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THREE DIFFERENT PESTICIDES

(CHLORPYRIPHOS-ETHYL,

2,4-DICHLOROPHENOXYACETIC ACID AND METALAXYL-M +

MANCOZEB) ON OXYGEN CONSUMPTION RATES AND BEHAVIORAL

RESPONSES OF ZEBRA CICHLIDS (*Amatitlania nigrofasciata*)

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by

MESUT AKPUNAR

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Approval of the Graduate School of Natural and Applied Sciences

Prof. Dr. Yaşar DÜRÜST
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Ekrem GÜREL
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Assoc. Prof. Dr. Hakan TÜRKER
Supervisor

Examining Committee Members

1- Prof. Dr. EKREM GÜREL ....

2- Assoc. Prof. Dr. HAKAN TÜRKER ....

3- Assist. Prof. Dr. MUHAMMET YILDIRIM ....

ABANT IZZET BAYSAL UNIVERSITY

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ABSTRACT

EFFECT OF THREE DIFFERENT PESTICIDES (CHLORPYRIPHOS-ETHYL, 2,4-DICHLOROPHENOXYACETIC ACID AND METALAXYL- M + MACOZEB) ON OXYGEN CONSUMPTION RATES AND BEHAVIORAL RESPONSES OF ZEBRA CICHLIDS (*Amatitlania nigrofasciata*)

AKPUNAR, Mesut
Master of Science, Department of Biology
Supervisor: Assoc. Prof. Dr. Hakan TURKER
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Pesticides are very toxic for fresh water ecosystem and generally derived from agricultural process for controlling pests, broadleaf plants and fungus to inhibit yield losses, protect crops, improve crop quality, simplify harvesting and reduce expenditure. Chlorpyriphos-Ethyl (CF-E), 2,4-Dichlorophenoxyacetic acid (2,4-D) and Metalaxyl-M+Mancozeb (MMM) mixture are the most widely used as insecticides, herbicide and fungicide, respectively, in Bolu region. In this study, male zebra cichlids (*Amatitlania nigrofasciata*) were exposed sublethal concentration of these three pesticides. Oxygen consumption rates and behavioral responses were determined under five different sublethal concentrations of three pesticides (CF-E; 0, 0.004, 0.008, 0.016, 0.032, 0.05 mg/l, 2,4-D; 0, 2.17, 4.35, 8.69, 17.38, 28.97 mg/l and MMM; 0, 0.16, 0.32, 0.64, 1.0, 1.6 mg/l) in similar weight of male zebra cichlids at 24±1°C for 96h. After the measurement of oxygen consumption rates by intermittent flow through computer controlled respirometry, routine metabolic rate and standard metabolic rate were calculated by statistical analysis. During the experimental period, oxygen consumption rates from three pesticides increased linearly as their concentration increased from 0.004 to 0.032 mg/l in CF-E, 2.17 to 17.38 mg/l in 2,4-D and 0.16 to 1.0 mg/l in MMM. Oxygen consumption rates decreased after at 0.032 mg/l in CF-E, 17.38 mg/l in 2,4-D and 1.0 mg/l in MMM. Cichlid fish exposed to pesticides showed abnormal behavioral activity such as, hyperexcitation, loss of equilibrium, irregular swimming, change in body color and finally sinking at the corner of test tank and staying motionless in there. Along the experiment, severe respiratory stress, rapid opercular activity, excessive mucus secretion and fatigue were also observed in the zebra cichlid.

Keywords: pesticides; toxicity; respirometry; oxygen consumption; behavioral dysfunction; zebra cichlid

ÖZET

ÜÇ FARKLI PESTİSİTİN (KLORİFOS-ETİL, 2,4-DİKLOROFENOKSİASETİK ASİT, METALAKSİL-M + MANKOZEP) ZEBRA CİKLETLERİNİN (*Amatitlania nigrofasciata*) OKSİJEN TÜKETİM ORANLARI VE DAVRANIŞSAL TEPKİLERİ ÜZERİNDEKİ ETKİLERİ

AKPUNAR, Mesut
Yüksek Lisans, Biyoloji Bölümü
Tez Danışmanı: Doç.Dr. Hakan TURKER
Kasım 2013, 50 sayfa

Pestisitler su ekosistemleri için oldukça toksik olan ve tarımsal faaliyetlerde yoğun olarak kullanılan maddelerdir. Zararlı böceklerin, yabani otların ve mantarların kontrol edilmesinde dolayısıyla ürün kaybını engelleme, mahsüllerin korunması, ürün kalitesinin arttırılması, hasadın kolaylaştırılması ve giderlerin azaltılmasında faydalanılan kimyasallardır. Klorifos-Etil (CF-E), 2,4-Diklorofenoksiasetik asit (2,4-D) ve Metalaksil-M+Mankozep (MMM) karışımı da sırasıyla bu kimyasalların Bolu da en yaygın kullanılanlarıdır. Bu çalışmada, erkek zebra ciklet balıkları (*Amatitlania nigrofasciata)* farklı sublethal konsantrasyonlardaki bu pestisitlere (CF-E; 0, 0.004, 0.008, 0.016, 0.032, 0.05 mg/l, 2,4-D; 0, 2.17, 4.35, 8.69, 17.38, 28.97 mg/l ve

MMM; 0, 0.16, 0.32, 0.64, 1.0, 1.6 mg/l) maruz bırakılmış ve bu üç pestisit için oksijen tüketim oranları ve davranışsal tepkiler belirlenmiştir. Deneylere 96 saat boyunca 24±1°C' de devam edilmiştir. Oksijen tüketim oranları bilgisayar kontrollü ve aralıklı akışlı respirometre ile ölçüldükten sonra rutin metabolik oran ve standart metabolik oran istatistiksel analiz ile hesaplanmıştır. Deney periyodu boyunca pestisitlerin konsantrasyonları arttıkça oksijen tüketim oranlarıda lineer bir şekilde arttı. CF-E'de 0.004 ten 0.032 mg/l'ye, 2,4-D'de 2.17 den 17.38 mg/l'ye, MMM'de ise 0.16 dan 1.0 mg/l'ye kadar oksijen tüketim oranı artmıştır. Fakat oksijen tüketimi 0.032 mg/l'den sonra CF-E'de, 17.38 mg/l'den sonra 2,4-D'de ve 1.0 mg/l'den sonra MMM'de azalmıştır. Pestisitlere maruz bırakılan ciklet balıkları anormal davranışsal aktiviteler göstermiştir. Örneğin; aşırı hassaslaşma, denge kaybı, düzensiz yüzme, vucut rengi değişimleri ve son olarak test ünitelerinin köşe kısımlarına çökerek orada öylece hareketsiz kalma gözlemlenen davranışsal bozukluklardır. Ayrıca deney süresince ağır solunum stresi, hızlı operkül hareketleri, artan mukus salgısı ve yorgunluk cikletlerde gözlemlenen bulgulardır.

Anahtar kelimeler: pestisitler; toksisite; respirometre; oksijen tüketimi; davranışsal anormallikler; zebra ciklet

TO MY PARENTS

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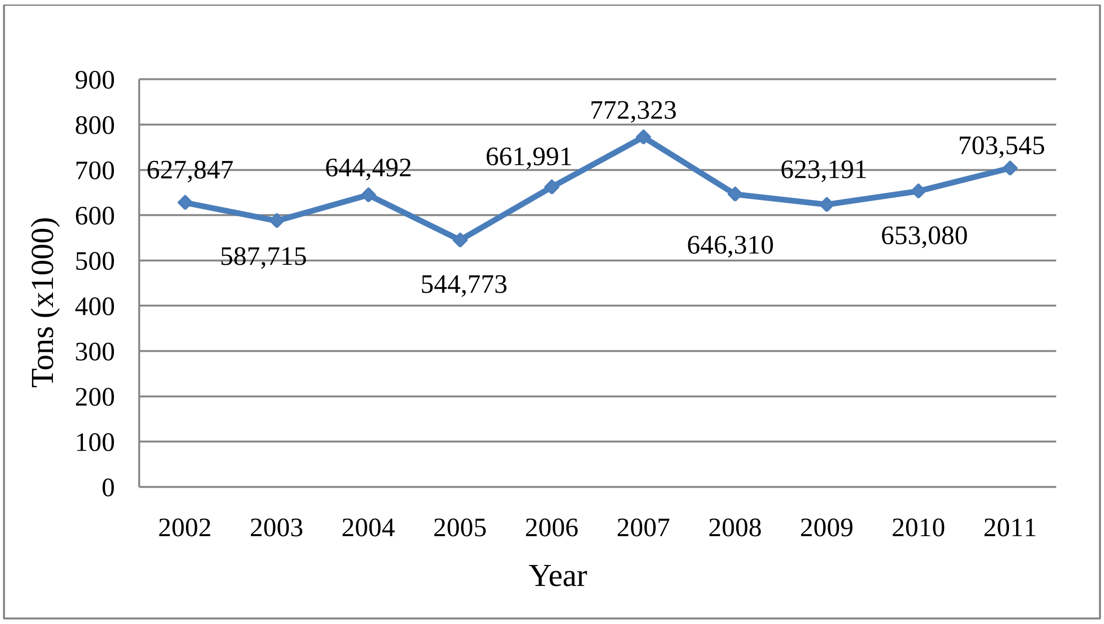
LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
CF-E	Chlorpyriphos- Ethyl
DDT	1,1-Trichloro-2,2-bis(4-DDT chlorophenoxyl)ethane
DO	Dissolved oxygen
MMM	Metalaxyl- M + Mancozeb mixture
OCR	Oxygen consumption rate
OP	Organophosphorous compounds
RMR	Routine metabolic rate
SMR	Standard metabolic rate
TUIK	Turkish Statistical Institute
2,4-D	2,4-Dichlorophenoxyacetic acid

1. INTRODUCTION AND LITERATURE REVIEW

At the present time; one of the most critical problem is rapidly increasing world population. According to the United Nations; every year world population is increasing about 78 million and until 2030, world population will be reached to 8 billion. If this scenario comes true, animal nutrients requirement will increase 2 fold in 20 years. Generally humans supply 20% of the animal protein from fish. Importance of seafood industry is derived from small amount of bait but high amount of yield; for example 100 kg bait gives 65 kg edible salmon meat, 20 kg chicken meat and 13 kg pig meat. Because of these reasons, while fish consumption amount was 0.7 kg in 1970, increased ten times and consumption reached 7.7 kg per capita in 2008 (Demir, 2011). In Turkey, fish production was more than 600 000 tons in marine and inland water in 2002, reached to 700 000 tons in 2011 and was projected to be 750 000 tons in 2013 (TUIK, 2011) (Table 1.1).

Table 1.1. Total fish production (including capture) in marine and inland water of Turkey.



The aquatic biota is continuously contaminated by xenobiotic pollutants such as; oil spills, heavy metals, detergents, fertilizers, sewage, pesticides (insecticides, herbicides, fungicides). A xenobiotic is a chemical which is found in an organism but which is not normally produced or expected to be present in it. Pesticides, for example, are one of the most important xenobitic in the aquatic system. They are known as chemicals used to control pests and prevent the spread of disease (Ecobichon, 2000). Pesticides can be classified based on the target organisims and include insecticides, herbicides, fungicides, algicides, nematicides, rodenticides, avicides, miticides, virucides and acaricides but pesticides do not include antibiotics. They are also classified by their chemical structure such as organophosphates, organochlorines, carbamates, pyrethroids, ureas, triazines and aryloxyalkanoic acids (Stenerson, 2004). In the last 50 years, pesticide usage has greatly increased to improve the food quality for the world population. However, pesticides have resulted to the environmental pollution. Agricultural pesticides usage are known as one of the most common contaminants in the world (Wang et al., 2011). Annually 3 million tons of pesticides is manufactured and each passing year production is rising about 1%. Tiryaki et al. (2010) summarized pesticide usage rates in the world (Figure 1.1).

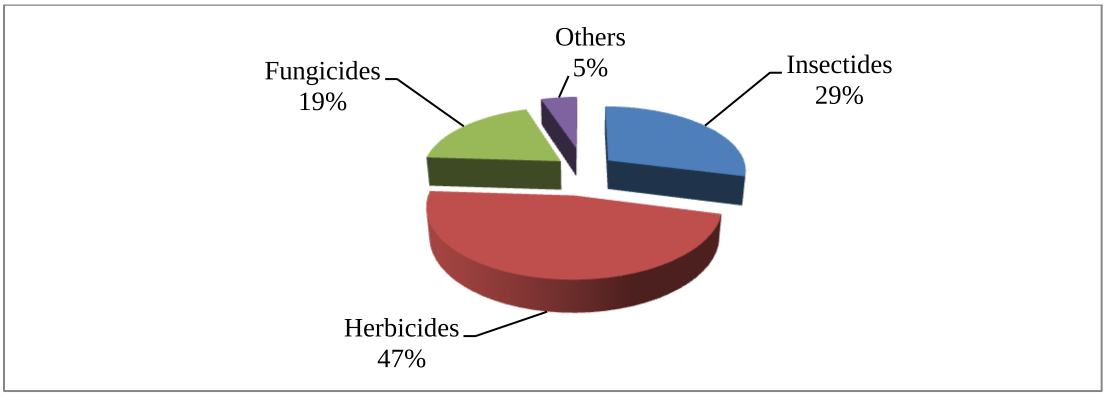


Figure 1.1. Pesticides usage in the world.

Agricultural crops and beneficial organisms are protected by conscious and technical usage that amount can be tolerated by ecosystem. In Turkey; 33 thousand tons of pesticide is used yearly. Karaca et al. (1996) reported pesticide usage in Turkey (Figure 1.2) and indicated that 230-250 million dolars are spent annually for this pesticides. The usage dose has continously rised in our country because of the unconscious applications and increased population of harmful species significantly. Insects have acquired resistance to pesticides. Consequently, new pesticides entered the market against these pests and diseases.

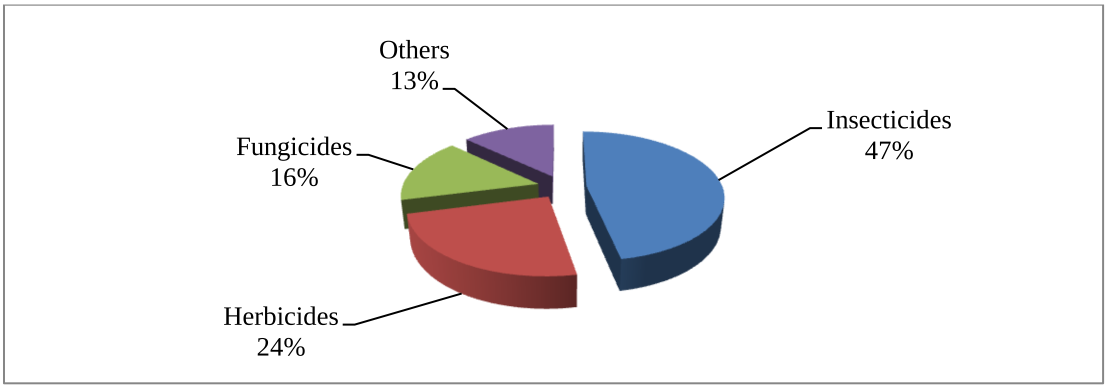


Figure 1.2. Pesticides usage in Turkey.

Pesticides usage amount in Bolu region was shown (Figure 1.3) according to Bolu Provincial Directorate of Food, Agriculture and Livestock data (1992-2012).

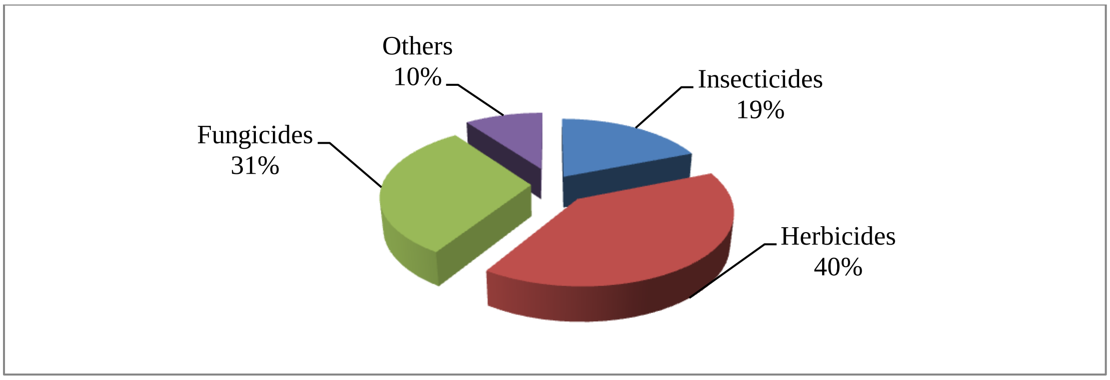


Figure 1.3. Pesticides usage in Bolu region.

Pesticides pollution is the most common problem in many regions of the world and derives from agricultural and industrial applications. Pesticide spraying amount is between 14-80% (Courshee, 1960; Cilgi and Jepson, 1992). Owing to both agricultural and industrial processes, some of these chemicals stay in the land and some of them reach into the aquatic systems by rain, wind and excess agricultural irrigation and such ways cause reduction of water quality. Pesticides, either pass to aquatic systems or remain in land ecosystem, living organisms in it affect positively or negatively. Generally fish population is negatively affected by pesticides because they directly affect dissolved oxygen concentration in the water (Adewoye, 2010).

Dissolved oxygen (DO) is the amount of oxygen (O_2) dissolved in water. DO measurements provide one of the best indicators of the health of water ecosystem, as oxygen is a necessary element for all forms of life, including aquatic life (Sanchez et al., 2007). Atmosphere contains nearly 21% oxygen gas and oxygen enters water through direct exchanges with the atmosphere. It is also produced as a byproduct of plant and phytoplankton photosynthesis. DO is used by plants and animals for respiration, and by aerobic bacteria in the process of decomposition. DO levels can also vary according to the time of day such as; weather, pollution, salinity and temperature (Spanou and Chen, 1999; Cox, 2003; Mullholand et al., 2005; Quinn et al., 2005). DO in water can range from 0-18 mg/l, but most natural water systems require 7-8 mg/l to support a diverse population. As DO levels drop below 5.0 mg/l, aquatic life is put under stress. A decrease in DO levels is typically associated with an organic pollutant. Thus, oxygen consumption and metabolic rates of fish decrease and behavioral abnormalities occur (Barbieri, 2008). Additionally, physiological deformities and death related to excess amount of pollutants.

Metabolism can be described as the system of chemical processes that keep something alive and include two processes: anabolism and catabolism. Anabolism is the process of building new body tissue for growth, maintenance and reproduction but catabolism is known as the breaking down of complex molecules into simpler ones to produce energy (Campbell and Reece, 2002). Depending on environmental conditions, metabolism of organisms can work at different rates. Metabolic rates are generally measured by direct and indirect calorimetry. Direct calorimetry mostly used for birds and mammals but direct calorimetry is not suitable for fish because fish are poikloterm animals that have low metabolic rate and heat producing capacity. The heat capacity of water is high, therefore the direct calorimetry is less sensitive than the indirect calorimetry. Indirect calorimetry based on oxygen consumption rate of fish and oxygen consumption rate is related to aerobic mitochondrial metabolism (Cech, 1990). Fish oxygen consumption level have been defined due to their physiological activity (Palanivelu et al., 2002). Thus, the oxygen consumption rate measurement of fish is important parameter to evaluate the pollution level. Furthermore, oxygen consumption rate measurement not only shows metabolic rate, but it also provides some clues for stress conditions in fish (Palanivelu et al., 2005).

In our experiment oxygen consumption rate of zebra cichlids were measured by respirometry in laboratory condition. Respirometry gives reliable and quantitative values. For example, how rapidly energy is wasted and oxygen is used by fish. The metabolic rate of the zebra cichlids in each chamber were measured as rates of O_2 uptake. Standard metabolic rate (SMR) and routine metabolic rate (RMR) of each fish were calculated by the system. Standard (basal, maintenance or resting) metabolism is known as minimum metabolic rate. The SMR is measured in organisms that are resting, fasting, and non-stressed period in a post-absorptive state. SMR should be calculated when fish are completely quiescent state in which they are consuming no energy for every metabolic activity such as; food uptake, reproduction, growth or struggle. RMR is the rate including normal activity. Fish represent generally quiescent state but modaretely active. Fish are fasting, non-streesed, modaretely active period in a post-absorptive state.

The behaviors of organisms are known as physical reactions shown against outside world. This physical reactions are controlled by nervous and endocrine systems. If the pollutants enter the water body; respiration, feeding, growth, reproduction, mucus secretion and survival rates of fish are affected negatively because fish are very sensitive to the environmental pollutants (Kane et al., 2005). In the past few years, mortality of fish has increased in various lakes, streams and ponds of around the world due to pesticides leak. Pesticide pollution can cause serious behavioral abnormalities due to physiological damages so fish are used as behavioral models because effect of many ecological stressors are easily observed and quantified in a controlled settings. Thus, they are ideal sentinels for behavioral tests of different stressors and toxic pollutants due to direct contact with the aquatic system (Dube and Hosetti, 2010; Palanivelu et al., 2005). Scott and Sloman (2004) illusrated physiological and environmental factors influence the behavior of fish (Figure 1.4).

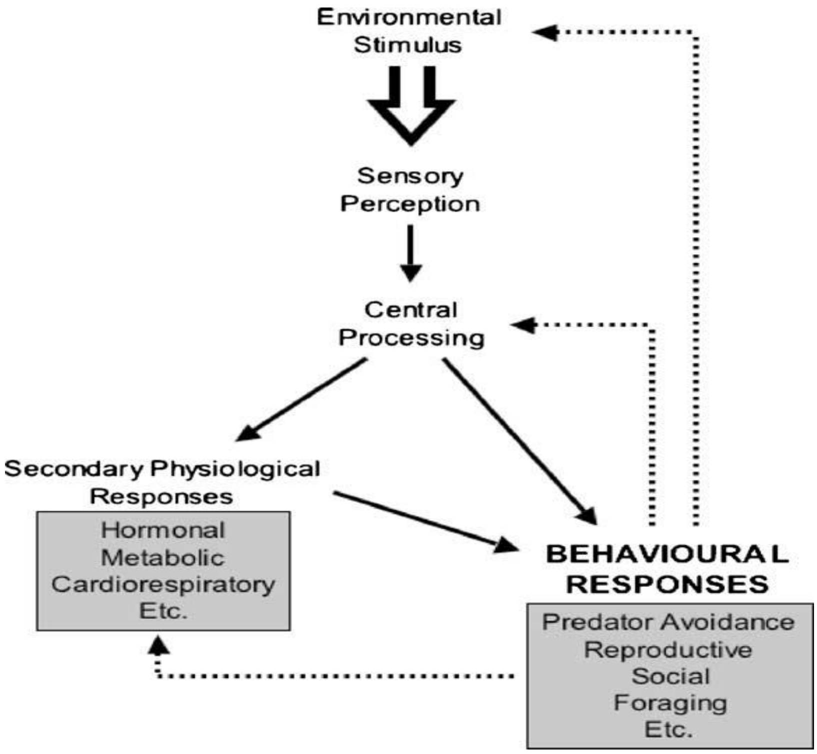


Figure 1.4. Effect of many environmental stimulus on fish behavior activity.

Monitoring and controlling of aquatic ecosystems are important because fish are still important source of protein and lipid for humans. For example; behavioral dysfunctions like less locomotor activity, loss of equilibrium, erractic swimming, upturned abdomen, air breathing, jumping give some clues about water quality and give some information about consumption of fish is healthy or not because maximum toxic substance accumulates in human body due to last link of food chain created by human. Hence, abnormal actions are accepted as alarm signals to protect human health.

Behavioral abnormalities and oxygen consumption rates of various fish species under exposure of pesticides have been reported by several researchers (Table 1.2).

Table 1.2. Summary of pesticides effects on fish oxygen consumption rates (OCR) and behavioral responses. [1]Organophosphate insecticide, [2] Pyrthroid insecticide, [3]Piscicide.

Pesticides	Species	Concentration	Effect on oxygen consumption rate	Effect on behavior	References
2,4-D	Pearl eartheater (*Geophagus brasiliensis)*	1, 5, 10, 25, 40 mg/l	OCR increased 2, 6 and 38% when exposed to 1, 5 and 10 mg/l then OCR decreased 26.22 and 59.01% to 25.0 and 40.0 mg/l	Not studied	Barbieri, 2008
CF-E	Mosquito fish (*Gambusia affinis)*	400, 500 µg/l	Not studied	Erractic swimming with jerky, loss of equilibrium, excess mucus secretion and hyperactivity	Rao et al., 2005
Methyl parathion[1]	Nile tilapia (*Oreochromis niloticus)*	0, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0 mg/l	OCR increased 4, 20 and 38.4% at 0.1, 0.5 and 1.0 mg/l, decreased 33.6, 35.2 and 42.4 % at 2.5, 5.0 and 10 mg/l and ammonium excretion increased 50, 150, 175, 208, 200 and 175%	Not studied	Barbieri et al., 2010
DDVP[1]	Grass carp (*Ctenopharyngodon idella)*	1.31, 1.09, 0.98, 0.65 mg/l	OCR decreased related to gill demage and AChE enzyme activity is inhibited	Not studied	Tilak and Kumari, 2008
Profenefos[1]	Common Carp (*Cyprinus carpio*)	0, 15.6, 31.2, 62.5, 125.0, 250.0 µg/l	Not studied	Loss of balance, staying motionless, lying laterally at the bottom, swimming in spiral fashion with jerks, revolving in water, opened mouth and rapid opercular movements	Ismail et al., 2009
Malathion[1]	Carp *(Labeo rohita)*	0.9, 9.0 µl/l	OCR increased at first 3 days at 9.0 µl/l then started to decrease. OCR decreased on day 1 and increased on day 5 and 10, decreased again on day 15 at 0.9 µl/l	Irregular, erractic and darting swimming, hyper excitability, loss of equilibrium, inactivation of AChE activity and hyper stimulation	Patil and David, 2008
Cypermethrin and Fenvalerate[2]	Sykess (Garra mullya)	0.06, 0,018,0.18 mg/l cypermethrin and 0.086, 0.026, 0.26 mg/l fenvalerate	OCR increased 36.7, 61.6 and 17.17% after 2,4 and 8h at 0.18 mg/l cypermethrin. OCR increased 26.38 and 52.5% after 2 and 4h at 0.26 mg/l fenvalerate	Not studied	Joshi and Kulkarni, 2007
Rotenone[3]	Rainbow Trout (*Oncorhynchus mykiss*)	1.5, 2.5, 3.0, 3.5 µg/l	Active and standard metabolic rate were 216 and 217 mg/O_2/kg/h for the control fish. 117 and 111 mgO_2/kg/h for the exposed fish	Loss of equilibrium, erractic swimming, sinking to the aquarium bottom and slow opercular movements in there and some fish died within 24 h at the highest concentration	Cheng and Farrell, 2006

In this experiment zebra cichlids (*Amatitlania nigrofasciata*) were used for experimental model. Zebra cichlid described by Albert Günther in 1867 belongs to family Cichlidae and endemic species of Central America. Zebra cichlid has vertical black and white stripes on their bodies, and resemble to that of a prisoner's uniform (Figure 1.5). They are found in a wide variety of environments such as, flowing waters of rivers or ponds and lakes. Zebra cichlid is an omnivore that consumes wide variety of food including brine shrimp, worms, insects, small fish, algae and some aquatic plants. Zebra cichlid is one of the most easily breeding species. Parental care is highly developed and continues for several weeks in this species. Natural habitats of the zebra cichlid, the pH is found to range from 6-8 and water temperature ranged from 23-29°C. Zebra cichlid is popular aquarium fish and also popular subject of many studies for fish behavior since discovered.

Figure 1.5. Appearance of male zebra cichlid fish.

The objective of this study was to investigate the effects of sublethal concentration of three pesticides (CF-E, 2,4-D and MMM) on oxygen consumption rates and behavioral responses of zebra cichlids under identical conditions. The results were analysed to determine if the oxygen consumption rate and behavioral response is changed with the increased amount of pesticides.

2. HISTORY AND DEFINITON OF THREE PESTICIDES

2.1. Organophosphate Chlorpyriphos-Ethyl (CF-E)

Organophosphate (OP) is a biodegradable organic compound which contains any esters of phosphoric acid or its derivatives and used as a pesticides, herbicides and nerve gases (Maroni et al., 2000; Fulton and Key, 2001). They are used mainly for pest controlling like an alternative to chlorinated hydrocarbons that persist in the environment and remain for a long time. Half of all pesticides used around the world contain OP that was developed in the 1930s (Fulton and Key, 2001). In 1932, German chemist Willy Lange described effect of OP on nervous system. Then this discovery inspired German chemist Gerhard Schrader, who used this compound like a pesticide. Since then, many pesticides, herbicides, fungicides and nerve gases have been derived from OP.

In 1999, Environmental Protection Agency issued that, OP pesticides are cheaper than others, broad spectrum synthetic chemical and also biodegradability is high. Therefore, OP production has been increased. In 2008, the National Marine Fisheries Service published a report and explained a plan for protecting Pacific salmon and steelhead against three toxic OP pesticides which were the chlorpyriphos, diazinon, and malathion and some rules imposed for example; aerial applications are prohibited within 30 meter of salmon and steelhead water, ground application prevented within 15 meter, buffer zone created about 0.6 meter is banned for agriculture and lastly, if wind speeds exceed to 16 km, application of these three pesticide can not perform. Due to OP pollutants, aquatic enviroments are at risk. Alteration in the chemical combination of water, generally affects the behaviors, metabolism, physiology and oxygen consumptions of fish. Therefore, monitoring of these pesticides are very important because they are dangerous for aquatic biota (Halappa and David, 2009).

Most common used OP contains chlorpyrifos, dichlorvos, parathion methyl, malathion, azinphos methyl, diazinon, phosmet, fenitrothion and tetrachlorvinphos. CF-E is a broad spectrum synthetic OP pesticide (Fulton and Key, 2001). They have been used at homes for household pests, mosquitoes and sometimes are used to control ectoparasites on animals. Like these, in agriculture CF-E inhibits acetylcholinesterase (AChE) enzyme activity and prevents destructive pests, effecting central nervous system, peripheral nervous system, respiratory system, and plasma cholinesterase (Oruç, 2009). Oruç (2010) found that LC_{50} values of CF-E on juvenile and adult nile tilapia (*O. niloticus*) as 0.99 mg/l and 0.154 mg/l, respectively. Kavitha and Rao (2008) determined that LC_{50} values of CF-E in mosquito fish (*G. affinis*) was 0.297 mg/l. CF-E is commonly used in Bolu region in agriculture to control pests, such as potato beetles, moths, european mole cricket, ants, cutworms, corn roorworms, flea beetles, hall scales, lice and etc. This pests damage fruit trees, vegetables, cereals, tobacco, corn, sunflowers, olive and vine.

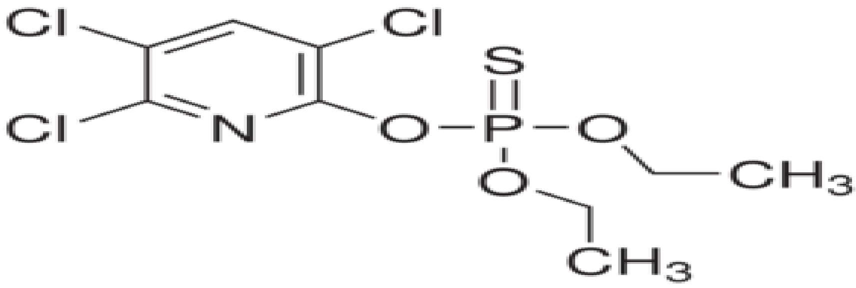
2.2. Physicochemical characteristic of CF-E

Korban was supplied local shop and was made by Koruma Klor Alkali San. ve Tic. A.Ş., İzmit/TURKEY.

Active ingredient: Chlorpyriphos-Ethyl

IUPAC name: *O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate*

Chemical structure:



Molecular formula: $C_9H_{11}Cl_3NO_3PS$

Molecular mass: 350.58

Melting point: 45.5-46.5°C

CAS number: 39475-55-3

Solubility in water: 0.0002%

Vapor pressure: 0.00002 mmHg

2.3. 2,4-Dichlorophenoxyacetic acid (2,4-D)

2,4-D is a chlorinated phenoxy compound which is a popular systemic herbicide. This herbicide was developed during World War II at British Rothamsted Experimental Station by Judah Hirsch Quastel and still in use today. It is easy to manufacture and that kills a lot of broadleaf plants (dicots) and grasses (monocots) such as dandelions, clover, thistles but generally has no effect on fruit trees, vegetables and crops. If 2,4-D absorbed by broadleaf plant which is translocate inside of plant and accumulate at the growth region (root and shoot apex) where it inhibits growth. 2,4-D's low cost has allowed to continue its usage today and now it is known as one of the most widely used herbicides in Turkey and in the world (Wauchope et al., 1992; Sarikaya and Yılmaz, 2003).

According to World Health Organization, 2,4-D is a Class II moderately hazardous pesticide. It's also an invaluable product in protecting public safety and infrastructure. Some formulations of 2,4-D are highly toxic to fish and some are less toxic. For example the LC_{50} in cutthroat trout (*Oncorhynchus clarki)* ranges between 1.0 mg/l to 100 mg/l (Stevens et al., 1991), for adult nile tilapia (*O. niloticus*) LC_{50} is observed 86.90 mg/l for 48h. In green sun fish (*Lepomis cyanellus*) 110 mg/l of 2,4-D does not affect the fish’s behavior (Gomez et al., 1998). Sarıkaya et al., (2002) determined the LC_{50} value of 2,4-D on tench (*Tinca tinca)* as 41.76 mg/l.

Like CF-E, 2,4-D were used too much in Bolu region and Turkey to control broadleaf plant such as earth smoke, cowherb, henbit, mallow, chickweed, spurges, knotgrass and etc.

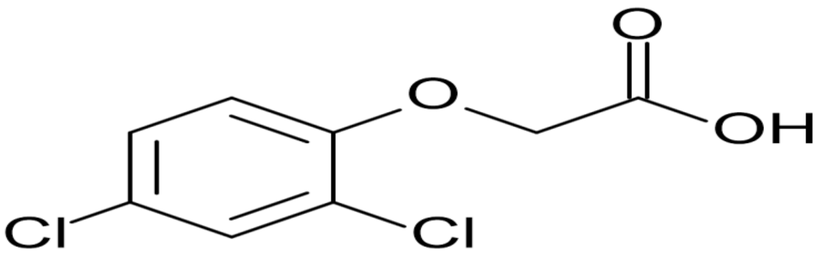
2.4. Physicochemical characteristic of 2,4-D

2,4-D was supplied from Plant Laboratory and was made by Sigma-Aldrich Co.P.O. Box 14508 St. Louis, MO 63178 USA

Active ingredient: 2,4-dichlorophenoxy acetic acid

IUPAC name: *(2,4-dichlorophenoxy) acetic acid*

Chemical structure:



Molecular formula: $C_8H_6Cl_2O_3$

Molecular mass: 221.0

Melting point: 136-140°C

CAS number: 94-75-7

Solubility in water : 0.05%

Vapor pressure : 0.000008 mmHg

2.5. Metalaxyl-M + Mancozeb (MMM)

MMM is a mixture of two systemic fungicide, which are used to kill or inhibit fungi or fungal spores. Fungi can cause severe damage in agriculture, causing in losses of crop and profit. MMM mixture uses for the control of a range of foliar diseases on potatoes, tomatoes, onions, brassicas, asparagus, grapes, cucurbits. MMM shows protective action and curative action, being absorbed through the leaves, stems and roots (Gullino et al., 2010). Mancozeb is fungicidal when contaminates to air. It is converted to an isothiocyanate and isothiocyanate inhibits the sulphahydral groups of fungi enzymes also some times mancozeb’s metal ion exchanges with fungi enzyme ion so activity of enzyme breaks down (Hamilton, 1995). Besides, Metalaxyl inactivates protein synthesis, growth and reproduction in fungi. This pesticide is rapidly uptaken (feature of metalaxyl) about 30 second by plant and translocated towards growing tips, helps to protect plant from oomycete family diseases for long period (feature of Mancozeb). Thus, plants are covered by MMM. MMM is much more toxic to warmwater fish than to coldwater fish. For example; LC_{50} are 24.0 mg/l in carp, 1.85 mg/l in rainbow trout, 2.2 mg/l in guppy for 48h (Hejduk and Svobodova., 1980). Atamanalp and Yanık (2003) determined the LC_{50} value of MMM on rainbow trout *(O. mykiss)* as 2.2 mg/l. MMM is a broad spectrum fungicide that controls destructive organisims such as, potato late blight, water mould, downy mildew and downy mildews on various vegetables, black rot of vines, damping off and etc.

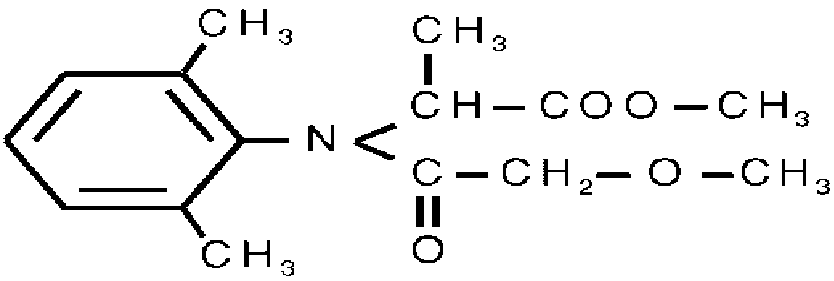
2.6. Physicochemical characteristic of MMM

Ridomil Gold MZ 72 was supplied local shop and was made by Koruma Klor Alkali San. ve Tic. A.Ş., İzmit/TURKEY.

Active ingredient: Metalaxyl- M

IUPAC name: *(Methyl N-(2-methoxyacetyl)-N-(2,6-xylyl)-DL-alaninate)*

Chemical structure:



Molecular Formula: $C_{15}H_{21}NO_4$

Molecular mass: 279.33

Melting Point: -38.7°C

CAS Number: 70630-17-0

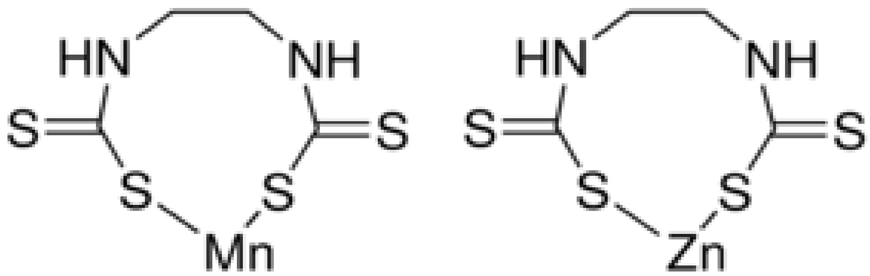
Solubility in water : 26,000 mg/l at 20°C

Vapor pressure : 3.3 mPa at 25° C

Active ingredient: Mancozeb

IUPAC name: *(Manganese ethylenebis(dithiocarbamate) (polymeric) complex with zinc salts)*

Chemical structure:



Molecular formula: $C_4H_6MnN_2S_4$;$C_4H_6ZnN_2S_4$

Molecular mass: 265.28;275.72

Melting point**:** 192-194ºC

CAS number: 8018-01-7 (8065-67-6)

Solubility in water : 6.20 mg/l

Vapor pressure : 0.013 mPa at 25°C

3. MATERIALS AND METHODS

3.1. Animal preparation

Adult male zebra cichlid fish (n=72; 11±0.5cm; 50±2.3g; Mean±SD) were reproduced in the Fish Biology Laboratory at Abant Izzet Baysal University, Bolu (Figure 3.1). Zebra cichlids were held in recirculation system (74x24x40cm/glass aquariums) for acclimation to the laboratory condition for 48h before the experiment (Figure 3.2). Acclimation units were filled with tap water. During acclimation period, each day about quater liters of water was removed in each aquarium. Temperature was stabilized to 24±1°C. The fish were kept normal day light and fed by commercial food. However feeding was stopped 48 hours ago, to prevent the accumulation of organic matters inside of the respirometry chambers also they were not fed throughout the experiment.

Figure 3.1. Fish breeding units in the Fish Biology Laboratory.

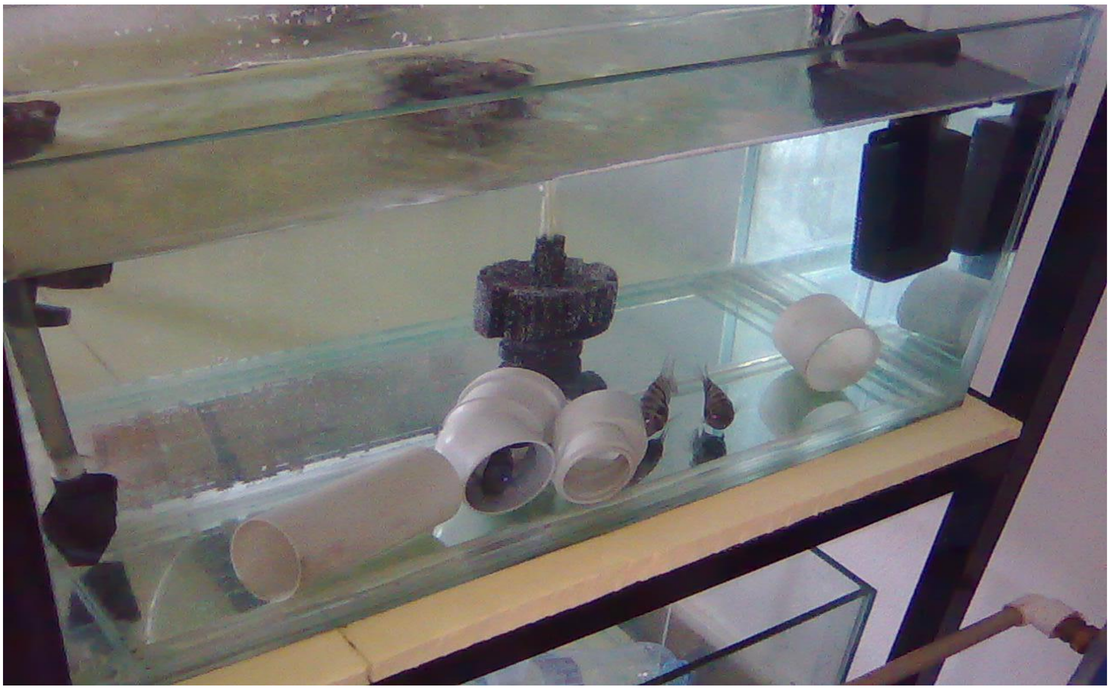


Figure 3.2. Acclimation units; during the acclimation period zebra fish were kept in these units.

3.2. Experimental design

In this study, after acclimation period, fish were exposed to different sublethal concentration of CF-E (0, 0.004, 0.008, 0.016, 0.032, 0.05 mg/l), 2,4-D (0, 2.17, 4.35, 8.69, 17.38, 28.97 mg/l) and MMM (0, 0.16, 0.32, 0.64, 1.0, 1.6 mg/l) to determine oxygen consumption rates and abnormal behavioral activities under 24±1°C and for 96h (Table 3.1). Oxygen consumption rate, behavioral responses and physiological deformities were studied in the experimental periods.

Table 3.1. Details of tested pesticides.

Commercial name	Active ingredient	Designation	Chemical name	Grade (%)	Supplier
Korban %25 WP	Chlorpyriphos-Ethyl	CF-E	O,O-diethyl-O-(3,5,6-trichlor-2-pyridyl) phosphorothioate	25%	Koruma Klor Alkali A.Ş. Ltd
Ridomil MZ %68	Metalaxyl-M+ Mancozeb	MMM	Methyl *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-D-alaninate + Zinc ion and manganese-ethylene-bis-dithio carbamate	64% + 4%	Koruma Klor Alkali A.Ş. Ltd
2,4-Dichlorophenoxyacetic acid %98	2,4-Dichlorophenoxyacetic acid	2,4-D	2',6'-Diethyl N-butoxymethyl1-2 chloroacetanilide	98%	Sigma-Aldrich Co.LLC

3.3. Oxygen consumption

Oxygen consumption rates were found by 3-chambered, computer controlled, intermittent flow respirometery (DAQ-PAC-G4S three-chamber intermittent flow respirometry system and sofware (Autoresp®) from Loligo Systems, Tjele/Denmark) (Figure 3.3). Reservoir of respirometery (150 l) was designed as 70x80x30 cm (Figure 3.4). Respirometery included 3 acrylic chambers. In the beginnig of each experiment, respirometery was cleaned and filled with tap water (Figure 3.5). Temperature was stabilized at 24±1°C. Before trial, fish were put into the chambers and waited 2h for handling stress. Before starting the experiment, chambers were closed without air bubbles and using galvanic probes oxygen consumption rate was measured, displayed and recorded in system software. Oxygen consumption rate values were taken sequentially the fish inside of the chamber every 10th min over periods of hours or days. Meanwhile the expriment respirometery tank was covered by a black curtain to prevent burst activity of fish (Figure 3.6). While the experiment, water in the respirometery was aerated with two air stones.

The working principle of the system consists of three phases; flush period, wait period, measuring period. First phase is flush period and this period is 240 seconds. Along this period recirculation pumps do not work but flush pumps are active and send water into the chambers so oxygen content increases. Second phase is waiting period, 300 seconds is wasted in this phase. During the waiting period recirculation pumps are active. Last phase is measuring period, fish respiration rate is measured by oxygen sensors from the reduction in oxygen content and recorded by system (Figure 3.7). Dissolved O_2 reduction is originated by fish consumption. Again the measuring period is followed by a flush period.

Advantages of this system are several; this measurement provides high resolution, metabolic wastes (CO_2 and NH_3) do not accumulate in the system, dissolved O_2 level does not decrease to a hypoxic level in the chambers, for long term measurements (24, 48, 72, 96 h) system is very suitable and environmental factors are kept under control such as, temperature, oxygen saturation and time.

The oxygen consumption ($mgO_2/kg/h$) of fish was calculated using the formula by computer software (AutoResp):

$MO_2 = \Delta C_W O_2 / \Delta t x VOL_{resp} / M$

$\Delta C_W O_2$= change in water oxygen concentration (mgO_2/l)

Δt= period of time (h)

VOL_{resp} = volume of respirometer’s chamber (l)

M = the mass of the fish (kg)

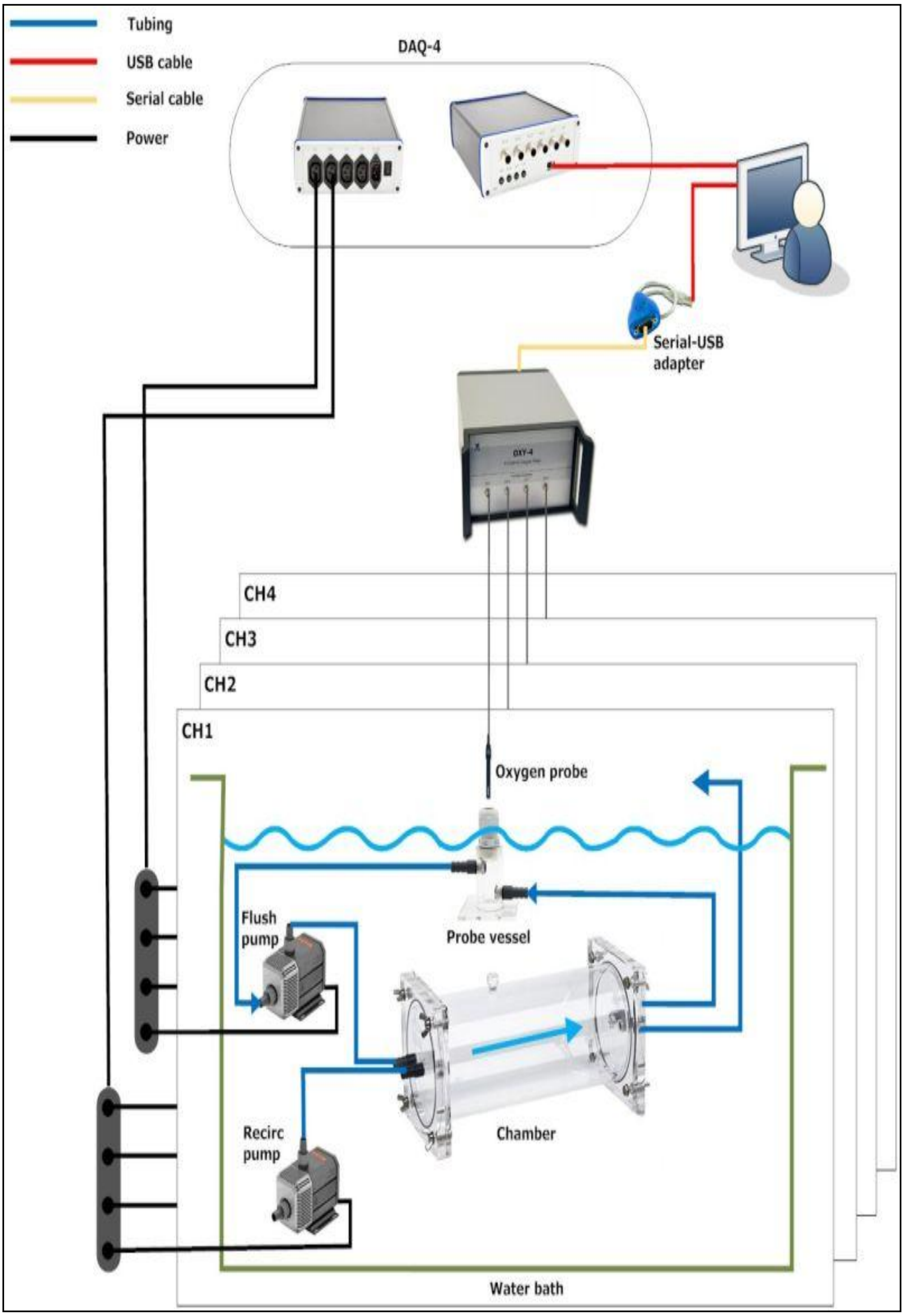


Figure 3.3. Schematic of intermittent flow through computer controlled respirometry.

Figure 3.4. Structure of respirometry tank with three chambers.

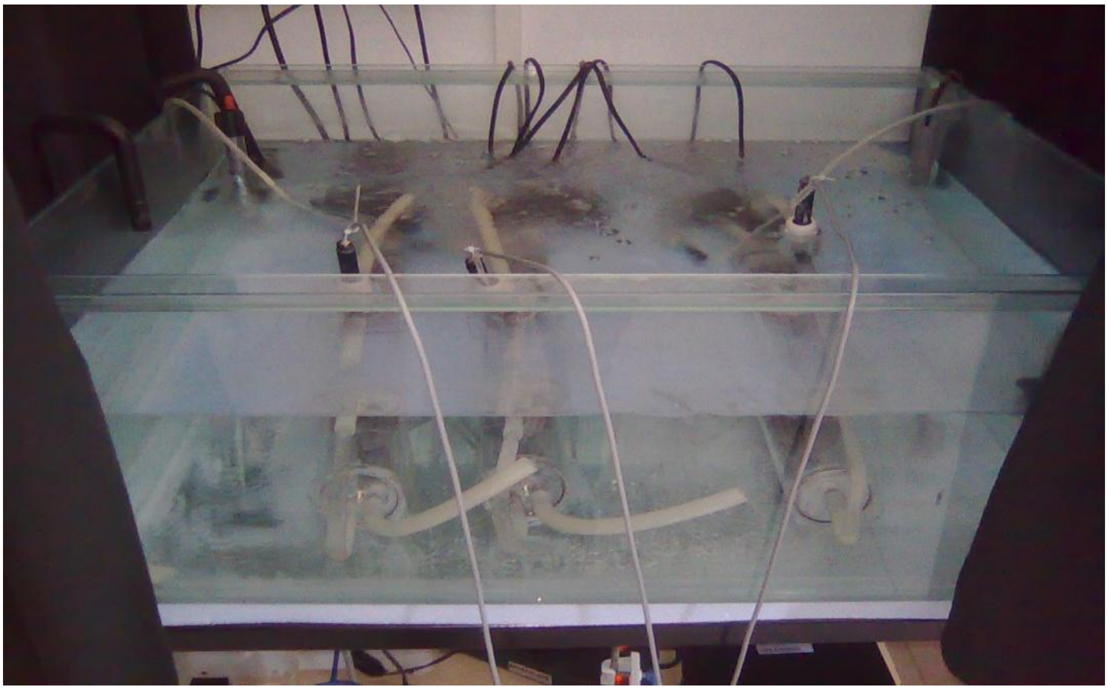


Figure 3.5. Respirometry setting after filling the tap water.

Figure 3.6. The tank of the respirometry was covered by black curtain to prevent burst activity during the experimental period.

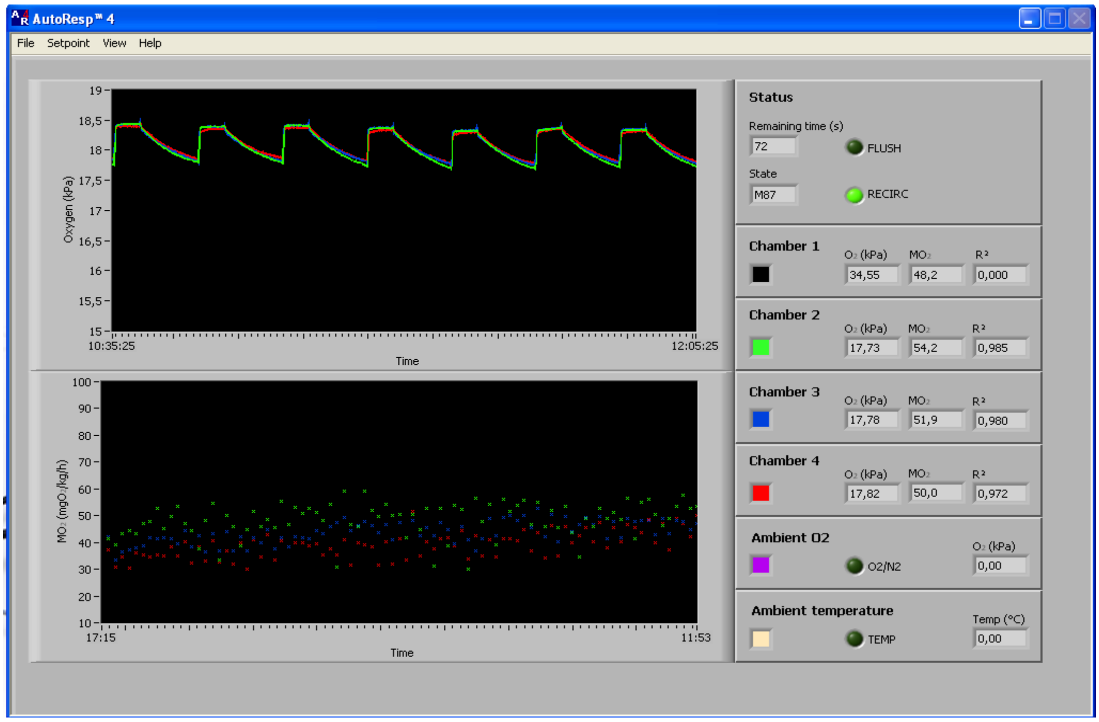


Figure 3.7. Screen view of AutoResp.

3.4. Water quality

Temperature, pH, dissolved oxygen, total hardness, electrical conductivity and alkalinity were determined and showed (Table 3.2). Temperature and dissolved oxygen were determined using the Dissolved Oxygen Meter (Model YSI 5000), pH was determined using the pH meter (Model Orion 290A) and electrical conductivity was determined conductivity meter (WTW Inolab Cond 730). Total alkalinity (Hach®) and total hardness (Aquadur®) were determined by water quality test strips.

Table 3.2. The physicochemical characteristics of tap water.

Temperature (°C)	24.0 ±1.0
Ph	7.1±0.1
Dissolved Oxygen (mg/l)	7.4±0.2
Total Hardness (mol/m^3) (25°d) (Ca^{2+} Mg^{2+})	> 4.5
Electrical Conductivity (µs/cm at 20°C)	629
Total Alkalinity (mg/l $CaCO_3$)	240

3.5. Statistical Analysis

Using extraction method SMR and RMR were statistically designated and this method commonly referred as Fourth-Spread method in Excel (Microsoft Office 2010). Boundaries of each of the quartiles in each raw data were calculated. Highest and lowest extreme values were removed and lower and upper boundries were set. RMR was taken upper part of boundry due to increased oxygen consumption and lower boundry was taken by SMR due to low oxygen consumption (Turker, 2011). All statistical tests were performed with SPSS software (Version 15, SPSS Inc., Chicago, IL, USA) and in all statistical tests $P<0.05$ was taken as the level of significance. RMR and SMR values were analysed with one-way ANOVA and Post Hoc comparisons of each concentration with control were performed with Dunnet's test.

4. RESULTS

4.1. Oxygen consumption Rates

RMR and SMR of male adult zebra cichlid fish were measured by intermittent flow through computer controlled respirometry with a high resolution in this experiment. Higher and lower extreme oxygen consumption values could be identified and removed from further analyses according to outlier detection method (Turker, 2011). Oxygen consumption rates were determined under five different sublethal concentrations of pesticides; (CF-E; 0, 0.004, 0.008, 0.016, 0.032, 0.05 mg/l, 2,4-D; 0, 2.17, 4.35, 8.69, 17.38, 28.97 mg/l and MMM; 0, 0.16, 0.32, 0.64, 1.0, 1.6 mg/l) were tested at 24±1°C for 96h.

RMR and SMR of control and exposed fish groups are presented with standard differences in (Table 4.1) under different sublethal concentrations of CF-E. The mean RMR and SMR in control fish was 77.4±7.8 and 26.6±6.8 mgO_2/kg/h±SE, respectively. The data indicates that in fish exposed to sublethal concentrations of CF-E, oxygen consumption was increased from 0.004 to 0.032 mg/l. RMR (131.8±8.6) and SMR (63.1±7.8) reached to highest values at 0.032 mg/l CF-E. RMR and SMR increased 70% and 137% at 0.032 mg/l according to control values

(Figure 4.1). However, exposure to 0.05 mg/l caused a decrease, the mean RMR and SMR was 83.1±8.1 and 45.7±2.2 mgO_2/kg/h±SE, respectively.

Table 4.1. The means (mgO_2/kg/h±SE) of RMR and SMR at under different sublethal concentrations of CF-E. Means±SE for metabolic rates in a column followed by the same letters are not significantly different (P<0.05) from each other according to Dunnet's test. ''n'' represents number of measurements along experimental period (96h).

Weight (g±SE)	Concentrations of CF-E (mg/l)	RMR±SE	n	SMR±SE	n
53.3±3.0	0	77.4±1.4a	31	26.6±0.7a	94
52.3±1.9	0.004	80.4±0.9a	28	42.1±0.4b	260
51.7±0.4	0.008	77.6±0.9a	37	42.8±0.3b	291
51.1 ±2.8	0.016	106.3±0.7b	69	64.4±0.5b	145
51.6±0.8	0.032	131.8±1.7b	25	63.1±0.5b	245
50.7±1.4	0.05	83.1±8.1b	101	45.7±2.2b	29

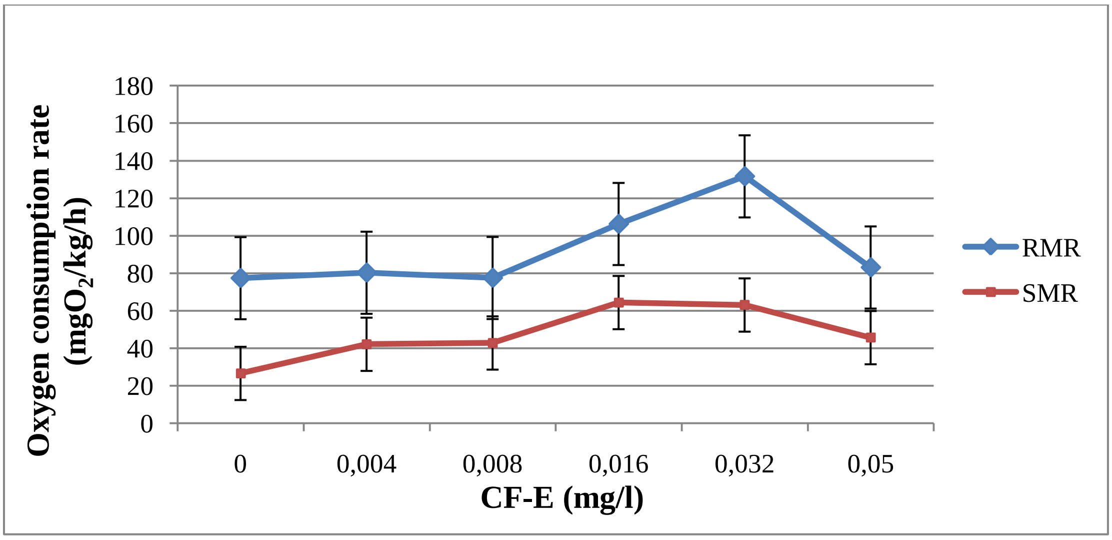


Figure 4.1. Relationship between CF-E concentrations and RMR and SMR after elimination of the outliers.

The result of 2,4-D on oxygen consumption rates with standard differences between control and exposed fish was detailed (Table 4.2). The data showed that in fish exposed to sublethal concentrations of 2,4-D, oxygen consumption was increased from 2.17 to 17.38 mg/l. RMR (136.7±12.3) and SMR (79.8±8.0) reached to highest values at 8.69 mg/l 2,4-D. RMR and SMR increased 76% and 200% at 8.69 mg/l according to control values (Figure 4.2). However, exposure to 28.97 mg/l caused a decrease, the mean RMR and SMR was 92.9±6.5 and 62.3±3.1 mgO_2/kg/h±SE, respectively.

Table 4.2. The means (mgO_2/kg/h±SE) of RMR and SMR at under different sublethal concentrations of 2,4-D. Means±SE for metabolic rates in a column followed by the same letters are not significantly different (P<0.05) from each other according to Dunnet's test. ''n'' represents number of measurements along experimental period (96h).

Weight (g±SE)	Concentrations of 2,4-D (mg/l)	RMR±SE	n	SMR±SE	n
53.3±3.0	0	77.4±1.4a	31	26.6±0.7a	94
52.2±2.1	2.17	98.5±8.4b	101	63.7±5.6b	128
51.4±1.8	4.35	99.8±0.8b	78	61.1±0.5b	188
51.1 ±1.1	8.69	136.7±1.4b	83	79.8±0.8b	93
51.1±2.1	17.38	120.3±1.1b	64	78.3±0.4b	176
51.8±1.5	28.97	92.9±6.5b	107	62.3±3.1b	35

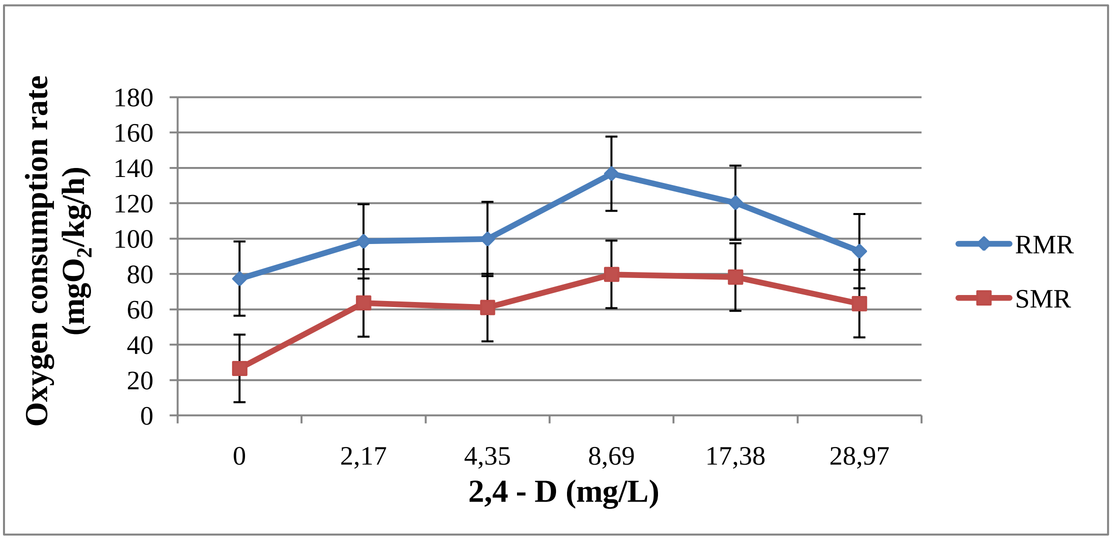


Figure 4.2. Relationship between 2,4-D concentrations and RMR and SMR after elimination of the outliers.

RMR and SMR of zebra cichlids showed with standard differences at under different sublethal concentrations of MMM (Table 4.3). Oxygen consumption was increased from 0.16 to 1.0 mg/l. RMR (124.7±2.3) and SMR (81.8±8.0) reached to highest values at 1.0 mg/l MMM. RMR and SMR increased 61% and 207% at 1.0 mg/l according to control values (Figure 4.3). However, exposure to 1.6 mg/l caused a decrease, the mean RMR and SMR was 85.0±4.6 and 62.0±3.4 $mgO_2/kg/h$±SE, respectively.

Table 4.3. The means ($mgO_2/kg/h$±SE) of RMR and SMR at under different sublethal concentrations of MMM. Means±SE for metabolic rates in a column followed by the same letters are not significantly different (P<0.05) from each other according to Dunnet's test. ''n'' represents number of measurements along experimental period (96h).

Weight (g±SE)	Concentrations of MMM (mg/l)	RMR±SE	n	SMR±SE	n
53.3±3.0	0	77.4±1.4a	31	26.6±0.7a	94
51.7±2.7	0.16	106.8±2.9b	21	37.1±1.2b	66
52.2±3.6	0.32	112.3±1.3b	56	51.3±0.9b	86
50.2 ±2.5	0.64	119.6±0.2b	36	80.6±0.3b	191
50.6±3.0	1.0	124.7±1.3b	35	81.8±0.4b	247
50.1±0.2	1.6	85.0±4.6b	139	62.0±3.4b	40

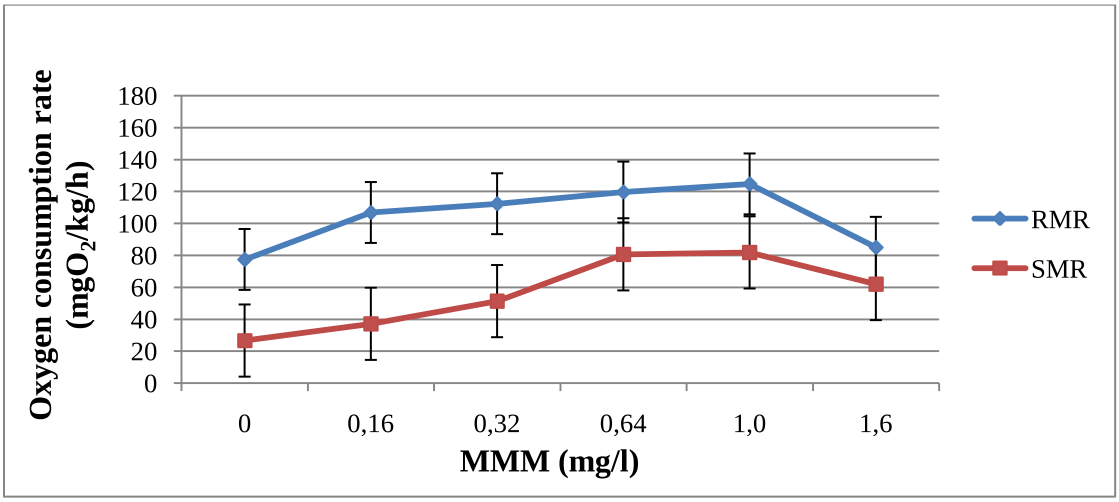


Figure 4.3. Relationship between MMM concentrations and RMR and SMR after elimination of the outliers.

Along the experimental period, oxygen consumption rates from these three pesticides increased linearly as their concentration increased but near the lethal concentration oxygen consumption rates started to decrease. Some measuring intervals were to high or to low as a result of stress. Those values have been automatically excluded from further data by using Fourth-Spread method. Seperation between routin and standard boundries for data with control group, CF-E, 2,4-D and MMM at 24±1°C were seen as a sample (Figure 4.4; 4.5; 4.6 and 4.7). RMR and SMR were calculated for each fish at each different sublethal concentrations and identical conditions after discarding the outliers from the all other data.

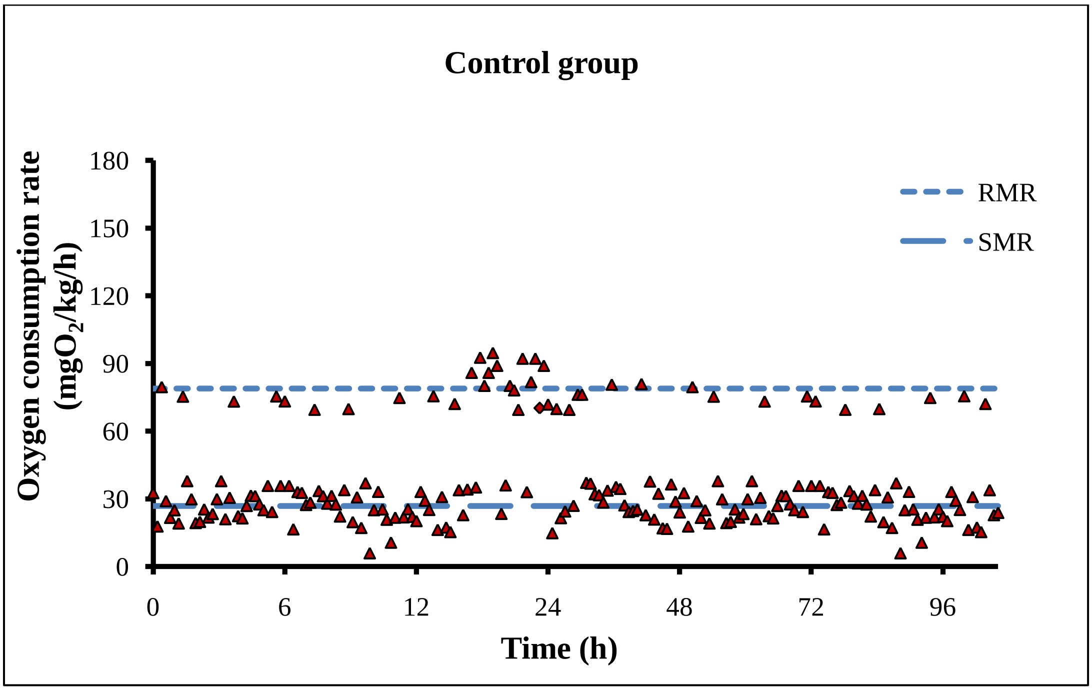


Figure 4.4. Oxygen consumptions of control fish at 24±1°C. The upper and lower horizontal line refer to routin metabolic rates (RMR) and standard metabolic rates (SMR).

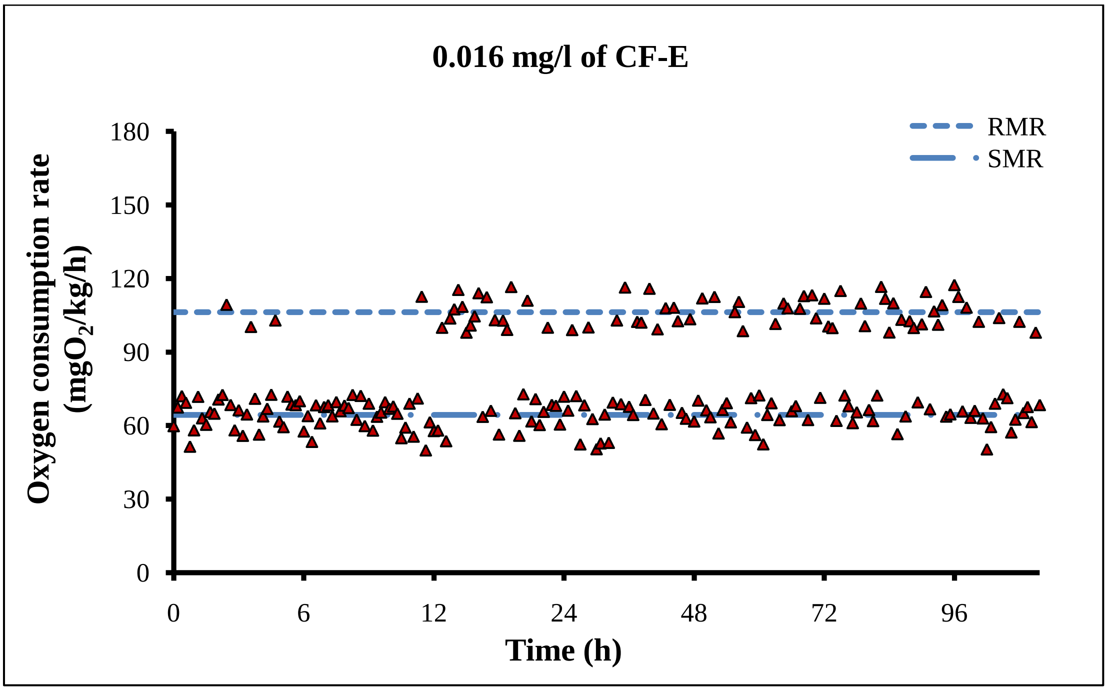


Figure 4.5. Oxygen consumptions of CF-E exposed fish at 24±1°C. The upper and lower horizontal line refer to routin metabolic rates (RMR) and standard metabolic rates (SMR).

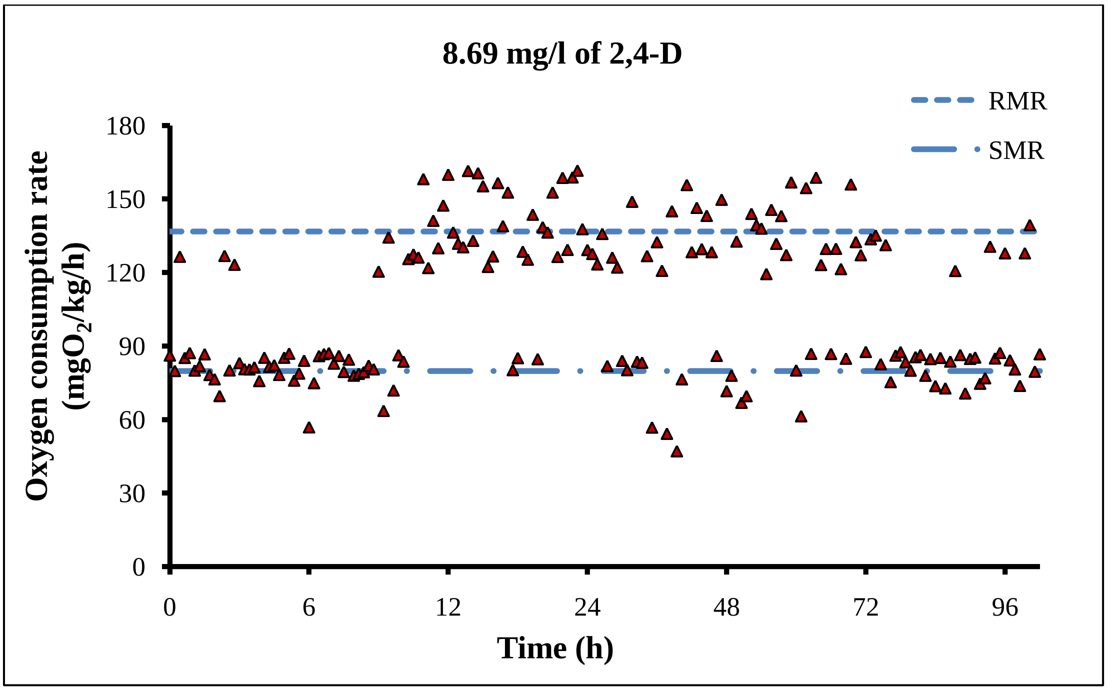


Figure 4.6. Oxygen consumptions of 2,4-D exposed at 24±1°C. The upper and lower horizontal line refer to routin metabolic rates (RMR) and standard metabolic rates (SMR).

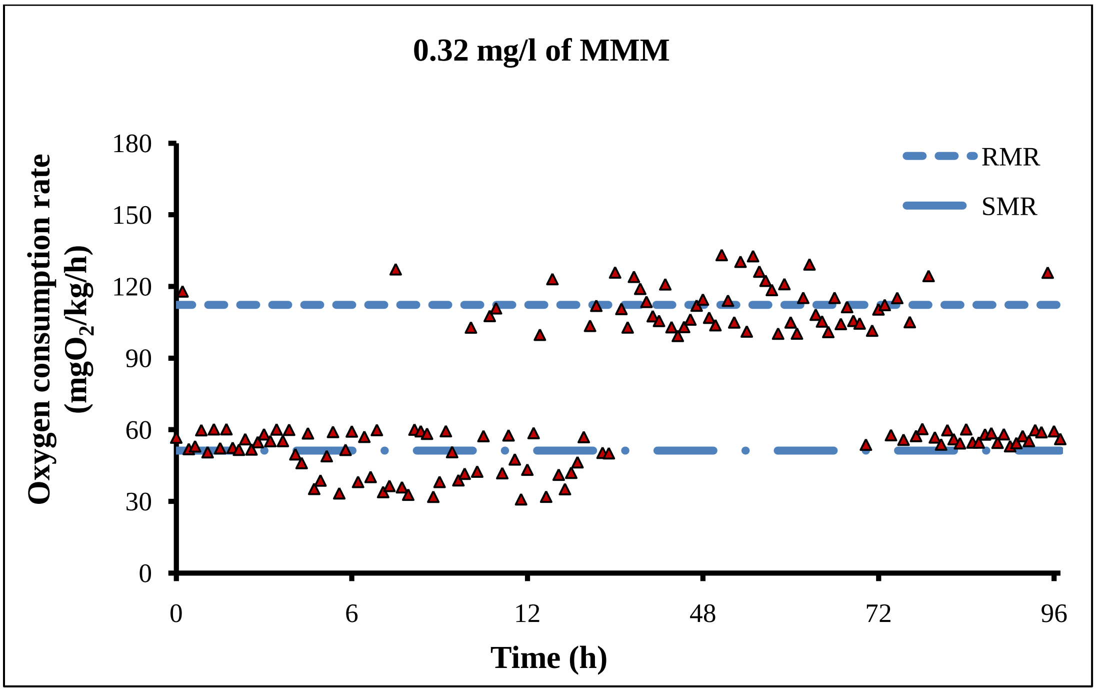


Figure 4.7. Oxygen consumptions of MMM exposed at 24±1°C. The upper and lower horizontal line refer to routin metabolic rates (RMR) and standard metabolic rates (SMR).

4.2. Behavioral Responses of Fish

In this experiment, behavioral responses of zebra cichlids were observed. In contrast to fish in the control group, the toxicosis symptoms of the fish after exposure to three pesticides included rapid gill movement, hyperexcitation, loss of equilibrium, irregular swimming, change in body color and finally sinking at the corner of test tank and staying motionless in there and also severe respiratory stress, rapid opercular activity, excessive mucus secretion and fatigue were observed in the zebra cichlids. Behavioral abnormalities were photographed and shown (Figure 4.8; 4.9 and 4.10).

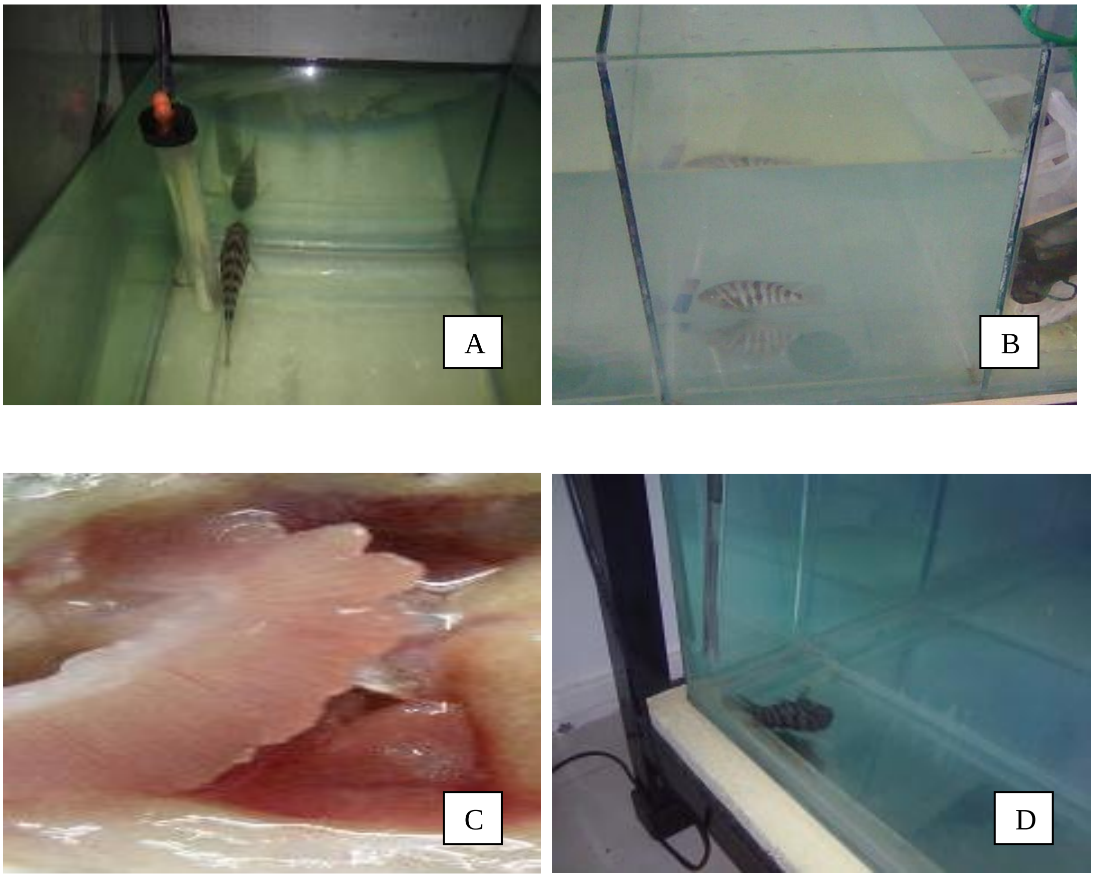


Figure 4.8. Effects of CF-E on the behavioral pattern of zebra cichlids exposed different sublethal concentrations for 96h.

(A); Activeness at 0.016 mg/l of CF-E.

(B); Color change at 0.032 mg/l of CF-E.

(C); Excess mucus secretion at 0.05 mg/l of CF-E.

(D); Lateral side movement at 0.05 mg/l of CF-E.

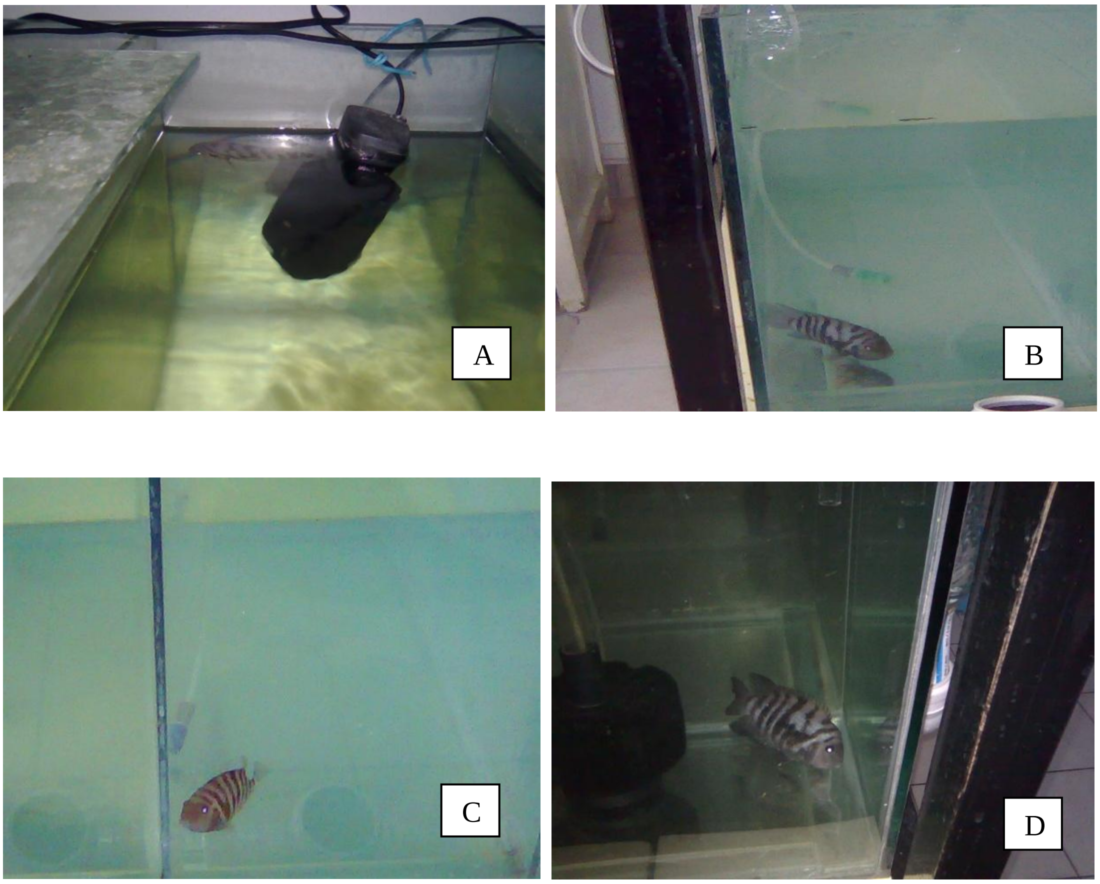


Figure 4.9. Effects of 2,4-D on the behavioral pattern of zebra cichlids exposed different sublethal concentrations for 96h.

(A); Swimming at the water surface at 28.97 mg/l of 2,4-D.

(B); Loss of balance at 17.38 mg/l of 2,4-D.

(C); Color change at 17.38 mg/l of 2,4-D.

(D); Lateral side movement at 28.97 mg/l of 2,4-D.

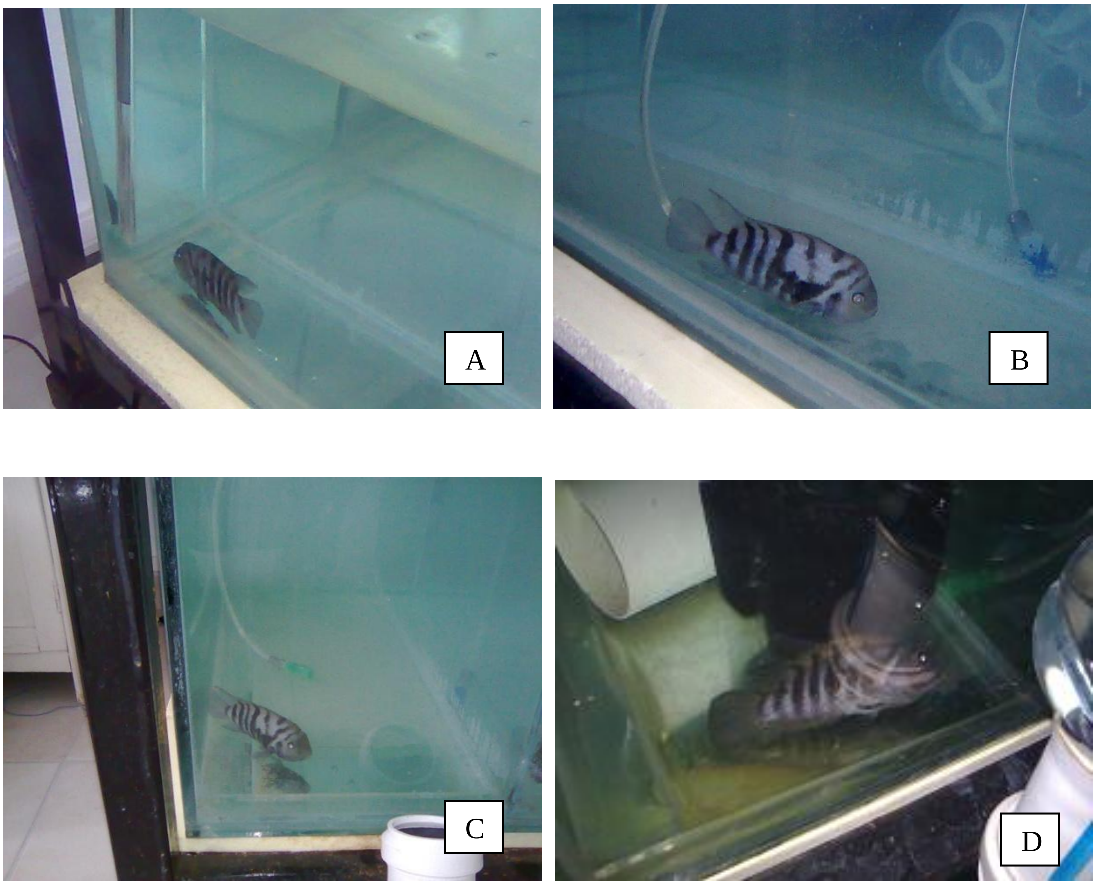


Figure 4.10. Effects of MMM on the behavioral pattern of zebra cichlids exposed different sublethal concentrations for 96h.

(A); Activeness at 0.64 mg/l of MMM.

(B); Loss of balance at 1.0 mg/l of MMM.

(C); Color change at 1.0 mg/l of MMM.

(D); Lateral side movement at 1.6 mg/l of MMM.

Effect of CF-E on the behavior of zebra cichlids were determined (Table 4.4). 0.004 and 0.008 mg/l of CF-E decreased the opercular ventilation rates but other parameters did not changed. 0.016 mg/l of CF-E decreased the opercular ventilation. Activeness and loss of balance started after 24h. Lateral side movement and color change started 48h and the last behavioral change was the mucus secretion increasing that started at 72h. 0.032 mg/l of CF-E highly impressed the fish behaviors, opercular ventilation decreased 30 beat in a minute. In 15 minutes, activeness started and fish waited motionless along the 96h. Loss of balance and color change started at 60 minutes. Black and white stripes on fish paled. Lateral side movement and mucus secretion started at 24h. Severe effect of CF-E was seen at 0.05 mg/l. Opercular ventilation decreased minimal level because of excess mucus secretion. Behavioral abnormalities were obviously seen. Activeness was the first symptom and started at the beginning of experiment. Loss of balance and color change started at 30 minutes and fish lost natural body color. Lateral side movement and excess mucus secretion started 60 minutes. Mostly, fish stayed motionless but when the fish swam, fish moved laterally.

Effect of 2,4-D on the fish behavior were presented (Table 4.5). 2.17, 4.35 and 8.69 mg/l of 2,4-D decreased the opercular ventilation rates but other parameters did not changed. 17.38 mg/l of 2,4-D decreased the opercular ventilation. Activeness, loss of balance and color change started at 24h. Lateral side movement and mucus secretion started at 48h. 28.97 mg/l of 2,4-D highly impressed the fish behaviors, opercular ventilation decreased 55 beat in a minute. In 60 minutes, activeness started and fish waited motionless along the 96h. Loss of balance and color change started at 60 minutes. Black and white stripes on fish paled. Lateral side movement and mucus secretion started at 24h.

Effect of MMM on the behavior of zebra cichlids were determined (Table 4.6). 0.16 and 0.32 mg/l of MMM decreased the opercular ventilation rates but other parameters did not changed. 0.64 mg/l of MMM decreased the opercular ventilation.

Activeness, loss of balance, lateral side movement and color change started at 48h. Mucus secretion increased little at 72h. 1.0 mg/l of MMM highly affected fish behavior, opercular ventilation decreased 70 beat in a minute after 96h. Activeness started at 24h and fish waited motionless along the 96h and also loss of balance and color change started at 24h. Lateral side movement and mucus secretion started at 48h. Black and white stripes on fish paled. Severe effect of MMM was seen at 1.6 mg/l. Opercular ventilation decreased minimal level because of excess mucus secretion. Behavioral abnormalities were obviously seen. Activeness and loss of balance were the first symptoms and started at the 60 minutes. Lateral side movement, excess mucus secretion and color change started at 48h and fish lost natural body color.

In this experiment, organophosphate CF-E showed very high toxicity, but MMM showed intermediate and 2,4-D showed low toxicity. For all exposure times, the rate of opercular activity decreased with increasing pesticides concentration because pesticides caused respiratory disstress by affecting respiratory centers of brain. Low pesticide concentrations did not changed other parameters. Locomotory behavior was affected negativelly. Especially, motionless reduced the energy consumption so fish survived in the pesticide contaminated water. Loss of balance may be associated with the ionic irregularities. Lateral side movement related to loss of balance. Color change and mucus secretion related to pesticide penetration. After pesticide contamination, natural body color disappeard and fish body had a gray color. Pesticides mainly entered the body from gills so excess amount of mucus secreted by gills and small amount secreted by fish body.

Table 4.4. Impact of CF-E on the behavioral pattern of zebra chiclid exposed different sublethal concentrations for 96h. Opercular ventilation rate is known as opercular beat in a minute* (---: Absent, --+: Mild, -++: Moderate, +++: Severe).

	0.004 mg/l							
Parameters	Control	15 min	30 min	60 min	24 h	48 h	72 h	96 h
Activeness	---	---	---	---	---	---	---	---
Loss of balance	---	---	---	---	---	---	---	---
Lateral side movement	---	---	---	---	---	---	---	---
Opercular ventilation*	113	120	100	104	95	83	80	72
Color change	---	---	---	---	---	---	---	---
Mucus secretion	---	---	---	---	---	---	---	---
	0.008 mg/l							
Activeness	---	---	---	---	---	---	---	---
Loss of balance	---	---	---	---	---	---	---	---
Lateral side movement	---	---	---	---	---	---	---	---
Opercular ventilation*	113	109	107	93	95	87	81	78
Color change	---	---	---	---	---	---	---	---
Mucus secretion	---	---	---	---	---	---	---	---
	0.016 mg/l							
Activeness	---	---	---	---	+--	+--	++-	++-
Loss of balance	---	---	---	---	+--	+--	++-	++-
Lateral side movement	---	---	---	---	---	+--	+--	+--
Opercular ventilation*	113	105	104	87	85	83	77	73
Color change	---	---	---	---	---	+--	+--	+--
Mucus secretion	---	---	---	---	---	---	+--	+--
	0.032 mg/l							
Activeness	---	++-	+++	+++	+++	+++	+++	+++
Loss of balance	---	---	---	+--	+--	++-	++-	+++
Lateral side movement	---	---	---	---	+--	+--	++-	+++
Opercular ventilation*	113	90	88	90	87	44	38	30
Color change	---	---	---	+--	+--	+--	+++	+++
Mucus secretion	---	---	---	---	+--	+--	+--	++-
	0.05 mg/l							
Activeness	---	+++	+++	+++	+++	+++	+++	+++
Loss of balance	---	---	+--	+--	++-	++-	+++	+++
Lateral side movement	---	---	---	+--	+--	++-	+++	+++
Opercular ventilation*	113	74	67	72	38	32	30	28
Color change	---	---	+--	+--	+--	++-	+++	+++
Mucus secretion	---	---	---	+--	+--	++-	+++	+++

Table 4.5. Impact of 2,4-D on the behavioral pattern of zebra chiclid exposed different sublethal concentrations for 96h. Opercular ventilation rate is known as opercular beat in a minute* (---: Absent, --+: Mild, -++: Moderate, +++: Severe).

	2.17 mg/l							
Parameters	Control	15 min	30 min	60 min	24 h	48 h	72 h	96 h
Activeness	---	---	---	---	---	---	---	---
Loss of balance	---	---	---	---	---	---	---	---
Lateral side movement	---	---	---	---	---	---	---	---
Opercular ventilation*	113	80	92	83	78	75	73	74
Color change	---	---	---	---	---	---	---	---
Mucus secretion	---	---	---	---	---	---	---	---

	4.35 mg/l							
Activeness	---	---	---	---	---	---	---	---
Loss of balance	---	---	---	---	---	---	---	---
Lateral side movement	---	---	---	---	---	---	---	---
Opercular ventilation*	113	86	83	81	78	79	77	72
Color change	---	---	---	---	---	---	---	---
Mucus secretion	---	---	---	---	---	---	---	---

	8.69 mg/l							
Activeness	---	---	---	---	---	---	---	---
Loss of balance	---	---	---	---	---	---	---	---
Lateral side movement	---	---	---	---	---	---	---	---
Opercular ventilation*	113	88	82	78	75	73	71	70
Color change	---	---	---	---	---	---	---	---
Mucus secretion	---	---	---	---	---	---	---	---

	17.38 mg/l							
Activeness	---	---	---	---	+--	++-	+++	+++
Loss of balance	---	---	---	---	+--	++-	++-	+++
Lateral side movement	---	---	---	---	---	+--	+--	++-
Opercular ventilation*	113	96	87	81	73	68	66	68
Color change	---	---	---	---	+--	+--	++-	+++
Mucus secretion	---	---	---	---	---	+--	+--	++-

	28.97 mg/l							
Activeness	---	---	---	+--	++-	+++	+++	+++
Loss of balance	---	---	---	+--	+--	++-	++-	+++
Lateral side movement	---	---	---	---	+--	+--	++-	+++
Opercular ventilation*	113	83	92	81	72	63	59	55
Color change	---	---	---	+--	+--	++-	++-	+++
Mucus secretion	---	---	---	---	+--	+--	+--	++-

Table 4.6. Impact of MMM on the behavioral pattern of zebra chiclid exposed different sublethal concentrations for 96h. Opercular ventilation rate is known as opercular beat in a minute* (---: Absent, --+: Mild, -++: Moderate, +++: Severe).

	0.16 mg/l							
Parameters	Control	15 min	30 min	60 min	24 h	48 h	72 h	96 h
Activeness	---	---	---	---	---	---	---	---
Loss of balance	---	---	---	---	---	---	---	---
Lateral side movement	---	---	---	---	---	---	---	---
Opercular ventilation*	113	96	97	88	93	91	90	88
Color change	---	---	---	---	---	---	---	---
Mucus secretion	---	---	---	---	---	---	---	---

	0.32 mg/l							
Activeness	---	---	---	---	---	---	---	---
Loss of balance	---	---	---	---	---	---	---	---
Lateral side movement	---	---	---	---	---	---	---	---
Opercular ventilation*	113	92	87	83	85	79	78	76
Color change	---	---	---	---	---	---	---	---
Mucus secretion	---	---	---	---	---	---	---	---

	0.64 mg/l							
Activeness	---	---	---	---	---	+--	+--	++-
Loss of balance	---	---	---	---	---	+--	+--	++-
Lateral side movement	---	---	---	---	---	+--	+--	+--
Opercular ventilation*	113	86	84	79	81	76	75	72
Color change	---	---	---	---	---	+--	+--	+--
Mucus secretion	---	---	---	---	---	---	+--	+--

	1.0 mg/l							
Activeness	---	---	---	---	+--	++-	+++	+++
Loss of balance	---	---	---	---	+--	++-	++-	+++
Lateral side movement	---	---	---	---	---	+--	++-	++-
Opercular ventilation*	113	83	82	87	78	76	69	70
Color change	---	---	---	---	+--	+--	++-	++-
Mucus secretion	---	---	---	---	---	+--	+--	++-

	1.6 mg/l							
Activeness	---	---	---	+--	++-	+++	+++	+++
Loss of balance	---	---	---	+--	+--	++-	++-	+++
Lateral side movement	---	---	---	---	+--	+--	++-	+++
Opercular ventilation*	113	77	74	71	68	63	60	61
Color change	---	---	---	---	+--	+--	++-	++-
Mucus secretion	---	---	---	---	+--	++-	++-	+++

5. DISCUSSION

In recent years, rapidly increasing human population has accelerated environmental pollution and agricultural runoff is a one of main source of pollution. In agriculture, more than 1400 different pesticide formulations are being used to control pests (Wang et al., 2011). These pesticides can pass to the aquatic systems by rain, wind and excess agricultural irrigation, only 0.015-6.0% of pesticide reaches to target but 94-99.9% of pesticide reaches to non-target organisms (Tiryaki et al., 2010). The harmful effect of pesticides on fish has attracted a lot of attention because they cause serious environmental disorders and increase the poisoning of fish and invertebrates (Barbieri et al., 2011). Primarily, fish and aquatic animals are exposed to pesticides in three main routes. First way is dermal, pesticide contaminated water can absorbed through the skin. Second way is oral that include drinking polluted water or capturing on pesticide contaminated prey. The last way is breathing, contaminated water directly uptaken by gills during the respiration (Satyavani et al., 2011).

The fish gills are multifunctional organs and especially pesticides enter into fish body through gills. Depending on the chemical properties of the pesticides, it may enter the circulatory system and be carried in body fluid, bind to proteins or dissolve in the blood. Thus, pesticides affects cell membranes of central and peripheral nervous system so enzymatic biotransformations are destroyed and this are caused to respiratory distress (Laurent and Perry, 1990). Reddy and Yellamna (1991) reported sublethal concentration of cypermethrin (0.004 mg) changed carbohydrate metabolism in liver, brain and gill tissues of fresh water teleost (*Tilapia* *mossambica*). A decrease in glycogen and pyruvate levels and increase in lactate amount was seen in all tissues so aerobic glycolytic pathway increased but mitochondrial metabolism decreased due to oxygen uptake and pyruvate movement.

First symptom of poisoning is known as oxygen consumption rate increases under sublethal concentrations but near the lethal concentrations, oxygen consumption rate decreases because of stress and excess mucus secretion from gills (Dube and Hosetti, 2010). Excessive secretion of mucus acts as a barrier between the body and polluted water to reduce its irritating effect (Susan et al., 2010; Barbieri, 2009; Barbieri et al., 2011; Joshi and Kulkarni, 2007; Marigoudar et al., 2009; Rao et al., 2003; Croux et al., 2002; Laurent and Perry, 1990). Barbieri (2008) in their study, pearl earthheater (*G. brasiliensis)* exposed to different concentration of 2,4-D. Oxygen consumption rate increased when exposed to 1, 5 and 10 mg/l then started to decrease at 25.0 and 40.0 mg/l of 2,4-D. Tilak and Kumari (2008) reported that, grass carp (*C. idella*) exposed to increasing concentration of DDVP so oxygen consumption rate decreased related to gill demage and inhibition of AChE enzyme activity. Barbieri et al., (2010) have reported that the 0.1, 0.5 and 1.0 mg/l of methyl parathion increased the oxygen consumption rate of Nile tilapia (*O. niloticus*) then started to decrease under 2.5, 5.0 and 10.0 mg/l. Joshi and Kulkarni (2007) used to sykes (*G. mullya*) to investigate effect of cypermethrin and fenvalerate. 0.18 mg/l cypermethrin and 0.26 mg/l fenvalerate increased the oxygen consumption rate. Cheng and Farrell (2006) in their experiment, rainbow trout (*O. mykis*) exposed to rotenone after exposure active and standard metabolic rate decreased under lethal concentration within 24h.

In this study, male zebra cichlid (*A. nigrofasciata*) were exposed sublethal concentration of three pesticides. Oxygen consumption rates from three pesticides increased linearly as their concentration increased from 0.45 to 3.6 mg/l in CF-E, 2.17 to 17.38 mg/l in 2,4-D and 0.16 to 1.0 mg/l in MMM. Oxygen consumption rates decreased after at 3.6 mg/l in CF-E, 17.38 mg/l in 2,4-D and 1.0 mg/l in MMM. When fish were exposed to sublethal concentrations of these three pesticides, various behavioral and physiological changes were observed such as, hyperexcitation, loss of equilibrium, irregular swimming, change in body color and finally sinking at the corner of test tank and staying motionless in there. Along the experiment; severe respiratory stress, rapid opercular activity, excessive mucus secretion and fatigue were observed in the zebra cichlids. Susan et al., (2010) reported that fenvalerate exposed three major carps (*L. rohita*, *Catla catla*, *Cirrhinus mrigala*) showed irregular, erractic, darting movements and loss of balance due to inhibition of AChE activity so acetylcholine accumulates in cholinergic synapses and cellular response changes. Patil and David (2008) have observed the malathion exposed carp (*L. rohita*) showed irregular, erractic and darting swimming, hyperexcitability, loss of equilirium, inactivation of AChE activity and hyperstimulation. Ismail et al., (2009) in their study, common carp (*C. carpio*) exposed to different concentration of profenefos and fish showed various abnormal behavior such as loss of balance, staying motionless, lying laterally at the bottom, swimming in spiral fashion with jerks, revolving in water, opened mouth and rapid opercular movements. Rao et al., (2005) used mosquito fish (*G. affinis*) to determine effect of CF-E. CF-E caused some irregular activity such as erractic swimming with jerky, loss of equilibrium, excess mucus secretion and hyperactivity.

6. CONCLUSION

Environmental pollutants, including pesticides, heavy metals, detergents, oil spils, fertilizers, sewage and other organics, cause serious risks to aquatic organisims. Especially, the harmful effects of pesticides on fish has attracted a lot of attention in recent years because pesticide usage has greatly increased to improved the food quality. Annually 3 million ton pesticides is manufactured and each passing year production is rising about 1%. However, 0.015-6.0% of pesticide reaches to target but 94-99.9% of pesticide reaches to non-target organisms (Tiryaki et al., 2010). High amount of pesticides reach to the aquatic systems by rain, wind, and excess agricultural irrigation. This study showed that the organophosphate CF-E toxicity was very high, but MMM showed intermediate and 2,4-D exhibited low toxicity. Pesticides had a profound impact on behavior and respiration in *A. nigrofasciata* in sublethal concentrations. Thus, they led to altered fish respiration and behavior. Oxygen consumption rate increased under sublethal concentrations but near the lethal concentration oxygen consumption decreased and pesticide contamination caused various behavioral alteration, including activeness, loss of balance, lateral side movement, reduction of opercular activity, color change and excess mucus secretion.

Consequently this experiment showed that, monitoring and controlling of aquatic ecosystems are very important and also zebra cichlids are ideal sentinels for respiration and behavioral tests because they show abnormalities very clear.

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