The in vitro effects of some pesticides on carbonic anhydrase activity of *Oncorhynchus mykiss* and *Cyprinus carpio carpio* fish

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Abstract

Systemic carbonic anhydrase (CA) inhibitors are among the most powerful agents to lower intraocular pressure. Unfortunately, their use is frequently accompanied by undesired side effects. Some are due to the relatively large amounts of drug that have to be systematically administered to inhibit the CA in the ciliary processes. The aim of the present work was to study in vitro effects of some pesticides on CA enzyme obtained from blood of fish, which play a key role in salt- and osmoregulation and acid–base balance in the fish, *Oncorhynchus mykiss* and *Cyprinus carpio carpio* living in freshwaters, and compared with CA inhibitors. CA activities were significantly inhibited by pesticides and inhibitors. I_{50} values of *O. mykiss* CA enzyme inhibited by lambda-cyhalothrin, deltametrin, diozinon, dorzolamide and brinzolamide were 6.05×10^{-4}, 1.48×10^{-5}, 6.84×10^{-3}, 3.82×10^{-5} and 1.80×10^{-6} mol/l, and that for *C. c. carpio* 6.86×10^{-4}, 4.70×10^{-4}, 3.92×10^{-3}, 8.34×10^{-6} and 1.42×10^{-6} mol/l, respectively. The pesticides used in this study inhibited the CA activity from different fish species to various degrees. It was found that the most effective inhibitor of CA enzyme within pesticides used was detrametrin. These findings observed in vitro could be useful in the understanding of the toxic effects that pesticides elicit on aquatic organisms in vivo.

1. Introduction

The zinc metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) catalyzes the reversible hydration of CO_2 with high efficiency, is widely distributed in nature, and also is a well-characterized enzyme in some living organisms. Changes in CA activity have been associated with altered metabolism, especially in diabetes mellitus. The observation that CAs are present in several pathogenic species suggests that the enzyme may be involved in microbial virulence [1]. CA has been intensely investigated since its discovery [2]. This ubiquitous enzyme is present in archaeo and eubacteria, green plants and animals [3]. CA catalyses a variety of reactions, including the reversible hydration of CO_2 to bicorbonate, as a physiological reaction, and some other processes [4] such as hydrolysis of aromatic and aliphatic esters, or the hydration of cyanate to urea, etc.

CA plays a crucial role in the excretion of metabolic CO_2 in all vertebrates, including fish. In fish, CO_2 produced in the tissues is rapidly hydrated to HCO_3^- following diffusion into blood, by the action of erythrocytic CA [5] and HCO_3^- represents almost 98% of the total carbon dioxide stored and transported in the plasma. At the respiratory epithelium (gills or skin), erythrocytic CA catalyses the rapid dehydration of HCO_3^- to molecular CO_2, which then diffuses passively into the ventilatory water stream. Moreover, the CO_2/HCO_3^- system constitutes one of the most important physiological buffers for acid–base regulation [6]. In teleosts, CA has been found in various tissues. It appears to be present in high concentrations in the gills [7,8], where it plays an important role in osmoregulation, nitrogen (ammonia) excretion, acid–base balance and gas exchange [9].

CA enzymes purified from various organisms have been shown to be inhibited by various compounds. Sulfonamides, like acetozolamide and heavy metals are considered as the strongest CA inhibitors [10]. In addition, some in vitro and in vivo studies showed that some antibiotics including ampicilin and gentamisin, some drugs, some chemicals like magnesium sulfate and, finally, some pesticides also inhibit CA enzyme activity to a wide range of degrees [11]. Many pesticides are being used in agriculture in order to improve the yield. Although the use of

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these chemicals caused a positive effect on crop production, certain pesticides, their residues, metabolites and/or contaminants have created many unforeseen adverse effects on the enviroment. Pesticides may be present in very low concentrations, which may not cause immediately detectable effects. However, this small amount of chemicals can cause sub-lethal damage to organism and this is more insidious and difficult to define than acute toxicity. Many chemicals, especially pesticides, at relatively low dosages affect the metabolism of biota by altering normal enzyme activity [12]. CA is of special concern because of physiological importance and thus could be particularly vulnerable to waterborne pollutants. However, there was a little information on CA sensitivity of aquatic organisma to pesticides. Generally, pesticides widely used in agriculture are one of the major pollutants for aquatic enviroments. Especially, this type of pollution is of great concern for freshwater organisms. CA is a good example for metal enzyme [13] and –SH rich enzyme [14], respectively, which play a pivotal role in teleost intestine and gill physiological functions such as salt- and osmoregulation and acid–base balance. It is interesting to note that these two epithelia are the first interfaces of the organism exposed to the aquatic environment, and for this reason a primary target for the action of environmental pollutants on fish. Therefore, alterations in their physiological functions by environmental pollutants could put the survival of the fish at risk [15]. Although a number of information is available in fish regarding effect of amides and metal ions on CA activity, very little information is available for pesticides on CA activity. In this study, I have investigated the effects of some pestisicides such as lambda-cyhalothrin: [(S)-α-cyano-3-phenoxybenzyl (Z)-(1R,3R)-3-(2-chloro-3,3,3-trifluorophenyl) 2,2-dimethyl cyclopropane carboxylate], deltametrin: [(S)-α-cyano-3-phenoxybenzyl (1R,3R)-3-(2,2-dibromovinyl) 2,2-dimethyl cyclopropane-carboxylate] and diozinon: [0,0-diethyl 0-2-isopropyl-4-methyl-6-pyrimidinyl thiophosphate], and the chemicals such as dorzolamide and brinzolamide, which are inhibitors of CA, on CA activity obtained from *Oncorhynchus mykiss* and *Cyprinus carpio carpio* fishes living in freshwaters. The inhibition power of these inhibitors has also been compared with pesticidies used. These findings will be useful in the understanding of the toxic effects that pesticides elicits on fish physiological functions.

2. Material and methods

2.1. Material

All chemicals used in this study were the best grade available and were used without further purification as they were obtained from Sigma Chemical Co. and Merck. Furthermore, pesticides were obtained from local companies licensed to sell the pesticidies. *O. mykiss* and *C. c. carpio* were trapped by nets from Manyas lake and Pınarbaşı stream in Balikesir/Turkey.

2.2. The preparation of blood samples and CA activity

Fish were held in aerated dechlorinated freshwater (8–15 °C) and fed a diet of crayfish and minnows. There were no signs of stress, nor mortalities among the fishes used in these experiments. Blood was collected by blind caudal puncture into a heparinized syringe and transferred into a tube containing heparin [16].

The blood samples were centrifuged at $2100 \times g$ for 20 min and the plasma and buffer coat were removed. After the packed red cells were washed twice with NaCl (0.9%), the erythrocytes were hemolyzed with chilled water. The ghost and intact cells were removed by centrifugation at $4\,°C$, $28250 \times g$ for 30 min. The pH of the hemolysate was brought to 8.7 with solid–Tris buffer. CA activity was measured using the method described by Wilbur and Anderson [17] with a slight modification. CO_2 hydrase activity as an enzyme unit (EU) was calculated by using the equation $[(t_0 - t_c)/t_c]$, where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

2.3. Inhibition of CA activity by some pesticides and inhibitors

Lambda-cyhalothrin: [(S)-α-cyano-3-phenoxybenzyl (Z)-(1R,3R)-3-(2-chloro-3,3,3-trifluorophenyl) 2,2-dimethyl cyclopropane carboxylate], deltametrin: [(S)-α-cyano-3-phenoxybenzyl (1R,3R)-3-(2,2-dibromovinyl) 2,2-dimethyl cyclopropane-carboxylate] and diozinon: [0,0-diethyl 0-2-isopropyl-4-methyl-6-pyrimidinyl thiophosphate], and chemicals such as dorzolamide and brinzolamide, which are inhibitors of CA, were evaluated for its effectiveness as inhibitors of CA activity obtained from *O. mykiss* and *C. c. carpio* fish. The structures of pesticides and inhibitors used are shown in Fig. 1.

Four millilitres reaction mixture contained 2 ml phenol red, 1.5 ml CO_2 solution, 0.3 ml buffer solution, 0.1 ml inhibitor at various concentrations and 0.1 ml enzyme solution. Sensitivity of CA to pesticides and inhibitors was examined by measuring CA activity in the presence of increasing concentrations of these pesticides and inhibitors. Table 1 has shown the activity values of CA measured in the absence and presence of pesticides and inhibitors. CA activities with the related pesticides and inhibitors were assayed by following the hydration of CO_2. Percent activity graphs were drawn from these results to find I_{50} values at five different inhibitor concentrations, which shows about 50% inhibition effect. In order to determine I_{50} values, regression analysis graphs were drawn by using percent inhibition values by an Excell program on a computer. The inhibition concentrations causing up to 50% inhibition were determined from the graphs. CA activity without pesticides and inhibitors was accepted as 100% activity. Each inhibition effects were repeated at least three times (Figs. 2 and 3).
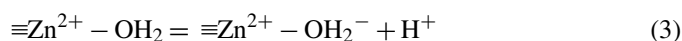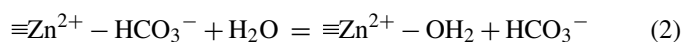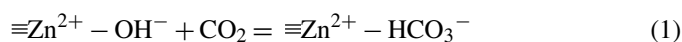
3. Results and discussion

Many compounds alter the activity of an enzyme by combining with it in a way that influences the binding of substrate and/or its turnover number. Substances that reduce an enzyme's activity in this way are known as inhibitors. Many inhibitors are substances that structurally resemble their enzyme's substrate but either do not react, or react very slowly, compared

Table 1
The activity values of CA in the absence and presence of pestisides and inhibitors

Pesticides							Inhibitors			
Diozinon		Lambda-cyhalothrin		Deltametrin			Brinzolamide		Dorzolamide	
Concentration (M) $\times 10^{+3}$	Activity	Concentration (M) $\times 10^{+4}$	Activity	Concentration (M) $\times 10^{+4}$	Activity		Concentration (M) $\times 10^{+7}$	Activity	Concentration (M) $\times 10^{+7}$	Activity
Cyprinus carpio carpio										
0.00	139	0.00	265	0	304		0	225	0	199
2.25	103	2.31	176	1.28	218		1	180	8	155
3.38	68	4.63	116	2.48	210		2	130	24	109
5.08	60	9.25	77	4.65	149		4	69	72	545
7.60	32	13.88	57	6.18	118		6	45	216	31
15.20	10	20.80	45	8.25	82		18	37	650	1
–	–	–	–	12.40	54		55	26	–	–
				Concentration (M) $\times 10^{+6}$						
Oncorhynchus mykiss										
0.00	192	0.00	292	0	206		0	584	0	1261
2.25	155	2.31	187	4.13	178		1	485	8	1200
3.38	138	4.63	125	8.30	148		2	462	24	1127
5.08	97	9.25	97	12.40	133		4	388	72	1014
7.60	75	13.88	70	13.80	85		6	208	216	889
15.20	47	20.80	0	24.80	65		18	206	650	387
–	–	–	–	–	–		55	88	–	–

with the substrate. Such inhibitors are commonly used to probe the chemical and conformational nature of a substrate-binding site as part of an effort to elucidate the enzyme's catalytic mechanism [18]. Many chemicals at relatively low dosages affect the metabolism of biota by altering normal enzyme activity, particularly inhibition of a specific enzyme [19]. The effects can be dramatic and systemic [20]. Indeed, CA isozymes are important enzymes in metabolism because they regulate pH in most tissues. CA inhibitors vary according to their affinity (K_i) of binding to a particular CA isozyme, potency (I_{50}) for inhibiting that isozyme, and physicochemical properties, which can influence their tissue distribution and scope of activity [21].

CA contains a catalytically essential Zn^{2+} ion, which is coordinated to three histidine residues. A fourth coordination site is occupied by an ionizable water molecule with a pK, near 7.0 [22]. Despite the wide range of catalytic efficiency of the CA isoenzymes, all are thought to follow a common mechanism of action, consisting of two distinct chemical steps. A Zn^{2+}-bound hydroxide ion or water molecule is implicated in both steps. For CO_2 hydration, the first step in catalysis is the attack of a Zn^{2+}-bound hydroxide on CO_2 to yield a Zn^{2+}-bound HCO_3^- species. The HCO_3^- is subsequently replaced by water to yield a Zn^{2+}-bound water molecule (Eqs. (1) and (2)). In a second, separate step, the original Zn^{2+}-bound hydroxide ion is regenerated by a hydrogen ion transfer step (Eq. (3)).

$$\equiv Zn^{2+} - OH^- + CO_2 = \ \equiv Zn^{2+} - HCO_3^- \tag{1}$$

$$\equiv Zn^{2+} - HCO_3^- + H_2O = \ \equiv Zn^{2+} - OH_2 + HCO_3^- \tag{2}$$

$$\equiv Zn^{2+} - OH_2 = \ \equiv Zn^{2+} - OH_2^- + H^+ \tag{3}$$

Eq. (3), the H^+ transfer step, appears to be rate limiting [23]. It has been reported that the most common inhibitors for CA are

several metal ions and various amides. These type inhibitors have been used by investigations for inhibition of CA activity obtained from some fish species, crabs and teleosts [10,24]. However, there was not much information available on inhibition of CA enzymes from aquatic organisms by pesticides. In this study, the inhibition of CA activity obtained from *O. mykiss* and *C. c. carpio* by pesticides such as lambda-cyhalothrin, deltametrin and diozinon, and inhibitors such as dorzolamide and brinzolamide has been investigated. These fish species were chosen due to being economically important in Turkey. I used the CA obtained from blood of fish used in this study.

I found that CA activity belonging to different fish species showed different inhibition power by pesticides and inhibitors, and also that not only amides but pesticides inhibit various fish CA enzymes. Table 2 shows I_{50} values of pesticides such as lambda-cyhalothrin, deltametrin and diozinon, and inhibitors such as dorzolamide and brinzolamide on carbonic anhydrase activity obtained from *O. mykiss* and *C. c. carpio*. The sensitivity of CA to pesticidies and inhibitors was different from one inhibitor to another. Sulfonamides inhibit fish CA activity quite strongly as pesticides were poor inhibitors of CA.

Table 2
I_{50} values of some pesticides and inhibitors on CA activity of *Oncorhynchus mykiss* and *Cyprinus carpio carpio*

Chemicals	*Cyprinus carpio carpio* (M)	*Oncorhynchus mykiss* (M)
Diozinon	3.92×10^{-3}	6.84×10^{-3}
Deltametrin	4.70×10^{-4}	1.48×10^{-5}
Lambda-cyhalothrin	6.86×10^{-4}	6.05×10^{-4}
Brinzolamide	1.42×10^{-6}	1.80×10^{-6}
Dorzolamide	8.34×10^{-6}	3.82×10^{-5}

Lambda-cyhalothrin

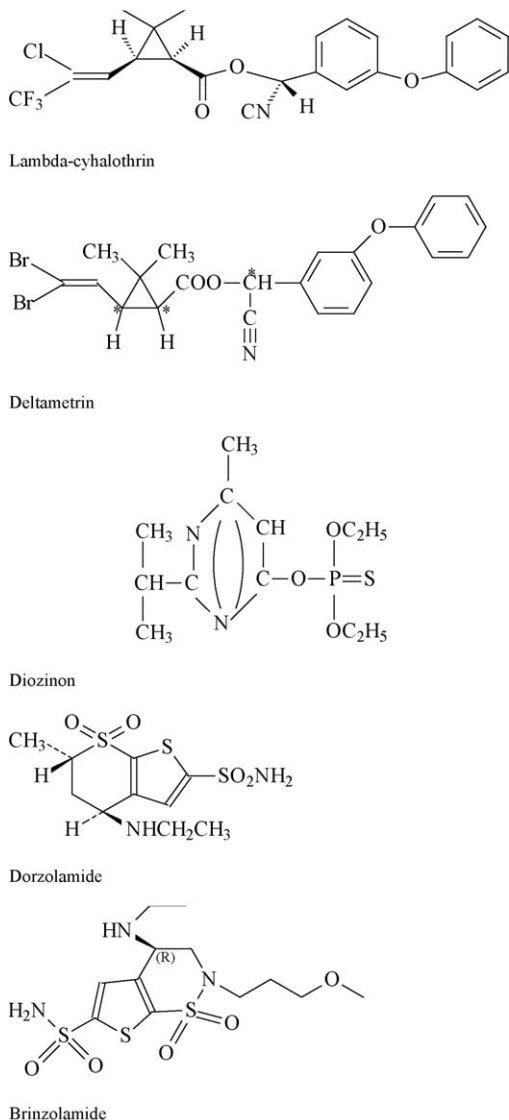Deltametrin

Diozinon

Dorzolamide

Brinzolamide

Fig. 1. The structure of pesticides and inhibitors.

The enzymatic activity observed with a specifice inhibitor may involve a single mechanism or be a result of an interplay of two or more mechanism of inhibitor action [25]. From Table 2, I_{50} values of *O. mykiss* CA enzyme inhibited by lambda-cyhalothrin, deltametrin, diozinon, dorzolamide and brinzolamide were found to be 6.05×10^{-4}, 1.48×10^{-5}, 6.84×10^{-3}, 3.82×10^{-5} and 1.80×10^{-6} M, and that for *C. c. carpio* 6.86×10^{-4}, 4.70×10^{-4}, 3.92×10^{-3}, 8.34×10^{-6} and 1.42×10^{-6} M, respectively. According to the results above, the most effective compound at inhibiting CA activity, for both *O. mykiss* and *C. c. carpio* were brinzolamide, followed by dorzolamide, deltametrin, lambda-cyhalothrin and diozinon, respectively. Işık et al. [12] investigated the effects of various pesticidies such as nuarimol, fenarimol, parathion-methyl and 2,4-dichlorophenoxy acetic acid on CA activity from some freshwater and seawater fish erythrocytes, and found that the pesticides used inhibited the CA activity from different fish species to various degrees. It was found that I_{50} values for nuarimol, fenarimol, parathion-methyl and 2,4-dichlorophenoxy
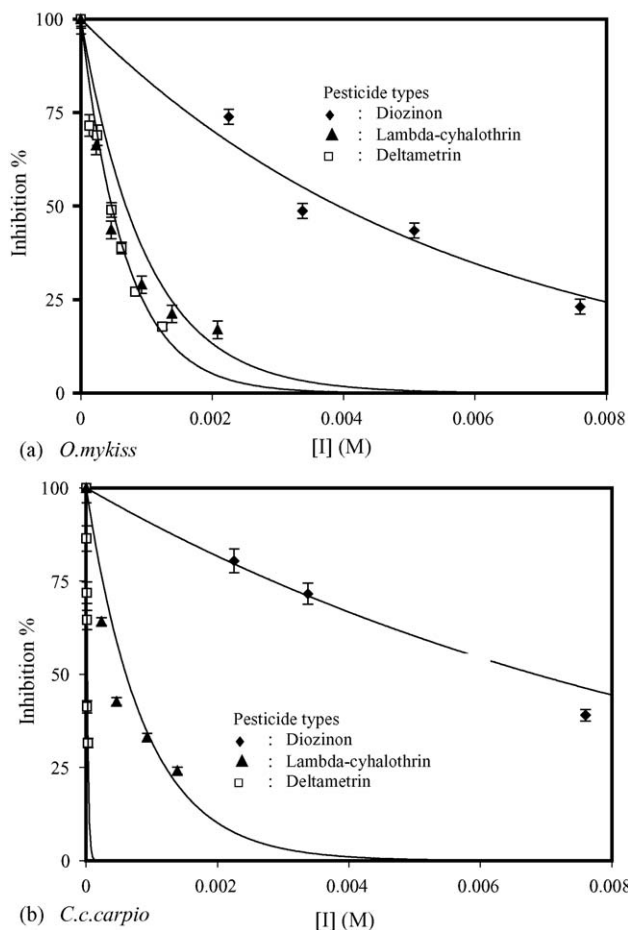

Fig. 2. The changing of percent inhibition vs. different inhibitor concentrations for various pesticides.

acetic acid pesticides were 0.38, 0.55, 2.9 and 2.72 mM for *C. carpio* CA; 0.28, 0.59, 2.45 and 1.73 mM for *Barbus barbus* CA; 0.23, 0.51, 1.77 and 1.26 mM for *O. mykiss* CA; 0.20, 0.18, 0.62 and 0.65 mM for *Scorpaena porcus* CA; 0.38, 0.37, 3.19 and 2.67 mM for *Diplodus vulgaris* CA, respectively. Işık et al. [12] reported that deltametrin pesiticide importantly decreases the CA activity as comparing with the pesticides. This result has shown that the deltametrin is an effective inhibitor of CA enzyme. When the results obtained for pesticides compared with inhibitors such as dorzolamide and brinzolamide of CA, it can be said that the inhibition power of pesticides is lower. As seen in Fig. 1, dorzolamide and brinzolamide are sulfonamides, which have –SO$_2$NH$_2$ groups. Dorzolamide's thienothiopyran ring has both an alkyamine group and a CH$_3$ substituent giving it two chiral carbons. Brinzolamide is the *R*-(+)enantiomer of 4-ethylamino-3,4-dihydro-2-(3-methoxypropyl)-2H-thieno [3,2-*e*]-1,2-thiazine-6-sulfonamide,1,1-dioxide, a thienothiazine sulfonamide with a free amine. It forms a suspension a physiologic pH. Brinzolamide is more lipophilic than dorzolamide at physiologic pH, where dorzolamide is uncharged. Lambda-cyhalothrin and deltametrin have –CN chromofor groups when diozinon has –O$_3$PS chromofor group. Sulfonamides such as dorzolamide and brinzolamide can inhibit CA by direct coordination of the anionic sulfonamide nitrogen with the catalytic
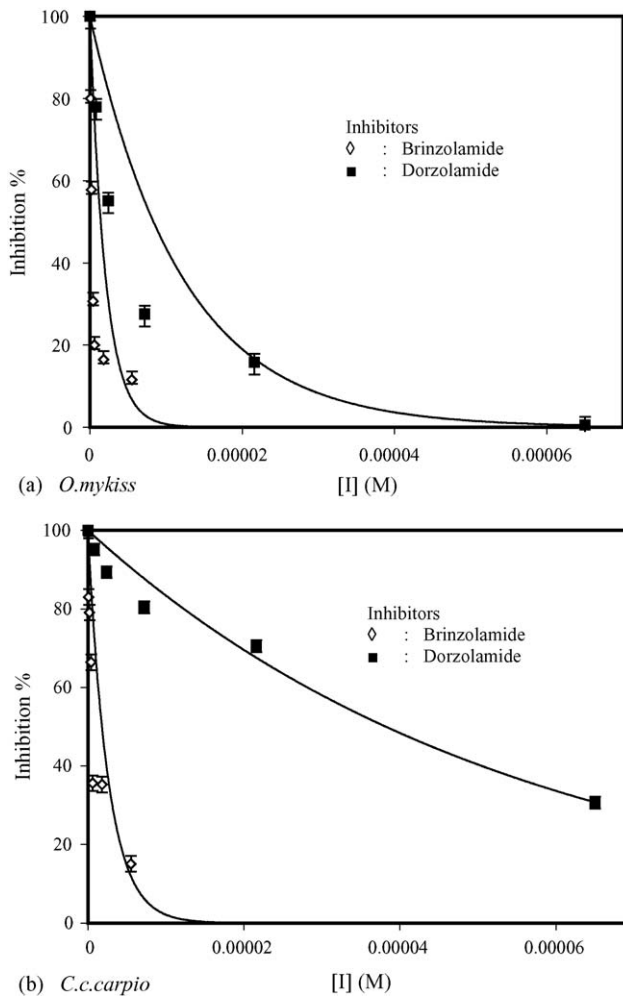
Fig. 3. The changing of percent inhibition vs. different inhibitor concentrations for various inhibitors.

zinc ion [26]. Differential sensitivity to chemical inhibition may be more common and widespread feature of the differences in CA structure among organisms. For example, metal ions are known to inhibit CA from a variety of aquatic organisms, and CA from different tissues in the same organims have been shown to have different sensitivities to these metals. Erythrocyte CAs in the catfish *Ictalurus punctatus*, the most abundant pool in fish were reported as having K_i values between 35 and 900 μM for Ag^+, Cd^{2+}, Cu^{2+} and Zn^{2+} [27]. In the eel, *Anguila anguila*, CA was more sensitive to metal inhibition than CA from intestinal brush border [15]. Also, the concentration of different metals and acetazolamide that inhibited gill CA of fish *I. punctatus* is markedly higher than that presented by estuarine carb *C. granulata*, suggesting that CA activity of the latter species could be relevant biomarker for monitoring enviromental pollution by heavy metals [10]. Differential sensitivity of fish CAs might be depending on a number of factors. It is possible that differences in inhibitions, rooted in the differences in binding affinity of the pesticides to the enzyme, are a result of species-specific isoforms. Differences in the sensitivity of CA to these pesticides may also have an impact on the ability of the intact organism to interact with its evironment.

4. Conclusions

The inhibitory effect of pesticides on CA activity, observed under in vitro experimental conditions, suggests that the pesticides might interfere with physiological functions in which gill CA is involved such as gas exchanges, acid–base balance, osmoregulation and clearance of the waste products from nitrogenous metabolism. Consequently, inappropriate use of pesticides endangers the balance of aquatic enviroment and are also a potential risk to animals and human health as well. The inhibition effects of three different pesticides and chemicals on fish CA were determined using the CO_2 hydratase method by plotting activity percent versus [pesticides or inhibitors]. Some pesticides, which are widely used for agricultural benefits, dramatically inhibit the erythrocyte CA enzymes of some freshwater fish. This minding is important, because pesticides generally contaminate not only the soil, but also water resources. I_{50} values of the pesticides or inhibitor exhibiting inhibition effects were found by means of these graphs. In conclusion, my data on the in vitro inhibitory effect of pesticides on CA activities of *O. mykiss* and *C. c. carpio* fishes have identified sub-cellular targets whose impairment can be hazardous to physiological functions such as osmoregulation and acid–base balance in fish. Moreover, because of the lack of toxicological data on CA in marine organisms, the sensitivity to pesticides of this enzyme reported here could be the starting point for further studies on this ubiquitous enzyme in the toxicological field. In fact, biochemical alterations are attractive as indicators of environmental health because they offer a rapid and sensitive means of monitoring the impact of chemicals on aquatic organisms. The only known physiological function of the CA is to facilitate the interconversion of CO_2 and HCO_3^-, and they therefore play key roles in diverse processes, such as physiological pH control and gas balance, calcification and photosynthesis. In addition, the inhibition of CA leads to decreased acidification of urine, and eventually metabolic acidosis [16]. Therefore, alterations in physiological functions by environmental pollutants could put the survival of the fish at risk. This work is of particular interest because it involves a variance of the structure-action relationship with respect to CA inhibition. Furthermore, the inhibition studies of these pesticides against these fish species may suggest the use of them as biomarker for monitoring enviromental pollution by pesticides. It is not yet possible to speculate on the mechanism of action of pesticides on CA activities measured in fish. Further studies about this matter would be beneficial in terms of the prevention of water pollutions.

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