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STUDY OF THE INFLUENCE OF NITRATES AND NITRITES ON EMBRYOGENESIS OF *DANIO RERIO*

An increase in the amount of environmental pollution caused by different hazardous elements and anthropogenic pressure complicates and decreases Kazakhstan's natural resources' stable ecological state. According to the Republic of Kazakhstan's bulletin on the state of the environment, the content of calcium and sodium nitrate and nitrite ions in water bodies grew by 13.5 and 7.5%, respectively, in the first half of 2022, indicating that their actual concentration exceeded the background class. At the same time, it was discovered that the level of nitrate and nitrite ions is surpassed not only in aquatic bodies, but also in the Republic of Kazakhstan's atmospheric air and soil. Furthermore, there was a decline in aquatic organism diversification and abundance in the analyzed water bodies, which could be attributed to nitrate-nitrite pollution and harmful effects on juveniles. As a result, the goal of this study was to look at the effects of nitrates and nitrites on Danio Rerio embryogenesis. This object was chosen over others for its lower maintenance expenses, rapid advancement of embryogenesis phases, transparency, and the comparatively small size of the embryos for successful visual observation. Based on our findings, the embryotoxic and teratogenic effects of calcium nitrate and sodium nitrite have been proven, resulting in the death of fish. Calcium nitrate and sodium nitrite at concentrations of 1, 10, and 100 MPC have been demonstrated to cause dose-dependent embryonic death. Furthermore, to a greater extent under the action of sodium nitrite. Teratogenic diseases were discovered in surviving larvae, including edema of the yolk sac and pericardium of the heart, curvature of the skeleton and asymmetry of the body, edema of the retina, and aberrant formation of the organs of the oral cavity. The findings acquired are theoretically and practically important in the fields of ecology, ecotoxicology, and aquatic organism biodiversity protection. They contribute to our understanding of the hazardous effects of naturally occurring nitrates and nitrites, as well as the need to employ Danio rerio embryos and larvae as test objects for bioindication of polluted water bodies.

Key words: ecology, zebrafish, nitrates, nitrites, emryotoxicity, teratogenicity, histostructure.

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Danio rerio эмбриогенезіне нитраттар мен нитриттердің әсерін зерттеу

Әртүрлі қауіпті факторлардың және антропогендік қысымның әсерінен қоршаған ортаның ластану деңгейінің артуы Қазақстанның табиғи ресурстарының тұрақты экологиялық жағдайын қиындатады және төмендетеді. Қазақстан Республикасының қоршаған ортаның жай-күйі туралы бюллетеніне сәйкес 2022 жылдың бірінші жартыжылдығында су айдындарындағы кальций мен натрий нитратының және нитрит иондарының мөлшері тиісінше 13,5 және 7,5%-ға өсті, яғни олардың нақты концентрациясы фондық деңгейден асып түсті. Сонымен қатар, нитрат пен нитрит иондарының деңгейі су айдындарында ғана емес, Қазақстан Республикасының атмосфералық ауасы мен топырағында да асып түсетіні анықталды. Оған қоса, зерттелетін су объектілерінде биологиялық алуантүрлілік пен су организмдерінің санының төмендеуі байқалды, бұл нитрат-нитритпен ластанумен және жас организмдерге токсикалық әсер етумен байланысты болуы мүмкін. Сондықтан бұл зерттеудің мақсаты нитраттар мен нитриттердің Danio rerio эмбриогенезіне әсерін зерттеу болып табылды. Басқа сынақ объектілерімен салыстырғанда бұл объектіні таңдау зерттеуге кететін шығындардың төмендігімен, эмбриогенез кезеңдерінің леуімен, эмбрион қабықшасының мөлдірлігімен және сәтті визуалды бақылау үшін эмбриондардың салыстырмалы түрде кішкентай өлшемімен түсіндіріледі. Алынған нәтижелеріміз негізінде кальций нитраты мен натрий нитритінің эмбриотоксикалық және тератогенді әсері анықталды, бұл ақыр соңында балықтардың өліміне әкеледі.1, 10 және 100 ШРК концентрациясында кальций нитраты мен натрий нитритінің әсері дозаға тәуелді эмбриондық өлімді тудыратыны көрсетілген, оның ішінде, натрий нитритінің әсерінен көбірек дәрежеде. Тірі қалған дернәсілдерде тератогенді бұзылыстар эмбрионның сарыуыз қапшығы мен жүрек перикардының ісінуі, қаңқаның қисаюы және дене ассиметриясы, көз торының ісінуі, ауыз қуысы мүшелерінің аномальды түрде қалыптасуы түрінде анықталды. Алынған мәліметтер экология, экотоксикология және су организмдерінің биоалуантүрлілігін сақтау саласында теориялық және практикалық маңызы бар. Белгілі бір дәрежеде олар табиғатта кең таралған нитраттар мен нитриттердің токсикалық әсері туралы білімді кеңейтеді, сонымен қатар ластанған су объектілерін биоиндикациялау үшін сынақ объектілері ретінде Danio rerio эмбриондары мен дернәсілдерін пайдалану қажеттілігін растайды.

Түйін сөздер: экология, зебрафиш, нитраттар, нитриттер, эмбриотоксинділік, тератогенділік, гистоқұрылым.

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Исследование влияния нитратов и нитритов на эмбриогенез *Danio rerio*

Повышение уровня загрязнения окружающей среды под воздействием различных опасных факторов и антропогенной нагрузки осложняет и снижает стабильное экологическое состояние природных ресурсов Казахстана. По данным бюллетеня РК о состоянии окружающей среды в первом полугодии 2022 года содержание нитрат- и нитрит-ионов кальция и натрия в водоемах увеличилось соответственно на 13,5 и на 7,5%, то есть фактическая их концентрация превысила фоновый уровень. При этом установлено, что уровень нитрат- и нитрит-ионов превышен не только в водоемах, но и в атмосферном воздухе и почве РК. Помимо этого наблюдалось снижение биоразнообразия и численности гидробионтов в исследованных водоемах, возможно, связанные с нитрат-нитритным загрязнением и токсическим влиянием на молодь. Поэтому целью данного исследования явилось изучение влияния нитратов и нитритов на эмбриогенез Danio Rerio. Выбор данного объекта по сравнению с другими тест-объектами обусловлен более низкими расходами на содержание, быстрым протеканием стадий эмбриогенеза, прозрачностью и относительно небольшим размером эмбрионов для успешного визуального наблюдения. На основании полученных нами результатов установлено эмбриотоксическое и тератогенное действие нитрата кальция и нитрита натрия, приводящее, в конечном счете, к гибели рыб. Показано, что воздействия нитрата кальция и нитрита натрия в концентрации 1, 10 и 100 ПДК вызывают дозозависимую смертность эмбрионов. Причем при воздействия нитрита натрия в большей степени. У выживших личинок обнаруживались тератогенные нарушения в виде отека желточного мешка и перикарда сердца, искривления скелета и асимметрии тела, отека сетчатки глаза и неправильного формирования органов ротовой полости. Полученные данные имеют теоретическое и практическое значение в области экологии, экотоксикологии и в сфере сохранения биоразнообразия гидробионтов. Они в известной степени расширяют знания о токсическом действии широкораспространенных в природе поллютантов – нитратов и нитритов, а также подтверждают факт необходимости использования эмбрионов и личинок Danio Rerio в качестве тест-объектов при биоиндикации загрязненных водоемов.

Ключевые слова: экология, зебрафиш, нитраты, нитриты, эмбриотоксичность, тератогенность, гистоструктура.

Introduction

Nitrites and nitrates are widely employed in the preservation of meat and other consumable products. Because of their use in intensive agriculture, animal husbandry, and wastewater discharges, they may reach the food chain as chemical contaminants in water [1]. Plants use nitrates as their primary nutrition in nature. The majority of nitrates used in commerce are inorganic fertilizers. Nitrates and nitrites are also employed in food preservation, some medications, and ammunition and explosives manufacturing [2]. Because decomposition or denitrification happens in modest amounts under aerobic circumstances, nitrate leaks into the aquifer in significant quantities. Nitrate is denitrified or nearly entirely degraded to nitrogen under anaerobic circumstances. Surface waters can also undergo nitrification and denitrification depending on temperature and pH. However, nitrate uptake by plants depletes nitrate stores in surface waters. Nitrates are found in the air mostly as nitrates and inorganic aerosols, but also as nitrate radicals and organic gases [3].

Nitrates are extremely mobile in soil and can migrate into groundwater due to their high water solubility and limited soil retention. Because nitrates and nitrites are not volatile, they can linger in water until they are absorbed by plants and other creatures [4]. Bacteria uptake ammonium nitrate, and nitrate decomposition is faster in anaerobic circumstances [5]. Nitrite can readily oxidize to nitrate, and nitrate is the more prevalent component in groundwater [6].

Calcium ions dominated precipitation by 13.5%, sodium ions by 7.5%, and actual concentrations of nitrite and nitrate anions, chemical oxygen consumption exceeded the background class, according to the Republic of Kazakhstan's information bulletin on the state of the environment in the first half of 2022. In urban wastewater discharges, exceeding quality criteria based on these indicators is most common [7].

The amount of nitrate and nitrite ions is exceeded not only in aquatic bodies, but also in the Republic of Kazakhstan's atmospheric air and soil, according to biotesting data. At the same time, the active expansion of industry, agriculture, and human life pollutes water, land, and the atmosphere even more [8-13].

Materials and reseach methods

The study's subject and the conditions for its content

The *Danio rerio* fish, which is widely used in science as a model object to establish various teratogenic effects of chemicals, pollutants, drugs, and so on, was chosen as the subject of study [14]. Individuals with sexual maturity and a length of 4 cm were used in the experiment. Females were distinguished from males by the presence of a large round belly and mild back lines, while males had a longer elongated body. The fish were kept in 15-liter aquariums with natural light, a saturation of about 80% oxygen, and a regular medium pH of 6.5-8. The ideal temperature for breeding *Danio rerio* fish is 25-28ºC. The diet consisted of dry food supplemented with vitamins and live A. crustaceans. To prevent fungal infections, methylene blue drops were added at each water change. 5 ml of the solution was added for every 10 l aquarium water for fish treatment. Males were kept apart from females and were only kept in the same aquarium during spawning.

Principles and conditions for obtaining embryos mastering the ZFET methodology

The Zebrafish embryotoxicity test (ZFET) is a method for determining the toxicity of compounds by observing their effects on zebrafish embryos.

It is based on the use of zebrafish embryos, which are extremely sensitive to harmful chemicals and grow quickly.

During testing, zebrafish embryos are immersed in a variety of hazardous solutions before their survival, growth, and development are assessed. Changes in these characteristics may indicate a substance's toxicity. The ZFET methodology, which can be used to analyze medications, insecticides, and other chemicals, is an alternative to established toxicity assessment methods such as animal testing. Figure 1 depicts the procedure for carrying out the ZFET methodology. The resulting embryos are first disseminated on Petri dishes, then fertilized embryos are picked using microscopy, and dead or coagulated eggs are eliminated. It is also useful for determining the environmental safety of substances and materials [15-19].

For the ZFET method to work properly, eggs must be quickly distributed among Petri dishes, and they must be at the zygote stage at the time of distribution (fertilized eggs) [20]. Embryos distributed, 10-15 pieces each, on prepared Petri dishes with a diameter of 90 mm that already contain solutions of the test substances in various concentrations. Every 12 hours, changes were observed. To compare, a portion of the eggs in the control filtered water were distributed. A Leica DMLB2 stereomicroscope and Micros MC20, were used to record and photograph each developmental change. The embryos' transparent chorion and the fry's transparent shell allow for observation of their development even after 72 hours.

Figure 1 shows photographs of *Danio rerio* embryos fixed at different stages of development, from an unfertilized egg to a hatched embryo at the 96-hour development stage.

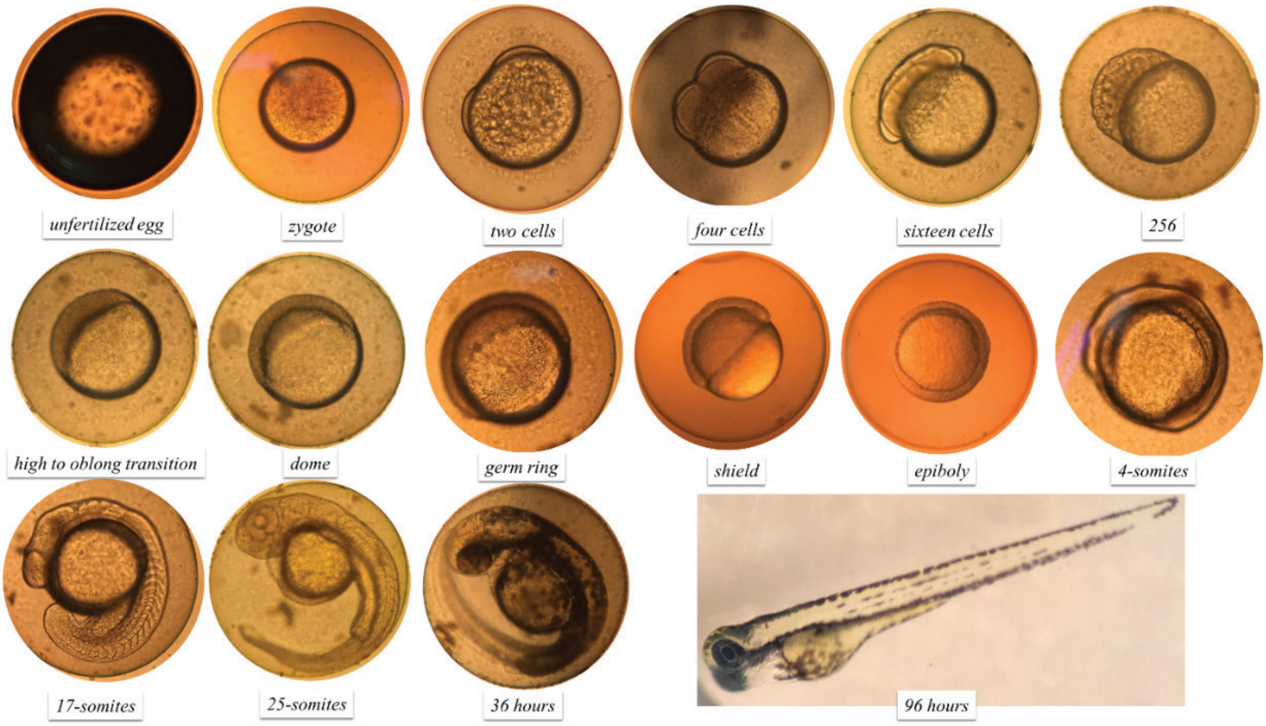

Figure 1 – *Danio rerio* fish embryogenesis at various stages berfore 96 hpf development

Method for preparation different concentrations of calcium nitrate and sodium nitrite solutions

$Ca(NO_3)_2$, also known as calcium saltpeter or calcium nitrate, is classified as dangerous to living organisms in the third class. The OECD recommended a maximum allowable concentration of 40 mg/dm^3 for them. Aqueous solutions of varying concentrations were prepared in accordance with the ecotoxicological protocol:

Control: pure filtered water
1 MPC: 40 (0.04) mg/dm^3 of $Ca(NO_3)_2$
10 MPC: 400 (0.4) mg/dm^3 of $Ca(NO_3)_2$
100 MPC: 4000 (4) mg/dm^3 of $Ca(NO_3)_2$

Calcium nitrate is used in the chemical industry, the match industry, industrial explosive production, and fertilizer production. It is used as a raw material in the production of reagents, as an additive to industrial concrete mixtures and mortars, and in the preparation of brines in industrial refrigeration. The amount of nitrates in Kazakhstan reservoirs exceeds 5.628 mg/dm^3.

$NaNO_2$, also known as the nitrite salt or sodium nitrite, is a white to slightly yellowish crystalline powder that is hygroscopic and readily soluble in water. It is a precursor to many organic compounds, including pharmaceuticals, dyes, and pesticides, but it is most well-known as the E250 food additive found in processed meats and fish products. Belongs to the first class of extremely dangerous substances. MPC of sodium nitrite is equal to 3.3 mg/dm^3:

Control: filtered water
1 MPC: 3,3 (0,0033g) mg/dm^3 of $NaNO_2$
10 MPC: 33 (0,033g) mg/dm^3 of $NaNO_2$
100 MPC: 330 (0,33g) mg/dm^3 of $NaNO_2$

The amount of nitrates in Kazakhstan reservoirs exceeds 0,342 mg/dm^3.

Marginal propensities to consume (MPCs) are established based on toxicological studies, risk assessments for human health, and environmental variables. Special experiments are carried out to study the hazardous qualities of the chemical as well as its influence on the human or animal body.

Based on the data acquired, safe levels of exposure to the chemical that do not result in harmful health effects are defined. MPCs can differ according on the type of substance, the mode of exposure to the body, the duration and frequency of exposure, and other factors. They are established at the legislative level and are used to control environmental and food quality [21-23].

Results of research and their discussion

Study the influence of calcium nitrate on the development of Danio Rerio

Table 1 illustrates the results of a study on the influence of calcium nitrates on the development of *Danio rerio* where the mortality rate of embryos in the control group is 3.3%, which is not higher than the OECD mortality rate [24-25].

In the experimental group exposed to $Ca(NO_3)_2$ at a concentration of 1 MPC, the lethality was 5.5%, which exceeded the control by 1.6 times. When exposed to $Ca(NO_3)_2$ at a concentration of 10 MPC, embryonic mortality was 11.2%, which exceeded the control values by 3.4 times. And when exposed to a higher dose of 100 MPC, the mortality of embryos was 32.6%, which is 9.9 times higher than the control level.

Table 1 – The percentage of mortality of *Danio rerio* embryos in the control group (clean filtered water) and varied amounts of calcium nitrate ($Ca(NO_3)_2$) observed every 12 hours

Period, hpf	Control	1 MPC	10 MPC	100 MPC
12	3.3%	3.3%	0%	4.4%
24	0%	2.2%	5.5%	0%
36	0%	0%	2.3%	5.8%
48	0%	0%	0%	6.9%
60	0%	0%	3.3%	6.6%
72	0%	0%	0%	8.9%
Total	3.3%	5.5% Mortality	11.2% Mortality	32.6% Mortality

Many variables and experimental groups were employed throughout the experiment to assure the experiment's reliability and repeatability. The arithmetic mean of mortality at 1, 10, and 100 MPC of calcium nitrate solutions was computed.

There was a control group and two different experimental groups in the experiment. The control group did not receive calcium nitrate, whereas the other experimental groups did. Throughout the experiment, many constants stayed constant. The time and duration that each zebrafish was exposed to calcium nitrate remained constant.

Study the influence of sodium nitrite on the development of Danio Rerio

The results of the study of the effect of sodium nitrites on the development of *Danio rerio* are shown in Table 2. The mortality of embryos in the control group is 3.3%, which does not exceed the mortality rate according to OECD data.

In the experimental group of $NaNO_2$ at a concentration of 1 MPC, the lethality of embryos was 5.5%, which is 1.6 times more than in the control; under the influence of $NaNO_2$ concentration of 10 MPC, the mortality of embryos was 14.8%, which is 4.4 times more than in the control. Moreover, exposure to a higher dose of 100 MPC resulted in a death rate of 42.5%, which is 12.8 times higher than in the control.

When compared to the previous table, the data clearly show that zebrafish exposed to sodium nitrite had a higher rate of mortality and physical deformities; however, both experimental groups experienced similar mortality rates, and embryos exposed to 100 MPC sodium nitrite had a much higher mortality rate. Embryos exposed to MPC 1 and 10 also hatched earlier than those that were not. These findings suggest that sodium nitrite contributed to aberrant embryonic development and inappropriate growth timing. These variables could have led to the failure of poorly developed organs, resulting in a greater death rate in embryos exposed to sodium nitrite.

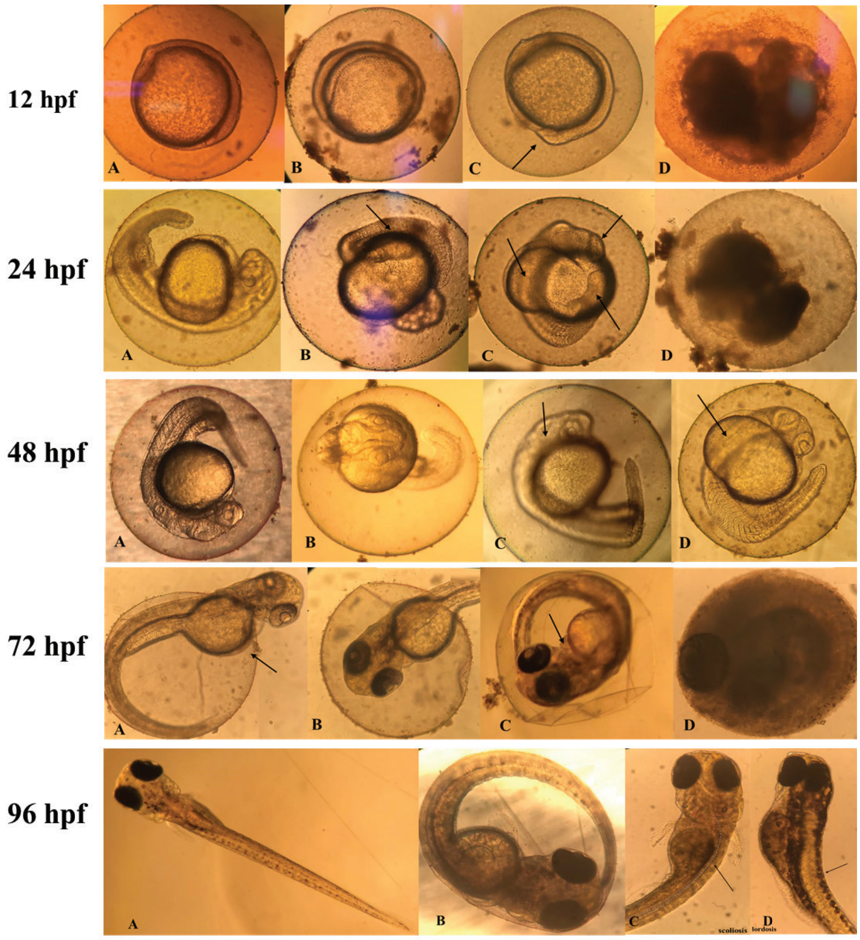

Figure 2 – *Danio rerio* embryos in a 12, 24, 48, 72, 96-hour developmental study at different concentrations of calcium nitrate. 12 hpf (A) – embryo in the control group, 12 hpf (B) – embryo at 1 MPC, 12 hpf (C) – embryo in the group with 10 MPC, start of protein coagulation, 12 hpf (D) – embryo at 100 MPC, complete coagulation and death of the embryo. 24 hpf (A) – embryo with normal development, 24 hpf (B) – embryo at 1 MPC solution, 24 hpf (C) – embryo at 10 MPC with yolk sac edema, 24 hpf (D) – dead embryo at 100 MPC. 48 hpf (A) – embryo in the control group with normal development, 48 hpf (B) – embryo at 1 MPC of calcium nitrate solution, 48 hpf (C) – embryo in the control group 10 MPC with developmental delay,48 hpf (D) – *Danio rerio* embryo at 100 MPC with yolk sac edema. 72 hpf (A) – control group at the hatching stage, 72 hpf (B) – group in 1 MPC solution, 72 hpf (C) – group with 10 MPC, the embryo with a developmental defect could not leave the chorion, 72 hpf (D) – group in 100 MPC solution completely coagulated. 96 hpf (A) – embryo in the control group with normal development, 96 hpf (B) – embryo at a concentration of 1 MPC with developmental delay, 96 hpf (C) – *Danio rerio* embryo in the group with 10 MPC, scoliosis is observed (indicated by an arrow), body asymmetry is indicated by a dashed line along the spine, 96 hpf (D) – *Danio rerio* embryo at 100 maximum concentration limit with observed lordosis.

Table 2 – The percentage of mortality of *Danio rerio* embryos in the control group (clean filtered water) and varied sodium nitrite ($NaNO_2$) concentrations observed every 12 hours

Period, hpf	Control	1 MPC	10 MPC	100 MPC
12	3.3%	5.5%	0%	6.7%
24	0%	0%	3.3%	8.9%
36	0%	0%	0%	0%
48	0%	0%	9.2%	4.7%
60	0%	0%	2.3%	13.9%
72	0%	0%	0%	8.3%
Total	3.3%	5.5% Mortality	14.8% Mortality	42.5% Mortality

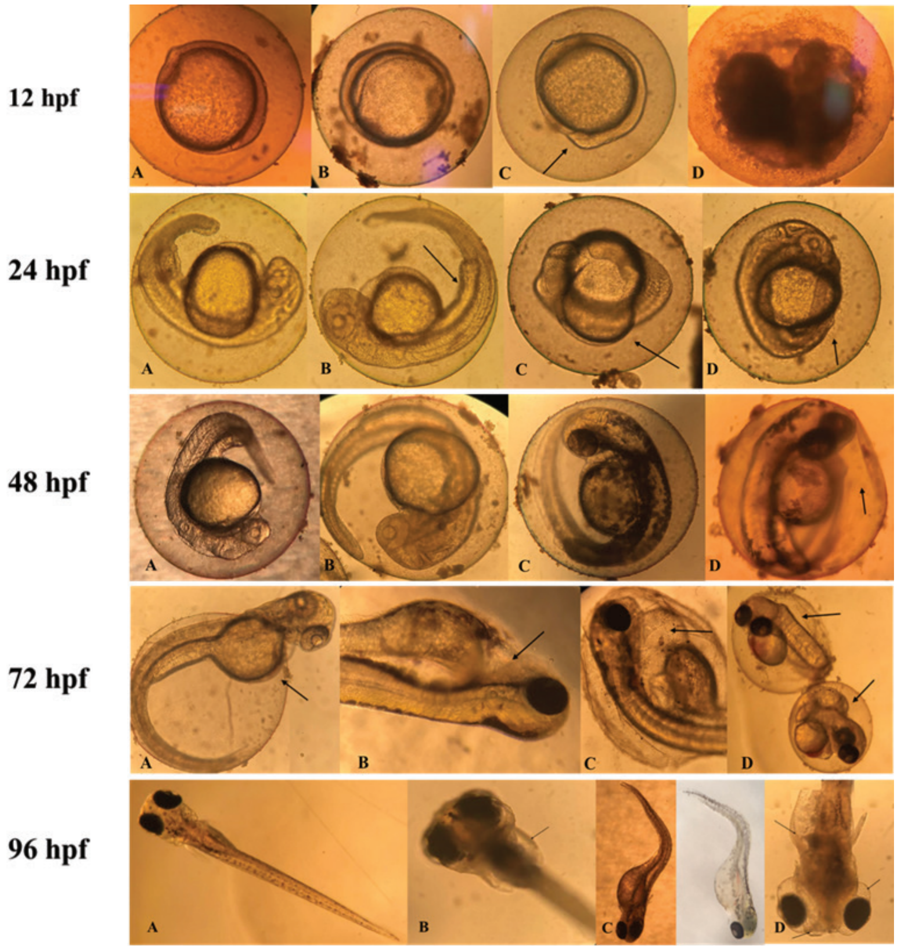

Figure 3 – Development of *Danio rerio* embryos after 12 hours, where A is the control group, B is the experimental group with 1 MPC $NaNO_2$, C is the group with 10 MPC $NaNO_2$ – the absence of somites is clearly visible, and D is the experimental group with 100 MPC $NaNO_2$, where the embryo perished due to coagulation. 24 hpf (A) represents normal development in the control group, 24 hpf (B) represents a group with 1 MPC $NaNO_2$ – yolk sac edema (marked by an arrow), 24 hpf (C) represents a group with 10 MPC $NaNO_2$ where yolk sac edema is visible (marked by an arrow), 24 hpf (D) represents an experimental group with 100 MPC $NaNO_2$ – developmental delay, abnormal somite division, the tail is not separated from the bodies. 48 hpf (A) represents normal development in the control group, 48 (B) represents 1 MPC $NaNO_2$, 48 (C) represents 10 MPC $NaNO_2$, and 48 (D) represents the experimental group with 100 MPC $NaNO_2$. MPC is the turbid content of the chorion (indicated by an arrow), the start of coagulation. 72 hpf (A) – a control group in which the embryo simply exits the shell (shown by an arrow). 72 hpf (B) – experimental group with 1 MPC of $NaNO_2$, there is pericardial edema and one eye is absent (shown by an arrow). 72 hpf (C) – 10 MPC $NaNO_2$ group, pericardial edema and yolk sac distortion (shown by an arrow), 72 hpf – 100 MPC group of $NaNO_2$, body curvature, tail deformity (indicated by an arrow), developmental delay. 96 hpf (A) – control group, 96 hpf (B) – experimental group 1 MPC of $NaNO_2$, immobile embryo, short fins (shown by an arrow), 96 hpf (C) – group of 10 MPC of $NaNO_2$, curvature of the body and tail, 96 hpf (D) – 100 MPC of $NaNO_2$, body asymmetry, fin length difference, enlargement of the eye, oral cavity malformations (marked by an arrow).

The arithmetic mean survival rates of zebrafish embryos in varied MPCs of calcium nitrate and sodium nitrite relative to the control are shown in Tables 3 and 4, with significant differences between control samples and solutions noted. The data were reported as a standard deviation, and $p \leq 0.05$ was utilized as the statistical significance level.

Table 3 compares the survival of *Danio rerio* embryos at various calcium nitrate concentrations to the control. The asterisks denote arithmetic means that differ significantly from the control group.

Table 4 compares the survival of *Danio rerio* embryos at various sodium nitrite concentrations to the control. The Student T-test method was employed to examine the significance of the differences between the control and experimental average groups.

Table 3 – Means of survival rate *Danio rerio* embryos at 12 hpf in different calcium nitrate MPCs in comparison with the control

	Control	1 MPC	10 MPC	100 MPC
12 hpf	10.0±0.00	9.6±0.13	10.0±0.00	9.3±0.27
24 hpf	9.6±0.13	9.3±0.13	9.6±0.13	9.0±0.23*
36 hpf	9.6±0.13	9.3±0.13	9.3±0.27	9.0±0.23*
48 hpf	9.6±0.13	9.3±0.13	9.0±0.23*	8.3±0.36*
60 hpf	9.6±0.13	9.3±0.13	8.6±0.13*	7.0±0.40*
72 hpf	9.6±0.13	9.3±0.13	8.6±0.13*	6.0±0.23*

Note: *p≤0.05, n=18 for each groups

Table 4 – Means of survival rate *Danio rerio* embryos in different sodium nitrite MPCs in comparison with the control

	Control	1 MPC	10 MPC	100 MPC
12 hpf	10.0±0.00	9.3±0.13	10.0±0.00	9.3±0.27
24 hpf	9.6±0.13	9.3±0.13	9.6±0.13	8.3±0.13*
36 hpf	9.6±0.13	9.3±0.13	9.6±0.13	8.3±0.13*
48 hpf	9.6±0.13	8.6±0.13*	8.6±0.13*	8.6±0.13*
60 hpf	9.6±0.13	8.6±0.13*	8.3±0.13*	6.3±0.36*
72 hpf	9.6±0.13	8.6±0.13*	8.3±0.13*	5.6±0.13*

Note: *p≤0.05. n=18 for each groups

The findings of this experiment are similar with previous studies in which zebrafish were exposed to nitrate or nitrite. Scientists discovered that exposing zebrafish embryos to nitrates and nitrites causes them to have aberrant developmental traits [26].

The data in photographs 2 and 3 show that embryos exposed to nitrates and nitrites are at risk of physical deformities such as curvature of the embryo's vertebral column, and the data in tables 3 and 4 show that the higher the concentration of the test substance, the greater the mortality. Embryos exposed to calcium nitrates and sodium nitrites hatched with bent dorsal canals, demonstrating that nitrates and nitrites caused the fish to develop improperly and the bone structure to develop differently than expected.

Conclusion

The investigation of the effects of calcium nitrates and sodium nitrites on the embryogenesis of the *Danio rerio* fish resulted in hypotheses. The embryotoxic and teratogenic effects of calcium nitrate and sodium nitrite were established based on the findings. Calcium nitrate and sodium nitrite at concentrations of 1, 10, and 100 MPC have been proven to cause dose-dependent embryonic mortality. Furthermore, calcium nitrates and sodium nitrites have been shown to impair the proper growth of fish in the following ways. Increased exposure to these toxins causes developmental defects such as yolk sac and pericardium swelling, spinal canal curvature, body asymmetry, immobility, eye edema and mouth malformations. Data comparison demonstrates that sodium nitrite is more embryotoxic and teratogenic than calcium nitrate. Thus, *Danio rerio* embryos, larvae, and mature species can be utilized as test items for bioindication of water pollution. The embryotoxic and teratogenic effects of calcium nitrate and sodium nitrite were established based on the results.

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