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# TOXICOLOGICAL EFFECTS OF METAL IONS AND SOME PESTICIDES ON CARBONIC ANHYDRASE ACTIVITY PURIFIED FROM BIGHEAD CARP (*HYPOPHTHALMICHTHYS NOBILIS*) GILL TISSUE

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**Abstract:** In this work, the total carbonic anhydrase (CA) enzymes were obtained from bighead carp (*Hypophthalmichthys nobilis*) gill tissue with a purification fold of 239.62, specific activity of 26128.18 EU $mg^{-1}$ and a yield of 44.04 % using Sepharose affinity column chromatography. For recording the CA purity, gel electrophoresis was performed in this part. The molecular weight (MW) of total CA enzyme was found 31 kDa. Additionally, the inhibitory effects of diverse pesticide compounds (carbaryl, carbofuran, permethrin, dimethoate, cypermethrin and λ-cyhalothrin) and heavy metals ($Cu^{2+}$, $Fe^{2+}$, $Pb^{2+}$, $Co^{2+}$) on CA bighead carp gill tissue CA enzyme activities were investigated and also the results calculated, and then plotted. The some pesticide compounds had $IC_{50}$ amounts in the range of 0.36 - 253.35 µM. Also, the metal ions used had $IC_{50}$ values ranging from 7.84 to 95.28 mm. As a result of these studies, a stable pH is set as pH: 8.0. CA inhibitors are target molecules in drug design studies.

**Keywords:** Bighead carp, gill, carbonic anhydrase, metals, pesticides, inhibition

## 1. INTRODUCTION

Bighead carp (*Hypophthalmichthys nobilis*) fish depends on a class of Cyprinidae. This species has 24 pairs of chromosomes and a genome size of about 0.9 GB. Annual carp production was 3.2 million tonnes worldwide in 2014, accounting for 98% of China's production. This fish is one of the most important and valuable aquaculture species in China, with annual production ranking fourth among freshwater species in 2012. However, considerable study has been carried out on large carp for breeding, several specific factors. Obstacles to traditional selectivity based on phenotypes, such as large-scale genomic resource scarcity, long reproductive cycles, and short markers that are strongly associated with growth rates (Marian & Krasznai, 1978; Bettoli et al., 1985).

Heavy metal ions (HM) accumulate in the major organs. Gill often collects HM and blood-retaining structures, while pyloric, gastric and intestinal tracts collect food-related elements. In fact, the highest concentrations of HM are found in fish, liver, gills and in some cases in the intestine. Zinc, copper, silver and cadmium ions enter the fish cells mainly through food. On the other hand, the water uptake is also essential, especially if the micronutrients are deficient in the body while HM concentrations are high in water. In addition, in vertebrate cells, HM ions can also enter through the cuticle. The role of each absorption method depends on the size of the animal's body, life cycle, feeding behavior and duration of exposure. For example, in various predatory arthropod cells, heavy metals mainly enter the body through food, while in filter feeders they are mostly composed of water (Durnam & Palmiter, 1981; Shallari et al., 1998).

Pesticides are economic poisons employed to regulate the effect of plants and noxious animals upon economy and our life. The effects of the vast majority are relatively nonselective and usage, therefore, may result in undesirable, even unanticipated, side effects. In 1943, Surber stated that "studies of the effects of pesticides on fish, on their food, and on various forms of wildlife are proceeding along operational lines. Pesticide compounds have now become an perfect section of our modern life and also are utilized to protect stored grain, flower gardens, agricultural land as well as to eradicate the pests transmitting perilous infectious diseases. Researchers and manufacturers are designing novel formulations of pesticide compounds

to meet the worldwide demand. Indeed, the applied pesticide compounds should just be toxic to the aim organisms, should be eco-friendly and biodegradable to some extent (Vogt, 1987; Farooq et al., 2012).

Carbonic anhydrase (CA) (EC 4.2.1.1) enzymes are metalloenzymes commonly found in virtually all living organisms, which recyclables the hydration / dehydration of $CO_2/HCO_3^-$. Apart from the reversible hydration reaction of $CO_2$ to bicarbonate, α-CAs also catalyze a variety of another reactions, like hydration from cyanate to carbamic acid or hydration from syamide to urea, hydration of aldehyde to gem-diols, hydrolysis of carboxylic esters or sulfonic esters. CA-I and CA-II are the two main isoforms of α-CAs found in the cytosol of mammalian red blood cells (Kirici et al., 2016; Öztaşkın et al., 2017; Boztas et al., 2019). The most important function of these isoenzymes takes place in the respiratory event by catalyzing the conversion of the metabolic product $CO_2$ molecule into $HCO_3^-$ compound in the tissue capillaries and the conversion of $HCO_3^-$ to $CO_2$ in the pulmonary pulmonary capillary. Studies have shown that CAI, CAII and most of other CA isoenzymes have important roles in various physiological processes such as $H^+$ production, acid-base homeostasis, pH balance, metabolic acidosis, and have demonstrated the association of abnormal levels or activities of these isoenzymes with human diseases. Therefore, CA isoenzymes are seen as potential therapeutic targets that can be inhibited in the therapy of most of diseases like gluocoma, edema, obesity, epilepsy, cancer, osteoporosis. Since CA isoenzymes have been discovered to be associated with many disorders, CA inhibitors are target molecules in drug design studies. Nowadays, studies for the identification of new and specific inhibitors of CA isoenzymes are continuing rapidly (Genc Bilgicli et al., 2019; Kaya et al., 2019; Koçyiğit et al., 2019).

In this study, CA enzyme was purified from bighead carp gill tissue with using chromatography, and we investigated the *in vitro* toxicological effects of some commonly used pesticides containing carbaryl, carbofuran, permethrin, dimethoate, cypermethrin and λ-cyhalothrin, heavy metal ions including $Cu^{2+}$, $Fe^{2+}$, $Pb^{2+}$, and $Co^{2+}$ on the purified CA enzyme.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

Carbaryl, carbofuran, permethrin, dimethoate, cypermethrin and λ-cyhalothrin were obtained from an agricultural pesticide shop. $NaAsO_2$, $CoCl_2$, $Pb(CH_3COO)_2$, $FeCl_2$, Sepharose-4B, $CuSO_4 \cdot 5H_2O$, protein assay reagents and chemicals for this study were purchased from SigmaAldrich (Taufkirchen, Germany).

### 2.2. Preparation of gill homogenate

Bighead carp gill tissue was obtained from the Hazara Sea in northwestern Iran. The gills were kept in ice. They are cut into 10 g pieces and stored at 20°C for later use. 0.9% NaCl was washed three times. To obtain gill homogenization, cut the fragments into small pieces and wash with liquid nitrogen using a 25 mm Tris HCl / 0.1 M $Na_2SO_4$ homogenous buffer (pH 8.7). The suspension was centrifuged for 20 minutes. At 13000 x g, this was done three times. This supernatant was used for subsequent analysis (Türkan et al., 2019a)

### 2.3. Purification of CA from bighead carp gill tissue

The homogenate pH of the gill tissue of bighead carp was adjusted to 8.7 with solid tris. The homogenate was plated and washed with Tris-HCl solution and 400 mL of 25 mM $Na_2SO_4$ (pH 8.7) which was performed as in previous studies (Cetin et al., 2019; Türkan et al., 2019b).

### 2.4. CA enzyme activity

CA enzyme activity was measured in two ways: the first is esterase activity which can be performed *in vitro* followed by spectrophotometric methods and the second is $CO_2$ hydrate activity, CA physiological activity. The activity of CA enzyme was similar to previous studies (Erdemir et al., 2018; Küçükoğlu et al., 2019).

### 2.5. Protein determination

The total CA enzyme were spectrophotometrically determined at 595 nm according to the Bradford assay. Bovine serum was used as a positive control (Maharramov et al., 2019).

### 2.6. Gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to investigate the purity of isoenzymes and to calculate the MW of the subunits. Indeed, 3 and 10% acrylamide was prepared for stacking SDS according to Laemmli's method. Denatured protein markers and enzyme samples were used for electrophoresis medium (Koçyiğit et al., 2018).

### 2.7. Stable and optimum pHs

To determine the optimum pH, CA activity was measured in 0.8 M sodium phosphate buffer ranging from pH 5.0-8.5 and 0.8 M Tris-HCl pH 7.5-8.5, 0.8 M Glycine-NaOH and pH 8.5-10.5. On the other hand, to determine stable pH, the activity of CA enzymes in these buffers was measured. Activity was measured over a 24-hour period during 4 days of incubation using PNA as substrate under standard conditions (Zengin et al., 2018).

### 2.8. Optimum temperature and optimum ionic strength

Various concentrations of glycine / NaOH buffer (pH 5.5) in the range of 0.1 to 1.1 mg were used to obtain the optimum ion resistance value of the enzymatic activities. Increase 10°C from 0°C to 80°C to determine the optimum temperature (Gulçin & Taslimi, 2018).

### 2.9. Inhibition assay

Inhibitory effects of metal ions ($Cu^{2+}$, $Fe^{2+}$, $Pb^{2+}$, $Co^{2+}$) and pesticide compounds (Carbaryl, carbofuran, permethrin, dimethoate, cypermethrin and λ-cyhalothrin) on the activity of CA carboxy-purified enzyme (gill tissue) bighead carp was evaluated. The metal ions were dissolved in water and the effects of enzyme inhibition were investigated and also $IC_{50}$ and $K_i$ values were calculated and plotted (Taslimi & Gulçin, 2018).

## 3. RESULTS

### 3.1. Characterization studies

In this work, the CA enzyme was purified from large carp gill tissue with specific activity of 26128.18 EU $mg^{-1}$, purification of 239.62, total activity of 2874.10 and yield of 44.04% using affinity column chromatography (Table 1).

SDS-PAGE was performed to determine the purity of the enzyme and the molecular mass of the subunit and single band was observed. The molecular mass of the subunit was found to be approximately 31 kDa. The results of studies on CA from gill tissue of large carp are shown in Figs. 1-5. The mass of the CA enzyme subunit was determined by SDS-PAGE and a single band was adsorbed. The molecular mass of CA was calculated to be about 31 kDa (Fig. 1).

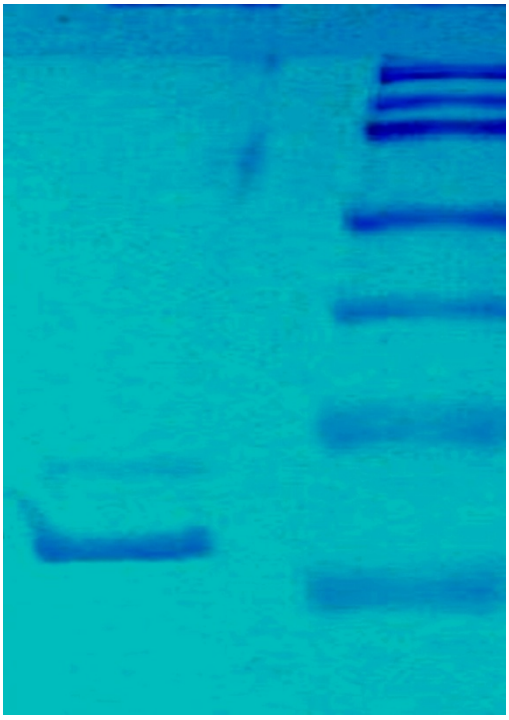

Figure 1. SDS polyacrylamide gel electrophoresis of bighead carp gill tissue

The optimum pH study was performed for CA enzyme purified from fish and their pH was determined using buffers 5, 6, 7, 8, 9 and 10 using buffers 5, 6, 7, 8, 9 and 10 (Fig. 2).As a result of studies, the most suitable ionic resistance for the CA enzyme purified from fish gills was determined as sodium phosphate buffer (pH 8.5, 0.8 M) (Fig. 3).

Table 1. Results of purification of CA enzyme from Bighead carp gill

| Purification step | Activity (EU $mL^{-1}$) | Total volume (mL) | Protein (mg $mL^{-1}$) | Total protein (mg) | Total activity (EU $mL^{-1}$) | Specific activity (EU $mg^{-1}$) | Yield (%) | Purification fold |
|---|---|---|---|---|---|---|---|---|
| Homogenate | 407.83 | 16 | 3.74 | 59.84 | 6525.28 | 109.04 | 100.00 | 1.00 |
| Sepharose-4B-L-tyrosine sulfanilamide affinity chromatography | 574.82 | 5 | 0.022 | 0.11 | 2874.10 | 26128.18 | 44.04 | 239.62 |

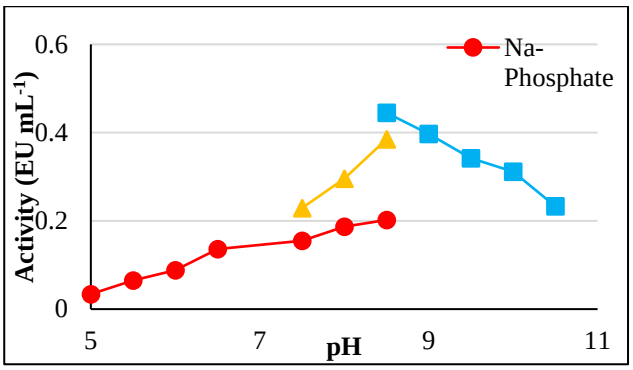

Figure 2. Optimum pH for from bighead carp.

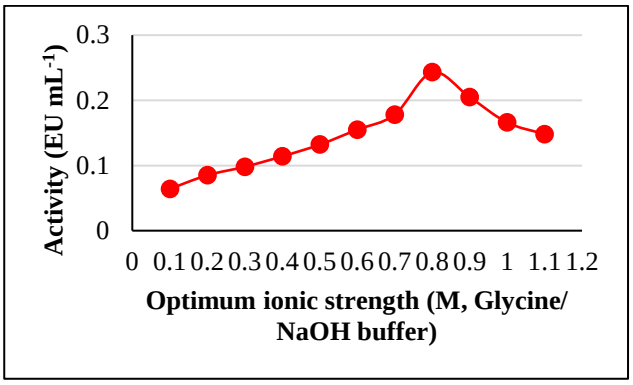

Figure 3. Optimum ionic strength (M, Tris-$SO_4$ buffer) for from bighead carp gill tissue.

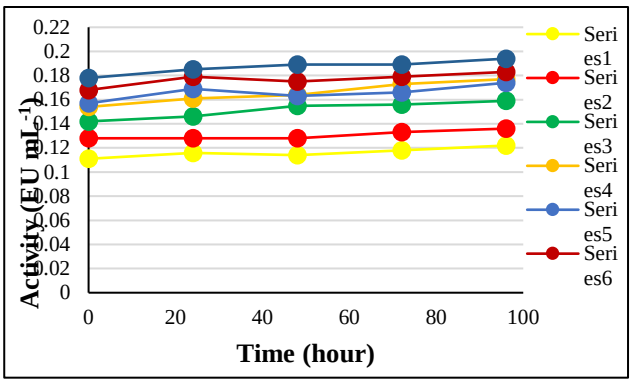

Figure 4. Stable pH graph of from bighead carp gill tissue for three days.

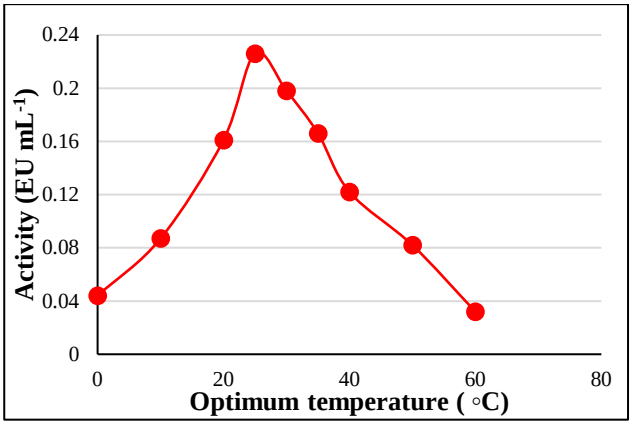

Figure 5. The effect of temperature on CA enzyme activity from bighead carp gill tissue.

In this study, a stable pH study was performed to determine the stable pH of pure CA enzyme. The results are shown in Fig. 4. As a result of these studies, a stable pH is set as pH: 8.0. In fact, 8.8 mM sodium phosphate (pH: 8.5) with optimum pH and appropriate ionic strength was used to determine the optimum temperature of pure CA enzyme. Activity measurement was performed at 0 to 60°C every 10°C. The results are shown in Fig. 5. As a result of these studies, the optimum temperature of 25°C was determined.

### 3.2 Inhibition results

CA inhibitor compounds are extensively utilized in clinical practice as an antihypertensive drug (Gülçin & Taslimi 2018). It was reported that CA inhibitors can provide a new therapy for infection, cancer, obesity, and Alzheimer's disease (Taslimi et al., 2017). In fact, there are more studies on purification of CA enzymes from diverse tissue cells, there are no reports on the purification and characterization of the enzyme from gill carp tissue. Therefore, the present work aims to purify and characterize the CA enzyme from gill tissue of the large carp for the first time and to investigate the effect of some heavy metal ions on enzyme activity. In this work, we determined the inhibitory effects of CA metal ions (Table 2). The metal ions used had $IC_{50}$ values ranging from 7.84 to 95.28 mm. The $IC_{50}$ values of some metal ions are as follows: $Co^{2+}$ (7.84 mM, $r^2$: 0.9624) < $Cu^{2+}$ (11.73 mM, $r^2$: 0.9680) < $Pb^{2+}$ (17.53 µM, $r^2$: 0.9612) < $Fe^{2+}$ (28.95 mM, $r^2$: 0.9812). Also, they recorded $K_i$ values in the range of 6.22 ± 1.02 - 33.63 ± 6.63 mM (Table 3). $K_i$ values were: $Co^{2+}$ (6.22 ± 1.02) < $Cu^{2+}$ (7.11 ± 2.04) < $Pb^{2+}$ (19.48 ± 4.57) < $Fe^{2+}$ (33.63 ± 6.63) (Table 2). The following metals, $Cu^{+2}$, $Fe^{+2}$, $Pb^{+2}$, and $Co^{+2}$ showed inhibitory effects on CA.

Additionally, we recorded the CA inhibition effects of some pesticide compounds (Table 3). The some pesticide compounds had $IC_{50}$ amounts in the range of 0.36 - 253.35 µM. $IC_{50}$ amounts of some pesticide compounds exhibited the following order: λ Cyhalothrin (0.36 µM, $r^2$: 0.9264) < Cypermethrin (13.38 µM, $r^2$: 0.9624) < Permethrin (88.08 µM, $r^2$: 0.9497) < Dimethoate (111.52 µM, $r^2$: 0.9698) < Carbaryl (126.43 µM, $r^2$: 0.9536) < Carbofuran (253.35 mM, $r^2$: 0.9133). Also, they obtained Ki values in the range of 0.45 ± 0.12-195.86 ± 35.75 µM (Table 3). $K_i$ values were: λ Cyhalothrin (0.45 ± 0.12) < Cypermethrin (18.33 ± 1.77) < Permethrin (75.47 ± 17.51) < Carbaryl (143.33 ± 57.33) < Dimethoate (162.53 ± 27.38) < Carbofuran (195.86 ± 35.75) (Table 3).

Table 2. $IC_{50}$ and $K_i$ values and inhibition types of some heavy metal CA obtained from bighead carp gill

| Metals | $IC_{50}$ (mM) | $r^2$ | $K_i$ (mM) | Inhibition type |
|---|---|---|---|---|
| $Cu^{2+}$ | 11.73 | 0.9680 | 7.11 ± 2.04 | Competitive |
| $Fe^{2+}$ | 28.95 | 0.9812 | 33.63 ± 6.63 | Uncompetitive |
| $Pb^{2+}$ | 17.53 | 0.9612 | 19.48 ± 4.57 | Uncompetitive |
| $Co^{2+}$ | 7.84 | 0.9624 | 6.22 ± 1.02 | Competitive |

Table 3. $IC_{50}$ and $K_i$ values and inhibition types of some pesticides on CA obtained from Bighead carp gill

| Pesticides | $IC_{50}$ (μM) | $r^2$ | $K_i$ (μM) | Inhibition type |
|---|---|---|---|---|
| Carbaryl | 126.43 | 0.9536 | 143.33 ± 57.33 | Uncompetitive |
| Carbofuran | 253.35 | 0.9133 | 195.86 ± 35.75 | Competitive |
| Permethrin | 88.08 | 0.9497 | 75.47 ± 17.51 | Competitive |
| Dimethoate | 111.52 | 0.9698 | 162.53 ± 27.38 | Uncompetitive |
| Cypermethrin | 13.38 | 0.9624 | 18.33 ± 1.77 | Uncompetitive |
| λ Cyhalothrin | 0.36 | 0.9264 | 0.45 ± 0.12 | Uncompetitive |

## 4. DISCUSSION

When we compared the results of this article with other articles, we found that they were good. In other articles they have tried different metal ions and different pesticides and obtained micromolar in their results (Gülçin & Taslimi 2018). Optimum pH and temperature values were obtained similar to previous articles. The CA enzyme has been cleared from plenty tissue cells including red blood cells and its kinetic properties in h-erythrocytes, fish gills and erythrocytes, saliva and rat blood cells, Plasmodium falciparum, insects, bones, leukocytes. CA has been reported to be partially characterized by plants, yeasts, and bacteria. Exposure to heavy metals is an important problem of environmental poisoning. Most heavy metal ions are toxic to animals, humans, and plants. Human cells are exposed to health risks associated with metals due to bioaccumulation (Daştan et al., 2017).

At low concentrations, copper, zinc, mercury, and cadmium ions influence morphological and physiological-biochemical parameters in some fishes. Such effects may include reduced immune status, changes in behavior, nutritional status and growth rate, digestive activity, nutrient absorption efficiency, and carbohydrate metabolism status. The accumulated Hg shows teratogenic, gonadotoxic, and mutagenic effects and damages protein, lipid, and peptide metabolism study. In aquatic invertebrates copper, zinc, mercury and cadmium alter the growth rate of morphological and physiological parameters, swimming speed, nutrient intake, respiratory rate, productivity, survival and life cycle (Durnam & Palmiter, 1981).

In the past decade, there has been a great deal of concern about industrial and agricultural contamination in aquaculture and the potential impacts of such contamination on human health as well as animal welfare. As seas and lakes, through rivers and other irrigation paths, become the final resting point for pollutants from plant effluents or agricultural uses, it is in such environments that we expect the first environmental catastrophe warnings to occur. Only the fish population in these environments are not endangered. Fish are part of the natural diet of aquatic mammals and birds, and increasingly, many humans are dependent on fish as a source of protein, directly and indirectly as animal feed for their pets. Organophosphorus pesticides are used for rapid degradation and the persistent nature of pest control in an agricultural field, but their wide range of harmful effects is far beyond the pest. This has been substantiated by facts that have been tested by a number of researchers using different concentrations of pesticides on organisms worldwide (Forbes et al., 2009).

Pugazhvendan et al., (2009) exposed *Ophicoephalus punctatus* to malathion at concentrations of 10, 12, 14, 16, 18, 20, 1.1 and 100 ppm for 7 days *in vitro* and for severe histological changes in brain, liver, ovary And fish tissues reported. Organophosphorus pesticides have a wide range of harmful effects in the living world. This has been corroborated by facts that have been tested by a number of researchers using different concentrations of pesticides on organisms worldwide. To save the living world from the harmful effects of these chemicals, as well as finding some alternative ways to control pests, it is important to do a great deal of research on the diversity of organisms using different concentrations of pesticides. Many relatively low-dose chemicals affect biomass metabolism by altering natural enzyme activity. With some of these interactions, there is a high reactivity that has a huge impact on the whole animal or plant. On the other hand, many chemicals mediate the activity of many enzymes to a moderate extent, and it is assumed that the ultimate debilitating effect on the whole organism is caused by a variety of nonspecific biochemical

functions (Pugazhvendan et al., 2009; Özbey et al., 2016).

## 5. CONCLUSION

In this work, the CA enzyme was purified from large carp gill tissue with specific activity of 26128.18 EU $mg^{-1}$, purification of 239.62, total activity of 2874.10 and yield of 44.04% using affinity column chromatography. The CA enzyme we purified in this work inhibited metal ions and pesticides well, and we wrote them all by comparison in the results part. The molecular mass of CA was calculated to be about 31 kDa. When we examined other articles, the molecular weight of the carbonic anhydrase enzyme was obtained around 30 KDa and it was similar to our result. In the present work, the CA enzyme was purified in one step by affinity chromatography of the gill tissue of the Bighead carp and we investigated the effects of *in vitro* toxicology using some pesticides and some heavy metal ions on the CA enzyme. After drawing the graph, the results were calculated. The results of inhibition of metal ions at mM level and pesticide level at micrometer level were obtained. The CA enzyme plays an important role in regulating the acid-base mechanism in fish cells by preparing the acid equivalent for exchange with the environment. In fact, unlike air-breathing vertebrate cells that regularly use respiratory compensation to regulate basal acid status, basal acid balance in fish is almost entirely dependent on direct exchange of base acid equivalent to the environment, which is a fuel offset.

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