/L $_{NaF}$ -19.23 mg/L $_{Na2SeO4}$), respectively (Table 8). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to the 1:1 mixture was (166.27 mg/L_{NaF}:9.07mg/L $_{Na2SeO4}$), with Fieller bounds (151.04 mg/L_{NaF}-8.24 mg/$L_{Na2SeO4}$:184.97 mg/L $_{NaF}$ -10.09 mg/L $_{Na2SeO4}$), respectively (Table 8). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 1.92. The mortality's NOEC and LOEC for the developing *Xenopus* embryos was (137.5$_{NaF}$:7.5 $_{Na2SeO4}$ mg/L) and (275$_{NaF}$:15 $_{Na2SeO4}$ mg/L) and the NOEC and LOEC for malformations was (33$_{NaF}$:1.8 $_{Na2SeO4}$ mg/L) and (66 $_{NaF}$:3.6 $_{Na2SeO4}$ mg/L). The minimum concentration to inhibit growth (MCIG) was found to be (66 $_{NaF}$:3.6 $_{Na2SeO4}$ mg/L) which can be observed in Figure13.

Figure 13

Overall concentration-response graph and growth graph for sodium fluoride : sodium selenate 1:1

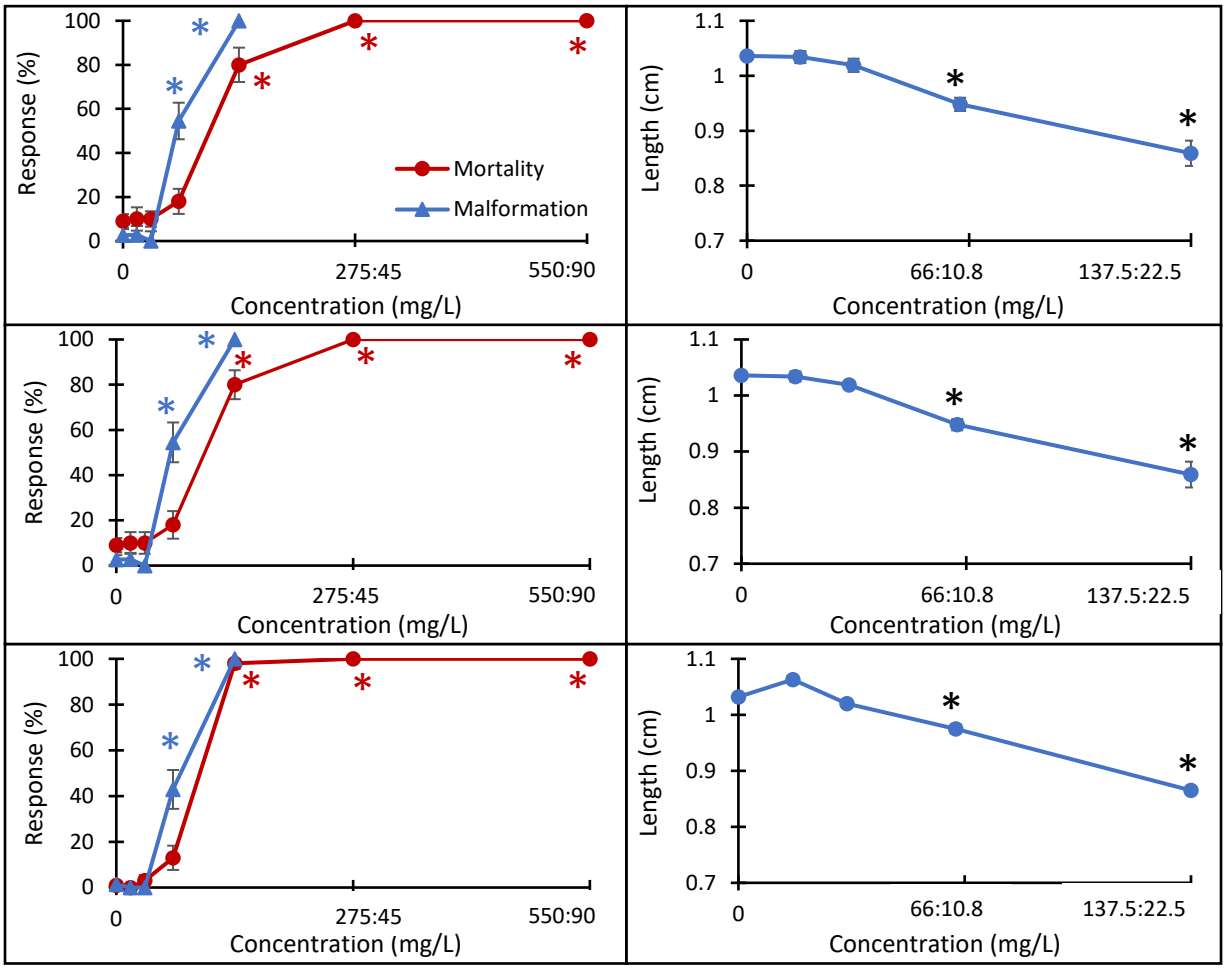


The left graph is a mortality and malformation concentration-response graph, and the right graph represents growth. The * indicate significant difference from controls at $p \leq 0.05$.

***Sodium Fluoride : Sodium Selenate* 1:3**

Figure 14

Concentration-response graphs and growth graphs for ***sodium fluoride : sodium selenate*** *1:3*



The top row is experiment 1, middle row is experiment 2 and the third row is experiment 3. The left graphs are mortality and malformation concentration-response graphs, and the right graphs represent growth. The * indicate significant difference from controls at $p \leq 0.05$.

Test 1's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 8.75% and 5.48%, respectively. The average pH measurement of FETAX was 7.73 ± 0.62, and the average pH measurement for the 1:3 (550:90) mixture solution was 8.12

± 0.61. The pH measurements that were taken for FETAX and the 1:3 mixture solutions were within the tolerance levels for this species. (Bantle & Sabourin, 1991).

The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to the 1:3 mixture was (119.24 mg/L_{NaF}:19.51 mg/L $_{Na2SeO4}$), with Fieller bounds (102.58 mg/L_{NaF}-16.79 mg/$L_{Na2SeO4}$:141.35 mg/L $_{NaF}$ -23.13 mg/L $_{Na2SeO4}$), respectively (Table 6). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to the 1:3 mixture was (76.73 mg/L_{NaF}:12.56 mg/L $_{Na2SeO4}$), with Fieller bounds (64.63 mg/L_{NaF}-10.58 mg/$L_{Na2SeO4}$:95.26 mg/L $_{NaF}$ -15.59 mg/L $_{Na2SeO4}$), respectively (Table 6).

The types of malformations that appeared in this test were loose guts, edemas, abdominal, kinked tail, bent notochord, eye, brain, and hemorrhage (Figure 15). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 1.55. The mortality's NOEC and LOEC for the developing *Xenopus* embryos was (66 $_{NaF}$:10.8 $_{Na2SeO4}$ mg/L) and (137.5 $_{NaF}$:22.5 $_{Na2SeO4}$ mg/L), and the NOEC and LOEC for malformations was (33 $_{NaF}$:5.4 $_{Na2SeO4}$ mg/L) and (66 $_{NaF}$:10.8 $_{Na2SeO4}$ mg/L). This showed that mortality occurred at a higher concentration than malformations did. The minimum concentration to inhibit growth (MCIG) was found to be (66 $_{NaF}$:10.8 $_{Na2SeO4}$ mg/L) which can be observed in Figure 14. The spacing between the mortality and malformation lines are close together and are very steep. From controls to highest concentration, there was under a 20% reduction in growth.

Test 2's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 8.75% and 2.74%, respectively. The average pH measurement of FETAX was 7.38 ± 0.08, and the average pH measurement for the 1:3 (550:90) mixture solution was 7.77 ± 0.07. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to the 1:3 mixture was

(98.395 mg/L_{NaF}:16.10 mg/L $_{Na2SeO4}$), with Fieller bounds (84.48 mg/L_{NaF}-13.82 mg/L $_{Na2SeO4}$:116.93 mg/L_{NaF}-19.13 mg/L $_{Na2SeO4}$), respectively (Table 6). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to the 1:3 mixture was (67.49 mg/L_{NaF}:11.04 mg/L $_{Na2SeO4}$), with Fieller bounds (58.03 mg/L_{NaF}-9.50 mg/L $_{Na2SeO4}$:82.23 mg/L $_{NaF}$ -13.46 mg/L $_{Na2SeO4}$), respectively (Table 6). The types of malformations that appeared in this test were loose guts, edemas, kinked tail, and bent notochord (Figure 15). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 1.46. The mortality's NOEC and LOEC for the developing *Xenopus* embryos was (66_{NaF}:$10.8_{Na2SeO4}$ mg/L) and (137.5_{NaF}:$22.5_{Na2SeO4}$ mg/L), and the NOEC and LOEC for malformations was (33_{NaF}:$5.4_{Na2SeO4}$ mg/L) and (66_{NaF}:$10.8_{Na2SeO4}$ mg/L). This showed that mortality occurred at a higher concentration than malformations did. The minimum concentration to inhibit growth (MCIG) was found to be (66_{NaF}:$10.8_{Na2SeO4}$ mg/L) which can be observed in Figure 14.

Table 6

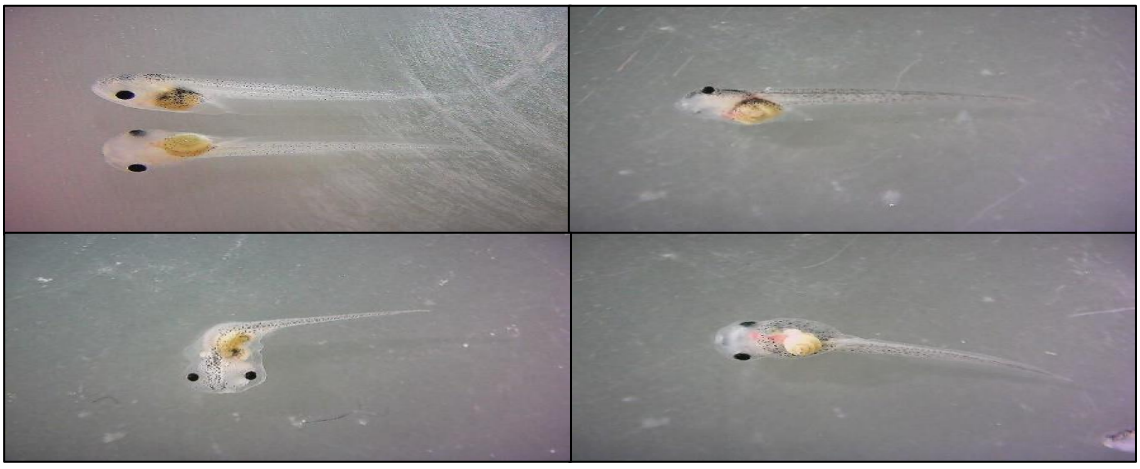
Results for three assays Xenopus laevis embryos exposed to mixture ratio 1:3 (550:90)

Overall results for toxicity assays (mg/L)							Mortality		Malformation		MCIG
Assay	Total embryos	LC50 $NaF:Na_2SeO_4$	(95% Fieller) $NaF:Na_2SeO_4$	EC50 (Malformation) $NaF:Na_2SeO_4$	(95% Fieller) $NaF:Na_2SeO_4$	T.I.	NOEC $NaF:Na_2SeO_4$	LOEC $NaF:Na_2SeO_4$	NOEC $NaF:Na_2SeO_4$	LOEC $NaF:Na_2SeO_4$	$NaF:Na_2SeO_4$
Test 1	320	119.24:19.51	102.58:16.79-141.35:23.13	76.73:12.56	64.63:10.58-95.26:15.59	1.55	66:10.8	137.50:22.5	33:5.4	66:10.8	66:10.8
Test 2	320	98.40:16.10	84.48:13.82-116.93:19.13	67.49:11.04	58.03:9.50-82.23:13.46	1.46	66:10.8	137.50:22.5	33:5.4	66:10.8	66:10.8
Test 3	320	90.75:14.85	81.02:13.26-102.52:16.78	72.99:11.94	63.58:10.40-89.82:14.70	1.24	33:5.4	66:10.8	33:5.4	66:10.8	66:10.8

TI = teratogenic index, NOEC = no observed effect concentration, LOEC = lowest observed effect concentration, MCIG = minimal concentration that inhibits growth

Figure 15

Malformations that appeared with Xenopus laevis that were exposed to ***sodium fluoride : sodium selenate*** *1:3*



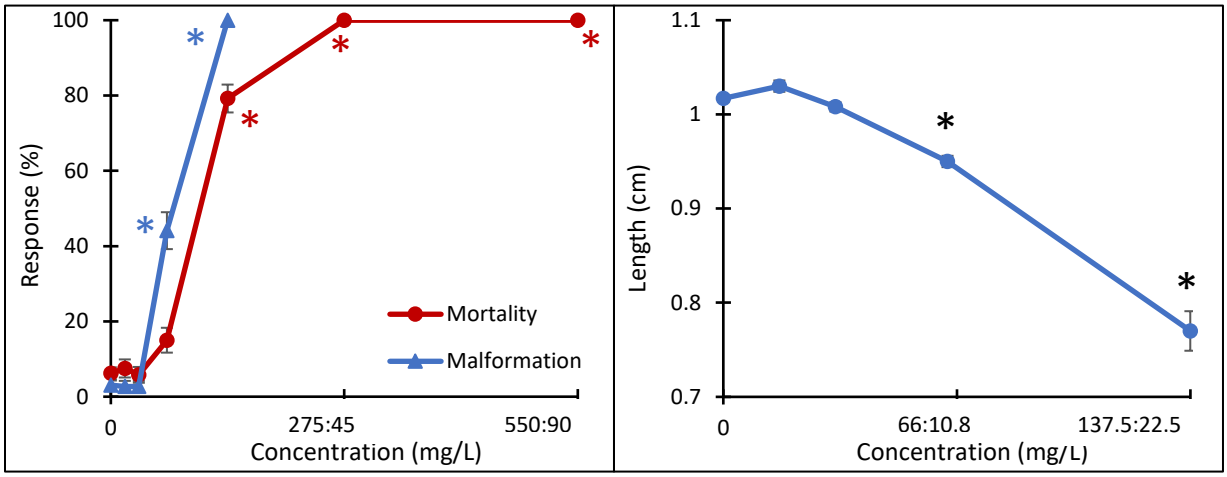
(Top left) Test 1-healthy control. (Top right) Test 1 66:10.8 mg/L- loose gut (Bottom left) Test 2 137.50:22.50 mg/L-stunted with loose gut, edema, bent notochord, and eye. (Bottom right) Test 1 66:10.80 mg/L-loose gut and edema.

Test 3's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 1.25% and 1.27%, respectively. The average pH measurement of FETAX was 7.41 ± 0.39, and the average pH measurement for the 1:3 (550:90) mixture solution was 7.61 ± 0.17. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to the 1:3 mixture was 16.50% (90.75 mg/L_{NaF}:14.85 mg/L $_{Na2SeO4}$), with Fieller bounds (81.02 mg/L_{NaF}-13.26 mg/L $_{Na2SeO4}$:102.52 mg/L_{NaF}-16.78 mg/L $_{Na2SeO4}$), respectively (Table 6). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to the 1:3 mixture was 13.27% (72.99 mg/L_{NaF}:11.94 mg/L $_{Na2SeO4}$), with Fieller bounds (63.58 mg/L_{NaF}-10.40 mg/L $_{Na2SeO4}$:89.82 mg/L_{NaF}-14.70 mg/L $_{Na2SeO4}$), respectively (Table 6). The types of malformations that appeared in this test were loose guts, edemas, kinked tail, and bent notochord (Figure 15). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 1.46. The mortality and malformation's NOEC and LOEC for the developing *Xenopus* embryos were (33_{NaF}:$5.4_{Na2SeO4}$ mg/L) and (66_{NaF}:$10.8_{Na2SeO4}$ mg/L). This showed that

mortality appeared at a lower concentration than malformations did. The minimum concentration to inhibit growth (MCIG) was found to be (66_{NaF}:$10.8_{Na2SeO4}$ mg/L) which can be observed in Figure 14.

Figure 16

Overall concentration-response graph and growth graph for sodium fluoride : sodium selenate 1:3



The left graph is a mortality and malformation concentration-response graph, and the right graph represents growth. The * indicate significant difference from controls at p < or equal to 0.05.

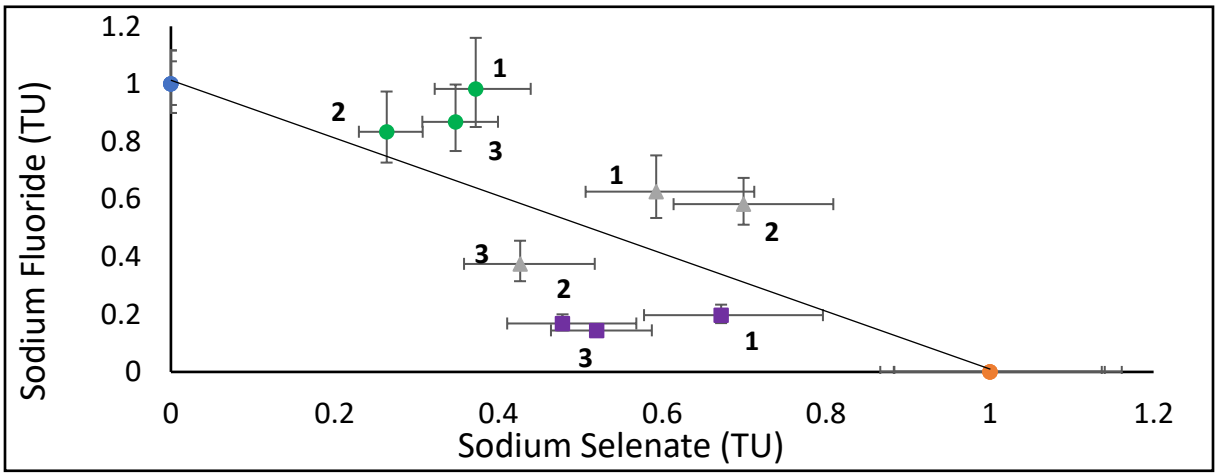
The overall mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 6.25% and 3.11%, respectively. The overall average pH measurement of FETAX was 7.50 ± 0.19, and the overall average pH measurement for the 1:3 (550:90) mixture solution was 7.83 ± 0.26. The mean 96-h LC_{50} for developing *Xenopus* embryos exposed to the 1:3 mixture was (102.69 mg/L_{NaF}:16.80 mg/L $_{Na2SeO4}$), with Fieller bounds (94.49 mg/L_{NaF}-15.46 mg/L $_{Na2SeO4}$:112.31 mg/L_{NaF}-18.38 mg/L $_{Na2SeO4}$), respectively (Table 8). The mean 96-h EC_{50} for developing *Xenopus* embryos exposed to the 1:3 mixture was (73.81 mg/L_{NaF}:12.08 mg/L $_{Na2SeO4}$), with Fieller bounds (66.88 mg/L_{NaF}-10.94 mg/L $_{Na2SeO4}$:82.94 mg/L_{NaF}-13.57 mg/L

$_{Na2SeO4}$), respectively (Table 8). The teratogenic index (96-h LC_{50} / 96-h EC_{50}) was 1.39. The mortality's NOEC and LOEC for the developing *Xenopus* embryos was (66_{NaF}:$10.8_{Na2SeO4}$ mg/L) and (137.5_{NaF}:$22.5_{Na2SeO4}$ mg/L), and the NOEC and LOEC for malformations was (33_{NaF}:$5.4_{Na2SeO4}$ mg/L) and (66_{NaF}:$10.8_{Na2SeO4}$ mg/L). This showed that mortality occurred at a higher concentration than malformations did. The minimum concentration to inhibit growth (MCIG) was found to be (66_{NaF}:$10.8_{Na2SeO4}$ mg/L) which can be observed in Figure 16.

Interaction Results (Isobole Diagrams)

Figure 17

Isobole diagram of toxic units for LC_{50} of Xenopus laevis experiments



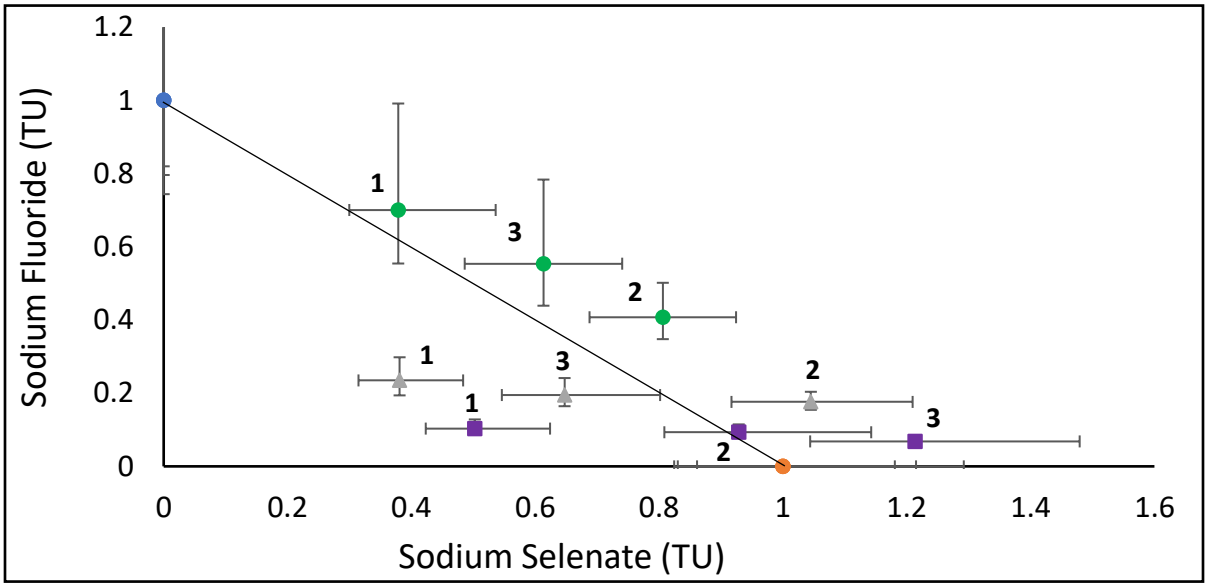
The green circle, grey triangle, and purple square represent 3:1 mixture tests, 1:1 mixture tests, and 1:3 mixture tests, respectively.

The Isobole diagram in Figure 17 shows representation of the LC_{50} of all experiments performed. The toxic units for sodium fluoride are located on the horizontal line while the toxic units of sodium selenate are located on the vertical line. With the 3:1 mixture, tests 1 and 3 showed response addition. Test 2, however, showed concentration response. Experiments 1, 2, and 3 showed toxic units of 1.36, 1.10, and 1.22, respectively. With the 1:1 mixture, experiments

1 and 2 showed response addition. Experiment 3, however, showed synergism. Experiments 1, 2, and 3 showed toxic units of 0.80, 1.22, and 1.28, respectively. With the 1:3 mixture, all experiments showed synergism. Experiments 1, 2, and 3 showed toxic units of 0.87, 0.65, and 0.66, respectively. This mixture was considered more toxic than the other mixtures.

Figure 18

Isobole diagram of toxic units for EC_{50} of Xenopus laevis experiments



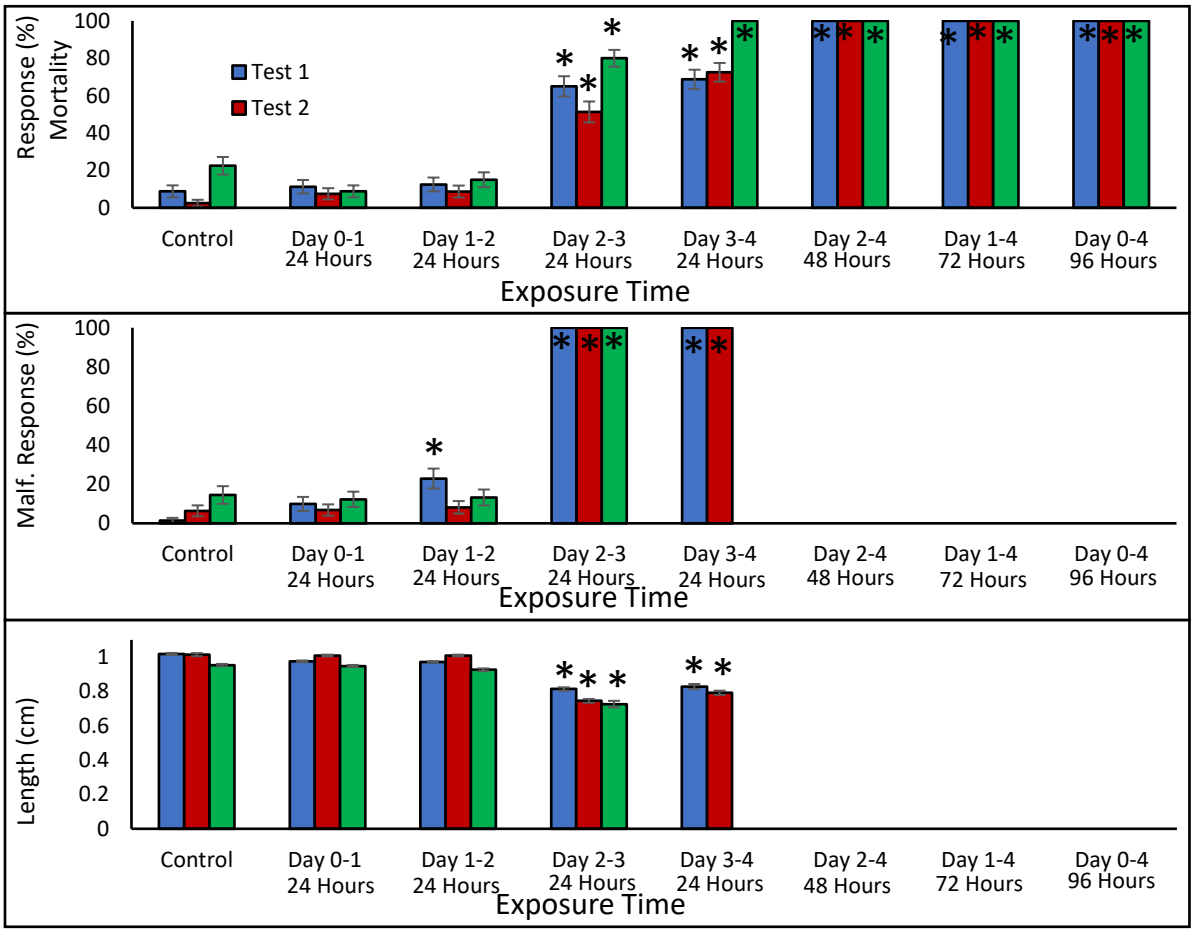
The green circle, grey triangle, and purple square represent 3:1 mixture tests, 1:1 mixture tests, and 1:3 mixture tests, respectively.

The Isobole diagram in Figure 18 shows representation of the EC_{50} of all experiments performed. With the 3:1 mixture, two experiments showed response addition. One experiment showed concentration response. With the 1:1 mixture, two experiments showed synergism. One experiment showed response addition. With the 1:3 mixture, each experiment showed a different kind of interaction; synergism, concentration response, and antagonism.

Duration Vs. Timed Exposure

Figure 19

Accumulation vs. Burst effect on Xenopus laevis using 100mg/L of sodium selenate



(Top) Mortality of *Xenopus* exposed to sodium selenate at different times. (Middle) Malformations of Xenopus exposed to sodium selenate at different times. (Bottom) Length measurements of Xenopus exposed to sodium selenate at different times. The * indicate significant difference from controls at $p \leq 0.05$.

Test 1's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 8.75% and 1.37%, respectively. The average pH measurement of FETAX was 7.07, and the average pH measurement for sodium selenate solution was 7.19. Test 2's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 2.50% and 6.41%, respectively. The average pH measurement of FETAX was 7.18, and the

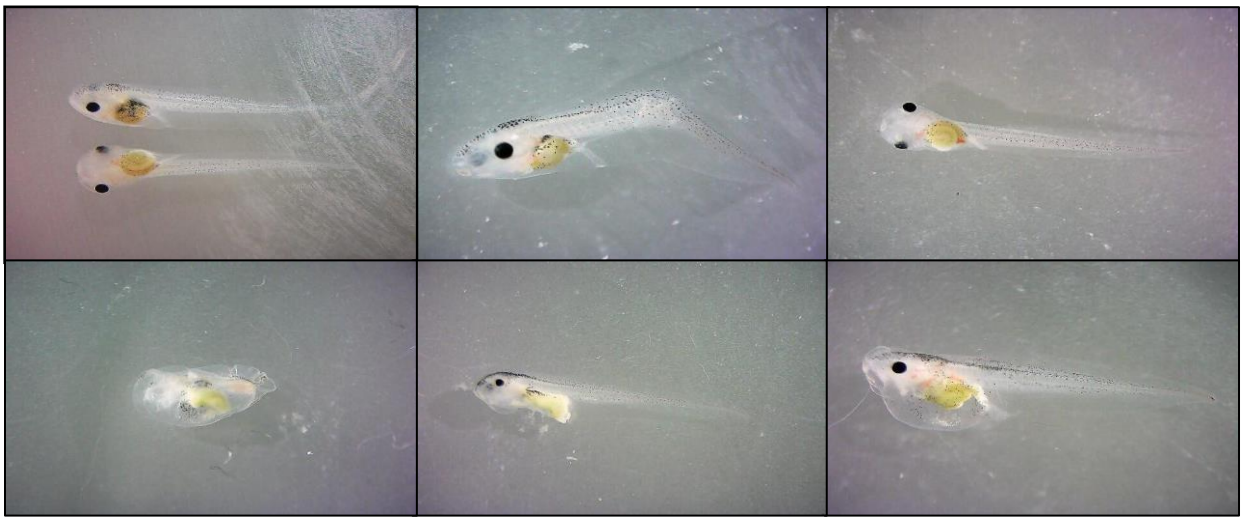
average pH measurement for sodium selenate solution was 7.27. Test 3's mortality and malformation responses for *Xenopus laevis* that were raised in FETAX solution were 22.50% and 14.52%, respectively. The average pH measurement of FETAX was 7.50, and the average pH measurement for sodium selenate solution was 7.52.

In all experiments mortality occurred 100% in days 2-4, 1-4, and 0-4 which can be seen in figure 19. In test 3, 100% mortality also occurred in the 24 h -Day 3-4. Throughout the experiments, day 0-1 and day 1-2 showed minimal mortality. Test 3 had higher mortality levels throughout the experiment compared to experiments 1 and 2. There was significant difference from controls in all duration time exposures as well as days 2-3 and 3-4.

As shown in Figure 19, all experiments showed malformations that occurred 100% in days 2-3 and days 3-4. Little malformation appeared in the earlier days of the experiment which suggests that they needed to progress in development for higher levels of malformations to appear. Minimal malformations were found in controls, day 0-1, and day 1-2. Most malformations that appeared occurred in the days 2-3 and days 3-4 and were loose guts, edemas, eye abnormalities, hemorrhages, kinked tails, and bent notochord (figure 20). There was significant difference from controls in days 2-3 and 3-4.

Figure 20

Malformations that appeared with Xenopus laevis that were exposed to 100 mg/L sodium selenate



(Top left) Test 1-healthy control *Xenopus* tadpole. (Top middle) Test 1 days 2-3-bent notochord and kinked tail. (Top right) Test 1 days 2-3-hemmorhage. (Bottom left) Test 2 days 3-4-severe abnormality. (Bottom middle) Test 2 days 3-4-stunted with loose gut, edema, and eye. (Bottom right) Test 1 days 3-4-hemmorhage and edema.

The length measurements showed a similar pattern throughout the three experiments. The controls, day 0-1, and day 1-2 showed little decrease in length growth. Day 2-3 showed the smallest length measurements between all the days. Day 3-4 also showed a significant length difference compared to the first two days in all the experiments. There was significant difference in length on days 2-3 and 3-4 compared to the controls.

Ambystoma **Results**

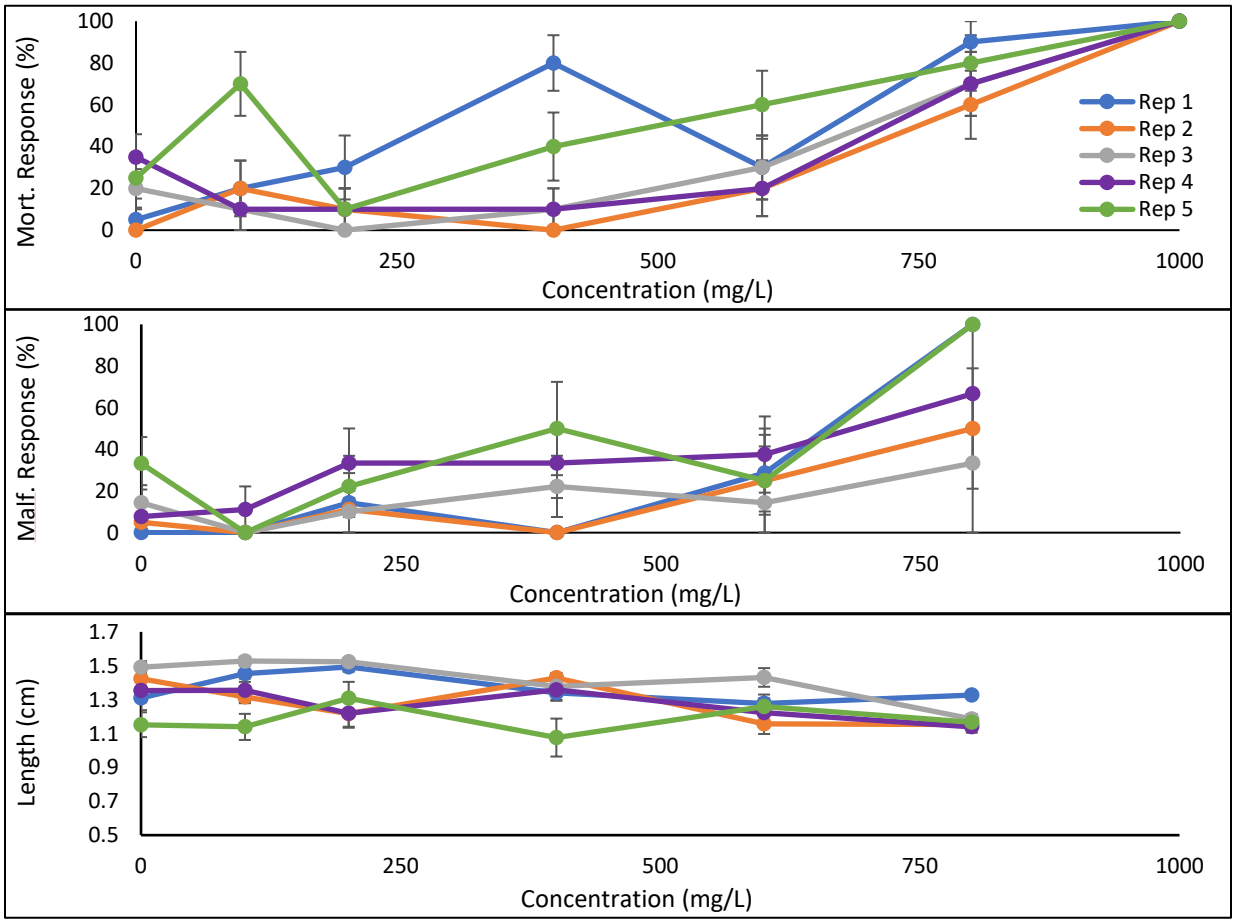
Sodium Fluoride : Sodium Selenate 1:0

Replicate 1's mortality and malformation responses for *Ambystoma maculatum* that were raised in FETAX solution were 5.00% and 0.00%, respectively. The average pH measurement of FETAX was 7.00 ± 0.16. Replicate 2's mortality and malformation responses for *Ambystoma maculatum* that were raised in FETAX solution were 10.00% and 5.56%, respectively. The

average pH measurement of FETAX was 7.00 ± 0.16. The average pH measurement in replicates 1 and 2 for sodium fluoride, sodium selenate, and mixture 20:1 solution was 7.88 ± 0.32, 7.21 ± 0.16, and 7.79 ± 0.29, respectively. Replicate 3's mortality and malformation responses for *Ambystoma maculatum* that were raised in FETAX solution were 20.00% and 12.50%, respectively.

Figure 21

Concentration-response graphs and growth graphs for sodium fluoride with Ambystoma maculatum

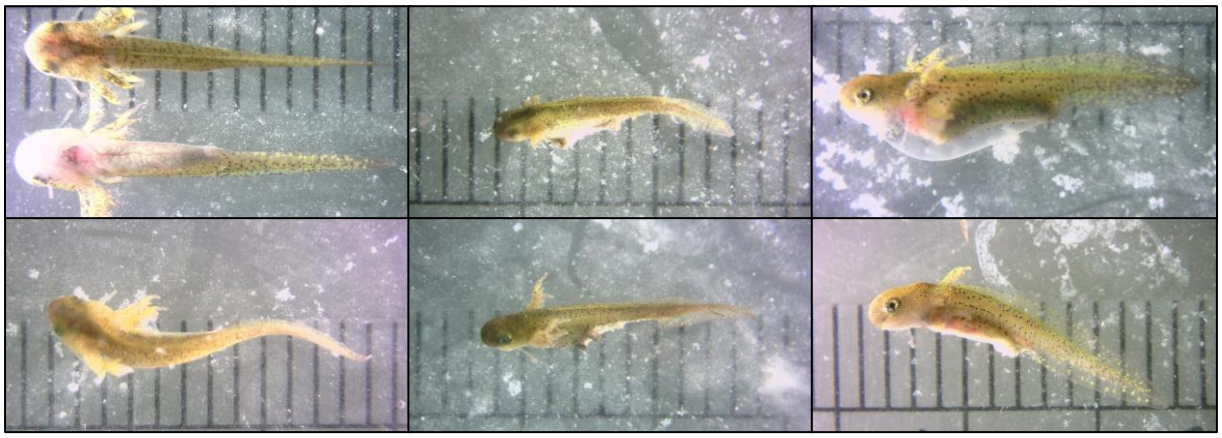


(Top) Mortality concentration response curve using five replicates with sodium fluoride. (Middle) Malformation concentration response curve using five replicates with sodium fluoride. (Bottom) Concentration growth curve using five replicates with sodium fluoride. The * indicate significant difference from controls at $p \leq 0.05$.

The average pH measurement of FETAX was 7.00 ± 0.20. The average pH measurement in replicate 3 for sodium fluoride, sodium selenate, and mixture 20:1 solution was 7.84 ± 0.15, 7.11 ± 0.10, and 7.69 ± 0.18, respectively. Replicate 4's mortality and malformation responses for *Ambystoma maculatum* that were raised in FETAX solution were 35.00% and 7.70%, respectively. The average pH measurement of FETAX was 7.06 ± 0.12. Replicate 5's mortality and malformation responses for *Ambystoma maculatum* that were raised in FETAX solution were 25.00% and 33.33%, respectively. The average pH measurement of FETAX was 7.06 ± 0.12 for replicates 4 and 5. The average pH measurement in replicates 4 and 5 for sodium fluoride, sodium selenate, and mixture 20:1 solution was 7.84 ± 0.08, 7.13 ± 0.19, and 7.58 ± 0.08, respectively.

Figure 22

Malformations that appeared with Ambystoma maculatum that were exposed to sodium fluoride



(Top left) Control-healthy embryo. (Top middle) 800 mg/L-stunted with bent notochord and gut abnormality. (Top right) 600 mg/L-stunted with edema and hemorrhage. (Bottom left) 800 mg/L-bent notochord. (Bottom middle) 400 mg/L-stunted with kinked tail and gut abnormality. (Bottom right) 400 mg/L-stunted with hemorrhage and gut abnormality.

The overall mean 12-day LC_{50} for developing *Ambystoma* embryos exposed to sodium fluoride was 540.39 mg/L, with lower and upper Fieller bounds being 476.42 and 614.41 mg/L, respectively (Table 7). Mortality in the *Ambystoma* reacted differently throughout the replicates.

When looking at Figure 21, replicates 2,3, and 4 behaved the most like to one another. The three replicates showed mortality increase significantly at 600 mg/L. Mortality continued to increase and hit 100% at 1,000 mg/L. Replicates 1 and 5 had abnormally high mortality results. Replicate 5 had a spike in mortality at 100 mg/L and then went back down at 200 mg/L. Then it continued to increase in mortality until it hit 100% at 1,000 mg/L. Replicate 1 increased in mortality at 100 mg/L which continued until it hit 400 mg/L. From there mortality went back down to 600 mg/L. Like the rest of the replicates, it hit 100% mortality at 1,000 mg/L.

Table 7

Results for Ambystoma maculatum exposed to sodium fluoride, sodium selenate, and 20:1 mixture

Overall results for toxicity assays (mg/L)							Mortality		Malformation		MCIG
Chemical	Total embryos	LC50 $NaF:Na_2SeO_4$	(95% Fieller) $NaF:Na_2SeO_4$	EC50 (Malformation) $NaF:Na_2SeO_4$	(95% Fieller) $NaF:Na_2SeO_4$	T.I.	NOEC $NaF:Na_2SeO_4$	LOEC $NaF:Na_2SeO_4$	NOEC $NaF:Na_2SeO_4$	LOEC $NaF:Na_2SeO_4$	$NaF:Na_2SeO_4$
Sodium Fluoride	320	540.39	473.42-614.41	862.26	658.11-1,366.61	0.63	600	800	600	800	NA
Sodium Selenate	320	53.06	42.15-68.51	388.37	260.02-776.05	0.14	10	100	10	100	NA
20:1 Mixture	320	528.24:2.64	444.02:2.22-639.61:3.20	NA	NA	NA	600:03:00	1,200:6	NA	NA	NA

TI = teratogenic index, NOEC = no observed effect concentration, LOEC = lowest observed effect concentration, MCIG = minimal concentration that inhibits growth

The overall mean 12-day EC_{50} for developing *Ambystoma* embryos exposed to sodium fluoride was 862.26, with lower and upper Fieller bounds being 658.11 and 1,366.61 mg/L, respectively (Table 7). The level of malformations that occurred for sodium fluoride fluctuated throughout all the replicates. The types of malformations that occurred in sodium fluoride were stunted growth, edemas, gut abnormalities, kinked tails, and bent notochords (Figure 22). When

looking for similarities, replicate 1 and 2 were similar up until 800 mg/L. Replicate 1 was 100% while replicate 2 was at 50% malformed. Replicate 3 showed the least malformations at the highest concentration at 33%. Replicate 4 had a steady level of malformations from concentrations 200 mg/L to 600 mg/L. When it hit 800 mg/L the malformations that occurred were at 67%. Replicate 10 had an increase of malformations up to 400 mg/L and then went back down to 600 mg/L. Besides replicate 1, 5 was the only other replicate that had 100% malformations occur at 800 mg/L. The length measurements for sodium fluoride treated *Ambystoma* were scattered across the board. Replicates 2 and 4 had the closest similarities whereas all the other replicates were nowhere near being similar. The only common occurrence between all replicates was that the length measurements increased at one point in time and then decreased. Replicate 5 showed the most abnormal measurements. Replicate 3 showed the closest outcome expected but still could be considered abnormal.

Length measurements were across the board for embryos exposed to sodium fluoride. Only replicates 2 and 4 had similar effects appear while the remaining replicates behaved more erratically. At one point in time, all concentrations had longer embryos in concentrations than in the controls which can be observed in Figure 21.

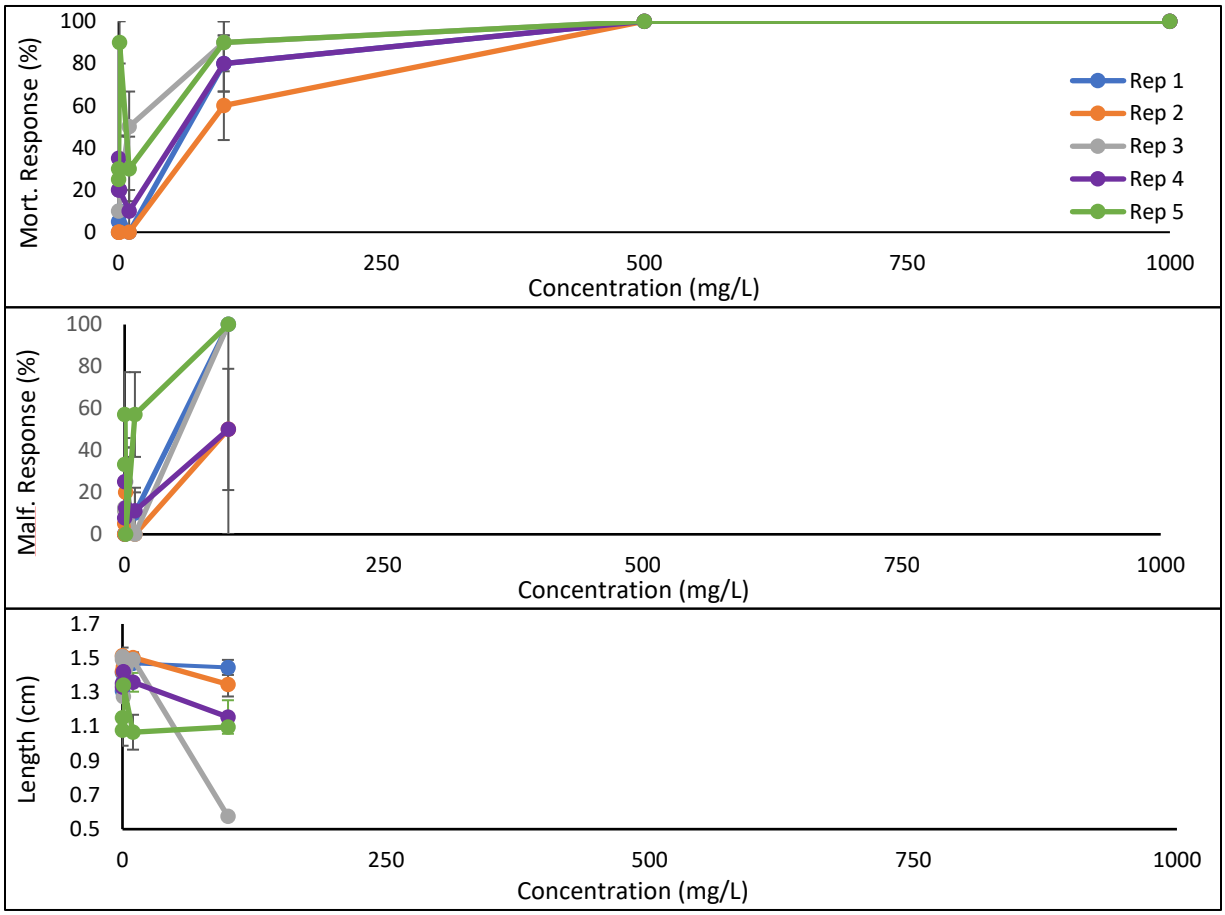
Sodium Fluoride : Sodium Selenate 0:1

The overall mean 12-day LC_{50} for developing *Ambystoma* embryos exposed to sodium selenate was 53.06, with lower and upper Fieller bounds being 42.15 and 68.51 mg/L, respectively (Table 7). Mortality in the *Ambystoma* exposed to sodium selenate reacted similarly in each replicate. When looking at Figure 23, replicate 5 had a 90% mortality rate at 1 mg/L than

any of the other replicates. All replicates showed high mortality rates at 100 mg/L. 100% mortality occurred with all replicates at 500 mg/L.

Figure 23

Concentration-response graphs and growth graphs for sodium selenate with Ambystoma maculatum



(Top) Mortality concentration response curve using five replicates with sodium selenate. (Middle) Malformation concentration response curve using five replicates with sodium selenate. (Bottom) Concentration growth curve using five replicates with sodium selenate. The * indicate significant difference from controls at $p \leq 0.05$.

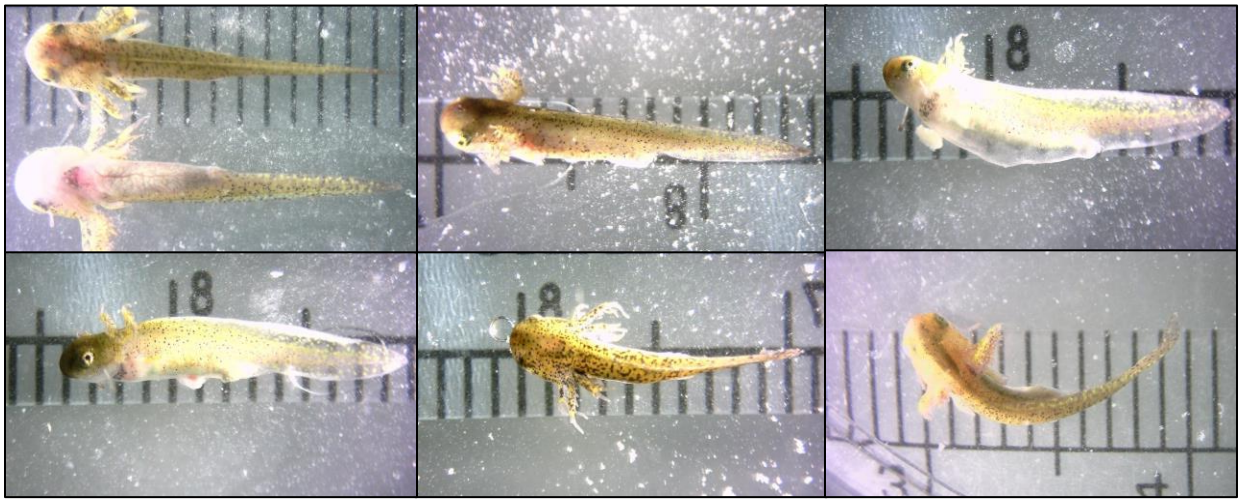
The overall mean 12-day EC_{50} for developing *Ambystoma* embryos exposed to sodium selenate was 69.21, with lower and upper Fieller bounds being 45.96 and 137.84 mg/L, respectively (Table 7). The level of malformations that occurred for sodium selenate fluctuated

throughout all the replicates. The types of malformations that occurred in sodium selenate were stunted growth, edemas, gut abnormalities, kinked tails, and bent notochords (Figure 24). When looking for similarities, replicates 1, 3, and 5 showed 100% malformation at 100 mg/L. Replicates 2 and 4 were similar and showed 50% malformation at 100 mg/L.

The length measurements for sodium selenate treated *Ambystoma* were scattered across the board decreased. Replicate 3 showed the smallest length measurements between all replicates. Replicate 1 showed the longest length measurements throughout all replicates. Replicates 3 and 4 showed similar decline in length from controls to 100 mg/L which can be observed in Figure 23.

Figure 24

Malformations that appeared with Ambystoma maculatum that were exposed to sodium selenate



(Top left) Control-healthy embryo. (Top middle) 100 mg/L-stunted with hemorrhage and abnormal gut. (Top right) 100 mg/L-stunted with edema. (Bottom left) 100 mg/L-stunted with hemorrhage, edemas, and gut abnormality. (Bottom middle) 0.1 mg/L-stunted with bent notochord. (Bottom right) 1 mg/L-stunted with bent notochord, kinked tail, and gut abnormality.

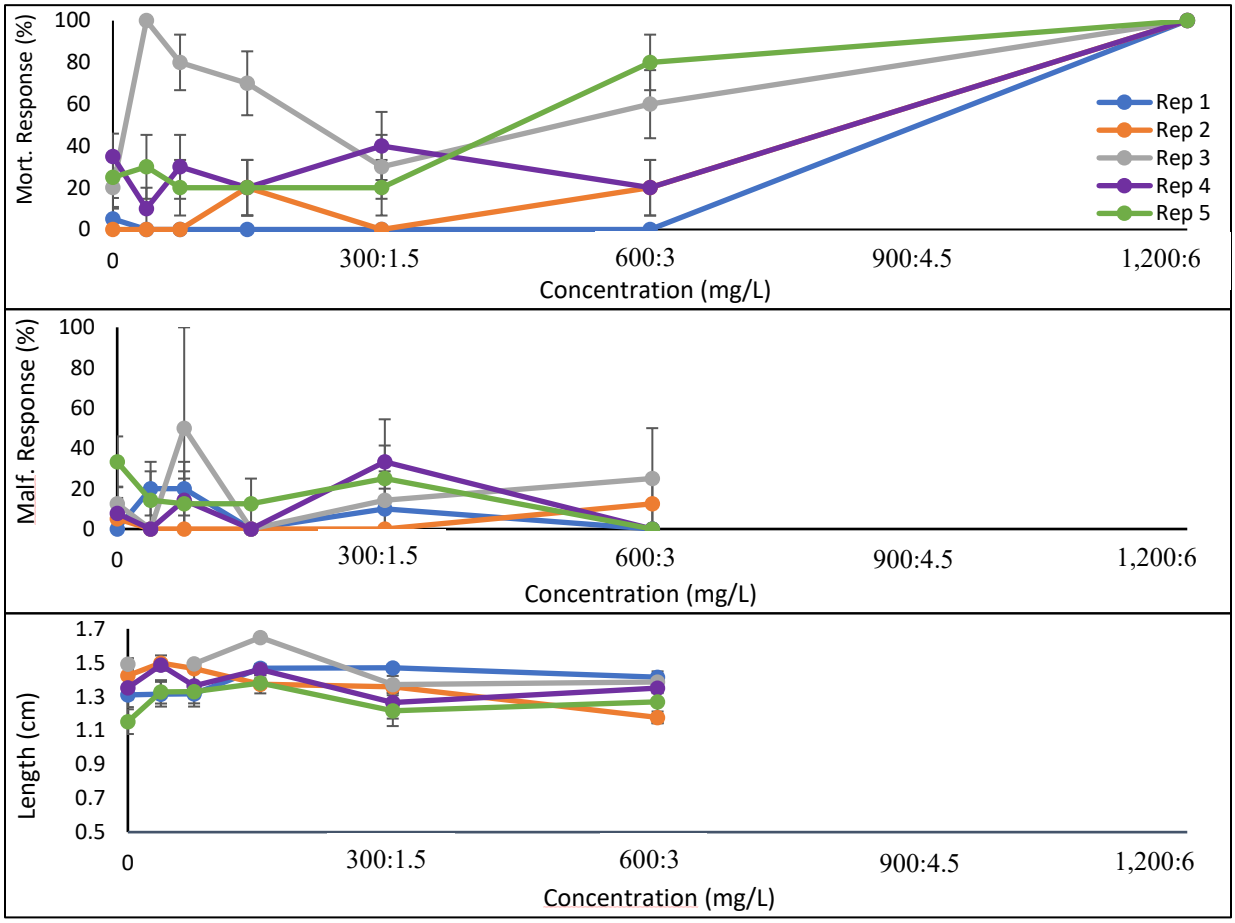
***Sodium fluoride : Sodium selenate* 20:1**

The overall mean 12-day LC_{50} for developing *Ambystoma* embryos exposed to 20:1 mixture was 44.02, with lower and upper Fieller bounds being 37.00 and 53.30 mg/L,

respectively (Table 7). Mortality in the *Ambystoma* exposed to 20:1 mixture reacted similar in each replicate. When looking at figure 25, replicate 5 had a 90% mortality rate at 1 mg/L than any of the other replicates. All replicates showed high mortality rates at 100 mg/L. There was 100% mortality that occurred with all replicates at 500 mg/L.

Figure 25

Concentration-response graphs and growth graphs for sodium fluoride : sodium selenate mixture 20:1 with Ambystoma maculatum

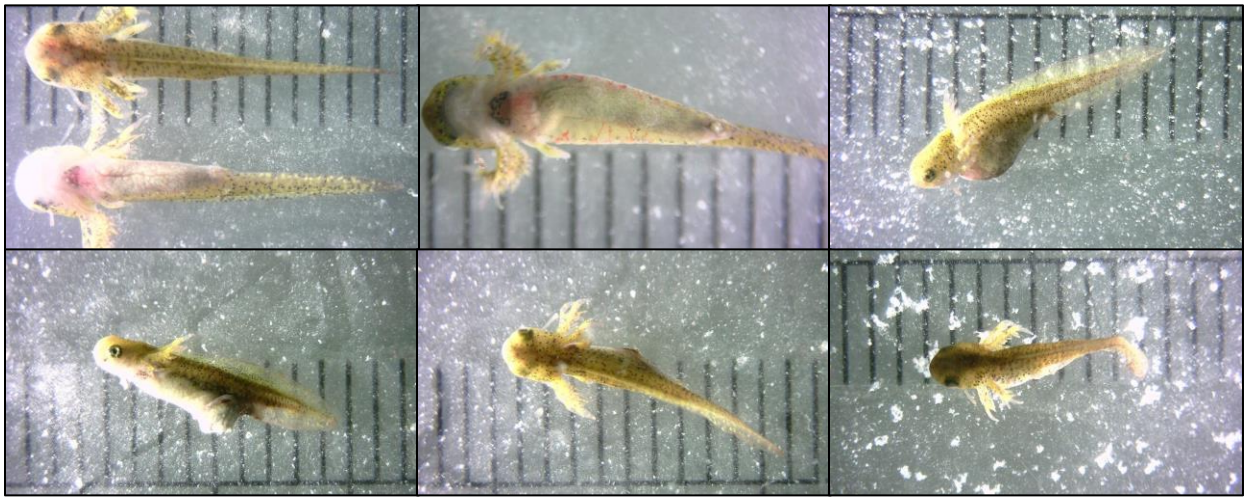


(Top) Mortality concentration response curve using five replicates with sodium fluoride : sodium selenate mixture 20:1. (Middle) Malformation concentration response curve using five replicates with sodium fluoride : sodium selenate mixture 20:1. (Bottom) Concentration growth curve using five replicates with sodium fluoride : sodium selenate mixture 20:1. The * indicate significant difference from controls at $p \leq 0.05$.

In the 20:1 mixture, there was no 12-day EC_{50} for developing *Ambystoma* embryos. The highest point of malformations occurred with replicate 3 at 50% malformed at the 6.25% concentration. Replicates 1,4, and 5 showed no malformations at (600:3 mg/L). Replicate 2 showed 5% of the embryos malformed in controls but did not see any more malformations until (600:3 mg/L) where 12.5% were malformed. The types of malformations that occurred in the 20:1 mixture was stunted growth, edemas, gut abnormalities, kinked tails, and bent notochords (Figure 26).

Figure 26

Malformations that appeared with Ambystoma maculatum that were exposed to sodium fluoride : sodium selenate 20:1



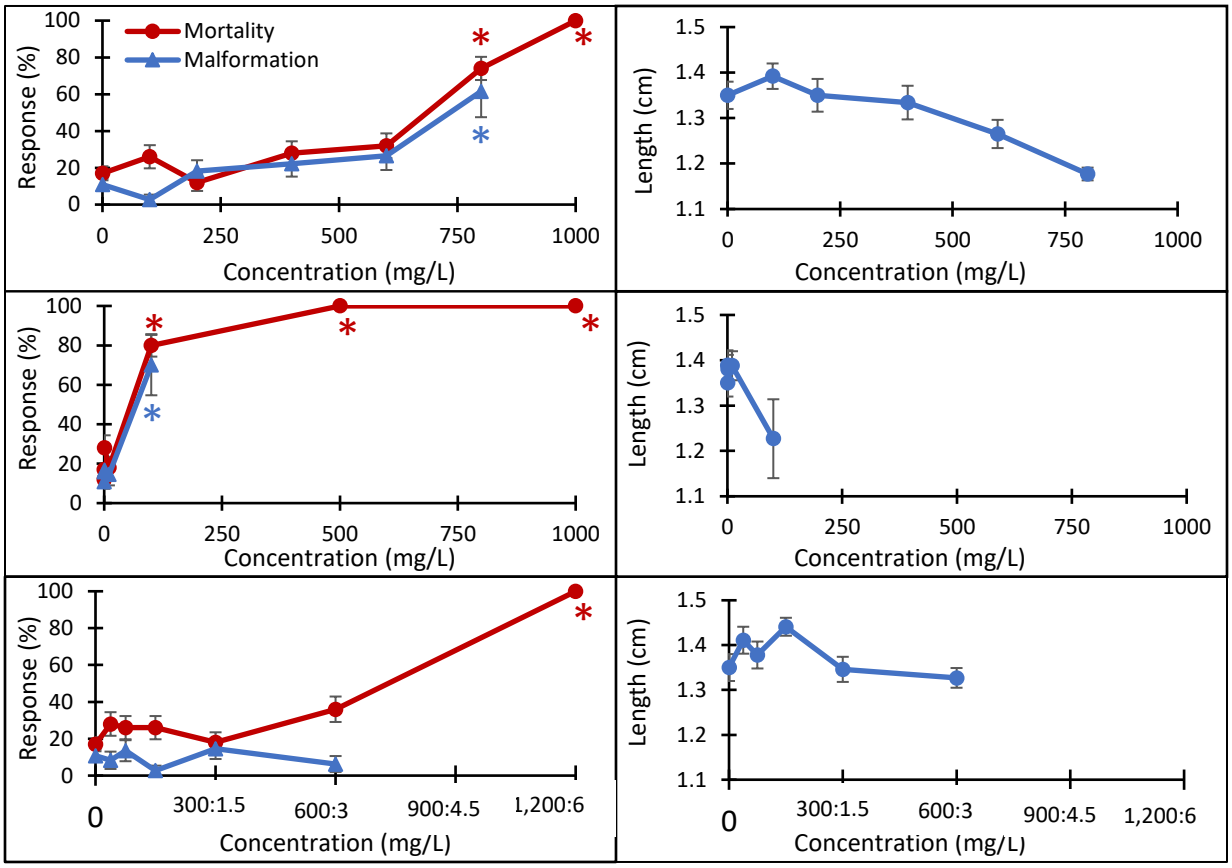
(Top left) Control-healthy embryo. (Top middle) 600:3 mg/L-hemorrhage and gut abnormality. (Top right) 300:1.5 mg/L-stunted with edema. (Bottom left) 150:0.75 mg/L-stunted with tail kink and gut abnormality. (Bottom middle) 37.5:0.19 mg/L-gut abnormality. (Bottom right) 300:1.5 mg/L-stunted with kinked tail and gut abnormality.

The length measurements for 20:1 mixture treated *Ambystoma* were like one another as the lengths of the embryos decreased. Replicate 2 showed the smallest length measurements between all replicates at (600:3 mg/L). Replicate 1 showed the longest length measurements throughout all replicates at (600:3 mg/L). Replicates 3 and 4 showed a similar decline in length

from controls to 100 mg/L. Looking at Figure 27, there was no significant difference from the controls. Embryos were longer in smaller concentrations but then shrunk in 300:1.5 mg/L.

Figure 27

Overall concentration-response graphs and growth graphs for sodium fluoride : sodium selenate mixture 20:1 with Ambystoma maculatum



The top row is experiment 1, middle row is experiment 2 and the third row is experiment 3. The left graphs are mortality and malformation concentration-response graphs, and the right graphs represents growth. The * indicate significant difference from controls at $p \leq 0.05$.

Discussion

Species Comparison of Sodium Fluoride.

The *Xenopus* and *Ambystoma* had similar LC_{50} and EC_{50} throughout the experiments. The Fieller bounds for the *Ambystoma* however had a larger range than the *Xenopus*. The teratogenic index was also similar between the two species. Rats showed deficits in learning and memory when exposed to sodium fluoride that exceeded 100 mg/L (Guth et al., 2020). Amphibians seem to have a higher tolerance to sodium fluoride compared to zebra fish embryos. Experiments from another article expressed that zebra fish embryos showed 80% mortality at 50 mg/L at 72 hours post fertilization (Di Paola, 2022).

Species Comparison of Sodium Selenate

The overall LC_{50} for *Xenopus* and *Ambystoma* showed that *Xenopus* embryos were 1.74-fold more sensitive to selenate. The EC_{50} however was 25.67-fold more sensitive. Because of the difference in EC50 without a difference in the LC50 the teratogenic index was higher for *Xenopus* than *Ambystoma* (2.01 and 0.14 respectively). In experiments performed using zebra fish embryos, 50% of the embryos perished while the surviving embryos exhibited severe deformities (Zhao et al., 2022). Experiments that used 40 mg/L sodium selenite on adult zebra fish saw 86% mortality at the 48-hour mark (Zhongchuang et al, 2019).

Timed Experiment Discussion

The purpose of these experiments was to determine if the duration of time exposed to sodium selenate, or the developmental stage of the embryos exposed to sodium selenate was more important. The developmental stage of the embryos was significant in levels of mortality and malformations that occurred. The embryos that were exposed for 24 hours between days 2-3

and 3-4 were severely malformed. The severely malformed embryos likely would have deceased soon thereafter. Embryos that were exposed for 24 hours for the first two days showed minimal mortality and malformations. This observation could show that the embryos have to reach a certain developmental stage where specific receptors are developed and effected by the sodium selenate. For future experiments on this topic, longer time periods could be performed to see if it continues to affect the embryos after 96 hours or if it stops affecting them at older stages of development.

Interactions Discussion

Table 8

Results for Xenopus laevis embryos exposed to sodium fluoride, sodium selenate, and mixture ratios 1:1, 1:3, and 3:1

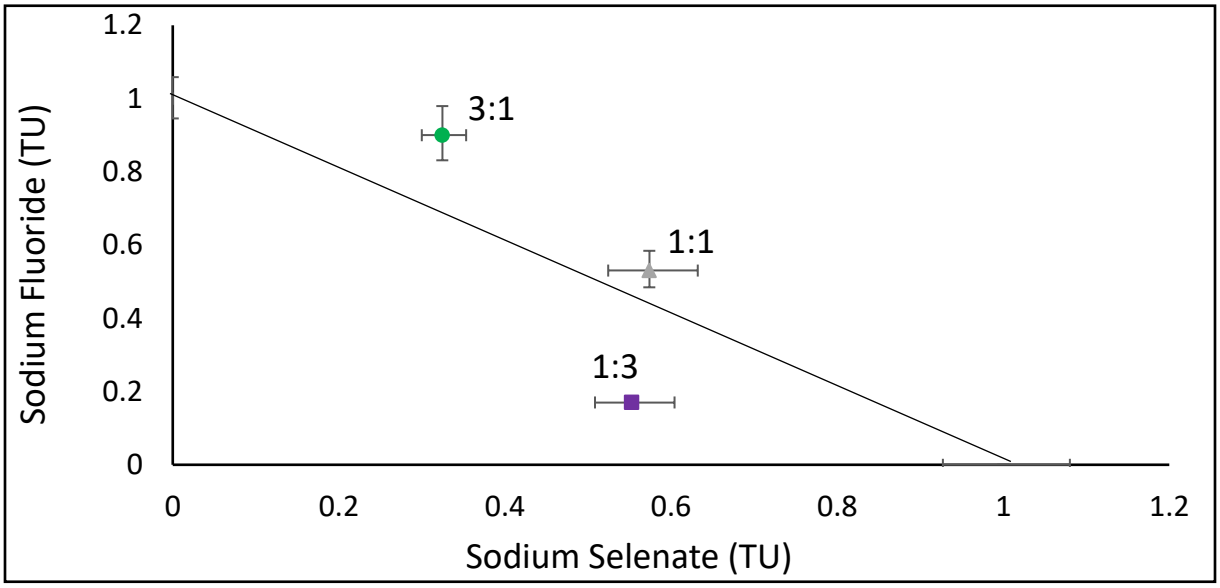
Overall results for toxicity assays (mg/L)							Mortality		Malformation		MCIG $NaF:Na_2SeO_4$
Assay	Total embryos	LC50 $NaF:Na_2SeO_4$	(95% Fieller) $NaF:Na_2SeO_4$	EC50 $NaF:Na_2SeO_4$	(95% Fieller) $NaF:Na_2SeO_4$	T.I.	NOEC $NaF:Na_2SeO_4$	LOEC $NaF:Na_2SeO_4$	NOEC $NaF:Na_2SeO_4$	LOEC $NaF:Na_2SeO_4$	
Sodium fluoride	960	603.36	570.58-638.61	819.42	710.101-989.596	0.74	200	400	200	400	400
Sodium selenate	960	30.41	28.20-32.85	15.13	13.716-16.818	2.01	10	20	1	10	10
3:1 (1,650:30)	960	542.85:9.9	501.60:9.10-590.87:10.7	451.94:8.2	396.00:7.20-529.98:9.60	1.20	412.50:7.5	825:15	198:3.6	412.50:7.5	99:1.8
1:1 (550:30)	960	320:17.5	292.38:15.9-352.50:19.2	166.27:9.1	151.03:8.20-184.97:10.10	1.92	137.50:7.5	275:15	33:1.8	66:3.6	66:3.6
1:3 (550:90)	960	102.69:16.8	94.49:15.5-112.31:18.4	73.81:12.1	66.88:10.9-82.94:13.60	1.39	66:10.8	137.50:22.5	33:5.4	66:10.8	66:10.8

TI = teratogenic index, NOEC = no observed effect concentration, LOEC = lowest observed effect concentration, MCIG = minimal concentration that inhibits growth

After observing the different interactions, sodium selenate was the most teratogenic overall with a teratogenic index of 2.01. Sodium fluoride was the least teratogenic overall with a teratogenic index of 0.74. When observing the interactions within the *Xenopus*, it was interesting to see sodium fluoride make sodium selenate more toxic in the 1:3 (remember ratios are always fluoride to selenate) mixture. However, when observing the 3:1 mixture, sodium selenate reduces the effects of sodium fluoride.

Figure 28

Isobole diagram of toxic units for overall LC50 of Xenopus laevis experiments



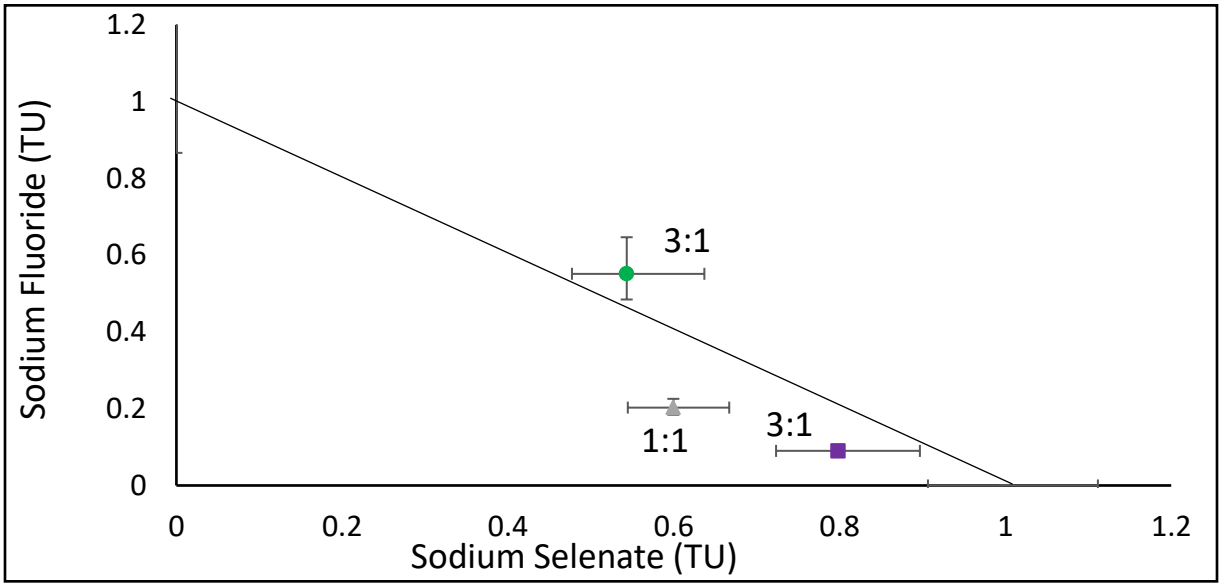
The green circle, grey triangle, and purple square represent 3:1 mixture tests, 1:1 mixture tests, and 1:3 mixture tests, respectively.

The 95% Fieller bounds were large compared to the *Xenopus* experiments that maybe related to the lower number of embryos per replicate and mixing clutches of embryos for experiments may have introduced more variability. Looking at Table 7, none of the *Ambystoma* experiments were had TI greater than 1 which indicate that the sodium fluoride and selenate and the mixture tested did not show malformations in high numbers. This indicates that the

salamander embryos are not as sensitive to malformations from fluoride and selenate. The EC_{50}, MCIG and teratogenic index for the 20:1 mixture was undetermined. Not enough testing was done beforehand to determine the proper ratio for the experiment. However due the fact fluoride is more common in the environment than selenate, the testing of the 20:1 mixture was useful in potential natural exposures. In experiments that used arsenic and chromium mixtures, the As(V):Cr(VI) mixture had a teratogenic index of 2.78 (Gardner, 2017) indicating that metals at least can induce some significant malformations.

Figure 29

Isobole diagram of toxic units for overall EC50 of Xenopus laevis experiments



The green circle, grey triangle, and purple square represent 3:1 mixture tests, 1:1 mixture tests, and 1:3 mixture tests, respectively.

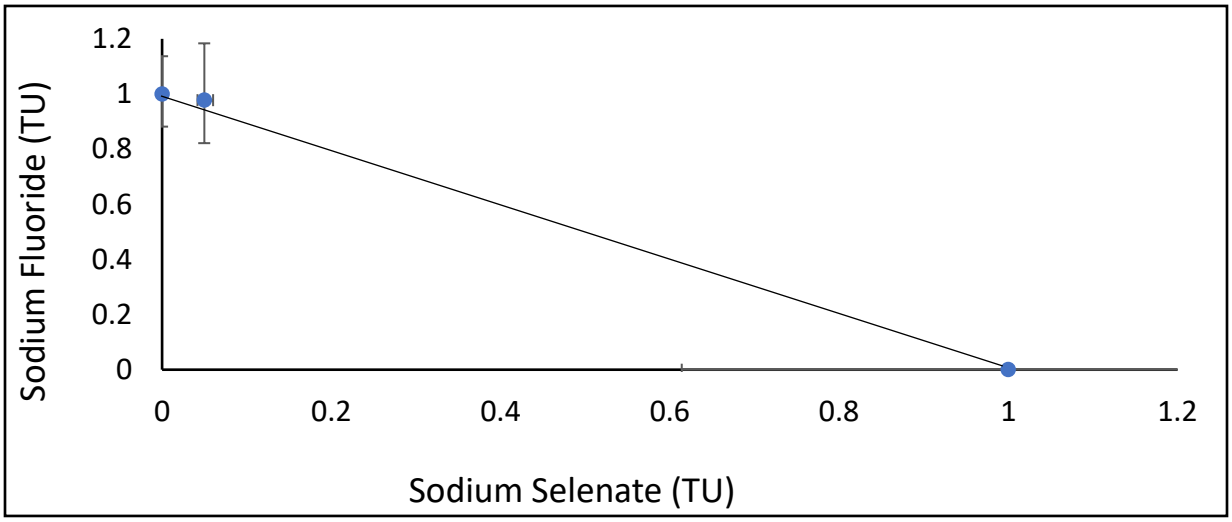
The Isobole diagram in Figure 28 shows the overall representation of the LC_{50} of all *Xenopus laevis* experiments performed. The 1:0 and 0:1 mixture had a toxic unit of 1.00 landing them on the axis lines. With the 3:1 mixture, the total toxic units were 1.22 and showed response addition. With the 1:1 mixture, the total toxic units were 1.10 and showed response addition.

With the 1:3 mixture, the total toxic units were 0.73 and showed synergism. Observing the 1:1 and 1:3 mixtures, the two chemicals had little effect on one another. This showed that sodium fluoride elevated the effects from sodium selenate.

The Isobole diagram in Figure 29 shows the overall representation of the EC_{50} of all *Xenopus laevis* experiments performed. With the 3:1 mixture, it showed response addition. Observing the mixture showed that the chemicals had little effect on one another. With the 1:1 and 1:3 mixture, both showed synergism which intel's that sodium fluoride elevated the effects of sodium selenate.

Figure 30

Isobole diagram of toxic units for overall LC50 of Ambystoma maculatum experiments



The Isobole diagram in Figure 30 shows the overall representation of the LC_{50} of all *Ambystoma maculatum* experiments performed. The 20:1 mixture had an overall toxic unit of 1.03. When determining the ratio for the mixture, miscalculations were made due to misguided observations. Due to this error, the mixture had twenty times more sodium fluoride than it should

have. This explains why the mixture appears close to the horizontal axis of the diagram. After looking at other literature sources showing there is more sodium fluoride in the environment than sodium selenate, it validates the ratio that was tested.

Species Comparison

Xenopus laevis and *Ambystoma maculatum* showed similar results when exposed to sodium fluoride. The LC_{50} and EC_{50} of *Xenopus* and *Ambystoma* exposed to sodium fluoride were similar in comparison. The teratogenic index for sodium fluoride in both species showed that it was non teratogenic because both were less than 1.5. With sodium fluoride, a significant difference in mortality and malformations in *Xenopus* was seen at 400 mg/L. In *Ambystoma*, a significant difference was not seen in mortality and malformation until 800 mg/L. The MCIG was determined in the *Xenopus* but not in the *Ambystoma*.

Xenopus laevis and *Ambystoma maculatum* showed similar results when exposed to sodium selenate. The LC_{50} of *Xenopus* and *Ambystoma* exposed to sodium selenate were similar in comparison. The EC_{50} of both species however were significantly different. The *Xenopus* experiments exposed to sodium selenate were teratogenic at 2.01 while the *Ambystoma* experiments were not teratogenic at 0.14. In *Xenopus*, a significant difference in mortality was seen at 10 mg/L and 20 mg/L in malformations. A significant difference was seen at 100 mg/L with mortality and malformations in the *Ambystoma*. Like the sodium fluoride experiments, *Xenopus* experiments were able to determine the MCIG but not in the *Ambystoma*.

Effects of Fluoride and Selenate on native amphibians

The effects sodium fluoride and sodium selenate showed on native amphibians such as *Ambystoma maculatum* had devastating outcomes. Multiple types of malformations were

observed that leave to believe that if the embryos were left in the environment, there would likely be no chance of them surviving to maturity. From observations, it seemed that the chemicals had a delayed effect on the *Ambystoma* embryos. It is possible that the jelly coat that surrounded the embryos was able to prevent some delay in effectiveness from the chemical exposures. This cannot be compared with the *Xenopus* because the jelly coat was removed with L-cysteine.

BIBLIOGRAPHY

Agudelo, M. H., Carbonell, B., Arenas, C., & Delgado, J. P. (2021). Ambystoma mexicanum, a model organism in developmental biology and regeneration: a colombian experience. *ResearchGate, 27*(1), 113-126. doi:10.15446/abc.v27n1.88309

Andrews, J. S., & Talmage, E. (2021). Phenological differences in wood frog and spotted salamander egg-mass onset and peak accumulation. *Northeastern Naturalist, 28*(4), 456-461.

Aoun, A. F. (2018). The fluoride debate: The pros and cons of fluoridation. *National Library of Medicine*, 171-180.

Arifa, M. R. (2019). Recent Advances in Dental Hard Tissue Remineralization: A Review of Literature. *International journal of clinical pediatric dentistry, 12*(2), 139-144. doi:https://doi.org/10.5005/jp-journals-10005-1603

Balint, B. K. (2021). Differences in the effects of sodium selenate and sodium selenite on the mortality, reproduction, lipid peroxidation and glutathione redox of Folsomia candida Willem 1902 (Collembola). *European Journal of Soil Biology, 107*, 1-7. doi:https://doi.org/10.1016/j.ejsobi.2021.103361

Bantle, J. A., & Sabourin, T. D. (1991). Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX). *American Society for Testing and Materials (ASTM) E1439-91, 11.06*, 1-11.

Bantle, J. A., Dumont, J. N., Finch, R. A., & Linder, G. (1991). *Atlas of Abnormalities: A Guide for the Performance of FETAX.* Stillwater, OK: Oklahoma State University Press.

Bantle, J. A., Fort, D. J., Rayburn, J. R., DeYoung, J., & Bush, S. J. (1990). Futher Validation of FETAX: Evaluation of the Developmental Toxicity of Five Known Mammalian Teratogens and Non-teratogens. *Drug and Chemical Toxicololgy, 13(4)*, 267-282.

Baxter, L. R. (2015). From effects of atrazine on egg masses of the yellow-spotted salamander (Ambystoma maculatum) and its endosymbiotic alga (Oophila amblystomatis). *PubMed*, 324-331.

Böswald, L. D. (2022). Current feeding practice of Xenopus laevis in a laboratory setting. *PubMed Central, 12*(9), 1163.

Browne, C. L., & Dumont, J. N. (1979). Toxicity of selenium to developing Xenopus laevis embryos. *Journal of Toxicity and Environment Health, 5*(4), 699-709.

Conant, R., & Collins, J. T. (1998). *A field guide to reptiles and amphibians of Eastern and Central North America.* Boston and New York: Houghton Mifflin Co.

Cope, R. (2017). Chapter 17 - Miscellaneous Inorganic Toxicants. *Veterinary Toxicology for Austalia and New Zealand*, 289-332. doi:https://pubchem.ncbi.nlm.nih.gov/compound/Sodium-Fluoride

Dawson, D. A., & Bantle, J. A. (1987). Development of a reconstituted water medium and initial validation of FETAX. *Journal of Applied Toxicology, 7*, 237-244.

D'Errico, M. C. (2020). Egg mass polymorphism in Ambystoma maculatum is not associated with larval performance or survival, or with cell density of the algal symbiont Oophila amblystomatis. *Evolutionary Ecology, 34*, 981-997.

DeYoung, D. J. (1991). Assessment of the developmental toxicity of ascorbic acid, sodium selenate, coumarin, serotonin, and 13-cis retinoic acid using fetax. *Drug and Chemical Toxicology, 14*(1&2), 127-141.

Di Paola, D. F. (2022). Environmental Toxicity Assessment of Sodium Fluoride and Platinum-Derived Drugs Co-Exposure on Aquatic Organisms. *Toxics, 10*(5), 272. doi:https://doi.org/10.3390/toxics10050272

Douglas, M. E., & Monroe, B. L. (1981). A comparative study of topographical orientation in ambystoma (Amphibia: Caudata). *Copeia, 1981*, 460-463.

Dumont, J. N., Schultz, T. W., Buchanan, M. V., & Kao, G. L. (1983). Frog Embryo Teratogenesis Assay: Xenopus (FETAX) — A Short-Term Assay Applicable to Complex Environmental Mixtures. *Environmental Science Research, 27*, 393-405.

Fainsod, A., & Kot-Leibovich, H. (2018). Xenopus embryos to study fetal alcohol syndrome, a model for environmental teratogenesis. *Biochemistry and Cell Biology, 96*(2), 77-87.

Fluoride: Nature's tooth decay fighter. (2009). *The Journal of the American Dental Association, 140*(1), 126.

Fort, D. J., Stover, E. L., Bantle, J. A., Rayburn, J. R., Hull, M. A., Finch, R. A., . . . Gaudet-Hull, A. M. (1998). Phase III interlaboratory study of FETAX, Part 2: Interlaboratory validation of an exogenous metabolic activation system for Frog Embryo Teratogenesis Assay-Xenopus (FETAX). *Drug and Chemical Toxicology, 21*(1), 1-14.

Foss, S. S., & Rayburn, J. R. (1997). Effects of culture duration on toxicity of ethanol to developing embryos of the grass shrimp, Palaemonetes pugio. *Environmental Contamination and Toxicology, 59*, 467-471.

Galdiero, E. A. (2017). Daphnia magna and Xenopus laevis as in vivo models to probe toxicity and uptake of quantum dots functionalized with gH625. *PubMed, 12*, 2717-2731.

Gardner, S. G. (2017). Developmental and interactive effects of arsenic and chromium to developing Ambystoma maculatum embryos: Toxicity, teratogenicity, and whole-body concentrations. *Journal of Toxicology and Environmental Health, 80*(2), 91-104.

Gates, J. E., & Thompson, E. L. (1981). Breeding habitat association of spotted salamanders (Ambystoma maculatum) in western maryland. *Journal of the Elisha Mitchell Scientific Society, 97*(3), 209-216.

Goh, E. H. (2003). Effects of fluoride on Xenopus embryo development. *Food and Chemical Toxicology, 41*(11), 1501-1508.

Gomez, C., & Echeverri, K. (2021). Salamanders: The molecular basis of tissue regeneration and its relevance to human disease. *Current Topics in Developmental Biology, 145*, 235-275.

Guth, S., Huser, S., Roth, A., Degen, G., Diel, P., Edlund, K., . . . Hengstler, J. G. (2020). Toxicity of fluoride: critical evaluation of evidence for human developmental neurotoxicity in epidemiological studies, animal experiments and in vitro analyses. *Archives of toxicology, 94*(5), 1375-1415. doi:https://doi.org/10.1007/s00204-020-02725-2

Harrison, R. (1969). *Harrison stages and description of the normal development of the spotted salamander, Amblystoma punctatum.* New Haven: Yale University Press.

Hassen, M. S., Morgan, A. M., Mekawy, M. M., Zaki, A. R., & Ghazi, Z. M. (2016). Teratogenic effect of cisplatin in rats and the protective role of sodium. *Experimental and Toxicologic Pathology, 68*, 277-287.

Heuring, W. L., & Mathis, A. (2014). Landmark learning by juvenile salamanders (Ambystoma maculatum). *Behavioural Processes, 108*, 173-176.

Homan, R. N., Holgerson, M. A., & Biga, L. M. (2018). A long-term demographic study of a spotted salamander (Ambystoma maculatum) population in central ohio. *Herpetologica, 74*(2), 109-116.

Hopkins, W. (2007). Amphibians as models for studying environmental change. *ILAR Journal, 48*(3), 270-277.

Horn, E. (2006). Xenopus laevis - a success story of biological research in space. *Advances in Space Research, 38*(6), 1059-1070.

Hossie, T. J., MacFarlane, S., Clement, A., & Murray, D. L. (2018). Threat of predation alters aggressive interactions among spotted salamander (Ambystoma maculatum) larvae. *Ecology and Evolution, 8*(6), 3131-3138.

Huang, R. Y., Pei, L., Liu, Q., Chen, S., Dou, H., Shu, G., . . . Fu, H. (2019). Isobologram Analysis: A Comprehensive Review of Methodology and Current Research. *Frontiers in Pharmacology, 10*, 1222. doi:https://doi.org/10.3389/fphar.2019.01222

Ireland, P. H. (1991). Separate effects of acid-derived anions and cations on growth of larval salamanaders of Ambystoma maculatum. *Copeia, 1991*(1), 132-137.

Ishibashi, S., Saldanha, F., & Amaya, E. (2017). Chapter 14-Xenopus as a model organism for biomedical research. *Basic Science Methods for Clinical Researchers*, 263-290.

Kerney, R. (2011). Symbioses between salamander embryos and green algae. *Symbiosis, 54*, 107-117.

Kleunen, M. V., Dawson, W., Bossdorf, O., & Fischer, M. (2014). The more the merrier: Multi-species experiments in ecology. *Basic and Applied Biology, 15*, 1-9.

Koriem, K. M., Shamsuri, R. B., & Ubaidillah, A. M. (2016). Evaluation of sodium fluoride toxicity in Schistosoma infected snails: assessment of antioxidants, antiapoptotic, hypoprotein and hypocholesterol activities. *Journal of parasitic diseases : official organ of the Indian Society for Parasitology, 40*(4), 1451-1458. doi:https://doi.org/10.1007/s12639-015-0711-z

Lanctot, C. M., Melvin, S. D., & Cresswell, T. (2017). Selenium speciation influences bioaccumulation in Limnodynastes peronii tadpoles. *Aquatic Toxicology, 187*, 1-8.

Leone, N. C., Geever, E. F., & Moran, N. C. (1956). *Acute and subacute toxicity studies of sodium fluoride in animals.* Public health reports, Washington.

Mann, R. M., & Bidwell, J. R. (2000). Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to Xenopus laevis and two Australian frogs. *Aquatic Toxicology, 51*(1), 19-29.

Martinez-Mier, E. A. (2012). Fluoride: Its Metabolism, Toxicity, and. *Journal of Evidence-Based Complementary & Alternative Medicine, 17*(1), 28-32. doi:10.1177/2156587211428076

Martini, F., Tarazona, J. V., & Pablos, M. V. (2012). Are Fish and Standardized FETAX Assays Protective Enough for Amphibians? A Case Study on Xenopus laevis Larvae Assay with Biologically Active Substances Present in Livestock Wastes. *The Scientific World Journal*.

Moretti, C. M., Bocchini, M. Q., Businelli, B. O., & Buonaurio, R. (2022). Sodium Selenate: An Environmental-Friendly Means to Control Tomato Bacterial Speck Disease. *Agronomy, 12*(6), 1-13. doi:10.3390/agronomy12061351

Moser, B., & Rayburn, J. (2007). Evaluation of Developmental Toxicity of Interaction betwen Caffeine and Pseudoephedrine Using Frog Embryo Teratogenesis Assay-Xenopus (FETAX). *Bios*, 1-9.

Mouche, I., Malesic, L., & Gillardeaux, O. (2017). FETAX assay for evaluation of developmental toxicity. *Methods in Molecular Biology, 1641*, 311-324.

Nelson, B. (1994). Interactions in developmental toxicology: a literature review and terminology proposal. *Teratology, 49*(1), 33-71. doi:https://doi.org/10.1002/tera.1420490107

(1994). *NTP Toxicity Studies of Sodium Selenate and Sodium Selenite (CAS Nos. 13410-01-0 and 10102-18-8) Administered in Drinking Water to F344/N Rats and B6C3F1 Mice.* Toxicity report series.

O'Rourke, D. (2007). Amphibians used in research and teaching. *ILAR Journal, 48*(3), 183-187.

Ozsvath, D. (2009). Fluoride and environmental health: a review. *Environmental Science and Bio/Technology, 8*, 59-79.

Pal, S., Samanta, P., Kole, D., Mukherjee, A. K., & Ghosh, A. R. (2018). Acute toxicity and oxidative stress responses in tadpole of skittering frog, Euphlyctis cyanophlyctis (Schneider, 1799) to sodium fluoride. *Bulletin of Environmental Contamination and Toxicology, 100*, 202-207.

Parker, T. T., & Rayburn, J. (2017). A comparison of electronic and traditional cigarette butt leachate on the development of Xenopus laevis embryos. *Toxicology Reports, 4*, 77-82.

Pecoraro, B. M., Leal, D. F., Frias-De-Diego, A., Browning, M., Odle, J., & Crisci, E. (2022). The health benefits of selenium in food animals: a review. *Journal of Animal Science and Biotechnology, 13*(58), 1-11. doi:https://doi.org/10.1186/s40104-022-00706-2

Podgorski, J., & Berg, M. (2022). Global analysis and prediction of fluoride in groundwater. *Nature Communications, 13*(4232), 1-9. doi:https://doi.org/10.1038/s41467-022-31940-x | www.nature.com/naturecommunications

Pollick, H. F. (2004). Water fluoridation and the environment: Current perspective in the United States. *INT J OCCUP ENVIRON HEALTH, 10*(3), 343-350.

Robinson, B. N., Krieger, K., Khan, F. M., Huffman, W., Chang, M., Naik, A., . . . Gaudino, M. (2019). The current state of animal models in research: A review. *International Journal of Surgery, 72*, 9-13.

Shirey, K., & Rayburn, J. (2013). Old world vs. new world: a preliminary comparison of the developmental toxicity of venom from two tarantula species, Grammostola rosea and Haplopelma lividum, using frog embryos from Xenopus laevis. *Allen Press, 84*(3).

Sobolev, O. I., Gutyj, B. V., Sobolieva, S. V., Borshch, O. O., Nedashkivsky, V. M., Kachan, L. M., . . . Guta, Z. A. (2020). Selenium in natural environment and food chains. *Ukrainian Journal of Ecology, 10*(4), 148-158.

Sorensen, H., Cedergreen, N., Skovgaard, M., & Streibig, J. C. (2007). An isobole-based statistical model and test for synergism/antagonism in binary mixture toxicity experiments. *Environmental and Ecological Statistics*, 383-397.

Stephen, R. C., Saville, D. J., & Watkinson, J. H. (1989). The effects of sodium selenate applications on growth and selenium concentration in wheat. *New Zealand Journal of*

Crop and Horticultural Science, 17(3), 229-237. doi:DOI: 10.1080/01140671.1989.10428037

Toxicology, N. R. (2000). *Scientific Frontiers in Developmental Toxicology and Risk Assessment* (Vol. 7). Washington (DC): National Academic Press. doi:https://www.ncbi.nlm.nih.gov/books/NBK225677/

Turtle, S. H. (2000). Embryonic survivorship of the spotted salamander (Ambystoma maculatum) in woodland and roadside vernal pools in Southeastern New Hampshire. *Journal of Herpetology 34*, 60-67.

Vitt, L. J., & Caldwell, J. P. (2013). *Herpetology: An introductory biology of amphibians and reptiles.* Burlington, Massachusetts: Academic Press.

Whitford, W. G., & Vinegar, A. (1966). Homing, survivorship, and overwintering of larvae in spotted salamanders (Ambystoma maculatum). *Copeia, 1966*(3), 515-519.

Wilzla, M., McNamara, S., & Horb, M. E. (2018). Generation and care of Xenopus laevis and Xenopus tropicalis embryos. *Methods in Molecular Biology, 1865*, 19-32.

Zhao, H., Zhu, Y., Zhao, Y., Wang, T., Li, H., Yang, J., . . . Wang, J. (2022). Alleviating effects of selenium on fluoride-induced testosterone synthesis disorder and reproduction toxicity in rats. *Ecotoxicology and Environmental Safety, 247*, 1-10.

Zhongchuang, L., Boning, C., Xiang, L., Hongyan, X., & Dongsheng, L. (2019). Toxicity assessment of artificially added zinc, selenium, and strontium. *Science of the Total Environment, 670*, 433-438. doi:https://doi.org/10.1016/j.scitotenv.2019.03.259

VITA

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